ISCHEMIC PRECONDITIONING OF THE MYOCARDIUM: ROLE OF ANGIOTENSIN II RECEPTORS

by

Roberto Jose Diaz

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DEDICATION

This thesis is dedicated to

my wife Gilda and my sons Roberto and David

who have continuously supported me, who have encouraged
me to pursue knowledge for the well-being of others and
who made this thesis possible

Ischemic Preconditioning of The Myocardium: Role of Angiotensin II Receptors

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CONDENSED ABSTRACT

Adenosine A_1, α_1-adrenergic and bradykinin B_2 receptors mediate ischemic preconditioning (IP) of the myocardium at least in part through a receptor-mediated protein kinase C (PKC) mechanism. Since angiotensin II (AII), which is released during ischemia, can activate PKC, it is possible that AII receptors participate in IP. To test this hypothesis, the ability of AII receptor stimulation or blockade to protect or abolish IP respectively, was assessed in isolated rabbit hearts. IP reduced infarct size significantly. Brief stimulation of AII receptors using AII (100nM) before the ischemic insult mimic the IP protection. Both non-selective inhibition of AII receptors with saralasin (1µM) and selective inhibition of AT_1 receptors with losartan (20µM) completely abolished IP. However, the IP protection was not blocked by PD-123,319 ditrifluoroacetate (10µM), a selective inhibitor of AT_2 receptors. Thus, activation of AT_1 receptors must occur for ischemic preconditioning to limit infarction.

ACKNOWLEDGMENT

PREFACE

Although current patient survival after cardiac surgery is high, there remain many patients who die because of ineffective intraoperative protection of the myocardium. Combined use of hypothermia and cardioplegia, two well-known myocardial preservation methods, provides surgeons a limited amount of time, usually about 90 minutes, to perform the intra-cardiac repairs. However, this duration is sometimes not enough to perform complicated procedures. In these cases, the duration of induced cardiac ischemia must be prolonged, increasing the risk of severe myocardial injury. Since necrosis produced by a period of myocardial ischemia and reperfusion, as well as arrhythmias on reperfusion can be substantially improved with ischemic preconditioning, the study of the cellular mechanism(s) responsible for the expression of this protective phenomenon becomes potentially important. Understanding the mechanism(s) of this phenomenon may allow the development of drugs designed to induce a powerful myocardial protection similar to ischemic preconditioning. The experimental studies described herein are intended to contribute to the understanding of the mechanism(s) of ischemic preconditioning.

ABSTRACT

Ischemic preconditioning (IP) is a process by which one or more brief episodes of ischemia followed by reperfusion confers a state of myocardial protection against injury induced by a subsequently sustained episode of ischemia and reperfusion. It is known that during ischemia substances such as adenosine, norepinephrine and bradykinin are endogenously released in the heart. Furthermore, cardiac adenosine A_1, α_1-adrenergic and bradykinin B_2 receptors are known to mediate IP at least in part through a receptor-mediated protein kinase C (PKC) activation mechanism. Because angiotensin II (AII) is also released during ischemia, and PKC can be activated through an AII receptor-mediated mechanism, it is possible to hypothesize that cardiac AII receptors participate in ischemic preconditioning.

To test this hypothesis, rabbit hearts were studied in an isolated buffer-perfused *in vitro* preparation. Initially, all hearts were subjected to 30 min of normoxic perfusion (stabilization period). Then, each heart was assigned to one of the heart groups and received an identical ischemic injury protocol consisting of 40 min of regional ischemia (at 37° C) followed by 60 min of reperfusion. Control hearts (C) received an additional 45 min of normoxic perfusion prior to the long ischemia to compensate for the duration of the IP cycles. IP was induced by subjecting each heart to three cycles of 5 min ischemia and 10 min reperfusion given prior to the 40 min ischemic period. In another group of hearts, activation of angiotensin II (AII) receptors was

accomplished by infusing AII (AngII, 100 nM) for five minutes followed by a drug-free period of normoxic perfusion before the long ischemia. Different control and/or IP hearts were treated with a constant infusion of i) [Sar^1,Val^5,Ala^8]-AII (saralasin, SAR, 1 µM), a nonselective AII receptor antagonist (C+SAR and IP+SAR, respectively), ii) losartan (LST, 20 µM), a selective AT_1 AII receptor antagonist (C+LST and IP+LST, respectively), or iii) PD-123,319 ditrifluoroacetate (PD, 10µM), a selective AT_2 AII receptor antagonist (IP+PD). Infarct size and left ventricular developed pressure were assessed. IP reduced infarct size [IP $5.2 \pm 1.2\%$ (mean $\pm$ S.E.M.), versus C $26.4 \pm 3.0\%$, $P < 0.001$], but it did not protect against post-ischemic ventricular dysfunction. Activation of AII receptors induced protection against infarction similar to IP (AngII $9.6 \pm 2.2\%$, $P < 0.01$ v control). Inhibition of AII receptors with saralasin blocked the protection of IP against necrosis (IP+SAR 29.7 $\pm 3.2\%$ versus IP, $P < 0.001$) while it did not increase infarct size in saralasin-treated control hearts (C+SAR 31.5 $\pm 3.9\%$ versus control, $P > 0.05$). Furthermore, inhibition of the AII AT_1 receptor subtype with losartan abolished the infarct size limiting effect of IP (IP+LST $27.4 \pm 3.0\%$ versus C+LST $29.3 \pm 2.0\%$, $P > 0.05$). Inhibition of the AII AT_2 receptor subtype with DP-123,319 ditrifluoroacetate did not have any effect on the protection induced by IP (IP+DP 7.9 ± 1.2 %, $P > 0.05$ versus IP group).

To further confim the *in vitro* results, we assessed the role of the AT_1 AII receptor in ischemic preconditioning in an *in situ* heart model. Anaesthetized adult rabbit hearts were initially subjected to 15 min of equilibration. Then, each heart was assigned

to one of four groups and subjected to an ischemic injury protocol consisting of 30 min of ischemia followed by 90 min of reperfusion. Control hearts (C) received an additional 15 min of stabilization to equalize the protocol time length with the IP heart protocol. IP hearts were subjected to 5 min of regional ischemia and 15 min of reperfusion prior to the long ischemia/reperfusion episode. In another two groups of C and IP hearts, AII receptors were inhibited with losartan (6 mg/kg bolus dose plus 3 mg/kg maintenance dose given for 25 min). Infarct size and mean arterial pressure (MAP) were assessed. IP reduced infarct size significantly (IP $13.1 \pm 2.7\%$ versus C $47.1 \pm 2.6\%$, $P<0.001$). Inhibition of the AT_1 AII receptor subtype with losartan completely blocked the IP protection against infarction (IP+LST $40.8 \pm 4.3\%$ versus C+LST $34.3 \pm 3.9\%$, $P > 0.05$). Even though sustained and significant decline in MAP was observed in rabbits with an intravenous infusion of losartan, it did not affect infarct size as evidenced by the absence of a relationship between MAP and infarct size in both control ($r=0.02$) and IP hearts ($r=0.35$). Therefore, the AT_1 but not the AT_2 AII receptor subtype participates in ischemic preconditioning. Activation of the AT_1 receptor subtype must occur before the prolonged ischemia for ischemic preconditioning to limit infarction.

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GLOSSARY

AC	Adenylyl cyclase
ACE	Angiotensin converting enzyme
Adenosine A_1	Adenosine receptor subtype A_1
α_1-adrenergic	Adrenergic receptor subtype alpha-1
AII	Angiotensin II
AngII	Angiotensin II-treated group
AI	Angiotensin I
ATP	Adenosine triphosphate
AT_1	Angiotensin II receptor subtype-1
AT_2	Angiotensin II receptor subtype-2
AV	Atrium-ventricular
Bradykinin B_2	Bradykinin receptor subtype B_2
C	Control heart group
$CaCl_2$	Calcium chloride
cAMP	Cyclic adenosine monophosphate
Cp	Paced control heart group
DAG	Diacylglycerol
DP	DP-123,319 ditrifluoroacetate
ET-1	Endothelin-1
HCO_3	Bicarbonate
I_{ca}	Calcium channel current
IP	Ischemic preconditioning
IPp	Paced preconditioned heart group
IP_3	Inositol triphosphate
KCl	Potassium chloride

KH_2PO_4	Potassium phosphate
LST	Losartan
LVDP	Left ventricular developed pressure
m	Milli
MAP	Mean arterial pressure
$MgSO_4$	Magnasium sulphate
min	Minutes
n	number of hearts
NaCl	Sodium chloride
Na^+/H^+ exchanger	Membrane channel NA^+/H^+ exchanger
$NaHCO_3$	Sodium bicarbonate
P	P value
P_aO_2	Arterial blood oxygen pressure
P_aCO_2	Anterial blood carbon dioxide pressure
PIP_2	Phosphoisonitol diphosphate
PKC	Protein kinase C
PLC	Phospholipase C
PLD	Phospholipase D
PLA_2	Phospholipase A_2
r	regression coefficient
SAR	Saralasin
SEM	Standard error of the mean

I. INTRODUCTION

In this introduction, an overview of ischemic/reperfusion injury, an analysis of the ischemic preconditioning phenomenon and a review of the cardiac renin-angiotensin system are provided to establish the formulation of the working hypothesis for the studies described herein.

1. ISCHEMIC/REPERFUSION INJURY

Changes in cardiac function [1], metabolism [2] and cellular structure [3] develop when coronary blood flow is greatly reduced (low flow ischemia) or absent (total ischemia). These changes are primarily the result of the lack of oxygen and substrate and can be reversible or irreversible upon the re-establishment of blood flow, depending on how long the myocardium is subjected to ischemia. Ischemic injury is defined as being reversible if restoration of arterial flow after an episode of occlusion is followed by restoration of function. In fact, cells obtained from papillary muscles and subjected to 15 min of ischemia are reversibly injured and after two minutes of reflow are indistinguishable by structural and chemical techniques [4, 5] from nonischemic left ventricular myocardium of the same animal [3]. On the other hand, irreversibly injured cells show significant changes when arterial flow is restored to them. Whalen et al [6] have shown that two minutes of arterial reperfusion after 40 min of ischemia result in marked swelling, almost explosive in character, in those cells which on ultrastructural analysis contain amorphous mitochondrial matrix

densities. Although the transition point from reversible to irreversible injury has not been defined, there are some ultrastructural findings that closely identify each of the two phases of myocardial ischemic injury. Some early ultrastructural changes that occur during ischemia are diminished cellular glycogen, relaxed myofibrils and isolated mitochondrial swelling. These changes are not detectable until about 10 to 15 min of myocardial ischemia. If the ischemic time is substantially prolonged the following changes then appear: 1) complete absence of cellular glycogen; 2) cell swelling with an increased sarcoplasmic space; 3) swollen mitochondria with an enlarged matrix and/or broken cristae; 4) amorphous densities in the matrix space of each mitochondrial profile [3]. In addition to the ischemia-induced changes in cell structure, reflow of previously ischemic cells may also contribute to the initial cell injury induced by myocardial ischemia, as evidenced by the appearance of significant ultrastructural cell changes such as explosive disruption of the sarcolemma upon reperfusion [7], massive cell swelling, myofibrillar contraction bands and deposition of calcium phosphate in the mitochondria in response to the sudden and massive intracellular calcium influx which the ischemically injured cells are not able to regulate [8-11]. All these ischemic and reperfusion-induced changes in cardiomyocyte ultrastructure have a negative effect on cell function and viability.

In addition to the structural changes, some metabolic changes are also induced by myocardial ischemia. The absence of oxygen supply to the myocardium induces a quick redistribution of

the intracellular energy sources [12]. The pathways of cardiac adenosine triphosphate (ATP) production changes in order to maintain its metabolic activities. Cardiomyocytes, which normally obtain the ATP necessary to satisfy their metabolic demand from aerobic metabolism, rapidly transform to anaerobic metabolism during anoxia. This transition involves inhibition of the citrate cycle, which in its active state produces 38 molecules of ATP per molecule of glucose, and activation of the glycolytic pathway, which generates only 2 ATP molecules per molecule of glucose or 3 molecules of ATP per molecule of glycogen [13]. Thus, ischemia produces a significant depletion of high energy phosphates as a result of an imbalance between heart tissue supply and demand for oxygen and substrates [2]. Because anaerobic metabolism is not as energy-efficient as aerobic metabolism, the myocardium has limited potential for survival during a long ischemic period. In addition to a limited production of ATP, intermediates of glycolysis (e.g. glucose-6-phosphate) and lactic acid accumulate in the myocardium [7] and may contribute to accelerate myocardial cell injury during sustained ischemia.

Finally, a number of cytotoxic oxygen free radicals (e.g. superoxide, hydrogen peroxide, hydroxy radicals), which are generated during ischemia and upon reperfusion, may also result in tissue injury as reviewed by Herman [14], Burton [15], Cohen [16], and Reimer [17]. Damage of myocardial tissue using exogenous free radical generators has been shown to mimic ischemic/reperfusion injury functionally and metabolically [18], as well as ultrastructurally [19].

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2.   Ischemic Preconditioning of the Myocardium

2.1   Definition, Characteristics and Species Differences.

Ischemic preconditioning (IP) of the myocardium can be defined as a protective phenomenon, induced by one or more brief episodes of global or regional ischemia and reperfusion, against ischemic/ reperfusion injury produced by a more prolonged episode of ischemia and reperfusion. This phenomenon, described first by Murry et al [20] provides a remarkable protection against myocardial infarction in many different species such as dogs [20-23], pigs [24, 25], rats [26-28] and rabbits [8, 29-42]. Ischemic preconditioning has been shown to protect against post-ischemic ventricular dysfunction in rat hearts [11, 43-46], however, it has not been demonstrated in rabbit hearts [29, 32, 47]. Furthermore, ischemic preconditioning appears to reduce the incidence of reperfusion ventricular arrhythmias in rat hearts [48-50] and dog hearts [51]. However, it did not limit the incidence of ventricular fibrillation in pig hearts [52]

Two modes of ischemic preconditioning have been described. Ischemic preconditioning produces an immediate protection (classical preconditioning) against infarction for a limited duration after the episodes of transient ischemia. Rabbit and rat hearts can remain preconditioned for up to one hour [41, 53], while dog hearts can be protected for a somewhat longer period of time [54]. In general, hearts are no longer protected by classical ischemic preconditioning beyond two hours from the initial

ischemic stimuli [41, 53, 54]. However, the protection against necrosis or reperfusion arrhythmias can be reinstated, by subjecting the heart to additional brief ischemia/reperfusion episodes, after protection can be expected to have disappeared (usually after one or two hours) [41]. Furthermore, the duration of the brief ischemic episode and the number of short ischemia/reperfusion periods required to trigger the IP protection (ischemic preconditioning threshold) against ischemic injury differ among species. Downey et al [33] found that two episodes of two minutes of ischemia did not trigger the protection in rabbits. However, Sandhu et al [55] have shown that a single 5 min of regional myocardial ischemia and 10 min of reperfusion produced a substantial protection against infarction in an in situ heart model [56]. Similarly, Yellon et al [28] found that a single five-minute cycle of transient ischemia was enough to protect the heart against infarction in rats. While rabbit and rat hearts require only one brief ischemia/reperfusion cycle to induce the IP protection against infarction [26, 56], dog and pig hearts appear to require 2 or more cycles of IP to induce the protection against infarction [23, 57].

Another mode of protection against ischemic/reperfusion injury which is similar to the protection obtained with classic IP has been observed in rabbit [58] and dog hearts [59] after 24 hours of the initial IP episode, without subjecting the heart to a new IP episode. This delayed protection, named 'second window of protection' or 'late phase of ischemic preconditioning', is considered a recapture of the initial IP protection. The mechanism(s) of the second window of protection is still unknown.

However, recent evidence suggest that an adenosine-triggered mechanism [60], and that an α_1-adrenergic mechanism involving protein synthesis may be involved [61]. The studies described herein were designed to investigate the classical ischemic preconditioning mechanism(s). Therefore, the late phase of ischemic preconditioning previously described is not within the scope of the present studies.

Controversy remains over whether or not the ischemic preconditioning phenomenon can be elicited in human hearts. However, an attempt to determine the ability of ischemic preconditioning to protect the human heart has been made by Yellon et al [62]. They examined human myocardial biopsy specimens obtained at the time of coronary artery bypass surgery. Fourteen patients were randomized into two groups. One group underwent a specific preconditioning protocol consisting of two 3-min episodes of aortic cross-clamping with 2 min of reperfusion between each episode; this was followed by a sustained period of ischemia consisting of 10 min of aortic cross-clamping while the heart was electrically fibrillated and distal aorto-coronary anastomoses were performed. The other group, a control group, consisted of patients who did not undergo intermittent aortic cross-clamping before the 10-min of ischemia. Yellon et al [62] found that preconditioned hearts had lower levels of ATP at the end of the intermittent cross-clamping and reperfusion than control hearts. However, ATP levels after prolonged ischemia were higher in preconditioned hearts than controls (12.0 μmol/g dry weight versus 6.8 μmol/g dry weight, respectively). Based on this first

piece of evidence (ATP levels in IP hearts during ischemia), Kloner and Yellon [63] suggested that a protective response against ventricular dysfunction may occur in humans. Although no other study has been designed and implemented in situ in human hearts to confirm this assumption, there are at least four published studies which support the concept that preconditioning may occur in humans: 1) angioplasty studies that show less ST segment shift and lactate production in subsequent balloon inflations, compared with the first coronary artery balloon inflation [64-67]; 2) clinical studies suggesting that angina prior to myocardial infarction confers acute beneficial effects [68-70]; 3) studies showing that acute tolerance to angina can develop [71, 72]; and 4) studies that have directly examined human tissue specimens taken from patients undergoing cardiac surgery in which biochemical and functional properties of ischemic preconditioning have been found [62, 73-76].

More recent studies performed in isolated rabbit [10] and rat cardiomyocytes [77] subjected to transient anoxic periods, as distinct from transient ischemic episodes, have revealed an ischemic preconditioning-like protective phenomenon as measured by the proportion of dead cells to living cells after a subsequent prolonged anoxic insult. In these experiments, isolated cardiomyocytes were pelleted, most of the supernatant was discarded to simulate loss of flow as in true ischemia, and a layer of mineral oil was placed on top of the cell to prevent oxygen exchange, making the cells anoxic. Although this model is not a real ischemic model, it does have the fundamental elements of myocardial ischemia, i.e. absence of flow, limited substrate availability and lactate accumulation. An

identical protective response to the animal cardiomyocytes has also been demonstrated in human cardiomyocytes [75]. These experiments suggest that isolated cardiomyocytes are capable of developing the same protective phenomenon. However, because this phenomenon appears to be multifactorial in its genesis, it is likely that other cells in addition to cardiomyocytes participate in the development of ischemic preconditioning in vivo and in vitro. The participation of bradykinin, produced mainly by mast cells, in ischemic preconditioning, provides some support for this idea [34, 46, 78].

2.2 Protection Against Ischemic/Reperfusion Injury by Ischemic Preconditioning (IP):

2.2.1 Protection Against Infarction:

Until 1986, it was accepted that sequential ischemic episodes in the heart would result in cumulative ischemic injury. This premise was supported by the notion of energy stores (ATP) in the heart. During ischemia, ATP levels were severely reduced over a few minutes. This decline gave rise to the assumption that any additional ischemic insult would induce a further decline in ATP stores in the hearts, and thus, the ischemic injury would become worse. This assumption prompted Geft et al [79] to assess this hypothesis in experimental animals. They studied the effect of cumulative ischemia in dog hearts. Three groups of dogs were subjected to 14-18 subsequent cycles of 5, 10 or 15 min of myocardial regional ischemia, respectively. Each ischemic episode was

followed by a 15-min reperfusion period. At the end of the ischemia/reperfusion cycles each heart was evaluated for necrosis using the tetrazolium staining technique. Although a few hearts developed some necrosis in all groups of dogs, the majority of them showed no necrosis at all even after 200 minutes of cumulative ischemia. The study by Geft et al [79] concluded that injury induced by short cycles of ischemia and reperfusion was not cumulative and suggested that intermittent reperfusion has a beneficial effect and may prevent necrosis. However, they could not explain why multiple, brief ischemic episodes interspersed whit equally brief reperfusion periods were not producing cumulative ischemic injury. It was not until Murry et al [20] performed an infarct size study in dogs, and obtained a reasonable explanation to this observation. Murry et al [20] subjected dogs to five transient ischemic episodes followed by an equal period of reperfusion before subjecting the same region at risk of infarction to either 40 min or 3 hours of regional ischemia followed by 2 hours of reperfusion. At the end of reperfusion the amount of necrosis developed as a result of ischemic/reperfusion injury was measured. They found that five cycles of transient ischemia and reperfusion, when given before a prolonged ischemia/reperfusion period in the 40 min of ischemia protocol, protected the hearts against the infarction observed in control hearts. They called this protective phenomenon "ischemic preconditioning".

2.2.2 Protection Against Ventricular Dysfunction.

A well-known deleterious effect of ischemia is the development of ventricular dysfunction, defined as the reduction in the cardiac performance as determined by a reduction in left ventricular developed pressure (LVDP = systolic pressure minus diastolic pressure), the pressure difference between systolic pressure and diastolic pressure. Short periods of ischemia lasting 5-10 min usually cause a moderate decrease in the LVDP, primarily attributed to a decrease in the left ventricular systolic pressure (LVSP). These short ischemic time periods have been used in many studies to induce the classic ischemic preconditioning phenomenon. Initially, Schott and Schaper [80] thought that stunning, a temporary reduction in ventricular function after a period of ischemia which persists for several hours, could be responsible for triggering the ischemic preconditioning response, thus suggesting that stunning and preconditioning were the same phenomenon seen from two different points of view. This assumption was based on the fact that stunning was being induced with the brief periods of ischemia and reperfusion used to precondition. Thus, the ischemic tolerance might be explained by reduced regional demand. However, Schott et al [24] observed only a modest reduction in myocardial oxygen consumption despite a nearly 5-fold decrease in infarct size. From these findings they concluded that the significant reduction observed in infarct size with ischemic preconditioning could not be explained simply by energy savings. Similar results were found by Lange et al [81] who subjected a group of in vivo dog hearts to three 5-min period of

ischemia, each ischemic period followed by 30 min of reperfusion, and found that a coronary occlusion as brief as 5 or 15 min leads to depression of endocardial contractile function in the reperfusion zone, but that these alterations are not cumulative after three repetitive coronary artery occlusions. In a more recent study, Matsuda et al [82] tested the ability of ischemic preconditioning to induce protection in the absence of stunning. In this study, a group of animals were subjected to short cycles of ischemia such that the ischemic episodes did not induce stunning and at the same time were long enough to reach the threshold to induce the ischemic preconditioning response. They were able to protect the hearts without inducing stunning [82]. In addition, Mitchell et al [45] also reported that cardiac ischemic preconditioning does not require myocardial stunning to induce protection. Such evidence indicates that the mechnaimis(s) of ischemic preconditioning and stunning are diffferent.

The aspect of the ischemic preconditioning phenomenon that attracts the most interest from cardiac surgeons is its ability to protect the heart against post-ischemic contractile dysfunction. In fact, several cycles of transient ischemia followed by reperfusion before the long ischemia/reperfusion episode protected isolated rat hearts from developing post-ischemic contractile dysfunction upon reperfusion [11, 43, 83]. Protection against post-ischemic ventricular dysfunction by preconditioning has also been found in rabbit hearts [47]. Furthermore, it is not yet known if ischemic preconditioning protects against post-ischemic contractile dysfunction in human hearts.

2.2.3 Protection Against Reperfusion Arrhythmias:

Another interesting effect induced by ischemic preconditioning is that it protects against reperfusion based ventricular arrhythmias. This hallmark of ischemic preconditioning has been included in the definition of the phenomenon. The protection against cardiac arrhythmias was first observed by Hagar et al [48]. They studied the effect of ischemic preconditioning on the development of reperfusion arrhythmias (ventricular fibrillation, ventricular tachycardia, ventricular extrasystole etc.) in dogs. They found that ischemic preconditioning reduced significantly the incidence of ventricular extra-systole, ventricular tachycardia, ventricular fibrillation and AV block. Although this effect of ischemic preconditioning has not been studied extensively, many different researchers have observed the same protective effect of ischemic preconditioning against reperfusion arrhythmias [49-51, 53, 84, 85]. Currently, the mechanism(s) responsible for the protection against reperfusion arrhythmias remains unknown. Recently, Seyfarth et al [57] suggested that a reduction in the amount of norepinephrine release during sustained ischemia, which they have found to be produced by transient ischemic episodes before the sustained ischemia, may explain the protection of ischemic preconditioning against reperfusion arrhythmias. This hypothesis is supported by the fact that ischemia-induced norepinephrine release is closely related to the incidence of ischemia-induced arrhythmias in rat hearts, since

pharmacological suppression of norepinephrine release abolished the incidence of ventricular arrhythmias during ischemia [86].

2.2.4 Effects on Cardiac Metabolism:

Many studies have analyzed the effects of ischemic preconditioning on myocardial glycolysis, glycogen stores and lactate accumulation [87, 88]. However, the exact mechanisms whereby ischemic preconditioning affects cardiac metabolic pathways are unknown.

During total myocardial ischemia an energy-efficient mechanism (aerobic metabolism) is almost completely inhibited and an alternate mechanism (anaerobic metabolism) which is less energy-efficient is activated. As a result of the activation of this anaerobic metabolism, glycolysis, a series of changes happen to the cardiomyocyte membranes, glycogen stores, ATP stores, and lactate production. The first glycolytic mechanism to be activated during ischemia is the non-ATP dependent transport of glucose inside the cells. Increased glycolytic influx is believed to come from a direct effect of the translocation of glucose transporters during ischemia (Glut-4), which are dependent on insulin and adenosine, from the cytosol to the membrane [89, 90]. Subendocardial ATP levels (μmol/g dry weight) are reduced by 60% at 10 min of normothermic ischemia (from 24.6±0.3 μmol/g dry weight to 9.1±0.8), and ATP levels are decreased even further to 6.9±0.7 μmol/g dry weight at 20 min of ischemia [91]. Because lactate is the final product of glycolysis, it accumulates rapidly from a baseline value of 12.1±0.7 μmol/g dry weight to 101.0±3.4 μmol/g dry weight at 10 min of

ischemia [91]. It has been demonstrated that ischemic preconditioning reduces tissue levels of ATP modestly before prolonged ischemia [92], and that it also significantly reduces the rate of ATP decline, at least in the first 10 min, from a baseline of 17.5 ± 0.5 μmol/g dry weight to 12.4 ± 0.5 μmol/g dry weight (about a 30 % reduction) at 10 min ischemia [91]. However, ATP levels were not different after 20 min of ischemia [91]. These data suggest that by reducing the rate of ATP decline in heart tissue the onset of irreversible injury during ischemia may be delayed. In addition, accumulation of glucose-1-phosphate, glucose-6-phosphate, and lactate have also been found to be significantly reduced by preconditioning, due to the reduced rate of glycogen breakdown and anaerobic glycolysis [91].

2.2.5   Effects on Intracellular pH:

Intracellular pH $[pH]_i$ describes concentration of hydrogen ions (H^+) inside the cell. During ischemia, changes in $[pH]_i$ depend on the cardiomyocyte's buffer capabilities and its ability to maintain an active extrusion of H^+. Since both H^+ and lactate are produced and accumulated during ischemia it is expected that $[pH]_i$ should fall quickly. Indeed, $[pH]_i$ falls rapidly in normal hearts subjected to a long period of ischemia [1]. Ischemic preconditioning induced by short cycles of ischemia and reperfusion has been shown to preserve intracellular pH as demonstrated by a slow rate of decline in intracellular pH in preconditioned hearts as compared to hearts only subjected to sustained ischemia [93, 94].

2.2.6   Differences in the Production of Ischemic Preconditioning Protection:

Recently, Lawson and Hearse [84] have shown that the protection of IP against arrhythmias shows a definite dose dependency, with a cumulative increase in protection against arrhythmias when the number of brief ischemia and reperfusion cycles used to produce IP is increased from one to three. In this context, dose is considered to be the number of IP cycles of fixed duration. Conceivably, the number and duration of transient ischemic episodes and the duration of reperfusion between them could all influence the amount of protection, but these separate variables have not been examined in detail. Sandhu et al [56] have shown that the ability of IP to suppress cAMP levels and norepinephrine released during sustained ischemia also shows a similar dose dependency. Based on those findings, Sandhu et al [95] hypothesized that differences between one-cycle IP and three-cycle IP in the susceptibility to blockade and/or in the ability to protect the heart against infarct may exist in rabbit hearts. These observations have been further investigated by Sandhu et al [55]. In that study, the ability of one-cycle IP versus three-cycle IP to protect the heart against infarction and the susceptibility of one-cycle IP versus three-cycle IP to blockade by a protein kinase C (PKC) inhibitor or an adenylyl cyclase (AC) activator were also compared to test the hipothesis of Sandhu et al [95]. Although the PKC inhibitors polymyxin B and chelerythrine, and also the AC

activator NKH477, partly blocked the effect of one-cycle IP against infarction in vivo, they were not able to block the protection induced by three-cycle IP. In addition, the protection against infarction induced by three-cycle IP was significantly greater than the protection induced by one-cycle IP. Thus, the ability of IP to protect the heart against infarction and the susceptibility of IP to blockade were both clearly dose-dependent. Three-cycle IP appeared to be a more powerful phenomenon than one-cycle IP as demonstrated by a significant protection against infarction and a lesser susceptibility to blockade by PKC inhibitors or AC activators.

2.3   Proposed Ischemic Preconditioning Mechanism(s):

It is well known that activation of cardiac receptors(adenosine, adrenergic, bradykinin, angiotensin II receptors) leads to downstream activation of a cascade of events which may involve selective activation of some specific enzymes (e.g. PLC, PLD, AC). These enzymes, in turn, break down specific substrates(e.g. phosphoinositol diphosphate, phosphatidylcholine, adenosine triphosphate) into different substances. These end products, called second messengers (e.g. diacylglycerol, inositol triphosphate, phosphatidic acid, cAMP, PKC, PKA) carry the different signals further down the cascade. The end result may be activation of specialized proteins (e.g. ion channels, sarcoplasmic reticulum channels or receptors). A more detailed description of these pathways is given later in this section when each pathway's role in ischemic preconditioning is disclosed.

Although the exact mechanism(s) of ischemic preconditioning is/are still unknown, there are some evolving hypotheses currently under investigation.

2.3.1 The Adenosine Hypothesis:

Acute ischemia triggers the release of several endogenous substances in the heart. Adenosine [96, 97], norepinephrine [98, 99] , bradykinin [100] and angiotensin II (AII) [100-102] are all released in the heart during ischemia. Furthermore, adenosine is released during ischemia [96] in relatively sufficient quantity (μM) to exert a physiologic effect.

Downey et al [80] have proposed that adenosine release may be the trigger event that mediates the ischemic preconditioning protective effect through the activation of adenosine A_1-receptors which are coupled to pertussis toxin-sensitive G protein (G_i). This inhibitory G_i protein then transmits the signal to induce protection. This hypothesis is supported by the following findings: 1) exogenous administration of adenosine can mimic the protective effect of ischemic preconditioning against necrosis in isolated rabbit hearts [103]; 2) Selective activation of adenosine A_1 receptors with exogenous administration of the agonist R(-)-phenyl-N[6]-isopropyl-adenosine (R-PIA) [103] or 2-chloro-N[6]-cyclopentyladenosine (CCPA) [7, 40] can also limit infarct size when given 10 min prior to a long ischemia. However, CCPA was not as protective in isolated rabbit cardiomyocytes [10]; 3) Selective inhibition of adenosine A_1 receptors with 8-(p-Sulfophenyl)theophylline in situ in rabbit hearts [103] and rabbit

cardiomyocytes [9] can abolish the protective effect of ischemic preconditioning; 4) Inhibition of the G_i protein with pertussis toxin blocks the protective effect of ischemic preconditioning [104]. Other investigators have produced protection with the selective activation of adenosine A_1 receptors with agonists using an isolated rabbit heart model but have not reduced necrosis with the administration of adenosine in rabbits[8]. Transient exposure to adenosine prior to a long anoxic period has also been shown to protect human cardiomyocytes against cell death [76]. In the rat heart, the protective effect of ischemic preconditioning both against necrosis [26, 105] and ventricular dysfunction [11] does not seem to be mediated through the activation of the adenosine A_1 receptor. This suggests that there are some species differences in terms of the trigger for ischemic preconditioning.

Adenosine, an intracellular nucleotide, was first thought to be the only substance directly responsible for mediating the ischemic preconditioning phenomenon. Adenosine may indeed participate in concert with other endogenous substances released or produced in the heart during ischemia to trigger the protective response in vivo. Thus, it is possible that the cardiac receptors for all these endogenous substances are also stimulated during ischemia and/or reperfusion. There is some evidence that suggests that α_1–adrenergic [43, 106, 107] and bradykinin B_2 [34, 46, 100] receptors participate in ischemic preconditioning. Exogenous administration of norepinephrine to rats can protect their hearts to the same extent as ischemic preconditioning [43]. In rabbit hearts, exogenous administration of bradykinin prior to ischemia [34]

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produces a similar protective effect against necrosis as exogenous administration of adenosine [103]. Although all these experiments were performed in different animal models or preparations, the results support the participation of these endogenous substances (adenosine, norepinephrine and bradykinin) in ischemic preconditioning. Although transient exposure to AII has been found to be cardioprotective in the isolated rabbit heart [108], which may suggest that activation of AII receptors might play a role in ischemic preconditioning, it is still not yet clear if AII plays a role in triggering the protective phenomenon.

2.3.2 The Norepinephrine hypothesis:

The norepineprine hypothesis, first proposed by Banerjee et al [43], is based on the fact that norepinephrine is released from intracardial nerve terminals during ischemia [98, 99]. Norepinephrine activates α_1-adrenergic receptors, which in turn activate the phospholipase C/diacylglycerol/protein kinase C (PLC/DAG/PKC) pathway. PKC purportedly stimulates the phosphorylation of protective protein(s), although currently no protective protein has been definitively identified. The norepinephrine hypothesis is supported by the following observations: 1) Exogenous administration of norepinephrine [43] or phenylephrine [106, 109], a selective α_1-adrenergic agonist, can mimic the protective effect of ischemic preconditioning against postischemic ventricular dysfunction in rats and against necrosis in rabbits.   2)   Induction of the release of endogenous catecholamines   mimics   ischemic   preconditioning   [107]   3)

Pretreatment with reserpine, a drug which depletes the nerve terminals of norepinephrine, prevents the induction of ischemic preconditioning [43]. 4) Blockade of α_1-adrenergic receptors with specific blockers (phentolamine, and BE-2254) abolishes the protection of ischemic preconditioning against ventricular dysfunction in rats [43]. These data suggest that activation of the α_1-adrenergic receptor during ischemia mediates the ischemic preconditioning protective effect, at least in rats.

The norepinephrine hypothesis has recently been challenged by Seyfarth et al [57], who showed that transient ischemic episodes did not induce release of norepinephrine in isolated rat hearts. The release of norepinephrine during the brief cycles of ischemia has been suggested as the stimulus responsible of the activation of α_1-adrenergic receptors and the consequent activation of PKC to induce protection in Banerjee's norepinephrine hypothesis. Furthermore, it has been shown that transient ischemic episodes reduce the release of norepinephrine during sustained ischemia. It is important to note that neither the administration of adenosine (100 μmol/L) nor of phenylephrine (10 μmol/L) as preconditioning stimuli reduce ischemia-induced norepinephrine release. In addition, blockade of adenosine A_1 receptors with 8-(p-Sulfophenyl)theophylline (10 μmol/L) and or blockade of α_1-adrenergic receptors with prazosin (1 μmol/L) do not abolish the effect of transient ischemia on ischemia-induced norepinephrine release [57]. Thus, more research is required to clarify the role of norepinephrine in ischemic preconditioning.

2.3.3 The PKC Translocation/Activation Hypothesis:

Selective cardiac stimulation of either adenosine A_1, α_1-adrenergic, bradykinin B_2 or AII receptors can independently activate different signal transduction pathways. However, it is possible for all of these receptors to activate protein kinase C (PKC) [108, 110-112]. This calcium-dependent PKC activation occurs presumably through the production of diacylglycerol (DAG) from the breakdown of phosphoinositol-diphosphate (PIP$_2$) by phospholipase C (PLC) (Figure I.1)

A role for phospholipase D (PLD) in ischemic preconditioning has also been suggested within the scope of the PKC hypothesis. PLD can be activated by different mechanisms as compared to PLC. AII is known to induce activation of PLD and also a sustained activation of PKC [113]. It has also been demonstrated in isolated rabbit hearts that PLD can be activated with adenosine [114]. PLD is also activated during ischemia [115].

Finally, there is some evidence to indicate that PKC may also be activated through PLD [31, 114]. Together, these findings suggest that the PLD-DAG-PKC pathway may be an important signal transduction cascade in the protection induced by ischemic preconditioning.

Myocardial ischemia is also known to induce activation of phospholipase A$_2$ (PLA$_2$) through which arachidonic acid is produced [116]. Thus, it is possible that PLA$_2$ may play a role in inducing the protection of ischemic preconditioning, presumably via AII formation during ischemia. Chen et al [117] have recently found in rat hearts that by inhibiting 12-lipoxygenase, an enzyme

which metabolizes arachidonic acid, with lipoxygenase inhibitors (nordihydroguaiaretic acid or eicosatetraynoic acid) during the ischemic preconditioning cycles, the protective effect of ischemic preconditioning or exogenous administration of a diacylglycerol analogue (1,2-dioctanoyl-sn-glycerol) against contractile dysfunction following 20 min of global ischemia was lost. These findings suggest that the protective effect of ischemic preconditioning and PKC activation on ischemic injury (as measured in rat hearts through post-ischemic contractile dysfunction) may be linked to the activation of the 12-lipoxygenase pathway.

Based on the fact that several receptors, which have the ability to activate PKC through the three phospholipases (PLD, PLC and PLA_2), have been found to participate in triggering the protective effect of ischemic preconditioning, Downey and coworkers [118] have formulated a new hypothesis that awards PKC a key role in IP. This hypothesis states that PKC must be initially translocated into the sarcolemma during the transient ischemia and reperfusion. Once in the membrane, translocated PKC is activated during the long ischemia to induce the protective effect of IP. Under these circumstances, the hypothesis states that any cardiac receptor activation which leads to PKC activation may, in theory, protect the myocardium to the same extend as ischemic preconditioning. Activation of cell membrane receptors (adenosineA_1, α_1-adrenergic, bradykinin B_2 and AII) known to activate PKC do indeed induce a cardioprotective effect similar to the protection by ischemic preconditioning [34, 43, 103, 108].
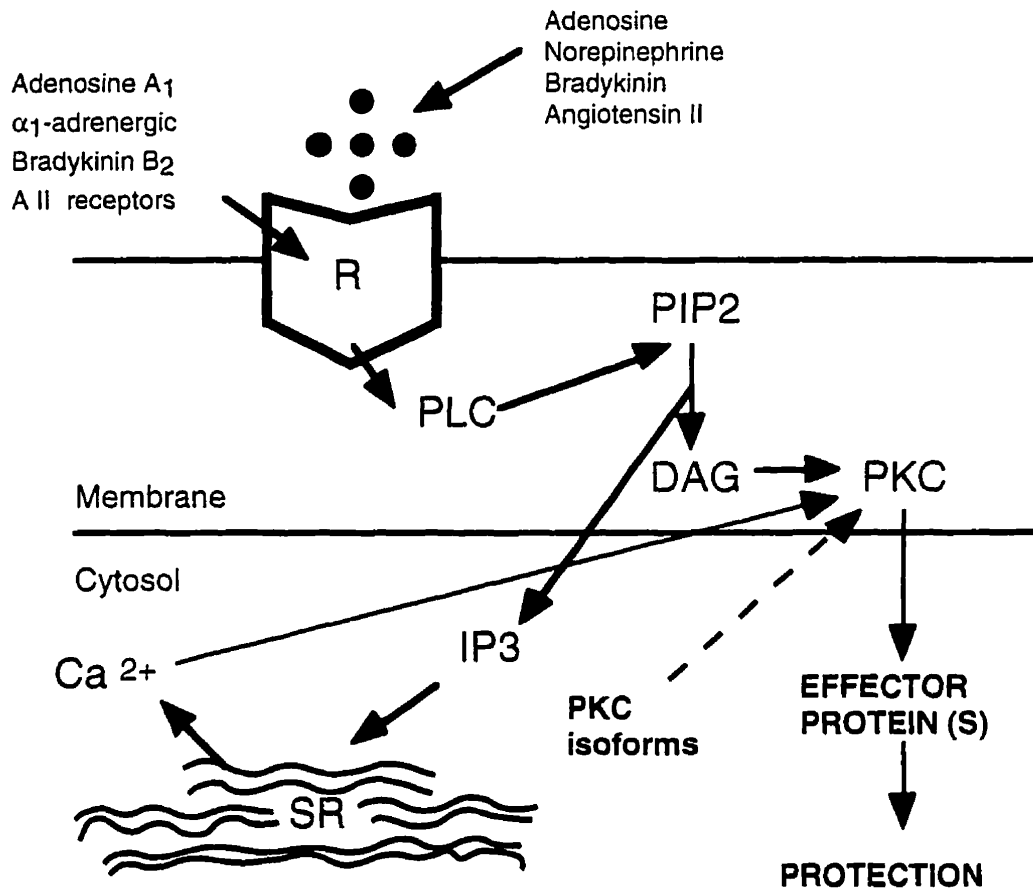
Figure I.1: This diagram shows different cardiomyocytes cell membrane receptors that can be activated during ischemia through the release of different endogenous substances. Individual activation of each receptor activates the plasma membrane enzyme phospholipase C (PLC), which in turn breakdown phospho-inositol-diphosphate (PIP$_2$) into two second messengers, inositol triphosphate (IP$_3$) which diffuses freely into the cytosol and interacts with the sarcoplasmic reticulum (SR) to release calcium (Ca $^{2+}$), and diacylglycerol (DAG), a known activator of protein kinase C (PKC) in the membrane [110]. Calcium-dependent and -independent PKC isoforms may translocate, as a result of an ischemic stimuli, into the cell membrane where they may be activated by DAG. PKC isoforms, once activated, are believed to induce the phosphorylation of protective proteins which are the end effectors responsible for the protection induced by transient ischemia and reperfusion (ischemic preconditioning) [118]. Thus, a multi-receptor triggering mechanism is thought to be responsible for this signal transduction cascade. PKC can also be activated through the production of DAG from the breakdown of phosphatidylcholine by the enzyme phospholipase D (not shown in the diagram) to produce phosphatidic acid that can be further metabolized by phosphatidic acid phosphohydrolase to form DAG [115].

Furthermore, the central role of PKC activation in this hypothesis is supported by a large body of circumstantial evidence which suggests that PKC is the key element in the induction of the ischemic preconditioning protection [37, 76, 118-124]. However, other investigators have not been able to block the protective effect of ischemic preconditioning using at least two PKC inhibitors, chelerythrine [27] and H7 [37].

In a recent study conducted in the laboratory in which my studies have been performed, it was demonstrated that inhibition of PKC activation with either chelerythrine or polymyxin B, prior and during three cycles of transient ischemia and reperfusion did not block the protective effect of three cycles of transient ischemia and reperfusion (5 min of ischemia followed by 10 min of reperfusion x 3) on necrosis in vivo in rabbit hearts [55]. However, in an otherwise identical one-cycle ischemic preconditioning protocol, chelerythrine and polymyxin B partly blocked such protection. Thus, it appears that PKC plays a significant role in ischemic preconditioning but some other signal transduction pathways are probably also involved.

2.3.4   The cAMP Hypothesis:

Cyclic adenosine monophosphate (cAMP), an intracellular second messenger formed from the dephosphorylation of ATP by the enzyme adenylyl cyclase (AC), has been shown to significantly increase in heart tissue during ischemia [125]. Ischemic preconditioning has been found to prevent the increase in cAMP levels in the hearts during the long ischemia [95]. This finding

stimulated Sandhu et al [56] to formulate a new hypothesis which may partially explain the mechanism of ischemic preconditioning. In cardiomyocytes, cAMP is formed from the breakdown of ATP by the enzyme adenylyl cyclase (AC). If AC is stimulated, as it occurs during ischemia, ATP is rapidly broken down to form cAMP, thus raising cAMP levels in the myocardium. Since a marked increase in cAMP during ischemia means a substantial reduction in ATP stores, thus low levels of cAMP during ischemia may be interpreted as the result of a slow down of the rate of ATP breakdown or inhibition of AC. Therefore, it is conceivable that preventing the rise in cAMP levels in the heart may slow down the rate of ATP decline during sustained ischemia. In addition, cAMP is a potent activator of protein kinase A, a kinase which can phosphorylate a number of effector proteins. Thus, low myocardial cAMP levels contribute to the IP protection by reducing PKC activity.

To test the hypothesis that low myocardial cAMP levels during ischemia are responsible for the protection induced by ischemic preconditioning, a study was performed in which in vivo rabbit hearts were treated with NKH477, a specific activator of adenylyl cyclase, to raise the levels of cAMP in preconditioned hearts and observe if the ischemic preconditioning protection against necrosis was abolished. The protection induced by one-cycle ischemic preconditioning (5 min ischemia followed by 10 min of reperfusion) was loss with the administration of NKH477. However, the protection obtained with three cycles of ischemic preconditioning could not be abolished by NKH477. These findings suggest that inhibition of PKA via reduction of cAMP levels could

possibly play a role in ischemic preconditioning. However, a direct assessment of the participation of PKA or an evaluation of any additional contribution of this pathway to the PKC pathway in ischemic preconditioning remains to be explored.

New evidence has recently emerged which supports the view that a calcium signaling pathway may also contribute to the protection of ischemic preconditioning. Meldrum et al [126] have found that short exposure to high extracellular calcium concentration (2.45 mM) obtained through exogenous infusion in the perfusate can produce equivalent protection against post-ischemic ventricular dysfunction as ischemic preconditioning in isolated rat hearts. In addition, PKC inhibitors were shown to abolish the calcium-mediated protective effect against contractile dysfunction [126]. These findings suggest that mobilization of intracellular Ca^{2+} contributes to the development of PKC mediated (receptor mediated) functional preconditioning in rat heart, a hypothesis formulated by Meldrum et al [126]. Similar results were obtained by Miyawaki and Ashraf [127] who were able to mimic the protection of preconditioning on contractile function by a transient increase on extracellular calcium concentration (5 min) followed by 10 min of washout given before the long ischemia/reperfusion episode in isolated rat hearts. In addition, chelerythrine, a PKC inhibitor, blocked the protective effect of transient exposure to high calcium concentration. More recently, Dekker et al [128] have demonstrated in arterially perfused papillary muscle that Ca^{2+} is the primary trigger for cellular uncoupling during ischemia in normal, preconditioned and metabolically inhibited myocardium.

They have also shown that ischemic preconditioning delayed $[Ca^{2+}]_i$ rise and cellular uncoupling during ischemia [128]. According to the cable theory, longitudinal R_t consists of intracellular (r_i) and extracellular (r_o) longitudinal resistances in parallel, where r_i is the series resistance of the intracellular space and the gap junctions and r_o is the resistance of the extracellular space [128]. During ischemia, cellular uncoupling can be appreciated as an increase of R_t that is caused by an increase of r_i. Dekker et al [128] have also shown that pretreatment of papillary muscle with 1mmol/L iodoacetate to inhibit anaerobic metabolism and minimize acidification during ischemia, produced significant advancement of the rise in intracellular calcium during ischemia. Since myocardial contracture, which is associated with increased intracellular Ca^{2+} during ischemia, is strongly indicative of irreversible ischemic damage and necrosis, it is conceivable that the protection of ischemic preconditioning against myocardial necrosis may be induced by a delay in $[Ca^{2+}]_i$ rise, uncoupling and contracture. Even though IP also delays the onset of severe intracellular acidosis during ischemia [93, 94], it appears, as evidenced by Dekker et al [128]'s metabolic experiments, that minimizing the acidification of intracellular pH is not a direct mechanism of protection for IP.

3. CARDIAC RENIN-ANGIOTENSIN SYSTEM

3.1 Endogenous Angiotensin II Production in the Heart:

The classical view of the hormonal system, that circulating AII was the only mediator of biological effects has changed in the last decade with the discovery of several local renin-angiotensin systems in various tissues including the adrenal glands [129], blood vessels [130], brain [131], kidneys [132] and heart [133]. There is evidence suggesting that local synthesis of angiotensins occurs within the myocardium. Lindpainter et al [134] have demonstrated in the atria and ventricles of hearts of rhesus monkeys sufficiently high concentrations of AI and AII to have biological effects. The level of AI has been shown to be increased in atria and ventricles of rats following nephrectomy [135], which suggests that the cardiac renin-angiotensin system may function separately from the circulating system. In rabbit hearts, Unger et al [136] have shown that the hearts of nephrectomized animals treated with Captopril, an angiotensin converting enzyme inhibitor, decreased the concentration of AII in the atria but had no effect in the ventricles, suggesting that AII may still be produced in the ventricles through an alternate mechanism. Furthermore, it has been recently demonstrated by Dostal et al [137] that AI and AII can be synthesized in culture media from cardiomyocytes and fibroblasts at physiologically relevant nanomolar concentrations. The presence of an intrinsic cardiac renin-angiotensin system is also supported by the demonstration that the other components of that system (renin, ACE and chymase) are present in the heart [138].

Renin and angiotensinogen mRNA have been detected in the heart of rat and mouse by Northern hybridization analysis, S_1 nuclease protection assay, and in situ hybridization [137, 139-141].

The cardiac renin-angiotensin cascade is shown in Figure I.2 Angiotensinogen, the primary precursor, is hydrolyzed to angiotensin I (AI) by the action of the aspartyl protease, renin [138]. Then, AI, a non-active decapeptide (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu), is converted to AII, an active octapeptide, by the cleavage of two carboxyterminal amino acids. This conversion can be realized in the heart by either the action of the angiotensin converting-enzyme (ACE) [142] or by the action of the enzyme chymase through the cleavage of the carboxyterminal His^9-Leu^{10} or Phe^8-His^9 [138], respectively. Most of the AII formed endogenously in the heart is thought to derive from the conversion of AI to AII by a specific serine protease, chymase [143]. AII can be further broken down to form angiotensin III, a peptide which is less biologically active than AII.

The presence of chymase in rabbit hearts, which is the species used for the studies presented herein, has not yet been assessed. However, two different groups of investigators have demonstrated the formation of AII from AI with the intervention of a chymase-like enzyme in monkey hearts [144] and dog hearts [145]. In addition, Urata et al [146] have been able to selectively identify a chymase-like enzyme in human hearts. These data indicate that heart chymase may be widely distributed across species, at least in mammals. However, the ability of a heart chymase to rapidly transform AI to AII may differ since many

similarities and differences exist among mammalian chymases with respect to peptide substrate specificity [138]. For example, human heart chymases [143, 147] readily hydrolyze the Phe^8-His^9 bond in AI to yield AII, but they cannot hydrolyze the Tyr^4-Ile^5 bond. However, rat chymase 1 preferentially hydrolyzes the Try^4-Ile^5 bond in AI but does not readily convert the prohormone to AII.

Angiotensin II is a powerful vasoconstrictor that also activates PKC via PLC and/or PLD, and is produced during ischemia in dog hearts [100, 102] and rat hearts [101]. Even though the exact pathophysiologic effects of the cardiac renin–angiotensin system during ischemia are not known, it is likely, based on the fact that myocardial ischemia enhances cardiac conversion of AI to AII and induces an AII-mediated coronary vasoconstrictor effect in isolated rat hearts [148], that activation of AII receptors may occur during ischemia.

3.2   Angiotensin II Membrane Receptors:

Angiotensin II receptor binding sites have been identified in sarcolemmal membrane preparations from rabbit [149], guinea-pig [150], bovine [151], avian [152], rat [153], porcine [150] and human myocardium [154]. AII receptors have also been identified, in the heart, in coronary vessels and sympathetic nerves using autoradiography [154]. In recent years, two putative AII receptors subtypes, AT_1 and AT_2 receptors, have been identified using selective ligands [155, 156]. Losartan (DuP753, MK954 or 2-nbutyl-4-chloro-5-hydroxymethyl-1-[(2'-(1H-tetrazol-5-yl)biphenyl-4-

PRO-RENIN

↓

RENIN ⟶ ▶ **ANGIOTENSINOGEN**

↓

ANGIOTENSIN I

ACE ⟶ ▶

CHYMASE

↓

ANGIOTENSIN II

↓

A III, A IV
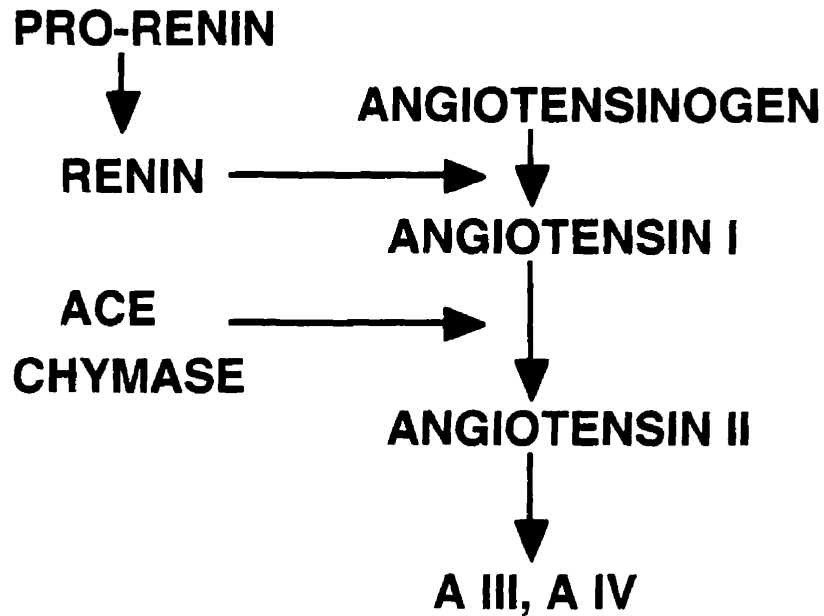
Figure I.2:   The renin angiotensin system. Angiotensinogen, the primary precursor, is hydrolyzed by the action of the enzyme renin (formed from pro-renin) to produce a ten-aminoacid polypeptide with no biological activity named angiotensin I (AI). In the presence of angiotensin converting enzyme (ACE) or the serine protease 'chymase', two amino acids of the AI molecule are cleaved to produce a very potent vasoconstrictor 8-aminoacid polypeptide called angiotensin II (AII). AII can be further degraded by other proteases to form AIII and AIV, which are both much less biologically potent peptides [133].

yl)methyl]imidazol), a nonpeptide AII receptor antagonist, binds specifically to the AT_1 receptor, whereas CGP 42112, PD-123177 and PD-123,319 have a high affinity for the AT_2 receptor. Both receptor subtypes, AT_1 and AT_2, are distinguished by two other characteristics: Firstly, AT_1 and AT_2 differ in their sensitivity to reducing agents, such as dithiothreitol (DTT), which decreases binding of AII by the AT_1 receptor, whereas binding by the AT_2 receptor is increased by this reducing agent [157, 158]. Secondly, coupling of AII receptors to guanine nucleotide binding proteins has been demonstrated for the AT_1 subtype but not for the AT_2 [159]. The AT_1 receptor belongs to the G-protein-coupled superfamily of receptors which are distinguished from other receptors by having seven transmembrane-spanning domains [160, 161] (Figure I.3).

The two subtypes of receptor, AT_1 and AT_2, have been identified in rabbit ventricular cardiomyocyte membranes [162]. The AT_1 subtype which has a K_d of 31 nM for losartan, whereas the AT_2 subtype shows a K_d of 0.5 nM for CGP 42112. Both receptor populations have an almost identical affinity for AII (1.5 and 1.2 nM for AT_1 and AT_2, respectively) and the ratio of AT_1/AT_2 is nearly equal (60:40) in monkey and rabbit hearts [163]. However, the proportion of AT_1 to AT_2 appears to be dependent on species, since a higher proportion of AT_1 receptors (greater AT_1/AT_2) has been observed in rats [163]. Most recently, Sechi et al [164] found equal proportions of AT_1 to AT_2 receptors from an 'in situ' binding assay on tissue sections obtained from fetal, neonatal and adult rats.

Most of the biological effects of AII in the heart have been attributed to the interaction of AII with the AT_1 receptor subtype [138, 158]. This receptor subtype (AT_1) has already been cloned and its molecular structure sequenced [138, 160]. Each AT_1 receptor is a 359 amino acid protein with a M_r of 41kd, but estimates of the molecular weight of the functional receptor range as high as 79 kd [138] (Figure I.3). This apparent discrepancy may be due to the fact that the protein is probably glycosylated. In the molecular structure of the AT_1 receptors, which include seven transmembrane-spanning domains, at least three potential consensus sites (branched structures in Figure I.3) for N-glycosylation have been found on the putative extracellular domains [138]. Each of the four extracellular regions also contains a cysteine residue [161], the function of which is not yet known.

3.3   Cellular Signaling Regulated by Angiotensin II Receptors:

There are several signal transduction pathways regulated by the AT_1 receptor subtype which have been elucidated in various types of tissues and cells [111, 112, 153, 165-168]. However, the signal tranduction pathways regulated by AT_2 receptors is still unknown. Most of the signaling pathways affected by AT_1 receptors are shown in Figure I.4.

The signal transduction pathways modulated by AT_1 receptors which have been studied intensively are the phospholipase C (PLC)/diacylglycerol (DAG)/protein kinase C (PKC) and adenylyl cyclase (AC)/cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) pathways. AT_1 receptors activate

phosphoinositide-specific PLC via a guanine nucleotide regulatory protein in chick cardiomyocytes[112] and rat neonatal cardiomyocytes [169]. PLC breaks down phosphoinositol diphosphate (PIP$_2$) to form inositol triphosphate (IP$_3$) and DAG. IP$_3$ mobilizes Ca^{2+} ions from intracellular stores (sarcoplasmic reticulum in the heart), and DAG activates the Ca^{2+}- and phospholipid-dependent enzyme PKC. DAG can also be transformed to phosphatidic acid by the action of the enzyme DAG-kinase. Furthermore, it is known that activation of AII receptors activates phospholipase D (PLD) and phospholipase A$_2$ (PLA$_2$) [166].

The most studied effect of AII on intracellular signaling in the myocardium is an acute increase in mean diastolic intracellular Ca^{2+} concentration [138]. This is accompanied by an increase in the frequency of Ca^{2+} transients, followed by a significant decrease in their amplitude [138]. In the heart, the positive inotropic effect of AII is mainly due to augmentation of inward Ca^{2+} current (I$_{ca}$) through L-type channels [142, 170, 171]. The subsequent decrease in the amplitude of Ca^{2+} transients following administration of AII can most likely, as suggested by Lindpainter and Ganten [138], be attributed to a negative feedback mechanism, possibly involving PKC. If this hypothesis is correct, then, in turn, the reduction in intracellular calcium concentration presumably occurring in preconditioned cardiomyocytes may be explained by the negative feedback induced by AII released during ischemia on intracellular calcium low release.

Angiotensin II Receptor (AT₁)
359 amino acids
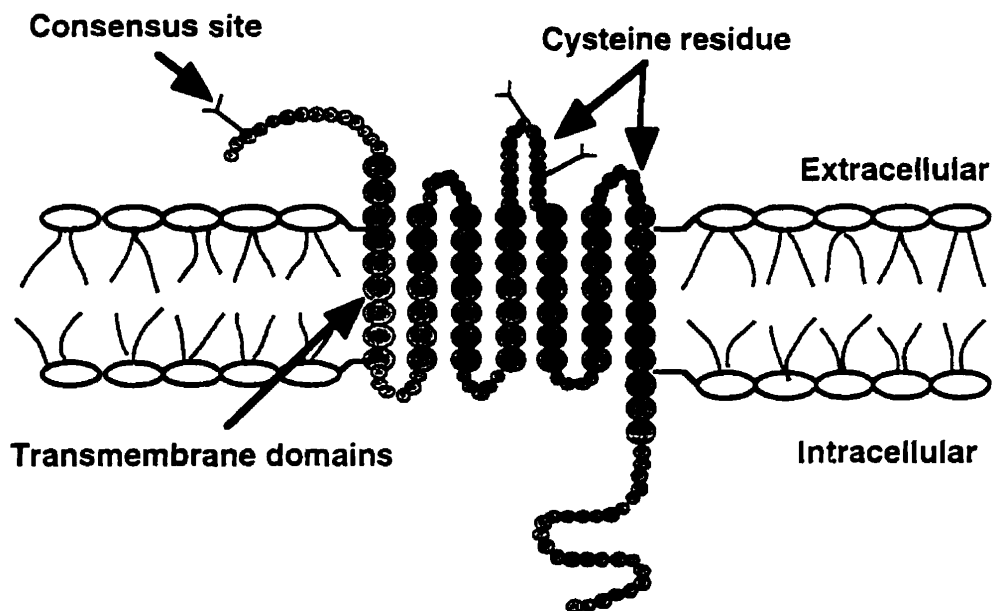
Figure I.3: Schematic of the AII AT₁ receptor subtype. The structure of this putative receptor has seven transmembrane-spanning domains. The molecular structure of this protein encodes a sequence of 359 amino acids. There are three potential consensus sites (branched structures) for N-glycosylation on the putative extracellular domains. Each of the four extracellular regions also contains a cysteine residue [161], the function of which is not yet known.

Angiotensin II Receptor

Angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe)
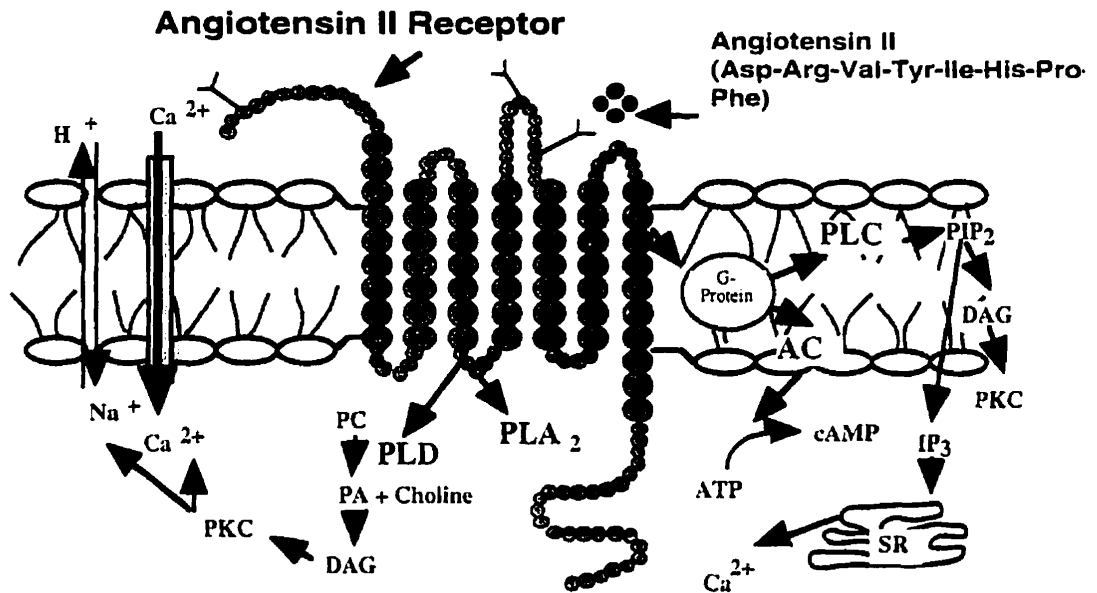
Figure I.4: Angiotensin II signaling in cardiomyocytes. Angiotensin II activates AT_1 and AT_2 receptor subtypes, but only the AT_1 receptor is known to couple to an inhibitory G_i protein. Activation of the AII AT_1 receptor subtype induces the activation of phospholipase C (PLC), phospholipase D (PLD) and phospholipase A_2 (PLA$_2$), thus producing inositol triphosphate (IP$_3$) and diacylglycerol (DAG) from phosphoinositol diphosphate (PIP$_2$), phosphatidic acid and choline from phosphatidylcholine (PC), or arachidonic acid from PC, respectively. Angiotensin II also stimulates an increase in current through L-type Ca^{2+} channel and activation of the Na^+/H^+ exchanger presumably through a PKC mechanism [134].

It appears that AII receptor stimulation activates an intracellular signal that affects the chloride channel in the membrane. This observation is supported by the findings of Endho et al [167] who have shown in rabbit ventricular cardiomyocytes, using the whole-cell voltage clamp technique, that exposure to AII increases chloride current via AT_1 receptors and that this effect depends on PKC activation [167]. Therefore, it is possible that chloride channels may contribute to the protection against necrosis induced by AII and that this channel could be a target effector protein in the signal transduction generated by ischemic preconditioning.

Another signaling pathway thought to be activated by AII stimulation via the AT_1 receptor subtype is the AII/tyrosine kinase pathway. A newly developed concept for guanine nucleotide protein-coupled receptors is the activation of intracellular second-messenger proteins via tyrosine phosphorylation[172]. For instance, AII stimulates rapid tyrosine phosphorylation and activation of phospholipase C-gamma-1 and the JAK family of intracellular kinases in cultured rat glomerular mesangial cells [173]. Both in cultured rat aortic smooth muscle cells and mesangial cells, the cellular tyrosine kinase c-src appears to play a critical role in the A II-stimulated tyrosine phosphorylation of PLC-gamma-1 and the generation of inositol triphosphate (IP_3) as evidenced by the inhibition of AII-mediated production of IP_3 and the intracellular release of Ca^{2+} produced by a blockade of the rapid tyrosine phosphorylation of PLC-gamma-1 [174]. These findings suggest that AII, working through the AT_1 receptor, stimulates tyrosine

phosphorylation and by this mechanism signals are conveyed from the cell surface to other sites inside the cell. Furthermore, AII stimulates the influx of calcium from the extracellular space in a dose-dependent fashion in rat aortic smooth muscle cells [175]. Suzuki et al [175] have shown that inhibition of tyrosine kinases with Genistein and tyrophostin, significantly suppresses the AII-induced Ca^{2+} influx and the AII-induced formation of choline. These results suggest that AII stimulates PLD due to Ca^{2+} influx from the extracellular space, and that tyrosine kinases are involved in the AII-induced Ca^{2+} influx, resulting in the promotion of phosphatidylcholine hydrolysis. A role for protein tyrosine kinase phosphorylation has been suggested based on evidence provided by experiments reported by Baines et al [176]. In these experiments, inhibition of tyrosine kinase activation with Genistein ($50\mu M$) during the prolonged period of ischemia, in a single-cycle IP buffer-perfused rabbit heart protocol blocked the protective effect of ischemic preconditioning against infarction (necrosis).

Angiotensin AT_1 receptors have also been found to be negatively coupled to adenylyl cyclase via an inhibitory G protein (G_i). Anand-Srivastava et al [153] have shown that myocardial tissue cAMP levels decline with AII stimulation in rabbit and rat hearts [153].

4. FORMULATION OF HYPOTHESES

In addition to adenosine, norepinephrine and bradykinin, the heart also produces AII during ischemia [100-102]. These findings strongly suggest that cardiac AII receptors are activated

during ischemia and that AII receptors may participate in triggering the protective effect of ischemic preconditioning against necrosis. A study performed in rabbits by Liu et al [108] has revealed that exposing isolated hearts to AII (100 nM) for 5 min to stimulate AII receptors produces a protective response against infarction equivalent to the protection obtained with ischemic preconditioning. Furthermore, this AII cardioprotection was blocked by co-administrating either losartan (10μM), an AT_1 AII receptor antagonist, or polymyxin B (50μM), a potent PKC inhibitor [108]. These data suggest that a short exposure to AII can be cardioprotective and that such cardioprotection may be mediated through PKC activation. Since AII can induce a protective mechanism against necrosis in such a short period (5 min), then it would be reasonable to think that 5 min of ischemia would provide enough time for the heart to become protected through an AII receptor mechanism. In addition, since AII is well-known to activate PKC in the heart [100-102], both through PLC/DAG/PKC and PLD/DAG/PKC pathways, then any PKC inhibitor given prior to the AII stimulus should be able to block the protective effect of AII pretreatment, as was demonstrated by Liu et al [108].

Furthermore, there are other findings which are in agreement with an AII receptor activation in ischemic preconditioning: 1) AII reduces cAMP in rabbit and rat myocardial sarcolemma [153]. Ischemic preconditioning induced with three cycles of 5 min ischemia followed by 10 min of reperfusion prevented a rapid increase of cAMP levels, which normally occurs after 10-30 min of ischemia, in rabbit hearts subjected to regional

normothermic (37°C) myocardial ischemia in an in vivo model [56]. 2) AII activates the Na^+/H^+ exchanger which pumps H^+ ions out of the cell in exchange of Na^+ ions, thus inducing an alkalization of the cytosol [93] that may possibly contribute to the AII-mediated cardioprotection against infarction. Ischemic preconditioning preserves intracellular pH during the long ischemia [93, 94]. 3) AII produces an increase in intracellular calcium through a PKC-independent L-type Ca^{2+} channel opening [138]. Transient exposure to a high concentration of extracellular calcium (2.45mM), which results in a slightly higher concentration of intracellular calcium, mimics the protective effect of ischemic preconditioning against ventricular dysfunction [126, 177]. Based on the evidence presented before, I have formulated my first hypothesis: "Cadiac AII receptors which are activated during the short cycles of ischemia and reperfusion by endogenously produced AII may trigger the ischemic preconditioning phenomenon in the myocardium."

It has been shown that inhibition of inhibitory G-proteins (G_i) with pertussis toxin abolishes the protection against infarction induced by ischemic preconditioning in rabbit heats [104]. These data suggest that the protection of ischemic preconditioning against necrosis may be mediated through G_i. Thus, I have formulated a second hypothesis as follows: "The myocardial AT_1 is the AII receptor subtype that participate in the triggering mechanism of ischemic preconditioning."

II. OBJECTIVE, HYPOTHESES, AND SPECIFIC AIMS

1. OBJECTIVE OF THIS STUDY:

The overall objective of this study was to determine the role of AII receptors in the triggering mechanism(s) of the ischemic preconditioning phenomenon in the myocardium.

1.1. Hypothesis 1:

"Activation of cardiac angiotensin II receptors by endogenously produced angiotensin II during the short ischemia/reperfusion episodes before a prolonged period of ischemia followed by reperfusion contributes significantly to the triggering mechanism of the protective effect of ischemic preconditioning against myocardial infarction."

To assess this hypothesis the following aims were established:

1.1.1 Aim 1:

To characterize the protective effect of ischemic preconditioning against infarction, in terms of infarct size in an isolated buffer-perfused rabbit heart model of regional myocardial ischemia.

1.1.2 Aim 2:

To assess the cardioprotective effect of angiotensin II receptor activation against infarction produced by a long

-41-

ischemia/reperfusion episode in the same *in vitro* rabbit heart model of regional myocardial ischemia previously described.

1.1.3   Aim 3:

To assess the ability of angiotensin II receptor blockade to abolish the protective effect of ischemic preconditioning against infarction using the same *in vitro* rabbit heart model of regional myocardial ischemia.

1.2   Hypothesis 2:

"The AT_1 angiotensin II receptor is the angiotensin II receptor subtype mediating the protection of ischemic preconditioning against myocardial infarction."

To assess this hypothesis the following aims were established:

1.2.1   Aim 1:

To determine the participation of each angiotensin II receptor subtype, AT_1 and AT_2, in ischemic preconditioning by assessing the ability of either AT_1 receptor or AT_2 receptor blockade to abolish the protective effect of ischemic preconditioning against infarction in the same *in vitro* rabbit heart model of regional myocardial ischemia previously described.

To confirm the participation the AII receptor subtypes in ischemic preconditioning in an in vivo model, I established the following aims 2 and 3.

1.2.2 Aim 2:

To characterize the protective effect of ischemic preconditioning against myocardial infarction in an in vivo open-chested rabbit heart model of regional ischemia.

1.2.3 Aim 3:

To determine the participation of the angiotensin II AT_1 receptor subtype in ischemic preconditioning of the myocardium by assessing the ability of an AT_1 receptor subtype blockade to abolish the protective effect of ischemic preconditioning against myocardial infarction in the *in vivo* rabbit heart model of myocardial regional ischemia.

III. ISCHEMIC PRECONDITIONING IN ISOLATED RABBIT HEARTS

The objective of this study was to characterize the protective effect of ischemic preconditioning against infarction, in terms of infarct size in an isolated buffer-perfused rabbit heart model of regional ischemia. Since it is virtually impossible to determine the specific effects of locally produced AII in vivo, it was necessary to develop an isolated heart model that eliminates any significant residual effects from non-cardiac renin–angiotensin systems during ischemia. Under these circumstances, any changes in infarct size or ventricular function should be the direct effect of specific pharmacological interventions on the heart.

1. MATERIALS AND METHODS

All rabbits were treated to conform with the *Guide for the care and use of laboratory animals* published by the US National Institutes of Health (NIH publication No. 85-23. revised 1996). The research protocol was approved by the Animal Care Committee of the Research Institute, The Hospital for Sick Children.

1.1 Surgical Preparation:

Hearts from New Zealand white rabbits (weight range, 3.0–3.5 kg) of either sex, were isolated and perfused in a modified Langendorff apparatus for this study (Figure III.1). To induce general anesthesia, each donor rabbit was given an intravenous

solution of pentobarbital (60 mg/kg) and heparin (200 IU/kg). This heparin-pentobarbital solution was divided in half and given as two intravenous bolus doses. The first bolus dose was given initially to induce anesthesia during which the rabbit was shaved on the anterior neck and left chest to prepare those areas for surgery. Five minutes after the first bolus, the second bolus was administered. Because this second bolus of anaesthesia usually produced respiratory arrest rapidly, the animal chests were opened through a midline sternotomy and the heart rapidly excised and quickly mounted (<30 seconds) onto an aortic cannula (ID: 4.0–4.2 mm) by its aortic root. Each heart was immediately perfused with a modified Krebs-Henseleit buffer solution containing 118.5 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 24.8 mM $NaHCO_3$, 1.2 mM KH_2PO_4, and 10 mM glucose. The perfusate used in the non-recirculating system was oxygenated with 95% O_2 – 5% CO_2 and maintained at 37°C, and perfusion was mainteined at a constant pressure of 75 mmHg.

A 2–0 silk suture was placed around a large branch of the left coronary artery and both ends pulled through the lumen of a polyethylene tubing (ID: 3mm, 8 cm long, Clay Adams Inc., Parsippany, NJ) to form a tourniquet-like device for performing coronary occlusions from the outside of the water-jacketed heart chamber. Subsequently, an intraventricular latex balloon filled with saline and connected to a pressure transducer (Model 800, Bentley Inc., Irvine, CA) was placed into the left ventricle via the left atrium. This was used to obtain data on left ventricular systolic and diastolic pressures and heart rate. Once the

PUMP

AIR TRAP

PERFUSION
PRESSURE
(75mmHg)

FILTER

HEATER

95%O2-5%CO2

TEMPERATURE
PROBE

SIDE PORT

HEART
CHAMBER

PRESSURE
TRANDUCER

RESERVOIRS

By Dr. Roberto J.
Diaz
Toronto, Ontario.
Canada

CORONARY
EFFLUENT
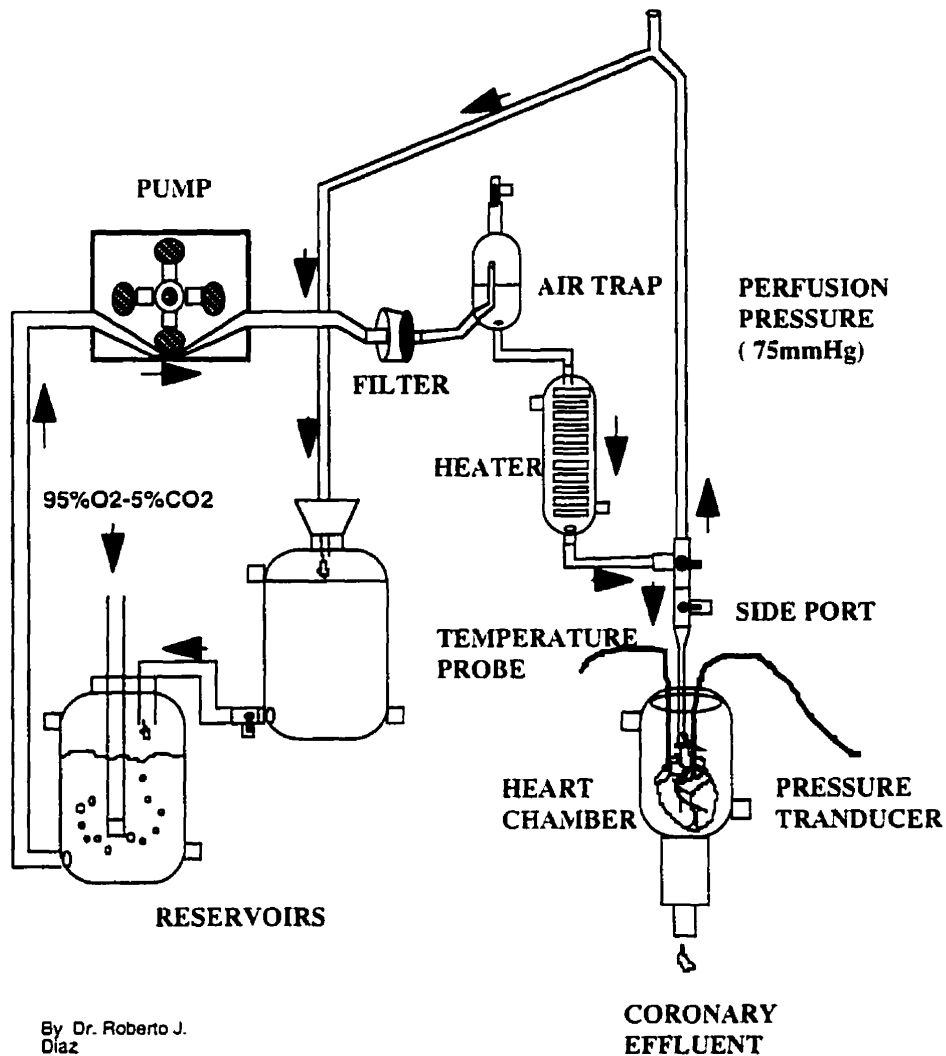
Figure III.1: The modified Langendorff perfusion apparatus. As shown in this picture, oxygenated buffer (37°C) is pumped up through tygon tubing into a glass air bubble trap. Then, the buffer circulates into a glass coiled heat exchanger from which it is then delivered directly into the heart through an aortic cannula. The heart is perfused at a constant pressure of 75 mmHg generated by a buffer column of 100 cmH2O. Any buffer overflow is then collected and returned to a water-jacketed glass container to be re-circulated. The dripping buffer coming from the heart is used to measure coronary outflow and then discarded. A latex balloon is inserted into the heart left ventricle to measure ventricular pressures and a temperature probe is placed into the pulmonary vein to measure the buffer temperature as surrogate for the actual myocardial temperature. The heart is maintained at a constant temperature (37°C) by placing it into a water-jacketed heart chamber.

heart was fully instrumented, it was placed into the water-jacketed heart chamber for temperature control. The heart temperature was kept constant at 37° C and continuously monitored using a Termo-Finer temperature probe (Model N4-1C008, Terumo Inc., Tokyo, Japan). The temperature probe was placed in the pulmonary artery root to measure coronary outflow temperature as a good surrogate of myocardial temperature. All drugs used in this study were infused through a side port located 12 cm away from the heart over the perfusion column.

Regional myocardial ischemia was induced by occluding a branch of the left coronary artery using the tourniquet-like device described above. Ischemia was confirmed by observing the following changes: 1) the collapse of the distal segment of the occluded coronary artery; 2) a marked decline in left LVDP; 3) a sudden reduction in coronary flow; 4) the appearance of localized ventricular akinesia in the territory supplied by the occluded coronary artery. Reperfusion of the ischemic area was confirmed by observing the refilling of the distal artery, a significant recovery in LVDP and coronary flow, and the resumption of synchronized ventricular contractions.

1.2   Ventricular Function Measurement:

Left ventricular function for each heart was assessed by measuring left ventricular systolic (LVSP) and end-diastolic pressure (LVEDP). Ventricular function was also assessed based on LVDP and heart rate throughout each experiment. These data was obtained by using an intraventricular latex balloon connected to a

pressure transducer. Heart rate was estimated from the pressure wave signal. These data was recorded and digitized using a computer software developed at The Hospital for Sick Children (Cardiac Pressure-Volume Loop Acquisition and Analysis Program, version 5.0). The volume of the latex balloon was initially adjusted to achieve end-diastolic pressure of 4–5 mmHg and kept constant throughout each experiment. Thus, any changes in end-diastolic pressure would reflect changes in myocardial distensibility.

1.3   Coronary Flow Measurements:

Since AII receptor stimulation induces coronary vasoconstriction, and its inhibition produces coronary vasodilatation, it was very important to measure coronary flow throughout each experiment to study the effect of ischemic preconditioning on coronary flow, to evaluate subsequent coronary responsiveness to each drug studied and to perform the dose-response studies. The coronary effluent dripping from each heart was collected over one minute and measured using a graduated cylinder at different time points during the aerobic perfusion (15, 30, 45, 60 min), ischemia (10, 20, 30, 40 min), and reperfusion (15, 30, 45, 60 min) periods.

1.4   Infarct Size Measurements:

1.4.1   Tetrazolium Staining Technique:

Infarct size was determined using the tetrazolium staining technique [95]. At the end of each experiment, the coronary artery

subjected to occlusion was re-occluded. The heart was then perfused with 5–10μm zinc-cadmium sulfide yellow fluorescent particles (Duke Scientific Inc., Palo Alto, CA) to identify the risk area (the area seen to be without particles under ultraviolet light), defined as the biventricular area subjected to ischemia and reperfusion. Each heart was then cross-sectioned into 4-5 slices 3-5 mm thick and incubated in a 1.25% solution of triphenyl tetrazolium chloride (TTC), made up with 0.2 M Tris buffer (pH 7.4), at 37°C for 10 min. Then, the heart slices were fixed in 10% formaldehyde. Tetrazolium salts require the presence of functional enzymatic systems such as NAD/NADH or NADP/NADPH. The enzymes NADH or NADPH are oxidized by an oxidase releasing free electrons that can be picked up by the tetrazole, which becomes reduced to a red formazan stain in normal myocardium, whereas the infarcted tissue remains unstained [178]. Thus, viable tissue was defined as the areas stained brick-red; dead tissue (necrosis) was defined as the areas that did not stain and looked white or tan in color. Subsequently, each stained heart was labeled with an assigned code to blind the planimetry measurements. From all heart slices, the total biventricular area at risk and necrotic areas were traced onto transparent acetate sheets and all areas planimetered by computer to calculate the percentage of the biventricular area that was at risk and the percentage of the area at risk that was necrotic in each heart as follows:

% of area at risk = risk area (mm^2)/total area (mm^2)

% of necrotic area = necrotic area (mm^2)/risk area (mm^2)

The absolute values (mm^2) for total area, area at risk and necrotic area were entered on an Excel spreadsheet in an IBM compatible personal computer to perform the calculation of the percentage of area at risk and the percentage of necrotic area.

1.4.2   Inter-observer Error Analysis:

Even though the planimetry measurements to calculate the amount of necrosis were performed by the same person within three days of each experiment, it was quite possible that an error in defining the limits of the area at risk and necrotic areas may have occurred during such measurements, resulting in either underestimation or overestimation. Therefore, an inter-observer error analysis was performed as follows: randomly assigned hearts from in vitro and in vivo experiments (n = 7) were blindly traced onto acetate sheets and re-planimetered by an experienced technician in computer planimetry, in addition to the first measurement. Then the two separate measurements for each heart were subjected to a regression analysis to determine how close each measure was to the other. Results from this inter-observer error analysis showed a significant correlation between the two measurements (r=0.89)

1.5   Experimental Protocol:

1.5.1   Stability Study:

To assess the stability of the preparation, two groups of baseline hearts, paced hearts (n = 7) and non-paced hearts (n = 7) , were instrumented and aerobically perfused with buffer solution for 160 min, thus to equalize this protocol to the ischemia/reperfusion experimental protocol in time. Paced hearts were electrically stimulated via the left atrium to produce a heart rate of 200 beats/min. These hearts did not receive any treatment. LVDP, heart rate and coronary flow were measured at 15 min intervals throughout the 160 min perfusion of each heart. Necrosis was also assessed in each heart at the end of each experiment using the tetrazolium staining technique previously described. The objective of performing two stability studies, with and without pacing, was to determine which method would be more stable, in terms of LVDP, for better characterization of the ischemic preconditioning phenomenon in subsequent experiments. Because it is known that transient rapid pacing can precondition the heart [179, 180], it was necessary to study the effect of atrial pacing ischemic preconditioning on the isolated rabbit heart model of regional ischemia.

1.5.2   Ischemic Preconditioning: Paced- Versus Nonpaced Heart Study:

Due to a moderate decline in heart rate observed after one-hour aerobic perfusion in the stability study, it was necessary to

study the effect of atrial pacing on the ischemic preconditioning phenomenon in this model. To address this issue, 30 hearts were subjected to a standard 15 min of aerobic perfusion followed by 40 min of normothermic regional ischemia (37° C) and 60 min of reperfusion (Fig.III.2). Paced control hearts (Cp, n=7) and nonpaced control hearts (C, n=8) were subjected to an additional 45 min of aerobic perfusion prior to the 40 min of ischemia in order to compensate for the ischemic preconditioning cycles. Preconditioned paced hearts (IPp, n=7) and preconditioned unpaced hearts (IP ,n=7) were subjected to three successive cycles of 5 min of regional ischemia followed by 10 min of reperfusion prior to the long ischemic insult. Paced control and preconditioned hearts were atrial-paced at 200 beats/min. The pacer was turned off during each ischemic episode because in pilot experiments (n = 3) where each heart was continuously paced at 200 beats/min, it was observed that the heart developed substantially more necrosis after the 60 min of reperfusion than either unpaced control hearts or intermittently paced control hearts, suggesting that continuous electrical atrial stimulation during ischemia may accelerate ischemic injury in this model. Left ventricular developed pressure and coronary flow was assessed every 15 min during aerobic perfusion and every 10 min during the prolonged ischemic episode. Infarct size was measured as a percentage of the area at risk that was necrotic using the TTC staining technique.

Experimental Protocol
Paced vs Nonpaced Hearts
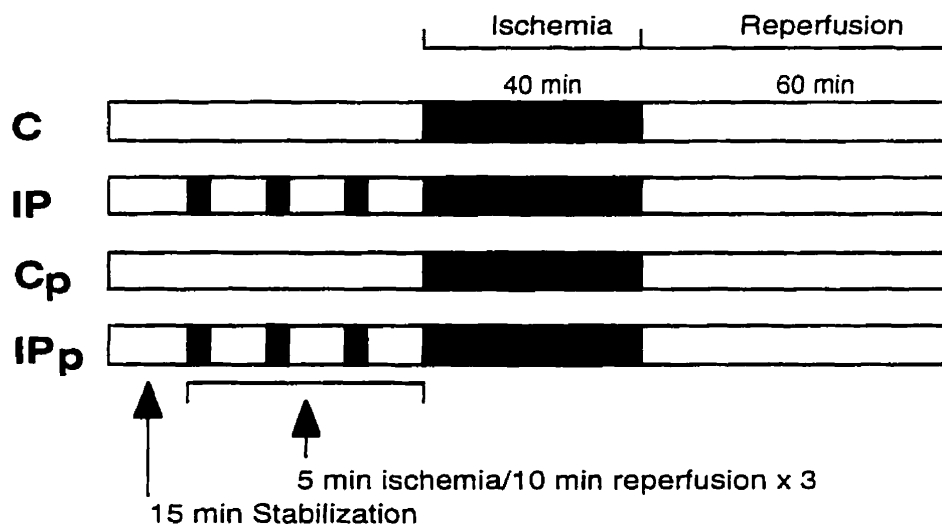
Figure III.2: Experimental protocol for the ischemic preconditioning study: paced versus nonpaced hearts. All hearts, paced control (Cp), paced preconditioned (IPp), nonpaced control (C) and nonpaced preconditioned (IP), received 15 min of aerobic perfusion followed by 40 min of normothermic regional ischemia (37°C) and 60 min of reperfusion. Control hearts received an additional 45 min of aerobic perfusion to compensate for the time used to induce preconditioning, to equalize all protocols in time. Preconditioned hearts were also subjected to three cycles of 5-min ischemia and 10-min reperfusion prior to the 40 min of normothermic regional ischemia and 60 min of reperfusion. Paced hearts, control and preconditioned, were atrial-paced at 200 beats/min. The pacer was turned off when ischemia started and turn on again when reperfusion started. Left ventricular developed pressure (LVDP) was assessed through the experiment and necrosis measured at the end of 60 min reperfusion.

1.6   Statistical Analysis:

All data are expressed as mean ± S.E.M. Factorial ANOVA was used to assess differences among the heart groups. Where appropriate, the Scheffé F-test was applied to determine whether a statistically significant difference (P < 0.05) existed between two groups. A regression analysis was performed between paced and nonpaced control and preconditioned heart groups to test for association between functional parameters (LVDP and heart rate) or coronary flow and infarct size. Regression analysis was also performed between all areas at risk and necrotic areas to test for any relationship between them.

2.   RESULTS

2.1   Stability Study:

All data (mean ± S.E.M.) for the stability study are summarized in Figure III.3. Both paced and nonpaced aerobically perfused hearts showed a significant (P < 0.05) decline in LVDP at the end of 160 min of aerobic perfusion (82. 1 ± 4.1 mmHg and 86.5 ± 4.8 mmHg, respectively) when compared to LVDP values at 15 min perfusion (110.7 ± 2.9 mmHg and 128.2 ± 3.6 mmHg, respectively). Furthermore, nonpaced hearts had a progressive and significant (P < 0.05) reduction in heart rate (from 170.4 ± 5.4 beats/min to 150.1 ± 8.5 beats/min) from 15 min to 160 min perfusion. Coronary flow also declined in both paced (from 85.1 ± 4.1 ml/min to 66.3 ± 2.7 ml/min) and nonpaced hearts (from 77.7 ± 2.4 ml/min to 60.5 ± 4.3 ml/min), from 15 min to 160 min
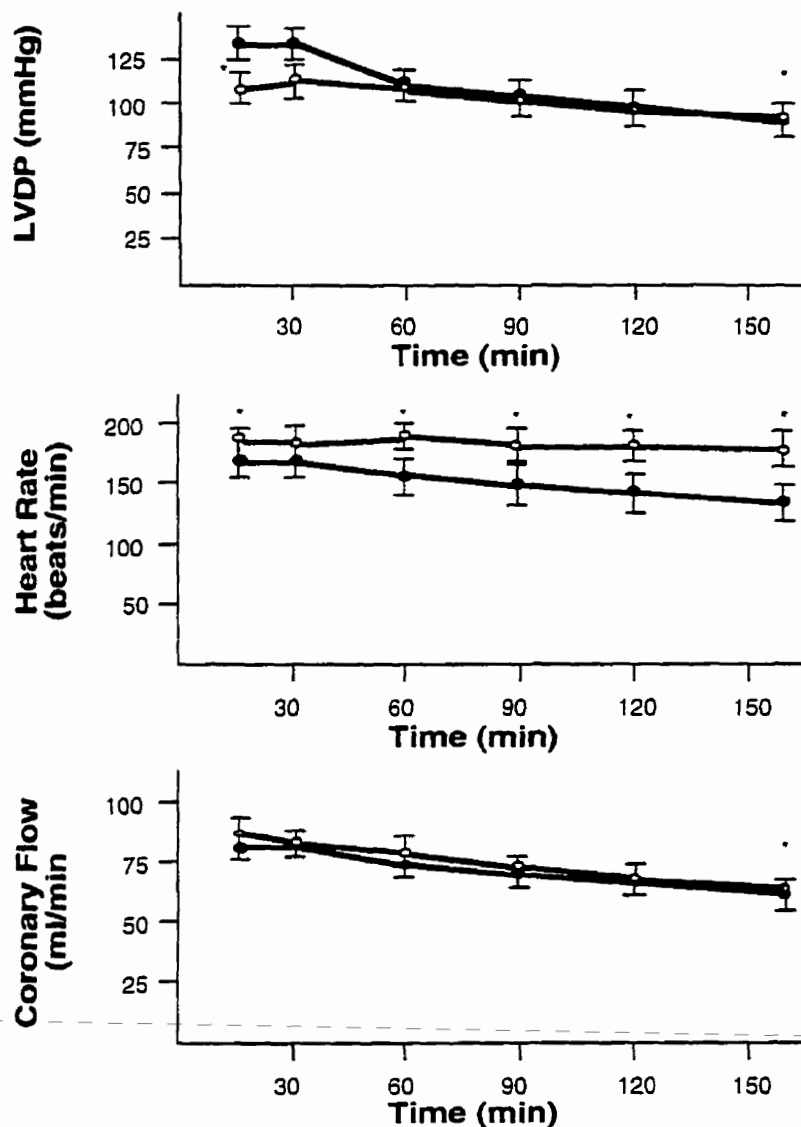
Figure III.3: Time-course graphs for left ventricular developed pressure (LVDP, upper graph), heart rate (central graph) and coronary flow (lower graph) in aerobically perfused paced (○) and non-paced (●) hearts. LVDP, heart rate and coronary flow decreased significantly in nonpaced hearts, by 33%, 12% and 22% of baseline, respectively, after 160 min. of perfusion as compared to baseline values at 15 min. perfusion ($P < 0.05$). Coronary flow also declined significantly in paced hearts after 160 min. of aerobic perfusion (23%) as compared to baseline values. Data are expressed as mean ± S.E.M. * $P<0.05$ versus baseline values at 15 min stabilization.

perfusion. There was no significant difference in the percentage of decline in coronary flow between paced and nonpaced hearts. As expected, paced and nonpaced hearts showed no necrosis, as assessed by TTC, at the end of the perfusion protocol.

2.2   Ischemic Preconditioning: Paced Versus Nonpaced Heart Study:

2.2.1   Infarct Size:

Infarct size data for this study are shown in Figure III.4. Ischemic preconditioning reduced infarct size significantly ($p < 0.01$) in the nonpaced hearts (IP $5.1 \pm 1.4\%$, mean $\pm$ SEM) as compared to nonpaced control hearts (C $26.1 \pm 3.4\%$). However, it did not induce any protection against infarction in paced hearts, Cp $19.7 \pm 3.2\%$ versus IPp $17.0 \pm 4.0\%$, $p > 0.05$. Since pacing the heart reduced the amount of necrosis in the control group, a statistical (Sheffe's test) was performed to assess if the reduction in necrosis was statistically significant. As a result of this analysis, it was found that there was no significant difference ($P>0.05$) in infarct size between paced control hearts ($19.7 \pm 3.2\%$) and nonpaced control hearts($26.1 \pm 3.4\%$). Representative photographs showing the effect of ischemic preconditioning on necrosis are presented in Figure III.5 and III.6.

2.2.2   Ventricular Function:

Left ventricular systolic and diastolic pressures (LVSP and LVDP, respectively), and coronary flow data are shown in Table

Ischemic Preconditioning Study
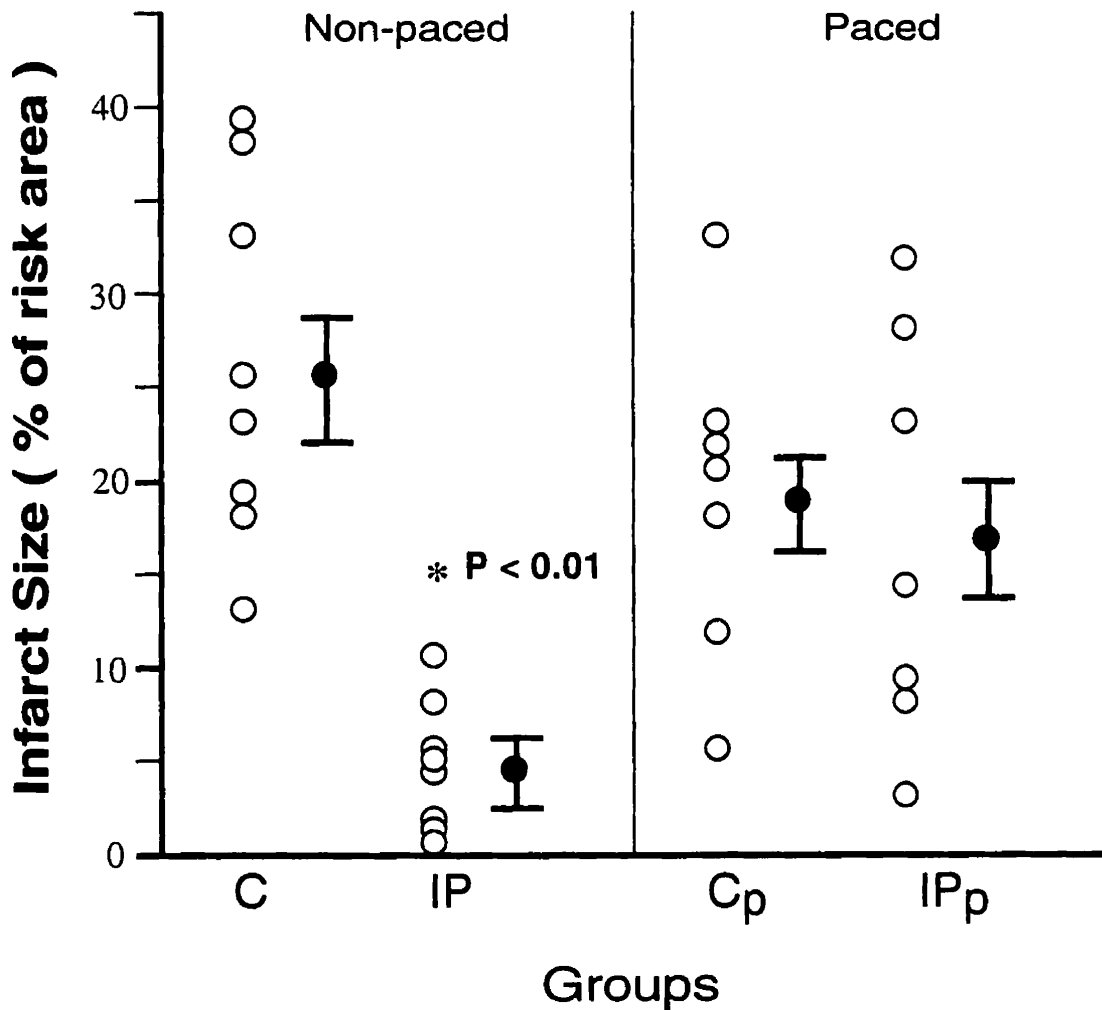
Figure III.4: This graph shows the infarct size measurements expressed as a percentage of risk areas. Ischemic preconditioning reduced infarct size significantly in nonpaced hearts, C 26.1 ± 3.4% versus IP 5.1 ± 3.4%. However, it did not protect against infarction in paced hearts, Cp 19.7 ± 3.2% versus IPp 17.0 ± 4.0%. Data are mean ± SEM. * P < 0.01 versus control group.
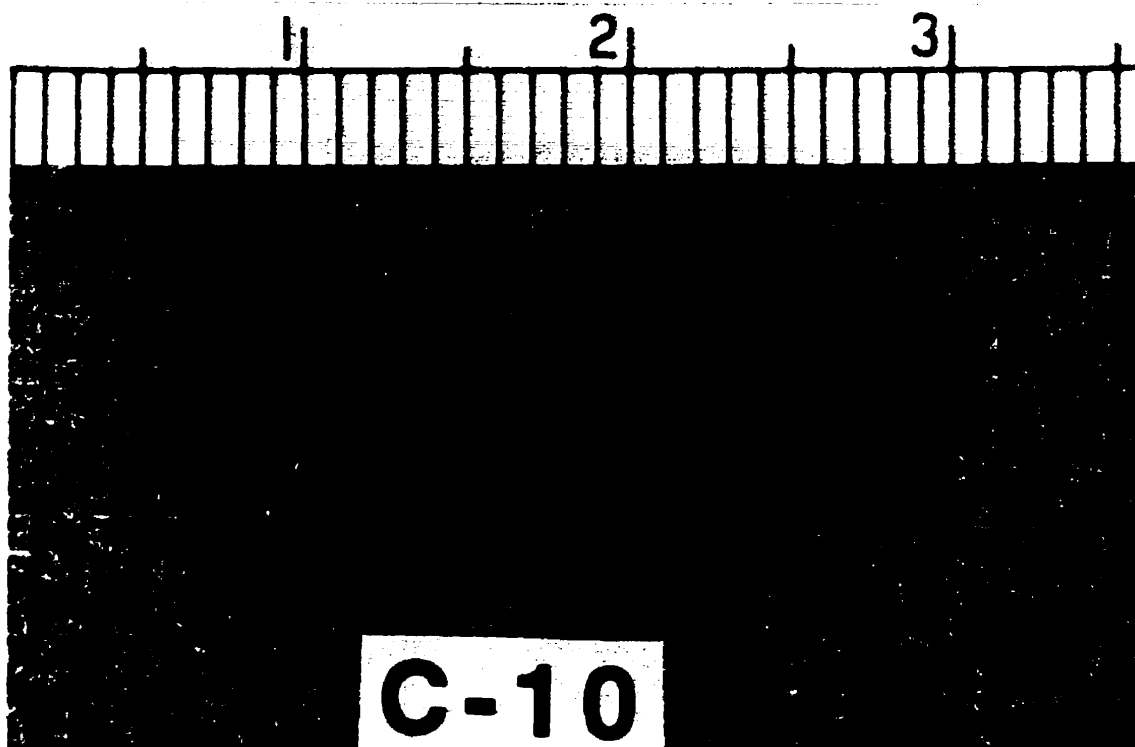
Fig. III.5: This is a representative slice from a control heart showing the TTC staining results obtained after 40 min of normothermic regional ischemia and 60 min of reperfusion. Viable tissue stained brick-red while necrotic tissue stained tan or white color. Region at risk was determined using yellow fluorescent particles which are not seen in this photograph. However, about 80% of the surface area shown in this heart slice picture was at risk.
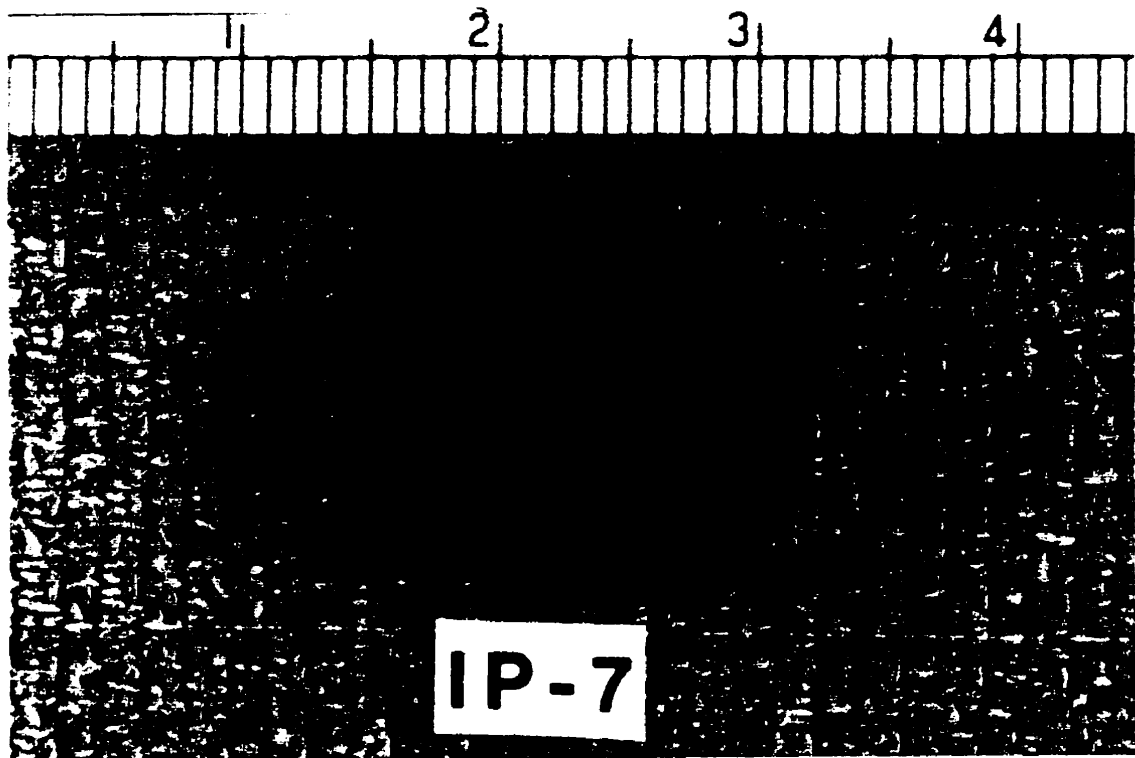
Fig. III.6: This is a representative slice from a preconditioned heart showing the TTC staining results obtained after 40 min of normothermic regional ischemia and 60 min of reperfusion. Viable tissue stained brick-red while necrotic tissue stained tan or white color. Region at risk was determined using yellow fluorescent particles which are not seen in this photograph. About 80% of the surface area was at risk in this heart slice.

III.1. Atrial pacing increased the rise in diastolic pressure in both paced control and preconditioned hearts at the end of 60 min reperfusion as compared to nonpaced control or preconditioned hearts. However, there was no difference in the changes in LVDP among all the groups.

Coronary flow declined significantly in all control and preconditioned groups (Table III.1). However, there was no significant difference between any of the groups compared (C and Cp, and IP and IPp hearts) and coronary flow values obtained at 160 min. of aerobic perfusion in the baseline hearts from the stability study.

A statistical regression analysis performed between paced and nonpaced control and preconditioned hearts showed no association between functional parameters (LVDP and heart rate) or coronary flow and infarct size ($r = 0.12$). In addition, a comparison performed of the area at risk with the necrotic area for all hearts did not show any relationship ($r = 0.32$) between them. Thus, in this study, infarct size was independent of the size of the area at risk.

3. **DISCUSSION**

The aims of the present study were to establish an isolated buffer-perfused rabbit heart model and to characterize the ischemic preconditioning phenomenon in that model. A model of regional ischemia in an isolated buffer-perfused rabbit heart preparation was developed (Figure III.1) in which a stable preparation was

Table III.1

Measurements of left-ventricular developed pressure (LVDP, mmHg)) heart rates (beats/min) and coronary flow (ml/min) for paced and non-paced control (C_p and C, respectively), paced and nonpaced preconditioned (IP_p and IP, respectively) heart groups.

Group	Measurement	15 min Stabilization	Before Ischemia	40 min Ischemia	60 min Reperfusion
C	LVDP	115.5 ± 3.1	98.4 ± 5.2	51.9 ± 5.3	66.1 ± 3.9
	Heart rate	174.1 ± 6.8	177.2 ± 8.8	160.6 ± 12.2	157.4 ± 10.0
	Coronary Flow	76.6 ± 5.0	62.7 ± 5.0	37.3 ± 4.0	44.9 ± 3.8
IP	LVDP	121.7 ± 4.1	92.7 ± 4.8	60.3 ± 3.7	67.5 ± 3.8
	Heart rate	170.0 ± 5.3	166.5 ± 5.7	150.7 ± 6.3	146.5 ± 6.6
	Coronary Flow	82.5 ± 2.3	74.0 ± 3.2	46.5 ± 3.4	50.0 ± 4.0
C_p	LVDP	100.8 ± 6.6	98.1 ± 6.6	58.2 ± 5.0	55.6 ± 4.7
	Heart rate	181.4 ± 1.4	179.9 ± 0.0	149.7 ± 9.8	180.0 ± 0.0
	Coronary Flow	87.5 ± 3.5	85.0 ± 3.8 *	57.5 ± 4.3 *	68.0 ± 3.3 *
IP_p	LVDP	104.0 ± 5.0	89.3 ± 5.8	61.7 ± 5.9	56.4 ± 3.9
	Heart rate	178.9 ± 0.8	169.3 ± 6.1	156.6 ± 8.5	184.0 ± 0.0 †
	Coronary Flow	84.5 ± 2.3	78.0 ± 2.4	57.0 ± 4.6	62.8 ± 3.6

IP = ischemic preconditioning. All data are mean ± S.E.M. * P < 0.05 versus the equivalent non-paced control value.
† P < 0.05 versus the equivalent non-paced IP.

demonstrated (Figure III.2). It was found that in hearts not subjected to atrial pacing (constant heart rate), ischemic preconditioning induced by three cycles of 5 min ischemia and 10 min of reperfusion protected the isolated hearts against ischemic/ reperfusion injury by reducing the amount of necrosis significantly (Figure III.3).

In the stability study, nonpaced rabbit hearts aerobically perfused in an isolated buffer-perfused heart model were found to have a consistent heart rate decline over time (Figure III.3). Because of early concerns about effects on the ischemic preconditioning phenomenon in this model resulting from such moderate fall in heart rate after 160 min of aerobic perfusion, it was critical to compare this group of nonpaced hearts with a second group of hearts in which aerobically perfused hearts were paced at 200 beats/min via the left atrium for 160 min. As a result, no significant difference was found on either LVDP or coronary flow between the two groups. In addition, in both groups of hearts, LVDP and coronary flow decreased significantly ($P<0.05$) after 160 min of aerobic perfusion. However, the levels to which LVDP and coronary flow fell did not jeopardize an effective perfusion of the myocardium or caused any ischemic injury to it as demonstrated by the absence of necrosis observed in all hearts at the end of the aerobic perfusion.

In characterizing the ischemic preconditioning phenomenon, it was found in pilot experiments that if the heart was paced via the left atrium continuously, including during the long ischemic period, infarct size was increased substantially as

compared to infarct size obtained in control hearts. The reason why necrosis increased with atrial pacing during ischemia in these experiments remains undetermined. However, it is quite possible that, since the demands for energy are increased during ischemia as more ATP is required for the uncoupling mechanism of the contractile apparatus, atrial pacing may have increased the demands for ATP by the ischemic myocardium, causing the depletion of energy reserves earlier. Infarct size was not affected when atrial pacing was used only during perfusion periods in control hearts, but it blocked the protective effect of ischemic preconditioning (Figure III.4). A possible explanation for not being able to precondition atrial-paced hearts may be that, in those experiments, there was low statistical power to detect a difference between control and preconditioned groups as demonstrated by a large variability in the infarct size data in both groups (Figure III.4). Nonetheless, the amount of protection obtained in preconditioned hearts not subjected to atrial pacing was consistent with the amount of protection observed in rabbit hearts with similar models, by other investigators[8, 29, 30, 103, 181].

It has been shown recently by Vegh et al[180] that a short period of rapid ventricular overdrive pacing (500 beats/min) mimics the protective effect of ischemic preconditioning against ventricular ectopic beats induced by a sustained coronary occlusion in dog hearts. In contrast, Marber et al[182] could not reduce infarct size in situ in hearts with a single 5 min period of rapid atrial pacing (300 beats/min). The atrial pacing experiments in the present study differ from those from Marber et al[182] and Vegh et

al[180] in that in the present study the hearts were paced at a much lower rate. Furthermore, in a recent study performed by Szilvassy et al[183] 5 min of ventricular overdrive pacing (500 beats/min) markedly attenuated the increase in left ventricular end-diastolic pressure induced by a longer period of ventricular overdrive pacing in anaesthetized rabbits. In that experiment[183], 5-min ventricular overdrive pacing slightly increased cGMP and profoundly elevated cAMP content in left ventricular tissue. However, when this ventricular pacing was preceded by a preconditioning ventricular overdrive pacing, the cAMP increase was significantly attenuated, whereas the cGMP increase was made larger[183]. Although these data suggest that pacing can induce protection, possibly through a mechanism related to ischemic preconditioning, paced hearts in the present study were not protected as determined by infarct size. An analysis of the levels of cAMP in paced control and/or preconditioned hearts was not performed in the present study. However, one may speculate that changes induced by atrial pacing on levels of cAMP in the heart might have interfered with the ischemic preconditioning protective signal(s) and blocked the protection against infarction observed in this study in hearts not subjected to atrial pacing.

Left ventricular developed pressure was assessed in this study in an attempt to determine if there is any significant protection against postischemic ventricular dysfunction by ischemic preconditioning in rabbit hearts. Ischemic preconditioning did not improve postischemic LVDP in this study, suggesting that, in the rabbit, the protection against postischemic

ventricular dysfunction is not present. In contrast to the present results, Cohen et al[47] found improved wall-motion and lower infarcts in rabbit hearts after transient coronary occlusions, suggesting an improvement in ventricular function with ischemic preconditioning. In addition, in an isolated blood-perfused rabbit heart model, Sandhu et al [29] have shown that a single cycle of 5 min ischemia followed by 10 min of reperfusion may improve postischemic ventricular function as suggested by a trend to lower end-diastolic pressure measured at the end of reperfusion in preconditioned hearts which was close to being significantly lower when compared to end-diastolic pressure in control hearts. Experiments performed more recently by Sandhu et al [55] showed that three cycles of transient ischemia and reperfusion produced a more robust phenomenon in terms of infarct-size limiting effect in an in vivo rabbit heart model of regional ischemia. These data suggest that it might be possible to find a significant improvement in end-diastolic pressure in preconditioned hearts by using three cycles of transient ischemia and reperfusion to induce the protective effect in an isolated blood perfused model.

In summary, ischemic preconditioning induced by three cycles of transient regional ischemia and reperfusion protects the heart against necrosis produced by a subsequent long period of ischemia and reperfusion in the isolated rabbit heart model. However, three-cycle ischemic preconditioning does not protect the hearts against postischemic ventricular dysfunction. Furthermore, atrial pacing blocks the protective effect of three-cycle ischemic preconditioning against necrosis in this model.

IV ANGIOTENSIN II RECEPTORS IN ISCHEMIC PRECONDITIONING: IN VITRO STUDY

The aims of this study were to determine the participation of AII receptors in triggering the ischemic preconditioning protection against infarction and to identify which AII receptor subtype is involved in ischemic preconditioning. To address these issues AII receptors were first stimulated prior to an ischemic/reperfusion injury episode to determine if such stimulation would result in protection against myocardial infarction. Then, any potential activation of AII receptors was completely blocked in preconditioned hearts to determine how important AII receptor activation was in producing the ischemic preconditioning protection against infarction. Subsequently, after AII receptors were found to participate in triggering the ischemic preconditioning phenomenon, selective inhibition of each AII receptor subtype, AT_1 and AT_2, was performed in preconditioned hearts to identify the relative involvement of each AII receptor subtype in ischemic preconditioning.

1. MATERIALS AND METHODS:

The surgical preparation and the ventricular function, the coronary flow and the infarct size measurement protocols used in this study were all identical to those used to characterize the ischemic preconditioning phenomenon protection against

infarction in the study "Ischemic Preconditioning in Isolated Rabbit Hearts" which has been described in detail in Chapter III.

1.1   Experimental Protocol:

1.1.1   Saralasin and Losartan Dose-Response Studies:

In these studies, the dose for saralasin and losartan that completely blocked any potential activation of AII receptor during three cycles of transient ischemia and reperfusion was determined based on two independent dose-response experiments performed in 28 isolated buffer-perfused rabbit hearts. Since rabbit hearts have AII receptors [162, 184] through which coronary flow can be modulated [185], the ability of increasing concentrations of saralasin (100 nM, 500 nM, 1 µM, n = 4 hearts per each dose) and losartan (100 nM, 1 µM, 10 µM and 20 µM, n = 4 hearts per each dose) to antagonize the effects of AII (100 nM) on coronary flow was tested as follows: Each heart was first aerobically perfused for 15 min to allow it to stabilize. Baseline for coronary flow was recorded at this time point. Then, the heart was exposed for 5 minutes to AII (100 nM) to obtain a baseline response (coronary flow reduction due to vasoconstriction) to the AII stimulus. The heart was then aerobically perfused for 20 min without any drug to allow recovery of coronary flow to baseline values. Subsequently, an infusion of either saralasin or losartan, at different concentrations, was initiated. After 10 min of aerobic perfusion, each heart treated with either one of these two AII receptor inhibitors received a challenge dose of AII to determine the degree

of receptor blockade at the different concentrations of each inhibitor. Each heart was given five AII doses. The last dose of AII that each heart received was given 20 min after the AII receptor inhibitor had been stopped, to assess the heart response to AII and rule out any desensitization of AII receptors.

The effect of increasing concentrations of saralasin and losartan on the coronary vasoconstrictor effect of AII (100 nM) are shown in Figures IV.1 and IV.2. Saralasin, a nonselective peptide AII antagonist, blocked the vasoconstrictor effect of AII on the coronary flow in a dose-dependent fashion and it blocked this effect completely at a concentration of 1 μM (Figure IV.1). At that concentration, saralasin also blocked the positive inotropic effect of AII completely. Similarly, losartan, a selective nonpeptide AII antagonist, inhibited the vasoconstrictor effect of AII in a dose-dependent fashion. Complete blockade of AT_1 receptors, as determined by the absence of coronary vasoconstriction, was consistently obtained with 20 μM losartan (Figure. IV.2). Even though it is likely that more than 80% of the AT_1 receptors were blocked by 10 μM losartan, this dose was obviously not enough to completely block the inotropic effect induced by 100 nM AII as evidenced by a consistently observed increase in systolic pressure. When the concentration of losartan was increased to 20 μM, as was done in the dose response experiments, the inotropic response to AII was completely abolished. None of the hearts used in the dose-response experiments experienced any loss of responsiveness to AII (tachyphylaxis) in terms of ventricular function or coronary flow.

In addition, another group of four hearts, which were aerobically perfused for 15 min, were used to test the specificity of receptor blockade by losartan. Each heart was initially exposed to endothelin-1 (ET-1, 1 nM) which was given through a side port over a period of one minute. This ET-1 dose was selected based of its moderate effect on coronary vasoconstriction, resulting in approximately 20% reduction in coronary flow (Figure IV.2), which would allow the heart to recover fully once the drug infusion was stopped. After baseline response to ET-1 was obtained and coronary flow has recovered, an infusion of losartan at 20 μM, a dose which completely blocked the AT_1 receptors, was initiated. Then, a second dose of ET-1 was co-administered to induce the same vasoconstrictor effect as in the baseline response, but in the presence of AT_1 receptor blockade. The main objective of these experiments was to isolate the effect of losartan and to demonstrate that other receptors remained fully functional even in the presence of losartan. ET-1 infusion (1 nM) produced an equivalent vasoconstrictor effect as AII (100nM), as determined by a moderate reduction in coronary flow, in the presence or absence of complete AT_1 receptor blockade (Figure IV.2).

1.1.2 Ischemic Preconditioning: Angiotensin II Receptors Study:

A total of 65 hearts were used to assess the role of AII receptors in ischemic preconditioning. These hearts were divided into eight groups and subjected to an initial period of 15 min of aerobic perfusion (stabilization period) followed by 40 min of normothermic (37° C) regional ischemia and 60 min of reperfusion

Saralasin Dose-Response
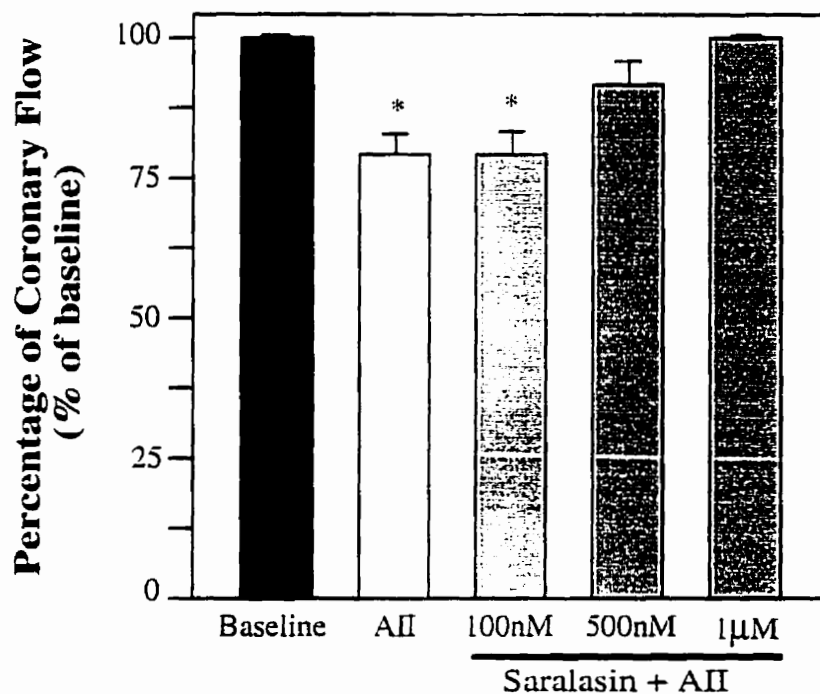
Figure IV.1: The effect of saralasin on the coronary vasoconstrictor response to AII (100 nM) is shown in this graph. AII alone produced a 22% reduction in coronary flow. This AII-mediated coronary reduction was completely abolished in the presence of 1 μM saralasin. Data are mean ± SEM. *P < 0.05 versus AII.

Losartan Dose-Response
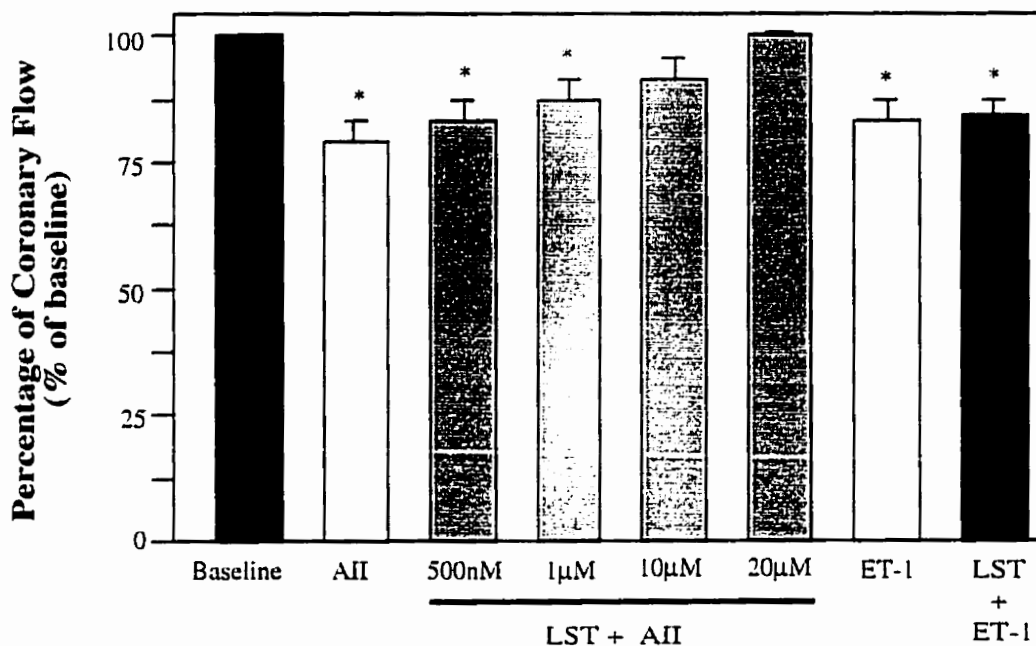
Figure IV.2: The effect of increasing the dose of losartan on coronary flow is shown in this graph. AII (100 nM) alone produced a 22% reduction on coronary flow. Low doses of losartan (500 nM and 1 μM) did not alter the vasoconstrictor effect of a challenging dose of AII. Although higher doses of losartan (10 and 20 μM) blocked the vasoconstrictor effect of AII completely, the AII positive inotropic effect (systolic pressure increase) was totally abolished only at 20 μM concentration of losartan. In addition, endothelin-1 (ET-1), a powerful vasoconstrictor, produced an equivalent reduction on coronary flow at 1 nM concentration (one minute exposure only), effect that was still present even in the presence of 20 μM losartan. Data are mean ± SEM. * P <0.05 versus baseline.

(Figure IV.3). Control hearts (C, n = 9) were subjected to the initial 15 min of stabilization followed by an additional 45 min of aerobic perfusion (total stabilization period = 60 min) before the 40 min of ischemia and 60 min reperfusion. The extra 45 min of aerobic perfusion was given to control hearts to compensate for the time required to precondition each heart and, thus to equalize the total length of the experimental protocol among all groups. Ischemic preconditioned hearts (IP, n = 9) were subjected to three cycles of 5 min normothermic regional ischemia followed by 10 min of reperfusion prior to the long ischemia. To test the ability of a transient activation of AII receptors to protect the myocardium, a third group of hearts was treated with AII (AngII group, n = 8). These AII-treated hearts were aerobically perfused for an additional 30 min after the initial 15 min stabilization (45 min altogether). At the end of this additional perfusion period each heart received a five-minute AII (100 nM) infusion followed by a ten-minute, drug-free, perfusion. This dose of AII was selected based on a recent study which showed that AII induced a cardioprotective effect when it was administered over a five-minute period before a long period of ischemia [108]. To assess the participation of AII receptors in inducing the ischemic preconditioning protection and to identify which AII receptor subtype is involved in ischemic preconditioning, three different AII inhibitors were given to five groups of hearts. These hearts were subjected to the same protocol as control and IP groups, except for the administration of AII receptor inhibitors. Thus, saralasin (1 μM), a specific AII receptor antagonist which inhibits both AT_1 and AT_2 receptor subtypes, was
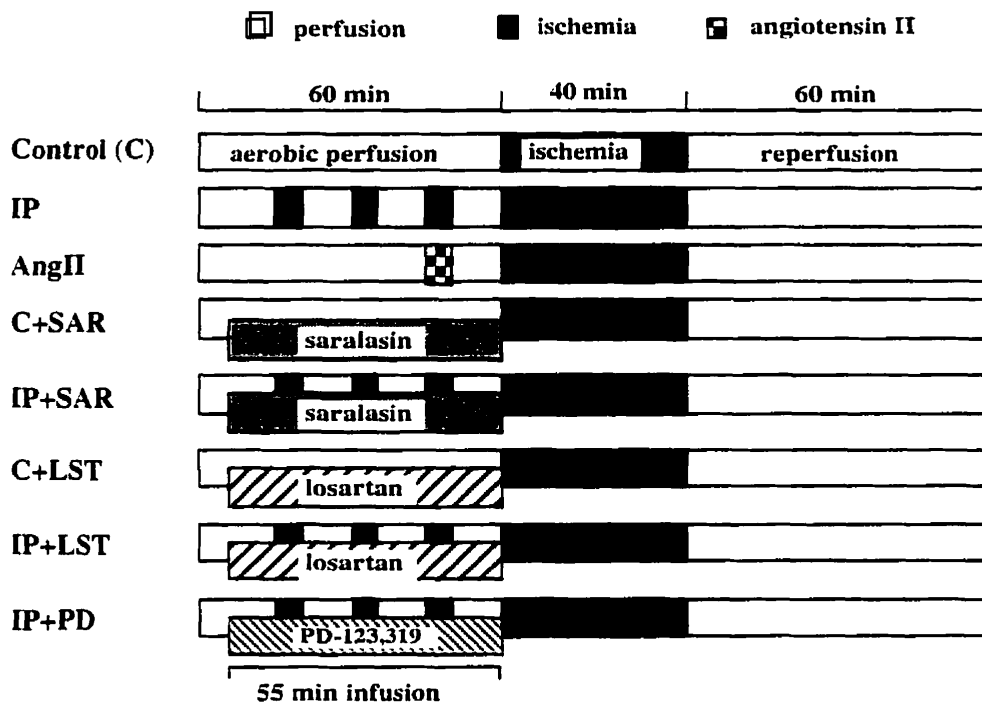
Figure IV3: Isolated rabbit heart experimental protocol used in the ischemic preconditioning study. Sixty-five hearts were subjected to 15 min of aerobic perfusion prior to any ischemic insult. Prolonged regional ischemia (40 min at 37°C) was followed by 60 min of reperfusion. Control (C) hearts were subjected to an additional 45 min (for a total of 60 min) of aerobic perfusion prior to the long ischemia. Ischemic preconditioned (IP) hearts were also subjected to three successive cycles of 5 min of regional ischemia and 10 min of reperfusion prior to the long ischemia. Heart in the AII-treated group (AngII), were pretreated with AII for a period of 5 min followed by a drug-free period of 10 min aerobic perfusion before the major ischemic insult. Other different groups of control and preconditioned hearts were treated with a constant infusion of either saralasin (C+SAR and IP+SAR) or losartan (C+LST and IP+LST). A single group of preconditioned hearts were also treated with a similar constant infusion of PD-123,319 ditrifluoroacetate (IP+PD). These continuous infusions were started 10 min prior to the first ischemic preconditioning cycle and ended just before the long ischemia.

given to control (C+SAR, n = 8) and IP (IP+SAR, n = 8) hearts for 55 min in a constant infusion, beginning at 5 min into the aerobic perfusion period and ending just before the long ischemia. Losartan (20 μM), a specific AT_1 receptor subtype inhibitor was given using the same infusion protocol as saralasin, to control (C+LST, n = 8) and IP (IP+LST, n = 9) hearts to assess the participation of AT_1 receptors in ischemic preconditioning. Similarly, a specific AT_2 receptor subtype inhibitor, PD-123,319 ditrifluoroacetate (PD, 10 μM)), was given using the same infusion protocol as in the other groups to IP (IP+PD, n = 6) hearts to assess the participation of AT_2 receptors in the phenomenon.

The dose used for saralasin and losartan were selected based on dose-response experiments. Since AT_2 receptors do not yet have any recognized biological function, the dose selected for PD was based on reports from other investigators [108, 186] who used the drug to selectively inhibit the AT_2 receptors.

1.1.3 Exclusions:

Exclusion criteria were established before the study began. These criteria were selected based on experience obtained in the laboratory in pilot experiments. The exclution criteria were as follows: 1) a heart that was not hung and perfused within 30 seconds; 2) a heart that would not achieve an acceptable LVDP (greater than 75 mmHg) after 15 min of aerobic perfusion; 3) any experiment in which an ischemic period was inadvertently extended; 4) any case where necrosis was not measured accurately due to staining failure; 5) any case where the heart fibrillated during ischemia; and 6) any heart in which there was air in the

aortic cannula or aortic root which could not be evacuated without affecting the perfusion pressure. Using these criteria, a small number of hearts were excluded from these studies for various reasons: One control, one IP, and two AII-treated hearts were excluded when an air bubble was found in the aortic root that restricted the coronary flow while the experiments were being carried out. One IP+PD heart was dismounted from the perfusion apparatus because of a perforation on the coronary artery subjected to occlusion. One IP+LST heart was excluded when it was realized that it had necrotic patches (presumably caused by air embolism not detected earlier in the experiment) outside the area at risk.

1.2   Chemical Reagents and Drug:

Angiotensin II and saralasin (Sigma Chemical Company Inc., St. Louis, MO, USA), were dissolved in small water aliquots (40 µg/80 µl and 5 mg/50 µl each, respectively) and stored at − 20° C. Both drugs were diluted, in 1 ml and 3 ml of oxygenated buffer, respectively. PD-123,319 ditrifluoroacetate (Research Biochemical International Inc., Natick, MA, USA) and losartan (a generous gift from DuPont Merck Inc., Wilmington, DE, USA) were dissolved in millipore-filtered water.

1.3   Statistical Analysis:

All data are expressed as mean ± S.E.M. Factorial ANOVA was used to assess difference among the heart groups. Where appropriate, the Scheffé F-test was applied to determine whether a statistically significant difference ($P < 0.05$) existed between two

groups. Regression analysis was performed to assess any relationship between risk area and necrotic area, and between LVDP and necrosis. Correlation analysis was also performed to assess for association between necrosis or LVDP and coronary-flow measurements.

2. RESULTS

2.1 Ischemic Preconditioning: Angiotensin II Receptors Study:

2.1.1 Infarct Size:

Infarct size (mean ± S.E.M.) for all groups and for each individual heart is shown in Figure. IV.4. Three cycles of ischemic preconditioning reduced infarct size significantly ($P < 0.001$), from 26.4 ± 3.0% of area at risk in the control group down to 5.1 ± 1.2% in the IP group. Protection was also achieved with the activation of AII receptors with a five-minute exposure to 100 nM AII (9.6 ± 2.2%, $P < 0.01$ v untreated control group) (Figure IV.4A). In addition, when AII receptors were competitively antagonized with saralasin prior to and during the preconditioning cycles, the protection against necrosis was lost (IP+SAR 29.7 ± 3.2%, $P < 0.001$ v untreated IP group) suggesting a participatory role of AII receptors in IP. Saralasin alone did not have any effect on necrosis (C+SAR 31.5 ± 3.9%, $P > 0.05$ v untreated control group) (Figure IV.4A). Furthermore, the protective effect of ischemic preconditioning against necrosis was also abolished when selective blockade of the AII receptors (AT_1) was accomplished with a constant infusion of 20 μM losartan, IP+LST 27.4 ± 3.0%, $P < 0.001$ v

untreated IP group. Losartan alone did not have any effect on necrosis (C+LST 29.3 ± 2.0%, P > 0.05 v untreated control group). However, selective blockade of AT_2 AII receptors with a constant infusion of 10 µM PD-123,319 ditrifluoroacetate did not block the effect of ischemic preconditioning on infarct size, IP+PD 7.9 ± 1.2%, P > 0.05 v untreated IP group (Figure IV.4B).

There was no relationship, indicated by a regression analysis, between risk area and infarct size for all control (r = 0.03) and all preconditioned (r = 0.01) hearts (Figure IV.5A and Figure IV.5B respectively).

2.1.2   Ventricular Function:

Data for LVDP and heart rate are presented in Table IV.1. Three cycles of ischemic preconditioning did not protect against ischemia-induced left ventricular dysfunction in the isolated heart model as evidenced by the absence of any significant improvement in the recovery of LVDP after 60 min. of reperfusion in the IP group as compared to the control group. Although the recovery of left ventricular function after ischemia was significantly (p < 0.05) improved with losartan in both control (C+LST) and IP (IP+LST) hearts at the end of 60 min reperfusion, this effect did not influence the outcome on necrosis since a regression analysis done between the two parameters (LVDP versus necrotic area) showed no relationship for both treated control (r = 0.12) and treated preconditioned (r = 0.28) hearts.
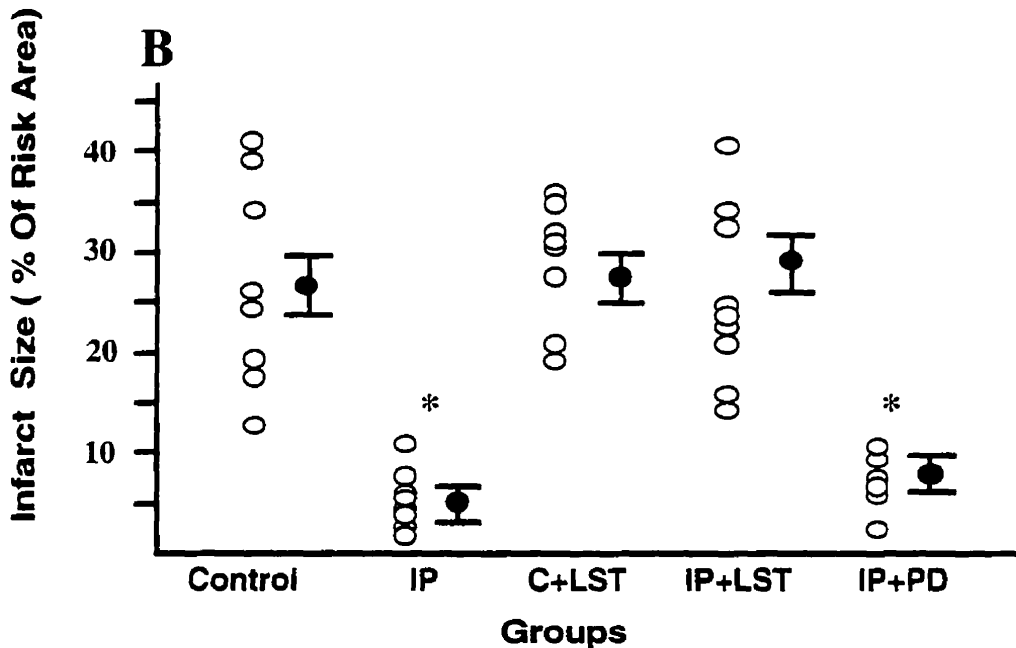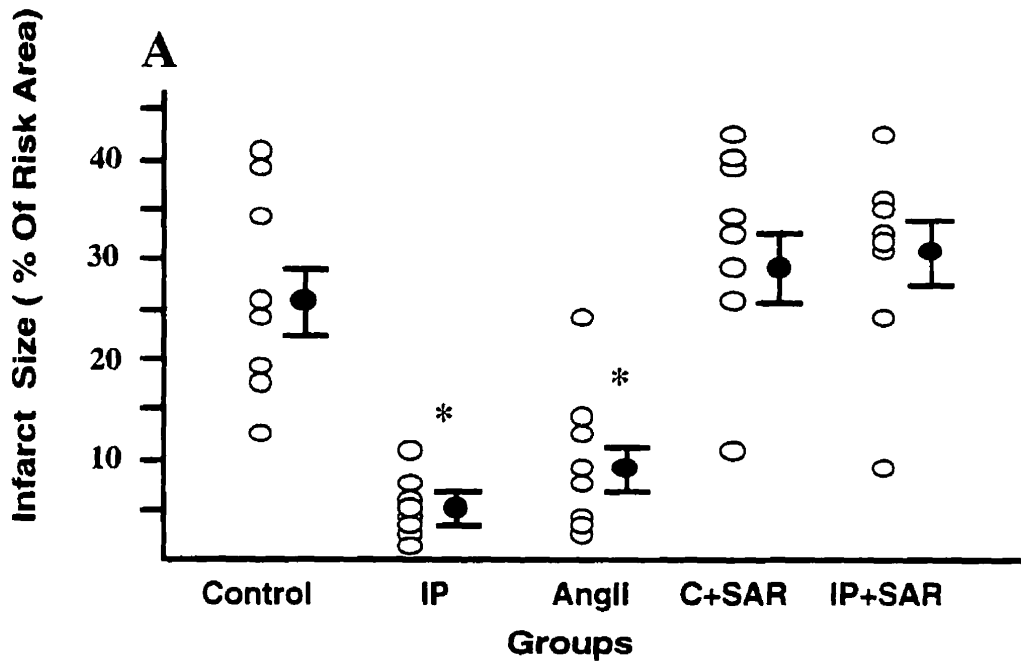
Figure IV.4: Infarct size measurements expressed as percent of the risk area (mean ± S.E.M.) for each group of hearts. Control (C, n = 9), preconditioned (IP, n = 9), AII-treated (AngII, n = 8), saralasin-treated (C+SAR and IP+SAR, n = 8/each), and losartan-treated (C+LST and IP+LST, n = 8 and n = 9, respectively), PD-123,319 ditrifluoroacetate-treated (IP+PD, n = 6) groups are shown (A and B, respectively). Ischemic preconditioning reduced infarct size significantly. Similarly, AII (100 nM) infusion reduced infarct size significantly. Furthermore, saralasin (1 µM), a potent AII receptor antagonist, blocked the protective effect of ischemic preconditioning. Similarly, losartan (20 µM), a selective AT_1 AII receptor antagonist, completely abolished the preconditioning protection against necrosis. PD (10 µM), a selective AT_2 AII receptor antagonist, did not alter necrosis in preconditioned hearts. * P <0.05 versus control group.

Changes in the inotropic state of the heart were observed in hearts treated with AII (data expressed as mean ± S.E.M.). Five-minute infusion of AII (100 nM) induced a significant increase in LVDP, from 100.0 ± 3.4 mmHg prior to the AII infusion (45 min. stabilization) up to 140 ± 3.5 mmHg ($P < 0.001$). Left ventricular diastolic pressure and heart rate did not change significantly in response to AII (3.9 ± 0.6 mmHg and 159.8 ± 6.2 beats/minute, respectively) as compared to their equivalent values at 45 min. stabilization (4.3 ± 0.3 mmHg and 173.0 ± 4.4 beats/minute, respectively).

2.1.3 Coronary Flow:

Coronary flow data (mean ± S.E.M.) are shown in Table IV.2. Coronary flow decreased significantly ($P < 0.05$) in untreated control, untreated IP, and AII-treated hearts after 60 min. of reperfusion as compared to values obtained at 15 min. of stabilization. There was no difference in coronary flow between any of the groups (at 60 min. of reperfusion) and the baseline group (at 160 min. of aerobic perfusion), indicating that the reduction in coronary flow observed in these groups was likely an inherent effect of the preparation. In AII-treated hearts, a five-minute infusion of AII (100 nM) reduced coronary flow by 15%, from 77.8 ± 3.0 ml/min (mean ± S.E.M.) at 45 min of stabilization to 66.3 ± 3.7 ml/min after 5 min of exposure to AII. The vasoconstrictor effect of AII did not disappear completely before the long ischemia as indicated by the partial recovery of coronary flow after 10 min of

A

B
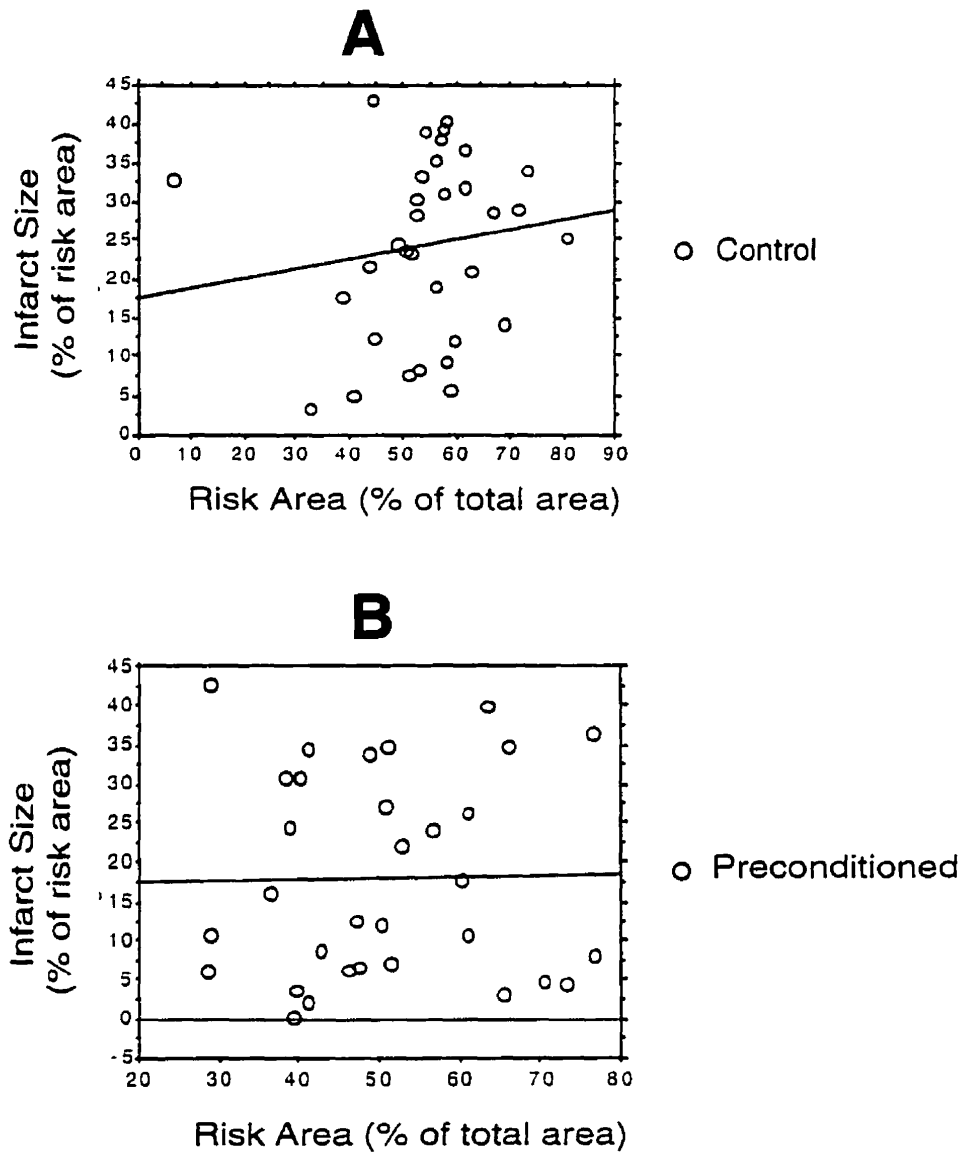
Figure IV.5: This graphs show the scattergram of a regression analysis performed to assess if there was any association between risk area (% of total area) and infarct size (% of risk area) in all control hearts (A) and preconditioned hearts (B). r values were 0.03 for control hearts and 0.01 for preconditioned hearts. Thus, in our experiments infarct size was independent of risk area.

Table IV.1

Measurements of left-ventricular developed pressure (LVDP, mmHg) and heart rates (beats/min) in isolated hearts.

Group	Measurement	15 min Stabilisation	Before Ischemia	40 min Ischemia	60 min Reperfusion
Control	LVDP	115.5 ± 3.1	98.4 ± 5.2	51.9 ± 5.3	66.1 ± 3.9
	Heart rate	174.1 ± 6.8	177.2 ± 8.8	160.6 ± 12.2	157.4 ± 10.0
IP	LVDP	121.7 ± 4.1	92.7 ± 4.8	60.3 ± 3.7	67.5 ± 3.8
	Heart rate	170.0 ± 5.3	166.5 ± 5.7	150.7 ± 6.3	146.5 ± 6.6
AngII	LVDP	119.9 ± 7.8	94.4 ± 4.8	55.2 ± 3.2	64.2 ± 3.7
	Heart rate	173.0 ± 4.4	159.8 ± 6.5	151.0 ± 6.8	149.4 ± 7.4
C+SAR	LVDP	100.0 ± 7.6	95.0 ± 5.7	57.6 ± 3.2	56.8 ± 6.4
	Heart rate	178.9 ± 3.9	167.2 ± 5.4	155.0 ± 5.6	160.7 ± 7.1
IP+SAR	LVDP	110.4 ± 7.0	92.9 ± 5.5	71.2 ± 6.7	70.2 ± 4.9
	Heart rate	176.2 ± 2.6	170.3 ± 5.0	154.2 ± 7.7	156.5 ± 8.8
C+LST	LVDP	126.9 ± 3.0 *	120.4 ± 3.9 *	75.2 ± 4.4 *	80.8 ± 3.4 *
	Heart rate	175.0 ± 3.2	162.5 ± 4.5	150.0 ± 6.5	150.0 ± 8.4
IP+LST	LVDP	122.5 ± 5.8	112.0 ± 3.5	72.8 ± 5.9 †	84.3 ± 3.7 †
	Heart rate	171.1 ± 3.0	160.0 ± 3.3	142.2 ± 7.0	142.2 ± 7.0
IP+DP	LVDP	109.2 ± 8.4	90.0 ± 6.1	52.9 ± 5.9	70.6 ± 5.5
	Heart rate	182.4 ± 7.1	172.9 ± 6.3	144.9 ± 10.0	154.3 ± 8.5

All data are means ± S.E.M. Before ischemia = end of 60 min aerobic perfusion or end of three IP cycles. IP = ischemic preconditioning; AngII = angiotensin II group; C = control; SAR = saralasin; LST = losartan; PD = PD 123,319 ditrifluoroacetate. * P < 0.05, versus the equivalent value in the control group.† P < 0.05, versus the equivalent value in the IP group.

drug-free aerobic perfusion (Table IV.3). Saralasin (1 µM) and losartan (20 µM), which completely blocked the vasoconstrictor effect of AII infusion in our dose-response experiments (Fig. 8-9), attenuated this reduction in coronary flow in treated control (C+SAR and C+LST) and in treated IP (IP+SAR and IP+LST) hearts. A similar effect was observed in hearts treated with PD-123,319 ditrifluoroacetate (IP+PD). However, there was no relationship between coronary flow and infarct size for both control (r = 0.1) and preconditioned (r = 0.38) hearts. This indicates that the high rate of coronary flow observed in hearts treated with these drugs is an independent variable not related to infarct size. Furthermore, the increase in coronary flow, which is attributed to a vasodilator response of AII receptor blockade, was sustained throughout the experimental protocol.

Table IV.2.
Coronary flow measurements obtained from isolated buffer-perfused rabbit hearts in the ischemic preconditioning study.

Groups	15 min Stabilization	Before Ischemia	40 min Ischemia	60 min Reperfusion
Control	76.6 ± 5.0	62.7 ± 5.0	37.3 ± 4.0	44.9 ± 3.8
IP	82.5 ± 2.3	74.0 ± 3.2	46.5 ± 3.4	50.0 ± 4.0
AngII	84.0 ± 4.5	72.5 ± 4.8	47.5 ± 4.8	49.5 ± 5.6
C+SAR	84.0 ± 1.5	82.0 ± 3.0 *	60.5 ± 3.7 *	70.5 ± 4.1 *
IP+SAR	90.0 ± 2.8	83.5 ± 3.4	69.1 ± 7.7 †	68.0 ± 3.9 †
C+LST	88.0 ± 3.5	85.5 ± 3.2 *	59.7 ± 3.8 *	65.8 ± 3.6 *
IP+LST	92.0 ± 3.6	94.6 ± 2.2 †	61.5 ± 3.5 †	71.6 ± 3.2 †
IP+DP	91.0 ± 3.3	90.0 ± 5.0 †	56.0 ± 5.1	69.3 ± 7.0 †

Data are means ± S.E.M., in milliliters per minute (ml/min). IP = ischemic preconditioning; AngII = angiotensin II; SAR = saralasin; LST = losartan; PD = PD-123,319 ditrifluoroacetate. * $P < 0.05$, versus the equivalent value in the control group. † $P < 0.05$, versus the equivalent value in the IP group.

3. DISCUSSION

The main objective of this study was to assess the role of AII receptors in ischemic preconditioning. There is substantial evidence suggesting the participation of at least three cardiac receptors, adenosine A_1[103], α_1-adrenergic[109, 187], and bradykinin B_2[34], in ischemic preconditioning. Activation of all these receptors is thought to lead to activation of PKC and consequently to the phosphorylation of one or more effector proteins. However, the diversity of known effects resulting from the activation of these receptors opens the possibility for the participation of other pathways in ischemic preconditioning. Since AII levels increase in the heart during ischemia[100-102], I hypothesized that AII receptors, which activate PKC, may participate in ischemic preconditioning. My results suggest that the AT_1 receptor subtype participates in the triggering mechanism of ischemic preconditioning, and that in the isolated rabbit heart model, activation of these receptors must occur during the short ischemia/reperfusion cycles for ischemic preconditioning to limit myocardial infarction. These conclusions are supported by my observations from the present study that activation of AII receptors causes infarct-limiting effects which resemble those of ischemic preconditioning, and selective blockade of AII receptors (AT_1 subtype) abolishes the protection of ischemic preconditioning against infarction.

Recently, Liu et al. [108] suggested that any receptor stimulation that activates PKC may induce a protective effect

similar to ischemic preconditioning. Their proposal is supported by some evidence that indicates that specific activation of adenosine A_1 [39, 103], α_1-adrenergic [109, 187], and bradykinin B_2 receptors [34] protects the heart against infarction. In our study in isolated hearts, specific stimulation of all AII receptors with five-minute exposure to AII (100 nM) rendered the hearts protected against a subsequent period of prolonged ischemia and reperfusion. These data confirm Liu's findings [108] which show that transient activation of AII receptors prior to ischemia can be as protective against ischemic injury as ischemic preconditioning. These authors demonstrated that this cardioprotective effect of AII could be reversed by inhibiting PKC activation with polymyxin B, suggesting a mediator role of PKC activation in such protection. However, AII may also activate other pathways that may be necessary to realize protection. For example, besides activating PKC, AII can also activate the Na^+/H^+ exchanger [138], which in the event of sustained ischemia may facilitate the extrusion of H^+ ions and therefore delay the onset of severe intracellular acidosis. Ischemic preconditioning has been found to preserve intracellular pH ($[pH]_i$) during sustained ischemia [94]. Prevention of ischemic acidosis appears to require PKC δ at the sarcolemma in the rat [43] and PKC translocation to the sarcolemma is thought to play a key role in ischemic preconditioning [118]. It can be speculated that the effect of AII on the Na^+–H^+ exchanger may contribute to the effect of ischemic preconditioning on $[pH]_i$, thus helping to protect the myocardium against sustained ischemia. In addition, AII-induced activation of PLD may also stimulate the activation of PKC [111]. It

has been recently shown that PLD activity increases significantly during ischemia as a result of one cycle of ischemic preconditioning in isolated cardiomyocytes [188], suggesting a role for the PLD - DAG - PKC pathway in ischemic preconditioning.

In this study, the participation of AII receptors in ischemic preconditioning has been addressed. Although there is evidence that the ischemic preconditioning response appears to be mediated via a pertussis-toxin–sensitive G-protein, G_i [104], and that the AT_1 but not the AT_2 AII receptor subtype is known to be coupled to G_i-proteins [138], a selective assessment of each receptor subtype's role in ischemic preconditioning was, nevertheless, performed. Blockade of AT_1 receptors with losartan, a highly selective non-peptide AT_1 receptor antagonist, completely abolished the ischemic preconditioning protection, whereas selective blockade of AT_2 receptors with PD-123,319 ditrifluoroacetate did not.

An interesting response observed in isolated buffer perfused hearts with the infusion of the selective AT_2 receptor inhibitor was that, at a dose of 10 μM, which is the dose used by other investigators [108, 186] to block this AII subtype, coronary flow increased significantly after 10 min infusion to levels similar to the increase induced by losartan. The increase in coronary flow was sustained throughout the experiment. Thus, coronary flow was significantly higher in preconditioned hearts treated with losartan and treated with PD 123,319 ditrifluoroacetate indicating that this agent, which blocks AT_2 receptors, reduces coronary vascular resistance through a vasodilator mechanism which involves the AT_2 receptor. To my knowledge, this is the first time that coronary

vasocontrictor properties for the AT_2 receptor are reported as a physiologic function. However, the reversibility of this AT_2 receptor-mediated vasodilator effect and the mechanism(s) involved in this response remain to be investigated.

In summary, transient activation of AII receptors protects the heart against infarction produced by a long episode of ischemia and reperfusion. The protective effect of AII is similar to the protective effect of ischemic preconditioning. When AT_1 receptors, but not AT_2 receptors, are blocked before and during the short ischemia and reperfusion cycles, the protective effect induced by ischemic preconditioning on necrosis is abolished. These data suggest that AT_1 but not AT_2 receptors participate in the triggering mechanism of ischemic preconditioning in the isolated rabbit hearts.

V. ROLE OF THE AT1 RECEPTOR IN ISCHEMIC PRECONDITIONING: IN VIVO STUDY

The aims of this study were to characterize the ischemic preconditioning phenomenon in an *in vivo* rabbit heart model of regional ischemia and to determine, in that model, the participation of the AT_1 AII receptor in ischemic preconditioning.

1. MATERIAL AND METHODS

1.1 Surgical Preparation:

New Zealand white rabbits (3.0 to 3.5 kg), of either sex, were selected for this *in vivo* study. General anesthesia was induced in each rabbit with an intravenous bolus infusion of pentobarbital (30 mg/kg). Each animal also received a dose of heparin (200 IU/kg) which was administered in the same bolus infusion of pentobarbital via an ear vein. The rabbit neck and chest were then shaved to prepare the skin for surgery. To maintain each animal deeply anaesthetized throughout the experiment, a 250 ml 5% dextrose solution which contained pentobarbital (2.8 mg/ml) and heparin (12 IU/ml) was given to each animal through an ear vein as follows: Initially, the infusion rate was maintained at 100 ml/hr, equivalent to 4.66 mg/min of pentobarbital and 20 IU/min of heparin, for 15 min. Then the infusion rate was reduced to 10-15 ml/hr, which is equivalent to a dose of pentobarbital of 10-12 mg/kg/hr Pedal and corneal reflexes were used to assess the

effectiveness of the anesthetic protocol. A midline longitudinal incision was performed on the anterior neck and blunt dissection was performed to expose the trachea. The trachea was then opened and a cannula of an appropriate size was inserted and kept in place with 2-0 silk sutures, to allow assisted ventilation. The animal was ventilated with 100% oxygen using a Harvard small animal ventilator (Harvard Apparatus, South Natick, MA) which was adjusted to deliver a tidal volume of 5 ml/kg. The left carotid artery was cannulated using a polyethylene tubing (Clay Adams Inc., Parsippany, NJ, USA) which was connected to a pressure transducer (Model 800, Bentley Inc., Irvine, CA, USA) to monitor arterial blood pressures. Arterial blood samples were regularly withdrawn from the arterial cannula to measure arterial blood and to determine each animal's acid-base status and oxygen saturation. Appropriate adjustments on the respirator settings were performed to keep arterial pH, PO_2, PCO_2 and HCO_3 within a physiological range. Each animal was then placed in a lateral position, resting on its right side, and a left thoracotomy was performed. The left lung was then gently displaced and protected with a wet gauze. The pericardium was opened and a 2-0 polypropylene suture attached to a curved needle was then passed around the left main coronary artery. Special care was taken to prevent any coronary vascular injury while the suture was being placed. Both suture ends were then pulled through a 3-4 cm length polyethylene tubing (Clay Adams Inc., Parsippany, NJ, USA), thus assembling a tourniquet-like device to perform the coronary occlusions. Another ear vein was also cannulated, using a butterfly 23-gauge needle, to

administer losartan. In each rabbit, body temperature was monitored through a needle temperature probe inserted into to a skeletal muscle through the skin.

1.2   Hemodynamic Measurements:

Mean arterial blood pressure (MAP) was monitored and recorded every 15 min throughout each experiment. The data were then digitized using computer software developed at The Hospital for Sick Children (Cardiac Pressure-Volume Loop Acquisition and Analysis Program, version 5.0). Heart rate was also calculated from the pressure signal and recorded together with the mean arterial pressure.

1.3   Infarct Size Measurements:

Myocardial necrosis was assessed using the TTC staining technique previously described in detail in the Chapter III. In brief, at the end of 90 min reperfusion, each heart was rapidly excised and mounted onto a Langendorff perfusion apparatus and perfused with normal saline for 2-3 minutes to washout all the blood residue. Then, the coronary artery branch previously used to perform the arterial occlusion was re-occluded to exclude the myocardium at risk from the infusion of 1-10 microns yellow fluorescent particles (Duke Scientific Inc., Palo Alto, CA) that followed. Then, each heart was sliced in 3-4 equal thickness slices and incubated in TTC for 15 min. Subsequently, the hearts slices were fixed in 10% formaldehyde and traced onto a transparent acetate sheet for further planimetry measurements and calculation

of the percentage of the myocardium that was necrotic in relation to the percentage of the myocardium that was at risk of infarction.

1.4   Experimental Protocol:

1.4.1   Stability Study:

Before any experimental group was studied a group of rabbits (sham operated group, n = 7) were surgically prepared and their mean arterial pressure (MAP) and arterial blood gases were monitored and measured every 30 min for a period of 150 minutes to assure that a stable preparation was maintained during that period of time and no infarct areas were detected as per TTC staining at the end of the 150 min stabilization period.

1.4.2   Losartan Dose-Response Study:

Since tissue AII levels increase with ischemia, it was necessary to be confident that all AT_1 receptors in the hearts were completely inhibited before and during the transient ischemia/reperfusion period. To address this issue, a dose-response experiment for the AT_1 receptor subtype antagonist was designed. Four rabbits were anaesthetized following exactly the same protocol used in the ischemic preconditioning study. An arterial cannula connected to a pressure transducer was placed in the aortic arch via the left carotid artery to monitor mean arterial pressure which served as the end point in this study since the effects of AII receptor activation on MAP is very well known. First, low doses of AII were tested to find an appropriate dose which the rabbit could

tolerate and would markedly increase MAP. Then, each rabbit received an intravenous dose of AII (1 nMol) via an ear vein to obtain a baseline response. After 5 min of stabilization, a loading dose of losartan (6 mg/kg) was given to the animal followed by a maintenance dose (3 mg/kg) which was given in an infusion for 25 min. Then, challenge doses of AII (1 nMol) were given in a bolus infusion at 10 min, 25 min and 55 min after the initial loading dose of losartan to assess the ability of that dose of losartan to block the AT_1 receptor subtype. In this experiment losartan was able to completely block the pressure effect of AII at 10 min, 25 min and 55 min after the initial dose (Figure V.1). However, it produced a significant fall in mean arterial pressure from 92.0 ± 6.6 mmHg at baseline down to 52.5 ± 14.3 mmHg at 10 min, 41.4 ± 9.2 mmHg at 25 min and 34.1 ± 5.5 mmHg at 55 min after the initial infusion of losartan. Due to the serious hypotensive effect of losartan at that dose I considered reducing the dose of losartan by half (3 mg/kg as the loading dose followed by 1.5 mg/kg as the maintenance dose given over 25 min, n=4)). Losartan, given at this lower dose, did not prevent the fall in mean arterial blood pressure nor did it block the inotropic effect of AII at 10 min and 25 min after the initial dose (Figure V.2). These rabbits were not challenged with a third dose of AII at 55 min due to the fact that this dose did not produce an effective blockade of the AT_1 receptors. Nonetheless, the losartan treated experiments were performed despite the hypotensive effect of losartan.

Losartan, given intravenously at a loading dose first (6 mg/kg) followed by a maintenance dose (3mg/kg given in an

infusion over 25 min), produced a complete blockade of the AT_1 receptor-mediated pressure effect of AII (1 nMol), at 10 min, 25 min and 55 min after the initial dose, as demonstrated by the lack of a large AII peak in Figure V.1. However, this dose of losartan induced a significant (P<0.05) decline in MAP, from 92.0 ± 6.6 mmHg at baseline down to 52.5 ± 14.3 mmHg at 10 min, 41.4 ± 9.2 mmHg at 25 min and 34.1 ± 5.5 mmHg at 55 min after the initial infusion of losartan (Figure V.1).

In a group of pilot experiments, a single dose of losartan (6 mg/kg bolus) was given intravenously without the maintenance dose. In these rabbits, MAP dropped significantly after 10 min and 25 min as was seen in previous group of rabbits treated with a bolus dose and a maintenance dose. AII peaks at 10 min and 25 min were absent suggesting an effective blockade of AT_1 receptors after 25 min. However, the increase in MAP, which was not present at 55 min with the combined dose (6 mg/kg bolus plus 3 mg/kg maintenance dose), appeared again at that time, thus reflecting a short half-life for losartan in rabbits.

Because 6 mg/kg of losartan produced a significant decline in mean arterial pressure in the closed-chested rabbit, I tried to find a lower dose which would minimize the decline in MAP. A dose of 3 mg/kg, half of the initially tested dose, was then given as a single dose at 5 min into the stabilization period to test its effect on MAP. This lower dose of losartan did not prevent the decline in MAP at 25 min after losartan was administered. In addition, the effectiveness of losartan to inhibit the positive inotropic effect of an intravenous infusion of AII (1 nMol) was substantially reduced
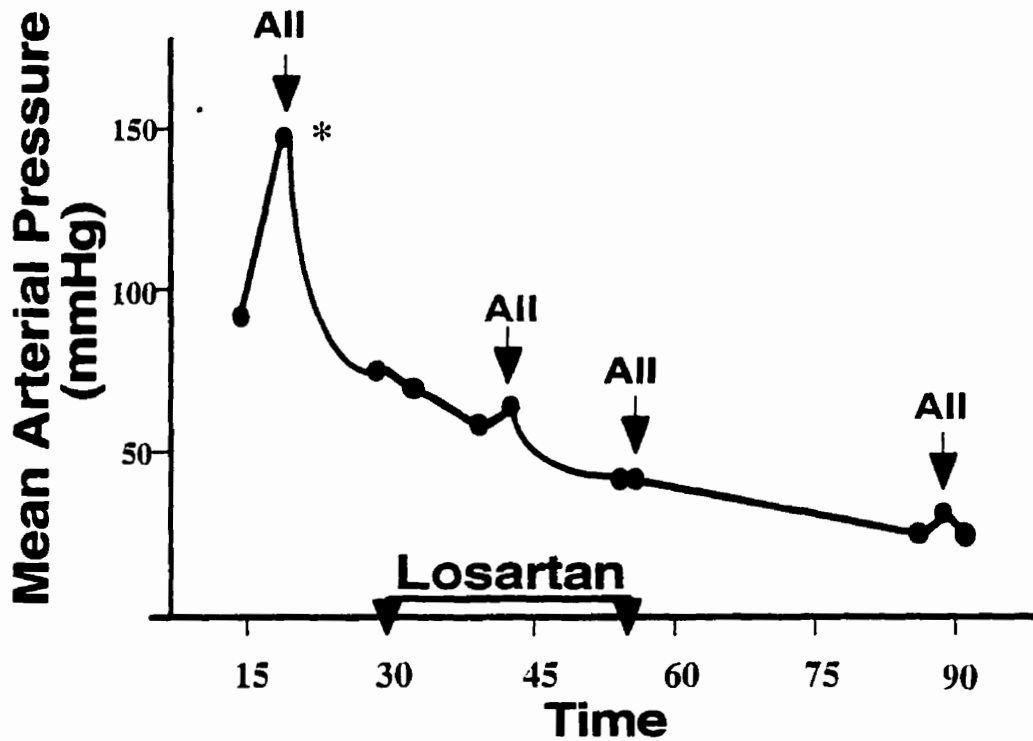
Figure V.1: This graph shows the inhibitory effect of one dose of losartan (loading dose=6mg/kg, maintenance dose=3mg/kg), a specific AT_1 receptor antagonist, in response to AII receptor stimulation with intravenous challenge doses of AII (1 nmol) given at 10 min, 25 min and 55 min after losartan was given. This dose of losartan completely blocked the AT_1 receptor-mediated pressure effect of AII for at least one hour as demonstrated by an almost flat response to AII at any time point selected. Data are mean ± SEM. * P<0.05 versus baseline.

as suggested by the presence of large AII peaks in the dose-response curve shown in Fig.V.2.

1.4.3 Enalaprilat Dose-Response Study:

It is known that some of the AII circulating in the blood stream is taken up by the heart and utilized in physiological processes. Thus, it is possible that the circulating pool of AII contributes significantly to the triggering of ischemic preconditioning in vivo. To address this issue, dose-response experiments were performed using increasing doses of enalaprilat, an angiotensin converting enzyme inhibitor the molecular structure of which does not include a sulfhydryl group (SH). The sulfhydryl radical has been associated with the cardioprotective properties of some ACE-inhibitors[102]. In the present study, twenty open-chested anaesthetized rabbits were ventilated and surgically prepared for tracheotomy . These rabbits were initially stabilized for 15 min before they were given a dose of AI (1 nMol) intravenously to increase the plasma levels of AII presumably via the angiotensin converting enzyme and to obtain dose-response baseline values. After a stabilization period (10 min) to allow the rabbit to recover from the AI, the animals were randomly selected to receive a dose of enalaprilat intravenously. Each dose of enalaprilat (1, 0.5, 0.25, 0.1, and 0.02 mg/kg) was tested on four different rabbits (n = 4/dose). One-third of the dose of enalaprilat was given in a bolus to achieve high plasma concentration quickly, then the other two-thirds of the dose was diluted in water and
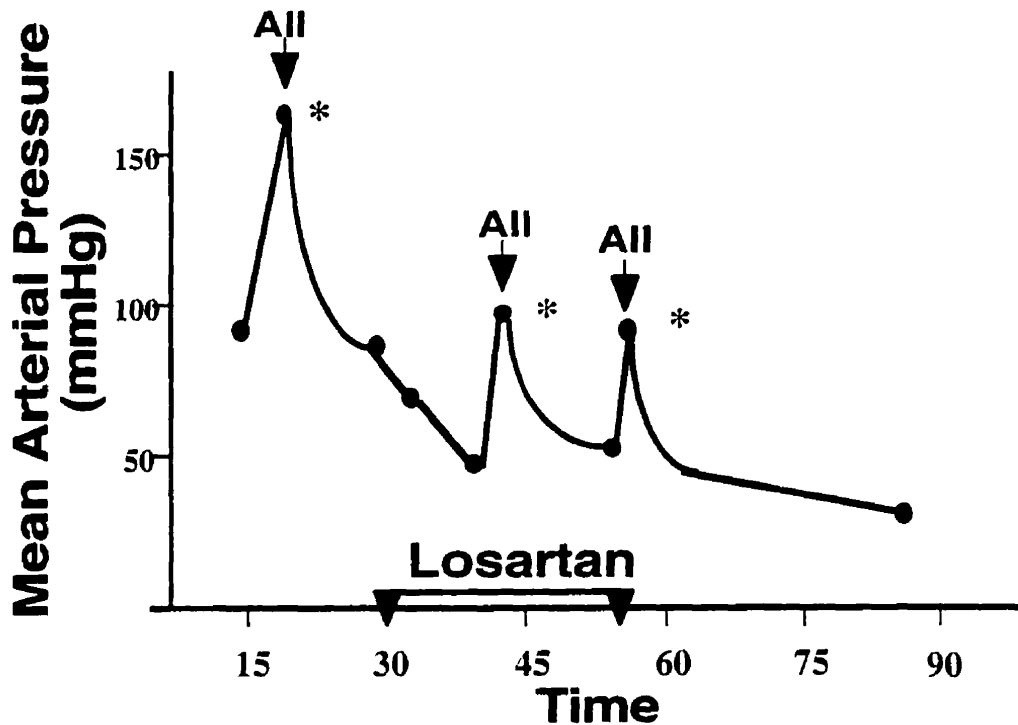
Figure V.2: This graph shows the inhibitory effect of one dose of losartan (3 mg/kg), a specific AT_1 receptor antagonist, in response to AII receptors stimulation with intravenous challenge doses of AII (1 nmol) given at 10 min and 25 min after losartan was given. This dose of losartan failed to completely block the AT_1 receptor-mediated pressure effect of AII as demonstrated by the appearance of peaks in the MAP in response to Ang II at any time point selected. Data are mean ± SEM. * P<0.05 versus baseline.

administered over a period of one hour. At the end of the 60 min, each rabbit was challenged with a repeat dose of AI to determine the inhibitory effect of each dose of enalaprilat on the angiotensin converting enzyme. Mean arterial pressure (MAP) and heart rate were monitored to assess the effect of both enalaprilat and AI in vivo.

As shown in Figure V.3, enalaprilat inhibited the angiotensin converting enzyme in a dose-dependent manner. The response to the administration of a challenge dose of AI, which was intended to increase the blood levels of AII and to cause MAP to rise, was only blocked by using high doses of enalaprilat. However, MAP decreased significantly (more than 40% of baseline) with the use of high doses of enalaprilat (Figure V.4). Because of this hypotensive effect of enalaprilat observed in vivo, this attempt to prove a role of the renin-angiotensin system in ischemic preconditioning was abandoned. Nonetheless, the study of the AT_1 AII receptor role in ischemic preconditioning in vivo were then performed using only losartan.

1.4.4 Ischemic Preconditioning: AT1 Angiotensin II Receptor Study:

The experimental protocol used in this study is shown in Figure V.5. Thirty-two in situ rabbit hearts were subjected to an initial 15 min of stabilization after the surgical procedures were completed. This stabilization period was followed by 30 min of normothermic (37°-37.5° C) regional ischemia and 90 min of reperfusion. The hearts were divided into four groups: The first
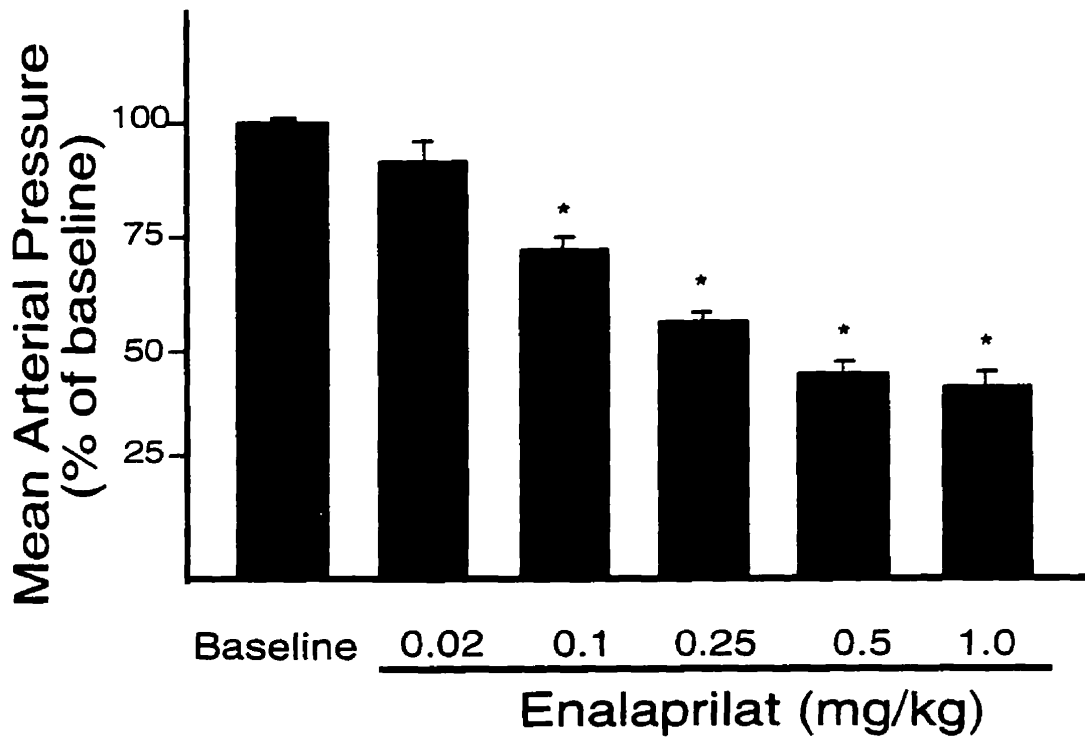
Figure V.3: This graph shows the inhibitory effect of different doses of enalaprilat on the angiotensin converting enzyme which are reflected by the decline in mean arterial pressure in a dose-dependent manner. * $P < 0.05$ versus baseline.
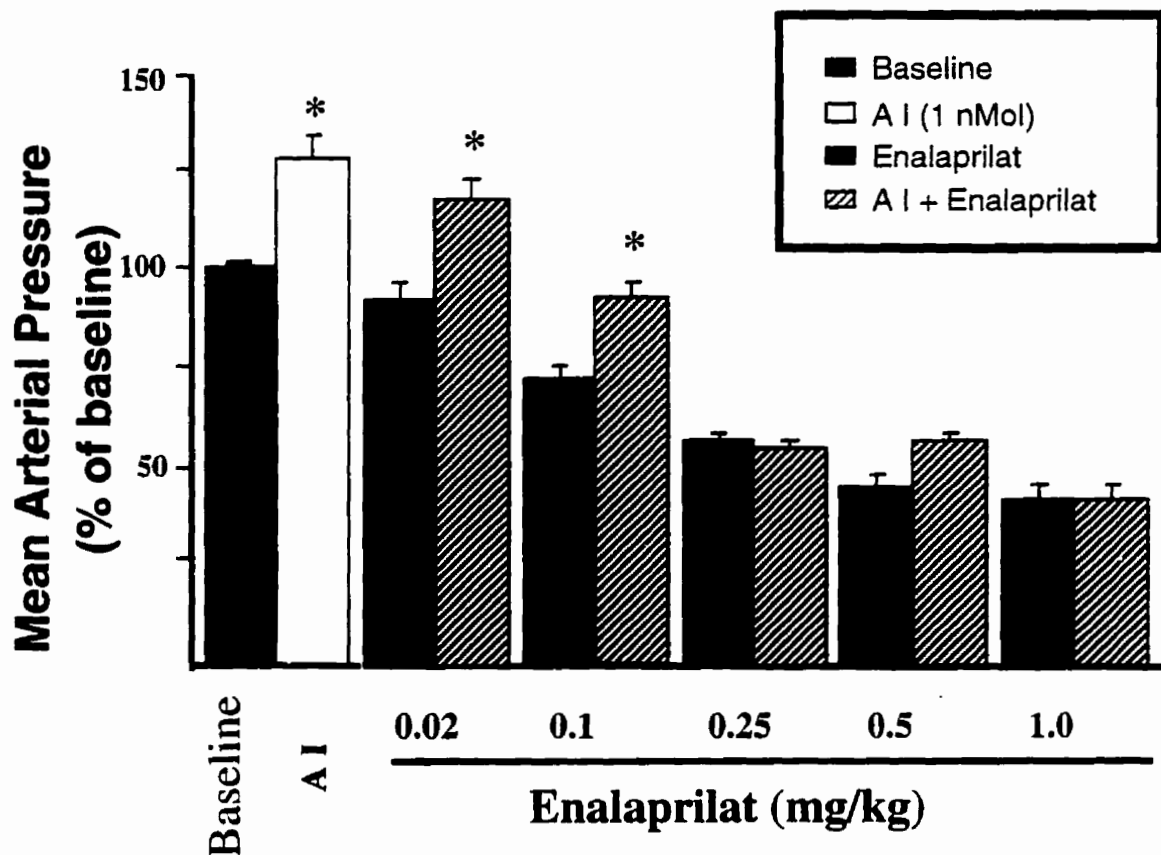
Figure V.4: This graph shows the effect of different inhibitory doses of enalaprilat (0.02, 0.1, 0.25, 0.5 and 1.0 mg/kg) on mean arterial pressure (expressed as percent of baseline) against the powerful positive inotropic response of angiotensin I (AI, 1 nmol). Enalaprilat (0.25, 0.5 and 1.0 mg/kg) was able to block completely the increase in mean arterial pressure caused by AI. * P<0.05 versus MAP values at each dose of enalaprilat.

two groups were designed to characterize the ischemic preconditioning phenomenon in an in vivo model. Control hearts (C, n = 8) received an additional 15 min of stabilization to parallel the time needed to precondition the hearts. Preconditioned hearts (IP, n = 8) were subjected to 5 min of regional ischemia followed by 10 min reperfusion prior to the 30 min ischemia. Since activation of AII AT_1 receptor subtype was found to play an important role in ischemic preconditioning in the isolated rabbit heart model, only this AII receptor subtype was assessed in this model to determine if what was observed in the isolated heart model was also true for the in situ heart. Two groups of hearts were studied: first, losartan-treated control hearts (C+LST, n = 7) which received an identical protocol as untreated control hearts. Hearts included in this group were given a loading dose of losartan (6 mg/kg) at 5 min of stabilization followed by a maintenance dose (3 mg/kg) given over a period of 25 min before the long ischemia. Second, losartan-treated preconditioned hearts (IP+LST, n = 8) were subjected to the same protocol as untreated preconditioned hearts and received an identical loading and maintenance dose of losartan as in losartan-treated control hearts. Although the losartan dose selected for these experiments was based on reported doses by other investigators [189], a dose-response experiment was performed to confirm the ability of that dose to produce a sustained blockade of the AT_1 receptor subtype (Figure V.1 and V.2). At the end of each experiment the heart was excised and mounted onto a Langendorff perfusion apparatus where it was perfused with saline to wash out all blood residue. Then, the heart was prepared for TTC staining to

In Situ Heart Experimental Protocol

Ischemic Preconditioning Study
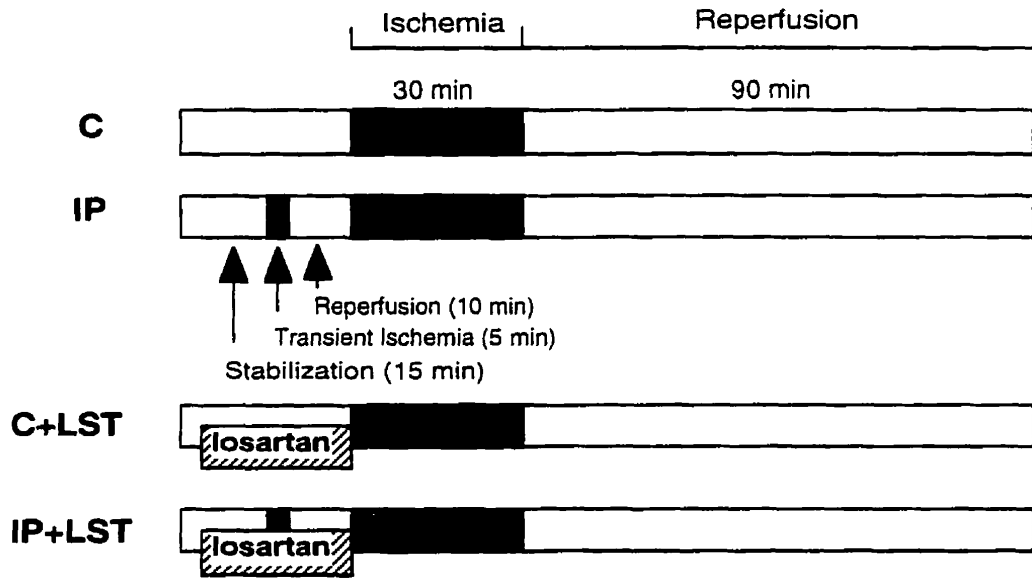
Figure V.5: Experimental protocol for the ischemic preconditioning: AII receptors study. All hearts received 15 min of stabilization followed by 30 min of regional ischemia and 90 min of reperfusion. Control hearts (C) received an additional 15 min of stabilization to parallel the protocol to the ischemic preconditioning protocol. Preconditioned hearts (IP) were also subjected to 5 min of regional ischemia followed by 10 min of reperfusion prior to the long ischemia/reperfusion period. Another two groups of hearts, losartan treated control (C+LST) and losartan treated preconditioned hearts (IP+LST), received the same protocol as their homologous group, respectively. Mean arterial pressure (MAP, mmHg) was measured throughout each experiment. Necrosis was assessed at the end of each experiment.

assess necrosis in the area at risk of infarction.

1.5   Chemical Reagents and Drug:

Angiotensin I (Sigma Chemical Company, St. Louis, MO), was dissolved in small water aliquots (0.6 µg/40 µL) and stored at - 20°C. AI aliquots were diluted in 1 mL of normal saline before being intravenously administered into the rabbit. Enalaprilat (vasotec™), 1.25 mg/mL in 2 mL vials, was obtained from the Hospital for Sick Children Pharmacy. Endothelin-1 (Sigma Chemical Company, St. Louis, MO) was dissolved in distilled water to make a stock solution (18 µg/8mL) and them diluted with Krebs buffer. Losartan (a generous gift from DuPont Merck Inc., Wilmington, DE) was dissolved in millipore-filtered water (40mg/5mL).

1.6   Statistical Analysis:

All data are expressed as mean ± S.E.M. Factorial ANOVA was used to assess differences among the heart groups. Where appropriate, the Scheffé F-test was applied to determine whether a statistically significant difference ($P < 0.05$) existed between two groups. Where it was appropriate, a student T-test was used to assess trends. Regression analysis was preformed to assess any relationship between risk area and necrotic area. Correlation analysis was also performed to assess for association between infarct size and mean arterial pressure measurements.

2. RESULTS

2.1 Stability study:

All data for the in vivo stability study are presented in Figure V.6. Mean arterial pressure (MAP) declined significantly (P<0.01) from 96.1±3.5 mmHg at 30 min of stabilization to 78.3±5.8 mmHg after 150 min. However, MAP was still high enough to assure an effective coronary perfusion pressure. In addition, heart rate did not change during the experiment suggesting that the rabbits were well anaesthetized and that the demand on the heart for blood was stable even with the slight drop in MAP. Body temperature did not have any significant change over the observation period as well (data are not shown in figure V.6). The anesthetic protocol used in these rabbits was sufficient to maintain each rabbit unconscious and without discomfort or pain throughout the experimental period. Arterial blood gases, pH, and hemoglobin were kept within the physiological range in each rabbit (range: PO_2= 288-300 mmHg, PCO_2=27.2 to 30.9 mmHg, HCO_3=20.2-21.4 MM/L, pH=7.36-7.49, and Hb=14-15 g%).

Each *in situ* heart subjected to the stability protocol was assessed for necrosis by tetrazolium staining of transversely sectioned heart slices at the end of the experiment. None of these hearts showed necrosis as determined by the TTC staining technique. Furthermore, none of these hearts developed
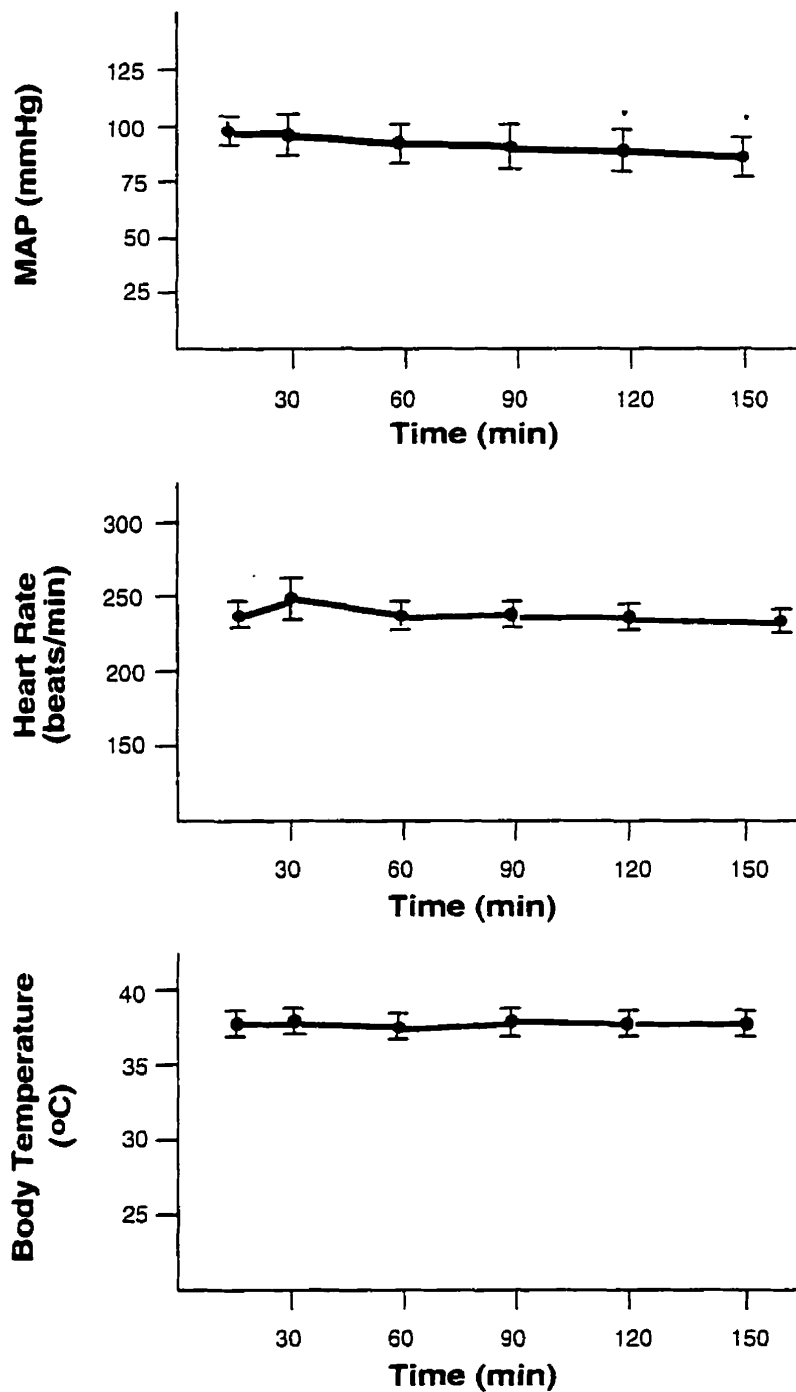
Figure V.6: These graphs show the mean arterial pressure (MAP, mmHg), heart rate (beats/min) and body temperature (°C) data for the stability study in an open-chested rabbit heart model. MAP decreased progressively throughout the 150-minute observation period. Heart rate and body temperature did not change over the same period of observation. * P<0.05 versus baseline at 30 min.

arrhythmias during the observation period. In summary, this open-chested in vivo rabbit heart model proved to be highly stable.

2.2   Ischemic Preconditioning: AT1 Angiotensin II Receptor Study:

2.2.1   Infarct Size:

Infarct size data for the in situ heart model are expressed as mean ± SEM and shown in Figure V.7. Ischemic preconditioning, induced by one single cycle of 5 min of regional ischemia and 10 min of reperfusion, protected the hearts against infarction [in vivo preconditioned hearts (IP) 13.1 ± 2.7%, P<0.001] when compared to in vivo control hearts (C 47.1 ± 2.6%). Losartan, given intravenously as described in the materials and methods section (loading dose of 6 mg/kg followed by a maintenance dose of 3 mg/kg given over a period of 25 min) blocked the protective effect of a single cycle ischemic preconditioning [in vivo losartan-treated preconditioned hearts (IP+LST) 40.8 ± 4.3%, P<0.05 versus IP], while it did not have any effect on necrosis in vivo in losartan-treated control hearts (C+LST 34.3 ± 3.9%, P>0.05 versus C).

A regression analysis performed on the infarct size data against area at risk indicates that there was no relationship between the two parameters as evidenced by an r value of only 0.16 for preconditioned hearts (Figure V.8A). However, a similar analysis demonstrated a weak correlation (r = 0.59) between infarct

size and area at risk in control hearts (Figure V.8B). As noted in Figure V.8B, this seemingly positive relationship is strongly influenced by a single outlier data point in the left bottom of the graph. Thus, a second regression analysis was performed without including the outlier point. Under these circumstances, the weak correlation between infarct size and area at risk vanished as evidenced by an r value of 0.21 (Figure V.8C). The overall statistical assessment of these data strongly suggest that an increase in infarct size was not dependent on an increase in the size of the area at risk for each heart in this study.

In addition, an analysis of variance performed on the area at risk data(Table V.1) demonstrated that there was no difference within the groups in terms of this parameter. Based on these data, it is possible to say that all hearts had areas at risk which were very close in size, thus providing a good basis for comparisons between the groups (Table V.1).
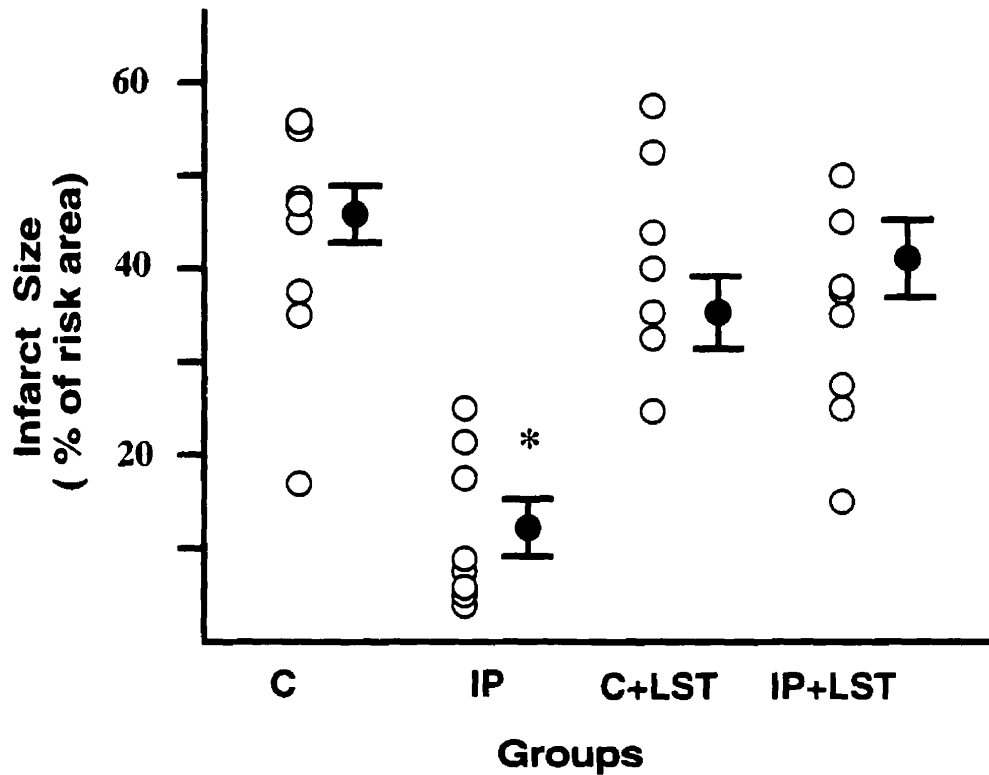
Figure V.7: This graph shows the percent of infarct size expressed as percent of area at risk (mean ± SEM) for in situ untreated and losartan-treated control heart groups (C and C+LST, respectively), and for untreated and losartan-treated preconditioned heart groups (IP and IP+LST, respectively) heart groups. As shown in the graph, a single cycle of 5 min of regional ischemia and 10 min of reperfusion significantly (P<0.01) reduced the percentage of infarction which otherwise would have been produced by a longer ischemic episode and reperfusion in untreated hearts. However, when AT_1 receptors were selectively inhibited in the hearts with losartan the protective effect of ischemic preconditioning against necrosis was lost. *P<0.05 versus control group.
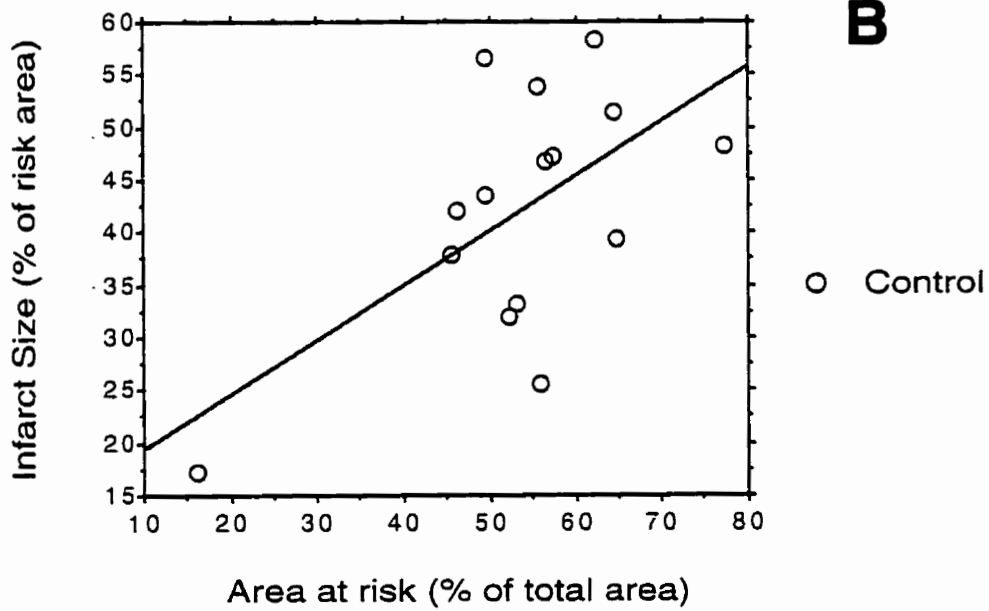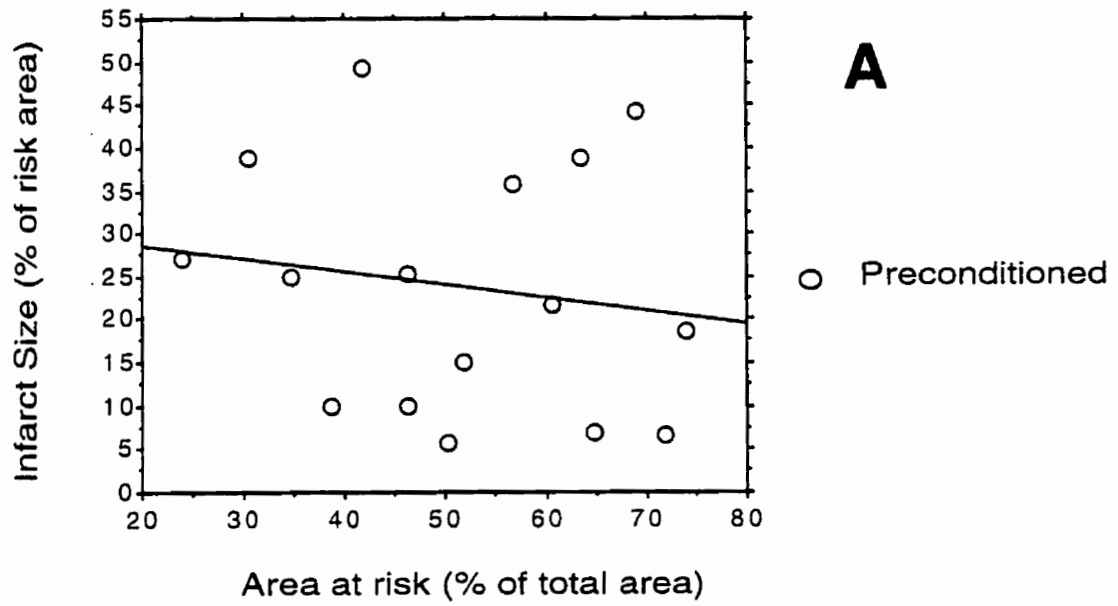
Fig. V.8
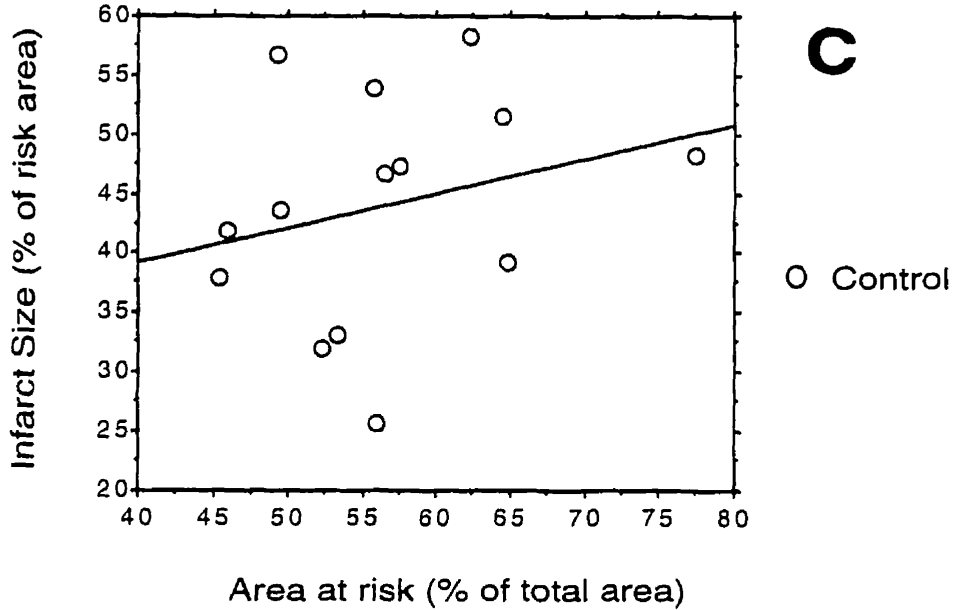
Figure V.8: A—This graph shows a regression analysis performed between all preconditioned heart infarct sizes (% of risk area) and areas at risk (% of total area). There was no relationship found between these parameters as evidenced by a regression coefficient (r) of only 0.16 (P>0.05). B— This graph depicts a regression analysis performed between control heart infarct size and area at risk. A weak relationship was detected between the two parameters as demonstrated by the slope of the relation and the regression coefficient (r) of 0.59 (P<0.05). C—This graph shows the same regression analysis done in all control hearts between infarct sizes and areas at risk, but this time an outlier shown on the left bottom part of graph B has been excluded based on the high sensitivity of this test to such data points. There was no relationship this time (without the outlier data point) between these parameters as evidenced by a very low regression coefficient (r) of 0.21 (P>0.05).

Table V.1
Infarct size (expressed as percent of risk area) and area at risk (expressed as percent of total area) are shown for all heart groups.

Group	Infarct Size (% of risk area)	Area at Risk (% of total area)
C	47.1 ± 2.6	58.0 ± 3.9
IP	13.1 ± 2.7*	56.6 ± 4.6
C+LST	34.3 ± 3.9	46.5 ± 5.8
IP+LST	40.8 ± 4.3	54.8 ± 2.5

C= untreated control; IP= untreated preconditioned; C+LST=losartan treated control; IP+LST=losartan treated preconditioned. * $P<0.05$ versus C.

2.2.2 Hemodynamic Data:

All hemodynamic data are shown as mean ± SEM in Table V.2. An statistical analysis performed on these data with factorial ANOVA (at 95% of confidence) showed that MAP was not significantly (P>0.05) different among untreated control heart group (C, 92.4 ± 3.2 mmHg), untreated preconditioned heart group (IP, 85.4 ± 4.7 mmHg), and losartan-treated control heart group (C+LST, 81.8 ± 7.2 mmHg) at 30 min of stabilization. However, it showed a significant (P<0.05) difference in MAP between the two preconditioned heart groups [losartan-treated preconditioned heart group (IP+LST) 61.9 ± 6.7 mmHg versus IP 85.4 ± 4.7 mmHg]. Even though the difference in MAP, at 30 min of stabilization, between C+LST and IP+LST groups did not reached statistical significance (P<0.10), a trend toward being significantly different was detected. In addition, a moderate decline in MAP, induced by losartan, in both C+LST and IP+LST groups was observed before the long ischemia; however, no significant difference in MAP was detected, at this point, between these two groups (C+LST 63.7 ± 10.2 mmHg versus IP+LST 51.4 ± 4.5 mmHg, P>0.05). MAP did not differ among all groups at the end of 30 min of ischemia (Table V.2). However, after 90 min of reperfusion, it was found that profound hypotension developed in the IP+LST group (MAP of 25.5 ± 1.4 mmHg). This marked hypotension showed to be significant difference, as determined by an unpaired Student T-test performed on the data, in MAP between IP+LST group and either C+LST group (41.4 ± 6.6 mmHg) or IP group (42.4 ± 4.0 mmHg). Furthermore, there was not significant changes in heart rate among the groups.

Table V.2
Measurements of mean arterial pressure (MAP, mmHg) and heart rate (beats/min) for untreated and losartan treated control (C and C+LST, respectively), untreated and losartan treated preconditioned (IP and IP+LST, respectively) heart groups.

Group	Measurement	15 min Stabilization	Before Ischemia	30 min Ischemia	90 min Reperfusion
C	MAP	92.4 ± 3.2	85.6 ± 5.7	65.2 ± 3.1	50.6 ± 3.7
	Heart rate	242.7 ± 8.7	239.2 ± 7.7	237.8 ± 7.2	203.5 ± 13.6
IP	MAP	85.4 ± 4.7	75.8 ± 5.5	55.8 ± 6.5	42.4 ± 4.0
	Heart rate	224.5 ± 9.5	252.3 ± 8.4	235.4 ± 7.3	195.1 ± 7.8
IP + LST	MAP	61.9 ± 6.7 *	51.4 ± 4.5	41.0 ± 3.4	25.5 ± 1.4 * †
	Heart rate	251.7 ± 9.5	250.0 ± 12.3	241.4 ± 6.3	207.1 ± 13.9
C + LST	MAP	81.8 ± 7.2	63.7 ± 10.2	49.9 ± 7.0	41.4 ± 6.6
	Heart rate	260.0 ± 5.1	253.3 ± 6.7	240.0 ± 5.2	210.3 ± 5.2

IP = ischemic preconditioning. All data are mean ± S.E.M. * $P < 0.05$ versus the equivalent untreated preconditioned value (ANOVA). † $P < 0.05$, versus C + LST group (student T-test)

3. DISCUSSION

The aim of this study was to determine the participation of AT_1 receptors in ischemic preconditioning in vivo. The data presented in the results section of this chapter appear to show that selective blockade of AT_1 receptors with losartan completely abolished the infarct-size limiting effect of ischemic preconditioning of the myocardium. These results seem to support the hypothesis that AT_1 AII receptors participate in ischemic preconditioning in vivo. However, because of hypotension associated with losartan, these findings need to be carefully interpreted

Selective inhibition of AT_1 angiotensin II receptors with losartan produced a moderate decline in MAP at 15 min of stabilization (Table V.2) in preconditioned hearts. This hypotensive effect of losartan was sustained and resulted in profound hypotension for the losartan-treated preconditioned hearts [MAP 25.5 ± 1.4 mmHg (mean ± SEM)] at 90 min of reperfusion (Table V.2). Based on these observations, one may argue that such profound hypotension may have limited coronary vascular flow and pressure to such extent that the myocardium may have been inadequately reperfused and possibly subjected to further ischemia during the 90 min of reperfusion period, thus increasing infarct size and eliminating the protection induced by ischemic preconditioning. Therefore, it is not possible to conclude that AT_1 receptors contribute to triggering the ischemic

preconditioning protection against infarction in vivo based on the data from these particular experiments.

Examining the mechanistic basis for the losartan-induced hypotension, it is noteworthy that in the present study, an intravenous dose of losartan, 6 mg/kg i.v. bolus followed by 3 mg/kg i.v. maintenance infusion, which completely blocked the positive inotropic effects (MAP increase) of an intravenous dose of AII (1 nMol), induced a significant ($p < 0.05$) and sustained hypotension (fall of mean arterial pressure of more than 25% of baseline) (Figure V.1) without affecting heart rate. Transient hypotension with the use of losartan i.v. has been reported in dogs by Wong et al[190] who showed in conscious normotensive dogs that 1 to 10 mg/kg, i.v. bolus of losartan resulted in a dose-dependent and transient decrease in blood pressure, which was accompanied by a decrease in heart rate. In contrast, in conscious rats with normal renin levels, losartan did not reduce or increase blood pressure even when administered in doses up to 100 mg/kg, i.v.[191].   These data suggest that AT_1 receptors may play an important role in vascular resistance in rabbits. However, in a group of rabbits in which losartan was administered at a dose where the inotropic effect of a challenge dose of AII i.v. (1 nmol), 3 mg/kg i.v. bolus followed by 1.5 mg/kg i.v. infusion, produced a similar hypotensive effect in rabbits (Figure V.12-13). This data can be interpreted in two different ways; one way is that the severe hypotensive effect of losartan in the rabbit may not be a direct effect of AT_1 blockade, and the other is that AT_1 receptors may be extremely important in vascular resistance regulatory mechanisms

so that blockade of a small percentage of receptors, as was expected to happen with low doses of losartan (3mg/kg i.v.), may produce a significant decrease of vascular resistance.

Based on recently reported differences in the susceptibility of ischemic preconditioning to blockade by selective cardiac bradykinin B_2 receptor blockade [34], by PKC inhibitors [55] or an AC activator [55], Sandhu et al [55] have suggested that ischemic preconditioning induced by one cycle of transient ischemia and reperfusion may be a weaker phenomenon, and therefore, more susceptible to blockade, than the preconditioning protection produced by three cycles of transient ischemia and reperfusion. In fact, three cycles of transient ischemia and reperfusion have shown to produce significantly greater protection against infarcts than a single cycle [55]. In the present study, a single 5-min cycle of transient ischemia and 10-min reperfusion produced a significant reduction on infarct size, from 47.1 ± 2.6 % (mean $\pm$ SEM) in control hearts to 13.1 ± 2.7 % in preconditioned hearts (P<0.001). These data are consistent with data obtained by Ytrehus et al[119], in an in vivo study where ischemic preconditioning was induced using one cycle of transient regional ischemia and reperfusion. In Ytrehus's study, ischemic preconditioning reduced infarct size significantly, from 37.8 ± 3.1% (mean $\pm$ SEM) in control to 7.3 ± 2.7% in preconditioned hearts. Similarly, these data are consistent with previous work done, in vivo, by Sandhu et al [55]. In that study, one-cycle ischemic preconditioning reduced infarct size significantly, from 60.1 ± 1.9% (mean $\pm$ SEM) in control hearts to 13.0 ± 2.9% in preconditioned hearts. Although infarct size for

control hearts appeared significantly higher in Sandhu's study than in either Ytrehus's or the current study, infarct size for preconditioned hearts for all three studies were similar.

In summary, one cycle of transient regional ischemia (5 min) followed by reperfusion(10 min) conferred protection against a subsequently prolonged ischemic/reperfusion insult in vivo in rabbit hearts. This protective effect of ischemic preconditioning against infarcts was apparently abolished by selective blockade of AT_1 AII receptors by losartan during the short transient ischemia and reperfusion episode. However, the development of severe hypotension by losartan precluded the formulation of inferences based on the infarct size data obtained in these in vivo studies.

VI. GENERAL DISCUSSION

1.   ROLE OF ANGIOTENSIN II RECEPTORS IN ISCHEMIC PRECONDITIONING.

The general objective of the present study was to assess the role of AII receptors in the triggering mechanism of ischemic preconditioning. This is the first study to demonstrate the participation of AII receptors (AT$_1$ subtype) in ischemic preconditioning of the myocardium [32]. Since myocardial infarction has been associated with a progressive increase in plasma levels of AII [102], it was necessary to developed a model which would eliminate any contributing effect of the systemic renin-angiotensin system. Thus, an isolated buffer-perfused rabbit heart model of regional ischemia was developed to assess the participation of AII in ischemic preconditioning. In this isolated heart model, ischemic preconditioning protected the heart against necrosis produced by a prolonged ischemic/reperfusion period. In addition, it was found that activation of cardiac AII receptors by short exposure to AII (100nM) produced a similar protection against infarcts as the ischemic preconditioning protection obtained with three cycles of transient ischemia and reperfusion. These data confirm the cardioprotective properties of AII receptor stimulation in isolated rabbit hearts reported early by Liu et al [108]. The results suggest that activation of AII occurs during the short transient ischemia and reperfusion prior to the long ischemia in preconditioned hearts and that such AII receptor activation plays a role in the triggering mechanism of ischemic

preconditioning, but they do not prove that AII receptors participate in ischemic preconditioning.

To demonstrate the participation of AII receptors in ischemic preconditioning, a complete specific receptor blockade was required to test the effectiveness of ischemic preconditioning to protect the myocardium against infarction under those circumstances. Specific blockade of AII receptors with saralasin, a specific competitive AII receptor antagonist, completely abolished the protective effect of three-cycles of ischemic preconditioning against necrosis in isolated rabbit hearts (Figure IV.4). These results directly demonstrate the involvemente of AII receptors in ischemic preconditioning. Since two AII receptor subtypes have been identified in rabbit hearts [192], I was interested in determining if both receptors were involved in ischemic preconditioning. Thus, AII receptors were then blocked using inhibitors to selectively target either AT_1 receptors with losartan or AT_2 receptors with PD-123,319. Selective blockade of AT_1 receptors but not AT_2 receptors completely abolished the protective effect of three-cycles ischemic preconditioning against infarction in isolated hearts (Figure IV.4). These data confirm the results obtained with saralasin and further determine the participation of the AT_1 AII receptors in ischemic preconditioning. Therefore, one may conclude that activation of AII receptors (AT_1) must occur for ischemic preconditioning to realize protection against infarction in isolated rabbit hearts.

Do AT_1 AII receptors play a role in ischemic preconditioning in vivo? I attempted to address this question by developing an in vivo open-chested rabbit heart model of regional myocardial ischemia and testing the effect of losartan on the protective effect of ischemic preconditioning in this model. In these studies, ischemic preconditioning induced by one cycle of transient ischemia and reperfusion produced a significant reduction on infarct size in preconditioned hearts as compared to control hearts. Furthermore, selective blockade of AT_1 AII receptors appeared to completely abolish the protective effect of one-cycle ischemic preconditioning against infarction, while it did not alter infarct size in control hearts in vivo (Figure V.7). Based on a regression analysis between MAP and infarct size which showed no relationship between the two parameters, and an analysis of variance performed among the groups which showed that losartan alone apparently did not modify infarct size, I could have concluded that activation of AII receptors are required for ischemic preconditioning to protect against infarction in vivo in rabbit hearts. However, observations of profound hypotension, as determined by MAP, in losartan-treated preconditioned hearts during the 90 min reperfusion phase following the long period of ischemia suggest that these hearts may have been inadequately reperfused and subjected to further ischemia, which may account for some of the necrosis found in these hearts. Nevertheless, combining the isolated and in vivo heart studies with other authors' work [100-102] in which AII production during ischemia has been documented, I believe a well balanced view is that

activation of AT_1 AII receptors by endogenously produced AII during ischemia in vivo contributes to triggering the ischemic preconditioning response, along with other receptors such as adenosine A_1, bradykinin B_2 and α_1-adrenergic receptors, against infarction in rabbit hearts.

2.   LIMITATIONS OF THE PRESENT STUDIES:

1) In the present studies tissue levels of AII and release of AII during ischemia were not examined. Although there is no direct evidence of the release of AII during ischemia in rabbit hearts, De Lannoy et al [193] have recently confirmed the production of AII in the interstitial fluid compartment of normoxically perfused isolated rat hearts. Furthermore, other groups of investigators have reported an increase in AII production in ventricular myocardium as a result of ischemia in rats [101] and dogs [100]. Ertl et al [102] have also reported an increase in plasma AII after coronary occlusion in dogs.

2) The TTC staining technique used to assess necrosis in the hearts was not validated in the present study against any other method of assessing myocardial necrosis (e.g. electron microscopic analysis). However, validation of this technique has been previously performed in the research groups of which I am a member and reported for both in situ [194] and isolated [14] rabbit heart preparations.

VII. <u>SUMMARY AND CONCLUSIONS</u>

1. <u>Objective of the Study:</u>

The overall objective of this study was to determine the role of AII receptors in the triggering mechanism(s) of the ischemic preconditioning phenomenon in the myocardium.

The participation of the angiotensin II receptor, and its subtypes, AT_1 and AT_2, in ischemic preconditioning of the myocardium was assessed in an isolated buffer-perfused rabbit heart model using regional ischemia. The hallmark effect of ischemic preconditioning, protection against infarct size, was used as the end point to assess the role of angiotensin II receptors in ischemic preconditioning. I found that the angiotensin AT_1 subtype but not the AT_2 receptor subtype participates in the triggering mechanism of ischemic preconditioning in isolated rabbit hearts.

1.1 <u>Hypothesis 1:</u>

"Activation of cardiac angiotensin II receptors by endogenously produced angiotensin II during the short ischemia/reperfusion episodes before a prolonged period of ischemia followed by reperfusion contributes significantly to the triggering mechanism of the protective effect of ischemic preconditioning against myocardial infarction."

I found that activation of angiotensin II receptors with a brief exposure (5 min) to exogenous AII (100 nM), instead of short cycles of ischemia and reperfusion, protected the heart against necrosis to the same extent as ischemic preconditioning. On the other hand, inhibition of angiotensin II receptors with two different angiotensin II receptor blockers, saralasin and losartan, completely abolished the protective effect of ischemic preconditioning, thus supporting hypothesis 1.

1.1.1 Aim 1:

To characterize the protective effect of the ischemic preconditioning phenomenon against infarction, in terms of infarct size in an isolated buffer-perfused rabbit heart model of regional myocardial ischemia.

The protective effect of ischemic preconditioning against necrosis was well characterized. Three cycles of transient ischemia followed by reperfusion protected the heart against necrosis produced by a subsequently prolonged episode of ischemia and reperfusion. This phenomenon was consistently and reliably produced in the isolated heart model with average infarct size reduction of 80% [untreated preconditioned heart group 5.1 ± 1.2% (mean±SEM), versus untreated control heart group 26.4 ± 3.0%, $P<0.001$]. Protection against contractile dysfunction was not seen in the isolated heart model.

1.1.2 Aim 2:

To assess the cardioprotective effect of angiotensin II receptor activation against infarction produced by a long ischemia/reperfusion episode in the same in vitro rabbit heart model of regional myocardial ischemia previously characterized.

I found that angiotensin II receptor activation alone is capable of triggering a protective mechanism against infarction in isolated rabbit hearts. Activation of angiotensin II receptors with 5 min exposure to 100 nM angiotensin II protected the heart against necrosis as evidenced by an average infarct size reduction of 67% [angiotensin II-treated heart group 9.6 ± 2.2% (mean±SEM) versus untreated control heart group 26.4 ± 3.0%, P<0.01].

1.1.3 Aim 3:

To assess the ability of an angiotensin II receptor blockade to abolish the protective effect of ischemic preconditioning against infarction using the same in vitro rabbit heart model of regional myocardial ischemia.

I found that activation of angiotensin II receptors participate in the triggering mechanism of ischemic preconditioning. Saralasin, a competitive angiotensin II receptor antagonist, completely blocked the protective effect of ischemic

preconditioning against necrosis in isolated rabbit hearts [saralasin-treated preconditioned heart group $29.7 \pm 3.2\%$ (mean±SEM) versus untreated preconditioned heart group $5.1 \pm 1.2\%$, $P<0.001$]. This evidence suggests that activation of angiotensin II receptors must occur for ischemic preconditioning to realize protection. This finding demonstrates a participatory role of angiotensin II receptors in ischemic preconditioning, but does not indicate which angiotensin II receptor subtype mediates the protection.

1.2   Hypothesis 2:

"The AT_1 angiotensin II receptor is the angiotensin II receptor subtype mediating the protection of ischemic preconditioning against myocardial infarction."

Selective inhibition of AT_1 receptors by losartan, but not AT_2 receptors by PD-123,319 ditrifluoroacetate, abolished the protective effect of ischemic preconditioning against necrosis in isolated buffer-perfused rabbit hearts, losartan-treated preconditioned heart group $27.4 \pm 3.0\%$ (mean±SEM) versus untreated preconditioned heart group $5.1 \pm 1.2\%$, $P<0.01$; and PD-123,319 ditrifluoroacetate-treated heart group $7.9 \pm 1.2\%$, versus untreated preconditioned heart group $5.1 \pm 1.2\%$, $P>0.05$. Ischemic preconditioning protection against necrosis was also blocked in vivo in rabbit hearts by selective inhibition of AT_1 receptors. These findings support hypothesis 2.

1.2.1 Aim 1:

To determine the participation of each angiotensin II receptor subtype, AT_1 and AT_2, in ischemic preconditioning by assessing the ability of either AT_1 receptor or AT_2 receptor blockade to abolish the protective effect of ischemic preconditioning against infarction in the same in vitro rabbit heart model of regional myocardial ischemia previously characterized.

I found that losartan, a selective AT_1 receptor antagonist, completely blocked the infarct size limiting effect of ischemic preconditioning in the isolated heart model. These data suggest that angiotensin II AT_1 receptor subtypes must be activated for ischemic preconditioning to protect against infarction in isolated hearts.

1.2.2 Aim 2:

To characterize the protective effect of ischemic preconditioning against myocardial infarction in an in vivo open-chested heart model of regional ischemia.

I have characterized the effect of a single cycle of transient ischemia and reperfusion to protect the myocardium against infarction produced by a subsequently prolonged ischemic/reperfusion episode in an in vivo rabbit heart model. Ischemic preconditioning reduced infarct size by an average of 72%

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in this model [untreated preconditioned heart group 13.1 ± 2.7% (mean±SEM), versus untreated control heart group 47.1 ± 2.6%, P<0.001].

1.2.3   Aim 3:

To determine the participation of angiotensin II AT_1 receptor subtype in ischemic preconditioning of the myocardium by assessing the ability of an AT_1 receptor subtype blockade to abolish the protective effect of ischemic preconditioning against myocardial infarction in the same *in vivo* rabbit heart model of myocardial regional ischemia previously described.

I found that, in the in vivo model, losartan, a selective AT_1 angiotensin II receptor antagonist, apparently abolished the protection of ischemic preconditioning against infarction [losartan-treated preconditioned heart group 40.8% ±4.3% (mean±SEM), versus untreated preconditioned heart group 13.1 ± 2.7%, P<0.05]. However, losartan produced profound hypotension in losartan-treated preconditioned hearts (MAP 25.5 ± 1.4 mmHg) at 90 min reperfusion, raising the possibility that inadequate reperfusion of the myocardium might account for the increase in necrosis found in these hearts. Based on these results, no inferences from these data can confirm or reject hypothesis 2.

VIII. <u>FUTURE DIRECTIONS</u>

Before the studies described herein were performed, adenosine A_1, α_1-adrenergic and bradykinin B_2 receptors were the only cardiac receptors demonstrated to be involved in triggering the ischemic preconditioning phenomenon. These new studies, which will be published, in part, in the Journal of Molecular and Cellular Cardiology [32], add a new receptor type to the group of cardiac receptors known to participate in ischemic preconditioning, thus opening up new possibilities for understanding how myocardial protection is produced. For example, it is conceivable that cell mechanisms such as AII-receptor-mediated opening of calcium L-type channel, activation of the Na^+/H^+ exchanger and calcium release from intracellular stores (e.g sarcoplasmic reticulum) may participate in the ischemic preconditioning mechanism of the myocardium. The exact role of these pathways in ischemic preconditioning of the myocardium remains to be explored.

Furthermore, it will be important to determine if the AT_1 receptor subtype participates in ischemic preconditioning of the myocardium in other species such as rat and dog. It has been shown in the present studies that AT_1 receptor participates in triggering the infarct size limiting effect of ischemic preconditioning, but the participation of AT_1 receptors in triggering the anti-arrhythmic effect or the protection against ventricular dysfunction remains to be explored.

Furthermore, it is also important to study the role of angiotensin II receptors, its participating downstream pathways and its target effector proteins, in ischemic preconditioning, in the isolated cardiomyocyte model in which the protection induced by simulated ischemia has been characterized by Armstrong et al[9]. There are three reasons that make the isolated cell model more advantageous, regarding the study of ischemic preconditioning mechanism(s) derived from the activation of angiotensin II receptors and others participating receptors (e.g. adenosin A_1, α_1-adrenergic, bradykinin B_2), than the other two model used in the present studies: 1) drugs which could be used to assess downstream mediators and end effectors are very expensive (e.g. calphostin C,) which limits its use in isolated buffer-perfused or in vivo heart preparations; 2) from the point of view of safety, it is more convenient to label isolated cardiomyocytes with small amounts of ^{32}P orthophosphate to assess end effector proteins (e.g. sarcoplasmic reticulum Ca^{2+}-ATPase, Phospholamban) rather than using large amounts of ^{32}P in whole organ perfusion model or in an in vivo model; 3) Direct measurement of electrophysiological changes induced by ischemic preconditioning through potential end effectors such as Ca^{2+} L-type, K_{ATP} channels, ryanodine receptor, Na^+/H^+ and Na^+/Ca^{2+} exchangers, can only be performed in isolated single cell using the whole-cell patch-clamp technique.

Furthermore, the present studies have triggered a number of other questions that currently remain unanswered. For example, it was shown in the isolated heart studies that coronary flow increases with both AT_1 or AT_2 receptor antagonist, losartan and

DP-123,319 respectively, suggesting the presence, in coronary vessels, of an AII receptor-mediated regulatory mechanism of blood flow. Further experiments are necessary to characterize this regulatory mechanism in coronary vessels. In addition, experiments performed using an angiotensin converting enzyme inhibitor (enalaprilat) showed that this drug effectively blocked the angiotensin converting enzyme in rabbits as evidenced by a significant decline in mean arterial pressure with a dose greater that 0.1 mg/kg, suggesting that, in fact, the renin-angiotensin component of arterial blood pressure is significant in rabbits. However, more research is needed to address this issue extensively.

IX. <u>REFERENCES</u>

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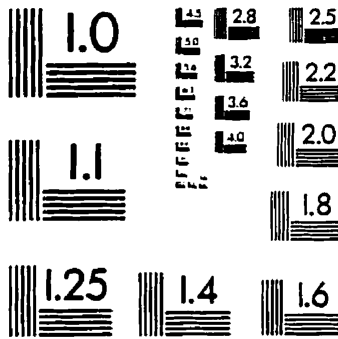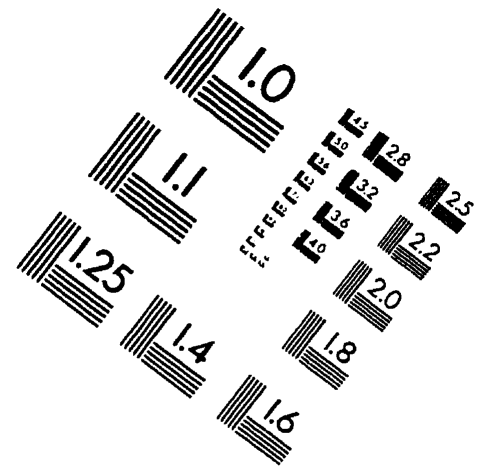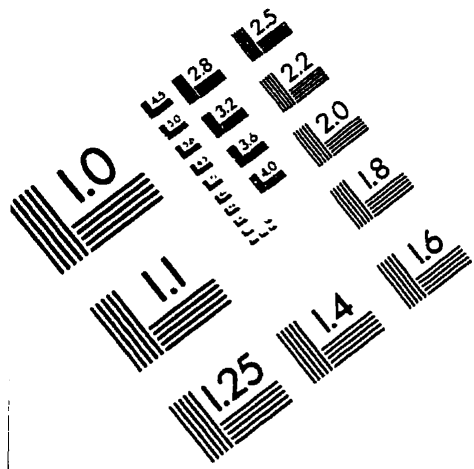
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IMAGE EVALUATION
TEST TARGET (QA-3)

150mm

6"