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A Novel Cardioprotective Therapy:

Adenosine and Lidocaine Solution
in an *In Vivo* Rat Model of Acute
Myocardial Ischemia-Reperfusion

Thesis submitted by Sarah J. Canyon BSc (Hons) JCU
in October 2003

For the degree of Doctor of Philosophy
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Abstract

Background: Recently, our laboratory demonstrated that an adenosine and lidocaine (AL) non-depolarizing cardioplegic arrest solution conferred superior protection during arrest and recovery compared with the hyperkalemic depolarizing St. Thomas' Hospital cardioplegic solution in isolated rat hearts. The aim of this thesis was to extend those findings by applying an adenosine and lidocaine (AL) solution at nonarresting concentrations before and during ischemia in an *in vivo* rat model of acute myocardial ischemia. No study has investigated the effect of AL combination treatment to reduce ischemic injury. Yet, previous studies in the 1990s have used the sequential and separate administration of lidocaine (2 mg/kg i.v.) and adenosine (150 μg/kg/ml/min i.c) as reperfusion therapy with conflicting results.

Methods: In all four studies, ischemia-reperfusion was achieved by placing a reversible tie around the left coronary artery of anaesthetized (sodium pentobarbital, 60 mg/ml/kg i.p.) and ventilated male Sprague-Dawley rats (300 - 400 g). The ischemic period lasted 30 min while reperfusion times were maintained for either 40 min or 120 min. Where applicable, a lead II electrocardiogram, heart rate, and systolic and diastolic pressures were recorded and mean arterial pressure and rate-pressure product calculated. The primary end points included infarct size, episodes and durations of ventricular arrhythmias, pH and changes in the concentration of ATP ([ATP]) and phosphocreatine ([PCr]) during ischemia-reperfusion recorded every 5 min. The level of statistical significance was $P < 0.05$.

Experimental design: The first two studies examined the cardioprotective potential of adenosine and lidocaine using the following treatment strategies: i) three AL solutions with varying concentrations of adenosine (A: 152, 305 and 457 μg/kg/min

plus L: 608 μg/kg/min i.v., n = 18) compared to saline controls (0.9% saline, n = 12), adenosine only (adeno-only, 305 μg/kg/min i.v., n = 8) and lidocaine only (lido-only, 608 μg/kg/min i.v., n = 8); all of these treatments were given 5 before ischemia and continued throughout 30 min ischemia but not reperfusion; and ii) the separate and sequential administration of adenosine (150 μg/kg/min i.v.) and lidocaine (2 mg/kg i.v) during reperfusion (n = 7); the sequential administration of AL solution (A: 305 μg/kg/min plus L: 608 μg/kg/min i.v.) of 5 min pretreatment and again 5 min before and during 30 min reperfusion, n = 6 and a 5 min pretreatment of AL solution (A: 305 μg/kg/min plus L: 608 μg/kg/min i.v.) continued throughout ischemia and 30 min of reperfusion (n = 6). In the third study, ^{31}P nuclear magnetic resonance was used to investigate the changes in [ATP], [PCr] and pH during ischemia (30 min) and reperfusion (40 min) with AL solution treatment (n = 6) or in controls (n = 7). In the fourth study, AL solution (A: 305 μg/kg/min plus L: 608 μg/kg/min i.v.) was compared to that of ischemic preconditioning (three 3 min cycles of ischemia-reperfusion) (IPC) (n = 6), the adenosine receptor A_1 agonist, 2-chloro-N6-cyclopentyladenosine (CCPA) (5 μg/kg i.v.) plus lidocaine (n = 6), and CCPA alone (n = 7).

Results: Seven of the 12 saline-control rats and 4 of the 8 Adeno-only treated rats died during the ischemic period from an episode of ventricular fibrillation. No deaths occurred in the Lido-only treated rats (n = 6) or in any group where AL solution was infused. Ventricular tachycardia (VT) occurred in 100% of saline controls (18 $\pm$ 9 episodes), 50% of the adeno-only group (11 $\pm$ 7 episodes), and 83% of lido-only treatment (23 $\pm$ 11 episodes). VT was also experienced in 60% of low-dose AL treated rats (2 $\pm$ 1 episodes) (P < 0.05), 57% of mid-dose AL (2 $\pm$ 1 episodes) (P < 0.05), and 67% of high-dose AL treated rats (6 $\pm$ 3 episodes). Ventricular fibrillation

(VF) occurred in 75% of saline controls (4 ± 3 episodes), 100% of adeno-only (3 ± 2 episodes), and in 33% lido-only treated rats (2 ± 1 episodes). Low-dose AL and mid-dose AL completely prevented VF from occurring during ischemia. The mean infarct size of mid dose-AL (38 ± 6%) was significantly reduced from saline controls (61 ± 5%), adeno-only (56 ± 4%), and lido-only (66 ± 8%) (P < 0.05) but not from low-dose AL (45± 9%) and high-dose AL animals (45 ± 6%).

The separate and sequential administration of lidocaine and adenosine resulted in 2 out of 7 deaths and ischemia-induced VT (6 ± 3 episodes, 4 ± 2 sec) was not prevented while VF (1 ± 0 episodes, 1 ± 0 sec) was reduced. Infarct size (52 ± 5%) was not significantly different from saline-controls (61 ± 5%). When AL was given at pretreatment, stopped for ischemia and resumed 5 min before reperfusion, infarct size reduction (67 ± 8%) and protection from ventricular arrhythmias (VT: 39 ± 23 episodes, 84 ± 49 sec; VF: 2 ± 1 episodes, 21 ± 8 sec) were lost; though, there were no deaths in this group. AL solution given continuously from pretreatment through ischemia and reperfusion provided similar protection to AL infusion during pretreatment and ischemia (41 ± 10% vs. 38 ± 6%, 2 ± 1 VT and 0 VF).

During ischemia, control [ATP] fell to 61% of baseline at 15 min and recovered 68% - 88% of baseline during reperfusion. AL treatment maintained [ATP] in a steady state throughout ischemia and reperfusion with changes ranging of 95 ± 7 % to 117 ± 10% of baseline. Control [PCr] was significantly reduced compared to AL treated hearts during ischemia at 10 min (62 ± 7 vs. 89 ± 9%), 15 min (45 ± 4% vs 81 ± 7%), 20 min (44 ± 9% vs. 92 ± 9%) and 30 min (45 ± 8% vs. 77 ± 7%) and during reperfusion at 10 min (44 ± 19% vs. 92 ± 9%) and 15 min (50 ± 8% vs. 90 ± 7%) (P < 0.05). The pH

of AL and control hearts were similar throughout ischemia ranging from pH 7.6 to 6.4 in control and pH 7.5 to 6.8 in AL hearts. Controls maintained a mean pH below baseline for the first 20 min of reperfusion (pH 7.1) while AL hearts pH recovered to baseline within the first 5 min of reperfusion (pH 7.4 ± 0.1).

Pretreating the heart before and during ischemia with AL or with CCPA plus lidocaine resulted in no deaths, and no lethal arrhythmias. Infarct size reduction in CCPA plus lidocaine treated rats (12 ± 4 %) was similar to ischemic preconditioning (11± 3%), whereas in AL- and CCPA- treated rats, the infarct size was 38 ± 6% and 42 ± 7% respectively.

Conclusions: i) The intravenous infusion of AL solution before or during 30 min was more cardioprotective than adenosine alone, lidocaine alone, or the separate and sequential infusion of adenosine and lidocaine; ii) the AL combination led to no death, virtually no episodes of VF, few episodes of VT, and a significantly reduced infarct size; iii) AL cardioprotection appears to be associated with preservation of high energy phosphates and a better balance between supply and demand during ischemic conditions; iv) low pH was not an indicator of myocardial damage in AL treated rats, and v) when adenosine was substituted with an adenosine A_1 receptor agonist, CCPA, plus lidocaine cardioprotection was significantly enhanced and similar to IPC. In summary, targeting adenosine receptors, especially the A_1 adenosine receptor, with lidocaine Na^+ fast channel modulation may offer a new combination therapy to delay myocardial damage during ischemia and prevent ischemia-induced arrhythmias.

Abbreviations

^{31}P	phosphorus-31
adeno	adenosine
ADP	adenosine diphosphate
AL	adenosine and lidocaine
AL solution	adenosine and lidocaine solution
AMP	adenosine monophosphate
APD	action potential duration
ATP	adenosine triphospate
bpm	beats per minute
Ca^{2+}	calcium ion
CCPA	2-chloro-N6-cyclopentyladenosine
CK	creatine kinase
Cr	creatine
D_2O	deuterium oxide
ECG	electrocardiogram
FID	free induction decay
H^+	hydrogen ion
HR	heart rate
Hrs	hours
i.c.	intracoronary
i.p.	intraperitoneal
i.v.	intravenous
K_{ATP}	ATP sensitive potassium channel
Lido	lidocaine hydrochloride
Map	mean arterial pressure
Mg^{2+}	free magnesium
min	minutes
Mito	mitochondrial
Na_i	intracellular sodium
NMR	nuclear magnetic resonance
o.d.	outer diameter
PCr	phosphocreatine
pH	$-log_{10} [H^+]$
P_i	inorganic phosphate
PKC	protein kinase C
PPA	phenylphosphoric acid
Ppm	parts per million
PVB	premature ventricular beat
rpp	rate pressure product
Sarc	sarcolemmal
SCF	saturation correction factor
Sec	seconds
solution	solution
T	tesla
TTC	triphenyltetrazolium chloride
VF	ventricular fibrillation
VT	ventricular tachycardia
VT+VF	sum of VT and VF

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Chapter 1. Introduction

Plate I. "Apoplexie du Coeur." Illustration by Jean Cruveilhier (1791-1874) in *Anatomie pathologique du corps human.*

(Leibowitz, 1970)

No understanding of the circulatory reactions of the body is possible unless we start first with the fundamental properties of the heart muscle itself, and then find out how these are modified, protected and controlled under the influence of the mechanisms – nervous, chemical, and mechanical – which under normal conditions play on the heart and blood vessels.

(Starling, 1920)

1.1. Cardioprotection

Worldwide an estimated 17 million deaths occur from cardiovascular disease each year (World Health Organization, 2000) and nearly 80 Australians die each day from heart conditions (Australian Institute of Health and Welfare, 1999). The twentieth century has seen extraordinary advances in cardiovascular medicine from interventional cardiology to coronary artery bypass surgery and heart transplantation. Yet, despite the advances, cardiovascular disease continues to be the most common cause of mortality and morbidity in the developed world and commands a multibillion dollar a year pharmaceutical industry. Regrettably, however, the application of potentially promising basic science rarely translates into clinical outcomes from human trials. The search for the "holy grail" of myocardial protection to prevent irreversible injury and minimize ventricular dysfunction has, however, not diminished in its importance or impetus in recent times. Indeed, discoveries such as ischemic preconditioning have opened more doors than they have closed and enhanced optimism towards finding the ultimate 'cardioprotective' mechanism. The term 'cardioprotection' is often used to describe therapeutic interventions or strategies that preserve or enhance the viability of the myocardium and its blood vessels during

ischemia and reperfusion thus limiting the extent of acute myocardial infarction (AMI) (Yellon and Baxter, 2000). The work presented in this thesis adds to the body of knowledge in the field of cardioprotection by testing the effects of adenosine and lidocaine solutions during regional myocardial ischemia in an *in vivo* rat model.

OVERALL HYPOTHESES

1. An adenosine and lidocaine solution (AL) will provide cardioprotection from ischemia-reperfusion injury by reducing mortality, decreasing ventricular arrhythmias and lowering infarct size.

2. Pharmacological preconditioning of the heart with AL solution will preserve ATP, phosphocreatine (PCr), and reduce the extent of acidosis (pH)

This introduction begins with a brief history highlighting some of the milestones in the understanding of heart physiology, followed by a discussion of myocardial energy sources and the events that characterize ischemia-reperfusion injury. Ischemic preconditioning is then described as the 'ultimate' paradigm of cardioprotection which has allured researchers into unraveling its underlying mechanism and into seeking to develop an equally protective pharmaceutical mimetic. As the the first trigger of ischemic preconditioning to be identified, adenosine and the mechanisms by which endogenous and exogenous adenosine address the injury and functional problems of ischemia-reperfusion are explored. Unlike adenosine, cardiac Na^+ channels have received little attention as targets of preconditioning, despite modifications of the membrane potential during early ischemia. Lidocaine is a Na^+ fast channel inhibitor and while it has not been implicated in ischemic preconditioning, it has potent anti-ischemia-reperfusion injury and anti-arrhythmic properties which are discussed. Next, the sequential use of lidocaine as an adjunct to adenosine reperfusion therapy is outlined followed by a discussion of the research aims.

1.2. A brief historical persective of the heart structure and function

Understanding of the heart has been evolving since the time of the ancient Greeks who dubbed it *kardia* hence the words cardiac, tachycardia, and bradycardia. Aristotle's observations of chick embryos led him to conclude that the heart was the most important organ of the body (Lyons and Petrucelli, 1987). Moreover, Aristotle considered the heart as the seat of intelligence, motion and sensation. By 200 AD the "father of experimental physiology," Galen, determined that arteries contained blood not air as previously thought (Leibowitz, 1970). However, while his investigations led to the understanding that the heart moved blood throughout the body, he believed the division between the left and right heart was porous not recognizing that blood left each ventricle through arteries (Opie, 1998). Against the popular view of the time, Vesalius (1514-1564) and Servetus (1511-1553) understood that the left and right heart were not porous and were distinctly separated by the septum (Herrlinger, 1970; Leibowitz, 1970). Servetus began to understand the separate circuitry of left and right heart blood flow and shortly thereafter, valve function was described by Cesalpino (1571) (Leibowitz, 1970; Opie, 1998). Harvey (1578-1657) laid the foundation for modern understanding of heart mechanics and circulation in his *Anatomical Treatise on the Motion of the Heart and Blood in Animals* (1628) (Leibowitz, 1970; Opie, 1998). While Harvey's work was at first widely rejected and many of his patients suffered fatal outcomes in his hands, by the end of the 17th century, his ideas based on observation and experiments came into favor. Throughout most of the 18th century, anatomical knowledge of the heart was refined based on Harvey's works.

By the end of the 19th century great strides in cardiac physiology took place which laid the foundations for the even greater distances covered throughout the 20th century.

By 1845, Purkinje and later His (1883) described the conduction tissues that bear their names (Granger, 1998). In 1883, Burdon-Sanderson and Page placed electrodes on the surface of the heart and provided the first monophasic action potential (Granger, 1998). Shortly thereafter, in 1887, the first human electrocardiogram (ECG) was recorded by Waller (1887). Einhoven aimed to correct Waller's waveforms with the then refined Lippmann capillary electrometer. He labeled the waves of his corrected curve PQRST and published this in 1895 (Hurst, 1998). By 1913, Einhoven designed a string galvanometer capable of precise measurements of faint electrical signals projected by the heart onto the body's surface furthering the field of electrocardiography (Granger, 1998).

Sidney Ringer (1835-1910) happened upon the essential role of calcium ions in muscle contraction almost by accident. The sodium chloride enriched tapwater initially used by Ringer also contained potassium chloride but this was unknown to Ringer at that time (Opie, 1998). Potassium chloride antagonizes calcium's effect on contraction. Eventually, Ringer discovered that an arrested heart could be reanimated by the addition of calcium chloride leading him to conclude that calcium was essential for the maintenance of contraction (Opie, 1998).

Table 1.1. Milestones and pioneers in cardiovascular physiology and medicine.
By the mid -1980s cardiovascular research increased exponentially and a large
number of highly valuable milestones have been achieved from coronary stents to
combination thrombolytic therapy and others too numerous to mention here

Date	Name	Country performed	Profession	Discovery
1628	William Harvey ("the father of physiology")	England	Physiologist	First describes blood circulation
1706	Raymond de Vieussens	France	Anatomy Professor	First describes the structure of the heart's chambers and vessels
1733	Stephen Hales	England	Clergyman and Scientist	First measures blood pressure
1816	Rene T. H. Laennec	France	Physician	Invents the stethoscope
1883	Sidney Ringer	England	Physiologist	Discovered that an arrested heart could be revived by addition of calcium chloride
1895	Oscar Langendorff	Germany	Physiologist	Describes isolated heart perfusion method to examine function and metabolism
1903	Willem Einthoven	Netherlands	Physiologist	Develops the electrocardiograph
1912	James B. Herrick	America	Physician	First describes heart disease resulting from hardening of the arteries
1938	Robert E. Gross	America	Surgeon	Performs first heart surgery
1949	William Bigelow	Canada	Surgeon	Showed open heart surgery could be extended in cold conditions
1951	Charles Hufnagel	America	Surgeon	Develops a plastic valve to repair an aortic valve
1952	F. John Lewis	America	Surgeon	Performs first successful open heart surgery
1953	John H. Gibbon	America	Surgeon	First uses a mechanical heart and blood purifier
1961	J. R. Jude	America	Cardiologist	Leads a team that performs the first external cardiac massage to restart the heart
1965	Michael DeBakey and Adrian Kantrowitz	America	Cardiologists	Implantation of mechanical devices to help a diseased heart
1967	Christiaan Barnard	South Africa	Surgeon	Performs the first whole heart transplant from one person to another
1977	Andreas Gruentzig	Switzerland	Physician	First angioplasty on a conscious patient
1982	Willem de Vries Robert Jarvik	America	Surgeon/Physician	Implants a permanent artificial heart into a patient

Also in the late 19th century, physiologists observed that occlusion of a major coronary artery led to death in a dog model (Braunwald, 2002). During that era, autopsies occasionally revealed thrombosis of coronary vessels and acute myocardial infarction leading pathologists to believe that the combination was fatal. However, Krehl (1901), a Viennese physician, challenged the fatal relationship reporting that coronary thrombosis is actually beneficial for survival from AMI (Leibowitz, 1970). By 1912, two Russian physicians, Obrastov and Strazheske and a Chicago physician, Herrick, described the clinical features and pathologic characteristics of AMI as well as distinguished between AMI and angina pectoris (Herrick, 1912; Leibowitz, 1970). As the 20th century progressed so did the observable prevalence with heart disease easily becoming the most common cause of death (Braunwald, 2002). As a result, a number of major milestones in the field of cardiovascular medicine have led to improved recovery and survival rates (Table 1.1). Yet, the challenge to better survival and recovery persists as the complex causes and effects of ischemia-reperfusion injury continue to be the subject of intense investigation.

1.3. Energy supply to the myocardium

During an average lifespan the human heart contracts about 3 billion times pumping approximately 500 million L of blood per kg of heart (Dobson, 2003). Continuous contraction of the human heart turns over a remarkable 3.5 kg of adenosine triphosphate (ATP) each day and the breakdown of ATP provides an immediate source of energy for muscle contraction, maintenance of ion gradients, and other vital functions (Fig. 1.1) (Opie, 1998; Dobson, 2003). Most of the ATP used by the heart is required for contraction (60 - 70%) including Ca^{2+} uptake in the sarcoplasmic reticulum, followed by active transport by the sodium-potassium pump (11 - 15%),

and maintenance of ion transfer for action potential generation or conduction impulse (< 5%) (Opie, 1998). Smaller amounts are needed for kinase reactions and for formation of cAMP.

The ATP is replenished by the complex demands of the heart and the mix of other fuels such as fatty acids, lactate and glucose from coronary circulation (Reimer and Jennings, 1992). Myocardial ATP replenishment occurs by 3 major pathways 1) oxidative phosphorylation, 2) glycolysis, and 3) the creatine kinase reaction.

The details of normal myocardial oxidative phosphorylation, glycolysis, and the creatine kinase reaction will not be discussed further here but excellent reviews of these systems have been provided by Opie (1998) and Reimer (1992).
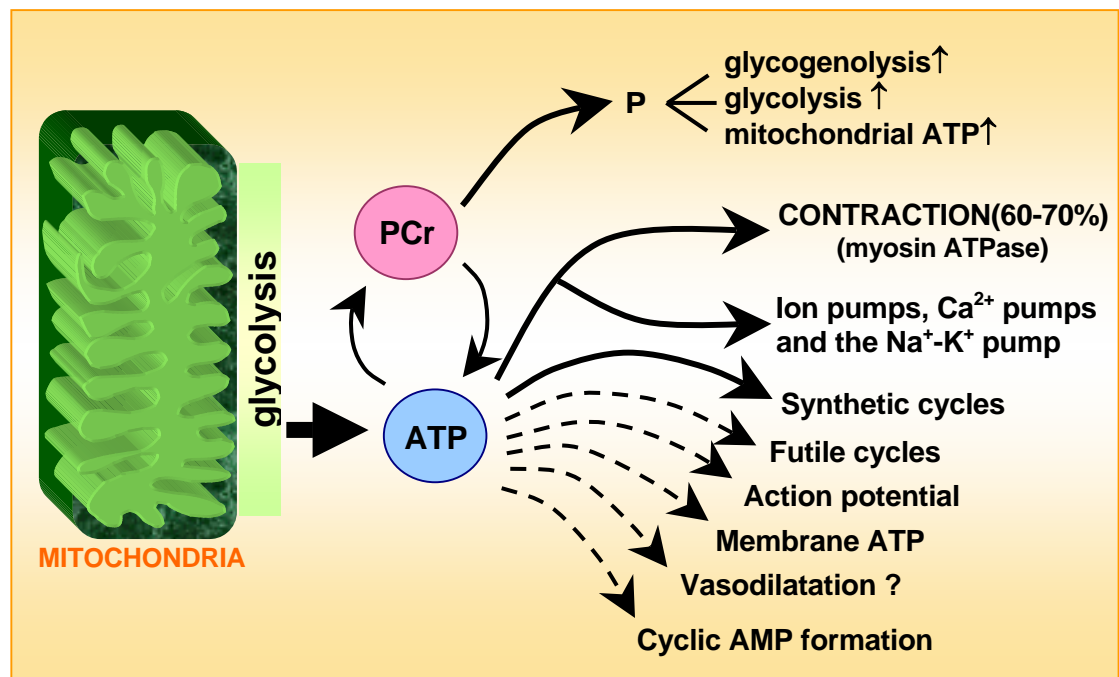
Figure 1.1. High energy phosphate, ATP and phosphocreatine (PCr), utilization in normal myocardium. (adapted from Opie, 1998).

1.4. Acute myocardial ischemia-reperfusion injury

Myocardial ischemia refers to a reduction in coronary blood flow such that myocardial oxygen supply is inadequate for the oxygen demands of the tissue (Jennings and Reimer, 1981). This section highlights some of the complex cellular changes during ischemia that contribute to reversible and irreversible cell damage. Acute and prolonged myocardial ischemia leads to a complex orchestration of metabolic and structural changes that ultimately affect the electrical integrity of the myocardium and microvasculature (Jennings et al., 1981; Opitz et al., 1995). The cellular composition of the myocardium is transformed to a state of reversible injury within 15 min of ischemia (Jennings and Reimer, 1981). During this time, myocytes undergo numerous changes including a decrease in high-energy phosphates and the adenine nucleotide pool, as well as glycogen depletion, lactate accumulation, acidosis and mild intracellular edema (Jennings and Reimer, 1981). Restoration of arterial flow during the 15 min window can revive reversibly damaged myocytes. Although, the sudden influx of oxygen at reperfusion restores aerobic metabolism and promotes salvage, it also exacerbates the reversible damage developed during ischemia, causes new damage, and may lead to myocardial stunning and postischemic dysfunction (Braunwald and Kloner, 1982; Bolli et al., 1989b; Jordan et al., 1999; Zhao et al., 2000). If reperfusion does not occur within the reversible period and ischemia persists, then subsequent metabolic alterations contribute to the transition from reversible to irreversible injury manifesting as necrosis or apoptosis (Jennings and Reimer, 1981). Necrosis develops with ischemic time as the ischemic wavefront extends from the subendocardium to the subepicardium (Reimer et al., 1977) and reperfusion is the most effective means of halting this 'wavefront of necrosis' (Hochmann and Choo, 1987). Despite the type of cell damage in either period, the electrical integrity of the heart is jeopardized and potentially fatal arrhythmias may

prohibit normal heart function or increase the likelihood of mortality (Opitz et al., 1998).

1.4.1 ACUTE ISCHEMIA

Complete occlusion of a coronary artery forces complete reliance on local metabolism to drive ATP synthesis as exogenous sources of fuel such as fatty acids and glucose can no longer reach the ischemic zone (Neely and Feuvray, 1981; Reimer and Jennings, 1992). Beginning with the cessation of coronary artery blood flow, tissue oxygen supply is severely reduced to the small quantities of oxygen in the lingering erythrocytes trapped in the capillaries or attached to myoglobin (Reimer and Jennings, 1992). The remaining oxygen supply is rapidly consumed resulting in its removal as the final electron acceptor in the electron transport chain, therefore, shutting down oxidative respiration in the mitochondria (Reimer and Jennings, 1992). Using NMR spectroscopy Whitman et al. (1983) showed the cessation of the mitochondrial electron transport 2 sec after the onset of global ischemia in the isolated rat heart model.

Ischemia induces energy metabolism to switch from aerobic pathways to anaerobic pathways (Morgan et al., 1959; Williamson, 1966; Braasch et al., 1968; Neely et al., 1976; Jennings and Reimer, 1981). Concurrent with the inhibition of oxidative mitochondrial metabolism, ischemia causes an immediate reduction in ATP, glucose-6-phosphate, and increases the availability of AMP and inorganic phosphate. In turn, these metabolites and modulators augment phosphorylase and phosphofructokinase activity causing an acceleration of anaerobic glycogenolysis and glycolysis with the concomitant production of lactate and protons (Reimer and Jennings, 1992). Jennings et al. (1986) reported a close association between ischemic death and a

sustained deficiency of ATP and an elevated osmotic load. In contrast, others have shown that slowing myocardial metabolism delays myocyte death during ischemia (Jones et al., 1982) and preserves the capacity of the myocardium to function (Kubler and Spieckermann, 1970; Hearse et al., 1975).

A major feature in ischemia, therefore, is the rapid rate of decline of ATP and PCr. Phosphocreatine is used to stabilize ATP content although the process is short-lived (Jones et al., 1976; Jeffery et al., 1989). During ischemia, anaerobic glycogenolysis becomes the primary ATP regenerating pathway in the ischemic cell. During severe ischemia, ATP drops from about 35% of initial baseline recordings by 15 min to less than 10% by 40 min of ischemia (Neely et al., 1973; Jennings et al., 1978; Reimer et al., 1981). In addition, the contractile force of ischemic cells decreases markedly alongside the ischemia-induced metabolic shift from aerobic to anaerobic metabolism (Jennings and Reimer, 1981).

In ischemic myocardium, as levels of ATP fall, ADP is broken down further into AMP which is further metabolized into inosine monophosphate (IMP) and adenosine. Adenosine production significantly correlates with the severity of ischemia (Deussen et al., 1988a; Deussen et al., 1988b) and acts to down-regulate the myocardial demand of the ischemic heart by promoting compensatory vasodilatation, slowing heart rate, and inhibiting sodium and calcium currents (Berne, 1963; Berne, 1980; Jennings et al., 1981; Olsson, 1988). These actions of adenosine and its use as a pharmacological cardioprotectant will be discussed in detail later.

While glycolytic ATP production has been shown to be beneficial during ischemia, the intracellular accumulation of metabolic end products such as lactate and protons [H$^+$], may exacerbate myocardial injury (Wissner, 1974; Neely and Grotyohann, 1984; van

Wylen, 1994; Cross et al., 1995a). Increasing lactate concentrations is believed to have a negative feedback effect on glycolysis while acidosis leads to increased Na^+/H^+ exchange with subsequent Na^+/Ca^{2+} exchange that results in elevated intracellular $[Na^+]$ and $[Ca^{2+}]$ (Neubauer et al., 1987). By inhibiting the Na^+/H^+ exchanger during ischemia (Tani and Neely, 1989; Murphy et al., 1991; Pike et al., 1993) and hypoxia (Anderson et al., 1990) many have confirmed this Na^+/H^+ exchanger action (Xaio and Allen, 2000). Intracellular Na^+ accumulation is further exacerbated by decreased Na^+/K^+ATPase activity due to the reduction of ATP synthesis (Cross et al., 1995b). Elevated intracellular Na^+ in ischemia may also occur through the voltage sensitive Na^+ fast channel (van Emous et al., 1997).

Apart from an association with sodium accumulation, elevated lactate concentrations have been shown to cause other deleterious effects in the ischemic cell. For example, lactate has been shown to alter the action potential and decrease overall myocardial muscle tension (Yatani et al., 1981). As well, the lactate anion contributes to mitochondrial swelling and decreases the phosphorylating capabilities of the mitochondria (Armiger et al., 1975).

Myocardial cellular acidosis may have multiple deleterious effects during ischemia (Williamson et al., 1976). Using NMR technology it has been demonstrated that pH as low as 6.1 can be tolerated for at least 20 -30 min ischemia (Cave et al., 2000). Nonetheless, protons are known to inhibit metabolic pathways (Williamson et al., 1976) and their accumulation has been shown to interfere with the contractile apparatus by displacing calcium binding sites on thin contractile filaments (Katz and Hecht, 1969). Acidosis has also been linked with ultrastructural changes such as nuclear chromatin aggregation and the formation of mitochondrial amorphous matrix densities (Ambrosio et al., 1987).

Increased calcium uptake by the myocardium is believed to occur in response to ischemia, reperfusion and catecholamine stimulation (Fleckenstein, 1971; Katz and Reuter, 1979; Farber, 1981; Nayler, 1981; Jennings et al., 1985; Murphy et al., 1988; Steenbergen et al., 1990; Barry, 1991). Fleckenstein (1971) first emphasized the pathological overload of calcium uptake coinciding with experimental myocardial necrosis and showed that calcium channel antagonists inhibited the process. Calcium overload during ischemia contributes to necrosis or dysfunction in at least four ways (Opie, 1998): i) phospholipases are activated by calcium which leads to cell membrane degradation (Lubbe et al., 1992); ii) elevated calcium contributes to the state of ischemic contracture, a state of sustained excess contraction (Steenbergen et al., 1990); iii) excessive calcium cycling in and out of the sarcoplasmic reticulum may lead to arrhythmias (e.g. after-depolarizations) (Reiter, 1964; Hondeghem and Katzung, 1984); iv) mitochondria become overloaded with calcium resulting in futile ATP use and increased oxygen demand leading to increased intracellular ischemia (Jennings et al., 1978; Farber, 1981; Leyssens et al., 1996).

Since 1935 it has been known that one of earliest consequences of severe ischemia is contractile dysfunction (Tennant, 1935; Tennant and Wiggers, 1935; Sayen et al., 1958; Tatooles and Randall, 1961) occurring within 10 sec after the onset of ischemia (Ross and Franklin, 1976; Harden et al., 1979). The primary cause of contractile dysfunction in ischemic hearts remains elusive. ATP depletion is thought to play a primary role as myocardial contraction is energy-dependent, however, the anomaly is that contractile failure occurs before the total tissue decline in ATP (Covell et al., 1967; Kanaide et al., 1982; Rauch et al., 1994). It is possible that small declines in compartmentalized ATP coupled to defects in energy transport may result in rapid

changes of contractile function (Kubler and Katz, 1977; Hearse, 1979). Similarly, small decreases in ATP may reduce Ca^{2+} influx across the sarcolemma and sarcoplasmic reticulum to promote impairment of contractility (Kubler and Katz, 1977). In addition, the accumulation of the products of ischemic metabolism are likely to have a role in contractile dysfunction including lactate (Tennant, 1935), intracellular acidosis (Katz and Hecht, 1969; Cobbe and Poole-Wison, 1980; Lee and Allen, 1991), and the buildup of inorganic phosphate (P_i) (Kubler and Katz, 1977; Lee and Allen, 1991).

In summary, the metabolic changes of severe ischemia result in reduced oxidative phosphorylation as metabolism shifts from aerobic glycolysis to anaerobic glygenolysis; stores of ATP and PCr are rapidly depleted as intracellular pH decreases, and failure of membrane pumps leads to calcium, sodium and water entry into the cell.

1.4.1.1 Development of cell death: The transition from reversible to irreversible injury

The severity and duration of ischemia determines the extent, rate, and type of injury endured by the myocardium (Jennings and Reimer, 1981). As already discussed, within the first 15-20 min of ischemia the injury is reversible, but contractility can be depressed for minutes to days (Reimer et al., 1977). By definition, reversible injury is the return of normal function and structure to a region of myocardial tissue that was previously, temporarily ischemic (Kloner and Jennings, 2001). The exact cause of the transition from reversible to irreversible injury during ischemia remains unknown. However, cell death is believed to be the consequence of a temporal combination of ischemic events such as: i) the critical loss of high energy phosphates (ATP, PCr); ii) membrane damage from mechanical or metabolic changes; iii) free radical formation,

iv) sodium and calcium overload and sodium pump inhibition (Reimer et al., 1977; Reimer and Jennings, 1992; Opie, 1998). Overall, the transition from reversible to irreversible injury is not likely to result from a single metabolic event but most likely, gradually develops as a process of interactions of many diverse mechanisms.

1.4.1.2. Ischemic electrophysiology

Within the ischemic zone, metabolic, ionic and autonomic fluctuations are not homogeneous (Gettes and Cascio, 1992; Coronel, 1994). The interface between ischemic and non-ischemic myocardium, the lateral border zone (Factor et al., 1981) can give rise to abnormal impulse conduction due to metabolic and ionic inhomogeneities on the border zone (Gettes and Cascio, 1992). In this transition zone, the rise in extracellular potassium and the fall in pH are less than in the central area of the ischemic zone (Hill and Gettes, 1980). Additionally, transmural inhomogeneities also exist. For instance, during coronary occlusion, the concentration of ATP and creatine phosphate are more reduced in the subendocardium than in the midmyocardium or subepicardium (Reimer et al., 1977; Lowe et al., 1983; Foilet et al., 1985). Likewise, Johnson et al. (1988) showed that during 10 min coronary artery occlusions, the change in potassium equilibrium potential varied from 20 to 35 mV in the ischemic zone center to 0 to 35 mV in the lateral margin. Changing levels of calcium and pH as well as α- and β-sympathetic agonists also give rise to variable electrophysiology across the ischemic zone (Gettes and Cascio, 1992). These changes are consistent with the findings of Reimer and coworkers who proposed the 'wavefront phenomenon of cell death' (Reimer et al., 1977). Necrosis develops within the area at risk extending from the subendocardium to the subepicardium in a wavefront pattern over occlusion time (Reimer et al., 1977).

Throughout the ischemic zone, potassium loss is a major contributor to ischemic depolarization (Janse and Wit, 1989; Coronel, 1994). During the early phases of acute ischemia a differential loss of K^+ ions from ischemic cells leads to a shift in the action potential to a less polarized state and a partial membrane depolarization (Gettes et al., 1963; Gettes and Cascio, 1992). The extent of depolarization and inhomogeneity of K^+ ions depends on the extent and duration of local ischemia (Janse and Wit, 1989; Coronel, 1994). While ischemic potassium efflux is not entirely understood (Reimer and Jennings, 1992), it has been demonstrated that potassium loss is depolarization-dependent (Shine, 1981), balances increased osmotic load (Jennings et al., 1986), occurs with co-ionic loss of negatively charged lactate and phosphate (Kleber, 1983), as well as inhibition of the Na^+/K^+ pump (Wilde and Aksnes, 1995), and occurs with activation of the ATP-dependent potassium channel (K_{ATP}) (Opie, 1998).

During ischemia, potassium ions are not washed away but accumulate outside ischemic cells. As well as depolarizing the resting membrane, extracellular potassium accumulation shifts the threshold potential, reduces the maximum rate of rise of the action potential upstroke, lowers the action potential amplitude and plateau potential, shortens the plateau duration, accelerates the slope of rapid repolarization, suppresses the oscillatory after-potentials induced by the increase in intracellular calcium and decreases the rate of spontaneous diastolic depolarization in Purkinje fibers (Weidmann, 1956; Gettes et al., 1963; Gettes and Cascio, 1992).

Potassium-related changes to the ischemic action potential are further exacerbated by the ischemic fall in intracellular pH (Kleber, 1983; Kleber, 1984; Kleber et al., 1987). Kleber et al. (1987) demonstrated that acidification of isolated guinea pig

hearts was immediately followed by K^+ accumulation while alkalinization was immediately followed by transient K^+ depletion (Kleber et al., 1987).

1.4.1.3. Arrhythmias arising from the ischemic myocardium

Arrhythmias arising from acute myocardial ischemia result from automaticity developing in nonnodal tissue and reentrant or triggered excitation (Gettes and Cascio, 1992; Janse, 1992; Wit and Rosen, 1992; Opie, 1998). Automaticity is the result of spontaneous diastolic depolarization from two or more independent centers of impulse formation (Scherf and Schott, 1973). Such ectopic beats caused by a pacemaker current can arise in the absence of any prior electrical activity (Di Francesco, 1985; Wit and Rosen, 1992; Opie, 1998). In contrast, triggered excitation results from abnormal impulse initiation in cardiac fibers precipitated by the preceding beat (Scherf and Schott, 1973). Triggered activity occurs under conditions where oscillations in membrane potential reach threshold either early during the repolarization phase of the action potential (early after-depolarizations) or following complete repolarization (delayed after-depolarizations) (Cranefield and Aronson, 1988; January and Fozzard, 1988).

 The conditions of reentrant excitation are initiated during early ischemia. Within minutes following acute coronary artery ligation, conduction velocity in the ischemic area decreases and myocyte activation is delayed, particularly in the subepicardium (Conrad et al., 1959; Durrer et al., 1961; Boineau and Cox, 1973; Scherlag et al., 1974). Spontaneous development of ventricular tachycardia and ventricular fibrillation coincides with increasing activation delay or slowing of conduction, (Scherlag et al., 1974) increased dispersion of refractory periods (Han and Moe, 1964; Naimi et al., 1977) and inhomogeneities in recovery of excitability (Factor et al.,

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1981; Gettes and Cascio, 1992). When the conducted impulse reaches an injured zone, it is slowed by the formation of abnormal action potentials in the injured zone. Because the slow rate of conduction delays the impulse until the refractory period of the normal impulse has passed, a re-entry impulse is possible (Opie, 1998).

The calcium overload that accompanies ischemia contributes to ischemic arrhythmogenesis. Through activation of the adrenergic nervous system β-adrenergic catecholamine stimulation of cyclic adenosine monophosphate (cAMP) is linked to cytosolic calcium overload (Lubbe et al., 1992). Further, three main adverse electrophysiologic effects result (Fig. 1.2) (Lubbe et al., 1992). First, delayed afterdepolarizations resulting from excess oscillations of cytosolic calcium and triggered automaticity may develop in otherwise quiescent ventricular muscle. Second, in depolarized fibers, cAMP can cause calcium–dependent slow responses which favor reentry conditions. Third, excess calcium can result in conduction slowing through intercellular uncoupling.
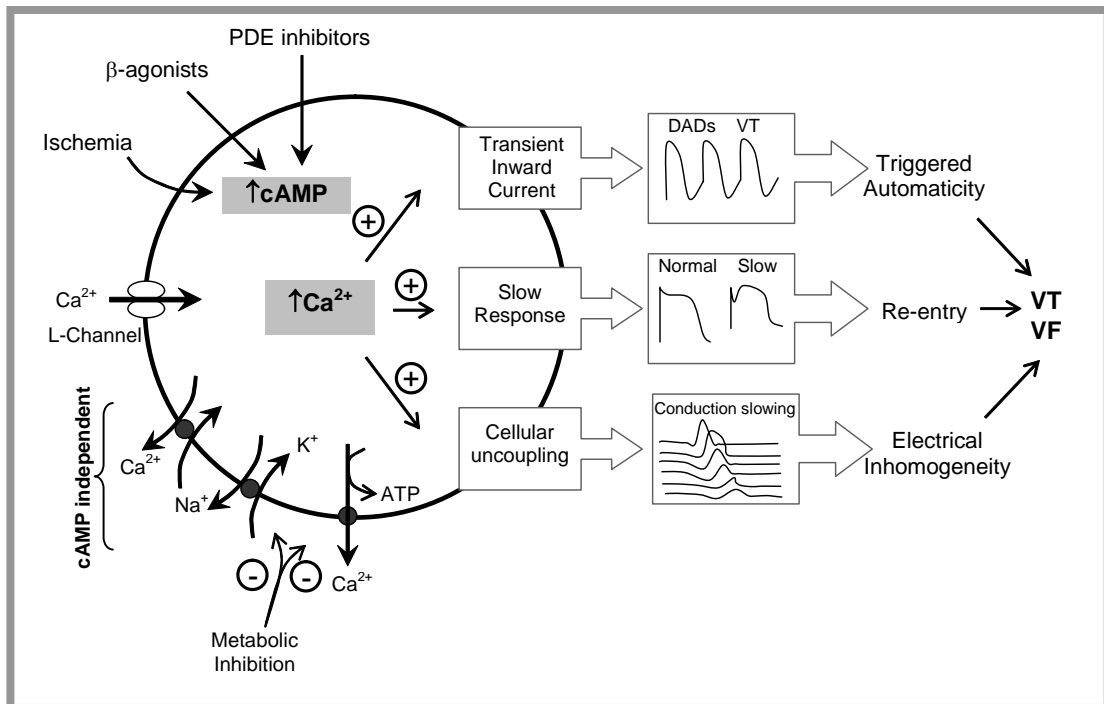
Figure 1.2. Effect of increased intracellular calcium on the genesis of ischemia-induce arrhythmias. Ischemia-induced increases in cytosolic calcium are linked with increases in cAMP and can predispose the myocardium to ventricular fibrillation (VF) and ventricular tachycardia (VT) (adapted from Opie, 1999).

1.4.2. REPERFUSION INJURY

While early reperfusion remains the most effective means of salvaging the myocardium from acute ischemia (Reimer et al., 1977; Hochmann and Choo, 1987), the sudden influx of oxygen paradoxically may lead to further necrosis, ventricular arrhythmias and death (Bolli et al., 1989a; Bolli et al., 1989b; Opitz et al., 1995; Opitz et al., 1998; Jordan et al., 1999; Zhao et al., 2000). The extent of reperfusion injury has also been linked to a cascade of inflammatory reactions including the generation and action of cytokines, leukocytes, platelets, reactive oxygen species (hydrogen peroxide) and free radicals (superoxide anions, hydroxyl and singlet oxygen) (Bolli et al., 1989a; Frangogiannis et al., 2002). Even if reperfusion occurs while cellular injury

is still reversible, recovery of contractile function may be delayed (Braunwald and Kloner, 1982). Often, reperfusion injury refers to the death of myocytes that were alive at the time of reperfusion as a result of one or more reperfusion-initiated events (Park and Lucchesi, 1999). Matsumura and colleagues demonstrated that myocytes that were viable at the start of reperfusion lost viability during the first 3 hours of reperfusion. (Matsumura et al., 1998). However, reperfusion has also been ascribed to metabolic functional and structural changes that are the consequence of restoring coronary arterial flow (Ganz, 1997). For example even during adequate coronary perfusion, myocytes have been shown to undergo necrosis as with catecholamine triggered stress (McManus et al., 1981; Rona, 1985) or loss of calcium homeostasis (Shen and Jennings, 1972; Vander-Heide et al., 1986). It has also been shown that myocytes may suffer programmed cell death through the process of apoptosis (Saraste et al., 1997), which may be initiated by the generation of reactive oxygen species with reperfusion (Semenza, 2000).

1.4.2.1. The 'oxygen paradox'

Following the observation that ventricular fibrillation can occur with reperfusion of canine hearts, Jennings et al. (1960) discussed the adverse structural and electrophysiologic changes associated with reperfusion of the ischemic canine heart (Jennings et al., 1960). Later, Hearse and coworkers found that reoxygenation led to cardiac enzyme release and alterations in myocardial ultrastructure; as a result, they developed the "oxygen paradox" hypothesis (Hearse et al., 1973; Hearse et al., 1975). The oxygen paradox refers to the concept that although oxygen is essential for tissue survival its restitution to previously ischemic myocardium may be injurious. Hearse et al. (1973) demonstrated that reoxygenated myocardium was characterized by myofibrillar hypercontracture and sarcolemmal disruption which may develop with

the onset of reperfusion. Nearly 20 years later, Ganote and colleagues (1990) proposed that this injury may be due to the reenergization of the myocardial tissue upon reoxygenation.

1.4.2.2. Free radical formation

As oxygen is restored to the myocardium, molecular oxygen is sequentially reduced to form reactive oxygen species such as superoxide anion and hydroxyl free radicals. A free radical is an atom or molecule that has one or more unpaired electrons in its outer orbital and therefore, is relatively unstable and highly reactive (Park and Lucchesi, 1999). These oxygen-derived free radicals may interact with cell membrane lipids and proteins to further myocardial cell damage and depress cardiac function. Their deleterious affects may be further exacerbated by the likelihood that the antioxidative defense of previously ischemic cells may have been reduced (Richard et al., 1990).

Support for free radical associated injury has been shown in animal models (Jolly et al., 1984; Bolli et al., 1989a; Bolli et al., 1989b) and in humans (Roberts et al., 1990; Beard et al., 1994). In the dog model of ischemia-reperfusion, agents that either scavenge or inhibit the formation of oxyradicals can improve recovery of contractile function following an ischemic insult (Myers et al., 1985; Gross et al., 1986; Przyklenk and Kloner, 1986; Bolli et al., 1987; Bolli et al., 1988; Farber et al., 1988; Sekili et al., 1993). However, there is controversy regarding the possible injurious role of free radical formation with reperfusion. Uraizee et al. (1987) and Gallagher et al. (1986) failed to demonstrate infarct size reduction following treatment with the free radical scavenger superoxide dismutase. While the differing results of these two studies may have been due to different ischemic times, there are others who have come to

the conclusion that an injurious role for the genesis of free radicals during reperfusion has not been proven (Ferrari and Hearse, 1997; Przyklenk, 1997).

1.4.2.3. Myocardial stunning

In a canine model of acute myocardial ischemia, Heyndrickx et al. (1975) discovered that a short ischemic insult of 5 min was followed by depressed mechanical function lasting for over 3 hours while a 15 min coronary occlusion led to 6 hours of left ventricular dysfunction. Of particular importance was that these short periods of ischemia did not lead to cell death and reduced postischemic function was not accompanied by any impairment of blood flow of the previously occluded vessel (Heyndrickx et al., 1978). Braunwald and Kloner (1982) described the condition as "myocardial stunning" because the mechanical function eventually recovers fully despite a *stunned* phase of "prolonged, postischemic dysfunction of viable tissue salvaged by reperfusion". Stunning is also believed to occur after ischemic periods which result in substantial necrosis (1-3hrs) (Kloner and Jennings, 2001). In this scenario, the cells that have not become necrotic and are still in a phase of reversible injury are believed to be stunned.

Examining stunned and necrotic tissue in an area at risk is extremely complex and with present methodology often yields little information about the mechanisms of stunning. So, the majority of knowledge regarding the mechanism of stunning comes from studies using reversibly injured tissue that is free of necrosis (Kloner and Jennings, 2001). Myers et al. (1985) and Bolli et al. (1989b) have shown that 50% to 70% of the stunning effect is due to short-lived O_2-derived free radicals that form in the first few minutes of reperfusion. Bolli and colleagues (1988) also provided the first direct evidence of the existence of O_2-derived free radicals using electron spin

resonance techniques. They found that most of the free radicals were released in the first 5 min of reperfusion. Because these results show that myocardial stunning is complicated by reperfusion, myocardial stunning is considered a form of reperfusion injury. Indeed others have presented evidence reporting that much of the stunning effect is partially prevented by treatment with free radical scavengers, superoxide dismutase and catalase (Gross et al., 1986; Przyklenk and Kloner, 1986).

The postischemic contractile failure upon reperfusion that hallmarks stunning after short ischemic insults may also be due to alterations in calcium homeostasis, as many have measured increased cytosolic calcium levels during early postischemic reperfusion (Brooks et al., 1995; Gao et al., 1995; Meissner and Morgan, 1995). One explanation for the relationship between calcium and stunning is that elevated internal cytosolic calcium may damage the contractile apparatus impairing the normal physiological response to calcium. The uptake of calcium during early reperfusion results from Na^+/Ca^{2+} exchange, entry via the L-channels (du Toit and Opie, 1992) and includes the movement of calcium entering or leaving the sarcoplasmic reticulum (du Toit and Opie, 1994). Furthermore, it has been proposed that calcium-mediated and free radical-mediated components of damage may be related to: i) inhibition of the sodium pump; ii) stimulation of the Na^+/Ca^{2+} exchanger and iii) a decreased rate of inactivation of the calcium current (Opie, 1991).

As in ischemia, calcium overload during reperfusion can seriously affect the myocyte through activation of a variety of proteases, lipases and phospholipases (Farber, 1981) and ATPases (Katz and Reuter, 1979; Farber, 1981; Nayler, 1981). Moreover, intracellular acidosis contributes Ca^{2+} overload via the Na^+/H^+ exchanger and Ca^{2+}/Na^+ exchanger (Grinwald, 1982; Lazdunski et al., 1985; Murphy et al., 1988).

1.4.2.4. Inflammation

Inflammation is thought to play a major role in reperfusion injury (Jordan et al., 1999; Zhao et al., 2000; Frangogiannis et al., 2002). By administering anti-inflammatory corticosteroids, Libby et al. (1973) presented the first evidence that inflammation is part of reperfusion injury by showing infarct size reduction in a canine model of acute myocardial infarction (Libby et al., 1973). Unfortunately, while corticosteroids inhibit the inflammatory process by decreasing the number of infiltrating leukocytes, they also delay healing and collagen deposition (Roberts et al., 1976). Hill and Ward (1969) were the first show complement as a major contributor to inflammation during myocardial reperfusion. The complement system initiates inflammation, destroys pathogens, aids in the clearance of immune complexes, and disrupts cell membranes.

The implication that neutrophil activation had a role in myocardial ischemia-reperfusion injury was first put forward by Romson and colleagues (1983) using histopathological analysis. They found a direct relationship between the duration of ischemia and infarct size with neutrophil accumulation in the myocardial tissue (Romson et al., 1983). Others have shown that neutropenia (neutrophil depletion) and inhibition of neutrophil adhesion may provide a cardioprotective effect (Simpson et al., 1988; Litt et al., 1989). Neutrophil adherence and activation is a complex process which intrinsically involves the endothelium partly because it serves as a matrix by which the neutrophils migrate to the inflammation site (Park and Lucchesi, 1999). Adherence of neutrophils to postischemic endothelial cells leads to capillary plugging and release of multiple inflammatory mediators such as proteolytic enzymes or reactive oxygen species all of which contributes to the "no reflow" phenomenon (Bernier et al., 1986; Frangogiannis et al., 2002).

1.5. Ischemic Preconditioning- a paradigm for cardioprotection

One of the most powerful protective strategies for myocardial protection involves inducing short periods of ischemia-reperfusion to protect from longer periods of ischemia-reperfusion injury in the phenomenon called ischemic preconditioning (IPC). In a landmark study, Murry et al. (1986) first described the IPC phenomenon in a canine model of acute myocardial ischemia. Brief ischemia-reperfusion cycles prior to a period of prolonged ischemia increased the myocardium's tolerance to an otherwise potentially lethal insult as evidenced by infarct size reduction (Murry et al., 1986; Reimer et al., 1986). Since Murry et al. (1986) first showed a dramatic reduction in myocyte necrosis (from 29% to 7% of the area at risk) following four 10-min episodes of ischemia in the dog, the phenomenon has been reported in a variety of species including rats and rabbits (Downey and Jordan, 1989; Li et al., 1990; Schott et al., 1990; Hagar et al., 1991) and humans (Muller et al., 1990; Yellon et al., 1993). Ischemic preconditioning has since been deemed the most powerful means of protecting ischemic myocardium (Lawson and Downey, 1993).

Despite overwhelming evidence in support of IPC (Lawson and Downey, 1993; Mentzer, 2000), surgeons remain reluctant to elicit brief ischemic insults to already diseased hearts prior to an ischemic intervention in the clinical setting. The main reason for this cautiousness is due to the lack of consensus on the duration and intensity of the IPC stimulus (Verdouw et al., 1995; Verdouw et al., 1997) and the risk of life-threatening postoperative contractile dysfunction in already sick patients (Heyndrickx et al., 1975; Braunwald and Kloner, 1982; Gross and Fryer, 1999; Mentzer, 2000). Though, ischemic preconditioning has been shown to protect against ventricular arrhythmias in most species tested including mice (Sakamoto et al., 1999), rats (Shiki and Hearse, 1987; Hagar et al., 1991; Wang et al., 2001),

rabbits (Cohen et al., 1994) and dogs (Kaszala et al., 1996), it has been shown that IPC does not protect from ventricular arrhythmias in a pig model of myocardial ischemia (Ovize et al., 1995; Shattock et al., 1996).

1.5.1. POSSIBLE MECHANISMS OF ISCHEMIC PRECONDITIONING

Ischemic preconditioning has become the paradigm of cardioprotection on which current injury-limiting pharmacological strategies are based (Fryer et al., 2002). Yet, the full mechanism of action of IPC remains unknown. Adenosine was the first arm of the IPC mechanism identified (Liu et al., 1991) and appears to act as both a trigger and mediator of ischemic preconditioning (Downey et al., 1993; Auchampach et al., 1997b; McCully et al., 2001). Adenosine acts on multiple receptor types and locations and has a wide range of effects making its precise mechanism for protection difficult to identify (Granger, 1997). By blocking adenosine receptors with a non-selective adenosine receptor antagonist (e.g. 8-p-sulfophenyltheophyline) during either a preconditioning stimulus or the prolonged ischemic episode abolishes any protective effect (Liu et al., 1991; Thornton et al., 1992; Tsuchida et al., 1993). The central role of adenosine appears to occur through cellular coupling of adenosine A_1 and/or A_3 receptors and PKC through the activation of ATP-sensitive potassium channels (K_{ATP}) by a G-protein mediated mechanism (Kirsch et al., 1990; Liu et al., 1991; Gross and Auchampach, 1992; Armstrong and Ganote, 1994; Liu et al., 1994; Headrick, 1996; Schulz et al., 2001). Adenosine's cardioprotective effects through receptor stimulation will be discussed in detail in the subsequent section on adenosine.

In addition to adenosine, there are a number of other potential receptor-dependent and independent triggers, intracellular signaling pathways and end effectors which

may be responsible for producing IPC-induced cardioprotection (Fig. 1.3) (Schulz et al., 2001). Receptor-dependent triggers include adenosine (Liu et al., 1991), bradykinin (Wall et al., 1994), G proteins (Thornton et al., 1993), phospholipases (Cohen et al., 1996), protein kinases (Ytrehus et al., 1994a), and opioids (Schulz et al., 2001). Receptor-independent triggers include free radicals (Das et al., 1999), nitric oxide (Lochner et al., 2000), tyrosine kinases (Fryer et al., 1999), and mitogen-activated protein kinases (Weinbrenner et al., 1997). Potential end effectors of IPC may involve energetic preservation and substrate metabolism, the Na^+/H^+ exchanger (Xaio and Allen, 2000), heat shock proteins (Sanada et al., 2001), cell volume regulation (Armstrong et al., 2001), and possibly tumor necrosis factor (Belosjorow et al., 1999). Others have reported that the repetitive acidosis accompanying the brief ischemic episodes may provide the IPC stimulus (Simkhovich et al., 1995; Lundmark et al., 1999). Figure 1.3 summarizes the current thoughts regarding the mechanistic pathways responsible for ischemic preconditioning.

1.5.1.1. ATP-sensitive potassium channels

The opening of both sarcolemmal and mitochondrial potassium channels has received increasing attention as both triggers and end effectors of IPC (Kong et al., 2001; Schulz et al., 2001). Cardiomyocyte ATP-sensitive potassium channels (K_{ATP} channels) were first identified by Noma (1983) in membrane patches prepared from isolated guinea pig ventricular myocytes. Noma (1983) hypothesized that opening of the sarcolemmal K_{ATP} channel (sarcK_{ATP} channel) may be an intrinsic cardioprotective response during ischemia that enhances ischemic action potential duration (APD) shortening through acceleration of phase 3 repolarization. Enhancing phase 3 repolarization would have the added benefit of inhibiting Ca^{2+} entry into the cell via L-

type channels thereby preventing Ca^{2+} overload and extending cell viability (Gross

and Peart, 2003). Cole et al. (1991) demonstrated that blocking sarcK_{ATP}
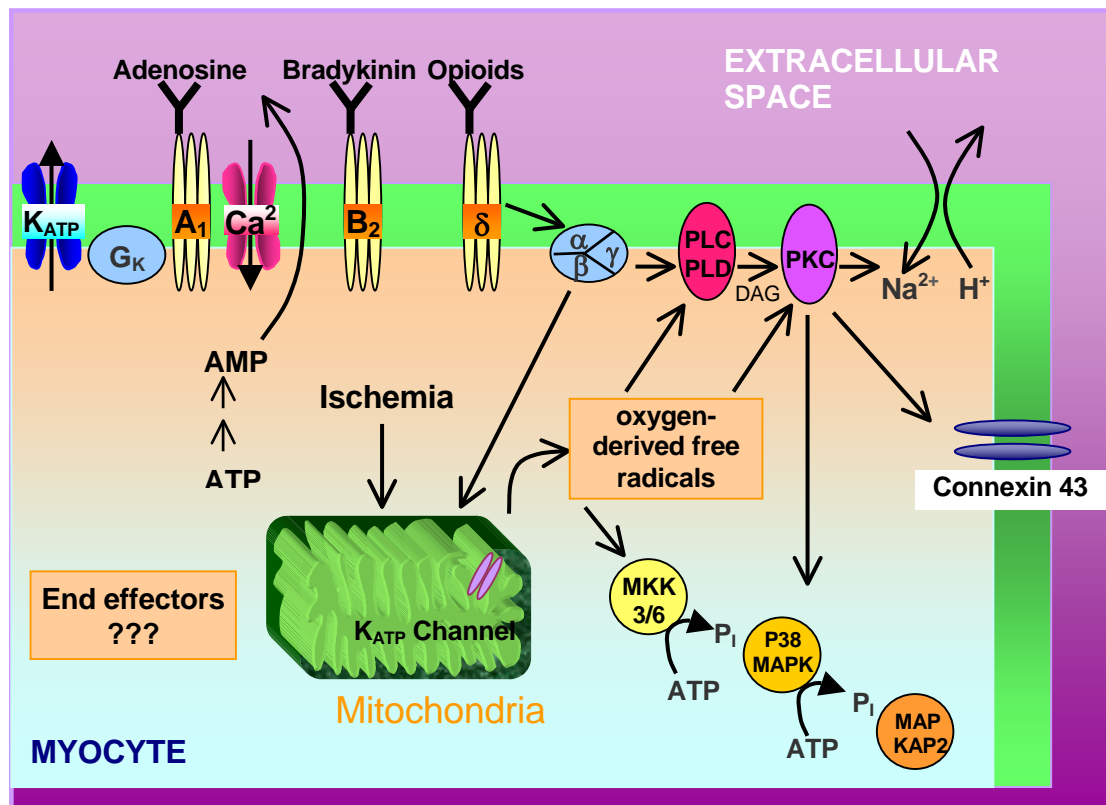
Figure 1.3. Schematic diagram of some current ideas on IPC signal transduction. Signal conduction in the early phase of ischemic preconditioning is likely due to receptor-dependent triggers such as adenosine, bradykinin, opioids, G-proteins, phospholipases and protein kinases; receptor-independent triggers such as free radical formation, and mitogen-activated protein activation; and potential end-effectors of IPC may be reduced energy demand, activation of the sodium-proton exchanger (Na^+/H^+), cell volume regulation, and tumor necrosis factor. Abbreviations: α, β, and γ symbols refer to different subunits on the G protein; DAG, diacylglycerol; G_K, refers to the G-protein associated with K_{ATP} channel opening; MAP, mitogen-activated protein; MKK, MAP kinase kinase; PKC, protein kinase C; PLC, phospholipase C; PLD, phospholipase D (adapted from Schulz et al., 2001).

channels with the nonselective K_{ATP} channel antagonist, glibenclamide, attenuated

ischemic APD shortening in isolated arterially perfused guinea pig right ventricular

wall preparation and resulted in impaired recovery of ventricular function during reperfusion. Conversely, in the same study it was reported that K_{ATP} channel opening by pinacidil resulted in accelerated APD shortening and improved recovery of ventricular function.

The hypothesis that APD shortening is one of the cardioprotective mechanisms of metabolic stress has gained support (Nichols et al., 1991; Edwards and Weston, 1993; Hearse, 1995) and it has been demonstrated that ischemic preconditioning leads to APD shortening (Schulz et al., 1994; Yao and Gross, 1994a). The first evidence for a role of the K_{ATP} channel in acute IPC in the canine heart was provided by Gross and Auchampach (1992). Moreover, Yao and Gross (1994a) demonstrated that the APD shortening could be inhibited glibenclamide. However, other than APD shortening, protection by K_{ATP} channel activation may result in hyperpolarization which may be an energy sparing mechanism (Nichols et al., 1991; O'Rourke, 2000; Suzuki et al., 2002) and may even lead to activation of the mitochondrial K_{ATP} channel (Gross and Peart, 2003).

The anomaly of K_{ATP} channel opening is that K^+ efflux is associated with harmful electrophysiological changes and ventricular arrhythmias (Gettes et al., 1963; Janse and Wit, 1989; Gettes and Cascio, 1992; Coronel, 1994). It may be assumed that activation of the K_{ATP} channel may enhance K^+ efflux during ischemia. However, Kanda et al. (1997) demonstrated that K_{ATP} channel activation by pinacidil in the *in situ* pig heart and the perfused rabbit intraventricular septum decreased the rise in K^+ efflux associated with ischemia. Recent work by Hu et al. (2003) indicates that excessive K^+ loss through the K_{ATP} channel may be down-regulated by adenosine-mediated PKC activation placing a 'brake' on ischemic K^+ efflux. Though this work

was performed in hippocampal CA1 neurons under metabolic stress, Hu et al.

suggests their findings have physiological implications in cardiac tissue.

Opening the mitochondrial K_{ATP} channel (mitoK$_{ATP}$ channel) has also been shown to

be an effective cardioprotective strategy and is believed to have a central role in IPC

(Yao et al., 1997; Fryer et al., 2000); although, unlike the sarcK$_{ATP}$ channel, the

mitoK$_{ATP}$ channel has not been cloned and its molecular structure remains unknown

(Gross and Peart, 2003). Furthermore, there is controversy regarding whether the

mitochondrial K_{ATP} channel is an end effector of IPC (Garlid et al., 1997; Liu et al.,

1998) or a primary trigger (Pain et al., 2000). The first direct evidence supporting the

role of the mitoK$_{ATP}$ channel was provided by Garlid et al. (1997). They showed that

diazoxide opening of mitoK$_{ATP}$ channels at concentrations that did not activate

sarcK$_{ATP}$ channels produced pronounced cardioprotection as evidenced by an

increasing time to ischemic contracture and enhancing functional recovery following

global ischemia and reperfusion in isolated rat hearts. Furthermore, these effects

were comparable to the effect of the K_{ATP} channel opener cromakalim. The

mechanism of action of mitoK$_{ATP}$ channels is likely to involve the translocation of

specific PKC isoforms which phosphorylate the channel and open it during the

transient ischemic insult leading to protection from the prolonged ischemic insult

(Wang and Ashraf, 1999; Liu et al., 2002; Gross and Peart, 2003). Additionally,

opening the mitoK$_{ATP}$ has been associated with free radical bursts which may act as a

trigger of cardioprotection (Vanden Hoek et al., 1998; Carroll et al., 2001; Forbes et

al., 2001).

1.5.2. PHASES OF ISCHEMIC PRECONDITIONING CARDIOPROTECTION

Cardioprotection from ischemic preconditioning occurs in two different time frames. An early phase ('classical' preconditioning) of protection lasts 1 to 2 hrs while a late phase of protection reappears at 18 – 24 hrs and lasts for up to 72 hrs (Baxter et al., 1997). The signaling pathways involved in early and late phase preconditioning are believed to share common elements; although, the early phase is thought to be the result of posttranslational modifications while the late phase may involve changes in gene expression and thus affect the quantity of cardioprotective proteins (Gross and Peart, 2003).

1.5.3. PROTECTIVE OUTCOMES OF ISCHEMIC PRECONDITIONING

Among its protective outcomes, IPC has been shown to affect the balance between ATP supply and ATP utilization (Murry et al., 1986; Reimer et al., 1986; Murry et al., 1990; Barry, 1991; Steenbergen et al., 1993; Zucchi et al., 1995; Kolocassides et al., 1996; Przyklenk and Kloner, 1998; Kawabata et al., 2000; Kuzmin et al., 2000; Mentzer, 2000). It has been shown that ischemic preconditioning reduces the rate of adenosine triphosphate (ATP) depletion (Murry et al., 1990; Volovsek et al., 1992), spares heart glycogen and decreases anaerobic glycolysis (Wolfe et al., 1993; Weiss et al., 1996) with reduced production of lactate (van Wylen, 1994; Jennings et al., 2001), hydrogen ions and inorganic phosphate (Kida et al., 1991; de Albuquerque et al., 1994; Garnier et al., 1996), and reduces intracellular Ca^{2+} influx by reduced stimulation of Na^+/H^+ and Na^+/Ca^{2+} exchange (Steenbergen et al., 1993) and intracellular Na^+ accumulation (Imahashi et al., 2001). In addition, others have shown that IPC reduces the accumulation of toxic catabolites (van Wylen, 1994).

1.6. Pharmacological targets based on the known mechanisms of ischemia-reperfusion injury and ischemic preconditioning

Over the past decade, considerable research has focused on pharmacological strategies to protect the myocardium from ischemia-reperfusion injury by targeting cell receptors (e.g., adenosine, opioid, α- and β-adrenergic, M_2 muscarinic, endothelin-1) (Lasley and Mentzer, 1998; Vinten-Johansen et al., 1999; Fryer et al., 2002), channels (e.g., Na^+ fast, sarcolemmal K_{ATP} and mitochondrial K_{ATP}, Cl^-, Ca^{2+}) (McCully et al., 1998), exchangers (e.g., Na^+/H^+, Na^+/Ca^{2+}) (Avkiran, 2001), heat shock proteins (Mubagwa and Willem, 2001), nitric oxide pathways (Vinten-Johansen et al., 1999; Zhao and Vinten-Johansen, 2002), and intracellular signaling pathways (e.g., protein kinase C, tyrosine protein kinase, guanylate cyclase and mitogen-activated protein kinase) from ischemia-reperfusion injury (Baxter and Ferdinandy, 2001). Chambers and Hearse (1999) have summarized the general areas interventions have targeted (Chambers and Hearse, 1999):

(i)   limiting cellular acidosis and the accumulation of toxic metabolites;

(ii)   modifying the rate of cellular energy depletion including increasing substrate delivery;

(iii)   manipulating ion imbalances;

(iv)   modifying the regulation of various enzymes and proteins;

(v)   preventing the loss of potassium and other essential ionic components together with other enzymatic cofactors and essential trace elements;

(vi)   limiting damage to nuclear material particularly during reperfusion;

(vii)   reducing the activation of lytic enzymes;

(viii) countering cell swelling and the accompanying membrane disruption that leads to severe ultrastructural changes.

1.7. Adenosine

The role of adenosine in the human heart has been studied for over 70 years (Drury and Szent-Gyorgi, 1929; Honey et al., 1930). Drury and Szent-Gyorgi (1929) administered intravenous bolus doses of adenosine to anesthetized dogs and observed consistent sinus rate slowing to approximately 50% of baseline. In 1930, Honey et al. reported that intravenous administration of adenosine to normal humans resulted in transient sinus bradycardia and atrioventricular conduction block. However, in patients with chronic atrial fibrillation, adenosine (0.5 to 100 mg) failed to terminate the arrhythmias. Honey et al. concluded that the therapeutic use of adenosine for the treatment of arrhythmias was not promising. In 1933, Jezzer et al. reported temporary termination of paroxysmal supraventricular tachycardia for 40 sec in a patient. Yet it wasn't until the 1950s when interest in adenosine was rekindled following the successful treatment of 214 episodes of paroxysmal supraventricular tachycardia with ATP (Somlo, 1955).

For over 20 years, adenosine has been recognized as a key component of the autoregulation of coronary and organ blood flow as an effective vasodilator (Berne, 1980; Feigl, 1983). Adenosine has been referred to as a "retaliatory metabolite" because it acts as a negative feedback regulator of the cells which counters the imbalance between tissue oxygen demand and oxygen supply (Newby, 1984). As a retaliatory metabolite adenosine reduces cellular work and initiates responses that redress the organ supply and demand mismatch through vasodilatation and reduction of ATP utilizing activities (Newby, 1984; Bruns, 1990; Belardinelli and Shyrock, 1992). As the oxygen supply-demand ratio falls and the levels of adenosine from cardiac myocytes are raised, adenosine acts to increase oxygen delivery by dilating coronary vessels (Newby, 1984; Sparks and Bardenheurer, 1986; Deussen and Schrader,

1991; Gorman et al., 1997) and by depressing heart rate, contractility and inhibiting

sympathetic neural activity (Schrader, 1990; Belardinelli and Shyrock, 1992).

Adenosine binds to at least four distinct receptor subtypes located on different cell

types including myocytes, neutrophils, and endothelial cells (Sommerschild and

Kirkeboen, 2000). Details of these subtypes will be discussed in upcoming sections.

In myocytes, adenosine or adenosine receptor agonists decrease glycolysis, ATP

depletion (Ely et al., 1985), acidosis (Lasley et al., 1990; Lasley and Mentzer, 1992;

Fralix et al., 1993), and lactate accumulation (Cave et al., 1993; Fralix et al., 1993;

van Wylen, 1994). Adenosine activation of the A_1 receptor appears to open K^+-

sensitive ATP channels, which may be an intrinsic energy sparing mechanism during

regional ischemia by stimulating outward potassium conductance while inhibiting

calcium conductance (Hu et al., 1999; Vinten-Johansen et al., 1999; O'Rourke,

2000). During ischemia, adenosine has been reported to inhibit catecholamine

release (Schrader et al., 1977; Carlsson et al., 1985), reduce adrenergic effects via

inhibition of adenylate cyclase (West et al., 1986), slow heart rate (Clemo et al.,

1987), slow the onset of ischemic contracture (Lasley et al., 1990), and inhibit Ca^{2+}

overload (Fralix et al., 1993) especially catecholamine induced Ca^{2+} overload (Fenton

et al., 1991).

Endogenous adenosine as well as exogenous adenosine administration results in

suppression of neutrophil superoxide generation, degranulation and neutrophil

adherence (Cronstein et al., 1983; Cronstein et al., 1985; Cronstein et al., 1992).

Additionally, adenosine has been linked to the generation of free radicals and

localized endothelial damage (Cronstein et al., 1986). Furthermore, adenosine

applied during pretreatment, ischemia and/or reperfusion has been shown to improve

cardiac functional recovery (Cronstein et al., 1986; Lasley and Mentzer, 1992; van

Wylen, 1994; Yao and Gross, 1994a; Lasley and Mentzer, 1995; Vander-Heide and Reimer, 1996; Granger, 1997; McCully et al., 1998) and reduce infarct size (Toombs et al., 1992; Yao and Gross, 1994b; Zhao et al., 1999).

Thus, adenosine's multiple down-regulating effects and broad-spectrum properties form the basis for its use as a cardioprotective agent (Vinten-Johansen et al., 1999). Adenosine has been utilized in a number of other cardiovascular interventions from cardioplegia to pretreatment and as a cardioprotectant during reperfusion (Olafsson et al., 1987; Kirsch et al., 1990; Liu et al., 1991; Downey, 1992; Downey et al., 1993; Zhao et al., 1993; Lasley et al., 1995; Lasley and Mentzer, 1995; Randhawa et al., 1995; Headrick, 1996; Dobson and Jones, 2003). While adenosine is used clinically as a first-line therapy for the elimination of supraventricular tachycardia (Di Marco et al., 1985; Wilbur and Marchlinski, 1997), many clinicians are reluctant to use adenosine for myocardial ischemia-reperfusion injury arguing poor cardioselectivity with potential to result in untoward systemic vasodilatation and hypotension before cardioprotection is manifest (Perrault and Menasche, 1999).

1.7.1. ADENOSINE SYNTHESIS DURING NORMOXIA, HYPOXIA AND ISCHEMIA

Adenosine is an endogenous purine nucleoside comprised of an adenine and ribose joined by a glycosidic bond. Adenosine has a ubiquitous presence in all cells as a precursor to the adenine nucleotides: ATP, ADP, and AMP (Engler and Gruber, 1992; Shyrock and Belardinelli, 1997). These adenine nucleotides may be released from a variety of cells (e.g. cardiomyocytes, endothelial cells, platelets, and neutrophils) or adrenergic nerve endings (Engler and Gruber, 1992). Under normal physiologic conditions, adenosine is generated both intracellularly and extracellularly in the heart by the hydrolysis of AMP to adenosine by membrane bound ecto-5'-

nucleotidases or cytosolic endo-5'-nucleotidases (Pearson et al., 1980; Gordon, 1986) and the catabolism of *S*-adenosyl homocysteine (Lloyd et al., 1988; Deussen et al., 1989; Kroll et al., 1993). Moreover, adenosine produced in the cytosol can enter the interstitial space by crossing the myocyte sarcolemma and enter endothelial cells through a nucleoside transporter (Conant and Jarvis, 1994) (Fig. 1.4).

During normal basal conditions, oxidative metabolism requires maximal oxygen extraction from coronary blood; hence, ATP synthesis is most effectively promoted by increasing coronary blood flow (Olsson, 1988). An interruption of oxygen delivery causes a disruption of the balance between ATP production and its consumption. As a result ATP concentrations decline while ADP and P_i levels increase (Opie, 1998). ADP is metabolized further to AMP which is further dephosphorylated forming adenosine (Bunger and Soboll, 1986; Smolenski et al., 1992). In conditions where there is net cardiac ATP catabolism resulting from decreased oxygen supply, as in hypoxia or ischemia, or accelerated ATP usage, as in excessive catecholamine drive, adenosine can be formed by a cytosolic 5'-nucleotidase activated by ADP, inhibited by ATP and that preferentially chooses AMP over IMP as substrate (Yamazaki et al., 1991; Engler and Gruber, 1992; Darvish and Metting, 1993; Shyrock and Belardinelli, 1997).

1.7.1.1. Vascular Adenosine Synthesis

Larger amounts of adenosine are produced by cardiomyocytes in the ischemic or hypoxic heart than are produced by the vascular endothelium (Bardenheurer and Schrader, 1986; Deussen et al., 1986; Borst and Schrader, 1991). However, adenosine derived from the endothelium has been shown to be an important site of adenosine production, for the ATP content of human endothelial cells is 2 to 3 times

higher than in other cell types including cardiomyocytes (Smolenski et al., 1994).

Endothelial cells employ all the key enzymes needed for the formation and

metabolism of adenine compounds (Pearson et al., 1980; Nees et al., 1985; Deussen

et al., 1986). Like cardiomyocytes and other cells, adenosine is also released from

endothelial cells during times of stress such as ischemia or hypoxia (Deussen et al.,

1986; Borst and Schrader, 1991).

The adenosine formation pathway differs in endothelial cells from cardiomyocytes.

Endothelial derived adenosine is chiefly formed by the *extracellular* metabolism of

AMP by 5'-ecto-nucleotidase rather than the *intracellular* breakdown of AMP by

cytosolic 5'-nucleotidase (Borst and Schrader, 1991; Smolenski et al., 1992).

1.7.1.2. Adenosine metabolism

As well as being an important source of adenosine, the endothelium is also a

metabolic barrier (Nees et al., 1985; Kroll et al., 1989). Exogenously administered

adenosine at concentrations below 1 μM do not cross the endothelial barrier to reach

the interstitium and therefore do not come into direct contact with the myocytes (Nees

et al., 1985; Kroll et al., 1989; Nees, 1989). The endothelium regulates adenosine

exchange between the myocytes and itself (Mullane and Bullough, 1995). Adenosine

is likely to be "compartmentalized" between myocyte and endothelial cells where it is

independently regulated by the endothelium's rapid metabolism of the nucleoside on

both the abluminal and luminal sides preventing exchange of adenosine between the

two compartments (Schrier, 1977; Nees et al., 1985; Kroll et al., 1989). Nees et al.

(1985) and Balcells et al (1992) used adenosine analogs that were polyadenylylated

on the ribose sugar or attached to dextran or carboxylated latex microspheres so that

the large size of the molecules prevented adenosine from leaving the vascular

compartment (Balcells et al., 1992). They found that the analogs induced many of the pharmacological properties of adenosine in the heart such as coronary vasodilatation, bradycardia, and decreased ventricular contractile activity and glycolytic flux. These activities were blocked by the nonselective adenosine antagonist, 8-SPT, indicating that the effects were mediated by adenosine receptors on the endothelial cell surface.
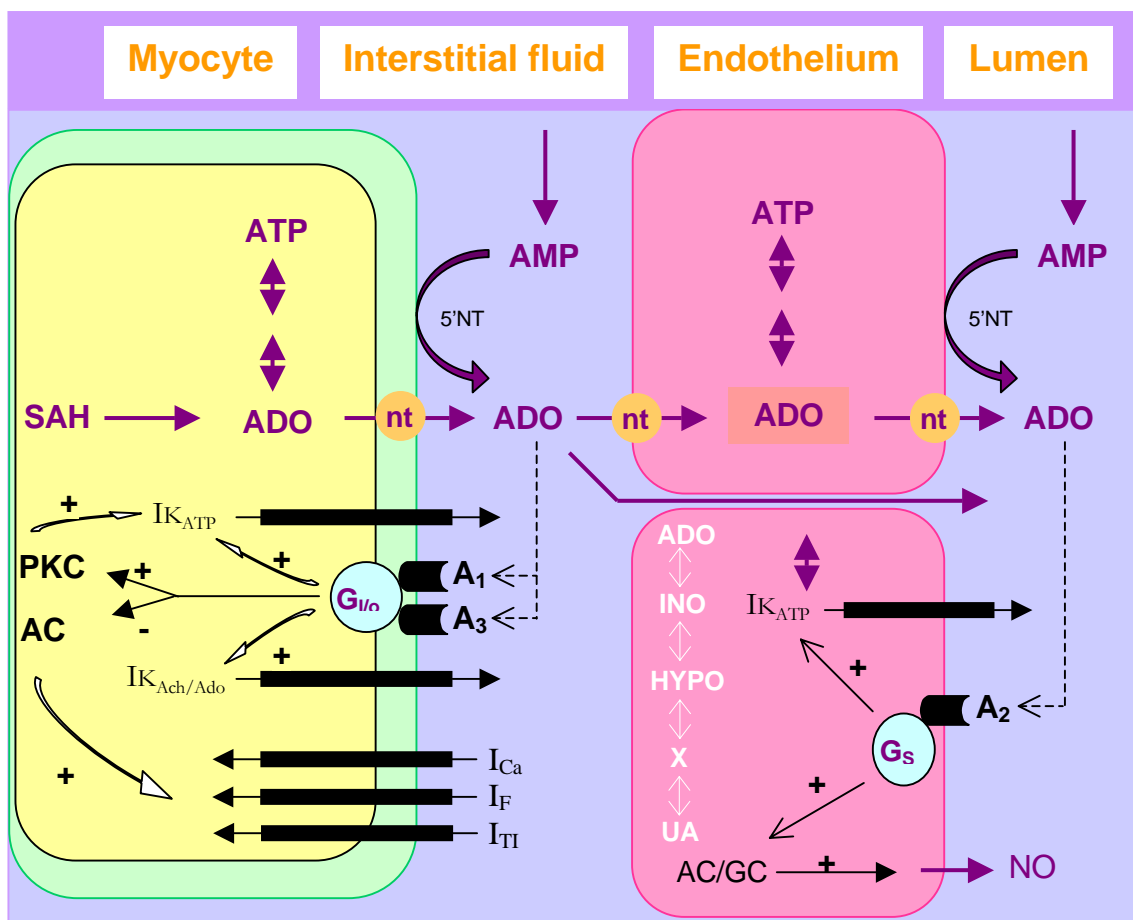
Figure 1.4. Adenosine formation, metabolism and receptor-effector coupling. Abbreviations: A_1, A_2, A_3, adenosine receptors; AC, adenylate cyclase; ADO, adenosine; AMP, adenosine monophosphate; GC, guanylate cyclase; $G_{i/o}$, inhibitory G protein; G_S, stimulatory G protein; HYPO, hypoxanthine, I_{KATP}, ATP-sensitive K^+ channel; $I_{Ach/Ado}$, inward rectifying K^+-channel current; I_{Ca}, L-type Ca^{2+} channels; I_F, time- and voltage-dependent inward current activated by hyperpolarization (pacemaker current); INO, inosine; I_{TI}, transient inward currents by catecholamines; 5'-NT, ecto-5'-nucleotidase; nt, facilitated diffusion; nitric oxide (NO); SAH, *S*-Adenosylhomocysteine; UA, uric acid; X, xanthine (adapted from de Jonge et al., 2000).

Extracellular adenosine is taken up into neighboring cells through a specific transmembrane nucleoside carrier system (Paterson and Oliver, 1971; Plagemann and Wohlhueter, 1980). At low concentrations (< 1 μM), adenosine is sequestered into the endothelial cells where 90-92% is rapidly phosphorylated by adenylate kinase and incorporated into adenine nucleotide pools or degraded (Nees et al., 1985; Deussen et al., 1988a; Kroll et al., 1989). However, during ischemic conditions when adenosine concentration rises from about 0.8 μM to about 2 μM, adenosine deaminase may preferably metabolize adenosine to inosine because it has a higher capacity for adenosine removal than adenosine kinase (Fox and Kelley, 1978; Deussen et al., 1988b), adenosine kinase requires ATP (Newby et al., 1983), and adenosine kinase is substrate inhibited at concentrations higher than 2 μM (Ashby and Holmsen, 1981). Likewise, intracellular adenosine is either salvaged through phosphorylation to AMP by adenosine kinase or deaminated to inosine by adenosine deaminase. Adenosine monophosphate is incorporated into the high-energy phosphate pool (Engler and Gruber, 1992). Inosine can diffuse into the extracellular space and be further catabolized to hypoxanthine, xanthine, and uric acid (Jennings and Reimer, 1981).

In summary, adenosine is either rephosphorylated to AMP by adenosine kinase or deaminated to inosine by adenosine deaminase or intracellular adenosine released from the cell and is taken up by a nucleoside transporter into endothelial cells and further metabolized. The effects of adenosine are localized and dissipate quickly due to adenosine's short half-life of less than 8-10 sec in human blood making exogenously administered adenosine 'the drug with the shortest half life' of any used in medicine (Moser et al., 1989).

1.7.2. PHARMACOLOGY OF ADENOSINE AND ADENOSINE RECEPTORS

In the mid-1960s the work of deGubareff and Slentor provided the first evidence for the existence of adenosine receptors by showing that caffeine antagonizes the effects of adenosine in heart tissue (Fredholm et al., 1994). Adenosine receptor stimulation has universal protective properties against ischemia-reperfusion injury affecting not only the heart but also the skeletal muscle, kidney, brain and intestine (Stromski et al., 1988; Daval et al., 1989; Engler, 1991). As already discussed briefly, adenosine's actions are mediated by binding to cell surface receptors on ventricular myocytes, supraventricular cells and to cells within the vascular compartment (Mullane and Bullough, 1995; Sommerschild and Kirkeboen, 2000). While, the adenosine A_1 and A_2 receptors were originally defined as eliciting decreases or increases in intracellular cyclic AMP (cAMP) levels in brain tissue (Londos and Wolfe, 1977; van Calker et al., 1979), advances in technology have allowed the cloning and sequence analysis of a number of adenosine receptors linked to the cAMP pathway in addition to the A_1 and A_2 receptors. Genetic analysis has shown that at least four subtypes (A_1, A_{2a}, A_{2b}, and A_3) are expressed in cardiomyocytes; although, the A_2 adenosine receptor is most prevalent in coronary vessels (Erion, 1993; Tucker and Linden, 1993; Auchampach and Bolli, 1999; Mubagwa and Willem, 2001). Separate activation of each adenosine receptor subtypes have been shown to couple to a complex intracellular signaling pathway (Sommerschild and Kirkeboen, 2000). Furthermore, the concomitant activation of these receptor subtypes by adenosine provokes a highly complex interrelated set of reactions (Sommerschild and Kirkeboen, 2000).

1.7.2.1. Adenosine A_1 receptor

Interest in the A_1 receptor was sparked with the finding by Liu and coworkers that IPC-induced cardioprotection could be abrogated by nonselective adenosine receptor antagonists (Liu et al., 1991). They also reported that A_1 specific agonists induced cardioprotection similar to IPC. Others have provided evidence of A_1 adenosine receptor cardioprotection (Lasley et al., 1990; Toombs et al., 1992; Yao and Gross, 1993; van Wylen, 1994; Zhao et al., 1994; de Jonge and de Jonge, 1999; de Jonge et al., 2002). Stimulation of the adenosine A_1-receptor subtype is known to confer protection via inhibitory G protein-coupled pathways which has been linked to the opening of sarcolemmal ATP sensitive K^+ channels, increased potassium conductance, action potential shortening and reduced Ca^{2+} entry into ischemic cells (Kirsch et al., 1990; Gross and Fryer, 1999).

The A_1 adenosine receptor is found in high concentrations in the heart, parts of the brain, spinal cord, testis, and kidneys where it is coupled to GTP-binding proteins (Shyrock and Belardinelli, 1997). Stimulation of the A_1 receptor in both atrial and ventricular cells mediates indirect anti(β)-adrenergic effects through reduction of cyclic adenosine monophosphate (cAMP) (Munshi et al., 1991; Belardinelli et al., 1995; Sommerschild and Kirkeboen, 2000). Activation of the A_1 adenosine receptor leads to G-protein linked inhibition of adenylyl cyclase which reduces cAMP and protein kinase phosphorylation of proteins including the L-type Ca^{2+}-channel (Belardinelli et al., 1995). In atrial and nodal cells, A_1 adenosine receptor activation increases an inward-rectifying potassium channel current, I_{K-Ado} (Belardinelli and Isenberg, 1983; Belardinelli et al., 1995).

In 1990, Kirsch et al. reported that A_1 receptors in rat ventricular myocytes were coupled to the ATP sensitive potassium (K^+_{ATP}) channel through G-proteins (Kirsch et al., 1990). Stimulation of the A_1 receptor activates K^+_{ATP} channels in isolated cardiomyocytes and causes an outward conductance of potassium inducing hyperpolarization of the cell membrane which may make the membrane more resistant to action potential formation (Trussel and Jackson, 1985; Belardinelli et al., 1995). The opening of K^+ channels by endogenous adenosine may be an intrinsic energy sparing mechanism during regional ischemia (Galinanes et al., 1992). Furthermore, K^+ efflux inhibits Ca^{2+} conductance thereby reducing Ca^{2+} uptake (Scholz et al., 1991; Zhu and Ikeda, 1993).

Adenosine A_1 receptor agonism is also linked to PKC activation and translocation and may lead mitochondria K_{ATP} channel opening (O'Rourke, 2000). Adenosine has also been reported to activate protein kinase C (PKC) in ventricular myocytes (Henry et al., 1996; Hu et al., 1996). An initial ischemic event, as in ischemic preconditioning, is believed to release adenosine which mediates the translocation of PKC from the cytosol to the cell membrane in close association with the adenosine receptor (Ytrehus et al., 1994a). Protection appears to be lost when PKC is released from the membrane back to the cytosol (Ytrehus et al., 1994a). Furthermore, protein kinase C inhibitors have been shown to block the infarct size limiting effects of ischemic preconditioning while activators (phorbol esters or diacylglycerol analogues) mimic cardioprotection (Speechly-Dick et al., 1994; Ytrehus et al., 1994a). There is increasing evidence that the mitochondrial K_{ATP} channel plays a cardioprotective role in the ischemic preconditioning mechanism linked with the opening of the A_1 linked K_{ATP} channel (McCullough et al., 1991; Auchampach et al., 1992; Sato et al., 2000).

In addition to adenosine's effect on the cardiomyocyte, activation of A_1 receptors also slows the sinoatrial (SA) nodal pacemaker rate (negative chronotropy), delays atrioventricular (A-V) nodal impulse conduction (negative dromotropy) and reduces atrial contractility (negative inotropy) (Lerman and Belardinelli, 1991; Shyrock and Belardinelli, 1997; Vinten-Johansen et al., 1999).

1.7.2.2. The A_{2a} and A_{2b} receptors

In contrast to A_1 adenosine receptor activation, the activation of A_2 adenosine receptors results in stimulation of adenylyl cyclase and increases in cAMP (Mullane and Bullough, 1995; Shyrock and Belardinelli, 1997) as well as in calcium dependent chloride conductance possibly through the stimulation of phospholipase C (Matsuura and Ehara, 1992; Yakel et al., 1993). Two subtypes of the A_2 adenosine receptors, A_{2a} and A_{2b}, have been identified in cardiovascular tissue (Rivkees and Reppert, 1992; Iwamoto et al., 1994; Ongini and Fredholm, 1996; Marala and Mustafa, 1998). Both A_{2a} and A_{2b} adenosine receptors are positively coupled to adenylyl cyclase and have been observed to antagonize the anti-adrenergic effects of A_1 receptors (Liang and Haltiwanger, 1995). However, recently, Norton and colleagues (Norton et al., 1999) reported A_{2a} receptor expression in ventricular cardiomyocytes. They showed that adenosine's A_1 activated anti-adrenergic effects were enhanced in the presence of A_{2a} selective antagonists; therefore, Norton and colleagues concluded that the A_{2a} receptor activation had pro-adrenergic effects counteracting the anti-adrenergic effects of A_1 activation. Moreover, the A_{2a} receptor has been implicated in mediating adenosine's vasodilation effect (Alberti et al., 1997; Shyrock and Belardinelli, 1997; Monopoli et al., 1998).

While A_{2b} receptors are thought to initiate similar actions to the A_{2a} receptors, a unique feature of the A_{2b} adenosine receptor response is that they also couple to mitogen-activated protein kinase pathways (Auchampach et al., 1997a) and may have a cardioprotective role in cardiac fibrosis inhibiting the growth of cardiac fibroblasts (Dubey et al., 1998).

The activation of A_2 adenosine receptors, in general, have been shown to be beneficial to the reperfused myocardium by inhibiting neutrophil (Cronstein et al., 1990; Wollner et al., 1993; Firestein et al., 1995; Jordan et al., 1997) and platelet activation (Hourani and Cusack, 1991) as well as preserving endothelial cell integrity and function. (Hourani and Cusack, 1991; Forman et al., 1993; Schlack et al., 1993; Zhao et al., 1993; Zhao et al., 1996). Upon A_2 stimulation neutrophil adhesion is attenuated by both selectin and integrin dependent mechanisms (Wollner et al., 1993; Bullough et al., 1994; Firestein et al., 1995). In addition, A_2 activation has inhibited neutrophil-derived oxygen metabolite formation (Cronstein et al., 1990). The inhibition of platelet aggregation by A_2 receptor activation may be mediated by the stimulation of adenylate cyclase activity (Hourani and Cusack, 1991).

1.7.2.3. Adenosine A_3 receptor

Cardioprotection by adenosine has generally been attributed to the A_1 and A_{2a} adenosine receptors (Lasley and Mentzer, 1992; Thornton et al., 1992; Toombs et al., 1992; Tsuchida et al., 1993; Yao and Gross, 1993); though, A_3 receptors are increasingly reported to be involved (Armstrong and Ganote, 1994; Liu et al., 1994; Strickler et al., 1996; Auchampach et al., 1997b; Thourani et al., 1999). Adenosine A_3 receptors have been identified in heart tissue and are believed to be expressed on endothelial cells and myocytes (Vinten-Johansen et al., 1999). Similar to the A_1

adenosine receptor, the activation of the A_3 adenosine receptor inhibits adenylate cyclase activity (Zhou et al., 1992; Salvatore et al., 1993) and stimulation of PKC (Armstrong and Ganote, 1994) and its effects may be mediated through K_{ATP} channels (Thourani et al., 1999). The cardioprotective effects of A_3 adenosine receptor activation include an ability to mimic or induce myocardial preconditioning, (Armstrong and Ganote, 1994; Liu et al., 1994; Tracey et al., 1997) reduce infarct size (Auchampach et al., 1997b) and myocardial stunning (Auchampach et al., 1997b) as well as attenuate postischemic dysfunction (Thourani et al., 1999). Some studies in rats have shown that both A_1 and A_3 receptors mimic IPC (Liem et al., 2001; de Jonge et al., 2002). Furthermore, Jordan et al. (1998) investigated the role of the A_3 receptor in neutrophil-endothelial cell interactions. The selective A_3 agonist C1-IB-MECA decreased neutrophil adherence to the coronary artery endothelium; though, the receptor-ligand interaction did not significantly inhibit superoxide anion generation by neutrophils in this study.

Some argue that research into the properties of A_3 activation has been hampered by lack of specific A_3 antagonists (Guo et al., 2001). Guo and coworkers (2001) showed that knock-out mice lacking functional A_3 adenosine receptors are more resistant to the development of irreversible ischemic injury leading them to conclude that A_3 receptors may not be necessary for the development of the early phase of IPC. They also proposed that the exacerbation of injury in A_3 negative mice may be related to A_3 expression in mast cells which may have led to the release of pro-inflammatory mediators (e.g. histamine, cytokines, proteolytic enzymes, etc.) (Salvatore et al., 2000; Tilley et al., 2000; Guo et al., 2001).

It has also been suggested that the A_3 adenosine receptor mediates A_1 adenosine receptor protection (Giannella et al., 1997). Hill and coworkers (1998) have argued

that the adenosine component of IPC is likely mediated through the A_1 receptor

because adenosine binding to the receptor is with greater affinity than the A_3 receptor

in rabbit hearts (K_iA_1=28 nM; K_iA_3= 532 nM) (Hill et al., 1998; de Jonge et al., 2002).

Though at present, the binding affinities have not been determined for rat hearts.

The results of Headrick et al. (1996) in isolated rat hearts appears to suggest that the

high interstitial levels of adenosine in isolated rat hearts during IPC strongly imply that

A_1 and A_3 receptors would both be maximally activated.

1.8. Lidocaine

Lidocaine is a commonly used local anesthetic and antiarrhythmic drug for the

treatment of ventricular arrhythmias that occur during cardiac surgery or myocardial

infarction (Lesnefsky et al., 1989; Kojima and Miura, 1991; Teo et al., 1993; Lee et

al., 1998). The drug was developed by Nile Lofgren in 1943 and marketed in 1948 as

part of a chemical search for compounds with cocaine-like anesthetic properties but

without the toxic and addictive side-effects of cocaine (Ritchie and Greene, 1990).

Lidocaine and other licensed local anesthetics were screened for anti-arrhythmic

effects during early anti-arrhythmia drug development (Hancox et al., 2000). Since

the 1960s lidocaine has been used for treatment of ventricular arrhythmias

associated with AMI (Gianelli et al., 1967; Kuller et al., 1972). However, the

appropriateness of the prophylactic use of lidocaine for the prevention of arrhythmias

has been hotly debated in recent years (Tan and Kong, 1999). In 1996, the American

College of Cardiology/American Heart Association recommended against the

prophylactic use of lidocaine in AMI (Ryan et al., 1996) following conflicting reports

regarding its association with increased mortality (Koster and Dunning, 1985;

MacMahon et al., 1988; Alexander et al., 1999; Sadowski et al., 1999). Lidocaine's

maligned association with increased mortality rates was mostly based on one study

which suggested asystole was the more common cause of death in lidocaine-treated patients (MacMahon et al., 1988; Tan and Kong, 1999). However, upon closer inspection, asystole was the cause of death in only 1 lidocaine-treated patient and in 1 control patient, yet asystole was more common in lidocaine-treated patients (26 of 2987) than control subjects (13 of 3037) (Koster and Dunning, 1985). Therefore, the cause of lidocaine's association with increased mortality rates is unclear. Even meta-analyses of pooled studies did not show a statistical difference between the mortality rate of lidocaine-treated patients and control subjects (MacMahon et al., 1988). Larger drug trials of other anti-arrhythmatics, such as encainide, flecaninide, and mexilitene, an oral congener of lidocaine, also resulted in increased mortality rate from proarrhythmic drug effect, leading to a more critical evaluation of anti-arrhythmics overall (Group of international investigators (Impact Research Group), 1984; Investigators from the Cardiac Arrhythmia Suppression Trial, 1989; Tan and Kong, 1999). The multi-center randomized GUSTO trials in the late 1990s revealed that lidocaine use has declined during the thrombolytic era though Alexander et al. (Alexander et al., 1999) concluded that its use may not be associated with increased mortality rates. In contrast, and in the same volume of the American Heart Journal, Sadowski et al. (1999) reported that while lidocaine reduces ventricular fibrillation but it may adversely affect mortality (Sadowski et al., 1999). These two opposing views, with many of the authors in the same collaborative group, have not been adequately and rigorously addressed.

Despite the controversy regarding mortality prevention, lidocaine remains effective at protecting against ischemia-induced ventricular arrhythmias (Hine et al., 1989b; Hine et al., 1989a; Barrett et al., 1995) and is recommended for the management of ventricular tachycardia and fibrillation (Hebbar and Huesten, 2002).

1.8.1. ANTIARRHYTHMIC PHARMACOLOGICAL EFFECTS OF LIDOCAINE

Lidocaine exerts its anti-arrhythmic effect by reducing the magnitude of the inward Na^+ current in cardiac muscle (Colatsky, 1982; Bean et al., 1983; Eisner and Lederer, 1983) (Fig. 1.5). In the heart, lidocaine reduces automaticity by decreasing the rate of diastolic (phase 4) depolarization through binding to the inactivated state of voltage-gated Na^+ channels (Balser et al., 1996; Roden, 2001). Lidocaine also prolongs refactoriness by delaying recovery of the inactivated Na^+ channel (Fig 1.6). Lidocaine rapidly dissociates from the inactivated state of the Na^+ channel therefore lidocaine exerts greater effects in depolarized (e.g. ischemic) and excitable tissues (Roden, 2001). Lidocaine has little effect on cardiac conduction at normal heart rates but may slow conduction during tachyarrhythmias and lidocaine's Na^+ blocking activity acts preferentially on ischemic myocardium. Lidocaine is not effective for the treatment of atrial arrhythmias possibly because atrial action potentials are shorter and Na^+ channels are in the inactivated state for less time (Hondeghem and Katzung, 1984).
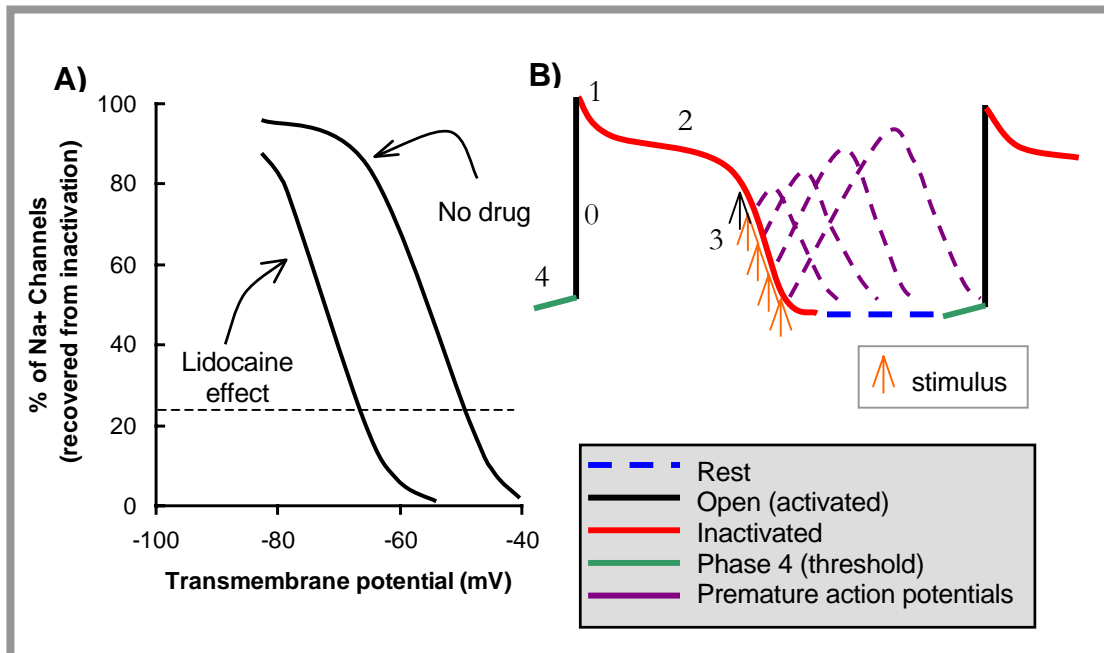
Figure 1.5. Effect of lidocaine on Na$^+$ channel recovery from inactivation in ventricular myocytes. A) Lidocaine shifts the relationship between the transmembrane potential and the degree of recovery of Na$^+$ channels to the left. The dotted line indicates 25% of the Na$^+$ channels have recovered from the inactivation state to the rest state. B) The arrows represent points of stimulus. The black arrow represents applying a premature stimulus while all the Na$^+$ channels are in the inactivated state and no upstroke results. However, as the action potential reporlarizes, some Na$^+$ channels recover from the inactivation state, which may allow Na$^+$ channel opening to occur. Also the phase 0 upstroke slope of the premature action potentials (*purple*) are greater with later stimuli because Na$^+$ channel recovery from inactivation is voltage-dependent (adapted from Rodan, 2001).

A. Normal ventricular action potential

Effective Refractory Period · no drug ▲ lidocaine

B. Lidocaine effect on action potential

action potential duration may be shortened

Rest
Open (activated)
Inactivated
Phase 4 (threshold)
Lidocaine effect

decreased phase 4 slope

threshold for excitability altered
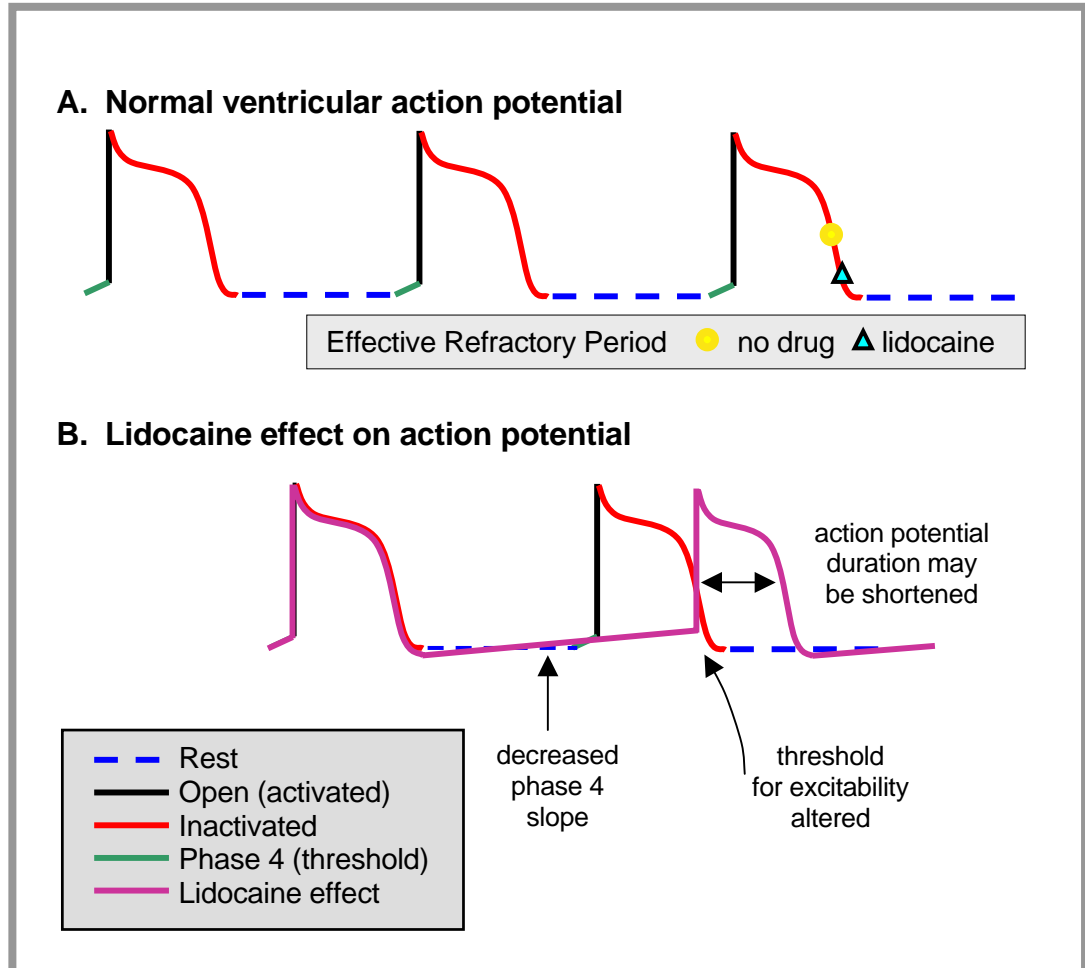
Figure 1.6. Diagram of the major features of lidocaine's effect on ventricular action potentials. A) The yellow circle indicates an arbitrary point at which it is assumed that a sufficient number of Na^+ channels have recovered from inactivation and can allow a premature stimulus to produce a propagated response. Block of inactivated Na^+ channels shifts the voltage dependence of recovery and delays the point at which a sufficient number of channels have recovered (*black and blue diamond*) resulting in prolonged refractoriness. B) Lidocaine reduces automaticity by decreasing the rate of diastolic (phase 4) depolarization, alters the threshold for excitability and may shorten the action potential duration (adapted from Roden, 2001).

Lidocaine appears to have little direct effect on the Na^+/Ca^{2+} exchanger (Eyolfson and Dhalla, 1989) though, lidocaine has been shown equivocally by a number of laboratories to inhibit Ca^{2+} conductance through the L-type Ca^{2+} channel (Volpe et al., 1983; Josephson, 1988; Murphy et al., 1991; Sugiyama and Muteki, 1994). Lidocaine suppresses arrhythmias by reducing the magnitude of the inward Na^+ current and shortening the cardiac action potential (Sheu and Lederer, 1985). The suppression of Na^+ ion entry also reduces intracellular Ca^{2+} entry which may contribute to its anti-arrhythmic effect (Sheu and Blaustein, 1992). As discussed earlier, an overload of calcium can have hazardous effects on myocytes (Nicotera et al., 1989;Barry, 1991) and the accumulation of Ca^{2+} can causes myofilament cycling leading to ectopic contractions (Steenbergen et al., 1990). Furthermore, calcium activates a variety of intracellular proteases, lipases, and phospholipases and augments ATP use by activating many ATPases (Tsokos and Bloom, 1977).

Lidocaine has also been shown experimentally to have Na^+ channel-independent effects which may contribute to its antiarrhythmic actions. In rat cardiomyocytes lidocaine has been reported to down-regulate the K_{ATP} channel (Olschewski et al., 1996). Down-regulating the K_{ATP} channel may contribute to lidocaine's antiarrhythmic effect through the attenuation of the ischemia-induced extracellular potassium accumulation which can lead to marked inhomogeneities of the refractory period and promote extrasystoles (Di Diego and Antzelevitch, 1993). Another Na^+ channel-independent effect of lidocaine was reported by Bernauer et al. (1995) who demonstrated that lidocaine attenuates adenosine release during reperfusion-induced arrhythmias only when ventricular fibrillation was abated in isolated rat hearts. The significance of this finding is unclear.

1.8.2. ADDITIONAL CARDIOPROTECTIVE PROPERTIES OF LIDOCAINE

Cardioprotection of the myocardium by lidocaine has been demonstrated in models of regional and global ischemia (Boudoulas et al., 1978; Nasser et al., 1980; Faria et al., 1983; Kyo et al., 1983; Matsumura et al., 1987). Lidocaine administration with ischemia-reperfusion has been reported to conserve high energy phosphates (Kojima and Miura, 1991;Butwell et al., 1993), attenuate myocardial acidosis (Kojima and Miura, 1991; Butwell et al., 1993), reduce intracellular Na^+ accumulation (Kojima and Miura, 1991; Butwell et al., 1993; van Emous et al., 1997), delay ischemic contracture (Butwell et al., 1993), and reduce infarct size (Lesnefsky et al., 1989; Vitola et al., 1997; Lee et al., 1998). Lidocaine has also been reported to suppress the decrease in mitochondrial ATP and pH common during ischemia and to restore ATP levels during reperfusion (Kojima and Miura, 1991).

Lidocaine may modulate the inflammatory response to ischemia-reperfusion injury by inhibiting the priming of human neutrophils and superoxide anion production with a suspected target site in a G_q–coupled signaling pathway (Vitola et al., 1997; Hollmann et al., 2001b). It has been demonstrated that lidocaine may afford protection to reperfused myocardium as a potent scavenger of hydroxyl radicals and singlet oxygen (Shlafer et al., 1982; Das and Misra, 1992). Reperfusion-induced oxygen-derived free radical generation is associated with postischemic contractile dysfunction and injury to the endothelium and microcirculation (Opie, 1989; Li et al., 1993; Sekili et al., 1993; Kaeffer et al., 1996; Gross and Fryer, 1999). Agents that either scavenge or inhibit the formation of superoxide anion and hydroxyl radical have been shown to improve recovery of contractile function in the dog following an ischemic insult (Myers et al., 1985; Gross et al., 1986; Przyklenk and Kloner, 1986; Bolli et al., 1987; Bolli et al., 1988; Farber et al., 1988; Sekili et al., 1993).

Lidocaine appears to have nonselective G protein activity (Xiong et al., 1999; Hollmann et al., 2001b) which may be related to its ability to inhibit neutrophil-mediated injury (Tomoda et al., 1990; Vitola et al., 1997; Fischer et al., 2001; Hollmann et al., 2001a) through suppression of the neutrophil respiratory burst (Hyvonen and Kowolik, 1998). In 1997, Nietgen and coworkers reported that lidocaine inhibited the G-protein coupled lysophosphatidate receptor (Nietgen et al., 1997). Lysophosphatidic acid (LPA) is an intercellular phospholipid mediator released by platelets and fibroblasts (Durieux and Lynch, 1993) with multiple actions linked to stimulation of inflammatory events such as platelet aggregation and neutrophil activation (Hollmann et al., 2001a).

1.9. Simultaneous adenosine and lidocaine for reperfusion therapy

By the mid-1980s, adenosine was well known to improve function and favorably affect biochemical alterations of myocardial ischemia and reperfusion, such as reducing ATP depletion during ischemia and improving ATP repletion during reperfusion (Ely et al., 1985; Mauser et al., 1985). Yet, conflicting reports were surfacing regarding adenosine's effect on the size of the experimental myocardial infarct. Olfasson and colleagues (Olafsson et al., 1987) showed that adenosine reduced reperfusion injury in a canine model of ischemia-reperfusion, as shown by infarct reduction following prolonged adenosine reperfusion therapy. Unable to repeat these findings, Homeister et al. (1990) examined Olfasson et al.'s protocol and identified that lidocaine, as well as morphine, diazepam and streptokinase, were also given to the animals. At the time lidocaine on its own had been shown to protect against myocardial ischemia-reperfusion injury (Schaub et al., 1977; Nasser et al., 1980; Lesnefsky et al., 1989) and influence neutrophil function (Schiffer et al., 1977; MacGregor et al., 1980; Peck et al., 1985) giving Homeister et al. good reason to

suspect that lidocaine may be contributing to the adenosine protection found by Olfasson and colleagues (Olafsson et al., 1987). Homeister et al. (1990) administered an intravenous bolus of lidocaine (2 mg/kg i.v.) in open-chest dogs 1 min before a 90 min occlusion of the left circumflex coronary artery and again 1 min before reperfusion followed by an adenosine intracoronary infusion (150 μg/kg/ml/min) at reperfusion for 1 hour. By comparing sequential treatment of adenosine and lidocaine at reperfusion against adenosine alone at reperfusion or lidocaine only treatment at reperfusion, Homeister and colleagues concluded that the sequential treatment of lidocaine and adenosine reduced infarct size (Homeister et al., 1990).

Other laboratories have attempted to confirm the findings of Homeister et al. with conflicting results. Using a similar canine model though administering adenosine intravenously, Vander-Heide and Reimer (1996) failed to reproduce Homeister et al.'s findings concluding that adenosine therapy (150 μg/ml/min i.v.) during reperfusion with or without lidocaine pretreatment did not limit infarct size after 90 min regional ischemia. They further reported that the rapid i.v. bolus of lidocaine failed to prevent lethal arrhythmias. In an attempt to investigate the issue further, Garratt et al. (1998) and Mahaffey et al. (1999) administered lidocaine and adenosine *sequentially and separately* in humans during balloon angioplasty and thrombolytic therapy respectively, but the results were again conflicting. Garratt et al. reported a potential benefit in 35 patients whereas a year later Mahaffey et al. in the larger trial entitled 'Acute Myocardial Infarction Study of Adenosine (AMISTAD)' involving 236 acute myocardial infarction patients concluded that the presence of lidocaine made no difference to the outcome of adenosine-treated patients in reducing infarct size.

Indeed, the clinical outcomes of the adenosine-treated group in the AMISTAD trials tended to be slightly worse than in the placebo group.

1.10. Summary of research aims

As stated in the beginning of this chapter the overall hypotheses are summarized as follows.

OVERALL HYPOTHESES

1. An adenosine and lidocaine solution (AL) will provide cardioprotection from ischemia-reperfusion injury by reducing mortality, decreasing ventricular arrhythmias and lowering infarct size.

2. Pharmacological preconditioning of the heart with AL solution will preserve ATP, phosphocreatine (PCr), and reduce the extent of acidosis (pH)

Recently, our laboratory demonstrated that an adenosine and lidocaine polarizing cardioplegic arrest solution conferred superior protection during arrest and recovery compared with hyperkalemic depolarizing St. Thomas' Hospital cardioplegic solution in isolated rat hearts (Dobson and Jones, 2003). The work presented in this thesis aimed to extend those findings by applying an adenosine and lidocaine solution (AL) as pretreatment and during ischemia in an *in vivo* rat model of acute myocardial ischemia.

The primary difference between the work of Homeister et al. (1990) and the use of adenosine and lidocaine in this thesis is that in the protocol of Homeister et al., adenosine and lidocaine were not given *simultaneously during the ischemic period.* Rather, the administration of adenosine and lidocaine was through *separate* routes at *separate* times and given *sequentially* at reperfusion. In the work presented in this

thesis, the use of adenosine and lidocaine was targeted specifically at ischemic injury based on our earlier findings using adenosine and lidocaine in a successful cardioplegia solution (Dobson and Jones, 2003). Likewise, lidocaine has never before been combined with an adenosine receptor agonist for protection from ischemia or reperfusion. The investigations presented here profile AL solution cardioprotection through the measurement of infarct size and ventricular arrhythmias (primary end-points) as well as changing concentrations of high-energy phosphates, ATP and phosphocreatine and pH.

This thesis is presented in the following sequence. Directly after a description of the materials and methods, the effect of AL solution on ventricular arrhythmias and cardiac death during 30 min ischemia is reported (Chapter 3). This is followed by an investigation of AL cardioprotection as ischemic vs reperfusion therapy (Chapter 4). Next, ^{31}P NMR spectroscopic analysis is used to evaluate the effect of AL solution on energetic metabolism and intracellular pH during acute myocardial ischemia-reperfusion (Chapter 5). To end the sequence of experiments presented in this thesis, pharmacological preconditioning from concomitant targeting of the adenosine A_1 receptor and modulation of sodium channels by lidocaine is shown to surpass AL solution cardioprotection (Chapter 6). Finally, this thesis concludes with a general discussion of all results.

Chapter 2. Materials and Methods
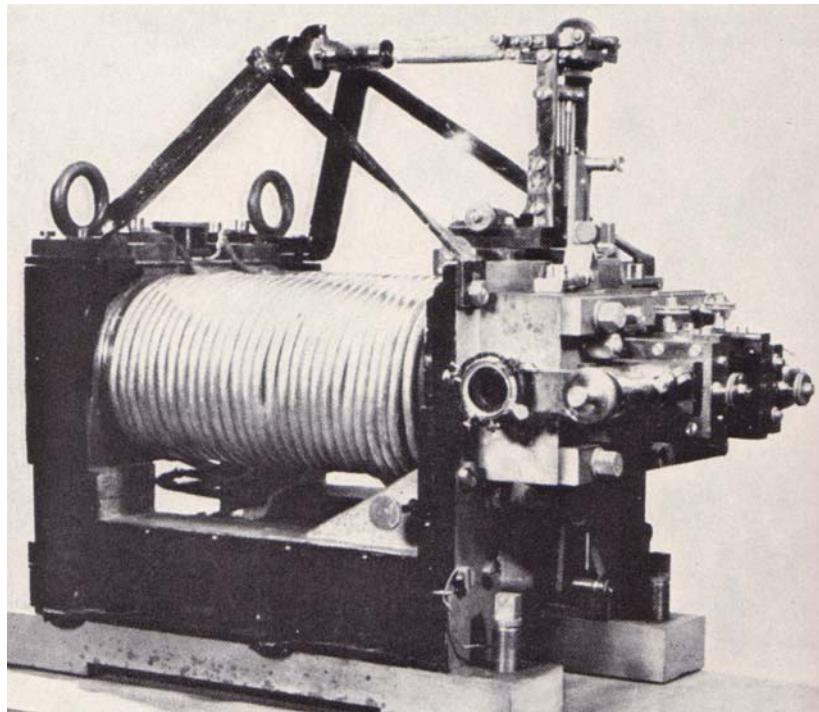
Plate II. Photograph of String galvanometer, 1903, invented by Willem Einhoven (1860-1927).

> *Einhoven describes the string galvanometer as "…a thin silver coated quartz fiber which is stretched like a string in a strong magnetic field. If an electric current is led through this quartz fiber, the fiber shows a movement which can be observed and photographed by means of a considerable magnification…"*

(Leibowitz, 1990)

2.1. Introduction

The experimental model used in all studies was the *in vivo* rat model of acute myocardial ischemia-reperfusion. A detailed explanation of the surgical preparation of the model including discussion on the measurement primary endpoints (mortality, ventricular arrhythmias, infarct size) and secondary endpoints (heart rate, mean arterial pressure and rate-pressure product) are presented. A description of the ^{31}P NMR method includes how the quantification of phosphorus compounds, pH and free Mg^{2+} in the *in vivo* rat heart was accomplished. Finally, the choice of statistical analysis for each experiment is given.

2.2. Animals and reagents

Male Sprague Dawley rats (330-400g) from the James Cook University Breeding Colony were fed *ad libitum* and housed in a 12-hour light/dark cycle. Animals were treated in accordance with the James Cook University Guidelines for use of 'Animals for Experimental Purposes' (Ethics approval number A557).

Adenosine (A9251 >99% purity), copper II pthalocyanine-tetrasulfonic acid tetrasodium salt (blue dye), 2-chloro-N6-cyclopentyladenosine (CCPA), deuterium oxide (D$_2$O), phenylphosphoric acid (PPA), sodium chloride (NaCl) and triphenyltetrazolium chloride (TTC) were obtained from Sigma Aldrich (Castle Hill, NSW). Nembutal (sodium pentobarbitone, 60 mg/ml) and lidocaine hydrochloride (2%) (ilium) were purchased from the local Pharmaceutical Supplies (Lyppard, Queensland). A 10% formalin solution phosphate buffer was made as needed (Drury and Wallington, 1967).

2.3. *In vivo* rat model of acute myocardial ischemia surgical protocol

On the day of the experiment, rats were anesthetized with an intraperitoneal injection (i.p.) of Nembutal (sodium pentobarbital; 60 mg/kg) (Lawson et al., 1993). Anesthetic was administered intraperitoneally as required throughout the protocol. A tracheotomy was performed and the animals were artificially ventilated at 75-80 strokes per min on humidified room air using a Harvard Small Animal Ventilator (Harvard Apparatus, Mass., USA). Blood pO_2, pCO_2 and pH were maintained in the normal physiological range and measured on a Ciba-Corning 865 blood gas analyzer. Body temperature was maintained at 37°C using a homeothermic blanket control unit (Harvard Apparatus, Mass., USA). The left or right femoral vein was cannulated using PE-50 tubing for drug infusions while the left femoral artery was cannulated for blood collection and to monitor blood pressure (UFI 1050 BP) using a MacLab.

Access to the heart was gained through a left thoractomy similar to that described by Selye et al (1960). A mid-line incision was made along the sternum through the epidermal layer exposing the rat's thoracic muscles. Blunt forceps were gently inserted between the left 4th and 5th ribs through the intercostal muscles. A transverse lateral incision was made by gently stretching the intercostal muscle with forceps and clipping the muscle with sharp surgical scissors. Upon visual inspection, the pericardium was identified and carefully opened. By applying gentle pressure to the right thorax, the heart was quickly exteriorized (<3 sec) through the blunt incision with the aid of cotton buds. A 6-0 suture was quickly threaded under the left coronary artery (LCA) located between the base of the pulmonary artery and left atrium. The LCA ties were attached to a custom designed snare occluder fastened to the cradle via a 50 cm teflon tube attached to a detachable 10 g weight (Fig. 2.1). By adding or

removing the weight, a constant ligation pressure could be applied and easily

released. Leads were implanted subcutaneously in a lead II electrocardiogram

(ECG) configuration (Fig. 2.2). Animals were then positioned in a specially designed

plexiglass cradle fitted to the occlusion device (Fig. 2.3). Rats stabilized for 15-20

minutes prior to occlusion. Ischemia was visually confirmed by regional cyanosis

downstream of the occlusion or changes in the ECG. The final set up of the *in vivo* rat

model is shown in Figures 2.3 and 2.4.
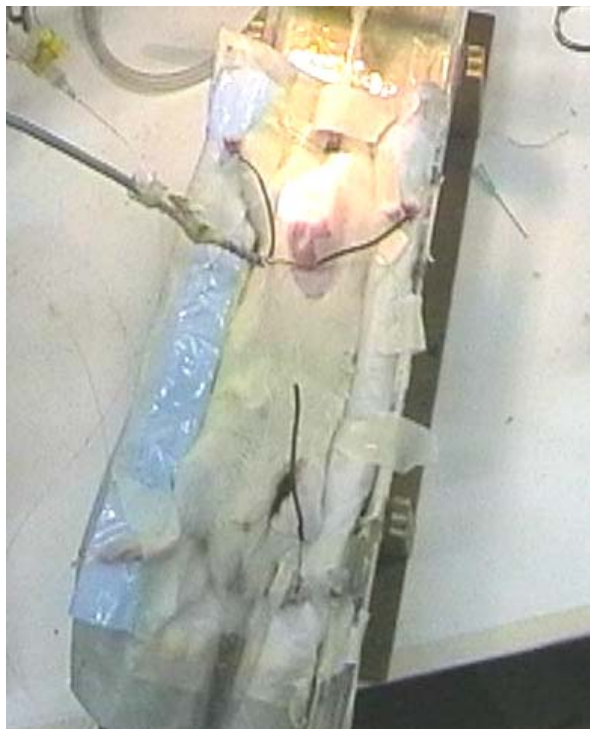
Figure 2.1. Photograph of the *in vivo* rat model of acute myocardial ischemia.
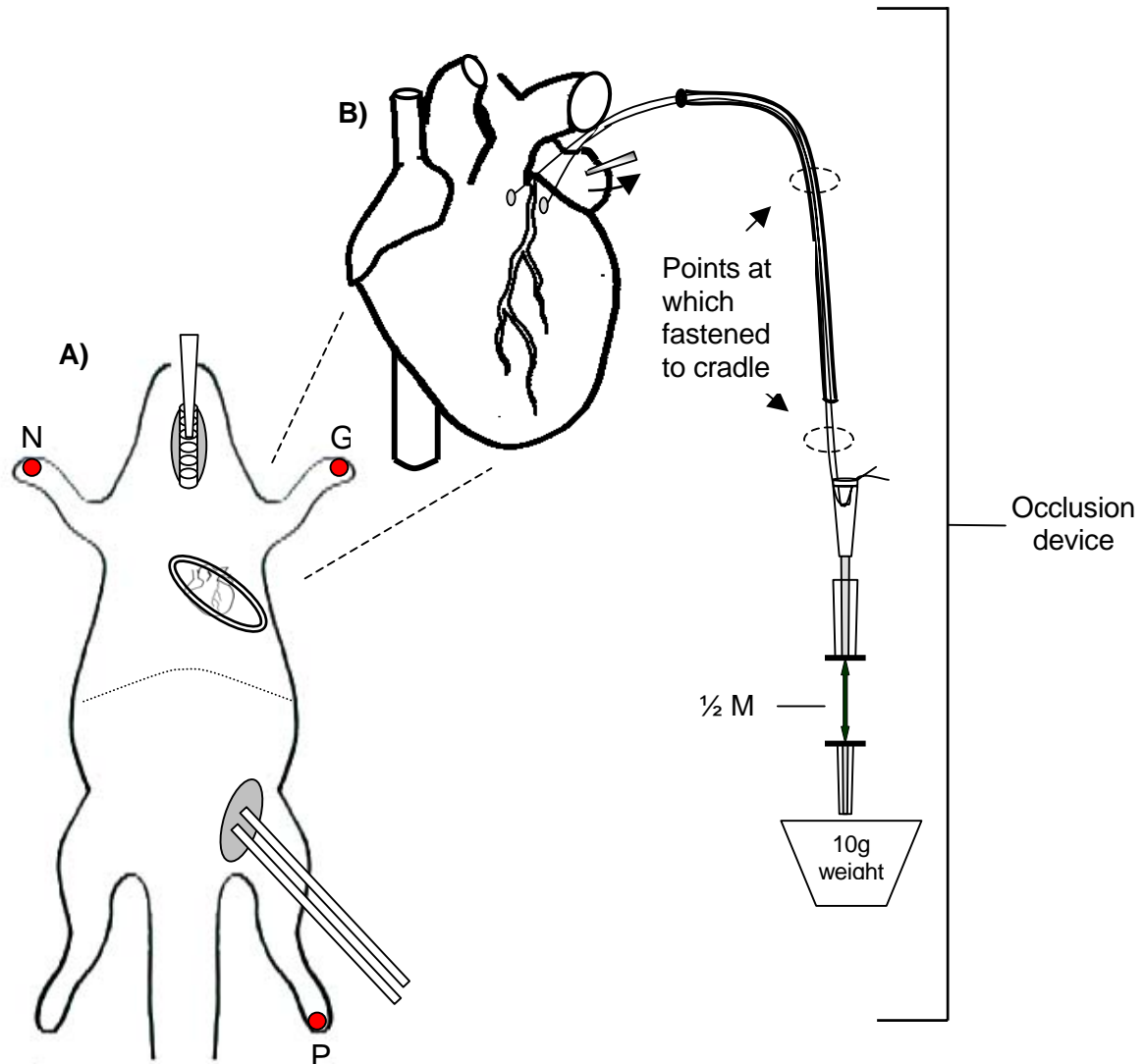
Figure 2.2. Diagram of the surgical preparation of rat *in vivo* model of myocardial ischemia. A) Depiction of rat following surgery. Position of tracheotomy, left thoractomy, femoral cannulation are shown; red circles indicate location of ECG lead placement; N – negative lead, P – positive lead, G - ground. B) Depiction of the occlusion device after suture is threaded under left coronary artery and into device. The occlusion device fastened to cradle and was ½ M long from the edge of the cradle to the weight.
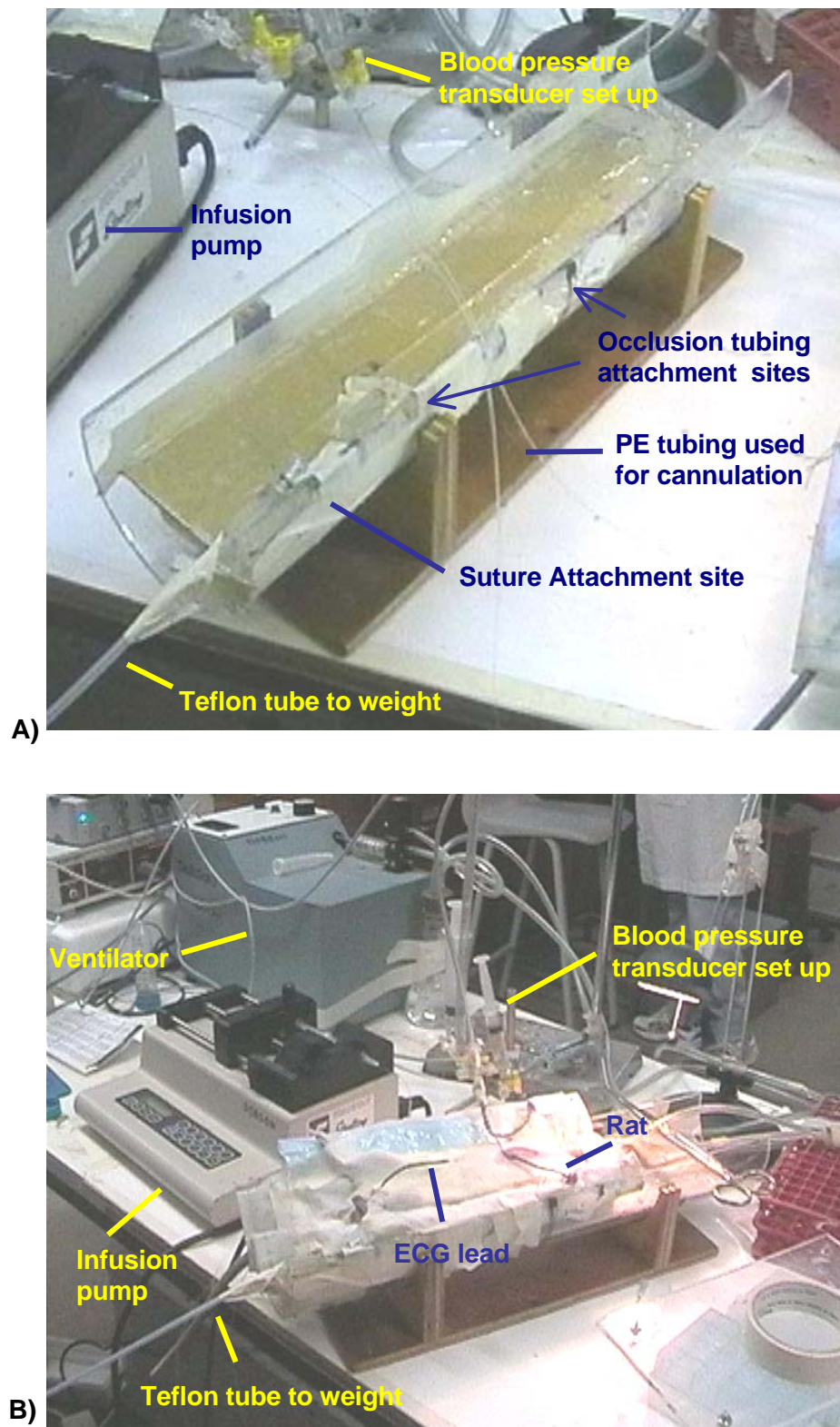
Figure 2.3 Photographs of the *In vivo* model set up. A) Perspex cradle in which rat is placed. B) *In vivo* bench with rat in cradle completely set up for an experiment.

Figure 2.4. The *in vivo* rat model of acute myocardial ischemia coupled to MacLab chart recording software.

2.4. Measurement of ischemic area at risk and infarct size

2.4.1. INTRODUCTION TO THE MAIN CONCEPTS OF INFARCT SIZE MEASUREMENT

The method for assessing the myocardial infarct size in experimental models of acute

myocardial ischemia commonly employs macroscopic staining by a blue colored dye

and triphenyltetrazolium chloride salt (TTC) (Fishbein et al., 1981; Bishop, 1984;

Vivaldi et al., 1985; Ytrehus et al., 1994b; Birnbaum et al., 1997; Schwarz et al.,

2000). Essentially, this method distinguishes between healthy and irreversibly injured

tissue. Firstly, the vascular bed previously supplied by the occluded artery is identified by flushing a colored dye (usually blue) through the coronary circulation during occlusion of the vessel. The tissue that remains uncolored is the tissue at risk of becoming necrotic and is termed the area at risk (AAR) or risk zone. Necrosis is determined by TTC staining. Upon incubation of the myocardial tissue in a TTC solution, the dehydrogenase enzymes and cofactors in viable tissue reduce the tetrazolium salts to form a brick-red colored formazin pigment. In contrast, irreversibly injured cells, lose dehydrogenase activity and therefore, do not cause a color change reaction with the TTC. Rather, TTC forms a white precipitate on the surface of necrotic tissue. The result is a demarcation between irreversibly injured tissue and viable tissue. In order for the demarcation to be achieved, there is a minimum period of reperfusion required to facilitate the process of increased membrane permeability and the loss of dehydrogenase enzymes and cofactors from the necrotic region (Fishbein et al., 1981). The minimum washout period in rat hearts is 120 min of reperfusion (Schwarz et al., 2000). The final infarct size measurement is expressed as the ratio of necrosis within the area at risk.

2.4.2 THE PROTOCOL USED FOR INFARCT SIZE MEASUREMENT

The protocol used in these studies is as follows. After 120 min of reperfusion, the coronary artery was reoccluded and the heart excised. Blue dye (Copper (II) Pthalocyanine-tetrasulfonic acid Tetrasodium salt, 3 ml) was flushed retrograde through the aorta at a flow rate of approximately 18 ml/min and allowed to circulate through the coronary vasculature to delineate the ischemic risk zone (Fig. 2.5). The heart was sliced transversely into 6 or 7 slices of uniform thickness (2mm) using a custom-made, equal spaced, multi-scalpel blade slicer (Fig. 2.5). The slices were weighed and digitally photographed (Fig. 2.6). Area measurements were made using

a digitized pen (Wacom) and the Image J (NIH) analysis program. The area left

unstained by the blue dye was defined as the left ventricular 'area-at-risk' (AAR/LV)

while the blue-stained region was the perfused area not at risk of suffering ischemic

damage. The slices were then incubated in a 1% solution of triphenyltetrazolium

chloride (TTC) at 37°C for 15 min (Fishbein et al., 1981), immersed in formalin and

photographed again (Fig. 2.6). The area of necrosis in the left ventricle (AN/LV) was

the region of the slice unstained by TTC (white) while the non-infarcted region was

the area of the slice stained by TTC (brick red). Infarct size of the left ventricle was

defined as the ratio of the area of necrosis (AN) to the area at risk (AN/AAR) and

expressed as a percentage.
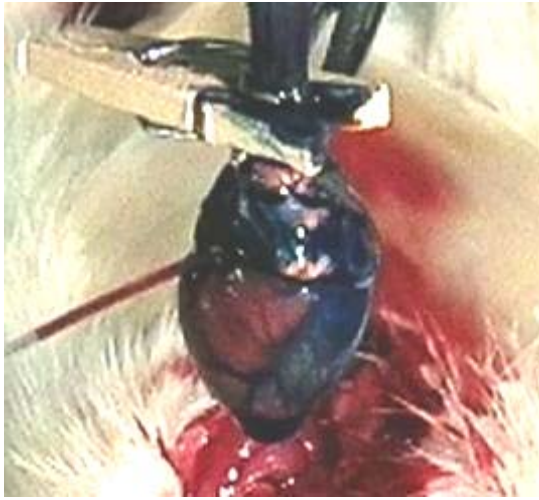
A) Demarcation of Risk Area

B) Slicer (aerial and side view)

C) Photography Platform

D) Slices in Glass Tray for Photography

Figure 2.5. Photographs of infarct size procedure components.
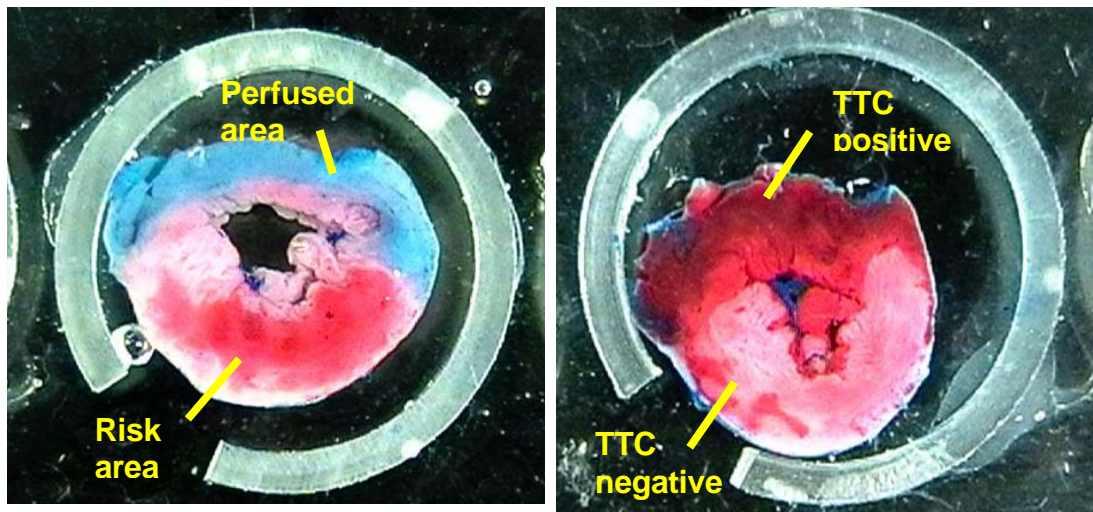
A) Area at Risk   B) Area of Necrosis

Figure 2.6. Examples of slices demarcated for infarct sizing.

2.5. Hemodynamic measurements

Hemodynamic measurements of heart rate, systolic and diastolic pressure, were collected throughout the protocol (section 2.3). From these, mean arterial pressure (systolic pressure – diastolic pressure/3 + diastolic pressure) and rate pressure product (heart rate x systolic pressure) were calculated. The rate pressure product is an important indicator of myocardial oxygen consumption of heart tissue.

2.6. Identification and analysis of arrhythmias

The *in vivo* rat model of acute myocardial ischemia is a widely used model for the production of experimental arrhythmias (Clark et al., 1980; Lepran et al., 1983; Barrett et al., 1995; Opitz et al., 1995; Janse et al., 1998; Opitz et al., 1998; Lu et al., 1999; Barrett et al., 2000). Using a lead II ECG tracing (section 2.3), the episodes of

premature ventricular beats (PVB), salvos, bigeminy, ventricular tachycardia (VT) and ventricular fibrillation (VF) were recorded, counted, and the duration of each episode of bigeminy, VT and VF were measured during 30 min ischemia and 30 min reperfusion. Examples of each type of arrhythmia are depicted in Figure 2.7. Arrhythmias were defined according to the Lambeth Conventions (Walker et al., 1988). Premature ventricular beats were defined as discrete and identifiable premature QRS complexes and an episode of bigeminy was recognized as a variant of PVBs and characterized by the minimum sequence: P, QRS, PVB, P, QRS, PVB. Salvos were defined as two or three consecutive PVBs while ventricular tachycardia was defined as a run of 4 or more consecutive ventricular premature beats. An episode of VF was defined as a signal where individual QRS deflections could not easily be distinguished from each other and rate could no longer be measured (Walker et al., 1988). The duration of each episode was measured in seconds and the sums of these during 30 min ischemia and 30 min reperfusion were analyzed. To overcome the occasional difficulty of identifying VF in a background of VT, the frequency and duration of both were summed and analyzed separately. For example, a VT with torsade de pointes morphology that converted to VF then reverted to VT without a clear-cut interface was included in the summed measurement (Opitz et al., 1995). Notwithstanding this limitation, every attempt was made to identify VT and VF as separate variables. Arrhythmias were analyzed separately during 30 min ischemia and the first 30 min of reperfusion.

A) Normal Rat ECG

B) ECG from ischemia

C) Premature Ventricular Beat (fusion beat)

D) Premature ventricular Beat

E) Bigeminy

F) Salvos

G) Non-sustained Ventricular Tachycardia

H) Sustained Ventricular Tachycardia

I) Ventricular Fibrillation
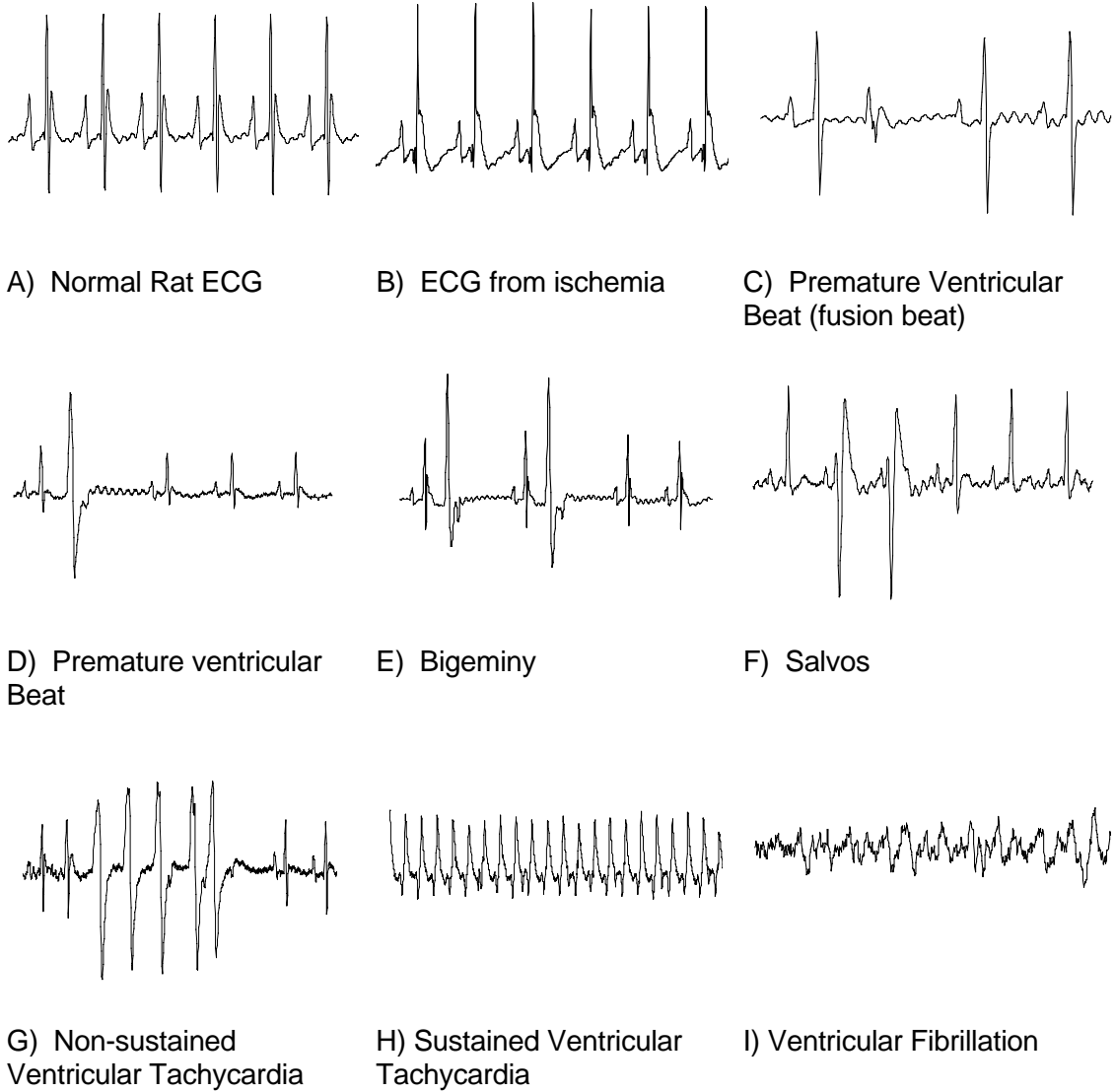
Figure 2.7. Examples of categorized arrhythmias taken from samples used in these studies.

Figure 2.8. Photograph of the Oxford 7.05-T superconducting magnet.

2.7. *In vivo* [31]P magnetic resonance spectroscopy of the rat heart

2.7.1. MODIFICATIONS TO SURGICAL PROTOCOL

Rats were surgically prepared as in section 2.3 with minor variations.

Electrocardiogram lead placement and recordings were omitted in nuclear magnetic

resonance studies. Surgically, the left thoractomy was modified so that 4 - 8mm of

ribs 4, 5 and/or 6 were removed to accommodate the surface coil. The intercostal

muscles of the removed ribs were cauterized and pressure was placed on bleeding

areas. Once stabilized, ($\approx$5 min), the surface coil (10mm o.d.) was placed over the

left ventricle within the chest cavity.

2.7.2. CALIBRATION OF SURFACE COIL SAMPLING DEPTH

The 3-turn surface coil was made of flexible Teflon coated copper wire (1.25 mm thick) tunable to ^{31}P provided enough flexibility to remain in contact with the heart while allowing movement (Fig. 2.9). A thin-walled latex balloon containing the external standard, 50 mM phenylphosphoric acid (PPA), was placed on the anterior side of the surface coil. Coil calibration required a self-contained multi-layered and multi-chambered device. This was designed for the purpose of measuring the sample depth of the coil (Fig. 2.10). The device was made out of Lexan high tensile inert plastic. Each layer was separated by 0.04 mm of plastic and the depth of each of the layers was 1.4mm (volume = 0.72 cm^3). Three layers were filled with a different phosphorus containing solution of the same concentration: 50mM PPA, trimethyl phosphate, and sodium phosphate. Fully relaxed ^{31}P spectrum was obtained and used to determine the relative contribution of each layer. The average thickness of the left ventricle beneath the coil was found to be 3.7 mm (n = 11). The signal arising from the consecutive layers of the multilayered phantom indicated that the sampling depth of the coil was ≈2 mm. This confirms that the coil sampled entirely from left ventricular myocardium and not into the ventricular chamber.

2.7.3. NMR SPECTROSCOPY

All ^{31}P NMR experiments were performed at 121.47 MHz in a 110-mm horizontal bore Oxford 7.05-T superconducting magnet (property of the School of Biomedical Sciences - JCU) (Fig. 2.8). The ^{31}P spectra were acquired using a Varian Inova NMR spectrometer. Homogeneity of the magnetic field was maximized on the ^{1}H free induction decay (FID) measured off resonance using an Oxford Instruments 15-channel shim supply. Radiofrequency pulses of 8-μs duration with a ≈ 40° flip angle

were applied at a 1-s interpulse delay. A total of 368 FIDs were averaged for each animal. Each FID was acquired over 0.4s with a spectral width of 8,000 Hz and a total of 12000 data points were averaged. An exponential line broadening factor of 30 Hz was applied to the ^{31}P NMR spectres, which were then fitted using the Varian Fitspec software. A typical phosphorus spectra for an *in vivo* rat heart is shown in Figure 2.11.
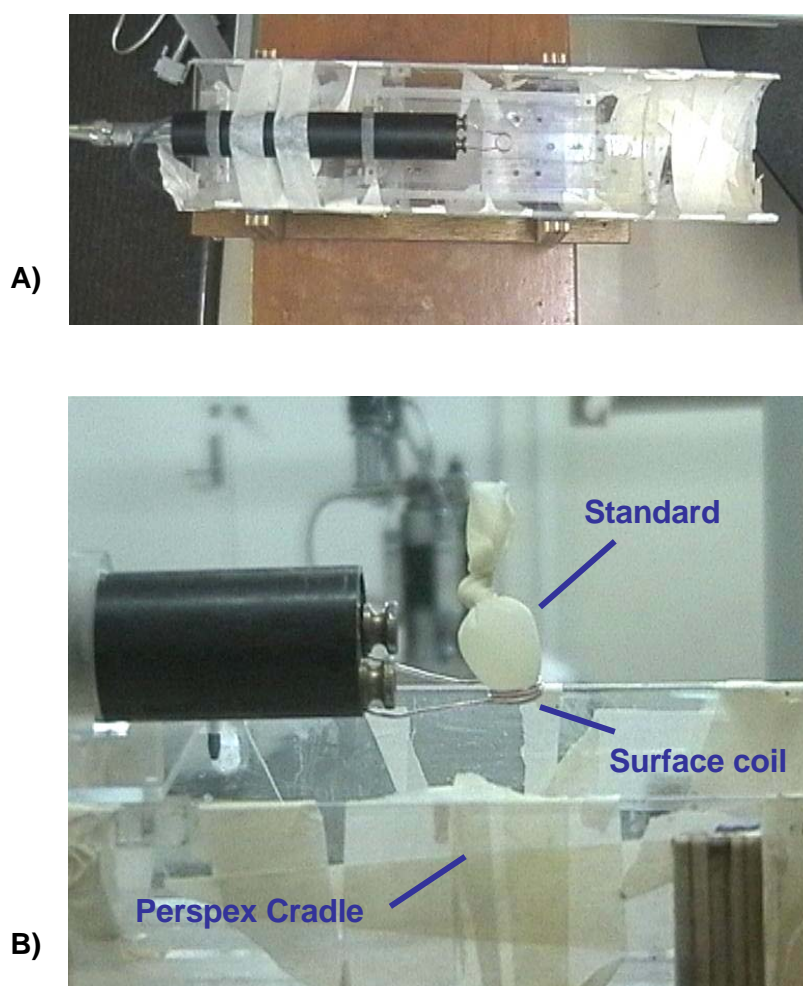
Figure 2.9. Photographs of surface coil placement upon Perspex cradle.
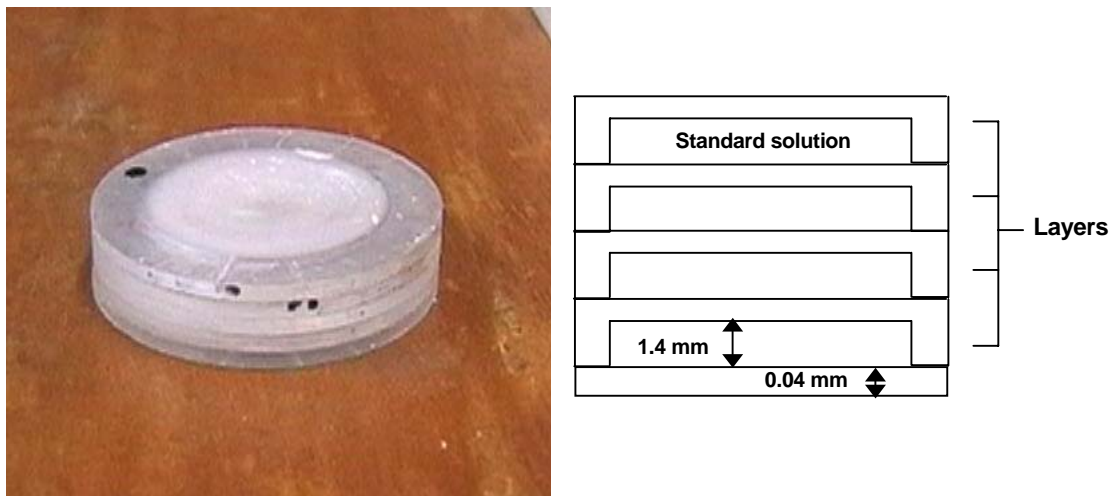
Figure 2.10. Multi-layered and multi-chambered device for coil sample depth calibration.
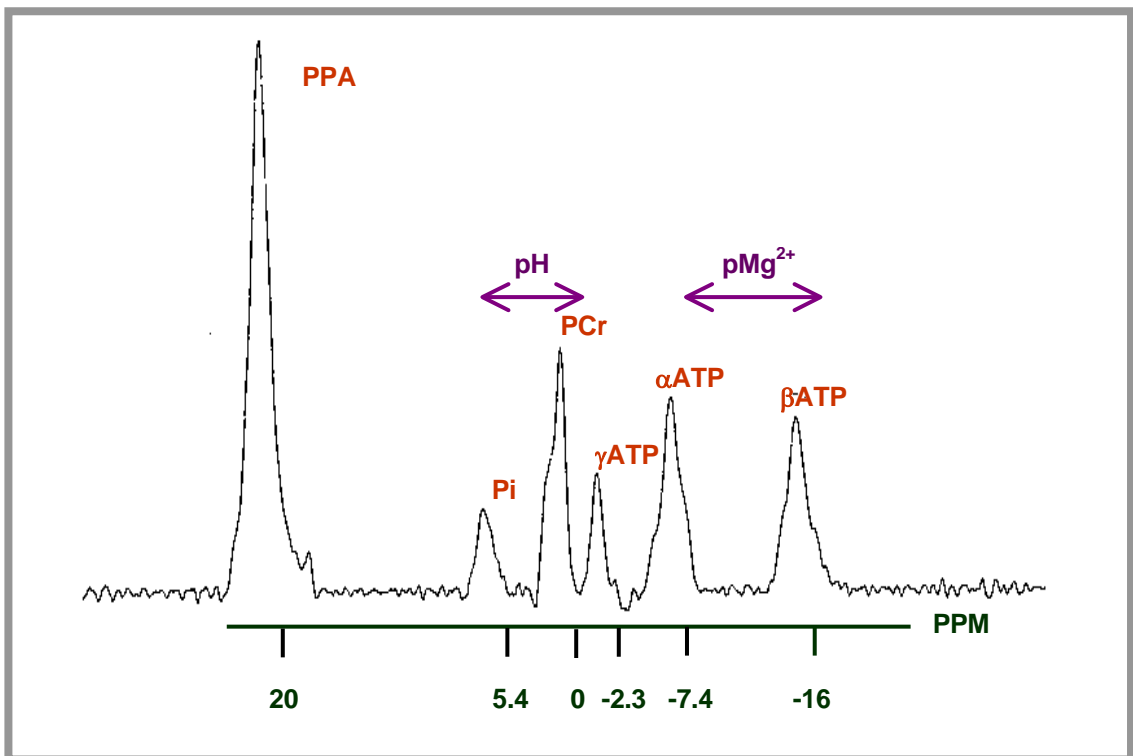
Figure 2.11. Example of typical phosphorus spectra from a non-ischemic rat heart used in this study.

Following integration, all peaks were multiplied by an experimentally derived saturation correction factor specific for each peak. These factors were determined by comparing peak integrals of partially relaxed spectra, obtained using the acquisition parameters described above, and the peak integrals of fully relaxed spectra (20-s interpulse delay). The mean values of individual phosphorus compounds found in the heart were used in NMR calculations for a 1-s acquisition delay. The mean correction factors for the 50 mM PPA external standard, P_i, PCr and βATP were 1.12 $\pm$ 0.03, 0.97 $\pm$ 0.08, 1.24 $\pm$ 0.04, and 0.87 $\pm$ 0.03 (n = 7) (Hitchins et al., 2001).

2.7.4. PHOSPHORUS QUANTIFICATION

The concentrations of P_i, PCr and ATP were determined using the external standard (50 mM PPA). The theory for using an external metabolic reference symmetrically positioned on the anterior side of the coil with the heart on the posterior was described in detail by Hitchins et al. (2001). The following equations present the theory.

Firstly, a phosphorus metabolite integral in heart is the observed signal (S) given by

$$S = \int_{allspace} \rho(x, y, z) \; X \; B_{1\,coil}(x, y, z)dV \qquad (1)$$

where ρ is the spin density distribution of the source signal, V is the volume where signal is measured and $B_{1\,coil}$ is the sensitivity of the coil system. The coil system sensitivity is generally the transverse field at a point in space normalized to the amplitude per mole of the phosphorus metabolite. The reference signal is the spatial distribution expressed as ρ = [PPA] X f_{PPA} (x, y, z). By taking f = 1 inside the sphere containing the PPA and f = 0 outside, then

$$S_{PPA} = [PPA] \int_{sphere} B_{1\,coil}(x, y, z)dV \qquad (2)$$

The tissue phosphorus signal (e.g. PCr) from *Eq. 1* above becomes

$$S_{PCr} = \int_{allspace} \rho_{PCr}(x, y, z) \times B_{1\,coil}(x, y, z)dV \qquad (3)$$

which leads to

$$[PCr] = \frac{S_{PCr}}{\int_{allspace} \rho_{PCr}(x, y, z) \times B_{1\,coil}(x, y, z)dV} \qquad (4)$$

assuming that PCr is uniformly distributed. If $f = 0$ outside, as for PPA in the sphere (*Eq. 2*), the volume integral is restricted to the convoluted volume of the heart. Therefore in this setting,

$$[PCr] = \frac{S_{PCr}}{\int_{heart} B_{1\,coil}dV} \qquad (5)$$

These equations affirm that PPA can be used as the external standard (*Eq. 2*) to determine tissue PCr (*Eq. 5*). The two fundamental assumptions of the method are that 1) [PCr] is uniformly distributed in the heart, and 2) the coil system sensitivity yields identical signal amplitude per mole ^{31}P between PPA and the phosphorus compounds in the heart. Therefore

$$\int_{heart} B_{1\,coil}dV = \int_{sphere} B_{1\,coil}dV \qquad (6)$$

If these assumptions are correct, then

$$[PCr] = \frac{S_{PCr} \times [PPA]}{S_{PP}} \tag{7}$$

The concentration of the PCr is then equivalent of micromoles of metabolite per gram wet weight in equal volumes given that 1 ml of water is equivalent to 1 g, and 1 ml tissue is equivalent to 1.1 g.

Following saturation correction, the phosphorus metabolite integrals were standardized from the saturation-corrected 50 mM PPA integral as follows

$$[P_i](\mu mol/g \ wet \ wt) = \frac{SCF_{Pi} \times integral_{Pi}}{SCF_{PPA} \times integral_{PPA}} \times 50 \ (mM) \tag{8}$$

$$[PCr](\mu mol/g \ wet \ wt) = \frac{SCF_{PCr} \times integral_{PCr}}{SCF_{PPA} \times integral_{PPA}} \times 50 \ (mM) \tag{9}$$

$$[ATP](\mu mol/g \ wet \ wt) = \frac{SCF_{ATP} \times integral_{ATP}}{SCF_{PPA} \times integral_{PPA}} \times 50 \ (mM) \tag{10}$$

2.7.5. INTRACELLULAR pH

The intracellular pH (pHi) was computed from the chemical shifts (δ) in parts per million (ppm) of P_i relative to PCr (0 ppm) in the ^{31}P spectra using the Henderson-Hasselbach equation adapted to NMR spectroscopy (Bak and Ingwall, 1992).

$$pH_i = 6.75 = \log (\delta - 3.25)/(5.69 - \delta) \tag{11}$$

2.7.6. FREE MAGNESIUM (MG^{2+})

The observed chemical shift difference ($\delta_{\alpha\beta}$; in ppm) between βP and αP resonances of ATP in the ^{31}P spectra was used for the calculation of intracellular free Mg^{2+} concentration ([Mg^{2+}]) in a modified form of the London equation (Golding and Golding, 1995):

$$[Mg^{2+}]_I = K_D \left[\frac{\delta_{\alpha\beta}\,(1 + \alpha) - (\delta_1 + \alpha\beta_2)}{\delta_3 + \beta\delta_4 - \delta_{\alpha\beta}\,(1 + \beta)} \right] \qquad (12)$$

where, $\alpha = H^+/K_H$ and $\beta = \alpha(K_D/K'_D)$. The definitions of the dissociation constants are as follows: K_H is the dissociation constant for the H$^+$/ATP^{4-} equilibrium, K_D is the dissociation constant for the ATP^{4-}/Mg^{2+} equilibrium and K'_D is the dissociation constant for the ATP^{3-}/Mg^{2+} equilibrium The parameters δ_1, δ_2, δ_3 and δ_4 were assigned published values of 10.600, 11.660, 8.165, and 8.52 ppm respectively; K_D was 9.0 X 10^{-5}, K_H was 3.4 X 10^{-7} M, and K'_D was 7.2 X 10^{-4} M (Golding and Golding, 1995).

2.8. General Experimental Design

The following contains some general similarities in the experimental design of each study presented, yet the specific experimental design for each study appears in its corresponding chapter. All rats were subjected to a pretreatment period, 30 min ischemia and a reperfusion period. All drugs were prepared on the day of the

experiment and delivered through the right or left femoral vein as required. In Chapters 3, 4 and 6, the primary end-points used to assess the cardioprotective effects of AL solution were infarct size, episodes and duration of ventricular arrhythmias and death. High mortality in the control group (>50%) was observed in our pilot studies as is common in the rat model of acute myocardial ischemia (Opitz et al., 1995). On the basis of the binomial distribution for episodes of ventricular fibrillation cited in the Lambeth Conventions, our study protocol required at least 4 animals in each group to survive for sufficient statistical power to test the primary end-points (Walker et al., 1988). The secondary end-points included heart rate, mean arterial pressure (systolic pressure – diastolic pressure/3 + diastolic pressure) and rate pressure product (heart rate x systolic pressure).

The effect of AL solution treatment on bioenergetics during myocardial ischemia was assessed in Chapter 5. The primary end-points were % change from baseline of pH, PCr and ATP over 30 min ischemia and 40 min reperfusion. In addition, P_i and intracellular Mg^{2+} measurements were taken and presented.   As in the other studies, the secondary end-points included heart rate, mean arterial pressure (systolic pressure – diastolic pressure/3 + diastolic pressure) and rate pressure product (heart rate x systolic pressure).

2.9. Statistical Analysis

All statistical analysis was carried out using SPSS statistical package (version 10.0.7). All values were expressed as means $\pm$ SE of the mean. Infarct size, hemodynamics and phosphorus metabolites were compared using a one-way analysis of variance (ANOVA) with the least significance difference (LSD) post hoc test. A Mann-Whitney U test was used for comparison of arrhythmia frequency and

duration because the variables of VT and VF are not normally distributed (Opitz et al., 1995). Correlations were tested using the Pearson's correlation coefficient (r). Statistical significance was defined as a P value of < 0.05.

Chapter 3. Protection against ventricular arrhythmias and cardiac death using an adenosine and lidocaine (AL) solution during acute myocardial ischemia-reperfusion
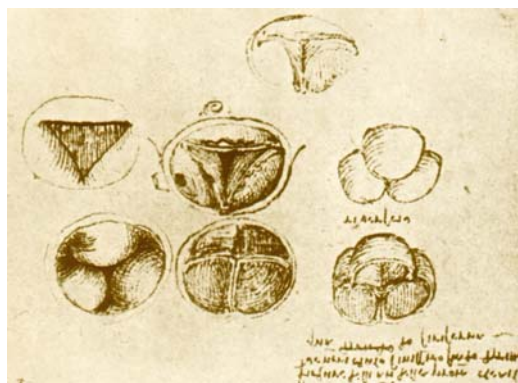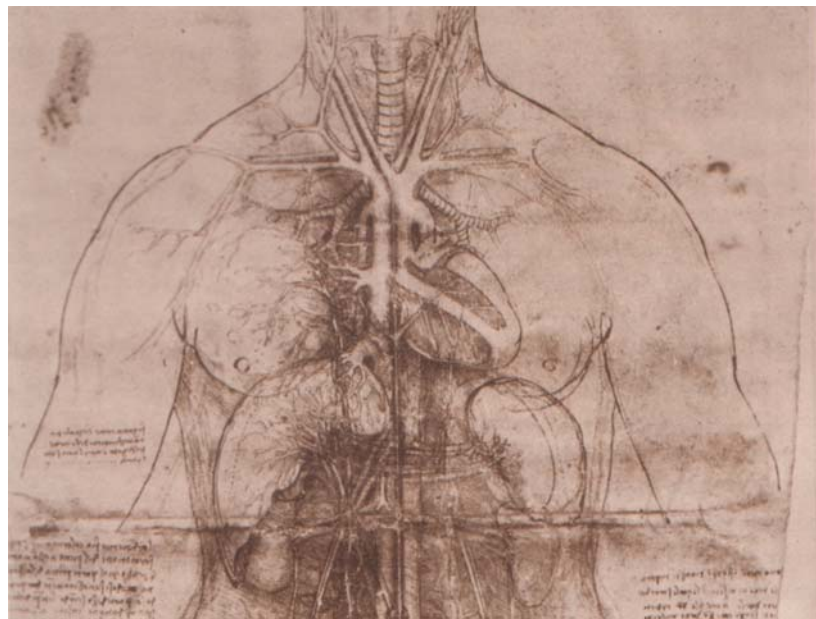
Plate III. Anatomical sketches of a "transparent" torso *(above)* by Leonardo Da Vinci (1452-1519).

(Leibowitz, 1970)

Plate IV. The first depiction of the origin of coronary vessels from the coronary sinus *(below)* also by Leonardo Da Vinci.

(Herrlinger, 1970)

3.1. Introduction

In the United States alone, acute ventricular arrhythmias are associated with over 450,000 sudden deaths each year (Huikuri et al., 2001). Therapeutic strategies designed for primary prevention of ventricular fibrillation (VF), tachycardia or cardiac arrest remain controversial as few trials have shown a survival benefit - an overview of 138 trials involving some 98,000 patients showed no survival benefit (Roden, 1994; Janse, 2003). Indeed, the Cardiac Arrhythmia Suppression Trial (CAST) demonstrated adverse outcomes including increased risk of death with the use of encainide and flecainide (Echt et al., 1991). Moreover, the prophylactic use of lidocaine in acute myocardial infarction is no longer recommended following conflicting reports regarding its association with increased mortality (MacMahon et al., 1988; Ryan et al., 1996; Alexander et al., 1999). Furthermore, trials involving the more popular amiodarone also found that its administration following acute myocardial ischemia demonstrated no survival benefit; however, when combined with beta-blockers a survival benefit was found (Janse, 2003).

As a general consensus, many antiarrhythmic drug therapies, including sodium-channel blocking agents, may exacerbate cardiac vulnerability through their proarrhythmic effects (Barrett et al., 1995; Barrett et al., 2000; Janse, 2003). Despite these limitations, lidocaine at therapeutic concentrations remains clinically effective at protecting against ischemia-induced ventricular arrhythmias by converting unidirectional block to bidirectional block (El-Sherif et al., 1977; Cardinal et al., 1981; Tosaki et al., 1988; Hine et al., 1989b; Li and Ferrier, 1991; Barrett et al., 1995; van Emous et al., 1997; Alexander et al., 1999; Sadowski et al., 1999), and has further advantages of attenuating the accumulation of intracellular Na^+ and Ca^{2+} during ischemia (van Emous et al., 1997), delaying intracellular acidosis by slowing ATP

utilization (van Emous et al., 1997), limiting neutrophil activation and platelet adhesion (Vitola et al., 1997) and reducing infarct size (Nasser et al., 1980).

Adenosine is another antiarrhythmic which has been reported in a few instances to be proarrhythmic (Pelleg, 2002). The drug is widely used to suppress supraventricular tachycardias mainly from its action to slow conduction through the atrioventricular node (Bellardinelli and Giles, 1988; Lerman and Belardinelli, 1991; Lerman et al., 2001; Pelleg, 2002). In addition, adenosine suppresses catecholamine triggered dysrhythmias through its antiadrenergic effect to reduce catecholamine-induced inward calcium current (Belardinelli et al., 1995). Adenosine also possesses many other broad-spectrum benefits, including dilating the coronary arteries (Ely and Berne, 1992), preconditioning the heart (Liu et al., 1991), reducing infarct size (Lasley et al., 1990; Lasley et al., 1995; Lasley and Mentzer, 1995), reducing reperfusion injury (Vinten-Johansen et al., 1999), and attenuating the inflammatory response (Vinten-Johansen et al., 1999).

The aim of this section was to test the hypothesis that administering adenosine and lidocaine (AL) in a single solution during regional ischemia in the *in vivo* rat model would reduce ventricular tachycardia and fibrillation and confer an improved survival benefit than either drug alone while reducing infarct size. In addition to their antiarrhythmic and anti-ischemic effects, part of the reasoning behind combining AL was that each drug is used clinically to target different regions of the arrhythmogenic heart. Adenosine targets the supra-ventricular region and lidocaine primarily targets the ventricles.

3.2. Experimental Design

Animals (n = 48) were randomly assigned into 6 treatment groups: (1) saline controls (0.9% saline, n = 12); (2) adenosine only (Adeno-only) (305 μg/kg/min, n = 8); (3) lidocaine only (Lido-only) (608 μg/kg/min, n = 8); (4) Low-dose adenosine with lidocaine (AL) (A: 152 μg/kg/min and L: 608 μg/kg/min, n = 6); (5) Mid-dose adenosine with lidocaine (AL) (A: 305 μg/kg/min and L: 608 μg /kg/min, n = 8) and (6) High-dose adenosine and lidocaine (AL) (A: 457 μg /kg/min and L: 608 μg /kg/min) (n = 6). The adenosine and lidocaine solutions were prepared on the day of the experiment in physiological saline (0.9%). Drugs were infused intravenously at 1 ml/hr (210 infusion pump, Stoelting, Illinois). All rats received continuous infusion for 5 minutes prior to and throughout 30 minutes of regional ischemia. The treatment was ceased when the coronary ligature was released at the onset of reperfusion after 30 min ischemia and animals reperfused for 120 minutes for infarct sizing (Fig. 3.1).

The method of arrhythmia identification and evaluation is described in detail in Chapter 2 section 2.6.

The primary end-points used to assess the cardioprotective effects of AL solution were mortality, episodes and durations of ventricular arrhythmias, and infarct size. The secondary end-points included heart rate, mean arterial pressure (systolic pressure – diastolic pressure/3 + diastolic pressure) and rate pressure product (heart rate x systolic pressure).
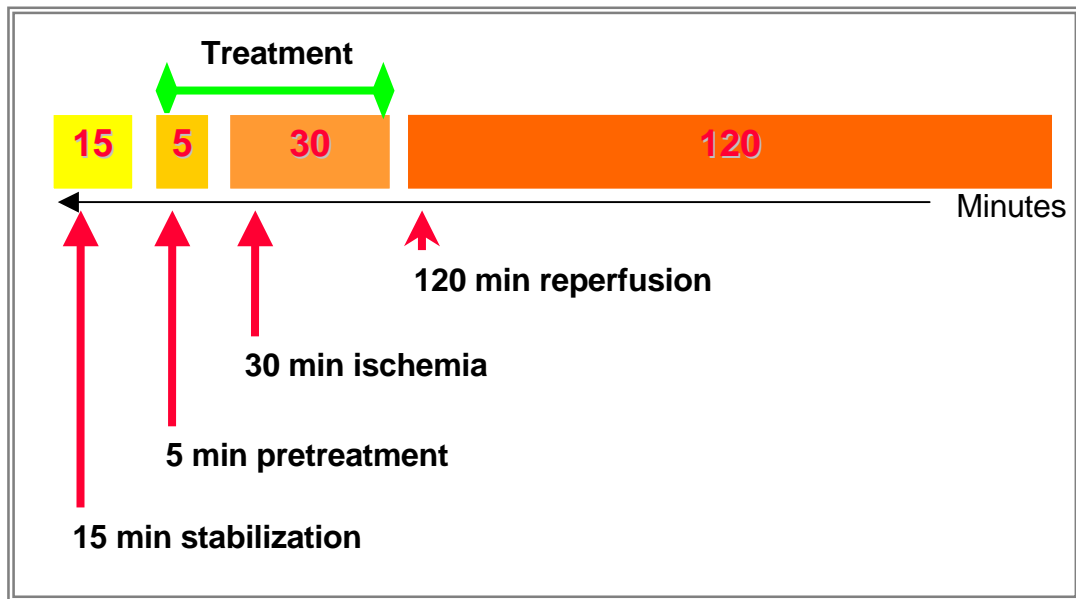
Figure 3.1 Chapter 3 treatment protocol.

3.4. Results

Four rats were excluded from this study: one animal's MAP was <70 mmHg before treatment (Lido-only), a second animal's ventilation tubing became clogged (Mid-dose AL group), a third rat from Lido-only group died before the end of the experiment from severe hypotension; no ventricular arrhythmias were involved and a fourth rat was excluded from the Low-dose AL group because of problems with occlusion device during blue dye perfusion (invaded risk space). Data from a total of 44 rats is reported and the mean body weight was 361 ± 3 g. No significant differences in rat weights were found between the groups.

3.4.1. MORTALITY

Seven of the 12 saline-control rats and 4 of the 8 Adeno-only treated rats died during the ischemic period from an episode of ventricular fibrillation. No deaths occurred in

the Lido-only treated rats (n = 6) or in any group where AL solution was infused (n = 18). Only data from surviving rats were further analyzed (n = 33).

3.4.2. ARRHYTHMIAS DURING ISCHEMIA

Arrhythmias occurred with great variability between groups. Treatment with Adeno-only, Lido-only or High-dose AL did not significantly change episodes or durations of arrhythmias of any type from Saline controls during ischemia or reperfusion. Episodes of PVBs occurred in all groups, Saline-controls (60 ± 22 episodes), Adeno-only (19 ± 4 episodes), Lido-only (104 ± 21 episodes), Low-dose AL (48 ± 10 episodes), Mid-dose AL (35 ± 12 episodes) and High-dose AL (57 ± 20), yet no group resulted in significant differences from Saline controls (Fig 3.2). Compared to Lido-only treatment, Low-dose AL and Mid-dose AL significantly reduced the number of PVBs (104 ± 21 vs. 48 ± 10 episodes and 35 ± 12 episodes) ($P < 0.05$). While, salvos occurred during the 30 min of ischemia in all treatment groups, Saline controls (15 ± 7 episodes), Adeno-only (10 ± 4 episodes), Lido-only (49 ± 21 episodes), Low-dose AL (20 ± 13 episodes), Mid-dose AL (11 ± 5 episodes) and High-dose AL (32 ± 20 episodes), no significant differences were found. Episodes of bigeminy also occurred in all groups, Saline controls (12 ± 4 episodes), Adeno-only (9 ± 3 episodes), Lido-only (23 ± 10 episodes), Low-dose AL (8 ± 3 episodes), Mid-dose AL (12 ± 5 episodes) and High-dose AL (14 ± 5 episodes) (Fig. 3.3). Over the 30 min of ischemia the cumulative durations of bigeminy were: Saline controls (23 ± 12 sec), Adeno-only (20 ± 7 sec), Lido-only (59 ± 26 sec), Low-dose AL (13 ± 7 sec), Mid-dose AL (20 ± 8 sec) and High-dose AL (63 ± 53 sec). No significant differences in bigeminy episodes and cumulative durations were found (Fig. 3.3).
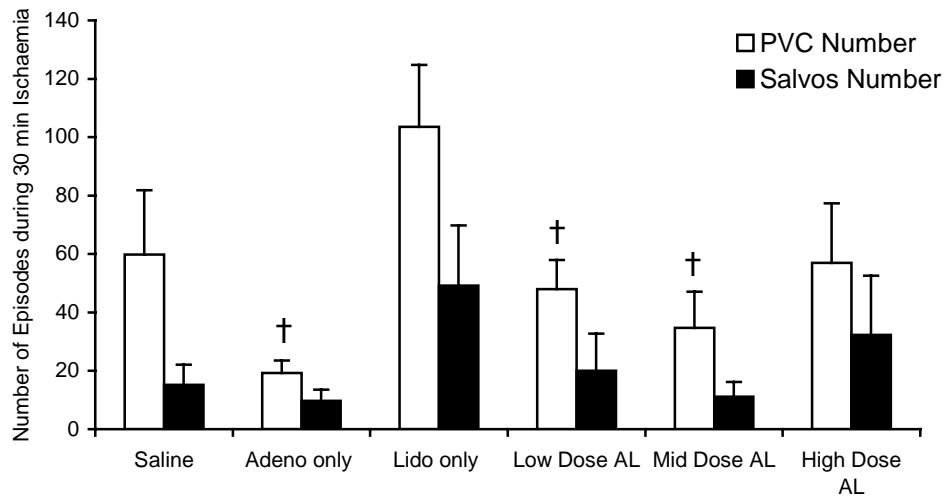
Figure 3.2. Episodes of premature ventricular beats (PVB) and salvos in saline-controls and the five treatment groups during 30 min ischemia. Significance was shown (†) between PVB in lidocaine-treated rats compared to Adeno-only and low- and mid-dose AL-treated rats (P < 0.05). Surviving rats: Saline, n=5; Adeno only, n=4; Lido only, n=6; Low dose AL, n=5; Mid dose AL, n=7; High dose AL, n=6.
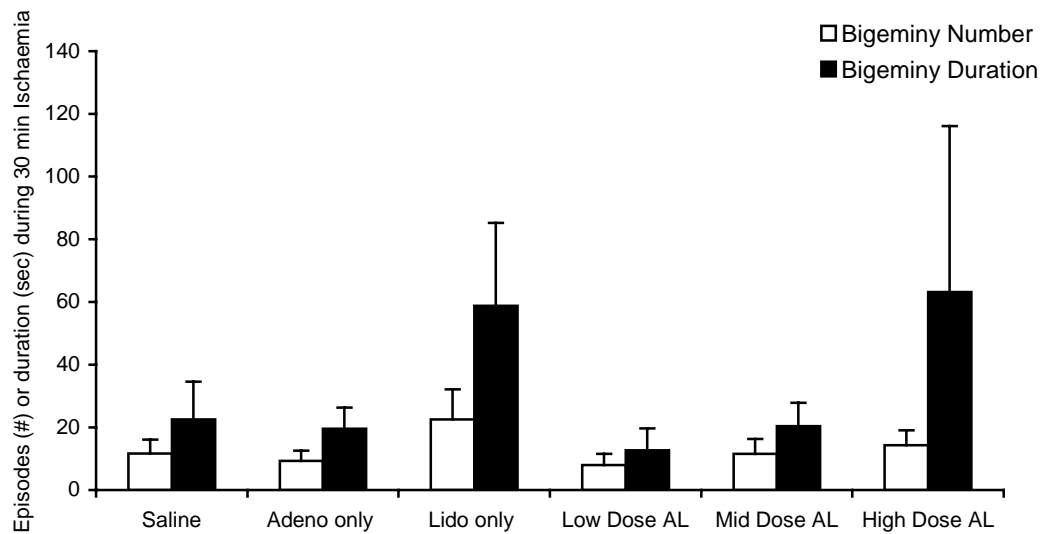
Figure 3.3. Episodes and durations of bigeminy in saline-controls and the five treatment groups during 30 min ischemia. Surviving rats: Saline, n=5; Adeno only, n=4; Lido only, n=6; Low dose AL, n=5; Mid dose AL, n=7; High dose AL, n=6.

The mean number of VT episodes in saline-controls was 18 ± 9 affecting 100% of animals while 75% experienced VF (4 ± 3 episodes) (Fig. 3.4). Treatment with Adeno-only resulted in VT in 50% of the rats tested (11 ± 7 episodes) and 100% of rats had VF (3 ± 2 episodes). In Lido-only treatment, ventricular tachycardia occurred in 83% (23 ± 11 episodes) and VF in 33% (2 ± 1 episodes) of rats tested. Of the subjects treated with Low-dose AL and Mid-dose AL solution, 60% and 57% of the treated rats, respectively, had at least 1 episode of VT (2 ± 1 episodes) while 67% of High-dose AL treated rats experienced 6 ± 3 episodes of VT. However, Low-Dose AL and Mid-dose AL *completely protected* against VF during ischemia, while only one rat treated with High-dose AL experienced VF (25.5 sec).
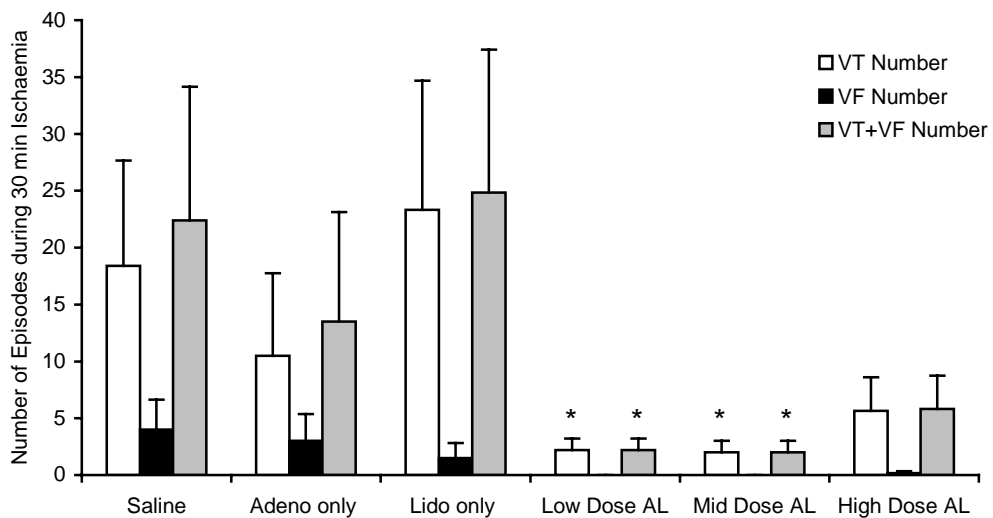
Figure 3.4. Sum of episodes of ventricular tachycardia (VT) and ventricular fibrillation (VF) in saline- controls and the five treatment groups during 30 min ischemia. There was no VF for low- or mid-dose AL treated rats, and virtually none in the high-dose AL. * P < 0.05 vs. saline controls. Surviving rats: Saline, n=5; Adeno only, n=4; Lido only, n=6; Low dose AL, n=5; Mid dose AL, n=7; High dose AL, n=6.
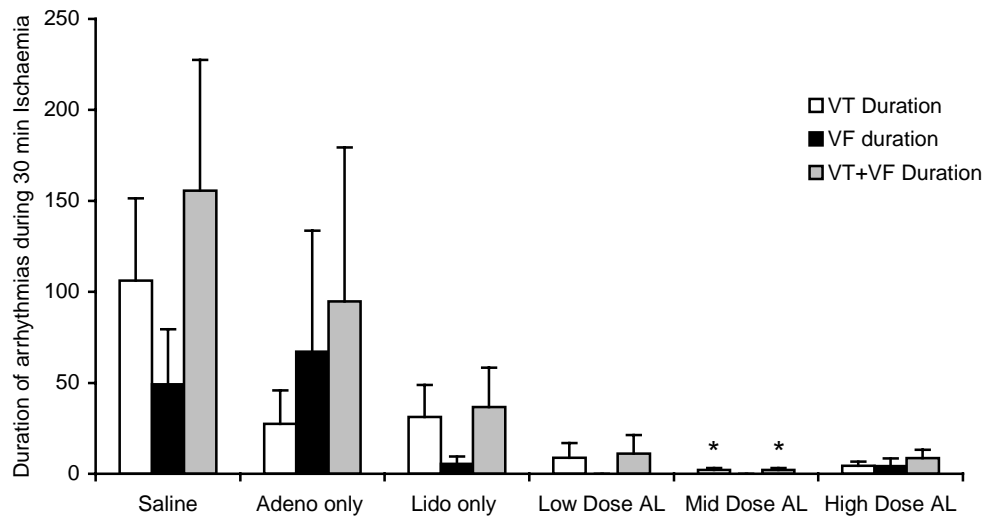
Figure 3.5. Sum of durations of ventricular tachycardia (VT) and ventricular fibrillation (VF) in saline controls and the five treatment groups during 30 min ischemia. * P < 0.05 vs. controls. Surviving rats: Saline, n=5; Adeno only, n=4; Lido only, n=6; Low dose AL, n=5; Mid dose AL, n=7; High dose AL, n=6.

Rats infused with Mid-dose AL solution experienced not only a significant reduction in VT's, but also a significant reduction in durations of VT (2 ± 1 sec) and VT+VF's (2 ± 1 sec) compared to saline-controls (P < 0.05) (Fig. 3.5). The durations of VT and VT + VF's for saline-controls were 106 ± 45 sec and 156 ± 72 sec and for Lido-only treatment were 31 ± 18 sec and 37 ± 22 sec respectively (Fig. 3.5). Low-dose AL resulted in mean VT duration of 9 ± 8 sec, and High-dose AL resulted in a mean VT durations of 4 ± 2 sec. In addition, infusion of Mid-dose AL solution significantly reduced the durations of the VT episodes compared to Adeno-only treated rats (27 ± 18 sec) (P < 0.05) (Fig. 3.5). It was noted that, with the exception of the Low-dose AL and Mid-dose AL solution treatments, a high variability in arrhythmia frequency and duration across the other treatment groups was apparent. Only the infusion of AL solution provided consistent reductions of VT or VF frequency or duration without large variability between samples.

3.4.3 EARLY REPERFUSION ARRHYTHMIAS

Within the first minute of reperfusion, 80% of Saline controls, 75% of the Adeno-only and 16% of Lido-only treated animals experienced at least one episode of VT of 0.6 to 35 sec duration. Neither treatment with Adeno-only or Lido-only differed significantly from each other, or from Saline-controls ($P < 0.05$). An episode of VF occurred in 1 of the 5 saline-controls within the first minute and lasted 16 sec. There were no episodes of VF in Adeno-only or Lido-only treatment groups during 30 min reperfusion. Rats treated with Low- or Mid-dose AL solution experienced no ventricular arrhythmias (PVB, Salvos bigeminy, VT or VF) at or during reperfusion. High-dose AL treatment prevented VT, VF salvos and bigeminy, yet 3 rats experienced 26 ± 23 episodes of PVB within the first 30 sec. The number of episodes of VT from Saline controls and the Adeno-only treated animals was found to be significantly higher than Mid-dose AL solution treated animals ($P < 0.05$). Additionally, the durations of VT and VT+VF durations in the Adeno-only group (11 ± 8 sec for both) were significantly longer than treatment with Mid-dose AL treatment ($P < 0.05$).

3.4.4. INFARCT SIZE

Mean area at risk (AAR/LV), areas of necrosis (AN/LV) and infarct size (AN/AAR) expressed as a percentage of left ventricle are shown in Figure 3.6a and 3.6b. The areas at risk for Saline controls, Adeno-only, Lido-only, Low-dose AL, Mid-dose AL and High-dose AL treated animals were $61 \pm 5\%$, $58 \pm 8\%$, $56 \pm 8\%$ $43 \pm 6\%$, $48 \pm 8\%$ and $66 \pm 8\%$ respectively, and not significantly different ($P < 0.05$) (Fig. 5a).
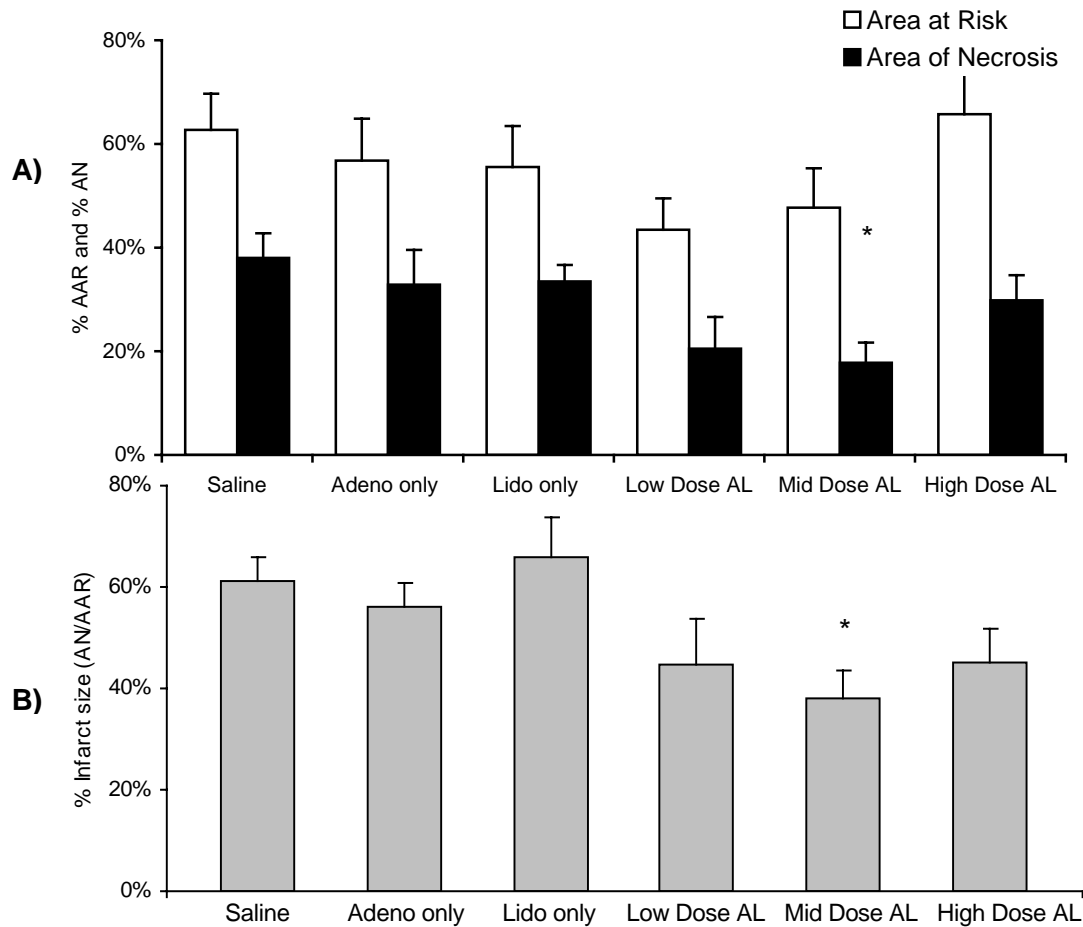
Figure 3.6. Effects of treatments on left ventricle necrosis and infarct size.
Areas at risk (AAR/LV) were not significantly different between groups A) Areas of
necrosis in the left ventricle (AN/LV) were significantly smaller with AL mixture
treatment. B) Infarct sizes (AN/AAR) in groups receiving AL solution treatment were
significantly smaller compared with saline-alone, adenosine-alone and lidocaine-
alone treatment groups. *$P < 0.05$. Surviving rats: Saline, n=5; Adeno only, n=4; Lido
only, n=6; Low dose AL, n=5; Mid dose AL, n=7; High dose AL, n=6.

Overall, the mean risk area was $55 \pm 0.03\%$ (n = 33). The area of necrosis in Mid-

dose AL treated animals was $18 \pm 4\%$ and significantly lower than Saline-controls (38

$\pm 5\%$), Adeno-only ($33 \pm 7\%$), and Lido-only ($33 \pm 3\%$) treatments ($P < 0.05$), but was

not different from Low-dose AL ($21 \pm 6\%$) or High-dose AL ($30 \pm 5\%$) (Fig. 3.6a). The

mean infarct size of Mid dose-AL ($38 \pm 6\%$) was also significantly reduced from

Saline controls (61 ± 5%), Adeno-only (56 ± 4%), and Lido-only (66 ± 8%) (P < 0.05) but not from Low-dose AL (45 ± 9%) and High-dose AL animals (45 ± 6%) (Fig. 3.6b). There was no significant difference in mean infarct size between Saline controls, Adeno-only, or Lido-only treatments (Fig. 3.6b).

3.4.5. SYSTEMIC HEMODYNAMICS

Heart rate (HR), mean arterial pressure (MAP) and rate-pressure product (RPP) are shown in Figure 3.7. There were no significant differences between groups at baseline prior to pretreatment. Following pretreatment at preocclusion, the heart rate of Lido-only (357 ± 16 bpm), Low-dose AL (328 ± 8 bpm), Mid-dose AL (327 ± 13 bpm) and High-dose AL (322 ± 9 bpm) were all significantly reduced compared to Saline controls (429 ± 13 bpm) and Adeno-only (405 ± 18 bpm). The preocclusion MAP and RPP of Saline controls (98 ± 9 mmHg, 36400 ± 3900 bpm.mmHg), Adeno-only (73 ± 18 mmHg, 24600 ± 8300 bpm.mmHg), and Lido-only (81 ± 12 mmHg, 25900 ± 5500 bpm.mmHg) were significantly greater than the Low-dose AL (49 ± 4 mmHg, 12000 ± 1200 bpm.mmHg), Mid-dose AL (45 ± 4 mmHg, 10000 ± 1000 bpm.mmHg) and High-dose AL (40 ± 3 mmHg, 8500 ± 900 bpm.mmHg). By 25 min ischemia, the heart rates of Lido-only (303 ± 22 bpm), Low-dose AL (305 ± 15 bpm), Mid-dose AL (314 ± 16 bpm) and High-dose AL (303 ± 18 bpm) treatments were significantly lower than Adeno-only treatment (405 ± 18 bpm) and Saline controls (427 ± 16 bpm) (P < 0.05). At 25 min ischemia, the MAP and RPP of Saline controls (98 ± 15 mmHg, 37100 ± 6700 bpm.mmHg) and Adeno-only (65 ± 9mmHg, 21900 ± 2300 bpm.mmHg) was maintained significantly higher than Lido-only (56 ± 4 mmHg, 13400 ± 1600 bpm.mmHg), Low-dose AL (54 ± 7 mmHg, 12500 ± 1700 bpm.mmHg), Mid-dose AL (49 ± 5 mmHg, 10500 ± 1800 bpm.mmHg) and High-dose AL (46 ± 6

91

mmHg, 9400 ± 1600 bpm.mmHg) ($P < 0.05$). Throughout reperfusion the hemodynamics in all groups rose toward pretreatment values. However, within the 120 min reperfusion period, no treatment reached starting baseline values in any group (Fig. 3.7).

The possibility of reduced hemodynamics at the start of ischemia, following pretreatment affecting infarct size reduction was examined using the Pearson's correlation coefficient for all treatments following pretreatment. No relationship was found between reduced heart rate, mean arterial pressure, rate-pressure product at pretreatment and infarct size reduction in any treatment group.
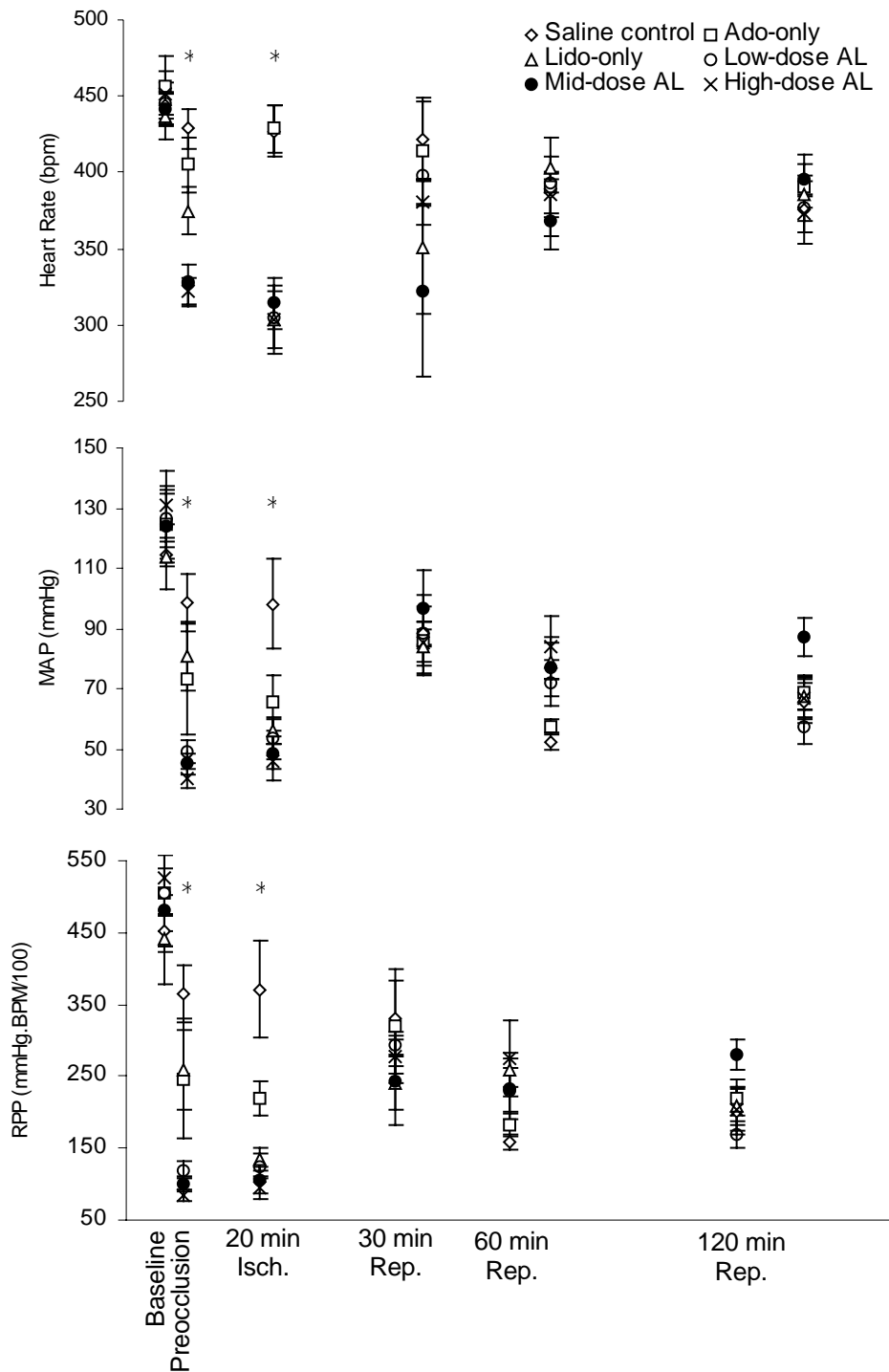
Figure 3.7. Hemodynamic changes in saline- controls and the five treatment groups at baseline, before occlusion, 20 min ischemia and 30, 60 and 120 min reperfusion. Measurements were recorded throughout pretreatment/preocclusion, ischemia and reperfusion. a) Heart rate (HR); b) Mean arterial pressure (MAP); c) Rate-pressure product (RPP). * P < 0.05. Surviving rats: Saline, n=5; Adeno only, n=4; Lido only, n=6; Low dose AL, n=5; Mid dose AL, n=7; High dose AL, n=6.

3.5. Discussion

This study demonstrates that an intravenous infusion of an AL solution administered 5 min before and during 30 min ischemia confers superior protection from death, ventricular arrhythmias and cell necrosis, than either drug alone in the *in vivo* rat model. In this study, 58% of saline-controls and 50% of adenosine-only treated animals died during ischemia secondary to ventricular fibrillation. In lidocaine-treated animals, there were no deaths although ventricular arrhythmias were greater than AL solution treatment and the mean infarct size was slightly larger than saline-controls (Fig. 3.6). In contrast, AL administration (at all combinations studied) prevented cardiac death, virtually abolished ventricular tachycardias and fibrillation, and reduced infarct size from 61 to 38% (Mid-dose AL) (Fig. 3.4, Fig. 3.5, and Fig. 3.6).

3.5.1. AL SOLUTION'S ANTIARRHYTHMIC ACTIONS AND SURVIVAL BENEFIT

Perhaps the most striking finding was that the infusion of AL solution resulted in no deaths while 58% of the saline-controls, 50% of the Ado-only treated animals died. Given adenosine's well-known role to potentiate the abolition of catecholamine triggered ventricular arrhythmias (Gorge et al., 1998; Schreieck and Richardt, 1999), and the nucleoside's ability to reduce myocardial injury when administered prior to and during regional or global ischemia (Thornton et al., 1992; Toombs et al., 1992; Du et al., 1993; Lasley and Mentzer, 1995; Vinten-Johansen et al., 1999), it was surprising that the adenosine only infusion failed to protect from death. Adenosine may have either failed to protect the heart from arrhythmias or, based on the higher relative durations of VF compared to durations in saline-controls, may have promoted arrhythmias. To my knowledge, death during ischemia with adenosine infusion has

not been reported before in the rat, dog, pig or human, and these unexpected results may relate to a proarrhythmic affect from the concentration administered. Furthermore, it is possible that these animals may have developed even larger infarcts than those measured among surviving animals. Unfortunately, the standard method of infarct size measurement with TTC cannot be achieved with at least 120 min of reperfusion which is impossible in dead animals.

3.5.1.1. Ischemia-induced arrhythmias

The remarkable ability of AL solution, especially the Mid-dose AL, to virtually abolish arrhythmias and reduce cell death may be linked to lower cardiac excitability and better stabilization of electrophysiological and metabolic functions in the AL-treated ischemic heart. Experimental support for lower demand was reflected in the reduced heart rate and other hemodynamic parameters in AL-treated groups (Fig. 3.7). Together with down-regulating the whole heart by targeting nodal tissue, atrial and ventricular myocytes, and perhaps the coronary vasculature (Ely and Berne, 1992; Wilson et al., 1993; Lu et al., 1999), the combination may stabilize myocardial excitability by activating adenosine A_1 receptor-linked opening of sarcolemmal ATP sensitive K^+ channels (Kirsch et al., 1990; Li et al., 1995). The increased potassium conductance in ischemic cells and accumulation of K^+ ions in the extracellular space, would lead to partial depolarization of resting membrane potential and cell excitability would decrease from the reduction of Na^+ ion fast channel availability (Janse and Wit, 1989; Wilde and Aksnes, 1995; Carmeliet, 1999). A partially depolarized resting membrane potential would promote lidocaine's binding to the inactivated Na^+ channel in ischemic cells (Cardinal et al., 1981; Barrett et al., 2000), and the reduction of Na^+ fast channel availability would slow Na^+ entry into these cells (Lu et al., 1999).

Shortening the action potential duration may also be associated with adenosine's opening of the K_{ATP} channels (and other K^+ channels) through accelerated phase 3 repolarization (Gross and Fryer, 2000), and separately by lidocaine's interaction with cardiac sarcolemmal L-type channels (Wilson et al., 1993; Lu et al., 1999). The overall effect of reducing action potential duration is to reduce Ca^{2+} entry, and improve Ca^{2+} handling in ischemic cells. Despite much controversy, involvement of sarcolemmal K_{ATP} channels during early myocardial ischemia have been implicated using the blocker glibenclamide (Kantor et al., 1990; Carmeliet, 1999; Gross et al., 1999), and the highly selective sarcolemma K_{ATP} blocker HMR-1883 (Billman et al., 1998; Toyoda et al., 2000). Mitochondria K_{ATP} channels is another potential sites of adenosine A_1 receptor activation (O'Rourke, 2000) as well as the sarcoplasmic reticulum (Mubagwa, 2002). Lastly, adenosine A_1 activation may confer cardioprotection by blunting the stimulatory effects of catecholamines (inhibiting sarcolemmal Na^+/H^+ and Na^+/Ca^{2+} exchangers), and inhibition of norepinephrine release from nerve terminals (Ely and Berne, 1992; Hayes, 2003). In summary, the antiarrhythmic effect and survival benefit of AL therapy may relate to a better matching of membrane excitability and myocardial metabolism in both ischemic and non-ischemic regions.

In addition, reentry arrhythmias could be reduced by AL's ability to reduce abnormal regional heterogeneity of action potential waveforms and refractory periods in the different chambers of the heart, including transmural, transepicardial and interventricular and supraventricular regions (Elharrar et al., 1977; Janse and Wit, 1989; Nattel, 1998; Janse, 2000). In the normal heart, heterogeneities in action potential durations and refractory periods are important to the synchronization of the heart as a pump (Burton and Cobbe, 2001; Nerbonne and Guo, 2002). Therefore,

reducing myocardial excitability and energy demand, and synchronizing action potential shortening and dispersion through better handling of intracellular Na^+ and Ca^{2+}, the combination of AL may limit the formation and dispersion of refractoriness in the ischemic and border zones. Since AL solution was infused before and during regional ischemia, further experiments are required to test this and to test if the antiarrhythmic therapy could break newly formed ventricular re-entry circuits when administered either as a bolus or intravenous infusion without prior treatment.

3.5.1.2. Reperfusion-induced Arrhythmias

Compared to ischemia-induced arrhythmias, reperfusion arrhythmias were minimal in this study overall. Reduced episodes of reperfusion arrhythmias in our study agree with the concept that the duration of ischemia inversely affects the episodes of subsequent reperfusion arrhythmias (Corr and Witkowski, 1983; Manning and Hearse, 1984). However, significant differences between treatment groups were found. Rats receiving adenosine-only or lidocaine-only experienced VT or VF upon reperfusion, while no animal that had received any dose of adenosine with lidocaine experienced reperfusion-induced arrhythmias. The genesis of early reperfusion-induced arrhythmias may be related to oxygen-derived free radicals (Ravingerova et al., 1999). Both adenosine and lidocaine alone have been shown to be protective against reactive oxygen species (Cronstein et al., 1992; Hyvonen and Kowolik, 1998). Therefore at the onset of reperfusion, AL solution may have attenuated the formation of reactive oxygen species.

3.5.2. PROARRHYTHMIC EFFECTS OF ADENOSINE AND LIDOCAINE ALONE

This study showed that both adenosine and lidocaine alone were potentially proarrhythmic. For example, infusion of adenosine alone (305 μg/kg/min) was not

effective at abating ventricular tachyarrhythmias in this rat model of regional

ischemia. Adenosine's main site of action in the heart is at the sino-atrial (SA) and

atrioventricular (AV) nodes where high concentrations of adenosine A_1 receptors are

expressed (Lerman and Belardinelli, 1991). While adenosine is not believed to effect

reentrant ventricular arrhythmias in the setting of myocardial ischemia, adenosine has

been shown to abate triggered ventricular arrhythmias through its antiadrenergic

effect (Wilbur and Marchlinski, 1997; Gorge et al., 1998; Lerman et al., 1999;

Schreieck and Richardt, 1999). In this study, adenosine-only infusion failed to protect

from arrhythmic death and ventricular tachycardia and fibrillation with 100% of the

surviving animals affected. These results were surprising given adenosine's well-

known ability to reduce myocardial injury when administered prior to and during

regional or global ischemia (Thornton et al., 1992; Toombs et al., 1992; Lasley et al.,

1995; Lasley and Mentzer, 1995; Vinten-Johansen et al., 1999). Based on the higher

relative durations of VF compared to durations in saline-controls, adenosine at the

concentration tested may have also promoted arrhythmias. Others have shown that

adenosine may initiate reentry arrhythmias and lead to premature ventricular

depolarizations (Wilbur and Marchlinski, 1997).

Similarly, lidocaine appears to have promoted arrhythmias in this study. While the

arrhythmias did not lead to cardiac death, there were a surprisingly high number of

episodes of premature ventricular beats, runs of bigeminy, and VT and VF during

ischemia (Fig. 3.2, Fig. 3.3, and Fig. 3.4). Interestingly, lidocaine treatment also led

to the highest incidence of PVBs (104 ± 21 episodes). Clinical trials have shown that

the risk for proarrhythmic events is highest in patients treated with sodium channel

blockers that develop premature ventricular beats during myocardial ischemia (Echt

et al., 1991; Reiffel et al., 1997; Bauer et al., 2003), yet a link between PVBs to

incidences of life-threatening ventricular fibrillation has not been established (Barrett et al., 1995).

The routine use of lidocaine to treat acute arrhythmias has come under scrutiny in recent years for two main reasons; a number of studies have shown that lidocaine increases electrical defibrillation threshold in a concentration dependent manner by slowing conduction and generating re-entry circuits (Campbell, 1994; Barrett et al., 2000), and others have implicated the drug in fatal bradyarrhythmias in patients with acute myocardial infarction (MacMahon et al., 1988; Hine et al., 1989b). The proarrhythmic effect of lidocaine is thought to develop by lidocaine slowing conduction to such a degree that reentry circuits are generated in the ischemic zone (Barrett et al., 2000).

However, despite its potential drawbacks, lidocaine is still used in the surgical setting to treat ventricular tachyarrhythmias in patients with acute myocardial ischemia when they interfere with hemodynamic status (Opie, 1995) as lidocaine is generally effective in abating ventricular arrhythmias (Barrett et al., 1995).

3.5.3. EFFECT ON INFARCT SIZE

Consistent with the observation of virtually no lethal arrhythmias in rats treated with AL solution, the infarct size was also significantly lower ($38 \pm 6\%$) in Mid-dose AL than in Saline-controls ($61 \pm 5\%$), Adeno-only ($56 \pm 5\%$) and Lido-only treatment ($66 \pm 8\%$) (Fig. 3.6). On the basis of the relationship between aortic diastolic pressure, coronary perfusion pressure, and myocardial oxygen supply, it was expected that any treatment-induced down-regulation of hemodynamics decrease in hemodynamic variables from pretreatment and throughout ischemia would correlate with infarct size reduction. However, our study failed to show a statistical difference between a

decrease in MAP or RPP and reduced infarct size. Interestingly lidocaine only and AL solution treatment groups incurred similar MAP and RPP during ischemia, yet infarct size outcomes were the most widely separated. The infarct sizes resulting from ischemia were more greatly reduced in the AL solution than from lidocaine only treatment, which appeared to not protect from infarction.

3.5.4. POSSIBLE MECHANISMS OF ACTION FOR AL SOLUTION IN ISCHEMIA AND REPERFUSION

Although the mechanism of protection from the AL combination was not investigated in this study, cardioprotection may be related to the synergistic effect of adenosine and lidocaine combined to reduce calcium entry into the myocardial cell. Overall, the data implies that each drug amplifies the effect of the other leading to a reduction in infarct size, episodes of ventricular arrhythmias and death compared to the administration of either drug alone. It is known that Ca^{2+} overload in the ischemic myocardium predisposes the tissue to injury by disturbing membrane linked ionic homeostasis and maintenance of the membrane potential which can lead to high incidences of arrhythmias (Lubbe et al., 1992; Curtis et al., 1993; Brooks et al., 1995). Reducing intracellular Ca^{2+} overload may be due to a complex interaction between adenosine and lidocaine targets involving the opening the A_1-mediated K_{ATP} channels (Vinten-Johansen et al., 1999) whilst blocking Na^+ channels having the overall effect of also reducing Na^+ entry and the activity of Na^+/Ca^{2+} exchanger (Eng et al., 1998; Lu et al., 1999). In addition, these actions may enhance cAMP-linked attenuation of VT (Lubbe et al., 1992; Lerman, 1993). Furthermore, Lu et al. (1999) have attributed inhibition of Ca^{2+} loading by lidocaine's blocking Na^+ entry more prominent in ischemic tissue thereby synchronizing myocardial cells and making reentrant arrhythmias less likely.

Furthermore, that no reperfusion arrhythmias were found in any of the AL solution treated rats, demonstrates that some protection extended into the reperfusion period. Yoshida et al. (2000) have shown in humans that reperfusion VT are most likely arrhythmias triggered by cAMP mediation rather than re-entrant electrical circuits.

As a critical loss of high energy phosphates such as ATP and PCr is strongly linked to the transition between reversible and irreversible injury (Jennings and Reimer, 1981;Reimer and Jennings, 1992), adenosine and lidocaine may have delayed ATP utilization and preserved PCr stores during ischemia to delay cell death therefore resulting in reduced apparent infarct size. Both adenosine (Liu et al., 1991; Fralix et al., 1993) and lidocaine (Matsumura et al., 1987; Wendland et al., 1993; van Emous et al., 1997) have been shown to delay the ischemia-induced decreases in [ATP].

3.5.5. CONCLUSION

This study has shown that intravenously administering a solution of adenosine and lidocaine before and during ischemia in the *in vivo* rat model led to exceptional protection from cardiac death, suppression of lethal arrhythmias and reduced tissue necrosis than either drug alone. The antiarrhythmic and other cardioprotective properties of AL solution during ischemia and reperfusion may involve opening the A_1 receptor-linked K_{ATP} channels and modulation of the voltage dependent Na^+ fast channels, improved intracellular Na^+ and Ca^{2+} handling and a combined effect on cAMP mediated ventricular arrhythmias. The new AL combinational therapy may have clinical relevance by reducing abnormal regional electrical heterogeneity and therefore life-threatening arrhythmias in the ischemic heart.

3.5.6. LIMITATIONS OF THE STUDY

Although remarkable survival benefit with co-administrating AL occurred in the rat model, attempts to develop better antiarrhythmic drug therapies is often hampered by translation from experimentally-induced ischemia to human patients with multiple underlying pathologies (Janse et al., 1998). One possible limitation may be the effect of AL therapy on hemodynamics. This would be less of a problem in the surgical setting or during percutaneous transluminal coronary interventions (balloon and stent) where hemodynamic changes could be avoided by using intracoronary infusions.

Lee et al. (1995) infused similar concentrations of adenosine (250-350 μg/kg) for 10 min in humans prior to elective cardiopulmonary bypass surgery without untoward effects. Indeed, it was found that adenosine pretreatment improved post-bypass left ventricular function compared to no treatment, and that benefit continued 40 hours postoperatively (Lee et al., 1995). However, arrhythmias were not investigated in their study. In addition, higher doses of adenosine have been used in other surgical settings without adverse effects. Lagerkranser et al. (1989) used a dose range of 60 - 350 μg /kg/min i.v. in patients undergoing surgery for cerebral aneurysm and found that adenosine-induced hypotension (MAP of 40-50 mmHg) did not affect cerebral oxygenation unfavorably. Furthermore, Tabrizchi (1997) reported that a dose of adenosine given at 300 μg/kg/min to sodium pentobarbital anesthetized rats did not significantly alter cardiac output.

Other considerations using animal models and their applicability to humans include differences in the scaling of mass-specific metabolic rates (Dobson, 2003), differences in timing of ischemia-induced arrhythmias and electrophysiological properties (Opitz et al., 1995) and differences in functional morphology such as in

collateral circulation (Schaper et al., 1992; Janse et al., 1998). Despite species differences, knowledge obtained from antiarrhythmic drug studies in mouse, rat, guinea-pig or rabbits, and their underlying electrophysiological and metabolic mechanisms of actions, have been instrumental in devising diagnostic and therapeutic strategies as part of the wider translational screening process linking basic research to clinical outcomes.

Chapter 4. AL cardioprotection: ischemic vs reperfusion therapy
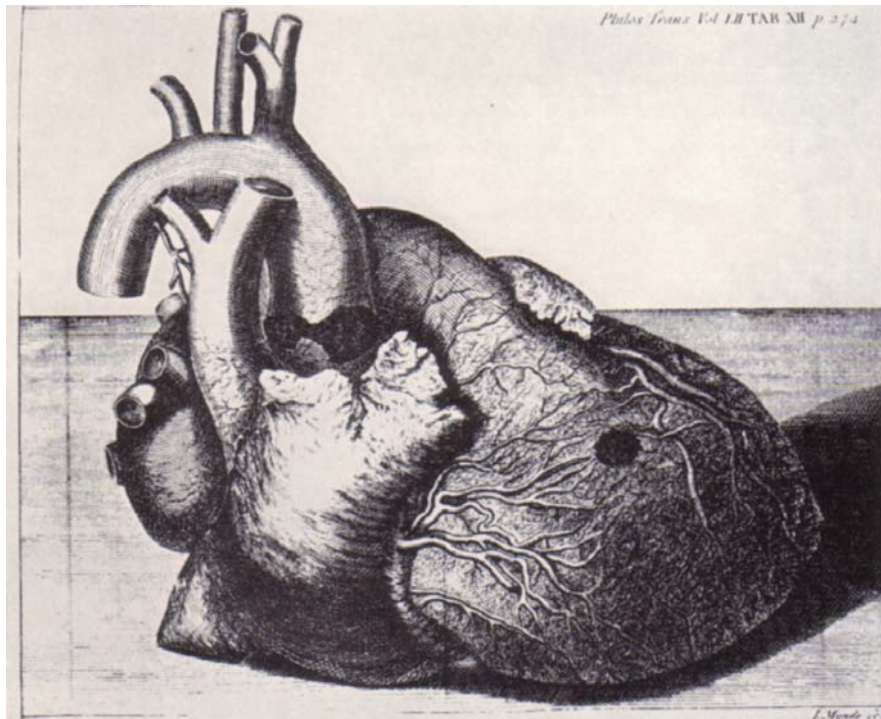
Plate V. Illustration of the ruptured heart of King George II (1683-1766); Frank Nichols (1699-1778) reported his post-mortem findings from the autopsy in the Philosophical Transactions of the Royal Society of London in 1761. King George II's death was probably caused by a coronary occlusion and the transverse fissure in the aortic trunk.

(Leibowitz, 1970)

4.1. Introduction

In Chapter 3, AL solution treatment was shown to be more cardioprotective than treatment with adenosine or lidocaine alone as evidenced by preventing mortality, ventricular fibrillation and reducing infarct size when given prior to and during ischemia. The present chapter extends these findings by investigating if AL solution protection would be effective if continued as a reperfusion therapy. Importantly, this chapter also highlights the differences between this work and the earlier study by Homeister et al. (1990) who used adenosine and lidocaine *separately and sequentially* to prevent reperfusion injury. Homeister and colleagues hypothesized that administration of exogenous adenosine at reperfusion would limit reperfusion injury only in the presence of lidocaine. Homeister et al.'s study was performed in recognition of the increased use of lidocaine in experimental protocols to prevent potentially lethal arrhythmias alongside reperfusion therapies such as exogenous adenosine.

As discussed in Chapter 1, the simultaneous use of adenosine and lidocaine as combinational therapy has received little or no experimentation *during* ischemia. Homeister et al. (1990) were the first to propose that adenosine reperfusion therapy is only effective in limiting infarct size if given in the presence of lidocaine using an *in vivo* canine model of acute myocardial infarction. Using a rabbit model of acute myocardial ischemia, Goto et al. (1991) investigated adenosine protection of reperfusion injury and reported that adenosine given during reperfusion failed to limit infarct size even when combined with lidocaine. In 1994, Schjott and colleagues examined lidocaine interaction with exogenous and endogenous adenosine in the isolated rat heart. They found that lidocaine failed to increase the physiologic

responses (aortic pressure or left ventricular developed pressure) to adenosine yet they did show that lidocaine did increase exogenous adenosine uptake.

Using a canine model and protocol similar to Homeister et al. (1990), Vander-Heide and coworkers (1986) were unable to repeat Homeister's findings and concluded that the concomitant use of adenosine and lidocaine during reperfusion did not offer any infarct limiting advantage. Whether or not this discrepancy was due to different routes of adenosine administration, intracoronary (Homeister et al., 1990) and intravenous (Vander-Heide et al., 1996), at the same dose (150 μg/kg/min) is still in question.

In humans, Garratt et al. (1998) concluded that adenosine and lidocaine have potential for benefit. Their protocol involved continuously infusing lidocaine from the point of anesthesia and throughout balloon angioplasty and administering adenosine 10 minutes prior to the balloon inflation and during reperfusion. In criticism of their own study these investigators report that the small sample size limited statistical confidence and that due to the study design they were unable to analyze the individual contributions of each drug to the measured end points. Using the data from the larger AMISTAD trial, Mahaffey et al. (1999) concluded that the continuous infusion of lidocaine given to adenosine-treated patients made no difference to the outcome of the adenosine-treated patients.   In summary, the issue of concomitant, *separate* and *sequential* administration of adenosine and lidocaine for reperfusion therapy remains unresolved.

The present chapter aims to 1) assess the ability of the AL solution to provide protection from ischemic injury vs. reperfusion injury or both and 2) to address the

level of protection afforded by AL solution from Chapter 3 against the separate and sequential infusion of adenosine and lidocaine similar to Homeister et al. (1990).

4.2. Experimental Design

All rats were subjected to a pretreatment period, 30 min of ischemia followed by 120 min of reperfusion. All drugs were prepared on the day of the experiment in physiological saline (0.9%) and delivered intravenously through either the left or right femoral vein as required. The saline-control animals and AL solution treated animals described in Chapter 3 are included for comparison. The AL solution contained 6.3 mg/ml adenosine (Ado) and 12.6 mg/ml lidocaine and was delivered at 1 ml/hr which converts to mass specific dosages of 305 μg/kg/min and 608 μg/kg/min for Ado and Lido respectively. The Mid-dose AL treated rats and saline-controls from Chapter 3 were included in the data analysis as the data for this chapter was collected within the same testing period. Overall, a total of thirty-two rats were included in this study.

Rats were randomly assigned to one of five different treatment regimes (Fig. 4.1): (1) Saline-controls (0.9% saline): saline was given 5 min before and throughout 30 min ischemia (n = 12); (2) AL solution: AL solution was administered 5 min before and throughout 30 min ischemia, though reperfusion (120 min) proceeded without any additional treatment (n = 7); (3) Lido, Ado-SEQ (*sequential*): a rapid bolus of lidocaine (2 mg/kg i.v.) was given 1 min before left coronary artery (LCA) ligation and another bolus at 1 min before reperfusion; adenosine (150 μg/kg/min i.v.) was infused 2 min before reperfusion and continued throughout 30 min of reperfusion (n = 7); (4) AL-SEQ: AL solution was given *sequentially* at two separate times, 5 min before but not throughout ischemia and then 5 min before reperfusion and throughout 30 min reperfusion (n = 6); (5) AL-PIR: AL solution was given continuously from

pretreatment (P), 5 min before ischemia, throughout ischemia (I) and throughout 30

min reperfusion (R) (n = 6).
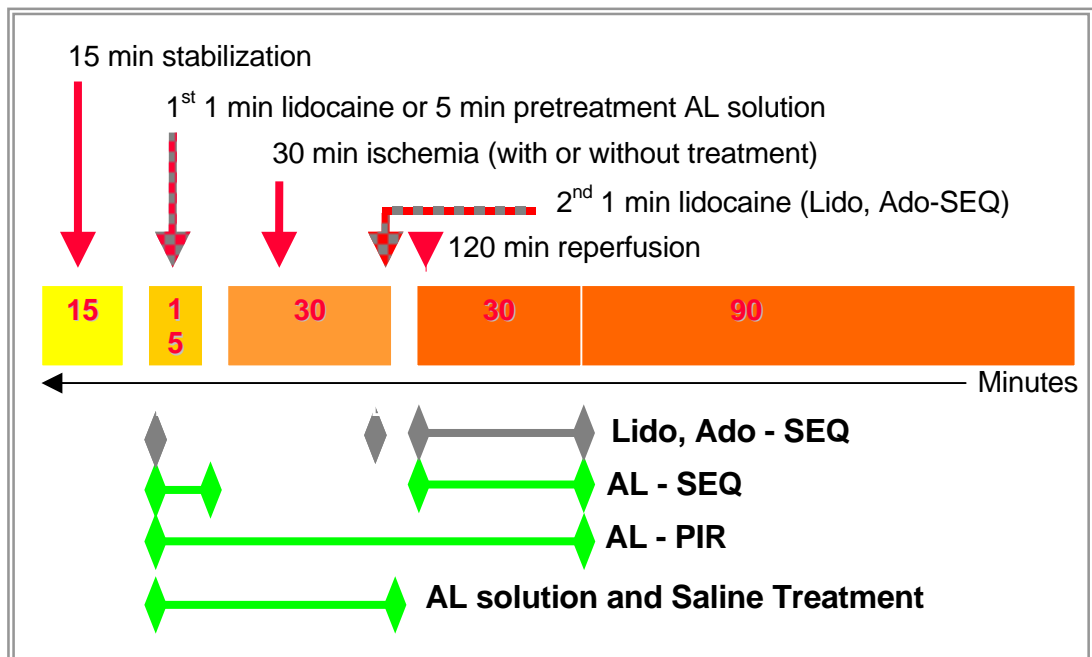
Figure 4.1. Chapter 4 treatment protocol.

The primary end-points used to assess the cardioprotective effects of AL solution

were infarct size, episodes and duration of ventricular arrhythmias and death. The

secondary end-points included heart rate, mean arterial pressure (systolic pressure –

diastolic pressure/3 + diastolic pressure) and rate pressure product (heart rate x

systolic pressure).

4.3. Results

4.3.1 MORTALITY

Death occurred secondary to ventricular fibrillation during the ischemic period. Seven of the 12 saline-control rats died during the ischemic period from an episode of ventricular fibrillation (n = 5 survived). No deaths occurred in AL solution infused animals (n = 7 survived). Pretreatment with a 2 mg/kg lidocaine bolus resulted in two deaths from ventricular fibrillation during ischemia before adenosine infusion commenced (Lido,Ado-SEQ) (n = 5 survived). In contrast, no deaths occurred from ischemia-induced arrhythmias in rats pretreated with 5 min of AL infusion, even though treatment was not extended through ischemia (AL-SEQ) (n = 6). Similarly no deaths were recorded in animals continuously infused with AL for 5 min pretreatment, 30 min ischemia and 30 min reperfusion (AL-PIR) (n = 6). Only data from surviving rats were further analyzed (n = 29).

4.3.2. ARRHYTHMIAS DURING ISCHEMIA: EPISODES AND DURATIONS

The episodes and durations of VT and VF from rats that survived ischemia are shown in Figure. 4.2. The mean number of episodes of ischemia-induced VT in saline-controls was 18 ± 9 affecting 100% of animals (Fig. 4.2), and 40% experienced VF (4 ± 3 episodes). In AL solution treated rats, 57% of subjects had at least 1 episode of VT (2 ± 1 episodes) while no rat experienced an episode of VF (Fig. 4.2). Rats infused with AL solution experienced not only a significant reduction in VT's, but also a significant reduction in durations of VT (2 ± 1 sec) and VT+VF's (2 ± 1 sec) compared to saline-controls. The durations of VT and VT + VF's for saline-controls were 106 ± 45 sec and 156 ± 72 sec (Fig. 4.2). Forty percent of the lidocaine-

pretreatment group (Lido,Ado-SEQ) experienced 6 ± 3 episodes of VT of 4 ± 2 sec

duration and 1 ± 0 episodes of VF of 1 ± 0 sec duration during ischemia (before

adenosine infusion) (Fig. 4.2).   The sum of VT and VF episodes and durations for

these groups were 7 ± 3 episodes and 4 ± 2 sec respectively. The lidocaine

pretreatment strategy (Lido,Ado-SEQ) did not significantly reduce episodes or

durations of VT or VF compared to saline-controls. In contrast, animals infused with

AL during 5 min pretreatment and continued throughout 30 min ischemia and

reperfusion (AL-PIR) experienced significantly reduced episodes and durations of VT

(2 ± 1 episodes, 2 ± 1 sec, 57% affected) and VT+VF (2 ± 1 episodes, 2 ± 1 sec)

compared to saline-controls (18 ± 9 episodes, 106 ± 45 sec, 100% affected and $22 \pm$

12 sec, 156 ± 72 sec, respectively) ($P < 0.05$). However, a 5 min pretreatment of AL

solution discontinued during ischemia (AL-SEQ) was not sufficient to prevent VF

episodes (2 ± 1 episodes, 21 ± 8 sec, 67% affected), or reduce VT (39 ± 23 episodes,

84 ± 49 sec, 83% affected) and VT+VF (40 ± 23 episodes, 104 ± 46 sec).

Importantly, only constant infusion of AL solution before and during ischemia

prevented episodes of VF during ischemia.
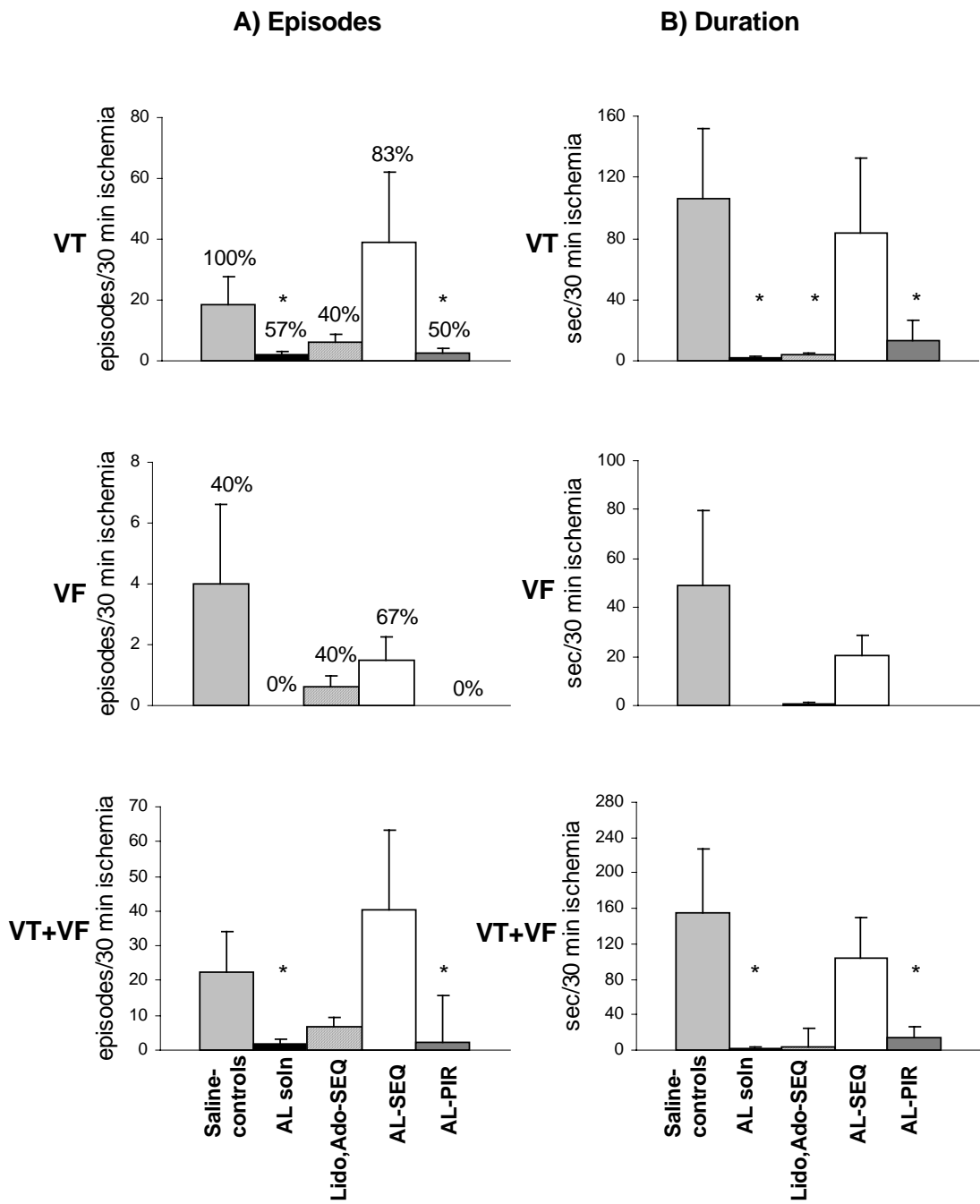
Figure 4.2. The episodes and duration of ventricular tachycardia (VT) and ventricular fibrillation (VF) and VT+VF during ischemia for surviving rats. These values represent overall sum of A) episodes and B) durations (sec) that occurred throughout the 30 min ischemic period. Surviving rats: saline-control, n = 5; AL solution, n = 7; Lido,Ado-SEQ, n = 5; AL-SEQ, n = 6, AL-PIR, n = 6. *P < 0.05.

4.3.2.1. Early Reperfusion Arrhythmias

Within the first minute of reperfusion, 80% of saline-controls experienced at least one episode of VT of 0.6 to 35 sec duration. An episode of VF occurred in 1 of the 5 saline-controls within the first minute and lasted 16 sec. Of the animals pretreated with a 2 mg/kg bolus of lidocaine before ischemia followed by another lidocaine bolus and adenosine infusion just before reperfusion (Lido,Ado-SEQ), 48% experienced VT and in 52% VF occurred. Overall there were 6 ± 3 episodes of VT+VF of 7 ± 4 sec duration within 1 min of reperfusion in the Lido,Ado-SEQ group. The number of episodes and the durations of VT, VF or VT+VF in the Lido,Ado-SEQ group were not significantly different from saline-controls ($P < 0.05$). In contrast, there were no reperfusion-induced arrhythmias in animals given AL solution in any sequence (AL solution, AL-SEQ, AL-PIR) which resulted in significantly reduced episodes in comparison to saline-controls and Lido, Ado SEQ treated animals ($P < 0.05$).

4.3.3. INFARCT SIZE

Mean area at risk (AAR/LV), areas of necrosis (AN/LV) and infarct size (AN/AAR) expressed as a percentage of left ventricle are shown in Figure 4.3. No significant differences were found in risk areas among all treatment groups and controls ($63 \pm 7\%$) (Fig. 4.3). The areas at risk for saline-controls, AL solution treated animals, lidocaine-bolus/adenosine infusion (Lido,Ado-SEQ), sequential AL infusion (AL-SEQ) and constant AL infusion (AL-PIR) were $63 \pm 7\%$, $48 \pm 8\%$, $55 \pm 5\%$, $44 \pm 8\%$ and $47 \pm 6\%$ respectively. The area of necrosis for saline-controls ($38 \pm 5\%$) was not significantly different from Lido,Ado-SEQ ($29 \pm 4\%$) and AL-SEQ ($29 \pm 5\%$) treatments (Fig. 4.3). In contrast, treatment with AL solution as a pretreatment and throughout ischemia (AL solution) or continued during 30 min reperfusion (AL-PIR)

significantly reduced the area of necrosis from controls to 18 ± 4% and 21 ± 6%,

respectively (P < 0.05) (Fig. 4.3). The mean infarct size was significantly reduced in

rats infused with AL solution (38 ± 6%) compared to saline-controls (61 ± 5%) and

AL-SEQ (P < 0.05) (Fig. 4.3). However, the mean infarct size for Lido,Ado-SEQ, AL-

SEQ and AL-PIR treated groups were 52 ± 5%, 67 ± 8%, and 41 ± 10% respectively

and not significantly different from saline-controls (61 ± 5%) (P < 0.05).
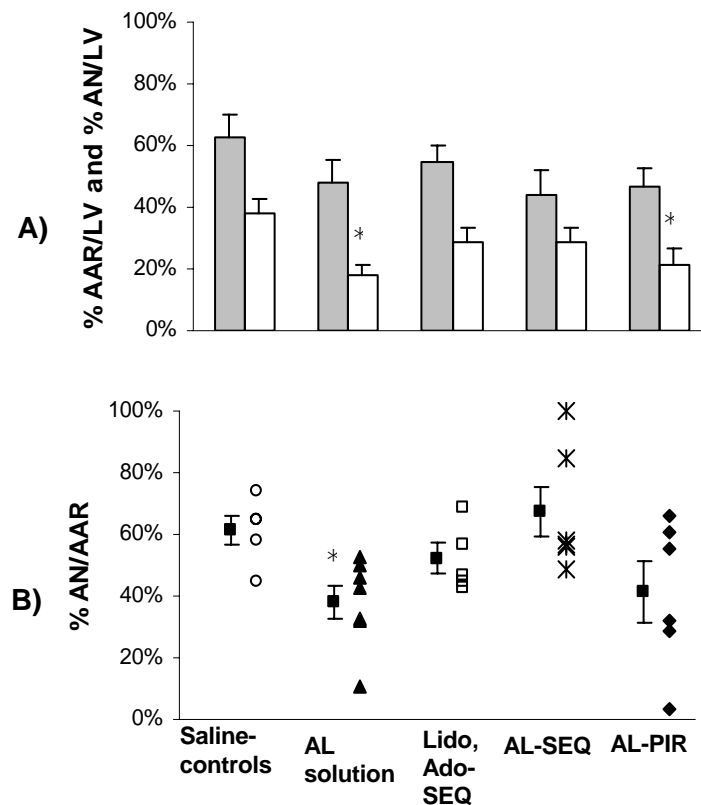
Figure 4.3. Effects of AL solution and sequential administration of adenosine and lignocaine during ischemia and/or reperfusion on infarct size. A) Percentages of areas at risk (AAR) and areas of necrosis (AN/LV). B) Infarct sizes (AN/AAR); Large symbols represent means ± SE for each group. Surviving rats: saline-control, n = 5; AL solution, n = 7; Lido, Ado-SEQ, n = 5; AL-SEQ, n = 6, AL-PIR, n = 6; *P < 0.05.

4.3.4. SYSTEMIC HEMODYNAMICS

Heart rate, MAP and RPP at baseline, after 5 min pretreatment, at 25 minutes ischemia, and at 30, 60 and 119 min of reperfusion were analyzed (Fig. 4.4). There were no significant differences among groups prior to any treatment at baseline. At pretreatment little change occurred to the HR, MAP and RPP in the saline-controls (429 ± 13 bpm, 98 ± 9 mmHg and 53379 ± 4168 bpm.mmHg) and lidocaine-bolus treatment group (Lido,Ado-SEQ, 376 ± 19, 110 ± 8 mmHg, 47907 ± 4734 bpm.mmHg). A significant reduction in HR, MAP and RPP occurred at pretreatment in animals receiving the AL solution (327 ± 13 bpm, 45 ± 4 mmHg, 26045 ± 3428 bpm.mmHg), AL-SEQ (346 ± 22 bpm, 49 ± 3 mmHg, 26291 ± 3502 bpm.mmHg), or AL-PIR (360 ± 6 bpm, 48 ± 3 mmHg, 25710 ± 1641 bpm.mmHg) compared to saline-controls or Lido,Ado-SEQ ($P < 0.05$). At 25 min ischemia, all hemodynamic variables continued to be reduced in AL solution (314 ± 16 bpm, 49 ± 5 mmHg, 26045 ± 3428 bpm.mmHg), and AL-PIR (323 ± 15 bpm, 49 ± 6 mmHg, 23877 ± 3522 bpm.mmHg); however, AL-SEQ hemodynamics increased significantly (404 ± 23 bpm, 82 ± 9 mmHg, 42047 ± 5777 bpm.mmHg) compared to AL solution and AL-PIR. No further significant changes in heart rate were found between groups throughout reperfusion. By 30 min of reperfusion, mean arterial pressures recovered in AL solution group (97 ± 13 mmHg) to values significantly higher than in Lido,Ado-SEQ (69 ± 5 mmHg), AL-SEQ (58 ± 4 mmHg) or AL-PIR treatment (55 ± 4 mmHg), yet not unlike saline-controls (88 ± 13). No significant differences in HR and RPP were found at 30 min reperfusion. At 60 min reperfusion, MAP dropped sharply in saline-controls (52 ± 3 mmHg) compared to AL solution (77 ± 9 mmHg) Lido,Ado-SEQ (88 ± 3 mmHg) and AL-PIR (80 ± 7 mmHg). The rate pressure product of saline controls decreased substantially at 60 min reperfusion (29909 ± 1251 bpm.mmHg) though the decrease
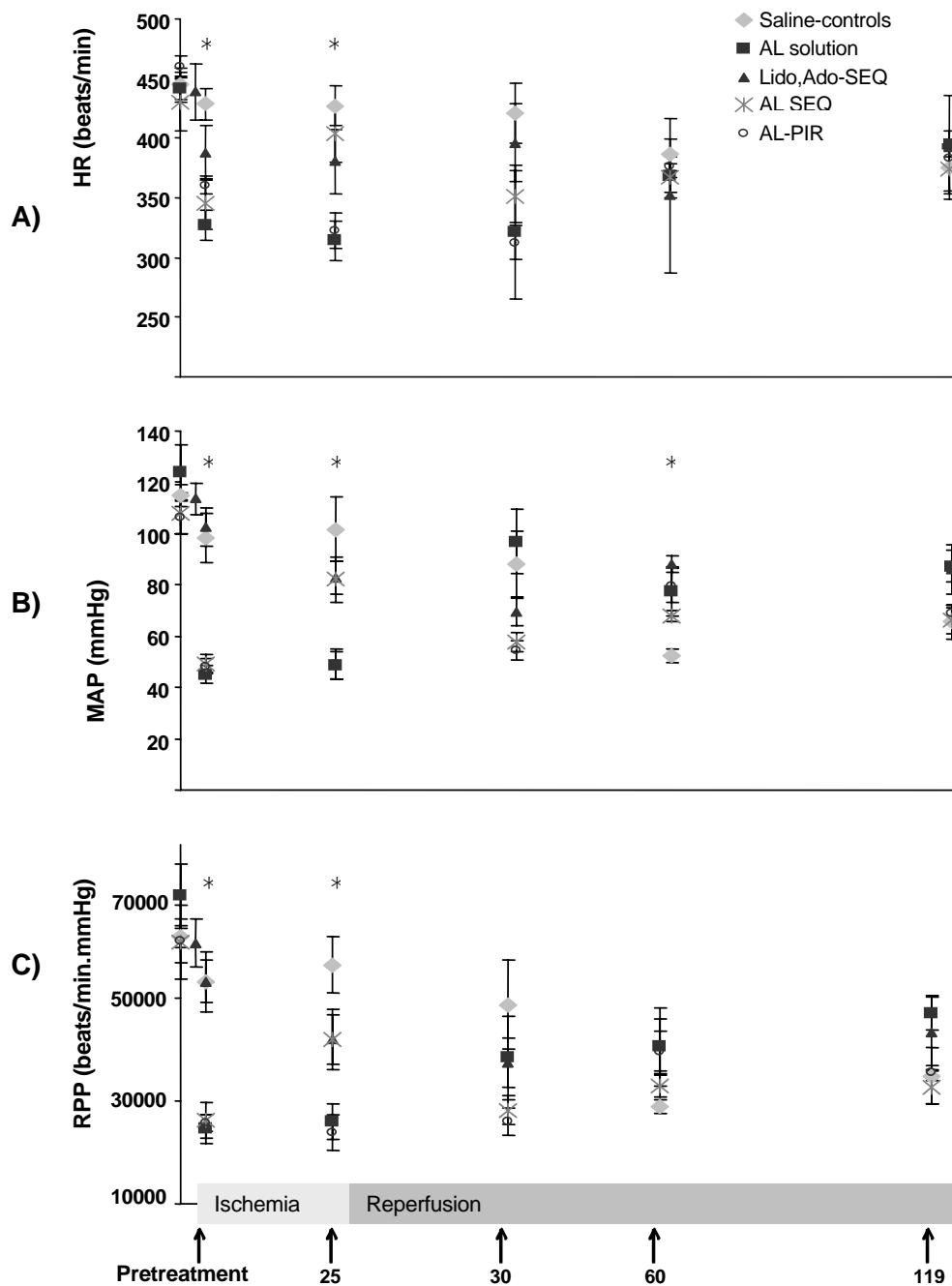
Figure 4.4. Hemodynamic changes for all surviving animals during the course of the first experiment. Measurements were recorded throughout pretreatment/preocclusion, ischemia and reperfusion. Shown, in order, baseline 0 min, following pretreatment, 25 min ischemia, 30 min, 60 min and 119 min reperfusion. A) Heart rate (HR); B) Mean arterial pressure (MAP); C) Rate-pressure product (RPP). Large symbols represent means ± SE for each group. Surviving rats: saline-control, n = 5; AL solution, n = 7; Lido, Ado-SEQ, n = 5; AL-SEQ, n = 6, AL-PIR, n = 6; *P < 0.05 for group effect.

was not significantly different than in other treatment groups. By 119 min reperfusion MAP was significantly improved in the AL solution (87 ± 6 mmHg) and the Lido,Ado-SEQ (86 ± 10 mmHg) compared with groups where AL solution was given for 30 min reperfusion (AL-SEQ 66 ± 5, and AL PIR, 69 ± 3) ($P < 0.05$).

4.4. Discussion

Intravenous infusion of AL solution before and throughout ischemia was associated with reduced mortality, reduced ventricular arrhythmias and lower infarct size. When the AL solution infusion was continued into reperfusion (AL-PIR), no differences were found compared to the AL solution group where infusion was stopped at reperfusion. Although sequential infusion of AL solution, at pretreatment and again prior to reperfusion, protected animals from mortality, this treatment strategy was unable to abate ventricular arrhythmias during ischemia and did not reduce infarct size compared to saline-controls. In addition, the results from this study, like Vander-Heide and Reimer (1996) and Mahaffey et al. (1999), appear to be in conflict with the work of Homeister et al. (1990). Adenosine and lidocaine treatment given *separately* and *sequentially* as reperfusion therapy in a similar protocol to Homeister et al. did not protect from mortality or reduce infarct size differently from saline-controls, yet ventricular arrhythmias were reduced during ischemia but not reperfusion.

This study's results were not in total conflict with Homeister and colleagues. While Homeister et al. did not report the effect of lidocaine or adenosine on mortality rates, they did exclude 6 dogs that received a rapid bolus of lidocaine (2 mg/kg i.v.) and five saline-controls because of intractable VF (Homeister et al., 1990). Presumably, these subject exclusions died during ischemia as no distinction was made with regard to the period in which the VF occurred.

In the present study, the infusion of a lidocaine bolus in Lido,Ado-SEQ failed to eliminate ventricular fibrillation and death. The data indicates that in an *in vivo* rat model of acute myocardial ischemia, a bolus of lidocaine at 2 mg/kg i.v. is not effective enough at abating arrhythmias to ensure no mortality during ischemia. In contrast, even when AL solution was only applied during 5 min pretreatment, there were no episodes of death, despite a variable amount of arrhythmias during ischemia. Overall, the AL solution was consistent in protecting from death from arrhythmias if applied at least at pretreatment.

Likewise during reperfusion, rats infused with AL solution experienced no early reperfusion-induced arrhythmias in any of the four protocols and 29 animals tested. On the contrary, rats receiving lidocaine bolus/adenosine infusion group (Lido,Ado-SEQ) experienced VT or VF early during reperfusion. It is puzzling that there wasn't a reduction in reperfusion arrhythmias with the *separate* and *sequential* adenosine and lidocaine compared to the reduction shown in all AL treated groups, for in both cases adenosine and lidocaine were in the presence of one another. One explanation might be related to the association between early reperfusion-induced arrhythmias with the genesis of oxygen-derived free radical production accompanying the sudden oxygen burst achieved with reperfusion (Ravingerova et al., 1999). Both adenosine and lidocaine alone have been shown to be protective against reactive oxygen species (Myers et al., 1985; Bolli et al., 1989b; Cronstein et al., 1992; Das and Misra, 1992; Hyvonen and Kowolik, 1998; Jordan et al., 1999). The AL solution may have attenuated the formation of reactive oxygen species such as hydrogen peroxide or free radical generation more effectively than the separate and sequential administration because the concentration of adenosine was higher in the AL solution than in the Homeister et al. protocol. Indeed most adenosine researchers prefer the

concentration of adenosine used by Homeister (150 µg/kg/ml/min i.c.) in experimental

models of acute myocardial ischemia whether or not the infusion is intracoronary

(Pitarys et al., 1991) or intravenous (Vander-Heide et al., 1996). However, as a point

of emphasis, no one has previously combined adenosine and lidocaine in a solution

primarily for treatment of ischemic injury.

The results of Chapter 3 indicate that an AL solution containing a mass specific dose

of adenosine at about 150 µg/kg/min was not as effective at reducing arrhythmias as

305 µg/kg/min adenosine with lidocaine. Lasley and Mentzer (1998) and Randhawa

et al. (1995) state that the doses of adenosine used must be large enough so that

adenosine is in contact with myocytes for adenosine receptor activation. The rapid

breakdown of adenosine by endothelium and red blood cells (≈ 8 secs) forms a

metabolic barrier preventing adenosine from reaching myocytes (Nees et al., 1985)

and could be a substantial limiting factor with intravenous administration of

adenosine.

The current study further illustrates that AL solution was most effective at reducing

ischemic injury through the infarct size changes associated with the treatments: AL

solution, AL-SEQ and AL-PIR. The infarct size was virtually unchanged when AL

solution was continued from ischemia into reperfusion (AL-PIR, $41 \pm 10\%$) compared

to AL solution given just as pretreatment and continued through ischemia (AL

solution, $38 \pm 6\%$). Coupling this result with the lack of infarct size reduction in the

AL-SEQ group, where AL treatment was primarily used to treat reperfusion, seems to

indicate that no additional protective effect by AL solution was afforded in the

reperfusion period. The effect of adenosine on infarct expansion from reperfusion

injury is controversial. Indeed, Homeister (1991) found that adenosine administered

during reperfusion only reduced infarct size in the presence of lidocaine while

Vander-Heide (1996) found no reduction of infarct size with either adenosine or the separate and sequential infusion of adenosine and lidocaine during reperfusion. Further, Lasley and Mentzer (1998) showed that the infarct limiting effect of adenosine was most effective when adenosine was continued throughout the ischemic period. The results of the present study suggest that AL solution primarily protects from ischemic injury and appears to have no effect on limiting infarct expansion during reperfusion.

4.4.1 CONCLUSIONS AND INTERPRETATION

This study shows that AL solution was most effective in protecting from ischemic injury if given at least as pretreatment and throughout ischemia in a rat model of acute myocardial ischemia-reperfusion. While minimizing reperfusion injury with adenosine has been a focus in recent years, treatment with AL solution before and during ischemia reinforces the concept that ischemia and reperfusion are composite events requiring an integrated therapeutic strategy tailored to treat the injury period.

Although the mechanism of protection from the AL combination was not investigated in this study, I postulate that a mechanistic synergy between adenosine and lidocaine action occurs that affords the myocardium protection. The actions of adenosine or lidocaine may be potentiated in the presence of one another combined with the changing conditions of ischemia. On a fundamental basis, adenosine and lidocaine may provide metabolic protection in the form of ATP and PCr preservation as well as protection from tissue acidosis. Studies of the separate effects of adenosine or lidocaine have reported reduced ATP and PCr utilization as well as delayed acidosis (Matsumura et al., 1987; Kojima and Miura, 1991; Butwell et al., 1993; Fralix et al., 1993). By protecting at the metabolic level ischemic injury may be delayed.

Adenosine and lidocaine solution may also reduce calcium entry into the myocardial cell. As indicated in Chapter 3 where AL solution groups where compared with adenosine alone and lidocaine alone, the data implied that each drug amplifies the effect of the other leading to a reduction in infarct size, episodes of ventricular arrhythmias and death compared to the administration of either drug alone because either drug alone was unable to induce these effects. It is known that Ca^{2+} overload in the ischemic myocardium predisposes the tissue to injury in part by disturbing membrane linked ionic homeostasis and maintenance of the membrane potential which can lead to high incidences of arrhythmias (Curtis et al., 1993; Brooks et al., 1995). Reducing intracellular Ca^{2+} overload is likely due to a complex interaction between adenosine and lidocaine targets involving the opening the A_1-mediated K_{ATP} channels (Vinten-Johansen et al., 1999) whilst blocking voltage-gated Na^+ channels having the overall effect of reducing Na^+ entry and the activity of Na^+/Ca^{2+} exchanger (Eng et al., 1998; Lu et al., 1999).

Furthermore, that no immediate reperfusion arrhythmias were found in any of the AL solution treated rats, demonstrates that protection extended to at least the first minutes of the reperfusion. Yoshida et al. (2000) have shown in humans that reperfusion VT are most likely arrhythmias triggered by cAMP mediation rather than re-entrant electrical circuits. Whereas Lu et al. (1999) have attributed inhibition of Ca^{2+} loading by lidocaine's blocking Na^+ entry which appears more prominent in ischemic tissue thereby synchronizing myocardial cells and making reentrant arrhythmias less likely. This extends the postulation to the possibility that the AL solution used in our study may have provided a primary window to reduce triggered (adenosine) and re-entrant (lidocaine) arrhythmias through an amplified reduction of

cytosolic Ca^{2+} during ischemia-reperfusion. Further studies are required to test this hypothesis.

Chapter 5. ^{31}P NMR spectroscopic analysis of the effect of AL solution on energetic metabolism and intracellular pH during acute myocardial ischemia
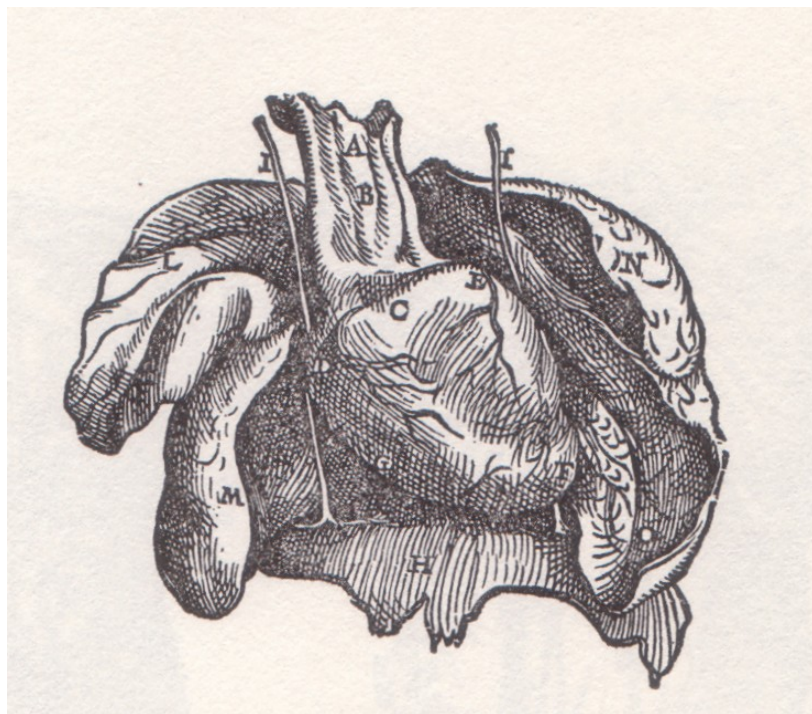
Plate VI. Heart within pericardium surrounded by the lungs and part of the diaphragm (1543), by Andreas Vesalius (1514-1564).

(Herrlinger, 1970)

5.1. Introduction

Phosphorus nuclear magnetic resonance (^{31}P NMR) is a noninvasive, nondestructive powerful tool for the investigation of cardiac energy metabolism during ischemia and reperfusion. It has been effectively used in humans (Bottomley, 1985) and commonly used in animal models (Grove et al., 1980; Jeffery et al., 1989; Bak and Ingwall, 1992; Mottet et al., 1994; Hoffenberg et al., 1996; Kolocassides et al., 1996; Meldrum et al., 1996; Omerovic et al., 1999; Hitchins et al., 2001; Jennings et al., 2001). With the onset of ischemia, when oxygen supply does not meet myocardial demand, phosphocreatine (PCr) rapidly declines immediately followed by a decrease in oxidative phosphorylation resulting in an overall decrease in ATP content. Concomitantly, intracellular pH falls (Reimer and Jennings, 1992). The substantial alterations in transmembrane and intracellular ion homeostasis resulting from an ischemic insult are associated with myocardial dysfunction (Grove et al., 1980; Neely and Feuvray, 1981; Jeffery et al., 1989; Steenbergen et al., 1993) and quantitative measurements of [ATP] provides a strong indication of cardiac viability (Jennings et al., 1986; Jennings et al., 2001). A key principle in a pretreatment strategy for ischemic cell preservation is to reduce myocardial oxygen demand such that the ATP requirements of the ischemic cells are also reduced because during ischemia, oxygen supplies are quickly depleted and not replenished. For example, β-adrenergic blocker treatment reduces blood pressure and decrease heart rate causing alteration in the supply-vs.-demand equation and as a result, protects ischemic cells (Opie, 1998). An effective metabolic therapy of ischemia should also reduce intracellular acidosis and lactate accumulation.

Many studies have shown a separate beneficial effect by both adenosine and lidocaine on cardiac energy metabolism (Matsumura et al., 1987; Kojima and Miura,

1991; Liu et al., 1991; Butwell et al., 1993; Fralix et al., 1993; Yao et al., 1997). Using ^{31}P NMR in isolated rat hearts, some researchers have shown that both adenosine and lidocaine separately are able to delay acidosis and lessen ATP and PCr utilization (Matsumura et al., 1987; Kojima and Miura, 1991; Butwell et al., 1993; Fralix et al., 1993). No study has, however, combined adenosine and lidocaine during ischemia and investigated the changes in [ATP], [PCr] and pH in heart.

Based on the results of Chapters 3 and 4, it was hypothesized that the mechanism of AL solution's effect on infarct size and ventricular arrhythmia reduction may be, in part, due to energy sparing and attenuation of the decrease in pH during ischemia. The following experiments were designed to determine whether the protective effects of AL solution could be attributed to a preservation of high-energy phosphates, PCr and ATP, and pH.

5.2. Experimental design

Animals (n = 13) were randomly assigned into 2 treatment groups: (1) Controls (n = 6) and (2) adenosine and lidocaine solution (AL solution) (n = 7). As in previous chapters, the adenosine and lidocaine solution contained 6.3 mg/ml adenosine and 12.6 mg/ml lidocaine and was prepared on the day of the experiment in physiological saline (0.9%). AL was infused intravenously at 1 ml/hr, which converted to mass specific dosages of 305 μg/kg/min and 608 μg/kg/min for adenosine and lidocaine respectively. All AL treated rats received a continuous infusion for 5 minutes prior to and throughout 30 minutes of regional ischemia. The treatment was ceased when the coronary ligature was released at the onset of reperfusion. Control animals did not receive any treatment. NMR spectra were obtained in 5 min intervals at baseline, during AL pretreatment and throughout 30 min ischemia and 40 min of reperfusion.
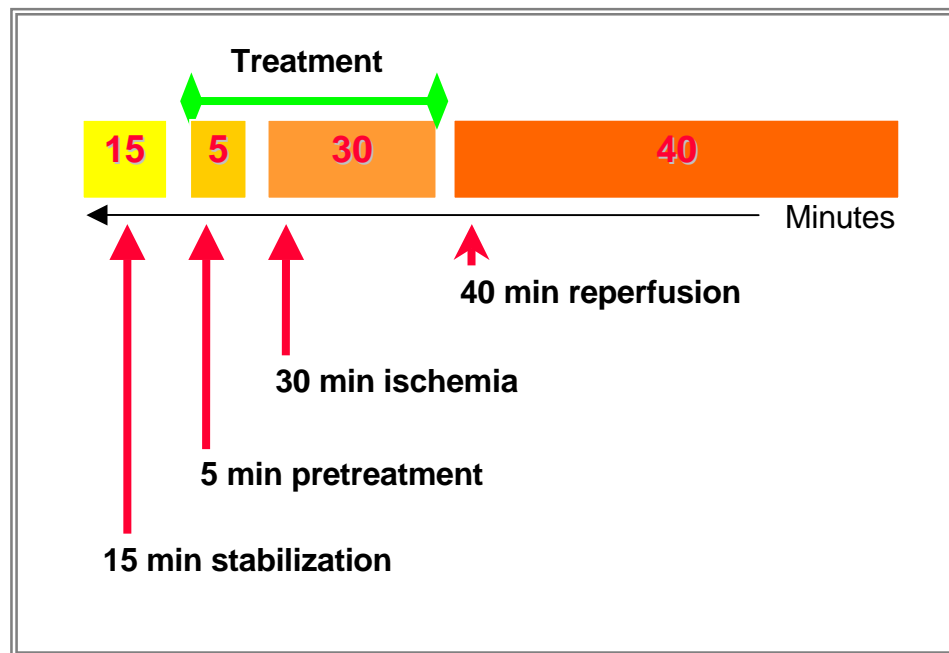
Figure 5.1. Chapter 5 treatment protocol.

5.3. Results

5.3.1. HEMODYNAMICS

The hemodynamic parameters of heart rate, mean arterial pressure and rate-pressure product are shown in Figure 5.2. At baseline there was no significant difference between groups. Five minutes of treatment with AL solution prior to ischemia resulted in a reduction of heart rate (256 ± 17 bpm), MAP (63 ± 6 mmHg), and RPP (23516 ± 7935 bpm.mmHg) compared to controls (332 ± 28 bpm, 117 ± 18 mmHg and 49383 ± 23392 bpm.mmHg respectively) ($P < 0.05$). Even though AL solution treatment was continued throughout ischemia, the significant reduction of hemodynamics was sustained only until 5 min ischemia. By 10 min ischemia all of control hemodynamic variables dropped to values not significant from AL solution

treatment during ischemia. However, by 20 min ischemia, the AL solution treatment group heart rate (219 ± 17 bpm) and RPP (22184 ± 8233 bpm.mmHg) were significantly lower than controls (333 ± 33 bpm and 42645 ± 18193 bpm.mmHg respectively) ($P < 0.05$). Reperfusion coincided with the discontinuation of AL solution treatment and resulted in an elevation of all hemodynamic variables in both groups, yet mean arterial pressure in the AL solution treatment group was significantly improved at 10 min (131 ± 8 mmHg) 15 min (134 ± 8 mmHg) and 35 min (132 ± 8 mmHg) reperfusion compared to controls (95 ± 16 mmHg, 100 ± 8 mmHg and 103 ± 9 mmHg) ($P < 0.05$). This trend towards better recovery was also seen in the change in RPP though the difference between controls was not significant.
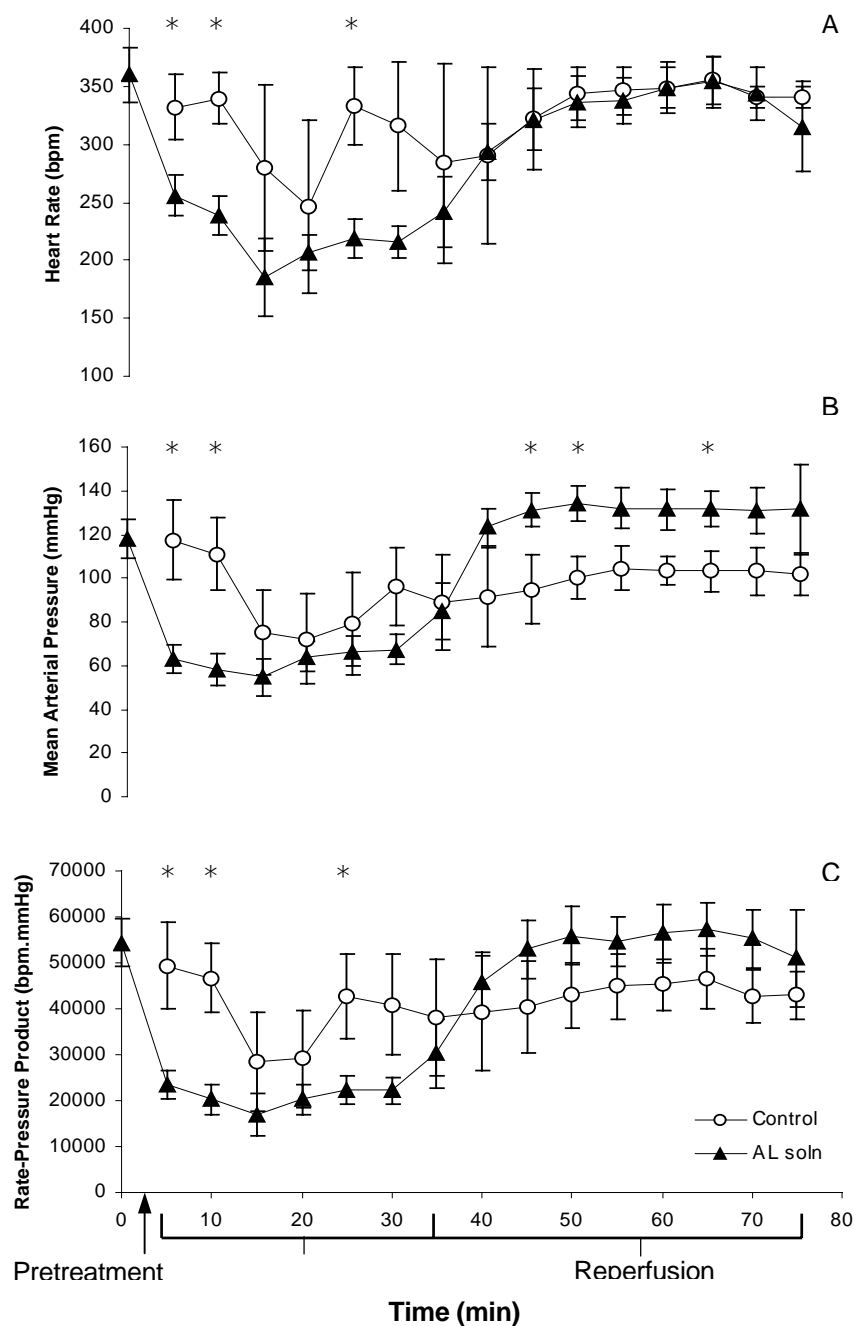
Figure 5.2. Hemodynamic parameters in control (no pretreatment) and AL solution rats at baseline (0 min AL solution; 5 min control), pretreatment (5 min AL solution), during ischemia (30 min) and reperfusion (30 min). A) Heart rate; B) Mean Arterial Pressure; and C) Rate-pressure product. All values are means ± S.E.M; * P < 0.05.

5.3.2. EFFECTS OF AL SOLUTION ON BIOENERGETIC RESPONSES TO ISCHEMIA-REPERFUSION

5.3.2.1. ATP Concentration ([ATP])
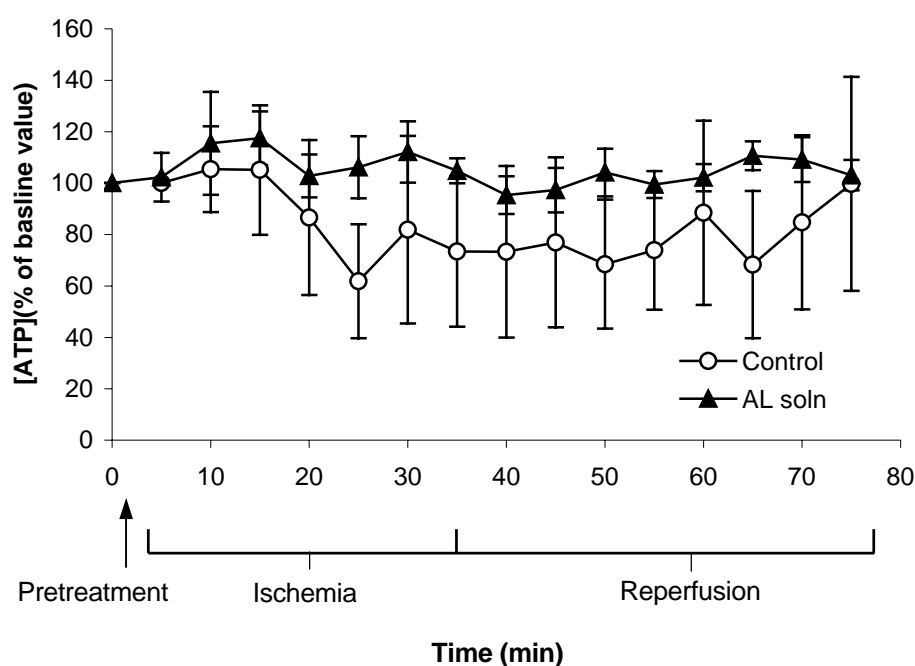
Figure 5.3. Percent changes in [ATP] from baseline in control (no pretreatment) and AL solution rats at baseline (0 min AL solution; 5 min control), pretreatment (5 min AL solution), during ischemia (30 min) and reperfusion (30 min). Percent change in [ATP] concentration from baseline. All values are means ± S.E.M; * P < 0.05.

The effect of AL solution treatment on [ATP] during ischemia-reperfusion is shown in

Fig. 5.3. Overall, the [ATP] in AL solution treated hearts did not change significantly

from baseline during ischemia and reperfusion with means ranging from 95 ± 7% of

baseline to 117 ± 10% of baseline. Controls showed little change during the first 10

min of ischemia (105% of baseline), by 15 min [ATP] fell to 61% of baseline followed

by a short recovery of [ATP] at 25 min ischemia which reached 81% of baseline. This

recovery was lost for the remaining 5 min of ischemia (73% of baseline). Throughout

most of reperfusion control hearts' [ATP] did not improve; during the first 20 min of reperfusion control [ATP] values stayed between 68% and 73% of baseline. Improvement towards controls' pre-ischemic values occurred at 25 min reperfusion where the mean % of baseline rose to 88%, yet this recovery was not sustained and dropped over the next 5 min to 68%. A second sharp recovery towards pretreatment values started at 30 min reperfusion and by at the end of the measured reperfusion period nearly matched baseline (40 min, 99%). However, due to higher variability in [ATP] measurements in the control group, the differences between the two groups were not statistically significant.

5.3.2.2. PCr concentration ([PCr])
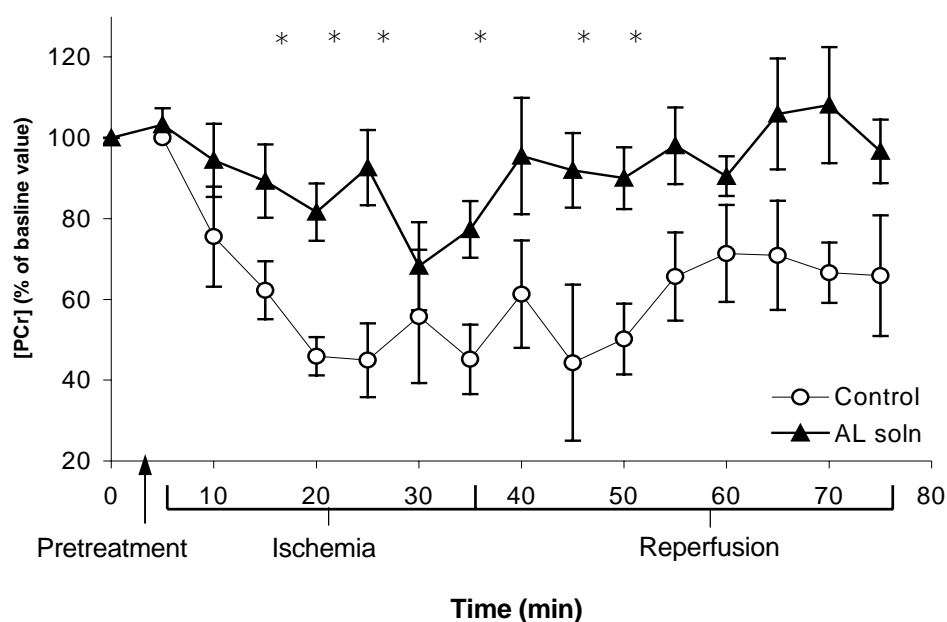
Figure 5.4. Changes in [PCr] in control (no pretreatment) and AL solution rats at baseline (0 min AL solution; 5 min control), pretreatment (5 min AL solution), during ischemia (30 min) and reperfusion (30 min). Percent change in [PCr] concentration from baseline. All values are means ± S.E.M; * P < 0.05.

The effect of AL solution treatment on [PCr] during ischemia-reperfusion is shown in Figure 5.4. Overall, AL solution treatment resulted in improved [PCr] preservation throughout ischemia and reperfusion compared to controls at all time points. Significant reductions in [PCr] in controls occurred during ischemia at 10 min (62 ± 7 vs. 89 $\pm$ 9%), 15 min (45 ± 4% vs 81 ± 7%), 20 min (44 ± 9% vs. 92 ± 9%) and 30 min (45 ± 8% vs. 77 ± 7%) and during reperfusion at 10 min (44 ± 19% vs. 92 ± 9%) and 15 min (50 ± 8% vs. 90 ± 7%) ($P < 0.05$). The first 15 min of ischemia resulted in a linear decline of [PCr] in both groups. Controls [PCr] decreased sharply from the onset of ischemia. In the first 10 min there was a 38% decline in [PCr] to 62% of baseline followed by another steep decrease at 20 min of ischemia to 44% of baseline. By 25 min of ischemia control heart's [PCr] increased slightly to 55% of baseline and then dropped 10% in the last 5 min of ischemia. For the first 10 min of reperfusion, control heart's [PCr] values ranged between 50% and 60%. From 15 to 30 min of reperfusion [PCr] increased to 70% of baseline where it remained relatively constant to 40 min of reperfusion. In the AL solution treatment group, within the first 15 min of ischemia [PCr] decreased to 80% of the initial baseline value. By 20 min ischemia, the [PCr] in the AL solution treatment group recovered to 90% of baseline and then decreased sharply in the next 5 min to 68% of baseline. Phosphocreatine recovered again by 30 min ischemia to 77% of baseline. Within 10 min of reperfusion the [PCr] in the AL solution treatment group improved towards the initial baseline value with no overshoot and remained relatively constant over the next 40 minutes ranging from 90 ± 7% to 108 ± 14% of baseline. Overall, AL solution treatment resulted in improved [PCr] preservation throughout ischemia and reperfusion compared to controls at all time points. Significant reductions in [PCr] in controls occurred at 15 and 25 min of ischemia and at 35, 45 and 50 min of reperfusion. The marked fall in [PCr] between 25 and 30 min would indicate that protection was

maintained because [ATP] remained at pretreatment levels. In contrast, the [PCr] and [ATP] in control hearts were significantly reduced during ischemia and reperfusion.

5.3.2.3. Inorganic phosphate ($[P_i]$)

The effect of AL solution treatment on $[P_i]$ during ischemia-reperfusion is shown in Figure 5.5. Elevation of $[P_i]$ during ischemia was comparable in both groups. During reperfusion, recovery of $[P_i]$ to pre-ischemic values was also statistically similar in both groups.
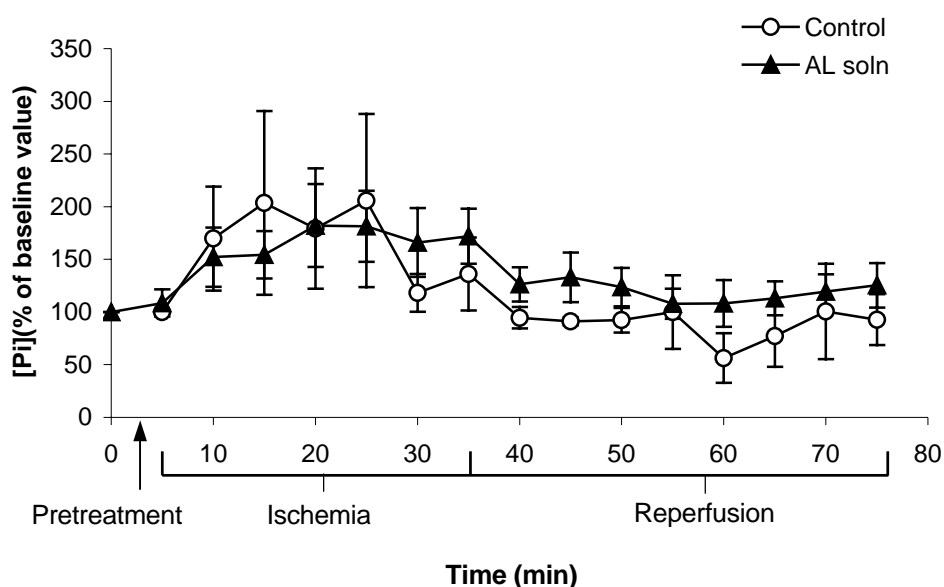
Figure 5.5. Changes in P_i peak integrals in control (no pretreatment) and AL solution rats at baseline (0 min AL solution; 5 min control), pretreatment (5 min AL solution), during ischemia (30 min) and reperfusion (30 min). Percent change in P_i concentration from baseline. All values are means $\pm$ S.E.M; p = n.s. for all values shown.

5.3.2.4. Intracellular pH
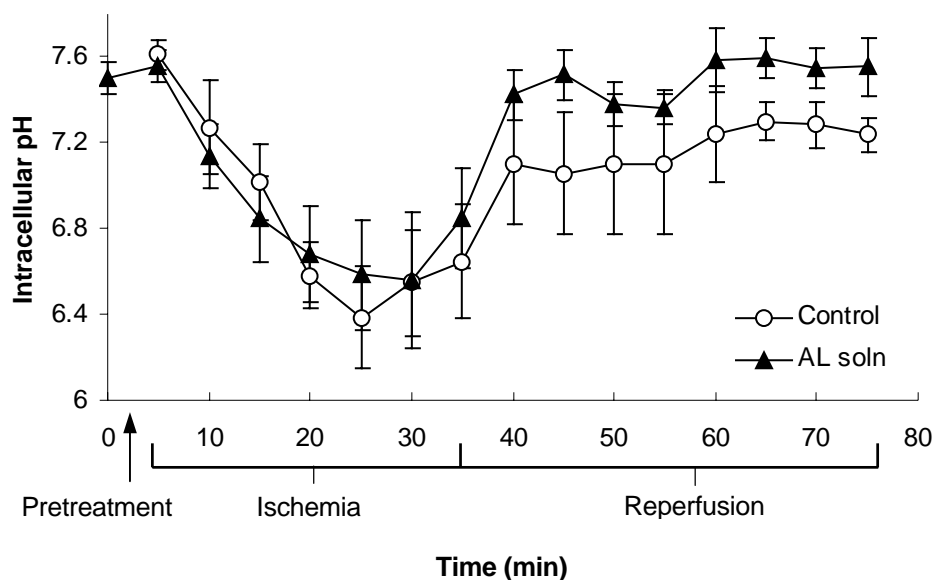
Figure 5.6. Changes in intracellular pH in control (no pretreatment) and AL solution rats at baseline (0 min AL solution; 5 min control), pretreatment (5 min AL solution), during ischemia (30 min) and reperfusion (30 min). All values are means $\pm$ S.E.M; p = n.s. for all values shown.

The effect of AL solution treatment on pH during ischemia-reperfusion is shown in

Figure 5.6. Thirty minutes of ischemia resulted in comparable changes in pH

between the AL solution treatment group and controls. At baseline the pH was 7.5

and 7.6 in the AL solution treatment group and controls respectively. AL

pretreatment caused a slight increase in pH to 7.55. This slight alkalynization

following pretreatment may be associated with the slight rise in [PCr] following pre-

treatment with AL (Fig. 5.4). With onset of ischemia, the pH markedly fell in both

groups for the first 20 min of ischemia declining to pH 6.4 $\pm$ 0.2 in control hearts and

leveling off at pH 6.8 $\pm$ 0.2 in AL solution treated hearts. Upon reperfusion, pH was

restored to baseline in AL hearts in the first 5 min (7.4 $\pm$ 0.1) whereas controls did not

rise above 7.1 for the first 20 min of reperfusion and for the remaining the last 20 min

of the measured reperfusion period pH improved to nearly 7.3 in controls.

5.3.2.5. Free magnesium ([Mg^{2+}])
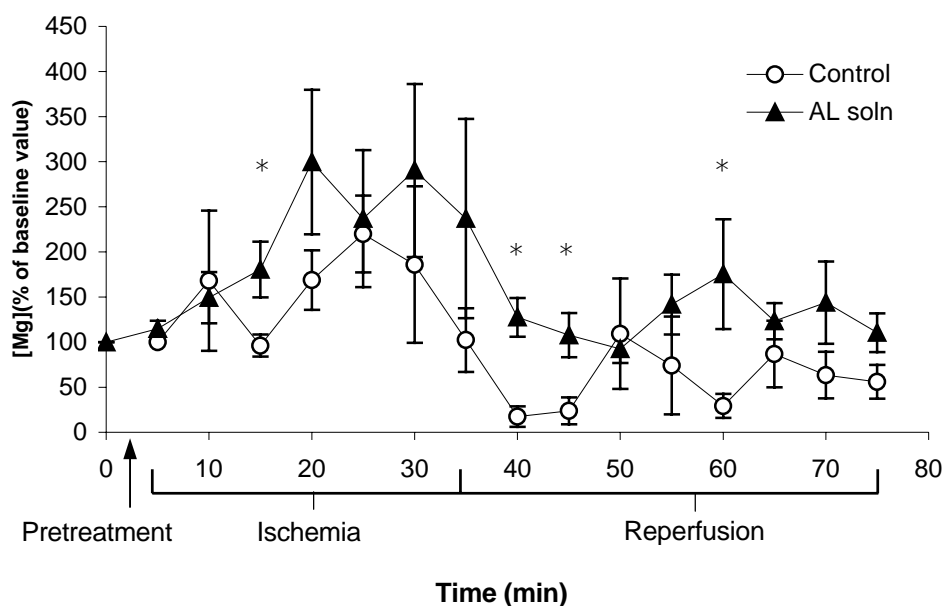
Figure 5.7. Changes in [Mg^{2+}] in control (no pretreatment) and AL solution rats at baseline (0 min AL solution; 5 min control), pretreatment (5 min AL solution), during ischemia (30 min) and reperfusion (30 min). Percent change in Mg^{2+} concentration from baseline. All values are means $\pm$ S.E.M; $P < 0.05$ for all values shown.

The effect of AL treatment on [Mg^{2+}] concentration is shown in Figure 5.7. During the

first 5 min of ischemia, [Mg^{2+}] in both groups was statistically similar; though, by 10

ischemia the control hearts [Mg^{2+}] sharply returned to $96 \pm 12\%$ of baseline while AL

solution treated hearts rose to $180 \pm 30\%$ of pre-ischemic values ($P < 0.05$). While

[Mg^{2+}] was not found to be statistically dissimilar throughout the rest of ischemia, in

general AL solution treatment resulted in higher overall trend in [Mg^{2+}]. Throughout

reperfusion, AL solution treatment led to an improved recovery of [Mg^{2+}]. In contrast,

control hearts suffered reductions of $[Mg^{2+}]$ significantly lower from AL solution

treatment hearts at 5 min (17 $\pm$ 11% vs. 127 $\pm$ 21%), 10 min (23 $\pm$ 15% vs. 107 $\pm$

24%) and 25 min (29 $\pm$ 13% vs. 175 $\pm$ 60%).

5.4. Discussion

5.4.1. METABOLIC FEATURES OF AL TREATED HEARTS COMPARED TO CONTROLS

In previous chapters it was shown that an AL solution given as a pretreatment and

throughout ischemia reduced ventricular arrhythmias, mortality, and infarct size. It

was proposed that AL solution may afford cardioprotection through preservation of

energetic metabolism and intracellular pH throughout ischemia. The present study

demonstrates that AL pretreatment continued through ischemia appears to preserve

[ATP] and [PCr] at the expense of a high intracellular acid load. Control hearts

exhibited the commonly observed metabolic features of prolonged ischemia using ^{31}P

NMR: 1) an initial sharp drop in [PCr] and pH with the onset of ischemia; 2)

concomitantly, [ATP] decreased when [PCr] was 40 - 55% of baseline values at 15

min ischemia; 3) while reperfusion resulted in a recovery of [PCr], [ATP] and pH, a

return to baseline was not achieved with these variables, and 4) an initial rise in $[P_i]$

and $[Mg^{2+}]$ (Grove et al., 1980; Jeffery et al., 1989; Reimer and Jennings, 1992). In

contrast, throughout ischemia and reperfusion, AL treated hearts closely maintained

baseline [ATP] and maintained [PCr] in a steady-state significantly higher than

controls delaying its decrease until 20 min ischemia. In addition, [PCr] recovery at

reperfusion significantly improved to near baseline values whereas controls did not.

Somewhat unexpectedly, based on the above results, P_i rose comparably in AL

treated and control hearts. Likewise, intracellular pH was not different between the

two groups. In both groups a rise in $[Mg^{2+}]$ during ischemia which recovered to baseline values during reperfusion was observed.

5.4.2. MYOCARDIAL PROTECTION BY AL TREATMENT: MAINTENANCE OF A MORE BALANCED ENERGETIC STEADY-STATE

In severe ischemia, tissue oxygen is only available for the first 8-10 sec (Sayen et al., 1958). Energy demand drives rapid phosphocreatine depletion while oxygen-deprived mitochondria can no longer support the [ATP] requirements of the cell. In severe ischemia, [ATP] can only be replenished from local supply of PCr or through glycogenolysis (Reimer and Jennings, 1992). Within the first 10 minutes, PCr is depleted and ischemic metabolism necessitates either anaerobic glycogenolysis for energy production or reduced ATP consumption (Reimer and Jennings, 1992; Opie, 1998). In the present study, the metabolic strategy of AL treatment to minimize ischemia-reperfusion stress was to preserve [ATP] as well as [PCr]. While the [ATP] in control hearts didn't begin to drop until after 10 min ischemia, the [PCr] in control hearts decreased steadily from the onset of ischemia. In contrast, AL treatment resulted in the [PCr] remaining in a steady-state for the first 20 min of ischemia. After this time there is a sharp decrease in [PCr] from 92% to 68% of baseline [PCr]. Even still, the sharp decline in [PCr] at by 25 min ischemia does not reach the lowest levels of [PCr] in ischemic control hearts (44%).

Within the first 5 min of reperfusion [PCr] in AL treated hearts recovered to 95% of baseline. Concurrently, the [ATP] was reduced no more than 5% from baseline throughout ischemia and reperfusion in AL treated hearts. In controls, [PCr] recovers only as much as 65% of baseline through most of reperfusion and 73% [ATP] during first 20 min of reperfusion.

The apparent steady-state levels of [ATP] and [PCr] in AL hearts during ischemia-reperfusion suggests that anaerobic glycogen utilization maintained [ATP] sufficiently such that [PCr] was also preserved. It, therefore, seems plausible that during ischemia, anaerobic ATP production was supported by glycogen as the principle fuel, which also maintained PCr in a steady-state via the creatine kinase (CK) equilibrium (Fig. 5.8) (Teague and Dobson, 1992).
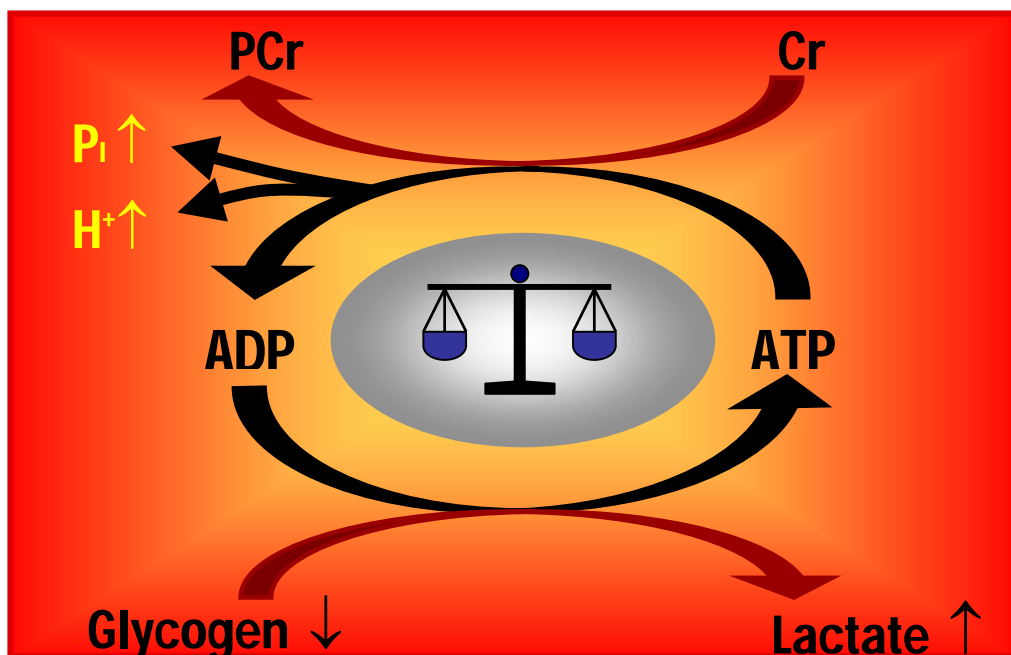
Figure 5.8. Schematic of the possible mechanisms of maintaining ATP and PCr in a metabolic steady-state during AL infusion throughout ischemia-reperfusion. AL cardioprotection appears to be associated with the glycogen supported preservation of ATP and PCr despite falling intracellular pH during ischemia. Protons (H$^+$) accumulate because of oxidative phosphorylation suppression and adenosine attenuation of the catecholamine response which leads to inhibition of the Na$^+$/H$^+$ exchanger.

Adenosine has been shown to slow [ATP] depletion during acute (Janier et al., 1993) and global ischemia (Fralix et al., 1993). Similarly, lidocaine has been shown to delay the decreased in [ATP] with ischemia (Matsumura et al., 1987; Wendland et al., 1993;

van Emous et al., 1997) as well as delay acidosis and reduce contractility in isolated rat hearts (van Emous et al., 1997). By comparing lidocaine reductions in RPP to Ca^{2+} free perfusions that induced reduced RPP, van Emous et al. were able to argue the positive effects of lidocaine on [ATP] and pH were not simply due to decreased contractility. They concluded that the beneficial effects to preserve [ATP] were more likely due to diminished consumption of ATP by the Na^+/K^+ ATPase as a result of reduced Na^+ accumulation from inhibition of the lidocaine sensitive Na^+ channel (van Emous et al., 1997).

The decreased energy expenditure in AL treated hearts, evidenced by reduced HR, MAP and RPP during ischemia, was likely to have contributed to the observed steady-states of [ATP] and [PCr] during ischemia. While no correlation was found between the reduction in cell death and reduced hemodynamics (Chapters 3), the possibility that down-regulation of hemodynamics may assist to reset the cells supply and demand ratio to delay cell death can't be dismissed.   Unfortunately, infarct size was not collected on the animals in this study. However, by maintaining [ATP] and [PCr] in a virtual steady-state, AL treatment appeared to improve the cellular energy balance which may explain the reduced infarct size reported in previous chapters. In AL hearts ischemic cell death may have been delayed by AL treatment such that cellular changes in the ischemic zone could not progress as rapidly towards irreversible injury as in control hearts.

In addition to maintaining steady-state metabolism, AL treatment appeared to lead to a more stable preparation overall as reflected by the lower standard errors seen in AL treatment effect on [ATP], [PCr] and pH compared to controls (Fig. 5.3, Fig. 5.4 and Fig. 5.6). The reason for the reduced variability in AL treated hearts is unclear as the treatments were given to the rats randomly (i.e. the decision to treat with either saline

or AL solution was made on a random basis), and variations in technique were highly unlikely given the level of experience prior to commencing the study. However, it appears that AL solution led to down-regulation and a better maching of supply and demand.

5.4.3. ACIDOSIS WITH CARDIOPROTECTION IN AL TREATED HEARTS

Anaerobic glycogenolysis leads to an accumulation of protons as well as lactate. However, the build-up of these two metabolites is known to cause cell damage (Cross et al., 1995a). Lactate is often associated with decreased contractile activity in the ischemic zone (Tennant and Wiggers, 1935), promotion of mitochondrial damage (Armiger et al., 1975), decreased action potential duration, and decreased glycolytic rate (Rovetto et al., 1975). In severely ischemic cells the pH can activate lysozymes causing irreversible tissue destruction (Opie, 1998). An excess of protons may also inhibit glycolytic enzymes (Williamson et al., 1976), damage myocardial ultrastructure (i.e. aggregation of nuclear chromatin, formation of mitochondrial amorphous matrix densities, etc) (Armiger et al., 1977) and lead to Na^+ and Ca^{2+} overload (Phillipson et al., 1982; Lazdunski et al., 1985). Furthermore, experimental episodes of acute myocardial ischemia in collateral deficient species such as rats (Schaper et al., 1992) results in a closed-system where proton accumulation is inevitable despite its origin and place of accumulation.

Since [ATP] hydrolysis is a major source of protons ($ATP^{4-} + H_2O \rightarrow ADP^{3-} + P_i^{2-} + H^+$), one might have expected a delay in acidosis in AL treated hearts at least in the first 10 minutes compared to controls. However, my results suggest that reducing acidosis was not a key factor for limiting ischemic injury (Fig. 5.8). This would agree with the work of Schaefer et al. (1995) who showed minimal differences in pH during

ischemia between preconditioned and glycogen-depleted/glucose treated isolated rat hearts despite contrasting functional recovery between the two groups. Preconditioned hearts had significantly improved recovered function while glycogen-depleted/glucose treated hearts' function was limited (Schaefer et al., 1995). Similary, Lundmark et al. (1999) agrees that present evidence regarding acidosis limitation as a requisite of infarct size reduction is only suggestive. They reported that transient repetitive acidosis prior to prolonged ischemia protected isolated rat hearts from ischemic injury and improved function upon reperfusion despite these hearts having had a significantly lower pH than both controls and preconditioned hearts.

Associated with the preservation of high energy phosphates, the negative chronotropic, inotropic and dromotropic effects also implicate the importance of adenosine activation of adenosine A_1 receptors (Hayes, 2003). It is further proposed that during ischemia, oxidative phosphorylation was suppressed and that activation of adenosine A_1 receptor could have resulted in catecholamine antagonism and consequently a fall in intracellular cyclic AMP which in turn may have led to an inhibition of the Na^+/H^+ and Na^+/Ca^{2+} exchangers (Munshi et al., 1991; Belardinelli et al., 1995; Sommerschild and Kirkeboen, 2000). While inhibition of the Na^+/H^+ exchanger reduces the rise in intracellular sodium, its been shown that proton accumulation is not enhanced presumably because there are multiple pathways by which H^+ can exit the cell (Pike et al., 1995; Gabel et al., 1997) such as through lactate-H^+ co-transport or with CO_2 efflux (Vanderberg et al., 1993). Interestingly, Pike et al. (1995) showed that Na^+/H^+ exchanger inhibition did not significantly alter pH levels; although, the attenuation of Na^+ accumulation was associated with reduced episodes of ventricular fibrillation.

This sequence of metabolic events helps explain the reduced pH levels with AL treatment during ischemia despite reduced ischemic injury. In control hearts acidosis appeared to accompany ATP hydrolysis. Though the change in pH in control animals was not significantly different for AL-treated heart, the latter may have differed by the adenosine A_1-linked chronotropic, inotropic and dromotropic effect of adenosine's action to lower demand in combination with Na^+ fast channel down-regulation in AL treated rats. The fall in intracellular pH is therefore in AL treated heart was not the 'villain' of myocardial ischemic injury but the outcome of a metabolic strategy to couple glycogen mobilization to ATP replacement during low or no-flow ischemia.

In this present study, AL treatment resulted in an improvement in pH observed during reperfusion. Two studies by Bak and Ingwall (1994 and 1998) provided evidence that intracellular acidosis may have a salutary effect on preserving the AMP pool due to inhibition of the cytosolic specific 5'-nucleotidase (5'-NT). Inhibition of 5'-NT leads to increased capacity for ATP resynthesis through limiting the conversion of AMP to purines (Bak and Ingwall, 1998). Retaining AMP during ischemia may then lead to improved ATP recovery with reperfusion.

On the other hand, in ischemic isolated hearts, Meldrum et al. (1996) did not show a difference in pH between controls and in adenosine treated hearts, while Fralix et al. (1993) saw a reduction in intracellular H^+ with adenosine treatment. The difference in the results of these two studies may be related to the fact that Meldrum et al.'s hearts had an 8 min washout following adenosine infusion prior to ischemia. Butwell et al. (1993) showed attenuation of pH with lidocaine treatment only took effect at 9 min ischemia. In the present study, the rate of the development of acidosis appeared slow slightly between 15 and 20 min ischemia. Moreover, controls did not regain

normal pH balance which may be a further indication of greater irreversible injury in controls vs. AL treated hearts.

5.4.4. INTRACELLULAR FREE MAGNESIUM ($[Mg^{2+}]$)

Free magnesium concentration is an important component of numerous enzymatic reactions including the sodium pump, myosin ATPase, oxidative phosphorylation, and various glycolytic enzymes. The magnesium complexes with compounds such as ATP (MgATP) are the true substrates for most metabolic reactions in the cell. Therefore, increases in $[Mg^{2+}]$ reflect decreases in magnesium bound ATP (Reimer and Jennings, 1992). As [ATP] is broken down during ischemia, intracellular magnesium may increase from about 0.6 mM to over 3 mM during severe ischemic episodes (Garfinkel et al., 1986; Kirkels et al., 1989). Baseline $[Mg^{2+}]$ values for both groups corresponded with the literature values (Garfinkel et al., 1986; Kirkels et al., 1989; Reimer and Jennings, 1992). Over the ischemic period in the present study, there was a parallel increase in $[Mg^{2+}]$ between both AL treated and control hearts. The $[Mg^{2+}]$ in AL treated hearts increased from 0.6 mM to 1.6 mM over the ischemic period while control hearts intracellular Mg^{2+} ranged from 0.6 mM to 1.2 mM. Curiously, while [ATP] was shown to remain in a steady-state in AL treated hearts, $[Mg^{2+}]$ increased more in the AL treated hearts than control. A possible explanation may be that the free Mg^{2+} may be linked to other ionic exchangers across the sarcolemma and mitochondria (Masuda et al., 1990). Changes in free Mg^{2+} transport may be associated with changes in intracellular P_i. Masuda et al. (1990) provided thermodynamic evidence favoring a net Mg^{2+}/ $H_2PO_4^{2-}$ exchange perhaps made up of Na^+/HPO_4^{2-} co-transport linked to a Na^+/Mg^{2+} co-transport across either the sarcolemma or mitochondria (Wohlrab, 1986) which may explain the changes in Mg^{2+} and P_i independent of changes in ATP in this study.

Increased $[Mg^{2+}]$ antagonizes Ca^{2+} channels and the Na^+/Ca^{2+} exchanger thereby attenuating ischemic-induced Ca^{2+} loading (Trosper and Philipson, 1983; Kimura, 1996). Although, there are conflicting reports regarding the underlying cardioprotective mechanism of elevated $[Mg^{2+}]$ (Headrick et al., 1998). Some studies report that inhibition of sarcolemmal Ca^{2+} fluxes may be the mechanism of action (James et al., 1987; Agus et al., 1989; Silverman et al., 1994) whereas others assert an intracellular site of action through better Ca^{2+} handling at the sarcoplasmic reticulum (Barbour et al., 1992; Terada et al., 1996).

5.4.5. CONCLUSIONS

This study shows that AL infusion before and during ischemia preserves steady-state concentrations of [ATP] and [PCr] at the expense of a high intracellular acid load. During ischemia it is suggested that anaerobic glycogen breakdown was the principle pathway supporting myocardial ATP requirements. In addition, down-regulation of hemodynamics as seen with AL treatment may have decreased energy expenditure and possibly reset the ischemic cells supply and demand ratio to delay cell death. The high intracellular acid load may have been associated with adenosine attenuation of cAMP and the subsequent inhibition of the Na^+/H^+ and Na^+/Ca^{2+} exchangers. Free magnesium and $[P_i]$ increased during ischemia with AL infusion despite no change in [ATP]. This may reflect changes to the differences in Mg^{2+} and P_i handling as a consequence of mitochondrial oxidative phosphorylation and altered transmembrane gradients.

5.4.6. LIMITATIONS WITH NMR SAMPLING OF *IN VIVO* RAT NMR

While ^{31}P NMR is an extremely powerful analytical tool for understanding real-time metabolic processes in the heart, there are some limitations specific to *in vivo* heart

models of acute myocardial ischemia. Theoretically, the quantification of ^{31}P NMR spectra involves measurement of the area under the peaks to derive the concentration of the metabolite of interest (Hoffenberg et al., 1996). However, a common source of error in *in vivo* heart models is the motion of the heart and signals arising from surrounding tissue, both of which can lead to a low signal to noise ratio making quantification of the peak areas difficult.

Another specific difficulty with ischemic hearts, relates to sample volume changes with edematous swelling (Askenasy et al., 1995). Significant intracellular (Jennings et al., 1985) and slight extracellular swelling (Tanaka et al., 1993) is known to result during reperfusion after an ischemic episode. This may cause changes to the conductivity of the sample, affecting the peak intensities (height) and the phase and width of peaks in the spectrum. At worst, this could lead to a loss of signal. While not specifically investigated, it is hypothesized that these osmotic changes may be related to the greater variability in high energy phosphate changes in control hearts vs. AL treated hearts.

A further limitation of acquiring ^{31}P NMR spectra of *in vivo* rat heart is the problem of overlapping resonances at the same position of the P_i peak. The two phosphates on 2,3-diacylphosphoglycerate (2,3-DPG) contained in blood can obscure the P_i signal. Overlapping resonances can lead to misidentification of the position of the P_i peak during ischemia and overestimation of P_i concentration (Hoffenberg et al., 1996). Inorganic phosphate is determined by the relative rates of ischemia induced hydrolysis of [PCr] and [ATP] and the acceleration of [ATP] degradation resulting from ischemic contracture. In this study, the change in [P_i] didn't reflect the [ATP] and [PCr] hydrolysis in control hearts. In control hearts [PCr] changed from the onset of ischemia while in AL treated hearts [PCr] remained in a steady state. While [ATP]

dropped in control hearts, the [ATP] remained constant in AL treated hearts.

However, the [P_i] change was similar in both groups during ischemia suggesting that

HEP utilization was similar in both groups, yet clearly it was not.

Chapter 6. Pharmacological preconditioning: Concomitant targeting of the adenosine A_1 receptor and sodium channels surpasses AL solution cardioprotection

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Plate VII. Diagrammatic relationship of the normal electrocardiogram (ECG) and the seven transmembrane action potentials of each cell type by Frank Netter. This diagram is just one example of the many outstanding cardiovascular illustrations by Frank Netter which are featured in his book.

(Netter, 1969)

6.1. Introduction

The heart possesses an extraordinary ability to 'remember' short episodes of sublethal ischemia-reperfusion (angina) which protects the myocardium and coronary vasculature from a subsequent lethal period of ischemia (infarction) (Murry et al., 1986). The phenomenon, known as ischemic preconditioning (IPC), is one of the most powerful means of attenuating cell death known (Yellon and Downey, 2002), and has been described in many tissues and organs (Przyklenk and Kloner, 1998). Ischemic preconditioning was first described in 1986 by Murry, Jennings and Reimer who reported an infarct size reduction from 29% to 7% in anaesthetised open-chested dogs after three brief episodes of ischemia-reperfusion and 40 min coronary artery occlusion (Murry et al., 1986). Two different time frames of preconditioning have subsequently been identified; an early "classical" window which lasts 1 to 3 hrs after the stimulus, and a "delayed" window which develops over many hours and can last up to 12 to 72 hours (Kuzuya et al., 1993; Przyklenk and Kloner, 1998; Bolli, 2000; Baxter, 2002).

Given the reluctance of most surgeons and clinicians to precondition an organ in the clinical setting (Vaage and Valen, 2003), the ultimate therapeutic goal has been to develop a pharmacological mimetic of IPC (Yellon and Downey, 2002). Potential 'triggers' include adenosine, acetylcholine, bradykinin, opioid, catecholamines, free radicals and nitric oxide (Lasley and Mentzer, 1998; Bolli, 2000; de Jong et al., 2000; Toyoda et al., 2000; Avkiran, 2001). Adenosine is of particular interest because in addition to 'triggering' early and delayed IPC (Baxter, 2002), it directly protects against acute cellular injury at multiple sites and levels during ischemia and reperfusion (Vinten-Johansen et al., 1999; Cohen et al., 2000; Yellon and Baxter, 2000; Post and Heusch, 2002). The physiological effects of adenosine are mainly

mediated by binding to four receptor subtypes (A_1, A_{2a}, A_{2b} and A_3) located on the surface of conduction cells, cardiomyocytes, cells in the microvascular compartment and on inflammatory cells (e.g. neutrophils) (Vinten-Johansen et al., 1999; Baxter, 2002). Stimulation of the adenosine A_1-receptor subtype is known to confer protection via inhibitory G protein-coupled pathways which has been linked to the opening of sarcolemmal ATP sensitive K^+ channels, increased potassium conductance, action potential shortening and reduced Ca^{2+} entry into ischemic cells (Gross and Fryer, 1999). More recently, the adenosine A_1 receptor 'trigger' has been linked to new targets including the mitochondria (O'Rourke, 2000) and sarcoplasmic reticulum (Mubagwa, 2002). Unlike adenosine, cardiac Na^+ channels have received surprisingly little attention as targets of preconditioning, despite voltage-gated Na^+ channels being highly dependent on the membrane potential which is modified during early ischemia (Balser et al., 1996; Carmeliet, 1999).

The previous studies led to the hypothesis that targeting the A_1 receptor in conjuction with lidocaine treatment may enhance cardioprotection. Therefore, the aim of the present study was to examine the pharmacological pretreatement effect of co-administering adenosine or A_1 agonist (CCPA) and the sodium channel modulator, lidocaine, in the *in vivo* rat heart during regional ischemia.

6.2. Experimental Design

Rats (n = 38) were randomly assigned to one of five groups: (1) Saline controls (0.9% saline) (n = 12) (from Chap 3); (2) IPC (n = 6); (3) adenosine and lidocaine solution (AL solution) (n = 7) (from Chap 3); (4) A_1 agonist (CCPA) plus lidocaine (n = 6). (5) CCPA alone (n = 7). Ischemic preconditioning was achieved using 3 cycles of ischemia/reperfusion with each transition lasting 3 min (Group 2). The AL solution

was prepared on the day and infused at 305 μg/kg/min and 608 μg/kg/min respectively (Group 3). Groups 1 and 3 rats received continuous infusion of saline or AL solution, respectively, for 5 min before and throughout 30 min of regional ischemia. At the onset of reperfusion the treatment was ceased. Group 4 rats were pretreated 5 min before ligation with a 5 min bolus of A_1 agonist CCPA (5μg/kg) and an infusion of lidocaine (608 μg/kg/min) which was continued throughout 30 min ischemia. Group 5 was treated with A_1 agonist (CCPA) alone 5 min before ligation. All animals were reperfused for 120 min for infarct size (Fig. 6.1). The primary end-points were death, episodes and duration of ventricular arrhythmias and infarct size. Infarct size is considered the "gold standard" of ischemic preconditioning (Przyklenk and Kloner, 1998). The hemodynamics collected throughout the study constituted the secondary end-points: heart rate, systolic pressure, mean arterial pressure (systolic pressure – diastolic pressure/3 + diastolic pressure), and rate pressure product (heart rate x systolic pressure).
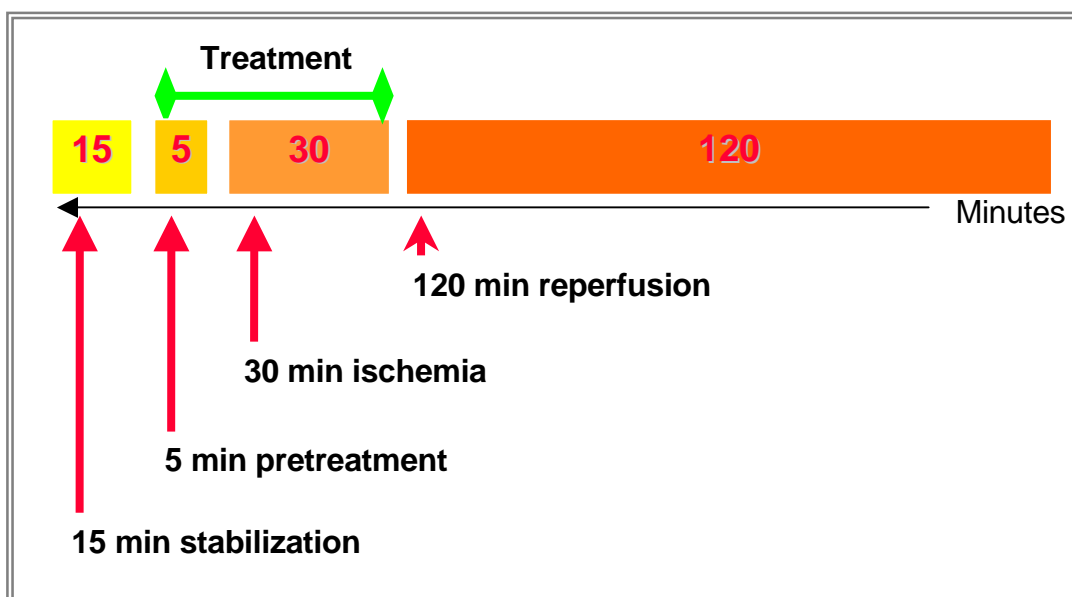
Figure 6.1. Chapter 6 treatment protocol.

6.3 Results

Seven of the 12 saline-controls (n=5 survived), 1 of the 7 IPC died (n=6 survived) and

2 of the 7 CCPA-rats (n = 5 survived) died during ischemia from ventricular

arrhythmias. None of the AL-treated rats (n = 7) or CCPA plus lidocaine-treated rats

(n = 6) died. Only data from surviving rats were further analyzed.
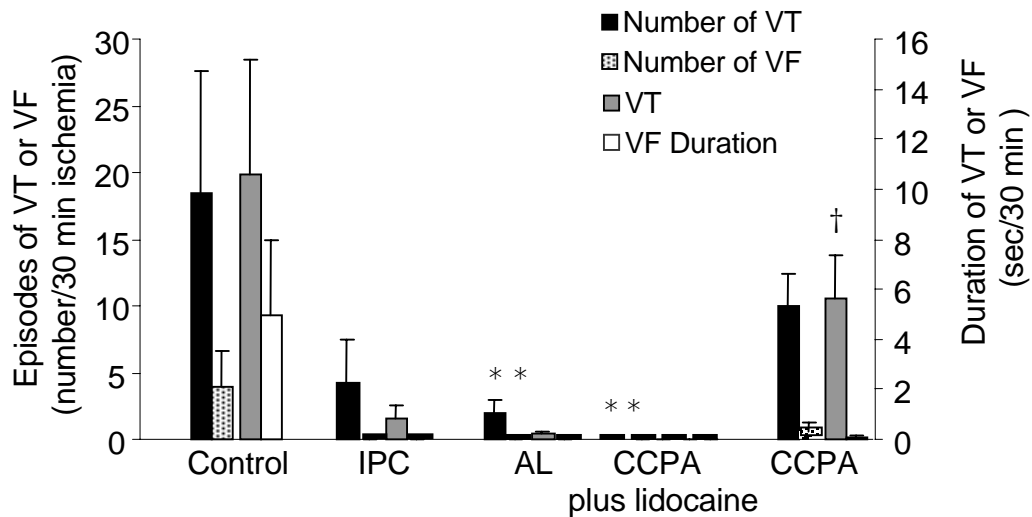
Figure 6.2. The episodes and duration of ventricular tachycardia (VT) and ventricular fibrillation (VF) and VT+VF during ischemia for surviving rats in all treatment groups. These values represent the overall sum of episodes and durations (sec) that occurred throughout the 30 min ischemic period. Surviving rats: saline-control, n = 5; ischemic preconditioning (IPC), n = 5, AL solution, n = 7, CCPA (5μg/kg) plus lido, n = 6 and CPA alone, (5μg/kg), n = 5. *P < 0.05 vs. control; †P < 0.05 vs. IPC.

Ischemia-induced ventricular arrhythmias are shown in Figure 6.2. Overall, the surviving rats in the saline-control group underwent 156 ± 72 sec of ventricular arrhythmias (VT, 106 ± 45; VF, 49 ± 30), while CCPA-treated animals tolerated 56 ± 18 sec of VT and no ventricular fibrillation (Fig. 6.2). Forty percent of the IPC treated-rats experienced 4 ± 3 episodes of VT for over 8 ± 6 sec. Pharmacological preconditioning with AL abolished VF and significantly reduced episodes and durations of VT (2 ± 1 sec) compared to controls (106 ± 45 sec). Within the AL-treated group, 42% of animals did not experience VT or VF. Treatment with CCPA plus lidocaine completely abolished VT and VF (Fig. 6.2). Immediately following ischemia, 80% of saline-controls, 60% of IPC-treated, and 100% of CCPA-treated rats experienced reperfusion tachycardias (Data not shown). No ventricular arrhythmias during reperfusion were experienced in rats preconditioned with AL solution or CCPA plus lidocaine.

The mean AAR/LV, AN/LV and AN/AAR are shown in Fig. 6.3. The areas at risk expressed as a percent of the left ventricle were not significantly different among the five groups, and on average comprised $58 \pm 2\%$ (Fig. 6.3). The areas of necrosis in saline-controls, AL solution, CCPA alone, IPC and CCPA plus lidocaine-treated rats were $38 \pm 5\%$, $18 \pm 4\%$, $24 \pm 3\%$, $7 \pm 2\%$ and $8 \pm 3\%$, respectively. The mean infarct size was $61 \pm 5\%$ for saline–controls, $38 \pm 6\%$ for AL solution, $42 \pm 7\%$ for CCPA, $11 \pm 3\%$ for IPC treated animals and 12 ± 4 for CCPA and lidocaine-treated rats (Fig. 6.3). IPC and pharmacological preconditioning with CCPA and lidocaine were not significantly different ($P < 0.05$).
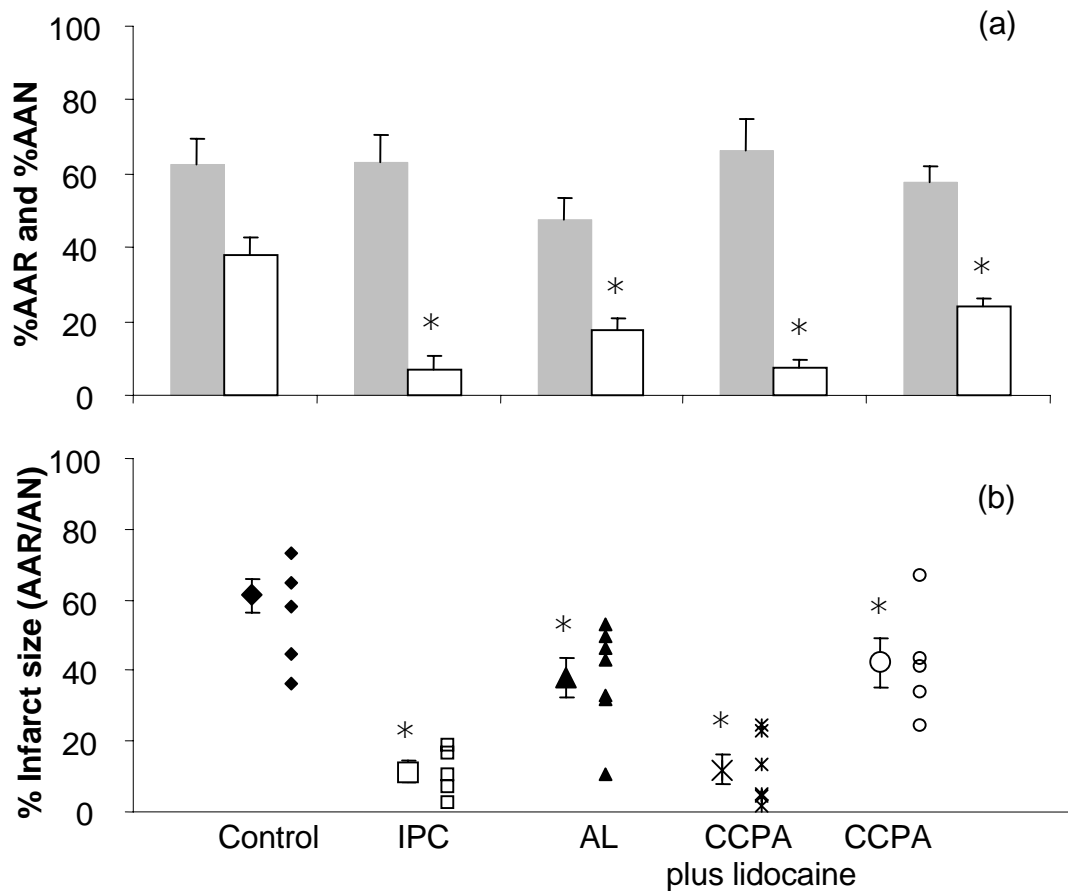
Figure 6.3. Effects of IPC, AL solution, CCPA plus lidocaine, CCPA alone on left ventricle necrosis and infarct size. Areas at risk (AAR/LV, shaded) were not significantly different between groups (a). Areas of necrosis in the left ventricle (AN/LV, unshaded) were significantly smaller with AL mixture treatment (b). Infarct sizes (AN/AAR) in groups receiving AL solution treatment were significantly smaller compared with all other treatment groups (B). Surviving rats: Saline-control, n = 5; ischemic preconditioning (IPC), n = 5; AL solution, n = 7; CCPA ($5\mu g/kg$) plus lido, n = 6; CCPA alone ($5\mu g/kg$), n = 5. *P < 0.05 vs. control.

The hemodynamic changes during pretreatment, ischemia and reperfusion are

shown in Table 6.1. Rats treated with AL solution or CCPA plus lidocaine had

significant reductions in heart rate, MAP and systolic pressure compared to saline-

controls and IPC. No significant differences in MAP were apparent between AL

solution or CCPA plus lidocaine, although heart rate was lower in the latter (Table

6.1). While hemodynamic measurements were lower than baseline after 2hrs

reperfusion in all groups, no group was significantly different from another.

Table 6.1. Hemodynamic parameters during ischemia-reperfusion.

Treatment		Baseline	Pre-occlusion	30 min Ischemia	120 min Reperfusion
Saline-controls	HR (bpm)	436 ± 13	433±15	391±31	381±30
	MAP(mmHg)	112±6	110±11	77±11	62±8
	Sys (mmHg)	139±6	137±11	104±6	86±12
IPC	HR (bpm)	438±9	416±16	414±7	379±8
	MAP mmHg)	130±8	90±19	92±14	69±6
	Sys (mmHg)	163±12	116±22	116±14	98±6
AL solution	HR (bpm)	497±13	332±14*†	316±17*†	395±11
	MAP mmHg)	123±11	46±4*†	52±6†	86±6
	Sys (mmHg)	159±11	75±7*†	86±8†	119±7
A_1 agonist (CCPA) plus lidocaine	HR (bpm)	436±18	270±14*†	172±26*†	347±17
	MAP mmHg)	110±12	49±4*†	44±2*†	72±5
	Sys (mmHg)	131±9	77±7*†	59±3*†	97±9
A_1 agonist (CCPA) only	HR (bpm)	421±15	336±29*†	308±73	367±12
	MAP mmHg)	114±6	89±10	91±10	71±2
	Sys (mmHg)	146±6	113±11	113±12	94±4

Surviving rats: Saline-control, n = 5; ischemic preconditioning (IPC), n = 5; AL solution, n = 7; A_1 agonist (CCPA, 5μg/kg) plus lido, n = 6; A_1 agonist only (CCPA, 5μg/kg), n = 5. Data are mean ± S.E.M.; *$P < 0.05$ vs. control. †$P < 0.05$ vs. IPC. MAP=mean arterial pressure, Sys=systolic pressure.

The possibility of rate-pressure product influencing infarct size was examined using the Pearson's Correlation coefficient (r) for all treatments 5 min before and during ischemia (5, 10, 15, 20 and 30 min ischemia) (Table 6.2). Three linear correlations were found involving IPC and CCPA alone treatment. There was a correlation between higher rate pressure products and lower infarct sizes in IPC at pretreatment (r=-0.88, P < 0.05) and CCPA alone at 10 min ischemia (r = -0.92, P < 0.05). At 15 min ischemia the opposite relationship was found in the IPC group where lower rate pressure products correlated with lower infarct sizes (r = 0.98, P < 0.05).

Table 6.2. This table shows Pearson' correlation coefficient (upper value) and the level of significance (in parentheses).

	Saline Controls (n=5)	IPC (n=5)	AL solution (n=7)	CCPA plus lido (n=6)	CCPA alone (n=5)
Pretreatment	0.15 (0.8)	-0.88 (0.05)*	-0.03 (0.94)	-0.17 (0.75)	0.11 (0.86)
5 min	-0.55 (0.33)	0.85 (0.07)	-0.03 (0.95)	-0.05 (0.93)	0.05 (0.94)
10 min	-0.45 (0.44)	0.07 (0.92)	-0.19 (0.68)	0.1 (0.85)	-0.92 (0.03)*
15 min	-0.72 (0.17)	0.98 (0.00)*	-0.24 (0.60)	0.51 (0.30)	-0.80 0.10
20 min	0.54 (0.35)	-0.57 (0.32)	-0.22 (0.63)	-0.01 (0.98)	0.27 (0.66)
25 min	0.26 (0.67)	-0.73 (0.16)	-0.09 (0.84)	0.43 (0.39)	0.28 (0.65)
30 min	0.01 (0.98)	-0.17 (0.78)	0.11 (0.82)	0.43 (0.40)	-0.27 (0.66)

Surviving rats: Saline-control, n = 5; ischemic preconditioning (IPC), n = 5; AL solution, n = 7; A_1 agonist (CCPA, 5μg/kg) plus lido, n = 6; A_1 agonist only (CCPA, 5μg/kg), n = 5. Data are mean ± S.E.M.; *P < 0.05.

6.4. Discussion

A goal for over two decades has been to develop a pharmacological mimetic that will reduce cell injury and death during acute ischemia. Nearly every IPC study has shown a profound reduction in infarct size, and most have reported a large reduction in the incidence of arrhythmias; while others (Murry et al., 1986; Ovize et al., 1995), have shown a proarrhythmic effect which may predispose the heart to stunning (Mentzer, 2000; Taggart and Yellon, 2002). This study showed that co-administration of A_1 receptor agonist, CCPA, and Na^+ fast channel modulator, lidocaine, 5 min before and during 30 min of left coronary artery ligation led to no deaths, no arrhythmias and a profound reduction in myocardial infarct size which was not significantly different to ischemic preconditioning (Fig. 6.3). Moreover, the combination of CCPA and lidocaine (and AL solution) surpassed IPC protection in abolishing ventricular arrhythmias and consequently mortality (Fig. 6.2).

Protection by adenosine or adenosine A_1 agonists with lidocaine is likely to occur at three integrated levels: electrophysiological, mechanical and metabolic. Electrophysiologically, the nearly complete abolition of ventricular arrhythmias may be linked to adenosine and lidocaine's ability to improve matching of atrial and ventricular electrical conduction and pump performance during ischemia (Fig. 6.2). It is well known that adenosine, by activating A_1 receptors, slows the sinoatrial (SA) nodal pacemaker rate (negative chronotropy), delays atrioventricular (A-V) nodal impulse conduction (negative dromotropy) and reduces atrial contractility (negative inotropy) (Lerman and Belardinelli, 1991). Adenosine's net effect appears greatest at the A-V node where it 30 times more effective in slowing conduction than SA pacemakers, which at therapeutic concentrations may reduce reentry arrhythmias (Pelleg and Kutalek, 1997). Indeed, adenosine is chiefly used by clinicians to abate

narrow complex supraventricular tachycardia but can also assist to a lesser degree in the management of ventricular tachycardia of aberrant origin (Pelleg and Kutalek, 1997). As an anti-arrhythmic, adenosine also inhibits the effect of catecholamines by reducing cyclic AMP and slowing Ca^{2+} influx (Lerman and Belardinelli, 1991).

Adenosine's actions on the heart may also be enhanced by lidocaine's effects on sodium channels and therefore excitability, which is potentiated in ventricular tissue by ischemic conditions (Li and Ferrier, 1991). Lidocaine at low concentrations reduces voltage dependent Na^+ entry and effectively resets the membrane potential to a more polarised voltage (i.e. limit the reduction in ischemic-induced maximum diastolic potential) (Barrett et al., 2000); and like adenosine, can shorten the action potential and thereby reduce Ca^{2+} entry into the cell (Lerman and Belardinelli, 1991). The overall effect appears to involve a down-regulation of myocardial metabolism during ischemia and improve pump performance in the post-ischemic period. Thus reduction of atrial and ventricular myocyte excitability, shortening of the action potential and slowing repolarization may explain how the combination of adenosine and lidocaine abolishes ventricular arrhythmias in the rat model and leads to a better electrical matching in both ischemic and non-ischemic regions of the heart. Furthermore, because sodium channels (and perhaps adenosine receptors) are also located in intercalated discs (Ruiz-Meana et al., 1999), the decreased membrane excitability with AL may reduce cell-to-cell coupling by modifying passive electrical properties and propagation of the action potential which would further benefit atrial-ventricular matching of conduction and pump performance. Recently Saltman and colleagues (2002) have shown that directly blocking gap junctions with heptanol preconditions the isolated rabbit heart.

Notwithstanding the cardioprotective effect of adenosine and lidocaine therapy, it is important to establish the direct effects of pretreatment on infarct-size reduction, and the indirect effects due to hemodynamic changes such as hypotension. To tease apart this complex question, let us assume that the *entire* infarct size reduction from 61% in saline-controls to 38% in AL-treated rats was due to hypotension. Since MAP was not significantly different between AL solution and CCPA plus lidocaine treatment (Table 6.2), the contribution of hypotension to infarct-size reduction in CCPA plus lidocaine rats could not exceed the fall from 61 to 38% (Fig. 6.3). Thus the maximal contribution of hypotension to infarct-size reduction in CCPA plus lidocaine treated rats would be 47% [(61-38)/(61-12) x 100], with the remaining 53% coming directly from the pharmacological therapy itself. This proposal would be consistent with the work of Casati et al., who showed that the protective action of CCPA activation of A_1 receptors in the *in vivo* rabbit model which was independent of changes to hemodynamics, including MAP (Casati, 1997). De Jong and colleagues (2000) also have shown in the isolated rat heart that the bradycardia effect of CCPA (Table 6.2) contributes little to infarct size reduction in the paced and non-paced heart.

In addition, when rate-pressure product (index of myocardial oxygen consumption) was plotted against the different treatments 5 min before and during ischemia (5 min, 10, 15, 20 and 30 min), some surprising relationships were identified (Table 6.2). Firstly, the profound reduction of infarct size in the CCPA plus lidocaine group was not even close to significantly related. Secondly, rather than a reduced rate pressure product leading to a reduced infarct size, it was found that a higher rate pressure product correlated with lower infarct sizes in the IPC group at pretreatment and in the CCPA alone group at 10 min ischemia. Quite the opposite was found in the IPC

group at 15 min ischemia where lower infarct sizes did correlate strongly with a lower rate pressure product. While sample size may be a factor in these findings it was not surprising in lieu of the above argument and the results of others cited above.

Lastly, in the clinical setting where acute systemic hypotension is to be avoided (Yellon and Baxter, 2000; Yellon and Downey, 2002), adenosine and lidocaine therapy could alternatively be administered via an intracoronary route. Novel adenosine agonists and/or allosteric modifiers with fewer hemodynamic effects could also be employed with Na^+ channel modulation and studies are underway to test their efficacy in larger animal models.

Chapter 7. Discussion
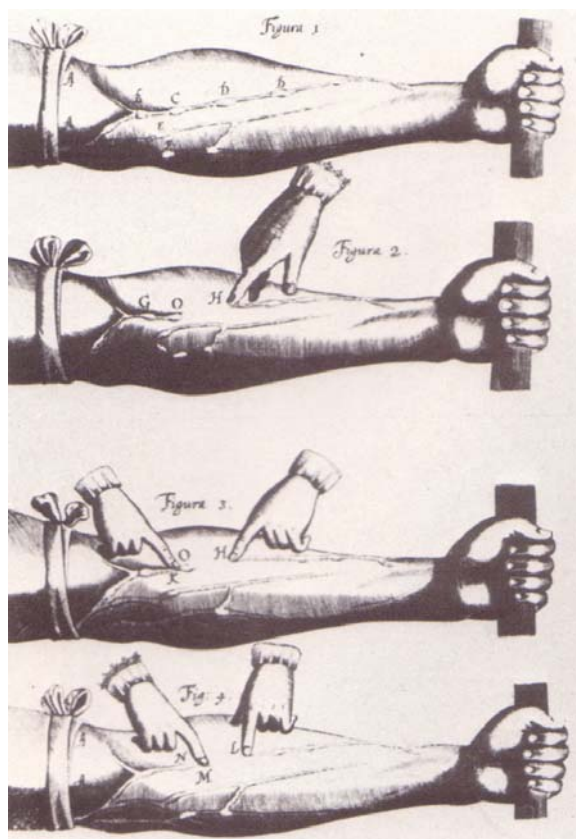
Plate VIII. William Harvey (1578-1657) used these woodcut prints to demonstrate the continuous circulation of blood within a contained system. This achievement has been deemed the most significant physiological and medical finding of the 17th century.

(Lyons, 1987)

7.1. Restatement of the Hypothesis

The main hypothesis was that an intravenous infusion of an adenosine and lidocaine solution would provide cardioprotection from ischemia-reperfusion injury by reducing mortality, decreasing ventricular arrhythmias and lowering infarct size. In addition, it was hypothesized that AL solution would preserve ATP and phosphocreatine, and reduce the degree of acidosis during ischemia-reperfusion.

While a few studies in the 1990s have used adenosine and lidocaine *separately* and *sequentially* as reperfusion therapy, no study has used adenosine and lidocaine combined in solution for pharmacological pretreatment and infusion during acute ischemia.

7.2. Summary of primary findings

An intravenous infusion of 3 different compositions of AL solution administered before and during ischemia offered superior protection from death, arrhythmias and tissue necrosis than either drug alone, or when lidocaine bolus preceded adenosine infusion (n = 44) (Chapter 3 and 4). When the AL solution infusion was given during reperfusion but not throughout ischemia (n = 6), ventricular arrhythmias occurred with increased frequency (VT: 39 ± 23 episodes, 84 ± 49 sec; VF: 2 ± 1 episodes, 21 ± 8 sec, Fig. 4.2) and its infarct size reduction ability was lost ($67 \pm 8\%$ vs. $38 \pm 6\%$, Fig. 4.3); although, no deaths occurred as a result of ischemia-induced VF. When AL infusion was continued on from ischemia into reperfusion (n = 6), no significant differences were found compared to the AL solution group where infusion was stopped at reperfusion (P < 0.05). These results suggested that AL solution exerts its cardioprotection mainly to delay ischemic injury. However, when adenosine and lidocaine treatment was given *separately* and *sequentially* as reperfusion therapy,

using a similar protocol to Homeister et al., it did not protect from mortality (29%), nor did it reduce infarct size ($52 \pm 5\%$) or reduce ischemia- or reperfusion-induced ventricular arrhythmias compared to saline-controls (Section 4.3.2.1 and 4.3.2.2) (Homeister et al., 1990).

From this thesis it was proposed that conservation of high-enrgy phosphates and improved cellular energy balance during early ischemia was likely to play a major role in the AL treatment's protective mechanism during early ischemia. AL combinational therapy maintained [ATP] in a steady state throughout ischemia and reperfusion with changes ranging of 95 ± 7 % to $117 \pm 10\%$ of baseline (Fig. 5.3). The concentration of [PCr] remained significantly elevated compared to controls throughout most of ischemia and early reperfusion (Fig. 5.4). Interestingly, treatment with AL solution did not change the development of ischemic acidosis; however, with reperfusion AL solution treated hearts rapidly returned to baseline pH (7.4) unlike controls which did not regained baseline pH during 40 min reperfusion (Fig. 5.6). On the whole, when AL infusion was continued throughout ischemia, the treatment resulted in significant preservation [ATP] and [PCr] at the expense of a high intercellular acid load during ischemia reperfusion. The low pH did not appear deleterious to heart function; rather, it reflected the shift from aerobic to anaerobic metabolism in ischemic cells with glycogen breakdown as the principle pathway to support myocardial ATP demand in regions of low- or no-flow ischemia. The accumulation of hydrogen ions were a consequence of the ischemia-induced suppression of oxidative phosphorylation, and possibly adenosine' indirect role to inhibit both Na^+/H^+ and Na^+/Ca^{2+} exchangers via a lower cyclic AMP levels and lidocaine's action to down-regulate the Na^+ fast channels, the effects of which have the potential to reduce Na^+ and Ca^{2+} loading.

Lastly, it was shown that co-administration of A_1 receptor agonist, CCPA, and a the Na^+ fast channel modulator, lidocaine, given as pretreatment 5 min before and during 30 min of left coronary artery ligation led to no deaths, no arrhythmias and a profound reduction in myocardial infarct size which was not significantly different to ischemic preconditioning (12 ± 4 % vs. 11 ± 3%). Moreover, the combination of CCPA and lidocaine surpassed IPC protection by abolishing ventricular arrhythmias (VT 4 ± 3 episodes, 8 ± 6 sec vs. 0 episodes) (Fig. 6.2) and mortality.

7.3. Possible mechanisms of action responsible for AL cardioprotection

The work in this thesis has inspired a virtual explosion of new hypotheses that may lead to a therapeutic benefit in the clinical setting. The following sections contain many ideas which are based on the known individual mechanisms of action of adenosine and lidocaine and are put forward in an effort to better understand the efficacy of AL solution.

7.3.1. REGULATION OF TRANSMEMBRANE ION DISTRIBUTION AND IMPROVED NA^+ AND CA^{2+} HANDLING

While the specific mechanisms for AL cardioprotection were not investigated in this thesis, they may involve adenosine's effect to cause negative chronotropy, negative inotropy and negative dromotropy (Ely and Berne, 1992), and lidocaine's ability to down-regulate voltage-dependent Na^+ fast channels in cardiac myocytes and possibly intercalated discs (Ruiz-Meana et al., 1999), and its negative inotropic effect via action potential shortening (Wilson et al., 1993; Lu et al., 1999). These primary electrophysiological effects may have 'downstream' metabolic consequences leading to reduced Na^+ and Ca^{2+} entry via multiple channels and exchangers (van Emous et al., 1997; Lu et al., 1999; Vinten-Johansen et al., 1999), and improved handling of

these cations by the sarcoplasmic reticulum (Mubagwa, 2002) and mitochondria
(Gross and Fryer, 1999).

Together with AL solution's ability to down-regulate the whole heart by targeting
nodal tissue, atrial and ventricular myocytes, and perhaps the coronary vasculature
(Ely and Berne, 1992; Wilson et al., 1993; Lu et al., 1999), the combination may
stabilize myocardial excitability by activating adenosine A_1 receptor-linked opening of
sarcolemmal ATP sensitive K^+ channels (Kirsch et al., 1990; Li et al., 1995). The
increased potassium conductance in ischemic cells and accumulation of K^+ ions in
the extracellular space, would lead to partial depolarization of resting membrane
potential and cell excitability would decrease from the reduction of Na^+ ion fast
channel availability (Janse and Wit, 1989; Wilde and Aksnes, 1995; Carmeliet, 1999).
The partially depolarized resting membrane potential would promote lidocaine's
binding to the inactivated Na^+ channel in ischemic cells (Cardinal et al., 1981; Barrett
et al., 2000), and the reduction of Na^+ fast channel availability would slow Na^+ entry
into these cells (Lu et al., 1999). Adenosine A_I activation (and perhaps A_3) may
further improve Na^+ and Ca^{2+} handling by blunting the stimulatory effects of
catecholamines (inhibiting sarcolemmal Na^+/H^+ and Na^+/Ca^{2+} exchangers), and
inhibition of norepinephrine release from nerve terminals (Ely and Berne, 1992;
Hayes, 2003).

AL solution's superior protection may also have come from shortening the action
potential duration (APD) through the combined effects of adenosine and lidocaine.
Opening of the K_{ATP} channels (and other K^+ channels) through enhanced phase 3
repolarization by adenosine could affect APD (Noma, 1983; Kirsch et al., 1990; Li et
al., 1995; Gross and Fryer, 1999), while lidocaine's interaction with cardiac
sarcolemmal Ca^{2+} channels may also shorten APD (Wilson et al., 1993; Lu et al.,

1999). Since excessive K^+ channel activation, and rapid APD shortening are known to cause arrhythmias (Coetzee et al., 2000), the AL combination may 'clamp' ischemic cells at more polarized potentials, than either adenosine or lidocaine alone (Dobson and Jones, 2003). As an anti-arrhythmic, adenosine also inhibits the effect of catecholamines by reducing cyclic AMP and slowing Ca^{2+} influx (Lerman and Belardinelli, 1991). Otherwise 'unchecked' depolarization of the resting membrane potential would lead to chaotic firing of some cells in the ischemic region and provide a substrate for arrhythmogenesis.

The complex interplay between adenosine and lidocaine to prevent rapid depolarization and APD shortening may involve i) adenosine's indirect effect on inactivation of voltage-sensitive Na^+ channels via increasing K^+ efflux which leads to a partial depolarization of the resting membrane potential and promotes lidocaine binding, ii) adenosine's protein kinase C-mediated, dynamin-dependent K_{ATP} channel internalization which reportedly acts like a brake on the rapid decline of excitability (Hu et al., 2003), and iii) lidocaine's voltage-dependent interactions with Ca^{2+} channels and its ability to partially inhibit K_{ATP} channels in cardiomyocytes (Olschewski et al., 1996). Thus, AL's overall effect to dampen excessive APD shortening and rapid decline in excitability may further assist to regulate Na^+ and Ca^{2+} entry and improve Ca^{2+} handling in ischemic regions of the heart.

Despite ongoing controversy (Gross and Peart, 2003), involvement of sarcolemmal K_{ATP} channels in early myocardial ischemia has been argued from studies using the blocker glibenclamide (Kantor et al., 1990; Carmeliet, 1999; Gross and Fryer, 1999), and the highly selective sarcolemma K_{ATP} blocker HMR-1883 (Billman et al., 1998; Toyoda et al., 2000). Other potential sites of adenosine's actions, and perhaps

lidocaine's actions, include the mitochondria (O'Rourke, 2000; Gross and Peart, 2003) and sarcoplasmic reticulum (Mubagwa, 2002).

In summary, reducing myocardial excitability and energy demand, and synchronizing action potential shortening and dispersion through better handling of intracellular Na^+ and Ca^{2+}, the combination of AL may limit the formation and dispersion of refractoriness in the ischemic and border zones. Since AL solution was infused before and during regional ischemia, further experiments are required to test if the antiarrhythmic therapy could break newly formed ventricular re-entry circuits when administered either as a bolus or intravenous infusion without prior pretreatment.

7.3.2. POSSIBLE ROLE FOR AL PROTECTION FROM INFLAMMATION INJURY

Inflammation injury was unlikely to have contributed to the measures of irreversible injury in this rat model of acute myocardial ischemia. Extending reperfusion times (up to 72 hours) could provide a great deal more insight into the boundaries of AL cardioprotection. Myocardial necrosis is considered to be fully evolved following 48 hours of reperfusion and shorter reperfusion times are known to yield differing results (Reimer, 1980). For instance, in this present research no additional protection was observed by extending AL solution through early reperfusion. Evidence of protection from reperfusion injury may have been blunted by the relatively short reperfusion period (120 min). The original work of Olfasson et al. (1987) administered adenosine for 60 min of reperfusion followed by 24 hours reperfusion and showed a reduction in infarct size. Interestingly, Homeister et al. (1990) stated an inability to reproduce these findings following 6 hours of reperfusion (Homeister et al., 1990). One wonders if the reason Homeister et al. wasn't able to repeat Olfasson et al.'s findings using just

adenosine may have been related to the shorter reperfusion time thereby not allowing the full effect of reperfusion to aid in recovery of some cells in the infarct zone.

Nevertheless, based on the known anti-inflammatory actions of adenosine and lidocaine individually, AL combined may also be useful to reduce the inflammatory response to ischemia-reperfusion injury. Adenosine is a potent modulator of the anti-inflammatory response by strongly inhibiting the activation of neutrophils, platelets and mononuclear leukocytes, which can lead to cytoxicity and endothelial dysfunction (Babbit et al., 1989; Pitarys et al., 1991; Jordan et al., 1999; Vinten-Johansen et al., 1999). Additionally, Zhao et al. (1999) have linked adenosine infusion at reperfusion with reduced PMN accumulation and reduced myocardial apoptosis. Recent work by Nakamura et al. (2000) verified this finding in rat hearts by showing that PMN accumulation was significantly correlated with the number of apoptotic cells.

Lidocaine also modulates a Na^+-channel independent inflammatory response by inhibiting the priming of human neutrophils and superoxide anion production with a suspected target site in a G_q–coupled signaling pathway (Vitola et al., 1997; Hollmann et al., 2001a; Hollmann et al., 2001b). Additionally, lidocaine inhibits intracellularly coupled lysophosphatidic acid (LPA) signalling (Hollmann et al., 2001a; Hollmann et al., 2001b). LPA is an intercellular phospholipid mediator with multiple actions linked to stimulation of inflammatory events such as platelet aggregation and neutrophil activation. As these events are related to the development of anatomic no reflow, AL solution may play a part initially reducing functional damage from ischemic injury and hinder the progression of anatomic no reflow (Engler et al., 1983; Golino et al., 1987; Olafsson et al., 1987).

In summary, the effects of AL combination to reduce ischemia-reperfusion injury could also be applied to reducing the adverse effects of the inflammatory process which includes attenuating the production of free radicals, reducing capillary plugging and minimizing direct injury to cardiomyocytes.

7.3.3. POSSIBLE CLINICAL SIGNIFICANCE AND LIMITATIONS OF AL INFUSION THERAPY

Despite advances in cardiovascular medicine, ischemic heart disease continues to be the leading cause of mortality and morbidity in the developed world and according to the World Health Organization acute coronary occlusion will be the most common cause of death in the entire world by 2020 (Yellon and Baxter, 2000). There are few effective treatments used in the clinical practice which preserve the viability of ischemic myocardium and limit the extent of an evolving myocardial infarction (Yellon and Baxter, 2000). Furthermore, clinical management of arrhythmias is also limited today by a lack of selective and safe antiarrhythmic drugs (Huikuri et al., 2001).

The work presented in this thesis may offer a new therapeutic combination which not only protects against ischemic injury but when targeting the adenosine A_1 receptor and voltage-gated Na^+ channels, the protection afforded equals ischemic preconditioning. Although, it is acknowledged, rat studies are often difficult to extrapolate to clinical scenarios for a number of reasons, including differences in mass-specific metabolic rate (Dobson and Himmelreich, 2002), electrophysiological properties (Opitz et al., 1998) and functional morphology such as collateral circulation (Schaper et al., 1992).

Indeed, because of the higher metabolic rate in the rat and the extremely short half-life of adenosine (8 sec) (Lasley and Mentzer, 1998), it appears that only the upper

range of adenosine concentrations have led to improved function or reduced necrosis in animal models (Ely and Berne, 1992; Yao and Gross, 1994b) and provided a therapeutic benefit to humans (Lagerkranser et al., 1989; Lee et al., 1995). Perhaps, the main problem limiting the use of AL and perhaps an A_1 agonist with lidocaine is the hypotensive effect observed in these studies. This concern could be minimized during surgical procedures or in the clinical setting through intracoronary infusion and the use of a highly specific A_1 agonist (Wilson et al., 1990). While much of the hypotensive effect originates from adenosine receptor stimulation, its worth noting that in humans, intracoronary infusions of up to 240 μg/kg/min adenosine cause minimal decreases in arterial pressure, heart rate or electrocardiographic variables and trials of novel adenosine agonists and allosteric modifiers with fewer hemodynamic effects are underway (Wilson et al., 1990). Similarly, intracarotid injections of adenosine of 1000 ug/ml in baboons have a profound effect to increase cerebral blood flow without any significant systemic side effects (Joshi et al., 2002).

Changing the dose of lidocaine may also be necessary for extrapolating these results in different models of coronary artery occlusion including humans. The maximum safe dose of lidocaine for humans is approximately 4 mg/kg i.v. (without epinephrine) and 7 mg/kg i.v. (with epinephrine). Lidocaine also has a short plasma half-life of approximately 8 minutes. Overall, a 70 kg adult should not receive more than around 300-500 mg cumulative dose of lidocaine. In our study, we omitted the standard rapid bolus of lidocaine (1-2 mg/kg) that usually precedes a continuous infusion (Opie, 1995) and opted for a lower dose (608 μg/kg/min) continuous infusion. Additionally, using lidocaine this way we aimed to avoid the reported proarrhythmic effects of lidocaine (Cardinal et al., 1981).

Another precaution in comparing data on rats and humans are differences in collateral circulation of the heart. However, since humans have a greater collateral circulation than the rat (Schaper et al., 1992), one might expect that the superior cardioprotection by AL infusion may have a greater effect in human patients. Further experiments are already underway to test this hypothesis.

7.4. Concluding remarks

In conclusion, this thesis has provided evidence that an adenosine and lidocaine solution is superior to either adenosine or lidocaine alone when administered 5 min before and during 30 min of regional ischemia in the *in vivo* rat model. Moreover, the concept may have therapeutic potential for the treatment of ischemic injury by targeting multiple sites including nodal cells, myocytes, endothelium and possibly the inflammatory response to injury. In addition, this thesis has identified a new potential pharmacological alternative to 'classical' ischemic preconditioning using the combination of an A_1 agonist with lidocaine.

In summary the potential use of adenosine and lidocaine or adenosine agonists plus lidocaine may involve:

    i.   Matching the action potential duration (refractoriness) to the ischemic-induced changes to conduction and excitability in the entire heart (nodal, atrial and ventricular myocyte) ,

    ii.   Changing the arrhythmogenic substrate and therefore underlying mechanisms for the genesis of arrhythmias in the ischemic regions and border zones such as better matching of membrane K^+ conductances, membrane potential, voltage dependent Na^+ entry during the upstroke of

the action potential, and less Ca^{2+} entry into the cell in areas of ischemia and border zones which are known to generate trigger activity,

iii.   Decreasing the electrical conductance across gap junctions,

iv.   Directly lowering the metabolic demand of the ischemic and non-ischemic tissue,

v.   Changing collateral flow to the myocardium, and

vi.   Protecting both the myocyte and coronary vasculature by attenuating neutrophil activation and platelet aggregation and adhesion as part of the inflammatory response to ischemia-reperfusion injury.

Though speculative, these possible sites of AL action open new windows into further investigations of acute ischemia-reperfusion injury, and attest to the power of basic science beginning with a question and ending with a question.

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Appendices

APPENDIX A

Table A1. Measurements for infarct size calculation (Chapter 3). Group numbers: 1 – Saline controls; 2 - Adeno-only; 3 - Lido-only; 4 - Low-dose AL; 5 - Mid-dose AL; 6 - High-dose AL.

Grp. No.	Left ventricle weight (g)	Left ventricle risk zone (rz)(g)	Left ventricle necrotic zone (nz) (g)	Infarct Size (rz/nz)
1	0.67	0.60	0.35	0.58
1	0.84	0.44	0.29	0.65
1	0.81	0.80	0.36	0.45
1	0.81	0.52	0.34	0.65
1	0.93	0.77	0.56	0.73
2	0.86	0.41	0.18	0.45
2	0.89	0.62	0.40	0.64
2	0.83	0.77	0.48	0.62
2	0.86	0.47	0.25	0.54
3	0.82	0.58	0.35	0.60
3	0.79	0.28	0.27	0.95
3	0.80	0.36	0.27	0.75
3	0.80	0.68	0.29	0.42
3	0.82	0.78	0.36	0.50
3	0.75	0.65	0.47	0.73
4	0.82	0.55	0.15	0.27
4	0.92	0.30	0.09	0.30
4	0.84	0.28	0.09	0.32
4	0.86	0.51	0.31	0.61
4	0.90	0.53	0.39	0.72
5	0.76	0.28	0.14	0.50
5	0.78	0.69	0.22	0.32
5	0.89	0.18	0.06	0.33
5	0.83	0.59	0.06	0.11
5	0.88	0.62	0.32	0.53
5	0.81	0.62	0.28	0.46
5	0.81	0.37	0.16	0.43
6	0.78	0.39	0.10	0.26
6	0.79	0.51	0.31	0.61
6	0.82	0.80	0.22	0.28
6	0.87	0.71	0.33	0.47
6	0.95	0.90	0.42	0.46
6	0.90	0.63	0.40	0.64

Table A2. Numbers of arrhythmias counted during ischemia (Chapter 3). For definitions of arrhythmias see Chapter 2. Group numbers: 1 – Saline controls; 2 - Adeno-only; 3 - Lido-only; 4 - Low-dose AL; 5 - Mid-dose AL; 6 - High-dose AL.

Grp No.	Premature ventricular beats	Bigeminy	Salvos	Ventricular Fibrillation (VF)	Ventricular Tachycardia (VT)	VT + VF
1	114	26	37	7	40	47
1	112	18	26	13	42	55
1	28	3	7	0	5	5
1	34	4	3	0	2	2
1	11	7	3	0	3	3
2	11	14	7	10	32	42
2	24	3	2	0	3	3
2	29	4	10	2	6	8
2	13	16	20	0	1	1
3	89	17	71	0	5	5
3	48	9	2	0	0	0
3	200	70	93	0	17	17
3	102	15	2	1	12	13
3	109	16	116	0	30	30
3	73	8	11	8	76	84
4	55	5	10	0	2	2
4	10	1	4	0	0	0
4	62	4	70	0	5	5
4	48	21	1	0	0	0
4	65	9	15	0	4	4
5	14	7	2	0	0	0
5	81	7	36	0	2	2
5	4	0	0	0	0	0
5	4	0	3	0	0	0
5	15	14	6	0	7	7
5	70	36	7	0	1	1
5	55	17	24	0	4	4
6	28	9	4	0	0	0
6	141	36	12	0	0	0
6	91	14	34	0	12	12
6	13	9	8	1	4	5
6	49	16	131	0	17	17
6	20	2	5	0	1	1

Table A3. Durations of arrhythmias counted during ischemia (Chapter 3). Group numbers: 1 – Saline controls; 2 - Adeno-only; 3 - Lido-only; 4 - Low-dose AL; 5 - Mid-dose AL; 6 - High-dose AL.

Group number	Bigeminy (sec)	VT (sec)	VF (sec)	VT+VF (sec)
1	67.30	163.57	124.19	287.76
1	28.16	230.34	122.04	352.38
1	3.05	131.61	0.00	131.61
1	8.66	2.17	0.00	2.172
1	5.82	3.67	0.00	3.67
2	35.08	82.04	266.31	348.35
2	2.46	15.36	0.00	15.36
2	18.43	7.39	2.63	10.01
2	22.35	5.18	0.00	5.18
3	65.22	11.26	0.00	11.26
3	10.87	0.00	0.00	0
3	184.90	10.81	0.00	10.81
3	46.94	32.27	7.80	40.07
3	21.15	16.13	0.00	16.13
3	23.51	117.03	24.70	141.73
4	3.20	0.92	0.00	0.92
4	0.78	0.00	0.00	0
4	3.00	2.67	0.00	2.67
4	18.81	0.00	0.00	0
4	37.53	40.71	11.11	51.82
5	4.97	0.00	0.00	0
5	38.92	2.20	0.00	2.20
5	0.00	0.00	0.00	0
5	0.00	0.00	0.00	0
5	16.69	3.26	0.00	3.26
5	47.85	1.26	0.00	1.26
5	34.21	7.75	0.00	7.75
6	7.33	0.00	0.00	0
6	327.75	0.00	0.00	0
6	11.86	9.36	0.00	9.36
6	9.37	3.29	25.54	28.83
6	20.98	12.93	0.00	12.93
6	1.48	0.82	0.00	0.82

Table A4. Numbers of arrhythmias counted during reperfusion (Chapter 3).
Group numbers: 1 – Saline controls; 2 - Adeno-only; 3 - Lido-only; 4 - Low-dose AL; 5 - Mid-dose AL; 6 - High-dose AL.

Grp. No.	Premature ventricular beats	Bigeminy	Salvos	Ventricular Fibrillation (VF)	Ventricular Tachycardia (VT)	VT + VF
1	11	0	2	0	0	0
1	0	0	0	1	0	1
1	6	0	0	3	1	4
1	2	0	0	3	3	6
1	0	0	0	1	0	1
2	3	2	2	0	0	0
2	2	0	0	1	0	1
2	2	0	1	2	0	2
2	4	0	2	2	0	2
3	0	0	0	0	0	0
3	0	0	0	0	0	0
3	0	0	0	1	0	1
3	0	0	0	0	0	0
3	19	0	0	0	0	0
3	2	0	2	0	0	0
4	0	0	0	0	0	0
4	0	0	0	0	0	0
4	0	0	0	0	0	0
4	1	0	1	0	0	0
4	0	0	0	0	0	0
5	0	0	0	0	0	0
5	0	0	0	0	0	0
5	0	0	0	0	0	0
5	0	0	0	0	0	0
5	0	0	0	0	0	0
5	0	0	0	0	0	0
5	0	0	0	0	0	0
6	0	0	0	1	0	1
6	4	0	0	0	0	0
6	0	0	0	0	0	0
6	0	0	0	0	0	0
6	139	3	2	0	0	0
6	10	1	0	5	1	6

Table A5. Durations of arrhythmias counted during reperfusion (Chapter 3).
Group numbers: 1 – Saline controls; 2 - Adeno-only; 3 - Lido-only; 4 - Low-dose AL; 5 - Mid-dose AL; 6 - High-dose AL.

Grp. No.	Bigeminy (sec)	VT (sec)	VF (sec)	VT+VF (sec)
1	0	0	0	0
1	0	0	0	0
1	12.09	0.41	0	0.41
1	0	0	15.54	15.54
1	0	23.24	0	23.24
2	9.3	0	0	0
2	0	0.87	0	0.87
2	0	34.95	0	34.95
2	0	6.6	0	6.6
3	0	0	0	0
3	0	0	0	0
3	0	0.6	0	0.6
3	0	0	0	0
3	0	0	0	0
3	0	0	0	0
4	0	0	0	0
4	0	0	0	0
4	0	0	0	0
4	0	0	0	0
4	0	0	0	0
5	0	0	0	0
5	0	0	0	0
5	0	0	0	0
5	0	0.36	0	0.36
5	0	0	0	0
5	0	0	0	0
5	0	0	0	0
6	0	0	0	0
6	0	0	0	0
6	0	0	0	0
6	0	0	0	0
6	450.62	0	0	0
6	0.53	14.43	0.41	14.84

Table A6. Heart rate taken at timepoints throughout ischemia and reperfusion (Chapter 3). Group numbers: 1 – Saline controls; 2 - Adeno-only; 3 - Lido-only; 4 - Low-dose AL; 5 - Mid-dose AL; 6 - High-dose AL.

Grp. No.	Baseline	Preocclusion	Ischemia (min) 25	Reperfusion (min) 30	60	119
1	409	419	423	352	374	356
1	465	434	463	465	394	328
1	424	385	371	366	348	340
1	439	442	457	461	426	444
1	486	464	422	461	389	410
2	448	436	386	429	395	397
2	483	396	427	454	424	392
2	492	429	459	461	446	440
2	401	359	443	312	298	332
3	433	365	347	399	411	395
3	420	336	293	355	388	344
3	452	360	317	372	393	391
3	373	345	301	406	429	399
3	463	409	356	435	413	403
3	481	433	206	139	384	379
4	433	365	347	399	411	395
4	403	332	340	376	391	389
4	494	325	314	410	384	388
4	474	307	299	388	349	328
4	396	317	255	425	420	391
5	418	359	332	1	357	411
5	429	339	272	295	293	367
5	407	278	288	403	395	382
5	482	348	335	406	315	371
5	449	305	316	338	387	390
5	466	295	266	391	411	449
5	443	364	390	419	423	397
6	420	349	318	376	360	357
6	418	313	221	345	354	357
6	480	348	320	376	379	366
6	483	299	344	369	418	360
6	421	297	283	368	359	363
6	488	326	334	446	445	437

Table A7. Systolic pressure taken at timepoints throughout ischemia and reperfusion (Chapter 3). Group numbers: 1 – Saline controls; 2 - Adeno-only; 3 - Lido-only; 4 - Low-dose AL; 5 - Mid-dose AL; 6 - High-dose AL.

Grp. No.	Baseline	Preocclusion	Ischemia (min) 25	Reperfusion (min) 30	60	119
1	150	161	163	74	82	95
1	146	99	151	141	79	75
1	145	123	111	84	69	67
1	125	123	115	146	71	118
1	134	118	119	118	75	101
2	163	157	121	113	91	92
2	185	99	109	139	78	82
2	155	86	80	105	73	94
2	115	65	71	73	72	100
3	140	89	91	141	132	93
3	110	77	70	101	80	73
3	157	103	87	102	109	70
3	116	99	64	139	121	124
3	166	128	103	111	113	108
3	169	158	68	94	101	100
4	140	89	91	141	132	93
4	154	83	99	119	101	106
4	158	66	85	124	78	93
4	187	84	107	107	82	70
4	113	76	58	103	90	84
5	143	107	100	171	104	149
5	150	75	65	93	96	149
5	111	61	70	125	137	98
5	193	81	100	162	77	105
5	172	72	90	94	143	116
5	188	58	49	100	74	106
5	143	66	94	131	134	112
6	147	54	68	100	110	80
6	131	53	38	99	81	72
6	124	74	101	116	119	87
6	191	60	90	108	138	89
6	187	77	85	129	87	115
6	188	87	78	127	138	111

Table A8. Diastolic pressure taken at timepoints throughout ischemia and reperfusion (Chapter 3). Group numbers: 1 – Saline controls; 2 - Adeno-only; 3 - Lido-only; 4 - Low-dose AL; 5 - Mid-dose AL; 6 - High-dose AL.

Grp. No.	Baseline	Preocclusion	Ischemia (min) 25	Reperfusion (min) 30	60	119
1	108	117	116	47	47	47
1	110	60	119	107	43	44
1	107	81	83	51	40	40
1	87	82	73	96	30	62
1	99	85	42	78	46	71
2	109	112	69	69	51	43
2	137	45	57	102	44	48
2	112	46	46	78	47	64
2	79	33	36	50	45	72
3	96	43	42	86	83	49
3	66	38	37	51	39	36
3	124	67	50	62	67	37
3	68	53	34	82	64	74
3	126	91	56	62	65	60
3	116	109	45	67	67	67
4	96	43	42	86	83	49
4	112	40	45	65	49	53
4	125	31	39	78	39	52
4	133	39	55	66	46	42
4	80	43	46	67	55	49
5	85	40	34	141	44	83
5	99	27	18	44	42	91
5	71	25	25	76	81	48
5	143	35	45	117	43	61
5	129	32	40	55	99	77
5	136	25	21	63	42	64
5	91	28	44	84	80	74
6	109	25	29	57	67	44
6	88	19	11	53	35	35
6	86	31	40	62	66	47
6	134	21	40	69	98	51
6	139	28	29	97	49	76
6	140	34	30	93	101	70

Table A9. Mean arterial pressure taken at timepoints throughout ischemia and reperfusion (Chapter 3). Group numbers: 1 – Saline controls; 2 - Adeno-only; 3 - Lido-only; 4 - Low-dose AL; 5 - Mid-dose AL; 6 - High-dose AL.

Grp. No.	Baseline	Preocclusion	Ischemia (min) 25	Reperfusion (min) 30	60	119
1	122	132	132	56	59	63
1	122	73	129	119	55	54
1	119	95	92	62	49	49
1	100	96	87	113	44	80
1	110	96	51	91	55	81
2	127	127	86	84	64	59
2	153	63	74	114	56	60
2	127	59	58	87	56	74
2	91	44	43	58	54	81
3	110	58	58	104	99	64
3	80	51	48	68	53	48
3	135	79	62	75	81	48
3	84	68	44	101	83	91
3	139	104	71	79	81	76
3	134	125	52	76	78	78
4	110	58	58	104	99	64
4	126	54	63	83	66	70
4	136	42	55	93	52	65
4	151	54	72	80	58	51
4	91	54	50	79	67	61
5	104	63	56	151	64	105
5	116	43	34	60	60	111
5	84	37	40	92	99	64
5	159	51	63	132	54	76
5	143	45	57	68	114	90
5	153	36	30	75	52	78
5	108	41	61	100	98	87
6	122	35	42	71	81	56
6	102	31	20	68	50	47
6	98	45	61	80	84	61
6	153	34	57	82	111	63
6	155	44	48	108	62	89
6	156	52	46	104	114	84

Table A10. Rate pressure product taken at timepoints throughout ischemia and reperfusion (Chapter 3). Group numbers: 1 – Saline controls; 2 - Adeno-only; 3 - Lido-only; 4 - Low-dose AL; 5 - Mid-dose AL; 6 - High-dose AL.

Grp. No.	Baseline	Preocclusion	Ischemia (min)	Reperfusion (min)		
			25	30	60	119
1	61379	67398	68759	26075	30471	33835
1	67774	42882	69645	65637	30950	24600
1	61322	47275	41326	30620	24126	22684
1	54866	54391	52685	67179	30230	52401
1	65158	54949	50264	54407	29269	41190
2	72968	68240	46820	48446	35910	36372
2	89206	39070	46365	63215	33101	32301
2	76266	36726	36566	48276	32481	41227
2	45981	23451	31594	22785	21603	33077
3	60610	32348	31672	56097	54081	36739
3	46357	25824	20511	35934	31088	25003
3	70746	37035	27546	38059	42948	27179
3	43287	34073	19385	56554	51814	49374
3	76590	52386	36561	48288	46660	43568
3	81481	68349	13964	13147	38718	38085
4	60610	32348	31672	56097	54081	36739
4	61884	27485	33596	44641	39290	41091
4	77948	21304	26722	50880	29876	35891
4	88416	25870	31916	41491	28458	23056
4	44749	24184	14888	43801	37757	32845
5	59917	38455	33082	171	37025	61030
5	64149	25251	17818	27437	28028	54633
5	45182	16934	20002	50213	54131	37559
5	92802	28247	33364	65548	24140	38819
5	77243	22087	28403	31872	55109	45244
5	87484	17033	13037	39235	30236	47737
5	63252	23970	36609	54905	56807	44580
6	61912	18660	21509	37630	39593	28631
6	54729	16719	8291	34257	28539	25682
6	59428	25716	32380	43596	45016	31781
6	92498	17905	31073	39767	57453	32084
6	78661	22905	24162	47512	31309	41563
6	91888	28353	25880	56777	61492	48251

APPENDIX B

Table B1. Measurements for infarct size calculation (Chapter 4). Group numbers: 1 – Saline controls; 2 – AL soln; 3 - Lido, Ado-SEQ; 4 - AL-SEQ; 5 - AL-PIR (see Chapter 4 "experimental design" for group abbreviation definitions).

Grp. No.	Left ventricle weight (g)	Left ventricle risk zone (rz)(g)	Left ventricle necrotic zone (nz) (g)	Infarct Size (nz/rz)
1	0.67	0.60	0.35	0.58
1	0.84	0.44	0.29	0.65
1	0.81	0.80	0.36	0.45
1	0.81	0.52	0.34	0.65
1	0.93	0.77	0.56	0.73
2	0.76	0.28	0.14	0.50
2	0.78	0.69	0.22	0.32
2	0.89	0.18	0.06	0.33
2	0.83	0.59	0.06	0.11
2	0.88	0.62	0.32	0.53
2	0.81	0.62	0.28	0.46
2	0.81	0.37	0.16	0.43
3	0.82	0.52	0.23	0.45
3	0.74	0.64	0.36	0.57
3	0.77	0.63	0.29	0.47
3	0.83	0.59	0.41	0.69
3	0.78	0.37	0.16	0.43
4	0.67	0.47	0.26	0.56
4	0.77	0.48	0.41	0.85
4	0.87	0.24	0.24	1.00
4	0.79	0.52	0.26	0.49
4	0.84	0.73	0.42	0.58
4	0.82	0.22	0.12	0.56
5	0.78	0.55	0.17	0.32
5	0.75	0.42	0.28	0.66
5	0.76	0.28	0.01	0.03
5	0.96	0.57	0.35	0.61
5	0.76	0.34	0.10	0.29
5	0.77	0.65	0.36	0.56

Table B2. Numbers and durations of arrhythmias counted during ischemia (Chapter 4). Group numbers: 1 – Saline controls; 2 – AL soln; 3 - Lido, Ado-SEQ; 4 - AL-SEQ; 5 - AL-PIR (see Chapter 4 "experimental design" for group abbreviation definitions).

Grp. No.	Number of			Duration (sec)		
	VT	VF	VT + VF	VT	VF	VT+VF
1	40	7	47	163.57	124.19	287.76
1	42	13	55	230.34	122.04	352.38
1	5	0	5	131.61	0	131.61
1	2	0	2	2.17	0	2.17
1	3	0	3	3.67	0	3.67
2	0	0	0	0	0	0
2	2	0	2	2.20	0	2.20
2	0	0	0	0	0	0
2	0	0	0	0	0	0
2	7	0	7	3.26	0	3.26
2	1	0	1	1.26	0	1.26
2	4	0	4	7.75	0	7.75
3	8	0	8	3.16	0	3.16
3	7	0	7	8.43	0	8.43
3	0	0	0	0	0	0
3	0	1	1	0	0.93	0.93
3	15	2	17	7.38	2.09	9.47
4	79	2	81	54.91	50.61	105.51
4	136	1	137	126.12	13.38	139.5
4	1	0	1	1.09	0	1.09
4	1	5	6	11.92	38.97	50.89
4	16	0	16	308.73	0	308.73
4	0	1	1	0	20.14	20.14
5	0	0	0	0	0	0
5	1	0	1	0.94	0	0.94
5	0	0	0	0	0	0
5	12	0	12	78.82	0	78.82
5	1	0	1	0.63	0	0.63
5	0	0	0	0	0	0

Table B3. Numbers and durations of arrhythmias counted during reperfusion (Chapter 4). Group numbers: 1 – Saline controls; 2 – AL soln; 3 - Lido, Ado-SEQ; 4 - AL-SEQ; 5 - AL-PIR (see Chapter 4 "experimental design" for group abbreviation definitions).

Grp. No.	Number of			Duration (sec)		
	VT	VF	VT + VF	VT	VF	VT+VF
1	0	0	0	0	0	0
1	1	0	1	0	0	0
1	3	1	4	0.41	0	0.41
1	3	3	6	0	15.54	15.54
1	1	0	1	23.24	0	23.24
2	0	0	0	0	0	0
2	0	0	0	0	0	0
2	0	0	0	0	0	0
2	0	0	0	0	0	0
2	0	0	0	0	0	0
2	0	0	0	0	0	0
2	0	0	0	0	0	0
3	11	0	11	10.4	0	10.4
3	0	0	0	0	0	0
3	4	0	4	4.85	0	4.85
3	0	15	15	0	18.99	18.99
3	1	0	1	1.04	0	1.04
4	0	0	0	0	0	0
4	0	0	0	0	0	0
4	0	0	0	0	0	0
4	0	0	0	0	0	0
4	0	0	0	0	0	0
4	0	0	0	0	0	0
5	0	0	0	0	0	0
5	0	0	0	0	0	0
5	0	0	0	0	0	0
5	0	0	0	0	0	0
5	0	0	0	0	0	0
5	0	0	0	0	0	0

Table B4. Heart rate taken at timepoints throughout ischemia and reperfusion (Chapter 4). Group numbers: 1 – Saline controls; 2 – AL soln; 3 - Lido, Ado-SEQ; 4 - AL-SEQ; 5 - AL-PIR (see Chapter 4 "experimental design" for group abbreviation definitions).

			Ischemia (min)	Reperfusion (min)		
Grp. No.	Baseline	Preocclusion	25	30	60	119
1	409	419	423	352	374	356
1	465	434	463	465	394	328
1	424	385	371	366	348	340
1	439	442	457	461	426	444
1	486	464	422	461	389	410
2	418	359	332	-	357	411
2	429	339	272	295	293	367
2	407	278	288	403	395	382
2	482	348	335	406	315	371
2	449	305	316	338	387	390
2	466	295	266	391	411	449
2	443	364	390	419	423	397
3	433	436	386	366	124	369
3	358	304	281	307	327	330
3	435	382	423	417	388	355
3	490	420	431	386	402	388
3	480	395	382	505	519	518
4	415	308	410	281	328	326
4	498	394	452	424	392	410
4	376	317	327	333	335	332
4	478	418	481	396	402	439
4	469	362	391	355	373	377
4	348	276	360	317	372	362
5	457	353	355	326	405	393
5	448	345	335	320	383	356
5	469	381	330	330	380	380
5	496	367	256	240	387	411
5	429	344	310	330	357	383
5	459	369	350	329	343	375

Table B7. Systolic pressure taken at timepoints throughout ischemia and reperfusion (Chapter 4). Group numbers: 1 – Saline controls; 2 – AL soln; 3 - Lido, Ado-SEQ; 4 - AL-SEQ; 5 - AL-PIR (see Chapter 4 "experimental design" for group abbreviation definitions).

Grp. No.	Baseline	Preocclusion	Ischemia (min) 25	Reperfusion (min) 30	60	119
1	150	161	163	74	82	95
1	146	99	151	141	79	75
1	145	123	111	84	69	67
1	125	123	115	146	71	118
1	134	118	119	118	75	101
2	143	107	100	171	104	149
2	150	75	65	93	96	149
2	111	61	70	125	137	98
2	193	81	100	162	77	105
2	172	72	90	94	143	116
2	188	58	49	100	74	106
2	143	66	94	131	134	112
3	126	157	121	88	95	104
3	138	112	96	112	129	129
3	129	123	125	90	126	88
3	146	139	113	73	112	88
3	151	148	93	110	107	137
4	134	57	134	64	84	69
4	169	94	113	93	89	81
4	103	61	79	80	82	106
4	158	87	120	81	101	104
4	144	73	73	75	85	71
4	129	76	95	84	96	94
5	119	66	98	95	121	98
5	152	95	90	102	138	106
5	123	61	65	89	109	87
5	160	74	38	59	88	79
5	128	64	71	76	88	105
5	116	69	72	74	86	84

Table B8. Diastolic pressure taken at timepoints throughout ischemia and reperfusion (Chapter 4). Group numbers: 1 – Saline controls; 2 – AL soln; 3 - Lido, Ado-SEQ; 4 - AL-SEQ; 5 - AL-PIR (see Chapter 4 "experimental design" for group abbreviation definitions).

Grp. No.	Baseline	Preocclusion	Ischemia (min) 25	Reperfusion (min) 30	60	119
1	108	117	116	47	47	47
1	110	60	119	107	43	44
1	107	81	83	51	40	40
1	87	82	73	96	30	62
1	99	85	42	78	46	71
2	85	40	34	141	44	83
2	99	27	18	44	42	91
2	71	25	25	76	81	48
2	143	35	45	117	43	61
2	129	32	40	55	99	77
2	136	25	21	63	42	64
2	91	28	44	84	80	74
3	93	112	69	54	70	69
3	110	89	58	51	76	92
3	81	74	90	51	84	55
3	101	93	87	48	74	57
3	123	120	68	78	74	102
4	96	30	102	34	56	44
4	111	43	82	65	59	51
4	63	35	52	41	51	68
4	108	44	85	48	66	68
4	94	37	52	50	58	46
4	81	31	59	42	57	57
5	86	33	49	43	74	58
5	104	46	49	50	92	66
5	86	34	33	41	64	51
5	112	35	21	33	64	50
5	99	38	34	36	53	63
5	72	33	40	42	57	54

Table B9. Mean arterial pressure taken at timepoints throughout ischemia and reperfusion (Chapter 4). Group numbers: 1 – Saline controls; 2 – AL soln; 3 - Lido, Ado-SEQ; 4 - AL-SEQ; 5 - AL-PIR (see Chapter 4 "experimental design" for group abbreviation definitions).

Grp. No.	Baseline	Preocclusion	Ischemia (min)	Reperfusion (min)		
			25	30	60	119
1	122	132	132	56	59	63
1	122	73	130	118	55	54
1	120	95	92	62	50	49
1	100	96	87	113	44	81
1	111	96	68	91	56	81
2	104	62	56	151	64	105
2	116	43	34	60	60	110
2	84	37	40	92	100	65
2	160	50	63	132	54	76
2	143	45	57	68	114	90
2	153	36	30	75	53	78
2	108	41	61	100	98	87
3	104	127	86	66	78	80
3	119	97	71	71	94	104
3	97	90	101	64	98	66
3	116	108	96	57	87	67
3	132	129	76	88	85	114
4	108	39	113	44	66	52
4	130	60	93	74	69	61
4	76	43	61	54	61	80
4	124	58	97	59	78	80
4	110	49	59	59	67	54
4	97	46	71	56	70	70
5	97	44	66	60	90	71
5	120	62	63	67	108	79
5	99	43	44	57	79	63
5	128	48	26	42	72	60
5	109	47	47	49	65	77
5	87	45	51	52	67	64

Table B10. Rate pressure product taken at timepoints throughout ischemia and reperfusion (Chapter 4). Group numbers: 1 – Saline controls; 2 – AL soln; 3 - Lido, Ado-SEQ; 4 - AL-SEQ; 5 - AL-PIR (see Chapter 4 "experimental design" for group abbreviation definitions).

			Ischemia (min)		Reperfusion (min)	
Grp. No.	Baseline	Preocclusion	25	30	60	119
1	61350	67459	68949	26048	30668	33820
1	67890	42966	69913	65565	31126	24600
1	61480	47355	41181	30744	24012	22780
1	54875	54366	52555	67306	30246	52392
1	65124	54752	50218	54398	29175	41410
2	59774	38413	33200	171	37128	61239
2	64350	25425	17680	27435	28128	54683
2	45177	16958	20160	50375	54115	37436
2	93026	28188	33500	65772	24255	38955
2	77228	21960	28440	31772	55341	45240
2	87608	17110	13034	39100	30414	47594
2	63349	24024	36660	54889	56682	44464
3	54706	68240	46820	32145	11749	38233
3	49575	34034	26826	34431	42275	42438
3	55915	47049	52790	37566	48822	31249
3	71751	58380	48841	28069	45104	34248
3	72258	58287	35487	55348	55324	70811
4	55611	17612	55063	18094	27569	22382
4	84177	37034	51065	39404	34744	33292
4	38571	19298	25875	26764	27370	35094
4	75476	36324	57624	32251	40602	45429
4	67230	26455	28455	26744	31788	26722
4	44771	21021	34200	26535	35627	33964
5	54602	23397	34897	31026	48795	38367
5	68185	32951	30041	32745	52917	37875
5	57689	23293	21490	29214	41545	33186
5	79346	27158	9641	14088	34211	32592
5	54732	21825	22134	24956	31434	40163
5	53325	25636	25060	24280	29421	31425

APPENDIX C

Table C1. Percent changes in βATP peak integrals from baseline in controls (group 1) and AL solution rats (group2). Controls did not receive pretreatment so for data analysis and graphical display group 1 data was compared with group 2 following pretreatment.

Group	time(min) →	Baseline or pretreatment								Ischemia		Reperfusion							
		0	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75
1	60203		100	126.92	102.65	50.23	22.52	49.92	33.50	23.43	49.94	16.90	38.82	40.00	24.21	30.90	33.90
1	70203		100	58.24	46.40	85.43	48.39	47.17	18.21	25.00	7.48	39.51	30.47	35.42	18.06	21.58	36.25
1	130203		100	100.77	91.37	101.66	89.79	80.62	97.30	79.45	87.42	88.50	102.05	88.81	93.80	147.78	209.30
1	200203		100	167.46	221.71	224.45	159.67	220.78	144.56	165.31	162.93	128.98	124.52	189.76	137.11	138.56	119.37
1	100203.1		100	64.38	62.74	30.55	26.71										
1	10203.2		100	114.69	105.74	27.34	23.98	10.68									
2	110203	100	84.38	108.30	124.03	98.45	92.38	101.30	98.14	72.41	78.12	96.47	123.00	114.50	87.98	102.24	107.80
2	120203	100	140.89	100.73	88.98	89.55	79.06	96.19	102.23	106.62	108.63	90.82	86.45	92.87	98.56	109.11	98.20
2	170203	100	100.51	80.83	106.05	108.09	138.10	121.44	94.01	75.63	85.92	112.58	111.84	106.94	104.00	113.87	108.27
2	180203	100	105.07	126.42	129.88	102.56	91.59	74.14	127.83	116.67	89.37	90.51	98.18	101.48	115.01	102.59	121.45
2	190203	100	61.74	90.31	118.48	87.80	82.60	135.67	105.04	84.49	68.11	71.49	84.56	75.82	116.19	129.31	103.60
2	210203.1	100	119.86	72.75	87.32	84.22	95.24	88.81	101.38	120.26	130.22	120.59	94.82	107.39	120.59	67.35	71.42
2	210203.2	100	103.54	228.78	167.75	148.85	163.78	166.98	-	90.70	120.44	146.40	96.94	115.94	132.13	139.16	110.55

Table C2. Percent changes in PCR peak integrals from baseline in controls (group 1) and AL solution rats (group2). Controls did not receive pretreatment so for data analysis and graphical display group 1 data was compared with group 2 following pretreatment.

βATP Group	time(min) →	Baseline or pretreatment			Ischemia					Reperfusion							
		0	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75
1	60203		100	126.92	102.65	50.23	22.52	49.92	33.50	23.43	49.94	16.90	38.82	40.00	24.21	30.90	33.90
1	70203		100	58.24	46.40	85.43	48.39	47.17	18.21	25.00	7.48	39.51	30.47	35.42	18.06	21.58	36.25
1	130203		100	100.77	91.37	101.66	89.79	80.62	97.30	79.45	87.42	88.50	102.05	88.81	93.80	147.78	209.30
1	200203		100	167.46	221.71	224.45	159.67	220.78	144.56	165.31	162.93	128.98	124.52	189.76	137.11	138.56	119.37
1	100203.1		100	64.38	62.74	30.55	26.71										
1	10203.2		100	114.69	105.74	27.34	23.98	10.68									
2	110203	100	84.38	108.30	124.03	98.45	92.38	101.30	98.14	72.41	78.12	96.47	123.00	114.50	87.98	102.24	107.80
2	120203	100	140.89	100.73	88.98	89.55	79.06	96.19	102.23	106.62	108.63	90.82	86.45	92.87	98.56	109.11	98.20
2	170203	100	100.51	80.83	106.05	108.09	138.10	121.44	94.01	75.63	85.92	112.58	111.84	106.94	104.00	113.87	108.27
2	180203	100	105.07	126.42	129.88	102.56	91.59	74.14	127.83	116.67	89.37	90.51	98.18	101.48	115.01	102.59	121.45
2	190203	100	61.74	90.31	118.48	87.80	82.60	135.67	105.04	84.49	68.11	71.49	84.56	75.82	116.19	129.31	103.60
2	210203.1	100	119.86	72.75	87.32	84.22	95.24	88.81	101.38	120.26	130.22	120.59	94.82	107.39	120.59	67.35	71.42
2	210203.2	100	103.54	228.78	167.75	148.85	163.78	166.98	-	90.70	120.44	146.40	96.94	115.94	132.13	139.16	110.55

Table C3. Percent changes in Pi peak integrals from baseline in controls (group 1) and AL solution rats (group2). Controls did not receive pretreatment so for data analysis and graphical display group 1 data was compared with group 2 following pretreatment.

PCR Group	time(min) →	Baseline or pretreatment 0	5	10	15	20	25	30	35	Reperfusion 40	45	50	55	60	65	70	75
1	60203	.	100	62.57	51.77	39.98	33.23	28.78	28.49	39.38	15	64.01	47.12	71.14	76.72	64.28	67.99
1	70203	.	100	44.1	44.49	45.1	42.61	51.49	33.97	54.17	27.1	27.09	63.22	62.2	44.62	77.25	32.92
1	130203	.	100	45.55	50.69	58.1	48.54	60.79	52.18	51.72	34.09	46.14	55.33	47.87	56.08	46.41	57.91
1	200203	.	100	87.27	66.94	61.9	87.5	115.41	65.99	100.01	101.09	63.53	97.01	104.31	106.14	78.54	104.71
1	100203.1	.	100	91.66	93.11	33.86	27.2	.	.	.	.	.	.	.	.	.	.
1	10203.2	.	100	122.01	66.56	36.72	30.55	22.42	.	.	.	.	.	.	.	.	.
2	110203	100	94.01	111.67	81.3	59.05	109.02	62.82	47.39	54.19	77.67	75.03	77.53	95.61	83.24	91.46	105.53
2	120203	100	107.1	80.7	49.61	92.64	72.3	72.07	69.15	92.64	81.54	85.88	87.68	87.76	84.76	91.85	83.71
2	170203	100	91.36	70.84	70.39	90.59	128.15	87.07	67.94	104.92	95.56	91.53	94.63	89.86	87.18	98.52	90.94
2	180203	100	122.22	71.22	104	102.96	102.07	89.11	90.49	63.52	95.37	95.01	115.93	103.21	82.63	180.34	117.25
2	190203	100	98.2	84.7	106.88	51.78	54.16	84.14	96.01	89.84	60.61	60.32	62.44	63.2	100.75	57.36	58.03
2	210203.1	100	100.06	107.94	119.88	87.19	99.23	6.14	98.82	172.07	139.42	125.46	135.99	96.08	182.6	122.4	108.61
2	210203.2	100	109.95	133.97	92.86	87.14	83.62	76.06	71.7	91.25	93.32	96.89	112.22	97.88	120.24	114.91	112.48

Note: columns 5–35 fall under the "Ischemia" heading; columns 40–75 fall under the "Reperfusion" heading.

Table C4. Percent changes in [PCR/[ATP] peak integrals from baseline in controls (group 1) and AL solution rats (group2). Controls did not receive pretreatment so for data analysis and graphical display group 1 data was compared with group 2 following pretreatment.

Pi Group	time(min) →	Baseline or pretreatment				Ischemia				Reperfusion							
		0	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75
1	60203	.	100	96.38	67.93	75.05	64.34	95.45	86.67	66.91	83.54	57.53	8.27	15.88	17.03	22.21	34.81
1	70203	.	100	188.77	173.92	168	212.31	92.91	238.35	115.19	86.27	110.34	178.7	.	155.71	228.15	152.71
1	130203	.	100	92.07	104.24	105.47	102.59	102.73	104.59	96.33	95.97	99.87	104.99	55.88	53.67	54.7	93.25
1	200203	.	100	100.58	106.27	109.17	115.26	111.08	114.49	100.03	98.58	101.12	108.02	97.16	82.72	97.08	90.28
1	100203.1	.	100	405.68	633.51	455.09	604.51	.	.	.	.	.	.	.	.	.	.
1	10203.2	.	100	134.82	135.49	162.16	135.41	188.54	.	.	.	.	.	.	.	.	.
2	110203	100	82.49	107.13	148.49	107.95	131.21	143.18	130.81	97.03	87.84	93.01	84.28	86.87	81.94	117.71	84.75
2	120203	100	86.34	105.85	103.14	127.65	130.25	135.33	139.41	107.74	89.33	89.76	85.47	73	80.47	94.57	85.09
2	170203	100	96.95	119.18	130.79	140.9	153.48	150.72	174.31	117.8	104.79	109.91	85.78	105.63	115.54	91.61	93.73
2	180203	100	95.08	114.72	119	132	132.76	132.71	134.64	115.43	96.6	101.5	87.6	94.8	90.39	84.38	98.26
2	190203	100	84.18	97.53	141.81	119.27	113.49	107.51	95.34	104	105.86	85.55	94.08	90.34	107.52	91.71	103.43
2	210203.1	100	153.65	260.58	154.05	257.12	254.91	.	237.5	120.43	213.51	201.52	133.68	67.94	110.39	156.36	190.73
2	210203.2	100	161.32	259.96	283.2	390.06	353.27	326.55	291.51	222.15	233.07	184.46	184.02	237.88	204.15	199.43	221.35

Table C5. Changes in pH peak integrals from baseline in controls (group 1) and AL solution rats (group2). Controls did not receive pretreatment so for data analysis and graphical display group 1 data was compared with group 2 following pretreatment.

Group	time(min) →	Baseline or pretreatment			Ischemia						Reperfusion							
		0	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75
1	60203		100	49.29	50.42	79.58	147.65	57.66	85.02	168.19	30.03	378.65	121.35	177.83	316.91	207.87	200.59
1	70203		100	75.74	95.87	52.81	88.07	109.17	186.56	216.64	362.34	68.59	207.48	175.64	247.03	357.92	90.81
1	130203		100	45.21	55.48	57.15	54.05	75.41	53.63	65.09	39.00	52.15	54.22	53.89	59.79	31.41	27.67
1	200203		100	52.11	30.19	27.58	54.80	52.28	45.64	60.49	62.04	49.26	77.90	54.97	77.42	56.69	87.72
1	100203.1		100	142.34	148.40	110.79	101.84										
1	10203.2		100	106.38	62.94	134.34	127.31	209.87									
2	110203	100	111.42	103.12	65.55	59.98	118.02	62.02	48.29	74.86	99.43	77.78	63.04	83.51	94.63	89.46	97.91
2	120203	100	76.02	80.12	55.75	103.45	91.45	74.93	67.64	86.89	75.07	94.55	101.42	94.49	86.01	84.18	85.25
2	170203	100	90.89	87.64	66.38	83.83	92.79	71.71	72.28	138.74	111.25	81.30	84.61	84.03	83.83	86.52	84.01
2	180203	100	116.29	56.33	80.05	100.38	111.42	120.16	70.79	54.44	106.69	104.95	118.08	101.70	71.82	175.74	96.52
2	190203	100	159.05	93.79	90.21	58.97	65.57	62.02	91.41	106.34	89.00	84.40	73.84	83.34	86.71	44.35	56.01
2	210203.1	100	83.50	148.35	137.28	103.51	104.21	6.91	97.50	143.09	107.06	104.05	143.45	89.49	151.43	181.75	152.09
2	210203.2	100	106.17	58.55	55.37	58.55	51.05	45.54	0.42	100.59	77.47	66.18	115.77	84.40	91.00	82.58	101.73

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Table C6. Changes in Mg^{2+} peak integrals from baseline in controls (group 1) and AL solution rats (group2). Controls did not receive pretreatment so for data analysis and graphical display group 1 data was compared with group 2 following pretreatment.

Group	time(min) →	Baseline or pretreatment			Ischemia						Reperfusion							
		0	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75
1	**60203**		7.67	6.36	6.82	6.48	6.35	6.35	6.31	6.55	6.81	6.31	6.43	.	7.22	7.2	7.14
1	**70203**		7.51	7.03	6.83	6.73	6.84	6.79	6.87	6.69	6.39	6.87	6.72	6.81	7.11	7.03	7.11
1	**130203**		7.56	7.27	7.14	7.27	7.23	7.37	7.28	7.66	7.41	7.4	7.35	7.33	7.34	7.43	7.45
1	**200203**		7.4	7.89	6.36	6.23	6.18	6.32	6.12	7.52	7.62	7.82	7.89	7.58	7.52	7.47	7.25
1	**100203.1**		7.6	7.66	7.38	6.48	5.57	.	.	.	.	.	.	.	.	.	.
1	**10203.2**		7.92	7.42	7.57	6.3	6.14	5.92	.	.	.	.	.	.	.	.	.
2	**110203**	7.46	7.33	6.9	6.3	5.99	5.6	5.48	5.78	7.24	7.46	7.41	7.53	7.46	7.78	7.42	7.58
2	**120203**	7.25	7.51	7.47	6.5	6.42	6.35	6.29	6.31	7.56	8.12	7.84	7.54	8.35	7.99	8	8.22
2	**170203**	7.53	7.43	7.39	6.48	6.42	6.12	6.09	7.01	7.45	7.38	7.68	7.17	7.18	7.53	7.55	7.57
2	**180203**	7.87	7.87	6.88	7.15	6.99	6.92	7.55	6.88	7.71	7.4	7.21	7.35	7.48	7.27	7.48	7.07
2	**190203**	7.58	7.74	7.22	7.11	7.62	7.55	7.55	7.62	7.77	7.35	7.26	7.03	7.77	7.35	7.19	7.27
2	**210203.1**	7.43	7.65	7.62	7.54	6.16	6.35	5.86	7.2	7.38	7.67	7.15	7.31	7.23	7.62	7.63	7.64
2	**210203.2**	7.37	7.38	6.51	.	7.16	7.21	7.1	7.12	6.85	7.23	7.11	7.62	7.62	7.62	7.54	7.53

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Table C7. Changes in heart rate (HR) from baseline in controls (group 1) and AL solution rats (group2). Controls did not receive pretreatment so for data analysis and graphical display group 1 data was compared with group 2 following pretreatment.

Group	time(min) →	Baseline or pretreatment								Ischemia			Reperfusion					
		0	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75
1	60203		100	554.08	113.69	172.1	191.01	259.42	193.44			384.77	340.05	0	244.56	136.55	105.92
1	70203		100	102.12	113.76	110.34	357.67	43.61	154.06			177.66	0	71.51	102.75	20.5	88.7
1	130203		100	110.52	72.7	60.67	76.93	55.87	92.11	63.97	68.16	53.2	54.18	52.54	70.2	118.87	56.7
1	200203		100	56.4	107.03	228.92	158.42	185.82	174.24	39.59	74.12	40.01	50.54	51.07	103.08	104.66	83.88
1	100203.1		100	79.06	123.83	155.3	320.79										
1	10203.2		100	106.03	46.54	285.27	213.43	571.16									
2	110203	100	113.49	190.45	308.48	732.23	561.34	665.44	891.7	188.04	163.76	65.39	102.04	147.79	137.66	79.04	198.5
2	120203	100	81.4	59.26	204.38	166.38	166.29	142.26	133.31	63.1	43.71	52.61	108.05	156.13	64.34	58.85	66.48
2	170203	100	98.15	147.96	213.61	196.18	490.13	631.92	115.77	186.11	73.02	103.99	89.44	96.34	182.52	132.98	68.96
2	180203	100	115.47	164.29	103.69	209.47	138.35	97.03	155.85	63.55	159.4	74.47	162.18	90.75	123.17	86.95	171.62
2	190203	100	146.79	180.08	160.15	151.67	130.21	127.49	206.16	178.21	199.65	130.23	244.7	160.49	107.54	145.98	111.13
2	210203.1	100	103.96	44.58	59.21	212.37	61.08	283.6	73.39	92.4	41.47	58.41	20.35	47.84	55.42	94.5	46.38
2	210203.2	100	144.25	258.77	213.34	428.46	110.25	84.25	83.12	121.28	73.59	163.98	265.34	528.39	192.7	408.57	110.32

Table C8. Changes in mean arterial pressure (MAP) from baseline in controls (group 1) and AL solution rats (group2). Controls did not receive pretreatment so for data analysis and graphical display group 1 data was compared with group 2 following pretreatment.

HR Group	time(min) →	Baseline or pretreatment 0	5	10	Ischemia 15	20	25	30	35	Reperfusion 40	45	50	55	60	65	70	75
1	60203	.	235	254	260	156	250	154	29	66	199	291	301	312	318	323	323
1	70203	.	317	359	367	362	362	356	353	346	342	336	331	340	341	342	347
1	130203	.	400	404	405	405	406	405	405	404	403	402	401	398	414	.	.
1	200203	.	317	296	356	310	316	349	350	344	344	343	351	346	350	355	353
1	100203.1	.	300	353	9	0	.	.	.	.	.	.	.	.	.	.	.
1	10203.2	.	424	372	.	.	.	.	.	.	.	.	.	.	.	.	.
2	110203	268	196	178	181	157	161	166	200	205	205	243	251	259	261	252	238
2	120203	338	269	275	42	238	261	247	246	313	336	343	341	329	345	337	350
2	170203	393	315	292	266	222	234	249	255	314	334	344	353	364	371	371	.
2	180203	356	217	201	186	165	180	183	186	269	304	320	334	345	352	356	358
2	190203	443	309	288	266	261	260	256	367	390	400	409	413	422	428	432	.
2	210203.1	361	249	224	174	226	259	210	314	332	351	358	370	372	369	374	.
2	210203.2	.	237	213	.	176	178	198	122	231	.	.	300	.	.	280	.

Table C8. Changes in mean arterial pressure (MAP) from baseline in controls (group 1) and AL solution rats (group2). Controls did not receive pretreatment so for data analysis and graphical display group 1 data was compared with group 2 following pretreatment.

MAP		Baseline or pretreatment		Ischemia						Reperfusion							
Group	time(min) →	0	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75
1	60203	.	49	42	44	65	57	49	42	28	27	57	84	88	91	95	98
1	70203	.	90	92	94	90	92	89	92	88	95	80	82	86	93	83	87
1	130203	.	120	123	118	123	126	123	123	114	124	121	117	119	111	110	.
1	200203	.	122	112	124	83	122	123	112	126	119	122	117	126	118	126	125
1	100203.1	.	150	158	71	.	0	.	158	.	.	.	.	.	.	.	.
1	10203.2	.	174	139	0	0	.	.	139	.	.	.	.	.	.	.	.
2	110203	105	51	39	39	45	47	47	39	49	88	105	115	119	121	117	118
2	120203	118	59	62	28	71	73	68	62	65	113	113	104	96	96	115	92
2	170203	128	70	61	61	61	64	69	61	72	126	131	132	135	136	135	136
2	180203	163	89	88	87	89	91	95	88	97	155	163	168	173	172	169	172
2	190203	121	79	78	78	83	88	90	78	139	147	151	156	147	145	144	153
2	210203.1	97	50	43	43	53	56	52	43	116	123	129	130	123	112	106	107
2	210203.2	95	43	38	46	46	47	51	38	56	112	126	133	131	137	134	137

Table C9. Changes in rate pressure product (RPP) from baseline in controls (group 1) and AL solution rats (group2). Controls did not receive pretreatment so for data analysis and graphical display group 1 data was compared with group 2 following pretreatment.

RPP		Baseline or pretreatment				Ischemia				Reperfusion							
Group	time(min) →	0	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75
1	60203	.	14930	13397	14356	13360	18540	10217	1159	2441	14713	28205	30018	32350	33996	35726	2441
1	70203	.	38143	41885	43697	40008	41800	40545	40044	42750	35397	34767	36089	40887	36971	38448	42750
1	130203	.	57529	59395	57857	60333	62191	60788	57932	62200	60662	59400	59887	57860	61153	.	62200
1	200203	.	48099	42526	53902	31603	48049	51744	53186	49776	51010	49326	53884	50171	53477	53949	49776
1	100203.1	.	51467	63307	1095	0	.	.	.	.	.	.	.	.	.	.	.
1	10203.2	.	86130	59670	0	.	.	.	.	.	.	.	.	.	.	.	.
2	110203	37744	16192	10380	10761	10603	11506	11721	13948	23502	26996	33859	35877	37569	36806	36265	23502
2	120203	48267	23410	23928	1813	26032	27969	24374	23018	43214	46813	44974	41650	40118	49682	40958	43214
2	170203	62188	31974	26019	24237	21631	23511	26590	28442	50970	54814	56872	59700	61650	62756	63382	50970
2	180203	66843	25973	24112	22415	21229	23131	24702	25767	49057	57269	61710	66551	68641	68402	70519	49057
2	190203	66649	34845	32738	31575	33305	35104	35433	64974	71933	74938	79234	76088	75723	75863	80850	71933
2	210203.1	44687	18166	13640	11009	18203	21313	17009	46642	51680	56852	58599	58467	54440	51097	52447	51680
2	210203.2	.	14052	11430	.	10356	12756	15090	8674	29000	.	.	44290	.	.	42960	29000

APPENDIX D

Table D1. Measurements for infarct size calculation (Chapter 6). Group numbers appear in a different order than in chapter: 1 – Saline controls; 2 – IPC; 3 - AL soln; 4 - A1 agonist (CCPA) alone; 5 –A1 agonist (CCPA) plus lidocaine.

Grp. No.	Left ventricle weight (g)	Left ventricle risk zone (rz)(g)	Left ventricle necrotic zone (nz) (g)	Infarct Size (rz/nz)
1	0.67	0.60	0.35	0.58
1	0.84	0.44	0.29	0.65
1	0.81	0.80	0.36	0.45
1	0.81	0.52	0.34	0.65
1	0.93	0.77	0.56	0.73
2	0.76	0.28	0.14	0.50
2	0.78	0.69	0.22	0.32
2	0.89	0.18	0.06	0.33
2	0.83	0.59	0.06	0.11
2	0.88	0.62	0.32	0.53
2	0.81	0.62	0.28	0.46
2	0.81	0.37	0.16	0.43
3	0.87	0.65	0.27	0.41
3	0.88	0.69	0.30	0.44
3	0.68	0.66	0.17	0.25
3	0.76	0.43	0.29	0.67
3	0.74	0.46	0.16	0.34
4	0.76	0.51	0.01	0.03
4	0.75	0.57	0.11	0.19
4	0.84	0.76	0.13	0.17
4	0.76	0.91	0.06	0.07
4	0.68	0.41	0.04	0.11
5	0.69	0.84	0.01	0.02
5	0.87	0.64	0.03	0.05
5	1.10	0.63	0.09	0.13
5	1.66	0.59	0.03	0.04
5	0.78	0.56	0.14	0.25
5	0.78	0.71	0.16	0.23

Table D2. Numbers and durations of arrhythmias counted during ischemia (Chapter 6). Group numbers appear in a different order than in chapter: 1 – Saline controls; 2 – IPC; 3 - AL soln; 4 - A1 agonist (CCPA) alone; 5 –A1 agonist (CCPA) plus lidocaine.

Grp. No.	Number of			Duration (sec)		
	VT	VF	VT + VF	VT	VF	VT+VF
1	40	7	47	163.57	124.19	287.76
1	42	13	55	230.34	122.04	352.38
1	5	0	5	131.61	0	131.61
1	2	0	2	2.17	0	2.17
1	3	0	3	3.67	0	3.67
2	0	0	0	0	0	0
2	2	0	2	2.20	0	2.20
2	0	0	0	0	0	0
2	0	0	0	0	0	0
2	7	0	7	3.26	0	3.26
2	1	0	1	1.26	0	1.26
2	4	0	4	7.75	0	7.75
3	11	0	11	14.69	0	14.69
3	6	0	6	92.72	0	92.72
3	18	3	21	65.4	2.98	68.38
3	4	0	4	14.69	0	14.69
3	11	0	11	92.72	0	92.72
4	0	0	0	0	0	0
4	4	0	4	30.61	0	30.61
4	0	0	0	0	0	0
4	0	0	0	7	0	7
4	17	0	17	3	0	3
5	0	0	0	0	0	0
5	0	0	0	0	0	0
5	0	0	0	0	0	0
5	0	0	0	0	0	0
5	0	0	0	0	0	0
5	0	0	0	0	0	0

Table D3. Numbers and durations of arrhythmias counted during reperfusion (Chapter 6). Group numbers appear in a different order than in chapter: 1 – Saline controls; 2 – IPC; 3 - AL soln; 4 - A1 agonist (CCPA) alone; 5 –A1 agonist (CCPA) plus lidocaine.

	Number of			Duration (sec)		
Grp. No.	VT	VF	VT + VF	VT	VF	VT+VF
1	0	0	0	0	0	0
1	1	0	1	0	0	0
1	3	1	4	0.41	0	0.41
1	3	3	6	0	15.54	15.54
1	1	0	1	23.24	0	23.24
2	0	0	0	0	0	0
2	0	0	0	0	0	0
2	0	0	0	0	0	0
2	0	0	0	0	0	0
2	0	0	0	0	0	0
2	0	0	0	0	0	0
2	0	0	0	0	0	0
3	5	0	5	0	29.09	29.09
3	3	0	3	0	3.43	3.43
3	1	0	1	0	9.74	9.74
3	1	0	1	0	23.14	23.14
3	2	0	2	0	23.59	23.59
4	2	0	2	18.62	0	18.62
4	4	0	4	10.34	0	10.34
4	0	0	0	0	0	0
4	0	0	0	0	0	0
4	1	0	1	2.19	0	2.19
5	0	0	0	0	0	0
5	0	0	0	0	0	0
5	0	0	0	0	0	0
5	0	0	0	0	0	0
5	0	0	0	0	0	0
5	0	0	0	0	0	0

Table D4a. Changes in heart rate during ischemia and reperfusion (Chapter 6).
Group numbers appear in a different order than in chapter: 1 – Saline controls; 2 –
IPC; 3 - AL soln; 4 - A1 agonist (CCPA) alone; 5 –A1 agonist (CCPA) plus lidocaine.

Grp. No.	Baseline	Pretreament	Ischemia (min)			Reperfusion (min)			
			10	20	30	10	20	30	40
1	416	426	245	387	364	347	370	372	364
1	422	460	192	454	280	405	420	465	426
1	417	380	206	441	432	369	371	358	370
1	440	446	471	456	451	445	450	457	444
1	485	455	426	426	426	422	435	405	401
2	380	362	319	327	324	381	361	338	359
2	423	358	316	281	267	312	289	288	289
2	413	281	295	308	269	385	414	403	390
2	481	359	315	332	330	329	322	381	347
2	453	305	305	309	340	364	369	339	339
2	465	296	271	269	272	412	365	388	380
2	445	366	369	398	384	425	433	423	420
3	362	226	315	390	366	358	326	355	360
3	434	331	341	427	382	363	377	397	406
3	428	396	379	106	386	391	403	393	403
3	443	370	219	354	389	344	307	331	363
3	436	357	301	383	385	409	430	416	403
4	461	462	449	463	414	414	411	402	410
4	411	361	451	430	433	414	395	407	403
4	442	431	428	398	395	403	396	387	394
4	423	415	325	313	406	303	383	397	376
4	454	412	437	390	420	438	407	378	401
5	459	242	145	159	166	193	209	222	241
5	496	318	237	237	234	247	261	290	330
5	413	231	183	193	189	212	222	246	273
5	452	289	68	219	51	226	247	277	302
5	369	249	199	207	192	201	211	212	205
5	426	292	75	205	200	235	246	270	304

Table D4b. Changes in heart rate during ischemia and reperfusion (Chapter 6).
Group numbers appear in a different order than in chapter: 1 – Saline controls; 2 – IPC; 3 - AL soln; 4 - A1 agonist (CCPA) alone; 5 –A1 agonist (CCPA) plus lidocaine.

	Reperfusion (min)							
Grp. No.	50	60	70	80	90	100	110	120
1	368	364	353	361	.	.	.	.
1	437	411	382	374	396	379	334	318
1	363	356	404	348	377	392	384	343
1	462	426	389	423	432	432	422	451
1	408	427	404	429	442	450	447	410
2	365	354	357	361	354	350	375	410
2	292	297	291	287	289	287	349	363
2	402	392	398	387	381	383	384	381
2	328	331	315	310	298	378	344	370
2	375	387	348	361	379	365	342	394
2	377	410	416	424	418	415	456	449
2	426	426	435	405	441	434	387	395
3	332	332	369	353	334	334	344	339
3	409	403	405	396	397	400	411	401
3	380	404	410	388	286	45	329	366
3	305	309	309	315	332	338	340	345
3	378	396	392	379	400	402	416	384
4	447	426	380	383	350	375	382	368
4	406	400	394	404	398	383	378	404
4	393	400	397	388	387	380	374	379
4	326	316	317	397	332	358	317	358
4	390	427	438	409	396	398	406	385
5	255	264	287	301	304	322	327	332
5	366	385	407	412	392	393	416	400
5	283	293	301	309	316	277	278	275
5	319	347	376	379	378	390	385	376
5	228	240	246	266	283	296	326	341
5	331	318	329	345	357	346	337	356

Table D5a. Changes in systolic pressure during ischemia and reperfusion (Chapter 6). Group numbers appear in a different order than in chapter: 1 – Saline controls; 2 – IPC; 3 - AL soln; 4 - A1 agonist (CCPA) alone; 5 –A1 agonist (CCPA) plus lidocaine.

Grp. No.	Baseline	Pretreament	Ischemia (min)			Reperfusion (min)			
			10	20	30	10	20	30	40
1	155	162	24.5	92	87	89	89	79	88
1	146	162	89	149	110	91	113	145	105
1	144	119	105	122	101	77	86	77	93
1	118	134	154	57	97	104	117	139	114
1	132	107	95	112	125	83	102	79	80
2	140	111	94	98	95	133	84	169	122
2	153	81	67	65	67	119	90	73	94
2	117	59	77	102	72	131	140	123	114
2	195	80	104	110	101	100	94	151	105
2	175	71	78	88	115	119	131	92	92
2	190	58	48	48	55	117	63	98	88
2	140	67	114	111	97	154	145	127	125
3	141	79	86	81	109	98	88	103	101
3	134	96	97	108	78	82	94	99	104
3	139	124	113	92	125	106	107	94	133
3	167	140	99	99	102	73	75	90	104
3	148	129	128	144	151	156	157	146	133
4	150	165	150	169	90	95	90	89	101
4	155	54	139	70	112	79	87	125	81
4	190	108	107	104	91	150	89	125	125
4	189	165	85	83	166	67	144	158	144
4	130	88	92	84	121	84	95	74	101
5	148	93	46	58	54	66	69	74	77
5	127	47	47	57	53	70	82	90	123
5	124	87	57	74	74	87	89	104	120
5	127	88	58	71	57	81	87	82	91
5	97	64	55	58	55	59	58	57	53
5	160	83	48	62	61	76	74	85	92

Table D5b. Changes in systolic pressure during ischemia and reperfusion (Chapter 6). Group numbers appear in a different order than in chapter: 1 – Saline controls; 2 – IPC; 3 - AL soln; 4 - A1 agonist (CCPA) alone; 5 –A1 agonist (CCPA) plus lidocaine.

Grp. No.	Reperfusion (min)							
	50	60	70	80	90	100	110	120
1	93	83	62	72	.	.	.	.
1	147	91	94	87	113	127	64	74
1	87	82	116	62	88	94	84	77
1	140	74	78	77	89	104	107	123
1	88	105	74	109	106	104	108	71
2	114	92	117	116	106	133	141	148
2	101	105	103	88	92	89	157	144
2	141	127	122	110	96	97	100	97
2	94	88	80	75	68	104	80	104
2	137	142	99	105	112	101	80	120
2	88	77	137	110	106	72	117	108
2	134	136	138	136	117	104	85	113
3	95	94	93	94	92	90	94	86
3	104	84	98	91	88	80	93	87
3	93	122	114	98	90	83	84	94
3	88	92	87	92	105	107	92	98
3	123	132	130	118	138	126	133	105
4	135	121	92	112	89	125	117	94
4	90	89	81	92	97	91	92	83
4	100	110	119	105	119	119	101	107
4	96	88	90	172	70	137	71	118
4	88	148	145	105	99	107	110	89
5	80	85	84	92	106	112	115	123
5	134	136	135	125	83	108	110	72
5	125	132	136	140	141	107	117	120
5	110	126	130	129	129	130	117	102
5	63	56	55	60	69	83	97	89
5	83	72	79	89	91	78	78	79

Table D6a. Changes in diastolic pressure during ischemia and reperfusion (Chapter 6). Group numbers appear in a different order than in chapter: 1 – Saline controls; 2 – IPC; 3 - AL soln; 4 - A1 agonist (CCPA) alone; 5 –A1 agonist (CCPA) plus lidocaine.

Grp. No.	Baseline	Pretreament	Ischemia (min)			Reperfusion (min)			
			10	20	30	10	20	30	40
1	105	116	13	63	17	49	53	44	48
1	110	124	52	114	81	62	83	111	70
1	105	76	56	91	73	48	56	46	58
1	80	93	112	28	55	59	69	91	64
1	97	73	67	79	92	53	69	48	50
2	77	44	33	33	32	70	36	108	57
2	87	31	13	18	18	58	43	28	41
2	71	20	27	36	24	79	93	73	62
2	143	37	44	48	45	56	53	106	60
2	131	31	35	42	56	79	86	54	49
2	137	25	19	20	23	74	38	61	51
2	85	29	33	51	44	97	90	86	70
3	85	46	50	53	75	68	60	69	66
3	95	60	63	78	51	54	64	65	67
3	100	88	84	65	94	76	72	63	99
3	115	97	57	69	76	48	50	61	71
3	104	90	78	101	105	106	107	96	93
4	111	126	114	132	53	55	49	48	55
4	113	30	103	40	75	46	51	80	41
4	127	63	65	53	54	99	40	70	70
4	129	117	53	53	126	32	107	114	99
4	89	60	61	58	88	57	62	46	69
5	106	52	21	28	28	34	38	42	44
5	92	29	28	35	32	42	47	49	70
5	73	37	22	31	32	41	42	51	63
5	90	50	15	39	30	46	51	46	54
5	59	31	26	29	27	29	28	26	24
5	108	44	24	34	35	48	47	57	64

Table D6b. Changes in diastolic pressure during ischemia and reperfusion (Chapter 6). Group numbers appear in a different order than in chapter: 1 – Saline controls; 2 – IPC; 3 - AL soln; 4 - A1 agonist (CCPA) alone; 5 –A1 agonist (CCPA) plus lidocaine.

Grp. No.	Reperfusion (min)							
	50	60	70	80	90	100	110	120
1	47	39	22	17	.	.	.	.
1	109	53	52	50	79	86	34	43
1	52	48	74	36	55	59	52	45
1	93	33	33	35	43	56	57	68
1	55	70	47	73	69	68	70	43
2	51	38	54	51	46	67	74	81
2	43	48	44	32	36	38	84	84
2	85	65	68	60	47	47	45	42
2	54	50	44	41	38	61	44	61
2	88	100	57	63	72	62	45	81
2	51	43	99	73	69	40	73	65
2	77	76	57	80	71	52	49	74
3	62	61	59	60	60	58	59	53
3	65	52	66	60	57	49	61	56
3	63	85	84	69	62	56	56	65
3	55	59	52	56	65	68	58	64
3	77	81	78	67	88	88	84	59
4	88	71	49	64	49	78	71	52
4	45	44	38	46	51	47	48	40
4	45	50	57	48	59	61	48	51
4	52	48	51	122	37	93	39	74
4	48	108	104	61	60	69	69	55
5	47	49	47	53	59	60	60	65
5	78	79	78	71	66	63	65	41
5	64	67	71	75	78	49	58	61
5	69	79	80	78	80	82	69	60
5	31	29	28	30	35	41	46	39
5	53	44	49	55	55	44	46	46

Table D7a. Changes in mean arterial pressure during ischemia and reperfusion (Chapter 6). Group numbers appear in a different order than in chapter: 1 – Saline controls; 2 – IPC; 3 - AL soln; 4 - A1 agonist (CCPA) alone; 5 –A1 agonist (CCPA) plus lidocaine.

Grp. No.	Baseline	Pretreament	Ischemia (min)			Reperfusion (min)			
			10	20	30	10	20	30	40
1	121	131	16.8	73	40	63	65	55	61
1	122	137	64	126	91	72	93	122	81
1	118	91	72	102	83	57	66	57	70
1	92	107	126	38	69	74	85	107	81
1	109	84	76	90	103	63	80	59	60
2	98	66	54	55	53	91	52	128	79
2	109	48	31	34	34	78	59	43	58
2	86	33	44	58	40	97	109	90	79
2	160	51	64	69	64	71	67	121	75
2	146	44	49	57	76	92	101	67	63
2	155	36	29	29	34	88	46	73	64
2	104	41	60	71	62	116	108	100	88
3	104	57	62	62	87	78	69	80	78
3	108	72	74	88	61	63	74	76	79
3	113	100	93	74	104	86	83	73	111
3	132	111	71	79	85	57	58	71	82
3	118	103	95	115	120	123	124	113	106
4	124	139	126	144	65	68	63	62	71
4	127	38	115	50	87	57	63	95	54
4	148	78	79	70	67	116	56	88	88
4	149	133	64	63	139	43	119	129	114
4	103	69	71	67	99	66	73	55	80
5	152	39	36	39	48	89	65	71	64
5	121	40	50	57	52	107	87	91	80
5	90	54	33	46	46	57	58	69	82
5	102	63	29	50	39	58	63	58	66
5	72	42	36	38	37	39	38	36	34
5	126	57	32	44	44	57	56	67	73

Table D7b. Changes in mean arterial pressure during ischemia and reperfusion (Chapter 6). Group numbers appear in a different order than in chapter: 1 – Saline controls; 2 – IPC; 3 - AL soln; 4 - A1 agonist (CCPA) alone; 5 –A1 agonist (CCPA) plus lidocaine.

Grp. No.	Reperfusion (min)							
	50	60	70	80	90	100	110	120
1	62	54	36	36	.	.	.	.
1	121	66	66	62	90	100	44	53
1	64	59	88	45	66	71	63	56
1	109	47	48	49	58	72	74	87
1	66	81	56	85	81	80	83	53
2	72	56	75	73	66	89	96	103
2	62	67	64	50	54	55	108	104
2	104	86	86	76	63	63	64	60
2	68	62	56	52	48	75	56	75
2	104	114	71	77	85	75	56	94
2	63	55	112	85	81	51	88	80
2	96	96	84	99	86	69	61	87
3	73	72	70	71	70	69	71	64
3	78	63	77	70	68	59	72	67
3	73	98	94	79	71	65	66	74
3	66	70	64	68	78	81	69	75
3	92	98	95	84	105	100	101	74
4	104	88	63	80	62	94	86	66
4	60	59	53	62	66	62	63	54
4	63	70	78	67	79	80	66	69
4	67	61	64	138	48	108	50	89
4	61	122	118	76	73	81	83	66
5	77	74	98	83	82	59	77	84
5	85	82	93	94	84	63	70	84
5	84	89	93	97	99	69	77	80
5	83	95	97	95	97	98	85	74
5	42	38	37	40	46	55	63	55
5	63	53	59	66	67	56	57	57

Table D8a. Changes in rate pressure product during ischemia and reperfusion (Chapter 6).   Group numbers appear in a different order than in chapter: 1 – Saline controls; 2 – IPC; 3 - AL soln; 4 - A1 agonist (CCPA) alone; 5 –A1 agonist (CCPA) plus lidocaine.

Grp. No.	Baseline	Pretreament	Ischemia (min)			Reperfusion (min)			
			10	20	30	10	20	30	40
1	64375	68941	125	35582	31594	30932	32850	29287	32092
1	61522	74583	17015	67560	30642	36986	47620	67306	44704
1	59825	45341	21607	53819	43711	28274	31907	27630	34458
1	51810	59848	72314	25990	43899	46212	52468	63514	50342
1	64075	48539	40644	47889	53270	35020	44560	31928	32235
2	53187	40296	29881	31863	30752	50761	30430	57083	43601
2	64869	29104	21014	18234	17877	37077	26146	20888	27055
2	48414	16677	22666	31470	19330	50481	57998	49488	44418
2	93766	28831	32900	36688	33364	32797	30361	57323	36443
2	79322	21483	23766	27077	39165	43305	48220	31182	31222
2	88167	17241	13045	12855	14812	48205	22935	38071	33589
2	62203	24463	41946	44124	37215	65545	62583	53531	52373
3	50998	17755	27116	31588	39814	35247	28836	36469	36176
3	58002	31752	32965	46115	1148	29580	35353	39115	42213
3	59435	48844	42747	9719	48417	41488	43040	37098	53747
3	74003	51624	21617	34997	39776	25250	23140	29808	37571
3	64397	45920	38537	55248	58004	63726	67487	60834	53479
4	69042	76134	67320	78062	37219	39260	36944	35930	41564
4	63792	19617	62609	30042	48443	32665	34484	50772	32788
4	83847	46462	45785	41342	36057	60309	35213	48182	49028
4	79736	68566	27626	25821	67289	20230	55252	62671	53970
4	58864	36271	39958	32610	50995	36809	38593	28018	40431
5	67965	22446	6579	9293	9002	12745	14511	16347	18516
5	63129	14773	11125	13409	12438	17251	21497	26142	40463
5	51167	20097	10328	14370	13862	18521	19736	25528	32702
5	57263	25483	3964	15556	2887	18388	21473	22797	27512
5	35902	16042	10979	11914	10524	11945	12238	12003	10880
5	68107	24261	3589	12796	12140	17728	18101	23075	27892

Table D8b. Changes in rate pressure product during ischemia and reperfusion (Chapter 6). Group numbers appear in a different order than in chapter: 1 – Saline controls; 2 – IPC; 3 - AL soln; 4 - A1 agonist (CCPA) alone; 5 –A1 agonist (CCPA) plus lidocaine.

Grp. No.	Reperfusion (min)							
	50	60	70	80	90	100	110	120
1	34239	30222	21949	26014	.	.	.	.
1	64232	37317	35994	32415	44575	48293	21454	23362
1	31524	29161	46664	21643	33219	36808	32306	26236
1	64436	31412	30158	32573	38432	45116	45059	55614
1	35960	44830	29812	46915	46650	46960	48081	29180
2	41758	32700	41691	41713	37620	46583	52685	60505
2	29510	31296	30003	25247	26425	25446	54859	52388
2	56808	49568	48414	42560	36439	37052	38433	36985
2	30804	29198	25195	23130	20121	39329	27458	38360
2	51443	54940	34269	37862	42433	36929	27292	47216
2	33310	31690	56972	46754	44062	29831	53264	48535
2	56918	57912	59858	55143	51413	44906	32738	44784
3	31649	31274	34317	33191	30585	30002	32311	29043
3	42618	33699	39862	35850	35055	31824	38081	34803
3	35397	49474	46927	37898	25561	3700	27620	34184
3	26845	28490	26931	29134	34729	36020	31221	33869
3	46306	52259	50947	44774	55388	50454	55386	40355
4	60493	51601	34724	42826	31159	46775	44591	34500
4	36378	35516	31850	37258	38448	34900	34673	33507
4	39281	44044	47307	40592	45860	45310	37873	40433
4	31455	27894	28589	68194	23119	49090	22408	42209
4	34190	63222	63656	42792	39194	42384	44605	34392
5	20483	22406	24111	27734	32236	36194	37715	40752
5	49140	52439	54959	51566	32567	42344	45971	28800
5	35356	38477	40731	43285	44711	29737	32463	32886
5	35165	43744	48855	48713	48773	50778	44856	38533
5	14298	13489	13522	16072	19520	24379	31530	30254
5	27308	23030	25916	30576	32585	26895	26234	28116