**Faculty of Medical and Health Sciences**
**School of Biomedical Sciences**
**Centre for Integrated Systems Biology & Medicine**


# Comparison of the Effects of Dietary Flavonoids and Statins on Lipopolysaccharide-Induced Vascular Inflammation

**By**

**Salmin Khalid Alshalmani**

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# Abstract

Numerous epidemiological studies indicate that flavonoid intake as part of a balanced diet confers beneficial health effects in man, including improved cardiovascular function, reduced incidence of cancer and amelioration of symptoms associated with inflammatory disorders (Boots *et al*., 2008). A recent area of interest that may be fruitful is the study of anti-inflammatory effects of flavonoids in combination with statins.

Porcine coronary artery (PCA) segments were incubated overnight at 37°C in modified Krebs-Henseleit solution with or without 1µg $mL^{-1}$ lipopolysaccharides (LPS), with either (0.1–10µM) quercetin, or 10µM quercetin 3′-suphate and 10µM quercetin-3-glucuronide, or with (0.01-10µM) epicatechins, 10µM catecchin and10µM epigallocatechin gallate. (0.03-3µM) simvastatins and 10µM pravastatin are also used in this study. In addition, since many quercetin-rich foods also contain significant amounts of myricetin, this flavonoid has also been examined.

After 16 to 18 hours, segments were prepared for isometric tension recording in Krebs-Henseleit solution. The segments were then exposed to cumulatively increasing concentrations of KCl and then U46619. Responses are shown as milliNewton or calculated as the concentration causing 50% of the maximum effect (-log $EC_{50}$). For nitrite measurement, segments of the PCA were incubated in DMEM at 37°C for 24 hours, with or without 1µg $mL^{-1}$ LPS. The nitrite content (nmol) of the bathing medium was determined by spectrophotometry using the Griess reaction, while inducible nitric oxide synthase was identified immunohistochemically. Differences between mean values were assessed by ANOVA (*post-hoc* Dunnett test).

Prolonged exposure to LPS caused hyporesponsiveness of the PCA associated with increase in nitrite production by a mechanism that appears to involve the induction of nitric oxide synthase. Nitrite content of the incubation medium increased 3 to 10-fold following exposure to LPS and inducible nitric oxide synthase was detected in the adventitia. The results indicated that all of the tested flavonoids and statins are able to suppress LPS-induced changes in vascular responses, nitrite production and expression of inducible nitric oxide synthase. While 10µM myricetin was inactive.

In conclusion I have demonstrated that quercetin, and its principal human metabolites and catechins oppose pro-inflammatory events in both endothelial cells and vascular smooth muscle cells. Possibly through a mechanism involving inhibition of NFkB. Since pre-treatment of the PCA with statins reduced LPS-induced changes in vasoconstrictor responses, suppressed the induction of nitric oxide synthase caused by LPS and the associated increase in nitrite production. It is unlikely that the effect of the statin involves direct inhibition of NOS. These findings are consistent with clinical studies suggesting that prior use of statins may afford protection against bacterial sepsis.

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# Disseminations of Results:

## *Papers*:

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S. Alshalmani, M. Taylor, S. Suri, P. Kroon, D. Hughes, S. Tribolo & V.G.Wilson. **Quercetin inhibits lipopolysaccharide-induced nitric oxide production in the porcine coronary artery**. 4th International Conference on Polyphenols and Health 7th to 11th December 2009. Harrogate International Centre, Harrogate-UK.

S. Alshalmani, M. Taylor, S. Suri, P. Kroon, D. Hughes, S. Tribolo & V.G.Wilson. **Myricetin antagonise the anti-inflammatory effect of quercetin in the porcine isolated coronary artery.** 4th International Conference on Polyphenols and Health 7th to 11th December 2009. Harrogate International Centre, Harrogate-UK.

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# Abbreviations:

| | |
|---|---|
| **AA** | arachidonic acid |
| **ABC** | avidin-biotin-peroxidase |
| **AF** | Arterial fibrillation |
| **ATP** | adenosine triphosphate |
| **ANOVA** | analysis of variance |
| **ATP K+ Channel** | adenosine triphosphate potassium sensitive channel |
| **BES** | bis-2-hydroxy-2-aminoethanesulfonic acid |
| **BK** | bradykinin |
| **CAD** | coronary artery disease |
| **CAM** | cell adhesion molecule |
| **cAMP** | cyclic adenosine monophosphate |
| **cGMP** | cyclic guanosine monophosphate |
| **CHF** | congestive heart failure |
| **COX** | cyclooxygenase |
| **CVD** | cardiovascular disease |
| **DMEM** | Dulbecco's modified Eagle medium |
| **DNA** | deoxyribonucleic acid |
| $\mathbf{E_{max}}$ | maximum response |
| **eNOS** | endothelial nitric oxide synthase |
| **ET-1** | endothelin-1 |
| **FPP** | farnesyl pyrophosphate |

| | |
|---|---|
| **GGPP** | Geranyl geranyl pyrophosphate |
| **GPP** | geranyl pyrophosphate |
| **HIMEC** | Human intestinal microvascular endothelial cells |
| **HMG CoA reductase** | 3-hydroxy-3-methyl-glutaryl-CoA reductase |
| **ICAM** | intercellular adhension molecule |
| **IHD** | ischemic heart disease |
| **IL-1β** | interleukin-1β |
| **IL-6** | interleukin-6 |
| **iNOS** | inducible nitric oxide synthase |
| **IRAK** | IL-4 receptor activated kinase |
| **KCl** | potassium chloride |
| **K-H** | Krebs- Henseleit solution |
| **LBP** | lipopolysaccharide binding protein |
| **LDL-C** | low density lipoprotein- cholesterol |
| **L-NAME** | $N^G$-nitro-L-arginine methyl ester |
| **LOX** | lipooxygenase |
| **LPS** | lipopolysaccharide |
| **mCD14** | membrane-bound CD14 |
| **MI** | myocardial infraction |
| **MMP-2** | matrix metalloprolinase-2 |
| **MyD88** | human myeloid differentiation factor-88 |
| **Myr** | myricetin |

| | |
|---|---|
| **NE** | norepinephrine |
| **NFκB** | nuclear factor κB |
| **NO** | nitric oxide |
| **NOS** | nitric oxide synthase |
| **oxLDL** | oxidized low density lipoprotein |
| **$PD_2$** | negative logarithm of EC50 |
| **PDGF** | platelet-derived growth factor |
| **$PI_3$** | phosphatidylinsositol-3 |
| **PG** | prostaglandins |
| **$PGH_2$** | prostaglandin $H_2$ |
| **$PGI_2$** | prostacyclin |
| **$PGF_{2\alpha}$** | prostaglandin $F_{2\alpha}$ |
| **$PLA_2$** | phospholipase $A_2$ |
| **Quer** | quercetin |
| **Que-glucu** | quercetin 3-glucuronide |
| **Que-sul** | quercetin 3'-sulphate |
| **SEM** | standard error of mean |
| **SMCS** | smooth muscle cells |
| **sCD14** | soluble CD14 |
| **SNP** | sodium nitroprusside |
| **SP** | substance P |
| **SREBP2** | sterol response element binding proteins |

| | |
|---|---|
| **TG-VLDL** | triglyceride- very low density lipoprotein |
| **TLR** | toll-like receptor |
| **TNFα** | tumor necrosis factor α |
| **TPP** | thiamine pyrophosphate |
| **TRAF6** | TNF receptor associated factor 6 |
| **$TXA_2$** | thromboxane $A_2$ |
| **U46619** | 9,11-Dideoxy-11a,9a-epoxymethanoprostaglandin $F_{2\alpha}$ |
| **VCAM** | vascular cell adhesion molecule |
| **VEGF** | vascular endothelial growth factor |
| **VLDL** | very low density lipoprotein |

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# Chapter 1

# General Introduction

Cardiovascular disease (CVD) remains the leading cause of death throughout the Western world and the second most common cause worldwide despite enormous progress in the prevention and treatment of cardiovascular diseases (Braunwald, 1997) . By the year 2020, it is estimated that nearly 40% of all deaths worldwide will be due to CVD, which is more than twice the percentage of deaths from cancer. In the last 30 years, hyperlipidemia has been identified as a major modifiable risk factor for CV death (Braunwald, 1997). Treatments aimed to modifying plasma cholesterol levels by dietary and pharmacological means. Dietary treatment focused on energy restrictions and modifying saturated fat intake. Statin drugs have been shown to very significantly reduce cardiovascular disease events in a number of large clinical trials (Scandinavian Simvastatin Survival Study (4S) Group, 1994). As a result, statins are now considered to represent one of the most powerful classes of agents for the treatment of cardiovascular diseases (Lefer *et al*., 2001). Originally, reductions in cardiovascular disease events and mortality and overall improved outcomes were attributed to dramatic reductions in circulating serum lipid levels that were mediated by inhibition of liver 3-hydroxy 3-methyl glutaryl coenzyme A (HMG-CoA) reductase. However, half of all coronary events occur in persons without overt hyperlipidemia (Braunwald, 1997). The search for new and better predictors of risk has led researchers to a broader understanding of the potential, for example of cardioprotective diet and pharmacological treatment that reflect an emerging appreciation of the importance of inflammation in atherogenesis and thrombosis. From a dietary perspective a number of epidemiological studies provide evidence that consumption of fruit and vegetables is associated with reduced risk of cardiovascular disease. This cardioprotective effect is might be due, at least in part, to minor components of fruit and vegetables called flavonoids (Cook and

Samman, 1996). Some epidemiologic studies suggest a cardioprotective role of flavonoids against coronary heart disease. One large clinical study indicated that flavonoids may reduce mortality from coronary heart disease (Hertog *et al.*, 1993) Various cohort studies indicated an inverse association between flavonoid intakes and coronary heart disease mortality (Hertog *et al.*, 1995; Knekt *et al.*, 1996; Skibola and Smith, 2000). Furthermore, epidemiologic studies suggest a protective role of dietary flavonoids against coronary heart disease (de Groot and Rauen, 1998). These studies are promising and indicate that flavonoids may be useful medical compounds. From a pharmacological perspective, statins are rapidly becoming frontline therapy for diabetes mellitus, hypertension, and other known cardiovascular disease risk factors. They are considered to act more broadly than simply by reducing plasma cholesterol levels, potentially via inhibiting inflammatory processes.

In this Introduction I will outline the evidence that inflammatory processes are important in the development of atherosclerosis, sepsis and other cardiovascular diseases. I will highlight the potential to use lipopolysaccharide *in vitro*, to model some of these changes. I will then discuss the chemistry, metabolism and biological actions of various flavonoids including quercetin and catechins. Finally, I will outline the evidence for statins, a key drug class in treatment of atherosclerosis, to influence inflammatory responses.

## 1.1 Pathology of Atherosclerosis

Atherosclerotic narrowing of the epicardial coronary arteries, together with thrombosis, platelet aggregation, and vasospasm, all contribute to the development of ischaemic heart disease (IHD). IHD results from an imbalance between perfusion (supply) and workload (demand) of the heart. Ischaemia not only involves oxygen insufficiency, but also reduced nutrient concentration and inadequate removal of metabolites. In more than 90% of cases, ischaemia is due to atherosclerosis. For this reason, ischaemic heart disease is often termed coronary artery disease and symptomatically can appear as either angina pectoris, acute coronary syndromes, such as myocardial infarction and heart failure, and also sudden cardiac death (Libby and Simon 2001).

Atherosclerosis, formerly considered as a bland lipid storage disease, actually involves an ongoing inflammatory response of the major conduit arteries of the heart, brain, legs and other major organs. It is now generally accepted that inflammation plays a fundamental role in mediating all stages of atherosclerosis, from initiation through progression and, ultimately, the thrombotic complications of the condition (Figure 1.1). Numerous clinical studies have shown that the biology of inflammation in atherosclerosis applies directly to human patients. Elevation in markers of inflammation predicts outcomes for patients with acute coronary syndromes, independently of myocardial damage. In addition, low-grade chronic inflammation, as indicated by levels of the inflammatory marker C-reactive protein, prospectively defines risk of atherosclerotic complications, thus adding to prognostic information provided by traditional risk factors. Moreover, certain treatments that reduce coronary risk also limit inflammation. In the case of

lipid lowering with statins, this anti-inflammatory effect does not appear to correlate with reduction in low-density lipoprotein levels (De Denus and Spinler, 2002; Giusti-Pavia *et al*., 2004; Kaesemeyer *et al*., 1999).

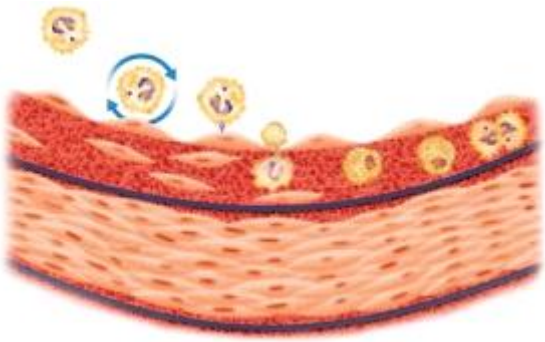
Growing evidence supports the role of local and systemic inflammation as a common pathophysiological mechanism in different cardiovascular diseases like congestive heart failure (CHF) or coronary artery disease (CAD). Elevated plasma levels of cytokines like interleukin (IL-) 6 and tumor necrosis factor- α (TNF- α) were not only consistently detected in patients with CHF (Levine *et al*., 1990; Lommi *et al*., 1997; Torre-Amione *et al*., 1996) but also in patients with stable or unstable angina and myocardial infarction (Gabriel *et al*., 2000; Ikeda *et al*., 2001; Kanda *et al*., 2000). Inflammatory mediators such as cytokines can modulate cardiac contractility and, therefore, might influence CHF progression (Kelly and Smith, 1997; Mann and Young, 1994; Niebauer, 2000). Cytokines such as IL-1, IL-6, and TNF-α exerte negative inotropic effects in isolated cells and hearts (Rietschel and Brade, 1992; Weisensee *et al*., 1993).

### 1.1.1 Role of the Inflammatory Process in Atherosclerosis

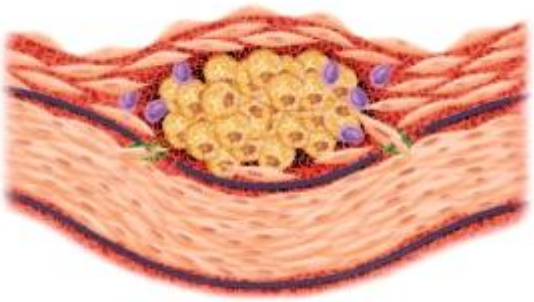
Inflammation is an orchestrated biological process, induced by microbial infection or tissue injury. A major trigger of inflammation is the recognition of microbes by specific receptors of the innate immune system, which play a crucial role in the induction of early signals initiating and establishing the inflammatory setting (Nathan, 2002). During inflammation, high concentrations of nitric oxide produced by inducible nitric oxide synthase in macrophages can result in oxidative damage. In such circumstances, activated macrophages greatly increase the simultaneous production of both nitric oxide and super-oxide anions. Nitric

oxide reacts with free radicals, thereby producing the highly damaging peroxynitrite that can directly oxidize LDL, resulting in irreversible damage to the cell membrane (Haenen *et al*., 1997).

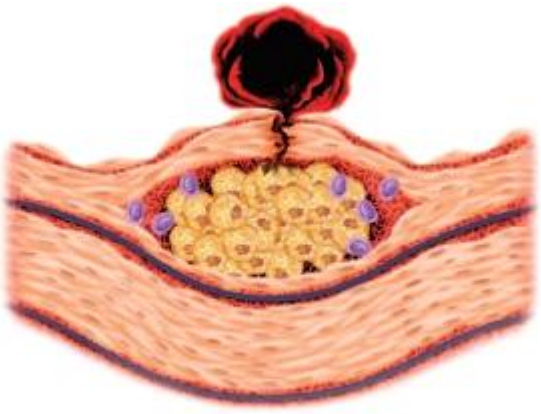
Inflammation plays a role in all stages of atherothrombosis. Early in the process, in response to oxidized low-density–lipoprotein cholesterol (LDL-C), injury, or infection, resident or circulating leukocytes bind monocytes to the site of a developing lesion. As they continue to ingest chemically modified lipids and lipoproteins, monocytes transform into activated macrophages and then become foam cells. More than half of all cells at the immediate site of plaque rupture are macrophages; they are the dominant type of atherosclerotic inflammatory cell infiltrate (Moreno *et al*., 1994). At the same time, other inflammatory cells, including activated T cells and mast cells, also attach themselves to the endothelium. All of these inflammatory cells eventually contribute to the formation of the atheromatous lesion, which consists of a lipid pool protected by a fibrous cap. The monocyte-macrophages release a variety of enzymes including metalloproteinases. These proteolytic enzymes can break down collagen in the fibrous cap, leaving it prone to rupture, and exposing the tissue factor and atherosclerotic debris beneath to arterial blood, inducing thrombosis. At the same time, smooth muscle cells (SMCs) secrete factors that recruit additional monocytes (Lefkowitz and Willerson, 2001). Local stimulation of SMCs in the artery wall can amplify the inflammatory response and promote a local procoagulant effect (Lefkowitz and Willerson, 2001; Libby and Simon, 2001).



A- Initiation of atherosclerosis



B- Progression of atherosclerosis.



C- Thrombotic complication of atherosclerosis

**Figure 1-1** Sequences in progression of atherosclerosis (Libby *et al*., 2002).

It has been proposed for several decades that infections may be responsible for the accelerated development of atherosclerosis. Numerous studies have shown an association between atherosclerosis and both viral and bacterial infections (Muhlestein, 1998) . IL-6 is an important regulator of the acute phase reaction and its proinflammatory effects are likely to adversely affect the cardiovascular system. NF-kB is a vital element in the production of proinflammatory proteins and is activated in patients with active CAD. However, recent findings suggest that NF-kB may have proinflammatory (Kempe *et al*., 2005) or anti-inflammatory actions (Sethi *et al.,* 2008) depending on the phase of the inflammatory process.

## 1.2 Proinflammatory Factors (Lipopolysaccharide)

Over the past decade it has become accepted that cardiovascular disease (e.g. atherosclerosis) has a significant inflammatory component (Hansson, 2005). High levels of plasma cholesterol, particularly associated with raised low density lipoprotein (LDL), is associated with raised plasma markers for inflammation, e.g. C-reactive protein, and expression of cell adhesion molecules on cells. In order to model some of these inflammatory changes *in vitro*, against which the potentially beneficial effects of flavonoids can be assessed, it is necessary to use known pro-inflammatory agents. Presently there are many agents used to elicit inflammation *in vitro,* lipopolysaccharide and oxidised low density lipoprotein (oxLDL). Epidemiological studies indicate that endotoxemia at levels as low as 50 pg/ml represents a strong risk factor for the development of atherosclerosis (Kiechl *et al.*, 2001; Wiedermann *et al*., 1999). A variety of gram-negative infections were associated with an increased risk of atherosclerosis (Kiechl *et al*.,

2001), supporting the hypothesis that endotoxin may be pathogenically linked to the development of atherosclerosis.

Lipopolysaccharide (LPS) is the main component of the outer membrane of gram-negative bacteria and is responsible for many of the pathophysiological effects. It consists of three domains, a lipid moiety (lipid A), a core region and an O-specific chain, of which lipid A is structurally the most conserved among different pathogenic bacteria and represents the toxic principle of LPS (Rietschel *et al.*, 1994). The presence of LPS in the systemic circulation causes a widespread activation of the innate immune response leading to the uncontrolled production of numerous inflammatory mediators, including tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6), by cells of the monocyte/macrophage lineage, as well as others such as nitric oxide produced by the endothelial cells, which act to cause a frequent fatal systemic inflammatory response called "septic shock" (Cinel and Opal, 2009).

Many studies indicated that either short-term or long-term infusion of LPS has been shown to depress the response to vasoactive agents and to induce hypotension in pigs *in vivo* (Datta and Magder, 1999). Effects of LPS in pigs not only demonstrated the vascular function change but also induced an accumulation in the plasma content of cycloxygenase products (Hellyer *et al*., 1997). Many of these effects have been replicated in human volunteers who have been exposed to a low dose of LPS – an experimental model of human endotoxaemia (O`Brien and Abraham, 2004).

Some of the inflammatory changes induced by LPS *in vivo* can also be observed in isolated cells. For example, LPS (10ng/ml) has been shown to induce TNF-α, interleukin-6 and interleukin 1ß in human peripheral blood mononuclear cells (Hougee *et al*., 2005; Richard *et al*., 2005). Higher concentrations (0.1-1µg/ml) have been shown to increase nitrite production in the macrophage cell line RAW 264.7 and mouse microglia (Chen *et al*., 2005; Kim *et al*., 2005). The model of LPS-treated J774.2 macrophages is widely used in studies of mechanisms of iNOS induction (Swierkosz *et al*., 1995; Szabo *et al*., 1993; Wu *et al*., 1995). Activation of J774.2 macrophages with LPS resulted in accumulation of nitrite in culture medium. This accumulation of nitrite reflected NO production due to induction of iNOS in LPS-treated cells (Wu *et al*., 1995).

Two glycoproteins are clearly implicated in the molecular and cellular basis of the interaction between endotoxin and macrophages (Morrison and Ryan, 1987; Ulevitch and Tobias, 1995). Firstly, lipopolysaccharide-binding protein (LBP), present in normal serum, recognizes and binds LPS with high affinity through its lipid A moiety (Schumann *et al*., 1990; Su *et al*., 1995). LPS-LBP complexes then activate cells through the second glycoprotein, membrane-bound CD14 (mCD14) to produce inflammatory mediators (Martin *et al*., 1994; Ulevitch and Tobias, 1995; Wright *et al*., 1990). Secondly, several reports have shown CD14 to be critical to the response of macrophages to low concentrations of LPS in the presence of LBP (Dentener *et al*., 1993; Kielian and Blecha, 1995; Maliszewski, 1991). The LPB: LPS complex is then capable of being transferred to TLR4, although full activation of the receptor requires the membrane receptor CD14 and an accessory protein, MD2 (Figure 1.2).

The engagement of the TLR4 complex results in the recruitment of several adapter proteins, including myeloid differentiation factor-88 (MyD88), TNF receptor associated factor (TRAF) and tumor necrosis factor (TNF), interleukin receptor-associated kinase (IRAK), creating a signaling complex that, in turn, leads to activation of downstream signaling through the mitogen-activated protein (MAP) kinase and phospahtidylinositol-3 (PI3) kinase pathways, and through the activation of the transcription factor, NF-κB. The consequence is the transcription of members of a family of early proinflammatory genes, including interleukin-1 (IL-1) and tumor necrosis factor (TNF), and their release from the cell. IL-1 and TNF then act on target cells through their own specific receptors, evoking further cellular responses that shape the phenotype of sepsis by, for example, activating coagulation through increased expression of tissue factor or inducing vasodilatation through upregulation of synthesis of nitric oxide catalyzed by inducible nitric oxide synthase (Brookes *et al.*, 2009).

In blood vessels, lipopolysaccharide (LPS) cause expression of inducible nitric oxide synthase (iNOS) consequently leading to NO overproduction and development of hyporeactivity to vasoconstrictor agonists (for review see (Stoclet *et al.*, 1993; Szabo, 1995; Thiemermann, 1997). Furthermore, higher concentrations of LPS (10-100 µg/ml) were reported by Wei (2006) to inhibit KCl and U46619-induced contractions of the porcine isolated coronary artery. This effect was associated with an increase in cellular cyclic GMP, an increase in TNF-α secretion and hyporesponsiveness to the vasodilators sodium nitroprusside, substance P and bradykinin (Wei, 2006).

It has been reported that low levels of endotoxin caused proinflammatory activation of human coronary artery endothelial cells and human carornary artery smooth cells, including the secretion of cytokines (Stoll *et al*., 2004 ) and enhanced expression of cell adhesion molecules (CAM) (Faure *et al.*, 2000). Endothelial activation by LPS leads to enhanced leukocyte adhesion and tissue recruitment from the circulation, a key regulatory step in the inflammatory response (see Figure 1a). Binion and colleagues reported that cultured human intestinal microvascular endothelial cells (HIMEC) are also strongly activated by bacterial LPS, as demonstrated by significantly enhanced CAM expression and increased leukocyte-binding activity (Binion *et al*., 1997). Although endothelial cells do not possess membrane-bounded CD14 (mCD14), complexes of soluble CD14 (sCD14) and LPS are able to stimulate cells including endothelial cells (Pugin *et al*., 1993) to produce these effects. The clinical significance of soluble CD14 is underlined by finding that elevated levels of LPS and soluble CD14 could be measured in patients with CHF (Anker *et al*., 1997; Niebauer *et al*., 1999; Rauchhaus *et al*., 2000). As described in monocytes and macrophages, endothelial cells are also believed to undergo activation through ligation of Toll-like receptor 4 (TLR4), a recently identified receptor and signal transducer of LPS (Aderem and Ulevitch, 2000; Zeuke *et al*., 2002). Ligation of TLR4 leads to recruitment of MyD88, phosphorylation of IL-1R associated kinase, oligomerization of TNFR-associated factor 6, and subsequent degradation of I-B. These events lead to the activation of NF-κB, and resultant transcription of immune responsegenes (Brookes *et al*, 2009) .

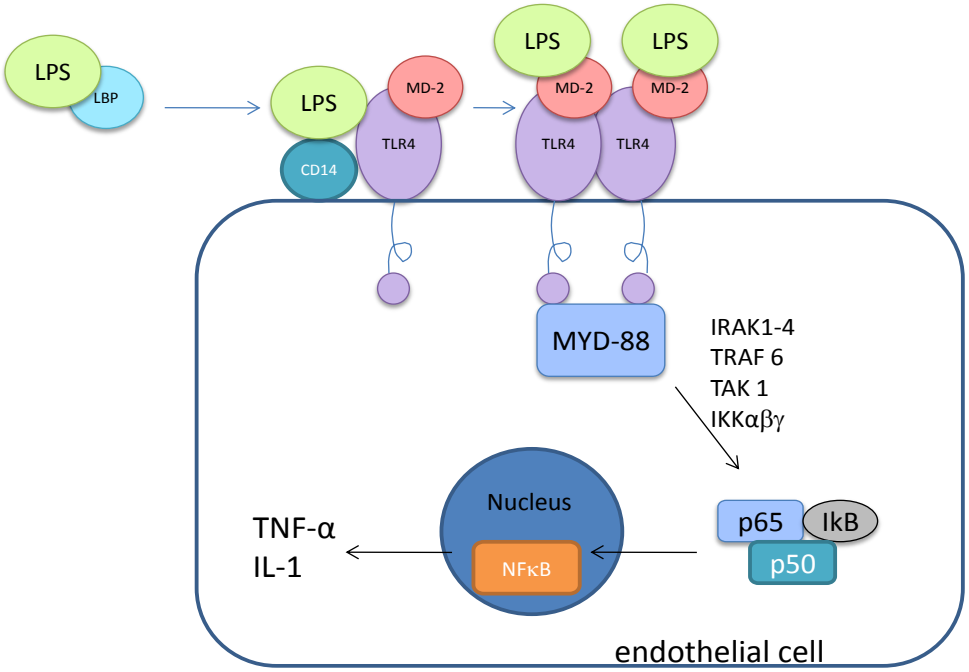



**Figure 1-2** Activation of endothelial cell TLR-4 by LPS
*Adapted from (Brookes et al., 2009).*

LPS is recognized by TLR-4 at the cell surface through interactions with several extracellular proteins. LBP delivers LPS to CD14. LPS then transfers to MD-2 to form an endotoxin- MD-2 complex which binds and activates TLR-4. Activation of TLR-4 induces a signalling cascade which leads to the activation of the transcription factor NF-κB, mediating the production of pro-inflammatory cytokines including TNF-α and IL-1.

**Abbreviation:** IRAK: interleukin receptor-associated kinase; TRAF: TNF receptor associated factor; MYD-88: myeloid differentiation factor-88.

Taken together, although many of the key symptoms of ischemic heart disease can be accounted for by inflammatory changes associated with the development of atherosclerosis in blood vessels, much of our understanding of the cellular events has depended on a study of the effect of lipopolysaccharide in immune cells, rather than the presumed pro-inflammatory factor oxidized LDL in vascular smooth muscle.

## 1.3 Flavonoids

Flavonoids belong to a group of natural substances, with variable phenolic structures. They are found in fruit, vegetables, grains, bark, roots, stems, flowers, tea, and wine (Middleton, 1998). They comprise a common flavan core, formed with 15 carbon atoms arranged in 3 rings ($C_6$-$C_3$-$C_6$) consisting of 2 aromatic ones linked through 3 carbons, usually forming an oxygenated heterocycle nucleus, the flavan nucleus (Bravo, 1998). The different patterns of this nucleus permit the classification of flavonoids into several subgroups, i.e., flavanols that use 2-hydroxy-2H-chromen-3-ol skeleton. These include the catechins, epicatechin and catechin gallate, flavonols; that have the 3-hydroxy-2-phenylchromone 4-one backbone and includes quercetin, myricetin and kaempferol, anthocyanidins (cyanidin, delphinidin), flavones (apigenin, diosmin), and flavanones (naringenin, hesperetin) (Bravo, 1998).

These natural products were known for their beneficial effects on health long before flavonoids were isolated as the effective compounds. More than 4000 varieties of flavonoids have been identified, many of which are responsible for the attractive colors of flowers, fruit, and leaves (de Groot and Rauen, 1998). Research on flavonoids received an added impulse with the discovery of the French paradox, i.e., the low cardiovascular mortality rate observed in Mediterranean populations, in association with red wine consumption and a high saturated fat intake. Numerous epidemiological studies indicate that flavonoid intake as part of a balanced diet confers beneficial health effects in man, including improved cardiovascular function, reduced incidence of cancer and amelioration of symptoms associated with inflammatory disorders (Boots *et al*., 2008).

Until recently, considerable attention has focused on the antioxidant properties of flavonoids, with the presumption that they scavenge free radicals and prevent deleterious changes to the vascular endothelium (Vita, 2005). However, the significance of this action has been called into question by reports that prolonged ingestion of non-flavonoid antioxidants failed to result in beneficial cardiovascular outcomes (Brigelius-Flohe *et al*., 2005; Devaraj and Jialal, 2005). Thus, other potentially relevant actions of flavonoids, including anti-inflammatory, anti-thrombotic and direct vascular effects (Middleton *et al*., 2000) , now need to be considered in greater detail. However, before commenting on the non anti-oxidant activities of flavonoids it is necessary to outline basic properties of vascular endothelium and smooth muscle that comprise blood vessels.

### 1.3.1 Nitric Oxide and Endothelial Function

The vascular endothelium is probably the most extensive tissue in the body, and its continuous smooth and non-thrombogenic surface forms a highly selective impermeable barrier (Desideri and Ferri, 2005; Furchgott and Zawadzki, 1980). A single layer of endothelial cells lines the entire vascular system, and normality of both endothelial cell structure and functions are of great importance in the maintenance of vessel wall integrity (Desideri and Ferri, 2005; Furchgott and Zawadzki, 1980). In this regard, endothelial cells actively regulate vascular reactivity by responding to mechanical forces and neurohormonal mediators with the release of a variety of relaxing and contracting factors (Desideri and Ferri, 2005; Furchgott and Zawadzki, 1980; Palmer *et al.*, 1988). The most important endothelium-derived vasodilator is nitric oxide (NO), an endogenous gas that is

synthesized by NO synthase (NOS) starting from L-arginine. After diffusion from endothelial to vascular smooth muscle cells, NO increases intracellular cyclic guanosine monophosphate (GMP) concentrations and then leads to vascular relaxation (Desideri and Ferri, 2005; Palmer *et al*., 1988).

NO is released from endothelial cells in response to shear stress, acetylcholine, and other stimuli and can profoundly affect both function and structure of the underlying vascular smooth muscle cells (Desideri *et al*., 2003; Ferri *et al*., 1999). Indeed, continuous production of NO by constitutive NOS maintains the vasculature in a state of vasodilation, whereas its phasic generation by inducible NOS can acutely dilate an artery in response to either physiological or pathological stimuli (Desideri *et al*., 2003; Desideri and Ferri, 2005; Ferri *et al.*, 1999; Furchgott and Zawadzki, 1980). However, NO is a reactive nitrogen compound and can be rapidly transformed into peroxynitrite ($ONOO^-$) by superoxide anion ($O_2^-$). In addition, $O_2^-$ and other endogenous oxidants are able to favour NOS uncoupling (Deanfield *et al*., 2007; Desideri and Ferri, 2005; Furchgott and Zawadzki, 1980). In turn, uncoupled NOS induce $O_2^-$ generation and then further decreases NO bioavailability and increases $ONOO^-$ formation (Deanfield *et al*., 2007; Desideri *et al*., 2003; Desideri and Ferri, 2005). Finally, augmented $ONOO^-$concentration decreases tetrahydrobiopterin, a fundamental cofactor in NO generation/activity, thereby further reducing NO bioavailability (Deanfield *et al*., 2007). Thus, an increased oxidative stress may result in a complete derangement of the NO system, with decreased NO bioavailability and a paradoxical NOS-related increment in oxidant generation.

Because of the antiatherogenic, antithrombotic properties of NO (Deanfield *et al*., 2007; Ferri *et al*., 2007; Ferri *et al*., 2006) and the proatherogenic prothrombotic

actions of endogenous oxidants (Deanfield *et al*., 2007; Desideri and Ferri, 2005; Ferri *et al*., 2007), a decreased NO bioavailability with increased oxidative and nitrosative stress will result not only in impaired endothelium-dependent vasorelaxation and blood pressure regulation but also in the acceleration of atherogenesis and onset of acute atherotrombotic events (Deanfield *et al*., 2007; Ferri *et al*., 1999; Ferri *et al*., 2007; Ferri *et al*., 2006; Palmer *et al*., 1988)**.** Thus, improved NOS activity and NO bioavailability and decreased oxidant generation, particularly of $O_2^-$ and $ONOO^-$, are both expected to protect the vessel wall and favour blood pressure homeostasis (Deanfield *et al.*, 2007; Desideri *et al*., 2003; Desideri and Ferri, 2005; Ferri *et al*., 1999; Ferri *et al*., 2006; Palmer *et al*., 1988).

### 1.3.2 Mechanism of vasorelaxation effect of flavonoids

The up-regulatory effect of flavonoids on NO levels occurs through either activation of endothelium nitric oxide synthase (eNOS) or by removing $O_2^-$ and thereby inhibiting consumption of NO (Benito *et al*., 2002; Huk *et al.*, 1998; Pechanova *et al*., 2004). Other than increasing eNOS activity (Benito *et al*., 2002), flavonoids may additionally induce eNOS expression (Hung *et al*., 2004; Olszanecki *et al*., 2002). As eNOS is a calcium-dependent enzyme, elevation of intracellular $Ca^{2+}$ has been suggested as the mechanism of the endothelium-dependent NO -mediated vasorelaxation by flavonoids (Akhlaghi and Brandy 2009; Andriambeloson *et al*., 1998; Martin *et al.*, 2002; Stoclet *et al*., 1999) (Figure 1. 3). Flavonoids likely increase intracellular $Ca^{2+}$ by stimulating both $Ca^{2+}$ entry from the extracellular milieu and $Ca^{2+}$ release from intracellular $Ca^{2+}$ stores (Martin *et al.*, 2002). Surprisingly, the rise of $Ca^{2+}$ by flavonoids occurs as a result of increased production of $O_2^-$ as application of superoxide dismutase plus catalase attenuated the $Ca^{2+}$

elevation (Durate *et al*., 1993). These results suggest that the effect of flavonoids on NO levels can occur both through stimulating $O_2^-$ production inside endothelial cells (stimulating eNOS activity), and through scavenging $O_2^-$ in the interstitial fluid (preserving NO). $NO^{\cdot}$ is generally produced by eNOS attached to the endothelium plasma membrane and delivered to smooth muscle cells where it manifests its biological functions (Sudano *et al*., 2006). In smooth muscle cells, $NO^{\cdot}$ activates guanylate cyclase which synthesizes cyclic GMP (cGMP), an important mediator of vasodilation (Figure 1.3) cGMP acts by activating protein kinase G which affects a number of target proteins including those involved in $Ca^{2+}$ channels, decreasing cytosolic $Ca^{2+}$ through activating endoplasmic reticulum $Ca^{2+}$ uptake and inhibiting extracellular $Ca^{2+}$ entry. The eventual low intracellular $Ca^{2+}$ in smooth muscle cells mitigates cellular contractility and yields relaxation (Figure 1.3). Other possible mechanisms of flavonoid vasorelaxation are inhibition of phosphodiesterases (PDE) and lowering $Ca^{2+}$ in smooth muscle cells (Akhlaghi and Brandy 2009).

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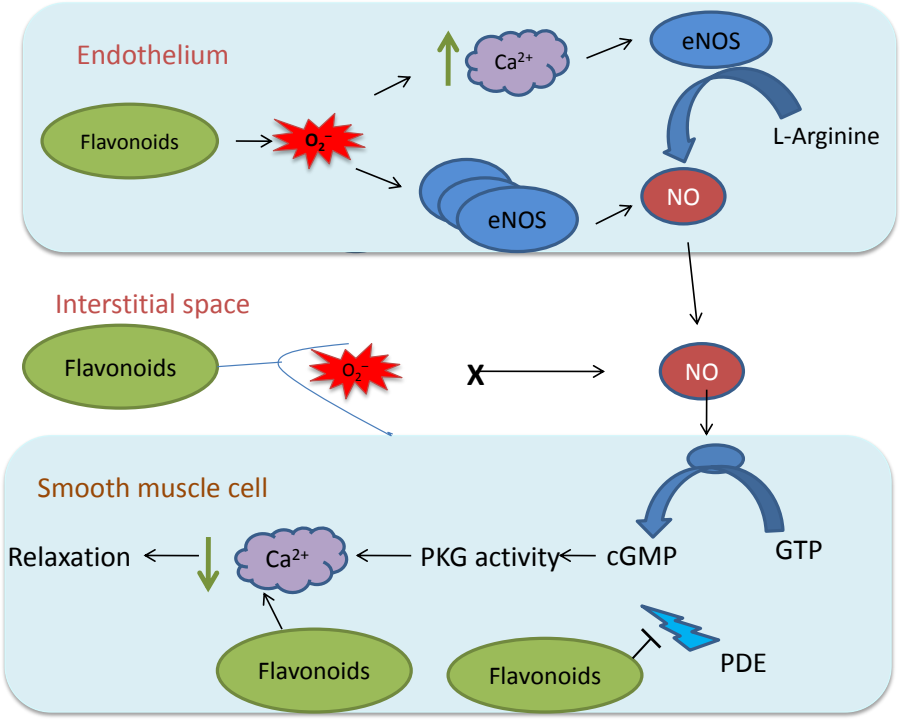



**Figure 1-3** Mechanism of vasorelaxant effects of flavonoids.
*Adapted from (Akhlaghi and Brandy 2009).*

Flavonoids may reduce vascular tone through stimulating $O_2^-$ production inside endothelial cells, and through scavenging $O_2^-$ in the interstitial fluid. In smooth muscle cells, by reducing cystolic $Ca^{+2}$.

**Abbreviation:** GTP: Guanosine-5'-triphosphate; cGMP: cyclic guanosine monophosphate; PDE: phosphodiesterases; PKG: protein kinase G.

### 1.3.3 Mechanism of anti-inflammatory effect of flavonoids

There have been several proposed cellular action mechanisms explaining *in vivo* anti-inflammatory activity of flavonoids. They possess antioxidative and radical scavenging activities. Certain flavonoids modulate the enzyme activities of arachidonic acid metabolizing enzymes such as phospholipase $A_2$, cyclooxygenase, and lipoxygenase and the nitric oxide producing enzyme, nitric oxide synthase. An inhibition of these enzymes by flavonoids reduces the production of AA, prostaglandins (PG), leukotrienes (LT), and NO, crucial mediators of inflammation. Thus, the inhibition of these enzymes exerted by flavonoids is definitely one of the important cellular mechanisms of anti-inflammation. Furthermore, in recent years, many lines of evidence support the idea that certain flavonoids are the modulators of gene expression, especially of proinflammatory gene expression, thus leading to the attenuation of the inflammatory response. At present, it is not known to what extent this proinflammatory gene expression contributes to the inflammatory response. However, it is evident that flavonoids show anti-inflammatory activity, at least in part, by the suppression of this proinflammatory gene expression. Flavonoids can also inhibit production of pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and interferon-γ (Marfella *et al*., 2006). Moreover, flavonoids and other polyphenols have shown inhibitory effects on expression of adhesion molecules such as ICAM-1, VCAM-1, and E-selectin (González-Gallego *et al*., 2007; Kris-Etherton *et al*., 2004; Manthey, 2000).

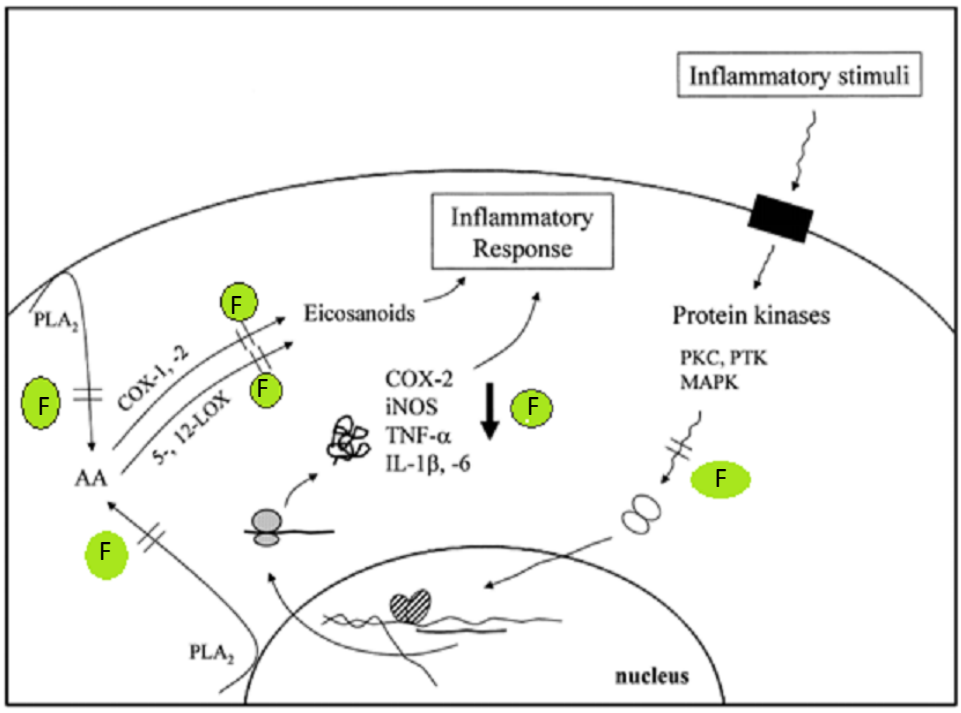



**Figure 1.4** The mechanism of anti-inflammatory effect of flavonoids.
*Adapted from (Kim et al; 2004).*

Flavonoids show anti-inflammatory activity by the suppression of these proinflammatory gene expressions and inhibit production of pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and interferon-γ, as well as chemotactic agents . Moreover, flavonoids have shown inhibitory effects on expression of adhesion molecules such as ICAM-1, VCAM-1, and E-selectin.

### 1.3.4 Quercetin

Quercetin is the major dietary flavonol found in abundance in onions, apples, broccoli, and berries (Hertog *et al.*, 1993). The daily intake of quercetin with a typical Western diet was estimated to range between 0 and 30 mg, with a median of 10 mg (Hertog *et al*., 1995). Consumption of flavonoids in general and quercetin in particular may be associated with a decreased risk of coronary heart disease and other degenerative diseases (Erdman *et al*., 2007). Chemically, quercetin is a 3, 3', 4', 5, 7-pentahydroxyflavone (Figure 1.5).

Potential health effects of bioactive compounds depend on their bioavailability following oral administration. In plant-derived foods, quercetin is mainly present as various glycosides. It has been repeatedly shown that the sugar moiety is a major determinant of the rate of intestinal absorption of quercetin (Lesser and Wolffram, 2007). In all species investigated so far, including humans, pigs, and rats, quercetin and its methylated derivatives with an intact flavonol structure (isorhamnetin, tamarixetin) are not present as free aglyca but only in the conjugated form (mainly glucuronide and sulfate conjugates). Data on bioavailability of quercetin aglycone or quercetin glycosides vary substantially between studies, according to the methods applied for quercetin measurement, and in different species (Lesser and Wolffram, 2007). In humans, absorption after oral supplementation ranged from 0 to over 50% of the administered dose (Hollman *et al.*, 1995; Hollman *et al*., 1996).

As to the biological activities of quercetin, *in vitro* studies indicate antioxidant, antiinflammatory, antithrombotic, anticarcinogenic, and vasodilatory actions

(Erdman *et al*., 2007). However, quercetin intervention trials in humans have so far shown inconclusive and even conflicting results (Erdman *et al.*, 2007; Williamson and Manach, 2005). Quercetin had favorable effects on a variety of antioxidant biomarkers, such as antioxidant enzymes, plasma antioxidant capacity, resistance to LDL oxidation, reduced lymphocyte DNA damage, and reduced urinary 8-OH-2'-deoxyguanosine. However, other studies did not support these data (Edwards *et al*., 2007; Kim *et al*., 2003). The majority of human studies used rather high doses of quercetin (and mostly only 1 concentration) and/or quercetin was not administered as a pure compound but via quercetin-rich foods/meals (e.g. white onions, onion soup) containing further phytochemicals, which may have influenced the results.

Anti-inflammatory effects have also been found *in vivo*, when quercetin was reported to suppress experimentally induced arthritis in rats (Mamani-Matsuda *et al*., 2006), and prevented gastric ulcer formation caused by the oral administration of absolute ethanol (Mizui *et al*., 1987). Mechanistically, It was reported that quercetin inhibited LPS-induced STAT-1 and NF-κB activations, and iNOS expression in activated macrophages (Hamalainen *et al*., 2007). Additionally, Chen et al have reported that quercetin inhibits IFNγ-induced STAT-1 activation in mouse BV-2 microglia (Chen *et al*., 2005). Various flavonoids, including quercetin, stimulate endothelial cells to release NO following elevation of intracellular calcium level [$Ca^{++}$] (Martin *et al*., 2002; Stoclet *et al*., 1999a) . Indeed, the infusion of flavonoids into the coronary circulation of isolated guinea pig hearts caused immediate vasodilatation. These responses were strongly inhibited by L-NAME, non-selective NOS inihibitor, which also abolished the response to bradykinin, a standard NO-dependent vasodilator (Chlopicki *et al*.,

1999). Interestingly, in contrast with the influence on iNOS induction, quercetin appeared to be a more potent endothelial NOS simulator than kaempferol and apigenin. Apparently, flavonoids stimulate endothelial eNOS and inhibit iNOS induction in LPS-treated macrophages by different mechanisms (Olszanecki *et al.*, 2002).

Fitzpatrick and colleagues demonstrated that wine and grape products such as quercetin and tannic acid cause endothelium-dependent relaxation in rat thoracic aorta (Fitzpatrick *et al.*, 1993), and there have been several subsequent reports of the pharmacological actions of flavonoids on vascular smooth muscle tone (Chen and Pace-Asciak, 1996; Durate *et al.*, 1993; Flesch *et al.*, 1998).

Numerous studies in the rat and human have demonstrated that dietary quercetin is partly absorbed into the body and accumulates as its glucouronides and sulphate conjugates in the blood. The amount of quercetin that remains biologically available may not be of sufficient concentration, theoretically, to explain the beneficial effects seen with the Mediterranean diet. However, Tribolo *et al* (2007) found that both quercetin and its metabolites (glucouronidated, sulphated and methylated) at physiological concentrations (2 µmol/L and 10 µmol/L) can inhibit the LPS-induced expression of cell adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1) and intercellular cell adhesion molecule-1 (ICAM-1) on human umbilical vein endothelial cells (Tribolo *et al.*, 2008b). More recently, Lodi and co-worker have established that quercetin and quercetin-3′-sulfate or quercetin-3-glucuronide inhibited NADPH oxidase-derived $O_2^-$ release and prevented the endothelial dysfunction induced by incubation with ET-1. These

data indicate that the conjugated metabolites could be responsible for the *in vivo* protective activity of quercetin on endothelial dysfunction (Lodi *et al*., 2009).

Myricetin (3,5,7,3',4',5'-hexahydroxyfavone), another naturally occurring flavonoid, differs from quercetin only by the addition of a hydroxyl at the 5`-OH of the phenyl moiety (Figure 1.5). It has been shown to exhibit a biphasic contractile response in pre-contracted rat thoracic aorta (Berger *et al*., 1992; Herrera *et al*., 1996). At low concentrations (<50 µM), it potentiates the responses to different contractile agents such as noradrenaline, high KCl and phorbol 12-myristate 13-acetate in rat aortic rings, whereas at higher concentrations, it exerts a vasorelaxant effect on vessels precontracted with these agents (Herrera *et al*., 1996). The potentiating effect of myricetin has been reported in a number of arteries from different species such as the rat tail and femoral arteries (Berger *et al*., 1992) or the rabbit pulmonary artery (Russell and Rohrbach, 1989). The mechanisms involved in the vasoconstrictor response are through an activation of $PGH_2$-$TXA_2$ receptors on vascular smooth muscle by the $TXA_2$ released from endothelium by a $Ca^{2+}$-sensitive activation of the arachidonic acid cascade (Jimenez *et al*., 1999).

## 1.3.5 Catechins

The catechins are polyphenolic compounds, and are found in green tea, chocolate, grapes and apples. Catechins can be found in common foodstuffs as well as in herbal remedies. A prospective cohort study (Zutphen Elderly study) provides evidence for a primary protective effect of flavanols (Buijsse *et al.*, 2006; Hertog *et al.*, 1993; Streppel *et al.*, 2009). Catechins belong to the flavan-3-ol class of flavonoids. The most abundant of the polyphenolic compounds in green tea is epigallocatechin gallate (EGCG), with other catechins such as epicatechin (EC), epigallocatechin (EGC) and epicatechin gallate (ECG) also present (Figure 1.6). The catechins have been reported to exhibit several beneficial health effects by acting as antioxidant (Bors and Michel, 1999), anticarcinogen (Guruvayoorappan and Kuttan, 2008), cardiopreventive (Heiss *et al.*, 2003), anti-microbial, anti-viral, (Selway, 1986) and neuro-protective agents (Bastianetto *et al.*, 2006 ).

The bioavailability of tea catechins appears to be relatively low. When healthy volunteers were given a single serving of 4.5 g of green tea solids dissolved in 500 mL of water, peak plasma concentrations of individual catechins (conjugated and unconjugated) were <2 µmol/L (Yang *et al.*, 1998a). Average peak plasma catechin concentrations (conjugated and unconjugated) in healthy volunteers given a single dose of 1.5 mmol of pure EGC, ECG or EGCG were 5.0, 3.1 and 1.3µmol/L, respectively (Van Amelsvoort *et al.*, 2001). These values represent peak plasma levels after high doses of green tea or pure catechins. Average plasma catechin concentrations are likely to be considerably lower. Because theaflavins and thearubigins are difficult to detect in blood or urine, there is little

information regarding the biotransformation or bioavailability of black tea polyphenols in humans or animals.

Upon ingestion, the catechins are rapidly and extensively metabolized in the intestines, liver and kidneys. The major biotransformation reactions of tea catechins are glucuronidation, sulfation and methylation (Bell *et al.*, 2000; Kim *et al.*, 2000); indeed, some of the variability in reported plasma concentrations may be due to the difficulties in measuring chemically modified as well as unaltered catechins.

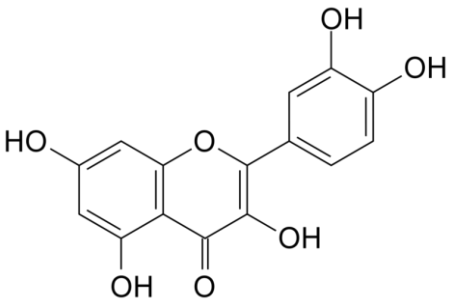
Many *in vitro* studies on catechins report mechanisms consistent with protection against degenerative diseases (Adcocks *et al.*, 2002; Huang *et al.*, 1999; Nakagawa *et al.*, 2002; Nie *et al.*, 2002). Many of these studies however used high concentrations of catechin and thus do not reflect typical catechin concentrations found in animal or human plasma. It is difficult to extrapolate these results to *in vivo* situations. Moreover, non galloylated catechins are present in plasma as conjugated forms (Kim *et al.*, 2000; Lee *et al.*, 2002; Piskula and Terao, 1998), except for EGCG and ECG, which are significantly unconjugated (Ullmann *et al.*, 2003). However, because of the lack of conjugated forms as standards or test compounds, it is not possible to test the in vitro biological effects of the conjugates.

They have been found to have anti-inflammatory properties, which may be due to their ability to inhibit tumor necrosis factor (TNF) synthesis (Yang *et al.*, 1998b), possibly by the inhibition of kinase(s) in signaling cascades, leading to activation

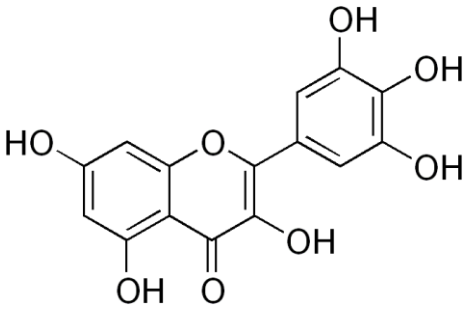
of certain transcription factors (Liang *et al*., 1999; Lin *et al*., 1999; Mukhtar and Ahmad, 1999).

The ability of the catechins to activate endothelial NOS and then improve NO bioavailability is likely to represent the primary mechanism underlying the blood pressure reduction observed in different human studies (Engler *et al*., 2004; Soares *et al*., 2002; Taubert *et al*., 2007), and the same mechanism has been strongly implicated as the main process responsible for the blood pressure-lowering effects exerted by either short-term (Engler *et al*., 2004) or long-term (Taubert *et al*., 2007) cocoa administration.

Oxidized LDL is a well recognized and the most studied risk factor for CVD. Many studies reported that catechins can inhibit the oxidation of LDL both *in vitro* and in animal studies (Ishikawa *et al*., 1997; Stangl *et al*., 2006; Sumpio *et al*., 2006; Tijburg *et al*., 1997; Yamanaka *et al*., 1997). It was also shown that EGCG alone has a lipoprotein bound antioxidant activity that is greater than that of tocopherol (Vinson *et al*., 1995). The addition of 2 to 20 μg/ml of EC, ECG, EGC, or EGCG to macrophages conserved the α-tocopherol content of LDL and delayed the onset of lipid peroxidation (Zhu *et al*., 1999).

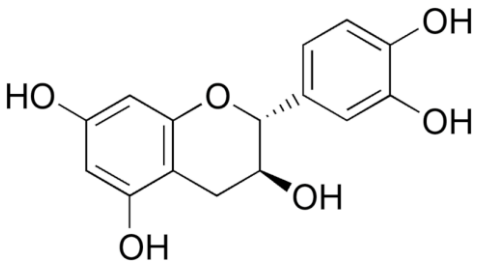



Quercetin

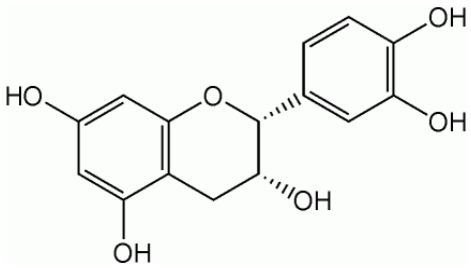



Myricetin

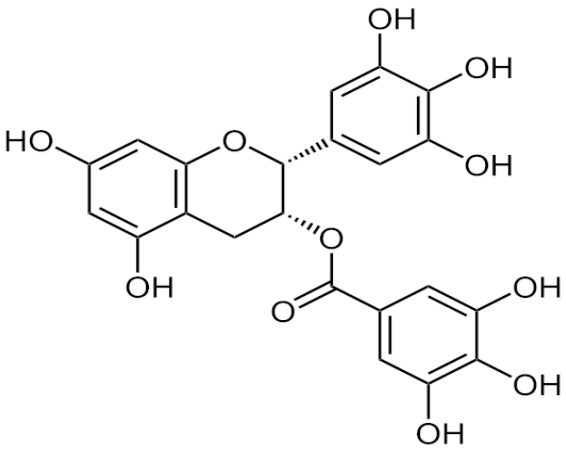
**Figure 1-5** Chemical structure of flavonols (Boots *et al.*, 2008).




- (+) Catechin




- (-) Epicatechin




Epigallocatechin gallate

**Figure 1-6** Chemical structure of flavanols (Boots *et al.*, 2008).

## 1.4 Statins

Statins (3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors) lower plasma cholesterol, are widely used in patients with hyperlipidemia and coronary artery disease, and are beneficial in the primary and secondary prevention of cardiovascular disease. Statins were shown to reduce cardiovascular morbidity and mortality in a number of large clinical trials, such as the Scandinavian Simvastatin Survival Study (4S) (Scandinavian Simvastatin Survival Study (4S) Group, 1994), the Cholesterol and Recurrent Events (CARE) (Sacks et al., 1996), the Long-term Intervention with Pravastatin in Ischemia Disease (LIPID) (The Long-Term Intervention with Pravastatin in Ischaemic Disease, 1998), the West of Scotland Coronary Prevention Study (WOSCOPS) (Shepherd *et al.*, 1995 ), the Air Force/Texas Coronary Atherosclerosis Prevention Study (AFCAPS/TexCAPS) (Downs *et al.*, 1998) and the Heart Protection Study (HPS) (Heart Protection Study Collaborative Group MRC/BHF Heart Protection, 2002).

There is increasing interest in the possibility that statin therapy may be protective in the condition of sepsis (Almog, 2003). Thus, a retrospective review of patients admitted to intensive care with bacteraemia showed a significant reduction in mortality in those receiving statin therapy at the time of admission (Liappis *et al.*, 2001). Additionally, a small prospective study of patients with acute bacterial infection found a reduced rate of development of severe sepsis in patients treated with statins (Almog *et al.*, 2004). Animal studies have shown beneficial effects on mortality of statin treatment given 12h and 1h before lipopolysaccharide (LPS) (Ando *et al.*, 2000), or 18h and 3h before caecal ligation and puncture (Merx *et*

*al*., 2004), although little is known of the underlying mechanisms for these beneficial effects.

Statins are the principal therapy for more than 25 million people at risk of cardiovascular disease worldwide. Statins lower blood cholesterol concentration and reduce the relative risk of coronary events by about 30% in both primary and secondary prevention. Statins are now widely prescribed to patients with ischemic heart disease, and it is increasingly clear that they have favourable effects unrelated to lipid lowering (Werner *et al*., 2002). For example, pravastatin has been claimed to reduce left-ventricular (LV) mass in hypertensive patients (Su *et al*., 2000) and in the 4S study patients receiving long-term simvastatin treatment had a reduced incidence of heart failure (Kjekshus *et al*., 1997). Statins have also recently been shown to prevent stroke (Heart Protection Study Collaborative Group, 2002), and the mechanism is likely to be multifactorial. Finally, in another study, statins were observed to reduce the incidence of atrial fibrillation (AF) in patients with coronary artery disease (Young-Xu *et al*., 2003).

Lovastatin is a fungal metabolite, of which pravastatin and simvastatin are semi-synthetic derivatives, whereas fluvastatin, atorvastatin and rosuvastatin are entirely synthetic (Hamelin and Turgeon, 1998). Lovastatin and simvastatin are of the lactone pro-drug form, whereas atorvastatin, fluvastatin and pravastatin are presented in the active (acid) form (Ishigami *et al*., 2001). The structures can be broadly divided into three parts (Gaw and Packard, 2000) an analogue of the target enzyme substrate, HMG-CoA; a complex hydrophobic ring structure that is covalently linked to the substrate analogue and is involved in binding of the statin to the reductase enzyme; side groups on the rings that define the solubility

properties of the drugs and therefore many of their pharmacokinetic properties (Figure 1.7). Lovastatin and simvastatin are relatively lipophilic compounds, while pravastatin and rosuvastatin are more hydrophilic as a result of a polar hydroxyl group and methane sulphonamide group, respectively (McTavish and Sorkin, 1991).

All statins are relatively hepatoselective with respect to inhibition of HMG-CoA reductase, an important property given that the majority of endogenous cholesterol production occurs in the liver. The mechanisms contributing to this hepatoselective effect are governed by the solubility profile of the statin. For lipophilic statins, passive diffusion through hepatocyte cell membranes is primarily responsible for efficient first pass uptake, while for hydrophilic statins extensive carrier-mediated uptake is the major mechanism (Hamelin and Turgeoun, 1998; Nezasa *et al*., 2003). While lipophilicity results in efficient hepatic shunting, the same property will result in ready passage through nonhepatic cell membranes. All statins are absorbed rapidly following administration, reaching peak plasma concentration ($T_{max}$) within 4 h. Simvastatin and lovastatin are mainly metabolized by cytochrome P450 (CYP) 3A, fluvastatin is metabolized by CYP2C9, and pravastatin is excreted largely unchanged.

There is a growing awareness that statins might have important anti-inflammatory effects, in addition to their lipid-lowering effects (Jialal *et al*., 2001; Yoshida *et al*., 2001). For example, cerivastatin inhibited firm adhesion of U-937 cells to IL-1-activated human umbilical vein endothelial cells while down-regulating surface expression of CD11a, CD18, and VLA4 and inhibiting actin polymerization in the U-937 cells (Yoshida *et al*., 2001). Rice and co-workers have demonstrated that

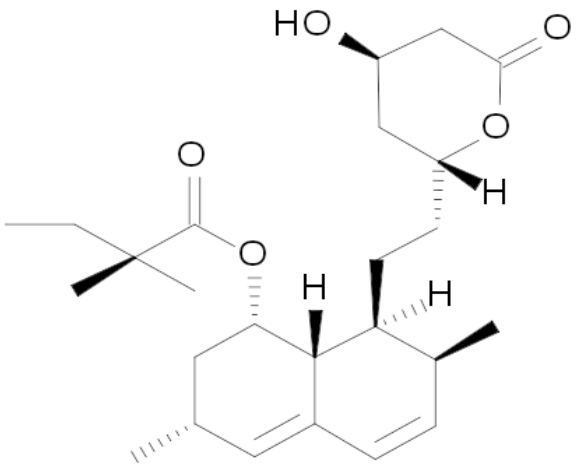
clinically relevant levels of endotoxin induce proinflammatory activation of intact human blood vessels. Endotoxin-induced release of IL-8 and induction of monocyte binding are blocked by atorvastatin, suggesting a beneficial effect of statins unrelated to cholesterol lowering. The mechanism for this inhibition of monocyte binding by atorvastatin appears to be reduced synthesis of mevalonate. Reduction in mevalonate formation could interrupt endotoxin signaling at a number of steps, leading to inhibition or down-regulation of chemoattractant molecules such as intercellular adhesion molecule-1 and vascular cell adhesion molecule; cytokines such as MCP-1 or IL-8; and/or production of reactive oxygen species (Rice *et al.,* 2003).

Several groups have now investigated the enticing possibility that these various anti-inflammatory and immune-modulatory effects might have utility in disease states beyond atherogenesis. Sparrow and colleagues demonstrated that simvastatin had a comparable anti-inflammatory effect to that of indomethacin in the carrageenan-induced foot pad oedema inflammatory model (Sparrow *et al.*, 2001). Statins have been shown to inhibit the production of TNF-α and inducible nitric oxide synthase by microglia and astrocytes (Pahan *et al.*, 1997). Statin treatment results in inhibition of NF-κB activity and subsequent reduction of the pro- inflammatory cytokines tumor necrosis factor (TNF)- and interleukin (IL)-6 (Ortego *et al.*, 1999; Weis *et al*., 2001; Zelvyte *et al*., 2002). Furthermore, statins inhibit lipopolysaccharide (LPS)-mediated activation of human peripheral mononuclear cells and endothelial cells (Neurauter *et al.*, 2003; Rice *et al*., 2003; Zeuke *et al*., 2002). Methe and colleagues demonstrated that statins exert direct regulatory effects on TLR4 expression in human monocytes that influences cellular activation. Statins reduce TLR4 surface expression on CD14 monocytes

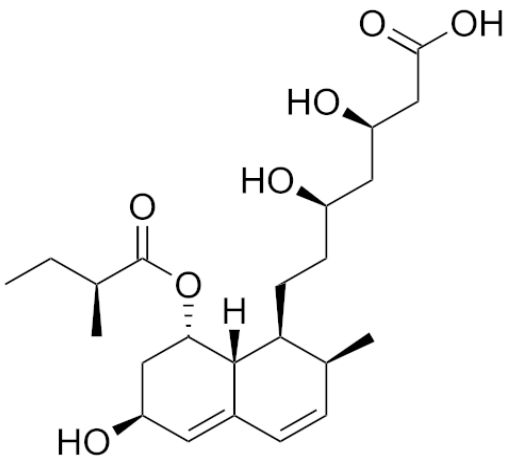
*in vivo* and *ex vivo* in a dose-dependent fashion, causing downregulation of IRAK-1 kinase activity and reduced expression of proinflammatory cytokines and B7-1 (Methe *et al.*, 2005).

### 1.4.1 Mechanism

Statin-induced inhibition of the enzyme HMG-CoA reductase leads to a reduction in the formation of mevalonate. As cholesterol and isoprenylated proteins are both products of mavelonate, HMG-CoA reductase inhibition can reduce levels of both cholesterol and isoprenylated proteins (e.g., Ras, Rho, Rac, Rap). Reduction of hepatic cholesterol synthesis activates SREBP2 (sterol response element binding protein 2), causing an increase in the transcription of the gene coding for the hepatic LDL receptor. Elevated numbers of LDL receptors lead to an increase in the clearance of circulating LDLs, the major mechanism by which statins reduce plasma LDL-C. In addition, reduced availability of hepatic free cholesterol and/or cholesteryl ester for incorporation into very low density lipoproteins (VLDLs) may reduce the production rate of these lipoproteins. On the other hand, inhibition of mevalonate synthesis also causes a decrease in the formation of other intermediates in the cholesterol synthetic pathway, such as farnesyl pyrophosphate, geranyl pyrophosphate, and gernyl geranylpyrophosphate. These isoprenoid-like molecules are involved in posttranslational modification of proteins, termed isoprenylation. Genarylgeneranylation is of particular interest, as it affects G protein–dependent cellular activation and numerous signaling pathways. Reduction of protein isoprenylation is the molecular mechanism underlying most lipid-lowering-independent effects of statins, collectively termed pleiotropic effects (Figure 1.8) (Alegret and Silvestre, 2006).




**Simvastatin**




**Pravastatin**

**Figure 1-7** Chemical structure of statins.

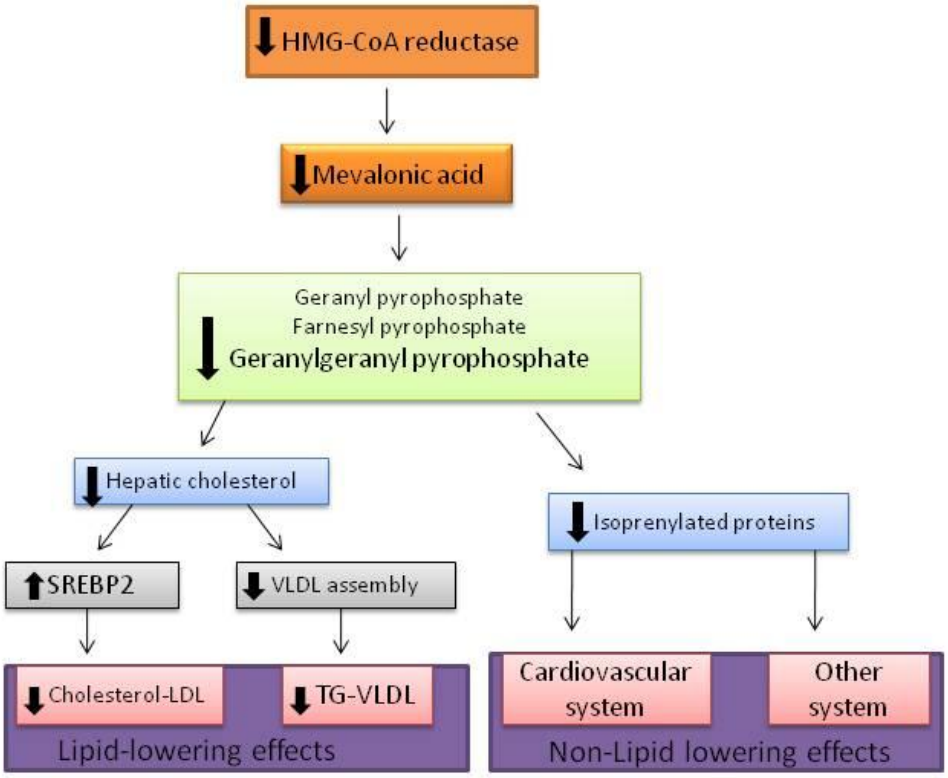



**Figure 1-8** Molecular mechanisms responsible for the lipid lowering and non-lipid lowering effects of statins. *Adapted from (Alegret and Silvestre, 2006).*

## 1.5 Aim of study

Although statins have an established major role in preventing the development of ischaemic heart disease and treating atherosclerosis, the widespread use is not without problems. The emerging evidence that dietary flavonoids may be of benefit in preventing ischaemic heart disease raises the question whether appropriate use of foods rich in quercetin or catechin could be used prior to the need for medical intervention. Basically, can we give specific dietary advice to the growing elderly population to promote better health?

To this end, I propose to

1. Optimize the conditions to develop suitable vascular models of inflammation to evaluate the effects of dietary flavonoids and statins.
2. To investigate the effect of quercetin, and two of the key metabolites in man, quercetin 3′-sulphate and quercetin 3-glucuronide, against LPS-induced changes in contractions of the porcine isolated coronary artery and the generation of nitric oxide.
3. To investigate the effects of flavanol type flavonoids (catechins, epicatechin and epigallocatechin gallate) on the vascular reactivity and the generation of nitric oxide of porcine isolated coronary artery exposed to LPS *in vitro*.
4. To examine the effect of simvastatin and pravastatin on LPS-induced changes in vascular responses and to investigate the combined effects of low concentrations of epicatechin and simvastatin on LPS-induced inflammatory changes on porcine isolated coronary artery function.

# Chapter 2

# Optimization of the *in vitro* assessment of LPS on vascular smooth muscle

## 2.1 Introduction

In this laboratory, when the porcine coronary artery is used in the pharmacological studies, it is usually stored in Krebs-Henseleit solution overnight at 4°C prior to performing the contractile experiment the following morning. However, for *in vivo* vasculature studies related to inflammatory process with LPS or other proinflammatory factors, vessels are generally stored at a physiological temperature, i.e. 37°C, over several hours. Therefore, before this work can begin it is essential that optimizing the conditions required for maintaining the properties of the blood vessels during prolonged storage (one or two days) at 37$^{o}$C is established.

The induction of inflammatory changes in cultured cells and isolated blood vessels often requires prolonged exposure to LPS (1-2 days). Cells are usually cultured in a chemically defined medium, e.g. Dulbecco's Modified Eagle Medium (DMEM), and the assays conducted using this medium (Schildknecht, 2004). In the case of isolated blood vessels, however, contractile experiments are usually conducted in Krebs-Henseleit solution (Bishop-Bailey *et al.*, 1997; O'Brien *et al.*, 2001). Previous work in this laboratory compared the effect of incubation of the porcine coronary artery overnight in previously gassed Krebs-Henseleit solution and DMEM (37$^{o}$C and in an atmosphere of 95%$O_2$, 5%$CO_2$) and surprisingly found little difference in the responsiveness of the vessel to KCl, U46619 and the endothelium-dependent relaxant substance P (Wei, 2006) . However, in further experiments Wei (2006) noted a 50% decline in the magnitude of the contractions to both KCl and U46619 when the period of incubation in Krebs-Henseleit solution was extended to 42 hours.

As reported by Rees (2007), the current use of phosphate/bicarbonate- buffered saline was instigated in 1932 by Krebs & Henseleit, for studies on isolated homogenates of mitochondria, i.e. intracellular organelles, from pigeon liver. During the 1950s, this medium was further developed by Krebs in his classical study on the analysis of oxygen consumption in tissue slices from different organs in a variety of animal species. Krebs later acknowledged that substrate depletion in isolated tissue/organ preparations over time was a consideration that had not been addressed in the composition of previous physiological solutions. Phosphate/bicarbonate buffers have been in use for sixty years and remain the most popular perfusion/preservation solutions for experimental studies on mammalian and human tissues/organs.

Although there are minor differences in the composition of the buffer between labs, the majority of investigators use a solution comprising NaCl (118.4 mM), KCl (4.7 mM), $MgSO_4.7H_2O$ (1.2 mM), $CaCl_2.2H_2O$ (2.5 mM), $NaHCO_3$ (24.9 mM) and $KH_2PO_4$ (1.2 mM). In addition 2g/L of glucose is added as an energy source for cell maintenance (Rees, 2007).

During the past 3 years a non-phosphate based solution (AQIX RS-I) has been marketed for the preservation and transportation of organ under normothermic conditions. Historically, large organs have been transported between transplantation centres in a variety of different preservation solutions generally maintained at low temperatures to reduce metabolic activity. However, problems arise from the need to subsequently reperfuse the organ and slowly elevate the temperature back to body temperature, which may give rise to generation of damaging free-radicals.

AQIX RS-I mammalian physiological solution has been developed over the last 20 years and designed with the aim of being a universal organ solution based upon the ' free' serum levels of the ionic species, while providing a suitable nutritive, buffered environment at room temperature. The concentration of ionic species in AQIX RS-I solution acknowledges the activity coefficients of each ionic species and not simply their total serum concentrations. Thus, the concentration of calcium ions, for example, is half (1.25 mM) that of Krebs-Henseleit solution because 50% of calcium in serum is protein-bound. A major distinguishing and novel feature of AQIX RS-I solution in comparison to conventional perfusion saline is the absence of inorganic phosphate which, for the past 60 years, has been used as the buffer vehicle in conventional, perfusion solutions. AQIX RS-I is osmotic to human serum (ca. 290 mOsmoles/L) and does not appear to necessitate the inclusion of plasma expanders as demonstrated by the fact that only minor changes (ca. 8%) in hydration occur during long term (i.e. 4-52 h) hypothermic perfusion of the isolated rat heart (Rees, 2007).

The main components of AQIX RS-1 solution are NaCl (110 mM), KCl (5.00 mM), $CaCl_2$ (1.25 mM) and $MgCl_2$ (0.45 mM). It also contains $NaHCO_3$ (25.0 mM), BES (5.00 mM), D-glucose (10.00 mM), glycerol (0.11 mM), glutamate (0.3 mM), glutamine (0.4 mM), aspartate (0.02 mM), carnitine (0.05 mM) and choline chloride (0.01 mM). In addition to thiamine pyrophosphate TPP (40.00 nM) and insulin (28.00 mIU) (Rees, 2007).

Initial studies using AQIX RS-I was focused on isolated, small animal tissues and organ bath system using oxygenated AQIX RS-I at 15-37°C. Such studies demonstrated functional viability of rat jejunum for 9 days, rat colon for 5 days, and

rabbit uterus for 7 days (Rees, 1989a). Interestingly, a direct comparison of the effect of Krebs Henseleit solution and AQIX RS-I on contractile responses of human colon revealed quantitative and qualitative differences in the magnitude of responses to carbachol, suggesting that AQIX RS-I may exert different effects on the electrical properties of electrically excitable tissues.

To date there have been no studies regarding the effect of AQIX RS-I on the contractile properties of vascular smooth muscle or on the effect of prolonged incubation in a static, non-perifused state. Therefore, I will compare vascular responses of the porcine coronary artery in experiments undertaken in either Krebs-Henseleit solution or AQIX RS-I. Then, I will then examine how the responses are affected by the medium used for storage, the temperature of incubation and the duration of incubation.

Previous studies have shown that the response of porcine isolated coronary artery to KCl and U46619 is significantly impaired following overnight exposure to a high concentration of lipopolysaccharides arising from the induction of nitric oxide synthase (Wei, 2006). To date most studies that have employed LPS to alter vascular responses *in vitro* have tended to used high concentrations of LPS a concentraction of 10-100 microgram per ml (Muller-Scheinitzer *et al*., 2004 ; O`Brien and Abraham, 2004). However, some researchers have found that only a low concentration of LPS is required to induce cytokine production or expression of adherence molecules in human coronary artery endothelial cells (Patton, 2006; Yang, 2005).

Previous discoveries have indicated that soluble serum components such as lipopolysaccharide binding protein (LBP) and CD14 influence the biological potency of bacterial LPS, and these findings have significantly advanced the understanding of the pathogenesis of gram negative bacterial diseases (Kielian and Blecha, 1995). Wei 2006 demonstrated that exposure of isolated porcine coronary artery to high concentrations of LPS (100μg/ml) for 42 hour in the presence of serum, potentiated the effects of LPS. It is interesting to examine what the responsiveness of porcine isolated coronary artery is following 42 hours (approximately two nights) exposure to low concentrations of LPS, and whether the serum factors enhance the responses of porcine coronary artery to LPS in terms of contractile function. However, as a prelude to the above studies it is important to optimize the conditions required for investigating the effects of the anti-inflammatory agents on LPS-induced pro-inflammatory changes in isolated blood vessels.

Thus, there are two major aims for this chapter. First, to undertake a direct comparison of the influence of Krebs-Henseleit solution and AQIX-RS-I on contractile responses of the porcine coronary artery. Second, to examine the influence of serum factors on the inhibitory effect of LPS on the coronary artery.

## 2.2 Materials and Methods

### 2.2.1 The influence of incubation temperature on the responsiveness of the porcine coronary artery

Porcine hearts were obtained from a local abattoir and transported to the laboratory in ice-cold Krebs-Henseleit (K-H) solution within one hour of death. The anterior descending branch of the porcine coronary artery was dissected and cleaned of fat and connective tissues. The arteries were then divided into 5mm long segments and placed in a sterile vial containing 2ml K-H solution with 2% ficoll and a combination of 60µg/ml benzyl penicillin and 20µg/ml streptomycin sulphate. The inclusion of the ficoll in the bathing medium was to prevent swelling of the tissue during storage. Prior to sealing the vials, the solution was gassed with 95% $O_2$ and 5% $CO_2$ for 5 minutes and then incubated overnight at either 4ºC or 37ºC for a minimum of 16 hours.

On the following day, segments were taken out of the incubation solution and prepared for isometric tension recording. Two stainless steel wire supports (0.4mm thick) were placed into the lumen of segments, one of which was linked by a cotton thread to a glass force-displacement transducer (Grass Model FT03); the other one was linked to a glass support. The segments were then placed into an organ bath filled with K-H solution and gassed with 95% $O_2$ and 5%$CO_2$. The temperature of the organ bath was maintained at 37ºC by a circulating water heater. The transducers were connected to an AD Instruments Quad Bridge pre-amplifier unit coupled to a Maclab 4e unit running Chart 3.4.5 and the results displayed on a Macintosh LCII computer.

Following 40 minutes equilibration in K-H solution, an initial resting tension of 100 mN was slowly applied to each segment. At the end of a further 40 min equilibration period, the resting tension had declined to approximately 40-60 mN. Three responses to 60mM KCl were elicited in each preparation until reproducible responses were obtained. Following washout, each preparation was exposed to cumulative concentrations of either KCl (6-60mM) or U46619 (1-200nM).

### 2.2.2 The influence of incubation medium on the responsiveness of porcine coronary artery

In order to define the influence of the incubation medium *per se*, responsiveness of porcine coronary artery following incubation overnight either in K-H solution or AQIX RS-I was examined. Porcine hearts were obtained from a local abattoir and transported to the laboratory in ice-cold AQIX RS-I solution within one hour. The anterior branches of the coronary artery were dissected from the hearts. The dissected segments were separately incubated either in the K-H solution (2ml) or in AQIX RS-I including an antibiotic mixture (60µg/ml benzyl penicillin and 20µg/ml streptomycin sulphate) and were thoroughly gassed before incubation. Samples were then stored either at 4ºC or 37ºC.

Following overnight exposure, the segments were prepared for the isometric tension recording test. Following 40 minutes equilibration either in K-H solution or AQIX RS-I, an initial resting tension of 100mN was slowly applied to each segment. At the end of a further 40 min equilibration period, the resting tension had declined to approximately 40-60mN. Three responses to 60mM KCl were

elicited in each preparation until reproducible responses were obtained. Following washout, each preparation was exposed to cumulative concentrations of either KCl (6-60mM) or U46619 (1-200nM). Then the preparations were exposed to substance P (10nM) and bradykinin (100nM), to assess the integrity of the vascular endothelium.

In another set of experiments, following reproducible responses to 60mM KCl, U46619 was added into the organ bath to elicit a sub- maximal response equivalent to approximately 60% of the response to 60mM KCl. After the tension was stable, the segments were then relaxed by cumulative concentrations of either sodium nitro- prusside (SNP, 1nM to 10μM) or isoprenaline (1nM to 1μM) to evaluate the response of the vessel to dilator agents.

### 2.2.3 The influence of incubation medium and period on the responsiveness of porcine coronary artery

The anterior branches of coronary arteries were dissected from pig hearts which were taken from a local abattoir in ice-cold K-H solution within 1 hour. The dissected porcine coronary artery segments were separately incubated in the K-H solution (2ml) or AQIX RS-I including an antibiotic mixture (60μg/ml benzyl penicillin and 20μg/ml streptomycin sulphate) and were thoroughly gassed before incubation. Samples were then stored either overnight or for two days at 37ºC. The medium was changed after one night incubation.

Following overnight incubation, the segments were prepared for the isometric tension recording test. Following 40 minutes equilibration either in K-H solution

or in AQIX RS-I, an initial resting tension of 100mN was slowly applied to each segment. At the end of a further 40 min equilibration period, the resting tension had declined to approximately 40-60 mN. Three responses to 60mM KCl were elicited in each preparation until reproducible responses were obtained. Following washout, each preparation was exposed to cumulative concentrations of either KCl (6-60mM) or U46619 (1-200nM). Then the preparations were exposed to substance P (10nM) and bradykinin (100nM), as described earlier, to assess the integrity of the vascular endothelium. Following two night incubation the segments were treated with the same manner with overnight incubation segments.

The results will show that the porcine coronary artery maintained vascular contractility following overnight incubation in K-H solution containing 2% ficoll and 60μg/ml benzyl penicillin and 20μg/ml streptomycin sulphate. However, in this study, a noticeable attenuation in the responses to KCl and U46619 was seen in the porcine isolated coronary artery segments incubated in AQIX RS-I at 37°C either overnight or for two nights. I conclude that the best method of preserving the vasculature for 18 hours is to use K-H solution. Therefore, Krebs-Henseleit (K-H) solution was used as incubation medium in subsequent studies.

### 2.2.4 The effect of exposure of porcine coronary artery to LPS in the absence or presence of 10% foetal calf serum overnight

Segments were incubated in 2ml Krebs-Henseleit solution (previously gassed with 95%$O_2$ and 5% $CO_2$ for 5 min.) including 2% ficoll and a combination of 60μg/ml benzyl penicillin and 20μg/ml streptomycin sulphate and the absence or

presence of lipopolysaccharides (LPS, E Coli O111:B4) at 100μg $mL^{-1}$ or 1μg $mL^{-1}$ in separate experiments with the absence or presence of 10% foetal calf serum (FCS). Following the incubation procedure, segments were taken out of the incubation solution and prepared for isometric procedure as described above.

### 2.2.5 The effect of exposure of porcine coronary artery to different concentrations of LPS

Segments of porcine coronary artery were separately incubated in 2ml K-H solution (including 10% FCS, 2% ficoll, 60μg/ml benzyl penicillin and 20μg/ml streptomycin sulphate) and exposed to 0.01, 0.1 and 1μg $mL^{-1}$ LPS respectively or a vehicle control. Following gassing with 95% $O_2$ and 5% $CO_2$ for 5 minutes, the samples were then sealed in sterile vials and incubated in an air incubator at 37$^{o}$C. After 18 hours incubation the segments were then prepared for the isometric tension recording as described previously. Following equilibration, the preparations were exposed to three applications of 60mM KCl to get reproducible response. Then, segments were exposed to cumulative concentrations of KCl (6-60mM) followed by cumulative concentrations of U46619 (1-200nM).

### 2.2.6 The effect of exposure of porcine coronary artery to 1μg $mL^{-1}$LPS for 42 hours

Segments of porcine coronary artery were incubated in K-H solution (including 10% FCS, 2% ficoll, 60μg/ml benzyl penicillin and 20μg/ml streptomycin sulphate) for 42 hours at 37°C with the absence or presence of 1μg $mL^{-1}$LPS. The medium was changed on the following morning. Following the incubation

procedure, the isometric test was used to examine the responsiveness to KCl and U46619.

### 2.2.7 The effect of removal of the endothelium on contractile responses of porcine coronary artery to KCl and U46619

In a further set of experiments the effect of 1μg $mL^{-1}$LPS was examined in contractile responses in endothelium-denuded segments of the coronary artery. The endothelium was removed by gently rubbing the lumen with the edge of a fine force tip; care was taken to ensure that the integrity of the endothelium was maintained.

## 2.3 Drugs and Solutions

The composition of Krebs-Henseleit solution was (mM): NaCl 118; KCl, 4.8; $MgSO_4$ .$7H_2O$, 1.2; $CaCl_2.2H_2O$, 1.3; $NaHCO_3$, 25.0; $KH_2PO_4$, 1.2. Benzyl penicillin, streptomycin sulphate, bradykinin, ficoll and lipopolysaccharide *E.coli.* O11:B4 were obtained from Sigma-Aldrich Company Ltd (Poole, Dosert, UK.). Substance P was obtained from Bachem (UK) Chemical Company (Delphe Court, Merseyside, UK). U46619 (9, 11-dideoxy-11a, 9a-epoxymethanoprostaglandin $F_{2\alpha}$) was obtained from Alexis Corporation (Nottingham UK). AQIX RS-I solution obtained from ICP bio Ltd, New Zealand. The composition of AQIX RS-I was NaCl (110 mM), KCl (5.00 mM), $CaCl_2$ (1.25 mM) and $MgCl_2$ (0.45 mM). It also contains $NaHCO_3$ (25.0 mM), BES (5.00 mM), D-glucose (10.00 mM), glycerol (0.11 mM), glutamate (0.3 mM), glutamine (0.4 mM), aspartate (0.02 mM), carnitine (0.05 mM) and choline chloride (0.01 mM), TPP (40.00 nM) and insulin (28.00 mIU).

### 2.4 Data Analysis

The contraction elicited by KCl and U46619 was expressed as milliNewtons force (mN) and is shown as mean± standard error of the mean (SEM). The maximum responses of constrictors were also expressed as mN force. The potency of the constrictor agents was determined as the negative logarithm of the concentration causing 50% of the maximum response ($pD_2$) by using a logistic equation (Kaleidagraph, Version 3.6 Synergy Software). In terms of vasodilators, the relaxations were expressed as percentages of the tone against U46619 and the sensitivity was determined by $pD_2$ if the maximum relaxation was achieved. All

responses were expressed as mean ±SEM. In most instances, differences of mean values between groups were compared using a Student's paired t-test (two tailed). Where there was more than one treatment condition assessed differences were analysed by ANOVA followed by *post hoc* Dunnett's test. A $p$-value < 0.05 was considered statistically significant.

## 2.5 Results

### 2.5.1 The influence of incubation temperature on the contractility of the porcine coronary artery

KCl and U46619 elicited concentration-dependent contractions of the porcine coronary artery. Figure 2.1 represents the effect of the incubation temperature on responses to KCl and U46619 in porcine isolated coronary artery. The results showed that the difference was not significant with response to the $E_{max}$ and sensitivity to KCl when the vessels were stored overnight either at 4ºC or 37ºC (Figure 2.1a). When the tissues were stored overnight at 37ºC, the magnitude in the responses to U46619 was not different statistically to that of those in the arteries incubated overnight at 4ºC. However, an attenuation (1.2-fold) in the sensitivity was seen in the preparation incubated at 37ºC (Figure 2.1b $pD_{2:}$ 4ºC group 7.86±0.07 vs. 37ºC group 7.5±0.2, $p$=0.07, n=10).

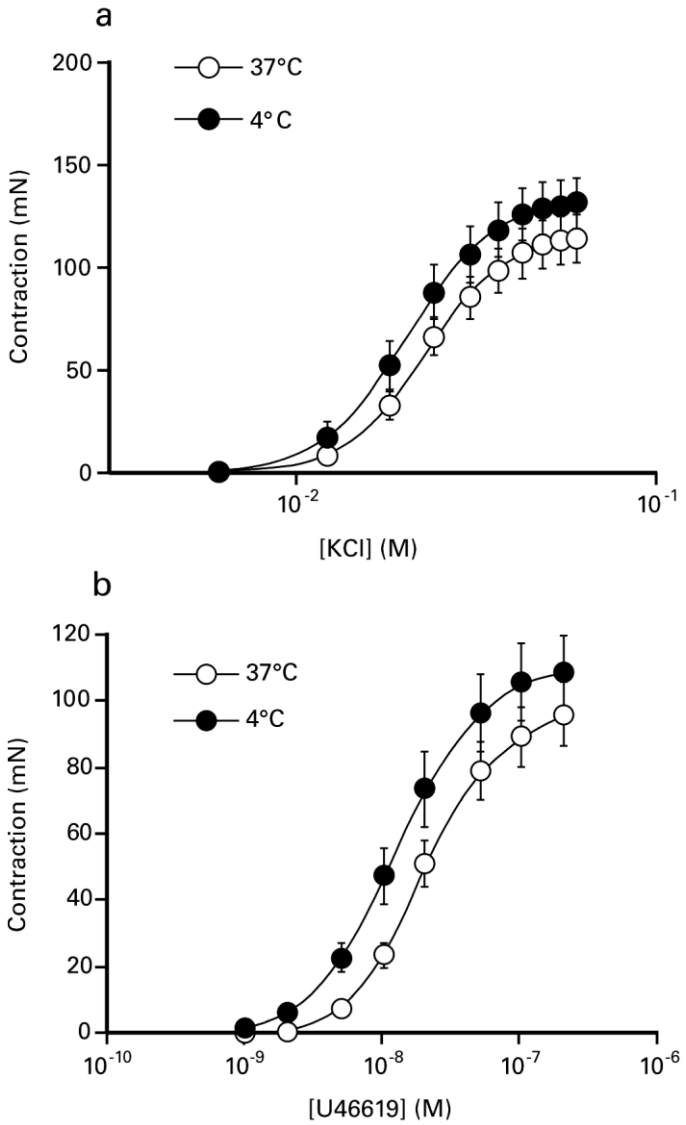



**Figure 2-1** Cumulative concentration response curves of (a) KCl and (b) U46619 on the porcine isolated coronary artery incubated overnight in K-H solution at 4ºC or 37ºC. Responses are expressed in milliNewtons and shown as mean ±SEM of 10-11 investigations.

**2.5.2 The influence of incubation medium on the responsiveness of the porcine coronary artery.**

Figure 2.2 shows digitized representative recording traces of the effects of 60mM KCl and cumulative concentrations of U46619 on porcine coronary artery stored overnight in Krebs-Henseleit solution or AQIX RS-I and studied in the respective solution. KCl and U46619 caused sustained, concentration-dependent contraction in either Krebs-Henseleit solution or AQIX RS-I. However, in 4/12 preparations segments studied in AQIX RS-1, spontaneous contractions to U46619 were observed (Figure 2.2).

There was a significant difference in the magnitude of contraction due to KCl. The magnitude of the response to KCl was greater in the arteries incubated in AQIX RS-I at 4 ºC (Figure 2.3a), However, the vasoconstrictor response to U46619 was reduced by 22±15.4%, reduced in the segments stored overnight in AQIX RS-I at 4ºC compared with those stored in Krebs-Henseleit solution (Figure 2.3c). The difference was not statistically significant with the $E_{max}$ and sensitivity to KCl and U46619 when the vessels were stored overnight either in K-H solution or AQIX RS-I at 37ºC (Figure 2.3b and 2.3d).

The responses of the preparations incubated overnight either in Krebs-Henseleit solution or AQIX RS-I at 37ºC to both endothelium-dependent relaxants, 10nM substance P and 100nM bradykinin, were reduced compared with tissues that were stored at 4 ºC in either Krebs-Henseleit solution or AQIX RS-I (Table 2.1). However, in terms of the endothelium-independent relaxants, sodium nitro prusside and isoprenaline, the percentage of relaxation to U46619 of the preparations were the

same in both conditions. There was a trend towards an increase in sensitivity ($pD_2$) for both agonists in the presence of AQIX RS-I but it did not attain statistical significance (Table 2.2).

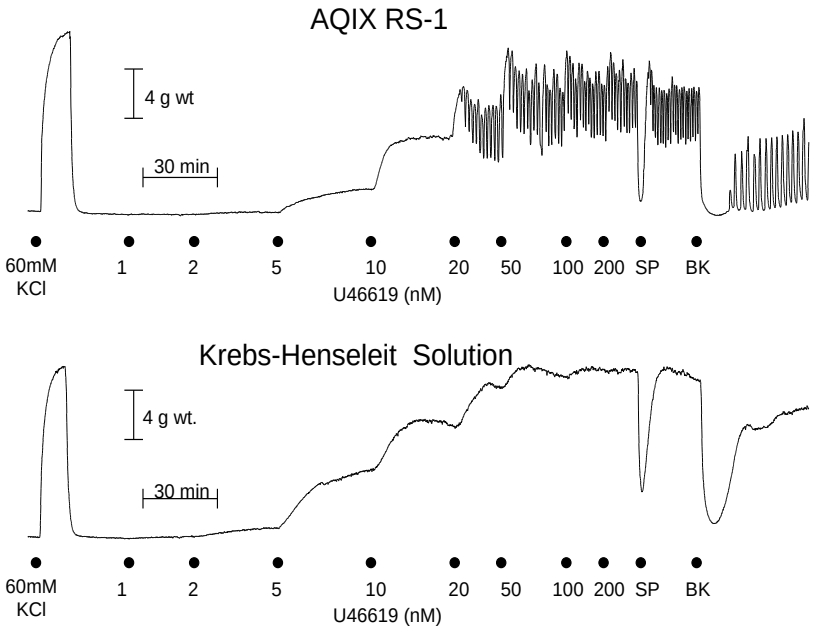



**Figure 2-2** The digitized representative recording traces of the effect of 60 mM KCl, cumulative response curves to U46619 (1nM-200 nM), substance P (10 nM) and bradykinin (100 nM) on the porcine isolated coronary artery stored overnight either in AQIX RS-I (Upper) or in Krebs-Henseleit solution (Lower).

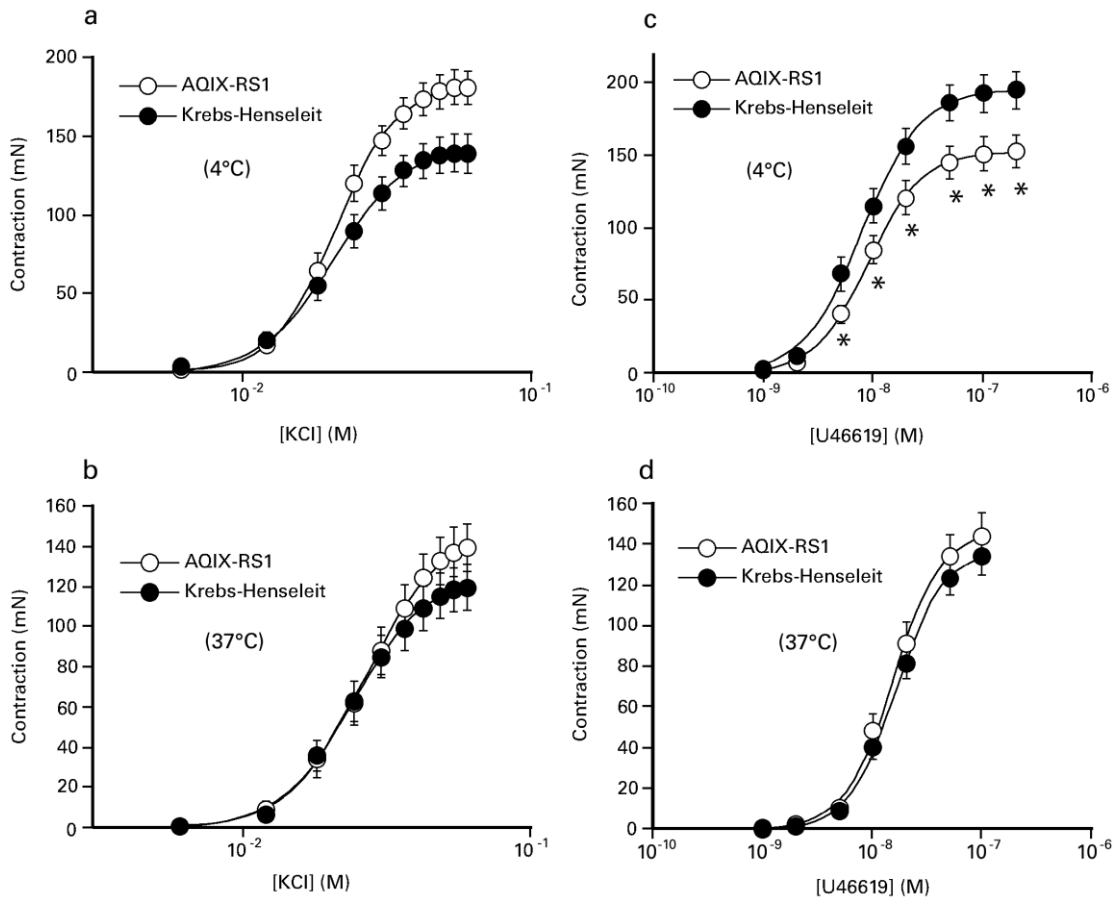



**Figure 2-3** The cumulative response curve of KCl (a, b) and U46619 (c, d) on the porcine isolated coronary incubated either in Krebs-Henseleit solution or AQIX RS-I at 4 or 37ºC overnight. Responses are expressed as milliNewton and shown as mean ± SEM of 12- 16 observations. *denotes a statistically significant# difference from control tissue by paired Student's t-test ($p$<0.05).

**Table 2-1** Percentage relaxation to substance P and bradykinin against U46619-induced contractions of the porcine isolated coronary artery following overnight storage at 4°C or at 37°C in either Krebs-Henseleit solution or AQIX RS-I.

| | **Substance P (10nM) % relaxation** | **Bradykinin (100nM) % relaxation** |
|---|---|---|
| **K-H solution (4°C)** | 72.4±1. | 96.9±1.5 |
| **K-H solution (37°C)** | 43.4±6.2* | 54.2±6.3* |
| **AQIX RS-I solution (4°C)** | 82.7±2.7 | 97.4±1.2 |
| **AQIX RS-I solution (37°C)** | 46.8±5.7* | 61.1±6.5* |

Data are shown as mean ± SEM of 12- 16 observations. * denotes a statistically difference from control tissue by paired Student's t-test ($p$<0.05).

**Table 2-2** Percentage relaxation to sodium nitroprousside and isoprenaline against U46619-induced contractions of the porcine isolated coronary artery following overnight storage at 4°C in either K-H solution or AQIX RS-I.

| | **Sodium nitroprusside** | | **Isoprenaline** | |
|---|---|---|---|---|
| | **% of U46619** | **$pD_2$** | **% of U46619** | **$pD_2$** |
| **K-H Solution** | 92.0±2.7 | 6.8±0.16 | 99.9±1.3 | 7.2±0.16 |
| **AQIX RS-I** | 93.7±1.6 | 7.2±0.21 | 102.0±2.3 | 7.46±0.1 |

Data are shown as mean ± SEM of 10 observations.

The experiments to date have shown that there is little difference in the response to KCl, U46619, SP and bradykinin when vessels are stored overnight at 37 ºC in either Krebs-Henseleit solution or AQIX RS-I. Previous work by Wei (2006) has shown that low concentrations of LPS may produce changes in vascular responses if the duration of incubation is two nights (approx 42 hours). Thus in this study I have examined the responsiveness of the arteries when stored for two nights in either AQIX RS-I or Krebs-Henseleit solution. However, in light of the fact that there were no major differences between Krebs and AQIX, I decided that further experiments comparing the different solutions should be limited to the incubation of the arteries; all tissue were collected in Krebs- Henseleit solution.

### 2.5.3 The influence of incubation medium and period on the responsiveness of the porcine coronary artery.

KCl and U46619 produced concentration-dependent contractions of the coronary artery stored overnight at 37ºC in either Krebs-Henseleit solution or AQIX RS-I. However, a further day of storage resulted in a proportion of preparations that failed to respond. In the case of preparations stored in Krebs-Henseleit solution for two nights only 12/16 preparation responded to the agents (> 40 mN), while only 6/16 preparations stored in AQIX RS-I solution for 2 days responded. Therefore, in the following figure (2.4) the data shown represent only these segment that responded to KCl (60mM) with contraction >40mN.

Figure 2.4a and 2.4b describe the concentration response curves of KCl and U466199 in porcine coronary artery incubated in AQIX RS-I overnight or for 40 hrs at 37ºC. A profound attenuation in the magnitude in the response to KCl and

U46619 was seen in the preparations incubated in AQIX RS-I for 40 hr compared to the preparations that were stored overnight in the same medium (Figure 2.4a, 4b). Figures 2.4c and 2.4d show the cumulative responses to KCl and U46619 in porcine coronary arteries incubated in Krebs-Henseleit solution overnight or for 40 hr at 37ºC. There were not statistical significant differences in the $E_{max}$ and sensitivity to KCl when the tissues were stored either overnight or for 40 hr. As shown in Table 2.3 there was no difference in the sensitivity of the preparations incubated at 37ºC either in AQIX RS-I or in Krebs-Henseleit solution to KCl. However, there is significant reduction in the sensitivity to U46619 of segments incubated in Krebs-Henseleit solution or in AQIX RS-I for 40 hr.

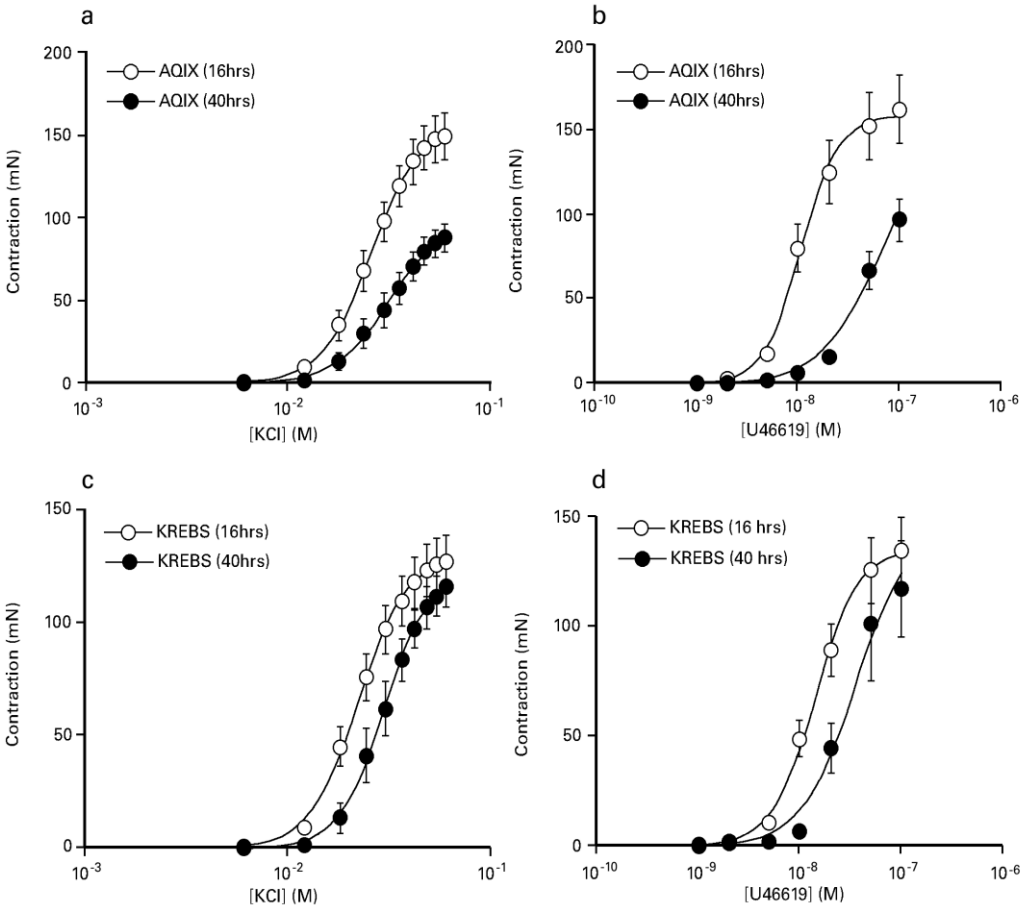



**Figure 2-4** Cumulative responses curve to KCl (a,c) and U46619 (b,d) of porcine isolated coronary arteries incubated in either Krebs-Henseleit solution or AQIX RS-I at 37ºC for 16 hours or 40 hours. Responses are expressed as milliNewton and shown as mean±SEM of 6-12 observations.

**Table 2-3** The effects of the incubation medium and incubation period on responses to KCl and U46619 in the porcine coronary artery.

| **Incubation Condition** | **KCl (n=12)** | | **U46619(n=6)** | |
|---|---|---|---|---|
| | **Max (mN)** | **$pD_2$** | **Max (mN)** | **$pD_2$** |
| Overnight In K-H solution | 124.6±11.7 | 1.64±0.03 | 132.2±14.7 | 7.8±0.09 |
| 40 hours In K-H solution | 113.6±15.6 | 1.49±0.05 | 114.6±21.5 | 7.5±0.17* |
| **Incubation Condition** | **KCl (n=9)** | | **U46619(n=6)** | |
| | **Max(mN)** | **$pD_2$** | **Max (mN)** | **$pD_2$** |
| Overnight In AQIX solution | 146.5±13.7 | 1.57±0.6 | 158.6±19.8 | 7.9±0.03 |
| 40 hours In AQIX solution | 86.4±7.8** | 1.45±0.04 | 94.6±11.6* | 7.0±0.13** |

The values shown represent the mean ± SEM of 6-12 investigations.
* denotes a statistically difference from control tissue by paired Student's t-test ($p$<0.05).

### 2.5.4 The effect of a high concentration of LPS on the porcine isolated coronary artery in presence or absence of 10% foetal calf serum.

Figure 2.5 illustrates the cumulative concentration response curves of KCl and U46619 in arteries exposed overnight (16-18 hours) to 100μg $mL^{-1}$LPS. As seen in the figure (2.5a and 2.5c) the responsiveness to KCl and U46619 was not reduced by exposure to 100μg $mL^{-1}$LPS when the preparations were incubated in Krebs-Henseleit solution only. In contrast, the $E_{max}$ to KCl was reduced by 30.5±10.2% (n=10) with a 1.2-fold reduction in the potency following exposure to 100μg $mL^{-1}$LPS and 10% foetal calf serum (FCS) (figure 2.5b). The maximum responses to U46619 were also attenuated by 25.7±8.2% (n=10), although the sensitivity was not changed in the arteries exposed to 100μg $mL^{-1}$LPS and 10% FCS (figure 2.5d).

Figure 2.6 depicts the responses curves of KCl and U46619 on porcine coronary artery exposed overnight to 1μg $mL^{-1}$LPS in the absence (Figure 2.6a and 2.6c) or presence (Figure 2.6.b and 2.6d) of 10% foetal calf serum. When the arteries were incubated in Krebs-Henseleit solution in the presence of 10% FCS, the maximum response to KCl was reduced to 51.9±5.4%, (n=8), with no change in the sensitivity (figure 2.6b). However, this effect was absent in the tissues incubated in 10% FCS free medium. Similarly, the contraction response curve of U46619 on the artery segments exposed to 1μg $mL^{-1}$LPS with 10% FCS was reduced to 60.7±4.9% ,( n=8) compared to the arteries segments that exposed to 1μg $mL^{-1}$ LPS only (figure 2.6d).

Following attainment of maximal contractions to U46619, the addition of 10nM substance P was associated with a relaxation (28.77±4.4%, n=8) that was

significantly (student`s paired t-test, $p$ <0.05) reduced in preparations previously exposed to 1μg $mL^{-1}$LPS (3.8±0.7%, n=8).

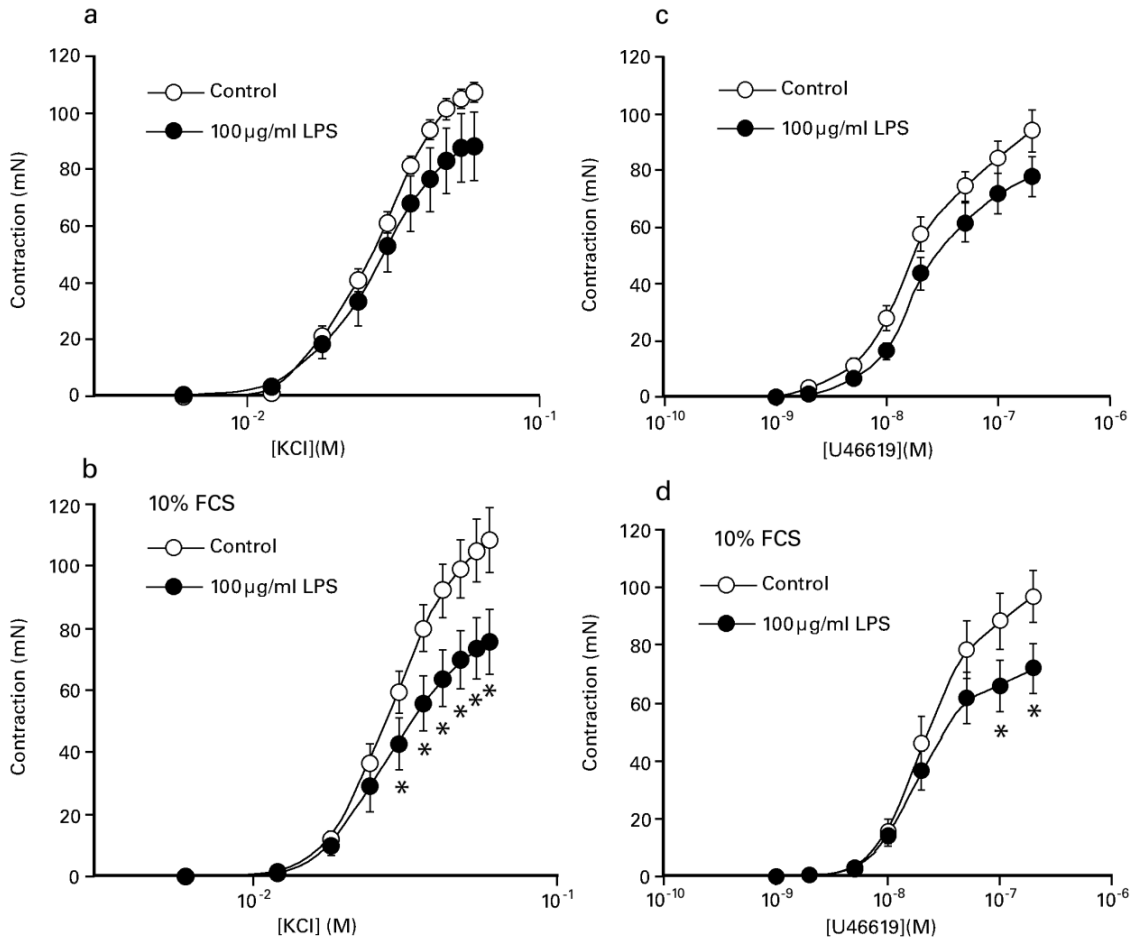



**Figure 2-5** Cumulative responses curves to KCl and U46619 in the porcine isolated coronary artery following 16-hour incubation in the presence or absence of 100µg $mL^{-1}$LPS in K-H solutions (a and c). Some preparations were exposed to 10% FBS (b and d). Responses to KCl or U46619 are expressed as mN and shown as mean ± SEM of 10 investigations. * denotes a statistically difference from control tissue by paired Student's t-test ($p$<0.05).

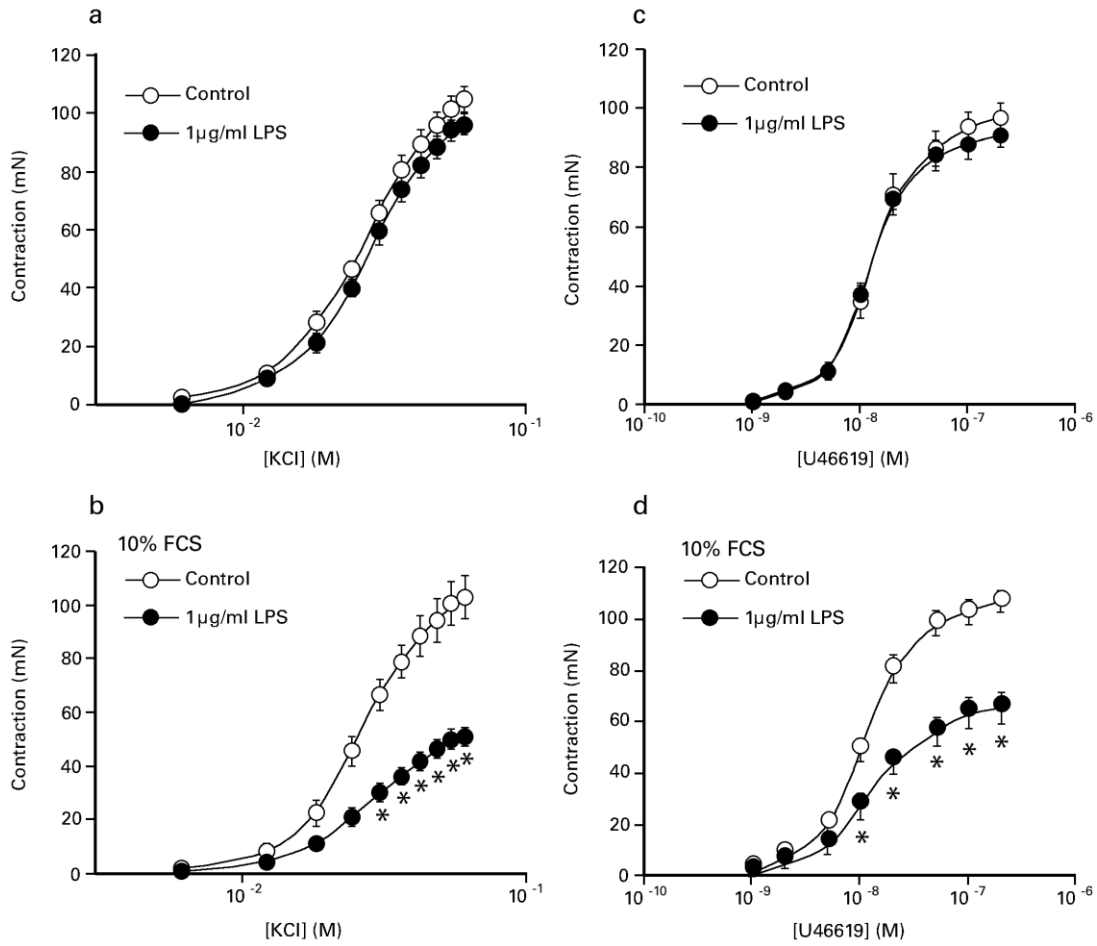



**Figure 2-6** Cumulative responses curves to KCl and U46619 in the porcine isolated coronary artery following 16-hour incubation in the presence or absence of 1µg $mL^{-1}$LPS in K-H solutions (a and c). Some preparations are exposed to 10% FBS (b and d). Responses to KCl or U46619 were expressed as mN and shown as mean ± SEM of 8 investigations. * denotes a statistically difference from control tissue by paired Student's t-test ($p$<0.05).

A wide range of concentrations of LPS were examined to determine the concentration needed to elicit a notable effect on the contractile function of porcine coronary artery. As seen from the table 2.4 neither 0.1μg $mL^{-1}$LPS nor 0.01μg $mL^{-1}$LPS had an effect on the contraction elicited by KCl and U46619. However, a significant attenuation in the sensitivity as well as in the maximum responses to KCl and U46619 was observed in the segments exposed to 1μg $mL^{-1}$LPS overnight (Table 2.4). Therefore 1μg $mL^{-1}$LPS was used for LPS to stimulate the artery in subsequent studies.

### 2.5.5 The effect of 1μg $mL^{-1}$LPS on the porcine isolated coronary artery incubated for 42hr presence of 10% foetal calf serum.

Since a pronounced effect of serum was established after 16 hours incubation, an experiment was designed to investigate the time-dependency of the serum enhancement effects on 1μg $mL^{-1}$LPS responses in segments of porcine coronary artery. Figure 2.7 shows statistically significant reduction in the maximum response of KCl to 57.4±3.4%, (n=8) of control and U46619 to 59.7±3.9% (n=8) of control of the segments exposed to 1μg $mL^{-1}$LPS for 42 hours in the presence of 10% FCS. At the end of the U46619-induced contractions response assay, the addition of 10nM substance P produced a transient relaxation (28.7±4.4%, n=8) in control preparations that was significantly reduced to (3.8±0.7, n=8) ($p$ <0.05) following LPS treatment.

### 2.5.6 The effect of removal of the endothelium on contractile responses of porcine coronary artery to KCl and U46619

In light of the finding that substance P induced relaxation was impaired by 1μg $mL^{-1}$ LPS I further examined the influence of endothelium-denudation and exposure to LPS on contractions to KCl and U46619. Endothelium denudation was confirmed by the absence of a relaxation to substance P following maximum contraction to U46619.

Figure 2.8 shows that after removal of endothelium, overnight exposure to 1μg $mL^{-1}$ LPS was still associated with a significant reduction in the maximum contractions to KCl (76.8±3.8% of control segments, n=8) and U46619 (71.2±4.6% of control segments, n=8).

**Table 2-4** The responses to KCl and U46619 of porcine isolated coronary arteries exposed to the different concentrations of LPS overnight in K-H solution in the presence of 10% FCS.

| | KCl | | U46619 | |
|---|---|---|---|---|
| | **Max (mN)** | **$pD_2$** | **Max (mN)** | **$pD_2$** |
| **Control (n=12)** | 138.8±12.7 | 1.51±0.02 | 140.4±12.7 | 7.77±0.15 |
| **0.01µg $mL^{-1}$LPS** | 135.8±10.7 | 1.47±0.02 | 141.0±4.9 | 7.70±0.06 |
| **0.1µg $mL^{-1}$LPS** | 132.2±13.7 | 1.54±0.01 | 132.2±11.7 | 7.81±0.05 |
| **1µg $mL^{-1}$LPS** | 66.1±3.2* | 1.53±0.04* | 66.6±5.7* | 7.57±0.06 |

Responses to KCl and U46619 were expressed as mN force and shown as mean ± SEM of 12 investigations. The difference between control and LPS groups was compared by ANOVA and considered as significant if $p<0.05$. *denotes a significant difference from Control tissues further compared by Dunnett's test.

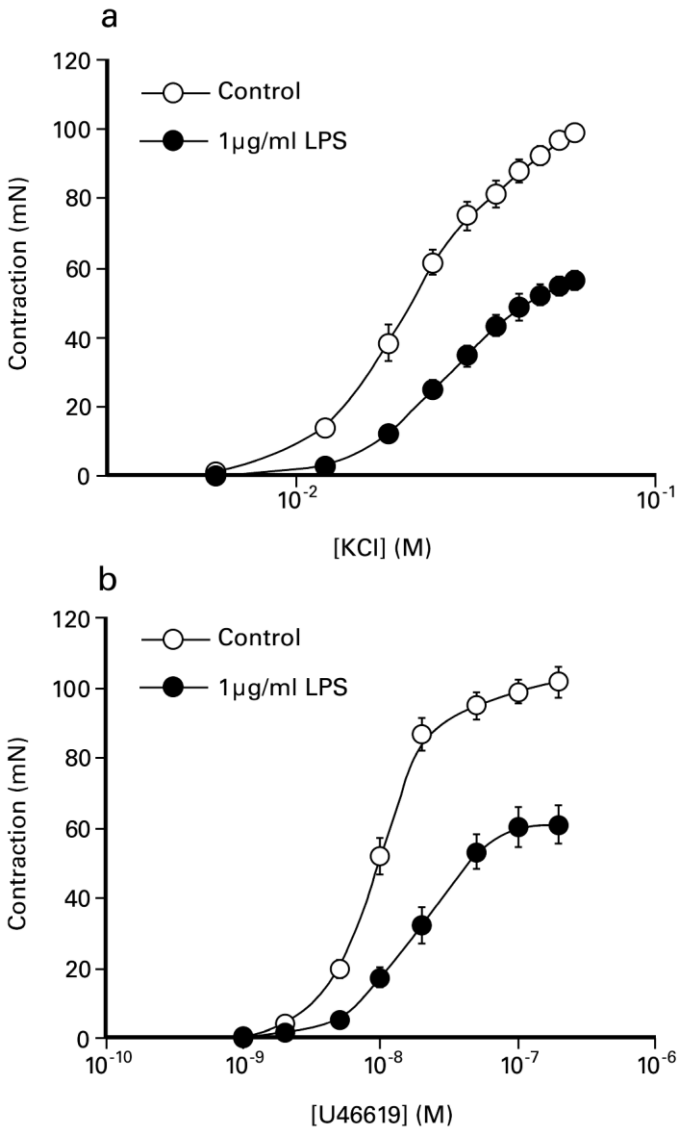



**Figure 2-7** The cumulative response curves of KCl (a) and U46619 (b) in the porcine isolated coronary artery following 42 hour incubation in the presence or absence of 1µg $mL^{-1}$LPS in K-H solutions. Responses to KCl or U46619 are expressed as mN and shown as mean ± SEM of 8 investigations.

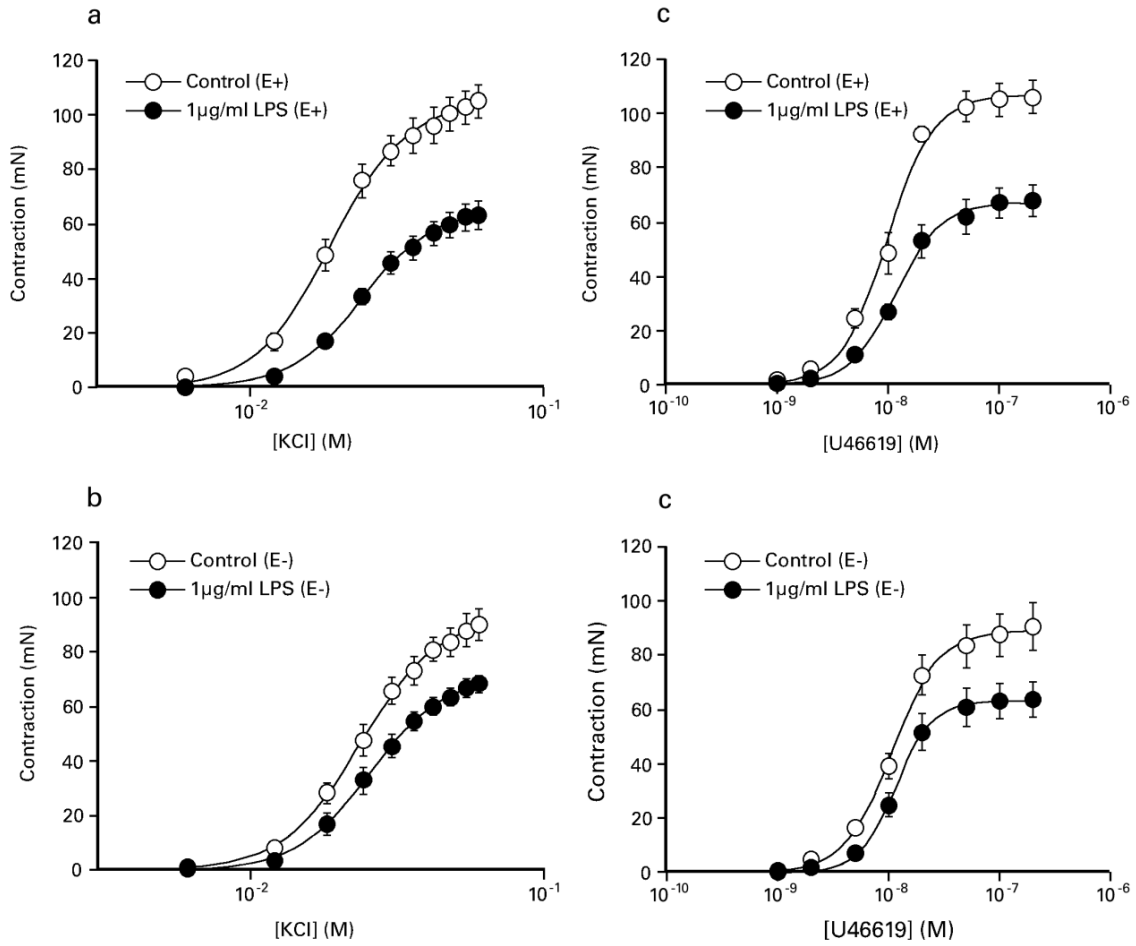



**Figure 2-8** The cumulative response curves of KCl and U46619 in endothelium-intact preparation (8a, 8c) and in endothelium-denuded preparations (8b, 8d) following 16 hour incubation in the presence or absence of 1µg $mL^{-1}$LPS in K-H solutions. Responses to KCl or U46619 are expressed as mN and shown as mean ± SEM of 8 investigations.

## 2.6 Discussion

In this chapter I have established that KCl and U46619-induced contractions were reduced following overnight incubation at 37°C in Krebs-Henseleit solution compared with segments stored at 4°C. The sensitivity of the preparations to KCl did not change. However, there was a 1.2-fold reduction in the sensitivity of the preparations to U46619. It has previously been reported that U46619-induced contractions were attenuated in segments following overnight incubation storage at 37°C (Wei, 2006a). Similarly, it was also demonstrated that the response of rat aorta segments to norepinephrine was attenuated following increasing the temperature in the organ bath (Musatafa *et al*., 2004). In an earlier study Ingemansson *et al*., (1995) demonstrated that when rat aorta was stored in University of Wisconsin solution at 22 ºC, for 36 hours, there was a significant decrease in vascular smooth muscle function. Another study by the same group demonstrated that vascular smooth muscle function can be fully preserved for 36 hours at 0.5 ºC (Ingemansson *et al*., 1996).

In terms of endothelium-dependent relaxation, I have established that substance P- and bradykinin-induced relaxations were impaired following overnight incubation at 37ºC. In a previous study, Wei 2006 found that substance P-induced relaxation was impaired following overnight incubation at 37ºC. They also reported that this reduction was fully prevented by a non-selective iNOS inhibitor L-NAME and a protein synthesis inhibitor, dexamethasone, indicating that induction of NO synthesis is mainly responsible for the inhibitory effect. It is well known that, in porcine coronary artery, the endothelium-dependent relaxation to agonists such as bradykinin, is mediated by a factor termed endothelium-derived hyperpolarization

factor (EDHF) by opening potassium channels in smooth muscle membrane, resulting in hypopolarization and consequently reduced calcium entry through voltage-dependent calcium channels (Hecker, 2000). Researchers indicated that exposure to low temperatures can impair the basal and stimulated release of endothelium-dependent relaxation factors (Bodelsson, 1989). However, Ingemansson et al (1996) found that no significant impairment in acetylcholine-induced relaxation was seen after storage rat aorta at 4°C for two days (Ingemansson *et al*., 1996). In contrast, Hashimoto and co-workers (1992) reported that, following 3 days of prolonged cold storing of porcine coronary arteries, endothelium-dependent relaxation to substance P was reduced (Hashimoto *et al*., 1992).

The results showed that there were no obvious difference between AQIX RS-I and K-H solution. But it was noted that some of the U46619- induced contraction showed phasic activity, rather than a sustained response, suggestion that AQIX RS-I solution may suppresses vascular tone (Figure 2.2). Additionally, Krebs-Henseliet solution, although unable to preserve endothelium-dependent relaxation, was able to preserve almost full contractile capacity after two days storage compared to AQIS-SR-I and this was due to the fact that Krebs solution contains double the amount of calcium compound with that present in AQIX RS-I. It was suggested that prolonged storage of smooth muscle cells in solutions containing too little calcium or none at all is harmful to contractile function (Ingemansson *et al* 1995). In contrast, Rees (1989a) reported that many isolated tissues have been either perifused or perfused with AQIX RS-I solution at room temperature and found to survive for several days without major loss of contractile function. This difference in the results may be due to the closed system that was used for incubating the blood vessel attenuating the

effect of AQIX RS-I solution (Rees 1989a). These preparations include rat uterus, rat heart, porcine kidney (Kay, 2007), porcine liver (Vekemans, 2009) and a variety of skeletal muscles. Despite the growing evidence for the potential advantages of a phosphate-free incubation medium, I found no clear advantage over Krebs- Henseleit solution. So the latter was used in further experiments.

In this chapter, I found that overnight exposure of segments from porcine coronary artery to either low or high concentrations of lipopolysaccharide in the presence of serum significantly impaired the contractile function of the segments, as well as the substance P-induced relaxation. Previous studies have shown the activation of iNOS in vascular smooth muscle cells of different species when stimulated by LPS over a relative by long culture period (Gabriel *et al*., 2000; MacNaul and Hutchinson, 1993; O'Brien *et al*., 2001; Shibano and Vanhoutte, 1993) or *in vivo* endotoxemia animals (Szabo *et al*., 1996; Wen and Han, 2000; Wu *et al*., 2004). Additionally, Fujii and his group found that injection of *E.coli* LPS was associated with a progressive decline in arterial pressure and a significant rise in serum nitrite–nitrate level, pulmonary NOS activity and upregulation of pulmonary iNOS protein expression these are attenuated by pre-treatment with P1/F1 peptide (Fujii *et al*., 2000). Moreover, a relatively moderate increase in cGMP production was induced in isolated porcine coronary artery under LPS stimulation which was abolished by L-NAME (Wei, 2006). In tissue culture studies, elevation of cGMP was also reported in rat aorta taken from LPS-injected rats (Fleming *et al*., 1991). LPS also impaired the endothelium-dependent vascular relaxation. Following exposure of porcine isolated coronary artery overnight or to 2 nights to either low or high concentration of LPS the response to substance P was impaired due to induction of iNOS by LPS

in endotheial cells (Gibreal *et al*., 2000). The concentrations of LPS in our study are similar to that reported to alter vascular reactivity in other blood vessels (O`Brien and Abraham, 2004; Takakura *et al*., 1994; Tsuneyoshi *et al*., 1996; Wylam *et al.*, 2001). In contrast, a previous study by Wei (2006) indicated that only a high concentrations of LPS (100μg $mL^{-1}$LPS) induced significant attenuation of the response of the porcine coronary artery segments to KCl and U46619.

Previous findings have indicated that soluble serum components such as lipopolysacchaides binding protein and CD14 influence the biological potency of bacterial LPS, and these findings have significantly advanced the understanding of the pathogenesis of gram negative bacterial diseases (Kielian and Bletcha, 1995). Serum enhanced the contractility depression induced by 1μg $mL^{-1}$LPS, in a similar way to that found with 100μg $mL^{-1}$LPS. Thus these results confirm the previous studies of Standiford *et al* (1990) and Pugin *et al* (1994) that LPS did not stimulate lung cells without serum (Pugin *et al*., 1994; Standiford *et al*., 1990).

It is known that LPS binding protein (LBP) binds to the lipid A region of LPS. The resulting LPS-LBP complex is recognised by both the membrane-bound (mCD14) and soluble form of CD14 ( sCD14) thereby enhancing the ability of LPS to activate myeloid, epithelial and endothelial cells (Kielian and Blecha, 1995). Kazunori and colleagues suggested that the ability of FCS to suppress LPS-induced activation of WEHI-3 cells is mainly dependent on the structure of the polysaccharide chain and also on the concentration of LPS employed. For example, they found that the LPS-induced production of NO and TNF-α by WEHI-3 cells was totally serum-independent, and that such serum factors as LBP or soluble CD14 might not be

necessary. They also found that FCS showed enhancing effects on the production of NO and TNF-α by the same cells stimulated with a low concentrations, (<100ng/ml) of LPS and rough mutant *Salmonella minnesota* Re LPS. In contrast, FCS inhibits TNF-α production by the same cells stimulated by a high concentration of smooth form LPS S LPS (>1000ng/ml) (Kazunori *et al*., 1999).

## 2.7 Conclusion

In summary, the results showed that there were no significant difference between AQIX RS-I and Krebs-Henseleit solution. But it was noted that overnight incubation of porcine isolated coronary artery in AQIX RS-1 showed reduction in the responses to KCl and U46619. I established an endotoxin-induced impaired vascular reactivity model *in vitro* by exposure of porcine coronary artery to 1μg $mL^{-1}$LPS overnight in presence of 10% foetal calf serum. The responses of porcine isolated coronary artery to a low concentration LPS is enhanced by serum, possibly due to the participation of sCD14 and LBP. In all further studies I prepare to use Krebs-Henseleit solution was used to store the segments at 37ºC and 1μg $mL^{-1}$LPS in the presence of 10% foetal calf serum was used to investigate the effects of flavonoids and statins on inflammatory changes in PCA.

# Chapter 3

# Comparison of the effects of quercetin, and related flavonoids, on LPS-induced changes in coronary artery *in vitro*

## 3.1 Introduction

Quercetin is a major flavonoid found in various foods, such as apples, onions and broccoli that are reported to possess a range of biological activities in isolated cells and tissues (Boots *et al.*, 2008; Halliwell, 2007). When ingested as a supplement by humans some of the effects noted for quercetin have been consistent with both the epidemiological and *in vitro* observations (Williamson and Manach, 2005). For example, Edwards and colleagues (2007) demonstrated that quercetin lowered blood pressure in hypertensive subjects, an effect potentially related to the reported direct vasodilator action of this flavonoid, and its metabolites, on isolated blood vessels (Edwards *et al.*, 2007; Perez-vizcaino *et al.*, 2002).

With respect to putative anti-inflammatory activity of quercetin, cytokine-induced or lipopolysaccharide (LPS)-induced production of nitric oxide and prostanoids in human macrophage cell lines have been reported to be sensitive to high concentrations (> 10 μM) of the flavonoid (Chen *et al.*, 2002; Hamalainen *et al.*, 2007). The mechanism for this protective effect of quercetin has been attributed to suppression of LPS-induced activation of NFκB, possibly linked to stabilization of the cytoplasmic NFκB/IκB complex (Hamalainen *et al.*, 2007) and specific inhibition of IκB kinase (Chen *et al.*, 2005) . In mouse microglia, quercetin (at approx 3 μM) exerted a greater inhibitory effect on LPS-induced nitrite production compared to that elicited by interleukin-1β (Chen *et al.*, 2005), thereby highlighting the selective nature of this action. The significance of these observations has, however, been called into question because the concentrations used greatly exceed plasma levels of the aglycone detected in humans (approx

30nM) and there is no information regarding the biological activity of key metabolites (Kroon *et al*., 2004).

It is well recognised that the vasculature is a significant component in the development of inflammatory responses. Endothelial cells exhibit signs of altered expression of cell adhesion molecules (Read *et al*., 1994), while vasoconstrictor responses are reduced under the influence of locally-generated dilator substances (Mitchell *et al*., 2007; van Gil *et al*., 2008). For example, nitric oxide production in blood vessels is increased by inflammatory stimuli and this is recognised as a major contributor to increased local blood flow (Mitchell *et al*., 2007). While there is recent information concerning the effect of quercetin and its metabolites on the expression of endothelial cell adhesion molecules (Tribolo *et al*., 2008a), there is no comparable study regarding the changes in smooth muscle contractions.

In the present chapter I have investigated the effect of quercetin, and two of the key metabolites in humans, quercetin 3'-sulphate and quercetin 3-glucuronide (Kroon *et al*., 2004; Wang and Morris, 2005), against LPS-induced changes in contractions of the porcine isolated coronary artery (Qi *et al*., 2007) and the generation of nitric oxide. In addition, since many quercetin-rich foods also contain significant amounts of myricetin, a 5' hydroxylated derivative of quercetin (Fusi *et al*., 2005; Hamalainen *et al*., 2007), this flavonoid has also been examined.

## 3.2 Materials and Methods

Porcine hearts were obtained from a local abattoir and placed in modified Krebs-Henseleit (K-H) solution maintained at 4°C before being transported to the laboratory. The anterior descending branch of the coronary artery was dissected from the hearts, cleaned of connective tissue, and then divided into 4 mm long segments.

### 3.2.1 Contraction Studies

For contraction experiments, single segments of the porcine coronary artery were incubated in 2ml K-H solution (previously gassed with 95% $O_2$ and 5% $CO_2$ for 5 min.) containing 2% ficoll and a combination of 60µg/ml benzyl penicillin and 20µg/ml streptomycin sulphate and 10% FCS. The solution also contained 1µg $mL^{-1}$ LPS, various concentrations of quercetin or a combination of LPS and the flavonoid (added 60 min before LPS) and then sealed in a sterilized glass vial and stored overnight at 37°C for 16-18 h. In some experiments the effect of 10µM myricetin, 10µM quercetin 3'-sulphate or 10µM quercetin 3-glucuronide in the presence of LPS was examined. In addition, the effect of co-incubation with a selective inhibitor of NFκB, Bay 11-7082 (10µM) (Pierce *et al*., 1997), against LPS-induced changes in the blood vessel was also assessed. Unless, indicated otherwise, all experiments were conducted on nominally endothelium-intact segments of the coronary artery. In a further set of experiments the effect of 1µM quercetin was examined against LPS-induced changes in contractile responses in endothelium-denuded segments of the coronary artery, prepared as described by

Suri *et al*., (2010). In all instances the segments were effectively paired so that each experimental condition had a control segment taken from the same animal.

After overnight storage, segments were removed from the incubation solution and placed in K-H solution (maintained at 37$^{o}$C and gassed with 95% $O_2$ and 5% $CO_2$) in a 15 ml isolated organ bath, prepared for isometric tension recordings as previously described (Qi *et al*., 2007) and allowed to equilibrate for 60 min. Contractions of the segment were measured using a Grass FT03 isometric force transducer connected to a MacLab unit coupled to a Macintosh LC4 computer running Chart 3.5. An initial resting tension of 80 mN was slowly applied to each segment at the end of the equilibration period and the recorded tension declined to 40-60 mN over a further 40 min period. Segments were repeatedly exposed to 60mM KCl for 15 min until reproducible contractions were observed. The preparations were exposed to cumulatively increasing concentrations of either KCl (6-60mM) or U46619 (a stable thromboxane-mimetic analogue, 9,11-dideoxy-9α,11α-methanoepoxyprostaglandin $F_{2\alpha,}$ 1-200 nM). When each preparation was exposed to a maximally-effective concentration of U46619, 10nM substance P was added to assess the integrity of the endothelium. In some experiments, the involvement of nitric oxide in the effect of LPS on contractile responses was investigated by adding 10μM 1400W, a selective inhibitor of inducible NOS (Garvey *et al*., 1997a), 30 min before constructing concentration-response curves to either KCl or U46619.

### 3.2.2 Nitrite Determination

For the measurement of nitrite ion accumulation, a marker for nitric oxide production (Kelm, 1999), two segments were incubated in each vial for 24 hr with aerated DMEM (without phenol red) containing 10% FCS, 1mM L-arginine, 60µg/ml benzyl penicillin and 20µg/ml streptomycin. The segments were exposed either to 1µg $mL^{-1}$ LPS, a flavonoid, or combination of LPS and flavonoid, as described above. Some preparations were also exposed to either 10µM 1400W, 1µM dexamethasone or 10µM Bay 11-7082 for 24 hr in the presence of 1µg $mL^{-1}$ LPS. After the incubation period, the segments were removed, briefly blotted on paper towel and weighed. Nitrite ion accumulation in the incubation medium was determined by the Greiss reaction as previously described (Ukil *et al*., 2006). Greiss reagent (500 µl of 1% sulphanilamide and 0.1% naphthylethylamine diamine in 5% hydrocholoric acid) was added to 500µl of the incubation medium and optical density at 550 nm was measured, using a UNICAM UV/VIS spectrophotometer. A standard concentration-optical density curve was calculated for every assay using sodium nitrite solution against a blank (500µl DMEM & 500µl Griess reagent). In a separate experiment I established that incubation of DMEM containing either the flavonoid, LPS or a combination of both (without the arterial segments) produced an optical density reading similar to the blank. The minimum detectable amount of nitrite was 375 nmoles.

### 3.2.3 Immunohistochemistry

Sections of the coronary artery incubated under the above conditions were prepared on a cryostat (5 µm thick) and stored frozen at -80$^{o}$C until required. The sections were then warmed to room temperature for 20 min and fixed in cold

acetone at -4°C for 20 min. To block endogenous peroxidase activity, sections were treated with blocking serum (2 drops of vectastain in 5ml phosphate buffer solution (pH 7.4) containing 2% w/v immunohistochemical grade bovine serum albumin) for 10 min at room temperature. The sections were then incubated for 1 hour with primary mouse antibodies: porcine-PECAM-1 (diluted 1:75), or rabbit inducible nitric oxide synthase (iNOS) (diluted 1:100). The sections were then washed in phosphate buffer solution, incubated for 10 min with biotinylated anti-mouse antibody in 10% NGS (Vectastain ABC Kit), washed in phosphate buffer solution, incubated for a further 5 min with ABC (avidin-biotin-peroxidase) reagent in phosphate buffer solution and, finally, washed again. Immunoreactive CD31 and iNOS were visualized by incubating the sections in Vector Red substrate (1 drop of levamisole solution to 5 ml of 200 mM Tris-HCl pH 8.2) for 30 min. Finally, sections were dehydrated and cover-slipped with DPX mounting medium. The observation and photographs were made using a light microscope (Leica DM4000B) and an imaging digital camera. Images were obtained using Openlab (improvision, UK).

## 3.3 Solutions and drugs

The composition of K-H solution was (in mM): NaCl, 118; KCl, 4.8; $MgSO_4.7H_2O$, 1.2; $CaCl_2.2H_2O$, 1.3; $NaHCO_3$, 25.0; $KH_2PO_4$, 1.2. Benzyl penicillin, streptomycin sulphate, ficoll, LPS (*Eschericha coli* O III: B4), Bay 11-7082 ((E)-3(4-methylphenylsulfonyl)-2-propenenitrile), sulphanilamide, N-(1-napthyl)-ethylene-diamine dihydrocholoride and quercetin dehydrate were all obtained from Sigma-Aldrich Company Ltd (Poole, Dorset, UK). Substance P was obtained from Bachem (UK). U46619 was obtained from Alexis Coporation

(Nottingham, UK). 1400W was obtained from Tocris Cookson Ltd (Avonmouth, UK). Dexamethasone sodium phosphate was purchased from Organon (Cambridge, UK). Dulbecco's Modified Eagle's Medium (DMEM) was supplemented with antibiotics (see above) and 2 mM L-glutamine (Gibco). The metabolites of quercetin, quercetin-3'-sulphate and quercetin-3-glucuronide, were prepared at the Institute of Food Research, Norwich (Needs and Kroon, 2006). Antibodies against rabbit iNOS (Santa Cruz Botechology, Santa Cruz, Califonia, USA) and mouse anti porcine CD31 (MCA1747, Serotec, Kidlington, UK) were also obtained. Quercetin, Bay 11-7082 and quercetin metabolites were dissolved in 100% DMSO at a concentration of 10mM (< 0.1% DMSO in final incubation medium), whereas dexamethasone was dissolved in absolute ethanol at a concentration of 10mM, all other drugs were dissolved in distilled water.

## 3.4 Data analysis and statistics

Contractions produced by U46619 and KCl were measured as milliNewtons force (mN). Responses were expressed as the maximum contraction ($E_{max}$), and potency determined as the negative logarithm of the concentration causing 50% of the maximum response (-log $EC_{50}$ or $pD_2$) using a logistic equation (Kaleidagraph, version 3.6 Synergy Software). The values are shown as the mean (± sem). The content of nitrite ions in the medium was calculated according to the equation deduced from the standard curve. The amount of nitrite ions in the medium was then divided by the wet weight of tissues to obtain the amount of nitrite ion production from each segment in the unit of nmoles per mg wet weight. In the majority of instances differences between mean force developed (mN) in segments from the same animal were assessed by a paired Student's t-test (two-

tailed). When responses were normalised, relative to either the maximum contractile response in the corresponding control segment, or pre-existing tone (dilator responses), then difference were assessed using a Wilcoxon test. Where there was more than one treatment condition assessed differences were analysed by ANOVA followed by post-hoc Dunnett's test. A $p$ value $< 0.05$ was considered statistically significant.

## 3.5 Results

### 3.5.1 Contraction Studies

Overnight incubation of segments with either 10μM quercetin alone or 10μM myricetin alone (followed by subsequent removal) was associated with a significant reduction, 18.6±3.7% (n=21) and 39.3±3.3% (n=7), respectively, in the contractions elicited by KCl (see Table 3.1). Responses to U46619 were also significantly reduced by 25.6±5.6% (n=7), following overnight exposure to 10μM myricetin alone (Table 3.1). Although exposure to 10μM quercetin did not significantly affect U46619-induced contractions (Table 3.1), substance P-induced relaxations were significantly reduced. Similarly, following overnight co-incubation of segments with 10μM Bay 11-7082, a selective inhibitor of NFκB (and subsequent removal), the maximum response to KCl and U46619 were significantly reduced with no alteration in the potency (Table 3.1). Table 3.1 also shows that overnight exposure to 1μM quercetin did not affect either KCl or U46619-induced contractions or substance P-induced relaxations.

KCl and U46619 elicited concentration-dependent contractions of the porcine coronary artery (Figure 3.1a and 3.1b), with a potency ($pD_2$) of 1.59±0.01 (n=11) and 7.96±0.05 (n=11), respectively. Overnight exposure of the porcine coronary artery to 1μg/ml LPS significantly reduced the maximum response to KCl to 68.8.±3.1% (n=11) of control without significantly altering the potency ($pD_2$ 1.55±0.02). Similarly, the maximum response to U46619 was reduced to 71.4±2.1% (n=11) of control with no alteration in the potency ($pD_2$ – 7.89±0.07). Following overnight co-incubation of segments with LPS and 10μM Bay 11-7082

(and subsequent removal), submaximal and maximal responses to both agonists were significantly increased (Figure 3.1), with the maximum contractions equivalent to 84.9±3.1% (KCl) and 89.8±2.6% (U46619) of the control responses (Figure 3.1). The potency of the agonists was not altered (data not shown). As shown in Figures 3.1c and 3.1d, LPS-induced suppression of the maximum responses to KCl (31.9±4.5%, n=12) and U46619-induced contractions (28.9±12.4%, n=12) were abolished following post-incubation exposure to 10μM 1400W, a selective inhibitor of inducible nitric oxide synthase.

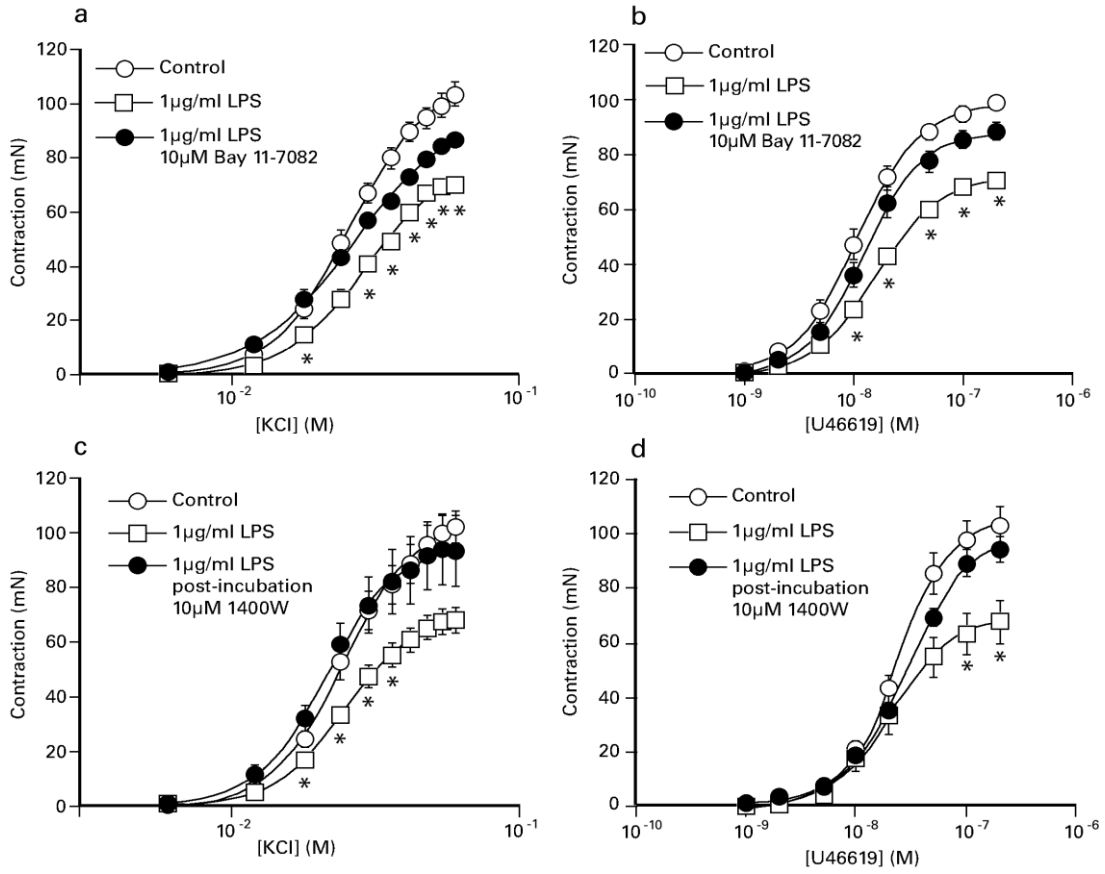



**Figure 3-1** The effect of overnight exposure of the porcine coronary artery to 1µg $mL^{-1}$ LPS with or without 10µM Bay 11-7082 on (a) KCl- and (b) U46619-induced contraction and 10µM 1400W on (c) KCl and (d) U46619-induced contraction. The responses shown are as mean ± SEM of 11-12 observations. *-($p$ <0.05) denote a statistically significant difference between the responses for the paired LPS-treated preparations.

**Table 3-1** Effect of Bay 11-7082, quercetin and myricetin on the maximum response (mN) and potency ($pD_2$) of KCl and U46619 contractions and SP-induced relaxation in isolated porcine coronary arteries incubated for 16 h in modified Krebs-Henseleit solution. Values shown are the mean ± SEM of 7-21 observations.

| | KCl | | U46619 | | SP |
|---|---|---|---|---|---|
| | Max (mN) | $pD_2$ | Max (mN) | $pD_2$ | % relaxation |
| Control (n=11) | 1015±4.3 | 1.59±0.01 | 96.8±2.3 | 7.96±0.04 | Nd |
| 10µM Bay 11-7082 | 83.5±3.3** | 1.57±0.02 | 86.1±2.0** | 7.91±0.08 | Nd |
| Control (n=21) | 124.6±9.2 | 1.54±0.02 | 120.6±9.6 | 7.67±0.07 | 37.1±4.9 |
| 10µM Quercetin | 98.1±6.7** | 1.62±0.03 | 109.8±6.7 | 7.88±0.06 | 24.1±3.1** |
| Control (n=7) | 121.6±7.3 | 1.68±0.04 | 110.8±6.4 | 8.11±0.08 | 16.7±7.6 |
| 10µM Myricetin | 73.5±5.2** | 1.68±0.01 | 82.6±7.2** | 7.97±0.08 | 7.2±1.4 |
| Control (n=8) | 94.4±6.3 | 1.54±0.02 | 93.2±4.1 | 8.01±0.03 | 38.8±2.0 |
| 1µM Quercetin | 9.63±0.64 | 1.52±0.02 | 9.50±0.42 | 8.01±0.03 | 35.2±2.5 |

*- ($p$ <0.05) and **- ($p$ < 0.01) denote a statistically significant difference from the paired control preparation.
nd – denotes not done

Overnight co-incubation of porcine coronary artery segments with 1μg $mL^{-1}$ LPS and 10μM quercetin (and subsequent removal) increased responses to both KCl and U46619 compared to that of LPS alone (Figure 3.2). In contrast, overnight incubation with 10μM myricetin did not affect LPS-induced inhibition of KCl and U46619-induded contractions (Figure 3.2). At the end of the U46619 concentration responses curve the addition of 10nM substance P produced a transient relaxation (25.9±5.6%, n=13) in control preparations (see also Table 3.1) that was significantly reduced ($p$ <0.01) following LPS-treatment (9.1±2.0%, n=13). As shown in Table 3.2, the inhibitory effect of LPS on substance P-induced relaxations was prevented by co-incubation with 1μM and 10μM quercetin. In contrast, substance P-induced relaxations were not significantly different between segments incubated overnight with either 1μg $mL^{-1}$ LPS or 1μg $mL^{-1}$ LPS and 10μM myricetin (Table 3.2).

**Table 3-2** Effect of quercetin, myricetin and Bay11-7082 compound on the maximum response (mN) and potency ($pD_2$) of KCl and U46619 contractions and SP-induced relaxation in isolated porcine coronary arteries incubated for 16 h in modified Krebs-Henseleit solution in the presence of 1μg $mL^{-1}$ LPS. Values shown are the mean ± SEM of 7-26 observations.

| | **KCl** | | **U46619** | | **SP** |
|---|---|---|---|---|---|
| | Max (% control) | $pD_2$ | Max (% control) | $pD_2$ | % Relaxation |
| LPS (n=13) | 60.1±3.5 | 1.62±0.03 | 71.2±3.4 | 7.72±0.06 | 9.35±1.9 |
| LPS 10μM Quercetin | 85.8±4.68* | 1.57±0.04 | 83.5±6.5* | 7.87±0.08* | 22.5±3.9* |
| LPS (n=7) | 58.6±5.9 | 1.64±0.04 | 70.3±4.4 | 7.89±0.06 | 5.4±0.6 |
| LPS 10μM Myricetin | 65.9±8.2 | 1.59±0.04 | 66.3±7.8 | 7.97±0.08 | 9.1±3.0 |
| LPS (n=26) | 62.6±3.0 | 1.59±0.03 | 77.6±3.4 | 7.72±0.03 | 8.6±1.4 |
| LPS 1μM Quercetin | 95.0±4.7** | 1.60±0.02 | 110.0±7.4** | 7.84±0.03* | 23.6±3.4** |
| LPS (n=13) | 64.0±4.8 | 1.53±0.04 | 75.5±3.4 | 7.73±0.04 | 7.9±2.0 |
| LPS 0.1μM Quercetin | 83.7±5.8** | 1.57±0.03 | 90.8±5.6* | 7.92±0.05* | 12.0±3.4 |
| LPS (n=10) 1μM Quercetin | 85.3±2.0 | 1.62±0.02 | 87.3±3.6 | 7.99±0.03 | 32.5.0±5.2 |
| LPS 1μM Quercetin 10μM Bay 11-7082 | 79.5±5.3 | 1.65±0.02 | 79.5±5.7 | 7.96±0.04 | 25.4±4.0 |

*- ($p$ <0.05) and **- ($p$ < 0.01) denote a statistically significant difference from the paired LPS preparation or the paired LPS & 1μM quercetin preparation.

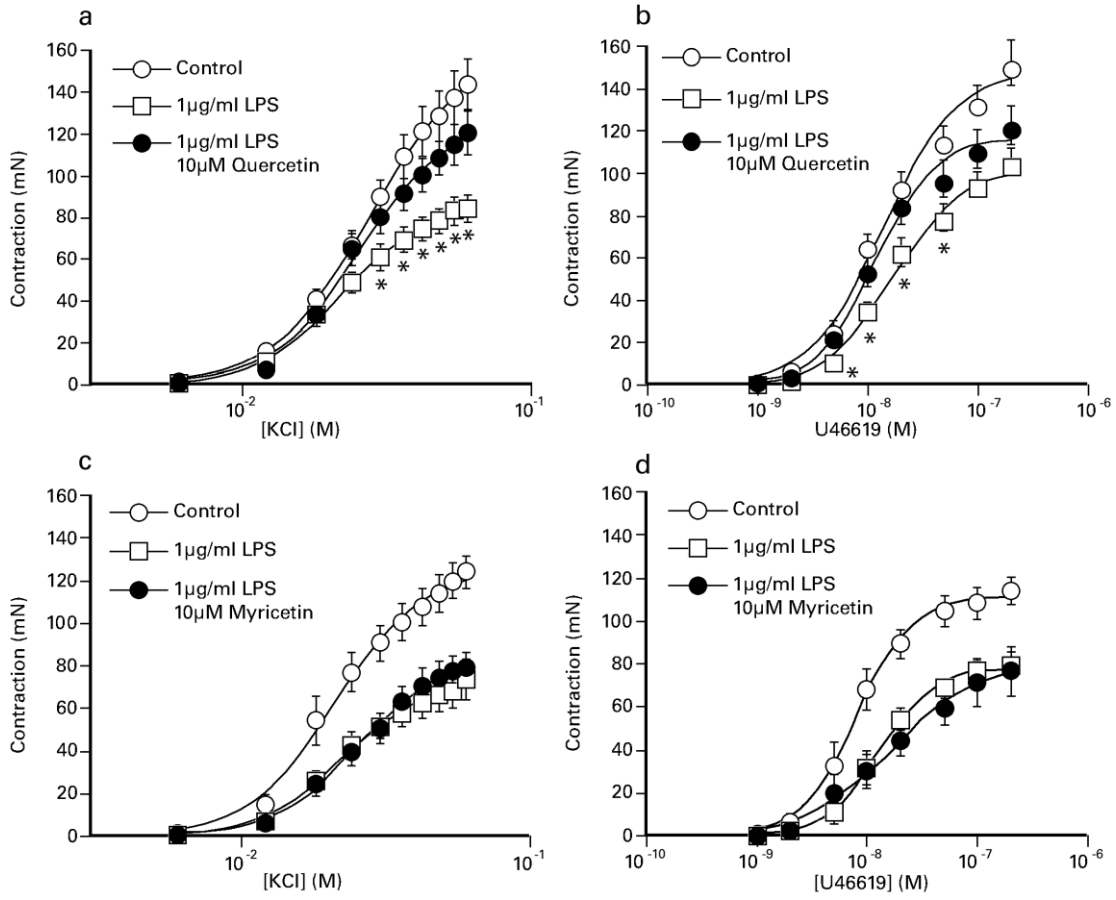



**Figure 3-2** The effect of overnight exposure of the porcine coronary artery to 1µg $mL^{-1}$ LPS, in the presence or absence of either (a, b) 10µM quercetin or (c, d) 10µM myricetin, on responses elicited by KCl and U46619. The responses shown are as mean ± SEM of 7-13 observations *- ($p$ <0.05) denote a statistically significant difference between the responses for the paired LPS-treated preparations.

Figure 3.3 shows that porcine coronary artery segments exposed overnight to a combination of 0.1μM quercetin with 1μg $mL^{-1}$ LPS, or 1μM quercetin with 1μg $mL^{-1}$ LPS, elicited larger contractions to both KCl and U46619 compared with segments exposed to 1μg $mL^{-1}$ LPS alone. Prior exposure to 1μM or 10μM quercetin prevented the inhibitory effect of 1μg $mL^{-1}$ LPS on substance P-induced relaxation and was associated with a small increase in the potency of U46619 (Table 3.2). The impairment of KCl and U46619 responses caused by 1μg $mL^{-1}$ LPS was also significantly reduced by prior exposure to either 10 μM quercetin-3'-sulphate or 10 μM quercetin-3-glucuronide (Figure 3.4). Similarly, substance P-induced relaxations following exposure to LPS were significantly larger when preparations were co-incubated with the metabolites (LPS 20.3±5.7 vs. quercetin-3`-sulphate 49.5±4.8, n=8) and (LPS 10.03±4.5 vs. quercetin-3-glucuronide 30.9±8.7, n=10). Figure 3.5 shows that while both 1μM quercetin and a combination of 1μM quercetin and 10μM Bay K 11-7082 were able to oppose the inhibitory effect of LPS on constrictor responses and endothelium-dependent relaxations, there was no difference between the two conditions (see Table 3.2).

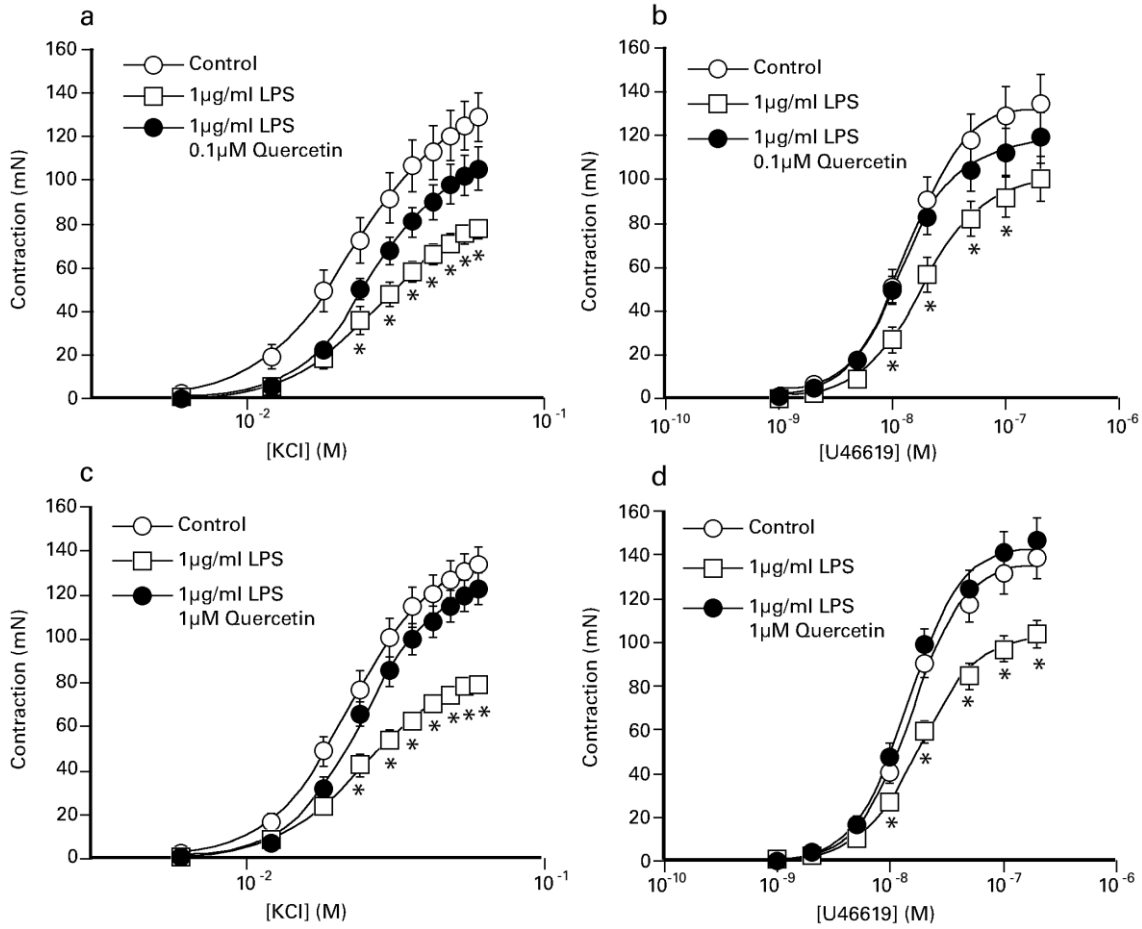



**Figure 3-3** The effect of overnight exposure of the porcine coronary artery to 1µg $mL^{-1}$ LPS, in the presence or absence of either (a, b) 0.1µM quercetin or (c, d) 1µM quercetin, on responses elicited by KCl and U46619. The responses shown are as mean ± SEM of 13-26 observations. *- ($p$ <0.05) denote a statistically significant difference between responses for the paired LPS-treated preparations.

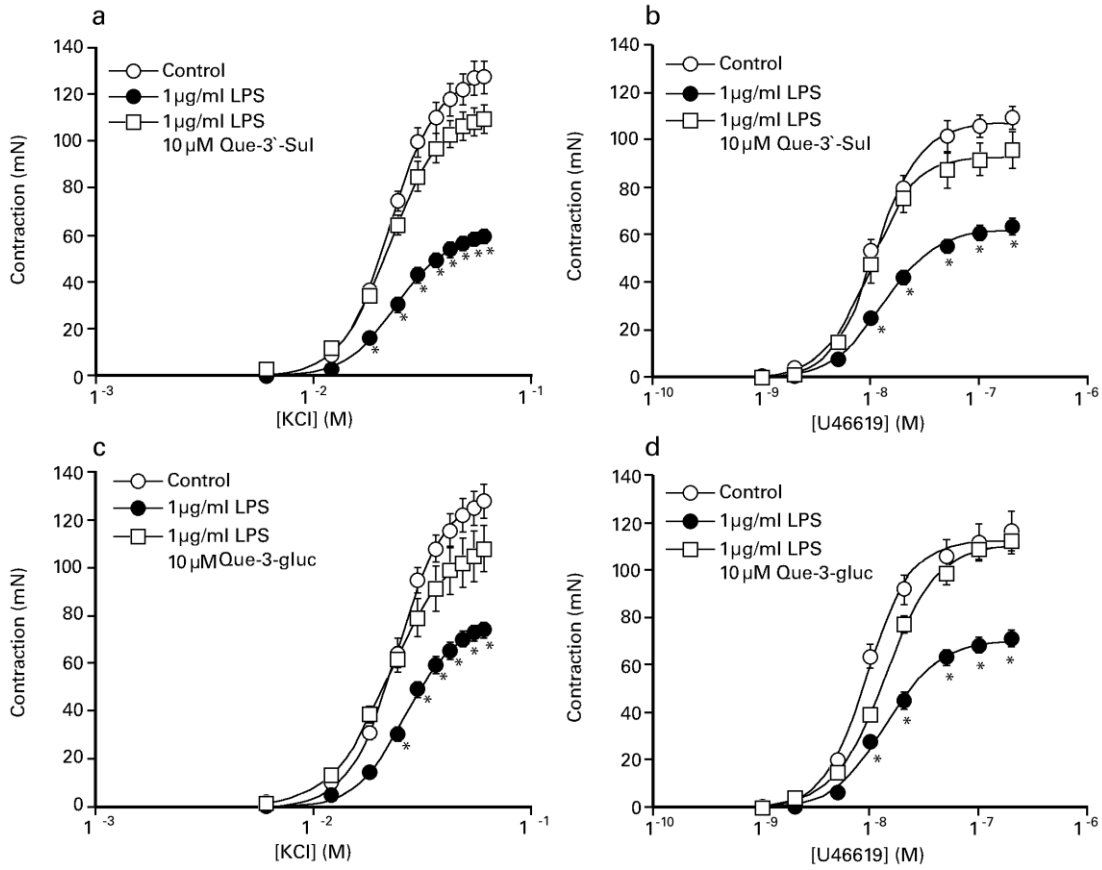



**Figure 3-4** The effect of overnight exposure of the porcine coronary artery to 1µg $mL^{-1}$ LPS, in the presence or absence of either (a, b) 10µM quercetin-3`-sulphate or (c, d) 10µM quercetin-3-glucuronide, on responses elicited by KCl and U46619. The responses shown are as mean ± SEM of 8-10 observations. *- ($p$ <0.05) denote a statistically significant difference between responses for the paired LPS-treated preparations.

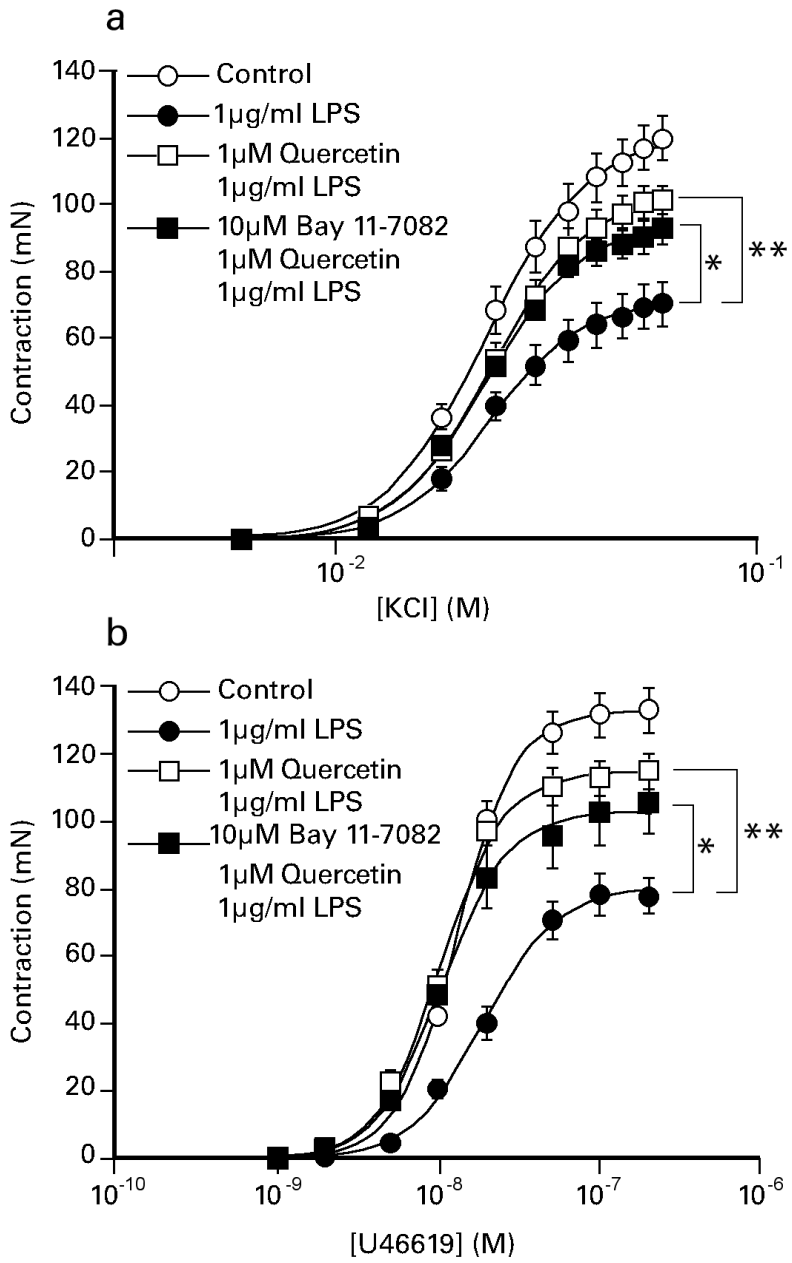



**Figure 3-5** The effect of overnight exposure of the porcine coronary artery to 1µg/ml LPS in the presence or absence of either 1µM quercetin, or 1µM quercetin and 10µM Bay K 11-7082 on responses to either (a) KCl or (b) U46619. The responses are shown as the mean ± SEM of 10 observations and significant differences between the maximum responses in preparations treated with LPS are shown as * ($p < 0.05$) or ** ($p < 0.01$) based on ANOVA followed by Dunnett post-hoc test.

### 3.5.2 Nitrite accumulation

Following overnight storage in DMEM control segments of the porcine coronary artery produced between 3 to 30 nmol nitrate/nitrite per mg wet weight. Exposure to 1μg $mL^{-1}$ LPS was associated with a 5 to 20-fold increase in the production of nitrite ions by the coronary artery (Table 3.3). Co-incubation of the porcine coronary artery with either 10μM 1400W or 1μM Dexamethasone reduced the LPS response by 77.6±5.3% (n=12) and 70.9±4.8% (n=12), respectively, while 10μM Bay 11-7082 abolished the response (Table 3.3).

While prior exposure to 10μM quercetin caused a significant 89.6±4.4% (n-12) reduction in LPS-induced nitrite production (Figure 3.7a), exposure to 10μM myricetin did not affect LPS-induced increase in nitrite production (Figure 3.7b). Table 3.3 shows that prior incubation with either 0.1μM and 1μM quercetin reduced LPS-induced nitrite production by 50.0±12.5% (n=9) and 73.1±12.0% (n=16), respectively. Similarly, exposure to 10μM quercetin 3'-sulphate and 10μM quercetin 3-glucuronide significantly reduced LPS-induced production by 77.0±5.9% (n=11) and 91.6±4.8% (n=11), respectively (Figure 3.8).

### 3.5.3 Immunohistochemistry

Evidence for the induction of nitric oxide synthase was provided by immunohistochemical examination of the porcine coronary artery. Figure 3.6 shows that control preparations express CD31 on endothelial cells but no evidence of inducible nitric oxide synthase. Segments treated overnight with 1μg $mL^{-1}$ LPS exhibited increased expression of endothelial CD31 and the appearance of inducible nitric oxide synthase in the tunica adventitia (but not the tunica media). Co-incubation with 10μM quercetin reduced LPS-induced expression of endothelial

CD31 and adventitial nitric oxide synthase (Fig 3.6). Similar observations were noted in three other preparations of the coronary artery.

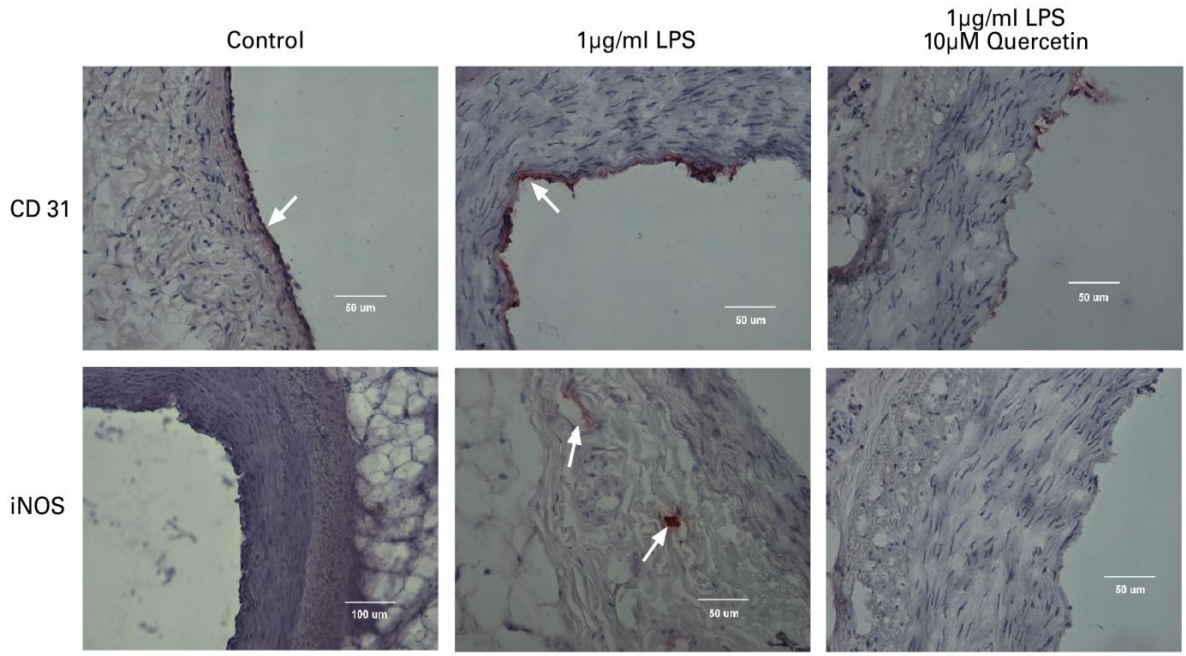



**Figure 3-6** Immunohistochemical localization of (upper panels) PECAM-1 (CD31) and (lower panels) inducible nitric oxide synthase (iNOS) in the porcine isolated coronary artery following incubation in modified Krebs-Henseleit solution for 16-18 hours at 37$^{o}$C without (Control) or with either 1µg mL$^{-1}$ LPS or 1µg mL$^{-1}$ LPS and 10µM quercetin (quercetin added 60 min before LPS).
Evidence for the presence of these proteins in either the endothelium or tunica adventitia is shown the presence of red staining either 100µm or 50µm.

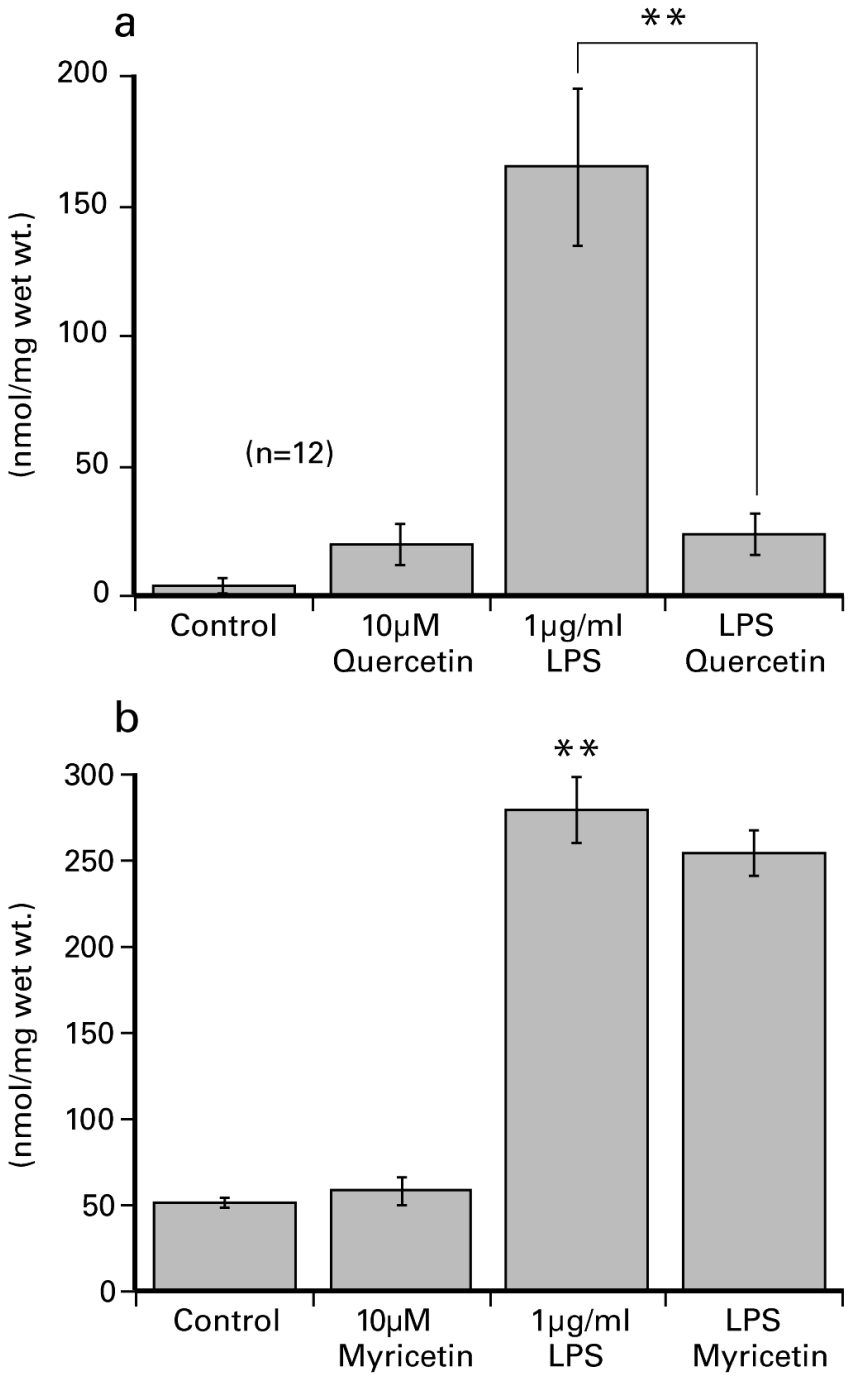



**Figure 3-7** The effect of 24 hour exposure to 1µg/ml LPS, (a) 1µg $mL^{-1}$ LPS plus 10µM quercetin and (b) 1µg/ml LPS plus 10µM myricetin on nitrite production in porcine coronary artery segments incubated in DMEM. The responses shown are the mean ± SEM of 8-12 observations. ** ($p$ <0.01) denotes significance difference from control preparations based on ANOVA followed by Dunnett post-hoc test.

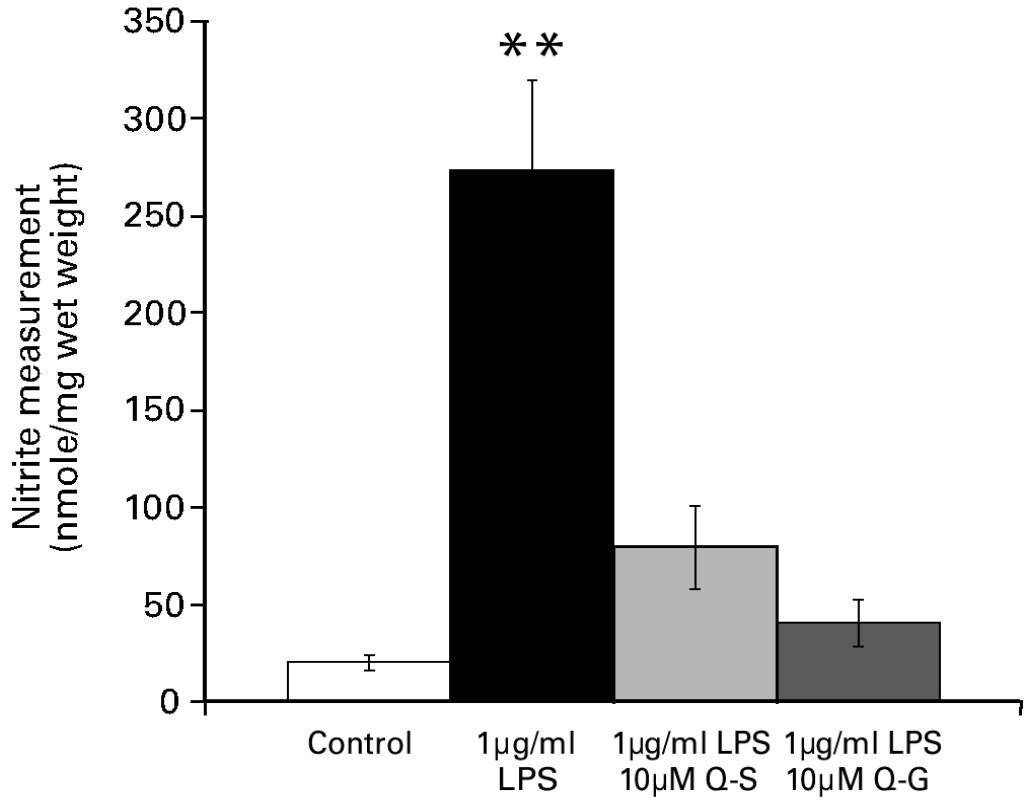



**Figure 3-8** Effect of inhibitors of nitric oxide synthase and various flavonoids on nitrite production of the porcine isolated coronary artery incubated for 16 h in modified Krebs-Henseleit solution in the absence or presence of 1µg $mL^{-1}$ LPS. Responses shown are the mean ± SEM of 9-16 observations. ** ($p$ <0.01) denotes significance difference from control preparations based on ANOVA followed by Dunnett post-hoc test.

Q-S quercetin-3`-sulphate
Q-G quercetin-3-glucuronides

**Table 3-3** Effect of inhibitors of nitric oxide synthase and various flavonoids on nitrite production of the porcine isolated coronary artery incubated for 24 h in DMEM in the absence or presence of 1μg $mL^{-1}$ LPS.

| | **Nitrite nmol/mg wet wt (n=12)** | **Nitrite nmol/mg wet wt (n=12)** | **Nitrite nmol/mg wet wt (n=9)** | **Nitrite nmol/mg wet wt (n=16)** |
|---|---|---|---|---|
| **Control** | 7.4±3.2 | 5.5±0.9 | 17.8±7.6 | 21.1±6.7 |
| **LPS** | 99.8±10.6 | 32.5±0.5 | 138±29.1 | 105±18.8 |
| **LPS & Flavonoid or Inhibitor** | 30.8±3.3** (10μM 1400W) | 6.4±0.9** (10 μM Bay 11-7082) | 70.6±20.0** (0.1μM Quercetin) | 37.4±10.2** (1μM Quercetin) |
| **LPS & Flavonoid or Inhibitor** | 33.1±5.6** (1μM Dexameth') | | | |

Values shown are the mean ± SEM of 9-16 observations. *- ($p$ <0.05) and **- ($p$ < 0.01) denote a statistically significant difference from the LPS preparation (paired Student's t-test) or ANOVA with a post-hoc Dunnett test.

Dexameth – Dexamethesone.

In light of the observations that quercetin, but not myricetin, reduced LPS-induced nitrite production in the coronary artery (Figure 3.7), I also examined the effect of prior exposure to 10μM myricetin against the inhibitory effect of 1μM quercetin. Figure 3.9 shows that 1μM quercetin alone reduced LPS-induced nitrite production by 88.4±2.4% (n=12) but was weakly active (23.6±17.1% reduction) in the presence of 10μM myricetin.

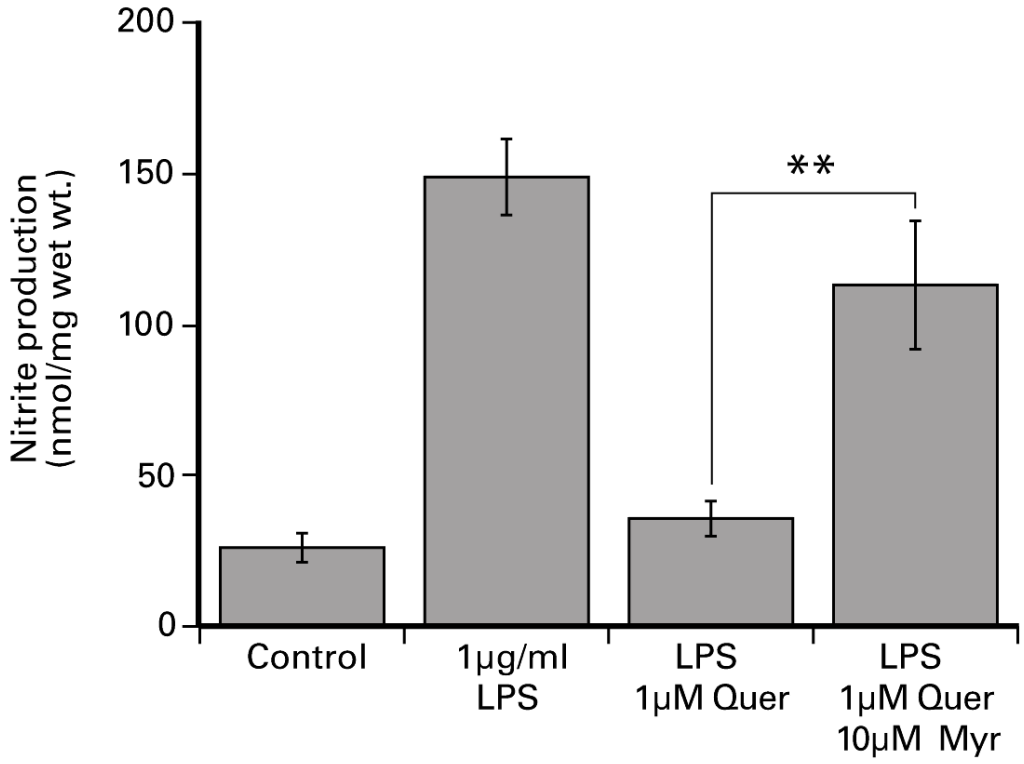



**Figure 3-9** The effect of 24 hour exposure to 1µg $mL^{-1}$ LPS, 1µg $mL^{-1}$ LPS plus 1µM quercetin (Quer) and 1µg/ml LPS with a combination of 10µM myricetin (Myr) and 1µM quercetin on nitrite production in porcine coronary artery segments incubated in DMEM. The values shown are the mean ± SEM of 12 observations. * ($p$<0.05) denotes a significant difference from control by ANOVA followed by Dunnette post–hoc test.

## 3.6 Discussion

The principal observation in this study is that quercetin and its metabolites, quercetin 3'-sulphate and quercetin 3-glucoronide, inhibit key inflammatory changes in the porcine isolated coronary artery induced by prolonged exposure to lipopolysaccharide. The basis of this potentially beneficial effect of the flavonoid can be largely attributed to prevention of the induction of nitric oxide synthase.

### 3.6.1 LPS-induced changes in the porcine coronary artery

Prolonged exposure of the coronary artery to LPS induced a significant reduction in contractile responses to both KCl and U46619, with a slightly greater effect against the former, and an impairment of endothelium-dependent relaxations to substance P. The effect of LPS on contractile responses is qualitatively similar to that noted in other studies (Gibreal *et al*., 2000; Piepot *et al*., 2000 ), but was not accompanied by a reduction in the potency of either agonist. In the case of substance P-induced relaxations, suppression of endothelium-dependent responses by LPS has been previously reported for the porcine coronary artery (Qi *et al*., 2007).

The above changes in vascular responsiveness were accompanied by increased production of nitrite ions (as determined by the Griess reaction) and immunohistochemical evidence for induction of nitric oxide synthase in the blood vessel. The adventitial location for inducible nitric oxide synthase in the coronary artery is similar to that reported in the rat aorta (Kleschyov *et al*., 1998), where exposure to LPS caused a 4 to 10-fold greater activity for nitric oxide production in the tunica adventitia compared to the tunica media. Pharmacological evidence for a

link between the biochemical and contractile changes detected was provided by the finding that post-incubation exposure to 1400W, a selective inhibitor of inducible nitric oxide synthase (Garvey *et al*., 1997b), reversed the impairment of contractile responses in the porcine coronary artery by LPS and reduced nitric oxide formation. Since the effect of 1400W on LPS-induced changes in the coronary artery were mimicked by inhibition of NFκB (with Bay 11-7082) it would appear that activation of this pathway precedes induction of nitric oxide synthase, as described in macrophages (Chen *et al*., 2005).

### 3.6.2 The effect of quercetin and quercetin metabolites

Quercetin, quercetin 3'sulphate and quercetin 3-glucuronide suppressed LPS-induced changes in contractile responses and the elevation in nitric oxide production in the coronary artery, with the aglycone exhibiting activity at concentrations as low as 0.1μM. The effect on contractile responses is qualitatively similar to that reported for eritoran, a Toll-like receptor 4 antagonist, against LPS in rat aortic segments (Ehrentraut *et al*., 2007), which suggests that these flavonoids may have therapeutic potential. In the case of quercetin, immunohistochemical evidence was also obtained that this effect was due to suppression of the induction of nitric oxide synthase in the tunica adventitia; which excludes the possibility of a direct inhibitory effect on the enzyme (cf. 1400W). Thus, the effect of quercetin is comparable to that observed for dexamethasone on nitric oxide production, which suggests an action primarily on the expression of the enzyme (Korhonen, 2002). Quercetin has been reported to inhibit LPS-induced nitric oxide production in various non-vascular cells, including RAW 264.7 macrophages (Chen *et al*., 2001), J774.1 macrophages (Hamalainen *et al*., 2007;

Raso *et al*., 2001) and mouse BV-2 microglia (Chen *et al*., 2005). Crucially, however, the potency of quercetin in the porcine isolated coronary artery is approximately 10-30-fold greater than that noted in cultured cells, where it is typically > 3μM. The efficacy of quercetin and its metabolites in this vascular model stands in marked contrast to that of myricetin, which failed to modify LPS-induced suppression of contractile responses.

Quercetin-induced changes of nitric oxide production superficially mirrors the effects observed on LPS-induced suppression of contractile responses. While this point is reinforced by the lack of effect of myricetin on LPS-induced nitrite/nitrate production, the precise relationship between biochemical events and functional changes is clearly complex. The highest concentration of quercetin examined (10μM) significantly impaired vasoconstrictor responses and substance P-mediated relaxations *per se* (see: Table 3.1). Thus, the overall effect of 10μM quercetin on LPS-induced changes in contractile responses is the sum of two opposing actions and less than that observed with 1μM quercetin - but comparable to that noted for the overall effect of 0.1μM quercetin. Also, it remains a possibility that the overall effect of LPS on contractile responses is product of the induction of several inflammatory mediators rather than just nitric oxide (see: (Qi *et al*., 2007).

It is well recognised that a key mediator of inflammatory responses in cells is the translocation of nuclear factor-κB (NFκB) from the cytoplasm to the nucleus and activation of numerous genes, including those for nitric oxide synthase and pro-inflammatory cytokines (Liu and Malik, 2006). In the case of endothelial and vascular smooth muscle cells NFκB has been linked to increased expression of cell

adhesion molecules (Read *et al*., 1994), the induction of nitric oxide synthase (Hattori *et al*., 2003) and associated with development of early atherosclerotic lesions (Hajra *et al*., 2000). Thus, stabilization of the NF-κB/IκB complex in the coronary artery could explain the protective effect of quercetin against LPS-induced changes in contractile responses, nitric oxide production and the expression of PECAM-1 (CD31) found in this study. This possibility is reinforced by the observation that a combination of quercetin and Bay K 11-7082, a known inhibitor of NFκB (Pierce *et al*., 1997) was no more effective against LPS-induced changes in the coronary artery than quercetin alone (see Figure 3.4), yet neither condition completely prevented the inhibitory effect of LPS. While the precise molecular target for this beneficial effect of quercetin was not investigated, the failure of myricetin to mimic the effect of quercetin on LPS-induced nitric oxide production, and to also 'antagonise' the effect of quercetin (see: Figure 3.7), indicates that these structurally-related flavonoids may prove useful in further studies. It is noteworthy that the lack of effect of myricetin on inflammatory responses in the coronary artery is not a selective effect for the vasculature. Blonska and colleagues (2003) noted that quercetin and kaempferol were capable of inhibiting LPS-induced production of IL-1β in RAW 264.7 cells but myricetin was inactive (Blonska *et al*., 2003).

Although the potency of the flavonoids against inflammatory events in the coronary artery is greater than that reported in macrophages, suggesting a selective action on the vasculature, the physiological relevance remains unclear. Quercetin is extensively metabolised in man and the concentrations used in this study exceeded the peak plasma levels of the aglycone (0.03μM) and the metabolites (3μM)

detected following dietary ingestion (Kroon *et al*., 2004; Wang and Morris, 2005). However, two recent studies raise the possibility that flavanoids can be either generated *in situ* from metabolites accumulated in activated cells (Kawai *et al*., 2008a) or even preferentially concentrated in cells (Kawai *et al*., 2008b), potentially reducing the threshold for physiological significance. Nonetheless, further studies on human blood vessels with lower concentrations of quercetin and the metabolites are warranted. In light of the striking difference between the effect of quercetin and myricetin on the porcine coronary artery, there is also a need to establish whether myricetin can inhibit the effect of quercetin in human vascular cells, effectively behaving as an 'antagonist'.

The finding that quercetin can oppose the proinflammatory effect of LPS on the vasculature may also hold therapeutic significance. Recently, quercetin has been shown to attenuate both the release of pro-inflammatory cytokines in response to LPS in mice and the associated lethality (Teng *et al*., 2009). Significantly, this effect of quercetin was manifest even when administered several hours after exposure to LPS, suggesting the possibility that a similar mechanism may occur in the vasculature.

## 3.7 Conclusion

In conclusion I have demonstrated that one of the major dietary flavonoids, quercetin, and its principal human metabolites oppose pro-inflammatory events in both endothelial cells and vascular smooth muscle cells. These effects of quercetin are evident at lower concentrations than previously reported in studies using other cell types and suggests a selective action on the vasculature, particularly against the induction of nitric oxide synthase. Further studies on human blood vessels are warranted to establish whether these observations are relevant to the well-documented beneficial effects of dietary flavonoids (Boots *et al.*, 2008; Halliwell, 2007).

# Chapter 4

# Comparison of the effect of catechins on LPS-induced changes in porcine isolated coronary artery *in vitro*

## 4.1 Introduction

Catechins are present in various species of plant, including the green tea plant, and they are known to exhibit potent anti-inflammatory, anti-oxidative and anti-carcinogenic effects (Beecher *et al*., 1999; Ishikawa *et al*., 1997; Junkun *et al*., 1997; Yang *et al*., 2001). Epidemiological studies have suggested that the intake of catechins correlates with risk reduction for coronary heart disease (Arts *et al*., 2001; Knekt *et al*., 2002). It has been reported that green tea consumption is associated with reduced mortality due to all causes and specifically due to cardiovascular diseases (Kuriyama *et al*., 2006; Suzuki *et al*., 2009). Interestingly, data from a population-based cohort study of 1,169 patients linked chocolate consumption with decreased mortality after myocardial infarction (MI) (Janszky *et al*., 2009). Furthermore, Ikeda and colleagues showed that habitual ingestion of green tea catechins from an early age prevented the development of spontaneous stroke in rat, probably by inhibiting the further development of high blood pressure at late ages (Ikeda *et al*., 2007). A prospective cohort study (Zutphen Elderly study) also provided evidence for a primary protective effect of flavanols (Buijsse *et al*., 2006; Hertog *et al*., 1993; Streppel *et al*., 2009).

Catechins belong to the flavan-3-ol class of flavonoids. The major tea catechins are epigallocathechin-3 gallate (EGCG), epigallocathechin (EGC), and epicathechin-3 gallate (ECG). Recent research has shown that inflammation plays a key role in coronary artery diseases (CAD) and other manifestations of atherosclerosis (Hansson, 2005). Epigallocatechin gallate has been particularly well studied and has been shown to modulate the secretion of a range of pro-inflammatory mediators implicated in the development of cardiovascular conditions. For example, EGCG has

been reported to inhibit lipopolysaccharide-induced TNF-α production in mouse macrophages (Yang *et al*., 1998b), the induction of inducible nitric oxide synthase in mouse macrophages (Lin and Lin, 1997) and IL-8 production by human keratinocytes (Tang and Meydani, 2001) or endothelial cells, (Chen *et al*., 2002; Tronperzinski et al., 2003). Al-Hanbali and colleagues (2009) reported that epicatechin suppresses IL-6, IL-8 and enhances IL-10 production with NF-kappaB nuclear translocation in a whole blood stimulated system.

The possible anti-inflammatory activity of the green tea catechins may, in large part, be accounted for by their antioxidant actions. EGCG has been found to inhibit the activity of the transcription factors AP-1 and NF-kappa B, both of which may mediate many inflammatory processes and both of which may be activated by reactive oxygen species. These effects are induced by the suppression of inflammatory factors including nuclear factor-kappa B (NF-κB), a multipotential promoter of inducible nitric oxide synthase (iNOS) and adhesion molecules (Yang *et al*., 2001). Moreover, it has been demonstrated that epigallocatechin-3-gallate blocks the induction of nitric oxide synthase by down-regulating lipopolysaccharide-induced activity of transcription factor nuclear factor-kB (Lin and Lin, 1997 ).

In contrast to the beneficial effects of catechins on vascular homeostasis described above, some studies reported that catechins can exert contractile effects in rat aorta and impair endothelium-dependent vasorelaxation (Sanae *et al.*, 2002; Shen *et al*., 2003). It was proposed that the inhibition of vasorelaxation by catechins could be due to inactivation of endothelium-derived NO (Sanae *et al*., 2002).

While the characteristics of the effects of green tea catechins have been well documented in various cell lines (Benelli *et al*., 2002), there is much less information regarding their acute effect on isolated blood vessels and relatively little information regarding their ability to modulate inflammatory events in the vasculature. Recent studies with flavonoid-containing beverages suggest that the benefits of these nutrients may relate, in part, to improved endothelial function. For example, Widlansky and colleagues found that acute epigallocatechin gallate supplementation improves endothelial function in humans with coronary heart disease (Widlansky *et al.*, 2007). Additionally, it was reported that epicatechin has vasorelaxant effects in rat mesenteric artery (Huang *et al*., 1998) and in rat aorta (Andriambeloson *et al.,* 1998; Benito *et al*., 2002) through the enhancement of nitric oxide production (Huang *et al*., 1999). Previous work in this lab found that acute exposure to quercetin caused endothelium-independent relaxation of porcine coronary artery that was associated with a selective enhancement of responses involving elevation of cyclic GMP (Suri *et al.*, 2010). Recently we have demonstrated that prolonged incubation with quercetin and two of its metabolites, quercetin-3`-sulphate and quercetin-3-glucuronide, inhibited the LPS-induced changes in endothelial and vascular responses and the elevation of nitrite ion production in porcine coronary artery (chapter 3). Therefore, the aim of this chapter is to investigate the effects of flavanol type flavonoids (catechins, epicatechin and epigallocatechin gallate) on the vascular reactivity and the generation of nitric oxide of porcine isolated coronary artery exposed to LPS *in vitro*.

## 4.2 Material and Methods

Porcine hearts were obtained from a local abattoir and transported to the laboratory in ice-cold Krebs-Henseleit bicarbonate buffer (K-H) solution within one hour. The anterior branch of the porcine coronary artery 5 mm length, 2-3 mm internal diameter was dissected and cleaned of fat and connective tissues.

### 4.2.1 Contraction Studies

For contraction experiments, single segments of the porcine coronary artery were incubated in 2ml K-H solution (previously gassed with 95% $O_2$ and 5% $CO_2$ for 5 min.) containing 2% ficoll and a combination of 60μg/ml benzyl penicillin and 20μg/ml streptomycin sulphate and 10% FCS. The inclusion of ficoll in the bathing medium was to prevent swelling of the tissue. The solution also contained 1μg $mL^{-1}$ LPS, various catechins or a combination of LPS and the flavanol (added 60 min before LPS) and then sealed in a sterilized glass vial and stored overnight at 37°C for 16-18 h. In some experiments the effect of various concentrations of epicatechin in the presence of LPS were examined. Unless, indicated otherwise, all experiments were conducted on nominally endothelium-intact segments of the coronary artery. In all instances the segments were effectively paired so that each experimental condition had a control segment taken from the same animal.

On the following day, segments were taken out of the incubation solution and prepared for isometric tension recording as described in Chapter 2. After repeated exposure to 60mM KCl to establish the reproducibility of responses, each

preparation was exposed to cumulative concentrations of potassium chloride (KCl, 6-60mM), and then to the stable thromboxane mimetic analog,9,11-dideoxy-11a,9a-epoxymethanoprostaglandin $F_2\alpha$ (U46619, 1-200nM) to evaluate the constrictor response of the tissue. After attainment of a stable response, the segments were then exposed to a single concentration of substance P (10nM).

### 4.2.2 Nitrite Determination

For the measurement of nitrite ion accumulation, a marker for nitric oxide production (Kelm 1999), two 5mm segments were incubated in each vial for 24 hr with aerated DMEM (without phenol red) containing 10% FCS, 1mM L-arginine, 60µg/ml benzyl penicillin and 20µg/ml streptomycin. The segments were exposed either to 1µg $mL^{-1}$ LPS, a flavanol, or combination of LPS and flavanol, as described above. Some preparations were also exposed to either 10µM myricetin, or combination of 0.01 µM epicatechin and 0.03µM simvastatin for 24 hr in the presence of 1µg $mL^{-1}$ LPS. After the incubation period, the segments were removed, briefly blotted on paper cloth and weighed. Nitrite ion accumulation in the incubation medium was determined by the Greiss reaction as previously described (Ukil *et al.*, 2006). Greiss reagent (500 µl of 1% sulphanilamide and 0.1% naphthylethylamine diamine in 5% hydrochloric acid) was added to 500µl of the incubation medium and optical density at 550 nm was measured, using a UNICAM UV/VIS spectrophotometer. A standard concentration-optical density curve was calculated for every assay using sodium nitrite solution against a blank (500µl DMEM & 500µl Griess Reagent). In a separate experiment we established that incubation of DMEM containing either the flavanol, LPS or a combination of both

(without the arterial segments) produced an optical density reading similar to the blank. The minimum detectable amount of nitrite was 375 nmoles.

## 4.3 Solutions and drugs

The composition of Krebs-Henseleit solution is (mM): NaCl 118; KCl, 4.8; $SO_4.7H_2O$, 1.2; $CaCl_2$ $.2H_2O$, 1.3; $NaHCO_3$, 25.0; $KH_2PO_4$, 1.2. Benzyl penicillin streptomycin sulphate, ficoll, LPS (*Eschericha coli* O III :B4) and the catechins were obtained from Sigma-Aldrich Company Ltd (Poole, Dorset, UK.). The catechins used were as follows; (+)-catechin, (+)-epicatechin and (-)-epigallocatechin gallate. Substance P was obtained from Bachem (UK). KCl (potassium chloride) and U46619 (9,11-dideoxy-11a,9a-epoxymethanoprostaglandin $F_2\alpha$) were obtained from Alexis Corporation (Nottingham, UK.). DMEM was supplemented with antibiotics (see above) and 2 mM L-glutamine (Gibco). All drugs were dissolved in distilled water.

## 4.4 Data analysis and statistics

Contractions produced by KCl and U46619 were measured as milliNewtons force (mN). Responses were expressed as the maximum contraction ($E_{max}$), and potency determined as the negative logarithm of the concentration causing 50% of the maximum response (-log $EC_{50}$ or $pD_2$) using a logistic equation (Kaleidagraph, version 3.6 Synergy Software). The values are shown as the mean (±SEM). The content of nitrite ions in the medium was calculated according to the equation deduced from the standard curve. The amount of nitrite ions in the medium was then divided by the wet weight of tissues to obtain the amount of nitrite ion production from each segment, as nmoles/mg wet weight. In the majority of instances differences between mean force developed (mN) in segments from the same animal were assessed by a paired Student's t-test (two-tailed). Where there was more than

one treatment condition assessed differences were analysed by ANOVA followed by post hoc Dunnett's test. A p-value $< 0.05$ was considered statistically significant.

## 4.5 Results

### 4.5.1 The effect of catechins alone

Prior to investigating the effects of the catechins on LPS-induced inflammatory changes in the coronary artery, it was necessary to establish the effect on vascular responsiveness of prolonged (overnight) exposure of the flavonoids. Table 4.1 shows that the overnight exposure of porcine isolated coronary artery to 10μM epicatachin (followed by subsequent removal) did not significantly changed the vascular response to KCl or U46619. In contrast overnight exposure to its isomer (10μM) catechin was associated with a significant reduction in the contractions of KCl and U46619 by 22.7±6.5%, (n=8) and 20.6±5.1%, (n=8), respectively without affecting the sensitivity to both agonists. Similarly, epigallocatechin gallate (EGCG) significantly affected the vascular response of the segments to KCl by 17.2±4.2%, (n=11) but its effect on the contractile response of U46619 failed to reach significance ($p$ = 0.07), as determined by Student t-test. The three compounds did not significantly change the relaxant effect of substance P (Table 4.1).

**Table 4-1** Effect of 10μM epicatechin, 10μM epigallocatechin gallate and 10μM catechin on the maximum response (mN) and potency ($pD_2$) of KCl and U46619 contractions and SP-induced relaxation in isolated porcine coronary arteries incubated for 16 h in modified Krebs-Henseleit solution.

| | KCl | | U46619 | | SP |
|---|---|---|---|---|---|
| | **Max (mN)** | **$pD_2$** | **Max (mN)** | **$pD_2$** | **% Relaxation** |
| **Control (n=12)** | 111.6±5.9 | 1.58±0.03 | 104.8±6.3 | 7.97±0.1 | 37.5±5.5 |
| **10μM Epicatechin** | 104.8±8.8 | 1.57±0.03 | 97.95±8.8 | 7.82±0.1 | 31.6±7.3 |
| **Control (n=11)** | 130.3±8.8 | 1.63±0.01 | 118.5±6.8 | 7.67±0.1 | 36.9±4.3 |
| **10μM EGCG** | 108.7±10.2** | 1.64±0.03 | 93.4±5.8 | 7.61±0.2 | 37.7±7.8 |
| **Control (n=8)** | 101.8±2.9 | 1.39±0.09 | 105.3±1.3 | 7.8±0.1 | 72.6 ±4.7 |
| **10μM Catechin** | 84.2±5.8** | 1.45±0.06 | 84. 2±4.2** | 7.8±0.04 | 70.9 ±6.1 |

Values shown are the mean ± SEM of 8-12 observations.
**- ($p < 0.01$) denote a statistically significant difference from the paired control preparation.

### 4.5.2 Effect of the catechins on 1μg $mL^{-1}$ LPS-induced changes in vascular responses of porcine coronary artery

Figure 4.1 shows the cumulative response curves of KCl and U46619 in porcine isolated coronary artery treated with 1μg $mL^{-1}$ LPS, a combination of 10μM epicatechin and 1μg $mL^{-1}$ LPS (figure 4.1a and 4.1b) or a combination of 10μM catechin and 1μg $mL^{-1}$ LPS (figure 4.1c and 4.1d), or a combination of 10μM epigallocatechin gallate and 1μg $mL^{-1}$ LPS (figure 4.1e and 4.1f). Overnight exposure to 1μg $mL^{-1}$ LPS significantly reduced the maximum responses to KCl and U46619 (to 71.3±4.9%, n=12 and 74.6±4.3% n=12, respectively, of control). Although exposure of the tissue to 10μM epicatechin alone failed to modify responses to either agonist (Table 4.1), co-incubation with 10μM epicatechin caused a significant increase in the contraction effects of KCl and U46619 (figure 4.1a and 4.1b). A significant reduction in the responses to 10nM substance P was noted in arterial segments exposed overnight to 1μg $mL^{-1}$ LPS (Control 36.5±5.5% vs. LPS 23.2±5.9% of U46619-induced tension, n=12). The inhibitory effect of LPS on substance P-induced relaxations was not observed if the segments were co-incubated with 10μM epicatechin (Table 4.2).

Figure 4.1c and 4.1d shows that overnight exposure of the porcine isolated coronary artery to 1μg $mL^{-1}$ LPS caused an approximate 40% reduction in the maximum response to KCl and a 30% reduction in the maximum response to U46619. The addition of the 10μM catechin 60 min before LPS prevented the changes in vascular responses. Substance P (10nM) caused 72.6±4.8%, (n=8) in control tissue, overnight exposure to 1μg $mL^{-1}$ LPS caused a significant reduction on the relaxation

induced by SP. However, the substance P-induced response was similar to the control in segments incubated with LPS and 10μM catechin (Table 4.2).

Figure 4.1e and 4.1f shows that overnight exposure of the porcine isolated coronary artery to 1μg $mL^{-1}$ LPS caused a 37±3.8%, (n=11) reduction in the maximum response to KCl and a 19±4.4% reduction in the maximum response to U46619. Attenuation of the responsiveness to KCl and U46619 in the segments exposed to LPS was prevented by co-incubation with 10μM EGCG. In terms of endothelium-dependent relaxation, substance P caused a 37±4.2% relaxation in control segments; this relaxant effect was impaired to 23±3.1% in presence of LPS, however, the presence of 10μM EGCG in the incubation medium prevented the inhibitory effect of LPS on SP-induced relaxation (Table 4.2).

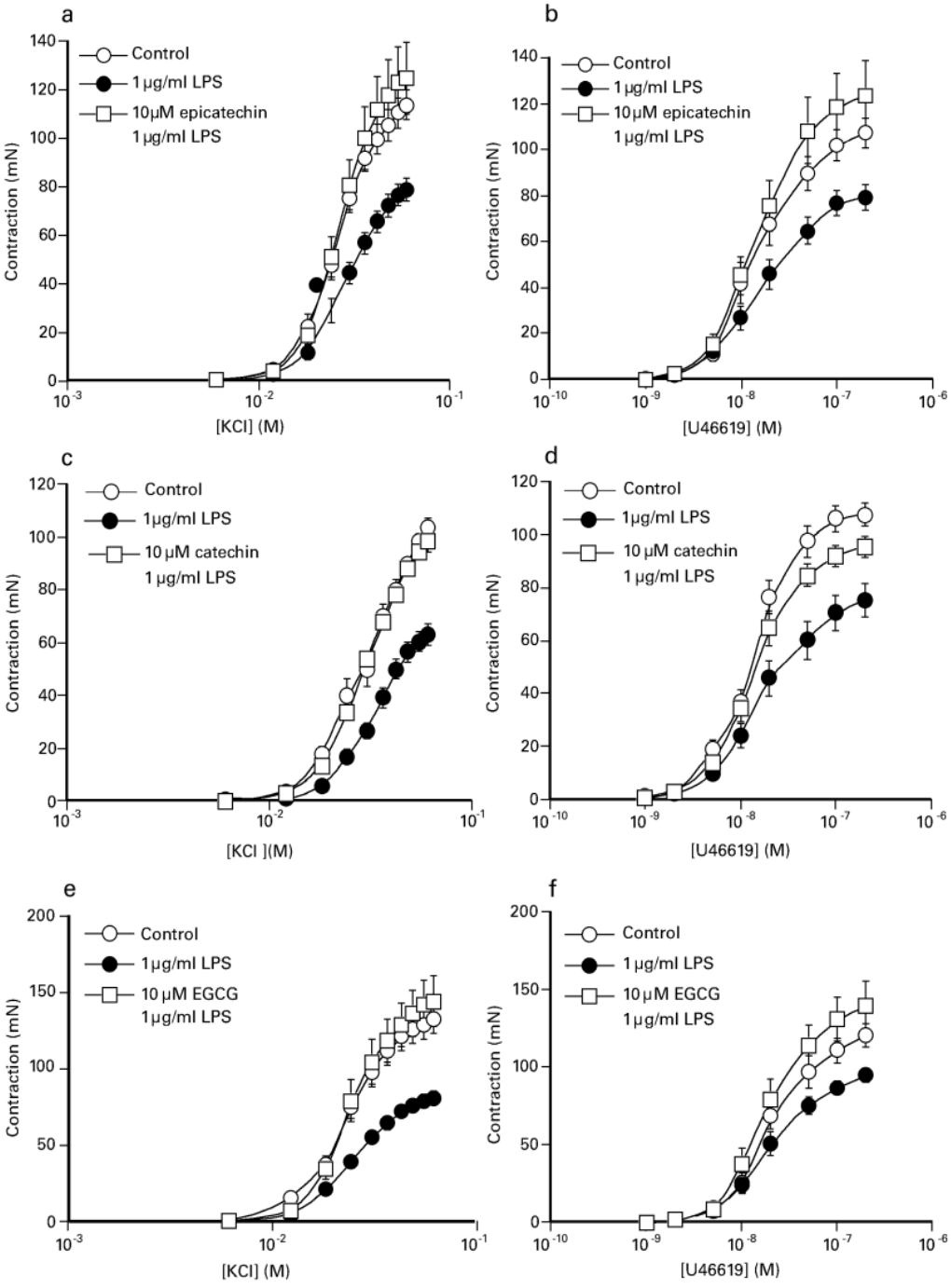



**Figure 4-1** The effect of overnight exposure of the porcine coronary artery to 1 $\mu g \cdot mL^{-1}$ LPS, in the presence or absence of either (a, b) 10µM epicatechin or (c, d) 10µM catechin, or (e, f) 10µM EGCG on responses elicited by KCl and U46619. The responses shown are as mean ± SEM of 8–12 observations.

Figure 4.2a and 4.2b show that LPS caused a significant 3-10-fold increase in the production of nitrite in the porcine isolated coronary artery and this effect was reduced by prior exposure to either 10μM catechin or 10μM epicatechin. Although neither catechin nor epicatechin alone affected the control segments in nitrite content, both flavanols significantly reduced the amount of nitrite induced by LPS (catechin 99.1±2.7%, n=11) (epicatechin 87.6±25.2 %, n=16).

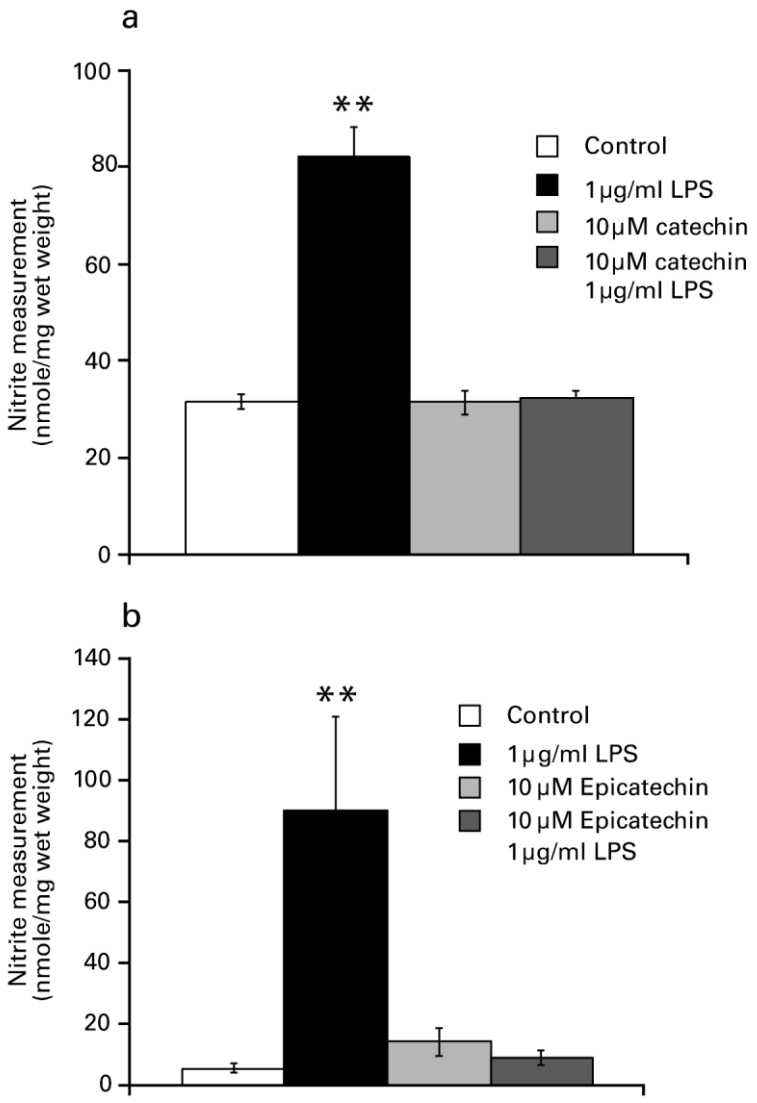



**Figure 4-2** The effect of 24 h exposure to 1 µg·mL$^{-1}$ LPS (a) 1 µg·mL$^{-1}$ LPS plus 10 µM catechin and (b) 1 µg·mL$^{-1}$ LPS plus 10 µM epicatechin on nitrite ion production in porcine coronary artery segments incubated in DMEM. The values shown are the mean ± SEM of 11–16 observations. $^{**}p < 0.01$ significant difference from control by ANOVA.

### 4.5.3 Effect of the epicatechins on 1μg $mL^{-1}$ LPS-induced changes on porcine coronary artery

As preliminary results showed that prolonged exposure to 10μM epicatechin alone did not change the vascular response to KCl and U46619 or the relaxant response to SP (Table 4.1), epicatechin was chosen to study in greater detail.

Figure 4.3 shows that porcine coronary artery segments exposed overnight to a combination of 1μM epicatechin with 1μg $mL^{-1}$ LPS, or to a combination of 0.1μM epicatechin with 1μg $mL^{-1}$ LPS, or to a combination of 0.01μM epicatechin with 1μg $mL^{-1}$ LPS elicited greater contraction to both KCl and U46619 compared to segments that exposed to 1μg $mL^{-1}$LPS alone. Additionally, prior exposure to low concentrations of epicatechin significantly inhibited the impairment effect of LPS on SP-induced relaxation (Table 4.2).

Incubation of segments from porcine coronary artery in DMEM alone for 24 hrs produced about 30nmoles/mg wet weight tissue of nitrite ions in the incubation medium, while exposure to 1μg $mL^{-1}$ LPS caused a 4-fold increase in nitrite ion production. Pre-incubation of the segments with either 1μM epicatechin or 0.1μM epicatechin reduced the LPS-induced nitrite by 95.8±4.9%, (n=8) and 98.1±4.5%, (n=8) respectively (Figure 4.4).

**Table 4-2** Effect of 0.1, 1 and 10μM epicatechin, 10 μM catechin and 10 μM epigallocatechin gallate (EGCG) on the maximum response (mN) and potency ($pD_2$) of KCl and U46619 contractions and SP-induced relaxation in isolated porcine coronary arteries incubated for 16 h in modified Krebs-Henseleit solution in the presence of 1μg $mL^{-1}$ LPS.

| | **KCl** | | **U46619** | | **SP** |
|---|---|---|---|---|---|
| | **Max (%control)** | **$pD_2$** | **Max (% control)** | **$pD_2$** | **% Relaxation** |
| **LPS (n=12)** | 71.30±4.9 | 1.52±0.03 | 74.6±4.3 | 7.74±0.07 | 23.17±5.9 |
| **LPS 10μM Epicatechin** | 109.3±10.55** | 1.56±0.02 | 117.3±14.6* | 7.83±0.05 | 38.7±0.01* |
| **LPS (n=14)** | 62.7±4.2 | 1.58±0.01 | 55.0±3.9 | 7.76±0.03 | 13.4±6.2 |
| **LPS 1μM Epicatechin** | 85.5±5.0** | 1.57±0.03 | 87.6±6.1** | 7.80±0.05* | 34.9±5.9** |
| **LPS (n=14)** | 62.7±4.2 | 1.58±0.01 | 55.0±3.9 | 7.76±0.03 | 13.4±6.2 |
| **LPS 0.1μM Epicatechin** | 101.4±8.4** | 1.57±0.03 | 109.9±11.8** | 7.76±0.06 | 30.1±5.3* |
| **LPS (n=8)** | 49.3±2.6 | 1.40±0.06 | 51.0±6.2 | 7.62±0.2 | 18.5±1.6 |
| **LPS 0.01μM Epicatechin** | 68.9±3.1** | 1.49±0.03 | 77.1±15.6** | 7.86±0.05 | 42.2±8.2** |
| **LPS (n=11)** | 63.3±3.9 | 1.60±0.02 | 81.0±4.4 | 7.73±0.1 | 23.9±3.1 |
| **LPS 10μM EGCG** | 107.2±8.1** | 1.63±0.03 | 115.5±10.8** | 7.73±0.08 | 38.1±4.1** |
| **LPS (n=8)** | 60.8±3.2 | 1.4±0.06 | 70.0±5.5 | 7.6±0.17 | 15.7±2.9 |
| **LPS 10μM Catechin** | 94.5±2.5* | 1.49±0.03 | 89.0±2.6** | 7.8±0.05 | 76.1±2.5** |

Values shown are the mean ± sem of 8-14 observations.* ($p$ <0.05) and ** ($p$ < 0.01) denote a statistically significant difference from the paired control preparation.

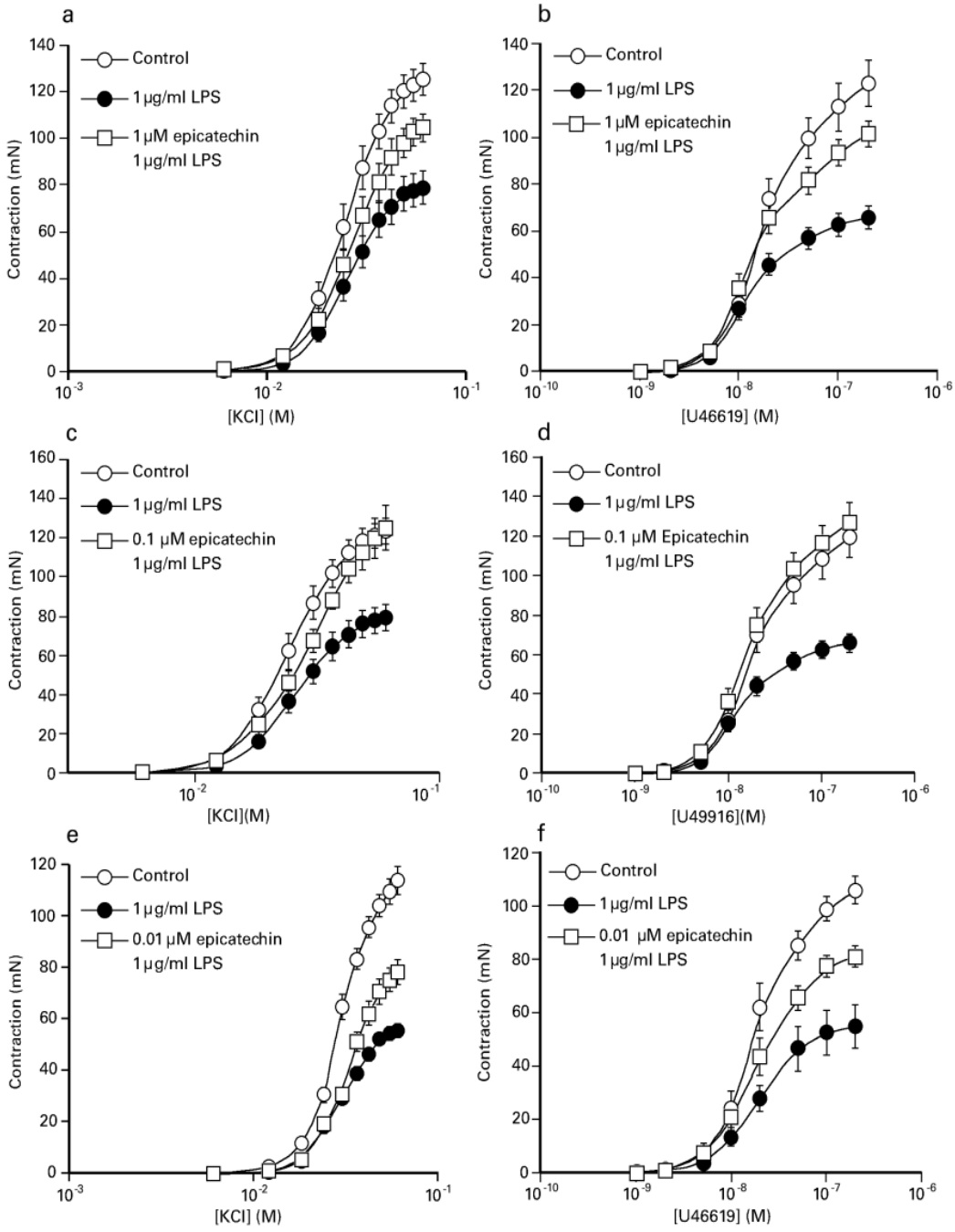



**Figure 4-3** The effect of overnight exposure of the porcine coronary artery to 1 µg $mL^{-1}$ LPS, in the presence or absence of either (a, b) 1µM epicatechin or (c, d) 0.1µM epicatechin or (e, f) 0.01µM epicatechin on responses elicited by KCl and U46619. The responses shown are as mean ± SEM of 8–14 observations.

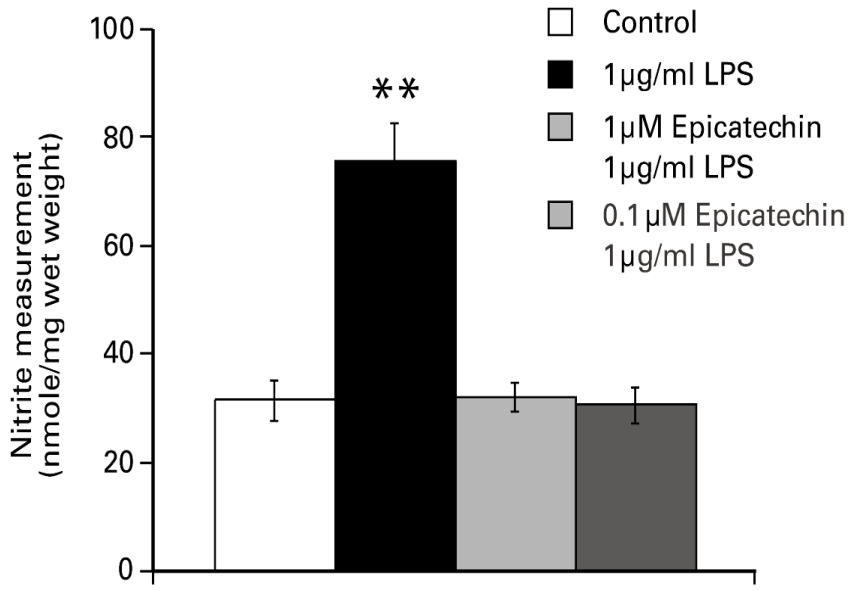



**Figure 4-4** The effect of 24h exposure to 1µg·mL$^{-1}$LPS, 1µg mL$^{-1}$LPS plus 1µM epicatechin and 1µg·mL$^{-1}$LPS plus 0.1µM epicatechin on nitrite ion production in porcine coronary artery segments incubated in DMEM. The values shown are the mean±SEM of 8 observations. $^{**}p < 0.01$ significant difference from control by ANOVA.

In chapter 3, I demonstrated that in contrast to quercetin, myricetin did not modify LPS-induced changes in contractile response. More significantly, myricetin failed to modify LPS-induced changes in nitrite ion production, but was able to block the ability effect of quercetin to do this. This was taken as evidence that myricetin and quercetin work at a common site. In the present study I repeated the myricetin/quercetin experiment on LPS-induced nitrite ion production, though on this occasion the magnitude of the antagonism was less than previously observed (see Figure 4.5a, compared with Figure 3.9). While exposure to 1µM quercetin alone caused a significant reduction (97.5±2.8%, (n=8) in LPS-induced nitrite ion production, prior exposure to 10 µM myricetin significantly reduced the inhibitory effects of quercetin (61±12.5%, n=8). To examine whether epicatechin behaves in the same way as quercetin, I examined this inhibitory effect of myricetin on flavanol type flavonoid (epicatechin). Figure 4.5b shows that 24 hour exposure of the segments to LPS was associated with 5-fold increase in nitrite production compared to the segment incubated in DMEM alone. In contrast to Figure 4.5a, prior exposure of the segment to 10µM myricetin did not affect the inhibitory effect of 1µM epicatechin on LPS-induced nitrite production.

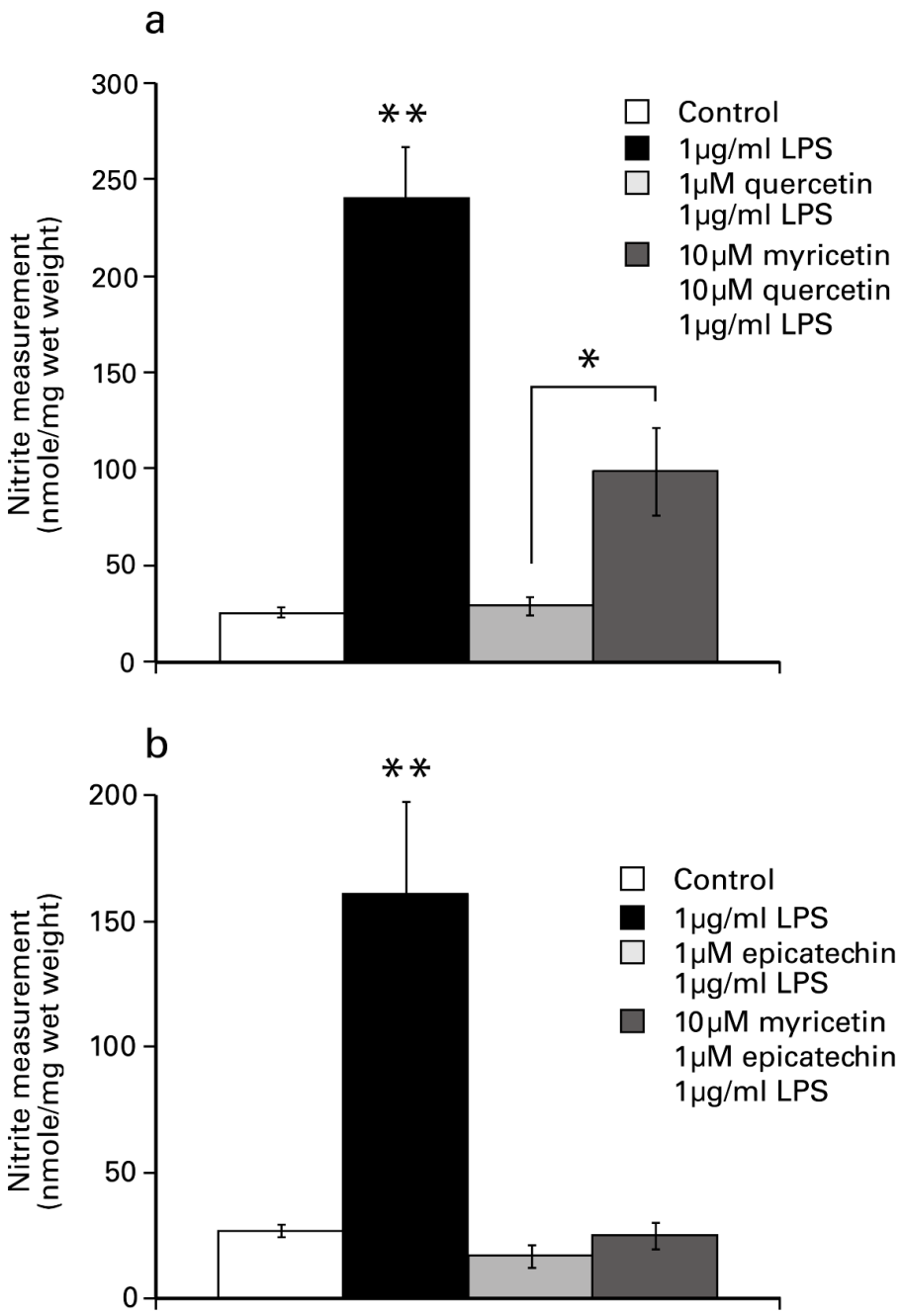



**Figure 4-5** The effect of 24 h exposure to 1µg·mL$^{-1}$LPS (a) 1µg mL$^{-1}$LPS plus 1µM quercetin and 1 µg mL$^{-1}$LPS with a combination of 10µM myricetin and 1µM quercetin and (b) 1µg mL$^{-1}$ LPS plus 1µM epicatechin and 1µg·mL$^{-1}$ LPS with a combination of 10µM myricetin and 1 µM epicatechin on nitrite ion production in porcine coronary artery segments incubated in DMEM.
The values shown are the mean ±SEM of 8-12 observations. $^{**}p < 0.01$ significant difference from control by ANOVA.

## 4.6 Discussion

The main finding in this chapter is that epicatechin, its steroisomer catechin and epigallocatechin gallate significantly suppressed the changes induced by 1µg $mL^{-1}$ LPS in porcine coronary artery. In keeping with the findings in previous chapters, prolonged exposure to LPS was associated with significant reduction in contractions elicited by KCl and U46619 and endothelium-dependent relaxation to substance P. Exposure to LPS was also associated with an increase in the generation of nitrite ions in the incubation medium. The hyporesponsiveness of coronary artery following exposure to LPS is generally attributed to the induction of the enzyme nitric oxide synthase, which results in a significant production of nitric oxide (Hauser *et al.*, 2005). In a previous chapter (chapter 3), I have shown that 1400W, a selective inhibitor of inducible nitric oxide synthase, abolished the effect of LPS.

In this chapter I have demonstrated that overnight incubation of porcine isolated coronary artery with 10µM epicatechin alone did not affect the contractile ability of the arteries to both KCl and U46619, However, both 10µM catechin and 10µM epigallocatechin gallate significantly reduced the vascular response of the arteries to KCl and U46619. In term of endothelium-dependent relaxations, none of the tested compounds have any effect on substance P. However, a previous study found that catechins prevented a substance P-induced hyperactive bladder in rats via the down regulation of ICAM and ROS (Chen *et al.*, 2004).

The results in this chapter showed that a high concentration (10µM) of epicatechin, catechin and epigallocatechin gallate suppressed the LPS-induced changes in endothelial and vascular responses. These results are in line with a study carried out by

Lin & Lin (1997); they examined the effects of EGCG on NO production from murine peritoneal macrophages and sought possible reaction mechanisms. Their results suggested that EGCG blocks early events of NOS induction, and the blocking could occur via inhibition of binding of transcription factor NF-κB to the iNOS promoter, thereby inhibiting the induction of iNOS transcription (Lin & Lin 1997). Also an *in vitro* study by Guruvayoorappan and Kuttan (+)-catechin significantly inhibited nitrite ion production and TNF-alpha production in LPS-stimulated macrophages (Guruvayoorappan and Kuttan, 2008).

While endothelial-derived NO from activation of constitutive NO synthase is important for maintaining vascular tone and homeostasis, higher concentrations of NO produced by iNOS from immune cells such as macrophages can cause oxidative damage. The results of this study showed the ability of epicatechin and its stereoisomer catechin to suppress the effect of LPS elevation in nitrite ion production. EGCG dose-dependently inhibited the expression of iNOS in lipopolysaccharide-activated macrophages by preventing the binding of nuclear transcriptional factor-kB (NF-kB) to the iNOS gene promoter and also reduced the activity of iNOS, thereby reducing toxic NO generation (Chan *et al*., 1997; Lin and Lin, 1997). Experimental and clinical studies suggest that tea catechins can significantly improve endothelial function, thereby providing an additional beneficial effect on patients with CVD. EGCG was shown to improve endothelial function and reduce blood pressure in hypertensive rats (Potenza *et al*., 2007). (–)-epicatechin reduces blood pressure in hypertensive patients and limits infarct size in animal models of myocardial ischemia–reperfusion injury. Recent results demonstrate the unique capacity of (–)-epicatechin to confer cardioprotection in the setting of a severe form of myocardial ischemic injury (Yamazaki, *et al.*, 2010).

Benito *et al* (2002) found that endothelium-dependent relaxation and cyclic guanosine-3',5'-monophosphate (cGMP) accumulation were greater in arterial tissue isolated from rats consuming de-alcoholized red wine or a catechin-rich diet compared with a control diet, and these effects were attributable to an increase in the activity, but not the expression of endothelial nitric oxide synthase (eNOS) (Benito *et al.,* 2002). Recent reports indicate that (–)-epicatechin can exert cardioprotective actions, which may involve endothelial nitric oxide synthase (eNOS)-mediated nitric oxide production in endothelial cells (Sanchez *et al*., 2010). Epicatechin had comparable effects on eNOS activity in cultured endothelial cells (Huang *et al*., 1999). Moreover, Lorenz and colleagues (2004) specifically examined the effects of EGCG on endothelial function. They observed that EGCG produces eNOS-dependent relaxation of isolated rat aorta and increases eNOS activity in cultured endothelial cells.

Myricetin and quercetin are structurly related flavonoids i.e. myricetin is 5-hydroxy quercetin. The results indicated that myricetin failed to mimic the effect of quercetin on LPS-induced nitric oxide production. It also antagonised the inhibitory effect of quercetin on LPS-induced nitrite. On the other hand, epicatechin is chemically different from myricetin; however, myricetin did not antagonise or reverse the effect of epicatchin on inhibition of nitrite ion induced by LPS.

## 4.7 Conclusion

In conclusion I have demonstrated that epicatechin, its steroisomers catechins and epigallocatechin gallate oppose pro-inflammatory events in both endothelial cells and vascular smooth muscle cells. These effects of catechins are evident at lower

concentrations than previously reported in studies using other cell types and suggests a selective action on the vasculature. Further work on human blood vessels is required to establish whether these observations are relevant to the well-documented beneficial effects of tea catechins (Arts *et al*., 2001).

# Chapter 5

# Comparison of the effect of simvastatin and pravastatin on LPS-induced changes in the porcine isolated coronary artery *in vitro*

## 5.1 Introduction

A burgeoning body of evidence, largely derived from retrospective studies, suggests that statins may be useful for preventing the development of sepsis in patients or treating the fulminate of the condition (Brookes *et al*., 2009; Gao *et al*., 2008; Terblanche *et al*., 2006). Liappis and colleagues were amongst the first to report that pre-admission use of statins, to lower cholesterol, was associated with a significant reduction in mortality rate amongst patients who developed bacteraemia in hospital (Liappis *et al*., 2001). Several other retrospective studies have provided general support for the notion that prior use of statins is associated with improved outcomes for patients with bacteraemia and suspected sepsis (Almog *et al*., 2004; Kruger *et al*., 2009; Schmidt *et al*., 2006). Taken together, these finding are consistent with accumulating data indicating that these drugs possess significant `anti-inflammatory` activity following cardiac and non-cardiac surgery (Clark *et al*., 2006; O'Neil-Callahan *et al*., 2005; Poldermans *et al.*, 2003; Schouten *et al.*, 2009).

The beneficial effect of statins in these clinical settings is generally through to be a function of inhibition of HMG CoA reductase, but to be unrelated to changes in plasma cholesterol. Instead, inflammation-associated changes in isoprenoid metabolism are thought to be suppressed by statins the so-called pleiotropic activity of this class of drugs (Brookes *et al*., 2009) that has been largely characterised from *in vitro* studies. For example, fluvastatin has been reported to inhibit lipopolysaccharide (LPS)-induced expression of nitric oxide synthase and the associated nitrite ion production in both cultured vascular smooth muscle cells and intact blood vessels (Wei *et al*., 2006), while simvastatin reduced LPS-induced cyclo-oxygenase-2 expression and postanoid production in a monocyctic cell line

U937 (Habib *et al*., 2007 ). Several studies have reported that statins can reduce expression of cell adhesion molecules on cultured human endothelial and vascular cells (Landsberger *et al*., 2007; Mulhaupt *et al*., 2003). Finally, several studies reported the anti-inflammatory cytokines in both smooth muscle cells (Ito *et al*., 2002) and cultured monocyes (Methe *et al*., 2005). These *in vitro* observations are also supported by findings in man, where simvastatin has been reported to inhibit LPS-induced elevation of plasma TNF-α and neutrophil oxidative burst (Pleiner *et al*., 2004).

Despite the wealth of evidence highlighting the anti-inflammatory activity of statins, doubts remain regarding its clinical relevance in septic shock, with a few reports indicating either no benefit (Yang *et al*., 2007) or even worse mortality outcomes (Fernandez *et al*., 2006). Moreover, statins have also been reported to enhance cytokine-induced expression of nitric oxide synthase and nitrite ion production in vascular smooth muscle cells (Chen *et al*., 2000; Wei *et al*., 2006; Yamamoto *et al*., 2003).

The impairment of endothelium-dependent relaxations and the development of hyporesponsiveness to vasoconstrictor agents are cardinal features of experimental and clinical sepsis in man (Pleiner *et al*., 2004). Since these changes are usually accompanied by an elevation in plasma nitrite ions, it is generally assumed that the increased expression of nitric oxide synthase in both vascular (Fernanades and Assreuy, 2008; Stoclet *et al*., 1999b) and non-vascular cells (Hollenberg and Cinel, 2009) makes a significant contribution to the condition. I have demonstrated that overnight exposure of the porcine isolated coronary artery to LPS is accompanied by

the increased expression of inducible nitric oxide synthase, impairment of endothelium-dependent relaxation to substance P and attenuation of vasoconstrictor response (Chapters 2, 3and 4). Thus, in the present chapter I have examined the effects of simvastatin and pravastatin on LPS-induced changes in vascular responses. Since overnight exposure of rat and human blood vessels to lovastatin has been reported to enhance vasoconstrictor response and impair endothelium-dependent relaxation (Roulett *et al*., 1995), I have also closely examined the effects of statins alone on vascular responses.

A recent area of interest that may be fruitful is the study of anti-inflammatory effects of tea polyphenols in combination with therapeutic agents (e.g. statins). In a recent study carried out by Naruszewicz *et al* (2007), it was demonstrated that a combination therapy of statin and flavonoid rich extract enhanced the reduction in cardiovascular risk markers in patients after myocardial infarction (MI) (Naruszewiczac *et al*., 2007). Therefore, I have investigated the combination effects of low concentration of epicatechin and simvastatin on LPS-induced inflammatory changes on porcine isolated coronary artery.

## 5.2 Material and Methods

Porcine hearts were obtained from a local abattoir and placed in modified Krebs-Henseleit (K-H) solution maintained at 4°C before being transported to the laboratory. The an interior descending branch of the coronary artery was dissected from the hearts, cleaned of connective tissue, and then divided into 4 mm long segments.

### 5.2.1 Contraction Studies

For contraction experiments, single segments of the porcine coronary artery were incubated in 2ml K-H solution (previously gassed with 95% $O_2$ and 5% $CO_2$ for 5 min.) containing 2% ficoll and a combination of 60µg/ml benzyl penicillin and 20µg/ml streptomycin sulphate and 10% FCS. The solution also contained 1µg $mL^{-1}$LPS, various concentrations of simvastatin or a combination of LPS and simvastatin (added 60 min before LPS) and then sealed in a sterilized glass vial and stored overnight at 37°C for 16-18 h. Similar experiments were also conducted in the presence of 10µM pravastatin. In order to better understand the action of simvastatin some segments were also incubated overnight in the presence of the vehicle, 1µg $mL^{-1}$LPS, a combination of LPS and 3µM simvastatin and combination of LPS, simvastatin and 100µM mevalonate. In this instance, it was also necessary to determine the effect of combination with 100µM mevalonate, against LPS-induced changes in the blood vessel. In another experiment, segments were exposed to a combination of 0.01µM epicatechin and 0.03µM simvastatin for 1hour followed by the addition of 1µg $mL^{-1}$ LPS to the incubation solution and incubated overnight in a pre-gassed modified K-H solution. In all instances the segments were effectively

paired so that each experimental condition had a control segment taken from the same animal.

After overnight storage, segments were removed from the incubation solution and placed in K-H solution (maintained at 37$^{o}$C and gassed with 95% $O_2$ and 5% $CO_2$) in a 15 ml isolated organ bath, prepared for isometric tension recordings as previously described (Qi *et al*., 2007) and allowed to equilibrate for 60 min. Contractions of the segment were measured using a Grass FT03 isometric force transducer connected to a MacLab unit coupled to a Macintosh LC4 computer running Chart 3.5. An initial resting tension of 80mN was slowly applied to each segment at the end of the equilibration period and the recorded tension declined to 40-60mN over a further 40 min period. Segments were repeatedly exposed to 60mM KCl for 15 min until reproducible contractions were observed. The preparations were exposed to cumulatively increasing concentrations of either KCl (6-60mM) or U46619 (a stable thromboxane-mimetic analogue, 9,11-dideoxy-9α,11α-methanoepoxyprostaglandin $F_{2\alpha,}$ 1-200 nM). When each preparation was exposed to a maximally-effective concentration of U46619, 10nM substance P was added to assess the integrity of the endothelium.

In order to establish whether the effects of simvastatin on LPS-induced changes in vascular responses can also occur if the drug is presented after the inflammatory stimuli, segments of porcine isolated coronary artery were incubated in the presence and absence of 1μg $mL^{-1}$LPS for 40 hours and 3μM simvastatin was added into the medium 16 hours after exposure to LPS (24 hour statin exposure time). After 40

hours incubation the segments were then prepared for the isometric tension recording as described above.

### 5.2.2 Nitrite Determination

For the measurement of nitrite accumulation, a marker for nitric oxide production (Kelm, 1999), two 4mm segments were incubated in each vial for 24 hr with aerated DMEM (without phenol red) containing 10% FCS, 1mM L-arginine, 60µg/ml benzyl penicillin and 20µg/ml streptomycin. The segments were exposed to either 1µg $mL^{-1}$LPS, simvastatin, or combination of LPS and simvastatin as described above. After the incubation period, the segments were removed, briefly blotted on paper cloth and weighed. The accumulation nitrite ions was determined by taking 500µl of the incubation medium adding 500µl Griess reagent and an optical density at 550nm was measured, using a UNICAM UV/VIS spectrophotometer. A standard concentration-optical density curve was calculated for every assay using sodium nitrite solution against a blank (500µl DMEM & 500µl Griess Reagent). In a separate experiment we established that incubation of DMEM containing either the statins, LPS or a combination of both (without the arterial segments) produced an optical density reading similar to the blank. The minimum detectable amount of nitrite was 375 nmoles. For comparative purposes, the effect of hydrophilic statin (pravastatin) on LPS-induced changes on porcine isolated coronary artery was also examined.

### 5.2.3 Immunohistochemistry

Sections of the coronary artery incubated under the above conditions were prepared on a cryostat (5µm thick) and stored frozen at -80$^{o}$C until required. The section

were then warmed to room temperature for 20 min and fixed in cold acetone at -4°C for 20 min. To block endogenous peroxidase activity, sections were treated with blocking serum (2 drops of vectastain in 5ml phosphate buffer solution (pH 7.4) containing 2%w/v immunohistochemical grade bovine serum albumin) for 10 min at room temperature. The sections were then incubated for 1 hour with primary mouse antibodies: porcine-PECAM-1 (diluted 1:75), or rabbit inducible nitric oxide synthase (iNOS) (diluted 1:100). The sections were then washed in phosphate buffer solution, incubated for 10 min with biotinylated anti-mouse antibody in 10% NGS (Vectastain ABC Kit), washed in phosphate buffer solution, incubated for a further 5 min with ABC (avidin-biotin-peroxidase) reagent in phosphate buffer solution and, finally, washed again. Immunoreactive CD31 and iNOS were visualized by incubating the sections in Vector Red substrate (1 drop of levamisole solution to 5 ml of 200 mM Tris-HCl pH 8.2) for 30 min. Finally, sections were dehydrated and cover-slipped with DPX mounting medium. The observation and photographs were made using a light microscope (Leica DM4000B) and an imaging digital camera. Images were obtained using Openlab (improvision, UK).

## 5.3 Solutions and drugs

The composition of K-H solution was (in mM): NaCl, 118; KCl, 4.8; $MgSO_4.7H_2O$, 1.2; $CaCl_2.2H_2O$, 1.3; $NaHCO_3$, 25.0; $KH_2PO_4$, 1.2. Benzyl penicillin, streptomycin sulphate, ficoll, LPS, R-(-) mevalonolactone and Pravastatin sodium were all obtained from Sigma-Aldrich Company Ltd (Poole, Dorset, UK). Substance P was obtained from Bachem (UK). U46619 was obtained from Alexis Coporation (Nottingham, UK). Dulbeccos Modified Eagle`s Medium (DMEM) was supplemented with antibiotics (see above) and 2 mM L-glutamine (Gibco). Statins, and mevalonolactone were dissolved in distilled water.

## 5.4 Data analysis and statistics

Contractions produced by U46619 and KCl were measured as mN force. Responses were expressed as the maximum contraction ($E_{max}$), and potency determined as the negative logarithm of the concentration causing 50% of the maximum response (-log $EC_{50}$ or $pD_2$) using a logistic equation (Kaleidagraph, version 3.6 Synergy Software). The values are shown as the mean (± SEM). The content of nitrite in the medium was calculated according to the equation deduced from the standard curve. The amount on nitrite in the medium was then divided by the wet weight of tissues to obtain the amount of nitrite production from each segment in the units of nmoles per mg wet weight. In the majority of instances differences between mean values were assessed by a Student's t-test (two-tailed). However, where there was more than one treatment condition assessed differences were analysed by ANOVA followed by post-hoc Dunnett's test. A $p$ value < 0.05 was considered statistically significant.

## 5.5 Results

### 5.5.1 The effect of simvastatin

Following overnight incubation of the isolated segments of porcine coronary artery at 37ºC in modified Krebs-Henseleit solution, KCl and U46619 produced concentration-dependent contractions of comparable sizes (Table 5.1) in the presence of maximally effective concentrations of U46619, 10nM substance P (SP) produced transient, endothelium-dependant relaxations (Table 5.1). Table 5.1 also shows that overnight exposure to 0.3- 30µM simvastatin (and subsequent removal) did not significantly alter either KCl-induced contraction or substance P-induced relaxations. However, overnight incubation of segments with 0.3-3µM simvastatin was associated with an increase in the potency of U46619 and an increase in the magnitude of the maximum contraction. In the case of 3µM simvastatin the increase in the maximum contraction to U46619 (36.4±8.3%, n=8) was statiscally significant ($p$<0.05). In a separate series of experiments, acute exposure of coronary artery segments to 3µM simvastatin (1 hour) did not affect either the potency or the magnitude of the maximum contractions to KCl and U46619. In addition, relaxation induced by substance P was unaffected by acute exposure to 3µM simvastatin (data not shown).

**Table 5-1** Effect of 30µM, 3µM and 0.3µM simvastatin on the maximum response (mN) and potency ($pD_2$) of KCl and U46619 contractions and SP-induced relaxation in isolated porcine coronary arteries incubated for 16 h in modified Krebs-Henseleit solution.

| | **KCl** | | **U46619** | | **SP** |
|---|---|---|---|---|---|
| | **Max (mN)** | **$pD_2$** | **Max (mN)** | **$pD_2$** | **% Relaxation** |
| **Control (n=8)** | 115.5±9.7 | 1.45±0.04 | 102.1±8.3 | 7.6±0.06 | 44.6±4.2 |
| **30µM Simvastatin** | 115.6±8.1 | 1.4±0.05 | 127.4±7.5 | 7.8±0.03** | 42.9±7.0 |
| **3µM Simvastatin** | 129.1±10.3 | 1.48±0.04 | 160.6±8.3** | 7.8±0.06** | 44.1±5.1 |
| **0.3µM Simvastatin** | 112.5±10.3 | 1.5±0.03 | 135.2±7.6* | 7.9±0.04** | 35.5±4.6 |

Values shown are the mean ± SEM of 8 observations. * ($p$ <0.05) and ** ($p$ < 0.01) denote a statistically significant difference from control by ANOVA.

Figure 5.1 shows that KCl and U46619-induced contractions of the porcine isolated coronary artery were significantly reduced to 73.9±3.4% (n=18) and 74.5±3.3% (n=18) of control, respectively, following overnight exposure to 1µg $mL^{-1}$LPS, with a small (1.8-fold), non-significant reduction in the potency of both agents. Following overnight co-incubation of segments with a combination of LPS and 3µM simvastatin (and subsequent removal), responses to both KCl and U46619 were significantly increased compared with that of LPS alone (Figure 5.1a and 5.1b); the maximum contraction to KCl and U46619 after exposure to LPS and 3µM simvastatin were 101.2±5.1% (n=18) and 105.9±4.5% (n=18) respectively. At the end of the U46619 contraction response curve, the addition of 10nM SP caused a transient relaxation (16.3±2.3%, n=18) in control preparations that was significantly impaired (10.9±3.2%, $p<0.05$) following 1µg $mL^{-1}$LPS treatment. The inhibitory effect of LPS on SP-induced relaxation was prevented by co-incubation with 3µM simvastatin (17.2±3.4%, n=18).

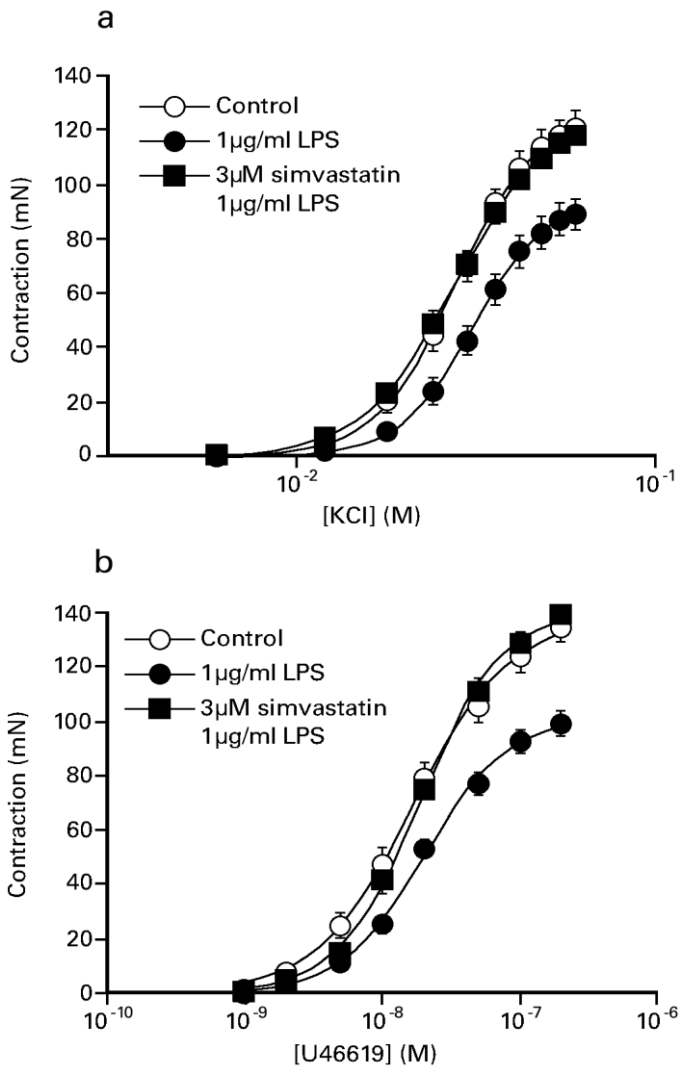



**Figure 5-1** The effect of overnight exposure of the porcine coronary artery to 1µg $mL^{-1}$ LPS, in the presence or absence of 3µM simvastatin on responses elicited by KCl (a) and U46619 (b). The responses shown are as mean ± SEM of 18 observations.

Table 5.2 shows the results from a further experiment to investigate the effect of lower concentration of simvastatin on LPS-induced changes in vascular responses. Overnight exposure of porcine isolated coronary artery to 1μg $mL^{-1}$LPS was associated with approximately 35% reduction in the maximum contractions to KCl and U46619 and a statistically significant 2-fold reduction in the potency of U46619. The combination of simvastatin (0.03μM and 0.3μM) and 1μg $mL^{-1}$ LPS was associated with a smaller impairment of maximum contractions (approximately 25%) to KCl and U46619 but this did not reach statistical significance. Interestingly, the 2-fold reduction in the potency of U46619 following exposure to 1μg $mL^{-1}$ LPS was not observed in the presence of LPS and simvastatin (Table 5.2). Also, the inhibitory effect of LPS on substance P-induced relaxation was prevented by co-incubation with either 0.3μM or 0.03μM simvastatin (Table 5.2).

**Table 5-2** Effect of 0.3 and 0.03μM simvastatin, on the maximum response (mN) and potency ($pD_2$) of KCl and U46619 contractions and SP-induced relaxation in isolated porcine coronary arteries incubated for 16 h in modified Krebs-Henseleit solution in the presence of 1μg $mL^{-1}$ LPS.

| | KCl | | U46619 | | SP |
|---|---|---|---|---|---|
| | **Max (% control)** | **$pD_2$** | **Max (% control)** | **$pD_2$** | **% Relaxation** |
| **Control** | 100 | 1.6±0.01 | 100 | 7.8±0.06 | 42.3±4.9 |
| **1μg/ml LPS** | 64.9±5.6** | 1.57±0.01 | 64.7±5.3** | 7.4±0.09** | 20.6±4.4** |
| **0.3μM Simvastatin 1μg/ml LPS** | 73.9±3.6** | 1.56±0.02 | 76.2±5.2** | 7.7±0.07 | 37.6±3.9 |
| **0.03μM Simvastatin 1μg/ml LPS** | 76.2±3.4** | 1.64±0.01 | 73.5±3.1** | 7.8±0.06 | 38.2±3.4 |

Values shown are the mean ± SEM of 11 observations. ** $p < 0.01$ denote a statistically significant difference from control by ANOVA.

Following 24 hours incubation of porcine coronary artery segments in DMEM, the nitric oxide content of the media was 15-20nmoles/mg wet weight tissue (Figure 5.2a). The inclusion of 1μg $mL^{-1}$ LPS in the media was associated with 7-10fold increase in the production of nitrite ions by coronary artery segments, while the inclusion of simvastatin (3μM) did not affect basal nitrite production. However, the addition of 3μM simvastatin prior to exposure to LPS was associated with a 94.7±5.7% (n=8) reduction in LPS-induced nitrite production. As shown in Figure 5.2b, co-incubation of the porcine coronary artery segments with either 0.3μM or 0.03μM simvastatin reduced the LPS-induced nitrite ions production by 92.4±6.6% (n=12) and 93.4±7.8% (n=12), respectively.

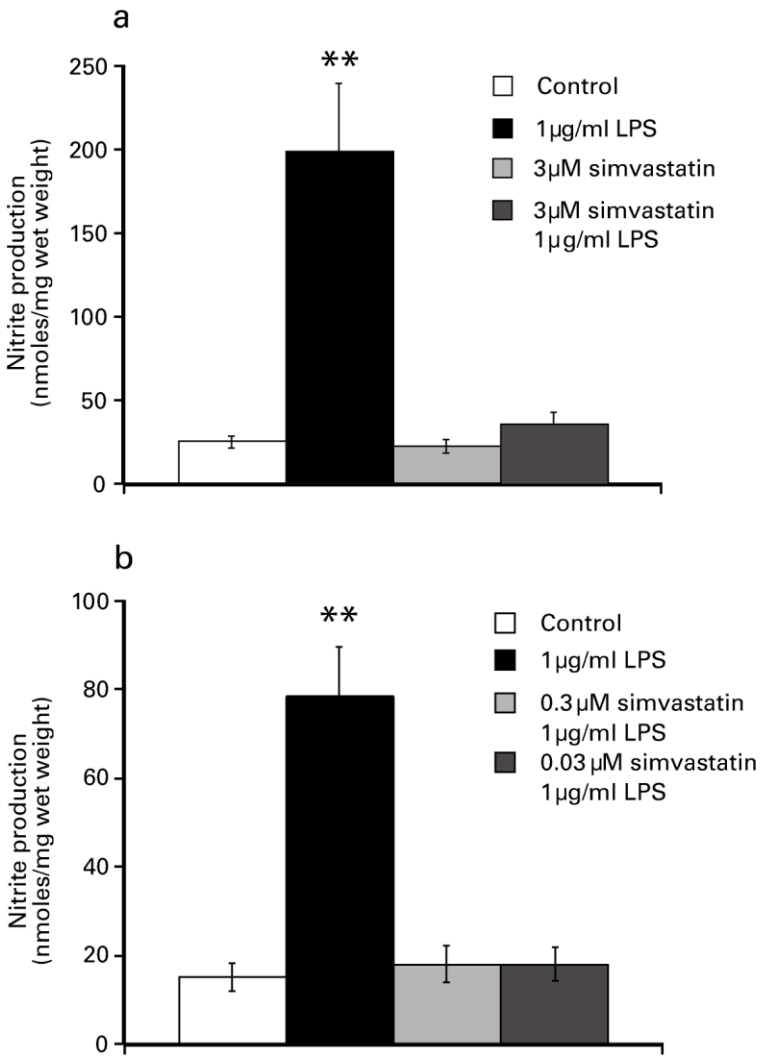



**Figure 5-2** The effect of 24 hour exposure to 1µg $mL^{-1}$ LPS, (a) 1µg $mL^{-1}$ LPS plus 3µM simvastatin and (b) 1µg $mL^{-1}$ LPS plus either 0.3µM or 0.03 µM simvastatin on nitrite production in porcine coronary artery segments incubated in DMEM. The values shown are the mean ± SEM of 8-12 observations. ** $p<0.01$ significant difference from control by ANOVA.

Immunohistochemical study showed strong evidence for the induction of nitric oxide synthase by LPS. As shown in Figure 5.3 which is representative of 4 experiments, immunohistochemical analysis showed that control preparations express CD31 on endothelial cells but provide no evidence of inducible nitric oxide synthase in the vessel. Treatment of segments of porcine coronary artery for 18 h with 1µg $mL^{-1}$ LPS exhibited increased expression of endothelial CD31 and the appearance of inducible nitric oxide synthase in the tunica adventitia (rather than the tunica media). Interestingly, in arterial segments co-incubation with 3µM simvastatin and LPS there was no evidence of adventitial nitric oxide synthase, while the intensity of endothelial CD31 detected by anti-CD31 antibody was comparable to that in control preparations (Figure 5.3).

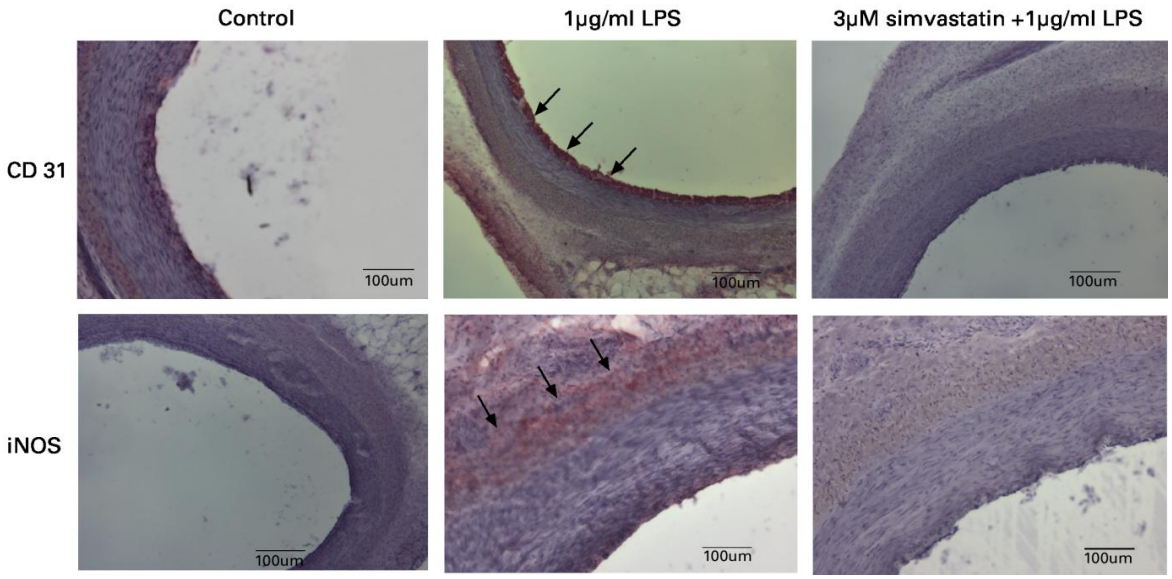



**Figure 5-3** Immunohistochemical localization of (upper panels) PECAM-1 (CD31) and (lower panels) inducible nitric oxide synthase (iNOS) in the porcine isolated coronary artery following incubation in modified Krebs-Henseleit solution for 16-18 hours at 37$^{o}$C without (Control) or with either 1µg mL$^{-1}$ LPS or 1µg mL$^{-1}$ LPS and 3µM simvastatin.

### 5.5.2 Effects of pravastatin

In light of the effect of simvastatin, a lipophilic statin, on LPS-induced changes in vascular responses, I also examined the effect of water-soluble statin, pravastatin. Overnight exposure to 10μM pravastatin alone (and subsequent removal) significantly altered the maximum contraction elicited by KCl and U46619. Pravastatin caused a significant increase in the contractile responses to KCl (Control 139.0±6.6mN vs. 10μM pravastatin 154.7±6.6mN, n=8) ($p$=0.02), but the response to U46619 (Control 149.0±4.9mN vs. 10μM pravastatin 169.5±7.5mN, n=8) just failed to reach statistical significance ($p$=0.06). The potency of KCl was unchanged; however, 10μM pravastatin significantly enhanced the potency of U46619. (Control 7.5±0.02. vs. 10μM pravastatin 7.6±0.03 (n=8) ($p$<0.05).

Figure 5.4 shows the cumulative concentration response curves to KCl and U46619 in porcine isolated coronary artery segments. Exposure to 1μg $mL^{-1}$LPS caused a significant reduction to the maximum response to KCl (34.5±5.6%, n=8) and to U46619 (31.2±5.4%, n=8) and these effects were significantly reduced by prior exposure to 10μM pravastatin without any alteration in the potency of either agent. Furthermore, exposure of coronary artery segments to 1μg $mL^{-1}$LPS was associated with a significant reduction in endothelium-dependent relaxations to substance P (control: 33.5±1.7% n=8; LPS: 13.1±0.6%, n=8) ($p$ <0.001) and prior exposure to 10μM pravastatin prevented this inhibitory effect on substance P-induced relaxation 34.5±1.4%, n=8) ($p$ <0.001).

Twenty-four hours incubation with 1μg $mL^{-1}$LPS significantly increased by 10-fold the production of nitrite ion in the incubation medium (Control, 26.6±2.4 nmoles mg

wet weight; LPS: 297±27.4 nmoles mg wet weight, n=8) and the presence of 10μM pravastatin reduced (91.5±3.0%, n=8; $P<0.0001$) nitrite accumulation in the presence of LPS (Figure 5.5).

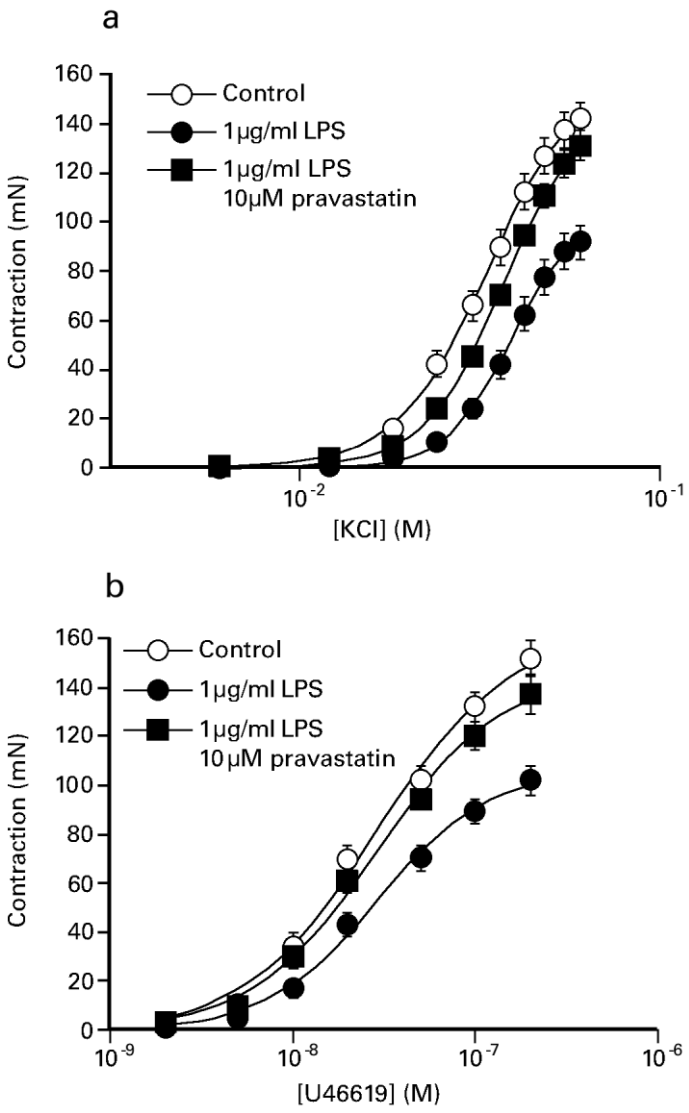



**Figure 5-4** The effect of overnight exposure of the porcine coronary artery to 1µg $mL^{-1}$ LPS, in the presence or absence of 10µM pravastatin on responses elicited by KCl (a) and U46619 (b). The responses shown are as mean ± SEM of 8 observations.

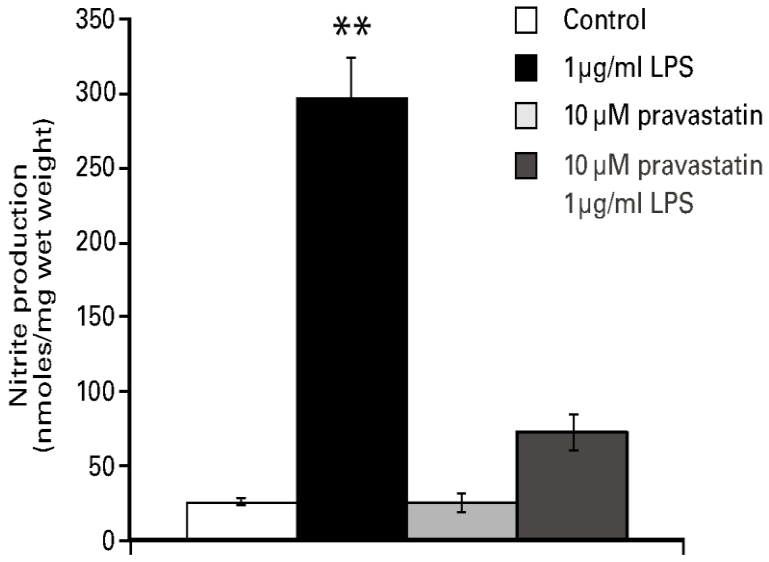



**Figure 5-5** The effect of 24 hour exposure to 1µg $mL^{-1}$ LPS, 1µg $mL^{-1}$ LPS plus 10µM pravastatin on nitrite production in porcine coronary artery segments incubated in DMEM. The values shown are the mean ±SEM of 8 observations. ** $p<0.01$ significant difference from control by ANOVA.

Immunohistochemical study showed strong evidence for the induction of nitric oxide synthase. As shown in Figure 5.6 immunohistochemical analysis showed that control preparations (n=4) express CD31 on endothelial cells but with no evidence of inducible nitric oxide synthase. Treatment of segments of porcine coronary artery for 18 h with 1µg $mL^{-1}$LPS increased expression of endothelial CD31 and the appearance of inducible nitric oxide synthase in the tunica adventitia (but not the tunica media). Co-incubation with 10µM pravastatin reduced LPS-induced expression of endothelial CD31 and adventitial nitric oxide synthase (Figure 5.6).

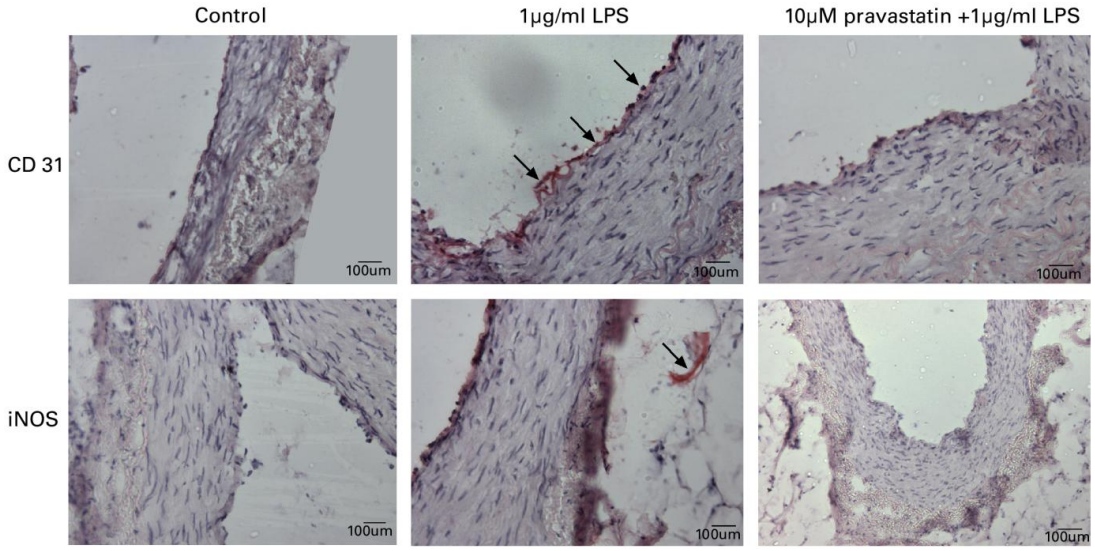



**Figure 5-6** Immunohistochemical localization of (upper panels) PECAM-1 (CD31) and (lower panels) inducible nitric oxide synthase (iNOS) in the porcine isolated coronary artery following incubation in modified Krebs-Henseleit solution for 16-18 hours at 37$^{o}$C without (Control) or with either 1µg mL$^{-1}$ LPS or 1µg mL$^{-1}$ LPS and 10µM pravastatin.

### 5.5.3 Effect of mevalonate

In order to assess the effect of mevalonate on the ability of statins to modulate LPS-induced changes in vascular responses, I established that overnight exposure to 100µM mevalonate alone did not affect either the potency or the maximum contraction eliciated by KCl and U46619 (Figure 5.7a and 5.7b). However, while overnight incubation of isolated coronary artery segments with 1µg $mL^{-1}$LPS was associated with a reduction in the maximum contractions to KCl by 62.8±2.5%., (n=7), the $E_{max}$ to U46619 was also decreased by 65.6±5.3%, (n=7) with no effect on the potency of KCl or U46619. Overnight incubation of isolated coronary artery segments with a combination of 100µM mevalonate and 1µg $mL^{-1}$LPS significantly enhanced the contractile response to KCl (Figure 5.8a), however, the effect of mevalonate failed to reach significance as determined by Student t-test on the response of U46619 (Figure 5.8b); 100µM mevalonate failed to affect the potency of KCl and U46619.

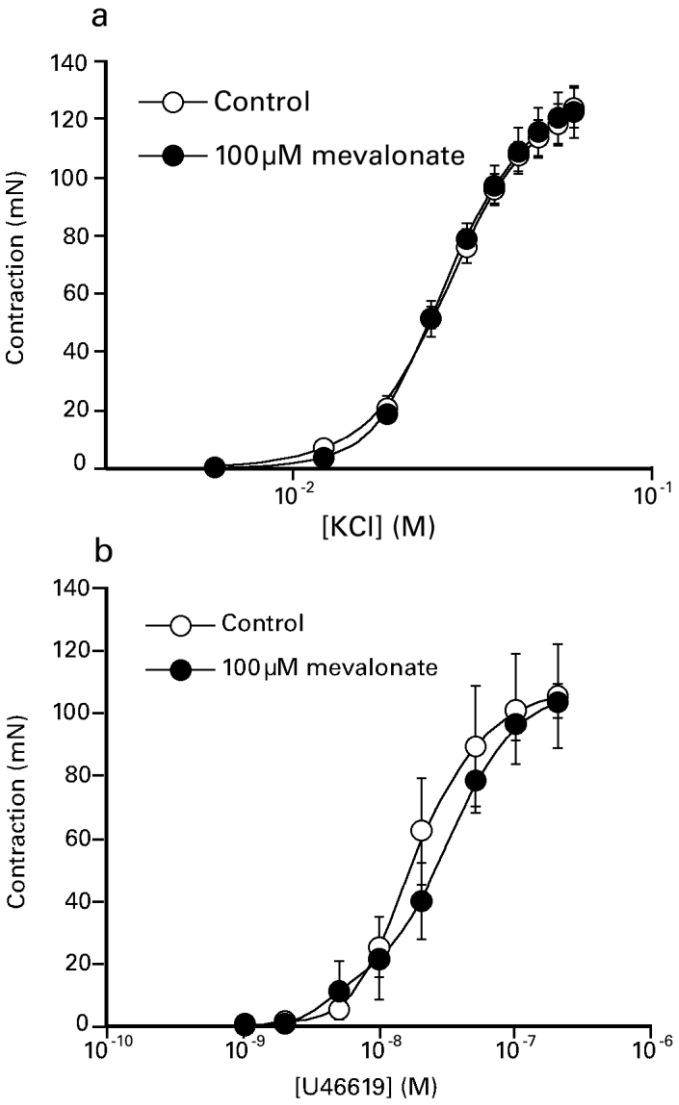



**Figure 5-7** The effect of overnight exposure of the porcine coronary artery to 100µM mevalonate (with subsequent removal) on responses elicited by KC (a) and U46619 (b). The responses shown are as mean ± SEM of 7 observations.

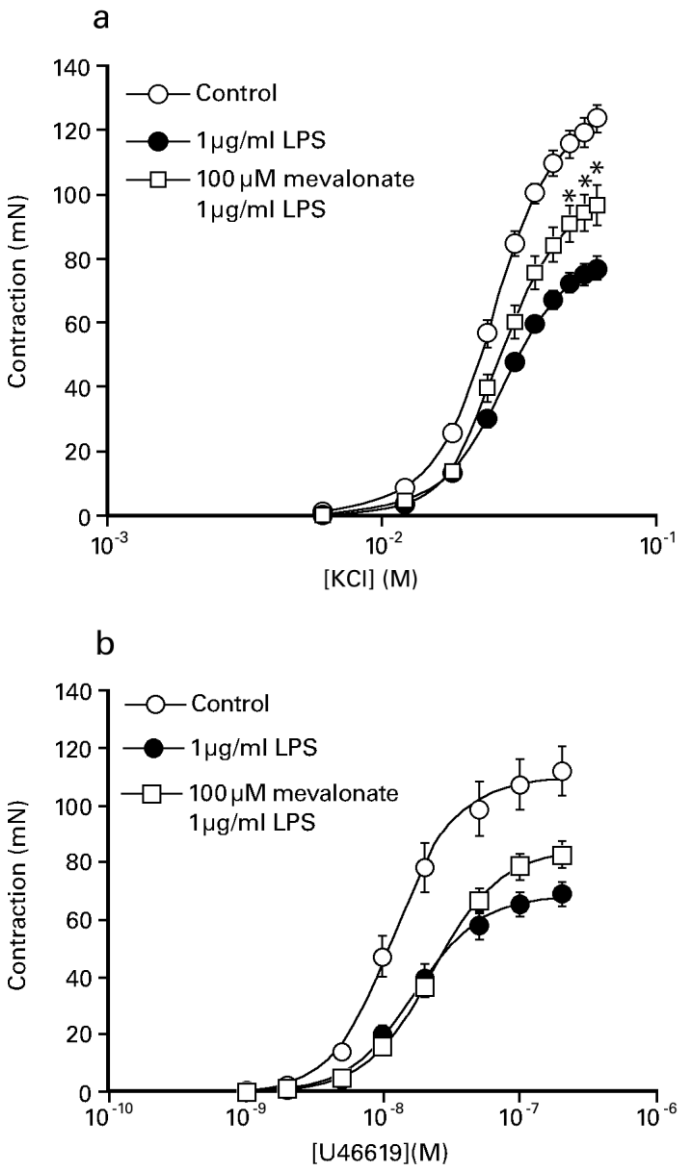



**Figure 5-8** The effect of overnight exposure of the porcine coronary artery to 1µg $mL^{-1}$ LPS with or without 100µM mevalonate (with subsequent removal) on on responses elicited by KC (a) and U46619 (b). The responses shown are as mean ± SEM of 7 observations. . *- ($p$ <0.05) denote a statistically significant difference between responses for the paired LPS-treated preparations.

To assess whether the effects of simvastatin described above were associated with inhibition of HMG-CoA reductase and subsequently reduced mevalonate levels in porcine coronary artery, the segments were exposed to LPS and simvastatin in the presence of mevalonate (100µM). As shown in figure 5.9a and 5.9b, mevalonate reversed the effect of simvastatin on KCl and U46619. Moreover, 10nM SP produced transient relaxation in control segments (39.8±6.1%, n=12). This relaxation effect was significantly reduced to (7.5±1.5%, n=12), in segments exposed to LPS alone. The inhibitory effect of LPS on SP-induced relaxation was significantly reversed when segments were treated with simvastatin (47.8±4.7%, n=12) and relaxation was impaired (8.1±1.5%, n=12) when 100µM mevalonate was incorporated in the incubation medium.

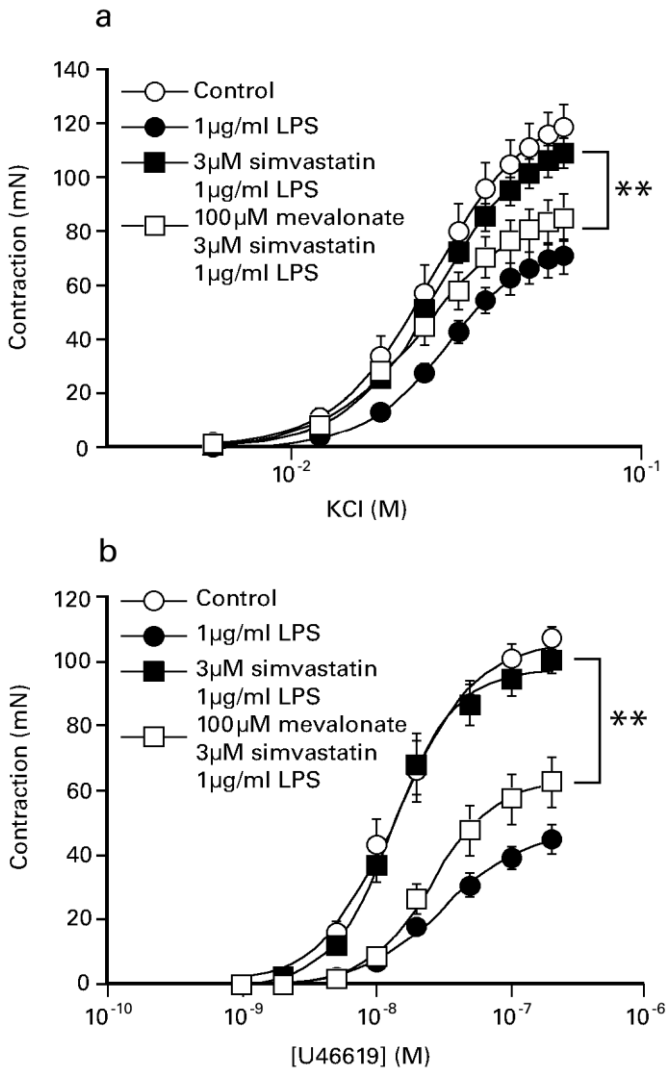



**Figure 5-9** The effect of overnight exposure of the porcine coronary artery to 1µg $mL^{-1}$ LPS in the presence or absence of either 3µM simvastatin, or 3µM simvastatin and 100µM mevalonate on responses to either (a) KCl or (b) U46619. The responses are shown as the mean ± SEM of 12 observations. *- ($p$ <0.05) denote a statistically significant difference between responses for the paired LPS-treated preparations.

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Incubation of segments in DMEM for 24 hrs produced 8-20nmoles per mg wet weight nitrite ions. Stimulation of the arteries with 1μg $mL^{-1}$ LPS caused a significant increase of 24-hr nitrite accumulation in the incubation medium. Addition of the 3μM simvastain or 0.3μM simvastain 60 min prior to LPS significantly inhibited the nitrite produced by LPS by 92.9±7.9%, (n=16) and 96.4±12.8%, (n=11) respectively. However addition of the combination of either 100μM mevalonate and 3μM simvastain or 100μM mevalonate and 0.3μM simvastain 60 min prior to LPS significantly inhibited the inhibitory effect of simvastatin on nitrite production by 31.5±12.8%, (n=16), and 51.3±17.2%, (n=11), respectively (Figure 5.10).

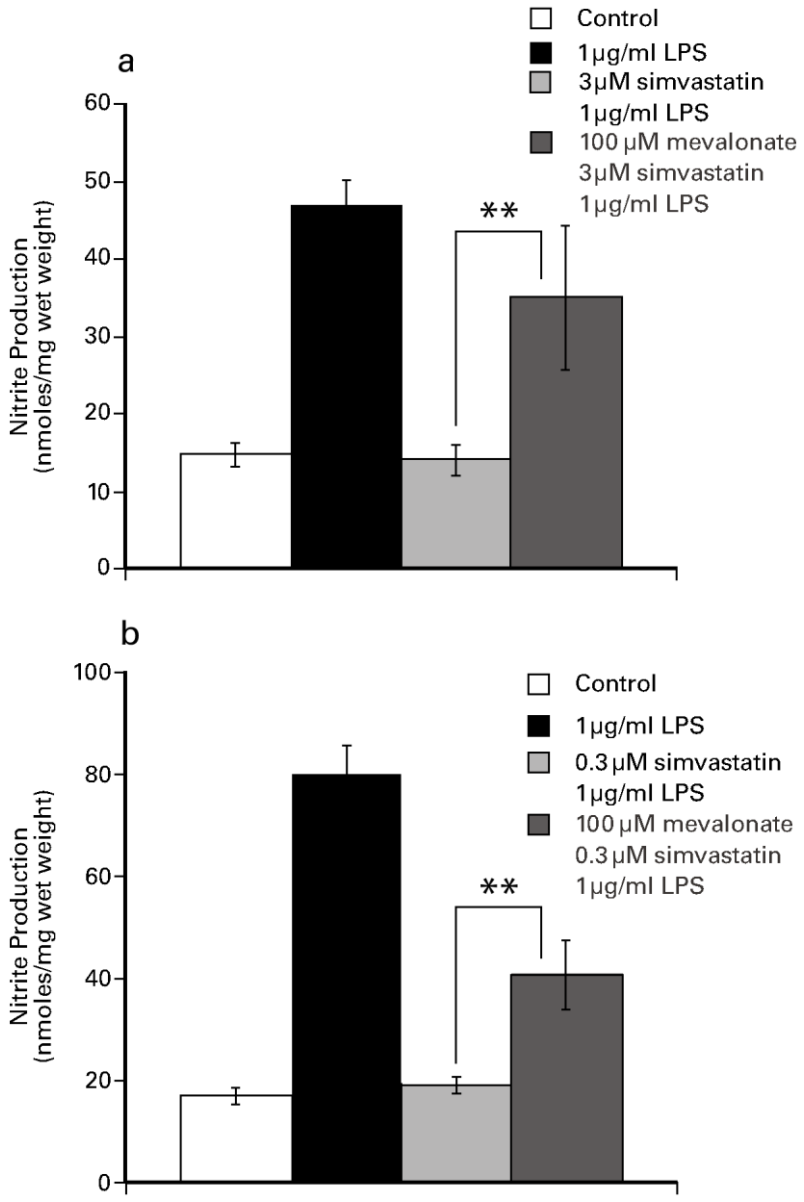


**Figure 5-10** The effect of 24 hour exposure to 1µg $mL^{-1}$ LPS, (a) 1µg $mL^{-1}$ LPS plus 3µM simvastatin and 1µg $mL^{-1}$ LPS with a combination of 100µM mevalonate and 3µM simvastatin and (b) 1µg $mL^{-1}$ LPS plus 0.3µM and 1µg $mL^{-1}$ LPS with a combination of 100µM mevalonate and 0.3µM simvastatin on nitrite production in porcine coronary artery segments incubated in DMEM. The values shown are the mean ± SEM of 11-16 observations. ** $p$<0.01 significant difference from control by ANOVA.

### 5.5.4 The effect of post-LPS exposure to simvastatin on vascular responses

All of the previous experiments have established that statins exert an anti-inflammatory effect on the porcine coronary artery if added *prior* to exposure to the inflammatory insult (LPS). In order to establish whether similar activity for simvastatin was manifest if added *after* exposure to LPS, the effect of 40hr exposure of porcine isolated coronary artery to 1µg $mL^{-1}$ LPS was determined. The segments were incubated in presence or absence of 1µg $mL^{-1}$ LPS for 40 hr in K-H solution at 37ºC (with an exchange of medium after 16 hr. In some experiments 3µM simvastatin was added after 16 hr and then the segments incubated for further 24 hr in the continued presence of LPS.

Following prolonged exposure (40hr) to 1µg $mL^{-1}$ LPS, KCl and U46619 produced concentration-dependent contractions that were significantly reduced in magnitude and potency. The maximum contractions to KCl and U46619 were reduced to 57.4±3.4% (n=8) and 59.7±3.9% (n=8), respectively, of responses in control preparations. In addition, the magnitude of substance P-induced relaxations was reduced from 36.1±4.1% (n=8) of U46619-induced tone to 15.1±3.6% (n=8) following exposure to LPS. The addition of 3µM simvastatin to the incubation medium for the last 24 hours significantly reversed the inhibitory effect of LPS on KCl and U46619 produced contraction (figure 5.11a and 5.11b). Furthermore, substance P-induced relaxations (43.1±3.5%, n=8) were comparable to control preparations.

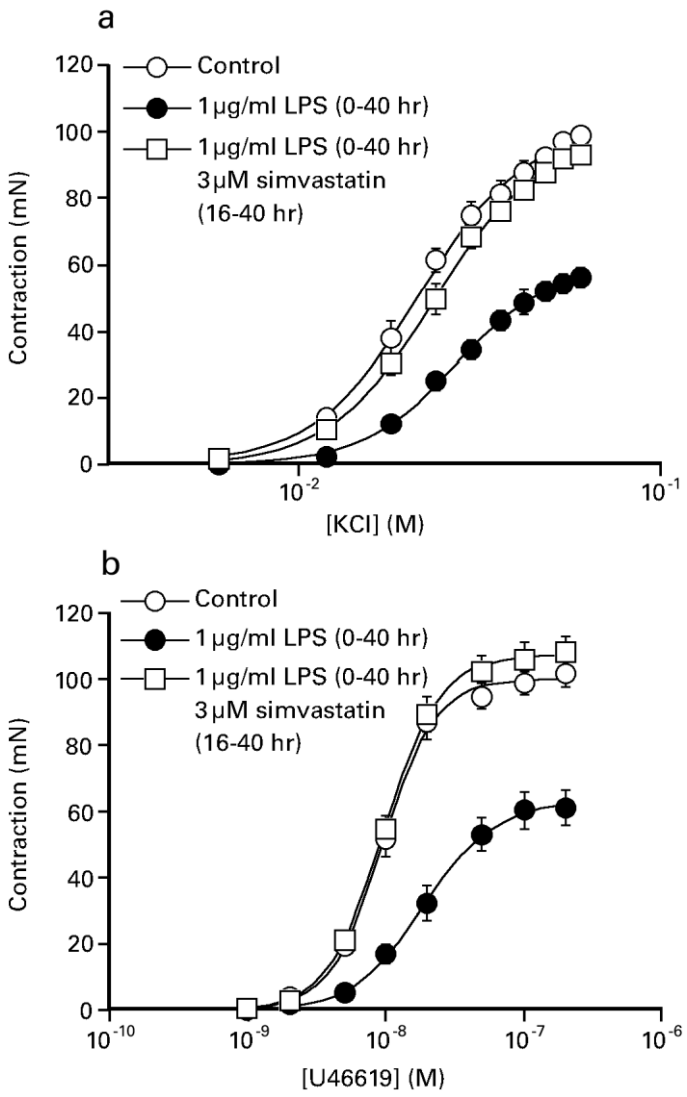


**Figure 5-11** The effect of (40hr) exposure of the porcine coronary artery to 1µg $mL^{-1}$ LPS, in the presence or absence of 3µM simvastatin on responses elicited by KCl (a) and U46619 (b). The responses shown are as mean ± SEM of 12 observations.

### 5.5.5 The effect of a combination of simvastatin and epicatechin on LPS-induced changes in contractile response.

In another experiment, segments were exposed to a combination of 0.01μM epicatechin and 0.03μM simvastatin for 1hour followed by the addition of 1μg $mL^{-1}$ LPS to the incubation solution and incubated overnight in pre-gassed modified K-H solution.

Figure 5.12 shows that KCl and U46619 elicited concentration-dependent contraction in segments of porcine coronary artery incubated overnight in Krebs-Henseleit solution. These contractions were impaired by overnight exposure of the segments to 1μg $mL^{-1}$ LPS. LPS reduced the maximum response to KCl (60.1±3.4% of Control, n=12) and to U46619 (57.7±4.2% of Control, n=12). Pre-treatment of the segments with 0.01μM epicatechin alone did not significantly affect the maximum responses to KCl and to U46619 in LPS-treated segments. Interestingly, combined incubation with epicatechin and simvastatin significantly prevented the inhibitory effect of LPS on contractions of KCl (83.5±3.6%, n=12, $p$=0.001) and U46619 (83.4±4.3%, n=12, $p$=0.001), as determined by ANOVA. Following attainment of maximal contractions to U46619, the addition of 10nM substance P was associated with a relaxation (49.8±6.4%, n=8) that was significantly reduced in preparations previously exposed to 1μg $mL^{-1}$LPS (10.5±2.5%, n=8). Pre-treatment of the segments with 0.01μM epicatechin alone or the combination incubation with epicatechin and simvastatin significantly prevented the inhibitory effect of LPS on substance P by (32.7±5.2%, n=12) and (37.0±6.0%, n=12) respectively.

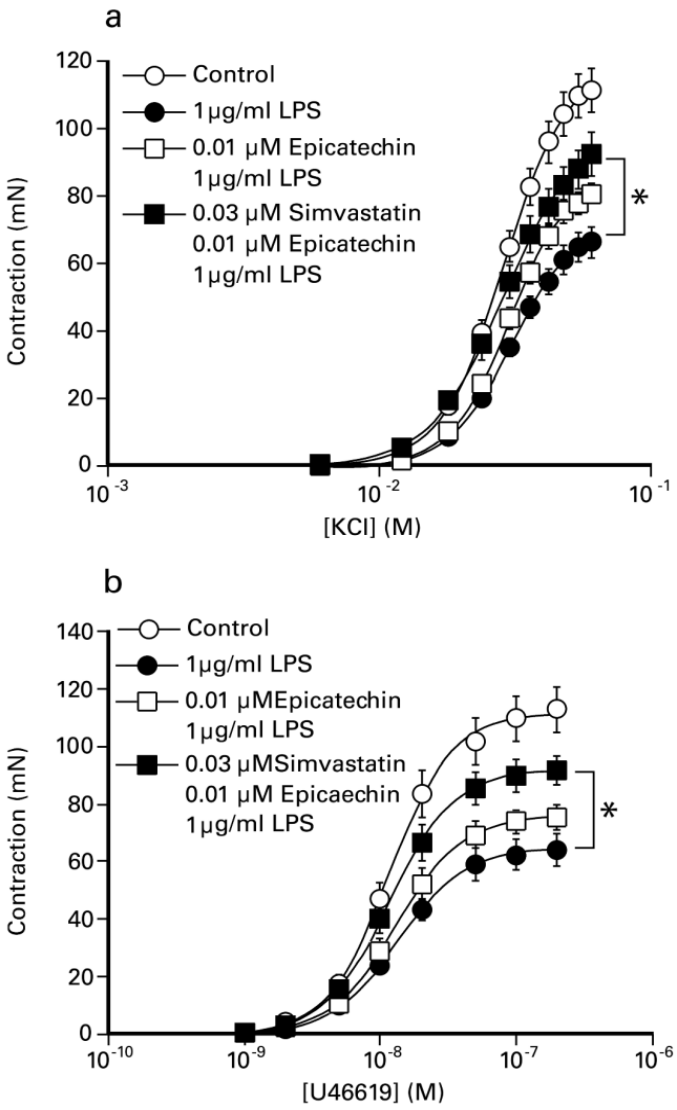


**Figure 5-12** The effect of overnight exposure of the porcine coronary artery to 1 µg $mL^{-1}$ LPS in the presence or absence of either 0.01µM epicatechin or 0.01µM epicatechin and 0.03 µM simvastatin on responses to either (a) KCl or (b) U46619. The responses are shown as the mean ± SEM of 12 observations. *$p$ < 0.05 significant differences between the maximum responses in preparations treated with LPS.

## 5.6 Discussion

As outlined in the Introduction, the evidence for an anti-inflammatory effect of statins is extensive and convincing, but is largely based on observations on cells maintained in cultured (see: Habib *et al*., 2006; Methe *et al*., 2005). While these findings also extend to cultured endothelial and vascular smooth cells, particularly in terms of the expression of cell adhesion molecules and other inflammatory markers (Landsberger *et al.* 2007; Wei *et al*., 2006; Mulhapht *et al*., 2006), there is little information regarding the impact of these drugs on the contractile properties of intact blood vessels. The results in this chapter represent one of the few studies that show the ability of statins to oppose inflammatory changes induced by LPS in a blood vessel.

### 5.6.1 Statins & LPS-induced changes in the coronary artery

In agreement with the observation in Chapter 3 & 4 that prolonged exposure to 1µg $mL^{-1}$ LPS was associated with a significant reduction in contractions to both KCl and U46619, and impairment of substance P-induced, endothelium-dependent relaxations. These effects were still evident 4-6 hours after removal of LPS. In addition, LPS caused an increase in both nitrite ion production (3-10-fold) and the expression of CD31 (an endothelial cell adhesion molecule) and also induced NOS in the adventitia of the vessels. In order to interpret the effect of statins on LPS-induced changes in the blood vessel, it was first necessary to examine the effect of prolonged exposure to the statin alone. While neither simvastatin nor pravastatin affected contractile responses to KCl or substance P-induced relaxations, contractions to U46619 were significantly increased (see Table 5.1). Interestingly,

the latter effect is qualitatively similar to that previously reported in both rat and human mesenteric arteries, where overnight incubation with lovastatin, and subsequent removal, was associated with increased potency of noradrenaline (Roulett *et al*., 1995). Thus, the effect of prolonged exposure to a statin on receptor-mediated contractile responses makes changes in KCl-induced contractions and SP-induced relaxations more reliable indicators of the modulation of inflammatory responses.

The mechanism underlying the selective effect of simvastatin and pravastatin on U46619-induced contractions is unclear. Even though prolonged inhibition of HMG CoA reductase by statins is associated with an increase in mRNA HMG CoA reductase and enzyme activity on removal of the drug (Puccetti *et al*., 2004; Laufs *et al*., 2005), it is unlikely that this factor is implicated. Specifically, after prolonged exposure to simvastatin there was no difference in the U46619-induced contractions between segments subsequently examined in simvastatin-free medium and those examined in the presence of simvastatin.

The key observation in this study is that high concentrations of simvastatin and pravastatin prevented the pro-inflammatory effect of LPS on the porcine isolated coronary artery. In the presence of the statin, LPS failed to reduce the maximum response to KCl, endothelium-dependent relaxations to substance P were not impaired and both nitrite ion production and the expression of CD31 were comparable to control preparations. The fact that simvastatin, a lipophilic agent, and pravastatin, a hydrophilic agent, were active in this model indicates that the anti-

inflammatory activity is a property of all statins and, therefore, similar to that reported in endotoxaemic human volunteers (Pleiner *et al*., 2004). Surprisingly, low concentrations of simvastatin (0.03-0.3μM), also inhibited LPS-induced nitrite ion production and prevented the impairment of substance P-induced relaxations, yet failed to prevent the reduction in KCl-induced contractions. Although I have previously demonstrated a close association between LPS-induced nitrite formation and suppression of contractile responses (Chapter 3), previous studies have highlighted that exposure of vascular smooth muscle to LPS also involves up-regulation in other vasoactive agents including production of both endothelin-1 and prostanoids (Bishop-Bailey *et al*., 1999; Wood *et al*., 1998). Indeed, earlier work in this laboratory has suggested a role for LPS-induced prostanoids, generated following the induction of COX-2, in supporting contractile responses in the porcine isolated coronary artery (Qi *et al*., 2007). Thus, it seems likely that the anti-inflammatory effect of statins involves a mechanism different from that of either quercetin or epicatechin. Taken together, since the anti-inflammatory effects of statins in the porcine isolated coronary artery was evident with concentrations lower than those known to occur with therapeutic doses of simvastatin (1μM; Lilja *et al*., 2004), the findings may have clinical significance.

### 5.6.2 Mechanism of action.

The principal target for the action of statins is inhibition of HMG CoA reductase (Ness and Chambers, 2000) and, interestingly, the activity of the enzyme has been reported to be elevated following exposure to LPS (Feingold *et al*., 1995; Memon *et al*., 1997). Although there is no direct evidence for the presence of the enzyme in

the porcine isolated coronary artery, it is possible to provide indirect support for the involvement of HMG CoA reductase by adding mevalonate to overcome the functional effect of the statin. As was the case for investigating the effect of the statins on LPS-induced changes in the blood vessel, it was necessary to first establish the effect of mevalonate alone on control preparations and LPS-exposed preparation. Overnight exposure to mevalonate alone did not affect KCl and U46619 responses in control preparations, but in LPS-treated segments caused a small, statistically significant increase in the maximum contraction to KCl (see Figure 5.8a). Nonetheless, the presence of mevalonate opposed the effect of simvastatin on LPS-induced changes to contractions induced by KCl and U46619 and also substance P-induced relaxations. These findings provide convincing evidence for a role of inhibition of HMG CoA reductase in the action of simvastatin.

A key observation in this chapter is that the anti-inflammatory action of simvastatin is also manifest if the drug is added *after* the inflammatory insult (Figure 5.11). Thus, changes in responses to KCl, U46619 and substance P produced by LPS could be reversed by exposure to simvastatin 16 hours after the onset of inflammation, which clearly makes these findings more clinically relevant. The vast majority of studies highlighting the anti-inflammatory actions of statins have tended to involve treatment *prior* to the inflammatory insult (see: Pleiner *et al*., 2004), but these findings provide support for the idea that statins could be used therapeutically in septic shock to improve outcomes (Brookes *et al*., 2009; Gao *et al*., 2008). This possibility raises the question as to mechanism underlying the effect of statins, since inhibition of the initiation of the inflammation is no longer an adequate explanation. It is noteworthy, therefore, that Reis and colleagues (2008) have suggested that

statins (and quercetin) may exert their primary anti-inflammatory effect by influencing protein breakdown at the level of the proteosome.

The final observation in this chapter is the finding that low, sub-therapeutic concentrations of simvastatin may synergise with dietary flavonoids (eg. epicatechin) to produce an anti-inflammatory effect on the vasculature. Qualitatively similar effects have been reported in isolated platelets (Franiak-Pietyga *et al*., 2009) and in patients (Naruszewicz *et al*., 2007). Thus, further experiments are warranted to establish whether this effect is also observed against other inflammatory markers (eg. LPS-induced cytokine production) and whether key flavonoid metabolites also possess the ability to interact with low concentrations of statins.

## 5.7 Conclusion

In summary, both simvastatin and pravastatin exhibit anti-inflammatory activity in intact blood vessels in manner comparable to that previously described for the flavonoids quercetin (Chapter 3) and epicatechin (Chapter 4). LPS-induced changes in vasoconstrictor and vasodilator responses, and the induction of inflammatory enzyme are suppressed by statins and, in the case of simvastatin, is manifest at concentrations lower than those currently used therapeutically to treat atherosclerosis. This effect appears to involve inhibition of HMG CoA reductase and, significantly is still apparent when administered after the onset of inflammatory processes. Taken together, the observations provide strong support for statins exerting important cholesterol-independent effects on the vasculature.

# Chapter 6

# General Discussion

The principal findings in this thesis are that quercetin and its metabolites, quercetin 3'-sulphate and quercetin 3-glucoronide, catechins and statins inhibit key inflammatory changes in the porcine isolated coronary artery induced by prolonged exposure to lipopolysaccharide.

## 6.1 LPS-induced changes in the porcine coronary artery

I have demonstrated that *in vitro* overnight incubation of porcine isolated coronary artery in Krebs-Henseleit solution with 1µg $mL^{-1}$LPS reproducibly induces substantial vascular hyporeactivity to KCl and to the thromboxane mimetic, U46619. Similar findings have also been reported in rat mesenteric artery (O'Brien *et al*., 2001), and in rat aorta (Wylam *et al*., 2001). While the LPS concentrations I used are similar to most other *in vitro* studies (Glembot *et al*., 1995; Muller *et al*., 1998; Scott *et al*., 1996), they still exceed those generally found in the plasma (mean peak 0.5 ng $mL^{-1}$) of septic humans (Danner *et al*., 1991). although concentrations as high as 10 ng $ml^{-1}$ have been reported in septic patients with meningitis (Brandizaeg *et al*., 1992), There has been one study of concentration-dependent depression of vascular contractility at much lower concentrations of LPS (1 – 100 ng $mL^{-1}$) in rat aorta rings incubated for 16 h in DMEM containing 1% FCS (Mckenna, 1990).

The addition of serum to the incubation medium enhanced and prolonged the effects of LPS. Serum contains LPS binding protein and soluble CD14 which have key roles in LPS-induced cellular activation and production of pro-inflammatory cytokines (Schiletter *et al*., 1995). LPS binding protein transfers LPS to soluble

CD14 and the newly formed LPS-CD14 complex can, in turn, activate endothelial and vascular smooth muscle cells to release transcription factors responsible for the induction of various proteins (Arditi *et al*., 1993; Loppnow *et al*., 1995). The situation is somewhat different in macrophages and monocytes which already contain membrane bound CD14 receptors that can be activated directly by LPS (Schiletter et al., 1995). However, CD14-independent mechanisms also contribute to LPS induction of iNOS expression and NO production in macrophages (Matsuno *et al*., 1998).

The continued presence of LPS within the incubation medium increased the reduction of vascular reactivity. The results indicated statistically significant reduction in the maximum response of KCl and U46619 of the segments exposed to 1μg $mL^{-1}$LPS for 42 hours in the presence of 10%FCS. A previous study, using an *in vitro* organ culture model of in rat aorta, reported that the expression of iNOS and COX-2 remained elevated over the entire 10-day duration in the presence of LPS (Bishop-Bailey *et al*., 1997).

Vascular hyporeactivity did not depend upon the presence of an intact endothelial layer, since denuding vessels of endothelium did not affect the level of vascular hyporeactivity following overnight exposure to LPS. This finding suggests that the medial and/or adventitial layers are principally responsible for continued hyporeactivity once the vessel has been transferred to the organ bath; an observation consistent with previous studies showing that an intact endothelium is not necessary for endotoxin-mediated vascular suppression (Hall *et al*., 1996; Julou-Schaeffer *et al*., 1990; Mckenna, 1990).

## 6.2 The effect of flavonoids and metabolites

Stimulation of inflammatory cells such as macrophages by bacterial endotoxins or inflammatory cytokines results in increased expression of inducible nitric oxide synthase (iNOS) and subsequent production of large amount of nitric oxide that is able to produce oxidative injury. The inhibition of this enzyme reduces the production of arachidonic acid, prostaglandins, leukeotrienes, and NO, which are crucial mediators of inflammation. Thus, the inhibition of these enzymes by flavonoids may be one of the most important mechanisms of their anti-inflammatory activity. Therefore, in this thesis I have investigate the major dietary flavonoids, quercetin and its main metabolites, quercetin-3`-sulphate and quercetin-3-glucurnide, and the catechins namely epicatechin, its isomer catechin and epigallocatechin gallate on LPS-induced inflammatory changes on porcine isolated coronary artery. The results indicated that, with the exception of myricetin, all tested flavonoids are able to suppress LPS-induced hyporesponsiveness in contractility and the elevation in nitric oxide production in the coronary artery, with the aglycone exhibiting activity at concentrations as low as 0.01µM. These results are in agreement with previous studies which found that flavonoids can inhibit lipopolysaccharide-induced iNOS gene expression, iNOS activity and nitric ion production in cultured macrophages (Chan *et al*., 1997; Sarkar and Bhaduri, 2001) and subsequently prevent oxidative damage. Moreover, the results indicated that quercetin suppressed the induction of nitric oxide synthase in the tunica adventitia, as demonstrated by an immunohistochemical approach. Interestingly, published data in rat aorta suggest that the adventitia is responsible for the majority of iNOS expression, NO production and medial hyporeactivity following exposure to LPS (Kleschyov *et al*., 1998; Zhangh *et al*., 1999). The results of this thesis suggest that quercetin, and its human metabolites

and the catechins may be considered as possible therapeutic agents for a variety of inflammatory diseases, such as atherosclerosis.

## 6.3 The effect of statins

Simvastatin and pravastatin suppressed the LPS-induced changes in endothelial and vascular responses, and suppressed the induction of nitric oxide synthase caused by LPS and the associated increase in nitrite ion production. These effects of simvastatin and pravaststin are similar to those noted in other studies. Simvastatin has been shown to decrease plasma levels of nitrate in vivo, in association with decreased iNOS activity (Giusti-Pavia *et al*., 2004). More recently, McGown *et al* 2010, reported that pravastatin reduced the increase in nitrite concentration induced by LPS in rat mesentry (McGown *et al*., 2010). Using immunohistochemistry I also reported that LPS caused increased expression of inducible nitric oxide synthase in the tunica advententia, however, co-incubation with either 3µM simvastatin or 10µM pravastatin significantly reduced LPS-induced expression of endothelial CD31 and adventitial nitric oxide synthase. Our results are in agreement with other studies, in that chronic administration of simvastatin has been shown to reduce production of NO via iNOS during inflammation induced by LPS (Alvarez de Sotomayer *et al*., 2008; Kang *et al*., 2004). Others have demonstrated that expression of iNOS in response to TNF-α and IL-1 is also reduced by statins (Madonna *et al*., 2005).

The observed vasculoprotective effects of statins are not related to their hydrophilic versus lipophilic properties, because both lipophilic statins (simvastatin) and hydrophilic statins (pravastain) exhibited similar effects.

HMG-CoA reductase catalyzes the conversion of HMG-CoA to mevalonate. Mevalonate metabolism yields a series of isoprenoid compounds including farnesyl pyrophosphate, geranyl geranylpyrophosphate, and squalene. The mevalonate pathway plays an important role in cell growth and signal transduction (Goldstein and Brown, 1990). Previous studies demonstrated that exogenous mevalonate completely prevented the inhibition of proliferation and migration of VSMCs induced by fluvastatin and the inhibition of the inhibition of preproendothin-1 transcription induced by simvastatin or atrovastatin (Herandez-Perera *et al*., 1998). It has been demonstrated that co-treatment with mevalonate completely reverses eNOS upregulation induced by simvastatin or atrovastatin (Laufs *et al*., 1997; Laufs *et al*., 1998). In this study, I observed that addition of mevalonate reversed the effect of simvastatin on LPS-induced changes in KCl and U46619-induced contractions, SP-induced relaxations and nitrite ion production. These effects of statins on LPS-induced changes appear to be mediated through specific inhibition of HMG-CoA reductase, since they were reversibly blocked by excess amounts of mevalonate. Recent studies suggest that, by blocking the mevalonate pathway, statins exerts pleriotropic effects on vascular cell function which may not be related directly to cholesterol synthesis (Laufs *et al*., 2001; Wassmann *et al*., 2001).

Several studies have investigated whether statins are most effective as a prophylactic treatment during sepsis (Merx *et al* 2004, Merx *et al* 2005). Our results indicate that addition of simvastatin to the incubation medium 16 hours after exposure to LPS reversed the inhibitory effect of LPS on KCl and U46619 produced contraction. Ando and colleagues (2000) found that cervistatin was ineffective when administered after LPS injection; however, in the same study, pre-treatment with cervistatin

reduced mortality rate. This effect of simvastatin was manifest even when administered several hours after exposure to LPS.

## 6.4 Further Possible Studies

The experiments of this thesis examined the vascular responses of the porcine isolated coronary artery only. Although a 30-40% reduction in the response to norepinephrine has been reported in an in vivo model of endotoxemia (Datta and Magder 1999), there is no literature that has directly compared the effects of LPS on vascular function of pigs ex vivo with that *in vitro*. Thus, it is hard to directly compare my results with other *in vivo* reports. Also, it is not clear whether other types of vascular bed, for example, the mesenteric artery or small resistant artery respond similarly to the stimulation by lipopolysaccharides. Nevertheless, the establishment of the inflammation in vascular model in this thesis provides the potential for investigating other type of vascular beds, for example, resistance arteries or veins, following exposure to the different inflammatory stimuli. Moreover, the induction of inducible nitric oxide synthase can be detected by using an immunohistochemical technique on this vascular model.

The concentrations of quercetin used in this study exceeded the peak plasma levels of the aglycone (0.03µM) and the metabolites (3µM) detected following dietary ingestion (Kroon *et al*., 2004; Wang and Morris, 2005). Nonetheless, further studies on human blood vessels with lower concentrations of quercetin and its metabolites are warranted. In light of the striking difference between the effects of quercetin and myricetin on the porcine coronary artery, there is also a need to establish whether

myricetin can inhibit the effect of quercetin in human vascular cells, effectively behaving as an 'antagonist'.

Further studies will be needed to determine the relative merits of flavonoid consumption as supplements or whether consumption of these compounds in whole foods and beverages is preferable, as is currently recommended by the American Heart Association (Kris-Etherton *et al.*, 2004).

Many large clinical studies conducted over the last two decades have shown that flavonoids exert positive influences on health and a diet rich in flavonoids alleviate and prevent many serious diseases. Flavonoids as antioxidants are ideal nutraceuticals for neutralizing stress-induced free radicals. Many other actions of flavonoids like in cancer prevention have been reported in the recent literature, but large clinical trials are necessary before these effects are proved.

The continual efforts will provide new insight into the anti-inflammatory activity of flavonoids, and might eventually lead to the development of a new class of anti-inflammatory agents based on the flavonoid molecule.

## 6.5 General Conclusion

In conclusion, I have established an impaired vascular reactivity model by exposing porcine coronary artery segments to 1μg/ml LPS overnight in Krebs-Henseleit solution at 37ºC in presence of 10% foetal calf serum. The responses of porcine isolated coronary artery to low concentrations of LPS is enhanced by serum, possibly due to the participation of sCD14 and LBP. I also have demonstrated that dietary

flavonoids, quercetin, and its principal human metabolites and catechins oppose pro-inflammatory events in both endothelial cells and vascular smooth muscle cells. Since pre-treatment of the porcine coronary artery with statin reduced LPS-induced changes in vasoconstrictor responses, suppressed the induction of nitric oxidec synthase caused by LPS and the associated increase in nitrite production. It is unlikely that the effect of the statin involves direct inhibition of NOS. These findings are consistent with clinical studies suggesting that prior use of statins may afford protection against bacterial sepsis.

*When Hippocrates, the Father of Medicine, said that "Let food be thy medicine, and let thy medicine be food" he was probably referring to foods like flavonoids.*

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