ADENOSINE AND LIDOCAINE (AL) AS A VASODILATOR IN CARDIAC PROCEDURES AND A STORAGE SOLUTION FOR VASCULAR BANKING

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DECLARATION ON ETHICS

The research presented and reported in this thesis was conducted within the guidelines for research and ethics outlined in the *National Statement on Ethics Conduct in Research Involving Human* (1999), the *James Cook University Policy on Experimentation Ethics Standard Practices and Guidelines* (2001), and the *James Cook University Statement and Guidelines on Research practice* (2001). Relevant research methodology reported in this thesis received clearance from the James Cook University Experimentation Ethics Review. Animal Ethics numbers A1535 and human ethics number H3663.

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ABSTRACT

Introduction

Cardiovascular disease is one of the most common causes of morbidity and mortality worldwide, and globally has contributed to more than 17 million deaths in a year. Coronary heart disease (CHD) alone is responsible for one in seven deaths in the US, and the mortality rate is expected to rise by 10% per year over the next 20 years. One of the invasive treatments for CHD is coronary artery bypass grafting (CABG), which aims to improve cardiac tissue perfusion by grafting another blood vessel to bypass the narrowed or blocked coronary artery. Currently the artery conduit is the standard choice for this procedure, however, the main concern for the use of artery conduits is that they have a high probability of inducing perioperative vasospasm. Therefore, it is crucial to maintain functionality of the conduit during harvest, pressure testing, storage and implantation.

The current strategy to prevent artery vasospasm involves a range of anti-spasmodic agents. Some of the most commonly used vasodilators in the surgical setting include Ca^{2+} antagonists (diltiazem, verapamil), nitrates (nitroglycerin, glyceryl trinitrate), and phosphodiesterase inhibitors (papaverine). However, the results remain unsatisfactory. Accordingly, the search for a vasorelaxation agent to reduce graft spasm remains an ongoing pursuit, which if successful may also be applicable to vascular surgery and neurosurgery. The aim of this thesis is to explore the use of adenosine and lidocaine combination as a potential vasodilator to improve arterial grafting using *in vitro* models.

Methods

In this thesis, vascular reactivity was assessed using two different *in vitro* methods: 1) Isometric force measurements for the isolated male rat aortic ring studies, and 2) Pressured myography for the isolated guinea pig mesenteric artery studies. Isometric force measurements of vasoreactivity were used as the basis for Chapters 3, 4 and 5; and for Chapter 6 after static cold storage. The pressure myography system was only used in Chapter 5.

Chapter 3 investigates the relaxation effect of adenosine as a single drug on rat aorta as well as its possible mechanisms of action. In this chapter, aortic rings were freshly harvested from adult male Sprague Dawley rats and equilibrated in an organ bath containing oxygenated, modified Krebs Henseleit (KH) solution (11 mM glucose, pH 7.4, 37°C). Isolated rings were pre-contracted sub-maximally with 0.3 μM norepinephrine (NE), and the effect of increasing concentrations of adenosine (1 to 1000 μM) was examined. The effect of antagonists on adenosine relaxation, such as N^G-nitro-L-arginine methyl ester (L-NAME), indomethacin, 4-aminopyridine (4-AP), glibenclamide, 5-hydroxydecanoate (5-HD), ouabain, 8-(3-chlorostyryl) caffeine (CSC) and 8-[4-[4-(4-chlorobenzyl)piperazide-1-sulfonyl)phenyl]]-1-propylxanthine (PSB-0788) were examined in intact and denuded aortic rings. Rings were dilated with 100 μM papaverine after each experiment to confirm viability.

In Chapter 4, lidocaine effects and mechanisms of action on rat aorta vasorelaxation were examined. Incremental concentrations of lidocaine (1 to 1000 μM) were administered and tested against 0.3 μM NE pre-contracted rat aorta. The effects of antagonists L-NAME, indomethacin, 4-AP, glibenclamide, 5-HD, ouabain, CSC and PSB-0788 were also examined against lidocaine relaxation. As in Chapter 3, rings were tested for viability after each experiment with maximally dilating 100 μM papaverine.

Chapter 5 focused on the effect of the combination of adenosine and lidocaine on rat aortic ring relaxation compared to each drug alone. Rings were pre-contracted sub-maximally with 0.3 μM norepinephrine, and the effects of increasing AL, A or L (up to 1.0 mM) were examined in intact and denuded rings. In this Chapter 5, the vasorelaxation effect of AL as a combination was further explored in the mesenteric artery of guinea pig. This study used the pressure myograph system to examine mesenteric conduit relaxation and the vascular dilatory response to adenosine, lidocaine and AL during luminal and abluminal administration. This methodology is often used to investigate small vessel function (diameter >60 μM) under near physiological conditions of pressure and flow by measuring vessel diameter and flow in time. Mesenteric artery segments were isolated from guinea pigs and mounted in an arteriograph containing KH solution and pressurized to 60 mmHg. Arteries were pre-constricted with 10^{-8} M vasopressin and AL, A or L was administered luminally or abluminally. Diameters were measured using video-microscopy.

Chapter 6 explores the potential use of AL as an additive in a vessel preservation solution. In this chapter, thoracic aortic vessels were harvested from 300-350 g

Sprague Dawley rats and transferred to a container with pre-cooled KH solution. Vessel segments were cleaned and cut in 3-mm length rings and stored at 4°C for six days in one of the following preservation solutions: 1) Krebs Henseleit (KH), 2) modified KH (low Ca^{2+}/high Mg^{2+}), 3) modified KH + adenosine-lidocaine (KH+AL), or 4) modified KH + AL and melatonin and insulin (KH+ALMI). After 6-day storage, physiological (contraction and relaxation) function of the preserved aortic rings was measured using an isometric force transducer. Contraction was induced by norepinephrine (NE; 0.3 μM) and potassium chloride (KCl; 60 mM). Vessel relaxation in response to acetylcholine (ACh; 10^{-6}-10^{-3} M) and sodium-nitroprusside (SNP; 10^{-6}-10^{-3} M) was tested after preconstriction with 0.3 μM NE. At the end of each experiment, rings were maximally dilated with 100 μM papaverine to confirm viability of the vessels.

Results

Adenosine induced a dose-dependent, triphasic relaxation response, and the mechanical removal of the endothelium significantly decreased adenosine relaxation above 10 μM. Interestingly, endothelial removal significantly reduced the responsiveness (defined as % relaxation per μM adenosine) by two-thirds between 10 and 100 μM, but not in the lower (1-10 μM) or higher (>100 μM) ranges. In intact rings, L-NAME, but not indomethacin, significantly reduced relaxation, suggesting a role of nitric oxide (NO) but not prostacyclin in adenosine endothelium-dependent relaxation. Antagonists of voltage-dependent K_v (4-AP), sarcolemmal K_{ATP} (glibenclamide) and mitochondrial K_{ATP} channels (5-HD) led to significant reductions in adenosine relaxation in both intact and denuded rings, with the Na^+/K^+-ATPase antagonist ouabain having little or no effect. Adenosine-induced relaxation appeared to involve the A_{2a} receptor, but not the A_{2b} subtype. In contrast to adenosine, lidocaine relaxation in intact rings was biphasic between 1 to 10 μM (Phase 1) and 10 to 1000 μM (Phase 2). Mechanical removal of the endothelium resulted in further relaxation, and at lower concentrations ring sensitivity (% relaxation per μM lidocaine) significantly increased 3.5 times compared to intact rings. The relaxing factor(s) responsible for enhancing lidocaine relaxation did not appear to be NO- or prostacyclin-dependent, as L-NAME and indomethacin had little or no effect on intact ring relaxation. In denuded rings, lidocaine relaxation was completely abolished by K_v channel inhibition and significantly reduced by antagonists of the MitoK_{ATP} channel, and to a lesser extent the SarcK_{ATP} channel. Curiously, A_{2a} subtype receptor antagonism significantly inhibited lidocaine relaxation above 100 μM, but not the A_{2b} receptor. In combination, adenosine and

lidocaine (AL) increased aortic relaxation from 21 to 100% (0.1-1.0 mM) and relaxation was endothelium-independent. Although adenosine alone was also a potent relaxant of aortic rings, unlike AL relaxation, it was partially endothelium-dependent.

Further investigation of AL effects on mesenteric artery showed that increasing luminal administration of AL in intact mesenteric artery segments produced a potent endothelium-independent dilation up to 90% (p<0.05). Adenosine dilation was endothelium-independent but not lidocaine, which produced 33% dilation only after endothelial removal. Extra-luminal AL and A led to 76% and 80% dilation in intact segments respectively, whereas L resulted in constriction (10-17%).

When exploring the potential use of AL as a preservation solution with 6-day cold storage in Chapter 6, it was found that AL addition in modified KH solution resulted in 100% recovery of NE contractile function in rat aorta, which was superior compared to KH solution alone (89% recovery). However, there was no further recovery in the KCl response over modified KH (76% recovery). A similar result was also shown with ALMI in modified KH, which led to 100% and 86% of contractile function recovery in response to NE and KCl, respectively. Furthermore, AL but not ALMI addition in modified KH significantly improved relaxation function compared to standard KH, with 93% recovery compared to 79% with modified KH alone after six days of storage. Maximal SNP relaxation following 6-day cold storage with either modified KH alone, modified KH with AL or with ALMI recovered 100%.

Conclusions

Adenosine is a potent vasodilator of aortic rings. Adenosine relaxation in NE-precontracted rat aortic rings was triphasic and endothelium-dependent above 10 μM, and relaxation involved endothelial nitric oxide (not prostanoids) and a complex interplay between smooth muscle A_{2a} subtype and voltage-dependent K_v, SarcK_{ATP} and MitoK_{ATP} channels. In contrast, lidocaine relaxation is not as potent as adenosine relaxation, but it appears to be significantly enhanced by endothelial removal, which did not appear to be NO- or prostacyclin-dependent. The unknown factor(s) responsible for enhanced relaxation was significantly reduced by K_v channel inhibition, MitoK_{ATP} channel inhibition, and A_{2a} subtype inhibition indicating a potential role for crosstalk in lidocaine's vasoreactivity. When combined, AL can dilate aortic rings and mesenteric artery segments by up to 90% regardless of whether the endothelium is intact. This may have potential translational significance of AL to improve conduit

protection in cardiac surgery, and other major surgeries where varying degrees of endothelial damage, vasoconstriction or vasospasm are known to occur. In addition, AL has a potential role as an adjuvant in preservation solutions since it improved vascular function after 6-day cold storage. AL addition in modified KH solution significantly improved NE-induced vascular contractility and ACh-induced relaxation compared to standard KH solution. This may indicate that AL improved endothelial preservation during storage, which was not achieved with standard preservation solution.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Cardiovascular disease is one of the major causes of morbidity and mortality worldwide, and globally has contributed to more than 17 million deaths in a year (Roth*, et al.*, 2015). In the USA, coronary heart disease (CHD) alone is responsible for one in seven deaths with a mortality rate of ~370,213 per year (Mozaffarian*, et al.*, 2016). More than 600,000 US citizens experience a new coronary attack each year, and around 300,000 will have a recurrent attack (Mozaffarian*, et al.*, 2016). CHD is expected to increase by 10% per year over the next 20 years, with a projected overall cost increase from $273 billion in 2010 to $818 billion in 2030 (Heidenreich*, et al.*, 2011).

A common treatment for CHD is coronary artery bypass grafting (CABG) and there are more than one million procedures being performed each year globally (DeFrances*, et al.*, 2008). CABG comprises the surgical attachment of a healthy artery or vein graft from the body, which bypasses the blocked portion of the coronary artery and creates a new path for oxygen-rich blood to be delivered to the heart muscle. Surgeons can bypass multiple coronary arteries during one surgery. As CABG surgery enters its fourth decade, the access to grafts and their perioperative protection remains a concern (Goldman*, et al.*, 2004b; Magee*, et al.*, 2008a).

The two major vessel types for bypass surgery are the arterial graft (internal thoracic or radial artery) and saphenous vein grafts (Shi*, et al.*, 2015). While arterial grafts have higher patency rates than saphenous vein grafts which are more prone for intimal hyperplasia (Sur*, et al.*, 2014), an ongoing problem is their increased tendency to spasm early in 4-10% of cases during the perioperative period (Cuminetti*, et al.*, 2017; El Tecle*, et al.*, 2016; Watanabe*, et al.*, 2013). Therefore, there is an unmet need to improve arterial graft protection from spasm during harvest, pressure testing, storage and implantation (Goldman*, et al.*, 2004b; He & Taggart, 2016a).

A range of anti-spasmodic agents has been examined in the literature to address arterial conduit dysfunction (Attaran*, et al.*, 2008). Some of the most commonly used vasodilators in the surgical setting include phosphodiesterase inhibitors (papaverine), nitrates (nitroglycerin, glyceryl trinitrate), and Ca^{2+} antagonists (diltiazem, verapamil)

(El Tecle, *et al.*, 2016; Erdem, *et al.*, 2015; Huh, *et al.*, 2016). Unfortunately, despite decades of investigations there remains no effective treatment based on translational research and clinical trials (Yagami, *et al.*, 2013; Yildiz, *et al.*, 2013). Accordingly, the search for a vasorelaxation agent to reduce graft spasm remains an ongoing pursuit, which if successful may also be applicable to vascular surgery and neurosurgery.

This chapter will provide a brief review on the structure of the arterial blood vessel wall and known mechanisms of vasospasm, and the ongoing search for pharmacological vasodilators that may provide alternative solutions to overcome graft spasm.

1.1 The structure and function of arterial blood vessels

Along with central nervous system (CNS) control of blood flow through arterial baroreceptors and direct innervation through the arterial tree (Thomas, 2011), several local factors influence the regulation of local blood flow including: 1) structural characteristics of the vasculature and wall structure (London & Pannier, 2010), 2) the intrinsic ability of the vascular endothelium and vascular smooth muscle to respond to various stimuli (Flammer, *et al.*, 2012; Triggle, *et al.*, 2012b), and 3) the role of ion channels in the regulation of vascular tone (Jackson, 2000).

1.1.1 Structural characteristics of the vasculature and wall structure

The vascular system is built from a vessel network comprising arteries, veins and capillaries (Ardalani, *et al.*, 2014). The arteries deliver oxygen (O_2)-rich blood from the heart to the entire body, through the aorta, small arteries and arterioles (Ardalani, *et al.*, 2014). The large arteries such as the aorta and carotid arteries are comprised of smooth muscle and elastic layers, and called elastic arteries (Rhodin, 1980; Wagenseil & Mecham, 2009). As the main arteries approach the organs, the size and diameter of the vessels decreases, the vessel wall becomes thinner, less elastic and mostly comprises the smooth muscle layer (muscular arteries) (Rhodin, 1980; Wagenseil & Mecham, 2009). In the aorta and large arteries, the contraction of smooth muscles largely contributes to vessel compliance, which is one of the key determinants of afterload. Meanwhile, the narrower small arteries and arterioles are largely responsible for peripheral vascular resistance regulation through contraction or relaxation of vascular smooth muscle, and are known as the resistance arteries (Martinez-Lemus, 2012; Touyz, *et al.*, 2013).

The veins, on the other hand, are thinner-walled vessels compared to arteries (Touyz*, et al.*, 2013). The dynamic nature of venous tone predominantly affects the capacity of systemic and regional circulation and therefore these are termed capacitance vessels. Ventricular filling and therefore preload is affected by venous blood pressure and the rate of venous return (Touyz*, et al.*, 2013). The exchange between the O_2 and metabolic CO_2 content of the blood and tissues occurs in the vast capillary network, which is supported by a single continuous endothelium layer with endothelial cell junctions acting as the pore system in tunica intima (Karnovsky, 1968). Arterioles together with capillaries, post capillary venules and muscular venules are called the microcirculation (Rhodin, 1980).

1.1.2 Layers of the vessel wall

In general, a blood vessel wall consists of three distinct layers: tunica intima, tunica media and tunica adventitia (Hurlimann*, et al.*, 2002) and is considered a dynamic structure that plays an important role in vascular contraction.

Tunica intima lies at the innermost side of the vessel and gets exposed directly to the lumen and consists of one continuous single layer of endothelial cells and basal lamina (Martinez-Lemus, 2012). Endothelial cells are organised in longitudinal orientation with thickness of approximately 0.5 µm (Rhodin, 1980). A subendothelial layer can be found in the aorta and large arteries, which contains a heterogeneous structural population including collagenous bundles, elastic fibrils, smooth muscles and fibroblasts (Orekhov*, et al.*, 2014).

Tunica media (middle layer) contains smooth muscle cells with layers of elastic lamina, which are connected together with a network of collagenous fibrils (Martinez-Lemus, 2012). Elastic lamina contains elastin that contributes to extensibility and recoil function of the vessel wall, which is particularly important to deal with pulsatile pressure (Keeley, 2013). The number of elastic laminae gradually decreases, while the smooth muscle percentage increases along the arterial system and becomes a major constituent in small arteries. In contrast, only a small number of smooth muscle cells are found in small veins and venules (Wagenseil & Mecham, 2009).

Tunica adventitia is the outermost layer of the vessel wall including the external elastic lamina, terminal nerve fibers, and connective tissues, which mostly comprises of fibroelastic tissues and macrophages (Eichmann & Brunet, 2014; Hurlimann*, et al.*,

2002; Majesky*, et al.*, 2011; Martinez-Lemus, 2012). The thickness of tunica adventitia may vary between vessels. In medium and large-sized veins, tunica adventitia contributes to 60% to 75% of the vessel wall, while only 10% is found in the wall of large arteries (Rhodin, 1980).

1.1.3 The endothelium

The endothelium is a single cell layer forming a continuous lining to all blood vessels in the body (Aird, 2007a, 2007b). In an adult human, the endothelial layer consists of around 10^{13} cells in total (Galley & Webster, 2004). As a multifunctional barrier, the vascular endothelium is dynamic and adaptive in response to spatial and temporal changes to meet regional demands (DeMaio*, et al.*, 2004; Mack*, et al.*, 2009). Indeed, endothelial cells have a specific phenotypic (structural and functional) heterogeneity that depends on the vascular size or site (Aird, 2007a, 2007b).

The physiological roles of the endothelium involve the complex balance between secretory, synthetic, metabolic, immunologic and haemostatic functions (Eliseyeva, 2013). Endothelial cells may serve as an endocrine "organ" by releasing vasoactive substances including nitric oxide, prostacyclin and endothelin, as well as factors involved in vasculogenesis and angiogenesis such as fibroblast growth factors, vascular endothelial growth factors, angiopoietins and matrix metalloproteinases (Adams & Alitalo, 2007; Triggle*, et al.*, 2012b). The endothelium also mediates immune and inflammatory reactions by activating signaling processes that are involved in leukocyte recruitment and extravasation or activation of T-cell memory (Pober & Sessa, 2007). Furthermore, endothelial cells can activate the coagulation system and downregulate anti-coagulation factors during pathological conditions such as sepsis and trauma (Levi*, et al.*, 2002).

The endothelium plays a major role in regulation of vascular tone by adjusting the diameter of the vascular wall through the release of vasoactive substances (Triggle*, et al.*, 2012b; Vanhoutte*, et al.*, 2017). Based on these "vasoactions", endothelium-released mediators are classified into three different categories: endothelium-derived relaxing factors (EDRFs), endothelium-derived hyperpolarizing factors (EDHFs), and endothelium-derived contracting factors (EDCFs) (Aird, 2007a, 2007b).

1.1.3.1 Endothelium-derived relaxing factors (EDRFs)

1.1.3.1.1 Nitric oxide

In early 1980s, Furchgott and Zawadzki showed precontracted vascular rings were relaxed in response to acetylcholine and the relaxation required the presence of an active endothelium and was independent of prostaglandins (Furchgott, 1984). This endothelium-dependent vasodilator was later identified as nitric oxide (NO). NO is a lipophilic free radical with single unpaired electron and has a short half-life of less than five seconds (Heinrich*, et al.*, 2013; Kang, 2014). As the key signaling molecule in vascular homeostasis, NO has important roles beyond vasorelaxation, which include cell proliferation, platelet aggregation and adhesion, as well as low density lipoprotein oxidation (Kang, 2014).

NO is regulated by the expression and activation of endothelial NO synthase (eNOS) that converts amino acid L-arginine to NO (Moncada*, et al.*, 1991) Endothelial NO synthase (eNOS) is constitutively expressed in all endothelial cells and its activities are closely related to intracellular Ca^{2+} level (Figure 1.1) (Koo*, et al.*, 2013). An increase in intracellular Ca^{2+} level allows interaction with protein calmodulin (CaM) to form Ca^{2+}/CaM complex that facilitates eNOS detachment from caveolin (Cav-1) leading to eNOS activation (Busse & Mulsch, 1990; Koo*, et al.*, 2013; Michel*, et al.*, 1997). Shear stress and most NO agonists, including bradykinin, acetylcholine (ACh), adenosine triphosphate (ATP), substance P, and thrombin, induce NO release by stimulating Ca^{2+} discharge from intracellular storage in the endoplasmic reticulum to trigger eNOS activation (Moncada & Higgs, 2006). In contrast, substances that reduce the intracellular Ca^{2+} level can prevent Ca^{2+}/CaM-provoked eNOS activation and reduce NO production (Thebault*, et al.*, 2011). Apart from intracellular store release, increased intracellular Ca^{2+} level can also be achieved through Ca^{2+} entry from the extracellular space (Zhang & Gutterman, 2011). Although the mechanisms are still unclear (Huang*, et al.*, 2011), Ca^{2+} entry to endothelial cells presumably involves store operated Ca^{2+} entry channels (SOCs) (Sundivakkam*, et al.*, 2013). Recent evidence suggest that SOCs are predominantly expressed as transient receptor potential (TRP) channels in endothelial cells, including TRPV4, TRPC4 and TRPC6 (Zhang & Gutterman, 2011) (Sundivakkam*, et al.*, 2013). In addition, several studies also reported Orai1 and Ca^{2+}-release activated Ca^{2+} channels (I_{CRAC}) contributed to transient Ca^{2+} entry in endothelial cells (Li*, et al.*, 2011).

Activation of eNOS may also be stimulated through Ca^{2+}-independent pathways. This can be accomplished at transcriptional and post-translational levels (Triggle*, et al.*, 2012b). At the transcriptional level, up-regulation of eNOS expression may occur due to biomechanical stimulation, such as shear stress, involving activation of key transcriptional proteins such as activator protein (AP)-1 and Kruppel-like factor (KLF)-2 (Boo*, et al.*, 2002; Koo*, et al.*, 2013). Meanwhile, post-translational modification includes eNOS phosphorylation at certain regulatory sites (Ser 617, Ser 635, Ser 1177, Ser 1179, Thr 495) (Triggle*, et al.*, 2012b), which is activated via protein kinases, including protein kinase A (PKA), protein kinase B (AKT), AMPK and calmodulin-dependent protein kinase II (Bae*, et al.*, 2003; Butt*, et al.*, 2000; Dudzinski & Michel, 2007).

After being released from the endothelium, NO diffuses to smooth muscle cells and binds to soluble guanylyl cyclase (sGC) on its heme and non-heme sites (Tsai & Kass, 2009). NO binding to sGC heme center catalyzes the conversion of guanosine triphosphate to cGMP up to several-hundred fold (see Fig. 1.1) (Martin*, et al.*, 2012; Oppermann*, et al.*, 2011). cGMP subsequently reduces intracellular Ca^{2+} level through several mechanisms: 1) blocking inositol triphosphate $(IP)_3$-sensitive intracellular Ca^{2+} release (Carrasco*, et al.*, 2004), 2) removal of intracellular Ca^{2+} via calcium pump (Yao & Huang, 2003), 3) halting extracellular Ca^{2+} influx (Tsai & Kass, 2009), and 4) stimulating Ca^{2+} re-uptake (Cornwell*, et al.*, 1991; Martínez-Ruiz*, et al.*, 2011). Reduction in intracellular Ca^{2+} decreases the myosin light chain kinase (MLCK) and/or increases myosin light chain phosphatase (MLCP) activities, leading to dephosphorylation of the 20-kDa regulatory light chain of myosin (MLC_{20}) (Morgado*, et al.*, 2012). As a result, tonic contraction of VSMC is abrogated and leads to vasorelaxation.

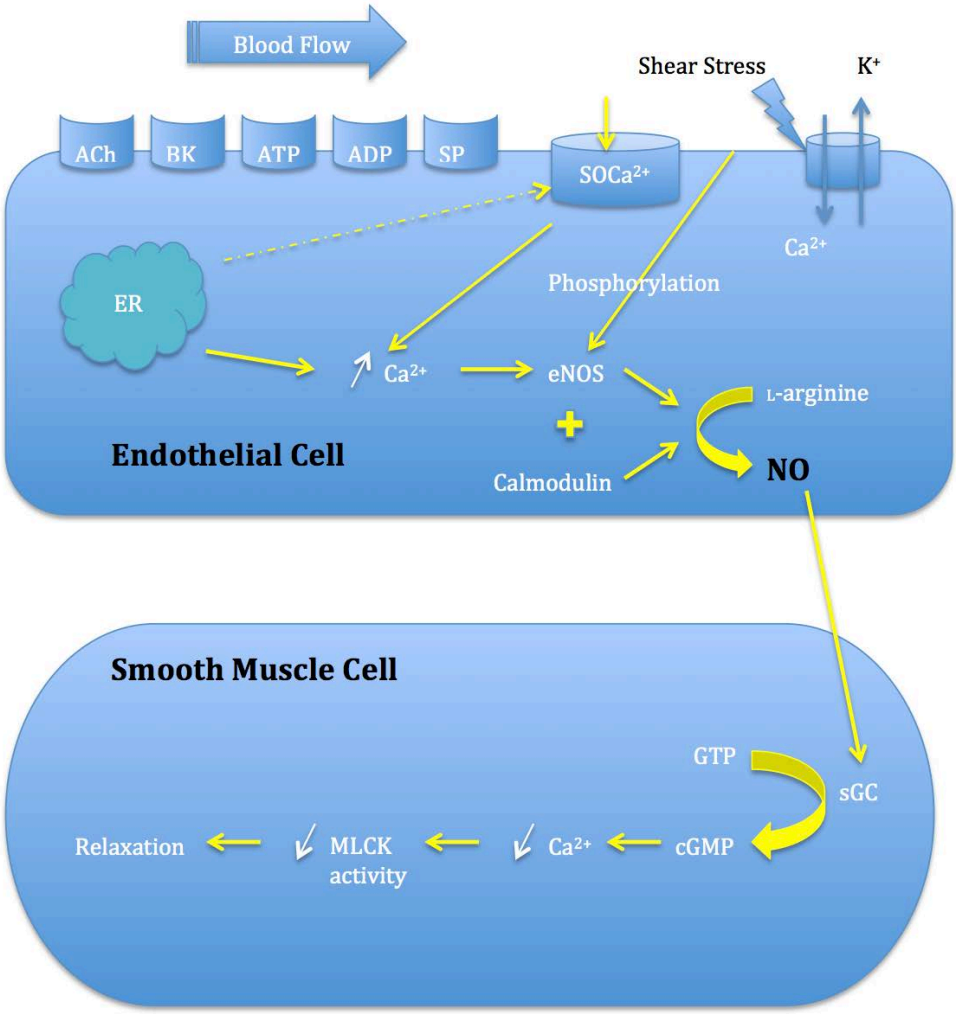

Figure 1.1 Endothelial nitric oxide production and it actions in the vascular smooth muscle cell. ACh= acetylcholine; BK= bradykinin; ATP= adenosine triphosphate; ADP= adenosine diphosphate; SP= substance P; $SOCa^{2+}$= store-operated Ca^{2+} channel; ER= endoplasmic reticulum; NO= nitric oxide; sGC= soluble guanylyl cyclase; cGMP= cyclic guanosine-3', 5-monophosphate; MLCK= myosin light chain kinase. *When Ca^{2+} stores of the endoplasmic reticulum are depleted, a signal is sent to $SOCa^{2+}$ channel which allows extracellular Ca^{2+} into the endothelial cell.

1.1.3.1.2 Prostacyclin

The production and release of NO is an important, but not the sole mechanism of endothelial-derived vasoregulation (Evgenov*, et al.*, 2006; Kang, 2014). Prostacyclin (PGI_2) was firstly identified (as PGX) by Moncada and coworkers in 1970s as a vasoactive substance that depended on an active vascular endothelium (Moncada*, et al.*, 1976). PGI_2 is a lipophilic metabolite synthesized from arachidonic acid, which easily diffuses through the endothelial membrane to act as an anticoagulant and local

vasorelaxant (Parkington*, et al.*, 2004). Similar to NO, PGI_2 is also released in response to shear stress, hypoxia, mild oxidative stress, bradykinin, serotonin, and growth factors (Kang, 2014; Toniolo*, et al.*, 2013).

PGI_2 synthesis is dependent on cyclooxygenase (COX) enzyme, of which two isoforms are currently recognized, COX-1 and COX-2 (Kirkbya*, et al.*, 2013). The COX-1 enzyme is expressed constitutively and mainly involved in physiological conditions and serves the 'housekeeping' function in endothelial cells. In contrast, COX-2 is an inducible form of COX that is increasingly expressed in pathological conditions, including during inflammation, angiogenesis and malignancy (Crofford, 1997; Félétou*, et al.*, 2011). However, whether prostacyclin production is majorly dependent on the COX-1 or COX-2 isoform has not been settled yet (Kirkbya*, et al.*, 2013). Ruan*, et al.* (2011) and others postulate the dominant role of COX-2 over COX-1 in prostacyclin biosynthesis, pointing out the potential risk of COX-2 inhibitor use for vascular disease development (Skarke & FitzGerald). Nonetheless, in physiological conditions, COX-1-dependent PGI_2 synthesis is apparently more important (Kirkbya*, et al.*, 2013), and this theory has been supported by experimental studies using immunohistochemistry (Belton*, et al.*, 2000; Kawka*, et al.*, 2007; Mitchell*, et al.*, 2006).

After its release, PGI_2 binds to prostanoid receptors in vascular smooth muscle cells and activates adenylate cyclase and stimulates cyclic adenosine monophosphate (cAMP) (Billington & Penn, 2003). An increase in cAMP subsequently activates protein kinase A, leading to vascular smooth muscle relaxation (Fetalvero*, et al.*, 2007; Stitham*, et al.*, 2004). Nevertheless, compared to NO, the role of PGI_2 as an endothelium-derived relaxing factor (EDRF) is less pronounced (Araújo*, et al.*, 2011; Vanhoutte*, et al.*, 2017). Verma and colleagues (2001) showed that blocking PGI_2 formation through COX-2 inhibition did not impede endothelial-dependent relaxation in healthy volunteers. However, PGI_2 contribution in endothelium-dependent vasodilation becomes more important when the NO level is diminished during pathological states (Sandoo*, et al.*, 2010a) or reduced due to ageing (Eisenach*, et al.*, 2014). In this state, PGI_2 plays a compensatory role to NO, and hence, chronic and acute inhibition of NO synthesis may lead to upregulation of COX-dependent PGI_2 production (Beierwaltes, 2002; Beverelli*, et al.*, 1997).

1.1.3.2 Endothelium-derived hyperpolarizing factors (EDHFs)

In 1980s, Chen et al. first demonstrated that the endothelium could hyperpolarize independent of EDRF. The existence of endothelium-derived hyperpolarizing factors (EDHFs) is postulated based on the fact that endothelium-dependent relaxation can be partially or totally resistant to NO (eNOS) or COX inhibition (Garland*, et al.*, 2011; Triggle*, et al.*, 2012b). The contribution of EDHFs becomes more pronounced as the vessel size decreases (Shimokawa*, et al.*, 1996), and hence, they play important roles in coronary, renal and small arterial vessel tone regulation (Garland*, et al.*, 2011; Quilley*, et al.*, 1997; Urakami-Harasawa*, et al.*, 1997). Along the same lines, eNOS expression in the mesenteric arteries decreases as the vessels becomes smaller, indicating a lesser role for NO function in these small resistant arteries. Indeed, in eNOS knock-out mice, EDHFs play a predominant role in endothelium-dependent vasodilation as a compensatory mechanism in the absence of endothelial NO (Chataigneau*, et al.*, 1999; Waldron*, et al.*, 1999). Recently, Spilk and colleagues (2013) have proposed a significant role of EDHFs in human skeletal muscle vasodilation during hypoxia (Spilk*, et al.*, 2013).

The specific mediator of EDHF regulation remains controversial and includes epoxyeicosatrienoic acid (EET), a cytochrome P450 of arachidonic acid (Archer*, et al.*, 2003; Eckman*, et al.*, 1998), myoendothelial gap junction (Sandow & Hill, 2000), potassium ions (Quignard*, et al.*, 1999), and hydrogen peroxide (H_2O_2) (Matoba*, et al.*, 2002; Shimokawa, 2010). However, instead of a single agent, it is clearer now that EDHFs incorporate complex processes involving a number of molecules (Giles*, et al.*, 2012). Zhang and Gutterman (2011) proposed that endothelial hyperpolarization predominantly involves two principal pathways, the classic pathway and the second pathway.

In the classic pathway, EDHFs are thought to induce vascular hyperpolarization through the activation of Ca^{2+}-activated K^+ channels (SK_{Ca}, IK_{Ca}) located in endothelial cells (Dora & Garland, 2013). The hyperpolarization is then conducted to vascular smooth muscle cells through myoendothelial gap junctions (Garland*, et al.*, 2011; Zhang & Gutterman, 2011). K^+ efflux via SK_{Ca} and IK_{Ca} raises extracellular K^+ level between endothelial cells and smooth muscle cells, which subsequently activates K_{IR}, K_{ATP} or Na^+/K^+ ATPase (Bełtowski & Jamroz-Wiśniewska, 2014). This process in turn hyperpolarizes smooth muscle cells decreasing Ca^{2+} influx via voltage-gated Ca^{2+} channels, and leads to vasorelaxation (Bełtowski & Jamroz-Wiśniewska, 2014). The

EDHF pathway is dependent on increased endothelial intracellular Ca^{2+}, particularly to activate the endothelial K_{Ca} channels considering these channels are not voltage dependent but depend on $[Ca^{2+}]_i$ elevation (Crane*, et al.*, 2003). Again, the increase of endothelial $[Ca^{2+}]_i$ can be accessed from intracellular store release or via extracellular entry through TRP channels (Zhang & Gutterman, 2011).

In the second pathway, alternatively, endothelial hyperpolarization may occur insensitive to SK_{Ca} and IK_{Ca} blockage (Edwards*, et al.*, 2010). This pathway is thought to involve endothelial releasing factors such as cytochrome P450-derived EET and superoxide anion metabolite, H_2O_2 (Zhang & Gutterman, 2011). EET appears to activate non-classical BK_{Ca} and K_{ATP} channels in human or rat vasculatures (Larsen*, et al.*, 2006; Lu*, et al.*, 2006). Similar to the classic pathway, hyperpolarization via EET is also a Ca^{2+}-dependent process (Zhang & Gutterman, 2011), especially because EET synthesis by cytochrome P450 requires an increase in Ca^{2+} level to stimulate the release of arachidonic acid (Griffith, 2004). Meanwhile, H_2O_2, so far, has been linked to activation of BK_{Ca}, K_{ATP}, K_{ir}, and K_v channels, dependent on the vessel type (Edwards*, et al.*, 2010). EET and H_2O_2 are found to co-exist in some vascular beds, such as porcine and human coronary artery, as well as rabbit mesenteric arteries (Larsen*, et al.*, 2008) (Weston*, et al.*, 2005; Zhang*, et al.*, 2007).

1.1.3.3 Endothelium-derived contracting factors (EDCFs)

1.1.3.3.1 Endothelin-1 (ET-1)

Two years after the discovery of EDRF by Furchgott and Zawadzki, the presence of endothelium-derived contracting factors (EDCFs) was first recognized (De Mey & Vanhoutte, 1982; De Mey & Vanhoutte, 2014). After purification and sequencing, Yanagisawa and coworkers (1988) identified one of the contracting factors as endothelin-1 (ET-1) (Rubanyi, 2011). ET-1 is a peptide consisting of 21-aminoacids (Yanagisawa*, et al.*, 1988), generated from a 38 amino acid precursor, proendothelin-1 after its conversion to big endothelin-1 by endothelin converting enzymes (ECEs) (De Mey & Vanhoutte, 2014; Grover*, et al.*, 1992; Haynes & Webb, 1994).

ET-1 effects are elicited through its binding to two different types of endothelin receptors (Arai*, et al.*, 1990; Sakurai*, et al.*, 1990), namely endothelin-A (ETA) and

endothelin-B (ETB) (Barton, 2011; Ohkita*, et al.*, 2012). The ETA subtype, mostly expressed in vascular smooth muscle, is known to primarily mediate vasoconstriction, whereas ETB may have biphasic actions dependent on the localization of the receptors (Barton, 2011; Faraci & Heistad, 1998). The activation of ETB receptors in vascular smooth muscle induces a profound vasoconstriction (Ohkita*, et al.*, 2012), especially in the arteriolar resistance and venous capacitance vessels (Haynes & Webb, 1994), or in disease states such as atherosclerosis and ischaemic heart disease (Dimitrijevic*, et al.*, 2009; Iwasa*, et al.*, 1999). While present in endothelial cells, ETB activation appears to facilitate EDRF and EDHF release leading to vasorelaxation, as well as to contribute to ET-1 clearance (Mazzuca & Khalil, 2012).

ET-1 is released continuously from endothelial cells and, to a lesser extent, from vascular smooth muscle cells, to maintain vascular tone (Thorin & Webb, 2010). As the most potent endogenous vasoconstrictor, ET-1 vasoregulation is interconnected with NO in several ways: 1) exogenous or endogenous NO inhibits ET-1 production and release (Junbao*, et al.*, 1999; Mitsutomi*, et al.*, 1999; Rapoport, 2014b), 2) increased NO level reduces response and sensitivity to ET-1 (Chen*, et al.*, 2003), 3) eNOS inhibition leads to increased ET-1 dependent vasoconstriction (Hilgers & De Mey, 2009; Rapoport, 2014a), 4) blocking ETA receptors leads to upregulation of eNOS expression and increased NO activity (Barton*, et al.*, 1998; Taner*, et al.*, 2001), and 5) ET-1 binding to ETA reduces eNOS expression and activity (Wedgwood & Black, 2005). This close ET-1/ NO interaction becomes the *Yin and Yang* of vascular function (Marasciulo*, et al.*, 2006). Indeed, alteration of this balance is evidently important in vascular dysfunction (Bourque*, et al.*, 2011) and the pathogenesis of cardiovascular diseases (Böhm & Pernow, 2007; Félétou*, et al.*, 2012; Tanaka*, et al.*, 2013). Recently, De Mey and Vanhoutte (2014) speculated about the presence of mechanisms beyond endothelial NO that may also interact with ET-1 regulation in vascular tone, and these include the sensorimotor neurotransmitter calcitonin gene-related peptide (CGRP). However, at this stage, available data supporting this contention are still limited.

1.1.3.3.2 COX-derived prostanoids

In addition to endothelial peptides (endothelin-1), moment-to-moment vascular tone is also regulated by contracting factor prostanoids derived from COX (Vanhoutte, 2011). Tang et al. (2005) showed that the EDCF activity was abolished in the aorta of COX-1, but not COX-2, knockout mice. Furthermore, inhibition of COX-1 (and not COX-2)

was shown to prevent endothelium-dependent vasoconstriction in diabetic rats (Shi*, et al.*, 2007), and the genomic expression of COX-1 increased in spontaneously hypertensive rats (Tang & Vanhoutte, 2008). These data suggest a predominant role of COX-1 in EDCF-mediated vasoconstriction (Vanhoutte, 2011).

After being released from endothelial cells, COX-derived contracting prostanoids diffuse to the adjacent vascular smooth muscle and activate the thromboxane-prostanoid (TP) receptors (Shi & Vanhoutte, 2014). The TP receptors are predominantly stimulated by thromboxane A_2, but may also be by a high level of endoperoxides (PGH_2) (Vanhoutte, 2011), COX-2 derived PGF_2 during ageing (Wong*, et al.*, 2009), and paradoxically, PGI_2, as seen in spontaneously hypertensive rats (Félétou*, et al.*, 2009). The activation of TP receptors stimulates production of IP_3 and DAG via activation of phospholipase C (Dorn & Becker, 1993; Suzuki*, et al.*, 2012), which increases the intracellular free Ca^{2+} through the opening of voltage-dependent and independent channels and leads to vasoconstriction (Suzuki*, et al.*, 2012). In addition, Liu et al. (2009) demonstrated that TP-evoked vasoconstriction could be achieved by averting eNOS activity via the RhoA/Rho-kinase pathway in rat carotid artery, which was also confirmed in rat aorta (Chan*, et al.*, 2009). This Ca^{2+}-independent pathway of TP-evoked vasoconstriction may also be important in vascular dysfunction and warrants further investigation (Liu*, et al.*, 2009).

1.1.4. The vascular smooth muscle cells (VSMCs)

The VSMCs comprise the contractile fibers (actin-myosin) normally located in the tunica media layer of blood vessels (Shinohara*, et al.*, 2012). The existence of VSMC heterogeneity has been uncovered ranging from contractile to synthetic/proliferative phenotypes (Beamish*, et al.*, 2010). The modulation of VSMC phenotypes depends on the development state or pathophysiological conditions. For example, the contractile phenotype is mostly expressed in healthy and mature VSMCs, while in developing arteries, the synthetic/proliferative phenotype is more dominant (Shinohara*, et al.*, 2012). During pathological conditions, the VSMC phenotype can reversibly swift to be more proliferative and migratory while losing its contractility function (Alford*, et al.*, 2011). The many factors that trigger the switching (phenotype modulation) have been proposed and these include extracellular matrix (Qin*, et al.*, 2000), platelet-derived growth factor (PDGF) and transforming growth factors (TGF) (Hirschi*, et al.*, 1998; Thomas*, et al.*, 2009), angiotensin II (Kim*, et al.*, 2005), microRNAs (Cheng*, et al.*,

2009; Davis*, et al.*, 2009), and collagen isotypes (Orr*, et al.*, 2009). However, at this stage, it is still difficult to conclude the actual mechanism of this phenomenon due to contradictory findings between laboratories (Alexander & Owens, 2012).

Like the endothelium, VSMCs have numerous other vascular functions and these include angiogenesis, immune and chemokine regulation (Hayes*, et al.*, 1998; Ma*, et al.*, 2007). However, the foremost role of VSMC is to regulate vascular tone and blood pressure to maintain adequate systemic and regional perfusion (Rensen*, et al.*, 2007). This is achieved by maintaining the balance between the cellular signaling pathways mediating force generation (contraction) and release (relaxation). The contraction and relaxation of VSMCs involves 3 distinctive mechanisms: 1) regulation of intracellular free Ca^{2+} ($[Ca^{2+}]_i$) level, 2) modulation of VSMC-Ca^{2+} sensitivity and 3) actin filament regulation (Akata, 2007a, 2007b; Hirano, 2007). These mechanisms will now be discussed in more detail.

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1.1.4.1 Contraction of VSMCs

The contraction of VSMCs is largely dependent on increases in intracellular $[Ca^{2+}]_i$ levels in VSMC. The basal vascular tone is maintained at $[Ca^{2+}]_i$ levels around 10^{-9} M, while VSMC contraction is initiated when $[Ca^{2+}]_i$ is elevated to 10^{-6} M (Koledova & Khalil, 2006). The two to three orders of magnitude $[Ca^{2+}]_i$ increase is generally achieved in two ways: 1) via Ca^{2+} influx from the extracellular compartment, and 2) through Ca^{2+} release from intracellular stores in the sarcoplasmic reticulum (Khalil, 2010) (see Figure 1.2). The first mechanism is the major source of $[Ca^{2+}]_i$ increase, which occurs through various Ca^{2+} channels or Ca^{2+} exchangers on membrane cells (Horowitz*, et al.*, 1996). Accordingly, the use of Ca^{2+} channel blockers inhibits vascular contraction or can lead to vasodilation (Cauvin*, et al.*, 1983; Timmermans*, et al.*, 1983). The release of Ca^{2+} from the sarcoplasmic reticulum is believed to occur in response to activation of inositol triphosphate (IP_3) or ryanodine (Ry) receptors (Rainbow*, et al.*, 2009). In addition, $[Ca^{2+}]_i$ can be increased by the simultaneous activation of a Ca^{2+}-induced Ca^{2+} release (CICR) mechanism (Breemen & Saida, 1989). This phenomenon has been observed in skinned cardiac cells from adult human, dog, cat, rabbit, and frog (Fabiato & Fabiato, 1978).

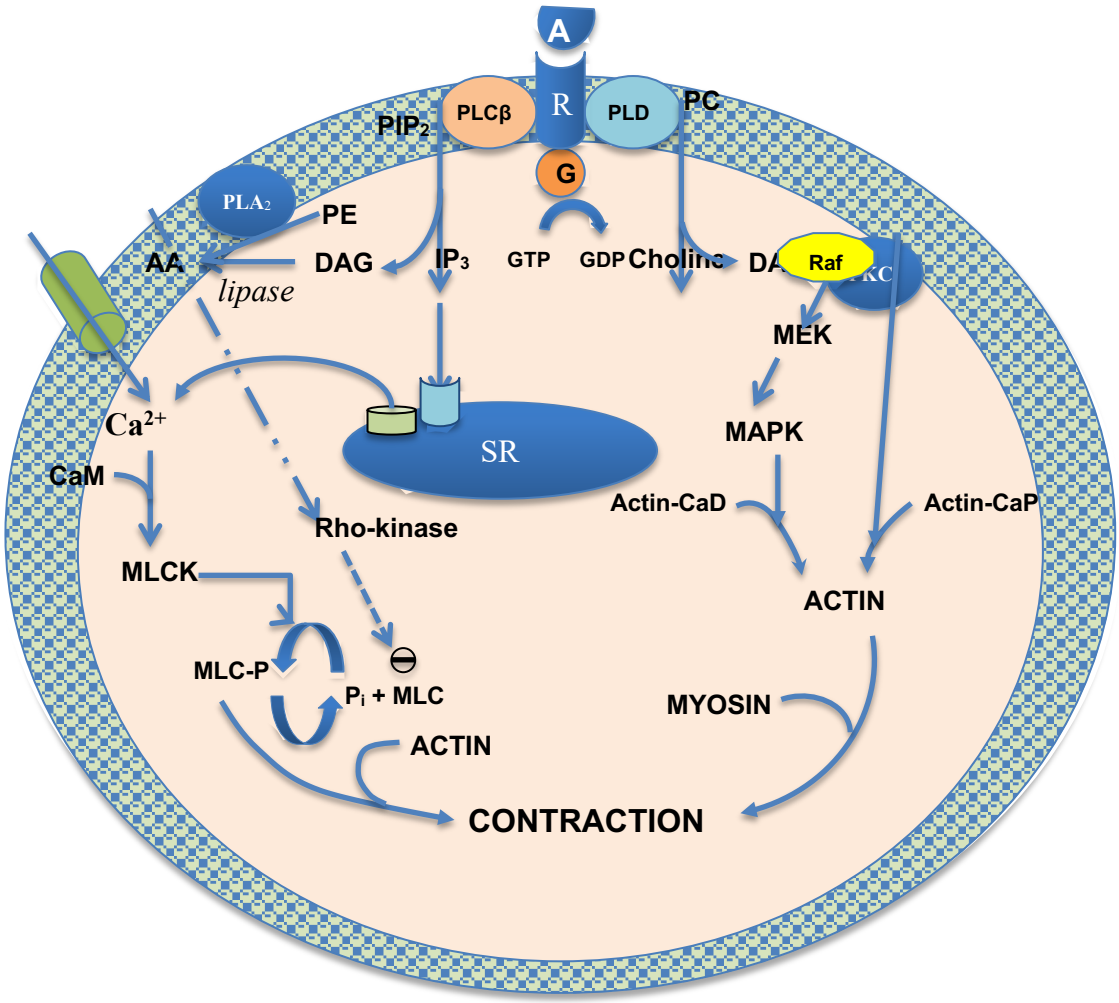

Figure 1.2 Mechanisms of VSMC contraction.

In VSMCs, $[Ca^{2+}]_i$ interacts with specific Ca^{2+}-binding proteins, calmodulin (CaM), which serves as a critical sensor for Ca^{2+} regulation (Koledova & Khalil, 2006). The Ca^{2+}/CaM complex activates 20-kDa myosin light chain (MLC_{20}) kinase, which leads to MLC_{20} phosphorylation (Van Lierop, *et al.*, 2002), which not only facilitates myosin monomers to gather into filaments but also enhances the actin-activated Mg^{2+}-ATPase activity (Horowitz, *et al.*, 1996). This process facilitates the actin-myosin binding leading to VSMC contraction (see Figure 1.2).

However, Ca^{2+}-dependent MLC phosphorylation is not the sole mechanism in VSMC contraction. Itoh and colleagues (1993) suggest other mechanisms that increase the myofilament force by changing the sensitivity to Ca^{2+} over a range of $[Ca^{2+}]_i$ levels (Itoh, *et al.*, 1993) or different states of MLC_{20} phosphorylation (Khalil & van Breemen, 1990) (Khalil, 2010). Ca^{2+} sensitization of myosin is believed to be modulated by the ratio of myosin light-chain kinase (MLCK) to myosin light chain phosphatase (MLCP) activity (Somlyo & Somlyo, 2003) (see Figure 1.2).

In addition to changing Ca^{2+} sensitization, Gunst and Zhang (2008) proposed another mechanism of increasing smooth muscle contraction involving polymerization of actin filaments (Gunst & Zhang, 2008). Actin polymerization is now recognized as an essential mechanism for tension development beyond actin-myosin interaction (Gunst & Zhang, 2008). Several reports have shown that pharmacological inhibition of actin polymerization leads to reduced VSMC contractile responses (Adler*, et al.*, 1983; Saito*, et al.*, 1996; Shaw*, et al.*, 2003). Recent studies by Moreno-Dominguez and colleagues have suggested the involvement of ROK and PKC pathways in regulation of actin filament polymerization (Moreno-Domínguez*, et al.*, 2013).

1.1.4.2 Relaxation of Vascular Smooth Muscle Cells (VSMCs)

Endothelium-derived NO initiates VSMC relaxation by activating the soluble guanylyl cyclase to generate cyclic guanosine monophospate (cGMP) (Qin*, et al.*, 2007). However, the intracellular events that occur downstream after cGMP formation remains controversial (Qin*, et al.*, 2007). One downstream target is the activation of cGMP-dependent protein kinase (PKG) (Burgoyne*, et al.*, 2012; Lincoln*, et al.*, 2001; Sausbier*, et al.*, 2000; Schlossmann*, et al.*, 2003) which can lead to vascular relaxation (Feil*, et al.*, 2003; Pfeifer*, et al.*, 1998).

The general consensus of VSMC relaxation, including the NO/cGMP/PKG pathways, is mediated by at least four major mechanisms: 1) decreases in $[Ca^{2+}]_i$ level, 2) calcium desensitization, 3) thin filament regularization (actin depolymerization), and 4) hyperpolarization of VSMC membrane (Morgado*, et al.*, 2012). VSMC membrane hyperpolarization is mostly mediated by PKG-modulation of K^+ channel activity in membrane cells (Morgado*, et al.*, 2012). K^+ channel blockade results in membrane depolarization leading to increased Ca^{2+} influx through voltage-gated Ca^{2+} channels; in contrast, K^+ opening leads to reduced Ca^{2+} influx (Brayden, 1996). The role of the specific K^+ channels in vessel tone regulation will now be discussed.

1.1.5 Role of potassium (K^+) channels and Na^+/K^+-ATPase in vascular tone

1.1.5.1 Role of K^+ channels in vascular tone

K^+ channels in the plasma membrane have profound effects on the regulation of vascular tone by regulating K^+ outflow, leading to smooth muscle cell hyperpolarization (Dick & Tune, 2010). Hyperpolarization triggers the closing of voltage-dependent Ca^{2+} channels (VDCC), reducing Ca^{2+} influx and resulting in vascular relaxation. Conversely, blocking K^+ channel causes smooth muscle cell depolarization, triggers VDCC opening, increases intracellular Ca^{2+} and leads to vasoconstriction (Dick & Tune, 2010).

K^+ channels are divided into three main families based on their different molecular structures. The number of transmembrane (TM) segments in their subunits divide K^+ channels into 1) 2TM, 2) 4TM and 3) 6TM classifications (Tang*, et al.*, 2004). These three main families of K^+ channels are further divided into subclasses of K^+ channels (Sandhiya, 2009):

- **2TM family**: these K^+ channels are also known as inward-rectifier K^+ channels (K_{IR}). Adenosine 5'-triphospate-sensitive K^+ channel (K_{ATP}) is one of the examples of this family.
- **4TM family**: these channels have two pore domains in their α-subunits. The channels include: TWIK, TREK, TASK, TALK, THIK and THRESK.
- **6TM family**: in this family there are voltage-activated K^+ (K_v) channels and Ca-activated K^+ (K_{Ca}) channels. There are three types of K_{Ca} channels: Small-conductance K_{Ca} (SK_{Ca}) channels, Intermediate-conductance K_{Ca} (IK_{Ca}) channels, and Large-conductance K_{Ca} (BK_{Ca}) channels.

Among the wide variety of K^+ channels, the major ones that play an important role in vascular reactivity and blood flow regulation are: K_v, K_{ATP}, K_{IR} and K_{ca} (Ko*, et al.*, 2008).

1.1.5.1.1 ATP-sensitive K^+ (K_{ATP}) channels

ATP-sensitive potassium (K_{ATP}) channels were first discovered in the heart 30 years ago (Nichols*, et al.*, 2013; Noma, 1983). K_{ATP} channels are believed to link cell

metabolism to membrane excitability and are involved in a wide range of physiological processes including control of vascular tone, hormone secretion and protection of cardiac and neuronal cells against ischaemic injuries (Nichols*, et al.*, 2013). The K_{ATP} channel is a large hetero-octamer of nearly 950 kDa, and is composed of four K_{IR} 6.1 or 6.2 as α-subunits, and four sulphonylurea receptor (SUR) proteins as β-subunits (Ko*, et al.*, 2008). Adenosine 5'-triposphate (ATP) inhibits the channels by binding to the α-subunit, while adenosine diphosphate (ADP) activates the channels by binding to the SUR domain (Nelson & Quayle, 1995). SUR was so-named from the β-subunits of the channels being sensitive to the K^+-channel blocker, sulphonylurea (Ocaña*, et al.*, 2004). Potassium channel openers such as pinacidil and levocromakalim also can activate the channels via these SUR subunits. In addition to vascular smooth muscle, K_{ATP} channels are found in heart (Zingman*, et al.*), brain (Ballanyi, 2004), pancreas (Remedi & Koster, 2010), skeletal muscle (Banas*, et al.*), and vascular endothelium (Figura*, et al.*, 2009; Martin*, et al.*, 2013).

The presence of K_{ATP} channels on vascular smooth muscle and endothelium have been reported in a number of different vascular beds including the aorta (Tang*, et al.*, 2005), coronary (Duncker*, et al.*, 1993) and mesenteric artery (Nelson, 1990). As mentioned above their primary role is the regulation of vascular tone (Daut*, et al.*, 1994; Nichols*, et al.*, 2013). Endogenous vasodilators such as calcitonin gene related peptide, prostacyclin, and adenosine are known to activate K_{ATP} channels via protein kinase A stimulation (Mannhold, 2004). Kuo and co-workers (1995) specifically showed that adenosine increased flow-induced relaxation in swine coronary artery by activating K_{ATP} channels in endothelium, which in turn release NO. They further found that flow-induced relaxation by adenosine was inhibited by 10^{-6} M sulfonylurea glibenclamide (Kuo & Chancellor, 1995). Endogenous vasoconstrictors, including endothelin-1, norepinephrine, and vasopressin, on the other hand, are shown to inhibit K_{ATP} channels (Park*, et al.*, 2005; Tan*, et al.*, 2007; Tsuchiya*, et al.*, 2002). While endothelin-1 and vasopressin-induced vasoconstriction involve blocking K_{ATP} channels via PKC-coupled reactions (Park, 2005; Tsuchiya, 2002), Tan and coworkers showed that norepinephrine inhibition of K_{ATP} channels is mediated by α2-adrenoceptor, possibly through inhibition of PKA activity (Tan*, et al.*, 2007).

Pharmacological modulation of K_{ATP} channels is largely studied using K^+ channel openers (KCOs) (Mannhold, 2004). KCOs such as cromakalim, levcromakalim, minoxidil, nicorandil, pinacidil and diazoxide cause K_{ATP} channels to become less sensitive to ATP inhibition, leading to increased K^+ efflux, hyperpolarization and

relaxation (Brayden, 2002; Mannhold, 2004). This effect to relax the vessel and increase flow can be blocked by 0.5-10 μM glibenclamide (Waldron & Cole, 1999), but not by K_{Ca} channel inhibitors (such as iberiotoxin and apamin) or low concentration of tetraethylammonium (TEA) (Brayden, 2002). In addition, K_{ATP} channels can be activated in pathophysiological states, including hypoxia (Quayle*, et al.*, 2006), septic shock (Buckley*, et al.*, 2006), acidosis (Xu*, et al.*, 2001), as well as during ischaemic-reperfusion to provide cellular protection (Okorie*, et al.*, 2011).

1.1.5.1.2 Voltage-activated K^+ (K_v) channels

Mammalian voltage-activated K^+ channels (K_v) were first cloned by Tempel and co-workers in 1988, a decade after Hodgkin and Huxley (1952) unveiled the electrical basis of cellular signaling (Jan & Jan, 2012). They are transmembrane proteins that enable the movement of K^+ ions down an electrochemical gradient (Pardo & Stuhmer, 2014). As voltage-gated channels, the shift between opening and closing of K_v channels is dependent on transmembrane voltage (Jensen*, et al.*, 2012). Jensen and colleagues (2012) further showed at the atomic level conformational shifting of K_v channels can be stimulated by altering the transmembrane voltage below (hyperpolarization) or higher than 0 mV (depolarization). The activation of K_v channels increases outward K^+ currents and hyperpolarizes the smooth muscle cells, reduces Ca^{2+} entry via closing of voltage-dependent Ca^{2+} channels (VDCC), and causes vasodilation. In contrast, K_v channel inhibition will cause cell depolarization, open the VDCC, increase $[Ca^{2+}]_I$, and cause vasoconstriction (Makino*, et al.*, 2011).

K_v channels are also recognized as delayed rectifier K^+ channels due to their delayed activation kinetics during the action potential. The term ‘rectifier’ comes from an electronic term when a circuit component conducts current on one direction compared to the other (Resta & Becchetti, 2010). The K_v channel consists of four polypeptides with each containing six transmembrane segments (S1-S6). The first four membranes are thought to control the channel’s pore opening and closing, with S4 being the voltage sensor of the channel (Miller, 2000). Each α-subunit is linked to additional β-subunits that determine the channel characteristics. The β-subunit was found to modify the inactivation kinetics of the K_v channel by accelerating the process (Pongs*, et al.*, 1999; Torres*, et al.*, 2007), and post-translational modification of the β-subunit by PKC phosphorylation plays a pivotal role in K_v channel’s inactivation and voltage sensitivity (David*, et al.*, 2012; Niwa & Nerbonne, 2010).

It is believed that there are multiple K_v channel subtypes expressed in smooth muscle cells as a number of investigators have reported differences in voltage-dependence activation (Jan & Jan, 2012), sensitivity against inhibitors (Marzian*, et al.*, 2013), as well as differences in gating properties (Niwa & Nerbonne, 2010). For example, tissue expression of $K_v1.5$, $K_v2.2$ and $K_{v\beta}4$ subunits have been reported in smooth muscle of canine artery (Ko*, et al.*, 2008), while $K_{v\alpha}1.2$, $K_{v\alpha}1.5$, $K_{v\beta}1.1$, $K_{v\beta}1.2$, $K_{v\beta}2.1$ and others have been reported in rat pulmonary artery (Cox, 2005; Standen & Quayle, 1998; Yuan*, et al.*, 1998).

The presence of the K_v channel and its properties in vascular smooth muscle and endothelial cells have been studied using the inhibitor 4-aminopyridine (4-AP) (Cox, 2005; Ko*, et al.*, 2008). 4-AP (0.5 – 5 mM) is the most selective known inhibitor for almost all K_v channels subtypes, except K_{v7} (Martelli*, et al.*, 2013; Novakovic*, et al.*, 2012; Yuan*, et al.*, 1998). 4-AP inhibits K_v channels in both open and closed forms, and it is believed that the 4-AP blocking site on the channel is at the internal side of the membrane cell (Bouchard & Fedida, 1995; Claydon*, et al.*, 2007).

K_v channels are abundantly expressed in vascular smooth muscle cells (Ko*, et al.*, 2010) including rat mesenteric (Lu*, et al.*, 2002), rat pulmonary artery (Michelakis*, et al.*, 2002), rat kidney (Carrisoza-Gaytán*, et al.*, 2010) and also rat aorta (Palacios*, et al.*, 2013). In contrast, there are fewer reports of the presence of K_v channels in vascular endothelium. However, Jow and colleagues (1999) reported the presence of K^+ outward current in vascular endothelium, which could be blocked by 4-AP, and this implied the presence of K_v channels in cultured human capillary endothelial cells. A more recent study by Millar and coworkers (2008) also showed the expression of K_v channels in endothelium of brain vasculatures (Millar*, et al.*, 2008).

1.1.5.2 Role of Na^+/K^+-ATPase in vascular tone

Potassium regulation in vascular tissue also arises from regulation of the Na^+/K^+ pump (Vedovato & Gadsby, 2014). Although, Na^+/K^+-ATPase is an integral protein that functions as an ion transporter like ion channels, it is categorized as an ion active pump since it is energized by ATP hydrolysis (Morth, 2011). When activated, channels permit ions to flow down their electrochemical gradients, whilst pumps actively push ions

against these gradients (Gadsby, 2009). The Na^+/K^+ pump expels three Na^+ ions out for two K^+ ions into the cell at the expense of ATP (Vedovato & Gadsby, 2014). The Na^+/K^+-ATPase function is important to maintain the high potassium and low sodium concentrations inside the cell, however, its activity decreases during aging (Maurya & Prakash, 2013). The Na^+/K^+-ATPase consists of two (α and β) subunits. The α-subunit acts as the binding site for cations, ATP or the inhibitor (ouabain); and also regulates the catalytic and transport function of the enzyme. Whereas, the β-subunit is believed to play an important role in maintaining the normal activity of the enzyme (Morth*, et al.*, 2009).

In vascular tissue, Na^+/K^+-ATPase is found in smooth muscle cells in rat (Oguchi*, et al.*, 1993), canine (Allen*, et al.*, 1986) as well as rabbit aorta (Gupta*, et al.*, 1994). The presence of the Na^+/K^+ pump has also been reported in isolated human endothelial cells (Duran*, et al.*, 2010). If the pump is activated the difference in pumping three Na^+ ions out compared to two K^+ ions pumped in leads to vascular smooth muscle membrane hyperpolarization which decreases intracellular Ca^{2+} availability, and relaxes the smooth muscle cells (Lockette*, et al.*, 1980), thus reducing vessel lumen size. In addition to its ion-pumping function, the Na^+/K^+ATPase role as a signal transducer has been recognised to mediate multiple protein-protein complexes (Reinhard*, et al.*, 2013).

1.2 Mechanism of vasospasm

Vasospasm refers to a temporary condition in which an arterial blood vessel spasms or constricts which can reduce blood flow and lead to ischaemia (Moukarbel & Weinrauch, 2012). Vasospasm occurs in the coronary arteries (Lanza & Crea, 2010), cerebral arteries (Siasios*, et al.*, 2013), ocular arteries (Gasser, 1989) and mesenteric arteries (White & Hassoun, 2013). The cause of arterial vasospasm is believed to involve at least one of three mechanisms: 1) endothelial dysfunction, 2) hyperactivity of VSMC to vasoconstrictive stimuli, and 3) sympathetic nerve dysfunction (Lanza*, et al.*, 2011).

1.2.1 Endothelial dysfunction

As discussed previously, the endothelium plays a crucial role in modulating vessel tone by releasing EDRFs (NO and PGI_2), EDHF, and EDCFs (endothelin-1) to maintain basal tone (Sandoo*, et al.*, 2010a). However, endothelial injury leads to an imbalance between these vasoactive mediators and vascular tone (Lanza*, et al.*, 2011). Endothelial injury leads to reduced NO, PGI_2 and EDHF release, and increases endothelin-1 production leading to impaired vasodilation and pronounced vasoconstriction (Virdis*, et al.*, 2010), causing vasospasm (Moukarbel & Weinrauch, 2012).

The vascular endothelium has other important functions such as vessel wall remodeling (Rudic*, et al.*, 1998), regulation of inflammatory mediators (Harrison*, et al.*, 2006) and anti-thrombotic factors (Vanhoutte, 2009; Wu & Thiagarajan, 1996). An imbalance between growth-inhibitors and growth-promoting factors, the pro- and anti-inflammatory mediators, the pro- and anti-coagulation factors, as well as the excessive reactive oxygen species (ROS) can occur from endothelial injury (Rajendran*, et al.*, 2013). Chronic endothelial dysfunction is associated with atherosclerosis (Bonetti*, et al.*, 2003; Davignon & Ganz, 2004), essential hypertension (Perticone*, et al.*, 2001), peripheral vascular disease (Vita & Hamburg, 2010) and stroke (Roquer*, et al.*, 2009). Oxidative stress is thought to be the common denominator of endothelial dysfunction (Chrissobolis*, et al.*, 2010; Schulz*, et al.*, 2011; Shrestha*, et al.*, 2018). Elevated ROS not only directly inactivates NO but also impairs its synthesis (Landmesser*, et al.*, 2006). In addition to oxidant stress, a range of factors can also contribute to endothelial dysfunction (Félétou & Vanhoutte, 2006). These include aging (Herrera*, et al.*, 2010), smoking (Lavi*, et al.*, 2007), dyslipidaemia (Guardamagna*, et al.*, 2009), hyperhomocyteinemia (Jin*, et al.*, 2007), and hypertension (Versari*, et al.*, 2009). Recently, it has been reported that accumulated chemotherapy may also induce arterial stiffness and endothelial dysfunction (Taniguchi*, et al.*, 2014). Medical interventions do not necessarily improve endothelial function, but they may recover part of these factors or symptoms (Félétou*, et al.*, 2011; Flammer*, et al.*, 2012).

1.2.2 Vascular Smooth Muscle Cell (VSMC) hyperactivity

It has been revealed that VSMC could provoke extreme responses to agents leading to vasospasm in a range of pathological conditions (Lacolley*, et al.*, 2012). Spasmodic agents may include catecholamines, serotonin, vasopressin, and also EDCF including COX-derived prostanoids (thromboxane A2) and endothelin-1 (Hoenicka*, et al.*, 2011; Sung*, et al.*, 2009).

Vascular hyper reactivity has been demonstrated in human Prinzmetal's angina *or* coronary artery spasm (Okumura*, et al.*, 1996), human diabetes (Fleischhacker*, et al.*, 1999; Guo*, et al.*, 2005) and in spontaneously hypertensive rats (de Oliveira Salgado & Krieger, 1982). The *in vivo* mechanism for abnormal vascular reactivity remains unknown. In diabetic and hypertensive animal models, Oliveira and colleagues have implicated the COX system (de Oliveira Salgado & Krieger, 1982; Guo*, et al.*, 2005), and Kadokami and colleagues has suggested enhanced Ca^{2+} sensitivity mediated by increased Rho-kinase or protein kinase C activities in abnormal coronary artery spasm (Kadokami*, et al.*, 1996; Kandabashi*, et al.*, 2000).

1.2.3 Sympathetic nerve innervation

Apart from endothelial dysfunction and vascular hyper-reactivity, increased sympathetic tone has also been implicated as one of the triggers of vasospasm (Brunet*, et al.*, 2014). Increased sympathetic stimulation can lead to elevated catecholamines, serotonin and vasopressin, leading to prolonged vasoconstriction (Guyenet, 2006). Immunohistochemical studies demonstrated that sympathetic nerves mainly project to the tunica adventitia and the border between the adventitia and tunica media of the vessels (Reddy*, et al.*, 2011). The density of sympathetic fiber innervation is apparently dissimilar in large elastic and small resistance arteries (Brunet*, et al.*, 2014). Large elastic aorta are poorly innervated (Storkebaum & Carmeliet, 2011), while very dense innervation is predominantly observed in skin and mesenteric arteries, and hence, these are more vulnerable to sympathetic stimulation (Luff*, et al.*, 2005; Ruocco*, et al.*, 2002). Barry and colleagues (2007) further showed that sympathetic innervation is evident in the adventitia of coronary, radial, ulnar and epigastric, but not in internal thoracic arteries (ITA), which may partly explain the better patency of ITA (Barry*, et al.*, 2007; He, 1999). However, more recently, Reddy and coworkers (2011)

found the presence of a sympathetic nerve area in ITA of men and women. However there is no direct contact between the VSMCs and sympathetic nerves which may contribute to the low incidence of spasm in ITA conduits (Reddy*, et al.*, 2011).

1.3 The importance of pharmacological vasodilation and vascular protection in cardiac surgery

As the prevalence of cardiovascular diseases grows worldwide, there is an increasing need to find new vasoactive agents (single or in combination) to maintain improved supply-demand balance in the heart (Gulati & Simari, 2009) and to ensure sufficient ventricular-arterial coupling and tissue oxygen supply following cardiac and major surgery (Ky*, et al.*, 2013). This thesis will address three different vascular conditions that continue to challenge surgeons, cardiologists and post-operative intensive care unit (ICU) specialists.

1.3.1 Preventing Arterial Graft Spasm

Internal mammary or radial arterial conduits are commonly used for CABG as they are easily accessible and have relatively low occlusion rates at around 8-10% after the first year (Desai*, et al.*, 2004; Tatoulis*, et al.*, 1999), and 13-15% after 8-10 years (Buxton*, et al.*, 2009; Goldman*, et al.*, 2004b). A potential limitation of arterial grafts is their high susceptibility of vasospasm during graft preparation (Gao*, et al.*, 2013; Watanabe*, et al.*, 2013), and peri-operative spasm which occurs in around 10% of cases (Ajani & Yan, 2007; Velez*, et al.*, 2001). Vasospasm may contribute to early myocardial ischaemia and myocardial infarction (Kaul & Ito, 2004).

Current dilators used in cardiac surgery to help overcome perioperative spasm are papaverine, calcium channel blockers (diltiazem and verapamil), organic nitrates (glyceryl-trinitrate and nitroglycerine), selective phospodiesterase III inhibitors (levosimendan), K^+ channel openers (levokromakalim, diazoxide, minoxidil sulphate), and alpha-adrenoceptor antagonist (phenoxybenzamine), lidocaine, and more recently the Rho-kinase inhibitor fasudil (Watanabe*, et al.*, 2013; Yildiz*, et al.*, 2013). The problem is many of these vasodilators have not been standardized and their use varies from surgeon to surgeon.

For example, topical papaverine, a phosphodiesterase inhibitor (Yildiz*, et al.*, 2013) is very common but it is acidic and short-lived in effect (Murakami*, et al.*, 2009), and there is growing evidence that papaverine may lead to endothelial dysfunction (Dipp*, et al.*, 2001; Gao*, et al.*, 2002; He, 1998; Mayranpaa*, et al.*, 2004). Gao and colleagues have shown that papaverine induces apoptosis and endothelial damage in canine and human internal thoracic or radial arteries especially at high concentrations (Gao*, et al.*, 2002). Mayranpaa has also shown reduced endothelial viability in the presence of papaverine in cultured human coronary endothelial cells (Mayranpaa*, et al.*, 2004). Since the integrity of endothelial cells is crucial for normal vascular function and post-graft patency, alternative strategies to prevent and protect spasm have been sought (Gao*, et al.*, 2002). To this end, in 2013, Watanabe and colleagues showed that fasudil had a very potent vasodilatory effect on the internal thoracic artery compared with conventional papaverine and it resulted in increased graft free flow (Watanabe*, et al.*, 2013). The group concluded that the Rho-kinase inhibitor may be a useful graft dilating agent in cardiac surgery, however it remains to be globally adopted. In summary, despite decades of investigating numerous pharmacological agents to relax arterial conduits for CABG surgery, there remains a need for alternate topical agents that are effective, safe and affordable.

1.3.2 Reducing Mesenteric Artery Vasoconstriction and Spasm

Vasoconstriction and/or spasm of the superior mesenteric artery that delivers blood to the intestine from the lower part of the duodenum through two-thirds of transverse colon, as well as the pancreas, can be life-threatening following cardiac surgery or other major surgery (Oldenburg W, 2004). Reduced blood flow can lead to acute mesenteric ischaemia (AMI). Although relatively rare, AMI arises from vasospasm, occlusion, and/or sudden hypoperfusion of the mesenteric vasculature (Oldenburg*, et al.*, 2004).

The clinical consequences of AMI can be catastrophic, including sepsis, bowel infarction, and death (Berland & Oldenburg, 2008). Based on autopsy data from the 1970-1980s, 12.9 per 100.000 population have been diagnosed with this condition (Acosta & Björck, 2014). Despite its low incidence, the mortality rate ranges from 70% to 100% (Schütz*, et al.*, 1998), and appears to be on the rise in the past few years (Berland & Oldenburg, 2008). The condition can be due to low cardiac output,

increased sympathetic tone causing mesenteric artery vasoconstriction, or embolic or thrombotic occlusion (Eker*, et al.*, 1999; Imanaka*, et al.*, 2006).

The management of AMI is difficult and there are few drug therapies to alleviate the condition. One method for non-occlusive AMI is continuous intravenous infusion of papaverine (Kahn, 2011; Oshikata*, et al.*, 2013). However, as mentioned above, the use of papaverine has been associated with side effects including cardiac tachyarrhythmias (Kahn, 2011). This would complicate its use in patients with underlying cardiac disease and, therefore, finding an alternative could play an important role in improving AMI survival rate. In summary, acute non-occlusive mesenteric ischaemia currently has no clear-cut effective management and has a poor prognosis as a result of low cardiac output, and it can be aggravated by on- or off-pump coronary artery bypass grafting (Katz*, et al.*, 2006).

1.3.3 Vascular Relaxation and Protection during Isolated Vessel Preservation

Vessel banking is becoming a popular experimental tool to provide vessel conduits for cardiovascular, vascular surgery or reconstructive surgery when they are otherwise limited options (Zatschler*, et al.*, 2009). Some of the problems with the current storage solutions and technologies are damage to endothelial cells which may promote low perfusion and graft vasculopathy (Wille*, et al.*, 2008; Winkler*, et al.*, 2016).

The main problem of vessel banking is the maintenance of vascular quality and function following extended storage and rewarming (Garbe*, et al.*, 2011). Although cold storage (0-4^{o}C) down-regulates tissue metabolism and provides some protection against endothelial damage, most of the damage occurs during the rewarming phase (Wille*, et al.*, 2008; Zatschler*, et al.*, 2009). Isolated aortic rings in cold storage with traditionally used high potassium depolarizing preservation solution such as University of Wisconsin (UW), Euro Collins (EC), and histidine-tryptophan-ketoglutarate (HTK) solution have failed to protect with contractility and relaxation loss after 72 hours of storage (Corner*, et al.*, 2003). The loss of contractile function of up to 40% is seen as early as 36-hours after cold storage using UW and Perfadex solution; and while Krebs solution could protect the contractility function, it also leads to 86% loss of endothelial-induced relaxation over similar time periods (Ingemansson*, et al.*, 1995).

Endothelial injury is believed to be the key factor of vascular dysfunction following isolated vessel or ring ischaemic preservation, since endothelial cells are vulnerable to oxidative stress (Kutchai & Geddis, 2001). As endothelial cells and smooth muscle interactions have key roles in optimal contraction-relaxation regulation and long-term patency (Davies & Hagen, 1995; Thatte & Khuri, 2001), endothelial dysfunction in cold high potassium depolarization storage solutions has been shown to lead to loss of relaxation, increased risk of atherosclerosis and intimal hyperplasia as well as reduced graft patency (Dobson*, et al.*, 2013a; Wilbring*, et al.*, 2011). Therefore, improved preservation solutions are required to maintain endothelial viability of isolated vessels during preservation and rewarming period if vessel banking is going to be clinically useful.

1.4 Potential alternative vasoactive agent for vasospasm prevention/dilation and vascular preservation

As already discussed, the major drawbacks with current antispastic strategies and vascular protection include less potency, insufficient endothelial protection and adverse effects on cardiac function. Finding a novel drug strategy may improve vascular outcomes. The topic of this present thesis is to explore the potential use of adenosine and lidocaine (AL) solution as a vasodilatory agent to prevent vasospasm and improve vascular preservation. What follows is a brief introduction into the properties of each drug followed by a summary of the work carried out on AL in cardiac surgery, as an anti-ischaemic and anti-inflammatory; and finally, the hypothesis to be tested in isolated blood vessels.

1.4.1 Adenosine

Adenosine is a naturally occurring purine nucleoside released from metabolically active cells mostly as a response to stress conditions such hypoxia or ischaemia (Berne, 1980; Layland*, et al.*, 2014; Peart & Headrick, 2007). It is formed either from adenosine monophosphate (AMP) dephosphorylation by 5'-nucleotidase or from S-adenosylhomocysteine (SAH) hydrolysis by SAH hydrolase (see Figure 1.3) (Linden, 2005; Obata, 2002; Sala-Newby*, et al.*, 1999). Obata (2002) showed that dephosphorylation of AMP is the major source of adenosine in hypoxic and ischaemic

rat heart.

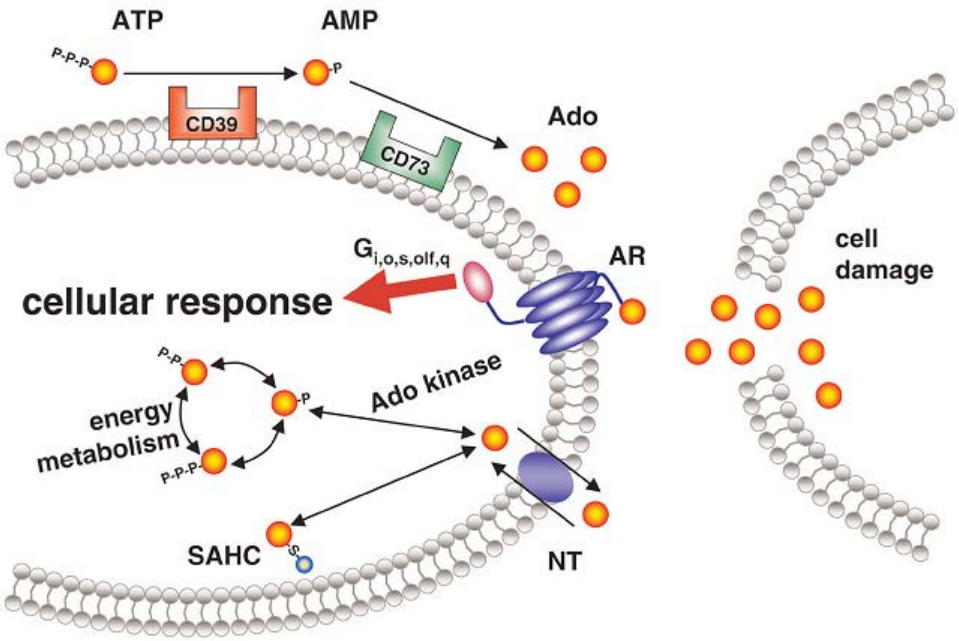

Figure 1.3 Sources of extracellular adenosine.
Intracellular hydrolysis of adenine nucleotides or S-adenosylhomocysteine (**SAHC**) yields adenosine that is released via specific nucleoside transporters (**NT**) or nonspecifically upon cell membrane damage. In the extracellular space, adenine nucleotides are hydrolyzed by ectoapyrase (**CD39**) and ecto-5'-nucleotidase (**CD73**). Adenosine binds to specific G-protein-coupled receptors, namely the adenosine receptors (AdoRs), which initiate various cellular responses (Carsten P Schepp and Jörg Reutershan, 2008).

Adenosine has many physiological functions including: 1) rapidly and reversibly slows heart rate and contraction (negative chronotropism and inotropism) (Belardinelli*, et al.*, 1995; Headrick*, et al.*, 2003; Peart & Headrick, 2007) 2) slows atrioventricular conduction (negative dromotropism) (Canyon & Dobson, 2004; Wagner*, et al.*, 1994), 3) possesses coronary vasodilatory properties (Vasu*, et al.*, 2013), 4) has preconditioning and anti-ischaemic properties involving activation of survival kinases via adenosine A_1 activation (Downey*, et al.*, 2007; Hausenloy & Yellon, 2006; Vinten-Johansen*, et al.*, 2005), 5) angiogenesis (Ernens*, et al.*, 2015; Feoktistov*, et al.*, 2003), 6) regulates activity in the sympathetic nervous system (Sousa*, et al.*, 2014) , 7) modulates inflammation (Antonioli*, et al.*, 2014; DeOliveira*, et al.*, 2017), and 8) has antithrombotic properties (Fuentes & Palomo, 2015). Adenosine has been called the retaliatory metabolite because it helps to regulate the oxygen supply and demand of heart and many other tissues and organs (Ådén*, et al.*, 2003; Alders*, et al.*, 2015; Peng*, et al.*, 2016). Adenosine actions and signaling is mediated by four G-protein–coupled

adenosine receptors (AR): A_1, A_{2a}, A_{2b}, and A_3 (Headrick*, et al.*, 2003; Peart & Headrick, 2007; Sheth*, et al.*, 2014). The four receptors involved and their role in regulating vascular relaxation and vascular tone in coronary vessels will now be discussed.

1.4.1.1 Adenosine physiological effects: the role of adenosine receptors

Adenosine receptors are a class of membrane-bound purinergic receptors called P1 receptors which are preferentially activated by adenosine in contrast to P2Y receptors that are preferentially activated by ATP (Jacobson & Gao, 2006). There are four main types of adenosine receptors (AdoRs), namely adenosine A_1, adenosine A_{2a}, adenosine A_{2b}, and adenosine A_3 receptors (Sheth*, et al.*, 2014). The AdoRs classification is based on the effect of different inhibitors, modulators with different affinities for adenosine involving G-protein coupled pathways (Bruns*, et al.*, 1986; Calker*, et al.*, 1979; Eltzschig*, et al.*, 2009b). The distribution of the AdoR subclasses throughout the body is diverse as shown in Table 1.1.

The A_1 receptors are highly expressed in nervous system and less so in skeletal muscle, spleen, liver and kidney (Fredholm*, et al.*, 2000). The A_{2a} receptors are dominantly found in striatum, spleen, thymus, olfactory bulb as well as leucocytes (Linden, 2001). In contrast, the A_{2b} receptors are abundantly distributed in caecum, colon, bladder, lung, eye and blood vessels, but also found in adipose tissue, adrenal glands, brain, kidney, and liver at low level (Fredholm*, et al.*, 2000).

Table 1.1 the adenosine receptor distributions and related effects
(Fredholm *et al.*, 2000)

Type	A_1	A_{2a}	A_{2b}	A_3
Species of cloning	Man, rat, mouse, dog, cow, rabbit, guinea pig, chicken	Man, rat, mouse, dog, guinea pig	Man, rat, mouse, chicken	Man, rat, mouse, dog, sheep, chicken
Organs with high expression	Brain, spinal cord, eye, adrenal gland, atria	Spleen, thymus, leukocytes, blood platelets, striatoplallidals GABA-nergic neurons, olfactory bulb	Caecum, colon, bladder	Testis (rat), mast cells (rat)

Effects of G-protein coupling	↓ cAMP ↑ IP_3/DAG (PLC) ↑arachidonate (PLA_2) ↑ choline (PLD)	↑ cAMP ↑ IP_3	↑ cAMP ↑ IP_3/DAG (PLC)	↓ cAMP ↑ IP_3/DAG (PLC)

Definition: ↑ increase; ↓ decrease of concentration

Historically, the intracellular signaling pathways of AdoRs are associated with the modulation of adenylyl cyclase activation, leading to either higher or lower intracellular cAMP levels (Fredholm*, et al.*, 2000). However, other intracellular pathways are involved (see fig. 1.4), including phospholipase C (PLC), potassium channels, calcium channels and mitogen-activated protein kinases (MAPKs) (Jacobson & Gao, 2006).

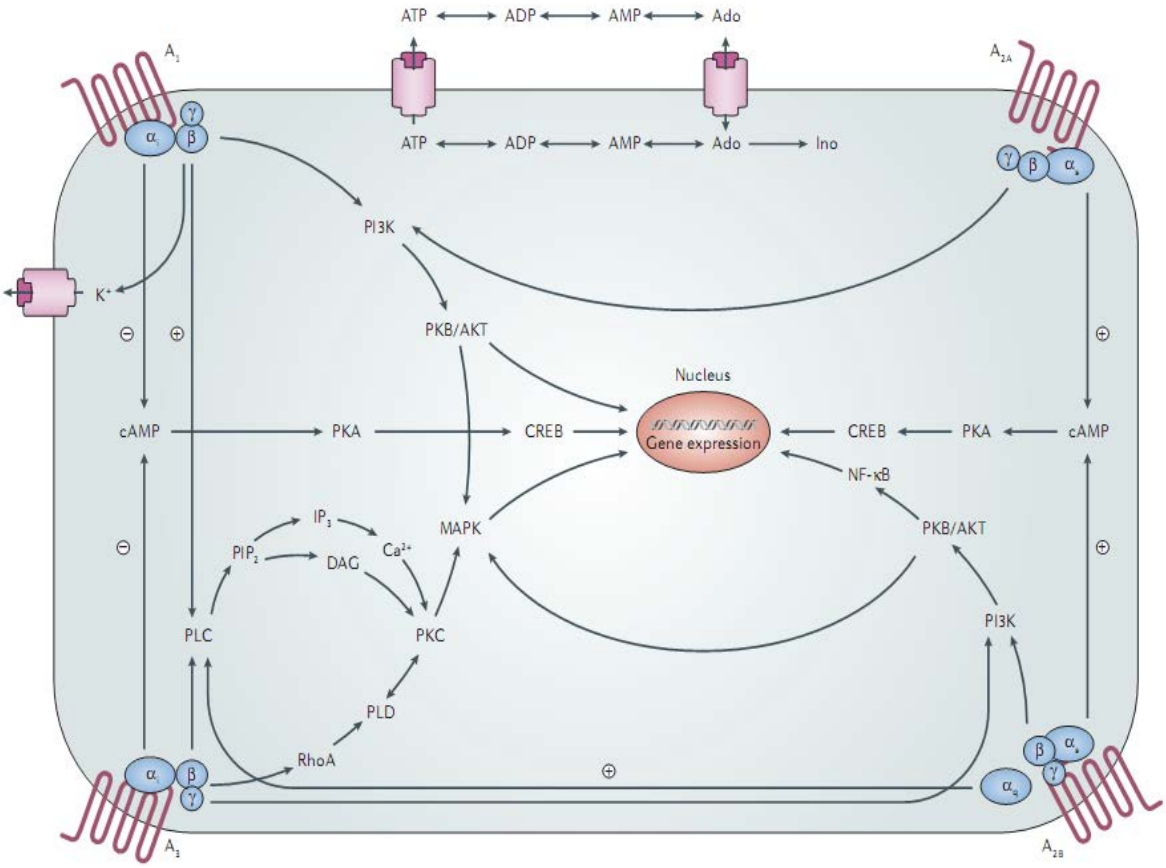

Figure 1.4 Adenosine receptor signaling pathways.
Activation of the A_1 and A_3 adenosine receptors (AdoRs) inhibits adenylyl cyclase activity through activation of pertussis toxin-sensitive G_i proteins and results in increased activity of phospholipase C (PLC) via $G_{\beta\gamma}$ subunits. Activation of the A_{2a} and A_{2b} AdoRs increases adenylyl cyclase activity through activation of Gs proteins. Activation of the A_{2a} AdoRs to induce

formation of inositol phosphates can occur under certain circumstances, possibly via the pertussis toxin-insensitive $G_{\alpha}15$ and $G_{\alpha}16$ proteins. A_{2b} AdoRs-induced activation of PLC is through G_q proteins. All four subtypes of AdoRs can couple to mitogen-activated protein kinase (MAPK), giving them a role in cell growth, survival, death and differentiation. CREB, cAMP response element binding protein; DAG, diacylglycerol; IP_3, inositol 1,4,5-trisphosphate; PI_3K, phosphatidylinositol 3-kinase; PIP, phosphatidylinositol-4,5-bisphosphate; PK, protein kinase; PLD, phospholipase D; NF-&B, nuclear factor-&B. (Jacobson & Gao, 2006)

A_1 receptor stimulation occurs from the inhibition of adenylate cyclase, indirect activation of potassium channels, indirect inactivation of calcium channels, alteration of inositol phosphates, or inactivation of phospholipase A_2 (Linden, 2001). Thus, in general, the A_1 receptor activation is mostly related to adenosine's inhibitory effects on physiological processes such as negative chronotropic, dromotropic, and inotropic (Shryock & Belardinelli, 1997) and antiadrenergic effects (Burgdorf*, et al.*, 2001) or even attenuation of norepinephrine-provoked adenylyl cyclase activation in cardiomyocytes (Belardinelli*, et al.*, 1995). Accordingly, adenosine's role in maintaining the oxygen supply-demand ratio is believed to be mostly mediated by activation of A_1 receptors (Babich*, et al.*, 2015)

As mentioned previously, the A_2 receptors are subdivided into A_{2a} and A_{2b} receptors based on differences in affinity to adenosine (Fredholm*, et al.*, 2000). The A_{2a} receptors have a higher affinity for adenosine compared to that of the A_{2b} receptors (Olanrewaju*, et al.*, 2000). The activation of the two A_2 receptors also involves cyclic AMP-dependent pathways including phospholipase C (Offermanns & Simon, 1995), P-type of Ca^{2+} channels (Umemiya & Berger, 1994), and MAPK (Fredholm*, et al.*, 2000). However, in contrast to the inhibitory action of A_1 receptors, the effect of A_2 receptors is predominantly stimulatory. In isolated cardiomyocytes, for example, they have positive inotropic, chronotropic and dromotropic actions (Dobson & Fenton, 1997; Stein*, et al.*, 1994) and believed partly due to coronary vasodilation and induced stretching of myocardium, which are largely associated with A_{2a} rather than A_{2b} receptor stimulation (Dobson & Fenton, 1997). In the central nervous system, an excitatory role of A_{2a} AdoRs on glutamate, GABA and acetylcholine activity has also been recognized (O'regan*, et al.*, 1992). This is supported by the fact that A_{2a} $AR^{-/-}$ knockout mice persistently showed declined exploratory behaviour, which implies a depressed central nervous system (Ledent*, et al.*, 1997).

Unfortunately, the physiological function of A_{2b} receptors has been difficult to assess. Several reports indicate a link between A_{2b} receptors with MAPK activation as well as

HMC-1 mast cells (Gao*, et al.*, 1999; Linden*, et al.*, 1999), which may suggest a role of A_{2b} receptors in the proliferation and differentiation of vascular smooth muscle cells, cell growth and proliferation (Jackson*, et al.*, 2011; Jonzon*, et al.*, 1985). However, these functions are also related to the adenosine A_3 receptor subtype (Hinze*, et al.*, 2012). It is thought that the lack of progress in understanding the physiological effects of A_{2b} activation most likely results from the lower affinity of A_{2b} towards adenosine and adenosine derivatives (Beukers*, et al.*, 2000; Gao*, et al.*, 2004). Fredholm (2001,2010) revealed that adenosine binding to A_{2b} was 30-80 less potent than the other subtypes (Fredholm, 2010; Fredholm*, et al.*, 2000). Thus, a higher level of adenosine is required to activate A_{2b} and a lower level of adenosine may be inadequate to trigger A_{2b} receptor activity (Eckle*, et al.*, 2008a; Eckle*, et al.*, 2008b; Grenz*, et al.*, 2008). Interestingly, it has been recently shown that in A_{2a} genetic depleted mice, that the A_{2b} receptors could be up-regulated to substitute A_{2a} receptor function (Teng*, et al.*, 2008).

Typically, the physiological concentration of adenosine is maintained about 1 μM, which is hardly sufficient to activate A_{2b} receptors (Hasko*, et al.*, 2008). Consequently, most A_{2b} receptor activation is found in pathological conditions, during which the generation of adenosine is elevated (Eckle*, et al.*, 2007; Eltzschig*, et al.*, 2009a; Fredholm, 2007).

The A_3 receptors appear to signal in a similar way to A_1 receptors, involving inhibition of adenylyl cyclase activity and/or stimulation of inositol 1,4,5-triphosphate as well as phospholipase C activity (Nishat*, et al.*, 2012). Due to their likeness in signaling pathways, the A_3 receptor-induced physiological effects are more or less equal to those of A_1 receptors (Ramkumar, 2000), especially in the cardiovascular system (Mubagwa & Flameng, 2001). The effects of A_3 receptor activation include cytoprotection, attenuation of pathophysiological intracellular calcium influx, reduced oxygen-derived radical formation, and inhibition of lipid peroxidation (Emanuelov*, et al.*, 2010; Shneyvays*, et al.*, 2000). Unlike the A_1 receptors, A_3 receptors experience more rapid phosphorylation along with desensitization (Palmer*, et al.*, 1996; Palmer & Stiles, 2000). It seems that A_3 receptor depalmitoylation increases their vulnerability to G protein-coupled receptor-induced phosphorylation (Palmer & Stiles, 2000).

1.4.1.2 Role of Adenosine in Vascular Tone

Adenosine and its analogs are involved in the regulation of vascular tone to varying degrees (Berne, 1980). A_{2a} and/or A_{2b} AdoRs are involved in adenosine-mediated vascular relaxation of coronary and aortic beds (Tabrizchi & Bedi, 2001), whereas A_1 AdoR involvement in the regulation of vascular tone is less clear. Adenosine's action on isolated rings from the pulmonary artery (Biaggioni*, et al.*, 1989; McCormack*, et al.*, 1989) and renal vasculature (Marraccini*, et al.*, 1996; Martin & Potts, 1994) are further complicated by it displaying a biphasic response in relaxation and contraction, which appears to be concentration-dependent. Despite decades of research, the role of the different adenosine receptor subtypes in vascular tone is still a matter of debate (reviewed by Tabrizchi and Bedi, 2001).

1.4.1.2.1 Adenosine-induced vasoconstriction

Using A_1 AdoR-knockout A_1 AdoR mice, Tawfik and colleagues showed that the activation of A_1 AdoRs caused a contraction in isolated aortic rings (Tawfik*, et al.*, 2005). According to the authors, the A_1 AdoRs interact with G_i- or G_o-regulatory proteins, which leads to inhibition of adenylate cyclase and a decrease in cAMP levels, as well as protein kinase A (PKA) activity, that appears to contribute to the adenosine-evoked vasoconstriction. Another study by Hansen and colleagues showed vasoconstriction involved G_i-proteins and activation of phospholipase C (Hansen*, et al.*, 2003). Shim et al., also reported that adenosine-induced vasoconstriction can be independent of a decrease in cAMP levels (Shim*, et al.*, 2002). These differences may be related to differences in distribution of adenosine receptors between tissues (Sheth*, et al.*, 2014).

1.4.1.2.2 Adenosine-induced vasodilation

The general consensus is that adenosine-induced vasodilation is exerted through the activation of the A_2 AdoRs (Fahim*, et al.*, 2001). While A_{2a} is thought to be predominant receptor subtype, A_{2b} may also play a role (Hinschen*, et al.*, 2003; Shryock*, et al.*, 1998). For example, a study using isolated guinea pig hearts shows that the A_{2a} AdoRs were involved in coronary vasodilation (Belardinelli*, et al.*, 1998); whereas, other studies suggest that vasodilation is mediated by activation of the A_{2b} AdoRs in human

small coronary arteries (Kemp & Cocks, 1999). The involvement of both A_{2a} and A_{2b} AdoRs in vasodilation is also supported by the finding that both subtypes are present on either or both endothelial and vascular smooth muscle cells (Olanrewaju & Mustafa, 2000). However, since the A_{2b} AdoRs have a lower affinity compared to the A_{2a} AdoRs for adenosine, it is believed that the activation of the A_{2b} receptors require high local concentrations of adenosine to result in vasodilation (Fredholm*, et al.*, 2000).

In addition, further complexity arises as large coronary arteries are less sensitive to adenosine than smaller ones and epicardial micro vessels are less responsive than those located in epicardium (Harder*, et al.*, 1979; Quillen & Harrison, 1992).

1.4.1.3 Contribution of endothelium to adenosine-induced vasodilation

The vasodilatory effect of adenosine has been linked to endothelial NO production by endothelial cells (Li*, et al.*, 1995). This has been confirmed when endothelial NO synthesis is inhibited (Okumura*, et al.*, 1992; Vials & Burnstock, 1993) or by removal of the endothelium (Iwamoto*, et al.*, 1994; Rose'Meyer & Hope, 1990).

The possible mechanisms for NO-induced adenosine relaxation include the following: 1) Activated endothelial A_{2a} receptors are coupled to K_{Ca} channels (Hein*, et al.*, 1999) which leads to K^+ efflux and Ca^{2+} influx and activates eNOS with subsequent NO synthesis and release (Ray & Marshall, 2006b). The production of NO leads to increased cGMP level which results in vascular relaxation (Olanrewaju & Mustafa, 2000). In addition, increased intracellular cAMP stimulates cAMP-dependent protein kinase A (PKA) activation, which, in turn, facilitates the phosphorylation of eNOS (Ray & Marshall, 2006b). 2) ATP-dependent potassium (K_{ATP}) channels have been shown to be activated by endothelial adenosine receptors, which leads to increased influx of Ca^{2+} and NO release (Leipert*, et al.*, 1992).

Another mechanism of adenosine's action is independent of the endothelium (Sato*, et al.*, 2005). In a study using denuded human coronary arteries, relaxation was shown by activation of K_{Ca} channels in vascular smooth muscle which leads to adenylate cyclase activation (Sato*, et al.*, 2005). Samples for this study were taken from patients with heart disease who probably have altered endothelial function. A separate study

on healthy adults has verified the importance of the endothelium in the vasorelaxant effect of adenosine in humans (Smits*, et al.*, 1995).

1.4.1.4 Contribution of K^+ channels and Na^+/K^+-ATPase to adenosine-induced vasodilation

Activation of vascular smooth muscle K_{ATP} channels in thoracic aorta (Kleppisch & Nelson, 1995), mesenteric arteries (Quayle*, et al.*, 1995) and gallbladder (Zhang*, et al.*, 1994a), are also involved in adenosine-evoked vasodilation (Tabrizchi & Bedi, 2001). K_{ATP} channels trigger the formation of cAMP and increase the activity of PKA (Brayden, 2002). In addition, the relaxation of rat aorta is found to be mediated, at least in part, by the activation of Na^+/K^+-ATPase, which is exerted through A_{2a} AdoR activation (Grbović & Radenković, 2003). Inhibition of the large-conductance calcium-activated potassium (K_{Ca}) channels in dog coronary arteries may attenuate adenosine–induced relaxation, suggesting their role in the vasodilator effect of adenosine (Cabell*, et al.*, 1994).

1.4.2 Lidocaine

Lidocaine was developed in 1943 as part of a chemical search for compounds with cocaine-like anaesthetic properties but without the toxic and addictive side-effects of cocaine (Bryce-Smith, 1960; Gordh*, et al.*, 2010; Heavner, 2007). Lidocaine acts as a Class 1B local anaesthetic and cell membrane stabilizer by blocking voltage-dependent sodium (Na^+) fast channels (Rosen & Danilo, 1980). In addition to its Class 1B anaesthetic function, lidocaine has been used as an intravenous antiarrhythmic agent that is widely used in the treatment and prevention of ventricular arrhythmias (Turan*, et al.*, 2000). Moreover, its function as a vasodilator has been identified, even though it's underlying mechanisms have not been completely elucidated (Gherardini*, et al.*, 1998a). Lidocaine also has non-Na^+ channel dependent effects which may involve N-type voltage-gated Ca^{2+} channels (Hiruma*, et al.*, 2008), K_v channels (Trellakis*, et al.*, 2006), K_{ATP} channels (Kinoshita*, et al.*, 2004), pore domain K channels TREK1 inhibition (Nayak*, et al.*, 2009), and mitoK_{ATP} channels (Tsutsumi*, et al.*, 2001), and believed to be responsible for its neuroprotection, anti-oxidant and anti-inflammatory effects (Lahat*, et al.*, 2008; Lee*, et al.*, 2010; Niiyama*, et al.*, 2005;

Tsutsumi, *et al.*, 2001; Wang, *et al.*, 2013). Lidocaine is also cardioprotective in cardiac surgery (Hinokiyama, *et al.*, 2003; Kim, *et al.*, 2014; Lee, *et al.*, 2011) by preventing ATP depletion (Fried, *et al.*, 1995; Soliman, *et al.*, 2012) and exerting anti-apoptotic actions during ischaemia (Kaczmarek, *et al.*, 2009). Of relevance to this thesis, lidocaine is reported to have vasodilatory effects but this remains very controversial (Kinoshita, *et al.*, 2001a; Kinoshita, *et al.*, 2004).

1.4.2.1 Lidocaine structure, metabolism and excretion

Lidocaine is a cationic amino amide drug that consists of a lipophilic end, 2,6-xylidine and connected to a hydrophilic end (diethylglycine) through an aminoamide link (Sweeney & Bromilow, 2006). Lidocaine metabolism greatly depends on microsomes in the liver. Studies on hepatic homogenates have shown that cytochrome P450 (CYP1A2) is involved in lidocaine degradation into monoethylglycinexylidide by N-de-ethylation through an oxidation process (Sweeney & Bromilow, 2006). Hydrolysis then takes place to convert the previous metabolite into 2,6-xylidine and other degradation products. Elimination of lidocaine and its metabolites occurs mainly through kidneys, and only a small amount is excreted via the intestines. Keenaghan and Boyes (1972) showed that more than 65% of lidocaine and the metabolites was found in the rat urine when administered either orally or intravenously, while only 0.5% accounts for intestinal elimination if lidocaine was administered orally, and none were found in the faeces if given intravenously.

1.4.2.2 Lidocaine effect on vascular tone

Lidocaine has been demonstrated to have biphasic vascular actions. It can produce either vasoconstriction or vasodilation in a concentration-dependent manner (Evans, *et al.*, 1997c; Johns, *et al.*, 1985).

1.4.2.2.1 Lidocaine-induced vasoconstriction

Preliminary studies have observed lidocaine-mediated vasoconstriction when administered intradermally especially at low concentration (Aps & Reynolds, 1976; Jorfeldt, *et al.*, 1970). This is in line with an *in vitro* study showing a vasoconstrictive

effect of lidocaine in isolated human arteries (Gherardini*, et al.*, 1995). The dose suggested for lidocaine-induced vasoconstriction is below 1.5 x 10^{-3} M. The vasoconstrictive effect of lidocaine is thought to be related to its interaction with K_{ATP} channels since lidocaine may impair relaxation induced by K_{ATP} channel activation (Kinoshita*, et al.*, 1999). It is suggested that lidocaine causes an increase in adenosine triphosphate (ATP) (Perlmutter*, et al.*, 1990a), therefore, with increased level of ATP, K_{ATP} -sensitive channels will be closed and depolarised (Rang*, et al.*, 2003). However, it seems that K_{ATP} channel is not the only pathway involved in the lidocaine vasoconstriction effect. Alteration of membrane potential as well as modulation of adrenergic action have been proposed by previous studies (Fleisch & Titus, 1973; Fukuda*, et al.*, 1980).

1.4.2.2.2 Lidocaine-induced vasodilation

Lidocaine at high concentration produces relaxation on blood vessels (Johns*, et al.*, 1985). This has been confirmed by *in vitro* studies using human radial and internal mammary artery rings (Gherardini*, et al.*, 1995; Jernbeck & Samuelson, 1993). Although one study showed a lidocaine vascular effect on human veins (Gherardini*, et al.*, 1996), vasodilation effects of lidocaine mainly affect arterioles (Turan*, et al.*, 2000). It is suggested that the vasodilation action of lidocaine was initiated at the concentration 30 μg/ml and was maximal at 2000 μg/ml (Perlmutter*, et al.*, 1990c).

It is believed that the lidocaine dilation action results partly from inhibition of the sympathetic adrenergic neurotransmission in the vascular wall (Hogan*, et al.*, 1998). The other mechanism suggested is a direct effect of lidocaine on vascular smooth muscle, since lidocaine is able to block Ca^{2+} influx required for contraction (Zhang*, et al.*, 1994b). Several reports have demonstrated that lidocaine inhibits Ca^{2+} entry through voltage and receptor gated channels as well as blocks the release of Ca^{2+} from intracellular stores (del Pozo*, et al.*, 1997; Tanaka*, et al.*, 2002). Consequently, lidocaine may attenuate transphosphorylation of myosin in the smooth muscle cells that fails the vascular smooth muscle constriction (Aberg & Wahlstrom, 1972). Regardless of a report showing weakened lidocaine-induced relaxation on endothelium intact arteries (Jernbeck & Samuelson, 1993), the lidocaine vasorelaxation effect is apparently independent of vascular endothelium (Gherardini*, et al.*, 1996). This in accordance with another paper by Turan *et al*. (2000) who reported

that removal of the endothelium did not significantly modify the lidocaine-induced relaxations.

1.4.2.2.3 Lidocaine modifies effects of other vasoactive agents

Lidocaine may differentially modify the vascular action of other agents. For example, lidocaine can inhibit noradrenaline-induced contractions, but it potentiates the caffeine-induced contraction in the vascular smooth muscle of rabbit aorta (Karaki*, et al.*, 1987). There is evidence that lidocaine inhibits endothelium-dependent relaxation induced by methacholine but not endothelium-independent vasodilation induced by sodium nitroprusside in rat isolated thoracic aorta (Johns, 1989). Several studies report that lidocaine is capable of impairing the relaxation mediated by K_{ATP} -sensitive channels (Kimoto*, et al.*, 2005; Kinoshita*, et al.*, 2003a; Kinoshita*, et al.*, 2004), supporting the role of K_{ATP} channels in the vasoconstriction effect of lidocaine. These studies observed attenuated relaxation with lidocaine at concentrations up to 100 mM, suggesting that higher concentrations are required to achieve vasodilation. Additionally, a recent finding showed that pH changes are possible to modify vasoactivity of lidocaine (Kinoshita*, et al.*, 2001a). One possible explanation could be that lidocaine acts through K_{ATP} channel activation, and changes in pH may result in the alteration of the ionization of ATP-sensitive K^+ protein (Kinoshita & Katusic, 1997).

1.4.3 Adenosine and lidocaine (AL) in combination

As mentioned previously, adenosine and lidocaine separately possess vascular actions, but their vasoactive effects as a combination (AL) have not been studied. It is the interest of this thesis to examine AL vasodilatory effect on isolated vessels and explore its potential use in vascular preservation. Current literatures have demonstrated AL benefits in cardiac surgery, as an anti-ischaemic and anti-arrhythmic agent, and in heart preservation solution. These benefits will be discussed in the following section.

1.4.3.1 AL in cardiac surgery

AL as a drug combination was first introduced as a cardioplegia by Dobson and Jones (2004). This formulation was based on the understanding that adenosine acts as a K_{ATP} opener, increasing K^+ efflux which can reduce the action potential duration in the heart, while lidocaine is a sodium fast channel blocker that works synergistically with adenosine by blocking the inward fast Na^+ current (Dobson, 2004). The adenosine-lidocaine combination arrests the heart just at the polarized state, at or near its resting membrane potential (Dobson, 2004). The main advantages of arresting the heart at or near resting membrane potential include: 1) less cellular activities leading to better energy preservation during ischaemia (Dobson, 2004), 2) less electrolyte and metabolic imbalances, thus, limiting cellular injuries (Cohen*, et al.*, 1995; Snabaitis*, et al.*, 1997), 3) less unwanted and toxic metabolites (ROS, lactate, ion H^+) are generated (Boutilier, 2001), and 4) reduced neutrophil activities, and hence reduced inflammatory reactions (Dobson, 2010).

The safety and efficacy of AL as a cardioplegia in high-risk patients of cardiac surgery has been demonstrated (Dobson*, et al.*, 2013a). O'Rullian*, et al.* (2008) reported a successful ±10-hour operation using AL for re-operative aortic and mitral valve replacement without systemic hyperkalaemia, haemodilution or significant tissue oedema on a 4-times redo elderly patient. Promising results were also demonstrated in the study by Jin*, et al.* (2008) using high (HPAL) and moderate (MPAL) K^+ concentrations with AL cardioplegia during hypothermia in randomized paediatric congenital heart patients. Jin and co-workers found no significant differences in arrest time, cardiopulmonary bypass (CPB) time, haematocrit value or fluid output compared to high potassium (HP) cardioplegia application. All three groups retrieved spontaneous rhythm after cross clamp removal, no temporary pacing support during operation, and there were no deaths or post-operative complications. However, AL with only 10mM K^+ (MPAL) cardioplegia resulted in higher systolic and pulse pressures after CPB, lower serum Troponin I value after 12-hour operation, reduced use of inotropes post-operatively, and less postoperative hospital time compared to other groups. In 2013, a randomised clinical trial showed that AL with magnesium and insulin in CABG patients led to significantly lower troponin and plasma lactate level, improved myocardial protection and cardiac index, less blood product requirement, and shorter ICU stays compared to patients who received the conventional Buckberg solution (Onorati*, et al.*, 2013b).

1.4.3.2 AL as an anti-ischaemic and anti-arrhythmic agent

In addition to cardiac surgery, AL has been shown to offer protection against ischaemia-reperfusion injury in animal models. (Canyon & Dobson, 2004, 2006). In the *in vivo* rat model, Canyon and Dobson (2004) showed that after 30 min of regional myocardial ischaemia, AL administration significantly improved survival with no deaths, compared to 58% mortality in control animals. AL treatment also significantly reduced myocardial infarct size, but this benefit was not conferred with either adenosine or lidocaine alone (Canyon & Dobson, 2004). The authors further showed that if the A_1 agonist 2-Chloro-N6-cyclopentyladenosine (CCPA) was used instead of adenosine and combined with lidocaine the infarct size fell to below 10%, similar to preconditioning (Canyon & Dobson, 2005). It is suggested that the anti-ischaemic effect of AL was associated with preservation of left ventricular high-energy phosphate (PCr and ATP) during ischaemia, indicating a downregulation of myocardial metabolism with AL (Canyon & Dobson, 2006). Lowering oxygen/energy demand appears to limit pathological milieu during ischaemia and following reperfusion (Dobson*, et al.*, 2013a).

Consistent with reduced infarct size, AL administration in the acute myocardial ischaemic model led to a 90% reduction in ventricular arrhythmias. In contrast, adenosine or lidocaine alone did not significantly reduce the arrhythmic events (Canyon & Dobson, 2004). The mechanism of AL's anti-arrhythmic action is unclear but may relate to synergistic effects of adenosine and lidocaine as both agents have anti-arrhythmic actions (Bush*, et al.*, 1989; Dobson*, et al.*, 2013a). Adenosine activates potassium channel ($I_K{}^+{}_{\mathbf{ADO,\ Ach}}$) opening leading to hyperpolarization and more stable myocardial excitability (Wilbur & Marchlinski, 1997). In addition, adenosine has an anti-adrenergic effect to counteract the stimulatory effects of catecholamines (Ely & Berne, 1992; Hayes, 2003). Whereas, lidocaine shortens the action potential by its inhibition of voltage-dependent Na^+ fast channels in cardiac myocytes (Ruiz-Meana*, et al.*, 1999), and through its interaction with sarcolemmal Ca^{2+} (Lu*, et al.*, 1999; Wilbur & Marchlinski, 1997). Prolonged hyperpolarization with adenosine or shortening the action potential with lidocaine may paradoxically become arrhythmogenic (Pelleg*, et al.*, 2002; Starmer*, et al.*, 1991), but the AL combination may "clamp" the membrane potential into a more polarized state compared to either adenosine or lidocaine alone

(Dobson & Jones, 2004). More recently, AL superiority to prevent fatal arrhythmias compared to each drug alone has also been shown following asphyxial cardiac arrest (Djabir & Dobson, 2013) and haemorrhagic shock in rats (Letson & Dobson, 2011b). The anti-ischaemic and anti-arrhythmic action of AL may find great utility if used as a vasodilator during cardiovascular surgery.

1.4.3.3 AL in heart preservation solution

AL cardioprotection has also been demonstrated in isolated rat heart preservation after six to eight hours of cold storage (Rudd & Dobson, 2009, 2011a, 2011b). In 2009, Rudd and Dobson showed that isolated rat hearts preserved in AL solution for six hours had significantly higher functional recovery compared to Celsior solution (68% vs 47% cardiac output). More prolonged (8-hour) cold storage with AL plus melatonin and insulin led to 78% recovery of full cardiac function compared to 4-25% recovery in those preserved in histidine-tryptophan-ketoglutarate (HTK) or Celsior solution. It is suggested that the AL combination stabilizes membrane polarity, reducing Na^+ and Ca^{2+} loading, and therefore limiting cellular damage and myocardial dysfunction during cold storage and reperfusion (Rudd & Dobson, 2011b). In accordance with this, no detectable troponin T and significantly lower lactate levels were found in AL-preserved hearts regardless of the prolonged cold static storage (Rudd & Dobson, 2011a, 2011b). Currently, the safe period for human heart storage is four to five hours (Rudd & Dobson, 2009). Thus, it would be interesting to examine if AL solution may offer the same protection in prolonged vascular preservation.

1.4.3.4 AL as an anti-inflammatory agent

AL protective effects appear to involve inhibition of the inflammatory response. Shi et al. (2012) showed that *in vitro*, AL combination reduced multiple polymorphonuclear neutrophil (PMN) activities to a greater extent compared to each drug alone. AL anti-inflammatory actions include suppression of superoxide generation, expression of adhesion molecules (CD11 and CD18), and PMN adherence and transmigration (Shi*, et al.*, 2012). In accordance with this, AL potent anti-inflammatory effects have been reported in an *in vivo* model of ventricular fibrillation-induced cardiac arrest (Granfeldt*, et al.*, 2013). Granfeldt and colleagues (2013) showed that resuscitation with AL not

only significantly improved cardiac function but also diminished leucocyte superoxide production following cardiac arrest in pigs. AL anti-inflammatory effects could be imperative to improve arterial graft protection when used as a vasodilator or to prevent endothelial dysfunction during vascular preservation.

1.5 General aim and hypothesis

The aim of this thesis is threefold:

1. To investigate the vasodilatory effects of adenosine (A), lidocaine (L) and adenosine-lidocaine (AL), and possible mechanisms of adenosine (A) and lidocaine (L) relaxation in isolated rat thoracic aortic rings.
2. To investigate the vasodilatory effects of A, L and AL on segments of guinea pig second order mesenteric arteries, with the future goal to reduce secondary complications from gut ischaemia during cardiac surgery.
3. To investigate the effect of AL and AL with antioxidants on six-day cold storage using rat thoracic aortic rings for future vessel preservation.

The following hypotheses will be tested:

1. Adenosine will relax precontracted isolated rat aortic rings dependent on endothelium. The relaxation may involve EDRF, K_v channels, K_{ATP} channels, Na^+/K^+-ATPase, A_{2a} and A_{2b} adenosine receptors (Chapter 3).

2. Lidocaine will relax precontracted isolated rat aortic rings independent of endothelium. The relaxation may involve EDRF, K_v channels, K_{ATP} channels, Na^+/K^+-ATPase, A_{2a} and A_{2b} adenosine receptors (Chapter 4).

3. Adenosine-lidocaine (AL) will relax precontracted isolated rat aortic rings independent of endothelium. The relaxation will be greater than A or L alone (Chapter 5).

4. A, L and AL will relax guinea pig mesenteric artery. Adenosine relaxation will be endothelium-dependent while L and AL will be endothelium-independent. Luminal and abluminal administration of A, L and AL will give the same relaxation on isolated mesenteric artery (Chapter 5).

5. AL and antioxidants can preserve isolated rat thoracic aortic rings for 6-day cold storage (Chapter 6).

CHAPTER 2
MATERIALS AND METHODS

2.1 INTRODUCTION

This thesis employed two *in vitro* methods to assess vascular reactivity: 1) Isometric force measurements for the isolated male rat aortic ring studies, and 2) Pressured myography for the isolated guinea pig mesenteric artery studies. This chapter will detail the experimental protocols including animal housing, anaesthesia and vessel preparation, drugs used, compositions and concentrations, and the method of statistical analysis.

2.2 ISOMETRIC FORCE MEASUREMENT

Isometric force measurements of vasoreactivity were used in Chapters 3, 4 and 5; and for Chapter 6 after static cold storage.

Isometric force measurement is useful in detecting the spasmogenic or vasodilatory effect of substances as well as their possible mechanisms of action on vascular segments. This technique requires basic pharmacological equipment, i.e. a force transducer, a water-jacketed organ bath and a data acquisition system. A schematic diagram and photograph of the experimental set-up are shown in Figures 2.1 and 2.2, respectively.

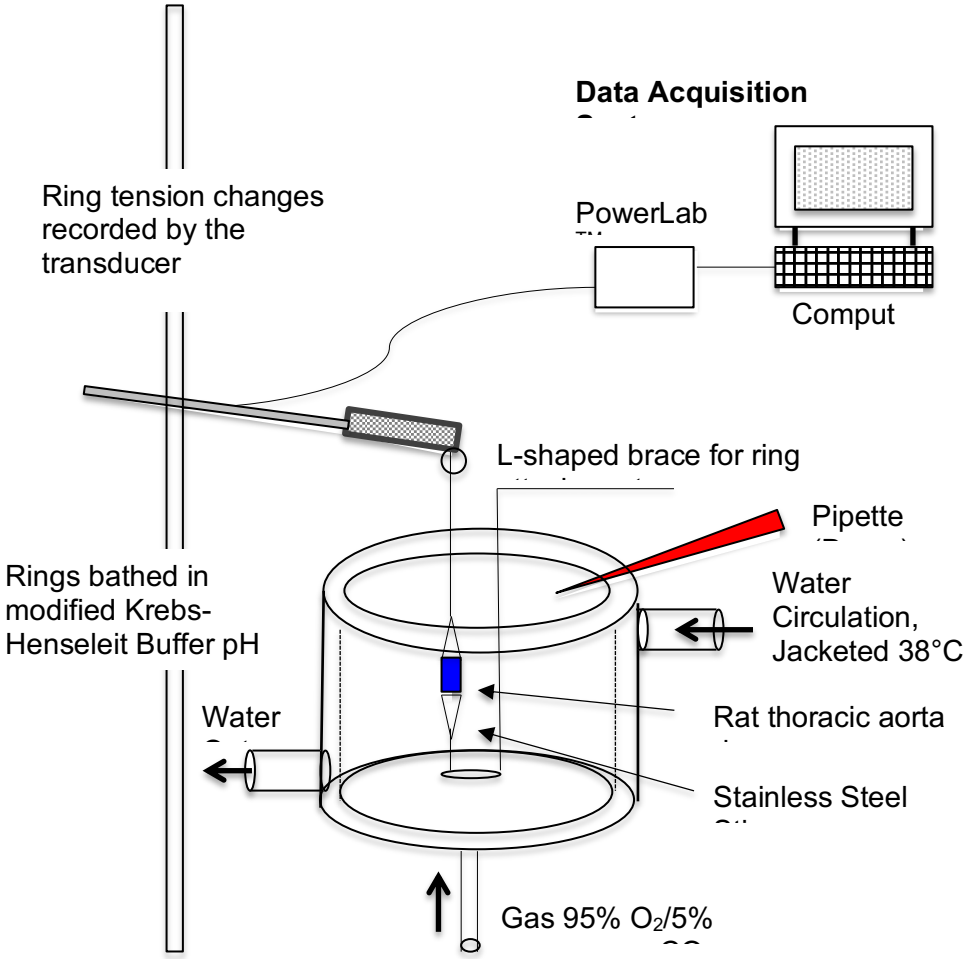

Figure 2.1 Diagram of isometric force measurement experimental setup.
The ring vessel was stretched between the triangle stirrups. One end was fixed on an L-shape brace and the other end was connected to a transducer. Ring tension changes recorded by the transducer were then sent to a PowerLab (ADInstrument) and portrayed on a computer.

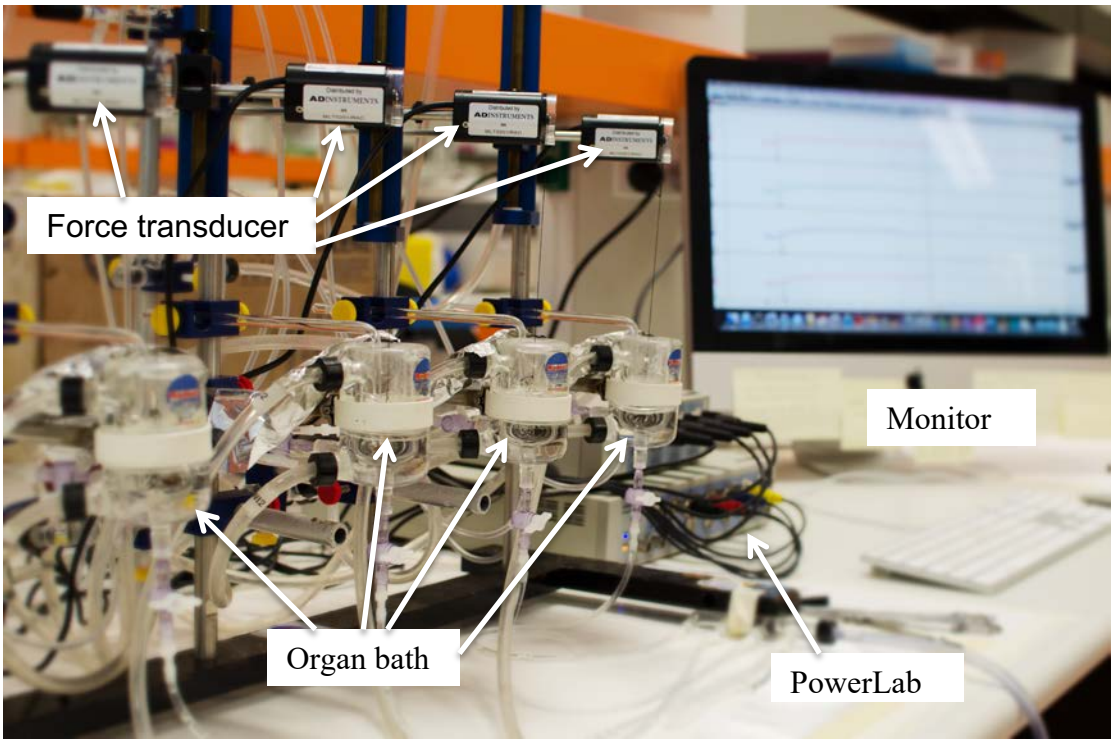

Figure 2.2 Photograph of the experimental setup for isometric force measurement.

2.2.1 Animal and drug preparation

Male Sprague Dawley rats (300-350g) from James Cook University breeding colony were fed *ad libitum* and housed in a 12-hour light/dark cycle. On the day of the experiment rats were anaesthetised with Na-thiopentone (100 mg/kg). Animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The studies were approved by James Cook University Animal Ethics Committee with approval number A1535. Lidocaine hydrochloride was sourced as a 2% solution from the local Pharmaceutical Suppliers (Lyppard, Queensland). All other chemicals, including adenosine (A9251 >99% purity), were purchased from Sigma Aldrich (Castle Hill, NSW).

2.2.2 Aortic ring preparation and organ bath tension measurements

2.2.2.1 Aortic ring preparation

Rat isolated aortic rings are well recognized in the literature as a robust model to investigate the vasoactive effects of substances, or their analogues (Grbović & Radenković, 2003; Prentice & Hourani, 2000; Tawfik*, et al.*, 2005).

Rats were anaesthetized as described in Section 2.2.1 and the thoracic cavity was opened and the thoracic aorta was removed and placed in an ice-cold solution of oxygenated Krebs Henseleit solution (118 mM NaCl, 4.7 mM KCl, 1.2 mM Na_2PO_4, 0.5 mM $MgCl_2$, 1.12 mM $CaCl_2$, 25 mM $NaHCO_3$, 0.03 mM EDTA) pH 7.4 with 11 mM glucose.

The rat thoracic aorta was carefully cleaned by dissecting the surrounding fat and connective tissue and cut into short segments of around 3-cm long. Isolated rings of 3-4 mm width were then cut from the segments (Broughton*, et al.*, 2010; Wang*, et al.*, 1996).

In those experiments that required the endothelium to be removed (denuded rings), this was achieved using the method of Broughton*, et al.* (2010) by gently rubbing the inside of the ring with a thin, smooth, stainless-steel rod. Successful removal of the endothelium was assessed by testing the aortic ring for a vasodilatory response to 10 μM acetylcholine (ACh) (Vo*, et al.*, 2005) as detailed in section *2.2.2.3 Endothelium function testing*. At the end of each experiment, the rings were tested for viability by being maximally dilated with 100 μM papaverine (Grbović & Radenković, 2003; Grbović*, et al.*, 2000b).

2.2.2.2 Aortic ring equilibration

Freshly prepared aortic rings (3-mm long) were equilibrated for 60 minutes in a standard 10 ml volume organ bath (Radnotti Glass, ADinstruments, NSW, AUS) filled with Krebs Henseleit (pH 7.4) containing 118 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgCl_2$, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$ and 0.03 mM EDTA with 11 mM glucose. The organ bath was continuously bubbled with 95% O_2 and 5% CO_2 at 37°C for 15 minutes (zero tension). The rings were vertically mounted on small stainless-

steel triangles, stirrups and connected to an isometric force transducer (PANLAB, distributed by ADInstruments as MLT 0201/RAD, NSW, AUS) coupled to a computer-based data acquisition system (PowerLab, ADInstruments) and data recording software LabChart 7 (ADInstruments Pty Ltd., Castle Hill, Australia).

The ring tension was manually adjusted to 1.5 g and equilibrated for 60 min. A tension of 1.5 g was chosen from the literature and preliminary studies showing that an optimum contraction was achieved at this tension following norepinephrine (NE) administration. During 60 min equilibration, the solution was changed at 15 minutes intervals. The aortic rings were then washed with freshly prepared Krebs Henseleit buffer pH 7.4 and the tension was readjusted to 1.5 g tension. Each preparation was sub-maximally contracted using 3 µl of 0.1 mM NE (0.3 µM final concentration) (Evans*, et al.*, 1997a; Zerkowski*, et al.*, 1993) and those aortic rings that failed to contract were discarded (Figure 2.3).

2.2.2.3 Endothelium function testing

When the increase of tension reached plateau level after 0.3 µM of NE administration, 10 µl of 10 mM acetylcholine (10 µM final concentration) was applied to confirm the presence or absence of an intact endothelium in all preparations (Vo*, et al.*, 2005). It is well-established that acetylcholine (ACh) will induce a rapid relaxation of pre-contracted rings if the endothelium is intact, and will have little or no effect if the endothelium is denuded (or damaged) leaving the rings in a contracted state (Furchgott & Zawadzki, 1980). In this thesis, aortic rings were considered intact if the relaxation induced by 10 µM ACh was greater than 80%, and the aortic ring was assumed to be denuded if relaxation was less than 10% (Vo*, et al.*, 2005). As mentioned above, in endothelium-denuded ring experiments, aortic rings were stripped by gently rubbing the intimal surface of the vessel segment with a thin, smooth stainless-steel probe (Broughton*, et al.*, 2010). After evaluation of endothelial function using ACh, organ baths were emptied and the rings were washed three times with Krebs Henseleit solution then left for another 30 min equilibration prior to experiments (Lewis*, et al.*, 2008) (Figure 2.3).

2.2.2.4 Concentration-dependent relaxation experiments

After 30 min equilibration, the ring tension was adjusted back to 1.5 g and then NE (0.3 µM) was re-applied. Before each experiment, the rings were contracted at least two

times until a reproducible contractile response was obtained. Each experiment started after 10-15 min stabilisation of tension because pilot studies showed that the increase in tension and plateau from 0.3 μM of NE was reached at 10 min and remained at this plateau level for over 60 min, the time course of each experiment. Adenosine, lidocaine or adenosine-lidocaine (AL) combination was added to the organ bath in a concentration-dependent manner (see 2.2.3 Drug concentrations used in the studies) and the change in tension of pre-contracted rings was assessed. At the end of each experiment, the rings were maximally dilated with 100 μM papaverine (Grbović*, et al.*, 2000b) (Figure 2.3).

2.2.3 Drug concentrations used in the studies

Adenosine, lidocaine or AL aliquots were freshly prepared in distilled water and made in three final concentrations; 1, 10 and 20 mM. Adenosine alone, lidocaine alone or AL concentration-response curves for intact and denuded aortic rings were obtained by cumulatively adding adenosine, lidocaine or AL 1, 5, 10, 50, 100, 500 and 1000 μM (final concentrations) into a 10 mL organ bath containing NE pre-contracted rings and the tension was measured (Figure 2.3). The AL concentration-response curves were obtained by adding the same concentrations in combination of adenosine and lidocaine. The volume of adenosine, lidocaine or AL added in the organ bath for each examined-concentration was the same.

2.2.4 Examination of adenosine or lidocaine mechanisms using specific inhibitors

In order to investigate the possible mechanisms of action of adenosine, lidocaine and AL relaxation in aortic rings (Chapters 3, 4 and 5), a number of specific drugs were employed to investigate the roles of an intact endothelium, endothelium-derived relaxation factors (EDRFs), potassium channels, Na^+/K^+ ion pump, and adenosine receptors. Isolated aortic rings were preincubated with the antagonists for 20-30 min before being contracted with NE followed by adenosine or lidocaine incremental dosing administration. Involvement of factors, channels, pumps and/or receptors would be indicated if their selective antagonists led to a significant reduction or abolishment of adenosine- or lidocaine-induced relaxation. These antagonists are outlined below.

1. The role of endothelium and EDRFs was examined using 100 μM N^G-nitro-L-arginine Methyl Ester (L-NAME) and 10 μM indomethacin. L-NAME is a nitric oxide synthase (NOS) inhibitor leading to inhibition of nitric

oxide (NO) production, while indomethacin is a cyclooxygenase (COX)-1 inhibitor that inhibits prostacyclin (PGI_2) release (Ilkka & Bengt Saltin, 2011). L-NAME and indomethacin were prepared in distilled water and applied in endothelium-intact rat aortic rings *only*.

2. The role of potassium channels was investigated using the following K^+ channel blockers:
    - 1 mM 4-aminopyridine (4-AP), a specific voltage K^+ (K_v) channel blocker. 4-AP was dissolved in distilled water.
    - 10 µM glibenclamide, a non-selective ATP-sensitive K^+ (K_{ATP}) channel blocker. Glibenclamide was dissolved in 100% dimethyl sulphoxide (DMSO).
    - 1 mM 5-hydroxydecanoate (5-HD), a specific mitochondrial ATP-sensitive K^+ (mitoK_{ATP}) channel inhibitor. 5-HD was dissolved in distilled water.
    - The role of Na^+/K^+-ATPase was investigated using 100 µM ouabain, a Na^+/K^+-ATPase inhibitor. Ouabain was dissolved in distilled water.
3. The role of adenosine receptors was investigated using 100 µM 8-(3-chlorostyryl) caffeine (CSC) and 10 µM 8-[4-[4-(4-chlorobenzyl)piperazide-1-sulfonyl)phenyl]]-1-propylxanthine (PSB 0788). CSC and PSB 0788 are highly selective for adenosine A_{2a} and A_{2b} receptors, respectively (Borrmann*, et al.*, 2009; Daly & Jacobson, 1995). CSC and PSB 0788 were dissolved in 100% DMSO.

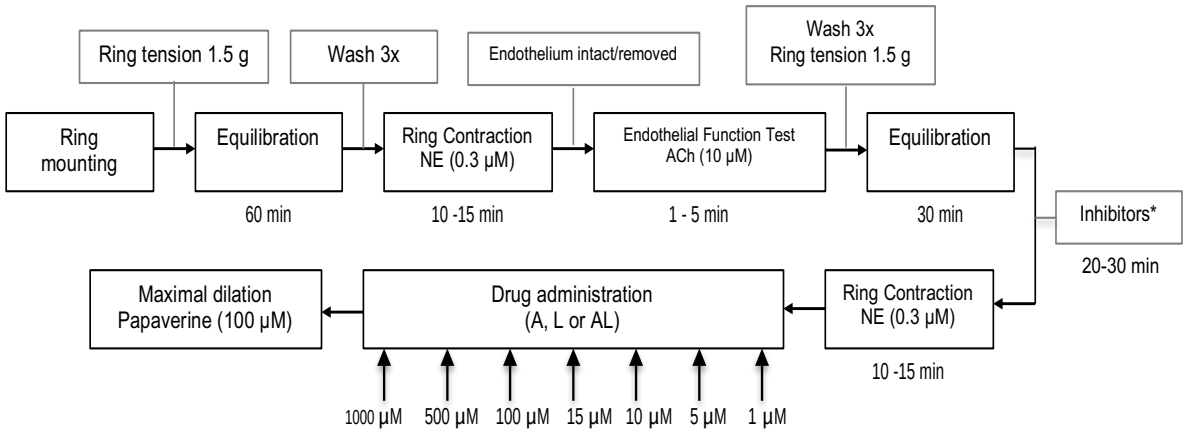

Figure 2.3 Diagram of experimental protocols of isometric force measurement.
* Inhibitors such as L-NAME, Indomethacin, 4-AP, 5-HD, Ouabain, CSC and PSB-0788 were only applied during experiments evaluating relaxation mechanisms of adenosine, lidocaine or AL.

2.3 PRESSURE MYOGRAPH SYSTEM

A pressure myograph system was the method used to examine mesenteric conduit relaxation in Chapter 5 and the vascular dilatory response to adenosine, lidocaine and AL during luminal and abluminal administration. The experimental design is based on the study of Doughty*, et al.* (1999). This methodology is often used to investigate small vessel function (diameter >60 μM) under near physiological conditions of pressure and flow by measuring vessel diameter and flow in time (Lu & Kassab, 2011).

2.3.1 Animal and drug preparation

Male guinea pigs (250-300 g) obtained from Flinders University were fed *ad libitum* and housed in a 12-hour light/dark cycle. Animals were treated in accordance with the Australian Code for the Responsible Conduct of Research at Flinders University. The ethics approval number for this study was 734/09. Lidocaine hydrochloride was sourced as a 2% solution (ilium) from the local Pharmaceutical Suppliers (Lyppard, Queensland). All other chemicals, including adenosine (A9251 >99% purity) and Arginine Vasopressin (AVP, >95%), were purchased from Sigma Aldrich (Castle Hill, NSW).

Animals were anaesthetised using ether then decapitated. A laparotomy was performed and the second order mesenteric arteries were dissected out according to the method of Ellis*, et al.* (2009).

2.3.2 Vessel isolation

Second order mesenteric artery branches were dissected and carefully transferred into physiological Krebs Henseleit solution (119 mM NaCl, 4.7 mM KCl, 1.18 mM KH_2PO_4, 1.17 mM $MgSO_4.7H_2O$, 2.5 mM $CaCl_2$, 22.8 mM $NaHCO_3$ and 0.026 mM EDTA) pH 7.4 with 5.5 mM glucose and continuously bubbled with 95% O_2 and 5% CO_2 (Ellis*, et al.*, 2009). The artery segment was carefully cleaned to remove fat and connective tissue then cut into at least 1 mm in lengths.

2.3.3 Vessel mounting in pressure myograph

The artery segment was mounted on transparent glass cannulae in a warm chamber filled with Krebs Henseleit solution and continuously bubbled with 95% O_2 and 5% CO_2 at 37^{o}C. Both ends of the segments were fixed on the cannulae with suture (Kenny*, et al.*, 2002). The transmural pressure was maintained using two reservoirs mounted on a column and connected to the cannulae. A pressure tranducer at one end of the artery allowed continual measurement of the intraluminar pressure. One of the reservoirs was filled with Krebs Henseleit gassed continuously with 5% CO_2 in 95% O_2 which constantly flowed through the artery throughout the experiment to give an intraluminal pressure of 60 mmHg and luminal flow of 100 µl/min (Kenny*, et al.*, 2002). Before the experiments were carried out, arteries were tested for leaks by clamping on tubes at both ends of the cannulae. If there was no decrease in pressure detected on the transducer for 15 seconds, then arteries were deemed leak-free. Arteries with leaks were discarded. The outer artery diameter was continuously measured through a NIKON TMS inverted microscope, which was portrayed using a video monitor (Doughty*, et al.*, 1999) (Figure 2.4, Figure 2.5).

Arteries were allowed to equilibrate for 30 min, then were constricted with 10^{-8} M AVP. AVP was used in the mesenteric artery experiments as the vasoconstrictor instead of NE since AVP produces greater maximum contraction on mesenteric arteries than NE (Karashima, 1981). Some of the artery segments were left intact and some were denuded using pumped air through the vessel (see section 2.3.4). Removal of endothelium was confirmed using 10^{-5} M ACh. Arteries that did not relax >10% of AVP-precontracted tone, were considered endothelium-denuded. Arteries were then washed with Krebs Henseleit solution and left for 30 minutes for re-equilibration. Following 30 min equilibration, precontraction was induced by 10^{-8} M AVP (Lei*, et al.*, 1993).

After tension stabilisation with AVP precontraction, the arteries then were exposed to adenosine, lidocaine or adenosine-lidocaine (AL) intraluminally or extraluminally in a concentration-dependent manner and the change in outer artery diameter was assessed. At the end of each experiment, the vessels were washed with calcium-free physiological solution containing 1 mM ethylene glycol tetraacetic acid (EGTA) to determine the maximal dilation of the vessels (Sokoya*, et al.*, 2006a).

2.3.4 Endothelium removal

After the mesentery artery was mounted, pressurized and checked for leaks, the denuding process commenced. One end of the cannulae tube (inflow) was connected to a 10 ml syringe attached to a pump machine and the other end (outflow) was opened. Air was then perfused into the lumen using the pump with a flow rate of 1000 µl/min. When 5 ml air had been infused, the outflow was clamped and the intraluminal pressure increased rapidly. When the lumen pressure reached 70 mmHg, flow rate was reduced to 2 µl/min and the vessel left pressurized for 10 minutes. The pump was then turned off and inflow to the Krebs Henseleit solution was opened. The outflow tube was opened to let the air bubbles out. When the vessel was free from air bubbles, the outgoing flow is switched to flowmeter. The denuded vessel was then left to equilibrate for 30 minutes.

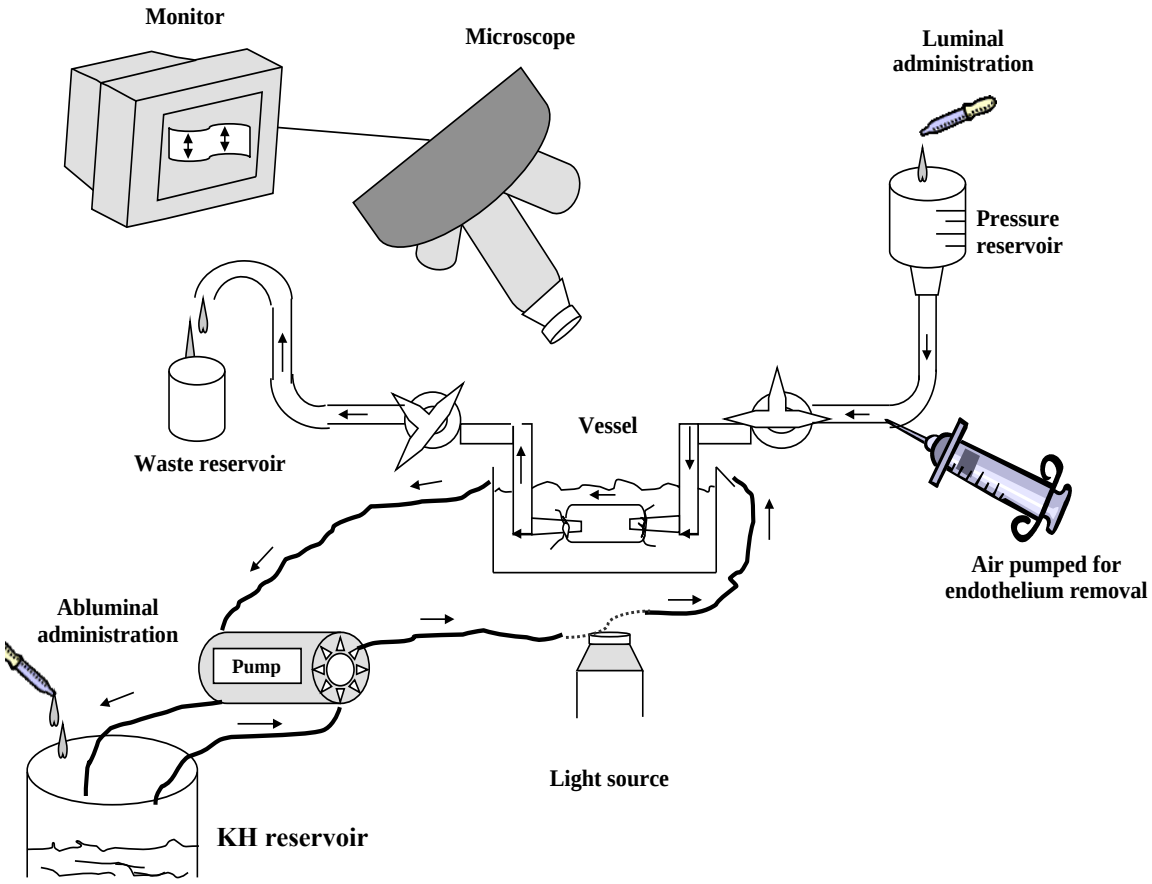

Figure 2.4 A schematic of experimental protocol using pressure myograph.
Ring vessel was mounted between the glass canulae. Transmural pressure was created by adjusting the height of the pressure reservoir and waste reservoir. Outer artery diameter was continuously measured through an inverted microscopy connected to a monitor. Drugs were delivered intraluminary through pressure reservoir and extraluminary through KH reservoir then pumped into the organ bath. Endothelium was removed by injecting high-pressured air into the vessel.

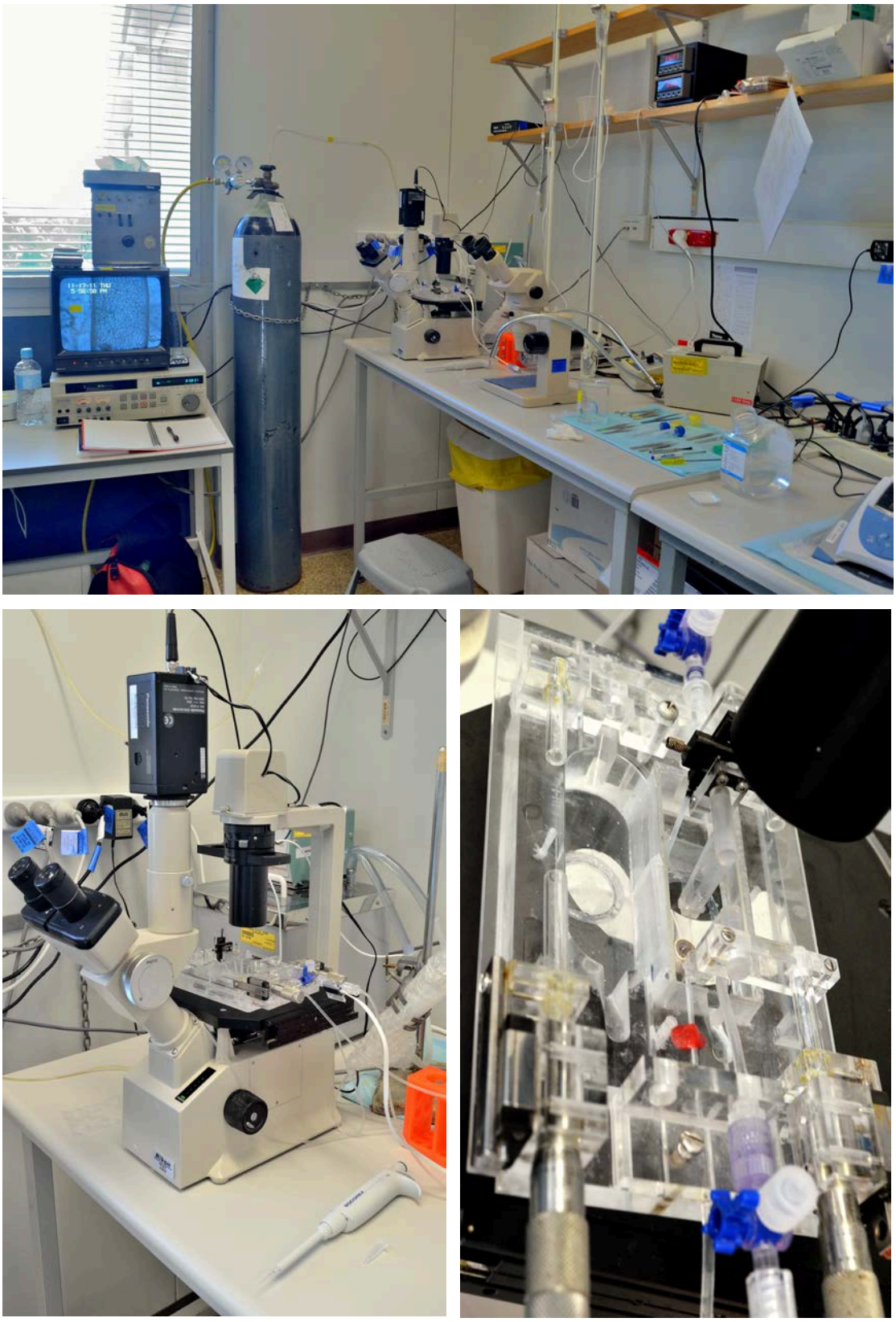

Figure 2.5 Pressure myograph system.

2.4 PRESERVATION WITH STATIC COLD STORAGE

The potential use of AL as an additive in vessel preservation solution was studied in Chapter 6. The aim was to examine if AL could improve preservation of vessel physiological function after six days of static cold storage. The physiological functions of the preserved vessels were measured using isometric force transducer (see Section 2.2; Figures. 2.1 and 2.2).

2.4.1 Animal and drug preparation

Animal and drug preparation were described in section 2.2.1.

2.4.2 Vessel preparation

Thoracic aortic vessels were harvested from 300-350 g male Sprague Dawley rats and transferred to a container with pre-cooled KH solution (118 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgCl_2$, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$ and 0.03 mM EDTA with 11 mM glucose). Vessel segments were cleared from surrounding adipose and connective tissue and cut in 3 mm length rings and stored at 4°C for six days in one of the following preservation solutions: 1) Krebs Henseleit (KH), 2) modified KH (low Ca^{2+}/high Mg^{2+}), 3) modified KH + adenosine-lidocaine (KH+AL), or 4) modified KH + AL and melatonin and insulin (KH+ALMI) solutions. Due to light sensitivity of melatonin, vessels stored in ALMI were covered with aluminium foil during storage.

2.4.3 Composition of preservation solutions

- KH: 118 mM NaCl, 4.7 mM KCl, 1.2 mM NaH_2PO_4, 2.5 mM $CaCl_2$,1.2 mM $MgCl_2$, 25 mM $NaHCO_3$, 0.03 mM Na-EDTA and 11 mM glucose.
- Modified KH: 118 mM NaCl, 4.7 mM KCl, 1.2 mM NaH_2PO_4, 0.22 mM $CaCl_2$, 2.6 mM $MgCl_2$, 25 mM $NaHCO_3$, 0.03 mM Na-EDTA and 11 mM glucose.
- Modified KH + AL: modified KH + 0.4 mM adenosine and 1 mM lidocaine.
- Modified KH +ALMI: modified KH + 0.4 mM adenosine and 1 mM lidocaine + 0.1 mM melatonin and 0.01 IU/ml insulin.

The AL composition was obtained from a previous study performed by Rudd and Dobson (2011b). The composition of A and L (400 μM and 1000 μM) which were twice

the AL cardioplegia, were used in their other study (Rudd & Dobson, 2011a) previously. The use of double concentrations of A and L in heart preservation, recovered a superior cardiac output (CO), heart rate, and developing pressure after eight hours cold storage (Rudd & Dobson, 2011b).

2.4.4 Vessel physiological function testing

After six days storage, the vessel rings were transferred to an organ bath filled with KH solution, gently warmed to 37°C, and subjected to function testing. Physiological (contraction and relaxation) function of the preserved aortic rings were measured using an isometric force transducer (PANLAB, distributed by ADInstruments as MLT 0201/RAD, NSW, AUS) as described in Section 2.2. Contraction was induced by norepinephrine (NE; 0.3 μM) and potassium chloride (KCl; 60 mM) to examine the receptor and non-receptor-induced contraction, respectively. Vessel relaxation in response to acetylcholine (ACh; 10^{-6}-10^{-3} M) and sodium-nitroprusside (SNP; 10^{-6}-10^{-3} M) was tested after preconstriction with 0.3 μM norepinephrine to assess endothelium-dependent and endothelium-independent relaxation, respectively (Veres*, et al.*, 2015). At the end of each experiment, rings were maximally dilated with 100 μM papaverine to confirm viability of the vessels (Grbović*, et al.*, 2000b) and the relaxation value would be used as a comparison to ACh and SNP relaxation. ACh is believed to act on muscarinic receptors which are G-protein-coupled receptors to produce relaxation via endothelial-derived NO production (Chataigneau*, et al.*, 1999), whereas SNP is an NO donor that induces vascular smooth muscle relaxation mainly through the activation of the soluble guanylate cyclase (sGC), and an increase in cyclic GMP (cGMP), although cGMP-independent mechanisms have also been reported (Cogolludo*, et al.*, 2001).

2. 5. STATISTICAL ANALYSIS

Values are reported as mean ± SEM. All data was tested for normality using *Kolmogorov-Smirnov* test, then tested for homogeneity of variances followed by one-Way ANOVA and coupled with Bonferroni post hoc test for individual data point comparisons.

CHAPTER 3. ADENOSINE RELAXATION IN ISOLATED RAT AORTIC RINGS AND POSSIBLE ROLES OF SMOOTH MUSCLE K_V CHANNELS, K_{ATP} CHANNELS AND A_{2A} RECEPTORS

Abstract:

Introduction: An area of ongoing controversy is the role of adenosine to regulate vascular tone in conduit vessels that regulate compliance, and the role of nitric oxide (NO), potassium channels and receptor subtypes involved. The aim of this chapter was to investigate adenosine relaxation in rat thoracic aortic rings, and the effect of inhibitors of NO, prostanoids, K_v, K_{ATP} channels, and A_{2a} and A_{2b} receptors.

Methods: Aortic rings were freshly harvested from adult male Sprague Dawley rats and equilibrated in an organ bath containing oxygenated, modified Krebs Henseleit solution (11 mM glucose, pH 7.4, 37°C). Isolated rings were pre-contracted sub-maximally with 0.3 μM norepinephrine (NE), and the effect of increasing concentrations of adenosine (1 to 1000 μM) were examined. The drugs L-NAME, indomethacin, 4-aminopyridine (4-AP), glibenclamide, 5-hydroxydecanoate, ouabain, 8-(3-chlorostyryl) caffeine and PSB-0788 were examined in intact and denuded rings. Rings were tested for viability after each experiment.

Results: Adenosine induced a dose-dependent, triphasic relaxation response, and the mechanical removal of the endothelium significantly deceased adenosine relaxation above 10 μM. Interestingly, endothelial removal significantly decreased the responsiveness (defined as % relaxation per μM adenosine) by two-thirds between 10 and 100 μM, but not in the lower (1-10 μM) or higher (>100 μM) ranges. In intact rings, L-NAME significantly reduced relaxation, but not indomethacin. Antagonists of voltage-dependent K_v (4-AP), sarcolemma K_{ATP} (glibenclamide) and mitochondrial K_{ATP} channels (5-HD) led to significant reductions in relaxation in both intact and denuded rings, with ouabain having little or no effect. Adenosine-induced relaxation appeared to involve the A_{2a} receptor, but not the A_{2b} subtype.

Conclusions: It was concluded that adenosine relaxation in NE-precontracted rat aortic rings was triphasic and endothelium-dependent above 10 μM, and relaxation

involved endothelial nitric oxide (not prostanoids) and a complex interplay between smooth muscle A_{2a} subtype and voltage-dependent K_v, $SarcK_{ATP}$ and $MitoK_{ATP}$ channels. The possible *in vivo* significance of the regulation of arterial compliance to left ventricular function coupling is discussed.

3.1 INTRODUCTION

Adenosine is a ubiquitous endogenous mediator that is activated in response to cellular ischaemic or hypoxic or shear stress (Burnstock & Ralevic, 2013; Ely & Berne, 1992; Fredholm*, et al.*, 2001; Jacobson & Gao, 2006; Mustafa*, et al.*, 2009). Adenosine exerts it cellular effects by binding to four major subtypes of the G-protein-coupled receptors; A_1, A_{2a}, A_{2b}, and A_3 which activate intracellular survival kinase pathways in a cell- and tissue-specific manner (Burnstock & Ralevic, 2013; Fredholm*, et al.*, 2001; Headrick*, et al.*, 2013; Jacobson & Gao, 2006). Through receptor-modulation and downstream signaling pathways adenosine alters coronary and peripheral vascular tone, cardiac function, brain and central nervous system signaling, sleep, the state of natural hibernation, ischaemic preconditioning, post-conditioning, inflammation, coagulation, angiogenesis, and cell proliferation and remodelling (Burnstock & Ralevic, 2013; Dobson*, et al.*, 2013a; Headrick*, et al.*, 2013; Mustafa*, et al.*, 2009).

An area of ongoing controversy is the role of adenosine to regulate vascular tone in the arterial tree, and the receptor subtypes involved. The subtype A_{2a} appears to be the predominant receptor in arterial vasodilation in mouse, rat, guinea pig, pigs and humans, however, the A_{2b} receptor has also been reported to dilate human coronary arteries (Kemp & Cocks, 1999), and possibly rat coronary arteries (Headrick*, et al.*, 2013). In the guinea pig, A_{2b} appears to predominate in the thoracic aorta to induce relaxation (Martin*, et al.*, 1993) and both A_{2a} and A_{2b} in the rat (Lewis & Hourani, 1997; Lewis*, et al.*, 1994a; Ponnoth*, et al.*, 2009). In addition, there is ongoing debate on the relative importance of an intact endothelium to adenosine relaxation in these vessels, and the role of nitric oxide (NO) and interplay between voltage-dependent transmembrane Na^+, K^+ and Ca^{2+} fluxes and signaling pathways. In the thoracic aorta, adenosine relaxation has been reported to be fully dependent (Lewis*, et al.*, 1994a; Newman*, et al.*, 1988), partially dependent (Headrick & Berne, 1990; Moritoki*, et al.*, 1990; Rose'Meyer & Hope, 1990; Yen*, et al.*, 1988) or not dependent on the presence of an intact endothelium (De Mey & Vanhoutte, 1981; Furchgott, 1983; Lewis*, et al.*, 1994a; Rubanyi & Vanhoutte, 1985). Adenosine vasodilation has also been linked to A_1 and A_{2a} receptor activation of endothelial production of NO and prostanoids (Ray & Marshall, 2006a), hyperpolarising factors (Mustafa*, et al.*, 2009), and a complex interplay between endothelial and smooth muscle mitochondrial and sarcolemmal K_{ATP} channels (Headrick & Berne, 1990; Sato*, et al.*, 2000; Taylor*, et al.*, 1988), and Na^+/K^+ ATPase activation (Grbovic & Radenkovic, 2003; Mustafa*, et al.*, 2009).

The aim of this chapter was to investigate adenosine relaxation in intact versus denuded rat thoracic aortic rings, and examine the effect of inhibitors of nitric oxide (NO), prostanoids, K_v channels, K_{ATP} channels, and adenosine A_{2a} and A_{2b} receptors. The rat thoracic aorta was chosen because of the ongoing debates about the mechanisms of adenosine relaxation, and its *in vivo* significance.

3.2 MATERIALS AND METHODS

3.2.1 Animals:

Male Sprague Dawley rats (300-350g, n=47) were prepared as described in Chapter 2.2.1.

3.2.2 Aortic ring preparation and organ bath tension measurements:

Rat thoracic aortic rings were prepared and tested as described in Chapter 2.2.2 and shown in Figure 2.3.

3.2.3 Adenosine relaxation in intact and denuded rat aortic rings

Adenosine was added into the oxygenated organ bath containing Krebs Henseleit solution to obtain 1, 5, 10, 50, 100, 500 and 1000 μM adenosine concentrations. The change in tension of pre-contracted intact or denuded rings was measured. The inhibitors used in this study were incubated in an organ bath 20-30 min before NE was administered followed by incremental administration of adenosine. These included: 1) 100 μM N^G-nitro-L-arginine Methyl Ester (L-NAME) (nitric oxide synthetase inhibitor) and 10 μM indomethacin (cyclooxygenase or prostaglandin inhibitor e.g. prostacyclin). NO and prostacyclin are two major endothelial derived relaxation factors (EDRF), and the inhibitors were only applied in endothelium intact aortic rings, and 2) 1 mM 4-aminopyridine (4-AP) (Non-selective voltage-dependent *K^+-channel* blocker of the K_{v1} to K_{v4} families rather than K_{v7} channels) (Mackie & Byron, 2008; Novakovic*, et al.*, 2006; Rautureau*, et al.*, 2002), 10 μM glibenclamide (Non-selective SarcK_{ATP} channel blocker) (O'rourke, 2000; Toyoda*, et al.*, 2000) and 1 mM 5-hydroxydecanoate (5-HD) (MitoK_{ATP} channel blocker) (Gross & Gross, 2007), and Na^+/K^+-ATPase inhibitor (100 μM ouabain) (Grbović & Radenković, 2003). These inhibitors were applied to intact endothelium rings in the presence of L-NAME and indomethacin, and without the

presence of L-NAME and indomethacin in denuded aortic rings. The adenosine A_{2a} receptor inhibitor was 100 μM 8-(3-chlorostyryl) caffeine (CSC) (Allende & Acevedo, 2011; Mathoôt*, et al.*, 1996), and the A_{2b} receptor inhibitor was 10 μM 8-(4-(4-(4-chlorobenzyl)piperazine-1-sulfonyl)phenyl)-1-propylxanthine (PSB-0788) (Borrmann*, et al.*, 2009). In rat striatal membranes, these antagonists have reported K_i values of 24 nM for CSC (Van der Walt*, et al.*, 2013) and 0.393 nM for PSB-0788 (Borrmann*, et al.*, 2009), and the micromolar concentrations used in the present study were based on previous published studies (Kalkan*, et al.*, 2007; Kataoka*, et al.*, 2007; Schiedel*, et al.*, 2013). The inhibitors were applied in endothelium intact and denuded aortic rings in an oxygenated medium. At the end of each experiment, the rings were tested for viability (or patency) by being maximally dilated with 100 μM papaverine, and relaxation was expressed as percentage of maximal relaxation to papaverine (Grbović & Radenković, 2003; Grbović*, et al.*, 2000b).

3.2.4 Statistics:

Values are expressed as mean ± SEM. Eight animals (n=8) were used for each group for seven measurement points using ANOVA analysis, and the number of rats was selected from *a priori* G-power analysis to achieve a level of 1.0. All data was tested for normality using *Kolmogorov-Smirnov* test. Relaxation responses to adenosine were analysed for homogeneity of variances followed by two-way ANOVA coupled with the Bonferroni post-hoc test for individual data point comparisons. The alpha level of significance for all experiments was set at $p<0.05$.

3.3 RESULTS

3.3.1 Intact versus Denuded Aortic Rings:

In endothelium-intact rat aortic rings, adenosine led to 10%, 21%, 29%, 60% and 81% relaxation at 10, 50, 100, 500, and 1000 μM adenosine concentrations, respectively (Figure 3.1). Adenosine relaxation in intact rings occurred in three linear phases (log scale); 0.96% per μM from 1 to 10 μM adenosine (Phase 1), 0.2% per μM from 10 to 100 μM adenosine (Phase 2), and 0.06% per μM from 100 to 1000 μM (Phase 3). After removing the endothelium, relaxation was reduced to 8%, 10%, 14%, 45% and 67% respectively, and was significant from 100 to 1000 μM. In denuded rings, adenosine relaxation was 0.72% per μM from 1 to 10 μM adenosine, 0.07% per μM from 10 to

100 μM adenosine (Phase 1), and 0.06% per μM in Phase 2 from 100 to 1000 μM. Thus, endothelial removal of rat aortic rings decreased the responsiveness (defined as % relaxation per μM) to around one-third between 10 and 100 μM, but not in the lower (Phase 1) or higher (Phase 3) ranges. (Figure 3.1).

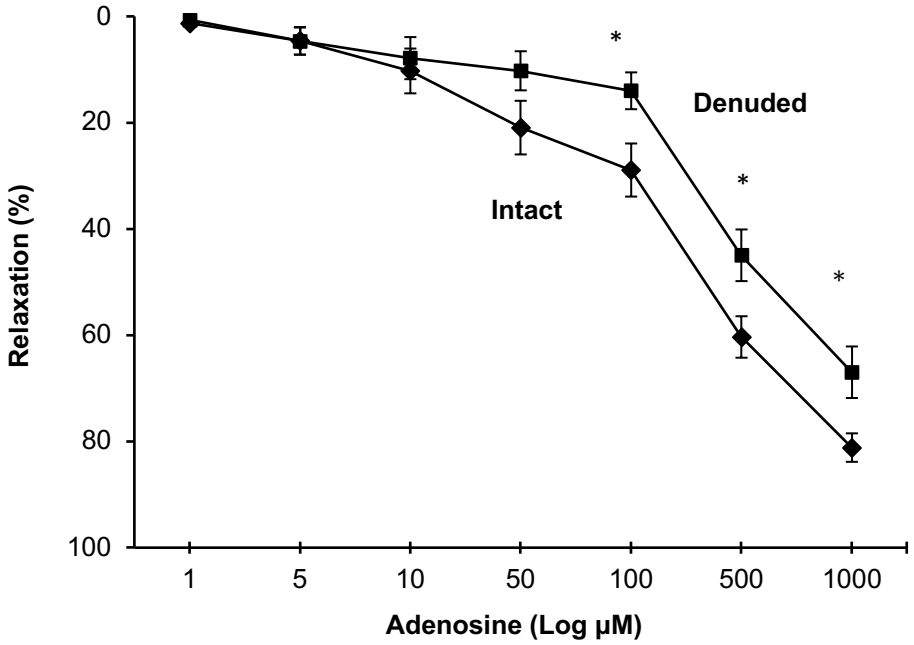

Figure 3.1 Concentration response curves to adenosine in intact and denuded isolated rat aortic rings.
Relaxation is expressed as percent of maximal relaxation to 100 μM papaverine. Points represent mean ± S.E.M of aortic rings from a total of seven animals. *p<0.05 statistical difference in responses between the intact and denuded rings. Symbols (◆) Intact rings (■) Denuded rings.

3.3.2 Effect of L-NAME and Indomethacin in intact aortic rings

Figure 3.2 shows that L-NAME and indomethacin significantly reduced adenosine relaxation at 50 to 1000 μM adenosine. At 50 μm, relaxation decreased from 26% to 11% or 42% (11/26 x 100) of the relaxation of intact controls. Thus at 50 μM adenosine 59% of relaxation was linked to L-NAME and indomethacin inhibition. At 100, 500 and 1000 μM adenosine concentrations, L-NAME and indomethacin contribution to inhibition were 53, 33 and 19% (Figure 3.2). In addition, experiments with L-NAME alone showed a similar inhibition, indicating that indomethacin had little or no significant inhibition (Figure 3.2). However, at 500 uM and 1000 uM adenosine there was a small difference of indomethacin from L-NAME but this was not significant (Figure 3.2).

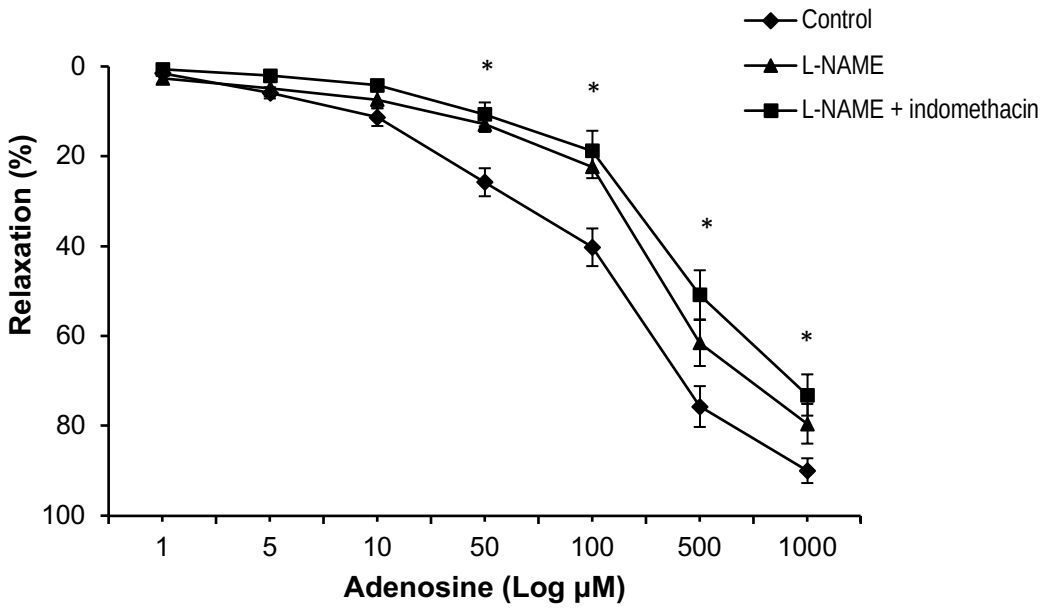

Figure 3.2 Concentration-response curves to adenosine with and without the presence of L-NAME alone and L-NAME + indomethacin in intact isolated rat aortic rings.
Relaxation is expressed as percent of maximal relaxation to 100 µM papaverine. Points represent mean ± S.E.M of aortic rings from a total of eight animals. *p<0.05 statistically different in the presence of L-NAME alone (▲) and L-NAME + indomethacin (■) compared to control on intact rings (◆).

The effect of K_v, sarcK_{ATP}, mitoK_{ATP} channels and Na^+/K^+-ATPase on adenosine relaxation in intact aortic rings is shown in Figure 3.3A-D. In order to eliminate the effect of NO- and prostacyclin-induced relaxation in intact rings, 100 µM L-NAME and 10 µM indomethacin were included in the controls.

3.3.3 Effect of K_v, SarcK_{ATP}, MitoK_{ATP} Blockers and Ouabain on Adenosine Relaxation in intact aortic rings

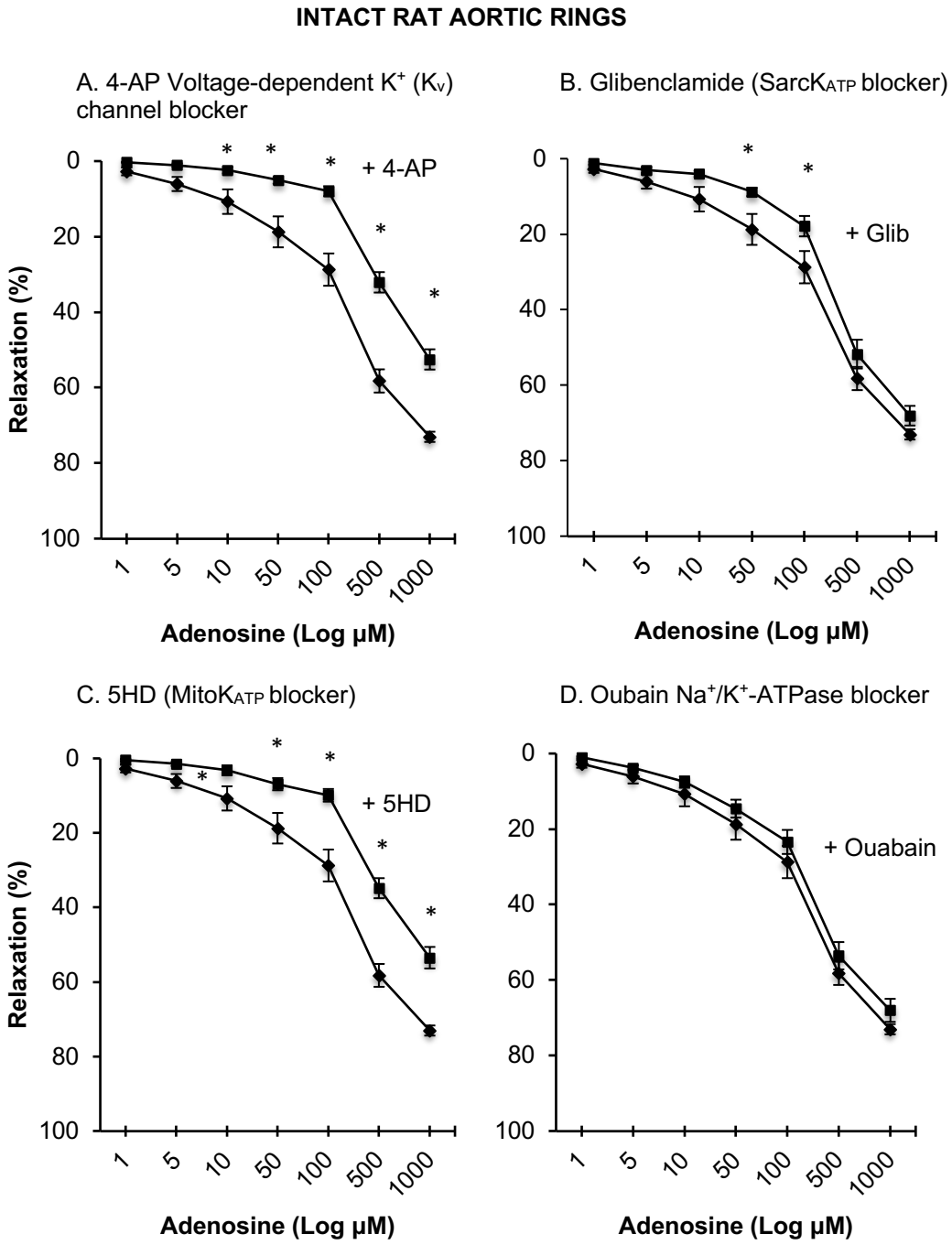

Figure 3.3 Concentration-response curves to adenosine with and without the presence of some specific ion channel blockers in intact isolated rat aortic rings.
[A] In the presence of 1 mM 4-aminopyridine (■). [B] In the presence of 1 mM 5-Hydroxydecanoate (■). [C] In the presence of 10 µM glibenclamide (■). [D] In the presence of 100 µM ouabain (■) compared to controls intact rings (◆). Relaxation is expressed as percent of maximal relaxation to 100 µM papaverine. Points represent mean ± S.E.M of aortic rings from a total of eight animals. *p<0.05 statistical difference in responses between the presence and the absence of inhibitors on intact rings.

K_v inhibition: The effect of pre-incubating intact rings with 1 mM 4-aminopyridine (4-AP) on adenosine relaxation is shown in Fig 3.3A. Percentage relaxation was 2.4, 5.1, 8.0, 32.1 and 52.5% for 10, 50, 100, 500 and 1000 μM adenosine, respectively. Expressed as a percentage contribution of adenosine relaxation relative to control intact rings, the K_v channel was responsible for 78%, 73%, 72%, 58.2% and 28% for 10, 50, 100, 500 and 1000 μM adenosine respectively, with greater relaxation between 10 to 100 μM (Figure 3.3A).

SarcK_{ATP} and MitoK_{ATP} Inhibition: The effect of glibenclamide on adenosine relaxation is shown in Figure 3.3B. Glibenclamide was not as striking as 4-AP but significantly decreased adenosine relaxation at 50 and 100 μM adenosine. The contribution of sarcK_{ATP} channel to adenosine relaxation was 63%, 53% and 38% at 10, 50 and 100 μM adenosine (Figure 3.3B). MitoK_{ATP} inhibitor, 5-hydroxydecanoate (5-HD), significantly led to a wider range of inhibition of adenosine relaxation compared to glibenclamide from 10 to 1000 μM, but the differences between the two blockers were not significant (Figure 3.3 C). The contribution of mitoK_{ATP} channel to adenosine relaxation was 70%, 63%, 65%, 40% and 27% at 10, 50, 100, 500 and 1000 μM adenosine level (Figure 3.3C).

Na^+/K^+-ATPase Inhibition Figure 3.3D shows that ouabain did not significantly change the inhibition produced by L-NAME and indomethacin in adenosine-induced relaxation at any given concentration, indicating that Na^+/K^+-ATPase contributed little extra to adenosine relaxation in endothelium intact aortic rings.

3.3.4 Effect of A_{2a} and A_{2b} Blockers in Intact and Denuded Aortic Ring

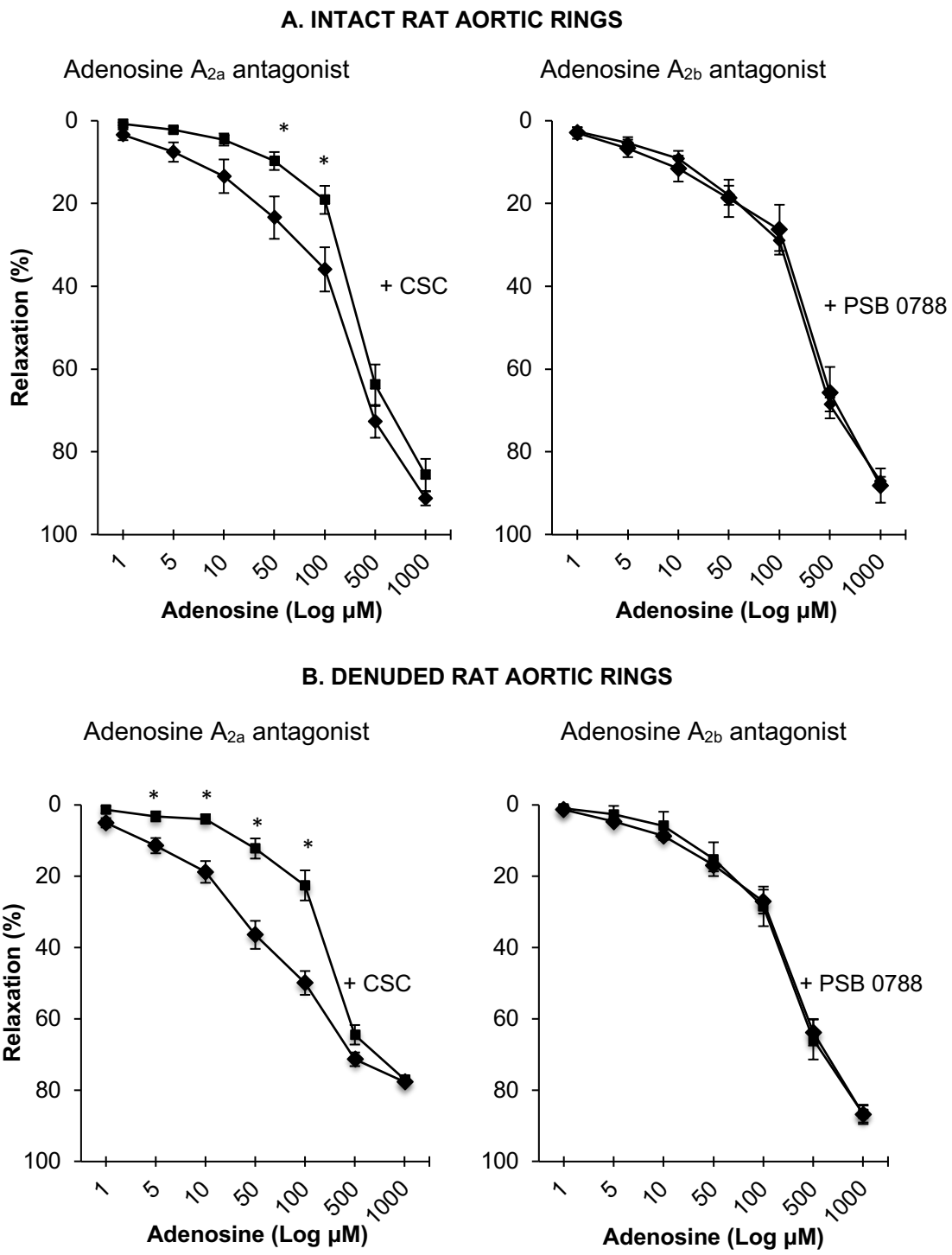

Figure 3.4 Concentration-response curves to adenosine with and without the presence of adenosine A_{2ab} receptor blockers in intact (A) and denuded (B)_isolated rat aortic rings.

In the presence of 100 µM 8-(3-Chlorostyryl) caffeine (■) or 10 µM PSB 0788 (■). Controls (intact and denuded rings) (◆). Relaxation is expressed as percent of maximal relaxation to 100 µM papaverine. Points represent mean ± S.E.M of aortic rings from a total of eight animals. *p<0.05 statistical difference in responses between the presence and the absence of inhibitors on intact rings.

Intact Rings:

L-NAME and indomethacin were not included in this experiment because it has been reported that NO or prostacyclin release are linked to adenosine $A_{2a,b}$ receptor activation (Hein*, et al.*, 1999). In the absence of any inhibitors, adenosine induced a rate of relaxation of about 10% for every 50 μM adenosine up to 100 μM, and ~25% relaxation per 50 μM from 100 to 1000 μM until 90% full relaxation (Figure 3.4A). Pre-incubating intact rings with adenosine A_{2a} receptor inhibitor, CSC, significantly reduced adenosine relaxation between 50 to 100 μM (Figure 3.4A). Although greater percentage falls in relaxation occurred at lower adenosine levels (e.g. 5 to 10 μM) these were not significantly different from controls (Figure 3.4A). The A_{2a} receptor was responsible for 71%, 66%, 59% and 47% adenosine relaxation at 5, 10, 50, and 100 μM adenosine, respectively. In direct contrast, adenosine A_{2b} receptor inhibitor, PSB 0788, did not change relaxation at any adenosine concentration studied (Figure 3.4B).

Denuded Rings:

In denuded rat aortic rings, incubation with A_{2a} blocker, CSC, showed a significant reduction of adenosine relaxation from 5 to 100 μM (Figure 3.4B). At 5, 10, 50 and 100 μM adenosine, the A_{2a} receptor was responsible for 72, 79, 66 and 55% reduction in relaxation. Similar to 4-AP and 5-HD, the A_{2a} receptor blocker did not inhibit adenosine relaxation at 500 uM and 1000 uM. In contrast, adenosine A_{2b} blocker, PSB 0788, had no effect to reduce adenosine-induced relaxation (Figure 3.4B).

3.3.5 Effect of K_v, SarcK_{ATP}, MitoK_{ATP} blockers and Ouabain on Adenosine Relaxation in Denuded Rings

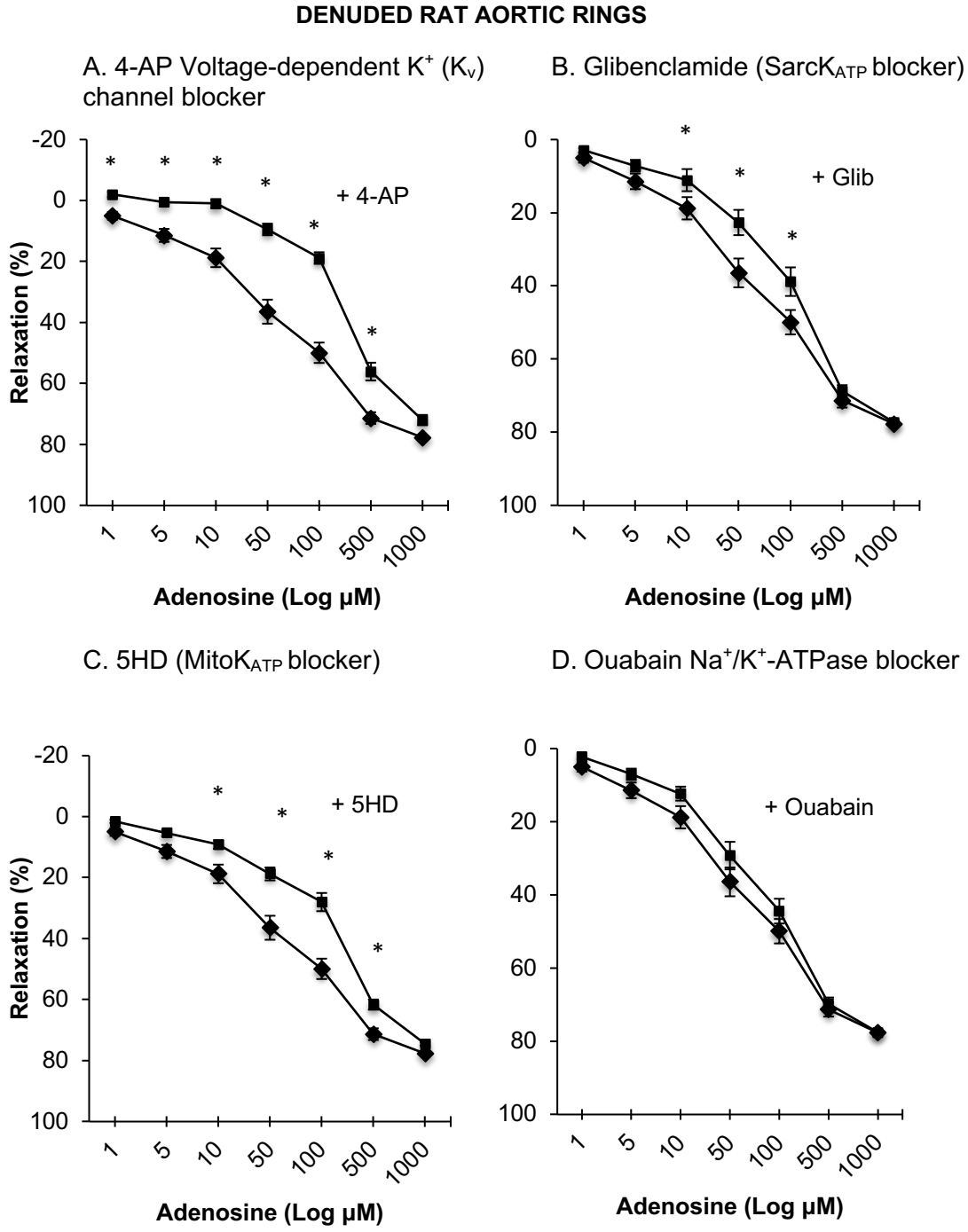

Figure 3.5 Concentration-response curves to adenosine with and without the presence of some specific ion channel blockers in denuded isolated rat aortic rings.
[A] In the presence of 1 mM 4-aminopyridine (■). [B] In the presence of 1 mM 5-Hydroxydecanoate (■). [C] In the presence of 10 µM glibenclamide (■). [D] In the presence of 100 µM ouabain (■). Control denuded rings (◆). Relaxation is expressed as percent of maximal relaxation to 100 µM papaverine. Points represent mean ± S.E.M of aortic rings from a total of eight animals. *p<0.05 statistical difference in responses between the presence and the absence of inhibitors on denuded rings.

In the absence of endothelium and blockers, adenosine relaxed rat aortic rings in a dose-dependent manner reaching 78% relaxation at the highest 1000 μM adenosine concentration (Figure 3.5). Pre-treatment with 4-AP significantly reduced relaxation from 1 to 500 μM adenosine but not at 1000 μM (Figure 3.5A). 4-AP nearly completely abolished adenosine-induced relaxation up to 10 μM adenosine with over 95% inhibition. At 50, 100 and 500 μM adenosine, the K_v channel was responsible for 74%, 62%, 21% of adenosine relaxation (Figure 3.5A).

The sarcK_{ATP} channel blocker, glibenclamide, also significantly reduced relaxation at 10, 50 and 100 μM adenosine levels (Figure 3.5B) indicating that the SarcK_{ATP} channel was responsible for 41%, 38% and 22% of adenosine relaxation, respectively. Mitochondrial K_{ATP} blocker, 5-HD, significantly reduced relaxation over a wider range than glibenclamide similar to intact rings (Figures 3.3BC and 3.5BC). The greatest effect of 5-HD was found at 10 to 100 μM. The contributions of the mitoK_{ATP} channel to adenosine relaxation were 51%, 48%, 44% and 14% at 10, 50, 100 and 500 uM adenosine levels, respectively. The Na^+/K^+-ATPase channel blocker ouabain, as in intact aortic rings, showed no significant effects to reduce adenosine-induced vasodilation at any given adenosine level (Figure 3.5D).

3.4 DISCUSSION

Despite decades of investigation, the mechanisms of adenosine relaxation in large elastic arteries such as the rat thoracic aorta, and smaller muscular resistance arterioles are not fully understood (Félétou & Vanhoutte, 2007; Furchgott, 1983; Headrick*, et al.*, 2013; Jacobson & Gao, 2006; Mustafa*, et al.*, 2009). This Chapter reports in isolated rat thoracic rings that adenosine vasodilation was: 1) triphasic and partially dependent on an intact endothelium, 2) regulated predominately by endothelial NO, not prostanoids, 3) dependent on opening smooth muscle K_v, SarcK_{ATP} and MitoK_{ATP} channels, 4) ouabain-insensitive (Na^+/K^+ ATPase), and 5) activated by the A_{2a} subtype, not A_{2b}. The possible interplay between these potassium channels and adenosine relaxation in denuded and intact aortic rings, and the *in vivo* significance will now be discussed.

3.4.1 Adenosine Relaxation involves an NO-Dependent Pathway

This study showed that L-NAME significantly reduced relaxation in intact rings and contributed up to 59% of adenosine relaxation with little or no effect of indomethacin

(Figure 3.2). In the rat aorta, endothelial NO is believed to induce vasodilation via cGMP- and cAMP-dependent protein kinase mechanisms, and the inhibition of Rho-kinase constrictor activity (Chitaley & Webb, 2002). The lack of a prostanoid effect in this study was surprising. In 2002, Ray and colleagues showed in an elegant series of studies, using a NO-sensitive electrode, that adenosine relaxation in the rat aorta produced a dose-dependent NO release from the endothelium (Ray*, et al.*, 2002). They further showed that A_1-receptor NO release was linked to endothelial prostacyclin release via a common cyclic AMP signaling pathway (Ray & Marshall, 2006a).

In contrast to the current study, Ray and colleagues used halothane-O_2 anaesthetized, hypoxic, male 200-250g Wistar rats, and aortic conduits of 10 mm in length which were longitudinally opened and the NO-sensitive electrode was directly in contact with the endothelial surface (Ray & Marshall, 2006a; Ray*, et al.*, 2002). Systemic hypoxia in their study was induced using 8% O_2 in N_2 for 5 min prior to aorta harvest, but the group did not specify the pO_2, pCO_2 or temperature of their bathing media. This is an interesting contrast, as in this thesis the thoracic aorta was harvested from normoxic, male 300-350g Sprague Dawley rats under thiopentone anaesthesia, and the isolated intact rings were 3-4 mm in length and fully oxygenated at all times. It is possible that prostanoid production in rat aortic rings is not activated during normoxia but during hypoxia. In 2001, Verma and colleagues also reported in healthy humans that COX-2–selective inhibition did not result in significant changes in endothelial vasodilator responses (Verma*, et al.*, 2001). Further work is required to examine these differences in different models.

3.4.2 Role of the Endothelium in Adenosine Relaxation

In the present study, adenosine vasodilation was partially endothelium-dependent (Fig 3.1), which is consistent with the earlier work of Yen and colleagues (Yen*, et al.*, 1988), Moritoki et al., (Moritoki*, et al.*, 1990), Headrick and Berne (Headrick & Berne, 1990) and Rose'Meyer and colleagues (Rose'Meyer & Hope, 1990) in rat and guinea pig thoracic aorta. However, in the present study adenosine relaxation was triphasic (Figure 3.1), and endothelial removal reduced ring relaxation 'responsiveness' between 10 to 100 μM adenosine (Phase 2) with little or no change to denuded ring sensitivity from 1 to 10 μM (Phase 1) or from 100 to 1000 μM (Phase 3) compared to intact rings (Figure 3.1). This triphasic nature of adenosine relaxation has not been reported before, and although the underlying mechanisms for the different sensitivities are not known, they appear to involve differential endothelial-smooth muscle

sensitivities to endothelial NO production, and smooth muscle A_{2a} receptor and voltage-dependent K_v and K_{ATP} channels (see below).

3.4.3 Role of Voltage-dependent K_v Channels in Adenosine Relaxation

The 4-AP experiments (~70-95% inhibition at 5 to 100 μM adenosine) demonstrated that the K_v channel has the potential to be a potent activator of adenosine relaxation in rat aortic rings. A similar change in intact and denuded rings (Figures 3.3A and 3.5A) suggests that the 4-AP effect was independent of endothelial NO production and was preferentially activated on vascular smooth muscle (Figure 3.3A). This data supports the study of Tammaro and colleagues who reported the presence of smooth muscle K_v channels in rat aorta (Tammaro*, et al.*, 2004), and that of Heaps and Bowles in swine coronary arteries who showed 4-AP-sensitive K^+ channels in adenosine relaxation (Heaps & Bowles, 2002). In addition, K_v channels have also been widely reported in regulating tone in smaller resistance vessels of cerebral and mesenteric vascular beds (Albarwani*, et al.*, 2003; Cole*, et al.*, 1996; Coleman*, et al.*, 2004; Mackie & Byron, 2008), and in vascular smooth muscle from larger rat pulmonary arteries (Archer*, et al.*, 2004). In conclusion, this data indicates that adenosine relaxation in isolated NE-precontracted rat aortic rings involved K_v channels with higher sensitivities found at lower adenosine levels. Further studies are required using more specific K_v channel isoform inhibitors (and agonists), and their membrane voltage dependence on relaxation (Smirnov*, et al.*, 2003) at low and high adenosine levels.

3.4.4 Contributions of SarcK_{ATP} and MitoK_{ATP} Channels to Adenosine Relaxation, and A_{2a} Receptor Activation.

The SarcK_{ATP} channel was shown to contribute to 14 to 63% of adenosine relaxation up to 100 μM adenosine (Figs 3.3B and 3.5B), and MitoK_{ATP} channels contributed to 22 to 70% relaxation up to 1000 μM adenosine in intact and denuded aortic rings (Figs 3.3C and 3.5C). The wider range of adenosine inhibition with MitoK_{ATP} channel blocker 5-HD indicates that it shifted the control relaxation curve more to the right than glibenclamide (Figs 3.3C and 3.5C). For example, at 10 and 100 μM adenosine, 5-HD led to 50% more inhibition than glibenclamide in intact rings (Figures 3.3B and 3.3C), and 17% and 29% more inhibition in denuded rings (Figures 3.5C and 3.5B). This difference may indicate differential contributions of the MitoK_{ATP} and SarcK_{ATP} channel activation to adenosine relaxation, however, 5-HD has been shown to exert effects independent of MitoK_{ATP} channels (Li*, et al.*, 2010) which may influence that

interpretation.

The glibenclamide data showing significant relaxation reduction (Figures 3.3B and 3.5B), albeit less potent than 5-HD (Figures 3.3C and 3.5C), is in contrast to the study of Husken and colleagues who reported no effect in rat aorta (Hüsken*, et al.*, 1997). However, their rings were bathed in a hypoxic, low-glucose medium. Similarly, Kemp and Cocks reported lack of a glibenclamide effect in coronary artery rings prepared from cardiac surgery patients (Kemp & Cocks, 1999). It appears therefore that glibenclamide-sensitive K_{ATP} channel activation and adenosine relaxation is dependent on the state of tissue oxygenation, prior disease states and possibly ischaemia.

Furthermore, Kemp and Cocks found that adenosine relaxation in their discarded human coronary artery rings was mediated largely by A_{2b} receptors (Kemp & Cocks, 1999), unlike A_{2a} receptors found in isolated rat aortic rings in the present study (Figs 3.4AB). Adenosine A_{2a} receptor activation and relaxation in rat aortic rings is consistent with the majority of studies in rabbit aorta and mesenteric and coeliac arteries (Kleppisch & Nelson, 1995), mouse hearts (Zatta & Headrick, 2005), and guinea pig, porcine and bovine coronary arteries (Conti*, et al.*, 1997; Lewis*, et al.*, 1994a; Shryock & Belardinelli, 1997). However, Lewis and colleagues reported in Wistar rat isolated aortic preparations that A_{2a} adenosine relaxation was entirely endothelium-dependent (Lewis*, et al.*, 1994a), not smooth muscle-dependent as was found in the present study (Figures 3.4A and 3.4B). In rat renal artery, Grbović and colleagues also showed that removal of the endothelium abolished A_{2a} adenosine relaxation, implicating endothelial relaxation factors such as NO for relaxation (Grbović*, et al.*, 2000b). These contrasting results may be due to differences in species, age, prior disease state, aortic ring preparation, presence of an endothelium and the bathing media. Another difference may be the type of artery; studying the larger arterial conduits versus smaller arteriolar resistance vessels which have very different functions (see below '*Limitations of the Present Study and Future Studies*'). It is noteworthy that Leal and colleagues found that A_{2a} and A_{2b} subtypes were abundant in all three layers of Wistar rat thoracic aorta wall (intima, media, and adventitia) (Leal*, et al.*, 2008), again illustrating the deep complexity of receptor and channel expression in the thoracic aorta.

3.4.5 Adenosine Regulation of Relaxation in Rat Aortic Rings: A Working Hypothesis

Although adenosine relaxation at different oxygenation states and pH, or from hypoxic animals, was not investigated in the current study, the results support the following scheme. Adenosine-linked NO production appeared to be a major endothelium-derived relaxing factor in intact rat aortic rings, not prostanoids, which sets the stage for endothelial-smooth muscle coupling. In denuded aortic rings, adenosine appears to activate A_{2a} receptors and trigger downstream opening of K_v and K_{ATP} channels located on smooth muscle resulting in membrane hyperpolarization, and relaxation, which may have involved common protein kinase signalling transduction pathways and crosstalk (Berwick*, et al.*, 2010; Brayden, 2002; Cole*, et al.*, 1996; Kleppisch & Nelson, 1995; Ko*, et al.*, 2008; Maimon*, et al.*, 2014; Quayle*, et al.*, 1997). Membrane hyperpolarization of only a few millivolts can lower cytosolic Ca^{2+} via reduced activity of membrane voltage-operated Ca^{2+} channels and reduced myofilament Ca^{2+} sensitivity (Akata, 2007a), resulting in smooth muscle relaxation. Partial support for this hypothesis in denuded rings comes from reports showing adenosine activation of K_v channels in pig coronary arterioles occurs via cAMP-dependent protein kinase (PKA) activation and vasodilatation (Heaps*, et al.*, 2008; Ko*, et al.*, 2010), and from Kleppisch and Nelson who showed that adenosine A_{2a} (not A_{2b}) activation opens K_{ATP} channels via the cAMP/PKA pathway in isolated rabbit mesenteric vascular smooth muscle cells (Kleppisch & Nelson, 1995). More recently, Maimon and colleagues showed in skeletal muscle arterioles that PKA signaling varies with pre-exposure to adenosine, and that PKA activation alone was not sufficient to dilate these arterioles, and required other Ca^{2+}-dependent mechanisms to facilitate vasodilation to adenosine (Maimon*, et al.*, 2014).

Another possible mechanism coupling adenosine A_{2a} receptor to opening K_v and K_{ATP} channels in rat denuded aorta rings may be via mitochondrial production of H_2O_2 (Bonnet & Archer, 2007; Krenz*, et al.*, 2002). H_2O_2 is a highly diffusible and signalling redox intermediate produced during mitochondrial phosphorylation of ADP to ATP, and is believed to trigger Ca^{2+} sparks that activate protein kinase pathways and adenosine relaxation (Dick*, et al.*, 2008; Sharifi-Sanjani*, et al.*, 2013). Dick and colleagues reported that H_2O_2 activated K_v channels and led to coronary vasodilation along with increases in myocardial metabolism (Dick*, et al.*, 2008), and Sharifi-Saniani and colleagues showed that adenosine A_{2a} receptor activation in mouse aorta during reactive hyperemia was coupled to smooth muscle K_{ATP} channels via the production of

H_2O_2 (Sharifi-Sanjani*, et al.*, 2013). It is possible that mitochondrial H_2O_2 bursts may also facilitate crosstalk between mito- and sarc-K_{ATP} channels in our model.

Lastly, activation of A_{2a} in rat aortic rings may also have occurred from adenosine's breakdown metabolite, inosine (via adenosine deaminase), which has recently been shown to be a functional agonist of the A_{2a} receptor (Welihinda*, et al.*, 2016). It is possible therefore that adenosine engages A_{2a} receptor to generate initial relaxation followed by a dual agonist-mediated response from inosine to amplify or prolong A_{2a} activation *in vivo*. While inosine is known to relax aortic rings (Chinellato*, et al.*, 1994), its dual action with adenosine has only been studied in inflammatory/immune cells (Welihinda*, et al.*, 2016).

3.4.6 Limitations of the Present Study and Future Studies

While all four major types of K^+ channels (K_v, K_{ATP}, K_{IR} and K_{Ca}) appear to be present in vascular endothelial and smooth muscle cells (Bonnet & Archer, 2007; Chen*, et al.*, 2006; Cole*, et al.*, 1996; Coleman*, et al.*, 2004; Edwards*, et al.*, 2010; Ko*, et al.*, 2008), the current study was limited to K_V and K_{ATP} channels in intact and denuded aortic rings. Furthermore, aortic ring relaxation was investigated in a high pO_2 environment and it would be of interest to investigate the effect of lowering pO_2 and changing pH. In addition, adenosine receptor characterization may have been more robust with the use of more than one A_{2a} and A_{2b} antagonist at appropriate concentrations. The isolated aortic ring preparation also lacks sympathetic neurohumoral innervations and the vasa vasorum, which makes translation to the intact vessel challenging. The *in vivo* significance of these results may relate to regulating compliance of the thoracic aorta as part of ventricular-arterial coupling (Jufri*, et al.*, 2015; Sandoo*, et al.*, 2010a; Triggle*, et al.*, 2012a). The thoracic aorta and other large arteries are compliance vessels and are continually subjected to different hemodynamic forces such as mechanical stretch due to pulsatile blood flow, and may adjust vascular tone by changing the balance of vasodilating and vasoconstricting factors and neurohumoral mechanisms (Jufri*, et al.*, 2015; Sandoo*, et al.*, 2010a; Triggle*, et al.*, 2012a). In contrast, smaller peripheral and coronary arterioles supply vascular beds and regulate flow by changing resistance to maintain adequate tissue oxygenation. Further studies are required to investigate the possible role of adenosine (and possibly inosine) and its various receptor subtypes to regulate compliance versus resistance vessels (including venous capacitance vessels) in different regions and vascular beds in the body.

3.5 CONCLUSIONS

This chapter showed that adenosine relaxation in NE-precontracted rat thoracic aortic rings was triphasic and partially endothelium-dependent and involved endothelial NO production with a complex interplay between smooth muscle A_{2a} subtype and voltage-dependent K_v, $SarcK_{ATP}$ and $MitoK_{ATP}$ channels, but not a prostanoid-dependent pathway.

CHAPTER 4. LIDOCAINE RELAXATION IN ISOLATED RAT AORTIC RINGS IS ENHANCED BY ENDOTHELIAL REMOVAL: POSSIBLE ROLE OF K_V, K_{ATP} CHANNELS AND A_{2A} RECEPTOR CROSSTALK

Abstract:

Introduction: Lidocaine is an approved local anaesthetic and Class 1B antiarrhythmic with a number of ancillary properties. The aim of this chapter was to investigate lidocaine's vasoreactivity properties in intact versus denuded rat thoracic aortic rings, and the effect of inhibitors of nitric oxide (NO), prostenoids, voltage-dependent K_v and K_{ATP} channels, membrane Na^+/K^+ pump, and A_{2a} and A_{2b} receptors.

Methods: Aortic rings were harvested from adult male Sprague Dawley rats and equilibrated in an organ bath containing oxygenated, modified Krebs-Henseleit solution, pH 7.4, 37°C. The rings were pre-contracted sub-maximally with 0.3 μM norepinephrine (NE), and the effect of increasing lidocaine concentrations was examined. Rings were tested for viability after each experiment with maximally dilating 100 μM papaverine. The drugs 4-aminopyridine (4-AP), glibenclamide, 5-hydroxydecanoate, ouabain, 8-(3-chlorostyryl) caffeine and PSB-0788 were examined.

Results: All drugs tested had no significant effect on basal tension. Lidocaine relaxation in intact rings was biphasic between 1 to 10 μM (Phase 1) and 10 to 1000 μM (Phase 2). Mechanical removal of the endothelium resulted in further relaxation, and at lower concentrations ring sensitivity (% relaxation per μM lidocaine) significantly increased 3.5 times compared to intact rings. The relaxing factor(s) responsible for enhancing ring relaxation did not appear to be NO- or prostacyclin-dependent, as L-NAME and indomethacin had little or no effect on intact ring relaxation. In denuded rings, lidocaine relaxation was completely abolished by K_v channel inhibition and significantly reduced by antagonists of the MitoK_{ATP} channel, and to a lesser extent the SarcK_{ATP} channel. Curiously, A_{2a} subtype receptor antagonism significantly inhibited lidocaine relaxation above 100 μM, but not the A_{2b} receptor.

Conclusions: This chapter shows that lidocaine relaxation in rat thoracic aorta was biphasic and significantly enhanced by endothelial removal, which did not appear to

be NO or prostacyclin dependent. The unknown factor(s) responsible for enhanced relaxation was significantly reduced by K_v inhibition, 5-HD inhibition, and A_{2a} subtype inhibition indicating a potential role for crosstalk in lidocaine's vasoreactivity.

4.1 INTRODUCTION

Lidocaine is a local amide-type cationic anaesthetic, which acts by blocking voltage-dependent Na^+ fast channels in excitable cells (EC50, 50-100 uM) (Opie & Gersh, 2012). At lower concentrations, lidocaine is an approved Class 1B antiarrhythmic (Wyman*, et al.*, 2004) and exerts anti-inflammatory (Hollmann*, et al.*, 2004b), neuroprotective (Butterworth & Hammon, 2002), energy-lowering (Seyfried*, et al.*, 2005), anti-ischaemic (Cassuto*, et al.*, 2006; Vinten-Johansen, 2004), anti-oxidant (Lee*, et al.*, 2010; Tomoda*, et al.*, 1990) and platelet-neutrophil interactive (Huang*, et al.*, 2009; Schmidt*, et al.*, 1997) properties.

Lidocaine has also been shown to exert a number of vasomodulatory properties in isolated vessels including: 1) endothelium-independent relaxation (Shan*, et al.*, 2004; Turan*, et al.*, 2000), and 2) vascular smooth muscle relaxation (Evans*, et al.*, 1997c; Johns*, et al.*, 1985; Turan*, et al.*, 2000) or contraction (Evans*, et al.*, 1997c; Gherardini*, et al.*, 1998a; Kinoshita*, et al.*, 2001a; Kinoshita*, et al.*, 2003a; Perlmutter*, et al.*, 1990a) properties. The apparent paradoxical nature of lidocaine on vascular smooth muscle is often explained as being dose-dependent with vasoconstriction of peripheral blood vessels occurring at low concentrations of lidocaine (~5 uM) and vasodilation at higher levels (> 50 uM) (Abe*, et al.*, 2000; Johns*, et al.*, 1985; Perlmutter*, et al.*, 1990a; Satoh*, et al.*, 2015). In the rat carotid artery, Kinoshita further proposed that lidocaine may impair the vasodilator response via the activation of ATP-sensitive K^+ channels which may be exacerbated by hypoxia (Kinoshita*, et al.*, 2003a). Earlier the same group showed that in pre-contracted denuded rat aortic rings that acidification promoted lidocaine relaxation and alkalization led to vasoconstriction (Kinoshita*, et al.*, 2001a).

These confounding effects of lidocaine vasoreactivity appear to be linked to differential modulation of multiple channels including Na^+ channels (Wyman*, et al.*, 2004), inwardly-rectifying K^+ channels (Josephson, 1988), Ca^{2+} channels (Shan*, et al.*, 2004; Tanaka*, et al.*, 2002) and/or K_{ATP} channels (Kinoshita*, et al.*, 2001a; Kinoshita*, et al.*, 2003a). Vasodilation may involve nitric oxide (NO) (Newton*, et al.*, 2007; Takaishi*, et al.*, 2013; Toda*, et al.*, 2007), redox regulation (Lee*, et al.*, 2010) and possible convergence of a multitude of downstream cAMP and cGMP signaling cascades that lead to changes in cytosolic Ca^{2+} (Scholz, 2002). Hollmann and colleagues, for example, identified lidocaine and G-protein coupled receptor systems as potential intracellular signaling mechanisms, and the G_q protein subunit as a possible common target (Hollmann*, et al.*, 2001b). In 2003, Benkwitz et al., also showed that the G_i

protein subunit was enhanced by lidocaine, and that it was potentiated adenosine A_1-receptor signaling (Benkwitz*, et al.*, 2003). The group proposed that lidocaine was not an A_1-receptor agonist but enhanced adenosine-A_1 receptor signaling separate from its local anaesthetic Na^+ channel properties (Benkwitz*, et al.*, 2003). The aim of the present study was to investigate the nature of lidocaine relaxation in isolated rat thoracic aortic rings, and examine the effect of inhibitors of NO, prostanoids, K_v channels, K_{ATP} channels, and adenosine A_{2a} and A_{2b} receptors. Adenosine A_2 receptors were chosen because they are widely known to modify vascular tone (Peart & Headrick, 2007), and may therefore be involved in possible crosstalk in lidocaine relaxation (Benkwitz*, et al.*, 2003).

4.2 MATERIALS AND METHODS

4.2.1 Animals:

Male Sprague Dawley rats (300-350g, n=72) were prepared as described in Chapter 2.2.1.

4.2.2 Aortic ring preparation and organ bath tension measurements:

Rat thoracic aortic rings were prepared and tested as described in Chapter 2.2.2 and shown in Figure 2.3.

4.2.3 Lidocaine relaxation in intact and denuded rat aortic rings: Experimental Protocol:

Lidocaine-HCl was added into the oxygenated organ bath containing KH solution to obtain 1, 5, 10, 50, 100, 500 and 1000 μM lidocaine concentrations. The change in tension of pre-contracted intact or denuded rings was measured. Responsiveness was defined as % relaxation per μM lidocaine. The inhibitors used in this study were incubated in organ bath 20-30 min before NE was administered followed by lidocaine incremental administration. These included 1) 100 μM N^G-nitro-L-arginine Methyl Ester (L-NAME) (nitric oxide synthetase inhibitor) and 10 μM indomethacin (cyclooxygenase or prostaglandin inhibitor e.g. prostacyclin). NO and prostacyclin are two major endothelial derived relaxation factors (EDRF), and the inhibitors were only applied in endothelium intact aortic rings. 2) 1 mM 4-aminopyridine (4-AP) (Non-selective

voltage-dependent *K^+-channel* blocker of the Kv1 to Kv4 families rather than Kv7 channels) (Mackie & Byron, 2008; Novakovic*, et al.*, 2006; Rautureau*, et al.*, 2002), 10 μM glibenclamide (Non-selective SarcK_{ATP} channel blocker) (O'rourke, 2000; Toyoda*, et al.*, 2000) and 1 mM 5-hydroxydecanoate (5-HD) (Non-selective MitoK_{ATP} channel blocker) (Gross & Gross, 2007), and Na^+/K^+-ATPase inhibitor (100 μM ouabain) (Grbović & Radenković, 2003). While 5-HD is commonly used in the literature as a specific MitoK_{ATP} channel blocker (Anastacio*, et al.*, 2013), Hanley and colleagues have shown that 5-HD is not a selective inhibitor of mitochondrial K_{ATP} channels but can act a substrate for the mitochondrial outer membrane enzyme acyl-CoA synthetase in the beta-oxidation pathway (Hanley*, et al.*, 2003), and it is also capable of playing a role as an inhibitor of sarcolemmal K_{ATP} channels in the presence of ATP (which was not the case in the current study) (Li*, et al.*, 2010). These inhibitors were applied to intact endothelium rings in the presence of L-NAME and indomethacin, and without the presence of L-NAME and indomethacin in denuded aortic rings. 3) The adenosine A_{2a} receptor inhibitor, 100 μM 8-(3-chlorostyryl) caffeine (CSC) (Allende & Acevedo, 2011; Jacobson*, et al.*, 1993; Mathoôt*, et al.*, 1996), and the A_{2b} receptor inhibitor, 10 μM 8-(4-(4-(4-chlorobenzyl)piperazine-1-sulfonyl)phenyl)-1-propylxanthine (PSB-0788) (Borrmann*, et al.*, 2009). These high affinity antagonists have been used in rodent studies with reported K_i values of 24 nM for CSC (Van der Walt*, et al.*, 2013) and 0.393 nM for PSB-0788 (Borrmann*, et al.*, 2009). CSC has also been shown to be 520-fold selective for A_{2a}-adenosine receptors in radioligand binding assays in the rat brain (K_i, 54 nM) with little or no effect on A_1 receptors (Jacobson*, et al.*, 1993). The inhibitors were applied to isolated rat aortic rings in an oxygenated medium. At the end of each experiment, the rings were tested for viability (or patency) by being maximally dilated with 100 μM papaverine, and relaxation was expressed as percentage of maximal relaxation to papaverine [(Grbović & Radenković, 2003; Grbović*, et al.*, 2000b).

4.2.4 Statistics:

Values are expressed as mean ± SEM. The number of rats was selected from *a priori* G-power analysis to achieve a level of 1.0. All data was tested for normality using *Kolmogorov-Smirnov* test. Relaxation responses to lidocaine were analysed for homogeneity of variances followed by two-way ANOVA coupled with the Bonferroni post-hoc test for individual data point comparisons. The alpha level of significance for all experiments was set at $p<0.05$.

4.3 RESULTS

4.3.1 Effect of Increasing Lidocaine on Relaxation in Intact and Denuded Rings

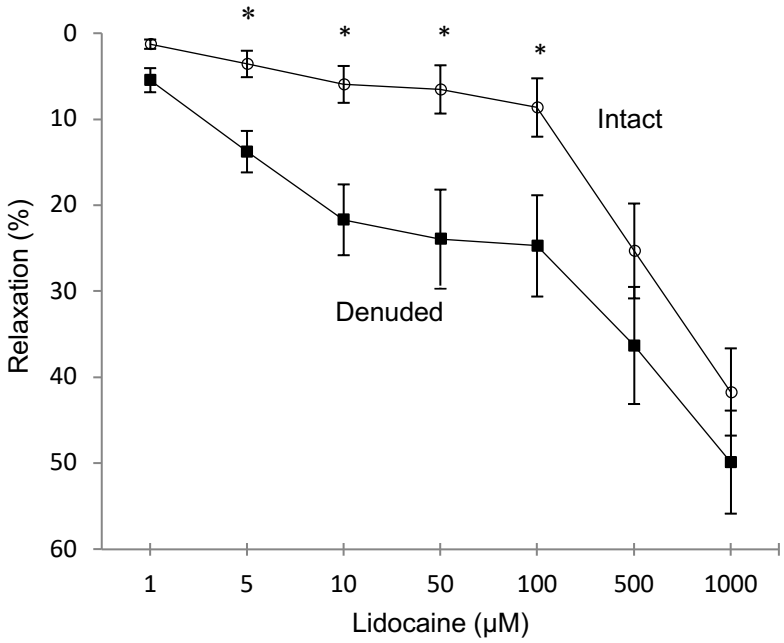

Figure 4.2 Concentration response curves to lidocaine in intact and denuded isolated rat aortic rings.
Relaxation is expressed as percent of maximal relaxation to 100 μM papaverine. Points represent mean ± S.E.M of aortic rings. *p<0.05 statistical difference in responses between the intact and denuded rings. Lidocaine concentrations are on a log scale. Total animals n=12

Intact Rings: The gram tension produced with NE administration in endothelium intact rings was not significantly different from denuded aortic rings. Lidocaine produced a concentration-dependent, biphasic relaxation relationship in intact and denuded rat aortic rings (Figure 4.2). The percentage relaxation in intact rat aortic rings was 1.3, 6.0, 8.6 and 41.7% at 1, 10, 100 and 1000 uM lidocaine respectively. The first relaxation phase was between 1-10 uM (Phase 1) and the second phase was from 10 to 1000 uM lidocaine (Phase 2) (log concentration scale) (Figure 4.2). The percentage relaxation per μM lidocaine (ring responsiveness) was 0.47% from 1 to 10 μM, 0.029% between 10 and 100 μM, and 0.037% increase per μM between 100 and 1000 μM lidocaine. The maximum relaxation from 1 to 1000 μM lidocaine in intact rings was 40.4%.

Denuded Rings: Removal of the endothelium significantly increased Phase 1 relaxation responsiveness from 0.47% to 1.80% per μM lidocaine between 1 and 10 μM (Figure 4.2). Interestingly, above 10 μM removing the endothelium had little or no significant effect on ring responsiveness to increasing lidocaine compared to intact rings. From 10 to 100 μM, % relaxation per μM was 0.033% and from 100 to 1000 μM was 0.028% (Figure 4.2). However, despite this similar responsiveness, at each lidocaine concentration up to 100 μM, the absolute percentage relaxation was significantly higher in denuded rings than intact rings. The absolute % relaxation in denuded rings was 5.5, 14, 22, 24, 25, 36 and 50% at 1, 5, 10, 50, 100, 500 and 1000 uM lidocaine, respectively (Figure 4.2). Thus, the effect of removing the endothelium was to significantly enhance ring *sensitivity* or *responsiveness* at lower lidocaine concentrations (1 to 10 μM) but not in the higher range (10 to 1000 μM), even though absolute relaxation values were significantly higher at each lidocaine concentration (1 to 1000 μM) in denuded versus intact rings (Figure 4.2).

4.3.2 Effect of L-NAME and Indomethacin in intact aortic rings

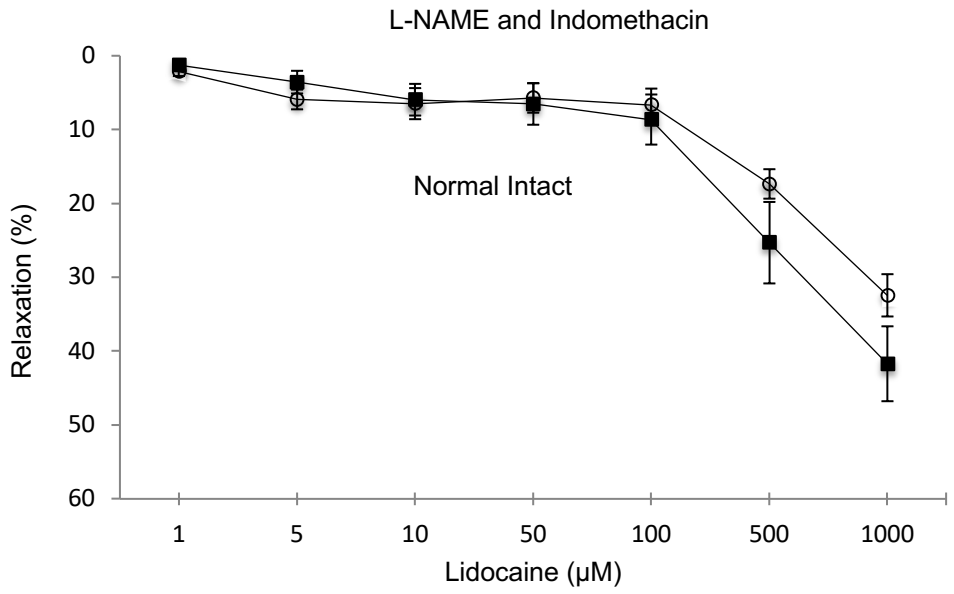

Figure 4.3 Concentration-response curves to lidocaine with and without the presence of L-NAME + indomethacin in intact isolated rat aortic rings.
Relaxation is expressed as percent of maximal relaxation to 100 μM papaverine. Points represent mean ± S.E.M of aortic rings. *p<0.05 statistically different in the presence of L-NAME + indomethacin compared to control on intact rings. Lidocaine concentrations are on a log scale. Total animals n=12.

In intact aortic rings, pre-treatment with L-NAME and indomethacin did not significantly change lidocaine relaxation from 1 to 1000 μM, although there was a trend towards inhibition at higher concentrations (Figure 4.3). Between 1 and 10 μM, the change in relaxation was 0.44% per μM, 0002% per μM between 10-100 μM and 0.029% per μM from 100 to 1000 μM. At 500 μM lidocaine, the % relaxation was 17% (32% lower than intact rings) and at 1000 μM lidocaine was 32% (24% lower than intact rings), but the differences were not significant (Figure 4.3).

4.3.3 Effect of K_v, SarcK_{ATP}, MitoK_{ATP} and Na^+/K^+ ATPase Antagonists on Relaxation in Intact and Denuded Rings

The effects of voltage-dependent K_v, SarcK_{ATP}, mitoK_{ATP} and Na^+/K^+-ATPase antagonists on lidocaine relaxation in intact rat aortic rings are shown in Figure 4.4.

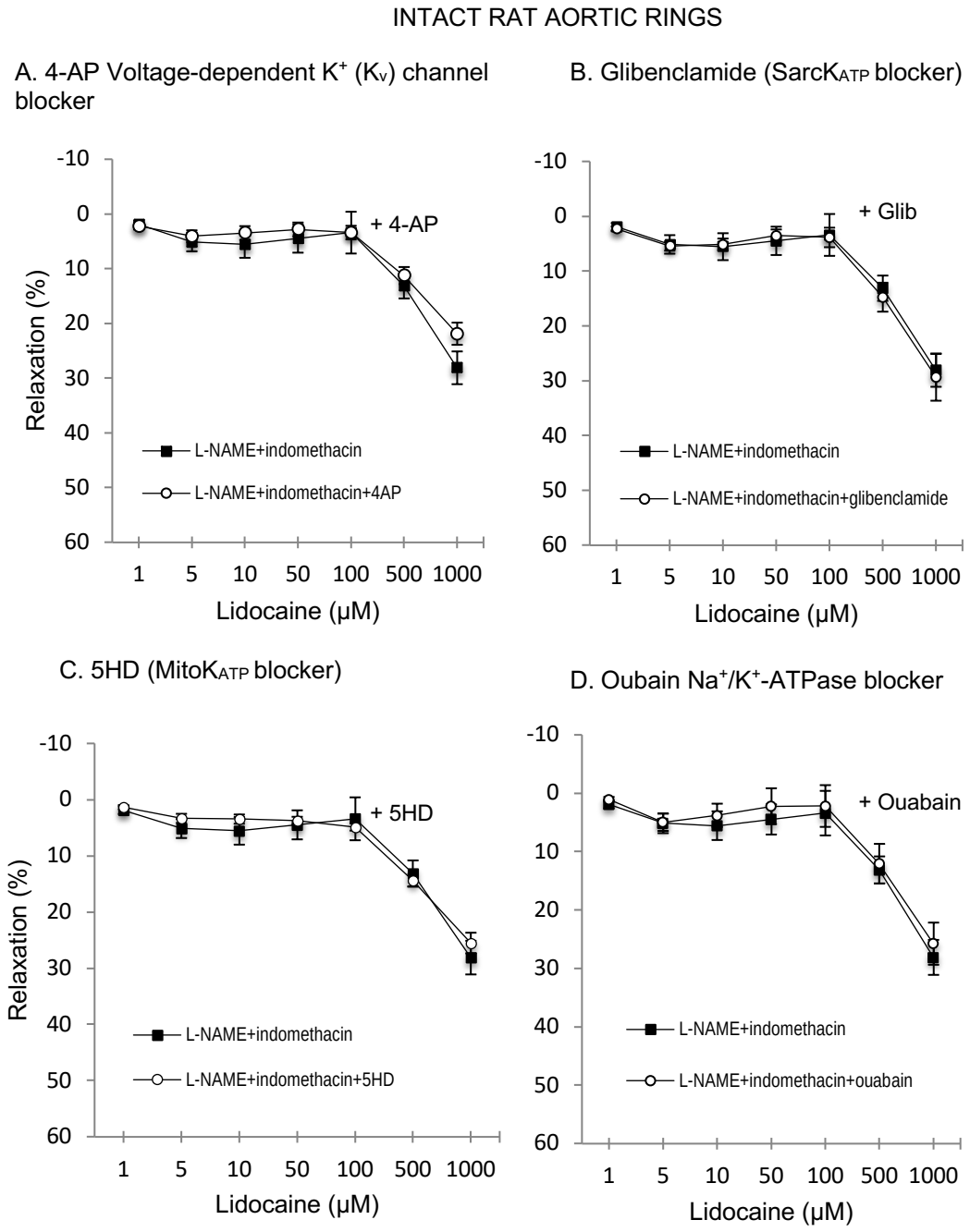

Figure 4.4 Concentration-response curves to lidocaine with and without the presence of specific ion channel blockers in intact isolated rat aortic rings.
[A] In the presence of 1 mM 4-aminopyridine. [B] In the presence of 1 mM 5-Hydroxydecanoate. [C] In the presence of 10 μM glibenclamide. [D] In the presence of 100 μM ouabain. Relaxation is expressed as percent of maximal relaxation to 100 μM papaverine. Points represent mean ± S.E.M of aortic rings in the presence of L-NAME and indomethacin. **p*<0.05 statistical difference in responses between the presence and the absence of inhibitors on intact rings. Lidocaine concentrations are on a log scale. Total animals n=16.

After pre-contracted with NE, ring basal tensions were 3.3 ± 0.09 g; 3.5 ± 0.17 g; 3.4 ± 0.09 g; 3.4 ± 0.14 g (n=8 each) for 4-AP, glibenclamide, 5-HD and ouabain groups, respectively; and not significantly different from NE with L-NAME and indomethacin controls (3.2 ± 0.19 g, n=8). In endothelial intact aortic rings, exposure of rings to these antagonists did not alter lidocaine-induced relaxation compared to controls (Figure 4.4).

DENUDED RAT AORTIC RINGS

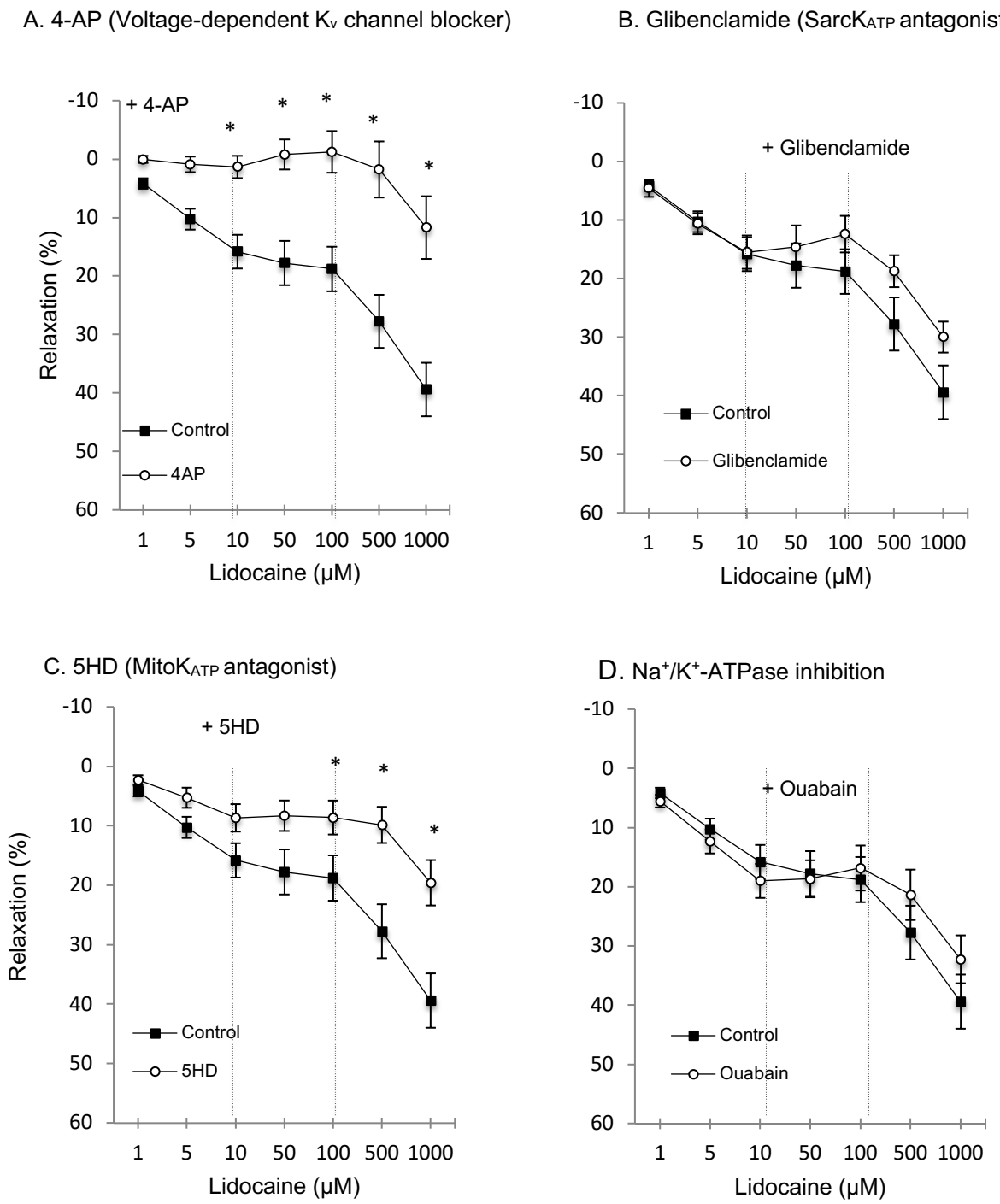

Figure 4.5 Concentration-response curves to lidocaine with and without the presence of specific ion channel blockers in denuded isolated rat aortic rings.
[A] In the presence of 1 mM 4-aminopyridine. [B] In the presence of 1 mM 5-Hydroxydecanoate. [C] In the presence of 10 μM glibenclamide. [D] In the presence of 100 μM ouabain. Relaxation is expressed as percent of maximal relaxation to 100 μM papaverine. Points represent mean ± S.E.M of aortic rings in the presence of L-NAME and indomethacin. *p<0.05 statistical difference in responses between the presence and the absence of inhibitors on intact rings. Lidocaine concentrations are on a log scale. Total animals n=16.

In denuded rings, the effect of 1 mM 4-AP was to totally abolish relaxation up to 500 μM after which relaxation was 12 ± 5% (n=8) compared to 39 ± 5% in denuded controls (i.e. 4-AP led to a 70% decrease in relaxation) (Figure 4.5A). 4-AP inhibition was significant from 1 to 1000 μM lidocaine (p<0.0001). The effect of glibenclamide (10 μM) had little or no effect on relaxation up to 10 μM lidocaine compared to denuded controls (Figure 4. 5B) and was ~20% lower at higher lidocaine concentrations; however, the differences were not significant. Exposure of denuded rings to 1 mM 5-HD led to ~50% decrease in lidocaine relaxation at 5 to 1000 μM lidocaine which was significant >50 μM (Figure 4.5C). The presence of 100 μM ouabain, a Na^+/K^+-ATPase channel inhibitor, had little or no significant effect on lidocaine-induced relaxation (Figure 4.5D).

4.3.4 Effect of A_{2a} and A_{2b} antagonists in intact and denuded rat aortic rings

The basal tension of NE-precontracted CSC group was 3.1 ± 0.16 g and PSB-0788 groups 3.7 ± 0.07 g (n=8 each) and not significantly different from controls (3.2 ± 0.19 g, n=8). Adenosine A_{2a} antagonist 8-(3-chlorostyryl) caffeine (CSC) significantly decreased lidocaine relaxation in the intact rat aorta at 100 to 1000 μM (Figure 4.6). Divergence began to occur at 50 μM lidocaine with relaxation values of 2, -2.8, -3.4 and 7.6% at 50, 100, 500 and 1000 μM lidocaine respectively. In direct contrast, the incubation with PSB-0788, an adenosine A_{2b} antagonist, did not modify lidocaine-induced relaxation curve at any concentration used in NE pre-contracted aortic rings (Figure 4.6).

In denuded rings, the basal tension of aortic rings with the presence of CSC (2.5 ± 0.15 g) or PSB-0788 (3.1 ± 0.18 g) was not significantly different. CSC had no effect on relaxation up to 10 uM lidocaine then strongly inhibited relaxation up to 500 μM (Figure 4.6). The maximum lidocaine relaxation was 13 ± 6%, which was significantly lower than control denuded rings (39 ± 5%, p<0.0001) (Figure 4.6). The adenosine A_{2b} receptor blocker PSB-0788 (10 μM) also decreased lidocaine relaxation by up to 50% but this effect was not significant (Figure 6). At 10, 100, and 1000 μM lidocaine, the relaxation percentages were 7 ± 1 %, 10 ± 2%, and 31 ± 3%, respectively compared to 16 ± 3%, 19 ± 4%, and 39 ± 5% in denuded controls (Figure 4.6).

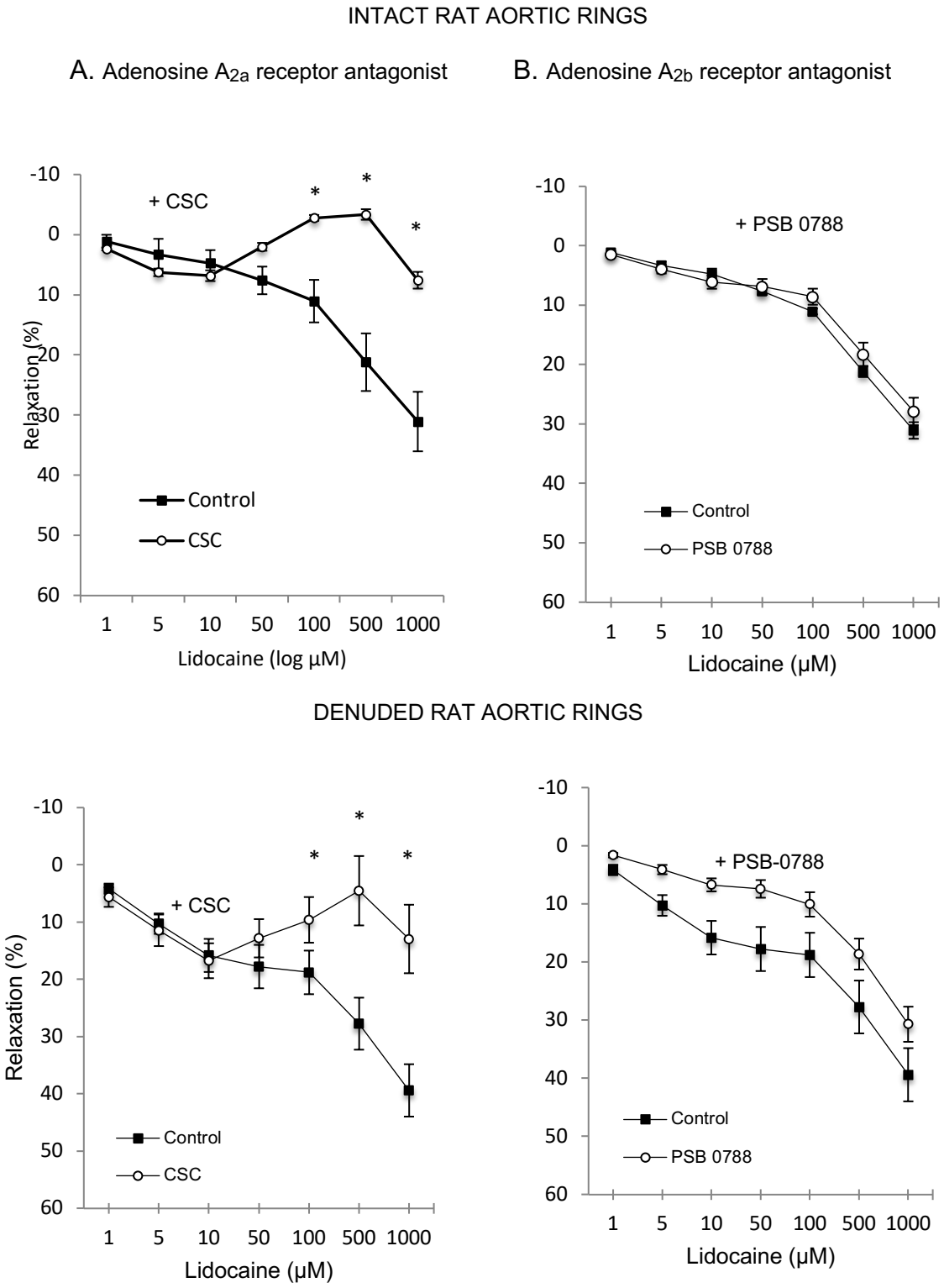

Figure 4.6 Concentration-response curves to lidocaine with and without the presence of adenosine A_{2ab} receptor blockers in intact and denuded isolated rat aortic rings.
[A] In the presence of 100 µM 8-(3-Chlorostyryl) caffeine. [B] In the presence of 10 µM PSB-0788. Relaxation is expressed as percent of maximal relaxation to 100 µM papaverine. Points represent mean ± S.E.M of aortic rings in the presence of L-NAME and indomethacin. *p<0.05 statistical difference in responses between the presence and the absence of inhibitors on intact rings. Lidocaine concentrations are on a log scale. Total animals n=16.

4.4 DISCUSSION

Despite decades of investigation, the mechanisms of lidocaine relaxation in the rat thoracic aorta, and muscular resistance arterioles are not fully understood (Abe*, et al.*, 2000; Evans*, et al.*, 1984; Johns*, et al.*, 1985; Perlmutter*, et al.*, 1990a; Satoh*, et al.*, 2015; Turan*, et al.*, 2000). This chapter reports in isolated rat thoracic rings, pre-contracted with NE, that lidocaine relaxation was: 1) biphasic from 1 to 10 μM and 10 to 1000 μM, 2) significantly enhanced by endothelial removal, particularly from 1 to 100 μM, 3) not significantly affected in the presence of L-NAME- and indomethacin in intact rings, 4) abolished by 4-AP in denuded rings and significantly reduced by 5-HD, and to a lesser extent glibenclamide, and 5) significantly reduced by A_{2a} subtype antagonist from 100 to 1000 μM, but not A_{2b}. The possible physiological significance of the biphasic nature of lidocaine relaxation, enhancement after endothelial removal, and potential role for crosstalk with the A_{2a} subtype and voltage-dependent K_v and K_{ATP} channels will be discussed.

4.4.1 Lidocaine Relaxation was Biphasic and Endothelial Dependent

The results show in oxygenated, glucose-containing Krebs Henseleit buffer, pH 7.4 at 37°C: 1) little or no change in relaxation in rat aortic rings at low lidocaine concentrations, and 2) a strong endothelial dependence which enhanced relaxation after its removal (Figure 4.2). The data suggest that the presence of an intact endothelium acted like a “brake” to reduce lidocaine relaxation, and upon its removal activated some putative factor to enhance relaxation. These findings are in contrast with those reported in rat cremaster skeletal muscle (Johns*, et al.*, 1985), epicardial porcine coronary arteries (Perlmutter*, et al.*, 1990a), human radial arterial rings (Jernbeck & Samuelson, 1993), human mammary arteries (Gherardini*, et al.*, 1995), and rabbit carotid arteries (Evans*, et al.*, 1997c; Gherardini*, et al.*, 1996), where lidocaine at low levels potentiated vasoconstriction, and at high concentrations led to relaxation. Jembeck and Samuelson further reported in isolated rings from radial arteries that lidocaine led to significantly stronger contractions after the endothelium was mechanically removed (Jernbeck & Samuelson, 1993). Reasons for the differences are not clear at present but may relate to species, age, mode of sacrifice, physiological state, pre-contractile conditions activating different channels and receptors (e.g. alteration of the membrane smooth muscle potential with high K^+ versus NE or phenylephrine to pre-contract isolated rings), tissue preparation, different endothelial removal procedures and possible damage, buffer conditions, temperature,

pO_2 availability, and the sequence of drug additions and concentrations. Another important difference is vessel type; the present study investigated the rat thoracic aorta, which is a large, highly elastic artery that normally offers little resistance to flow but assists in coupling the heart, as a pump and pressure-generator, to the arterial system by changing aortic compliance not resistance (Dobson, 2015a).

That lidocaine relaxation occurred from 1 to 1000 uM is consistent with the study of Shan and colleagues who showed that lidocaine relaxed phenylephrine or KCl (60 mM) precontracted rat aortic rings in a concentration-dependent manner (Shan*, et al.*, 2004). However, their study differed because they showed lidocaine relaxation was not significantly modified by endothelium removal*,* and their aortic rings were obtained from rats sacrificed by stunning and cervical dislocation, not anaesthesia (Shan*, et al.*, 2004). The present study also agreed with Turan and colleagues who showed lidocaine relaxed phenylephrine-precontracted rabbit thoracic aorta intact and denuded rings, however, when lidocaine (1 to 100 μM) was applied 15 min before the addition of phenylephrine it produced contractions at high concentrations (up to 10 mM), and endothelium removal did not significantly affect contractile activity. This example demonstrates the dynamics of the pre-contractile state and the importance of specifying the sequence of drug administration, which can produce different results. Further studies are required to investigate these discrepancies in the thoracic aorta of rat and other species prepared from different modes of sacrifice, different precontracted states and basal tone.

4.4.2 Lidocaine Relaxation Enhancement involves an Endothelium-Smooth Muscle Coupling and possible activation of K_v and K_{ATP} channels

Since lidocaine relaxation displayed a strong endothelial-dependence (Figure 4.2), it suggested a possible role for NO release or activation of the cyclo-oxygenase pathway and/or their interactions with the adrenoreceptors on vascular smooth muscle. Surprisingly, there was little or no effect of either L-NAME and indomethacin on lidocaine relaxation (Figure 4.3) indicating that the putative relaxing factor after endothelial removal was neither NO nor prostacyclin. Other unknown factor(s) must be released upon endothelial removal to enhance lidocaine relaxation. Another possibility is endothelial-dependent activation of smooth muscle voltage-dependent K_v channels and/or smooth muscle mitochondrial K_{ATP} channels since 4-AP completely abolished

relaxation (Figures 4.3A and 4.4A) and 5-HD led to ~50% inhibition in denuded rings (Figures 4.4C and 4.5C).

Enhanced lidocaine relaxation may also have come from changing the cellular redox state and reactive oxygen species (ROS) derived from NAD(P)H oxidases (Hsieh*, et al.*, 2014; Tsai & Jiang, 2010), as it has been reported that lidocaine at higher concentrations protects against ROS attack in rabbit abdominal aorta (Lee*, et al.*, 2010). Rogers and colleagues further showed that 4-AP-sensitive K_v channels are redox sensitive and contribute to H_2O_2-induced coronary vasodilation (Rogers*, et al.*, 2007). In summary, enhanced lidocaine relaxation after endothelial removal does not appear to involve the direct activation of NO or prostanoid-linked pathways, and other relaxing factors and downstream signaling pathways, possibly involving K_v and/or 5-HD sensitive K_{ATP} channels, are involved.

4.4.3 Smooth Muscle Adenosine A_{2a} modulation may also be involved in the Enhanced Lidocaine Relaxation

The present study also suggests an intriguing possibility for enhancing lidocaine relaxation may be activation of the A_{2a} receptor on vascular smooth muscle. A surprising result was that lidocaine relaxation above 50 μM in intact and denuded rat aortic rings was significantly inhibited by 75 to 100% in the presence of A_{2a} blocker 8-(3-chlorostyryl) caffeine (CSC) (Figure 4.6). This implies that the A_{2a} receptor may be involved in the presence or absence of an intact endothelium. Assuming CSC has high specificity for A_{2a} receptors (Jacobson*, et al.*, 1993), this antagonist may reduce lidocaine relaxation from one or more of the following mechanisms: 1) Directly or indirectly increasing Ca^{2+} influx from extracellular sources such as L-type Ca^{2+} channels (Amberg & Navedo, 2013), 2) increasing the release of Ca^{2+} from intracellular stores (e.g. sarcoplasmic or endoplasmic) to increase cytosolic free Ca^{2+}, and/or 3) increasing myofibrillar contractile sensitivity to existing free Ca^{2+} (signaled via the RhoA/Rho kinase pathway), increasing cross-bridge cycling and development of force (Tsai & Jiang, 2010; Webb, 2003). Possible crosstalk between A_{2a} receptors and lidocaine may also involve transmembrane domains of adenyl cyclase and other downstream signaling pathways to alter intracellular free Ca^{2+} and/or myofibrillar sensitisation.

Little or no data exist on adenosine and lidocaine interactions in intact rat aortic rings or endothelial-vascular smooth muscle interactions. Adenosine A_{2a} and A_{2b} receptors are present on vascular endothelium and smooth muscle of many vessels (Hein*, et al.*, 2013;

Kemp & Cocks, 1999) and when activated can lead to vasodilation. A_{2a} receptor vasodilation is thought to involve: 1) endothelial NO production which activates smooth muscle guanylyl cyclase via opening K_{IR} channels (Hein*, et al.*, 2013), and/or 2) more direct smooth muscle A_{2a} receptor activation which in turn stimulates mostly G_s proteins (and Gq) and cAMP signaling pathways to reduce intracellular Ca^{2+} levels (Hein*, et al.*, 2013; Linden, 2001). In addition, adenosine A_{2a} activation may activate sarcolemma Ca^{2+} channels and regulate influx in large elastic arteries and resistance vessels. Stella and colleagues showed that activation of A_2 receptors stimulates protein kinase A to inhibit L-type Ca^{2+} channels in rod photoreceptors resulting in a decreased Ca^{2+} influx (Stella Jr*, et al.*, 2002). Gubitz and colleagues have proposed dual A_{2a} signaling involving the activation of both N- and P-type calcium channels by different G proteins and protein kinases in some nerve terminals (Gubitz*, et al.*, 1996). Goncalves and colleagues showed that adenosine A_{2a} receptors facilitated Ca^{2+} uptake through class A calcium channels in rat hippocampal CA3 region (Goncalves*, et al.*, 1997).

Interestingly, Benkwitz and colleagues also showed that higher concentrations of lidocaine (1000 uM) in hamster oocytes potentiated $G\alpha_i$-coupled A_1 receptor signaling by reducing cyclic AMP production in a dose-dependent manner through an unidentified mechanism (Benkwitz*, et al.*, 2003). The authors proposed that lidocaine was not an A_1-receptor agonist *but enhanced adenosine-A_1 receptor signaling*. They argued that lidocaine interacted with a pool of already activated Gαi present in the cytoplasm and thereby facilitated its ability to inhibit adenylate cyclase leading to lower cAMP (Benkwitz*, et al.*, 2003). The present study did not examine adenosine A_1 receptor antagonism. It can be concluded from the current study that A_{2a} receptor may have enhanced lidocaine relaxation activation by directly effecting vascular smooth muscle (Figure 6), and this may have occurred by reducing intracellular Ca^{2+} and/or myofibrillar contractile sensitization in intact isolated rat aortic rings, although the underlying mechanisms remain to be identified. Further studies are required to investigate the role of adenosine and lidocaine on membrane Ca^{2+} channel modulation in isolated rat aortic rings.

4.4.4 Limitations of the Study and Possible Physiological Significance

The present study examined lidocaine relaxation in isolated rat thoracic rings using length-tension experiments and a number of antagonists of NO, prostanoids, K_v, K_{ATP} and A_2 receptors under normoxic and normal pH conditions from healthy rats. Before definitive conclusions can be drawn regarding the nature of unknown relaxation

factor(s), it would be important to examine separately and in combination other drug antagonists and agonists of NO, prostanoids, K_v, Sarc- and Mito-K_{ATP} channels and A_2 receptors on lidocaine relaxation in intact and denuded rings. Furthermore, to gain greater mechanistic insight into the nature of voltage-dependent K^+ channels and lidocaine vasorelaxation electrophysiological experiments would be essential. Leukotrienes, and leukotriene synthase inhibitors, may also be of interest because they have been shown to modulate rat aortic ring relaxation (Lawson*, et al.*, 1989). Possible physiological significance of the present study relates to lidocaine's effect to regulate *in vivo* compliance such as ventricular-arterial coupling functions linking the heart as a pump to tissue perfusion (Bellien*, et al.*, 2010; Marti*, et al.*, 2012). However, further *in vivo* studies are required to test this hypothesis. This work may have clinical applicability on the ancillary properties of lidocaine at the site of injection during infiltration, nerve block, or epidural anaesthesia (Johns*, et al.*, 1985), and on damaged endothelium such as in plaque formation, arterial and venous conduit protection for cardiopulmonary bypass grafting (Gur*, et al.*, 2012a), prevention of vascular spasm during neurosurgery (Li*, et al.*, 2012), lowering elevated intracranial pressure (Zeiler*, et al.*, 2015), lidocaine cardioplegia (Baraka*, et al.*, 1993; Dobson*, et al.*, 2013a), and other surgical applications (Dobson, 2015a).

4.5 CONCLUSIONS

This chapter showed in isolated, oxygenated NE precontracted rat aortic rings that lidocaine relaxation was biphasic from 1 to 10 uM and 10 to 1000 uM. Furthermore, lidocaine relaxation was found to be significantly enhanced by endothelial removal, which did not appear to be NO or prostacyclin dependent. The putative unknown factor(s) responsible for enhanced relaxation may involve activation of smooth muscle voltage-sensitive K_v and 5-HD sensitive channels or pathways, and possible crosstalk with A_{2a} subtype receptor at higher lidocaine concentrations.

CHAPTER 5. ADENOSINE AND LIDOCAINE (AL) COMBINATION DILATES INTIMALLY DAMAGED RAT THORACIC AROTIC RINGS AND GUINEA PIG MESENTERIC ARTERIES: POSSIBLE SIGNIFICANCE TO CARDIAC SURGERY

Abstract:

Introduction: New pharmacotherapies are required to improve vessel graft protection and prevent vasoconstriction and spasm in CABG surgery. Chapters 3 and 4 studied adenosine (A) and lidocaine (L) relaxation in rat aortic rings and reported a possible crosstalk between L relaxation and adenosine A_{2a} receptor inhibition. The aim of Chapter 5 was to examine the effect of AL combination compared to A and L alone on relaxation in intact and denuded rat aortic rings and in guinea-pig pressurized mesenteric arterial segments.

Methods: Aortic rings were harvested from Sprague-Dawley rats and equilibrated in an organ bath containing modified Krebs Henseleit (KH) solution, pH 7.4, 37°C. Rings were pre-contracted sub-maximally with 0.3 μM norepinephrine, and the effects of increasing AL, A or L (up to 1.0 mM) were examined in intact and denuded rings. Mesenteric artery segments were isolated from guinea pigs and mounted in an arteriograph containing KH solution and pressurised to 60 mmHg. Arteries were preconstricted with 10^{-8} M vasopressin and AL, A, or L was administered luminally or abluminally. Diameters were measured using video-microscopy.

Results: In intact rat aortic rings, AL increased relaxation from 21 to 100% (0.1-1.0 mM) and relaxation was endothelium-independent. Adenosine alone was also a potent relaxant of aortic rings but, unlike AL relaxation, it was partially endothelium-dependent. In intact mesenteric artery segments, increasing luminal AL produced a potent endothelium-independent dilation (up to 90%). Adenosine dilation was endothelium-independent but not lidocaine, which produced 33% dilation only after endothelial removal. Extra-luminal AL and A led to 76% and 80% dilation in intact segments respectively, whereas L resulted in constriction (10-17%).

Conclusions: AL can dilate aortic rings and mesenteric artery segments by up to 90% regardless of whether the endothelium is intact.

5.1 INTRODUCTION

Endothelial damage is common after open surgical or endoscopic conduit harvesting, pressure-testing, storage and implantation in patients undergoing coronary artery bypass graft (CABG) surgery (Dobson*, et al.*, 2013b; He, 2005; Kopjar & Dashwood, 2016). Endothelial dysfunction is a major factor responsible for loss of graft patency after one to five years (Fukui*, et al.*, 2010; He & Taggart, 2016b; Ruel*, et al.*, 2004). Intimal damage is involved in the imbalance between endothelium-dependent and endothelium-independent derived vasodilators and vasoconstrictors that can lead to endothelial-smooth muscle decoupling, vasoconstriction, vasospasm, local inflammation/thrombosis and possible intimal hyperplasia/stenosis (Al-Sabti*, et al.*, 2013 ; Cartier*, et al.*, 1993b; Félétou, 2016; Goldman*, et al.*, 2004a; He, 2005). The major vasodilators currently include nitric oxide, prostacyclin, bradykinin, adenosine and endothelium-derived hyperpolarising factor, and the major vasoconstrictors include endothelin-1, angiotensin II and reactive oxygen/nitrogen species (Félétou, 2016; He & Taggart, 2016b; Sandoo*, et al.*, 2010b).

An area of ongoing controversy is the role adenosine to regulate vascular tone in a number of vessel types, and their endothelial dependence (Arsyad & Dobson, 2016a). Chapter 3 showed that in rat aortic rings, adenosine relaxation was endothelium-dependent above 10 μM, and involved endothelial nitric oxide production but not prostacyclin. Another vasoreactive modulator, lidocaine, has a number of paradoxical properties ranging from smooth muscle relaxation to constriction (Evans*, et al.*, 1997b; Gherardini*, et al.*, 1998b; Kinoshita*, et al.*, 2001b; Kinoshita*, et al.*, 2003b; Perlmutter*, et al.*, 1990b). Chapter 4 showed that lidocaine relaxation in rat aortic rings was significantly enhanced by endothelial removal, which did not appear to be NO or prostacyclin-dependent (Arsyad & Dobson, 2016b). That study identified an unknown factor(s) responsible for enhanced lidocaine relaxation, and implicated the involvement of the adenosine A_{2a} receptor (Arsyad & Dobson, 2016b). Given the potential crosstalk between A and L, the aim of Chapter 5 was to examine the effect of AL combination compared to A and L alone on the relaxation properties of intact and denuded rat thoracic aortic rings and guinea-pig pressurized mesenteric arterial segments. Mesenteric arterial segments were included because mesenteric vasoconstriction and gut ischemia-reperfusion injury during major surgery can be particularly lethal with mortality rates of over 70% (Hasan*, et al.*, 2004; Sastry*, et al.*, 2014).

5.2 MATERIAL AND METHODS

5.2.1 Animals

Adult male Sprague-Dawley rats (300-350g) and guinea-pigs (250-300g) were prepared as described in Chapter 2.2.1 and 2.3.1, respectively.

5.2.2 Aortic ring preparation and organ bath tension measurements

Rat thoracic aortic rings were harvested and prepared as described in Chapter 2.2.2 and shown in Figure 2.3.

AL, A and L were added to the organ bath to obtain 1, 5, 10, 50, 100, 500 and 1000 μM concentrations. At the end of each experiment, rings were tested for viability with 100 μM papaverine using the method of Grbović and colleagues (Grbović*, et al.*, 2000a), and relaxation was expressed as a % maximal relaxation to papaverine.

5.2.3 Guinea-pig mesenteric artery segments

The arterial perfusion system coupled to microscopy and video monitoring is described by Sokoya and colleagues (Sokoya*, et al.*, 2006b) and outlined in Chapter 2.3.2 and 2.3.3.

After 30 min equilibration in the pressure myograph, arterial segments were pre-constricted with 10^{-8} M arginine vasopressin (AVP). Some segments were left intact, and others denuded by pumping air into the vessel lumen at a flow rate 1ml/min for 10 minutes (Sokoya*, et al.*, 2006b). Removal of the endothelium was confirmed with 10^{-5} M acetylcholine. After diameter stabilization, the arteries were exposed to AL, A or L luminally or abluminally (1 to 1000 μM) and the change in outer artery diameter was measured. At the end of each experiment, vessels were washed with calcium-free Krebs Henseleit solution containing 1 mM ethylene glycol tetraacetic acid (EGTA) to determine the maximal dilation of the vessels (Sokoya*, et al.*, 2006b). Preliminary experiments found that, unlike aortic rings, 100 μM of papaverine led to inconsistent maximum dilation in mesenteric segments. For mesenteric artery experiments, the effects of A, L and ALM on vasodilation (or relaxation) were expressed % maximal dilation to calcium-free Krebs Henseleit solution containing 1 mM EGTA. At each

concentration of ACh, the percentage dilation was calculated according to the following equation:

$$\text{Percentage Dilation} = (D_{ACh} - D_{base})/(D_{max} - D_{base}) * 100$$

where D_{ACh} is the diameter of the artery after luminal administration of A_{Ch}, D_{base} is the baseline diameter of the artery before addition of ACh, D_{max} is the maximal diameter of the artery in the presence of calcium-free Krebs Henseleit solution (containing 1 mM EGTA).

5.2.4 Statistics

Values are expressed as mean ± SEM. The number of rats and guinea-pigs were selected from a priori G-power analysis to achieve a level of 1.0. All data was tested for normality using *Kolmogorov-Smirnov* test. Relaxation responses to adenosine, lidocaine and AL were analysed for homogeneity of variances followed by two-way repeated measures ANOVA coupled with the Bonferroni post-hoc test for individual data point comparisons. The alpha level of significance for all experiments was set at $p<0.05$.

5.3 RESULTS

5.3.1 Effect of Increasing AL on Relaxation in Rat Aortic Rings

AL produced a dose-dependent relationship in intact and denuded rat aortic rings (Figure 5.1A). In intact rings, AL had little effect below 100 μM (<5% relaxation) and increased relaxation to 76% above 100 μM. Curiously, when the experiment was repeated but started at 100 μM (Figure 5.1B), rather than 1 μM, (Figure 1A), relaxation was higher (21% vs 3.7%), and maximum relaxation after 1000 μM was 98% compared to 76% after 1 to 1000 μM serial dilution (Figure 1A). The data also show that serial additions of AL from 1 to 1000 μM vs. 100 to 1000 μM lead to different maximum relaxations at 100 μM (4 vs. 14% relaxation) and 1000 μM (76 versus 98% dilation), indicating a tachyphylaxis-like response in the first experiment (Figure 5.1A) but not in the latter (Figure 5.1B). Similar relaxation profiles were found in rings with the endothelium removed. Another interesting feature of Figure 5.1B was that at the AL-induced relaxation at the cardioplegic concentrations (250 to 1000 μM AL) (Figure

5.1B), showed a slight curvilinear effect that was more pronounced after removing the endothelium (greater dilation at a given AL concentration), but this was not significant.

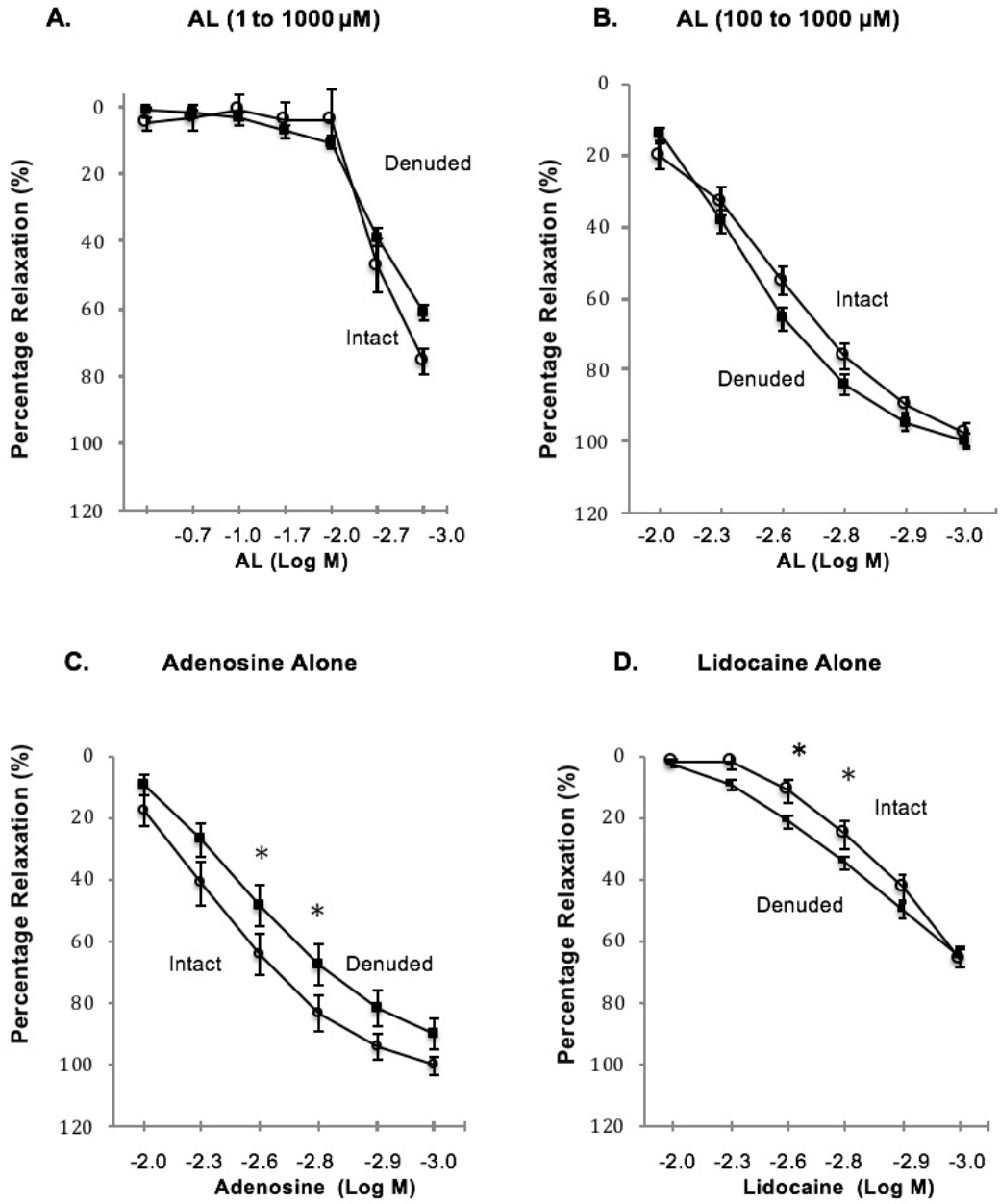

Figure 5.1. Dose-response curves of adenosine-lidocaine (AL), adenosine (A) and lidocaine (L) in isolated intact and denuded rat thoracic aortic rings.
A. AL (1-1000 µM, log scale). **B**. AL (100-1000 µM, log scale). **C.** Adenosine (100-1000 µM) and **D.** Lidocaine (100-1000 µM). Values are mean ± S.E.M for aortic rings from 8 animals. **p*<0.05 adenosine endothelium intact and denuded groups.

5.3.2 Effect of A and L Alone on Relaxation in Rat Aortic Rings

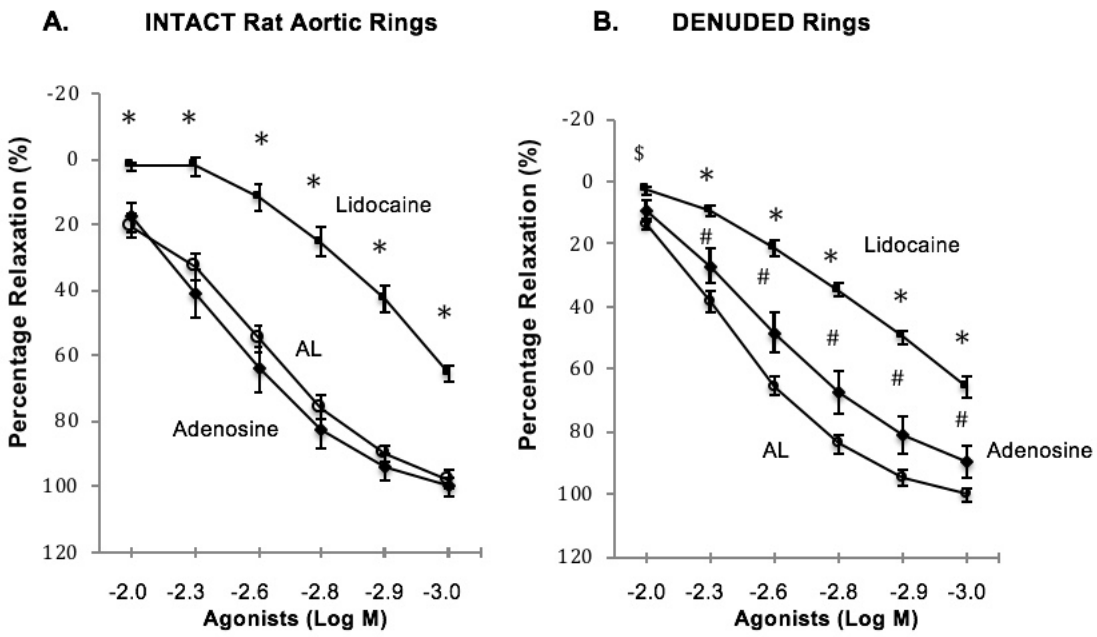

Figure 5.2 Comparison of dose-response curves to AL, Adenosine and Lidocaine in isolated intact (A) and denuded (B) rat thoracic aortic rings.
Values are mean ± S.E.M for aortic rings from 8 animals. *p<0.05 lidocaine group and other groups. # p<0.05 adenosine group and AL group.

Increasing adenosine from 100 to 1000 μM significantly increased relaxation by 82% in intact rings, and by 81% in denuded rings from baseline (Figure 5.1C). The effect of removing the endothelium significantly reduced relaxation by 23 to 18% at 400 and 600 μM respectively (Figure 5.1C). Lidocaine alone increased relaxation by 63% in intact and denuded rings, and endothelial removal significantly enhanced dilation 1.5 times at 400 μM and 1.3 times at 600 μM lidocaine compared to intact rings (Figure 5.1D). Figures 5.2A and 5.2B represents the combined data in intact and denuded rat aortic rings. In intact rings, AL relaxation was not significantly different from adenosine, and both were significantly higher than lidocaine alone. In denuded rings, adenosine relaxation was significantly lower for a given concentration, lidocaine relaxation was significantly higher, and there was no change in AL relaxation after endothelial removal.

Guinea-Pig Mesenteric Artery

5.3.3 Effect of luminal AL, A and L on endothelium intact and denuded segments

For all mesenteric artery experiments, there was no significant difference in AVP-stimulated constriction between the groups in either intact or denuded segments. In intact arterial segments, increasing luminal AL or adenosine led to over a 10-fold increase in dilation from 8% to 84% (Figure 5.3A). The AL or A treatments were not significantly different from each other. In contrast, increasing intraluminal lidocaine concentration produced a 2.6 fold constriction, and was significantly different from all other groups (Figure 5.3A).

In denuded segments, increasing luminal AL and adenosine followed a similar pattern to the intact artery with 90% dilation from 1 to 1000 μM (Figure 5.3B). Removal of the endothelium led to a dramatic and significant change in mesenteric arterial reactivity to lidocaine. Instead of modest constriction in intact segments, increasing lidocaine led to a 4.7-fold increase in dilation from 1 to 1000 μM (Figure 5.3B). Lidocaine's maximum dilation response after denudation was 42% at 1000 μM compared to 9% at 1.0 μM (Figure 5.3B).

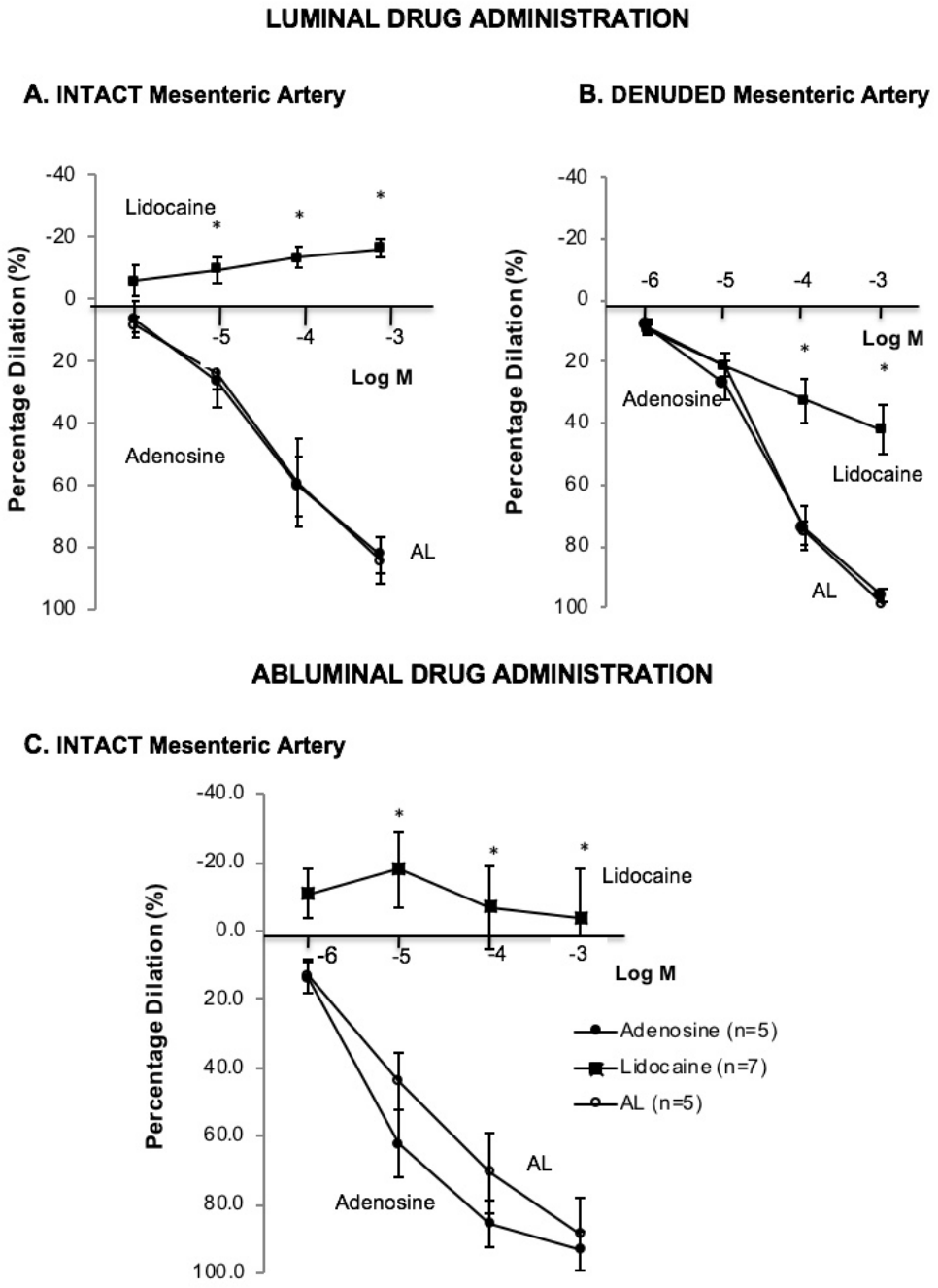

Figure 5.3 Effects of luminal AL, adenosine and lidocaine administration on guinea-pig intact (A) and denuded (B) mesenteric artery administered luminally, and (C) abluminal adminstration on intact mesenteric artery segments.
A and B: Drugs were delivered luminally (A and B) and abluminally (C) in a dose dependent manner. Values are mean ± S.E.M for mesenteric rings from 5-7 animals. *p<0.05 lidocaine and other groups.

5.3.4 Effects of abluminal AL, A and L on intact mesenteric segments

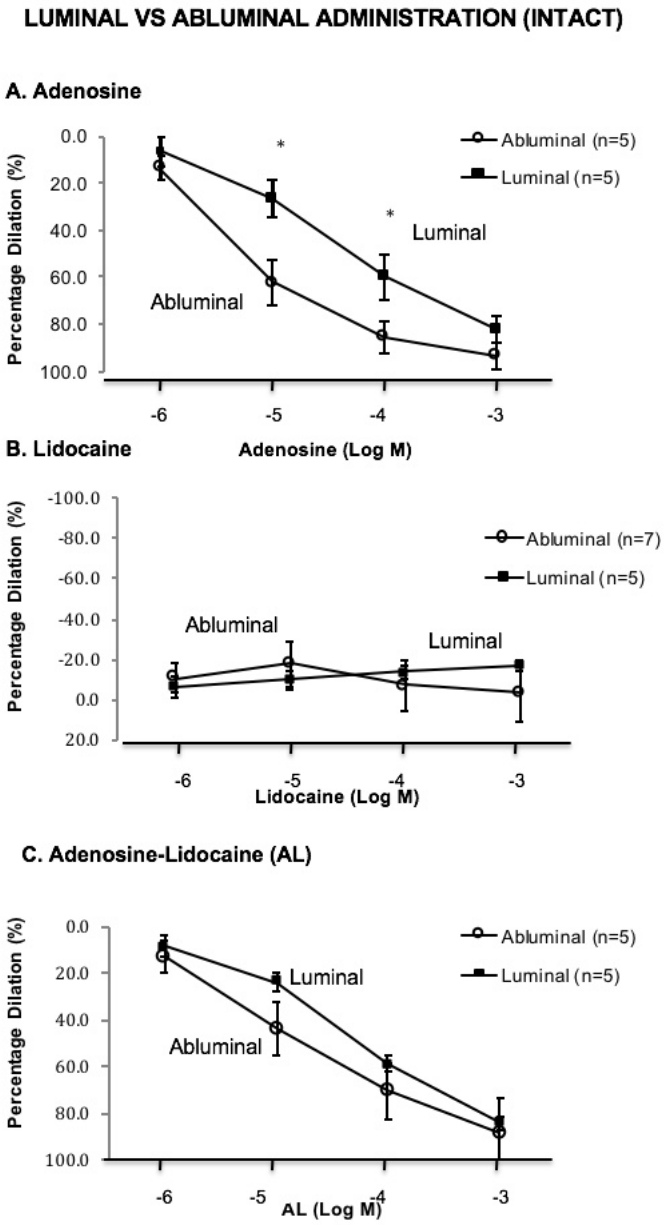

Figure 5.4 Luminal versus Abluminal Comparison between adenosine (A) and lidocaine (B) and AL (C) in intact guinea-pig mesenteric artery.
Values represent mean ± S.E.M of mesenteric rings from 5-7 animals. **p*<0.05 statistical difference in responses between luminal and abluminal adenosine administration.

The effect of increasing abluminal AL produced 76% dilation from 1 to 1000 μM (Figure 5.3C). Similarly, adenosine led to a significant increase in dilation of 80% (Figure 5.3C). The differences between AL and adenosine were not significant. Increasing lidocaine from 1 to 10 μM produced an increase in constriction from 10% to 17% with

vessel diameter slowly dilating to baseline (Figure 5.3C). The comparative effects of AL, adenosine or lidocaine on luminal verses abluminal administration in intact mesenteric segments are summarized in Figures 5.4A and 5.4C. The data show that adenosine relaxation is significantly enhanced when administered abluminally (Figure 5.4A), while no differences were found in lidocaine administration (Figure 5.4B). Abluminal versus luminal AL administration, like adenosine, produced up to 45% more dilation over a range of AL concentrations, however, the differences were not significant (Figure 5.4C).

5.4 DISCUSSION

New pharmacological strategies are required to improve autologous supplementary arterial and venous graft protection in CABG surgery (Kopjar & Dashwood, 2016). This chapter reports the following: 1) In rat aortic rings, AL combined produced a strong relaxation response that was endothelium-independent, 2) Adenosine relaxation in intact rings was similar to AL, but differed by being partially endothelium-dependent, 3) Lidocaine relaxation was significantly less than AL and adenosine, but like adenosine was endothelium-dependent, 4) In intact mesenteric arterial segments, luminal administration of AL or adenosine produced a strong vasodilation (up to 90%) and was endothelium-independent, whereas lidocaine dilation was significantly less and occurred only after endothelial removal, and 5) Abluminal AL and adenosine produced 80% dilation, and lidocaine produced 10 to 17% constriction. These differences will now be discussed.

5.4.1 AL relaxation was endothelial-independent

Two interesting results of the present study were: 1) AL was a powerful dilator in isolated rat thoracic aortic rings and pressurized guinea-pig mesenteric segments, and 2) relaxation/dilation was endothelium-independent. The finding that AL was a strong relaxant in rat aorta, regardless of whether the intima was damaged or not, suggests that AL may find utility in harvesting and perfusing conduits for revascularisation. Harvest, storage and implantation are times when the conduit vessel is highly vulnerable to intimal damage and potential failure (Cartier*, et al.*, 1993a; Chiavarelli*, et al.*, 1982; Dashwood & Tsui, 2013). Thus, maintaining optimal vascular tone in harvested vessels may lead to improved perfusion and reduce myocardial dysfunction.

Interestingly, the relaxation effect of AL in aortic rings and mesenteric artery segments

was not a synergistic effect of adenosine and lidocaine but was dominated by adenosine and a complex dilation-contraction behavior from lidocaine (Figures 5.2 and 5.3). Currently, the underlying mechanisms of AL's action are not known. It is important to recognize that the aortic ring experiments and pressurized artery experiments were different with regards to drug application. In the rings, drugs have access to both the endothelium and smooth muscle whereas in the pressurized vessels, they were applied to either the endothelium or smooth muscle. This may be particularly important for AL combination because it appears that there are receptors for both adenosine and lidocaine on both cell types (see below).

5.4.2 Rat thoracic aortic rings

In rat thoracic aorta, adenosine relaxation has been reported in the literature to be either fully endothelium dependent, partially dependent or have no dependency see (Arsyad & Dobson, 2016a). Adenosine vasodilation is believed to involve a complex interplay between endothelial A_{2a} subtype receptor activation, nitric oxide (NO), prostanoids (e.g Prostaglandins and thromboxane A_2), hyperpolarising factors, and voltage-dependent K^+ channels (Headrick*, et al.*, 2011). Chapter 3 revisited the question and found that relaxation in isolated rat thoracic aortic rings was endothelium-dependent, and involved activation of smooth muscle A_{2a}, endothelial NO production, and voltage-dependent K_v, K_{ATP} channels (sarcoplasmic and mitochondrial), but not prostanoid production (Arsyad & Dobson, 2016a). The present study confirmed adenosine's partial endothelium-dependency, but the focus was on the higher concentrations that appear in AL cardioplegia.

Lidocaine reactivity in isolated rat thoracic rings is also controversial (Arsyad & Dobson, 2016b). The present study showed that by removing the endothelium it led to significant enhancement of lidocaine dilation (Figure 5.1D). In Chapter 4, a putative relaxation factor(s) was proposed to explain this anomalous effect, and showed it was not NO- or prostacyclin-dependent (Arsyad & Dobson, 2016b). However, dilation was significantly reduced in the presence of K_v and MitoK_{ATP} inhibition in denuded rings (Arsyad & Dobson, 2016b), and interestingly by adenosine A_{2a} subtype antagonism (above 100 µM lidocaine) in intact and denuded rings, indicating a potential role for voltage-dependent K^+ channels and crosstalk with adenosine receptor activation (Arsyad & Dobson, 2016b). Further studies are required to identify and understand the nature of this putative lidocaine relaxation factor, which appears to be activated upon denudation (Figure 5.1D).

5.4.3 Mesenteric artery segments

In contrast to rat aortic rings, luminal adenosine relaxation was endothelium-independent in mesenteric arterial segments (Figures 5.3A and 5.3B). A possible reason for the difference is because adenosine in the isolated rat aortic rings was reaching receptors on both the endothelium and smooth muscle but not in the pressurized arteries. That luminal infusion of adenosine elicits an endothelium-independent dilation indicates the presence of smooth muscle adenosine receptors. This curious result supports the 2005 study of Radenković and colleagues in the rat mesenteric artery (Radenković*, et al.*, 2005), who reported that dilation was partly induced by activation of smooth muscle A_{2a} receptors and mediated via the opening a mixed population of smooth muscle K^+ channels, and possibly the Na^+/K^+-ATPase pump (Radenković*, et al.*, 2005). Unfortunately, there appear to be only a handful of studies on the effect of adenosine on mesenteric artery vasoreactivity.

In the current study, increasing luminal lidocaine concentration also resulted in complex behaviour (Figures 5.3A and 5.3B). In intact mesenteric artery segments, lidocaine produced a constriction (~10%), and denudation produced an opposite effect i.e. a significant dilation (42% relaxation at 1000 μM) (Figure 5.3B), which was similar to what was found in rat aortic rings. It appears that the lidocaine-induced 'constricting' factor(s) originated from the intact endothelium, and the 'dilation' factor(s) from vascular smooth muscle. In the abluminal lidocaine experiments, it is also possible that lipid-soluble lidocaine diffuses through to the endothelium, and the only way to remove the 'constricting' factor(s) is to remove the endothelium. Further, the addition of AL suggests that the effect of the lidocaine-mediated 'constricting' factor is overcome by the adenosine-mediated dilation.

Complex lidocaine vasoreactivity has also been reported in human internal mammary artery (IMA), radial artery (RA) and saphenous vein segments (Gur*, et al.*, 2012b). Gur and colleagues reported that lidocaine at low concentrations (10^{-9} to $10^{-7.5}$ M resulted in ~40% dilation in IMA and RA segments isolated from 20 patients, and constriction at higher concentrations (>$10^{-7.5}$ M) (Gur*, et al.*, 2012b). In saphenous vein segments, the group also reported a 24% dilation at 10^{-9} to 10^{-7} M lidocaine, and a dose dependent constriction above 10^{-7} M (Gur*, et al.*, 2012b). Unfortunately, there are only a few studies on the effect of lidocaine on the mesenteric artery. In 1975, Sovac and colleagues found that intra-arterial injection of lidocaine was a poor mesenteric dilator in dogs (Sovak*, et al.*, 1975), which is consistent with the current study's data (Figure

5.3A). Lastly, abluminal AL and A administration produced greater dilation than luminal delivery (Figures 5.4A and 5.4C) with no change in lidocaine reactivity (Figure 5.4B). Unfortunately, this experiment was not performed on denuded mesenteric segments.

5.4.4 Possible clinical translational significance

The findings of the present study have the potential to translate and improve graft protection in patients undergoing CABG surgery (Al-Sabti*, et al.*, 2013 ; Dashwood & Tsui, 2013; Magee*, et al.*, 2008b; Wallitt*, et al.*, 2007b). AL solution at cardioplegia concentrations (0.25 to 1 mM) may further improve protection of the heart and conduits during reperfusion when vessels are susceptible to spasm, particularly radial arterial conduits (Ruel*, et al.*, 2004). Translation may be achievable because the AL cardioplegia is in clinical use in the USA and Italy, and two prospective randomized trials have shown superiority over high potassium solutions (Onorati*, et al.*, 2016b; Onorati*, et al.*, 2013a). The uniqueness of the AL cardioplegia resides in arresting the heart at its natural or resting membrane potential (5 mM K^+), not unnatural depolarizing potentials from high potassium solutions (>15 mM K^+) (Dobson*, et al.*, 2013b). Thus, the AL cardioplegic solution may not only arrest and protect the heart but may protect the newly implanted grafts with the potential to improve graft patency.

AL solution may also find clinical utility as a topical (abluminal) or intravenous antispasmotic agent for the internal mammary artery (IMA), which is commonly used *in situ* for CABG surgery (Harskamp*, et al.*, 2008b; He & Taggart, 2016b). Similar techniques may also apply to the *in situ* protection of the gastro-epiploic artery (a branch of celiac trunk), which is less commonly used today because it is particularly prone to vasospasm (He & Taggart, 2016b). AL topical use could also be used in neurosurgery where cerebral arterial spasm can lead to ischemic neurological deficits. Lastly, the finding that AL dilates mesenteric artery segments helps explain recent trauma work which showed that small-volume intravenous infusion of AL and magnesium (ALM) led to increased blood flow and local pO_2 to the gut after non-compressible haemorrhage and shock in the rat (Dobson & Letson, 2016a; Letson & Dobson, 2017a). Protecting the gut is important during trauma or major surgery because it is the "motor of multiple organ failure" and responsible for triggering, heightening, and perpetuating the systemic inflammatory response (Dobson, 2015b; Patel*, et al.*, 2016).

5.4.5 Limitations and future studies

A major limitation of the present study was that the arterial rings and mesenteric segments were from healthy rats and guinea-pigs, and it would be clinically important to investigate segments from human conduit arteries and saphenous veins used in cardiac surgery, and the underlying pathogenesis of vasospasm. Another limitation is that rat thoracic aorta was used, which is a large, highly elastic compliance vessel that normally offers little resistance to flow in contrast to smaller peripheral and coronary arterioles (Arsyad & Dobson, 2016a). From a mechanistic viewpoint, studies are required to investigate the possible cross-talk between A and L that leads to relaxation in the presence and absence of an intact endothelium, and include the effect of key modulators, ion channel activators and inhibitors and protein/mRNA analysis of candidate receptors. Since the net effect of this dynamic system is to regulate vascular reactivity via intracellular Ca^{2+}, A and L crosstalk may be revealed from electrophysiological, immune-histochemical and qRT-PCR techniques targeting microdomain Ca^{2+} signaling receptor sites and pathways in denuded and intact arterial segments (Earley & Brayden, 2015). It would also be important to investigate the effect of AL on changing the membrane potential of endothelial and smooth muscle cells during relaxation because the cardioplegia confers its superior protection by keeping the heart at its resting membrane potential (Dobson*, et al.*, 2013b; Sloots & Dobson, 2010a).

5.5 CONCLUSIONS

Chapter 5 concludes that AL solution above 100 μM produced a concentration-dependent relaxation/dilation in rat aorta and guinea-pig mesenteric artery segments with or without an intact endothelium. AL may find translational utility to improve conduit protection in CABG surgery, and other major surgeries, where varying degrees of endothelial damage, vasoconstriction or vasospasm are known to occur.

CHAPTER 6. DEVELOPING A PRESERVATION SOLUTION FOR VESSEL STORAGE WITH ADENOSINE AND LIDOCAINE COMBINATION

Abstract:

Introduction: Current methods of cold storage protection on vessel function remains unsatisfactory. Cold storage with traditionally used preservation solutions, such as University of Wisconsin (UW), EuroCollins (EC), and histidine-tryptophan-ketoglutarate (HTK) solution, can result in dramatic losses of vascular contractility and relaxation after 72 hours of storage. Therefore, new pharmacotherapies are required for vessel graft protection and to prevent vasoconstriction and spasm of the graft.

Methods: Thoracic aortic vessels were harvested from 300-350 g Sprague Dawley rats and transferred to a container with pre-cooled KH solution. Thoracic aortic rings (n=40) were then assigned to one of the following groups: 1. Fresh preparation (controls), 2. Krebs Henseleit (KH), 3. Modified KH (low Ca^{2+}/high Mg^{2+}), 4. Modified KH + adenosine + lidocaine (KH+AL), 5. Modified KH + adenosine + lidocaine + melatonin + insulin (KH+ALMI). With the exception of controls that were immediately subjected to vessel function analysis after harvesting, other groups were kept in the assigned preservation solutions at 4°C for six days. After 6-day storage, the vessel rings were then transferred to an organ bath filled with KH solution, gently warmed to 37°C, and then subjected to vessel function analysis.
Contraction responses to potassium chloride (KCl; 60 mM), norepinephrine (NE; 0.3 μM), and relaxation responses to acetylcholine (ACh; 10^{-6}-10^{-3} M) and sodium nitroprusside (SNP; 10^{-6}-10^{-3} M) were evaluated using an isometric force transducer. At the end of each experiment, rings were maximally dilated with 100 μM papaverine to confirm viability of the vessels and the relaxation value would be used as a comparison to ACh and SNP-induced relaxation.

Results: Following 6-day storage (4°C) in KH solution, the aortic rings only recovered 46% and 34% of their contractility responses to NE and KCl, respectively. Modified KH with low Ca^{2+}/high Mg^{2+} improved recovery of contractile function with 89% and 76% return with NE and KCl use, respectively. Meanwhile, AL storage in modified KH

recovered 100% of NE contractile function, but there was no further recovery in the KCl response compared to modified KH alone (76% recovery). Similar results were found with ALMI in modified KH, which led to 100% and 86% of contractile function recovery in response to NE and KCl, respectively.

After 6-day storage in standard KH, aortic rings only recovered 42 % of ACh relaxation function, while modified KH storage led to 79% relaxation recovery. AL addition in modified KH significantly improved relaxation function, with 93% recovery after six days. In contrast, ALMI addition did not improve relaxation response to ACh compared to modified KH alone, and led to 70% of aortic recovery after six days in cold storage. Maximal SNP relaxation following 6-day cold storage with KH solution was only 63% compared to controls. Modified KH, with AL or with ALMI returned 100% of maximal SNP relaxation.

Conclusions: After 6-day cold storage in standard KH, vascular contractile and relaxation functions were considerably impaired and lowering Ca^{2+}/Mg^{2+} ratio (modified KH) was shown to improve vascular preservation of isolated rat aortic rings. The addition of AL in modified KH further increased protection, especially in response to NE. Melatonin and insulin added to AL conferred no additional protection.

6.1 INTRODUCTION

CABG or vascular injury sometimes requires preservation of vessels for revascularization or replacement of infected vessels for longer than a few hours (Bisdas*, et al.*, 2010; Fahner*, et al.*, 2006). The preserved vessels are often stored in cold medium for up to several days (Wille*, et al.*, 2008). However, current methods of cold storage protection on vessel function after 24-48 hours remains unsatisfactory (Zatschler*, et al.*, 2009). Cold storage with traditionally used preservation solutions, such as University of Wisconsin (UW), EuroCollins (EC), and histidine-tryptophan-ketoglutarate (HTK) solution, can result in dramatic losses of vascular contractility and relaxation after 72 hours of storage (Corner*, et al.*, 2003), and Krebs Henseleit solution alone results in 86% loss of endothelial-induced relaxation after 36 hour of cold storage (Ingemansson*, et al.*, 1995).

Adenosine and lidocaine (AL) combination solution has been studied in the isolated rat heart preservation model, and it was shown that AL addition in low Ca^{2+}/high Mg^{2+} Krebs Henseleit solution (modified KH), with or without melatonin and insulin, resulted in recovery of 78% cardiac output following 6-8 hours of static cold storage (Rudd & Dobson, 2011a, 2011b). This was significantly greater compared to traditional preservation solution, such as Celsior and HTK which could only recover 25% and 4% respectively (Rudd & Dobson, 2011b).

The aim of Chapter 6 was to investigate the preservation effects of AL in Krebs Henseleit (KH), modified KH, and modified KH, with and without antioxidants melatonin and insulin, on rat aorta physiological function after 6-day cold static storage.

6.2 EXPERIMENTAL DESIGN

6.2.1 Animal preparation:

Thoracic aortic vessels were harvested from 300-350 g Sprague Dawley rats as outlined in Chapter 2.2.1 and 2.4.2 and transferred to a container with pre-cooled KH solution. Thoracic aortic rings (n=40) were then assigned to one of the following groups:

1. Fresh preparation (controls)
2. Krebs Henseleit (KH)
3. Modified KH (low Ca^{2+}/high Mg^{2+})
4. Modified KH + adenosine + lidocaine (KH+AL)
5. Modified KH + adenosine + lidocaine + melatonin + insulin (KH+ALMI)

With the exception of controls that were immediately subjected to vessel function analysis after harvesting, other groups were kept in the assigned preservation solutions at 4°C for six days. After 6-day storage, the vessel rings were then transferred to an organ bath filled with KH solution, gently warmed to 37°C, and then subjected to vessel function analysis. The contraction and relaxation responses of preserved aortic rings were compared with those of controls.

6.2.2 Vessel physiological function testing

Vessel physiological function testing has been described in detail in Chapter 2. Briefly, at the end of cold storage, preserved rings were then mounted and stretched to an optimal resting tension in oxygenated KH solution at 37°C. Contraction responses to potassium chloride (KCl; 60 mM), norepinephrine (NE; 0.3 μM), and relaxation responses to acetylcholine (ACh; 10^{-6}-10^{-3} M) and sodium nitroprusside (SNP; 10^{-6}-10^{-3} M) were evaluated using an isometric force transducer (PANLAB, distributed by ADInstruments as MLT 0201/RAD, NSW, AUS) coupled to a computer-based data acquisition system (PowerLab, ADInstruments) and data recording software LabChart 7 (ADInstruments Pty Ltd., Castle Hill, Australia). At the end of each experiment, rings were maximally dilated with 100 μM papaverine to confirm viability of the vessels and the relaxation value would be used as a comparison to ACh and SNP-induced relaxation. ACh is believed to act on muscarinic receptors which are G-protein-coupled receptors to produce relaxation via endothelial-derived NO production (Chataigneau, *et al.*, 1999), whereas SNP is an NO donor that induces vascular smooth muscle

relaxation mainly through the activation of soluble guanylate cyclase (sGC), and an increase in cyclic GMP (cGMP), although cGMP-independent mechanisms have also been reported (Cogolludo*, et al.*, 2001)

6.2.3 Statistical analysis

Values are reported as mean ± SEM. NE and KCl-induced contraction were expressed as the mean of gram tension results from the number of animals in each group, while ACh and SNP-induced relaxation responses were expressed as percentage of maximal relaxation to 100 μM papaverine. All data was tested for normality using *Kolmogorov-Smirnov* test. Contraction responses to NE and KCl, and relaxation responses to ACh and SNP were analysed for homogeneity of variances followed by one-way ANOVA and coupled with Bonferroni post hoc test for individual data point comparisons.

6.3 RESULTS

6.3.1 Vasoconstrictive response to norepinephrine (NE) and potassium chloride (KCl) with different preservation solutions

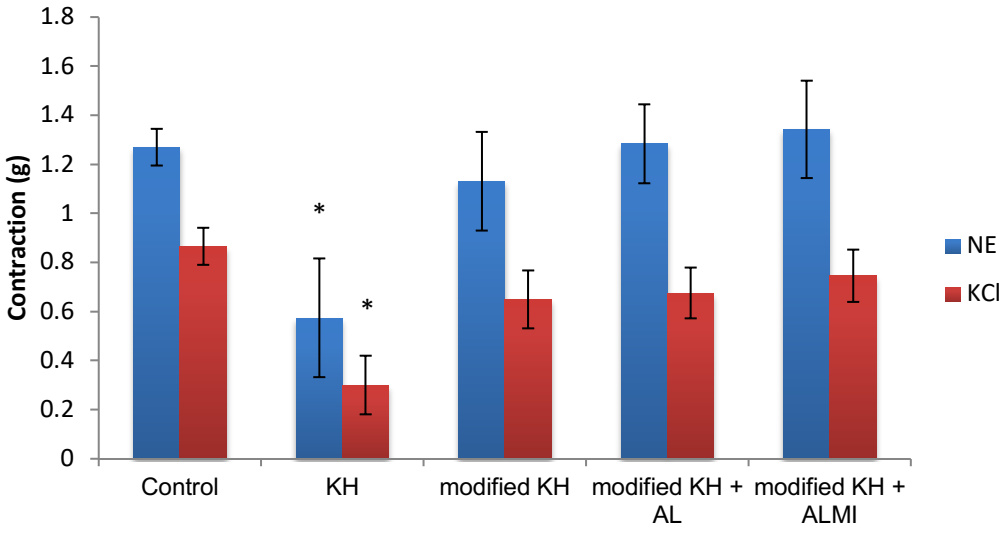

Figure 6.1. Isolated rat aortic ring contraction response to norepinephrine or KCl after 6-day storage compared to fresh preparation (control).
All values are expressed as mean ± S.E.M of aortic ring relaxation from 8 animals. *p<0.05 statistical difference between KH and control groups.

Contractile function of cold preserved rat aortic rings was tested in response to NE and KCl precontracted rings to indicate the receptor and non-receptor-stimulated constriction, respectively. In freshly harvested aortic rings NE and KCl (both controls) increased aortic ring tension to 1.27 ± 0.07 and 0.87 ± 0.08 g tension, respectively (Figure 6.1). Following 6-day storage (4°C) in KH solution, the aortic rings only produced 0.58 ± 0.24 and 0.30 ± 0.12 g tension in response to NE and KCl, respectively, indicating 46% and 34% recovery of their contractility responses. Low Ca^{2+}/high Mg^{2+} (modified KH) improved recovery of contractile function with 89% and 76% return with NE and KCl use, respectively. Meanwhile, AL storage in modified KH recovered 100% of NE contractile function, but no further recovery in the KCl response compared to modified KH alone (76% recovery). Similar results were found with ALMI in modified KH, which led to 100% and 86% of contractile function recovery in response to NE and KCl, respectively (Figure 6.1).

6.3.2 Vasorelaxation response to acetylcholine (ACh) and sodium nitroprusside (SNP) with different preservation solutions

Relaxation function of preserved aortic rings was tested in response to ACh and SNP to measure the endothelium-dependent and endothelium-independent relaxation function, respectively.

Freshly prepared aortic rings (controls) produced a relaxation in response to ACh in a concentration-dependent manner. Percentages of relaxation were 4.8 ± 1.36%, 10.3 ± 2.57%, 38.8 ± 13.58%, 62.2 ± 11.67%, 76.6 ± 8.24%, 79.2 ± 6.67%, and 68.0 ± 9.47% after the administration of ACh at concentration of 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}, and 10^{-3} M, respectively (Figure 6.2A). After 6-day storage in KH solution, aortic relaxation responses were reduced to 28.8 ± 12.61%, 33.5 ± 13.38%, 25.6 ± 11.79%, and 28.6 ± 11.18% at ACh concentration of 10^{-6} to 10^{-3} M but the reduction was only significant at concentrations 10^{-5} and 10^{-4} M. Compared to standard KH, modified KH (with low Ca^{2+} and high Mg^{2+}) improved relaxation to 58 ± 10.06%, 62 ± 10.32%, 47 ± 9.21%, and 37 ± 10.79% at ACh concentration of 10^{-6} to 10^{-3} M; however, this was not statistically significant. The addition of AL in modified KH further improved relaxation at ACh concentration of 10^{-6} to 10^{-5} M to 71 ± 3.78% and 75 ± 3.34%, respectively, and this was significantly different from standard KH (p<0.05). Interestingly, ALMI addition did not improve relaxation response compared to modified KH alone, and resulted in 51 ± 6.35%, 52 ± 7.42%, 45 ± 7.74% and 41 ± 8.20% relaxation at ACh concentration of 10^{-6}, 10^{-5}, 10^{-4} and 10^{-3} M (Figure 6.2A).

When comparing maximum relaxation from ACh (endothelial NO-induced) relaxation, a value of 81.5 ± 7.1% was found in fresh prepared aortic rings (controls) following precontraction with NE. After 6-day storage in standard KH, aortic rings only recovered 42 ± 13.68% of ACh relaxation function (Figure 6.2B), while modified KH storage led to 79 ± 9.97% relaxation recovery. AL addition in modified KH significantly improved relaxation function compared to standard KH, with 93 ± 3.59% recovery after six days. In contrast, ALMI addition in modified KH did not improve relaxation response to ACh compared to modified KH alone, and led to 70 ± 6.28% of aortic recovery after six days in cold storage (Figure 6.2B).

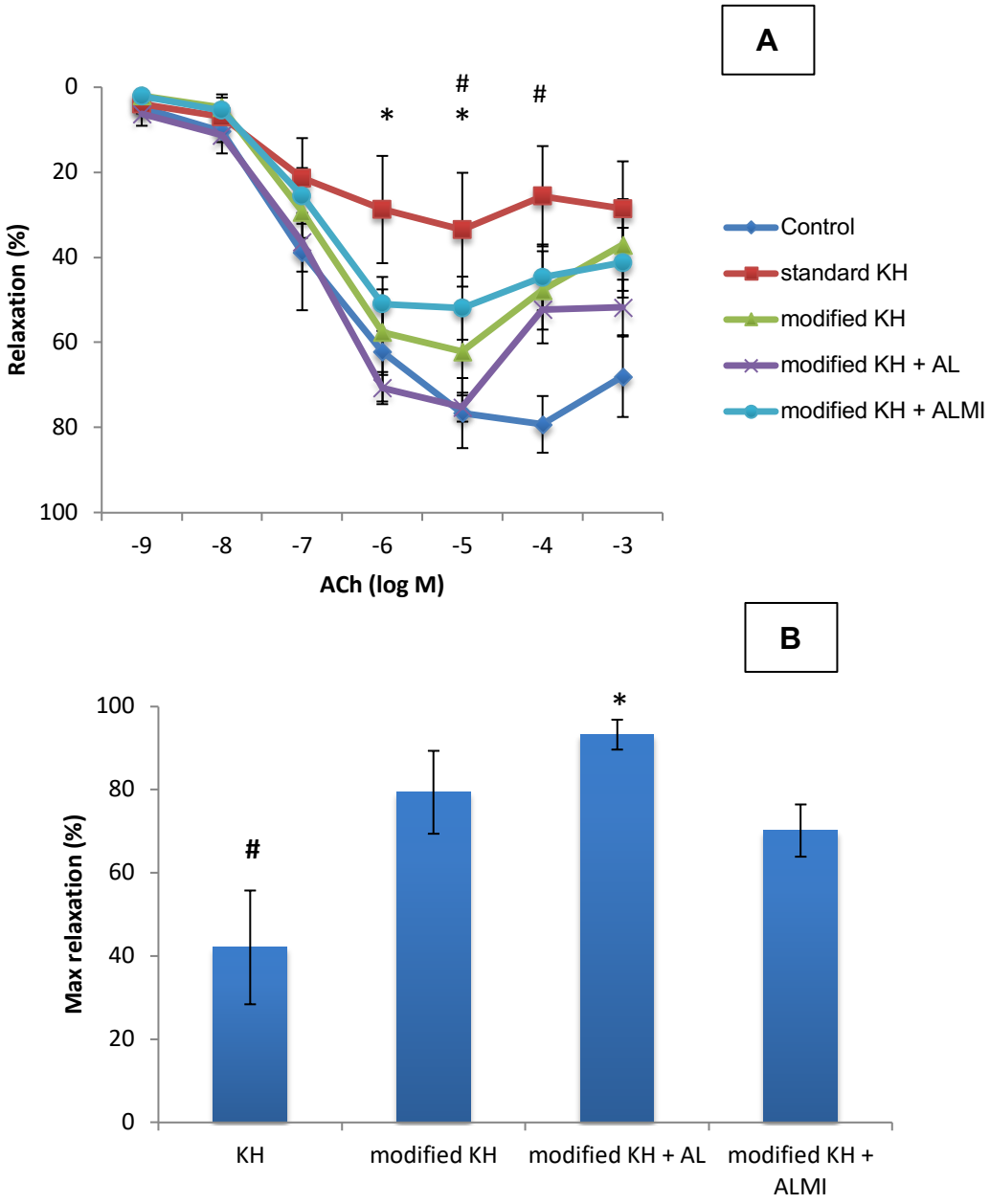

Figure 6.2. Isolated rat aortic ring relaxation response to acetylcholine (endothelial NO-induced relaxation) after 6 day storage compared to control.
(A) Concentration-response curve for ACh-induced relaxation in aortic rings. Relaxation is expressed as percent of relaxation to 100 μM papaverine. (B) ACh-induced maximal relaxation in KH solution group compare to the one in modified KH solution group. Relaxation is expressed as percent of control group maximal relaxation. Points represent mean ± S.E.M of aortic ring relaxation from 8 animals. *p<0.05 statistical difference between KH and modified KH + AL group. #p<0.05 statistical difference between KH and control group.

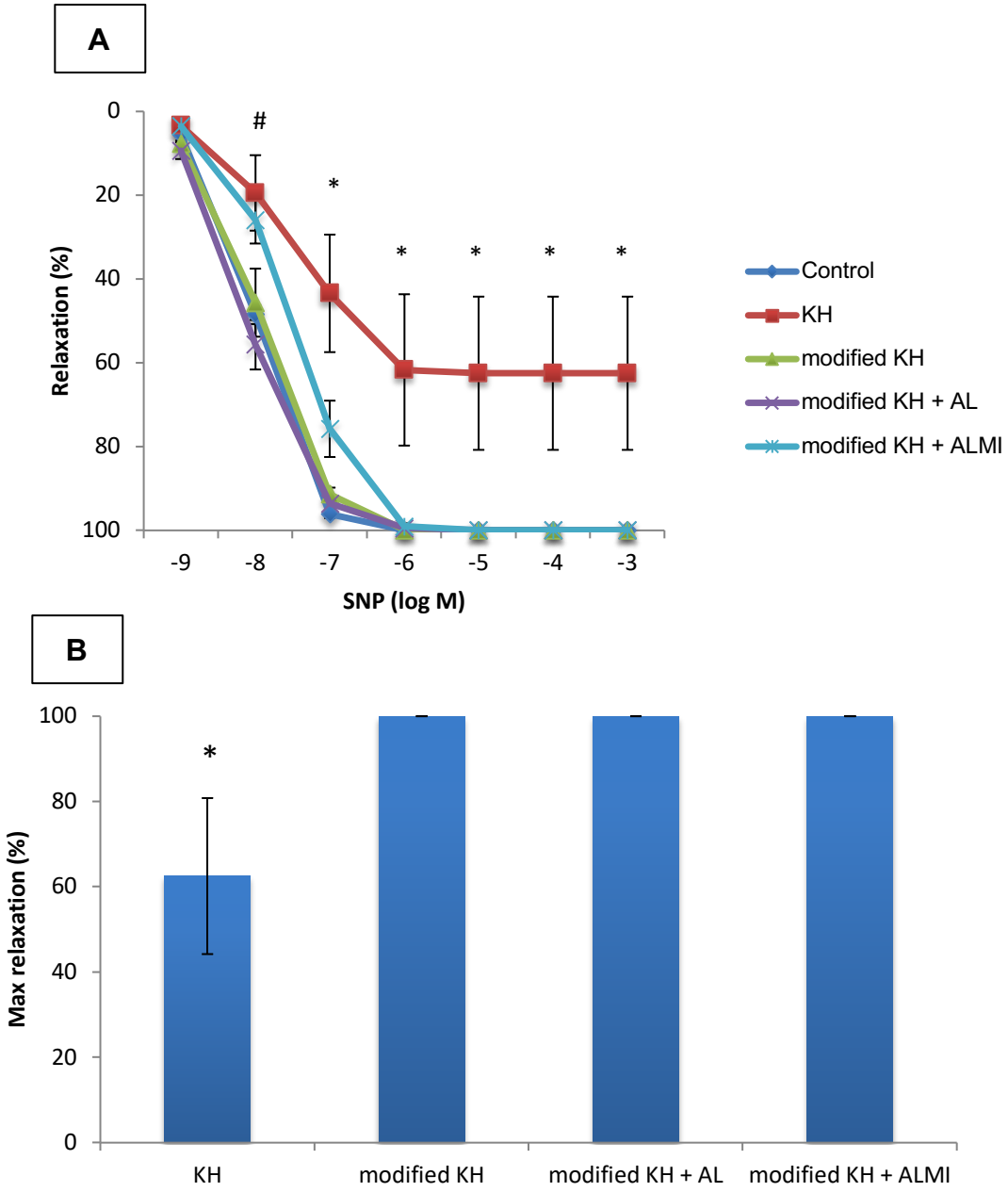

Figure 6.3. Concentration-response curve for SNP (smooth muscle NO-induced relaxation) after six day storage compared to control.
(A) Concentration-response curve for SNP-induced relaxation in aortic rings. Relaxation is expressed as percent of relaxation to 100 μM papaverine. (B) SNP-induced maximal relaxation in different groups compare to maximal relaxation of control group. Relaxation is expressed as percent of control group maximal relaxation. Points represent mean ± S.E.M of aortic ring relaxation from 8 animals. *p<0.05 statistical differences between KH and the other groups. # p<0.05 KH group statistically differ from control and modified KH + AL groups

In response to SNP, control aortic rings relaxed in a concentration-dependent manner from 5.6 ± 1.39% relaxation at SNP concentration of 10^{-9} M, 48.4 ± 2.32% at 10^{-8} M, 96.2 ± 0.90% at 10^{-7} M, and reached 100% at 10^{-6} -10^{-3} M (Figure 6.3A). After 6-day preservation in KH solution, aortic relaxation to SNP was significantly attenuated, with maximum relaxation of 63 ± 18.07% at concentration 10^{-6} to 10^{-3} M. In contrast, the use of modified KH with or without AL produced relaxation response to SNP similar to that of control at any concentration regardless of prolonged (6-day) storage. Interestingly, the addition of ALMI slightly reduced relaxation response compared to modified KH alone, but only at SNP concentration 10^{-8} to 10^{-7} M. All three modified KH solutions (with or without AL or ALMI) led to 100% relaxation starting at SNP concentration 10^{-6} M (Figure 6.3A). Again, Figure 6.3B shows that maximal SNP relaxation following 6-day cold storage with KH solution was only 63% compared to controls. Modified KH, with AL or with ALMI returned 100% of maximal SNP relaxation (Figure 6.3B).

6.4 DISCUSSION

Vessel graft preservation can alter the physiological and biochemical properties of the vascular wall which can affect vasoreactivity (Chow & Zhang, 2011). Thus, optimizing vascular response with the addition of certain physiological activities to preservation solutions is of particular clinical interest (Corner*, et al.*, 2003; Garbe*, et al.*, 2011; Wille*, et al.*, 2008). After 6-day cold storage in standard KH solution, rat aortic rings recovered 34-46% of contractile functions and 42-63% of relaxation responses. Modified KH with low Ca^{2+}/high Mg^{2+} content increased contraction and relaxation functions of preserved aortic rings by almost twofold. The addition of AL and ALMI led to 100% recovery of NE-induced contraction with modified KH, but KCl contraction was not improved. AL addition in modified KH also led to a 16% increase in endothelium-dependent relaxation (ACh sensitive). These results will now be discussed.

6.4.1 The effect of standard KH versus modified KH (low Ca^{2+} and high Mg^{2+} content) on aortic preservation

The results show that after six days of cold storage, aortic rings preserved in standard KH (Ca^{2+}=2.5 mM, Mg^{2+}=1.2mM) recovered 46% and 34% of their contractility in response to NE and KCl, respectively (Figure 6.1). NE-induced vasoconstriction is obtained through alpha-adrenergic receptor stimulation (Young*, et al.*, 1988), while KCl-induced vasoconstriction occurs via a receptor-independent Ca^{2+}-dependent response on vascular tissue (Amerini*, et al.*, 1995). Thus, the data suggests that

standard KH was insufficient to protect adrenergic-dependent and receptor independent-Ca^{2+} contractions of rat aorta, and resulted in greater than 54-66% loss of contractility after six days of cold storage. In addition to contraction loss, 6-day preservation with standard KH in the present study only led to 42% and 63% relaxation function in response to ACh and SNP, respectively (Figures 6.2B and 6.3B).

Earlier studies of Ingemansson*, et al.* (1995) showed that standard KH solution was the only solution that could preserve 90-100% contractility of cold stored rat aorta after 36 hours, compared to standard Euro-Collins, University of Wisconsin (UW), and Perfadex solutions. The contractile maintenance properties of KH were thought to be provided by its Ca^{2+} content (1.5 mM) (Ingemansson*, et al.*, 1995). Ingemanson et al. further showed that adding Ca^{2+} content (1.5 mM) in UW, Perfadex and Euro-Collins solutions improved the maintenance of contractile function of rat infrarenal aorta after 24-36 hours storage compared to the Ca^{2+}-free solutions (Ingemansson*, et al.*, 1997; Ingemansson*, et al.*, 1996). However, Ingemansson (1997; 1995; 1996) also reported the opposite in rat infrarenal aortic rings and showed that the recovery response was poor (22% recovery) following 36 hours storage in KH solution, and significantly worse compared to those stored in Ca^{2+}-free solution such as UW or Perfadex solution (Ingemansson*, et al.*, 1995). Whether the Ca^{2+} content in KH solution negatively impacts vessel endothelial function is controversial because it depends on the other actives present in the preservation solution, and what vessel is being studied. Baker and Neely and colleagues addressed this issue in the isolated rat heart and found that the optimum concentration of Ca^{2+} for perfusion or cold storage was 0.015 to 0.3 mM for buffered KH solution to reduce reperfusion injury from Ca^{2+} loading in the cells (Baker*, et al.*, 1991; Tani & Neely, 1990).

In the present study, lowering Ca^{2+} and raising Mg^{2+} (Ca^{2+}=0.22 mM, Mg^{2+}=2.6 mM) showed a significant improvement on recovery in rat aortic rings after cold storage with 76 - 89% recovery after six days (Figure 6.1). Furthermore, modified KH solution improved relaxation response to ACh and SNP; from 42% and 63% in standard KH, increased to 79% and 100% in modified KH. The concentration of Ca^{2+}/Mg^{2+} in this study was also found to be highly protective in a number of studied such as the isolated rat heart preservation in Rudd & Dobson studies (2011b), the isolated heart of Foreman and colleagues after prolonged cold storage (Foreman*, et al.*, 1985), and after cardioplegic arrest (Brown Jr*, et al.*, 1991; Fukuhiro*, et al.*, 2000; Robinson & Harwood, 1991). Fukuhiro*, et al.* (2000) further showed that lowering Ca^{2+} in addition

to high Mg^{2+} in a cardioplegic solution was beneficial to reduce intracellular Ca^{2+} overloading, leading to significantly greater cardiac recovery.

In summary, low Ca^{2+} and high Mg^{2+} modified KH solution used in the present study may have improved recovery after six days cold storage by: 1) reducing Ca^{2+} cell loading (Iseri & French, 1984), 2) improving endothelial function (Tiruppathi*, et al.*, 2002), and 3) maintaining vascular integrity (Tiruppathi*, et al.*, 2002). Further functional, histological and electrophysiological studies are required to test these possibilities.

6.4.2 The effect of AL or AL with melatonin and insulin (ALMI) in modified Krebs-Henseleit on preserved aortic rings

Adenosine and lidocaine (AL) solution was first developed as a normokalaemic cardioplegia for human cardiac surgery (Dobson & Jones, 2004) and this polarized arrest concept was modified by lowering Ca^{2+}, increasing Mg^{2+} and adding the antioxidants melatonin and insulin for cold static preservation (Rudd & Dobson, 2011a). This chapter also examined if AL or AL plus melatonin and insulin in modified KH conferred extra protection to vascular preservation in cold static storage.

AL in modified KH solution*:* In the present study, AL administration in modified KH solution was beneficial to slightly improve NE-induced contraction (from 89% to 100%), while the potassium-induced contraction remains unchanged (77% from baseline contraction) after 6-day cold storage (Figure 6.1). The vasorelaxation response to SNP was 100%, showing unaltered endothelium-independent vasodilation function (Figure 6.3.), and this response was similar to that of modified KH without AL. However, AL addition increased vascular endothelial-induced vasorelaxation from 79% to 93% (Figure 6.2) and significantly greater than that of standard KH, indicating a significant improvement of endothelial function with AL treatment (with low Ca^{2+} and high Mg^{2+} KH) despite the prolonged cold storage. This result may be clinically important since endothelial cells are more vulnerable to ischaemic damage compared to smooth muscle (Mankad*, et al.*, 1997). Previously, Hashimoto et al (1992) has shown that three days of cold anoxic storage obliterated endothelial function in porcine coronary arteries, while endothelial-independent vascular reactivity was less affected (Hashimoto*, et al.*, 1992). In addition, impaired integrity of human vascular endothelial cells was observed only after three hours of hypoxic and hypothermic storage (Hidalgo*,*

et al., 1996). Apart from hypoxia, exposure to cold storage may also induce endothelial injury through increased intracellular calcium and/or iron accumulation (Haddad*, et al.*, 1999; Rauen & de Groot, 2002). Parolari (2002) also listed a number of other acute and chronic mechanisms responsible for impaired vessel wall vasoreactivity and cold storage. These include mitochondrial dysfunction and increased reliance on anaerobic metabolism with acidosis, activation of free radical production, oxidant damage and apoptosis (Parolari*, et al.*, 2002). If prolonged, the hypoxic injury process becomes irreversible and consequent cellular death occurs (Stempien-Otero*, et al.*, 1999). The present thesis did not examine the mechanism of improved contractility and/or relaxation in AL in Modified KH. However, further studies may include a histological and electrophysiological examination and/or biomarker analysis to confirm the presence/absence of functional endothelial or vascular smooth muscle damage compared to rings preserved in modified KH alone.

6.5 CLINICAL SIGNIFICANCE

A functional endothelium is critical for preservation of grafts and long-term patency after surgery (Massa*, et al.*, 1994). To date, methods for vascular preservation have not been standardized (Brockbank & Taylor, 2006) and generally rely on surgeon and/or clinical experience, which focuses on hypothermic storage with multiple additives to reduce vascular graft injury (Brockbank & Taylor, 2006). However, hypothermia itself is known to damage the vascular endothelium (Rauen & de Groot, 2004). The design of any new hypothermic static storage solution needs to prevent the unwanted effects. Previously, AL has been demonstrated to maintain ionic balance near resting membrane voltage state during pharmacological arrest (Dobson & Jones, 2004), prevent acidosis (Rudd & Dobson, 2011a), attenuate free radicals (Shi*, et al.*, 2012), and maintain high-energy compounds during ischaemia (Canyon & Dobson, 2006). This chapter showed that AL in modified KH led to significant protection compared to modified KH alone and may be potentially useful for clinical translation of human vascular grafts for cardiac and other major vascular surgery.

6.6 LIMITATIONS AND FUTURE STUDIES

The major limitation of the present study was that the aortic rings used in the experiments are not normally used in clinical settings for grafts. It would be clinically important to investigate segments from human conduit arteries and saphenous veins used in cardiac surgery.

Future studies are require to further investigate AL preservation solution compared with other traditionally used preservation solutions, such as University of Wisconsin (UW), EuroCollins (EC), and histidine-tryptophan-ketoglutarate (HTK) solution. It would also be important to investigate histological changes of the vessels after the cold storage.

6.7 CONCLUSION

After 6-day cold storage in standard KH, vascular contractile and relaxation functions were considerably impaired and lowering Ca^{2+}/Mg^{2+} ratio (modified KH) was shown to improve vascular preservation of isolated rat aortic rings. The addition of AL in modified KH further increased protection, especially in response to NE. Melatonin and insulin added to AL conferred no additional protection.

CHAPTER 7
GENERAL DISCUSSION

7.1 Restatement of the Problems and General Aims

There are around one million coronary artery bypass graft (CABG) surgeries performed each year around the world (Mangiacapra*, et al.*, 2011). An ongoing problem with CABG surgery is the patency of venous and arterial conduits (Goldman, 2004). Although arterial grafts may provide superior long-term patency, they tend to spasm from their thicker muscular walls during harvesting, preparation, grafting and in the post-operative period (Rehman*, et al.*, 2013). Vasospasm can lead to post-operative myocardial infarction and mortality (Lanza*, et al.*, 2011; Wakabayashi*, et al.*, 2008). Vasodilators and anti-spasmodics are not standardized and may vary from surgeon to surgeon (Kitamura*, et al.*, 2011). Therefore, there is a need for improved pharmacological strategies to protect the vessel and prevent spasm and to achieve optimal patency of arterial and/or venous grafts during and post-surgery (Goldman*, et al.*, 2004b; Taggart, 2013; Wallitt*, et al.*, 2007a).

This thesis examined the effect of adenosine and lidocaine (AL) solution to relax and protect arterial conduits in rat and guinea pig models, and possible storage. AL was first developed as a non-depolarising cardioplegia (Dobson, 2010; Dobson*, et al.*, 2013a), and is currently used in adult and paediatric surgery (Jin*, et al.*, 2008; Onorati*, et al.*, 2016a; Onorati*, et al.*, 2013b). AL has been found to possess anti-inflammatory (Shi*, et al.*, 2012), anti ischaemic effects, and lowers energy demand (Canyon & Dobson, 2004, 2006). In addition, animal studies have observed improved coronary flow with AL cardioplegia (Sloots & Dobson, 2010b). In an attempt to begin to translate AL protection into cardiac surgery to anti-spasmodic and vessel preservation, the aims of this thesis were three-fold:

1. To investigate the vasodilatory effects and possible mechanisms of adenosine (A), lidocaine (L) and adenosine-lidocaine (AL) relaxation on isolated rat thoracic aortic rings.
2. To investigate the vasodilatory effects of A, L, and AL on segments of guinea pig second order mesenteric artery, with the future goal to reduce secondary complications from gut ischaemia during cardiac surgery.
3. To investigate the effect of AL and AL solution with antioxidants on 6-day cold storage using rat thoracic aortic rings for future vessel banking.

7.2 Major Findings

The main findings of this thesis were:

1) In Chapter 3, adenosine was shown to induce concentration-dependent relaxation in NE-precontracted isolated rat aortic rings at 1-1000 μM. The maximum relaxation was >80% in endothelium-intact rat aortic rings at 1000 μM. Endothelium removal did not abolish adenosine relaxation but significantly attenuated it (p<0.05). The study further showed that adenosine-induced relaxation in intact rings was mediated by NO, prostacyclin, potassium channels (K_v, sarcK_{ATP} and mitoK_{ATP}), and adenosine A_{2a} receptors were involved. However, in endothelium-denuded rings, adenosine relaxation involved potassium channels (K_v, sarcK_{ATP} and mitoK_{ATP}), and adenosine A_{2a} receptors. In Chapter 4, lidocaine also elicited a concentration-dependent relaxation in isolated rat thoracic aorta with a maximum relaxation of 42% in endothelium-intact aortic rings (p<0.05), but less potent than adenosine. Interestingly, endothelium denudation significantly increased lidocaine relaxation at concentrations of 5-100 μM, although, the maximum relaxation was not significantly altered (50% at 1000 μM). Another interesting result from this study was that lidocaine relaxation in endothelium-intact aortic rings involved adenosine A_{2a} receptor activation, implying crosstalk between adenosine receptor activation and lidocaine relaxation. In denuded rings, it was shown that increased lidocaine relaxation involved K_v channels, mitoK_{ATP} channels, and adenosine A_{2a} receptors activation. In Chapter 5, the AL vasodilatory effect was compared to adenosine and lidocaine alone in rat aortic rings. It is found that AL and adenosine alone relaxed intact isolated rat aortic rings up to ~100% (p<0.05), compared to 65% relaxation with lidocaine. A most interesting finding was that the relaxation induced by AL was different to that of adenosine since it was not significantly reduced by endothelium removal. However, AL relaxation was distinguished from that of lidocaine alone (p<0.05), since AL relaxation is much more potent. In endothelium-denuded aortic rings, AL maximum relaxation was 100%, compared to 90% with adenosine (p<0.05) and 65% with lidocaine (p<0.05).
2) In Chapter 5, the vasodilatory effects of adenosine, lidocaine and AL were investigated in mesenteric artery of guinea pigs during intraluminal and abluminal administration. Intraluminal administration of adenosine and AL concentration 10^{-6}-10^{-3} M produced a marked vasodilation (>80%) on AVP-precontracted mesenteric arteries (p<0.05). Adenosine and AL relaxation was not attenuated with endothelium removal (Fig. 5.3 AB). Interestingly, luminal lidocaine produced

no relaxation in endothelium intact but 40% relaxation in endothelium denuded mesenteric arteries (Fig. 5.3AB), indicating lidocaine relaxation was endothelial-dependent (i.e. removing endothelium increases relaxation). The maximal relaxation with lidocaine after endothelium removal was 42% (p<0.05). In the second part of the study, it was found that abluminal application of adenosine and AL produced ~90% relaxation (p<0.05), indicating that topical/intraperitoneal administration has a potent effect. Meanwhile, abluminal application of lidocaine led to no significant relaxation.

3) In Chapter 6, the preservation effects of AL and AL with antioxidant melatonin and insulin (ALMI) in modified KH solution (low Ca^{2+}/high Mg^{2+}) were investigated in rat aortic rings after 6-day cold storage. This preservation cocktail was chosen after the isolated heart preservation studies of Rudd and Dobson (2011b). After six days of cold preservation, modified KH solution alone significantly improved return of contractility by a factor of two in both NE and KCl contracted aortic rings compared to standard KH solution. Cold AL and ALMI in modified KH solution fully recovered NE contractile function, but little or no further recovery was found in the KCl response over modified KH. Recovery of endothelium-dependent relaxation (ACh response) was 42% with cold standard KH alone. This study found that recovery was significantly improved with low Ca^{2+}/high Mg^{2+} modified KH (80%), with AL (93%) and with ALMI (70% recovery). Meanwhile, smooth muscle dependent relaxation (SNP response) was 60% recovered with cold standard KH, while 100% recovery of SNP relaxation was obtained with Ca^{2+}/high Mg^{2+} modified KH alone, with AL or with ALMI.

The results of each chapter will now be discussed in detail.

7.3 Adenosine relaxation on rat aortic rings and possible mechanisms

Adenosine has become one of the most extensively studied vasodilators in vascular biology, including in isolated aorta (Headrick & Berne, 1990; Prentice*, et al.*, 2001; Prentice & Hourani, 2000; Ray & Marshall, 2006b; Rose'Meyer & Hope, 1990). However, many aspects of the mechanisms by which adenosine induces relaxation are still controversial, including the role of endothelium (Headrick & Berne, 1990; Lewis*, et al.*, 1994b; Newman*, et al.*, 1988; Rose'Meyer & Hope, 1990), K^+ channels (Grbović & Radenković, 2003; Ray & Marshall, 2006b), and adenosine receptor subtypes (Kemp & Cocks, 1999; Ponnoth*, et al.*, 2009).

The present study supports the concept that adenosine relaxation in rat aorta is mediated predominately by A_{2a} and not A_{2b} receptors, since the A_{2a} receptor blocker caused a significant (up to 50%) reduction in relaxation in endothelium-intact aortic rings (Fig. 3.4A). In addition, blocking A_{2a} receptor in denuded rings led to a more pronounced (up to 79%) reduction in adenosine relaxation, suggesting a greater role of A_{2a} receptor located in smooth muscle of isolated rat aorta (Fig. 3.4B). However, it must be noted that blocking the A_{2a} receptors did not completely abolish adenosine relaxation, indicating other receptors/pathways, such as A_1 or A_3 subtypes may be involved in the isolated rat aorta. Notwithstanding these contributions, the presence of A_{2a} receptors in both endothelial cells of rat aorta (Lewis*, et al.*, 1994b) and vascular smooth muscle (McPherson*, et al.*, 2001) attest to the importance of this receptor subtype in adenosine relaxation.

A_{2a} receptor vasodilation involves endothelial NO production, which activates smooth muscle guanylyl cyclase via opening Kir channels (Hein*, et al.*, 2013). Another mechanism believed to be central in controlling relaxation is A_{2a} activation of K^+ channel opening, which inhibits Ca^{2+} influx through L-type Ca^{2+} channels, inactivates IP_3 receptor in sarcoplasmic reticulum, and inhibits myosin light chain kinase to induce smooth muscle relaxation (Taylor*, et al.*, 1999). MitoK_{ATP} channel activation is also thought to be involved and may also relax vascular smooth muscle cells via the generation of reactive oxygen species release (Krenz*, et al.*, 2002), which subsequently stimulate K_{Ca} activation leading to hyperpolarization of smooth muscle cells (Xi*, et al.*, 2005).

Further examination with specific K^+ channel inhibitors showed that in addition to L-NAME and indomethacin, adenosine relaxation in endothelium intact aorta was significantly inhibited by 4-AP (K_v channel blocker) with ~20% reduction, glibenclamide (sarcK_{ATP}) with ~10% reduction, and 5-HD (mitoK_{ATP}) with ~20% reduction (Fig. 3.3). These specific inhibitors also significantly attenuated adenosine relaxation in endothelium denuded rat aortic rings (Fig.3.5). This indicates that activation of K_v, sarcK_{ATP} and mitoK_{ATP} channels plays an important role in endothelium dependent and independent relaxation of adenosine. The role of K_v and sarcK_{ATP} in adenosine-induced relaxation has been implicated in earlier studies in swine coronary arteries (Heaps & Bowles, 2002; Hein & Kuo, 1999), perfused mouse cremaster arterioles (Maimon*, et al.*, 2014) and isolated rat aorta (Ray & Marshall, 2006b). The new finding in this present study is that adenosine relaxation was also mediated by mitoK_{ATP} channels in both endothelium intact and denuded rat aorta. Although the underlying

mechanisms remain elusive, there is an implication that mitoK_{ATP} channel activation relaxes vascular smooth muscle cells through reactive oxygen species release (Krenz*, et al.*, 2002), which subsequently stimulate K_{Ca} activation leading to hyperpolarization of smooth muscle cells (Xi*, et al.*, 2005).

The results of 4-AP studies indicated that, in addition to the opening of SarcK_{ATP} and mitoK_{ATP} channels, adenosine relaxation in denuded rat aortic rings also appeared to involve K_v channels (Fig.3.5). K_v channels have been implicated in relaxation in earlier studies in swine coronary arteries (Heaps & Bowles, 2002; Hein & Kuo, 1999), perfused mouse cremaster arterioles (Maimon*, et al.*, 2014) and rat aorta (Ray & Marshall, 2006b). However, no study has reported that adenosine relaxation in *intact* rat aorta was also mediated by K_v, SarcK_{ATP} and mitoK_{ATP} channels (Figs 3.3), as well as in denuded rings (Fig. 3.5), which indicates adenosine may be useful if applied intraluminally or topically to protect and prevent an artery from spasm. Although the underlying mechanisms for smooth muscle and endothelium crosstalk remain elusive, relaxation appears to involve a complex interplay between endothelial NO dependent pathway(s) (not indomethacin-dependent) and smooth muscle A_{2a} subtype, voltage-dependent K_v , SarcK_{ATP} and MitoK_{ATP} channels. Further work needs to examine this crosstalk and linkages with K_{IR} and K_{Ca} channels in both endothelium-intact and denuded rings.

7.4 Lidocaine relaxation on rat aortic rings

In the literature, lidocaine displays unusual properties including relaxation or constriction depending upon its concentration (Gherardini*, et al.*, 1995; Johns*, et al.*, 1985; Perlmutter*, et al.*, 1990a) and whether the vessel is pre-constricted or dilated (Turan*, et al.*, 2000). In Chapter 4, lidocaine was found to elicit a biphasic concentration dependent relaxation in NE-preconstricted rat aortic rings, and that this relaxation was significantly augmented with the mechanical removal of the endothelium at lower concentrations of lidocaine (5–100 μM) (Fig. 4.2). The effect of denudation to increase relaxation suggests that relaxation was largely mediated by vascular smooth muscle activation, and that the intact endothelium appears to strongly inhibit this response. The factor responsible for keeping a brake on lidocaine vasoreactivity in the intact ring is unknown.

In this thesis it was found that lidocaine relaxation does not appear to be NO- or

prostacyclin-dependent, as L-NAME and indomethacin had little or no effect on intact ring relaxation (Fig. 4.3). However, in denuded rings, relaxation was found to be completely abolished by K_v channel inhibition (p<0.05) and significantly reduced by antagonists of the MitoK_{ATP} channel, and to a lesser extent the SarcK_{ATP} channel. Lidocaine direct action on smooth muscle is generally believed to be mediated by its blockage on Ca^{2+} influx through L-type Ca^{2+} channels, resulting in smooth muscle relaxation (del Pozo*, et al.*, 1997; Shan*, et al.*, 2004; Tanaka*, et al.*, 2002). However, based on this present result, the Ca^{2+} influx may be indirectly inhibited by smooth muscle K^+ channel activation, most likely through K_v channels and mitoK_{ATP} to a lesser extent. Currently, most reports have implicated K_{ATP} channel modulation in lidocaine-induced vasoaction (Kimoto*, et al.*, 2005; Kinoshita*, et al.*, 2001a; Kinoshita*, et al.*, 1999; Kinoshita*, et al.*, 2003a). This present study revealed that only 5-HD sensitive (mitoK_{ATP}) but not glibenclamide-sensitive SarcK_{ATP} channel activation had a contribution in lidocaine relaxation (Fig. 4.5). The mechanism of K_v and mitoK_{ATP} channel activation mediating lidocaine-induced vasorelaxation warrants further investigation.

In addition, the present study found that A_{2a} subtype receptor antagonism significantly inhibited lidocaine relaxation above 100 μM (p<0.05) (Fig. 4.6A), but not the A_{2b} receptor (Fig. 4.6B). While this implicates A_{2a} subtype receptor in lidocaine relaxation, other factors must be involved because the blocking A_{2a}'s effect was more pronounced in the higher range of lidocaine concentrations (Fig. 4.6A). Nevertheless, this is a new finding, and it is possible that the relaxing factor is linked to multiple receptors and channels (e.g. K_v and mitoK_{ATP}), including a role for the A_{2a} receptor subtype. It is also interesting that lidocaine (or its analogue) has been reportedly linked to G-protein coupled receptor (GPCR) activation (Hollmann*, et al.*, 2004a; Nietgen*, et al.*, 1998), and Xiong and colleagues in 1999 found that lidocaine binding to GPCRs modulates G-protein mediated K^+ and Ca^{2+} currents in rat anterior pituitary cells (Hollmann*, et al.*, 2001a; Xiong*, et al.*, 1999), which may have relevance to the present study. Benkwitz and colleagues also showed that higher concentrations of lidocaine (1000 uM) in hamster oocytes potentiated Gαi-coupled A_1 receptor signaling by reducing cyclic AMP production through an unidentified mechanism (Benkwitz*, et al.*, 2003). They proposed that lidocaine interacted with a pool of already activated Gαi present in the cytoplasm, and thereby facilitated its ability to inhibit adenylate cyclase leading to lower cAMP (Benkwitz*, et al.*, 2003). It would have been interesting to study A_1 antagonism in the present rat aortic model. In summary, the A_{2a} receptor subtype may have enhanced lidocaine relaxation activation by directly affecting vascular smooth muscle, and this

may have occurred by reducing intracellular Ca^{2+} and/or myofibrillar contractile sensitization in intact isolated rat aortic rings, although the underlying mechanisms remain to be identified.

7.5 AL relaxation was endothelium-independent, and possible significance to cardiac surgery.

In Chapter 5, one of the striking findings was that AL produced ~100% relaxation (p<0.05) of intact rat aortic rings at 1000 μM (Figs. 5.1B and 5.2A) compared to ~100% for adenosine (Figs. 5.1C and 5.2A) and 66% relaxation from lidocaine (Figs. 5.1D and 5.2A). This potent relaxation with AL was maintained after the endothelium was physically removed while adenosine relaxation was significantly attenuated (Fig.5.1). In endothelium-denuded aortic rings, adenosine maximal relaxation was 90% (Figs. 5.1C and 5.2B) while AL relaxation was 100% (p<0.05) (Figs. 5.1B and 5.2B). Lidocaine relaxation remained at around 66% in denuded aortic rings at maximum concentration (1000 μM) and was significantly less than A or AL (Figs. 5.1D and 5.2B).

The finding that AL relaxation in rat aortic rings was endothelium-independent has possible clinical translation potential to protect arterial vessels and conduits from spasm in cardiac surgery. Endothelial damage and spasm are known to occur during harvest, pressure testing, storage and implantation, which can affect the clinical immediate and post-operative outcome such as ischaemia-reperfusion injury, arrhythmias and infarction (Goldman*, et al.*, 2004b; Magee*, et al.*, 2008a; Wallitt*, et al.*, 2007a) (Harskamp*, et al.*, 2008a). As mentioned in the introduction there is no standardized strategy to prevent perioperative graft spasm (He*, et al.*, 2008; Yildiz*, et al.*, 2013).

However, based on adenosine's ability to relax aortic rings makes it another candidate for reducing spasm in the clinical setting given that there was around 90% relaxation in denuded rings at its highest concentration (Figs. 5.1D and 5.2B). There are however reasons why AL may be preferred in the clinical setting given that the combination has been shown to protect the human heart as a cardioplegia (Dobson*, et al.*, 2013a; Jin*, et al.*, 2008; Onorati*, et al.*, 2013b), as well as AL's ability to reduce arrhythmias and ischaemia-reperfusion injury (Canyon & Dobson, 2004; Djabir & Dobson, 2013; Letson & Dobson, 2011a), AL's ability to reduce inflammation (Dobson & Letson, 2016b; Shi*,*

et al., 2012), AL's ability to protect the endothelium (Letson & Dobson, 2017b, 2018), and ALM's ability to lower energy demand (Dobson & Letson, 2016b). The mechanism for AL to reduce injury after trauma is not known but believed to involve improved central-cardiovascular and endothelial coupling (Dobson & Letson, 2016b), and the differential activation of tissue-specific master-genes of metabolism and mitochondrial health that control biological time (Dobson and Letson, unpublished data). Adenosine alone or lidocaine alone does not have these protective properties (Dobson & Letson, 2016b).

The mechanism of how AL can elicit a potent relaxation regardless of damaged endothelium is unknown. One can only speculate from the data in this thesis that AL relaxation appears to involve interactions between the adenosine A_{2a} subtype and modulation of K_v and mitoK_{ATP} channels, although the mechanistic differences from adenosine alone or lidocaine alone are difficult to separate at this time (Chapter 3 and Chapter 4, respectively). However, the involvement of endothelium and EDRF (NO/PGI_2) were only implicated in adenosine relaxation but not lidocaine relaxation, which raises the question if AL relaxation was also mediated partly by EDRF in intact rings, although not a dominant pathway because no loss of relaxation was found with AL (or A to a lesser extent) after the endothelium was physically removed. Future studies are required to elucidate AL relaxation in the presence of specific blockers, including EDRF, adenosine receptors subtypes (A_1, A_{2a} and A_3) and K^+ channel inhibitors.

7.6 Adenosine and AL elicited an endothelium-independent relaxation in isolated guinea pig mesenteric artery, whereas lidocaine produced endothelium-dependent vasoconstriction

Similar findings were found when AL, adenosine and lidocaine were examined in isolated mesenteric conduits of the guinea pig. It was found that increasing luminal AL produced a significant and potent endothelium-independent dilation (up to 90%) (Figs. 5.3A, and 5.3B). Similarly, adenosine dilation was endothelium-independent but not lidocaine, which produced 33% dilation only after endothelial removal (Figs. 5.3A and 5.3B). Extra-luminal AL and adenosine led to 76% and 80% dilation in intact segments respectively, whereas lidocaine resulted in constriction (10-17%) (Fig. 5.3C).

Although endothelium-dependent relaxation of adenosine has been reported in

mesenteric arteries (Hiley*, et al.*, 1995; Tabrizchi & Lupichuk, 1995; Vuorinen*, et al.*, 1992), the majority of studies have failed to confirm the importance of an intact endothelium, including in rats (Prentice*, et al.*, 1997; Radenkovic, 2005) and in rabbits (Mathieson & Burnstock, 1985). This is consistent with the finding that EDRF (NO and PGI_2) was not found to play a role in adenosine relaxation in mesentery beds (Hiley*, et al.*, 1995; Hogan*, et al.*, 1998; Radenkovic, 2005). Thus, it is generally agreed that adenosine relaxation in guinea pig mesenteric arteries largely depends on non-endothelial process. This is different from adenosine relaxation in rat aortic rings (Chapter 3), which showed a partial dependency of adenosine relaxation on functional endothelium (Fig. 3.1), and a role of EDRFs (NO and PGI_2) in adenosine vascular relaxation. The difference in endothelium dependence between aortic and mesenteric relaxation may derive from: 1) different functions of the vascular beds; i.e. conductance versus resistance vessels (Lüscher*, et al.*, 1992), 2) different distribution of adenosine receptor subtypes (Shryock & Belardinelli, 1997), and 3) different cellular pathways, such as hyperpolarizing factors and specific ion channels (Lüscher*, et al.*, 1992; Zygmunt*, et al.*, 1995)

Again these findings may have clinical relevance given the potential problem of acute gut hypoperfusion ischaemia, ileus, and potential infection during major surgery such as CABG surgery (Gabe, 2001). The mesenteric arterial system supplies the intestine from the lower part of the duodenum through two-thirds of the transverse colon and pancreas (Asfar*, et al.*, 2003; Jacobson, 1982). Although the incidence of mesenteric ischaemia is rare (<3%), the mortality rate ranges from 46-100% (Nilsson*, et al.*, 2013; Pang*, et al.*, 2012). Currently, the standard vasodilator used to prevent gut ischaemia is papaverine (Kahn, 2011; Oshikata*, et al.*, 2013). However, the use of papaverine has been associated with side effects, one of the most common is cardiac tachyarrhythmias (Kahn, 2011). It should be noted that lidocaine infusion is known to significantly reduce ileus and hospital stay in a number of major surgeries (McCarthy*, et al.*, 2010). Future studies are required to investigate cardiac effects of adenosine and AL as potential vasodilators in *in vivo* models of non-occlusive mesenteric ischemia. For example, AL could protect arterial vessels and conduits in cardiac surgery, and possibly as an infusion during surgery to protect the gut from ilieus or ischaemic episodes. Recently, it was shown that AL(M) infusion led to increased blood flow to the gut in the rat model of haemorrhagic shock *in vivo* compared to controls (Letson & Dobson, 2017b), which would be wholly consistent with the vasodilatory properties of AL found in isolated mesentery segments in this thesis.

7.7 Development of a low Ca^{2+}/high Mg^{2+} AL addition for graft preservation

Since there is no consensus on a graft storage solution for CABG and other types of vascular graft surgery, it was decided in Chapter 6 to examine if AL and AL plus antioxidants insulin and melatonin could provide protection in a low Ca^{2+}/high Mg^{2+} (Ca^{2+}=0.22 mM, Mg^{2+}=2.6 mM) Krebs Henseleit (KH) modified solution. One of the most commonly used physiological solutions is Krebs Henseleit, which partially mimics ionic contents of mammalian blood (Bailey & Ong, 1978; Lockwood, 1961). AL alone in modified KH produced the best recovery (ACh response) after six days storage, with no additional advantage of having melatonin and insulin present (Fig. 6.2). Interestingly, recovery of smooth muscle-dependent relaxation (assessed by SNP response) was 60% in cold standard KH compared to 100% recovery in Ca^{2+}/high Mg^{2+} modified KH alone, with AL or with ALMI (all $p<0.05$), indicating low Ca^{2+}/high Mg^{2+} was a key factor in protecting smooth muscle contraction and relaxation after six days storage at 4°C (Fig. 6.3). Further functional, histological, biochemical and electrophysiological work is required in this area, but it appears that a graft harvest and storage solution with AL in a low Ca^{2+}/high Mg^{2+} may have translational potential for CABG and possibly neurosurgery.

7.8 Possible limitations and future studies

The primary objective of this thesis was to develop a novel pharmacological vasodilator combination for cardiac surgery, and to understand the possible mechanisms of relaxation or dilation in the isolated rat aorta, and mesenteric segments. Originally the aim was to examine adenosine, lidocaine and AL on fresh discarded human left internal mammary artery (LIMA) or radial artery segments from cardiac surgeons but this failed to occur due to lack of conduit availability from the cardiovascular department. The study therefore focused on rat aortic rings. The rat aorta was chosen because it was an established model to investigate the vasoactivity of drugs (Grbović & Radenković, 2003; Prentice & Hourani, 2000; Tawfik*, et al.*, 2005). Although studies of this type may not translate to humans (Rosenfeldt*, et al.*, 1999), the rat aortic model is relevant because it mimics many of the functional and morphological endothelial changes that occur in human grafting during excision, pressure testing, storage, implantation and after anastomoses (Andriambeloson*, et al.*, 2001). However, it must be appreciated in the translation process that the thoracic aorta is a large elastic

conduit artery, which has different functional properties to smaller muscular arteries and arterioles (Dobson*, et al.*, 2017).

Despite these potential limitations, future studies based on the data in the present thesis may progress be to the clinical scenario and investigate the AL solution on vessel protection and spasm in cardiac surgery. This may include: 1) testing topical applications of the AL solution to the dissected LIMA *in situ* prior to grafting, a muscular vessel particularly prone to spasm during cardiac surgery, 2) infusing the AL solution through arterial conduits, and possibly venous conduits, prior to pressure testing, and storage, and 3) investigating AL in the storage graft solution to protect endothelium-smooth muscle integrity prior to performing the bypass operation.

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