INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI

A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor MI 48106-1346 USA
313/761-4700 800/521-0600

Université d'Ottawa · University of Ottawa

APOPTOSIS IN THE ISOLATED PERFUSED RAT HEART: INVOLVEMENT OF REPERFUSION, OXIDANTS AND PROTEIN SYNTHESIS

David C. Dean

A Thesis Submitted to the School of Graduate Studies at the University of Ottawa in Partial Fulfillment of the Requirements for the Degree of Master of Science in Physiology

Supervisor: Dr. Henry Fliss

David C. Dean, Ottawa, Canada, 1996

National Library of Canada

Bibliothèque nationale du Canada

Acquisitions and Bibliographic Services

Acquisitions et services bibliographiques

395 Wellington Street
Ottawa ON K1A 0N4
Canada

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence

Our file Notre référence

The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

L'auteur conserve la propriété du droit d'auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-26315-0

Canadä

ABSTRACT

Reperfusion of an ischemic myocardium has been shown to cause increased injury to the post-ischemic myocardium. This injury is visible by an increase in the resting tension of the heart and decreased contractility. It remains unknown if this injury is a delayed result of ischemia, or results from the reperfusion of an ischemic area, or a combination of both. Studies exploring the mechanisms of ischemia/reperfusion (IR) injury are abundant and have shown that oxidants, as well as accumulation of metabolic byproducts, play major roles in IR.

The isolated perfused heart model has been used very successfully in the past, allowing examination of IR, as well as the application of agents to combat IR injury. However, in the last few years there has been increased interest in a newly discovered component of IR. Apoptosis has recently been determined to occur in the post ischemic heart *in vivo* by this laboratory, as well as others. It remains unknown whether active cell death contributes to the decrease in myocardial performance characteristic of IR, however, the manipulation of apoptosis using various agents has been successfully documented in many different systems, including cardiac myocytes. Therefore, we sought to determine for the first time if: 1) Apoptosis occurs in the isolated buffer perfused heart and 2) If manipulation of apoptosis would protect against IR injury.

Apoptosis was characterized using DNA agarose gel electrophoresis and In-Situ-End-Labelling (ISEL). Together, these provide significant evidence of apoptosis, in the isolated hearts. Thirty minutes ischemia, followed by 4 hours of reperfusion with Krebs-Ringer buffer provided abundant apoptosis in cardiomyocyte nuclei (11 of 14 hearts

displayed DNA "ladders" and positive ISEL), coupled with significant IR injury, in the hearts (IR GROUP). Neither apoptosis nor performance injury was detected in the uninterrupted perfused (no ischemia) CONT GROUP. Known inhibitors of apoptosis, including antioxidants (DTT GROUP, Zn^{2+} INJECTED GROUP), manipulators of protein synthesis (CYCLO GROUP) and metals (Zn^{2+}PYR GROUP) were applied to the perfusate to determine their effect on both active cell death, as well as IR injury. All proven inhibitors of apoptosis did protect the IR hearts from active cell death. However, only the DTT GROUP and the CYCLO GROUP showed protection from IR injury. The DTT GROUP provided increased LVDP, decreased aortic root pressure, and decreased diastolic pressure vs. the IR GROUP (ANOVA $p<0.05$). The CYCLO GROUP also provided increased LVDP and decreased diastolic pressure (ANOVA, $p<0.05$).

The involvement of thiols in active cell death was explored. Neither glutathione (GSH) nor protein sulfhydryls (PSH) were different among any of the groups (excepting Zn^{2+}PYR and MCI) (ANOVA), providing evidence that the abundant apoptosis in the IR group may not be related to thiol level.

DEDICATION

This work is dedicated to my parents, Peggy and Alan Dean. It is only because of their encouragement, generosity and patience that this, my greatest accomplishment, was possible. My hope is that they will benefit from this work in some way.

ACKNOWLEDGEMENTS

Dr. Henry Fliss:

I am eternally indebted to Dr. Henry Fliss for his patience, understanding and for this tremendous opportunity. His relentless dedication and genuine love of science is truly a gift to us all.

Debbie Gattinger:

Debbie gave me a hand when mine were full. She worked out the bugs in many of the protocols which were used in this work. I sincerely hope that Debbie is rewarded by science as much as she rewards science.

Michel Menard:

Mike (he likes it, he likes it, Mikey likes it!) is unfortunately no longer with this lab, but one thing is for sure, his talents remain. He is perhaps the most patient and giving person I have ever had the fortune of encountering. He taught me virtually all of the techniques used in this work.

Lindsay "Turkey" Angus:

It is beyond me how Lindsay does such great work and deals with me at the same time. Her love of great results (and cookies), teddy bears (and cookies) and cookies (and cookies) made her both a privilege and a joy to work with.

Clare Howlett:

This visitor from CHEO came with stories of NO, NO and for the last time, NO. Although not part of our lab, she is without question, part of our lab. And then some.

Dr. B. Korecky:

Without the generous donation of the Langendorff apparatus by Dr. Korecky, this study could not have been accomplished. As well, Dr. Korecky was instrumental in the initial stages of this work by providing not only the equipment, but the laboratory space in which the work was completed. A very distinguished scientist, Dr. Korecky has remained an influential component of the Department of Physiology.

Dr. Frank Kolar:

Although this Czechoslovakian scientist was here for only a brief stay, he generously donated his very precious time to myself. Specifically, he aided this study by teaching me how to make the cellophane balloons necessary for the monitoring of cardiac function, as well as the set-up and calibration of the Grass recorders.

Marika Masika:

Marika was also instrumental in teaching the basic procedure in the isolation, as well as the perfusion, of rat hearts.

TABLE OF CONTENTS

Abstract	II
Dedication	IV
Acknowledgements	V
Table of Contents	VII
List of Abbreviations	X
List of Figures	XII
Title and Introduction	1
1.0 Myocardial Ischemia	1
2.0 Cardiac Ischemia and Resulting Injury	2
2.1 Ischemia	2
2.2 Contractile Failure	7
2.3 Calcium Overload	8
2.4 Pathophysiology of a Human Myocardial Infarct	8
3.0 Reperfusion Injury	10
3.1 Reperfusion Injury-General Comments	10
3.2 Oxygen Paradox	14
3.3 Oxygen Free Radical Generation in Reperfusion	14

3.4 Free Radical Action and Injury in the Myocardium 17

4.0 General Comments on Cell Death 19

4.1 Necrotic Cell Death 19

4.2 Necrotic Cell Death in the Ischemic Reperfused Heart 23

4.3 Apoptosis-General Comments 23

4.4 The Role of Oxidants in the Apoptotic Process 24

4.5 The Role of De Novo Protein Synthesis in Apoptosis 25

4.6 Apoptosis in the Reperfused Myocardium 26

4.7 Involvement of Zinc in Apoptosis 28

4.8 Zinc Induced Metallothionein Expression 29

Project Objectives 31

Methods 33

Langendorff Isolated Perfused Heart 33

Tissue Homogenization and Freezing 38

DNA extraction and Electrophoresis 38

In-Situ-End-Labelling (ISEL) 39

Bradford Assay for Protein Concentration 40

Dithio-Nitrobenzoic (DTNB) Assay for Thiol Concentration 40

Zn^{2+} Induced MT Overexpression 41

Metallothionein Column Chromatography 41

Sephadex Column Chromatography 42

Statistical Analysis 42

Results 43

Control Group 43

Reperfused Group 43

Antioxidant Treatment (DTT) 46

Monochloramine Group 49

Zinc Pyrithione Group 52

Zinc Injection 55

Cycloheximide Group 60

Thiol Content 63

DNA Electrophoresis 65

In-Situ-End-Labelling (ISEL) 68

Discussion 72

Apoptosis as a Component of Ischemia Reperfusion Injury 72

The Involvement of Apoptosis on Myocardial Contractility 75

Signalling Pathways in Apoptosis 77

The Involvement of Protein Oxidation in Apoptosis 77

GSH as a Signal for Apoptosis 79

Manipulation of Apoptosis using Antioxidants 80

Involvement of Protein Synthesis in Apoptosis 84

Involvement of Zinc in Apoptosis 85

References 86

LIST OF ABBREVIATIONS

ADP - adenosine diphosphate

ANOVA- analysis of variance

AMP- adenosine monophosphate

ATP- adenosine triphosphate

BSA- bovine serum albumin

CP- creatine phosphate

CYCLO- cycloheximide

DTNB- dithio-nitrobenzoic acid

DMSO- dimethyl sulfoxide

DNA- deoxyribonucleic acid

DTT- dithiothreitol

ESR- electron spin resonance spectroscopy

GSH- glutathione

GSSG- glutathione disulfide

HEP- high energy phosphate

HSP- heat shock protein

IR- ischemia reperfusion

ISEL- in-situ-end-labelling

KR- Krebs Ringer Buffer

LVDP- left ventricular developed pressure

MI- myocardial ischemia

MPO- myeloperoxidase

MT- metallothionein

NAC- N-acetylcysteine

NAD- nicotinamide-adenine dinucleotide

NADP- nicotinamide-adenine dinucleotide phosphate

OFR- oxygen free radical

PBS- phosphate buffered saline

PMN- polymorphonuclear leukocyte

PSH- protein sulphydryls

RNA- ribonucleic acid

ROS- reactive oxygen species

SAS- statistical analysis software

SR- sarcoplasmic reticulum

TCA- trichloroacetic acid

TSH- total sulphydryls

LIST OF FIGURES

FIGURE	Page
Figure 1 Xanthine/Xanthine Oxidase System	13
Figure 2 Typical Langendorff Tracings	34
Figure 3 Perfusion Protocol	37
Figure 4 IR Group vs. Control Group Performance	44
Figure 5 IR vs. Control Group LVDP	45
Figure 6 DTT Group Performance	47
Figure 7 Antioxidant Groups LVDP	48
Figure 8 Monochloramine Group Performance	50
Figure 9 Monochloramine Group LVDP	51
Figure 10 Zinc Pyrithione Group Performance	53
Figure 11 Zinc Pyrithione Group LVDP	54
Figure 12 Metallothionein Elevation	56
Figure 13 Sephadex Column Chromatography	57
Figure 14 Zinc Injected Group Performance	58
Figure 15 Zinc Injected Group LVDP	59
Figure 16 Cycloheximide Group Performance	61
Figure 17 Cycloheximide Group LVDP	62
Figure 18 Thiol Data	64
Figure 19 DNA Gel Electrophoresis	67
Figure 20 ISEL Pictures	69

APOPTOSIS IN THE ISOLATED PERFUSED RAT HEART MODEL: INVOLVEMENT OF REPERFUSION, OXIDANTS AND PROTEIN SYNTHESIS

Introduction

1. Myocardial Ischemia - General Background

Myocardial ischemia (MI) results from a decrease, or complete loss, of blood circulation in a region of the heart. It can be the result of a mobilized thrombus, or any form of occlusion in the coronary vasculature, and its severity is proportional to both the size of the ischemic region as well as the duration of the ischemic episode. Most postmortem examinations find that the predominant cause of myocardial infarction causing death is a thrombus superimposed on preexisting atherosclerotic disease (1-4). The mobilized thrombus is almost always due to the prior disruption of an atherosclerotic plaque (5-9). Once the vasculature is occluded, the affected area receives a significantly decreased oxygen and nutrient supply, and experiences impaired waste removal. Depending upon the abundance of collateral circulation, the ischemic area may not receive any oxygen or nutrients, and removal of waste products may cease entirely.

Clinical treatment of MI includes thrombolytic agents, percutaneous transluminal angioplasty, or bypass surgery to return blood flow to the region. Ironically, reperfusion of an ischemic region, although essential for tissue salvage, has been shown to actually cause additional tissue injury (10-14). Thought to be due primarily to the generation of

oxygen radicals early in the reperfusion period (15-18), this form of tissue damage is appropriately termed "reperfusion injury".

Since this thesis explores possible functional and biochemical consequences of ischemic injury, the mechanisms of cardiac disfunction resulting from ischemia will be described below. The various components of MI will be described beginning with an overview of ischemia. Reperfusion injury will be discussed in the context of oxygen free radical (OFR) generation and its effects. The pathophysiological conditions which exist in the human heart during the development of scar tissue will complete the section on cardiac disfunction.

2. Cardiac ischemia and resulting injury

2.1 Ischemia

Cardiac ischemia is comprised of many components. Hypoxia results when the supply of oxygen is not sufficient to meet the demands of the tissue and is thought to be the most injurious element of ischemia, but decreased nutrient supply as well as metabolic waste accumulation also contribute to the overall damage. The severity of cardiac ischemia is dependant upon the degree, and duration, of flow interruption. Collateral circulation may be available to provide decreased but adequate amounts of oxygen, nutrients, and waste removal to the ischemic tissues. In circumstances of high collateral blood flow, partial reduction of perfusion has been shown to cause decreased

oxygen consumption accompanied by reduced contractile performance, but without a corresponding reduction in the high-energy phosphate (HEP) content in isolated perfused rabbit hearts (19). Similarly, restriction of coronary flow during inotropic stimulation prevented the anticipated increase in contractile effort (20). These studies clearly implicate limited oxygen delivery as a modulator of reduced myocardial contractile function. This alteration of function appears to be independent of a detectable deficit in high-energy phosphate stores.

In the human, life is dependent upon uninterrupted contractile function of the heart. The average heart contracts 100,000 times in one day, pumping over 18,000 liters at a systolic pressure of 120 mmHg. For this, the myocardium is almost entirely dependant upon aerobic metabolism for the creation of adenosine triphosphate (ATP) by mitochondrial respiration.

The mitochondria occupy about 35% of the volume of a myocyte (21), reflecting their importance to contractile function. Mitochondrial respiration occurs through use of the electron transport chain within the inner membrane and is closely coupled to oxidative phosphorylation. In the absence of adenosine diphosphate (ADP), little oxygen is used but very little ATP is produced. However, when the myocardium is contracting vigorously, a high concentration of ADP is present and O_2 consumption is high, producing large amounts of ATP.

In the ischemic myocardium (due to cessation of coronary flow) the remaining oxygen in any erythrocytes trapped within the capillaries, or bound to myoglobin, is quickly consumed. Without oxygen as the final electron acceptor, the activity of the

electron transport chain decreases dramatically. In fact, the electron transport chain has been shown to come to a standstill within the first 2 seconds of global ischemia in isolated rat hearts (22). Nicotinamide adenine dinucleotide (NAD) reduction to $NADH_2$ has also been found to begin within the initial 2 sec of ischemia (23,24). Glycolysis is accelerated (due to an increase in both adenosine monophosphate (AMP) and inorganic phosphate, and a decrease in ATP and citrate) but quickly slows as glyceraldehyde-3-phosphate dehydrogenase activity is inhibited by increasing concentrations of NADH, H^+ and lactate (25,26). In either moderate ischemia or high-flow anoxia this does not occur, as both exogenous glucose and waste product removal are supplied to the tissue (27,28). However, even if tissue is continually cleansed of waste, and glucose supply is maintained, anaerobic glycolysis remains a very inefficient supplier of high-energy phosphates. Only 2 molecules of ADP can be phosphorylated to ATP from the catabolism of glucose to lactate. If glycogen is available, then an additional ATP molecule results, producing 3 ATP's per glucose. Compared to the 38 molecules of ATP created by the complete oxidation of glucose to CO_2 and H_2O during aerobic metabolism, anaerobic glycolysis can meet no more than 7% of the HEP requirements of the normal working heart (27).

With continuing ischemia and the ultimate cessation of aerobic metabolism and the inhibition of anaerobic glycolysis, the myocardium turns to the extremely limited stores of HEP. Creatine phosphate (CP), ATP and ADP are all stored in the tissues, albeit in very small quantities. CP has been found to be reduced by 33% after only 5 sec of ischemia (29) and when completely exhausted, the adenylate kinase reaction (2ADP $\leftrightarrow$ ATP +

AMP) and the remaining stored ATP is sufficient for only 30 sec of normal heart contractile function (30). Although the HEP demand is reduced by the depression of contractile function within 15 sec of ischemic onset, electrical and other metabolic activities (such as Ca^{2+} transport by the SR and sarcolemma, activity of the sarcolemmal Na^+, K^+-ATPase, fatty acid CoA synthetase and the mitochondrial ATP-ase) continue to consume ATP. Therefore in summary, the major metabolic effect of myocardial ischemia is that HEP demands greatly exceed the rate of production leading to a rapid decline in HEP's within the ischemic myocardium. CP stores are depleted within the first few seconds of ischemia (31,32) and myocardial ATP declines nearly as quickly. Dog studies showed that myocardial ATP was reduced to 35% of control values in severe regional ischemia within 15 minutes and below 10% by 40 minutes (32,33).

After 10 minutes of severe ischemia, both ATP and ADP decline while AMP levels increase through the adenylate kinase reaction. AMP may be further catabolized to adenosine via the enzyme 5'-nucleotidase which is then converted to inosine by adenosine deaminase. Both adenosine and inosine readily diffuse into the extracellular space where inosine may be catabolized to hypoxanthine and xanthine (by the enzymes nucleotide phosphorylase and xanthine oxidase, respectively) see Figure 1. Histochemical analysis has located these enzymes intracellularily within endothelial cells (34). Upon reperfusion, xanthine is then able to enter a pathway which generates tissue damaging oxygen radicals (discussed in section 3.3). In severe ischemia, nucleosides increase at a rate coinciding with the decrease in adenine nucleotides. Therefore ischemia causes not only the depletion of HEP's, but also the degradation of the adenine nucleotide pool

(33,35).

Metabolic end products such as lactate, inorganic phosphate and purine bases (end products of adenine nucleotide catabolism) accumulate in ischemic tissue. Lactate, the end product of anaerobic glycolysis, has been shown to alter action potentials and decrease the tension in guinea pig ventricular myocytes (36) as well as cause mitochondrial swelling (37) and inhibition of mitochondrial phosphorylation (38). H^+ also accumulates as a result of several catabolic processes such as glycolysis, lipolysis and ATP hydrolysis, and drives the pH of the tissue down into acidosis (39-41). A low pH in ischemic cardiac tissue may protect the threatened HEP by decreasing the contractile function of the heart (39,42,43). However, myocardial acidosis inhibits various metabolic pathways, including enzymes of the anaerobic glycolytic pathway, thus limiting the amount of ATP which can be synthesized from either glycogen or glucose in the early stages of ischemia. The resulting increase in $[H^+]_i$, elevates Na^+/H^+ exchange activity, leading to a calcium overload in the ischemic myocardium (discussed in section 2.3).

The myocardium does contain small stores of energy in the form of triglycerides (44), and as a result acyl CoA and acyl carnitine may accumulate in the heart during the initial stages of ischemia (45,46). However, the complete oxidation of fatty acids to water and CO_2 occurs in the mitochondria and cannot proceed in the absence of oxygen.

2.2 Contractile failure

Cardiac contractility decreases almost instantaneously upon the onset of ischemia. In fact, contractile force is measurably decreased within the initial 10 sec of ischemia (47,48) and is one of the earliest consequences of severe ischemia (49-51). It remains unknown why there is such a marked reduction in cardiac contractility so early in the ischemic episode. Logically, one would attribute this to tissue depravation of HEP, but it has been determined that the onset of contractile disfunction occurs prior to any substantial decrease in ATP (52,53). The notion that it is a small pool of ATP which may be specifically depleted has been proposed (54,55) and there is evidence that ATP may play a modulatory role in the control of passive ion fluxes across cellular membranes (55). A slight decrease in ATP may inhibit Ca^{2+} influx across the sarcolemma and from the SR thus impairing cardiac contraction and promoting early contractile failure.

The accumulation of metabolites has also been hypothesized to cause the premature contractile impairment. Hydrogen ions may compete with Ca^{2+} for the calcium binding site on troponin (56) but again, the decreased contractility has been observed prior to any significant measurable decrease in pH_i (57-59). More convincingly, high-flow anoxia (allowing metabolite removal) has produced the same decrease in *in vitro* cardiac contractility as seen in ischemia (acidosis would also proceed more slowly because of the presence of buffers in the perfusate). Koretsune et al. (60) proposed that contractility is decreased due to impaired cross-bridge formation by inorganic phosphates (61) but as with the other factors, contractile failure preceded any measurable rise in inorganic phosphate. From these results, researchers have proposed that early contractile failure

is the result of a loss of intravascular pressure.

2.3 Calcium overload

Ischemia has been proposed to result in an increase in cytosolic calcium (62-64). This may be the result of increased Ca^{2+} influx from either the sarcoplasmic reticulum (SR) or from extracellular spaces (65). A decrease in ATP may be one cause of the calcium overload due to decreased active Ca^{2+} sequestering and extrusion. As earlier stated, in the ischemic myocardium it is the Na^+/H^+ exchange that is thought to play the principal role in pH regulation. This protein removes hydrogen ions, but at the expense of increasing $[Na^+]_i$. It has therefore been proposed that the Ca^{2+} enters the cell as a result of Na^+ extrusion by the Na^+/Ca^{2+} exchange. This is particularly harmful to the myocyte because calcium is known to activate a variety of proteases, lipases and phospholipases (66) as well as increase the ATP deficit by activating various ATPases (62,63). Calcium also inhibits oxidative phosphorylation in the mitochondria (67) and causes hyper-contraction of the myocytes leading to contraction band necrosis (discussed in section 2.4).

2.4 Pathophysiology of a Human Myocardial Infarct

A myocardial infarct develops after about 40 minutes of ischemia in the human. The result is an area of cell death in which contractile function has ceased permanently, surrounded by the portions of the ischemic "area at risk" which remain viable, and are potentially salvageable. The ultimate size of the infarct depends on the fate of the "area

at risk", as cell death increases with time and extends the boundaries of the infarct.

A typical human myocardial infarct affects the left ventricle and may be transmural (involving the full thickness of the wall) or subendocardial (limited to the inner half of the myocardium). During the healing process, the infarct undergoes a series of changes beginning with the appearance of a cyanotic bruise. Cellular edema sets in followed by an inflammatory response with leukocyte infiltration within the first 24 hours of ischemia. The release of cardiac enzymes occurs, followed by tissue degradation and removal of necrotic fibers. Gradually fibrous connective tissue replaces the necrotic muscle and the wall undergoes subsequent thickening over a 6 week period as the scar is developed.

Myocardial infarction significantly decreases ventricular contractility as a result of the complete loss of contraction in the infarcted zone, and decreased contractility in the boundary of the infarct (area at risk). Functionally, myocardial infarction of the left ventricle results in:

1) Reduced contractility of the ventricle.
2) Abnormal wall motion.
3) Altered ventricular wall compliance.
4) Reduced stroke volume.
5) Diminished ejection fraction.
6) Elevated ventricular end-systolic and end-diastolic volumes.
7) Increased left ventricular end-diastolic pressure.

Areas of cardiac infarction typically do not display any functionality due to the necrotic state of the tissue. This leads to a reduction in the overall contractility of the ventricle (measured by this study as a decreased LVDP) and abnormal wall motion (because some parts of the ventricle may not contract). This disfunction is manifested in a reduced stroke volume and because the end-systolic and end-diastolic volumes are elevated, a decreased ejection fraction. Areas which were ischemic and are not infarcted display indications of a calcium overload. Altered ventricular wall compliance and increased left ventricular end-diastolic pressures result from the hypercontracture of the cardiomyocytes due to elevated intracellular calcium.

Section 3

3.1 Reperfusion Injury-General Comments

Although vital to tissue salvage, reperfusion of previously ischemic myocardium has been shown to cause injury. Studies have determined that following reperfusion, various cardiac disorders occur in the myocardium. Cardiac arrhythmias (68-71), altered metabolism (71-73) and decreased contractile performance (74-77) all occur following reperfusion of an ischemic heart. First defined by Rosenkranz and Buckburg (78) as referring "to those metabolic, functional and structural consequences of restoring coronary flow that can be avoided or reversed by modification of the conditions of reperfusion", reperfusion injury implies the manifestations of injury which may not have been present immediately before reperfusion. Lethal reperfusion injury refers to the death of myocytes which were viable prior to reperfusion.

An alternative explanation for reperfusion injury is that the cellular damage which occurs during reperfusion (explosive cell swelling, enzyme washout, massive calcium overload, and contraction band necrosis) is a result of injuries which occurred during ischemia and are postmortem manifestations of an injury made possible by the presence of large volumes of plasma or calcium (79,80). Therefore, reperfusion simply accelerates the development of injuries sustained during the ischemia.

If one considers that lethal reperfusion injury is a result of reperfusion alone, or that reperfusion superimposes a new injury upon damage which occurred during ischemia, then there are three pathways which are possible mechanisms of reperfusion injury.

First, the cellular acidosis which exists during ischemia may contribute to a calcium overload by Na^+/H^+ exchange followed by Ca^{2+}/Na^+ exchange (see section 2.3). With reperfusion, contraction band necrosis and mitochondrial calcification can result from this calcium overload (81-83). The hyper-contracture of the myocytes may then contribute to the rupture of the sarcolemma. However, it has been proposed that much of the Ca^{2+} may enter the cell through already existing gaps in the disrupted sarcolemma (84). If this is correct, then a small Ca^{2+} overload may induce hyper-contracture of the myocytes, furthering sarcolemmal destruction.

As discussed in section 2.3, a calcium overload may also have serious consequences for myocytes through activation of various proteases, lipases, phospholipases, and ATPases (62,63,66). Also, ATP synthesis (by mitochondrial oxidative phosphorylation and ATP synthetase) is inhibited by the precipitation of Ca^{2+} with phosphate and its subsequent accumulation in the matrix space of the reoxygenated

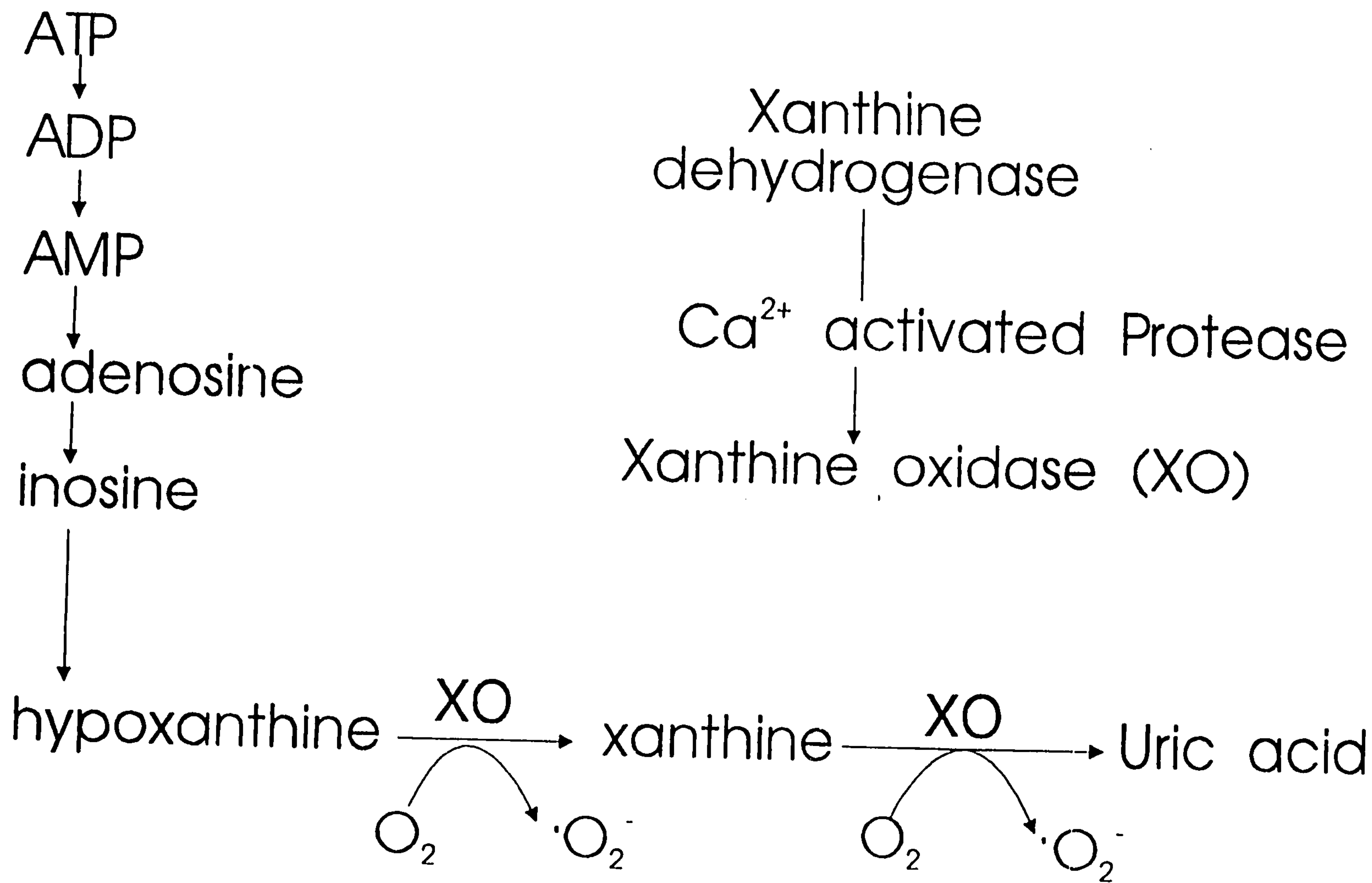
mitochondria. Compared to the modest calcium overload detected during ischemia (discussed in section 2.3), reperfusion induced Ca^{2+} overload cannot be reversed by further reperfusion. In contrast, the ischemia-induced Ca^{2+} overload has been determined to occur during the reversible stages of cellular injury, and can be eliminated by reperfusion (85-89), restoring normal Ca^{2+} homeostasis. For this reason, Ca^{2+} overload during ischemia is thought to be non-lethal.

The second pathway by which reperfusion may induce cellular injury is through the increased osmotic gradient known to occur during ischemia. This elevation in intracellular osmotic pressure is the result of accumulated catabolites which draw in plasma water when presented with unlimited flow. The combination of osmotic swelling with an already weakened cellular membrane may result in rupture of the membrane (90-92).

Third, free radicals from many sources are known to be produced upon reperfusion. As shown above, adenine nucleotide catabolism during myocardial ischemia results in an increase in hypoxanthine and xanthine. Upon reperfusion, the reintroduction of oxygen results in the production of oxygen free radicals (OFR) such as the superoxide radical ($\cdot O_2^-$). A Ca^{2+} activated protease is used to convert xanthine dehydrogenase to xanthine oxidase, allowing production of the radical to occur. This system is summarized in Figure 1 and is a major component of the oxygen paradox, discussed below. OFR's have been proposed to injure the sarcolemma (93) and thus may contribute to the cellular damage which occurs during reperfusion. Moreover, polymorphonuclear leukocytes (PMN's) accumulate in the damaged area and release injurious oxygen free radicals.

FIGURE 1. Ischemia is proposed to cause: A) The accumulation of hypoxanthine from the catabolism of adenine nucleotides during ischemia. B) The conversion of xanthine dehydrogenase to xanthine oxidase (XO) by a Ca^{2+} activated protease (the Ca^{2+} derives from the calcium overload typical of cardiac ischemia). Upon reperfusion, the superoxide radical ($\cdot O_2^-$) forms from the XO catalyzed reactions converting hypoxanthine to xanthine and then xanthine to uric acid.

Figure 1



3.2 The Oxygen Paradox

The oxygen paradox occurs in reperfused hearts following a period of hypoxia. As shown in isolated perfused hearts, perfusion with normoxic buffer following an hypoxic episode causes extensive cell swelling, contraction band necrosis and massive enzyme release (94,95). Reoxygenation of anoxic cultured myocytes has shown similar consequences (96,97). These studies have shown that it is the withdrawal, and subsequent return of oxygen, not the return of flow, to the ischemic tissue which causes the cellular disruption. Thus, in the presence of cyanide or dinitro-phenol to inhibit cellular respiration, reoxygenation of hypoxic tissue has not resulted in similar cell damage (98,99), suggesting the involvement of OFR's.

A "free radical" is by definition, a molecule with a very reactive unpaired electron. Free radical damage has been implicated in the oxygen paradox, although in some models anti-oxidants have proven ineffective (100). At present, it is believed that OFR's may be produced by a variety of mechanisms, and are not the result of the oxygen paradox alone.

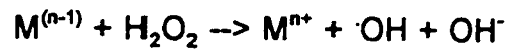
3.3 Oxygen Free Radical Generation in Reperfusion

Many reactive oxygen metabolites are produced in various biological systems which are potentially harmful to living cells (101). Superoxide anion ($\cdot O_2^-$), the hydroxyl radical ($\cdot OH$), and hydrogen peroxide (H_2O_2) are all generated in living tissues. In addition,

the OFR's released from leukocytes to attack harmful bacteria (102) have proven to damage the affected tissues (103). OFR's have been implicated in reperfusion injury following cerebral and intestinal ischemia (104-106), circulatory shock (107), organ transplantation (107), skin flap grafting (108), and pulmonary hyperoxia (109). There has been extensive research over the past several years to determine if OFR's are generated in the reperfused myocardium and through which mechanisms.

Free radicals have been detected in the initial few seconds of reperfusion in isolated perfused hearts, and intact dog hearts subjected to ischemia and reperfusion (110-112) using electron spin resonance (ESR) spectroscopy. Their source remains controversial, but it appears that most are derived from superoxide as the addition of superoxide dismutase prevents this occurrence (69,113-117). The superoxide anion can be produced by a number of mechanisms: the xanthine oxidase reaction, mitochondrial respiration, arachidonic acid metabolism, catecholamine oxidation, and NADPH oxidase in neutrophils. Similarlily, hydrogen peroxide (H_2O_2) may be produced by the xanthine oxidase reaction as well, but also from the reduction of O^{2-} by the enzyme superoxide dismutase. The subsequent accumulation of H_2O_2 is prevented by the antioxidant enzymes catalase and glutathione peroxidase, both of which result in the reduction of H_2O_2 to water. However, ischemia is reported to reduce the activity of the above enzymes (118,119), and when combined with increased superoxide production, the endogenous defenses of the cell may be overwhelmed. If this occurs, $^{\cdot}OH$ may be produced from the combination of $^{\cdot}O_2$- with a metal (eg. Fe^{3+} or Cu^{2+}), or H_2O_2 with a similar metal, through the Haber-Weiss reaction:

$$\cdot O^{2-} + M^{n+} \rightarrow O_2 + M^{(n-1)+}$$



$$M^{(n-1)} + H_2O_2 \rightarrow M^{n+} + \cdot OH + OH^-$$

$$\cdot O^{2-} + H_2O_2 \rightarrow O_2 + \cdot OH + OH^-$$

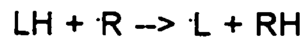
Although OFR's may originate from many sources, the two which have received the most attention are the xanthine oxidase reaction as well as neutrophils which have accumulated in the injured tissue. As detailed in section 3.2, the xanthine oxidase mechanism of OFR generation produces $\cdot O_2^-$ and H_2O_2 from the oxidation of hypoxanthine to xanthine and then to uric acid (93). However, this mechanism is species specific and although likely to occur in humans (120), dogs (121-123) and rats (124,125), is unlikely to cause lethal reperfusion injury in other species like rabbits (126) and pigs (127) in which xanthine oxidase activity is undetectable biochemically.

However, accumulated neutrophils are believed to be a ubiquitous feature of reperfusion in all species. A component of the inflammatory response, neutrophils release potent OFR's to degrade tissue in preparation for scar formation. As neutrophils produce $\cdot O_2^-$ (through the NADPH oxidase system), H_2O_2 (by the dismutation of superoxide), and hypochlorous acid (HOCl by the myeloperoxidase system, MPO) they may injure nearby viable cells as well as the cells which were targeted. Neutrophil granules also contain a number of proteolytic and lipolytic enzymes such as elastase, collagenase, acid hydrolases, and phospholipase A_2 which may be released into the extracellular space, inducing a reperfusion induced degradation of tissue (128-132). In addition, neutrophils

may become lodged in capillaries, plugging the vasculature and increasing the injury (133).

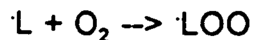
3.4 Free Radical Action and Injury in the Myocardium.

Regardless of source, the production of OFR's is harmful to many components of the affected tissue. One of the key challenges of OFR investigation is determining the target for oxidative damage. For example, the primary target of $\cdot O_2^-$ and $\cdot OH$ may be polyunsaturated fatty acids (134,135). Thus, the polyunsaturated fatty acids of membrane phospholipids may incur OFR damage and subsequently lose fluidity causing reduced membrane fluidity and decreased protein function. This proceeds through the following reaction, where LH is the fatty acid and .R is the reactive oxidant.



$$LH + \cdot R \rightarrow \cdot L + RH$$

The combination of the fatty acid and the reactive oxidant creates a lipid radical ($\cdot L$) which then can react readily with oxygen to form a peroxy radical ($\cdot LOO$).



$$\cdot L + O_2 \rightarrow \cdot LOO$$

The peroxy radical is free to attack a nearby fatty acid, forming hydroperoxide (LOOH) and another lipid radical.

$$LH + {}^{\cdot}LOO \dashrightarrow LOOH + {}^{\cdot}L$$

Damage to membrane lipids may alter the conformation (therefore the function) of membrane-bound protein complexes like ion channels and pumps. This reaction may propagate until two radicals unite to terminate the chain reaction, forming a non-radical product (LOOL or LL). Alternately, a radical may be quenched by reacting with a stabilizing agent such as α-tocopherol (136).

Proteins may also be directly damaged by OFR's. Chain scission, amino acid oxidation and creation of disulfide bonds may result from protein exposure to OFR's, all of which impair proper function. The most likely targets are the thiol and aromatic ring containing amino acids cysteine, methionine, histidine, tryptophan, phenylalanine, and tyrosine (137-144). OFR's have been reported to interfere with the function of the Ca^{2+}ATPase (142) of the SR, and the Na^{+}, K^{+}ATPase (145-147) which could contribute to impaired myocyte contractility and altered action potential propagation, respectively. The Ca^{2+}ATPase contains 26 cysteine residues, accounting for almost 95% of the sulfhydryl groups in the SR making it the most vulnerable target. Nucleic acids are also targets of OFR's. Hydroxyl radicals may cleave RNA or DNA strands by attacking the ribose-phosphate backbone of the molecule as well as modifying the nitrogenous bases (148-150).

CELL DEATH

4.0 General Comments on Cell Death

Cell death was defined by Buja et al. (151) as being the "irreversible loss of vital cellular structure and function". Cell death is a physiological process which occurs during organogenesis in embryos, in the final stages of the cell cycle in adults, and as a pathological response to various injuries (151). Investigation into this fundamental phenomenon has revealed that there are two distinct forms of cell death, necrosis and apoptosis.

4.1 Necrotic cell death

When cells die immediately upon exposure to an overwhelming insult, no preliminary morphological alterations are detectable. An example of instantaneously occurring cell death is the chemical fixation of tissues. However, in living organisms, cells suffering from irreversible cellular injury do exhibit morphologically and metabolically detectable changes. These events are exaggerated by the degradative reactions that follow cell death. The process by which cells die following irreversible cellular injury in living organisms is termed necrotic cell death.

Cells dying necrotically, usually swell rather than shrink. Cellular fragmentation occurs in the late stages and results from autolysis (activation and subsequent release of lysosomal enzymes), and heterolysis (due to actions of inflammatory cells infiltrating

necrotic tissue) following cell death. Various forms of necrosis are known to occur. Coagulation necrosis results from the denaturation and coagulation of cellular proteins and is the pattern of pathological cell death which normally occurs in response to prolonged ischemia, hypoxia, chemical toxins, infections, and trauma (152). Colliquative necrosis occurs in tissue which has a very low level of protein (eg. brain), and results in the rapid liquification of tissues due to both the poor protein content and the abundance of hydrolytic enzyme activity (151).

It appears that the transition between reversible and irreversible injury in necrosis ("the point of no return") is closely related to both HEP and ATP depletion (153) in the myocardium. These metabolic changes are accompanied by a characteristic series of morphological changes as well. Cytoplasmic edema manifested by a decrease in cytoplasmic density precedes blebbing of the basal membrane and subsequent nuclear chromatin condensation. During these stages, the cell remains salvageable. Irreversible cellular injury occurs with additional morphological changes such as advanced alterations in nuclear chromatin; mitochondrial lesions including amorphous matrix (flocculent) densities (composed of osmiophilic aggregates of lipid and protein), linear densities (fusion of mitochondrial cristae) and dense calcium phosphate deposits; and physical defects (breaks) in both the plasma and the organelle membranes (154,155). Because these changes are all consistent with progressive membrane damage, injuries applied to membranes are thought to be a key event in the pathological process of irreversible cellular injury in necrosis. There has been extensive research into the pathophysiology of membrane injury and it has been determined that 3 stages are involved, briefly: (1)

initially discrete alterations in the ionic transport systems of membranes, (2) membrane permeability is increased (non-specific) and (3) there is finally a distinct physical membrane disruption.

As indicated in section 3.3, various forms of cell injury are accompanied by the increased production of OFR's, which then are known to cause the disruption of membranes and proteins described above. Thus, the plasma membrane and the membranes of organelles become abnormally permeable and significantly altered as a result of disruptive phospholipid metabolism, which is magnified by the effects of amphipathic lipids and lipid peroxidation.

The cytoskeletal filaments may also become injured and indirectly damage membranes. Normally the filaments stabilize membranes and modulate function of the plasma membrane by connecting it with the interior of the cell (156,157). An example of this in the *in vitro* ischemic myocardium is the cytoskeletal protein vinculin which was reported to decrease during ischemia, causing membrane damage (158). Postulated to be a result of proteases, the precise cause of cytoskeletal damage has yet to be determined. It is also possible that the cytoskeletal-membrane connections may be involved. In any event, it is likely that disruptions in the cytoskeleton further destabilize the already altered plasma membrane and increase the prevalence of actual membrane disruption during severe swelling. In the ischemic myocardium, cells progressively swell due to intracellular water accumulation caused by the increasing osmotic load (due to metabolic end product accumulation) on the cell. The cellular swelling provides a stimulus for membrane disruption (90). In this case, rupture of the cell membrane represents the

final stage in the progression of a damaged, but still salvageable, cell into a state of irreversible injury.

It is thought that the moderate Ca^{2+} overload which occurs in ischemic hearts and the much more significant Ca^{2+} overload which occurs upon reperfusion (see section 2.3) plays an important role in irreversible injury. Briefly, calcium overload may occur because of: (1) altered Ca^{2+} membrane permeability of the slow Ca^{2+} channel and other calcium transporters, as well as (2) a decrease in ATP dependant Ca^{2+} reuptake into the SR and (3) a release of calcium from the mitochondria. The increase in Ca^{2+} may have several deleterious effects, including activation of phospholipases and proteases (promoting membrane damage), activation of ATPases (furthering ATP depletion), and mitochondrial calcium accumulation (reducing ATP production).

Therefore, in summary, cellular necrosis occurs when the cell is exposed to an environment, or an insult, which it cannot survive. The cell swells, then lyses as a result of both membrane failure and increased water influx. The membrane may fail either as a result of direct injury from OFR's and toxic species, or indirectly from cytoskeletal damage effects, and the increased osmotic load. Regardless of the cause, membrane disruption is considered to be the transition stage between reversible injury (cell still viable) and irreversible injury. A calcium overload is often correlated with this process.

4.2 Necrotic death in the Ischemic Reperfused Heart

If the ischemic myocardium is reperfused (either by spontaneous physiological means or by clinical intervention) during the early, reversible, stages of ischemic injury, the cells may eventually recover. However, if the reperfusion occurs after the "point of no return" (usually membrane disruption), the restored blood flow causes massive calcium overload, rapid structural failure and cell lysis leading to necrotic death termed contraction band necrosis (80,159,160).

4.3 Apoptosis-General Comments

Apoptosis has emerged recently in the forefront of medical research. It has been implicated in many diseases such as cancer (161,162), AIDS (163-167), stroke (168-171) and neurodegenerative disorders such as Huntingtons, Parkinsons and Alzheimers (172-177). In contrast to the different forms of necrotic cell death, cells undergoing apoptosis proceed through a genetically predetermined set of steps in the process of cellular suicide. The morphological features of this form of active cell death have been observed for over one hundred years. These include cell shrinkage with loss of neighboring contacts, chromatin condensation, nuclear fragmentation followed by cellular budding and subsequent digestion by dedicated phagocytes (thereby avoiding an inflammatory response). The DNA of apoptotic cells is typically fragmented at intervals of 180-200 base pairs as a result of specific endonuclease cleavage (178,179), allowing simple detection by DNA electrophoresis. These events are genetically defined and are thought to spare the surrounding tissue the exposure of the dangerous cytosolic contents

released during necrotic cell death (180). This distinct form of cell death was virtually ignored until 1972 when Kerr et al. (181) re-examined this phenomenon and named it apoptosis. Generally, active cell death has been believed to require active RNA and protein synthesis. However, it is now thought that the process of apoptosis may be more complicated and is carefully regulated by a balance between activators and suppressors of apoptosis (182-185).

Of the numerous known activators of apoptosis the two most relevant to this study are OFR's and agents capable of affecting protein synthesis. A number of proteins such as ced-3, ced-4 (ced stands for cell death defective) and p53 (183) may be turned on by oxidants (186,187), endocrine factors (188) or glucocorticoids (189) and are known to be involved in apoptosis. Due to their relevance to this project, the influence of OFR's and protein synthesis will be discussed below, respectively.

4.4 The role of Oxidants in the Apoptotic Process

Oxidants have been shown to play an important role in the mediation of apoptosis. Oxidant generating mechanisms (radiation, metabolism, ischemia, neutrophil activation, active overstretching) have all been shown to induce apoptosis (177,190-195) while antioxidants have been proven particularly effective in protecting against programmed cell death (196-199). For example, antioxidants can protect against apoptosis in lymphocytes (200,201), thymocytes (202-204), neurons (199,205,206), myocytes (195,207), and endothelial cells (208,209), possibly by altering gene expression, as has recently been shown with the antioxidant N-acetylcysteine (NAC). Interestingly, rather than acting as a

scavenger of OFR's, NAC was found to activate a suppressor of apoptosis, thereby inhibiting apoptosis.

The endogenous cellular antioxidant and reductant, glutathione (GSH), may also play a role in apoptosis. Decreased levels of GSH, signaling the presence of oxidants, may contribute to the activation of apoptosis. However, the role of GSH may be more complex than originally thought. For example, a recent study (210) has shown that GSH is decreased in human monocytic cells during programmed cell death without the intracellular or extracellular accumulation of oxidized GSH, glutathione disulfide (GSSG). In this study it was found that GSH was extruded during apoptosis and the observed decrease in GSH was not oxidant-mediated. In another study, the depletion of GSH failed to induce immediate programmed cell death (211). One study found decreased apoptosis, but increased necrosis, following GSH depletion (212). It is therefore likely that GSH does not play a direct role in some forms of apoptosis (213,214), but may do so in others.

4.5 The Role of *De Novo* Protein Synthesis

The role of *de novo* protein synthesis in apoptosis is presently controversial. Some apoptotic signalling pathways appear to require the synthesis of new proteins (215-218). However, other models of apoptosis apparently do not. For example, one study has suggested that *de novo* protein synthesis (in thymocytes) is primarily responsible for suppressor activity and any promotion of apoptosis due to a translational inhibitor is a result of the decrease in suppressor expression (219). This suggests that the apoptotic apparatus is always active in this model, but is continually depressed due to apoptotic

inhibitors, and indicates that proteins regulating the apoptotic process (both suppressor and enhancer) are continually expressed and need only activation to commence their task. It was determined by this study that the translational inhibitors may only be delaying the onset of apoptosis and that any protective effect observed may be temporary. Therefore, in summary, the role of *de novo* protein synthesis in apoptosis is not likely the creation/activation of a single set of proteins as previously thought. Instead, the synthesis of proteins which either inhibit or promote apoptosis may be balanced to produce the desired effect.

4.6 Apoptosis in the Reperfused Myocardium

It is widely accepted that necrotic death is abundant both during ischemia and following reperfusion in the myocardium. As described in section 4.1, necrosis results from a severe cellular trauma and causes the destruction of the cell membrane and subsequent release of cellular contents (151,174). This poses a threat to neighboring cells as some of the spilled contents are toxic, as well as proteolytic. Presently, there is a great deal of interest in whether apoptosis is also present in the ischemic and reperfused myocardium. Apoptosis in *in vivo* ischemic cardiomyocytes has been documented (220,221), while programmed cell death in the reperfused myocardium has been found in rabbits (207), rats (10,221) and recently, humans (222,223). It is now apparent that apoptosis is likely to be the predominant form of early cell death in the ischemic myocardium (220,221,223), or reperfused heart (221), and can occur as early as 2-3 hours following the commencement of the ischemic episode (220,221).

The acute effect of cardiac myocyte apoptosis on cardiac function is unknown. Although this form of cell disintegration ultimately results in the demise of the myocyte, it remains speculative as to what stage, or amount, of apoptosis is required before a global myocardial performance deficit becomes detectable. As well, to date no one has examined the performance characteristics of a single apoptotic myocyte.

The importance of blood borne agents (such as neutrophils) in reperfusion induced apoptosis has been questioned. In both the reperfused and the permanently occluded heart (no reperfusion), neutrophil activity increased similarly over normal heart tissue (221). Briefly, that study involved the ligature of the left anterior descending coronary artery, with various durations of occlusion and reperfusion. Reperfusion was obtained by removal of the ligature. In the reperfused myocardium, apoptosis was observed after only one hour of reperfusion following a 45 minute occlusion, in the permanently occluded myocardium, it was necessary to maintain the occlusion for a period of 2 hours and 45 minutes before apoptosis was visible. In this study, myeloperoxidase activity (an index of neutrophil activation) of the permanently ischemic and reperfused regions did not differ, suggesting that neutrophils were able to infiltrate the ischemic region without the aid of reperfusion during this time period. Thiol levels were significantly lower in both the ischemic and reperfused myocardium, possibly the result of neutrophil OFR's generated upon reperfusion. Another study (224) found that as the duration of ischemia increased, MPO activity increased as well. Neutrophil accumulation was quantified and found to be accurately represented by MPO activity, providing evidence that neutrophil activity may be similarly elevated in ischemic and reperfused tissues. A study by Buerke et al. (10)

used Insulin like Growth Factor-I to decrease neutrophil adhesion after myocardial ischemia and showed a correlation between the decrease in neutrophil accumulation and a decrease in reperfusion induced apoptosis. It therefore appears likely that the OFR's released during the inflammatory response increase apoptosis and that activated neutrophils may have a significant role in this process. However, this is controversial as one study found neutrophils did not potentiate apoptosis in reperfused rabbit hearts (207). To date no study has attempted to examine apoptosis in an isolated organ model, such as the isolated perfused heart in which neutrophils would not be expected to play a role. Removal of blood born agents would allow study of only the intrinsic modulators of apoptosis while also permitting the exploration of the mechanisms of induction of programmed cell death.

4.7 Involvement of Zinc in Apoptosis

Apoptosis has been found to be attenuated by metals such as Zn^{2+} (225-230), Cu^{2+} (231), Ca^{2+} (229,230,232,233), Fe^{2+} (208,231) and Cd^{2+} (231,234). Of these, the role of Zn^{2+} is particularly intriguing. It is well documented that Zn^{2+} can inhibit apoptosis, possibly by inhibiting the Ca^{2+}-dependent endonuclease responsible for DNA fragmentation in cells (228). Interestingly, in the brain model of ischemia induced apoptosis, the CA3 region of the hippocampus appears to be resistant to apoptotic cell death, and is also the region which contains the highest amounts of intracellular Zn^{2+}.

In addition to inhibiting endonucleases, zinc may also block apoptosis by preventing the production of OFR's. For example, a recent study used Zn^{2+} to prevent the

generation of OFR's by inhibiting the catalysts necessary for OFR generation (235) showing that Zn^{2+} protected cardiac tissue following an ischemic episode by decreasing the formation of ·OH radicals. Studies have found that Zn^{2+} reduced OFR generation, as detected by spin trap techniques (236), presumably by blocking the ability of Cu^{2+} and Fe^{2+} to catalyze OFR generating redox reactions. This antioxidant property of Zn^{2+} may therefore protect against apoptosis, in view of the well established ability of OFR to cause apoptosis in many cell types (195,199,201,202,237).

As well as inhibiting specific endonucleases and oxidant generating mechanisms involved in apoptosis, zinc may function directly as an antioxidant by binding to, and protecting, the vulnerable sulfhydryl groups (eg. cysteine residues) in proteins. This has been most successfully documented by studies using the enzyme δ-aminolevulinate dehydratase (238). A very strong relationship was found between thiol status and enzyme activity. As the protein thiols were oxidized, enzyme activity decreased. Addition of zinc prevented this oxidation and the subsequent functional impairment. This protection has been attributed to the prevention of thiol oxidation in cysteine residues. The antioxidant abilities of zinc may therefore decrease apoptosis by preventing oxidative stress.

4.8 Zinc induced metallothionein expression

Still another possible mechanism for protection against apoptosis by Zn^{2+} is by the synthesis of metallothionein (MT). Numerous studies have shown that Zn^{2+} can induce the synthesis of this thiol-rich, metal-binding protein (239,240). The amino acid

composition of MT consists of 25-30% cysteine residues. MT is present in four different isoforms, none of which contains any disulfide bonds under normal reduced conditions. MT I and II are present in the brain and the peripheral tissues. Type III is located almost exclusively in the brain, but is present in minute quantities in the pancreas and the intestine. Isoform IV is found only in tissues containing stratified epithelial cells. Because MT is so rich in thiols, it possesses strong antioxidant properties (241-243).

Studies have confirmed that MT can serve as an antioxidant (242,244,245). Transgenic mice overexpressing MT have shown a decreased susceptibility to the potent oxidant NO (242), suggesting that MT thiols served as antioxidants. Although numerous tissue culture studies have demonstrated a similar effect in vitro (246), to the best of our knowledge no one has successfully induced MT overexpression in the rat myocardium (247).

Interestingly, the oxidation of MT thiols can release the bound metals. Seven zinc atoms may be bound to the thiols of a single MT protein, all of which are in groups of cysteine residues called zinc clusters. The oxidation of these clusters forms disulfide bonds and liberates the bound zinc (143,243,248). Since free zinc is known to provide antioxidant protection, it may be that this mobilization of zinc offers a second form of protection against apoptosis.

Therefore, this thesis hypothesizes that apoptosis may be detrimental to the acute post-ischemic cardiac performance measured by this study. As well, this work explored the possibility that, by modulating apoptosis, the post-ischemic decrease in performance would also be altered.

PROJECT OBJECTIVES:

In view of the increasing importance of apoptosis as a mechanism of cell death in the myocardium, it is clear that a better understanding of the mechanisms of apoptosis may offer opportunities for effective therapeutic intervention. Unlike the *in vivo* model of myocardial ischemia, where numerous systemic functions may obscure the myocardial processes, an *in vitro* model offers a better opportunity to investigate apoptosis specifically in the myocytes.

Therefore, I explored the role of apoptosis in ischemic injury in an isolated buffer-perfused rat heart model. The isolated perfused heart allows easy monitoring of myocardial function without the confounding influences of normal blood constituents such as leukocytes, erythrocytes, platelets, endocrine factors, plasma proteins and fibrinogen, all of which may obscure the apoptotic process. The Langendorff apparatus permits easy and accurate manipulation of the conditions involving ischemia and reperfusion, as well as the introduction of therapeutic agents.

Perhaps the greatest benefit of the Langendorff apparatus is that it permits the monitoring of contractile performance. This allows an accurate and rapid determination of cardiac function, and the effects on performance of various agents.

Apoptotic manipulation by oxidants and antioxidants, as well as inhibitors of protein synthesis was attempted in this study, and the performance of the hearts was correlated with a number of biochemical parameters such as protein oxidation and DNA fragmentation.

The objectives of this study were therefore to:

1) Develop an in vitro model of ischemia-induced apoptosis in an isolated perfused rat heart.

2) Using the in vitro model, determine the effects of compounds known to inhibit apoptosis such as antioxidants and inhibitors of protein synthesis.

3) Determine if there is a correlation between apoptosis and contractile performance.

METHODS

Langendorff isolated perfused heart

Male sprague Dawley rats (275-325g) were anesthetized using sodium pentobarbital injection (65 mg/kg body weight) and their hearts were chilled and rapidly excised. The hearts were cannulated and perfused retrogradely through the aorta at a rate of flow required to provide an aortic root pressure of 50-55 mm Hg, using a peristaltic pump, with Krebs Ringer (KR) buffer (120mM NaCl, 10mM glucose, 4.8mM KCl, 2.5mM $CaCl_2$, 1.2mM $MgSO_4$, 1.2mM KH_2PO_4, 25mM $NaHCO_3$, 0.5mM EDTA) at 37°C, bubbled with 5% CO_2 in 95% oxygen. Hearts were paced using a grass stimulator at 280 bpm. A water filled cellophane balloon inserted through the mitral valve, was used to monitor both the peak systolic and end-diastolic pressures using pressure transducers and a Grass recorder/polygraph. The left ventricular developed pressure (LVDP) was calculated as the difference between the peak systolic and end-diastolic pressures, with higher values representing increased contractility and thus a greater stroke volume *in vivo*. Typical tracings appear in Figure 2 and indicate systolic and diastolic pressures. The perfusion pressure was monitored visually using a mercury manometer. All hearts began the ischemic insult with a perfusion pressure between 50-55 mm Hg, a minimum peak contractile force of 80 mm Hg and a resting tension of 8 to 12 mm Hg following a 15 minute equilibration period.

FIGURE 2. A photograph of a typical tracing showing the contractile properties of the isolated perfused rat heart. The maximal height obtained represents the peak tension associated with systolic pressure while the minimum pressure indicates the resting tension, or diastolic pressure. Seen below the tracing is the time scale, where each large line represents one minute. At t=9 min, the heart was rendered ischemic for a period of 6 minutes. The contractility is determined by relation to the scale (in mmHg) This tracing does not represent data collected for any of the protocols in this study and is shown merely as an example of the recordings taken.

The ischemic insult consisted of a 30 minute period of complete cessation of flow to the heart. During ischemia, the heart was maintained at 37°C in a thermostat-regulated chamber while humidified nitrogen was passed through the chamber to maintain an oxygen deprived environment. Following the ischemic insult, hearts underwent 4 hours of reperfusion. Rat hearts were subjected to various treatment protocols to monitor the effect on apoptosis. The number of hearts per group varied (minimum of three). The groups were as follows:

Control (CONT): Hearts were perfused with KR buffer without ischemia

Reperfused (IR): Hearts were subjected to ischemia and reperfusion

DTT (DTT): the reducing agent and antioxidant dithiothreitol (10 uM-final concentration) was included to the perfusate for the first hour of reperfusion

Monochloramine (MCl): the oxidant monochloramine (0.1 uM-final concentration) was added to the perfusate for the first 3 h of reperfusion.

Zn^{2+}-pyrithione (Zn PYR): the zinc ionophore Zn^{2+}-pyrithione (0.1 uM-final concentration) was added to the perfusate for 30 min prior to ischemia and for the first 30 min of reperfusion.

Cycloheximide (CYCLO): 10 uM (final concentration) of the protein synthesis inhibitor cycloheximide was added to the perfusate for 30 min prior to ischemia and for the entire 4 hr

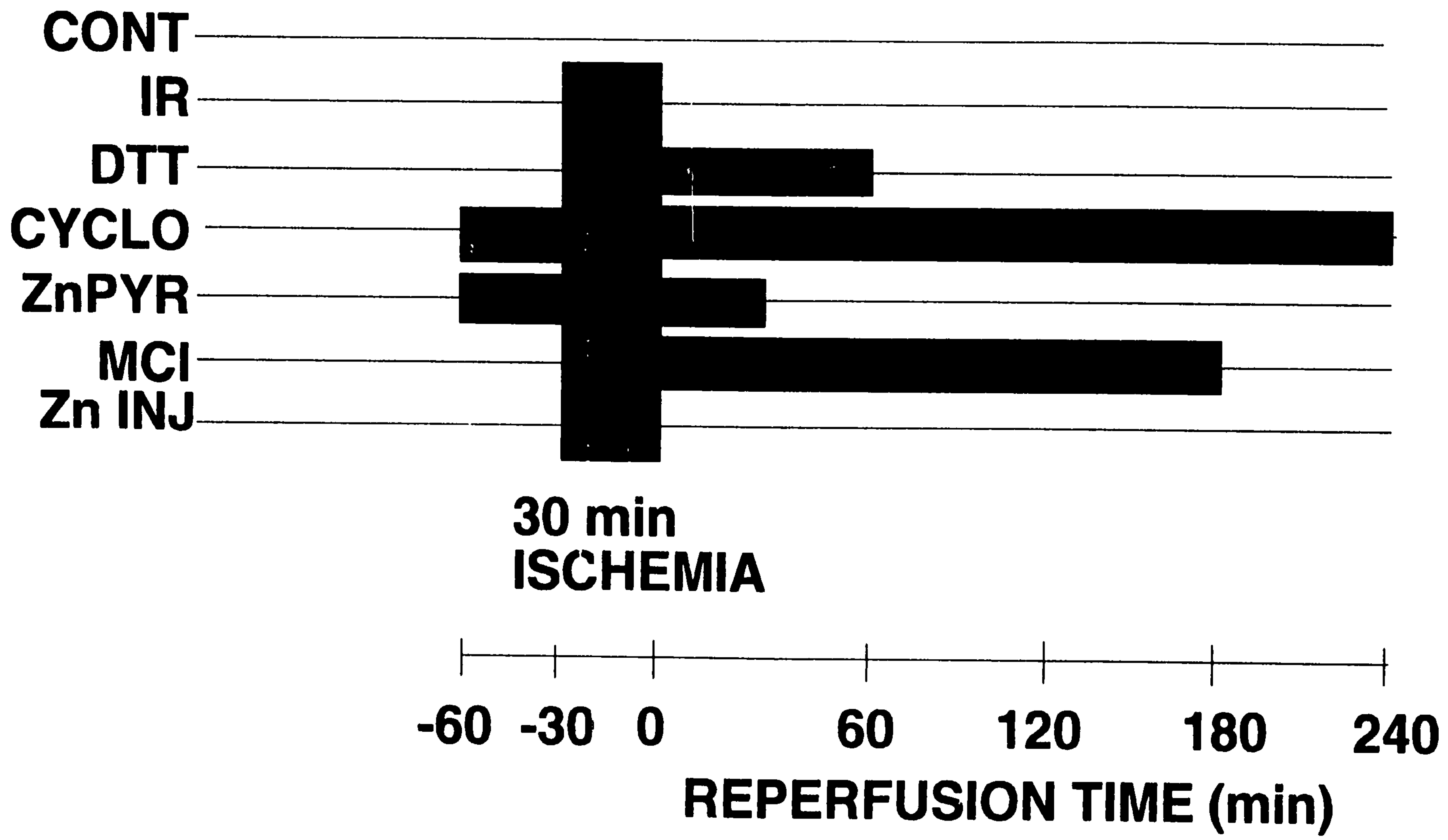
duration of the reperfusion.

Zn^{2+} Injected (Zn INJ): rats were injected intraperitoneally with zinc (10 mg Zn/kg BW in 3mL sterile saline) 18 h prior to excision of the hearts. Hearts were subjected to ischemia and reperfusion as in the IR group.

Non-Perfused (NP): hearts were removed from untreated rats and were immediately frozen for future analysis without any perfusion.

The protocols described above are summarized in Figure 3

FIGURE 3. The treatments appearing on the left side of the diagram were applied during the time indicated by the solid bars. The shaded vertical bar represents 30 minutes ischemia while the thin horizontal lines represent perfusion (pre-ischemic and reperfusion). CONT GROUP hearts were not subjected to ischemia while the IR GROUP and the Zn INJECTED GROUP did not receive any treatment (beyond the ischemic episode) during the experimental protocol. DTT, CYCLO, ZnPYR and MCI treated hearts received the respective treatment during the time indicated by the solid horizontal bars.


PERFUSION PROTOCOL
CONT
IR
DTT
CYCLO
ZnPYR
MCI
Zn INJ
30 min
ISCHEMIA
-60
-30
0
60
120
180
240
REPERFUSION TIME (min)

Tissue homogenization and freezing

Following reperfusion, the hearts were chilled rapidly, and were cut transversely into 2 parts, apical and basal. The apical portion was frozen rapidly over dry ice in preparation for cryostat sectioning and In-Situ-End-Labelling (ISEL). The basal part was homogenized using a polytron at 10,000 rpm for 30 seconds in an equal volume of phosphate buffered saline (PBS) at 0°C, containing 0.5 mM PMSF and 10% glycerol. The crude homogenate was frozen for future DNA extraction and electrophoresis, and DTNB assays (see below).

DNA extraction and electrophoresis

Frozen homogenates (100 ul) were treated with 1.25 ml of lysis buffer (10 mM Tris-HCl, 10 mM EDTA, 75 mM NaCl, 0.5% SDS, pH 8.0) at room temperature for 15 minutes in a 1.5 ml Ependorff tube and then spun in a microcentrifuge at 13,000g for 15 minutes. The supernatant containing the fragmented DNA, was separated from the viscous pellet which contained the intact DNA. RNAse (100 ug) was added to the supernatant which was then incubated for 30 minutes at 37°C. Proteinase K (100 ug) was then added and the supernatant incubated at 60°C. The DNA was precipitated overnight in 50% ethanol, 0.5 N NaCl, at -20°C. The precipitated DNA was collected by spinning at 13,000g for 15 minutes at 4°C, and was then dried at room temperature for 30 minutes. The pellet was resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and the DNA was extracted with 50% phenol/chloroform and chloroform washes. The ethanol precipitation

was repeated (minimum of 1 hour incubation) at -20°C and the DNA was centrifuged, dried and dissolved in 50 ul of TE buffer. The DNA concentration was determined spectrophotometrically at 260 nm, and a ratio of 260/280 nm =2.0 was used as an index of purity. DNA (10 ug) was subjected to electrophoresis on agarose gels (1.5%) at 100V in TAE buffer (400 mM Tris, 300 mM Acetic acid, 20 mM EDTA, pH 8.0). The gel was stained with 0.5 ug/ml ethidium bromide at room temperature for 30 minutes then photographed over an ultraviolet light source.

In-Situ-End-Labelling (ISEL)

Previously published procedures (249-251) provided the basis for the ISEL technique used. All reagents were products of Sigma Chemical (St. Louis, MO) or BDH (Toronto, Canada). Frozen tissue sections were simultaneously thawed and fixed in 1% glutaraldehyde for 15 min at room temperature (RT), washed two times for 5 minutes each with phosphate buffered saline (PBS), then permeabilized with methanol/acetone (1:1) for 10 minutes at RT. Following two more PBS washes, the sections were incubated for 15 minutes at RT with 20 ug/ml proteinase K in 25 mM Tris-HCl (1ml/section), pH 6.6. After two water washes of 15 minutes each, the sections were stained with Hoechst 33258 (0.05 ug/ml) at RT for 30 min in the dark. Three more PBS washes (1 min) were done and the sections were then incubated in a buffer containing 200mM potassium cacodylate, 2 mM $CoCl_2$, 0.25 mg/ml bovine serum albumin, 25 mM Tris-HCl, pH 6.6, 10 µM biotin-16-dUTP (from Boehringer Mannheim Canada, Laval, Quebec), and 25 units of terminal transferase (Boehringer) in a humidified chamber at 37° for one hour. The

sections were washed in PBS three times for 1 minute each at RT to stop the reaction and were then incubated with 1 ml of a solution containing avidin-FITC (2.5 ug/ml fluorescein isothiocyanate-avidin), 4X saline-sodium citrate buffer, 0.1% Triton X-100, and 5% powdered milk, for 30 min at RT protected from light. The sections were washed 3 times with PBS prior to coverslipping in "anti-fade" solution (1 mg/ml p-phenylenediamine, 90% glycerol, in PBS). The sections were viewed under a Zeiss Axiophot florescence microscope. Positive control sections were treated with 5 units/ml DNAse for 1 minute at 37°C prior to terminal transferase treatment.

Bradford Assay for Protein Concentration

The concentration of protein in solutions was determined spectrophotometrically at 595 nm using the Bio-Rad kit and bovine serum albumin (BSA) as a standard.

Dithio-Nitrobenzoic Acid (DTNB) Assay for Thiol Concentration

Aliquots of homogenate were clarified by centrifugation, brought to 5 mg/ml protein concentration and analyzed for total thiol (TSH), glutathione (GSH) and protein sulfhydryl (PSH) content using DTNB. For total thiol content, the reaction mixture consisted of homogenate, 1 mM DTNB, and 6M guanidine-HCl in 50 mM Tris-HCl (pH 8.2). The mixture was incubated in the dark for 30 min and then read at 412 nm against 6M guanidine-HCl and DTNB blanks. To measure GSH, samples of homogenate at 5 mg protein/ml were precipitated with 5% trichloroacetic acid (TCA) at 4°C for 15 minutes. The samples were then centrifuged at 13,000 g for 5 minutes. The supernatant was removed,

its pH was adjusted to 8.2 with NaOH, and it was then reacted with DTNB alone.

Protein sulfhydryl content was determined by subtracting the GSH content from the TSH. A molar extinction coefficient of 13,600 was used for the calculation, and all values were reported as amount of thiol per mg protein.

Zn^{2+}-induced MT Overexpression

Rats in the "Zn^{2+}-injected" group were injected with zinc (10mg/Kg body weight) in the form of $ZnCl_2$ (BDH) 18 hours prior to the ischemic episode. The amount of zinc injected was based on a previously published study examining the effect of zinc injection on ischemia induced infarct size in dog hearts (252). Zinc was dissolved in 3 ml of sterile saline and injected intraperitoneally. The blood level of zinc was not measured and it was assumed that all of the injected zinc was accommodated by MT overexpression. A dose response curve was not deemed necessary.

Metallothionein Determination Assay

Heart homogenates were diluted to 5 mg/ml protein and were analyzed for metallothionein content according to the published procedure of Eaton and Toal (253). Briefly, homogenates from Zn^{2+} injected rats were placed in boiling water for 2 min to denature the heat-labile proteins, then centrifuged (13,000 g) for 2 min, leaving MT as the only soluble metal binding protein. The supernatant was removed and incubated with a solution containing ^{109}Cd (10 mM Tris-HCl, pH 7.4, 2 ug/ml Cd^{2+} at 1 uCi/ml). The mixture was allowed to react for 10 minutes then 2% hemoglobin was added to complex the

excess ^{109}Cd. The mixture was boiled again to precipitate the Cd-hemoglobin, and was spun leaving Cd-labelled MT in the supernatant. The pellet was discarded and the radioactivity in the supernatant analyzed with a scintillation counter. The amount of metallothionein was then calculated. Sephadex column chromatography was used to verify that MT was the only ^{109}Cd binding protein remaining in the supernatant (see below).

Sephadex Column Chromatography

Column chromatography was used to characterize the Cd-binding proteins in the supernatant following the ^{109}Cd treatment described above. For control purposes, authentic MT (500 µg) was reacted with ^{109}Cd, and was applied to a sephadex G75 column with a length of 32 cm and a width of 1.2 cm. Fractions of the eluate were collected and were assessed for both ^{109}Cd (scintillation counter) and protein content (absorbance at 220 nm).

Statistical Analysis

Students t-tests were completed using Sigmaplot for Windows software produced by Jandel Scientific. Cardiac performance was analyzed using a split-plot ANOVA on SAS software, while thiol data (Fig 18) and MT overexpression (Fig 12) was examined using a Newman-Keuls ANOVA on Kwikstat software. Only p values of less than 0.05 were considered significant. Error bars represent standard error of the mean (SEM).

Results

Cardiac Performance

Control Group

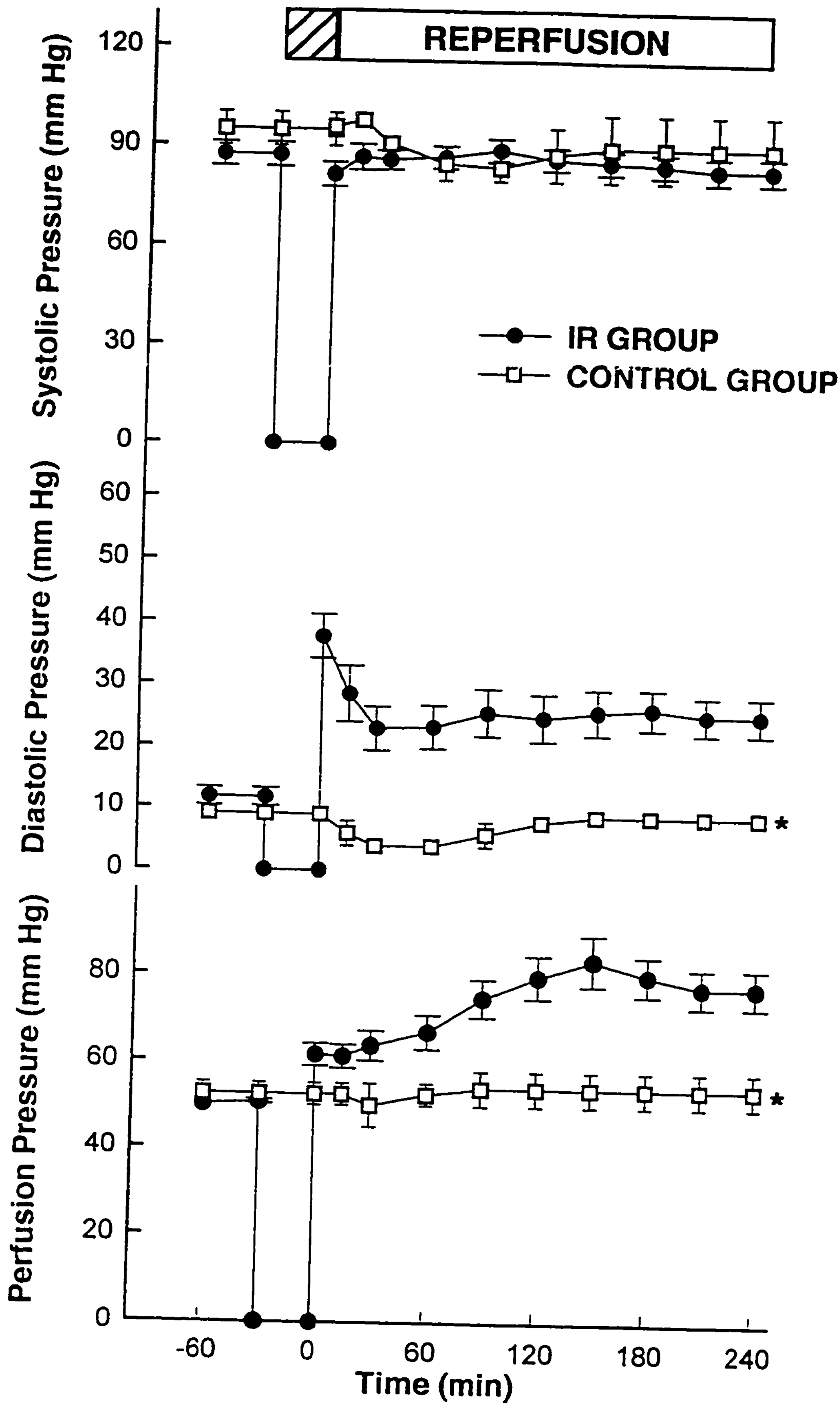
Hearts could be perfused for up to 5 hours without displaying any changes in any of the monitored parameters (Figure 4). Both end-diastolic (resting tension) and peak systolic pressure remained at their initial levels providing a constant left ventricular developed pressure (LVDP) (Figure 5). The perfusion pressure also remained unchanged.

Reperfused Group

Hearts which were rendered ischemic for 30 minutes and subsequently reperfused for a 4 hour period, displayed significant contractile disfunction (Figure 4). Perfusion pressure was significantly elevated from the pre-ischemic values ($p<0.05$, ANOVA), as was the resting tension ($p<0.05$, ANOVA), indicating cardiac injury. Although peak systolic pressure was unchanged, the increase in resting tension resulted in a significant ($p<0.05$, ANOVA) decrease in the contractility of the myocardium (LVDP) (Figure 5).

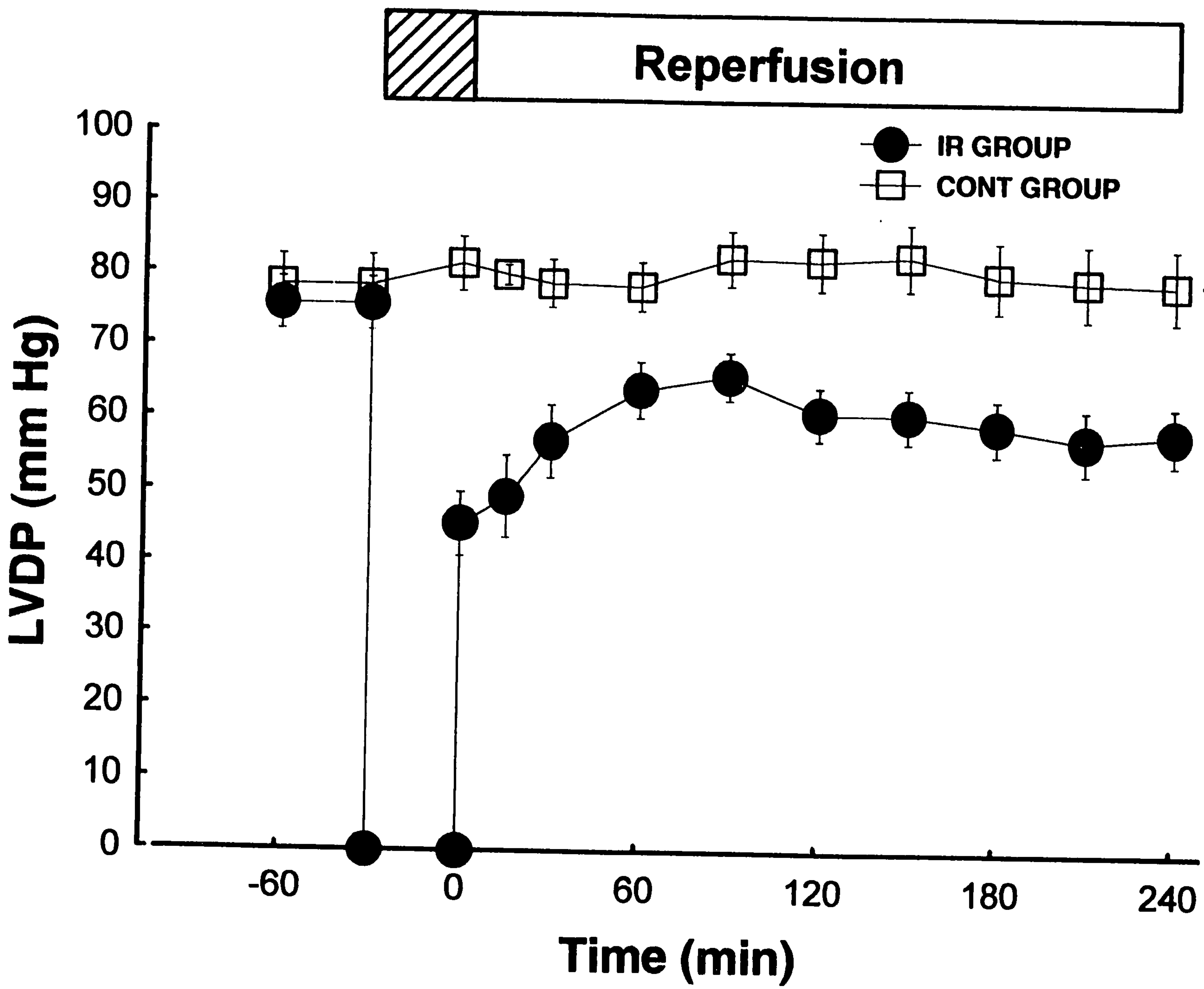
FIGURE 4. Cardiac performance of the CONT GROUP and the IR GROUP is shown in mm Hg vs. time in minutes. Rat hearts were perfused with KR buffer, and the systolic (top panel), diastolic (middle panel), and perfusion (bottom panel) pressure monitored as described in the Methods section.The hatched bar above systolic pressure indicates the period of ischemia while the clear bar indicates the duration of the subsequent reperfusion.

CONT GROUP hearts did not undergo an ischemic episode and were continually perfused. The performance of CONT GROUP hearts (□) was stable and did not change significantly throughout the entire perfusion (n=4). IR GROUP hearts (●) displayed characteristics typical of reperfusion injury following the ischemic episode (n=23). Both the diastolic and perfusion pressures were significantly elevated (*, $p<0.05$, ANOVA) in IR hearts vs. the IR GROUP.



* significant @ p<0.01 (ANOVA) vs. IR GROUP

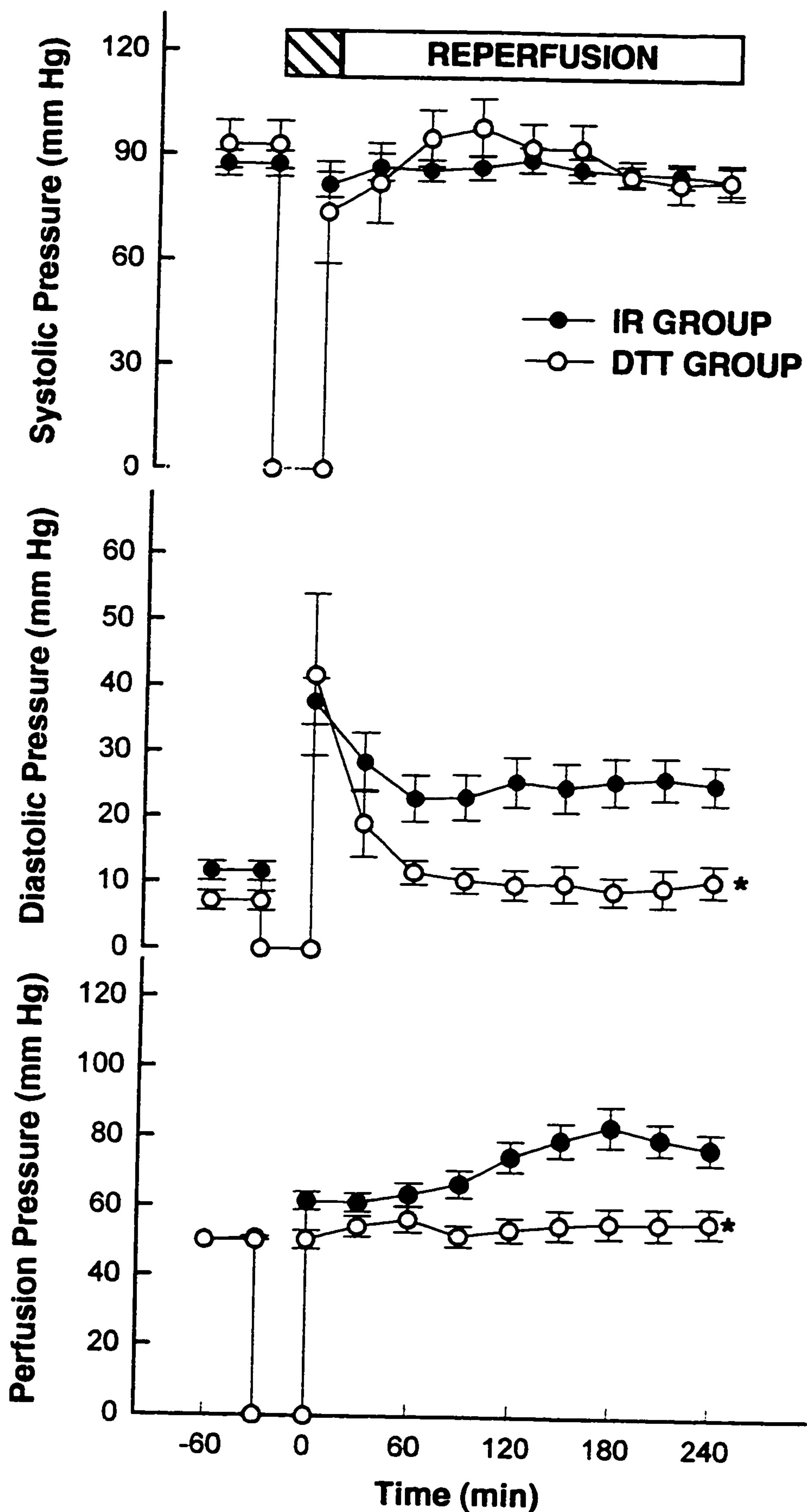
FIGURE 5. LVDP (mm Hg) vs. time. LVDP is the difference between peak systolic and diastolic pressure. CONT GROUP (□) hearts remained at their initial level of performance, while IR GROUP hearts (●) showed decreased LVDP (*, $p<0.05$, ANOVA) following the ischemic episode (indicated by the hatched bar on top).


Reperfusion
IR GROUP
CONT GROUP
LVDP (mm Hg)
100
90
80
70
60
50
40
30
20
10
0
-60
0
60
120
180
240
Time (min)
*
* significant @ p<0.05 (ANOVA) vs. IR GROUP

Antioxidant Treatment

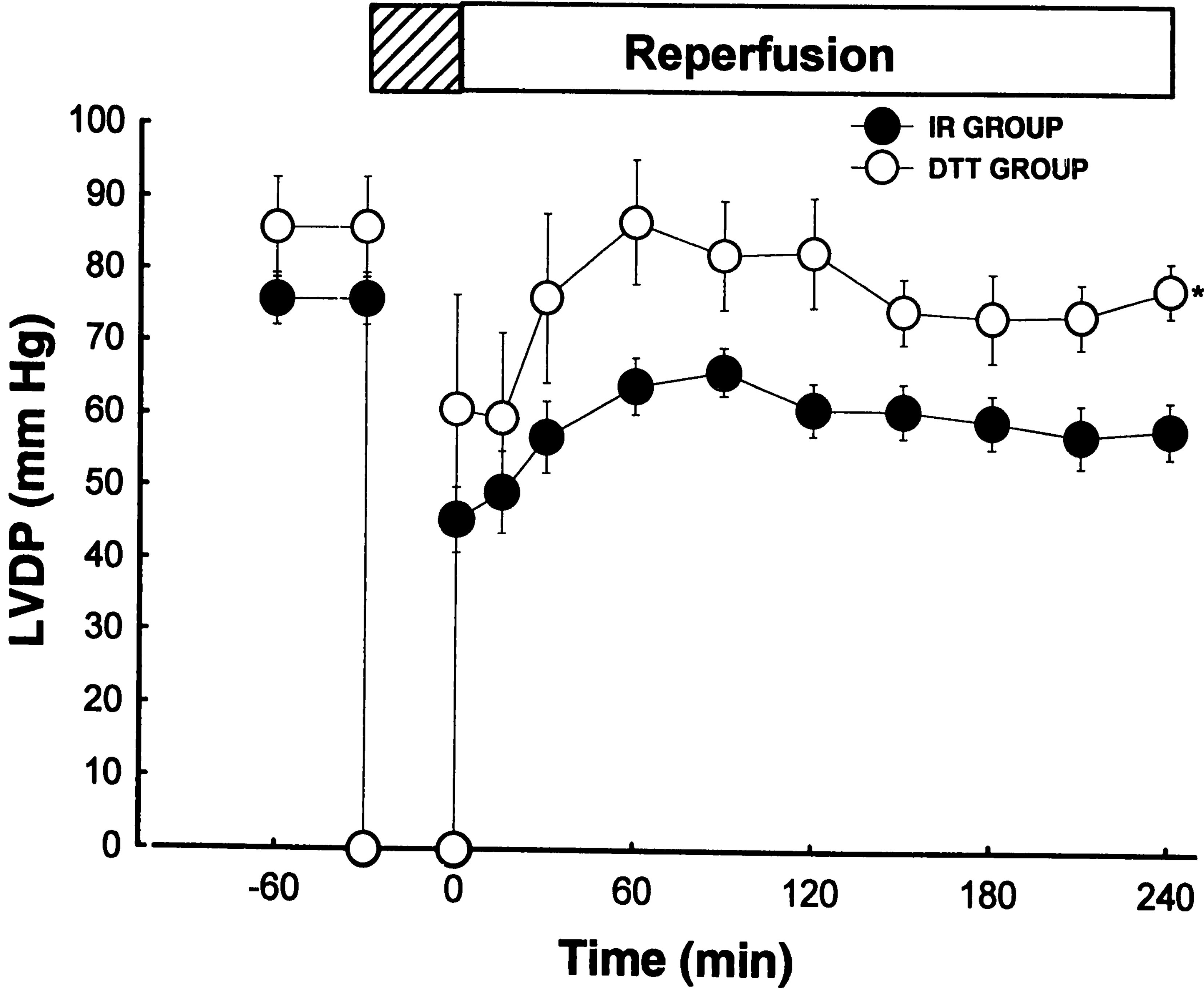
The ability of antioxidants to protect against ischemia/reperfusion-induced contractile disfunction was examined with the DTT group (Figure 6). Addition of DTT to the perfusate for the first hour of reperfusion significantly lowered the diastolic pressure and significantly increased LVDP (Figure 7) and perfusion pressure when compared to the reperfused group ($p<0.05$, ANOVA).

FIGURE 6. Cardiac performance of the DTT GROUP (O) (n=7) compared to the IR GROUP (●). Systolic pressure was unchanged with DTT treatment, but both diastolic and perfusion pressures were decreased as a result of the antioxidant application (*, $p<0.05$, ANOVA) for the initial hour of reperfusion, showing that DTT is capable of protecting tissues from the decrease in performance due to an ischemic injury.



* significant @ p<0.05 (ANOVA) vs. IR GROUP

FIGURE 7. LVDP with the antioxidant treatment, DTT (n=7). The DTT GROUP (O) displayed significantly improved contractility over the IR GROUP (●) (* p<0.05, ANOVA).

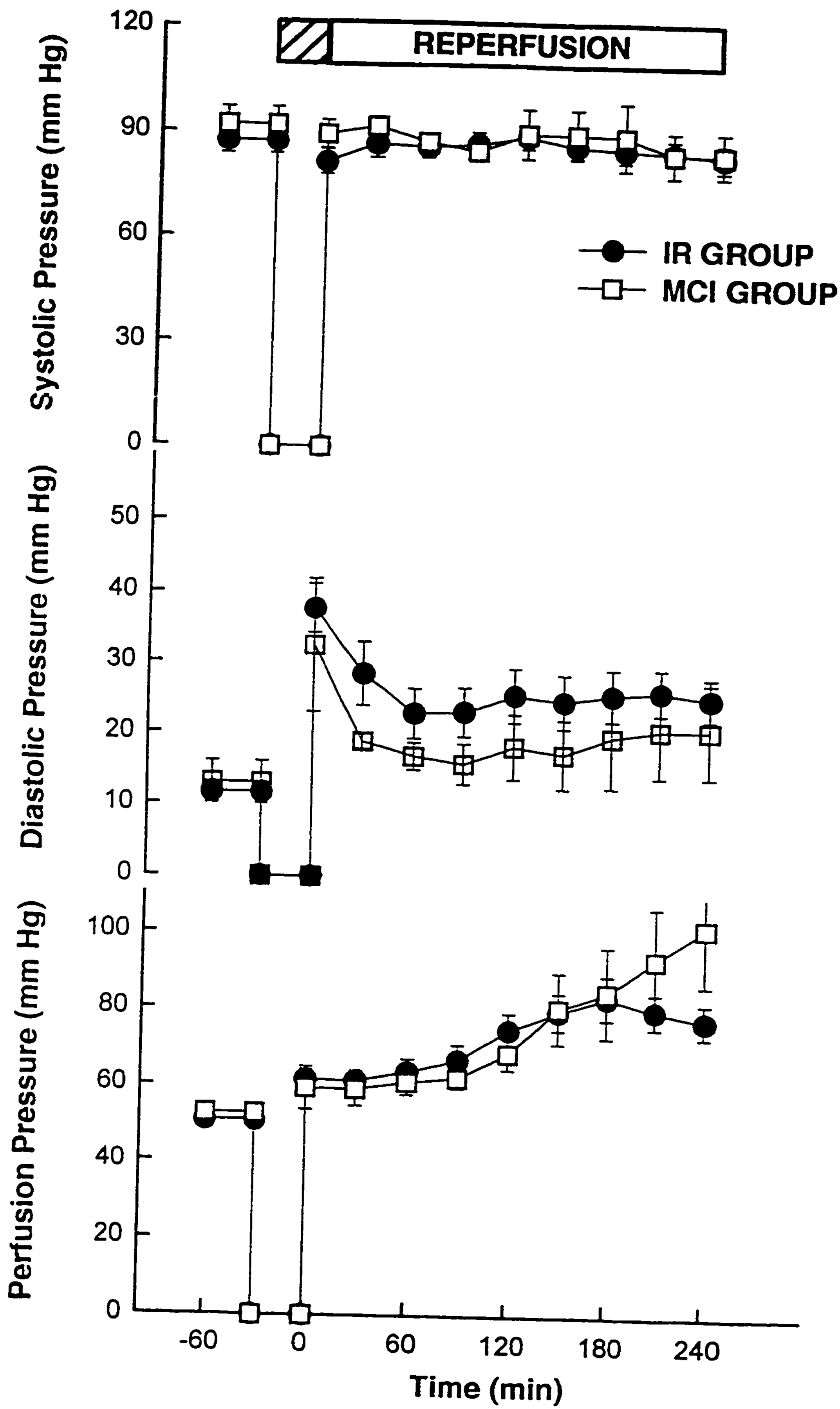


* significant @ $p<0.05$ (ANOVA) vs. IR GROUP

Monochloramine Group

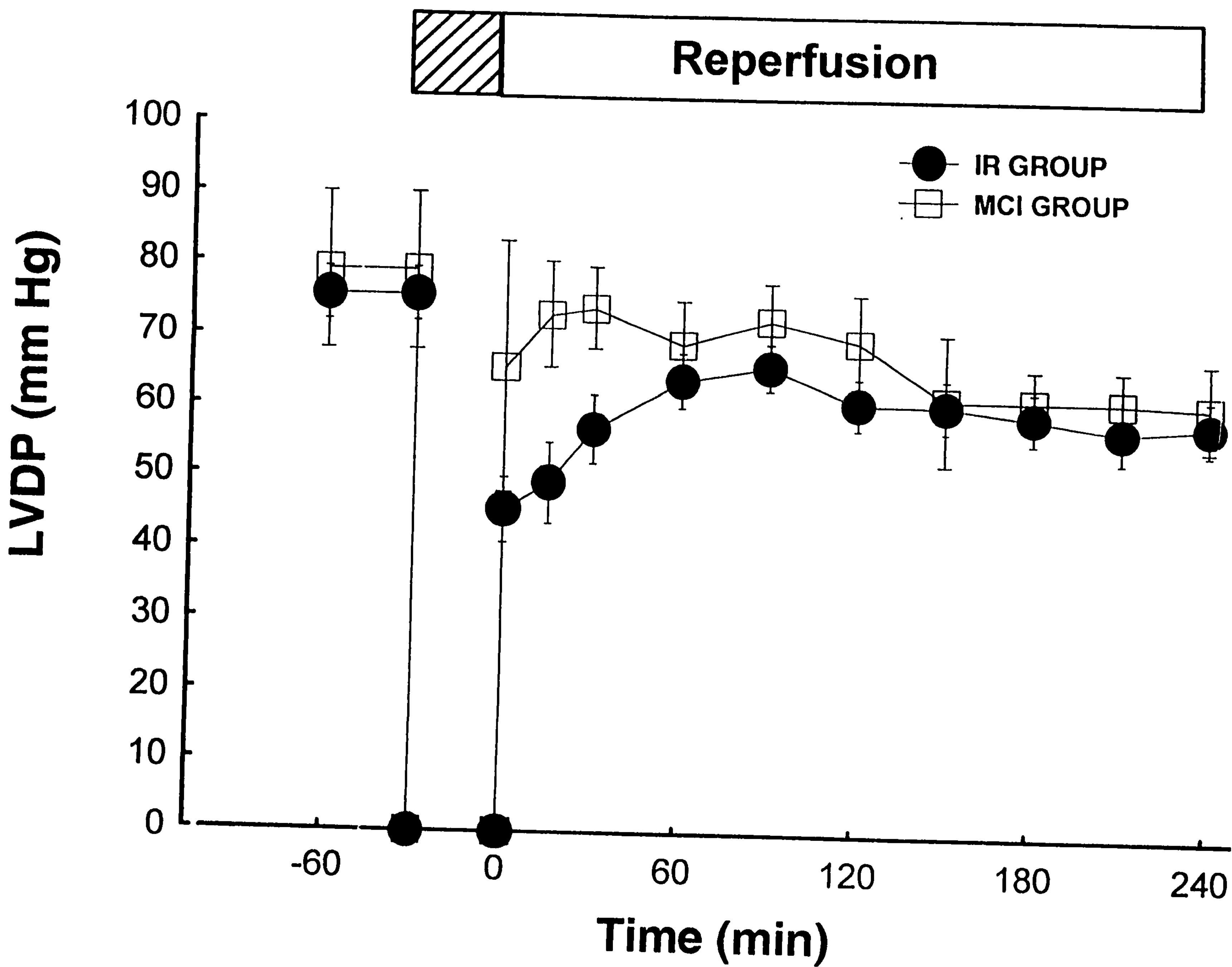
Monochloramine (0.1 uM), a potent and membrane permeant oxidant, was added to the perfusate for the initial 3 hours of reperfusion in order to simulate an inflammatory response. Monochloramine addition did not significantly change cardiac performance under these conditions (Figure 8). In comparison with the reperfused group, the systolic, diastolic, LVDP (Figure 9) and perfusion pressures of the MCl group remained unaltered.

FIGURE 8. Cardiac performance of isolated hearts exposed to the oxidant MCI (n=4) for the first 3 h of reperfusion. No significant difference between MCI GROUP (□) and IR GROUP (●) was detected for any measured parameter during the perfusion protocol.



REPERFUSION
Systolic Pressure (mm Hg)
120
90
60
30
0
IR GROUP
MCI GROUP
Diastolic Pressure (mm Hg)
50
40
30
20
10
0
Perfusion Pressure (mm Hg)
100
80
60
40
20
0
-60
0
60
120
180
240
Time (min)

FIGURE 9. LVDP of the MCI GROUP (□) (n=4) versus the IR GROUP (●). No significant difference was observed indicating that the potent oxidant did not further aggravate contractility of the isolated hearts under the conditions of the perfusion protocol.



Reperfusion
IR GROUP
MCI GROUP
LVDP (mm Hg)
100
90
80
70
60
50
40
30
20
10
0
-60
0
60
120
180
240
Time (min)

Zinc Pyrithione Group

Zinc pyrithione (a zinc ionophore) facilitates zinc entry into the cells. The inclusion of 0.1 uM Zn^{2+}-pyrithione in the perfusate for 30 min prior to ischemia, and for the first 30 minutes of reperfusion, did not significantly modify the perfusion, systolic or diastolic pressures (Figure 10), or the LVDP (Figure 11), of hearts when compared to the IR group. An increase in intracellular Zn^{2+} therefore failed to improve post-ischemic performance.

FIGURE 10. Cardiac performance of the Zn^{2+} PYRITHIONE GROUP (□) compared to the IR GROUP (●). No significant alterations in cardiac performance (systolic, diastolic, or perfusion pressure) were observed with the addition of 0.1uM Zn^{2+} Pyrithione to the isolated hearts for 30 min pre and post ischemia (n=7).

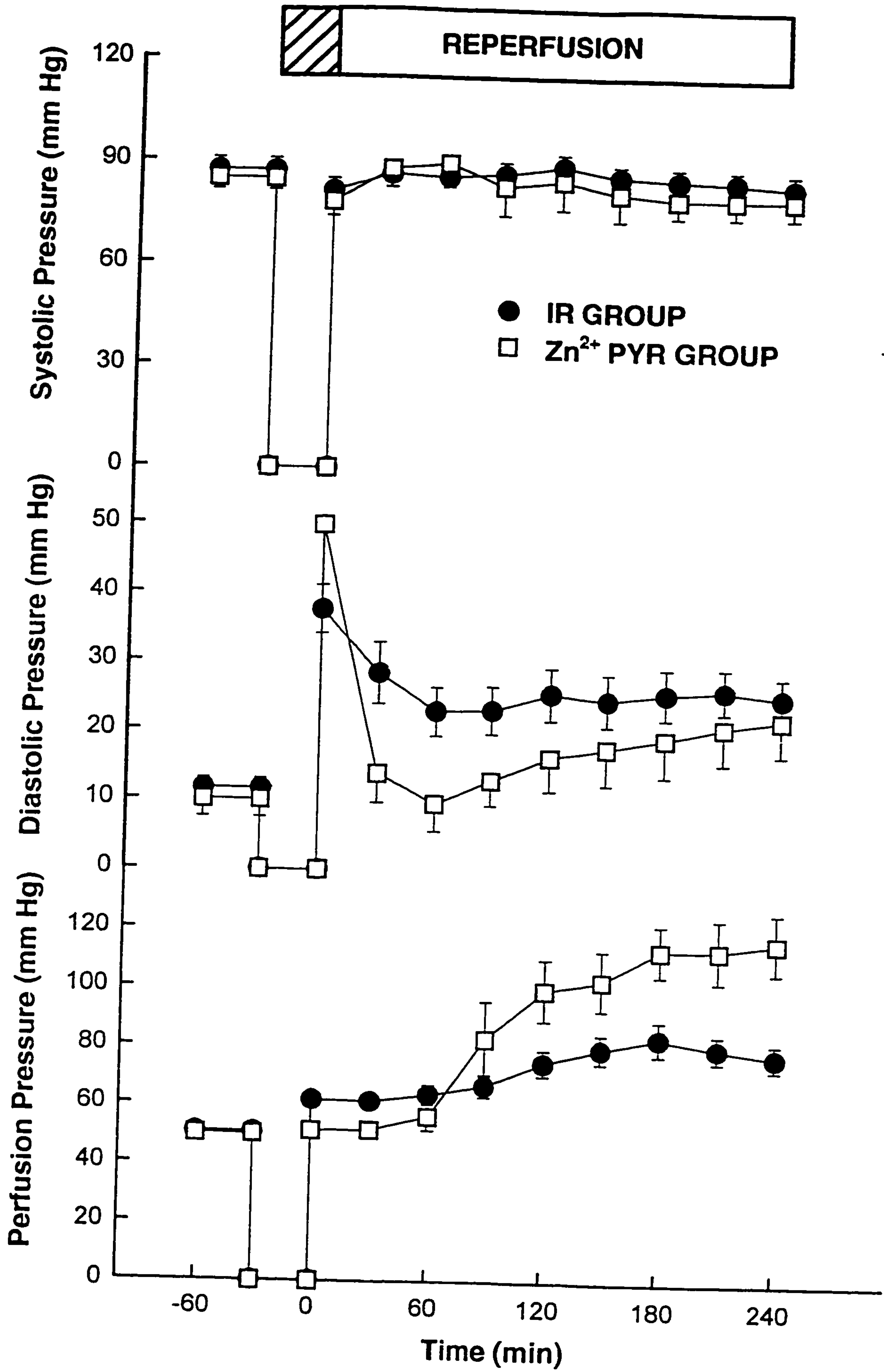
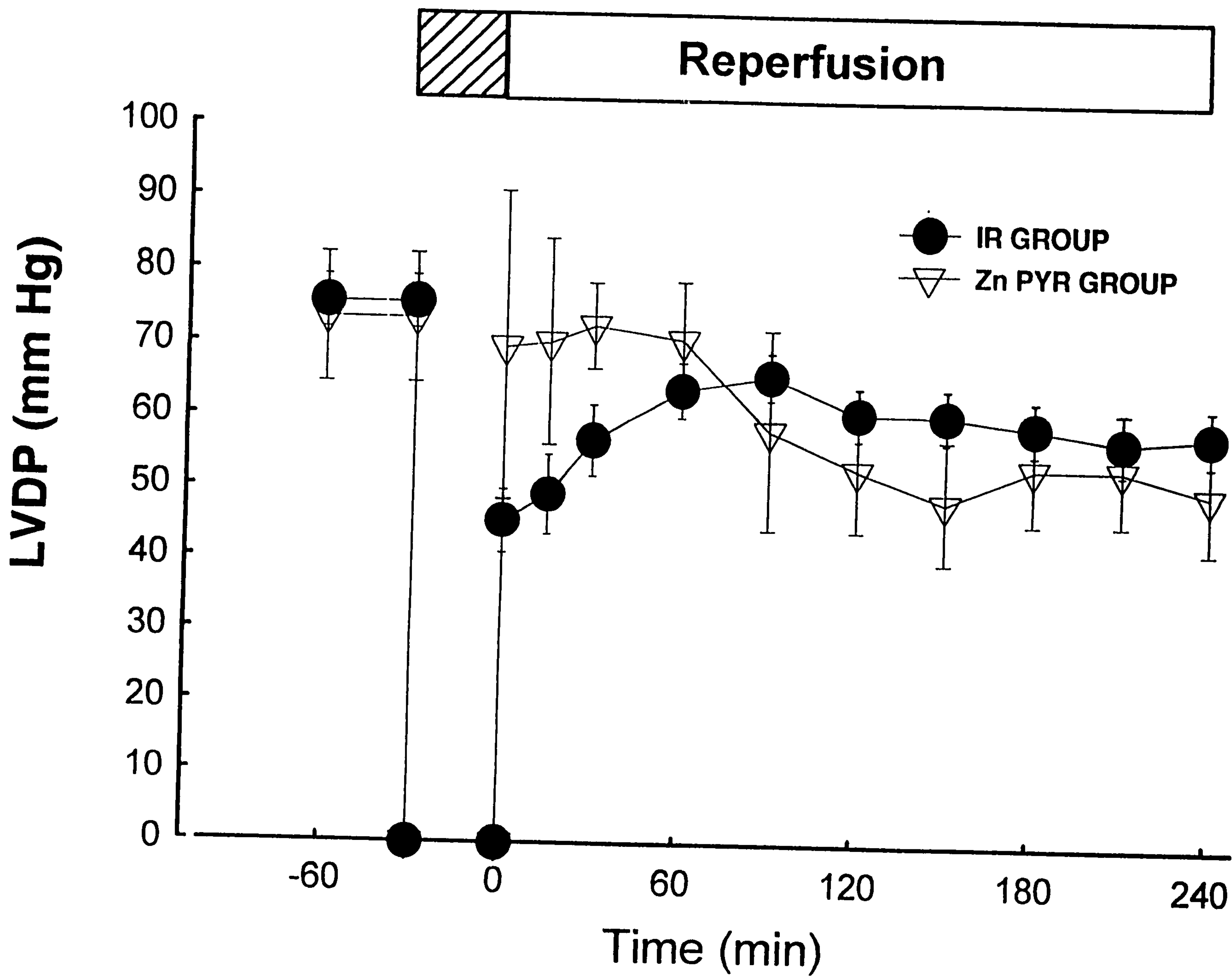

REPERFUSION
Systolic Pressure (mm Hg)
120
90
60
30
0
IR GROUP
Zn2+ PYR GROUP
Diastolic Pressure (mm Hg)
50
40
30
20
10
0
Perfusion Pressure (mm Hg)
120
100
80
60
40
20
0
-60
0
60
120
180
240
Time (min)

FIGURE 11. The LVDP of the Zn^{2+} PYR GROUP (▿) was not significantly different from the IR GROUP (●).



Reperfusion
LVDP (mm Hg)
100
90
80
70
60
50
40
30
20
10
0
IR GROUP
Zn PYR GROUP
-60
0
60
120
180
240
Time (min)

Zinc Injected Group

The effects of intraperitoneal Zn^{2+} injection on metallothionein expression in rat hearts was examined as a function of time. A maximal elevation in metallothionein content was observed, using the MT determination assay as described in Methods, at 18 hours following Zn^{2+} injection (10 mg/kg) (Figure 12). These data are similar to those of Klein et al. (254) who determined that MT showed maximal elevation at 18 hours in the rat liver. Sephadex column chromatography verified that MT was the sole ^{109}Cd binding protein recorded by the assay (Figure 13). A previous study by Klein et al. (254) showed a similar elution volume for MT. However, the intracellular elevation of this endogenous antioxidant did not significantly improve either the performance of reperfused hearts (Figure 14) or LVDP (Figure 15) when compared to the IR group (n=7), although the performance was highly variable (for unknown reasons). Perhaps there was variability in the zinc injection and some rats were better able to accommodate the excess zinc, leading to better antioxidant protection. To ascertain if there truly is no difference more hearts should be examined. Thus, in this model of ischemia/reperfusion, neither MT overexpression nor zinc injection appears to affect cardiac performance.

FIGURE 12. The maximal expression of MT occurred at 18 h following the injection of 10 mg/Kg body weight Zn^{2+} (in the form of $ZnCl_2$). MT expression was unchanged from the control condition (1.85±0.16 nmol/mg protein, n=11) at 4 h (1.86±0.33 nmol/mg protein, n=2), was approximately double by 12 h (4.25±0.97 nmol/mg protein, n=3) and more than quadruple at 18 h (9.03±1.19 nmol/mg protein, n=8). By 24 h, MT expression had returned to control levels (2.26±0.33 nmol/mg protein, n=14). Significance (ANOVA) was attained at 18 hours only.

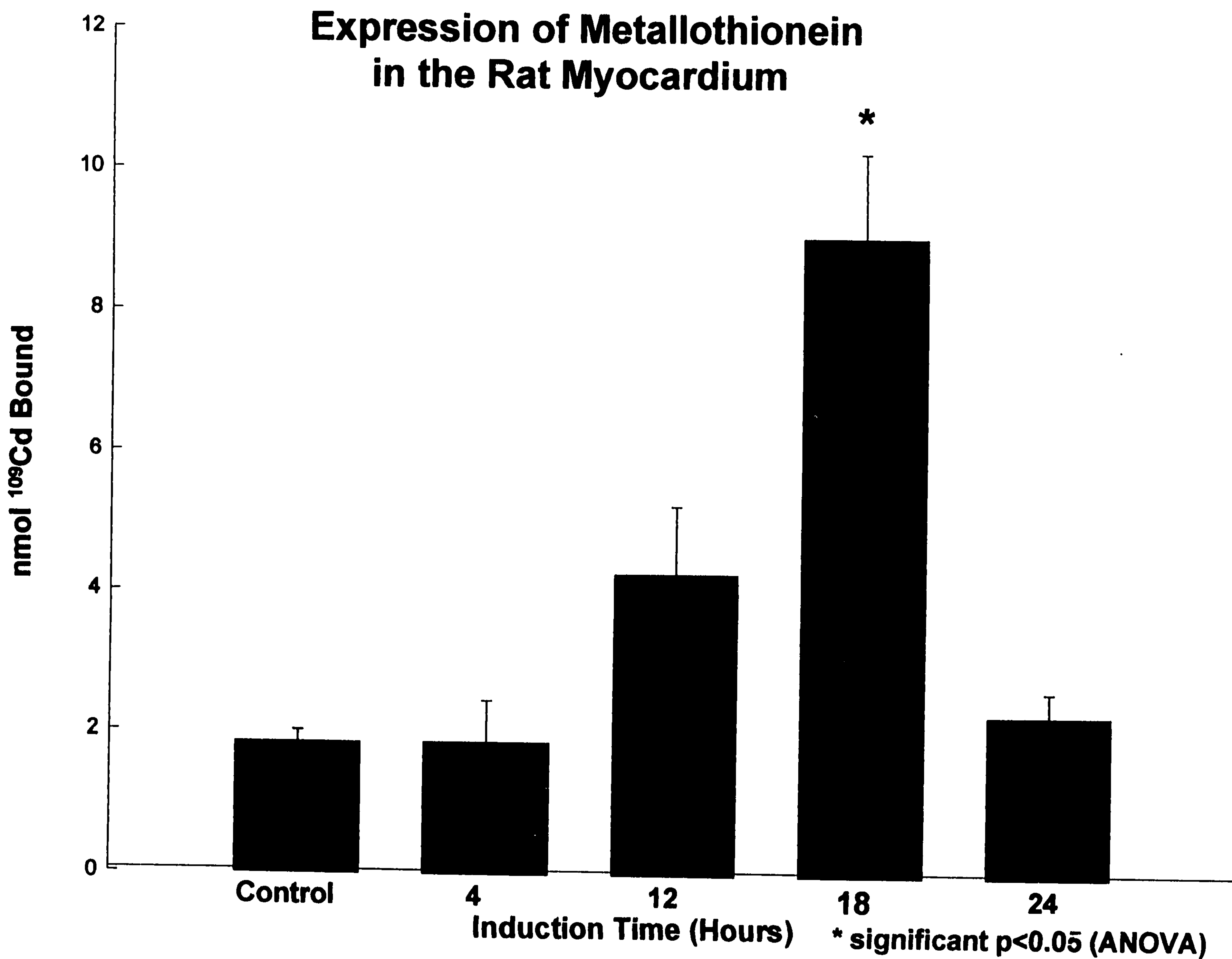

Expression of Metallothionein
in the Rat Myocardium
*
nmol ¹⁰⁹Cd Bound
12
10
8
6
4
2
0
Control
4
12
18
24
Induction Time (Hours)
* significant p<0.05 (ANOVA)

FIGURE 13. Sephadex G-75 column chromatography indicated that MT was the only heat-stable, ^{109}Cd-binding molecule following the ^{109}Cd-MT isolation assay. 500 µg of ^{109}Cd labelled MT ("Authentic Metallothionein",●) was applied to the column and determined to elute in fraction 34. Unbound ^{109}Cd (▲) migrated slower than MT, showing a peak in fraction 53. Samples (100 µl) which had undergone the MT extraction ("Sample Metallothionein,▼) showed a single peak which co-migrated with authentic MT, providing strong evidence that the MT assay was not affected by unbound ^{109}Cd or other metal binding molecules. Samples which underwent the MT isolation assay, but were not radiolabelled, did not show a peak in any fraction (■).

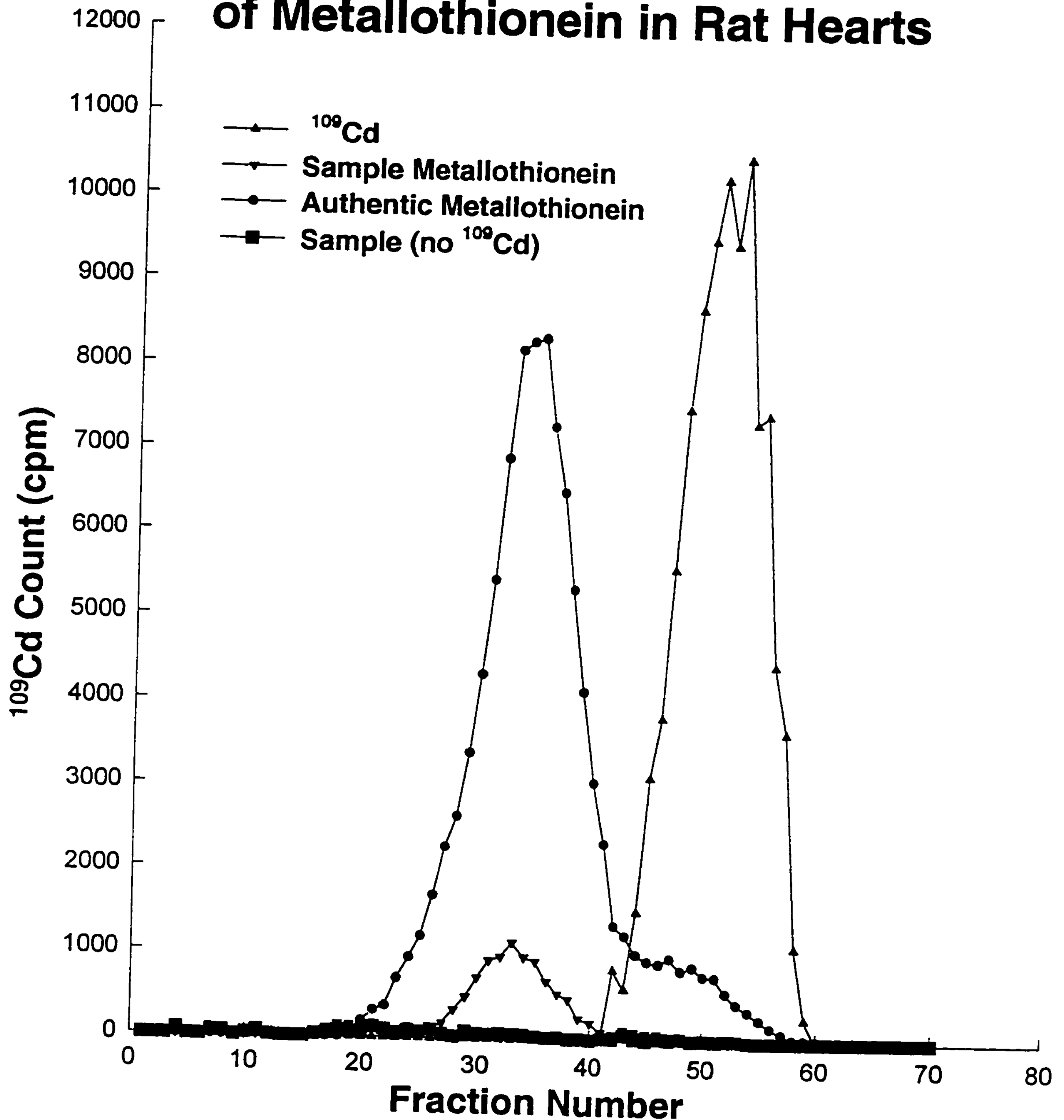

Column Chromatography
of Metallothionein in Rat Hearts
109Cd
Sample Metallothionein
Authentic Metallothionein
Sample (no 109Cd)
109Cd Count (cpm)
Fraction Number
12000
11000
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
0
10
20
30
40
50
60
70
80

FIGURE 14. Performance of the Zn^{2+} INJECTED GROUP (◊) (n=7). No measure of cardiac performance (systolic, diastolic, perfusion pressures) was significantly altered due to zinc injection when compared to the IR GROUP (●).

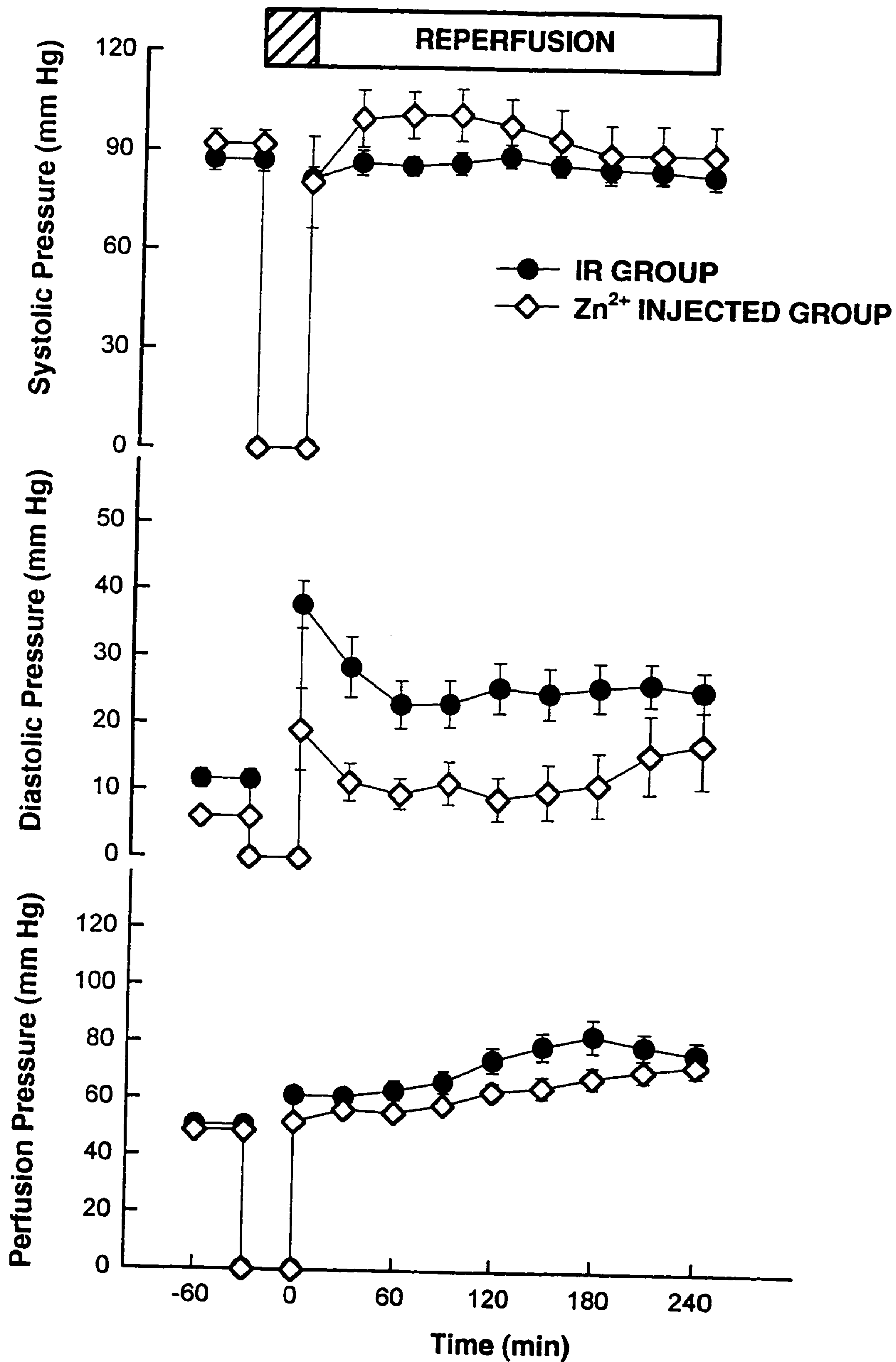
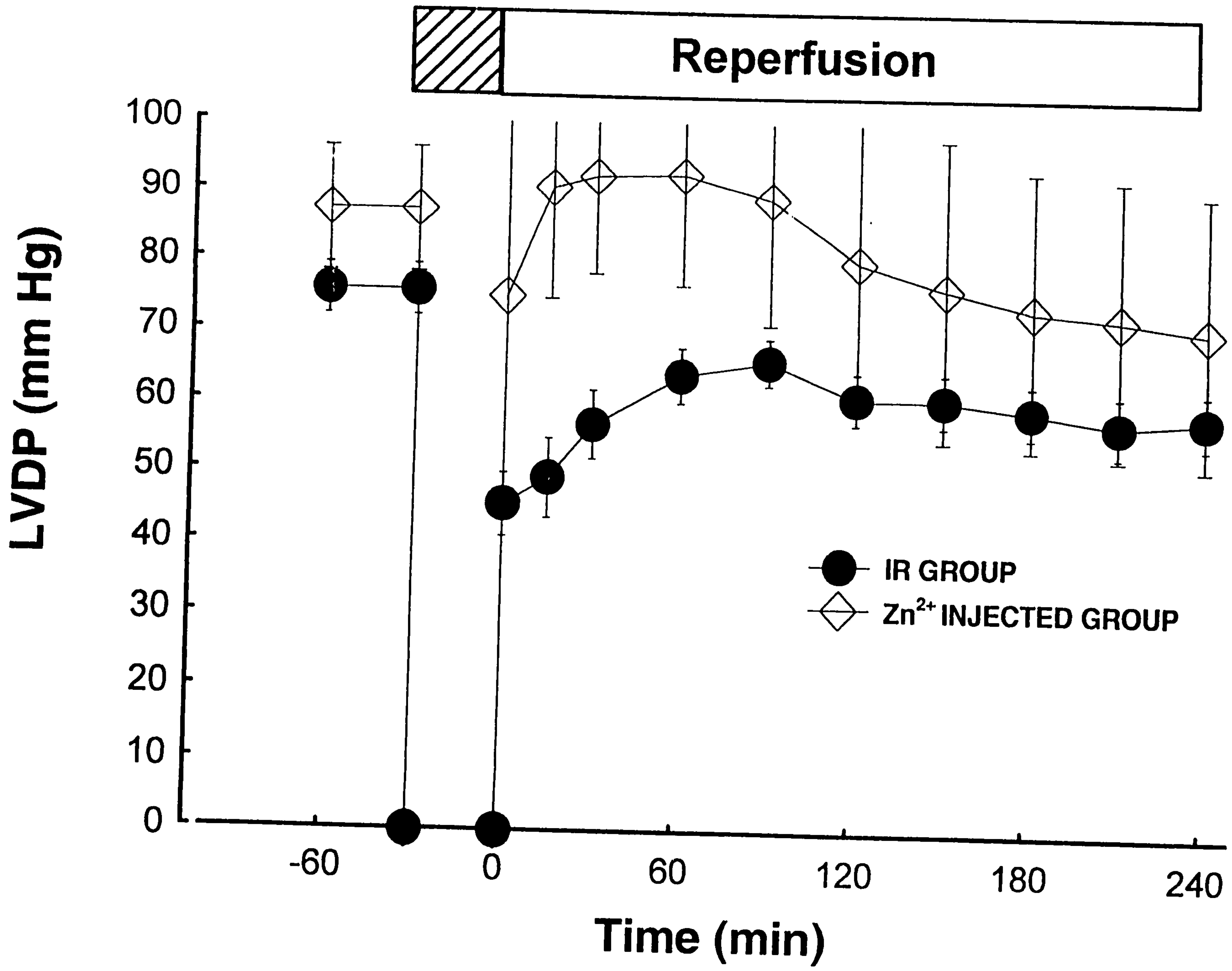

REPERFUSION
Systolic Pressure (mm Hg)
120
90
60
30
0
IR GROUP
Zn2+ INJECTED GROUP
Diastolic Pressure (mm Hg)
50
40
30
20
10
0
Perfusion Pressure (mm Hg)
120
100
80
60
40
20
0
-60
0
60
120
180
240
Time (min)

FIGURE 15. The LVDP of the Zn^{2+} INJECTED GROUP (◊) was not significantly different from the IR GROUP (●). Therefore, zinc injection (n=7) did not modify cardiac contractility of the post-ischemic hearts.

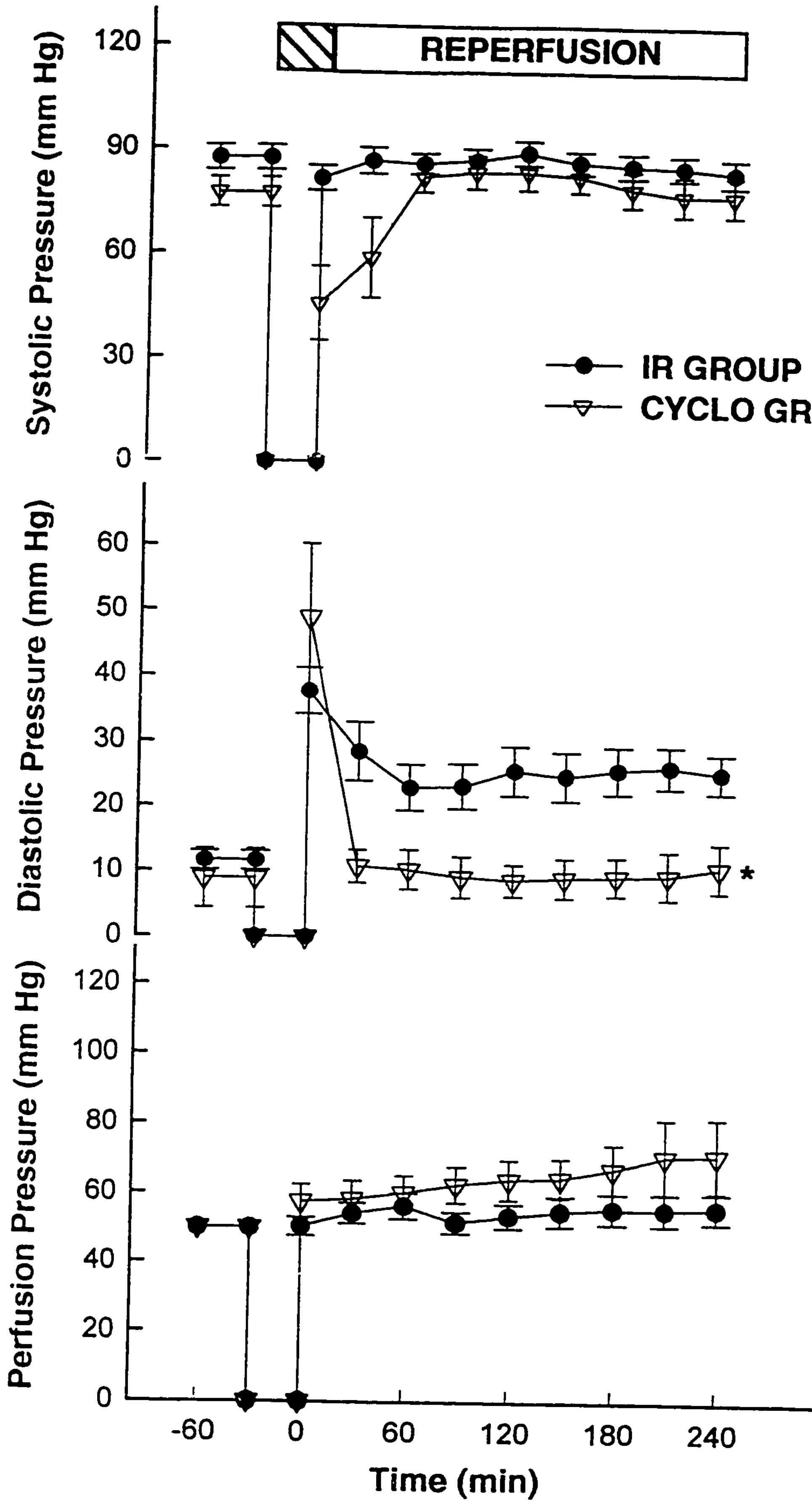


Reperfusion
LVDP (mm Hg)
100
90
80
70
60
50
40
30
20
10
0
IR GROUP
Zn^{2+} INJECTED GROUP
-60
0
60
120
180
240
Time (min)

Cycloheximide Group

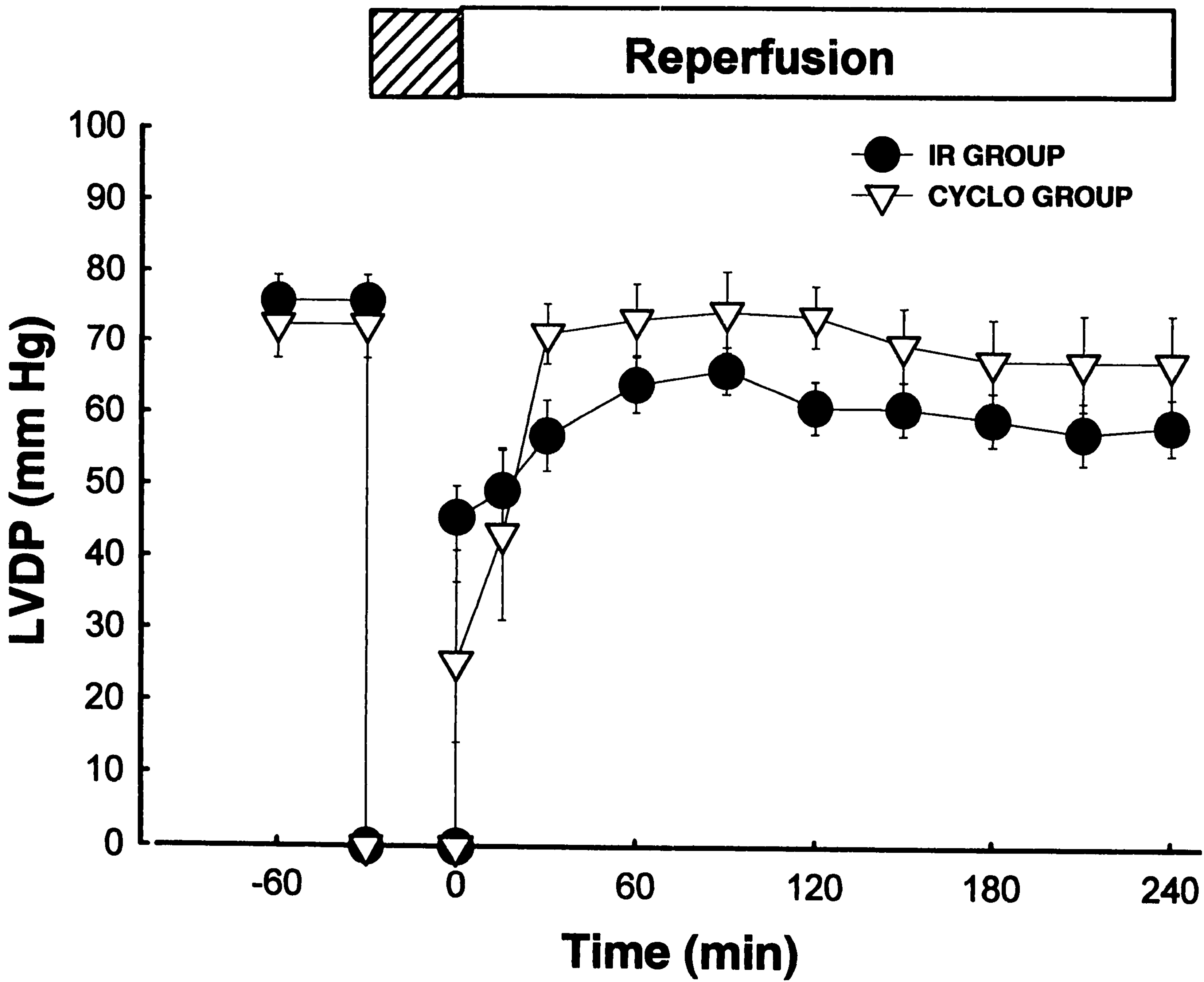
Inclusion of a protein synthesis inhibitor, cycloheximide (10 uM), in the perfusion buffer for 30 min prior to ischemia and for the entire reperfusion period prevented the increase in resting tension ($p<0.05$, ANOVA) but did not affect either the systolic or perfusion pressures when compared to the reperfused group (Figure 16 (n=9)). LVDP was also unaffected by cycloheximide (Figure 17). Addition of the solvent vehicle alone (dimethyl sulfoxide, DMSO, 1.4 mM) did not affect performance when compared to IR hearts.

FIGURE 16. The diastolic performance of the CYCLO GROUP (∇) was significantly improved ($p<0.05$, ANOVA) over the IR GROUP (●). Neither the systolic nor the perfusion pressures were altered as a result of the application of cycloheximide 30 minutes prior to, and for 4 h following, the ischemic episode (n=9).



* significant @ $p<0.05$ (ANOVA) vs. IR GROUP

FIGURE 17. The LVDP of the CYCLO GROUP (∇) (n=9) was not significantly different from the IR GROUP (●), indicating that cycloheximide did not affect the post-ischemic cardiac contractility of the isolated hearts.



Reperfusion
IR GROUP
CYCLO GROUP
LVDP (mm Hg)
0
10
20
30
40
50
60
70
80
90
100
-60
0
60
120
180
240
Time (min)

Thiol Content

DTNB assays indicated that only the application of Zn^{2+} PYR (140.4±19.8 nmol/mg) and the injection of Zinc (121.1±18.9 nmol/mg) altered total thiol levels (TSH). CONTROL (60.3±7 nmol/mg) and IR groups (55.9±15.0 nmol/mg) were not different indicating that the period of ischemia did not threaten total cellular thiols. There was no difference between any group in measures of GSH providing evidence that apoptosis is not related to GSH level.

Only Zn^{2+} PYR (112.0±4.7 nmol/mg) perfusion elevated PSH thiols over the IR group (37.5±17.8 nmol/mg). Protein sulfhydryls were not decreased as a result of the ischemic episode as the amount of thiols in the IR group was not different from the CONT group (44.6±11.5 nmol/mg). Neither the application of DTT, CYCLO, nor Zinc INJECTION affected PSH level (58.4±3.6 nmol/mg and 55.5±8.2 nmol/mg, 71.1±15 nmol/mg, respectively). The application of the potent membrane permeable oxidant monochloramine to the perfusate significantly reduced PSH level (9.1±4.2 nmol/mg) indicating that MCl perfusion may have provided protection from apoptosis by inducing necrotic death.

NON-PERFUSED group was not included in the statistical analysis as it consisted of a n of 2.

The n for each group is as follows:

NON-PERFUSED n=2 DTT n=9 Zn PYR n=4

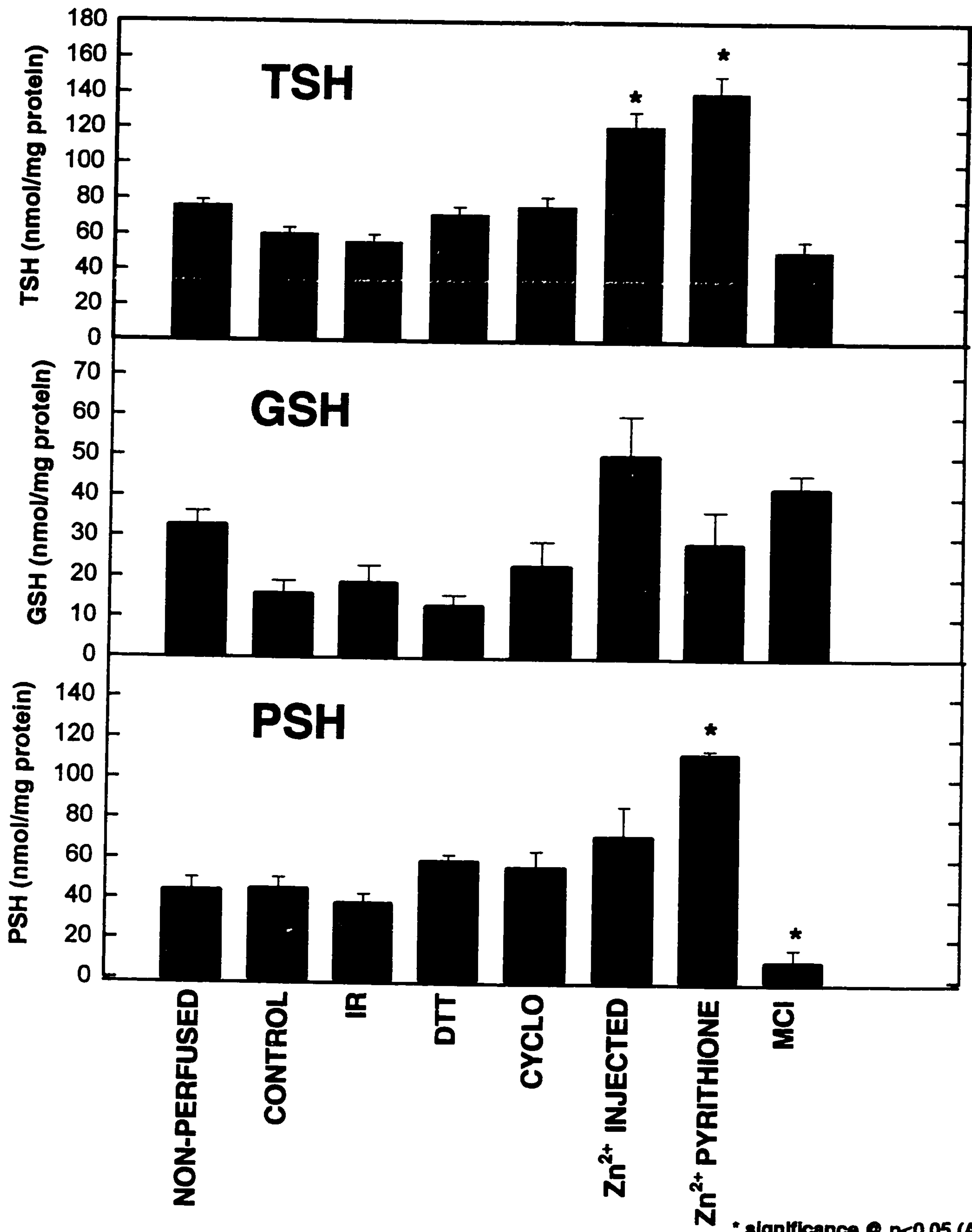
CONTROL n=4 CYCLO n=4 MCl n=3

IR n= 13 Zn INJ n=5

FIGURE 18. Thiol levels in perfused hearts. The top panel shows total sulfhydryl levels (TSH) for all protocols, while the middle and bottom panels represent glutathione (GSH) and protein sulphydryls (PSH), respectively. TSH levels were not changed significantly by either the ischemic episode or various treatments, as the total sulphydryl level in CONT (60.35±3.5 nmol/mg protein), IR GROUPS (55.8±4.14), DTT (71.33±4.4), CYCLO (76.0±5.3), were not significantly different. However, Zn^{2+} INJECTION (121.1±8.4) and Zn^{2+} PYRITHIONE (140.4±9.4) GROUPS showed significantly increased ($p<0.05$, ANOVA) TSH levels. GSH levels were not different in the IR GROUP (18.5±4.4 nmol/mg protein) compared to the CONT GROUP (15.7±3.2) indicating that in our model, ischemia and reperfusion did not alter GSH. As well various treatments did not alter GSH level as Zn^{2+} INJECTION (50.6±10.0), MCI (42.1±3.6), DTT (12.9±2.7), CYCLO (22.8±6.1), and Zn^{2+} PYRITHIONE (28.3±8.2) all failed to alter cellular GSH. The PSH level of CONT (44.6±5.7), IR (37.4±4.9), CYCLO (55.5±8.2), DTT (58.4±3.6) and Zn^{2+} INJECTION (71.1±15.0) were not significantly different. However exposure of the perfused hearts to Zn^{2+} PYRITHIONE (112±2.3) and MCI (9.0±6.3) significantly altered PSH levels. Statistics were not done on the NON-PERFUSED group because of the small n.

The n for each group is as follows:

NON-PERFUSED GROUP n=2
CONT GROUP n=4
IR GROUP n=13
DTT GROUP n=9
CYCLO GROUP n=4
Zn^{2+}PYRITHIONE GROUP n=4
MCI GROUP n=3
Zn^{2+}INJECTED GROUP n=5



TSH
TSH (nmol/mg protein)
180
160
140
120
100
80
60
40
20
0
GSH
GSH (nmol/mg protein)
70
60
50
40
30
20
10
0
PSH
PSH (nmol/mg protein)
140
120
100
80
60
40
20
0
*
NON-PERFUSED
CONTROL
IR
DTT
CYCLO
Zn2+ INJECTED
Zn2+ PYRITHIONE
MCI
* significance @ p<0.05 (ANOVA)

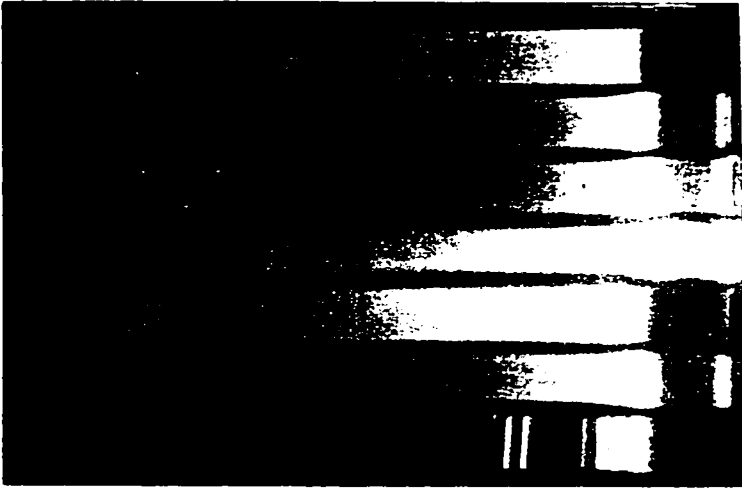
DNA Electrophoresis

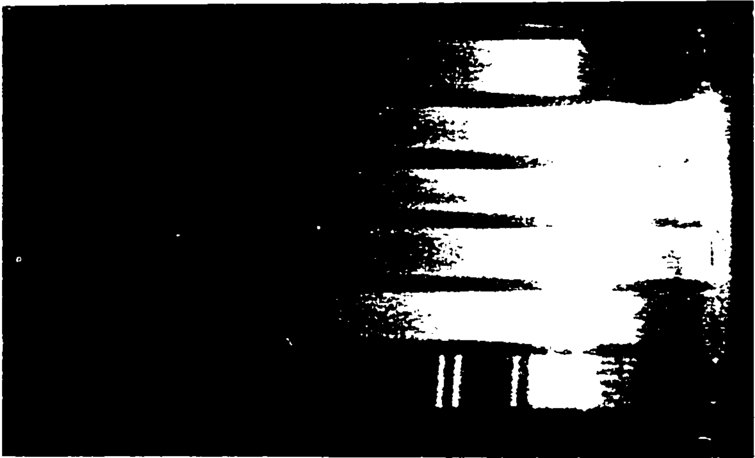
DNA electrophoresis on agarose gels provided evidence for apoptosis in hearts subjected to ischemia-reperfusion (Figure 19). The presence of ladders indicated DNA fragmentation in multiples of 180-200 base pairs in 19 of 22 hearts in the reperfused group (lane 4). It is clear that in both the non-perfused (lane 2) and the control hearts (lane 3), DNA fragmentation typical of apoptotic death is not present. This indicates that it is the ischemia/reperfusion itself, and not the isolation or the perfusion protocols which causes the observed apoptotic DNA fragmentation. Hearts treated with DTT showed apoptotic fragmentation in only 2 of 10 hearts (lane 5). Some DNA smearing was evident in lanes 4 and 5 (reperfused and DTT groups, respectively). Smearing is indicative of the random DNA fragmentation typical of necrotic death. The non-perfused and control groups (lanes 2 and 3, respectively) did not display the smearing, showing that the myocardial DNA remains relatively intact during the extraction and perfusion protocols. Hearts treated with cycloheximide (lane 6) also showed protection from apoptosis (only 2 of 6 hearts showed apoptotic DNA fragmentation), supporting a role for *de novo* protein synthesis in the apoptotic mechanism in this model. Zn^{2+} pyrithione perfusion also provided protection from apoptotic fragmentation, with ladders in only 2 of 6 hearts (lane 10).

Zinc injection (Lane 11) and the subsequent MT overexpression in hearts protected against programmed cell death as apoptotic DNA fragmentation was visible in only 2 of 8 hearts. Ironically, protection was also exhibited by monochloramine perfusion. The presence of this potent and membrane permeant oxidant in low amounts (0.1 uM) showed

strong protection against apoptosis (only 1 of 12 hearts displayed apoptotic laddering, lane 12). However, this "protection" may be misleading since MCI increased the necrotic cell death. Shorter durations of untreated reperfusion (2 and 3 h, n=3 each) did not result in apoptosis as visualized by agarose electrophoresis (not shown).

FIGURE 19. Agarose DNA gel electrophoresis was used to examine the presence of apoptosis in the myocardial DNA following the various treatments. DNA was extracted from isolated perfused hearts and was subjected to electrophoresis as described in Methods. Unless otherwise stated, 10 ug of DNA was loaded into each lane. Internucleosomal fragmentation typical of apoptosis is illustrated by the "ladder" pattern of thymocyte DNA (3 ug) in Lane 1 and Lane 8 (taken from gamma-irradiated rats). Lane 2 shows that *in vivo*, apoptosis is not found in the NON-PERFUSED GROUP rat heart. Isolation and 5 h perfusion of the hearts does not cause an increase in apoptosis over the *in vivo* state, as "ladders" are not found in the CONT GROUP (Lane 3). The internucleosomal fragmentation visible in the IR GROUP (Lane 4) indicates that 30 minutes of ischemia, followed by 4 hr reperfusion causes apoptosis. Lanes 5 and 6 represent DTT (10 umol/L for first hour of reperfusion) and CYCLO GROUPS (10 umol/L for 30 minutes prior, and 4 h following, 30 minutes ischemia), respectively. Both DTT and CYCLO GROUPS were devoid of apoptosis. However, the DTT GROUP appears to have significant necrotic death visualized by the dominant "smearing" (random DNA fragmentation) in Lane 5. Lanes 9, 10, 11, and 12 show NAC (0.1 umol/L for the initial 60 min of reperfusion), Zn^{2+} PYRITHIONE (1 umol/L for 30 min prior to, and following, ischemia), Zn^{2+} INJECTED (10 mg/Kg BW injected 18 h prior to ischemic episode) and MCI GROUPS (1 umol/L for initial 3 hours of reperfusion), respectively. All show protection from apoptosis, but the MCI GROUP displayed large amounts of necrotic cell death. Lanes 7 and 13 show Hind III fragments of lambda phage DNA (1 ug, Sigma) , with an arrowhead indicating molecular weight of 564.


1 2 3 4 5 6 7

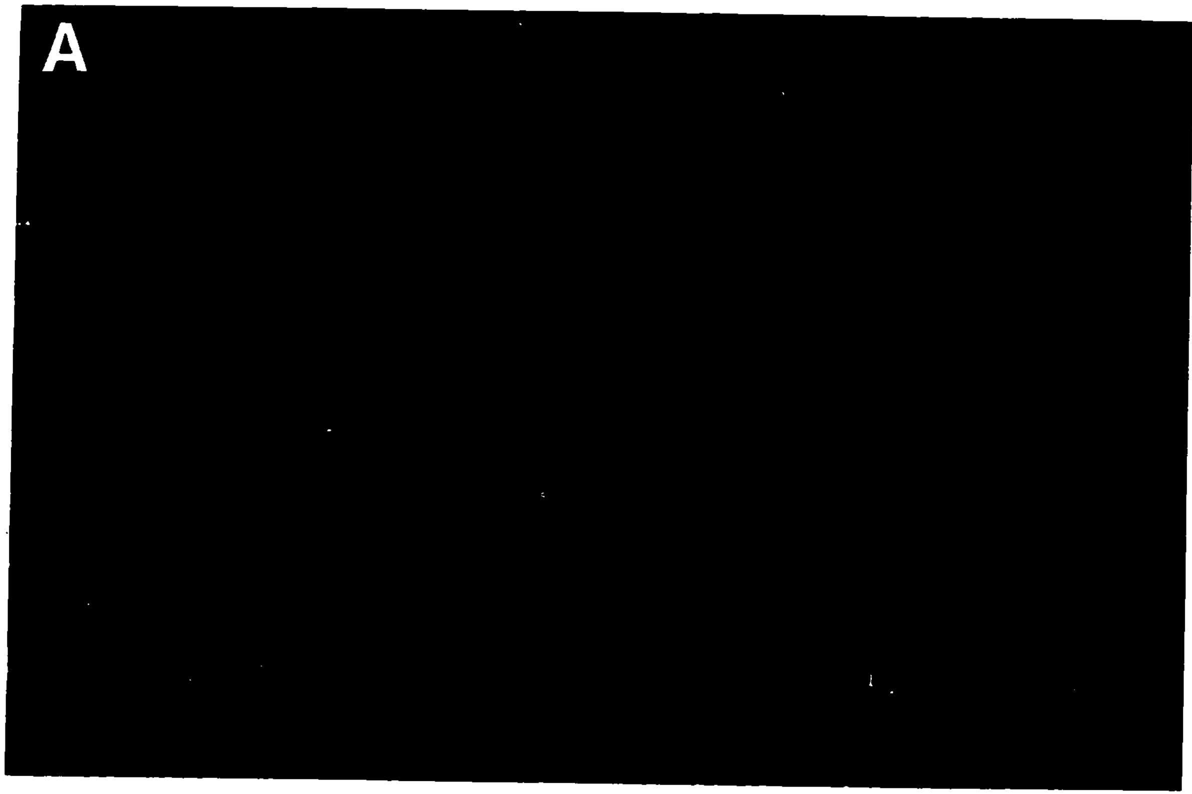

8 9 10 11 12 13

In-Situ-End-Labelling (ISEL)

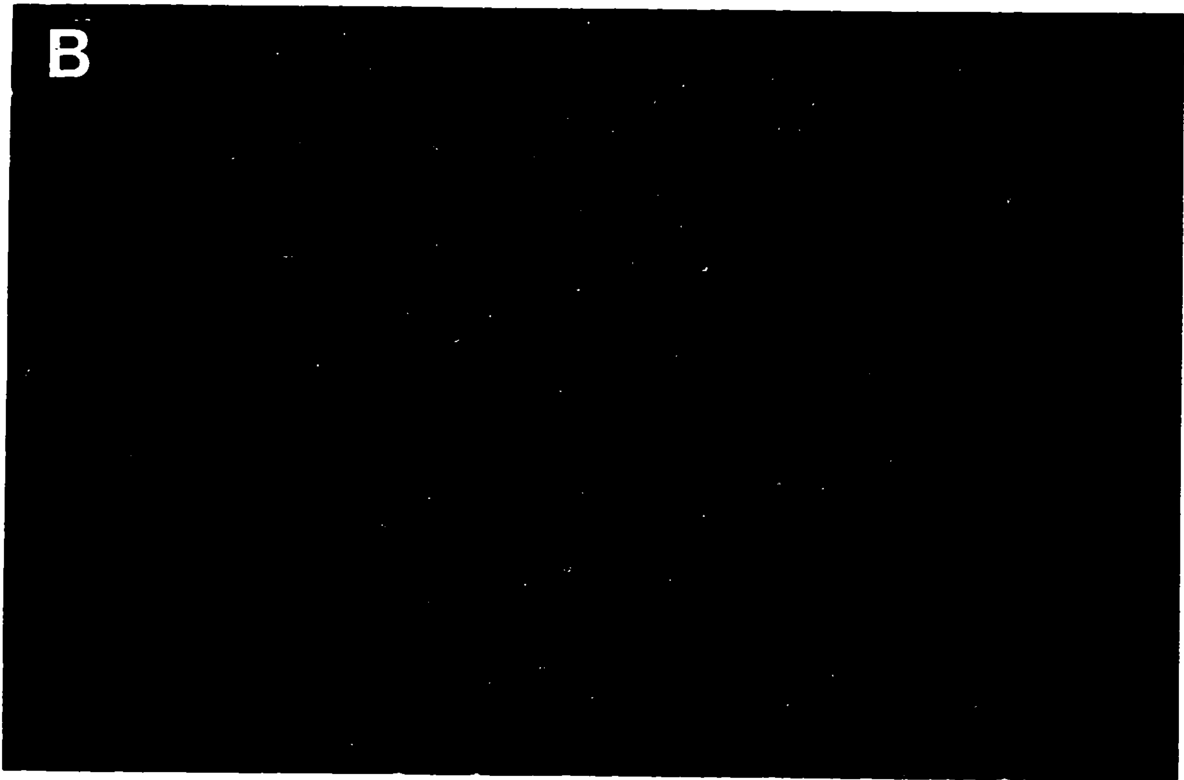
In-Situ-End-Labelling is a histochemical technique used to label double strand DNA nicks typical of apoptosis in cryostat sections. The fluorescent labelling in the nuclei is detectable with a fluorescent microscope.

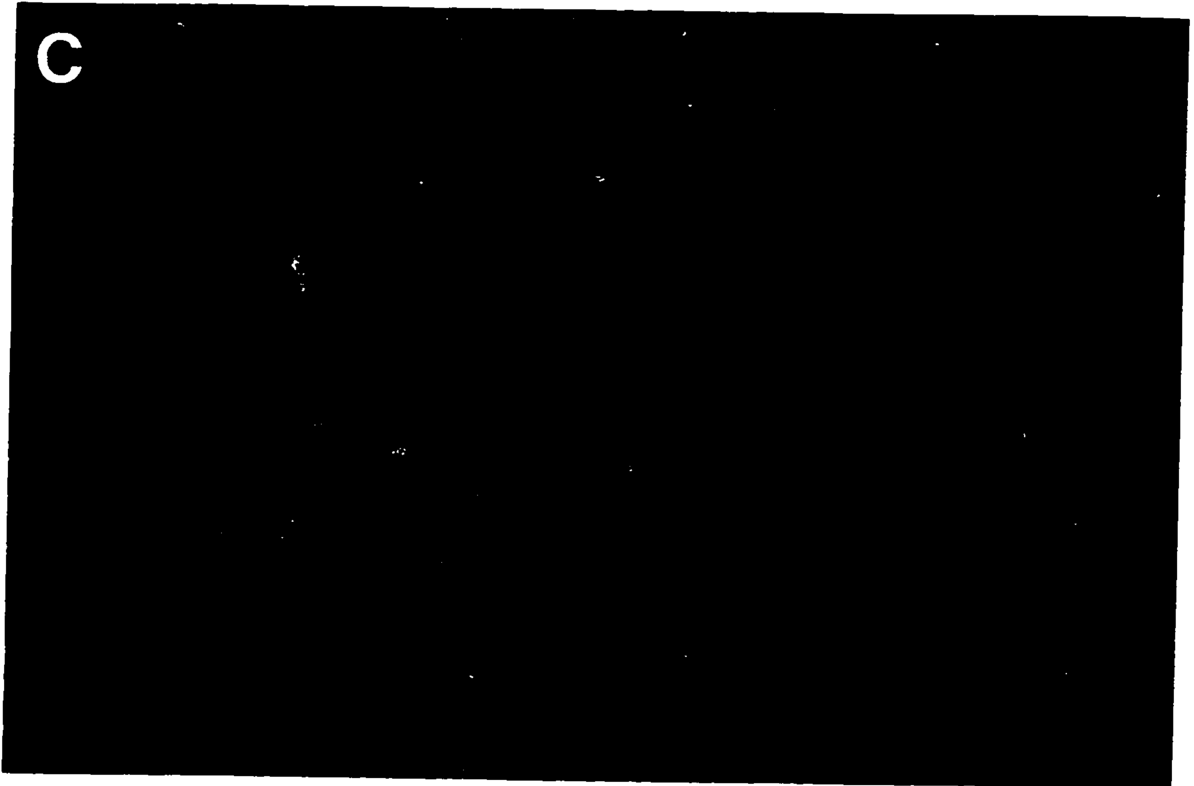
The ISEL data (Figure 20) supported the gel electrophoresis results, showing a high number of fluorescent nuclei in the IR GROUP (Panel C). The fluorescent nuclei appeared to be isolated in cardiomyocytes. The CONT GROUP did not show any evidence of apoptosis as indicated by an absence of labelled nuclei (Panel A). Reperfused hearts which were treated with DTT (Panel E) or with cycloheximide (Panel F), showed a greatly diminished number of fluorescent nuclei in comparison with the IR group. Panels B and D show a global nuclear stain indicating that nuclei are indeed present in ISEL-negative cells and that the result is not due to an absence of nuclei.

FIGURE 20. ISEL staining of myocardial sections. Cryosections were prepared and stained as outlined in Methods. Panel A, C, E and F represent sections stained by ISEL, while Panel B and D show global nuclear staining with Hoechst. Apoptosis is absent in the CONT GROUP (Panel A), however, following the ischemia/reperfusion protocol, apoptosis is abundant in the IR GROUP (Panel C). DTT (Panel E) and CYCLO GROUP sections (Panel F) showed protection from apoptosis. All ISEL sections support the data obtained by DNA agarose electrophoresis. Panel A,B original magnification = X 200. Panel C,D,E,F original magnification X 400. Scale bar is as indicated.

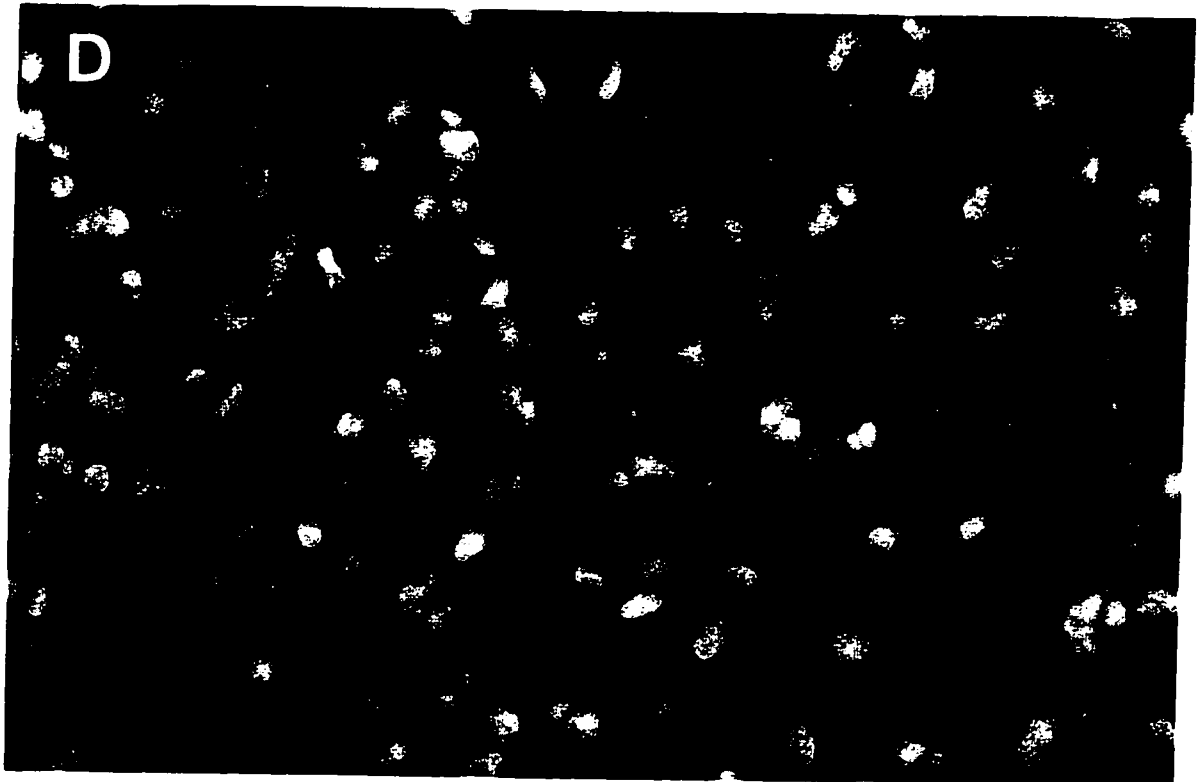


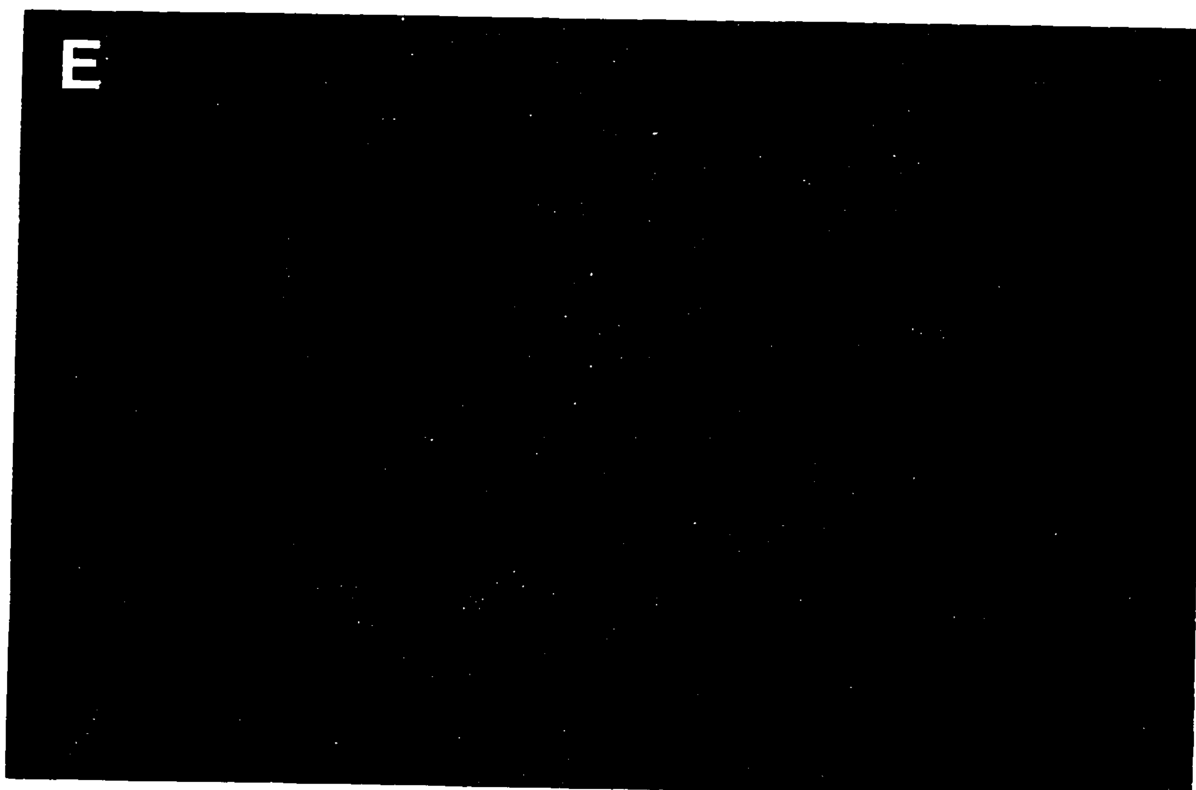
5um



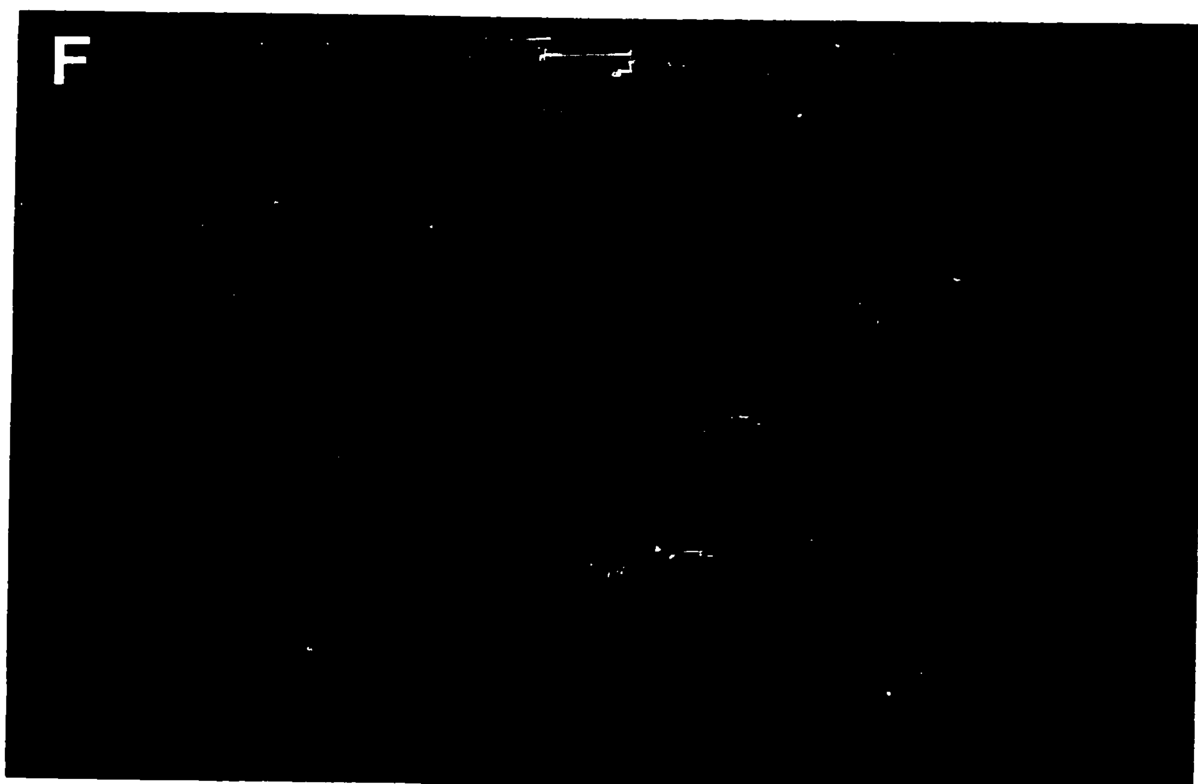


2um





2 um



NOTE TO USERS

Page(s) were not included in the original manuscript and are unavailable from the author or university. The manuscript was microfilmed as received.

70-71

UMI

DISCUSSION

Apoptosis as a Component of Ischemia-Reperfusion Injury

This study provides the first *in vitro* evidence of the involvement of programmed cell death in ischemia/reperfusion injury in the rat heart. It was shown that 30 minutes of ischemia is sufficient to cause the symptoms of reperfusion injury in isolated rat hearts as indicated by the decrease in LVDP and an increase in the diastolic pressure of the post-ischemic heart. Coupled with this functional injury, I detected clear evidence of apoptosis in the hearts following 4 h of reperfusion. Programmed cell death was ascertained using ISEL as fluorescent nuclei in tissue sections, and DNA "ladders" in multiples of approximately 180 base pairs in DNA agarose gel electrophoresis. Because ISEL labels all double stranded DNA nicks, a positive ISEL alone is not in itself, a strong apoptotic indicator. Although necrosis does not typically cleave selectively at the internucleosomal locations (255,256), it nevertheless cleaves DNA into ISEL-sensitive fragments. As a result, the use of the ISEL technique alone for the identification of apoptosis is still controversial (257). However, coupled with agarose DNA electrophoresis, the two techniques are believed to provide a strong indication of both the presence, and quantity of apoptosis. My observation of a possible correlation between apoptosis and impaired contractility raises the exciting possibility that there may be a direct effect of programmed cell death on normal myocardial function.

Apoptotic DNA fragmentation occurs at specific locations as a result of the activation of specific endonucleases which cleave DNA at internucleosomal sites, giving

the appearance of a ladder pattern in agarose gels. Presently, it remains unknown which endonuclease(s) are responsible for the DNA cleavage, although DNAse 1 and 2 have been implicated in various apoptotic systems (179,258). How these become activated is also not established. Apoptosis may commence from a variety of signals (eg. oxidation) which then begin the program resulting in DNA fragmentation and ultimately, apoptotic cell death.

Recently, there have been several *in vivo* studies which have reported apoptosis in the reperfused heart (220,259,260). The findings of this study are, however, the first indications of *in vitro* apoptosis in the isolated heart. The presence of programmed cell death in a buffer-perfused system indicates that external factors are not essential for apoptosis. Intrinsic factors alone are sufficient for ischemia/reperfusion induced apoptosis in this model as the removal of blood-borne factors (eg. neutrophils) did not prevent programmed cell death. Therefore, the isolated perfused heart model has the potential to be a very useful model in the study of the intrinsic mechanisms of apoptosis following an ischemic episode in the heart.

Cell death is the most critical consequence of either ischemia or reperfusion induced injury. Therefore, the ability of various interventions to modulate programmed cell death in this model may lead to therapeutic treatments designed to combat ischemic and reperfusion injury. In contrast to necrotic cell death, apoptosis proceeds through a cascade of predictable steps which may permit possible intervention to arrest, or reverse, this process. Recent data suggest that apoptosis may be reversible (261,262) and that some cell types may be rescued from apoptosis after the process has already begun. The

present study, by providing strong evidence that apoptosis contributes to the decline in cardiac function following reperfusion of an ischemic area, raises the exciting possibility that contractile function may be preserved or restored with anti-apoptotic agents.

Apoptosis has been shown to be the predominant and early form of cell death in reperfused human (222,223) and rat (220,221) hearts. Kajstura et al. (1996) (220) recently reported pronounced programmed cell death as early as 2 h following ischemia. Apoptosis subsequently peaked at 4.5 h, and subsequently declined. However, necrotic death was virtually non-existent prior to 6h, at which time it became the predominant form of cell death and remained significantly elevated for 48 h following ischemia (220). Work with human tissue from both autopsy and explanted hearts has verified this in humans as well. Veinot et al. (1996) (223) showed, using ISEL and DNA agarose gels, extensive apoptosis in recently reperfused human heart tissue. After prolonged reperfusion (between 1 and 5 days) , no ISEL, but much necrosis, was detectable. These studies imply that apoptosis is the major form of cardiac cell death within the initial few hours of ischemia and reperfusion, and its decline is matched by an increase in necrotic death over a period of days. Although significant differences do exist between the *in vivo* studies and our *in vitro* conditions, it is likely that apoptosis is the major form of cell death in our model as well. The duration of our reperfusion (4 h), is well below the 6 h required by Kajstura et al. (1996) (220) to observe necrotic death in a neutrophil rich environment. Moreover, we did not detect extensive DNA smearing in our agarose gels, an indicator of necrosis. Therefore, the possibility that ISEL has indicated necrotic death is unlikely in our model. One could speculate that programmed cell death may injure adjacent

cardiomyocytes, leading to the widespread necrosis at a latter time (263).

The Involvement of Apoptosis on Myocardial Contractility

This study produced a number of conflicting data. Some agents (eg. DTT, cycloheximide) showed protection against apoptosis with an improvement in performance, while others showed protection without enhancement of performance (Zn^{2+}-Pyrithione, MCI, Zn^{2+} injected). There may be a number of explanations for this paradox. Apoptosis is known to be induced by numerous distinct mechanisms. For example, it is possible that ischemia/reperfusion induced injury may affect a complex set of cardiomyocyte functions, some of which trigger apoptosis, while others cause contractile disfunction without apoptosis. If tissues are protected from both apoptosis and the decreased LVDP characteristic of reperfusion injury, then it is likely that the agents which provided apoptotic protection also protected the myocardial tissues against injury to the non-apoptotic functions, such as Ca^{2+}-ATPase, or membrane disruption. Thus, although widespread apoptosis may ultimately decrease contractile performance in the heart, the extent of the decline in LVDP seen in the IR group may not be attributed solely to apoptosis.

It remains to be determined whether our *in vitro* model reflects the effect of apoptosis on contractility. To date, no model has successfully documented the effect of apoptosis on myocyte performance. One can therefore only speculate on the contribution that apoptosis may have on cardiac contractility. The principal difficulty in establishing the apoptotic/contractile performance relationship lies in the fact that apoptotic

cardiomyocytes may retain contractile function for an indeterminate length of time after the onset of apoptosis. Although nuclear DNA is clearly fragmented, there is no evidence indicating that a myocyte cannot function (either impaired or normally) in the initial stages of programmed cell death. Another laboratory has attempted to examine whether this occurs in apoptotic cardiac myocytes. In personal communications with an expert in cardiac apoptosis, Dr. Anversa, he indicated that his laboratory has unsuccessfully attempted to explore the functional abilities of a single cardiac papillary myocyte. Without the study of the affect of apoptosis on a single myocyte, direct conclusions pertaining to the affect of apoptosis on contractility cannot be made. However in his opinion, an apoptotic myocyte may remain functionally active, at least acutely.

Another complicating factor is the number of nuclei in cardiomyocytes. Cardiac myocytes may be binucleated and thus may undergo apoptosis in one nucleus, but not the other (264). How this affects myocyte contractility is unknown, but it is presently thought that apoptotic DNA fragmentation must occur in both of the nuclei of a binucleated myocyte before apoptotic cell death results, although there are presently no published data to support this. Thus a myocyte may appear to be apoptotic, but may remain fully functional. Therefore, although the present study demonstrates an apparent correlation between the extent of apoptosis and contractile dysfunction, it remains to be determined whether the decrease in contractility observed by this study is, in fact, due to apoptosis. Therefore, in this model, the decrease in LVDP may result from cellular damage independent of apoptosis.

Signalling Pathways in Apoptosis

The Involvement of Protein Oxidation in Apoptosis

The finding that apoptosis was not detectable at 2 h following reperfusion in our model is not surprising. The removal of blood-borne agents, such as neutrophils, likely reduced the stress to the tissues substantially. This is supported by results obtained by an *in vivo* study of reperfusion-induced apoptosis completed in our laboratory. In contrast to the present study, the *in vivo* project (221) showed a significant reduction in both GSH and PSH content in the reperfused tissues, indicating a substantial oxidative stress. Such an insult may arise from the aggregation and subsequent activation of invading neutrophils in the heart. Myeloperoxidase activity was found to be significantly increased in that model indicating the elevated presence of neutrophils after 4 h of ischemia or reperfusion. This likely sustained the oxidative burden on the myocardium, compounding cellular thiol oxidation.

The lack of GSH or PSH depletion following the ischemic episode in our model implies that the oxidative stress may be substantially decreased at the end of the 4 h *in vitro* perfusion protocol compared to the initial minutes of reperfusion. Previous studies with similar models have shown that OFR generation in reperfused myocardium peaks within the first few minutes of reperfusion and then rapidly returns to normal levels (18,265-267). Moreover, protein oxidation (as measured by carbonyl levels) was shown to be over four-fold greater than control levels at 5 minutes of reperfusion (268). However, by 15 minutes these levels were reduced to 150% of controls. Therefore, it is not entirely

surprising that an oxidative stress was not detected at the termination of the 4 h reperfusion protocol in our study. OFR's generated during those first minutes may, therefore, be sufficient to activate the genetic machinery responsible for apoptosis but insufficient to provide levels of oxidation beyond which the tissues can recover during the 4 h reperfusion.

Although oxidative damage was not detectable, Ca^{2+} injury was evident following the ischemic episode. Typically, in I/R injury, the Ca^{2+}-ATPase is injured by radicals. This causes a decrease in Ca^{2+} reuptake into the SR, increasing the resting tension of the muscle (increased free $[Ca^{2+}]$ initiates cross bridge cycling) with a corresponding decrease in LVDP. Moreover, an elevation of $[Ca^{2+}]_i$ has been proposed to activate the endonucleases involved in apoptosis, providing a mechanism through which I/R may induce apoptosis (179,269). However, this remains controversial since calcium does not appear to play a role in some models of apoptosis (230,232,270).

Another reason for the apparent absence of protein oxidation in our apoptotic model may be the synthesis of enzymes. Protein synthesis is known to occur in the isolated heart (271-273), and it is likely that in our study, some of the proteins synthesized in the reperfused hearts were thiol-reducing enzymes. A previous study using an isolated perfused rat heart model showed that mRNA for heat shock protein 70 (HSP 70) was increased between 5 and 13 fold due to oxidative stress (274). HSP 70 is a thiol reducing enzyme and may reduce disulfide bonds in oxidized proteins. It is therefore possible that the synthesis of HSP 70, or similar stress proteins, may have reversed the initial oxidative injury to the proteins.

In summary, ischemia/reperfusion was found to significantly impair the function of isolated reperfused hearts in our model. The reperfused hearts displayed decreased performance typical of oxidative injury, resulting in an overall decline in the contractility (LVDP) of the hearts and an increase in the diastolic pressure (the increase in the resting tension was likely due to Ca^{2+} overload). Although oxidative stress has been linked to programmed cell death in other models of ischemic/reperfusion injury, our results indicate that although apoptosis is abundant following 4 h of reperfusion, there does not appear to be any sustained evidence of thiol depletion at the end of the perfusion protocol. It is possible that the actions of disulfide reducing enzymes synthesized during the reperfusion, returned the oxidized thiol levels to pre-ischemic levels. In view of previous data, and our DTT protection studies, it seems unlikely that oxidative stress did not contribute to apoptosis in our model. Instead, it appears likely that the initial transient production of OFR's known to be associated with reperfusion (18,265-267), contributed to the induction of apoptosis, although it did not result in permanent protein oxidation.

GSH as a Signal for Apoptosis

In our *in vitro* model, apoptosis seems to occur independently of GSH level, supporting recent findings by others (210,211,213,214,275). Control hearts indicated a potentially (statistics could not be examined due to small group size) decreased GSH with respect to non-perfused hearts, but did not show evidence of programmed cell death. As well, IR hearts, which showed extensive apoptosis, had GSH levels which were not significantly different from control hearts. Moreover, the addition of DTT to the perfusate

prevented apoptosis, but did not elevate GSH levels. Therefore, these results indicate that the cellular GSH content (at the termination of the 4 h reperfusion protocol) does not appear to correlate with apoptosis in our model. However, as stated above, although the present study failed to show GSH oxidation following the I/R protocol, it is possible that such oxidation may have occurred at an earlier time.

An additional factor which tends to complicate any attempted apoptosis-GSH correlation is the fact that depletion of cellular glutathione may be the result of a decrease in cysteine availability, and may be entirely independent of oxidative stress. Thus, Brunet et al. (276) have shown a massive release of GSH following reperfusion of an isolated heart. This same release may be occurring in our model which, when combined with decreased GSH synthesis over the approximately 5 hours the hearts are isolated, provides the depleted GSH content independent of an oxidative threat.

Manipulation of Apoptosis using DTT

Previous studies have shown that agents which have the ability to alter cellular thiol status have proven effective in preventing active cell death. The present study provides additional evidence that antioxidants may protect against apoptosis, suggesting that oxidants may be involved in apoptosis. Hearts treated with the antioxidant DTT for the first hour of reperfusion, were protected from a decrease in cellular thiols, and deterioration in cardiac performance. Moreover, apoptosis was only visible in 2 of the 8 DTT-treated hearts examined (in contrast to IR hearts which exhibited apoptosis in 11 of 14 hearts). The ability of DTT to protect against oxidants is well established. Eley et al.

(1989) (277) showed that DTT can protect against neutrophil oxidants in the same model used in the present study. DTT may exert its protective effect at the level of Ca^{2+} regulation (eg. the SR Ca^{2+}ATPase).

The vulnerability of the cardiac SR Ca^{2+}-ATPase to thiol oxidation is well documented (278-280), and it is thought that the cysteine residues in this protein can be the target of oxidants, causing Ca^{2+} mediated contractile disfunction, as was shown by Eley et al. (142,277) using cardiac muscle. The levels of both Ca^{2+} uptake by the SR and the phosphatase activity of the SR Ca^{2+}ATPase declined upon oxidant treatment in isolated rat hearts (142). Simultaneous changes in the migration pattern of Ca^{2+}ATPase in SDS-polyacrylamide gels suggested that the structure of the Ca^{2+}ATPase was modified by the oxidative stress. However, subsequent treatment with DTT resulted in a complete functional recovery of both the myocardium and the Ca^{2+}ATPase activity. This study provided strong evidence that the function of the Ca^{2+}ATPase was impaired by oxidants and that this injury could be reversed with DTT.

It is therefore likely that the contractile disfunction observed in the present study is also due to disruption of Ca^{2+} homeostasis (281-283), causing an increase in $[Ca^{2+}]_i$ which then increases myocardial resting tension. The addition of DTT following the ischemic period in our study likely protected the sensitive thiols of the Ca^{2+}ATPase from the reperfusion-associated burst, preventing the decrease in LVDP seen in the IR group. Although both GSH and PSH were unaffected following DTT treatment, it appears likely that DTT protected cardiac contractility through either direct scavenging of oxidants or the reduction of oxidized disulfides, including those of the Ca^{2+}ATPase.

DTT may have anti-apoptotic actions beyond its known antioxidant abilities. Recently, 2,3-dimercaptopropanol, an agent closely related to DTT, was shown to suppress both protein synthesis and cell death in PC12 sympathetic neurons (284). Therefore, it remains to be elucidated if in our model DTT is acting in a similar fashion. The suppression of apoptosis shown following DTT treatment may provide important pharmacologic therapy of myocardial apoptosis in the reperfused heart.

Hearts from rats that had been pre-injected with zinc, showed a marked resistance to the decline in TSH after reperfusion. This is likely due to an increased expression of MT, an endogenous thiol-rich, zinc-chelating protein which is overexpressed in these animals in response to the injected zinc (10mg/kg body weight). Each MT molecule contains 7 zinc atoms in cysteine-bound clusters. When exposed to an oxidative stress, the thiols in MT are oxidized, releasing the bound zinc (143,248). Therefore, MT has antioxidant properties. However, despite the thiol protection provided by zinc injection, as well as a marked protection against apoptosis, no functional (cardiac performance) difference was seen in hearts overexpressing MT.

Protection against apoptosis by zinc injection was observed in 6 out of 8 hearts. This may have occurred through several mechanisms (see section 4.7) but is likely due to zinc induced MT overexpression in the tissues which elevated the antioxidant capabilities of the cell. In addition, free zinc also has the ability to inhibit the Ca^{2+} dependant endonucleases required for apoptotic DNA fragmentation. OFR generation is also inhibited by Zn^{2+} in the isolated heart (235). Zinc may interfere with Cu^{2+} and Fe^{2+} catalyzed reactions which have been shown to result in $^{\cdot}OH$ generation. Therefore, in the

presence of an oxidative stress, MT may have released its bound zinc allowing any of the above mechanisms of apoptotic protection to occur.

The involvement of neutrophils in apoptosis remains unresolved (see section 4.6). In an attempt to simulate the additional oxidative burden likely to be imposed by invading neutrophils, we introduced the neutrophil oxidant monochloramine into the perfusate during reperfusion. The dramatic increase in apparent necrotic cell death, illustrated by the increased DNA smearing, suggests that neutrophil oxidants may exacerbate the injury to the myocardium to the point where necrosis is the predominant form of cell death. A recent study from this laboratory has shown that neutrophils do not appear to participate in apoptosis in ischemic human myocardium (223). This study confirmed that early apoptosis occurred in the absence of neutrophils, and that the later influx of neutrophils was associated with necrotic death. The present study therefore provides support for these *in vivo* observations.

Involvement of Protein Synthesis in Apoptosis

Cycloheximide, an inhibitor of protein synthesis was also shown to provide protection against apoptosis in our study. Cycloheximide prevented the decrease in cellular thiols while also preventing an elevation of resting tension. The ability of cycloheximide to preserve cellular thiols was demonstrated elsewhere as well. Ratan et al. (285) have proposed that, by inhibiting protein synthesis, cycloheximide lowers cysteine incorporation into *de novo* synthesized proteins, thereby increasing its availability for cellular GSH production. However, another study has reported that most of the

cysteine required for GSH resynthesis derives from methionine, and not from the catabolism of GSH as previously thought (286). Although the influence of protein synthesis inhibitors on apoptosis varies between cell types, in our model, programmed cell death was consistently prevented with cycloheximide. Currently, it appears that in the heart, the application of cycloheximide protects against apoptosis, however its role is controversial (7575).

The involvement of protein synthesis in our model of apoptosis appears likely. It is apparent that *de novo* protein synthesis contributes to the apoptotic mechanism in the heart. While the role of protein synthesis in apoptosis is well documented, to date no study has explored the affect of *de novo* protein synthesis on myocardial performance following an ischemic injury. As shown in the introduction, the regulation of apoptosis is apparently regulated by a continuous competition between apoptosis-promoting proteins (eg. p53, proteases, ect...) and apoptosis-inhibiting proteins (eg. bcl-2, ect...). It is premature to speculate on the nature of this balance in the heart, but our data suggest that synthesis of apoptosis-promoting proteins is required to cause apoptosis in this tissue. Thus, cycloheximide inhibits the synthesis of these proteins, preventing the appearance of apoptosis.

Involvement of Zinc in Apoptosis

Similar to many previous studies (225-230), our study shows that the inclusion of zinc in the perfusate offers protection from programmed cell death. As detailed in section 4.7, zinc may inhibit apoptosis by three mechanisms. Firstly, Zn^{2+} may

inhibit a Ca^{2+}-dependant endonuclease which is responsible for the DNA fragmentation typical of apoptosis (228). Secondly, zinc has been shown to inhibit the catalysts necessary for ROS generation (235), most likely by blocking the ability of Cu^{2+} and Fe^{2+} to catalyze ROS generating redox reactions. The third mechanism by which zinc may inhibit active cell death is through direct antioxidant action. Zinc is capable of binding directly to cellular thiols (eg. those on cysteine residues) providing protection against thiol oxidation (238), thereby maintaining the normal function of the protein. In the present study similar protection was achieved by perfusion with zinc pyrithione. Zinc pyrithione is a zinc ionophore which rapidly transports zinc into the cell. In our study, protein sulfhydryl levels were significantly protected at the end of the zinc perfusion protocol, confirming the role of zinc as an antioxidant. Interestingly, GSH levels were unaffected by zinc perfusion, possibly as a result of the low affinity of Zn^{2+} for non-protein thiols.

In conclusion, the present study provides cellular evidence that ischemia/reperfusion can cause apoptosis in the isolated reperfused rat heart model. Although the mechanisms regulating this apoptosis remain unresolved, our data suggest that oxidative stress may contribute to this phenomenon. The ability of DTT and zinc to provide protection against this form of cell death provides support for an oxidative mechanism. Moreover, the protection provided by these agents raises the possibility that they may contribute a potentially affective therapeutic treatment against myocardial apoptosis

REFERENCES

1. Davies, M. J., N. Woolf, and W. B. Robertson. 1996. Pathology of acute myocardial infarction with particular reference to occlusive coronary thrombi. *Br. Heart J.* 38:659-664.

2. Lee.J.T., , Ideker.R.E., and Reimer.K.A.. 1981. Myocardial infarct size and location in relation to the coronary vascular bed at risk in man. *Circulation* 64:526-534.

3. Roberts, R., and Y. Ishikawa,. 1972. Enzymatic estimation of infarct size during reperfusion. *Mod. Concepts Cardiovasc. Dis.* 41:7-10.

4. Silver, M. D.,, G. Baroldi,, and F. Mariani,. 1980. The relationship between acute occlusive coronary thrombi and myocardial infarction studied in 100 consecutive patients. *Circulation* 61:219-227.

5. Chapman, I., 1987. Morphogenesis of occluding coronary artery thrombis. *Arch. Pathol.* 80:256-261.

6. Davies, M. J. and A. C. Thomas. 1985. Plaque fissuring-the cause of acute myocardial infarction, sudden ischemic death, and crescendo angina. *Br. Heart J.* 53:363-373.

7. Falk, E. 1983. Plaque rupture with severe pre-existing stenosis precipitating coronary thrombosis. Characteristics of coronary atherosclerotic plaques underlying fatal occlusive thrombi. *Br. Heart J.* 50:127-134.

8. Friedman, M., 1971. The coronary thrombosis:It's origin and fate. *Human Pathol.* 2:81-128.

9. Ridolphi, R. L., and G. M. Hutchins,. 1977. The relationship between coronary arter lesions and myocardial infarcts: Ulceration of atherosclerotic plaques precipitating coronary thrombosis. *Am. Heart J.* 93:468-496.

10. Buerke, M., T. Murohara, C. Skurk, C. Nuss, K. Tomaselli, and A. M. Lefer. 1995. Cardioprotective effect of insulin-like growth factor I in myocardial ischemia followed by reperfusion. *Proc. Natl. Acad. Sci. USA* 92:8031-8035.

11. Coudray, C., F. Boucher, S. Pucheu, J. De Leiris, and A. Favier. 1995. Relationship between severity of ischemia and oxidant scavenger enzyme activities in the isolated rat heart. *Int. J. Biochem. Cell Biol.* 27:61-69.

12. Manché, A., S. J. Edmondson, and D. J. Hearse. 1995. Dynamics of early postischemic myocardial functional recovery: Evidence of reperfusion-induced injury. *Circulation* 92:526-534.

13. Raschke, P., P. Massoudy, and B. F. Becker. 1995. Taurine protects the heart from neutrophil-induced reperfusion injury. *Free Radic. Biol. Med.* 19:461-471.

14. Ko, T., H. Otani, H. Imamura, K. Omori, and C. Inagaki. 1995. Role of sodium pump activity in warm induction of cardioplegia combined with reperfusion of oxygenated cardioplegic solution. *J. Thorac. Cardiovasc. Surg.* 110:103-110.

15. Alberola, A., L. Such, F. Gil, R. Zaragoza, and E. J. Morcillo. 1991. Protective Effect of N-Acetylcysteine on Ischaemia-Induced Myocardial Damage in Canine Heart. *Naunym Schmiedebergs Arch. Pharmacol.* 343:505-510.

16. Maulik, N., M. Watanabe, D. Engelman, R. M. Engelman, V. E. Kagan, E. Kisin, V. Tyurin, G. A. Cordis, and D. K. Das. 1995. Myocardial adaptation to ischemia by oxidative stress induced by endotoxin. *Am. J. Physiol.* 269:C907-C916.

17. Jeroudi, M. O., C. J. Hartley, and R. Bolli. 1994. Myocardial reperfusion injury: Role of oxygen radicals and potential therapy with antioxidants. *Am. J. Cardiol.* 73:2B-7B.

18. Ambrosio, G., J. L. Zweier, C. Duilio, P. Kuppusamy, G. Santoro, P. P. Elia, I. Tritto, P. Cirillo, M. Condorelli, M. Chiariello, and J. T. Flaherty. 1993. Evidence that mitochondrial respiration is a source of potentially toxic oxygen free radicals in intact rabbit hearts subjected to ischemia and reflow. *J. Biol. Chem.* 268:18532-18541.

19. Marshall, R. C.,, W. W. Nash,, M. M. Bersohn,, and G. A. Wong,. 1987. Myocardial energy production and consumption remain balanced during positive inotropic stimulation when coronary flow is restricted to basal rates in rabbit heart. *J. Clin. Invest.* 80:1165-1171.

20. Marshall, R. C., and D. Y. Zhang,. 1988. Correlation of contractile dysfunction with oxidative energy production and tissue high energy phosphate stores during partial coronary flow disruption in rabbit heart. *J. Clin. Invest.* 82:86-95.

21. Page, D. L., and L. P. McCallister,. 1973. Quantitative electron microscopic description of heart muscle cells. Application to normal, hypertrophied and thyroxin-stimulated hearts. *Am. J. Cardiol.* 31:172-181.

22. Whitman, G.,, R. Kieval,, and L. Wetstein,. 1983. The relationship between global myocardial redox state and high energy phosphate profile. A phosphorus-31 nuclear magnetic resonance study. *J. Surg. Res.* 35:332-339.

23. Barlow, C. H. and B. Chance. 1976. Ischemic areas in perfused rat hearts: Measurement by NADH fluorescence photography. *Nature* 193:909-910.

24. Barlow, C. H., A. H. Harken, and B. Chance. 1977. Evaluation of cardiac ischemia by NADH fluorescence photography. *Ann. Surg.* 196:737-740.

25. Mochizuki, S. and J. R. Neely. 1979. Control of glyceraldehyde-3-phosphate dehydrogenase in cardiac muscle. *J. Mol. Cell. Cardiol.* 11:221-236.

26. Neely, J. R. and H. E. Morgan. 1974. Relationship between carbohydrate and lipid metabolism and the energy balance of heart muscle. *Annu. Rev. Physiol.* 36:413-459.

27. Kobayashi, K. and J. R. Neely. 1979. Control of maximum rates of glycolysis in rat cardiac muscle. *Circ. Res.* 44:166-175.

28. Neely, J. R., J. T. Whitmer, and M. J. Rovetto. 1975. Effect of coronary blood flow on glycolytic flux and intracellular pH in isolated rat hearts. *Circ. Res.* 37:733-741.

29. Hori, S., K. Fukuda, S. Kyotani, T. Satoh, Y. Ohnishi, S. Inoue, N. Aikawa, S. Handa, M. Bessho, and Y. Nakamura. 1990. A rapid fall in the redox ratio after blood flow deprivation in the beating heart (Abstract). *Circulation* Suppl.III:III-38.

30. Jennings, R. B., K. A. Reimer, M. L. Hill, and S. E. Mayer. 1981. Total ischemia in dog hearts in vitro. 1. Comparison of high-energy phosphate production, utilization and depletion and of adenine nucleotide catabolism in total ischemia in vitro vs. severe ischemia in vitro. *Circ. Res.* 49:892-900.

31. Braasch, W., S. Gudbjarnason, P. S. Puri, K. G. Ravens, and R. J. Bing. 1968. Early changes in energy metabolism in the myocardium following acute coronary artery occlusion in anesthetized dogs. *Circ. Res.* 23:429-438.

32. Jones, C. E., J. X. Thomas, J. C. Parker, and R. E. Parker. 1976. Acute changes in high-energy phosphates, nucleotide derivatives, and contractile force in ischemic and nonischemic canine myocardium following coronary occlusion. *Cardiovasc. Res.* 10:275-282.

33. Reimer, K. A., M. L. Hill, and R. B. Jennings. 1981. Prolonged depletion of ATP because of delayed repletion of the adenine nucleotide pool following reversible myocardial ischemic injury in dogs. *J. Mol. Cell. Cardiol.* 13:229-239.

34. Rubio, V. R., T. Wiedmeier, and R. M. Berne. 1972. Nucleoside phosphorylase: Localization and role in the myocardial distribution of purines. *Am. J. Physiol.* 222:550-555.

35. Jugdutt, R. I., G. M. Hutchins, B. H. Bulkley, and L. C. Becker. 1979. Myocardial infarction in the conscious dog: Three-dimensional mapping of infarct, collateral flow and region at risk. *Circulation* 60:1141-1150.

36. Tanaka, H., Y. Habuchi, T. Furukawa, J. Morikawa, and M. Yoshimura. 1994. Modulation of sodium current by lactate in guinea pig ventricular myocytes. *Cardiovasc. Res.* 10:1507-1512.

37. Arminger, L. C., R. N. Seelye, D. Phil, J. G. Elswijk, V. M. Carnell, D. C. Benson, J. B. Gavin, and P. B. Herdson. 1975. Mitochondrial changes in dog myocardium induced by lactate in vivo. *Lab. Invest.* 33:502-508.

38. Kahles, H., M. M. Gedhard, V. A. Mezger, H. Nordbeck, C. J. Preuse, and P. G. Spieckermann. 1996. The role of ATP and lactic acid for mitochondrial function during myocardial ischemia. *Basic Res. Cardiol.*

39. Cobbe, S. M. and P. A. Poole-Wilson. 1980. The time of onset and severity of acidosis in myocardial ischemia. *J. Mol. Cell. Cardiol.* 12:745-760.

40. Lange, R., R. A. Kloner, M. Zierler, N. Carlson, M. Seiler, and S. F. Khuri. 1983. Time course of ischemic alterations during normothermic and hypothermic arrest and its reflection by on-line monitoring of tissue pH. *J. Thorac. Cardiovasc. Surg.* 86:418-434.

41. McNamara, J. J., J. R. Soeter, G. T. Suehiro, R. J. Anema, and G. T. Smith. 1974. Surface pH changes during and after myocardial ischemia in primates. *J. Thorac. Cardiovasc. Surg.* 67:191-194.

42. Cobbe, S. M. and P. A. Poole-Wilson. 1980. Tissue acidosis in myocardial hypoxia. *J. Mol. Cell. Cardiol.* 12:761-770.

43. Steenbergen, C., G. Delleuw, T. Rich, and J. R. WIlliamson. 1977. Effects of acidosis and ischemia on contractility and intracellular pH of rat heart. *Circ. Res.* 41:849-858.

44. Van der Vusse, G. J. and R. S. Reneman. 1983. Cardiovascular Metabolism. In Cardiovascular Metabolism. A.J.Drake-Holland and M.I.M.Noble, editor. John Wiley & Sons, London. 215-237.

45. Prinzen, F. W., G. J. Van der Vusse, T. Arts, T. Roeman, and W. A. Coumans. 1984. Accumulation of nonesterified fatty acids in ischemic canine myocardium. *Am. J. Physiol.* 247:H264-H272.

46. Van der Vusse, G. J., T. H. M. Roeman, F. W. Prinzen, W. A. Coumans, and R. S. Reneman. 1982. Uptake and tissue content of fatty acids in dog myocardium under normoxic and ischemic conditions. *Circ. Res.* 50:538-546.

47. Harden, W. R., C. H. Barlow, M. B. Simson, and A. H. Harken. 1979. Temporal relation between onset of cell anoxia and ischemic contractile failure. *Am. J. Cardiol.* 44:741-746.

48. Ross, J. Jr. and D. Franklin. 1976. Analysis of regional myocardial function, dimensions, and wall thickness in the characterization of myocardial ischemia and infarction. *Circulation* 53:188-192.

49. Sayen, J. J., W. F. Sheldon, G. Peirce, and P. T. Kuo. 1958. Polarographic oxygen, the epicardial electrocardiogram and muscle contraction in experimental acute regional ischemia of the left ventricle. *Circ. Res.* 6:779-798.

50. Tatooles, C. L. and W. C. Randall. 1961. Local ventricular bulging after acute artery occlusion. *Am. J. Physiol.* 201:451-456.

51. Tennant, R. and C. J. Wiggers. 1935. The effect of coronary occlusion on myocardial contraction. *Am. J. Physiol.* 112:351-361.

52. Covell, J. W., P. E. Pool, and E. Braunwald. 1967. Effects of acutely induced ischemic heart failure on myocardial high energy phosphate stores. *Proc. Soc. Exp. Biol. Med.* 124:126-131.

53. Kanaide, H., R. Yoshimura, N. Makino, and M. Nakamura. 1982. Regional myocardial function and metabolism during acute coronary artery occlusion. *Am. J. Physiol.* 242:H980-H989.

54. Hearse, D. J. 1979. Oxygen deprivation and early myocardial contractile failure: A reassessment of the possible role of adenosine triphosphate. *Am. J. Cardiol.* 44:1115-1121.

55. Kubler, W. and A. M. Katz. 1977. Mechanism of early "pump" failure of the ischemic heart: Possible role of adenosine triphosphate and inorganic phosphate accumulattion. *Am. J. Cardiol.* 40:467-471.

56. Katz, A. M. 1973. Effects of ischemia on the contractile processes of heart muscle. *Am. J. Cardiol.* 32:456-460.

57. Jacobus, W. E., I. H. Pores, S. K. Lucas, M. L. Weisfeldt, and J. T. Flaherty. 1982. Intracellular acidosis and contractility in the normal and ischemic heart as examined by 31P NMR. *J. Mol. Cell. Cardiol.* 14:13-20.

58. Lai, F. and J. Scheuer. 1981. Early changes in myocardial hypoxia: Relations between mechanical function, pH and intracellular compartmental metabolites. *J. Mol. Cell. Cardiol.* 7:289-303.

59. Weiss, J., G. S. Couper, B. Hiltbrand, and K. L. Shine. 1984. Role of acidosis in early contractile dysfunction during ischemia. Evidence from pH measurements. *Am. J. Physiol.* 247:H760-H767.

60. Koretsune, Y., M. Corretti, H. Kusuoka, and E. Marban. 1977. Mechanism of early ischemic contractile failure: Inexcitability, metabolite accumulation, or vascular collapse. *Am. J. Cardiol.* 40:467-471.

61. Kusoka, H., M. L. Weisfeldt, J. L. Zweier, W. E. Jacobus, and E. Marban. 1986. Mechanism of early contractile failure during hypoxia in intact ferret heart: Evidence for modulation of maximal Ca2+-activated force by inorganic phosphate. *Circ. Res.* 59:270-282.

62. Katz, A. M. and H. Reuter. 1979. Cellular calcium and cardiac cell death. *Am. J. Cardiol.* 44:188-190.

63. Nayler, W. G. 1981. The role of calcium in the ischemic myocardium. *Am. J. Pathol.* 102:262-270.

64. Poole-Wilson, P. A., D. P. Harding, P. D. V. Bourdillon, and M. A. Tones. 1984. Calcium out of control. *J. Mol. Cell. Cardiol.* 16:175-187.

65. Shine, K. I., A. M. Douglas, and N. V. Ricchiuti. 1978. Calcium, strontium, and barium movements during ischemia and reperfusion in rabbit ventricle. Implications for myocardial preservation. *Circ. Res.* 43:712-720.

66. Farber, J. L., K. R. Chien, and S. Mittnacht. 1981. The pathogenesis of irreversible cell injury in ischemia. *Am. J. Pathol.* 102:271-281.

67. Tsokos, J. and S. Bloom. 1977. Effects of calcium on respiration and ATP content of isolated, leaky, heart muscle cells. *J. Mol. Cell. Cardiol.* 9:823-836.

68. Okabe, E., S. Takahashi, M. Norisue, N. H. Manson, R. C. Kukreja, M. L. Hess, and H. Ito. 1993. The effect of hypochlorous acid and hydrogen peroxide on coronary flow and arrhythmogenesis in myocardial ischemia and reperfusion. *Eur. J. Pharmacol. Environ. Toxicol. Pharmacol.* 248:33-39.

69. Kirshenbaum, L. A. and P. K. Singal. 1993. Increase in endogenous antioxidant enzymes protects hearts against reperfusion injury. *Am. J. Physiol.* 265:H484-H493.

70. Jabr, R. I. and W. C. Cole. 1995. Oxygen-derived free radical stress activates nonselective cation current in guinea pig ventricular myocytes: Role of sulfhydryl groups. *Circ. Res.* 76:812-824.

71. Goldhaber, J. I., S. Ji, S. T. Lamp, and J. N. Weiss. 1989. Effects of exogenous free radicals on electromechanical function and metabolism in isolated rabbit and guinea pig ventricle. Implications for ischemia and reperfusion injury. *J. Clin. Invest.* 83:1800-1809.

72. Elliott, A. C., G. L. Smith, D. A. Eisner, and D. G. Allen. 1992. Metabolic changes during ischaemia and their role in contractile failure in isolated ferret hearts. *J. Physiol. (Lond.)* 454:467-490.

73. Jennings, R. B. and K. A. Reimer. 1991. The cell biology of acute myocardial ischemia. *Annu. Rev. Med.* 42:225-246.

74. Chen, W., S. Gabel, C. Steenbergen, and E. Murphy. 1995. A redox-based mechanism for cardioprotection induced by ischemic preconditioning in perfused rat heart. *Circ. Res.* 77:424-429.

75. Marber, M. S., R. Mestril, S. -H. Chi, M. R. Sayen, D. M. Yellon, and W. H. Dillmann. 1995. Overexpression of the rat inducible 70-kD heat stress protein in a transgenic mouse increases the resistance of the heart to ischemic injury. *J. Clin. Invest.* 95:1446-1456.

76. Plumier, J. -C. L., B. M. Ross, R. W. Currie, C. E. Angelidis, H. Kazlaris, G. Kollias, and G. N. Pagoulatos. 1995. Transgenic mice expressing the human heat shock protein 70 have improved post-ischemic myocardial recovery. *J. Clin. Invest.* 95:1854-1860.

77. Galiñanes, M., C. S. Lawson, R. Ferrari, G. A. Limb, N. W. Derias, and D. J. Hearse. 1993. Early and late effects of leukopenic reperfusion on the recovery of cardiac contractile function: Studies in the transplanted and isolated blood-perfused rat heart. *Circulation* 88:673-683.

78. Rosenkranz, E. R. and G. D. Buckberg. 1983. Myocardial protection during surgical coronary reperfusion. *J. Am. Coll. Cardiol.* 1:1235-1246.

79. Jennings, R. B. and K. A. Reimer. 1981. Lethal myocardial ischemic injury. *Am. J. Pathol.* 102:241-255.

80. Jennings, R. B. and K. A. Reimer. 1983. Factors involved in salvaging ischemic myocardium: Effect of reperfusion of arterial blood. *Circulation* 68:I25-I36.

81. Jennings, R. B., H. K. Hawkins, J. E. Lowe, M. L. Hill, S. Klotman, and K. A. Reimer. 1978. Relation between high energy phosphate and lethal injury in myocardial ischemia in the dog. *Am. J. Pathol.* 92:187-214.

82. Kloner, R. A., C. E. Ganote, D. Whalen, and R Jennings,B.. 1974. Effect of a transient period of ischemia on myocardial cells: II. Fine structure during the first few minutes of reflow. *Am. J. Pathol.* 74:399-422.

83. Shen, A. C. and R. B. Jennings. 1972. Myocardial calcium and magnesium in acute ischemic injury. *Am. J. Pathol.* 67:417-440.

84. Reimer, K. A. and R. B. Jennings. 1992. Myocardial ischemia, hypoxia, and infarction. In The heart and cardiovascular system. H.A. Fozzard, R.B. Jennings, E. Haber, A.M. Katz, and H.E. Morgan, editors. Raven Press, Ltd., New York. 1875-1972.

85. Lee, H. C., R. Mohabir, N. Smith, M. R. Franz, and W. T. Clusin. 1988. Effect of ischemia on calcium-dependant fluoresence transients in rabbit hearts containing indo 1. *Circulation* 789:1047-1059.

86. Lee, H. C., N. Smith, R. Mohabir, and W. T. Clusin. 1987. Cytosolic calcium transients from the beating mammalian heart. *Proc. Natl. Acad. Sci. USA* 252:C441-C449.

87. Marban, E., M. Kitakaze, Y. Koretsune, D. T. Yue, V. P. Chacko, and M. M. Pike. 1990. Quantification of Ca2+i in perfused hearts: Critical evaluation of the 5F-BAPTA and nuclear magnetic resonance method as applied to the study of ischemia and reperfusion. *Circ. Res.* 66:1255-1267.

88. Marban, E., M. Kitaze, H. Kusuoka, J. K. Porterfield, D. T. Yue, and V. P. Chacko. 1987. Intracellular free calcium concentration measured with 19F NMR spectroscopy in intact feret hearts. *Proc. Natl. Acad. Sci. USA* 84:6005-6009.

89. Steenbergen, C., E. Murphy, L. Levy, and R. E. London. 1987. Elevation in cytosolic free calcium concentration early in myocardial ischemia in perfused rat heart. *Circ. Res.* 60:700-707.

90. Jennings, R. B., K. A. Reimer, and C. Steenbergen. 1986. Myocardial ischemia revisited. The osmolar load, membrane damage, and reperfusion. *J. Mol. Cell. Cardiol.* 18:769-780.

91. Vander Heide, R. S. and C. E. Ganote. 1987. Increased myocyte fragility following anoxic injury. *J. Mol. Cell. Cardiol.* 19:1085-1103.

92. Vander Heide, R. S., D. Rim, C. M. Hohl, and C. E. Ganote. 1990. An in vitro model of myocardial ischemia utilizing isolated rat myocytes. *J. Mol. Cell. Cardiol.* 22:164-181.

93. McCord, J. M. 1985. Oxygen derived radicals in post ischemia tissue injury. *N. Eng. J. Med.* 312:159-163.

94. Ganote, C. E., J. Worstell, and J. P. Kaltenbach. 1976. Oxygen-induced enzyme release after irreversible myocardial injury. *Am. J. Pathol.* 84:327-350.

95. Hearse, D. J., S. M. Humphrey, W. G. Naylor, A. Slade, and D. Border. 1975. Ultrastructural damage associated with reoxygenation of the anoxic myocardium. *J. Mol. Cell. Cardiol.* 7:315-324.

96. Hohl, C., A. Ansel, R. Altschuld, and G. P. Brierley. 1982. Contracture of isolated rat heart cells on anaerobic to aerobic transition. *Am. J. Pathol.* 242:H1022-H1030.

97. Roeske, W. R., M. DeLuca, and J. S. Ingwall. 1978. Factors influencing enzyme release from cultured fetal mouse hearts deprived of oxygen and glucose. *J. Mol. Cell. Cardiol.* 10:907-919.

98. Higgenson, L. A., D. S. Beanlands, R. C. Nair, V. Temple, and K. Sheldrick. 1983. The time course and characterization of myocardial hemorrhage after coronary reperfusion in the anesthetized dog. *Circulation* 67:1024-1031.

99. Ganote, C. E., J. McGarr, S. Y. Liu, and J. P. Kaltenbach. 1980. Oxygen-induced enzyme release. Assesment of mitochondrial function in anoxic myocardial injury and effects of the mitochondrial uncoupling agent 2.4-dinitrophenol (DNP). *J. Mol. Cell. Cardiol.* 12:387-408.

100. Vander Heide, R. S., P. A. Sobotka, and C. E. Ganote. 1987. Effects of the free radical scavenger DMTU and mannitol on the oxygen paradox in perfused rat hearts. *J. Mol. Cell. Cardiol.* 19:615-625.

101. Fridovich, I. 1978. The biology of oxygen radical. *Science* 201:875-880.

102. Weiss, A. B. and A. F. Le Buglio. 1982. Biology of disease: Phagocytes-generated oxygen metabolites and cellular injury. *Lab. Invest.* 47:5-18.

103. Schreiber, R.D. and H.J. Muller-Eberhard. 1979. Immunologic Mechanisms of Renal Disease. Churchill Livingston, London. 67 pp.

104. Fleishman, K. E., T. Brand, H. S. Sharma, D. J. Duncker, L. M. A. Sassen, E. O. McFalls, P. D. Verdouw, and W. Schaper. 1990. Gene expression in a preconditioning model. *Circulation* 82:III-464.

105. Granger, D. N., G. Rutili, and J. M. McCord. 1981. Superoxide radicals in feline intestinal ischemia. *Gastroenterology* 81:22-29.

106. Parks, D. A., G. B. Bulkley, D. N. Granger, S. R. Hamilton, and J. M. McCord. 1982. Ischemic injury in the cat small intestine. *Gastroenterology* 82:9-15.

107. Parks, D. A., G. B. Bulkley, and D. N. Granger. 1983. Role of free radicals in shock, ischemia, and organ preservation. *Surgery* 94:428-432.

108. Manson, P. N., R. M. Anthenelli, M. J. Im, G. B. Bulkley, and J. E. Hoopes. 1983. The role of oxygen free radicals in ischemic tissue injury in island skin flaps. *Ann. Surg.* 198:87-90.

109. Crapo, J. D., B. A. Freeman, B. E. Barry, J. F. Turrens, and S. L. Young. 1983. Mechanisms of hyperoxic injury to pulmonary microcirculation. *Phys.* 26:170-176.

110. Bolli, R., B. S. Patel, M. O. Jeroudi, E. K. Lai, and P. B. McCay. 1988. Demonstration of free radical generation in "stunned" myocardium of intact dogs with the use of the spin trap alpha-phenyl N-tert-butyl nitrone [published erratum appears in J Clin Invest 1988 Nov;82(5):following 1807]. *J. Clin. Invest.* 82:476-485.

111. Arroyo, C. M., J. H. Kramer, B. F. Dickens, and W. B. Weglicki. 1987. Identification of free radicals in myocardial ischemia/reperfusion by spin trapping with nitrone DMPO. *FEBS Lett.* 222:101-104.

112. Garlick, P. B., M. J Davies, D. J. Hearse, and T. F. Slater. 1987. Direct detection of free radicals in the reperfused rat heart using electron spin resonance spectroscopy. *Circ. Res.* 187:757-760.

113. Zweier, J. L. 1988. Measurement of superoxide-derived free radicals in the reperfused heart. Evidence for a free radical mechanism of reperfusion injury. *J. Biol. Chem.* 263:1353-1357.

114. Pereira, B. M., P. H. Chan, P. R. Weinstein, and R. A. Fishman. 1990. Cerebral protection during reperfusion with superoxide dismutase in focal cerebral ischemia. *Adv. Neurol.* 52:97-103.

115. Chan, P. H., R. A. Fishman, M. A. Wesley, and S. Longar. 1990. Pathogenesis of vasogenic edema in focal cerebral ischemia: Role of superoxide radicals. *Adv. Neurol.* 52:177-183.

116. Johansson, M. H., J. Deinum, S. L. Marklund, and P. O. Sj:oquist. 1990. Recombinant human extracellular superoxide dismutase reduces concentration of oxygen free radicals in the reperfused rat heart. *Cardiovasc. Res.* 24:500-503.

117. Yang, G., P. H. Chan, J. Chen, E. Carlson, S. F. Chen, P. Weinstein, C. J. Epstein, and H. Kamii. 1994. Human copper-zinc superoxide dismutase transgenic mice are highly resistant to reperfusion injury after focal cerebral ischemia. *Stroke* 25:165-170.

118. Plateel, M., M. P. Dehouck, G. Torpier, R. Cecchelli, and E. Teissier. 1995. Hypoxia increases the susceptibility to oxidant stress and the permeability of the blood-brain barrier endothelial cell monolayer. *J. Neurochem.* 65:2138-2145.

119. Arduini, A., A. Mezzetti, E. Porreca, D. Lapenna, J. DeJulia, L. Marzio, G. Polidoro, and F. Cuccurullo. 1988. Effect of ischemia and reperfusion on antioxidant enzymes and mitochondrial inner membrane in perfused rat heart. *Biochim. Biophys. Acta* 970:113-121.

120. Zweier, J. L., P. Kuppusamy, S. Thompson-Gorman, D. Klunk, and G. A. Lutty. 1994. Measurement and characterization of free radical generation in reoxygenated human endothelial cells. *Am. J. Physiol.* 266:C700-C708.

121. Korthuis, R. J., D. N. Granger, M. I. Townsley, and A. E. Taylor. 1985. The role of oxygen-derived free radicals in ischemia-induced increases in canine skeletal muscle vascular permeability. *Circ. Res.* 57:599-609.

122. Richard, V. J., C. E. Murry, R. B. Jennings, and K. A. Reimer. 1988. Therapy to reduce free radicals during early reperfusion does not limit the size of myocardial infarcts caused by 90 minutes of ischemia in dogs. *Circulation* 78:473-480.

123. Ikeda, H., Y. Koga, T. Oda, K. Kuwano, H. Nakayama, T. Ueno, H. Toshima, L. H. Michael, and M. L. Entman. 1994. Free oxygen radicals contribute to platelet aggregation and cyclic flow variations in stenosed and endothelium-injured canine coronary arteries. *J. Am. Coll. Cardiol.* 24:1749-1756.

124. Basu, D. K. and M. Karmazyn. 1987. Injury to rat hearts produced by an exogenous free radical generating system. Study into the role of arachidonic acid and eicosanoids. *J. Pharmacol. Exp. Ther.* 242:673-685.

125. Cohen, N. L., C. L. Keen, B. Lönnerdal, and L. S. Hurley. 1985. Effects of varying dietary iron on the expression of copper deficiency in the growing rat: anemia, ferroxidase I and II, tissue trace elements, ascorbic acid, and xanthine dehydrogenase. *J. Nutr.* 115:633-649.

126. Ooiwa, H., T. Miura, T. Iwamoto, T. Ogawa, R. Ishimoto, T. Adachi, and O. Iimura. 1992. Superoxide dismutase attenuated post-ischaemic contractile dysfunction in a myocardial xanthine oxidase deficient species. *Clin. Exp. Pharmacol. Physiol.* 19:119-125.

127. De Jong, J. W., P. van der Meer, A. S. Nieukoop, T. Huizer, R. J. Stroeve, and E. Bos. 1990. Xanthine oxidoreductase activity in perfused hearts of various species, including humans. *Circ. Res.* 67:770-773.

128. Mason, D. Y., E. M. Cramer, J. -M. Massé, R. Crystal, J. -M. Bassot, and J. Breton-Gorius. 1991. Alpha$_1$-antitrypsin is present within the primary granules of human polymorphonuclear leukocytes. *Am. J. Pathol.* 139:623-628.

129. Desrochers, P. E., K. Mookhtiar, H. E. Van Wart, K. A. Hasty, and S. J. Weiss. 1992. Proteolytic inactivation of α_1-proteinase inhibitor and α_1-antichymotrypsin by oxidatively activated human neutrophil metalloproteinases. *J. Biol. Chem.* 267:5005-5012.

130. Desrochers, P. E. and S. J. Weiss. 1988. Proteolytic inactivation of alpha-1-proteinase inhibitor by a neutrophil metalloproteinase. *J. Clin. Invest.* 81:1646-1650.

131. Ottonello, L., P. Dapino, M. Scirocco, F. Dallegri, and C. Sacchetti. 1994. Proteolytic inactivation of alpha-1-antitrypsin by human neutrophils: Involvement of multiple and interlinked cell responses to phagocytosable targets. *Eur. J. Clin. Invest.* 24:42-49.

132. Davies, J. M. S., D. A. Horwitz, and K. J. A. Davies. 1994. Inhibition of collagenase activity by *N*-chlorotaurine, a product of activated neutrophils. *Arthritis Rheum.* 37:424-427.

133. Engler, R. L., G. W. Schmid-Schonbein, and R. S. Pavelec. 1983. Leukocyte capillary plugging in myocardial ischemia and reperfusion in dogs. *Am. J. Pathol.* 111:98-111.

134. Bielski, B. H. J., R. L. Arudi, and M. W. Sutherland. 1983. A study of the reacitivity of perhydroxy radical/superoxide ion with unsaturated fatty acids. *J. Biol. Chem.* 258:4759-4761.

135. Fridovich, I. 1985. Biological effects of the superoxide radical. *Arch. Biochem. Biophys.* 240:500-508.

136. Hogg, N., J. Joseph, and B. Kalyanaraman. 1994. The oxidation of α-tocopherol and trolox by peroxynitrite. *Arch. Biochem. Biophys.* 314:153-158.

137. Yuan, G., M. Kaneko, H. Masuda, R. B. Hon, A. Kobayashi, and N. Yamazaki. 1992. Decrease in heart mitochondrial creatine kinase activity due to oxygen free radicals. *Biochim. Biophys. Acta* 1140:78-84.

138. Stadtman, E. R. 1993. Oxidation of free amino acids and amino acid residues in proteins by radiolysis and by metal-catalyzed reactions. *Annu. Rev. Biochem.* 62:797-821.

139. Fliss, H. and G. Docherty. 1987. Oxidation of methionine in proteins of isolated rat heart myocytes and tissue slices by neutrophil-generated oxygen free radicals. In Myocardial Ischemia. N.S. Dhalla, I.R. Innes, and R.E. Beamish, editors. Martinus Nijhoff Publishing, Boston. 85-98.

140. Fliss, H., H. Weissbach, and N. Brot. 1983. Oxidation of methionine residues in proteins of activated human neutrophils. *Proc. Natl. Acad. Sci. USA* 80:7160-7164.

141. Eley, D. W., B. Korecky, H. Fliss, and M. Désilets. 1991. Calcium homeostasis in rabbit ventricular myocytes: disruption by hypochlorous acid and restoration by dithiothreitol. *Circ. Res.* 69:1132-1138.

142. Eley, D. W., J. M. Eley, B. Korecky, and H. Fliss. 1991. Impairment of cardiac contractility and sarcoplasmic reticulum Ca^{2+} ATPase activity by hypochlorous acid: reversal by dithiothreitol. *Can. J. Physiol. Pharmacol.* 69:1677-1685.

143. Fliss, H. and M. Ménard. 1992. Oxidant-induced mobilization of zinc from metallothionein. *Arch. Biochem. Biophys.* 293:195-199.

144. Fliss, H., D. A. Hubatsch, and T. Tatsumi. 1993. Sulfhydryl oxidation and zinc mobilization in reperfused myocardium in vivo. *J. Mol. Cell. Cardiol.* 25(suppl.I):S100.(Abstr.)

145. Shattock, M. J. and H. Matsuura. 1993. Measurement of Na^{+}-K^{+} pump current in isolated rabbit ventricular myocytes using the whole-cell voltage-clamp technique: Inhibition of the pump by oxidant stress. *Circ. Res.* 72:91-101.

146. Kako, K. J. 1987. Free radical effects on membrane protein in myocardial ischemia/reperfusion injury. *J. Mol. Cell. Cardiol.* 19:209-211.

147. Weiss, S. 1986. Oxygen, ischemia, inflammation. *Acta Physiol. Scand.* 126:9-37.

148. Har-El, R. and M. Chevion. 1991. Zinc(II) protects against metal-mediated free radical induced damage: studies on single and double-strand DNA breakage. *Free Radic. Res. Commun.* 12/13:509-515.

149. Held, K. D. 1986. Role of free radical scavengers in radiation protection of DNA. In Free Radicals, Aging, and Degenerative Diseases. Alan R. Liss, Inc., 527-568.

150. Salgo, M. G., K. Stone, G. L. Squadrito, J. R. Battista, and W. A. Pryor. 1995. Peroxynitrite causes DNA nicks in plasmid pBR322. *Biochem. Biophys. Res. Commun.* 210:1025-1030.

151. Buja, L. M., M. L. Eigenbrodt, and E. H. Eigenbrodt. 1993. Apoptosis and necrosis: Basic types and mechanisms of cell death. *Arch. Pathol. Lab. Med.* 117:1208-1214.

152. Farber, J. L. 1982. Biology of disease: membrane injury and calcium homeostasis in the pathogenesis of coagulative necrosis. *Lab. Invest.* 47:114-123.

153. Hillis, L. D. and E. Braunwald. 1977. Myocardial ischemia. *N. Eng. J. Med.* 296:971-978.

154. Searle, J., J. F. R. Kerr, and C. J. Bishop. 1982. Necrosis and apoptosis: distinct modes of cell death with fundamentally different significance. *Pathol. Annu.* 17:229-259.

155. Trump, B. F., J. M. Valigorsky, and J. H. Dees. 1973. Cellular changes in juman disease: a new method of pathological analysis. *Hum. Pathol.* 4:89-109.

156. Burridge, K., K. Fath, T. Kelly, G. Nuckolls, and C. Turner. 1988. Focal adhesions: transmembrane junctions between the extracelluair matrix and the cytoskeleton. *Ann. Rev. Cell Biol.* 4:487-525.

157. Luna, E. J. and A. L. Hitt. 1992. Cytoskeleton-plasma membrane interactions. *Science* 258:955-964.

158. Steenbergen, C., M. L. Hill, and R. B. Jennings. 1987. Cytoskeletal damage during myocardial ischemia: changes in vinculin immunofluorescence staining during total in vitro ischemia in canine heart. *Circ. Res.* 60:478-486.

159. Majno, G. and I. Joris. 1995. Apoptosis, oncosis, and necrosis: An overview of cell death. *Am. J. Pathol.* 146:3-15.

160. Reimer, K. A., R. B. Jennings, and A. H. Tatum. 1983. Pathobiology of acute myocardial ischemia: metabolic, functional and ultrastructural studies. *Am. J. Cardiol.* 52:72A-81A.

161. Schmandt, R. and G. B. Mills. 1993. Genomic components of carcinogenesis. *Clin. Chem.* 39:2375-2385.

162. Kornbluth, R. S. 1994. The immunological potential of apoptotic debris produced by tumor cells and during HIV infection. *Immunol. Lett.* 43:125-132.

163. McConkey, D. J. and S. Orrenius. 1994. Signal transduction pathways to apoptosis. *Trends Cell Biol.* 4:370-374.

164. Pace, G. W. and C. D. Leaf. 1995. The role of oxidative stress in HIV disease. *Free Radic. Biol. Med.* 19:523-528.

165. Petito, C. K. and B. Roberts. 1995. Evidence of apoptotic cell death in HIV encephalitis. *Am. J. Pathol.* 146:1121-1130.

166. Greenspan, H. C. and O. Arouma. 1994. Could oxidative stress initiate programmed cell death in HIV infection? A role for plant derived metabolites having synergistic antioxidant activity. *Chem. Biol. Interact.* 91:187-197.

167. Gougeon, M. -L., S. Garcia, J. Heeney, R. Tschopp, H. Lecoeur, D. Guetard, V. Rame, C. Dauguet, and L. Montagnier. 1993. Programmed cell death in AIDS-related HIV and SIV infections. *AIDS Res. Hum. Retroviruses* 9:553-563.

168. Linnik, M. D., R. H. Zobrist, and M. D. Hatfield. 1993. Evidence supporting a role for programmed cell death in focal cerebral ischemia in rats. *Stroke* 24:2002-2008.

169. MacManus, J. P., A. M. Buchan, I. E. Hill, I. Rasquinha, and E. Preston. 1993. Global ischemia can cause DNA fragmentation indicative of apoptosis in rat brain. *Neurosci. Lett.* 164:89-92.

170. Manev, H., A. Kharlamov, and D. M. Armstrong. 1994. Photochemical brain injury in rats triggers DNA fragmentation, p53 and HSP72. *Neuroreport* 5:2661-2664.

171. MacManus, J. P., I. E. Hill, E. Preston, I. Rasquinha, T. Walker, and A. M. Buchan. 1995. Differences in DNA fragmentation following transient cerebral or decapitation ischemia in rats. *J. Cereb. Blood Flow Metab.* 15:728-737.

172. Coyle, J. T. and P. Puttfarcken. 1993. Oxidative stress, glutamate, and neurodegenerative disorders. *Science* 262:689-695.

173. Weyrich, A. S., T. M. McIntyre, R. P. McEver, S. M. Prescott, and G. A. Zimmerman. 1995. Monocyte tethering by P-selectin regulates monocyte chemotactic protein-1 and tumor necrosis factor-α secretion. Signal integration and NF-kappaB translocation. *J. Clin. Invest.* 95:2297-2303.

174. Barr, P. J. and L. D. Tomei. 1994. Apoptosis and its role in human disease. *Bio/Technology* 12:487-493.

175. Thompson, C. B. 1995. Apoptosis in the pathogenesis and treatment of disease. *Science* 267:1456-1462.

176. Portera-Cailliau, C., J. C. Hedreen, D. L. Price, and V. E. Koliatsos. 1995. Evidence for apoptotic cell death in Huntington disease and excitotoxic animal models. *J. Neurosci.* 15:3775-3787.

177. Bonfoco, E., D. Krainc, M. Ankarcrona, P. Nicotera, and S. A. Lipton. 1995. Apoptosis and necrosis: Two distinct events induced, respectively, by mild and intense insults with *N*-methyl-D-aspartate or nitric oxide/superoxide in cortical cell cultures. *Proc. Natl. Acad. Sci. USA* 92:7162-7166.

178. Gottlieb, R. A., H. A. Giesing, R. L. Engler, and B. M. Babior. 1995. The acid deoxyribonuclease of neutrophils: A possible participant in apoptosis-associated genome destruction. *Blood* 86:2414-2418.

179. Yao, M., A. Keogh, P. Spratt, G. Dos Remedios, and P. C. Kiessling. 1996. Elevated DNase I levels in human idiopathic dilated cardiomyopathy: An indicator of apoptosis. *J. Mol. Cell. Cardiol.* 28:95-101.

180. Martin, S. J., D. R. Green, and T. G. Cotter. 1994. Dicing with death: Dissecting the components of the apoptosis machinery. *Trends Biochem. Sci.* 19:26-30.

181. Kerr, J. F. R., A. H. Wyllie, and A. R. Currie. 1972. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br. J. Cancer* 26:239-257.

182. Collins, M. K. L. and A. L. Rivas. 1993. The control of apoptosis in mammalian cells. *Trends Biochem. Sci.* 18:307-309.

183. Steller, H. 1995. Mechanisms and genes of cellular suicide. *Science* 267:1445-1449.

184. Castle, V. P., K. P. Heidelberger, J. Bromberg, X. Ou, M. Dole, and G. Nuñez. 1993. Expression of the apoptosis-suppressing protein *bcl-2*, in neuroblastoma is associated with unfavorable histology and N-*myc* amplification. *Am. J. Pathol.* 143:1543-1550.

185. Talley, A. K., S. Dewhurst, S. W. Perry, S. C. Dollard, S. Gummuluru, S. M. Fine, D. New, L. G. Epstein, H. E. Gendelman, and H. A. Gelbard. 1995. Tumor necrosis factor alpha-induced apoptosis in human neuronal cells: Protection by the antioxidant *N*-acetylcysteine and the genes *bcl-2* and *crmA*. *Mol. Cell. Biol.* 15:2359-2366.

186. Spector, A., G. -M. Wang, R. -R. Wang, W. -C. Li, and N. J. Kleiman. 1995. A brief photochemically induced oxidative insult causes irreversible lens damage and cataract II. Mechanism of action. *Exp. Eye Res.* 60:483-493.

187. Shimizu, S., Y. Eguchi, H. Kosaka, W. Kamiike, H. Matsuda, and Y. Tsujimoto. 1995. Prevention of hypoxia-induced cell death by Bcl-2 and Bcl- xL. *Nature* 374:811-813.

188. Tilly, J. L. and K. I. Tilly. 1995. Inhibitors of oxidative stress mimic the ability of follicle-stimulating hormone to suppress apoptosis in cultured rat ovarian follicles. *Endocrinology* 136:242-252.

189. Caron-Leslie, L. -A. M., R. B. Evans, and J. A. Cidlowski. 1994. Bcl-2 inhibits glucocorticoid-induced apoptosis but only partially blocks calcium ionophore or cycloheximide-regulated apoptosis in S49 cells. *FASEB J.* 8:639-645.

190. Delic, J., H. Magdelénat, C. Barbaroux, M. P. Chaillet, B. Dubray, E. Gluckman, A. Fourquet, T. Girinsky, and J. M. Cosset. 1995. *In vivo* induction of apoptosis In human lymphocytes by therapeutic fractionated total body irradiation. *Br. J. Radiol.* 68:997-1003.

191. Dewey, W. C., C. C. Ling, and R. E. Meyn. 1995. Radiation-induced apoptosis: Relevance to radiotherapy. *Int. J. Radiat. Oncol. Biol. Phys.* 33:781-796.

192. Eissner, G., F. Kohlhuber, M. Grell, M. Ueffing, P. Scheurich, A. Hieke, G. Multhoff, G. W. Bornkamm, and E. Holler. 1995. Critical involvement of transmembrane tumor necrosis factor-α in endothelial programmed cell death mediated by ionizing radiation and bacterial endotoxin. *Blood* 86:4184-4193.

193. Buttke, T. M. and P. A. Sandstrom. 1994. Oxidative stress as a mediator of apoptosis. *Immunol. Today* 15:7-10.

194. Kroemer, G., P. Petit, N. Zamzami, J. L. Vayssière, and B. Mignotte. 1995. The biochemistry of programmed cell death. *FASEB J.* 9:1277-1287.

195. Cheng, W., B. S. Li, J. Kajstura, P. Li, M. S. Wolin, E. H. Sonnenblick, T. H. Hintze, G. Olivetti, and P. Anversa. 1995. Stretch-induced programmed myocyte cell death. *J. Clin. Invest.* 96:2247-2259.

196. Verhaegen, S., A. J. McGowan, A. R. Brophy, R. S. Fernandes, and T. G. Cotter. 1995. Inhibition of apoptosis by antioxidants in the human HL- 60 leukemia cell line. *Biochem. Pharmacol.* 50:1021-1029.

197. Ratan, R. R., T. H. Murphy, and J. M. Baraban. 1994. Oxidative stress induces apoptosis in embryonic cortical neurons. *J. Neurochem.* 62:376-379.

198. Enokido, Y. and H. Hatanaka. 1993. Apoptotic cell death occurs in hippocampal neurons cultured in a high oxygen atmosphere. *Neuroscience* 57:965-972.

199. Greenlund, L. J. S., T. L. Deckwerth, and E. M. Johnson,Jr.. 1995. Superoxide dismutase delays neuronal apoptosis: A role for reactive oxygen species in programmed neuronal death. *Neuron* 14:303-315.

200. Fang, W., J. J. Rivard, J. A. Ganser, T. W. LeBien, K. A. Nath, D. L. Mueller, and T. W. Behrens. 1995. Bcl-x_L rescues WEHI 231 B lymphocytes from oxidant-mediated death following diverse apoptotic stimuli. *J. Immunol.* 155:66-75.

201. Buttke, T. M. and P. A. Sandstrom. 1995. Redox regulation of programmed cell death in lymphocytes. *Free Radic. Res.* 22:389-397.

202. Bustamante, J., A. F. G. Slater, and S. Orrenius. 1995. Antioxidant inhibition of thymocyte apoptosis by dihydrolipoic acid. *Free Radic. Biol. Med.* 19:339-347.

203. Stefanelli, C., I. Stanic, F. Bonavita, C. Muscari, C. Pignatti, C. Rossoni, and C. M. Caldarera. 1995. Oxygen tension influences DNA fragmentation and cell death in glucocorticoid-treated thymocytes. *Biochem. Biophys. Res. Commun.* 212:300-306.

204. Slater, A. F. G., C. S. I. Nobel, E. Maellaro, J. Bustamante, M. Kimland, and S. Orrenius. 1995. Nitrone spin traps and a nitroxide antioxidant inhibit a common pathway of thymocyte apoptosis. *Biochem. J.* 306:771-778.

205. Rothstein, J. D., L. A. Bristol, B. Hosler, R. H. Brown,Jr., and R. W. Kuncl. 1994. Chronic inhibition of superoxide dismutase produces apoptotic death of spinal neurons. *Proc. Natl. Acad. Sci. USA* 91:4155-4159.

206. Sheen, V. L. and J. D. Macklis. 1994. Apoptotic mechanisms in targeted neuronal cell death by chromophore-activated photolysis. *Exp. Neurol.* 130:67-81.

207. Gottlieb, R. A., K. O. Burleson, R. A. Kloner, B. M. Babior, and R. L. Engler. 1994. Reperfusion injury induces apoptosis in rabbit cardiomyocytes. *J. Clin. Invest.* 94:1621-1628.

208. Abello, P. A., S. A. Fidler, G. B. Bulkley, and T. G. Buchman. 1994. Antioxidants modulate induction of programmed endothelial cell death (apoptosis) by endotoxin. *Arch. Surg.* 129:134-141.

209. Tsukada, T., K. Eguchi, K. Migita, Y. Kawabe, A. Kawakami, N. Matsuoka, H. Takashima, A. Mizokami, and S. Nagataki. 1995. Transforming growth factor β1 induces apoptotic cell death in cultured human umbilical vein endothelial cells with down-regulated expression of bcl-2. *Biochem. Biophys. Res. Commun.* 210:1076-1082.

210. Ghibelli, L., S. Coppola, G. Rotilio, E. Lafavia, V. Maresca, and M. R. Ciriolo. 1995. Non-oxidative loss of glutathione in apoptosis via GSH extrusion. *Biochem. Biophys. Res. Commun.* 216:313-320.

211. Ogita, K. and Y. Yoneda. 1995. Detection of DNA binding activities of transcription factors with different protein motifs in nuclear extracts of murine brain by using gel-retardation electrophoresis. *J. Neurochem.* 64:1431-1439.

212. Fernandes, R. S. and T. G. Cotter. 1994. Apoptosis or necrosis: Intracellular levels of glutathione influence mode of cell death. *Biochem. Pharmacol.* 48:675-681.

213. Jones, D. P., E. Maellaro, S. Jiang, A. F. G. Slater, and S. Orrenius. 1995. Effects of *N*-acetyl-L-cysteine on T-cell apoptosis are not mediated by increased cellular glutathione. *Immunol. Lett.* 45:205-209.

214. Sato, N., S. Iwata, K. Nakamura, T. Hori, K. Mori, and J. Yodoi. 1995. Thiol-mediated redox regulation of apoptosis: Possible roles of cellular thiols other than glutathione in T cell apoptosis. *J. Immunol.* 154:3194-3203.

215. Tortosa, A., R. Rivera, and I. Ferrer. 1994. Dose-related effects of cycloheximide on delayed neuronal death in the gerbil hippocampus after bilateral transitory forebrain ischemia. *J. Neurol. Sci.* 121:10-17.

216. Findik, D., Q. Song, H. Hidaka, and M. Lavin. 1995. Protein kinase A inhibitors enhance radiation-induced apoptosis. *J. Cell. Biochem.* 57:12-21.

217. Araki, S., I. Tsuna, K. Kaji, and H. Hayashi. 1994. Programmed cell death in response to alkyllysophospholipids in endothelial cells. *J. Biochem.* 115:245-247.

218. Bertrand, R., E. Solary, P. O'Connor, K. W. Kohn, and Y. Pommier. 1994. Induction of a common pathway of apoptosis by staurosporine. *Exp. Cell Res.* 211:314-321.

219. Chow, S. C., I. Peters, and S. Orrenius. 1995. Reevaluation of the role of *de novo* protein synthesis in rat thymocyte apoptosis. *Exp. Cell Res.* 216:149-159.

220. Kajstura, J., W. Cheng, K. Reiss, W. A. Clark, E. H. Sonnenblick, S. Krajewski, J. C. Reed, G. Olivetti, and P. Anversa. 1996. Apoptotic and necrotic myocyte cell deaths are independent contributing variables of infarct size in rats. *Lab. Invest.* 74:86-107.

221. Fliss, H. and D. Gattinger. 1995. Apoptosis and protein oxidation in ischemic and reperfused rat myocardium. *Circ. Res.* (In Press):

222. Itoh, G., J. Tamura, M. Suzuki, Y. Suzuki, H. Ikeda, M. Koike, M. Nomura, T. Jie, and K. Ito. 1995. DNA fragmentation of human infarcted myocardial cells demonstrated by the nick end labeling method and DNA agarose gel electrophoresis. *Am. J. Pathol.* 146:1325-1331.

223. Veinot, J. P., D. A. Gattinger, and H. Fliss. 1996. Early apoptosis in human myocardial infarcts. *Hum. Pathol.* (In Press):

224. Barone, F. C., D. B. Schmidt, L. M. Hillegass, W. J. Price, R. F. White, G. Z. Feuerstein, R. K. Clark, E. V. Lee, D. E. Griswold, and H. M. Sarau. 1992. Reperfusion increases neutrophils and leukotriene B_4 receptor binding in rat focal ischemia. *Stroke* 23:1337-1348.

225. Sunderman, F. W.,Jr. 1995. The influence of zinc on apoptosis. *Ann. Clin. Lab. Sci.* 25:134-142.

226. Telford, W. G. and P. J. Fraker. 1995. Preferential induction of apoptosis in mouse $CD4^{+}CD8^{+}\alpha\beta TCR^{lo}CD3\epsilon^{lo}$ thymocytes by zinc. *J. Cell. Physiol.* 164:259-270.

227. Fliss, H. and D. Hubatsch. 1994. Zinc mobilization and apoptosis in reperfused rat myocardium. *J. Free Radic. Biol. Med.* 2(3):H4.(Abstr.)

228. Lohmann, R. D. and D. Beyersmann. 1994. Effects of zinc and cadmium on apoptotic DNA fragmentation in isolated bovine liver nuclei. *Environ. Health Perspect.* 102 Suppl. 3:269-271.

229. Treves, S., P. L. Trentini, M. Ascanelli, G. Bucci, and F. Di Virgilio. 1994. Apoptosis is dependent on intracellular zinc and independent of intracellular calcium in lymphocytes. *Exp. Cell Res.* 211:339-343.

230. Jiang, S., S. C. Chow, M. J. McCabe,Jr., and S. Orrenius. 1995. Lack of Ca^{2+} involvement in thymocyte apoptosis induced by chelation of intracellular Zn^{2+}. *Lab. Invest.* 73:111-117.

231. Wolfe, J. T., D. Ross, and G. M. Cohen. 1994. A role for metals and free radicals in the induction of apoptosis in thymocytes. *FEBS Lett.* 352:58-62.

232. Kluck, R. M., C. A. McDougall, B. V. Harmon, and J. W. Halliday. 1994. Calcium chelators induce apoptosis--Evidence that raised intracellular ionised calcium is not essential for apoptosis. *Biochim. Biophys. Acta* 1223:247-254.

233. Magnelli, L., M. Cinelli, A. Turchetti, and V. P. Chiarugi. 1994. Bcl-2 overexpression abolishes early calcium waving preceding apoptosis in NIH-3T3 murine fibroblasts. *Biochem. Biophys. Res. Commun.* 204:84-90.

234. Ishido, M., S. T. Homma, P. S. Leung, and C. Tohyama. 1995. Cadmium-induced DNA fragmentation is inhibitable by zinc in porcine kidney $LLC\text{-}PK_1$ cells. *Life Sci.* 56:PL351-PL356.

235. Powell, S. R., D. Hall, L. Aiuto, R. A. Wapnir, S. Teichberg, and A. J. Tortolani. 1994. Zinc improves postischemic recovery of isolated rat hearts through inhibition of oxidative stress. *Am. J. Physiol.* 266:H2497-H2507.

236. Searle, A. J. F. and A. Tomasi. 1982. Hydroxyl free radical production in iron-cysteine solutions and protection by zinc. *J. Inorg. Biochem.* 17:161-166.

237. Jacobson, M. D. and M. C. Raff. 1995. Programmed cell death and Bcl-2 protection in very low oxygen. *Nature* 374:814-816.

238. Seehra, J. S., M. G. Gore, A. G. Chaudry, and P. M. Jordan. 1981. Gamma aminolevulinic acid dehydratase. The role of sulfhydryl groups in gamma aminolevulinic dehydratase from bovine liver. *Eur. J. Biochem.* 114:263-269.

239. Hart, B. A., R. M. Prabhu, J. D. Eneman, C. C. Durieux-Lu, A. M. L. Janssen, and P. J. A. Borm. 1995. Oxidant resistance of cadmium-adapted human lung fibroblasts. *Toxicology* 98:1-13.

240. Wan, M., R. Heuchel, F. Radtke, P. E. Hunziker, and J. H. R. Kägi. 1995. Regulation of metallothionein gene expression in Cd- or Zn-adapted RK-13 cells. *Experientia* 51:606-611.

241. Ebadi, M., P. L. Iversen, R. Hao, D. R. Cerutis, P. Rojas, H. K. Happe, L. C. Murrin, and R. F. Pfeiffer. 1995. Expression and regulation of brain metallothionein. *Neurochem. Int.* 27:1-22.

242. Schwarz, M. A., J. S. Lazo, J. C. Yalowich, W. P. Allen, M. Whitmore, H. A. Bergonia, E. Tzeng, T. R. Billiar, P. D. Robbins, J. R. Lancaster,Jr., and B. R. Pitt. 1995. Metallothionein protects against the cytotoxic and DNA-damaging effects of nitric oxide. *Proc. Natl. Acad. Sci. USA* 92:4452-4456.

243. Kröncke, K. -D., K. Fehsel, T. Schmidt, F. T. Zenke, I. Dasting, J. R. Wesener, H. Bettermann, K. D. Breunig, and V. Kolb-Bachofen. 1994. Nitric oxide destroys zinc-sulfur clusters inducing zinc release from metallothionein and inhibition of the zinc finger-type yeast transcription activator LAC9. *Biochem. Biophys. Res. Commun.* 200:1105-1110.

244. Hidalgo, J., L. Campmany, M. Borras, J. S. Garvey, and A. Armario. 1988. Metallothionein response to stress in rats: role in free radical scavenging. *Am. J. Physiol.* 255:E518-E524.

245. Matsubara, J., Y. Tajima, and M. Karasawa. 1987. Metallothionein induction as a potent means of radiation protection in mice. *Radiat. Res.* 111:267-275.

246. Hidalgo, J., A. García, A. M. Oliva, M. Giralt, T. Gasull, B. González, H. Milnerowicz, A. Wood, and I. Bremner. 1994. Effect of zinc, copper and glucocorticoids on metallothionein levels of cultured neurons and astrocytes from rat brain. *Chem. Biol. Interact.* 93:197-219.

247. Hidalgo, J., M. Borràs, J. S. Garvey, and A. Armario. 1990. Liver, brain, and heart metallothionein induction by stress. *J. Neurochem.* 55:651-654.

248. Fliss, H. and M. Ménard. 1991. Hypochlorous acid-induced mobilization of zinc from metalloproteins. *Arch. Biochem. Biophys.* 287:175-179.

249. Gavrieli, Y., Y. Sherman, and S. A. Ben-Sasson. 1992. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J. Cell Biol.* 119:493-501.

250. Hammer, B., W. Davis Parker,Jr., and J. P. Bennett,Jr.. 1993. NMDA receptors increase OH radicals *in vivo* by using nitric oxide synthase and protein kinase C. *Neuroreport* 5:72-74.

251. Sei, Y., D. K. J. E. Von Lubitz, A. S. Basile, M. M. Borner, R. C. -S. Lin, P. Skolnick, and L. H. Fossom. 1994. Internucleosomal DNA fragmentation in gerbil hippocampus following forebrain ischemia. *Neurosci. Lett.* 171:179-182.

252. Lal, A. 1991. Effect of zinc sulphate on infarct size in experimental myocardial infarction in dogs. *Indian J. Med. Res.* 94:316-319.

253. Eaton, D. L. and B. F. Toal. 1982. Evaluation of the Cd/hemoglobin affinity assay for the rapid determination of metallothionein in biological tissues. *Toxicol. Appl. Pharmacol.* 66:134-142.

254. Klein, D., S. Sato, and K. H. Summer. 1994. Quantification of oxidized metallothionein in biological material by a Cd saturation method. *Anal. Biochem.* 221:405-409.

255. Gold, R., M. Schmied, G. Giegerich, H. Breitschopf, H. P. Hartung, K. V. Toyka, and H. Lassmann. 1994. Differentiation between cellular apoptosis and necrosis by the combined use of *in situ* tailing and nick translation techniques. *Lab. Invest.* 71:219-225.

256. Kressel, M. and P. Groscurth. 1994. Distinction of apoptotic and necrotic cell death by in situ labelling of fragmented DNA. *Cell Tissue Res.* 278:549-556.

257. Grasl-Kraupp, B., B. Ruttkay-Nedecky, H. Koudelka, K. Bukowska, W. Bursch, and R. Schulte-Hermann. 1995. *In situ* detection of fragmented DNA (TUNEL assay) fails to discriminate among apoptosis, necrosis, and autolytic cell death: A cautionary note. *Hepatology* 21:1465-1468.

258. Tempel, K. -H. and A. Ignatius. 1993. Toxicological studies with primary cultures of chick embryo cells: DNA fragmentation under the influence of DNase I-inhibitors. *Arch. Toxicol.* 67:318-324.

259. Fliss, H. and D. Gattinger. 1995. Apoptosis and protein oxidation in ischemic or reperfused rat myocardium in vivo. *J. Mol. Cell. Cardiol.* 27:A23.(Abstr.)

260. Sharov, V. G., H. N. Sabbah, H. Shimoyama, A. V. Goussev, M. Lesch, and S. Goldstein. 1996. Evidence of cardiocyte apoptosis in myocardium of dogs with chronic heart failure. *Am. J. Pathol.* 148:141-149.

261. Oppenheim, R. W., L. J. Houenou, J. E. Johnson, L. -F. H. Lin, L. Li, A. C. Lo, A. L. Newsome, D. M. Prevette, and S. Wang. 1995. Developing motor neurons rescued from programmed and axotomy-induced cell death by GDNF. *Nature* 373:344-346.

262. Houenou, L. J., P. L. Turner, L. Li, R. W. Oppenheim, and B. W. Festoff. 1995. A serine protease inhibitor, protease nexin I, rescues motoneurons from naturally occurring and axotomy-induced cell death. *Proc. Natl. Acad. Sci. USA* 92:895-899.

263. Cheng, W., J. Kajstura, J. Nitahara, B. Li, K. Reiss, Y. Liu, W. A Clark, S. Krajewski, J. Reed, G. Olivetti, and P. Anversa. 1996. Programmed Myocyte Cell Death Affects the Viable Myocardium after Infarction in Rats. *Exp. Cell Res.* 226:316-327.

264. Kawano, H., R. Okada, Y. Kawano, and N. Sueyoshi. 1994. Apoptosis in acute and chronic myocarditis. *Jpn. Heart J.* 35:745-750.

265. Ferrari, R., C. Ceconi, S. Curello, A. Cargnoni, O. Alfieri, A. Pardini, P. Marzollo, and O. Visioli. 1991. Oxygen free radicals and myocardial damage: Protective role of thiol-containing agents. *Am. J. Med.* 91 Suppl. 3C:95S-105S.

266. Ceconi, C., S. Curello, A. Cargnoni, G. M. Boffa, and R. Ferrari. 1990. Antioxidant protection against damage during cardiac ischemia and reperfusion: effect of dimercapto-propanol. *Cardioscience* 1:191-198.

267. Kukreja. R. C., K. E. Loesser, A. A. Kearns, S. A. Naseem, and M. L. Hess. 1993. Protective effects of histidine during ischemia-reperfusion in isolated perfused rat hearts. *Am. J. Physiol.* 264:H1370-H1381.

268. Poston, J. M. and G. L. Parenteau. 1992. Biochemical effects of ischemia on isolated, perfused rat heart tissues. *Arch. Biochem. Biophys.* 295:35-41.

269. Matsubara, K., M. Kubota, K. Kuwakado, H. Hirota, Y. Wakazono, A. Okuda, R. Bessho, Y. W. Lin, S. Adachi, and Y. Akiyama. 1994. Variable susceptibility to apoptosis induced by calcium ionophore in hybridomas between HL-60 promyelocytic and CEM T-lymphoblastic leukemia cell lines: Relationship to constitutive Mg^{2+}-dependent endonuclease. *Exp. Cell Res.* 213:412-417.

270. Duke, R. C., R. Z. Witter, P. B. Nash, J. D. -E. Young, and D. M. Ojcius. 1994. Cytolysis mediated by ionophores and pore-forming agents: Role of intracellular calcium in apoptosis. *FASEB J.* 8:237-246.

271. Currie, R. W. 1986. Synthesis of stress-induced protein in isolated and perfused rat heart. *Biochem. Cell Biol.* 64:418-426.

272. Currie, R. W. 1987. Effects of ischemia and perfusion temperature on the synthesis of stress-induced (heat shock) proteins in isolated and perfused rat hearts. *J. Mol. Cell. Cardiol.* 19:795-808.

273. Currie, R. W. 1988. Protein synthesis in perfused rat hearts after in vivo hyperthermia and in vitro cold ischemia. *Biochem. Cell Biol.* 66:13-19.

274. Kukreja, R. C., M. C. Kontos, K. E. Loesser, S. K. Batra, Y. -Z. Qian, C. J. Gbur,Jr., S. A. Naseem, R. L. Jesse, and M. L. Hess. 1994. Oxidant stress increases heat shock protein 70 mRNA in isolated perfused rat heart. *Am. J. Physiol.* 267:H2213-H2219.

275. Radi, R., M. Rodriguez, L. Castro, and R. Telleri. 1994. Inhibition of mitochondrial electron transport by peroxynitrite. *Arch. Biochem. Biophys.* 308:89-95.

276. Brunet, J., M. J. Boily, S. Cordeau, and C. Des Rosiers. 1995. Effects of *N*-acetylcysteine in the rat heart reperfused after low-flow ischemia: Evidence for a direct scavenging of hydroxyl radicals and a nitric oxide-dependent increase in coronary flow. *Free Radic. Biol. Med.* 19:627-638.

277. Eley, D. W., B. Korecky, and H. Fliss. 1989. Dithiothreitol restores contractile function to oxidant-injured cardiac muscle. *Am. J. Physiol.* 257:H1321-H1325.

278. Boraso, A. and A. J. Williams. 1994. Modification of the gating of the cardiac sarcoplasmic reticulum Ca^{2+}-release channel by H_2O_2 and dithiothreitol. *Am. J. Physiol.* 267:H1010-H1016.

279. Black, S. C., J. H. McNeill, and S. Katz. 1991. Calcium transport activity of rat cardiac sarcoplasmic reticulum isolated in the presence of dithiothreitol. *J. Pharmacol. Methods* 25:53-60.

280. Koshita, M., K. Miwa, and T. Oba. 1993. Sulfhydryl oxidation induces calcium release from fragmented sarcoplasmic reticulum even in the presence of glutathione. *Experientia* 49:282-284.

281. Steenbergen, C., E. Murphy, J. A. Watts, and R. E. London. 1990. Correlation between cytosolic free calcium, contracture, ATP, and irreversible ischemic injury in perfused rat heart. *Circ. Res.* 66:135-146.

282. Tani, M. 1990. Effects of anti-free radical agents on Na+, Ca2+, and function in reperfused rat hearts. *Am. J. Physiol.* 259:H137-H143.

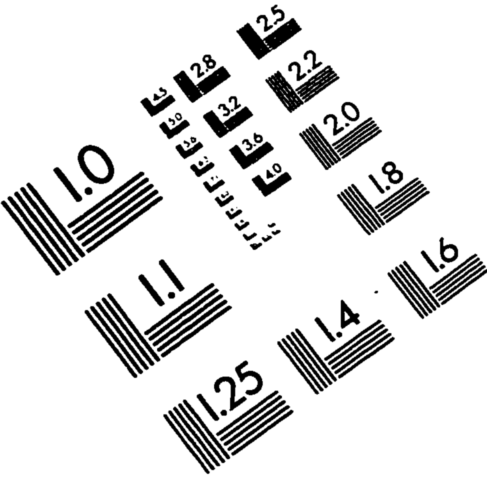
283. Gailis, L., W. G. Nayler, and S. E. Harding. 1990. Reversible elimination of myofibrillar Ca2+ sensitivity by diamide and other sulfhydryl reagents: comparison with reversible contracture produced in intact cells. *Can. J. Physiol. Pharmacol.* 68:1170-1175.

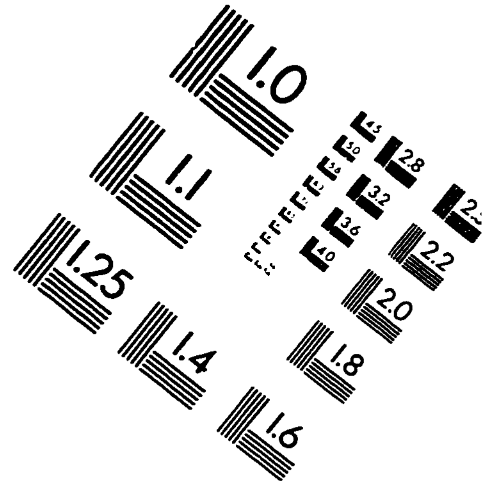
284. Yan, C. Y. I., G. Ferrari, and L. A. Greene. 1995. *N*-acetylcysteine-promoted survival of PC12 cells is glutathione- independent but transcriptional-dependent. *J. Biol. Chem.* 270:26827-26832.

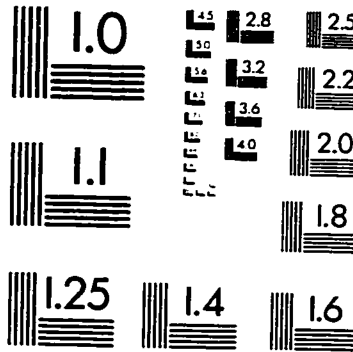
285. Ratan, R. R., T. H. Murphy, and J. M. Baraban. 1994. Macromolecular synthesis inhibitors prevent oxidative stress-induced apoptosis in embryonic cortical neurons by shunting cysteine from protein synthesis to glutathione. *J. Neurosci.* 14:4385-4392.

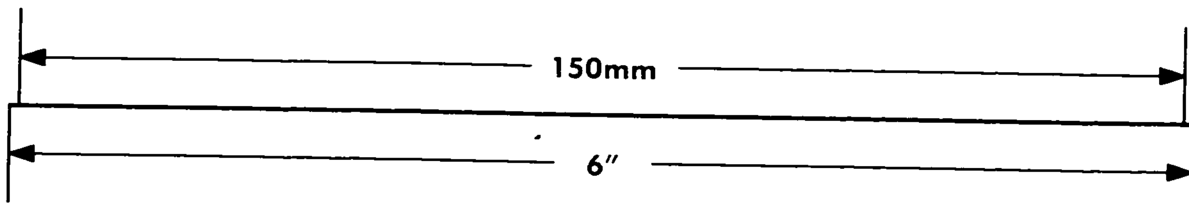
286. Bilzer, M. and B. Lauterburg. 1991. Glutathione metabolism in activated human neutrophils: stimulation of glutathione synthesis and consumption of glutathione by reactive oxygen species. *Eur. J. Clin. Invest.* 21:316-322.

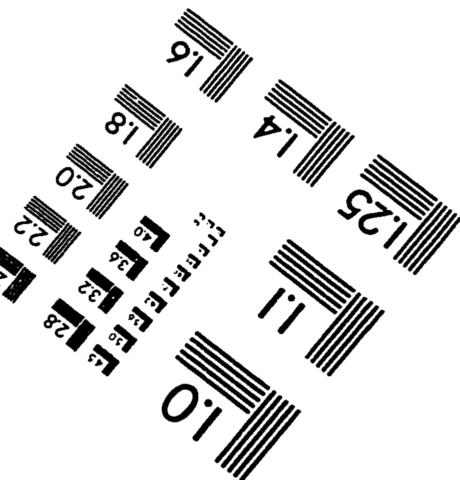
IMAGE EVALUATION TEST TARGET (QA-3)











APPLIED IMAGE . Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989

© 1993, Applied Image, Inc., All Rights Reserved