Abstract

Reperfusion of the ischemic myocardium occurs in nearly 2 million people annually in the United States as a complication of cardiovascular disease, in patients experiencing cardiac arrest, myocardial infarction or undergoing cardioplegic arrest during cardiac surgery. Various levels of low flow are induced by such ischemic events, most notably in the moments following ischemia. However, the post-ischemic low flow period has hardly been examined in the literature. The work presented in this dissertation explores the role of reactive oxygen species (ROS) generation in global ischemia, post-ischemic low flow, and subsequent full reperfusion of the myocardium. This dissertation consists of three parts. In Chapter 2, an isolated buffer-perfused rat heart model was employed to explore both low pressure and low coronary flow as interventions in global cardiac ischemia. The ROS burst was observed upon full reperfusion in rat hearts, with differences in ROS generation between the cellular and vascular compartments. Differences in recovery of left ventricular (LV) function were also observed, with LV functional preservation at 10% low flow, but not at 0.5% low flow intervention. In Chapter 3, a novel model of the isolated heart is described, in which whole blood is recirculated throughout experimentation. Blood-perfused and buffer-perfused hearts were noted to respond similarly to global ischemia and low flow intervention. Also, a method describing detection of the ROS burst at reperfusion in blood-perfused hearts is
described. A characteristic acute, short-lived formation of hydrogen peroxide was
discovered in blood-perfused hearts subjected to ischemia and reperfusion. In Chapter 4,
the neutrophil elastase inhibitor Sivelestat is examined for putative cardioprotective
properties. Sivelestat was noted to possess dramatic infarct-sparing properties in the
isolated heart, along with the ability to preserve LV function. ROS production was
examined, and Sivelestat was shown to reduce ROS in ischemic-reperfused hearts and
also in hypoxic-reoxygenated aortic endothelial cells. The aim of Chapter 4 is to
introduce Sivelestat to the heavily investigated field of cardiac pharmacology.
Dedicated to my little sisters, Heidi and Hannah
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Chapter 1. Introduction

1.1 Low coronary flow following myocardial ischemia

Reperfusion of the ischemic myocardium occurs in nearly 2 million people annually in the United States as a complication of cardiovascular disease, in patients experiencing cardiac arrest, myocardial infarction or undergoing cardioplegic arrest during cardiac surgery [1]. Various levels of low flow are induced by such ischemic events, most notably in the moments following ischemia [2, 3, 4]. In the case of cardiac arrest, which affects over 300,000 people each year, cardiopulmonary resuscitation (CPR) is the tool of first responders [5]. The return of spontaneous circulation (ROSC) must be attained in order to salvage heart tissue and function, but the survival-to-hospital discharge rate for out-of-hospital cardiac arrests (OHCA) patients remains fixed below 10%. External defibrillation is a valuable, but incomplete approach to cardiac arrest, as over 70% of OHCA are not a result of ventricular fibrillation or tachycardia [6]. During resuscitation efforts, the heart experiences a period of low pressure compressions, which induces low coronary flow [7]. This period of reduced myocardial perfusion induced during resuscitation efforts following global ischemia is poorly understood. Hearts subjected to low pressure/low flow intervention may experience different recovery of left ventricular (LV) function than hearts reperfused at full pressure without a preceding
resuscitative period of low pressure [8]. It is not known if this period of low pressure is beneficial or detrimental to heart function, and the subcellular mechanisms that are activated during this period are scantly defined. A major theme of this dissertation is to examine the influence of one ubiquitous set of subcellular signaling molecules on post-ischemic low flow, that of reactive oxygen species.

1.2 The Role of Reactive Oxygen Species in Myocardial Ischemia and Reperfusion

At reperfusion, the reintroduction of oxygen to the ischemic myocardium contributes to myocardial injury by generating a burst of reactive oxygen species (ROS). Heavily studied ROS include superoxide (O$_{2}^-$), hydroxyl radical (OH$^\cdot$), and hydrogen peroxide (H$_2$O$_2$) [9]. The release of oxygen-derived free radicals concurrent with the reintroduction of oxygen contributes to the pathophysiology of ischemia-reperfusion (IR) injury [10, 11, 12, 13]. For instance, the reperfusion ROS burst is implicated in subsequent organ failure and death in humans who have experienced cardiac arrest and myocardial reperfusion upon ROSC [14]. In addition to cellular injury through oxygen radical-mediated damage of cellular [12] and mitochondrial [15] structural components, reactive oxygen species signal neutrophil infiltration [16] and are implicated in endothelial dysfunction [17] and smooth muscle injury [18]. Specifically, ischemia-reperfusion impairs endothelium-dependent vasorelaxation through production of ROS [18]. In addition to the macrocirculation, ischemia-reperfusion impairs endothelium-dependent relaxation of coronary microvessels [19]. Electron spin resonance
characterization of vascular xanthine and NAD(P)H oxidase activity in patients with coronary artery disease: relation to endothelium-dependent vasodilation. Excess hydrogen peroxide production in response to reperfusion of the ischemic myocardium damages the life cycle of vascular smooth muscle cells [20], and reduction of ROS upon reperfusion attenuates myocyte and endothelial cell injury following ischemia-reperfusion [21, 22]. The study of ROS generation remains a strong focus of investigators of myocardial ischemia-reperfusion injury.

1.3 Sub-cellular sources of reactive oxygen species

The ubiquitous enzyme xanthine oxidoreductase (XOR) remains a heavily studied source of free radicals in the reperfused myocardium, both in basic research and clinical trials [23] (comprehensive reviews in [24] and [25]. Pharmacologic targeting of xanthine oxidase in the heart during IR continues to generate a wealth of clinical inquiry and remains an active field of pharmacologic research [4, 26, 27]. XOR is localized in the coronary vascular endothelium and in smooth muscle and interstitium in rat myocardium [28], and is present in the vascular smooth muscle, luminal endothelium [29] and myocytes of human cardiac muscle [3, 30, 31, 32]. It is interconvertible between its two forms, xanthine dehydrogenase (XDH), which reduces NAD+, and xanthine oxidase (XO), which transfers an electron to molecular oxygen during conversion of hypoxanthine to xanthine and again during conversion of xanthine to uric acid, twice forming the free radical superoxide [33, 34]. During ischemia in the heart, XDH is
converted by proteases to the XO form [35]. Ischemia also causes buildup of byproducts of anaerobic metabolism, including hypoxanthine [36]. Upon the reintroduction of oxygen at reperfusion, the increased concentrations of activated xanthine oxidase and its substrate, hypoxanthine, contribute to the burst of free radicals that occurs upon reperfusion [37, 38]. XO-derived superoxide has the effect of scavenging endothelium-derived nitric oxide (NO) and forming peroxynitrite [39], thereby contributing directly to post-ischemic endothelial dysfunction in the heart [40, 41].

NADPH oxidase is a superoxide-producing enzyme that was originally discovered as the source of the phagocytic oxidative burst [42], and is involved in gene regulation and expression in the vasculature [43]. Non-superoxide-producing xanthine oxidoreductase is converted to the superoxide-producing form xanthine oxidase by ROS produced from NADPH oxidase [44]. Vascular smooth muscle cells express NADPH oxidase 1 and 4 (Nox1, Nox4), while cardiomyocytes express Nox2 and Nox4, but not Nox1 [9]. NADPH oxidase is also implicated in ischemia-reperfusion injury of vascular cells in the heart [45]. ROS from NADPH oxidase induces apoptosis of vascular smooth muscle cells [46]. In support of this finding, blockade of NADPH oxidase enzyme protects cardiomyocytes from hypoxia-reoxygenation injury [47]. NADPH oxidase is inhibited effectively by apocynin and thus is an important pharmacologic target for prevention of ischemia-reperfusion injury [48].
In this dissertation, NADPH oxidase and xanthine oxidase enzymes were examined as important sources of ROS generation in the settings of post-ischemic low flow and full-flow reperfusion.

1.4 Investigating the neutrophil elastase inhibitor Sivelestat for putative cardioprotection

Despite intense investigation, there is no drug in the United States that has been approved for reduction of infarct size following cardiac ischemia-reperfusion (IR) [49]. In the earliest stages of reperfusion, apoptosis occurs first in the endothelium of small coronary vessels, and then becomes uniform after 2 hours in the myocytes of isolated rat hearts [50]. In this way, apoptosis is first initiated by myocardial ischemia and then actualized during reperfusion, leading to infarction [51]. An important cardioprotective strategy is the preservation of vascular endothelial function to reduce infarct formation following ischemia-reperfusion. This approach holds great promise for clinical-translation studies in humans [52].

Sivelestat, the neutrophil elastase inhibitor, is currently in use in Japanese clinics for the treatment of acute lung injury and acute respiratory distress syndrome [53], and has recently been shown to preserve heart function and endothelium-dependent vasoreactivity when applied during reperfusion of post-ischemic isolated rat hearts. Kambe et al published data also using buffer-perfused isolated rat hearts showing a 35%
recovery of left ventricular (LV) developed pressure versus controls (16%) when Sivelestat was infused continuously during the first 10 min of full reperfusion [54].

Certain studies assert that Sivelestat mediates vasorelaxation independent of endothelium. Maeda et al. indicated that this mechanism lies in the vasculature by demonstrating that Sivelestat selectively inhibits calcium sensitization to a receptor agonist in porcine vascular smooth muscle strips with or without endothelium, without affecting calcium-induced contraction [55]. Amemori et al. reiterated this work showing that Sivelestat induces endothelium-independent vasorelaxation in pre-contracted human gastric arteries [56]. Sivelestat also protects the heart in situations where neutrophils are present. Akiyama et al. showed attenuation of myocardial stunning in swine with post-ischemic infusion of sivelestat [57]. Ueno et al. demonstrated a reduction in IR injury with application of sivelestat in a heart transplantation model using dogs [58]. Toyama et al. reported an association of Sivelestat infusion with improved fractional area of change in the left ventricle of pediatric patients who underwent cardiovascular surgery with cardiopulmonary bypass, demonstrating that Sivelestat is protective in at least one setting of ischemia-reperfusion in humans [59].

The influence of Sivelestat on ROS production in the myocardium is not known, nor are the precise mechanisms of Sivelestat in cardioprotection following prolonged global ischemia. Based upon the scientific literature outlined above, it is likely that Sivelestat possesses cardioprotective mechanisms, both dependent on, and independent of, neutrophil elastase inhibition. This dissertation investigates the unknown neutrophil-independent cardioprotective mechanisms of Sivelestat. The purpose of chapter 4 is to
introduce the putative cardioprotective properties of Sivelestat to the heavily studied field of cardiac pharmacology.

1.5 The isolated whole blood-perfused heart

Multiple experimental models are required in order to study ischemia-reperfusion in the heart. The isolated rat heart preparation, first described in 1897 by Oscar Langendorff [60], continues to be a useful model. This heart preparation is particularly relevant for isolating myocardial response from peripheral neural, endocrine and vascular influences [61, 62, 63]. The buffer-perfused Langendorff heart model has been used extensively in studies of myocardial ischemia and reperfusion (IR). Key findings demonstrated in this model include a burst of reactive oxygen species production at reperfusion [64] and impaired left ventricular (LV) function due to stunning or infarction during reperfusion [65, 66].

There are, however, inherent limitations when using the buffer-perfused Langendorff rat heart to study IR generated ROS production and oxidant stress. Buffer-based acellular solutions require high oxygen tension and coronary flow rates several times the physiologic rate to compensate for their low oxygen carrying capacity [60, 61, 67]. The resulting high oxygen tension can potentially increase ROS production, particularly with reoxygenation of the ischemic myocardium [68]. The absence of the blood borne antioxidant capability in buffer-perfused hearts can further contribute to an
increased ROS effect [69, 70, 71]. All of these factors may exacerbate IR tissue damage. In the study of ROS formation and myocardial IR the blood-perfused heart offers significant advantages over the buffer-perfused heart, including the ability to oxygenate the heart under physiologic oxygen delivery conditions. In this chapter we describe a novel recirculating blood-perfused isolated rat heart model using autologous whole blood from the same animal and the ability to measure plasma H$_2$O$_2$ and tissue oxidant stress following IR.

In this dissertation, it is my desire to contribute a small amount of understanding to the subcellular ROS response of hearts to post-ischemic low flow. I hope that such results can be further extended to studies in the whole animal.
Chapter 2: Intervention in global myocardial ischemia with low pressure perfusion causes modification of cellular and vascular reactive oxygen species at full reperfusion

2.1 Introduction and Hypothesis

Low coronary flow is a common condition in post-ischemic hearts. The objective of this study was to investigate vascular and intracellular ROS formation in response to the progression of global ischemia, low flow and full reperfusion in the heart. Though reactive oxygen species are implicated in damage resulting from ischemia and reperfusion in the heart, the influence of post-ischemic low flow on ROS production is not known. An understanding of how post-ischemic low flow influences ROS production in the cellular compartments is essential for the development of therapies targeted at improving LV function following insult. We hypothesized that intervening in global zero-flow ischemia with low pressure perfusion prior to full-flow reperfusion would improve recovery of left ventricular function through a reactive oxygen species-mediated mechanism.
2.2 Methods

2.2.1 Isolated Heart Preparation

Male Sprague-Dawley rats (350-450 g) supplied by Harlan (Indianapolis, IN) were used in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996) and the approval of the Ohio State University Laboratory Animal Resources Committee. Hearts were isolated and perfused in the Langendorff mode as described in Palmer et al. [72].

Rats were anesthetized with intra-peritoneal sodium pentobarbital (50 mg/kg). The right superficial jugular vein was isolated and heparin (1000 U/kg) was administered. The trachea was cannulated with a 16-ga catheter attached to a rodent ventilator (Harvard Apparatus, South Natick, MA) set to provide adequate ventilation with room air. A midsternal thoracotomy was performed to expose the heart and to cannulate the aorta. Following rapid cannulation of the aorta, retrograde coronary perfusion with Krebs-Henseleit (K-H) buffer (1.25 mM CaCl2, 11 mM glucose, 112 mM NaCl, 25 mM NaHCO3, 5 mM KCl, 1.2 mM MgSO4, 1 mM K2PO4, and 0.2 mM octanoic acid, bubbled with 95% O2 / 5% CO2, pH of 7.4) was initiated in situ. Hearts were quickly excised from the chest and transferred to the Langendorff apparatus and perfused with warmed (37.4° C) K-H buffer at a constant pressure of 85 mmHg.
2.2.2 Tissue surface fluorescence

The heart perfusion apparatus was housed within a black cabinet that, when closed, was designed to be impermeable to visible light. The temperature controlled glass chamber within which the heart was positioned was equipped with a water-tight glass tube that rested against the left ventricle. The glass tube was designed to hold a single two-way, excitation/emission, fiberoptic cable attached to a photomultiplier tube, which was attached to a fluorometer positioned external to the cabinet. A 150 watt Xenon lamp light source was focused on a fiber optic cable 6 mm in diameter and sequentially focused on a photomultiplier tube (model HC 120-05MOD-6308; Hamamatsu PTM Assembly, Japan). The excitation and emission optical fibers were combined into a single cable for epifluorescence measurements. Within the fluorometer the single fiberoptic cable was split into excitation and emission branches. The fluorometer was equipped with two linked four-chambered filter wheel assemblies, one at the excitation end and one at the emission end of the photomultiplier tube. Precisely weight-balanced fluorescent light filters could be placed within each chamber to allow for select bands of the fluorescent light source at both the excitation and emission side of the photomultiplier tube. The two filter wheel assemblies were linked by a single axle that was driven by a motor designed to turn the axle at up to 1500 rotations per minute. In this way, each one of the four chambers within the excitation side of the filter wheel assembly was dynamically linked to its partner on the emission side. Similarly, since the rate of decay of fluorescence energy is on the femtosecond scale, each excitation/emission filter pair was linked in near simultaneous excitation and emission of a specific band of fluorescent energy. As the
axle was rapidly rotated, up to four fluorophores could be excited and the emission light captured in near-simultaneous fashion with a ratiometric tissue fluorometer (RADNOTI). Light exposure causes oxidation of fluorescent molecules that leads to decay of the fluorescent emission light. In order to minimize photooxidation of the fluorophores located within the tissue, lamp shutters were driven automatically to expose the left ventricle for only 6 seconds out of each minute of perfusion. Fluorescence emission data was collected through an analog-to-digital converter and recorded using a modified programming language (Workbench PC, Strawberry Tree) [73].

2.2.4 Detection of ROS and redox state with fluorophores

In the presence of the enzyme horseradish peroxidase (HRP), Amplex Red is converted to resorufin in response to the reactive oxygen species hydrogen peroxide. Amplex Red and its product resorufin are confined to the vasculature of the heart and thus will not cross the endothelial barrier. This confinement allows Amplex Red to rapidly perfuse through the heart and wash out in the coronary effluent. The rapid washout time requires the user to continually infuse Amplex Red/HRP as the heart is being perfused so as to avoid loss of fluorescent resorufin signal. Resorufin was excited at 535 nm, with a filtered bandwidth of 35 nm, and emission light was captured at 595 nm, bandwidth 45 nm.
The fluorescent species dihydrofluorescein diacetate (fluorescein) is sensitive to most oxidases, and the acetate-free form of the molecule becomes fluorescent in the presence of reactive oxygen species. This fluorescence can only occur after the molecule crosses the myocyte cellular wall and the acetate moieties of the molecule are cleaved by intracellular esterases. As a result, the acetate-free molecule is sensitive to reactive oxygen species that are produced within the myocyte cytosol only. Fluorescent fluorescein remains trapped within the cell cytosol and thus does not experience the washout that occurs with resorufin [74]. Fluorescein was excited at 455 nm, with a filtered bandwidth of 70 nm, and emission light was captured at 556 nm, bandwidth 20 nm.

The term “autofluorophore” refers to any fluorescent molecule that exists naturally within a cell [75]. The autofluorescent coenzyme nicotinamide adenine dinucleotide, abbreviated NAD+, is present in all mammalian cells, and functions as an energy store in redox metabolism. NAD+ accepts two electrons from the oxidation of glucose and fatty acids, forming NADH,H+, simply termed NADH by the literature. NADH is fluorescent, while NAD+ is not. An active electron transport chain found on the membrane of cardiac mitochondria will oxidize NADH to NAD+, generating ATP through oxidative phosphorylation. The onset of ischemia causes an immediate buildup of NADH as the electron transport chain ceases to accept electrons from NADH. The buildup of NADH causes an increase in the NADH/NAD+ ratio, which is observed as an increase in the autofluorescence emission intensity of NADH. Monitoring the NADH autofluorescence of the functioning left ventricle gives an excellent reflection of
intramitochondrial redox state [76]. NADH was excited at 330 nm, bandwidth 80 nm, and emission light was captured at 470 nm, bandwidth 10.

2.2.5 Fluorescent microscopy

Confocal fluorescent microscopy was utilized to ensure that each fluorophore was present within the proper tissue compartment. In separate experiments, isolated hearts were paralyzed with the negative inotrope 2,3-butanedione monoxime (BDM, 50 mM) [77]. Hearts were perfused in a large petri dish on the stage of a Zeiss LSM 510 META Laser Scanning Microscope equipped with an AxioCam digital microscope camera. Perfusate was maintained at 37° C with a thermocoupler (Harvard Apparatus) placed in line with the perfusate tubing. Fluorescein was excited with a single photon Argon laser set to 488 nm wavelength excitation, and emission light was collected beyond a 543 nm band pass filter. Amplex Red was infused continuously at 25 nM concentration with horseradish peroxidase as described above. Resorufin was excited using a Helium Neon (HeNe) laser at 543 nm, and emission light was collected beyond a 545 nm band pass filter. During loading of hearts with Amplex Red and HRP, Vybrant DiD (Invitrogen) was also loaded, which is a redox-insensitive permanent fluorescent label of endothelial cells. DiD was excited with a single photon Helium Neon (HeNe2) laser at 633 nm, and emission light was collected beyond a 650 nm band pass filter. Photographs were taken using META software (Carl Zeiss Microscopy, LLC).
The use of fluorescein as an intracellular ROS probe in tissue surface fluorometry was proven in concept with fluorescent microscopy. Hearts paralyzed with BDM showed an intracellular uptake of fluorescein that was not observed in vessels or endothelial interstitial space. Upon loading hearts with dihydrofluorescein diacetate, bright fluorescein fluorescence was observed to be localized within the cellular compartments of the functional syncytium of cardiomyocytes (See Figure 2.1). Resorufin and DiD fluorescence was observed to be localized within the vascular compartments of the left ventricle (See Figure 2.2).
Figure 2.1 Parallel chains of myocytes at the left ventricular surface of the intact heart. Fluorescein was localized within myocytes. Magnification = 200X.
Figure 2.2. Resorufin and Did fluorescence were colocalized within the vasculature. Resorufin fluorescence (purple) was located within the blood vessels of the left ventricle, along with DiD (magenta, DiD is a vascular marker). White areas indicate high colocalization coefficient (greater than 0.99) between resorufin and DiD. Magnification=200x.
2.2.5 Experimental protocol

To test the hypothesis that intervening in global zero-flow ischemia with low pressure perfusion would improve recovery of LV function through a ROS-mediated mechanism, hearts were randomized to two groups (N = 4 per group). After the infusion of fluorophores, hearts received 1) 20 minutes of global no-flow ischemia, or 2) 3 minutes of global no-flow ischemia followed by 17 minutes of low pressure perfusion at 20 mm Hg generated by a pulsatile infusion pump (Harvard Apparatus) operating at 200 pumps per minute. Flow generated by the pulsatile pump was between 2 and 3 mL per minute. After ischemia or low flow, hearts from both groups were immediately reperfused at 85 mm Hg perfusion pressure for 30 minutes.

2.2.6 Left ventricular function

A saline-filled latex balloon attached to a pressure transducer was inserted into the left ventricle for measurements of left ventricular (LV) contractile function. The hearts were positioned in a temperature-controlled (37.4º C) glass chamber. Coronary flow rates were measured using an in-line flow probe. The LV balloon volume was inflated at the beginning of the experiment to a left ventricular end diastolic pressure of 5 mmHg. LV pressure was continuously sampled at a frequency response of 45 Hz and digitally processed by a heart performance analyzer (Digi-Med, Micro-Med, Louisville, KY). Continuous measures of LV function were derived by computer algorithm: LV systolic
pressure, LV end diastolic pressure, heart rate, and dP/dt\textsubscript{max}. Developed pressure (systolic – diastolic pressure), and rate-pressure product (developed pressure × heart rate) were calculated.

### 2.2.7 Infusion of fluorophores

Prior to ischemia, hearts (n=4/group) were perfused via a side cannula situated directly above the aorta for 15 minutes with 25 µM fluorescein. Hearts were infused in the same manner with 25 nM Amplex Red (Invitrogen) and 5 mU/mL horseradish peroxidase (HRP). Amplex Red and HRP were also infused throughout low flow and reperfusion. All fluorophore concentrations noted were the final concentrations entering the heart. Each fluorophore was infused through a side-arm stopcock directly above the heart at 20 times the final concentration, with infusion rate set to a 1:20 ratio to the coronary flow rate of the krebs-henseleit perfusate. Relative fluorescent emission output was time-averaged every 30 seconds. Time-resolved emission data for each oxidant probe was taken as a fraction of the average reading for ten minutes prior to ischemia (the baseline period) and called F/F\textsubscript{0}. 


2.2.8 Data analysis

Data are presented as mean ± S.E. Indices of LV function and fluorescence were calculated and analyzed by one-way analysis of variance (ANOVA) with Tukey’s post-hoc test to determine significance of results at p<0.05.

2.3 Results

![Graph showing rate pressure product](image)

Figure 2.3. Rate pressure product. **p<0.01

Rate pressure product was significantly reduced in hearts subjected to low pressure intervention at the end of 30 minutes of reperfusion, as compared to control
ischemia (low pressure vs. RPP = 17001 ± 1870 vs. control ischemia RPP = 6797 ± 3723).

Figure 2.4. **Peak reperfusion fluorescence.** **p<0.01**

Measurements were taken as the maximum fluorescence reading observed within 5 minutes of the onset of full reperfusion. Low pressure intervention following 3 min of global ischemia caused a significant increase, upon reperfusion with full pressure, in resorufin fluorescence (low pressure F/F₀ = 0.348 ± 0.081 vs. control ischemia F/F₀ = 0.178 ± 0.080) and a significant decrease in fluorescein fluorescence (low pressure F/F₀ = 0.04 ± 0.018 vs. control ischemia F/F₀ = 0.650 ± 0.124) as compared to hearts that received control ischemia (20 min of global ischemia) prior to reperfusion.
Figure 2.5. NADH fluorescence. *p<0.05

NADH fluorescence dropped significantly at the onset of low pressure perfusion, and remained significantly low throughout low pressure intervention as compared to hearts that received control ischemia. NADH fluorescence was significantly decreased at the end of reperfusion in hearts that received low pressure intervention as compared to hearts that received control ischemia prior to reperfusion (low pressure F/F₀ = 0.904 ± 0.025 vs. control ischemia F/F₀ = 1.021 ± 0.036).
2.4 Discussion

Contrary to our hypothesis, this study revealed that intervention in global ischemia with low pressure perfusion is deleterious to recovery of heart contractile function. LV recovery is possibly coupled to reperfusion ROS generation, as observed in the significant changes in fluorescence within the cytosol and vasculature. Low pressure intervention also induced alterations in redox metabolism, as indicated by changes in the intracellular NADH:NAD+ ratio.

Perfusion pressure during low pressure perfusion was 37% of baseline. In a similar study employing a longer duration of ischemia (15 min) before low flow intervention, Klawitter et al. suggested that post-ischemic coronary flow levels below 1% of baseline values resulted in worse bioenergetic recovery than in hearts subjected to 20 min of global ischemia [78]. Within the same study, however, the authors reported improved LV functional recovery from ischemia by application of coronary flow at 10% of baseline values. The authors of that study concluded that a period of post-ischemic low flow improved myocardial function in isolated rat hearts only if a certain level of low flow was met. In contrast, the magnitude of coronary flow during low pressure intervention in our model was 18 ± 0.2% of baseline values, a value that was greater than in the Klawitter study, but that, unlike in the Klawitter study, resulted in reduced indices of LV functional recovery. However, Klawitter et al. enforced a longer duration of index ischemia (15 min) and a shorter duration of post-ischemic low flow (5 min).
It is possible that recovery from low pressure intervention at 18% of baseline coronary flow values is dependent upon the duration of low pressure applied to the isolated heart. Just as the duration of global ischemia is inversely related to the recovery of LV function after reperfusion, the duration and magnitude of low pressure perfusion may operate under the same principle \[72\]. It may be that as post-ischemic low pressure perfusion is extended, myocardial viability is reduced. Evidence against this may exist, however, as reported in a study by Ferrari et al. using isolated rabbit hearts \[79\]. The authors demonstrated a 92.3% preservation of developed pressure at the end of an hour of reperfusion after 10 minutes of global ischemia and subsequent 230 minutes of low coronary flow at 10% of baseline values. They noted a very low (43%) recovery of developed pressure in hearts that did not receive global ischemia prior to 240 min of 10% hypoperfusion. The authors contend that 10 minutes of global ischemia served as a stimulus for preconditioning or hibernation protection during the subsequent prolonged low flow period. Neither 3 min nor 10 min of global ischemia fit the definition of preconditioning, which is achieved through brief stuttered episodes of ischemia and reperfusion prior to prolonged ischemia \[80\]. However, 3 min of global ischemia as used in our model is very brief, and the shorter duration of our low flow period may not be a strong enough stimulus for hibernation. This idea is supported by our observed reduction in NADH/NAD+ during the low pressure period itself, which indicated continued oxygen utilization by mitochondria.

Generation of vascular ROS (hydrogen peroxide) was observed at full reperfusion in both groups, but was significantly greater in the low pressure group. The generation of
excess vascular reperfusion ROS by low pressure hearts may be explained by greater endothelial activation, particularly from the superoxide-producing enzyme NADPH oxidase. Ischemia-reperfusion triggers the release of superoxide from this vascular source, which is reduced to hydrogen peroxide by superoxide dismutase [81]. It is unclear from this study why reduction of intracellular ROS was associated with a worse recovery of LV function. The sources of intracellular hydrogen peroxide (through superoxide dismutation by S.O.D.), chiefly the mitochondrial ETC and the cytosolic xanthine oxidase enzyme [82], may be inhibited by low pressure intervention. The partial prevention of NADH buildup that we observed during low pressure perfusion lowers the availability of NADH for ROS-generating dehydrogenases [83], potentially limiting deleterious effects to tissue function. If so, this reasoning is in contrast to our results, but may be yet explained by examining the global ischemia period prior to low pressure.

Surface NADH/NAD+ fluorescence is a measure of mitochondrial energy state [84]. The low pressure state in the heart is a form of partial reperfusion, but may become an ischemic state as oxygen demand is only partially met. The small, but significant, reduction of NADH levels during low pressure perfusion indicates that substrate for complex I on the electron transport chain (ETC) is being consumed, and ATP is being produced through maintenance of the mitochondrial electrochemical gradient. This modified redox state during low pressure perfusion led to less NADH availability at reperfusion. Using isolated rat hearts, Stoner et al. demonstrated that the return of myocardial contractile function following post-ischemic low flow reperfusion depended upon critical levels of reductive capacity in the form of NADH [85]. The reduction in the
intracellular ROS burst in our study was correlated with a reduction in the final level of NADH fluorescence within the left ventricle. These results are similar to findings describing a compartmentalized divergence in reperfusion ROS production following ischemia-reperfusion in buffer-perfused rat hearts. Stoner et al. noted a decrease in intracellular ROS concurrent with an increase in extracellular ROS production in hearts reperfused with hypoxic perfusate [86]. In earlier work, Stoner et al. reported a strong positive correlation between the magnitude of the intracellular ROS burst seen at initial reperfusion and the final NADH fluorescence measured at the LV surface [85].

A reduction in available NADH before full reperfusion may lead to a reduction in NADH later in reperfusion, as it is consumed by the ETC to restore mitochondrial membrane potential. The reduction in NADH/NAD$^+$ at the end of 30 minutes of reperfusion may indicate that energy expenditure is being shifted away from myofilament relaxation through actin-myosin-ATPase and towards non-contractile processes such as ion homeostasis [84]. This would associate the reduction of final LV functional recovery with the observed final reduction of NADH/NAD$^+$ in the low pressure group.

2.5 Limitations and Future Directions

Though this study established the concept of compartmentalization of ROS production following low pressure, putative sources of ROS generation were not examined in this study, which was a limitation for inference to the origin of the
mechanism of altered ROS production in post-ischemic hypoperfusion. The putative sources of ROS generation during hypoperfusion will be examined in detail in Chapter 3. Patterns of ROS generation *during* the low pressure period will need to be examined for elucidation of the influence of hypoperfusion on recovery of LV function. Modification of the global ischemia duration may shed further light on the influence of ROS production on recovery of LV function following post-ischemic hypoperfusion.
Chapter 3: Blockade of NADPH oxidase, but not xanthine oxidase, during post-ischemic low flow preserves recovery of myocardial function

3.1 Introduction and Hypothesis

In this study, low pressure intervention was replaced with low flow intervention. Critical atrial opening pressures may not always be achieved with low pressure intervention. Using a model of forced low flow ensured that critical opening pressures were always overcome, and hearts were always perfused with identical levels of low flow within groups. NADPH oxidase (Nox) is a superoxide-producing enzyme that resides in the vascular endothelium of the heart, and is a major source of free radical-induced injury following ischemia and reperfusion [45, 46]. NADPH oxidase has long been implicated in ischemia-reperfusion dysfunction of the endothelium. Specifically targeting NADPH oxidase isoforms holds distinct advantage over current therapies against oxidative damage in the heart, because such targeting would prevent the formation of ROS directly [48].

Xanthine oxidoreductase is a vascular and cellular enzyme that is converted to xanthine oxidase during ischemia [35], and becomes a source of superoxide upon
ischemia and reperfusion [32]. We hypothesized that blockade of NADPH oxidase and xanthine oxidase would preserve myocardial function through reduction of ROS generation during post-ischemic low flow and upon subsequent full reperfusion in isolated rat hearts.

3.2 Methods

3.2.1 Langendorff isolation and tissue surface fluorescence

Rat hearts were isolated, perfused and monitored as in Chapter 2. Metrics of LV function were taken as percents of pre-ischemic values. Fluorescein, Resorufin and NADH fluorescence were recorded as in Chapter 2. Fluorescence values were taken as fractions of pre-ischemic values (F/F₀), and were examined during the global zero-flow time period, the low flow period, and the reperfusion period.

3.2.2 Ischemia-reperfusion (I-R) protocol

After the infusion of fluorophores, hearts were randomized to 9 groups (N = 4 per group. Hearts in groups 1-3 received 20 min of global no-flow ischemia followed by infusion during the first 5 min of full-flow reperfusion with vehicle (Group 1); the NADPH oxidase inhibitor apocynin, 100 µM (Group 2); or the xanthine oxidase inhibitor
oxypurinol, 500 µM (Group 3). Hearts in groups 4-6 received 6 min of global no-flow ischemia followed by 14 min of very low flow at 0.5% of baseline coronary flow values with infusion during very low flow and during the first 5 min of full-flow reperfusion with vehicle (Group 4); the NADPH oxidase inhibitor apocynin (Group 5); or the xanthine oxidase inhibitor oxypurinol (Group 6). Hearts in groups 7-9 received 6 min of global no-flow ischemia followed by 14 min of low flow at 10% of baseline coronary flow values with infusion during low flow and for the first 5 min of full-flow reperfusion with vehicle (Group 7); the NADPH oxidase inhibitor apocynin (Group 8); or the xanthine oxidase inhibitor oxypurinol (Group 9). After ischemia or ischemia plus low flow, hearts from all groups were immediately reperfused at 85 mmHg perfusion pressure for 15 minutes (See Figure 3.1).

**Figure 3.1:** Experimental protocol.
3.2.3 Data Analysis

Data are presented as mean ± S.E. Indices of LV function and fluorescence were calculated and analyzed in series with one-way analysis of variance and with significance set at p<0.05.

3.3 Results

3.3.1 LV Function

Before ischemia, there were no significant differences between groups in end diastolic pressure (EDP), heart rate, dP/dt, rate pressure product (RPP), developed pressure or coronary flow.
In vehicle- and apocynin-treated hearts, 10% low flow, but not 0.5% low flow, improved recovery of rate pressure product (RPP), as compared to control ischemia. Within the block of hearts that received vehicle only, percent recovery of RPP was significantly improved in the group of hearts that received 10% low flow prior to reperfusion, as compared to control ischemia (10% low flow RPP = 87.5 ± 1.3% vs. control ischemia RPP = 71.8 ± 6.2%). Similarly, within hearts that received apocynin, RPP recovery was significantly improved in the group of hearts that received 10% low flow prior to reperfusion, as compared to control ischemia (10% low flow RPP = 99.5 ± 0.65% vs. control ischemia RPP = 86.3 ± 4.8%). Oxypurinol did not significantly
improve recovery of RPP, as compared to vehicle, when infused in control ischemia, 0.5% low flow or 10% low flow hearts.

**Figure 3.3: Developed pressure.** *p*<0.05

Within hearts that received vehicle only, 10% low flow significantly improved the percent recovery of developed pressure (Dev-P), as compared to control ischemia (10% low flow Dev-P = 84.5 ± 2.72% vs. control ischemia CF = 74.7 ± 0.88%). In hearts treated with 0.5% low flow and 10% low flow, neither apocynin or oxypurinol caused a significant difference in recovery of developed pressure, as compared to control ischemia.
In vehicle- and apocynin-treated hearts, neither 0.5% nor 10% low flow improved recovery of dP/dt_{max}, as compared to control ischemia. Oxypurinol improved recovery of dP/dt_{max} in 0.5% low flow hearts (0.5% low flow dP/dt_{max} = 101 \pm 1.00\% vs. control ischemia dP/dt_{max} = 91.5 \pm 1.26\%), and decreased recovery of dP/dt_{max} in 10% low flow hearts (10% low flow dP/dt_{max} = 75.5 \pm 0.500\% vs. control ischemia dP/dt_{max} = 91.5 \pm 1.26\%), as compared to control ischemia.
In vehicle- and apocynin-treated hearts, 10% low flow, but not 0.5% low flow, improved recovery of coronary flow (CF), as compared to control ischemia. In hearts that received vehicle only, percent recovery of coronary flow (CF) was significantly greater in the 10% low flow group as compared to control ischemia (10% low flow CF = 98.6 ± 2.2% vs. control ischemia CF = 79.0 ± 7.4%, p<0.05). In hearts that received apocynin, CF recovery was significantly greater in the group of hearts that received 10% low flow prior to reperfusion, as compared to control ischemia (10% low flow CF = 99.6 ± 1.96% vs. control ischemia CF = 86.0 ± 1.68%, p<0.01). Oxypurinol did not significantly change recovery of CF, as compared to vehicle, when infused in control ischemia, 0.5% low flow or 10% low flow hearts.

Figure 3.5: Coronary Flow. *p<0.05, **p<0.01
Figure 3.6: Apocynin improved the recovery of LV function, but oxypurinol did not.

*p<0.05, ***p<0.001

In hearts that received control ischemia or 0.5% low flow, neither oxypurinol nor apocynin significantly improved recovery of LV function, as compared to vehicle. Within hearts that received 10% low flow, recovery of RPP was very significantly increased in hearts that received apocynin, as compared to vehicle only (apocynin RPP = 99.5 ± 0.65% vs. 87.3 ± 1.49%). Within hearts that received 10% low flow, apocynin significantly improved the percent recovery of developed pressure, as compared to vehicle only (apocynin Dev-P = 98.5 ± 4.33% vs. vehicle Dev-P = 84.5 ± 2.72%). In hearts given 10% low flow, oxypurinol did not significantly change recovery of RPP and Dev-P, as compared to vehicle-treated hearts.
3.3.2 Fluorometry

During global ischemia, there were no significant differences between groups in NADH, fluorescein or resorufin fluorescence. In all groups, the onset of global ischemia caused an increase in NADH fluorescence.

3.3.2.1 NADH changes during low flow intervention

Figure 3.7: 10% low flow causes significant oxidation of NADH in vehicle-treated hearts. **p<0.01
During low flow intervention, NADH fluorescence in vehicle only hearts was significantly decreased in hearts given 10% low flow as compared to control ischemia (End of low flow values: 10% low flow F/F₀ = 0.874 ± 0.016 vs. control ischemia F/F₀ = 1.03 ± 0.005).

Figure 3.8: NADH oxidation during low flow. **p<0.01

10% low flow causes significant oxidation of NADH in vehicle-treated hearts (Figure 3.7), but this trend was abolished in apocynin- and oxypurinol-treated hearts: there was no difference in NADH fluorescence during the low flow period in 0.5% low flow and 10% low flow hearts treated with either apocynin or oxypurinol, as compared to control ischemia.
3.3.2.2 NADH recovery at the end of full reperfusion

Figure 3.9: NADH fluorescence changes at the end of full reperfusion.

*Np<0.05, **p<0.01

NADH fluorescence at the end of full-flow reperfusion was significantly decreased in vehicle-treated hearts given 0.5% low flow as compared to control (0.5% low flow F/F₀ = 0.445 ± 0.003 vs. control ischemia F/F₀ = 0.553 ± 0.025). This trend was abolished in apocynin-treated hearts, and reversed in oxypurinol-treated hearts: NADH fluorescence in oxypurinol-treated hearts was significantly increased in 0.5% low flow hearts as compared to control ischemia (0.5% low flow F/F₀ = 0.599 ± 0.042 vs. control ischemia F/F₀ = 0.464 ± 0.001). NADH fluorescence was also significantly increased in
oxypurinol-treated hearts given 10% low flow as compared to control ischemia (10% low flow $F/F_0 = 0.635 \pm 0.031$).

3.3.2.3: Fluorescein changes during low flow intervention

Figure 3.10: Cellular ROS are decreased during 10% low flow in vehicle-treated hearts. $p<0.0001$

In hearts treated with vehicle only, fluorescein fluorescence during low flow was significantly reduced as compared to control ischemia in 10% low flow hearts (10% low flow $F/F_0 = 0.381 \pm 0.025$ vs. control ischemia $F/F_0 = 0.903 \pm 0.051$).
10% Low Flow Causes a Decrease, and Oxypurinol during 0.5% low flow causes an increase, in Intracellular ROS. During low flow intervention, fluorescein fluorescence in apocynin-treated hearts was also significantly decreased in hearts given 10% low flow as compared to control ischemia (10% low flow F/F₀ = 0.678 ± 0.016 vs. control ischemia F/F₀ = 0.822 ± 0.010). Fluorescein fluorescence was significantly increased during 0.5% low flow in hearts treated with oxypurinol, as compared to control ischemia (0.5% low flow F/F₀ = 1.18 ± 0.062 vs. control ischemia F/F₀ = 0.822 ± 0.021).
3.3.2.4 Fluorescein Changes During Full Reperfusion

Figure 3.12: The burst of intracellular ROS at reperfusion.

10% Low Flow with Vehicle or Apocynin Decreases, and 0.5% Low Flow with Oxypurinol Increases, the Burst of Intracellular ROS at Reperfusion. In hearts treated with vehicle only, maximum fluorescein fluorescence during the reperfusion burst was reduced in 10% low flow hearts as compared to control ischemia (10% low flow F/F₀ = 0.592 ± 0.032 vs. control ischemia F/F₀ = 1.08 ± 0.063, p<0.001). In hearts treated with apocynin, maximum fluorescein fluorescence during the reperfusion burst was reduced in 10% low flow hearts as compared to control ischemia (10% low flow F/F₀ = 0.883 ± 0.012 vs. control ischemia F/F₀ = 1.17 ± 0.031, p<0.001). In hearts treated with
oxypurinol, maximum fluorescein fluorescence during the reperfusion burst was significantly increased in 0.5% low flow hearts as compared to control ischemia (0.5% low flow F/F₀ = 2.07 ± 0.386 vs. control ischemia F/F₀ = 1.21 ± 0.052). *p<0.05, ***p<0.001

3.3.2.5 Resorufin Fluorescence Changes During Low Flow Intervention

In hearts treated with vehicle only, maximum resorufin fluorescence during low flow was not significantly different between ischemia types. In hearts treated with apocynin, maximum resorufin fluorescence during low flow was significantly reduced as compared to control ischemia in 10% low flow hearts (10% low flow F/F₀ = 0.938 ±...
0.021 vs. control ischemia $F/F_0 = 1.04 \pm 0.008$). In hearts treated with oxypurinol, maximum resorufin fluorescence during low flow was not significantly in 0.5% low flow hearts and 10% low flow hearts, as compared to control ischemia. **p<0.01

### 3.3.2.6 Resorufin Fluorescence Changes During Full Reperfusion

Figure 3.14: The vascular ROS burst at reperfusion.

In hearts treated with vehicle only, maximum resorufin fluorescence during the reperfusion burst was significantly increased as in 10% low flow hearts, compared to control ischemia (10% low flow $F/F_0 = 1.15 \pm 0.046$ vs. control ischemia $F/F_0 = 0.969 \pm 0.029$, $p<0.05$). Conversely, in hearts treated with apocynin, maximum resorufin
fluorescence during the reperfusion burst was not significantly different in 10% low flow hearts or 0.5% low flow hearts, as compared to control ischemia. Similarly in hearts treated with oxypurinol, maximum resorufin fluorescence during the reperfusion burst was not different between ischemia types. *p<0.05

Figure 3.15: Summary of extracellular (resorufin) and intracellular (fluorescein) ROS bursts at full reperfusion in vehicle-only hearts.

3.4 Discussion

Our results in this study are the first to show the phenomenon of post-ischemic conditioning with low flow in the isolated heart. This study demonstrates that the
alteration of ROS generation occurs in a way that is dependent upon the location of the cellular compartment.

In summary, vascular H$_2$O$_2$ fluorescence was increased and intracellular ROS was decreased during the reperfusion ROS burst in the 10% low flow group in hearts that received vehicle or apocynin. This was accompanied by improvements in the recovery of coronary flow and rate pressure product. These results support our initial hypothesis that blockade of vascular NADPH oxidase with apocynin would reduce vascular ROS and mediate an improvement in recovery of LV function. However, the results do not support our hypothesis that oxypurinol would reduce ROS and mediate an improvement in LV function.

Our results show that ROS are generated during global ischemia and again during low flow reperfusion. An increase in intracellular ROS after the onset of ischemia was observed by an increase in fluorescein fluorescence. This is in agreement with Becker et al, who demonstrated increased intracellular superoxide generation during the hypoxic phase of hypoxic-reoxygenated cardiomyocytes [87]. The onset of ischemia caused an acute increase in NADH. Treatment with 10% low flow in this study caused significant oxidation of NADH during the low flow state in vehicle-treated hearts. In support of this finding, Kay et al. demonstrated decreased NADH fluorescence on the surface of isolated rat hearts exposed to low-flow reperfusion following regional ischemia [88]. In general, low flow reperfusion at 10% of baseline coronary flow resulted in an onset of LV contractions with a very low heart rate (less than 60 bpm, data not shown), indicating the
incomplete generation of ATP for contraction from the oxidative phosphorylation on the ETC. A partial reduction in NADH fluorescence would be expected.

In this study, vascular NADPH oxidase and cellular xanthine oxidase enzymes were examined as important sources of ROS generation in the settings of post-ischemic low flow and full-flow reperfusion. Vascular ROS was increased, and cellular ROS decreased at reperfusion, revealing a compartmentalized ROS response to low flow intervention. Stoner et al. demonstrated a compartmentalized generation of ROS production in the heart. In isolated rat hearts exposed to global ischemia, hypoxic reperfusion (20% oxygenated perfusate) led to a decrease in intracellular ROS and an increase in vascular ROS, as compared to reperfusion with 95% oxygenated perfusate. The authors concluded that initial high oxygen at reperfusion promotes intracellular ROS production, while low oxygen promotes vascular ROS [86]. In support of this concept, using spin-trapping of superoxide in coronary effluent, Angelos et al. demonstrated an increase in ROS production in ischemic hearts reperfused with hypoxic (2% oxygen) perfusate, as compared to 95% oxygen perfusate [89]. Though oxygen delivery was not measured in this study, hearts were reperfused at full flow with 95% oxygen-bubbled perfusate. There is no indication that hearts in our study were reperfused with hypoxic buffer. That compartmentalized ROS generation is similar at reperfusion in hearts given hypoxic buffer and 10% low flow intervention is an observation worth further examination. For instance, Rae et al. hypothesized the idea of post-ischemic conditioning of the myocardium in CPR-type low flow states [7]. This conditioning may occur in a way that is dependent upon the interplay between oxygen delivery and ROS generation.
Our results show that xanthine oxidase (XO) inhibition was unsuccessful in scavenging reactive oxygen species, or in preserving LV function, during IR in the heart. This is in contrast to findings by Kinugasa et al., who demonstrated a reduction in hydroxyl radical and improvement in recovery of developed pressure and dP/dt with XO inhibition with allopurinol in post-ischemic isolated rat hearts [90]. In our study, the intracellular ROS burst was increased upon reperfusion following 0.5% low flow with oxypurinol. It is likely that at such a low flow, oxypurinol reached a toxic dose as it built up in the heart over 14 minutes of low flow. Future studies involving post-ischemic low flow should use oxypurinol in lower doses. However, this result may highlight the importance of maintaining a precise level of low flow when pharmaceutical treatment is applied to the post-ischemic myocardium. Treatment that may be cardioprotective at one level of coronary flow may be toxic at a lower level.

XO is inhibited by allopurinol (and its isomer oxypurinol), an isomer of the naturally occurring XO substrate hypoxanthine [25]. The use of allopurinol as an anti-ischemic drug in clinical trials of cardiac patients was just reported by Noman et al, who found that high doses of allopurinol reduced angina pain onset triggered by exercise [2]. XO activity inhibition by allopurinol attenuated hypoxia-reoxygenation-induced ROS production by preventing activation of extracellular signal-regulated protein kinase (ERK) in rat myocytes [91]. The same study reported a reduction of infarct size associated with a complete attenuation of XO activity by application of allopurinol in post-ischemic isolated rat hearts. However, the role of allopurinol as an infarct-inhibiting drug in myocardial ischemia-reperfusion (IR) remains controversial. Clinical trials
employing XO inhibition by allopurinol during coronary bypass surgery have reported conflicting results. One study reported decreased mortality in 169 bypass patients when allopurinol was applied during surgery [92], while a concurrent study showed extensions of infarct in 90 patients when allopurinol was applied chronically after myocardial infarction [93]. At times conflicting, these results do point to a mechanism of XO-induced endothelial dysfunction by superoxide production upon reperfusion of the ischemic myocardium [94, 95].

In summary, this study confirmed that low flow intervention in global ischemia alters ROS generation upon full reperfusion. This is coupled with an alteration of ROS production during the low flow period itself, which may play an important role in ROS generation at full reperfusion.

3.5 Limitations and Future Directions

Though NADH was monitored as a reflection of mitochondrial redox state, this study did not examine mitochondrial ROS, which are an important source of free radicals in the ischemic-reperfused myocardium [96]. Also, different doses of oxypurinol were not used to examine a dose-response of heart function. Finally, hearts were only reperfused for 15 minutes, while many studies require an hour of full reperfusion for evaluation of LV function.
In future studies, full reperfusion of the heart should be extended to one hour in order to accurately compare results with those found in the majority of published studies that examine LV functional recovery. Infarct size should be measured to determine the influence of post-ischemic low flow on tissue scavenging. Furthermore, mitochondrial ROS should be examined with dihydroethidium to address a component of mitochondrial ROS generation in low flow. Finally, since oxygen content and ROS production are intimately linked in post-ischemic myocardium [86], oxygen delivery and consumption should be key measures in future studies.
Chapter 4: Sivelestat attenuates reperfusion injury in the post-ischemic myocardium through reactive oxygen species-mediated and nitric oxide synthase-mediated mechanisms

4.1 Introduction and Hypothesis

There is currently no drug on the market in the United States that is approved for reduction of myocardial infarction following ischemia (global or regional) in the heart, and so the search for cardioprotective drugs remains a heavily investigated field [49]. Recently, the neutrophil elastase inhibitor Sivelestat (ONO-5046) has been shown to be cardioprotective in several animals studies [57, 58] and in at least one setting in humans [59]. Interestingly, Kambe et al. recently showed that Sivelestat preserves LV function when infused after global ischemia in the Langendorff buffer-perfused heart, a model of the functioning heart that is nearly bereft of neutrophils [54]. The neutrophil-independent cardioprotective mechanisms of Sivelestat are unknown. We hypothesized that infusion of the neutrophil elastase inhibitor following ischemia in the isolated rat heart would reduce myocardial infarction and preserve LV function in reperfusion through a reactive oxygen species (ROS)-mediated mechanism.
4.2 Methods

4.2.1 Isolated buffer perfused rat hearts

Male Sprague-Dawley rats (400-500 g) purchased from Harlan Laboratories (Indianapolis, IN) were cared for in accordance with the National Institute of Health (NIH) guidelines and the approval of the Institutional Animal Care and Use Committee. Hearts were isolated and perfused in the Langendorff mode and perfused with buffer as previously described (20). Rats were anesthetized with intraperitoneal sodium pentobarbital (70 mg/kg) and heparin (1,000 U/kg). The trachea was cannulated with a 16-gauge angiocath attached to a rodent ventilator (Harvard Apparatus, South Natick, MA. Animals were ventilated with room air at 70 respirations per min with a 2.5 mL stroke volume. A mid-sternal thoracotomy was performed to expose the heart and isolate the aorta. The aorta was cannulated in situ and hearts were excised. Retrograde perfusion of the coronary arteries was immediately initiated with warmed (37 °C) modified Krebs-Henseleit buffer (1.25 mM CaCl2, 11 mM glucose, 112 mM NaCl, 25 mM NaHCO3, 5 mM KCl, 1.2 mM MgSO4, 1 mM K2PO4, and 0.2 mM octanoic acid, bubbled with 95% O2 / 5% CO2, pH 7.4) at a constant perfusion pressure of 75 mm Hg. A saline-filled latex balloon attached to a pressure transducer was inflated to 5-10 mm Hg in the left ventricle (LV) for measurements of LV contractile function. The heart was positioned inside a temperature controlled glass chamber at 37 °C. Coronary flow was monitored by use of an in-line small animal flow meter (Model T206, Transonic Systems Inc., Ithaca, NY)

4.2.2 Left Ventricular Function

LV pressure was continuously sampled at 30 Hz and digitally processed with a Digi-Med Heart Performance Analyzer (HPA-210a, Micro-Med, Inc., Louisville, Kentucky). Heart rate, dP/dtmax, LV systolic pressure and LV end diastolic pressure were derived by computer algorithm. Developed pressure was calculated as the difference between systolic and end diastolic pressures. Rate pressure product (RPP) was calculated as the product of heart rate and developed pressure. Hearts that did not achieve an average RPP of at least 20,000 and an average dP/dtmax of at least 3,000 in the pre-ischemic baseline stabilization period were excluded from further experimentation.

4.2.3 Experimental Protocol

Global ischemia was induced by completely occluding perfusion flow to the heart. Hearts were randomly assigned to 4 ischemia groups (n=8 per group): Group 1: 25 min of global ischemia; Group 2: 28 min of ischemia; Group 3: 25 min of ischemia followed by 3 min of low flow with vehicle at 4 mL per min; Group 4: 25 min of ischemia followed by 3 min of low flow with sivelestat (100 µg per mL) at 4 mL per min (See Figure 4.1).
Vehicle and sivelestat infusion were administered through a side-port directly above the aorta by a PHD 2000 Programmable pump (Harvard Apparatus, Holliston, MA). Hearts were reperfused for 60 min at 75 mm Hg.

Figure 4.1 Experimental Protocol.

4.2.4 Acetylcholine-induced change in coronary flow

Following 60 min of reperfusion, hearts were infused with 1.0 µM acetylcholine chloride for one minute and then switched back to normal perfusion fluid. At this point the recovery in coronary flow was recorded as the maximum coronary flow value within
one minute of reflow with normal perfusion fluid. Since perfusion pressure was maintained at 75 mmHg, increases and decreases in coronary flow were a reflection of endothelial relaxation and constriction, respectively.

4.2.5 Infarct size

Following IR, hearts were stained with triphenyltetrazolium chloride (TTC) for measurement of infarct size, using the method of Ferrera et al. [97]. Using this method, Ferrera et al. concluded that one hour reperfusion is enough to assess function and infarct size with TTC staining in Langendorff rat model. Hearts were frozen at -80 °C, sliced in 2 mm sections, incubated in TTC (1.0 % w/v) at 37 °C for 10 min per side to allow mitochondrial uptake of TTC, and then fixed in formalin prior to photomicrography. Living tissue appears brick red and infarcted tissue appears pale pink or white and after TTC staining. Infarct size was measured using MetaVue imaging software (version 6.2r6, Universal Imaging Corp.).

4.2.6 Creatine Kinase Release

Coronary effluent was collected from all hearts before ischemia (i.e. in baseline) at at 10, 30 and 60 minutes of full reperfusion. The effluent was assayed for creatine kinase (CK) content using a standard spectrophotometric assay kit (Stanbio Laboratory,
Boerne, TX). CK catalyzes the phosphorylation of ADP to ATP. Through a series of enzymatically coupled reactions, NADH is produced at a rate directly proportional to CK activity. Reagent containing nucleotides in buffer was added to the sample. Sample was warmed to 37.4°C and the absorbance increase of NADH at 340 nm was measured each minute for 3 minutes using a UV-Vis spectrophotometer. Values of CK activity in U/L are derived based upon the molar absorptivity coefficient of NADH at 340 nm (0.00622). One unit (U) per liter (L) of CK activity is that amount of CK which can oxidize one µmol of NADH per minute.

4.2.7 Blockade of endothelial nitric oxide synthase (eNOS) in isolated hearts

4.2.7.1 Infarct Size

In a third set of experiments, additional isolated hearts subjected to 25 min of ischemia and 3 min of drug infusion at 4 mL per min then received full reperfusion for two hours. During 3 min of drug infusion, hearts were directly infused with either a) vehicle, b) sivelestat (100 µg per mL), c) the non-specific NOS blocker N-nitro-L-arginine methyl ester (L-NAME, 100 µM), or d) L-NAME + sivelestat (N=4 per group). Infarct size was measured following two hours of full reperfusion as detailed above. LV function was also monitored as detailed above.
4.2.7.2 Tissue reactive oxygen species generation

In a duplicate block of experiments (as in 4.2.7.1), hearts were collected at the end of reperfusion for measurements of ROS production under fluorescent microscopy, as in Khan et al. [98]. After reperfusion, hearts were embedded in optimal cutting temperature gel, sliced to 5 µm thick in a cryotome and placed on glass slides. Dihydroethidium (DHE, 10 µM), which becomes fluorescent upon reaction with superoxide [99], was topically applied to each tissue section and slides were incubated in a light-impermeable chamber at 37° C for 30 min. After rinsing with PBS, each section was stained with DAPI and coverslipped. Slides were photographed using a Nikon (Tokyo, Japan) Eclipse TE 2000-U microscope equipped with an X-Cite 120 Fluorescence Illumination System. Photographs of oxyethidium fluorescence were taken under a rhodamine filter (green excitation 550 nm, red emission 573 nm). Fluorescent intensity, which positively correlates with superoxide generation in tissue, was quantified using MetaMorph image analysis software (Molecular Devices, Sunnyvale, CA). Fluorescent units were reported as a function of optical density (OD).

4.2.8 Hypoxic-reoxygenated bovine aortic endothelial cells

To determine the influence of sivelestat on ROS production in endothelial cells, bovine aortic endothelial cells (BAECs) were subjected to hypoxia-reoxygenation in the presence of the spin trap DMPO. BAECs were cultured in low glucose DMEM (Gibco)
supplemented with 10% FBS (Millipore), Penicillin/Streptomycin (Gibco) and 0.1% of Endothelial Cell Growth Supplement (ECGS, Millipore). Cells were cultured in T-75 cm² (Corning) tissue culture dishes at 37°C in a humidified environment of 5% CO2 and 21% O2. When the cells achieved 80-85% confluency, the cells were washed with PBS (Gibco), trypsinized and pelleted at 1500 rpm for 5 min. The cells were redissolved in Low Glucose DMEM without phenol red to achieve a cell count of 107 cells /ml and used for further analysis. BAECs diluted to 5x106 cells per mL in media were placed in a heated chamber at 37.0°C and flushed with 100% nitrogen for 45 min. Following the rapid addition of 5,5-dimethyl-1-pyrroline-N-oxide (DMPO, 50 mM final concentration) endothelial cells were then flushed with oxygen (95% O2 / 5% CO2, pH 7.4) for one minute at 37.0°C in the presence of a) no drug, b) sivelestat (100 µg per mL final concentration), d) the xanthine oxidase inhibitor oxypurinol (500 µM) or d) superoxide dismutase (SOD, from bovine erythrocytes, Sigma-Aldrich Co., St. Louis, MO). Endothelial cells were then transferred to a quartz flat-cell inside an EPR-300 X-band (9.7 GHz) spectrometer. Second peak amplitude of the DMPO-OH spectrum was quantified and taken as an indication of the magnitude of superoxide production.

4.2.8 Data Analysis

Data was expressed as mean ± SEM. Statistical significance between groups was calculated by one-way ANOVA followed by Tukey’s Multiple Comparison Test. A p value of 0.05 or less was considered statistically significant.
Infarct size (I.S.) was significantly reduced in the Sivelestat group after one hour of reperfusion compared to hearts receiving 25 min of ischemia (Sivelestat I.S. = 11±2% vs. 25 min of ischemia I.S. = 47±2%).
Sivelestat significantly preserved LV tissue as seen through TTC staining. Left ventricle slices were stained with TTC. Whiter areas indicate regions of tissue infarction, and pink and red areas indicate viable, functional tissue.

4.3.2 Sivelestat preserves LV functional recovery

LV functional recovery was calculated as percent recovery from ischemia and reperfusion relative to baseline (pre-ischemic) values. Administration of Sivelestat significantly improved recovery of developed pressure (Dev-P) at 60 min of reperfusion.
compared to hearts receiving 25 min of ischemia (Sivelestat Dev-P = 50±3% vs. 25 min ischemia Dev-P = 32±3%). Also, administration of Sivelestat significantly improved recovery of dP/dt_{max} at 60 min of reperfusion compared to hearts receiving 25 min of ischemia (Sivelestat dP/dt_{max} = 61±3% vs. 25 min ischemia dP/dt = 36±4%) (See Figure 4.4).

![Bar chart showing recovery of developed pressure and dP/dtmax](image)

**Figure 4.4 Sivelestat given after global ischemia preserves developed pressure and dP/dt after an hour of full reperfusion. #p<0.0001**

There were no significant differences in recovery of developed pressure of dP/dt_{max} between groups of hearts that did not receive sivelestat.

Administration of Sivelestat significantly improved recovery of rate pressure product (RPP) at 60 min of reperfusion as compared to hearts receiving 25 min of ischemia (Sivelestat RPP = 45±4% vs. 25 min ischemia RPP = 28±3%) (See Figure 4.5)
There were no significant differences in recovery of RPP between groups of hearts that did not receive Sivelestat.

**Figure 4.5** Sivelestat significantly preserves recovery of rate pressure product (developed pressure x heart rate). **p<0.01**

### 4.3.3 Sivelestat preserves vasoreactivity

Administration of Sivelestat significantly improved recovery of coronary flow (C.F.) following acetylcholine infusion at 60 min of reperfusion as compared to hearts receiving 25 min of ischemia (Sivelestat C.F. = 8.4±1.8% vs. 25 min ischemia C.F. = 3.4±0.5%) (See Figure 4.6). There were no significant differences in recovery of
coronary flow following acetylcholine infusion between groups of hearts that did not receive Sivelestat.

![Figure 4.6 Acetycholine-induced vasodilation. **p<0.01](image)

Acetylcholine-induced vasodilation is significantly preserved in hearts treated with Sivelestat following ischemia.

4.3.4 Creatine Kinase Release

Total creatine kinase (CK) release was significantly reduced in hearts treated with Sivelestat during 3 min of post-ischemic low flow as compared to 25 min of ischemia.
only (Sivelestat CK = 475 ± 52 U/L vs. 25 min ischemia CK = 744 ± 73 U/L) (See Figure 4.7).

Figure 4.7 Creatine kinase release. **p<0.01

Creatine kinase release was significantly reduced by treatment with Sivelestat at 10 min and 60 min of full reperfusion.

4.3.5 Oxygen Radical Formation

Sivelestat reduces oxygen radical formation in hypoxic-reoxygenated bovine aortic endothelial cells. In BAECs exposed to 45 min of hypoxia followed by reoxygenation, the spin adduct DMPO-OH was significantly reduced in cells treated with 100 µg per mL Sivelestat, compared to untreated endothelial cells, after 20 min
(Sivelestat 2nd peak ht. = $3.373 \times 10^6 \pm 2.706 \times 10^5$ vs. vehicle 2nd peak ht. = $4.619 \times 10^6 \pm 2.993 \times 10^5$), 30 min (Sivelestat 2nd peak ht. = $3.167 \times 10^6 \pm 1.675 \times 10^5$ vs. vehicle 2nd peak ht. = $4.608 \times 10^6 \pm 2.691 \times 10^5$) and 40 min (Sivelestat 2nd peak ht. = $3.129 \times 10^6 \pm 1.105 \times 10^5$ vs. vehicle 2nd peak ht. = $4.414 \times 10^6 \pm 2.938 \times 10^5$) of reoxygenation. Oxypurinol significantly reduced DMPO-OH formation at all time points, but not as effectively as S.O.D. S.O.D. significantly reduced DMPO-OH at all time points, compared to vehicle, Sivelestat, and oxypurinol (See Figure 4.8).

**Figure 4.8 Sivelestat reduced DMPO-OH formation.** **p<0.01, ***p<0.001**

Sivelestat significantly reduced DMPO-OH formation at 20, 30 and 40 min of reoxygenation as compared to control.
4.3.6 Blockade of endothelial NOS with L-NAME

After 2 hours of full reperfusion the percent infarct size (I.S.) was significantly less in hearts treated with Sivelestat compared to L-NAME-treated hearts (Sivelestat I.S. = 18.6±3.2% vs. L-NAME I.S. = 49.8±2.7%). This Sivelestat-mediated reduction in infarct size was lost in the L-NAME + Sivelestat-treated hearts (58.8±1.0%) (See Figure 4.9).

![Figure 4.9 L-NAME reverses the infarct-sparing effects of Sivelestat. ***p<0.001](image)

The percent recovery of rate pressure product (RPP) after one hour of full reperfusion was significantly greater in Sivelestat-treated hearts compared to vehicle-treated hearts (Sivelestat RPP = 47 ± 5% vs. vehicle RPP = 28 ± 5%) and to L-NAME +
Sivelestat-treated hearts (Sivelestat RPP = 47 ± 5% vs. L-NAME + Sivelestat RPP = 26 ± 3%). Percent recovery of dP/dt after one hour of full reperfusion in Sivelestat-treated hearts was significantly greater than in L-NAME-treated hearts (Sivelestat dP/dt = 50 ± 5% vs. L-NAME dP/dt = 33 ± 3%, p<0.01) and L-NAME + Sivelestat-treated hearts (24 ± 3%) (See Figure 4.10).

Figure 4.10 L-NAME reverses Sivelestat’s protective effects on LV function. *p<0.05

4.3.7 Superoxide Production in explanted LV Tissue

Hydroethidine fluorescence was low in baseline (See Figure 4.11). Sivelestat significantly reduced the optical density of oxyethidium fluorescence, indicative of a
reduction in superoxide production, as compared to 25 min ischemia hearts (Sivelestat OD = 1.59 ± 0.01 vs 25 min ischemia OD = 1.62 ± 0.003, p<0.05). Coinfusion of L-NAME with Sivelestat in low flow prior to reperfusion abolished the reduction in superoxide formation (L-NAME+Sivelestat OD = 1.63 ± 0.0007) (See Figures 4.12 and 4.13).

Figure 4.11 Hydroethidium fluorescence in baseline, before ischemia. Superoxide production was low in baseline, as indicated by dim HE fluorescence. Magnification=200x.
Figure 4.12: Sivelestat significantly reduced oxyethidium fluorescence in LV sections. This effect which was reversed by coinfusion with L-NAME, *p<0.05. Magnification=200x.
4.4 Discussion

Our results confirm that Sivelestat significantly reduces infarct size, LV stunning and preserves endothelium-dependent vasorelaxation, in the isolated heart independent of neutrophils. Our study is the first to demonstrate the infarct-reducing power of sivelestat in any animal model in the heart, although prior studies using sivelestat in rats have shown a reduction in cellular necrosis following transient cerebral ischemia [100, 101]. Interestingly, any cardioprotection offered by Sivelestat is lost with NOS inhibition. Sivelestat reduced superoxide production in LV sections, but ROS was restored to control
ischemia levels when NOS enzymes were inhibited with L-NAME in the presence of Sivelestat. In further studies using hypoxic-reoxygenated bovine aortic endothelial cells, Sivelestat treatment during hypoxia resulted in a reduction of the superoxide (DMPO-OH) signal during reoxygenation.

Kambe et al published data also using buffer-perfused isolated rat hearts showing a 35% recovery of left ventricular (LV) developed pressure versus controls (16%) when sivelestat was infused continuously during the first 10 min of full reperfusion [54]. Our LV functional results are in agreement with Kambe et al, which to our knowledge is the only other study to have employed sivelestat in a model of ischemia-reperfusion in hearts in the absence of neutrophils. Sivelestat also protects the heart in situations where neutrophils are present. Akiyama et al. showed attenuation of myocardial stunning in swine with post-ischemic infusion of sivelestat [57]. Ueno et al. demonstrated a reduction in IR injury with application of sivelestat in a heart transplantation model using dogs [58]. Toyama et al. reported an association of Sivelestat infusion with improved fractional area of change in the left ventricle of pediatric patients who underwent cardiovascular surgery with cardiopulmonary bypass, demonstrating that Sivelestat is protective in at least one setting of ischemia-reperfusion in humans [59].

In the presence of Sivelestat, myocardial protection was abolished along with an associated restoration of ROS generation by NOS inhibition with L-NAME. Nitric oxide reversibly inhibits mitochondrial respiration by binding to cytochrome c oxidase in competition with oxygen on complex IV of the ETC [102]. The reaction of NO’ with O2•−...
causes formation of the cytotoxic reactive nitrogen species peroxynitrite, ONOO’, which contributes to reperfusion injury. The formation of ONOO’ is prevented if NOS are inhibited, which leads to the preservation of LV contractile function in the isolated rat heart, as shown originally by Yasmin et al. [103]. It is possible that scavenging of superoxide by Sivelestat increases NO’ bioavailability through prevention of ONOO’ formation. Though we did not measure ONOO’ formation, it is logical to assume that if Sivelestat reduced superoxide production in the heart after ischemia-reperfusion, ONOO’ formation may also have been reduced. This explanation may be disappointing, as exogenous antioxidants have not shown promise in extensive trials attempting to reduce infarct size following IR in humans [104, 105]. However, repeated exposure to ONOO’ also decreases the ability of the heart to respond properly to vasorelaxing agents [106]. It is likely that, along with superoxide reduction, Sivelestat preserved sensitivity to nitric oxide in our study, as endothelium-dependent relaxation was preserved. It is widely accepted that reactive oxygen species are implicated in oxidative injury to the endothelium following ischemia-reperfusion [107]. Therefore, it is likely that Sivelestat exerts protection through reduction of vascular ROS. The effect of Sivelestat on intracellular ROS production at reperfusion is less clear, but may involve reduction in ROS on the ETC through reduced reaction with NO’.

Our data confirm that co-infusion of the reversible nitric oxide synthase (NOS) blocker L-NAME prior to full reperfusion results in a loss of the infarct-sparing and LV function-preserving effects of Sivelestat. In agreement with Kambe et al., Sivelestat significantly preserved endothelium-dependent vasorelaxation following IR. That study
showed a 15% increase over controls in coronary flow in response to acetylcholine infusion at the end of reperfusion in hearts treated with Sivelestat. Coronary flow was not different between groups in our study at the onset of, or at the end of, reperfusion (data not shown), suggesting that Sivelestat actually prevented the formation of ROS, rather than simply causing an increased expulsion of ROS from the myocardium through an increase in coronary flow. Minor differences in the timing of administration and the dose of Sivelestat between this study and that of Kambe et al. may limit comparisons of our results. However, using a model that is appropriate for demonstration of the neutrophil-independent properties of Sivelestat, our results confirm, as do those of Kambe et al., that Sivelestat preserves vasodilatation in the coronary endothelium of isolated buffer-perfused rat hearts subjected to IR.

In contrast to our conclusion that Sivelestat exerts protective modulation directly on the endothelium, certain studies assert that Sivelestat mediates vasorelaxation independent of endothelium. Maeda et al. indicated that this mechanism lies in the vasculature, by demonstrating that Sivelestat selectively inhibits calcium sensitization to a receptor agonist in porcine vascular smooth muscle strips with or without endothelium, without affecting calcium-induced contraction [55]. Amemori et al. reiterated this work showing that Sivelestat induces endothelium-independent vasorelaxation in pre- contracted human gastric arteries [56]. However, Takayama and Kohsuke concluded that Sivelestat exerted vasodilatory effects on guinea-pig airways by both NO- and epithelium-dependent and –independent mechanisms. They showed that Sivelestat inhibited substance P-induced contraction of tracheal ring preparations and that this effect
was significantly attenuated by either removal of epithelium or blockade of NOS by L-NAME. In contrast, the same effect was not observed in guinea-pig bronchial ring preparations, suggesting that Sivelestat has a region-dependent affect in pulmonary smooth muscle [108]. Okajima et al. demonstrated that Sivelestat reduced IR-induced liver injury, an effect that was completely inhibited by pre-treatment with L-NAME, concluding that Sivelestat was dependent on endothelium-dependent production of NO [109]. Care should be taken in extending the above results in other organ systems to the heart, as the same mechanisms may not be active.

There were no differences in recovery of LV function between hearts that received 25 min of global ischemia and hearts that received 3 min of low flow with vehicle following the same duration of global ischemia. This may suggest, at the very least, that short episodes of post-ischemic low flow preserve the recovery of LV function after the onset of full reperfusion. Sivelestat treatment during this brief post-ischemic low flow period dramatically improved recovery of LV function and significantly spared the LV from infarction. The infarct-sparing effects of Sivelestat were reversed with simultaneous NOS enzyme inhibition, suggesting that NOS enzyme function is essential during even a 3 minute period of post-ischemic low flow. These results show that the period of post-ischemic low flow can be exploited for significant cardioprotection. It is unknown how longer periods of low flow would effect LV functional recovery, and if longer periods can be exploited for protection of LV contractile function and tissue. Much investigation remains to be completed in order to fully describe the subcellular
protective and harmful mechanisms activated in the brief period of low flow following global ischemia in the myocardium.

4.5 Limitations and Future Directions

The idea that preservation of NO bioavailability may lead to myocardial salvage from IR is widely accepted and heavily studied [52]. However, due to the dearth of studies examining the effects of Sivelestat on the heart in situations of IR, additional studies are needed to elucidate the exact mechanism(s) of protection exerted by Sivelestat.

For instance, a direct measure of NO production by coronary endothelium is needed to confirm our data that sivelestat enhances bioavailability of NO. Also, it is not known if Sivelestat reacts directly with endothelial NOS, possibly mediating eNOS coupling of electron transfer from the reductase domain to the heme of the oxygenase domain of the dimer. Future studies should attempt to address these questions, and to do so using a range of concentrations of sivelestat.

Summary: Sivelestat possesses mechanisms of protection that are independent of neutrophil mediation, but likely dependent upon prevention of reactive oxygen species formation and possibly upon preservation of nitric oxide synthase function in the heart.
Due to wide availability and current use in humans, Sivelestat remains a promising translational pharmaceutical for treatment of infarct reduction following myocardial IR.

Investigations of ROS production in the buffer-perfused heart are limited. The buffer-perfused heart does not contain neutrophils, which are a key source of ROS generation in ischemia-reperfusion. Also, the high oxygen content of the perfusate does not accurately reflect the \textit{in vivo} oxygen content and carrying capacity of blood. The description of the blood-perfused model of the isolated heart in Chapters 5 and 6 shows our attempts to remedy these limitations.
Chapter 5: Measurement of Hydrogen Peroxide and Oxidant Stress in a Recirculating Whole Blood Perfused Rat Heart Model

5.1 Introduction and Hypothesis

In the study of ROS formation and myocardial IR the blood-perfused isolated heart offers significant advantages over the buffer-perfused heart, including the ability to oxygenate the heart under physiologic oxygen delivery conditions. In this chapter we describe a novel recirculating blood-perfused isolated rat heart model using autologous whole blood from the same animal and the ability to measure plasma H$_2$O$_2$ and tissue oxidant stress following IR. We hypothesized that recovery of left ventricular function in isolated blood-perfused would be greater than in isolated buffer-perfused rat hearts following ischemia and reperfusion.
5.2 Methods

5.2.1 Isolated Blood-Perfused Heart Preparation

Male Sprague-Dawley rats (400-500 g) purchased from Harlan Laboratories (Indianapolis, IN) were cared for in accordance with the National Institute of Health (NIH) guidelines and the approval of the Institutional Animal Care and Use Committee. Animals were orally intubated after i.p. injection of pentobarbital (60 mg/kg) and ventilated (model 683, Harvard Apparatus, MA) with room air at a rate of 35 strokes/min, a tidal volume of 8 mL/kg, and a positive end expiratory pressure of 3 cm H$_2$O. The right femoral artery and the right jugular vein were cannulated with 24-gauge catheters for blood withdrawal and drug administration, respectively. The core body temperature was maintained at 37 °C by a thermistor-controlled heat lamp. Heparin (1,000 Units•kg$^{-1}$) was administered intravenously and allowed to circulate for 10 minutes. Blood (10 mL•kg$^{-1}$) was withdrawn over 30 seconds from the right femoral artery with concurrent infusion of an equal volume of saline into the right jugular vein. Withdrawn blood was added to the recirculating perfusion apparatus and allowed to circulate. A mid-sternal thoracotomy was performed to expose the heart and isolate the aorta. Following cannulation of the aorta, the heart was immediately excised and perfused with blood on the recirculating perfusion apparatus. Additional blood was collected from the chest cavity, filtered through nylon mesh (37 micron pore size) and added to the recirculating perfusion apparatus for a total circuit blood volume of 16 mL.
Modifying a previously described model [110], blood was pumped from the bottom of the reservoir through two nylon mesh inline filters (37 micron pore size) by a peristaltic roller pump (Cole Parmer Instrument Co., Vernon Hills, IL) (See Figure 4.1). The circuit included an inline flow transducer (Transonic Systems Inc, Ithaca, NY), for continual measurement of coronary blood flow (CBF), and a 3.0 mL oxygenator. The oxygenator consisted of highly gas-permeable silicone tubing (I.D. = 0.89 mm), coiled around a central rod and incased in a plastic housing. A continual flow of 95% oxygen and 5% CO₂ was administered to the interior of the oxygenator, thereby allowing rapid diffusion of oxygen into the recirculating blood. Blood flowed through water-jacketed tubing heated to 37° C before entering the aorta of the heart. Perfusion pressure was monitored in-line directly above the heart.
Figure 5.1: Schematic of recirculating blood perfusion apparatus

Autologous effluent blood from the heart is collected in the plastic reservoir. Excess blood submerges the heart as it circulates. Blood is circulated through the peristaltic pump and through two parallel inline filters, the flow transducer and then the silicone
tubing membrane oxygenator. Tubing volume of the oxygenator is 3.0 mL. Oxygenated blood is heated to 37° C in 0.5 mL of water-jacketed tubing before it enters the aorta of the isolated heart. Two inline ports are placed above the heart, one for drawing blood for measurements of PO₂ and the other to allow measurement of perfusion pressure.

5.2.2 Left Ventricular Function

A saline-filled latex balloon attached to a pressure transducer was inflated to 5-7 mm Hg in the left ventricle (LV) for measurements of LV function and inflated. Heart rate, dP/dt max, systolic and end diastolic pressures were continuously monitored and recorded using analog-to-digital converter boxes (Digi-Med HPA-210a, Micro-Med, Inc., Louisville, Kentucky). Rate pressure product (RPP) was calculated as the product of heart rate and developed pressure.

5.2.3 Experimental Protocol

Initial coronary blood flow was set at 3.0 mL min⁻¹ and was adjusted to maintain perfusion pressures between 50 and 60 mm Hg. Following a short stabilization period, hearts were subjected to one of two protocols; sham or ischemia-reperfusion (IR). The sham group received normal perfusion for 120 minutes. The IR group received 35 minutes of no-flow global ischemia, followed by 60
minutes of reperfusion at baseline coronary blood flow levels. After two hours, hearts in both groups were flash-frozen in liquid nitrogen. Frozen tissue was stored at -80°C for measurements of reduced glutathione and thiobarbituric acid reactive substances (TBARS).

5.2.4 Blood Chemistry

Hemoglobin, oxygen tension (PO2) and pH of circulating blood were measured at set intervals using a blood gas analyzer (Instrumentation Laboratory Gem Premier 3000) and a co-oximeter (Instrumentation Laboratory 682 Co-oximeter, IL Company, Lexington, MA). Myocardial oxygen consumption (MVO2) was calculated as the difference in oxygen content directly above and below the perfused heart, and multiplied by coronary flow rate, according to the Fick method. MVO2 and blood pH were measured at baseline and at 10, 30 and 60 min of reperfusion in IR hearts and at corresponding times in the sham group.

5.2.5 Plasma Hydrogen Peroxide Content

Hydrogen peroxide (H2O2) was measured in plasma using the method reported by Dikalov et al., with modifications [111]. During experimentation, 100 µL of effluent blood was collected at baseline in both groups, at 1, 3, 5, 7, 9, 15, 30, 45 and 60 min of
reperfusion in the IR group, and at 69, 90 and 120 min of perfusion in the sham group (See Figure 4.2). Effluent blood was immediately mixed with 1 µL of 2 M NaN₃ (an inactivator of endogenous catalase), horseradish peroxidase [112] and 1 µL of 0.15 M deferoxamine mesylate (an iron chelator) [113]. Collected blood was centrifuged at 1000 g for 5 min at 4° C and the plasma fraction was immediately stored at -80° C until measurement. Fresh standards of hydrogen peroxide were prepared with absorbance at 240 nm, using an extinction coefficient of 43.6 M⁻¹cm⁻¹ [114]. Assay buffer (pH 7.4) consisted of NaCl 145 mM, KCl 4.86 mM, NaH₂PO₄ 5.7 mM, CaCl₂ 0.54 mM, MgSO₄ 1.22 mM, glucose 11 mM, deferoxamine mesylate 0.1 mM and horseradish peroxidase 0.1 U/mL. Amplex Red (Aldrich Chemical Company, Allentown, PA) was measured in samples and standards (Ex 530 nm, Em 590 nm) at 37° C every two min for 20 min in a Synergy HT microplate reader (BioTek, Winooski, VT) in the presence of hydrogen peroxide and horseradish peroxidase. The absorbance was recorded as the peak value at the linear portion of the absorbance vs. time curve for each sample.
5.2.6 Glutathione

Tissue reduced glutathione, GSH, was measured using the method of Rahman, et al. [115]. Briefly, frozen left ventricle tissue homogenate (10% w/v) was incubated at room temperature in 0.1 M phosphate buffer (pH 7.5) containing 5 mM EDTA, 0.5 mM 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), 0.5 mM NADPH and 2 U/mL glutathione reductase. The change in absorbance of the thio-nitrobenzene (TNB) chromophore at 412 nm as an indicator of GSH content in the supernatant was measured every 30
seconds over 3 minutes in a microplate reader kept at room temperature. GSH for each sample was normalized to LV mass.

5.2.7 Thiobarbituric acid reactive substances (TBARS)

Thiobarbituric acid reactive substances (chiefly malondialdehyde) were assayed from frozen tissue using the method of Ohkawa et al. [116] with modifications [117]. Briefly, supernatant from frozen left ventricular tissue homogenate was incubated with 4, 6-dihydroxy-2-mercaptopurimidine (TBA) at 95° C for one hour. After centrifugation, supernatant was mixed with n-butanol/pyridine (15:1 v/v). The organic fraction was reserved for measurement of TBARS. Total fluorescence of the malondialdehyde-TBA adduct was measured in a microplate reader (Ex 530 nm, Em 590 nm) against known TMP standards. TBARS content was normalized to the protein content in each sample using the Bradford assay (Bio-rad Life Science, Hercules, CA).

5.2.8 Isolated buffer perfused heart preparation

A separate group of hearts were isolated and perfused in the Langendorff mode and perfused with buffer as we have previously described [118]. Briefly, rats were anesthetized with i.p. sodium pentobarbital (60 mg/kg) isolated and hung on the perfusion column similar to the blood perfused heart. Coronary perfusion consisted of modified
Krebs-Henseleit buffer (1.25 mM CaCl₂, 5.5 mM glucose, 112 mM NaCl, 25 mM NaHCO₃, 5 mM KCl, 1.2 mM MgSO₄, 1 mM K₂PO₄, and 0.2 mM octanoic acid, bubbled with 95% O₂ / 5% CO₂, pH of 7.4) at a constant perfusion pressure of 85 mmHg without recirculation. A subgroup of these hearts underwent 25 minutes of global ischemia followed by 60 minutes of reperfusion.

5.2.9 Data Analysis

Group values are expressed as means plus or minus standard error. Two-sided t-tests followed by Welch’s correction for unequal variance were used to compare means between sham and IR groups. Statistical significance was set at p < 0.05.

5.3 Results

5.3.1 LV Function and Blood Chemistry

A comparison of oxygen measurements in blood and buffer perfused hearts shows the significant increase in myocardial oxygen delivery in the blood perfused hearts despite lower flow and lower partial pressure of oxygen. The baseline rate pressure product was lower in the blood perfused heart; however, contrary to our hypothesis, LV
recovery as a % of baseline was similar in both models following 25 minutes of ischemia and 60 minutes of reperfusion (See Table 5.1).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Blood-perfused hearts (n=12)</th>
<th>Buffer-perfused hearts (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PO₂, mm Hg</strong></td>
<td>77 ± 9*</td>
<td>634 ± 9*</td>
</tr>
<tr>
<td><strong>[Hb], g/dL</strong></td>
<td>9.55 ± 0.27</td>
<td>0</td>
</tr>
<tr>
<td><strong>Flow, mL/min</strong></td>
<td>3.75 ± 0.10*</td>
<td>14.32 ± 0.39</td>
</tr>
<tr>
<td><strong>O₂ Content, mL O₂/dL</strong></td>
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<td>1.97 ± 0.03</td>
</tr>
<tr>
<td><strong>O₂ Delivery, mL/min</strong></td>
<td>0.430 ± 0.018*</td>
<td>0.281 ± 0.008</td>
</tr>
<tr>
<td><strong>MVO₂, µL/min</strong></td>
<td>69 ± 4*</td>
<td>207 ± 7</td>
</tr>
<tr>
<td><strong>RPP, mm Hg/min</strong></td>
<td>16,882 ± 1,461*</td>
<td>34,451 ± 2,105</td>
</tr>
<tr>
<td><strong>Cardiac Efficiency, µL O₂/min</strong></td>
<td>251.8 ± 24.2*</td>
<td>165.5 ± 7.0</td>
</tr>
<tr>
<td><strong>RPP Recovery, %</strong></td>
<td>59.96 ± 8.29</td>
<td>60.57 ± 14.29</td>
</tr>
</tbody>
</table>

Mean ± S.E., *p<0.01.

Table 5.1: Baseline parameters of left ventricular (LV) function
Figure 5.3 $dP/dt_{\text{max}}$. **p<0.01

$dP/dt_{\text{max}}$ was significantly depressed throughout the reperfusion period in IR hearts as compared to the Sham group.
Figure 5.4 EDP. **p<0.01

EDP was significantly elevated throughout reperfusion in the IR group, as compared with the sham group.
Blood pH was similar between groups at baseline (IR 7.36 ± 0.02 vs. Sham 7.39 ± 0.03) but was significantly decreased at 10 min and 30 min of reperfusion in the IR group compared to the Sham group.

Myocardial oxygen consumption did not differ between groups at measured time points (60 min values: IR 68.6 ± 5.6 µL/min vs. Sham 70.4 ± 6.4 µL/min). Hematocrit in the Sham group was 23.1 ± 1.0 at baseline and 22.7 ± 1.3 at 120 min. In the IR group, hematocrit was 21.2 ± 1.5 at baseline and 22.4 ± 1.6 at 120 min. At the end of the 60 min reperfusion period, plasma free hemoglobin was not different between groups (IR 0.38 ± 0.16 g/dL vs. Sham 0.43 ± 0.10 g/dL).
5.3.2 Plasma Hydrogen Peroxide Production in Reperfusion

Figure 5.6 Plasma H$_2$O$_2$ content. *p<0.05, **p<0.01, ***p < 0.001

Plasma H$_2$O$_2$ peaked during early reperfusion and gradually decreased in IR hearts. No increase in H$_2$O$_2$ was seen in the Sham group throughout 120 min of perfusion. Plasma hydrogen peroxide concentration peaked early in reperfusion and remained significantly increased throughout reperfusion in the IR group compared with the sham group. At the end of the 60 min reperfusion period plasma H$_2$O$_2$ remained significantly higher in the IR group.
5.3.3 Tissue glutathione and thiobarbituric acid reactive substances content

At the end of reperfusion, IR hearts had significantly lower left ventricle tissue reduced glutathione content compared to sham hearts (IR 228 ± 18 µg/g LV vs. Sham 322 ± 23 µg/g LV).

There was no statistical difference in left ventricular thiobarbituric acid substances (TBARS) content between sham and ischemia-reperfusion hearts (IR 3.2 ± 0.6 nmoles/mg LV vs. Sham 2.6 ± 0.5 nmoles/mg LV).

Figure 5.7 Tissue glutathione content. **p<0.01
5.4 Discussion

In this paper we describe a recirculating autologous whole blood-perfused isolated rat heart model for the study of reactive oxygen species (ROS) production following global ischemia and reperfusion. We demonstrate a technique to measure H$_2$O$_2$ in the circulating plasma as an indicator of ROS production at reperfusion. In this blood-perfused model following IR, we note a significant rise of plasma hydrogen peroxide, a reduction in tissue antioxidant capacity and increased LV dysfunction. After 25 minutes of global ischemia, LV function recovered to about 60% of baseline function, similar to previously published studies in isolated hearts using both blood and buffer perfusion [119], though caution must be exercised when comparing buffer-perfused to blood-perfused hearts in the study of IR [120]. Blood pH was reduced at 10 min and 30 min of reperfusion due to ischemia-induced lactic acidosis, but recovered to baseline levels by the end of 60 min of reperfusion, indicating a resumption of aerobic metabolism of the heart during reperfusion [121, 122]. These findings, although relatively similar to previous reports in the buffer-perfused isolated heart, were obtained under physiologic blood-perfused conditions.

**Model advantages over other Langendorff models:** Whole blood perfusion of the heart affords a number of physiologic advantages over the more commonly used buffer-perfused Langendorff heart. The greatest physiologic advantage lies in the normal oxygen content of the perfusate, which in our model is autologous blood. Blood PO$_2$ at baseline in this model was 77 ± 9 mm Hg, which is more physiologic and far less than the
PO2 values seen in the perfusate of buffer-perfused hearts (634 ± 9 mm Hg). Friedman et al. reported PO2 values of 550 vs. 150 mmHg in buffer-perfused vs. blood-perfused isolated rat hearts, respectively [123]. Due to the higher oxygen delivery, coronary flow rates in the recirculating blood-perfused model are lower and more physiologic [124]. Coronary flow rates in the present model were one-quarter of those observed in crystalloid-perfused heart models (3.8 ml/min vs. 14.3 ml/min). Despite the lower flow rates and lower oxygen tension of the blood, the increased oxygen content due to hemoglobin allows for normal myocardial oxygen delivery [125]. Despite the higher myocardial oxygen delivery in the blood perfused hearts, myocardial oxygen consumption and consequently oxygen extraction (blood 16 % vs. buffer 74%) were lower than buffer perfused hearts. This is in part due to the higher work load (higher rate pressure product) in the buffer perfused hearts. However, work load alone does not account entirely for these differences in myocardial oxygen consumption, as cardiac efficiency as defined by the ratio of rate pressure product to MVO2 was higher in the blood perfused heart. This data suggests that the buffer perfused heart uses oxygen less efficiently than the blood perfused heart. These oxygen measures were derived pre-ischemia to highlight the differences between blood and buffer perfused hearts and thus do not reflect the post-ischemic condition. An important distinction between the models is the mode of perfusion. Due to the recirculating nature of the described blood perfused heart, perfusion is flow regulated, whereas perfusion in the buffer perfused heart is pressure regulated. However, despite the differences in perfusion, the oxygen and flow needed to support the heart in each model are vastly different.
This re-circulating blood model also affords significant advantages over previously described blood-perfused isolated heart models. The use of the same rat for both the isolated heart and the necessary blood to perfuse the circuit significantly reduces animal use. Previously described models all require multiple animals for blood donation or a separate support animal for the isolated heart [126]. Compared with models that depend on a support rat for oxygen delivery and perfusion, our model avoids the potential negative impact of the metabolic state of the anesthetized support rat on the isolated heart. Utilization of fresh autologous blood also eliminates potential immunologic reactions due to interspecies blood transfusion. Unlike isolated erythrocyte preparations, all components of the blood, including leukocytes are present. This is important in the study of various sources of ROS formation during reperfusion.

\textit{H}_2\textit{O}_2\textit{ and tissue oxidation:} \textit{H}_2\textit{O}_2\textit{ concentration was measured in the circulating blood at various time points as an indicator of ROS formation. To measure \textit{H}_2\textit{O}_2\textit{ in the blood, we modified an established method [127]. The blood samples were immediately treated with NaN3, to block catalase and peroxidase, and with deferoxamine to stop continued \textit{H}_2\textit{O}_2\textit{ production through iron surface chemistry [128]. The primary source of \textit{H}_2\textit{O}_2\textit{ during early reperfusion is through dismutation of the short-lived superoxide reactive oxygen species, which is produced from a variety of sources [129]. In the present study, a rapid increase in \textit{H}_2\textit{O}_2\textit{ concentration was observed during early reperfusion, with a gradual decrease over the 60 min reperfusion period. The early increase in \textit{H}_2\textit{O}_2\textit{ production seen in our blood-perfused model is similar to the early increase in ROS production seen in the first few minutes of reperfusion in buffer-
perfused hearts [130]. Brown et al. reported that maximal hydrogen peroxide production occurred 10 min following reperfusion in isolated rat hearts subjected to 20 min of global ischemia [131].

In our model, myocyte membrane lipid peroxidation and reduced glutathione (GSH) were examined in whole tissue homogenate as markers of oxidative stress. GSH was reduced in IR hearts in this model, an observation that is in agreement with studies done previously in isolated hearts. Singh et al. reported GSH tissue content values of 380 µg/g in two hr sham-operated pig hearts, similar to 322 µg/g after two hours of sham perfusion in our model [132]. Lipid peroxidation, measured with TBARS, was not significantly increased at the end of the reperfusion period. A similar finding was noted by Coudray et al. who did not find differences in lipid peroxidation between ischemic and non-ischemic isolated buffer-perfused rat hearts [133]. Similarly, Janssen et al. noticed an increase in oxidized glutathione, but no increase in lipid peroxidation, during reperfusion of globally ischemic rat hearts [134]. These markers of oxidant stress following IR in our model respond similarly to those reported in the literature.

5.5 Limitations

Due to the recirculating nature of the blood and the absence of other organs (liver and kidney), metabolites can accumulate over time. Like other Langendorff models, this preparation is stable for a limited period of time, as LV function gradually declines.
Sham hearts were stable for 120 min, with no loss of left ventricular function over this time period. In preliminary studies, we noted LV function preservation for up to 4 hours under normal perfusion. However, in other experiments we noted an increase in perfusion pressure over time in this constant flow model, due to increased coronary resistance accompanied by increasing LV dysfunction. The exact source of this increase in coronary resistance is unclear, but is likely due to scavenging of nitric oxide due to exposure to free hemoglobin. The recirculation of blood by a peristaltic pump over time causes some cell lysis. Free plasma hemoglobin levels were measured at the end of experimentation and higher free hemoglobin levels were correlated with higher coronary resistance (data not shown).

This recirculating blood-perfused isolated heart model allows for the study of ROS production under physiologic oxygen conditions following myocardial ischemia and reperfusion. This model offers significant advantages over established models of isolated buffer and blood perfused hearts. These advantages include the use of whole autologous blood, the use of only one animal per experiment, physiologic oxygen delivery and the ability to measure hydrogen peroxide concentration in the blood.
Chapter 6. Low Flow Intervention in the Blood-Perfused Isolated Heart

6.1 Introduction and Hypothesis

It is unclear how intervention in ischemia with low blood flow will affect recovery of left ventricular function. This study utilized the same small volume autologous whole blood-perfused isolated rat heart model to test the hypothesis that, following ischemia, low flow intervention in global ischemia prior to full flow reperfusion improves recovery of LV function.

6.2 Methods

The same methods as in chapter 4 were used to isolate and perfuse each rat heart. After successful isolation, hearts were randomized to three groups (N=6 per group): 1) 25 min of global no-flow schema before full reperfusion, 2) 15 minutes of global no-flow ischemia followed by 10 minutes of low coronary perfusion at 1% of baseline coronary flow values prior to full reperfusion, or 3) 15 minutes of global no-flow ischemia followed by 10 minutes of low coronary perfusion at 10% of baseline coronary flow.
values prior to full reperfusion. The duration of full reperfusion was 60 minutes in all three groups. LV function was continuously monitored as previously described in this manuscript.

6.3 Results

The 10% low flow group (N=6) had significantly higher recovery of developed pressure (Figure 5.1) and rate pressure product (Figure 5.2) (p <0.001 with ANOVA) during reperfusion than the Control group (N=7). The very low flow (1%) group (N=6) was no different from the Control group (no low flow) in recovery of either parameter of LV function.
Recovery of developed pressure was improved at the end of ischemia in isolated blood-perfused hearts subjected to low flow intervention.
Rate Pressure Product in Isolated Blood-perfused Hearts subjected to 2 levels of Low Flow or Control (25 min) Ischemia

<table>
<thead>
<tr>
<th>Time, minutes</th>
<th>0 2 0 4 0 6 0 8 0</th>
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<tr>
<td>mm Hg per min</td>
<td>0 5000 10000 15000 20000 25000 30000</td>
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- 25 min Ischemia (N=7)
- 10% Low Flow (N=6)
- 1% Low Flow (N=6)

Recovery of rate pressure product (RPP) is significantly improved in hearts subjected to low flow intervention.

6.4 Discussion and Future Directions

This small study measured LV recovery with a model of the isolated perfused heart that is more physiologic than the buffer-perfused isolated heart, both in oxygen delivery and antioxidant capacity. The results support the idea that low flow may also be protective in a whole animal model of cardiac arrest and resuscitation.
This brief study also emphasizes the important role of a short period of low flow to significantly improve left ventricular functional recovery. This is in support of earlier work in the buffer perfused heart [78]. However, whole blood has a large reservoir of antioxidant capacity that is not present in buffer-perfused hearts [135]. This antioxidant capacity may render the production of ROS less important than oxygen delivery in the recovery of LV function. Conversely, physiologic levels of oxygen delivery may provide substrate for enhanced production of damaging ROS [136]. It is simple to speculate that ROS production may be similarly reduced after low flow in this blood-perfused model.

In this study, 10% low flow, but not 1% low flow, improved recovery of LV function following 25 min of global no-flow ischemia. These results suggest that the level of coronary flow induced by intervention in ischemia is related to LV recovery.

Future work should include a measure of ROS production, such as H₂O₂, as was performed in the buffer-perfused heart. Oxygen content should be measured in future studies of low flow with whole blood perfusion to determine the influence of oxygen delivery and consumption during low flow on LV functional recovery following insult.
Chapter 7. Conclusions

This dissertation has revealed the importance of reactive oxygen species generation during post-ischemic low flow and subsequent full reperfusion on the preservation of LV recovery. The preservation of LV function extends to hearts that are perfused with whole blood. Mitochondrial ROS generation must be examined in order to fully elucidate the subcellular ROS response to post-ischemic low flow states. Many studies, both basic and clinical, have explored ROS reduction as a strategy for infarct reduction and LV functional preservation following cardiac insult, mostly to mixed results. ROS generation during low flow and full reperfusion is undoubtedly a dynamic process among the various cellular and subcellular compartments. For example, though ROS production has been linked to the pathogenesis of cardiac failure, recent evidence indicates that ROS are also essential to physiologic regulation of cardiac contractility \[137\]. Additionally, superoxide itself regulates vascular tone by acting through multiple signalling pathways \[138\]. Any strategy aimed at reducing ROS in the myocardium must then be aimed at maintenance of ROS homeostasis, without disruption of cellular homeostasis. Because the myocyte is inhabited by enzymes that consume free radicals at diffusion-limited rates (for instance, superoxide dismutase, SOD), any strategy aimed at ROS reduction should be focused on inhibiting the sources of ROS, rather than simply scavenging ROS, lest the cellular antioxidant pool become overwhelmed \[139\].
Low flow is both a mechanical and metabolic intervention to the ischemic heart. Reperfusion of oxygen causes the reintroduction of electrons to the respiratory chain of mitochondria, leading to overproduction of reactive oxygen species and calcium overload. A slow reintroduction of oxygen may reduce the release of ROS and also reduce calcium overload [140]. It is also possible that slower oxygen reintroduction can extend metabolic acidosis into the reperfusion period, thus preventing opening of the mitochondrial permeability transition pore, which is a known signal for cell death [141]. Conversely, the reintroduction of low coronary flow may preserve LV function through mechanical means. Flow-mediated nitric oxide release from aortic endothelial cells is independent of intracellular calcium, suggesting that low flow itself may stimulate NO release independent of LV contractile state [142]. Coronary artery bypass grafting (CABG) patients showed attenuation of the inflammatory response when pump flow was increased by 20% the normal rate during hypothermia [143]. In general, myocardial postconditioning modalities involve protection of the coronary endothelium, among many other mechanisms. Though it is well known that endothelium-dependent and independent vasodilatation is critical to tissue oxygen delivery, the interplay of oxygen reintroduction and endothelial protection afforded by post-ischemic low flow is a complex process that warrants much further investigation [144, 145].

In Chapter 2, intervention with low-pressure pulsatile flow was detrimental to recovery of heart function, while in Chapter 3, intervention with constant low flow was beneficial. However, in contrast to our findings, CABG patients given a pulsatile flow component during surgery showed attenuation of the whole body inflammatory response,
as compared to patients given non-pulsatile myocardial perfusion [146]. These potentially conflicting results do suggest that the mode of low flow is important in effecting myocardial recovery. Constant low flow ensured that critical opening pressures for coronary septa would be achieved, highlighting the importance of coronary perfusion during low flow states. Low flow states that do not achieve critical opening pressures with resuscitation efforts may actually be harmful to recovery of heart function at reperfusion [147]. In support of this idea, intervention with 0.5% low flow was not beneficial to recovery of LV function. The results in Chapters 4 and 5 further demonstrated that recovery of LV function is protected with 10% low flow intervention in a more physiologic model, that of the blood-perfused isolated heart. However, this LV preservation was not achieved with 1% low flow. Taken together, these results show that the amount of coronary perfusion is important if recovery of heart function is to be preserved.

7.1 Limitations and Future Directions

A major limitation evident in studies of myocardial ischemia and reperfusion is the lack of a regimented approach to the phenomenon. Multiple different models, types, durations, post-ischemic treatment modalities and sub-cellular systems are described in the literature, making inference of the results of any one study difficult to extend to others. Without scientific consensus, experimental results have little chance of success upon extension to clinical situations. Clearly, a systematic approach to IR must be
developed in order to reach consensus for clinical treatment of post-ischemic low flow conditions.

A multitude of questions remain regarding post-ischemic low flow in the heart. For instance, intracellular calcium overload is central to the pathophysiology of ischemia-reperfusion injury. It is completely unknown how calcium handling is affected by post-ischemic low flow. It is possible that a certain level of low flow can affect gradual restoration of calcium homeostasis without calcium overload. To test this idea, a cell-permeable, calcium-sensitive dye such as fura-2 acetoxymethylester can be infused into the isolated heart, and fluorescence measured at the left ventricular wall [148]. In this dissertation, cellular and vascular sources of ROS were examined, but not the response of mitochondrial ROS generation to post-ischemic low flow. A UV-active superoxide indicator specific to mitochondria has recently been demonstrated in isolated murine hearts, and could be employed to examine that cellular compartment [149]. Additionally, energy storage molecules such as ATP can be measured to test the effects of post-ischemic low flow on cellular metabolism. The studies undertaken in this dissertation were all performed using hearts isolated from healthy Sprague-Dawley rats. The effects of post-ischemic low flow on isolated hearts harvested from animals subjected to induced left ventricular failure may be divergent from the effects observed in healthy animals. Many questions remain regarding the effects of Sivelestat. It is clear that Sivelestat causes a relatively mild decrease in ROS generation at reperfusion, and that this effect is related to nitric oxide synthase function. However, it unclear if Sivelestat prevents uncoupling of nitric oxide synthases directly or acts upstream of NOS enzymes. Nitric
oxide bioavailability is reduced in rats with streptozotocin-induced diabetes mellitus through uncoupling of endothelial nitric oxide synthase (eNOS). If Sivelestat prevents eNOS uncoupling, then NO bioavailability may be enhanced by treatment with Sivelestat. Finally, the results presented here showed that Sivelestat preserved endothelium-dependent vasoreactivity to acetylcholine (Ach). It is unclear how Sivelestat affects endothelium-independent vasoreactivity. As such, it would be valuable to determine the coronary flow response of Sivelestat-treated isolated hearts to an endothelium-independent vasodilator such as sodium nitroprusside [150].

It is clear that, aside from the importance of the level of coronary perfusion, there are subcellular processes that can be manipulated with post-ischemic low flow, both as an interventional strategy, and as a strategy for post-ischemic drug delivery. A full description of vascular, cellular, mitochondrial and hematologic responses to low flow must be provided in order to elucidate proper protective strategies for use in the whole animal. It is my hope that the results presented in this dissertation might be the starting point for investigation towards a more dynamic cardioprotective strategy against ischemia and reperfusion in the heart.
Reference List


(11) Zweier JL, Duke SS, Kuppusamy P, Sylvester JT, Gabrielson EW. Electron paramagnetic resonance evidence that cellular oxygen toxicity is caused by the...


(16) Chen XL, Zhang Q, Zhao R, Ding X, Tummala PE, Medford RM. Rac1 and superoxide are required for the expression of cell adhesion molecules induced by tumor necrosis factor-alpha in endothelial cells. *J Pharmacol Exp Ther* 2003 May;305(2):573-80.


(49) Downey JM, Cohen MV. Why do we still not have cardioprotective drugs? *Circ J* 2009 July;73(7):1171-7.


(105) Ferreira R. The reduction of infarct size--forty years of research--second of two parts. *Rev Port Cardiol* 2010 July;29(7-8):1219-44.


(107) Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc Natl Acad Sci U S A* 1990 February;87(4):1620-4.


(133) Coudray C, Pucheu S, Boucher F, De LJ, Favier A. Ischemia and reperfusion injury in isolated rat heart: effect of reperfusion duration on xanthine oxidase,


