CARDIOPROTECTION BY A NOVEL ORGANIC NITRATE DURING ISCHEMIA-REPERFUSION INJURY

by

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ABSTRACT

Quynh-Lang Nguyen: Cardioprotection by a novel organic nitrate during ischemia-reperfusion injury. MSc. Thesis, Queen's University, Kingston, ON, September 2001.

Glyceryl Trinitrate (GTN), the prototypic organic nitrate, is used in the treatment of acute myocardial infarction since it decreases myocardial oxygen requirements and its coronary vasodilator effects can result in increased regional blood flow. In experimental models of cardiac ischemia-reperfusion (I/R) injury, several pharmacological agents have demonstrated cardioprotective effects, including GTN. However, there remains no "gold standard" for the treatment of acute I/R injury, because *in vitro* reports are conflicting and many potential cardioprotective agents have been disappointing in clinical trials. Recently, a novel organic nitrate (GT 015) was shown to have neuroprotective properties in *in vitro* and *in vivo* rat models of transient cerebral ischemia. This thesis examined whether the neuroprotective properties of GT 015 extends to the myocardium. Rat hearts were isolated and perfused in Langendorff mode at constant flow. After an equilibration period, the left coronary artery (LCA) was occluded, followed by reperfusion. Drug treatments (DMSO, GTN or GT 015) were initiated at two distinct time points: (i) prior to and throughout the 45 min. period of LCA occlusion (LCAO) (protection) or (ii) prior to and throughout the 90 min. reperfusion period (salvage). The perfusate was assayed for lactate dehydrogenase (LDH) activity as an index of cell damage, and infarct size was assessed by computerized planimetric analysis of heart slices after triphenyltetrazolium chloride (TTC) staining. Left coronary artery occlusion was associated with a 9-fold increase in LDH release. Both GTN and GT 015 attenuated LDH release in a dose-dependent manner during the ischemic period when administered prior to and throughout the period of LCAO. However, only GT 015 reduced LDH release during reperfusion and decreased infarct size, whereas GTN had no effect. When GTN and GT 015 were infused prior to and throughout reperfusion, both drugs decreased LDH release during the reperfusion period. Furthermore, administration of the putative metabolite of GT 015, GT 152, prior to and throughout reperfusion, also attenuated LDH release during the reperfusion period. However, whereas GTN, GT 015 and GT 152 all decreased LDH release, only GT 015 and GT 152 reduced infarct size when administered prior to and throughout reperfusion. In a series of mechanistic studies, we measured cGMP formation in the coronary effluent of drug-treated hearts. The results suggest that GT 015-mediated cardioprotection involves mechanisms other than, or in addition to, cGMP elevation.

CO-AUTHORSHIP

This thesis was based on research conducted by the candidate Quynh-Lang Nguyen, under the supervision of Dr. Brian Bennett, Ph.D.

All data were obtained and analysed by Quynh-Lang Nguyen, with the following exception:

The data from those sections in Chapter 2 in which cGMP formation was measured from samples of the coronary effluent collected during perfusion of the isolated hearts, were obtained by Diane J. Anderson.

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DEDICATION

Dedicated with love to my parents

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1,2-GDN	glyceryl-1,2-dinitrate
1,3-GDN	glyceryl-1,3-dinitrate
AAR	area at risk
ADP	adenosine diphosphate
ATP	adenosine triphosphate
cGMP	3', 5'-cyclic guanosine monophosphate
CPP	coronary perfusion pressure
DMSO	dimethylsulfoxide
GTN	glyceryl trinitrate
GT 015	2,3-dinitroxy-3-(2,3-bis-nitroxypropyldisulfanyl)-propane
GT 152	1-nitroxy-2,3-epithiopropane
I/R	ischemia-reperfusion
K_{ATP}	ATP-sensitive potassium channel
LCA	left coronary artery
LCAO	LCA occlusion
LDH	lactate dehydrogenase
L-NAME	L-nitroarginine methyl ester
MI	myocardial infarction
NADH	β-nicotinamide adenine dinucleotide reduced form
NHE-1	sodium-hydrogen exchanger isoform type 1
NO	nitric oxide
NOS-III	NO synthase isoform type III
SOD	superoxide dismutase
TTC	triphenyltetrazolium chloride

CHAPTER 1: General Introduction

1.1 Statement of the research problem

Ischemic heart disease remains the most common cause of organ-specific death in industrialized countries (World Health Organization, 1998). Intense research has been, and continues to be conducted to determine the cellular and molecular mechanisms leading to irreversible cell death (myocardial infarction, MI) following an acute ischemic insult. The clinical potential of developing effective cardioprotective agents is great: to reduce mortality in critically ill patients and to limit infarct size, scar formation and to improve the prognosis for the uncomplicated infarction. However, despite a wealth of experimental knowledge, no single therapeutic agent has gained widespread acceptance as an effective treatment for the ischemic heart, mainly because the complex interplay of multiple events involved in ischemia-induced myocardial damage makes it difficult to define a singular mechanism which can be uniformly targeted (Piper *et al.*, 1990).

1.2 Ischemia-reperfusion (I/R) injury

1.2.1 Hypoxia versus Ischemia

Ischemia is defined as an imbalance between the supply and demand ratio of oxygen and metabolic substrates (e.g. glucose) by the heart, and is often associated with the atherosclerotic narrowing of the coronary arteries due to factors such as high blood pressure (hypertension), hyperlipidemia (including high cholesterol levels) and smoking. Ischemic (depriving a tissue of oxygen and substrates by flow restriction) and hypoxic (removing oxygen from the perfusate/blood) injury are the most common

types of cell injury in clinical medicine, however the two terms should not be used interchangeably because these two methods of oxygen deprivation have several significant differences. For example, only ischemic tissue accumulates various metabolites and undergoes sustained pH changes, i.e. acidosis due to the release of hydrogen ions following the breakdown of ATP and glycolysis. Another significant difference between hypoxia and ischemia is the absence of energy substrate in ischemic tissue. During an ischemic insult, in contrast to hypoxia, delivery of substrate to the tissue ceases and glycolytic metabolism is limited. Conversely, during hypoxia, glycolytic energy production can proceed. In ischemic tissues, anaerobic energy generation will cease after glycolytic substrates become depleted or the metabolic process is inhibited by the accumulation of metabolites. Clearly there are several differences between ischemic injury and hypoxic injury and overall, tissue injury develops more rapidly during ischemia than hypoxia (Lucchesi, 2001)

1.2.2 Ischemic cell injury

Ischemia initiates a continuum of molecular and cellular changes that progress in severity, and without reperfusion, will ultimately compromise vital structural and biochemical components and culminate in cell death. The metabolic and functional changes occurring during the early minutes of ischemia are reversible, such that cell injury can be arrested and damaged cells can undergo repair if oxygen and metabolic substrates are made available by restoring perfusion (reperfusion). This process is referred to as *reversible ischemic injury* (Lucchesi, 2001). The amount of salvageable muscle depends on: (i) the duration of the ischemic insult, (ii) the size of the obstructed vessel, (iii) the degree of obstruction and (iv) the adequacy of pre-existing collateral

vessels (Vyden and Takano, 1978). As ischemia progresses, however, tissue damage becomes more severe, recovery takes longer, and reperfusion no longer has beneficial effects for the damaged cell. This is referred to as *irreversible ischemic injury*. A central factor contributing to this injury is membrane damage. Loss of mitochondrial membrane function, increased permeability to extracellular molecules, and plasma membrane ultrastructural defects occur during the earliest stages of irreversible injury (Lucchesi, 2001).

The aforementioned impairments to membrane structure, function and composition leads to both fluid and electrolyte alterations and to cytoplasmic, organelle and cellular swelling. Fluid alterations involve an increased permeability to water flowing into the cell, and changes in the normal homeostatic balance of ions such as calcium (Ca^{2+}), sodium (Na^{+}), potassium (K^{+}), magnesium (Mg^{2+}), chloride (Cl^{-}) and protons (H^{+}) (Theroux, 1999a).

1.2.3 Reperfusion injury

Myocardial ischemia of limited duration (<20 min.) followed by reperfusion is accompanied by functional recovery without morphologic or biochemical evidence of irreversible tissue injury (Lucchesi, 2001). Paradoxically, reperfusion of cardiac tissue that has been subjected to an extended period of ischemia (>45 min) results in a phenomenon known as "reperfusion injury" (Lucchesi, 2001). Reperfusion injury was originally defined by Hearse *et al.* (1973) as the sudden release of intracellular constituents (mainly measured as creatine kinase or lactate dehydrogenase) from heart tissue upon reoxygenation after a period of hypoxia (Hearse *et al.*, 1973). Today, reperfusion injury is much more broadly defined as any adverse change that occurs

upon the return of oxygen into a previously deprived tissue, and that cannot be directly attributed to the preceding hypoxic or ischemic episode. Results from experimental animals and isolated hearts indicate that timely reperfusion (re-supply of normal levels of oxygen and substrates, and washout of waste products) following an ischemic insult is clearly the most effective means to prevent the progression of ischemic cell necrosis after coronary artery occlusion. In the clinical setting, reperfusion treatments in acute situations include percutaneous procedures or bypass surgery to relieve the obstructive plaque, and with procedures including administration of thrombolytic agents to dissolve occluding thrombi and antiplatelet and anticoagulant therapy to prevent their progression (Theroux, 1999b). However, under certain conditions, the restoration of tissue perfusion to irreversibly damaged cells may exacerbate injury and cause it to progress at a rapid rate. In contrast to cell death progressing over the course of hours in the presence of a sustained ischemic insult, reperfusion significantly accelerates the rate (minutes) of the conversion from a reversible to irreversible state. The accelerated change in status of the tissue can be observed almost immediately on the electrocardiogram and as a loss of cytosolic enzymes and morphologic evidence of cell death (Lucchesi, 2001). Therefore, reperfusion may be considered a double-edged sword; reoxygenation or reflow, while essential for survival of the tissue, may actually exacerbate rather than diminish cell injury.

Whereas it is widely accepted that extensive changes occur upon reperfusion of ischemic tissue, disagreements exist as to whether these changes reflect new injury or are simply unmasking damage that occurred during the period of oxygen deprivation (Heusch and Schulz, 1997; Schaper and Schaper, 1997). There is yet no way to tell whether irreversible injury is a consequence of the ischemia or the reperfusion, since tissue which is not reperfused must eventually die. Interestingly, however,

experimental evidence suggests that there are recognizable ultrastructural differences in the appearance of tissue subjected to ischemia of sufficient duration with or without reperfusion. Ischemic, nonreperfused tissue is essentially normal in its appearance, whereas tissue from the heart that is reperfused after 60 min ischemia has features characteristic of irreversible injury and cell death (Lucchesi, 2001). Therefore, there must be a component of myocardial cell damage which occurs during the reintroduction of molecular oxygen and/or restoration of blood flow to the previously ischemic heart, thereby extending the region of myocardial injury beyond that due to the ischemic insult. Definite proof for or against the existence of reperfusion injury is difficult to obtain, and would require the demonstration of a new phenomenon occuring at the time of reoxygenation. As Opie (1989) suggested, support of reperfusion injury could be demonstrated by tissue salvage using a pharmacological intervention administered only at the time of reoxygenation (Opie, 1989). For simplicity, the term ischemia-reperfusion (I/R) injury will be used for the remainder of this discussion to describe the overall deleterious consequences of an acute ischemic insult followed by a period of reperfusion.

Ischemia-reperfusion injury can be characterized in terms of four distinct events: (a) dysrhythmias appearing at the time of reperfusion; (b) the progressive return of contractile function upon reperfusion (myocardial stunning); (c) lethal reperfusion-induced injury in which normal or reversibly injured cells, at the end of the ischemic insult, are transformed rapidly into irreversibly injured cells and progress to cell death; and (d) accelerated appearance of cellular changes that resemble the characteristics of necrotic tissue (Lucchesi, 2001). All of these consequences of I/R injury are important in the clinical setting as they limit the efficacy of anti-ischemia therapy and reperfusion (Ambrosio and Tritto, 1999).

1.2.4 Cellular and molecular mechanisms of ischemia/reperfusion injury

1.2.4.1 Introduction: The big "3" of I/R injury

There are many factors that contribute to I/R injury, and these factors seem to synergistically cause cellular injury in the heart. Although many working hypotheses have been proposed by different researchers, three main theories are generally discussed relating to the onset of irreversible damage following an ischemic insult: (a) a critical loss or severe reduction of ATP, (b) damage by oxygen free radicals, and (c) a critical overload of cellular calcium by failure of the calcium pumps.

1.2.4.2 ATP depletion

Intracellular ATP levels are thought to determine the turning point between reversible and irreversible cellular injury, because maintenance of intracellular homeostasis is energy dependent. For example, the Ca^{2+} ATPase and the interaction of actin and myosin are ATP-dependent (Kitakaze and Hori, 2001). Many investigators believe that there exists a critical energetic threshold defining the "point of no-return" or limit of reversible injury (Schwartz *et al.*, 1984; Piper *et al.*, 1990). An apparent ATP threshold (1-2 mmol/g wet weight) for post-ischemic recovery has been described for ischemic dog myocardium (Kubler and Spieckermann, 1970; Jennings *et al.*, 1978), ischemic rat hearts (Taegtmeyer *et al.*, 1985) and anoxic cultured rat cardiomyocytes (Schwartz *et al.*, 1984). Subsequently, Allen and Orchard observed that a 90% decrease in ATP is associated with the irreversible deterioration of the myocardium, and suggested the idea that depletion of ATP content in reperfused myocardium may be a critical factor in the process of irreversible injury (Allen and Orchard, 1987). If, however, adenosine is administered throughout the ischemic and reperfusion periods, a significant increase of ATP synthesis is obtained, probably through stimulation of

glucose metabolism. Recovery of intracellular ATP via administration of ribose or adenosine after reperfusion injury does not promote recovery of contractile function, suggesting that the recovery of intracellular ATP levels does not necessarily improve contractile function (Taegtmeyer *et al.*, 1985). Thus, there is consensus that depletion of ATP levels are important for the determination of irreversible cellular injury, but may not be involved in reversible cellular injury such as myocardial stunning (Kitakaze and Hori, 2001).

1.2.4.3 Free Radicals and the oxygen paradox

The reintroduction of oxygen into the previously oxygen-deprived heart after a period of hypoxia or anoxia in the isolated perfused heart is associated with the "oxygen paradox". The "oxygen paradox" is characterized by an abrupt alteration in the integrity of the myocardial cell and an immediate decline or absolute loss of myocardial contractile function along with an abrupt increase in resting tension (contracture) and ultrastructural changes (Lucchesi, 2001).

The basis of the oxygen paradox is this: although required for maintaining cell viability, molecular oxygen is a major contributor to I/R injury. There is ample indirect evidence that oxygen free radicals and related reactive oxygen species are formed by leukocytes and endothelial cells, to a limited degree during myocardial ischemia, and which markedly increases during reperfusion. Free radicals are reactive chemical species with an unpaired electron in their outer orbitals. Free radicals derived from oxygen include the superoxide radical anion, which can form hydrogen peroxide in a reaction catalyzed by superoxide dismutase (SOD). Hydrogen peroxide may undergo one of two reactions, either detoxification by glutathione-S-peroxidase or catalase, or it may undergo the Fenton/Haber-Weiss reaction to yield the hydroxyl anion. The latter is

highly reactive, penetrates readily across the cell membranes and is most likely the offending reactive species (Lucchesi, 2001). Normally, there are intracellular scavenging systems that protect the cell from free radical mediated injury. These endogenous defense systems include superoxide dismutase (which dismutates superoxide to oxygen and water) and catalase (which reduces hydrogen peroxide to oxygen and water) (Hearse *et al.*, 1994).

There are multiple subcellular sources of oxygen-derived free radicals including lipid peroxides derived from the peroxidation of cell membrane lipids, metabolism of arachidonic acid, and hydrogen peroxide formed during oxygen metabolism. Futhermore, mitochondria can become both a source and a target for toxic oxygen radicals and cell injury. Free radical species may also be derived from extracellular sources, including production from activated neutrophils that infiltrate the ischemic/reperfused zone as part of the inflammatory response. The detrimental effects of free radicals can be studied with *in vitro* experimental models, since tissues or organs subjected to oxygen deprivation and reoxygenation have the capacity to generate cytotoxic reactive oxygen species (Lucchesi, 2001).

All components within the cell are subject to attack by free radicals. Targets for attack include: (1) lipids in the cellular membrane, especially those containing unsaturated double bonds and (2) membrane enzymes, pumps and proteins, such as Na^+/K^+ -ATPase and Ca^{2+} channels (Lucchesi, 2001). Thus, an excessive oxidant stress such as that produced by a prolonged ischemic insult followed by reperfusion may disrupt the cell membrane and related enzymatic functions essential for continued tissue viability. Over an extended period, oxidative stress causes vascular remodeling, such as thickening of the intima and plaque rupture (Akizuki *et al.*, 1985). These effects may be very important in the genesis of acute myocardial infarction.

While free radical species are generated within the normal myocardium, the deleterious effects of free radicals in myocardial injury are thought to relate to an increase in free radical formation, greater toxicity of the species formed, and defects in the endogenous defense/scavenging systems during ischemic stress which are normally responsible for protection of the cell (Ambrosio and Tritto, 1999).

1.2.4.4 Calcium overload

The role of Ca^{2+} overload in myocardial post-ischemic-reperfusion injury has been confirmed by many researchers (Shen and Jennings, 1972; Nayler, 1981; Marban *et al.*, 1987). When myocardial ischemia occurs, an insufficient supply of oxygen depresses mitochondrial oxidative phosphorylation activity. This leads to a rapid depletion of high energy phosphate stores and to an accumulation of H^+ and inorganic phosphates, causing cellular acidosis (Ferrari *et al.*, 1985). Cytoplasmic acidosis activates Na^+/H^+ exchange via the accumulation of H^+, with a prompt increase of intracellular Na^+ levels. Inhibition of the Na^+/K^+ ATPase activity also contributes to an elevation of intracellular Na^+ levels (van Echteld *et al.*, 1991). Subsequently, Na^+ -Ca^{2+} exchange is activated to work in the reverse mode, to expel Na^+ out of the cell in exchange for extracellular Ca^{2+} leading to the development of intracellular Ca^{2+} overload (Figure 1). Furthermore, activation of L-type Ca^{2+} channels on the sarcoplasmic membrane may also contribute to Ca^{2+} overload (du Toit and Opie, 1992). The process of reperfusion is also accompanied by an increase in the level of cytoplasmic Ca^{2+}. Upon reperfusion, restoration of myocardial pH by washout of accumulated H^+ generates a gradient which activates the Na^+/H^+ exchanger. The subsequent increase in intracellular Na^+ activates the Na^+/Ca^{2+} exchanger, resulting in

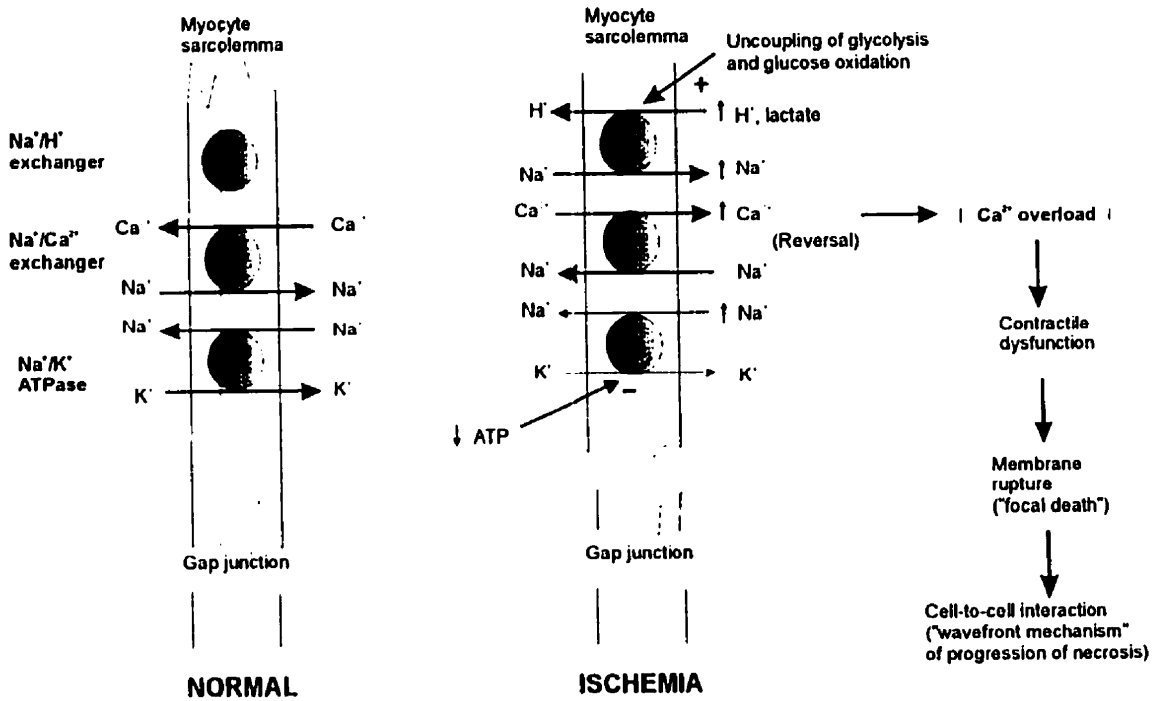


FIGURE 1. SEQUENCE OF INTRACELLULAR EVENTS ASSOCIATED WITH MYOCARDIAL CELL ISCHEMIA AND PROGRESSION TO CELL NECROSIS.

In the normal myocyte, the ATP-dependent Na^+/K^+ pump maintains a Na^+ gradient which drives the Na^+/Ca^{2+} exchanger, which removes Ca^{2+} from the intracellular compartment. The Na^+/H^+ exchange system is non operative. When myocardial ischemia occurs, an insufficient supply of oxygen depresses mitochondrial activity and leads to a rapid depletion of ATP, uncoupling of glycolysis and glucose oxidation, and an accumulation of H^+ causing cellular acidosis. Cytoplasmic acidosis activates the Na^+/H^+ exchange, with a prompt increase in Na^+ levels. Inhibition of Na^+/K^+ ATPase activity also contributes to an increase of intracellular Na^+ levels. Subsequently, Na^+/Ca^{2+} exchange is reversed, leading to the development of intracellular Ca^{2+} overload. Ca^{2+} overload initiates the progression of cell necrosis: contracture, sarcolemma rupture, cell death and the spread of necrosis through cell-to-cell interactions across the gap junctions. (Modified from Theroux, 1999a)

Ca^{2+} overload (Brooks *et al.*, 1995; Meissner and Morgan,1995). In addition, Ca^{2+} overload may be attributed to depressed sarcolemmal Na^{+}/K^{+} ATPase and/or Ca^{2+} ATPase activity, or increased Ca^{2+} entry via the L-type Ca^{2+} channel (du Toit and Opie, 1992). Although calcium ions are vital in mediating cardiac excitation-contraction coupling, an excessive amount of intracellular calcium produces deleterious effects on myocardial function (e.g. hypercontracture), structure (e.g. disruption of cytoskeletal structures), and metabolism (Ambrosio and Tritto, 1999). The latter is a result of accumulation of Ca^{2+} in the mitochondria during respiration, which reduces the energy available for formation of ATP and eventually leads to irreversible injury to the mitochondria (Carafoli, 1985). The toxic effects of Ca^{2+} overload also include abnormalities in energy production and utilization, electrophysiological derangement, disruption of membrane integrity, and ultrastructural changes. Several studies have shown that Ca^{2+} overload plays a major role in myocardial ischemic cell damage and cardiac dysfunction, probably as a consequence of activation of different Ca^{2+}-dependent phospholipases, proteases (calpains) and other hydrolytic enzymes, as well as oxidative stress (Vork *et al.*, 1993; Weglicki and Low, 1987). It appears that oxidative stress as a consequence of oxygen free radical formation due to metabolic abnormalities in the myocardium, plays an important role in the development of Ca^{2+} overload and cardiac dysfunction in ischemic heart disease. Elevated levels of both free radicals and Ca^{2+} contribute synergistically to cardiac dysfunction (Dhalla *et al.*, 2001). The burst of reactive oxygen species formation in the early stages of reperfusion of the ischemic heart can induce defects in key cellular organelles, resulting in cardiac dysfunction (see section 1.2.4.3). Clearly, defects in several Ca^{2+}-dependent pumps with eventual Ca^{2+} overload must play a major role in the abnormal cardiac excitation-contraction coupling observed during I/R injury. Furthermore, increased

intracellular Ca^{2+} levels would stimulate the production of free radicals by the conversion of xanthine dehydrogenase to xanthine oxidase in the myocardium (Downey *et al.*, 1988; Akizuki *et al.*, 1985.

1.2.5 Other factors that contribute to I/R injury

1.2.5.1 Circulating catecholamines

Catecholamines such as norepinephrine (NE) may be involved with I/R injury. When myocardial ischemia occurs, presynaptic vesicles release NE via the accumulation of Na^+. Norepinephrine activates both α- and β-adrenoceptors: α-adrenoceptor stimulation increases intracellular Ca^{2+} levels and causes coronary vasoconstriction and β-adrenoceptor stimulation increases myocardial oxygen consumption (Kitakaze and Hori, 2001). These factors may cause deterioration of myocardial contractile and metabolic functions during ischemia and reperfusion. Indeed, many experimental and clinical studies reveal that β-blockers have anti-ischemic effects (see section 1.3.3.2).

1.2.5.2 Microcirculatory disturbances

Even if ischemic myocardium is reperfused by the once occluded coronary artery after acute myocardial infarction, coronary microvasculature does not necessarily receive adequate flow (Kitakaze and Hori, 2001). This is called the "no reflow phenomenon" and has been reported to predict the size of the myocardial necrosis and functional recovery in experimental models, as well as in patients with acute myocardial infarction (Ito *et al.*, 1995; Kloner *et al.*, 1974). The no-reflow phenomenon may be caused by myocardial cell injury, platelet plugging, leukocyte adhesion, and increases

in the tone of small coronary vessels. Kloner *et al.* reported that ischemia >40 min. is associated with no-reflow (Kloner *et al.*, 1974).

1.2.5.3 Endothelin

Endothelin (ET) is an endogenous endothelium-derived substance which appears to be a vasodilator (ET-2 and ET-3 subtypes) at physiologically low concentrations, and a potent vasocontrictor in several pathologies associated with a rise in ET-1 plasma levels (Brunner, 1997; Brunner *et al.*, 1992). Increased ET-1 levels have been measured in patients with angina and acute myocardial infarction (Brunner *et al.*, 1992). However, the role of ET in experimental models of I/R injury remains controversial, since the effects of ET receptor antagonists on infarct size are conflicting (Watanabe *et al.*, 1991; Krause *et al.*, 1994; Watanabe *et al.*, 1998). Endothelin may be deleterious to ischemic hearts by inducing coronary vasoconstriction, Ca^{2+} overload or leukocyte or platelet activation (Kitakaze and Hori, 2001). However, the precise mechanisms are not clear at present.

1.2.5.4 Apoptotic versus necrotic cell death

Apoptosis can be defined as cellular suicide involving specialized initiation and execution mechanisms within the cell (Buja *et al.*, 1993). Based on cellular pathophysiology, the classification of cell death has focused on two major entities: necrosis (more accurately referred to as "oncosis", which describes the process of death by cell lysis) and apoptosis. The basic difference between the two forms of cell death can be summarized as destruction of the cell by an external stimulus in necrosis, versus self-destruction in response to unfavorable conditions in the case of apoptosis

(Haunstetter and Izumo, 2001). This difference results in different morphological, biochemical and diagnostic features (Table 1). A cell can undergo apoptosis in reaction to several different stimuli, including ligands at the cell membrane or DNA-damaging agents, but the common final mechanism involves activation of a characteristic subgroup of proteases, called caspases (Pijl *et al.*, 1993a). Once activated, caspases cleave many cellular proteins leading to characteristic morphological alterations of the dying cell. In contrast, necrotic (oncotic) cell death seems to be triggered by energy depletion, since maintenance of cellular integrity requires ATP (Leist and Nicotera, 1997). Deregulated ion transport induces water shifts leading to increased cytoplasmic volume and swelling of intracellular organelles, such as mitochondria and the nucleus (Majno and Joris, 1995). Membrane rupture/lysis is the irreversible consequence that kills the cells.

It is unclear whether the mode of cell injury and death in ischemic myocardium is a result of apoptotic or necrotic cell death. Whereas apoptosis and necrosis are two distinct forms of cell death, they may occur simultaneously in tissues or cell cultures exposed to the same stimulus. The decision as to whether myocytes enter apoptosis or necrotic injury in response to ischemia or other stimuli seems to be determined by the energy status of the cell (Brooks *et al.*, 1995; Kloner *et al.*, 1974). Ischemia leads to the depletion of ATP within a few minutes, resulting in defects in energy dependent intracellular signaling and cellular homeostatic mechanisms regulating cell volume and ion pumps. The result is cell swelling and rupture not amenable to regulation. A review of the literature illustrates several inconsistencies: whereas some studies describe the occurrence of apoptosis in ischemic myocardium during coronary occlusion (Yaoita *et al.*, 1998), others describe the development of apoptosis only during reperfusion of previously ischemic myocardium (Mocanu *et al.*, 2000; Gottlieb *et al.*, 1994).

TABLE 1. FEATURES OF APOPTOTIC AND NECROTIC CELL DEATH

	Apoptosis	Necrosis
Cell morphology	Cell shrinkage Cell fragmentation	Cell swelling
Nuclear morphology	Chromatin condensation Nuclear fragmentation	Nuclear swelling
Membrane alterations	Intact membrane	Membrane rupture
Caspase activation	Yes	No
Energy dependence	Yes	No

Modified from Haunstetter and Izumo, in Heart Physiology and Pathophysiology (2001).

Conversely, necrotic cell injury with cell swelling has been repeatedly described as the dominant pattern of myocardial ischemic injury (Antoine *et al.*, 1997; Kitakaze *et al.*, 1997).

Cardiac myocytes are terminally differentiated cells that are not believed to possess an adequate regenerative potential (Soonpaa and Field, 1998). Therefore myocyte loss, be it necrotic or apoptotic, acute or chronic, will irreversibly reduce the pool of contractile cells. Although surviving myocytes may compensate for this loss by cellular hypertrophy and increased functional capacity, progressive myocyte depletion may overwhelm compensatory mechanisms, and clinical cardiac disease ensues (Haunstetter and Izumo, 2001).

1.3 Pharmacological agents with anti-ischemic properties

1.3.1 Therapeutic strategies and limitations

Myocardial cell protection and prevention of cell necrosis have been therapeutic targets for a long time. However, the complex interplay of neural, hormonal and local factors that control cardiac function complicates the search for cardioprotective agents. The potential success of any therapy designed to salvage reversibly ischemic myocardium depends on: (1) the presence of viable ischemic cells and (2) adequate perfusion at the microvascular level in the affected area (i.e. area-at-risk) to permit delivery of the agent (Vyden and Takano, 1978). However, a major limitation of clinical treatment in acute situations is time: there is a time delay in the application of cardioprotective agents, and in reaching their therapeutic effect in the absence of aerobic reserve of the myocardial cell (Theroux, 1999b). Furthermore, considerable controversy still exists as to the efficacy of these different drug treatments, since some

investigators have been unable to duplicate the cardioprotective effects observed by other groups (Richard *et al.*, 1988).

Since most experimental studies involve the sudden occlusion of a coronary artery in what was previously healthy tissue, this differs greatly from the complex and progressive development of human cardiovascular disease with its underlying vascular perturbations and genetic and environmental components. These differences might contribute to the disappointing fact that so many drugs have failed when tested in man (Hearse, 1988). Despite these limitations and challenges, the burden of treating morbidity and mortality of heart diseases for individual patients, as well as the social and economical burden, fuels the exhaustive efforts of investigators. In experimental models, three different approaches have been used to achieve cardioprotection in ischemic hearts: (1) cardioprotection *before* the onset of ischemia, (2) cardioprotection *during* ischemia and reperfusion and (3) drugs to treat coronary heart failure *after* acute myocardial infarction.

1.3.2 Cardioprotection before the occurrence of ischemia

1.3.2.1 Rationale

A subset of the population are at greater risk for acute myocardial infarction, primarily due to the presence of "high-risk factors" including hyperlipidemia, smoking, obesity, stress, diabetes and hypertension. For these individuals, there is a need for effective pretreatment before the onset of myocardial ischemia to attenuate ischemia-reperfusion injury (Kitakaze and Hori, 2001). Increasing ischemic tolerance before the onset of myocardial ischemia is analogous to immunization for infectious diseases. The different approaches that have been employed to induce cardioprotection prior to

an acute ischemic insult include plaque rupture prevention, induction of collateral circulation, and ischemic preconditioning.

1.3.2.2 Plaque rupture prevention

Acute infarction is known to be precipitated by rupture of the atherosclerotic plaque within the coronary vessel, followed by accumulated platelet aggregation. Hyperlipidemia is a major risk factor for plaque formation. Cholesterol lowering therapy, such as oral administration of pravastatin (an inhibitor of HMG-CoA reductase, an enzyme required for cholesterol synthesis) has been shown to lower the incidence of cardiac death in high risk patients (Sacks *et al.*, 1996).

1.3.2.3 Collateral vessel formation

Alternatively, cardioprotection has been attempted through efforts to promote collateral circulation to the ischemic myocardium. Growth factors, including fibroblast growth factor, tumor growth factor beta and vascular endothelial growth factor, are known to promote angiogenesis (i.e. proliferation and migration of vascular smooth muscle cells) and may be beneficial in promoting the formation of new vessels to supply ischemic zones of the heart. Furthermore, heparin and adenosine administration may enhance angiogenesis via induction of the aforementioned growth factors (Schaper, 1991; Watanabe *et al.*, 1998)

1.3.2.4 Ischemic preconditioning and ATP-sensitive potassium channels

In the last several years, a great deal of interest has focused on the phenomenon of ischemic preconditioning as a mechanism of protecting cells from ischemic injury. Ischemic preconditioning was first described by Murry *et al.* in 1986,

and refers to a phenomenon in which a single or multiple brief periods of ischemia protect the heart from a subsequent prolonged ischemic insult (Murry *et al.*, 1986). In various studies in different species, ischemic preconditioning has been shown to limit infarct size by 10 to 20 % of the risk area in the reperfused myocardium following an acute ischemic insult (Xi *et al.*, 1998; Gross and Fryer, 1999; Theroux, 1999b; Pijl *et al.*, 1993b). There are thought to be two "windows" of protection afforded by ischemic preconditioning: the first window or "early phase" of protection lasts 1 to 3 hours after the initial preconditioning stimulus, and a second window or "late phase" appears between 12 to 24 hours later and may last for up to 3 days (Watts *et al.*, 1987; Yellon and Baxter, 1995). The mechanism underlying this method of cardioprotection remains controversial, but is thought to be receptor mediated: activation of the adenosine A_1 receptor with subsequent activation of phospholipase C, and activation of protein kinase C (PKC). Protein kinase C is then thought to phosphorylate and increase the permeability of ATP-sensitive potassium (K_{ATP}) channels (Pijl *et al.*, 1993b; Ford *et al.*, 1998, and Baxter *et al.*, 1994)

An abundance of experimental evidence in the last few years has identified two types of K_{ATP} channels: one on the surface of the sarcolemma (sarc K_{ATP}) and a mitochondrial K^+ channel (mito K_{ATP}). These channels couple cellular metabolism with membrane excitability and have been implicated in the regulation of vascular tone and cardioprotection. Experimental evidence using drugs selective for the mito K_{ATP} channel (e.g. diazoxide at submicromolar concentrations) suggests the mitochondrial potassium channel to be the primary mediator of cardioprotection (Garlid *et al.*, 1997; Gross and Fryer, 1999; Gross and Auchampach, 1992; Yellon and Gross, 1995). The primary endogenous ligand of K_{ATP} channels is intracellular ATP, which blocks channel activity, whereas intracellular ADP serves as the major channel activator (Noma, 1983).

Thus, in normal cells, K_{ATP} channels are believed to be silent, and open under conditions of metabolic stress with changes in the ATP/ADP ratio i.e. during an acute ischemic insult (Figure 2).

1.3.3 Cardioprotection during ischemia and reperfusion

1.3.3.1 Endogenous factors that cause cardioprotection

1.3.3.1.1 Adenosine

Adenosine is produced by cardiomyocytes and endothelial cells, and is known to be cardioprotective. Activation of adenosine A_1 and A_2 receptors results in attenuation of the release of catecholamines and β-receptor-mediated hypercontraction and Ca^{2+} overload, and increased coronary blood flow and inhibition of platelet and leukocyte activation, respectively (Hori and Kitakaze, 1991). Stimulation of A_2 receptors activates adenylyl cyclase to produce cyclic adenosine monophosphate (cAMP). Increased cAMP levels may have several beneficial effects, including relaxation of coronary vascular smooth muscle and opening of K_{ATP} channels. Furthermore, stimulation of A_2 receptors has been reported to inhibit the platelet aggregation responsible for small coronary microembolizations in coronary arteries, which may be the cause of no-reflow (Kitakaze *et al.*, 1991).

1.3.3.1.2 Role of nitric oxide in the heart: cardioprotective or cardiotoxic?

Nitric oxide (NO) produces both protective and detrimental effects in a variety of systems (Schulz and Wambolt, 1995). In the normal heart, nitric oxide (NO) is synthesized from oxygen and L-arginine by the coronary endothelium, cardiac myocytes and endocardial endothelial cells, all of which possess calcium-dependent NO synthase (NOS-III). The physiological release of small quantities of NO in the heart

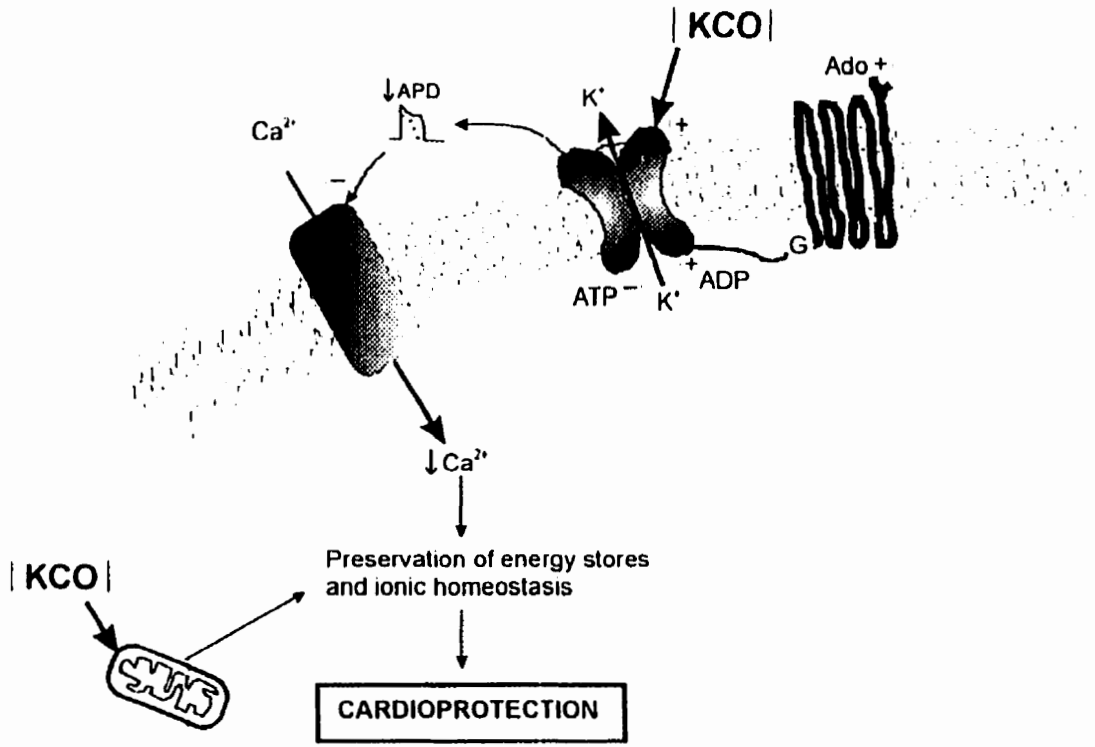


FIGURE 2. POTASSIUM CHANNEL OPENERS (KCO) MODULATE SARCOLEMMAL AND MITOCHONDRIAL K_{ATP} CHANNELS IN CARDIAC MYOCYTES.

KCO activate sarcolemmal K_{ATP} channels, leading to K^+ efflux, shortening of the action potential duration (APD), and a decrease in Ca^{2+} influx. KCO also activate K_{ATP} channels in mitochondria, resulting in membrane depolarization and in prevention of mitochondrial Ca^{2+} overload. Adenosine (Ado) has been recognized as a channel activator through a direct action via a guanosine triphosphate binding protein (G). K_{ATP} channels opening in ischemia occur through a change in the ADP/ATP ratio at the channel site. Cardioprotection may involve both types of K_{ATP} channels. Modified from Jahangir *et al.*, in Heart Physiology and Pathophysiology (2001).

provides for the maintenance of coronary vasodilator tone, and inhibition of platelet aggregation and the adhesion of neutrophils and platelets to the vascular endothelium (Brunner, 1997). Furthermore, NO has direct negative inotropic and chronotropic effects on cardiac muscle. These effects may be cardioprotective by decreasing heart rate, afterload and/or contractility to improve the oxygen supply/demand ratio (Vyden and Takano, 1978). Recent studies have shown that basal release of NO from rat hearts was diminished after ischemia and reperfusion (Maulik *et al.*, 1995) and the detrimental consequences of impaired NO release were abolished by providing exogenous NO donors (Pabla *et al.*, 1995). Although debate still exists, several lines of evidence suggest that NO reduces myocardial contractile function and improves metabolic function in the ischemic heart (Node *et al.*, 1996; Schulz and Wambolt, 1995).

During reperfusion following myocardial ischemia , there is an acute and excess production of NO that has been implicated in cellular reperfusion injury. Excess NO production may be through: (i) increased shear stress (that is a strong stimulus for NO formation) along the endothelium during reflow; (ii) increased intracellular Ca^{2+} that stimulates the Ca^{2+}-dependent NOS (NOS-III) in cardiac myocytes, coronary endothelial cells and endocardium; and (iii) reintroduction of oxygen during reperfusion which is a substrate for NO synthesis (Pabla and Curtis, 1996). The deleterious effects of NO have been shown by a reduction of infarct size following ischemia and reperfusion *in vivo* when a NOS inhibitor (L-NAME) is administered (Schulz and Wambolt, 1995; Hotta *et al.*, 1999). Futhermore, NO may be detrimental because it may react with molecular oxygen to produce peroxynitrite, which is very harmful to cells. However, the general consensus is that in terms of ischemia and reperfusion injury, the beneficial actions of NO outweigh the deleterious ones (Maulik *et al.*, 1995).

Recently, a great deal of effort has focused on elucidating the possible role NO may play in the ischemic myocardium. The beneficial effects of organic nitrates (widely believed to undergo a biotransformation reaction to release NO) on the ischemic myocardium is believed to result from dilation of coronary arteries and peripheral vessels, leading to an improved nutritional and oxygen status, together with a reduction in work load. Nitrates may also produce an overall improvement of metabolic parameters, as seen by a reduction in lactate levels, an increase in tissue energy charge and a reduction in NADH levels following administration of drugs (e.g. 8-Bromo-cGMP, sodium nitroprusside (SNP), glyceryl trinitrate (GTN) and cysteine) which increase cGMP levels in myocardial tissue (Barger *et al.*, 1995; Laustiola *et al.*, 1983a; Laustiola,1985; Laustiola *et al.*, 1983b; Laustiola *et al.*, 1984). It is thought that the role of NO in cardiac pathophysiology may involve free radicals (which inactivate NO), metabolic enzymes (by inhibiting an overdrive of anaerobic metabolism) and intracelluar calcium homeostasis (Maulik *et al.*, 1995). Another mechanism by which NO may induce cardioprotection in the ischemic heart is through activation of K_{ATP} channels. Nitric oxide has been shown to open K_{ATP} in isolated mesenteric arteries (Murphy and Brayden,1995), in isolated pancreatic islets (Antoine *et al.*, 1997), and in mitochondria isolated from rat hearts (Crestanello *et al.*, 2000).

1.3.3.1.3 Acidosis

Cellular acidosis is thought to be a natural defense mechanism against myocardial I/R injury. Kitakaze and colleagues have observed that hydrogen ions block Ca^{2+} channels, Na^{+}/Ca^{2+} exchange and antagonize Ca^{2+} overload in the myocardium (Kitakaze *et al.*, 1997). Therefore, transient cellular acidosis seems to attenuate I/R

injury, restore myocardial function, and limit cellular necrosis (Kitakaze and Hori, 2001).

1.3.3.2 Rationale for use of pharmacological agents during ischemia and reperfusion

Clinically, the goal of treatment for acute myocardial infarction is to reperfuse the occluded coronary artery, either by percutaneous transluminal coronary angioplasty or percutaneous transluminal coronary recanalization (Kitakaze and Hori, 2001). However, these interventions only seem to limit infarct size to a modest extent because of the diminution of a beneficial effect by reperfusion injury. Thus, there is a need to find adjunctive therapy to treat ischemia and reperfusion injury directly.

Coronary artery disease and acute myocardial infarction result in an imbalance between the normal oxygen requirements of the contracting heart and oxygen delivery through the narrowed coronary arteries. Thus, therapeutic strategies have generally been aimed at a reduction of myocardial oxygen needs or interventions aimed to improve oxygen delivery. Initial efforts to protect the ischemic heart were mainly aimed at a reduction of myocardial oxygen needs by acting on the major determinants of oxygen consumption of the beating heart: heart rate, the inotropic state, afterload and preload (Theroux, 1999b). Indeed, beta-blockers (e.g. atenolol) may have anti-ischemic effects through reduced oxygen demand and blunting of sympathetic tone to prevent lethal arrhythmias and epinephrine-induced platelet aggregation involved in thrombus formation (see section 1.3.4.1).

In considering the temporal and spatial charateristics of ischemic injury, it is well established that the injury does not evolve uniformly throughout the myocardium; regional differences in metabolism and energy requirement render the endocardium the most vulnerable to injury. Thus, cell death and tissue necrosis will develop first in the

endocardium and spread as a "wavefront" toward the epicardium (Chambers and Hearse, 2001). This wavefront phenomenon of myocardial ischemic death was first described by Reimer and Jennings in 1979, and may underly the mechanism of cardioprotection afforded by drugs like beta-blockers, which are thought to redistribute coronary blood flow from the epicardium toward the endocardium, the core of the ischemic region (Reimer and Jennings, 1979).

Greater success in the treatment for acute myocardial infarction has been obtained with interventions directed to improve oxygen delivery. For example, the atherosclerotic plaque may be relieved with percutaneous procedures or with bypass surgery, or corrected with reperfusion procedures including thrombolytic agents to dissolve the occluding thrombi and antiplatelet and anticoagulant therapy to prevent their progression (Theroux, 1999a).

Because the mediators of I/R injury are multifactorial (see section 1.2.4), the rationale for mediating cardioprotection during ischemia and reperfusion is (1) to use many drugs that inhibit each deleterious factor or (2) to use one drug that inhibits many deleterious factors. In the clinical setting, the latter seems to be more plausible.

1.3.3.2.1 Calcium channel blockers

Calcium channel blockers have been investigated as potential cardioprotective agents. Cardioprotection by calcium channel blockers *in vitro* has been reported by many groups (Pijl *et al.*, 1993a; Watts *et al.*, 1987; Grover *et al.*, 1990a; Hamm and Opie, 1983). The beneficial effects have been ascribed to the ability of these agents to reduce myocardial oxygen demand by reducing contractile force, left ventricular afterload, and heart rate, as well as to improve oxygen supply by vasodilation of stem arteries and collaterals (Held *et al.*, 1989). In addition, at the cellular level, calcium

channel blockade may prevent a detrimental accumulation of calcium in ischemic mitochondria and slow the loss of oxidative phosphorylation (Bowser *et al.*, 1998). Furthermore, a free radical scavenging effect has been reported for a number of calcium channel blockers (Czarnowska *et al.*, 1998; Ross *et al.*, 1998). However, a major disadvantage of the calcium channel blockers is that their anti-ischemic activity is accompanied by negative inotropic responses; i.e. cardiodepression at cardioprotective concentrations (Grover *et al.*, 1990a).

Clinical therapy with calcium antagonists including verapamil, nifedipine and diltiazem have consistently failed to show any significant benefical effect on mortality in patients with acute myocardial infarction and was even associated with a trend toward an *increase* in mortality in some trials. Based on these observations, calcium antagonists are not recommended as primary therapy for unstable angina or acute MI (Held *et al.*, 1989).

1.3.3.2.2 Anti-radical therapies

Experimental evidence has shown radical production contributes to the generation of myocardial contractile dysfunction, and this dysfunction is restored by administration of SOD or xanthine oxidase inhibitors such as oxypurinol (Gross *et al.*, 1986; Maie *et al.*, 1991; Reimer *et al.*, 1990; Bolli, 1988). However, these results are controversial in that other researchers have been unable to reproduce the cardioprotection observed by these groups (Richard *et al.*, 1988; Kinsman *et al.*, 1988). Furthermore, despite a number of positive reports, there are studies failing to demonstrate the deleterious effects of oxygen-free radicals in the extension of myocardial injury (Engler and Gilpin, 1989; Uraizee *et al.*, 1987). Indeed, the TIMI (Thrombolysis In Myocardial Infarction) study revealed that 10 mg/kg of SOD did not

attenuate infarct size in 120 patients with acute myocardial infarction (Flaherty *et al.*, 1994). This surprising result may be attributable to the fact that oxygen-derived free radicals may not contribute to the formation of myocardial necrosis to an appreciable extent *in vivo*, or the therapeutic time window of SOD may be narrow in patients with acute myocardial infarction.

1.3.3.2.3 Sodium-Hydrogen exchange inhibitors

Recently, a great deal of interest has focused on anti-ischemic therapy beyond the traditional approach of decreasing myocardial oxygen needs or increasing oxygen supply. During ischemia, the rate of glycolysis is increased, whereas glucose and free fatty acid oxidation are impaired. With reperfusion, fatty acid oxidation recovers more promptly, inhibiting glucose oxidation. Thus there is a metabolic mismatch between glycolysis and glucose oxidation that results in accumulation of lactate, pyruvate, and hydrogen (protons) ions (Theroux, 1999a; Theroux, 1999b). The accumulation of hydrogen ions activates the Na^+/H^+ exchange (NHE-1) system. The system results in H^+ extrusion from the cell in exchange for Na^+, resulting in cell swelling and Na^+ exchange for Ca^{2+}; Ca^{2+} overload, cell contracture, sarcolemmal bleb formation and rupture follow (Theroux, 1999a). Since the NHE-1 system may indirectly lead to Ca^{2+} accumulation, inhibitors such as amiloride and cariporide may prevent or delay cell death. Indeed, results of several *in vitro* studies in isolated myocytes and heart preparations, and *in vivo* studies in pigs and rats have suggested NHE-1 exchange inhibition is an effective means to prevent lethal reperfusion injury and dysrhythmia, and to improve myocardial contractile dysfunction (Ferrari *et al.*, 1985; Shen and Jennings, 1972; Bowser *et al.*, 1998; Held *et al.*, 1989). Furthermore, administration of the NHE-1 specific inhibitor cariporide (HOE 642) *in vitro*, has been shown to reduce

infarct size and enzyme release (Tritto *et al.*, 1998). However, whereas Rohmann *et al.* have recently reported that cariporide reduces infarct size in pigs when applied either pre- or post-ischemia (Rohmann *et al.*, 1995), most groups have only reported infarct-limiting effects of cariporide when administered *prior* to the acute ischemic insult (Garcia-Dorado *et al.*, 1997; Klein *et al.*, 1997; Miura *et al.*, 1997).

Based on these promising experimental results, (Ambrosio and Tritto, 1999) in 1999 the first major clinical investigation to test the efficacy of NHE-1 inhibition on management of acute coronary syndromes was reported. In the GUARD During Ischemia Against Necrosis (GUARDIAN) phase II/III clinical trial, almost 12,000 patients with acute coronary syndromes at risk of myocardial necrosis were treated with cariporide and assessed for mortality and myocardial infarction. The results suggest that administration of cariporide attenuates myocardial reperfusion injury and may be effective in the management of complications (e.g. left ventricular dysfunction) following acute myocardial infarction (Theroux, 1999b). However, while NHE-1 inhibitors represent the most promising cardioprotective agent at present, more long term studies are needed to determine whether NHE-1 inhibitors reduce mortality.

1.3.3.2.4 K_{ATP} channel modulators

Potassium channel openers (e.g. cromakalim, diazoxide) improve the post-ischemic recovery of myocardial function and diminish infarct size (Grover *et al.*, 1989; Grover *et al.*, 1990b; Gross and Fryer, 1999). Conversely, in the isolated ischemic/reperfused heart, administration of K_{ATP} antagonists such as glibenclamide (glyburide) and sodium 5-hydroxydecanoate (5-HD) have been shown to abolish drug-induced cardioprotection (Ferdinandy *et al.*, 1995a; Ferdinandy *et al.*, 1995b; Csont *et al.*, 1999). Cardioprotection by K_{ATP} channel openers is independent from changes in

collateral blood flow, suggesting a direct cytoprotective action on the cardiomyocyte. Whereas the precise mechanism of K_{ATP} channel-mediated cardioprotection remains unclear, it is thought that opening of mito K_{ATP} results in an influx of K^+ into the mitochondria. The consequences of this K^+ influx may include: (i) an expansion of mitochondrial matrix volume (leading to activation of the electron transport chain and stimulation of metabolism to restore ATP levels) (Holmuhamedov *et al.*, 1998), (ii) membrane depolarization, thereby decreasing the driving force for Ca^{2+} through the mitochondrial Ca^{2+} uniport (Crestanello *et al.*, 2000; Holmuhamedov *et al.*, 1998) and (iii) a reduction in the inner mitochondrial membrane potential established by the proton pump (Pijl *et al.*, 1993b). In a 1999 multicentre, randomized European clinical trial (Clinical European Studies in Angina and Revascularization, CESAR 2), 20 mg twice daily oral nicorandil (a K_{ATP} channel opener) was administered to patients with unstable angina. The results suggested that nicroandil has beneficial anti-dysrhythmic and anti-ischemic actions (Patel *et al.*, 1999). Whereas these results suggest modulation of K_{ATP} channels is a novel pharmacological approach with significant clinical potential, more large-scale clinical trials are necessary before these ion channel modulators are accepted into clinical medicine.

1.3.3.2.5 Anti-apoptosis therapy

Increasing evidence indicates that apoptosis is a feature of several cardiac disease states, including ischemic heart disease. Apoptosis has been an appealing target for anti-ischemia therapy for several reasons. Firstly, apoptosis is a regulated form of cell death for which antiapoptotic mechanisms are available in the myocyte. Second, antiapoptotic interventions may be effective after the apoptotsis-inducing stimulus has reached the cell, thus increasing the time window for intervention. Thirdly,

therapeutic approaches may be developed that chronically strengthen antiapoptotic regulatory pathways or weaken proapoptotic mechanisms, thus providing long-term cardiac protection against any apoptotic stimulus (Haunstetter and Izumo, 2001).

In animal studies, persistent ischemia or transient ischemia followed by reperfusion have been associated with myocyte apoptosis as evidenced by TUNEL (TdT-UTP nick end labeling, which allows for detection of individual apoptotic nuclei) staining and demonstration of internucleosomal DNA fragmentation (Gottlieb *et al.*, 1994). It has been reported that the inhibition of apoptosis by caspase inhibition in a rat model of myocardial infarction reduces infarct size and protects against lethal reperfusion injury (Yaoita *et al.*, 1998; Pijl *et al.*, 1993a)

1.3.3.3 Nitrates in the treatment of cardiovascular disease

A variety of nitrites and nitrates have been widely used clinically in the treatment of angina pectoris for more than 100 years. The anti-anginal effects of glyceryl trinitrate (GTN) are believed to be based on the drug-induced decrease in preload and afterload, improvement of coronary collateral flow, dilation of stenotic coronary arteries, and inhibition of platelet aggregation (Ahlner *et al.*, 1991; Harrison and Bates, 1993).

All nitrates used as vasodilators in the treatment of angina have a similar molecular structure with the nitrate ester bond (R-O-NO_2) as an essential feature. All organic nitrate esters share similar pharmacological properties, including basic mechanism of action, due to the similarites in their chemical structure.

In a number of large scale randomised clinical trials of GTN administered intravenously after an acute myocardial infarction, the benefit of nitrates was relatively small, although some evidence of a slight reduction in short-term mortality was seen in

two trials (the Fourth International Study of Infarct Survival, ISIS-4, and Gruppo Intaliano per lo Studio dell Soprawivenza nell'Infarto miocardico, GISSI-3).

1.3.3.4 Signal transduction pathways of organic nitrates

The current state of knowledge suggests that organic nitrates act as inactive prodrugs that require a complex metabolic process for their activation. These metabolic changes occur predominantly in the intracellular space after penetration of the smooth muscle cell membrane. The biotransformation of GTN results in the formation of dinitrate metabolites glyceryl-1,2-dinitrate (1,2-GDN) and glyceryl-1,3-dinitrate (1,3-GDN) and either inorganic nitrite anion (NO_2^-) or nitric oxide (NO) (Figure 3). Since experimental evidence indicates that not all biotransformation reactions of organic nitrates lead to vascular smooth muscle relaxation, the terms "clearance-based" and "mechanism-based" biotransformation may be used to differentiate between the possible functional consequences of organic nitrate biotransformation. Thus, clearance-based biotransformation is associated with metabolism of GTN to the inactive nitrite anion, and mechanism-based biotransformation is associated with metabolism of GTN to an active metabolite such as NO (Bennett *et al.*, 1994). Nitric oxide is believed to be the pharmacologically active intermediate produced by GTN biotransformation (Katsuki *et al.*, 1977). However, some researchers propose that S-nitrosothiol compounds may be intermediates of GTN bioactivation, since experimental evidence has shown that NO reacts with several thiol-containing compounds including cysteine and glutathione to form products that activate guanylyl cyclase in broken cell preparations (Ignarro *et al.*, 1980). Investigating the proposed formation of S-nitrosothiol intermediates is limited by inadequate technology to measure the intracellular production of these intermediates during smooth muscle relaxation.

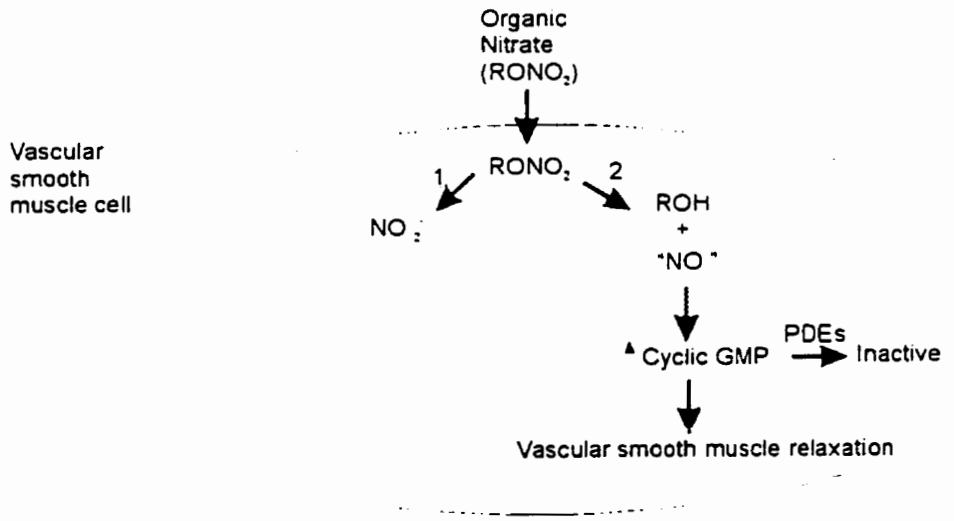


FIGURE 3. BIOTRANSFORMATION PATHWAYS OF ORGANIC NITRATES IN VASCULAR SMOOTH MUSCLE.

Clearance based biotransformation is illustrated by ① and mechanism-based biotransformation is illustrated by ②.

Regardless of the nature of the active intermediate that is formed, it is generally accepted that biotransformation of GTN is essential for its pharmacological activity.

It is believed that activation of the soluble form of guanylyl cyclase (GC) is essential for the signal transduction of NO following organic nitrate biotransformation (Katsuki *et al.*, 1977). Activation of GC results in accumulation of 3',5'-cyclic guanosine monophosphate (cyclic GMP or cGMP), which in turn activates type I cyclic GMP-dependent protein kinase(PKG-I). PKG-1 subsequently phosphorylates key proteins that regulate vascular tone (Ahlner *et al.*, 1991). Termination of cGMP activity is achieved through hydrolysis by cyclic nucleotide phosphodiesterases (PDEs), specifically the PDE1 and PDE5 isoforms (Pyne *et al.*, 1996).

1.3.4 Cardioprotection after acute myocardial infarction (MI)

1.3.4.1 A pharmacological approach to treating MI: Effect of beta-blockers and ACE-inhibitors

From a pharmacological perspective, treatment after acute MI targets the neurohormonal factors that are thought to exacerbate the severity of chronic heart failure, including catecholamines, renin-angiotensin and cytokines. Indeed, β-receptor blockers and angiotensin-converting enzyme inhibitors have been proven to be effective in the treatment of chronic heart failure.

Beta-blockers have been tested for therapeutic efficacy in clinical trials. In the ISIS-1 (First International Study of Infarct Survival) phase III clinical trial, a 1986 randomized study of 16,027 cases of suspected myocardial infarction in which patients received 100 mg/day atenolol for one week, there was a significant reduction in short term mortality (within the first two weeks of the trial). Other investigators have shown angiotensin-converting enzyme (ACE) inhibitors (e.g. enalapril) have cardioprotective

effects *in vitro*. In trials of acute therapy (ISIS-4, 1995; GISI-3, 1994) in patients both with and without evidence of left ventricular dysfunction, ACE inhibitors reduced mortality at four to six weeks in two different patient populations and with two different ACE inhibitors, captopril (ISIS-4) and lisinopril (GISI-3).

Alternatively, nitric oxide, adenosine and endothelin receptor antagonists have all been proven to provide beneficial effects in the treatment of chronic heart failure, although further clinical trials are needed.

1.3.4.2 Molecular and mechanical approaches in the treatment of MI

Other strategies for the treatment of chronic heart failure are through attempts to compensate for the loss of myocardium and to decrease myocardial fibrosis. One method is through gene targeting. Many investigators are focusing on finding a master gene to regulate the proliferative capability of cardiomyocytes, which if successful would be an effective treatment for the failing heart. Another method that could potentially be used is the transplantation of cardiomyocytes to failing hearts. However, further investigation is needed to find a way to change human embryonic stem cells to cardiomyocytes (Kitakaze and Hori, 2001).

Finally, another interesting approach for the treatment of chronic heart failure is the artificial heart. Since the need for donor hearts far exceeds their availability, it would be clinically very relevant to develop artificial hearts to support failing hearts (Kitakaze and Hori, 2001).

1.4 Experimental models for the study of cardiovascular function and disease

1.4.1 Selecting the most appropriate model and end-points for the study of I/R injury

In the study of cardiovascular function, both under conditions of health and disease, there is a vast range of experimental models available, as well as a vast spectrum of measurable indices of function and injury. Investigative models range from studies in man with severely ill patients or healthy volunteers, through a vast array of animal models (i.e. large and small mammalian hearts) to the other extreme where single cells (Wahler *et al.*, 1990), molecules or ions may be under investigation (Marban *et al.*, 1987). Heart researchers have a sprectrum of endpoints to measure cardiovascular function and malfunction. For example, in the study of ischemic heart disease and myocardial infarction, investigators may wish to measure contractile, biochemical, physiological and morphological consequences (Table 2).

Each experimental model, each species and each end-point has its own inherent advantages and disadvantages and an appreciation of these is critical to the selection of the most appropriate study system for the particular question under investigation. Determining the best experimental model of a human condition such as myocardial ischemia requires a number of decisions and compromises, especially in relation to obtaining the optimal balance between the quantity and quality of the data produced vs. the relevance of the data to the condition under investigation. In general, the further one moves away from the study of human tissues, the greater becomes the quantity, quality and reproducibility of the data and the lower becomes the cost and time-to-result. Unfortunately, especially when a disease process such as ischemia is the focus of study, this is usually offset by the model becoming increasingly less relevant to the human condition (Marban *et al.*, 1987). As a starting point for the study

TABLE 2. MARKERS USED TO ASSESS CARDIAC ISCHEMIA-REPERFUSION INJURY

BIOCHEMICAL	CONDUCTANCE	LYTIC	MECHANICAL	PATHOLOGICAL
Mitochondrial function	Electrocardiograms	Release of intracellular enzymes	Contractility	Infarct size
Oxygen utilization	Dysrhythmias	Release of myoglobin	Peak and resting tension	Myocardial edema
Energy metabolism			Coronary flow	Capillary permeability
Calcium transport and content			Cardiac output	Ultrastructural and histochemical changes
			Systemic blood pressure	
			Ventricular pressure	

From Kehrer and Starnes (1989).

of potential cardioprotective agents, many investigators have chosen the isolated perfused small mammalian heart as a model because it represents the optimal compromise between the quantity and quality of data that can be acquired vs. clinical relevance (Figure 4) (Schaper and Schaper, 1997).

1.4.2 The isolated perfused heart: Advantages and Disadvantages

By far the most routinely used model in physiological and pharmacological studies of the heart is the method for the perfusion of an isolated mammalian heart introduced by Langendorff in 1895. Almost all preparations used today are variations of the nonworking Langendorff preparations in which the isolated heart is perfused retrogradely through the aorta under constant pressure or flow (Kehrer and Starnes, 1989).

The analysis of the pathogenesis of ischemia-reperfusion injury in the heart *in situ* is hampered by the great complexity of the process and the knowledge that many factors are known to be involved in the natural course of injury development. Although many details have been elucidated from studies on the ischemic heart *in situ*, the basic mechanisms are more likely to be identified by searching for key events in models of reduced complexity (i.e. the isolated, prefused heart) (Node *et al.*, 1996).

There are many advantages to use of the isolated heart. At a practical level, this model provides a highly reproducible preparation which can be studied quickly and in large numbers at relatively low cost. Furthermore, a broad spectrum of biochemical, physiological, morphological and pharmacological indices can be measured in the absence of confounding effects of other organs, the systemic circulation and a host of peripheral complications such as circulating neurohormonal factors. This is a major investigational advantage in that peripheral responses can be separated from cardiac

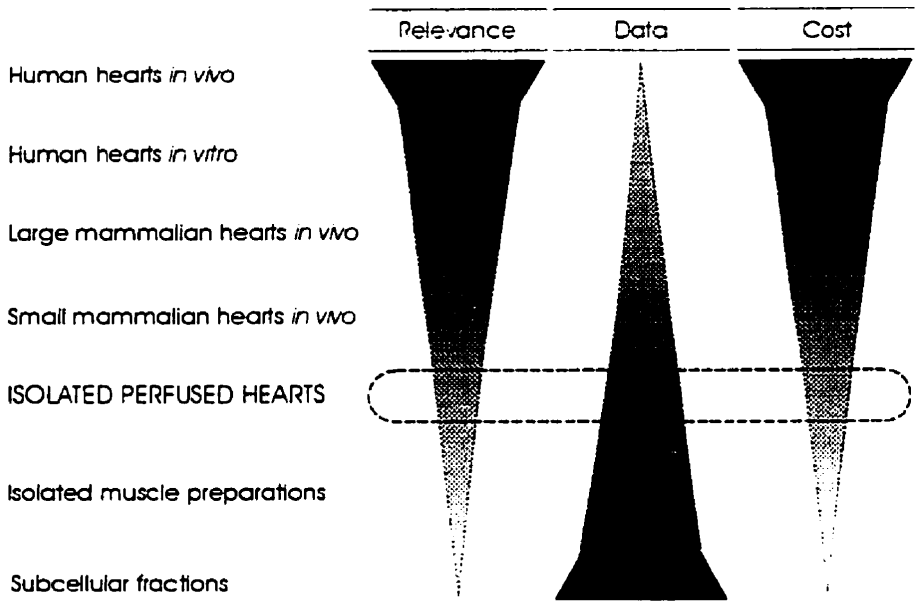


FIGURE 4. DIFFERENT MODELS FOR THE STUDY OF CARDIOVASCULAR FUNCTION AND DISEASE.

The isolated perfused small mammalian heart represents the optimal compromise between the quantity and quality of data that can be acquired vs. its clinical relevance. (From Hearse and Sutherland, 2000)

responses, and the response of the heart to metabolic and pharmacological interventions can be carefully studied.

Some disadvantages of this preparation are that as a denervated model, the isolated heart is one step further removed from the *in vivo* state. Also, the isolated heart is a constantly deteriorating preparation, although it is nonetheless capable of study for several hours.

1.4.3 Choosing the species best for perfusion

The hearts from any mammalian species, or non-mammalian hearts such as those from frogs and birds, may be perfused. However, isolated perfusion of large animal hearts such as pigs, monkeys, sheep, dogs and even man, are less frequently used on account of the high cost, greater variability, large volumes of perfusion fluids and cumbersome equipment that is required (Schaper and Schaper, 1997).

Although a variety of small mammalian species have been used as sources of hearts for isolated-perfused preparations, including rabbits, guinea-pigs, hamsters and mice, the rat is by far the best characterized and most frequently used species. The rat heart is easier to handle then smaller hearts such as the mouse, and does not experience complications with anesthesia as observed in species such as the rabbit (Sutherland and Hearse, 2000; Patel *et al.*, 1993). The anatomy of the rat heart also makes it a particularly good model for the study of regional ischemia. The two ventricles and septum of the rat heart are supplied by right and left coronary arteries arising from the ascending aorta. Thus, this model has the advantages of perfusing the myocardium through its own capillary bed, maintaining physiological temperatures, and allowing (or through electrical pacing) the heart to beat at a rate similar to that of the intact animal (Kehrer and Starnes, 1989). Most importantly, the coronary vasculature of

the rat heart is devoid of collateral flow between the left and right coronary arteries, whereas the guinea-pig heart is totally collateralized, effectively preventing the study of regional ischemia in this species.

1.5 Rationale, hypotheses and objectives

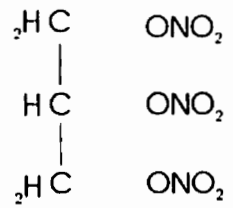
Despite a wealth of experimental knowledge and several promising reports in both *in vitro* and clinical studies, there is yet no pharmacolgical therapy which is considered to be the "gold standard" for use in the treatment of I/R injury. Glyceryl trinitrate has been used in the treatment of acute myocardial infarction since it decreases myocardial oxygen requirements and its coronary vasodilator effects can result in increased regional blood flow. Furthermore, a direct myocardial anti-ischemic effect of GTN has recently been reported (Csont *et al.*, 1999). However, GTN-induced cardioprotection in experimental models has usually been reported when GTN was infused immediately prior to the ischemic insult; this represents a major limitation to its clinical use, since there is often a time delay after acute MI before the administration of pharmacological therapy. Most significantly, whereas numerous investigators have shown that GTN may attenuate necrotic cell death since it attenuates the release of intracellular enzymes such as lactate dehydrogenase, there is little or no evidence that GTN reduces infarct size (Csont *et al.*, 1999; Ferdinandy *et al.*, 1995b).

Recently, a novel organic nitrate (GT 015) has been shown to be neuroprotective in *in vivo* (middle cerebral artery occlusion, MCAO) and *in vitro* (isolated hippocampal slices) models of cerebral ischemia (GoBang Therapeutics, unpublished data). Interestingly, GT 015 attenuated LDH release and reduced infarct size when administered 4 hours after the onset of MCAO, suggesting a direct cytoprotective

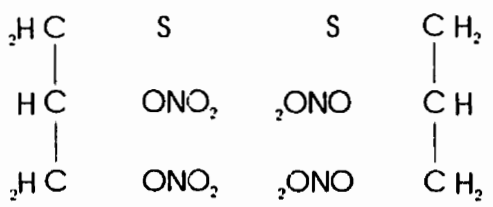
effect. The chemical structure of GT 015 is similar to that of GTN, although it is thought that the disulfide bond of GT 015 may afford unique properties including radical scavenging ability (Figure 5). Another compound, GT 152, is thought to be a metabolite of GT 015, that is formed after cleavage of the disulfide bond (Figure 6). GT 152 demonstrates similar pharmacological properties with GT 015 with respect to vascular relaxation, increased cGMP accumulation in isolated blood vessels, and neuroprotection in the malonate model of neuronal injury and cognition enhancement in a model of scopolamine-induced impairment of memory and learning (GoBang Therapeutics, unpublished data). Although the precise mechanism of irreversible cell death following an ischemic insult may show some tissue variability, the general pathogenesis of the insult is similar in many organs (Yellon *et al.*, 1984). Thus, we wished to answer the question: *do the protective effects of GT 015 extend to cell types other than in the brain?*

Hypothesis 1: The novel organic nitrate GT 015 reduces the severity of post-ischemia reperfusion injury in isolated rat hearts when administered prior to and throughout the period of ischemia. If GT 015 has cardioprotective properties, infusion of the drug prior to and throughout the period of ischemia should produce favourable effects on the end points we have selected to assess the severity of I/R injury; namely, LDH release and infarct size. We also predicted that administration of GTN would decrease LDH release, but have little effect on infarct size.

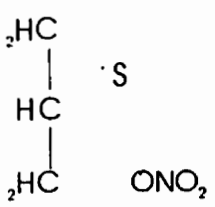
Objective 1 was to characterize the isolated perfused heart model and determine the optimal durations of ischemia and reperfusion to consistently produce severe injury that could be measured by LDH release and infarct size quantification. It



GLYCERYL TRINITRATE (GTN)



GT 015



GT 152

Figure 5. CHEMICAL STRUCTURES OF GLYCERYL TRINITRATE (GTN) AND THE NOVEL ORGANIC NITRATES GT 015 AND GT 152.

GTN and GT 015 are structurally similar with the exception that in GT 015, one nitrate ester bond of GTN has been replaced by a disulfide bond, attached to a second glycerol backbone. GT 152 is a putative metabolite of GT 015.

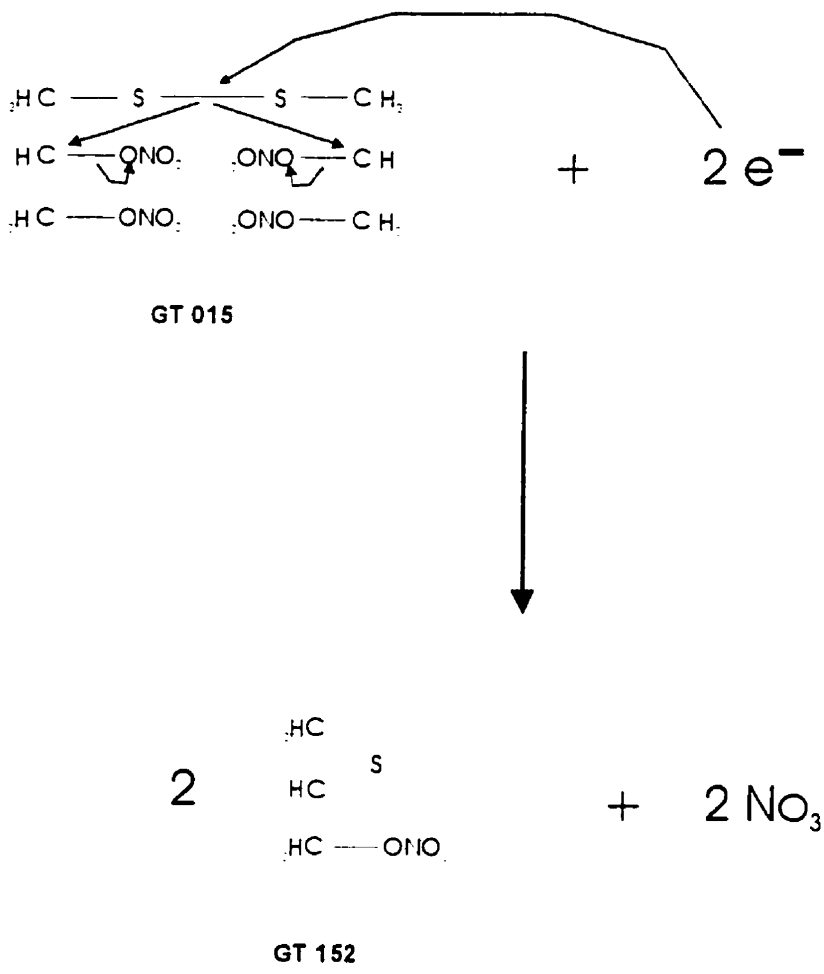


FIGURE 6. PROPOSED BIOTRANSFORMATION PATHWAY OF GT 015.

The chemical reaction that may result in conversion of GT 015 to GT 152 is initiated by electron attack on the disulfide bond of GT 015. Alternatively, the reaction may be initiated by radical species with an unpaired electron in the outer orbital. Cleavage of the disulfide bond produces an unpaired electron on each sulfur atom. This electron may then initiate an intramolecular reaction at the second carbon atom on the glycerol backbone, resulting in the formation of a 3-membered ring structure (GT 152) and the release of nitrate ion (NO_3^-).

was important to detemine a period of ischemia of sufficient duration to produce irreversible cellular injury, but which would not compromise the stability of the preparation during reperfusion.

Objective 2 was to compare the cardioprotective effects of the novel organic nitrate, GT 015, and the prototype organic nitrate, GTN, when infused prior to and throughout the period of ischemia. Cardioprotection was assessed as an attenuation of LDH release and a reduction of infarct size as compared to untreated hearts.

The results presented in Chapter 2 suggest that both GT 015 and GTN produce dose-dependent cardioprotection when administered prior to and throughout the period of acute ischemia. However, whereas both agents attenuated LDH release during the period of ischemia, only GT 015 reduced LDH release during reperfusion, when drug infusion had ceased. These results suggest that GT 015 may directly *protect* some myocardial cells and prevent the transition between reversible and irreversible cellular injury during an acute ischemic insult. Furthermore, GT 015 significantly reduced infarct size whereas GTN had no effect. These results were consistent with the majority of previous experimental studies in which GTN was administered just prior to an acute ischemic insult. It was observed that both GT 015 and GTN increased cGMP levels, but only GT 015 reduced infarct size, suggesting that the cardioprotection afforded by GT 015 involves mechanisms other than, or in addition to, increased cGMP levels.

Hypothesis 2: GT 015 decreases the severity of post-ischemic reperfusion injury when administered prior to and throughout the period of reperfusion. If GT 015 does indeed directly protect some cells from becoming irreversibly injured, administration of GT 015 prior to reperfusion but after the onset of ischemia should reduce the severity of post-ischemia reperfusion injury. If it is assumed that reperfusion injury represents a unique phenomenon, pharmacological intervention at the time of reperfusion may protect cells from additional injury associated with reperfusion itself. In other words, GT 015 may *salvage* some cells that have been reversibly injured by the ischemic insult. Conversely, GTN was not expected to have any beneficial effects.

Objective 3 was to compare the cardioprotective effects (by LDH release and infarct size quantification) of GT 015 and GTN in isolated rat hearts when administered just prior to and throughout the reperfusion period. We also wished to determine the effects of a putative metabolite of GT 015, GT 152, on the severity of post-ischemia reperfusion injury when GT 152 was administered just prior to and throughout reperfusion.

The data demonstrated that GT 015 is cardioprotective when administered prior to and throughout reperfusion. This was a significant finding, since there are few reports of pharmacological intervention at the onset of reperfusion having cardioprotective effects. Administration of GT 152 was also associated with a decrease in LDH efflux, as we predicted. Surprisingly, however, we observed that GTN also significantly attenuated LDH release during reperfusion. However, only GT 015 and its metabolite GT 152 reduced infarct size.

Since it is widely accepted that the pharmacological actions of organic nitrates involve an increase in cGMP levels, we also performed a series of mechanistic studies

to examine whether the cardioprotective effects of GT 015 and GTN involve an increase in cGMP formation. When the drugs were administered prior to and throughout ischemia, both GTN and GT 015 were shown to increase cGMP formation during ischemia and reperfusion. Because GTN did not have any effect on infarct size, this suggests the mechanism of GT 015-mediated cardioprotection, when administered prior to and throughout an acute ischemic insult, is at least partly independent of cGMP. When we measured cGMP efflux from hearts treated with GTN, GT 015 and GT 152 prior to and throughout reperfusion, we observed that GTN and GT 015 significantly increased cGMP levels in the coronary effluent during ischemia. During reperfusion, however, only GTN increased cGMP levels. Since GTN, GT 015 and GT 152 were all observed to decrease LDH release during reperfusion when administered just prior to and throughout the reperfusion period, but all did not increase cGMP levels, there is probably another mechanism involved. Furthermore, whereas GTN did significantly increase cGMP both during ischemia and reperfusion when administered prior to reperfusion, it did not reduce infarct size. This further suggests that cardioprotection in our model is, at least to some extent, cGMP-independent.

CHAPTER 2: Cardioprotection by a novel organic nitrate during ischemia/reperfusion injury is cGMP-independent

2.1 Introduction

A great deal of interest in cardiovascular research has focused on identifying novel pharmacological therapy for the treatment of ischemic heart disease. The isolated perfused rat heart is a well characterized and widely used model for studying myocardial ischemia-reperfusion (I/R) injury *in vitro*, and has proven to be an indispensable tool for the discovery of potential cardioprotective agents. However, despite an exhaustive body of work and many promising reports, there remains no single therapeutic agent that has gained widespread acceptance for the treatment of acute myocardial infarction. Furthermore, many agents which have demonstrated cardioprotective properties in *in vitro* models have limited clinical potential, since these agents were only shown to have beneficial effects when administered prior to the acute ischemic insult. Clearly, there is a need to continue the search for an effective treatment of myocardial I/R injury. Specifically, there is a need for an agent which can be administered just prior to or at the onset of reperfusion, that could potentially protect vulnerable cells and/or salvage reversibly injured cells, and prevent them from undergoing the transition to irreversible injury.

Organic nitrates, of which glyceryl trinitrate (GTN) is the prototype, have demonstrated anti-ischemic effects *in vitro*, but have been disappointing in clinical trials of acute myocardial infarction. Biotransformation of organic nitrates to an active species, presumably nitric oxide (NO), is generally accepted as a requirement for their pharmacological effect. Furthermore, NO is believed to initiate a downstream signal transduction cascade, with cGMP as a central component of this signalling pathway.

GoBang Therapeutics Ltd. have recently synthesized a proprietary library of compounds which are structurally similar to GTN. One of these compounds, GT 015, has been shown to possess anti-ischemic properties in both *in vivo* and *in vitro* models of stroke. Whereas GT 015 is a much less potent vasodilator than GTN and is thought to spontaneously release NO, it is not yet clear by which mechanism(s) GT 015 protects cells from ischemic injury. However, it is highly plausible that cGMP may be involved in the signaling pathway of GT 015, due to its structural similarity with GTN.

2.2 Materials and Methods

2.2.1 Preparation of isolated perfused hearts

Male Sprague-Dawley rats between 300 to 450 grams were pretreated with heparin (1000 units/kg body weight, i.p.), and then anaesthetized five minutes later with 100 mg/kg i p sodium pentobarbitone (Somnotol™, BIMEDA-MTC Health Inc., Cambridge, ON). The heart was rapidly excised by the following procedure: the abdominal cavity was opened by transverse incision with scissors; the diaphragm was transected and lateral incisions were made along both sides of the rib cage and the anterior chest wall was folded back. The pericardium and other filamentous tissues of the mediastinum were gently removed and the lungs and other chest contents were gently pushed toward the back to identify the point at which the pulmonary veins join the left atrium. Two 3/0 silk sutures, attached to a tapered curved needle, were passed through the posterior heart, under the left coronary artery (LCA) a few millimeters distal to the point of origin in the aorta. The heart was then cut away from the body by a single incision.

The excised heart was immediately immersed in a petri dish containing ice-cold (4°C) Krebs-Henseleit buffer solution containing 0.5 mL of Heparin (1000 units/mL) and contractions were allowed to cease within a few seconds. Any attached connective tissue, thymus or lung were carefully removed using fine surgical scissors. Using a pair of fine-tipped forceps, the heart was transferred to the perfusion apparatus and the aorta was carefully slipped onto the grooved tip of the perfusion cannula and secured in place with a ligature. Using a standard peristaltic pump calibrated to deliver perfusate at a flow rate of 8 mL/min, retrograde perfusion was initiated according to the Langendorff technique (Figure 7) at 37°C with an oxygenated Krebs-Henseleit solution containing in mmol/L: NaCl 118, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 1.3, KH_2PO_4 1.2, $NaHCO_3$ 25, Dextrose (D-Glucose) 11 and Dextran (clinical grade, obtained from Sigma Chemical Co., St. Louis, MO) 40 grams/L. The temperature of the perfusate and the heart were maintained at 37°C using water jacketed tubing warmed by a water bath and a high-tech heart chamber (Harvard Apparatus, St. Laurent, QC), respectively. The coronary perfusion pressure was monitored by a calibrated pressure transducer connected to the perfusion line and displayed on a computer using the Chart® program (Figure 7).

Contractions resumed within 10 seconds of perfusion and both the heart itself and the overflow effluent were allowed to clear of all traces of blood. Normoxic perfusion was continued for 25 minutes to remove all blood, to equilibrate the substrate concentration in the perfusion medium with those in the interstitial fluid and to allow the heart to recover from the period of anoxia associated with excision and initiation of perfusion.

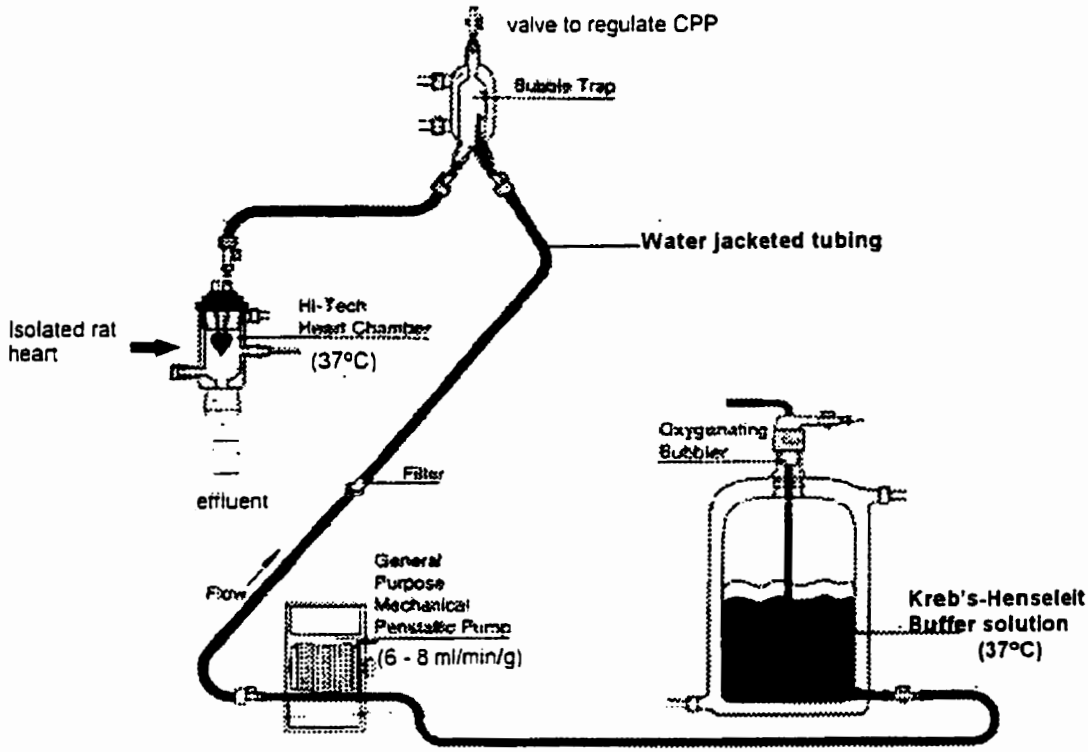


FIGURE 7. CONSTANT FLOW MODEL FOR PERFUSION OF THE ISOLATED RAT HEART.

CPP, coronary perfusion pressure.

2.2.2 Ischemia-reperfusion protocol

For regional ischemia, temporary occlusion of the left coronary artery was induced by placing a short piece of plastic tubing on the surface of the heart and tightening one of the two sutures positioned around the LCA around the tubing. Left coronary artery occlusion lasted 45 minutes, at which time the suture was removed using a razor blade and reperfusion at normal flow was initiated for 90 min. Throughout the ischemia-reperfusion period, continuous timed samples of the coronary effluent were collected and kept on ice. After 15 min of equilibration, coronary flow, heart rate and perfusion pressure was recorded. At the end of the reperfusion period, the LCA was re-occluded and 0.5 mL of 1% Evan's Blue dye (dissolved in saline) was slowly infused into the heart, via the aotic cannula, to stain the area of myocardium perfused by the patent coronary artery. Thus the area-at-risk (AAR) for infarction was determined by negative staining.

2.2.3 Drug solutions and infusion protocol

When drugs were infused, they were included in a separate beaker of Kreb's. The drug solutions used were: (a) vehicle (0.04% dimethyl sulfoxide, DMSO), (b) 10 to 50 μM GTN, (c) 10 to 50 μM GT 015 and (d) 50 μM GT 152. All drugs were dissolved in DMSO, warmed to 37°C, and oxygenated at the beginning of the experiment. The dead volume in the system was determined and drug infusion was initiated at the appropriate time, taking this volume into consideration.

2.2.4 Assessing the severity of I/R injury

2.2.4.1 Lactate dehydrogenase (LDH) assay

Lactate dehydrogenase catalyzes the oxidation of lactate in the following reaction:

$$\text{Lactate} + NAD^+ \rightarrow \text{pyruvate} + NADH + H^+$$

During a period of ischemia, blockade of cellular ATP production (due to oxygen and metabolic substrate deficiency) occurs which leads to a loss of contractile function followed by a loss of cellular integrity. As a result, intracelluar enzymes such as LDH and creatine kinase are released and become detectable in the extracellular space. On the morning of the assay, the following solutions are made fresh and kept on ice until used: 0.00635 grams pyruvate and 0.005 grams NADH, each dissolved in phosphate buffer (0.1 M, pH 7.4) in separate tubes to make up final volume of 5 mL. To each 1.1 mL of perfusate, 1.5 mL of phosphate buffer and 200 μL NADH solution are added and all samples are incubated at 25°C for 5 – 10 minutes. To initiate the reaction, 200 μL of pyruvate solution is added and the progress of the reaction is measured by a decrease in absorption at 340 nm (Bergmeyer and Bernt, 1974).

2.2.4.2 Triphenyltetrazolium chloride (TTC) staining

After the coronary artery occlusion ischemia-reperfusion protocol was completed, the heart was dried, weighed and placed in the freezer (- 25°C) overnight. The frozen heart was then placed in a plastic mold and sectioned into 1 mm transverse sections from the apex to base (6 -7 slices/heart) using razor blades (American Safety Razor of Canada, Newmarket, ON). The slices were allowed to defrost, then incubated at 37°C with 1% w/v triphenyltetrazolium chloride (TTC) in 0.1 M phosphate buffer (pH

7.4) for 10 - 15 min. The stained slices were then fixed in 10% v/v formaldehyde solution to distinguish stained viable tissue (stains brick red) and unstained (stains pale yellow/white) necrotic tissue (Joyeux *et al.*, 1998).

2.2.4.3 Quantification of infarct size

The preserved heart slices were then placed in a saline filled petri dish and positioned over a dark background (to enhance contrast) under a colour camera. Digital images of the slices were then captured by the camera which was connected to a computer running the imaging software MCID-M5® (version 4 for Windows NT). The images were then analyzed by computerized planimmetry using the Bioquant® analysis software. Infarct size was expressed as infarct area (negative staining after TTC staining) as a percent of the area-at-risk (negative staining after Evan's Blue dye). This is a well characterized and widely used method to determine infarct size experimentally (Joyeux *et al.*, 1999)

2.2.5 Measurement of cyclic GMP activity by radioimmunoassay

Cardiac cGMP formation was measured in control and drug treated hearts from samples of the coronary effluent using radioimmunassay (RIA). The RIA is a sensitive and specific method that is based upon competition of the cyclic nucleotide (i.e. cGMP) with isotopically labeled nucleotide derivatives for binding sites on a specific antibody. The detectability/sensitivity range for the cGMP RIA is in the range of 0.01 to 1.0 picomoles per tube (Steiner *et al.*, 1972).

2.2.6 Data analysis

Data were presented as the mean ± standard error of the mean (s.e.m). Lactate dehydrogenase release was expressed as units (where one unit equals a 0.01 absorbance change) per millilitre of effluent per minute per gram of heart tissue weight. Cyclic GMP efflux in the effluent was expressed in femtomoles per millileter of effluent per minute. Differences between treatment groups were analysed by the appropriate statistical test as indicated. P values less than 0.05 were considered statistically significant.

2.3 Results

2.3.1 Characterization of the isolated perfused heart model

The isolated hearts were equilibrated by normoxic perfusion for 25 min and the coronary perfusion pressure (CPP) was allowed to stabilize between 50 to 70 mmHg (Figure 8). Coronary flow and heart rate were measured after 15 min. Flow rate was maintained between 6 to 8 ml/min and the heart rate ranged between 250 to 350 bpm. Hearts were excluded if the HR was < 250 bpm or if the CPP was < 50 mmHg at the end of the equilibration period.

Left coronary artery occlusion (LCAO) was associated with a significant increase in LDH release which peaked within the first 10 minutes of ischemia and decreased over the remaining 45 min period of LCAO (Figure 9). Upon reperfusion, LDH release increased to a lesser extent than upon LCAO but remained above control levels throughout the 90 min of reperfusion (Figure 9). Occlusion of the left coronary artery was confirmed by an increase in perfusion pressure (Figure 8) and by negative staining

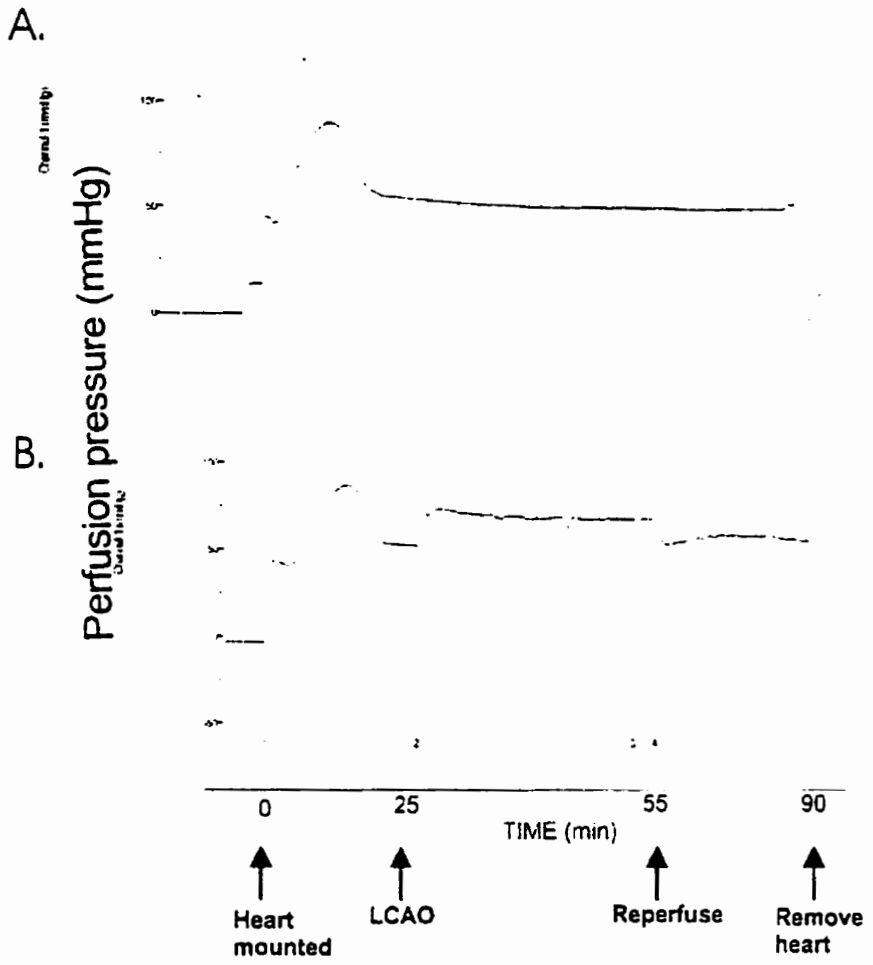


FIGURE 8. REPERESENTATIVE TRACINGS OF CARDIAC PERFUSION PRESSURE (CPP) GENERATED BY THE ISOLATED PERFUSED HEART.

A pressure transducer connected in-line with the perfusion system monitored CPP generated by the isolated heart. During the 25 min equilibration period, the perfusion pressure was allowed to stabilize between 50 to 70 mmHg. In control hearts (A), the CPP remained unchanged throughout the remaining period of normoxic perfusion. In ischemic hearts (B), left coronary artery occlusion (LCAO) was associated with a 10 to 15 mmHg increase in CPP. CPP remained elevated during the 30 min of LCAO then decreased to the pre-ischemic level upon release of the occlusion (reperfusion).

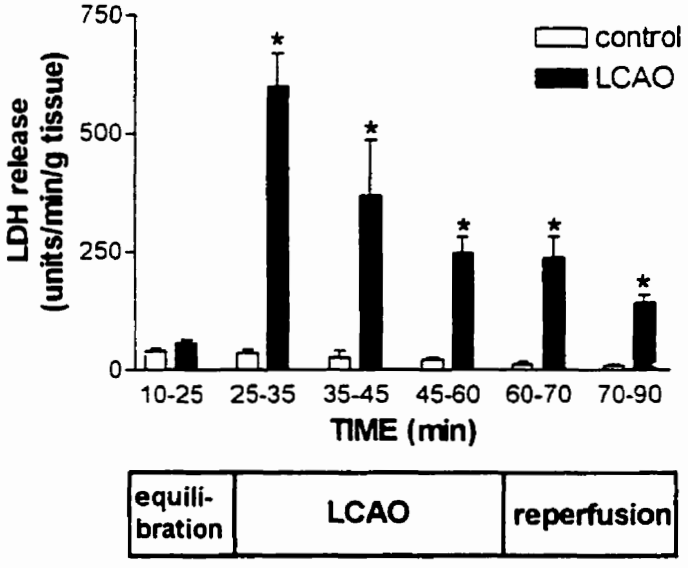


FIGURE 9. LACTATE DEHYDROGENASE (LDH) RELEASE FROM ISOLATED PERFUSED RAT HEARTS.

Control hearts (n = 4) were subjected to 90 min of normoxic perfusion and other hearts (n = 6) were made ischemic after 25 min of equilibration by occlusion of the left coronary artery (LCAO) for 35 min followed by 30 min normoxic reperfusion. In control hearts, LDH release was highest during the equilibration period and gradually decreased throughout the remainder of the experiment. LCAO was associated with a 6-fold increase in LDH release within the first 10 min of regional ischemia. Upon reperfusion, LDH release did not increase further but remained significantly above control levels. Data represent mean ± s.e.m. * $p < 0.05$ vs control, student's t-test.

with Evan's Blue dye (Figure 10) when the artery was re-occluded at the end of the experiment.

We assessed the per minute release of LDH for three different durations of regional ischemia. There was no significant difference between the total per minute LDH release after 35, 40 or 45 minutes of LCAO (Figure 11). However, triphenyltetrazolium (TTC) staining of the hearts after these three different durations of ischemia showed that 45 min of LCAO was the minimal duration of ischemia required to consistently produce a quantifiable infarct (data not shown).

Different durations of reperfusion after 30 min of regional ischemia were also compared (Figure 12). We observed the total per minute release of LDH was significantly increased after 90 min and 120 min of reperfusion, with a maximal release rate after 90 min. However, for reperfusion durations > 90 min, the contractile function of the heart was severely impaired by the end of the experiment. Therefore, we decided to use a protocol consisting of 25 min equilibration, 45 min ischemia and 90 min reperfusion for subsequent experiments.

2.3.2 Cardioprotection by a novel organic nitrate (GT 015) and GTN when infused prior to and throughout the ischemic period

Having determined the optimal ischemia-reperfusion protocol for our experimental system, we proceeded to assess whether GT 015 and GTN could reduce the severity of post-ischemia reperfusion injury when infused just prior to and throughout the period of LCAO. Isolated hearts were randomly assigned to receive one of three treatments (DMSO, GTN or GT 015) for 10 min prior to and throughout the 45

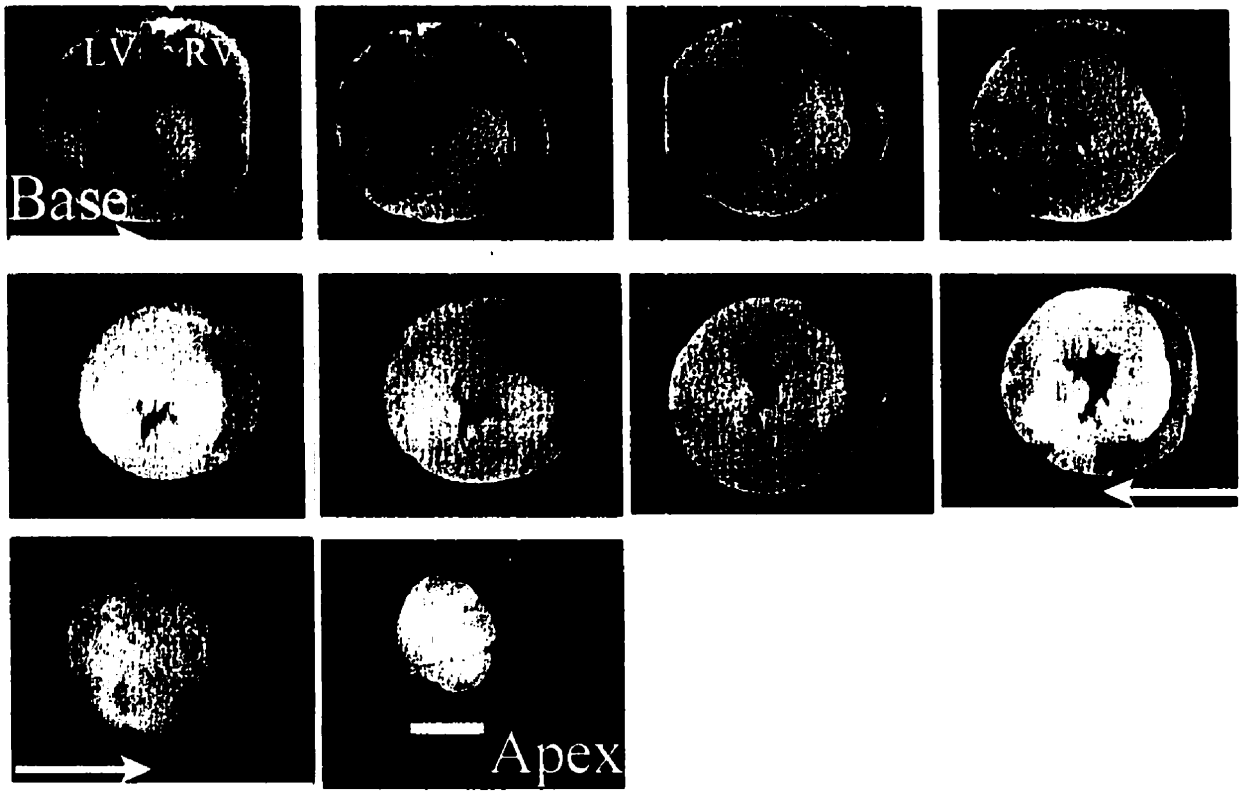


FIGURE 10. INFUSION OF EVAN'S BLUE DYE TO DELINEATE THE AREA-AT-RISK (AAR) FOR INFARCTION.

Digital images of transverse slices from the base (point of origin of the aorta) to the apex of a single heart in which 0.1 % Evan's Blue dye has been injected into the aortic cannula. At the end of the ischemia-reperfusion protocol, the left coronary artery was re-occluded and Evan's Blue dye was infused into the aortic cannula. Areas that stain blue represent myocardial tissue which is still being perfused. Negative staining represents the AAR for infarction. White scale bar represents 5 mm. *LV*, left ventricle; *RV*, right ventricle.

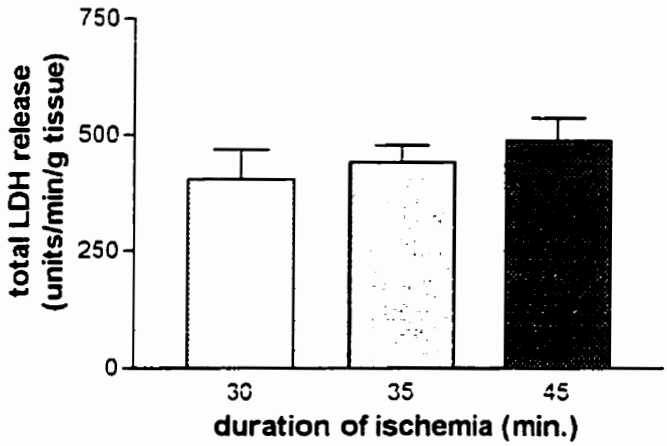


FIGURE 11. EFFECT OF DIFFERENT DURATIONS OF REGIONAL ISCHEMIA ON LDH RELEASE FROM ISOLATED PERFUSED HEARTS.

Hearts were isolated and perfused for 25 min. Regional ischemia was induced by LCAO. The total per minute LDH release was compared for three durations of LCAO (n = 6 for all groups). There was no significant difference in LDH release after 30, 35 or 45 min of LCAO. Data represent mean ± s.e.m.

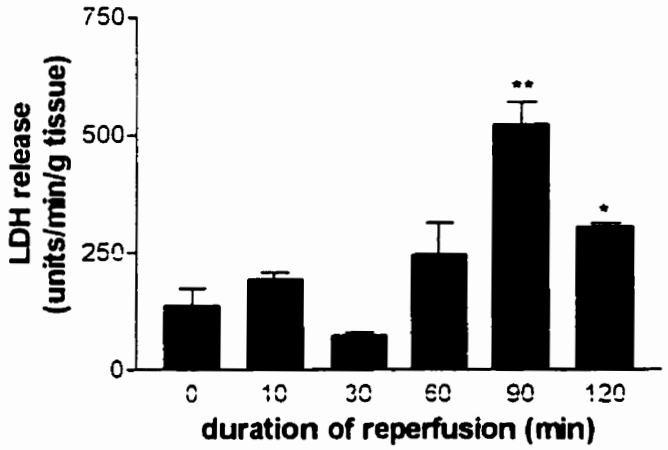


FIGURE 12. EFFECT OF DIFFERENT DURATIONS OF REPERFUSION ON LDH RELEASE FROM ISOLATED PERFUSED HEARTS.

Isolated hearts were allowed to equilibrate and were subjected to 35 min of regional ischemia before reperfusion was initiated. The total per minute LDH release after different durations (0, 10, 30, 60, 90 or 120 min) of normoxic reperfusion were compared. LDH activity was significantly increased after 90 min and 120 min of reperfusion. Data represent mean ± s.e.m. *$p < 0.01$ and ** $p < 0.001$ versus 0 min reperfusion, n = 3 for all groups, one-way ANOVA, Tukey-Kramer post-hoc test.

min of regional ischemia induced by LCAO. Some control hearts were infused with vehicle but did not undergo LCAO. Drug infusion had no effect on CPP during ischemia, even at the highest dose (50 μM) administered (Figure 13). However, during reperfusion, CPP was observed to increase rapidly as contractile function became severely impaired. GT 015 appeared to prevent the loss of contractility as compared to vehicle and GTN-treated hearts (Figure 13 and Table 3).

Lactate dehydrogenase release from control hearts that did not undergo LCAO was 33 ± 3 units/min/g tissue during the equilibration period and decreased gradually over the course of the experiment (Figure 14). Occlusion of the LCA (with DMSO treatment) was associated with a 9-fold increase in LDH release versus basal levels (453 ± 40 units/min/g vs. 50 ± 5 units/min/g) (Figure 14). During reperfusion, the per minute LDH release was lower than during the ischemic period, but remained 4-fold above basal levels (Figure 14). Infusion of GTN and GT 015 resulted in dose-dependent decreases in LDH release during the ischemic period (Figure 14). However, during reperfusion only GT 015 reduced LDH release in a dose-dependent manner, whereas GTN treatment had no effect (Figure 14).

Infarct size after TTC staining was quantified and compared for the different treatment groups. Infarcts at the end of the ischemia-reperfusion protocol were visible upon gross examination of the transverse slices (Figure 15). Infarct size was quantified by computerized planimmetry. Left coronary artery occlusion for 45 min followed by 90 min reperfusion produced infarcts that were 55 ± 4 % of the area-at-risk (AAR) (Figure 16). Administration of GTN 10 min prior to and throughout the ischemic period did not significantly reduce infarct size (47 ± 2 % of the AAR) (Figure 16). Conversely, GT 015

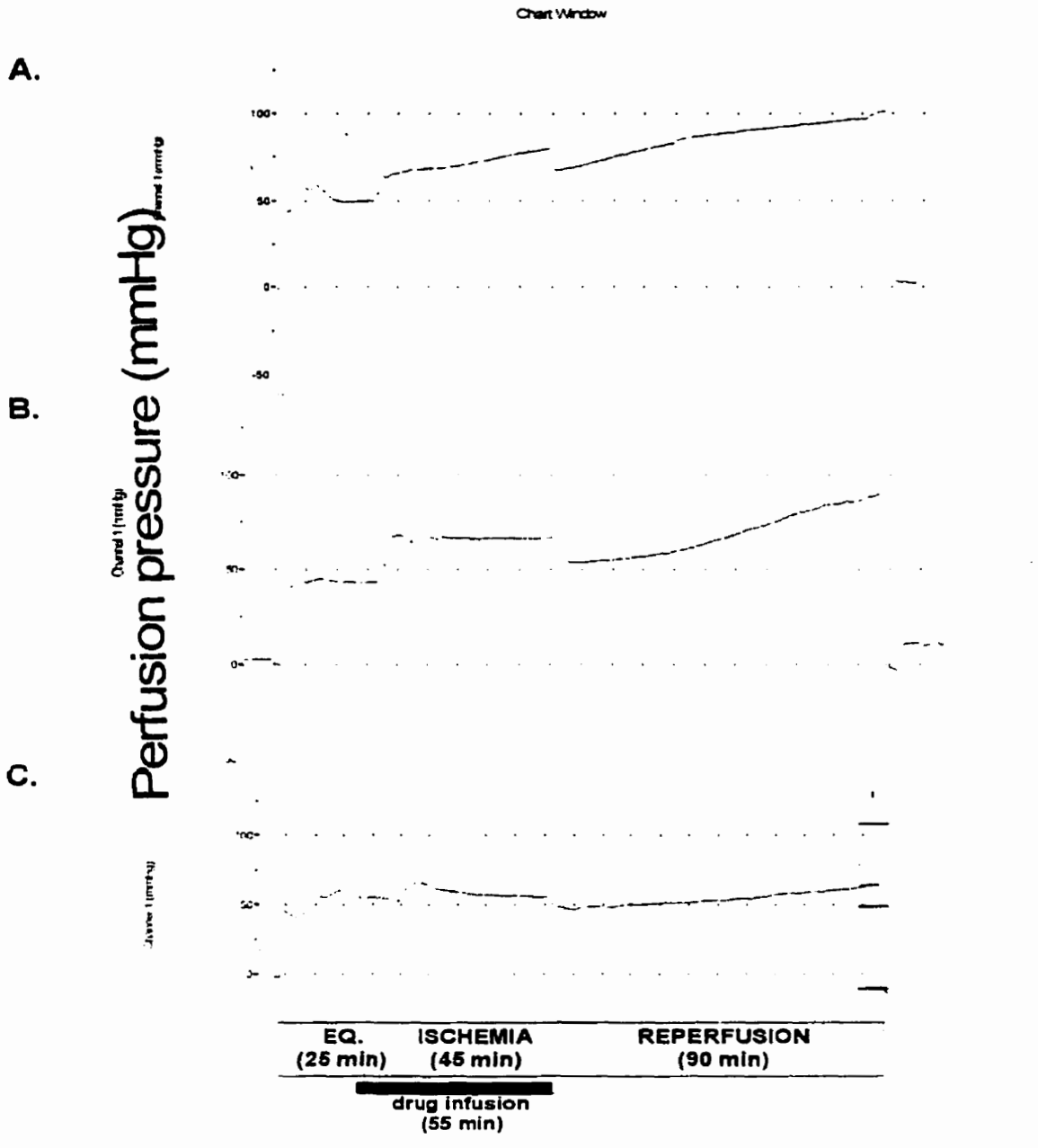


FIGURE 13. REPRESENTATIVE TRACINGS OF CORONARY PERFUSION PRESSURE (CPP) GENERATED BY ISOLATED PERFUSED HEARTS WITH DRUG ADMINISTRATION PRIOR TO AND THROUGHOUT ISCHEMIA.

Hearts were mounted and allowed to equilibrate for 15 min, at which point drug administration (A, DMSO; B, GTN (50 μM); C, GT 015 (50 μM)) 10 min prior to and throughout the 45 min of left coronary artery occlusion (LCAO). LCAO was associated with a 10 to 15 mmHg increase in CPP, which returned to the pre-ischemic level upon reperfusion. Drug administration had no effect on CPP during ischemia. During reperfusion, a rapid increase in CPP was observed. GT 015 appeared to prevent the increase in CPP, whereas vehicle and GTN treatment had no effect.

TABLE 3. COMPARISON OF DRUG EFFECTS ON CORONARY PERFUSION PRESSURE AT THE END OF THE POST-ISCHEMIC REPERFUSION PERIOD IN ISOLATED RAT HEARTS

		CORONARY PERFUSION PRESSURE (mm Hg)		
TREATMENT GROUP	n	EQUILIBRATION[a]	REPERFUSION[a]	
			0 min	90 min
control + DMSO	6	65 ± 5	60 ± 5	65 ± 4
LCAO + DMSO	6	63 ± 4	62 ± 6	120 ± 20*
LCAO + GTN (50 μM	6	60 ± 12	62 ± 10	125 ± 15*
LCAO + GT 015 (50 μM)	6	61 ± 4	60 ± 6	80 ± 10

[a] each value represents the mean ± s.e.m

Values in each column (equilibration and after 0 and 90 min reperfusion) were compared by one-way ANOVA and Tukey-Kramer post-hoc was used for individual comparisons between treatment groups

* significantly different than control + DMSO group ($p < 0.05$)

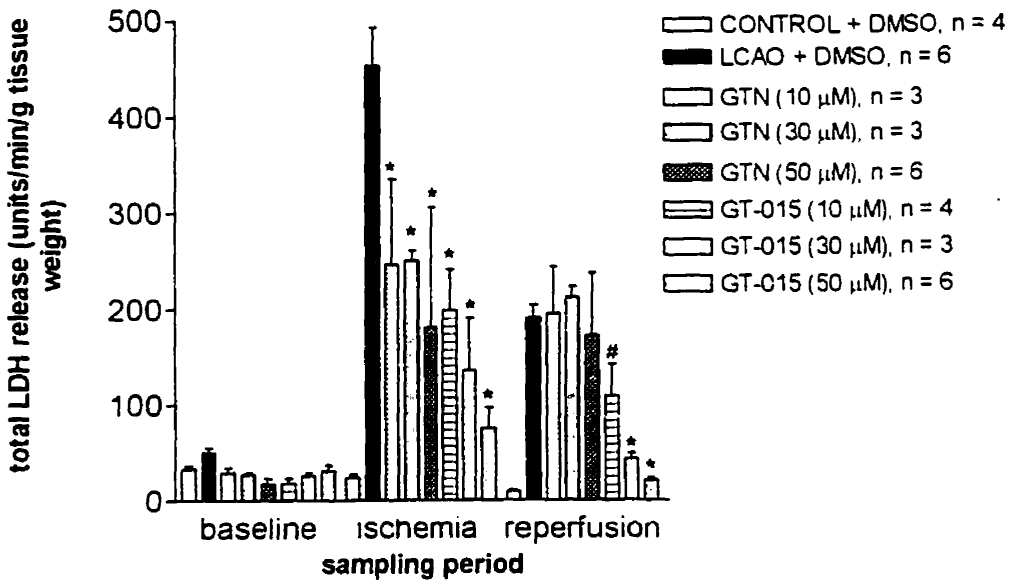


FIGURE 14. DOSE-DEPENDENT CARDIOPROTECTION BY GT 015 AND GTN WHEN INFUSED 10 MIN PRIOR TO AND THROUGHOUT 45 MIN OF REGIONAL ISCHEMIA.

Total lactate dehydrogenase (LDH) release from isolated hearts during 25 min equilibration (baseline), 45 min left coronary artery occlusion (LCAO) (ischemia) and 90 min reperfusion. Hearts were allowed to equilibrate for 15 min before drug (0.04 % DMSO; 10, 30 or 50 μM GTN; 10, 30 or 50 μM GT 015) infusion was initiated for 10 min prior to and throughout LCAO. Some control hearts received vehicle treatment but did not undergo LCAO (control + DMSO). Occlusion of the LCA resulted in a 9-fold increase in LDH release during ischemia. Both GTN and GT 015 attenuated this release in a dose-dependent manner (* $p < 0.001$ vs. LCAO + DMSO, one-way ANOVA, Tukey-Kramer post-hoc test). During reperfusion, LDH release from vehicle treated hearts was 3-fold higher than basal levels. Only GT 015 produced a dose-dependent reduction in LDH release during the reperfusion period, whereas GTN had no significant effect (# $p < 0.01$ and * $p < 0.001$ vs. LCAO + DMSO, one-way ANOVA, Tukey-Kramer post-hoc test). All data represent the mean ± s.e.m.

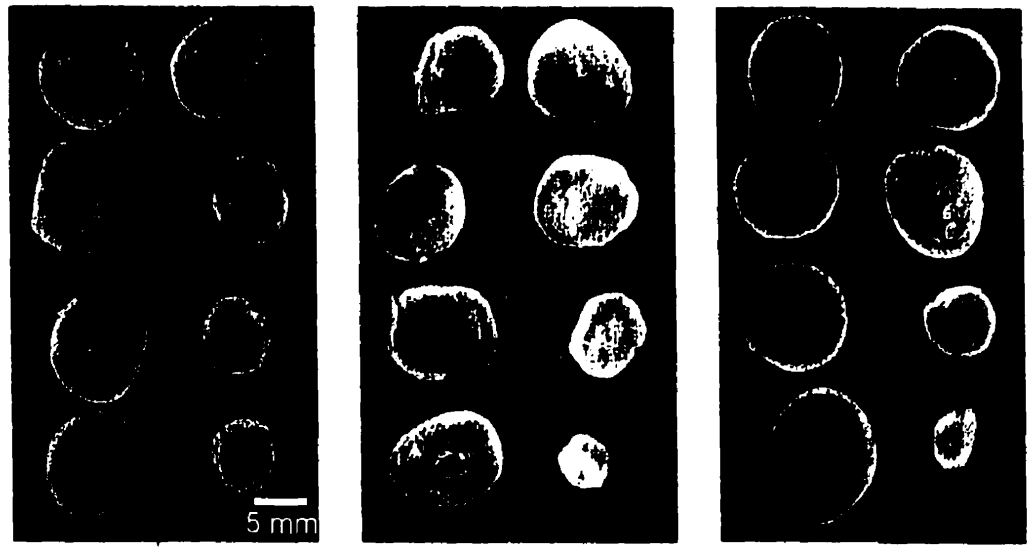


FIGURE 15. EFFECT OF DRUG TREATMENT PRIOR TO AND THROUGHOUT THE ISCHEMIC PERIOD ON INFARCT SIZE IN AFTER TRIPHYENYLTETRAZOLIUM (TTC) STAINING IN ISOLATED PERFUSED HEARTS.

At the end of the ischemia-reperfusion protocol, the effect of drug treatment (DMSO, 50 μM GTN or 50 μM GT 015) on infarct size was assessed. The left coronary artery was re-occluded (including control hearts that did not undergo an initial period of LCAO) and 0.1 % Evan's Blue dye was infused to delineate the area-at-risk (AAR) for infarction. Negative staining after Evan's Blue dye represents the AAR. The hearts were dried, weighed and frozen overnight. Transverse slices (1 mm) were made and the slices were incubated with 1 % TTC at 37°C for 15 min. Viable tissue stains brick red and necrotic/ischemic tissues appears pale white. Gross examination of the suggests GT 015 treatment reduces infarct size. *LCAO*, left coronary artery occlusion; *DMSO*, dimethylsulfoxide.

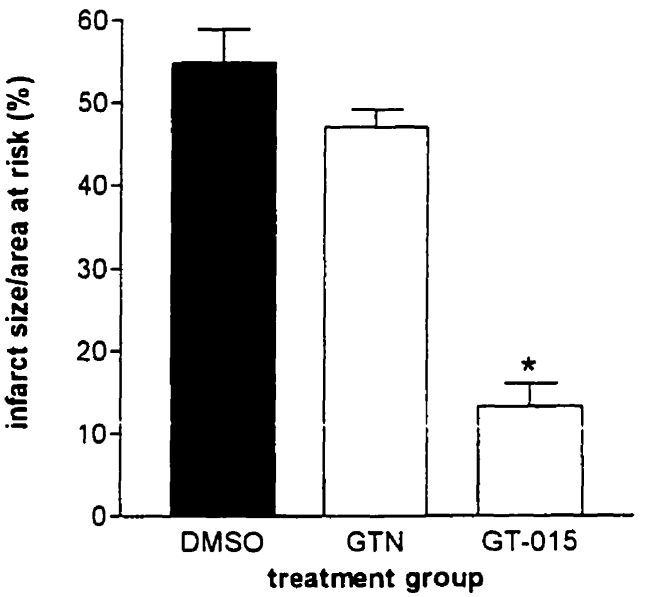


FIGURE 16. EFFECT OF DRUG TREATMENT PRIOR TO AND THROUGHOUT ISCHEMIA ON INFARCT SIZE IN ISOLATED PERFUSED HEARTS.

Hearts were isolated and perfused as described in the Methods. Drug administration (DMSO (n = 5), 50 μM GTN (n = 5) or 50 μM GT 015 (n = 6)) was initiated 10 min prior to and throughout the 45 min of ischemia. After 90 min of reperfusion, the area-at-risk (AAR) was determined by Evan's Blue dye and the hearts were dried, weighed and frozen. The hearts were thawed and incubated with TTC to determine the infarct area. Infarct size was quantitated by computerized planimmetry and expressed as a % of the AAR. Administration of GT 015 significantly reduced infarct size whereas GTN had no effect. * $p < 0.01$ vs DMSO treatment, one-way ANOVA, Tukey-Kramer post-hoc test. All bars represent the mean ± s.e.m.

reduced infarct size by 75 % vs. vehicle treatment (13 ± 2 % of the AAR, $p < 0.01$) (Figure 16).

2.3.3 Cardioprotection by GTN, GT 015 and GT 152 when infused prior to and throughout the reperfusion period

Using the same materials and methods as described in section 2.3.2, the effect of drug administration 10 or 15 min prior to and throughout the 90 min reperfusion period was assessed. In addition to GTN and GT 015, we also examined the effect of GT 152 (a putative metabolite of GT 015) administration on LDH release and infarct size. We used a dose of 50 μM for all drugs, based on the fact that this concentration produced a maximal cardioprotective effect in the previous study.

Coronary perfusion pressure (CPP) was monitored throughout the experiments. Drug administration had no effect on CPP and there was no difference in CPP between treatment groups at the end of the reperfusion period (Figure 17).

Figure 18 illustrates the effect of drug treatment on LDH release. The control data were presented previously in Figure 14. Left coronary artery occlusion with vehicle treatment was associated with a 10-fold increase in LDH release during ischemia (620 ± 150 units/min/ml/g during LCAO vs. 60 ± 10 units/min/ml/g during equilibration) (Figure 18). Drug administration within the last 10 or 15 min of LCAO did not significantly attenuate LDH release during the ischemic period (Figure 18). However, GTN, GT 015 and GT 152 all reduced LDH release during the reperfusion period ($p < 0.01$) (Figure 18).

Analysis of infarct size after TTC staining indicates that whereas GTN reduced LDH release, it had no effect on infarct size (63 ± 6 % of the AAR for GTN vs. 54 ± 6 % of the AAR for vehicle treated) (Figure 19). Conversely, both GT 015 and its metabolite

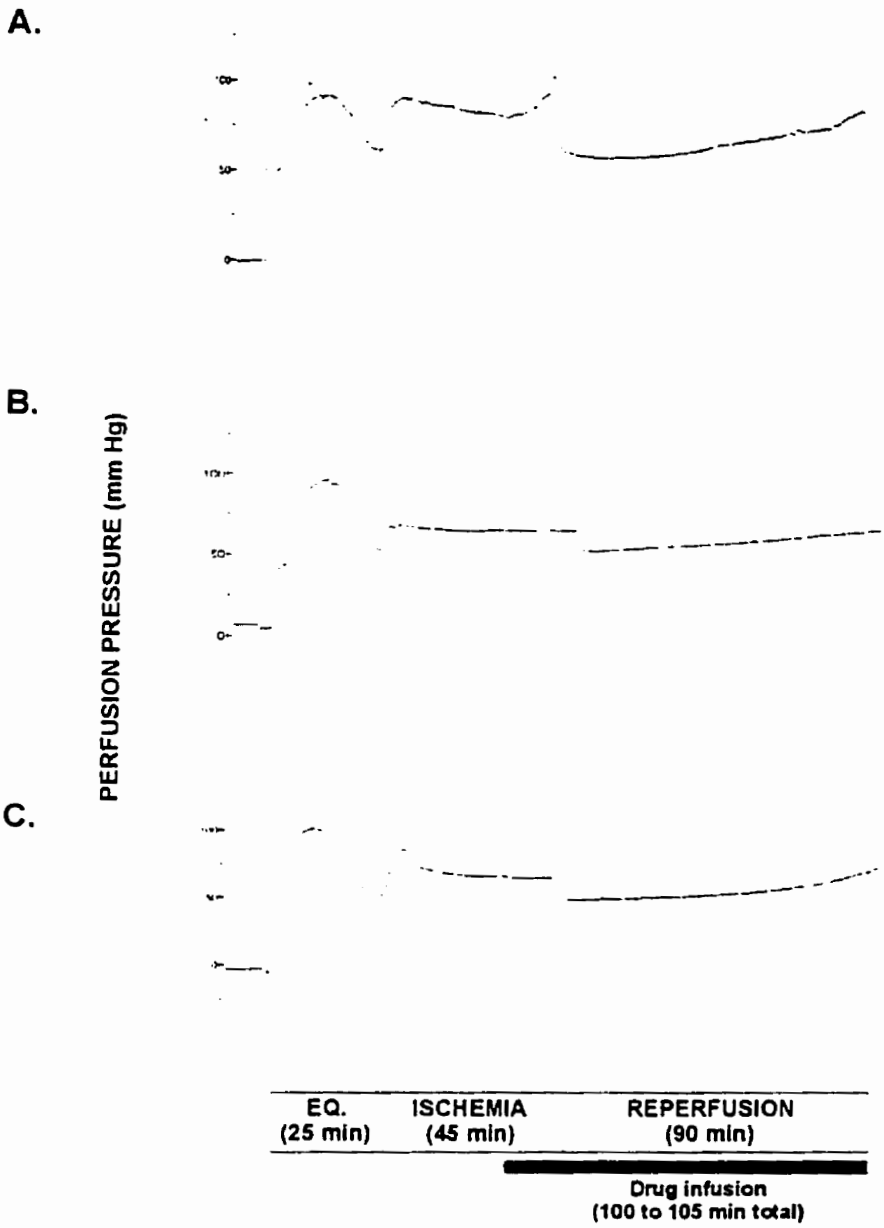


FIGURE 17. REPRESENTATIVE TRACINGS OF CORONARY PERFUSION PRESSURE (CPP) GENERATED BY ISOLATED PERFUSED HEARTS WITH DRUG ADMINISTRATION PRIOR TO AND THROUGHOUT REPERFUSION.

Hearts were mounted, equilibrated for 25 min and ischemia was induced by LCAO. LCAO was associated with a 10 to 15 mm Hg increase in CPP. After 30 to 35 min of ischemia, drug administration (A, DMSO; B, 50 μM GTN; or C, 50 μM GT 015) was initiated and allowed to continue throughout the 90 min of reperfusion. Drug administration had no apparent effect on CPP.

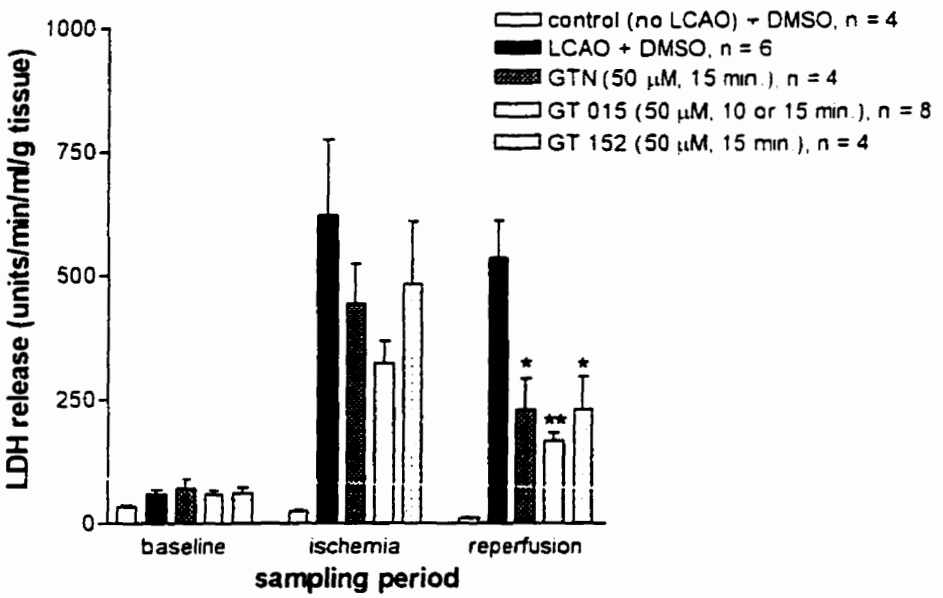


FIGURE 18. EFFECT OF DRUG TREATMENT PRIOR TO AND THROUGHOUT REPERFUSION ON LACTATE DEHYDROGENASE (LDH) RELEASE FROM THE ISOLATED PERFUSED HEART.

Hearts were isolated and perfused in section 2.2. Drugs (DMSO, 50 µM GTN, GT 015 or GT 152) were infused 10 to 15 min prior to and throughout the 90 min of reperfusion. Left coronary artery occlusion with vehicle treatment resulted in a 10-fold increase in LDH release versus the basal level. LDH release during ischemia was unchanged by drug administration. During reperfusion, GTN, GT 015 and GT 152 all significantly reduced LDH release. All bars represent the mean ± s.e.m. * $P < 0.01$, ** $p < 0.001$ vs. vehicle treatment, one-way ANOVA, Tukey-Kramer post-hoc test.

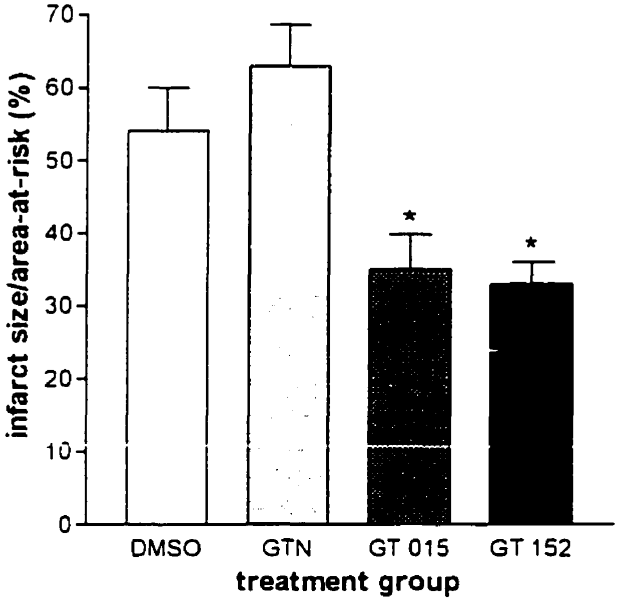


FIGURE 19. EFFECT OF DRUG ADMINISTRATION JUST PRIOR TO AND THROUGHOUT REPERFUSION ON INFARCT SIZE IN ISOLATED PERFUSED HEARTS.

Hearts were isolated and perfused as previously described. Drug solutions (DMSO, n = 5; 50 μM GTN, n = 4; 50 μM GT 015, n = 6; 50 μM GT 152, n = 3) were infused 10 to 15 min prior to and throughout the 90 min reperfusion period. Infarct size was quantified after TTC staining. GTN treatment had no effect on infarct size. Conversely, both GT 015 and GT 152 significantly reduced infarct size. All bars represent the mean ± s.e.m.

* $p < 0.05$ vs. vehicle treated hearts, one-way ANOVA, Tukey-Kramer post-hoc test.

GT 152 significantly reduced infarct size by 35 % and 39 %, respectively ($p < 0.05$) (Figure 19). Figure 20 represents images from four separate hearts, each receiving one of the four different drug treatments.

2.3.4 Mechanistic studies: measurement of cGMP efflux.

Samples (0.5 mL) of the coronary effluent were collected for measurement of cGMP activity by radioimmunoassay, as described in the Methods (see section 2.2.5).

Basal cGMP release for the vehicle, GT 015 and GT 152 hearts was an average of 9 ± 1 fmol/ml/min during the equilibration period. There was significantly higher basal cGMP levels in the GTN-treated group during equilibration (Figure 21). Drugs were administered 10 min prior to and throughout the period of LCAO, and both GTN (50 μM) and GT 015 (50 μM) were observed to increased cGMP efflux by 2-fold vs. vehicle treatment during the period of LCAO ($p < 0.05$) (Figure 21). During reperfusion, GT 015 at concentrations of 30 μM and 50 μM increased cGMP levels in the coronary effluent by 3.5-fold, and GTN caused an almost 5-fold increase ($p < 0.05$) (Figure 21).

Figure 22 shows the results when 50 μM of GTN, GT 015 or GT 152 were infused 10 to 15 min prior to and throughout the reperfusion period. Administration of both GTN and GT 015 was associated with a doubling of cGMP efflux vs. basal release ($p < 0.05$) during the period of LCAO (Figure 22). During reperfusion, however, only GTN significantly increased cGMP release (13 ± 1 fmol/ml/min for GTN vs. 6 ± 2 fmol/ml/min for DMSO treatment) (Figure 22). GT 152 did not appear to have a significant effect on cGMP efflux from the isolated perfused hearts.

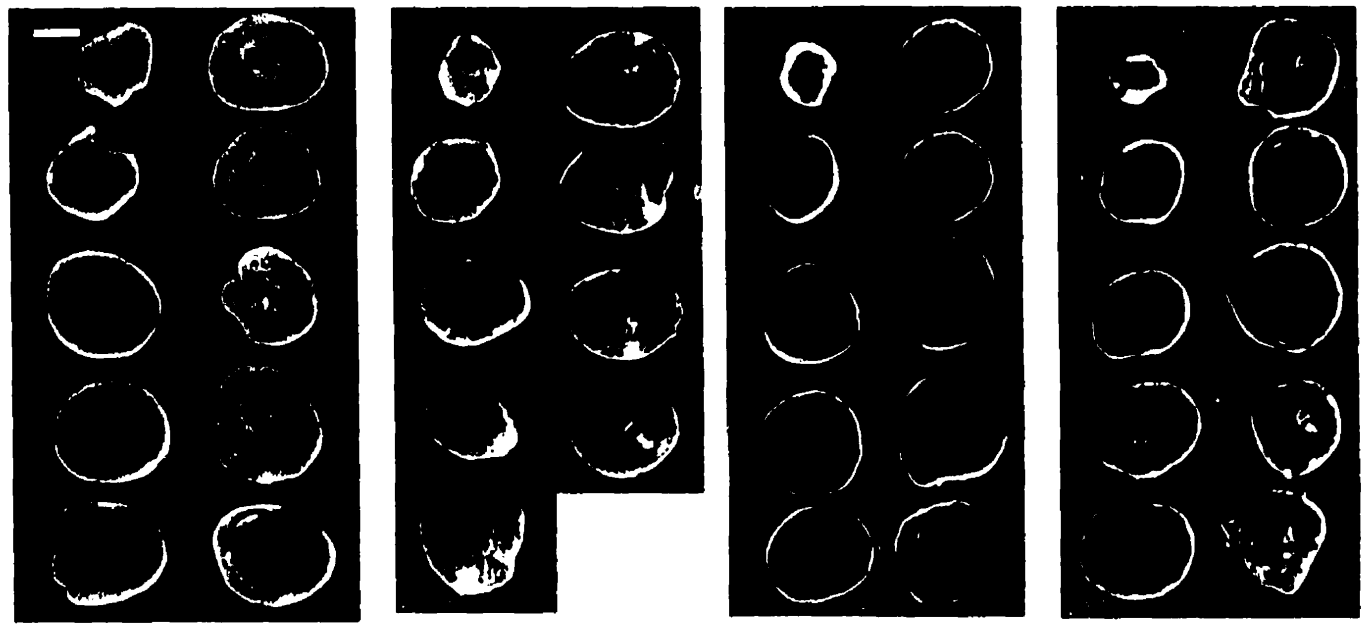


FIGURE 20. EFFECT OF DRUG TREATMENT PRIOR TO AND THROUGHOUT REPERFUSION ON INFARCT SIZE AFTER TTC STAINING IN ISOLATED PERFUSED HEARTS.

At the end of the ischemia-reperfusion protocol, the effect of drug treatment (DMSO, 50 μM GTN, 50 μM GT 015 and 50 μM GT 152) on infarct size was assessed. Drugs were infuseded 10 to 15 min prior to and throughout the 90 min of reperfusion following 45 min of regional ischemia. Evan's Blue dye was injected to delineate the AAR for infarction (negative staining) and TTC staining was performed to distinguish ischemic (white) and normal (red) tissue. Differences in infarct size are difficult to observe upon gross examination because the AAR shows considerable variability between hearts. All images are at the same magnification. White scale bar represents approximately 5 mm

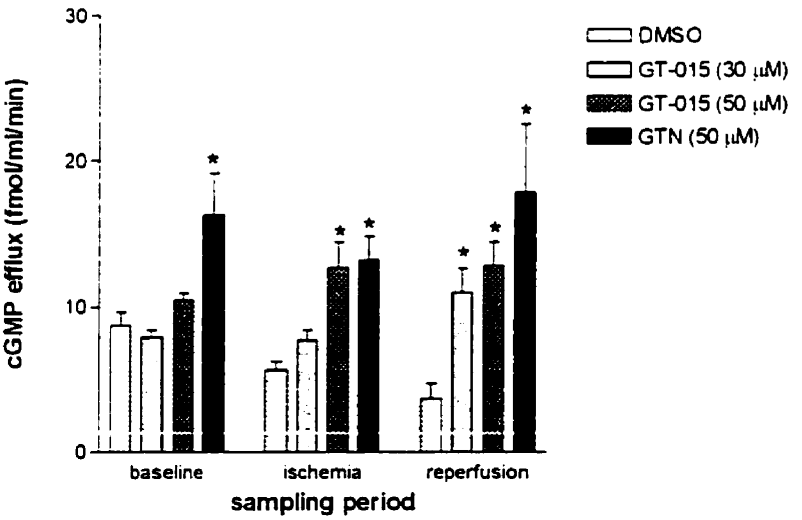


FIGURE 21. EFFECT OF DRUG ADMINISTRATION PRIOR TO AND THROUGHOUT ISCHEMIA ON cGMP EFFLUX FROM ISOLATED PERFUSED HEARTS.

Samples of the coronary effluent were collected for measurement of cGMP activity by radioimmunoassay as described in section 2.2.5. During equilibration, there were significantly higher cGMP levels measured in the group of hearts that were to receive GTN. During ischemia, 50 μM GT 015 and 50 μM GTN both increased cGMP release by 2-fold vs. vehicle treated hearts (* $p < 0.05$). During reperfusion, GT 015 infusion resulted in a dose-dependent increase in cGMP efflux and GTN produced an almost 5-fold increase. All bars represent the mean ± s.e.m, n = 3 for all treatment groups, one-way ANOVA, Tukey-Kramer post-hoc test.

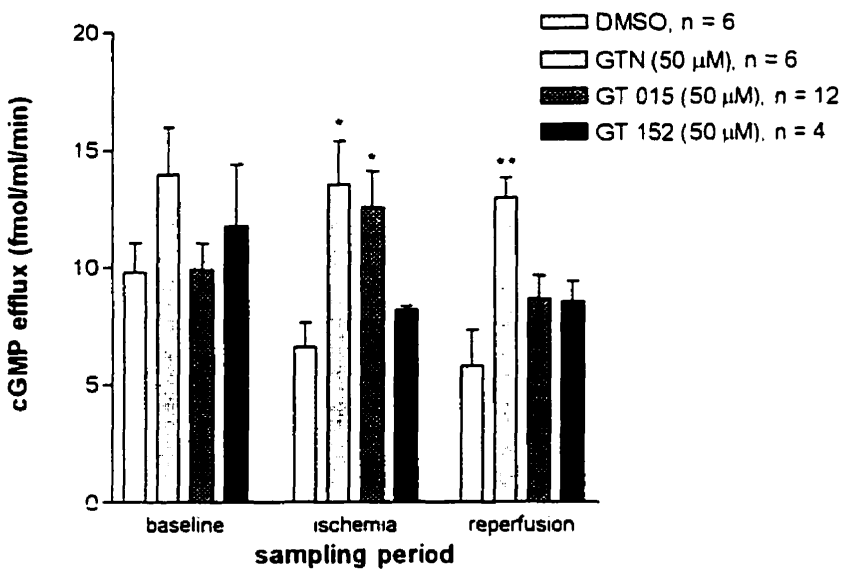


FIGURE 22. EFFECT OF DRUG ADMINISTRATION PRIOR TO AND THROUGHOUT REPERFUSION ON cGMP EFFLUX FROM ISOLATED PERFUSED HEARTS.

Samples of the coronary effluent were collected and analyzed for cGMP content by radioimmunassay as described in 2.2.5. During equilibration (baseline), there was no difference in cGMP release between the treatment groups. During the period of ischemia, both GTN and GT 015 significantly increased cGMP efflux by 2-fold (* $p < 0.05$). During the reperfusion period, GTN increased cGMP efflux by 2-fold (** $p < 0.01$), whereas GT 015 and GT 152 did not have a significant effect. All bars represent the mean ± s.e.m, one-way ANOVA, Tukey-Kramer post-hoc test.

2.4 Discussion

Our first objective in answering the question of whether a novel pharmacological agent (GT 015) could decrease the severity of post-ischemia reperfusion injury, was to characterize our constant-flow model for perfusion of isolated rat hearts. Indeed, Sumeray and Yellon (1998) have emphasized the importance of a thorough process of characterization of models of I/R injury, before meaningful conclusions can be drawn from the results they generate (Sumeray and Yellon, 1998).

We determined that for our experimental model, the optimal durations of regional ischemia and reperfusion were 45 min and 90 min, respectively. This choice was based on the observation that whereas 30 min of left coronary artery occlusion produced a significant and measurable increase in LDH release (Figure 10), 45 min was the minimum duration of ischemia needed to produce measurable infarcts. Furthermore, the reperfusion period was selected based on the fact that the per minute release of LDH was greatest after 90 min (Figure 11), but contractile function was not as severely impaired as was seen with reperfusion longer than 90 min. Indeed, the isolated perfused heart as an *ex vivo* preparation will deteriorate over time, at a rate of about 5-10% /hr (Sutherland and Hearse, 2000). Also, 25 min of equilibration was determined to be sufficient time for the heart to recover (i.e. to establish stable coronary perfusion pressure and heart rate) after the period of anoxia associated with the surgery.[1]

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[1]The period of anoxia associated with excision of the hearts and initiation of perfusion was always less than 2 minutes (hearts were excluded if there were problems with the surgery or transfer). By performing the surgery in close proximity to the perfusion apparatus and with practice, the whole procedure was performed quickly to prevent unintentional ischemic preconditioning. Awan *et al.* (1999) have reported that normothermic transfer times up to 3 min will not precondition the isolated rat heart (Awan *et al.*, 1999)

In previous studies, investigators studying I/R injury *in vitro* have used regional ischemic durations of anywhere between 30 min to 2 hr, and reperfusion periods between 30 min up to 4 hr after the period of regional ischemia (Joyeux *et al.*, 1999; al Makdessi *et al.*, 1999; Csont *et al.*, 1999). Differences in the duration of reduced oxygen and substrate delivery may explain some of the conflicting reports regarding the ability of drugs to improve the recovery of heart tissue after a period of ischemia. For example, protective effects have been reported with superoxide dismutase and catalase after 90 min (Jolly *et al.*, 1984) but not 3 hr (Gallagher *et al.*, 1986) of ischemia. Although it may very well be that the use of different model systems played a role in the contrasting effects, it is also possible that reactive oxygen species are involved only after short periods of ischemia. Thus, there may be time-dependent effects associated with ischemic periods of different durations, and the investigator must be aware of this fact when drawing conclusions based on his/her experimental protocol.

The length of reperfusion is another model-related factor that has considerable influence on the occurrence and mechanism of injury, both *in vivo* and *in vitro* (Reimer *et al.*, 1993). A body of evidence suggests that some of the damaging factors that are thought to be involved in the pathophysiology of reperfusion injury, such as peroxidized lipids and oxygen radicals, produce deleterious effects that are proportional to the length of time reperfusion is performed before an index of damage is measured. Clearly, studies are needed to follow various indices of injury as a function of time of reperfusion.

We selected our model and end-points of injury based on a thorough review of the literature. As discussed previously (see section 1.4.1), the isolated perfused heart

represents the optimal compromise in the conflict between the quantity and quality of data that can be acquired vs. its clinical relevance, and offers many experimental advantages (see section 1.4.2) (Sutherland and Hearse, 2000). The rat was selected as the model species because it is already well characterized in the literature and offers several advantages, most notably the fact that the anatomy of the coronary vasculature allows for the study of regional ischemia in this species (see section 1.4.3). It must be said, however, that some researchers have reservations about the suitability of the rat heart for the study of infarct size limitation of acute myocardial infarction. Hearse *et al.* contend that in the absence of collateral flow (as in rat hearts), cell death is inevitable in tissue that is severely ischemic and claim the rat heart may be an inappropriate model in which to study drug-induced tissue salvage during sustained ischemia (Hearse *et al.*, 1988). They also suggest studies of infarct size limitation are more relevant in canine preparations that offer the advantages of size, well documented biology and pathophysiology, and a substantial collateral blood supply that most resembles the clinical situation in humans with ischemic heart disease. However, these researchers acknowledge that the reduced cost and complexity of small animal preparations vs. canine preparations is a major investigational advantage. Furthermore, there are many reports of pharmacological limitation of infarct size in rats with permanent coronary occlusion and the rat may be a highly relevant model of sudden thrombotic occlusion in young adults where few collaterals are likely to exist (Hearse *et al.*, 1988).

We decided to use a constant flow model, instead of constant pressure, because constant flow perfusion adds an additional element of constancy to an experiment; that is, autoregulatory mechanisms are overridden and it does not automatically alter the amount of perfusate delivered to the whole heart when there are

changes in heart rate or work or when regional ischemia is imposed (Sutherland and Hearse, 2000). Using a constant flow model, we could instantaneously confirm occlusion of the left coronary artery by an increase in coronary perfusion pressure (since the same volume of perfusate is forced through a smaller perfusion bed).

The markers used to quantitate cardiac ischemia-reperfusion injury examine mechanical, electrical and biochemical functions (see Table 2). It is likely that the endpoint chosen to assess I/R injury will also influence conclusions regarding the mechanism and severity of this injury. Hearse (1988) made the important observation that both the rate and extent of myocardial recovery during reperfusion are important factors to consider when assessing the effect of "protective" agents (Hearse, 1988). Namely, interventions which increase the rate of recovery might ultimately be damaging by shunting cellular resources away from critically damaged sites. Alternatively, a drug may delay the onset of cell death rather than reduce the ultimate extent of this death (a problem with inappropriately short experiments) (Hearse *et al.*, 1988). It is important, therefore, to determine whether the recovery observed during the first few hours after an acute ischemic insult and reperfusion is maintained permanently (Kehrer and Starnes, 1989). Indeed, in several clinical trials, morbidity and mortality after myocardial infarction have been monitored weeks after the cessation of therapy. In addition, it is important to actually study the mechanism of any apparent cardioprotective effects. Further studies may reveal that the drug-induced cardioprotection is due to antidysrhythmic activity rather than some specific effect on ischemic or reperfusion injury (Kehrer and Starnes, 1989).

Two of the most widely reported indices to measure the severity of I/R injury are the lytic release of the intracellular enzyme lactate dehydrogenase, and quantification

of infarct size after triphenyltetrazolium chloride staining. Clearly, one must select the end-points that are most appropriate for the question under investigation, while appreciating the inherent advantages and limitations of the selection. Infarct size as a percent of the "zone at risk" is widely accepted as a sensitive index of cardiac damage. Controversy over infarct size limitation is partially based on the failure of many studies to account for the heterogeneity of the area-at-risk (i.e. to assess the ultimate infarct size in relation to the highly variable tissue mass initially at risk for infarction) (Hearse *et al.*, 1988). Indeed, in our studies we expressed infarct size as a percent of the AAR because we observed considerable variability in the area-at-risk for infarction (determined by negative staining with Evan's Blue dye) in different perfused hearts. This heterogeneity in the AAR may be partially attributed to the different anatomical arrangements of the left coronary artery in rats (Figure 23). An important consideration when discussing the AAR is that the zone of underreperfusion at the end of the ischemic insult may not accurately reflect the risk zone measured at the onset of ischemia. That is, the volume of underreperfused tissue may change during the evolution of I/R injury since re-infarction and vasoconstriction may increase the underreperfused volume (Hearse *et al.*, 1988).

Furthermore, some investigators argue that infarct size assessment by dye staining procedures, such as triphenyltetrazolium chloride staining, are not as reliable as histological examination. Freeman *et al.* (1990) and Reimer and Jennings (1984) have suggested that macroscopic staining with TTC may not be sufficiently sensitive to detect the gradient of viability in small areas of infarcted myocardium, especially in the irregular "border-zone" (see discussion below) between viable and necrotic tissue (Reimer and Jennings, 1979; Freeman *et al.*, 1990). However, histological examination

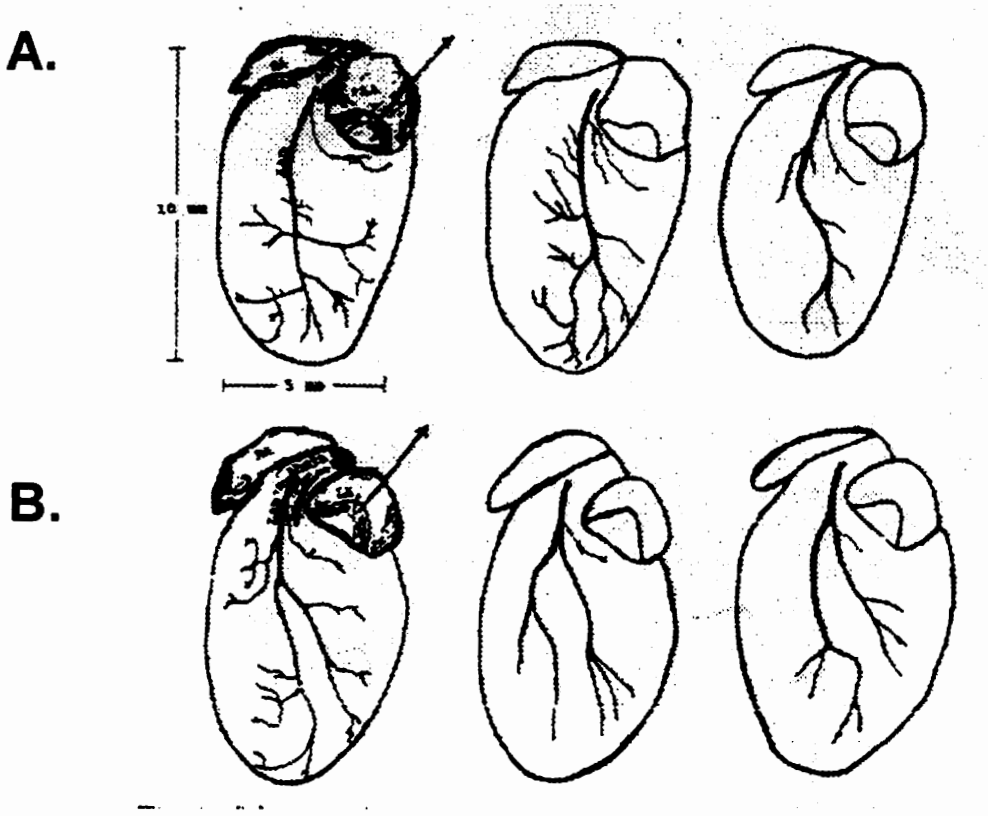

FIGURE 23. SCHEMATIC OF THE ANATOMICAL PATTERNS OF THE LEFT CORONARY ARTERY (LCA) IN RATS.

Schematic drawing of the two major anatomical patterns of the LCA in rats, illustrating the importance of expressing infarct size as a percent of the highly variable area-at-risk for infarction. The two common patterns of the LCA are a major singluar (A) or a major bifurcation (B). Note that in either case, there are branches of variable size and number arising from the LCA. From Michael *et al.* (1995).

has certain limitations: it is costly, time consuming and requires expertise in the analysis of specimens (Holmbom *et al.*, 1993). Furthermore, the histological method has been shown to give an underestimation of infarcted volume due to shrinkage of the infarcted myocytes (Ebrahimi *et al.*, 1990). Clearly, no method is completely accurate. However, TTC staining has been reported to reliably permit quantification of infarct size as early as 30 minutes after coronary occlusion in the rat (Vivaldi *et al.*, 1985; Fishbein *et al.*, 1981; Holmbom *et al.*, 1993)

The concept of a border-zone is based on the fact that within the spectrum of ischemic injury, there should exist zones were flow is so reduced that cell death is inevitable, and in addition, there may be peripheral areas where the ischemia is so mild that the cells can continue to function and survive (Hearse and Yellon,1981). Between these two extremes there may be a zone of intermediate injury where flow is sufficient to prevent, for a time at least, the transition from reversible to irreversible injury. These so-called "jeoparized" cells may represent a target for interventions designed for tissue salvage and reduction of infarct size (Hearse and Yellon,1981).

We expected that occlusion of the left coronary artery would induce the cellular and morphological changes associated with ischemic injury, with the ultimate consequence of cell lysis and release of intracellular enzymes. Lactate dehydrogenase exists as five different isozymes (LDH_1 to LDH_5) and is well known as a protein whose release into the circulation is pathognomic for cardiac infarction (Poston and Parenteau, 1992). Clinically, LDH levels increase slowly and peak 4 to 5 days post-infarction (Poston and Parenteau, 1992). We assumed, and based on previous studies, that any released LDH would remain in the tissue and not appear in the effluent until "washout" by the process of reperfusion. However, the LDH release

profile that we consistently observed was a sudden and marked release of LDH within 10 min of LCAO and a smaller secondary peak at the onset of reperfusion (Figures 8, 13, 17). This peak release early in ischemia was unexpected, but has been reported by other investigators (Poston and Parenteau, 1992; Sumeray and Yellon, 1998) For example, groups have reported a sudden and marked increase (Poston and Parenteau, 1992), a slight and gradual increase (al Makdessi *et al.*, 1996; Manning *et al.*, 1980), a weak increase (Herscher *et al.*, 1984), or no change (Ganote and Kaltenbach, 1979), in LDH release immediately after coronary artery occlusion. Unfortunately, few of these researchers have suggested a possible reason for the unexpected LDH release profile, only to say that "it is puzzling". Poston and Parenteau (1992) suggest that a probable explanation for the rise in LDH early in ischemia is *de novo* synthesis of active enzyme under conditions of altered NAD:NADH ratios, since levels of the enzyme are known to be under such control in other tissues (Poston and Parenteau, 1992; Everse and Kaplan, 1973). Likewise, these researchers suggest that the lower levels observed during reperfusion are probably a result of proteolysis of the enzyme and that a steady-state balance between synthesis and proteolysis is maintained by the NAD:NADH ratio (Poston and Parenteau, 1992). However, these researchers used isolated rat hearts perfused in the Langendorff "working mode" (i.e. cannulation of the left atrium) and induced global ischemia as opposed to regional ischemia. Thus, whether their explanation applies to our model is unclear. It is possible that tightening the suture around the coronary artery may produce mechanical (shear) stress in cells being perfused by the patent artery (because we are using a constant flow system, during regional ischemia the same volume of perfusate is being forced through a much smaller perfusion bed). The result may be "explosive cell swelling" and lysis in areas outside of

the actual ischemic zone (i.e. cells in the "border-zone"). Indeed, after Evan's Blue staining in some hearts, negative staining was seen as diffuse, patchy areas throughout the left ventricle, and on a few occasions the right ventricle appeared to be part of the area-at-risk. This suggests that there may have been damage in areas outside of the zone of tissue made ischemic by occlusion of the left coronary artery.

In this thesis, we examined the potential cardioprotective effects of the novel organic nitrate, GT 015, in a characterized model for perfusion of isolated rat hearts, and compared the effects of GT 015 to the prototypical organic nitrate, GTN. In the majority of previous studies, a reduction in the severity of post-ischemia reperfusion injury has been observed with pharmacological interventions just prior to or at the onset of ischemia. Indeed, GTN has recently been reported to attenuate LDH release when administered prior to an acute ischemic insult in isolated rat hearts (Csont *et al.*, 1999).

When we administered GTN and GT 015 10 min prior to and throughout a 45 min period of regional ischemia, we observed that both agents produced a dose-dependent decrease in LDH release during the ischemic insult. However, interestingly, only GT 015 attenuated LDH release during the reperfusion period, after drug administration had ceased (Figure 13). Furthermore, GT 015 caused a marked reduction in infarct size, whereas GTN had no effect (Figure 15). When we measured the levels of cGMP in the coronary effluent, we observed that both GTN and GT 015 increased cGMP content, although only GT 015 had an effect on infarct size and LDH release during reperfusion (Figure 20). Together, these results suggest that GT 015 is cardioprotective when administered prior to and throughout an acute ischemic insult, and that GT 015-mediated cardioprotection may involve mechanisms other than, or in addition to, cGMP elevation.

Prolonged ischemia followed by reperfusion is known to induce contractile dysfunction, possibly mediated by intracelluar calcium overload (Dhalla *et al.*, 2001). When we examined the perfusion pressure tracings from vehicle-treated hearts after 45 min of ischemia and 90 min of reperfusion, there was evidence of myocardial "stunning" (i.e. reversible inhibition of contractile function). Whereas GTN administration prior to and throughout reperfusion did not improve post-ischemic contractile recovery, GT 015 did appear to attenuate the deterioration of the heart and inhibit hypercontracture (as assessed by the CPP at the end of 90 min reperfusion).

Because GT 015 showed cardioprotective effects when administered prior to the ischemic insult, we wished to determine whether it could also decrease the severity of post-ischemia reperfusion injury when administered just prior to reperfusion. This study was particularly important in determining the potential usefulness of GT 015 as a cardioprotective agent, since in the clinical setting it would most likely be administered *after* an acute myocardial infarction had already occurred. We also administered the agent GT 152, a putative metabolite of GT 015 formed after cleavage of the disulfide bond (Figure 6).

Administration of GTN, GT 015 and GT 152 at a concentration of 50 μM, 10 to 15 min prior to and throughout the 90 min of reperfusion following a 45 min ischemic insult, produced some unexpected results. While we were not surprised to find that none of these agents significantly reduced LDH release during the ischemic period, the fact that GTN attenuated LDH release during reperfusion was highly unexpected since it did not attenuate LDH release when administered prior to and throughout ischemia. However, analysis of infarct size revealed that whereas GTN reduced LDH release, it did not have any effect on infarct size (Figure 18). Conversely, both GT 015 and GT

152 attenuated LDH release during reperfusion and both significantly reduced infarct volume as a percent of the area-at-risk (Figure 18).

The fact that GTN attenuated LDH release but did not have any effect on infarct size suggests that GTN administration prior to and throughout reperfusion may delay the onset of cell death rather than reduce the absolute extent of injury. It may be that the hemodynamic effects of GTN (i.e. vasodilation to improve oxygen and substrate delivery to the ischemic zone during reperfusion) delays the progression of irreversible cell injury during reperfusion. However, the tissue may already be "condemned" (Hearse and Yellon, 1981) for irreversible injury and cell death, and GTN has no beneficial effect in this regard. We suggest that some of the ischemic cells may be alive in terms of membrane integrity during the post-ischemic reperfusion period (thus low levels of LDH were detected), but dead in relation to their primary role; that is, contraction. This may explain why GTN administration seemed to decrease LDH release but had no infarct-limiting effect.

Although the results from the previous study suggested that GT 015-mediated cardioprotection is at least partly independent of cGMP, the mechanisms of ischemia-induced injury and reperfusion-induced injury (assuming they are two distinct phenomena) may involve different mediators and mechanisms. Thus, we measured cGMP levels in hearts treated just prior to and throughout reperfusion to determine whether cGMP is associated with drug-mediated cardioprotection when administered prior to reperfusion. We observed that both GTN and GT 015 increased cGMP levels during ischemia, but only GTN had an effect during reperfusion (Figure 21).

As discussed previously (see section 1.3.3.5; Figure 3), it is widely accepted that GTN undergoes biotransformation to an active metabolite, believed to be NO.

Nitric oxide is thought to initiate the downstream signaling cascade that results in increased cGMP levels (Ahlner *et al.*, 1991). Nitric oxide has been implicated as a deleterious mediator of myocardial reperfusion injury, possibly through the formation of the peroxynitrite radical (Hotta *et al.*, 1999). If NO is in fact deleterious to myocardial cells during post-ischemia reperfusion, it may have played a role in the development of the infarcts we observed in GTN-treated hearts. The fact that GT 015 and GT 152 did not significantly increase cGMP levels during the reperfusion period, but still attenuated LDH release and most significantly, reduced infarct size, suggests that elevated cGMP levels may not be an important factor for GT 015-mediated cardioprotection. Furthermore, elevated cGMP/NO may in fact *increase* the severity of post-ischemia reperfusion injury in our model.

The fact that GT 015, and its putative metabolite GT 152, both attenuated LDH release and reduced infarct size but did not significantly increase cGMP, suggests that GT 015-mediated cardioprotection (during reperfusion) is at least partly independent of cGMP. A possible mechanism of GT 015-mediated cardioprotection may involve scavenging of reactive free radicals generated in the myocardium during reperfusion (Figure 6). Clearly, further mechanistic studies are needed to elucidate the underlying mechanisms of how GT 015 mediates cardioprotection during I/R injury (see Future Directions).

In conclusion, we have found that the novel organic nitrate, GT 015, reduces the severity of post-ischemia reperfusion injury. Although the exact mechanism of GT 015-mediated cardioprotection remains to be defined, the results of this thesis suggest that GT 015 may be a promising pharmacological therapy in the prevention of cellular injury due to an acute ischemic insult. Furthermore, GT 015 was cardioprotective even when

administered after an ischemic insult, suggesting it may have exciting clinical potential as treatment after acute myocardial infarction.

CHAPTER 3: Summary, conclusions and future directions

Glyceryl trinitrate is the prototypical organic nitrate, and has been used clinically for over a century. The ongoing interest to elucidate the mechanisms of myocardial ischemia-reperfusion injury, and to identify agents that may reduce its severity, have led researchers to test the cardioprotective ability of several pharmacological drugs, including GTN. However, despite a number of promising reports *in vitro* (isolated perfused hearts from different animals), *in vivo* (studies in intact animals) and in clinical trials, there remains, as yet, no therapeutic agent that has gained widespread acceptance for the treatment of acute cardiac ischemic injury. Thus, this thesis research was conducted to answer the question "*Does a novel organic nitrate reduce the severity of post-ischemia reperfusion injury?*"

Our results suggest that the novel organic nitrate, GT 015, reduces the severity of ischemia-reperfusion injury when administered prior to and throughout an acute ischemic insult or *just prior to and throughout reperfusion, after a prolonged ischemic insult*. The clinical significance of the latter finding is based on the fact that one of the most critical aspects in treating ischemic myocardium is the time delay between the transient occlusion of a coronary vessel (e.g. by a thrombotic occlusion) and when the patient presents at the hospital for reperfusion treatment, by transluminal angioplasty or thrombolysis. Thus, GT 015 may have greater clinical potential than other agents such as GTN, which has not been consistently shown to improve I/R injury in experimental models when administered at the onset of reperfusion, and has been disappointing in clinical trials when adminstered after myocardial infarction. While we acknowledge the limitations of our model and end-points of injury (namely, LDH release as a marker of

necrotic cell death and infarct size quantification by TTC staining), our promising results suggest that further studies with GT 015 as a novel cardioprotective agent are warranted.

The results presented in this thesis address the basic question of whether GT 015 has any beneficial effects in an *in vitro* model of ischemia-reperfusion injury. Clearly, further studies are needed to validate these findings, and to elucidate the mechanism of GT 015-mediated cardioprotection. Future directions should include more studies to characterize the time-dependent effects of GT 015. For example, the drug could be administered to isolated hearts at the onset of and throughout reperfusion. Such studies would not only demonstrate how late after an acute ischemic insult GT 015 may be administered and still have cardioprotective properties, but administration of the drug at reperfusion could also help clarify whether reperfusion injury is a distinct phenomenon. That is, if reperfusion itself has deleterious effects independent of the acute ischemic insult that precedes it, then pharmacological intervention at the time of reperfusion may salvage some tissue or prevent further injury (Opie, 1989).

Although the isolated perfused heart offers many experimental advantages and is a valuable tool for basic studies of pharmacological agents, it would be necessary to repeat the experiments we have presented here first in a "working-heart" model and then in *in vivo* studies. It will be interesting to observe how the presence of neurhormonal factors and circulating catecholamines in the intact animal affect both the progression of I/R injury and GT 015-mediated cardioprotection.

Clearly, more mechanistic studies are needed to fully understand how GT 015 reduces the severity of post-ischemia reperfusion injury. Recently, a great deal of

interest has focused on understanding the role of ATP-sensitive K^+ channels in myocardial ischemic injury (see section 1.3.2.4). In a series of preliminary studies, we assessed whether the K_{ATP} channel blocker glibenclamide (30 μM, infused 10 min prior to and throughout infusion of GTN (50μM) or GT 015 (50 μM)) reverses the cardioprotective effects of GTN and GT 015 when these agents were infused prior to and throughout the ischemic period. The preliminary results (n = 2) suggest that glibenclamide reverses GTN-mediated and GT 015-mediated reduction of LDH release during ischemia (infarct size was not quantitiated). These results agree with a recent report that the anti-ischemic effect of GTN is blocked by gibenclamide (Csont *et al.*, 1999)

Whereas it would be necessary to test GT 015 in more complex models of I/R injury to increase the clinical relevance of the results, future *mechanistic* studies could involve more simplified models such as isolated cardiomyocytes. It would be very interesting (and technically feasible) to determine the involvement of mitochondria during the evolution of I/R injury, since maintenance of mitochondrial function is fundamental for the normal performance and survival of cells that have a high-energy requirement, such as the beating cardiomyocyte (Mathur *et al.*, 2000; Regitz *et al.*, 1984). Several studies have been performed using fluorescent dyes, for example JC-1 (5,5',6,6'-tetrachloro-1,1'3,3'-tetra-ethylbenzimidazolocarbocyanine iodide), in cultured cardiomyocytes to detect changes in mitochondrial membrane potential during ischemia and reperfusion (Mathur *et al.*, 2000). These studies have implicated the collapse of the mitochondrial membrane potential as a molecular mechanism associated with reperfusion injury to the heart (Duchen *et al.*, 1993; Di Lisa *et al.*, 1995). Incubation of GT 015 with cultured myocytes subjected to hypoxic conditions, and measurement of

changes in mitochondrial membrane potential using JC-1, could provide insight as to whether the mechanism of GT 015-induced cardioprotection involves an improvement of mitochondrial function.

Conclusions:

This thesis described the effect of pharmacological intervention in a model of myocardial ischemia-reperfusion injury. The results of this work suggest that the novel organic nitrate, GT 015, reduces the severity of post-ischemia reperfusion injury, and that its cardioprotective effects extend beyond the actions of the prototypical organic nitrate, GTN. Furthermore, it appears that GT 015-mediated cardioprotection involves mechanisms other than, or in addition to, cyclic GMP elevation. This author suggests that GT 015 may scavenge reactive oxygen species generated during reperfusion, although further studies both *in vitro* and *in vivo* are needed. If future mechanistic and toxicological studies in the intact animal validate the findings presented here, GT 015 may indeed represent a novel therapeutic strategy to prevent the morbidity and mortality of acute myocardial infarction.

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