Purine Participation in the Vasomotor Effect of Hypochlorous Acid in the Porcine Coronary Artery

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Abstract

Neutrophils are part of the body's defence mechanism against microorganisms. They produce their anti-inflammatory action by producing different reactive oxygen species (ROS). Hypochlorous acid (HOCl), generated by neutrophils, plays a major role in killing pathogens and mediating inflammation. It is known that nucleotides and nucleosides are also released during inflammation. Inflammation leads to dysfunctional purinergic signalling, which is linked to cardiovascular diseases such as hypertension, diabetes, atherosclerosis, and thrombosis. However, it is unclear whether purine nucleotides are involved in HOCl's vasomotor response. Understanding vascular control mechanisms is essential for developing new therapeutic approaches to cardiovascular diseases such as hypertension and atherosclerosis. The main purpose of this study was to investigate the direct vasomotor effects of HOCl on the porcine coronary artery (PCA) as well as the mechanisms involved in this response. Further investigation was conducted to examine the possibility that purine receptors were involved in the response produced by HOCl by using different purinergic receptor antagonists. Moreover, the role of pannexin 1 and connexin channels (through which ATP may be released) in PCA's response to HOCl was investigated. It was also investigated whether HOCl released ATP from cultured human coronary artery endothelial cells. Moreover, the pharmacological profile of exogenous ATP and adenosine on PCA was examined for similarities with that of HOCl.

Segments of the PCA were placed in an organ bath for isometric tension recording. They were incubated in the absence or presence of different antagonists and inhibitors, pre-contracted with U46619 (thromboxane A_2 receptor agonist), then exposed to HOCl (100 and 500 μM). In some segments, the endothelium was removed to investigate the

endothelium-dependency of the responses, and in some segments, the perivascular adipose tissue (PVAT) was retained.

At 100 μM, HOCl induced an initial transient endothelium-dependent relaxation which returned to baseline; thereafter, a second slow relaxation independent of the endothelium was observed for 60 minutes. A transient endothelium-dependent relaxation response was also induced by 500 μM HOCl, followed by endothelium-dependent contraction; slow endothelium-independent relaxation was observed over 60 minutes. Nitic oxide synthase (NOS) inhibitors also inhibited the rapid relaxation in response to 100 and 500 μM HOCl and the contraction to 500 μM HOCl on the PCA. Based on these findings, it appears that NO might play a role in the rapid relaxation and contraction of HOCl. The HOCl-induced rapid relaxation and slow relaxation over 60 minutes were blocked in tissues precontracted with KCl instead of U46619. This indicates that the relaxation of PCA rings produced by HOCl is mediated by a mechanism that is sensitive to membrane hyperpolarization. Moreover, PVAT exerts an anti-contractile effect on the HOCl response.

An adenosine P1 receptor blocker, 8-(p-sulphophenyl) theophylline, inhibited the rapid relaxation of both 100 and 500 μM HOCl and the contraction of 500 μM HOCl, indicating that adenosine receptors may be involved. In the presence of suramin (P2 receptor antagonist), rapid relaxation at 100 μM HOCl was blocked, whereas rapid relaxation at 500 μM HOCl was still evident. However, suramin blocked the contraction to 500 μM HOCl. This may indicate that P2 receptors play a role in the response to HOCl. The study excluded a subset of P2 purinoceptors (P2X1, $P2Y_1$, and P2X4) because specific antagonists for these receptors were found not to affect HOCl responses. The effect of HOCl was not prolonged or altered in any way by ARL67156 (ecto-ATPase inhibitor).

However, apyrase (hydrolysis nucleotides) abolished the response to both concentrations of HOCl. Probenecid and carbenoxolone (pannexin 1 and connexin channel blocker) inhibited contraction to 500 μM HOCl, consistent with HOCl-induced purine release via pannexin 1 and/or connexin channels, with purines acting at vasomotor P2 receptors. The concentration of extracellular ATP in samples of bathing fluid from cultured human coronary artery endothelial cells (HCAECs) was measured directly by a luminescence ATP assay kit (Abcam, Cat#ab113849). It found that exposure of HCAECs to 100 μM HOCl resulted in a significant increase in ATP levels within 1 minute. It is consistent with the observation that HOCl-induced rapid endothelium-dependent relaxation of PCA may occur through its actions on P2 and/or P1 receptors (subsequent to the release of ATP from the endothelium and breakdown of ATP to adenosine).

Investigation of the responses to exogenous adenosine (30 μM) and ATP (100 μM) showed that the response to adenosine consisted of a largely endothelium-independent relaxation response, whereas the response to ATP additionally included a transient rapid relaxation phase that was partially dependent on the endothelium. Neither L-NAME nor suramin affected the relaxation response to adenosine or ATP. Adenosine and ATP responses were significantly inhibited by 8-SPT and apyrase, which is consistent with the inhibitory effects of 8-SPT and apyrase on HOCl responses in the PCA.Together, these results suggest that P1 and P2 purine receptors may participate in the vasomotor response of the PCA to HOCl. Additionally, the evidence suggests that connexin and pannexin 1 channels are involved in ATP release in response to HOCl. It is evident that purinergic signalling plays an important role in inflammatory responses and vascular contractility as a result of this interaction.

Dedication

This thesis is dedicated to my beloved brother, Emad. Your memory lives on in every step I take and on every page of this work. Your spirit, kindness, and encouragement have always inspired me. Even though you are no longer with us, your influence continues to guide me. You are deeply missed. May your soul rest in peace.

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In closing, I would like to express my deepest love and appreciation for my son hero, Abdulmalik. I have been continuously inspired by your smiles and enthusiasm. In spite of the difficulties encountered during this work, I was motivated by the knowledge that I was doing it for you. My ambition and resilience are fuelled by your presence in my life. I'm thankful for your light. I love you.

Publications

1- **Baghdadi A,** Dunn WR, Ralevic V. (2019).

Effect of Hypochlorous Acid on the Vascular Contractility of the Porcine Coronary Artery. UK Purine Club 10th Anniversary Symposium 2019, Sheffield, UK.

2- **Baghdadi A,** Dunn WR, Ralevic V. (2021).

Purine involvement in hypochlorous acid effects on vascular contractility of the porcine coronary artery. Pharmacology 2021, Online, worldwide.

3- Alqarni A, & **Baghdadi A.** (2022).

Functional role of adenosine via KATP channels in cerebral capillary endothelial cells and pericytes. Purinergic Signalling, Journal Club.

4- **Baghdadi A**, Dunn WR, Ralevic V. (2022).

Purine participation in the effect of hypochlorous acid on the porcine coronary artery's vascular contractility. Pharmacology 2022. ACC Convention Centre, Liverpool, UK.

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List of Abbreviations

8-SPT	8-(p-sulphophenyl) theophylline
ABC	ATP-binding cassette
AC	Adenylyl cyclase
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanine monophosphate
CNS	Central nervous system
COX	Cyclooxygenase
DAG	Diacylglycerol
DAMPs	Danger-associated molecular patterns
ECM	Extracellular matrix
EDH	Endothelium-derived hyperpolarising
EDRF	Endothelium-derived relaxation factor
E-NTPDase	Ecto-nucleoside triphosphate diphosphohydrolase
ET	Endothelin
GPCR	G protein-coupled receptor
GTP	Guanosine-5'-triphosphate
HCAECs	Human coronary artery endothelial cells
HOCl	Hypochlorous acid
IL-β	Interleukin-1β
IP_3	Inositol 1,4,5-triphosphate
K_{ATP}	ATP-sensitive K^+ channels
K_{Ca}	Large conductance Ca^{2+}-activated K^+ channels
K_V	Voltage-gated K^+ channels

LDL	Low-density lipoprotein
L-NAME	N^G-Nitro-L-arginine methyl ester
L-NMMA	N^G-monomethyl-L-arginine
MLC	Myosin light chain
MLCP	Myosin light chain phosphatase
MPO	Myeloperoxidase
NADPH	Nicotinamide adenine dinucleotide phosphate
NOS	Nitric oxide synthase
PAMPs	Pathogen-associated molecular patterns
PCA	Porcine coronary artery
PCR	Polymerase chain reaction
PGD synthase	Prostaglandin D synthase
PGD_2	Prostaglandin D_2
PGE synthase	Prostaglandin E synthase
PGE_2	Prostaglandin E_2
PGF synthase	Prostaglandin F synthase
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PGH_2	Prostaglandin H_2
PGI synthase	Prostacyclin synthase
PGI_2	Prostacyclin
PKC	Protein kinase C
PKG	cGMP-dependent protein kinases
PLA_2	phospholipase A_2
PLC	Phospholipase C
PMN	Polymorphonuclear neutrophils
PVAT	Perivascular adipose tissue
ROS	Reactive oxygen species

SR	Sarcoplasmic reticulum
TNF-α	Tumour necrosis factor-α
TXA_2	Thromboxane A_2
U46619	9,11-dideoxy-11α,9α-epoxymethanoprostaglandin F2α
UDP	Uridine diphosphate
UTP	Uridine triphosphate
VSMC	Vascular smooth muscle cell

Chapter 1 General Introduction

1.1 Blood Vessel Structure

The majority of blood vessels, except capillaries, consist of three layers: the tunic intima, the tunica media, and the tunica externa, from inner to outer layer respectively, as shown in Figure 1-1 (Lilly, 2014). Endothelial cells comprise the main component of the tunic intima. They prevent blood components from leaving the blood vessel and produce paracrine, autocrine, and endocrine hormones that regulate permeability and blood flow through the vascular wall (Cherng et al., 2017). The middle layer, the tunica media, contains vascular smooth muscle cells (VSMCs). These have two important proteins in their cytoplasm, myosin (thick filaments) and actin (thin filaments), to control contraction. The tunica media has elastic tissue and collagen. In VSMCs, the sarcoplasmic reticulum (SR) acts as a Ca^{2+} store (Zhou et al., 2018). The outer layer is the tunica externa, which consists of adventitial fibroblasts and fibrous connective tissue that synthesises collagen, elastin, and extracellular matrix (ECM) proteins. These components help cells proliferate, migrate, and deposit collagen in response to tissue injury and stretch (Cherng et al., 2018). The adventitia exhibits both lymphatic and nerve networks. The perivascular cells are closely associated with the adventitia, especially in the coronary arteries and aorta (Brown et al., 2014; Majesky, 2015). Perivascular tissue consists of lymphatic vessels, adipocytes, perivascular nerves, and stromal cells. Adventitia and periadventitial cells are interconnected by microvessels, nerves, and migratory cells, which influence vascular homeostasis, physiology, and disease progression (Majesky, 2015). Perivascular adipose tissue (PVAT) surrounds the adventitia in most blood vessels and lies on its exterior, with no clear anatomical barrier separating it from the adventitia (Brown et al., 2014). PVAT is also referred to as the fourth layer of the blood

vessel or the "tunica adiposa". PVAT is widely distributed throughout the body, except in cerebral circulation. It is present in the walls of large arteries and veins, resistance vessels, and skeletal muscle microvessels. Different vasoactive compounds are produced from these layers, which will be discussed in detail below.

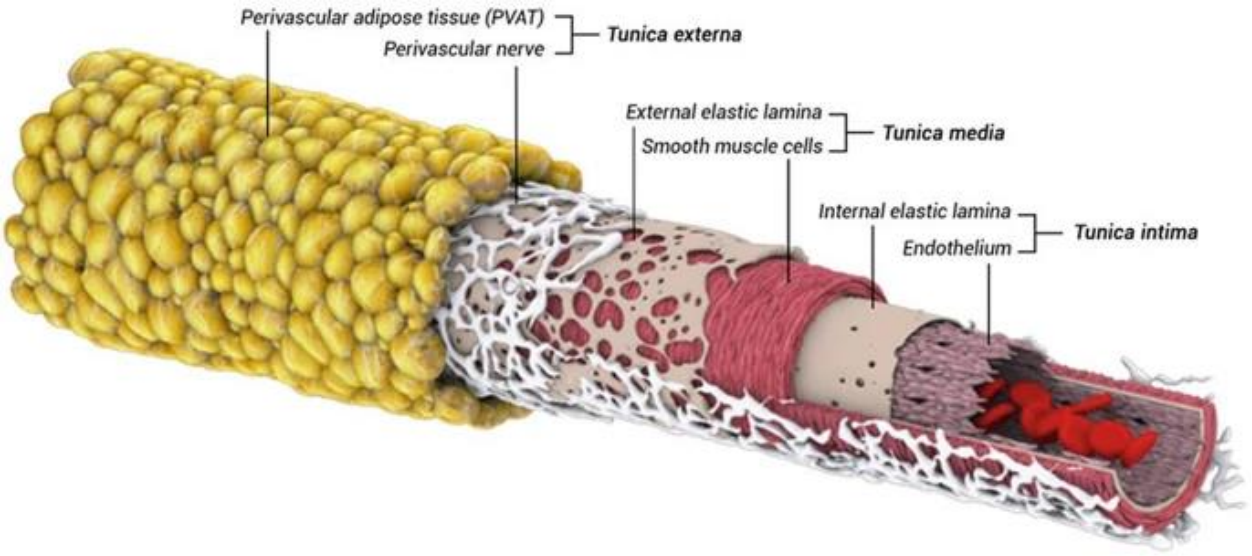


Figure 1-1. The layers of the vascular wall are the tunica intima, the tunica media, the tunica externa, and PVAT. Figure adapted from Daly (2019).

1.1.1 Endothelium

Blood and intact tissue are separated by the endothelium that controls several physiological and pathological processes, including cell adhesion, inflammation, coagulation, vascular tone, and permeability (Hinsbergh, 2012). The endothelium produces many mediators that regulate blood flow by changing vascular diameter. These substances include nitric oxide (NO), angiotensin II, prostacyclin, endothelin, and endothelium-derived hyperpolarising (EDH) factor (Kharbanda & Deanfield, 2001; He, 2005; Pirahanchi & Brown, 2019).

1.1.1.1 Nitric Oxide

A factor called endothelium-derived relaxation factor (EDRF) was proposed to explain acetylcholine's ability to induce relaxation of arterial smooth muscle cells by releasing it from intact endothelial cells. However, the researchers were not able to positively identify the specific EDRF. They excluded the possibility that EDRF was prostacyclin, bradykinin, cAMP, or adenosine (Furchgott & Zawadzki, 1980). The reason for this is that they either do not relax aortic preparations or cause only minimal relaxation. Moreover, adenosine and cAMP were excluded from consideration since their maximum relaxation effect was significantly less than that of acetylcholine (Furchgott & Zawadzki, 1980; Furchgott et al., 1984; Ignarro et al., 1987). Later, NO was identified as one of the factors involved in the release of EDRF from endothelial cells (Palmer et al.,1987).

NO is produced from nitric oxide synthase (NOS). There are three forms of NOS: neuronal, inducible, and endothelial (nNOS, iNOS, and eNOS, respectively). nNOS is constitutively expressed in specific neurons in the brain. Ca^{2+} and calmodulin regulate the enzyme's activity. Cells generally do not express iNOS, but bacterial lipopolysaccharide, cytokines, and other factors can stimulate its expression. As soon as it is expressed, iNOS is active and is not controlled by intracellular Ca^{2+} levels (Förstermann & Sessa, 2012). Endothelial cells are the primary source of eNOS expression; it has also been identified in some neurons and in kidneys, placentas, cardiomyocytes, and platelets. Ca^{2+} regulates eNOS activity in a manner similar to that of nNOS. eNOS gene expression appears to be quantitatively regulated by shear stress and cell proliferation. Various agonists (e.g., bradykinin, adenosine, adenosine

diphosphate (ADP)/adenosine triphosphate (ATP), and thrombin) stimulate eNOS enzymatic activity in part by increasing intracellular free Ca^{2+} levels (Arnal et al., 1999).

NOS is present in an inactive form in caveolae at the plasma membrane, attached to caveolin. Active NOS results from an increase in Ca^{2+} in the endothelium with the detachment of caveolin from NOS (Kharbanda & Deanfield, 2001).

NOS uses the substrate L-arginine and several co-factors, including nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide, flavin mononucleotide, and tetrahydrobiopterin, to produce NO and L-citrulline (Cherng et al., 2018). NO, a diffusible gas, can enter both lumens and VSMCs. In smooth muscle, NO binds to the heme component of guanylyl cyclase, guanosine triphosphate (GTP) is cleaved into two phosphate groups and cyclic guanine monophosphate (cGMP). An increase in cGMP leads to cGMP-dependent protein kinase G(PKG) activation, which has several actions on VSMCs (Cherng et al., 2018). Activated PKG decreases Ca^{2+} through four mechanisms as shown in Figure 1-2: 1. The voltage-dependent Ca^{2+} channels are blocked, thereby reducing Ca^{2+} influx into the cells. 2. An increase in ATP-dependent Ca^{2+} efflux is caused by the activation of plasma membrane Ca^{2+} ATPases. 3. Blocking of inositol 1,4,5-triphosphate (IP_3) receptors to block entry of Ca^{2+} from the SR to the cytosol. 4. Sarcoplasmic Ca^{2+} ATPases (SERCA) are activated, which increases the ATP-dependent transport of Ca^{2+} from the cytoplasm to the SR. Decreased Ca^{2+} levels leads to the activation of myosin light chain phosphatase (MLCP) which causes vasodilatation (Khalaf et al., 2019).

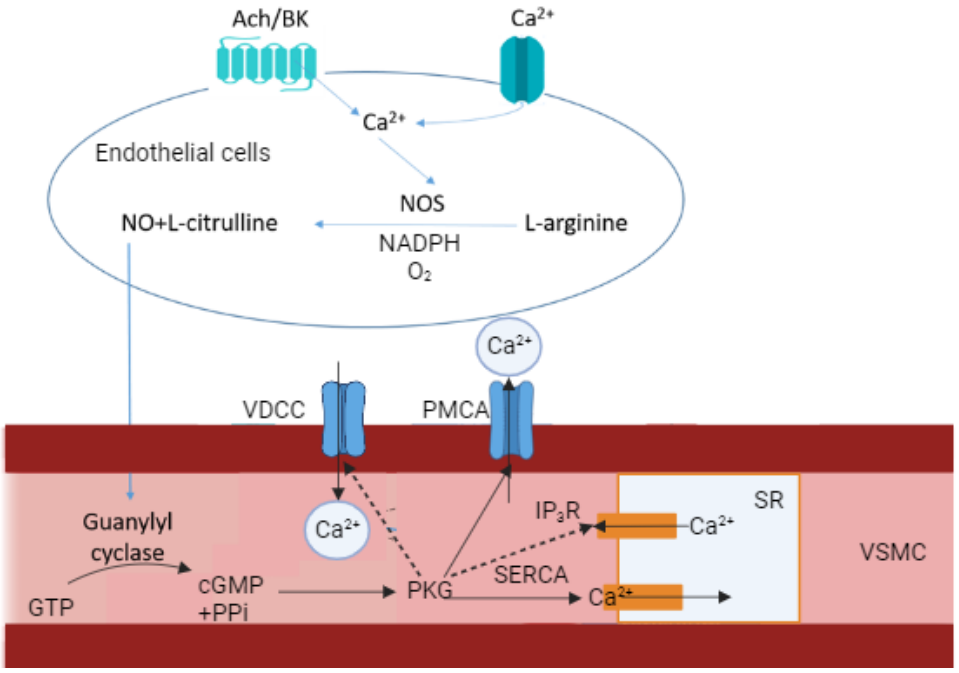


Figure 1-2. Endothelial cells produce nitric oxide as a result of activating eNOS. cGMP and vasorelaxation are caused by NO diffusion in smooth muscle cells. Adapted from Khalaf et al. (2019). (VDCC): voltage-dependent Ca^{2+} channels, (PMCA): plasma membrane Ca^{2+} ATPases, (IP_3R): inositol triphosphate receptors, and (SERCA): sarcoplasmic Ca^{2+} ATPases.

NO has other actions, including inhibition of platelet aggregation, leucocyte migration, cell proliferation, and cellular adhesion (Vallance, 2001). When released into the vascular lumen, NO inhibits the aggregation of platelets and their adhesion to the vascular wall (Cheung et al., 1997). Also, NO has the potential to inhibit leukocyte adherence to the vessel wall by interfering with CD11/CD18's ability to bind to the endothelial cell surface or by inhibiting the expression of CD11/CD18 on leukocytes (Kubes et al., 1991). It has also been shown that NO inhibits DNA synthesis, and proliferation in vascular smooth muscle cells. cGMP may be responsible for these antiproliferative effects (Nakaki et al., 1990). The presence of NO derived from the

endothelium plays a significant role in initiating endothelium-dependent relaxations when stimulated by a variety of agonists.

NO release has been demonstrated in many other blood vessels, including in the porcine coronary artery (PCA) (Kilpatrick & Cocks, 1994), and human brachial arteries (Harasawa et al., 1997). NO has a lesser effect on relaxing vessels when vessels are smaller. Since larger arteries express more of eNOS, they are more dependent on NO-mediated, endothelium-dependent dilation. Due to their increase of VSM and connective tissue proteins than smaller vessels, large vessels require adequate NO to dilate multiple layers of smooth muscle, or to allow downstream of smaller vessels to dilate (Laughlin et al., 2003). This has been observed in other research, including that ACh-dependent relaxation is mediated primarily by NO in superior mesenteric arteries (unstretched luminal diameter 650 microns) pre-contracted by phenylephrine rather than mesenteric resistance arteries (unstretched luminal diameter 200 microns) pre-contracted by phenylephrine which is not sensitive to NO (Hwa et al., 1994). It is similar to what Tschudi et al. (1991) found. The study indicates that endothelium-dependent vasorelaxation to clonidine (α_2-adrenergic agonist) has a dependency on NO in intramyocardial porcine coronary resistance arteries, though maximal relaxation in coronary resistance arteries is less pronounced than in large coronary arteries (i.e. porcine epicardial coronary arteries) in response to clonidine (Richard et al., 1990).

Furthermore, NO is spontaneously released from endothelial cells under basal conditions (Shimokawa et al., 1996). There is continuous secretion of NO to maintain vascular tone. NG monomethyl-L-arginine (L-NMMA), which is an L-arginine analogue,

is one of several NOS inhibitors that have been used to study the role of NO. Adding L-NMMA to the isolated ring of rabbit aorta causes endothelium contraction (Vallance, 2001), while an increase in blood pressure results from the injection of L-NMMA to experimental animals intravenously. Infusion of L-NMMA into the brachial artery in the forearms of humans also causes vasoconstriction (Vallance et al., 1989). These observations indicate that basal NO release acts to suppress vascular smooth muscle tone.

1.1.1.2 Prostaglandins

Endothelial cells also produce thromboxane A_2 (TXA_2), prostacyclin (PGI_2), and other prostaglandins including prostaglandin E_2 (PGE_2), prostaglandin D_2 (PGD_2) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) through the action of cyclooxygenase (COX) (Wong & Vanhoutte, 2010). There are two isoforms of COX (COX-1 and COX-2) that catalyse the conversion of arachidonic acid to the inactive endoperoxide prostaglandin H_2 (PGH_2) by phospholipase A_2 (PLA_2). Afterwards, PGH_2 can be transformed by different synthases into PGI_2, TXA_2 , PGE_2, or PGD_2 as shown in Figure 1-3 (Michael, 2023).

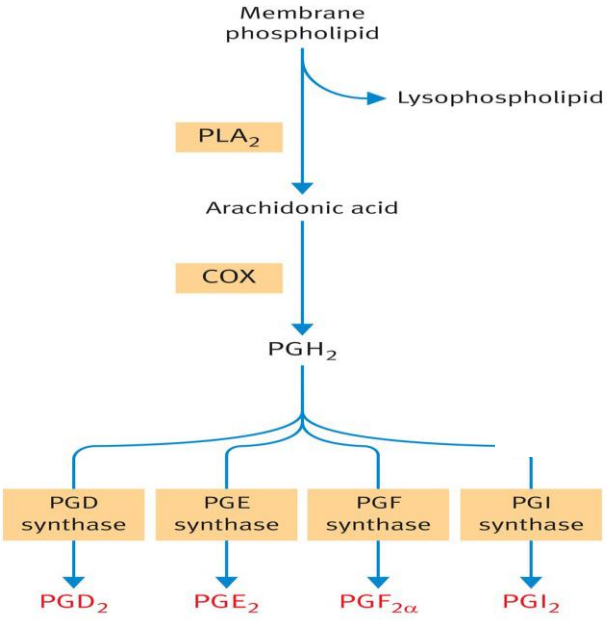


Figure 1-3. Cyclooxygenase (COX) and prostaglandins synthesis. Adapted from Michael (2023).

PGI_2 and TXA_2 are generally considered the most important cyclooxygenase metabolites. PGI_2 is a vasodilator which acts to bind to IP_3, a G protein-coupled receptor (GPCR), to increase cyclic adenosine monophosphate (cAMP) levels, leading to efflux of Ca^{2+} from cytosol and potassium channel (K^+) mediated hyperpolarisation. Increased cAMP also inhibits platelet aggregation and cell proliferation in the case of cell injury (Lau & Lui, 2021). Under normal conditions, there is a constant secretion of PG_2 to help maintain vascular integrity (He, 2005; Cherng et al., 2018).

TXA_2 is a vasoconstrictor and increases platelet aggregation. Under normal conditions, the vasodilator effect of prostacyclin is dominant, but this scenario changes in pathological states where vasoconstrictor prostanoids become more prevalent (Kharbanda & Deanfield, 2001).

COX-1 is present at relatively stable levels in many types of tissues and cells, including platelets, VSMCs, and endothelial cells. COX-2, in contrast to COX-1, is present in

inflammatory sites as well as in the brain, kidneys, and the endothelium of vessels (Morita, 2002). Various studies have shown that optimal blood pressure and platelet function require a balance between NO and PGI_2 *in vivo*. One study concluded that celecoxib (a selective COX-2 inhibitor) and indomethacin (a non-selective COX inhibitor) do not affect blood pressure in healthy mice, but if NO generation is inhibited, these drugs can significantly raise blood pressure. Accordingly, NO appears to play a protective role against the effects of non-steroidal anti-inflammatory drugs (NSAIDs) on blood pressure (Anning et al., 2006).

1.1.1.3 Endothelin (ET)

Endothelin is a pre-prohormone produced from the activating of ET-converting enzyme. It has three active forms: ET-1, ET-2 and ET-3. ET-1 and ET-2 are potent vasoconstrictors, while ET-3 is a vasodilator. There are two G-protein receptor targets, ET_A receptor (ET_AR)and ET_B receptor (ET_BR) (He, 2005; Cherng et al., 2017). ET-1 acts as vasoconstrictor, promotes cell proliferation, and causes endothelium-dependent contraction (Barton, 2011).

In endothelial cells, ET_BR are expressed and cause vasodilation by increasing the secretion of vasodilator as NO and PGI_2. They also accelerate clearance of circulatory ET-1 from kidney and lungs (Rodríguez-Pascual et al., 2011). In contrast, ET_AR is highly expressed in smooth muscle and heart tissue. It acts as a vasoconstrictor by activating phospholipase C, increasing IP_3 and diacylglycerol (DAG) levels, and subsequently activating protein kinase C (PKC) and increasing Ca^{2+} secretion from the SR (Malmsjö et al., 2007; Rodríguez-Pascual et al., 2011).

1.1.1.4 Endothelium-Dependent Hyperpolarisation

In addition to NO and PGI_2, an endothelial pathway called endothelium-dependent hyperpolarisation (EDH) can lead to VSMC relaxation (Pirahanchi & Brown, 2019). EDH increases $[Ca^{2+}]i$ in endothelial cells, and as a result, activates and opens Ca^{2+} dependent K^+ channels (K_{Ca}), specifically intermediate conductance (IK_{Ca}) and small conductance (SK_{Ca}) channels. This does not include the large conductance KCa (BK_{Ca}) channel. EDH responses are sensitive to charybdotoxin, a non-selective inhibitor of BK_{Ca} and IK_{Ca}, and apamin, a specific inhibitor of SK_{Ca} channels but not iberiotoxin (a specific inhibitor of BK_{Ca} channels) (Busse et al., 2002). In addition, BK_{Ca} channels are present in VSMCs and not in endothelial cells (Félétou & Vanhoutte, 2009). Activation of these channels will lead to endothelial cell hyperpolarisation (Félétou & Vanhoutte, 2009).

There are different suggestions for EDH causing VSMC relaxation. First, in endothelial cells, arachidonic acid metabolises into three important substances: COX, which is responsible for synthesising a different type of PG as PGI_2 and TXA_2; lipoxygenase to produce oxygenated fatty acid and leukotrienes; and cytochrome P450 (CYP450) enzymes (Weintraub et al., 1994). CYP450 metabolises arachidonic acid to epoxygenases and hydroxyeicosatetraenoic acids. Epoxygenases synthase four regioisomeric epoxyeicosatrienoic acids (EETs) (14,15-, 11, 12-, 8,9-, and 5,6-) which are vasodilators while hydroxyeicosatetraenoic acid acts as a vasoconstrictor (Fleming, 2004; Félétou & Vanhoutte, 2009). 11,12- and 5,6-EET were considered the main EDH factor that independent vasodilator to NO and PGI_2 (not changed or partially inhibited by

NO and PGI_2 inhibitors) (He, 2005). However, there is disagreement regarding this because EET activates BK_{Ca} rather than IK_{Ca} and SK_{Ca} channels, which do not account for EDH (Félétou & Vanhoutte, 2004; Fleming, 2004). EET can consider an intracellular second messenger to begin hyperpolarisation by regulating Ca^{2+} influx to endothelial cells and increasing K^+ channel activity (Busse et al., 2002).

Secondly, endothelial cell hyperpolarisation can be achieved by increased K^+ in the sub-endothelial space, which activates rectifying K^+ (K_{IR}) and Na^+-K^+ ATPase. The hyperpolarisation occurs subsequent to K_{Ca} channel activation (Félétou & Vanhoutte, 2009).

Thirdly, hyperpolarisation can be transmitted through gap junctions between endothelial cells and smooth muscle cells (Félétou & Vanhoutte, 2009). Moreover, EET may have a role in gap junction communication between endothelial cells and VSMCs (Busse et al., 2002) and diffuse to VSMCs to activate BK_{Ca} and cause relaxation (Félétou & Vanhoutte, 2009).

Fourthly, it has been shown that hydrogen sulphide (H_2S) is a major EDH formed in vascular endothelial cells through the action of cystathionine γ-lyase (CSE), a protein that is Ca^{2+} calmodulin-dependent. CSE activates ATP-sensitive, small conductance, and intermediate conductance K^+ channels. Other enzymes, such as cystathionine β-synthase and 3-mercaptopyruvate sulphurtransferase, have also been demonstrated to generate H_2S in various tissues (Mustafa et al., 2011; Wang et al., 2015).

1.1.2 Vascular Smooth Muscle

The contraction of VSMC results from different physiological responses from hormones and from paracrine, autocrine, and sympathetic nervous systems which alter the diameter of blood vessels and lead to alterations in resistance to flow (Cherng et al., 2018). This is an important factor regulating blood pressure and allowing the control of blood flow to different organs (Zhou et al., 2018). VSMCs have two phenotypes: synthetic proliferative and differentiated contractile. The main role of synthetic proliferative VSMCs (also referred to as secretory) is to produce ECM, collagen and elastin, which are essential for migration and proliferation as a result of any pathological or physiological changes, such as in injury, inflammation, hypertension, or pregnancy. The second phenotype, which is contractile, plays an important role in muscle contraction (Zhou et al., 2018).

A cardiovascular system is composed of the heart and blood vessels (arteries, capillaries, veins). Under various conditions, heart rate and cardiac output are affected by the body's need for oxygen and nutrients. The autonomic nervous system, hormones, and other factors regulate heart rate and contractility in order to respond rapidly to changing tissue requirements (Gordan et al., 2015). In the heart, coronary arteries run along the myocardium. They are primarily responsible for supplying blood to and draining blood from the heart's tissues, hence regulating blood pressure (Ogobuiro & Tuma, 2018). NO is released by the endothelium of the coronary artery, which relaxes the smooth muscles. The endothelium is required for both normal coronary artery dilation and vascular growth, repair, and thrombosis resistance. There is also a cardiac

response to reduced pressure independent of endothelial function. Moreover, in response to sympathetic and parasympathetic activation, adrenergic and muscarinic receptors regulate vasoconstriction or vasodilation of coronary arteries Schelbert, 2010; Reynolds et al., 2022). Coronary artery disease is caused by a restriction in the flow of blood through the coronary arteries, which leads to malfunctioning of heart muscle. Coronary artery disease is primarily caused by atherosclerosis (Ogobuiro & Tuma, 2018).

1.1.2.1 Relaxation and Contraction Mechanism of VSMCs

Intracellular free Ca^{2+} concentration largely determines the contractile status of VSMCs. It can be released from the extracellular space into the cytoplasm though voltage-gated Ca^{2+} channels or receptor-operated Ca^{2+} channels on the plasma membrane that open to allow the influx of Ca^{2+} from the extracellular space, down its concentration gradient (Cherng et al., 2018).

Voltage-gated calcium channels are a group of calcium-selective proteins present in both excitable and nonexcitable cells. Calcium entery affects membrane potential properties by depolarizing cells and an increase in excitability in general. A wide variety of physiological processes are controlled by voltage-gated calcium channels, such as contraction, gene expression, hormone secretion, and neurotransmitter release. A significant role for voltage-gated calcium channels is to regulate vascular tone and blood pressure, and their malfunction is associated with a variety of cardiovascular diseases, such as hypertension and arrhythmias (Bidaud et al., 2006; Catterall, 2011). It is possible to categorize voltage-gated calcium channels into two categories based on

their thresholds of activation: low voltage activated (LVA) and high voltage activated (HVA). There are four types of voltage-gated calcium channels based on their pharmacological characteristics: T-, L-, N-, P/Q- and R-types (Bidaud et al., 2006). However, receptor-operated Ca^{2+} channels function differently. The activation of these processes is triggered by the binding of ligands to a range of G-protein-coupled receptors on the surface of cells. A change in membrane potential does not affect this binding. Activating receptor-operated Ca^{2+} channels allow Ca^{2+} to enter the cell, which initiates a series of reactions that cause muscles to contract (McFadzean & Gibson, 2002).

Ca^{2+} can also be released from the SR (Khalil, 2010). When a ligand or hormone binds to a GPCR that activates phospholipase C, two second messengers are produced: diacylglycerol (DAG) and IP_3. DAG activates PKC, while IP_3 causes Ca^{2+} to be released from the SR (Webb, 2003).

Once Ca^{2+} level rises in cytoplasm, myosin light chain (MLC) kinase is activated as a result of the interaction between Ca^{2+} and calmodulin which then phosphorylates MLC to enable cross-bridge cycling to happen. Furthermore, Ca^{2+} sensitisation maintains smooth muscle contraction by inhibiting myosin phosphatase enzyme (Khalil, 2010).

The Ca^{2+} sensitising mechanism occurs when phospholipase C activates the GTP-binding protein RhoA, as a result of the activation of a guanine nucleotide exchange factor (RhoGEF). Rho kinase activity inhibits myosin phosphatase activity and, subsequently, the light chain of myosin cannot be dephosphorylated, as seen in Figure 1-4 (Webb, 2003).

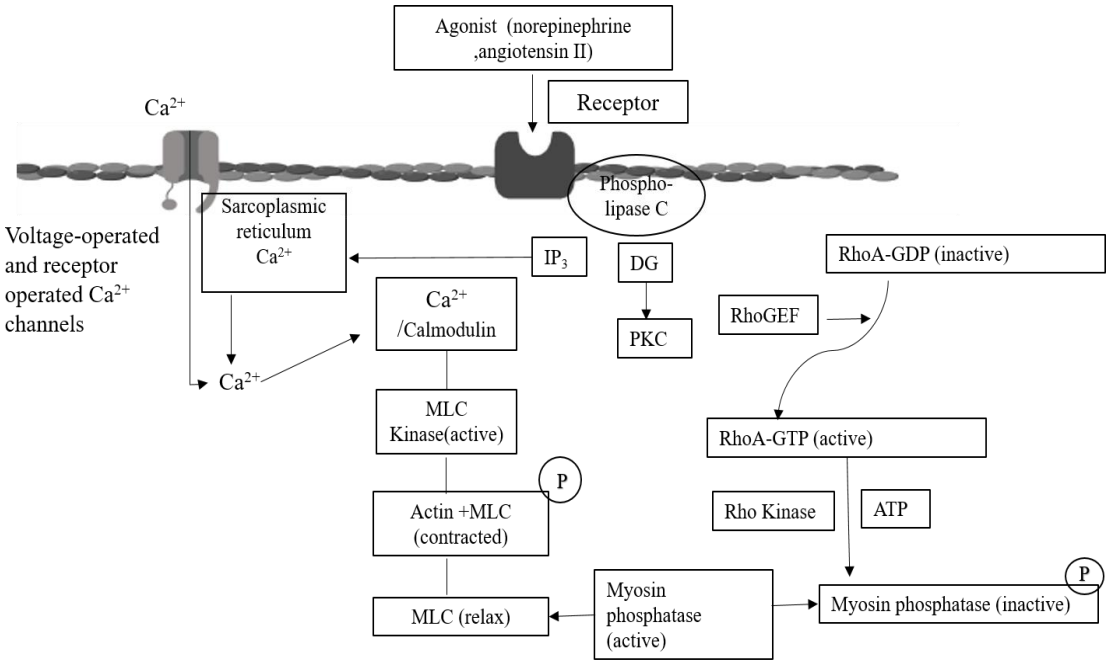


Figure 1-4. Vascular smooth muscle contraction and Ca^{2+} sensitisation. An agonist (neurotransmitter, hormone) stimulates smooth muscle contractions by binding to a specific receptor. This binding results in an increase in PLC; PLC produces DAG and IP_3 as potent second messengers from the membrane lipid phosphatidylinositol 4,5-bisphosphate. As a result of IP_3 binding to specific receptors on the SR, Ca^{2+} is released. Ca^{2+} and DAG activate PKC, which phosphorylates specific target proteins. Myosin light chain kinase is activated when calcium binds to calmodulin. Myosin light chain is phosphorylated by this kinase, and, in conjunction with actin, cross-bridge cycling occurs, resulting in the contraction of smooth muscle cells. Adapted from Webb (2003).

Smooth muscle relaxation can result either by increased MLC phosphatase activity or by decrease in intracellular Ca^{2+} concentration. The removal of Ca^{2+} can be achieved through Ca, Mg_ ATPases by efflux two Ca^{2+} to the luminal side of the SR and pumping the remaining Ca^{2+} into the cytoplasm. Plasma membrane Na^{+}/Ca^{2+} exchangers contribute to the decrease of intracellular Ca^{2+} levels. In addition, receptor- and voltage-operated Ca^{2+} channels close, subsequently reducing Ca^{2+} entry into the cell (Webb, 2003)

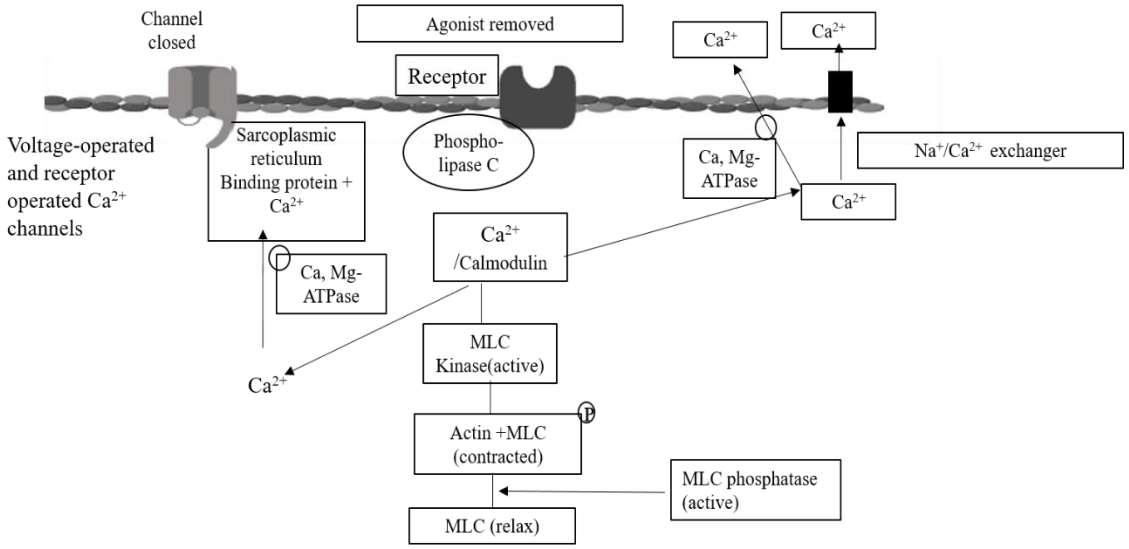


Figure 1-5. Vascular smooth muscle relaxation. Smooth muscle relaxation is either caused by removing the contractile stimulus or by a substance that inhibits the contractile mechanism directly. Relaxation requires a decrease in intracellular Ca^{2+} concentration and an increase in MLC phosphatase activity. By removing Ca^{2+} from the cytosol via Ca,Mg-ATPases, as well as Na^{+}/Ca^{2+} exchangers located on the plasma membrane. In addition, receptor- and voltage-operated Ca^{2+} channels in the plasma membrane are closed, resulting in a reduction in Ca^{2+} entry. Adapted from Webb (2003).

1.1.3 Adventitia

The adventitia is the outermost layer of the blood vessel wall and is composed of different types of cells, including fibroblasts, sympathetic and parasympathetic nerves, immune cells (macrophage and dendritic), progenitor cells, and pericytes. The adventitia is important for maintaining vascular wall function by releasing, storing, integrating, and retrieving various regulators. Moreover, the adventitia is able to control vascular structure and function from “outside-in” in response to inflammatory, hormonal and environmental changes. For example, ischaemia causes different adventitia cells (such as fibroblasts progenitor and immune cells) to increase cell proliferation, ECM adhesion protein levels, and the secretion of cytokines and chemokines (Stenmark et al., 2013).

The fibroblasts mainly fibrillate collagens I and III in the adventitia and regulate ECM composition. In addition, the adventitia acts as a primary step in vascular inflammation, which supports the “outside-in” hypothesis which states that inflammation is initiated in the adventitia which releases innate immune cells, mainly macrophage and dendritic cells, which then move to the medial and intimal layers (Stenmark et al., 2013).

1.1.4 Perivascular Adipose Tissue (PVAT)

PVAT is adipose tissue covering most blood vessels. Several vasoactive factors are released from PVAT around most blood vessels (Cheng et al., 2018). As with other adipose tissue deposits, PVAT is comprised of several types of cells, including adipocytes (white or brown), lymphocytes, macrophages, vasa vasorum endothelial cells, fibroblasts, mesenchymal stem cells, and nerve endings (Szasz et al., 2013). In different blood vessels, adipose tissue of PVAT composition varies considerably. A thick layer of brown and white fat cells surrounds the aorta. There is very little PVAT in the rodent tail artery or in the cerebral artery. There is a deep mass of fat covering the renal arteries, while the small mesenteric arteries are surrounded mainly by white PVAT (Daly, 2019). The coronary PVAT appears to consist of a mixture of white and brown adipocytes with characteristics more similar to brown than white in animal models, and it is more similar to white in humans (Grigoras et al., 2019). The composition of PVAT varies, however, depending on the physiological state (Ortega et al., 2015). It has been shown that PVAT around atherosclerotic aortae attracts immune cells such as T cells and macrophages, thereby producing proinflammatory cytokines during atherogenesis (Ortega et al., 2015).

PVAT releases a variety of bioactive molecules termed adipokines (or adipocytokines) that influence the contraction, proliferation, and migration of VSMCs. There are several types of adipokines, such as adiponectin, leptin, resistin, and hepatic growth factor. During contraction, PVAT provides mechanical protection for vessels from surrounding tissues. Additionally, PVAT has been demonstrated to have vasodilator effects, which are impaired in individuals with conditions such as metabolic syndrome or hypertension (Brown et al., 2014). Although PVAT reveals dynamic effects on the tone of the vessels, no single factor has been identified as responsible for this vasodilator effect. The effect has been referred to as the "PVAT-derived relaxing factor". Several compounds have been proposed as making up the factor, including NO, H_2S, and prostacyclin (Brown et al., 2014).

It is also known that PVAT has contractile effects on blood vessels. PVAT contains many components of the renin-angiotensin system and norepinephrine, which controls blood pressure (Mamiya, et al., 1991; Hermenegildo et al., 2005). A reactive oxygen species (ROS) called superoxide is also produced by PVAT, which increases the contraction response of mesenteric arteries to nerve stimulation (Gao et al., 2006). PVAT was recently reported to have contractile effects, particularly in obese individuals. The "adipose-derived contracting factor" has been identified as the molecule responsible for PVAT's contractile effects in obesity (Meyer et al., 2013). Although PVAT has contractile effects on vessels, the effect depends on the organism's physiology as well as on location. For example, the PVAT of lean mice exhibits a greater contractile ability than that of obese mice, and the PVAT of thoracic mouse aorta exhibits a greater contractile ability than that of abdominal or mesenteric PVAT (Meyer

et al., 2013). The PVAT of coronary artery does not have an anticontractile effect, but it can attenuate endothelial-dependent relaxation and increase the contraction of the vascular system in obese individuals (Owen et al., 2014; Ahmad et al., 2017; Fernández-Alfonso et al., 2017; Fernández-Alfonso et al., 2018).

PVAT has been linked to cardiovascular disease risk factors. Smoking, for example, has been shown to increase inflammation of PVAT by increasing P2X7 receptor inflammasome activity (Rossi et al., 2014). Another cardiovascular disease, systemic lupus erythematosus, has also been linked to vascular calcification and increased PVAT in the aorta (Shields et al., 2013).

In summary, PVAT produces a number of molecules that may have an autocrine or paracrine effect. It is known that PVAT is closely associated with inflammation and that it releases several cytokines, including tumour necrosis factor (TNF)-α and interleukins (IL) 1, 6, and 8. It also produces ROS (like superoxide, H_2O_2), H_2S, as well as hormones such as prostaglandins and angiotensin (Brown et al., 2014).

1.2 Purinergic Signalling

1.2.1 General Background

Signalling molecules include extracellular purines (i.e., adenosine, ADP, and ATP) and pyrimidines, namely uridine diphosphate (UDP) and uridine triphosphate (UTP), which bind to cell surface receptors known as purine receptors to function in a variety of biological processes (Ralevic & Burnstock, 1998; Burnstock, 2007). As a result of the initial suggestion that nucleotide receptors exist on cell surfaces, it has become

increasingly clear that purines and pyrimidines, such as ATP, ADP, UTP, and UDP, serve many functions beyond serving as intracellular energy sources. They are now recognised as key extracellular signalling molecules (Erlinge & Burnstock, 2008; Burnstock et al., 2010)

Since the mid-1950s, comprehensive research has demonstrated that ATP can be released from a wide range of cell types through autocrine and/or paracrine mechanisms. The term 'purinergic' was introduced by Burnstock in 1971 to describe nerves in the autonomic nervous system that release ATP rather than noradrenaline or acetylcholine when stimulated. A broader definition of purine signalling has been adopted since then (Lohman et al., 2012).

Based on extensive research into the effects of purine nucleotides and nucleosides on various tissues, the first classification of purinergic receptors was made in 1978. Early in the 1990s, purine and pyrimidine receptors were cloned and characterised (Burnstock, 2017). It is reported that P1 purinoceptors are more responsive to adenosine and AMP than to ADP and ATP, and that they can be inhibited selectively and competitively by methylxanthines, including caffeine and theophylline. The International Union of Basic and Clinical Pharmacology (IUPHAR) nomenclature subcommittee had classified P1 purinoceptors A_1, A_{2A}, A_{2B}, and A_3 subtypes, which all belong to the GPCR family (Pawson et al., 2014; Ralevic, 2020). In contrast, P2 purinoceptors are more sensitive to purine nucleotides (ATP and ADP) than to AMP and adenosine (Burnstock et al., 2010; Kennedy, 2021). P2 receptors also respond to pyrimidine nucleotides and nucleotide sugars, including UTP, UDP, and UDP-glucose (Ralevic, 2020). According to IUPHAR,

P2 receptors are divided into two structurally and functionally distinct classes: ligand-gated ionotropic P2X receptors and G protein-coupled P2Y receptors. The P2X receptor has seven subtypes (P2X1–7), and these ligand-gated ion channels are activated by extracellular ATP (Burnstock et al., 2010; Pawson et al., 2014). P2X receptors permeabilise to cations, resulting in Ca^{2+} influx and depolarisation. There are eight subtypes of G protein-coupled P2Y receptors: $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$. These receptors are triggered by a range of nucleotides, including ATP, ADP, UTP, UDP, and UDP-glucose. $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors are linked to G_q proteins, which causes an increase in inositol triphosphate (IP_3) by stimulating phospholipase C (PLC), thereby causing an increase in intracellular Ca^{2+} levels and activation of PKC. In contrast, $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$ receptors are coupled to Gi proteins, inhibiting adenylyl cyclase (AC) activity and reducing intracellular cAMP levels. PLC and AC are activated by $P2Y_{11}$ receptors that are associated with both G_s and G_q proteins (Stephen et al., 2009; Nishimura et al., 2017; Ralevic, 2020).

1.2.2 Classification of Purine and Pyrimidine

1.2.2.1 P1 (Adenosine) Receptors

The adenosine/P1 receptor family includes A_1, A2A, A_{2B}, and A_3 adenosine receptors, as established by a combination of molecular, biochemical, and pharmacological studies. Adenosine receptors are GPCR and composed of seven transmembrane domains, each made up of approximately 21 to 28 amino acids. Protein N-termini are oriented towards the extracellular side of the membrane, while protein C-termini face the cytoplasmic side (Ralevic & Burnstock, 1998).

A_1 have a negative association with adenylyl cyclase via the $G_{i/o}$ protein. An important signalling pathway of the A_1 receptor involves inhibiting adenylyl cyclase, resulting in a decrease in cAMP levels (Ralevic & Burnstock, 1998; Ralevic, 2020). The activation of PLC is another subsequent to activation of A_1 receptors that promotes increase in the production of IP_3 and DAG, thereby affecting Ca^{2+} mobilisation (Ralevic & Burnstock, 1998). As IP_3 increases cytosolic Ca^{2+} levels, several signalling pathways are activated, including the PKC family, PLA_2, Ca^{2+}-dependent K^+ channels, and NOS. The coupling of A_1 receptors to ATP-sensitive K^+ (K_{ATP}) channels has been demonstrated in porcine coronary arteries cause vasodilatation (Merkel et al., 1992; Dart & Standen, 1993). A_1 receptors are involved in a variety of biological processes. According to Olsson and Pearson (1990), A_1 receptors mediate cardiac depression through their antagonistic chronotropic, dromotropic, and inotropic effects, causing bradycardia and heart block.

A_{2A} and A_{2B} receptors are known to transmit signals by activating adenylyl cyclase, resulting in an increase in cAMP (Ralevic & Burnstock, 1998). Many tissues, including the immune system, platelets, the central nervous system (CNS), as well as vascular smooth muscle and the endothelium, contain A_{2A} receptors (Ralevic & Burnstock, 1998). Both smooth muscle and endothelial cells contain A_{2A} receptors, which are associated with vasodilation. In the PCA, A_{2A} receptors can mediate relaxation (Conti et al., 1993). There is evidence that A_{2B} receptors exist in isolated cells and tissues, such as fibroblasts, glial cells, airway smooth muscle, the gastrointestinal tract, and the circulatory system (Ralevic & Burnstock, 1998). Human mast cells are activated by A_{2B} receptors (Feoktistov & Biaggioni, 1996), suggesting an important role for A_{2B} receptors in allergic and inflammatory diseases. It has been reported that A_{2B} receptors play an

important role in inflammation, fibrosis, angiogenesis, and tumour progression (Goulding et al., 2017). As mentioned, vascular A_{2B} receptors have been identified in both smooth muscle and endothelial cells. In some vessels, such as guinea pig pulmonary arteries (Szentmiklósi et al., 1995), A_{2B} receptors appear to exert vasodilation, but not in others where A_{2A} receptors predominate (Ralevic & Burnstock, 1998). Both A_{2A} and A_{2B} receptors involve the production of NO by endothelial cells of coronary arteries, which cause vasodilation through the activation of K_{ATP} channels and possible activation of p38 mitogen-activated protein kinase (Sanjani et al., 2011).

It has been demonstrated that the A_3 receptor inhibits the activity of adenylyl cyclase (Sanjani et al., 2011). The physiological function of A_3 receptor remains largely unknown, even though it is highly expressed in the lung, heart, aorta and liver tissues of humans, with lower levels in the brain (Zhao et al., 2000). A_3 receptors may be involved in cardioprotection during ischaemic events as well as in preconditioning during ischaemia-reperfusion injuries (Armstrong & Ganote, 1994; Liu et al., 1994; Stambaugh et al., 1997). It has been shown that A_3 receptors are involved in the regulation of coronary blood flow in isolated mouse hearts (Talukder et al., 2002). P1 receptor selective agonists and antagonists are shown in Table 1-1 along with their main distributions and signalling mechanisms.

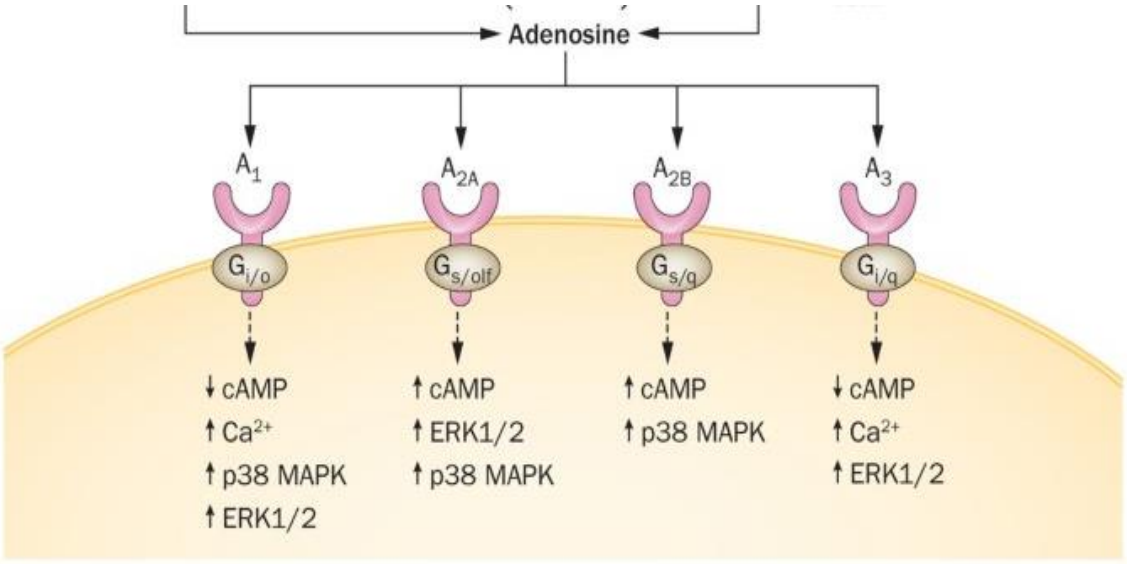


Figure1-6. Adenosine signalling pathway. Adapted from Antonioli et al., (2015)

Receptor	Main distribution	Selective agonists	Selective antagonists	Transduction mechanisms
A_1	brain, spinal cord, testis, heart, autonomic nerve terminals	CPA, CCPA	DPCPX, MRS1754, WRC-0571	$G_{i/o}$ ↓cAMP ↑IP_3 ↑K^+ ↓Ca^{2+}
A_{2A}	brain, heart, lung, spleen	CGS21680, HE-NECA	KF17837, SCH58261, ZM241385,	G_S ↑cAMP
A_{2B}	large intestine, bladder	NECA (non-selective)	enprofylline, MRS17541, MRS 1706	G_S ↑cAMP
A_3	lung, liver, brain, testis, heart	IB-MECA, 2-Cl-IB-MECA	MRS1220, L-268605, MRS1191, MRS1523	$G_{i/o}$ ↓cAMP

Table 1-1. Signalling and Pharmacological Properties of Adenosine (P1) Receptors
Adapted from Burnstock (2007)

1.2.2.2 P2 (ATP) Receptors

1.2.2.2.1 P2X Receptors

In 1994, the first cDNAs coding for P2X receptor subunits were identified (Valera et al., 1994; North, 2002). Each P2X receptor subunit is composed of a singular polypeptide chain that creates two transmembrane helical regions, surrounded by intracellular C- and N-termini, and extracellular loop. These subunits are homomeric and heteromeric, non-selective cationic channels, with high permeability to Ca^{2+} (Kennedy, 2021).

The receptors serve as ATP-gated ion channels that are capable of rapidly and selectively permeating several cations (Na^+, K^+, and Ca^{2+}) (Bean, 1992; Dubyak & el-Moatassim, 1993). This is consistent with their distribution on excitable cells, such as neurons, smooth muscle cells, and glial cells, which facilitates the rapid transmission of excitatory neurotransmission into ATP. In contrast, P2Y receptors respond more slowly to ATP, involving interaction with G proteins as well as second messenger systems. There are seven clones of P2X receptors (P2X1 through P2X7) (Ralevic & Burnstock, 1998; Ralevic & Dunn, 2015; Ralevic, 2021).

P2X1 functions as a cation-selective channel with a minimal selectivity for both Na^+ and K^+, while exhibiting a relatively high permeability for Ca^{2+} (Burnstock, 2007). In vascular smooth muscle, P2X1 receptors appear to be the most significant subtype of P2X, although P2X4 receptors may also be expressed (Soto et al., 1996). In most smooth muscle preparations, the ATP and α,β -meATP-mediated contractile responses are rapidly desensitised (Burnstock & Kennedy, 1985). In the cardiovascular system, P2X1 receptors are widely expressed, including in the smooth muscle of most blood vessels and in platelets. When ATP is released in conjunction with noradrenaline, a sympathetic neurotransmitter, the P2X1 in blood vessels is activated, leading to the depolarisation and contraction of smooth muscles (Ralevic, 2015)

In contrast to the P2X1 receptor, the P2X2 receptor exhibits minimal or no desensitisation and a considerably higher sensitivity to inhibition by extracellular Ca^{2+} than the P2X1 receptor (Evans et al., 1996). P2X2 receptor mRNA can be found in the adrenal medulla, bladder, intestine, brain, spinal cord, and vas deferens, with the

pituitary gland and vas deferens exhibiting the highest levels (Brake et al., 1994). In arterial smooth muscle, P2X2 receptor immunoreactivity has been demonstrated (Ralevic, 2015). The distribution of the P2X3 receiver is extremely limited; it is expressed only by a specific group of sensory neurons, including nodose, trigeminal, and dorsal root ganglia. It is not found in sympathetic neurons, CNS neurons, or smooth muscle (Lewis et al., 1995). Sensory neurons express P2X2 and P2X3 subtypes that mediate local depolarisation and propagate nociceptive signals (Giniatullin & Nistri, 2023).

P2X4 receptors function as Ca^{2+}-selective channels. P2X4 receptors become increasingly permeable to larger organic cations such as N-methyl-D-glucamine with prolonged ATP application. P2X4 receptors are less susceptible to desensitisation than P2X1 and $P2X_2$ receptors (Kanellopoulos et al., 2021). There are several tissues in which the P2X4 receptor is expressed, including the salivary glands, brain, spinal cord, adrenal glands, testis, lung, bladder, and vas deferens (Bo et al., 1995; Collo et al., 1996; Séguéla et al., 1996; Kanellopoulos et al., 2021; Sophocleous et al., 2022). Additionally, it has been shown that endothelial cells release ATP as a result of shear stress. P2X4 receptor acts as a mediator of endothelium-dependent vasodilatation by regulating Ca^{2+} influx and NO release in endothelial cells, which is essential for maintaining blood pressure (Tang et al., 2008; Ralevic, 2012).

The P2X5 receptor exhibits minimal desensitisation and is not activated by α,β-meATP. Inhibition of these receptors can be accomplished by suramin and PPADS at concentrations similar to those that inhibit P2X2 receptors. A high level of P2X5 mRNA

is found in skeletal muscle (Ralevic & Burnstock, 1998). The P2X6 mRNA distribution is predominantly found in the CNS, predominantly in the cerebellar Purkinje cells and ependyma (Burnstock, 2014).

The P2X7 receptor, which was originally cloned from rat brains and macrophages, exhibits unique properties upon activation (Surprenant et al., 1996). In response to activation, the membrane depolarises rapidly, and cations are reversibly influxed. However, sustained activation increases permeability, allowing bidirectional transport of ions such as sodium, K^+, and Ca^{2+}, as well as small molecules. The activation of P2X7 receptor in human macrophages, as well as in human leukaemic lymphocytes, triggers the activation of phospholipase D, although the mechanism by which this occurs is unknown. Human macrophages have also been shown to release the inflammatory cytokine interleukin-1β (IL-1β) after activation of the P2X7-like receptor. The presence of this receptor may have a physiological or pathophysiological significance. The P2X7 receptor is found in bone marrow cells, including monocytes/macrophages, granulocytes, B lymphocytes, and brain macrophages (Ralevic & Burnstock, 1998). On endothelial cells of the mammary artery, radial artery, and saphenous vein, P2X1, P2X4, and P2X7 receptors were identified by immunohistochemistry (Ray et al., 2002; Wang et al., 2002; Wu et al., 2023). P2X4 and P2X7 receptors were reported to be immunocytochemically expressed in bovine aortic endothelial cells (Ralevic, 2012). P2X receptor selective agonists and antagonists are shown in Table 1-2 along with their main distributions and signalling mechanisms.

Table 1-2. Signalling and pharmacological properties of P2X

Receptor	Main distribution	Selective agonists	Selective antagonists	Transduction mechanisms
P2X1	smooth muscle, platelets, cerebellum, dorsal horn spinal neurons	α,β-meATP, L-β,γ-meATP	NF023, NF279, NF449 PPADS, MRS2159, MRS2220	intrinsic cation channel (Ca^{2+} and Na^{+})
$P2X_2$	smooth muscle, CNS, retina, chromaffin cells, autonomic and sensory ganglia	-	-	intrinsic ion channel (particularly Ca^{2+})
P2X3	sensory neurones, some sympathetic neurons	A317491	NF110, TNP-ATP, A317491, RO-3, RO-85, AF-353, spinorphin, gefapixant (AF-219, MK-7264), BLU-5937	intrinsic cation channel
P2X4	CNS, testis, colon	-	5-BDBD NC-2600	intrinsic ion channel (especially Ca^{2+})
P2X5	proliferating cells in skin, gut, bladder, thymus, spinal cord	-	-	intrinsic ion channel
P2X6	CNS, motor neurons in spinal cord	-	-	intrinsic ion channel
$P2X_7$	apoptotic cells in, for example, immune cells, pancreas, skin	-	AZ11645373, AZ10606120, AZD9056, A740003, A438079	intrinsic cation channel and a large pore with prolonged activation

Adapted from Burnstock, 2007; Burnstock & Ralevic, 2014; Kennedy, 2021.

1.2.2.2.2 P2Y Receptors

The first $P2Y_1$ receptors were discovered in 1993, and since then, several other subtypes have been identified via homology cloning (Webb et al., 1993). Human P2Y receptors can be classified into eight categories: $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$. Some numbers are not included, which indicate non-mammalian receptors or with sequence similarity to P2Y receptors, but without evidence that they are nucleotide responsive (Burnstock, 2007). The P2Y receptor consists of 308 to 377 amino acids, yielding a mass of 41 to 53 kDa following glycosylation (Ralevic & Burnstock, 1998).

They are composed of seven helical transmembrane domains of hydrophobic amino acids; these domains are connected via three intracellular loops at the C-terminus and three extracellular loops at the N-terminus (Ralevic & Burnstock, 1988; Kennedy, 2021). Several studies (Erb et al., 1995; Barnard & Simon, 2001) have demonstrated that four amino acid residues of TM6 and TM7 are necessary for agonist binding, potency, and specificity. According to their endogenous ligands, they are preferential to adenine nucleotides ($P2Y_1$, $P2Y_{11}$, $P2Y_{12}$ and $P2Y_{13}$ receptors) and to uracil nucleotides or sugars ($P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{14}$ receptors) (von Kügelgen, 2006). On the basis of their alignment in sequence and effector coupling, P2Y receptors may also be divided into $P2Y_1$-like and $P2Y_{12}$-like families (von Kügelgen, 2006). Costanzi et al. (2004) reported that the $P2Y_1$-like family binds to Gq protein and participates in the PLC pathways. This subgroup comprises $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$. There is also evidence that the $P2Y_{11}$ receptor is capable of interacting with the G_s protein, which

stimulates AC (Communi et al., 1997). On the other hand, the $P2Y_{12}$-like family is able to bind to the Gi protein, leading to the suppression of AC (Jacobson et al., 2012). $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$ are included in this subgroup. Compared to $P2Y_1$ and $P2Y_{12}$ receptors, there is little sequence homology between these two subfamilies. For instance, the sequence identity between $P2Y_1$ and $P2Y_{12}$ receptors is only 20%. However, sequence identity within the same subfamily is significantly higher. For example, the sequence identity between $P2Y_{12}$ and $P2Y_{14}$ receptors is 45% (Jacobson et al., 2012).

Initial sequences of the $P2Y_1$ receptor were found in late-embryonic chick brain (Webb et al., 1993), and subsequently, related sequences were found in humans. The cloned $P2Y_1$ has been found to be expressed in a variety of tissues, including smooth muscle, endothelium, and neurons. Ca^{2+} dependent activation of endothelial NOS, EDRF, and EDH are released by the receptor, which mediates muscle relaxation. The $P2Y_1$ receptor also stimulates the production of endothelial prostacyclin, although this appears to have minimal impact on vasodilation, particularly under physiological conditions. ADP-induced platelet aggregation is also mediated by the $P2Y_1$ receptor (Kennedy & Burnstock, 1985; Ralevic & Burnstock, 1998; von Kügelgen, 2006).

The expression of $P2Y_2$ receptors has been identified in various organs, including the spleen, testes, kidney, liver, lung, heart, and brain (Lustig et al., 1993). In the vascular system, $P2Y_2$ receptors are typically present in the endothelium. Activation of the $P2Y_2$ receptor leads to an increase in the synthesis and release of arachidonic acid, prostaglandins, and NO. A number of inflammatory mediators, such as interleukin-1β

(IL-1β), TNF-α, and interferon-γ have been found to increase the expression of $P2Y_2$ receptors in smooth muscle cells (Vera Ralevic & Burnstock, 1998; Burnstock, 2007).

$P2Y_4$ is predominantly expressed in the placenta and to a lesser extent in the lungs and smooth muscles of vascular vessels (Erlinge et al., 1998). In addition, the human $P2Y_4$ receptor exhibits a high affinity for UTP over ATP and does not respond to nucleoside diphosphate (Ralevic & Burnstock, 1998; Kügelgen, 2006).

The $P2Y_6$ receptors, found in mice, rats, and humans, show selectivity for UDP. The expression of $P2Y_6$ mRNA is evident in many tissues, such as the placenta, thymus, lung, stomach, intestine, spleen, mesentery, heart, and aorta (Chang et al., 1995; Communi et al., 1996; Ralevic & Burnstock, 1998; Kügelgen, 2006).

In contrast to other P2Y receptors, $P2Y_{11}$ stimulates both phosphoinositide and AC pathways. $P2Y_{11}$ receptor mRNA has been detected in spleen and intestine tissues (Communi et al., 1997). There is a high expression of the $P2Y_{11}$ receptor in immunocytes, suggesting that it may be involved in the differentiation of these cells (Ralevic & Burnstock, 1998; Burnstock, 2007).

$P2Y_{12}$ receptors can be found in certain brain regions, as well as in glial cells, cerebral capillary endothelial cells, smooth muscle cells, and chromaffin cells (Kügelgen, 2006; Burnstock, 2007). In addition, $P2Y_{12}$ receptors are expressed in both platelets and neurons. The receptor plays a crucial role in platelet aggregation (Dorsam & Kunapuli, 2004). Vasoconstriction occurs in human blood vessels when the $P2Y_{12}$ receptor is activated (Wihlborg et al., 2004).

The $P2Y_{13}$ receptor is found in haematopoietic cells and neuronal cells. As with the $P2Y_{12}$ receptor, it is activated by analogues of ADP. There is expression of $P2Y_{13}$ gene in the spleen, the heart, placenta, the liver, bone marrow, T cells, lungs, and different parts of the brain (Kügelgen, 2006; Burnstock, 2007).

In humans, the $P2Y_{14}$ receptor is highly expressed in adipose tissue and in placenta, intestine, and stomach tissues (Chambers et al., 2000). Several types of UDP sugars activate this receptor, including UDP-glucose, UDP-galactose, UDP-glucuronic acid, and UDP-N-acetylglucosamine (Kügelgen, 2006; Burnstock, 2007). Table 1-3 shows the main distributions, natural agonists, agonists, antagonists, and signalling mechanisms of P2Y receptors. Table 1-4 describes the functional expression of P1 and P2 receptors on smooth muscle and endothelial cells of porcine and human vessels that mediate vasoconstriction (+) and vasodilation (−). Moreover, Table 1-5 illustrates P1 and P2 receptors expression on smooth muscle and endothelial cells in different blood vessels using PCR and immunohistochemistry techniques.

Table 1-3. Signalling and Pharmacological Properties of P2Y

Receptor	Main distribution	Natural agonists	Selective agonists	Selective antagonists	Transduction mechanisms
$P2Y_1$	Epithelial and endothelial cells,platelets, immune cells, osteoclasts, brain	ADP, ATP	MRS2365	MRS2179 MRS2279 MRS2500	$G_{q/11}$ PLC-β, IP_3, DAG, Ca^{2+}
$P2Y_2$	immune cells, epithelial and endothelial cells, kidney tubules, osteoblasts	UTP, ATP	2-thioUTP MRS2698 INS37217	AR-C118925XX	$G_{q/11}$ PLC-β, IP_3, DAG, Ca^2
$P2Y_4$	endothelial cells, placenta, spleen, thymus	UTP, ATP	-	-	$G_{q/11}$ PLC-β, IP_3, DAG, Ca^2
$P2Y_6$	airway epithelial cells, placenta, t cells, thymus, microglia	UDP	PSB0474 MRS2693 MRS2782 INS48823 5-	MRS2578	$G_{q/11}$ PLC-β, IP_3, DAG, Ca^2
$P2Y_{11}$	spleen, intestine, granulocytes	ATP, ADP	AR-C67085	NF340	$G_{q/11+}$ + G_s PLC-β, IP_3, DAG, Ca^2 ↑cAMP
$P2Y_{12}$	platelets, glial cells	ADP	-	clopidogrel ticlopidine prasugrel ticagrelor cangrelor	$G_{i/o}$ ↓cAMP
$P2Y_{13}$	spleen, brain, lymph nodes, bone marrow, erythrocytes	ADP	-	MRS2211	$G_{i/o}$ ↓cAMP
$P2Y_{14}$	placenta, adipose tissue,stomach, intestine, discrete brain regions, mast cells	UDP UDP-glucose	α,β-difluoro methylene-UDP 2-thio-UDP-glucose	PPTN	$G_{i/o}$ ↓cAMP

Adapted from Burnstock, 2014; Kennedy, 2021.

Table 1-4. P1 and P2 Receptors on Smooth Muscle and Endothelial Cells in Different Blood Vessels Mediating Vasoconstriction (+) and Vasodilatation (−)

	P1 Receptors		**P2X Receptors**		**P2Y Receptors**	
Coronary vessels	**SM**	**EC**	**SM**	**EC**	**SM**	**EC**
Functional expression						
Pig	A^1, A_{2A}, A_{2B} (−)	A_{2A} (−)	$P2X_1$ (+)		$P2Y_2$ (+) $P2Y_{14}$ (+)	$P2Y_1$ (−)
Human	A_{2A}, A_{2B} (−)		$P2X_1$ (+)		$P2Y_2$ (+)	
Rat		P1 (−)				$P2Y_1$ (−)
Guinea pig		A_1, A_{2A}, A_3 (−)			P2Y (−)	$P2Y_{2\text{-}4}$ (−)

Adapted from various studies Keef et al., 1992; Merkel et al., 1992; Vials & Burnstock, 1993, 1994; Matsumoto et al.,1997b; Simonsen et al., 1997; Kemp & Cocks, 1999; Lynch et al., 2006; Malmsjö et al., 2000, 2007; Yambolieva et al.,2000; Olanrewaju et al., 2000; Van der Giet et al., 2002; Rubio & Ceballos, 2003; Olivecrona et al., 2004; Shen et al., 2004; Sato et al., 2005; Teng et al.,2005; Rayment et al., 2007b; Burnstock & Ralevic, 2014; Abbas, 2017; Burnstock, 2017; Abbas et al., 2018)

Table 1-5. P1 and P2 Receptors Expression on Smooth Muscle and Endothelial Cells in Different Blood Vessels Using PCR and Immunohistochemistry Techniques

Coronary vessels	P1 Receptors		P2X Receptors		P2Y Receptors		References
	SM	EC	SM	EC	SM	EC	
PCR							
Porcine coronary small arteries		A_{2A}		P2X4		$P2Y_1$ $P2Y_2$ $P2Y_4$ $P2Y_6$	Zhou et al., 2013
Cultured porcine coronary artery smooth muscle cell	A_1 A_{2A} A_3				$P2Y_2$		Shen et al., 2005; Shen et al., 2004
Cultured porcine coronary artery endothelial cells (PCAEC)		A_{2A} A_{2B}					Olanrewaju et al., 2000
Human coronary arteries			P2X1		$P2Y_1$ P2Y2 P2Y4 P2Y6		Malmsjö et al., 2000
Cultured human coronary artery endothelial cells (HCAEC)		A_{2A} A_{2B}				$P2Y_2$ $P2Y_{11}$	Olanrewaju et al., 2000; (Peng & Shen, 2020)
Immunohistochemistry							
Porcine coronary small arteries						$P2Y_6$	Zhou et al., 2013
Mice proximal part of the left descending artery					$P2Y_6$	$P2Y_2$	Haanes et al., 2016

1.3 Mechanisms of ATP Release

The same mechanisms that release ATP from cells will also release UTP from cells but for clarity this discussion refers principally to ATP (and purine nucleotides).The release of ATP from myocardial cells was observed by Forrester and Williams in 1977 (Lohman et al., 2012). Various types of cells in the vessel wall, including endothelial cells, perivascular sympathetic nerves, smooth muscle cells, and circulating erythrocytes, release ATP (Lohman et al., 2012). Understanding of the mechanisms that control purine nucleotide release in the vascular wall is crucial for the development of new therapeutic strategies for vascular diseases. Extracellular ATP release can occur from both excitatory/secretory tissues and nonexcitatory tissues. Excitatory tissues such as neurons, chromaffin cells, platelets, mast cells, and pancreatic acinar cells. Synaptic vesicles and chromaffin granules are the structures in which these substances are stored. ATP is found in a variety of nonexcitatory tissues, including endothelial and epithelial cells, fibroblasts, hepatocytes, astrocytes, monocytes, lymphocytes, red blood cells, and chondrocytes (Lazarowski et al., 2003; Lazarowski et al., 2011). There is a wide range of high intracellular ATP concentrations of 3–10 mM. As compared with ATP, UTP concentrations interocular were 10–30% of those determined for ATP (Lazarowski & Harden, 1999). Since ATP is rapidly hydrolysed by ecto-ATPases and ecto-apyrases, in the extracellular environment, ATP concentrations are typically much lower than inside cells. Therefore, under normal physiological conditions, ATP concentrations outside cells are only about 10 nM (Feng et al., 2020; Greiner & Glonek, 2021).

It is likely that different mechanisms regulate nucleotide release, given that nucleotides have been detected in a variety of conditions and cell types. Several pathways have been identified as contributing to nucleotide release, including vesicular exocytosis, ATP-binding cassette (ABC) transporters, the plasma membrane F_1/F_0-ATP synthase, connexin hemichannels, and pannexin channels (Lazarowski et al., 2003; Lohman et al., 2012) (Figure 1-7).

Vesicular exocytosis

Vesicular exocytosis is a cellular process in which proteins synthesised in the endoplasmic reticulum and Golgi apparatus are packaged into intracellular vesicles (Figure 1-7). These vesicles become fused with the plasma membrane, allowing soluble proteins and ATP to be released into the extracellular membrane. The secretory cell undergoes this process frequently, and it is crucial in maintaining cellular balance by releasing soluble proteins, signalling molecules, and neurotransmitters (Lohman et al., 2012; Dosch et al., 2018).

ABC Transporters

ABC transporters transport a variety of molecules through the plasma membrane, such as cholesterol, lipids, and hydrophobic drugs, using the energy provided by ATP hydrolysis (Figure 1-7). Some members of the ABC transporter family, such as the cystic fibrosis transmembrane conductance regulator, the multidrug resistance gene product, and the sulfonylurea receptor may play a role in the release of ATP in a variety

of cell types, including VSMCs, endothelial cells, erythrocytes, and platelets (Lazarowski et al., 2003, 2011; Lohman, 2012).

F_1F_0-ATPase

The inner mitochondrial membrane establishes an electrochemical proton gradient during oxidative phosphorylation, which occurs in the mitochondria. This gradient is utilised by mitochondrial F_1/F_0-ATP synthase, also known as ATP synthase, to release ATP (Figure 1-7). It is composed of a membrane-bound F_0 subunit that transports proton and a catalytic F_1 subunit that synthesises ATP. Recent evidence suggests that ATP synthase is present at the plasma membrane of various types of cells, including vascular endothelial cells, contrary to what was initially believed. In order to produce ATP at the extracellular surface, a proton gradient across the plasma membrane is necessary, and this may have an impact on intracellular pH and the activity of other proteins (Lohman et al., 2012).

Connexins (Cx) Hemichannels

Connexins (Cx) are proteins that form gap junctions which permit cell-to-cell communication by creating channels between adjacent cells. These channels transport signalling molecules such as ATP and electrical currents into the extracellular space (Lohman et al., 2012) (Figure 1-7). These hemichannels can be opened by various stimuli, such as changes in Ca^{2+} levels, depolarisation of the membrane, mechanical stimulation, and metabolic inhibition (Quist et al., 2000; Contreras et al., 2003; Gomes et al., 2005; Thimm et al., 2005). Several studies have shown that connexin hemichannels

are involved in the release of ATP by endothelial cells, smooth muscle cells, and monocytes, thus affecting vascular tone. Blood vessel smooth muscle cells and endothelial cells abundantly express the four connexin isoforms Cx37, Cx40, Cx43, and Cx45 (Haefliger et al., 2004; Hakim et al., 2008). Further, connexin hemichannels are implicated in inflammatory and atherosclerotic conditions. An increase in Cx43 hemichannel activity and release of ATP into the plasma membrane trigger an inflammatory response (Robertson et al., 2010; Ley et al., 2011).

Pannexin Channels

Pannexins (Panx) are a protein family that shares similar characteristics with connexins and that play a role in the formation of gap junctions (Figure 1-7). In the endoplasmic reticulum and Golgi, pannexins, specifically pannexin 1, Panx2, and Panx3, form channels and are transported to the cell surface. In contrast to connexins, pannexins do not form gap junctions but rather channels for the movement of molecules between intra- and extracellular spaces. In addition, pannexins are not affected by Ca^{2+} as connexins are (Lohman et al., 2012). In numerous studies, pannexin 1 channels have been shown to play a role in the release of ATP from various cell types. These include glial cells, astrocytes, neurons, epithelia of the airways, T cells, taste cells, and smooth muscle cells of the vascular system (Schenk et al., 2008; Thompson et al., 2008; Ransford et al., 2009; Celetti et al., 2010; Woehrle et al., 2010; Seminario-Vidal et al., 2011; Gödecke et al., 2012). ATP release from pannexin 1 channels in the smooth muscle cells of small arteries and arterioles may have a significant impact on blood flow regulation (Lohman et al., 2012).

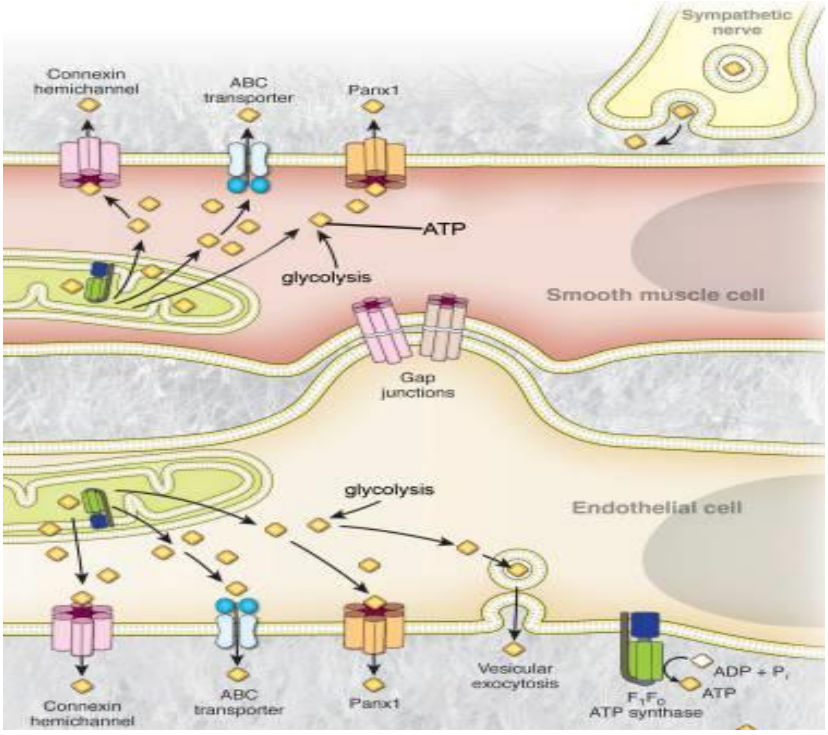


Figure 1-7. Mechanisms of ATP release from cells of the blood vessel wall. Adapted from Lohman et al. (2012).

1.4 Metabolism of Nucleotides

The biological activities of ATP, UTP, ADP, UDP, and UDP-glucose are controlled by membrane-bound enzymes that regulate nucleotide hydrolysis and phosphorylation following their release to extracellular. These nucleotides are subject to a cascade of extracellular hydrolysis which results in the formation of nucleosides and free phosphates (Pastor-Anglada et al., 1998). A variety of ectoenzymes are capable of breaking down nucleotides, including the ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase or CD39-family of ecto-apyrases) family, the ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPP) family, the

glycosylphosphatidylinositol (GPI)-anchored ecto-5′-nucleotidase (CD73); and the GPI-anchored alkaline phosphatases (APs). On the cell membrane, these enzymes have their active sites facing the extracellular media. They are particularly active in areas with high levels of extracellular nucleotides and require divalent cations such as Ca^{2+} or Mg^{2+} as well as an alkaline pH to be effective (Zimmermann, 2000).

The ENTPD gene family encodes eight NTPDase proteins. NTPDases 1, 2, 3, and 8 are typical cell surface enzymes with an extracellular catalytic site. NTPDases 5 and 6 may be cleaved to become soluble enzymes. Both NTPDases 4 and 7 are located within the cell (Robson et al., 2006). NTPDases 1, 2, 3, and 8 hydrolsis nucleoside 5-triphosphates and nucleoside 5-diphosphates for example, ATP to ADP and ADP to AMP sequentially (Baqi, 2015). In contrast to these enzymes. E-NPP (divided into three subunits E-NPP 1-3) converts ATP directly to AMP in the absence of significant release of ADP and release of pyrophosphate (PPi) (Baqi, 2015). ecto-5′-nucleotidase (CD73) or one of four AP isoforms catalyses AMP hydrolysis to adenosine and phosphate. Despite the fact that all of them hydrolyse nucleoside triphosphates equally, the rates of hydrolysis for nucleoside diphosphates differ between the subtypes.

1.5 Inflammation

Inflammation can be defined as a protective immune response triggered by different factors, such as pathogens, injury, toxic substances and chronic diseases. When tissue is affected by any of these factors, the body activates leukocyte chemotaxis toward the site of inflammation using the circulatory system and an innate inflammatory response (Chen et al., 2018).

The mechanism of inflammation can be summarised as follows. 1) Activation of germline-encoded pattern-recognition receptors, which are sensitive to cellular damage caused by the presence of microorganisms though a specific part of the microbial structure, called pathogen-associated molecular patterns (PAMPs), or an endogenous signal from an internal injury, called danger-associated molecular patterns (DAMPs). 2) Activation of inflammatory pathways by secretion of inflammatory mediators known as proinflammatory cytokines, which include IL-1β, IL-6, and TNF-α, which act on their receptors: IL-1 receptor, IL-6 receptor, and the TNF receptor. These mediators facilitate the inflammation process by changing endothelial cell permeability and recruiting neutrophils and other leucocytes to the site of inflammation. 3) Release of inflammatory markers such as inflammatory cytokines, including ILs, colony stimulating factors, transforming growth factors, TNFs, interferons and chemokines, from immune cells, such as monocytes and macrophages, inflammatory proteins and enzymes, such as C-reactive proteins, and other inflammatory markers including antioxidant enzymes and other antioxidant defence mechanisms which influence oxidative stress (Ahmed, 2011; Chen et al., 2018).

At the site of inflammation in blood vessels, a change in function and morphology of the endothelial cells is the first step of the inflammation cascade that leads to the recruitment of different types of cells and proinflammatory factors (Trepels et al., 2006).

Leukocytes (with rolling, adhesion to vascular endothelium and migration) are considered the first type of cell attracted to the site of injury, followed by monocytes and lymphocytes (Osborn, 1990).

Neutrophils produce their anti-inflammatory action by producing different ROS.

Leukocytes secrete several enzymes, including NADPH oxidase, at the plasma membrane to generate the superoxide anion (O_2^-). Consequently, O_2^- dismutases to hydrogen peroxide (H_2O_2) (Rayner et al., 2014). Myeloperoxidase (MPO) is considered a hemeglycoprotein enzyme that is present mainly in neutrophils and can be released to catalyse the reaction of H_2O_2 with a halide – chloride – to form the reactive oxygen intermediate hypochlorous acid (HOCl) (Aratani, 2018). HOCl is considered an oxidising agent that acts as a microbicidal agent able to kill most pathogens (Pullar et al., 2000).

However, MPO encourages host tissue damage by generating various diffusible oxidants (Zhang et al., 2001). Studies show that MPO has a role in the development of atherosclerosis through different mechanisms: MPO catalyst oxidises the protein moiety of low-density lipoprotein (LDL) that is generated from damaged cardiovascular tissue to oxidised LDLs (OxLDL) (Zhang et al., 2001). Ox-LDL and the oxidant HOCl encourage lipoprotein accumulation; therefore, they are taken by the express scavenger receptor as CD36 in macrophage, which is a key component in atherosclerotic lesion and fatty streak development and facilitates foam cell formation (also known as lipid-laden macrophages). Foam cells have a role in the inflammatory process and atherosclerotic plaque formation, producing growth factors and cytokines such as TNF-α, IL-1β and the macrophage colony stimulating factor (Delporte et al., 2013). As a result of phagocytic cell accumulation in the cardiovascular wall, fatty streaks that consist of macrophage foam cells narrow the blood vessels (Heinecke, 2006).

A recent study indicates that MPO plays a direct role in endothelial dysfunction by reducing NO bioavailability. Atherosclerotic plaques contain MPO, which consumes NO

and promotes endothelial dysfunction. MPO produces oxidants that can inhibit the activity of NOS through chlorination of arginine, which reduces its availability as a substrate. This results in decreased NO production and impaired relaxation of the vascular system. Additionally, MPO reduces NADPH availability, an essential cofactor for NO synthesis, in the endothelial membrane and inhibits NO synthesis by altering the localization of endothelial NOS (Nicholls & Hazen, 2005).

Inflammation plays a significant role in the progression of coronary artery disease. As a result of smoking, high blood pressure, high cholesterol, diabetes, or simply ageing, chronic inflammation occurs, and the coronary endothelium loses its vasodilator as a result of impaired NO release and antithrombotic properties (Fioranelli et al., 2018). Endothelial dysfunction is associated with inflammatory coronary artery disease (Tousoulis et al., 2008). When risk factors are present, the vascular endothelium responds by activating PKC and nuclear factor-kB. Thus, the angiotensin-converting enzyme is activated, angiotensin II is produced, and adhesion molecules are expressed on the surface of endothelial cells. As a result, endothelial dysfunction occurs in conduit coronary arteries. The intima may thicken, plaque may form, and ultimately the plaque may be disrupted. It is believed that three components of circulation, namely monocytes, platelets, and lymphocytes, in conjunction with endothelial and smooth muscle cells, contribute to the development of atherosclerotic lesions. Proinflammatory cytokines are also crucial in the development of atherosclerosis (Tousoulis et al., 2008).

1.6 Role of Purine and Pyrimidine Nucleotides in Inflammation

Damage to cells caused by inflammation may lead to the release of nucleotides, such as ATP, which act on smooth muscle vasocontractile receptors to cause vasoconstriction (Burnstock & Ralevic, 2014). It has been demonstrated that mechanical stimuli, such as osmotic and shear stress, can trigger the release of ATP from different cells, such as endothelial cells, smooth muscle cells, cardiomyocytes, and erythrocytes, through channels mediated by connexins and pannexins (Dahl, 2015; Mikolajewicz et al., 2018). Nucleotides are also released during inflammation by inflammatory cells, such as leukocytes and platelets. Therefore, these mechanisms are likely to play an important role in the physiology and pathophysiology of cardiovascular disease. As a result of inflammation, purinergic signalling is dysfunctional, which has been linked to cardiovascular diseases such as hypertension, diabetes, atherosclerosis, and thrombosis (Ralevic, 2020). Human endothelial cells have been observed to selectively induce purinergic receptors, such as $P2Y_6$, in response to inflammatory stimuli, and their activation or inhibition appears to influence inflammation (Burnstock, 2017).

$P2Y_2$ receptors in human macrophages enhance the oxidative burst caused by ATP and UTP. During cell clearance, phagocytes migrate towards dying cells, recognise and uptake these dying cells, and degrade them. In various physiological and pathological processes, the clearance of apoptotic cells is crucial; inflammation and autoimmune diseases can be caused by the insufficient clearance of apoptotic cells. Recent studies

suggest that apoptotic cells release "find-me" signals in order to attract phagocytes and facilitate efficient engulfment of the cells. Additionally, these signals influence the surrounding cellular environment and coordinate the clearance process (Medina & Ravichandran, 2016). Apoptotic cells release a variety of nucleotides that can function as find-me signals, such as ATP and UTP. While the cell membrane is still intact, these nucleotides are released by pannexin 1 channels through the plasma membrane. During apoptosis, the C-terminal tail of pannexin 1 is cleaved by effector caspases, resulting in the opening of the channel and the release of nucleotides into the extracellular membrane. The specific mechanism of action by which pannexin 1 releases ATP/UTP is discussed above (1.3). The release of ATP and UTP triggers phagocyte chemotaxis, as evidenced by the decrease in migration and clearance following inhibition of the $P2Y_2$ receptor (Elliott et al., 2009; Medina & Ravichandran, 2016). According to Elliott et al., ATP released by apoptotic cells acts as a find-me signal to recruit monocytes and macrophages. Apyrase and $P2Y_2$ receptor-deficient tissues reduce monocyte/macrophage recruitment to apoptotic cell supernatants. Accordingly, the $P2Y_2$ receptor may be involved in purinergic-mediated macrophage chemotaxis.

The endothelial $P2Y_1$ receptor plays an important role in vascular inflammation. This receptor is specifically influenced by inorganic polyphosphate, which is released by bacteria. According to *in vivo* studies, inorganic polyphosphate exhibits prothrombotic effects (Dinarvand et al., 2014). Certain mouse models have shown reduced atherosclerotic lesions and plaque areas occupied by macrophages after the deletion of $P2Y_1$ receptors (Erlinge & Burnstock, 2008).

Even though P2 receptor-mediated endothelium activation releases nitric oxide, which inhibits inflammatory cells, endothelium activation can also trigger significant proinflammatory effects (Erlinge & Burnstock, 2008). In addition to stimulating neutrophil adhesion, ATP also triggers the release of proinflammatory cytokines such as IL-6, IL-8, growth-regulated oncogene α, and monocyte chemoattractant protein-1. In endothelial cells, UTP and ATP induce proinflammatory vascular cell adhesion molecule-1 expression by activating the $P2Y_2$ receptor. As a result, monocytic cells are more likely to adhere to human coronary endothelial cells (Dawicki et al., 1995; Seye et al., 2003). Furthermore, it has also been reported that the $P2Y_{12}$ receptor expressed on VSMCs can promote proinflammatory responses in vitro via a thrombin-induced pathway (Hechler & Gachet, 2015).

Neutrophils express the P2X1 receptor, which facilitates neutrophil chemotaxis in response to stimulants and contributes to neutrophil migration. In recent research, the $P2X_1$ receptor has been associated with acute inflammation, specifically during endotoxemia in mice, when neutrophils emigrate from venules (Lecut et al., 2009).

It has been discovered that P2X7 receptors play a significant role in inflammation, and antagonists of these receptors have been investigated as potential treatments for chronic systemic inflammatory disease (Erlinge & Burnstock, 2008; Kennedy, 2021) Several cell types, including fibroblasts, microglia, mast cells, and dendritic cells, are capable of releasing proinflammatory factors such as TNF and IL-6 via the P2X7 receptor. A key component of the NOD-like receptor family pyrin domain-containing protein 3 (NLRP3) is a multiprotein complex responsible for activating caspase-1 and

releasing proinflammatory cytokines, such as IL-1β and interleukin-18 (IL-18). As a result of various stimuli, including PAMPs and DAMPs, the NLRP3 inflammasome is activated, resulting in the initiation of an inflammatory cascade. P2X7 receptor is also involved in activating the NLRP3 inflammasome, which releases IL-1β. As a result, the P2X7 receptor plays a significant role in promoting both proinflammatory and anti-inflammatory responses, making it a potential therapeutic target in the treatment of inflammatory conditions (Virgilio et al., 2017).

1.7 Aims and Objectives

By the action of the heme enzyme MPO, HOCl is produced and released in areas of inflammation by active neutrophils and other immune cells (Rayner et al., 2014). Inflammatory diseases are closely associated with excessive HOCl generation during chronic inflammation (Zhang et al., 2001). The presence of high concentrations of purine nucleotides and nucleosides in the intracellular environment is known, but whether these nucleotides contribute to the vasomotor response to HOCl is still unclear. Also, little is known about the vasomotor response to HOCl (Ralevic & Dunn, 2015). In this study, the proximal end of PCA, which is a large vessel, was used to investigate inflammatory processes. Porcine cardiovascular systems are notable for their anatomical and physiological similarities to human cardiovascular systems, making them an ideal model for cardiovascular research. Importantly, coronary arteries represent the site of major pathological events in humans, such as coronary artery disease (Crisóstomo et al., 2016). Furthermore, inflammation often starts in small arteries and extends to larger arteries as a disease progresses (Lusis, 2000). It is

possible to generate important insights into the progression and potential severity of diseases such as atherosclerosis by studying the influence of inflammation on larger arteries such as the proximal end of PCA. Consequently, this approach might provide a more comprehensive picture of the inflammation process throughout the body. This study aimed to gain a better understanding of vascular inflammation and purine receptor involvement in the response produced by HOCl. The objectives of the study were:

- To investigate the direct effects of different concentrations of HOCl on the PCA.
- Understand the mechanism by which HOCl mediates response, specifically through mechanical removal of the endothelium, different NOS inhibitors or prostaglandin inhibitions, K^+ channel inhibitions, cAMP and cGMP inhibitions. Moreover, examine whether PVAT contributes the response to HOCl.
- To examine the role of purine nucleotides in the response induced by HOCl, P1, P2 receptor antagonists, Ecto-ATPase Inhibitor, apyrase (Hydrolyses Nucleotides), and ATP assays were used.
- To investigate whether pannexin 1 and connexin channels (through which ATP can be released) were involved in the response to HOCl in PCA.
- To investigate the direct effect of the exogenous adenosine and ATP on PCA.
- To examine the direct effects of a single concentration of ATP and adenosine in the presence of different receptor antagonists, which have been shown to alter the response to HOCl.

Chapter 2 Materials and Methods

2.1 Organ Bath Technique

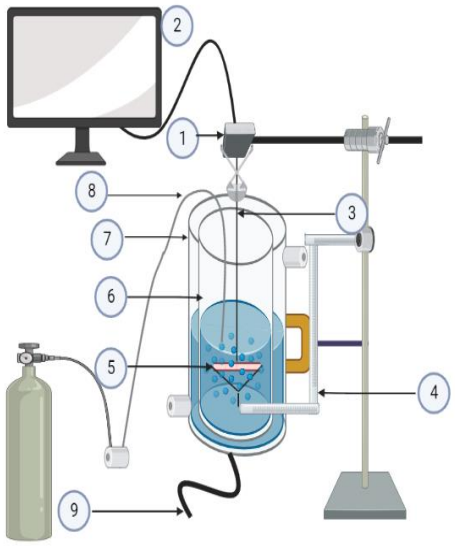
In pharmacological research, the organ bath technique is widely used as a means of examining how isolated tissues respond to different pharmacological and physiological conditions (Jespersen et al., 2015). For more than 150 years, this technique has been a cornerstone of pharmacology. As a result of its flexibility, scientists around the world have been able to explore and better understand receptor activities and signal transduction processes. A multitude of conditions have been treated through these techniques, including hypertension, diabetes, gastroenteritis, and asthma. It is still important to use the organ bath method for drug development (Upchurch & Iaizzo, 2022).

This technique is particularly impressive since it allows the tissue to remain alive and fully functional. It is vital to observe physiological responses such as contractions and relaxations in order to gain a deeper understanding of the physiological processes of the body. This technique combines multiple steps such as drug-receptor interaction, tissue function, and signal transduction. Moreover, the organ bath technique preserves tissue function, so pharmacological variables can be calculated in a way that is more representative of how drugs interact with tissue. In pharmacological research, this approach offers a more realistic and holistic understanding of drug actions (Jespersen et al., 2015; Upchurch & Iaizzo, 2022).

2.1.1 Preparing PCA Rings

Hearts were isolated from male and female pigs (large white hybrid pigs, 4–6 months old, weighing ~50 kg). The hearts were immediately delivered to the laboratory in an ice-cold Krebs–Henseleit solution from a local abattoir. The Krebs–Henseleit solution contained 118 mM NaCl, 4.8 mM KCl, 1.1 mM $MgSO_4$, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 12mM D-glucose, and 1.25mM $CaCl_2$ and was gassed with 5% CO_2 and 95% O_2. A crude dissection was carried out to isolate the proximal part of the coronary artery (diameter 4.06 ± 0.73 mm), which was then placed in the pre-gassed Krebs–Henseleit solution and refrigerated overnight at 4 °C. Fine dissection was carried out the following day to remove connective tissue and PVAT. The coronary arteries were then cut to approximately 5 mm in length and suspended in a 20 ml multichannel organ bath. Organ baths were filled with 20 ml of Krebs–Henseleit solution that was gassed constantly with 5% CO_2 and 95% O_2 and sustained at 37 °C (Figure 2-1).

A



B

Figure 2-1. Organ bath tissue set up depicted as follows: (1) transducer, (2) PowerLab recording system connected to transducer, (3) thread attached the top hook to the transducer, (4) glass rod connected to the bottom hook, (5) blood vessel between hooks, (6) Kreb–Henseleit buffered organ bath, (7) thermostatic jacket, (8) gas tube delivering 95% oxygen and 5% carbon dioxide, and (9) drain (A). Multichannel of organ bath (B).

2.1.2 Vasomotor Responses of Porcine Isolated Coronary Arteries

Each artery segment was connected—via a thread—to an isometric force transducer (ADInstruments ML T050/A force transducer (0–50 g). A Quad Bridge amplifier unit was used to connect the PowerLab recording system (ADInstruments, Oxfordshire, UK) to the transducers. Any change in tension was measured using the GraphPad program (Version 9, GraphPad Software, Inc., San Diego, CA, USA).

Coronary artery rings were tensioned to ~10 g and left for approximately 30–40 minutes to allow for equilibration. Afterward, the viability of the artery was assessed

using 60 mM potassium chloride (KCl). When a stable contraction to KCl was achieved after approximately 6 minutes, the tissue was washed and left for 30 o 45 minutes to allow for its return to baseline tension, then the second KCl wash was added. Thereafter, U46619 (9,11-dideoxy-11α,9α-epoxymethanoprostaglandin F2α), which is a thromboxane A_2 analogue, was used to contract vessels to ~50%–60% of the second KCl response. It was determined that the concentration of U46619 required to produce this level of contraction varied depending on the conditions (for example, endothelium-denuded and L-NAME). Accordingly, the concentration was adjusted accordingly, as shown in the results.

2.1.3 Experimental Protocols

2.1.3.1 Protocol for Bradykinin Response

As described previously (2.1.1), the PCA data were collected and processed. U46619 was employed following the addition of KCl twice in order to increase vascular tone to a level that is 50%–60% of that produced by KCl. A concentration-response curve for bradykinin (0.1 nM–3 μM) was constructed once a stable tone had been attained. The endothelium dependence mechanism of response to bradykinin was examined by removing the endothelium (described later in 2.1.3.3) and then adding bradykinin (0.1 nM–3 μM) cumulatively.

2.1.3.2 General Protocol of HOCl, ATP, Adenosine Response

After inducing tone with U46619, the segments were allowed to equilibrate for 20 minutes in order to stabilise the tone. Therefore, the relevant testing compounds (HOCl: 100 and 500 μM, ATP: 100 μM, and adenosine: 30 μM) were added, and 60 minutes of incubation were allowed. Adjacent segments from the same coronary artery were used as controls. KCl was used instead of U46619 to contract vessels in some experiments.

2.1.3.3 Protocol of Endothelium-Denuded Experiment

In some experiments, the endothelium was removed by gently rubbing the innermost surface of the artery segments on Krebs–Henseleit wet tissue with forceps before placing them in the organ bath (Rayment et al., 2007). Substance P (100 nM) confirmed that this methodology was effective. Endothelium-denuded arteries responded to substance P with less than 10% of the contraction induced by U46619 (Berkestedt et al., 2008).

2.1.3.4 Protocol of Inhibitors and Antagonists

These compounds were added immediately after the second KCl wash and allowed to sit for the duration of the experiment. During that period, U46619 was added to the contract vessels, and the relaxation responses (HOCl, ATP, or adenosine) of the vessels were measured. The table below shows the inhibitors and antagonists used in this protocol.

Table 2-1. Concentrations of Inhibitors and Antagonists Used

Group	Inhibitor or antagonist	Concentration	Reference
Nitric oxide inhibitors			
NOS inhibitor	N^G-Nitro-L-arginine methyl ester (L-NAME)	300 µM	Wong et al., 2014
	N^G-Nitro-D-arginine methyl ester (D-NAME)	300 µM	Wong et al., 2014
	N^G-monomethyl-L-arginine (L-NMMA)	300 µM	Motta, et al., 1999
	N^G-monomethyl-D-arginine (D-NMMA)	300 µM	Bray & Quast, 1991
	Haemoglobin	10 µM	Chen et al., 1988
NO scavenger	2-phenyl-4,4,5,5,-tetramethylimidazoline-1-oxyl 3-oxide (PTIO)	300 µM	Quignard et al., 1999
Prostaglandin inhibitors			
Cyclooxygenase inhibitor	Indomethacin	10 µM	Malinowski et al., 2008
K^+ channel blockers			
K_{Ca} channels inhibitor	Tetraethylammonium chloride (TEA)	10 mM	Ahmad et al., 2020; Donovan et al., 2017
ATP-sensitive K+ channels (K_{ATP}) blocker	Glibenclamide	1 µM	Hedegaard et al., 2014; Mellemkjær & Nielsen-Kudsk, 1994),
KV7 channel blocker	10,10-bis(4-pyridinylmethyl)-9(10H)-anthracenone (XE991)	10 µM	Hedegaard et al., 2014
cAMP and cGMP inhibitors			
Guanylyl cyclase inhibitor	1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ)	10 µM	Hayabuchi et al., 1998
Adenylyl cyclase inhibitor	9-(tetrahydro-2-furanyl)-9H-purin-6-amine (SQ22536)	100 µM	Yu et al., 2014

P1 receptor antagonists			
Adenosine antagonist	8-(p-sulphophenyl) theophylline (8-SPT)	100 µM	King et al., 1990
A_{2a} inhibitor	4-(-2-[7-amino-2-{2-furyl} {1,2,4}triazolo{2,3-a} {1,3,5}triazin-5-yl-amino]ethyl)phenol (ZM 241358)	100 µM	Hasan et al., 2000
A_1 antagonist	8-cyclopentyl-1,3-dipropylxanthine (DPCPX)	10 nM	Hussain & Mustafa, 1995
P2 receptor antagonists			
Non-selective P2 antagonist	suramin	100 µM	Alefishat et al., 2015
	Pyridoxal-5′-phosphate-6-azo-phenyl-2,4-disulfonate (PPADS)	10 µM	Alsaqati et al., 2014
$P2Y_1$ receptor antagonist	2'-Deoxy-N^6-methyladenosine 3',5'-bisphosphate tetrasodium salt (MRS2179)	10 µM	Alefishat et al., 2013
Non-selective P2Y antagonist	Reactive blue 2	30 µM	Matsumoto et al., 1997
$P2Y_2$ receptor antagonist	5-(7-chloro-4H-1-thia-3-aza-benzo[f]-4-yl)-3-methyl-6-thioxo-piperadin-2-one (AR-C 118925XX)	10 µM	Kemp et al., 2004
P2X1 receptor antagonist	4,4′,4″,4‴-(carbonylbis(imino-5,1,3-benzenetriylbis(carbonylimino))) tetrakis-benzene-1,3-disulfonic acid and (NF449)	10 µM	Alefishat et al., 2015
P2X1 receptor desensitising agent	α,β-methylene ATP	10 µM	Alefishat et al., 2015

P2X4 antagonist	(1-(2,6-dibromo-4-isopropyl-phenyl)-3-(3-pyridyl) urea (BX430)	10 µM	Pasqualetto, et al., 2023
Ecto-ATPase inhibitor			
	6-N,N-diethyl-d-β-γ-dibromomethylene adenosine triphosphate (ARL67156)	100 µM	Rayment et al., 2007
Nucleotides hydrolysis			
	apyrase	100 units/ml	Houston et al., 1984
Pannexin and connexin 43 channel blocker			
Pannexin 1 channel blocker	probenecid	1 mM	Kuroiwa et al., 1993
Pannexin 1 and connexin channels blocker	carbenoxolone	100 µM	Wong et al., 2014

2.1.3.5 Protocol for Effect of Perivascular Adipose Tissue

To assess the effect of PVAT on HOCl response, approximately 0.3 grams of PVAT were taken from the proximal parts of fresh the PCAs and left attached to the tissue or carefully removed and stored in Krebs–Henseleit solution until later re-added to the bath at the beginning of the experiment or following the second wash of contraction of vessels with KCl. In this study, 0.3 g of PVAT was used based on preliminary laboratory studies to produce a significant, reproducible response in the PCA (Owen et al., 2013; Ahmad et al., 2017).

2.2 Cell Culture

2.2.1 Human Coronary Artery Endothelial Cells (HCAECs)

HCAECs were obtained from ScienCell Research Laboratories (CA, USA) (passage 6-9) and were cultured in endothelial cell medium from ScienCell Research Laboratories consisting of 500 ml of basal medium, 25 ml of fetal bovine serum (Cat. #0025), 5 ml of penicillin/streptomycin solution (Cat. #0503), and 5 ml of endothelial cell growth supplement (Cat. #1052). In a T-75 flask, the cells were grown in incubator at 37 °C in 5% CO_2 until they reached 80%–90% confluency. The cells were then passaged every 3 to 4 days, typically (Figure 2-2). Dulbecco's phosphate-buffered saline (Ca^{2+}, Mg^{2+}, and phenol red-free, Cat. #0303) was swirled around the flask to remove any residual bovine serum that could inhibit trypsin activity. To ensure complete detachment of the cells from the culture flask, 3 ml of 0.05% trypsin/ethylenediaminetetraacetic acid (EDTA) (0.05% trypsin and 0.5 mM EDTA) solution was added for 3–5 minutes. In order to stop trypsinisation, the cell suspension was diluted in culture medium and centrifuged for 5 minutes at 1000 RPM to collect the cells. Following the suspension of the cell pellet in a suitable volume of fresh culture medium, the cells were aliquoted into a new flask containing 15 ml of the medium. Using the TC20 automated cell counter from Bio-Rad (Hercules, CA, USA), 10 µL of the suspension was mixed with 10 µL of 0.4% trypan blue dye and then loaded into the chamber of the counting slide according to the equation:

% viable cells = number of unstained cells/total number of cells × 100

A total and live cell count of cells per ml was displayed, and a diluting calculator was used to determine the volume required to achieve the desired cell count. HCAECs were seeded in a 6-well cell culture plate at a density of 2.5 x 10^5 cells/well.

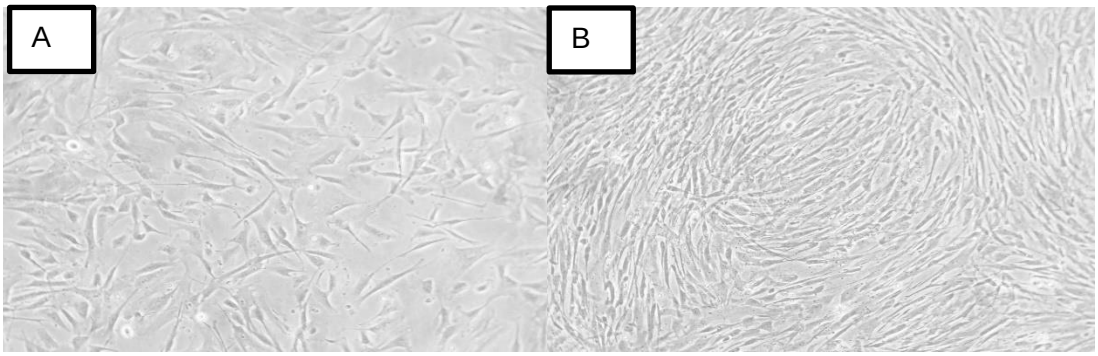


Figure 2-2. Image of cultured human coronary artery endothelial cells (HCAECs), magnification power 10×, Scale bar = 500 µm, n = 6. Passage 7 in Day 2 (A) and in Day 4 (B) (ScienCell Research Laboratories)

2.2.2 ATP Assay

A luminescent ATP assay kit (Abcam, Cat#ab113849) was used to assess the effect of 100 µM HOCl on ATP levels at 1 and 5 minutes. An ATP luminescent assay protocol consists of taking a sample of the bathing solution, adding luciferase enzyme and luciferin, and measuring emitted light by using microplate luminometer. Cells were seeded in a 6-well cell culture plate at a density of 2.5 x 10^5 cells/well. After 24 h, cells were treated with 10 µL of endothelial cell medium (as vehicle control) and 10 µL (equal concentration of 100 µM) of HOCl then incubated for 1 and 5 minutes. Finally, 100 µL of bathing solution from each well was aliquoted in a 96-well plate. As a result of irreversible inactivation of ATP-degrading enzymes

(ATPases) by detergent, the luminescent signal obtained is representative of the extracelluar levels of ATP. The protocol for the luminescent ATP assay is as follows:

In the experiment, standard ATP was added to the standard wells, and control media into the control wells on the same plate containing the cells designated for analysis. Following this, a detergent solution was added to stabilise the ATP, and the mixture was incubated for 5 minutes. Subsequently, a substrate solution was added to the mix and was left to incubate for another 5 minutes. After the incubation periods, the plate was stored in a dark environment for 10 minutes to prevent photodegradation. Finally, the plate was analysed using a luminescence reader to determine the presence and quantity of the ATP in the samples.

2.3 Statistical Analysis

Data are expressed as the mean ± standard error of the mean (SEM) of at least six independent experiments where *n* is the number of animals. GraphPad Prism (version 7, GraphPad Software, La Jolla, CA, USA) was used to generate time-response curves and concentration-response curves for bradykinin. The response to bradykinin was measured as percentage of the U46619-induced contraction. Data were expressed as log concentration-response plots. The concentration–response curves for bradykinin were fitted to a sigmoidal curve. The negative log of concentration required to produce half the maximal contraction of the induced tone (pEC_{50}) and maximum response contraction (R_{max}) were measured from the fitted curves. The contraction and relaxation responses were expressed as a percentage (%) of those induced by U46619-induced tone (measured as the increase above baseline). Any

change induced by HOCl, ATP, or adenosine was calculated as a change from the pre-contracted U46619 levels. For the HOCl, ATP, and adenosine responses, tension was recorded at 1-minute intervals for the first 10 min and then at 5 min intervals for a further 50 min (60 min in total). To compare differences between two groups, a two-tailed, unpaired Student's *t*-test was used. One-way ANOVA was performed to assess differences between three or more groups. Statistical significance was defined as *p* values less than 0.05 ($p < 0.05$). Asterisks (*) indicate $p < 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$.

2.4 Drugs and Chemicals

Except for ZM241385, DPCPX, AR-C 118925XX, and BX430 which were obtained from Tocris-Cookson (Bristol, UK). SQ22536 and reactive blue 2 were purchased from MedChemExpress (Monmouth, USA). All drugs were purchased from Sigma-Aldrich (Poole, UK). HOCl, haemoglobin, XE991, carbenoxolone, and adenosine salt were kept in a refrigerator. KCl, indomethacin, TEA, glibenclamide, 8-SPT, ZM241385, DPCPX, reactive blue 2, probenecid, and BX430 were kept at room temperature. All stock solutions were frozen at −20 °C.

PTIO, ODQ, indomethacin, glibenclamide, ZM241358, DPCPX, and BX430 were dissolved in ethanol. Stock U46619 solutions were dissolved to 10 mM in ethanol, and further dilutions of the stock solutions were made using distilled water. AR-C 118925XX was dissolved in DMSO, and probenecid was dissolved in NaOH (1 M). All further stock dilutions were made using distilled water.

Chapter 3 Characterisation of the Vasomotor Effects of HOCl in Porcine Coronary Artery

3.1 Introduction

Neutrophils and other leukocytes produce different ROS in their anti-inflammatory action. They secrete several enzymes, including NADPH oxidase at the plasma membrane to generate the superoxide anion (O_2^-). Consequently, O_2^- dismutases to hydrogen peroxide (H_2O_2) (Rayner et al., 2014). MPO is a haemoprotein enzyme that is present mainly in neutrophils and can be released to catalyse the reaction of H_2O_2 with a halide, chloride, to form the reactive oxygen intermediate HOCl (Aratani, 2018). HOCl is an oxidising agent that acts as a microbicidal agent able to kill most pathogens (Pullar et al., 2000).

Studies show that MPO has a role in the development of atherosclerosis, and HOCl produced in atherosclerotic blood vessels is one of the most important oxidants in rabbit atherosclerotic vessels (Stocker et al., 2004). HOCl is a known to consume NO, a critical component of normal endothelial function, thus reducing the bioavailability of NO through the formation of peroxynitrite and promoting atherosclerosis. Reports show that MPO and HOCl are known to reduce the bioavailability of NO by acting as its catalytic sink and via blocking nitric oxide synthesis. Reducing arginine, which is the substrate for NOS as a result of arginine chlorination. All these factors lead to reduced endothelial NO formation (Stocker et al., 2004; Nicholls & Hazen, 2005).

Human atherosclerotic vessels contain MPO which is frequently closely associated with endothelial cells (Sugiyama et al., 2001). MPO is transported across endothelial cells, resulting in MPO accumulation in the ECM (Baldus et al., 2001).

Endothelium-derived nitric oxide is impaired by atherosclerosis, and its related diseases, such as diabetes mellitus and hypercholesterolemia. The effects of atherosclerosis include impaired vasodilation, as well as endothelial dysfunction. Evidence indicates that oxidative stress in the vascular system may contribute to endothelial dysfunction (Beckman et al., 1990; Stocker et al., 2004).

When neutrophils are activated, they generate approximately 150–425 μM HOCl/h (Weiss et al., 1982; Kettle & Winterbourn, 1994), whereas at sites of inflammation this amount can reach up to 5 mM (Epstein & Weiss, 1989). The oxygen consumption of activated neutrophils indicates that low millimolar concentrations of HOCl can be achieved *in vivo* (Winterbourn et al., 2000). Accordingly, the present investigation used 100 and 500 μM HOCl to investigate its vasomotor effects in blood vessels. Other studies have considered how HOCl incubation impacts vascular contractility and have shown that it impairs endothelium-dependent relaxation (Schraufstätter et a., 1990; Zavodnik et al., 2001; Stocker et al., 2004; Liu et al.,2017), but relatively few have investigated its direct vasomotor effects, which are of interest in understanding the early events involved in the MPO/HOCl-induced inflammatory response.

This study may provide important insights into the pathogenesis of cardiovascular diseases, as atherosclerosis by exploring the effects of HOCl on the vasculature and elucidating its underlying mechanisms. To the best of my knowledge, this is the first study to compare the direct vasomotor responses mediated by different concentrations of HOCl in isolated PCA. Very few studies have looked at the direct

effects of HOCl in blood vessels, and none have looked at its effects on PVAT.

Aim and objectives: The aim of this chapter was to better understand vascular inflammation. The objectives were, first, to investigate the direct vasomotor effects of two HOCl concentrations, 100 and 500 μM, on the PCA; second, to understand the mechanism of response mediated by HOCl, specifically through mechanical removal of the endothelium, using different NOS inhibitors or prostaglandins inhibition, K^+ channel inhibition, and cAMP and cGMP inhibition; third, to investigate whether PVAT plays a role in the multiphasic response to HOCl.

3.2 Materials and Methods

The materials and methods employed were as described in Chapter 2.

3.3 Results

3.3.1 Responses to Bradykinin

Concentration-dependent relaxations were produced by bradykinin (0.1 nM–3 μM) and examined to confirm that the endothelium was not damaged during fine dissection in the PCA pre-contracted with U46619 with pEC_{50} of 7.36 ± 0.09 and Rmax = 73.6 ± 3.8% (n = 9) (Figure 3-1A) and pEC_{50} 8.26 ± 1.1 and Rmax = 98 ± 3.3% (n = 6) (Figure 3-1B). Bradykinin responses were endothelium-dependent since mechanical removal of the endothelium abolished the bradykinin-induced relaxations ($p < 0.0001$) (Figure 3-1B). These findings are consistent with other studies done in the PCA where the values were as follows: pEC_{50} values 8.50 ± 0.08 in females and

8.38 ± 0.08 in males (Wong et al., 2014); 8.2 ± 0.1 (Weston et al., 2005); and 8.03 ± 0.05 (Danser et al., 2000). The mechanical disruption of the endothelium abolished the relaxant effect of bradykinin, which is consistent with the findings of others that bradykinin exerts vascular relaxation through the endothelium in the PCA (Cherry et al., 1982; Weintraub et al., 1994).

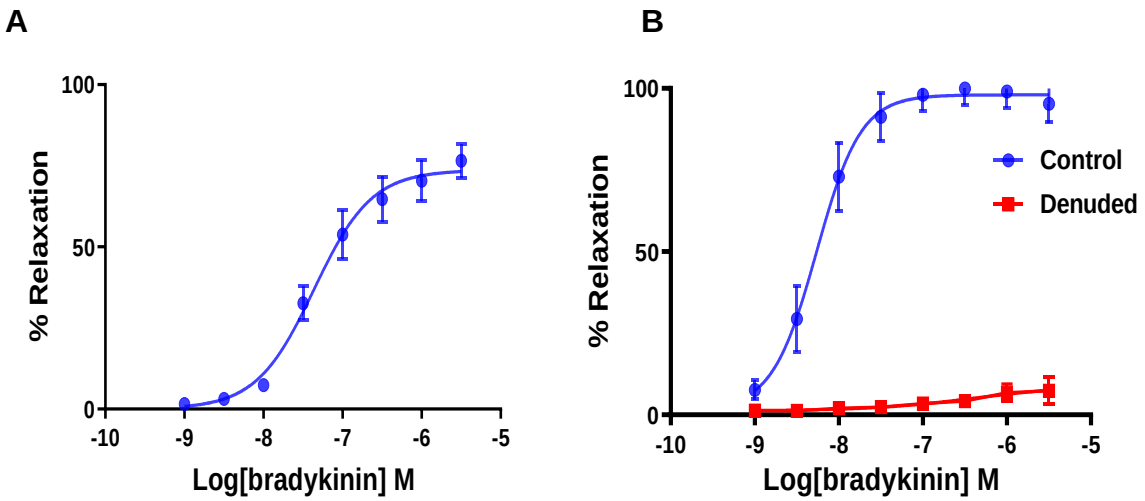


Figure 3-1. Relaxation responses to bradykinin in porcine coronary artery rings. (A) Bradykinin relaxations produced in U46619 pre-contracted the PCA (*n* = 9). (B) Bradykinin relaxations determined in intact or endothelium-denuded artery segments (*n* = 6). Relaxation responses were expressed as a percentage of artery contraction to U46619.

3.3.2 Response to HOCl

To determine the effect of HOCl on the PCA, different concentrations (100 and 500 μM HOCl) were added to the PCA rings pre-contracted with U46691. At 100 μM, the HOCl induced a transient initial relaxation (62.45 ± 13.87%) which lasted 2–4 minutes and then returned to baseline after 5–6 minutes; after that, further relaxation was observed for the remaining 60 minutes (103.63 ± 3.94%) (*n* = 7) (Figure 3-2). A rapid and transient relaxation response (28.77 ± 11.48%) was also induced by 500

μM HOCl, lasting about 40 seconds, followed by contraction which peaked at between 6 and 10 minutes (52.39 ± 23.27%), and then slow relaxation was observed (97.80 ± 4.20%) (*n* = 7) (Figure 3-2A). Importantly, there were no qualitative differences in the responses to the different concentrations of HOCl when expressed either as a percentage of U46619 or in grams (see Appendix for data expressed as grams).

Figure 3-2B shows an expanded time course of the first 6 min of the responses to HOCl in order to show more clearly the rapid relaxation responses. In control PCA segments pre-contracted with U46619 but with no addition of HOCl, artery tone remained stable for the 60 min duration of the experiment (*n* = 6) (Figure 3-2A). The levels of pre-contraction of the PCA achieved with U46619 (expressed as % of KCl) in control, 100 and 500 μM HOCl groups were 65 ± 5.5% (*n* = 6), 69 ± 3.2% (*n* = 7) and 67 ± 4.8% (*n* = 7), respectively, and there was no significant difference between them (*p* = 0.97).

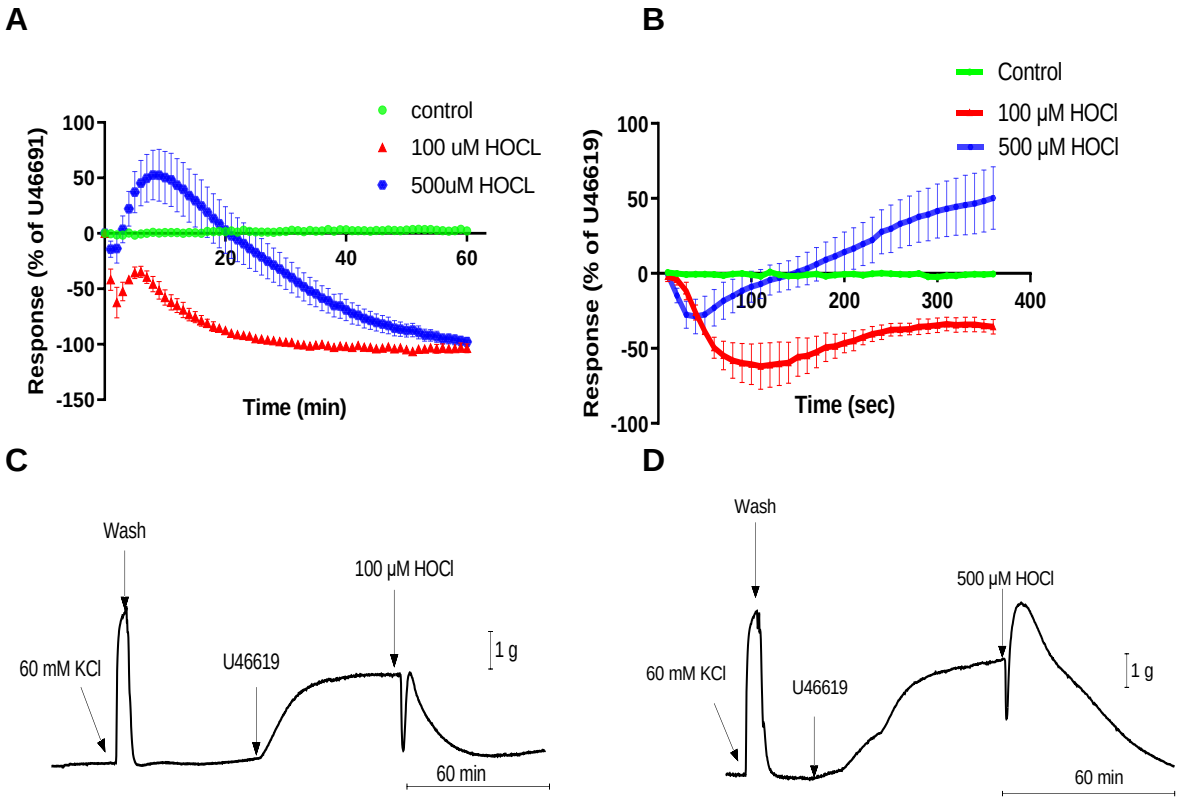


Figure 3-2. Responses of porcine coronary arteries induced by HOCl at different concentrations: control (*n* = 6), 100 µM (*n* = 7), and 500 µM (*n* = 7): (A) responses measured for 60 minutes at 1-minute intervals; (B) expanded time course of the first 6 minutes measured at 10-second intervals (360 seconds) to show the initial rapid relaxation; (C–D) representative trace for: (C)100 µM HOCl and (D) 500 µM HOCl. Artery segments were pre-contracted with U46619.

3.3.3 Characterisation of HOCl Responses

3.3.3.1 Endothelium Involvement in HOCl Responses

In endothelium-denuded PCA, the transient relaxation observed in the PCA with intact endothelium to 100 µM HOCl at 2 min (70 ± 4.5%, *n* = 6) was abolished. Moreover, the relaxation at 60 minutes was statistically significantly larger in the PCA

with denuded endothelium (111 ± 3.2 %, *n* = 6) compared to endothelium-intact PCA (97 ± 1.5%, *n* = 6) (*p* = 0.002) (Figure 3-3A).

At a concentration of 500 µM HOCl, the transient rapid relaxation that was observed in the PCA with intact endothelium (57.5 ± 5.5%, *n* = 6) was also blocked when the endothelium was removed; the contraction in endothelium-denuded arteries was blocked too (54.7 ± 8% (*n* = 6). The relaxation at 60 minutes was the same in the PCA without endothelium (102.2 ± 4.1%, *n* = 6) as in endothelium-intact PCA (93.8 ± 2.8%, *n* = 6) (*p* = .12) (Figure 3-3B).

The levels of U46119 pre-contraction (% of KCl) achieved in the denuded endothelium in the 100 and 500 µM HOCl groups were 55.4 $\pm$ 4.5% and 60 $\pm$ 2%, respectively (both *n* = 6). Moreover, with the endothelium intact, the contraction levels with U46619 were 60 ± 5% and 59 ± 3.3% for 100 and 500 µM HOCl, respectively (both *n* = 6). There was no significant difference in tone in the denuded and intact endothelium for any of the 100 and 500 µM HOCl experimental groups (*p* = 0.47 and 0.76, respectively). The concentration of U46619 used to pre-contract the coronary arteries was lower and statistically significant in the denuded endothelium (4.3 $\pm$ 0.4 nM) than in intact endothelium (7.5 $\pm$ 0.9 nM, *n* = 6, and 6) (*p* < 0.007).

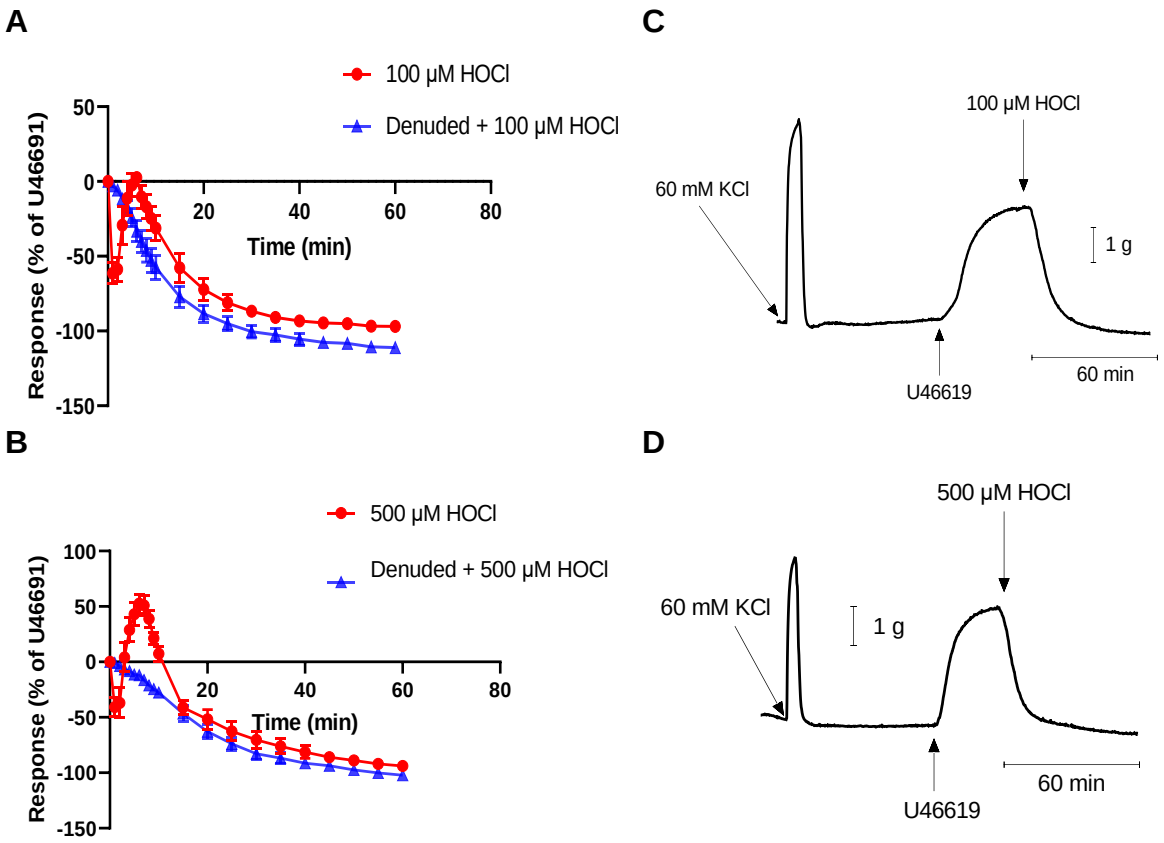


Figure 3-3. Porcine coronary artery responses evoked by (A) 100 µM HOCl and (B) 500 µM HOCl in endothelium-intact and denuded vessels (*n* = 6). (C–D) representative trace for: (C) 100 µM HOCl and (D) 500 µM HOCl in denuded endothelium. Artery segments were pre-contracted with U46619.

3.3.3.2 Nitric Oxide Involvement

3.3.3.2.1 Effects of L-NAME and D-NAME on HOCl Responses

Initial experiments showed that endothelium removal abolished the transient relaxation to 100 and 500 µM and pronounced contraction of 500 µM HOCl. The later slow relaxations were still present. Endothelium produces several mediators, including NO. Therefore, the NO synthase inhibitor L-NAME (300 µM) was used to investigate whether NO was involved.

Notably, L-NAME has been shown to increase responses to vasoconstrictors (Loomis et al., 2005), and, accordingly, the concentration of U46619 used to pre-contract the coronary arteries was significantly lower in arteries with L-NAME (3.5 $\pm$ 0.5 nM) than in those without (7.5 ± 0.9 nM) (*n* = 6, and 6) (*p* = 0.001) (Table 3-1). Tone levels (% of KCl) achieved with U46619 in the absence of L-NAME in the 100 and 500 µM HOCl groups were 55.4 $\pm$ 1.8% and 61.6 $\pm$ 3.3%, respectively (all *n* = 6). Moreover, with L-NAME, the contraction levels with U46619 were 57.0 $\pm$ 1.8% and 60.8 $\pm$ 1.8% for the 100 and 500 µM HOCl groups, respectively (all *n* = 6). Furthermore, there were no significant differences in tone in the presence and absence of L-NAME for any of the 100 and 500 µM HOCl experimental groups (*p* = 0.426 and 0.832, respectively).

L-NAME (300 µM) abolished the rapid relaxation and contraction observed at 100 µM HOCl (66.8 $\pm$ 9.0%, *n* = 6) and (2.5 $\pm$ 3.8%, *n* = 6). In addition, the relaxation observed at 60 minutes of HOCl (control: 99.28 ± 2.3%, *n* = 6) was significantly decreased in the presence of L-NAME (54.5 ± 4.97%, *n* = 6) (*p* < 0.0001) (Figure 3-4A).

L-NAME also blocked the contraction produced by 500 µM HOCl (57.5 ± 5.5%, *n* = 6), while rapid relaxation was replaced by a pronounced relaxation (94.1 ± 3%, *n* = *6*). Slow relaxation in the presence of L-NAME (103.9 ± 2.3%, *n* = 6) was not significantly different compared to the control group at 60 minutes (93.8 ± 2.8%, *n* = 6) (*p* = 0.11) (Figure 3-4B).

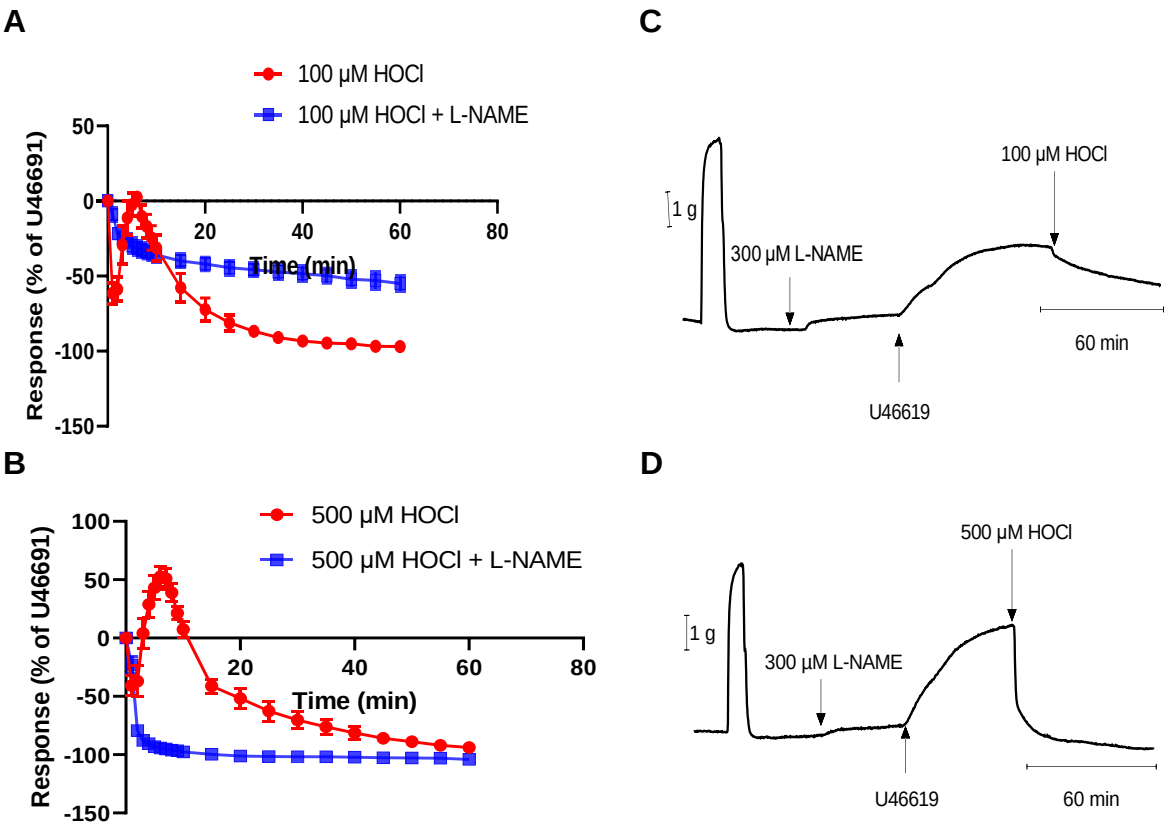


Figure 3-4. Porcine coronary artery responses produced by (A) 100 µM HOCl, and (B) 500 µM HOCl in the absence or presence of 300 µM L-NAME (*n* = 6). (C–D) representative trace for: (C) 100 µM HOCl and (D) 500 µM HOCl in presence of L-NAME. Artery segments were pre-contracted with U46619.

HOCl at 100 µM failed to evoke any response in the presence of D-NAME (300 µM) (the inactive form of L-NAME) (Figure 3-5A). Moreover, D-NAME blocked the initial relaxation and subsequent contraction to 500 µM HOCl (Figure 3-5B). Relaxation after 60 minutes was similar in the presence of D-NAME (80.4 ± 11.7%, *n* = 6) compared to the control group (83.1 ± 12.1%, *n* = 6) (*p* = 0.87) (Figure 3-5B).

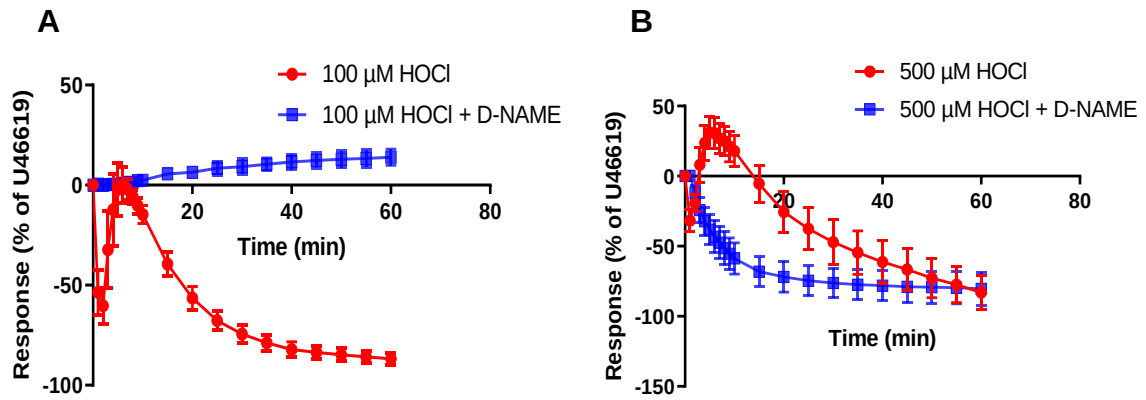


Figure 3-5. Porcine coronary artery responses were produced by (A) 100 µM HOCl, and (B) 500 µM HOCl in the absence or presence of 300 µM D-NAME (*n* = 6). Artery segments were pre-contracted with U46619.

Table 3-1. Concentration of U46619 Required to Pre-constrict Artery Segments in Experimental Groups and Level of U46619-Induced Tone Achieved

Experimental groups		**Concentration of U46619 required to pre-constrict PCA (nM)**	**Level of U46619-induced tone achieved (expressed as % of KCl)**	***n***	***p***
HOCl	100 µM	8 ± 1.5	69 ± 3.2%	7	ns
	500 µM	7.5 ± 2.3	67 ± 4.8%	7	ns
Denuded endothelium		4.3 ± 0.4	60 ± 2%	12	**
L-NAME		3.5 ± 0.5	58.5 ± 2.5%	12	***
D-NAME		3.4 ± 0.4	62.5 ± 3.2%	12	ns
L-NMMA		5.6 ± 1.5	58.2 ± 2%	16	**
D-NMMA		2.3 ± 0.75	56 ± 1.5%	14	ns
Haemoglobin		9.6 ± 3.5	59.5 ± 4.6%	12	ns
PTIO		58.5 ± 14.3	63.5 ± 6.2%	12	**
ODQ		17.5 ± 2.7	70.5 ± 8.3%	12	ns
SQ22536		10.4 ± 1.2	66.5 ± 3.6%	12	ns
PVAT attached		16.6 ± 4.8	65.4 ± 1.2%	15	ns
PVAT re-addition (before KCl)		8.3 ± 2.4	70.4 ± 1.2%	12	ns

PVAT re-addition (after KCl)	10.2 ± 1.7	68.5 ± 3.8%	12	ns
Indomethacin	7.4 ± 2.6	67.4 ± 5.8%	6	ns
TEA	14.8 ± 2.2	66.4 ± 1.5%	17	ns
XE991	17.7 ± 2.4	60.5 ± 7.2%	12	ns
8-SPT	20.9 ± 2.3	59.6 ± 5.4%	12	ns
ZM 241358	15.5 ± 4.2	66.4 ± 3.8%	12	ns
DPCPX	20.2 ± 5.1	63.5 ± 8.2%	13	ns
Suramin	22 ± 4.3	68.5 ± 4.2%	13	**
PPAD	29.5 ± 9	56.7 ± 5.5%	13	ns
MRS2179	15.5 ± 2.7	69 ± 2.3%	15	ns
NF449	17.2 ± 3.3	68.4 ± 6.5%	18	ns
α, β-methylene ATP	18.3 ± 4.2	67 ± 1.5%	12	ns
probenecid	15.8 ± 5.2	67.2 ± 2.8%	13	ns
carbenoxolone	17.5 ± 1.4	73.4 ± 2.2%	12	ns
BX430	17.7 ± 2.4	65.5 ± 6.4%	12	ns
ARL67156	10.5 ± 0.9	73.4 ± 3.3%	12	ns
apyrase (100 units/ml)	2.5 ± 0.3	77.9 ± 2.4%	12	****
apyrase (10 units/ml)	9.6 ± 3.4	70.3 ± 3.6%	12	*

Note. P value shows a difference in the concentration. The comparisons are with respective controls carried out at the same time as the test compounds. ns: not signifinant. Asterisks (*) indicate *p < 0.05, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$. Statistical analysis was performed using *t*-test.

3.3.3.2.2 Effect of L-NMMA and D-NMMA on HOCl Responses

Despite the fact that L-NAME is typically used to block the production of NO, it has recently been demonstrated that NO may be released from the guanidino nitro group of L-NAME (Liu et al., 2019). Moreover, there is evidence that L-NAME interacts with iron-containing enzymes (Das et al., 1999). The use of L-NMMA is based on the fact that it is not clear whether NO plays a role in the HOCl response when L-NAME is employed (Goldsmith et al., 1995).

Similar to L-NAME, L-NMMA is known to increase the response to vasoconstrictors

(Bruck et al., 2001). Therefore, the concentration of U46619 used for pre-contracting the coronary arteries was significantly lower in those containing L-NMMA (5.6 ± 1.5 nM) as compared to those without (25.6 ± 5.9 nM) (n = 6, and 6) (p = 0.0049). The levels of tone (% of KCl) achieved with U46619 in the 100 and 500 μM HOCl groups were 52.8 ± 1.7% and 63.6 ± 2.4%, respectively (all n = 6). In the presence and absence of L-NMMA, there were no significant differences in tone between the 100 and 500 μM HOCl experimental groups (p = 0.72 and 0.42, respectively).

L-NMMA (300 μM) abolished the rapid relaxation observed at 100 μM HOCl (100.4 ± 13.6%, n = 6). In addition, the later relaxation at 60 minutes to HOCl (105.9 ± 3.2%, n = 6) was also eliminated in the presence of L-NMMA (4.9 ± 5.3%, n = 6) (p = 0.0001) (Figure 3-6A).

Moreover, L-NMMA significantly decreased the rapid relaxation observed with 500 μM HOCl (56.4 ± 3.8%, n = 10) compared to the L-NMMA group (18.1 ± 9.4%, n = 10) (p = 0.0006) (Figure 3-6B). L-NMMA blocked the contraction produced by 500 μM HOCl (45.9 ± 7.8%, n = 10). The prolonged relaxation following at 60 minutes was markedly reduced (Figure 3-6B).

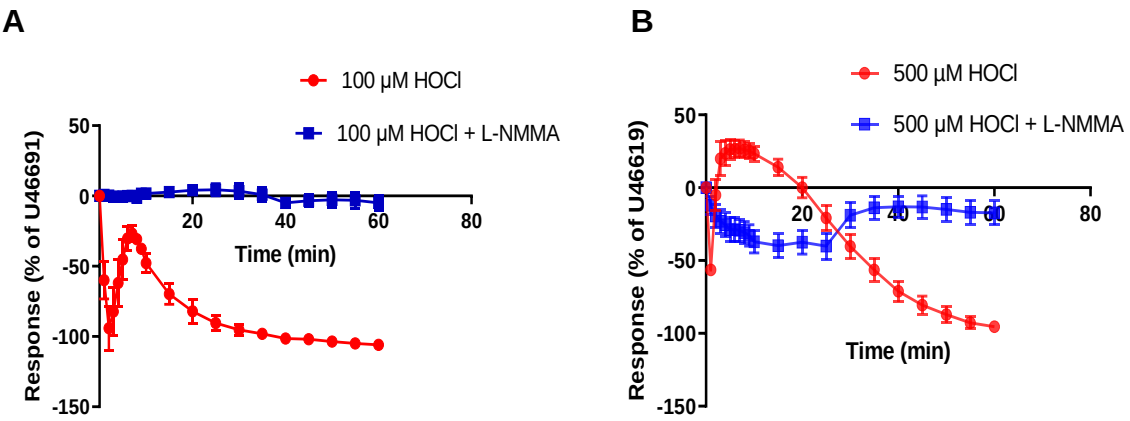


Figure 3-6. Porcine coronary artery responses were produced by (A) 100 µM HOCl (*n* = 6), and (B) 500 µM HOCl in the absence or presence of 300 µM L-NMMA (*n* = 10). Artery segments were pre-contracted with U46619.

D-NMMA (300 µM) abolished the rapid relaxation observed at 100 µM HOCl (70.3 ± 3.9%, *n* = 6). In addition, the prolonged relaxation of HOCl of 90.3 ± 5.1% (*n* = 6) was eliminated in the presence of D-NMMA (4.7 ± 1%, *n* = 6) (*p* < 0.0001) (Figure 3-7A). D-NMMA blocked the contraction produced by 500 µM HOCl (49.8 ± 2.8%, *n* = 6). The prolonged relaxation following at 60 minutes was markedly reduced (Figure 3-7B). Figure 3-7. Porcine coronary artery responses were produced by (A) 100 µM HOCl, and (B) 500 µM HOCl in the absence or presence of 300 µM D-NMMA (*n* = 6). Artery segments were pre-contracted with U46619.

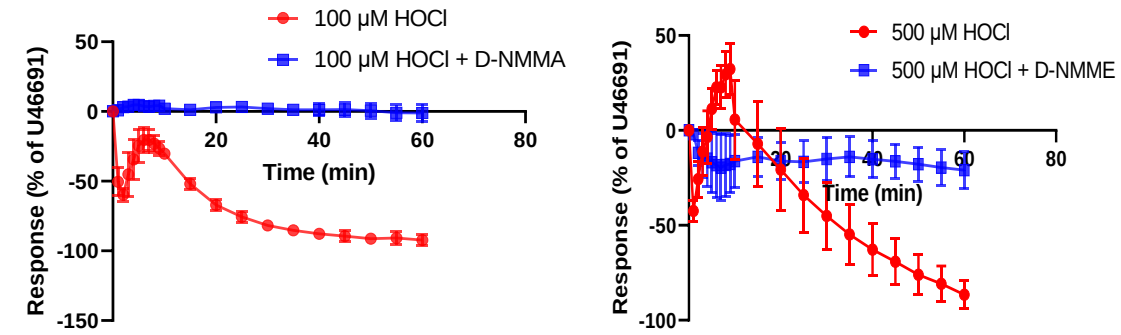


Figure 3-7. Porcine coronary artery responses were produced by (A) 100 μM HOCl, and (B) 500 μM HOCl in the absence or presence of 300 μM D-NMMA (*n* = 6). Artery segments were pre-contracted with U46619.

3.3.3.2.3 Effect of Denuded Endothelium and L-NAME on HOCl Responses

Figure 3-7 shows that 300 μM L-NAME inhibits the rapid relaxation and pronounced contraction induced by added 100 and 500 μM HOCl in arteries with intact endothelium. That endothelium removal alone had a similar inhibitory effect (Figure 3-3) suggests that the endothelium is involved in the inhibition by L-NAME of the rapid relaxation and contraction. With regard to the prolonged relaxation, this was more pronounced in the presence of L-NAME than in controls and in endothelium-denuded PCA (Figure 3-3) and was unaffected by endothelium removal, suggesting an involvement of the smooth muscle.

The relaxation observed at 60 minutes of 100 μM HOCl (control: 99.28 ± 2.3%, *n* = 7) was significantly decreased in the presence of L-NAME in the PCA segments with intact endothelium (54.5 ± 4.97%, *n* = 7) and in denuded the PCA (48.5 ± 12.2%, *n* = 7) ($p < 0.002$) (Figure 3-8A).

Moving to 500 μM HOCl, relaxation at 60 minutes was comparable in the control group (93.8 ± 2.8%, *n* = 6), with the presence of L-NAME in intact PCA (99.2 ± 2.3%, *n* = 6) and with L-NAME in denuded PCA (101 ± 1.2%, *n* = 6) ($p = 0.24$) (Figure 3-8B).

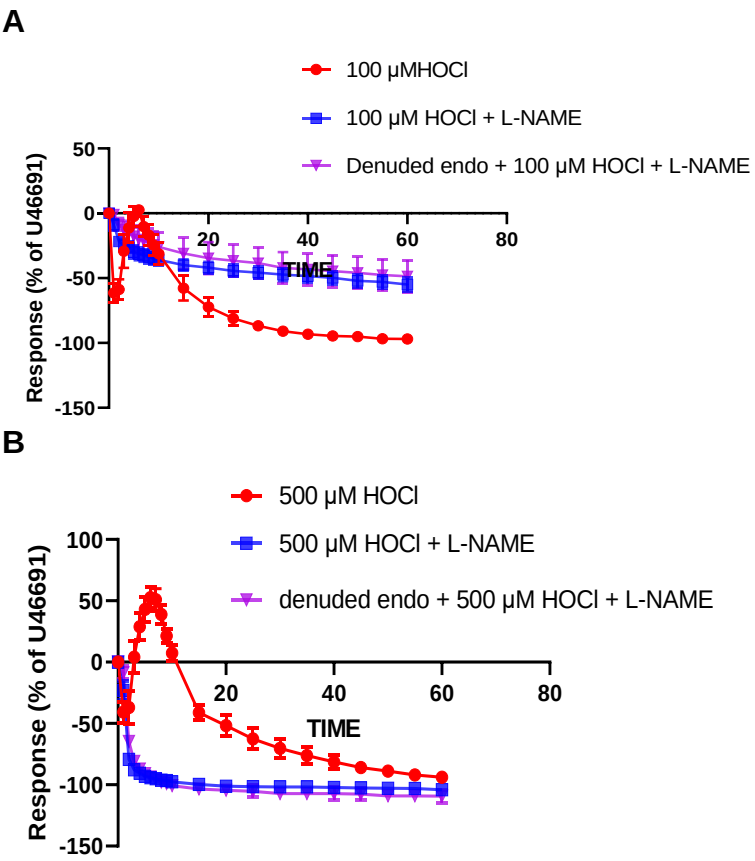


Figure 3-8. Porcine coronary artery responses were produced by (A) 100 μM HOCl, and (B) 500 μM HOCl: in the absence or presence of 300 μM of L-NAME and in denuded endothelium in the presence of 300 μM L-NAME (*n* = 7). Artery segments were pre-contracted with U46619.

3.3.3.2.4 Effect of Haemoglobin on HOCl Response

It is known that haemoglobin inhibits and destroys NO activity by reaction with oxyHb; after that, oxyHb reacts with NO to form metHb and nitrate (Ignarro, 1989; Helms & Kim-Shapiro, 2013). Haemoglobin (10 μM) caused a loss of both the 100 μM and 500 μM HOCl response (Figure 3-9A and B). According to Helms and Kim-Shapiro (2013), HOCl and its conjugate base (OCl^-) can conjugate to the heme moiety of haemoglobin (Hb). As a result, it affects and can abolish the HOCl response.

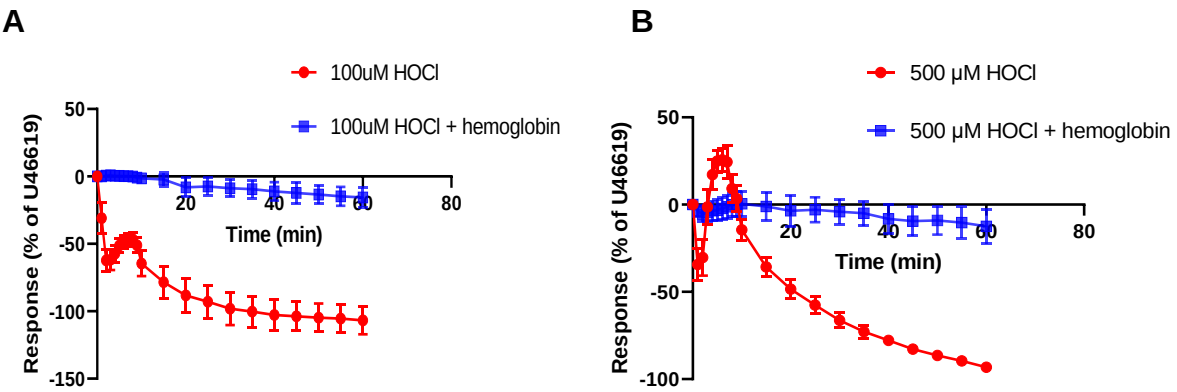


Figure 3-9. Porcine coronary artery responses were produced by (A) 100 µM HOCl, and (B) 500 µM HOCl in the absence or presence of 10 µM haemoglobin (*n* = 6). Artery segments were pre-contracted with U46619.

3.3.3.2.5 Effect of PTIO on HOCl Response

To examine further the possible involvement of NO in the responses to HOCl, the NO scavenger PTIO (300 µM) (Yoshida et al., 1997) was used.

PTIO (300 µM) abolished the rapid relaxation observed at 100 µM HOCl (69.1 ± 6.6%, *n* = 6). In addition, the relaxation observed at 60 minutes of HOCl (control: 106 ± 1.7%, *n* = 6) was not significantly different in the presence of PTIO (106.6 ± 2.2%, *n* = 6) (*p* = 0.85) (Figure 3-10A).

PTIO also blocked the rapid relaxation observed at 500 µM HOCl (32.6 ± 5.2%, *n* = 6) (*p* = 0.0001) and also reduced the contraction produced by 500 µM HOCl (50.2 ± 9.9%, *n* = 6) compared to the control group (22.2 ± 2.4%, *n* = 6) (*p* = 0.021) (Figure 3-10B). Slow relaxation in the presence of PTIO (102.4 ± 2.5%, *n* = 6) was significantly increased compared to the control group at 60 minutes (91.9 ± 3.5%, *n* = 6) (*p* = 0.037) (Figure 3-10B).

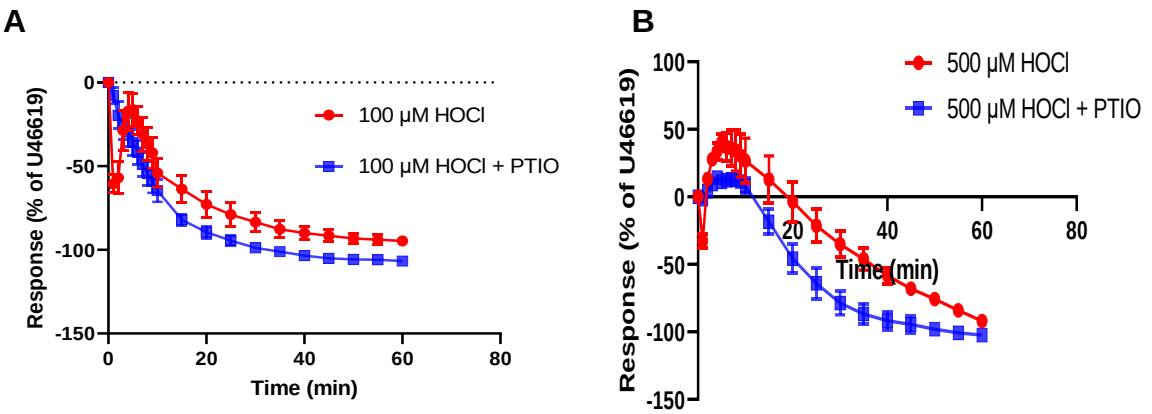


Figure 3-10. Porcine coronary artery responses were produced by (A) 100 µM HOCl, and (B) 500 µM HOCl in the absence or presence of 300 µM PTIO (*n* = 6). Artery segments were pre-contracted with U46619.

3.3.3.3 Prostaglandin Involvement

3.3.3.3.1 Effect of Indomethacin on HOCl Responses

Part of the pronounced relaxation response to the 500 µM HOCl was independent of NO. It is possible that this was due to the release of vasodilator prostanoids from the smooth muscle (Sandoo et al., 2010). Prostaglandin involvement was examined using indomethacin (prostaglandin inhibitor).

At 500 µM HOCl, the transient relaxation, high contraction, and slow relaxation in the presence of indomethacin (10 µM) were 51.8 ± 5.2%, 11.3 ± 10% and 98 ± 1.8% (*n* = 6), respectively. Similar responses were produced in control arteries 39.8 ± 2.6%, 5.5 ± 8.7% and 96.9 ± 1.5% (*n* = 6), respectively, without any significant change (*p* = 0.06, *p* = 0.66 and *p* = 0.62) (Figure 3-11).

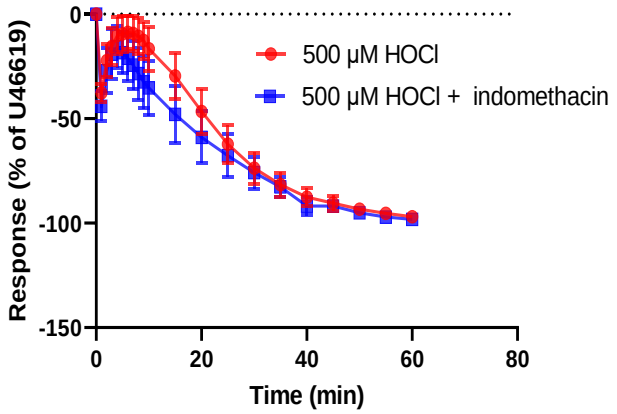


Figure 3-11. Porcine coronary artery responses produced by 500 µM HOCl in the absence or presence of 10 µM indomethacin (*n* = 6). Artery segments were pre-contracted with U46619.

3.3.3.4 Effect of High K^+ and K^+ Channel Blockers

3.3.3.4.1 HOCl Response on PCA Rings Pre-contracted With KCl

There exists a possibility that the release of a hyperpolarising factor from the endothelium mediates the rapid relaxant effect of HOCl on PCA rings. Thus, depolarisation with KCl that entirely prevents the hyperpolarisation of both the endothelial and the smooth muscle cells was used to investigate the HOCl effect in PCA rings pre-contracted with KCl (Weintraub et al., 1994).

At 100 µM, the HOCl response on the PCA pre-contracted with KCl was completely blocked compared to the PCA pre-contracted with U46619 (Figure 3-12A). The rapid relaxation and slow relaxation in the PCA pre-contracted with U46619 were 83.2 ± 0.9% and 80.8 ± 18.4% respectively (*n* = 6) compared to PCA pre-contracted with KCl 7.2 ± 1.4% ($p < 0.0001$) and 8.8 ± 4.4 % ($p = 0.002$) (*n* = 6).

Moreover, 500 µM HOCl in KCl pre-contracted artery showed blocked rapid

relaxation and slow relaxation observed at 60 minutes compared to the PCA pre-contracted with U46619. In contrast, the pronounced contraction observed in response to U46619 pre-contracted (41.7 ± 4.3%, *n* = 6) was evident in the KCl pre-contracted artery (51.8 ± 5.2%, *n* = 6) (*p* = .16). (Figure 3-12B). Notably, the concentration of KCl that was required to produce 60% to 80% of the second KCl contraction was 28.3 ± 1.6 mM (*n* = 12). The pre-contraction produced by the addition of KCl was 68 ± 1.2% (*n* = 6) compared to the PCA pre-contracted by U46619 62.5 ± 2.4% (*n* = 6).

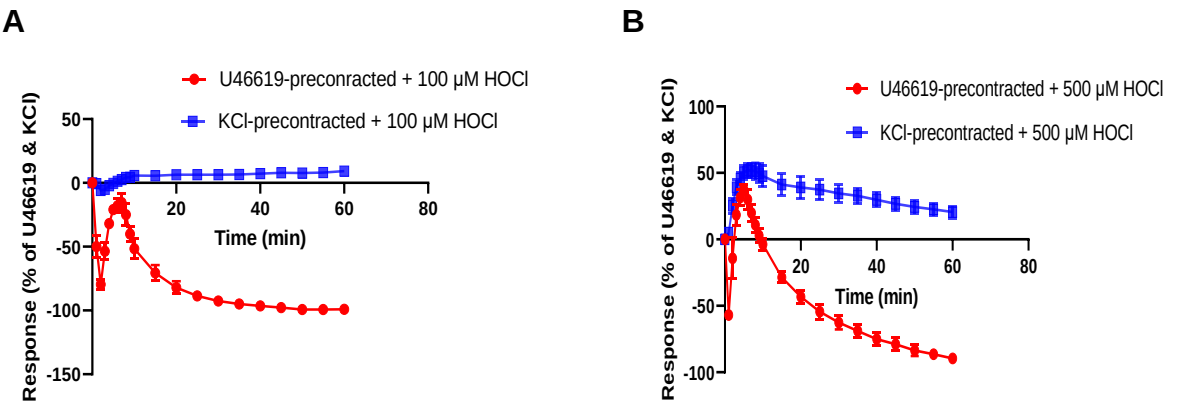


Figure 3-12. Porcine coronary artery responses were produced by (A) 100 µM HOCl, and (B) 500 µM HOCl. Artery segments were pre-contracted with KCl or U46619 (*n* = 6).

3.3.3.4.2 Effect of K^+ Channel Blockers on HOCl Response

Cellular membrane voltage is regulated by K^+ channels, which in increased or decreased activity results in hyperpolarisation or depolarisation of the membrane. Consequently, K^+ channels play a crucial role in the activity of voltage-dependent Ca^{2+} channels and, subsequently, in the contraction of smooth muscle. The K^+ channel subunits are expressed by more than 75 genes in humans, making them the

most diverse group of ion channels. Smooth muscle contains four major types of K^+ channels: large conductance Ca^{2+}-sensitive (K_{Ca}), ATP-sensitive (K_{ATP}), inwardly rectifying (K_{ir}) and voltage-gated (K_V) (Stott et al., 2014).

3.3.3.4.2.1 Effect of TEA on HOCl Response

EDH can lead to vascular smooth muscle cell relaxation through endothelial cells hyperpolarisation by increasing K^+ in the sub-endothelial space, which activates K_{Ca}, K_{IR} and K_{ATP} (Nagao & Vanhoutte, 1992). For that reason, TEA has been used as an inhibitor of K_{Ca} channels.

The transient relaxation of the PCA segments evoked by 100 µM HOCl was still pronounced in the presence and absence of TEA (10 mM) (45.8 ± 5.2, *n* = 9) (61.8 ± 5.8%, *n* = 9) (*p* = 0.60) respectively. Additionally, the relaxation at 60 minutes (98.6 ± 1.8 %, *n* = 9) was higher in the control compared to the TEA group (88.7 ± 5.3 %, *n* = 9) (83.7 ± 5.3 %, *n* = 9) (*p* = 0.01) (Figure 3-13A).

At 500 µM HOCl, the transient relaxation and slow relaxation was still pronounced in the presence and absence of TEA. In the presence of TEA HOCl produced a more pronounced contraction 107.8 ± 8.4 %, (*n* = 8) compared to that in the control at 29.3± 3.6 % (*n* = 8) (P< 0.0001) (Figure 3-13B).

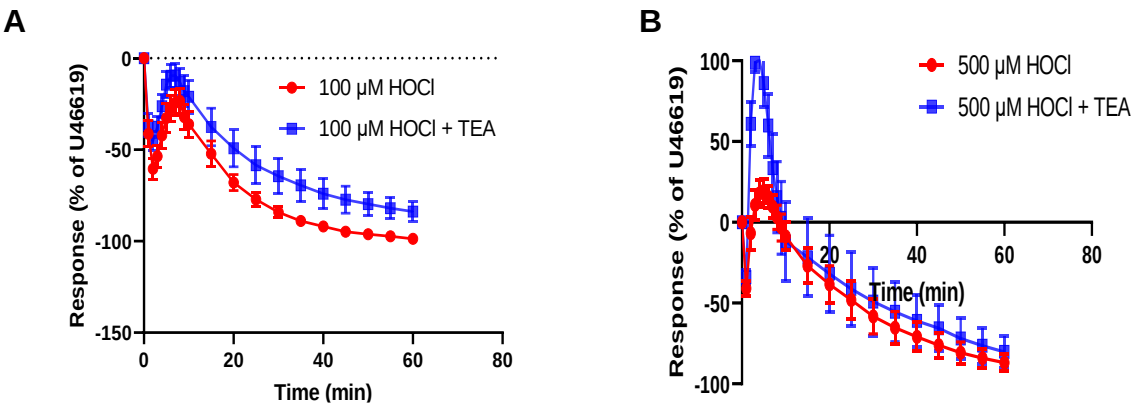


Figure 3-13. Porcine coronary artery responses were produced by (A) 100 µM HOCl (*n* = 9) and (B) 500 µM HOCl (*n* = 8) in the absence or presence of 10 mM TEA. Artery segments were pre-contracted with U46619.

3.3.3.4.2.2 Effect of glibenclamide on HOCl Response

In coronary VSMCs, K_{ATP} play an important role in regulating coronary circulation. A decrease in the intracellular concentration of ATP, such as under hypoxia or inflammation, activates these K^+ channels. This results in an increase in K^+ outflow from the cell, hyperpolarisation and relaxation of vascular smooth muscle (Mellemkjær & Nielsen-Kudsk, 1994). In an attempt to determine whether K_{ATP} is involved in the prolonged vasodilation of HOCl response, glibenclamide, a specific inhibitor of K_{ATP}, was used.

Glibenclamide (1 µM) did not alter transient or slow relaxation at 60 minutes of 100 µM. The transient relaxation in the control group was 53.5 ± 4%, *n* = 6, whereas the transient relaxation in the glibenclamide group was 54.6 ± 4.7%, *n* = 6 (*p* = 0.86). In the glibenclamide group, slow relaxation was 100.9 ± 1.5% (*n* = 6) compared to 97.8 ± 2.2% in the control group (*p* = 0.27) (Figure 3-14A).

Moreover, there was no difference between responses in the presence or absence of

glibenclamide at 500 μM HOCl; with glibenclamide, the transient relaxation was 31.2 ± 3.7% (*n* = 6), and without it, it was 30 ± 3.6% (*n* = 6) (*p* = 0.81). The subsequent contraction was 40.8 ± 4.1% (*n* = 6) in the control compared to 37.6 ± 5.4% (*n* = 6) in the presence of glibenclamide (*p* = 0.64). HOCl showed no difference in the later relaxations (*p* = 0.55) in the presence or absence of glibenclamide (*n* = 6) (Figure 3-14B).

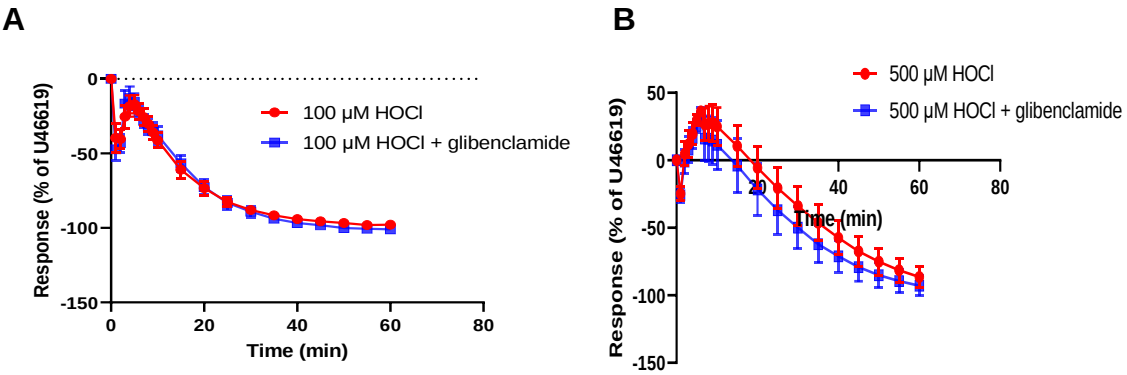


Figure 3-14. Porcine coronary artery responses produced by (A) 100 μM and (B) 500 μM HOCl in the absence or presence of 1 μM glibenclamide (*n* = 6). Artery segments were pre-contracted with U46619.

3.3.3.4.2.3 Effect of XE991 on HOCl Response

Recent evidence suggests that K_V channels play a role in the control of the tone of vascular smooth muscles, with changes in the function of the channels contributing to the development of various vascular diseases (Stott et al., 2014). Hedegaard et al. (2014) suggested that hypoxic vasodilation in the PCA is primarily mediated by the smooth muscle $K_V7.4$ and $K_V7.5$ channels, with smaller contributions from other K channels. To check whether K_V7 channel was involved, XE991 was used.

The rapid relaxation evoked by 100 μM HOCl in the absence of XE991 (10 μM) was

74.5 ± 6.5% (*n* = 6) with no change compared to 66.4 ± 3.2% (*n* = 6) in the presence of XE991 (*p* = 0.29) (Figure 3-15A). Similarly, the later relaxation response at 60 minutes in the absence or presence of XE991 was similar at 96.3 ± 3.1% (*n* = 6) and 98.9 ± 2.4% (*n* = 6) (*p* = 0.36), respectively (Figure 3-15A).

The early relaxation and subsequent contraction at 500 μM HOCl alone were 44.2 ± 5.5% (*n* = 6) and 58.9 ± 5.6% (*n* = 6) respectively, but not significantly different in the presence of XE991 at 45.8 ± 4.2% (*n* = 6) and 64.2 ± 4% (*n* = 6) respectively (*p* = 0.83 and *p* = 0.44). A similar difference was seen in the later relaxation after 60 minutes in the presence of XE991 at 98 ± 2.5% (*n* = 6) compared to that in the control group of 89.7 ± 2.8% (*n* = 6) (*p* = 0.054) (Figure 3-15B).

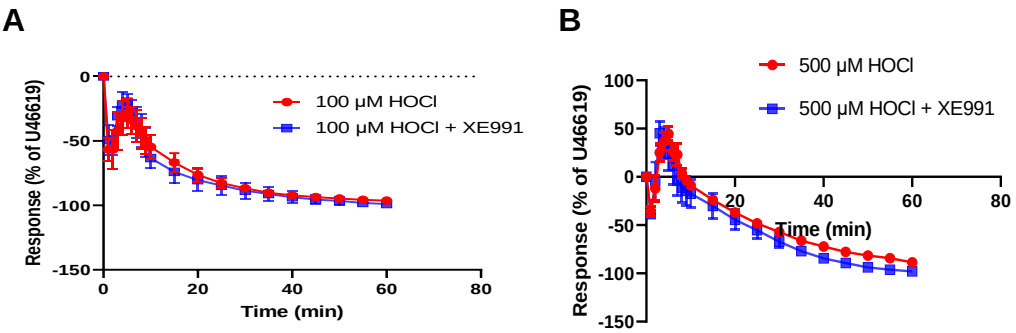


Figure 3-15. Porcine coronary artery responses produced by (A) 100 μM and (B) 500 μM HOCl in the absence or presence of 10 μM XE991 (*n* = 6). Artery segments were pre-contracted with U46619.

3.3.3.5 cGMP and cAMP Involvement

cGMP and cAMP in the vascular system play an important role in blood vessel relaxation in smooth muscle cells. Therefore, ODQ (guanylyl cyclase inhibitor) and SQ22536 (adenyl cyclase inhibitor) were used to investigate whether cGMP and cAMP are involved in the response to HOCl.

ODQ (10 μM) neither altered the transient relaxation nor slowed relaxation at 60 minutes after the addition of 100 μM HOCl. The transient relaxation in the control was 50 ± 5.5% (n = 6) compared to the ODQ 43.6 ± 8% (n = 6) (p = 0.52). The slow relaxation was 89.3 ± 3.8% (n = 6) in the ODQ group compared to the control 94.7 $\pm$ 3.5% (n = 6) (p = 0.32) (Figure 3-16A).

In addition, there was no difference between responses in the presence of ODQ and in control at 500 μM HOCl; in the presence of ODQ, the transient relaxation was 30 ± 4.8% (n = 6) and in control was 35.5 ± 5% (n = 6) (p = 0.45); the subsequent contraction in the presence of ODQ was 41 ± 9.6% (n = 6) compared to the control response of 23 ± 10.6% (n = 6) (p = 0.23). The later relaxation in the presence and absence of ODQ was 87.5 $\pm$ 2.8% (n = 6) and 88.5 ± 3.4% (n = 6), respectively, without significant change (p = 0.83) (Figure 3-16B).

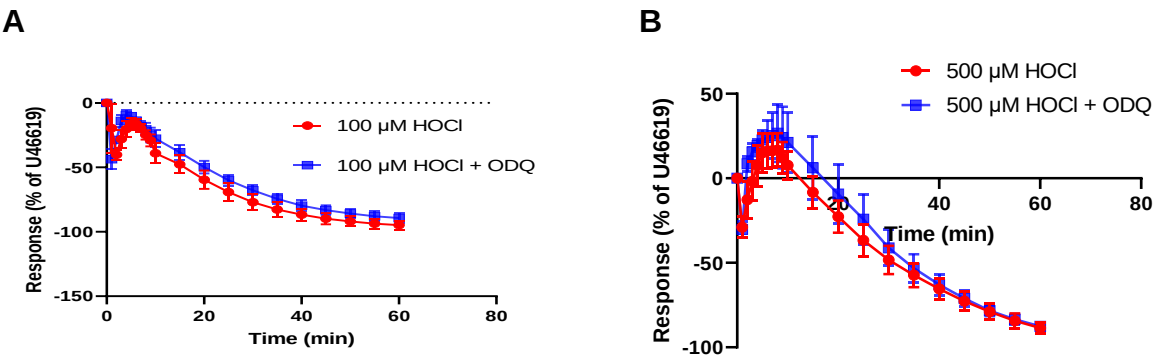


Figure 3-16. Porcine coronary artery responses were produced by (A) 100 µM HOCl, and (B) 500 µM HOCl (*n* = 6) in the absence or presence of 10 µM ODQ. Artery segments were pre-contracted with U46619.

Moving to SQ22536, 10 µM SQ22536 did not show any effect on HOCl response. The transient relaxation in the control was 69 ± 6.6%, (*n* = 6) compared to the SQ22536 group's 63.6 ± 5.7% (*n* = 6) (*p* = 0.54). The slow relaxation was 93.4 ± 4.9% (*n* = 6) in the SQ22536 group compared to the control's 94.6 ± 2.8% (*n* = 6) (*p* = 0.83) (Figure 3-17A).

At 500 µM HOCl, in the presence of SQ22536, the transient relaxation was 43.6 ± 5.2% (*n* = 6) and in control was 38.57 ± 4.5% (*n* = 6) (*p* = 0.49). The subsequent contraction in the presence of SQ22536 was 58 ± 3.6% (*n* = 6) compared to the control response of 48.7 ± 3.7% (*n* = 6) (*p* = .1). The later relaxation in the presence and absence of SQ22536 was 99.8 ± 1.8% (*n* = 6) and 91.5 ± 3.2% (*n* = 6), respectively, without significant change (*p* = 0.05) (Figure 3-17B).

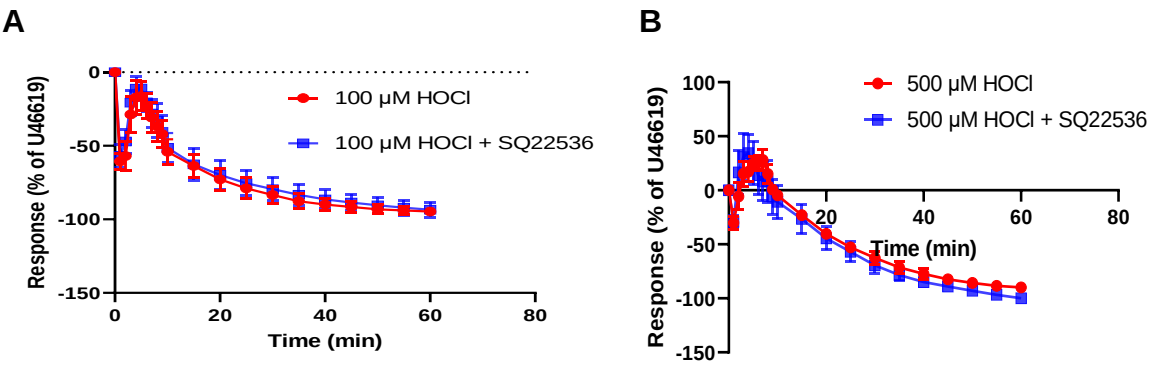


Figure 3-17. Porcine coronary artery responses were produced by (A) 100 µM HOCl, and (B) 500 µM HOCl (*n* = 6) in the absence or presence of 10 µM SQ22536. Artery segments were pre-contracted with U46619.

3.3.3.6 Perivascular Adipose Tissue Affects HOCl Response

3.3.3.6.1 Effects of Attached PVAT on HOCl Response

It's understood that PVAT influences vascular function through endocrine and paracrine pathways. A wide range of substances are released, including adipokines, cytokines and chemokines, NO, and H_2S. As a result, these substances can reach both the VSMCs and the endothelial cells of blood vessels (Gao et al., 2007; Man et al., 2020). It has been demonstrated that PVAT secretes factors that either promote or inhibit vascular contraction. PVAT-derived relaxing factors (PDRFs), also known as anticontractile factors, include a variety of substances such as NO and H2S, H2O2, PGI2, as well as adipocytokines such as leptin, apelin, adiponectin, and omentin. There is evidence that these factors may result in PVAT's anti-contractile effects through mechanisms independent or dependent on the endothelium. Several pathways are involved in PVAT's regulation function at the level of VSMCs, including BKCa, cGMP-dependent protein kinases (PKG), ATP-sensitive channels and

voltage-gated K+ channels (Ahmed et al., 2023).

In contrast, PVAT also releases pro-contractile factors, known as PVAT-derived contracting factors (PDCFs), including prostaglandins, superoxide anion, angiotensin II, catecholamines, chemerin, and resistin. Particularly under conditions such as obesity, hypertension, and diabetes, these factors can contribute to PVAT's pro-contractile effects. A number of mechanisms have been identified as responsible for their effect, including activation of Rho-kinase in VSMCs, inhibition of K+ channels, and an increase in ROS production (Ahmed et al., 2023).

PCAs with and without attached PVAT were used to investigate whether PVAT would affect the response to HOCl, especially the rapid contraction. To study the effects of PVAT on HOCl responses, the effects of 100µM and 500µM HOCl were investigated in the PCAs in the presence (+) and absence (−) of attached PVAT.

At 100 µM HOCl, PVAT (+) did not affect the rapid relaxation seen in PVAT (−) (52 $\pm$ 8%, n = 8) (51 $\pm$ 11%, n = 8) respectively (p = 0.98). Return to baseline (or contraction) was blocked in PVAT (+); contraction in PVAT (−) was 22.1 $\pm$ 9.7% (n = 8) compared to PVAT (+) where there was no contraction or return to baseline (p = 0.04). Additionally, the relaxation at 60 minutes was significantly reduced in the PVAT (+) compared to the PVAT (−) (58.6 $\pm$ 13%, n = 8) (92.5 $\pm$ 4%, n = 8) (p = 0.03) (Figure 3-18A).

Figure 3-18B shows that PVAT (+) inhibited the contraction observed at 500 µM HOCl (0.12 ± 6.9%, n = 7) compared to PVAT (−) (61.8 ± 9%, n = 7) (p = 0.0002). In

addition, the relaxation observed at 60 minutes of 500 µM HOCl (90.5 ± 9%, *n* = 7) was significantly reduced in PVAT (+) compared to PVAT (−) (58.9 ± 7.5%, *n* = 7) (*p* = 0.02) respectively (Figure 3-18B).

Notably, maximum force produced in response to 60 mM KCl was not statistically different between PVAT (+) and PVAT (−) rings (in grams: 11.2 ± 0.4, 11.6 ± 0.9 for PVAT (+) and PVAT (−), respectively, *n* = 8, and 7, *p* = 0.7).

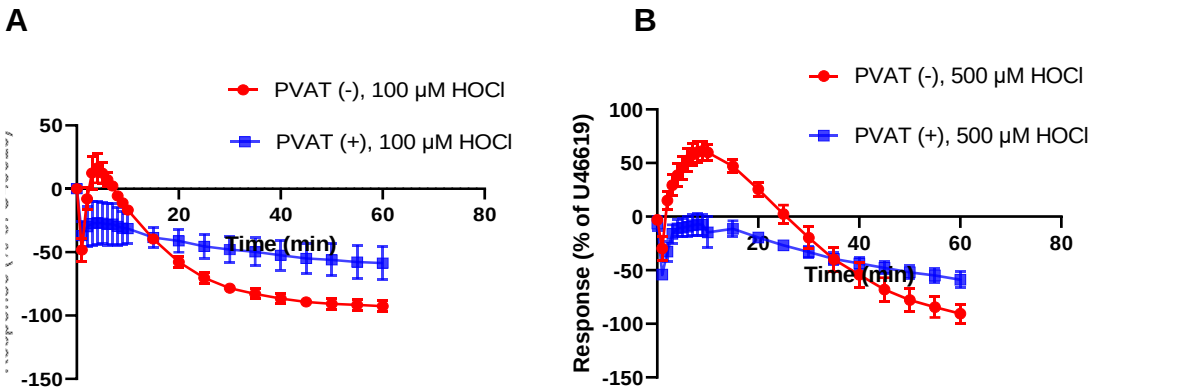


Figure 3-18. Porcine coronary artery responses were produced by (A) 100 µM HOCl (*n* = 8), and (B) 500 µM HOCl (*n* = 7) in the absence of PVAT (−) and presence of PVAT (+). Artery segments were pre-contracted with U46619.

3.3.3.6.2 Effects of PVAT Re-Addition, Before and After KCl, on the HOCl Response

In this experiment, PVAT dissected from the PCA was re-added into the organ bath instead of leaving it attached PVAT. If attached PVAT has an effect, it could be due to some mechanical effect on HOCl contraction, while if taken off and re-added, the effect is mainly due to PVAT itself.

At 100 µM HOCl, PVAT (+) added before KCl blocked the rapid relaxation that was

observed at 100 µM HOCl (51 ± 10.4%, *n* = 6). Additionally, the relaxation at 60 minutes as measured by adding PVAT before KCl was significantly lower than in the control group (62.7 ± 14.4%, *n* = 6) (93.8 ± 2.3%, *n* = 6) (*p* = 0.03) respectively (Figure 3-19A).

Added PVAT (+) after KCl also eliminated the rapid relaxation that was noted at 100 µM HOCl. In addition, the relaxation observed at 60 minutes of 100 µM HOCl was significantly decreased in PVAT added after KCl (20.7 ± 5.7%, *n* = 6) (*p* < 0.0001), respectively (Figure 3-19A).

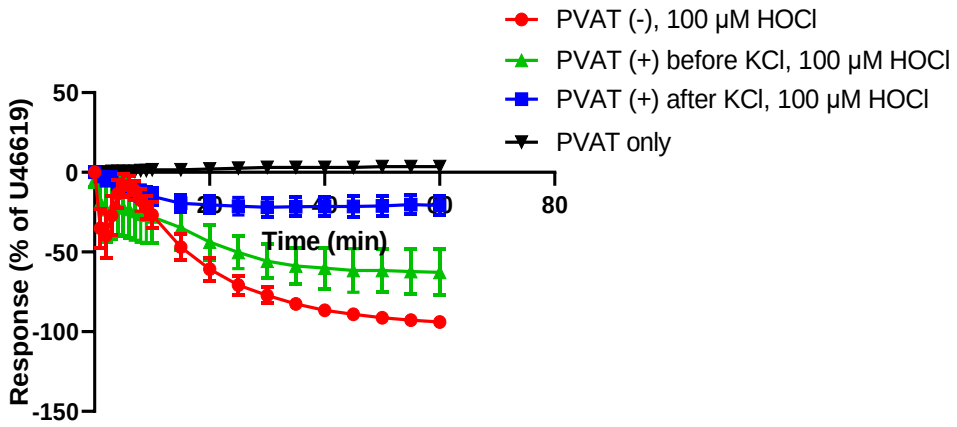
Similarly at 500 µM HOCl, PVAT (+) added before or after added KCl blocked the rapid relaxation and pronounced contraction that was observed at 500 µM HOCl (45.8 ± 2.9%, *n* = 6) (45.8 ± 12.4%, *n* = 6) respectively (Figure 3-19B).

The relaxation observed at 60 minutes of 500 µM HOCl was significantly decreased in PVAT added after KCl (67 ± 6.9%, *n* = 6) compared to the control (113.2 ± 10.5%, *n* = 6) (*p* = 0.004) (Figure 3-19A). On the other hand, there was no significant difference in relaxation at 60 min between control and PVAT (+) added before KCl (107.6 ± 12.3%, *n* = 6) (*p* = 0.7) (Figure 3-19B).

When PVAT was added after the second KCl wash to segments of the PCA at baseline tone, a slow, time-dependent contraction occurred that peaked at 30 minutes (12.1 ± 1.4% of the KCl contraction) (*n* = 6) (Figure 3-20).

Table 3-2, Table 3-3, Table 3-4, Table 3-5, and Table 3-6 summarize the effects of the various inhibitors on multiphasic response to HOCl.

A



B

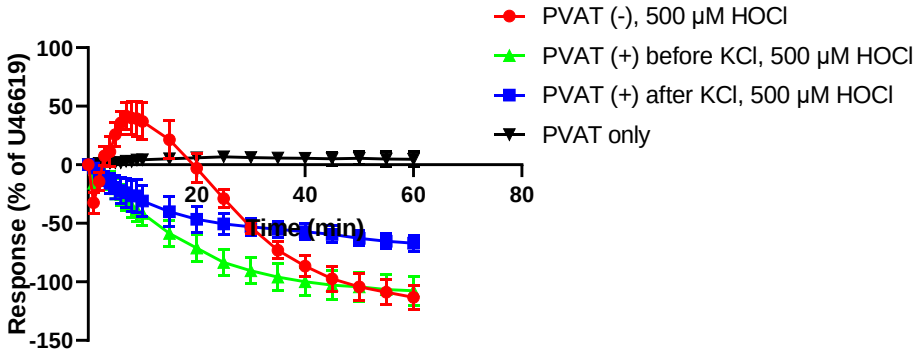


Figure 3-19. Porcine coronary artery responses were produced by (A) 100 µM HOCl (*n* = 6), and (B) 500 µM HOCl (*n* = 6) in the absence of PVAT (−) and by adding PVAT (+) before (*n* = 6) and after (*n* = 6) adding KCl. Artery segments were pre-contracted with U46619.

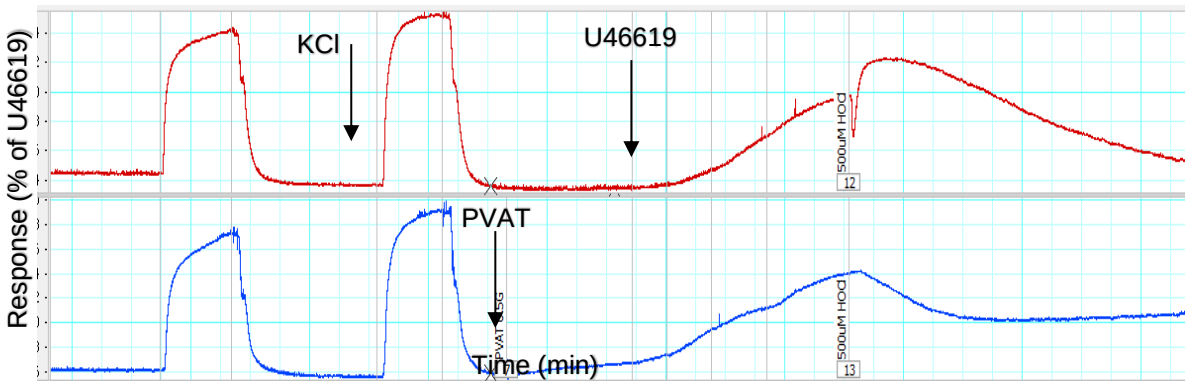


Figure 3-20. A slow, time-dependent contraction occurred that peaked at 30 minutes when PVAT was added after second KCl wash to segments of the PCA at 500 µM HOCl. Artery segments were pre-contracted with U46619.

Table 3-2. Effect of Various Inhibitors on Rapid Relaxation of 100 μM HOCl

Antagonist	**Concentration**	**Control**	**Treatment**	***n***	***p***
endothelium denuded		70.0 ± 4.5%	5.7 ± 2.2%	6	<0.0001
L-NAME (NOS inhibitor)	300 μM	66.8 ± 9.0%	8.9 ± 4.4%	6	<0.0001
D-NAME (inactive form of NOS inhibitor)	300 μM	76.1 ± 3.1%	0.1 ± 0.7%	6	<0.0001
L-NMMA (NOS inhibitor)	300 μM	100.4 ± 13.6%	0.2 ± 2.5 %	6	<0.0001
D-NMMA (inactive form of NOS inhibitor)	300 μM	70.3 ± 3.9%	4.7 ± 2.0%	6	<0.0001
PTIO (NO scavenger)	300 μM	69.1 ± 6.6%	7.6 ± 4.7%	6	<0.0001
PCA rings pre-contracted with KCl	28.3 ± 1.6 mM	83.2 ± 0.9%	7.2 ± 1.4%	6	<0.0001
TEA (inhibitor of K_{Ca} channels)	10 mM	61.8 ± 5.8%	45.8 ± 5.2%	9	0.60
glibenclamide (inhibitor of K_{ATP})	1 μM	53.5 ± 4.0%	54.6 ± 4.7%	6	0.86
XE991 (KV7 channel antagonist)	10 μM	74.5 ± 6.5%	66.4 ± 3.2%	6	0.29
ODQ (guanylyl cyclase inhibitor)	10 μM	50.0 ± 5.5%	43.6 ± 7.9%	6	0.52
SQ22536 (AC inhibitor)	10 μM	69.0 ± 6.6%	63.6 ± 5.7%	6	0.54
attached PVAT		52.0 ± 8.0%	51.0 ± 11%	8	0.98
PVAT re-addition before KCl		51.0 ± 10.4%	8.9 ± 5.1%	6	0.015
PVAT re-addition after KCl		51.0 ± 10.4%	5.1 ±3.0%	6	0.001

Note. Statistical significance was defined as *p* values less than 0.05 in *t*-test.

Table 3-3. Effect of Various Inhibitors on Slow Relaxation of 100 μM HOCl

Antagonist	Concentration	Control	Treatment	*n*	*p*
endothelium denuded		97.0 ± 1.5%	111 ± 3.2 %	6	0.002
L-NAME (NOS inhibitor)	300 μM	99.28 ± 2.3%	54.5 ± 4.97%	6	<0.0001
D-NAME (inactive form of NOS inhibitor)	300 μM	86.8 ± 13.9%	13.9 ± 4.0%	6	<0.0001
L-NMMA (NOS inhibitor)	300 μM	105.9 ± 3.2%	4.9 ± 5.3%	6	0.0001
D-NMMA (inactive form of NOS inhibitor)	300 μM	90.3 ± 5.1%	4.7 ±1.0%	6	<0.0001
PTIO (NO scavenger)	300 μM	106 ± 1.7%	106.6 ± 2.2%	6	0.85
the PCA rings pre-contracted with KCl	28.3 ± 1.6 mM	80.8 ± 18.4%	8.8 ± 4.4%	6	0.002
TEA (inhibitor of K_{Ca} channels)	10 mM	98.6 ± 1.8 %	83.7 ± 5.3%	9	0.01
glibenclamide (inhibitor of K_{ATP})	1 μM	97.8 ± 2.2%	100.9 ± 1.5%	6	0.27
XE991 (KV7 channel antagonist)	10 μM	96.3 ± 3.1%	98.9 ± 2.4%	6	0.36
ODQ (guanylyl cyclase inhibitor)	10 μM	94.7 ± 3.5%	89.3 ± 3.8%	6	0.32
SQ22536 (AC inhibitor)	10 μM	94.6 ± 2.8%	93.4 ± 4.9%	6	0.83
attached PVAT		92.5 ± 4.0%	58.6 ± 13.0%	8	0.03
PVAT re-addition before KCl		93.8 ± 2.3%	62.7 ± 14.4%	6	0.03
PVAT re-addition after KCl		93.8 ± 2.3%	20.7 ± 5.7%	6	<0.0001

Note. Statistical significance was defined as *p* values less than 0.05 in *t*-test.

Table 3-4. Effect of Various Inhibitors on Rapid Relaxation of 500 μM HOCl

Antagonist	Concentration	Control	Treatment	*n*	*p*
endothelium denuded		57.5 ± 5.5%	1.0 ± 0.3%	6	<0.0001
L-NAME (NOS inhibitor)	300 μM	57.7 ± 5.5%	94.1 ± 3.0%	6	0.0005
D-NAME (inactive form of NOS inhibitor)	300 μM	37.1 ± 5.6%	0.2 ± 0.6%	6	0.0003
L-NMMA (NOS inhibitor)	300 μM	56.4 ±3.8%,	18.1 ± 9.4%	10	0.0006
D-NMMA (inactive form of NOS inhibitor)	300 μM	54.5 ± 5.0%	0.8 ±1.5%	7	<0.0001
PTIO (NO scavenger)	300 μM	32.6 ± 5.2%	1.7 ± 0.1%	6	0.0001
indomethacin (prostaglandin inhibitor)	10 μM	51.8 ± 5.2%	39.8 ± 2.6 %	6	0.06
PCA rings pre-contracted with KCl	28.3 ± 1.6 mM	58.9 ± 3.6%	5.1 ± 3.2%	6	<0.0001
TEA (inhibitor of K_{Ca} channels)	10 mM	41.5 ± 4.7%	33.8 ± 4.2%	8	0.24
glibenclamide (inhibitor of K_{ATP})	1 μM	30 ± 3.6%	31.2 ± 3.7%	6	0.81
XE991 (KV7 channel antagonist)	10 μM	44.2 ± 5.5%	45.8 ± 4.2%	6	0.83
ODQ (guanylyl cyclase inhibitor)	10 μM	35.5 ± 5.0%	30 ± 4.8%	6	0.45
SQ22536 (AC inhibitor)	10 μM	38.57 ± 4.5%	43.6 ± 5.2%	6	0.49
attached PVAT		45.8 ± 3.2%	54.1 ± 4.0%	7	0.13
PVAT re-addition before KCl		45.8 ± 2.9%	15.7 ± 7.6%	6	0.004
PVAT re-addition after KCl		45.8 ± 2.9%	2.9 ± 1.8%	6	<0.0001

Note. Statistical significance was defined as *p* values less than 0.05 in *t*-test.

Table 3-5. Effect of Various Inhibitors on Pronounced Contraction of 500 μM HOCl

Antagonist	Concentration	Control	Treatment	*n*	*p*
endothelium denuded		54.7 ± 8.0%	blocked	6	<0.0001
L-NAME (NOS inhibitor)	300 μM	57.5 ± 5.5%	blocked	6	<0.0001
D-NAME (inactive form of NOS inhibitor)	300 μM	33.8 ± 11.5%	blocked	6	0.0011
L-NMMA (NOS inhibitor)	300 μM	45.9 ± 7.8%	blocked	10	<0.0001
D-NMMA (inactive form of NOS inhibitor)	300 μM	57.1 ± 20.7	blocked	7	<0.0001
PTIO (NO scavenger)	300 μM	50.2 ± 9.9%	22.2 ± 2.4%	6	0.02
indomethacin (prostaglandin inhibitor)	10 μM	5.5 ± 8.7 %	11.3 ± 10%	6	0.66
the PCA rings pre-contracted with KCl	28.3 ± 1.6 mM	41.7 ± 4.3%	51.8 ± 5.2%,	6	0.16
TEA (inhibitor of K_{Ca} channels)	10 mM	29.3 ± 3.6%	107.8 ± 8.4%	8	<0.0001
glibenclamide (inhibitor of K_{ATP})	1 μM	40.8 ± 4.1%	37.6 ± 5.4%	6	0.64
XE991 (KV7 channel antagonist)	10 μM	58.9 ± 5.6%	64.2 ± 4%	6	0.44
ODQ (guanylyl cyclase inhibitor)	10 μM	23.0 ± 10.6%	41 ± 9.6%	6	0.23
SQ22536 (AC inhibitor)	10 μM	48.7 ± 3.7%	58 ± 3.6%	6	0.1
attached PVAT		61.8 ± 9%	0.12 ± 6.9%	7	0.0002
PVAT re-addition before KCl		45.8 ± 12.4%	blocked	6	<0.0001
PVAT re-addition after KCl		45.8 ± 12.4%	blocked	6	<0.0001

Note. Statistical significance was defined as *p* values less than 0.05 in *t*-test.

Table 3-6. Effect of Various Inhibitors on Slow Relaxation of 500 μM HOCl

Antagonist	Concentration	Control	Treatment	*n*	*p*
endothelium denuded		93.8 ± 2.8%	102.2 ± 4.1%	6	0.12
L-NAME (NOS inhibitor)	300 μM	93.8 ± 2.8%	103.9 ± 2.3%	6	0.11
D-NAME (inactive form of NOS inhibitor)	300 μM	80.4 ± 11.7%	83.1 ± 12.1%	6	0.87
L-NMMA (NOS inhibitor)	300 μM	95.5 ± 3.7%	17.2 ± 8.2%	10	<0.0001
D-NMMA (inactive form of NOS inhibitor)	300 μM	83.5 ± 7.0%	17.7 ± 10.7%	7	0.0002
PTIO (NO scavenger)	300 μM	91.9 ± 3.5%	102.4 ± 2.5%	6	0.037
indomethacin (prostaglandin inhibitor)	10 μM	96.9 ± 1.5%	98 ± 1.8%	6	0.62
the PCA rings pre-contracted with KCl	28.3 ± 1.6 mM	89.6 ± 3.5%	20.5 ± 4.6%	6	<0.0001
TEA (inhibitor of K_{Ca} channels)	10 mM	86.8 ± 5.4%	80.2 ± 9.7%	8	0.56
glibenclamide (inhibitor of K_{ATP})	1 μM	86.4 ± 7.7%	92.9 ± 7.6%	6	0.55
XE991 (KV7 channel antagonist)	10 μM	89.7 ± 2.8%	98.0 ± 2.5%	6	0.54
ODQ (guanylyl cyclase inhibitor)	10 μM	88.5 ± 3.4%	87.5 ± 2.8%	6	0.83
SQ22536 (AC inhibitor)	10 μM	91.5 ± 3.2%	99.8 ± 1.8%	6	0.05
attached PVAT		90.5 ± 9.0%	58.9 ± 7.5%	7	0.02
PVAT re-addition before KCl		113.2 ± 10.5%	107.6 ± 12.3%	6	0.73
PVAT re-addition after KCl		113.2 ± 10.5%	67.0 ± 6.9%	6	0.004

Note. Statistical significance was defined as *p* values less than 0.05 in *t*-test.

3.4 Discussion

The purpose of this experiment was to investigate the effects of different concentrations of HOCl producing different responses in the PCA pre-contracted by U46619 and investigate the mechanisms involved. Studying this question can contribute to unravelling the role of HOCl in diverse cardiovascular disorders by providing insights regarding how HOCl impacts vascular function. HOCl, a reactive oxidant produced by immune cells, plays an important role in the normal function of the vascular system by protecting against pathogens. Nevertheless, excessive HOCl production has been linked to diseases such as hypertension and atherosclerosis (Stocker et al., 2004). As a result, it would be helpful to understand how varying concentrations of HOCl affect vascular function for the development of potential therapeutic approaches and understanding the pathogenesis of diseases (Stocker et al., 2004).

3.4.1 HOCl Produced a Multiphasic Response in PCA

In U46619 pre-contracted PCA, different concentrations of HOCl produced differing responses. Rapid and transient relaxation, which returned to baseline followed by slow relaxation over 60 minutes, was produced using 100μM HOCl, while rapid and transient relaxations, contraction, and slow relaxation were observed with the higher concentration of HOCl of 500 μM.

The concentrations of 100 and 500 μM HOCl used in the present study are those used by Stocker et al. (2004). However, to the best of my knowledge, this is the first

study to compare the direct vasomotor responses mediated by different concentrations of HOCl in isolated PCA. Only a few studies have examined the direct effects of HOCl on blood vessels. In isolated perfused guinea pig hearts, HOCl (1 μM) has been shown to cause a reduction in coronary blood flow (Leipert et al., 1992). A concentration of 1 mM to 0.1 M HOCl in sheep pulmonary arteries exhibited contractions under resting force while mediating relaxation in pulmonary arteries pre-contracted with serotonin (Turan et al., 2000). Low concentrations of HOCl (100 nM to 100 μM) did not cause pulmonary arteries to contract, whereas high concentrations (1 mM to 0.1 M) caused contractions that were concentration-dependent. Conversely, when HOCl was added at a high concentration (1 M) to pulmonary arteries pre-contracted with 10 μM serotonin, vasodilation was observed (Turan et al., 2000).

3.4.2 Endothelium Removal Abolishes Rapid Relaxation

Endothelium removal abolished the transient relaxation and pronounced contraction in response to HOCl at both 100 and 500 μM, but slow relaxation was largely unaffected. The results indicate that smooth muscle is involved during slow relaxation, while contraction and rapid transient relaxation are entirely dependent on the endothelium. The slow relaxation observed at 100 μM HOCl was slightly greater in segments of artery without endothelium, possibly because the endothelium acts as a barrier that limits access of HOCl to the smooth muscle cells. There is evidence that the removal of the endothelium increases vasocontractile responses as a result of factors such as the removal of barrier function as well as an ongoing release of

endothelial NO which normally opposes contraction (Shipley & Muller-Delp, 2005; Turan et al., 2000). The endothelium could similarly act as a barrier to relaxation in the present study.

As in the current study, the slow vasodilator effect of various concentrations of HOCl (1 mM to 1 M) was observed in sheep endothelium-denuded pulmonary artery segments that had been pre-contracted with serotonin (Turan et al., 2000).

Other studies have shown that HOCl can alter endothelial function. In one, rat aortic segments were incubated in HOCl 50 μM for 60 min (Zhang et al., 2001). Next, phenylephrine was added as a contracting agent, and acetylcholine, which is an endothelium-dependent vasodilator, failed to produce relaxation. To confirm that relaxation inhibition is not due to muscle dysfunction, sodium nitroprusside, added as a NO donor, successfully produced relaxation (Zhang et al., 2001). This finding confirms that HOCl alters the function of endothelium, consequently affecting the release of endothelium-dependent vasodilators such as NO.

This finding is similar to those of other studies showing that bradykinin, and other endothelium-dependent vasodilators such as acetylcholine, fail to produce relaxation in arteries after pre-treatment with HOCl (Stocker et al., 2004; Weston et al., 2005). As an exogenous NO donor, diethylamine NONOate (DEANO) NO, and sodium nitroprusside was added to cause relaxation consistent with the relaxation inhibition of HOCl not caused by muscle dysfunction in segments of thoracic rabbit aorta (Zhang et al., 2001; Stocker et al., 2004). Moreover, HOCl can reduce NO bioavailability, an incubation of PCA with HOCl for four hours impairs bradykinin endothelial-dependent vasorelaxation primarily by inhibiting NO signalling because L-NAME, an inhibitor of

NOS, blocks its effect (Harper et al., 2023).

Regarding the effect of HOCl on the endothelium, HOCl at 25 µM can increase the permeability of cultured bovine aortic endothelial cells through the oxidation of sulfhydryl residues, increased intracellular zinc concentrations, and oxidised glutathione; as a result of zinc mobilisation, junctions undergo integrity loss, cell retractions, and shortened actin filaments of cytoskeletal in the endothelium (Tatsumi & Fliss, 1994).

A key finding of the present study is that HOCl evokes endothelium-dependent vasomotor responses of rapid relaxation followed by constriction in the PCA, which could be important in the inflammatory response following the release of MPO from neutrophils. The endothelium produces a number of vasorelaxant and contractile factors, including NO, EDH, ATP, prostaglandins, and endothelin (see Introduction). Thus, this study aimed to further characterise endothelium-dependent responses, starting with the possible involvement of NO.

3.4.3 NO Involvement

An inhibition of rapid relaxation in response to 100 µM HOCl was observed in association with L-NAME, a NOS inhibitor that blocks the conversion of L-arginine into L-citrulline and NO, which indicates the participation of NO in this reaction. The mechanism by which HOCl might stimulate the release and production of NO is unclear. Previous studies have shown that HOCl (10 and 50 µM, for 1 hour) does not alter endothelial NOS protein level or activity, even though the time after HOCl

exposure (1 hour) (Zhang et al., 2001) was much longer than the 1 minute in which the rapid relaxation occurred in the PCA. On the other hand, the rapid relaxation was replaced by a pronounced relaxation at 500 µM HOCl.

In the present study, L-NAME also blocked the contraction of 100 and 500 µM HOCl. This was consistent with the fact that the contraction of HOCl was endothelium-dependent. Functional antagonism is unlikely to have caused the attenuation of the contraction to HOCl by L-NAME (and the other NOS inhibitors discussed below) in the present study. L-NAME and other NOS inhibitors (such as L-NMMA) are known to increase responses to vasoconstrictors (Bone et al., 1997), and this was the case in the present study, as the concentration of U46619 used to pre-contract the coronary arteries was lower in arteries with L-NAME and L-NMMA than in those without. However, in these experiments, the U46619 pre-contracted tone of the coronary artery segments was carefully controlled, and there was no significant difference in tone based on the presence or absence of L-NAME and L-NMMA (see Results). In addition, functional antagonism would not explain the appearance of a pronounced relaxation of HOCl (500 µM) when contraction was inhibited.

Although L-NAME is typically used to inhibit NO production, it has recently been shown that NO can be released from the guanidino nitro group of L-NAME (Liu et al., 2019). This is the possible reason for the large relaxation observed in the presence of L-NAME and 500 µM HOCl. Importantly, another iNOS inhibitor, mercaptoethylguanidine, which has a similar structure to L-NAME, has been shown to scavenge HOCl. Therefore, it is possible that L-NAME also inhibits HOCl

vasomotor responses in the PCA by acting as a scavenger of HOCl (Whiteman et al., 1999). For that reason, this study used a structurally unrelated NOS inhibitor (L-NMMA) and the enantiomers D-NAME and D-NMMA to see if the effects are specific to L-NAME and whether HOCl (500 µM) is associated with the release of NO from L-NAME.

D-NAME is an inactive enantiomer of L-NAME (Liu et al., 2019), and in this study, 100 µM HOCl failed to evoke any response when added to arteries in the presence of D-NAME. Moreover, D-NAME blocked the initial relaxation and contraction of 500 µM HOCl; relaxation after 60 minutes was equal in the presence of D-NAME compared to the control group. D-NAME has been widely used as a negative control for L-NAME, but there are some studies which suggest that it too can inhibit NO production: Pechánová et al. (1999) and Babál et al. (2000) argued that D-NAME inhibits NOS in the aorta and heart and, as a result, elevates blood pressure, causes left ventricular hypertrophy and myocardial fibrosis, and increases DNA concentration in rats at the same plateau level but with significantly slower onset than L-NAME. In the present study, the pronounced inhibition of the HOCl responses with D-NAME, which was greater than that produced with L-NAME, makes actions through selective inhibition of eNOS seem unlikely.

L-NMMA (300 µM) abolished the rapid and prolonged relaxation observed at 100 µM HOCl. Moreover, L-NMMA significantly decreased the rapid relaxation observed in 500 µM HOCl; L-NMMA blocked contraction to 500 µM HOCl. In addition, the prolonged relaxation observed at 60 minutes was blocked in the presence of L-

NMMA. L-NMMA acts as an L-arginine analogue that inhibits NO synthesis, resulting in increased blood pressure, while D-NMMA, considered an inactive form of L-NMMA, does not inhibit NO synthesis in the endothelium (Whittle et al., 1989). As with D-NAME, the actions of D-NMMA were unexpected and suggest that these compounds are not acting through selective inhibition of eNOS under the conditions of the present study. It is possible that the NOS inhibitors chemically react with HOCl to neutralise/inactivate 100 µM HOCl and to release NO from 500 µM HOCl to produce an augmented relaxation response. Peterson et al. (1992) suggested that L-NAME and L-NMMA evoke their response through mechanisms other than NO synthase inhibition, for example by inhibition of electron transfer via iron centre, therefore affecting interaction with several enzymes. Thus, the effects of NOS inhibitors are not always due to blocking NOS. For example, L-NMMA can inhibit amiloride and dibutyryl cyclic AMP, which cause endothelium-independent vasodilatation (Peterson et al., 2003).

As a result of conflicting information about the mechanism of action of NO synthase inhibitors, other inhibitors were used, such as haemoglobin and PTIO.

A cell-free system has demonstrated that haemoglobin (Hb) inhibits the release of soluble guanylyl cyclase–stimulated NO and certain nitro vasodilators and inhibits endothelium-dependent relaxation (Martin et al., 1985; Chen et al., 1988). Haemoglobin blocks the acetylcholine sustained relaxation, but not the transient hyperpolarisation of the rat aorta and rat main pulmonary artery pre-contracted with noradrenaline (Chen et al., 1988). Maitra et al. (2011) claimed that HOCl reacts with

different forms of Hb through a different pathway, including the subsequent generation and degradation of oxoferryl intermediates. As a result of the reaction between HOCl and the heme moiety of Hb, the heme is degraded, and free iron is released, which likely explains why haemoglobin abolished the HOCl responses in the PCA.

PTIO, which acts as a NO scavenger, blocked the rapid relaxation, and pronounced contraction that was observed at 100 and 500 µM HOCl. This is in line with the endothelium-dependency of these components of the vasomotor response to HOCl and with a possible involvement of NO.

3.4.4 Prostaglandin Involvement

The prolonged relaxation response to both 100 and 500 µM HOCl was endothelium-independent. It is possible that this is due to the release of vasodilator/contractile prostanoids, likely from the smooth muscle (Sandoo et al., 2010). Prostaglandin involvement was examined using indomethacin (prostaglandin inhibitor). In the present study, indomethacin did not alter the HOCl responses.

Indomethacin is an NSAID that inhibits cyclooxygenase, which is essential for prostaglandin synthesis (Shacter et al., 1991). Wasil et al. (1987) and Shacter et al. (1991) stated that indomethacin and other NSAIDs inhibit HOCl formation through interfering with MPO activity, specifically the oxidising of Cl^-. Pekoe et al. (1982a) and Pekoe et al. (1982b) demonstrated that indomethacin acts on the intermediate oxidant of MPO as H_2O_2 and Cl^- rather than acting as a scavenger of HOCl. This

study confirms that finding, as adding indomethacin did not affect the HOCl response.

3.4.5 Hyperpolarisation Involvement

Endothelium releases different relaxation factors, such as NO, EDH, and prostacyclin. It is believed that hyperpolarisation occurs because of increased efflux of K^+ ions from VSMCs following the activation of K^+ channels. Upon hyperpolarisation, voltage-dependent Ca^{2+} channels close, thereby reducing Ca^{2+} entry into smooth muscle cells and resulting in vasorelaxation (Weintraub et al., 1994; Félétou & Vanhoutte, 2006). In the PCA, evidence suggests that K^+ channels are involved in EDHs (Nagao & Vanhoutte, 1992).

In the PCA, high K^+ (60 mM) produced sustained contractions. At a concentration of 10 nM, bradykinin did not induce relaxation while at a concentration of 100 nM, bradykinin-induced relaxation of the rings contracted with high K^+. Nitro-L-arginine (NOS inhibitor) abolishes relaxation of vessels pre-contracted with high K^+ but does not inhibit the relaxation of vessels pre-contracted with U46619 (Nagao & Vanhoutte, 1992). Thus, endothelium-dependent relaxations of the PCA are caused by two distinct mechanisms: NO production and hyperpolarisation of smooth muscle cells. Relaxation that is resistant to nitro-L-arginine is caused by membrane hyperpolarisation, while NO plays the main role in the relaxation of tissues contracted with high K^+ levels (Nagao & Vanhoutte, 1992). In smooth muscle cells, resting potential is normally retained as a result of a balance between K^+ ions moving into the cell and Na^+ ions leaving the cell. As a result, it is creating an

electrical gradient across the cell membrane. The addition of KCl increases the concentration of K^+ ions within a cell, reducing the electrical potential difference across the endothelium and smooth muscle cell membrane, resulting in voltage-gated Ca^{2+} channels in the cell membrane opening, allowing Ca^{2+} ions into the cell; this leads to inhibition of relaxation due to EDH caused by K^+ and thus to vasocontraction (Cowan & Cohen, 1991).

One way to block a hyperpolarisation factor and know whether it is involved in the HOCl relaxation response is by using KCl as pre-contractile agent instead of U46619. As a result of high K^+ levels in the PCA, hyperpolarisation of the smooth muscle is blocked (Nagao & Vanhoutte, 1992). Using this approach, it was found that rapid relaxation and slow relaxation over 60 minutes were attenuated in tissues pre-contracted with KCl in the presence of HOCl (100 and 500 µM). In the present study, It hypothesised that the relaxation of PCA rings produced by HOCl is mediated by a mechanism which is sensitive to membrane hyperpolarisation. This could involve NO, because NO has been shown to be a mediator of endothelium-dependent hyperpolarisation (Cohen et al., 1997) although there is also evidence that relaxation of PCA rings pre-contracted with U46619 proceeds through a mechanism that is unaffected by NOS inhibitors but is inhibited when pre-contracted with KCl, which opposes hyperpolarisation (Cowan & Cohen, 1991; Nagao & Vanhoutte, 1992; Weintraub et al., 1994).

In the current study, exposing the rings to elevated K^+ produced a tone equivalent to that produced by U46619 (as well as presumably inhibiting membrane

hyperpolarisation); since tone was well maintained, this is unlikely to have caused the inhibition of responses.

Smooth muscle membrane potential and vascular tone are regulated by changes in K^+ channel activity (Brayden, 1996). The involvement of K^+ in HOCl response was examined using different K^+ channel blockers, namely TEA (K_{Ca} channels blocker), glibenclamide (inhibitor of K_{ATP}), and XE991 (KV7 channel blocker). Surprisingly, none of these agents had an effect on HOCl response.

3.4.6 cAMP and cGMP Involvement

Multiple types and functions of VSMCs in the cardiovascular system are controlled by cyclic GMP. The process involves the production of NO in the endothelium through eNOS and subsequent transfer of NO to VSMCs in the blood vessels, where NO-sensitive guanylyl cyclase is activated in order to release cGMP. The concentration of cGMP is increased, which activates cGMP protein kinase type I, resulting in VSMC relaxation (Lehners et al., 2018). Further, adenyl cyclase catalyzes the conversion of ATP to cAMP, causing smooth muscle relaxation through the activation of different signalling pathways such as cAMP-Dependent Protein Kinase, which is responsible for phosphorylating MLCK. As a result, the affinity of calmodulin-Ca^{2+} complexes decrease, resulting in tissue relaxation (Murray, 1990). Neither ODQ (guanylyl cyclase inhibitor) nor SQ22536 (adenyl cyclase inhibitor) had any effect on the HOCl response. It is known that NO can act through cGMP-dependent pathways that catalyse the conversion of GTP into cyclic GMP. As a result of this, PKG and its downstream targets are activated (Ignarro,

1989). In this context, it would be expected that, if L-NAME influences HOCl responses, ODQ would also affect HOCl responses. However, there was a lack of inhibitory effect of ODQ on the HOCl responses. Furthermore, evidence indicates that NO can signal through pathways other than cGMP, specifically through protein S-nitrosylation, which influences the activity of a wide variety of metabolic enzymes and proteins (Romero-Aguirregomezcorta et al., 2014). The influence of NO on bovine oocyte meiosis has not been shown to be related to the cGMP/PKG pathway (Romero-Aguirregomezcorta et al., 2014). NO impacts vascular tone through multiple pathways, including the activation of sGC and downstream signalling cascades and the S-nitrosylation of various proteins involved in Ca^{2+} regulation and receptor function (Zhao et al., 2015).

3.4.7 Anti-contractile Effect of PVAT

PVAT is considered a potent therapeutic target because of its special position and capability to release a variety of vasoactive molecules. These molecules affect the tone of the vessels (Man et al., 2020). The main finding from the experiments in the present study is that PVAT exerts anti-contractile effects on HOCl response. PVAT elicits its anti-contractile effects via the following mechanisms: (1) through endothelium-dependent relaxation resulting in the production of perivascular adipose tissue-derived relaxation factor, which causes NO to be released and K^+ channels to be opened, leading to the relaxation of the endothelium; (2) PVAT releases H_2O_2 and induces sGC through an endothelium-independent mechanism (Gao et al., 2007; Man et al., 2020); and (3) adipokines released by PVAT, including leptin,

adiponectin, omentin, and chemerin, in addition to cytokines such as interleukins and TNF-α and gaseous molecules like H_2S and H_2O_2, play a role in the regulation of vascular function and pathophysiology (Mattu & Randeva, 2013).

It can be concluded from the data presented in this chapter that different concentrations of HOCl produce different responses in the vascular wall. The transient relaxation and pronounced contraction to HOCl at both 100 and 500 μM responses are mediated by both the endothelium and NO. Despite the fact that high concentrations of purine nucleotides exist intracellularly (Ralevic & Dunn, 2015), it is unclear whether they play a significant role in the vasomotor response produced by HOCl. The following chapter discusses purine receptor involvement in this response.

Chapter 4 Purine Involvement in HOCl Effects on Vascular Contractility of the Porcine Coronary Artery

4.1 Introduction

A defensive immune response to infections, accidents, toxic chemicals, or chronic diseases may be characterised as inflammation. If any of these factors affect tissue, the body triggers leukocyte chemotaxis through the circulatory system towards the site of inflammation, as well as an endogenous inflammatory response (Chen et al., 2017). There is a common mechanism that underlies inflammatory responses: 1) pattern receptors on cell surfaces detect inflammatory stimuli; 2) inflammation pathways are activated; 3) markers of inflammation are secreted; and 4) inflammatory cells are recruited (Ahmed, 2011; Chen et al., 2017) . Though nucleotides, such as ATP, ADP, and UTP, are primarily intracellular, they can be released into extracellular fluids through a variety of mechanisms. Firstly, ATP and other nucleotides can be derived from damaged or dead tissues or in response to inflammatory stimuli. This acts as a danger signal associated with an enhanced immune response. Additionally, they are secreted in response to a variety of stresses, including pathogen invasion, hypoxia, and mechanical stimulation (stretching, shear stress). Mechanisms of the release include vesicular transport, exocytosis, and membrane channels, including pannexins, connexins, and ATP-binding cassette (ABC) transporters (Burnstock & Boeynaems, 2014; Hechler & Gachet, 2015). Purinergic signalling maintains the physiological function of different organ systems under normal conditions in a highly regulated manner. A dysfunction in any element of the purinergic signalling pathway contribute to the pathogenesis of disease (Rai, 2022).

HOCl is a strong oxidant released in areas of inflammation by active neutrophils and associated immune cells. Excessive HOCl generation during chronic inflammation increases host tissue damage, which is closely associated with inflammatory diseases. It is known that high concentrations of purine nucleotides are present inside cells and that they can be released from cells during inflammation to act as find-me signals, but whether purine nucleotides participate in the vasomotor response of HOCl is unclear (Pullar et al., 2000; Zhang et al., 2001; Rayner et al., 2014; Ralevic & Dunn, 2015). When inflammation occurs, the generation and release of adenosine is also greatly enhanced (Haskó et al., 2008; Barletta et al., 2012). Again, it is unclear whether they are involved in the vasomotor response to HOCl. For this reason, this chapter investigated the involvement of purine nucleotides and nucleosides in the HOCl vasomotor response.

In the PCA, adenosine P1 and P2 receptors play a significant role in mediating relaxation and contraction. PCA smooth muscle cells and endothelial cells express A_2 adenosine receptors; the activation of these receptors results in a relaxing effect (King et al., 1990; Abebe et al., 1994; Rayment et al., 2007). There is evidence that adenosine release and A_{2A} receptor activation in smooth muscle are involved in the ADP-induced relaxation of smooth muscles (Ralevic & Dunn, 2015). There is evidence that the PCA expresses $P2Y_2$ receptors associated with smooth muscle contractions (Rayment et al., 2007).

Aim and objectives: The aim of this study was to further understand vascular inflammation. The objective was to investigate purine nucleotide involvement in the

response produced by HOCl, using P1, and P2 receptor antagonists, Ecto-ATPase Inhibitor, apyrase, and ATP assay.

4.2 Materials and Methods

The materials and methods were as described in chapter 2.

4.3 Results

4.3.1 P1 (Adenosine) Receptor Involvement

4.3.1.1 Effect of 8-(p-Sulphophenyl) theophylline (Adenosine Antagonist)

The effects of adenosine receptor antagonists on HOCl response were investigated. The presence of 8-SPT (100 µM) blocked the rapid relaxation response induced by 100 mM HOCl (72.6 ± 4.6%, *n* = 6), while the slow relaxation response observed under control conditions (94.5 ± 5.5%, *n* = 6) remained in the presence of 8-SPT (89 ± 6.3%, *n* = 6) (*p* = 0.53) (Figure 4-1A).

At a concentration of 500 µM HOCl, the transient rapid relaxation that was observed in PCA with HOCl (42.7 ± 4.3%, *n* = 6) was blocked when 8-SPT was added; the contraction (57.3 ± 5.9%, *n* = 6) was blocked too in the presence of 8-SPT (Figure 4-1B). The relaxation at 60 minutes with 8-SPT (89 ± 6.3%, *n* = 6) was comparable to that in the control (89.9 ± 4.4%, *n* = 6) (*p* = 0.91) (Figure 4-1B). Based on the actions of 8-SPT it was decided to use selective P1 receptor antagonists in order to identify the subtypes of P1 receptor involved in the response to HOCl.

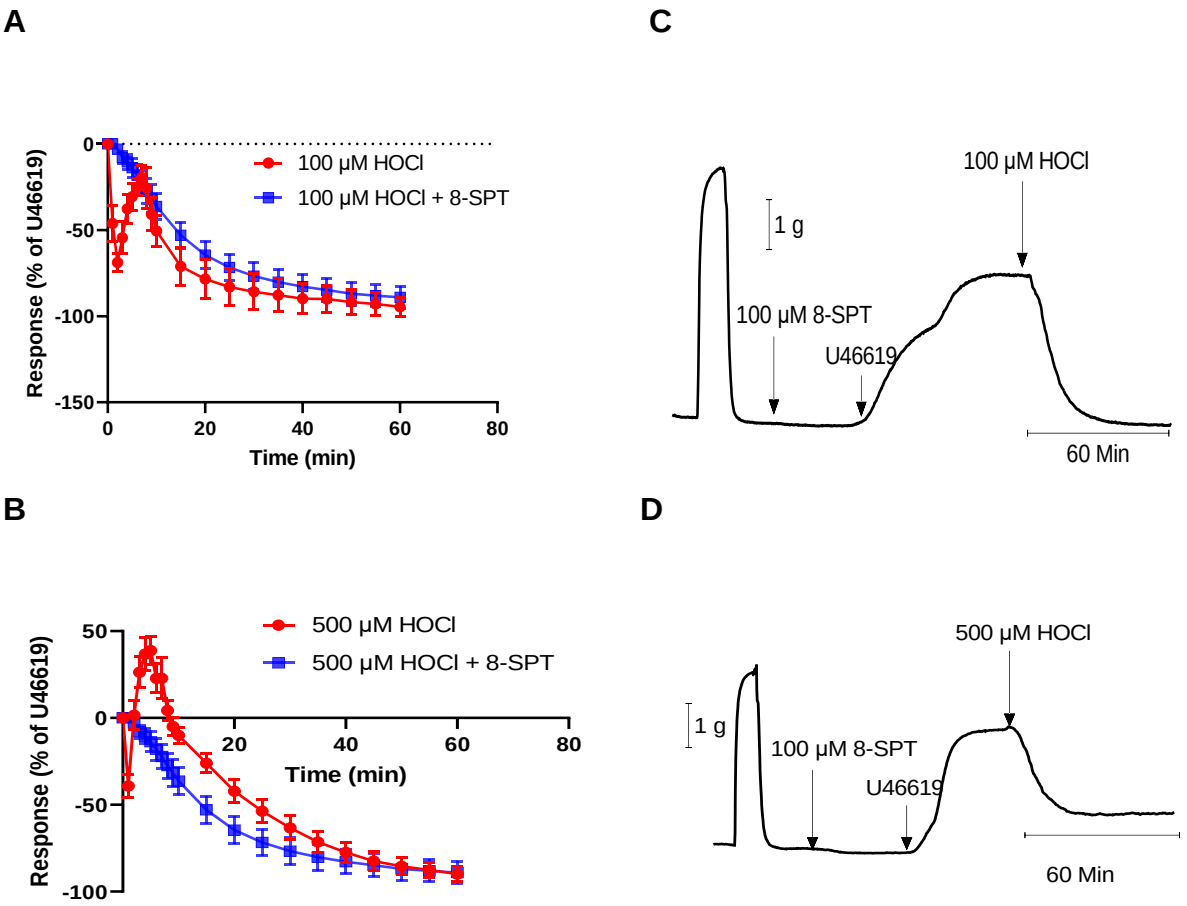


Figure 4-1. Porcine coronary artery responses produced by (A) 100 µM HOCl (*n* = 6) and (B) 500 µM HOCl (*n* = 6) in the absence or presence of 100 µM 8-SPT. (C–D) representative trace for: (C) 100 µM HOCl and (D) 500 µM HOCl in the presence of 8-SPT. Artery segments were pre-contracted with U46619.

4.3.1.2 Effect of ZM 241385 (A_{2A} Antagonist)

The transient relaxation effect of the PCA segments evoked by 100 µM HOCl was still pronounced in the presence and absence of ZM 241385 (1 µM) (56.6 ± 2.6%, *n* = 6) (64 ± 3.5%, *n* = 6) (*p* = 0.12) respectively. Additionally, the relaxation at 60 minutes (99.7 ± 2.8%, *n* = 6) was similar in the PCA with ZM 241385 compared to the control (102.5 ± 1.9%, *n* = 6) (*p* = .43) (Figure 4-2A).

At 500 µM HOCl, the transient relaxation, contraction, and slow relaxation in the

presence of ZM 241385 were 40.5 ± 5.4%, 59.9 ± 8.1% and 99.1 ± 1.3% (*n* = 6), respectively. Similar responses were produced in control arteries at 37.2 ± 5.1%, 41.9 ± 7.7% and 94.1 ± 3.4% (*n* = 6), respectively, without any significant change (*p* = 0.66, *p* =0.13 and *p* = 0.2) (Figure 4-2B).

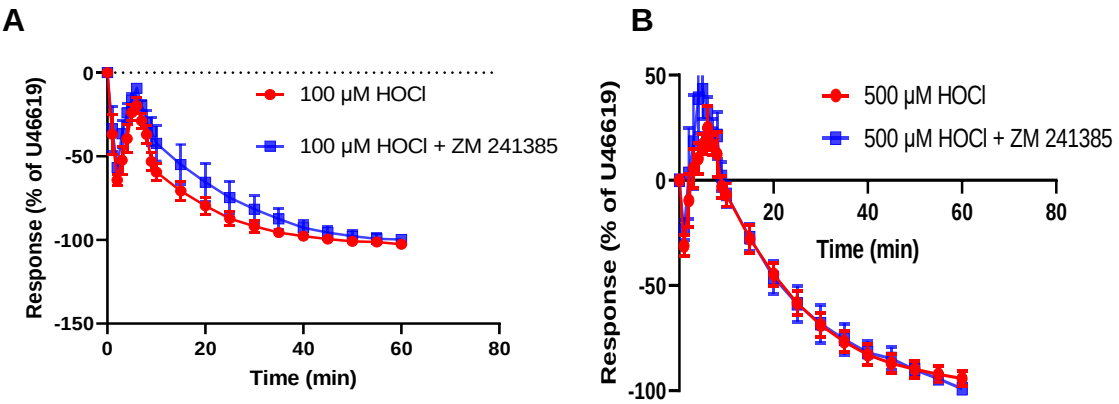


Figure 4-2. Porcine coronary artery responses produced by (A) 100 µM HOCl (*n* = 6) and (B) 500 µM HOCl (*n* = 6) in the absence or presence of 1 µM ZM 241358. Artery segments were pre-contracted with U46619.

4.3.1.3 Effect of DPCPX (A_1 Receptor Antagonist)

At 100 µM HOCl, 100 Nm DPCPX did not alter the response of HOCl; rapid relaxation was 56.8 ± 9.3% (*n* = 7) in the absence of DPCPX compared to 61.1 ± 6.3% (*n* = 7) in the presence of DPCPX (*p* = 0.7). Likewise, the later relaxation responses at 60 minutes in the absence or presence of DPCPX were similar at 100.4 ± 2.6% (*n* = 7) and 93 ± 2.5% (*n* = 7) (*p* = 0.06), respectively (Figure 4-3A).

With 500 µM HOCl, the rapid relaxation and subsequent contraction were still evident in the presence of DPCPX (28 ± 3.3%, *n* = 6, and 43.4 ± 14%, *n* = 6) compared to the control (29.6 ± 2.8%, *n* = 6, and 41 ± 11.5%, *n* = 6) (*p* = 0.71 and *p* = 0.89). In addition, the later relaxation response was similar in the control artery and

in the presence of DPCPX (respectively 81.8 ± 4%, *n* = 6; 85.7 ± 4%, *n* = 6) (*p* = 0.51) (Figure 4-3B).

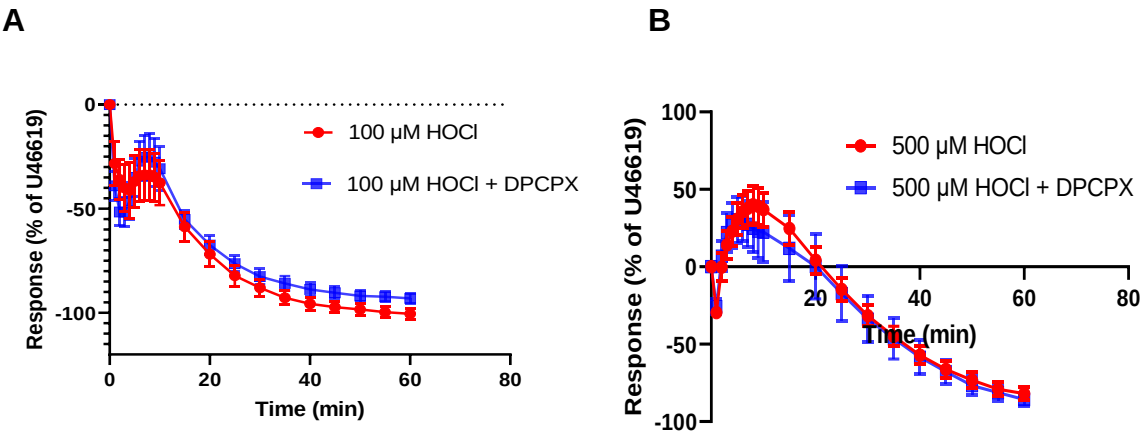


Figure 4-3. Porcine coronary artery responses produced by (A) 100 µM HOCl (*n* = 7) and (B) 500 µM HOCl (*n* = 6) in the absence or presence of 100 nM DPCPX. Artery segments were pre-contracted with U46619.

4.3.2 P2 (ATP, ADP, UTP, and UDP) Receptor Involvement

4.3.2.1 Effect of Suramin (Non-selective P2-Purinoceptor Antagonist)

Suramin (100 µM) blocked the rapid relaxation observed in response to 100 µM HOCl, and the slow relaxation response observed under control conditions (90.7 ± 6.3%, *n* = 6) was still present in the presence of suramin (102.6 ± 1.7%, *n* = 6) (*p* = 0.1) (Figure 4-4A).

In contrast, the rapid relaxation observed in response to 500 µM HOCl (50.5 ± 5.9%, *n* = 7) was evident in the presence of suramin (50.2 ± 8.2%, *n* = 7) at 40 seconds (*p* = 0.97). The contraction observed in control tissues in response to HOCl (64.3 ± 9.1%, *n* = 7) was blocked by suramin. The later relaxation response observed under control

conditions (79.9 ± 7.9%, *n* = 7) was still present in the presence of suramin (97.1 ± 4.4%, *n* = 7) (*p* = 0.08) (Figure 4-4B).

Since the actions of suramin suggest a possible involvement of P2 receptors in the response to HOCl, the next experiments used selective P2 receptor antagonists with an aim of identifying the P2 receptor subtypes involved.

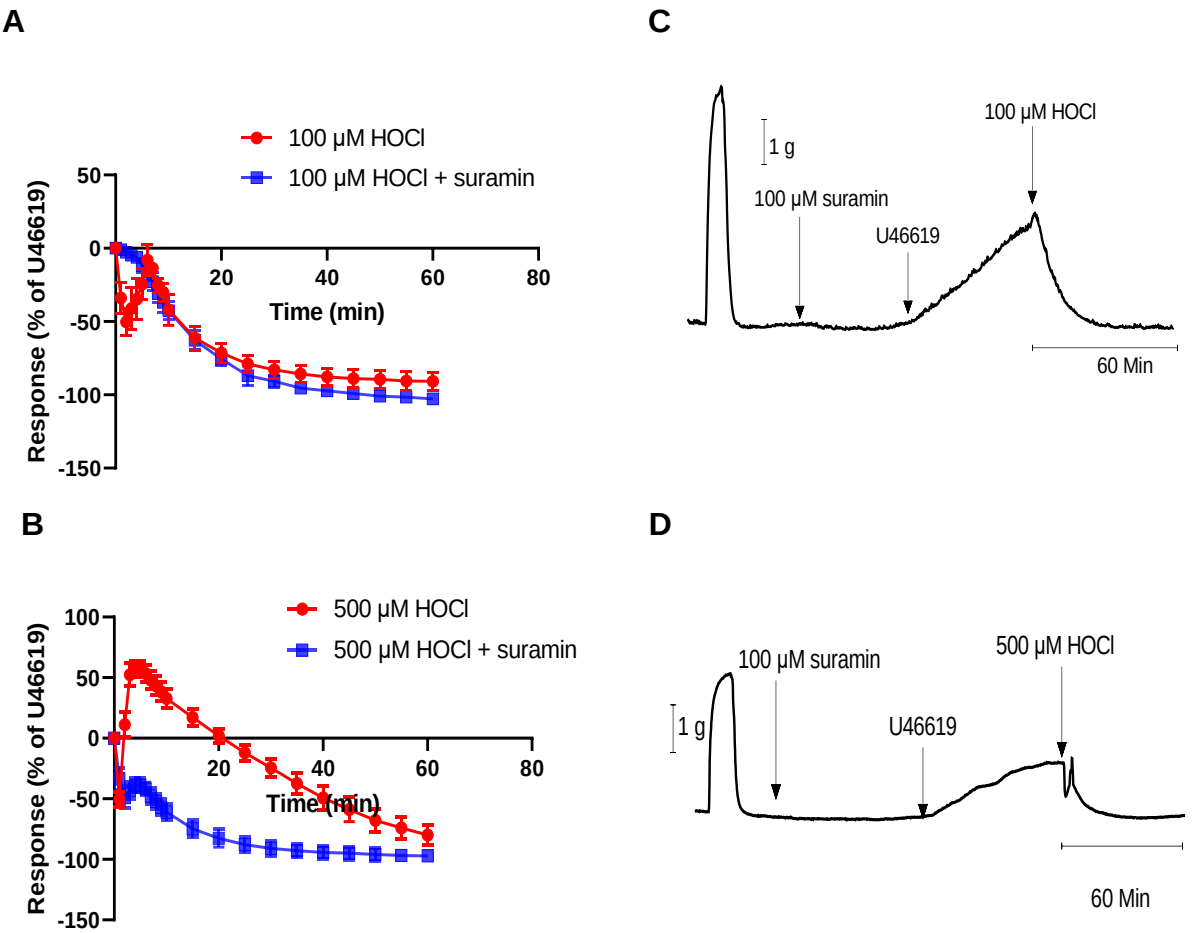


Figure 4-4. Porcine coronary artery responses are produced by (A) 100 µM HOCl (*n* = 6) and (B) 500 µM HOCl (*n* = 7) in the absence or presence of 100 µM suramin (*n* = 6). (C–D) representative trace for: (C) 100 µM HOCl and (D) 500 µM HOCl in the presence of suramin. Artery segments were pre-contracted with U46619.

4.3.2.2 Effect of PPADS (Non-selective P2-Purinoceptor Antagonist)

PPADS (10 μM) neither altered the transient relaxation nor slowed relaxation at 60 minutes of 100 μM HOCl (67.8 ± 4.6%, *n* = 7; 97.7 ± 1.2%, *n* = 7) compared to the control (67.6 ± 5%, *n* = 7; 97.9 ± 0.9%, *n* = 7) (*p* = 0.97; *p* = 0.93, respectively) (Figure 4-5A).

In addition, there was no difference between responses in the presence of PPADS and the control at 500 μM HOCl; in the presence of PPADS, the transient relaxation was 45.6 ± 5.7% (*n* = 6) and in the control was 49 ± 7.1% (*n* = 6) (*p* = 0.71). The subsequent contraction in the presence of PPADS was 56.3 ± 14.7% (*n* = 6) compared to that of the control, which was measured at 44.9 ± 12% (*n* = 6) (*p* = 0.56). The later slow relaxation in the presence and absence of PPADS was 81.9 ± 6.3% (*n* = 6) and 86.4 ± 3.5% (*n* = 6), respectively, without notable change (*p* = 0.54) (Figure 4-5B).

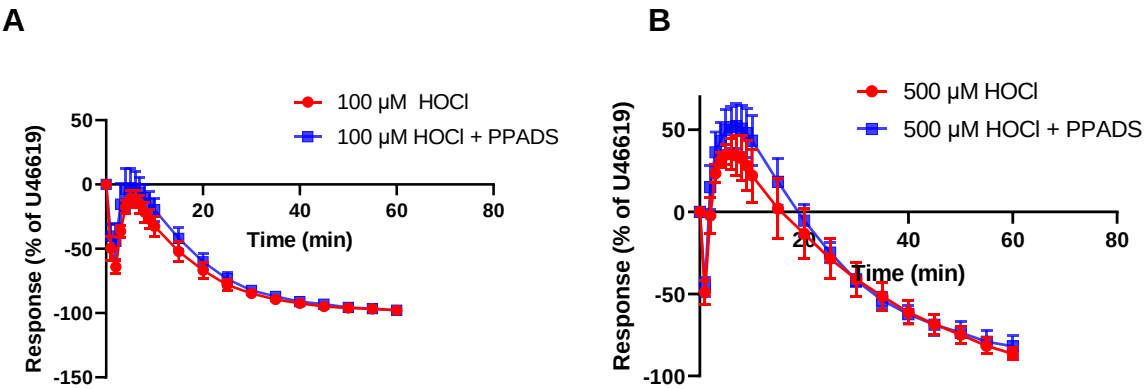


Figure 4-5. Porcine coronary artery responses produced by (A) 100 μM HOCl (*n* = 7) and (B) 500 μM HOCl (*n* = 6) in the absence or presence of 10 μM PPADS. Artery segments were pre-contracted with U46619.

4.3.2.3 Effect of MRS2179 (P2Y1 Receptor Antagonist)

The transient relaxation effect of the PCA segments evoked by 100 µM HOCl at 2 minutes was still pronounced in the presence and absence of MRS 2179 (10 µM) (respectively 75.3 ± 6.6%, *n* = 9; 80.1 ± 6.2%, *n* = 9) (*p* = 0.61). Additionally, the relaxation at 60 minutes (95.3 ± 2.5%, *n* = 9) was similar in the PCA with MRS 2179 compared to the control (103.36 ± 4.3%, *n* = 9) (*p* = 0.12) (Figure 4-6A).

At 500 µM HOCl, the transient relaxation, contraction, and slow relaxation in the presence of MRS 2179 were 58 ± 9.1%, 27.9 ± 13.2% and 88.1 ± 2.8% (*n* = 6) respectively. Similar responses were produced in control arteries: 61.5 ± 8.4%, 20.2 ± 6.4% and 88.8 ± 5% (*n* = 6), respectively, without significant change (*p* = 0.78, *p* = 0.61 and *p* = 0.9) (Figure 4-6B).

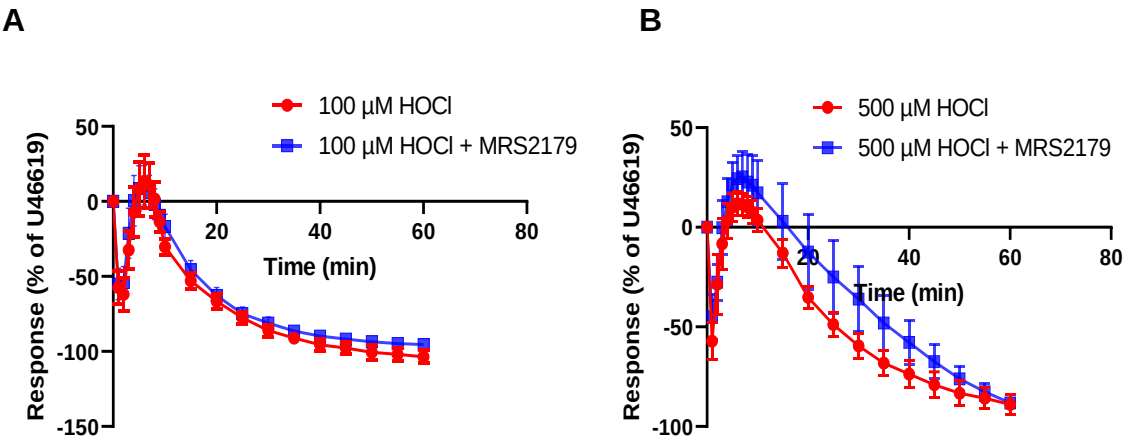


Figure 4-6. Porcine coronary artery responses produced by (A) 100 µM HOCl (*n* = 9) and (B) 500 µM HOCl in the absence or presence of 10 µM MRS 2179 (*n* = 6). Artery segments were pre-contracted with U46619.

4.3.2.4 Effect of Reactive Blue 2 (Non-selective P2Y Receptor Antagonist)

At 100 μM HOCl, 30 μM reactive blue 2 did not alter the response of HOCl; the pronounced relaxation was 74.5 ± 6.5% (*n* = 6) in the absence of reactive blue 2 compared to 66.4 ± 3.2% (*n* = 6) in its presence (*p* = 0.29). Likewise, the later relaxation response at 60 minutes in the absence or presence of reactive blue 2 were similar at 96.3 ± 1.3% (*n* = 6) and 98.9 ± 2.4% (*n* = 6) (*p* = 0.36), respectively (Figure 4-7A).

With 500 μM HOCl, the rapid relaxation and subsequent contraction were still observed in the presence of reactive blue 2 (45.8 ± 4.2%, *n* = 6, and 65.8 ± 3.8%, *n* = 6) compared to the control, which was 44.2 ± 5.5% (*n* = 6) and 58.9 ± 5.6% (*n* = 6) (*p* = 0.83 and *p* = 0.33, respectively). In addition, the later relaxation response was similar in the control artery and in the presence of reactive blue 2 (respectively 92.7 ± 1.5%, *n* = 6; 98 ± 2.5%, *n* = 6) (*p* = 0.12) (Figure 4-7B).

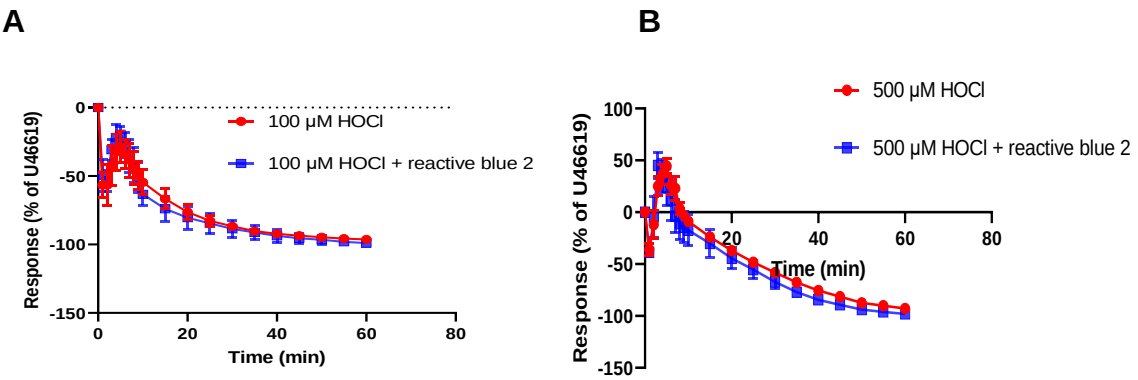


Figure 4-7. Porcine coronary artery responses produced by (A) 100 μM HOCl (*n* = 6) and (B) 500 μM HOCl (*n* = 6) in the absence or presence of 30 μM reactive blue 2. Artery segments were pre-contracted with U46619.

4.3.2.5 Effect of AR-C118925XX (P2Y2 Receptor Antagonist)

AR-C118925XX (10 μM) was dissolved in DMSO. Consequently, a loss of response was observed in both AR-C118925XX and control groups at 100 μM and 500 μM HOCl. (Figure 4-8A and B). Based on the results of the current experiment, It concluded that DMSO cannot be used as a solvent in experiments because it results in a loss of response to HOCl. Since many antagonists, such as those targeting the P2X7 receptor, use DMSO as a solvent, It was unable to use them.

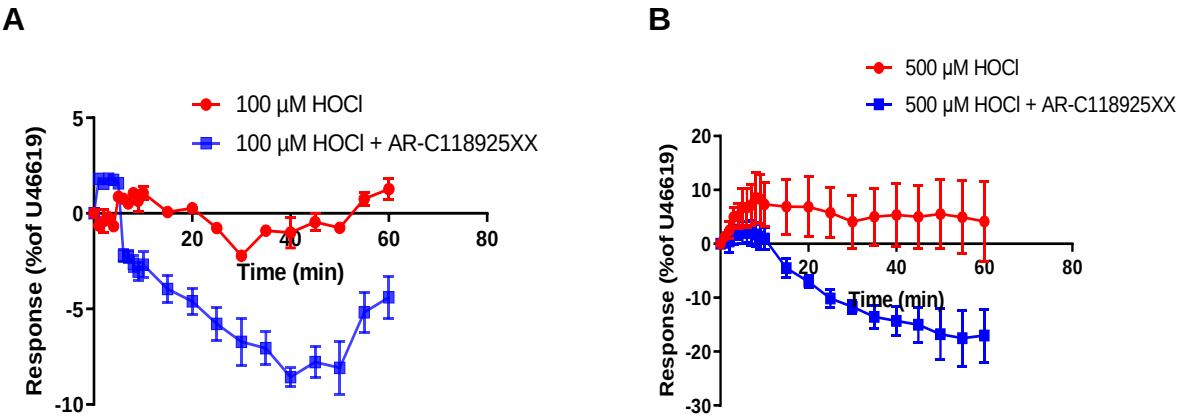


Figure 4-8. Porcine coronary artery responses produced by (A) 100 μM HOCl and (B) 500 μM HOCl in the absence or presence of 10 μM AR-C118925XX dissolved in DMSO (*n* = 4). Artery segments were pre-contracted with U46619.

4.3.2.6 Effect of NF449 and α,β-Methylene ATP (P2X1 Receptor Antagonist and P2X1 Receptor Desensitising Agent)

4.3.2.6.1 NF449

NF449 (10 μM) neither altered the transient relaxation nor slowed relaxation at 60 minutes of 100 μM (85 ± 3.7%, *n* = 6; 88.4 ± 5%, *n* = 6) compared to the control (84.5 ± 5.4%, *n* = 6; 97.1 ± 1%, *n* = 6) (*p* = 0.93, *p* = 0.12, respectively) (Figure 4-9A).

In addition, there was no difference between responses in the presence of NF449 and the control at 500 µM HOCl; in the presence of NF449, the transient relaxation was 63 ± 3.3% (*n* = 8) and in the control was 40.5 ± 7.2% (*n* = 8) (*p* = 0.05). The subsequent contraction in the presence of NF449 was 82.9 ± 15.1% (*n* = 8) compared to that of the control, which was 94.6 ± 9.1% (*n* = 8) (*p* = 0.49). The later relaxation in the presence and absence of NF449 was 91.6 ± 2.4% (*n* = 8) and 92.1 ± 5.1% (*n* = 8), respectively, without any change (*p* = 0.94) (Figure 4-9B).

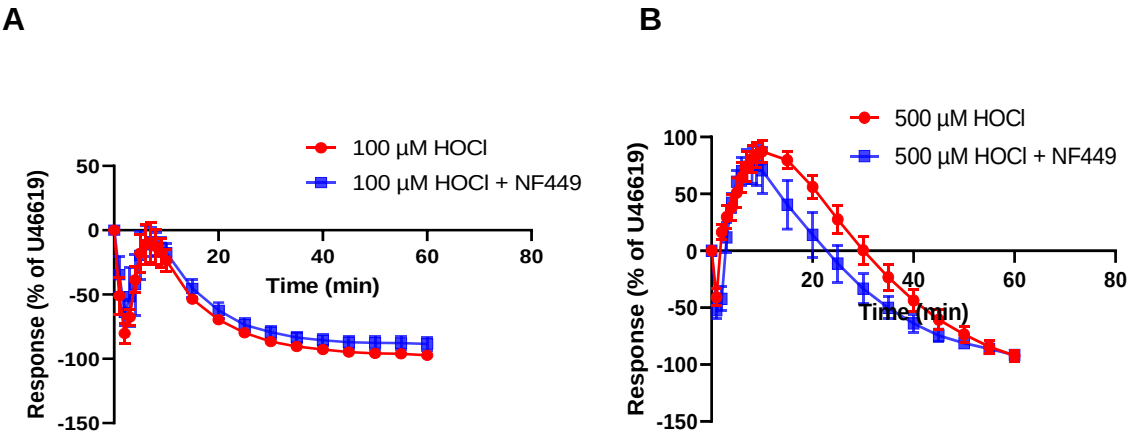


Figure 4-9. Responses of porcine coronary arteries treated with HOCl at different concentrations: (A) 100 µM (*n* = 6) and (B) 500 µM (*n* = 8) in the absence or presence of 10 µM NF449. Artery segments were pre-contracted with U46619.

4.3.2.6.2 α,β-Methylene ATP

At 100 µM HOCl, 10 µM α,β-methylene ATP did not alter the response in PCA; the pronounced relaxation was 64.2 ± 8.1% (*n* = 6) in the absence of α,β-methylene ATP compared to 67.5 ± 8% (*n* = 6) in the presence of α,β-methylene ATP (*p* = 0.77). Likewise, the later relaxation responses at 60 minutes in the absence or presence of α,β-methylene ATP were similar at 89.4 ± 5.6% (*n* = 6) and 91.5 ± 8.8% (*n* = 6) (*p* =

0.84), respectively (Figure 4-10A).

With 500 µM HOCl, the rapid relaxation and subsequent contraction were still evident in the presence of α,β-methylene ATP (59.6 ± 10.7%, *n* = 6, and 47.1 ± 11.5%, *n* = 6) compared to the control (56.4 ± 9.7%, *n* = 6, and 55.2 ± 13.7%, *n* = 6) (*p* = 0.82 and *p* = 0.65). In addition, the later relaxation response was similar in the control artery and in the presence of α,β-methylene ATP (respectively 79.7 ± 4.6%, *n* = 6; 89 ± 3.7%, *n* = 6) (*p* = 0.15) (Figure 4-10B).

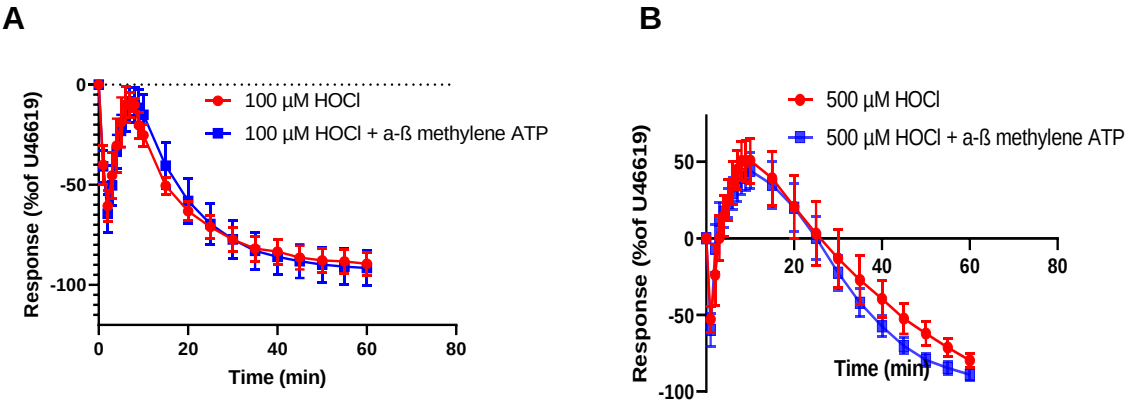


Figure 4-10. Porcine coronary artery responses produced by (A) 100 µM HOCl and (B) 500 µM HOCl in the absence or presence of 10 µM α,β-methylene ATP (*n* = 6). Artery segments were pre-contracted with U46619.

4.3.2.7 Effect of BX430 (P2X4 Receptor Antagonist)

BX430 (10 µM) failed to alter the rapid relaxation evoked by 100 µM HOCl. The rapid relaxation was 74.8 ± 6.5% (*n* = 6) in the absence of BX430 compared to 66.4 ± 3.2% (*n* = 6) in its presence (*p* = 0.29) (Figure 4-11A). Likewise, the later relaxation responses at 60 minutes in the absence or presence of BX430 were similar at 96.3 ± 3.1% (*n* = 6) and 98.9 ± 2.4% (*n* = 6) (*p* = 0.36), respectively (Figure 4-11A).

At 500 µM HOCl alone, the early relaxation and subsequent contraction were 44.2 ± 5.5% (*n* = 6) and 58.9 ± 5.6% (*n* = 6) respectively, which were not significantly different in the presence of BX430 (45.8 ± 4.2%, *n* = 6, and 64.2 ± 4%, *n* = 6, respectively; *p* = 0.83 and *p* = 0.44). Similar, the later relaxation after 60 minutes was not statistically significantly different in the presence of BX430 (98 ± 2.5%, *n* = 6) compared to the control group (89.7 ± 2.8%, *n* = 6) (*p* = 0.05) (Figure 4-11B).

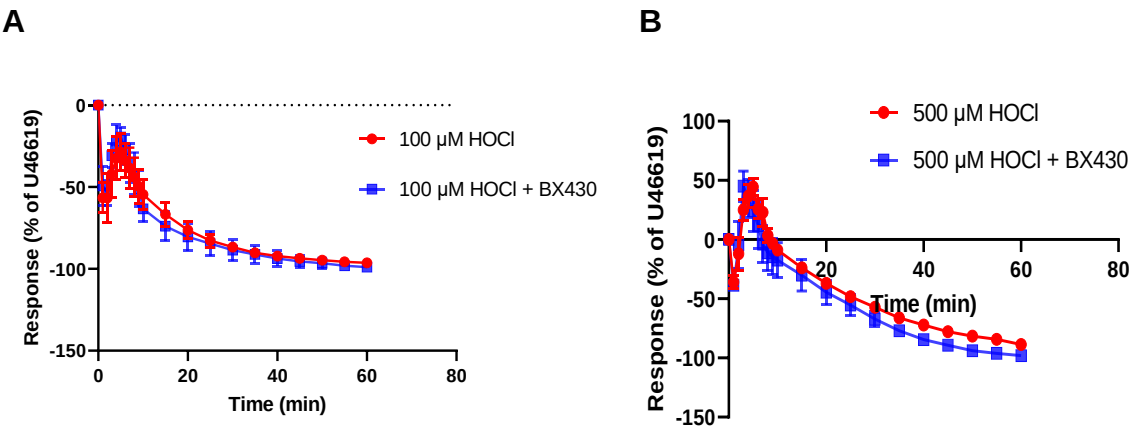


Figure 4-11. Porcine coronary artery responses produced by (A) 100 µM HOCl (*n* = 6) and (B) 500 µM HOCl (*n* = 6) in the absence or presence of 10 µM BX430. Artery segments were pre-contracted with U46619.

4.3.2.8 Effect of Probenecid (Pannexin 1 Channel Blocker)

Suramin can inhibit connexin and pannexin channels (Qiu & Dahl, 2008). For that reason, probenecid and carbenoxolone were used to investigate whether the effect of suramin involved connexin channels. Probenecid (1 mM) had no effect on the initial relaxation response to 100 µM HOCl (78.5 ± 2.1%, n = 7) compared to the control (69.7 ± 7.1%, n = 7) (p = 0.25). The later relaxation after 60 minutes was significantly increased in the presence of probenecid (105.8 ± 1.0%, n = 7) compared to the control group (94.7 ± 3.9%, n = 7) (p = 0.01) (Figure 4-12A).

In the presence of probenecid, the initial rapid relaxation to 500 µM HOCl were 47.1 ± 8.1% (*n* = 6) and in the control group were similar at 52.3 ± 10.8% (*n* = 6) (*p* = 0.7). The subsequent contraction to 500 µM HOCl were 29.8 ± 12.1% (*n* = 6) which was significantly reduced in the presence of probenecid 11.1 ± 13.1% (*n* = 6) (*p* = 0.04). The later slow relaxation after 60 minutes was significantly increased in the presence of probenecid (104.5 ± 1.7%, *n* = 6) compared to HOCl alone (82.1 ± 5.4%, *n* = 6) (*p* = 0.002) (Figure 4-12B).

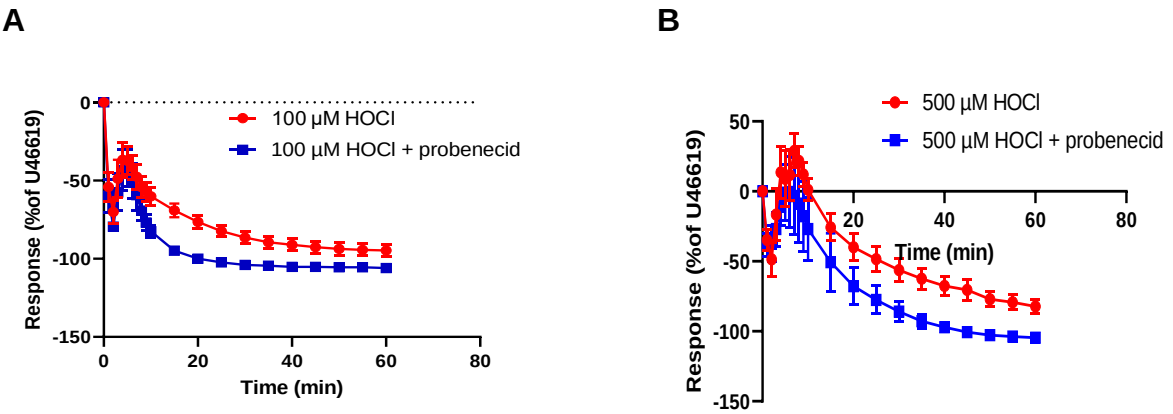


Figure 4-12. Porcine coronary artery responses produced by (A) 100 µM HOCl (*n* = 7) and (B) 500 µM HOCl (*n* = 6) in the absence or presence of 1 mM probenecid. Artery segments were pre-contracted with U46619.

4.3.2.9 Effect of Carbenoxolone (Pannexin 1 and Connexin Channels Blocker)

Carbenoxolone (100 µM) failed to block the rapid relaxation evoked by 100 µM HOCl, as is shown in Figure 4-13A. On the other hand, carbenoxolone failed to return vessels to baseline (50.2 ± 8.9%, *n* = 6) compared to controls (22.2 ± 7.7%, *n* = 6) (*p* = 0.03). Moreover, HOCl produced a larger relaxation at 60 minutes in the presence of carbenoxolone compared to HOCl alone (114.1 ± 2.1%, *n* = 6; 98.8 ±

1.6%, *n* = 6, respectively) (*p* = 0.0002) (Figure 4-13A).

At 500 µM HOCl alone, the early relaxation was 51.6 ± 5.2% (*n* = 6) in the control, which was not significantly different in the presence of carbenoxolone (41.2 ± 6.1%, *n* = 6) (*p* = 0.22). However, subsequent contraction was inhibited in the presence of carbenoxolone (1.7 ± 7.4%, *n* = 6) compared to the control (25.6 ± 5.5%, *n* = 6) (*p* = 0.03). The later relaxation after 60 minutes significantly increased in the presence of carbenoxolone (104.4 ± 1.3%, *n* = 6) compared to the control group (89.3 ± 2.3%, *n* = 6) (*p* = 0.0002) (Figure 4-13B).

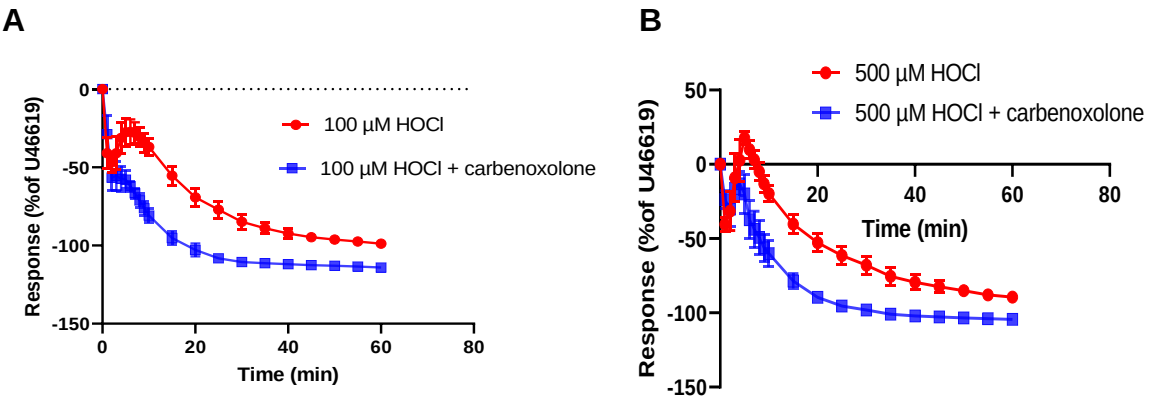


Figure 4-13. Porcine coronary artery responses produced by (A) 100 µM HOCl (*n* = 6) and (B) 500 µM HOCl (*n* = 6) in the absence or presence of 100 µM carbenoxolone. Artery segments were pre-contracted with U46619.

4.3.2.10 Effect of ARL67156 (Ecto-ATPase Inhibitor)

Extracellularly, ATP is rapidly hydrolysed by E-NTPDases to ADP and AMP, which are subsequently converted to adenosine. An effective inhibitor of ecto-ATPase, ARL67156 is widely used in tissue preparations to inhibit ATP hydrolysis (Durnin et al., 2016). In the present study, ARL67156 was used to determine whether ATP is

involved in any of the components of the HOCl response; if so, increased responses would be expected when ARL67156 is used to inhibit the hydrolysis of ATP.

ARL67156 (100 μM) did not alter transient or slow relaxation at 60 minutes of 100 μM HOCl. The transient relaxation in the control group was 41 ± 9.2% (*n* = 6), whereas that in the ARL67156 group was 46.8 ± 9.1% (*n* = 6) (*p* = 0.66). In the ARL 67156 group, slow relaxation was 91.5 ± 2% (*n* = 6) compared to 93.5 ± 1.6% (*n* = 6) in the control group (*p* = 0.48) (Figure 4-14A).

Moreover, there was no difference between responses in the presence and absence of ARL67156 at 500 μM HOCl; with ARL67156, the transient relaxation was 28 ± 3.3% (*n* = 6), and, without it, it was 29.6 ± 2.8% (*n* = 6) (*p* = 0.71). Regarding the subsequent contraction, this was 41 ± 11.5% (*n* = 6) in the control compared to 34.5 ± 15.2% (*n* = 6) in the presence of ARL67156 (*p* = 0.74). HOCl (500 μM) showed no difference in the later relaxations (*p* = 0.51) in the presence and absence of ARL67156 (*n* = 6) (Figure 4-14B).

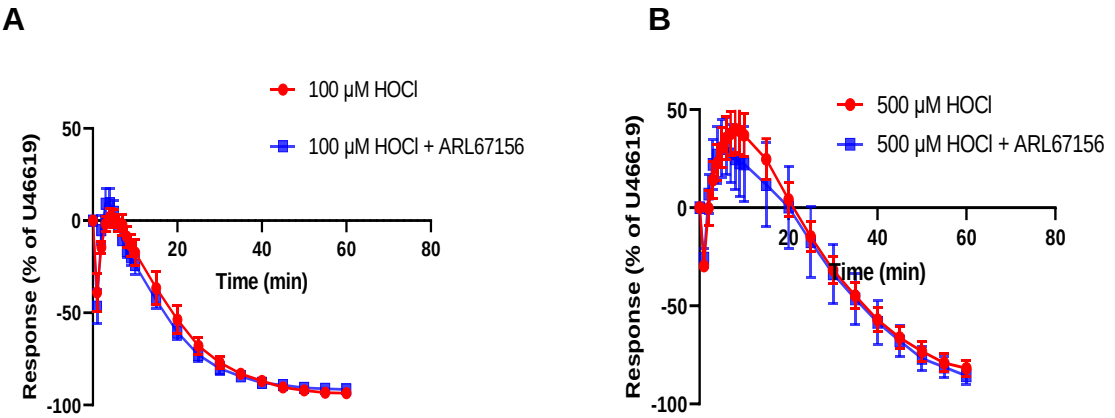


Figure 4-14. Porcine coronary artery responses produced by (A) 100 μM HOCl (*n* = 6) and (B) 500 μM HOCl (*n* = 6) in the absence or presence of 100 μM ARL67156. Artery segments were pre-contracted with U46619.

4.3.2.11 Apyrase Effect (Hydrolyses Nucleotides)

4.3.2.11.1 Effect of Apyrase (100 units/ml)

At 100 μM HOCl, 100 units/ml of apyrase completely blocked the HOCl response compared to the control. The rapid relaxation and slow relaxation in the PCA control group were 58.7 ± 5% and 104.5 ± 9.7%, respectively (n = 6), compared to the apyrase group at 4.6 ± 1.3% ($p < 0.0001$) and 10.8 ± 3.4% ($p < 0.0001$) (n = 6) (Figure 4-15A).

Apyrase abolished the rapid relaxation observed at 500 μM HOCl (0.7 ± 0.5%, n = 6) compared to the control group (48.4 ± 5.1%, n = 6) ($p < 0.0001$). Moreover, apyrase blocked the contraction produced by 500 μM HOCl (24.9 ± 4.6%, n = 6) compared to present of apyrase (10.7 ± 2.8%, n = 6) ($p = 0.02$). In addition, the later relaxation at 60 minutes to HOCl (96.5 ± 6.2%, n = 6) was eliminated in the presence of apyrase (7 ± 9.6%, n = 6) ($p < 0.0001$) (Figure 4-15B). The concentration of U46619 used to pre-contract the PCA was significantly lower in arteries with apyrase, which has been shown to enhance responses to vasoconstrictors (Table 3-1).

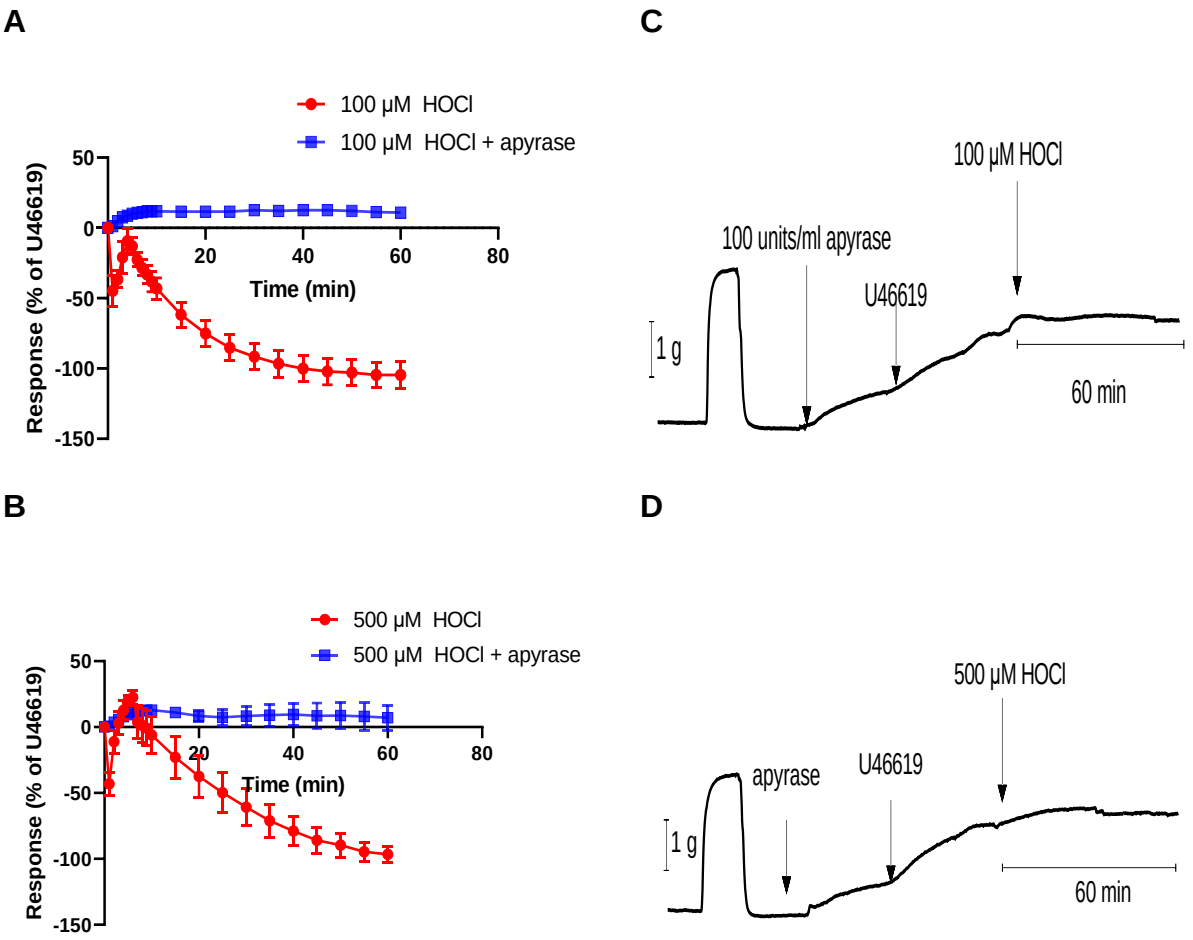


Figure 4-15. Responses of porcine coronary arteries that were treated with HOCl at (A) 100 µM (*n* = 6) and (B) 500 µM (*n* = 6) in the absence or presence of 100 units/ml apyrase. (C–D) Representative trace for: (C) 100 µM HOCl and (D) 500 µM HOCl in the presence of apyrase. Artery segments were pre-contracted with U46619.

4.3.2.11.2 Effect of Lower Concentration of Apyrase (10 units/ml)

Apyrase (10 units/ml) did not alter the response of the initial relaxation response to 100 µM HOCl (51.9 ± 6.6%, *n* = 6) compared to the control (51.5 ± 7.1%, *n* = 6) (*p* = 0.96). The later relaxation after 60 minutes did not change in presence of apyrase (101.2 ± 4.8%, *n* = 6) compared to the control group (93.1 ± 2.7%, *n* = 6) (*p* = 0.17) (Figure 4-16A).

In the presence of apyrase, the initial pronounced rapid relaxation and subsequent contraction to 500 µM HOCl (47.2 ± 4%, *n* = 6, and 50.6 ± 11.7%, *n* = 6) were similar to those observed in the control (respectively 44.7 ± 6.6%, *n* = 6, and 30.7 ± 6.8%, *n* = 6), (*p* = 0.75 and *p* = 0.17). The later relaxation after 60 minutes was not significantly changed in the presence of apyrase (98.9 ± 3.2%, *n* = 6) compared to HOCl alone (94.5 ± 6.8%, *n* = 6) (*p* = 0.57) (Figure 4-16B).

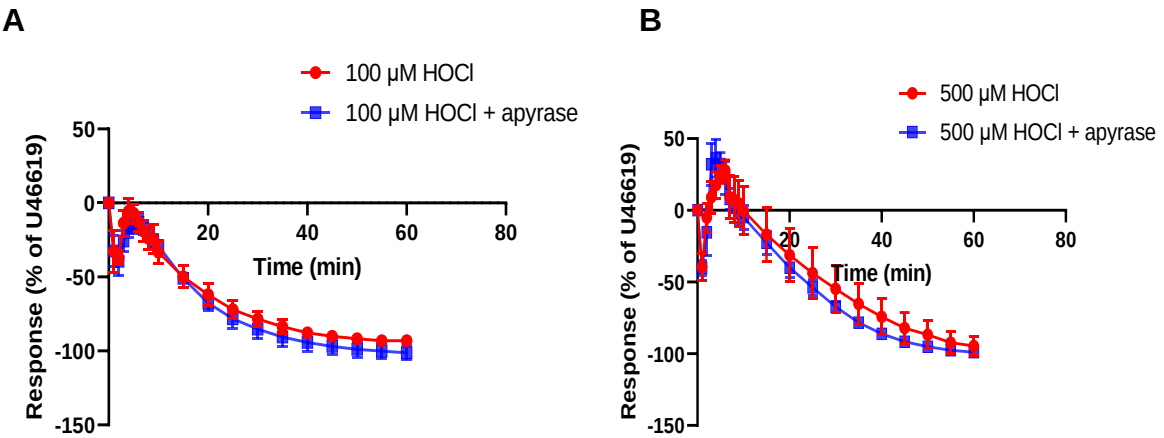
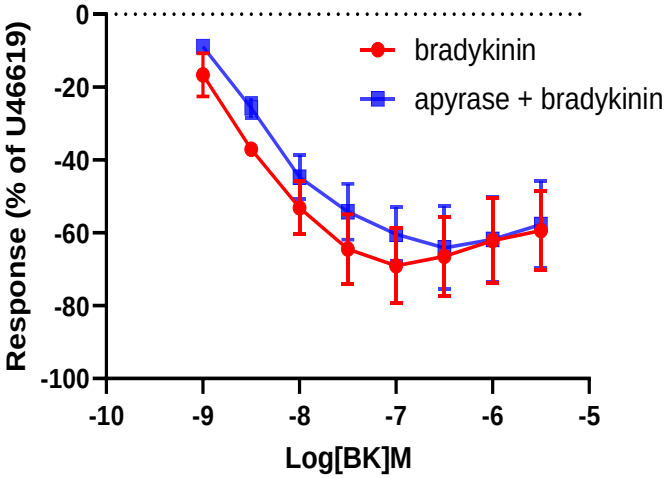


Figure 4-16. Responses of porcine coronary arteries treated with HOCl at (A) 100 µM (*n* = 6) and (B) 500 µM (*n* = 6) in the absence or presence of 10 units/ml apyrase.

4.3.2.11.3 Effect of Apyrase on Bradykinin Response

To confirm that inhibition of HOCl response by apyrase is not due to a non-specific inhibition of vascular smooth muscle relaxation, apyrase was tested against bradykinin, which is an endothelium-dependent relaxing agent in the PCA (Cherry et al., 1982; Weintraub et al., 1994). Concentration-dependent relaxations were produced by bradykinin (1 nM–3 µM) in PCA pre-contracted with U46619, with a pEC_{50} of 8.5 ± 0.4 and R_{max} = 64.5 ± 4.5% (*n* = 6) which was not changed in arteries with added apyrase, with a pEC_{50} of 8.4 ± 0.5 and R_{max} = 61.3 ± 4.8% (*n* = 6) (Figure



4-17)

Figure 4-17. Responses of porcine coronary arteries that were treated with bradykinin in the absence or presence of 100 units/ml apyrase (*n* = 6).

4.3.2.12 HOCl Releases ATP From Cultured Human Coronary Artery Endothelial Cells

To investigate the effect of HOCl treatment on ATP production, an ATP assay kit was used(Abcam, Cat#ab113849) to measure ATP levels in the samples.

The results show that the mean ATP level in the supernatant of cells with added 100 µM HOCl at 1 min was 0.18 ± 0.07 nmol, which was significantly higher than the mean ATP level in the control group (0.12 ± 0.06 nmol) (*n* = 9) (*p* = 0.02). These findings suggest that HOCl has a positive effect on ATP production at 1 minute, which is consistent with the time of maximum rapid relaxation (Figure 4-18).

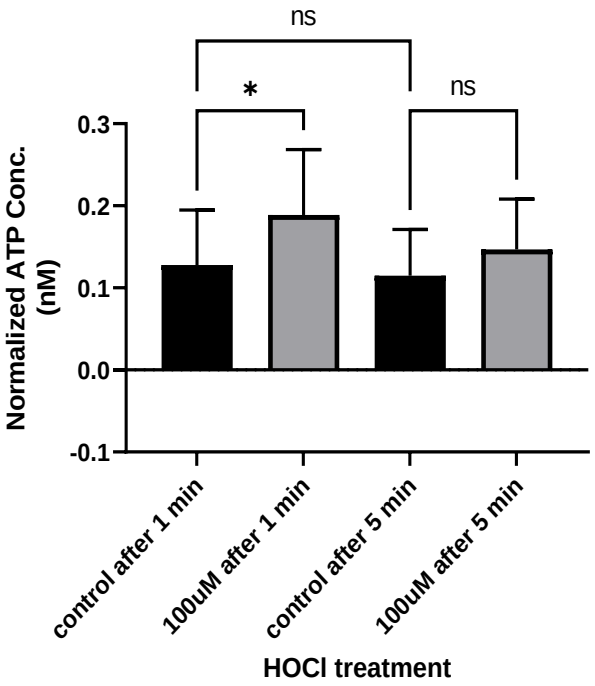


Figure 4-18. ATP release from cultured human coronary artery endothelial cells (HCAECs) by 100 µM HOCl at 1 and 5 minutes (n = 9). *p < 0.05 (two-way ANOVA).

Table 4-1. Effect of Various Inhibitors on Rapid Relaxation of 100 μM HOCl

Antagonist	**Concentration**	**Control**	**Treatment**	***n***	***p***
8-SPT (adenosine receptor antagonist)	100 μM	72.6 ± 4.6%	2.9 ± 1.2%	6	<0.0001
ZM 241385(A_{2A} receptor antagonist)	1 μM	64.1 ± 3.5%	56.6 ± 2.6%	6	0.12
DPCPX (A_1 receptor antagonist)	100 nM	56.8 ± 9.3%	61.1 ± 6.3%	7	0.7
suramin (non-selective P2 receptor antagonist)	100 μM	57.7 ± 10.0%	2.8 ± 1.8%	6	0.0003
PPADS (non-selective P2 receptor antagonist)	10 μM	76.6 ± 5.0%	67.8 ± 4.6%	7	0.97
MRS2179 ($P2Y_1$ receptor antagonist)	10 μM	80.1 ±6.2%	75.3 ±6.6%	9	0.61
reactive blue 2(P2Y receptor antagonist)	30 μM	74.5 ± 6.5%	66.4 ± 3.2%	6	0.29
NF449 (P2X1 receptor antagonist)	10 μM	84.5 ± 5.4%	85.0 ± 3.7%	6	0.93
α,β-methylene ATP (P2X1 receptor desensitising agent)	10 μM	64.2 ± 8.1%	67.5 ± 8.0%	6	0.77
BX430 (P2X4 receptor antagonist)	10 μM	74.8 ± 6.5%	66.4 ± 3.2%	6	0.29
probenecid (pannexin 1 channel blocker)	1 mM	69.7 ± 7.1%	78.5 ± 2.1%	7	0.25
carbenoxolone (pannexin 1 and connexin channels blocker)	100 μM	60.6 ± 3.6%	62.1 ± 9.6%	6	0.88
ARL67156 (ecto-ATPase inhibitor)	100 μM	41.0 ± 9.2%	46.8 ± 9.1%	6	0.66
apyrase (hydrolyses nucleotides)	100 units/ml	58.7 ± 5.0%	4.6 ± 1.3%	6	<0.0001
apyrase (hydrolyses nucleotides)	10 units/ml	51.5 ± 7.1%	51.9 ± 6.6%	6	0.96

Note. Statistical significance was defined as *p* values of less than 0.05 (using *t*-test).

Table 4-2. Effect of Various Inhibitors on Slow Relaxation of 100 μM HOCl

Antagonist	**Concentration**	**Control**	**Treatment**	***n***	***p***
8-SPT (adenosine receptor antagonist)	100 μM	94.5 ± 5.5%	89.0 ± 6.3%	6	0.53
ZM 241385(A_{2A} receptor antagonist)	1 μM	102.5 ± 1.9%	99.7 ± 2.8%	6	0.43
DPCPX (A_1 receptor antagonist)	100 nM	100.4 ± 2.6%	93.0 ± 2.5%	7	0.06
suramin (non-selective P2 receptor antagonist)	100 μM	90.7 ± 6.3%	102.6 ± 1.7%	6	0.1
PPADS (non-selective P2 receptor antagonist)	10 μM	97.9 ± 0.9%	97.7 ± 1.2%	7	0.93
MRS2179 ($P2Y_1$ receptor antagonist)	10 μM	103.36 ± 4.3%	95.3 ± 2.5 %	9	0.12
reactive blue 2(P2Y receptor antagonist)	30 μM	96.3 ± 1.3%	98.9 ± 2.4%	6	0.36
NF449 (P2X1 receptor antagonist)	10 μM	97.1 ± 1.0%	88.4 ± 5.0%	6	0.12
α,β-methylene ATP (P2X1 receptor desensitising agent)	10 μM	89.4 ± 5.6%	91.5 ± 8.8%	6	0.84
BX430 (P2X4 receptor antagonist)	10 μM	96.3 ± 3.1%	98.9 ± 2.4%	6	0.36
probenecid (pannexin 1 channel blocker)	1 mM	94.7 ± 3.9%	105.8 ± 1.0%	7	0.01
carbenoxolone (pannexin 1 and connexin channels blocker)	100 μM	98.8 ± 1.6%	114.1 ± 2.1%	6	0.0002
ARL67156 (ecto-ATPase inhibitor)	100 μM	93.5 ± 1.6%	91.5 ± 2.0%	6	0.48
apyrase (hydrolyses nucleotides)	100 units/ml	104.5 ± 9.7%	10.8 ± 3.4%	6	<0.0001
apyrase (hydrolyses nucleotides)	10 units/ml	93.1 ± 2.7%	101.2 ± 4.8%	6	0.17

Note. Statistical significance was defined as *p* values of less than 0.05 (using *t*-test).

Table 4-3. Effect of Various Inhibitors on Rapid Relaxation of 500 μM HOCl

Antagonist	**Concentration**	**Control**	**Treatment**	***n***	***p***
8-SPT (adenosine receptor antagonist)	100 μM	42.7 ± 4.6%	Blocked	6	<0.0001
ZM 241385(A_{2A} receptor antagonist)	1 μM	37.2 ± 5.1%	40.5 ± 5.4%	6	0.66
DPCPX (A_1 receptor antagonist)	100 nM	29.6 ± 2.8%	28.0 ± 3.3%	6	0.71
suramin (non-selective P2 receptor antagonist)	100 μM	50.5 ± 5.9%	50.2 ± 8.2%	7	0.97
PPADS (non-selective P2 receptor antagonist)	10 μM	49.0 ± 7.1%	45.6 ± 5.7%	6	0.71
MRS2179 ($P2Y_1$ receptor antagonist)	10 μM	61.5 ± 8.4%	58.0 ± 9.1%	6	0.78
reactive blue 2(P2Y receptor antagonist)	30 μM	44.2 ± 5.5%	45.8± 4.2%	6	0.83
NF449 (P2X1 receptor antagonist)	10 μM	40.5 ± 7.2%	63.0 ± 3.3%	8	0.057
α,β-methylene ATP (P2X1 receptor desensitising agent)	10 μM	56.4 ± 9.7%	59.6 ± 10.7%	6	0.82
BX430 (P2X4 receptor antagonist)	10 μM	44.2 ± 5.5%	45.8 ± 4.2%	6	0.83
probenecid (pannexin 1 channel blocker)	1 mM	52.4 ± 10.8%	47.1 ± 8.1%	6	0.7
carbenoxolone (pannexin 1 and connexin channels blocker)	100 μM	51.6 ± 5.2%	41.2 ± 6.1%	6	0.22
ARL67156 (ecto-ATPase inhibitor)	100 μM	29.6 ± 2.8%	28.0 ± 3.3%	6	0.71
apyrase (hydrolyses nucleotides)	100 units/ml	48.4 ± 5.1%	Blocked	6	<0.0001
apyrase (hydrolyses nucleotides)	10 units/ml	44.7 ± 6.6%	47.2 ±4.0%	6	0.75

Note. Statistical significance was defined as *p* values of less than 0.05 (using *t*-test).

Table 4-4. Effect of Various Inhibitors on Contraction of 500 μM HOCl

Antagonist	Concentration	Control	Treatment	*n*	*p*
8-SPT (adenosine receptor antagonist)	100 μM	57.3 ± 5.9%	Blocked	6	<0.0001
ZM 241385(A_{2A} receptor antagonist)	1 μM	41.9 ± 7.7%	59.9 ± 8.1%	6	0.13
DPCPX (A_1 receptor antagonist)	100 nM	41.0 ± 11.5%	43.4 ± 14.0%	6	0.89
suramin (non-selective P2 receptor antagonist)	100 μM	64.3 ± 9.1%	Blocked	7	<0.0001
PPADS (non-selective P2 receptor antagonist)	10 μM	44.9 ± 12.0%	56.3 ± 14.7%	6	0.56
MRS2179 ($P2Y_1$ receptor antagonist)	10 μM	20.2 ± 6.4%	27.9 ± 13.2%	6	0.61
reactive blue 2(P2Y receptor antagonist)	30 μM	58.9 ± 5.6%	65.8± 3.8%	6	0.33
NF449 (P2X1 receptor antagonist)	10 μM	94.6 ± 9.1%	82.9 ± 15.1%	8	0.49
α,β-methylene ATP (P2X1 receptor desensitising agent)	10 μM	55.2 ± 13.7%	47.1 ± 11.5%	6	0.65
BX430 (P2X4 receptor antagonist)	10 μM	58.9 ± 5.6%	11.1 ± 13.1%	6	0.044
probenecid (pannexin 1 channel blocker)	1 mM	29.8 ± 12.1%	47.1 ±19.1%	6	0.04
carbenoxolone (pannexin 1 and connexin channels blocker)	100 μM	25.6 ± 5.5%	1.7 ± 7.4%	6	0.03
ARL67156 (ecto-ATPase inhibitor)	100 μM	41.0 ± 11.5 %	34.5 ± 15.2%	6	0.74
apyrase (hydrolyses nucleotides)	100 units/ml	24.9 ± 4.6%	10.7 ± 2.8%	6	0.02
apyrase (hydrolyses nucleotides)	10 units/ml	30.7 ± 6.8%	50.6 ± 11.7%	6	0.17

Note. Statistical significance was defined as *p* values of less than 0.05 (using *t*-test).

Table 4-5. Effect of Various Inhibitors on Slow Relaxation of 500 μM HOCl

Antagonist	**Concentration**	**Control**	**Treatment**	***n***	***p***
8-SPT (adenosine receptor antagonist)	100 μM	89.9 ± 4.4%	89.0 ± 6.3%	6	0.91
ZM 241385(A_{2A} receptor antagonist)	1 μM	94.1 ± 3.4%	99.1 ± 1.3%	6	0.2
DPCPX (A_1 receptor antagonist)	100 nM	81.8 ± 4.0%	85.7 ± 4.0%	6	0.51
suramin (non-selective P2 receptor antagonist)	100 μM	79.9 ± 7.9%	97.1 ± 4.4%	7	0.08
PPADS (non-selective P2 receptor antagonist)	10 μM	86.4 ± 3.5%	81.9 ± 6.3%	6	0.54
MRS2179 ($P2Y_1$ receptor antagonist)	10 μM	88.8 ± 5.0%	88.1 ± 2.8%	6	0.90
reactive blue 2(P2Y receptor antagonist)	30 μM	92.7 ± 1.5%	98.0 ± 2.5%	6	0.12
NF449 (P2X1 receptor antagonist)	10 μM	92.1 ± 5.1%	91.6 ± 2.4%	8	0.94
α,β-methylene ATP (P2X1 receptor desensitising agent)	10 μM	79.7 ± 4.6%	89.0 ± 3.7%	6	0.15
BX430 (P2X4 receptor antagonist)	10 μM	89.7 ± 2.8%	98.0 ± 2.5%	6	0.05
probenecid (pannexin 1 channel blocker)	1 mM	82.1 ± 5.4%	104.5 ± 1.7%	6	0.002
carbenoxolone (pannexin 1 and connexin channels blocker)	100 μM	89.3 ± 2.3%	104.4 ± 1.3	6	0.002
ARL67156 (ecto-ATPase inhibitor)	100 μM	81.8 ± 4.0%	85.7 ± 4.0%	6	0.51
apyrase (hydrolyses nucleotides)	100 units/ml	96.5 ± 6.2%	Blocked	6	< 0.0001
apyrase (hydrolyses nucleotides)	10 units/ml	94.5 ± 6.8%	98.9 ± 3.2%	6	0.57

Note. Statistical significance was defined as *p* values of less than 0.05 (using *t*-test).

4.4 Discussion

As a result of inflammation, high levels of nucleotides and nucleosides are released into the extracellular space (Burnstock & Ralevic, 2014). Through mechanisms that include connexin and pannexin channels, ATP can be secreted by endothelial cells and smooth muscle cells via mechanical stimuli such as shear stress (Dahl, 2015; Mikolajewicz et al., 2018). The main finding from this chapter is that the use of 8-SPT suggests the participation of adenosine (P1) receptors in the vasomotor response to HOCl, while the use of suramin and apyrase indicates the possible involvement of nucleotide release and actions at P2 receptors. Probenecid and carbenoxolone inhibited contraction to 500 μM HOCl, consistent with HOCl-induced purine release via pannexin 1 and/or connexin channels, with purines acting at vasomotor P2 receptors. Detection of HOCl-induced ATP release from HCAECs is consistent with the suggestion that ATP could be responsible for the rapid relaxation to HOCl through its actions on endothelial P2 receptors and the release of NO.

4.4.1 Adenosine Receptor Involvement

A non-selective adenosine receptor blocker (Barnes, 2013), 8-SPT blocked rapid relaxation of 100 μM HOCl as well as rapid relaxation and contraction of 500 μM HOCl, suggesting a possible involvement of adenosine. King et al. (1990) suggested that porcine coronary arteries contain adenosine A_2 receptors, which mediate dilation by adenosine and different adenosine analogues and are competitively blocked by 8-SPT. Moreover, PCA and cultured porcine coronary smooth muscle cells express A_1 receptors, which induce contraction of vascular smooth muscle by upregulating PKC

(Marala & Mustafa, 1995b, 1995a; Nayeem & Mustafa, 2002). Moreover, in the PCA, A_{2A} receptors can mediate relaxation (Conti et al., 1993)

Since the HOCl rapid relaxation and contractile responses were blocked by 8-SPT, selective adenosine receptor antagonists ZM 241385 (A_{2A} receptor antagonist) and DPCPX (A_1 receptor antagonist) were used. As these antagonists target specific subtypes of adenosine receptors, It investigated how they might modulate the response to HOCl.

Neither ZM2 41385 nor DPCPX altered the response to HOCl (Poucher et al., 1995). ZM 241385 has been successfully used in the PCA by others at the same concentration that have been used to block A_{2A} receptors. According to Hasan et al. (2000), ZM 241385 (1 µM) significantly inhibited relaxation responses induced by adenosine receptor agonist 5′-N-carboxamido adenosine (NECA) and CGS21680 in PCA pre-contracted with prostaglandin $F_{2\alpha}$.

DPCPX is an A_1 selective antagonist (Hussain & Mustafa, 1995). A_1 receptor was found to be linked to guanylyl cyclase in rat and bovine coronary arteries and to K^+ channels in PCAs (Hussain & Mustafa, 1995). Since neither ZM 241385 nor DPCPX altered the responses to HOCl, this raises the possibility that the effects of 8-SPT were due to actions other than the blocking of adenosine receptors. In addition to adenosine receptor antagonism, 8-SPT has another mechanism of action: the inhibition of phosphodiesterase enzymes which are required to break down cyclic nucleotides (such as cAMP and cGMP). Because of this, 8-SPT results in an increase in cyclic nucleotides in smooth muscle cells, leading to relaxation of the

tissue (Barnes, 2013). As a result, if such a mechanism is present here, relaxation to HOCl should be larger. However, this does not occur.

4.4.2 P2 Receptor Involvement

Suramin blocked rapid relaxation of 100 μM HOCl, while the rapid relaxation observed at 500 μM HOCl was evident in the presence of suramin. On the other hand, the contraction in response to 500 μM HOCl was blocked by suramin. Suramin is used to treat trypanosomiasis, inflammation and cancer and to protect cells from injuries resulting from toxic chemicals and ischaemia (Kharlamov et al., 2002; McGeary et al., 2008). It is also a non-selective P2 purine receptor antagonist (Sahu et al., 2017). The present study showed that suramin blocked the rapid relaxation of 100 μM HOCl and contraction observed at 500 μM HOCl. This could indicate the participation of P2 receptors in the HOCl response. Notably, suramin is not effective in blocking all P2 receptors. As an example, suramin can inhibit $P2Y_2$ receptors in concentrations of up to 100 mM, but not $P2Y_4$ or $P2Y_6$ receptors (Charlton et al., 1996; Rayment et al., 2007). Moreover, P2X2 and P2X5 receptors respond to suramin and PPADS, while P2X4 and P2X6 receptors do not respond to suramin (Collo et al., 1996).

However other, non-P2 receptor-mediated actions of suramin could be involved. According to Sahu et al. (2017), sulfonate groups present in suramin act as scavengers of HOCl free radicals and as powerful antioxidants of nitric oxide and hydrogen peroxide free radicals, which are considered toxic, especially when they react with superoxide radicals to form a very reactive peroxynitrite anion ($ONOO^-$).

Chi et al. (2014) stated that suramin inhibits different types of cell membrane channels, including Ca^{2+} release channels and N-methyl-D-aspartate (NMDA) gated ion channels, as a cystic fibrosis transmembrane conductance regulator. The study illustrated the activation of a hemichannel resulting from the elimination of extracellular Ca^{2+}, which is blocked in the presence of suramin.

Neither MRS 2179 nor reactive blue 2 and BX430 seem to have any effect on the HOCl response. MRS 2179 acts as a $P2Y_1$ receptor antagonist (Baurand et al., 2001; Baurand & Gachet, 2003; Lenain et al., 2003). Typically, reactive blue 2 is known as a antagonist of $P2Y_4$ receptors; however, P2X receptor effects and non-P2 effects have also been identified (Lee et al., 2006; Kennedy, 2007).

Neither NF 449 nor α,β-methylene ATP altered the response to HOCl. NF449 is a selective P2X1 receptor antagonist. P2X1 receptors are expressed on platelets and atrial and smooth muscle in other organs and mediate thrombus stability under stress. They regulate sympathetic mediated vasoconstriction and blood flow (Vial & Evans, 2005). α,β-Methylene ATP is a stable analogue of ATP that acts as a potent agonist at P2X1 receptors located in smooth muscle, and so acts as a vasoconstrictor (Ralevic et al., 1997; Vial & Evans, 2005). Following their activation, P2X1 receptors rapidly desensitise (Ralevic et al., 1997). The P2X1 receptor is present in the smooth muscle cells of the PCA, and its activation causes vasoconstriction (Burnstock, 1990). Therefore, P2X1 may be involved in the contractile response to HOCl. Because α,β-methylene ATP is relatively resistant to hydrolysis, it is commonly used to desensitise P2X1 receptors in investigations of

their roles.

Moreover, in addition to being P2X4 antagonist, BX430 is widely expressed on the surface of the endothelial cells of the vascular system, as well as in the CNS, and has significant importance in the control of vascular tone. P2X4 is also present in macrophages and in microglia, where it contributes a significant role in the generation of inflammatory factors (Ase et al., 2015). The conclusion which can be drawn from the use of MRS 2179, reactive blue 2, NF449, α,β-methylene ATP, and BX430 is that, if suramin blocks the response to HOCl through antagonism of vascular P2 receptors, then these receptors are not P2X1, $P2Y_1$ or P2X4 receptors.

Naturally, the question arises as to whether suramin's effects on HOCl were caused by its effects on P2 purinoceptors or through other mechanisms, fot that reason, another non-selective P2 (PPADS) have been used. The fact that PPADS, a structurally different antagonist of P2 purinoceptors (Lämmer et al., 2011), did not mimic the effects of suramin illustrates this point. A variety of ligand-gated (P2X1, P2X2, P2X3, P2X5, and P2X7) and G-protein-coupled receptor subtypes ($P2Y_1$ and $P2Y_4$) can be inhibited by PPADS (Lämmer et al., 2011). It is generally believed that PPADS has a potency similar or slightly greater than that of suramin, although the P2X7 receptor is much less sensitive, and the P2X4 receptor is completely insensitive to it (Lämmer et al., 2011).

Suramin can inhibit connexin and pannexin channels (Qiu & Dahl, 2008). Connexins allow communication between cells, while pannexins can release ATP under hypotonic conditions (Scemes et al., 2007). The channel gating of both connexin and

pannexin is affected by different factors, including pH and Ca^{2+} (Nielsen et al., 2012). It is possible that HOCl causes a release of purines from vascular cells via pannexin 1 and/ or connexin channels, and that the extracellular purines mediate the changes in tone seen in the present study as well as being involved in other components of the inflammatory response *in vivo*. For that reason, probenecid and carbenoxolone were used to investigate whether the effect of suramin involved pannexin 1 and/ or connexin channels.

Probenecid is a pannexin 1 hemichannel blocker and therefore inhibits the release of ATP. It has been used as a treatment for gout by inhibiting an anion transporter and urate reuptake, thereby increasing uric acid excretion (Silverman et al., 2008). Carbenoxolone inhibits pannexin 1 and connexin gap junction channels that regulate ATP release and play an essential role throughout the body. For example, pannexin 1 in the immune system controls the release of ATP from apoptotic cells to enhance the clearance of death cells and control noradrenergic vasoconstriction (Michalski & Kawate, 2016). Moreover, carbenoxolone inhibits ATP release from mechanical stress through blocking connexin channels (Luckprom et al., 2011). Both probenecid and carbenoxolone had no significant effect on the rapid relaxation to HOCl. Moreover, both probenecid and carbenoxolone also caused a reduction in the contraction to 500 μM HOCl, this may indicate that HOCl induces purine release from cells via pannexin 1 and/or connexin channels, with the purines acting at vasomotor P2 receptors. Consistent with this is the attenuation of the HOCl-induced contraction by suramin. Notably, suramin, probenecid, and carbenoxolone enhanced slow relaxation due to functional antagonisms as they blocked contraction. Therefore, the other effect which

is slow relaxation will be enhanced.

ARL67156, an ecto-ATPase inhibitor, failed to prolong or alter in other ways any component of the effect of HOCl. In both coronary and ear porcine arteries, the contractile response to 1mM UDP was reported to be unaffected by the ecto-ATPase inhibitor ARL67156 when it was incubated for 15 minutes prior to adding an agonist. In parallel experiments, ARL67156 failed to alter the relaxation of ADP in PCAs (Rayment et al., 2007). On the other hand, ARL67156 significantly increased contractions in coronary and ear arteries when treated with 300 μM UTP (Rayment et al., 2007). It has been demonstrated that ARL67156 is not effective in blocking any nucleotidases when the substrate ATP is present in high concentrations (Lévesque et al., 2007; Durnin et al., 2016). Furthermore, based on the concentrations commonly used (50–100 μM), ARL-67156 only partially inhibits the activities of mouse and human recombinant NTPDase1, NTPDase3, and NPP1. Moreover, it has been demonstrated that ARL-67156 has no impact on NTPDase 2 and only weak effects on NTPDases 1 and 3 (Müller et al.,2006; Lévesque et al., 2007). The explanations provided may help to explain why ARL67156 failed to alter the effect of HOCl.

In this study, the effect of apyrase (10 and 100 units/ml) on HOCl response in the PCA was examined. Surprisingly, apyrase (100 units/ml) abolished the response to both concentrations of HOCl. Apyrase at 10 units/ml had no effect. ecto-ATPase apyrase hydrolyses ATP and ADP (Kammoe et al., 2021), which appear to be involved in the HOCl response in the PCA.

The effect of apyrase on ROS generation by polymorphonuclear neutrophils (PMNs) was investigated by Bertelli et al. (2011); they found that apyrase can decrease ROS production in neutrophils, which play an important role in immune responses. In order to reduce ROS production, apyrase removes extracellular ATP, which is known to stimulate ROS production in neutrophils. Apyrase reduces ATP levels, thereby decreasing the activity of NADPH oxidase, an enzyme complex responsible for producing ROS such as HOCl in neutrophils.

According to Madry et al. (2018), apyrase affects the morphology and function of microglial cells in the brain, resulting in alterations in their shape and activity; these cells are normally involved in monitoring the brain for signs of infection or damage. Unlike previous assumptions, their study showed that these changes were not caused by ATP depletion but rather by a high concentration of K^+, which caused significant cell depolarisation. Notably, a high concentration of K^+ also blocked the relaxant responses to HOCl in the present study (see Chapter 3, Figure 3-12). Also, apyrase alone at 100 units/ml produced a sustained contraction of the PCA (Figure 4-15, C, D). K^+ in the apyrase solution could, therefore, account for the effect of apyrase. However, it is worth noting that the study of Madry et al. (2018) used a higher dose of apyrase (200 units/ml) compared to the dose applied in this experiment (100 units/ml). The concentration at which apyrase was applied is a critical consideration when comparing the effects of apyrase across various studies. For example, it has also been found that 2 units/ml of apyrase significantly decreases contractions induced by 100 μM UDP, UTP, ADP, or ATP in mouse bladder strips (Kammoe et al., 2021). When endothelium is exposed to NS, cell

membrane integrity is compromised, resulting in the release of ATP. As a result of incubation of a rat aorta segment in a normal saline solution, the endothelial-dependent relaxation to carbachol (500 nM), an acetylcholine analogue in the tissues pre-contracted with phenylephrine (500 nM), was reduced, but endothelial function was retained when rat aortas were incubated in NS with apyrase (4 units/ml) (Cheung-Flynn et al., 2019).

Apyrase (100 units/ml) was also tested for its effects on the endothelium-dependent relaxant response to the vasoactive peptide bradykinin in the PCA. Apyrase did not significantly affect the response to bradykinin, suggesting that the effects of apyrase on HOCl is not due to non-specific effects on vasorelaxant function and so may be specific to ATP pathways. Moreover, preventing hyperpolarization with high extracellular K^+ in Krebs solution in bovine epicardial coronary arteries (68 mM) and PCA (125 mM) did not affect the relaxation of bradykinin, which is consistent with the finding that high concentrations of K^+ in apyrase did not affect bradykinin response (Nagao & Vanhoutte, 1992; Kilpatrick & Cocks, 1994; Drummond & Cocks, 1996). PCA pre-contracted with K^+ (25 mM) relaxed completely in response to bradykinin as well (Cowan & Cohen, 1991). Nevertheless, other studies have found that PCA pre-contracted with KCl reduced bradykinin response (Weintraub et al., 1994; Hernanz et al., 1999; Tomioka et al., 2001). Therefore, endothelium-dependent relaxation of bradykinin in PCA results from two distinct mechanisms: the production of NO and the hyperpolarization of smooth muscle cells (Weintraub et al., 1994).

A study by Csóka et al. (2015) investigated the role of the enzyme CD39, which is a membrane-bound apyrase, in sepsis. The study found that CD39 improved survival in mice with microbial sepsis by attenuating systemic inflammation. The authors suggested that the anti-inflammatory effects of CD39 are due to its ability to hydrolyse extracellular ATP and ADP, which are known to promote inflammation. In addition, converting ATP and ADP into adenosine reduces inflammation and improves survival in sepsis. Overall, these findings provide insight into the complex interactions between apyrase and vascular function. Further research is needed to fully elucidate the mechanism of action of apyrase and its potential therapeutic applications.

To investigate the role of ATP in HOCl response, an ATP assay kit (Abcam, Cat#ab113849) was used to measure ATP levels in samples of the bathing fluid from cultured HCAECs. The assay kit is a luminescent assay that uses enzyme luciferase to generate light in proportion to the amount of ATP present in the sample. It found that exposure of HCAECs to 100 μM HOCl resulted in a significant increase in ATP levels within 1 minute. This finding is consistent with the observation that ATP could cause the rapid relaxation by actions at endothelial P2 receptors and the release of NO of the PCA in the organ bath experiments. The mechanisms of HCAEC-induced ATP release could include pannexins (as discussed above) and could also be through lysis. At concentrations of 10–20 μM, HOCl induces membrane dysfunction. Conversely, at higher levels exceeding 50 μM, the lysis process becomes prevalent (Schraufstätter et al., 1990). My results suggest a potential involvement of purinergic receptors in the response to HOCl, as these receptors are known to play a key role

in the modulation of vascular function. The involvement of purinergic receptors in the response to HOCl warrants further investigation and could provide new insights into the regulation of vascular function.

With the data presented in this chapter, it can be concluded that HOCl mediates PCA responses by releasing ATP through actions at endothelial P1 and P2 receptors. Based on the assumption that P1 and P2 receptors are involved in HOCl response, it will be useful to discuss in the next chapter the direct effects of ATP and adenosine on the PCA in order to determine if they have the same vasomotor effect as that obtained from HOCl on the PCA. Antagonists of the response to HOCl may also affect the direct actions of ATP and adenosine on PCA.

Chapter 5 Effect of ATP and Adenosine on PCA Contractility

5.1 Introduction

Adenosine and ATP play a crucial role in cardiovascular function. Adenosine acts as a potent vasodilator and inhibits platelet aggregation, while ATP has a key role in regulating vascular tone and blood flow. These molecules are involved in various physiological and pathophysiological processes in the cardiovascular system (Ralevic, 2021).

ATP is released from endothelial cells in response to various stimuli, including shear stress, hypoxia, and ischaemia (Bodin & Burnstock, 2001). ATP binds to P2 receptors on VSMCs, causing vasodilation or vasoconstriction (Burnstock & Ralevic, 2014). Specific subtypes of P2X and P2Y receptors that these nucleotides interact with have been identified (Kennedy et al., 2013). ATP is also involved in the regulation of angiogenesis, which is the formation of new blood vessels in response to tissue hypoxia. ATP can promote angiogenesis by stimulating endothelial cell proliferation and migration, which helps increase blood flow to hypoxic tissues (Burnstock & Ralevic, 2014).

Adenosine, on the other hand, is produced in response to ischaemic stress and plays a critical role in regulating coronary blood flow (Mustafa et al., 2009). Adenosine receptors (predominantly A_{2A} but also A_{2B}) are found in high concentrations in the coronary vasculature, and the binding of adenosine to these receptors causes vasodilation of the coronary arteries, leading to an increase in blood flow to the myocardium (Olanrewaju & Mustafa, 2000). This mechanism helps improve myocardial perfusion and oxygen delivery during ischaemic conditions,

making it an important component of the ischaemic preconditioning response (Mustafa et al., 2009). Additionally, A_{2A} has been shown to inhibit platelet aggregation, reducing the risk of thrombosis and potentially preventing the development of cardiovascular disease (Fredholm et al., 2011).

It has been documented that A_1 and A_{2A} proteins are expressed in the left anterior descending artery of porcine hearts, while A_1, A_{2A} and A_{2B} proteins are expressed in the coronary arteries. A_{2A} is primarily responsible for endothelial-independent coronary vasodilation, while A_{2B} may play a minor role (Zhang et al., 2021). By reverse transcription PCR and western blot analysis, it has been demonstrated that A_{2A} and A_{2B} receptors are present in cultured HCAEC and porcine coronary artery endothelial cells and that they cause endothelium relaxation (Olanrewaju et al., 2000). PCA smooth muscle cells express both P2X1 and $P2Y_2$ receptors (Burnstock, 2017). In the PCA, there is an expression of mRNA encoding $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors, whereas immunoblotting indicates that $P2Y_6$ receptors are present in these tissues. The PCA exhibits $P2Y_2$ receptors coupled to smooth muscle contractions (Rayment et al., 2007). Moreover, $P2Y_{14}$ receptor expression in the PCA, which is primarily activated by UDP-glucose, contributes to the contraction of the muscle (Abbas et al., 2018).

Understanding the roles of adenosine and ATP in cardiovascular function is essential for the development of new treatments for cardiovascular disease. In Chapter 4, there was some evidence for an involvement of purines in the effect of HOCl through the inhibitory actions of 8-SPT, suramin and apyrase. To investigate this further, this

chapter focuses on the direct effects of adenosine and ATP on PCA relaxation. Many studies have examined the cumulative responses to adenosine and ATP in the PCA and other vascular tissues (King et al.,1990; Rubin et al., 2000; Kuo & Hein, 2001; Rayment et al., 2007). On the other hand, when moving to an inflammatory condition and HOCl release, these responses appear to occur in a sudden, abrupt manner (Chen et al., 2018). The time-course profile of response when a large amount of ATP is introduced at a specific moment, particularly in an inflammatory condition, is limited (Manica et al., 2018). By exploring the underlying mechanisms of adenosine- and ATP-induced relaxation, it may be possible to gain a better understanding of the potential similarities and differences between their effects and those of HOCl. This in turn will inform understanding of whether adenosine and ATP are potential mediators of the response to HOCl.

Aim and objectives: The aim of this study was to enhance our knowledge of vascular inflammation and the role of ATP and adenosine in inflammation. The objective of the study was to investigate the direct effects of a single concentration of ATP and adenosine on the intact and denuded-endothelium PCA by using the organ bath technique and to evaluate the effects of different receptor antagonists and inhibitors including L-NAME, 8-SPT, suramin and apyrase, which were shown in previous chapters to alter the response to HOCl, on the direct effects of a single concentration of ATP and adenosine.

5.2 Methods and Materials

The methods and materials were as described in Chapter 2.

5.3 Results

5.3.1 Adenosine Effect on PCA

5.3.1.1 Relaxant Effect of Adenosine on PCA

Figure 5-1 displays that adenosine (30 µM) produced a relaxation in U46619-contracted PCA rings. The highest response of adenosine was 67.9 ± 7.4% at about 15 minutes (*n* = 8). The relaxation observed at 60 minutes was 48.8 ± 8.1% (*n* = 8).

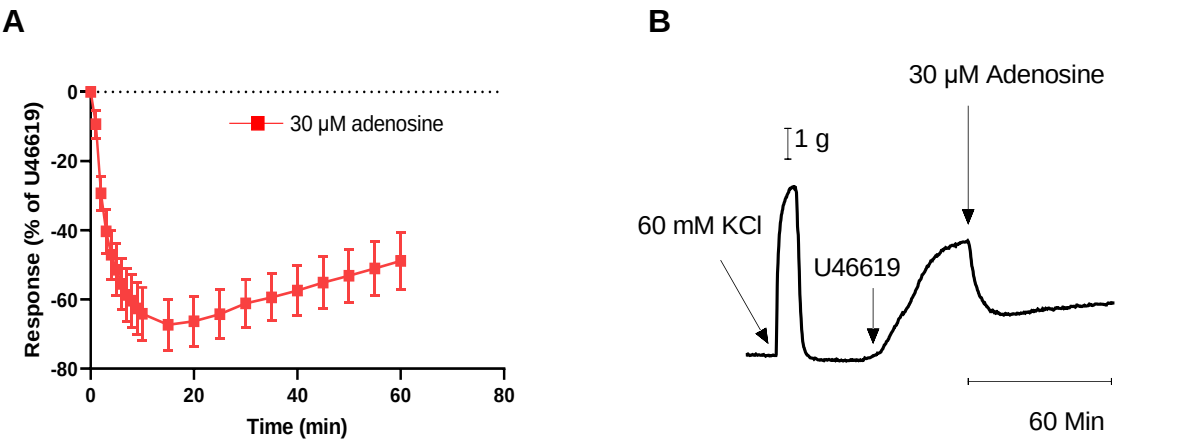


Figure 5-1. (A) Porcine coronary artery responses are produced by 30 µM adenosine (*n* = 8). Artery segments were pre-contracted with U46619. (B) Original traces showing the responses of the PCA to 30 µM adenosine in a vessel pre-contracted with U46619.

5.3.1.2 Endothelium Involvement in Adenosine Responses

The relaxation in PCA with intact endothelium to 30 µM adenosine at 15 min (81.5 ± 7.8%, *n* = 6) was still pronounced in the PCA with denuded endothelium (75.8 ± 10.3%, *n* = 6) (*p* = .6). Moreover, the relaxation at 60 minutes in the PCA with denuded endothelium was less pronounced but not significantly different (56.4 ± 15.4%, *n* = 6) compared to endothelium-intact PCA (71.8 ± 10.3%, *n* = 6) (*p* = 0.42)

(Figure 5-2).

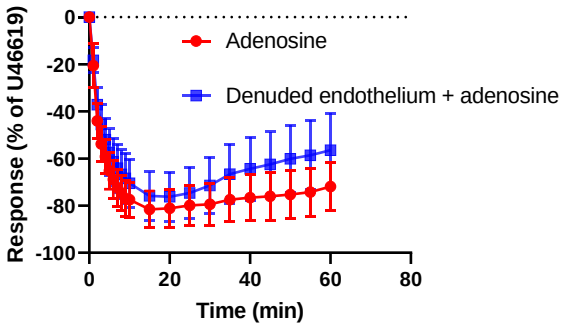


Figure 5-2. Porcine coronary artery responses evoked by 30 μM adenosine in endothelium-intact and -denuded vessels (*n* = 6). Artery segments were pre-contracted with U46619.

5.3.1.3 Effect of L-NAME on Adenosine Response

L-NAME (300 μM), a NOS inhibitor, did not affect the relaxation observed at 15 minutes of 30 μM adenosine. In the presence of L-NAME, the maximum relaxation was 32.3 ± 5.4% (*n* = 6) and in the control 28.8 ± 5.2% (*n* = 6) (*p* = .65). In addition, the relaxation observed at 60 minutes of adenosine (control: 18.8 ± 4.6%, *n* = 6) was similar in the presence of L-NAME (27.4 ± 2.7%, *n* = 6) (*p* = .14) (Figure 5-3).

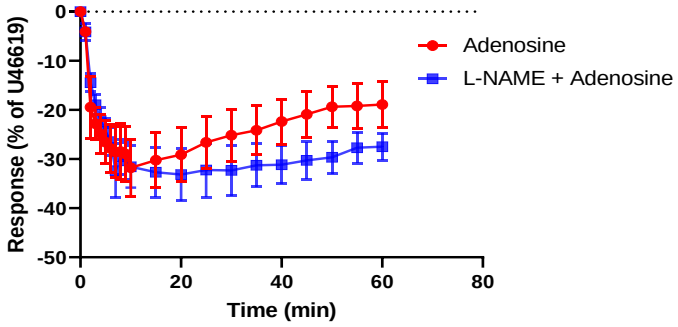


Figure 5-3. Porcine coronary artery responses produced by 30 μM adenosine in the absence or presence of 300 μM L-NAME (*n* = 9). Artery segments were pre-contracted with U46619.

5.3.1.4 Effect of 8-SPT on Adenosine Response

The presence of 8-SPT (100 µM), which is a non-selective adenosine receptor blocker (Barnes, 2013), reduced the relaxation response induced by 30 µM adenosine (48.9 ± 9.7%, *n* = 6) compared to the relaxation response observed under control conditions (83.2 ± 7.1%, *n* = 6) (*p* = 0.01) (Figure 5-4).

The relaxation after 60 minutes was reduce but not significantly different in the presence of 8-SPT (35.8 ± 12.2%, *n* = 6) compared to the control group (67.3 ± 8.4%, *n* = 6) (*p* = 0.06) (Figure 5-4).

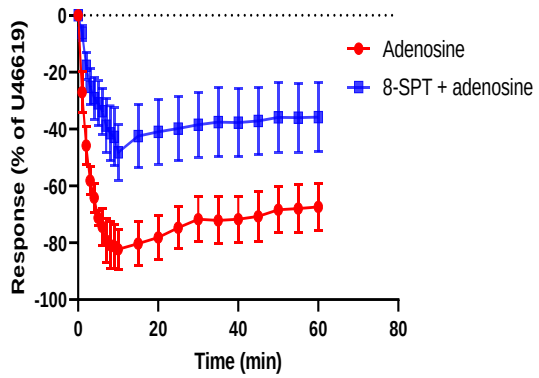


Figure 5-4. Porcine coronary artery responses produced by 30 µM adenosine in the absence or presence of 100 µM 8-SPT (*n* = 6). Artery segments were pre-contracted with U46619.

5.3.1.5 Effect of Suramin on Adenosine Response

One hundred micromolar suramin (a non-selective P2 purine receptor antagonist; Sahu et al., 2017) did not alter the response of adenosine; the rapid relaxation was 50.2 ± 10.7% (*n* = 6) in the absence of suramin compared to 29.3 ± 9.3% (*n* = 6) in the presence of suramin (*p* = 0.17). Likewise, the relaxation responses to adenosine

at 60 minutes in the absence or presence of suramin were similar at 58.3 ± 13.13% (*n* = 6) and 73.7 ± 12.3% (*n* = 6) (*p* = 0.41), respectively (Figure 5-5).

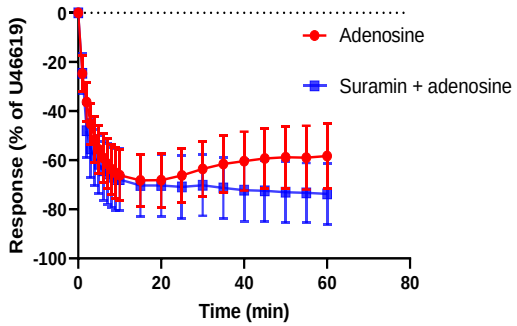


Figure 5-5. Porcine coronary artery responses produced by 30 μM adenosine in the absence or presence of 100 μM Suramin (*n* = 6). Artery segments were pre-contracted with U46619.

5.3.1.6 Effect of Apyrase on Adenosine Response

The adenosine relaxation at 15 minutes, 74 ± 12.1% (*n* = 6), was completely blocked by 100 units/ml of apyrase, 19.0 ± 4.9% (*n* = 6) (*p* = 0.001). The relaxation at 60 minutes in the control group was 70.9 ± 13.1% (*n* = 6) compared to the apyrase group's 7.5 ± 4.9% (n = 6) (*p* = 0.001) (Figure 5-6). In arteries with apyrase, which is known to enhance the response to vasoconstrictors (25%–42%), the concentration of U46619 used to pre-contract the PCA was significantly lower (Table 3-1).

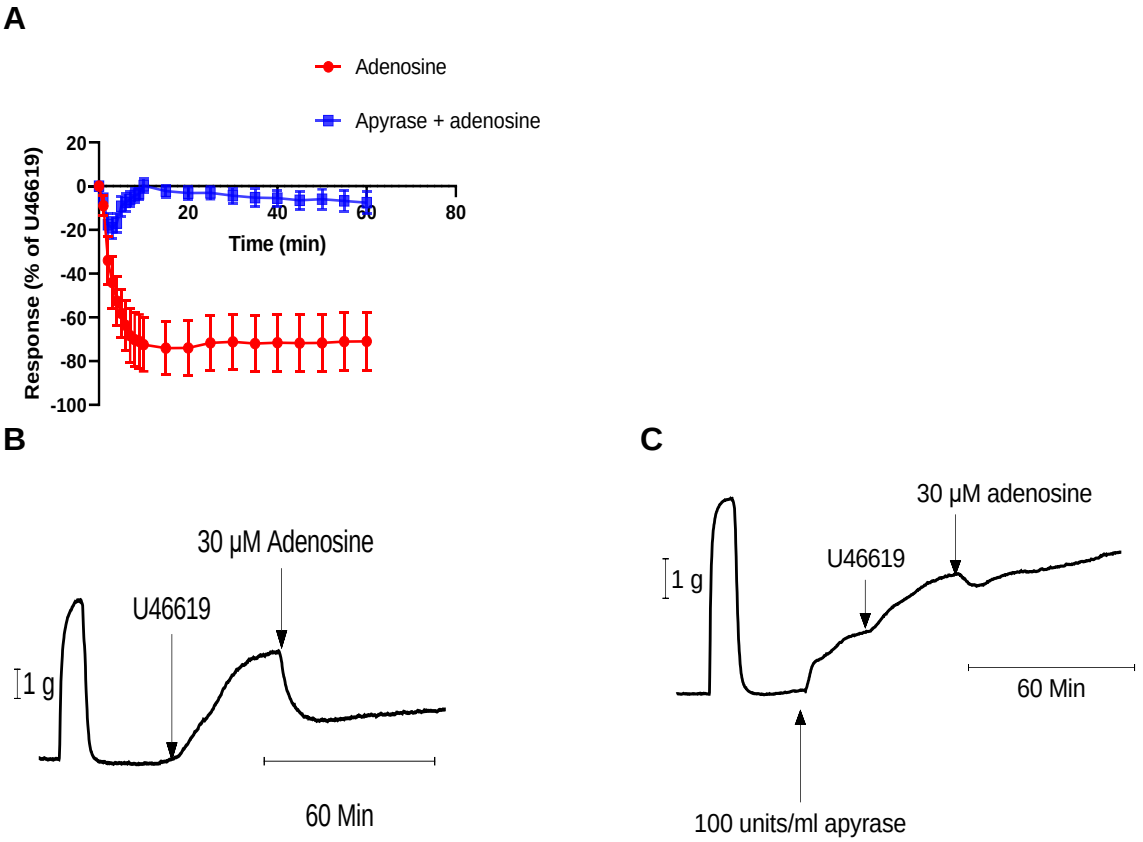


Figure 5-6. (A) Responses of porcine coronary arteries treated with 30 μM adenosine in the absence or presence of 100 units/ml apyrase (*n* = 6). Original traces showing responses of PCA to 30 μM adenosine in vessels pre-contracted with U46619 in the absence (B) and presence (C) of 100 units/ml apyrase.

5.3.2 ATP Effect on PCA

It was found that ATP at 100 μM caused relaxation of U46619 pre-contracted rings. Rapid relaxation response to ATP occurred within 1 minute, followed by sustained relaxation over 59 minutes. In some experiments, rapid relaxation was absent; therefore, rapid relaxation at 1 minute (if present), maximum relaxation (approximately 15 minutes), and 60-minute relaxation were reported.

5.3.2.1 Relaxant Effect of ATP on PCA

The results depicted in Figure 5-7 indicate that when ATP was applied at a concentration of 100 µM, it caused relaxation in U46619 pre-contracted rings. The rapid relaxation response to ATP occurred at 1 minute, with a percentage of 50.2 ± 10.7% (*n* = 6). The maximum relaxation response to ATP was 88.3 ± 7.1% (*n* = 6). At 60 minutes, the observed relaxation was 84.4 ± 8.3% (*n* = 6).

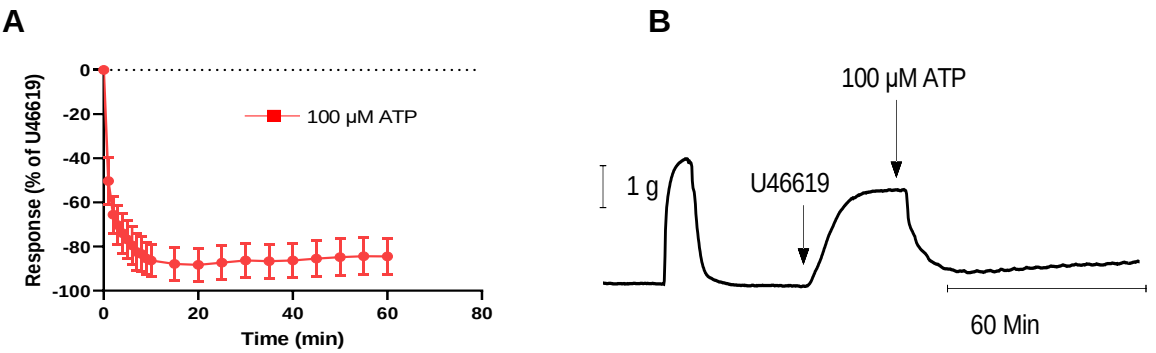


Figure 5-7. (A) Porcine coronary artery responses produced by 100 µM ATP (*n* = 8). Artery segments were pre-contracted with U46619. (B) Original traces showing the responses of the PCA to 30 µM adenosine in vessels pre-contracted with U46619.

5.3.2.2 Endothelium Involvement in ATP Responses

The rapid relaxation response of the PCA with intact endothelium to ATP at a concentration of 100 µM (measured at 1 minute) was 68.9 ± 4.3% (*n* = 8). It was blocked after removing the endothelium: 16.5 ± 5.3%, *n* = 8 ($p < 0.0001$). The maximum relaxation response of the PCA with intact endothelium to ATP at a concentration of 100 µM (measured at ~15 minutes) was 82.2 ± 8.9% (*n* = 6). Even after removing the endothelium, the relaxation response was larger, with a percentage of 97.1 ± 6.0% (*n* = 6), and this difference was not statistically significant

(p = 0.19). At 60 minutes, the relaxation response in endothelium-denuded PCA was 95.2 ± 7.0% (n = 8), which was bigger but not significantly different compared to endothelium-intact PCA, which showed a response of 72.4 ± 11.5% (n = 8) (p = 0.11) (Figure 5-8).

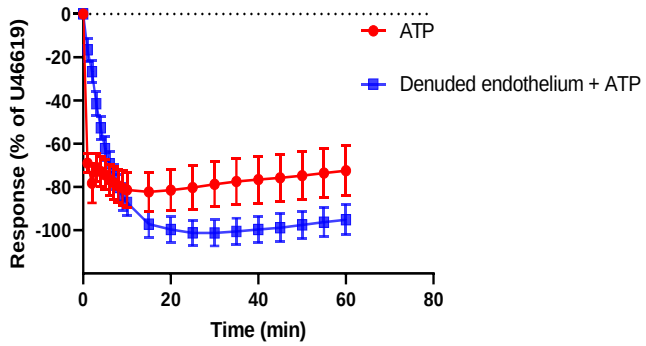


Figure 5-8. Porcine coronary artery responses evoked by 100 μM ATP in endothelium-intact and -denuded vessels (n = 8). Artery segments were pre-contracted with U46619.

5.3.2.3 Effect of L-NAME on ATP Response

As a result of the presence of L-NAME, the maximum relaxation was 62.5 ± 14.9% (n = 8) while the maximum relaxation in the control group was 79.9 ± 7.5% (n = 8) (p = 0.31). Further, the relaxation observed at 60 minutes of ATP (control: 64.6 ± 11.2%, n = 8) was not significantly different in the presence of L-NAME (56.6 ± 17.1%, n = 8) (p = 0.7) (Figure 5-9).

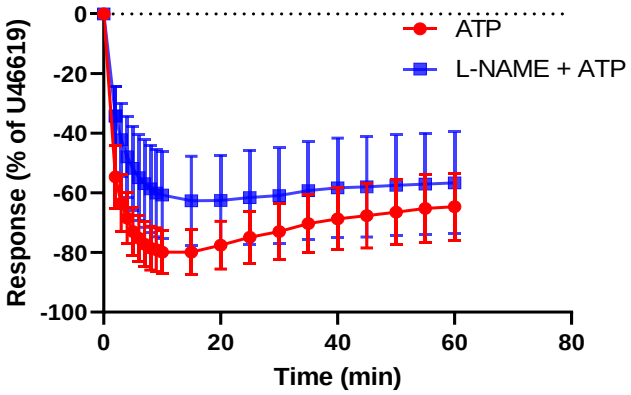


Figure 5-9. Porcine coronary artery responses produced by 100 μM ATP in the absence or presence of 300 μM L-NAME (*n* = 8). Artery segments were pre-contracted with U46619.

5.3.2.4 Effect of 8-SPT on ATP Response

The presence of 8-SPT (100 μM) reduced the rapid relaxation response caused by 100 μM ATP (33.2 ± 7.43%, *n* = 6) as compared to the relaxation response observed under normal conditions (64.3 ± 8.4%, *n* = 6) (*p* = 0.02) (Figure 5-10). It also reduced the maximum relaxation response caused by 100 μM ATP (36.6 ± 7.8%, *n* = 6) as compared to the relaxation response observed under normal conditions (84.1 ± 6.1%, *n* = 6) (*p* = 0.0009) Furthermore, after 60 minutes, the relaxation response to ATP in the presence of 8-SPT (25.7 ± 10.3%, *n* = 6) was significantly lower than that in the control group (68.8 ± 12.6%, *n* = 8) (*p* = 0.03) (Figure 5-10).

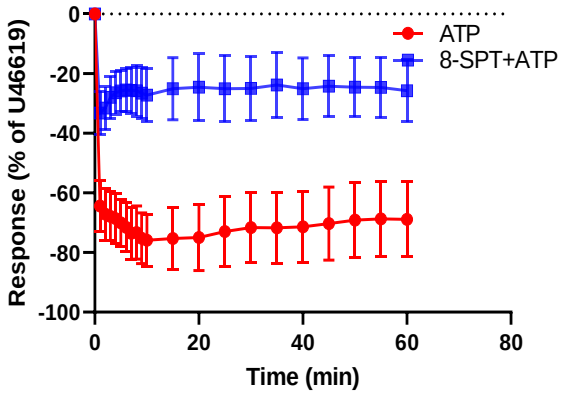


Figure 5-10. Porcine coronary artery responses produced by 100 µM ATP in the absence or presence of 100 µM 8-SPT (*n* = 6). Artery segments were pre-contracted with U46619.

5.3.2.5 Effect of Suramin on ATP Response

The presence of 100 µM suramin did not alter the response of PCA to ATP; the maximum relaxation was 87.8 ± 7.3% (*n* = 6) in the absence of suramin compared to 101.3 ± 1.3% (*n* = 6) in the presence of suramin (*p* = 0.09). Likewise, the relaxation responses at 60 minutes in the absence or presence of suramin were similar at 84.4 ± 8.3% and 102.6 ± 1.6% (*n* = 6) (*p* = 0.05), respectively (Figure 5-11).

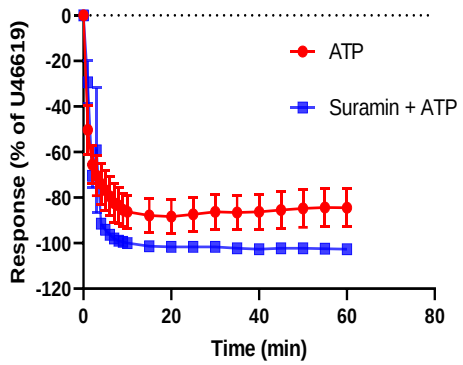


Figure 5-11. Porcine coronary artery responses produced by 100 µM ATP in the absence or presence of 100 µM Suramin (*n* = 6). Artery segments were pre-contracted with U46619.

5.3.2.6 Effect of Apyrase on ATP Response

The presence of 100 units/mL of apyrase completely inhibited the maximum ATP response when compared to the control group, with relaxation of 8.8 ± 2.1% and 92.2 ± 1.1% respectively ($p = 0.0005$) ($n = 6$). At 60 minutes, the control group exhibited a relaxation of 78.5 ± 11.0% ($n = 6$), whereas the apyrase group showed a significantly lower relaxation of 4.4 ± 4.9% ($p = 0.0001$) (Figure 5-12).

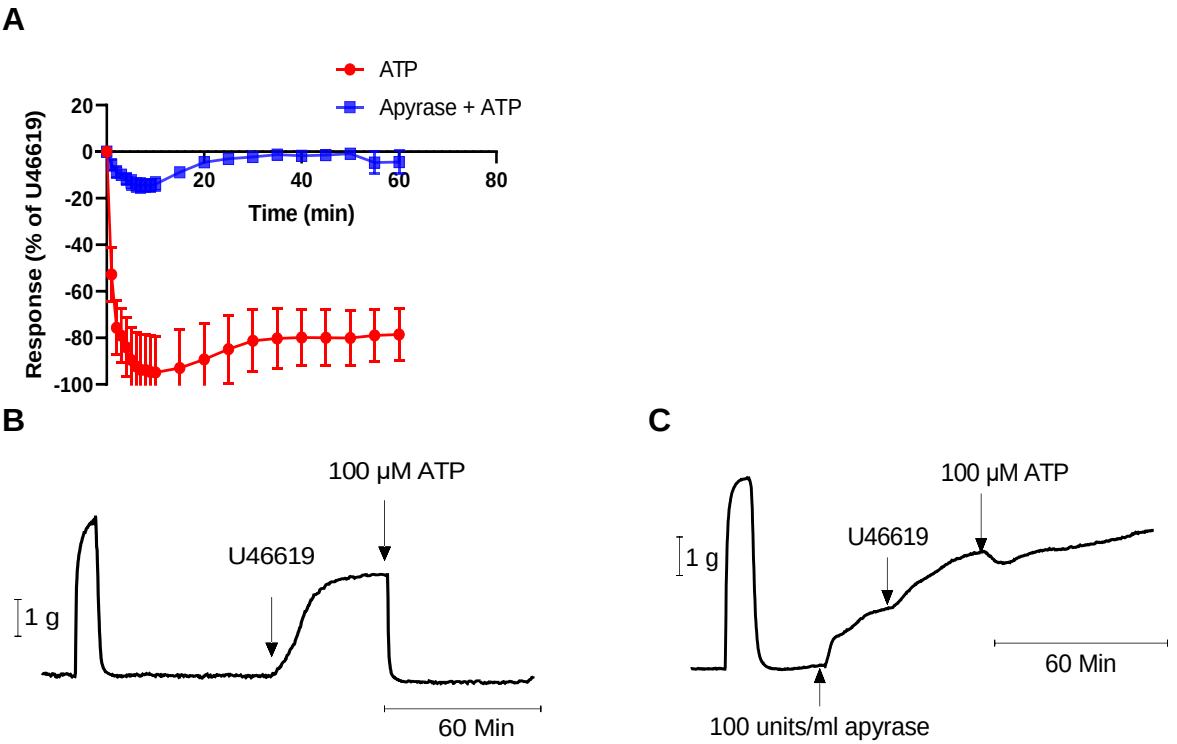


Figure 5-12. (A) Responses of porcine coronary arteries that were mediated by 100 µM ATP in the absence or presence of 100 units/ml apyrase ($n = 6$). Original traces showing the responses of PCA to 100 µM ATP in vessels pre-contracted with U46619 in the absence (B) and presence (C) of 100 units/ml apyrase.

Table 5-1. Effect of Various Inhibitors on Maximum Relaxation of 30 μM Adenosine

Antagonist	Concentration	Control	Treatment	*n*	*p*
endothelium denuded		81.5 ± 7.8%	75.8 ± 10.3%	6	0.6
L-NAME (NOS inhibitor)	300 μM	28.8 ± 5.2%	32.3 ± 5.4%	6	0.65
8-SPT (adenosine receptor antagonist)	100 μM	83.2 ± 7.1%	48.9 ± 9.7%	6	0.01
suramin (non-selective P2 receptor antagonist)	100 μM	68.2 ± 10.6%	70.2 ± 12.8%	6	0.9
apyrase (hydrolyses nucleotides)	100 units/ml	74.0 ± 12.1%	19.0 ± 4.9%	6	0.001

Note. Statistical significance was defined as *p* values of less than 0.05 in *t*-test.

Table 5-2. Effect of Various Inhibitors on Maximum Relaxation of 100 μM ATP

Antagonist	Concentration	Control	Treatment	*n*	*p*
endothelium denuded		82.2 ± 8.9%	97.1 ± 6.0%	6	0.19
L-NAME (NOS inhibitor)	300 μM	79.9 ± 7.5	62.5 ± 14.9 %	6	0.31
8-SPT (adenosine receptor antagonist)	100 μM	84.1 ± 6.1%	36.6 ± 7.8%	6	0.0009
suramin (non-selective P2 receptor antagonist)	100 μM	87.8 ± 7.3%	101.3 ± 1.3%	6	0.09
apyrase (hydrolyses nucleotides)	100 units/ml	92.2 ± 1.1%	8.8 ± 2.1%	6	0.0005

Note. Statistical significance was defined as *p* values of less than 0.05 in *t*-test.

Table 5-3. Concentration of U46619 Required to Pre-constrict Artery Segments in Experimental Groups for Relaxation Induced by 30 Mm Adenosine and Level of U46619-Induced Tone Achieved

Experimental group	**Concentration of U46619 required to pre-constrict PCA (nM)**		**Level of U46619-induced tone achieved (expressed as % of KCl)**	***n***	***p***
	Control	**Treatment**			
denuded endothelium	7.5 ± 1.4	3.5 ± 0.1	68.3 ± 4.2%	6	0.01
L-NAME (300 µM)	29.7 ± 3.1	11.8 ± 1.7	66.5 ± 4.5%	9	0.0001
8-SPT (100 µM)	10.1 ± 1.0	11.6 ± 1.7	71.6 ± 2.4%	6	0.45
suramin (100 µM)	14.4 ± 1.9	48.0 ± 7.5	78.5 ± 4.2%	6	0.001
apyrase (100 units/ml)	13.2 ± 1.6	4.5 ± 1.7	72.5 ± 1.3%	6	0.04

Note. Statistical significance was defined as *p* values of less than 0.05 in *t*-test.

Table 5-4. Concentration of U46619 Required to Pre-constrict Artery Segments in Experimental Groups for Relaxation Induced by 100 μM ATP and Level of U46619-Induced Tone Achieved

Experimental group	Concentration of U46619 required to pre-constrict PCA (nM)		Level of U46619-induced tone achieved (expressed as % of KCl)	*n*	*p*
	Control	Treatment			
denuded endothelium	13.7 ± 1.9	6.8 ± 0.7	68.8 ± 2.8%	6	0.05
L-NAME (300 μM)	14.9 ± 2.6	6.8 ± 0.9	71.5 ± 3.8%	7	0.01
8-SPT (100 μM)	11.6 ± 1.4	15.5 ± 1.2	75.6 ±.3.9%	8	0.05
suramin (100 μM)	10.9 ± 1.6	43.2 ± 5.6	77.5 ± 7.2%	6	<0.0001
apyrase (100 units/mL)	10.3 ± 0.9	2.3 ± 0.7	79.5 ± 4.3%	6	<0.0001

5.4 Discussion

The main findings of this chapter are that the responses observed with a single concentration of adenosine (30 μM) and ATP (100 μM) consist of an endothelium-independent relaxation response to adenosine (maximal at approximately 15 minutes) and for ATP a partially endothelium-dependent rapid relaxation phase (at ~1 min), followed by a more sustained relaxation. Furthermore, L-NAME and suramin did not affect either the adenosine or the ATP relaxation response. Both 8-

SPT and apyrase had significant inhibitory effects on the adenosine and ATP responses, which is in line with their inhibitory effects on HOCl responses in the PCA.

It is advantageous to use a single concentration because it allows investigation of the specific effects of ATP at a given concentration while reducing the risk of complications associated with the desensitisation of receptors as can occur with cumulative concentration-response curves. Notably, even the response to a single concentration reverse, as was seen with adenosine and ATP in the present study, could be due to receptor desensitisation. This phenomenon occurs quite frequently with GPCRs and involves a variety of elements that change over time. The process of fast desensitisation occurs when GPCR kinase enzymes phosphorylate the receptors. The result is that the receptors preferentially bind to molecules known as arrestins. This combination prevents the receptor from binding with the G-protein, which affects the receptor's normal function (Sheth et al., 2014).

Purine nucleotides can be moved by two-way nucleoside transporters into the extracellular space (Sheth et al., 2014). There are two types of nucleoside transporters: equilibrative nucleoside transporter and concentrative nucleoside transporter. Nucleosides are transported across the cell membrane by passive equilibrative nucleoside transporter depending on the gradient of its concentration. By contrast, concentrative nucleoside transporter transports nucleosides against its gradient of concentration through sodium (Na^+) (Sheth et al., 2014; Antonioli et al., 2019).

5.4.1 Mechanism of Adenosine Relaxation

Adenosine was tested at a concentration of 30 μM and was found to significantly relax the isolated PCA segments. This concentration was selected because it is similar to the EC_{50} values for adenosine reported by others: EC_{50} = 8.0 ± 0.9 μM (Rubin et al., 2000), pEC_{50} (5.61 ± 0.089) (Rayment et al., 2007), and EC_{50} = 5.51 + 0.13 (King et al.,1990).

This study examined the relaxation response to adenosine in endothelium-denuded rings. According to the findings, adenosine relaxation was unaltered in endothelium-denuded rings, suggesting that the relaxation effects of adenosine are predominantly mediated through the activation of adenosine receptors on smooth muscle. This result is consistent with those reported by Abebe et al. (1994) and Rayment et al. (2007), who found that adenosine and adenosine analogues mediate relaxation mainly through A_{2A} (Mustafa et al., 2009) of the PCA, which was mainly independent of the presence of an intact endothelium.

In this study, the impact of the NO pathway on adenosine-induced relaxation of the PCA was investigated. The findings show that L-NAME did not affect the response of endothelium-intact rings to adenosine. This implies that the relaxation responses initiated by adenosine in endothelium-intact coronary rings were largely not linked to NO. Others have shown that L-NAME can reduce the vasodilatory effect of PCA at a lower concentration of adenosine (≤0.1 μM) (Kuo & Hein, 2001). However, a

significant dilation to greater concentrations of adenosine (1 μM) remained after the endothelium was removed or in the presence of L-NAME (Kuo & Hein, 2001). According to Kuo and Hein, the relaxation effect of adenosine is facilitated by the release of NO by endothelial cells at low concentrations. Conversely, at higher concentrations of adenosine, smooth muscle K_{ATP} channels are primarily responsible for dilation.

Using 8-SPT, an antagonist of adenosine receptors, It found that the adenosine relaxation response was significantly reduced. Consistent with this, one study found that adenosine and its analogues produce relaxation in the PCA, and these relaxations are inhibited by 8-SPT (King et al., 1990). The findings suggest that adenosine A_2 receptors play a significant role in mediating these relaxations.

Studies have examined adenosine receptor expression in porcine, human and rodent coronary arteries and have provided valuable insights into the functional and expression profiles of adenosine A_{2A} and A_{2B} receptors in these tissues. For example, adenosine receptors are distributed differently in coronary arteries. According to Wang et al. (2005), A_1, A_{2A}, and A_{2B} receptors are also expressed in the arterioles and venules of porcine heart. Studies have shown that A_{2A} and A_{2B} receptors mediate adenosine-induced dilation of mouse coronary arteries (Talukder et al., 2003). In human coronary arteries, adenosine induces relaxation, most likely via A_2 receptors (Ramagopal et al., 1988). Kemp and Cocks (1999) provided evidence that adenosine mediates the relaxation of human small coronary arteries through interaction with A_{2B} receptors. In summary, these reports suggest that

adenosine A_{2A} receptors play a significant role in mediating relaxations in the PCA.

In the present study, suramin, a non-selective P2 receptor antagonist, was evaluated in adenosine-induced relaxation. The data indicate that suramin has no significant effect on the relaxation response to adenosine, indicating that P2 receptors do not play a significant role in adenosine-induced relaxation. This finding supports a study by Vials and Burnstock (1994) that examined the effects of suramin (30 μM) on vasodilator responses to adenosine and ATP in the coronary vasculature of rats. The authors found that suramin did not inhibit the relaxation caused by adenosine (except at a low concentration of adenosine, 5×10^{-11} M).

As a result of the addition of apyrase, the response to adenosine was inhibited. ATP and ADP are hydrolysed by apyrase to AMP; adenosine is not hydrolysed by apyrase (Kammoe et al., 2021). In this study, an unexpected finding was that apyrase blocked adenosine-induced relaxation in the PCA. It is unlikely to affect adenosine relaxation. Notably, commercial apyrase preparations contain high K^+ concentrations (Madry et al., 2018). Therefore, the effect seen on microglial cannot be attributed to the enzymatic activity of apyrase. Consequently, there is a significant depolarisation of the cell due to this high K^+ concentration (Madry et al., 2018). The presence of high levels of K^+ significantly impacts vascular contractility; in addition, apyrase alone results in an increase in tone (see result). Adenosine reportedly failed to relax K^+ pre-contracted canine large coronary arteries even at concentrations of up to 100 μM (White & Angus, 1987).

5.4.2 Mechanism of ATP Relaxation

The direct effect of ATP on the PCA was investigated using a single concentration of 100 μM. The concentration selected was similar to previous EC_{50} values for adenosine reported by others: pEC_{50} of 5.06 ± 0.29 in Rayment et al. (2007) and EC_{50} = 5.39 + 0.10 in King et al.(1990), although these studies used cumulative concentration-response curves. In the present study, the mechanism by which ATP-mediated vasodilation occurs was mediated by adenosine acting on P1 receptors because the response was blocked by 8-SPT, in line with the involvement of A_{2A} receptors shown by Rayment et al. (2007). Additionally, the results indicate that there was no significant contribution by P2 receptors to the ATP-induced vasodilation of the PCA, as shown by the lack of effect of suramin. The results suggest that ATP may cause relaxation by being converted to adenosine or through ATP-induced release of adenosine, as discussed by Rayment et al. (2007).

The present study found that rapid relaxations to ATP were partially endothelium-dependent. In line with this, Lee et al. (1990) demonstrated that relaxations induced by injected ATP as a bolus dose over 2 minutes in the coronary vascular system of guinea pigs was endothelium-dependent. On the other hand, Rayment et al. (2007) examined the effects of cumulative concentrations of ADP and ATP on the relaxation of pre-contracted PCA by using concentrations between 1 and 100 μM. Both ADP and ATP were found to cause concentration-dependent relaxation of the PCA. Neither ADP nor ATP relaxations were significantly affected by the endothelium, indicating that no endothelial mechanism is involved in the relaxation process. It is

possible that these observed differences can be attributed to the different administration methods (dose versus continuous infusion) used. In addition, in the present study, endothelium removal affected only the ATP rapid relaxation response that was observed at 1 min, while the remainder of the response remained unchanged, suggesting that the ATP response on the PCA only partially involves the endothelium.

ATP responses were not altered by L-NAME, suggesting that ATP may induce relaxation by signalling other than NO that is partially dependent of the endothelium. A study in the isolated hearts of guinea pigs concluded that NO has little or no effect on ATP-induced vasodilation (Brown et al., 1992). Vasodilation instigated by ATP happens due to the production of adenosine and activation of guanylyl cyclase in smooth muscle (Kelm & Schrader, 1990).

In this study, 8-SPT (a non-selective antagonist of the adenosine receptor) inhibited the relaxation to ATP, indicating that ATP may induce relaxation through adenosine release, as suggested by Rayment et al. (2007), but could also involve ATP metabolism to adenosine. According to Bunger et al. (1975) and Erga et al. (2000), 8-SPT significantly reduces ATP-mediated coronary vasodilation in the isolated hearts of guinea pigs. Similarly, the adenosine receptor blockers 8-SPT and CGS 15943A cause the same level of inhibition of the coronary vasodilator effects of both adenosine and ATP (Kroll & Schrader, 1993). In contrast, there are studies that found that adenosine receptor antagonists do not significantly reduce the effects of ATP on coronary vasodilation. The infusion of xanthine amine congener (XAC), a

potent adenosine receptor blocker, inhibited adenosine-induced coronary vasodilation but did not significantly change ATP responses in guinea pig isolated perfused heart (Brown et al., 1992). According to Vials and Burnstock (1994), 8-SPT was not able to affect the vasodilator response to ATP in a significant way.

The addition of suramin did not result in any significant changes in the ATP response. Suramin also acts as a ectonucleotidase inhibitor in addition to a P2 receptor antagonist (Bonan et al., 1999). It has been demonstrated that suramin blocks ecto-ATPase activity in various contexts, including in the bladder of guinea pigs, in endothelial cells, and in blood cells. These findings suggest that ecto-ATPase enzymes and P2-purinoceptors have similar binding domains. As a result, suramin may disrupt the degradation of ATP and/or interfere with the transmission of purinergic signals. As suramin inhibits the degradation of ATP, its potency as an antagonist may be undervalued when tested against agonists such as ATP, ADP, and AMP, which are substrates for ectonucleotidases (Bonan et al., 1999). However, this is unlikely to explain its lack of effect in the present study, which is more likely to be because ATP acts at suramin-insensitive receptors, likely A_{2A} adenosine receptors (Rayment et al., 2007). A study was conducted in perfused rat hearts to investigate the effect of suramin on vasodilator responses to ATP and 2-methylthio-ATP (2-meSATP) (Vials & Burnstock, 1994). In that study, it was found that suramin, at a concentration of 100 μM, inhibited the vasodilator response to low concentrations of ATP (0.05 μM and 0.005 μM), but not higher concentrations of ATP. The study demonstrated that suramin inhibited relaxation induced by 2-

meSATP in the perfused rat hearts, suggesting that it may be an effective inhibitor of P_2Y purinoceptors (Vials & Burnstock, 1994).

Apyrase inhibited the relaxation response of ATP, which suggests that apyrase increased the breakdown of ATP. This effect may be caused by enzymatic activity of apyrase or by depolarization of the cell as a result of high K^+ concentrations. In guinea pig and rabbit coronary arteries, ATP relaxation was reported to be affected by changes in K^+ levels. The hyperpolarisation of ATP was reduced or abolished as K^+ levels increased (Keef et al., 1992).

The conclusion of this chapter revolves around the relaxation response to a single concentration of adenosine (30 μM) and ATP (100 μM). Further, 8-SPT and apyrase significantly decreased the responses to adenosine and ATP. The findings on the effect of 8-SPT suggest that adenosine A_2 receptors play a significant role in mediating these relaxations. Moreover, the chapter shows that apyrase can affect vascular function either because of its enzymatic activity or because of its high concentration of K^+.

Chapter 6 General Discussion

MPO is an enzyme in immune cells that produces HOCl. Activated immune cells and neutrophils release it during inflammation or when the body responds to damage (Flouda et al., 2021). The production of HOCl is essential for the body's natural defence against bacteria and other pathogens. However, excess HOCl can damage tissues after prolonged exposure in chronic inflammation. For example, atherosclerosis and coronary artery disease as well as myocardial infarction are diseases associated with chronic inflammation (Flouda et al., 2021). This study characterized the direct vasomotor response to HOCl and demonstrated the possible involvement of purine nucleotides and nucleosides in the response in PCA.

The findings indicate that HOCl induces rapid relaxation and contraction dependent on the endothelium, followed by sustained relaxation independent of the endothelium in the PCA. The study found that purinergic signalling plays a role in these responses. It may involve ATP, which promotes the release of NO, a potent vasodilator and component of vascular homeostasis. Adenosine (P1) receptors appear to be involved in this process, as evidenced by the use of 8-SPT. Suramin was used to investigate the possible involvement of P2 receptors. The use of probenecid and carbenoxolone suggests that HOCl induces the release of purines from cells via connexin and pannexin 1channels, with the purines acting on vasomotor P2 receptors. A subset of P2 purinoceptors (P2X1, $P2Y_1$ or P2X4) was excluded from participation in the study since specific antagonists for these receptors were not found to affect HOCl responses. Compared to untreated HCAECs, HOCl-treated HCAECs released higher levels of ATP into the culture medium after 1 minute.

6.1 ATP Release in Inflammation

It is known that ATP is released from damaged, necrotic, and apoptotic cells and from inflammatory cells. The same mechanisms that release ATP from cells will also release UTP from cells (see Introduction) but for clarity this discussion refers principally to ATP (and purine nucleotides).

Necrotic cells release ATP, which is recognised by inflammatory cells. Extracellular ATP acts as a "find-me" signal to mobilise phagocytes, which eliminate necrotic cells (Elliott et al., 2009; Ravichandran, 2011). Apoptotic cells release ATP through pannexin-1 channels. It has long been known that ATP serves as a signal to inflammatory cells that directs them to the site of injury as damage-associated molecular patterns, or "danger signals" (Ayna et al., 2012). It has been demonstrated, in particular, that ATP released by apoptotic cells attracts monocytes to remove apoptotic cells. Not only is ATP released by apoptotic cells important for phagocyte recruitment, but it is also essential for macrophages to secrete IL-1 (Lauber et al., 2004; Qu et al., 2011; Ravichandran, 2011; Ayna et al., 2012). Inflammation is associated with the release of nucleotides as a result of the significant difference between intracellular and extracellular spaces in terms of nucleotide content. During infection, PAMPs are expressed, causing inflammatory cells to release ATP. Microglia, macrophages, and monocytes release ATP through vesicular exocytosis in response to different stimuli, such as ionomycin and lipopolysaccharide. Additionally, ATP can also be released by connexin 43 and pannexin-1 channels in monocytes and macrophages (Dosch et al., 2018).

Inflammatory cytokine and bacteria load are increased when ATP activates P2X7 receptors (Elliott et al., 2009). As well as monocytes, PMNs play an important role in the acute phase of the inflammatory response to PAMPs. It has been demonstrated that PMNs release ATP when activated with N-formyl Met-Leu-Phe, a potent chemoattractant factor, and lipopolysaccharide (Eltzschig et al., 2006).

Hypoxia stimulates the production of inflammatory cytokines through the activation of hypoxia-inducible factors. Hypoxia can also cause inflammation by promoting the release of ATP. As a result of hypoxia, endothelial cells release ATP through the activation of phosphoinositide 3-kinases and Rho-associated protein kinases. Prostaglandins and NO are released as a result of the released ATP, leading to vasodilation (Dosch et al., 2018).

This study looked at the vasomotor response of blood vessels to a specific stimulus, namely HOCl, that is released in different conditions such as inflammation, sepsis, and hypoxia, which can occur in inflammation; it looked specifically at the possible involvement of ATP, and adenosine which can be formed form ATP metabolism.

In the present study, ATP and adenosine were used as single concentrations rather than cumulative response curves because inflammation conditions result in responses that are sudden with abrupt onsets (Chen et al., 2018), in contrast to cumulative responses. This study was focused on what happens when a large amount of ATP is released at once, a process that is poorly understood, especially in inflammation (King et al., 1990).

6.1 Endothelium and NO Involvement in the Rapid Relaxation and Contraction of HOCl Response

As demonstrated in Chapter 3, HOCl at 100 μM and 500 μM evoked a multiphasic response on the PCA. This consisted of rapid and transient relaxation, a return to baseline, and slow relaxation over 60 minutes for 100 μM HOCl. The higher concentration of HOCl used, 500 μM, exerted the same effect; however, after rapid relaxation, the return to baseline was replaced by a contraction which preceded the slow relaxation. Vasodilation is the first phase of acute response to inflammation, which is triggered by the endothelium's release of vasodilating agents. At this point, increased blood flow can contribute to the arrival of immune cells at the site of inflammation and restore tissue function. Blood vessel contraction is the second phase of this response, as a protective mechanism against excessive blood flow and tissue damage in cases of inflammation. Thirdly, the response involves prolonged vasodilation as a result of the release of factors that counteract the vasoconstriction (Sherwood & Toliver-Kinsky, 2004; Villano et al., 2009). The investigation in Chapter 3 demonstrated that vascular smooth muscle is involved during slow relaxation, while contraction and rapid transient relaxation are entirely dependent on the endothelium. The rapid relaxation in response to 100 and 500 μM HOCl and the contraction response to HOCl 500 μM on the PCA are also influenced by NOS inhibitors. These findings indicate the possible involvement of NO in the rapid relaxation and contraction response. However, the actions of inactive enantiomers (D-NAME, and D-NMMA) were unexpected and suggest that these compounds are not acting through selective inhibition of eNOS under the conditions of the present

study. A precontracted tissue containing KCl instead of U46619 inhibited the HOCl-induced rapid relaxation and slow relaxation over a period of 60 minutes. It appears that the relaxation of PCA rings caused by HOCl occurs through a mechanism that is sensitive to membrane hyperpolarization.

Chapter 3 provides new insight into the impact of HOCl on vascular contractility. The study explored the direct effects of single HOCl concentrations on the PCA as well as the role of smooth muscle and the endothelium. As opposed to previous studies, which primarily examined the effects of HOCl on responses to other vasodilators/vasoconstrictors after incubation for a specific time (Zhang et al., 2001; Stocker et al., 2004; Harper et al., 2023), it is interesting to focus on the potential role of HOCl in compensating impaired endothelium-dependent relaxation, a condition that it is typically known to induce (Leipert et al., 1992; Stocker et al., 2004; Harper et al., 2023). HOCl has been well established to disrupt the endothelium's relaxation (Stocker et al., 2004). However, it was shown in the present study that HOCl may also induce a direct relaxation response. In the event that such a mechanism exists *in vivo*, it may serve to balance the detrimental effects of HOCl on the endothelium, thereby maintaining or even promoting vascular relaxation despite these impairments. The role of HOCl in endothelial and vascular smooth muscle function and vascular health should be investigated in greater depth in future research.

Chapter 3 indicates that HOCl induces endothelium-dependent vascular responses of rapid relaxation followed by contraction in the PCA, which can play a role in the inflammatory response following the release of MPO by neutrophils, monocytes and

macrophages. Gaining a more comprehensive understanding of the endothelium's involvement in regulating vascular tone is important, particularly in the context of inflammation where HOCl is generated by activated neutrophils. The endothelium could be part of a vasomotor (vasodilatation) response that occurs in inflammation to attract inflammatory cells to the injured area. On the other hand, HOCl-induced endothelium-dependent relaxation is transient, so the prolonged relaxation, which was endothelium-independent, may be more important in this context.

In this context, the HOCl-induced release of NO from the endothelium in the rapid relaxation phase may work in the preservation of vascular equilibrium during inflammation. Moreover, HOCl induced relaxation in the PCA rings through a mechanism that appears to be sensitive to changes in membrane hyperpolarisation. As reported by Cowan and Cohen (1991), NO may play a role in this process due to its established role as a mediator of EDH. These mediators induce several advantageous effects within the circulatory system, such as controlling vascular tone, the proliferation of VSMCs, and inflammation. The present study differs from previous work in showing that HOCl inhibits NO activity by altering eNOS activity, resulting in impaired vascular function (Stocker et al., 2004; Zhang et al., 2001). The chlorination of L-arginine by HOCl leads to the depletion of the substrate needed for the activity of eNOS (Zhang et al., 2001). It is likely that this difference is due to variations in experimental conditions or approaches, such as concentration and time of incubation.

Purine nucleotides, including adenosine and ATP, play an important role in

extracellular signalling, and they are involved in a variety of physiological and pathological processes, including vasomotor responses, and in inflammation. Thus, it would be relevant to investigate their contribution to the PCA's response to HOCl. This objective formed the basis for Chapters 4 and 5.

6.2 Purinergic Receptor Involvement in HOCl Response

The findings in Chapter 4 provide important insights into the role of purinergic signalling in HOCl's effects on vascular responses in the PCA. An adenosine P1 receptor blocker, 8-SPT, inhibited the rapid relaxation of 100 and 500 µM HOCl and the contraction of 500 µM HOCl, indicating that adenosine (P1) receptors may be involved. As well as this, suramin blocked rapid relaxation of 100 µM HOCl, while the rapid relaxation observed at 500 µM HOCl was evident in the presence of suramin. On the other hand, the contraction to 500 µM HOCl was blocked by suramin. Suramin can inhibit connexin and pannexin channels (Qiu & Dahl, 2008). In this study, probenecid and carbenoxolone inhibited HOCl-induced contraction of 500 µM HOCl as did suramin, suggesting that HOCl may release purines through connexin and pannexin 1channels. Carbenoxolone has different cellular targets and also acts as an inhibitor of connexin (50–100 µM/L) and pannexin 1 channels (EC_{50} = 1–4 µM/L) (Li et al., 2013; Willebrords et al., 2017). It has been suggested, however, that higher concentrations of carbenoxolone (100 µM) may have non-specific effects on the membrane (Goto et al., 2002). According to Bruzzone et al. (2005), the concentration of carbenoxolone required to affect pannexin channels is slightly lower than that required to affect connexin channels. Based on this information and

concentration 100 µM was used, it seems that carbenoxolone blocks both connexin and pannexin 1 channels. Cx37 (EC), Cx40 (EC and VSMC), and Cx43 (EC and VSMC) are the main connexins expressed in the PCA (Gaete et al., 2014). The inhibitory activity of probenecid on Pannexin 1 channel currents is concentration-dependent (IC_{50} of ~150 µM). However, it has no effect on connexin channel currents regardless of concentration (Silverman et al., 2008). The use of suramin, probenecid, and carbenoxolone provided information regarding the possible involvement of nucleotide release and actions at P2 receptors in HOCl response and, therefore, a deeper understanding of how HOCl affects the PCA, demonstrating that both P1 and P2 purinergic receptors are involved in this process.

When purine nucleotides are released in inflammation, a variety of outcomes are triggered. These include facilitating the migration of inflammatory cells, promoting T-helper cell differentiation, stimulating the release of chemokines, cytokines and growth factors, initiating the formation of oxygen and nitrogen radicals, and enhancing the elimination of pathogens (Virgilio et al., 2020). In the context of inflammation, the primary purinergic receptors implicated are $P2Y_1$, $P2Y_2$, $P2Y_6$, P2X4, and P2X7, while in the P1 subfamily, the most relevant ones are A_{2A}, A_{2B}, and A_3 (Virgilio et al., 2020).

Consequently, suramin can affect HOCl independently of its effects on P2 purinoceptors. Accordingly, another antagonist of P2 purinoceptors, PPADS, which has a structure different from that of suramin (Charlton et al., 1996), did not replicate the effects of suramin. It is possible that suramin's influence on HOCl may occur

through mechanisms unrelated to P2 purinoceptors, but it should be noted that the lack of effect of PPADS on connexin and pannexin 1channels (Ziganshin et al., 1994) suggests suramin's inhibition of HOCl response may be dependent on its actions involving the connexin and pannexin 1channels.

An important finding of this study is the inhibition of HOCl responses by apyrase. The role of apyrase, an enzyme that degrades ATP and ADP to AMP (Kammoe et al., 2021), indicates the importance of purinergic signalling (in particular the activation of P2 receptors) in mediating HOCl's effect on vascular function. There was an intriguing finding that apyrase blocked all components of the HOCl response (rapid relaxation, contraction, and prolonged relaxation), and almost completely blocked it, in contrast to antagonists of P1 and P2 receptors, as well as various inhibitors (e.g. the pannexins inhibitors) had always inhibited one or two of the components and had shown partial inhibition. Madry et al. (2018) demonstrated that apyrase affects the morphology and function of microglial cells in the brain. According to their study, microglial cells, which are responsible for monitoring the brain for signs of infection or damage, were altered by apyrase treatment; these changes were not a result of ATP depletion but rather of a high concentration of K^+ that caused a significant depolarisation of the cells. This may affect microglial activity and surveillance. As shown in Figure 3-10 in Chapter 3, a high concentration of K^+ also inhibited relaxation responses to HOCl. According to these results, apyrase may affect HOCl response through ectonucleotidase activity or by a high K^+ concentration.

In summary, inflammation is characterised by an increase in the production of

reactive species such as HOCl by neutrophils (Flouda et al., 2021). Chapter 4 suggests that HOCl may release ATP during inflammation, which then influences vascular function by acting on purinergic receptors.

A notable release of ATP from HCAECs after HOCl administration provides compelling evidence that ATP plays an important role in HOCl responses. HOCl-induced responses may be mediated by ATP through its actions on endothelial P2 receptors, which is consistent with ATP's known role as a signalling molecule. It highlights ATP's potential role in the rapid relaxation to HOCl, which was endothelium-dependent and involved ATP promoting the release of NO, a potent vasodilator and key component of vascular homeostasis. Pharmacological investigations suggest that $P2Y_1$ and $P2Y_2$ receptors are primarily responsible for endothelium-dependent vasodilation through the release of NO, EDH, and prostaglandins. This response is also mediated by $P2Y_6$ and $P2Y_{12}$ receptors, to a lesser extent. (Kennedy et al., 2013).

6.3 ATP and Adenosine Direct Effect

Chapters 3 and 4 extensively examined HOCl's direct effects on the PCA. According to the results, HOCl's effect may be mediated by purine release, which acts on endothelial P2 receptors causes NO release, and endothelial P1 receptors. The pharmacology of vasomotor responses to exogenous adenosine and ATP were examined directly in Chapter 5 based on the findings.

The expression of purinergic receptors in the PCA has been extensively described in

the literature. In the PCA, mRNA encoding $P2Y_4$ and $P2Y_6$ receptors as well as $P2Y_2$ receptors are associated with smooth muscle contraction (Rayment et al., 2007). $P2X_1$ and $P2Y_2$ receptors are expressed on the smooth muscle of the PCA, and their activation causes vasoconstriction (Burnstock, 2007). $P2Y_1$ has been found in the endothelial cells of most blood vessels; it contributes to vasodilation through the activation of endothelial NOS and the production of EDRF and EDH (Ralevic & Burnstock, 1998).

It has been suggested that A_1 receptors, alongside A_2 receptors, are located on smooth muscle exhibiting vasorelaxation in the PCA (Merkel et al., 1992; Dart & Standen, 1993; Abebe et al., 1994). In cultured endothelial cells of the PCA, both A_{2A} and A_{2B} receptors reportedly contributed to the release of NO, but it was suggested that A_{2A} receptors are dominant (Olanrewaju & Mustafa, 2000). Following this research, it was found that the smooth muscle is responsible for A_{2A}-mediated relaxation (Rayment et al., 2007). There are multiple P1 subtypes involved in endothelium-dependent coronary vasodilation in guinea pigs; A_1 facilitates the release of both NO and prostaglandin I_2, whereas A_{2A} and A_3 receptors act predominantly through NO (Rubio & Ceballos, 2003; Burnstock & Pelleg, 2015).

Adenosine caused an endothelium-independent relaxation response, while ATP caused a partially endothelium-dependent relaxation response. It is noteworthy that neither L-NAME nor suramin affected the relaxation response to adenosine or ATP. In light of this observation, it seems unlikely that adenosine and ATP directly activate P2 purinergic receptors in the PCA, or that they do so in a manner that is sensitive to

inhibition by either L-NAME- or suramin-insensitive P2 receptors; as an example, the effects of suramin on ATP-mediated responses may be complicated by the fact that suramin is not capable of blocking all P2 receptors. For example, at concentrations up to 100 μM, suramin inhibits $P2Y_2$ receptors but does not affect $P2Y_4$ or $P2Y_6$ receptors (Charlton et al., 1996; Rayment et al., 2007). In order to further substantiate this interpretation, additional studies using P2 receptor antagonists as PPADS will be required.

Both 8-SPT and apyrase inhibited the responses of adenosine and ATP responses. The effect of 8-SPT suggests that adenosine receptors (P1) are involved in the relaxation response, whereas the effect of apyrase suggests that released adenosine (or adenosine formed from the metabolism of released ATP) also contributes to this response, confirming the importance of purinergic signalling in vascular response.

In conclusion, Chapter 5 demonstrated distinct but overlapping effects of adenosine and ATP on PCA relaxation, which overlap with those described in Chapters 3 and 4, thus contributing to understanding of HOCl vasomotor regulation in coronary arteries. Rather than focusing on HOCl's effects on the PCA, this chapter discussed adenosine and ATP's direct effects on the PCA.

6.4 Clinical Implications

In terms of clinical implications, understanding the role of purinergic signalling in inflammation-induced vascular dysfunction may guide the development of targeted therapies.

The disease of atherosclerosis is characterised by the development of characteristic lesions known as atheromatous plaques. This is the most prevalent pathological state in peripheral arterial, coronary artery, and cerebrovascular disease. In the majority of experimental studies, purinergic signalling is implicated in atherogenesis (Ferrari et al., 2015; Ferrar et al., 2021). VSMC proliferation and migration and endothelial dysfunction are induced by $P2Y_{12}$ receptor activity, and inflammatory cell activity is induced by $P2Y_{12}$ receptor activity. Furthermore, ADP-induced platelet activation and platelet aggregation may be amplified and maintained by $P2Y_{12}$ receptor activity (Gao et al., 2019). Inhibiting $P2Y_{12}$ receptor may be effective in treating other cardiovascular diseases, such as atherosclerosis. Studies in patients with ischaemic stroke or transient ischaemic attack associated with atherosclerosis have suggested that inhibition of the $P2Y_{12}$ receptor may be a more effective antiplatelet agent than other antiplatelet drugs. The inhibition of $P2Y_{12}$ receptor or gene deletion has also been demonstrated to attenuate the initiation and progression of atherosclerosis in experimental studies (Gao et al., 2019; Wernly & Zhou, 2020).

As apyrase inhibits the HOCl response, it may be used in the treatment of vascular disorders caused by inflammation. Additionally, there is a possibility that ATP levels could be used as a biomarker for inflammation-induced vascular dysfunction since

ATP is released during inflammation and has an effect on vascular function through P2 receptors. Using idiopathic nephrotic syndrome as an example, Bertelli et al. (2011) showed that the nucleotide pathway plays a critical role in the generation of ROS by polymorphonuclear leukocytes. A substantial proportion of ROS (30%–40%) was produced by ATP. As a result of adding apyrase to the medium, ROS levels decreased by 40%, indicating that ATP plays a crucial role in modulating ROS production. They also found that ATP-selective $P2X_7$ antagonists, such as Brilliant Blue G, reduced ROS generation in the same manner as apyrase (Bertelli et al.). Moreover, when inflammation is uncontrolled, organ failure occurs, resulting in sepsis. Csóka et al. (2015) revealed that ectonucleotidase CD39 reduces inflammation and organ damage while enhancing survival rates in sepsis.

Sepsis is characterised by neutrophil dysregulation, which results in abnormal migration patterns, including increased MPO levels, a biomarker associated with sepsis (Martín-Fernández et al., 2021). MPO has been recognised as a biomarker associated with sepsis in intensive care units (Schrijver et al., 2017). As well, 2-chlorofatty aldehyde and 2-chlorofatty acid, both stable chlorinated lipids in inflammation which correlate with HOCl, indicate neutrophil involvement in acute respiratory distress syndrome in septic patients (Schrijver et al., 2017). Septic shock, which is characterised by significant hypotension and vasodilation, has been shown to be associated with increased plasma ATP levels (Dosch et al., 2018), suggesting that HOCl may play a role in the release of ATP, but its possible interaction with a variety of cellular processes warrants further investigation.

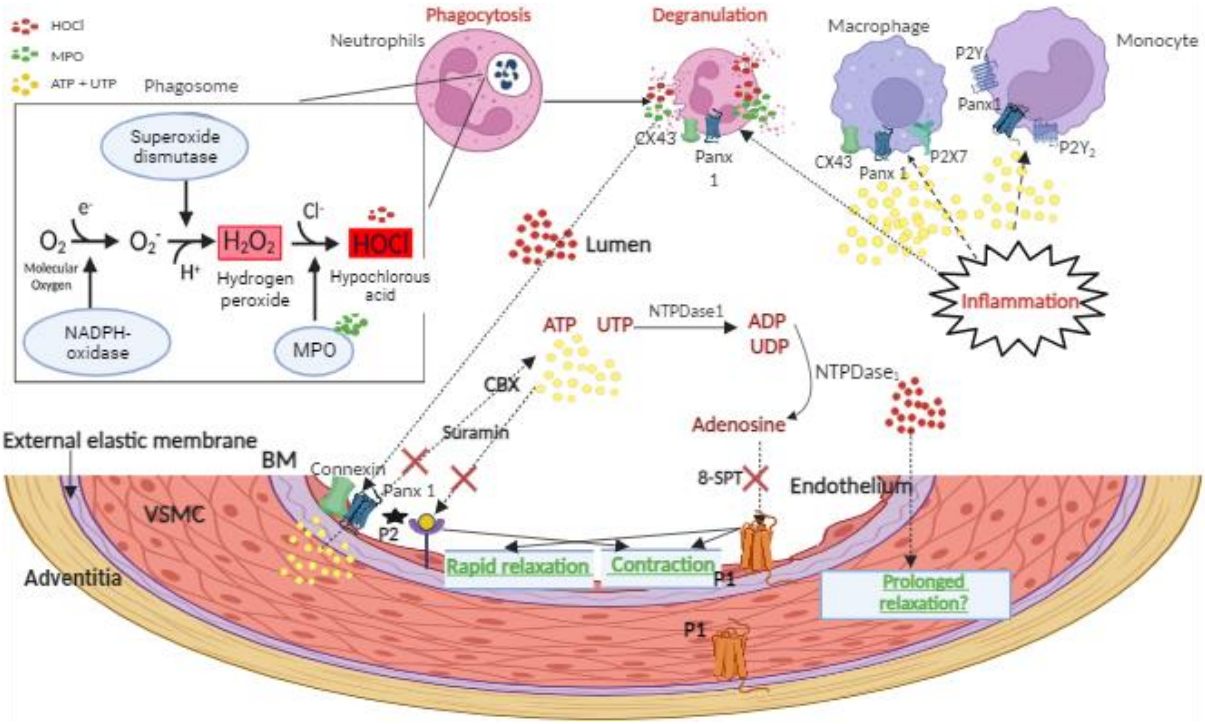


Figure 6-1. Hypothesised schematic of the effect of HOCl on PCA and ATP release from different types of inflammatory cells (Jacob et al., 2013).BM: basement membrane; CBX: carbenoxolone; HOCl: hypochlorous acid; MPO: myeloperoxidase; NADPH: Nicotinamide adenine dinucleotide phosphate; VSMC: vascular smooth muscle cell.* At 100 μM of HOCl, P2 causes relaxation, whereas at 500 μM HOCl, it causes contraction. Adapted from Le et al., (2019).

6.5 Future Directions in Identifying the Role of Purine Receptor in HOCl Response

By providing insights into how HOCl affects vascular function, this study can contribute to unravelling the role of HOCl in diverse cardiovascular disorders. Hypertension and atherosclerosis have been linked to excessive levels of HOCl production (Stocker et al., 2004). Inflammation results in the release of large

amounts of nucleotides and nucleosides into the extracellular space (Burnstock & Ralevic, 2014). Therefore, it would be beneficial to understand how varying concentrations of HOCl affect vascular function. There is uncertainty as to whether nucleotides and nucleosides are involved in the vasomotor response to HOCl. Therefore, the purpose of this thesis was to investigate the involvement of purine nucleotides and nucleosides in the vasomotor response to HOCl.

The study found that purinergic signalling is involved in these responses. ATP plays a role in this process, as it promotes the release of NO, a powerful vasodilator and component of vascular homeostasis. The use of 8-SPT indicates that adenosine (P1) receptors are involved in this process. Suramin, probenecid, and carbenoxolone (pannexin 1 and connexin channel blocker) inhibited the contraction to 500 μM HOCl, consistent with HOCl-induced purine release via pannexin 1 and/or connexin channels, with purines acting at vasomotor P2 receptors. An HOCl assay will be conducted in order to investigate the potential inhibition of the HOCl response by compounds such as suramin, 8-SPT, and apyrase. These compounds could attenuate HOCl response by targeting purinergic receptors. In addition, these compounds are also capable of directly neutralizing HOCl, thereby inhibiting its response without interfering with purinergic receptors.

Although the current study examined the proximal end of the coronary arteries, which are large arteries, inflammation is often initiated in smaller vessels (Lusis, 2000). It is possible to gain a deeper understanding of these early inflammatory changes in smaller vessels through the use of myography. Myography experiments

can be used to investigate the vasomotor response of small arteries to HOCl. Integrating data from both large and small arteries can provide a more comprehensive understanding of vascular inflammation and its progression. This will allow for the development of targeted interventions for the treatment of cardiovascular diseases. P2X7 receptors have attracted considerable attention due to their pronounced role in inflammation and immunity, and antagonists of these receptors may be effective in treating chronic systemic inflammatory diseases (Erlinge & Burnstock, 2008; Kennedy, 2021). This study did not address this receptor due to solvent-related issues, but its examination could be extremely valuable. Investigating the P2X7 receptor is crucial because it is well known that the P2X7 receptor plays a crucial role in promoting both pro-inflammatory and anti-inflammatory responses, making it a potential therapeutic target for the treatment of inflammatory conditions (Virgilio et al., 2017). Developing new approaches for treating conditions such as sepsis and other chronic systemic inflammatory diseases may be possible by blocking the P2X7 receptor (Erlinge & Burnstock, 2008; Kennedy, 2021).

Furthermore, Western blotting and PCR could be used to investigate the role of nucleotides and nucleosides in HOCl response. PCR provides insight into the gene expression levels of purinergic receptors by measuring the amount of a specific mRNA. It is possible to detect purinergic receptors involved in the response to HOCl by Western blotting, a method used for the analysis of protein expression. In order to investigate the expression of purinergic receptors, for example, P2X7, in response to HOCl, Western blotting and PCR could be used. Using Western blots, these

receptors can be detected and quantified at the protein level. HOCl could be used to compare the levels of these receptors in cells treated with HOCl to cells untreated with HOCl in order to determine whether it affects their expression. Moreover, it is possible to measure the expression of these receptors at the mRNA level using PCR, particularly quantitative PCR. In addition, PCR can be used to determine whether purinergic antagonists that affect HOCl responses in organ bath techniques, such as suramin, 8-SPT, and apyrase, impact the expression of these receptors.

It is possible to use knockout technique to inactivate or "knock out" specific genes of purinergic receptors in vessels. As an example, it can be tested on isolated coronary arteries by knocking out the genes for these receptors (Haanes et al., 2016). It is possible that this method provide insight into whether these receptors are required to respond to HOCl.

It is unclear what role apyrase plays in the HOCl response. Apyrase hydrolyses both ATP and ADP (Kammoe et al., 2021). Madry et al. (2018) reported that ATP/ADP depletion is not responsible for the decrease in microglial activity after exposure to apyrase, but rather an increase in K^+ levels. Inhibition of ectonucleotidase or elevated K^+ levels may have been responsible for the inhibitory effects of apyrase on HOCl response. K^+ (10 mM) is capable of attenuating the vasodilation caused by adenosine in hamster cremaster muscle (Lamb & Murrant, 2015). Adenosine has a relaxation effect on the hog carotid artery that decreases with an increase in K^+ concentration (Herlihy et al., 1976). Future experiments might measure vasodilation by treating PCA with adenosine and different K^+ concentrations. In addition, it could

study the role of ATP/ADP signalling in the effects of apyrase. It may be possible to use fluorescent probes that can detect ATP or ADP (Kreiter et al., 2020) in order to measure changes in ATP/ADP signalling after treating cells with apyrase, use this information to determine whether the effects of apyrase are due to changes in the ATP/ADP signalling pathway or to other causes.

In future studies, cumulative dose-response methods have significant potential, particularly for studying adenosine and ATP effects on PCA. The advantage of this approach is that it provides an understanding of the dose-response relationship, the plateau, and the maximum response that can be achieved. Moreover, conducting these experiments in a cumulative manner makes them more efficient because extensive data can be collected within a single experimental setup. There are, however, a number of challenges associated with this approach, notably receptor desensitization. As a result of repeated exposure to increasing doses, adenosine receptors may become less responsive, potentially leading to attenuated responses at higher doses.

Considering that the effect of most antagonists and inhibitors used in this study is on rapid relaxation and contraction mediated by HOCl, future research may focus on examining the prominent sustained relaxation. Various signal transduction and/or effector mechanisms, including PKC, PLA2, and the BK_{Ca} channel, may be involved in the vascular response to HOCl (Richard et al., 1990; Tschudi et al., 1991; Hwa et al., 1994). This focus could provide a more balanced and comprehensive understanding, particularly as sustained vasodilation is considered an important

marker in inflammatory responses (Zanoli et al., 2020).

In this study, the main findings have been derived from in vitro studies, which provide valuable insight into purinergic signalling and vascular responses to HOCl. Further study is required to determine whether these findings are physiologically and clinically relevant. In light of these findings, follow-up studies will be required in order to determine the role of purinergic signalling and the mechanisms underlying its effects in order to identify novel therapeutic targets for cardiovascular diseases.

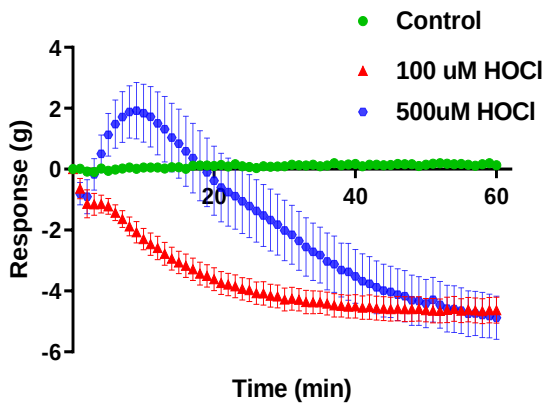
6.6 Conclusion

This study investigated the role HOCl in vascular responses which are considered part of the inflammatory response. Immune cells produce HOCl to fight pathogens, but excessive amounts can affect functional/vasomotor response.

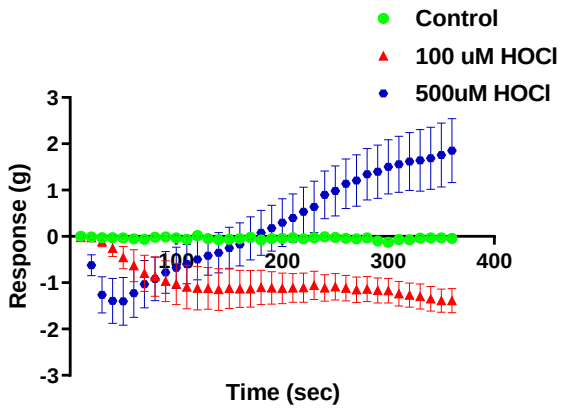
In PCA, HOCl induces endothelium-dependent action including rapid relaxation and contraction, followed by endothelium-independent sustained relaxation. According to the study, P1 and P2 receptors may be involved in the PCA's response to HOCl. Furthermore, the evidence suggests that connexin and pannexin 1channels may be involved in the release of ATP in response to HOCl. It is clear that purinergic signalling plays an important role in inflammatory responses as a result of this interaction. This study increases our understanding of the role of purinergic signalling in inflammation. The research provides a solid foundation for future studies aimed at targeting purinergic signalling to control inflammation in the cardiovascular system.

Appendices

A



B



Responses of porcine coronary arteries expressed as grams that mediated by HOCl at different concentrations: control (n=6), 10 μM (n=7), 100 μM (n=7) and 500 μM (n=7): (A) responses measured for 60 minutes at 1 min intervals; (B) expanded time course of the first 6 minutes measured at 10 second intervals (360 seconds) to show the initial rapid relaxation.

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