UNIVERSITY OF ALBERTA

ROLE OF PEROXYNITRITE IN ISCHEMIA-REPERFUSION INJURY IN THE ISOLATED RAT HEART

BY

WAHIDA YASMIN

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of MASTER OF SCIENCE

DEPARTMENT OF PEDIATRICS

EDMONTON, ALBERTA

FALL 1996

ISBN 0-612-18339-4

UNIVERSITY OF ALBERTA

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NAME OF AUTHOR: **WAHIDA YASMIN**

TITLE OF THESIS: **E OF PEROXYNITRITE IN ISCHEMIA-ERFUSION INJURY IN THE ISOLATED RAT HEART**

DEGREE: **MASTER OF SCIENCE**

YEAR THIS DEGREE GRANTED: **1996**

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **ROLE OF PEROXYNITRITE IN ISCHEMIA-REPERFUSION INJURY IN THE ISOLATED RAT HEART** submitted by WAHIDA YASMIN in partial fulfillment of the requirements of the degree of MASTER OF SCIENCE

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ABSTRACT

Reperfusion of ischemic hearts results in the release of superoxide anion and nitric oxide (NO) that are likely to form peroxynitrite ($ONOO^-$). Release of $ONOO^-$ was determined in isolated rat hearts perfused with Krebs-Henseleit buffer containing L-tyrosine, which reacts with $ONOO^-$ to form dityrosine. Dityrosine was detected by fluorescence spectroscopy in the coronary effluent during the acute reperfusion of globally ischemic hearts and was abolished by the NO synthase inhibitor N^G-monomethyl-L-arginine (L-NMMA), the NO donor S-nitroso-N-acetyl-D,L-penicillamine (SNAP), or the combination of superoxide dismutase (SOD) and catalase. Moreover, improvement in the recovery of mechanical function was observed with L-NMMA, SNAP and SOD mimetic (MnTBAP) during reperfusion. These data indicate that reperfusion of the ischemic heart causes the production of $ONOO^-$. Inhibiting its biosynthesis or antagonizing its oxidant actions are possible protective measures in myocardial ischemia-reperfusion injury.

ACKNOWLEDGEMENTS

To my supervisor, Dr. Rick Schulz, I express deep gratitude for his knowledgeable guidance, encouragement and patience during the course of this study. I consider myself very fortunate to have very close supervision from such an inquisitive explorer in the field of nitric oxide. I also acknowledge his personal support, understanding and adjustment in my favour, which helped me through on various occasions.

I express my appreciation to Dr. Gary Lopaschuk for his valuable suggestions, support and encouragement in and outside my committee. I am also thankful to him for letting me use the fluorometer and UV spectrophotometer in his lab.

I would like to thank Dr. Marek Radomski for his support and helpful discussions as my committee member. His valuable suggestions regarding the preparation of peroxynitrite are gratefully acknowledged.

I thank Dr. Nasreen Mesaeli for helping me with setting up an intraventricular balloon technique.

To Dr. Susan Jacobs, who allowed me to use her infusion pump throughout my experiments, I am very thankful.

To Dr. Sandy Clanachan, his valuable suggestions are appreciated.

I thank Mr. Ken Strynadka for conducting high performance liquid chromatography technique.

My very special thanks are due to Donna Panas, Karen Dodge and Kassim Abou-Chehade for their help on different occasions. I also appreciate help from Fadi Khadour and Po-Yin Cheung.

I thank Mary-Jo Boeglin who helped me through various administrative details in my program.

My thanks to the Canadian International Development Agency (CIDA) for providing me with special assistance towards my graduate studies.

I am thankful to the Faculty of Medicine at the University of Alberta for the 75th Anniversary Award.

I gratefully acknowledge the financial support from the Heart and Stroke Foundation of Alberta.

Finally, I am grateful to my husband, Anisur Rahman and my children, Ridi and Parash for their understanding, inspiration, and loving support which helped me to achieve this degree.

Dedicated
to

My parents, Amjad & Fahmida
My husband, Anis
&
My children, Ridi & Parasah

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ABBREVIATIONS

L-NMMA	N^G-monomethyl-L-arginine acetate salt
MnTBAP	Mn(III) tetrakis (4-benzoic acid) porphyrin
NO	Nitric oxide
NOS	Nitric oxide synthase
$O_2^{-\cdot}$	Superoxide anion
$ONOO^-$	Peroxynitrite
Rx	Reaction
SNAP	S-nitroso-N-acetyl-D,L-penicillamine
SOD	Superoxide dismutase

CHAPTER I

INTRODUCTION

A) Myocardial ischemia and reperfusion and the role of oxygen-derived radicals

Myocardial ischemia and reperfusion: Definitions

Myocardial ischemia is a state when the reduction of coronary flow is so severe that the supply of oxygen to the myocardium is inadequate for the oxygen demands of the tissue (Opie, 1991). Early reperfusion is an absolute prerequisite for the survival of severely ischemic tissue. However, it has been suggested that reperfusion itself may have deleterious effects on the ischemic myocardium (Hearse, 1977). Thus, *myocardial reperfusion injury* has been defined as cellular damage resulting from the reintroduction of molecular oxygen at the time of organ reperfusion (Lucchesi, 1990). The basic hypothetical concept of 'reperfusion injury' is that reversible and irreversible manifestations of injury after a temporary episode of ischemia may not be caused directly by the state of cellular functions existing immediately before the moment of reperfusion but rather may result from some deleterious aspects of reperfusion *per se* (Jolly, 1984). Cardiac myocytes which are viable at the end of the ischemic interval are subjected to

unfavourable conditions such as oxidative stress related to reperfusion and the act of reperfusion itself leads to cell damage (Lucchesi, 1990). In addition to oxidative stress, metabolic changes occur ie. uncoupling of glycolysis from glucose oxidation and increase in fatty acid oxidation (Lopaschuk et al, 1990; Lopaschuk et al, 1993), which are detrimental to the heart. Other proposed pathways are cellular acidosis which could contribute to reperfusion injury by increasing calcium via changes in ion pump such as sodium/hydrogen and calcium/sodium changes (Grinwald, 1982; Lanzdunski et al, 1985).

The consequences of myocardial reperfusion on ischemic myocytes depends on the condition of the affected myocytes and adjacent microvasculature at the time of reperfusion (Jennings and Reimer, 1983). A brief period of ischemia causes reversible myocyte injury as early restoration of flow prevents myocardial infarction and permits recovery of normal myocardial structure, function and metabolism. However, studies have demonstrated that complete recovery is not immediate. A number of experiments have shown that profound metabolic and functional abnormalities may persists for hours or days after as little as 5 to 15 min of coronary occlusion in experimental animals (Weiner et al, 1976; Reimer et al, 1981; Kloner et al, 1983). The characteristic features of *reversibly injured myocytes* which recover rapidly (within minutes to hours) include: partial loss of glycogen, relaxation of myofibrils, mild margination of nuclear chromatin, mild cell swelling and loss of normal mitochondrial matrix granules. This reversible recovery of structural, metabolic and contractile function can also be delayed for hours to days which is evidenced by the slow recovery of ATP content, myocardial stunning and reperfusion arrhythmias. The consequences of *irreversible injury* are explosive cell

swelling, formation of sarcolemmal blebs associated with sarcolemmal disruption, massive increase in intracellular calcium, myofibrillar disruption, contraction band necrosis, release of cytosolic constituents such as creatinine kinase, lactate dehydrogenase and other enzymes and cofactors into the systemic circulation and microvascular damage.

The phenomenon of stunning was first defined from observations that post-ischemic contractile dysfunction persists for hours or days after a brief (5-15 min) period of ischemic injury even though the previously ischemic myocardium is completely viable. This phenomenon was reported by Heyndrix et al (1975) and later termed "myocardial stunning" by Braunwald and Kloner (1982). On reperfusion, the ischemic myocardium manifests sarcolemmal disruption, mitochondrial swelling and contraction band necrosis (Jennings and Ganote, 1974). In vitro studies have placed emphasis upon the need to control the conditions of reperfusion and the composition of perfusate to prevent myocardial dysfunction (Buckberg, 1986; Kloner et al, 1989; Kitakaze et al, 1988). Several studies have implicated the role of oxygen-derived radicals in the pathogenesis of reperfusion abnormalities such as: reperfusion injuries (Thompson and Hess, 1986; McCord, 1985), stunned myocardium (Bolli, 1988; Bolli et al, 1989) and reperfusion arrhythmias (Manning and Hearse, 1984; Bernier et al, 1986). A number of studies have shown that the acute release of oxygen-derived radicals in the heart during reperfusion of the ischemic myocardium contributes to myocardial stunning (Bolli, 1990; Marban, 1991).

Biochemistry of oxygen-derived radicals

Oxygen-derived radicals constitute a class of chemical compounds in which an unpaired electron occupies the outer orbital of the oxygen molecule (Goldheiber and Weiss, 1992). These can greatly vary in their chemical reactivity. They are also by-products of cellular metabolism during the tetravalent reduction of molecular oxygen to water. Under normal physiologic conditions, mitochondrial respiration is a significant source in generating superoxide anion ($O_2^{-\cdot}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$). It has been also observed that mitochondria also maintains the cellular redox status by eliminating $O_2^{-\cdot}$ of both mitochondrial and cytosolic origin (Guidot et al, 1995). The hydroxyl radical is thought to be the most directly cytotoxic species of oxygen derived radicals (McCord 1985). The oxidation of reduced components of the electron transport chain such as ubiquinone may generate $O_2^{-\cdot}$ through the univalent transfer of an electron to molecular oxygen (Figure 1.1, Rx. a). In biological tissues, $O_2^{-\cdot}$ is quickly reduced to H_2O_2 by the antioxidant enzyme superoxide dismutase (Figure 1.1, Rx. b). Hydroxyl radical can be formed by the Haber-Weiss reaction (Haber and Weiss, 1934) when $O_2^{-\cdot}$ and H_2O_2 combine to form O_2, OH^- and $OH^{\cdot}$ radical (Figure 1.1, Rx. c). Another way of producing $OH^{\cdot}$ radicals is via the Fenton reaction (Figure 1.1, Rx. d) where H_2O_2 accepts an electron from a reduced metal ion such as Fe^{2+} or Cu^{2+}. Superoxide anion plays a critical role here because it is the primary reducing agent to replenish the transition metals (M) such as Fe^{2+} following its oxidation to Fe^{3+}.

The formation of oxidant species

a. The reduction of molecular oxygen to superoxide anion

$$O_2 + e^- \Rightarrow O_2^{-\bullet}$$

b. Dismutation of superoxide anion by superoxide dismutase

$$2O_2^{-\bullet} + 2H^+ \Rightarrow H_2O_2 + O_2$$

c. The Haber-Weiss Reaction

$$O_2^{-\bullet} + H_2O_2 \Rightarrow O_2 + HO^- + HO^\bullet$$

d. The Fenton reaction

$$O_2^{-\bullet} + M^{n+1} \Rightarrow M^n + O_2$$

$$M^n + H_2O_2 \Rightarrow M^{n+1} + HO^- + HO^\bullet$$

Figure 1.1: Formation of oxidant species are shown in 4 steps: a) molecular oxygen is reduced to form superoxide anion ($O_2^{-\bullet}$), b) dismutation of $O_2^{-\bullet}$ by superoxide dismutase causes formation of H_2O_2, c) production of $OH^\bullet$ from H_2O_2 by Haber-Weiss reaction and d) in presence of metal ion (M), formation of $OH^\bullet$ from $O_2^{-\bullet}$ by Fenton reaction.

Under physiological conditions $O_2^{-\cdot}$ is a relatively weak oxidizing agent and is incapable of lipid peroxidation (Halliwell et al, 1984). Much of the oxidative injury that occurs is thought to depend upon the formation of $OH^{\cdot}$. In contrast to $O_2^{-\cdot}$, $OH^{\cdot}$ is an extremely reactive molecule and has been shown to be capable of initiating lipid peroxidation in vitro (Fong et al, 1973). However, in the absence of a catalyst, the Haber-Weiss reaction is too slow to be of biological importance as a source of $OH^{\cdot}$ (Halliwell, 1976). Iron is an important catalyst for the production of $OH^{\cdot}$ in biological systems (Aust et al, 1982). Under physiological conditions, however, levels of free iron are negligible as it is strictly controlled by the cell and is normally completely bound in the form of ferritin. It is therefore debatable whether there is sufficient free iron present to catalyze the Fenton reaction (Gutteridge and Halliwell, 1990).

In normal conditions these oxidative species are effectively neutralized by endogenous antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase. Other important cellular constituents known to participate in the defense mechanism are glutathione, ascorbate (vitamin C), α-tocopherol (vitamin E) and selenium, a cofactor for the enzyme glutathione peroxidase.

There is now a considerable evidence that oxygen-derived radicals are in large part responsible for reperfusion injury following ischemia in different organs including heart (Jolly et al, 1984), brain (Flamm, 1978), small intestine (Granger et al, 1981), liver (Adkinson et al, 1986), kidney (Paller et al, 1984), pancreas (Sanfey, 1984), skeletal muscle (Korthius et al, 1985) and skin (Manson et al, 1983). Several studies have demonstrated that oxygen-derived radical species, including superoxide anion ($O_2^{-\cdot}$), are

formed during reperfusion of the ischemic heart and peak in concentration within the first minute of reperfusion (Zweier et al, 1989; Bolli et al, 1988; Garlick et al, 1987). Superoxide anion must be transformed into more reactive oxidant species such as the hydroxyl radical in order to cause stunning, yet direct evidence for such pathways in the heart is lacking. In the absence of ischemia and reperfusion, exposure of hearts to oxidant generating systems cause irreversible cardiac injury similar to that caused by ischemia and reperfusion (Thompson and Hess, 1986).

Mechanisms of free radical injury

The deleterious effects of oxygen-derived radicals in biological systems are numerous due to their multiple targets (Halliwell and Gutteridge, 1985; Freeman and Crapo, 1982; Fridovich, 1978; Richard et al, 1994). Deoxyribonucleic acid base modification and strand scission occurs following exposure to oxygen-derived radicals. These radicals can also damage proteins by chemical modification of amino acid residues, leading to conformational changes, fragmentation, or cross linking. This denatures the protein, rendering it more susceptible to hydrolysis. The consequences of these changes include the loss of enzyme activity and altered function of ion channels, transporters and receptors. An important target of free radicals are lipids, perhaps most importantly resulting in the peroxidation of membrane lipids. The reaction of oxygen-derived radicals with lipid is initiated through their abstraction of a hydrogen atom from the unsaturated fatty acid side chain to form a carbon-centred lipid radical. Double bond rearrangement

results in the formation of a more stable conjugated diene which may then react with molecular oxygen to form a peroxy radical. This peroxy radical is highly reactive and is capable of removing hydrogen atoms from adjacent fatty acids, thus propagating the chain reaction of oxidative injury. If the above reactions are allowed to continue unabated, such reactions may result in decreased membrane fluidity, disruption of membrane-bound enzyme and transport systems and alteration of cell permeability. The end result is cell destruction.

Sources of oxygen derived radicals

Increased production of oxidant species can occur during myocardial ischemia as a result of a variety of events. Superoxide may be produced in the conversion of hypoxanthine to uric acid by the enzyme xanthine oxidase (McCord, 1985). In non-ischemic cells xanthine is oxidized by xanthine dehydrogenase which reduces NAD^+ (Batteli et al, 1972). In ischemic cells, an increase in intracellular levels of Ca^{2+} can increase the conversion of xanthine dehydrogenase to xanthine oxidase. Xanthine oxidase has been identified in parenchymal cells of many tissues including capillary endothelium (Jarash et al, 1981). Romson and coworkers (1983) first reported that neutrophils are a source of oxygen radical production during the course of myocardial ischemia and reperfusion. The NADPH-dependent oxidase system on the membrane surface of the neutrophils is a highly efficient source of $O_2^{-\cdot}$ (Tauber et al, 1979). This enzyme when activated catalyzes the reduction of oxygen to $O_2^{-\cdot}$ and H_2O_2. Hydrogen peroxide in the

presence of myeloperoxidase and chloride ions forms hypochlorous acid (HOCl). Thus, as a result of infiltration of the microvasculature with neutrophils (Engler et al, 1986), different kinds of oxidant species are formed, including $O_2^{-\cdot}$, H_2O_2, $OH^{\cdot}$ and OCl^- (hypochlorous anion). Increased intracellular levels of Ca^{2+} as a result of ischemia may also activate phospholipase C to stimulate arachidonic acid metabolism which generates oxidant species as by-products (Karmazyn et al, 1985). Other sources include catecholamine metabolism (Singal et al, 1980).

Evidence of oxygen derived radicals during ischemia and reperfusion

Methods used to measure oxygen derived radicals include electron paramagnetic resonance spectroscopy and techniques based upon chemiluminescent reactions between free radicals and specific substrates. A number of studies have shown a sudden increase in free radical production following reperfusion of globally ischemic isolated hearts (Zweier et al, 1987; Zweier et al, 1988). Using electron paramagnetic resonance spectroscopy, Zweier et al (1989) demonstrated that there is a burst of free radical generation during the early seconds of postischemic reperfusion. Zweier et al (1989) performed both direct and spin trapping measurements (using 5,5'-dimethyl-1-pyrroline-N-oxide, DMPO) of the time course and mechanism of radical generation in isolated perfused rabbit hearts. Reperfusion-associated radical generation was maximum after approximately 30 min of global ischemia, with peak radical concentrations observed between 10-20 seconds of reperfusion, then gradually decreased until after 5 min there

was almost no detectable signal. Bolli et al (1989) used electron paramagnetic resonance spectroscopy to investigate the free radical hypothesis in stunned myocardium. In this study an open chest dog model was used in which the heart was subjected to 15 min occlusion of the left anterior descending coronary artery. A free radical trapping agent, α-phenyl *N-tert*-butylnitrone (PBN, 50 mg/kg), was infused intravenously. Electron paramagnetic resonance signals characteristic of radical adducts of α-phenyl *N-tert*-butylnitrone appeared in the coronary venous blood during ischemia and increased markedly during the first minute of reperfusion. This signal was inhibited by the simultaneous infusion of SOD and catalase which was associated with an improvement of mechanical function. These results provided evidence using an in vivo model to support the hypothesis that oxygen radicals play a causal role in myocardial stunning following brief ischemia.

B) Nitric oxide and nitric oxide synthases

Nitric oxide (NO) is an important bioregulatory molecule which is involved in diverse physiological and pathological processes of the body. It was first discovered as endothelium-derived relaxing factor (EDRF) by Furchgott and Zawadzki (1980). Palmer et al (1987) recognized the chemical identity of EDRF as nitric oxide. Nitric oxide has also been identified as a neurotransmitter (Garthwaite et al, 1989; Bredt et al, 1990) and as a cytotoxic factor in the immune system (Nathan, 1992).

The enzymes responsible for the synthesis of NO from L-arginine (Moncada et al,

1991) are known as nitric oxide synthases (NOS). There are at least three distinct enzymes which include a neuronal form (nNOS or NOS type I), one found in endothelial cells (eNOS or NOS type III) and an inducible form which was originally found in macrophages (iNOS or NOS type II). The NO synthases are unique cytochrome P450-like hemoproteins which exist as dimers, are calmodulin-dependent or calmodulin-containing, and which bind NADPH, FAD, FMN, tetrahydrobiopterin and prosthetic heme to catalyze the five electron oxidation of L-arginine to NO and citrulline. The neuronal and endothelial forms of the enzymes are constitutively expressed and are Ca^{2+}-calmodulin dependent. In contrast, the macrophage enzyme is Ca^{2+}-independent and requires de novo synthesis when cells are stimulated by bacterial endotoxin and/or pro-inflammatory cytokines. The distribution and functions of the three NO synthases are summarized in Table 1.1.

The normal heart synthesizes NO from oxygen and L-arginine by a Ca^{2+}-dependent NOS which has been identified as the endothelial isoform (eNOS or NOS Type III). This Ca^{2+}-dependent NOS is present in coronary endothelium (Cocks et al, 1985), endocardial endothelium (Schulz et al, 1991), cardiac myocytes (Schulz et al, 1992; Balligand et al, 1995) and cardiac neurons (Klimaschewski et al, 1992). The physiological production of small quantities of NO in the heart acts to maintain coronary vasodilator tone, inhibit platelet aggregation and the adhesion of neutrophils and platelets to vascular endothelium (see Moncada and Higgs, 1993 for review). Moreover, NO also modulates cardiac muscle function (Schulz and Triggle, 1994) with negative inotropic (Brady et al, 1993; Shaw and Lewis, 1993) and chronotropic (Balligand et al, 1993) actions.

Table 1.1: Nitric oxide synthases (NOS)

NO synthases	Functions	Distribution
endothelial (eNOS) Constitutive Ca^{2+}-dependent	• regulates blood pressure regional blood flow and myocardial contractility • dilates vascular and other smooth muscle • regulates proliferation of vascular smooth muscle • inhibits platelet aggregation and adhesion of platelet or leucocytes to endothelium	endothelium, epithelium of several tissues (e.g. bronchial tree), neurons in the brain (e.g. pyramidal cells of the hippocampus), cardiac myocytes, platelets, mitochondria (?)
neuronal (nNOS) Constitutive Ca^{2+}-dependent	• neurotransmitter substance released upon activation of excitatory amino acid receptors and non-adrenergic non-cholinergic nerves • modulator of skeletal muscle contractility • dilator of smooth muscles	brain, spinal chord, peripheral nervous system, pituitary, kidney, adrenal medulla, male sex organ, skeletal muscle
macrophage (iNOS) Inducible Ca^{2+}-independent	• cytotoxic agent produced by macrophages to kill tumour cells and pathogens (fungi, virus, parasites and bacteria) • produces large amount of NO which causes hypotension during septic shock • de novo expression upon exposure to pro-inflammatory cytokines	macrophages, neutrophils, megakaryocytes, endothelial cells, vascular smooth muscle cells, cardiac myocytes, endocardial endothelial cells, chondrocytes, keratinocytes, respiratory and retinal pigment epithelial cells, cerebellar granular cells, glial cells, fibroblasts, hepatocytes, islets of Langerhans

C) Formation of peroxynitrite

Peroxynitrite ($ONOO^-$) is an important chemical species which may account for some of the cytotoxic effects of NO. In 1990, Beckman et al demonstrated that NO combines with $O_2^{-\cdot}$ to form $ONOO^-$ ($k = 6.7 \times 10^9\ M^{-1}\ s^{-1}$), at a rate three-fold faster than the dismutation of $O_2^{-\cdot}$ by superoxide dismutase (Figure 1.2, Rx. 1). Peroxynitrite has a pKa of 6.8 at 25°C (Radi et al, 1991a) and is therefore rapidly protonated at physiological pH to form peroxynitrous acid (Beckman et al, 1990) (Figure 1.2, Rx. 2). It can be formed under biological conditions where both NO and $O_2^{-\cdot}$ are generated, such as by activated macrophages which are a major source of $ONOO^-$ (Ischiropoulos et al, 1992). The other potential loci in vivo where simultaneous generation of $O_2^{-\cdot}$ and NO may cause formation of peroxynitrite are: stimulated neutrophils (Carreras et al, 1994), macrophages (Ischiropoulos et al, 1992), neuronal cells (Lafon-Cazal et al, 1993) and vascular endothelial cells (Kooy and Royall, 1994).

Peroxynitrous acid is highly unstable and its degradation by either heterolytic or homolytic cleavage results in the formation of strong oxidants (Koppenol, 1992; Pryor and Squadrito, 1995), including nitronium ion (NO_2^+) (Figure 1.2, Rx. 3) and an intermediate with hydroxyl radical character (Figure 1.2, Rx. 4). There is evidence that $ONOO^-$ or its degradation products damages cell membranes and proteins (Beckman et al, 1990; Koppenol et al, 1992; Yu et al, 1994). The peroxynitrite anion itself reacts rapidly with cysteine and methionine residues (Radi et al, 1991a; Moreno and Pryor, 1992) mediates oxidation of deoxyribose (Beckman et al, 1990) and DNA (King et al, 1992).

Production and fate of peroxynitrite

Formation:		
Rx. 1:	$^{-}$:O-O• + •NO ⇒ $ONOO^-$ ($k = 6.7 \times 10^9\ M^{-1}\ sec^{-1}$)	*peroxynitrite*
At physiological pH:		
Rx. 2:	$ONOO^- + H^+ \Rightarrow$ ONO-OH	*peroxynitrous acid*
Fates of peroxynitrous acid:		
Rx. 3:	ONO-OH ⇒ NO_2^+ + OH^- *nitronium ion*	*(heterolytic cleavage)*
Rx. 4:	ONO-OH $\overset{Cu/Fe}{\Rightarrow}$ "•NO_2 ••• •OH"	*(homolytic cleavage)*

Figure 1.2: Production and fate of peroxynitrite ($ONOO^-$) is shown in 4 different reactions (Rx.): Rx. 1 shows formation of $ONOO^-$ from nitric oxide (NO) and superoxide anion ($O_2^{-\cdot}$). In Rx. 2 protonation of $ONOO^-$ cause formation of ONOOH. Heterolytic and homolytic cleavage of ONOOH form nitronium ion (NO_2^+) and hydroxyl ion ($OH^\cdot$) respectively which are shown in Rx. 3 and 4.

Peroxynitrite has been shown to induce lipid peroxidation (Radi et al, 1991b), inhibits mitochondrial electron transport (Radi et al, 1994), inactivates amiloride sensitive sodium channels (Bauer et al, 1992), α1-antiproteinase (Moreno and Pryor, 1992) and aconitase (Castro et al, 1994). Both $ONOO^-$ and $\cdot NO_2$ react with tyrosine to form nitrotyrosine and dityrosine (Ischiropoulos et al, 1992b; Van der Vliet et al, 1994). It has also been suggested that $ONOO^-$ mediates tissue injury in immunocomplex-stimulated pulmonary edema (Mulligan et al, 1991), pulmonary emphysema (Moreno and Pryor, 1992), neurotoxicity (Cazevielle et al, 1993), atherogenesis (Darley-Usmar et al, 1992) and microbial killing (Denicola et al, 1993). Thus, $ONOO^-$ is increasingly recognized as a biologically generated reactive and toxic species.

It has been suggested that $ONOO^-$ may be responsible for ischemia reperfusion (Schulz and Wambolt, 1995; Naseem et al, 1995) and hypoxia-reoxygenation injury (Mathies et al, 1992) of the heart, although direct evidence for this has not been shown. Ischemia itself stimulates an increase in NO formation in the heart, (Depré and Hue, 1994; Zweier et al, 1995; Node et al, 1995) and during reperfusion after controlled coronary hypoperfusion in dogs, its breakdown products in plasma, nitrite and nitrate, remain elevated (Node et al, 1995). The conditions at reperfusion may be ideal for the release of excess quantities of NO in the heart. The concomitant release of both NO and $O_2^{-\cdot}$ at reperfusion may be deleterious to heart function (Figure 1.3). The change in shear stress during reintroduction of flow at reperfusion is a strong stimulus for NO release from the vascular endothelium as seen in the reactive hyperaemic response in the coronary circulation which is blocked by NOS inhibitors (Kostic and Schrader, 1992).

Possible mechanism of peroxynitrite formation

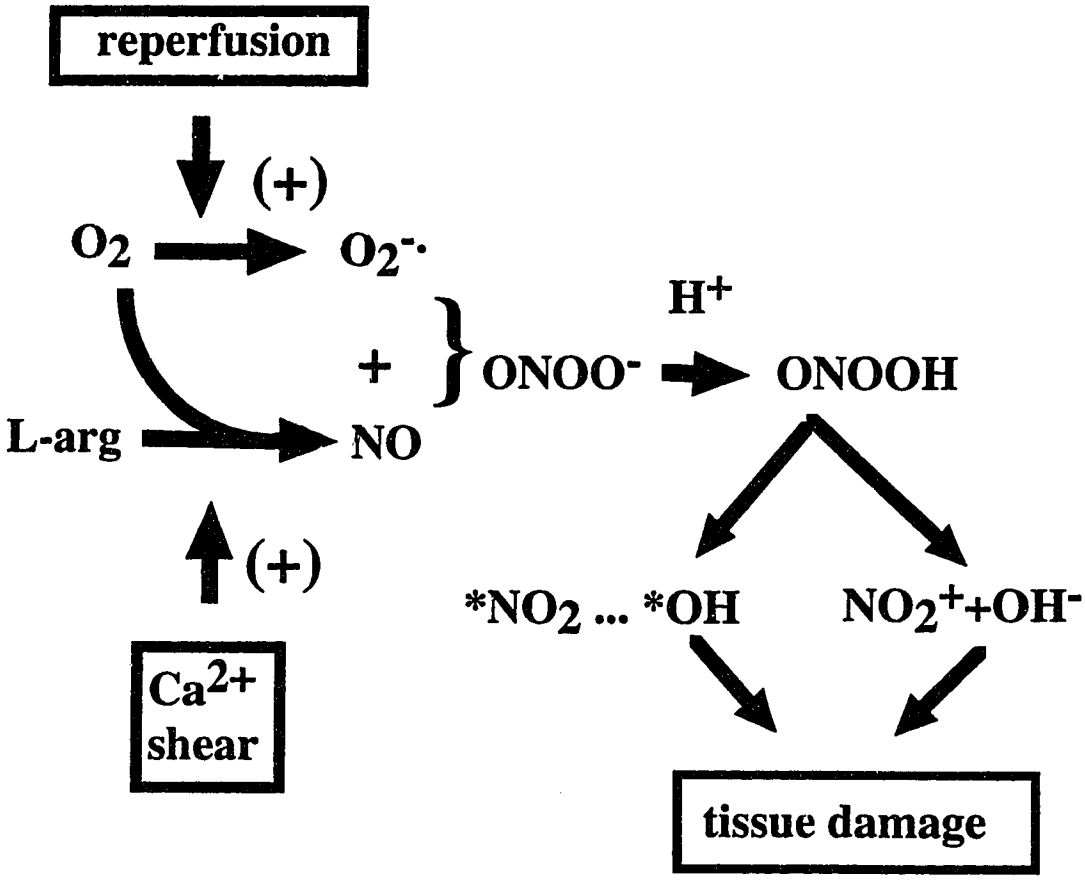


Figure 1.3: Schematic presentation of possible mechanism of peroxynitrite formation during myocardial ischemia reperfusion. At reperfusion, change in shear stress, rise in Ca^{2+}, reintroduction of O_2 thermodynamically favor the formation of nitric oxide (NO) from L-arginine (L-arg). NO rapidly reacts with superoxide anion ($O_2^{-}\cdot$) to form peroxynitrite ($ONOO^-$). $ONOO^-$ protonates to form peroxynitrous acid (ONOOH) which then decomposes homolytically and heterolytically and causes tissue damage.

Moreover, the reintroduction of oxygen should thermodynamically favour the formation of NO from L-arginine. Also the rise in myocardial cytoplasmic Ca^{2+} which occurs during ischemia (Barry, 1991) could stimulate NO formation by Ca^{2+}-dependent NOS. The endothelial NOS present in cardiac myocytes and the coronary and endocardial endothelium could contribute to a burst of NO at reperfusion.

Ischemia also induces production of both intracellular $O_2^{-\cdot}$, which can escape through anion channels to the extracellular space (Lynch and Fridovich, 1978), and extracellular $O_2^{-\cdot}$ may be produced by circulating neutrophils and xanthine oxidase released from the liver (Yokoyama et al, 1990). NO rapidly reacts with $O_2^{-\cdot}$ both intracellularly and in the vascular lumen to form $ONOO^-$ (Blough and Zafirou, 1985; Beckman et al, 1990). Because the rate of $ONOO^-$ formation depends upon the product of $O_2^{-\cdot}$ and NO concentrations, each tenfold increase in their individual concentrations results in a hundredfold increase in the rate of $ONOO^-$ production (Beckman et al, 1990). Thus, the earlier concept that oxygen derived radical-associated acute stunning during reperfusion of the ischemic heart, which was originally considered to be mediated by $O_2^{-\cdot}$ and Fenton chemistry to form other more toxic free radicals, may occur through the acute production of $ONOO^-$ from the formation of both $O_2^{-\cdot}$ and NO at reperfusion.

Reaction of peroxynitrite with L-tyrosine

Peroxynitrite can be detected by its reaction with L-tyrosine to generate a fluorescent product, dityrosine (Vliet et al, 1994), which can be detected either by spectrophotofluorometry or high performance liquid chromatography. The mechanism of dityrosine formation includes the generation of a tyrosyl radical, radical isomerization followed by diradical reaction and finally enolization (Giulivi and Davies, 1994). Dityrosine was first recognized 30 years ago (Gross and Sizer, 1959) and can be found in residues of several proteins as a product of: a) UV radiation of calmodulin and troponin C (Malencik et al, 1990), b) exposure of insulin and urease to oxidant species (ie. bolus addition of H_2O_2 in a molar ratio of 10:1) (Amado et al, 1994), and c) incubation of lysozyme with peroxidases (Amado et al, 1994). Evidence suggests that oxidative modification is explicitly the common element involved in dityrosine formation (Giulivi and Davies, 1994). Hence, we applied the reaction of L-tyrosine with $ONOO^-$ to form dityrosine as a method in an isolated rat heart model to detect whether $ONOO^-$ may be released as a result of ischemia and reperfusion.

D) Protection against myocardial ischemia/reperfusion injury

Superoxide dismutase

Superoxide dismutase (SOD), discovered by McCord and Fridovich (1969), represents

a family of enzymes which by dismutation eliminates $O_2^{-\cdot}$ (see Figure 1.1, Rx. b) from biological systems (see review by Omar et al, 1992). A number of studies with spin-trapping probes confirm that a variety of free radicals are generated in the reperfused heart (Zweier et al, 1988; Bolli et al, 1989). Moreover, if hearts are perfused with SOD, all trapped radical signals are attenuated, suggesting that they all are derived secondarily from superoxide or the spin traps only detect a subset of free radicals (Zweier et al, 1987).

However, the role of SOD in protecting the heart from myocardial ischemia-reperfusion injury has been controversial. SOD has been reported to be protective in stunned canine myocardium (Myers et al, 1985; Przyklenk and Kloner, 1986; Gross et al, 1986), however all of these studies employed less than 20 min of ischemia. In contrast, subsequent studies (Nejima et al, 1989; Przyklenk and Kloner, 1989) reported no protective effects of the combination of SOD and catalase on the functional recovery of dog hearts subjected to 90 and 120 min of ischemia, respectively. Also SOD was shown to have no protective effect on infarct size following ischemia and reperfusion (Patel et al, 1990; Nejima et al, 1989; Przyklenk and Kloner, 1989). Also a bell shaped dose response has been observed, where SOD at higher concentrations loses its effectiveness and may enhance the extent of reperfusion injury (Bernier et al, 1989; Omar et al, 1991). Mao et al (1993) demonstrated that in presence of very low concentrations of Fe^{2+}, increasing amounts of SOD can result in the production of excess amounts of hydroxyl radicals as monitored by spin-trapping techniques using electron paramagnetic resonance. They also observed that SOD-catalse conjugates results in as inhibition of the Fenton

reaction in an in vitro system and offers much greater protection in an isolated working heart model of ischemia-reperfusion injury. There are other possible explanations as to why SOD failed in some animal models: SOD may have failed because of subsequent formation of H_2O_2, which has shown to cause in activation of glyceraldehyde-3-phosphate-dehydrogenase in an isolated rat heart model perfused with low concentrations of H_2O_2 (Chatham et al, 1989). Moreover, SOD may not have ready access to critical compartments in the organ, or superoxide may simply not contribute to myocardial dysfunction and tissue necrosis (Omar et al, 1992). For example, Cu,Zn-SOD has a relatively short half life in the plasma of 6-10 min (McCord and Wong, 1979) and has a negative charge at physiological pH (Omar et al, 1992); both of these factors could reduce the delivery of the enzyme to sites of oxygen radical generation. Its high molecular weight (30-50 kD) makes it membrane impermeable (Omar et al, 1992). Recent research has focused on the development of SOD mimetics of low-molecular weight and which are positively charged, so that they are membrane permeable. Black et al (1994) demonstrated the protective effects of a synthetic, manganese based organic SOD mimetic (SC-52608) in a canine model of regional myocardial ischemia and reperfusion.

Nitric oxide synthase inhibitors

a) in vitro

In vitro experimental findings have demonstrated that N^G-monomethyl-L-arginine (L-

NMMA), an analog of L-arginine, is a competitive inhibitor of the NOS (Palmer and Moncada, 1989; Mayer et al, 1989; Moncada et al, 1991). L-NMMA was shown to inhibit the formation of NO in endothelial cells (Palmer et al, 1988) and vascular tissues (Rees et al, 1989). This compound has been an effective agent in the study of the biological implication of the L-arginine:NO pathway in the cardiovascular system (Moncada et al, 1991). L-NMMA induced an endothelium-dependent constriction of rabbit aortic rings, indicating that there is a continuous release of NO which maintains a dilator tone in this tissue (Palmer et al, 1988). Furthermore, L-NMMA inhibited endothelium-dependent relaxation induced by acetylcholine and substance P (Palmer et al, 1988; Rees et al, 1989), which could be reversed by L-arginine but not D-arginine (Rees et al, 1989).

Findings that endocardial endothelial cells release a factor like EDRF which showed negative inotropic properties in the isolated papillary muscle preparation (Smith et al, 1991) and that these cells possess Ca^{2+}-dependent NOS activity (Schulz et al, 1991), first suggested that NO plays a significant role in myocardial contractility. The physiological release of small quantities of NO in the heart provides for the maintenance of coronary vasodilator tone, inhibition of platelet aggregation and the adhesion of neutrophils and platelets to the vascular endothelium (see review by Moncada and Higgs, 1993). Furthermore, NO has direct negative inotropic and chronotropic effects on cardiac muscle (see review by Schulz and Triggle, 1994). Schulz and Wambolt (1995) demonstrated the potential detrimental role of NO in myocardial ischemia reperfusion injury using L-NMMA in isolated perfused rabbit hearts. Hearts were subjected to global, no-flow

ischemia and it was shown that L-NMMA and another competitive inhibitor of NOS, L-N^G-nitro-L-arginine methyl ester (L-NAME), improved the recovery of mechanical function during reperfusion. The protective action of L-NAME was reversible with excess L-arginine, but not D-arginine, suggesting that their effect was due to inhibition of NO synthase (Schulz and Wambolt, 1995). Inhibitors of NO synthase have been found to be protective in different in vitro models of myocardial ischemia and reperfusion injury (Naseem et al, 1995; Depré et al, 1995; Zweier et al, 1995 and Woolfson et al, 1995). Inhibitors of NO synthase have also been found to be protective in an in situ model of myocardial ischemia and reperfusion injury in the blood perfused heart (Patel et al, 1993). How inhibition of NO production protects the heart from reperfusion injury is yet unclear, with both adenosine-dependent mechanisms (Woolfson et al, 1995) and an improvement in glucose metabolism during ischemia (Depré et al, 1995) indicated. There are also studies suggesting that NOS inhibitors are either not protective or potentially worsen recovery of function (Beresewicz et al, 1995). This points to a protective role of endogenous NO in preventing I/R injury (see below).

A. In vivo

In anesthetized rabbits, intravenous adminstration of L-NMMA, but not D-NMMA, induces an increase in blood pressure that can be reversed by L-arginine but not D-arginine (Rees et al 1989). L-NMMA has also been shown to cause an increase in blood pressure in anesthetized rats (Whittle et al, 1989). This compound when infused in human brachial artery also attenuates vasodilation induced by acetylcholine (Vallance et

al, 1989a). In the rat gastric microcirculation, L-NMMA but not D-NMMA reduces mucosal blood flow which was reversed by L-arginine (Pique et al, 1989). The available evidence, therefore, indicates that the function of cardiovascular system is constantly dependent on the generation of NO.

Exogenous Nitric Oxide Donors

In addition to the protective action of NOS inhibitors, subvasodilatory concentrations of NO donors (Siegfried et al, 1992) or L-arginine (Weyrich et al, 1992) are known to ameliorate cardiac injury following regional ischemia and reperfusion in vivo. Schulz and Wambolt (1995) showed a partial protection with L-arginine in myocardial ischemia-reperfusion injury of isolated rabbit hearts. The mechanism by which L-arginine and NO donors are protective is still unresolved. In vivo studies (Siegfried et al, 1992; Weyrich et al, 1992) suggest that the NO provided by NO donors or by the metabolism of L-arginine to NO by NOS, inhibits neutrophil and/or platelet activation. Recently, specific concentration-dependent protection from the deleterious actions of $ONOO^-$ and other free radicals has been shown by the use of NO or NO donor compounds such as the nitrosothiols. Nitrosothiols prevented the impairment of endothelium-dependent vasodilation in isolated perfused hearts caused by infusion of $ONOO^-$ (Villa et al, 1994) and also inhibited stimulation of platelet aggregation by $ONOO^-$ (Moro et al, 1994). Moreover, $ONOO^-$-induced lipid peroxidation (Rubbo et al, 1994) and damage to fibroblast cultures (Wink et al, 1994) was antagonized by NO donors. It was suggested that exogenously provided NO protects from $ONOO^-$-mediated damage by terminating

$ONOO^-$-induced lipid radical chain propagation (Rubbo et al, 1994) and/or by decreasing intracellular Ca^{2+} levels by stimulating cGMP production (Nakashima et al, 1986). During myocardial reperfusion, concomitant production of NO and $O_2^{-\cdot}$ to form $ONOO^-$ can be detrimental, yet the provision of exogenous NO through NO donors may overcome some of the harmful effects of $ONOO^-$.

E) Conclusion

It is unknown whether $ONOO^-$ may be formed during myocardial ischemia and reperfusion injury. This study suggests that the generation of $ONOO^-$ may contribute to the pathogenesis of ischemia-reperfusion injury in isolated rat hearts. Pharmacological modulation of $ONOO^-$ or antagonizing the cytotoxic actions of $ONOO^-$ cause improve recovery of myocardial function following ischemia reperfusion injury.

F) Thesis hypothesis and objectives

Thesis hypothesis:

- acute myocardial dysfu[illegible]owing reperfusion of the ischemic heart involves the acute release of NO and $O_2^{\bullet-}$ forming $ONOO^-$

Thesis objectives:

- to detect the release of $ONOO^-$ during ischemia-reperfusion in the isolated heart

- to determine the dose-response effect of a NO synthase inhibitor in preventing ischemia-reperfusion injury and whether inhibition of NO synthase reduces the signal related to $ONOO^-$ formation

- to determine the effects of agents which reduce superoxide levels on improving myocardial function following ischemia and reperfusion as well as their effects o $ONOO^-$ formation

- to determine effects of an exogenous NO donor from ischemia-reperfusion injury of the heart and its effects [illegible] $ONOO^-$ formation

CHAPTER II

MATERIALS AND METHODS

A) Materials

N^G-monomethyl-L-arginine acetate (L-NMMA) was a gift from the Wellcome Research Laboratories (Beckenham, UK). S-nitroso-N-acetyl-D,L-penicillamine (SNAP) was purchased from Alexis Corporation, San Diego, CA. Bovine Cu-Zn superoxide dismutase and catalase were purchased from Boehringer Mannheim Laval, Quebec. Latex balloons (size 4) were purchased from Radnoti Glass Technology, Monrovia, CA. Mn(III) tetrakis (4-benzoic acid) porphyrin (MnTBAP) was kindly provided by Dr. Csaba Szabo, Children's Hospital Medical Center, Cincinnati, OH. The following reagents used for the experiments were obtained from VWR Canlab or Sigma: D-glucose, KCl, NaCl, $CaCl_2$, KH_2PO_4, $MgSO_4$, L-tyrosine, $NaNO_2$, NaOH, $NaHCO_3$, H_2O_2. $Mn(IV)O_2$ was purchased from Aldrich Chemical Company, Milwaukee, WI.

B) Animal model

Adult male Sprague-Dawley rats weighing 250-300g were used throughout the studies. These animals were maintained in the Health Sciences Laboratory Animal

Services of the University of Alberta. Animals were provided normal rat lab chow and water *ad libitum.* This investigation conforms with the *Guide to the care and use of experimental animals* published by the Canadian Council on Animal Care (revised 1993).

C) Preparation of peroxynitrite and decomposed peroxynitrite

Both peroxynitrite and decomposed peroxynitrite were prepared by the method as described previously (Halfpenny and Robinson, 1952; Villa et al 1994). The entire procedure was done in a cold room under reduced light conditions to decrease the likelihood of $ONOO^-$ decomposition. An ice-cold aqueous solution of $NaNO_2$ (2 M) and a solution containing nitric acid (11.1 M) and H_2O_2 (8.2 M) were filled into two separate 10 ml syringes connected with a Y-piece tubing. The syringe contents were simultaneously discharged (< 1 sec) into a rapidly stirring solution of 6 ml of ice-cold NaOH (4.2 M). Excess H_2O_2 was removed by treating the mixture with 2 g of granular $Mn(IV)O_2$ (60-230 mesh) for 2 min with stirring and the solution was filtered through Whatman #54 filter paper. The concentration of $ONOO^-$ was determined by UV spectroscopy (Figure 2.1) using a Beckman DU 65 spectrophotometer (λ_{max} = 302 nm, ε = 1670 L x M^{-1} x cm^{-1}; Hughes and Nicklin, 1968). The final concentration of $ONOO^-$ achieved in this process varied between 130-200 mM. Decomposed $ONOO^-$ was prepared in the similar way, except that the solutions of $NaNO_2$ and H_2O_2 in nitric acid were not captured in the NaOH solution. After 5 minutes, at which time $ONOO^-$ was completely decomposed, 6 ml of NaOH (4.2 M) was then added. This solution was also treated with

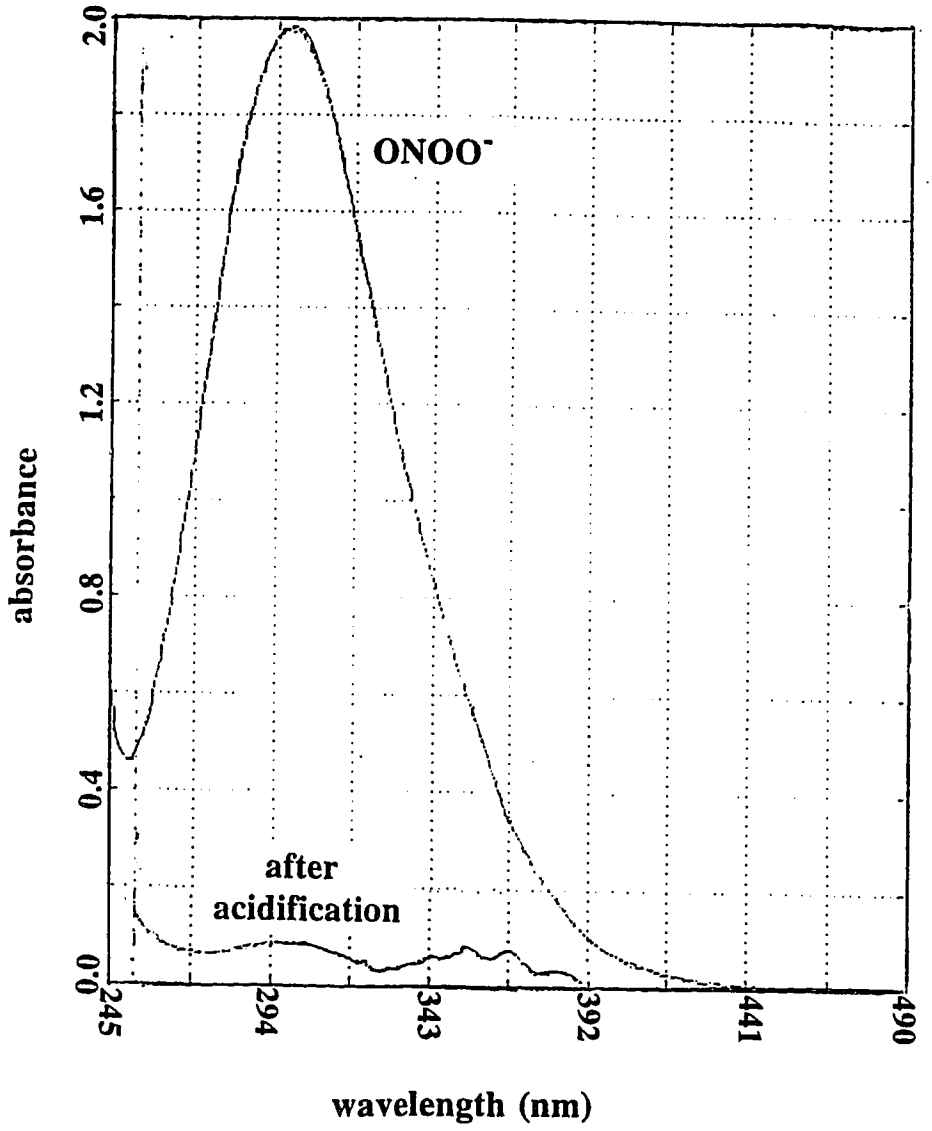


Figure 2.1: Ultraviolet absorbance spectrum of peroxynitrite ($ONOO^-$). Top trace shows spectrum of peroxynitrite solution and bottom trace shows its decomposition following acidification to pH <2. Solution of $ONOO^-$ (200 mM approx.) synthesised as in methods was diluted 1:200 with deionized water for UV analysis to a final volume of 1.0 ml. Acidified solution of $ONOO^-$ was prepared by adding 20 µl of 2 M HCl.

$Mn(IV)O_2$ to remove excess H_2O_2 and filtered. The absence of $ONOO^-$ in this solution (referred to as "decomposed $ONOO^-$") was verified by UV spectroscopy. The stock solutions of $ONOO^-$ and decomposed $ONOO^-$ were aliquoted and stored at -80°C for up to two weeks.

D) Synthesis of dityrosine

Dityrosine was synthesized in the laboratory of Dr. Brian Dunford (Department of Chemistry, University of Alberta), according to a published method (Amado et al, 1984; Heincker et al, 1993) and its purity was verified by high performance liquid chromatography (Giulivi et al, 1993; Giulivi et al 1994).

E) Measurement of dityrosine by fluorescence spectroscopy

Peroxynitrite reacts with L-tyrosine to form a fluorescent product, dityrosine (Vliet et al, 1994). In order to check the sensitivity and linearity of this reaction, solutions of L-tyrosine (0.3 and 2 mM) prepared in 50 mM phosphate buffer (pH 6.0) were reacted with authentic $ONOO^-$ (10-1000 µM) to produce dityrosine. To dissolve L-tyrosine, phosphate buffer was first warmed for 5 minutes to 37°C and then L-tyrosine was added. $ONOO^-$ (60-200 µl) to give final concentrations of 0, 10, 30, 100, 300 or 1000 µM, or the equivalent volume of decomposed $ONOO^-$ was added to the top of the 400 µl of L-tyrosine solution and vortexed immediately. The reaction mixture was incubated for 30

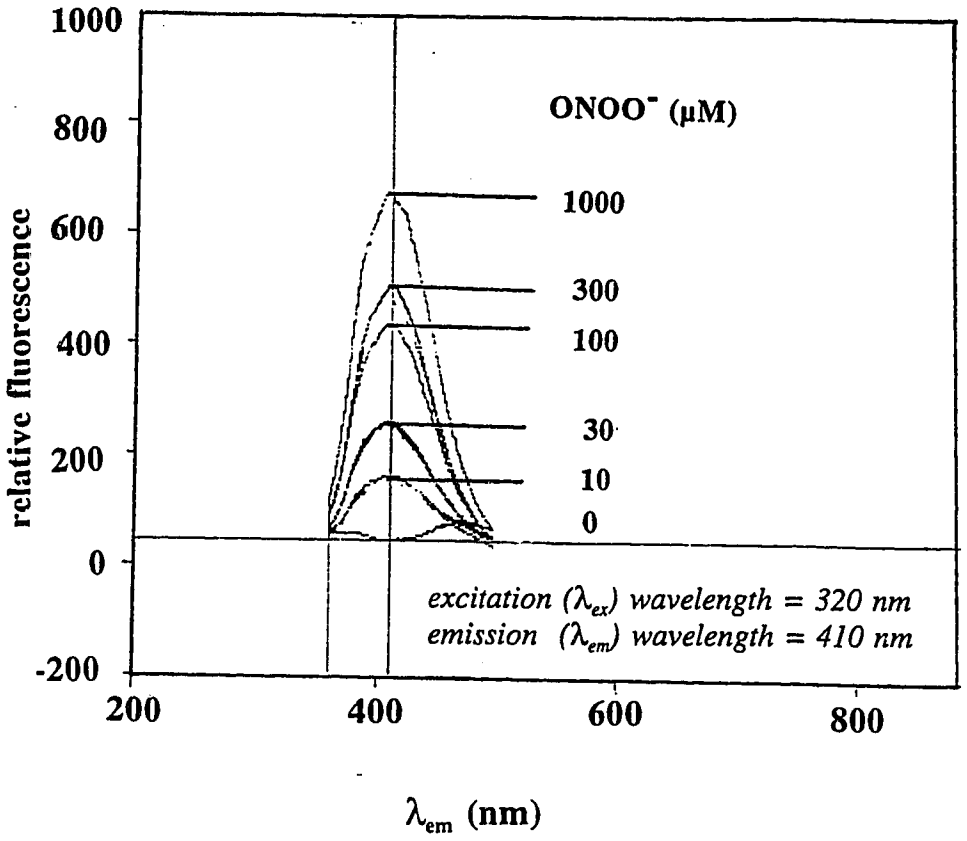


Figure 2.2: Typical spectrophotofluorometric spectra of dityrosine produced as a result of reaction of 2 mM L-tyrosine prepared in 50 mM phosphate buffer (pH 6) incubated for 30 min at 37°C with different concentrations of $ONOO^-$. Excitation slit width was 5 nm and emission slit width was 10 nm.

min at 37°C. Samples were then placed on ice and protected from light until analysis. The formation of dityrosine in the incubate was analyzed by fluorescence spectroscopy (λ_{ex} = 320 nm and λ_{em} = 410 nm; slit widths: excitation slit = 5 nm and emission slit = 10 nm; Vliet et al, 1994) at room temperature using a Shimadzu RF 5000 spectrophotofluorometer. Solutions of authentic dityrosine standard (0.01-1.0 μM) prepared in either 50 mM phosphate buffer (pH 6.0) or in Krebs-Henseleit solution (pH 7.4) showed a linear relationship between fluorescence intensity and concentration as analyzed using linear regression analysis (data not shown). The detection limit for dityrosine in either buffer was 0.01 μM. A spectrophotofluorometric scan showing the relative fluorescence of dityrosine formed by reaction between peroxynitrite and L-tyrosine is shown in Figure 2.2.

F) Detection of dityrosine by high performance liquid chromatography

A sample from the same aliquot was also analyzed directly by high performance liquid chromatography (Giulivi and Davies, 1993; Giulivi and Davies, 1994) to determine its dityrosine content. Samples were chromatographed using a reverse phase Bondapak C18 column (5 μm particle size, 4.6 x 30 cm). Solvent A was methanol:water:trifluoroacetoacetic acid (25:75:0.1, v/v), and solvent B was acetonitrite:water:trifluoroacetoacetic acid (80:20:0.1, v/v). The following elution program was used (Giulivi and Davies, 1994): solvent A was run with a flow rate of 0.8 ml/min for 10 min; from 10 min until 20 min a gradient of solvent B from 0 to 4% was followed

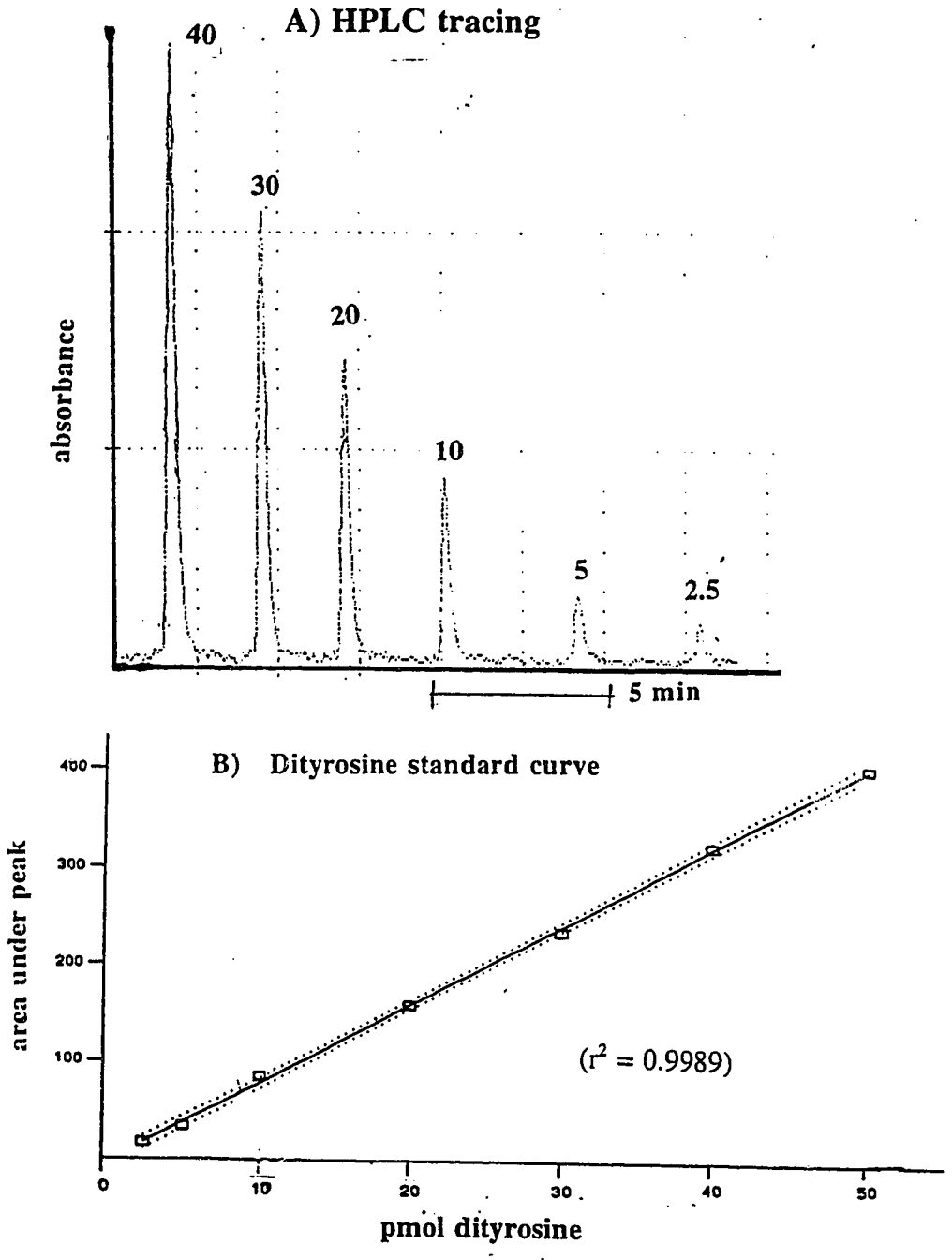


Figure 2.3: A) A typical high performance liquid chromatogram of repeated injections of different concentrations of dityrosine standard using a fluorescence detector (λ_{ex} = 315 nm; λ_{em} = 410 nm). Numbers beside peaks indicate pmol dityrosine injected. Retention time of dityrosine is 4.73 min. B) Dityrosine standard curve produced by HPLC analysis. Linear correlation between amount of dityrosine standard injected (pmol) and area under peak.

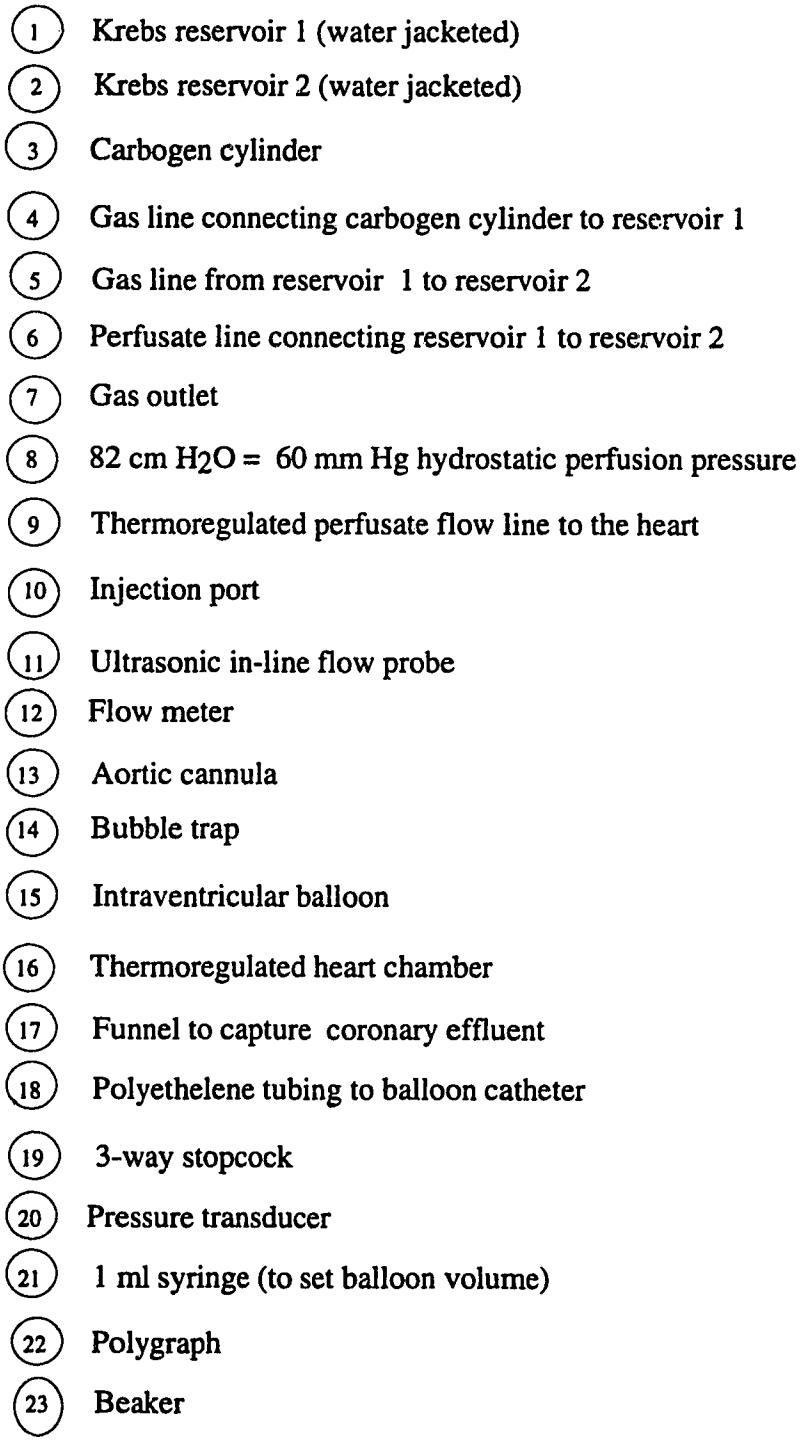
by running solvent B (100%) for a final 20 min. Detection of dityrosine was carried out using a fluoroscence detector at λ_{ex} = 315 nm and λ_{em} = 420 nm. Using these conditions dityrosine has a retention time of 4.73 min and a detection limit of 0.5 pmol. A typical chromatogram showing repeated injections of dityrosine (2.5-40 pmol) to establish a standard curve is shown in Figure 2.3.

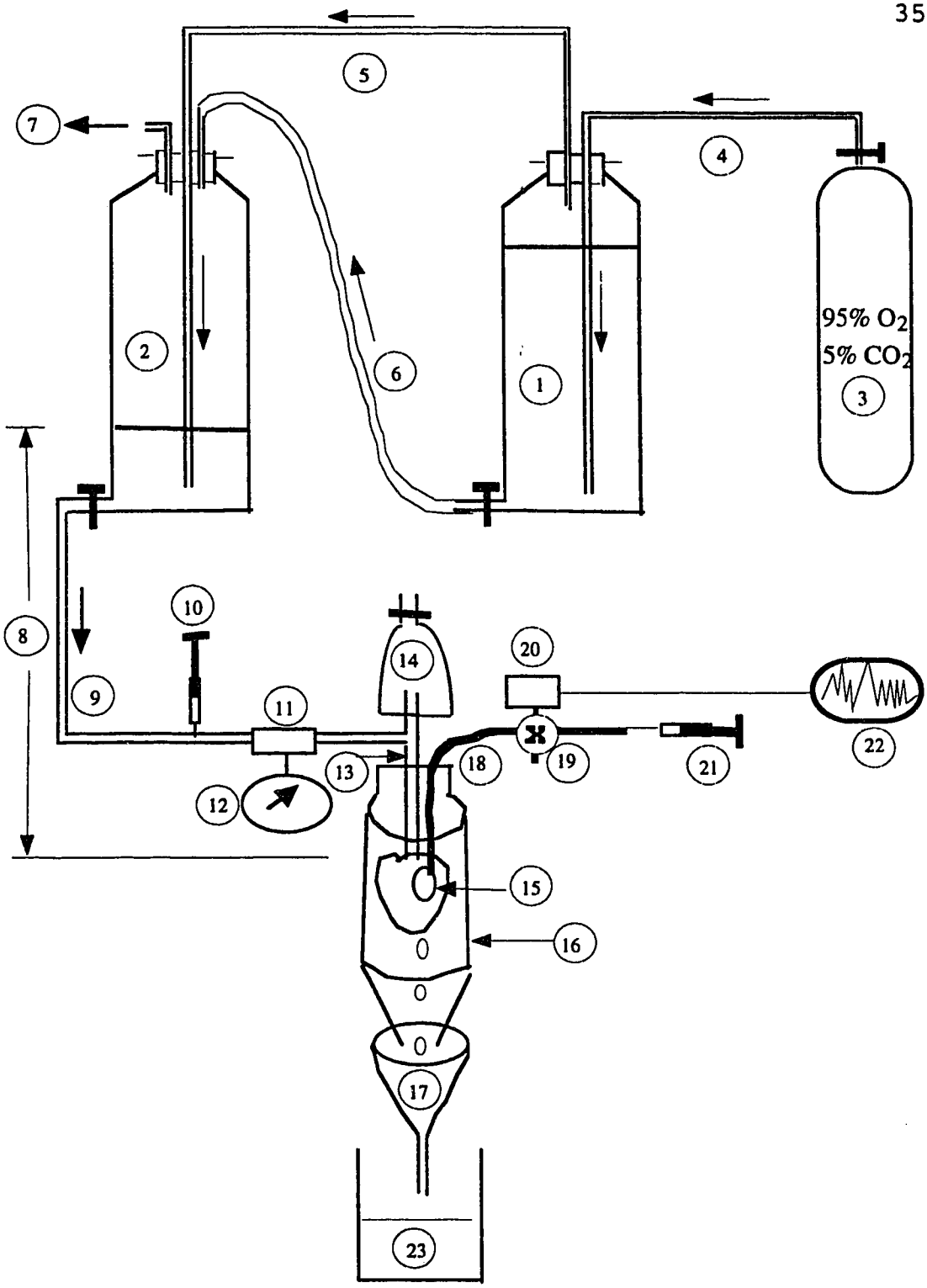
G) Heart perfusions

The method for perfusion of the isolated rat heart was derived from Langendorff (1895) (see Figure 2.4). The classical "Langendorff preparation" is a technique in which the aorta is cannulated and the coronary vessels are perfused by introducing perfusate retrogradely into the aorta. This preparation does not perform external mechanical work as the isolated working heart (Neely et al, 1967), however it does develop ventricular pressure.

The reservoir for delivery of the perfusion fluid consisted of two water-jacketed, temperature-controlled supply vessels (4L capacity each). The perfusion fluid was continuously gassed in both vessels with a mixture of 95% O_2 and 5% CO_2. Reservoir 1 was used to maintain a constant hydrostatic perfusion pressure of the perfusion fluid in reservoir 2 by continuously refilling by siphonic action reservoir 2 such that a constant fluid level was maintained. The distance between the fluid level of reservoir 2 and the base of the heart was kept constant to maintain a constant hydrostatic equivalent pressure of 60 mm Hg. Reservoir 2 was connected to a thermoregulated heart chamber by a

Figure 2.4: Schematic of the isolated Langendorff rat heart set-up




95% O_2
5% CO_2
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23

water-jacketed tubing to supply perfusion fluid to the heart. A water-jacketed glass heart chamber which sealed around the cannula served to maintain the heart at 37°C. Below the heart chamber a funnel was attached for collecting the coronary effluent.

Adult male Sprague-Dawley rats (250-300 g) were anesthetized by intraperitoneal injection of sodium pentobarbital (60 mg/kg). The chest was opened by right thoracotomy and the heart was rapidly excised and placed in ice-cold Krebs-Henseleit solution. The aorta was cannulated and retrograde Langendorff perfusion was begun with oxygenated Krebs-Henseleit solution (Krebs and Henseleit, 1932). The perfusate was maintained at 37°C and was continuously gassed with 95% O_2/5% CO_2 to give a final pH of 7.4. Perfusion pressure was maintained at 60 mm Hg (82 cm H_2O). The composition of the perfusion buffer was (in mM): NaCl (118), KCl (4.7), $MgSO_4$ (1.2), KH_2PO_4 (1.2), $CaCl_2$ (2.5), $NaHCO_3$ (25), glucose (11), EDTA (0.5) and L-tyrosine (0.3). The latter substrate reacts with $ONOO^-$ to form the fluorescent product dityrosine (Vliet et al. 1994). The perfusate was prepared on the day before the experiment with the exception of glucose and L-tyrosine which were added to the buffer on the day of the experiment. To avoid precipitation, L-tyrosine was first dissolved in 2 ml of 1 N NaOH and then added to 4 L of perfusate.

Spontaneously beating hearts were used for these studies so as to determine any potential consequences of changes in endogenous myocardial NO production on heart rate (Balligand et al, 1993). Isovolumetric left ventricular pressure was measured using a bailoon method (Gottlieb and Magnus, 1904). This setup consists of a balloon catheter attached to a mechano-electric pressure transducer. A small latex balloon fixed to a

polyethelene catheter tip (tied up with 40 silk and then sealed with water insoluble glue) was inserted through the left atrium and pushed through the mitral valve into the left ventricle (Grupp and Grupp, 1984). To measure heart rate and left ventricular developed pressure, the balloon was connected by a 3-way stopcock to a TSD 104 blood pressure transducer (Harvard Apparatus, Saint-Laurent, Quebec). The other end of the stopcock was connected to a one ml syringe filled with deionized water. To achieve an end diastolic pressure of 8-12 mm Hg, the balloon was filled using the syringe with approximately 0.1 ml of water. Special care was taken to ensure no air bubbles were filled into the balloon. To eliminate air bubbles, the balloon was let to hang downwards from the transducer so that all air bubbles could rise into the transducer dome. This process was facilitated by tapping the catheter and rapidly compressing and decompressing the balloon. By this air bubbles were also removed from the transducer dome. To fit the volume of the left ventricle of rats, balloons of 3-5 mm in diameter (when filled at zero pressure) were found to be appropriate. The transducer was connected to a Grass polygraph recorder (Model 7D, Grass Instruments, Quincy, Mass.) and heart rate and left ventricular pressure were monitored. Coronary flow was measured by an in-line ultrasonic flow probe (Model APO2N, Transonic Systems Inc.; Ithaca, NY) positioned proximal to the perfusion cannula and connected to a T206 ultrasonic blood flow meter. During ischemia changes in left ventricular pressure were continuously registered. The time to onset of ischemic contracture was defined by a 4 mm Hg increase above baseline in left ventricular balloon pressure. The increase above baseline pressure from the onset of ischemia to that determined at the end of ischemia was defined as the

magnitude of ischemic contracture.

H) Infusion of $ONOO^-$ into the isolated heart

To determine whether dityrosine could be detected in the coronary effluent as a result of the reaction of L-tyrosine with $ONOO^-$, authentic $ONOO^-$ was infused into hearts perfused aerobically with Krebs' buffer containing 0.3 mM L-tyrosine. Following a 15 minute period of equilibration, $ONOO^-$ (or decomposed $ONOO^-$) was infused using a Hamilton syringe into the aortic cannula via a sideport (50 µL over 30 seconds) in different concentrations such that the final concentration of $ONOO^-$ in the perfusate entering the heart was 10, 50 or 150 µM. A five minute period was allowed between each bolus infusion. Coronary effluent samples were collected at 15 s intervals for 2 min from the start of infusion and were kept on ice until analyzed for dityrosine by fluorescence spectroscopy.

I) Ischemia-reperfusion protocol

Hearts were subjected to an ischemia reperfusion protocol which consisted of 25 min of aerobic perfusion, 20 min of global, no-flow ischemia induced by clamping the aortic inflow line, and 30 min of aerobic reperfusion subsequent to reopening the clamp. The whole isolated heart system was water-jacketed to maintain the temperature at 37°C throughout the entire protocol. In preliminary experiments a thermistor was inserted in

the pulmonary artery to measure the temperature of the heart during the entire protocol and it was determined that it remained at 37°C throughout the experiment (data not shown). In additional series of hearts different drugs were infused using a syringe infusion pump (Model 600-000, Dover, Mass) at a rate of 115 µl/min assuming an average coronary flow rate of 12 ml/min (dilution factor = 1:100) connected to the aortic side arm cannula to give the following final concentrations:

a. N^G-monomethyl-L-arginine acetate (1-100 µM)

N^G-monomethyl-L-arginine acetate was dissolved in deionized water and infused during the final 10 minutes of aerobic perfusion and throughout the reperfusion period.

b. Superoxide dismutase (50 U/ml) and catalase (50 U/ml)

Superoxide dismutase and catalase dissolved in saline and were infused in combination during the final 5 min of aerobic perfusion and for the first 5 min of reperfusion.

c. S-nitroso-N-acetyl-D,L-penicillamine (0.02-0.2 µM)

S-nitroso-N-acetyl-D,L-penicillamine (SNAP) was first dissolved in 200 µl of 1N NaOH and then added to 4.8 ml of saline and was infused during the final 5 min of aerobic perfusion and for the first 5 min of reperfusion.

d. Mn(III) tetrakis (4-benzoic acid) porphyrin (100 µM)

Mn(III) tetrakis (4-benzoic acid) porphyrin was dissolved at first in 500 µl of 0.1N NaOH and 4.5 ml of saline added and was infused during the final 5 min of aerobic perfusion and for the first 5 min of reperfusion.

For all hearts, the coronary effluent was collected for 30 s intervals ending at 15 and 25 min of aerobic reperfusion and at 30 second intervals for the first two minutes of reperfusion and then for 30 second intervals ending at 5, 15 and 30 min of reperfusion. The samples were kept on ice until the end of the experiment, allowed to return to room temperature, and dityrosine fluorescence was measured within 15 min. Coronary flow was monitored during the collection intervals and was used to express the release of dityrosine in relative fluorescence intensity units per ml of effluent.

I) Statistical Analysis

Data were expressed as mean ± SEM of n experiments. Student's paired or unpaired t-test, or analysis of variance followed by Dunnett's test, were used to compare individual means. A value of $p<0.05$ was considered statistically si[illegible]nt.

CHAPTER III

RESULTS

A) Formation of dityrosine from authentic $ONOO^-$ and L-tyrosine

In 50 mM phosphate buffer (pH 6.0, 37°C) synthetic $ONOO^-$ (10-1000 μM) reacted with L-tyrosine (0.3 mM) in a concentration-dependent manner and revealed a fluorescent signal with λ_{ex} and λ_{em} maxima characteristic for dityrosine as previously shown (Vliet et al, 1994). The same reaction mixture, whether analysed by fluorescence spectroscopy (Figure 3.1a) or by high performance liquid chromatography (Figure 3.1b), showed a similar concentration-dependent response profile. Furthermore, there was a strong linear correlation between the amplitude of the fluorescent signal relating to dityrosine and the concentration of dityrosine determined in the same aliquot by high performance liquid chromatography (r^2 = 0.9790) (Figure 3.1c). Similar results were observed using a higher concentration of the substrate L-tyrosine (2.0 mM, Figure 3.2a-c). Dityrosine standard (0.01-1.0 μM) was analysed by fluorescence spectroscopy in either 50 mM phosphate buffer (pH 6.0) or in Krebs-Henseleit solution (pH 7.4). Identical λ_{em} maxima at 410 nm (measured at λ_{ex} = 320 nm) and an excellent linear correlation between fluorescence signal amplitude and concentration of dityrosine were obtained using either phosphate buffer (r^2 = 0.9994, Figure 3.3a) or Krebs-Henseleit

Figure 3.1: Reaction of L-tyrosine with $ONOO^-$ to form dityrosine. Concentration-dependent formation of dityrosine in 50 mM phosphate buffer (pH 6.0) containing 0.3 mM L-tyrosine after 30 min incubation at 37°C with $ONOO^-$ (●, 10-1000 μM) or decomposed (dec.) $ONOO^-$ (○), as measured by: A) fluorescence spectroscopy (n=3) or B) high performance liquid chromatography (n=3). C) Linear regression analysis between the amplitude of the dityrosine fluorescence signal measured in the incubation mixture and the mass of dityrosine detected in the same sample using high performance liquid chromatography. If no error bar is shown the error is within the height of the symbol.

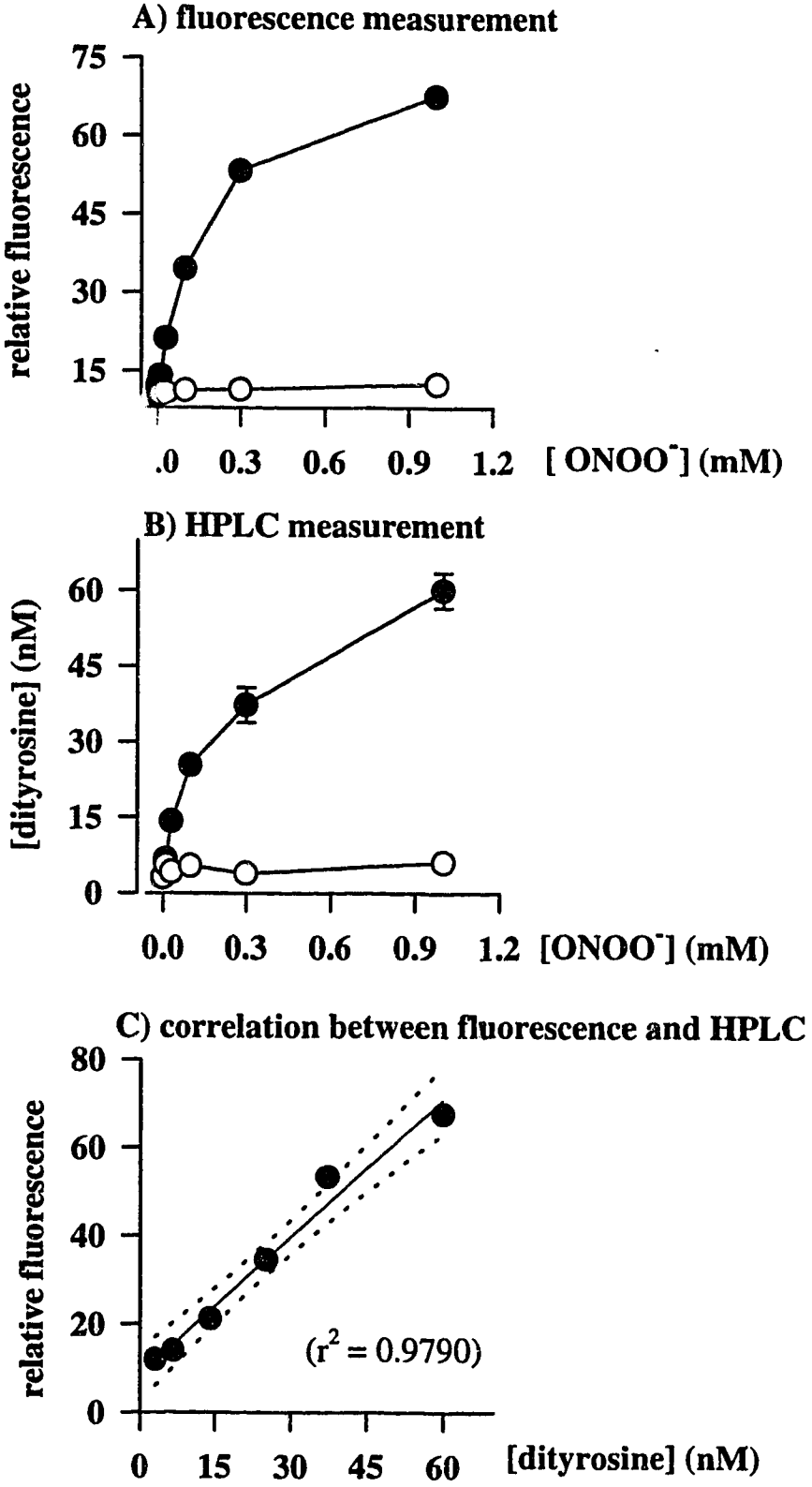
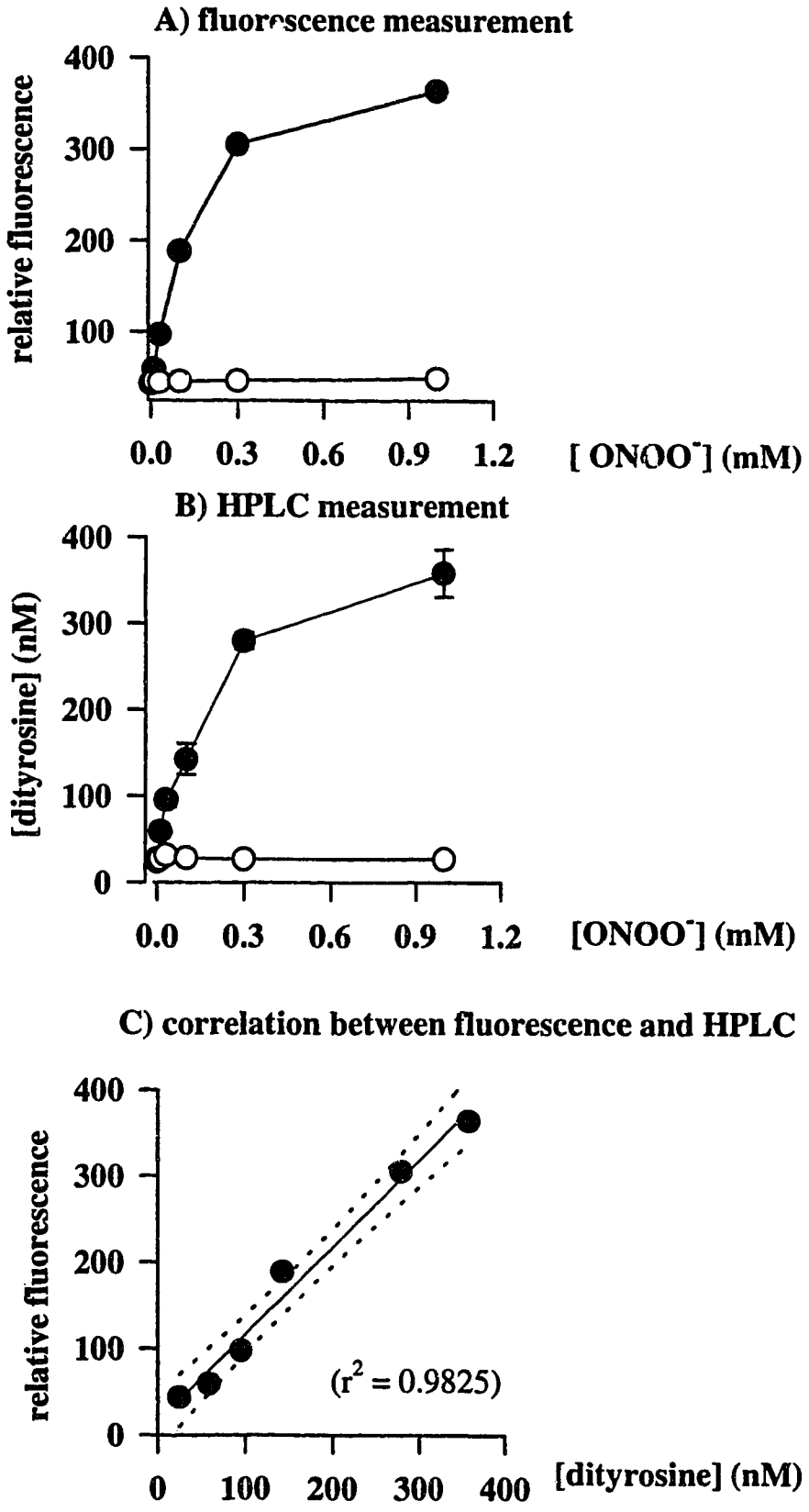

A) fluorescence measurement
relative fluorescence
75
60
45
30
15
.0
0.3
0.6
0.9
1.2
[ONOO⁻] (mM)
B) HPLC measurement
[dityrosine] (nM)
60
45
30
15
0
0.0
0.3
0.6
0.9
1.2
[ONOO⁻] (mM)
C) correlation between fluorescence and HPLC
relative fluorescence
80
60
40
20
0
(r² = 0.9790)
0
15
30
45
60
[dityrosine] (nM)

Figure 3.2: Reaction of L-tyrosine with $ONOO^-$ to form dityrosine. Concentration-dependent formation of dityrosine in 50 mM phosphate buffer (pH 6.0) containing 2.0 mM L-tyrosine after 30 min incubation at 37°C with $ONOO^-$ (●, 10-1000 μM) or decomposed (dec.) $ONOO^-$ (○), as measured by: A) fluorescence spectroscopy (n=3) or B) high performance liquid chromatography (n=3). C) Linear regression analysis between the amplitude of the dityrosine fluorescence signal measured in the incubation mixture and the mass of dityrosine detected in the same sample using high performance liquid chromatography. If no error bar is shown the error is within the height of the symbol.


A) fluorescence measurement
relative fluorescence
400
300
200
100
0.0
0.3
0.6
0.9
1.2
[ONOO⁻] (mM)
B) HPLC measurement
[dityrosine] (nM)
400
300
200
100
0
0.0
0.3
0.6
0.9
1.2
[ONOO⁻] (mM)
C) correlation between fluorescence and HPLC
relative fluorescence
400
300
200
100
0
(r² = 0.9825)
0
100
200
300
400
[dityrosine] (nM)

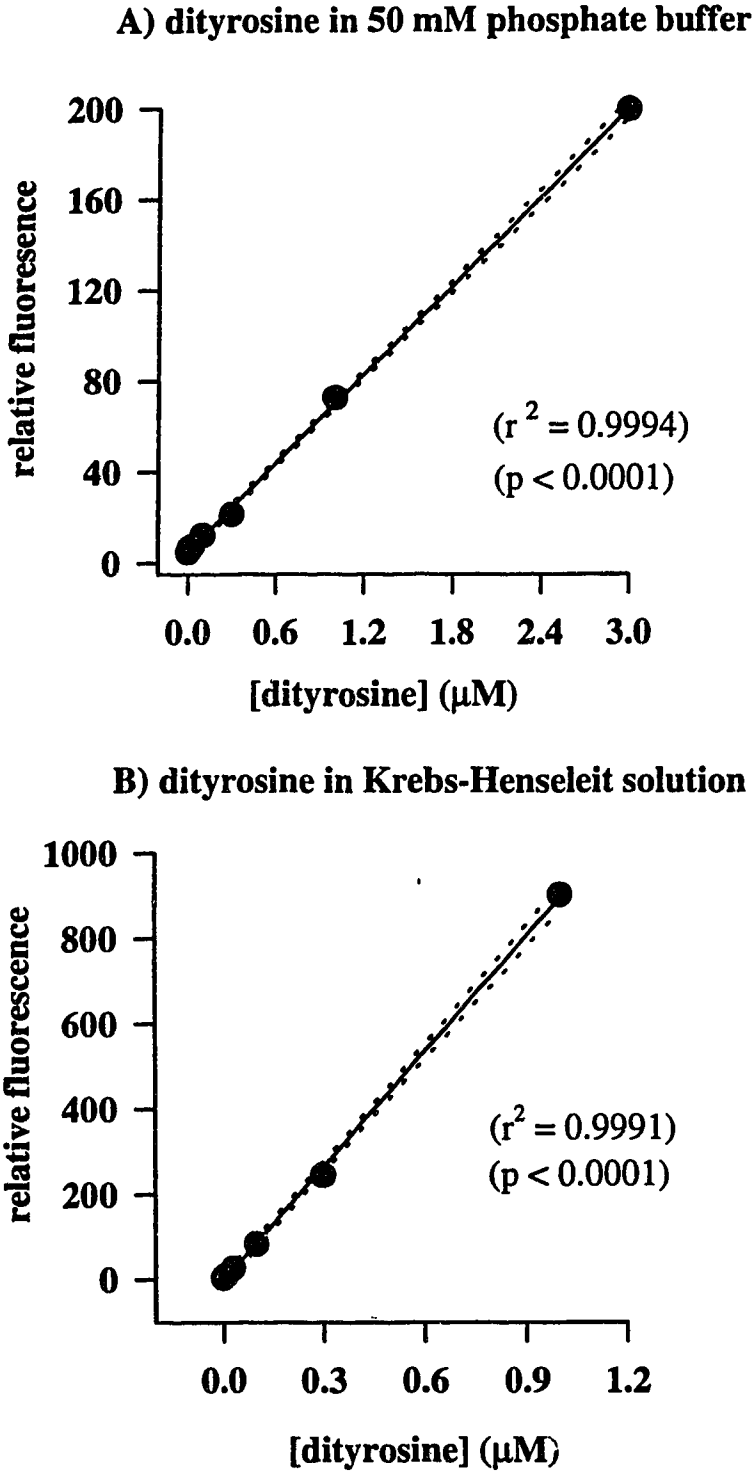


Figure 3.3: Dityrosine standard measured by fluorescence spectroscopy in: A) 50 mM phosphate buffer (pH 6.0) or B) Krebs-Henseleit solution.

solution (r^2 = 0.9991, Figure 3.3b). A greater fluorescence amplitude of dityrosine was obtained in Krebs-Henseleit solution compared to phosphate buffer. Reaction of 0.3 mM L-tyrosine with 10 mM H_2O_2 for 30 min at 37°C showed no formation of dityrosine as determined by fluorescence spectroscopy (data not shown).

B) Formation of dityrosine from exogenous $ONOO^-$

It was next determined whether L-tyrosine could be used as a detector for $ONOO^-$ formation, through the production of dityrosine, in the isolated, perfused heart preparation. Isolated rat hearts were perfused with Krebs-Henseleit buffer supplemented with L-tyrosine (0.3 mM). Synthetic $ONOO^-$ was infused through the aortic cannula to determine whether dityrosine could then be detected in the coronary effluent. As $ONOO^-$ is known to act as a vasodilator (Villa et al, 1994; Liu et al, 1994), and in order to verify the biological activity of our preparation of it, bolus doses of $ONOO^-$ (10-150 μM) were infused over 30 seconds into the aortic cannula of the isolated heart. $ONOO^-$ caused a concentration-dependent increase in coronary flow (Figure 3.4) which was rapid in onset, returned to baseline within two minutes and is consistent with the known coronary vasodilator action of $ONOO^-$ (Villa et al, 1994). In contrast, bolus infusion of decomposed $ONOO^-$ had no effect on coronary flow (Figure 3.4). Figure 3.5 shows the production of dityrosine as measured by its fluorescent signal determined in the coronary effluent which was collected during the bolus injection of $ONOO^-$ or decomposed ONOO.

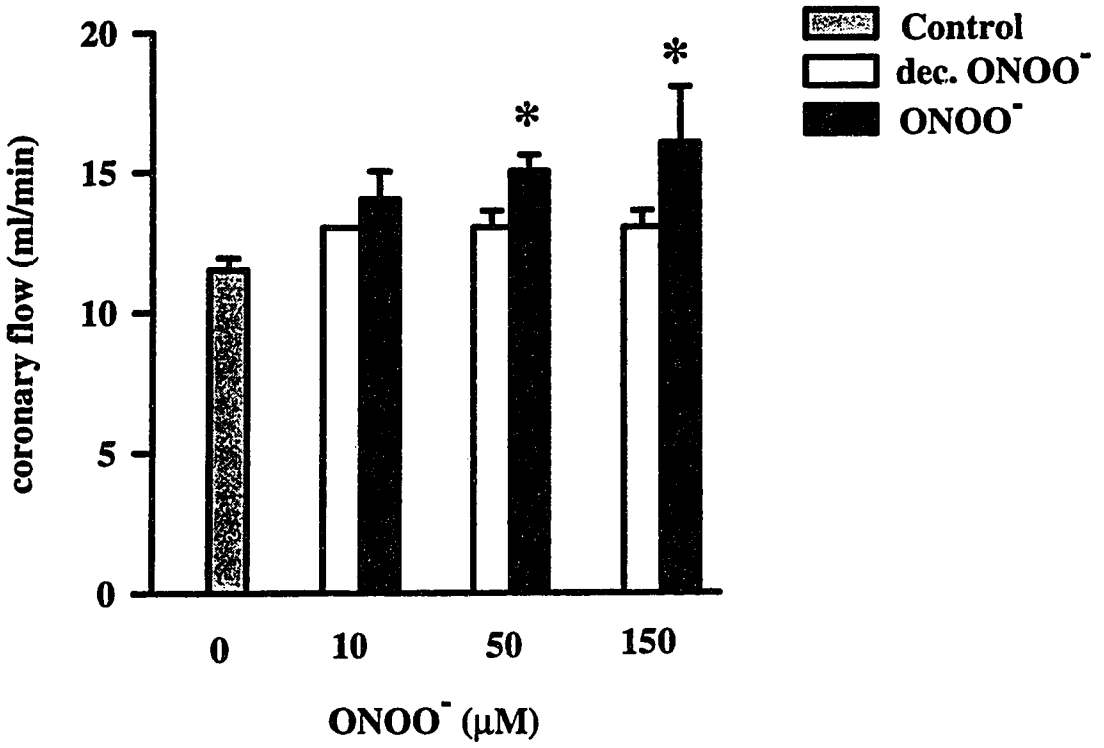


Figure 3.4: Effect of infusion of exogenous $ONOO^-$ into isolated perfused rat hearts on coronary flow. $ONOO^-$ (10-150 μM) or decomposed (dec.) $ONOO^-$ were infused as a 30 second bolus into aerobically perfused hearts (n=3). Grey bar: control baseline flow during aerobic perfusion. Open bars: peak change in flow after decomposed $ONOO^-$. Filled bars: peak change in flow after $ONOO^-$.

* $p<0.05$ versus control using Student's paired t-test.

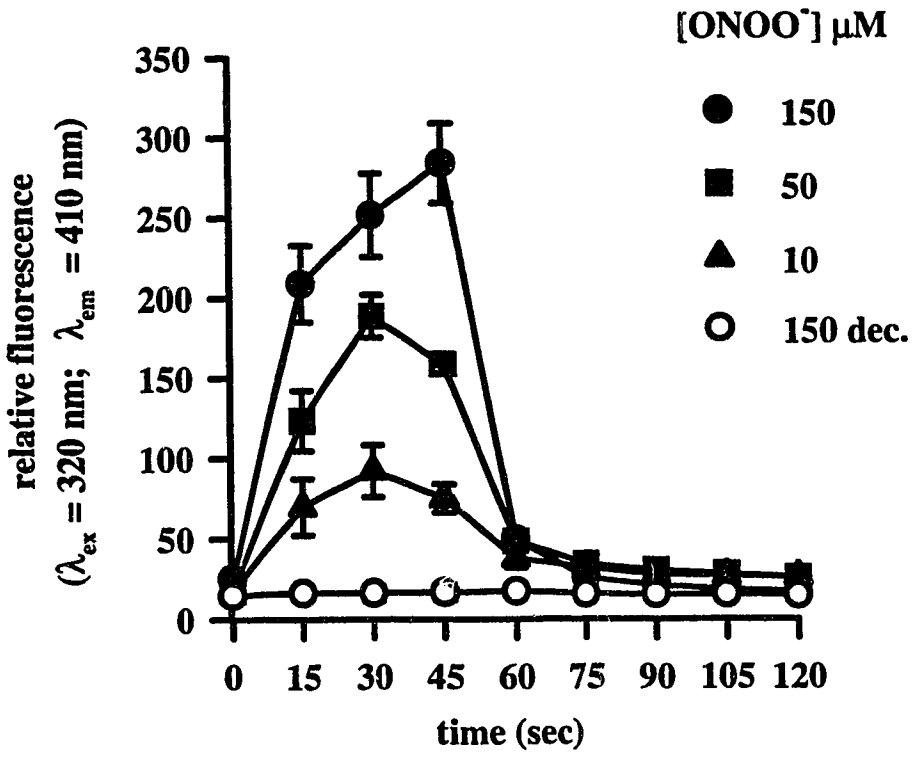


Figure 3.5: Effect of infusion of synthetic $ONOO^-$ into isolated rat hearts on the formation of dityrosine in the coronary effluent. Hearts were perfused with Krebs-Henseleit solution containing 0.3 mM L-tyrosine and coronary effluent samples were taken at 15 second intervals beginning from the start (t=0) of a 30 second bolus injection of 10, 50, or 150 μM $ONOO^-$ or decomposed (dec.) $ONOO^-$. n=3 hearts per group. If no error bar is shown the error lies within the height of the symbol.

In the concentration range of 10-150 μM, $ONOO^-$ caused a rapid and concentration-dependent increase in dityrosine fluorescence which peaked at 30-45 s following injection. In contrast, injection of decomposed $ONOO^-$ caused no change in dityrosine fluorescence (Figure 3.5).

C) Effect of L-NMMA on ischemic contracture

During ischemia in control hearts, the onset of ischemic contracture began within 15.5 ± 0.8 min from the start of ischemia (Figure 3.6a) and the magnitude of contracture rose to a maximum of 38.3 ± 4.3 mmHg (Figure 3.6b). L-NMMA delayed the time to onset of ischemic contracture so that it was significantly greater than control hearts at 10 and 100 μM (Figure 3.6a). Moreover, L-NMMA at 10 and 100 μM, but not 1 μM, significantly decreased the magnitude (Figure 3.6b).

D) Effect of L-NMMA on the recovery of mechanical function of reperfusion

L-NMMA (10 μM, but not 1 or 100 μM) caused a rapid and significant improvement in the recovery of post-ischemic mechanical function (heart rate x left ventricular developed pressure) throughout the 30 min reperfusion period compared to control hearts (Figure 3.7). Measured at 30 min reperfusion, recovery of the rate-pressure product was 10.9 ± 1.4 vs. 3.0 ± 1.5 mmHg x min^{-1} x 10^{-3} ($p< 0.05$) in 10 μM L-NMMA

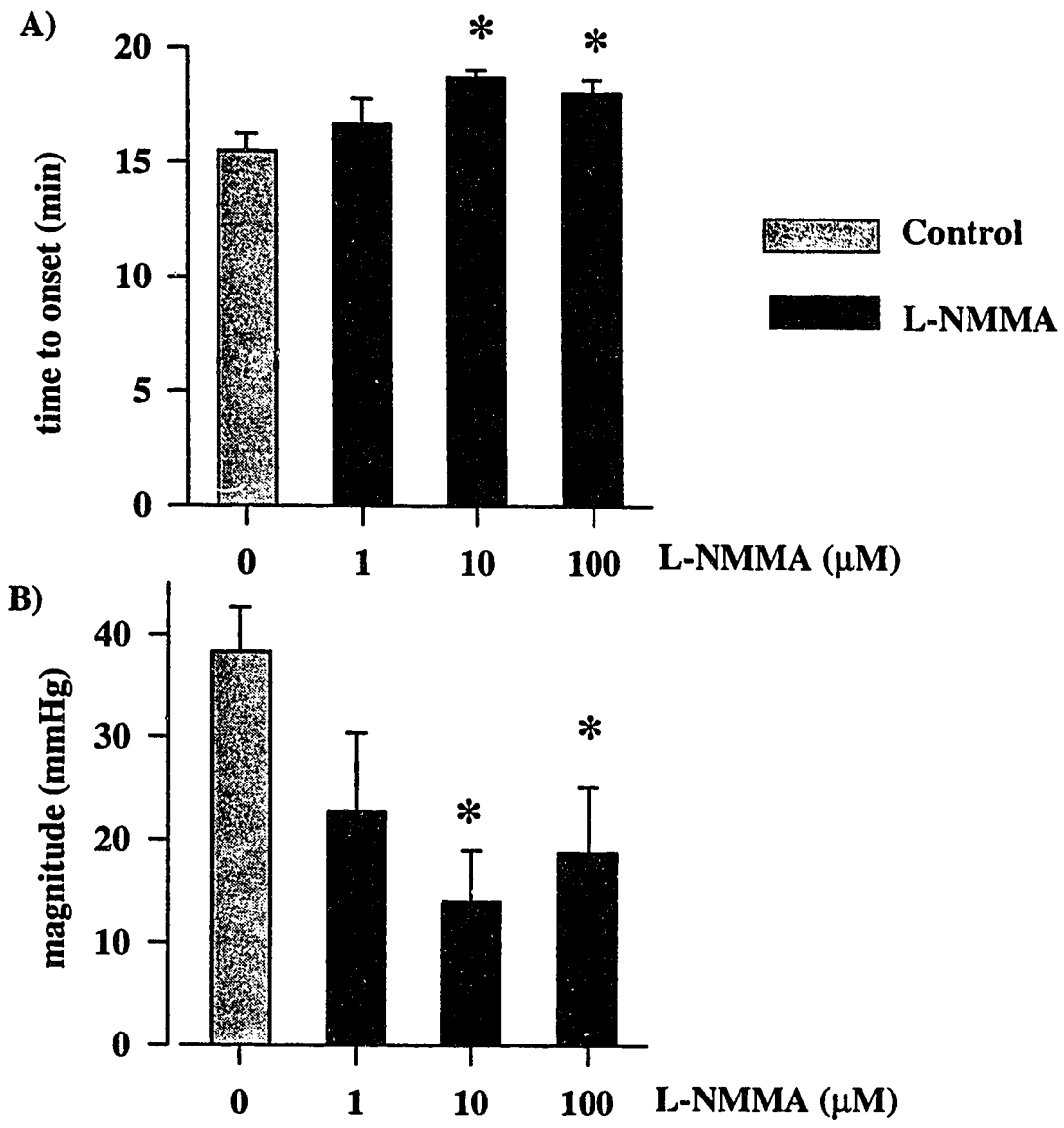


Figure 3.6: Effects of L-NMMA on the: A) time to onset and B) magnitude of ischemic contracture. Hearts were subjected to 25 min of aerobic perfusion followed by 20 min of global ischemia. Infusion of L-NMMA began 10 min prior to the onset of ischemia. n=6 hearts per group. *$p<0.05$ versus control using one-way analysis of variance followed by Dunnett's test for pairwise comparisons.

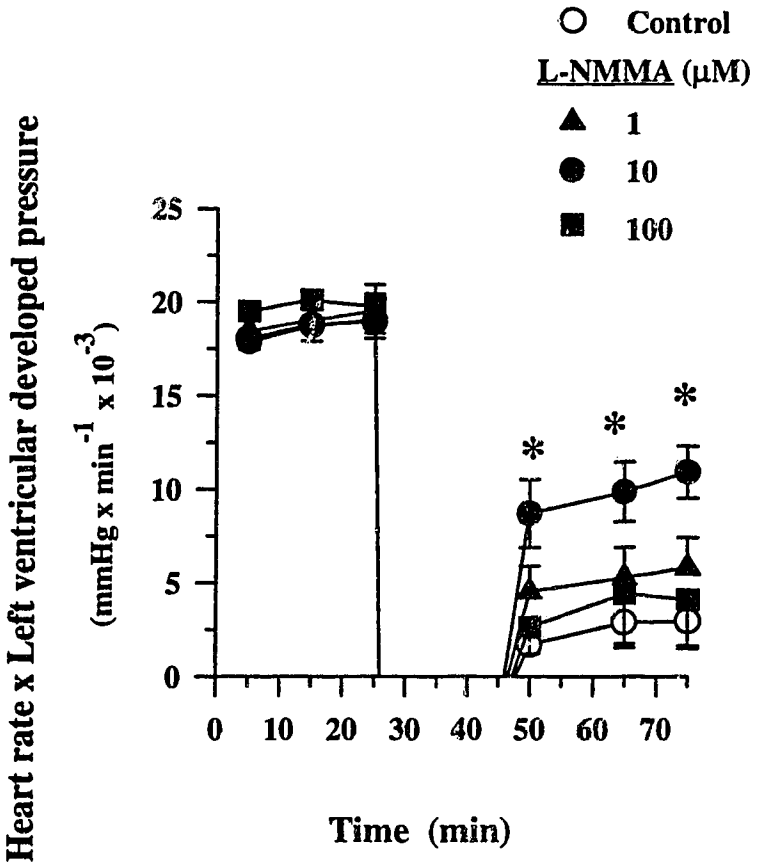


Figure 3.7: Effects of L-NMMA on mechanical performance of hearts before and after 20 min of global, no-flow ischemia and 30 min reperfusion. Mechanical function is shown as the heart rate x left ventricular developed pressure product for control hearts or hearts subjected to 1, 10, or 100 μM L-NMMA added 10 min prior to ischemia and throughout 30 min of reperfusion.

n=6 hearts per group. *$p<0.05$ versus control using one-way analysis of variance followed by Dunnett's test for pairwise comparisons.

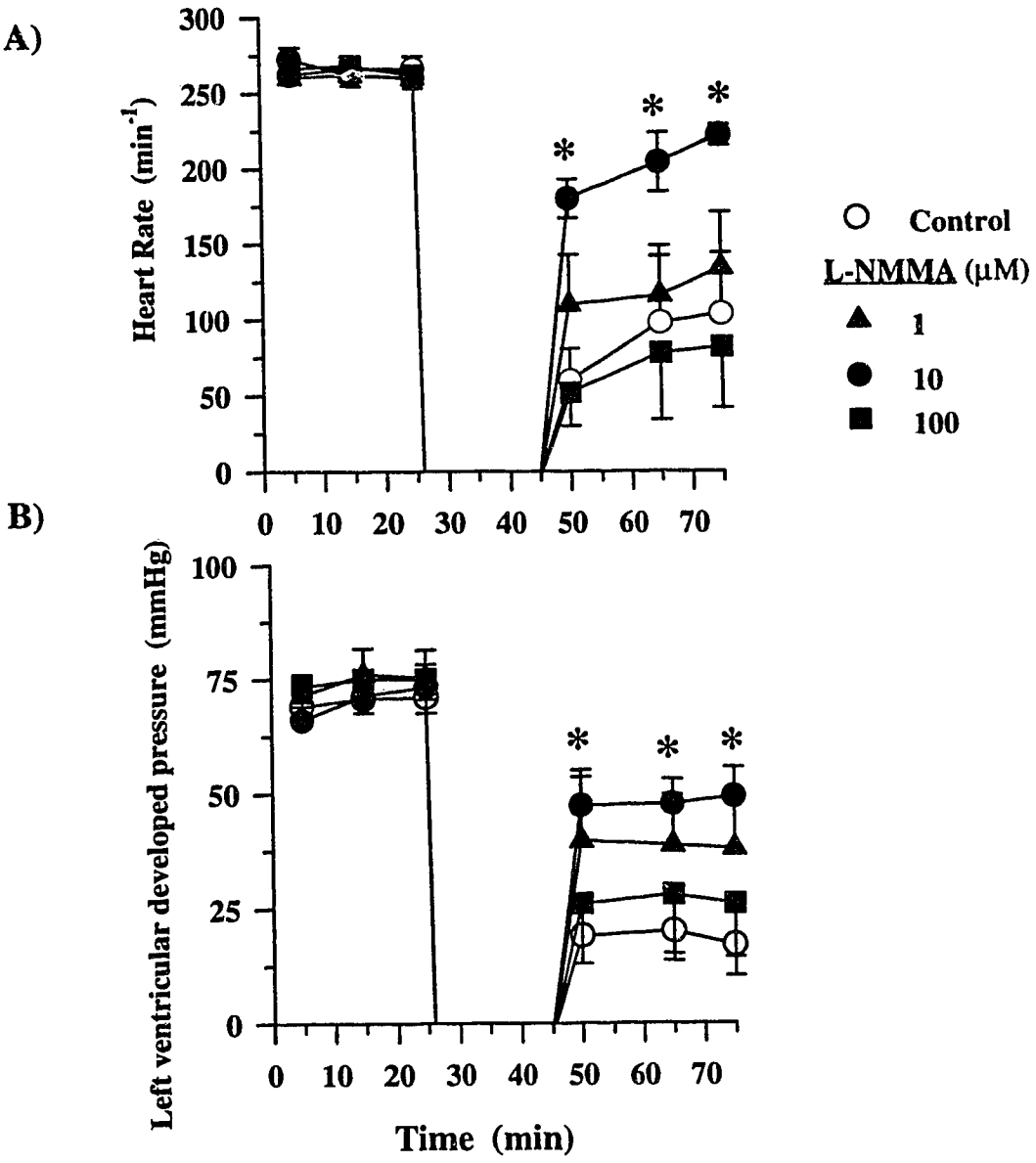


Figure 3.8: Effects of L-NMMA on mechanical performance of hearts before and after 20 min of global, no-flow ischemia and 30 min reperfusion. Mechanical function is shown as: A) heart rate and B) left ventricular developed pressure for control or hearts subjected to 1, 10, or 100 μM L-NMMA added 10 min prior to ischemia and throughout 30 min of reperfusion.

n=6 hearts per group. *$p<0.05$ versus control for heart rate using one-way analysis of variance (ANOVA) followed by Dunnett's test for pairwise comparisons. For LVDP graph one-way ANOVA did not show significant differences. However, Student's unpaired t-test for control vs. 10 μM L-NMMA was significantly different at all time points (*$p<0.05$).

treated hearts and in controls, respectively. This represents a recovery of 58 ± 9 % and 16 ± 8 % of preischemic function, respectively ($p<0.05$). The recovery of mechanical rapid and persistent improvement of both heart rate (Figure 3.8a) and left ventricular developed pressure (Figure 3.8b). Additional hearts were perfused with 3 or 30 μM L-NMMA. Expressed as a percentage of pre-ischemic function, the recovery of the rate-pressure product at 30 min reperfusion showed a bell-shaped, concentration-dependent protective action of L-NMMA with a maximum protective effect at 10 μM (Figure 3.9).

E) Effect of L-NMMA on coronary flow

L-NMMA at 1 and 3 μM had no significant effect on coronary flow during aerobic perfusion, whereas between 10-100 μM it caused a significant decrease in baseline coronary flow (Table 3.1). L-NMMA (1-100 μM) did not change either left ventricular developed pressure or heart rate during aerobic perfusion (data not shown).

Table 3.2 gives the ratio of mechanical function (heart rate x left ventricular developed pressure product) to coronary flow during aerobic perfusion and after 5, 15 and 30 min of reperfusion. In control hearts, a marked four to eightfold decrease in this ratio was evident throughout the reperfusion period, suggesting an inefficient matching between mechanical function and coronary flow. L-NMMA (1-100 μM) caused a concentration-dependent improvement in this ratio which peaked to near pre-ischemic values at 10 μM. At concentrations of 30 and 100 μM L-NMMA, however, the improvement in matching between mechanical function and coronary flow was no longer evident.

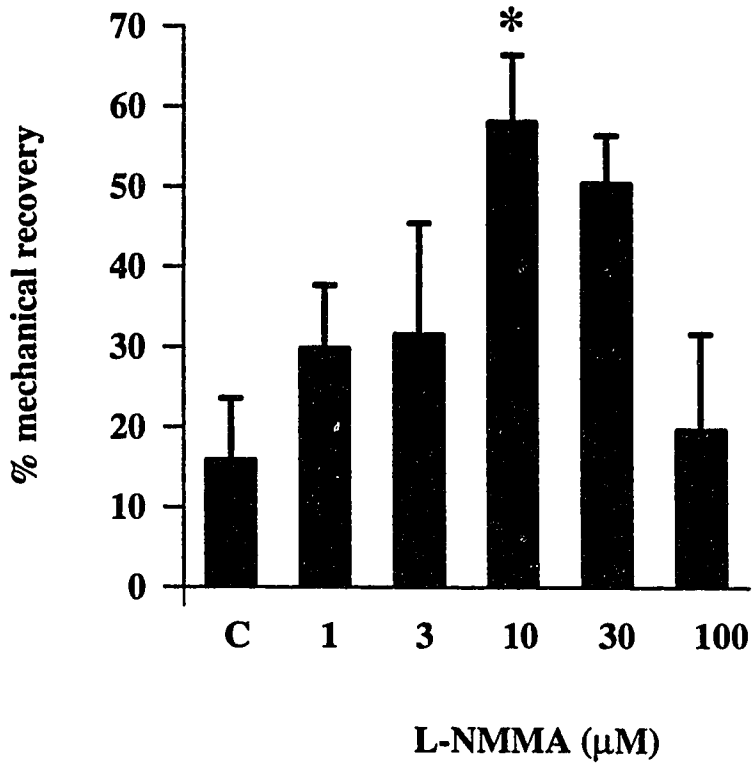


Figure 3.9: Concentration-dependence of the protective action of L-NMMA on the recovery of mechanical function in ischemic-reperfused hearts. Values are expressed as the percentage recovery of the product of heart rate x left ventricular developed pressure after 30 min reperfusion compared to pre-ischemic function at 25 min aerobic perfusion. C denotes control hearts (without L-NMMA). n=6 hearts per group. *$p<0.05$ versus control using one-way analysis of variance followed by Dunnett's test for pairwise comparisons.

Table 3.1: Effect of L-NMMA on coronary flow during aerobic perfusion in isolated rat hearts before or 10 min following continuous infusion of L-NMMA.

	Coronary flow (ml/min)	
	Baseline flow	*10 min after*
Control	12.7±0.4	12.7±0.4
L-NMMA (μM)		
1	12.3±0.6	11.2±0.4
3	11.6±0.4	11.4±0.4
10	11.3±0.4	9.8±0.5*
30	12.2±0.6	9.8±0.5*
100	11.0±0.5	9.7±0.3*

Values represent the mean± s.e.m of control (baseline) values and 10 min after the start of infusing vehicle or L-NMMA. n=6 hearts per group.

*$p<0.05$ versus baseline value using Student's paired t-test.

Table 3.2: Effect of L-NMMA on the functional recovery of the ratio of (heart rate x left ventricular developed pressure product) to coronary flow during reperfusion

	Heart rate x pressure product / coronary flow (mmHg x ml^{-1})			
	Aerobic Perfusion	*Time of Reperfusion (min)*		
		5	15	30
Control	1.52 ± 0.07	0.18 ± 0.07	0.33 ± 0.16	0.38 ±0.18
L-NMMA (μM)				
1	1.53 ± 0.10	0.38 ± 0.1	0.54 ± 0.15	0.78 ± 0.24
3	1.71 ± 0.06	0.74 ± 0.22*	0.87 ± 0.36	0.86 ± 0.36
10	1.67 ± 0.15	0.73 ± 0.11*	1.26 ± 0.28	1.51 ± 0.26*
30	1.62 ± 0.08	0.60 ± 0.19	0.89 ± 0.30	1.35 ± 0.25
100	1.81 ± 0.12	0.21 ± 0.15	0.44 ± 0.28	0.52 ± 0.34

Values represent the mean ± s.e.m of the ratio of (heart rate x left ventricular developed pressure: coronary flow) in isolated hearts at 15 min aerobic (pre-ischemic) perfusion and at 30 min reperfusion following 20 min of global, no-flow ischemia. n=6 hearts per group.

*$p<0.05$ versus control using one-way analysis of variance followed by Dunnett's test for pairwise comparisons.

F) Detection of dityrosine at reperfusion and effect of L-NMMA

As L-NMMA is an inhibitor of endogenous NO formation, we examined its action on endogenous $ONOO^-$ formation during reperfusion following ischemia. In control hearts, reperfusion caused the rapid increase in the generation of dityrosine in the coronary effluent (expressed as a percentage to its background level in the same heart during aerobic perfusion) to a peak at 30 seconds of 167 ± 14% ($p<0.002$) which then declined to baseline by 5 min reperfusion (Figure 3.10) and remained there until the end of the 30 min reperfusion period (data not shown). L-NMMA (1, 10, and 100 μM) significantly inhibited the peak in the dityrosine signal seen at 30 s reperfusion to 119 ± 5, 113 ± 7, and 114 ± 4% of baseline values, respectively, which was significant for all concentrations of L-NMMA ($p<0.05$).

G) Effects of superoxide dismutase and catalase

Figure 3.10 shows the actions of superoxide dismutase (50 U/ml) and catalase (50 U/ml) on ischemic contracture, recovery of mechanical function and dityrosine formation in ischemic-reperfused hearts. The combination of superoxide dismutase and catalase did not reduce the time to onset or significantly alter the magnitude of ischemic contracture (Figure 3.11a), nor did it improve the recovery of mechanical function during reperfusion (Figure 3.11b). In contrast, upon reperfusion the formation of dityrosine in the coronary effluent was significantly reduced by superoxide dismutase and catalase (Figure 3.11c).

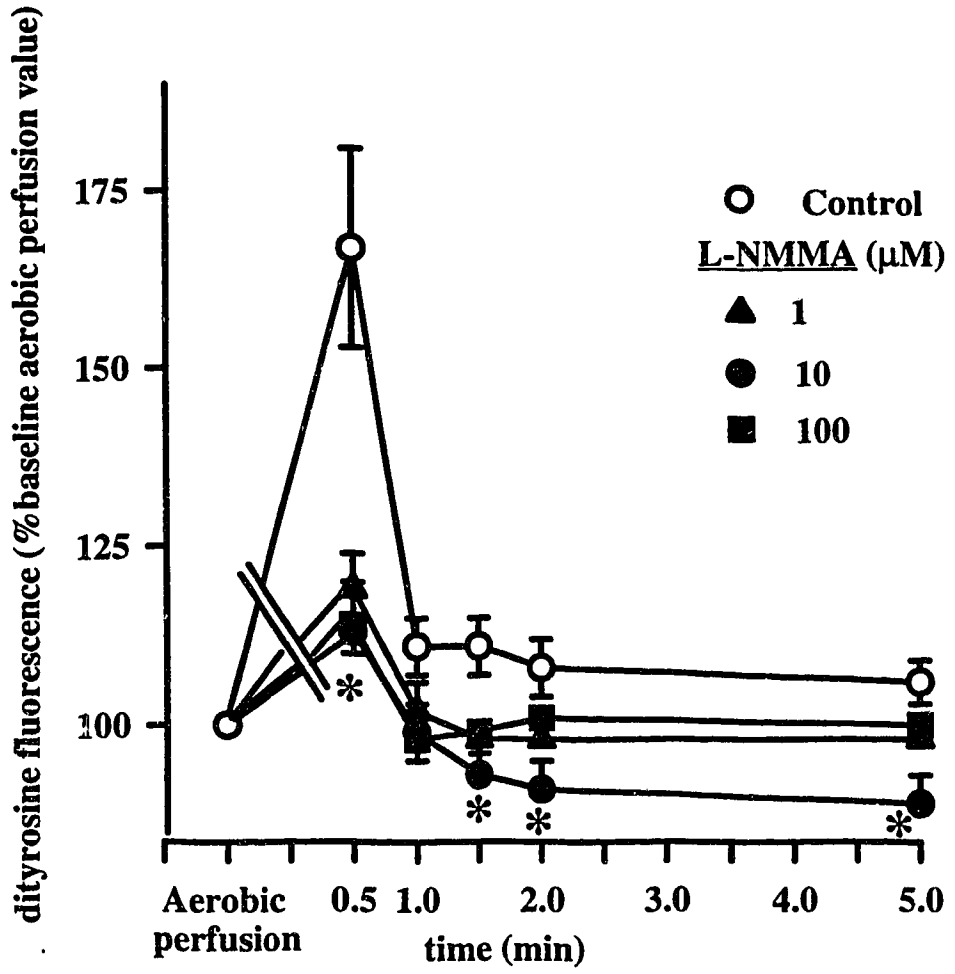
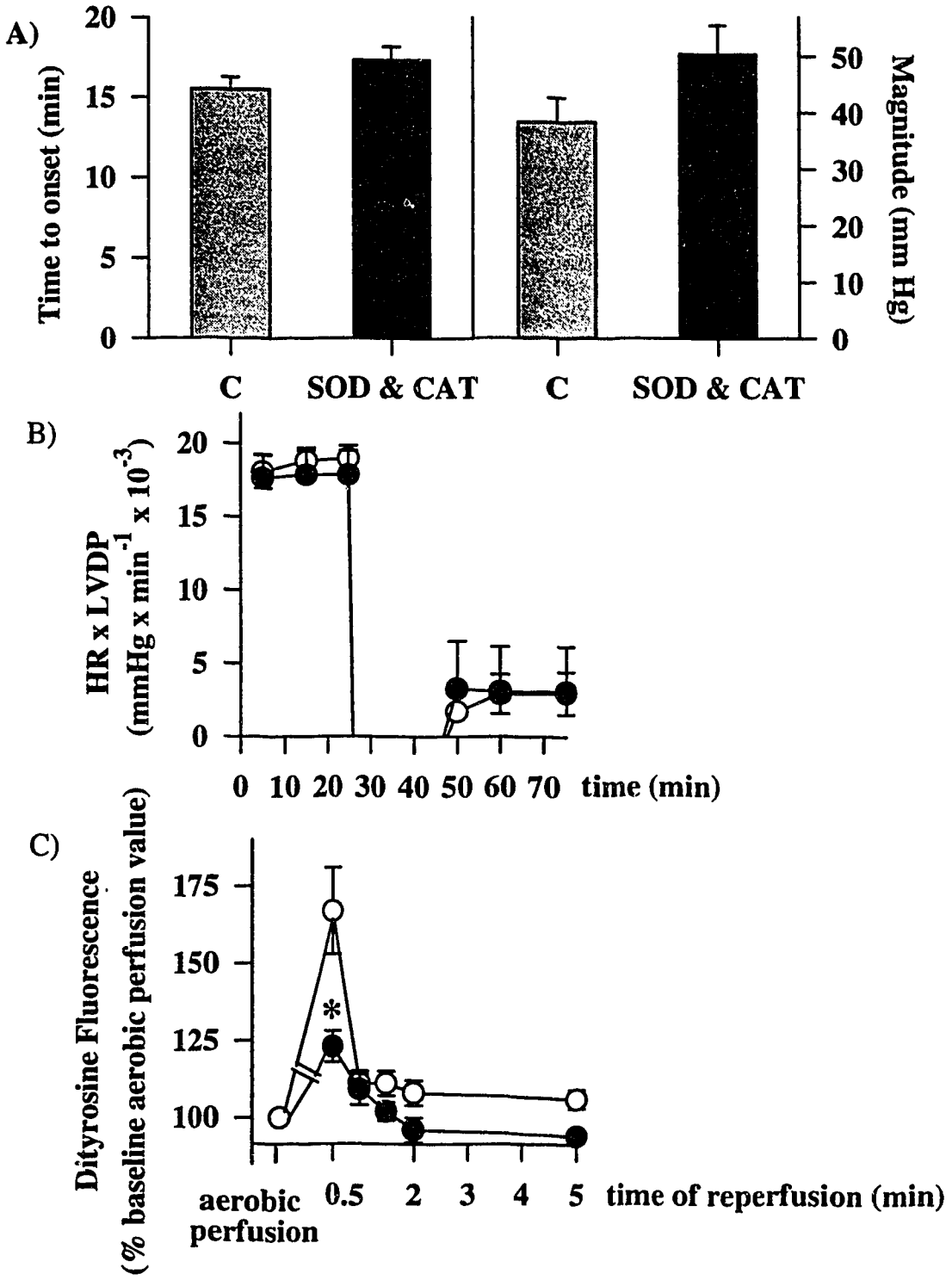


Figure 3.10: Release of dityrosine into the coronary effluent, measured by fluorescence spectroscopy, during reperfusion of hearts subjected to 20 min of global, no-flow ischemia and effect of L-NMMA. Values are expressed as a percentage of baseline coronary effluent fluorescence measured during aerobic perfusion immediately prior to the onset of ischemia. L-NMMA (1, 10, or 100 μM) was infused 10 min prior to the onset of ischemia and throughout the reperfusion.

n=6 hearts per group. *$p<0.05$ vs. control (for all concentrations of L-NMMA at 0.5 min reperfusion) or as indicated using one-way analysis of variance followed by Dunnett's test for pairwise comparisons.

Figure 3.11: Effects of superoxide dismutase (SOD, 50 U/ml) and catalase (CAT, 50 U/ml) (●) infused 5 min prior to the onset of ischemia and for the first 5 min of reperfusion in hearts subjected to 20 min of global, no-flow ischemia on: A) time to onset (left panel) and magnitude of ischemic contracture (right panel), B) recovery of mechanical function (heart rate x left ventricular developed pressure = HR x LVDP) and C) release of dityrosine into the coronary effluent. C or (O) denotes control hearts. n=6 hearts per group.

*$p<0.05$ versus control using Student's unpaired t-test.


A)
Time to onset (min)
20
15
10
5
0
Magnitude (mm Hg)
50
40
30
20
10
0
C
SOD & CAT
C
SOD & CAT
B)
HR x LVDP
(mmHg x min-1 x 10-3)
20
15
10
5
0
0 10 20 30 40 50 60 70 time (min)
C)
Dityrosine Fluorescence
(% baseline aerobic perfusion value)
175
150
125
100
*
aerobic perfusion
0.5 2 3 4 5 time of reperfusion (min)

H) Effects of exogenous NO by infusion of SNAP

The NO donor SNAP was infused at 0.02 and 0.2 μM into hearts 5 min prior to the onset of ischemia and for the first 5 min of reperfusion. At these concentrations, SNAP had no significant effect on coronary flow during the aerobic perfusion period prior to the onset of ischemia (Table 3.3). As shown in Figure 3.12a, SNAP at both 0.02 and 0.2 μM significantly increased the time to onset and reduced the magnitude of ischemic contracture (Figure 3.12a). In contrast, SNAP at 0.2 μM (but not 0.02 μM) significantly enhanced the recovery of post-ischemic mechanical function to 58 ± 12 % of pre-ischemic function, as measured at 30 min of reperfusion (Figure 3.12b). Infusion of SNAP inhibited the formation of dityrosine in the coronary effluent during reperfusion in a concentration-dependent manner (Figure 3.12c).

I) Effects of SOD mimetic MnTBAP

The low molecular weight SOD mimetic, MnTBAP (100 μM) was infused into hearts 5 min prior to the onset of ischemia and for the first 5 min of reperfusion. This concentration reduced the magnitude of ischemic contracture and delayed its time to onset (Figure 3.13a). It also caused a significant improvement (56 ± 13 % measured at 30 min of reperfusion) of mechanical function in contrast to control hearts (Figure 3.13b). MnTBAP caused strong interference in the fluorometric assay of dityrosine. No fluorescent dityrosine was detectable after 5 min of infusion at 25 min aerobic perfusion

and during reperfusion. To see whether this effect was due to its $ONOO^-$ scavenging property, 100 μM of MnTBAP was added to 2 μM of authentic dityrosine. MnTBAP completely blocked dityrosine fluorescence (data not shown).

Table 3.3: Effect of SNAP on coronary flow during aerobic perfusion in isolated rat hearts before or 5 min following infusion of SNAP

	Coronary flow (ml/min)	
	Baseline	*5 min after*
Control	11.8±0.4	11.2±0.6
SNAP (μM)		
0.02	11.2±0.5	11.0±0.5
0.2	11.8±0.3	11.5±0.4

Values represent the mean ± s.e.m of control (baseline) values and 5 min after the start of infusing vehicle or SNAP.

n=6 hearts per group.

Figure 3.12: Effects of 0.02 μM or 0.2 μM SNAP infused 5 min prior to the onset of ischemia and for the first 5 min of reperfusion in hearts subjected to 20 min of global, no-flow ischemia on: A) time to onset (left panel) and magnitude of ischemic contracture (right panel), B) recovery of mechanical function (heart rate x left ventricular developed pressure = HR x LVDP) and C) release of dityrosine into the coronary effluent. C or (O) denotes control hearts.

n=6 hearts per group.

*$p<0.05$ versus control using one-way analysis of variance followed by Dunnett's test for pairwise comparisons.

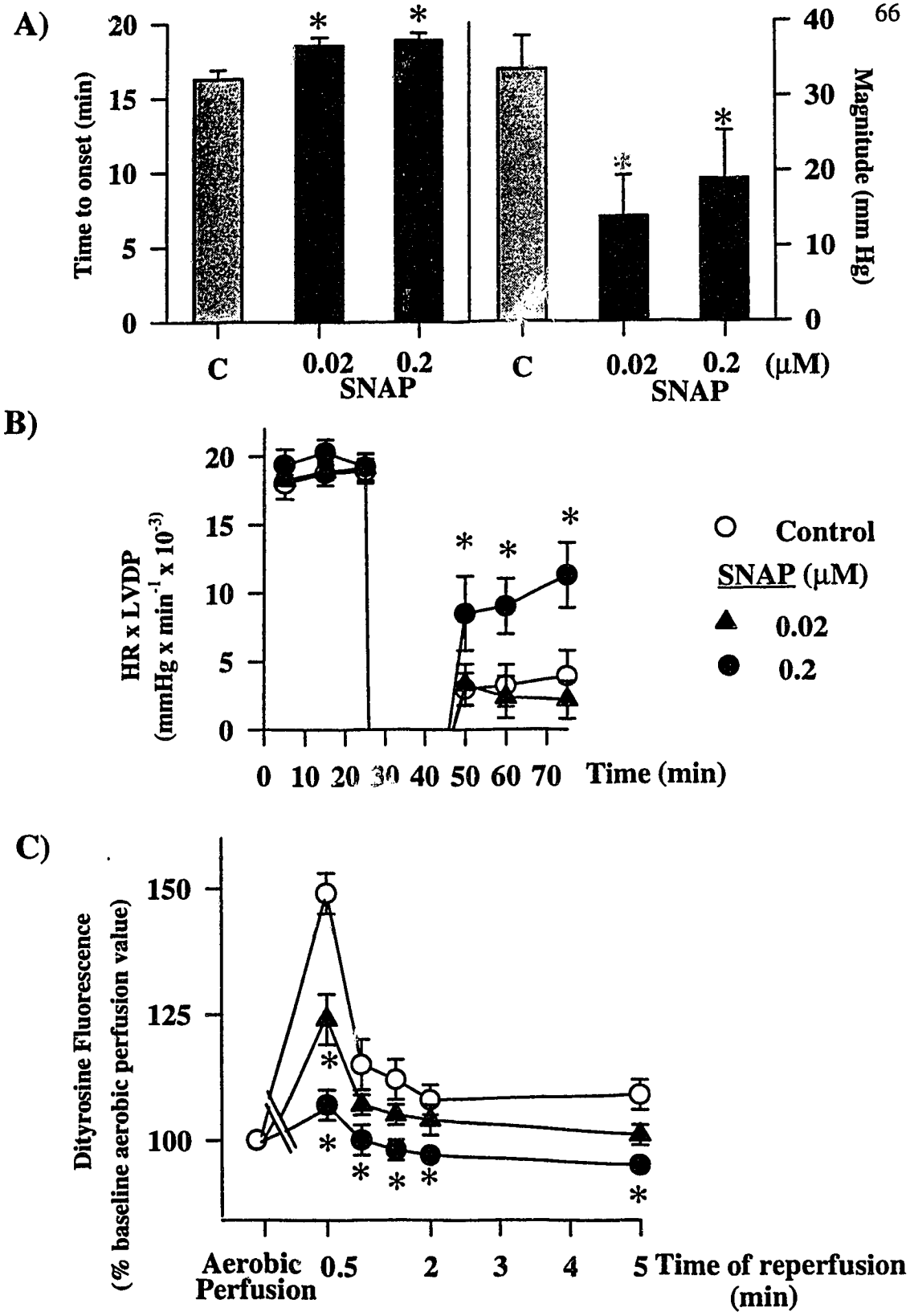
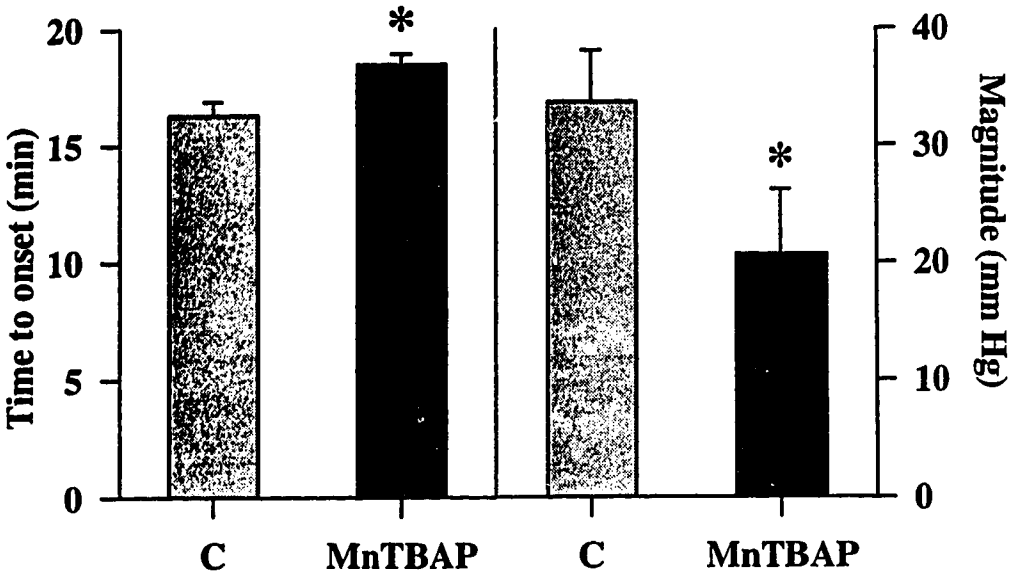
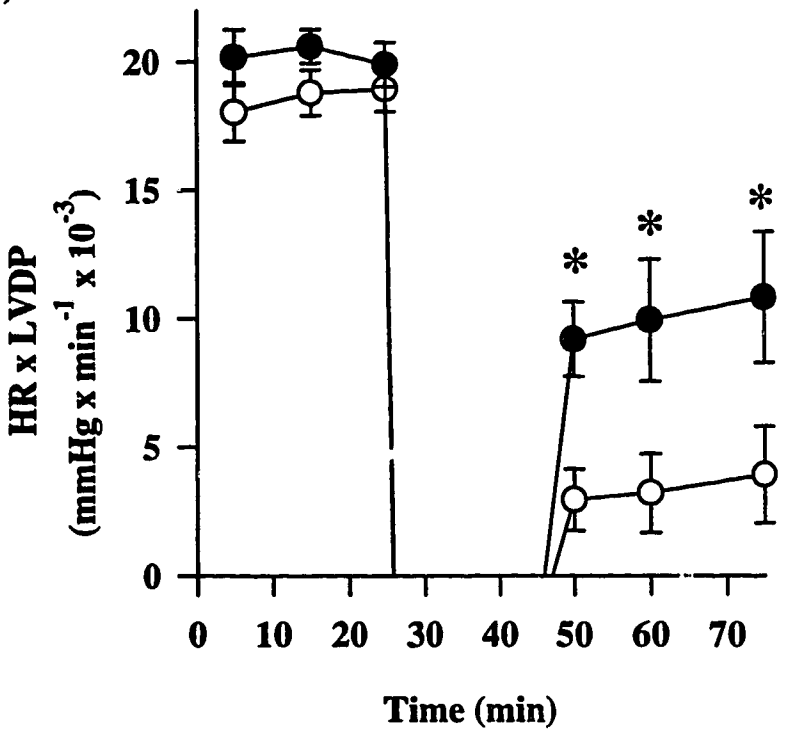

A)
Time to onset (min)
Magnitude (mm Hg)
C
0.02
0.2
SNAP
(μM)
B)
HR x LVDP
(mmHg x min-1 x 10-3)
Control
SNAP (μM)
0.02
0.2
Time (min)
C)
Dityrosine Fluorescence
(% baseline aerobic perfusion value)
Aerobic Perfusion
Time of reperfusion (min)

Figure 3.13: Effects of 100 μM MnTBAP (●) infused 5 min prior to the onset of ischemia and for the first 5 min of reperfusion in hearts subjected to 20 min of global, no-flow ischemia on: A) time to onset (left panel) and magnitude of ischemic contracture (right panel) and B) recovery of mechanical function (heart rate x left ventricular developed pressure = HR x LVDP). C or (○) denotes control hearts. n=6 hearts per group. *$p<0.05$ vs. control using unpaired t-test.

A)


Time to onset (min)
Magnitude (mm Hg)
20
15
10
5
0
40
30
20
10
0
*
*
C
MnTBAP
C
MnTBAP

B)


HR x LVDP
(mmHg x min-1 x 10-3)
20
15
10
5
0
*
*
*
0
10
20
30
40
50
60
70
Time (min)

CHAPTER IV

DISCUSSION

A) General

Ischemia-reperfusion injury in the isolated rat heart resulted in the increased generation of dityrosine, which is formed by the reaction of $ONOO^-$ with L-tyrosine (Vliet et al, 1994; Yasmin and Schulz, 1995). Since the generation of dityrosine was inhibited by L-NMMA, a specific inhibitor of NOS, or by superoxide dismutase, which removes $O_2^{-\cdot}$, these results strongly indicate that $ONOO^-$ was generated during ischemia-reperfusion injury. The hypothesis that generation of $ONOO^-$ contributes to the development of mechanical dysfunction of the myocardium subjected to ischemia and reperfusion was examined. This hypothesis was tested by examining the actions of L-NMMA, superoxide dismutase, and SNAP on the development of this dysfunction and comparing this to their effects on $ONOO^-$ formation (Yasmin and Schulz, 1995). In accordance with this hypothesis, Schulz et al (1995) recently observed a concentration and time-dependent depression of myocardial mechanical function in isolated rat hearts subjected to the continuous infusion of synthetic $ONOO^-$.

$ONOO^-$ has a number of potentially detrimental actions including lipid peroxidation (Rubbo et al, 1995), inhibition of mitochondrial respiration (Radi et al, 1994) and aconitase activity (Castro et al, 1994), release of iron from ferritin (Reif and

Simmons, 1991), oxidation of sulfhydryl moieties (Radi et al, 1991), and nitration of protein-associated tyrosine (Beckman et al, 1994), all or some of which could contribute to myocardial stunning.

B) Methodological considerations

a) Isolated Langendorff rat heart model

In this study, the Langendorff isolated rat heart model had been used to study the role of peroxynitrite in ischemia reperfusion injury. This method, first devised by Oscar Langendorff (1895), permits investigation of the mechanical activity of the isolated heart. This simple technique employs a retrograde perfusion of the coronary arteries via an aortic canula. This model is advantageous in that it is useful to study the formation of reactive oxygen species during ischemia-reperfusion injury and also it allows to determine the pharmacological effects of various drugs on myocardial contractile function, heart rate and coronary vascular tone.

b) Detection of $ONOO^-$ by reaction with L-tyrosine

To detect formation of endogenous $ONOO^-$ a simple spectrofluorometric technique has been applied for the first time in a biological system. Peroxynitrite reacts with L-tyrosine to form the strongly fluorescent product dityrosine (Vliet et al, 1994) which can

be detected both by spectrophotofluorometry and high performance liquid chromatography. The fluorometric method was preferred over high performance liquid chromatography because it was easier to perform and samples could be analyzed for the fluorescent signal within minutes of doing the experiments. This method was also verified with high performance liquid chromatography, showing a good linear correlation between the two methods of analysis. The reaction between L-tyrosine and $ONOO^-$ was not due to effects of $ONOO^-$ vehicle as no fluorescence signal for dityrosine was detected after reaction of L-tyrosine with decomposed $ONOO^-$. Heinecker et al (1993) suggested that myeloperoxidase secreted by activated neutrophils can utilize H_2O_2 to oxidize L-tyrosine to tyrosyl radical, yielding dityrosine. In this study reaction of 1 mM of H_2O_2 with 300 μM L-tyrosine did not result in the formation of any dityrosine (data not shown). Moreover, the buffer perfused isolated heart was devoid of neutrophils which reduced the likelihood of H_2O_2 production. However, the homolytic cleavage of peroxynitrous acid also causes nitration of L-tyrosine forming nitrotyrosine (Vliet et al, 1994). Detection of dityrosine was preferred over nitrotyrosine because of the high fluorescence of the former allows for a better detection limit.

The reaction between L-tyrosine and $ONOO^-$ is very rapid at physiological pH (Vliet et al, 1994). This effect was reflected by the immediate detection of dityrosine in the coronary effluent during infusion of exogenous $ONOO^-$ into the coronary circulation of aerobically perfused hearts with Krebs-Henseleit buffer containing L-tyrosine. During reperfusion of ischemic hearts, fluorescence of dityrosine in the coronary effluent peaked within 30 seconds from the onset of reperfusion. This signal rapidly returned to baseline

within 2 min of reperfusion and after 5 min there was no detectable signal of dityrosine above baseline. This method therefore, is comparable as a detection method for the rapid release of oxygen derived radicals which were detected by electron paramagnetic resonance spectroscopy in the first minute of reperfusion of ischemic hearts (Zweier et al, 1989; Bolli et al, 1989).

C) Effects of different drugs in improving myocardial functions as well as reducing $ONOO^-$ formation

a) NOS inhibitor (L-NMMA)

The appearance of the dityrosine fluorescent signal during reperfusion was blocked with a specific inhibitor of NO synthase, L-NMMA, indicating that the formation of dityrosine in the coronary effluent was dependent upon the biosynthesis of endogenous NO. Infusion of hearts with L-NMMA resulted in a marked bell-shaped, concentration-dependent improvement in the recovery of mechanical function during reperfusion. Optimal protection by L-NMMA occurred at 10 μM, suggesting that a partial inhibition of NO synthesis is required to protect the heart from myocardial stunning. The protective action of L-NMMA is dose-dependent and has a bell shape and is likely related to the detrimental (generation of $ONOO^-$ from increased amounts of NO and $O_2^{-\cdot}$ generated during reperfusion) and beneficial (physiological mediator of a continuous coronary vasodilator tone) actions of NO in the heart. Similar bell-shaped concentration-response

relationships for inhibitors of NO synthase in preventing the consequences of excess production of NO during endotoxaemia (Nava et al, 1991) and during cytokine-mediated depression of cardiac mechanical function (Schulz et al, 1995) have been observed. Therefore, it may be very important to select a concentration of NO synthase inhibitor which has minimal effect on basal NO production yet reduces the excess release of NO at reperfusion.

Measuring dityrosine in the coronary effluent underestimates $ONOO^-$ production at reperfusion owing to the efficiency of the reaction of $ONOO^-$ with L-tyrosine, which is estimated on the basis of results presented here to be <0.1%. Moreover, dityrosine may only reflect the intravascular production of $ONOO^-$. Therefore, although 1 μM L-NMMA reduced the formation of dityrosine in the coronary effluent, we do not know whether the production of $ONOO^-$ throughout the entire heart was significantly reduced. At this concentration of L-NMMA, no functional protection was seen during reperfusion. This suggests that either significant $ONOO^-$ production, undetected as dityrosine formation in the coronary effluent, may have occurred under these conditions or that there is a dissociation between the degree of functional protection and the formation of $ONOO^-$ at reperfusion. A more direct means to measure $ONOO^-$ production in myocardial tissue at the time of perfusion would be necessary to resolve this.

In 1995 Schulz and Wambolt observed that L-NMMA and N^G-nitro-L-arginine methyl ester (L-NAME) improved the recovery of mechanical function following global, no-flow ischemia in isolated working rabbit hearts, an effect which was specific to inhibition of NO synthase as the protection was abolished in the presence of excess L-

arginine, but not D-arginine. Several points need to be addressed in regards to the concentration-dependent actions of L-NMMA in myocardial ischemia-reperfusion injury. Firstly, in this study, protection by L-NMMA was maximal at 10 μM, a concentration which significantly inhibited coronary flow during aerobic perfusion (Table 3.1). At higher concentrations the protective action of L-NMMA was lost, although this was not accompanied by the further reduction of coronary flow during aerobic perfusion. The constant coronary perfusion pressure model we used here may have masked any further reduction in coronary flow to higher concentrations of L-NMMA.

Recently, Depré et al (1995) showed that L-NMMA (from 0.001 to 10 μM, but not at 100 μM) provided significant protection against ischemic injury in isolated rabbit hearts perfused at constant flow and subjected to low-flow ischemia. In agreement with their study, a decrease in the magnitude and an increase in the time to onset of ischemic contracture with L-NMMA, albeit at higher concentrations, was observed. However, in this study a protective action of 100 μM L-NMMA on the development of ischemic contracture was seen, whereas it failed to improve functional recovery during reperfusion. This suggests that events which occurred at reperfusion in this study were of equal or greater importance than those during ischemia, which would be predicted in a model of no-flow ischemia compared to low-flow ischemia. Although Depré et al (1995) showed that the protection afforded by L-NMMA may have been due to the enhancement of glycolysis during ischemia itself, they could not rule out a possible protective action of L-NMMA during early reperfusion. Interestingly, Schulz and Wambolt (1995) observed in their global no-flow model of ischemia of isolated rabbit heart that administration of

NOS inhibitor prior to ischemia did not protect the myocardium by preventing metabolic changes during ischemia itself, as both the increase in myocardial lactate and the depletion of endogenous glycogen were unaltered by L-NAME.

Other mechanisms for the protective action of NOS inhibitors in myocardial ischemia and reperfusion have been suggested. These include both an adenosine-dependent protective action of the NO synthase inhibitor L-NAME used at the concentration of 30 μM (Woolfson et al, 1995). At this concentration L-NAME abolishes endothelium-dependent NO production (Rees et al, 1990). At 30 μM L-NAME stimulates the release of adenosine in the heart (Woolfson et al, 1995; Kostic and Schrader, 1992), perhaps as a compensatory vasodilator mechanism when NO synthesis is abolished. It is also important to consider that L-NAME is at least tenfold more potent than L-NMMA as an inhibitor of endothelial NO production (Rees et al, 1990). The maximum protection with L-NMMA in this study was found at 10 μM, which is approximately equipotent to 1 μM L-NAME (Rees et al, 1990). Therefore, a 30-fold lower concentration of L-NAME "equivalent" was added to that which stimulates adenosine release (Woolfson et al, 1995; Kostic and Schrader, 1992). Others have found that L-NAME was protective in myocardial ischemia-reperfusion injury at the single concentration which was tested (1000 μM; Zweier et al, 1995). It may be possible that this protective action was due, in part, to non-selective actions of L-NAME at this concentration, including its unique action (L-NMMA does not have any such activity) as a muscarinic receptor antagonist (observed at concentrations ≥ 100 μM, which is a supramaximal concentration to inhibit NOS activity; see Buxton et al, 1993) and/or by the stimulation of adenosine release (Woolfson

et al, 1995; Kostic and Schrader, 1992).

b) Superoxide dismutase and catalase

The effects of removing $O_2^{-\cdot}$ and hydrogen peroxide by SOD and catalase, respectively, were examined. In contrast to the inhibition of NOS, this treatment failed to reduce ischemic contracture or improve the recovery of mechanical function of the heart, although it effectively inhibited the formation of dityrosine in the coronary effluent, similar to the results of 1 μM L-NMMA. The protective action, or lack thereof, the combination of SOD and catalase in myocardial ischemia-reperfusion injury is controversial. The combination of SOD and catalase (24 U/ml each) was protective in a Langendorff rat heart model of ischemia-reperfusion injury (Ohsuzu et al, 1989). Also Ataka et al (1989) has shown the protective action of the combined adminstration of SOD and catalase (40 U/ml and 4 U/ml respectively) in improving the functional recovery in a working rat heart model of myocardial ischemia-reperfusion injury. In contrast to these findings it has also been shown that SOD at a dose range of 20-80 U/ml was not protective in isolated rat hearts subjected to ischemia and reperfusion (Hatori et al, 1992; Sjoquist et al, 1993). The insufficient protection by the combination of SOD and catalase is consistent with these previous studies. It is worth to point out, however, that SOD does not readily cross the cell membrane (Omar et al, 1992) and therefore this treatment may not have affected the intracellular generation of $ONOO^-$ but only that which was released into the vascular lumen.

c) Superoxide dismutase mimetic (MnTBAP)

The effect of SOD on ischemia-reperfusion injury was therefore re-examined using a low molecular weight SOD mimetic, MnTBAP (Faulker et al, 1994), which has better access to the intracellular compartment. In a canine model of regional ischemia, Black et al (1994) demonstrated that a manganese based SOD mimetic, SC-52608, protects against myocardial ischemia reperfusion injury by reducing infarct size. To date, no data has been presented in an isolated heart model using these agents.

In this study, in contrast to superoxide dismutase and catalase, MnTBAP (100 μM) provided significant protection against myocardial ischemia reperfusion injury by reducing ischemic contracture and improving the recovery of mechanical function to a similar extent as L-NMMA (10 μM). Szabo et al (1996) reported that MnTBAP may also act as a scavenger of $ONOO^-$, as it inhibited the oxidation of dihydrorhodamine-123 by $ONOO^-$. Moreover, it protected against the suppression of mitochondrial respiration in J774 cells exposed to $ONOO^-$ (Szabo et al, 1996). However, MnTBAP was not compatible with the spectrophotofluorometric detection of dityrosine at reperfusion, as this drug caused strong fluorometric interference. This interference was verified by adding MnTBAP to a solution of authentic dityrosine standard, the fluorescence signal of which was also inhibited by MnTBAP. Verification of the ability of MnTBAP to inhibit dityrosine formation at reperfusion may require the use of high performance liquid chromatography to detect dityrosine in the coronary effluent. Thus in this study the effects of MnTBAP in improving myocardial function after ischemia-reperfusion injury

could be due to its actions as a SOD mimetic, however, some recent evidence suggests that it may have additional effects as a scavenger of $ONOO^-$ (Szabo et al, 1996). Further study is necessary to resolve this issue.

d) NO donor (SNAP)

Infusion of the NO donor SNAP, in concentrations which did not affect coronary flow, also caused a concentration-dependent improvement in the recovery of myocardial function which was evidenced by both the reduction of ischemic contracture and the increase in mechanical function following ischemia and reperfusion. Moreover, infusion of SNAP reduced the formation of dityrosine from $ONOO^-$ released at reperfusion. This may have been a result of the competitive antagonism by NO of the reaction between tyrosyl radical intermediates (which are produced during exposure of L-tyrosine to $ONOO^-$) to form dityrosine (Vliet et al, 1995).

These results must be compared with the known protective actions of subvasodilatory doses of the NO synthase substrate L-arginine (Weyrich et al, 1992; Schulz and Wambolt, 1995; Amrani et al, 1995) and NO donors (Siegfried et al, 1992), and agents which stimulate endogenous NO formation (ie. acetylcholine, see Richard et al, 1995) in myocardial ischemia-reperfusion injury. It is likely that *in vivo*, NO-mediated prevention of neutrophil adhesion to the endothelium and/or platelet activation and adhesion may become important, especially in the later (hours) stages of reperfusion. Concentration-dependent protection by NO and NO donor compounds of the deleterious

effects of $ONOO^-$ and other free radicals have been observed in the heart (Villa et al, 1994), platelets (Moro et al, 1994), fibroblasts (Wink et al, 1994) and lipid membranes (Rubbo et a, 1994). The protection of L-arginine and NO donors in the setting of myocardial reperfusion may be due to the ability of NO to terminate $ONOO^-$-mediated lipid radical chain propagation (Rubbo et al, 1994), decrease elevated cytosolic Ca^{2+} levels by stimulating cyclic GMP production (Nakashima et al, 1986), or prevent $ONOO^-$-mediated impairment of endothelium-dependent vasodilation in the coronary circulation (Moro et al, 1994).

D) Isoforms of NO synthase contributing to reperfusion injury

The isoform(s) of NOS responsible for the rapid increase in NO production in the heart during ischemia and reperfusion is unknown. Although it can be speculated, the rapid increase in L-NMMA-inhibitable dityrosine production at reperfusion is likely a consequence of the Ca^{2+}-dependent NOS normally present in coronary vascular (Cocks et al, 1985) and endocardial endothelial cells (Schulz et al, 1991), cardiac myocytes (Schulz et al, 1992; Balligand et al, 1995), as well as in some cardiac neurons (Klimaschewski, 1992), as the time course of the experiment is too short to permit expression of inducible, Ca^{2+}-independent NOS in the isolated heart. Indeed rapid changes in NO production have been shown to occur within minutes of the onset of ischemia (Depré and Hue, 1994). It has previously been observed that the freshly isolated rat heart possesses only Ca^{2+}-dependent NOS activity and requires at least 2 hr exposure

to endotoxin and/or pro-inflammatory cytokines in vitro before inducible NOS activity is measurable in the myocardium (Schulz et al, 1992; Schulz et al, 1995).

E) Clinical Relevance

The introduction of potent thrombolytic agents and balloon angioplasty has resulted in reperfusion therapy in the treatment of developing myocardial infarction. The present data provide evidence for a role of endogenous $ONOO^-$ in myocardial ischemia-reperfusion injury during the first minute of reperfusion. As a result, any therapeutic intervention intended to effectively limit reperfusion injury should be aimed at interfering with the production or actions of $ONOO^-$ and must be initiated before or immediately upon reperfusion. Given the current understanding of the mechanisms of the reperfusion injury, it is evident that protection of the post-ischemic myocardium may be accomplished through the following means: by inhibiting NO synthase, reducing $O_2^{-\cdot}$ formation, or by providing exogenous NO. Low concentrations of a NOS inhibitor may be useful therapeutically but complete inhibition of NO synthesis may be deleterious because of NO's physiological roles in regulating thrombogenicity (Radomski et al, 1990; Radomski, 1995) and vasodilation. An increase in NO by-products have been observed in the coronary sinus blood of patients following cross-clamp release during cardiac by-pass surgery (Hattler et al, 1994) and in piglet hearts subjected to hypoxia-reoxygenation injury (Mathies et al, 1992). It can be speculated that the use of NOS inhibitors at low doses to limit the excess production of NO and $ONOO^-$ during the acute reperfusion phase

following thrombolytic therapy or after cardioplegia during cardiac surgery may only be necessary and prudent during the first minutes of reperfusion. After this time it is likely that provision of L-arginine (to reverse the action of the NO synthase inhibitor) or NO donors would be of benefit due to the cardioprotective (vasodilator, anti-neutrophil and anti-platelet) actions of NO.

F) Future directions

For future studies, using a combination of drugs which reduce both the formation of NO and $O_2^{-\cdot}$, may be more effective in improving post-ischemic myocardial function then the partial protection seen with either drug alone. As $ONOO^-$ causes both hydroxylation and nitration of tyrosine, further studies need to be done in detecting changes in protein tyrosine residues by using an immunohistochemical technique (Beckman et al, 1994). One also needs to identify the most likely targets and the mechanisms by which $ONOO^-$ causes injury to the heart. Moreover, to find out the specific NOS isoform involved in $ONOO^-$ formation, mice genetically deficient in endothelial NOS (Huang et al, 1995), brain NOS (Huang et al, 1994) or inducible NOS (Xie et al, 1995) by homologous recombination could provide valuable information when used in newly developed models of ischemia reperfusion injury in mice (Michael et al, 1995).

G) Conclusions

In summary, this study demonstrated that myocardial ischemia-reperfusion injury results in the generation of $ONOO^-$, detected by the formation of dityrosine in the coronary effluent at reperfusion. This injury was prevented by partial inhibition of NOS, removal of $O_2^{-\cdot}$, or by the provision of low amounts of exogenous NO. These experimental treatments represent new pharmacological approaches in the treatment of myocardial ischemia-reperfusion injury. These results support the hypothesis that the formation of $ONOO^-$ at reperfusion may contribute to the pathogenesis of the mechanical dysfunction of the heart. Whether the generation of $ONOO^-$ is a generalized phenomenon affecting various regional vascular beds as a result of ischemia-reperfusion injury remains to be studied.

CHAPTER V

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