ENDOTOXIN INDUCED MUSCLE WASTING IN AVIAN AND MURINE SKELETAL MUSCLE

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For the soul of my mother

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Abstract

This project was aimed to elucidate the sub-cellular and molecular regulation of Lipopolysaccharide (LPS) induced muscle protein turnover (protein synthesis (PS) and protein degradation) in two *in vitro* models, C2C12 murine myotubes and avian primary skeletal muscle cell line. In addition, the effect of natural challenge of chicken with *Salmonella* serotypes gallinarium or Enteritidis on mRNA expression levels in skeletal muscle was assessed.

LPS (1 μgml^{-1}) transiently decreased PS rate by 50% compared with control cells. This effect was mediated via decreased phosphorylation of translation initiation mediators ($p70^{S6K}$, 4E-BP1 and eIF-4E). This effect was preceded by decreased Akt and mTOR phosphorylation. Although, LPS significantly increased p38, Erk1/2 and their down stream target Mnk1, however, this effect was not sufficient to abolish LPS-induced decreased PS.

The role of Akt and MAPKs (p38 or Erk1/2) was verified using specific pathway inhibitors. Inhibition of Akt by LY0294002 (PI3-K/Akt inhibitor) dramatically decreased PS by 80% compared with control cells. Incubation of C2C12 myotubes with SB203580 (p38 inhibitor) or with PD098059 (MEK/Erk inhibitor) alone significantly decreased the PS rate at the 3 h time point by -63 $\pm$ 12.48% and -64 $\pm$ 5.05% respectively compared with control cells ($P < 0.01$).

In contrast, LPS (1 μgml^{-1}) significantly increased the chymotrypsin-like enzyme at all the time points. This effect was preceded by a significant increase in the I*k*B-α phosphorylation and nuclear translocation of NF-kB, and significant increase in TNF-α, atrogin-1, MuRF1 and TLR4 mRNA expression. Of note, increased atrogin-1 mRNA is the prominent feature of our septic model. The data presented in chapter 4 and 5 showed that, there is no absolute correlation between the expression levels of atrogens (atrogin-1 and MuRF1) and the overall proteolytic activity in LPS-stimulated C2C12 myotubes.

The beneficial roles of the curcumin were evaluated LPS-stimulated C2C12 myotubes for 3 h. Incubation of C2C12 myotubes with LPS (1 μgml^{-1}) and curcumin (25 μM) significantly decreased the LPS-induced chymotrypsin-

like enzyme activity. This effect was mediated via decreased p38 and I*k*B-α phosphorylation. Although, curcumin blocked LPS-induced decreased Akt and p70S6K phosphorylation and significantly increased Erk1/2 phosphorylation, however, curcumin still had no effect on LPS-induced decreased protein synthesis.

The effect of the LPS on the muscle protein turnover in the avian primary skeletal muscle was summarised in chapter (7). Incubation of avian primary skeletal cells with LPS (1 μgml^{-1}) for 3 h, significantly decreased the proteasomal activity and increased PS rate. The difference in response to LPS between C2C12 myotubes and avian primary skeletal muscle cells could be attributed to the different incubation parameters mainly the presence of insulin in case of avian primary cells.

Finally, the effect of natural challenge of chicken with *S*. Gallinarum or *S*. Enteritidis on skeletal muscle mRNA expression was summarised in chapter 9. Natural challenge of chicken with *S*. Gallinarum or *S*. Enteritidis had no effect on the expression of many atrophic genes in chicken skeletal muscle (gastrocnemius and pectoral muscle).

The data collected from this project showed that, LPS is a strong catabolic stimulus significantly decreased PS along with increased protein breakdown rates in skeletal muscle. This effect was mediated via two main pathways PI3-K/Akt and MAPKs (p38 or Erk1/2) and the cross talk between them is exists. The better understanding of these signalling cascades and their cross talk will be the starting point for developing the appropriate and safe therapeutic intervention in order to decrease the sepsis-induced muscle proteolysis.

Publications

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Tarabees R, Rauch C, Barrow PA, and Loughna PT. The role of the nitric oxide (NO) in LPS-induced muscle wasting in skeletal muscle. Presented in the pathogenesis of Infectious Diseases conference, Prato, Italy (6-9/10), 2010 (oral presentation).

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Tarabees R, Rauch C, Barrow PA, and Loughna PT. Potential *in vitro* anabolic effects of LPS. Presented in the 4th CBCB symposium Dynamic cellular process, University of Nottingham (2008).

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Abbreviations

4E-BP1	4E-Binding protein
Akt	Protein kinase B
BSA	Bovine serum Albumin
C2C12	Murine-derived Myoblast
DM	Differentiating media
DMEM	Dulbecco′s modified Eagle′s medium
E1	Ubiquitin-activating enzyme
E2	Ubiquitin-conjugating enzyme
E3	Ubiquitin-protein ligase
ERK1/2	Extracellular regulatory Kinase 1,2
eEF	Eukaryotic elongation factor
eIF	Eukaryotic initiation factor
FAM	6-carboxyl-flourescein
GM	Growth media
GMEB-1	Glucorticoids modulatory element binding protein
GSK-3β	Glycogen-synthase kinase
HS	Horse serum
HSP	Heat-shock protein
IGF-I	Insulin-like growth factor-I
IL	Interleukin
IFN-γ	Interferon-gamma
INOS	Inducible nitric oxide synthase
i.p	Interaperitoneal
i.v	Intravenous
LPS	Lipopolysaccharide
MAFbx	Muscle atrophy F-box
MAPK	Mitogen-activated protein kinase
M-CK	Muscle creatine kinase

mTOR	Mammalian target of rapamycin
Mnk1	MAPK-integrating kinase 1
MuRF1	Muscle ubiquitin RING-finger 1
NED	N-napthylethylenediamine dihyrochloride
NO	Nitric oxide
NOS	Nitric oxide synthase
NF-kB	Nuclear factor Kappa B
PAMPS	Pathogen-associated molecular patterns
PS	Protein synthesis
PI3-K	Phosphatydilinostol-3-kinase
RT-PCR	Reverse transcriptase-PCR
SDS	Sodium dodecyl sulfate
TCA	Trichloroacetic acid
TLR4	Toll like receptor-4
TNF-α	Tumor necrosis factor
TRAF6	Tumor necrosis factor (TNF) receptor–associated factor-6
UPS	Ubiquitin-proteasome system

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Declaration

I herby confirm that, the work contained in the thesis was obtained from the experimental work primarily done by myself, only in the PS (chapter 3) experiment, Paul Loughna and Derek gave a hand in the preparation and lysis of the samples.

1 General Introduction

1.1 Introduction

Salmonella enterica is a facultative intracellular pathogen capable of causing disease in a broad range of host species. Amongst *S. enterica* serotypes, *S*. Typhimurium and *S.* Enteritidis are the most important salmonellae and can cause enteritis in many species, and these serotypes are responsible for the majority of *Salmonella* food-borne infections in man (for more details, Kaiser *et al.,* 2000).

The genus *Salmonella* includes more than 2,463 serotypes. Historically, *S*. *enterica* serovar Typhimurium is the most common serovar isolated from poultry and it covers 40% of all *Salmonella* isolations from poultry, followed by *S. enterica* serovar Enteritidis (6%), serovar Pullorum (4%) and serovar Gallinarum (3%) (Sojka *et al.,* 1977). However, during the 1980s, the *S. enterica* serovar Enteritidis phage type4 (PT4) became the predominant serovar isolated from poultry.

The classification of the genus *Salmonella* has been initiated from the initial one serotype one species model proposed by the Kauffmann and White Scheme (Kauffmann 1963, 1966, 1972, 1978) based on the somatic antigen (O), flagellar antigen (H) and in some cases capsular antigen (Vi). Further advances in *Salmonella* taxonomy occurred in 1973 when Corsa *et al* (1973) demonstrated by DNA-DNA hybridisation that, all serotypes and subgenera I, II and IV of *Salmonella* and all serotypes of *Arizona* were related and are in fact a single species. The single exception, is *S. bongori* (previously known as subspecies V), with DNA-DNA hybridisation showed to be a separate species (Reeves *et al.,* 1989). *Salmonella enterica* has been divided

into six subspecies (Le Minor and Propoff 1987; Reeves *et al.,* 1989). The following table illustrated the *Salmonella* classification

Table (1.1) *Salmonella* species, subspecies, serotypes and their usual habitats, Kauffmann-White scheme.

Salmonella species and subspecies	No. of species	Usual habitat
S. enterica subsp. *enterica* (I)	1,454	Warm-blooded animals
S. enterica subsp. *salamae* (II)	489	Cold-blooded animals and environment
S. enterica subsp. *arizona* (IIIa)	94	Cold-blooded animals and environment
S. enterica subsp. *diarizonae* (IIIb)	324	Cold-blooded animals and environment
S. enterica subsp. *houtnae* (IV)	70	Cold-blooded animals and environment
S. enterica subsp. *indica* (VI)	12	Cold-blooded animals and environment
S. bongo (V)	20	Cold-blooded animals and environment
Total	2,463	

1.2 Morphology of *Salmonella*

Salmonella are Gram negative (G-ve) bacteria predominantly motile by peritrichous flagella, non-encapsulated, non-sporulated, bacilli, 2-5 µm long and 0.7-1.5 µm wide (Krieg and Holt 1984). The organism has the ability to grow under both aerobic and anaerobic conditions in ordinary media at 37 °C, and develops small colonies 2-4 mm in diameter, which are smooth and homogenous. The metabolic features of *Salmonella* usually include the utilization of citrate as a singular carbon source and the production of gas from glucose (Krieg and Holt 1984; Andrews *et al.,* 1995).

1.3 Distribution

Salmonella serotypes are distributed worldwide, and can infect many mammals and birds, and the main route of infection is the ingestion of contaminated foodstuff (faecal oral route). In poultry some *Salmonella* serotypes such as *S.* Enteritidis and *S.* Typhimurium infect the ovaries, and therefore they can be isolated from eggs and may be transmitted vertically to the next generation (Guan *et al.,* 2006). Only *S. enterica* subspecies *enterica* is of a clinical significance, and its subspecies include the pathogen associated with the typhoid fever (Bhan *et al.,* 2005). The other subspecies and *S. bongori* are usually isolated either from environment or reptiles and therefore are of little clinical important (Bopp *et al.,* 2003).

The intestinal tract of humans and animals, particularly poultry and swine is considered as the main reservoir for *Salmonella*. The microorganism is excreted in the faeces and transmitted by insects and other creatures, soil, water, and kitchen surfaces. Eggs, poultry and meat products are considered the most important source of S*almonella* infection in humans, with the *S.* Typhimurium and *S.* Enteritidis being the most commonly isolated food-borne serovars (Jay 1996). Moreover, during recent years several countries

have reported a high incidence rate of *Salmonella* in animals feed especially bone and meat meal and feed.

1.4 Pathogenesis of salmonellosis

There are two main groups of the genus *Salmonella* from the pathogenesis perspective. The first group produces a systemic disease, and in the absence of systemic disease, they colonise the intestine poorly, do not contaminate the carcass surface, and are not often implicated in human food poisoning. This group includes *Salmonella* serovars Pullorum, and Gallinarium, which causes pullorum disease and fowl typhoid in poultry respectively (Chao *et al.,* 2007).

The second group produces food poisoning in humans and is able to produce systemic disease under certain circumstances when the host immunity is decreased such as during parturition, in lay, in very young or old animals, or after some viral infection (McMeechan *et al.,* 2007). This group consists of *Salmonella enterica* mainly serovars Typhimurium and Enteritidis. These serovars can colonise in the gut and cause gastroenteritis infection in a wide range of hosts including humans.

Understanding of the pathogenesis of *Salmonella* is mainly derived from the studies using the *S*. Typhimurium-infected rat model (Barrow 2007). The severity of the infection depends up on many factors including the virulence of the *Salmonella* strain, the route of the infection, the innate resistance, the immune response of the host and the genetic background of the bird. The forms of systemic infection in chickens encompass three main phases. The first is invasion via the gastrointestinal tract. The second is the establishment of systemic infection (intracellular infection of the macrophages). Finally, clearance of the infection by the immune system, or the carrier state may develop (Chappell *et al.,* 2009). The main route of *Salmonella* infection in animals and humans is the oral route. Following

ingestion, a proportion of the organisms resist the low pH of the stomach, and reach the distal ileum and caecum and invade the mucosa and replicate in the submucosa and Peyer's patches (Carter and Collins 1974). In young chickens extensive bacterial infection occurs in the caeca (Cooper *et al.,* 1994). In addition, invasion can occur via M cells with the contribution of the invasion genes within pathogencity island 1 (SP-1) or via the transportation of the bacteria within CD18-expressing phagocytes from the gastrointestinal tract to the bloodstream (Vazquez-Torres *et al.,* 1999). Following the systemic infection, the microorganism reaches the phagocytes of the liver, spleen and the bone marrow. Activation of the bacterial surface or the presence of opsonising serum antibodies improve the uptake of micrograms by phagocytes (Liang-Takasaki *et al.,* 1983; Saxen *et al.,* 1987). The majority of *Salmonella* are coupled with macrophages and polymorphonuclear phagocytes and the ability of the *Salmonella* to survive in and grow in these cells is one of the most important determinants of *Salmonella* virulence (Hoiseth and Stocker 1981; Lissner *et al.,* 1983; Fields *et al.,* 1986; Dunlap *et al.,* 1991; Mastroeni *et al.,* 1995; Richter-Dahlfors *et al.,* 1997).

There is a considerable variation in virulence for day-old chickens between strains inoculated via the oral route, not associated with the phage type of the strain (Smith *et al.,* 1985). The mortality following the oral route inoculation varies from 0-100 percent with some breeds of birds more susceptible than others do. Moreover, there is no correlation between oral and parenteral virulence, and the cause of death is most likely a combination of anorexia and dehydration resulting from general malaise and diarrhoea. of note, diarrhoea is not observed in chickens inoculated intramuscularly (Barrow *et al.,* 1987).

1.5 Host Resistance to Salmonellosis

There is a paucity in the understanding of the mechanism of natural resistance and long-term protective immunity to Salmonellosis in chickens.

Innate immunity is the non-specific first line of defence against the invading pathogens**.** Innate immunity dose not provide the host with a long-lasting immunity. It dose not specifically react with the microbial pathogens via receptors, and can not serve to distinguish between the different types of pathogens (Medzhitov and Janeway 2000). The innate immune system includes macrophages, neutrophils, mast cells, dendritic cells, and natural killer cells, for more details see (Kirby *et al.,* 2002). The recognition of LPS by the pathogen recognition receptors (PRR) mainly Toll-like receptor-4 (TLR-4), is the first step in the signal transduction and later stimulation of the innate immune system followed by the release of the pro-inflammatory and chemokines (Totemeyer *et al.,* 2003) including the tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) (Lembo *et al.,* 2003). In addition, the cellular immunity plays a significant role in host defence against *Salmonella* mainly controlled by the T lymphocytes (direct effect cytotoxic T lymphocytes (CTLs) and indirect effect T helper (Th) (Lillehoj 1993; Schat 1994). The role of cell-mediated immune mechanisms in systemic clearance of *S.* Enteritidis has been recently investigated in chickens (Farnell *et al.,* 2001). Also, clearance of *S.* Typhimurium in chickens is correlated with high cell-mediated responses (delayed type hypersensitivity reaction) and not with antibody levels (Hong *et al.,* 2006). Thus, the assumption that cell-mediated immunity plays a more important part than humoral responses in protection against *Salmonella* is still the most acceptable dogma (Mastroeni *et al.,* 1993; Barrow 2007).

Recent studies have highlighted the role of Th1 in immunity against *Salmonella* infection. The interperitoneal administration of interferon-gamma (IFN-γ) minimises the organ colonisation after *S*. Enteritidis infection (Farnell *et al.,* 2001). High levels of IFN-γ are correlated with high clearance rates (Beal *et al.,* 2004). The high levels of IFN-γ are also associated with a strong T-cell response (Withanage *et al.,* 2005). In contrast, IFN-γ knockout mice and mice administrated with neutralising antibodies to IL-12 attenuate host protection against Salmonella attenuated strain. Therefore, the Th1 cytokines, which enhance cell-mediated responses are more important than

the humoral responses in the protection against *Salmonella* infection (Eckmann and Kagnoff 2001; Raupach and Kaufmann 2001). The role of early $CD8^+$ T cells in the protection against primary *Salmonella* infection in young chicks has been shown (Berndt and Methner 2001). In contrast, the role of IgA responses and polymorhonuclear leukocytes as a key players in intestinal clearance of *Salmonella* is still confusing (Nagaraja and Rajashekara 1999). IgA plays a significant role against intestinal colonisation as show follows. Chemical bursectomy of chickens infected with *S.* Enteritidis that mainly affects the cells types other than B cells resulted in an increase in the faecal excretion and higher caecal counts of *Salmonella,* while having normal counts in internal organs (Desmidt *et al.,* 1998). In contrast, surgical removal of the bursa (affects mainly B-cell maturation) has no effect on the clearance rate of *S*. Typhimurium between the bursectomised and non-bursectomised chickens (Beal *et al.,* 2006). Combined, this suggests that the antibody response is not essential for gut clearance. While, in case of poultry, the situation is not similar. In chickens, heterophilic granulocytes accumulate in the propria mucosae of the caeca within 18 h post experimental infection with *S.*Enteritidis field strain (Van Immerseel *et al.,* 2000a, 2000b), but in case of infection with *S.* Typhimurium it is accompanied by acute enteropathogenic responses characterized by expression of CXC chemokines and a polymorhonuclear (PMN) cell influx (Withanage *et al.,* 2005). Heterophils in response to *S.* Enteritidis infection have been shown to up-regulate mRNA expressions for pro-inflammatory chemokines, IL-6 and the anti-inflammatory cytokine transforming growth factor-β4, whereas expression of IL-18 and IFN-γ was down-regulated (Kogut *et al.,* 2003).

1.6 The role of cytokines in *Salmonella* pathogenesis

Cytokines are a large and diverse family of polypeptides produced mainly by cells of the immune system. Cytokines are signalling molecules involved in cell communication and signal transduction between the cells. Infection with *Salmonella* enhances the production of the early stage

cytokines including IFN-γ (Ramarathinam *et al.,* 1991; Klimpel *et al.,* 1995), TNF-α (Arnold and Holt 1996). IFN-γ is responsible for the activation of anti-microbial activity of macrophages in the case of infection with *S.* Typhimurium (Foster *et al.,* 2003).

TNF-α enhanced the immunity and decreased the number of *S*. Typhimurium in the peritoneal cavity of infected rats, for more detail see (Nakano *et al.,* 1990). Moreover, TNF-α contributed to the maturation of the dendritic cells in mice orally infected with *Salmonella* (Lembo *et al.,* 2003). Beside IFN-γ and TNF-α, infection with *S.* Typhimurium stimulated the production and release of many other cytokines with various functions such as IL-6, IL-8, and IL-12. IL-6 mRNA significantly increased in intestinal tissue following the secondary infection with *S*. Typhimurium (Withanage *et al.,* 2005). *S*. Typhimurium also up-regulates both IL-1β and IL-6 in avian epithelial cells (Kaiser *et al.,* 2000).

IL-6 is a pro-inflammatory cytokine involved in the activation of T and B Lymphocytes and the development of macrophages (Hirano 1998; Lynagh *et al.,* 2000). IL-8 is an inflammatory chemokine secreted by several body cells, in particular macrophages. IL-8 is chemoattractant attracting the neutrophils to the site of infection and thus is known as neutrophils chemotactic factor, for further details see Köhidai and Csaba (1998). IL-12 is mainly produced by the dendritic cells (Kalinski *et al.,* 1997). IL-12 known as T cell stimulating factor stimulates the growth and the function of the T cells, involved in the differentiation of the naïve T cells to Th0 cells, which later develop into Th1 or Th2 cells. The production of IL-12 enhances the cell-mediated immune response against *S.* Typhimurium (Yrlid *et al.,* 2000). With regard to the role of cytokines in LPS-induced muscle wasting of particular interest is the role of TNF-α and IL-6. Further details about their function are given later.

1.7 The clinical features in chickens infected orally with virulent strain strains of *Salmonella* Typhimurium and Pullorum

Salmonella infection in poultry is still a major problem worldwide. Many *Salmonella* outbreaks with the mostly frequent isolated serovars S. Typhimurium, *S*. enteritidis, *S*. Gallinarum, *S*. Pullorum, *S*. newport, *S*. Cerro, *S*. Montevideo, and *S*. Agona have been reported worldwide (Konrad et al., 1994). The severity of the infection depends on many factors including, the strain of Salmonella, the standard of hygiene, age of the bird, route of infection, and immune status of the birds. Generally the greatest severity of infection was seen in young birds; the younger the birds the higher mortality rate. For instance, a higher mortality rate was seen in young birds infected with pullorum disease and fowl typhoid, while the adult birds develop the carrier state and the infection persists in the reproductive tract of the birds (Chao et al., 2007), and are involved in vertical transmission (Gast 1997).

The general signs of infection in chicks are depression, loss of appetite, ruffled feathers, dehydration, thirst, and yellow or green diarrhoea. The mortality rate is higher especially in young chicks (one to three weeks old chicks) (Wigley *et al.,* 2002). In the adult birds, the signs vary from depression, weight loss, decreased egg production, and low fertility rate. The post-mortem lesions include grey nodules in lungs, liver, gizzard wall and heart, intestinal or caecal inflammation, splenomegaly, caecal cores, and urate crystals in ureters. Of note, diarrhoea is absent in chickens inoculated intramuscularly (Barrow *et al.,* 1987).

The mortality rate varies according to the strain of *Salmonella*, age and the immune status of the host. For example Jones and associates have reported a 60% mortality rate in experimentally infected 3 week old outbreed chickens with *S*. Gallinarum (Jones *et al.,* 2001). Infection with *S*. Pullorum causes a considerable mortality, but is less frequent in birds more

than a week old (Wigley *et al.,* 2001). Infection of chickens with *S. entreica* serovar Typhimurium and Enteritidis is associated with transient systemic infection mainly in newly hatched chicks (Barrow 2000).

The microorganism can be isolated from the alimentary tract and most organs other than blood, breast muscle, yolk sac, and brain where a little isolation was made. The number of isolated *Salmonella* increased when birds became clinically infected with *Salmonella.* The presence of the organism in the viscera is not a result of septicaemia as low numbers were isolated from the blood demonstrating that *Salmonella* is mainly an intracellular parasite (Suter and Ramseiar 1964; Popiel and Turnbull 1985). The high counts in the lung were likely to be raised from intracellular locations rather than oral infection. In the case of *Salmonella* Pullorum, the development of the carrier cases is the most common feature of the disease in birds. The microrgansim infects the reproductive tract, and persists until sexual maturity leading to reproductive tract abnormalities and infection of progeny without disease (Chappell *et al.,* 2009).

1.8 Food-borne infection

The members of genus *Salmonella* are one of the most important pathogenic bacteria that are identified to cause mass food-poisoning incidents (Krieg and Holt 1984). Human *Salmonella* infection is still a major problem, in terms of both morbidity and economic cost (Barnass *et al.,* 1989). Amongst the genus *Salmonella*, S. *enterica* serovar Enteritidis has become a worldwide problem, arising in the main in poultry (Rodrigue *et al.,* 1990). There are nearly 70 *Salmonella* associated deaths each year in the UK (Anon. 1997). In 1985 there were 80,000 cases notified in the UK rising to just over 90.000 in 1997, but falling to about 68,000 in 2000, with highest rates of infection in ages over 70 and under 20, particularly infants (Cogan and Humphrey 2003). Human food poisoning occurs because of consumption of

contaminated poultry products (meat and eggs) with *S. enterica* especially *S.* Typhimurium and *S.* Enteritidis is considered as a major public health problem. Salmonellosis is a food-poisoning syndrome, caused by consumption of contaminated food and meat and their by-products with pathogenic *Salmonellae.* The disease is a serious bacterial toxi-infection syndrome associated with gastroenteritis and fever, and less frequently with vomiting, these symptoms usually last 4-7 days (Jay 1996). The disease is self-limiting and the majority of cases recover without treatment, but it can be more serious in the elderly, infants and people with chronic conditions. If *Salmonella* gets into the bloodstream, it can be serious or even life threatening, and a course of antibiotics is the usual treatment.

1.9 Stimulation of host immune defence and lipopolysaccharide

The immune and non-immune cells have the ability to recognise the pathogens and pathogens particles by secreting several proteins called Toll-like receptors (TLRs) which are similar to that were first discovered in Drosophila (Delneste *et al.,* 2007). Mammals secrete a dozen of proteins homologous to Toll protein secreted by Drosophila, and these proteins were scattered in many tissues and skeletal muscle is one (Carpenter and O'Neill 2007). TLRs are considered the first line of defence against the bacterial cell components including LPS (Gao *et al.,* 2008). Skeletal muscle and C2C12 myocytes produce mRNAs for TLR1 to TLR7, but not TLR9 or TLR10, thus skeletal muscle, whether *in vivo* or *in vitro* can respond to a wide array of bacterial cell wall components including LPS, Flagellin, Lipopeptides, single and double strand RNA, viruses, but not bacterial DNA (Frost *et al.,* 2006). TLR4 is a member of a family of receptors that recognise pathogen-associated molecular patterns (PAMPS) mainly LPS. LPS can be recognised by TLR4 and the role of TLR2 is crucial in mice harbouring a mutation of TLR4 (Frost *et al.,* 2006). The recognition of LPS by TLR4 in cultured skeletal muscle cells (C2C12) is the same either in the absence or in the presence of serum

components (such as LPS-binding protein and soluble CD14) (Frost *et al.,* 2002)**.**

Lipopolysaccharide (LPS) or bacterial endotoxin are an integral part of Gram-negative bacterial cell wall, and are very strong activators of the innate immune system recognised mainly by TLR4 (Raetz 1990). LPS binds to the plasma LPS-binding protein and the complex is associated with CD14 (Alexander and Rietschel 2001). The interaction of LPS with TLR4 is the first step in the signal transduction and later stimulation of inflammatory cytokine gene expression (Kaisho and Akira 2000).

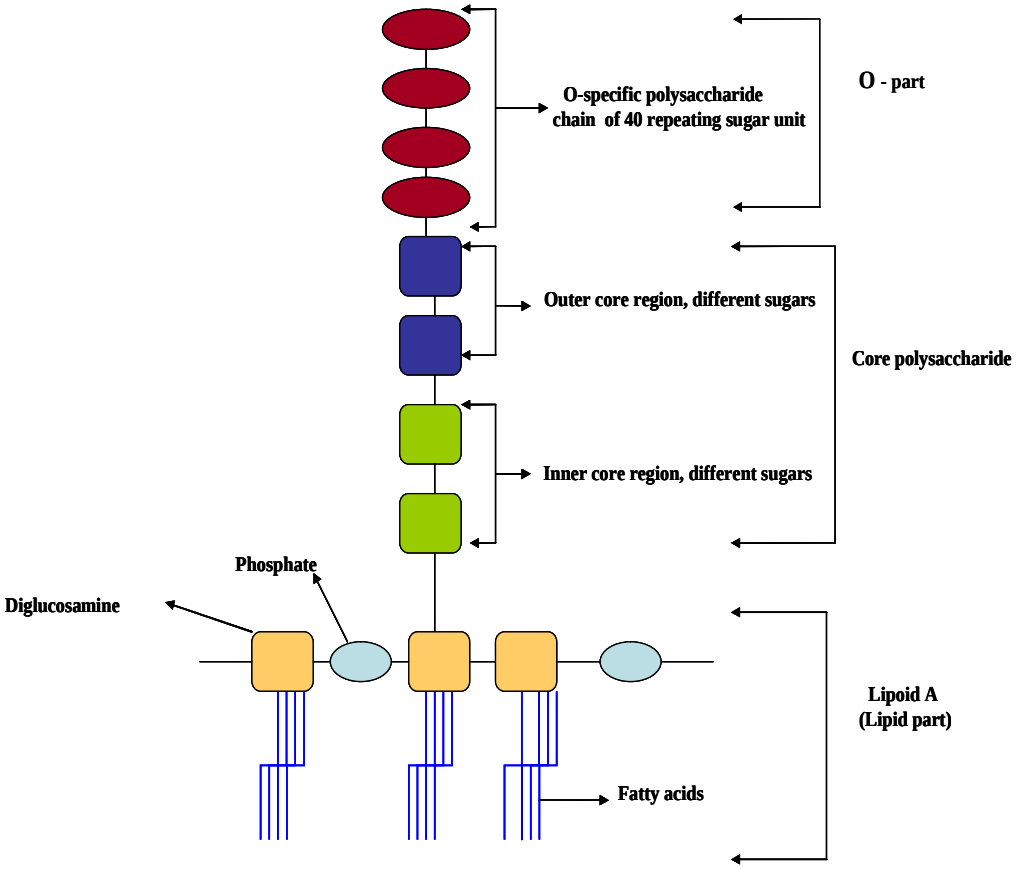


Figure (1-1) Schematic diagram illustrates the structure of Lipopolysaccharide molecule. LPS composed of three parts. Lipid core (lipid A, responsible for biological effect of LPS and can alone reproduce its toxicity), core polysaccharide part, and O-part (variable part responsible for LPS heterogeneous response among bacterial species).

The LPS molecules are composed of three main parts (Fig. 1-1). The first is the lipid core (lipid A), which is responsible for most of the biological effects of LPS and can alone produce its toxicity (Raetz 1996; Wyckoff *et al.,* 1998). The second part, is core oligosaccharide, and the third part is O-antigen, which is the only variable part of LPS molecule and it is the main cause of the heterogeneity of the response to LPS from different strains of bacteria (Rietschel and Brade 1992; Rietschel *et al.,* 1994). The interaction of the LPS with cells such as macrophages, granulocytes, dendritic cells or mast cells leads to the formation and liberation of many inflammatory mediators that are essential for the early innate and subsequent adaptive anti-bacterial defence (Beutler *et al.,* 2003; Freudenberg *et al.,* 2003). However, these strong inflammatory responses to LPS can themselves be threatening and may lead to endotoxic shock and even death (Freudenberg *et al.,* 2008). The development of LPS hypersensitivity is mediated by IFN-γ or IFN-α-β depending on the inducing microbial agent (Freudenberg *et al.,* 2003). For example, the sensing of LPS by TLR4 leads to activation of nuclear factor-kB (NF-kB) and mitogen-activated protein kinase (MAP kinase) (JNK, P38) pathways (Medzhitov 2001), which in turn leads to stimulation of a number of host defence genes as pro-inflammatory cytokines IL-1, IL-6, IL-8, IL-12 and other chemokines and co-stimulatory modules CD80 and CD86 (Medzhitov *et al.,* 1997; Takeuchi *et al.,* 1999; Schnare *et al.,* 2001).

Administration of LPS affects the transcription and release of several pro-inflammatory cytokines. Interperitoneal (i.p) injection of LPS significantly increased the expression of mRNA of TNF-α and IL-1β. IL-1β is a member of IL-1 super family which has role in the immune defence against infection as a pro-inflammatory cytokine (Lang *et al*., 2003). Also, direct injection of LPS significantly increased the expression of IL-6 mRNA in skeletal muscle (Frost *et al.,* 2006). LPS increased IL-6 and IL-6 mRNA as well as increased mRNA for other inflammatory cytokines and decreased the expression of IGF-I mRNA in septic skeletal muscle (Lang and Frost 2005). In addition, LPS stimulated NO synthase-2 (NOS2) mRNA expression in C2C12 myoblasts and primary

cultures of human myocytes (Frost *et al.,* 2004). The enzyme nitric oxide synthase 2 (NOS2) which is often called inducible NOS (iNOS), is a member of NOS family first described in macrophages and now it has been detected in nearly every cell type; playing a central role in the inflammatory response to infection or tissue damage (Galea and Feinstein 1999). Further details on how LPS affecting the muscle protein balance (protein synthesis and protein degradation) are given later in details.

1.10 Structure of skeletal muscle

Skeletal muscles comprise 60-70% of the whole lean body mass, and the main function of the muscle is the contraction and hence movement. Skeletal muscle is considered as the natural reserve of amino acids and energy for time of need (Frost and Lang 2008). Skeletal muscle is considered as an important component of the body's response towards sepsis and injury because of its bulk. A minor change in muscle protein metabolism has a major impact on whole body metabolism. The reduction of muscle protein content can affect the myofibrillar compartment, and this has important implications in term of wound healing, morbidity, and mortality.

Skeletal muscle composed of repeated bundles of muscle fibres separated by a connective tissue called perimysium. Each fascicle is made up of a muscle fibre, which is separated by a connective tissue called endomysium. Muscle fibres are multinucleated and straighted in appearance. The muscle fibre is composed of subunits called myofibrils; each myofibril is made of myofilaments. The myofilaments are composed mainly of two proteins, the thick filament (myosin) and the thin filament (actin). The specific arrangement of both thin and thick filament forms the main contraction unit of the muscle (sarcomere) (Fig. 1-2).

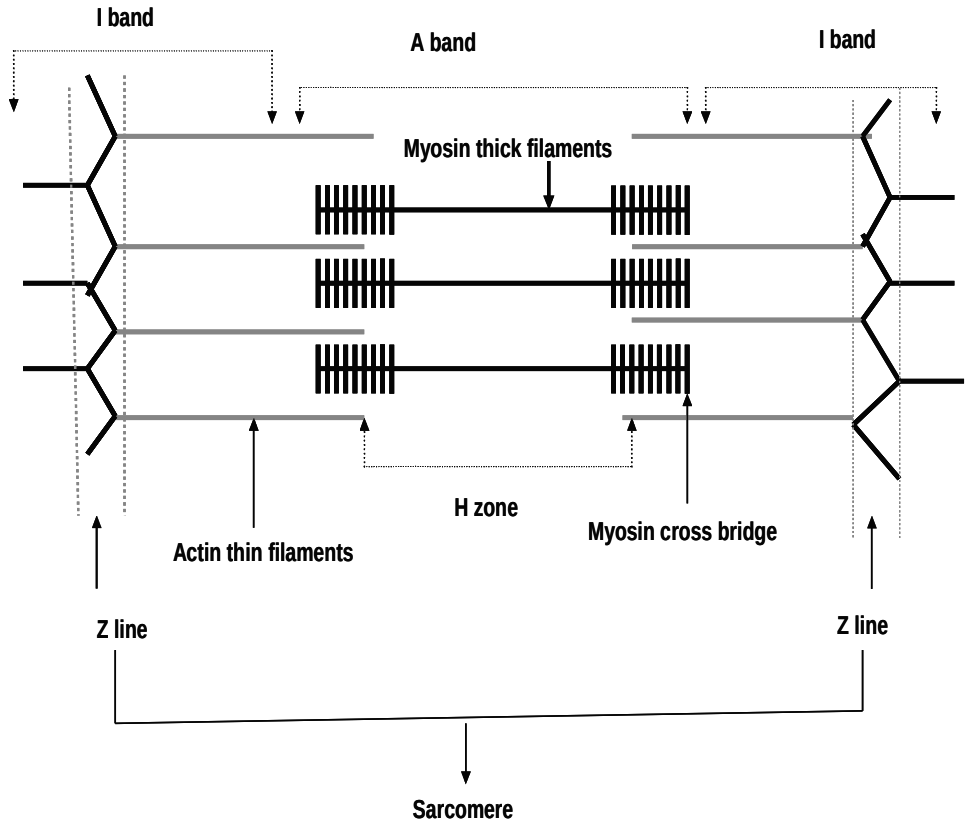


Figure (1-2) diagram illustrates the structure of the myofibrils and the arrangement of the thin filaments (actin) and thick filament (myosin) that forms the contraction unit (sarcomere).

The myosin molecule is composed of 6 polypeptides, 2 heavy chains and 4 light chains. The heavy chains contain the myosin head that interacts with actin to force the muscle to contract (Scott *et al.,* 2001). The myosin heavy chain is considered as the mechanochemical enzyme, or motor protein. In the head region it contains the ATP binding sites that serves as an enzyme (ATPase) for the hydrolysis of ATP to the ADP and inorganic phosphate, and this provides the energy required for the muscle contraction (Stevens *et al.,* 2006). In the presence of the ATP, the myosin head binds and pulls the actin filament along this allowing the sarcomere to shorten. Thus, the interaction between the actin and myosin represents the basis for force generation and contractile functions.

Skeletal muscles can be categorised according their colour into red and white muscle fibre. In addition, muscle fibres can be classified according to their contraction capability into fast and slow twitch-muscle fibres. To be more concise, the muscle can be subdivided into two broad groups, slow-twitch (type-1) and fast-twitch (type-2), the latter can be subdivided into tye-2A, type-B and type-2X fibre. The fast twitch muscle fibre responds more quickly the stimulus and expresses a more rapid relaxation compared with the slow-twitch fibre. In addition, type-2X fibres utilise the glycolysis as a means of cellular respiration (Zierath and Hawley 2004), while slow-twitch fibres are largely dependent upon the mitochondrial respiration. The better understanding the difference of the muscle fibres composition helps in the better understanding the muscle plasticity and how the skeletal muscle can respond to a wide verity of stimuli.

1.10.1 C2C12 murine-derived myotubes and avian primary skeletal muscle cell cultures

Skeletal muscle is made up of many muscle fibres which differ in their physiological and metabolic parameters. The skeletal muscle can diversely respond to many stimuli and this response termed as muscle plasticity. Two main models that have been extensively used in muscle research will be employed in the present studies, the C2C12 murine-derived myotubes model and the primary cell line model (avian primary skeletal muscle cell line).

1.10.1.1 C2C12 murine-derived fully differentiated myotubes

C2C12 murine-derived myotubes is a well-established commercial model used extensively in muscle research and is a sub clone of differentiated C2 cells (adult C3H leg muscle) (Yaffe and Saxel 1977; Blau *et al.,* 1985). C2C12 myoblasts and fully differentiated myotubes have been extensively used as *in vitro* model in many muscle research works. This because C2C12 myoblasts and myotubes have the same functional operating receptor recognition

system as skeletal muscle. These cells express the mRNA of TLR1 through TLR4 and not TLR9 or TLR10 (Frost and Lang 2008). Therefore, the C2C12 cells can respond to the external stimuli similarly as the skeletal muscle.

1.10.1.2 The avian primary skeletal muscle cells

The *in vitro* culture of the skeletal muscle as a primary cultures established cell lines has previously been demonstrated (Yaffe 1968). The primary cultures of skeletal muscle have been used as a model to study of the muscular and neuromuscular diseases and parasitic cytogenesis (Fong *et al.,* 1990; Metzinger *et al.,* 1993; Passaquin *et al.,* 1993; Bouzakri *et al.,* 2003; Guimaraes *et al.,* 2008). Herein, we have used the avian primary skeletal muscle cells that were obtained from the broiler chickens. These birds have been genetically selected for rapid growth and in particular the accretion of muscle mass (Nakashima *et al.,* 2009). These chickens are characterised by a lower protein degradation rate than that observed in layers (Hayashi *et al.,* 1985; Maeda *et al.,* 1987). This because of the fractional rates of protein synthesis in the broiler chicken are greater than in the layers (Maeda *et al.,* 1984). In the context, there is no clear data regarding the direct effect of LPS on the protein turnover in these cells. Thus, we assume that, the effect of the LPS will different in these cells compared with the C2C12 myotubes model.

1.11 Protein turnover

Protein synthesis, involves two main steps at the molecular levels: transcription and translation (Fig. 1-3). Transcription is the formation of mature messenger RNA (mRNA) from the DNA (nuclear code) in the nucleus, while the translation is the formation of specific protein from the mRNA. The translation step of protein synthesis includes three main phases: initiation, elongation, and termination which are regulated by eukaryotic initiation, elongation and releases factors, respectively (Proud 2007). The rate of protein turnover varies between tissues; with some tissues like liver and gut having a greater rate of protein breakdown and synthesis compared to skeletal muscle, and this variation in addition has been demonstrated in the fractional protein synthesis rate between individual proteins (Wagenmakers 1999). Planas *et al.* have demonstrated that the rate of protein synthesis was higher in the liver and diaphragm and lower in skeletal muscle of septic rat compared to the control (Planas *et al.,* 1995). Increased rate of protein synthesis in liver was derived from accelerated formation of the acute phase protein (Frost and Lang 2008). In addition, the difference in protein synthesis rates was varied according to species, age, status of nutrition, and the method of measurement. Within the same species i.e. rats, the protein synthesis rate was varied in individual tissues with the highest rate was seen in the pancreas and the lowest was in muscle (Waterlow 2006). The study carried out by Marway *et al*., (1993) demonstrated that the rate of protein synthesis was significantly decreased from the mouth to rectum. In skeletal muscle, a little part of the discrepancy in the protein turnover rates can be attributed to fibre-type composition of the muscle (Maltin *et al.,* 1989). Catabolic insults like sepsis altered the protein synthesis rate according the muscle fibre type, Vary and Kimball demonstrated that sepsis decreased the protein synthesis in fast-twitch but not in the slow-twitch muscle (Vary and Kimball 1992). Similarly, *in vivo* studies using C2C12 septic model, Frost *el al*., have demonstrated that LPS alone could not change the protein synthesis while in

combination with IFN-γ it induced 80% decrease in protein synthesis (Frost *et al.,* 2009).

Under normal conditions, the total lean body mass is maintained by the balance between the protein synthesis and protein degradation. In case of catabolic diseases such as sepsis, the protein degradation rate is increased while the protein synthesis in decreased (Rennie *et al.,* 1983; Sjolin *et al.,* 1990). The major portion of protein breakdown was originated primarily from the skeletal muscle. Sepsis decreased synthesis of myofibrillar and sarcoplasmic proteins (Vary *et al.,* 1996) and this effect was mediated via the alteration of the translation initiation step of protein synthesis mainly decreasing the availability of the active eukaryotic initiation factor-4G (eIF-4G)-eIF-4E complex (Vary and Kimball 2000).

In skeletal muscle, the proteins can be categorised into two major groups, sarcoplasmic and myofibrillar proteins (myosin and actin), and recently the importance of the mitochondrial protein has been given more consideration (Stump *et al.,* 2003). The protein turnover rates between individual proteins in the same group are still contradictory. Zak *et al*. (1977) have demonstrated that myosin heavy chain, α-actinin and tropomyosin turnover faster than actin and myosin light chains. While, Fiorotto *et al*. (2000) showed that under nutrition caused a similar 30% decreases in synthesis rates of seven different myofibrillar proteins. Increased net protein degradation was resulted primarily from accelerated rate of amino acid fluxes from the tissues (Biolo *et al.,* 2000). It has been previously demonstrated that amino acids act as signalling molecules enhancing protein synthesis by accelerating the translation initiation (Vary and Kimball 2000). Leucine (Leu), is one of the amino acids that has been shown to regulate the protein synthesis. Leu enhanced protein synthesis acting by enhancing the phosphorylation of 4E-binding protein1 (4E-BP1) and $p70^{S6K}$, and thus it enhance the association of the eIF-4E with eIF-4G and switch on the mRNA

translation initiation (Crozier *et al.,* 2005). Further details about these factors are given later in this chapter.

Amino acids are the precursor of protein synthesis and the end product of the protein breakdown. Two main methods have been used to measure the protein synthesis *in vivo* using labelled amino acid tracer, the constant infusion method and flooding dose method. In case of constant infusion method protein synthesis is measured by calculating the plateau labelling of the tracer and the increase in tissue incorporation of tracer at a single point at the end of the infusion (Smith and Rennie 1996). While, the flooding methods includes the administration of a large (flooding) dose, to equilibrate the tracer in all the intra-and extracellular amino acids pools i.e. using large pools of leucine or phenylalanine (Smith *et al.,* 1992). Fractional protein breakdown rates have been measured by discerning dilution of free intracellular amino acids. This technique offers the study of both the protein synthesis and protein breakdown in one study (Phillips *et al.,* 2002; Zhang *et al.,* 2002). *In vitro*, protein synthesis has been generally determined by measuring the direct incorporation of ^{3}H-phenylalanine into protein (Vandenburgh *et al.,* 1991; Frost *et al.,* 2009), and the protein degradation determined by measuring the chymotrypsin-like enzyme activity (proteasomal activity) (Orino *et al.,* 1991; Whitehouse and Tisdale 2003).

1.11.1 Protein turnover in C2C12 murine-derived myotubes and the avian primary skeletal muscle cells

Protein turnover is the ratio between the protein synthesis and the protein degradation. In normal conditions, all the body proteins are in a constant state of turnover. Sepsis is an acute inflammatory condition characterised by accelerated rate of protein degradation much of which originated from the skeletal muscle and decreased rate of protein synthesis. (Jepson *et al.,* 1986; Hummel *et al.,* 1988; Ash and Griffin 1989; Hasselgren *et al.,* 1989; Gracey *et al.,* 2004). The accelerated rate of protein degradation

from the skeletal muscle aimed mainly to afford the liver with the required amino acids to form the acute phase protein and for the production of glucose by gluconeogenesis. Skeletal muscle has the ability to respond to various external stimuli including LPS, and this effect is mainly mediated through TLRs mainly TLR4. Thus, skeletal muscle can respond directly to LPS, the majority of injected LPS has been taken by the liver, and a considerable amount has been taken by the muscle, thus muscle is considered a significant store for LPS (Mathison *et al.,* 1980). LPS stimulated cytokines production and release in both classical immune and non-immune cells including, skeletal muscle (Frost *et al*., 2003). Direct injection of LPS in the skeletal muscle of the dog significantly increased the level glucose-6-phoshate, phosphocreatine and ATP within 5 min (Myrvold *et al.,* 1975), and mRNA of TNF-α and IL-1β mRNA within 30 min (Lang *et al*., 2003). In addition, LPS directly and indirectly through TNF-α autocrine manner stimulated the IL-6 mRNA production and release in septic skeletal muscle (Frost *et al.*, 2006; Crossland *et al.*, 2008). Regarding the *in vitro* studies, the use of C2C12 myotubes is the most suitable and acceptable model to evaluate the effect of LPS on skeletal muscle. The C2C12 fully differentiated myotubes are behave and respond to the external stimuli in a similar way to that of skeletal muscle. LPS in combination with IFN-γ was able to induce the nitric oxide (NO) production (Williams *et al.,* 1994), and decreased the protein synthesis rate by 80% (Frost *et al.,* 2009) in C2C12 myotubes. The accelerated protein degradation in muscle is mainly due to the increased activity of ubiquitin-proteasome pathway (Hershko and Ciechanover 1998) and this effect is mainly associated with increased activity of both atrogin-1 and MuRF1 mRNA (E3 ubiquitin ligases) (Li *et al*., 2003; Li *et al*., 2005; Jin and Li 2007). Further details regarding the adverse effects of sepsis on skeletal muscle protein metabolism are given later.

1.12 The effect of LPS (bacterial endotoxin) on the protein synthesis and degradation

In normal conditions, the balance between the rate of muscle protein turnover and the muscle protein synthetic rate governs the maintenance of skeletal muscle protein metabolism. The loss of lean body mass is one of the most important consequences of severe infection and sepsis (Macallan *et al*., 1996). Sepsis, endotoxin, excess inflammatory cytokines, and glucocorticoid administration are characterised by a reduction in the rate of muscle protein synthesis (Jepson *et al.,* 1986; Hummel *et al.,* 1988; Ash and Griffin 1989; Hasselgren *et al.,* 1989; Gracey *et al.,* 2004) and/or an increased rate of protein breakdown. The mechanism by which the protein synthesis is decreased is either transcriptional or translational (Macallan *et al.,* 1996), with the translational regulation being the foremost mechanism in acute sepsis (Jepson *et al.,* 1986). Skeletal muscle is considered as a significant store for LPS (Mathison *et al.,* 1980), and can respond directly to LPS (Frost *el al* 2006). The administration of LPS in sub-lethal doses causes activation of cytokines cascades, with rapid release of large amounts of TNF-α into the circulation which in turn associated with the development of the a classical acute phase response (Sharma *et al.,* 1992). In addition, LPS significantly increased the expression level of many cytokines mRNA including TNF-α and IL-1β mRNA. TNF-α and IL-1β are two main mediators involved in muscle wasting Lang *et al*. (2003). Elevated levels of IL-1β and TNF-α significantly induce muscle wasting via activation of atrogin-1 in a p38-dependent manner (Li *et al.,* 2005; Li *et al.,* 2009). LPS administration was associated with a 10% loss of body weight in the first 24 h post-treatment (Ash and Griffin 1989; Hasselgren *et al.,* 1989), and this effect was because of the reduction of food intake (Sharma *et al.,* 1992; Macallan *et al.,* 1996). In contrast, LPS significantly decreased the protein synthetic rate in the skeletal muscle of neonatal pigs (Orellana *et al.,* 2002). Similarly, *in vitro* a mixture of LPS and IFN-γ significantly decreased the protein synthesis rate in C2C12 cells by 80% (Frost *et al.,* 2009), and LPS alone significantly decreased the rate of PS by a

60% compared with the control in the same cell line (Russell *et al.* 2010). Together, the regulation of LPS-induced muscle wasting requires the interaction of many signalling cascades and transcriptional factors. This makes the overall elucidation of these process is a hard task requires further investigation in order to develop an appropriate therapeutic intervention.

The major proteolytic systems implicated in skeletal muscle atrophy in many catabolic models are lysosomal, calcium-dependent and the ATP-dependent ubiquitin-proteasome pathway (Finn and Dice 2006). The ATP dependent ubiquitin-proteasome pathway is considered the predominant biological mechanism controlling the protein degradation in myofibrillar proteins in skeletal muscle (Attaix *et al.,* 2005). Further details on the role of these proteasomal systems are given in details later.

The decrease in body lean mass is mainly because of the loss of skeletal muscle protein (Goldberg *et al.,* 1988). The biochemical mechanism of muscle wasting involves complex interactions among several mediators, which are not fully understood. Therefore, a better understanding of these interactions is important for developing successful therapies to retard the loss in lean body mass and lessen morbidity and mortality (Fernandez-Celemin *et al.,* 2002). Further details about how LPS-induced decreased protein synthetic rate and the possible contribution of Akt and other translation initiation mediators mainly (mammalian target of rapamycin (mTOR), eIF-4E and 4E-BP1 are given later.

1.13 Signalling pathways regulating protein synthesis

Muscle wasting is a common result of severe catabolic diseases such as sepsis, cancer, and AIDS. Sepsis is an inflammatory condition that causes severe and rapid loss of body lean mass, much of which originates from skeletal muscle (Hasselgren 1995). LPS are an integral part of cell wall

components of Gram-negative bacteria, and are potent activators of the innate immunity system through recognition by TLR4 (Raetz 1990).

The dynamic balance between rates of protein synthesis (PS) and protein degradation determines skeletal muscle protein mass. To date, most studies investigating the effects of LPS upon skeletal muscle protein metabolism have examined the regulation of catabolic pathways involved in muscle protein loss (Potter *et al*., 2001; Ravindranath *et al*., 2003; Cai *et al*., 2004). Recently, two main *in vitro* studies (Frost *et al*., 2009; Russell *et al*., 2010) and one *in vivo* study (Orellana *et al*., 2002) have examined the effect of LPS on PS. The regulation of PS in skeletal muscle can be at either the transcriptional or translational level (Macallan *et al.,* 1996), but, the latter is the main mechanism in acute conditions (Jepson *et al.,* 1986).

One of the most studied anabolic pathways is the phosphatidylinositol 3-kinase (PI3-K)/Akt pathway. Akt (protein kinase B), is a family of serine-theronine protein kinases (Datta *et al*., 1999) which are stimulated by a number of receptor tyrosine kinases and often mediated by the action of PI3-K (Walsh 2006). Akt has also been shown to be activated by *Salmonella enterica* serovar Typhimurium in epithelial cells (Steele-Mortimer *et al.,* 2000). In skeletal muscle, acute activation of Akt for 2 weeks leads to a dramatic increase in the skeletal muscle size, which occurs via an increase in the PS pathways (Lai *et al*., 2004)**.** The involvement of PI3K-Akt pathway and the mTOR, and their down stream targets $P70^{S6K}$ and 4E-BP1 in the process of PS was reviewed in (Glass 2005), which plays a crucial role in the up-regulation of PS (Wang *et al.,* 2001). Akt mainly regulated the protein synthesis via the regulation of the translation initiation process (Fig. 1-4).

The process of PS translation is conventionally divided into three main stages: initiation elongation and termination. mTOR controls the initiation and elongation process stage of translation (Wang and Proud 2006)

through its downstream effectors, including ribosomal S6Kinase (S6K1) and 4E-BP1 (Wang and Proud 2006).

The mitogen Activated Protein Kinases (MAPKs) family plays an indispensable role in the regulation of cell growth and differentiation. *In vitro* stimulation of MAPK-integrating kinase1 (Mnk1) (downstream target of both p38 and Erk1/2 MAP kinases) regulates the translation initiation process through eIF-4E (Fukunaga and Hunter 1997). Further details about these signalling cascades and their roles are given later.

1.13.1 Role of Akt in Protein synthesis

Akt (Protein kinase B), is a serine/theronine kinase that is stimulated by a variety of growth factors including insulin through the PI3-K-dependent mechanism (Chan *et al.,* 1999; Walsh 2006). Currently, three mammalian isoforms of high degree of sequence homology (Akt1/PKBα, Akt2/PKBβ and Akt3/PKBγ) have been identified (Brodbeck *et al*., 1999 2001). These isoforms vary in their functions. Akt1 and Akt2 are ubiquitously expressed, whereas the expression of Akt3 is mainly restricted to heart, kidney, brain, testis, lung and skeletal muscle (Brodbeck *et al*., 1999; Brodbeck *et al*., 2001). Akt1 is most important to the growth both *in utero* and after birth (Cho *et al.,* 2001), while Akt2 is required for insulin to maintain normal glucose homeostasis. The absence of Akt2 significantly decreased the insulin stimulated glucose uptake in muscle and the whole glucose disposal from the body (Cho *et al.,* 2001). In skeletal muscle acute activation of Akt for 2 weeks increased the skeletal muscle size, which occurs via an increase in the PS pathways (Lai *et al.* 2004). Moreover, nearly in all the previous studied models of muscle hypertrophy, the amount of phosphorylated Akt was higher throughout the hypertrophy process. In case of Insulin-induced hypertrophy model for instance, stimulation of myotubes with insulin associated with activation of PI3-K/Akt pathway, which in turn leads to the stimulation of mTOR and its downstream targets $P70^{S6K}$ and 4E-BP1, this promotes the protein synthesis

via increased translation initiation and elongation (Terada *et al.,* 1994; Lin and Lawrence 1996; Rommel *et al.,* 2001).

Akt has the ability to phosphorylate and change the activity of many vital metabolic targets. Thus, Akt is considered as the key player in the regulation of muscle protein synthesis and degradation signals for more details see (Glass 2005; Wu *et al.,* 2010). Regarding the role of Akt in the PS process, Akt stimulates the protein synthesis in many ways. First, activation of Akt, phophorylates and inhibits the glycogen synthase kinase 3β (GSK-3β), (Jefferson *et al.,* 1999), the phosphorylation of GSK-3β releases its inhibition of the translation initiation factor eIF-2B (Rhoads 1999) and this switches on the translation initiation step pf PS. Second, another possible *in vivo* mechanism*,* Akt stimulates $P70^{S6K}$ and 4E-BP1 most likely via mTOR and the phosphorylation of 4E-BP1 facilitates the binding of eIF-4E to eIF-4G and provokes translation initiation induction fully described by Glass (2003). Increased Akt1 and Akt2 activity *in vivo* significantly increased the phosphorylation of $p70^{S6K}$, glycogen accumulation and muscle fibre hypertrophy (Cleasby *et al.,* 2007), and inhibits the dexamethasone-induced expression of atrogin-1 in cultured myotubes (Sandri *et al.,* 2004). In addition, constitutive Akt activity increased basal and insulin-induced glucose and amino acids uptake and protein synthetic rate in L6 myotubes (Hajduch *et al.,* 1998; Ueki *et al.,* 1998). Akt plays a pivotal role in the prevention of the muscle atrophy; phosphorylation of Akt suppresses the expression of atrogin-1/MAFbx (Muscle Atrophy F box) and MuRF1 (Muscle Ring Factor) through Foxo (Forked head box O) transcription family-dependent way (Bodine *et al*., 2001; Sandri *et al*., 2004; Whitman *et al*., 2005). Activation of Akt inactivates Foxo proteins through ser-thr phosphorylation; Foxo translocated to cytosol, degraded by the proteasome, and thus suppresses the expression of atrogin-1 and MuRF1 (Fig. 1-4).

Atrogin-1 and MuRF1 are skeletal muscle specific E_3 ubiquitin ligases that involved in muscle atrophy nearly in all muscle atrophy models (Plas and

Thompson 2003). Conversely, inhibition of the Akt activity activates the Foxo proteins, which later translocated to the nucleus, and activate atrogin-1 and MuRF1 transcription (Van Der Heide *et al.,* 2004). For example, *in vivo* LPS-induced sepsis decreased Akt phosphorylation and subsequently activated the expression of atrogin-1 mRNA in the rat skeletal muscle (Crossland *et al*., 2008). There are many recent findings suggesting that, decreased activity of PI3-K/Akt signalling pathways leads to muscle atrophy (Bodine *et al.,* 2001), and reduced the size of myotubes (Rommel *et al*., 2001). In contrast, induction of Akt activity can modulate muscle structure and cellular signalling. Increased Akt activity increases muscle fibre size and muscle mass in adult muscle (Lai *et al*., 2004; Izumiya *et al.,* 2008; Ouchi *et al.,* 2008). In addition, activation of Akt1 can prevent denervation-induced muscle atrophy in adult skeletal muscle (Pallafacchina *et al.,* 2002), and can produce type IIb myofibre atrophy and increase muscle strength (Izumiya *et al.,* 2008). For more details on how the Akt activity controls both the hypotrophy and atrophy signalling pathways for more details see Glass (2005) and Wu *et al*. (2010). Although, activation of Akt has been involved in the regulation of muscle protein synthesis and the prevention of muscle protein breakdown, however recent studies showed that proteolysis induced factor (PIF-1) significantly increased the proteasomal and lysosomal proteolytic activity via transient activation of Akt and Akt inhibition by LY0294002 prevents PIF-induced protein degradation (Russell *et al.,* 2008). They argue this finding that, transient Akt phosphorylation was aimed to increase PS and hence increase the production of the proteasome subunits involved in the muscle proteolysis. Moreover, inhibition of Akt prevents PIF-induced phosphorylation and proteasomal degradation of I*K*Bα and nuclear translocation of NF-kB (Wyke *et al.,* 2005), and Akt activation could mediated PIF-1-induced E3 expression via NF-kB manner (Li *et al*., 2009). Thus, the proper activation of Akt is pivotal in the regulation of muscle protein metabolism and muscle mass (Wu *et al.,* 2010).

1.13.2 Role of mTOR in Protein synthesis

The mammalian target of rapamycin (mTOR), is a 289KD serine/threonine protein kinase that regulates many cellular functions including cell growth, cell proliferation, cell motility, cell survival, protein synthesis, and transcription (Asnaghi *et al.,* 2004; Hay and Sonenberg 2004). mTOR is considered as a central key regulator of cell development (Schmelzle and Hall 2000).

The protein synthesis in all eukaryotic cells is a highly regulated multitask process, which involves the interaction between several mediators, and mTOR is one of the key elements regulating this process. mTOR plays a pivotal role as a regulator of protein synthesis and translation initiation (Gingras *et al.,* 2001). mTOR controls the protein synthesis by two mechanisms. Firstly, mTOR controls the transcription of ribosomal proteins and the synthesis of ribosomal and RNA transfer (Hardwick *et al.,* 1999; Powers and Walter 1999). Anabolic stimuli including, growth stimulating factors and amino acids increased mTOR phosphorylation, and this activates ribosomal S6 kinase ($p70^{S6K}$) and the binding protein eIF-4E (4E-BP1) (Fig. 1-4). mTOR stimulates the activity of $p70^{S6K}$ and inactivates the 4E-BP1; this stimulates the protein translation initiation (Hara *et al.,* 1998; Proud 2004). The activity of $p70^{S6K}$ is required to maintain the normal size of the muscle fibre (Ohanna *et al.,* 2005). Rapamycin (mTOR specific inhibitor) inhibits $p70^{S6K}$ phosphorylation and this decreases the activity of the 40S ribosomal protein S6, this suggests that mTOR regulates the translation initiation step of protein synthesis (Gingras *et al*., 2001). Increased mTOR phosphorylation enhanced the cap-dependent translation and formation of eIF4F complex via the phosphorylation and subsequent inactivation 4E-BP1 (Schmelzle and Hall 2000). Therefore, mTOR is considered as a pivotal player the regulation of protein synthesis via the control of the translation initiation process (Asnaghi *et al.,* 2004) (Fig. 1-4).

mTOR has been shown to control the activity of two diverse signalling pathways: the first is the cell survival pathway through its upstream regulators PI3-K, and the second is the apoptotic pathway through the phosphorylation and inhibition of anti-apoptotic Bcl-2 protein enhancing the programmed cell death (Haldar *et al*., 1998; Chadebech *et al*., 1999). In case of skeletal muscle, LPS–induced protein synthesis decrease are mainly mediated through PI3-K/Akt/mTOR and their downstream substrates $P70^{S6K}$ and 4E-BP1 and this has been demonstrated in many *in vitro* and *in vivo* studies (Kimball *et al.,* 2003; Lang and Frost 2005; Orellana *et al.,* 2007; Eley *et al.,* 2008). Combined, the activity of Akt/mTOR and their downstream target 4E-BP1 and eIF-4E are involved in the regulation of protein synthesis and skeletal muscle growth (Bodine *et al.,* 2001; Matheny and Adamo 2009).

1.13.3 Role of translation initiation mediators (eIF-4E and 4E-BP1) in Protein synthesis

Protein synthesis in the eukaryotic cell is a complex multi-steps process. The production of protein from the transcripted mRNA involves three distinct steps initiation, elongation and termination. A number of protein factors regulate the process of engagement of mRNA by the ribosome functionally classified as eukaryotic initiation factors (eIFs) (Shah *et al.,* 2000). In addition, a group of eukaryotic elongation factors (eEFs) and releasing factor (RF) are responsible for regulation of the elongation and termination process respectively. Two main steps are crucial in the control of protein synthesis *in vivo.* First, the formation of 43S preinitiation complex and this step is regulated by a group of eukaryotic factors, mainly (eIF) 2 and eIF-2B. The formation of this complex decreased in skeletal muscle in case of sepsis because of the diminished activity of eIF-2B (Vary *et al*., 1994; Voisin *et al*., 1996; Vary *et al*., 1998). Second, the binding of mRNA with the 43S preinitiation complex which is mediated by eIF-4E (subunit of eIF-4F) to form eIF4E-mRNA complex, this is followed by the binding of the eIF-4G, eIF-4A

with eIF-4E-mRNA to from the active eIF-4F complex. The formation of the eIF-4F complex allows the cap-dependent translation initiation to goes on (Rhoads *et al*., 1994; Lang and Frost 2007; Rhoads 2009). Consequently, increased eIF-4E phosphorylation correlated with improved protein synthesis rate in stimulated cell cultures with various anabolic stimuli (Morley and Traugh 1990). LPS altered the phosphorylation of 4E-BP1 and consequently decreased the protein translation initiation via decrease the availability of free eIF-4E was shown in (Fig. 1-4)

In skeletal muscle, sepsis reduced the amount of the active eIF4E-eIF4G complex by 65% compared to the control (Lang and Frost 2007). In skeletal muscle, the cap-dependent translational translation repressor protein (4E-BP1) controls the interaction between the eIF4E mRNA and eIF4G to form the active eIF-4 G complex. The binding of 4E-BP1 to eIF-4E limits its availability to form the eIF4E-eIF4G active complex and this effect is withdrawn by the phosphorylation of 4E-BP1 (Flynn and Proud 1996). The 4E-Binding Protein (4E-BP1) is one of a family of small, acid-and heat-stable proteins forms inactive complex with eIF-4E (Bennett *et al.,* 1994).

4E-BP1 is the main translational repressor protein in skeletal muscle; it regulates the availability of eIF-4E. The decrease in protein synthesis during sepsis is mainly due to the defect in translational efficiency (translation initiation) (Vary *et al.,* 1994; Jurasinski *et al.,* 1995), not the reduction in elongation (Vary and Kimball 1992). Increased activity related phosphorylation of 4E-BP1 release it form the eIF-4E and this later facilitates the formation of eIF-4F active complex. Hence, 4E-BP1 considered as the main regulator of the cap-dependent translation initiation in skeletal muscle. Sepsis and endotoxaemia decreased 4E-BP1 phosphorylation and this limits the availability of eIF-4E to from the active eIF-4E complex (Lang *et al*., 2000; Svanberg *et al*., 2000; Lang and Frost 2004). In addition, sepsis increased the amount of inactive eIF4E 4E-BP1 complex in gastrocenmius muscle by 80% compared with the control non-septic rat muscle (Lang and Frost 2007). *in vitro* , Atherton and associates demonstrated that, cyclic stretch increased

the activity related phosphorylation of 4E-BP1 in L6 cells despite there being a decrease in the protein synthesis (Atherton *et al.,* 2009). Whereas, Frost and his colleagues showed that, LPS alone could not alter the status of 4E-BP1, while LPS with IFN-γ was able, and this effect was sufficient to induce a 80% decrease in PS rate in C2C12 myotubes (Frost *et al.,* 2009). Many studies have shown a low correlation between the amount of eIF-4E associated with 4E-BP1 and the formation of eIF4E-eIf-4G active complex (Vary and Kimball 2000; Shen *et al.,* 2002), and this suggests that the regulation of dissociation of 4E-BP1 from the eIF-4E is differentially regulated from the association of eIF4F with eIF4G (Shen *et al.,* 2005). This suggests that the translation initiation is mostly dependent upon the formation of eIF4E-eIF4G active complex not on the amount of phosphorylated 4E-BP1. Therefore, the paradoxical data regarding the effect of the endotoxin on the phosphorylation status of 4E-BP1 and its effect on the PS need further deep investigation The dogma that, measuring both the effect of LPS on the anabolic signalling and the PS together in the same model under the same conditions is the appropriate approach that we have undertake in our C2C12 model of sepsis.

1.13.4 Protein synthesis in skeletal muscles From LPS to mitogen-activated protein kinases (MAPKs)

The mitogen-activated protein kinases (MAPKs) family is considered the most important intercellular signalling system involved in the regulation of many processes such as, cell growth, differentiation, and survival (Seger and Krebs 1995). MAPKs play an important role in interorgan communication in many physiological and behavioral phenomena through various cytokines (Kahn *et al.,* 2005; Long and Zierath 2006). In muscle the effect of MAPKs is controversial, some think that it is mitogenic and prevents differentiation, others believe that it is not involved in differentiation, while others still think that it may promote differentiation (Eran Gredinger *et al.,* 1998).

1.13.4.1 P38 MAP kinase and muscle protein metabolism

p38 MAPK has been recognised as one of the MAPKs signalling family that is activated in response to a wide array of environmental stress factors (Lang and Frost 2005), including ultraviolet light (Han *et al.,* 1994; Yeow *et al.,* 2002), osmotic stress (Moriguchi *et al.,* 1995; Shapiro and Dinarello 1995), heat shock, and inflammatory cytokines (Haneda *et al.,* 1995; Raingeaud *et al.,* 1995; Karem *et al.,* 1996; Kohn *et al.,* 1996; Modur *et al.,* 1996; Cuenda *et al.,* 1997; Hajduch *et al.,* 1998; Igarashi *et al.,* 1999; Hong *et al.,* 2006). Four members of p38 MAP Kinases have been characterised: p38 (p38α) (Han *et al.,* 1994; Hong *et al.,* 2006), p38β (Jiang *et al.,* 1996), p38γ (Erk6, SAPK3 stress- activated protein kinase) (Lechner *et al.,* 1996; Cuenda *et al.,* 1997; Li and Reid 2000), and p38δ (SAPK4) (Jiang *et al.,* 1997; Kumar *et al.,* 1997). Among the p38 MAPK isoforms, p38α is a well identified and expressed in most cell types (Remy *et al.,* 2009). Activation of the p38 pathway may accompanied with many terminal changes in cell function, including modulation of gene expression, cytoskeletal reorganisation, cell cycle arrest, the immune response and cell death (New and Han 1998).

In skeletal muscle, increased p38 activity has been reported in many pro-catabolic diseases. The role of p38 in sepsis mediated muscle protein breakdown revealed from, TNF-α and IL-1, known mediators of muscle wasting (Jackman and Kandarian 2004) stimulates the activity of p38 and enhances the nuclear translocation of NF-kB (Zarubin and Han 2005). Increased p38 activity associated with increased expression level of the atrogin-1 mRNA (Li *et al.*, 2005), whereas, the increase of nuclear translocation of NF-kB is associated with increased expression of MuRF1 mRNA (Cai *et al*., 2004). In contrast, inhibition of the p38 activity significantly decreased the TNF-α-induced up-regulation of atrogin-1 mRNA not MuRF1 (Jin and Li 2007). Therefore, the inhibition of p38 is considered as one of the most therapeutic targets to lessen or minimise the sepsis induced muscle protein breakdown (Fig. 1-5).

1.13.4.2 ERK1/2 and muscle protein metabolism

The MAPKs signalling factors include two closely related MAP kinases, known as extracellular-signal-related kinase 1 (Erk1, p44) and 2 (Erk2, p42). Activation of these Erks is involved in the regulation of many cellular functions including cellular proliferation and differentiation (Olson 1992; Pages *et al.,* 1993). The activation of the Erk pathway has been recognised as a part of the early biochemical events that are initiated by LPS treatment of macrophages or their infection by virulent and attenuated *Salmonella* strains (Procyk *et al.,* 1999). It has been reported that, the activation of p38 and Erk occurred within 15 min after stimulation with LPS, and rapidly returned to normal basal levels within 30 min (Cho *et al.,* 2007). Increased Erk activity has been reported in response to TNF-α, a catabolic factor that activated nearly in all catabolic conditions (Tantini *et al.,* 2002). In contrast, it has been shown that, p38 and Erk activation was involved in the PS process *in vitro* mediated via Mnk1 (MAPK-integrating kinase1/ MAPK-interacting kinase) (Fukunaga and Hunter 1997; Waskiewicz *et al*., 1997; Scheper and Proud 2002). Moreover, Plaisance and associates demonstrated that activation of Erk1/2 and its downstream target Mnk1 mediated the TNF-α-induced protein synthesis in C2C12 myotubes (Plaisance *et al.,* 2008). Although, the physiological function of Mnk1 remains relatively unknown, however, several studies have demonstrated that one of the Mnk1 substrates is the eIF-4E (Gingras *et al*., 1999). Combined, this suggests that activation of Erk can modulate many cellular processes such as protein synthesis and protein degradation. Taken together, the MAPKs (p38 or Erk1/2) signalling is considered as one of the signalling cascade involved in LPS-induced muscle wasting. Moreover, inhibition of their activities can be considered as a therapeutic intervention in sepsis-induced muscle wasting.

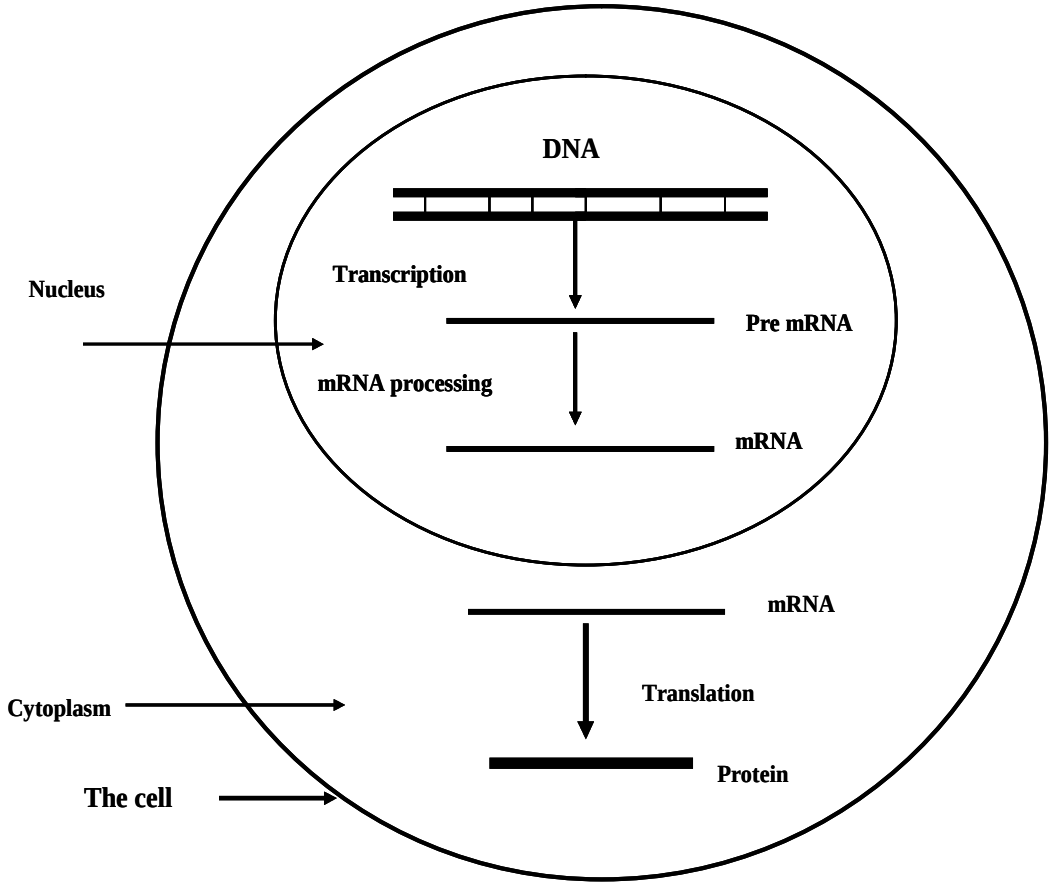


Figure (1-3) Schematic diagram illustrates the transcriptional and translational regulation of protein synthesis. DNA transcribed into mRNA in the nucleus, and mature mRNA leaves the nucleus and then translated into a protein in the cytoplasm. LPS-induced decreased protein synthesis via the alteration of protein translation initiation.

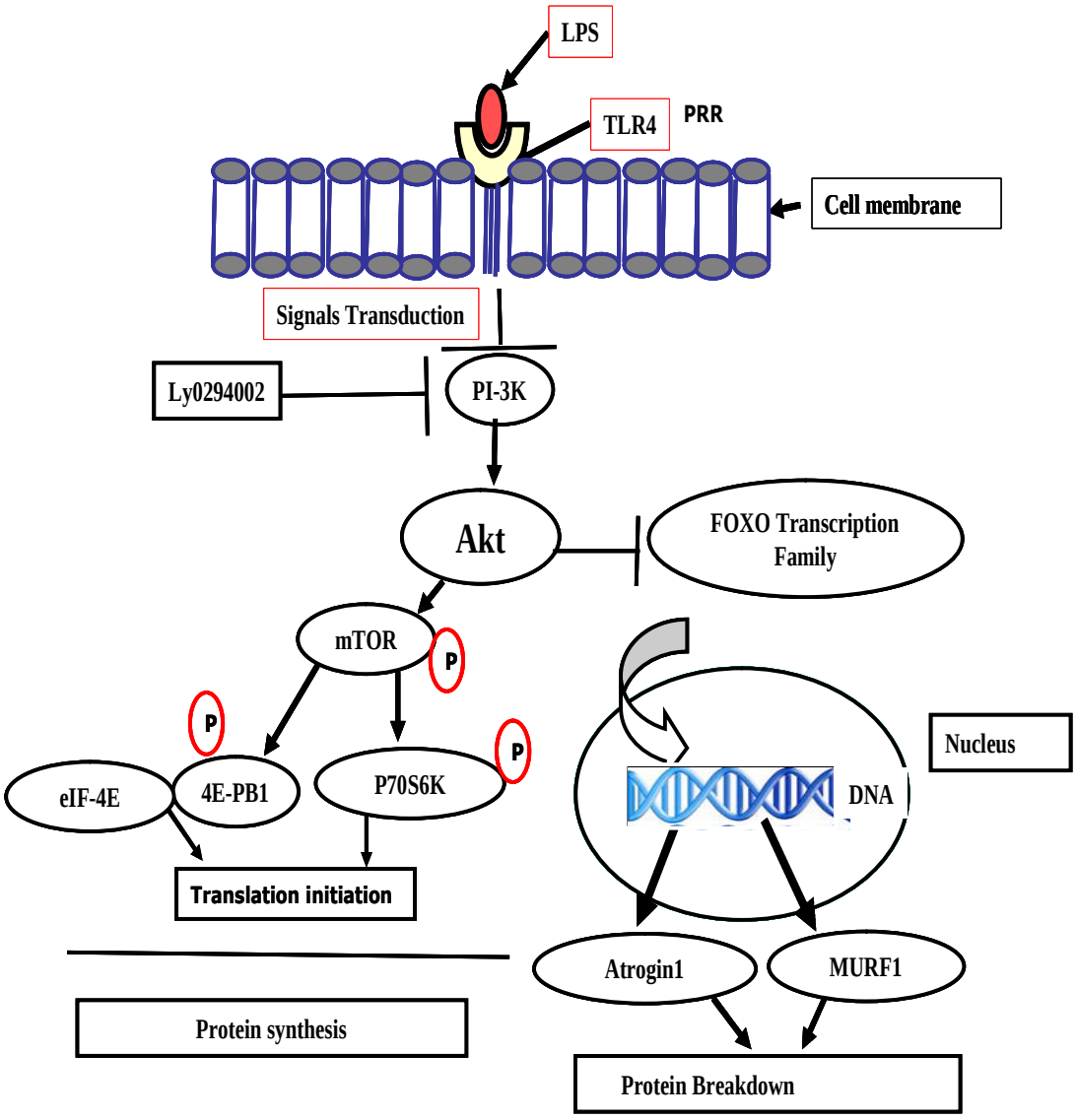


Figure (1-4) Schematic diagram shows the signalling pathways that control the protein synthesis and degradation. LPS-TLR4 legend formation followed by signal transduction. LPS decreased Akt activity. Decreased Akt activity altered the protein synthesis via decreased phosphorylation of mTOR and its downstream targets $p70^{S6K}$, 4E-BP1 (translation initiation mediators). Decreased phosphorylation of 4E-BP1 inhibits the formation of eIF-4F complex and consequently decreased the translation initiation. In contrast, decreased Akt activity significantly increased protein degradation via up-regulation of the ubiquitin ligases (atrogin-1 and MuRF1), and this is regulated via Foxo-dependent way. This diagram is adopted from (Glass 2005).

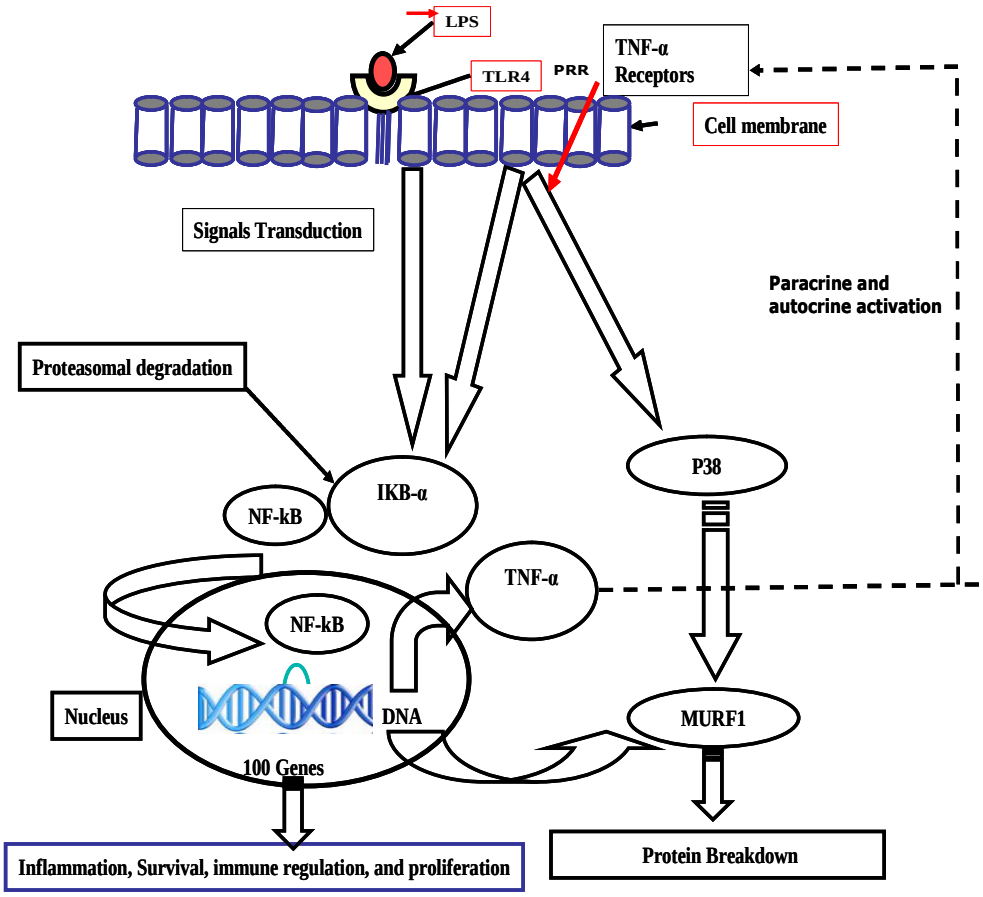


Figure (1-5) Schematic diagram showing the regulation of protein degradation in particular TNF-α signalling pathway and the involvement of I*k*B-α, NF-kB and ubiquitin ligases (atrogin-1 and MuRF1). LPS via TLR4, enhanced p38 phosphorylation and TNF-α secretion. TNF-α mediated protein turnover via p38 mediated atrogin-1 up-regulation and via I*k*B/NF-kB signalling cascade it mediates the MuRF1 mRNA up-regulation. TNF-α secreted has a paracrine and autocrine functions. The activation of the ubiquitin ligases (MuRF1) leads to acceleration of the muscle protein breakdown rate.

1.14 LPS significantly increases the protein degradation in cultured myotubes

Muscle wasting is one of the well-known features of severe acute conditions such as sepsis, cancer, AIDS, (Hasselgren 1995; Tisdale 1997). Sepsis is an inflammatory condition that causes severe and rapid loss of body protein, much of which originates from skeletal muscle (Rosenblatt *et al.,* 1983), and other catabolic diseases are well characterised by a reduction in the rate of muscle protein synthesis and an increase in the rate of protein breakdown (Jepson *et al.,* 1986; Hummel *et al.,* 1988; Ash and Griffin 1989; Hasselgren *et al.,* 1989; Gracey *et al.,* 2004).

Loss of lean body mass results in the main from accelerated protein degradation via the ubiquitin-proteasome pathways (Jagoe and Goldberg 2001). The degradation of the protein by these mechanism started with marking of the targeted protein particle by a chain of ubiquitin molecules, and later degrading of the protein in the central core of 26S proteasome (Hershko and Ciechanover 1998). Selecting a particular protein to be ubiquitinated is primarily the function of ubiquitin ligases (E3 proteins). There are three E proteins known to regulate ubiquitin conjugation in catabolic states: E3α, atrogin-1 and MuRF1. Atrogin-1 and MuRF1 are rate limiting for muscle protein loss in many catabolic conditions. The expression of those two genes is up-regulated in various models of muscle atrophy, in particular sepsis (Bodine *et al.,* 2001). Individual knockout of atrogin-1 or MuRF1 results in attenuation of denervation-induced muscle atrophy (Bodine *et al.,* 2001). This suggests that atrogin-1 and MuRF1gene are crucial to muscle protein loss, and makes these genes as important targets for therapeutic intervention in order to lessen the adverse outcomes of sepsis mainly morbidity and mortality (Jin and Li 2007).

Concentrated research has been done to elucidate of the signalling cascade that control the expression of these two ubiquitin ligases. Akt is one of the most studied pathway, Akt regulates the activity of the Foxo, Foxo a transcription factor family has been shown to control atrogin-1 and MuRF1 expression (Glass 2005). Various anabolic and catabolic signals either activate or suppress Akt. Anabolic signals such as IGF-I activate Akt, resulting in the suppression of atrogin-1 and MuRF1expression. Conversely, catabolic signals such as glucocorticoids suppress the Akt activity resulting in the up-regulation of atrogin-1 and MuRF1 expression (Lai *et al.,* 2004; Sandri *et al.,* 2004) (Fig. 1-4). Moreover, NF-kB, an inflammatory stimulus-activated transcription factor that mediates muscle protein through multiple components of the ubiquitin proteasome pathway the up-regulation of MuRF1 expression (Li and Reid 2000; Li *et al.,* 2003). Recently, TNF-α via p38 MAPK-dependent mechanism up-regulated atrogin-1 mRNA in C2C12 myotubes and inhibition of p38 blocked this effect (Li *et al.,* 2005). p38 increased activity has been reported in a number of pro-catabolic states (Childs *et al.,* 2003; Koistinen *et al.,* 2003; Di Giovanni *et al.,* 2004). Take in consideration the fact that p38 and NF-kB are activated by TNF-α and IL-1β, two known mediators of muscle catabolism (Jackman and Kandarian 2004). Moreover, the inflammatory conditions induce oxidative stress through the enhancing the production of free radicals such as NO (nitric oxide) (Buck and Chojkier 1996) which have been linked to the pathogenesis of muscle wasting. The mechanism how nitric oxide (NO) mediates muscle wasting remains largely unknown but previously shown that LPS stimulate the activity of NoS (Griffiths *et al.,* 1995; Ravindranath *et al.,* 2003). Nitric oxide (NO) is involved in both inflammatory and non inflammatory-induced muscle catabolism through either decreasing protein synthesis or enhancing proteolysis.

1.15 Signalling pathways involved in protein degradation

There are many signalling cascades and transcription factors families involved in sepsis-induced muscle protein turnover. These factors includes, TNF-α, NF-kB, IL-6 and proteasome.

1.15.1 The role of TLR4 in the LPS signals transduction

Skeletal muscle can respond directly to various microbial components during the course of infection. These molecules represent the pathogen-associated molecular patterns (PAMPS) that can be recognised by the host defence system (Netea *et al.,* 2004). TLRs are a transmembrane signalling proteins considered to be the main class in recognising of the PAMPS (Underhill and Ozinsky 2002; Kopp and Medzhitov 2003). TLR considered as the first line of defence against bacterial cell components, such as LPS, lipopeptides, as well as RNA and DNA from viruses and proteins from protozoa in a relatively specific manner (Leaver *et al.,* 2007). Mouse C2C12 cell line and the mouse skeletal muscle express the mRNA of TLR1 through TLR4 and not TLR9 or TLR10 (Frost and Lang 2008). TLR4 is an essential component in the host defence through the stimulation of cytokines production. LPS and heat-shock proteins (Hsps) are recognized by TLR-4 (Akira and Hemmi 2003). The recognition of LPS requires TLR4 and TLR4-accessory molecule, MD-2 (Shimazu *et al.,* 1999). The interaction of the PAMPS with the TLR-4 (TLR-PAMP interaction) leads to activation of the nuclear factor (NF-kB), a critical component in many pro-inflammatory pathways and the innate host defence, through the tumor necrosis factor (TNF) receptor–associated factor-6 (TRAF6) signalling pathway in the presence of specific adaptors (MyD88 and Mal) (Akira 2000). This interaction of TLR4 with LPS leads to production and release of many catabolic factors including TNF-α and NF-kB.

1.15.2 The role of TNF-α in sepsis-induced muscle protein breakdown

Tumour necrosis factor-α (TNF-α) is a pro-inflammatory cytokine, was originally called cachectin, and is an intensively studied cytokines plays an indispensable role in the host defence against invading pathogens (Tisdale 1997). Also, high levels of TNF-α have been reported in most catabolic states (Garcia-Martinez *et al.,* 1995), and TNF-α over expressing in transgenic animals are associated with accelerated rate of protein degradation (Kubota *et al.,* 1997). In contrast, administration of anti-TNF-α antibodies or of TNF-α soluble receptor attenuates this catabolic response (Sharma *et al.,* 1992; Ksontini *et al.,* 1998). It has been shown that, TNF-α directly stimulates muscle protein loss in cultured myotubes (Li *et al.,* 1998), and decrease protein synthesis (Lang *et al.,* 1996; Cooney *et al.,* 1999). In addition, the direct infusion of TNF-α decreases the protein synthetic rate (Cooney *et al.,* 1999; Lang *et al.,* 2002). TNF-α generates its catabolic effect through various ways. TNF-α acts directly on muscle via its receptors to induce muscle catabolism (Langen *et al.,* 2001; Ladner *et al.,* 2003; Lang and Frost 2005) via the up regulation of the ubiquitin-proteosome pathway in particular atrogin-1 (Li and Reid 2000). TNF-α can stimulate skeletal muscle catabolism either by modifying hormones that regulate protein turn over such as IGF-I (Tracey *et al.,* 1990; Tracey and Cerami 1993; De Rossi *et al.,* 2000), or by increasing the production of other cytokines (Tracey and Cerami, 1993) or by inducing anorexia (Tracey *et al.,* 1990). Stimulation of myotubes with TNF-α, leads to stimulation of Reactive Oxygen Species (ROS) production in skeletal muscle fibres (Alisantosa *et al.,* 2000) and activates redox-sensitive transcription factors and protein kinases such as, NF-kB and MAPKs (P38, Erk1/2 and JNK) (Garg and Aggarwal 2002). TNF-α induces up-regulation of UbcH2 gene ($E2_{k20}$ protein) mediated via NF-kB (Li *et al.,* 2003), and atrogin-1 mRNA via p38 MAPK dependent mechanism (Li *et al.*, 2005). Furthermore, high concentrations of TNF-α inhibit PI3-K and consequently its downstream targets (Hotamisligil *et al.,* 1996), this later increases the expression of atrogin-1 mRNA through Akt/Foxo4-dependent mechanism (Moylan *et al*., 2008). In contrast, TNF-α inhibits insulin stimulated protein synthesis via

Erk1/2 dependent ways (Williamson *et al.,* 2005). This effect was mediated via the regulation of binding of capped mRNA to the 43S preinitiation complex. TNF-α is involved in the sepsis-induced 80% increase of the inactive eIF4E 4E-BP1 complex and 65% decrease in the eIF4E eIF4G active complex (Lang and Frost 2007). Thus, TNF-α is considered as a key element regulating the translation initiation and the protein synthesis, and this effect is still unchanged even when septic rats were administrated insulin (Vary *et al.,* 2001).

Although the previous mentioned studies have shown that TNF-α is a strong catabolic stimulus significantly decreasing protein synthetic rate and increased protein turnover either *in vivo* or *in vitro*. However, Plaisance *et al*. (2008) have demonstrated that incubation of C2C12 myotubes with TNF-α significantly increased protein synthesis mediated either via Erk1/2-Mnk1-eIF-4E signalling cascade, or via Akt and its stimulation targets, GSK-3β, $p70^{S6K}$ and 4E-BP1, without changing the protein breakdown rate and with out changing cell number.

1.15.3 The potential role of NF-kB and I*k*B-α involvement

NF-kB is a transcriptional factor family involved in the regulation of many cellular functions such as, innate immune response, cellular proliferation and differentiation and cell survival (Baldwin 1996). NF-kB, is more classically involved in the transcriptional regulation of many genes involved in the immune response, cell growth and cell death (Li *et al.,* 1998). NF-kB activation is defined as a momentary response mainly initiated by the phosphorylation and subsequent degradation of the NF-kB inhibitory protein (IKB-α) leading to nuclear import of NF-kB, and accumulating with the resynthesis of the I*k*B-α and subsequent activation of the transcription factor (Ladner *et al.,* 2003) (Fig. 1-5). LPS stimulates the degradation of The I*k*B-α in C2C12 (Frost *et al*., 2002; Frost *et al*., 2003), but I*K*B-α is rapidly resynthesized to moderate the response of NF-kB. The rapid (with in 4 h)

activation of NF-kB in septic muscle during suggesting that NF-kB plays a role in the pathogenesis of this (Penner *et al.,* 2001). Sepsis increased the activity of NF-kB (Penner *et al*., 2001), and NF-kB blockade significantly decreased the muscle protein breakdown in C2C12 (Li and Reid 2000). NF-kB activation mediated cytokines-induced skeletal muscle protein loss (Baldwin 1996; Ladner *et al*., 2003). Moreover, the transient activation of NF-kB is responsible for the predominant early response of the rat to the LPS (Penner *et al.*, 2001; Frost *et al*., 2003). In addition, TNF-α stimulates the activity of NF-kB (von Haehling *et al.,* 2002), and this inhibits the myocyte differentiation in C2C12 cells (Guttridge *et al.,* 2000; Langen *et al.,* 2001; Ladner *et al.,* 2003). Increased NF-kB activity in isolated muscles is associated with increased the muscle atrophy via increased the amino acids excretion and tyrosine turnover (Cai *et al*., 2004). NF-kB mediated sepsis-induced muscle breakdown via up-regulation of MuRF1 not atrogin-1, and NF-kB inhibition significantly decreased the proteasomal expression associated with muscle atrophy (Wyke *et al.,* 2004).

In summary, NF-kB is an important transcription factor significantly increased in most catabolic conditions which mediates muscle protein turnover via MuRF1 up-regulation. This makes NF-kB a therapeutic target to minimise the adverse consequences of sepsis.

1.15.4 The role of IL-6 in sepsis-induced muscle protein degradation

Interleukin-6 (IL-6) is an important cytokine that possessing both pro- and anti-inflammatory properties (Ikemoto *et al.,* 2000) and is mainly secreted from classical immune tissues such as, liver, spleen (Luo *et al.,* 2003), and non-immune tissues including skeletal muscle with various local and systemic effects. The role of IL-6 in the regulation of muscle protein degradation in various muscle wasting conditions is still controversial. Some studies showed no role (Garcia-Martinez *et al.,* 1994; Williams *et al.,* 1998), whereas others proved its contribution (Strassmann *et al.,* 1992; Haddad *et*

al., 2005). IL-6 produced locally in muscle may play a role in the regulation of acute phase protein production in liver (Luo *et al*., 2003). Skeletal muscle produces and releases many cytokines including IL-6 in response to various stimuli including LPS. LPS and TNF-α increased the expression of IL-6 mRNA in a way mediated via the increased activity of NF-kB (Zhang *et al.,* 1990; Dendorfer *et al.,* 1994). Recently, reactive oxygen species (ROS) significantly increased the expression and release of IL-6 mRNA especially in differentiated myotubes, and the activation of p38 and I*K*B-α and subsequent activation of NF-kB is essential for the ROS-IL-6 release (Kosmidou *et al.,* 2002). Although, the precise role of IL-6 in skeletal muscle *in vitro* is still unclear. However, it has been shown that IL-6 enhances insulin-stimulated glucose uptake (Stouthard *et al.,* 1996) and glycogen synthesis (Weigert *et al.,* 2004; Weigert *et al.,* 2005) in myotubes via activation of AMP-activated protein kinase (AMPK) (Carey *et al.,* 2006). How the inflammatory stimuli including TNF-α, IL-1β and LPS stimulate the IL-6 production and the signalling cascades involved in this mechanism is showed in (Fig. 1-6).

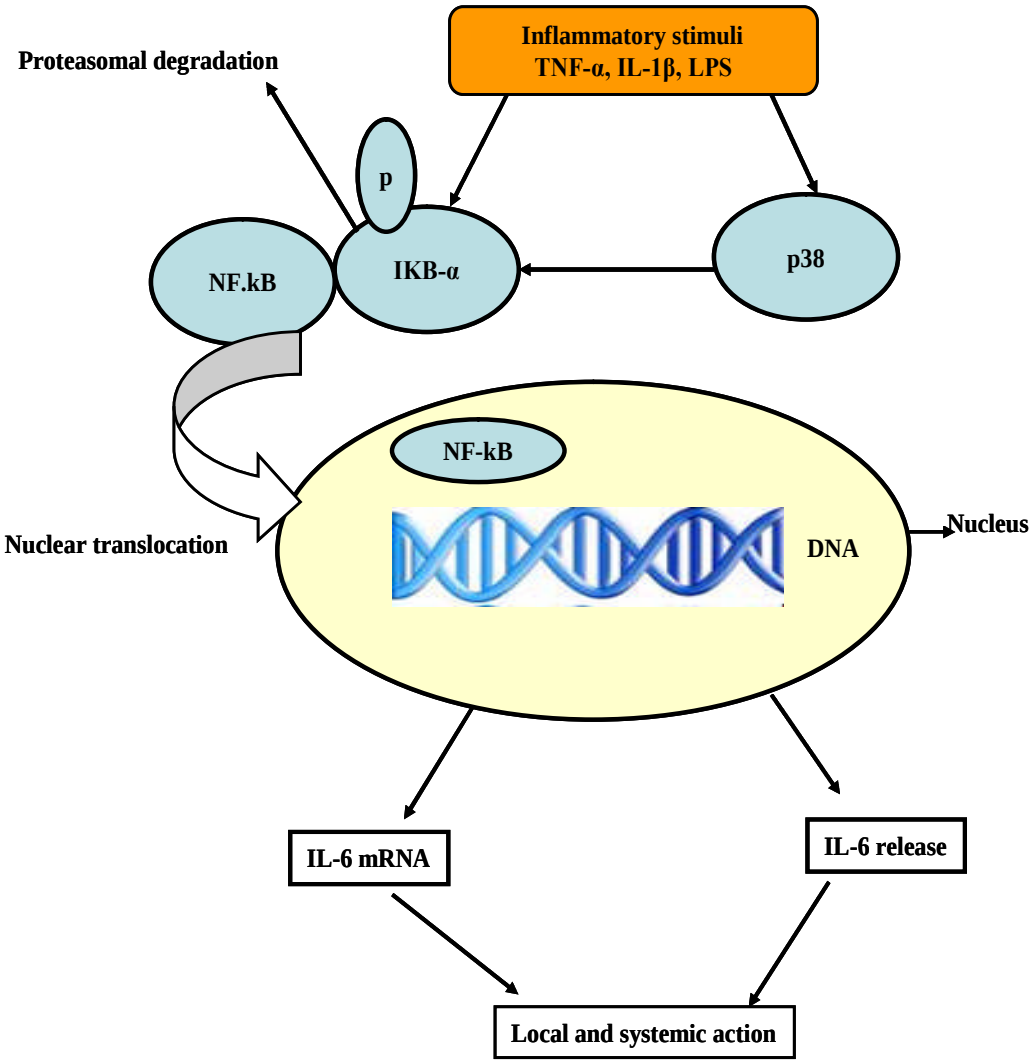


Figure (1-6) Schematic diagram illustrates the possible mechanism of IL-6 activation. LPS and TNF-α significantly increased the phosphorylation of p38 and I*k*B-α, and later enhanced the nuclear translocation of NF-kB. NF-kB enhanced the transcription of many genes including IL-6. IL-6 secreted locally at skeletal muscle has many local and systemic actions.

1.15.5 The role of the proteasome in the skeletal muscle protein degradation

There is a balance between the rate of the rate of muscle protein synthesis and the rate of muscle protein breakdown, and this balance determine the skeletal muscle growth (Goll *et al.,* 1992). In skeletal muscle, one of the main mechanisms involved in the atrophying process is the activation of the proteolytic pathways (Dean *et al.,* 1997; Grune *et al.,* 1997; Chevion *et al.,* 2000). The protein degradation in the eukaryotic cells requires the activation of one or more of the major four catabolic systems, the caspases, cathepsins, calcium-dependent calpains, and the ubiquitin-proteasome system (UPS).

1.15.5.1 Caspase-mediated protein degradation

Caspases, or cysteine-aspartic proteases or cysteine-dependent aspartate-directed specific proteases are a family of cysteine proteases that specifically cleave the C-terminal bond of aspartate residues, which plays an essential role in many cellular processes including, apoptosis, necrosis and inflammation For review see (Chang and Yang 2000). There are three classes of caspases exist; initiator caspases, capable of activating the procaspases; effector caspases, responsible for the proteolysis of cellular proteins (Buendia *et al.,* 1999); and inflammatory caspases, aiding in the formation of cytokines (Wang *et al.,* 1998). The precise role of caspases in regulation of skeletal muscle proteolysis is still relatively unclear. However, it has been recently shown that caspase-3 plays an important initial role in the skeletal muscle protein degradation via cleaving of actomyosin *in vitro* (L6 myotubes) and in gastrocnemius muscle of rat (Du *et al.,* 2004). In addition, this study has shown that inhibition of caspase-3 activity suppresses acute diabetes-induced accelerated muscle proteolysis and the accumulation of actin fragments. Furthermore, mice deficient in caspase-3 were protected from

the denervation induced muscle atrophy (Plant *et al.,* 2009). Similarly, in C2C12 myotubes, attenuation of the activity of caspase-3 and caspase-8 significantly decreased the protein degradation and increased the protein synthesis: for more details see (Russell *et al*., 2010). In contrast, Wei *et al* demonstrated that, the mRNA and processed active caspase-3 activity are still unchanged in skeletal muscle after a septic challenge (Wei *et al.,* 2005). In conclusion, although the most important catabolic pathway activated in most cases of muscle protein breakdown is the ubiquitin-proteasome pathway (Lecker *et al.,* 1999). However, the contribution of the caspase still exists and further studies are required both *in vivo* and *in vitro* to speculate this role.

1.15.5.2 Lysosomes-mediated protein degradation

Cathepsins also referred to as lysosomal proteolytic enzymes, a member of the lysosomal cysteine protease family. The cathepsins members include, cathepsins A, G (serine protease), cathepsins D, E (aspartic protease) and cathepsins L. Elevated cathepsins enzyme levels in serum have been linked to many pathological conditions including Alzheimer's, cancer and arthritis. Although, the limited input of the lysosomal pathway in muscle protein breakdown in case of sepsis. However, it has been shown that the activation of the lysosomal enzymes and the ubiquitin proteasome pathways may be involved in the sepsis-induced muscle protein turnover (Drew *et al.,* 1988; Mitch and Goldberg 1996). In addition, Helliwell and associates have shown that increased labelling for lysosomal enzymes and ubiquitin have been seen in fibre atrophy and the collapse of cytoskeleton (Helliwell *et al.,* 1998). Furthermore, elevated level of the cathepsins mRNA has been reported in many catabolic conditions including sepsis (Hummel *et al.,* 1988; Voisin *et al.,* 1996; Deval *et al.,* 2001). In contrast, incubation of septic rat skeletal muscle with curcumin and cathepsin L inhibitor significantly decreased the cathepsin activity by 40% compared with the control group (Poylin *et al.,* 2008). Although, the findings of Deval *et al*. (2001) with the

other's findings are encouraging the use of the elevated cathepsin L mRNA as a clinical marker of muscle loss, yet the potential role of cathepsins in sepsis induced muscle breakdown *in vitro* needs further investigation.

1.15.5.3 Calpain-mediated protein degradation

The major catabolic pathway activated in most catabolic cases is the proteasome pathway and is reviewed in detail later. The accelerated rate of muscle protein breakdown has been prevented by inhibition of the activity of 20S proteasome (Tawa *et al.,* 1997; Jagoe *et al.,* 2002). Although, proteasome can degrade many proteins within the muscle, yet the proteasome was unable to degrade the intact myofibrils, while the protease activity of ubiquitious calpains μ-calpain and m-calpain was able (Huang and Forsberg 1998). The calpains are a family of calcium-dependent, non-lysosomal cysteine proteases. The expression of the two ubiquitous calpains, μ-calpain and m-calpain has been nearly detected in all tissues, and they play a pivotal role in many cellular functions including cell differentiation (Cottin *et al.,* 1994), cell cycle (Raynaud *et al.,* 2004), cell migration and adhesion (Glading *et al.,* 2002; Franco *et al.,* 2004). Considered as targets of calpain increased activity, several studies demonstrated that increased calapin activity affects the activity of several kinases, phosphatases, membrane associated proteins and transcription factors for more details see (Goll *et al.,* 2003). *In vitro* activation of calpains initiated the degradation of many myofibrillar proteins including desmin, filamen, C-proteins, tropomysin, troponin T, troponin I, titin and nebulin (Koohmaraie 1992). In contrast, calpains do not degrade the α actin, α actinin, myosin heavy chain (Tan *et al.,* 1988). Inhibition of m-calpain was associated with a 30% reduce in skeletal muscle protein degradation full reviewed in (Huang and Forsberg 1998).

The potential role of the calapin in the sepsis induced muscle wasting has been demonstrated in many studies (Bartoli and Richard 2005; Costelli *et al*., 2005; Smith *et al*., 2008). Sepsis increased the cellular concentrations of

Ca^{2+} (Fischer *et al.,* 2001), and calpain has been shown to be activated by calcium (Goll *et al.,* 2003). The activated calpain cleaved the myofibrils and released the myofilaments which later ubiquitinated and degraded by the 26S proteasome (Solomon and Goldberg 1996; Huang and Forsberg 1998; Solomon *et al*., 1998; Hasselgren and Fischer 2001). Therefore, the activation of the calpain is considered as the initial step for the activation of 26S proteasome (Menconi *et al.,* 2004; Smith and Dodd 2007; Smith *et al.,* 2008). In conclusion, although the previous studies have shown the importance of the caspases, cathepsins and calcium-dependent calpains in sepsis-mediated protein breakdown, yet the ubiquitin proteasome pathway is still the most important proteolytic system activated in response to many muscle wasting conditions.

1.15.5.4 Ubiquitin-proteasome pathway

Proteasome is present in the nucleus and the cytosol of all eukaryotic cells examined and within the cytoskeleton (Scherrer and Bey 1994). It represents 1% of the cell protein (Tanaka *et al.,* 1986). The degradation of muscle protein in eukaryotic cells was mediated by several specialised systems, mainly the ubiquitin-proteasome system (UPS), which is the most active system. In general, the proteasome is a multicatalytic high molecular weight protease complex responsible for ubiquitin-dependent protein degradation within the cells fully reviewed in (Peters 1994; Coux *et al.,* 1996; Hilt and Wolf 1996). The proteasome is able to do complete proteolysis of protein substrates and activation of protein function through limited proteolytic process (Palombella *et al.,* 1994). The physiologically active form of the ubiquitin-proteasome system is the 26S proteasome. 26S proteasome is a large proteolytic complex that hydrolyses protein conjugates, mainly composed of 19S cap structure attached to 20S core (Hayter *et al.,* 2005). The 20S (700kDa) proteasome is the active proteolytic machinery in which the proteolysis takes place; 20S can degrade peptide and certain protein substrates independently of the presence of ATP (Fig. 1-7). The 20S

proteasome thought to have five main catalytic peptidase activities, the trypsin-like, chymotrypsin-like, peptidylglutamyl peptidase, branched-chain amino acid-preferring, and small neutral amino acid-preferring activities (Orlowski *et al.,* 1993), amongst the chymotrypsin- like activity is the most important (Fenteany and Schreiber 1996). Increased ubiquitin-proteasome activity has been observed in many catabolic diseases including sepsis (Jin and Li 2007). In addition, in muscle atrophy the unregulated ubiquitin proteasome activity was significantly blocked by the proteasomal inhibitor (MG-132) (Tawa *et al.,* 1997). The most important ubiquitin proteasome system involved in the degradation of skeletal muscle protein is the ubiquitin ligases (Hershko and Ciechanover 1998). Further details about the involvement of the ubiquitin ligases in the process of muscle protein breakdown are in the following part about the role of atrogin-1 and MuRF1.

1.15.6 Skeletal muscle protein degradation from LPS to atrogin-1 and MuRF1

Loss of lean body mass results predominantly from accelerated protein degradation rate via the ubiquitin-proteasome pathways (Jagoe and Goldberg 2001). Selecting a particular protein to be ubiquitinated is primarily a function of ubiquitin ligases (E3 proteins). The ubiquitin ligases protein (also called an E3 ubiquitin ligases) is a protein in combination with an E2 (Ubiquitin-conjugating enzyme) which causes the attachment of ubiquitin to a lysine on a target protein substrates for degradation by proteasome. The E3 proteins are a rate limiting for ubiquitin conjugation (Bodine *et al.,* 2001). The E3α proteins, atrogin-1 and MuRF1 are rate limiting for muscle protein loss in many catabolic conditions. Increased expression of E3α proteins has been shown in sepsis (Voisin *et al*., 1996; Fischer *et al*., 2000). The activity of atrogin-1 and MuRF1 is reciprocal to the activity of Foxo transcription family, and Akt activity controls the Foxo transcription family activity. Decreased activity of Akt in C2C12 myotubes was shown to activate (dephosphorylates) Foxo transcription family, which in turn increases the activity ubiquitin E3

ligases mainly atrogin-1 and MuRF1 (Dehoux *et al*., 2003; Sandri *et al*., 2004; Stitt *et al*., 2004) and vice versa (Royle *et al*., 2003; Sandri *et al*., 2004).

LPS injection was associated with a dramatic increase in the expression of muscle genes atrogin-1 and MuRF1, and mice harbouring mutation in both atrogin-1 and MuRF1 are resistant to denervation-induced muscle atrophy (Bodine *et al.,* 2001). Interestingly, the both groups of knockout mice appeared phenotypically normal with normal growth patterns, weight, and morphology under normal conditions (Bodine *et al.,* 2001). This gives the evidence that both atrogin-1 and MuRF1 are pivotal for muscle degradation process across the various catabolic conditions (Lecker *et al.,* 2004). Many studies concerning the regulation of ubiquitin proteasome system reported that TNF-α and IGF-I play a pivotal role in its regulation.

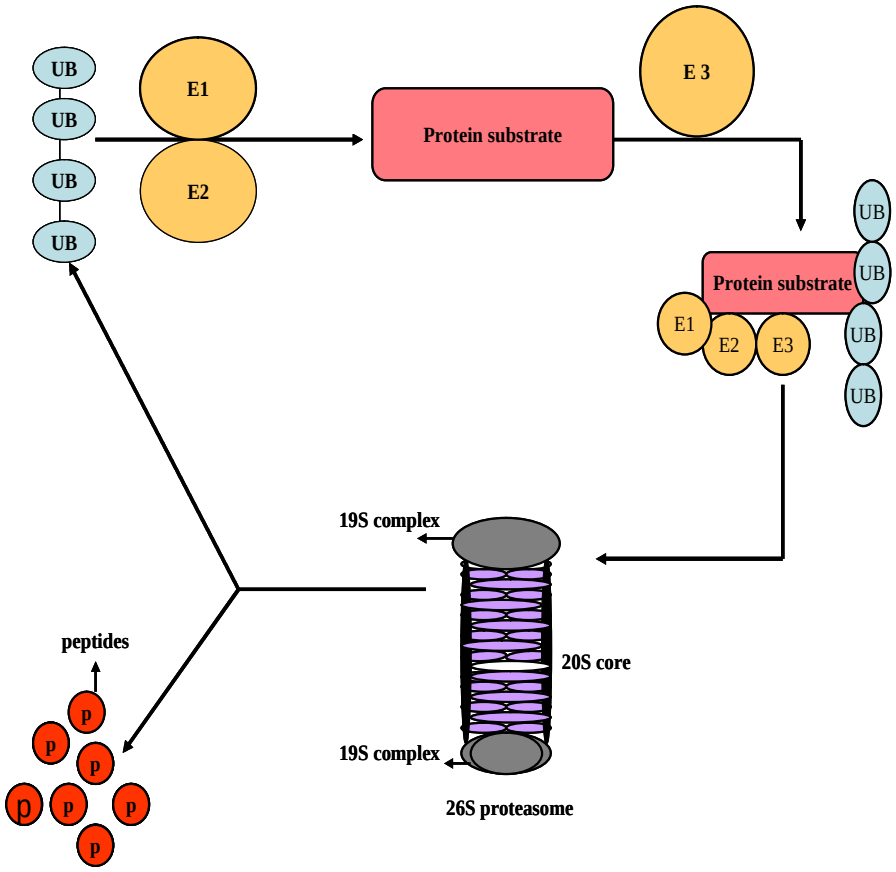


Figure (1-7) Schematic diagram showing the process of protein ubiquitination and subsequent degradation in the 26S proteasome. The protein of interest labelled with four ubiquitin molecules and later it degraded in the central core of 26S proteasome. This process fully controlled by the activity ubiquitin ligases protein (atrogin-1 and MuRF1). The figure is adopted from (Kessler 2006).

1.15.6.1 Atrogin-1/ MAFbx (Muscle atrophy F box)

The 42KDa protein atrogin-1 is a muscle specific ubiquitin ligase plays a fundamental role in muscle proteolysis (Bodine *et al.,* 2001). Atrogin-1 is a part of Skp, Cullin and F-box (SCF) containing complex group of ubiquitin ligases (Gomes *et al.,* 2001), that catalysing the ubiquitination of proteins destined for proteasomal degradation (Deshaies 1999). The linking of protein substrate with the rest of ubiquitin ligases is the responsibility of the F-box protein (Cardozo and Pagano 2004). While, SKp1 is required for stabilising of this link *in vivo* (Gomes *et al.,* 2001). The presence of atrogin-1 sequences in the muscle nucleus suggests the role of atrogin-1 as a cell-signalling molecule added to its role in muscle proteolysis (Sandri *et al*., 2004). The atrogin-1 controls the activity of many substrates particularly, calcineurin A (Li *et al.,* 2004) and MyoD (Tintignac *et al.,* 2005), which are integrated in the maintenance or restoring of muscle mass. Calcineurin A is responsible for the activation of nuclear factor activated T cell (NFAT) family members and leads to muscle hypertrophy (Li *et al.,* 2004). MyoD is a muscle specific transcription factor required for skeletal muscle differentiation (Edmondson and Olson 1993; Kitzmann *et al.,* 1998; Tintignac *et al.,* 2004) and myogenic stem cell function in skeletal muscle (Megeney *et al.,* 1996). MyoD initiates the expression of muscle specific genes and consequently cells start entering a myogenic lineage (Weintraub 1993). The atrogin-1 degrades the MyoD in proliferating myoblasts (Tintignac *et al.,* 2005). Recently, it has been demonstrated under the experimental conditions proposed by Lagirand-Cantaloube and associates, that MyoD is a target of atrogin-1 in skeletal muscle atrophy (Lagirand-Cantaloube *et al.,* 2009). Atrogin-1 is the most important ubiquitin ligase that activated in most cases of muscle atrophy (Bodine *et al*., 2001; Gomes *et al*., 2001), and its knockout significantly decreased the muscle protein turnover (Bodine *et al*., 2001). There are many *in vitro* studies regarding the effect of sepsis and other catabolic stimuli on the atrogin-1 mRNA expression and regulation (Li *et al*., 2005; Jin and Li

2007; Murton *et al*., 2009). Whereas, the regulation of atrogin-1 *in vitro* still unclear and needs further investigation. There are many signalling control the activity of atrogin-1 either *in vivo* or *in vitro.* Glass demonstrated that Akt is the key regulator of the atrogin-1 mRNA expression in skeletal muscle mediated via Foxo transcription family (Glass 2005). In addition, TNF-α significantly increased the atrogin-1 mRNA up-regulation mediated via p38 (Li *et al*., 2005; Jin and Li 2007). Yet, there are many hidden aspects regarding the regulation of atrogin-1 activity in skeletal muscle in case of sepsis need further investigation.

1.15.6.2 MuRF1 (Muscle Ring Finger Protein 1)

MuRF1 is a 40KDa ubiquitin ligase exclusively expressed in skeletal and cardiac muscles (Bodine *et al.,* 2001). MuRF1 is mainly composed of RING domain, amino-terminal end and two coiled–coil domains in its central region (Centner *et al.,* 2001). Similar to atrogin-1, MuRF1 plays a role in regulation of cell signalling pathways (McElhinny *et al.,* 2002). Because of the expression of atrogin-1 and MuRF1 governed by the activity of Foxo family of transcription factors. Prolonged sepsis increased the expression of atrogin-1 not MuRF1 mRNA (Frost *et al.,* 2007). In contrast, the activation of NF-kB is associated with the up-regulation of the MuRF1 not the atrogin-1 mRNA (Cai *et al.,* 2004). Thus, the separation between the role of atrogin-1 and MuRF1 and the factors that regulating their activity became increasingly important. MuRF1 plays an important role in the ubiquitination and degradation of titin (single polypeptide, important in the contraction of striated muscle tissues), and may be other proteins in the Z-band resulting in releasing of actin and myosin (Wray *et al.,* 2003). In contrast, mice lacking MuRF1 were protected from denervation-induced muscle atrophy to a lesser degree than mice harbouring a negative mutation in atrogin-1 (Bodine *et al*., 2001). In response to glucocorticoids, MuRF1 interacts with glucorticoids modulatory element binding protein-1(GMEB-1), a nuclear protein regulates transcription in response to glucocorticoids (McElhinny *et al*., 2002). MuRF1 degrades

troponin 1 in cardiomyocytes (Li *et al.,* 2004). The interaction between the MuRF1 and the myofibrillar proteins (titin and troponin) may explain the role of the MuRF1 in muscle atrophy. MuRF1 interacts with the giant muscle proteins such as nebulin and titin (Centner *et al.,* 2001; Witt *et al.,* 2005). Furthermore, MuRF1 plays a role in the maintaining of energy metabolism of skeletal muscle by ubiquitination and degradation of the CK (Koyama *et al*., 2008). LPS-induced toxaemia in rodents resulted in the reduction of total Akt protein levels, decreased cytosolic Foxo1 and Foxo3 phosphorylation, and increased levels of atrogin-1 protein and MuRF1 mRNA in skeletal muscle (Crossland *et al*., 2008). Recently, the LPS-induced toxaemia in rats was associated with increased levels of both atrogin-1 and MuRF1 mRNA in skeletal muscle not in myocardium muscle (Murton *et al.,* 2009).

Taken together, we can conclude that the most important pathway activated in most cases of muscle atrophy is the ubiquitin proteasome pathway. In addition to the direct role of both of atrogin-1 and MuRF1 in the muscle protein degradation, MuRF1 plays an essential role in maintaining the energy metabolism in skeletal muscle. Furthermore, considering the atrogin-1 and MuRF1 as therapeutic targets is one of the most important ways to minimise the sepsis-induced protein breakdown. However, the absolute use of both atrogin-1 and MuRF1 and a unique markers of muscle proteolysis either in vivo or in vitro is considered relatively incorrect for more details reviewed in (Attaix and Baracos 2010).

1.16 LPS stimulates the release of Creatine kinase (CK)

Creatine kinase/phosphocreatine kinase is an enzyme that catalyses the conversion of creatine to phosphocreatine utilizing ATP in a reversible reaction. Although, the mechanism of CK release from the muscle is still unclear. However, CK release has been used as an index of muscle damage in the *in vitro* and *in vivo* studies (Maglara *et al.,* 2003). The relationship between the availability of muscle ATP and increased efflux of CK has been

established (Jackson *et al.,* 1984). Skeletal muscle considered as the most important reserve of ATP and respond to various catabolic stimuli by decreasing the energy consumption. Skeletal muscle lack creatine kinase (CK) exhibited no physiological or pathological changes, but significant alteration of the energy metabolism has been shown (van Deursen *et al.,* 1993; Ventura-Clapier *et al.,* 1995). LPS significantly increased the glucose-6-phosphate, phosphocreatine and ATP in the skeletal muscle within 5 min (Myrvold *et al.,* 1975). To date, the studies concerning the creatine kinase are mainly focused on the relationship between the creatine and the energy metabolism in skeletal muscle. Koyama and associates recently demonstrated that, MuRF1 maintain the energy metabolism of the muscle by ubiquitination and degradation of CK for more details (Koyama *et al.,* 2008). Furthermore, Zhao *et al* showed that MuRF1 ubiquitinated and later degraded the oxidised form of the creatine kinase *in vitro*, this mean that, under abnormal conditions the skeletal muscle decreases the energy consumption mainly via degradation of the creatine kinase mediated most likely via MuRF1 (Zhao *et al.,* 2007).

1.17 LPS-stimulates nitric oxide (NO) production in skeletal muscle

Nitric oxide (NO) is one of the few known gaseous signalling molecules synthesized from the amino acid L-arginine and oxygen in a reaction catalysed by NO synthase enzymes (NOS). NO is an important messenger molecule taking a part in many physiological and pathological processes, for example as an antiproliferative agent for smooth muscle cells (Baumgarten *et al.,* 2001). In skeletal muscle, NO increases the blood flow (Clark *et al.,* 2003) and glucose uptake (Etgen *et al.,* 1997; Bradley *et al.,* 1999). NO is toxic to infectious agents and when it reacts with superoxide to form NO intermediates it can cause tissue damage (Murrant and Reid 2001). Skeletal muscle produces nitric oxide as part of the host immune defence to kill the invading microorganisms, but on the other

hand, excess NO production leads to muscle wasting (Sambe *et al.,* 1998). Skeletal muscle contains three forms of NOS, two of these isoforms, NOS1 and NOS3, are constitutively expressed.

NOS2 (known as inducible NOS (iNOS)) is expressed in skeletal muscle in response to injury and repair stimulated by bacterial cell wall components (Wehling *et al.,* 2001). In response to LPS administration, there is an abundant increase in the NOS2 protein in skeletal muscle fibres between 6 and 48 h (Hussain *et al.,* 1997). Furthermore, restoring the dysfunction induced by LPS in respiratory muscle by NOS inhibitor is also a proof for the role of NOS in muscle wasting (Sambe *et al.,* 1998). Recently, Frost *et al* demonstrated that LPS was able to increase the NOS2 mRNA expression four folds comparing with the control group in both myoblasts and myotubes (Frost *et al*., 2004). The expression of NOS2 mRNA was transient with maximal increase at 6 h and this expression is a TLR4 dependent. Moreover, they showed that the increased NOS2 mRNA expression was mainly through the proteasomal activity and LPS-NOS2 mRNA increased activity blocked by MG-132 (proteasomal inhibitor). Although, there is no precise limit for the NO production that can damage the skeletal muscle, yet further investigation are required in order to lessen the adverse consequences of sepsis.

1.18 General Hypothesis

The previous literatures have demonstrated that the process of sepsis induced muscle protein turnover *in vivo* involves the interaction of many signalling cascades and transcriptional factors. This makes overall anticipation of their role is a hard task requires further investigation. In addition, *in vivo* it has been shown that sepsis induced decreased PS and increased protein breakdown was mediated via decreased translation initiation step of protein synthesis and increased ubiquitin proteasome system activity respectively. In the context, there is no clear data regarding the correlation between the changes in the anabolic and catabolic signalling streams and the changes in

protein synthesis and degradation. In addition, there is no clear collective data about the direct contribution of Akt and MAPKs (p38 or Erk) signalling cascades in this process. Moreover, the scarcity of data regarding the effect of LPS on the primary skeletal muscle cells makes the understanding of this effect is increasingly important. The effect of natural challenge with S*almonella* serotypes (Gallinarium or Enteritidis) is still unclear and requires further investigation. Herein, this project was aimed to obtain a comprehensive idea about the subcellular and molecular regulation of LPS-induced muscle protein turnover in two *in vitro* septic models (C2C12-murine myotubes and avian primary skeletal muscle cells) and how the natural challenge with *Salmonella* serotypes (Gallinarium or Enteritidis) will affect the skeletal muscle gene expression. Furthermore, the beneficial roles of curcumin as a natural compound in sepsis were evaluated. The overall exploitation of this project is the creation of a suitable therapeutic intervention in order to lessen or minimise the adverse effects of sepsis.

1.19 Gaps in the understanding

The previous chapter emphasizes a number of gaps within the literatures regarding the direct effects of the LPS on protein synthesis and protein degradation in both C2C12 myotubes and avian primary skeletal muscle cells.

First, although LPS significantly decreased the PS in septic skeletal muscle (*in vivo*), however, there is no clear data regarding the *in vitro* effect, and the obtained data are relatively contradictory.

Secondly, the direct contribution of the Akt and MAPKs (p38 or Erk1/2) signalling cascades in the LPS-induced muscle protein turnover is still unclear, and there is no collective data about their role in the same model of sepsis.

Thirdly, the transcription regulation of LPS-induced muscle protein turnover and the potential role of Akt and MAPKs (p38 or Erk1/2) signalling cascades require further verification.

Fourthly, there are no clear data about the direct effect of curcumin on protein metabolism (PS and PD) *in vitro*. Curcumin (diferuloymethane), one of the components of spice turmeric (Curcuma longa) that has been used extensively in the traditional Indian medicine to treat many diseases.

Fifthly, there are no clear data about the signalling cascades involved in the LPS-induced NO production in both C2C12 myotubes and avian primary skeletal muscle cells.

Finally, the direct effect of natural challenge with *Salmonella* on mRNA expression levels in chicken skeletal muscle is still largely unclear.

1.20 The study's objectives:

- To measure the direct effect of the LPS on the PS in C2C12 myotubes and the potential role of various signalling cascades including Akt and MAPKs (p38 or Erk1/2) (Chapter 3).
- To verify the effect of LPS on these signalling cascades using specific pathways inhibitors (Chapter 3).
- To assess the direct effect of the LPS on the chymotrypsin-like enzyme activity and the direct involvement of Akt and MAPKs (p38 or Erk1/2) was assessed using specific pathway inhibitors (Chapter 4).
- The transcriptional regulation of the LPS-induced muscle protein turnover and the potential role of the Akt and MAPKs (p38 or Erk1/2) signalling cascades was assessed using specific pathway inhibitors (Chapter 5).
- To assess the beneficial effects of curcumin in case of sepsis (direct effect on the protein synthesis and protein breakdown) (Chapter 6).

- To characterise the effect of LPS on the protein turnover (protein synthesis and degradation) in avian primary skeletal muscle cells (Chapter 7).
- The LPS-induced nitric oxide (NO) production in both (C2C12 myotubes and avian primary skeletal muscle cells) and the potential role of Akt and MAPKs (p38 or Erk1/2) were evaluated (Chapter 8).
- To access the effect of the natural challenge with two *Salmonella* serovars (Gallinarium or Enteritidis) on chicken skeletal muscle protein metabolism *in vivo* (Chapter 9).

2 General Materials and Methodology

2.1 Cell line and animal experiment

2.1.1 C2C12 Murine skeletal muscle myotubes

C2C12 murine myoblasts (Yaffe and Saxel 1977; Blau *et al.,* 1985) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) Foetal Bovine Serum (FBS) and antibiotics (Penicillin and streptomycin and glutamine) (growth medium, GM) in 5% CO_2 at 37 °C. Upon reaching 80% confluence, differentiation was induced by changing the culture medium to DMEM medium supplemented with 2% (v/v) horse serum (differentiating medium, DM). Cells were then maintained in the DM for up to six days, the DM was changed every other day until the formation of myotubes (multinucleated myotubes) was confirmed morphologically using Leica microscope at the day six of differentiation (Li *et al.,* 2008) (Fig. 2-1). Of note, in all the experiments of C2C12 myotubes, cells were treated at the day six of differentiation.

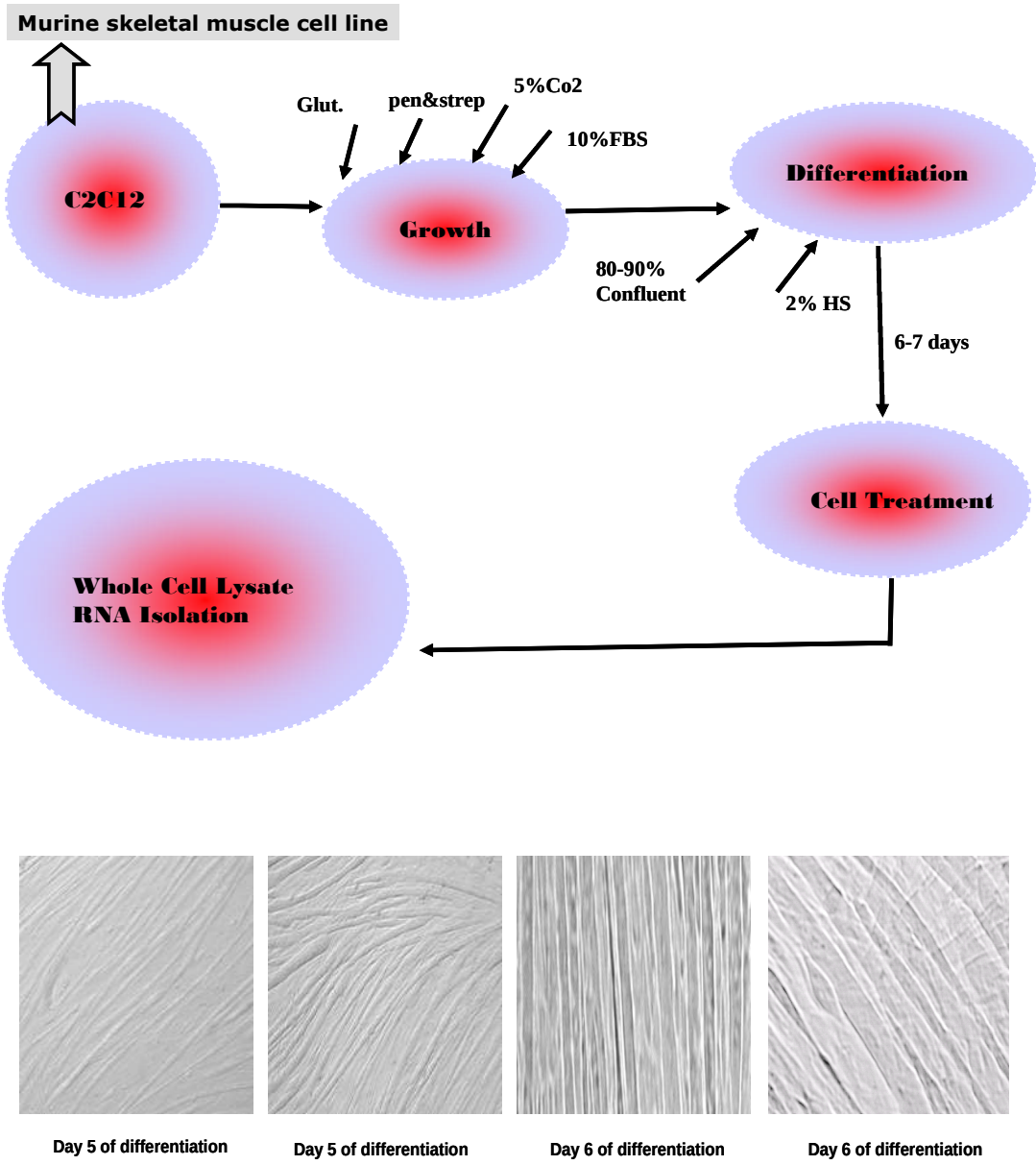


Figure (2-1) Schematic diagram illustrating the process of C2C12 myoblasts growth and differentiation (5 and 6 day of differentiation) and the experimental steps.

2.1.2 Avian primary skeletal muscle cells

Cells were a kind gift from Prof. Kin Chow Chang (SVMS, University of Nottingham). Cells were obtained from 4 weeks old Ross 308 broiler chicks, for further information how these cells prepared see appendix. Cells were grown in GM containing DMEM/F-12 (1:1), supplemented with 10% (v/v) Horse serum (Invitrogen), 4% (v/v) Chicken embryo extract (Biosera), 1% (v/v) Penicillin and streptomycin and 1% (v/v) Insulin-transferrin-selenium (Halevy *et al.,* 2004). The cells were then maintained in this media until the formation of myotubes was confirmed morphologically using Leica microscope. All the experiments were carried out at the day five of differentiation. Further information about how these cells prepared are given in the appendix.

2.2 Cell lysis and Western blotting

C2C12 murine myotubes or avian primary skeletal muscle cells were treated with LPS (10 $ngml^{-1}$, 100 $ngml^{-1}$ and 1 μgml^{-1}) in case of C2C12 myotubes, and with LPS (1 μgml^{-1}) in case of avian primary skeletal muscle cells. Cells were then washed three times in cold Phosphate buffer saline (PBS), then lysed in lysis buffer (76.5 mMTris HCL (pH 6.8), glycerol 10% (v/v), SDS 2% (w/v)) supplemented with 1 mM Sodium metavanedate ($NaVO_3$), 10 mM leupeptin, 1 mM AEBSF (pefabloc^{TM}), supplemented with the protease inhibitors (Roche protease mini cocktail). In case of the NF-kB experiments lysis buffer was used to isolate the cytoplasmic fraction from the nuclear fraction (at low speed centrifugation at 2500 rpm) using Eppendorff Centrifuge 5417R, composed of 10 mM Tris-HCL (pH7.9), 1 mM EDTA, 60 mM KCL, 1 mM DTT, 0.075% (v/v) NP40 and 1mM $NaVO_4$, supplemented with protease inhibitor mixture. The supernatant was then combined with an equal volume of SDS-PAGE Laemmli buffer (4% (w/v) SDS, 20% (v/v) glycerol, 0.004% (w/v) bromphenol blue and 0.125 M Tris HCL) with final pH 6.8 and fractionated on a 12% SDS-PAGE gel. The protein concentration of samples

was measured by the BCA kit (Pierce). For immunoblotting, 25 µg of whole-cell lysates were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (PAGE) (Rauch and Loughna 2005; Atherton *et al*., 2009) and transferred onto Hypond-LPF Low Fluorescent PVDF membrane (GE Healthcare). The membrane were then blocked overnight in 5% non-fat dry milk PBS 0.01% Tween-20. The membranes were then probed with the appropriate primary antibodies diluted in 2% (w/v) Bovine Serum Albumin in PBS. Horseradish peroxidase-conjugated secondary antibodies were used to detect the primary antibodies by the enhanced chemiluminescence system (GE Healthcare). Bands on the X-ray films were quantified using densitometry software (Quantity one software, Bio-Rad) (Fig. 2-2). The primary antibodies used in our experiment were pErk1/2 (Thr202/Tyr204), pAkt1/2/3 (Ser473), pp38 (Thr180/Tyr182), Erk1(C-16), Akt1/2 (N-19), p38, p*I*B-α Rabbit polyclonal (Ser32), I*K*B-α Rabbit Polyclonal antibody, PNF-kB p50 (ser337) and HRP–conjugated secondary antibody (Anti-rabbit HRP conjugated IgG) (Santa Cruz) and Phosphoplus p70S6 kinase (Thr389, Thr421/Ser424) Antibody Kit, Phospho-4E-BP1 (Ser65), Phospho-eIF-4E (Ser209) and Phospho-mTOR (Ser2448) (Cell Signalling). β-actin has been used as loading control. The data were normalized to the β-actin as a loading control. Further information about the dilutions of the primary and secondary antibodies is given in the appendix.

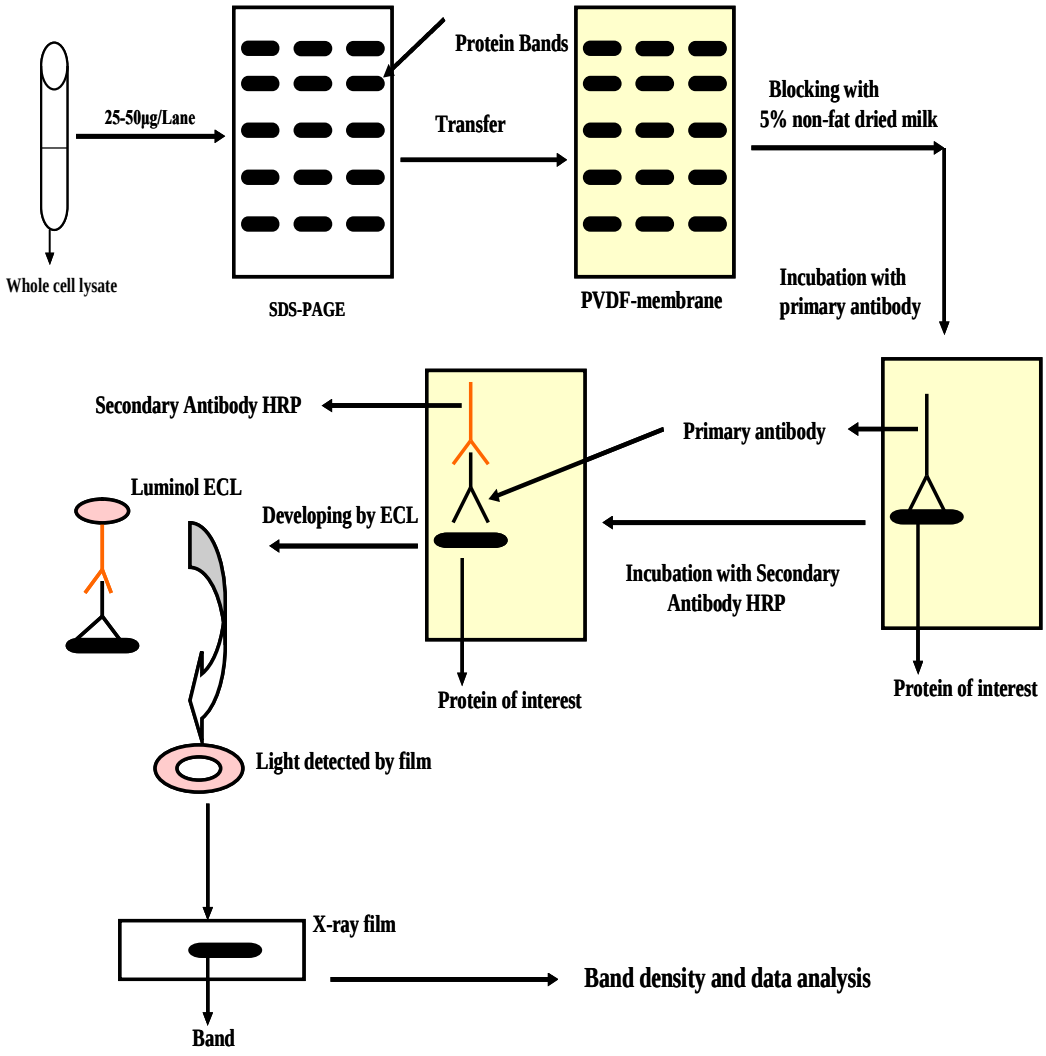


Figure (2-2) Schematic diagram illustrating the western Blotting technique. First, the proteins were fractionated on 3% (w/v), 10% (w/v) and 12% (w/v) SDS-PAGE gel. The proteins were then transferred on PVDF membrane using a Semi-dry blotter. The PVDF membranes were then incubated with the appropriate antibodies. The membranes were then developed with a chemiluminescence system. The density of the band was measured by Quantity one software and normalized to β-actin expression.

2.3 Chicken experiment (*in vivo*)

This experiment was a joint experiment with one of our group. The birds were mainly kept to evaluate the effect of natural challenge with different *Salmonella* serotypes on the immune response of birds. Three groups of these birds were selected for the skeletal muscle samples collection as follows. Thirty one-day-old specific–pathogen free Ross 308 broiler chicks were divided into three groups (10 chicks each). The birds were allocated in three separate rooms receiving sterile drinking water. At day 20, the birds were given free access to drinking water containing vancomycin (100 μg/ml), and the birds were then treated individually with 0.5 ml of (40 mg/ml) vancomycin. The vancomycin is an antibacterial drug that inhibits the intestinal bacterial growth. At day 21, two groups of birds were orally infected with 0.3 ml of $3x10^8$ CFU/ml *S*. Enteritidis (*SE*) and *S.* Gallinarium (*SG*) while the third group received 0.3 ml nutrient broth and was kept as control. The commercial feed (in powder without antibiotics) and drinking water (without antibiotics) were both available ad libitum. Cleaning and feeding patterns were maintained to prevent the cross contamination throughout the experiment. At day 4-post infection, five birds were selected and the birds were killed by cervical dislocation. Five skeletal muscle samples were aseptically collected form the petrol and gastrocnemius muscles and immediately frozen in liquid nitrogen and kept for RNA isolation and RT-PCR experiments. All the animal experiments were carried out according the Home Office rules and regulations.

2.4 Chymotrypsin like-enzyme activity assay

The 20S proteasomal activity was determined by measuring the chymotrypsin-like enzyme activity according to the method previously described by Orino *et al* (1991) and recently by Whitehouse and Tisdale (2003). The proteasomal activity was determined by the release of 7-amino-

4-methylcoumarin from the fluorogenic peptide succinyl-LLVY-7-amino-4-methylcoumarin (Sigma).

C2C12 myoblasts and the avian primary skeletal muscle cells were grown, differentiated and maintained until the formation of the myotubes was confirmed morphologically as previously described. Fully differentiated C2C12 myotubes were then treated either with LPS (1 μgml^{-1}) alone (*Salmonella Typhimurium* cell culture tested, Sigma) or with LPS in combination with inhibitors or with the inhibitors alone for 5, 30 min, 1, 3, or 18 h. In case of the avian primary skeletal muscle cells, cells were treated with LPS (1 μgml^{-1}) alone or with LPS in combination of the inhibitors for 3 h only. Cells were then washed with cold sterile PBS three times, then lysed in 500 µl lysis buffer (20 mM Tris HCL, pH 7.5, 5 mM $MgCl_2$, 50 mM DTT and 2 mM ATP and sonicated three times with pulses of 15 seconds with 10 seconds intervals. The cells were then spun down at 15.000 rpm for 15 min at 4 $^{\circ}$C. The supernatant was removed and the protein content was determined using Bradford assay method using BSA as standard. Each 200-µl final reaction volume, 10 µl fluorescent substrate (N-Succinyl-Leu-Leu-Val-Tyr7-Amido-4-Methylcoumarin (Sigma), and 10-100 µg systolic protein in 50 mM Tris-Hcl (pH 8) were added. Then samples were added in duplicate to into a 96 well plate. The plates were read on a flourimeter (BMG flourogenic plate reader) set for 360 nm excitation and 460 nm emission. The obtained results were normalised to the protein content of each sample. The chymotrypsin-like enzyme activity was measured per µg protein.

2.5 Protein Assay Method (Stoscheck 1990)

a- Main method used to determine the protein content of samples (BCA and Bradford protein assay Method), BSA (Bovine Serum Albumin) was used as standard.

b- **Standard curve preparation**

Stock solution= 2 mg/ml BSA.

The other concentrations prepared as µg protein/ml

1.8 mg /ml: 270 µl stock + 30 µl H_2O

1.5 mg/ml: 150 µl stock + 50 µl H_2O

1.2 mg/ml: 180 µl stock + 120 µl H_2O

0.9 mg/ml: 150 µl 1.8 mg/ml + 150 µl H_2O

0.6 mg/ml: 100 µl 1.2 mg/ml + 100 µl H_2O

0.3 mg/ml: 100 µl 0.9 mg/ml + 200 µl H_2O

0.1 mg /ml: 100 µl 0.3 mg/ml + 200 µl H_2

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	0	0	0.1	0.1	0.3	0.3	0.6	0.6	0.9	0.9	1.2
B	Blank	1	1									1.2
C	Blank	2	2									1.5
D	Blank	3	3									1.5
E	Blank	4	4									1.8
F	Blank	5	5									1.8
G		6	6									2.0
H		7	7									2.0

Figure (2-3) Diagram illustrating the layout of one of BCA protein Assay experiments.

1-7 = Samples & added water (10 µl each)

0-2.0 = Standard & added lysis buffer (10 µl each)

Blank = water & added lysis buffer (10 µl each)

200 µl of the reagent was added to each well, measure the absorbance and calculate the amount of protein (µg/µl) from the standard curve.

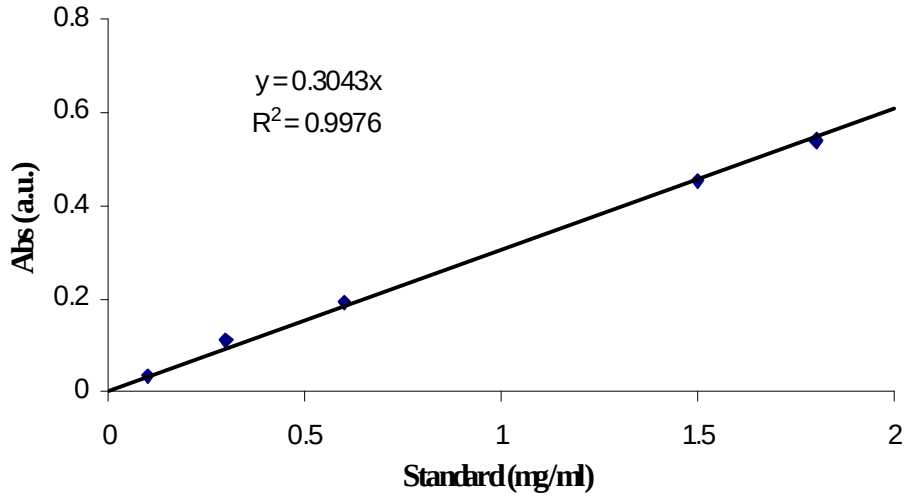


Figure (2-4) graph illustrates the standard curve obtained from one of the protein Assay (BCA). The amounts of proteins (µg/µl) were detected from the equation showed on the graph. The equation corresponding to a linear fit of the absorbance

2.6 Colorimetric Determination of Creatine Kinase Activity (CK) at 340 nm

C2C12 murine myoblasts were grown and differentiated as previously described above. The media was shifted to 2% (v/v) HS DMEM medium without phenol red 24 h before the experiment. Cells were then treated with LPS (1 μgml^{-1}) for 5, 30 min, 1, 3 and 18 h. The conditioned media was collected and stored at -80 $^{\circ}$C. The test was carried out according to the manufacture's protocol. Briefly, 100 µl company's reagent (BioAssay Systems) was added into each well of the 96 wells plate (calibrator and the samples), then mixed, and incubated at room temperature at 37 C. The plate was then at 340 nm once after 20 min incubation and again after 40 min. The CK activity was obtained from the following equation;

CK (U/L) = $(OD_{40\ min} - OD_{20\ min)} / (OD_{calibrator} - OD_{H2O})$ X 150

OD, optical reading at 340 nm after 20 min and 40 min

One unit of CK will transfer 1 µM of phosphocreatine to ADP per min at pH 6.0.

2.7 Protein Synthesis (PS) in Myotubes

Fully differentiated C2C12 myotubes and avian primary skeletal muscle cell were treated with LPS (1 μgml^{-1}) alone, or with LPS (1 μgml^{-1}) in combination with the inhibitors or with the inhibitors alone. 2 µCi ^{3}H-phenylalanine (GE Healthcare) in the case of C2C12 myotubes or ^{3}H-Tyrosine (PerkinElmer) in case of avian primary skeletal muscle cells was added for 1 h before terminating the experiment. The PS was expressed as incorporation of ^{3}H-phenylalanine per milligram of total 5% Trichloroacetic acid (5% TCA) percipitable protein as previously described by (Vandenburgh *et al.,* 1991). Briefly, the cells were washed three times with cold PBS, 500 µl 5% (w/v) TCA was added to cells. The plates were then scraped and washed twice with 400 µl 5% (w/v) TCA. The samples were then placed on ice for 1 h. The protein

pellet was then washed three times to remove free ^{3}H-phenylalanine and then dissolved in 400 µl 0.1 MNaOH. Samples were then heated in 0.1% (w/v) sodium dodecyl sulfate (SDS) for 2 h at 70 °C to solubilise the precipitate. The protein concentration of the samples was determined by the Bradford protein Assay kit-using BSA (Peirce) as standard. The ^{3}H-phenylalanine or ^{3}H-tyrosine incorporation was measured by Liquid scintillation analyzer (Apackard Bioscience). The obtained values were normalised to the protein content of each sample. The values were expressed as counts per µg-precipitated protein per minute (CPM/µg protein).

2.8 Measurement of nitric oxide (NO) using the Griess assay

Nitric oxide production was measured by analysing the supernatant fluid for the presence of nitrite, using the standard Griess assay method (Ding *et al.,* 1988; Sung *et al.,* 1991). The absorbance was read at 540 nm. Serial dilutions of sodium nitrite (Sigma) were used to determine a standard curve. The test was performed as follow:

1- **Composition of the solutions**

1-NED Solution (0.1% (w/v) N-napthylethylenediamine dihyrochloride in water)

2-Sulfanilamide Solution (1% (v/v) Sulfanilamide in 5% (v/v) phosphoric acid).

3-Nitrite standard (0.1 M sodium nitrite in water)

All the reagent were obtained from Sigma company UK

2- **Procedures of the test**

NO release was determined in a 96-well plate by mixing 50 µl medium with 50 µl of the Sulfanilamide solution, followed by incubation at room temperature for 10-15 min, 50 µl of the NED solution was then added, with subsequent incubation at room temperature for 10-15 min. The absorbance was then read at 540 nm (Fig. 2-5). The NO concentration was derived from the standard curve. Serial dilutions of sodium nitrite were used to determine a standard curve (Fig. 2-6). The obtained results were normalised to the protein

content of each sample. The protein content of the samples was determined by the BCA method using BSA as standard.

	1	2	3	4	5	6	7	8	9	10	11	11
A	S1	S1	S1	**1**	**1**							
B	S2	S2	S2	**2**	**2**							
C	S3	S3	S3	**3**	**3**							
D	S4	S4	S4	**3**	**3**							
E	S5	S5	S5	**4**	**4**							
F	S6	S6	S6	**5**	**5**							
G	S7	S7	S7	**6**	**6**							
H	Media (0**µM**)	Media (0**µM**)	Media (0 **µM**)	**7**	**7**							

Figure (2-5) Schematic illustration of the plate layout of the nitric oxide experiment

S1-S7. Standard prepared with twofold six serial dilutions starting from (125 µM Nitrite to 0 µM Nitrite)

1-7. Samples

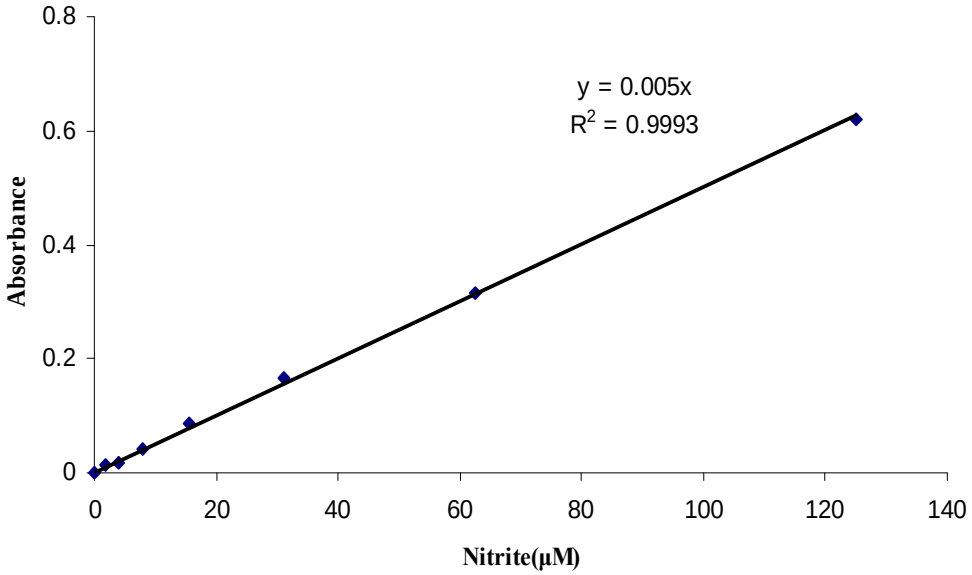


Figure (2-6) graph illustrates the standard curve obtained from one of the NO experiment. The concentration of nitrite (µM) in each sample was obtained from the equation showed on the graph. The equation corresponding to a linear fit of the absorbance.

2.9 TNF-α and IL-6 enzyme-linked immunosorbant assay

C2C12 myotubes were grown and maintained as previously described. The cells were then treated with LPS (1 μgml^{-1}) for 5, 30 min, 1, 3, or 18 h respectively. The conditioned media were collected and stored at -80 °C. TNF-α and IL-6 cytokine levels in the conditioned media were measured using the TNF-α and IL-6 ELISA Ready-set-Go kit (eBioscience) according to the manufacture's procedures. Briefly 100 μl/well of the media or standard were added to the appropriate wells, the plate was sealed and kept overnight at 4°C. After washing the wells, 100 μl/well of the detection antibody was added, sealed and left for 1 h at room temperature. The wells were then washed, followed by addition of 100 μl/well of Avidin-HRP secondary antibody was added, reseal the plate for 30 min at the room temperature, then 50 μl/well of the substrate were added followed by 50 μl/well of stop solution. Absorbance reading was done at 450 nm (Fig. 2-7). The obtained readings were normalised to the protein content of each sample. The protein content of the samples was determined by BCA method using BSA as standard.

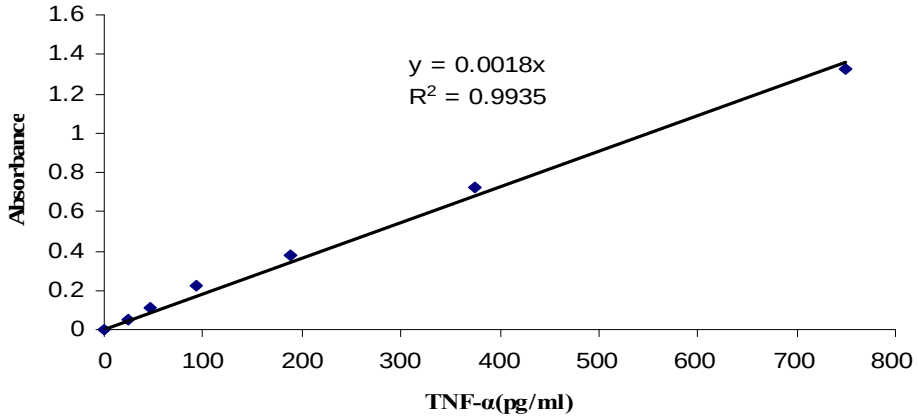


Figure (2-7) graph illustrates the standard curve for level of TNF-α cytokine. The concentration of TNF-α in each sample was obtained from the equation showed on the graph. The equation corresponding to a linear fit of the absorbance.

2.10 Total RNA Extraction from the C2C12 myotubes and avian primary skeletal muscle cells

C2C12 myoblasts and avian primary skeletal muscle cells were grown, maintained and differentiated as described above. Cells were then treated according the experiment (either with LPS (1 μgml^{-1}) alone, or with LPS (1 μgml^{-1}) in combination with the inhibitors or with the inhibitor alone. Cells were then washed three times with cold PBS, and then lysed and the RNA isolated using Nucleospin® RNA II (Fisher Scientific) following the manufacturer's instructions. Briefly, 350 µl Lysis buffer + 3.5 µl β-mercaptoethanol were added to lyse cells, and then the lysate was filtrated by centrifuging at 11,000xg (SIGMA (1-14), Scientific Laboratory Supplies (SLS)) for 1 min. The binding of RNA was adjusted by mixing with 350 µl 70% Ethanol and mixed. Samples were then spun at 11,000xg for 30 seconds. Desalted the silica membrane by adding 350 µl Membrane Desalting Buffer (MDB). The columns were then centrifuged at 11,000xg (SIGMA 1-14, SLS) for 1 min. Then, 95 µl DNase reaction mixtures were added to digest the DNA for 15 min at room temperature. The RNA bound to the membrane was then washed three times with the RA2 solution, and later the it was eluted in 60 µl RNase-free water by centrifuging the columns at 11,000xg (SIGMA 1-14, SLS) for 1 min. RNA concentration was accessed using the Nanodrop at 260 and 280 nm absorbance (Okamoto and Okabe 2000), and then stored at -80 $^{\circ}$C for the cDNA synthesis.

2.11 Total RNA Extraction from chicken skeletal muscle using Trizol reagent

Total RNA was extracted using the standard protocol (Jones *et al.,* 2004). Briefly, approximately 100 mg of frozen muscle samples were homogenised using a gentle MACS dissociator (Miltenyi Biotec) in 1 ml ice-cold Trizol (Invitrogen) for 1 min. To the homogenate, 0.2 ml chloroform was

added and shaken vigorously for 15 seconds. After incubation for 5 min at room temperature, the homogenate was then centrifuged at 12000xg (HERAEUS PIC 17, Thermo Scientific) at 2-8 °C for 15 min. The aqueous phase was then removed and kept in clean sterile eppendorff tube. To the aqueous phase, 0.25 ml isopropanol and 0.25 ml high salt precipitation solution (0.8 M Sodium Citrate/ 1.2 M NaCL) was added. After incubation for 10 min at room temperature the mixture was next centrifuged at 12000xg (HERAEUS PIC 17, Thermo Scientific) for 10 min at 2-8 °C. The RNA pellet was then washed with 75% ethanol before being centrifuged at 7500xg (HERAEUS PIC 17, Thermo Scientific) for 5 min at 2-8 °C. After discarding the ethanol the pellet was then air dried and resuspended in 30 µl RNase-free water. The RNA recovered was quantified using the Nanodrop at 260 and 280 nm absorbance (Okamoto and Okabe 2000), and the quality of the extraction monitored by the 260:280 nm ratios, and the RNA was then stored at -80 °C.

2.12 cDNA Synthesis

Total RNA was Transcripted into complementary DNA (cDNA) using the Transcriptor First Strand cDNA Synthesis kit (Roche®). Following the manufacture's procedures, 1 µg total RNA, 2 µl of random hexamer primer (60 µM final concentration), 2 µl Deoxynucleotide Mix (dNTPs) (1 mM final concentration), 0.5 µl protector RNase inhibitor (RNase) (20 U/µl final concentration), 0.5 µl Transcriptor Reverse Transcriptase (RT) (10 U/µl final concentration) and 4 µl RNA buffer and the solution was completed to 20 µl final volume with Rnase-free in PCR tube. Then the tube was placed in thermal block cycler PCR for 10 min at 25 °C followed by 30 min at 55°C. Table (2-1) showed how to prepare 50 CDNA samples and the amount of reagents required.

Table (2:1) Table showing the amount of each vial required to prepare 50 cDNA samples using Transcriptor First Strand cDNA Synthesis kit, (Roche®). The reagents showed in the table were completed in PCR tube to 20 µl final volume with Rnase free water.

cDNA Required Reagents			
No of Samples **50**	**Vail**	**Amount (µl) from each vial**	**Total (µl) from each vial**
RNA Buffer		**4.0**	**200**
Transcriptor Reverse Transcriptase (RT)		**0.5**	**25**
Deoxynucleotide Mix (dNTPs)		**2.0**	**100**
Random hexamer primer		**2.0**	**100**
Protector Rnase inhibitor		**0.5**	**25**

The following tables show the nucleotides sequences of the primers and the probes that were used in the RT-PCR experiments and their accession number and hyperlink to the gene bank.

Table (2:2) Primer sequences (5'-3') used for mRNA quantification by reverse transcriptase-PCR (RT-PCR) in the case of C2C12 murine skeletal muscle cells (Roche)**.** The probes were obtained from Roche universal probe library.

Name	Probe number	Forward	Reverse
B-actin (NM_007393.3)	**#64**	CTAAGGCCAACCGTGAAAAG	ACCAGAGGCATACAGGGACA
MuRF1 (AF294790)	**#105**	GCCAACGACGTCTTCCAG	GGACAACCTCGTGCCTACA
Atrogin-1 (AF441120)	**#53**	AGTGAGGACCGGCTACTGTG	GATCAAACGCTTGCGAATCT
TNF-α (NM_013693.2)	**#49**	TGCCTATGTCTCAGCCTCTTC	GAGGCCATTTGGGAACTTCT
TLR4 (NM_021297.2)	**#2**	GGACTCTGATCATGGCACTG	CTGATCCATGCATTGGTAGGT

All the probes were labelled by (5'-FAM-3'-TAMARA). The primers and the probes for IL-6, IL-8 and IL-10 were obtained from (Smith *et al.,* 2005), while the other chicken primers and probes obtained from (Nakashima *et al.,* 2009). The expression of mRNA was quantified by RT-PCR using a LightCycler480 instrument (Roche). The expression of 18S was used as an internal control. The premiers were designed using **GenScript Real-time PCR (TaqMan) Primer Design software**. The accession number of the genes used in both the primary cell experiment and the chicken skeletal muscle experiment were Chicken18S mRNA (**AF173612**), Chicken TNF-α, Chicken Atrogin mRNA (NM_**001030956**), Chicken Caspase-3 mRNA (**AF083029**), Chicken Cathepsin mRNA (**U18083**), Chicken Ubiquitin mRNA (**X02650**), Chicken IL-6 mRNA(**AJ250838**), Chicken IL-8 mRNA (**AJ009800**) and Chicken IL-10 mRNA (**AJ621614**)

Table (2:3) Primer sequences (5'-3') used for mRNA quantification by reverse transcriptase-PCR (RT-PCR) used in the case of avian primary skeletal muscle chicken cells and muscle from live chicken bought from Eurogentec company.

Name	Forward	Reverse	Probe
18S (AF173612)	TGGCAAATGCTTTCGTCGCTTT	TGTGCCGCTAGAGGTGAAATT	GCCCTCCTCCTGGTTTCAG
C-TNF-α	CCCTTCTGAGGCATTTGGAA	CAGCCTGCAAATTTTGTCTTCTT	CAGCGTTCGGAGTGGGCT
C-Atrogin (NM_001030956)	TTGCTGCAGACCCTCTACAC	GCCTGGTGATTTGAATGTTG	TTGCCGACCCGTTGAACCAA
C-Caspase-3 (AF083029)	AGCTGAAGGCTCCTGGTTTA	TCTGCCACTCTGCGATTTAC	TCCTGGCGTGTTCCTTCAGCA
C.Cathepsine (U18083),	AAGCTCTGTGGCACTTTCCT	GTCAAAGGTATCCGGCAAAT	CAGGAGCCTTGGGTCCACCC
C-Ubiquitin (X02650),	GGATGCAGATCTTCGTGAAA	GGATCTTGGCCTTCACATTT	CACCCTTGAGGTTGAGCCCAGTG
C-IL-6 (AJ250838),	GCTCGCCGGCTTCGA	GGTAGGCTGAAAGGCGAACAG	AGGAGAAATGCCTGACGAAGCTCTCCA
C-IL-8 (AJ009800),	TGGCACCGCAGCTCATT	TCTTTACCAGCGTCCTACCTTGCGACA	GCCCTCCTCCTGGTTTCAG
C-IL-10 (AJ621614)	CATGCTGCTGGGCCTGAA	CGTCTCCTTGATCTGCTTGATG	CGACGATGCGGCGCTGTCA

2.13 RT-PCR

The fold change in mRNA level was quantified by the Light Cycler 480 (Roche Company). cDNA was obtained as previously mentioned above form the reverse transcription reaction of mRNA. Real-time PCR analyses were performed in triplicate using 96-well plate using 2x master mix (Roche Company). Two microliters of cDNA were used as a template for real-time PCR; 1.2 µl forward primer (5 µM), 1.2 µl reverse primer (5 µM), and 0.7 µl probe (5 µM) were added to 10 µl Roche 2x master mix and 4 µl Rnase free water. Reactions were performed in 20 µl volume reaction volume under the following conditions 1 cycle for the pre-incubation and the activation of the fast start taq DNA polymerase, followed by denaturation of the DNA at 95 °C for 10 min. Then 45 cycles for amplification at (95 °C for 10 sec, 60 °C for 15 sec and 72 °C for 01 sec) and 1 cycle for cooling at 40 °C for 10 sec. The changes in mRNA levels of gene of interest relative to β-Actin mRNA was determined by advanced relative quantification method using the Light cycler 480 (LC480 1.5.0.39**)**. The amount of the target mRNA was normalised to the endogenous reference and expressed as a relative to the calibrator according to Pfaffl (2001). β-Actin has been used as a reference gene in case of C2C12 myotubes, while in case of the avian primary skeletal muscle cells 18s has been used. Note that, the expression levels of β-Actin and 18S were not affected by treatment in all experiments. Examples of the C_T values for some genes in different experiments were shown in appendix.

2.14 Pathway inhibitors

C2C12 murine myoblasts and the avian primary skeletal muscle cells were cultured, maintained and differentiated until the formation of myotubes was confirmed morphologically as previously described above. To the full differentiated myotubes, the Toll-like Receptor 4 Inhibitor, 5 μgml^{-1} (Polymyxin B, Sigma) was added 2 h before adding LPS, while LY0294002, 50 µM (PI3-K/Akt inhibitor, Cell signaling), PD098059, 40 µM (MEK/Erk1/2 inhibitor, Cell Signaling), MG-132, 40 µM (proteasomal inhibitor, Sigma) and SB203580, 10 µM (P38 inhibitor, Sigma) were added 1 h before adding LPS (1 μgml^{-1}). The optimal doses of inhibition was obtained from the previous literatures: Polymyxin B (Frost *et al.,* 2006), SB203580 (Li *et al.,* 2005; Huang *et al.,* 2007; Jump *et al.,* 2009), MG-132 (Frost *et al.,* 2003), PD098059,(Shi *et al*., 2002; Dehoux *et al*., 2003; Plaisance *et al.*, 2008) and for LY0294002 (Plaisance *et al.,* 2008) with modification that we used higher dose according to the manufacture recommendation for PD098059 and LY0294002. Afterwards the cells treated as described above. Moreover, the theses kinases inhibitors especially, p38 and Erk1/2 inhibitors are selective for their respective kinase pathways in C2C12 cells (Frost *et al*., 2003). LY0294002 is a potent mTOR inhibitor (Brunn *et al.,* 1996). Curcumin (Sigma) was dissolved in DMSO and various concentrations (12, 25 and 50 µM) were used in the experiment. The following diagram illustrates the how these inhibitors block their relevant pathway (Fig. 2-8).

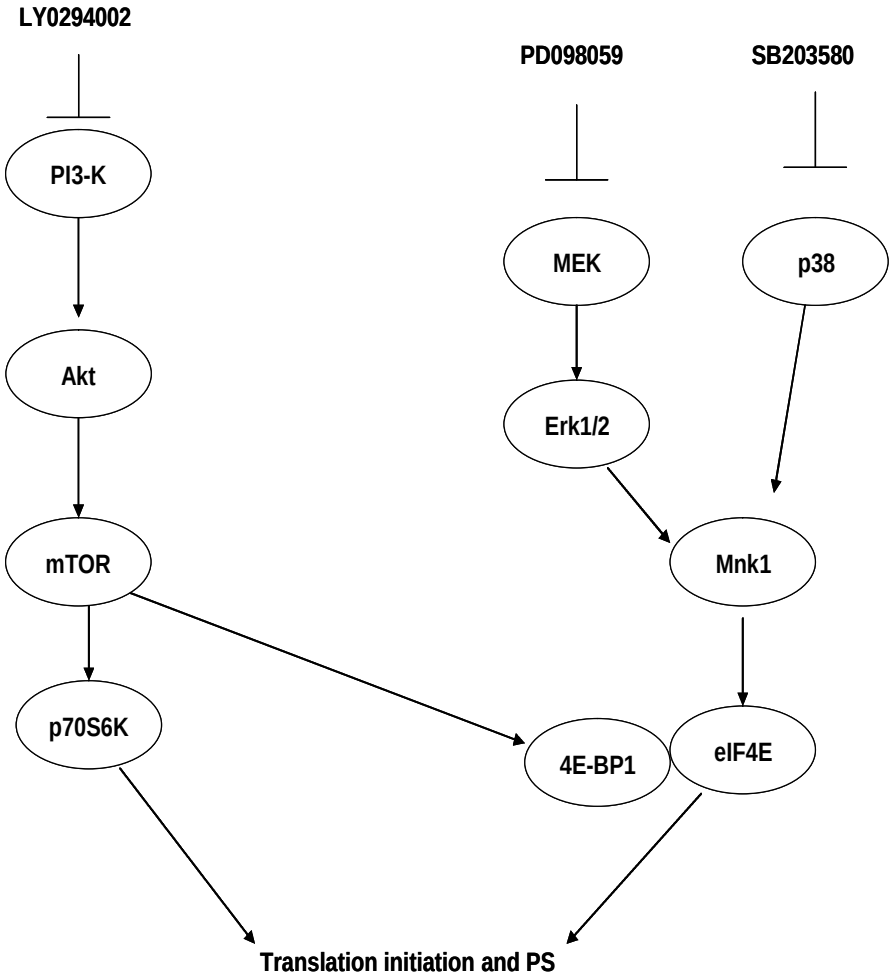


Figure (2-8) Diagram showing how the inhibition of Akt and MAPKs (p38 or Erk1/2) can modulate the PS. Inhibition of PI3-K/Akt activity by LY0294002 decreased PS through the inhibition of translation initiation mediated via decreased mTOR, p70S6K and 4E-BP1 phosphorylation. In contrast inhibition of MEK/Erk1/2 by PD098059, and inhibition of p38 by SB203580 altered the PS via inhibition of MnK1 induced eIF-4 phosphorylation and hence this inhibits the translation initiation.

2.15 Statistics

Data are expressed as the means ± S.E.M unless stated in each graph. The statistical significant differences among the groups were assessed by (one and two-way analysis of variance with Turkey's *post hoc* testing to locate statistically significant difference at $P < 0.05$ for the normally distributed data. To allocate the difference between two groups, Student's *t-test* was used. While in case of nonparametric data Kruskal-Wallis Test has been used with the Mann-Whitney test to locate the difference between two groups. A probability below 0.05 ($P < 0.05$) considered significant. All the statistical analysis was done by Prism-4 (Graph Pad), Minitab 15 Statistical Software and GenStat 12th Edition, University of Nottingham.

3 Endotoxin transiently inhibits protein synthesis through Akt and MAPK mediating pathways in C2C12 myotubes

3.1 Introduction

Muscle wasting is a common syndrome associated with severe catabolic diseases such as sepsis, cancer and AIDS. Sepsis is an inflammatory condition that causes severe and rapid loss of body protein, much of which originates from skeletal muscle (Hasselgren 1995). LPS are an integral part of cell wall components of Gram-negative bacteria, and are potent activators of the innate immune system through recognition by TLR4 (Raetz 1990). TLR4 is a part of a large family of receptors that recognize PAMPS, and plays an indispensable role in the transduction of the LPS signal and subsequently the stimulation of inflammatory cytokine gene expression (Kaisho and Akira 2000). The dynamic balance between rates of PS and PD governs the skeletal muscle protein mass maintenance.

To date most studies investigating the effects of sepsis upon skeletal muscle protein metabolism have examined the regulation of catabolic pathways that lead to accelerated rate of protein loss (Frost *et al.,* 2003). Clinical studies in humans and in a variety of animal models clearly demonstrate that PD contributes to the loss of skeletal muscle protein and impaired contractile function observed in response to sepsis or endotoxin administration (Owens *et al.,* 1994). In a number of clinical conditions and experimental models, it has been shown, however, that a change in rate of PS is the primary initiator of altered muscle mass (Vary 1998; Lang *et al.* 2002; Lang and Frost 2007). The few *in vivo* studies examining LPS and muscle PS (Vary and Kimball 1992; Lang *et al.,* 2000) are contradictory while some

suggest a suppression of PS; others do not. To date, two studies have examined the direct effect of LPS on PS in cultured muscle cells *in vitro* (Frost *et al.,* 2009; Russell *et al.,* 2010). Frost and associates (2009) have shown that, LPS alone has no effect on protein synthesis, but the combination of LPS and IFN-γ induced a 80% decrease in the PS. In contrast, Russell *et al.* (2010) demonstrated that LPS alone induced a 50-60% decrease in PS in the same cell line.

PS in skeletal muscle is regulated at the transcriptional or translational level (Macallan *et al.,* 1996), with the translational control is the common mechanism in most acute responses (Jepson *et al.,* 1986). One of the most extensively studied anabolic pathways is the PI3-K/Akt pathway. Akt/protein kinase B, is a member of a family serine/threonine protein kinases (Datta *et al.,* 1999) which are stimulated by a number of receptor tyrosine kinases and often this was mediated by the action of PI3-K (Walsh 2006). Akt has been shown to be activated by *Salmonella enterica* serovar Typhimurium in the epithelial cells (Steele-Mortimer *et al.,* 2000). In skeletal muscle acute activation of Akt for 2 weeks significantly increased the skeletal muscle fibre size, and this effect produced in the main through an increase in the PS pathway (Lai *et al.,* 2004). The contribution of the PI3-K/Akt pathway and their down-stream effectors including mTOR, $P70^{S6K}$ and 4E-BP1 in the regulation of skeletal muscle mass has been reviewed in detail by Glass (2005). The translation of mRNA into protein is conventionally divided into three main stages: initiation, elongation and termination. mTOR controls the initiation and elongation stages of the protein translation (Wang and Proud 2006) through its downstream effectors including ribosomal S6Kinase (S6K1) and 4E-BP1 (Wang and Proud 2006). The direct contribution of the MAPKs family and their proposed downstream target Mnk1 in the regulation of the translation initiation process through eIF-4E has also been shown by Fukunaga and Hunter (1997).

A number of studies have measured the changes in the signalling pathways activation that may regulate PS without direct measurement of this process itself. Recent studies suggest that in some cases changes in the activation of the pathways that regulate translation may not always correlated with the expected changes in PS (Miranda *et al.,* 2008; Atherton *et al.,* 2009). Thus, a better understanding of the role of signalling pathways and in particular that of the Akt and MAPKs (p38 or Erk1/2) in sepsis is important for developing successful therapies in order to retard the loss in lean body mass and reduce morbidity and mortality (Lai *et al.,* 2004; Stitt *et al.,* 2004). For these reasons, our study aimed to examine the subcellular mechanisms responsible for the LPS-associated changes in PS in murine C2C12 murine myotubes, and the possible role of Akt/MAPK signalling pathways. In addition, we measured the direct effect of LPS alone and with specific pathway inhibitors on PS as a short-term (3 h) or long-term (18 h) response.

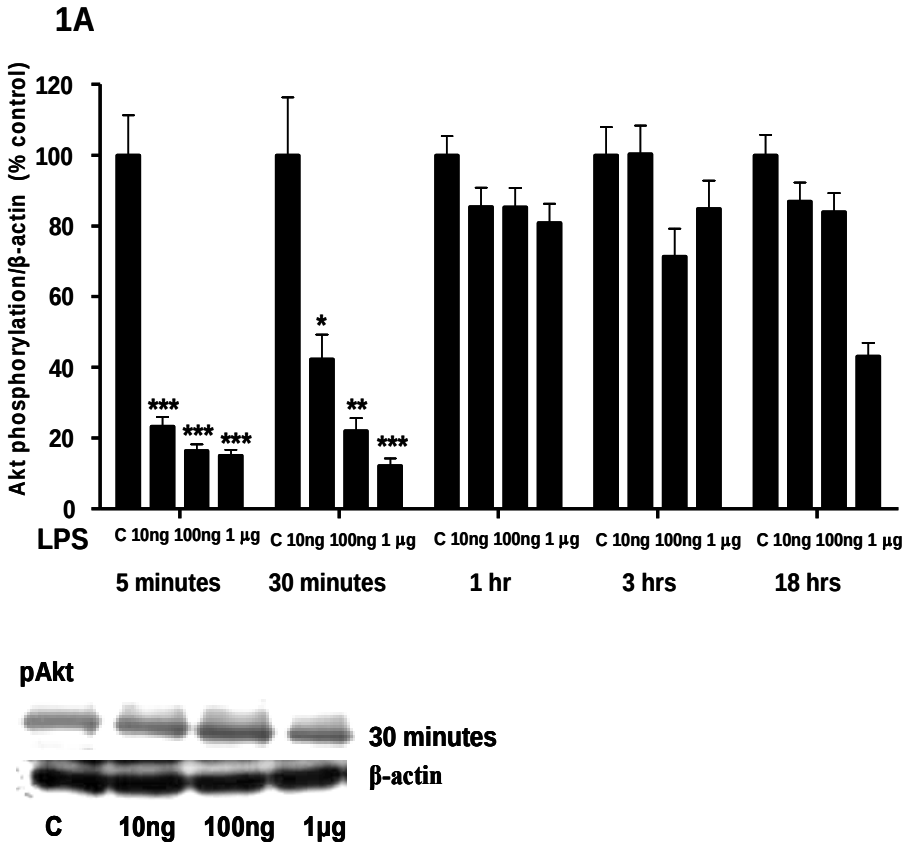
3.2 Hypothesis

LPS has previously been shown to induce decrease in PS i*n vivo*, and this effect was mediated via decreased translation initiation step of protein synthesis. Thus we anticipated that this effect could be the same *in vitro*. C2C12 myotubes have been extensively used in muscle research because they can respond to external stimuli (LPS) similarly equal to skeletal muscle. In addition, it has previously shown that the activity of PI3-K/Akt and MAPKs (p38 or Erk1/2) can regulate the PS mainly through the control of translation initiation step of PS. Therefore, the aim of this chapter is to investigate the direct of LPS on PS (measured as ^{3}H-phenylalanine incorporation/μg 5% TCA precipitable protein) and the subcellular signalling molecules involved in this effect in particular the role of Akt and MAPKs (p38 or Erk1/2) was investigated.

3.3 Results

3.3.1 LPS induced changes in the activity of Akt and mTOR

To characterise the effect of LPS on the signalling pathways mediating the protein synthesis, C2C12 myotubes were incubated at various concentrations of LPS (10 $ngml^{-1}$, 100 $ngml^{-1}$ and 1 μgml^{-1}) at a number of time points (5, 30 min, 1, 3 and 18 h). The effect of LPS on the activity-related phosphorylation state of Akt was presented in (Fig. 3-1*A*). LPS induced a rapid but transient decrease in the Akt phosphorylation lasting only for 30 min. LPS significantly decreased the Akt phosphorylation at 5 and 30 min in a dose-dependent manner (Fig. 3-1*A*). Later the LPS had no effect on Akt phosphorylation compared with the control levels at other time points (Fig. 3-1*A*). In contrast, LPS induced a long time and dose–dependent decrease in mTOR phosphorylation at all the time points starting early at 5 min and continued to the 18 h time point (Fig. 3-1*B*).


1A
Akt phosphorylation/β-actin (% control)
120
100
80
60
40
20
0
***
***
***
*
**
***
LPS
C 10ng 100ng 1 µg
C 10ng 100ng 1 µg
C 10ng 100ng 1 µg
C 10ng 100ng 1 µg
C 10ng 100ng 1 µg
5 minutes
30 minutes
1 hr
3 hrs
18 hrs
pAkt
30 minutes
β-actin
C
10ng
100ng
1µg

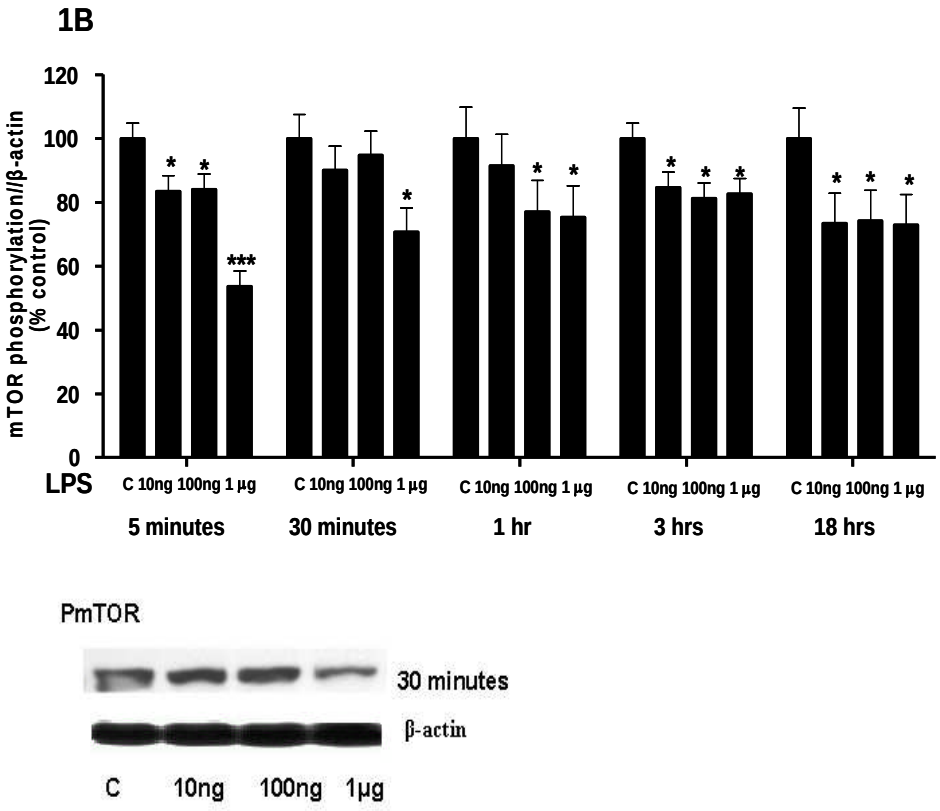
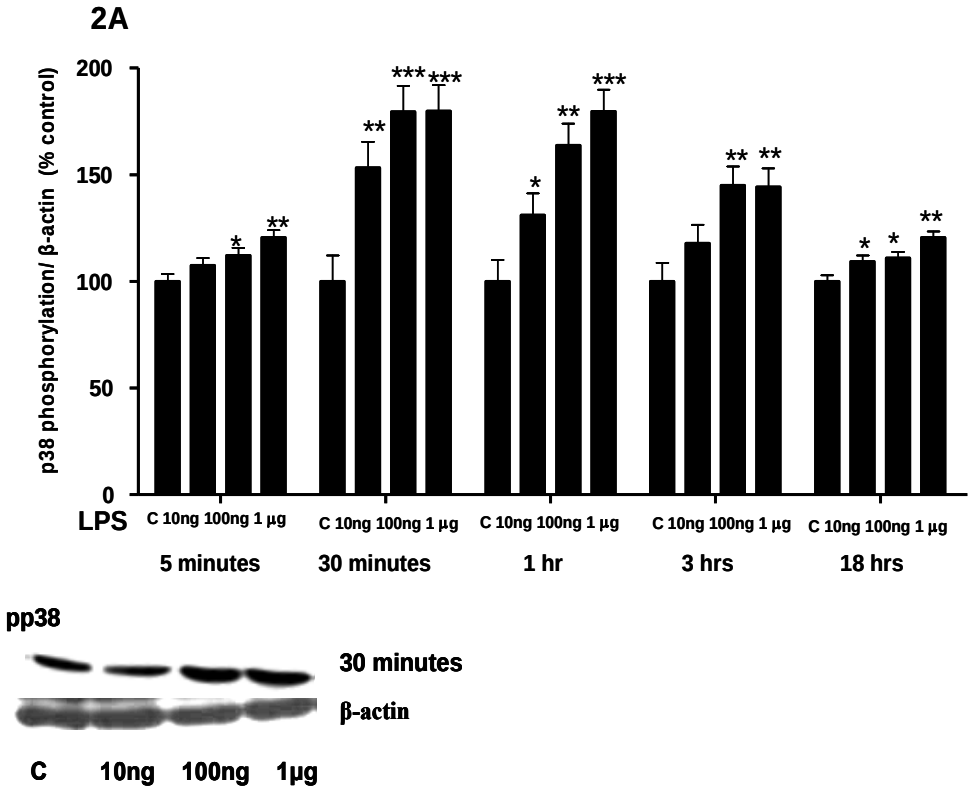
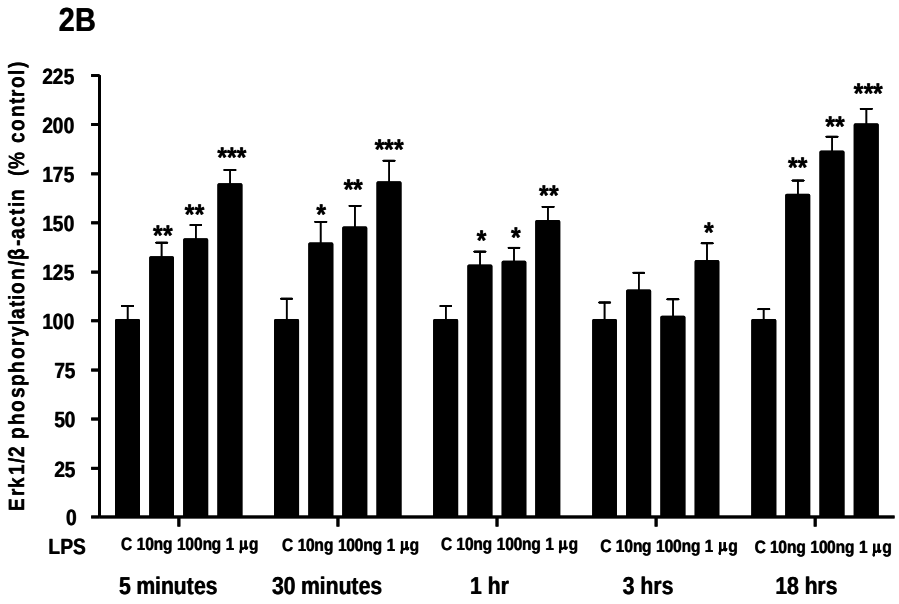


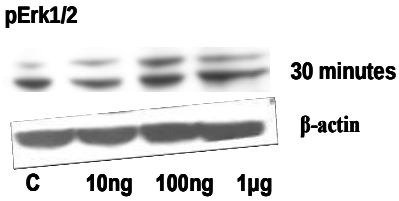
Figure (3-1) Time and dose-dependent changes in **A**) phospho-Akt and **B**) phospho-mTOR. C2C12 myotubes were incubated with LPS (10 $ngml^{-1}$, 100 $ngml^{-1}$ and 1 µg/ml) for (5, 30 min, 1, 3, or 18 h). Values represented as means ± SEM (percentage of control) normalised to β-actin as internal loading control. n = 3. Bars carry asterisks are statistically different from the control cells ($^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$). The representative blots illustrated the effect of LPS (10 $ngml^{-1}$, 100 $ngml^{-1}$ and 1 μgml^{-1}) on the activity-related phosphorylation of Akt and mTOR at 30 min.

3.3.2 LPS induced changes in P38, Erk1/2 and their down-stream target Mnk1 activity-related phosphorylation

To investigate the possible role of the other signalling pathways known to regulate the anabolic pathways, the level of phosphorylated p38, Erk1/2 and their substrate Mnk1 were measured in response to LPS administration (Figs. 3-2*A*, 3-2*B* and 3-2*C*). LPS-induced increases in p38 activity-related phosphorylation at all time points peaked at 30 min, 1 h and 3 h time points (Fig. 3-2*A).* The effect of the LPS on the phospho-Erk1/2 is summarised in (Fig. 3-2*B*). Incubation of the C2C12 myotubes with LPS (10 $ngml^{-1}$, 100 $ngml^{-1}$ and 1 μgml^{-1}) significantly induced a dose-dependent increase in Erk1/2 phosphorylation at all the time points with the exception of the 3 h time point (Fig. 3-2*B*). In Figure (3-2*C*) the activity-related phosphorylation of the Mnk1 was presented. Mnk1 has previously been known as *in vitro* downstream target of the MAPKs (p38 or Erk1/2). Incubation of the C2C12 myotubes with LPS (10 $ngml^{-1}$, 100 $ngml^{-1}$ and 1 μgml^{-1}) increased the Mnk1 phosphorylation particularly at 1 h and 3 h time points with no further changes observed at the other time points (Fig. 3-2*C*).


2A
p38 phosphorylation/ β-actin (% control)
200
150
100
50
0
LPS
C 10ng 100ng 1 µg
5 minutes
30 minutes
1 hr
3 hrs
18 hrs
pp38
30 minutes
β-actin
C
10ng
100ng
1µg


2B
Erk1/2 phosphorylation/β-actin (% control)
225
200
175
150
125
100
75
50
25
0
LPS
C 10ng 100ng 1 µg
5 minutes
30 minutes
1 hr
3 hrs
18 hrs


pErk1/2
30 minutes
β-actin
C
10ng
100ng
1µg

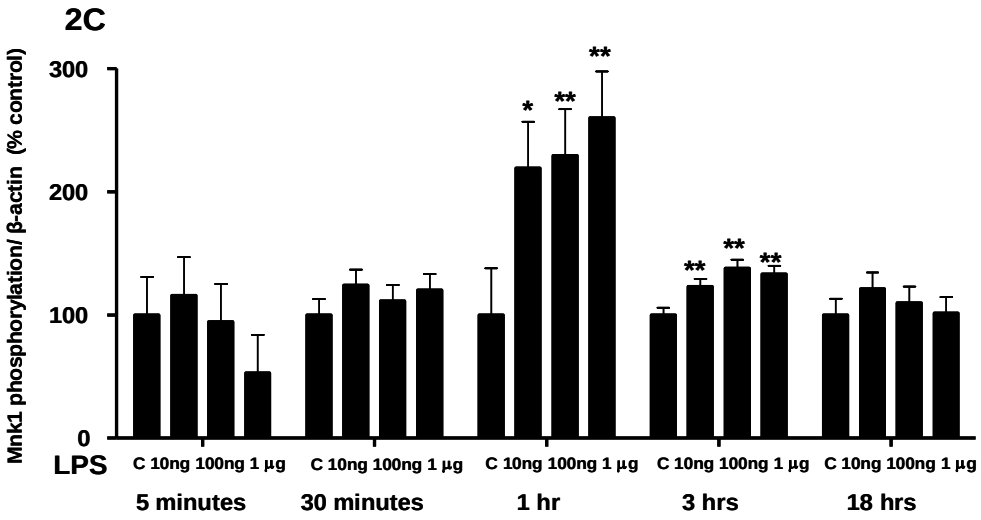
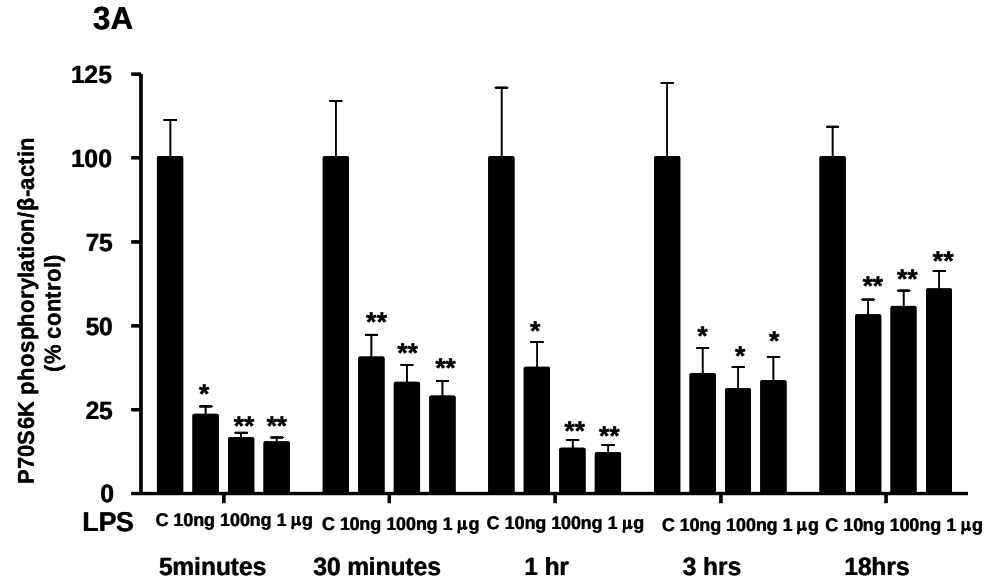
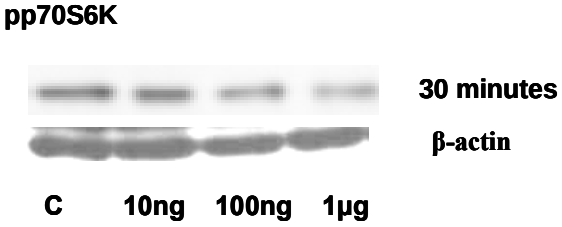


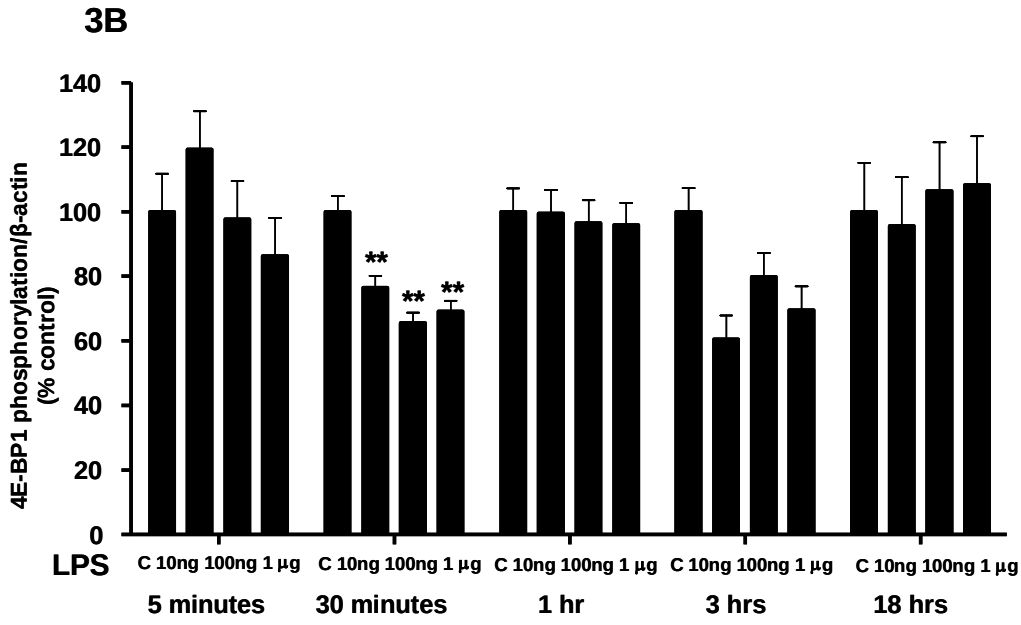
Figure (3-2) Time and dose-dependent changes in **A**) phospho-P38, **B**) phospho-Erk1/2 and C) phospho-Mnk1. C2C12 myotubes were incubated with LPS (10 $ngml^{-1}$, 100 $ngml^{-1}$ and 1 μgml^{-1}) for (5, 30 min, 1, 3, 0r 18 h. Values are represented as means $\pm$ SEM (percentage of control) normalised to β-actin as an internal loading control. n = 3. Bars carry asterisks are statistically different from the untreated control cells ($^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$). The representative blots illustrated the effect of LPS (10 $ngml^{-1}$, 100 $ngml^{-1}$ and 1 μgml^{-1}) on the activity-related phosphorylation of p38 and Erk1/2 at 30 min time point.

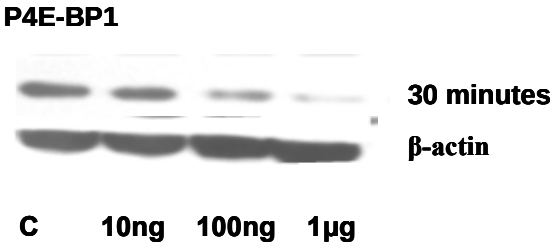
3.3.3 The effect of LPS on the regulation of translation initiation signalling molecules in C2C12 myotubes

In vivo, it has previously been shown that, LPS and sepsis significantly altered the PS via the alteration of the translation initiation step of the protein synthesis. The effect of the LPS (10 $ngml^{-1}$, 100 $ngml^{-1}$ and 1 μgml^{-1}) administration on the translation initiation signalling streams particularly $p70^{S6K}$, 4E-BP1 and eIF-4E is summarised in (Figs. 3-3*A*, 3-3*B* and 3-3*C*). Incubation of the C2C12 myotubes with LPS significantly decreased the $p70^{S6K}$ phosphorylation in a dose-dependent manner at the time points, and this effect was maximally seen at the 5 min and 1 h time points (Fig. 3-3*A*). In contrast, LPS only decreased the 4E-BP1 phosphorylation at the 1 h time point (Fig. 3-3*B*). The decrease in 4E-BP1 phosphorylation at the 1 h time points was then followed by a decrease in the eIF-4E phosphorylation at 3 h time point (Fig. 3-3*C*).


3A
P70S6K phosphorylation/β-actin (% control)
125
100
75
50
25
0
LPS
C 10ng 100ng 1 µg C 10ng 100ng 1 µg C 10ng 100ng 1 µg C 10ng 100ng 1 µg C 10ng 100ng 1 µg
5minutes
30 minutes
1 hr
3 hrs
18hrs


pp70S6K
30 minutes
β-actin
C
10ng
100ng
1µg


3B
4E-BP1 phosphorylation/β-actin (% control)
140
120
100
80
60
40
20
0
**
**
**
LPS
C 10ng 100ng 1 µg
C 10ng 100ng 1 µg
C 10ng 100ng 1 µg
C 10ng 100ng 1 µg
C 10ng 100ng 1 µg
5 minutes
30 minutes
1 hr
3 hrs
18 hrs


P4E-BP1
30 minutes
β-actin
C
10ng
100ng
1µg

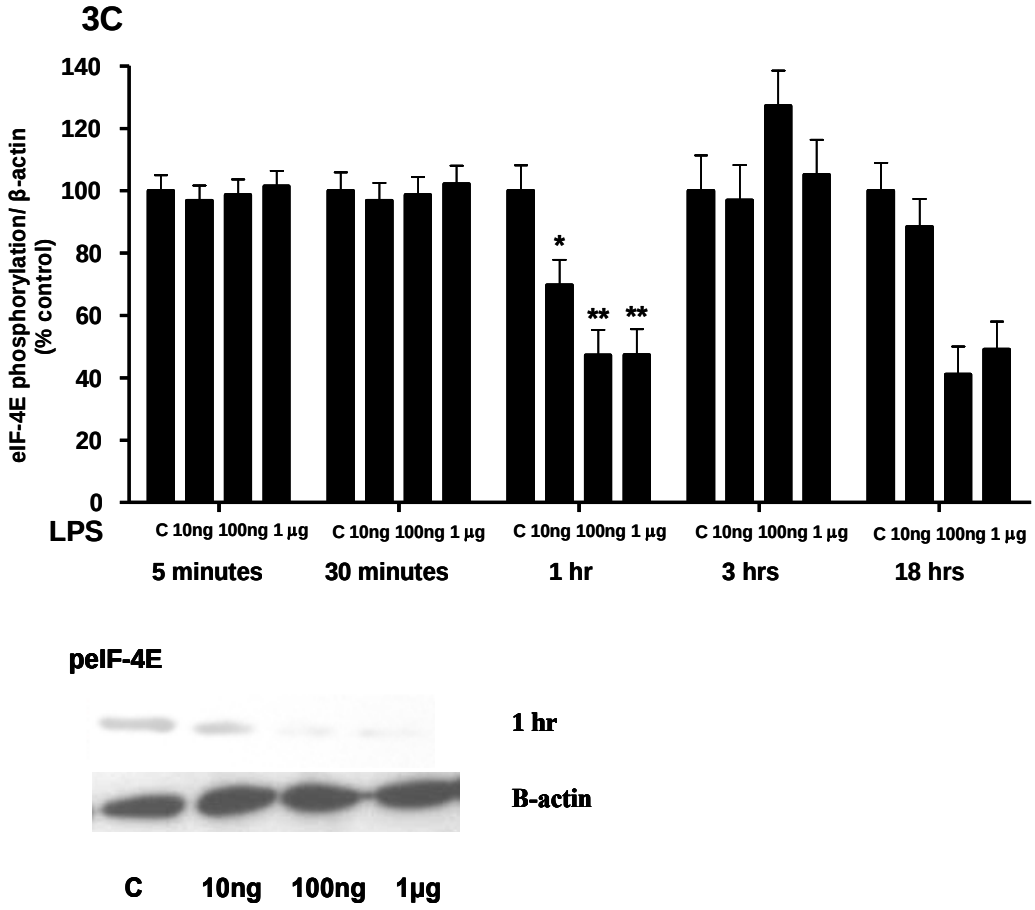


Figure (3-3) Time and dose dependent changes in **A**) phospho-P70^{S6K}; **B**) phospho-4E-BP1 and **C**) phospho-eIF-4E. C2C12 myotubes were incubated with LPS (10 ngml^{-1}, 100 ngml^{-1} and 1 µgml^{-1}) for (5, 30 min, 1, 3, or 18 h). Values represented as means ± SEM (percentage of control) normalised to β-actin as an internal loading control. n = 3. Bars carry asterisks are statistically different from control cells ($^{*}P < 0.05$ and $^{**}P < 0.01$). The representative blots illustrated the effect of LPS (10 ngml^{-1}, 100 ngml^{-1} and 1 µgml^{-1}) on the activity-related phosphorylation of p70^{S6K}, 4E-BP1 and eIF-4E at 30 min, 30 min and 1 hr time points respectively.

3.3.4 The effect of LPS on the Muscle cell PS rate

From the previous results, we can conclude that LPS significantly decreased the anabolic signalling pathway (Akt/mTOR and their down stream targets). Thus, the second aim was to measure the direct effect of LPS on the PS rate (expressed as incorporation of ^{3}H-phenylalanine per microgram of total trichloroacetic acid (5%TCA) Precipitable protein) after stimulation of C2C12 murine myotubes with LPS (1 $\mu g ml^{-1}$) for 3 h (short-term effect) and 18 h (long-term effect). Our result showed that at 3 h there was a significant decrease in PS rate by 49% ($P < 0.01$) compared with the control cells) (Fig. 3-4. Table.1) with no significant change was observed at 18 h time point (Fig. 3-6*A*).

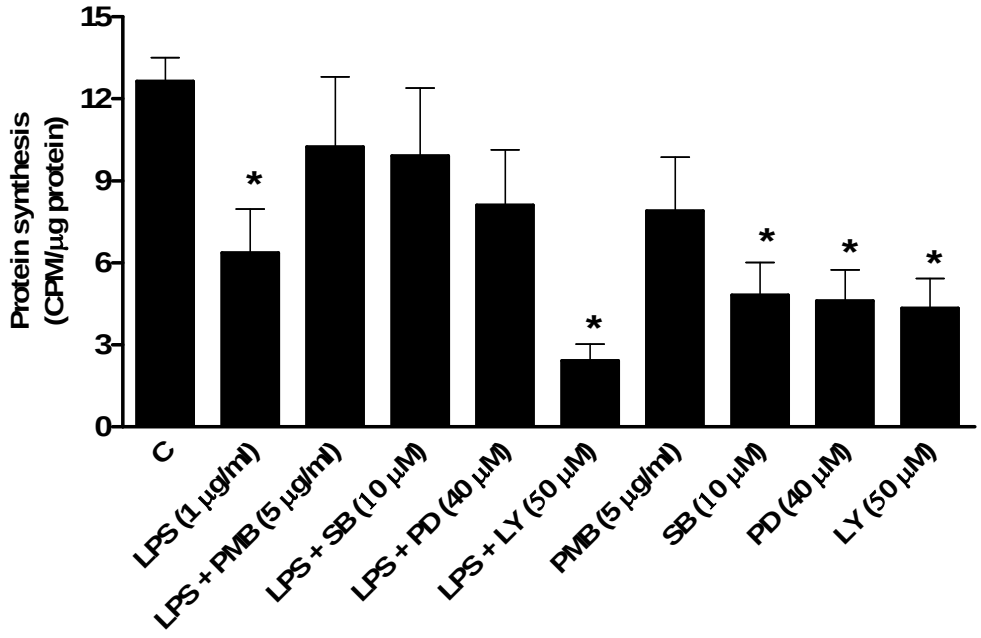


Figure (3-4) Changes in the protein synthesis (PS) counts/µg protein. C2C12 myotubes were incubated either with LPS (1 μgml^{-1}) alone or with LPS and Polymyxin B (LPS neutralising agent) (PMB, 5 μgml^{-1}), or LPS and SB203580 (p38 inhibitor) (SB, 10 µM), or LPS and PD098059 (MEK/Erk inhibitor) (PD, 40 µM), or LPS and LY0294002 (PI3K/Akt inhibitor) (LY, 50 µM), or with Polymyxin B (PMB, 5 μgml^{-1}), SB203580 (SB, 10 µM), PD98059 (PD, 40 µM), or LY0294002 (LY, 50 µM) alone for 3 h. The protein synthesis rate was determined by measuring the rate of ^{3}H-phenylalanine incorporation into the cell protein count per minute (CPM)/µg protein. Values represented as means ± SEM. Bars carry asterisks are statistically different from control cells ($^{*}P < 0.05$). n = 6

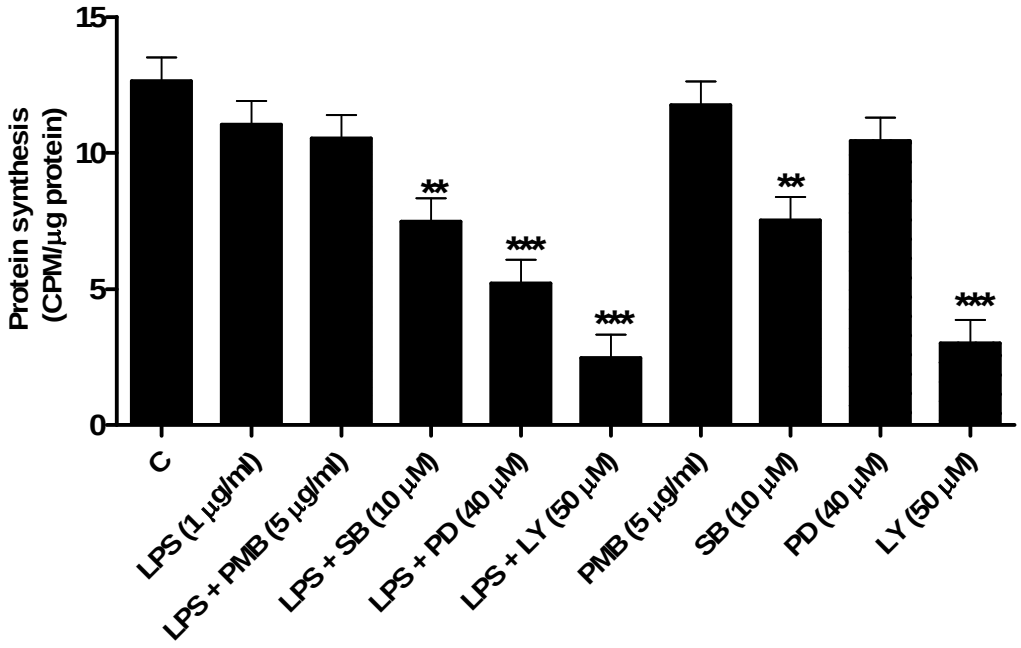


Figure (3-5) Changes in the protein synthesis (PS) counts/µg protein. C2C12 myotubes were incubated either with LPS (1 µgml^{-1}) alone or with LPS and Polymyxin B (LPS neutralising agent) (PMB, 5 µgml^{-1}), or LPS and SB203580 (p38 inhibitor) (SB, 10 µM), or LPS and PD098059 (MEK/Erk inhibitor) (PD, 40 µM), or LPS and LY0294002 (PI3-K/Akt inhibitor) (LY, 50 µM), or with Polymyxin B (PMB, 5 µgml^{-1}), SB203580 (SB, 10 µM), PD98059 (PD, 40 µM), or LY0294002 (LY,50 µM) alone for 18 h. The protein synthesis rate was determined by measuring the rate of ^{3}H-phenylalanine incorporation into the cell protein (count per minute (CPM)/µg protein. Values represented as means ± SEM. Bars carry asterisks are statistically different from control cells ($^{**}P < 0.01$ and $^{***}P < 0.001$). n = 6.

Table (3:1) the decrease percentage of PS rate compared to the control. C2C12 myotubes were incubated either with LPS (1 μgml^{-1}) alone or with LPS (1 μgml^{-1}) in combination with the inhibitors, Polymyxin B (PMB, 5 μgml^{-1}), SB203580 (p38 inhibitor) (SB, 10 μM), PD098059 (MEK/Erk inhibitor) (PD, 40 μM) and LY0294002 (PI3-K/Akt inhibitor) (LY, 50 μM) for 3 and 18 h. Values are represented as means ± SEM (percentage of control). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ represent the statistical difference compared with the control group.

Time point	Treatment				
	LPS	LPS+PMB	LPS+SB	LPS+PD	LPS+LY
3 h	49± 9.29%*	8 ± 15.54%	23±8.14%	39± 14.08%	81±7.42%**
18 h	14±9.93%	16±12.96%	41±7.2%**	59±2.06%***	80±1.91%***

Table (3-2) the decrease percentage of PS rate compared to the control. C2C12 myotubes were incubated with inhibitors Polymyxin B (PMB, 5 μgml^{-1}), SB203580 (p38 inhibitor) (SB, 10 μM), PD098059 (MEK/Erk inhibitor) (PD, 40 μM) or LY0294002 (PI3-K/Akt) (LY, 50 μM) alone for 3 and 18 h. Values are represented as means ± SEM (percentage of control). $P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ represent the statistical difference compared with the control group.

Time point	Treatment			
	PMB	SB	PD	LY
3 h	37±20.47%	63±12.48%**	64±5.05%**	66±6.04%**
18 h	8±11.08%	40±6.68%*	18±11.77%	76±2.065%***

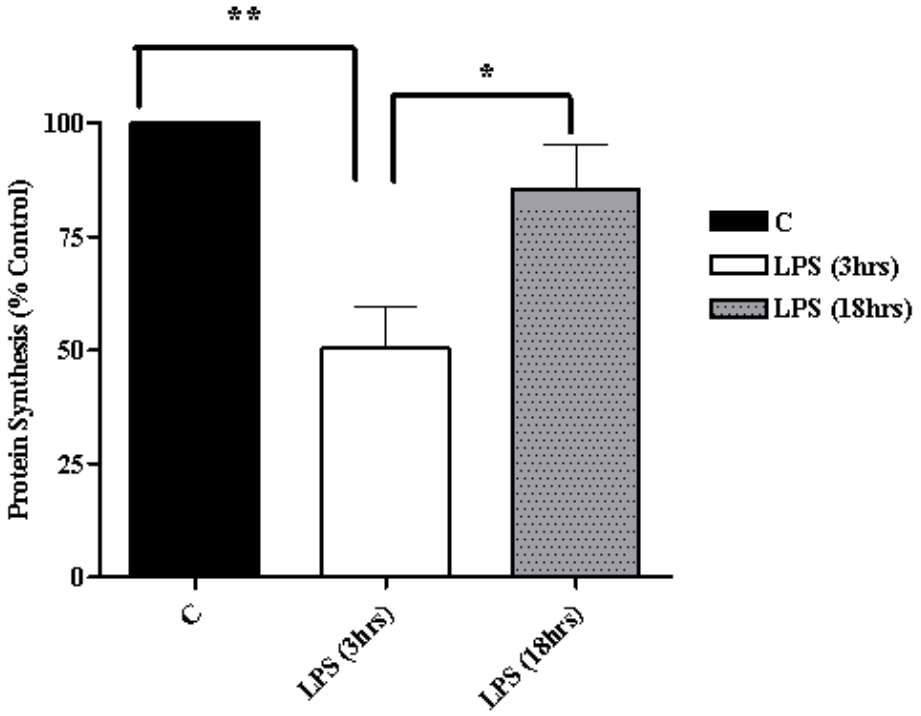


Figure (3-6) Changes in the protein synthesis (PS) measured as ^{3}H-phenylalanine incorporation/µg protein. C2C12 myotubes were incubated with LPS (1 µgml^{-1}) for 3 or 18 h. Values represented as means ± SEM (percentage of control). Bars carry asterisks are statistically different from control and 18 h LPS-treated cells (*P < 0.05 and $^{**}P$ < 0.01). n = 6

3.3.5 The effect of inhibitors on the muscle cell PS rate

The effect of LPS and signalling pathways was verified using specific pathway inhibitors. Polymyxin B (PMB, 5 μgml^{-1}); LY0294002 (LY, 50 μM), PD098059 (PD, 40 μM) and SB203580 (SB, 10 μM) were added to fully differentiated C2C12 alone or with LPS (1 μgml^{-1}). Incubation of C2C12 myotubes either with LPS in combination with PMB or with PMB alone showed no changes in the PS rate compared to the control level at both 3 h and 18 h time points (Figs. 3-4 and 3-5. Tables. 1 and 2). Incubation of C2C12 myotubes with LPS and SB203580 (Figs. 4 and 5. Table. 1 and 2) significantly decreased the PS at 18 h by 41% ($P < 0.01$), but not at 3 h, while incubation of cells with SB203580 alone significantly decreased protein synthesis at 3 h by 63% ($P < 0.01$) and at 18 h by 40% ($P < 0.05$) (Figs. 4 and 5. Table. 2). Incubation of cells with LPS and PD98059 had no effect on PS at 3 h (Fig. 4. Table. 1), while it significantly decreased the PS rate by 59% ($P < 0.01$) at 18 h time point (Fig. 5. Table. 1). In contrast, incubation of cells with PD98059 alone significantly decreased PS by 64% ($P < 0.05$) at 3 h time point (Fig. 4. Table. 2), with no further changes were observed at the 18 h time point (Fig. 3-5. Table. 2). Finally, incubation of cells with LPS with LY0294002 (LY, 50 μM) induced a significant reduction in PS by 81% ($P < 0.01$) at 3 h and by 80 % ($P < 0.001$) at 18 h compared with the control cells respectively (Figs. 3-4 and 3-5, Table. 1). Similarly, incubation of cells with LY0294002 (LY, 50 μM) alone induced a significant decrease in PS by 66% ($P < 0.01$) and 76% ($P < 0.001$) compared with the control untreated cells at 3 and 18 h time points respectively (Figs. 3-4 and 3-5. Table. 2).

The efficiency of inhibition was verified by the western blotting. Our western blotting results have shown that incubation of cells with LPS and LY0294002 or with LPS and PD098059 completely abolished the activity related phosphorylation of Akt and Erk1/2 with no effect on the total Akt or the total Erk1/2 respectively (Figs. 3-7*A*, 3-7*C* and 3-7*D*). While, incubation of

cells with LPS and SB203580 had no effect on the activity related phosphorylation of p38 (Figs. 3-7*B* and 3-7*D*). Interestingly, Incubation of cells either with LPS and SB203580 (p38 inhibitor) or with LPS and PD098059 (MEK/Erk1/2 inhibitor) significantly decreased the Akt phosphorylation ($P < 0.05$) (Figs. 3-7*A* and 3-7*D*) compared to the control cells. While, incubation of cells with LPS in combination with Polymyxin B (LPS neutralising agent) had no effect on the activity related phosphorylation of Akt, p38 and Erk1/2 compared with the control cells (Figs. 3-7*A*, 3-7*B*, 3-7*C* and 3-7*D*).

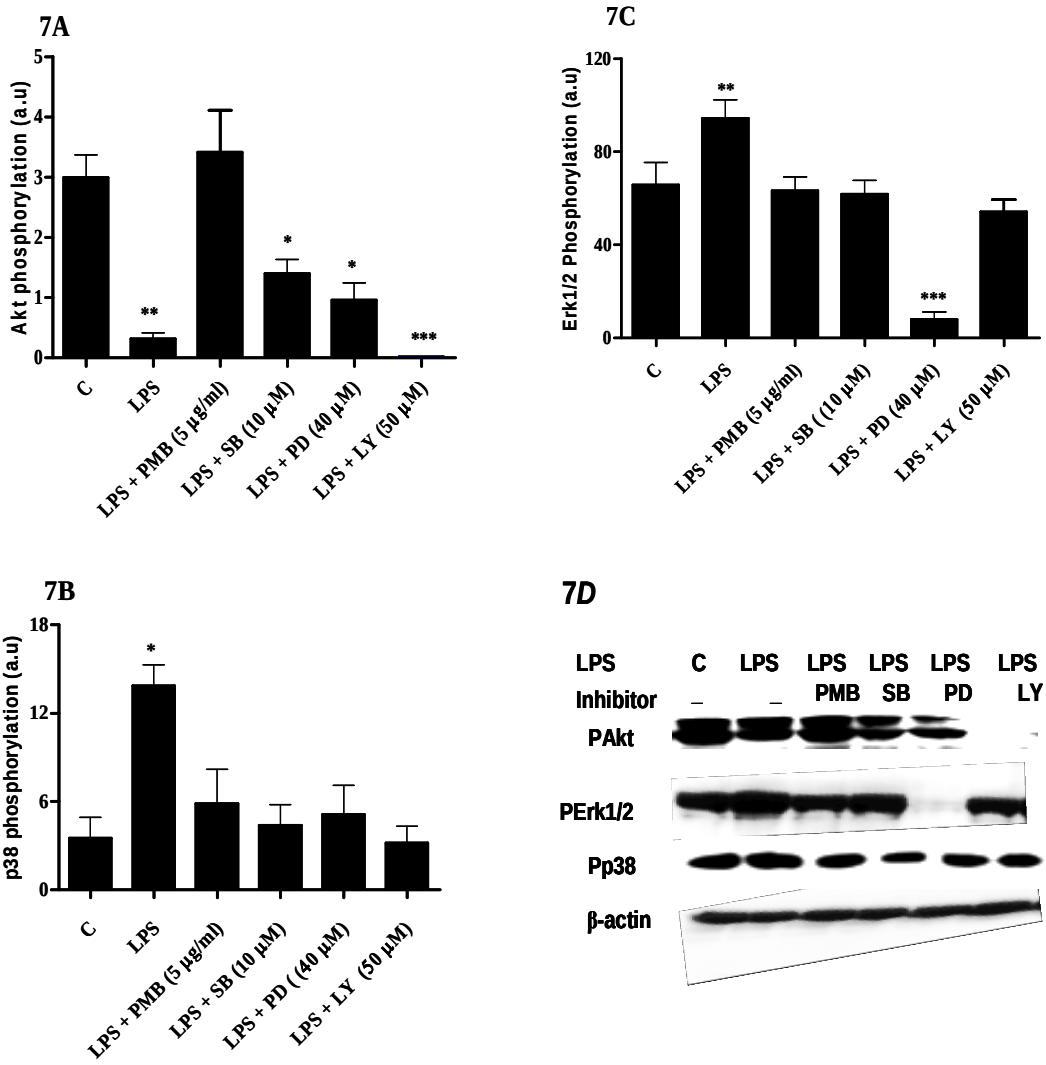


Figure (3-7) Changes in the **A**) phospho-Akt, **B**) phospho-p38, **C**) phospho-Erk1/2 and **D**)representative blots of phospho-Akt, Erk1/2, and p38 and β-actin as internal loading control. C2C12 myotubes were incubated either with LPS (1 μgml^{-1}) alone or with LPS and polymyxin B (LPS neutralising agent, 5 μgml^{-1}); LPS and SB203580 (p38 inhibitor, 10 µM); LPS and PD098059 (MEK/Erk inhibitor, 40 µM); or LPS and LY0294002 (PI3-K/Akt inhibitor, 50 µM). Values are represented as means ± SEM normalised to β-actin as an internal loading control. Bars carry asterisks are statistically different compared with control cells ($^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$). n = 4.

3.4 Discussion

Sepsis is an acute inflammatory condition characterized by accelerated rate of protein turnover and decreased protein synthetic rate (Hasselgren 1995). *In vivo* it has been shown that endotoxin induced a significant decrease in the rate of PS in the gastrocnemius muscle of the rat (Lang *et al.,* 2000). It has also been observed that sepsis itself significantly decreased the rate of PS in the gastrocnemius but not, however, in the slow-twitch soleus muscle (Vary and Kimball 1992). While, *in vitro* the effect of LPS was varied, with Frost *et al.,* (2009) showing no changes in PS rate when the LPS used alone in C2C12 differentiated myotubes, but the subsequent study of Russell *et al*., (2010) showing a significant decrease in the PS rate in response to LPS alone in the same cell line. Our data showed that LPS significantly decreased the PS rate in C2C12 fully differentiated myotubes by 50% but that this effect was transient (3 h) and the PS returned to the control levels by18 h time point. These findings are consistent with the findings of Russell *et al*. (2010). Studies in adult animal model of sepsis have shown that, LPS challenge significantly decreased the skeletal muscle protein synthesis, a reduction occurs as early as 4 h and maintained for 24 h (Vary and Kimball 1992; Lang *et al.,* 2000). While in case of *in vitro* model of sepsis, Russell *et al*. (2010) demonstrated that, endotoxin decreased skeletal muscle protein synthesis between at 4 h post LPS administration, while Frost *et al.,* showed that, LPS alone could alter the skeletal muscle protein synthesis rate either at 10 h or at 14 h time points (Frost *et al.,* 2009). Thus, we assume that the effect of endotoxin on the skeletal muscle protein metabolism is the same either *in vitro* or *in vivo*. LPS has been demonstrated to decrease muscle protein content most likely via decreasing the rate of PS and the translation efficiency (Jepson *et al.,* 1986; Ash and Griffin 1989; Macallan *et al.,* 1996; Cooney *et al.,* 1997). The effect of LPS was diminished by the LPS neutralizing agent polymyxin B (PMB) (Frost *et al.,* 2006). LPS significantly increased TLR4 mRNA in a time-dependent manner and Polymyxin B abolished this effect (Chapter 5, Figs. 5-4*A* and 5-4*B*). This

strongly suggesting that TLR-4 is the main signalling cascades involved in the LPS-signals transduction in our septic model (Kaisho and Akira 2000).

The process of PS divided into three distinct stages initiation, elongation and termination. Sepsis-induced inhibition of PS is a prime result of the decrease in the translation efficiency not the decrease in the number of ribosome (Vary and Kimball 1992; Cooney *et al.,* 1999; Lang *et al.,* 2000). The process of mRNA translation regulation is primarily the function of a group of eIFs. The active eIF-4F complex controls the peptide-chain initiation by regulating the recruitment of the 43S pre-initiation complex to mRNA (Pain 1996). The formation of the active eIF-4F complex depends on the availability of eIF-4E, and the interaction between of eIF-4G and eIF-4E is controlled partially by the eIF-4E binding protein (BP) 1 (Hara *et al.,* 1997). Increased 4E-BP1 phosphorylation releases it from eIF-4E, and this facilitates the formation of active eIF-4F complex (Lang and Frost 2005). Herein, LPS significantly decreased the basal phosphorylation of 4E-BP1 and subsequently decreased the availability of eIF-4E. These changes in 4E-BP1 and eIF-4E phosphorylation are suggestive of a fall in PS and similar to changes observed *in vivo* (Vary and Kimball 1992).

The activation of $p70^{S6K}$ kinase is essential to obtain a normal muscle fibre size (Ohanna *et al.,* 2005). A significant time and dose-dependent decrease in activity-related phosphorylation of $p70^{S6K}$ is observed in response to LPS administration (Fig. 3-3*A*) which correlates well with *in vivo* studies (Vary and Kimball 1992; Lang *et al.,* 2000) but is in contrast to the finding of Frost *et al.* (2009) who observed no such decrease in response to LPS alone. Combined, LPS decreased the phosphorylation of 4E-BP1 and this effect was associated with an increased amount of the inactive 4E-BP eIF-4E complex. In addition, LPS significantly decreased $p70^{S6K}$ phosphorylation. These findings are consistent with the previous findings the previously reported *in vivo* (Lang *et al*., 2000; Lang and Frost 2005). Thus, LPS significantly decreased PS rate

either *in vivo* or *in vitro* via the alteration of the translation initiation of mRNA (Vary and Kimball 1992; Cooney *et al.,* 1999).

Upstream regulators of protein synthesis are known to include Akt and the MAPKs (p38 or Erk1/2). Although, in the present study, LPS significantly decreased the phosphorylation of Akt for only 30 min, whereas, its downstream target mTOR exhibited a decreased activity-related phosphorylation over a longer time period, this suggests that LPS mediated its effect on PS mainly through the Akt/mTOR and their down-stream targets including $p70^{S6K}$, eIF-4E and 4E-BP1 (Gingras *et al.,* 1999; Lang *et al.,* 2000; Bodine *et al.,* 2001; Orellana *et al.,* 2002; Lang and Frost 2005). Decreased mTOR phosphorylation has been previously shown in many *in vitro* studies. Lang and Frost (2005) have demonstrated that administration of LPS significantly decreased the basal phosphorylation of mTOR by 50% in rat skeletal muscle. Therefore, mTOR is considered as one of the LPS downstream targets molecule, and any alteration of mTOR activity acts as a point of bifurcation leading to stimulation or inhibition of protein synthesis mostly likely via 4E-BP1 (Gingras *et al.,* 2001). In the same study they have demonstrated that one of the downstream targets of mTOR is the 4E-BP1. Combined, these data indicate that mTOR is one of the key elements regulating the protein synthesis. mTOR is involved in LPS-induced alteration of translation initiation via decreased phosphorylation of the translation initiation repressor (4E-BP1). Conversely, Frost and associates have shown that LPS alone could not abolish mTOR phosphorylation or protein synthesis in myocytes (Frost *et al.,* 2004). In order to further investigate the role of Akt inhibition on PS, we used LY0294002 (PI3-K/Akt inhibitor) (Plaisance *et al.,* 2008). Incubation of C2C12 myotubes either with LPS and LY0294002 or with LY0294002 alone dramatically decreased the PS rate (Figs. 3-4 and 3-5), indicating that Akt plays a key role in the regulation of PS in skeletal muscle. Decreased Akt total protein has been seen in rodent model of sepsis (Crossland *et al.,* 2008) as well as in C2C12 model of sepsis (Sandri *et al.,* 2004; Stitt *et al.,* 2004). Akt is a well known upstream regulator of translation

initiation, increased Akt phosphorylation increases both eIF-2B and eIF-4F assembly via the phosphorylation and subsequent inactivation of GSK-3β and 4E-BP1 (Nader *et al.,* 2002). Therefore, the dogma is, LPS significantly mediated decreased skeletal muscle protein synthesis *in vivo* and *in vitro* via the same mechanism (decreases the protein translation efficiency).

The MAPK family is considered a fundamentally important ubiquitous intracellular signalling system involved in the regulation of many cellular processes including cell growth, differentiation and survival (Seger and Krebs 1995). In the present study, LPS significantly increased the p38 and Erk1/2 phosphorylation at all time points in a dose-dependent manner (Figs. 3-2*A* and 3-2*B*). Increased muscle p38 phosphorylation has been shown in many pro-catabolic conditions including acute quadriplegic myopathy (Di Giovanni *et al.,* 2004), neurogenic atrophy (Di Giovanni *et al.,* 2004), aging (Williamson *et al.,* 2003) and sepsis (Li *et al.,* 2005; Jin and Li 2007). Although similar increases in p38 phosphorylation have been observed in rats administered LPS, a diminished Erk1/2 and p38 phosphorylation was found by vary *et al*., (2004) in which chronic abdominal sepsis had been induced. Interestingly however, in the later study, these falls in Erk1/2 and p38 phosphorylation were prevented when the activity of TNF-α was inhibited by the administration of TNF binding protein to the rats with sepsis. One of the downstream targets of both p38 and Erk1/2 MAP kinases is Mnk1 (Fukunaga and Hunter 1997; Waskiewicz *et al.,* 1997). Mnk1 has been shown to be involved in the process of PS in the main via its down-stream target eIF-4E (Gingras *et al.* 1999). The data presented in Figures (3-2*A*, *B* and *C*) have shown that, LPS significantly increased p38 and Erk1/2 phosphorylation and this effect was associated with increased basal phosphorylation of Mnk1, suggesting that Mnk1 considered as one of the MAPKs (p38 or Erk1/2) substrates (Fukunaga and Hunter 1997; Waskiewicz *et al.* 1997). Although, LPS increased the phosphorylation of MAPKs (p38 or Erk1/2) and Mnk1, but this effect was not sufficient to prevent the LPS-induced significant decrease in PS at the 3 h time point. The most important step in the translation is the

formation of cap-binding complex, and this step is depends upon the formation of eIF-4F complex (eIF-4E/eIF-4G). Increased eIF-4E phosphorylation enhanced its binding with eIF-4G, and this process is enhanced by hyperphosphorylation of 4E-BP1. Herein, LPS underphosphorylated 4E-BP1, this impair the formation of eIF-4F complex, and at this point phosphorylation of eIF-4E cannot occur because the eIF-4E is no longer a substrate for Mnk1 reviewed in (Pyronnet 2000). Our data showed that inhibition of MAPKs (p38 or Erk1/2) activity by SB203580 or PD98059 alone respectively significantly decreased the rate of PS (Figs. 3-4 and 3-5), suggesting that MAPKs directly involved in the regulation of PS preassembly via Mnk1/eIF-4E cascade. The involvement of the MAPK in the regulation of PS has been shown in several studies (Kimball *et al.,* 1998; Kleijn and Proud 2000).

MAPK regulates PS in two possible ways. First, MAPK can phosphorylate the eIF-4E via Mnk1 and eIF-4E phosphorylation regulates the translation initiation (Waskiewicz *et al.*, 1997). Secondly, MAPK may be involved in the regulation of the formation of eIF-4F complex (Kleijn and Proud 2000). Surprisingly, incubation of the cells with both SB203580 (p38 inhibitor) and PD098059 (MEK/Erk inhibitor) alone significantly decreased the PS rate, but in combination with LPS, no such decrease in the rate of PS has been observed at the 3 h time point. The possible explanation, in normal conditions, the PS maintained at the basal level (control), but when the cells incubated with SB203580 and PD98059 alone, it significantly decreased PS rate. This effect was probably mediated via decreased Mnk1 and eIF-4E basal phosphorylation (Waskiewicz *et al.* 1997). Incubation of C2C12 myotubes with PD098059 alone was associated with decreased eIF4G phosphorylation and consequently eIF4E:eIF4G association formation and this effect was associated with decreased PS (Williamson *et al.,* 2005). Whereas, in case of combination with LPS, LPS significantly increased MAPK phosphorylation and subsequently Mnk1 phosphorylation. Although, this effect was not sufficient to inhibit LPS-induced decreased PS rate, but this is somewhat modulates the effect of LPS

on PS rate. There is no absolute correlation between the eIF-4E phosphorylation and PS. In addition, there is no absolute correlation between the activity of p38 and eIF-4E phosphorylation. Arsenite and anisomycin exposure diminishes the translation rate, while eIF-4E phosphorylation is increased (Wang *et al.,* 1998). This eIF-4E increased phosphorylation can be attributed to stress-induced p38 increased activity (Pyronnet 2000). In contrast, other cellular stress including heat shock, are accompanied by a decrease in eIF-4E despite of p38 increased activity (Duncan *et al.,* 1987). Nevertheless, it remains unclear and needs further investigation, whether MAPK regulates PS by mechanism(s) other than the regulation of Mnk1 and eIF-4E phosphorylation (Shen *et al.* 2005).

The cross talk between the PI3-K/Akt and MAPKs (p38 or Erk1/2) pathways has been demonstrated in many tissues (Rommel *et al.* 1999; Hausenloy *et al.* 2004; Drummond *et al.* 2009). Inhibition of mTOR by rapamycin significantly decreased the Erk1/2 phosphorylation and the activation of both mTORC1 and Erk1/2 signalling pathways is critical for stimulation of protein synthesis in human skeletal muscle (Drummond *et al.,* 2009). In addition, the activation of the Erk1/2 activates and phosphorylates rpS6 ser235/236 leading to enhanced translation initiation (Pende *et al.,* 2004; Roux *et al.,* 2007). The western blotting presented in (Figs. 3-7*A*, *B*, C and *D*) showed that, Incubation of C2C12 myotubes with LPS in combination with LY0294002 significantly blocked Akt phosphorylation with no effect on the total Akt (Figs. 3-7*A* and 3-7*D*). Similarly, incubation of C2C12 myotubes with LPS plus PD98059 (MEK/Erk inhibitor) significantly blocked Erk1/2 phosphorylation with no such effect on the total Erk1/2. In contrast, incubation of cells with LPS plus SB203580 (p38 inhibitor) could not diminished LPS-induced p38 phosphorylation, with no such effect on Akt and Erk1/2 basal phosphorylation. This finding suggesting that, SB203580 had no effect on p38 phosphorylation but it mainly acted through the inhibition of the p38 downstream activity Kumar *et al.* (1999). The data presented in this chapter showed that, LPS alone significantly decreased the protein synthetic

rate in C2C12 myotubes (measured as ^{3}H-phenylalanine incorporation/μg 5% TCA precipitable protein) by 50%, and this effect was mediated most likely via Akt/mTOR signalling cascade and the alteration of the translation initiation efficiency. However, the direct involvement of MAPKs (p38 or Erk1/2) in the regulation of global PS has been shown.

3.5 Conclusion

The data presented in this chapter are considered important because it companies the effect of LPS on both the anabolic signalling and the direct protein synthetic rate. These data showed that, LPS mediated the 50% decrease in the global PS rate most likely via decreasing the translation initiation efficiency. The effect of LPS on PS was similar either *in vivo* or *in vitro*. The direct involvement of the MAPK in the regulation of PS and the cross talk with PI3-K/Akt signalling pathway has been shown. Yet, further investigation are required to elucidate the mechanism of MAPKs (p38 or Erk1/2) regulating PS.

4 LPS-induced the proteasomal activity in C2C12 myotubes via MAPK signalling pathway

4.1 Introduction

Skeletal muscle comprises 60-70% of the total lean body mass, and is considered as the main pool for the amino acids as an energy substrate in case of increased need in catabolic conditions including sepsis. The decrease in the lean body mass in case of acute catabolic conditions such as sepsis, cancer and AIDS (Hasselgren 1995; Tisdale 1997), is most likely due to a reduction in the rate of muscle protein synthesis and/or an increased rate of protein breakdown (Jepson *et al.,* 1986; Hummel *et al.,* 1988; Ash and Griffin 1989; Hasselgren *et al.,* 1989; Lai *et al.,* 2004), with the latter being the predominant mechanism in most catabolic conditions. Sepsis is an inflammatory condition which causes severe and rapid loss of body protein, much of which originates from skeletal muscle (Rosenblatt *et al.* 1983; Jin and Li 2007). Accelerated muscle protein degradation in the case of sepsis provides the necessary amino acids to form the acute phase protein in liver (Frost and Lang 2008).

The main catabolic system involved in the muscle protein turnover is the ubiquitin-proteasome pathways (UPS) (Jagoe and Goldberg 2001). Degradation by this mechanism involves marking of the target protein by four ubiquitin molecules, followed by breakdown in the central core of the 26S proteasome (Hershko and Ciechanover 1998). The physiologically active form of the ubiquitin-proteasome system is the 26 S proteasome. 26S proteasome is a large proteolytic complex that hydrolyses the protein conjugate, which composed of the 19S cap structure attached to the 20S core (Hayter *et al.,* 2005). The 20S (700kDa) proteasome is the active proteolytic machinery in

which the proteolysis takes place independently of the presence of ATP. The 20 S proteasome has five main catalytic activities including the chymotrypsin-like activity (Fenteany and Schreiber 1996). The chymotrypsin-like enzyme activity is considered as the major proteolytic activity of the 20S proteasome (Gomes-Marcondes *et al.,* 2002). Increased ubiquitin-proteasome activity has been reported in many catabolic diseases including sepsis (Jin and Li 2007), and the use of specific proteasome inhibitor (MG-132) has been used to inhibit the muscle proteolysis in many muscle-wasting conditions (Tawa *et al.,* 1997), providing a further evidence of the importance of ubiquitin-proteasome system in sepsis.

LPS-induced muscle wasting involves the input of many transcriptional factors and signalling pathways including Akt and MAPKs (p38 or Erk1/2) and NF-kB. The PI3-K/Akt pathway is considered as a key regulator of both muscle protein synthesis and degradation signals (reviewed by Glass and Wu (Glass 2005; Wu *et al.,* 2010). Anabolic stimuli such as insulin and IGF-I activate Akt, which leads to suppression of atrogin-1 and MuRF1 expression via a Foxo-dependent pathway. Conversely, catabolic signals such as glucocorticoids deactivate Akt leading to up-regulation of atrogin-1 and MuRF1 expression (Lai *et al.,* 2004; Sandri *et al.,* 2004). Atrogin-1 and MuRF1 are E3 ubiquitin ligases secreted mainly by skeletal muscle, and their up-regulation has been demonstrated in many atrophic models including sepsis (Bodine *et al.,* 2001; Jin and Li 2007). Inflammatory mediators stimulate the activity of atrogin-1 and muRF1 via many signalling cascades including Akt and MAPK streams. Reduced Akt activity during starvation in the absence of growth factors and in sepsis, leads to removal of Foxo inhibition and an increase in atrogin-1 and MuRF1 mRNA expression (Sacheck *et al.,* 2004; Stitt *et al.,* 2004; Crossland *et al.,* 2008). Thus, the PI3-K/Akt pathway is generally considered a key regulator of not only the muscle protein breakdown but also protein synthesis and muscle fibre size (Rommel *et al.,* 2001; Stitt *et al*., 2004; Glass 2005).

The regulation of the atrogin-1 and MuRF1 is in a part regulated by MAPK activity mainly p38 MAPK. The MAPK family is considered the most important intracellular signalling system involved in the regulation of many processes such as cell growth, differentiation and survival (Seger and Krebs 1995). p38 MAPK has been recognised as one of the MAPKs signalling factors that is activated in response to environmental stress factors (Lang and Frost 2005), including ultraviolet light (Han *et al.,* 1994; Yeow *et al.,* 2002), osmotic stress (Moriguchi *et al.,* 1995; Shapiro and Dinarello 1995), heat shock and inflammatory cytokines (Haneda *et al.,* 1995; Raingeaud *et al.,* 1995; Karem *et al.,* 1996; Kohn *et al.,* 1996; Modur *et al.,* 1996; Cuenda *et al.,* 1997; Hajduch *et al.,* 1998; Igarashi *et al.,* 1999; Hong *et al.,* 2006). Increased p38 phosphorylation and NF-kB translocation is another possible mechanism which has been implicated in LPS-induced muscle wasting in many catabolic conditions. TNF-α and IL-1β, are two known mediators of muscle catabolism mediating the muscle wasting via p38 and NF-kB (Jackman and Kandarian 2004). Increased p38 activity has been reported in a number of pro-catabolic conditions including sepsis (Childs *et al.,* 2003; Koistinen *et al.,* 2003; Di Giovanni *et al.,* 2004). P38 mediated TNF-α up-regulation of atrogin-1 mRNA in C2C12 myotubes (Li *et al.,* 2005), and in septic skeletal muscle of rat (Jin and Li 2007) and SB203580 (p38 inhibitor) significantly diminished this effect. Another MAPK, Erk1/2 has also been implicated in the cellular proliferation and differentiation (Olson 1992; Pages *et al.,* 1993), and it has been recognised as a part of the early biochemical events that are initiated by LPS treatment of macrophages or their infection by virulent and attenuated *Salmonella* strains (Procyk *et al.,* 1999). TNF-α is a well known catabolic factor that has been shown to activate Erk (Tantini *et al.,* 2002). Mnk1 is one of the downstream targets of both p38 and Erk activation *in vitro* (Fukunaga and Hunter 1997; Waskiewicz *et al.* 1997; Scheper and Proud 2002) and several studies have demonstrated that one of the Mnk1 substrates is eIF-4E (Gingras *et al.* 1999). Therefore, the activation of Erk1/2 increased the protein synthesis in cultured C2C12 myotubes via the activation Mnk1 (Plaisance *et al.,* 2008). Taken together, the activation of Erk1/2 has been

implicated in many cellular processes including the regulation of protein synthesis. in the data presented in chapter 3 showed that, LPS (1 μgml^{-1}) alone transiently decreased the muscle protein synthetic rate in C2C12 myotubes by 50% at 3 h time point, and this effect mediated via Akt and MAPKs (p38 or Erk1/2). In contrast, the direct contribution of MAPKs (p38 or Erk1/2) in LPS-induced muscle proteolysis still unclear and needs further investigation.

NF-kB is a member of the transcriptional protein family that regulates the expression of many genes involved in a broad range of cellular processes including, immune responses, cell growth and cell death (Baeuerle and Baltimore 1996; Karin 1998). There is considerable evidence that NF-kB stimulates the protein loss via the UPS (Li and Reid 2000; Li *et al.,* 2003), mainly through the action of MuRF1 (Cai *et al.,* 2004). The NF-kB is located in the cytoplasm bound to its inhibitory protein I*K*B-α. TNF-α stimulated the nuclear translocation of the NF-kB in the differentiated myotubes via the activation and later proteasomal degradation of the I*K*B-α (Li *et al.,* 1998), and LPS significantly increased the NF-kB activation and binding in skeletal muscle (Penner *et al.* 2001; Jin and Li 2007).

The involvement of complex signalling cascades in LPS-induced muscle protein breakdown makes the overall elucidation of these mechanism difficult. Therefore, the better understanding of these mediators and their contributions in muscle protein breakdown is important for developing successful therapies to retard the loss in lean body mass and lessen morbidity and mortality (Lai *et al.,* 2004). Moreover, there is a scarcity of data regarding the effect of these signalling modulations on the LPS-induced proteasomal activity (measured as chymotrypsin-like enzyme activity) *in vitro*. Therefore, we addressed the hypothesis that, LPS-increased the proteasomal degradation of muscle protein via Akt and MAPKs (p38 or Erk1/2). Furthermore, we anticipated that inhibition of these pathways will affect the proteasomal activity. In this study, LPS-treated C2C12 myotubes for a period

ranged from 5 min to 18 h were used. This model has been used to evaluate the effect of endotoxin on muscle protein degradation, and the possible contribution of various signalling cascades mainly Akt and MAPKs (p38 or Erk1/2).

4.2 Hypothesis

The data presented in chapter 3 have shown that, LPS transiently decreased PS in C2C12 myotubes and this effect was mediated via the two main signalling cascades PI3-K/Akt and the MAPKs (p38 or Erk1/2). In addition, the data collected from previous studies have shown that, LPS significantly decreased Akt activity and this was associated with increased Foxo activity, the major transcription family factors involved in the activation of the muscle proteolysis. Furthermore, increased p38 activity *in vivo* has been shown to mediate the muscle proteolysis via activation of atrogin-1. Therefore, the aim in this chapter is to elucidate the direct effect of LPS on muscle proteolysis (measured as chymotrypsin-like enzyme activity), and the role of PI3-K/Akt and MAPKs (p38 or Erk1/2) was verified by specific pathway inhibitors. LPS (1 $\mu g ml^{-1}$) stimulated C2C12 myotubes have been used to test our hypothesis.

4.3 Results

4.3.1 LPS significantly increased the chymotrypsin-like enzyme activity

LPS (1 μgml^{-1}) significantly increased the chymotrypsin-like enzyme activity ($P < 0.01$) compared with the control untreated cells at 5, 30 min, 1, 3 and 18 h, with a peak at 30 min and 1 h time points (Fig. 4-1*A*. Table. 4-1). While, incubation of cells with LPS plus proteasomal inhibitor (MG-132, 40 µM) significantly inhibited the chymotrypsin-like enzyme activity by 80% ($P < 0.001$) compared with the control cells at all the time points (Fig. 4-1*B*. Table. 4-1). Incubation of the cells with LPS in combination with Toll-like receptor 4 inhibitor (Polymyxin B, 5 μgml^{-1}) completely prevented the LPS-induced chymotrypsin-like enzyme activity at all time points except at 30 min (Fig. 4-1. Table. 4-1).

4.3.2 PI3-K inhibitor (LY0294002) delayed the LPS-induced proteasomal activity

Incubation of the C2C12 myotubes with LPS (1 μgml^{-1}) and LY0294002 (LY, 50 µM) prevented the increased chymotrypsin-like enzyme activity observed in response to LPS alone at 5 and 30 min time points. At later time points (1, 3, and 18 h) LPS still induced a significant increase in the proteasomal activity compared with control cells despite the presence of LY0294002 (Fig. 4-1*D*. Table. 4-1).

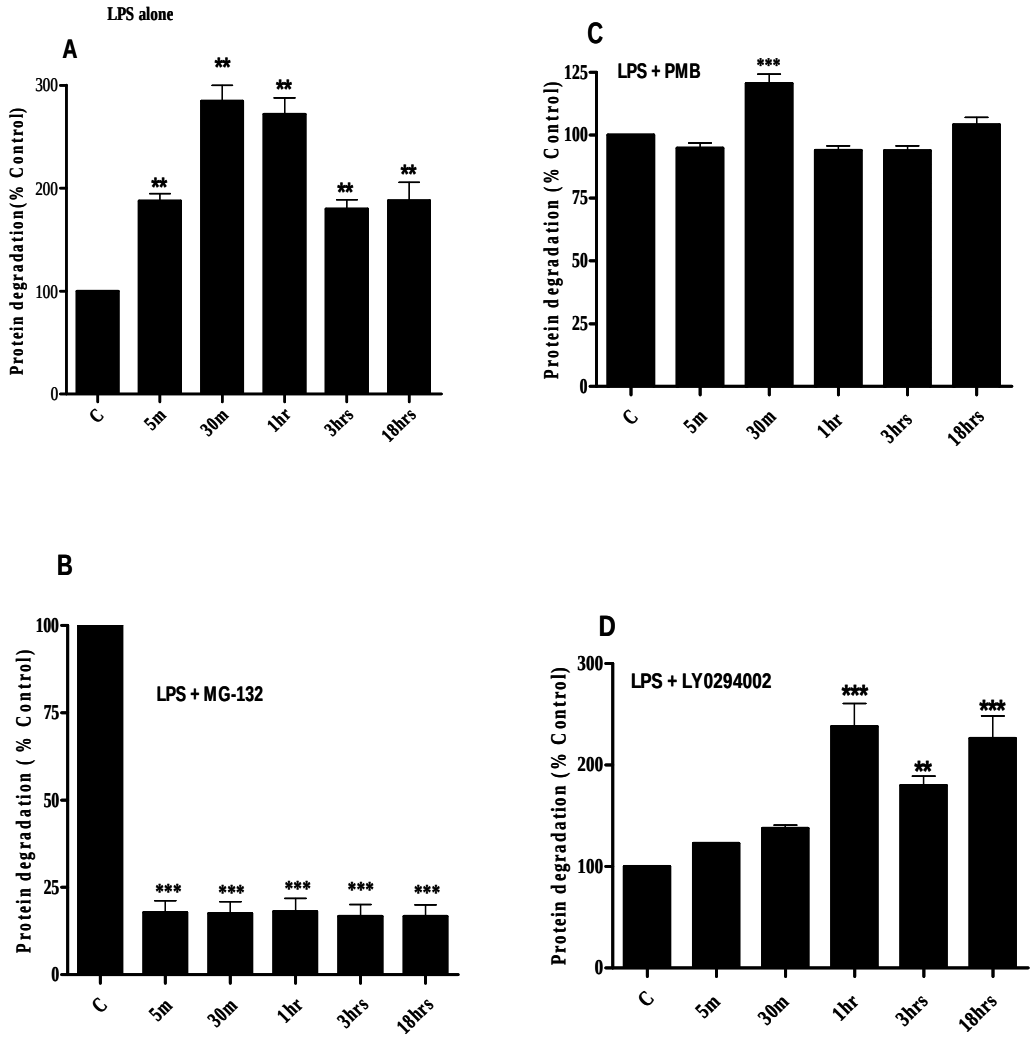


Figure (4-1) Time-dependent changes in chymotrypsin-like enzyme activity (proteasomal activity). C2C12 myotubes were incubated either with **A**) LPS (1 μgml^{-1}) alone; **B**) LPS (1 μgml^{-1}) and MG-132 (40 µM); **C**) LPS (1 μgml^{-1}) and Polymyxin B (LPS neutralising agent) (PMB, 5 μgml^{-1}); **D**) LPS (1 μgml^{-1}) and LY0294002 (PI3K/Akt inhibitor) (Ly, 50 µM) for 5, 30 min, 1, 3, or 18 h. Values are represented as means ± SEM (percentage of control). Bars carry asterisks are statistically different from the control cells ($^{**}P < 0.01$ and $^{***}P < 0.001$).n = 6

4.3.3 Inhibition of the MAPKs (p38 or Erk1/2) significantly decreased the LPS-induced proteasomal activity

Incubation of the C2C12 myotubes either with LPS (1 $\mu g ml^{-1}$) and p38 inhibitor (SB203580, 10 μM), or with LPS and MEK/Erk inhibitor (PD098059, 40 μM) significantly decreased the chymotrypsin-like enzyme activity ($P < 0.001$) compared with the control cells at all time points (Figs. 4-2*A*, 4-2*B*. Table. 4-1). In contrast, incubation of C2C12 myotubes either with SB203580 (SB, 2, 5 and 10 μM) and PD098059 (PD, 10, 20 and 40 μM) alone hade no effect on the chymotrypsin-like enzyme activity (Fig. 4-3).

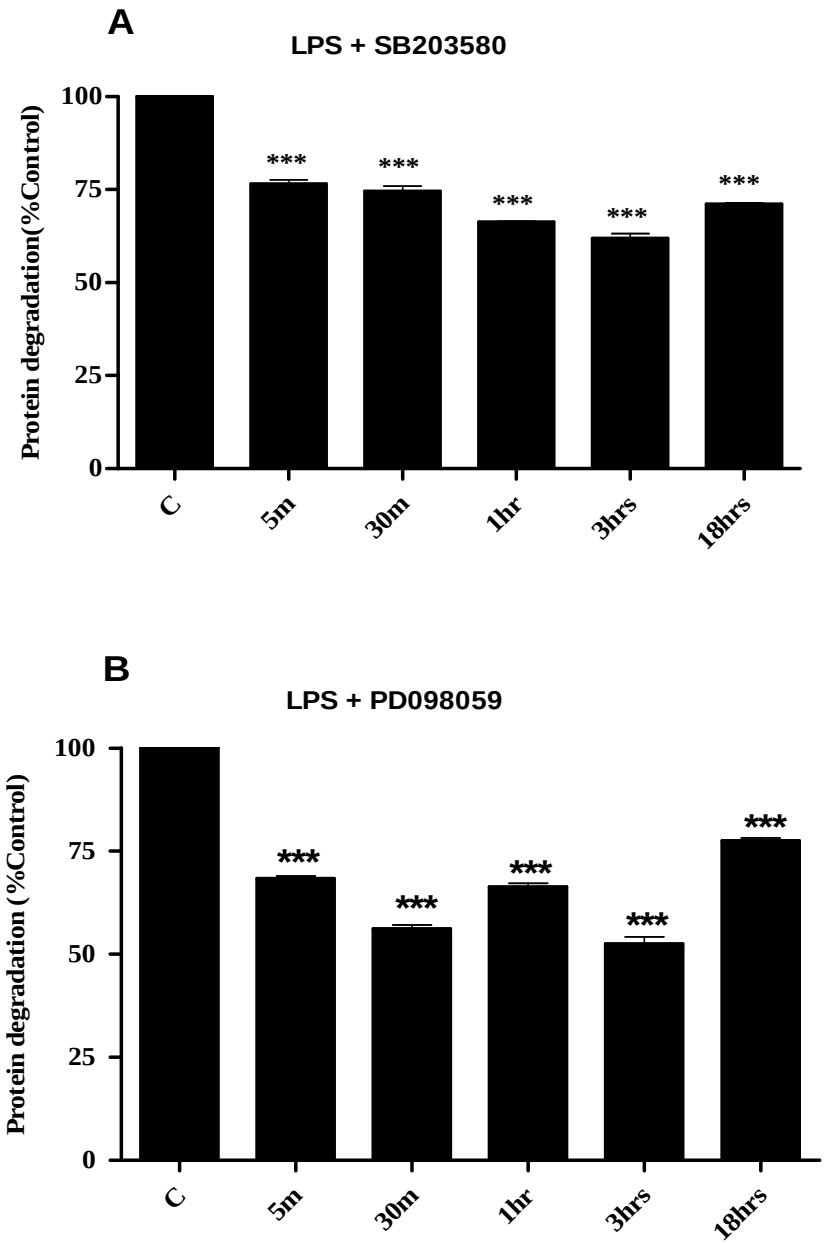


Figure (4-2) Time-dependent changes in the chymotrypsin-like enzyme activity (proteasomal activity). C2C12 myotubes were incubated either with **A**) LPS (1 μgml^{-1}) and SB203580 (P38 inhibitor) (SB, 10 μM); or **B**) LPS (1 μgml^{-1}) and PD098059 (MEK /Erk1/2 inhibitor) (PD, 40 μM) for 5, 30 min, 1, 3, or 18 h. Values are represented as means ± SEM (percentage of control). Bars carry asterisks are statistically different from the control cells ($^{***}P < 0.001$). n = 6

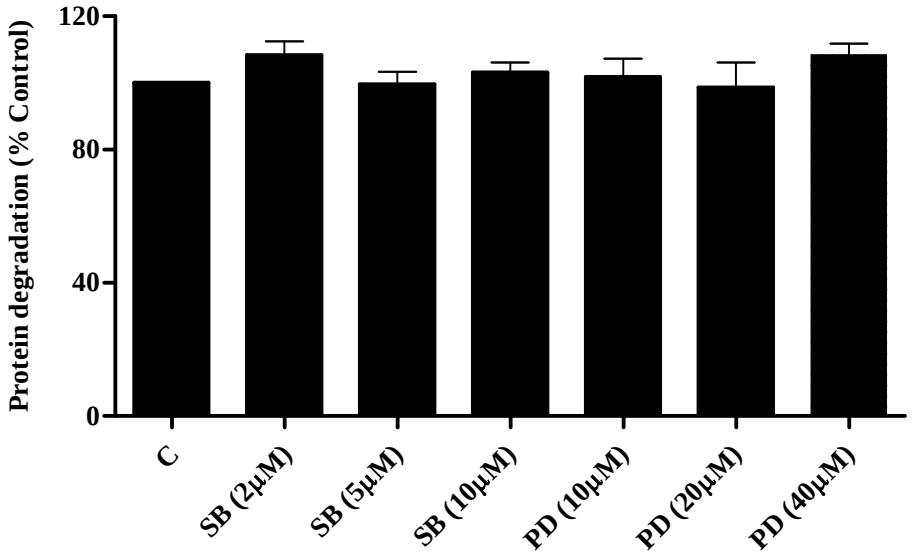


Figure (4-3) The dose-dependent changes in the chymotrypsin activity (proteasomal activity). C2C12 myotubes were incubated either with SB203580 (p38 inhibitor) (SB, 2, 5 and 10 μM) or PD098059 (MEK/Erk1/2 inhibitor) (PD, 10, 20 and 40 μM) alone for 3 h. Values are represented as means ± SEM (percentage of the control). n = 6

4.3.4 LPS significantly increased the media concentration of TNF-α and IL-6

Incubation of the C2C12 myotubes with LPS (1 $\mu g ml^{-1}$) significantly increased the level of TNF-α by 94 % ($P < 0.05$), 212% ($P < 0.05$), 287% ($P < 0.01$), 184% ($P < 0.01$) and 103% ($P < 0.05$) at 5 min, 30 min, 1 h, 3 h and 18 h time points respectively compared with the control cells (Fig. 4-4*A*). In contrast, LPS (1 $\mu g ml^{-1}$) transiently increased the media concentration level of IL-6 only at the 18 h time point by 169% ($P < 0.001$) compared with the control cells, and this effect is significant compared with the LPS-treated cells (Fig. 4-4*B*).

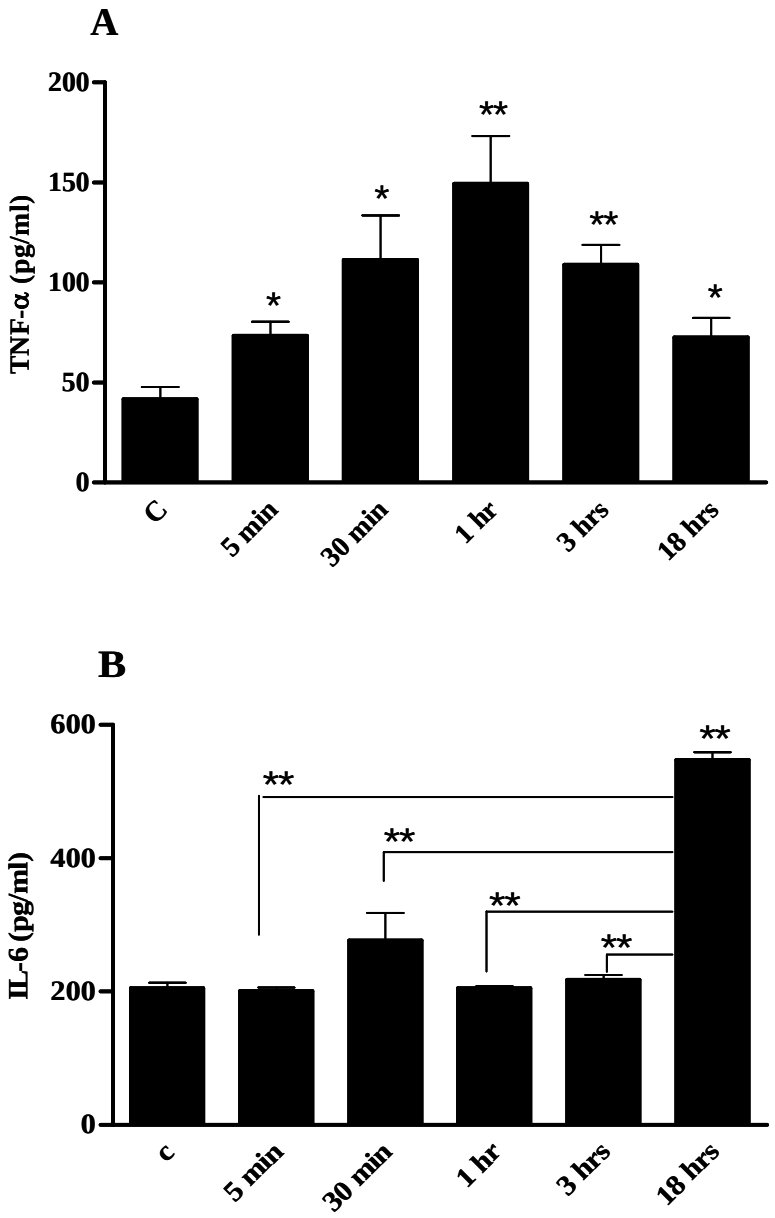


Figure (4-4) The changes in concentration levels of **A**) TNF-α and **B**) IL-6 cytokines levels. C2C12 were incubated with LPS (1 μgml^{-1}) for (5, 30 min, 1, 3 and 18 h). The conditioned media were then collected and the concentration levels of TNF-α and IL-6 was assessed by ELISA. Values are represented as means ± SEM. Bars carry asterisks are statistically different from the control and LPS-treated cells as shown in the graph ($^{*}P < 0.05$ and $^{**}P < 0.01$) n = 6

4.3.5 LPS increased the media concentration of the creatine kinase (CK)

The creatine kinase activity was measured in the conditioned media of control (untreated) and LPS-treated cells. LPS (1 μgml^{-1}) significantly increased the creatine kinase activity at 5, 30 min and 1 h (P < 0.01) compared with control cells with a peak at 5 min time point with no significant effect was observed at the 3 or 18 h time points (Fig. 4-5).

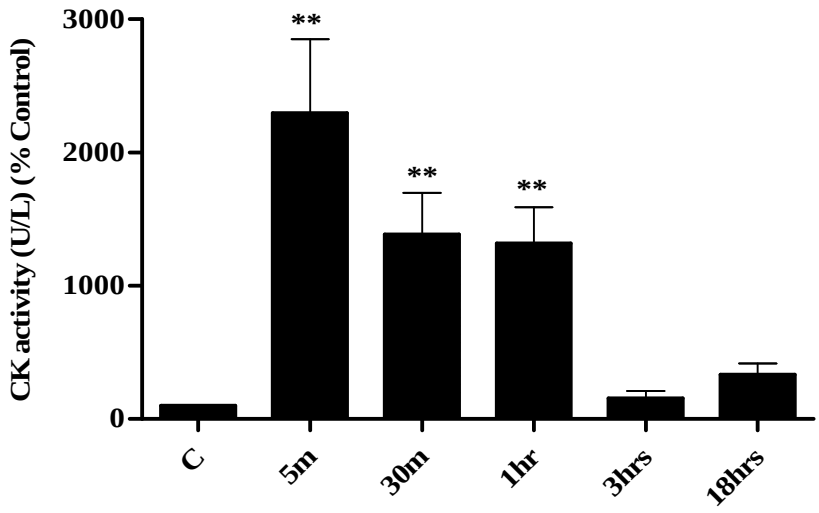
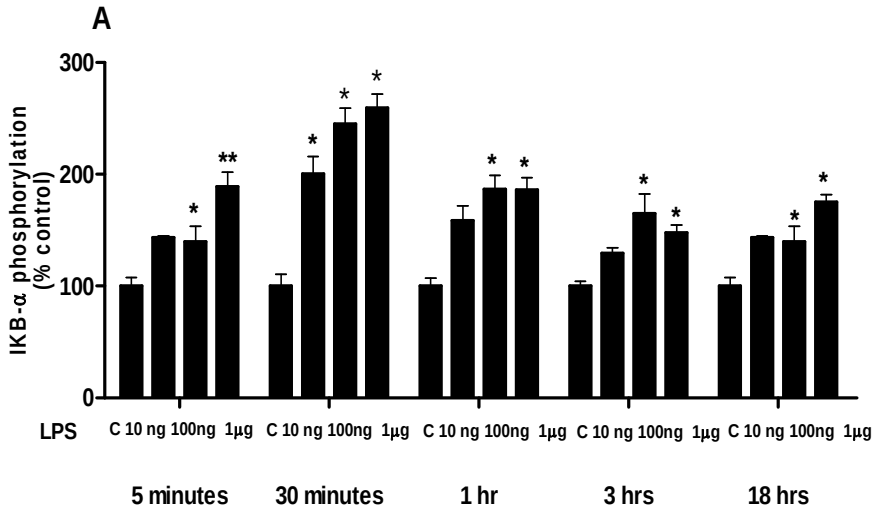


Figure (4-5) LPS-induced changes in creatine kinase activity (CK). C2C12 myotubes were incubated with LPS (1 μgml^{-1}) for (5, 30 min, 1, 3, or 18 h), and the conditioned media were then collected and M-CK activity was measured according to the material and methods. Values are represented as means ± SEM (percentage of the control). Bars carry asterisks are statistically different from the control cells ($^{**}P$ < 0.01). n = 5. One unit of CK will transfer 1 μ mole of phosphate from phosphocreatine to ADP per min at pH 6.0.

4.3.6 LPS significantly increased the I*k*B-α phosphorylation

Western blotting data showed that, LPS particularly (100 $ngml^{-1}$ and 1 μgml^{-1}) significantly increased the phosphorylation of I*k*B-α at all the time points ($P < 0.05$) compared to the control cells. This effect was accompanied by decreased level of total I*k*B-α. LPS significantly decreased I*k*B-α at all time points particularly at the 3 and 18 h time points ($P < 0.05$) compared to the control cells (Figs. 4-6*A* and 4-6*B*).



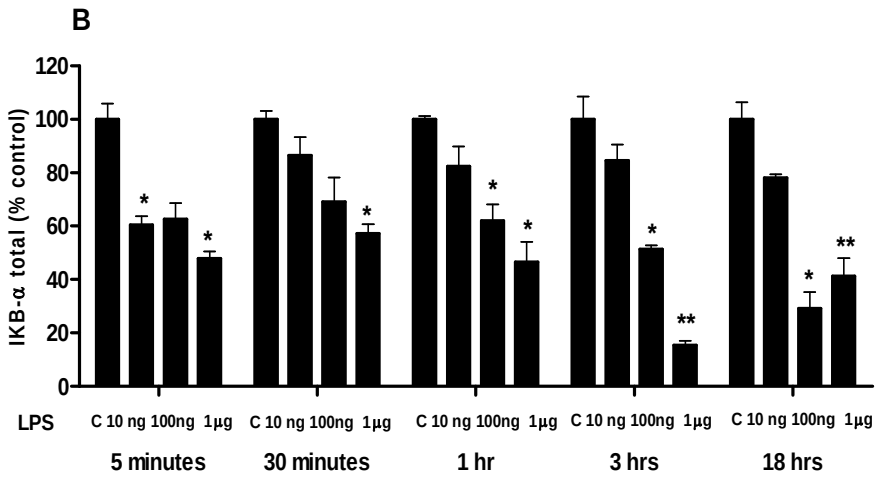


Figure (4-6) Time and dose-dependent changes in **A**) phospho-I*k*B-α and **B**) total I*k*B-α. C2C12 myotubes were incubated with LPS (10 $ngml^{-1}$, 100 $ngml^{-1}$ and 1 μgml^{-1}) for (5, 30 min, 1, 3, or 18 h). Values are represented as means ± SEM (percentage of the control) normalised to β-actin as an internal loading control. Bars carry asterisks are statistically different from the control cells ($^{*}P < 0.05$ and $^{**}P < 0.01$) n = 3

4.3.7 LPS significantly increased the nuclear translocation of NF-kB

Although LPS increased the nuclear translocation of NF-kB (visual inspection) however this effect is still insignificant compared with control cells. The maximal effect of LPS was seen at 18 h time point (Fig. 4-7).

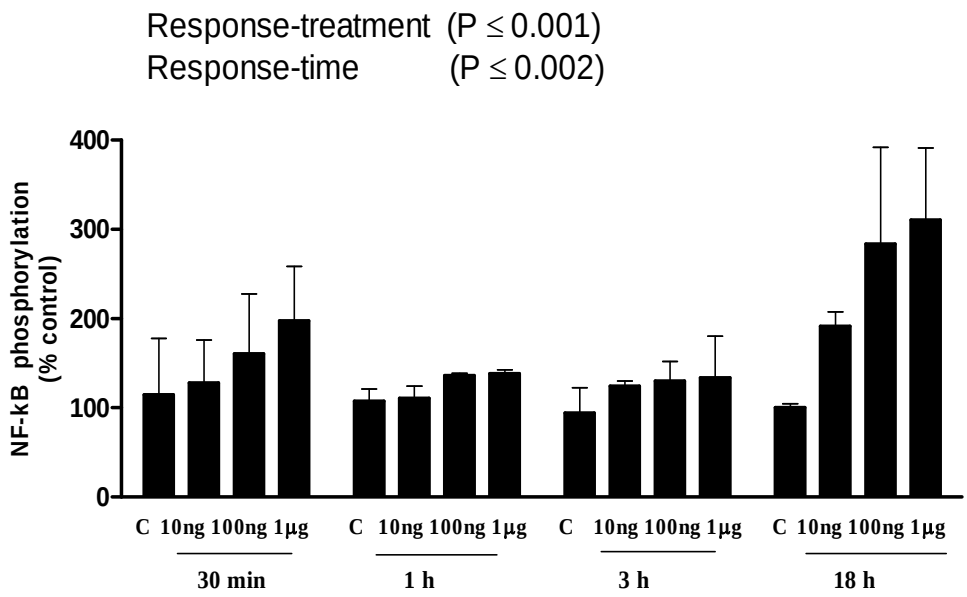


Figure (4-4-7) Time and dose-dependent changes in the NF-kB (nuclear portion). C2C12 myotubes were incubated with LPS (10 $ngml^{-1}$, 100 $ngml^{-1}$ and 1 μgml^{-1}) for (5, 30 min, 1, 3 and 18 h). Values are represented as median ± IQR. n = 4.

Table (4:1) Time changes in the chymotrypsin-like enzyme activity (proteasomal activity). C2C12 myotubes were incubated either with A) LPS (1 μgml^{-1}) alone; B) LPS (1 μgml^{-1}) and MG-132 (proteasomal inhibitor) (40 µM); C) LPS (1 μgml^{-1}) and Polymyxin B (LPS neutralising agent) (PMB, 5 μgml^{-1}); D) LPS (1 μgml^{-1}) and LY0294002 (PI3K/Akt inhibitor) (LY, 50 µM); LPS (1 μgml^{-1}) and SB203580 (p38 inhibitor) (SB, 10 µM); or LPS (1 μgml^{-1}) with PD098059 (MEK/Erk inhibitor) (PD, 40 µM) for (5, 30 min, 1, 3 and 18 h). Values are represented as means ± SEM (percentage of the control). Asterisks represent the statistical difference compared with control cells ($^{**}P < 0.01$ and $^{***}P < 0.001$). n = 6

Treatment	Time points					
	C	5 min	30 min	1hr	3 hrs	18 hrs
LPS alone	100	187 ± 7.23**	284 ± 15.26**	271 ± 15.87**	179 ± 9.24**	187 ± 17.88**
LPS and MG-132	100	16± 3.32***	17± 3.37***	18 ± 3.68***	16 ± 3.34***	16 ± 3.37***
LPS and PMB	100	94 ± 2.10	120 ± 3.82**	93 ± 1.79	93 ± 1.91	104 ± 2.94
LPS and SB	100	76 ± 1.04***	74 ± 1.44***	66 ± 0.18***	61 ± 1.30***	71 ± 0.35***
LPS and PD	100	68 ± 0.59***	56 ± 0.93***	66 ± 0.87***	52 ± 1.74***	77 ± 0.79***
LPS and LY	100	122 ± 0.86	137 ± 2.87	238 ± 22.87**	179± 9.03**	226 ± 21.86***

4.4 Discussion

The data presented in this chapter demonstrated that, 1) LPS is a strong catabolic stimulus which significantly increased the proteasomal activity measured as chymotrypsin-like enzyme activity at all time points between 5 min and 18 h; 2) LPS-induced chymotrypsin activity is totally inhibited by MG-132 (proteasomal inhibitor) at all time points and also by Polymyxin B (LPS neutralising agent) with the exception of the 30 minute time point; 3) Akt inhibition delayed the LPS-induced increased chymotrypsin activity and these increase was not elevated until after 1 h; 4) Inhibition of MAPKs (p38 and Erk1/2) in combination with LPS significantly decreased the chymotrypsin-like enzyme activity to below control level; 5) LPS significantly increased the media concentration levels of TNF-α cytokine at all the time points examined, and IL-6 only at the 18 h time point; 6) LPS transiently increased the creatine kinase activity for only 1 h after LPS administration; and 7) LPS significantly increased the phosphorylation and subsequent proteasomal degradation of the I*K*B-α, followed by the nuclear translocation of the NF-kB.

Sepsis is an acute inflammatory condition characterised by excessive production of pro-inflammatory cytokines such as TNF-α (Vary and Kimball 2000). The data presented in this chapter showed that, LPS significantly increased the proteasomal functional activity measured as increased chymotrypsin-like enzyme activity. MG-132 a specific proteasome inhibitor (Frost *et al.,* 2003) dramatically decreased the LPS-induced chymotrypsin-like enzyme activity by 80% compared with control cells (Fig. 4-1*B*). This suggests that LPS–induced muscle protein breakdown was mediated via the activation of ubiquitin-proteasome pathway. These results are consistent with the findings of Russell *et al*. (2010). The proteasome is a multicatalytic high molecular weight protease complex responsible for ubiquitin dependent protein breakdown within cells (Peters 1994; Coux *et al.,* 1996; Hilt and Wolf 1996). LPS has been shown to bind to the proteasome subunits, and activation

of these subunits has been involved in degradation of incorrectly folded protein or those specially tagged for degradation (Hirsch and Ploegh 2000; Reis *et al.,* 2008). LPS significantly increased TLR4 mRNA expression in a time-dependent manner and Polymyxin B (LPS neutralising agent) (Frost *et al.,* 2006) significantly abolished this effect (chapter 5, Figs. 5-4*A* and 5-4*B*). Herein, incubation of C2C12 with LPS in combination with Polymyxin B neutralises the LPS effect on the proteasomal activity, suggesting that LPS mediated muscle proteolysis is mainly via TLR4 (Frost *et al.,* 2006).

The process of muscle proteolysis is a multifactorial process involving the interaction of many signalling cascades. At the present time, the overall potential contribution of these signalling is still unclear, and further investigation are still required to verify this role.

Akt/protein kinase B is considered as a central regulator of not only muscle protein synthesis but also muscle turnover (Glass 2005). In skeletal muscle, activation of the Akt significantly increased the muscle protein synthetic rate and hence the muscle fibre size (Lai *et al*., 2004). In contrast, decreased Akt activity significantly increased muscle protein degradation via the ubiquitin ligases activation in a Foxo-dependent manner (Van Der Heide *et al.,* 2004). The data presented in this chapter showed that, incubation of C2C12 myotubes with LPS in combination with LY0294002 (PI3-K/Akt inhibitor) (Walker *et al.,* 2000) significantly increased the chymotrypsin-like enzyme activity. In Chapter 3, incubation of C2C12 myotubes with LY0294002 alone or with LPS significantly decreased the protein synthesis rate by 80% (chapter 3, Figs. 3-4 and 3-5). Taken together, Akt inhibition can modulate many fundamental processes in particular protein metabolism (PS and PD) and cell growth.

The MAPK family is considered as an important intracellular signalling system involved in the regulation of many cellular processes including cell growth, differentiation and survival (Seger and Krebs 1995). Increased p38

activity has been reported in many pro-catabolic conditions and sepsis is one (Jackman and Kandarian 2004). Sepsis-induced ubiquitin-proteasomal activity in skeletal muscle is regulated in the main via p38 increased activity (Li *et al*., 2005). In contrast, p38 blockade by SB203580 significantly decreased TNF-α-induced atrogin-1 mRNA up regulation (Jin and Li 2007). Herein, incubation of myotubes with LPS in combination with SB203580 (p38 specific inhibitor) (Li *et al*., 2005) significantly decreased the chymotrypsin-like enzyme activity suggesting that, p38 increased activity is a part of LPS-induced muscle protein turnover (Jackman and Kandarian 2004).

Another MAPK member is Erk1/2. Increased Erk1 (p44) and Erk2 (p42) phosphorylation has been involved in the cellular proliferation and differentiation processes (Olson 1992; Pages *et al.,* 1993), and it has been recognised as a part of the early biochemical events that are initiated by LPS treatment of macrophages or their infection by virulent and attenuated *Salmonella* strains (Procyk *et al.,* 1999). Furthermore, Erk1/2 increased activity has been reported in LPS-induced lung injury (Carter *et al.,* 1999). Erk1/2 activation in skeletal muscle mediated TNF-α induced stimulation of stress-related protein (nuclear factor-kB) (Traenckner *et al.,* 1995). In contrast, Erk1/2 phosphorylation has been involved in the regulation of the protein synthesis in a mechanism most likely via Mnk1/eIF-4E mechanism (Waskiewicz *et al.* 1997; Williamson *et al.* 2005). In chapter 3 (Figs. 3-2*A* and 3-2*B*), LPS significantly increased Erk1/2 phosphorylation and incubation of C2C12 cells with PD098059 (MEK/Erk inhibitor) (Shi *et al.,* 2002; Plaisance *et al.,* 2008) significantly decreased muscle protein synthesis rate. In this chapter, incubation of C2C12 myotubes with LPS and PD098059 (40 μM) significantly decreased LPS-induced proteasomal activity, suggesting that Erk1/2 activation is crucial to LPS-induced muscle protein breakdown.

Interestingly, incubation of the cells with SB203580 (p38 inhibitor) and PD098059 (MEK/Erk inhibitor) had no effect on the proteasomal activity compared to the control level. However, incubation of the cells with these

inhibitors along with LPS significantly decreased the proteasomal activity below the control basal levels. Of note, incubation of cells with these inhibitors alone significantly decreased PS rate compared with control cells (Figs. 3-4 and 3-5); however, this effect was minor compared with that of Akt inhibition. Similarly, PD098059 (MEK/Erk inhibitor) blocked protein synthesis in TNF-α stimulated C2C12 myotubes, however, this effect compared with LY0294002 (PI3-K/Akt inhibitor) considered minor (Plaisance *et al.,* 2008). Under normal condition their basal proteasomal activity was maintained by the basal p38 and Erk1/2 activity, and inhibition of these MAPKs despite the presence of LPS will decrease the proteasomal activity.

Increased levels of TNF-α and IL-6 have been demonstrated in rat skeletal muscle in response to sepsis (Frost *et al.* 2002; 2003; Crossland *et al.* 2008; Borge *et al.* 2009). LPS significantly increased the synthesis and production of cytokines in many tissues including skeletal muscle (Frost *et al*., 2002). TNF-α has previously been reported to increase the IL-6 mRNA expression level in C2C12 myoblasts after 8 h (Frost *et al.,* 2002). In our study, a significant release of the TNF-α was observed after 5 min of LPS administration, which was followed by a significant release of IL-6 at 18 h. LPS has been shown to increase the IL-6 release in skeletal myocytes either directly or in directly via TNF-α autocrine manner (Frost *et al.,* 2006), with the latter being probably the main mechanism in our model of sepsis. TNF-α has been considered for a long period as a catabolic factor, significantly increased in many animal and human catabolic conditions. However, it has been recently shown that, TNF-α significantly increased protein synthesis in C2C12 cells (Plaisance *et al.,* 2008). Of note, LPS induced a significant increase in TNF-α levels in the conditioned media at all time points, and this effect was correlated with increased proteasomal activity. Together, this suggests that TNF-α plays a significant role in LPS-induced muscle proteolysis in C2C12 myotubes. Increased TNF-α concentration was associated with increased E_3 ubiquitin ligases activity in particular atrogin-1 (Li *et al.,* 2005). In addition, increased circulating concentrations levels of TNF-α was associated with

decreased eIF-4G phosphorylation (Lang and Frost 2007) and this inhibits the formation of eIF-4F complex which in turn alters the translation initiation. Although, the effect of MAPKs (p38 or Erk1/2) inhibition on the circulating concentrations of TNF-α is not addressed herein, however, inhibition of theses MAPKs significantly decreased the LPS-induced muscle proteolytic activity. In addition, pre incubation of C2C12 cells with PD098059 (MEK/Erk inhibitor) attenuated the effect of TNF-α on insulin-stimulated protein synthesis (Williamson *et al.,* 2005). Taken together, this makes the inhibition of MAPKs (p38 or Erk1/2) as a possible target for drug discovery to minimise the sepsis-induced muscle wasting.

With regard to the role of IL-6 in various muscle-wasting conditions with some studies have shown a significant role (Strassmann *et al.,* 1992; Haddad *et al.,* 2005) where others have not (Garcia-Martinez *et al.,* 1994; Williams *et al.,* 1998). IL-6 significantly increased the expression of Eα-II ubiquitin protein, the predominant protein expressed in the skeletal muscle in cancer cachexia. Therefore, IL-6 may have presumably enhanced the rate of skeletal muscle protein breakdown mediated via activation of ubiquitin-proteasome pathway (Acharyya *et al.,* 2004; Kwak *et al.,* 2004). The direct infusion of IL-6 into skeletal muscle *in vivo* has been associated with increased muscle protein turnover rate and a 17% decrease in the myofibrillar protein (Haddad *et al.,* 2005). Moreover, rat administrated IL-6 for 1 week showed skeletal muscle atrophy (Janssen *et al.,* 2005). In contrast, IL-6 has many beneficial effects such as increasing fatty acids oxidation in rat skeletal muscle (Bruce and Dyck 2004) and in L6 myotubes (Petersen *et al.,* 2005). In addition, it stimulates lipolysis and fatty acids oxidation in humans (van Hall *et al.,* 2003). In the present study, TNF-α concentration levels are significantly increased in the media as early as 5 min after LPS administration and continued for 18 h, while IL-6 media concentration was observed at 18 h time point. However the proteasomal activity was markedly raised after 5 min of LPS administration. These data suggest that, in the present model of sepsis

the release of TNF-α plays a significant role in LPS-induced increased proteasomal activity, and the potential role of IL-6 in this process is minimal.

The creatine kinase (CK) is considered as an essential enzyme for energy metabolism that reversibly produces phosphocreatine and ATP (Bessman and Carpenter 1985; Wallimann *et al.* 1992; Koyama *et al.* 2008). Although, the mechanism of CK release from the muscle is still relatively unclear, however, CK release has been used as an index of muscle breakdown in the *in vitro* and *in vivo* studies (Maglara *et al.,* 2003). The relationship between the availability of muscle ATP and increased efflux of CK has been established by Jackson *et al* (1984). Under abnormal conditions like malnutrition skeletal muscle decreases its energy consumption mainly by degradation of CK via the MuRF1 mediated mechanism (Koyama *et al.,* 2008). Herein, LPS significantly increased the release of CK at 5, 30 min and 1 h with a peak at 5 min time point (> 20 fold). Increased CK concentration suggesting that, LPS significantly increased the muscle energy consumption via enhancing the formation of the phosphocreatine and ATP (Bessman and Carpenter 1985). Therefore, LPS-induced increased CK level in the muscle tissue could contributes to the high availability of the energy supply (Seraydarian and Abbott 1976) and the impairment of the contractile functions of the muscle (Wegmann *et al.,* 1992).

NF-kB is a member of a transcriptional protein family which has been involved in the regulation of many cellular processes including immune responses, cell growth and cell death (Baeuerle and Baltimore 1996; Karin 1998). In addition, NF-kB stimulates the protein loss via stimulation of the ubiquitin proteasome pathway (Li and Reid 2000; Li *et al*., 2003), mainly MuRF1 mRNA expression (Cai *et al.* 2004). TNF-α stimulates the nuclear translocation of the NF-kB in the differentiated myotubes via the activation and later proteasomal degradation of the I*k*B-α (Li *et al.,* 1998). LPS significantly increased the NF-kB activation and binding in skeletal muscle (Penner *et al*., 2001; Jin and Li 2007). Our findings showed that, LPS

significantly increased the phosphorylation and later proteasomal degradation of I*k*B-α, which in turn followed by the nuclear translocation of NF-kB. Collectively, LPS-induced muscle protein breakdown (measured as chymotrypsin-like enzyme activity) mostly via two probable mechanisms. Firstly, phosphorylation and proteasomal degradation of I*k*B-α followed by nuclear translocation of NF-kB. Second, via decreased Akt phosphorylation and increased phosphorylation MAPKs (p38 or Erk1/2), and later stimulation of ubiquitin ligases mainly atrogin-1 and MuRF1.

4.5 Conclusion

LPS is a strong catabolic stimulus significantly increased the release of many pro-inflammatory and inflammatory cytokines in skeletal muscle tissue. LPS-induced proteasomal cleavage of muscle protein via the MAPKs (p38 or Erk1/2) signalling cascade. Furthermore, the LPS-induced release of TNF-α plays a crucial role in LPS-induced proteasomal activity. Finally, IL-6 release had no role in this process.

5 The transcriptional regulation of muscle protein breakdown in LPS-stimulated C2C12 myotubes

5.1 Introduction

Skeletal muscle is the main store of amino acids and energy substrate in the time of need such as sepsis. Skeletal muscle comprises 60-70% of the total lean body mass (Frost and Lang 2008). The decrease in the muscle protein in the case of acute catabolic conditions such as sepsis, cancer, AIDS (Hasselgren 1995; Tisdale 1997), is mostly the result of the reduction in the muscle protein synthetic rate and/or the increase in the muscle protein breakdown rate (Jepson *et al.,* 1986; Hummel *et al.,* 1988; Ash and Griffin 1989; Hasselgren *et al.,* 1989; Lai *et al.,* 2004). Sepsis is an acute inflammatory condition characterised by severe and rapid loss of body protein, much of which originates from the skeletal muscle (Rosenblatt *et al.,* 1983), and this effect was aimed to provide the liver with the necessary amino acids to form the acute phase protein (Frost and Lang 2008).

Skeletal muscle can respond directly to wide array of stimuli during the course of infection, these stimuli known as the PAMPS. These molecules can be recognised by the host defence system mainly TLRs (Netea *et al.,* 2004). TLRs are transmembrane signalling proteins that are important in recognising PAMPS (Underhill and Ozinsky 2002; Kopp and Medzhitov 2003), and are considered as the first line of defence against the bacterial cell wall components, such as LPS, lipopeptides, as well as RNA and DNA from viruses and proteins from protozoa in a relatively specific manner (Leaver *et al.,* 2007). Mouse C2C12 cell line and mouse skeletal muscle express the mRNA of TLR1 through TLR4 but not TLR9 or TLR10 (Frost and Lang 2008). TLR4 is an essential component in the host defence through the stimulation of cytokine

production. LPS or bacterial endotoxin is the main component of Gram-negative bacterial cell wall, recognized mainly by TLR4 (Akira and Hemmi 2003).

The ubiquitin-proteasome system is the main catabolic pathway involved in many inflammatory-induced muscle wasting conditions including sepsis (Attaix *et al.,* 1998; Jagoe and Goldberg 2001), and a significant reduction in muscle proteolysis in many animal catabolic models has been reported with specific proteasome inhibitors (Jagoe and Goldberg 2001). Atrogin-1 and MuRF1 interacts with $E2_{14K}$ to attach ubiquitin to protein substrates according to the N-end rule (Lecker *et al.,* 1999). Atrogin-1 and MuRF1 are muscle specific genes, and their expression has been up-regulated in many animal models of muscle atrophy including sepsis (Bodine *et al.,* 2001), and individual knockout of atrogin-1 or MuRF1 gene resulted in the attenuation of denervation-induced muscle atrophy (Bodine *et al.,* 2001). This makes these genes important targets for therapeutic intervention (Jin and Li 2007).

The regulation of theses proteolytic pathways involves the interaction between many signalling cascades and transcriptional factors; this makes the overall elucidation of these processes a difficult task. The PI3-K/Akt signalling cascade is the main pathway controlling the activity of these two genes. Increased Akt activity leads to decreased expression of atrogin-1 and MuRF1 (Bodine *et al*., 2001; Sandri *et al*., 2004; Stitt *et al*., 2004), in a mechanism regulated via the Foxo transcription family (for more details see Glass 2005).

MAPKs (p38 or Erk1/2) and NF-kB play a crucial role in sepsis-induced muscle protein turnover. Increased activity of MAPK p38 and NF-kB translocation has been implicated in the up-regulation of the atrogin-1 and MuRF1. Moreover, increased p38 activity has been reported in many pro-catabolic conditions and sepsis (Childs *et al.,* 2003; Koistinen *et al.,* 2003; Di Giovanni *et al.,* 2004). TNF-α and IL-1β mediated the muscle wasting in many catabolic conditions primarily via p38 and NF-kB (Jackman and Kandarian

2004), and the increased p38 phosphorylation has been shown to mediate TNF-α induced up-regulation of atrogin-1 mRNA in C2C12 myotubes (Li *et al.,* 2005), and in septic skeletal muscle of rat (Jin and Li 2007).

The fact that the regulation of LPS-induced muscle protein turnover involves the interaction between many signalling cascades and the cross talk between them is no doubt exist. This makes the understanding of this process more difficult and requires further investigation. In addition, there are no collective *in vitro* data regarding the effect of these second-messenger inhibition on the mRNA activity of atrogens (atrogin-1 and MuRF1), TNF-α and TLR4 mRNA. Therefore, the LPS-stimulated C2C12 septic model was used to evaluate this effect, and various compounds were used to verify this effect.

5.2 Hypothesis

The data collected in chapter 3 and 4 have shown that, LPS is a strong catabolic stimulus transiently decreased PS by nearly 50%, and increased muscle proteolysis all time points. This effect involves the interaction between many signalling streams in particular PI3-K/Akt and MAPKs (p38 or Erk) streams. The data collected in chapter 3 and 4 have shown that, inhibition of Akt was associated with dramatic decrease in PS and increase in protein breakdown, and the cross talk with MAPKs (p38 or Erk) has been revealed. In addition, inhibition of theses signalling molecules significantly altered the effect of LPS. In the context, there is collective data about the transcriptional regulation of LPS-induced muscle protein turnover, and the role of these signalling streams. Herein, we hypothesized that LPS-induced muscle proteolysis includes the activation of ubiquitin proteasome system in particular ubiquitin ligases (atrogin-1 and MuRF1), and inhibition of Akt and MAPKs (p38 or Erk) can modulate this effect. Therefore, the TNF-α, atrogin-1, MuRF1 and TLR4 mRNA was measured in LPS-stimulated C2C12 myotubes over various time points ranged from 30 min to 18 h.

5.3 Results

5.3.1 LPS alone or with inhibitors significantly up-regulated TNF-α m RNA tested by RT-PCR analyses

Incubation of differentiated C2C12 murine myotubes with LPS alone or with specific pathway inhibitors but not Polymyxin B significantly increased TNF-α mRNA expression level (Figs. 5-1*A*, 1*B*, 1*C*, 1*D* and 1*E*). LPS alone transiently increased TNF-α mRNA expression at 1 h by 1856 % ($P < 0.01$) compared with the control cells (Fig. 5-1*A*. Table. 5-1), with no further changes noticed at the other time points. In contrast, Polymyxin B (PMB, 5 $\mu g ml^{-1}$) completely abolished this effect (Fig. 5-1*B*. Table. 5-2). Incubation of C2C12 myotubes with LPS in combination with LY0294002 (LY, 50 μM**)** significantly increased the TNF-α mRNA by 291% at 18 h ($P < 0.05$) compared to control cells (Fig. 5-1*C*, Table. 5-3) with no further changes noticed at any other time points. Incubation of C2C12 with LPS and SB203580 (SB, 10 μM) transiently increased the TNF-α at 3 h by 2510% ($P < 0.001$) compared with control cells (Fig. 5-1*D*. Table. 5-4). Surprisingly, incubation of C2C12 myotubes with LPS plus PD098059 (40 μM) (Erk1/2 specific inhibitor) significantly increased TNF-α mRNA by 1706% ($P < 0.05$), 970% ($P < 0.05$) and 221% ($P < 0.05$) at the 1, 3, 12 h respectively compared with control cells with a peak at the 3 h (Fig. 5-1*E*. Table. 5-5).

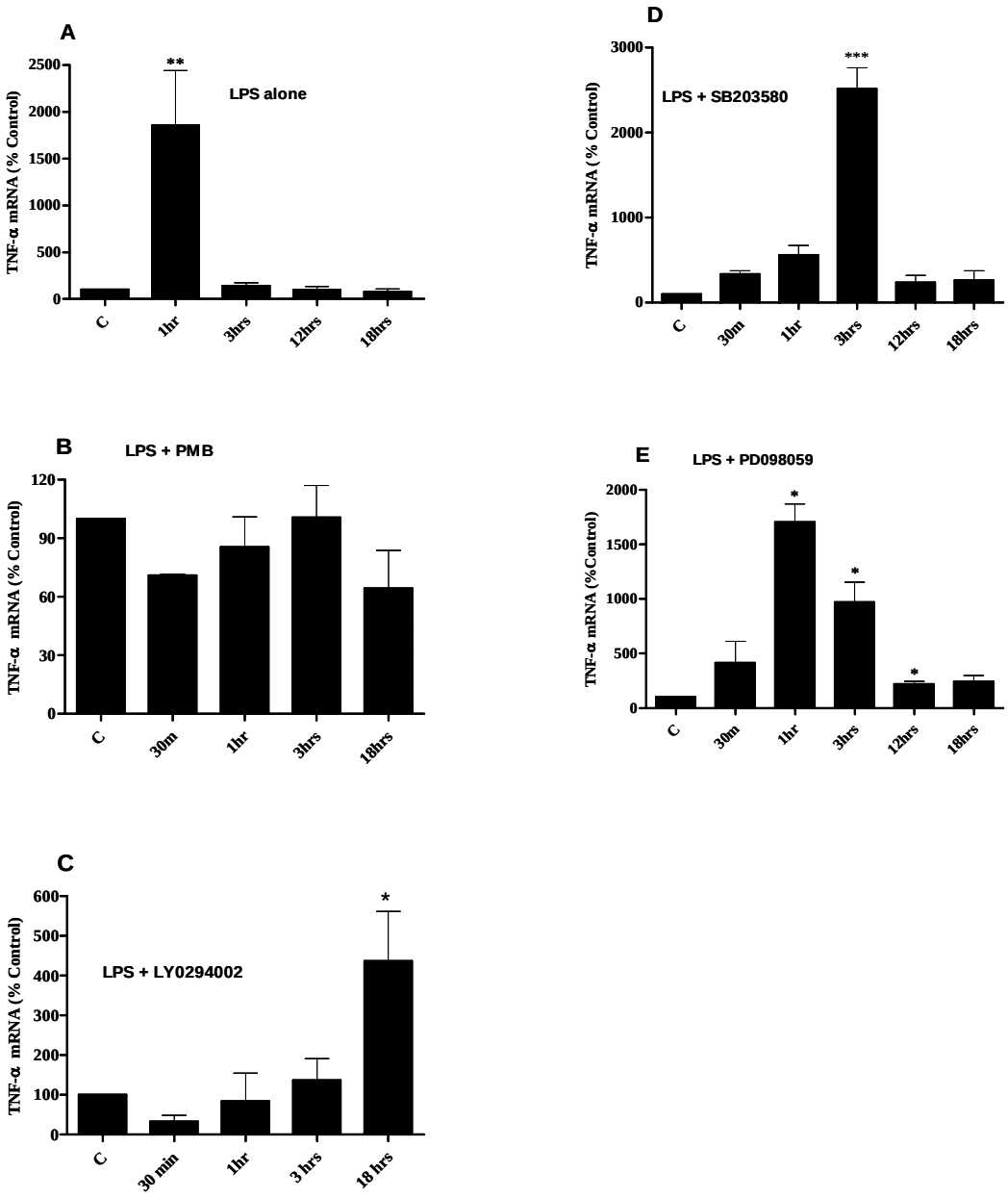


Figure (5-1) Time dependent changes in TNF-α mRNA. C2C12 myotubes were incubated either with **A**) LPS (1 μgml^{-1}) alone, **B**) LPS (1 μgml^{-1}) with Polymyxin B (LPS neutralising agent) (PMB, 5 μgml^{-1}), **C**) LPS (1 μg/ml) and LY294002 (PI3K/Akt inhibitor) (LY, 50 μM), **D**) LPS (1 μgml^{-1}) and SB203580 (p38 inhibitor)(SB, 10 μM) and **E**) or LPS (1 μgml^{-1}) and PD098059 (MEK/Erk inhibitor) (PD, 40 μM). Values are represented as means ± SEM (percentage of control). Bars carry asterisks are statistically different from control cells ($^{*}P < 0.05$ and $^{**}P < 0.01$) n = 3. Values normalized to β-actin mRNA as described in material and methods

5.3.2 LPS alone or with pathway specific inhibitors significantly increased atrogin-1 mRNA

Incubation of C2C12 myotubes with LPS (1 $\mu g ml^{-1}$) alone significantly increased atrogin-1 mRNA in a time-dependent manner by 200% ($P < 0.05$), 283% ($P < 0.05$) and 257% ($P < 0.01$) at the 3, 12 and 18 h time points respectively compared with untreated control cells (Fig. 5-2*A*. Table. 5-1). LPS neutralising agent (PMB, 5 $\mu g ml^{-1}$) failed to inhibit the effect of LPS particularly at the 18 h time point (Fig. 5-2*B*. Table. 5-2), LPS significantly increased atrogin-1 mRNA by 167% ($P < 0.05$) compared with untreated cells. Incubation of cells with LPS and LY0294002 (LY, 50 μM) significantly increased atrogin-1 mRNA by 506% ($P < 0.05$) and 1649% ($P < 0.01$) compared with control cells at the 3 and 18 h time points respectively (Fig. 5-2*C*. Table. 5-3). In contrast, the atrogin-1 mRNA expression exhibited a long time course when C2C12 myotubes were incubated with LPS and SB203580 (SB, 10 μM), atrogin-1 mRNA significantly increased in a time-dependent manner with a peak at the 12 and 18 h time points by 663% and 670% ($P < 0.01$) compared with control cells (Fig. 5-2*D*. Table. 5-4). Likewise, incubation of C2C12 myotubes with LPS in combination with PD098059 (PD, 40 μM) significantly increased atrogin-1 mRNA by 375% ($P < 0.05$), 306% ($P < 0.01$), and 317% ($P < 0.01$) compared with control cells at the 3, 12, 18 h time points respectively (Fig. 5-2*E*. Table. 5-5).

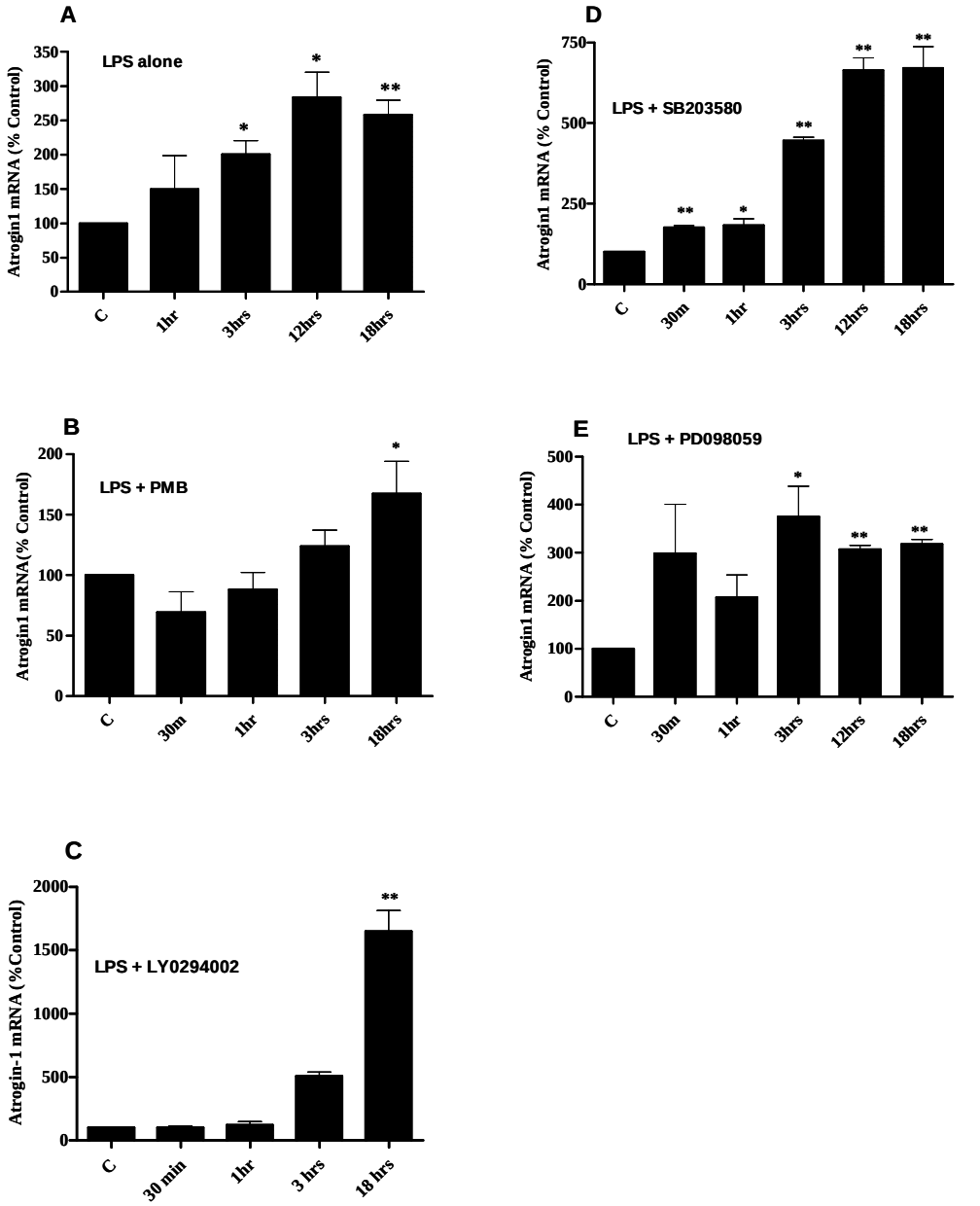


Figure (5-2) Time-dependent changes in atrogin-1 mRNA. C2C12 myotubes were incubated either with **A**) LPS (1 μgml^{-1}) alone, **B**) LPS (1 μgml^{-1}) and Polymyxin B (LPS neutralising agent) (PMB, 5 μgml^{-1}), **C**) LPS (1 μgml^{-1}) and LY0294002 (PI3K/Akt inhibitor) (LY, 50 μM), **D**) LPS (1 μgml^{-1}) and SB203580 (p38 inhibitor)(SB, 10 μM), or **E**) LPS (1 μgml^{-1}) and PD098059 (MEK/Erk inhibitor) (PD, 40 μM). Values are represented as means ± SEM (percentage of control). Bars carry asterisks are statistically different from control cells ($^{*}P < 0.05$ and $^{**}P < 0.01$) n = 3. Values normalized to β-actin mRNA as described in material and methods.

5.3.3 LPS transiently increased MuRF1 mRNA and significantly increased its expression in combination with SB203580

The expression of MuRF1 mRNA exhibited a different course to that of atrogin-1 mRNA. LPS alone significantly increased MuRF1 mRNA transiently at 18 h time points by 155% ($P < 0.05$) compared with control cells with no further changes were noticed at any other time points (Fig. 5-3*A*. Table. 5-1). Surprisingly, MuRF1 exhibited a long time course when the C2C12 myotubes incubated with LPS plus SB203580 (Fig. 5-3*D*. Table. 5-4). MuRF1 mRNA expression was increased in a time-dependent manner with a peak at the 18 h (280%, $P < 0.01$ compared with control cells). No further changes were noticed with the other pathway specific inhibitors (Figs. 5-3*B*, 5-3*C* and 5-3*E*).

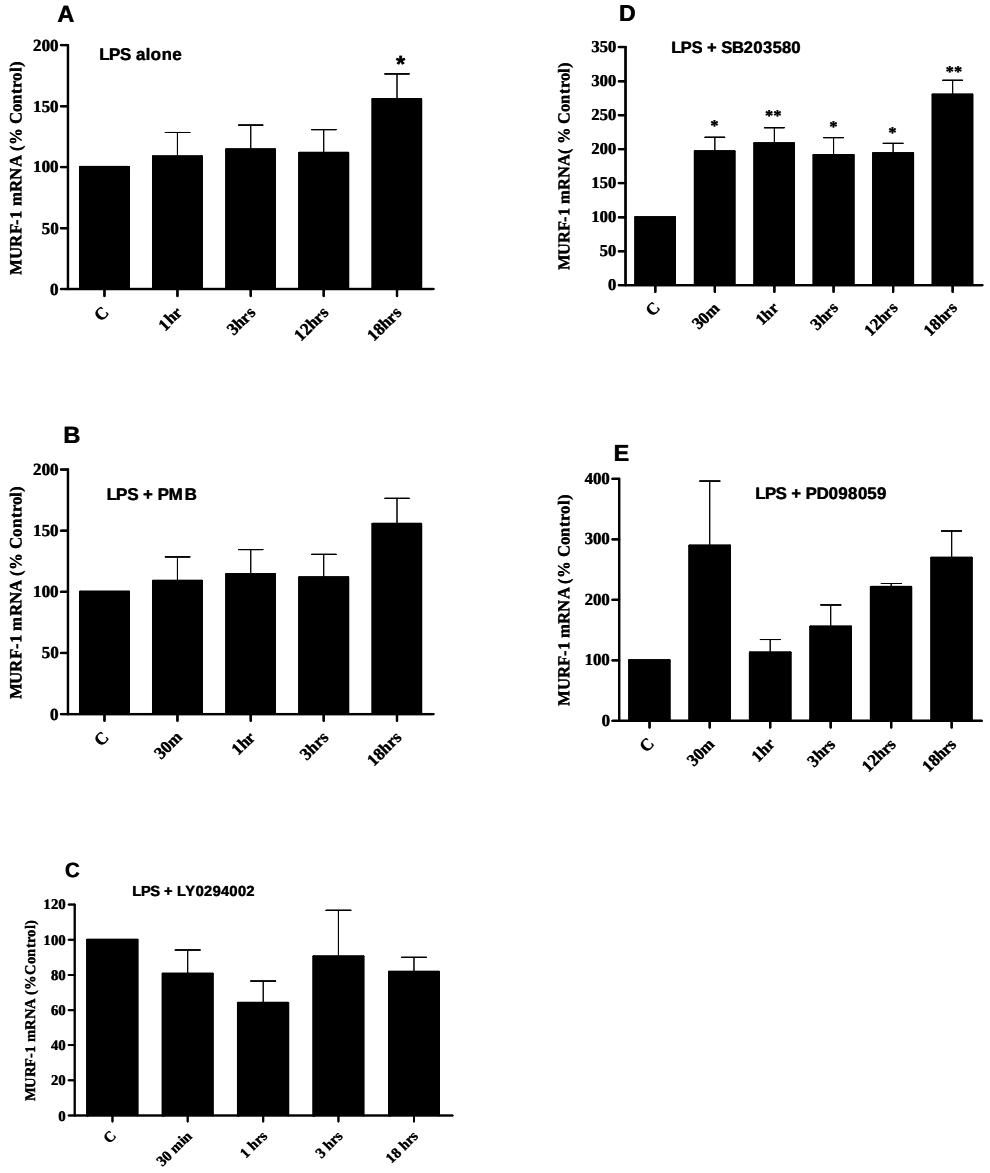


Figure (5-3) Time dependent changes in MuRF1 mRNA. C2C12 myotubes were incubated either with **A**) LPS (1 μgml^{-1}) alone, **B**) LPS (1 μgml^{-1}) and Polymyxin B (LPS neutralising agent) (PMB, 5 μgml^{-1}), **C**) LPS (1 μg/ml) plus LY0294002 (PI3K/Akt inhibitor) (LY, 50 μM), **D**) LPS (1 μgml^{-1}) and SB203580 (p38 inhibitor) (SB, 10 μM), or **E**) LPS (1 μgml^{-1}) and PD098059 (MEK/Erk inhibitor) (PD, 40 μM). Values are represented as means ± SEM (percentage of control cells). Bars carry asterisks are statistically different from control cells ($^{*}P < 0.05$ and $^{**}P < 0.01$). n = 3. Values normalized to β-actin mRNA as described in material and methods.

5.3.4 LPS-stimulated TLR4 mRNA expression only abolished by LPS neutralising agent (Polymyxin B)

LPS significantly increased TLR4 mRNA in a time-dependent manner with a peak at the 12 and 18 h time points (155%, $P < 0.05$ compared with control cells) (Fig. 5-4*A*. Table. 5-1). In contrast, Polymyxin B (PMB, 5 μgml^{-1}) LPS neutralising agent completely abolished this effect (Fig. 5-4*B*. Table. 5-2). While incubation of cells with LPS and LY0294002 significantly increased TLR4 mRNA transiently at 18 h by 509% ($P < 0.01$) compared with control cells (Fig. 5-4*C*. Table. 5-3). Surprisingly, SB203580 significantly magnified this effect. TLR4 mRNA significantly increased in a time-dependent manner by 1130% and 1026% ($P < 0.001$) compared with untreated control cells at the 12 and 18 h time points (Fig. 5-4*D*, Table 5-4). Similarly, inhibition of Erk1/2 activity significantly increased TLR4 mRNA in a time-dependent manner with a peak at the 18 h (683%, $P < 0.001$ compared with control cells) (Fig. 5-4*E*. Table. 5-4).

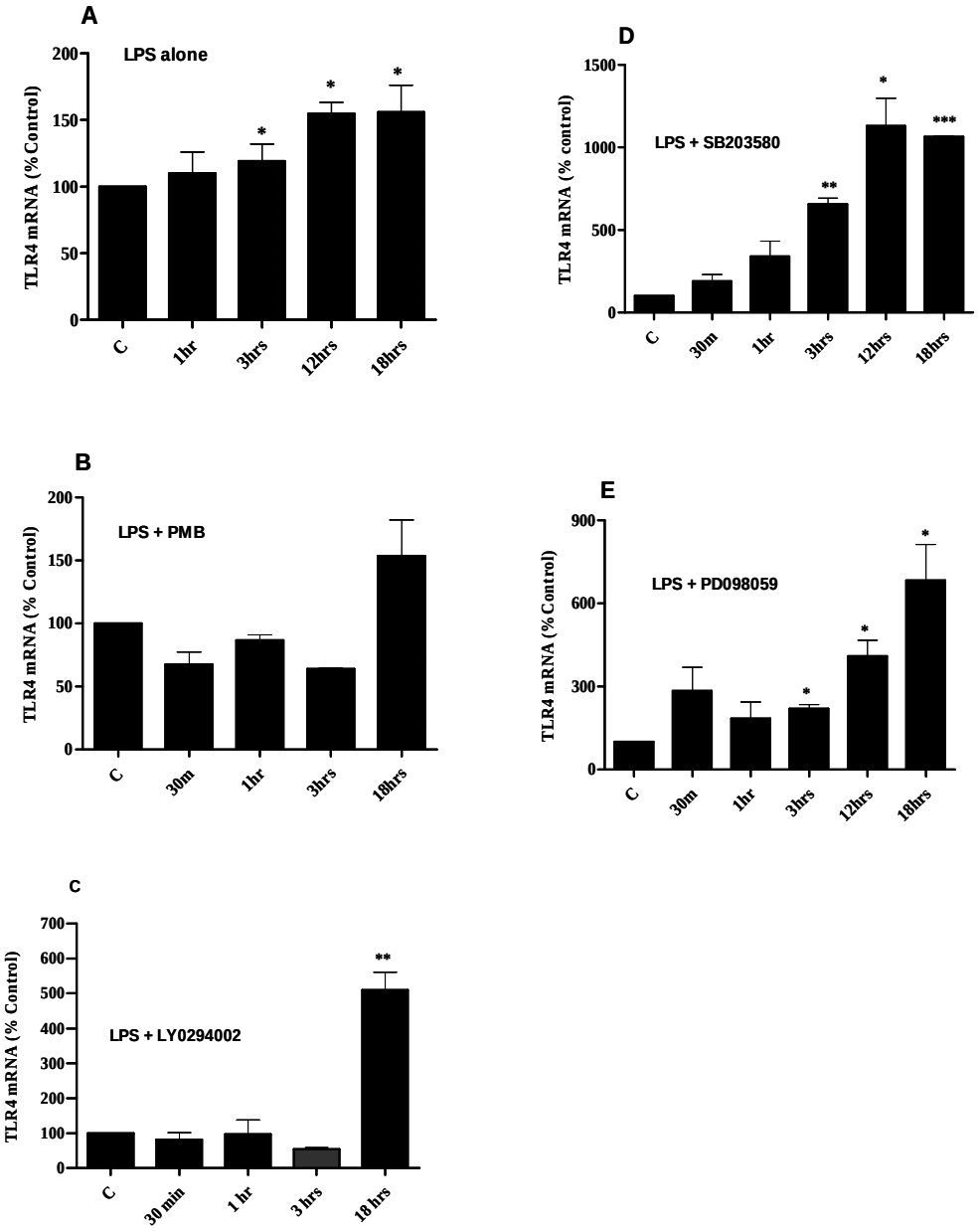


Figure (5-4) Time dependent changes in TLR4 mRNA. C2C12 myotubes were incubated either with **A**) LPS (1 μgml^{-1}) alone, **B**) LPS (1 μgml^{-1}) and Polymyxin B (LPS neutralising agent) (PMB, 5 μgml^{-1}), **C**) LPS (1 μgml^{-1}) and LY2094002 (PI3K/Akt inhibitor) (LY, 50 μM), **D**) LPS (1 μgml^{-1}) and SB203580 (p38 inhibitor) (SB, 10 μM), or **E**) LPS (1 μgml^{-1}) and PD098059 (ME/Erk inhibitor) (PD, 40 μM). Values are represented as means ± SEM (percentage of control cells). Bars carry asterisks are statistically different from control cells ($^{*}P < 0.05$ and $^{**}P < 0.01$). n = 3. Values normalized to β-actin mRNA as described in material and methods.

Table (5:1) Changes in TNF-α, atrogin-1, MuRF-1 and TLR4 mRNA expression level. C2C12 were incubated with LPS (1 μgml^{-1}) for (30 min, 1, 3, 12 and 18 h). Values are expressed as means (percentage of control). Asterisks represent the statistical difference compared with the control cells ($^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$). n = 3

Gene mRNA	Time post LPS administration				
	C	1 h	3 h	12 h	18 h
TNF-α	100	1858**	138	98	78
Atrogin-1	100	149	200*	283*	257*
MuRF1	100	108	114	111	155
TLR4	100	110	119*	154*	155*

Table (5:2) Changes in TNF-α, atrogin-1, MuRF-1 and TLR4 mRNA expression C2C12 were incubated with LPS (1 μgml^{-1}) and Polymyxin B (LPS neutralising agent) (PMB, 5μgml^{-1}) for (30 min, 1, 3 and 18 h). Values are expressed as means (percentage of control). Asterisks represent the statistical difference compared with the control cells ($^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$).n = 3

Gene mRNA	Time post LPS + PMB				
	C	30 min	1 h	3 h	18 h
TNF-α	100	71	85	100	64
Atrogin-1	100	69	88	123	167*
MuRF1	100	111	101	96	145
TLR4	100	67	86	63	153

Table (5:3) Changes in TNF-α, atrogin-1, MuRF-1 and TLR4 mRNA expression level. C2C12 were incubated with LPS (1 μgml^{-1}) and LY0294002 (PI3-K/Akt inhibitor) (LY, 50 μM) for (30 min, 1, 3 and 18 h). Values are expressed as means (percentage of control). Asterisks represent the statistical difference compared with the control cells ($^{*}P < 0.05$ and $^{***}P < 0.001$).n = 3

Gene mRNA	Time post LPS + LY0294002				
	C	30 min	1 h	3 h	18 h
TNF-α	100	32	83	136	291
Atrogin-1	100	102	122	509*	1649*
MuRF1	100	80	64	90	81
TLR4	100	81.38	96.51	54.21	509.16**

Table (5:4) Changes in TNF-α, atrogin-1, MuRF-1 and TLR4 mRNA expression level. C2C12 were incubated with LPS (1 μgml^{-1}) and SB203580 (p38 inhibitor) (SB, 10 μM) for (30 min, 1, 3, 12 and 18 h) .Values are expressed as means (percentage of control). Asterisks represent the statistical difference compared with the control cells ($^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$). n = 3

Gene mRNA	Time post LPS + SB203580					
	C	30 min	1 h	3 h	12 h	18 h
TNF-α	100	334	559	2150***	238	262
Atrogin-1	100	176**	182*	445*	663*	670*
MuRF1	100	196*	208**	191*	193*	280**
TLR4	100	189	339	655**	1130***	1026***

Table (5:5) Changes in TNF-α, atrogin-1, MuRF-1 and TLR4 mRNA expression level.C2C12 were incubated with LPS (1 μgml^{-1}) and PD098059 (MEK/Erk inhibitor) (PD, 40 μM) for (30 min, 1, 3, 12 and 18 h). Values are expressed as means (percentage of control). Asterisks represent the statistical difference compared with the control cells ($^{*}P < 0.05$ and $^{**}P < 0.01$).n = 3

Gene mRNA	Time post LPS + PD098059					
	C	30 min	1 h	3 h	12 h	18 h
TNF-α	100	415	1706*	970*	221*	241
Atrogin1	100	298	207	375*	306**	317**
MuRF1	100	289	112	155	221	269
TLR4	100	283	184	240	409	683**

5.4 Discussion

The data presented here is considered as a comprehensive study aimed mainly to elucidate the effect of endotoxin alone and endotoxin plus specific pathway inhibitors on the transcription regulation of muscle protein breakdown *in vitro* using C2C12 myotubes septic model. The major findings of the study can be summarised as the following 1) LPS is a strong stimulator of TNF-α, atrogin-1, MuRF1 and TLR4 mRNA expression in C2C12 differentiated myotubes; and Polymyxin B (PMB) neutralises this effect; 2) LY0294002 (PI3-K/Akt inhibitor) significantly increased TNF-α, atrogin-1 and TLR4 mRNA but not MuRF1; 3) SB203580 (p38 inhibitor) significantly magnified the effect of LPS and it significantly increased the TNF-α, atrogin-1, MuRF1 and TLR4 mRNA expression in a time-dependent manner; 4) PD098059 (MEK/Erk1/2 specific inhibitor) significantly increased only TNF-α, atrogin-1 and TLR4 mRNA expression but not the MuRF1 and finally 5) the up-regulation of atrogin-1 is the main feature in our model of sepsis and it might be the key regulator of LPS-induced muscle protein breakdown.

Skeletal muscle comprises nearly 45% of the body weight and nearly around 70% of sepsis-induced body protein loss originates from the skeletal muscle (Plank *et al.,* 1998). Sepsis-induced protein wasting was derived mainly from the imbalance between the rate of protein synthesis and protein degradation. The Loss of muscle mass in sepsis is derived primarily from the accelerated muscle protein degradation rate via the ubiquitin-proteasome pathways (Jagoe and Goldberg 2001). Selecting a particular protein to be ubiquitinated is the function of ubiquitin ligases. The ubiquitin ligases protein (also called an E3 ubiquitin ligases) is a protein in combination with an E2 (Ubiquitin-conjugating enzyme) which causes the attachment of ubiquitin to a lysine on a target protein substrates for degradation in the proteasome (Lecker *et al.,* 1999). The ubiquitin ligase proteins atrogin-1 and MuRF1 are a rate limiting for muscle protein loss in many catabolic conditions. The muscle

expression of these proteins has been increased during sepsis (Voisin *et al.,* 1996; Fischer *et al.,* 2000). The activity of atrogin-1 and MuRF1 is reciprocal to the activity of Foxo transcription family. The activity of Foxo transcription family is mainly controlled by Akt activity. Decreased activity of Akt in C2C12 myotubes was shown to activate Foxo transcription family, which in turn increases the activity expression of muscle specific ubiquitin ligases atrogin-1 and MuRF1, and vice versa. LPS injection was associated with dramatic increase in the expression of muscle specific genes atrogin-1 and MuRF1 (Bodine *et al.,* 2001). During muscle atrophy, atrogin-1 inhibits the binding of Met-tRNA to the 40S ribosome via the ubiquitination and later proteasomal degradation of the eukaryotic initiation factor 3, subunit 5 (eIF3-F), while MuRF1 targets the myosin heavy chain protein, troponin and other myofibrillar proteins (Clarke *et al.,* 2007), and suppressed the muscle protein synthesis (Koyama *et al.,* 2008).

Herein, LPS significantly increased the levels of TNF-α, atrogin-1, MuRF1 mRNA, this is consistent with the findings of (Frost *et al*., 2002), and this effect was mediated mainly via TLR4. LPS is considered as a potent stimulus of cytokines synthesis and accumulation of their mRNA occurs in the classical immune cells (Shindoh *et al.,* 1995; Saghizadeh *et al.,* 1996), and in the skeletal muscle mediated via TLR4 (Frost *et al.,* 2006). These data are inconsistent with the finding that LPS alone fail to induce TNF-α release in C2C12 myotubes and thus, TNF-α expression is considered as a generalised response of skeletal muscle to LPS administration (Fernandez-Celemin *et al.,* 2002). TNF-α is a pro-inflammatory cytokine originally called cachectin and is one of the intensively studied cytokines plays an indispensable role in the host defence against invading pathogens (Tisdale 1997). Elevated circulating levels of TNF-α have been reported in many catabolic conditions including sepsis (Cooney *et al.,* 1999), and accompanied with a reduction in skeletal muscle protein and a loss of muscle mass (Williamson *et al.,* 2005). TNF-α induced muscle catabolism either by decreasing protein synthesis and myogenesis in C2C12 myoblasts (Guttridge *et al.,* 2000; Langen *et al.,* 2001) or increasing the

contractile protein proteolysis particularly myosin heavy chain via activation of ubiquitin-proteasome pathway (Li and Reid 2000) in a mechanism mediated via activation of p38 (phosphorylation) and NF-kB activation (Fernandez-Celemin *et al.,* 2002; Li *et al.,* 2005). Conversely, it has recently been shown that TNF-α significantly increased protein synthesis in a dose-dependent manner via MEK signalling cascade in C2C12 myotubes (Plaisance *et al.,* 2008). LPS significantly increased TLR4 mRNA which was diminished by Polymyxin B in cultured skeletal muscle cells. Although, Lang *et al* failed to detect any changes in TLR4 mRNA in septic skeletal muscles of rat (Lang *et al.* 2003), however the subsequent study of Frost and others demonstrated that LPS stimulated expression of TLR4 mRNA in both C2C12 myoblasts and differentiated myotubes (Frost *et al.,* 2004). Hence, LPS affects cytokine expression at multiple levels including transcription (Learn *et al.,* 2000), translational (Rausch *et al.,* 1994) processing and secretion (Mehta *et al.,* 2001), an effect mediated most likely via TLR4 signalling cascade.

Akt has been shown to regulate the expression of atrogin-1 and MuRF1 mRNA in a mechanism mediated via the Foxo pathway. Decreased Akt activity was associated with increased atrogin-1 and MuRF1 expression (Glass 2005). The atrogin-1 mRNA significantly increased when the C2C12 myotubes were incubated with LPS in combination with LY0294002. This result is consistent with the result of Moylan *et al*., (2008), blocking of PI3-K/Akt pathway with wortmannin could not diminish the expression of atrogin-1 mRNA (+340%). Moreover, inhibition of Akt by LY029004 significantly increased atrogin-1 mRNA in dexamethasone-stimulated C2C12 cells (Sacheck *et al.,* 2004). Conversely, transient activation of Akt was involved in the PIF (proteolysis induced factor)-induced muscle proteolysis and inhibition of Akt by LY029004 significantly decreased the proteolytic activity (measured as chymotrypsin-like enzyme activity for more details see (Russell *et al.,* 2008). This suggests that Akt is the a crucial key element regulating not only the protein synthesis but also protein degradation in skeletal muscle (Glass 2005). The fact that the expression of atrogin-1 is one the predominant features of our septic models

argue the impotence of the E_3 atrogin-1 during the atrophy process. This makes atrogin-1 is a target for future drug discovery to decrease sepsis-induced muscle wasting.

P38 MAPK has been known as a potential regulator of muscle catabolism, and elevated p38 activity in skeletal muscle has been reported in many pro-catabolic conditions (Tracey 2002; Childs *et al.,* 2003; Di Giovanni *et al.,* 2004). P38 mediated the TNF-α up-regulation of atrogin-1 mRNA not MuRF1 in rat skeletal muscle (Li *et al*., 2005; Jin and Li 2007). SB203580, a selective p38 inhibitor (Cuenda *et al.,* 1995), failed to block the LPS up-regulation of TNF-α, atrogin-1, MuRF1 and TLR4 mRNA. These findings are inconsistent with the findings of Li *et al.*, (2005), that SB203580 significantly blocked the TNF-α-p38 mediated up-regulation of the atrogin-1 mRNA in skeletal muscle (Li *et al*., 2005). The western blotting data (chapter 3, Figs. 3-7*B* and 3-7*D*)) showed that SB203580 had no effect on the p38 activity related phosphorylation, and this is consistent with the findings of Kumar *et al*., (1999). In contrast, SB203580 significantly decreased the Akt phosphorylation compared with untreated cells ($P < 0.05$). Therefore, the assumption is that incubation of C2C12 myotubes with LPS and SB203580 significantly decreased Akt, and Akt is considered as the regulatory key of muscle protein degradation mediated most likely via atrogin-1 (Glass 2005).

Beside p38, Erk1/2 is an another MAPK that is involved in many cellular functions including cellular proliferation and differentiation (Olson 1992; Pages *et al.,* 1993). In skeletal muscle, Erk1/2 activation has been associated with controversial functions. TNF-α induced muscle loss is primarily associated with the activation of Erk1/2 MAPK, which in turn stimulates stress-related protein NF-kB (Traenckner *et al.,* 1995; Ueki *et al.,* 2004). Conversely, the role of the Erk1/2 in the stimulation of translation initiation via Mnk1 downstream eIF-4E has been shown by Waskiewicz *et al*., (1997). Inhibition of p38 and Erk1/2 significantly increased the up-regulation of TNF-α, atrogin-1 and MuRF1 mRNA expression. These data is inconsistent with the findings of

Sacheck *et al.* that inhibition of MEK has no effect on the atrogin-1 mRNA level (Sacheck *et al.,* 2004). This data showed the direct contribution of the MAPKs (p38 or Erk1/2) in LPS-induced atrogens up regulation and consequently muscle proteolysis. The significant increase in the atrogin-1 mRNA was the main feature of our septic model. The western results presented in chapter 3 (Figs. 3-7*B* and 3-7*C*) showed that, inhibition of the Erk1/2 and p38 significantly decreased the Akt phosphorylation ($P < 0.05$) compared with the control cells (Figs. 3-7*A* and 3-7*D*). This suggests that there is a cross talk between these various signalling pathways. The crosstalk between the Akt and Erk1/2 signalling pathways has been demonstrated in many studies (Hausenloy *et al.,* 2004; Drummond *et al.,* 2009). Drummond *et al* (2009) demonstrated that, inhibition of mTOR activity significantly decreased the Erk1/2 phosphorylation, and the activation of both mTORC1 and Erk1/2 signalling pathways is required for the stimulation of protein synthesis in human skeletal muscle. In contrast, inhibition of MAPKs (p38 or Erk1/2) might alter PS in an effect mediated via the Mnk1/eIF-4E pathway (Fukunaga and Hunter 1997; Waskiewicz *et al.,* 1997; Plaisance *et al.,* 2008). The LPS-induced muscle protein breakdown was thus mediated mainly via the Akt, and Akt is considered as the central regulator of both muscle protein synthesis and muscle protein breakdown (Glass 2005). Although, inhibition of MAPKs (p38 or Erk1/2) significantly increased the activity of ubiquitin ligases mainly atorgin-1 and too less extend MuRF1, this effect was mediated mainly via decreased Akt activity. The data presented in chapter 4 and 5, showed that, there is no absolute correlation between the proteasomal proteolytic activity (chymotrypsin-like enzyme activity) and the expression of atrogens (atrogin-1 and MuRF1) in our septic model. In other words, inhibition of MAPKs (p38 or Erk1/2) significantly decreased LPS-induced proteasomal activity (Fig. 4-2), while it significantly increased the expression of atrogens in particular atrogin-1 (Fig. 5-2). This makes the use of the atrogen-1 and MuRF1 expression as a marker of muscle proteolysis is a poor indicator. Recently it has been reviewed in Attaix and Baracos (2010), the controversial observations regarding the link between muscle proteolysis and the

expression of these atrogens. However, the obtained data suggests that the atrogin-1 is the key player in many cases of muscle wasting including our C2C12 model, cancer (Gomes *et al.,* 2001), and sepsis (Li *et al.,* 2005), and its transcriptional regulation involves the interaction of many signalling pathways. This makes these genes the target for further therapeutic intervention in order to retard the adverse outcomes of sepsis (Jin and Li 2007).

5.5 Conclusion

LPS significantly increased the production and release of cytokine in both immune and non immune tissues. LPS significantly increased TNF-α, atrogin-1, MuRF1 and TLR4 in C2C12 differentiated myotubes. There was a cross-talk between the MAPKs (p38 or Erk1/2) and Akt signalling pathways. The most important signalling cascade involved in LPS-mediated up-regulation of TNF-α, atrogin-1, MuRF1 and TLR4 mRNA is the TLR4/Akt signalling cascade. The predominant ubiquitin protein ligases unregulated in our model of sepsis (C2C12 myotubes) is atrogin-1. The data presented in this chapter highlighted the importance of atrogin-1 knockout as an effective therapy in order to lessen the protein turnover in case of sepsis.

6 Curcumin prevented the LPS-induced protein degradation in C2C12 myotubes

6.1 Introduction

Sepsis is an acute catabolic condition resulting in severe consequences including, increased rate of protein degradation and/or decreased rate of protein synthesis (Jepson *et al.,* 1986; Hummel *et al.,* 1988; Ash and Griffin 1989; Hasselgren *et al.,* 1989; Lai *et al.,* 2004). The increased rate of protein degradation is mediated via increased ubiquitin-proteasome activity pathway (Jagoe and Goldberg 2001) and for more details regarding the contribution of ubiquitin and ubiquitin ligases in this process see Hershko and Ciechanover (1998). Briefly, the targeted protein first linked and marked by a chain of four ubiquitin molecules, and later it degraded in the 26S proteasome central core. Sepsis is associated with increased secretion of TNF-α and IL-1β (Zarubin and Han 2005), which in turn increases the activity of p38 and NF-kB leading to an excessive muscle protein degradation (Jackman and Kandarian 2004). This effect mediated via the up-regulation of the ubiquitin ligases (atrogin-1 and MuRF1) mRNA. The regulation of the skeletal muscle protein degradation is a complicated process, involved the interaction between many signalling pathways, but the predominant mechanism in case of catabolic diseases involves TNF-α/p38, atrogin-1 (Li *et al.*, 2005), and the TNF-α/I*k*B-α/ MuRF1 pathways (Li & Reid, 2000; Cai *et al.*, 2004).

Curcumin is the principle curcuminoid of the popular Indian spice turmeric, has been used for a long time in the traditional Indian medicine to treat many diseases. During the last two decades, many studies have been carried out to explore the beneficial effects of curcumin, and nearly all these studies have been promising. Curcumin inhibits the p38 activity (Carter *et al.*, 2003) and consequently this inhibits the up-regulation of atrogin-1 ubiquitin

ligase in C2C12 myotubes (Li *et al.,* 2005). Moreover, curcumin inhibits the NF-kB activity mediated by decreasing phosphorylation and proteasomal degradation of I*K*B-α, and this keeps the NF-kB bound to its inhibitory protein I*K*B-α in the cytoplasm (Jobin *et al.,* 1999), leading to a considerable decrease in the inflammation-stimulated muscle protein degradation mediated via p38 and NF-kB pathways. In addition, along with these positive effects of curcumin, it has no known toxic effects, even at very high doses (Chainani-Wu 2003).

IL-6 is a cytokine which has pro and ant-inflammatory properties (Ikemoto *et al.,* 2000), and is mainly secreted from the classical immune tissues, such as liver, spleen (Luo *et al.,* 2003), and septic skeletal muscle (Frost *et al.,* 2002). The data regarding the role of IL-6 in the regulation of muscle protein degradation in various muscle wasting conditions are still contradictory with some studies showed no role (Garcia-Martinez *et al.,* 1994; Williams *et al.,* 1998), while others suggested a contribution (Strassmann *et al.,* 1992; Haddad *et al.,* 2005). Taking into consideration, the results of these studies and the growing importance of IL-6 in the regulation of muscle protein metabolism, the understanding of the regulation of IL-6 production in muscle becomes increasingly important. Developing appropriate and safe therapeutic intervention that decreases the complications of sepsis with minimal side effects has become increasingly important. In addition, there are a paucity of studies regarding the direct effect of curcumin on the rate of muscle protein degradation and protein synthesis *in vivo*. Therefore, our study aimed to explore the direct effect of three curcumin concentrations (12, 25 and 50 µM) on the protein synthesis and protein degradation in the LPS-C2C12 myotubes as an *in vitro* model of sepsis. Our study showed that, curcumin (25 µM) significantly decreases the LPS-induced protein degradation (measured as chymotrypsin-like enzyme activity) with no effect on the LPS-induced decreased PS (measured as ^{3}H-tyrosine incorporation/µg 5% TCA Precipitable protein) in the same time with no side effects on the myotubes.

6.2 Hypothesis

The data presented in chapters 3, 4 and 5 have shown that, LPS is a strong catabolic agent increased the protein breakdown and decreased the protein synthesis particularly at the 3 h time point. This effect involves the activation of ubiquitin ligases in particular atrogin-1 and MuRF1. In addition, LPS significantly increased the production of pro-inflammatory cytokines in skeletal muscle mainly TNF-α. The role of Akt and MAPKs (p38 or Erk) has been shown. Curcumin has been shown to have anti-inflammatory effect mediated via inhibition of p38 activity. *In vivo* studies have demonstrated that curcumin significantly decreased atrogin-1 ubiquitin ligase and this effect was mediated via decreased p38 activity. Furthermore, there is no data regarding the direct role of curcumin on protein synthesis and protein breakdown *in vitro*. Therefore, three curcumin concentrations (12, 25 and 50 μM) were used in 3 h LPS stimulated C2C12 myotubes to investigate the beneficial effects of curcumin.

6.3 Results

6.3.1 Curcumin increases the Akt phosphorylation

The first experiment was to assess the effect of LPS alone and LPS in combination with curcumin (12, 25 and 50 μM) and curcumin alone on the Akt activity-related phosphorylation. The data showed that LPS alone significantly decreased the Akt phosphorylation by -25% ($P < 0.05$) compared with the control cells (Figs. 6-1 and 6-5*A*). While, incubation of C2C12 myotubes with LPS and curcumin (12, 25 and 50 μM) significantly increased the Akt phosphorylation compared with LPS-treated cells but this effect is still insignificant compared with the control cells. Interestingly, incubation of cells with LPS along with curcumin (25 μM) significantly increased Akt phosphorylation by 43% ($P < 0.05$) compared with control cells, while incubation of cells with curcumin (25 μM) alone significantly increased the Akt phosphorylation by 62% ($P < 0.01$) compared to control cells.

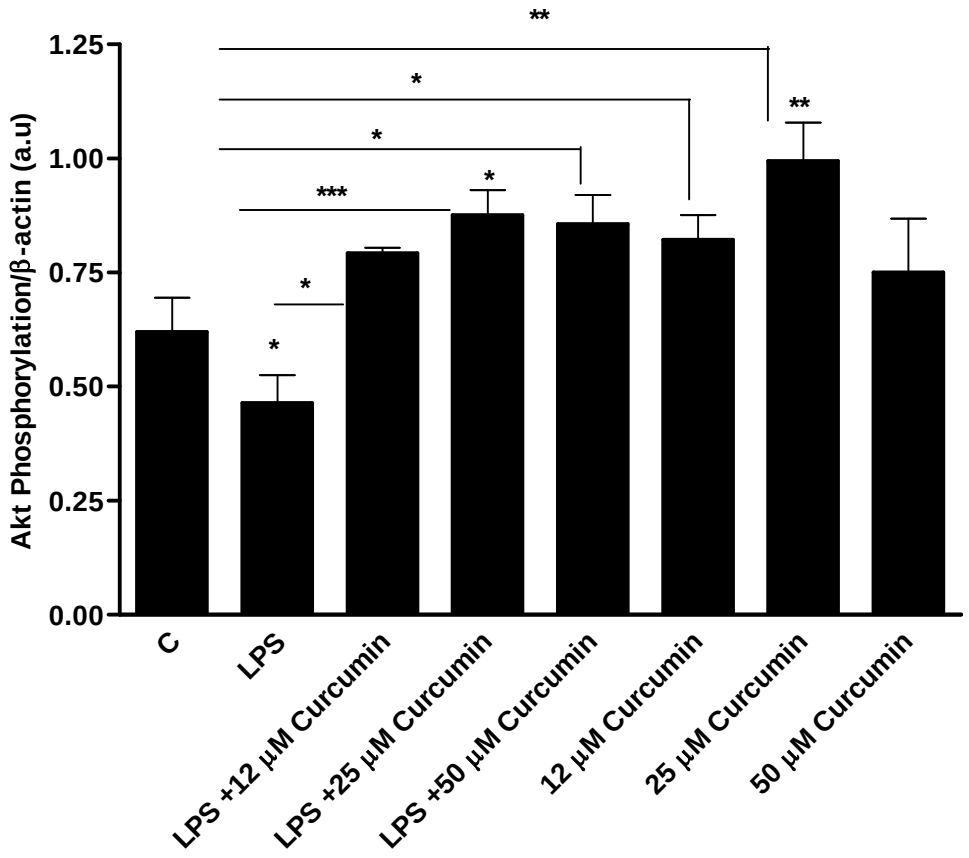


Figure (6-1) LPS and curcumin induced changes in phospho-Akt. C2C12 myotubes were incubated either with LPS (1 μgml^{-1}) alone, or with LPS (1 μgml^{-1}) and Curcumin (12, 25 and 50 μM), or Curcumin (12, 25 and 50 μM) alone for 3 h. Values are represented as means ± SEM arbitrary unit normalised to β-actin as an internal loading control. Bars carry asterisks are statistically significant from control and LPS-treated cells ($^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$) n = 5

6.3.2 Curcumin blocked the LPS-induced decreased ribosomal protein (70KD) P70^{S6K} Phosphorylation

The level of phosphorylated P70^{S6K} has been measured to evaluate the effect of curcumin on the translation initiation step of PS in C2C12 myotubes. The data presented in (Figs. 6-2 and 6-5*B*) showed that, LPS alone significantly decreased p70^{S6K} phosphorylation by -31 % ($P < 0.05$) compared to control cells, while incubation of C2C12 myotubes with LPS and curcumin (12 and 25 µM) could not shift this effect to the normal basal levels (Fig. 6-2). Notably, incubation of C2C12 myotubes with LPS along with curcumin (50 µM) significantly decreased the P70^{S6K} activity by -56% ($P < 0.01$) compared with the control cells (Figs. 6-2 and 6-5*B*).

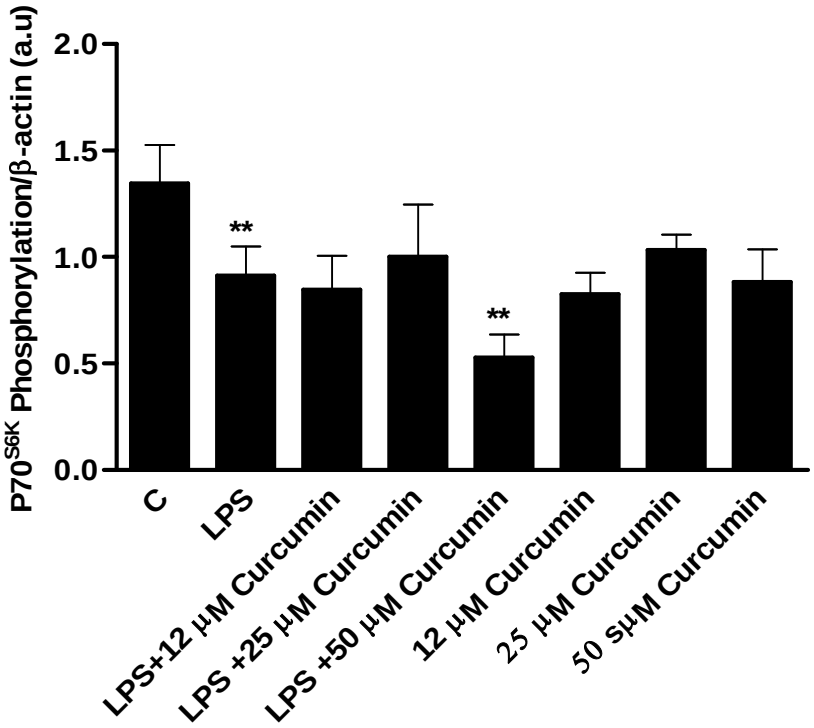


Figure (6-2) LPS and curcumin induced changes phospho-$P70^{S6K}$. C2C12 myotubes were incubated either with LPS (1 μg/ml), or with LPS (1 $\mu g ml^{-1}$) and Curcumin (12, 25 and 50 μM), or with Curcumin (12, 25 and 50 μM) alone for 3 h. Values are represented as means ± SEM arbitrary unit normalised to β-actin as an internal loading control. Bars carry asterisks are statistically significant from control cells ($^{**}P < 0.01$). n = 5

6.3.3 The effect of curcumin on the activity-related phosphorylation of p38 and Erk1/2

The effect of LPS and LPS with curcumin and curcumin alone on the phospho-p38 and phospho-Erk1/2 was summarised in (Figs. 6-3*A*, 6-3*D*, 6-5*C* and 6-5*D*). Incubation of cells with LPS alone significantly increased p38 phosphorylation by 44% ($P < 0.01$) compared to the control cells. While, LPS with curcumin (12, 25 and 50 μM) significantly decreased the p38 phosphorylation ($P < 0.001$) compared with the LPS-treated group (Figs. 6-3*A* and 6-5*C*). Similarly, incubation of C2C12 myotubes with curcumin (12, 25 and 50 μM) alone significantly decreased p38 phosphorylation compared to the LPS-treated. Regarding the phospho-Erk1/2, incubation of cells with LPS for 3 h significantly increased the Erk1/2 phosphorylation by 94% ($P< 0.01$) compared with the control cells. Likewise, incubation of cells with LPS and curcumin (12, 25 and 50 μM) significantly increased the phosphorylation of Erk1/2 by (100% ($P < 0.05$), 56% ($P < 0.001$) and 144% ($P < 0.01$), respectively) compared to the control cells. Similarly, The Erk1/2 phosphorylation has been increased significantly when the cells were incubated with curcumin (25 μM) by 95% ($P < 0.05$) and curcumin (50 μM) by 128% ($P < 0.05$) compared to the control cells (Figs. 6-3*B* and 6-5*D*).

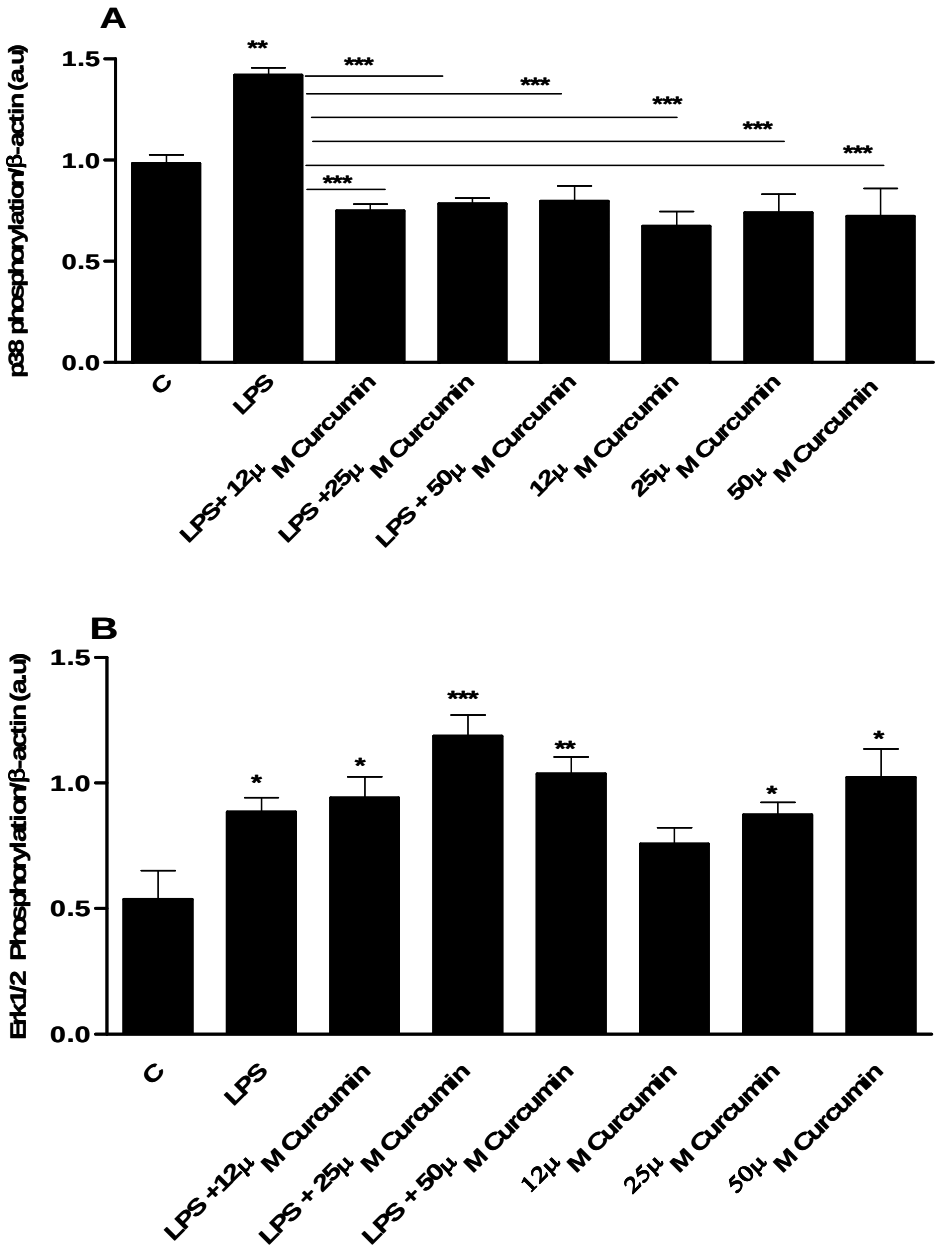


Figure (6-3) LPS and curcumin induced changes in **A**) phospho-p38 and **B**) phospho-Erk1/2. C2C12 myotubes were incubated either with LPS (1 μgml^{-1}) alone, or with LPS (1 μg/ml), or with Curcumin (12, 25 and 50 μM), and Curcumin (12, 25 and 50 μM) alone for 3 h. Values are represented as means ± SEM arbitrary unit normalised to β-actin as an internal loading control. Bars carry asterisks are statistically significant from control and LPS-treated cells ($^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$). n = 5

6.3.4 The effect of curcumin on the I*k*B-α phosphorylation

Incubation of C2C12 myotubes with LPS significantly increased the activity related phosphorylation of I*k*B-α by 84% ($P < 0.05$) and decreased the total I*k*B-α by -22% ($P < 0.05$) compared with control cells (Figs. 6-4*A* and 6-4*B*). Incubation of cells with LPS and curcumin (12 and 25 μM) significantly decreased the I*k*B-α phosphorylation by -31% ($P < 0.01$) and -28% ($P < 0.05$) compared to the LPS-treated cells (Figs. 6-4*A* and 6-5*E*). Incubation of C2C12 myotubes either with LPS in combination with curcumin (12, 25 and 50 μM) or with curcumin (12, 25 and 50 μM) alone had no effect on either I*k*B-α phosphorylated and total level compared with control cells (Figs. 6-4*A*, 6-4*B* and 6-5*E*).

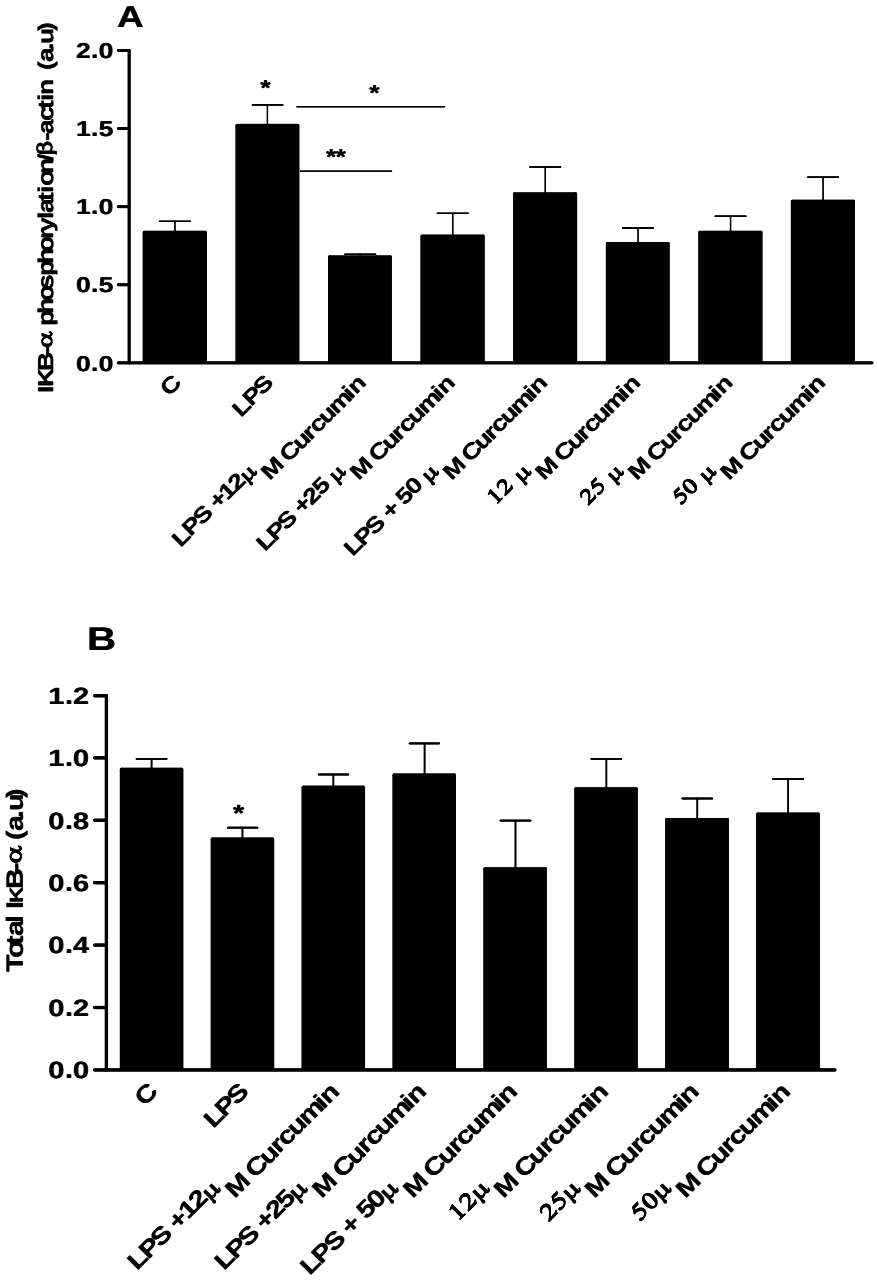


Figure (6-4) LPS and curcumin induced changes in A) phospho-IkB-α and B) total I*k*B-α. C2C12 myotubes were incubated either with LPS (1 μgml^{-1}) alone, or with LPS (1 μg/ml) and Curcumin (12, 25 and 50 μM), or with Curcumin (12, 25 and 50 μM) alone for 3 h. Values are represented as means ± SEM arbitrary unit normalised to β-actin as an internal loading control. Bars carry asterisks are statistically significant from control and LPS-treated cells ($^{*}P < 0.05$ and $^{**}P < 0.01$), n = 5

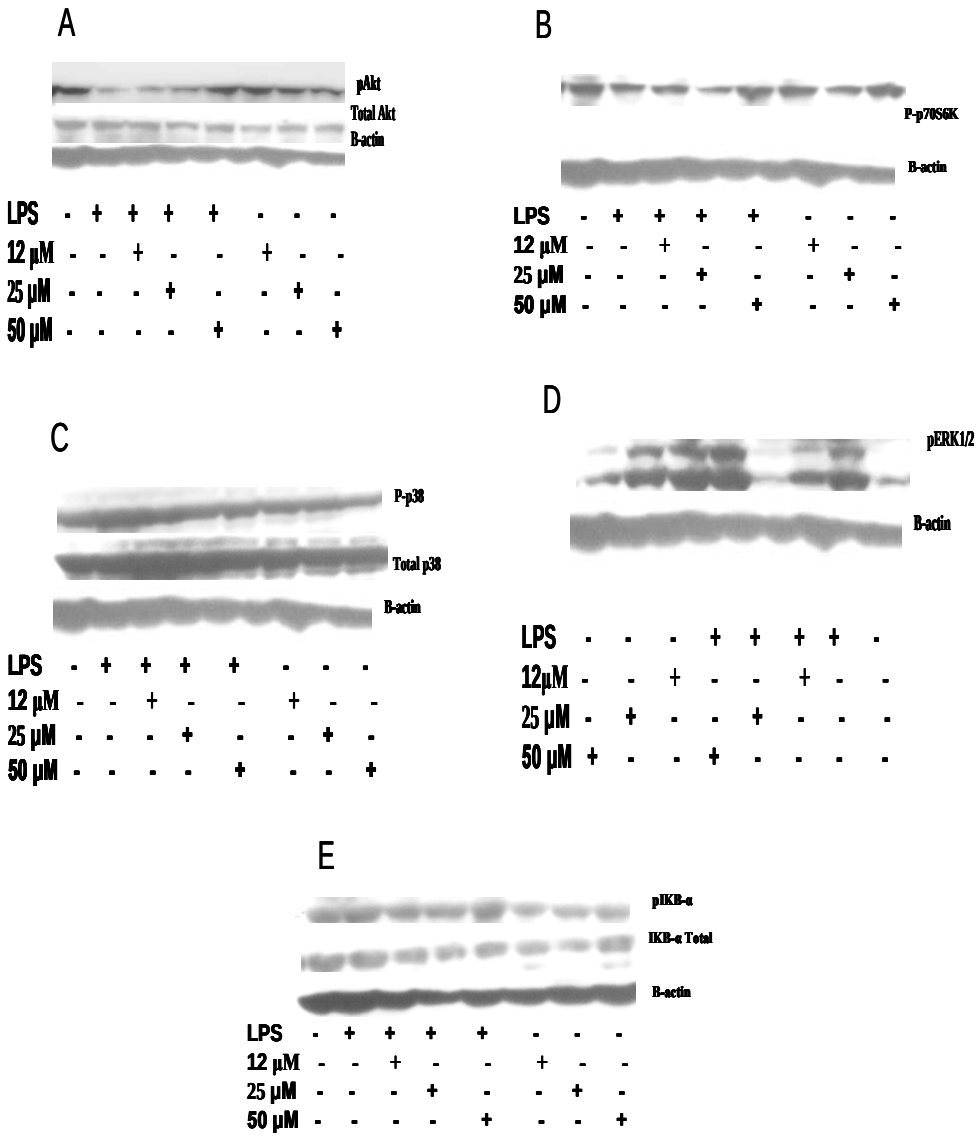


Figure (6-5) Representative blots of A) Akt, B) $p70^{S6K}$, C) p38, D) Erk 1/2 and E) I*k*B-α. C2C12 myotubes were incubated either with LPS (1 $\mu g ml^{-1}$) alone, or with LPS (1 $\mu g ml^{-1}$) and Curcumin (12, 25 and 50 µM), or with Curcumin (12, 25 and 50 µM) alone for 3 h. values are normalised to the β-actin as an internal loading control.

6.3.5 Curcumin decreases the level of TNF-α and IL-6 cytokines

Incubation of cells with LPS significantly increased the production of TNF-α cytokine by 58% ($P < 0.05$) compared with the control cells. While, incubation of cells with LPS in combination with curcumin (12, 25, 50 μM) significantly decreased the TNF-α production by 51%, 58% and 56% respectively ($P < 0.001$) compared with the LPS-treated cells. Interestingly incubation of cells with LPS and curcumin (25 μM) significantly decreased the TNF-α production by -38% ($P < 0.01$) compared to the control group. Incubation of cells with curcumin (25 and 50 μM) alone significantly decreased the TNF-α production by -48% and -46% respectively ($P < 0.01$) compared with the control cells (Fig. 6-6). Regarding the IL-6, our data in (Fig. 6-7) showed that, LPS significantly increase the IL-6 cytokine by 175% ($P < 0.001$) compared to the control cells. Likewise, incubation of cells with LPS and curcumin (12, 25 μM) significantly increased the IL-6 production by 70% and 78% respectively ($P < 0.05$) compared to the control cells. Comparing this effect LPS-treated cells, incubation of cells with LPS along with curcumin (12, 25 and 50 μM) significantly reduced the LPS mediated up-regulation of IL-6 by -37%, -40%, and -70% respectively ($P < 0.001$,). Finally, incubation of cells with curcumin (12 and 25 μM) significantly reduced LPS-induced IL-6 production by -50% ($P < 0.001$) compared with control cells.

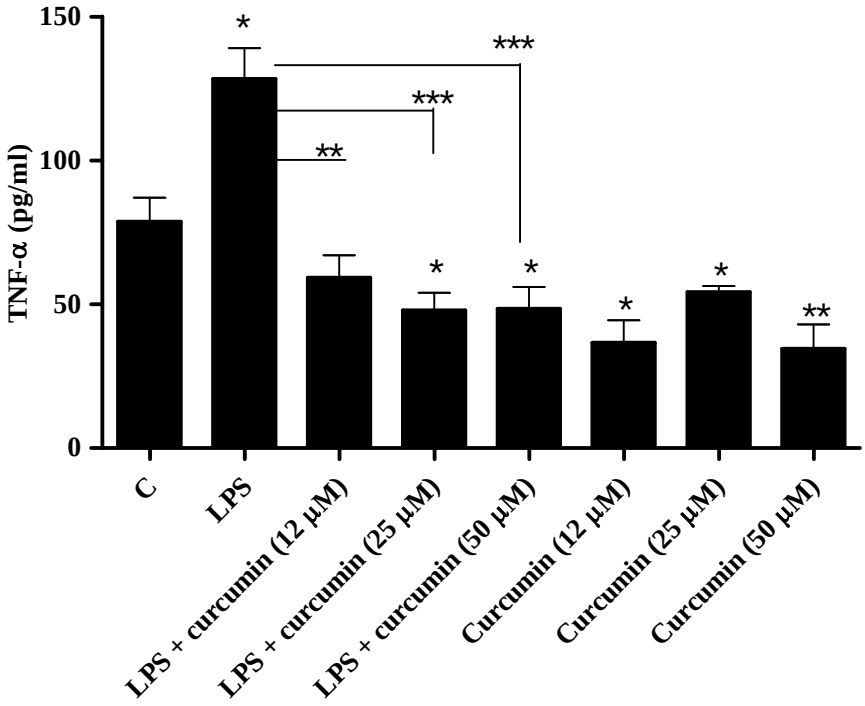


Figure (6-6) Effect of LPS and Curcumin on the level of TNF-α cytokine. C2C12 myotubes incubated either with LPS (1 µgml^{-1}), or with LPS (1 µgml^{-1}) and curcumin (12, 25 and 50 µM), or with curcumin (12, 25 and 50 µM) alone for 3 h. Values are represented as means ± SEM. Bars carry asterisks are statistically significant from control and LPS-treated cells ($^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$). n = 6

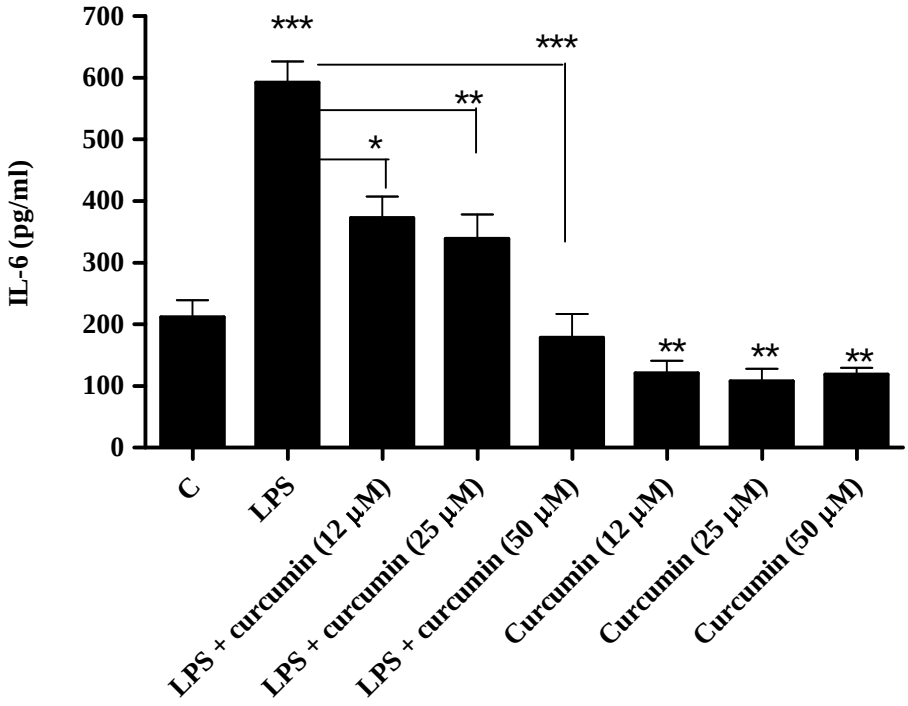


Figure (6-7) Effect of LPS and Curcumin on the media concentration levels of IL-6. C2C12 myotubes were incubated either with LPS (1 µgml^{-1}) or with LPS (1 µgml^{-1}) and curcumin (12, 25 and 50 µM), or with curcumin (12, 25 and 50 µM) alone for 3 h. Values are represented as means ± SEM. Bars carry asterisks are statistically significant from control and LPS-treated cells ($^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$). n = 6

6.3.6 Incubation of C2C12 myotubes with LPS and Curcumin (25 μM) decreases the LPS-induced chymotrypsin-like enzyme activity

Incubation of C2C12 cells with LPS alone significantly increased chymotrypsin-like activity by 37% ($P < 0.01$) compared with control cells. While, incubation of cells with LPS along with curcumin (12 and 50 μM) significantly increased chymotrypsin-like activity by 39% ($P < 0.05$) and 58% ($P < 0.001$) compared with control cells (Fig. 6-8) respectively. Interestingly, incubation of cell with LPS and curcumin (25 μM) significantly decreased the chymotrypsin-enzyme like activity compared to cells treated LPS alone and LPS with curcumin (12 and 50 μM) (Fig. 6-8). Incubation of cells with curcumin (12 and 25 μM) alone has no effect on the chymotrypsin-enzyme like activity (Fig. 6-8), while curcumin (50 μM) alone significantly increased chymotrypsin-like enzyme activity by 64% ($P < 0.05$) compared to the control cells (Fig. 6-8).

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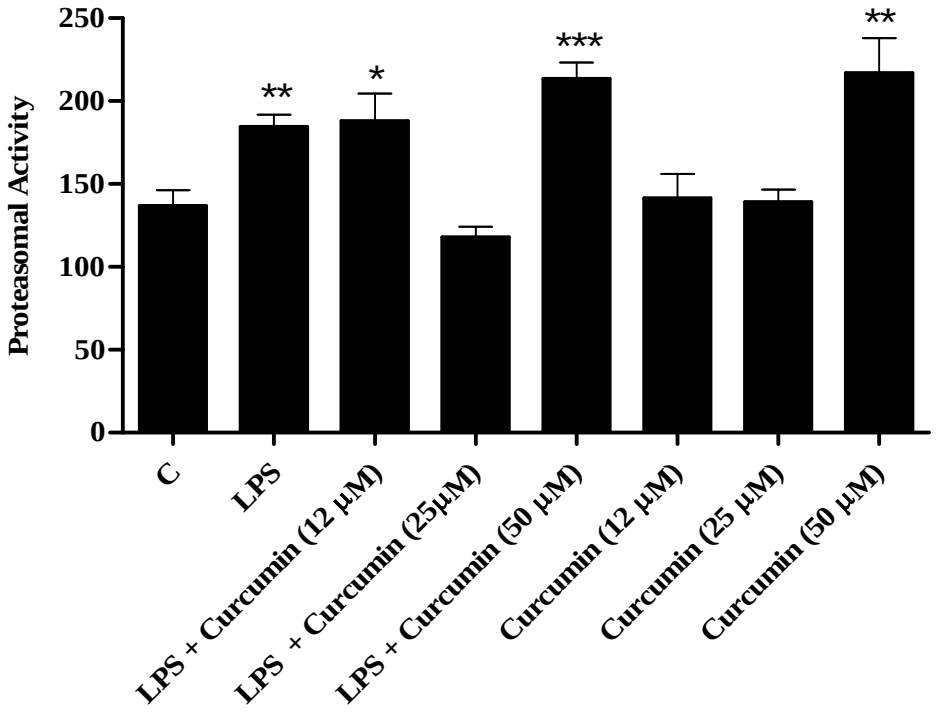

Figure (6-8) Effect of LPS and Curcumin on the chymotrypsin-like enzyme activity. C2C12 myotubes incubated either with LPS (1 µgml^{-1}), or with LPS (1 µgml^{-1}) and curcumin (12, 25 and 50 µM), or with curcumin (12, 25 and 50 µM) alone for 3 h. Values are represented as means ± SEM. Bars carry asterisks are statistically significant from control cells ($^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$), n = 6

6.3.7 Curcumin had no effect on protein synthesis rate in C2C12 myotubes

The final experiment was to elucidate the direct effect of curcumin on the PS rate in LPS-stimulated C2C12 myotubes model. Incubation of C2C12 with LPS alone decreased the PS by -20% ($P > 0.05$) compared with control cells (Fig. 6-9). Whereas, incubation of cells with LPS in combination with curcumin (25 μM) significantly decreased the PS rate by -59% ($P < 0.05$) compared with the control cells (Fig. 6-8). Likewise, incubation of cells with curcumin (50 μM) alone significantly decreased the PS rate (measured as ^{3}H-Tyrosine incorporation) by -35% ($P > 0.5$) compared with the control cells (Fig. 6-9).

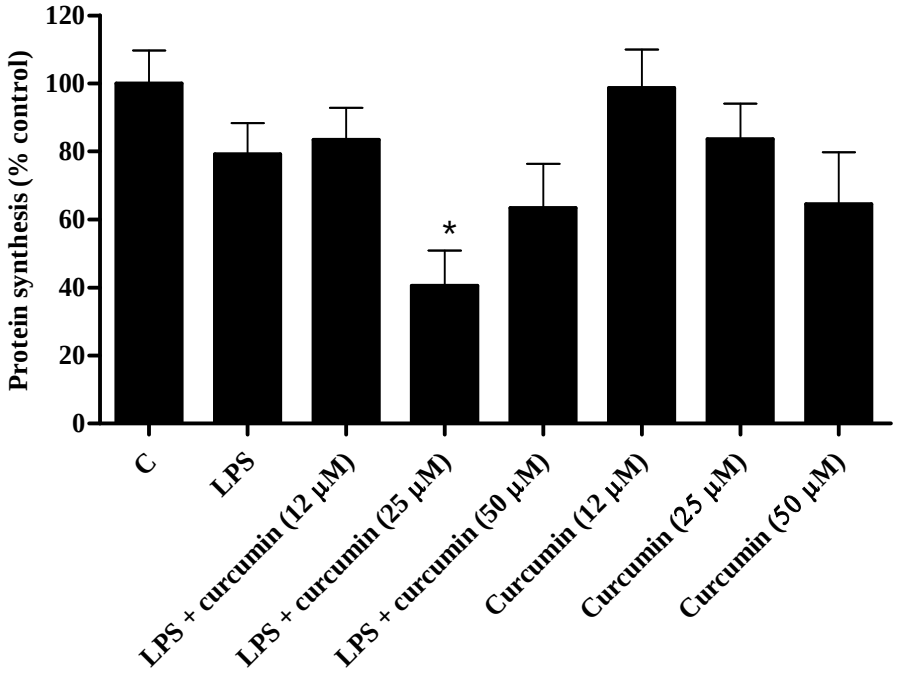

Figure (6-9) Effect of LPS and Curcumin on protein synthesis rate. C2C12 myotubes were incubated either with LPS (1 μgml^{-1}) or with LPS (1 μgml^{-1}) and curcumin (12, 25 and 50 μM) or with curcumin (12, 25 and 50 μM) alone for 3 h. Values are represented as means ± SEM. Bars carry asterisks are statistically significant from the control cells ($^{*}P < 0.05$) n = 6

6.4 Discussion

Decreased lean body mass is a well known outcome of sepsis. This decrease results originally from an accelerated rate of protein degradation and decreased rate of PS. The ubiquitin-proteasome pathway is most important catabolic pathway activated in many acute catabolic conditions (Bodine *et al.*, 2001). There is has been a remarkable increase in our knowledge regarding the factors involved in maintenance the skeletal muscle protein balance during the last two decades however, a better understandings is needed to develop appropriate therapeutic interventions to lessen the adverse effects of sepsis is needed. Thus, the aim was to investigate the direct role of curcumin in maintaining the balance between the protein degradation and PS rates. We have used the LPS-stimulated C2C12 myotubes model to test our hypothesis.

The obtained data along with others *in vivo* studies (Jin and Li 2007; Poylin *et al.,* 2008) demonstrated the value of curcumin as a possible intervention for sepsis-induced muscle wasting. Our model is an *in vitro* model which has shown for the first time the direct role curcumin in preventing the protein degradation with no effect on the LPS-induced decreased PS. Our data have shown that:1) curcumin significantly increased the phosphorylation of Akt, while it could not change the LPS-induced decreased $P70^{S6K}$ phosphorylation; 2) curcumin significantly decreased the LPS-induced increased P38 activity, while it significantly increased the Erk1/2 phosphorylation; 3) Curcumin could not abolish the LPS-induced decreased PS rate in C2C12 myotubes; 4) curcumin significantly decreased the LPS-induced I*K*B-α phosphorylation and prevented the LPS-induced degradation of total I*k*B-α; 5) curcumin significantly blocked LPS-induced TNF-α and IL-6 cytokines production; 6) only curcumin (25 μM) significantly decreased the chymotrypsin-like activity in an effect mediated possibly via the p38 and I*K*B-α pathways.

Although, Our data have shown that only curcumin (25 μM) decreased the LPS-induced accelerated proteasomal activity with no effect on the LPS-induced decreased PS, this suggests curcumin as a potential therapy in sepsis-induced muscle wasting with no side effects even at high doses (Chainani-Wu 2003). Curcumin abolished the LPS-induced proteolytic activity and hence it restore LPS-induced muscle breakdown. This effect was similar to that obtained *in vitro*; curcumin significantly decreased LPS-induced decreased protein content in gasterocnemius muscle (Jin and Li 2007). The data in presented herein and in chapter 3 (Fig. 3-1*A*) with others (Crossland *et al.,* 2008; Frost *et al.,* 2009) have shown that, LPS is a potent catabolic stimulus which suppresses Akt and its down-stream effectors mTOR and the translation initiation mediator ($p70^{S6K}$, eIF-4E and 4E-BP1). Decreased Akt phosphorylation leads to increased protein breakdown via the activation of the ubiquitin-proteasome system mainly atrogin-1 ubiquitin ligase (Sandri *et al.*, 2004; Stitt *et al.*, 2004). Herein, curcumin effectively blocked the LPS-induced decreased Akt phosphorylation, moreover curcumin (25 μM) alone significantly increased Akt phosphorylation in C2C12 myotubes (*in vitro*), while in case of the *in vivo* study that carried out by Jin and Li (2007), curcumin could not alter the LPS-induced decreased Akt activity in mice skeletal muscle. Increased Akt has been implicated in many fundamental cellular processes including protein synthesis via mTOR and their downstream molecules (translation initiation mediators mainly, $p70^{S6K}$, 4E-BP1 and eIF-4E) for more details see (Glass 2005; Wu *et al.,* 2010).

Increased Erk1/2 activity has also been implicated in the stimulation of the protein translation initiation through a pathway that most likely involves the phosphorylation of eIF-4E mediated through the Mnk1 phosphorylation (Waskiewicz *et al.* 1997; Ueda *et al.* 2004). Herein, curcumin (12, 25 and 50 μM) significantly increased Erk1/2 phosphorylation despite the presence of LPS. In addition, curcumin (25 and 50 μM) alone significantly increased Erk1/2 phosphorylation. In chapter 3, we have shown that inhibition of Akt and Erk1/2 significantly decreased the PS in C2C12 myotubes. In this chapter,

although curcumin significantly increased Akt and Erk1/2 phosphorylation, however this effect was not sufficient to abolish the catabolic effect of LPS on the PS. Incubation of the cells with LPS alone decreased the PS by -20% ($P > 0.05$) compared with the control cells. This suggesting that, the correlation between the activity of anabolic signalling and the PS does not always exist (Miranda *et al.* 2008; Atherton *et al.* 2009). Taken together, LPS is a strong catabolic stimulus altering muscle protein metabolism mainly via decreased PS and increased PD rates.

Increased p38 phosphorylation has been reported in many pro-catabolic conditions including limb immobilization (Childs *et al.,* 2003), Type 2 diabetes (Koistinen *et al.,* 2003) and aging (Williamson *et al.,* 2003). The results included in this chapter showed that, LPS is a strong stimulus of p38 activity, this is consistent with the finding of Jin and Li (2007). The positive correlation between the increased p38 and increased atrogin-1 mRNA has been demonstrated in many *in vivo* studies (Li *et al.,* 2005; Jin and Li 2007). Moreover, increased NF-kB activity in skeletal muscle has been shown during sepsis (Penner *et al.,* 2001) or in cultured myotubes treated with pro-inflammatory cytokines (Li and Reid 2000; Ladner *et al.,* 2003). NF-kB a transcriptional factor family protein which when bound to NF-kB inhibitory protein (I*k*B-α) plays a significant role in the sepsis-induced muscle wasting (Cai *et al.,* 2004). Taken together, LPS significantly increased the phosphorylation of p38 and I*k*B-α, while curcumin blocked the LPS-induced p38 and I*k*B-α phosphorylation. This suggests that, curcumin can inhibit many proteolytic pathways activated in sepsis (Poylin *et al.*, 2008), and this leading to a significant reduction in the LPS-induced proteasomal activity.

LPS significantly increased the TNF-α and IL-6 production in skeletal muscle in a time and dose-dependent way, and incubation of cells with TNF-α has been shown to increase the expression of IL-6 mRNA (Frost *et al.,* 2002). The results regarding the role of TNF-α in muscle protein degradation are controversial. Some studies (Costelli *et al.,* 1993; Garcia-Martinez *et al.,* 1993),

demonstrated that, TNF-α enhanced of protein degradation in rat skeletal muscle, and this effect is mediated via p38 and NF-kB pathway (Jin and Li 2007). Recently, Plaisance *et al*. (2008) demonstrated that TNF-α significantly increased the protein synthesis in myotubes, and this effect was mediated via the stimulation of the Akt/Erk1/2 and their down-stream translation initiation mediators. Similarly, IL-6 plays a controversial role, with some studies showing its contribution while the other does not. Herein, curcumin significantly altered the LPS-stimulated TNF-α and IL-6 production (Figs. 6-6 and 6-7) and this effect is associated with significant reduction in the chymotrypsin-like activity especially with curcumin (25 μM). This suggests that, the LPS-induced TNF-α and IL-6 production in C2C12 has been linked to the muscle protein degradation, and the reverse of muscle protein can be achieved when these cytokines are blocked.

Thus, although curcumin (25 μM) significantly decreased LPS-induced proteasomal activity but this had no positive effect on the LPS-induced decreased PS, nevertheless, these effects are still promising but further *in vitro* investigation are needed. Of note, this effect of curcumin on skeletal muscle protein balance (decreasing protein degradation) in addition to the *in vivo* findings of Polyin *et al*. (2008), does not encourage the use of curcumin as a drug of muscle wasting in sepsis but encourages using curcumin in a preventive capacity. Although, herein curcumin (25 μM) significantly decreased the protein degradation in LPS-stimulated C2C12 myotubes, a similar finding obtained from *in vitro* studies, yet, further investigation are required before applying the curcumin as a drug in septic patients, and to validate the beneficial effects of curcumin obtained from our study and other *in vivo* studies (Jin & Li, 2007; Poylin *et al.*, 2008).

6.5 Conclusion

LPS is a potent catabolic stimulus significantly induced muscle proteolysis along with decrease rate of protein synthesis. This effect was mediated mainly via increased p38 and I*k*B-α phosphorylation along with the liberation of many pro-inflammatory cytokines in particular TNF-α and IL-6. Curcumin (25 μM) significantly abolished this effect and thus leads to significant reduction in the proteasomal activity. In contrast, although curcumin significantly increased the phosphorylation of Akt and Erk1/2, however this effect was not sufficient to abolish the LPS-induced decreased PS.

7 Avian primary skeletal muscle cell resist the effect of LPS

7.1 Introduction

Primary cultures of skeletal muscle are considered as an ideal model for studying terminal cellular differentiation, and extensively used for the study of the *in vitro* myogenesis (Richler and Yaffe 1970; Braun *et al.,* 1989). The primary cultures of skeletal muscle have been widely used in the study of animal models of muscular and neuromuscular diseases and parasitic cytogenesis (Fong *et al.,* 1990; Metzinger *et al.,* 1993; Passaquin *et al.,* 1993; Bouzakri *et al.,* 2003; Guimaraes *et al.,* 2008). Under normal conditions, the balance between the rate of protein degradation and synthesis controls the maintenance of the lean body mass. Sepsis is an acute inflammatory condition characterised by accelerated rate of protein degradation with or without decreased rate of PS.

The regulation of the muscle protein metabolism has been measured either *in vitro* using C2C12 murine skeletal myotubes (Frost *et al*., 2009; Russell *et al*., 2010), or *in vivo* using a septic rat model (Lang *et al*., 2000; Vary and Kimball 2000; Lang and Frost 2007), and in primary cultured myotubes (Vandenburgh and Kaufman 1980; Janeczko and Etlinger 1984; Gulve and Dice 1989). The skeletal muscle cells can be grown as a primary cultures (Konigsberg 1979), with similar features as the embryonic muscle, for more details see (Fischbach *et al.,* 1974; Sandra and Przybylski 1979; Siegelbaum *et al.,* 1984). Therefore, cultured muscle cells are considered as a unique model to study various catabolic conditions where the net nitrogen balance can range from positive to negative(Gulve and Dice 1989). *In vivo* studies have shown that, LPS significantly decreased the PS rate in the gastrocnemius muscle of rat (Lang *et al.*, 2000). In addition, sepsis significantly decreased

the rate of PS in the gastrocnemius but not in the slow-twitch soleus muscle (Vary and Kimball 1992). *In vitro* studies using C2C12 myotubes remain controversial. Frost *et al*. (2009) showed that LPS alone had no effect on PS in C2C12 myotubes, while LPS in combination with IFN-γ significantly decreased PS by 80%. In alter study by Russell *et.al* (2010) a significant 50-60% decreases in the PS rate in response to LPS in the same cell line was shown. Previous *in vitro* and *in vivo* studies demonstrated that sepsis increased the rate of protein degradation and decreased the rate of PS acting on the translation initiation level (Vary *et al*., 1994; Jurasinski *et al*., 1995; Lang *et al*., 2000; Lang and Frost 2007), not the reduction in the elongation (Vary and Kimball 1992). Similarly, the available data about the regulation of the protein breakdown in primary chick myotubes (Vandenburgh and Kaufman 1980; Janeczko and Etlinger 1984; McElligott and Dice 1984) and in myoblasts from the L6 rat cell line (Ballard 1982; Ballard and Francis 1983; Ballard *et al.,* 1986), propose that both protein synthesis and degradation are subjected to regulation. This regulation has been carefully compared by Ballard (1982). The process of protein synthesis includes three main steps: initiation, elongation, and termination. Akt is one of the most extensively studied anabolic signalling pathways. Increased Akt activity is associated with skeletal muscle hypertrophy via increased protein synthesis (Lai *et al*., 2004). In contrast, decreased Akt activity has been demonstrated in burn-induced muscle atrophy (Sugita *et al*., 2005). The direct contribution of Akt in both muscle atrophy and hypertrophy has been discussed in details in (Glass 2005; Wu *et al.* 2010). The translation initiation step of protein synthesis controlled by a group of eIFs. The eIF-4E is bound to 4E-BP1. This limits the availability of eIF-4E for the formation of eIF4E/eIF4G active complex. This effect is withdrawn by the phosphorylation of 4E-BP1 (Flynn and Proud 1996). In sepsis the amount of eIF4E/4E-BP1 inactive complex is increased in gastrocnemius muscle by 80% and later it significantly decreased the amount of the active eIF4E/eIF4G active complex by a 65% (Lang and Frost 2007).

LPS increases the skeletal muscle protein breakdown via stimulation of the ubiquitin proteasome pathway (Hershko and Ciechanover 1998). This effect is preceded by increased cytokines synthesis and accumulation of their mRNA in the skeletal muscle mediated via TLR4 (Frost *et al*., 2002). It has previously been shown that, LPS significantly increased the release and production of TNF-α and IL-1β (Vary 1998; Lang *et al*., 2003) two main mediators implicated in the muscle proteolysis. However, it still unclear, how the avian primary skeletal muscle cells respond to LPS.

The use of primary cells has been used to mimic the *in vivo* state and generate more physiologically relevant data and has become a common technique. Once differentiation has occurred, cultured skeletal muscle myotubes may respond to factors that modulate protein degradation and synthesis in adult skeletal muscle (Vandenburgh and Kaufman 1980).

In the previous chapters (3 & 4), that the data presented showed that LPS increases the chymotrypsin-like enzyme activity and decreases the rate of PS in C2C12 myotubes. LPS significantly decreased the rate of PS by 50% but this effect is transient lasting for 3 h, and is mediated by TLR4 and the Akt signalling cascades. It was also demonstrated that, LPS increased the synthesis and the release of many cytokines including, TNF-α and IL-6. Finally, LPS increased the mRNA level of the atrogin-1, MuRF1 and TLR4. In the present chapter the aim was to explore the effect of LPS on the avian primary skeletal muscle protein metabolism (protein synthesis and breakdown). Therefore, LPS (1 $\mu g ml^{-1}$)-stimulated avian primary skeletal muscle cells model was used for 3 h and 18 h. Prof. Kin Chaw Chang generously provided the avian primary skeletal muscle cells.

7.2 Hypothesis

In the previous chapters, the data presented have shown that LPS mediated increased protein breakdown and decreased protein synthesis. This effect involves the interaction of many signalling pathways including PI3K/Akt and MAPKs (p38 or Erk). In contrast, there is no clear data regarding the effect of LPS on primary skeletal muscle cells. We assume that the effect of LPS on avian primary skeletal muscle will not be the same. Therefore, LPS stimulated avian primary skeletal muscle cells septic model for 3 h has been used. We have only one time point (3 h) because the maximal changes in PS in C2C12 myotubes were observed at this time point.

7.3 Results

7.3.1 LPS effect on Akt, 4E-BP1 and eIF-4 phosphorylation

Incubation of the primary skeletal muscle with LPS for 3 h decreased Akt and eIF-4E phosphorylation but this effect was not significant compared to the untreated control cells (Figs. 7-1*A* and 7-1*C*). While, LPS significantly increased the activity related phosphorylation of 4E-BP1 ($P < 0.01$) compared to the untreated control cells (Fig. 7-1*B*).

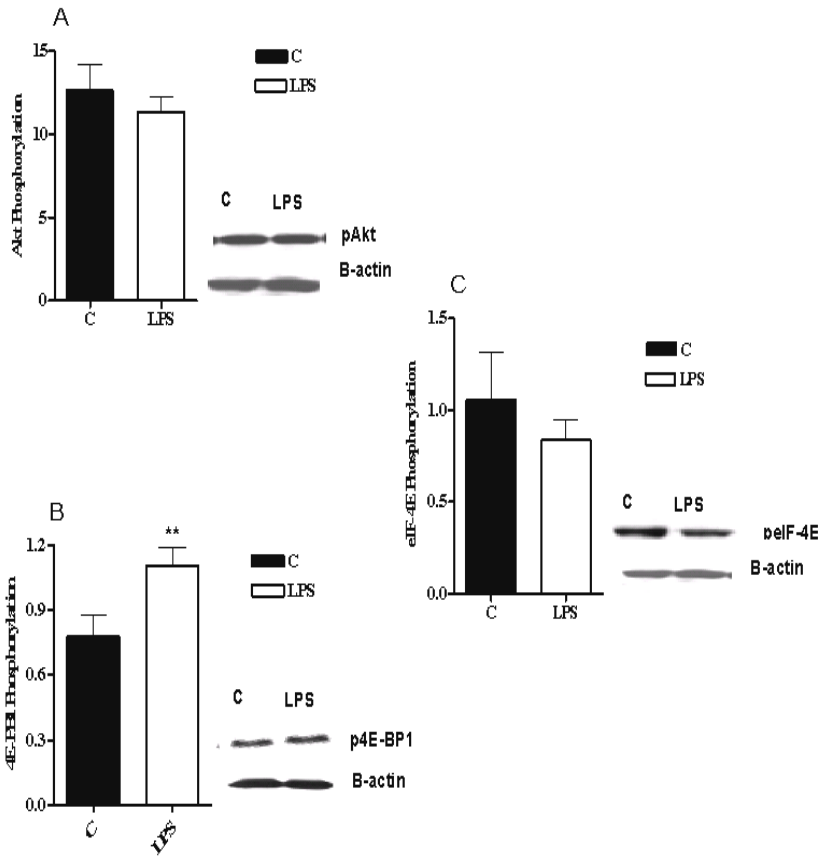

Figure (7-1) Changes in **A**) phospho-Akt , **B**) phospho-4E-BP1, and **C**) phospho-eIF-4E. Avian primary skeletal muscle cells were incubated with LPS (1 $\mu g ml^{-1}$) for 3 h. Then western blotting method was used to measure the level of phosphorylated protein. Values are represented as arbitrary unit means ± SEM normalized to the levels of β-actin as an internal loading control. Bars carry asterisks are statistically significant from control cells ($^{**}P < 0.01$). n = 6

7.3.2 LPS significantly decreased the proteasomal activity

LPS decreased the chymotrypsin-like enzyme activity ($P < 0.05$) compared with the untreated control cells (Fig. 7-2). The effect of LPS on the chymotrypsin-like enzyme activity verified using specific inhibitors. MG-132, the specific proteasome inhibitor decreased the chymotrypsin-like enzyme activity ($P < 0.001$) compared with the untreated control cells. Similarly, incubation of the cells with LPS and Polymyxin B (5 $\mu g ml^{-1}$), or with LPS and curcumin (12 μM) significantly decreased the chymotrypsin-like enzyme activity ($P < 0.01$ and $P < 0.001$ compared to the control cells respectively) (Fig. 7-2). In contrast, incubation of the primary skeletal muscle cells with the LPS and LY0294002 (50 μM) significantly increased the chymotrypsin like activity compared with the untreated control cells ($P < 0.05$), and compared with the LPS-treated cells ($P < 0.001$) (Fig. 7-2). Incubation of cells with LPS and SB203580 (SB, 10 μM) or PD098059 (PD, 40 μM) had no effect on the chymotrypsin-like enzyme activity (Fig. 7-2).

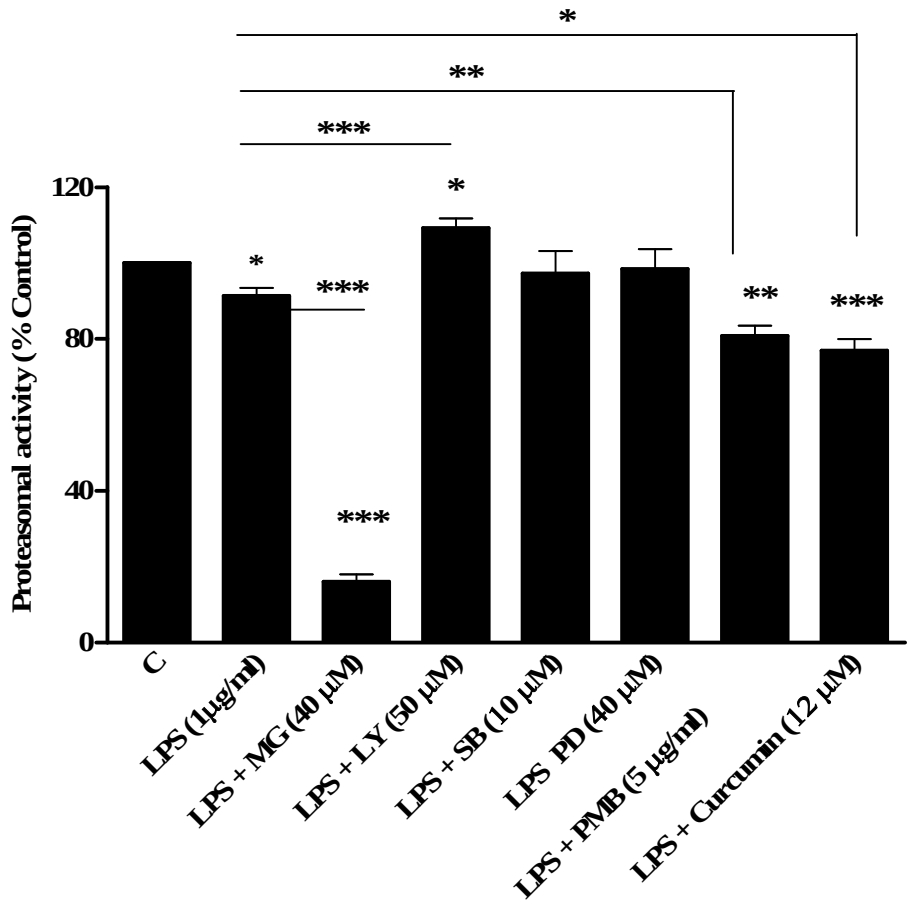

Figure (7-2) Changes in chymotrypsin-like enzyme activity (proteasomal degrdation of muscle protein). Avian primary skeletal muscle cells were incubated either with LPS (1 µgml^{-1}) alone, LPS and MG-132 (proteasomal inhibiotr) (40 µM), LPS and LY0249002 (PI3-K/Akt inhibitor) (LY, 50 µM), LPS and SB203580 (p38 inhibiotr) (SB, 10 µM), LPS and PD098059 (MEK/Erk inhibitor) (PD, 40 µM), LPS and Polymyxin B (LPS neutralisng agnet) (PMB, 5 µgml^{-1}), or LPS andcurcumin (12 µM) for 3 h. Values are represented as means ± SEM (percentage of control). Bars carry asterisks are statistically significant from the control cells ($^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$). n = 3

7.3.3 LPS has no effect on the TNF-α mRNA

Incubation of the avian primary skeletal muscle with LPS alone or in combination with Polymyxin B (5 μgml^{-1}), SB203580 (SB, 10 μM), PD098059 (PD, 40 μM), or LY0294002 (LY, 50 μM) had no effect on the TNF-α mRNA expression level (Fig. 7-3).

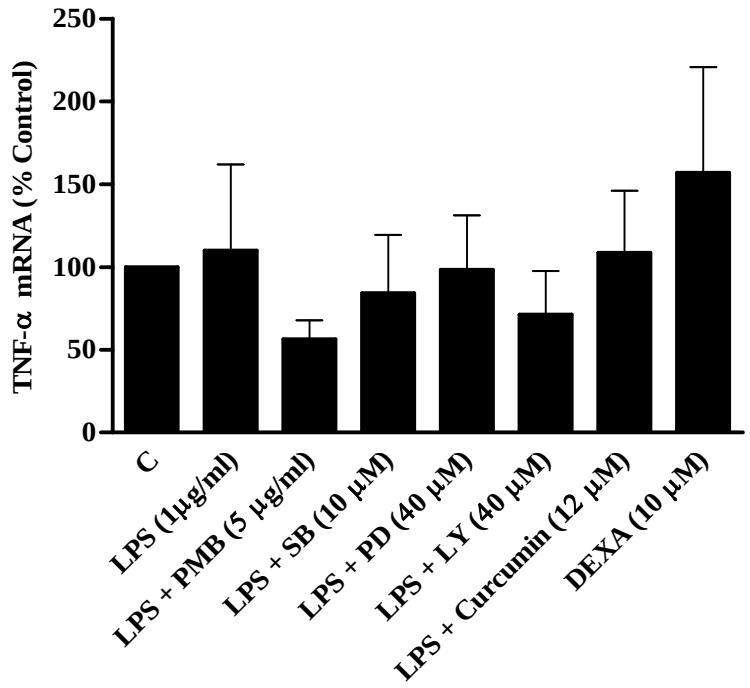

Figure (7-3) Changes in TNF-α mRNA. Avian primary skeletal muscle cells were incubated either with LPS (1 μgml^{-1}) alone, Dexamethasone (DEXA, 10 μM), LPS and LY0249002 (PI3K/Akt inhibitor) (LY, 50 μM), LPS and SB203580 (p38 inhibitor) (SB, 10 μM), LPS and PD098059 (MEK/Erk inhibitor) (PD, 40 μM), LPS and Polymyxin B (LPS neutralsing agent) (PMB, 5 μgml^{-1}), or LPS and curcumin (12 μM) for 3 h. Values are represented as means ± SEM (percentage of control). n = 3

7.3.4 LPS has no effect on the atrogin-1 mRNA activity

Incubation of cells with LPS alone had no effect on atrogin-1 mRNA. Likewise, incubation of cells with LPS in combination with Polymyxin B (PMB, 5 $\mu g ml^{-1}$), SB203580 (SB, 10 μM) and PD098059 (PD, 40 μM) had no effect on the atrogin-1 mRNA. On the contrary, incubation of the cells with LPS and LY0294002 (LY, 50 μM) significantly increased atrogin-1 mRNA by 309.9 ± 84.62% compared with untreated control cells ($P < 0.01$), and compared with LPS-treated cells ($P < 0.001$). The effect of LPS on atrogin-1 mRNA was compared against dexamethasone, where it significantly increased atrogin-1 mRNA by 475.1 ± 85.36% ($P < 0.001$) compared with the untreated control cells (Fig. 7-4).

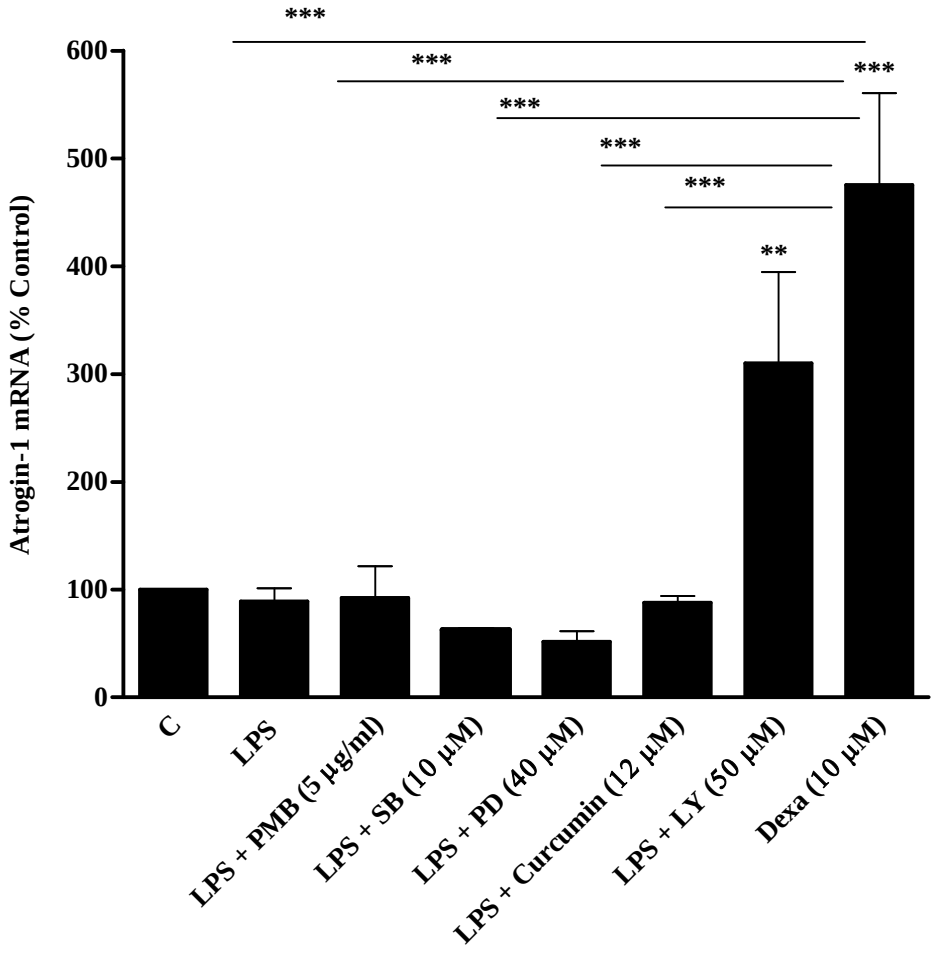

Figure (7-4) Changes in atrogin-1 mRNA expression. Avian primary skeletal muscle cells were incubated either with LPS (1 µgml^{-1}) alone, Dexamethasone (DEXA, 10 µM), LPS and LY0249002 (PI3-K/Akt inhibitor) (LY, 50 µM), LPS and SB203580 (p38 inhibitor) (SB, 10 µM), LPS and PD098059 (MEK/Erk inhibitor) (PD, 40 µM), LPS and Polymyxin B (LPS neutralsing agent) (PMB, 5 µgml^{-1}), or LPS and curcumin (12 µM) for 3 h. Values are represented as means ± SEM (percentage of control). Bars carry asterisks are statistically different the control cells ($^{**}P < 0.01$ and $^{***}P < 0.001$). n = 3

7.3.5 LPS has no effect on the expression level of the IL-6, caspase-3 and ubiquitin mRNA

Incubation of cells with LPS alone or in combination with specific inhibitors had no effect on the expression levels of the IL-6 mRNA (Fig. 7-5), caspase-3 mRNA (Fig. 7-6) and ubiquitin mRNA (Fig. 7-7).

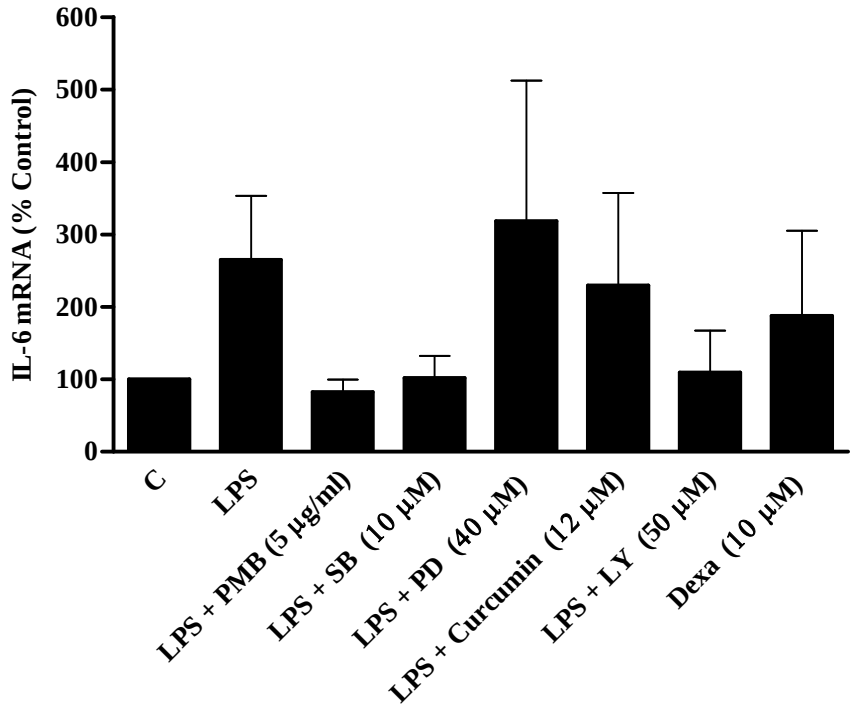

Figure (7-5) Changes in IL-6 mRNA expression. Avian primary skeletal muscle cells were incubated either with LPS (1 µgml^{-1}) alone, Dexamethasone (DEXA, 10 µM), LPS and LY0249002 (PI3-K/Akt inhibiotr) (LY, 50 µM), LPS and SB203580 (p38 inhibitor) (SB, 10 µM), LPS and PD098059 (MEK/Erk inhibitor) (PD, 40 µM), LPS and Polymyxin B (LPS neutralsing agent) (PMB, 5 µgml^{-1}), or LPS and curcumin (12 µM) for 3 h. Values are represented as means ± SEM (percentage of control). n = 3

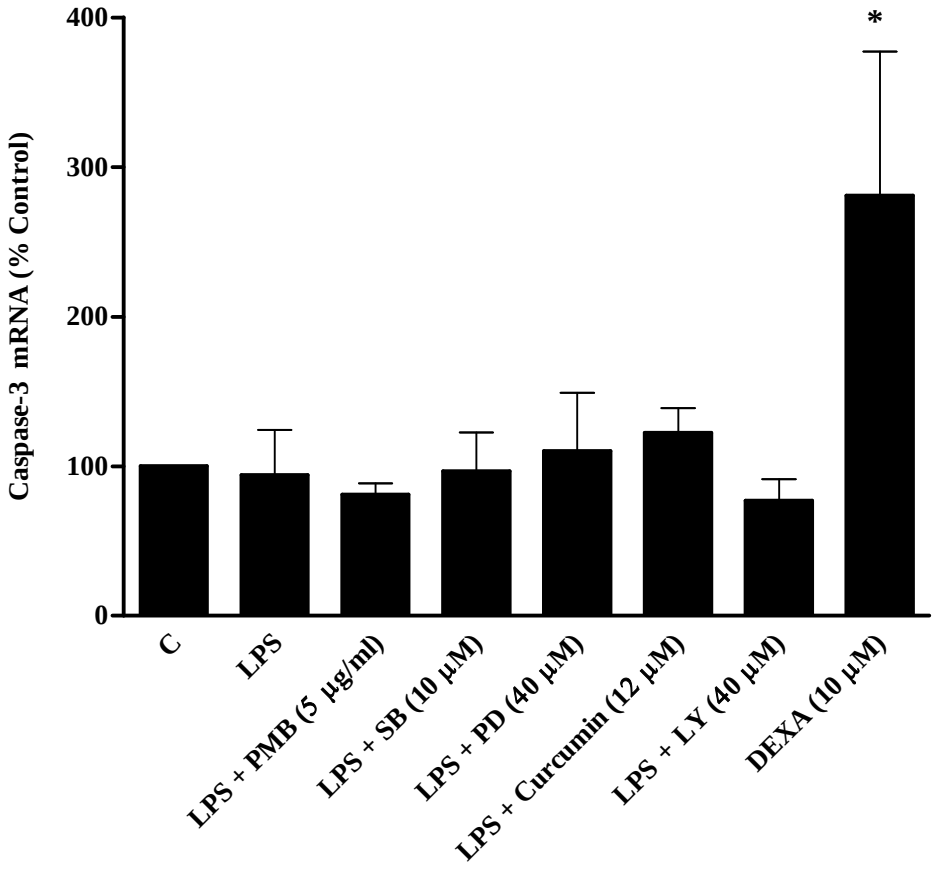

Figure (7-6) Changes in caspase-3 mRNA expression Avian primary skeletal muscle cells were incubated either with LPS (1 µgml^{-1}) alone, Dexamethasone (DEXA, 10 µM), LPS and LY0249002 (PI3K/Akt inhibitor) (LY, 50 µM), LPS and SB203580 (P38 inhibitor) (SB, 10 µM), LPS and PD098059 (MEK/Erk inhibitor) (PD, 40 µM), LPS and Polymyxin B (LPS neutralsing agent) (PMB, 5 µgml^{-1}), or LPS and curcumin (12 µM) for 3 h. Values are represented as means ± SEM (percentage of control). Bars carry asterisks are statistically different from control cells ($^{*}P < 0.05$). n = 3

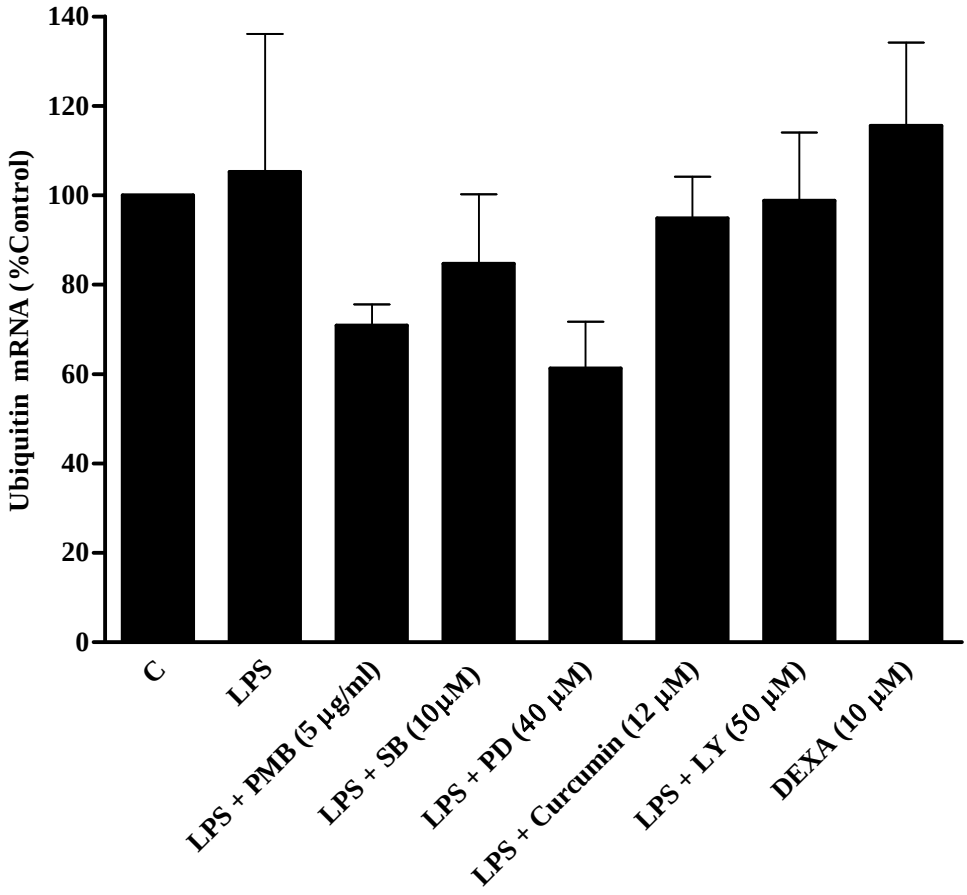

Figure (7-7) Changes in Ubiquitin mRNA expression. Avian primary skeletal muscle cells were incubated either with LPS (1 $\mu g ml^{-1}$) alone, Dexamethasone (DEXA, 10 µM), LPS and LY0249002 (PI3K/Akt inhibitor) (LY, 50 µM), LPS and SB203580 (p38 inhibitor) (SB, 10 µM), LPS and PD098059 (MEK/Erk inhibitor) (PD, 40 µM), LPS and Polymyxin B (LPS neutralisng agent) (PMB, 5 $\mu g ml^{-1}$), or LPS and curcumin (12 µM) for 3 h. Values are represented as means $\pm$ SEM (percentage of control). n = 3

7.3.6 LPS significantly increased the rate of PS

The data presented before showed that LPS significantly decreased the chymotrypsin-like enzyme activity and that it has no effect on the major catabolic mediators. Therefore, the next experiment was mainly to evaluate the direct effect of LPS on the rate of PS expressed as percentage ^{3}H-Tyrosine incorporation/ μg 5% TCA precipitable protein. It was found that LPS increased the rate of PS by 251 % ($P < 0.001$) after 3 h treatment (Fig. 7-8) with no further change was observed at the 18 h time point.

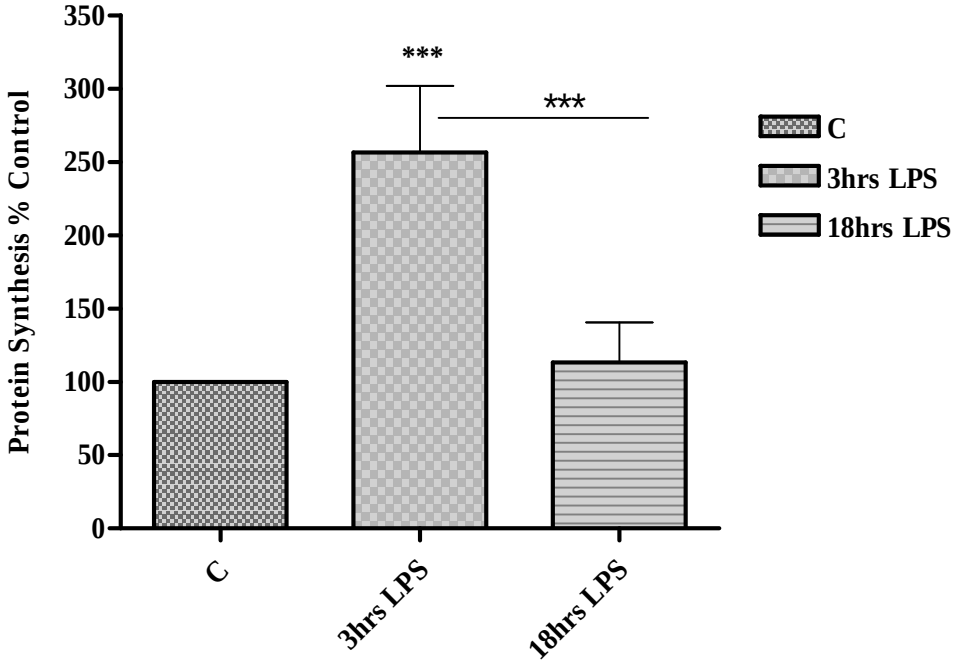

Figure (7-8) Changes in PS rate expressed as the direct incorporation of ^{3}H-Tyrosine. Avian primary skeletal muscle cells were incubated with LPS (1 μgml^{-1}) alone for 3 and 18 h. Values are represented as means ± SEM (percentage of control). Bars carry asterisks are significantly significant from the control and 18 h LPS-treated cells ($^{***}P < 0.001$). n = 6.

7.4 Discussion

The data presented here in this chapter suggest that, the effect of LPS on the avian primary skeletal muscle cells is not the same as what we have seen in the C2C12 myotubes (chapters 3, 4; 5 and 6). Herein, the effect of LPS on the avian primary skeletal muscle cells can be summarised as the following 1) LPS significantly decreased the chymotrypsin-like enzyme activity; 2) LPS significantly increased the protein synthetic rate; 3) LPS has no effect on the mRNA levels of TNF-α, IL-6, atrogin-1, caspase-3 and ubiquitin.

The regulation of muscle protein synthesis and degradation in cultured myotubes have been measured as a response to various anabolic stimulus such as serum, insulin and insulin-like growth factors (Vandenburgh *et al.,* 1991), foetal bovine serum, human or bovine colostrums (Ballard 1982), and multiplication-stimulating activity (Janeczko and Etlinger 1984). These data presumes that, both protein synthesis and degradation are subject to regulation. Incubation of cultured myotubes (primary cultures of chicken embryo skeletal muscles) with 10%(v/v) horse serum decreased the protein turnover compared with serum free media (Janeczko and Etlinger 1984).

The data presented in this chapter demonstrated that LPS significantly decreased the chymotrypsin-like enzyme activity in avian primary skeletal muscle cells. In contrast, we have shown in chapter 4 (Fig 4-1*A*) that LPS significantly increased its activity in a time-dependent manner in C2C12 murine myotubes. Although, LPS is a strong catabolic stimulus significantly increased the muscle proteolysis (Jepson *et al.,* 1986; Hummel *et al.,* 1988; Ash and Griffin 1989; Hasselgren *et al.,* 1989; Lai *et al.,* 2004). However, avian primary skeletal robustly resist this effect. Interestingly, incubation of the avian primary chicken skeletal muscle cells with LPS for 3 h significantly

increased the PS ($P < 0.001$). While, in case of C2C12 myotubes (chapter 3) LPS significantly decreased the rate of PS at 3 h by 50% ($P < 0.05$) (Fig. 3-4). Also, LPS in combination with and IFN-γ significantly induced 80% decrease in the (Frost *et al.,* 2009). The data obtained either from *in vivo* (Lang *et al*., 2000; Vary and Kimball 2000; Lang *et al*., 2002; Lang and Frost 2007; Crossland *et al*., 2008), or *in vitro* C2C12 myotubes (Frost *et al*., 2009) studies have demonstrated that, LPS altered the muscle protein synthesis via Akt signalling pathway and later impaired the translation initiation mediators. In the chapter 3, LPS decreased the Akt/mTOR phosphorylation and later decreased the translation initiation molecules ($p70^{S6K}$, 4E-BP1 and eIF-4E) in C2C12 myotubes (Figs. 3-1*A*, 1*B*, 3-3*A*, 3*B* and 3*C*). In this chapter, LPS decreased the Akt phosphorylation and significantly increased 4E-BP1 Phosphorylation (Figs. 7-1*A* and 7-1*B)*. Increased 4E-BP1 phosphorylation increases the availability of eIF-4E to form the eIF-4F complex. The formation of eIF-4f complex initiates the binding of the mRNA to the 43S preinitiation complex (43 S ribosome and eIF-4F complex), and this step is considered as rate-limiting step in translation initiation (Shen *et al.,* 2005). Therefore, we assume that LPS-induced increased PS in avian primary skeletal muscle model was initiated primarily from increased 4E-BP1 phosphorylation and consequently the translation initiation.

The data collected from our previous experiments in chapters 3 & 4 showed that, LPS significantly increased the production and accumulation of mRNA of many cytokines and atrogens in skeletal muscle including TNF-α, atrogin1 and MuRF1. Increased TNF-α mRNA contents in C2C12 myoblasts has been shown in LPS-stimulated myoblasts (Frost *et al*., 2002). TNF-α significantly increased atrogin-1 mRNA up-regulation via p38 (Jin and Li 2007) and Foxo4 (Moylan *et al.,* 2008). Atrogin-1 is a muscle specific ubiquitin ligase significantly up regulated in many muscle-wasting models (Bodine *et al.* 2001). LPS significantly increased the up-regulation of atrogin-1 and MuRF1 mRNA (Wray *et al.,* 2003). Nevertheless, in this chapter LPS had no effect on the level of TNF-α, atrogin-1 and ubiquitin mRNA in avian primary skeletal

muscle cells. This suggesting that, there is a correlation between the expression of atrophic genes and the proteasomal activity in LPS-stimulated avian primary skeletal muscle cells inconsistent with the findings of Moylan *et al.,* (2008) and ours in the chapter 4. This discrepancy in response to LPS has been shown in different muscle fibre types. LPS decreased PS in the fast-twitch muscle fibre but not in the slow-twitch muscle (Vary and Kimball 1992).

Caspase-3 was involved in significant morphological changes of apoptosis including chromatin condensation, DNA fragmentation and cytoskeletal destruction (Hengartner 2000). Caspase-3 activation is considered as the step in sepsis-induced muscle proteolysis *in vitro* and in skeletal muscle via the cleavage of actinomysin (Du *et al.,* 2004). In this chapter, LPS had no effect on the caspase-3 mRNA in avian primary skeletal muscle cells (Fig. 7-6). This result is consistent with the findings of Wei *et al* that, sepsis did not alter the caspase-3 activity and mRNA level (Wei *et al.,* 2005).

The role of IL-6 in the sepsis induced muscle breakdown has been reviewed in details in the chapter 2 and chapter 5. In this chapter we have shown that LPS had no effect on IL-6 mRNA (Fig. 7-5). In contrast, LPS significantly increased IL-6 mRNA in C2C12 myoblasts (Frost *et al*., 2002). In addition, LPS increased the IL-6 media level in C2C12 myotubes (chapter 4, Fig. 4-*4*B).

Taken together, the effect of LPS is seems to a cell-specific, yet further investigation are required to verify this. The avian primary skeletal muscle cells were derived from broiler chickens. These animals have been genetically selected for rapid growth and accelerated muscle mass (Nakashima *et al.,* 2009) because of the lower protein degradation rate than that observed in layers (Hayashi *et al.,* 1985; Maeda *et al.,* 1987). Of note, the avian primary skeletal muscle cells were incubated with insulin transferrin selenium, while

C2C12 myotubes were incubated with DMEM media supplemented with serum and antibiotics. Therefore, the most appropriate explanation for this difference in response between C2C12 myotubes and avian primary cells could be attributed to the presence of insulin in the media of the avian primary skeletal muscle cells. Insulin is a strong anabolic stimulus has previously been shown to induce muscle hypertrophy mediated via acceleration of translation initiation. One limitation must be drawn that is: we have tested the effect of LPS on the avian primary skeletal muscle cells for only 3 h. This because, our previous results in chapter 3 showed that LPS maximal effect was seen at the 3 h time point. Therefore, further investigation are potentially required to establish an acceptable theory about the effect of endotoxin on the primary skeletal muscle cells protein metabolism.

7.5 Conclusion

Our study showed that, LPS effect on muscle protein of the primary skeletal muscle cells was not same as in C2C12 myotubes. LPS significantly decreased chymotrypsin-like enzyme activity and increased the PS rate. LPS had no effect on the mRNA levels of TNF-α, atrogin-1, caspase-3, ubiquitin and IL-6. The response to LPS is might be a cell specific response, although further investigation are required to verify this hypothesis.

8 Inhibition of second-messenger signalling modulates endotoxin-induced nitric oxide production in skeletal muscle cells

8.1 Introduction

Nitric oxide (NO) is an important intracellular signalling molecule with many diverse physiological and pathological effects. NO is synthesized from the oxidation of L-arginine, a process regulated by the nitric oxide synthase (NOS) class of enzymes (Frost *et al*., 2004). In skeletal muscle, NO regulates many diverse functions including force production, regulation of the blood flow, myocyte differentiation, respiration and glucose homeostasis (Stamler and Meissner 2001).

LPS is the main component of the cell wall of Gram-negative bacteria, and is a potent activator of the host innate immune system including cytokine production through recognition by TLR4 (Raetz 1990). LPS has previously been shown to increase NOS2 mRNA expression in C2C12 myoblasts (Frost *et al*., 2004), and NOS2 protein in skeletal muscle fibres (Hussain *et al.,* 1997).

LPS-induced signal transduction involves the interaction of several signalling cascades and in particular PI3-K/Akt and MAPKs (p38 or Erk1/2). Akt, is a central key element involved in the regulation of many fundamental cellular processes including protein synthesis and cell growth (Wu *et al.,* 2010). LPS alone can decrease Akt activity in rodent skeletal muscle (Crossland *et al.* 2008) and in C2C12 myotubes (chapter 3). In addition, we have also shown that LPS significantly increased p38, Erk1/2 phosphorylation and induced the nuclear translocation of NF-KB (Chapter 4).

Curcumin has been used for a long time in traditional Indian medicine to treat many diseases. Curcumin inhibits p38 activity (Carter *et al.*, 2003), and consequently inhibits the ubiquitin ligases activity in C2C12 myotubes (Li *et al.,* 2005). Moreover, curcumin has anti-NF-kB activity (Jobin *et al.,* 1999). How the inhibition of these signalling cascades can modulate the LPS-induced NO production in skeletal muscle is still largely unclear. Thus, LPS-induced NO production was measured as μM of nitrite as an indicator of NO production in the conditioned media of LPS-stimulated C2C12 myotubes and avian primary skeletal muscle cells. The intracellular pathways initiated by LPS were investigated using specific inhibitors. In addition, the inhibitory effect of curcumin as anti-p38 and NF-kB activity on the LPS induced nitric oxide production was assessed using three different concentrations (12, 25 and 50 μM).

8.2 Hypothesis

The LPS-induced muscle protein turnover in C2C12 myotubes includes the production and accumulation of many pro-inflammatory cytokines in skeletal muscle in particular TNF-α, while, the role of IL-6 is considered as minor. In contrast, there is a paucity of data about the role of nitric oxide (NO) in this process. In addition, there is no data regarding the signalling cascades involved in the nitric oxide production, and how inhibition of these second-messengers can modulate the nitric oxide production. Therefore, two septic models (C2C12 myotubes and avian primary skeletal muscle cells) were used to investigate these signalling streams and their inhibition can alter the LPS induced nitric oxide production.

8.3 Results

8.3.1 LPS increases nitric oxide production

Incubation of the C2C12 myotubes with LPS (1 μgml^{-1}) significantly increased the production of nitric oxide at 5 min ($P < 0.01$) and at 1 h and 3 h time points ($P < 0.05$) with no changes observed at any other time points (Fig. 8-1).

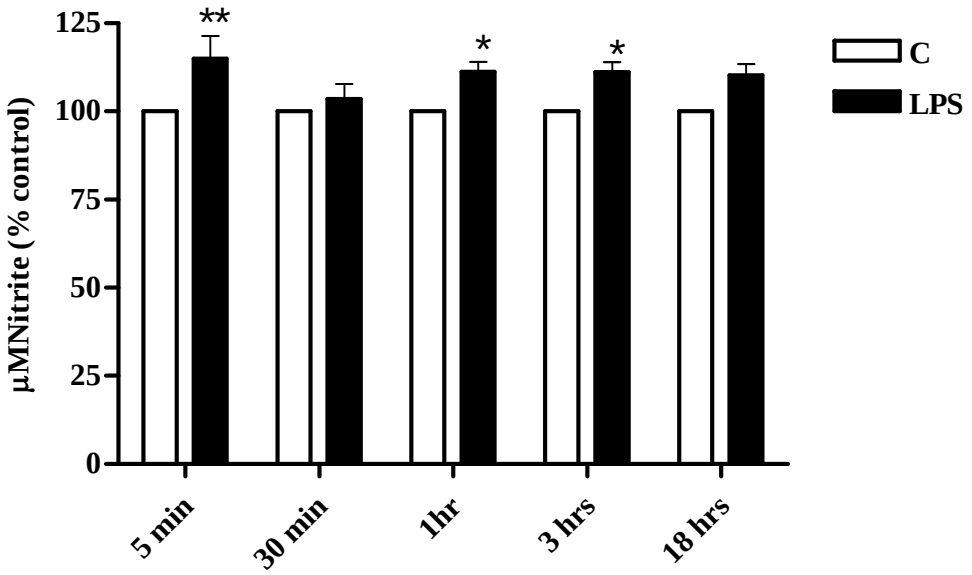

Figure (8-1) LPS (1 μgml^{-1})-induced nitric oxide (NO) production in C2C12 myotubes. Cells were incubated with LPS for various time points (namely 5, 30 min, 1 h, 3 h and 18 h) and the NO concentration was assessed in the conditioned media (as in the material and methods section). Data are expressed as means ± SEM (percentage of control). n = 6. Bars carrying asterisks are significantly significant from control $^{*}P < 0.05$ and $^{**}P < 0.01$.

8.3.2 Inhibition of second-messenger signalling blocked LPS-induced NO production in C2C12 myotubes

Incubation of C2C12 myotubes with LPS in combination with Polymyxin B (LPS neutralising agent), LY0294002 (PI-3K inhibitor), SB203580 (p38 inhibitor) and PD098059 (MEK/Erk inhibitor) abolished the effect of LPS at both 1 and 3 h time points compared with LPS-treated cells (Figs. 8-2*A* and 8-2*B*).

8.3.3 Curcumin decreases nitric oxide production in C2C12 myotubes

From the previous experiment the maximal effect of LPS was seen at 3 h. Thus, the effect of curcumin on NO production was also measured at 3 h. Incubation of C2C12 myotubes with LPS for 3 h significantly increased NO production compared to untreated cells ($P < 0.05$), while incubation of the cells with LPS along with curcumin (12, 25 and 50 μM) abolished the LPS-induced NO production. Similarly, incubation of the cells with curcumin (12, 25 and 50 μM) alone had no effect on the LPS-induced NO production (Fig. 8-3). Curcumin even at higher doses had no adverse effect on myotubes upon morphological testing (data not shown).

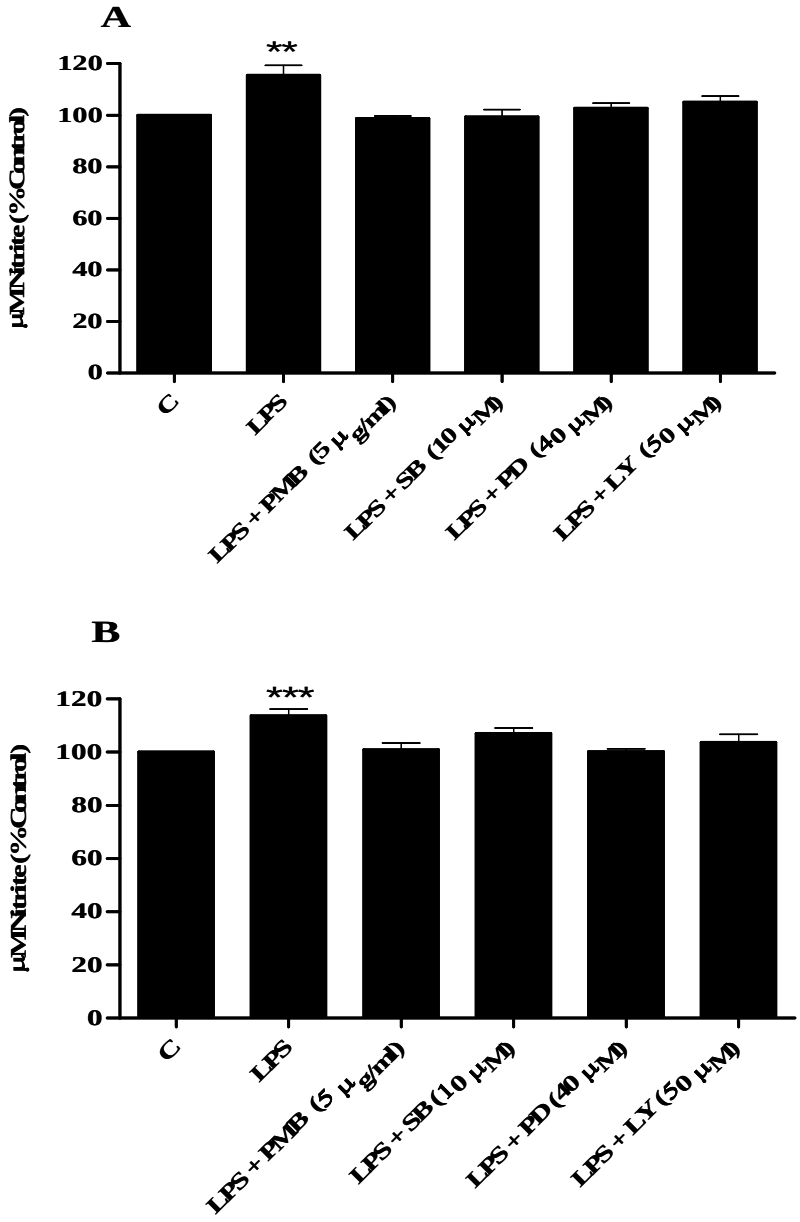

Figure (8-2) Effect of LPS alone and LPS combined with second-messenger inhibitors on NO production, A) Effect of 1 h incubation; B) effect of 3 h incubation. C2C12 myotubes were incubated either with LPS alone, or with LPS and Polymyxin B (LPS neutralising agent) (PMB, 5 µgml^{-1}), LPS and SB203580 (p38 inhibitor) (SB, 10µM), LPS and PD098059 (MEK/Erk inhibitor) (PD, 40 µM), or LPS and LY0294002 (PI3-K/Akt inhibitor) (LY, 50 µM) Data are expressed as means ± SEM (percentage of control). n = 6. Bars carry asterisks are significantly different from control and LPS-treated cells as shown in graph ($^{**}P < 0.01$ and $^{***}P < 0.001$).

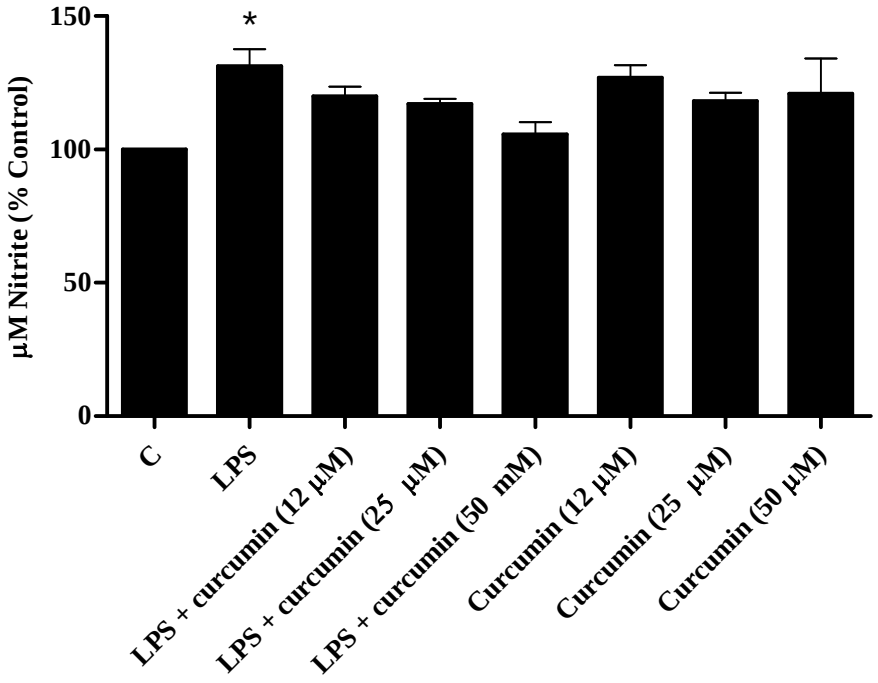

Figure (8-3) Effect of LPS alone and LPS in combination with curcumin (12, 25 and 50 µM) and curcumin alone on NO production. C2C12 myotubes were incubated either with LPS (1 $\mu g ml^{-1}$) alone, or with LPS and curcumin (12, 25 and 50 µM), or with curcumin (12, 25 and 50 µM) alone for 3 h. Data expressed as means ± SEM (percentage of control). n = 6. Bars carrying asterisks are significantly significant compared with control and LPS-treated cells as shown in figure, $^{*}P < 0.05$

8.3.4 LPS alone and with inhibitors increase nitric oxide production in avian primary skeletal muscle cell line

The effect of LPS on NO production in avian skeletal muscle cells was measured at 3 h. Incubation of cells with LPS alone increased NO production by 31% compared with the control cells. Incubation of the cells with LPS in combination either with Polymyxin B (PMB, 5 $\mu g ml^{-1}$), or LY0294002 (LY, 50 µM), or PD098059 (PD, 40 µM), or SB203580 (SB, 10 µM), or curcumin (12 µM) increased the NO production by 31% ($P < 0.01$), 50% ($P < 0.001$), 37% ($P < 0.01$), 41% ($P < 0.001$) and 35% ($P < 0.001$), respectively compared with the control cell (Fig. 8-4).

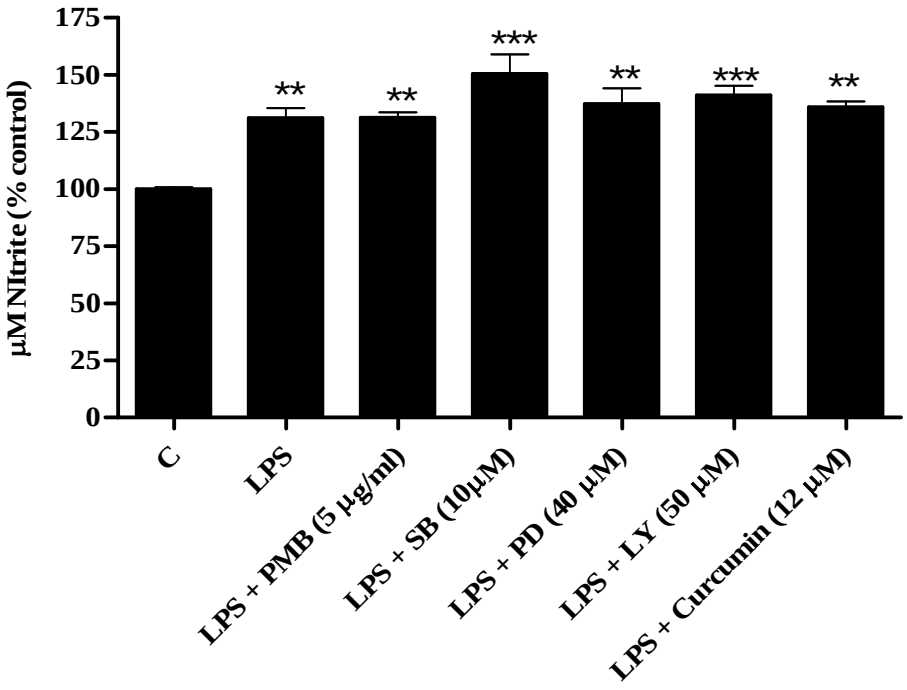

Figure (8-4) Effect of LPS alone and LPS combined with second messenger inhibitors on NO production**.** Avian primary skeletal muscle cells were incubated with LPS (1 $\mu g ml^{-1}$) alone, or with LPS and Polymyxin B (LPS neutralising agent) (PMB, 5 $\mu g ml^{-1}$); LPS and SB203580 (p38 inhibitor) (SB, 10 µM), LPS and PD098059 (MEK/Erk inhibitor) (PD, 40 µM), LPS and LY0294002 (PI3K/Akt inhibitor) (LY, 50 µM), or LPS and curcumin, 12 µM for 3 h. Data are expressed as means ± SEM (percentage of control); n = 3. Bars carrying asterisks are significantly significant from control cells, $^{**}P < 0.01$ and $^{***}P < 0.001$.

8.3.5 The effect of the inhibitors alone on NO production

C2C12 fully differentiated myotubes were incubated with either the inhibitors alone or with various concentrations of DMSO (2 and 5 µlml^{-1}) for 3 h. There were no significant changes in NO production (Fig. 8-5).

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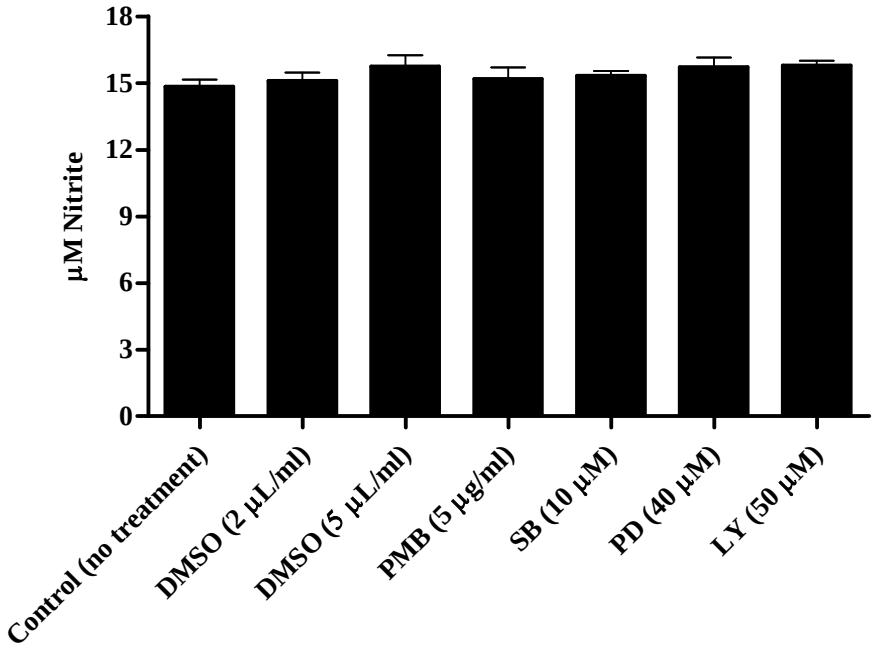

Figure (8-5) The effect of various inhibitors on the NO production. C2C12-murine derived myotubes were incubated with either no treatment, DMSO (2 µlml^{-1}), DMSO (5 µlml^{-1}), Polymyxin B (LPS neutralising agent) (PMB, 5µgml^{-1}), SB203580 (p38 inhibitor) (SB, 10 µM), PD098059 (MEK/Erk inhibitor) (PD, 40 µM), or LY0294002(PI3K/Akt) (LY, 50 µM) for 3 h, and media collected and NO assay was done as in material and method section. Values are represented as means ± SEM. n = 4

8.4 Discussion

The C2C12 murine-derived cell line is a well-established myocyte model that has been used extensively in skeletal muscle research (Sharples *et al*., 2010). Our study demonstrated that, LPS (1 $\mu g ml^{-1}$) alone significantly increased the NO production at 5 min, 1 h and up to 3 h. In contrast, Williams *et al*., (1994) observed that LPS alone did not induce NO production in C2C12 myocytes and myotubes. LPS–induced NO production can cause cellular damage (Frost and Lang 2008). Skeletal muscle releases NO as a part of the host immune response to kill invading micro-organisms, but excess NO production can contribute to muscle wasting and impair the contractile functions of the muscle (Sambe *et al*., 1998). However, depending on its concentration, studies have also shown that NO plays a significant role in the process of muscle regeneration including the myogenic–programme activated by insulin-like growth factor (IGF)-II (Kaliman *et al.,* 1999; Carrasco *et al.,* 2002), or the stimulation of myoblast fusion and satellite cell activation (Pisconti *et al.,* 2006). In addition, Sciorati *et al*., (2006) demonstrated that NO exerts multiple actions aimed at limiting muscle damage.

Herein, this moderate increase in the NO production in LPS-stimulated C2C12 myotubes model is most likely to be a part of the host immune response (Sambe *et al.* 1998). LPS-induced NO production in our septic model is consistent with the findings of Fernandez *et al*.(2002) that neither TNF-α nor IFN-γ increased the NO in C2C12 myotubes, while both together induced marked increase in NO (47-fold) compared with control. This suggests that LPS is a strong catabolic stimulus increased NO production alone in C2C12 myotubes. The fact that Polymyxin B (LPS neutralising agent) abolished the effect of LPS and inhibited the production of NO, suggested that LPS-induced NO production in C2C12 myotubes is mediated via TLR4 signalling cascade. The role of PI3-K/Akt and MAPKs (p38 or Erk1/2) signalling pathways in the LPS-induced NO production was also evaluated. Incubation of the cells with

LPS along with LY0294002 (PI3-K/Akt inhibitor) completely blocked the LPS-induced NO production at 1 and 3 h time points.

Akt is a serine/threonine kinase ubiquitously expressed in many tissues with Akt1 and Akt2 predominantly expressed in skeletal muscle. Akt activity regulates many functions in skeletal muscle such as muscle contraction, muscle glucose metabolism, muscle protein synthesis and degradation (for review see, Wu *et al.,* 2010). There is however, a paucity of data regarding the relationship between the Akt activity and NO production in skeletal muscle. JP05-induced NO production in the brain endothelial cells is mediated via Akt and Erk activation (Son *et al.* 2010). In addition, Akt has been shown to be implicated in the regulation of iNOS in the vascular smooth muscle cells (Isenovic *et al.,* 2002), and Wang and associates have demonstrated that iNOS and endothelial nitric oxide synthase (eNOS) are downstream targets of Akt activation (Wang *et al.,* 2004). In addition, Kim *et al*. demonstrated that LY0294002 (PI3-K/Akt inhibitor) significantly inhibited the LPS-induced macrophage NO production via NF-kB signalling cascades not PI3-K (Kim *et al*., 2005).

The MAPKs plays an important role in many physiological and behavioural phenomena through various cytokines (Kahn *et al.,* 2005; Long and Zierath 2006). Our results show that, inhibition of p38 or Erk1/2 blocked LPS-induced NO production and are consistent with the findings of Frost *et al*., (2004). Our data are the first that show that direct inhibition of PI3-K/Akt and MAPKs (p38 or Erk1/2) can modulate NO production in skeletal muscle.

Curcumin is the principal curcuminoid of the popular Indian spice turmeric (Ammon and Wahl 1991), and has been used for more than a century in the traditional Indian medicine. During the last two decades, many studies have explored the beneficial effects of curcumin. Curcumin has anti-p38 activity (Carter *et al.,* 2003), and anti-NF-kB activity (Wang *et al.,* 1999) and can thus act to decrease inflammation-stimulated muscle protein

degradation. In addition, curcumin has no known toxic effects even at very high doses (Chainani-Wu 2003). Our data showed that curcumin significantly blocked LPS-induced NO production compared with LPS-treated cells. We have shown that curcumin blocked LPS-induced p38 phosphorylation (chapter 6).This suggested a direct role of p38 in the LPS-induced NO production in C2C12 myotubes. Although, we have shown for the first time that, inhibition of p38 activity modulated the LPS-induced NO production in C2C12 myotubes. However, further investigation are still required to validate this effect *in vivo*.

The primary cultures of skeletal muscle have been used as a model to study of the muscular and neuromuscular diseases and parasitic cytogenesis (Fong *et al.,* 1990; Metzinger *et al.,* 1993; Passaquin *et al.,* 1993; Bouzakri *et al.,* 2003; Guimaraes *et al.,* 2008). In our study, incubation of avian primary skeletal muscle cells with LPS alone for 3 h increased NO production. Thus, LPS is considered as a potent stimulus of NO in avian primary skeletal muscle cells. Surprisingly however, none of the specific inhibitors we used could abolish this effect in these cells, suggesting that the regulation of NO production is different in these avian primary cells to that in the C2C12 cell line. This response might be due to the fact that these cells were derived from the broiler chickens. These animals have been genetically selected for rapid growth and in particular the accretion of muscle mass (Nakashima *et al.,* 2009). These chickens are characterised by a lower protein degradation rate than that observed in layers (Hayashi *et al.,* 1985; Maeda *et al.,* 1987). Thus, the fractional rates of protein synthesis in the broiler chicken are greater than in the layers (Maeda *et al.,* 1984). The discrepancy in response to LPS has been shown in different muscle fibres (fast and low twitch muscle). LPS administration significantly increased the NOS activity in the gastrocnemius muscle (fast twitch muscle) but not in the soleus (slow twitch muscle) (Vary and Kimball 1992; Kapur *et al.,* 1997; el-Dwairi *et al.,* 1998). Taken together, this might explain the diversity in response to LPS. Although, LPS is generally catabolic, however, the avian primary skeletal muscle cells may be resisting this effect. We have observed that, incubation of the avian primary skeletal

muscle cells with LPS significantly decreased the proteasomal activity and increased the protein synthesis (chapter 3&4). Although, this study shows the diverse modulation of NO production in LPS-induced sepsis model. Further investigation are still required to explore the effect of LPS on the NO production not only in broilers but in muscle from chickens with a different genetic background such as layers.

8.5 Conclusion

LPS is a strong activator of NO production in both C2C12 myotubes and avian primary skeletal muscle cells. The regulation of NO production in C2C12 myotubes involves the interaction of many signalling cascades, mainly Akt and MAPKs (p38 or Erk1/2). Inhibition of these signalling cascades modulated NO production in the C2C12 myotubes, while in the chicken skeletal muscle cells it had no effect. Thus, we suggest that the regulation of NO production is a cell-specific process. Although, we have shown inhibition of second-messenger signalling modulated NO production, further investigation is still required to test this hypothesis.

9 Effect of natural challenge with *Salmonella* Gallinarium and *Salmonella* Enteritidis on the skeletal muscle protein metabolism

9.1 Introduction

Salmonella is a Gram-negative rod-shaped enterobacteria mainly found in the intestine of human, and animals including birds. *Salmonella* can produce a broad range of clinical symptoms range from a symptomatic carrier state to life-threatening sepsis (Leveque *et al*., 2003), this makes *Salmonella* as a major issue in relation to poultry industry (Barrow 2007), in particular *S. enterica* serovar Enteritidis (Rodrigue *et al.,* 1990).

According to the pathogenesis, there are two main groups of the genus *Salmonella.* The first group consists of, *S. enterica* serovar Typhimurium and serovar Enteritidis (non-typhoidal *Salmonella*), non-host specific, can colonise in the gut and causes food poisoning in human. The second group (typhoidal *Salmonella*), host-specific *Salmonella*, including *S.* Pullorum and *S.* Gallinarium which causes pullorum disease and fowl typhoid in poultry (Chao *et al.,* 2007).

There are many factors determine the severity of the *Salmonella* infection including, the strain of *Salmonella*, route of infection, bird's strain, age of the bird and the immune status of the bird. The younger the bird the higher mortality rate is. Experimental infection of 3 weeks old chicks with *S.* Gallinarium results in 60% mortality rate (Jones *et al.,* 2001), while *S*. Pullorum causes substantial mortality but less frequent mortality in birds of more than a week old (Wigley *et al*., 2001). In case of *S*. pullorum infection, *S.* pullorum persists in the reproductive tract until sexual maturity and then

infects the eggs leading to vertical transmission another economic problem (Barrow 2007).

The current knowledge about the *Salmonella* pathogenesis has been derived in the main from the experimental infection studies using *S.* Typhimurium-infected mice model. In contrast, the situation is still relatively unclear concerning *Salmonella* infected chicken model (Barrow 2007). Generally, salmonellosis in chicken includes three main phases.

9.2 Phases of avian salmonellosis

Generally, avian systemic salmonellosis has three main phases including, intestinal invasion, establishment of the systemic infection in macrophages, and clearance by the immune system (Chappell *et al.,* 2009). During the intestinal invasion phase, following the faecal-oral infection, *Salmonella* adapt to the acidic pH to pass through the crop via proventriculus to gizzard reaching the intestine. In the small intestine there are lymphoid aggregates and Peyer's patches. There are no M cells in the lymphoid aggregates, and their role in *Salmonella* infection still unclear (Smith and Beal 2008). Following the infection of GI tract with *S.* Typhimurium, a rapid influx of heterophils and inflammation occurs in contrast to infection with *S.* Pullorum (Henderson *et al.,* 1999).

The second phase is the development of systemic infection that followed the invasion phase. *Salmonella* is taken up by the macrophages and dendritic cells via the lymphatic system to the spleen and liver (Mastroeni and Menager 2003). The ability of the *Salmonella* to survive in the macrophages depends upon *Salmonella* pathogencity island 2 (SP1-2) type III secretion system (Hensel 2000). Complete attenuation of systemic infection with *S*. Gallinarum, *S*. Pullorum, or *S*. Typhimurium occurs in the absence of a functional SPI-2 system (Jones *et al.,* 2001; Wigley *et al.,* 2002; Jones *et al.,* 2007). Therefore, survival of the *Salmonella* in the macrophages is the first

step in the initiation of systemic infection in both mammals and birds (Barrow *et al.,* 1994).

The clearance, persistence, or death phase is a phase following the invasion and the establishment of systemic infection. The fate of *Salmonella* infection in chicken is either, clearance or control of the replication of bacteria in resistant inbred chicken, or later clearance the infection via adaptive immunity (Chappell *et al.,* 2009).

9.3 The role of cytokines in the pathogenesis of salmonellosis

Cytokines are a large and diverse family of polypeptides mainly produced by the immune cells, acting as signalling molecules involved in the cell communication and signals transduction. Cytokines are considered as a key regulators of the host response to intracellular pathogens (Liles and Van Voorhis 1995). Cytokines stimulate immune cell migration, proliferation, and interactions; this enhances the host immunity against the invading microorganism (Kaufmann 1993; Liles and Van Voorhis 1995). *Salmonella* components (LPS, flagellin, porins, and outer membrane proteins) stimulate the immune system and later enhance cytokine expression (Henderson *et al.,* 1996; Ciacci-Woolwine *et al.,* 1998; Wilson *et al.,* 1998). Following systemic infection, *Salmonella* become localised in the large blind caecum. The caecal tonsils are considered as the largest secondary lymphoid organ of the chicken GI tract and the main target for most *Salmonella* serovars (Withanage *et al*., 2005). Experimental chicken infection with *S*. Typhimurium induces expression of pro-inflammatory cytokines and chemokines IL-1β, IL-6, CXCLi1, and CXCLi2 (previously known as IL-8) (Withanage *et al.,* 2004).

Following oral infection of mice with *S*. Typhimurium, IFN-γ stimulates macrophage anti-bacterial activity (Ramarathinam *et al.,* 1991). IFN-γ significantly decreased the severity of *Salmonella* infection in mice if administrated in the early days after challenge with a virulent strain (Gould

and Sonnenfeld 1987; Muotiala and Makela 1990), and treatment of mice with the anti-IFN-γ significantly increased the severity of the infection (Muotiala and Makela 1990).

Regarding the effect of salmonellosis on TNF-α expression, the collected data are controversial. With some studies have showed no systemic release of TNF-α has been detected in *Salmonella* infected mice (Kumazawa *et al.,* 1991) or calves (Peel *et al.,* 1990), while others showed it has been detected during *Salmonella*-infection in mice (Klimpel *et al.,* 1995). TNF-α may have a role in the host immune response against salmonellosis by its cytotoxic activity against *Salmonella*-infected cells (Klimpel *et al.,* 1990). The depletion of TNF-α significantly increase the severity of *Salmonella* infection and decreases host survival (Nauciel and Espinasse-Maes 1992; Gulig *et al.,* 1997). TNF-α plays an important role in the recall of the immune system and contributes to the maturation of the dendritic cells in mice orally infected with *Salmonella* (for further information see ,Lembo *et al.,* 2003). Concerning the effect of the TNF-α on the muscle protein metabolism, see chapter (1) for more details.

IL-6 is a multifunctional pro-inflammatory cytokines with similar functions in both mammals and chickens (Kaiser *et al.,* 2000). IL-6 stimulates the synthesis of acute phase protein, differentiation of B-lymphocytes, cytotoxic T cells and the growth of T cells (Hirana 1994). Invasion of avian cells with *S*. Typhimurium and *S*. Enteritidis significantly increased the production of IL-6 mRNA, while S. Gallinarium caused no increase (Kaiser *et al.,* 2000). S. Enteritidis down regulates IL-6 mRNA in chicken peripheral blood mononuclear cells (Kaiser *et al.,* 2006). Additionally, incubation of the chicken heterophils with LPS significantly increased IL-6 mRNA (Kogut *et al.,* 2005). In addition, higher levels of IL-6 mRNA have been observed at day 14-post vaccination with S. Enteritidis vaccine (Okamura *et al.,* 2004) which is very late for pro-inflammatory cytokine. In conclusion, IL-6 plays a controversial role in the immunity against *Salmonella* infection.

Several other cytokines with a controversial role have been studied in the pathogenesis of *Salmonella* infection, including IL-8 (Carvajal *et al.,* 2008; Cheeseman *et al.,* 2008; Figueiredo *et al.,* 2009; Popa *et al.,* 2009; Salerno-Goncalves and Sztein 2009), and IL-15 (Nishimura *et al.,* 2000; Eckmann and Kagnoff 2001; Nishimura *et al.,* 2001; Ashkar *et al.,* 2009). Up to date, there are no studies regarding the effect of *Salmonella* infection on the expression level of these cytokines in skeletal muscle. Thereby, our final experiment was aimed to explore the effect of natural *Salmonella* infection on the expression levels of many genes mRNA including TNF-α, atrogin-1, Il-6, IL8, IL-10, ubiquitin and caspase-3 mRNA.

9.4 Hypothesis

The data continued in the previous chapters have demonstrated that when C2C12 myotubes incubated with LPS, a significant decrease in protein synthesis and increase in protein breakdown was observed. In contrast, avian primary skeletal muscle seems to be resistant to LPS either because their origin or different incubation condition (presence of insulin in case of primary cells). In addition, the data regarding natural challenge with *Salmonella* were related to the stimulation of immune system, no clear data regarding the effect of natural challenge on skeletal muscle protein metabolism. Therefore, skeletal muscles (pectoral and gastrocnemius) from two different groups of birds were challenged with two *Salmonella* serotypes (Gallinarium or Enteritidis) were investigated by RT-PCR to evaluate the effect of natural challenge on muscle protein metabolism.

9.5 Results

9.5.1 Natural infection with *S*. Gallinarum and *S*. Enteritidis significantly decreased TNF-α mRNA

Concerning the expression of TNF-α mRNA, there was no expression detected in the pectoral muscle. While, in case of gastrocnemius muscle, infection with *Salmonella s*erovars Gallinarum or Enteritidis significantly decreased TNF-α mRNA by nearly 80% ($P < 0.01$) compared to untreated control group (Fig. 9-1).

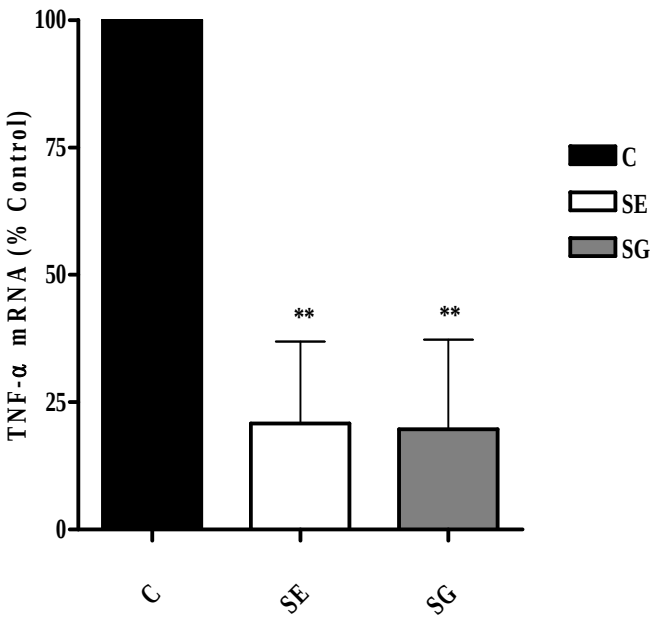

Figure (9-1) Changes in TNF-α mRNA expression rate in the gastrocnemius muscle as a result of natural infection with *Salmonella* Enteritidis (SE) or *Salmonella* Gallinarum (SG) for 4 days. Values represented as means ± SEM (percentage of control). Stars represent significance difference control chicken (**$P < 0.01$). n = 5. Data were normalised to 18S mRNA (previously mentioned in material and methods).

9.5.2 Natural infection with *S*. Gallinarum and *S*. Enteritidis significantly decreased atrogin-1 mRNA

Challenge of the chicks with *Salmonella* serovars Gallinarum or Enteritidis had no effect on the atrogin-1 mRNA in pectoral muscle, while it significantly deceased its expression in the gastrocnemius muscle by nearly 50% and 75% ($P < 0.001$) compared with the control group (Figs. 9-2*A* and 9-2*B*).

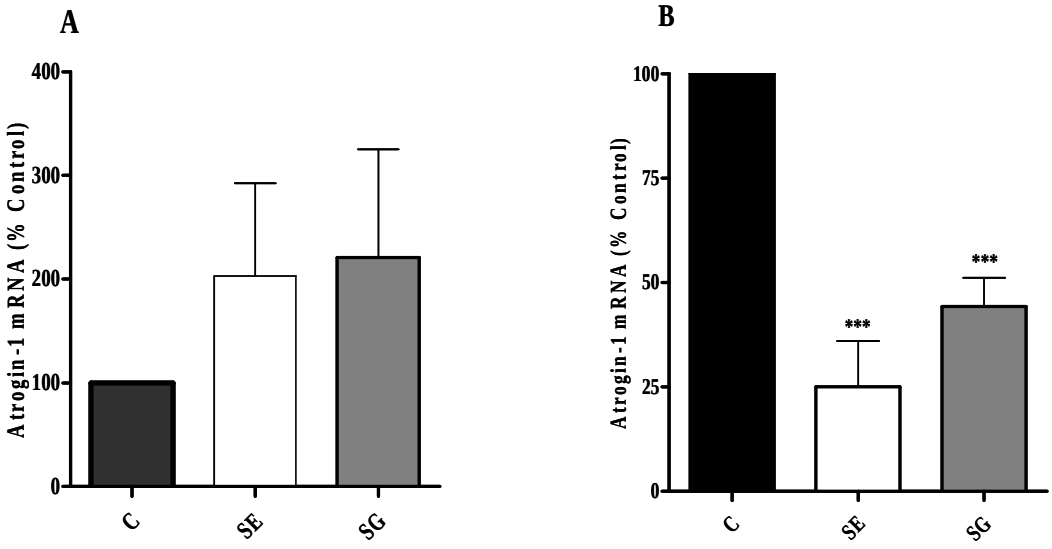

Figure (9-2) Changes in atrogin-1 mRNA expression rate in A) the pectoral (Breast muscle) and B) gastrocnemius muscle as a result of natural infection with *Salmonell*a Enteritidis (SE) or *Salmonella* Gallinarum (SG) for 4 days. Values are represented as means ± SEM (percentage of control). Stars represent significance difference from control chicken ($^{***}P < 0.001$). n= 5

9.5.3 Natural infection with *S*. Gallinarum and *S*. Enteritidis significantly decreased caspase-3 mRNA

Interestingly, the expression of the caspase-3 mRNA was significantly decreased in the both the pectoral and the gastrocnemius. In the pectoral muscle, challenge of the birds with *S.* Gallinarum or *S.* Enteritidis significantly decreased the caspase-3 mRNA by nearly 50% and 60% respectively ($P < 0.001$) compared with control group. While, in the gastrocnemius muscle the caspase-3 mRNA expression was significantly decreased by nearly 70% and 60% respectively, ($P < 0.01$) compared with control group (Figs. 9-3*A* and 9-3*B*)

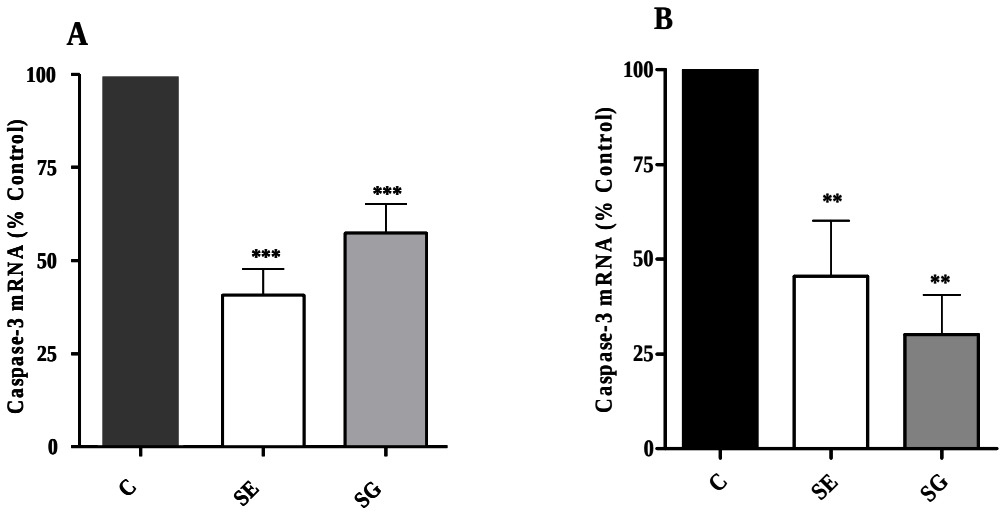

Figure (9-3) Changes caspase-3 mRNA expression rate in A) the pectoral (Breast muscle) and B) gastrocnemius muscle as a result of natural infection with *Salmonell*a Enteritidis (SE) or *Salmonella* Gallinarum (SG) for 4 days. Values represented as means ± SEM (percentage of control). Stars represent significance difference from control chicken ($^{**}P < 0.01$ and $^{***}P < 0.001$). n = 5

9.5.4 Natural infection with *S*. Gallinarum or *S*. Enteritidis had no effect on the ubiquitin mRNA expression level

There were no significant changes detected in the expression level of the ubiquitin mRNA in both the pectoral and gastrocnemius muscle compared with the untreated control group (Figs. 9-4*A* and 9-4*B*).

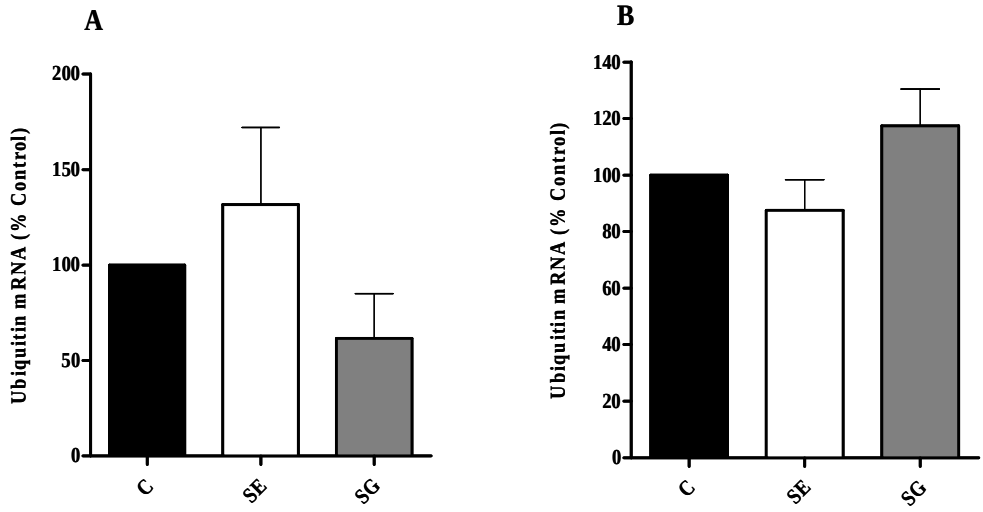

Figure (9-4) Changes in ubiquitin mRNA expression rate in A) the pectoral (Breast muscle) and B) gastrocnemius muscle as a result of natural infection with *Salmonell*a Enteritidis (SE) or *Salmonella* Gallinarum (SG) for 4 days. Values represented as means ± SEM (percentage of control). .n = 5

9.5.5 Natural infection with *S*. Gallinarum or *S*. Enteritidis significantly decreased the IL-6 mRNA expression

We could not detect any expression of IL-6 mRNA in the pectoral muscle. In contrast, challenge with *S*. Gallinarum or *S*. Enteritidis significantly decreased the IL-6 mRNA in gastrocnemius muscle by nearly a 60% ($P < 0.01$) and a 90% ($P < 0.001$) compared with the control group (Fig. 9-5*A*).

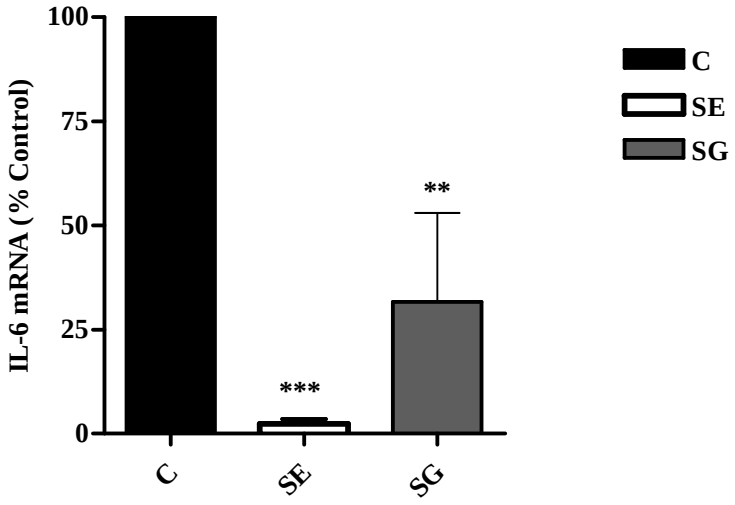

Figure (9-5) Changes in the IL-6 mRNA expression rate in gastrocnemius muscle as a result of natural infection with *Salmonell*a Enteritidis (SE) or *Salmonella* Gallinarum (SG) for 4 days. Values represented as means ± SEM (percentage of control). Stars represent significance difference from untreated control chicken ($^{**}P < 0.01$ and $^{***}P < 0.001$). n = 5

9.6 Discussion

In the present chapter, two groups of chicken were challenged with *Salmonella* serovars *S*. Gallinarium (host-specific serotype) and *S*. Enteritidis (non-host specific serotype). The result obtained from this study in total showed that natural challenge with *Salmonella* serovars Gallinarum and Enteritidis significantly decreased the expression of TNF-α, IL-6, atrogin-1, caspase-3 and ubiquitin mRNA in chicken skeletal muscle.

These *Salmonella* serovar Enteritidis colonise the caecum and is responsible for human food-borne mainly via consumption of contaminated chicken's egg and meat (Berndt *et al.,* 2007). *S*. Gallinarum dose not colonise the intestine but the point of invasiveness, *S*. Gallinarum more invasive than S. Enteritidis (Bumstead and Barrow 1993).

Salmonella infection significantly changes the expression of many cytokines, particularly in the intestine and caecum of the birds (Berndt *et al.,* 2007; Cheeseman *et al.,* 2008; Fasina *et al.,* 2008; Haghighi *et al.,* 2008). Challenge of chicken with *S.* Typhimurium results in the expression of many cytokines in many cells including macrophages and many other tissues including the cecal tonsils, ileum, spleen and liver (Beal *et al.* 2004; Withanage *et al.* 2004; Withanage *et al.* 2005). In addition, the pattern of cytokine expression was correlated with the host resistance against *S.* Typhimurium (Haghighi *et al.,* 2008). The first cytokines stimulated by *Salmonella are* IFN-γ, IL-12 and IL-8 and their main role is the protection against *Salmonella*, while IL-4 and IL-10 down regulate the pro-inflammatory responses to the bacterium (Eckmann and Kagnoff 2001).

To date, nearly all the studies concerning the immune response and the host resistance have focused on the immune tissues including liver, spleen and tonsils, whereas in the present context the effect of the natural

infection on the skeletal muscle's gene expression remains largely unclear. Skeletal muscle has been shown to produce cytokines in the same way as the immune cells in response to various stimuli (Frost *et al*., 2003). Moreover, LPS significantly increased the synthesis of many cytokines including IL-1β, TNF-α and IL-6 in muscle (Frost *et al*., 2002; Lang *et al*., 2003; Huey *et al*., 2008). In addition, the pro-inflammatory cytokines mainly IL-6 produced locally by muscle and thus have a systemic role (Steensberg *et al.,* 2000; Pedersen *et al.,* 2001; Febbraio and Pedersen 2002).

TNF-α has a cytotoxic activity against *Salmonella*-infected cells (Klimpel *et al.,* 1990), and promotes the maturation of the dendritic cells in mice orally infected with *Salmonella* (Lembo *et al.,* 2003). The depletion of TNF-α significantly increased the severity of *Salmonella* infection and decreased the host survival (Nauciel and Espinasse-Maes 1992; Gulig *et al.,* 1997). In the case of skeletal muscle TNF-α is considered as a pro-inflammatory cytokine involved in the process of muscle wasting (Frost and Lang 2005). The excessive production of TNF-α is associated with enhanced rate of muscle protein breakdown both *in vitro* (Li *et al.,* 2000) and *in vivo* (Flores *et al.,* 1989; Goodman 1991). Herein, the expression of the TNF-α mRNA is significantly decreased; this suggests that natural challenge with these *Salmonella* serovars could not induce significant TNF-α release. The possible explanation is that, skeletal muscle samples were collected 4 days post infection. In the study carried out by Arnold and Holt (1996) have shown that the maximal effect of TNF-α was seen in SE infected hens 24 hr and 10 days after infection. Therefore, further time points are still required in order to build a complete notion about the direct link between natural challenge with *Salmonella* and the local release of TNF-α in skeletal muscle.

IL-6 a multifunctional pleiotropic pro-inflammatory cytokine, stimulating the synthesis of acute phase protein, differentiation of B-lymphocytes, cytotoxic T cells and the growth of T cells (Hirana 1994). In our study, chickens infected with *S*. *enterica* serovars Enteritidis and Gallinarum

caused a significant decrease in the IL-6 mRNA in gastrocnemius muscle by nearly a 60% ($P < 0.01$) and 90% ($P < 0.001$) respectively compared with the control group. This indicates that the down-regulation of IL-6 mRNA in skeletal muscle seems to be a specific effect of *S*. Gallinarum and S. Enteritidis. The production of cytokines by macrophages and tissues, including caecal tonsils, ileum, spleen and liver has been reported in chicken following challenge with *Salmonella* (Beal *et al.,* 2004; Withanage *et al.,* 2004; Withanage *et al.,* 2005). In caecal tonsils, challenge with *S*. Typhimurium significantly increased IL-6 by day 21 post infection (pi) (Withanage *et al.,* 2005). This explains why we could not find any significant increase in lL-6 mRNA in our model at day 4pi. Similarly, in the same tissue (caecal tonsils), there is no changes in the level of IL-6 mRNA were observed at day 1, day 3 and day 5pi with *S*. Typhimurium (Haghighi *et al.,* 2008). Of note, the severity of infection of infection was greater in younger chickens compared with older ones. Herein, we have used *S*. Typhimurium and Enteritidis serovras and the infection of chickens with theses *S.* serovar is associated with transient systemic infection mainly in newly hatched chicks (Barrow 2000). Thus, we assume that older birds (21 days) are more resistant and this explains why natural infections with serovars could not trigger any pro-inflammatory reactions in skeletal muscle of infected chickens. Moreover, the experimental infection of chickens with these serovars was insufficient to induce enough circulation of ether the microrganism or its toxin. The fact that, *Salmonella* is an intracellular organism (Suter and Ramseiar 1964; Popiel and Turnbull 1985). Therefore, the number of isolated microorgnism form the visceral organs was correlated with the severity of infection. In case of skeletal muscle, there is a scarcity of data about the effect of natural infection with *Salmonella* on the IL_6 mRNA level. However, the data obtained from *in vitro* studies have demonstrated that Stimulation of C2C12 myoblasts directly with LPS significantly increases the production and release of IL-6 (Frost *et al*., 2002; Lang *et al*., 2003). In chapter 4 have shown that, LPS significantly increased the production of IL-6 in C2C12 a consistent findings with that obtained by Strle *et al*. (2007). In skeletal

muscle, excessive production of IL-6, IL-1β and TNF-α alter the protein balance by changing the rate of protein synthesis and protein degradation (Argiles *et al.,* 2005; Frost and Lang 2005). Although, IL-1β and TNF-α have a pro-inflammatory properties, IL-6 possesses both the pro- and anti-inflammatory properties (Tilg *et al.,* 1997) and excessive production of IL-6 significantly increased the expression of the anti-inflammatory cytokines like IL-10 (Steensberg *et al.,* 2003). The direct infusion of IL-6 has been implicated in the induction of muscle protein catabolism in rats (Haddad *et al.,* 2005). The fact that natural challenge of chicken with *Salmonella* was associated with decreased TNF-α and IL-6 mRNA expression in skeletal muscle suggests that natural infection with *S*. Gallinarum and *S*. Enteritidis does not trigger strong immune or inflammatory responses in skeletal muscle.

Caspase activity is considered as the central step of the apoptotic pathway and elimination of the caspase activity significantly delays or prevents apoptosis (Earnshaw *et al.,* 1999). Caspase-3 is involved in remarkable morphological changes of apoptosis including chromatin condensation, DNA fragmentation and cytoskeletal destruction (Hengartner 2000). Caspase-3 cleaves actinomyosin *in vitro* and *in vivo* (Du *et al.,* 2004), and this facilitates the actomyosin degradation by the ubiquitin proteasome system. Thus, increased caspase-3 activity is the first step in skeletal muscle protein breakdown. Our findings showed that natural infection with *S*. Gallinarum and *S*. Enteritidis significantly decreased the caspase-3 mRNA expression, suggesting that natural infection does induce neither any apoptotic activity nor any actomyosin degradation in the skeletal muscle of chicken

Ubiquitination is the predominant mechanism implicated in protein degradation and includes the activation of specific ubiquitin enzymes E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme) and E3 (ubiquitin-protein ligase) (Hough *et al.,* 1987). The predominant ubiquitin enzyme, which determines the specificity of the degraded protein is the E3

ubiquitin-protein ligase (Hershko and Ciechanover 1998). The ubiquitination process, in addition controls various cellular processes including cell cycle, vesicle trafficking and signal transduction (Hough *et al.,* 1987). The expression of the atrogin-1 mRNA is only limited to the skeletal muscle and atrogin-1 knockout significantly blocked the denervation-induced muscle wasting on the skeletal muscle (Bodine *et al.,* 2001). Others and we in chapter 4 have shown that, LPS stimulated the expression of atrogin-1 mRNA in skeletal muscle *in vivo* and *in vitro* (Gomes *et al*., 2001; Jin and Li 2007). The expression of atrogin-1 mRNA has been dramatically increased in many other catabolic conditions like food deprivation (Jagoe *et al*., 2002), and denervation (Bodine *et al*., 2001). Therefore, the use of ubiquitin mRNA is considered as suitable marker for ubiquitin-proteasome activation The fact that challenge with *S*. Gallinarum and *S*. Enteritidis significantly decreased the expression of atrogin-1 in gastrocnemius muscle by 50% and 75% respectively ($P < 0.001$) compared with control group respectively, this suggests that natural infection had a little or no effect on the skeletal muscle protein. Combined, natural challenge could not induce muscle proteolysis (measured as expression of the atrophic genes), this emphasises the importance of presence of LPS (main part of Gram negative bacterial cell wall) rather than the presence of the live bacteria in the circulation. Therefore, the data presented in this chapter suggest that natural challenge with *Salmonella* serotypes could not induce significant increase in the circulating levels of LPS and thus it had no effect on muscle protein. In the study carried out by Barrow and associates (1999), natural challenge of commercial ducks with the *Salmonella* strains (Typhimurium and Enteritidis), these strains are invasive and been isolated from different oranges with a variable counts, however, almost no or low level of LPS IgG was detected by ELISA. The data presented in this chapter was not similar to that we have obtained in cultured C2C12 myotubes. Thus, further investigation are still needed to verify this discrepancy taken in the consideration the difference in relation to the muscle fibre type. Previous studies have demonstrated that the response of the muscle protein metabolism to catabolic stimuli was

differed according to the muscle fibre type (for more details see,Vary and Kimball 1992). In the context there is a paucity of data about the effect of natural infection with *Salmonella*, and all the data about the effect of LPS on skeletal muscle were obtained from rat and mice studies (mentioned in details in the previous chapters). Therefore, we assume that the appropriate model will be direct injection of chicken with LPS.

9.7 Conclusion

In the present study, natural infection with two invasive *Salmonella enterica* serovars Gallinarium and Enteritidis significantly decreased the expression of many cytokines including TNF-α and IL-6 mRNA and many atrophic genes such as atrogin-1, caspase-3 and ubiquitin mRNA. Thus, we think that this model is not the appropriate model to study the pathogenesis of muscle wasting in chicken, and to build up a hypothesis about the natural infection a better model most likely will be either maintaining the birds for longer or direct intramuscular injection of the chicken with the LPS.

10 General Summary

10.1 General summary

As stated in the end of the introduction, the specific aims of the study were:

1- To examine the direct effect of the LPS (bacterial endotoxin) alone on the signalling pathways that control the process of the protein synthesis in C2C12 murine myotubes involving PI3-K/Akt and MAPKs (p38 or Erk1/2) signalling cascades and their downstream targets i.e. mTOR, $p70^{S6K}$, 4E-BP1 and eIF-4E.

2- To examine the rate of protein synthesis (PS) (expressed as a direct ^{3}H-phenylalanine incorporation/µg 5% TCA perceptible protein) after 3 h (short-time response) and 18 h (long-term response) LPS exposure.

3- To examine the effect of LPS on muscle protein degradation (measured as chymotrypsin-like enzyme activity) and the signalling cascades that involved in C2C12 murine myotubes septic model.

4- To validate the effect of LPS on protein synthesis and protein degradation by using specific signalling pathways inhibitors.

5- To examine the beneficial effects of curcumin (as a natural compound) on the rates of protein synthesis and protein degradation in LPS-stimulated C2C12 murine myotubes for 3 h.

6- To examine LPS-induced production of TNF-α, IL-6 and nitric oxide in the conditioned media of LPS-stimulated C2C12 myotubes.

7- To examine the effect of LPS on the protein metabolism of the avian primary skeletal muscle cells.

8- To assess the effect of natural infection of chicken with *S*. Gallinarum or *S*. Enteritidis on the skeletal muscle protein metabolism.

10.2 The effect of the LPS on the anabolic signalling and the PS rate in C2C12 myotubes

The aim stated in chapter 3, was to examine the subcellular mechanisms involved in LPS-induced decreased PS rate. This include the study of intracellular signalling molecules (second messengers) involving i.e. Akt, mTOR, 4E-BP1, eIF-4E, p38, Erk1/2 and Mnk1.

10.2.1 Anabolic Signalling

In chapter 3, LPS-stimulated C2C12 murine derived myotubes model was used to examine our hypothesis. Different LPS concentrations (10 $ngml^{-1}$, 100 $ngml^{-1}$ and 1μgml^{-1}) were used at different time points (namely 5, 30 min and 1, 3 and 18 h).

LPS significantly decreased Akt phosphorylation, this effect was started early at 5 min with a peak at 30 min ($P < 0.001$) in a time and dose-dependent manner. While, mTOR phosphorylation decreased over a longer time course lasting up to 18 h in a time and dose-dependent manner. This effect was followed by decreased $p70^{S6K}$, 4E-BP1 and eIF-4E phosphorylation. This effect suggests that, LPS-induced decreased PS was mediated via alteration of the translation initiation step of protein synthesis to which Akt is considered as a key regulator of this process. In addition, it suggests that mTOR is considered as bifurcation point for the activation of both $p70^{S6K}$ and 4E-BP1 *in vitro*. This effect is similar to that obtained from in vivo studies (Lang *et al.,* 2007; Bentzinger *et al.,* 2008).

The effect of LPS on the MAPKs (p38, Erk1/2 and Mnk1) activity-related phosphorylation was measured in chapter 3. LPS significantly increased the phosphorylation of MAPKs (p38 and Erk1/2) in a time and dose-dependent manner. While, their downstream target Mnk1

phosphorylated after 1 h and lasting for 3 h. Increased Mnk1 phosphorylation was related to eIF-4E phosphorylation and hence increases the translation initiation step of PS process. However, this effect was not sufficient to inhibit the transient decrease in PS after 3 h LPS exposure. In contrast, increased Mnk1 phosphorylation following MAPKs (p38 or Erk1/2) phosphorylation here in and in other studies suggested that, Mnk1 is the downstream target of MAPKs (p38 or Erk1/2) *in vitro*.

10.2.2 Direct protein synthesis

The data presented in chapter 3 showed that incubation of the C2C12 myotubes with LPS (1 $\mu g ml^{-1}$) alone transiently decreased the PS rate by 50% compared with the control cells ($P < 0.05$) at the 3 h time point (short-term response). Afterwards, the PS returned to the normal basal levels at the 18 h. This effect was correlated with the decreased activities of the anabolic signalling molecules mainly Akt and its down-stream targets (mTOR, $p70^{S6K}$, 4E-BP1 and eIF-4E). This effect is similar to that obtained from many *in vivo* studies. Thus, we can conclude that the LPS-induced decreased PS in skeletal muscle is a multifactorial process and involving the input of many signalling cascades, and that a cross talk exists between them. This makes the overall elucidation of this process a hard task which requires further investigation.

10.3 The effect of LPS on the protein degradation

In chapter 3 the data presented have demonstrated that LPS transiently decreased PS and this effect involved Akt/mTOR/ $p70^{S6K}$, 4E-BP1 and eIF-4E signalling molecules, and the input of MAPKs (p38 or Erk1/2) signalling cascade was essential. Therefore, the requisite was to elucidate the direct effect of LPS on protein breakdown (chymotrypsin-like enzyme activity).

The data presented in chapter 4 showed that LPS (1 $\mu g ml^{-1}$) significantly increased the chymotrypsin-like enzyme activity ($P < 0.001$)

compared with the control cells at all time points. This effect was preceded by increased phosphorylation and subsequent decreased amount of the total I*k*B-α. This effect was then followed by the nuclear translocation of the NF-kB. In addition, LPS significantly increased TNF-α media concentrations at all the time points (5, 30 min, 1, 3 and 18 h). This was followed by increased media concentration of IL-6 after 18 h. Increased circulating concentration levels of TNF-α in LPS-treated cells at all the time points was correlated with increased proteasomal activity, suggesting that TNF-α is involved in the LPS-induced muscle turnover herein. In addition, incubation of the cells with LPS significantly increased the CK for 1 h. This suggests that, LPS might alter the energy metabolism of the skeletal muscle via increased production and release of CK. These data with that presented in chapter 3 have demonstrated that LPS-induced muscle protein imbalance in C2C12 myotubes was mediated through decreased protein synthesis together with increased protein breakdown rates. The LPS-induced decrease in PS was mediated mainly through alteration of the translation initiation, while PD was mediated via the activation of the ubiquitin-proteasome system. In addition, the deactivation of one signalling cascade may alter the activation of many diverse cellular functions, i.e. deactivation of Akt significantly decreased PS and increased PD. The data presented in chapter 3 and 4 have shown that, the mechanism of LPS-induced protein turnover requires the activation of many signalling streams. These signalling streams are cross talked with other. The better understanding of this cross talk will help in the developing of the appropriate therapeutic intervention that ameliorates the sepsis-induced muscle wasting.

10.4 Transcriptional regulation of LPS-induced proteasomal activity

The transcriptional regulation of LPS-induced proteasomal activity was addressed in chapter 5. We examined the effect of LPS on the mRNA expression levels of TNF-α, atrogin-1, MuRF1 and TLR4. C2C12 myotubes

were incubated with LPS (1 μgml^{-1}) up to 18 h. LPS increased the levels of TNF-α, atrogin-1, MuRF1 and TLR4 mRNA in a time-dependent manner. These results suggested that skeletal muscle can respond directly to LPS, and that the LPS increase the synthesis and the accumulation of cytokines in skeletal muscle mediated by the TLR4 signalling pathway.

The combined results of chapter 3 & 4 and 5 demonstrate that, LPS is a potent catabolic stimulus that increases protein breakdown and decreases protein synthesis.

10.5 Validation of the effect of LPS on the protein degradation and protein synthesis

The results that obtained in the chapter 3, 4 and 5 raised the following questions what are

1- The signalling cascades involved in LPS-induced decreased of PS rate and increased rate of muscle protein breakdown in C2C12 myotubes.
2- The potential role of TLR4 in the LPS signal.
3- The direct role of the ubiquitin-proteasome system in the LPS-induced muscle protein breakdown.
4- What are the contributions of MAPKs (p38 or Erk1/2).

The components of these questions were examined in the chapters 3, 4 and 5.

The *in vitro* studies showed that TLR4 plays the main role in the signal transduction induced by LPS. In chapter 3, 4 and 5 incubation of C2C12 myotubes with LPS along with Polymyxin (LPS neutralising agent) (5 μgml^{-1}) significantly decreased the chymotrypsin-like enzyme activity, and abolished the effect of LPS on PS. In addition, it significantly blocked LPS induced up-regulation of TNF-α, MuRF1 and TLR4 mRNA while it partially inhibited atrogin-1 mRNA expression at 18 h time point. Taken together, these results indicate that TLR4 is the main pathogen recognition receptor involved in LPS signals transduction in muscle cells.

The role of the ubiquitin-proteasome system was evaluated by the MG-132 (specific proteasomal inhibitor). Incubation of the cells with LPS in combination with MG-132 significantly blocked the LPS-induced increased chymotrypsin-like enzyme activity below the normal basal levels (Fig. 4-1*B*). This suggests that the proteasomal pathway is the main proteolytic system involved in the LPS-induced muscle protein turnover. This result consistent with the other findings that, the proteasome is the main catabolic system involved in LPS-induced muscle proteolysis and inhibition of the proteasomal activity significantly blocked this effect.

The role of Akt was verified using LY0294002 (PI3-K/Akt inhibitor). Incubation of C2C12 myotubes with LPS in combination with LY0294002 delayed LPS-induced up-regulation of TNF-α and atrogin-1 mRNA (18 and 24 h post LPS administration). Moreover, inhibition of Akt significantly increased the atrogin-1 mRNA expression level; this suggests that atrogin-1 is the main ubiquitin ligase involved in LPS-induced protein breakdown. Furthermore, inhibition of Akt alone or with LPS significantly decreased the PS rate by 80% compared with untreated cells ($P < 0.001$). Together, these data demonstrated that Akt pathway is a key regulator of both of the protein synthesis and the protein degradation in skeletal muscle.

Although, incubation of the cells with LPS along with either SB203580 (p38 inhibitor) or PD098059 (MEK/Erk inhibitor) significantly decreased the proteasomal activity, it significantly increased the expression of TNF-α and atrogin-1 mRNA. Thus, it seems that there is no absolute correlation between the activity of these atrophic genes and the proteasomal activity. Similarly, the inhibition of MAPKs decreased the PS rate; this gave the evidence for the direct involvement of MAPKs (p38 or Erk1/2) in the protein synthesis process. In addition, the cross talk between the PI3-K/Akt and MAPKs (p38 or Erk1/2) was revealed by the western blotting technique. The data presented in chapter 3 showed that, inhibition of MAPKs (p38 or Erk1/2) significantly

decreased the phosphorylation of Akt ($P < 0.05$). Therefore, we assumed that, the direct involvement of MAPKs (p38 or Erk1/2) in the PS was mediated via the regulation of both Akt and Mnk1 signalling cascades. These results demonstrated the importance of the Akt as a key regulator not only for the protein synthesis but also for protein degradation. In addition, there is a cross talk between these signalling cascades; this makes the understanding of the LPS-induced decreased PS rate a complicated matter that requiring further investigation. Collectively, these results suggest the importance of MAPKs (p38 or Erk1/2) in the regulation of many diverse cellular processes in both normal and disease conditions.

The data presented in Chapter 3, 4 and 5 showed that LPS is a potent catabolic stimulus accelerates the rate of muscle proteolysis (expressed as chymotrypsin-like enzyme activity) and decreases the rate of PS (^{3}H-phenylalanine incorporation/μg 5%TCA precipitable protein). This effect was mediated via Akt and MAPKs (p38 or Erk1/2) and their downstream targets. There is however there is more than one limit in this study. Firstly, the effect MG-132 (proteasomal inhibitor) on the level TNF-α, atrogin-1, MuRF1 and TLR4 mRNA was not addressed. To answer this question, we have addressed whether there is a correlation between the proteasomal activity and the mRNA expression level of the ubiquitin ligases (atrogin-1 and MuRF1). We assume that the dramatic decrease in the proteasomal by 80% ($P < 0.001$) in the presence of LPS and MG-132 compared with LPS alone (chapter 4) associated with decreased ubiquitin ligases activity.

The second limit was that, we did not address the effect of second-messenger inhibition on the media concentration of TNF-α. The answer of this should be the topic of future research. The third limit concerning the NF-kB-DNA binding activity by the Electrophoresis mobility shift assay (EMSA). In this study, we have determined only the nuclear translocation of NF-kB rather than electro mobility shift assay (EMSA). EMSA has bee used as a indication of the translocated NF-kB is already bound to DNA and this

enables the alteration of many genes transcription. However, in the study carried out by Jin and Li, the absolute correlation between the NF-kB binding activity and the increased ubiquitin ligases activity is not always the case (Jin and Li 2007). Jin al Li demonstrated that, although curcumin significantly abolished the sepsis-induced atrogin-1, however it had no effect on the NF-kB DNA binding activity.

10.6 The role of curcumin in LPS-induced muscle protein turnover

Data presented in chapter 3, 4 and 5 showed that the LPS-induced decreased PS and increased protein degradation rates is a multifactorial process involving the interaction of many signalling cascades in particular Akt and MAPKs (p38 or Erk1/2) and their downstream effectors. In addition, the cross talk between them has been shown in chapter 3 and the inhibition of these signalling cascades differentially modulated the LPS-induced muscle protein turnover. Additionally, many studies that showed the value in curcumin as an anti-inflammatory, anti p38 activity and anti-NF-kB activity natural compound. Curcumin has previously been shown to decrease the TNF-α-p38 mediated up-regulation of atrogin-1 *in vivo.* However, the direct effect of curcumin on protein degradation and PS *in vitro* is still unclear and needs further investigation. This was the main aim of chapter 6.

The data presented in chapter 6 showed that, curcumin has anti-p38 and anti I*K*B-α activity. This effect was associated with decreased LPS-induced proteasomal activity especially curcumin (25 μM). Surprisingly, although curcumin abolished the LPS-induced decreased Akt and $P70^{S6K}$ phosphorylation and significantly increased Akt and Erk1/2 phosphorylation, however, this effect was not sufficient to provoke any significant changes in LPS-induced decreased PS rate. The data presented in the chapter 6 along with that in chapter 3 demonstrated that, LPS is a strong catabolic stimulus that significantly decreased the PS in C2C12 myotubes. In addition, the value

of curcumin as preventive should be considered and further studies are required to validate the curcumin as therapy in human.

10.7 The effect of LPS on the avian primary skeletal muscle cells

This was addressed in chapter 7. LPS significantly decreased chymotrypsin-like enzyme activity ($P < 0.05$) and increased the PS rate at 3 h ($P < 0.001$) compared with untreated and 18 h LPS-treated cells. This was not surprising LPS had no effect on the expression level of TNF-α, IL-6, atrogin-1, caspase-3, ubiquitin mRNA, but it significantly increased the phosphorylation 4E-BP1 switching on the translation initiation of PS. Inhibition of Akt, significantly increased the atrogin-1 mRNA level and this suggests again that Akt is the main signalling pathway involved in this process. In conclusion, the data generated in chapter 7 suggesting that effect of LPS is cell specific, and further investigation are still required to validate this effect with different primary skeletal muscle cells. The possible explanation for this discrepancy in response to LPS is that, the avian primary skeletal muscle cells have been derived from the broiler chickens that with different genetic background. These chicken were grown up to give a high body mass in a short period of time (few weeks), characterised by high protein synthesis rate and low protein breakdown rate. This suggests that these birds might robustly resistant the effect of LPS. An interesting point concerns the difference in the ability of specific inhibitors to inhibit the individual intracellular pathways in different cell types. Another possible cause could be the presence of the Insulin in case of avian primary cells, and insulin has previously been known as an anabolic stimulus significantly increased the PS via the acceleration of the translation initiation. This has been shown in the data collected (Fig. 7-1*B*). Although the cells were incubated with LPS, however, the 4E-BP! Phosphorylation has been increased and this means that the translation initiation is switches on. Therefore, further investigation are required to verify the responses in avian primary skeletal muscle cells using different

incubation conditions (without insulin) and compare this effect with that of the C2C12 cells.

10.8 Modulation of the nitric oxide production in LPS-stimulated skeletal muscle cells

In chapter 3, 4, 5 and 6, the presented data demonstrated that LPS significantly increased protein breakdown and decreased protein synthesis in C2C12 myotubes, but not in avian primary skeletal muscle cells. In addition, we have shown that LPS increased the synthesis and production of many cytokines in skeletal muscle. In chapter 8 we have addressed the effect of the LPS on nitric oxide production and how the inhibition of second-messenger signalling can modulate this effect.

The results obtained in chapter 8 demonstrated that LPS increased the nitric oxide production in C2C12 myotubes and avian primary skeletal muscle cells. However, this transient moderate increase in nitric oxide production suggested that nitric oxide has a minor role in LPS-induced protein breakdown.

There are no clear data regarding the role of different signalling cascades in, particular PI3-K/Akt and MAPKs (p38 or Erk1/2) in LPS-induced nitric oxide production. Therefore, the effect of inhibition of these signalling molecules was elucidated. Inhibition of these second messengers abolished the effect the LPS-induced nitric oxide production in C2C12 myotubes but not in the avian primary skeletal muscle cells. This discrepancy could result from the fact that the avian primary skeletal muscle cells were derived from broiler chickens of different genetic background. In addition, the data obtained *in vivo* showed that, LPS altered the PS and NOS2 activity in the fast twitch muscle (gastrocnemius) not in the slow twitch muscle (soleus).

Taken together, the data presented in chapter 6 have shown that the inhibition of second-messenger signalling modulated the nitric oxide production in C2C12 myotubes but not in the avian primary skeletal muscle cells. However, further investigation are still required to verify this effect in different primary cell lines including skeletal muscle obtained from layers.

The main limit we did not measure the effect of LPS on the NOS mRNA (the enzyme required for the production of nitric oxide). This is could be answered the presence of the nitric oxide in the media suggesting that the NOS mRNA is biologically translated into an active compound (nitric oxide), herein we have found that LPS increased NO production in the media and we assume that this effect was preceded by increased NOS mRNA levels.

10.9 The effect of natural challenge with S. Gallinarum or S. Enteritidis

The data presented in chapter 7 along with these of chapter 3 & 4 raised the following question: will natural challenge of broiler chicken with *Salmonella* affect the skeletal muscle protein metabolism.

Natural challenge with *S*. Gallinarum or *S*. Enteritidis decreased the expression of TNF-α, IL-6 and caspase-3 mRNA, while it has no effect on the ubiquitin mRNA. There was no expression of IL-8 or IL-10 mRNA detected in the skeletal muscle of chicken. The data presented in this chapter showed that, the challenge with *Salmonella* serotypes was not associated with any pro-inflammatory responses in skeletal muscle. Moreover, this particular model is not the most appropriate model for the studying the relationship between the salmonellosis and the muscle wasting syndrome. In addition, this emphasises the importance of LPS presence in the circulation rather than the live bacteria in order to detect significant alteration of muscle protein metabolism.

10.10 Future work

In the current study, the main aim was to elucidate the direct effect of LPS on the muscle protein synthesis and degradation and the signalling streams that involved in. Our data demonstrated that:

1- LPS alone decreased the PS rate by 50% and increased the protein degradation rate simultaneously in C2C12-murine derived myotubes. Therefore, herein our septic model has shown that LPS induced muscle-wasting was a result of accelerated rate of muscle proteolysis along with decreased rate of PS a similar effect to that obtained in vivo studies.

2- Akt is the key element involved in the control of both protein synthesis and protein degradation signalling streams.

3- LPS alone increased the TLR4 mRNA *in vitro.* Thus, LPS mediated its effect via TLR4.

4- LPS increased the cytokines synthesis and accumulation in the cultured skeletal muscle myotubes in particular TNF-α.

5- The ubiquitin proteasome system in particular atrogin-1 is the main proteolytic system involved in LPS-induced muscle proteolysis in C2C12 myotubes.

6- There is no absolute correlation between the activity of atrophic genes (atrogin-1 and MuRF1) and the overall proteasomal activity especially in case of MAPKs (p38 or Erk1/2) inhibition. However, elevated levels of atrogin-1 mRNA is still the predominant feature of our septic model.

7- Cross talk exists between MAPKs (p38 or Erk1/2) and PI3-K/Akt signalling cascades in protein synthesis.

8- Curcumin (25 μM) significantly decreased proteasomal activity in LPS-stimulated C2C12 myotubes. In contrast, it had no beneficial effects on PS.

9- LPS is a strong catabolic stimulus increased NO production in both two septic model (C2C12 myotubes and avian primary skeletal muscle cells). The transient and moderate increase in NO production was seems to be a part of the host immune response toward LPS and had no or minimal effect on muscle protein turnover. In addition, the LPS-induced NO production was modulated by the second-messenger inhibition in C2C12 murine-derived myotubes not in the avian primary skeletal muscle cells.

10- LPS significantly decreased the proteasomal activity and increased the PS rates in the avian primary skeletal muscle cells.

11- Natural challenge of chicken with *Salmonella* is not the appropriate model to study the pathogenesis of muscle wasting in salmonellosis.

Therefore, the future work will be

1- The effect of sepsis on the other proteolytic system activity in particular, the effect of sepsis on the calpain activity and how far the second-messenger inhibition will modulate this effect. This because, increased calpain activity has been linked to increased proteasomal activity, and considered as an essential step for disassembly of sarcomere components in particular actin and myosin. This afterwards facilitates the ubiquitination and proteasomal activation of the actin and myosin.

2- The effect of various concentrations of recombinant IL-6 on protein metabolism in the C2C12 myotube and avian primary skeletal muscle cells. There is no clear data about the role of IL-6 on muscle protein turnover.

3- The effect of LPS on short and long-lived proteins degradation rates and the potential role of second-messenger inhibition of particular that of Akt and MAPKs (p38 or Erk1/2). This because the myofibrillar proteins comprises two third of the whole skeletal muscle protein. In addition, evaluation of this effect will leads to a comprehensive

overview about the overall LPS-induced muscle proteolysis. This in turn helps in developing an appropriate therapeutic intervention.

4- The effect of intramuscular injection of LPS in chicken and how it will affect the skeletal muscle protein metabolism. Herein, the use of natural challenge model with *Salmonella* serotypes was not associated with any pro-inflammatory effects in skeletal muscle.

5- The effect of *Salmonella* Systemic infection in chicken and explore the effect on muscle protein metabolism.

6- The effect of viral infection of skeletal muscle upon the muscle protein metabolism. In addition, elucidation of the signalling cascades that possibly involved in these process.

7- The incubation of skeletal muscle specimen (*in vitro*) with various concentrations of LPS and recombinant IL-6 and to explore the effect on the muscle protein metabolism.

8- The effect of endotoxin on the activity of skeletal muscle satellite cells. The value of the satellite cells in the process of skeletal muscle regeneration has been shown in many studies.

9- The relationship between nitric oxide production and muscle protein metabolism using NO donors and NO specific inhibitors to build up a comprehensive idea about the NO contribution in LPS-induced muscle proteolysis.

10- Microarray transcriptional analysis of LPS-stimulated cultured myotubes (C2C12-murine derived myotubes and avian primary skeletal muscle cells) and septic chicken skeletal muscle (*in vivo*) to built a comprehensive overview about the effect of sepsis in different *in vivo* and *in vitro* models.

11- The proteomic analysis of LPS-stimulated cultured skeletal muscle myotubes and septic chicken skeletal muscle. This important in determining which protein is abundantly increased or decreased in response to sepsis (identification by peptide mass mapping) using the database search program.

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Appendix

1-Extraction and culture of primary chicken muscle cells.

Cut 1 centimetre square of tissue (skeletal muscle), and wash in sterile. Then the tissue was chopped with sterile scalpel as fine as possible and then it transfer into a falcon tube with collection media. Then 30 ml of sterile PBS were added, the falcon tube was shacked and left to settle and pour off PBS. This process was repeated with the same amount of sterile PBS. Then pour the PBS and replaced with 30 ml of the collection media, then the tube was centrifuged at 1200 rpm for 5 min at room temperature. This step was repeated again, and then pours the supernatant and split the tissue into 2 or 3 falcon tubes (depending on how much the tissue), adds to each tube 30 ml of the Dissociation media supplemented with 1% pronase (1.4 mg/ml) to dissociation media (300ul of pronase). The falcon tubes were then Put in roller at 4 degrees for 2 hours. To each tube, half the pronase added before (150ul) was added and left to roll overnight at 4 degrees. In the next day, the tubes were left to settle for 5 min. Then the content of the tubes were transferred with a pipette to a new 50 ml falcons and then centrifuge 1200rpm for 6 min. Remove the supernatant and add 20 ml 10% fetal calf serum (FCS) + P/S (penicillin/streptomycin) + fungizone mix and Centrifuge again. This step was repeated again and centrifuge. Then, tip off liquid and resuspend the pellet in 20 ml chicken culture media (CCM) supplemented with extra fungizol 1% (200 ul for 20 ml). Then, transfer 20 ml of the content to a new collagen coated flask T75 (of note, rinse with PBS the collagen coated flask before transferring the cells, then incubate the flasks at 37 $^{\circ}$C 5% CO2 for one day and then change media. The first harvest of these is the avian primary skeletal muscle cell.

The composition of the media:

1-Collection media:

DMEM+glutamax

1% P/S

1% fungizone

2-Dissociation media:

DMEM/F-12(1:1)

1% P/S

1% fungizone

0.5ml of ciprofloxacin per 500ml of media.

3-Chicken culture media (CCM)

DMEM/F-12(1:1)

5% FCS

1% P/S

1% insulin-transferrin-selenium

2% Chicken embryo extracts (CCE)

2-Table show the dilutions of the primary and secondary antibodies

Antibody's name	Company	Catalogue number	Dilution
4E-BP1 (ser65)	Cell signalling	#9451	1:1000
β-actin	Cell Signalling	#4970	1:1000
Anti-rabbit IgG HRP-linked Antibody	Cell Signalling	#7074	1:2500
Akt1/2/3	Santa Cruz	Sc-1619	1:500
Phospho-Akt1/2/3	Santa Cruz	R:sc-7985-R	1:500
Erk1/2	Santa Cruz	Sc-93	1:500
Phosph-Erk1/2 (Thr202/Tyr204)	Santa Cruz	Sc-16982	1:500
P38α	Cell signalling	#2371	1:1000
p-p38 (Thr180/Tyr182)	Santa Cruz	R:sc-17852-R	1:500
IkB- α (C-21)	Santa Cruz	Sc-371	1:500
Phospho- IkB- α	Santa Cruz	Sc-7977	1:500
Phosphoplus p70S6Kinase (Thr389, Thr421/Ser424) Antibody Kit	Cell signalling	#9430	1:1000
Phospho-eIF4E (Ser209)	Cell Signalling	#9741	1:1000
Phospho-mTOR (Ser2448)	Cell Signalling	#2971	1:1000
Phospho-NF-kB	Santa Cruz	sc-33022	1:500

Table represents the C_T values for LPS alone. C2C12 myotubes were treated with LPS alone for various time points

Gene	LPS alone				
	C	1 h	3 h	12 h	18 h
TNF-α	34.64336	30.19144	34.55953	35.58322	34.93385
Atrogin-1	25.74232	26.49701	22.85326	22.27426	25.41691
MuRF1	31.17995	31.11185	31.22126	31.49557	30.33128
TLR4	28.33817	28.24394	28.29932	28.29369	27.54996

Table represents the C_T values for LPS and SB203580 (p38 inhibitor). C2C12 myotubes were treated with LPS in combination with SB203580 for various time points

Gene	LPS + SB203580					
	C	30 m	1 h	3 h	12 h	18 h
TNF-α	34. 31817	35.18493	33.89269	31.75619	36.61537	35.67907
MuRF1	32.21235	29.71146	29.56991	29.68474	30.66979	29.63457
TLR4	32.23125	26.76082	25.89496	25.50416	25.09408	24.66654

Table represents the C_T values for LPS and PD098059 (MEK/Erk inhibitor). C2C12 myotubes were treated with LPS in combination with SB203580 for various time points.

Gene	LPS + PD098090					
	C	30 m	1 h	3 h	12 h	18 h
TNF-α	34. 21817	35.38521	33.13956	22.28769	32.14607	36.27319
Atrogin1	31. 21881	26.29536	26.51023	25.21639	26.34388	26.38619
TLR4	32.12233	26.6181	27.02856	26.48498	26.06186	25.60501

Table represents C_T values fro some genes that used in the avian primary skeletal muscle cells. Cells were treated with LPS alone or LPS in combination with inhibitors for 3 h.

Gene	Treatment							
	C	LPS	LPS+ PMB	LPS+ SB	LPS+ PD	LPS+ LY	LPS+ Curcumin	Dexa
Atrogin-1	28.62	28.4	28.71	28.96	30.49	27.87	28.68	27.090
Caspase	26.48	26.52	26.62	26.34	27.44	26.69	26.10	25.83
Ubiquitin	22.50	22.36	22.84	22.46	24.12	22.34	22.47	23.00

Table represents C_T values for some genes that used in the chicken experiment. Chicken were challenged with *Salmonella* Gallinarium (SG) or *Salmonella* Enteritidis (SE) for 4 days.

Gene	Treatment		
	C	SG	SE
Atrogin-1	26.24894	28.11036	28.15522
Caspase-3	29.10884	30.56068	35.05839
Ubiquitin	25.53715	23.75329	33.10558