# EVALUATION OF A NOVEL SODIUM-CALCIUM EXCHANGE INHIBITOR, SEA0400, IN THE JCR:LA-*cp* RAT, A MODEL OF THE METABOLIC SYNDROME AND CARDIOVASCULAR DISEASE

BY

CANDACE Y.W. LEE

A Thesis submitted to the Faculty of Graduate Studies,

in Partial Fulfillment of the Requirements for the Degree of:

DOCTOR OF PHILOSOPHY

Department of Physiology

University of Manitoba

Winnipeg, Manitoba

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

*****

COPYRIGHT PERMISSION PAGE

# Evaluation of a Novel Sodium-Calcium Exchange Inhibitor, SEA0400, in the JCR:LA-*cp* Rat, a Model of the Metabolic Syndrome and Cardiovascular Disease

BY

Candace Y.W. Lee

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

Dedicated to

Dr. Larry V. Hryshko

## TABLE OF CONTENTS

## TABLE OF CONTENTS (continued)

Page

## TABLE OF CONTENTS (continued)

## TABLE OF CONTENTS (continued)

Page

## TABLE OF CONTENTS (continued)

Page

## ACKNOWLEDGEMENTS

I would like to thank:

**Dr. Larry Hryshko**, my advisor, for having introduced me to this fascinating area of research since the Fourth International Conference in Sodium-Calcium Exchange in 2001, for his invaluable guidance and support throughout my studies on the physiology and pharmacology of cardiac sodium-calcium exchange, and for having made my studies so educational and enjoyable;

**Dr. Naranjan Dhalla**, my mentor, for having enriched my education with the excellent opportunity of participating in the TACTICS (Tailored Advanced Collaborative Training in Cardiovascular Science for Postdoctoral Fellows & Clinician Scientists) Program, and for his invaluable guidance and support in my studies on myocardial ischemia/reperfusion injury;

Members of my PhD Advisory Committee, **Dr. Ratna Bose** (Dept. of Pharmacology), **Dr. Ian Dixon** (Dept. of Physiology), **Dr. John Ducas** (Section of Cardiology, Dept. of Medicine), and **Dr. Grant Pierce** (Dept. of Physiology) for their advice and guidance during my studies;

External Examiner of my PhD thesis, **Dr. Michael Artman** (Professor and Head, Dept. of Pediatrics, University of Iowa) for his valuable comments;

**All colleagues in Dr. Hryshko's Laboratory and Dr. Dhalla's Laboratory** for their friendship, academic stimulation, and kind support in various parts of my project – special thanks to **Drs. Neeraj Visen, Raja Singh, Mark Hnatowich, Alex Omelchenko**; and **Hoa Dinh Le**;

**Dr. John Foerster** (Executive Director of Research, St. Boniface Research Centre) for his kind support and encouragement on many occasions;

**Dr. Peter Liu** (University of Toronto), Program Director of the TACTICS Program, for his guidance and support during my TACTICS fellowship;

**Dr. Peter Backx** (University of Toronto) and **Dr. Morris Karmazyn** (University of Western Ontario), members of my TACTICS Advisory Committee, for their helpful suggestions on my research project;

**Dr. Marielle Scherrer-Crosbie** (Cardiac Ultrasound Unit, Massachusetts General Hospital, Harvard University, U.S.A.) for her expert guidance in murine echocardiography during my elective training at the M.G.H., and for her valuable advice through correspondence during my PhD studies;

**Dr. Geneviève Derumeaux** (Laboratoire d'Echocardiographie, Hôpital Louis Pradel, Lyon, France) for her valuable input during my elective training in murine

echocardiography, which took place at the opportune time of her visit to the Cardiac Ultrasound Unit at the Massachusetts General Hospital, and for her helpful suggestions through subsequent correspondence;

**Dr. Peter Zahradka** (Department of Physiology) for his valuable advice during my graduate studies and for having kindly provided me with access to equipment in his laboratory for quantification of lactate dehydrogenase in rat coronary effluent;

**Dr. Jim Russell** (Professor Emeritus, Dept. of Surgery, University of Alberta) for his helpful suggestions on my research project utilizing the JCR:LA-*cp* rats;

**Dr. Ted Cuddy** (Professor Emeritus, Faculty of Medicine) for his longstanding support and for the excellent opportunities of working with him during my clinical training and in the Manitoba Follow-up Study;

**Dr. Randy Aitken** (Director of the R.O. Burrell Facility) for his kind support and helpful suggestions, and **all members of staff** of the Facility for their outstanding professional assistance;

**Dr. Carmen Morales** (Director of the Immunoprotein Laboratory, University of Manitoba) and **members of staff** of the Laboratory for their collaboration and expert assistance with quantification of rat C-reactive protein;

**Mrs. Mary Cheang** (Biostatistical Consulting Unit, University of Manitoba) for her advice on statistics during my studies and for her professional assistance with the statistical analyses pertaining to *in vivo* drug evaluation;

My former Residency Program Directors at the University of Manitoba: **Dr. Kenneth Van Ameyde** (Internal Medicine) and **Dr. Kevin Wolfe** (Cardiology) for their longstanding support and encouragement;

My mentors at Queen's University: **Dr. Robert Hudson** (Division of Endocrinology and Metabolism, Department of Medicine) and **Dr. Hugh Gorwill** (Department of Obstetrics and Gynaecology) for their inspiration in research;

The **Canadian Institutes of Health Research (CIHR)** and the **TACTICS Strategic Training Program in cardiovascular research (partnership between the CIHR and the Heart and Stroke Foundation of Canada)** for fellowship support, which has been instrumental in making my full-time research training possible;

My significant other for his unconditional support and everlasting love;

My grandmother, parents, and sister for their encouragement and support;

Last but not least, I gratefully acknowledge the wisdom of Buddha in guiding my life.

## LIST OF FIGURES

## LIST OF FIGURES (continued)

## LIST OF FIGURES (continued)

Figure Page

## LIST OF FIGURES (continued)

Figure Page

## LIST OF FIGURES (continued)

## LIST OF TABLES

## LIST OF ABBREVIATIONS

| | |
|---|---|
| 2-D | Two-dimensional |
| mM | Millimolar |
| μM | Micromolar |
| nM | Nanomolar |
| ACS | Acute coronary syndromes |
| $Ca^{2+}_i$ | Intracellular calcium |
| $Ca^{2+}_o$ | Extracellular calcium |
| CABG surgery | Coronary artery bypass graft surgery |
| CAD | Coronary artery disease |
| CASTEMI trial | Calderet in ST Elevation Myocardial Infarction trial (1) |
| *cp* | corpulent |
| CRP | C-reactive protein |
| *db* | diabetes |
| DMSO | Dimethylsulfoxide |
| $dP/dt_{max}$ | Maximum rate of pressure development |
| $dP/dt_{min}$ | Maximum rate of pressure decline |
| DT | Deceleration time |
| E/A ratio | Ratio of the early maximum filling velocity (E velocity) to the atrial velocity (A velocity) of left ventricular diastolic filling (2) |

## LIST OF ABBREVIATIONS (continued)

| | |
|---|---|
| $EC_{50}$ | Concentration of drug that produces 50% of maximal effect (E = effect observed at concentration C) (3) |
| ECG | Electrocardiogram |
| ELISA | Enzyme-linked immunosorbent assay (4) |
| ESCAMI trial | Evaluation of the Safety and Cardioprotective Effects of Eniporide in Acute Myocardial Infarction trial (5) |
| EXPEDITION trial | Sodium-hydrogen Exchange Inhibition to Prevent Coronary Events in Acute Cardiac Conditions trial (6) |
| *fa* | fatty |
| FS | Fractional shortening |
| Gly | Glycine |
| GUARDIAN trial | Guard During Ischemia Against Necrosis trial (7) |
| HDL | High-density lipoprotein |
| KB-R7943 | 2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl]isothiourea methanesulphonate |
| $I_1$ inactivation | Intracellular sodium ($Na^+_i$) dependent inactivation |
| $I_2$ regulation | $Ca^{2+}$-dependent regulation |
| $IC_{30}$ | 30% inhibitory concentration |
| $IC_{50}$ | 50% inhibitory concentration |
| IR(I) | Ischemia/reperfusion (injury) |
| IRS | Insulin resistance syndrome |
| IU | International Units |

## LIST OF ABBREVIATIONS (continued)

| | |
|---|---|
| i.v. | intravenous |
| JCR:LA-*cp* rat | James C. Russell corpulent rat |
| LDH | Lactate dehydrogenase |
| LV | Left ventricle/left ventricular |
| LVdevP | Left ventricular developed pressure |
| LVdiasP | Left ventricular diastolic pressure |
| LVEDD | Left ventricular end-diastolic dimension |
| LVESD | Left ventricular end-systolic dimension |
| LVH | Left ventricular hypertrophy |
| LVSP | Left ventricular systolic pressure |
| M-mode | Motion-mode (echocardiography) |
| MetS | Metabolic syndrome |
| MCC-135 | 5-methyl-2-(1-piperazinyl) benzenesulfonic acid monohydrate |
| MI | Myocardial infarction |
| Min | Minutes |
| $Na^+_i$ | Intracellular sodium |
| $Na^+_o$ | Extracellular sodium |
| NBC | Sodium-bicarbonate co-transporter |
| NCEP ATPIII | National Cholesterol Education Program's Adult Treatment Panel III report (of the United States) |
| NCX | Sodium-calcium exchange(r) |

## LIST OF ABBREVIATIONS (continued)

| | |
|---|---|
| NCX1 | Sodium-calcium exchanger isoform 1 |
| NCX1.1, NCX 1.3 | Splice variants of sodium-calcium exchange isoform 1 |
| NHE | Sodium-hydrogen exchange(r) |
| NHE-1 | Sodium hydrogen exchanger isoform 1 |
| NPY | Neuropeptide Y |
| *ob* | obese |
| ObR | Leptin receptor |
| PAI-1 | Plasminogen activator inhibitor-1 |
| PCI | Percutaneous coronary intervention |
| Phe | Phenylalanine |
| PW Doppler | Pulsed-wave Doppler |
| PW | Posterior wall |
| RV | Right ventricle or right ventricular |
| SD | Standard deviation |
| SEM | Standard error mean |
| SEA0400 | 2-[4-[(2,5-difluorophenyl)methoxy]phenoxy]-5-ethoxyaniline |
| SM-15811 | 3,4-dihydro-2(1H)-quinazolinone derivative |
| SN-6 | 2-[4-(4-nitrobenzyloxy)benzyl]thiazolidine-4-carboxylic acid ethyl ester |
| SR | Sarcoplasmic reticulum |
| ST | Septal thickness |

## LIST OF ABBREVIATIONS (continued)

| | |
|---|---|
| STEMI | ST-segment elevation myocardial infarction |
| TG | Triglyceride |
| TMS | Transmembrane segments |
| $V_{max}$ | Maximal velocity |
| VF | Ventricular fibrillation |
| VLDL | Very-low-density lipoprotein |
| *vs* | versus |
| VT | Ventricular tachycardia |
| XIP | Exchange inhibitory peptide |

## ABSTRACT

The cardiac $Na^+$-$Ca^{2+}$ exchanger (NCX) plays an important role in regulating $Ca^{2+}$ under physiological and pathophysiological conditions. In its forward mode of operation, which is the predominant mode under physiological conditions, it extrudes $Ca^{2+}$ that enters the cardiac myocyte through L-type $Ca^{2+}$ channels on a beat-to-beat basis. During ischemia/reperfusion (IR), increased intracellular $Na^+$ favours a reduction of $Ca^{2+}$ efflux through the forward mode and an increase in $Ca^{2+}$ influx through the reverse mode of NCX, resulting in $Ca^{2+}$ overload and consequent IR injury (IRI). Pharmacological inhibition of the reverse mode of NCX represents an attractive therapeutic strategy for cardioprotection. This thesis focuses on the evaluation of a novel NCX inhibitor, 2-[4-[(2,5-difluorophenyl)methoxy]phenoxy]-5-ethoxyaniline (SEA0400), in attenuating IRI in a polygenetic model of the metabolic syndrome and cardiovascular disease, the JCR:LA-*cp* rat. The major objectives were: (1) to investigate the efficacy of SEA0400 in attenuating IRI in this clinically relevant animal model, using the Langendorff-perfused isolated heart technique; (2) to test the concept of prolonged *in vivo* administration of SEA0400 as a chronic cardioprotective therapy and to assess any potential effects of SEA0400 on left ventricular (LV) function by serial transthoracic echocardiography. The main findings are: (1) an increased sensitivity to myocardial ischemia in the obese JCR:LA-*cp* rats (10-13 months) as compared with lean controls; (2) a lack of cardioprotective effects of SEA0400 against IRI in obese rats, based on cardiac performance and release of lactate dehydrogenase during early reperfusion, under our experimental conditions; (3) the stability of LV systolic and diastolic function during prolonged continuous intravenous administration of SEA0400

in obese rats over 4 weeks. In addition, serum C-reactive protein levels and echocardiographic features of the JCR:LA-*cp* rats were characterized for the first time. In conclusion, the obese JCR:LA-*cp* rats are more susceptible to myocardial IRI as compared with lean controls, consistent with previous findings at an earlier stage of life in this strain. While prolonged administration of SEA0400 was feasible and did not significantly affect LV function as assessed by echocardiography, SEA0400 did not confer significant cardioprotection against IRI in the obese JCR:LA-*cp* rats under the experimental conditions of this study.

# 1. REVIEW OF THE LITERATURE

## 1.1 Physiological Role of the Cardiac Sodium-Calcium Exchanger

The cardiac sodium-calcium exchanger (NCX1) is a transsarcolemmal protein that plays an important role in $Ca^{2+}$ homeostasis (8-11). It is a member of the NCX family, which consists of 3 distinct proteins: NCX1, NCX2, and NCX3 (10,12). These 3 proteins have approximately 70% of their respective amino acid sequences in common (13). NCX1 is ubiquitously distributed, whereas NCX2 and NCX3 are found in brain and skeletal muscle (10). Sodium-calcium exchange activity was first described in guinea pig atria by Reuter and Seitz in 1968 (14) and the exchanger was later cloned by Nicoll *et al* in 1990 (15). The current topological model of NCX1 is illustrated in Figure 1 (10,16-18). The NCX1 protein, which is composed of 938 amino acids (110 kDa), consists of 9 transmembrane segments (TMS) and a large intracellular loop connecting TMS 5 and 6 (17,19,20). The TMS, especially the α-repeat regions (21,22), are involved in ion transport; whereas regions of the large intracellular loop, such as the exchange inhibitory peptide (XIP) region (23,24) and regulatory $Ca^{2+}$ binding sites (25,26), are involved in regulation (12,13).

There is considerable evidence indicating that $Na^+$-$Ca^{2+}$ exchange is the primary mechanism for transsarcolemmal $Ca^{2+}$ removal and that, quantitatively, this mechanism removes the same amount of $Ca^{2+}$ that enters through L-type $Ca^{2+}$ channels on a beat-to-beat basis (9,27). In the forward mode (or $Ca^{2+}$ efflux mode) of NCX, which is the dominant mode under physiological conditions, $Na^+$ influx is coupled to $Ca^{2+}$ efflux in a $3Na^+$:$1Ca^{2+}$ stoichiometric ratio (11,28) (Figure 2), utilizing the $Na^+$ gradient established by the $Na^+/K^+$-ATPase (20). Surprisingly, however, a recent study

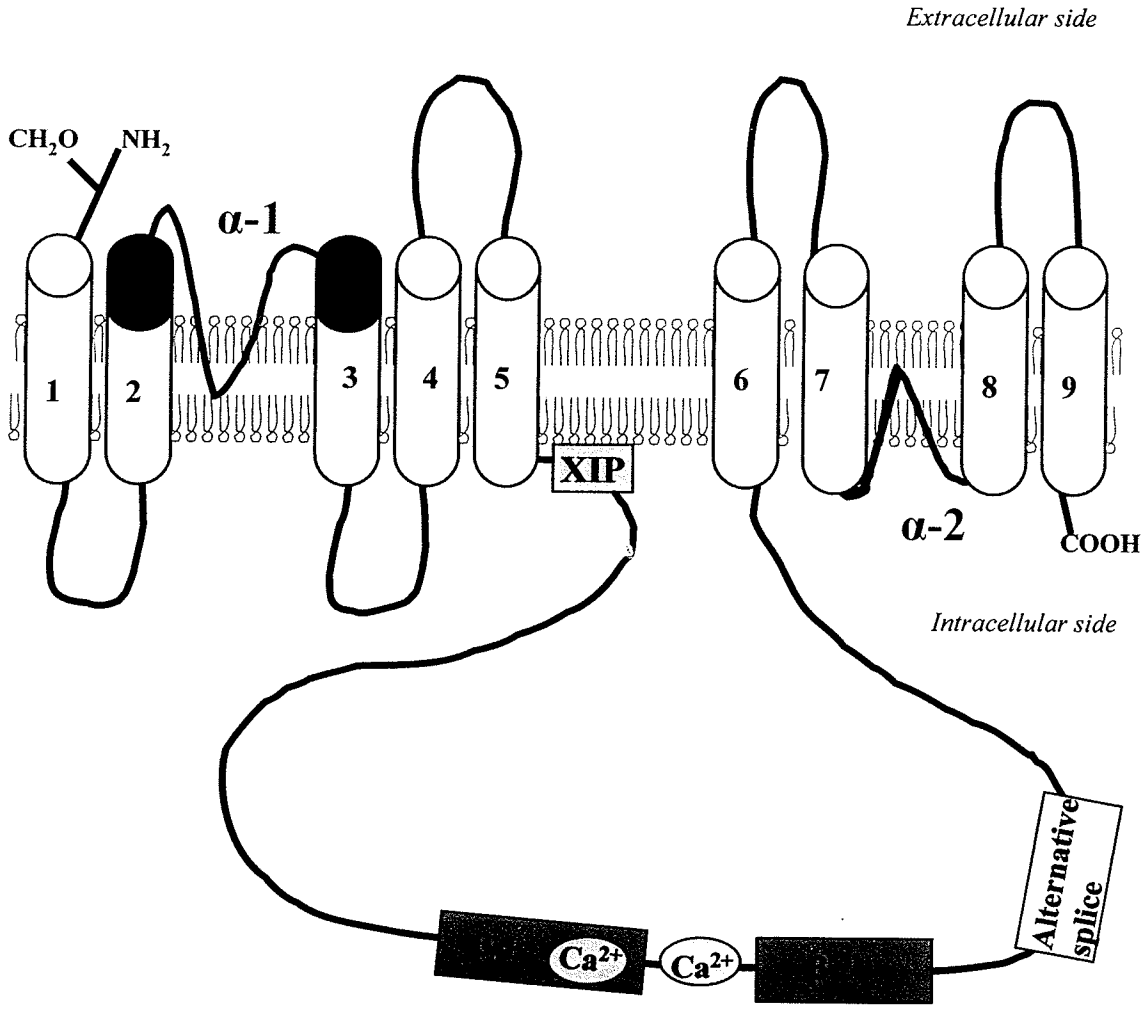



**Figure 1. Topographic Model of the Cardiac Sodium-Calcium Exchanger.**
(Redrawn based on references #10 and 16-18.)

by Henderson *et al* (29) has demonstrated survival of mice following cardiac-specific knockout of the exchanger, indicating that alternative $Ca^{2+}$ efflux mechanisms must exist (29). While the sarcoplasmic reticulum $Ca^{2+}$-ATPase and the mitochondrial $Ca^{2+}$ uniporter can sequester intracellular $Ca^{2+}$, these transport systems cannot extrude $Ca^{2+}$ to maintain an overall intracellular $Ca^{2+}$ balance (9) (Figure 2). The only identified parallel $Ca^{2+}$ efflux pathway in cardiac muscle is the sarcolemmal $Ca^{2+}$-ATPase, although it remains unclear as to whether this transport system has the capacity to match $Ca^{2+}$ influx levels during cardiac excitation (30). Notably, in a recent study by Hurtado *et al* (31,32), the technique of RNA interference was successfully applied as a novel strategy in interfering with NCX expression in neonatal myocytes and spontaneous contractions of the myocytes were observed, albeit at a lower frequency, even after severe depletion of NCX. In addition, sarcoplasmic $Ca^{2+}$ pump expression was found to have increased 3 times, as an adaptive response to the depletion of NCX (32). Taken together, findings from these studies using distinct techniques suggest an important, but not crucial, role of the NCX in cardiac excitation-contraction coupling.

Being a bi-directional electrogenic transporter, the cardiac NCX also allows $Ca^{2+}$ entry through its reverse mode (or $Ca^{2+}$ influx mode), and the direction of transport is influenced by the membrane potential and the intracellular and extracellular concentrations of $Na^+$ and $Ca^{2+}$ (11,33). In contrast to its relatively insignificant role under physiological conditions in the adult myocardium, the reverse mode of NCX becomes much more important in the presence of elevated intracellular sodium ($Na^+_i$), such as during ischemia/reperfusion, heart failure, and digitalis toxicity (12,20,34-38), where NCX allows entry of 1 $Ca^{2+}$ in exchange for extrusion of 3 $Na^+$ (12).

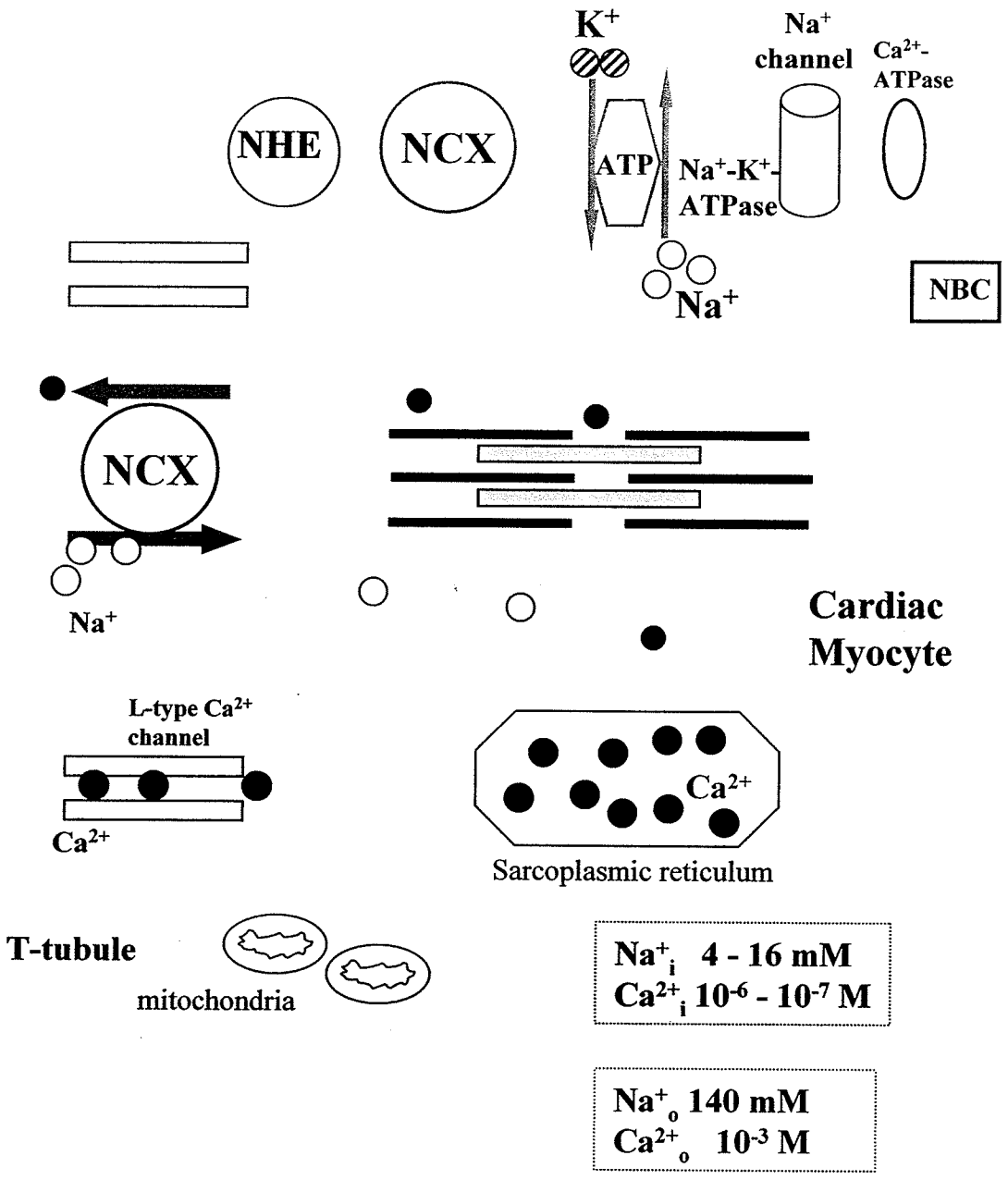



**Figure 2. Role of the Cardiac Sodium-Calcium Exchanger (NCX) Under Physiological Conditions.**

Sodium-calcium exchange activity is regulated by a number of factors, such as $Na^+$ and $Ca^{2+}$, phosphorylation, phosphatidylinositol-4,5-bisposphate ($PIP_2$) and pH (12,17,33). Intracellular sodium ($Na^+_i$) dependent inactivation (also known as $I_1$ inactivation), which was initially described by Hilgemann in 1990 (39), refers to the ability of $Na^+_i$ to facilitate or induce the transition of NCX into an inactive state (12,40); whereas, $Ca^{2+}$-dependent regulation (or $I_2$ regulation) describes the stimulation of NCX activity by cytoplasmic $Ca^{2+}$ (12,39).

Physiological concentrations of extracellular and intracellular $Na^+$ and $Ca^{2+}$ in cardiac muscle are illustrated in Figure 2. Notably, $[Na^+]_i$ has been found to be higher in the rat and the mouse (41-45), as compared with $[Na^+]_i$ in other mammalian species, such as the rabbit or the guinea pig (45,46). The greater $[Na^+]_i$ in the rat has been attributed to a higher rate of $Na^+$ influx (with differential contribution from $Na^+$ channels, NHE, and NCX) rather than reduced efflux (43). In the cardiac myocyte, $Na^+$ homeostasis is achieved through a fine balance between $Na^+$ influx and efflux. Pathways for $Na^+$ influx include $Na^+$ channels, $Na^+/Ca^{2+}$ exchange, $Na^+/H^+$ exchange, $Na^+/Mg^{2+}$ exchange, $Na^+/HCO_3^-$ co-transport (NBC), and $Na^+/K^+/2Cl^-$ co-transport (Figure 3), with the first two being the major pathways under physiology conditions (20). In contrast, sodium efflux occurs primarily through the $Na^+/K^+$-ATPase, which extrudes 3 $Na^+$ in exchange for the influx of 2 $K^+$ at the expense of ATP (20,47).

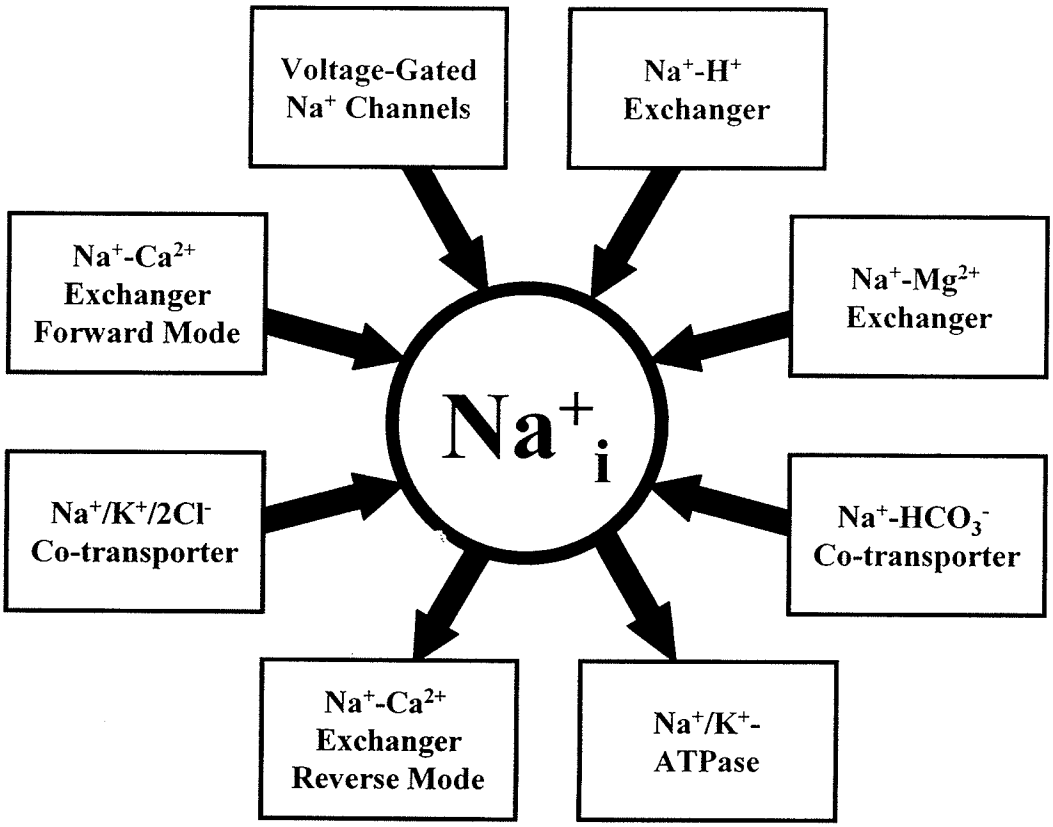



**Figure 3. Pathways of Influx and Efflux of $Na^+$.**

## 1.2 Roles of Intracellular Sodium and Calcium in Ischemia/Reperfusion Injury: Focus on the $Na^+$-$H^+$ Exchanger and the $Na^+$-$Ca^{2+}$ Exchanger

### *1.2.1 The Sodium-Hydrogen Exchanger and $Na^+$ Overload During IRI*

The cardiac $Na^+$-$H^+$ exchanger (NHE) is a transsarcolemmal protein that is important in the regulation of intracellular pH and cell volume (20,48,49). Of the nine isoforms that have been identified to date (50), NHE-1 is the primary subtype found in mammalian heart (48,51). In contrast to its relatively quiescent state under physiological conditions, NHE-1 becomes rapidly stimulated in the setting of intracellular acidosis (51). During myocardial ischemia, anaerobic metabolism rapidly produces intracellular acidosis, which activates the NHE through a pH sensor on the cytoplasmic surface of the exchanger (51), resulting in the extrusion of $H^+$ in exchange for $Na^+$ influx in a 1:1 ratio (34,49,52) (Figure 3). This elevation in $[Na^+]_i$ alters the reversal potential of the sarcolemmal NCX, decreasing calcium efflux (forward mode) and, depending on severity, may favour $Ca^{2+}$ influx (reverse mode) (12,34,35,53,54). Additional $Na^+$ may also enter the cardiac myocyte via NHE on reperfusion, as the outwardly directed $H^+$ gradient becomes augmented during the washout of extracellular $H^+$ (34,55). In addition, $[Na^+]_i$ accumulation is aggravated by $Na^+$ influx through $Na^+$ channels (56-58) and the sodium-bicarbonate co-transporter (59,60), as well as a reduction in $Na^+$ efflux through the $Na^+/K^+$-ATPase (20) (Figure 4). Intracellular $Na^+$ overload alters the reversal potential for NCX resulting in reduced $Ca^{2+}$ efflux and, depending on severity, may lead to $Ca^{2+}$ influx through the reverse mode of transport and $Ca^{2+}$ overload (12,20). Other than stimulation by ischemia-induced acidosis, NHE activity may also be increased by endothelin-1, angiotensin II, $\alpha_1$-adrenergic agonists,

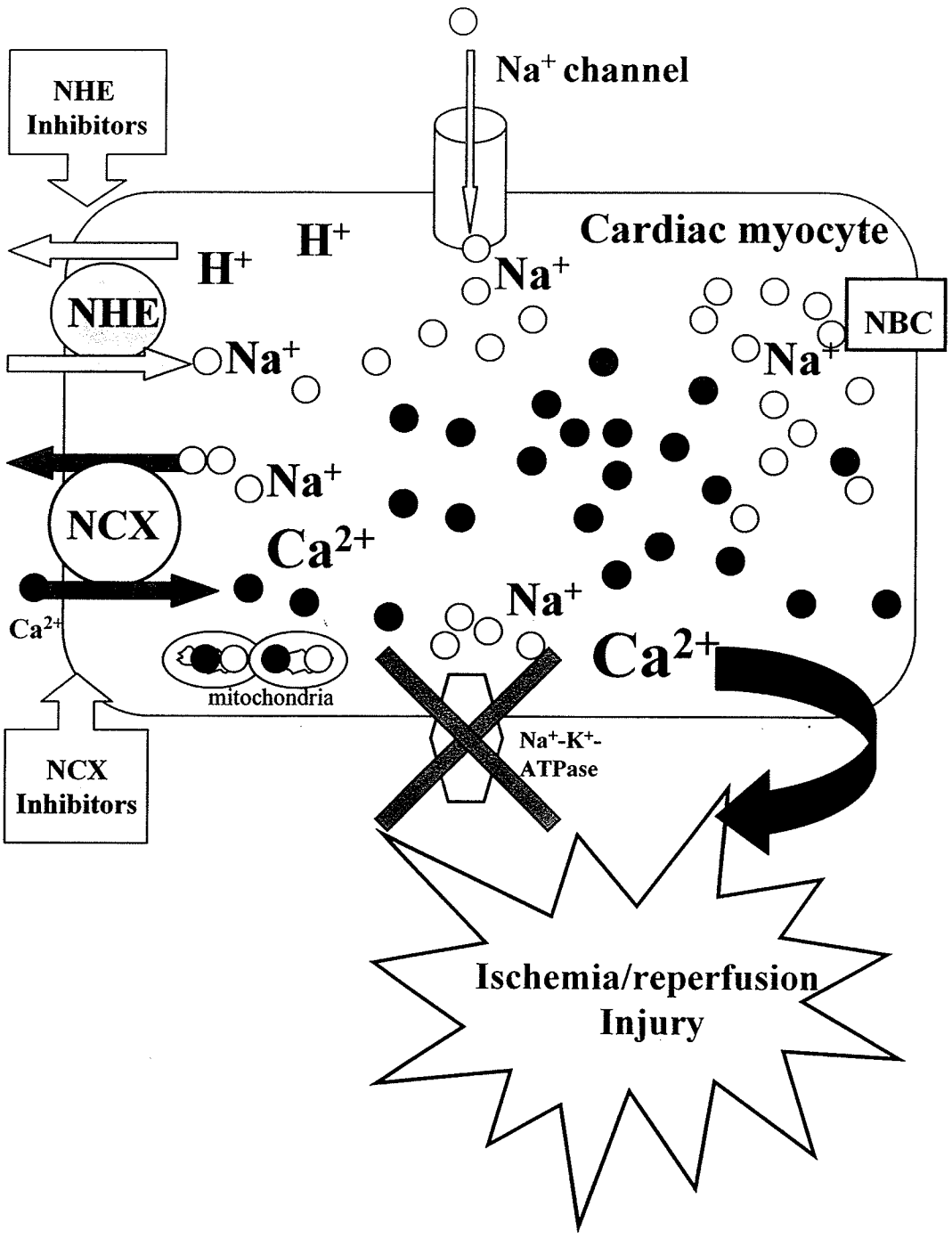



**Figure 4. Role of the Sodium-Hydrogen Exchanger (NHE) and the Sodium-Calcium Exchanger (NCX) During Myocardial Ischemia/Reperfusion.**

thrombin, and ischemic metabolites, through paracrine or autocrine mechanisms (51). Moreover, increased synthesis of NHE may occur in response to myocardial ischemia or ischemia-induced metabolites, such as hydrogen peroxide and lysophosphatidylcholine (61).

Intracellular $Na^+$ overload may also cause water influx into the cytosol (62-64), in addition to its deleterious effects with respect to $K^+$ loss (65-67) and energetics (35). During ischemia, products of anaerobic metabolism increase the osmotic load inside the myocyte and in the interstitial space (63,68). On reperfusion, the washout of osmotically active molecules leads to the generation of an osmotic gradient, favoring influx of water into the myocyte (63). In the presence of concomitant stresses, ischemic insults, and/or hypercontracture, the myocyte becomes more susceptible to osmotic challenge (62). Ultimately, myocardial edema may contribute to stunning, reperfusion arrhythmias, and ventricular remodeling (63). (Figure 5) Moreover, $[Na^+]_i$ overload may induce functional impairment of the mitochondrial membrane, affect ATP production, and result in contractile dysfunction during reperfusion (69). (Figure 5) Additional factors may influence $Na^+$ handling and susceptibility to IR injury, such as pre-existing cardiac pathologies (70), age- (71) and gender-related differences (72). For instance, increased $Na^+_i$ accumulation and ventricular dysfunction have been reported in hypertrophied rat hearts subjected to low-flow ischemia as compared with control (70). Impaired $Na^+$ handling with significant increase in $Na^+_i$, as compared with younger adult rats, has been documented in Langendorff-perfused isolated hearts of 'middle-aged' and senescent Fisher 344 rats subjected to global ischemia (71). Recently, a murine isolated perfused heart study has demonstrated significantly increased ischemia-induced $Na^+$

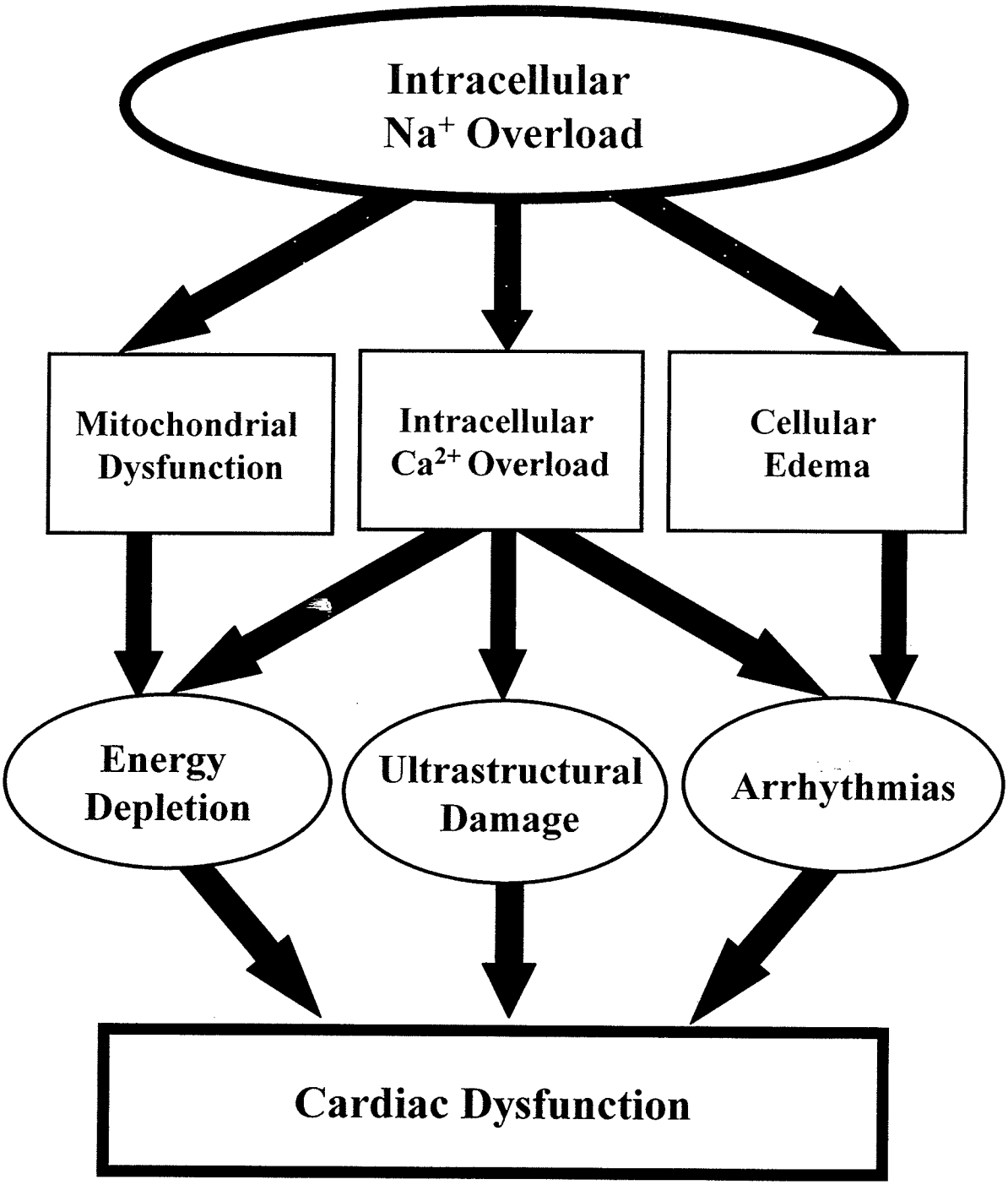



**Figure 5. Schematic Depiction of Consequence of Intracellular $Na^+$-Overload.**

accumulation by $^{23}$Na-NMR spectroscopy in hearts from male as compared those from female rats, and the mechanism of which was postulated to be mediated through a nitric-oxide dependent mechanism and a difference in $Na^+$ influx (72).

### *1.2.2 The Reverse Mode of NCX*

Calcium influx through the reverse-mode of NCX (Figure 3) is an important contributor to $Ca^{2+}$ overload and consequent hypoxia/reoxygenation or ischemia/reperfusion injury (53,73-77) (Figure 6). Consequences of $Ca^{2+}$ overload may include arrhythmogenesis (54,78), deranged energy production and utilization, disrupted membrane integrity, and ultrastructural changes (54,55,79). These deleterious effects are likely mediated through activation of various enzymes, such as proteases, phospholipases, as well as other factors including oxidative stress (54,80,81). In the context of NCX and IRI, reactive oxygen species have been shown to be necessary for rapid reactivation of NCX after inhibition by ischemia in a hypoxia/reoxygenation model utilizing guinea pig ventricular myocytes (82). In a recent study by Eigel *et al* (83), pre-treatment of cultured guinea pig ventricular myocytes with an antisense oligonucleotide (to suppress NCX protein expression) was found to inhibit apoptosis induced by hypoxia/reoxygenation, suggesting a potentially important role of NCX in initiating apoptosis in hypoxia/reoxygenation injury. Since, in addition to necrosis, apoptosis has been recognized as another mechanism of IR-induced cell death (84-86), this finding may have therapeutic implications. Moreover, the reverse mode of NCX is involved in the propagation of IR-induced injury (87): once hypercontracture develops, it may propagate via the passage of $Na^+$ through gap junctions from myocyte to myocyte, with subsequent $[Na^+]_i$-mediated $Ca^{2+}$ influx through the reverse mode of NCX (79,87,88).

Ultimately, myocardial IRI may manifest as stunning, microvascular dysfunction, reperfusion arrhythmias, and myocardial cell death or necrosis (89-91).

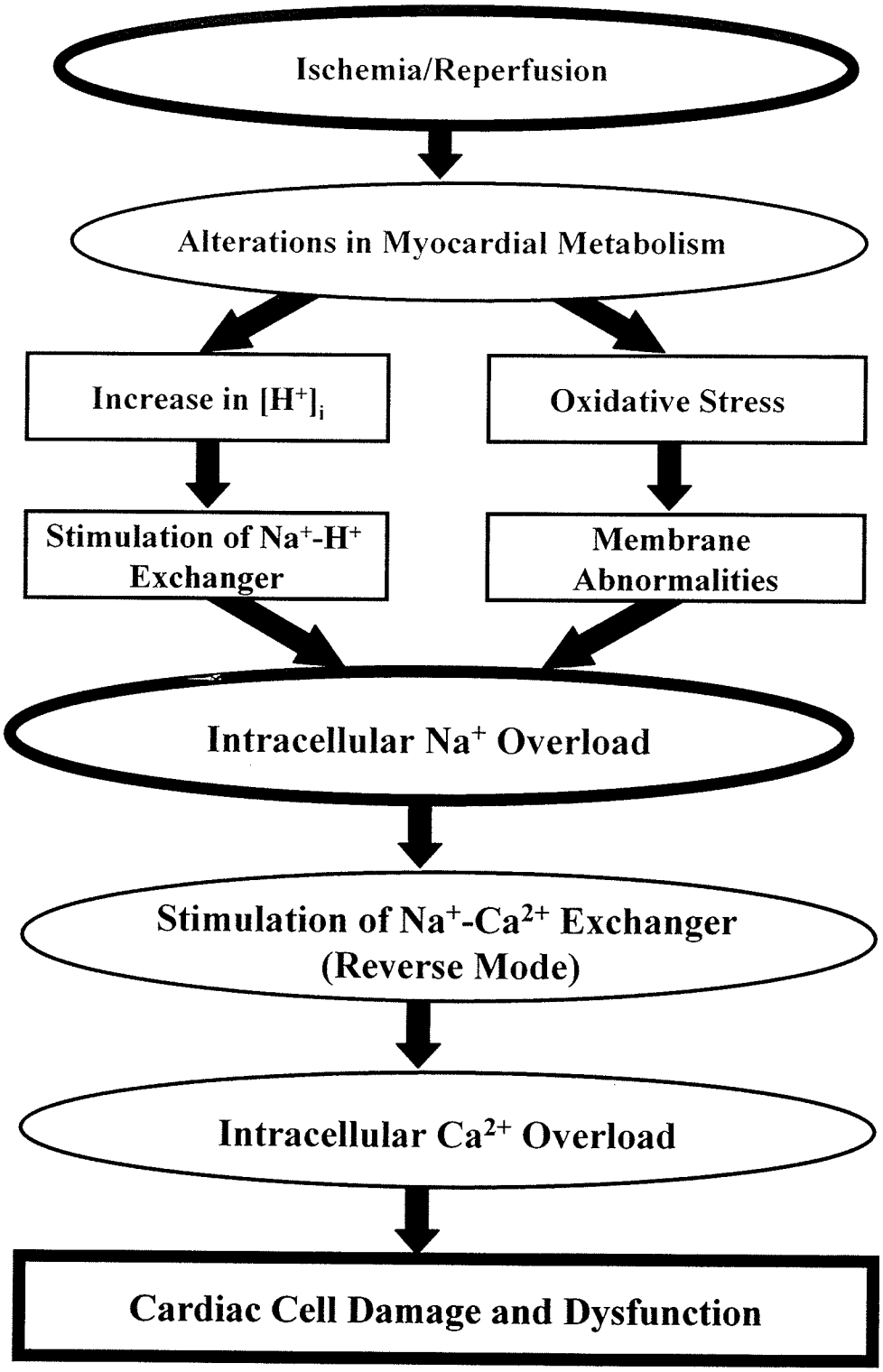



**Figure 6. Proposed Sequence of Events Occurring due to Ischemia/ Reperfusion.**

Other mechanisms include changes in myocardial metabolism, microvascular and endothelial cell dysfunction, disruption of the sarcolemma, generation of oxygen free radicals, and activation of platelets, neutrophils, and complement (54,90,91).

The pathophysiological role of the reverse mode of NCX in hypoxia/reoxygenation or ischemia/reperfusion injury was suggested in earlier studies which employed ionic manipulations (92) or non-selective compounds with inhibitory effects on NCX (53,73,93). Subsequently, the generation of transgenic mice and heterozygous knock-out mice has substantially facilitated testing of these hypotheses. Specifically, overexpression of NCX has been shown to increase sensitivity to IRI in male, but not female, transgenic mice (94); whereas heterozygous knock-out mice exhibited attenuation of contractile dysfunction and reduction in infarct size after ischemia and reperfusion, as compared with wild-type mice (95). Notably, other studies that utilized molecular biological techniques, such as, antisense oligonucleotides (to inhibit NCX expression and function) (75), or pharmacological approaches with novel selective inhibitors of the reverse mode of NCX (96-98) also supported the notion that the reverse mode of NCX is an important mechanism for $Ca^{2+}$ influx during IRI (99). The consistency of the conclusions from these studies, despite their distinct methodologies, has further strengthened this notion.

### *1.2.3 Pharmacological Inhibition of NHE as a Therapeutic Strategy*

Inhibition of NHE has been extensively investigated as a therapeutic strategy for attenuating IR injury (100-104). The first experimental evidence supporting NHE inhibition as a cardioprotective strategy was reported by Karmazyn in 1988 (52), when amiloride, an NHE inhibitor available at that time with relatively non-specific actions (101), was tested in isolated rat hearts subjected to low-flow ischemia followed by reperfusion (52). Enhanced post-ischemic contractile recovery was demonstrated, with the maximal benefit derived when amiloride was given during both ischemia and reperfusion (52). Subsequent studies which evaluated amiloride and its derivatives have confirmed the cardioprotective effects of NHE inhibition in a number of experimental models of IRI (73,105-108). Notably, reduction in $[Na^+]_i$ accumulation was shown to be an important mechanism for the observed cardioprotection (105,109,110), in addition to attenuation of $Ca^{2+}$ overload (111,112).

Subsequently, benzoylguanidines, which are more potent and selective inhibitors of NHE-1, were synthesized (104,113). Beneficial effects have been demonstrated in multiple animal models using a variety of endpoints, including reduction in ischemic rigor contracture (114,115), attenuation in ventricular dysfunction or improvement in functional recovery (52,56,110,116-121), decrease in cardiac enzyme release (56,122-125), reduction in infarct size (114,123,126-131), decrease in ventricular arrhythmias (51,110,114,118,132-134), attenuation of endothelial dysfunction (128,135), reduction in mitochondrial damage (121) and apoptosis (136), as well as preservation or improvement of cellular energetics (116,118,137,138). Attenuation of platelet swelling (122,139) and neutrophil activity (51,123) have also been reported, which may be of

particular relevance in IR, where platelets (140) and neutrophils (141) play major roles in the pathophysiology of IRI. Cariporide has been tested in senescent rats (Langendorff-perfused isolated heart model) where attenuation in coronary and myocardial dysfunction and reduction of cardiac enzyme release were observed (142). In addition, beneficial effects of NHE inhibition in cardiac allograft preservation have been demonstrated in a porcine model of cardiac transplantation (143,144), as well as in an isolated working heart model in rats (145). Furthermore, oral administration of cariporide has also shown great promise in attenuating the acute response to IR in a regional ischemia model of anesthetized rats (146), and in reducing infarct size in rabbits subjected to coronary ligation, with or without further aggravation with an atherogenic diet (131). In these cariporide-treated rabbits, attenuation of both lipoprotein abnormalities and endothelial dysfunction were observed and the mechanism has yet to be defined (131). In addition, alterations in NHE activity may occur in the presence of underlying disease, such as diabetes (147,148). Specifically, reduced NHE activity has been reported in chemically-induced models of diabetes (147,149), which may be an endogenous protective mechanism against IRI (148,150). Direct measurement of $[Na^+]_i$ by $^{23}Na$-magnetic resonance spectroscopy in isolated hearts from streptozotocin-induced diabetic rats has shown less $[Na^+]_i$ accumulation (by 48.1%) in diabetic hearts during zero-flow ischemia as compared with non-diabetic control, and this observation was attributed to reduced activities of NHE (150). In another study utilizing isolated perfused hearts of genetically diabetic rats, NHE inhibition with ethylisopropylamiloride has been shown to attenuate elevations in $[Na^+]_i$ and $[Ca^{2+}]_i$, limit acidosis, and preserve ATP during ischemia, even in the face of impaired NHE

activity (151). More recently, in a rat model of hypertrophied myocardium (induced by aortic banding) subjected to simulated conditions of cardiac surgery with cardioplegic arrest, the salutary effects of cariporide have also been demonstrated, including reduction of ischemic contracture, attenuation of post-ischemic diastolic dysfunction, and reduction in cardiac enzyme release (152).

Even though the cardioprotective effects of NHE inhibitors were demonstrated whether NHE inhibition was initiated before or after the onset of ischemia (at the time of reperfusion) in experimental studies, more consistent benefits were observed with the former approach (153-156). This difference in efficacy may be explained by the previous observation that NHE contributed substantially to $Na^+$ influx immediately on reperfusion (157), hence the availability of NHE inhibitors at adequate concentrations in the vicinity of the cardiac myocytes at the moment of reflow would be a critical issue. Understandably, the degree of difficulty in achieving this immediate access would be expected to increase as the model, subject, or environment of study increases in complexity, in the ascending order of isolated cardiac myocytes, isolated heart, whole animal, cardiac patients undergoing reperfusion by elective surgery or percutaneous coronary intervention, and unstable cardiac patients undergoing emergency reperfusion.

Despite the overwhelming evidence in support of a cardioprotective role of NHE inhibition (52,56,73,105,108,116,122,127,131,132,142,143,158-163) in experimental settings and some initial promising results in humans (164), subsequent large clinical trials have demonstrated less encouraging findings. Specifically, in the study by Rupprecht *et al* (164), 100 patients with anterior myocardial infarction undergoing

percutaneous transluminal coronary angioplasty (PTCA) were randomized to cariporide *vs* placebo (administered prior to reperfusion). The cariporide-treated group was found to have a reduction cardiac enzyme release and attenuation in ventricular dysfunction at 3 weeks post-PTCA (Table 1). In contrast, in the GUARDIAN (GUARd During Ischemia Against Necrosis) trial (7), which evaluated the efficacy and safety of cariporide in over 11,000 patients with acute IR in various clinical settings of risk, cardioprotection was mainly observed in the subgroup of patients undergoing high-risk coronary artery bypass graft (CABG) surgery (7,165). The discrepancy observed here mirrors those of the aforementioned experimental studies in that clinical situations which enabled the delivery of the NHE inhibitor to the myocardium prior to reperfusion resulted in improved outcomes. More recently, the efficacy and safety of NHE inhibition in the prevention of myocardial infarction or death in patients undergoing CABG were further evaluated in the EXPEDITION (sodium-hydrogen EXchange inhibition to Prevent coronary Events in acute cardiac conDITIONs) trial, with the primary composite end-point of death or myocardial infarction (MI) at day 5 (6,166). Five thousand seven hundred and seventy CABG patients with risk factors for perioperative myocardial ischemia were randomized to either intravenous cariporide or placebo (6,166) (Table 1). Preliminary results showed a significant reduction in death or MI at day 5, but the effects in the 2 components of the composite end-point were 'heterogeneous', with an increased mortality rate observed at day 5 in the cariporide group as compared with the placebo group (6). In addition, a significant increase in the overall rate of cerebrovascular events was found in the cariporide group as compared with the placebo group (6). Pending the final report of the study, further investigations

| | Rupprecht *et al*[164] | GUARDIAN[7] | ESCAMI[5] | EXPEDITION[6] |
|---|---|---|---|---|
| **No. of subjects** | 100 | 11,590 | 1,389 | 5,770 |
| **Clinical settings** | Direct PTCA for anterior STEMI | Unstable angina, non-STEMI, high-risk CABG & PCI | Thrombolytic therapy/primary PTCA for STEMI | CABG + risk factors for peri-operative ischemia |
| **NHE inhibitor *vs* placebo** | Cariporide (40 mg i.v. bolus before reperfusion) | Cariporide (20, 80, 120 mg/1h i.v. q8h) after admission or before CABG/PCI | Eniporide (10-min i.v. infusion before reperfusion) | Cariporide (180 mg/1h pre-operative loading, followed by 40 mg/h over 24 h and then 20 mg/h over 24 h) |
| **Main findings** | •Improved LV function<br>•Reduced enzyme release (CK, CK-MB, LDH) | •1° endpoint (death/MI assessed after 36 days) N.S.<br>•25% reduction in death or MI in CABG subgroup (120-mg dose)<br>•'no increases in clinically serious adverse events' | •1° efficacy endpoint (infarct size estimated by α-HBDH) N.S.<br>•Overall, no effect on clinical outcome | •↓ death/MI at day 5, maintained until month 6<br>•'heterogeneous' effects in end-point components (see text)<br>•significant ↑ in overall rate of cerebrovascular events in cariporide group vs. placebo group |

***α-HBDH*** *alpha-hydroxybutyrate dehydrogenase;* ***1°*** *primary;* ***CABG*** *coronary artery bypass graft surgery;* ***CK*** *creatine kinase;* ***CK-MB*** *creatine kinase isoenzyme;* ***ESCAMI*** *Evaluation of the Safety and Cardioprotective Effects of Eniporide in Acute Myocardial Infarction;* ***EXPEDITION*** *sodium-hydrogen EXchange inhibition to Prevent coronary Events in acute cardiac conDITIONs;* ***GUARDIAN*** *GUARd During Ischemia-Against Necrosis;* ***i.v.*** *intravenous;* ***LDH*** *lactate dehydrogenase;* ***N.S.*** *not significant;* ***PCI*** *percutaneous coronary intervention;* ***PTCA*** *percutaneous transluminal coronary angioplasty;* ***STEMI*** *ST-elevation myocardial infarction.*

**Table 1. Summary of Clinical Trials on Sodium-Hydrogen Exchange Inhibitors**

into the pathophysiological mechanisms for these unexpected findings would likely be important for future research in this area (167,168). It is speculated that the possibility of dose-related adverse effects (be it agent-specific or class-specific) will need to be considered, as the dosing regimen of the EXPEDITION trial (preoperative loading dose 180 mg over 1 hour, followed by 40 mg/h over the first 24 hours and then 20 mg/h over the second 24 hours) was higher than that of the GUARDIAN trial, as inferred from the estimations made by Weber *et al* (169) based on a population pharmacokinetic model of cariporide. Moreover, in review of the ESCAMI trial (5) (a randomized, double-blind, placebo-controlled trial phase 2 trial, Table 1) which evaluated eniporide, another NHE inhibitor, in patients with acute ST-elevation myocardial infarction who underwent thrombolytic therapy or primary angioplasty, a statistically non-significant excess of deaths at 6 weeks (3.7 % placebo, 6.9% 50-mg, 4.6 % 100-mg, 5.1% 150-mg, 8.8% 200-mg groups) and stroke (0.2 % placebo, 0% 50-mg, 0.7% 100-mg, 1.8% 150-mg, 2.2% 200-mg groups) was noted in the eniporide-treated patients. This trend toward an excess of deaths and stroke was felt to be due to chance (5,84) at that time in light of the absence of such findings in 2 other clinical studies (7,164), including the GUARDIAN trial. Taken together, clinical data to date appear to be in keeping with the cardioprotective effects of NHE inhibition demonstrated in the experimental settings, provided that the drug was given under the specific clinical circumstance that ensures its presence (and at an adequate concentration) on reperfusion – with the caveat of safety issues that remain to be addressed.

In a recent experimental study (170), post-ischemic administration of cariporide was shown to exert limited effects on $Na^+_i$ and $pH_i$ in rats, providing

mechanistic insight into the lack of therapeutic benefit observed in some studies which employed post-ischemic dosing regimens. In addition, in a model of anoxia/reoxygenation injury utilizing isolated rat cardiac myocytes, Schäfer *et al* (171) reported that simultaneous inhibition of NHE and NBC was necessary for protecting cardiac myocytes against reoxygenation-induced hypercontracture, although such combination strategy was not found to be effective in a global ischemia model in isolated rat hearts (60). Regardless of the therapeutic strategy employed, the importance of timely reperfusion cannot be overemphasized (154,172).

Emerging data on the use of NHE inhibitors in experimental settings of cardiac resuscitation appear promising, both in the isolated rat heart (173-175) and in closed-chest models of resuscitation in the rat (173,175) and the pig (176-178). Salutary effects include attenuation of ischemic contracture (173,174,178), spontaneous defibrillation (173,175,179) or reduction in ventricular arrhythmias, amelioration of ventricular dysfunction (175,177), and reduction in mortality (175). Cariporide has also been shown to augment the efficacy of chest compression in one report (180) and improve response to resuscitation in another study (177). Moreover, there is preliminary evidence of increased benefits of the combination of cariporide and epinephrine over either agent alone (178) for resuscitation of cardiac arrest induced by ventricular fibrillation (VF), with a decrease in ischemic contracture and favourable effects on coronary perfusion pressure. In another recent abstract, cariporide was found to decrease the adverse effects of epinephrine (181). In a swine model of resuscitation, cariporide was shown to attenuate ventricular dysfunction after induced VF and the

authors proposed that NHE inhibition may have potential utility in patients with severe LV dysfunction who require electrophysiological testing (182).

## 1.3 Therapeutic Potential of Novel $Na^+$-$Ca^{2+}$ Exchange Inhibitors in Attenuating Myocardial Ischemia/Reperfusion Injury

Given the role of NCX in IRI, selective inhibition of its reverse mode of transport represents an attractive therapeutic strategy for cardioprotection (11,78,183-185) (Figure 3). Novel NCX inhibitors have gained increasing attention in recent years as potential cardioprotective agents against IRI (11,185,186). Enthusiasm for this pharmacological target likely stems from a combination of factors, including the recent discovery of compounds that inhibit the reverse mode of NCX (11,183,187-191), the mixed results that have emerged from clinical trials evaluating the therapeutic potential of sodium-hydrogen exchange (NHE) inhibitors as novel cardioprotective agents (5-7,164,165,167), the lack of therapies against IRI that have been successfully translated from bench to bedside (172), and the established prominent roles of NCX in cardiac physiology and pathophysiology (8-10,192,193).

Historically, pharmacological inhibition of NCX had been problematic due to the dearth of reverse-mode-specific inhibitors (12), which would be ideal for targeting $Ca^{2+}$ influx through the NCX – an important mechanism for $Ca^{2+}$ overload in IRI (81). Even though amiloride analogs (*e.g.*, 3',4'-dichlorobenzamil), divalent and trivalent cations (*e.g.*, $Ni^{2+}$, $Cd^{2+}$, and $La^{3+}$), anti-arrhythmic agents (*e.g.*, bepridil), and organic molecules are some examples of inhibitors of the NCX, they also act on other transporters and ion channels at low doses and are thus non-specific for the NCX (8,12,13). In 1996, a compound, 2-[2-[4-(4-nitrobenzyloxy)-phenyl]ethyl]isothiourea methanesulphonate (KB-R7943), was reported to inhibit NCX with potency in the micromolar range of concentrations and selectivity for the reverse mode of NCX

(187,188). Subsequently, the efficacy of KB-R7943 in reducing cardiac IRI has been shown, including improvement in post-ischemic contractile dysfunction (76,194-196); reduction in propagation of myocyte hypercontracture (87); decrease in cardiac enzyme release (74,77,196,197) or infarct size (98,198); reduction in reperfusion arrhythmias in most studies (194,196,199-201), with exceptions (202,203); and preservation of energy (204). It is noteworthy that KB-R7943 has also been shown to be cardioprotective when given on reperfusion (*i.e.*, after the onset of ischemia) (194,198). This may have implications in potential clinical applications, as the onset of ischemic symptoms typically precedes treatment in most cases, whereas initiation of therapy prior to ischemia may only be applicable in a limited number of clinical settings, such as prior to elective cardiac surgery. Moreover, cardioprotective effects of KB-R7943 in senescent rat myocardium (administered as a 10-minute infusion on reperfusion in the isolated heart) have been reported (196), as well as in models of experimental resuscitation, based on 2 abstracts (205,206). For instance, in a rat model of ventricular fibrillation (VF) and closed-chest resuscitation where attenuation of post-resuscitation myocardial dysfunction was demonstrated (206).

Despite its cardiac (38,74,76,77,87,96,98,194-196,198,199,201,204,206-208), neural (209-212), and renal (213-215) protective effects, KB-R7943 has shown several limitations, including its inhibitory effects on certain ion channels and receptors (189,207,216-220), increases in cellular enzyme release when higher doses were administered (77,221), and negative hemodynamic effects (98,198,199,222,223). Moreover, the lack of benefit of KB-R7943 has also been documented in several studies (58,202,203,224), including preliminary evidence in diabetic rats (203,224). The

mechanism of this discrepancy remains elusive. Finally, while most studies suggested that KB-R7943 was selective for the reverse mode of NCX (187,188,199,225), this mode selectivity was not observed under bi-directional ionic conditions (226).

In 2001, a more potent and selective NCX inhibitor, 2-[4-[(2,5-difluorophenyl)-methoxy]phenoxy]-5-ethoxyaniline (SEA0400), was reported to exhibit protective properties in both *in vitro* and *in vivo* models of cerebral ischemia with potency in the nanomolar range of concentrations (189). Its inhibitory potency was 10 to 100 times greater than that of KB-R7943 (97,183,189,219). In NCX1 transfectants [Chinese hamster lung fibroblasts, CCL-39 (191)] where the rates of $[Na^+]_i$ dependent $^{45}Ca^{2+}$ uptake were measured, the $IC_{50}$ values of SEA0400, SN-6 [2-[4-(4-nitrobenzyloxy)-benzyl]thiazolidine-4-carboxylic acid ethyl ester, another novel NCX inhibitor described in 2002 (227,228)], KBR-7943, $Ni^{2+}$ were 0.06, 3.2, 4.9, and 42 μM, respectively (207,228,229). In addition, SEA0400 has been shown to preferentially inhibit the reverse mode of NCX as compared with the forward mode (228,230,231); and had negligible effects on other membrane transporters, ion channels, and receptors in both neuronal (189) and cardiac (219) preparations. Subsequent experimental studies have documented cardioprotective effects of SEA0400, including reduction in infarct size in the isolated Langendorff-perfused rabbit heart subjected to coronary ligation and reperfusion (98); attenuation of IR-induced ventricular dysfunction in the isolated rabbit and rat hearts (97,98); a decrease in the incidence of VF and mortality in an IR-arrhythmia model of anesthetized rats subjected to coronary ligation (97); attenuation of LV dilation and dysfunction at 1 week post-MI (232); attenuation of stunning in anesthetized dogs (233); and enhanced recovery of contractile function after zero-flow

ischemia in a guinea-pig model of coronary-perfused right ventricular tissue preparation, as reported in a recent abstract (234). The cardioprotective effects of SEA0400 will be discussed further in the next section.

## 1.4 SEA0400

Among the newer NCX inhibitors described to date, 2-[4-[(2,5-difluorophenyl)-methoxy]phenoxy]-5-ethoxyaniline (SEA0400), a benzyloxyphenyl derivative (13), appears particularly promising as a potent and selective inhibitor of the NCX. This compound was developed by Taisho Pharmaceutical Co., Ltd. (Saitama, Japan) and was first described in 2001 (189). In this section, experimental data on SEA0400 in the published literature to date will be reviewed and its therapeutic potential as a cardioprotective agent will be discussed [a part of chapter 1 of the thesis, with a focus on SEA0400, has been published (235)].

### *1.4.1 Chemistry*

SEA0400, as first described by Matsuda *et al* (189), was identified by screening a compound library for inhibition of $Na^+$-dependent $Ca^{2+}$ uptake in cultured astrocytes and isolated cardiac sarcolemmal vesicles. Its chemical structure is shown in Figure 7 (189). It has a low solubility in water (189). As described in several studies (97,98,189,231) that evaluated this compound *in vitro*, SEA0400 was dissolved in dimethyl sulfoxide (DMSO) with a final vehicle concentration of $\leq$ 0.1 % before application in experiments. For *in vivo* studies, SEA0400 has been administered intravenously as a lipid emulsion with the vehicle serving as control (97,189,232,233,236-239), or orally (238).

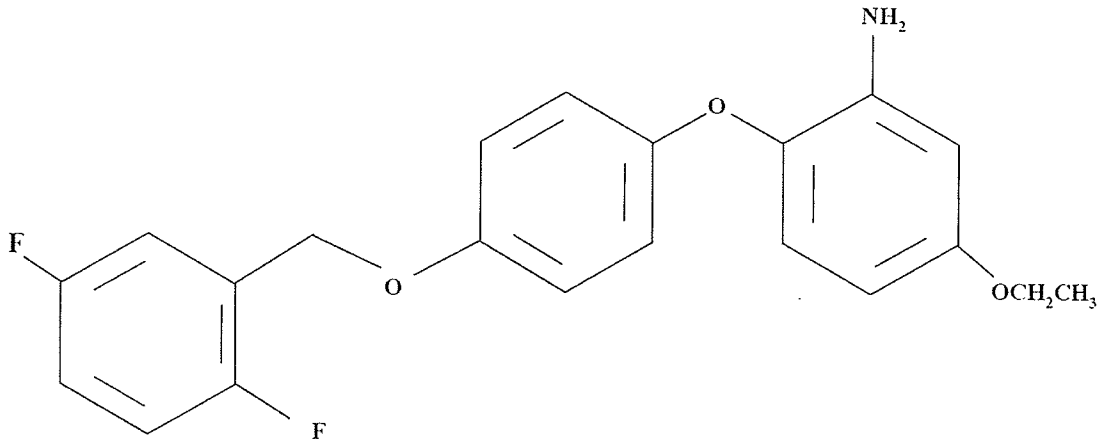



**Figure 7. Chemical Structure of SEA0400.**
(Redrawn based on reference #189.)

### *1.4.2 Potency and Mode Selectivity*

SEA0400 inhibited $Na^+$-dependent $Ca^{2+}$ uptake in neurons, astrocytes, and microglia with $IC_{50}$'s of 33 nM, 5.0 nM, and 8.3 nM, respectively (189). Notably, this inhibition of $Na^+$-$Ca^{2+}$ exchange was up to 100 times more potent than KB-R7943 [$IC_{50}$'s = 3.8 μM, 2.0 μM, 3.1 μM, respectively] (189), an agent first described in 1996 as a potent and selective $Na^+$-$Ca^{2+}$ exchange inhibitor (187,188). In canine sarcolemmal vesicles and in cultured rat myocytes, SEA0400 inhibited NCX activity with $IC_{50}$ values of 90 and 92 nM, respectively (189). In isolated guinea-pig ventricular myocytes examined by the whole cell voltage clamp technique, Tanaka *et al* reported that SEA0400 concentration-dependently inhibited NCX current with a 10-fold higher potency as compared with KB-R7943 (> 80% inhibition of NCX current was achieved with 1 μM SEA0400 and 10 μM KB-R7943), and that the $EC_{50}$ value of SEA0400 for the inward and outward NCX current was 40 nM and 32 nM, respectively (219). In addition, Takahashi *et al* (97) studied the effects of SEA0400 in canine sarcolemmal vesicles and rat cardiac myocytes, and found that SEA0400 inhibited $Na^+$-dependent $Ca^{2+}$ uptake with $IC_{50}$ values of 90 and 92 nM, respectively. In those experiments, SEA0400 was estimated to be 80 and 100 times more potent than KB-R7943, which exhibited $IC_{50}$'s of 7 μM in canine cardiac sarcolemmal vesicles and 9.5 μM in rat cardiac myocytes, respectively (97).

Similar inhibitory potency was also observed for the cloned cardiac $Na^+$-$Ca^{2+}$ exchanger, NCX1.1, expressed in *Xenopus laevis* oocytes, where activity was evaluated by the giant excised patch technique: SEA0400 inhibited outward NCX currents with $IC_{50}$ values of 78 nM and 23 nM for peak and steady state currents, respectively (231).

In contrast, considerably less inhibitory potency (in the micromolar range) was observed for inward currents (231). This preferential inhibition of SEA0400 for outward currents was also observed by other investigators (228,240). In CCL39 fibroblasts transfected with NCX1, SEA0400 at 0.01 – 1 μM inhibited the outward current in a concentration-dependent manner with $IC_{50}$ of 0.028 μM, whereas pretreatment with 0.3 – 10 μM did not significantly affect inward currents (240). In addition, Iwamoto *et al* (240) evaluated the effects of SEA0400 on inhibition of $Na^+_i$-dependent $^{45}Ca^{2+}$ uptake into CCL39 cells stably transfected with NCX isoforms (NCX1, NCX2, NCX3) or the $K^+$-dependent $Na^+$-$Ca^{2+}$ exchanger (NCKX2). It was shown that SEA0400 concentration-dependently inhibited the initial rate of $^{45}Ca^{2+}$ uptake into NCX1 and NCX2 transfectants ($IC_{50}$'s = 0.056 and 0.98 μM, respectively) (240). In contrast, the $^{45}Ca^{2+}$ uptake into NCX3 and NCKX2 was not affected (240). These findings suggest that SEA0400 preferentially inhibits NCX1 (240).

In a recent study of the effects of interferon-γ on NCX activity in cultured rat microglia, utilizing ratio-imaging of fura-2 fluorescence for measurement of $[Ca^{2+}]_i$ (241), SEA0400 at 10 nM inhibited the $Na^+$-dependent increase in $[Ca^{2+}]_i$ in cultured microglia by 50 %, with potency similar to that previously reported (189).

### *1.4.3 Selectivity for NCX*

Several studies have investigated the selectivity of SEA0400 for the NCX. Matsuda *et al* (189) studied the effect of SEA0400 on store-operated $Ca^{2+}$ entry (SOCE) in cultured astrocytes. At concentrations $\leq$ 3 μM, SEA0400 did not inhibit SOCE, although inhibition was detected at 10 μM (189). The effects of SEA0400 on several ion transporters and ion channels were also evaluated and, at concentrations $\leq$ 3 μM, SEA0400 was found to have negligible affinities for $Na^+$ channels, $Ca^{2+}$ channels, $K^+$ channels (including the $K_{ATP}$ channel), norepinephrine transporter, and 14 receptors (including adenosine receptors, adrenergic receptors, glutamate receptors, muscarinic acetylcholine receptors, bradykinin receptors, leukotriene $B_4$ receptors, platelet-activating factor receptors); and did not affect the activities of NHE, $Na^+/K^+$-ATPase, $Ca^{2+}$-ATPase and 5 enzymes, including phospholipase $A_2$, phospholipase C, 5-lipoxygenase, inducible nitric-oxide synthetase, and constitutive nitric-oxide synthetase (189). At higher concentrations, SEA0400 inhibited the dihydropyridine site and the diltiazem site of the L-type $Ca^{2+}$ channel, as well as site 2 of the sodium channel, in rat cerebral cortex, with estimated $IC_{50}$ values of 10.8 μM, 20.1 μM, and 13.8 μM, respectively (189). At 30 μM, SEA0400 inhibited [$^3$H]leukotriene $B_4$ and [$^3$H]nisoxetine binding (189).

SEA0400 at 1 μM was reported to have no effect on $Na^+$ current, L-type $Ca^{2+}$ current, inwardly rectifying $K^+$ current, and delayed rectifier $K^+$ current in isolated guinea-pig ventricular myocytes (219). However, at 10 μM, SEA0400 partially inhibited the L-type $Ca^{2+}$ current (219). In isolated canine ventricular myocytes, SEA0400 (1 μM) did not exert any significant effects on the amplitude of L-type $Ca^{2+}$

current, as assessed by the whole-cell voltage clamp technique, or the shape or magnitude of the $Ca^{2+}$ transients, as measured by fura-2-based ratiometric fluorescence (242).

### *1.4.4 Other Electrophysiological Effects*

In canine ventricular papillary muscles and Purkinje fibres, SEA0400 (1 μM) led to a significant reduction in the amplitude of both early and delayed afterdepolarizations (242).

In guinea-pig ventricular myocytes, SEA0400 (1 – 100 μM) has been shown not to induce any significant change in the action potential configurations (237). In guinea-pig RV tissue preparations, SEA0400 did not exert any significant effects on the action potential under normal conditions or during ischemia, when shortening of the action potential duration occurred (234). However, on reperfusion, it facilitated the recovery of the action potential duration (234). In an intact guinea-pig model of aconitine-induced arrhythmias, SEA0400 (10 mg/kg) was shown to cause no significant change in the durations of ventricular arrhythmias (243). In a preliminary report (244) utilizing acutely dissociated rat neocortical neurons, SEA0400 (10 μM) was demonstrated to have no effects on glutamate receptor/channel-mediated currents as assessed by nystatin-perforated patch recording configuration. In another preliminary

report (245), SEA0400 was noted to have no effects on the beating rate of guinea-pig right atria and action potential configuration of rabbit sinoatrial node.

In the *in vivo* canine study by Nagasawa *et al*, SEA0400 1.0 mg/kg i.v. did not significantly affect PR interval, QT interval, QTc interval (QT interval corrected for heart rate), or RR interval, but a slight prolongation was detected in the QRS duration at 9 minutes after SEA0400 was given: 59 ms in the SEA-treated group *vs* 57 ms in the control group ($p < 0.05$) (239).

Preliminary results in coronary-perfused guinea-pig RV tissue preparation documented that SEA0400 did not exert any significant effects on the action potential under normal conditions (234). In another abstract (245), SEA0400 was reported to have no significant effects on action potential parameters in the guinea pig but shortened the late plateau in the rat and mouse ventricle. Finally, in studies employing heart tubes from NCX knock-out mice, SEA0400 was observed to depress $Ca^{2+}$ transients, indicating actions beyond that of NCX inhibition (220). In our laboratory, SEA0400 was without contractile effects in electrically-stimulated rabbit papillary muscles (unpublished observation).

### *1.4.5 Cardioprotective Effects Against Ischemia/Reperfusion Injury*

In a model of $Ca^{2+}$ paradox injury utilizing cultured rat myocytes reported by Takahashi *et al* (97), SEA0400 at 1 μM and 3 μM attenuated $Ca^{2+}$ paradox induced cell death. Specifically, the myocyte survival rate was significantly increased from 32.1 % (control) up to 57.9% (1 μM) and 66.1 % (3 μM). In that model, SEA0400 was more than 10 times as potent as KB-R7943, which also prevented cell death at 10 μM (97).

Tanaka *et al* (234) studied the effects of SEA0400 in tissue preparations of coronary-perfused guinea-pig right ventricle under conditions of zero-flow ischemia followed by reperfusion. Preliminary results showed that SEA0400 at 1 μM had no effect on the diminution in contractile force during ischemia but 'abolished' the increase in resting tension (234). During reperfusion, significant improvement in recovery from contractile dysfunction was noted (234).

In Langendorff-perfused isolated rat hearts, Takahashi *et al* (97) evaluated SEA0400 under constant pressure (65 mmHg) conditions, using a protocol of low-pressure perfusion (7 cm $H_2O$ for 60 minutes) followed by normal perfusion for 60 minutes. One of three drugs (SEA0400, KB-R7943, or cariporide) was added to the perfusion buffer immediately after return to normal perfusion for 10 minutes (97). It was shown that SEA0400 (0.3 μM and 1 μM) significantly improved recovery of left ventricular developed pressure from 32.1 % (control) to 66.7% and 66.8%, respectively, after reperfusion (97). In an IR arrhythmia model of anesthetized rats (97), SEA0400 at 1 mg/kg, administered at 1 minute before reperfusion (by releasing suture for 10 minutes after a 5-minute coronary occlusion), significantly reduced the incidence of VF from 80% (control) to 30%, and mortality rate from 70% (control) to 20%. In addition,

SEA0400 at 0.3 mg/kg also decreased the mortality rate to 20% (97). There was no effect on the incidence of ventricular tachycardia at any dose examined (97). This latter finding was confirmed in a recent study by Nasagawa *et al* (239), who demonstrated in an *in vivo* canine model of IR-induced ventricular arrhythmias that SEA0400 had no significant effects on these arrhythmias, although the basis for the difference in the efficacy of SEA0400 toward VF in these 2 studies is unclear. The investigators suggested that inter-species difference might be a potential explanation, although the precise mechanism remains to be determined (239).

SEA0400 was evaluated in the rabbit by Magee *et al* (98) using the Langendorff-perfused isolated heart technique under constant pressure conditions (80 mmHg). The isolated hearts were subjected to 30 minutes of regional ischemia (by coronary artery ligation) and 120 minutes of reperfusion. SEA0400 was shown to reduce infarct size in a concentration-dependent manner ($EC_{50}$ 5.7 nM) when it was administered 30 minutes before regional ischemia, with the maximum reduction in infarct size being 75% (at 0.1 μM SEA0400). Importantly, similar reduction in infarct size was observed, whether perfusion with SEA0400 (1 μM) was started at 30 minutes before regional ischemia or at 1 minute before reperfusion, and was continued until completion of 120 minutes of reperfusion (98).

Yoshiyama *et al* (232) evaluated the cardioprotective effects of SEA0400 on myocardial IRI in rats, using transthoracic echocardiography for assessment of changes in cardiac structure and function at 1 week following myocardial infarction induced by coronary ligation. It was found that SEA0400-treated rats (1 mg/kg i.v. administered at 5 minutes before coronary ligation) had significantly less increase in end-diastolic

dimension and volume, and significantly less reduction in ejection fraction as assessed by echocardiography, in comparison with the vehicle-treated group (232). Moreover, statistically significant reduction in infarct size and attenuation of mRNA expression of plasminogen activator inhibitor-1 (PAI-1) were observed in non-infarcted areas of the LV in the SEA0400-treated group, as compared with the vehicle-treated group (232).

SEA0400 has been shown to protect the canine heart against myocardial stunning (233). In anesthetized dogs, stunning was induced by occlusion of the left anterior descending coronary artery for 15 minutes followed by 4 hours of reperfusion. SEA0400 at 0.3 or 1 mg/kg i.v., administered as a bolus at 1 minute before reperfusion, significantly improved the recovery of myocardial segment shortening from 36% (in vehicle-treated group) to 65 % and 72%, respectively (233).

### *1.4.6 Other Models of Cardiovascular Pathology*

The role of the reverse mode of vascular NCX in salt-sensitive hypertension has been recently investigated (238,246). In a rat model of salt-sensitive hypertension, SEA0400 was shown to increase arterial blood flow, suggesting a vasodilatory effects (238). SEA0400 decreased arterial blood pressure in salt-sensitive rat models, but not in normotensive or spontaneously hypertensive rats (238). A single oral dose of SEA0400 (1-10 mg/kg) reduced arterial pressure in deoxycorticosterone acetate (DOCA)-salt hypertensive rats in a dose-dependently manner (238). Prolonged administration of

SEA0400 (3 or 10 mg/kg for 3 weeks) prevented the onset of hypertension, vascular hypertrophy and renal dysfunction (238). In Sprague-Dawley rats treated with long-term ouabain, SEA0400 (1 or 10 mg/kg) was noted to suppress blood pressure in a dose-dependent manner (238). In addition, it was observed that SEA0400 significantly lowered salt-sensitive hypertension in vascular NCX1.3-transgenic mice but not in mice that overexpressed an SEA0400-insensitive mutant, consistent with the notion that the reverse mode of vascular NCX1 is involved in the development of salt-sensitive hypertension (247).

In a preliminary report (248), SEA0400 (as well as KB-R7943) was shown to concentration-dependently inhibit catecholamine secretion from bovine adrenal chromaffin cells, through attenuation of acetylcholine-mediated elevation in $[Ca^{2+}]_i$. The potential significance of this finding awaits further delineation.

In anesthetized dogs with digitalis-induced ventricular tachycardia, SEA0400 3 mg/kg i.v. bolus was reported to suppress tachyarrhythmias 'immediately' after SEA0400 was administered and to reduce sinus rate; however, atrioventricular block and asystole were observed in 2 dogs (239).

Recently, NHE has been shown to be active under basal conditions in intracerebral arterioles in rats and to regulate cerebral arteriolar tone through $Na^+/K^+$-ATPase and NCX (249). Moreover, arteriolar constriction induced by NHE inhibitors was completely abolished in the presence of SEA0400 and ouabain in that study (249). These findings may have implications in the future therapeutic applications of NHE and NCX inhibitors.

### *1.4.7 Mechanisms of Action*

The mechanisms of action of SEA0400, or of novel NCX inhibitors in general, have been an intense area of investigation, as the availability of selective NCX inhibitors in recent years has afforded opportunities not only in studying the physiological and pathophysiological roles of NCX, but also in evaluating the prospect of utilizing NCX as a therapeutic target. With regard to the latter goal, mechanistic insights are important for any attempt in optimizing the benefit to risk ratio of such therapy and, probably, toward future discovery and development of NCX inhibitors with improved potency, selectivity, and safety profile. As reviewed in section 1.4.2, previous studies on the mode-selectivity of SEA0400 has demonstrated mixed findings. Such differences may be reconciled by the observation that the apparent mode selectivity of SEA0400 is likely explained by its ability to stabilize the $Na^+_i$-dependent inactive state of NCX (230,231), with its greatest effectiveness under conditions of increased $Na^+_i$, as in the setting of ischemia and reperfusion (230,231). Findings from mutagenesis studies are in support for this hypothesis (230,240) and suggest that Phe-213, Gly-833, and residues that remove $Na^+_i$-dependent inactivation are important for the inhibitory effects of SEA0400 (240). Putative interaction domains for benzyloxyphenyl derivatives have been recently proposed and a potential mechanism of ion transport inhibition through blockade of 'pores formed within the membrane regions' was postulated (13). Moreover, in CCL39 fibroblasts transfected with NCX1, Iwamoto *et al* (240) evaluated the inhibitory properties of SEA0400 and reported that SEA0400 significantly increased the half-maximal $Ca^{2+}$ concentration ($K_{m(Ca)}$) value from 0.22 mM (control) to 0.39 mM, and that it significantly decreased the corresponding maximal velocity ($V_{max}$) from 24

nmol/mg/30 s (control) to 11 nmol/mg/30 s. These results suggest the possibility of mixed (competitive and noncompetitive) inhibition (240).

Recently, Beaugé and Dipolo (250) evaluated SEA0400 in internally dialyzed squid giant axons and confirmed the 'enhancement' of $Na^+_i$-dependent inactivation. In addition, these investigators proposed that the mechanism of action of SEA0400 involves $Na^+_i$-$H^+_i$ synergism, which is influenced by ATP, and that the site of action of SEA0400 is likely intracellular (250).

In the salt-sensitive hypertensive state, elevated cardiotonic steroids inhibit $Na^+/K^+$-ATPase in smooth muscle cells, resulting in increased $[Na^+]_i$ (251), which reduces $Ca^{2+}$ efflux through the forward mode of NCX and favours $Ca^{2+}$ influx through the reverse mode. This results in an increase in $[Ca^{2+}]_i$ and consequent vasoconstriction and elevation of systemic blood pressure (238,251).

### *1.4.8 Preliminary Pharmacokinetics and Metabolism*

In the initial report on SEA0400 in attenuating reperfusion injury in cerebral ischemic models (189), Matsuda *et al* reported that SEA0400 rapidly cleared from plasma and that penetration of SEA0400 was 'excellent' in the brain. When injected at 1 mg/kg and 3 mg/kg i.v., the maximal concentrations of SEA0400 in the brain were reported to be 2.7 and 7.3 μM, respectively (189). In the rat heart, tissue concentrations of SEA0400 at 0.3 and 1 mg/kg were found to be 0.75 and 2.1 μg/g tissue, respectively, at 1 minute after i.v. infusion (97). In the study by Nagasawa *et al* (239) in anesthetized dogs, free plasma concentration of SEA0400 (1.0 mg/kg, administered i.v. at 10 minutes before coronary ligation) was 1.10, 0.56, 0.33 μg/ml at 10, 20, and 40 minutes after administration, respectively, as determined by a liquid chromatography mass spectrometric/tandem mass spectrometric method. In another canine study, Takahashi *et al* (233) showed that at 30 minutes after i.v. administration of SEA0400 at 0.3 and 1 mg/kg, plasma concentrations were 0.11 and 0.32 μg/ml, corresponding to 0.29 and 0.87 μM, respectively. The investigators indicated that these concentrations were adequate for inhibiting NCX and that a single bolus of i.v. SEA0400 would inhibit $Ca^{2+}$ overload mediated through the reverse mode of NCX (233).

### *1.4.9 Toxicology*

Matsuda *et al* (189) evaluated the neuroprotective effects of SEA0400 in rats, both *in vitro* and *in vivo*, and did not observe any cell toxicity (up to the time of assay) or any significant effects of SEA0400 on mean blood pressure, regional cortical blood flow, or body temperature (when given at 3 mg/kg bolus i.v. followed by 3 mg/kg/hr for 2 hours).

Administration of SEA0400 under physiological conditions did not exert any significant effects on hemodynamics or cardiac contractile function (97,98). This topic will be further reviewed under 'DISCUSSION'.

In addition, Ogata *et al* (236) commented on the absence of significant effects in renal function by administration of SEA0400 (3 mg/kg, i.v.) to sham-operated rats (controls for rats subjected to acute renal ischemia by transient occlusion of left renal artery and vein at 2 weeks following unilateral nephrectomy).

### *1.4.10 Effects on the Nervous System and the Renal System*

In a model of $Ca^{2+}$ paradox-like injury utilizing cultured astrocytes, SEA0400 (at concentrations > 0.1 μM) attenuated injury, decreased production of reactive oxygen species in a concentration-dependent manner, and reduced apoptosis (189,252,253). Concentration-dependent reduction in DNA ladder formation was observed and nuclear condensation was almost completely blocked by SEA0400 at 1 μM (189). Very recently,

SEA0400 (0.3 – 1 μM) has been shown to confer protective effects against sodium-nitroprusside-induced apoptosis in cultured rat microglia (254).

In an anesthetized rat model of transient middle cerebral artery (MCA) occlusion, SEA0400 at 3 mg/kg i.v. bolus administered immediately after MCA occlusion, followed by 3 mg/kg/hr continuous infusion for 2 hours, significantly decreased infarct volume in the cerebral cortex and striatum, as compared with vehicle (189). In addition, SEA0400 (3 and 10 mg/kg i.v.) has been shown to significantly suppress edema after the induction of radiofrequency lesions in rat brains (255).

Ogata *et al* (236) studied the effects on SEA0400 on ischemic acute renal failure in rats subjected to transient occlusion of the left renal artery and vein (by clamping ) followed by reperfusion, at 2 weeks after contralateral nephrectomy. SEA0400 (0.3, 1, and 3 mg/kg, i.v.) or vehicle administered by slow bolus injection at 30 minutes before renal vascular occlusion was shown to dose-dependently attenuate IR-induced renal dysfunction and histological damage (236). In addition, SEA0400 (at 3 mg/kg i.v.) almost completely suppressed IR-induced increases in renal endothelin-1 contents after reperfusion, as compared with vehicle-treated rats with acute renal failure (236). Furthermore, administration of SEA0400 3 mg/kg i.v. post-ischemia also attenuated IR-induced renal dysfunction (236).

SEA0400 (0.2 and 1 μM) has also been shown to confer protection to cultured porcine tubular cells against hypoxia/reoxygenation-induced cell injury *in vitro* (236). In another study, Iwamoto *et al* (240) reported that SEA0400 protected porcine renal tubular cells expressing wild-type NCX1 against hypoxia/reoxygenation-induced cell injury (this effect was not observed in cells expressing SEA0400-insensitive mutants).

### *1.4.11 Comparison with Other NCX Inhibitors*

In CCL-39 fibroblasts transfected with NCX1, the inhibitory effects of different NCX inhibitors on the rates of $[Na^+]_i$ dependent $^{45}Ca^{2+}$ uptake were measured and the $IC_{50}$ values of SEA0400, SN-6 [a novel benzyloxyphenyl derivative with preferential inhibition of the reverse mode of NCX (191)], KB-R7943, and $Ni^{2+}$ were 0.06, 3.2, 4.9, and 42 μM, respectively (207,228,229). In NCX 1 transfectants, SEA0400 has been estimated to be '70 times more inhibitory' than KB-R7943 ($IC_{50}$'s of 0.056 μM and 4.1 μM, respectively) (240).

In addition to increased potency, SEA0400 has also been demonstrated to be more selective for NCX as compared with KB-R7943 (189,219). However, as both SEA0400 and KB-R7943 have been shown to depress $Ca^{2+}$ transients at low concentrations in electrically stimulated heart tubes from NCX1 knock out mouse embryos (220), both agents are not entirely specific for NCX. Isoform selectivities also differ among the 3 benzyloxyphenyl derivatives (13). SEA0400 primarily inhibits NCX1, with mild inhibitory action on NCX2 and no effect on NCX3 (240). SN-6 also inhibits NCX1 more (by 3 to 5 fold) than NCX2 and NCX3 (191). In contrast, KB-R7943 has been shown to inhibit NCX3 3-fold more than its effects on NCX1 and NCX2 (229).

With regard to comparisons of efficacy, in the study by Magee *et al* (98), SEA0400 was shown to be more efficacious than KB-R7943 in reducing infarct size in the Langendorff-perfused isolated rabbit heart subjected to regional ischemia, with 75% and 40% reduction in infarct size by 1 μM SEA0400 and 1 μM KB-R7943, respectively (98). In addition, in the IR model of Langendorff-perfused isolated rat hearts reported

by Takahashi *et al* (97), SEA0400 was > 10 times as potent as KB-R7943 and cariporide in attenuating cardiac dysfunction.

Moreover, SEA0400 ($\leq$ 1 μM) did not show any negative effects on cardiac function in Langendorff-perfused isolated rabbit hearts, in contrast to the deterioration in cardiac function and coronary flow observed with KB-R7943 at concentrations > 1 μM (98). In Langendorff-perfused isolated rat hearts, SEA0400 ($\leq$ 1 μM) did not exert any significant effects on heart rate and coronary flow, whereas KB-R7943 at 10 μM led to a significant reduction in heart rate and a significant increase in coronary flow as compared with control (DMSO) (97). Notably, negative effects of KB-R7943 on hemodynamics have also been reported in previous studies (198,199,222,223).

### *1.4.12 Comparison with NHE Inhibitors*

In Langendorff-perfused isolated rat hearts subjected to hypoperfusion followed by normal perfusion, Takahashi *et al* (97) demonstrated that SEA0400 (0.3 and 1 μM) significantly improved the post-ischemic recovery of LV developed pressure. Even though such benefit was also observed using cariporide (3 μM), its potency was 10 times less than that of SEA0400 in this model (97). In the IR-arrhythmia model of anesthetized rats (97), SEA0400 was > 3 times as potent as cariporide in reducing the incidence of ventricular fibrillation (VF), and the mortality rate was significantly reduced by SEA0400, as compared with a trend in the reduction of mortality in the cariporide-treated group. In addition, Magee *et al* (98) indicated that, in their experience, the efficacy of SEA0400 in cardioprotection was similar to that of zoniporide and cariporide, but was greater than that of eniporide - with maximum reduction in infarct size of 75%, 83%, 85%, and 58% for SEA0400, zoniporide, cariporide, and eniporide, respectively (127). Based on the above findings, it appears that the efficacy of SEA0400 in attenuating myocardial IRI is at least equivalent to that of NHE inhibition, pending confirmation in other animal models. Overall, these results are consistent with those of a previous study which demonstrated that either KB-R7943 or cariporide, when administered before ischemia, conferred cardioprotection in a rabbit model of global IRI (198). However, when either compound was administered on reperfusion, only KB-R7943 afforded benefit (198).

### *1.4.13 Comparison with Other Drugs*

SEA0400 was compared with lidocaine in the IR arrhythmia model of anesthetized rats (97), and lidocaine (1 mg/kg and 3 mg/kg) did not show any significant protection against IR arrhythmias whereas SEA0400 reduced the incidence of VF and mortality as discussed.

## 1.5 Other Novel NCX Inhibitors

In 2002, preliminary data on another mode-selective NCX inhibitor, 2-[4-(nitrobenzyloxy)benzyl]thiazolidine-4-carboxylic acid ethyl ester (SN-6), was described (256). It also had additional action as a nitric oxide donor and exhibited cardioprotective effects in Langendorff-perfused guinea pig hearts (256). Iwamoto *et al* (191) has demonstrated that SN-6 preferentially inhibits the reverse mode of NCX (by $Na^+_i$-dependent $^{45}Ca^{2+}$ uptake) in CCL39 cells transfected with NCX1, NCX2, and NCX3 with $IC_{50}$ values 2.9, 16, 8.6 μM, respectively, with potentiation of inhibitory effects under conditions of ATP depletion ($IC_{50}$ = 0.78 μM in ATP depleted NCX1 transfectants). Further investigation documented protective effects of SN-6 against hypoxia/reoxygenation-induced damage in renal tubular cells overexpressing NCX1 with an $IC_{50}$ value of 0.63 μM (191). It has been postulated that the properties of this compound may derive from its interaction with the XIP region of the NCX molecule (191), which is thought to play an important role in $Na^+_i$-dependent inactivation of NCX (12). This compound may have a future role as an anti-ischemic agent (257).

Recently, a potent NCX inhibitor, SM-15811, a 3,4-dihydro-2(1H)-quinazolinone derivative, was discovered, with an $IC_{30}$ value of 0.017 μM (190). In cardiac myocytes, it inhibited $Na^+$-dependent $Ca^{2+}$ influx in a concentration-dependent manner (190). Its mechanism of action is not known. In addition, phenoxypyridine derivatives have been synthesized and tested in CCL 39 cells expressing NCX1.1 for their inhibitory activity against forward mode and reverse mode of NCX (258). Two of these derivatives were reported to have a high selectivity for the reverse mode of NCX ($IC_{50}$ values = 0.60 and 0.79 μM, the latter was a 6-{4-[(3-flurobenzyl)-

oxy]phenoxy}nicotinamide derivative). More recently, the synthesis and the structure-activity relationships of 6-{4-[(3-flurobenzyl)oxy]phenoxy}nicotinamide derivatives were described (259). In particular, *N*-(3-aminobenzyl)-6-{4-[(3-flurobenzyl)-oxy]phenoxy}nicotinamide was reported to inhibit the reverse mode of NCX1.1 expressed in CCL39 cells with an $IC_{50}$ value of 0.24 μM (259) and was further utilized for designing a series of benzyloxyphenyl derivatives ($IC_{50}$ values of the 2 most potent derivatives against the reverse mode of NCX were 0.22 μM) (260). [In some of the more recent publications described above (258-260), reference was also made to the chemical structure of a patented compound (261) in the context of benzyloxyphenyl derivatives with inhibitory effects on the NCX.] Based on the $IC_{50}$ values described in these recent reports [with the possible exception of SM-15811 described in one publication (190)], SEA0400 remains the most potent NCX inhibitor described to date.

Apart from inhibitors with predominant actions against the NCX, another compound, 5-methyl-2-(1-piperazinyl) benzenesulfonic acid monohydrate (MCC-135), has been described to act on both the NCX and the sarcoplasmic reticulum (SR) (262). Specifically, in a recent study, MCC-135 was shown to suppress $Ca^{2+}$ overload, which was attributed to inhibition of $[Na^+]_i$-dependent influx of $Ca^{2+}$ via the reverse mode of NCX, and to confer cardioprotective effects in Langendorff-perfused isolated rat hearts (263). This represents an additional mechanism to findings in an earlier study (262), which demonstrated lusitropic effects of MCC-135 on the diabetic myocardium, attributable to an increase in SR $Ca^{2+}$ uptake and a reduction in SR-leak (262). Notably, this compound is being evaluated in phase II clinical trials in the settings of the acute coronary syndromes (1,264) and heart failure (265). Preliminary results of the Calderet

in ST Elevation Myocardial Infarction (CASTEMI) trial was reported at the American College of Cardiology Annual Scientific Session 2004 (1). It was an international, multicentre, randomized, placebo-controlled trial designed to evaluate whether i.v. caldaret, when used in the setting of primary percutaneous coronary intervention (PCI) for acute ST-elevation myocardial infarction (STEMI), could lead to further reduction in infarct size and improvement in LV function (1). Three hundred and eighty-seven patients were randomized to receive a 48-hour infusion (initiated 'during' PCI) of high-dose caldaret (172.5 mg i.v.), low-dose caldaret (57.5 mg i.v.), or placebo (1). No difference in infarct size was detected between the groups, as assessed by single photon emission computed tomography (1). In patients TIMI (Thrombolysis in Myocardial Infarction trial) grade 0 (complete occlusion) or grade 1 (some penetration of contrast agent beyond the obstruction but without distal perfusion) flow (266,267) and anterior myocardial infarction, high-dose caldaret was associated with significant decreases in LV end-systolic and end-diastolic volumes, as compared with placebo, at days 7 and 30 (1). High-dose caldaret was also associated with a significantly greater improvement in global ventricular function at day 7 (although the difference was no longer statistically significant at day 30) and a significant decrease in cardiac markers (1). No abnormalities in biochemical, hemodynamic, or electrocardiographic parameters were detected in patients treated with caldaret (1). In addition, further analysis of this study, as reported in a recent abstract (268), demonstrated that significant left ventricular dysfunction with ejection fraction $\leq$30% occurred less frequently in the subgroup of patients who presented with anterior myocardial infarction and were treated with caldaret pre-PCI, suggesting that this compound may be of benefit to patients with more

extensive infarcts. Based on these findings, it appears that caldaret may have a potential role as an adjunctive cardioprotective agent in the context of the acute coronary syndromes and thus warrants further investigation.

## 1.6 The Metabolic Syndrome (or the Insulin Resistance Syndrome)

In light of the promising cardioprotective effects demonstrated by novel NCX inhibitors, it is speculated that such therapy would be of the greatest therapeutic potential in individuals with increased risk for coronary artery disease, such as those with the metabolic syndrome. The metabolic syndrome (MetS), also known as the insulin resistance syndrome (IRS) or syndrome X, consists of a cluster of metabolic risk factors, with insulin resistance being the primary defect (269,270). Insulin resistance occurs primarily in skeletal muscles (271), with potential involvement of adipose tissues (272), and may be overcome by compensatory hyperinsulinemia (271). Even though hyperglycemia may be prevented by compensatory hyperinsulinema, this state is associated with the atherogenic metabolic abnormalities (271). It was first recognized by Reaven in 1988 as a multiplex risk factor for cardiovascular disease (269). There have been a number of proposed definitions or diagnostic criteria for this syndrome (273-277). For instance, the National Cholesterol Education Program's Adult Treatment Panel III report (ATPIII) definition (275), as recently reviewed (274,277), identified 5 characteristics in this syndrome: abdominal obesity (waist circumference > 102 cm in men or > 88 cm in women), hypertriglyceridemia [triglyceride (TG) $\geq$ 1.7 mmol/L or $\geq$ 150 mg/dL], low high-density lipoprotein (HDL) cholesterol (< 1.0 mmol/L or < 40 mg/dL in men; < 1.3 mmol/L or < 50 mg/dL in women), high blood pressure ($\geq$ 130/$\geq$ 85 mmHg) , and elevated fasting glucose ($\geq$ 6.1 mmol/L or $\geq$ 110 mg/dL). A clinical diagnosis can be made when the cut-points for 3 or more of the 5 characteristics are met (275). In contrast, the World Health Organization requires the demonstration of insulin resistance (as defined by type 2 diabetes; impaired fasting glucose; impaired glucose

tolerance; or, in the presence of a normal fasting glucose, a glucose uptake below the lowest quartile for background population under hyperinsulinemic, euglycemic conditions) as one of the diagnostic criteria, in conjunction with 2 other risk factors (273). The latter may include high blood pressure (≥ 140 mmHg systolic or ≥ 90 mmHg diastolic) and/or the need for antihypertensive medications, plasma TG ≥ 150 mg/dL (≥ 1.7 mmol/L), HDL cholesterol < 35 mg/dL (0.9 mmol/L) in men or < 39 mg/dL (1.0 mmol/L) in women, BMI > 30 kg/$m^2$ and/or waist to hip ratio > 0.9 in men, > 0.85 in women, urinary albumin excretion rate ≥ 20 μg/min or albumin-to-creatinine ratio ≥ 30 mg/g (277). Notably, for both the NCEP ATPIII and the WHO criteria, the MetS can still be diagnosed in the setting of type 2 diabetes (277).

Even though the MetS and IRS are often used interchangeably, the emphasis of the two terms differ in that IRS highlights the concept of insulin resistance being the fundamental defect, whereas the MetS emphasizes the clinical manifestations and the associated risks (278). While the definition of MetS can be readily applied without having to demonstrate insulin resistance by objective testing (which is not widely available in common clinical settings) (277), it may have some limitations in applying the obesity criteria in different populations (279) and in identifying insulin resistance in apparently healthy individuals (280). However, given the many common features among the various sets of diagnostic criteria, it is likely that individuals at risk will be identified by one method or another (281). For the purpose of this thesis, the term 'metabolic syndrome' will be used throughout for consistency, recognizing that the clinical components in this definition would be an extrapolation to the experimental

setting and that the 'insulin resistance syndrome' would also be very appropriate as an alternative term.

Individuals with the MetS are at elevated risk of developing diabetes (almost half of the population-attributable risk) (277), cardiovascular disease (277,282-291), and increased mortality from cardiovascular disease and all causes (292). The 10-year risk for developing cardiovascular disease ranges from 10 to 20% (277). In those who have clinically manifest vascular disease, the presence of the MetS has been associated with advanced vascular damage (293). In addition, in type 2 diabetes, which is the predominant type in the adult population (274,294), the MetS is associated with an almost 5-fold increase in the risk for developing cardiovascular disease (295) and is also a predictor of increased all-cause and cardiovascular mortality (296). Individuals with 4 to 5 metabolic abnormalities have been shown to be more likely to develop coronary events and new onset diabetes, as compared with those with 2 or 3 abnormalities (287).

In Canada, the overall prevalence of the MetS has been reported to be 25.8 % in one study which randomly sampled > 1200 individuals of different ethnic backgrounds from 4 communities in Ontario and Alberta, using the definition of NCEP ATPIII (297). Individuals with the MetS were found to have a higher burden of atherosclerosis (as assessed by B-mode carotid ultrasound), more cardiovascular disease, and higher levels of plasminogen-activator-inhibitor-1 (a marker of fibrinolytic dysfunction), the latter may, at least in part, explain the increased risk of cardiovascular disease in the MetS (297). In a registry of patients referred for urgent and elective percutaneous coronary intervention in Nova Scotia (298), the prevalence of MetS was recently reported, in an abstract, to be 40.1%. In these patients with the MetS, higher

rates of rehospitalization for any cardiac cause and of CABG surgery were observed as compared with patients without the MetS (298). Moreover, in another study which enrolled consecutive patients (mean age 58 years, 793 men and 315 women) undergoing elective coronary angiography in Québec, 51% of them were found to have the MetS, as defined by the NCEP ATPIII criteria (299). These patients had more severe coronary artery disease (CAD) on angiography as compared with those without the MetS (299). Other studies have also documented increased prevalence of overt CAD or subclinical atherosclerosis in the setting of the MetS (285,300). Notably, even in the presence of angiographically 'normal' coronary arteries, coronary blood flow has been shown to be impaired in the presence of the MetS (301). The magnitude of this tremendous burden of cardiovascular disease is further magnified by the fact that the prevalence of the MetS increases with age (> 40% in those aged ≥ 60 years) (302) and that the MetS affects populations worldwide (279,302), especially in light of the growing epidemic of obesity (277,303,304).

Six components of the MetS have been identified by the NCEP ATPIII as being components that specifically pertain to cardiovascular disease – the primary clinical outcome of MetS (277). They include abdominal obesity, atherogenic dyslipidemia [typically hypertriglyceridemia, low HDL, and small dense LDL (305)], increased blood pressure, insulin resistance with or without glucose intolerance, proinflammatory state, and prothrombotic state (277). Abdominal obesity, which manifests as increased waist circumference, is the type of obesity with the strongest association with the MetS (277). Previous studies have described the 'hypertriglyceridemic waist', which was defined as waist circumference ≥ 90 cm in the

presence of elevated triglyceride $\geq$ 2 mmol/L, and its utility in predicting of a triad of atherogenic metabolic abnormalities, including hyperinsulinemia, hyperapolipoprotein B, and small dense LDL, in men (306), as well as coronary artery disease (306). In the Quebec Health Survey, the 'hypertriglyceridemic waist' was present in 19% of a sample of 907 adult non-diabetic male and was accompanied by the highest levels of fasting insulin and total cholesterol to HDL cholesterol ratios in the study (307). Other studies have also documented abdominal obesity as a risk factor for coronary artery disease (308), myocardial infarction (309), stroke (308), hypertension (310), and type 2 diabetes (303,311). In the presence of obesity and physical inactivity, insulin resistance with hyperinsulinemia commonly occurs before the onset of hyperglycemia (303). Moreover, intra-abdominal fat has been shown to be independently associated with each one of the 5 diagnostic criteria of the MetS, suggesting its potential involvement in the pathophysiology of the MetS (312). In particular, the release of various products (such as nonesterified fatty acid, inflammatory cytokines, PAI-1, and leptin) from adipose tissue has been a major focus of research (304). As the simple measurement of waist circumference is positively correlated with intra-abdominal obesity (311), it provides a simple and practical way to assess the cardiovascular risk associated with obesity in the clinical setting (310,313).

The prothrombotic state of the MetS is characterized by elevated fibrinogen and PAI-1 (277). Fibrinogen is an acute-phase reactant that increases in response to a rise in cytokines (277) and has been shown to be predictive of future risk of MI and stroke in epidemiological studies (314). Plasminogen activator inhibitor-1, is an endogenous inhibitor of the clot-dissolving tissue-type plasminogen (t-PA) and

urokinase activator (314), and is a marker of hypofibrinolysis in the MetS in human (315). Among the various risk markers, C-reactive protein (CRP), a marker of inflammation and cardiovascular risk, appears most promising (314,316,317) and has gained intense research and clinical attention on its utility as a biomarker of cardiovascular risk in clinical settings and, potentially, as a marker of therapeutic efficacy (318-320). The latter appears particularly promising in the context of lipid lowering with HMG-CoA reductase inhibitors as revealed in recent clinical trials (321,322). Notably, CRP is commonly found to be elevated in the setting of the MetS (277,323). The mechanism may be attributed to obesity, especially abdominal fat accumulation (324), as increase in CRP may be mediated through inflammatory cytokines released from adipose tissues (277). Moreover, CRP also significantly correlated with insulin resistance (323,325).

The remarkable research progress in CRP can be traced back to 1930 when Tillet and Francis (326) first noted, in the sera of individuals with acute pneumonia, a substance which formed a precipitate with or 'reacted with' a distinct fraction derived from pneumococci (this fraction was the third one derived from pneumococci and was designated as 'Fraction C'). Subsequently, this substance was characterized as a protein by MacLeod and Avery (327), as reviewed in (328). C-reactive protein is made up of five 23-kDa non-glycosylated subunits that are arranged in a cyclic configuration (329,330). It is synthesized and released primarily in the liver (318,329). Under physiological conditions, CRP is present only in a trace amount in blood (318). However, its synthesis is stimulated by cytokines in response to tissue injury or infection within hours (328,331). Moreover, local production of CRP has also been demonstrated

in smooth muscle cells of human coronary arteries, especially in vessels with active atherosclerotic lesions, but not in end-stage plaques (332).

The availability of high-sensitivity CRP (hs-CRP) assays has improved the precision of measurements in the clinical setting and has given this test the attribute toward the prediction of cardiovascular events (316,333). In multiple prospective epidemiological studies, hs-CRP was demonstrated to predict the risk of MI, stroke, peripheral vascular disease, and sudden cardiac death (314,334,335). It has also been shown to be a stronger predictor of cardiovascular events than low-density lipoprotein (LDL) cholesterol (336), and provide additional prognostic information in conjunction with LDL measurement or to the Framingham Risk Score in the prediction of cardiovascular risks (336,337). Moreover, it may play a direct role as a mediator of atherosclerotic disease (338-343). Elevated CRP is commonly seen in the MetS, with an age-adjusted prevalence of 29% in one study (344), and is a marker of the associated proinflammatory state (277). In particular, CRP level has been shown to correlate with the various components of the MetS, such as fasting insulin, microalbuminuria, and impaired fibrinolysis (319). It also adds prognostic information on cardiovascular risk at all levels of the MetS (345), and predicts the risk for the development of type 2 diabetes (334,346). Of note, it has been postulated that inflammation may be a common pathophysiological basis for insulin resistance, the MetS, and atherosclerosis (347).

Current management strategies of the MetS include correction of underlying metabolic risk factors, with lifestyle modification being the first-line therapy, and control of other major cardiovascular risk factors (348). If lifestyle modification alone is ineffective, pharmacological therapy may be considered, such as in the management

of dyslipidemia and hypertension (348,349). In the absence of diabetes, the use of insulin-sensitizing agent is not recommended at the present time, given the lack of data on reduction of cardiovascular risks (348). Even though prophylactic use of low-dose aspirin is recommended for intermediate-risk and high-risk individuals, with the goal of ameliorating the prothrombotic and proinflammatory state of the MetS (348), there is clearly a dearth of cardioprotective therapies that specifically address the pathophysiology of ischemia/reperfusion injury, both in the context of the MetS and in cardiovascular medicine in general. Conceivably, myocardial protection in individuals with the MetS may be of particular clinical relevance, in light of the prevalence of the MetS (which will very likely further increase with aging of our population), their predisposition toward the development of both cardiovascular disease and type 2 diabetes (277), and the potential risks of having repeated episodes of ischemia and reperfusion during their lifetime (such as in the setting of acute coronary syndromes and revascularization procedures).

## 1.7 The JCR:LA-*cp* Rat

Given the growing impact of the MetS and the associated cardiovascular disease, and the dearth of clinical therapies against myocardial IRI, clinically relevant animal models of the MetS would greatly facilitate evaluation of novel compounds for cardioprotection. As rodents share a very similar natural history and manifestations of the MetS with humans, they can be utilized as an 'accelerated version' of the MetS in human (350). Among the various rodent models of hyperinsulinemia, such as the JCR:LA-*cp* rat, the Zucker *fa/fa* rat, the Zucker diabetic *fa/fa* rat, the *ob/ob* mouse and the *db/db* mouse (351-356), the JCR:LA-*cp* rat is unique in that it has not only the typical metabolic abnormalities, but also well-characterized, spontaneous cardiovascular complications (351,352,357-366). The mutated gene in this strain was identified by Koletsky (367) and was later designated as *cp* by Hansen (368), as reviewed by Russell and Graham (352). It was later found to be due to a point mutation of the leptin receptor at amino acid 763, leading to a stop codon in the extracellular domain of the leptin receptor and consequent absence of leptin receptors in the plasma membrane (352,369,370). As leptin, the obese gene product (371), is a hormone with that serves to reduce appetite, enhance energy consumption, inhibit insulin secretion and improve insulin sensitivity, along with other actions (372), leptin dysregulation in the *cp/cp* rats results in hyperphagia and marked hypersecretion of insulin (352). The basis of the hyperphagia could be attributed to the absence of leptin-mediated inhibition of neuropeptide Y, an important stimulant of eating, in the *cp/cp* rats (373). Rats that are homozygous recessive for the corpulent, *cp*, gene, exhibit severe hyperinsulinemia, morbid obesity (Figure 8), marked elevation of very-low-density lipoprotein (VLDL),

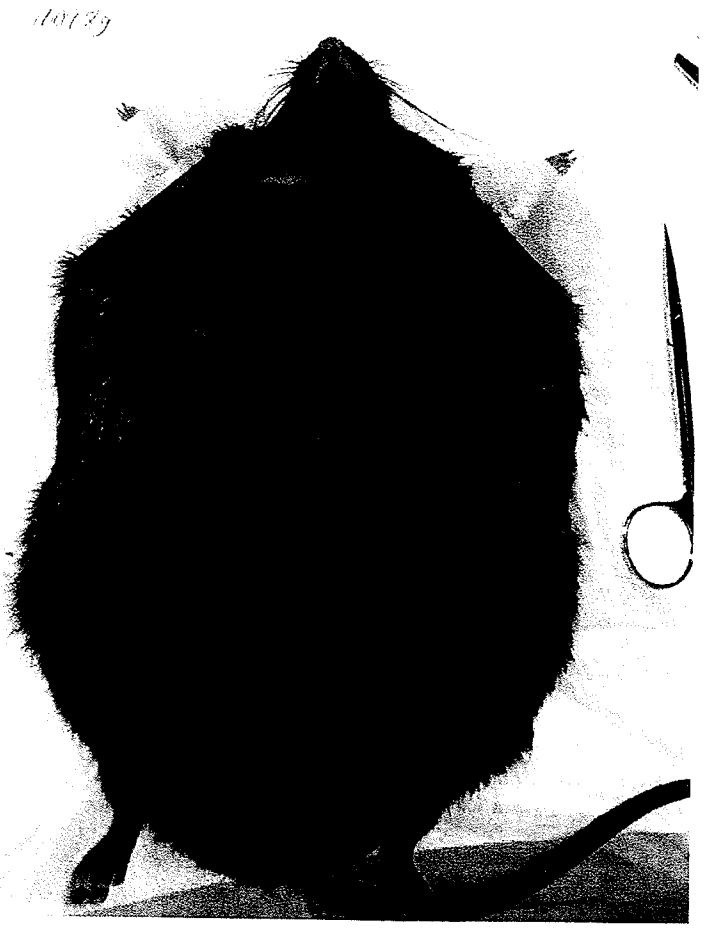


**Figure 8. The Obese JCR:LA-*cp* Rat at 12 Months.**

hypertriglyceridemia, glucose intolerance, hyperleptinemia, and cardiovascular disease; whereas +/+ or +/*cp* rats are lean and metabolically normal (352).

Spontaneous cardiovascular complications, which are unique features in the *cp/cp* rats, include atherosclerosis, myocardial ischemia and infarction (352,354). These lesions have been confirmed on necropsy and have been well characterized on the basis of pathology (352,362). For example, advanced intimal lesions of the aortic arch have been observed in almost all *cp/cp* male rats by 9 months of age (352,362). The genetic basis of the metabolic and cardiovascular abnormalities has not been well defined, although a polygenetic basis has been postulated (352). In addition, these rats have been described as being susceptible to stress, to the extent that stress-induced spontaneous myocardial ischemia and myocardial infarction have occurred (352,362). Even mild stress induced by transient restraint of the obese rat could lead to rapid accumulation of non-esterified fatty acid in blood (374). The sensitivity of the obese rats to global ischemia at 6 months and 9 months of age has also been well described in Langendorff-perfused isolated hearts (361). Moreover, the obese rats have been shown to have abnormal vascular function and impaired ability to compensate for vasoconstriction, especially with advancing age (365). Plasminogen activator inhibitor-1 expression in culture of aortic rings *in vitro* and PAI-1 levels in blood were found to be elevated in another study (375), suggesting a potential role of hypofibrinolysis in the pathophysiology of the MetS and atherothrombosis in this animal model. Of note, the incidence of cardiovascular disease in the obese rats has been demonstrated not to correlate with the extent of dyslipidemia, but rather, it correlated with the degree of insulin resistance and glucose intolerance (376).

The insulin-resistant JCR:LA-*cp* rat has been shown to have preserved myocardial $Ca^{2+}$-ATPase activity and sarcoplasmic reticular function, in contrast to animal models of chemically-induced diabetes (with low insulin levels) which are often found to have cardiomyopathy (377,378). Given the constellation of metabolic and cardiovascular features mimicking their human counterparts, this strain of rat may serve as a valuable model for evaluation of cardioprotective therapies against acute IRI in the context of the MetS.

# 2. STATEMENT OF THE PROBLEM AND HYPOTHESES

## 2.1 Statement of the Problem

2.1.1 The metabolic syndrome is a prevalent public health problem in Canada (297,379) and worldwide (279), and is anticipated to increase in magnitude as our population ages and the obesity epidemic prevails (303,380). The constellation of atherogenic risk factors in the MetS substantially increases the risks for both cardiovascular disease and type 2 diabetes (277), with the latter being a 'risk equivalent' to CAD (275,381).

2.1.2 As atherosclerosis is a chronic process which progresses with time, potentially punctuated with episodes of acute coronary events, it is anticipated that individuals with coronary artery disease would be at risk for repeated episodes of IRI during their lifetime. Since the MetS confers increased cardiac risk, adjunctive cardioprotective therapies against IRI, in conjunction with timely reperfusion (168), may have particular therapeutic potential in this population.

2.1.3 Despite the wealth of data accumulated over the past 3 decades on compounds that exhibited cardioprotective properties against IRI in experimental settings, none has been successfully translated into routine clinical practice (172). To date, there continues to be a need for such

adjunctive cardioprotective therapies in spite of recent advances in both pharmacological and catheter-based reperfusion strategies for the management of acute coronary syndromes (168,381).

2.1.4 Pharmacological inhibition of the NHE as a strategy for myocardial protection is one of the few examples where remarkable research progress has been made (168) - from the first report of cardioprotection in the experimental setting (52) to completion of 3 large-scale clinical trials (5-7) within only 15 years – but, unfortunately, was hampered by preliminary evidence of serious adverse events (6), which remain to be addressed. Whether inhibition of the NCX, a downstream target in relation to NHE, might provide an alternative strategy for myocardial protection remains to be further investigated, although experimental data to date appear promising (97,98,233).

2.1.5 One of the challenges in translating experimental findings to clinical applications is related to limitations of animal models (382). For instance, healthy animals may have more reserve to withstand drug toxicities and, therefore, during drug evaluation, they may not manifest certain adverse effects that may otherwise occur under pathophysiological conditions (383). Similarly, findings in young animals are not necessarily applicable to senescent animals (and elderly persons) due to age-related changes in the cardiovascular system (384). A clinically relevant animal model with

parallel characteristics and pathologies as those in its humans counterpart would be particularly valuable for assessment of the efficacy (382) and safety (383) of novel therapies.

2.1.6 Delays in drug administration, which commonly occur for various reasons in clinical settings (381), may partly explain the lack of efficacy of cardioprotective agents observed in some clinical trials of NHE inhibitors (154). Successful delivery of a cardioprotective agent to the jeopardized myocardium in the presence of an occluded artery is a major challenge, especially when time is of the essence in salvaging the myocardium (381,385).

2.1.7 While experimental data on the cardioprotective effects of NCX inhibition appears promising in models of acute IR, the utility of chronic administration of an NCX inhibitor was unknown (at the start of this project), which may have potential clinical relevance. Recently, the reverse mode of NCX has been shown in experimental models to play a central role in the pathophysiology of salt-sensitive hypertension (238) and, conceivably, NCX inhibitors may be of therapeutic potential both as a chronic anti-hypertensive agent and as a prophylactic drug against IRI. The latter may circumvent the problem of delay in drug delivery, as it is anticipated that a steady drug level would have been achieved in the circulation prior to the onset of ischemia.

2.1.8 Before contemplating chronic use of NCX inhibitors, one fundamental issue that needs to be addressed is whether such strategy would compromise the forward mode of NCX, which plays an important physiological role in the extrusion of $Ca^{2+}$ that enters the cardiac myocyte through L-type $Ca^{2+}$ channels on a beat-to-beat basis (27). While the novel NCX inhibitor, SEA0400, has been described as the most potent and selective NCX inhibitor available to date (13), whether chronic use of NCX inhibitors under physiological conditions is indeed innocuous to basal ventricular function requires formal evaluation.

2.1.9 One established method of non-invasive evaluation of ventricular function is echocardiography, both in the experimental (386-390) and the clinical settings (391,392). Whether such a technique is applicable to the JCR:LA-*cp* rat is unknown, in light of its stress-prone nature and its morbid obesity. The former may constitute a form of stress and impose a cardiac risk, whereas the latter may introduce technical difficulties in image acquisition, as is commonly observed in humans (393).

2.1.10 While the identification of novel biomarkers in predicting cardiovascular risk has been an intense area of research in clinical cardiovascular medicine (314) and the MetS (334), with particular promise demonstrated by CRP (316), it is unknown whether CRP would have a potential role as a clinically-relevant biomarker in pre-clinical drug evaluation. At the time of

writing, baseline CRP level of the JCR:LA-*cp* rat is not available in the existing literature.

## 2.2 Hypotheses

2.2.1 The JCR:LA-*cp* rat would be a valuable animal model for evaluation of SEA0400 as a cardioprotective agent in the context of the MetS and IRI.

2.2.2 Transthoracic echocardiography would be feasible and practical in the JCR:LA-*cp* rat, despite its stress-prone nature and morbid obesity. Similarly, prolonged prophylactic therapy with SEA0400 via an osmotic pump would be a feasible approach for experimentation.

2.2.3 *In vivo* administration of SEA0400 under physiological conditions would have negligible effects on cardiac function, as the reverse mode of NCX does not play a significant role under physiological conditions.

2.2.4 The senescent, obese JCR:LA-*cp* rat, a model of the MetS and cardiovascular disease in the elderly, would be more sensitive to myocardial ischemia as compared with the lean control.

2.2.5 Pharmacological inhibition of the reverse mode of NCX with SEA0400 would be cardioprotective against IRI in this polygenetic model of the MetS.

2.2.6 Baseline CRP would be significantly elevated in the senescent, obese rat (in light of its severe metabolic abnormalities and atherosclerosis) as compared with the lean control and may have potential utility in pre-clinical drug evaluation.

## 2.3 Objectives

Utilizing the obese JCR:LA-*cp* rats and the lean controls, this study was conducted to achieve the following objectives:

2.3.1 to assess the feasibility of transthoracic echocardiography in this strain;

2.3.2 to characterize baseline cardiac structure and function by echocardiography;

2.3.3 to evaluate the effects of prolonged administration of SEA0400 *in vivo*, with a focus on left ventricular function as assessed serially by echocardiography,

2.3.4 to assess the sensitivity of the senescent myocardium to ischemia and reperfusion in JCR:LA-*cp* rats, using the Langendorff-perfused isolated heart technique,

2.3.5 to assess the efficacy of SEA0400 in attenuating IRI at the level of the isolated heart,

2.3.6 to establish baseline C-reactive protein levels in this strain.

# 3. MATERIALS AND METHODS

## 3.1 Animals

The experimental protocol was approved by University of Manitoba Protocol Management & Review Committee in accordance to guidelines set forth by the Canadian Council on Animal Care.

All JCR:LA-*cp* rats (obese and lean control) were bred in an established colony at the University of Alberta (352) and were transported to the University of Manitoba by airfreight. To minimize stress, an acclimatization period of $\geq$ 1 week was introduced prior to any experimentation. This period was typically much longer as the rats were purchased in large numbers to ensure adequate supply for the entire project, given the scarcity of this strain.

Only male rats were studied to minimize variability in background cardiac risk, in light of previously reported differences between male and female JCR-LA:*cp* rats. Specifically, male *cp/cp* rats exhibit more severe hyperinsulinemia and glucose intolerance (394), more severe vascular dysfunction (352), more advanced atherosclerotic and myocardial lesions (352,376), but less severe dyslipidemia (376) as compared with *cp/cp* females. Rats were maintained on a 12:12-hour light-dark cycle and were housed in pairs in polycarbonate cages with wood chip bedding. A standard rodent diet with water was freely available.

Rats of the 2 distinct age groups were studied: 3 – 6 and 10 – 13 months. The former group was utilized for *in vivo* evaluation of SEA0400 over a 4-week period followed by *in vitro* testing using the Langendorff-perfused isolated heart technique.

The latter group was utilized for *in vitro* evaluation of SEA0400, and these rats will be considered as 'senescent' since *cp/cp* rats have been described to have a reduced lifespan (352,395). These older rats were utilized as a model of the MetS in the elderly, given the increasing prevalence of the MetS with age (302).

## 3.2 Anesthesia

Isoflurane was the anesthetic agent used for all experiments, primarily based on previously reported favourable experience with volatile anesthesia in the *cp/cp* rats (352). For all *in vitro* and *in vivo* experiments, anesthesia was administered exclusively by the staff of the R.O. Burrell Facility of the St. Boniface General Hospital Research Centre according to the standard operating procedures of the Facility.

For induction of anesthesia, the rat was gently introduced into a 4-litre jar containing 4-5% isoflurane with oxygen at 2L/min, with subsequent titration of isoflurane (1.5 – 2%) to maintain anesthesia.

## 3.3 The Study Drug (SEA0400)

The compound, 2-[4-[(2,5-difluorophenyl)methoxy]phenoxy]-5-ethoxyaniline (SEA0400), was obtained from Taisho Pharmaceutical Co., Ltd. (Saitama, Japan) in the forms of a powder (for *in vitro* experiments) and an emulsion (containing the active compound and the vehicle for *in vivo* experiments). It was stored in tight and light-resistant containers, at 1 – 10°C, according to the instructions on the Material Safety Data Sheet on SEA0400 (396).

The vehicle, which was a lipid emulsion containing soybean oil 20%, vitelline lecithin 2.4%, glycerine 2.1%, deionized water, was also obtained from the manufacturer for *in vivo* studies. A stock solution (10 mM) was made from the SEA0400 powder (diluted in DMSO, with a final concentration of DMSO < 0.1%) and was used for preparing the required dose of SEA0400 on the day of the experiment, using precautions to minimize exposure to light and to heat (396).

## 3.4 Implantation of Osmotic Pumps

This route of drug administration was chosen to maximize bioavailability and to avoid fluctuations in drug level (397) in *in vivo* studies utilizing the *cp/cp* rats.

The osmotic pump consists of 3 concentric cylindrical layers: an outermost, rigid layer of semipermeable membrane made of cellulose materials; a middle layer of

supersaturated salt solution; and an innermost, flexible layer of impermeable drug reservoir (397). After implantation, interstitial fluid enters the pump through the semipermeable membrane (driven by the osmotic gradient between the supersaturated salt solution and the interstitial fluid) (397). Due to fluid expansion within the fixed volume enclosed by the rigid outermost layer, the drug reservoir (within the flexible innermost layer) became compressed and the drug was delivered out of the pump at the same rate as fluid enters the pump (397).

Subcutaneous implantation of osmotic pumps was performed by a designated member of staff in the R.O. Burrell Facility of the St. Boniface Research Centre, according to the instructions and a video of the surgical implantation procedure provided by DURECT Corporation (Cupertino, CA), the manufacturer of the ALZET® osmotic pumps (398) (Figure 9), where all the osmotic pumps were purchased. The osmotic pumps were filled through the delivery portal leading to the reservoir of each pump, with SEA0400 (emulsion containing the active compound and the vehicle) or the vehicle alone, by means of a filling tube connected to a drug-filled syringe at the other end (397). SEA0400 or vehicle was delivered through the same port and access into the venous system of the rat was achieved by cannulation of the right jugular vein. The total duration of continuous i.v. drug infusion was 28 days. The dose of SEA0400 (1 mg/kg i.v. continuously over each day) was chosen based on the experience reported by Takahashi *et al* (97).

6 Echo-Control Rats:

Baseline Echo — Follow-up Echo (at weeks 1, 2, 3, 4)

↓ ↓ ↓ ↓ ↓

---

**20 rats – implantation of osmotic pumps**
**(containing SEA0400 + vehicle *vs.* vehicle alone)**

**Baseline Echo — Follow-up Echo (at weeks 1, 2, 3, 4)**

↓ ↓ ↓ ↓ ↓

---

↑

**Pump implantation**

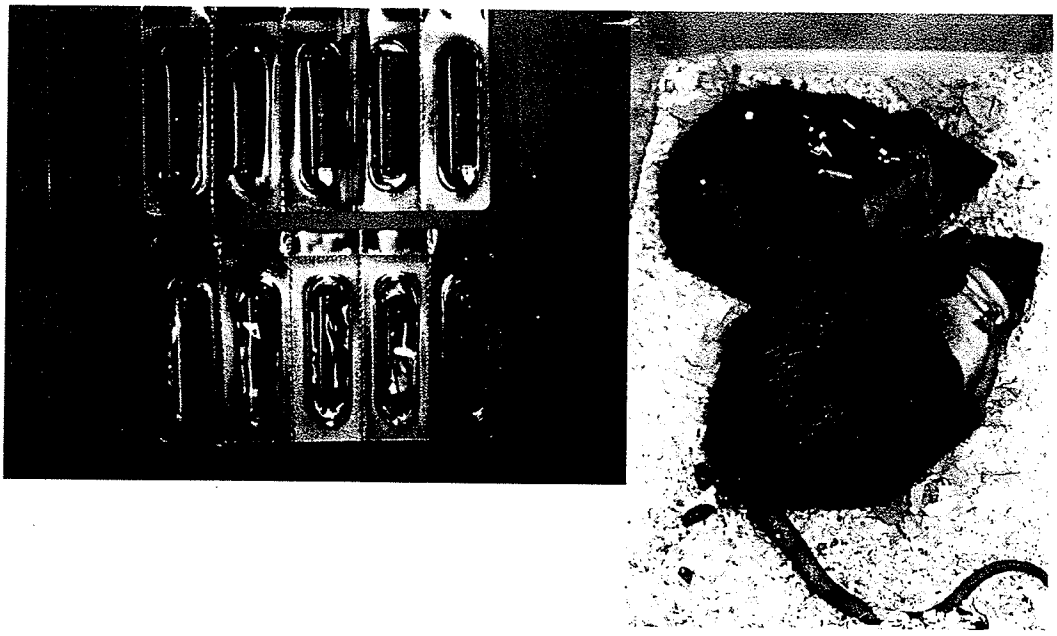


**Figure 9. Experimental Protocol for *In Vivo* Evaluation of SEA0400 (upper panel). ALZET® osmotic pumps (lower left). Obese JCR:LA-*cp* rats on post-operative day 1 following osmotic pump implantation (lower right).**

## 3.5 Transthoracic Echocardiography in Rats

On the day of echocardiography, the rat was anesthetized with isoflurane and was placed on a heating pad to avoid hypothermia (352,399) (Figure 10). The ultrasound medium was also pre-warmed. The hair on the rat's chest was shaved to optimize image quality. The rat was then placed in the left lateral decubitus position for image acquisition from the parasternal window (388,400,401) (Figure 11).

Imaging was performed at a depth of 2 cm (388). Standard views include the parasternal long-axis view, the parasternal short-axis view, and the apical 4-chamber view. A 12-MHz transducer was used to acquire images (SONOS 5500, Philips Medical Systems, Markham, ON). Two-dimensionally-guided M-mode images (at 150 mm/sec) were acquired from the parasternal window at the level of the papillary muscles (401-403) (Figure 11, upper panel). Pulsed-wave Doppler signal of mitral inflow was obtained from the apical window under 2D-guidance (Figure 11, lower panel). To minimize the duration of exposure of the rats to volatile anesthesia, all images were recorded on magneto-optical disks (Hewlett-Packard, Palo Alto, CA) for analysis using the same ultrasound system at a later date. The duration of exposure to anesthesia of each rat was also recorded.

Motion-mode (M-mode) measurements included LV end-diastolic dimension (LVEDD), LV end-systolic dimension (LVESD), septal thickness (ST), and posterior wall (PW) thickness, and the duration of each cardiac cycle (for determination of heart rate - an attempt for continuous electrocardiographic monitoring was also made at the beginning of the project, but the quality of the signal was suboptimal and therefore was not used). Motion-mode measurements were made from leading edge to leading edge

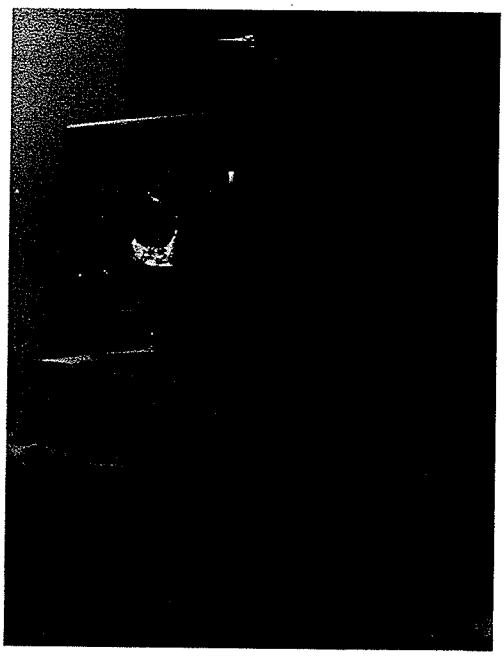


**Figure 10. Transthoracic Echocardiographic in the JCR:LA-cp Rat.**
Upper panel: The rat was anesthetized with isoflurane and was placed in the left lateral decubitus position.
Lower panel: Experimental setup (note heating pad in the left lower corner).

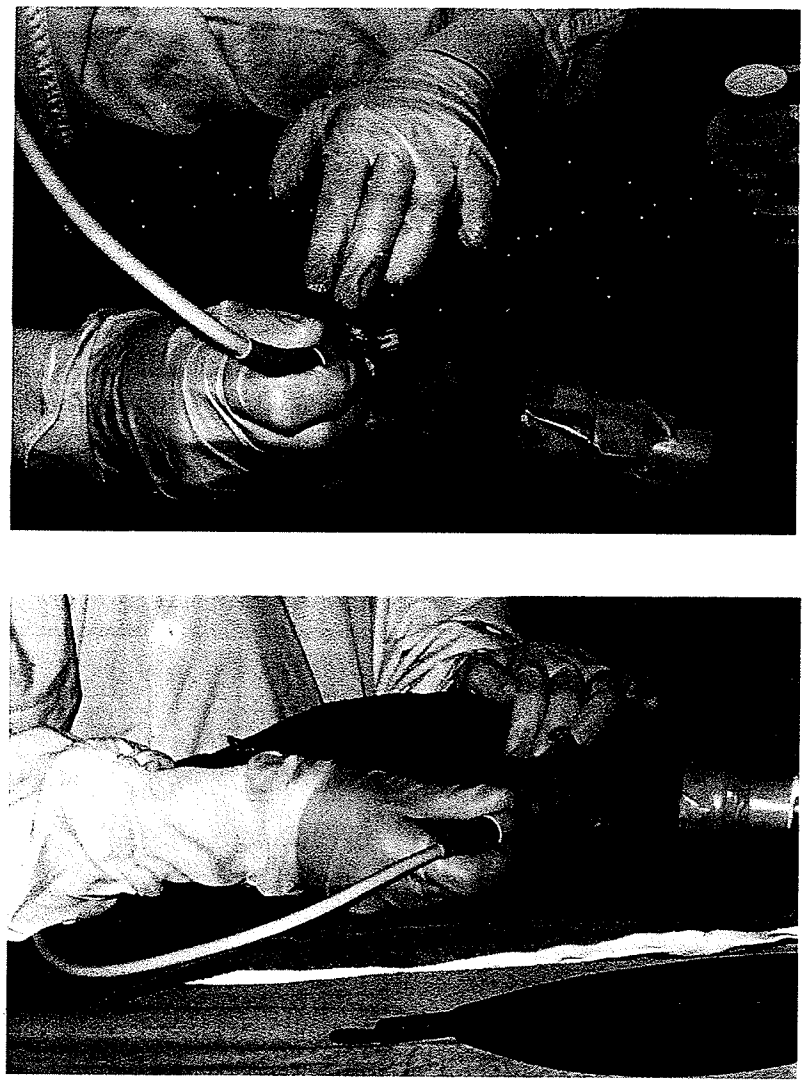


**Figure 11. Image Acquisition by Echocardiography.**
Upper panel: parasternal window
Lower panel: apical window

(according to the recommendations of the American Society of Echocardiography) (401,404).

Left ventricular systolic function was estimated by calculation of fractional shortening, using the formula (LVEDD - LVESD)/LVEDD (405). Left ventricular mass was calculated by the following formula which has been validated in rats (386,406), with the assumption of a spherical LV geometry (407): LV mass = 1.04 x [(LVEDD + PW + ST)$^3$ - LVEDD$^3$], where 1.04 g/ml = specific gravity of muscle, LVEDD = LV end-diastolic dimension (in cm), PW = end-diastolic posterior wall thickness (in cm), ST = septal thickness (in cm). The following mitral inflow parameters was obtained by the pulsed-wave Doppler technique, using the smallest sample volume (set at 0.06 cm) at the tip of the mitral valve leaflets (408): E velocity, A velocity, E/A ratio, and deceleration time (2). An average of 3 readings (390) was used for all parameters, where possible. Echocardiographic studies were analyzed in a blinded fashion to minimize bias (409).

Baseline echocardiography was performed on 26 obese rats and 12 lean rats to characterize cardiac structure and function. The former were utilized for *in vivo* drug evaluation, whereas the latter were sacrificed at 10 to 13 months of age for isolated heart experiments.

The obese rats were randomized to one of following 3 groups within a few days after baseline echocardiograms were obtained:

1) osmotic pump implantation + 4-week continuous i.v. infusion of SEA 1 mg/kg/day,

2) osmotic pump implantation + 4-week continuous i.v. infusion of vehicle,

3) no intervention [no osmotic pump implantation and no SEA/vehicle to assess if the procedure of osmotic pump implantation alone has any effects on ventricular function, in light of the previously reported stress-prone nature of these rats (352,362)].

After osmotic pump implantation, follow-up echocardiograms were obtained at the end of weeks 1, 2, 3, and 4 (Figure 9). After 4 weeks, all 26 obese rats were sacrificed for isolated heart experiments as described in the next section. Inspection of the osmotic pumps was made post-mortem to confirm emptying of reservoir.

## 3.6 Perfusion of Isolated Rat Hearts

Retrograde perfusion of the isolated rat heart was performed according to Langendorff (410). A protocol of global ischemia followed by reperfusion was employed to simulate IR during cardiac surgery (411), as outlined in Figure 12. The isolated heart experiments were initially performed under constant pressure conditions (80 mmHg). Unfortunately, because of technical problems with the pressure transducer that could not be easily resolved, the project was interrupted and had to be re-started on a different system (under constant flow conditions as detailed below). All isolated heart experiments reported in this thesis were performed using the latter system.

On the day of the experiment, the JCR:LA-*cp* rat (male, obese *cp/cp* or lean control +/+ or +/*cp*) were anesthetized with isoflurane at 30 minutes following pre-treatment with intra-peritoneal injection of heparin at 40 IU/kg for anticoagulation in the animal facility. The heart was excised and the aorta was cannulated for retrograde perfusion according to Langendorff (410), using an established perfusion system (412). Calibration of the perfusion system was performed prior to the start of the first experiment of each day.

The final composition of the perfusion solution was as follows (in mM): 120 NaCl, 20 $NaHCO_3$, 1.25 $CaCl_2$, 4.5 KCl, 1.2 $MgCl_2$, 1.2 $KH_2PO_4$, 10 dextrose (pH 7.4 at 37°C), modified from (361). The concentration of $CaCl_2$ was lowered from 1.8 to 1.25 mM approximately one third into the course of the studies, due to excessive ventricular arrhythmias, which will be detailed under section 5 'DISCUSSION'. All the results reported in this thesis were based on experiments using 1.25 mM of $CaCl_2$. The perfusion rate was maintained constant at 10 ml per minute.

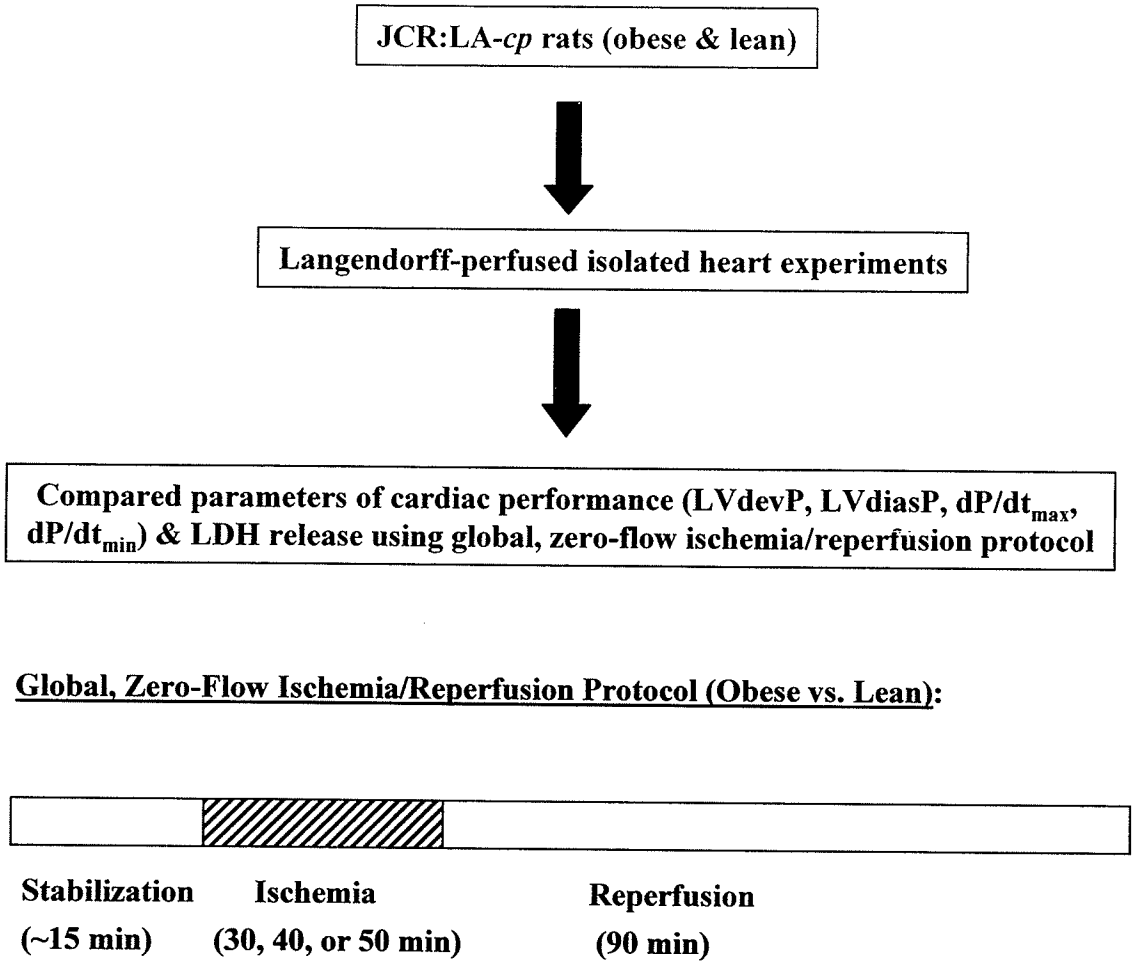
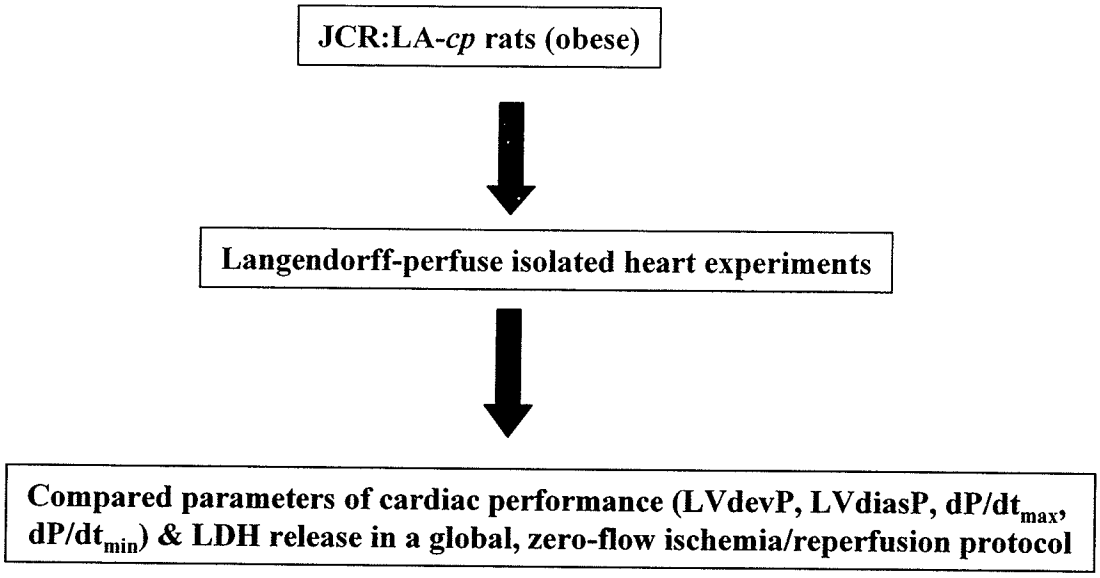



**Figure 12. Protocol of Langendorff-Perfused Isolated Heart Experiments Assessing Sensitivity to Ischemia (Obese *vs.* Lean Rats).**

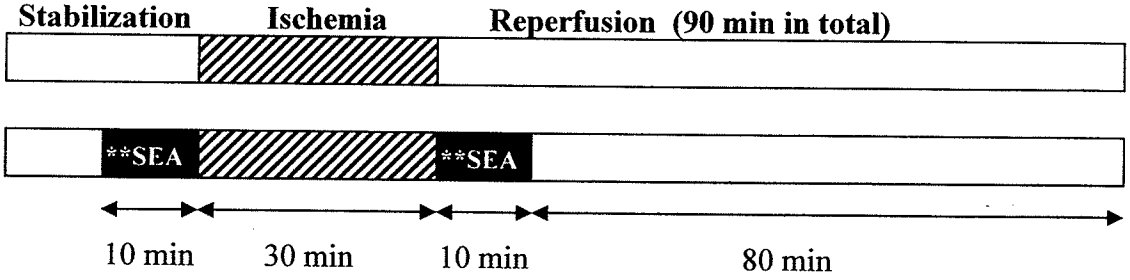
Once perfusion was established, the heart was electrically stimulated at 5 Hz (Harvard 6002 stimulator, Harvard Apparatus, Holliston, MA) to achieve a constant heart rate of 300 beats per minute throughout the experiment. The left atrium was incised and an unstressed, hand-made, plastic balloon (using commercial plastic wrap) was inserted into the LV through the mitral valve. The balloon was attached to a pressure transducer (Model 1050BP; BIOPAC Systems Inc., Goleta, CA) (412). Left ventricular diastolic pressure (LVdiasP) was maintained at $\leq$ 10 mmHg during the stabilization period (of approximately 15 minutes) by adjusting the size of the balloon. Once stabilization was achieved, the balloon remained isovolumic for the rest of the experiment. Global ischemia was induced by turning off the peristaltic pump and reperfusion was achieved by restarting the pump.

For assessment of sensitivity to myocardial ischemia, an ischemic period of 30, 40, or 50 minutes was used. The experimental protocol is presented in Figure 12. In preliminary experiments, 30 minutes of global ischemia resulted in moderate functional recovery (in the range of 40%). This duration was used in the evaluation of SEA0400 in the isolated hearts of obese rats. Three concentrations of SEA0400 were evaluated, 100 nM, 300 nM, 1 μM, based on cardioprotective efficacy demonstrated in previous studies (97,98).

The experimental protocol is presented in Figure 13. SEA0400 infusion was initiated immediately before the onset of global ischemia as a 10-minute infusion and again for 10 minutes at the start of reperfusion. In control experiments, an equivalent amount of DMSO used for dissolving SEA0400 was added to the solution for perfusion during the entire experiment.




**Global, Zero-Flow Ischemia/Reperfusion Protocol (*DMSO vs. SEA0400):**




* *For control, the perfusate contains DMSO (the equivalent amount for dissolving SEA0400) throughout the entire experiment.*

** *SEA0400 100 nM, 300 nM, or 1 μM*

**Figure 13. Protocol of Langendorff-Perfused Isolated Heart Experiments for Evaluating SEA0400.**

Cardiac performance was assessed by the following parameters: LV developed pressure (LVdevP), LV diastolic pressure (LVdiasP); and maximum rates of isovolumic pressure development and decline, $dP/dt_{max}$ and $dP/dt_{min}$, respectively (as calculated by the Acknowledge 3.5.3 software for Windows, BIOPAC System Inc., Goleta, CA) (412).

The following predefined exclusion criteria were employed:

1) LVSP < 60 mmHg at the end of the stabilization period

2) irreversible ventricular arrhythmias at the end of the stabilization period (up to 30 minutes from the time of excision of rat heart).

Attempts were also made to assess the effects of pre-ischemic administration alone and post-ischemic administration alone. As illustrated in Figure 14 (upper panel), there was a considerable distance between the drug chamber (wrapped in aluminum foil to minimize exposure of SEA0400 to light) and the chamber for the isolated heart (red arrow) in the Langendorff-perfusion system, introducing some difficulties in conducting these additional protocols (which will be detailed under 'RESULTS'). An alternative method of drug delivery via an infusion pump within a short distance from the isolated heart (Figure 14, lower panel, orange arrow) was also attempted. Preliminary results were also suboptimal and will be further discussed under 'RESULTS'.

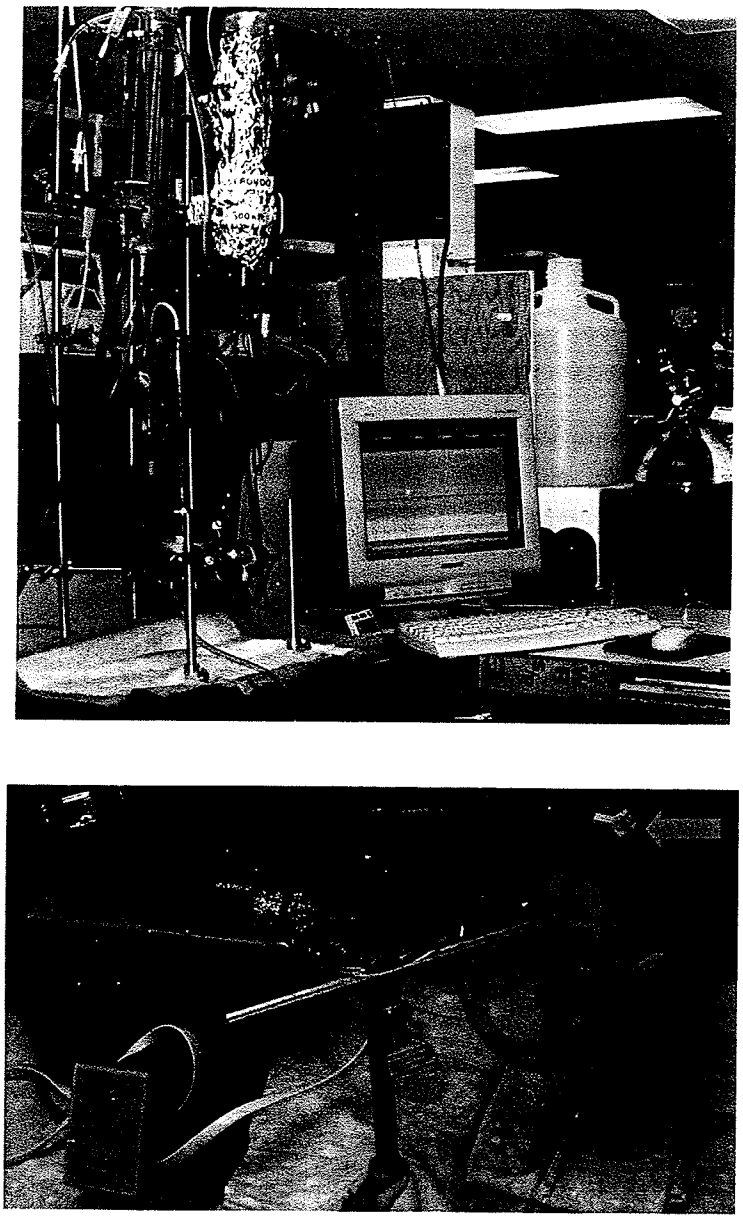


**Figure 14. Perfusion System for Isolated Heart Experiments.**
Upper panel: The SEA0400-containing chamber was wrapped with aluminum foil to minimize exposure of SEA0400 to light.
Lower panel: Alternative position for drug infusion via infusion pump was attempted. (The arrow indicates the level where SEA0400 entered the perfusion system.)

## 3.7 Measurement of Non-fasting Blood Glucose

Immediately after excision of rat heart for Langendorff-perfusion experiments, a drop of rat blood was placed on a new test strip of the glucometer (ACCU-CHEK® Compact System, Roche Diagnostics, Laval, PQ) for measurement of blood glucose level in the non-fasting state.

## 3.8 Measurement of Lactate Dehydrogenase in Coronary Effluent

Coronary effluent was collected to measure lactate dehydrogenase (LDH), a cytosolic enzyme, for estimation of the extent of myocardial injury (413). During initial experiments, the following schedule of frequent collection was employed to assess the timing of occurrence of peak LDH release (2 drops of coronary effluent per tube): baseline (in triplicate), 40 tubes immediately on reperfusion (collected at the maximum possible rate to capture peak LDH release), and then 40 tubes at 2-minute intervals for the rest of reperfusion. Samples were placed in liquid nitrogen and were stored in the freezer at -80°C until assay, using commercially available kits (Oxford Biomedical Research, Inc., Oxford, MI). The assays were performed according to the manufacturer's instructions (414) and quantification of colour development (as an indicator of the amount of LDH) was made using a Molecular Devices ThermoMax

microplate reader (Molecular Devices Corporation, Sunnyvale, CA). The colorimetric assay measured the amount of LDH release upon membrane damage or cytolysis and was based on the following reaction catalyzed by LDH (414):

$$NAD^+ + \text{lactate} \xrightarrow{\text{LDH}} \text{pyruvate} + NADH$$

where NAD(H) = nicotinamide-adenine dinucleotide (reduced) (4)

NADH was then reoxidized in a reaction coupled with the reduction of another substance, resulting in the formation of a formazan dye which was bright red in colour. A standard curve was constructed based on the average absorbance of the controls at pre-defined dilutions (the reconstituted control vial contained a known amount of LDH) and this standard curve was subsequently used for determining the concentration of LDH in the experimental wells of the microplate containing rat coronary effluent (414).

Based on initial results (n = 7), which demonstrated an early peak of LDH at the beginning of reperfusion (in the first tube n = 6, in the second tube n = 1), the frequency of collection was reduced: 16 tubes immediately on reperfusion and 1 tube every 10 minutes until the end of reperfusion. Based on further analysis of samples from 36 rats, it was determined that the amount of the LDH released as assessed in the collection of the first 7 tubes of coronary effluent, would provide the most representative data and therefore this value of 'LDH release during early reperfusion' will be reported in this thesis.

## 3.9 Quantification of Rat C-Reactive Protein

Exsanguinated rat blood from the aorta was collected (415) immediately on excision of the heart for Langendorff-perfusion experiments. Blood was allowed to clot for 30 minutes prior to centrifugation at 1000 x g for 10 minutes, according to the instructions from the manufacturer of the C-reactive protein kit (BD Biosciences, Mississauga, ON). The serum was gently aspirated with a pipette into a plastic tube, which was placed in liquid nitrogen until the end of the experiment. The sample was then transferred to the freezer for storage at - 80°C until assay.

For this solid phase sandwich enzyme-linked immunosorbent assay (ELISA) (416), an antibody specific for rat C-reactive protein was coated on a 96-well plate and the immobilized antibody bound any CRP present in samples or standards added into the wells. After a period of incubation followed by washing of the wells, a horseradish peroxidase conjugated anti-rat CRP was added to form an antibody-antigen-antibody 'sandwich' (416). Following another incubation period, the wells were again washed and a substrate solution containing 3,3',5,5'-tetramethylbenzidine was added which led to the production of colour in proportion to the amount of CRP present (416). The colour could then measured on a spectrophotometer or on an ELISA reader (416).

## 3.10 Measurement of Abdominal Girth

Measurement of abdominal girth in rat was made under general anesthesia immediately before dissection, using a piece of thread (Figure 15), which was later measured against a ruler. Care was taken to avoid excessive tension when placing the thread against the skin to optimize accuracy.

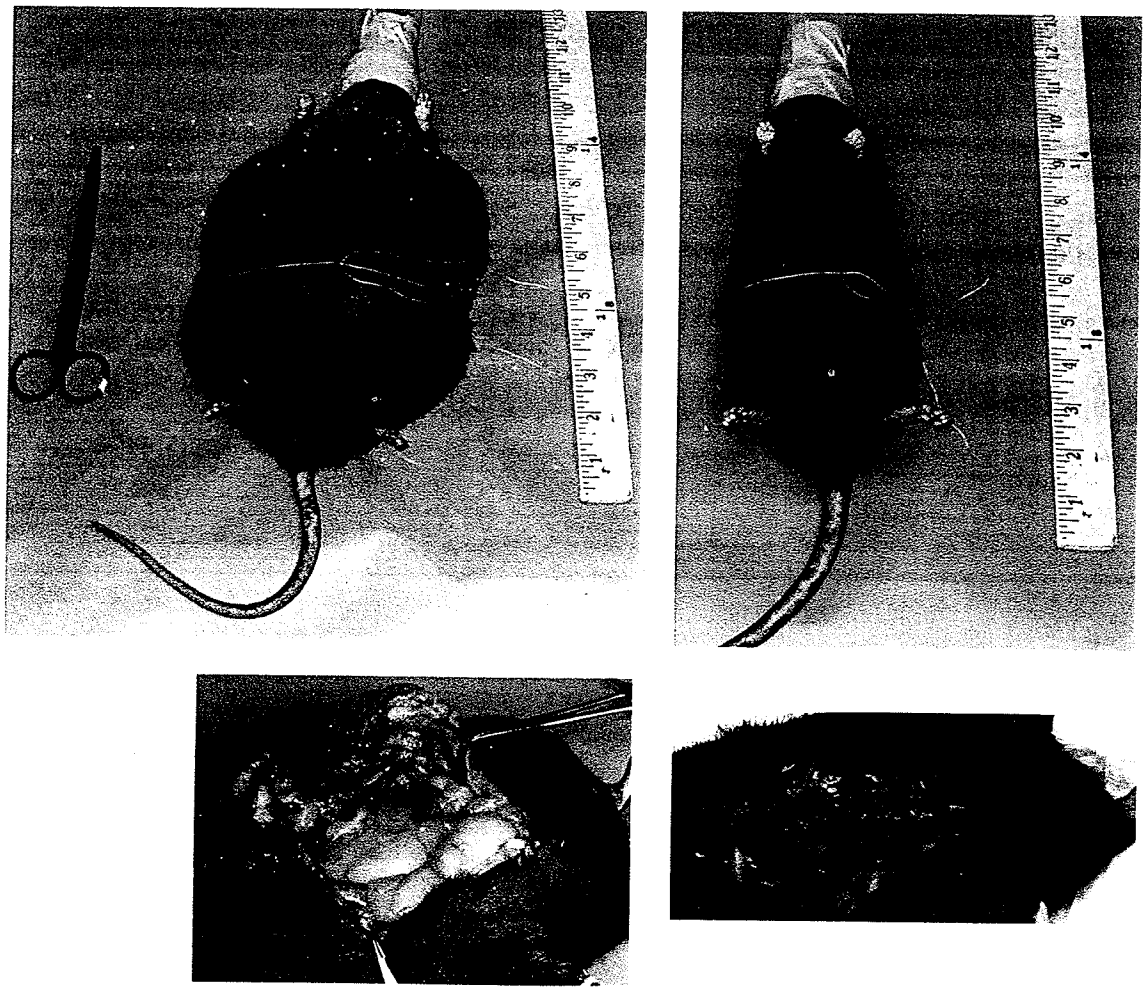


**Figure 15. The JCR:LA-cp Rat (12-13 Months), Obese and Lean.**
Upper panel: Measurement of Abdominal Girth.
Lower panel: Illustration of Intra-abdominal Adipose Tissue.

### 3.11 Statistical analysis

For comparison of baseline characteristics, 2-tailed independent t-test was used. Statistics pertaining to echocardiography (where blinding was used in the study protocol for drug evaluation and some of the comparisons between obese and lean rats) were performed with the professional assistance of the Biostatistical Consulting Unit at the University of Manitoba. Echocardiographic parameters of the SEA0400-treated group, the vehicle-treated group, and the no-intervention group at baseline and during weekly follow-up for a total of 4 weeks were analyzed using repeated measures analysis of variance followed by a least square means test for multiple comparisons.

For comparison between contractile parameters before and after infusion of SEA0400, 2-tailed paired t-test was employed. For analysis of other data obtained from isolated heart experiments, 2-tailed independent t-test was used for comparison between groups (417) and analysis of variance was used for multiple comparisons between groups at pre-specified time points (end of stabilization; end of ischemia; 30, 60, and 90 minutes after reperfusion). The following statistical software was employed: Microsoft® Excel Version 2002 (Microsoft Corporation, Redmond, WA), electronic worksheets from the textbook by Bolton and Bon (418), and ORIGIN® version 7.5 (OriginLab Corporation, Northampton, MA). Bonferroni correction was applied in multiple comparisons between groups to maintain the overall type I error at 0.05 (419), where applicable. The ORIGIN® software (420) was employed for correlation analysis and for making graphical presentations of all statistical data.

Statistical significance was defined as $p < 0.05$ for all statistical tests described in this thesis. Standard deviation (S.D.) will be presented for baseline characteristics and echocardiographic measurements, whereas standard error mean (S.E.M.) will be used for presentation and graphing of data on isolated heart experiments.

# 4. RESULTS

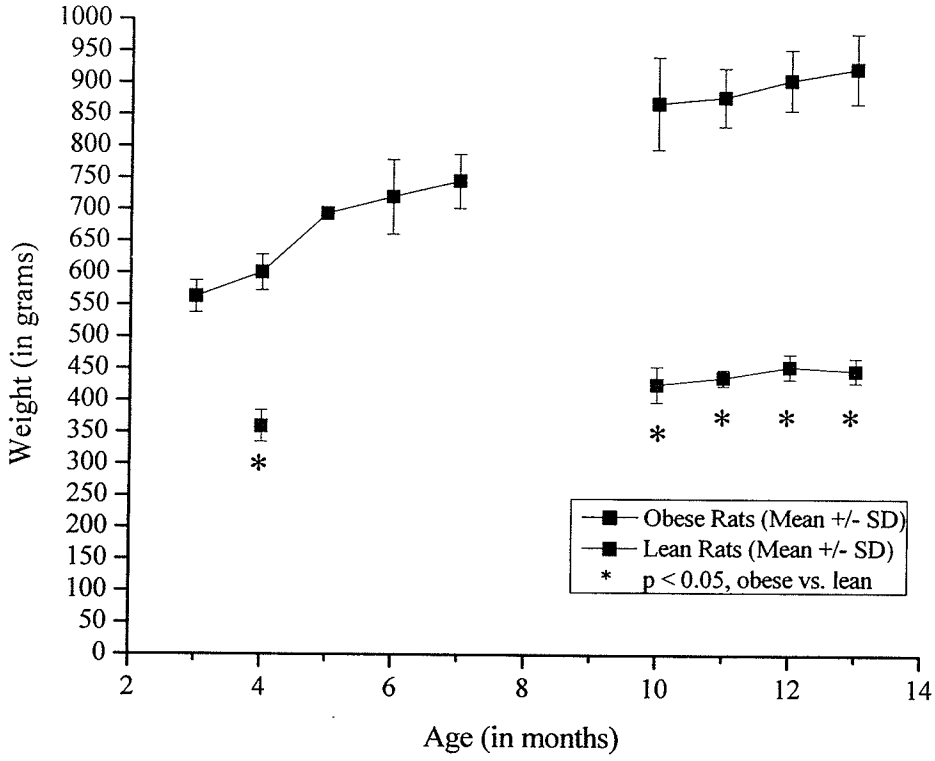
## 4.1 Baseline Characteristics of the JCR:LA-*cp* Rats

### *4.1.1 Age*

In total, 131 male JCR:LA-*cp* rats (107 obese, *cp/cp*, and 24 lean, +/+ or +/*cp*) were involved in this study. They were evaluated at 2 stages of their lives, 3 - 6 months (*in vitro* and *in vivo* experiments) and 10 - 13 months (*in vitro* experiments only). There were no stress-related deaths. Five rats were euthanized due to skin disease (n = 1), dehydration or gastrointestinal problems (n = 2); and respiratory distress within the first week following implantation of osmotic pumps (n = 2). The latter will be detailed in section 5.3.

### *4.1.2 Weight*

A comparison of the available weights of obese *vs* lean rats is presented in Figure 16. In 6 cases where serial weights were available without the confounding weights of the SEA0400/vehicle-filled osmotic pumps, specifically the 'no-intervention' group, the data at both weeks 1 and 4 were included in the statistical analysis. A statistically significant difference was detected at all time points (4, 10, 11, 12, 13 months, $p < 0.05$, obese *vs* lean) where comparisons were made.




| Age (months) | Weights of Obese Rats | | Weights of Lean Rats | |
|---|---|---|---|---|
| | Mean ± SD (g) | n | Mean ± SD (g) | n |
| 3 | 563 ± 25 | 5 | N/A | 0 |
| 4 | 601 ± 28 | 8 | 360 ± 25 | 12 |
| 5 | 694 ± 2 | 2 | N/A | 0 |
| 6 | 720 ± 59 | 22 | N/A | 0 |
| 7 | 745 ± 43 | 16 | N/A | 0 |
| 8 | N/A | 0 | N/A | 0 |
| 9 | 788 | 1 | N/A | 0 |
| 10 | 868 ± 73 | 25 | 427 ± 28 | 10 |
| 11 | 878 ± 46 | 23 | 438 ± 13 | 4 |
| 12 | 905 ± 48 | 24 | 455 ± 20 | 5 |
| 13 | 923 ± 55 | 9 | 449 ± 19 | 3 |

**Figure 16. Weights of Obese Rats *vs* Lean Rats.**

### *4.1.3 Abdominal Girth*

The available mean abdominal girth measurements of obese rats and lean rats are presented in Figure 17. Obese rats at 6 months of age had a significantly smaller abdominal girth than those at 10 months of age ($p < 0.05$). When comparisons were made between obese rats and lean rats, a statistically significant difference was detected at all time points evaluated (10, 11, 12, 13 months, $p < 0.05$ for comparison at each time point).

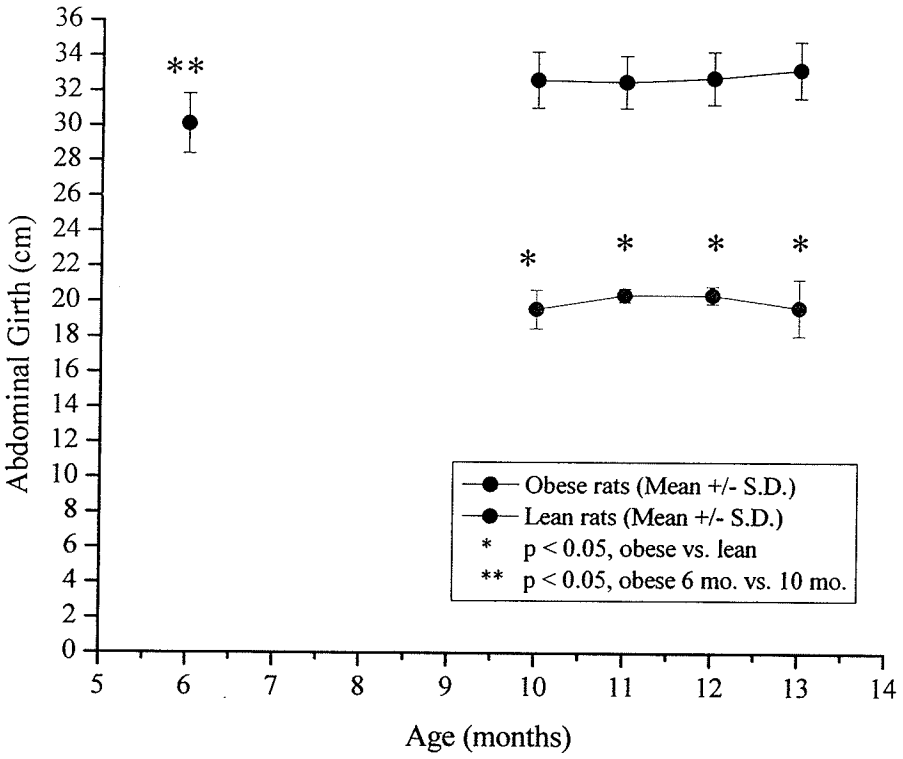
In 73 obese rats and 22 lean rats, both weight and abdominal girth were available. Correlation analysis was performed and the correlation coefficients (r) between abdominal girth and body weight for obese rats and for lean rats were 0.72 ($p < 0.0001$) and 0.53 ($p < 0.05$), respectively (Figure 18).

### *4.1.4 Non-fasting Glucose*

The mean ± SD of available non-fasting glucose levels of obese ($n = 35$) and lean ($n = 13$) rats were 13.4 ± 3.0 and 14.0 ± 1.8 mmol/L, respectively (Figure 19). There were no significant differences between the 2 groups.

### *4.1.5 C-Reactive Protein*

The mean ± SD C-reactive protein levels of obese rats and lean rats were 234.7 ± 25.2 and 228.4 ± 28.3 μg/mL, respectively (Figures 20 and 21). There were no statistically significant differences between the 2 groups.




| Age (months) | Abdominal Girth of Obese Rats | | Abdominal Girth of Lean Rats | |
|---|---|---|---|---|
| | Mean ± SD (cm) | n | Mean ± SD (cm) | n |
| 6 | 30.1 ± 1.7 | 4 | N/A | 0 |
| 10 | 32.6 ± 1.6 | 24 | 19.6 ± 1.1 | 10 |
| 11 | 32.5 ± 1.5 | 21 | 20.4 ± 0.4 | 4 |
| 12 | 32.7 ± 1.5 | 15 | 20.4 ± 0.5 | 5 |
| 13 | 33.2 ± 1.6 | 9 | 19.7 ± 1.6 | 3 |

**Figure 17. Abdominal Girth of Obese *vs* Lean Rats.**

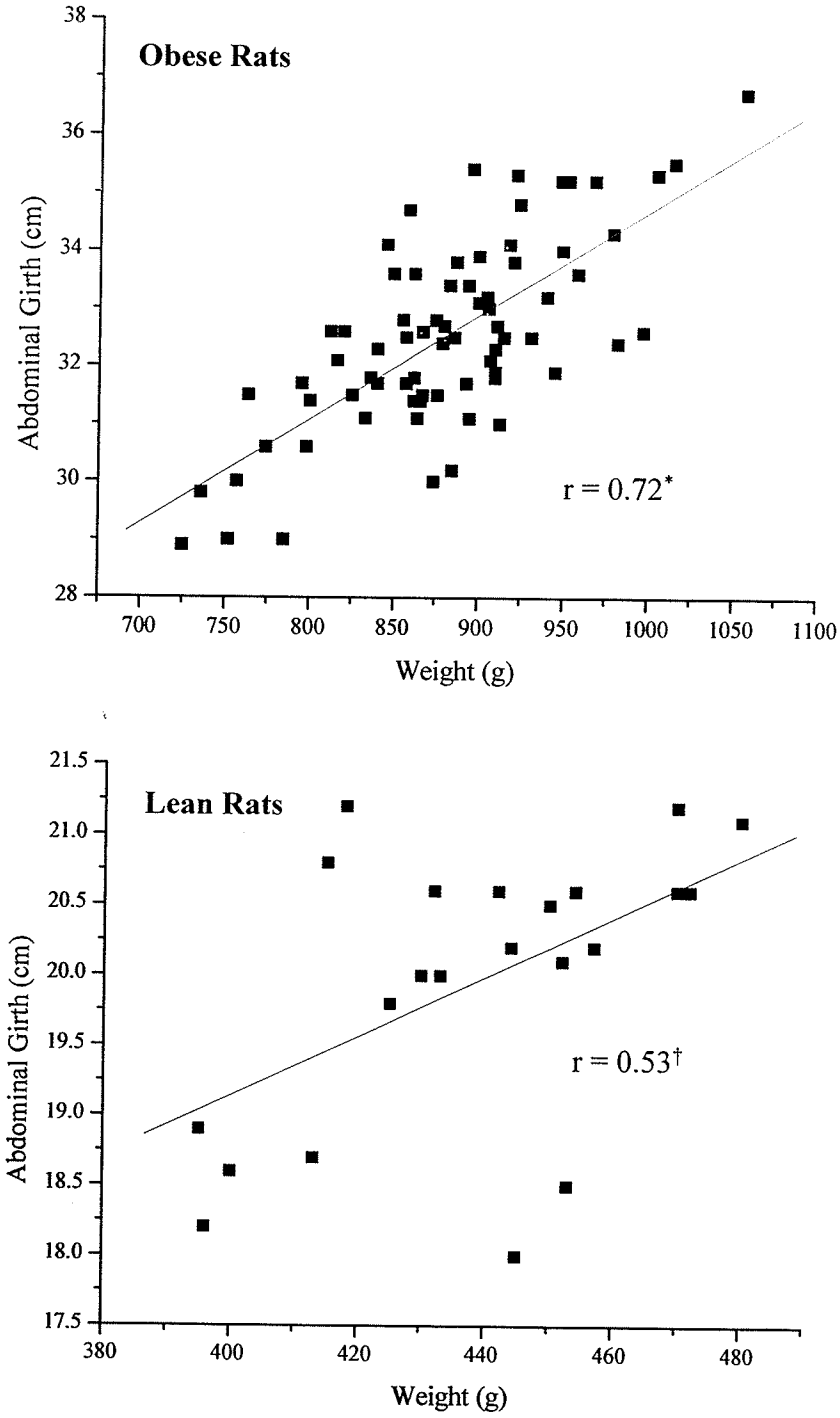



**Figure 18. Correlation Between Abdominal Girth and Weight.** Obese Rats (upper panel) and Lean Rats (lower panel). r = correlation coefficient. $^{*}p < 0.0001$, $^{\dagger}p < 0.05$.

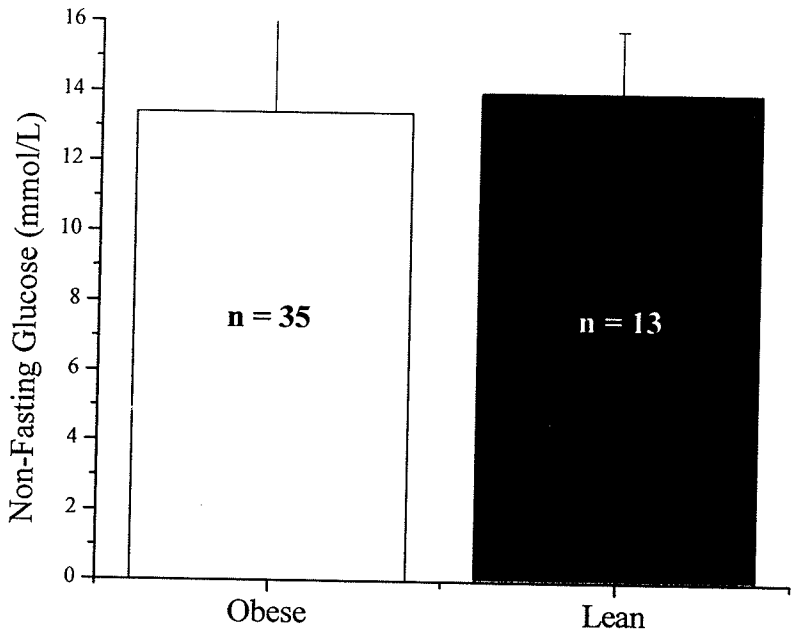



**Figure 19. Non-fasting Glucose of Obese *vs* Lean Rats.**
Values shown are means and standard deviations.

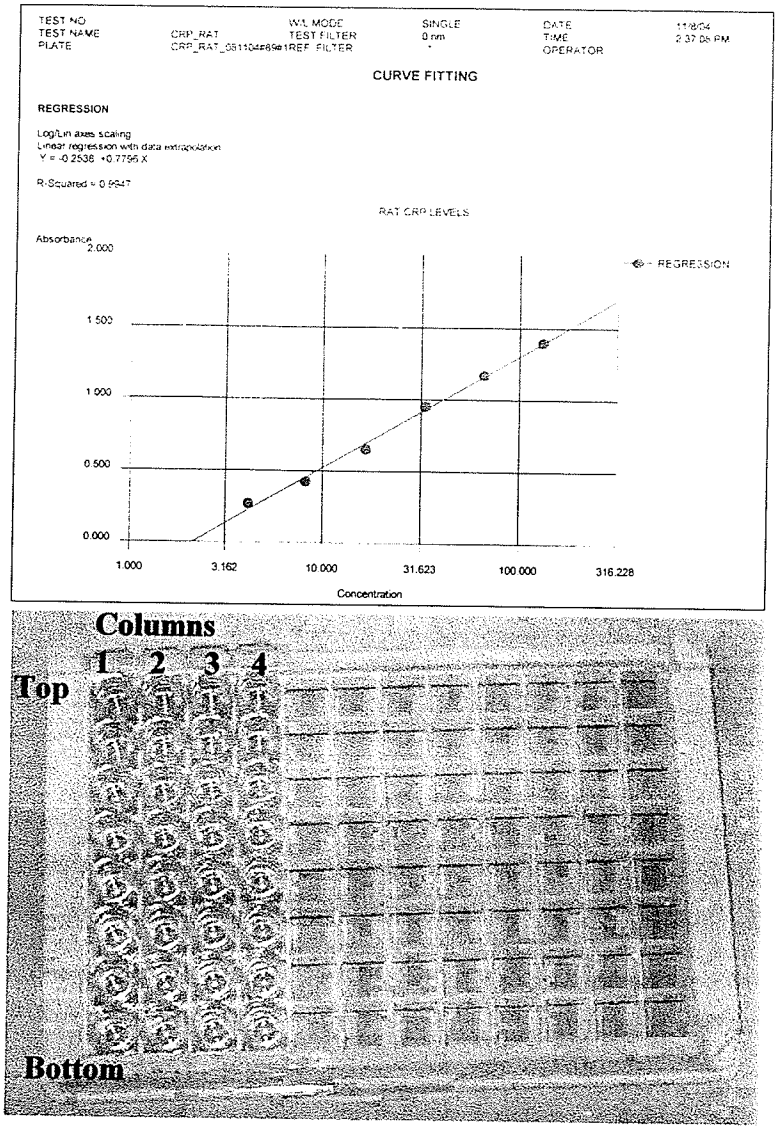



**Figure 20.** **Quantification of Rat Serum C-reactive Protein.**
Upper panel: Standard curve.
Lower panel: Colour reaction (columns numbered from left to right): standard samples in decreasing concentration (6 wells starting from top of column 1); 13 samples of lean rats (bottom 2 wells of column 1, all of column 2, top 3 wells of column 3); 13 samples of obese rats (bottom 5 wells of column 3, all of column 4).

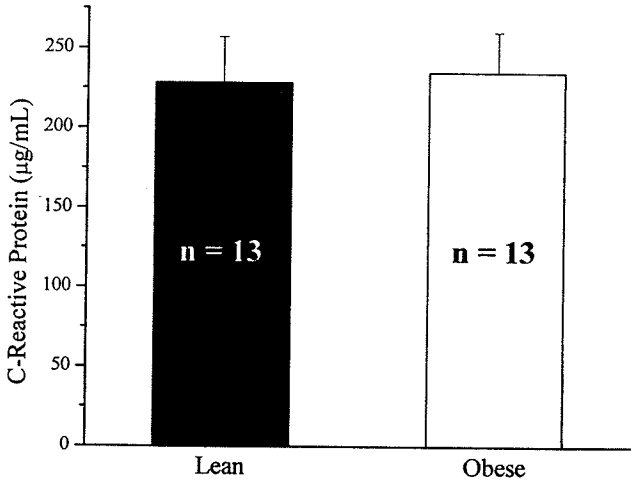



**Figure 21. Baseline C-Reactive Protein of Obese Rats *vs* Lean Rats.**
Values shown are means and standard deviations.

## 4.2 Characterization of Echocardiographic Features of the JCR:LA-*cp* Rats

### *4.2.1 Feasibility*

Transthoracic echocardiography was performed on 26 obese rats and 12 lean rats at baseline. There was no apparent morbidity or mortality. The duration of time required for anesthesia was typically less than 15 minutes.

### *4.2.2 Ventricular Dimensions and Wall Thicknesses*

Baseline data of obese rats (O) and lean rats (L) are presented in Table 2. An example of the echocardiographic images of the obese rats and the lean rats, along with their phenotypic appearance, is shown in Figure 22.

Heart rate and the duration of time required for anesthesia were similar between the 2 groups. Notably, the heart rates of the obese rats and the lean rats, 379 ± 27 and 371 ± 26 beats per minute, respectively, were in the physiological range for rats (421). Significant differences (mean ± SD) were detected in septal thickness (0.17 ± 0.01 *vs* 0.13 ± 0.01 cm, $p < 0.0001$, O *vs* L), posterior wall thickness (0.17 ± 0.01 *vs* 0.14 ± 0.01 cm, $p < 0.0001$, O *vs* L), and LV mass (0.76 ± 0.10 vs 0.60 ± 0.07 g, $p < 0.0001$, O *vs* L), consistent with findings of left ventricular hypertrophy. When the above LV mass was indexed with body weight, there was a significant difference in the reversed direction (LV mass/body weight, mean ± SD): 1.17 ± 0.00 *vs* 1.67 ± 0.00 mg/g ($p < 0.0001$, O *vs* L). No significant difference was observed in ventricular dimensions.

| **Results** (mean ± SD): | **Obese** (n = 26) | **Lean** (n = 12) | **P value** |
|---|---|---|---|
| Weight (g) | 652 ± 68 | 360 ± 25 | *<0.0001* |
| Heart rate (bpm) | 379 ± 27 | 371 ± 26 | 0.37 |
| Anesthesia time (min) | 11.3 ± 3.5 | 10.8 ± 3.4 | 0.68 |
| Septal thickness (cm) | 0.17 ± .01 | 0.13 ± .01 | *<0.0001* |
| LV end-diastolic dimension (cm) | 0.68 ± .04 | 0.71 ± .05 | 0.06 |
| Posterior wall (cm) | 0.17 ± .01 | 0.14 ± .01 | *<0.0001* |
| LV end-systolic dimension (cm) | 0.39 ± .04 | 0.38 ± .03 | 0.54 |
| Fractional shortening | 0.43 ± .04 | 0.47 ± .03 | *<0.01* |
| LV mass (g) | 0.76 ± .10 | 0.60 ± .07 | *<0.0001* |
| LV mass/Weight (mg/g) | 1.17 ± .00 | 1.67 ± .00 | *<0.0001* |
| *E/A | 1.54 ± .78 | 1.77 ± .56 | *0.02* |
| *Deceleration time (s) | 0.05 ± .02 | 0.04 ± .01 | 0.29 |

*16 obese, 11 lean

**Table 2. Echocardiography in the JCR:LA-*cp* Rats: Comparison of Baseline Characteristics of Obese (3 - 6 Months) *vs* Lean (4 Months) Rats.**

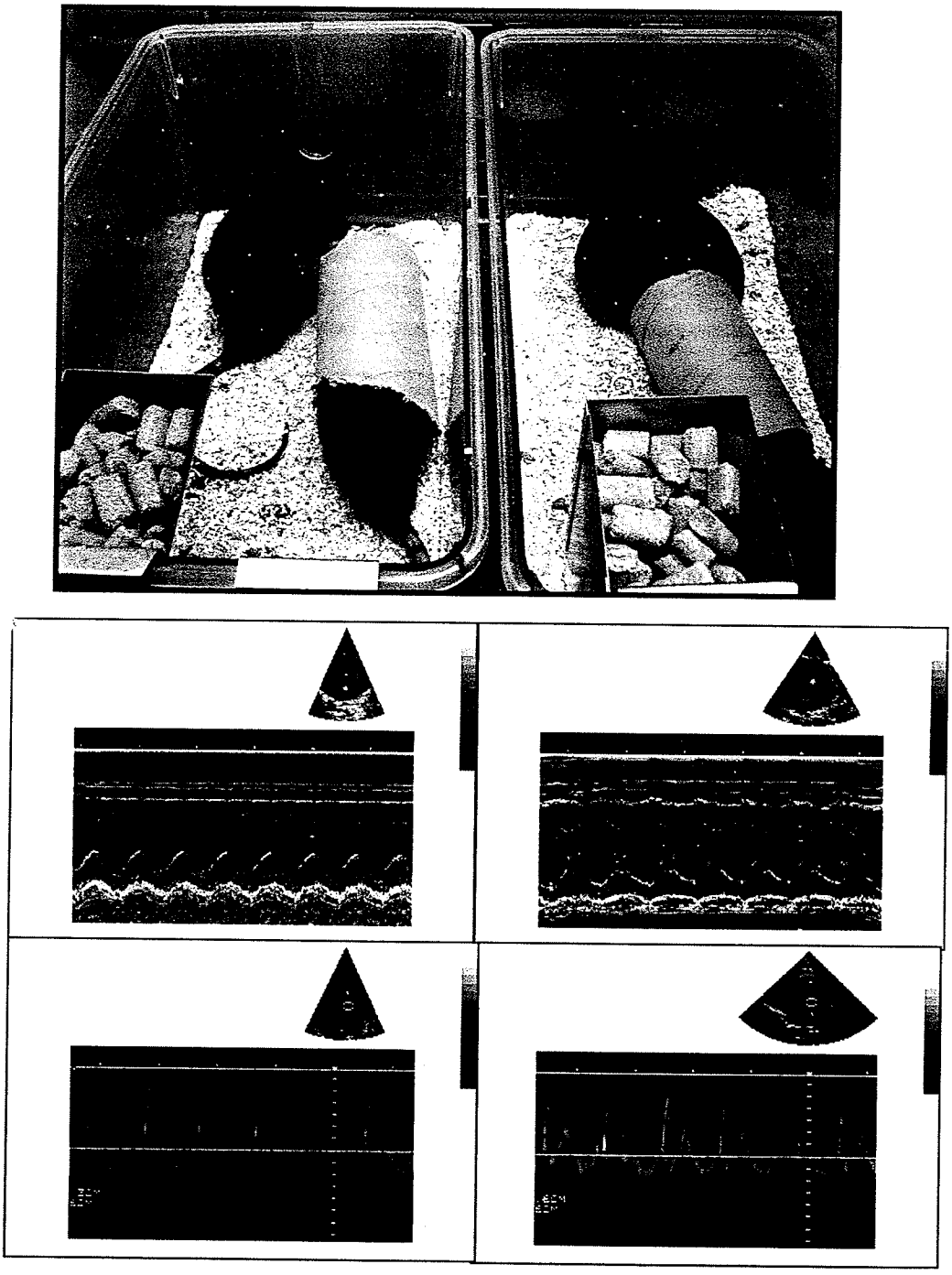


**Figure 22. Phenotypic and Echocardiographic Features of the JCR:LA-*cp* Rats, Obese (Right) vs. Lean (Left).**

Due to logistical difficulties in coordinating schedules (of echocardiography, anesthesia, and osmotic pump implantation) and the fact that the *cp/cp* rats were relatively scarce in supply [as they represented the 25% of the offspring of heterozygous rats and were themselves infertile (352)], the intended age range for the echocardiographic studies, 4 to 5 months, had to be extended to 3 to 6 months. In contrast, all the lean rats were of 4 months of age. As the impact of the above on the echocardiographic measurements was unclear (specifically, whether the above would have contributed to the findings of thicker walls and larger LV mass in the obese rats was unknown), a subsequent analysis was performed focusing on 10 obese rats at 3 to 4 months of age (3 months, n = 5; 4 months, n = 5) *vs* 12 lean rats (4 months). The data are presented in Table 3. A similar pattern of findings was still observed with the comparisons in wall thicknesses and LV mass remain significant. Specifically, in the comparison between most closely age-matched obese and lean rats: septal thickness 0.16 *vs* 0.13 cm ($p < 0.0001$), posterior wall 0.16 *vs* 0.14 cm ($p < 0.0001$), LV mass 0.70 *vs* 0.60 g ($p < 0.01$), and LV mass/weight 1.21 *vs* 1.67 mg/g ($p < 0.0001$).

| **Results** (mean ± SD): | **Obese** (n = 10) | Lean (n = 12) | **P value** |
|---|---|---|---|
| Weight (g) | 577 ± 28 | 360 ± 25 | *<0.0001* |
| Heart rate (bpm) | 384 ± 30 | 371 ± 26 | 0.26 |
| Anesthesia time (min) | 14.5 ± 2.6 | 10.8 ± 3.4 | *0.01* |
| Septal thickness (cm) | 0.16 ± .01 | 0.13 ± .01 | *<0.0001* |
| LV end-diastolic dimension (cm) | 0.68 ± .04 | 0.71 ± .05 | 0.18 |
| Posterior wall (cm) | 0.16 ± .01 | 0.14 ± .01 | *<0.0001* |
| LV end-systolic dimension (cm) | 0.37 ± .04 | 0.38 ± .03 | 0.68 |
| Fractional shortening | 0.46 ± .03 | 0.47 ± .03 | 0.39 |
| LV mass (g) | 0.70 ± .07 | 0.60 ± .07 | *<0.01* |
| LV mass/Weight (mg/g) | 1.21 ± .00 | 1.67 ± .00 | *<0.0001* |
| *E/A | 1.54 ± .53 | 1.77 ± .56 | 0.09 |
| *Deceleration time (s) | 0.05 ± .02 | 0.04 ± .01 | 0.21 |

*9 obese, 11 lean

**Table 3. Further Analysis of Baseline Characteristics Focusing on 10 Obese Rats (3 - 4 Months) *vs* Lean Rats (4 Months).**

### *4.2.3 Systolic and Diastolic Function*

Systolic function was assessed by the calculated fractional shortening (FS), which showed a statistically significant difference between the 2 groups (mean ± SD): 0.43 ± 0.04 *vs* 0.47 ± 0.03 ($p < 0.01$, O *vs* L, Table 2). However, when the mean FS of the 10 obese rats at 3 - 4 months of age (0.46 ± 0.03) was compared with that of the lean rats at 4 months of age (0.47 ± 0.03), the difference was no longer significant (Table 3).

Assessment of diastolic function, as estimated from mitral inflow parameters, was possible in 16 of the 26 obese rats, and 11 of the 12 lean rats. The main reasons for not being able to obtain these parameters in some rats included complete fusion of the E wave and the A wave as a result of high heart rate and technical difficulties. Comparison of the E/A ratio and the deceleration time between the obese rats ($n = 26$) and the lean rats ($n = 12$) showed with a statistically significant reduction in the E/A ratio in the obese rats: 1.54 ± 0.78 and 1.77 ± 0.56 ($p = 0.02$, O *vs* L), suggesting the possibility of impaired relaxation (2), although the numeric increase in deceleration time (0.05 ± 0.02 *vs* 0.04 ± 0.01, O *vs* L) did not reach statistical significance ($p = 0.29$). Further analysis focusing on the results of 10 best-matched obese rats in terms of age (3 to 4 months) to the 12 lean rats (4 months) showed a numeric decrease in E/A ratio in the obese rats (O *vs* L, $p = 0.09$): 1.54 ± 0.53 and 1.77 ± 0.56, and the findings on deceleration time were similar to the overall analysis as shown above (0.05 ± 0.02 *vs* 0.04 ± 0.01, $p = 0.21$).

## 4.3 Serial Echocardiographic Assessments of Cardiac Function during Prolonged *In Vivo* Administration of SEA0400 in the JCR:LA-*cp* rat

### *4.3.1 Feasibility*

Twenty-six obese JCR:LA-*cp* rats (male, *cp/cp*, 3 – 6 months) were involved in this part of the study. Osmotic pumps were implanted in 20 obese JCR:LA-*cp* rats which were randomized to a 4-week i.v. continuous infusion of SEA0400 1 mg/kg/day (n = 13) or vehicle (n = 7). Six rats were randomly assigned to the 'no-intervention' group (no osmotic pump implantation and no SEA0400/vehicle) as a control group to assess for any potential effects of osmotic pump implantation alone.

Of these 26 rats, 2 rats (1 SEA-treated, 1 vehicle-treated) were euthanized after osmotic pump implantation due to respiratory distress. These 2 rats were noted by the staff of the animal facility on day 4 after the procedure to exhibit unusual behavior. One rat appeared lethargic, dehydrated, stressed, and lost its appetite. Its breathing appeared laboured. A decision was made to euthanize the rat. (An echocardiogram was not performed for diagnostic purposes prior to euthanization of the 2 rats, as the incidents occurred during my absence at the American Heart Association Scientific Sessions in 2003.) After euthanization, the thorax of the rat was opened and the position of the catheter was checked by the personnel who performed the implantation procedure. It was reported to be unremarkable. A blood smear was obtained to screen for evidence of infection and was also reported to be unremarkable. The cause of the deterioration of this rat was unclear. The second rat showed similar signs, but to a lesser extent, as compared to those of the first rat and was treated empirically with enrofloxacin 10mg/kg

intramuscularly for 3 days. Because of a lack of improvement, the second rat was also euthanized. After euthanization, the catheter of the osmotic pump was again examined by the personnel who performed the procedure and was reported to be unremarkable. However, the thoracic cavity of this second rat was noted to be full of clear liquid and the possibility of heart failure was contemplated at that time. However, since a formal necropsy was not performed, the exact causes of death of these 2 rats remain speculative. Data from these 2 rats were excluded from analysis in this section on drug evaluation, although their baseline echocardiographic findings have been included in the comparison between obese rats *vs* lean rats in the previous section.

### *4.3.2 Weight and Heart Rate*

The baseline weights (mean ± SD) of the rats in SEA0400-treated group, the vehicle-treated group, and the 'no-intervention' group were 672 ± 66, 631 ± 60, 652 ± 82 grams, respectively, which were significantly different (p values as listed in Table 4).

All the heart rates presented in Table 4 were within the physiological range of heart rate for rats (421). Statistically significant differences were present in some of the comparisons, although biological significance, if any, was unclear.

| Parameter over 4 weeks (mean±SD) | SEA0400 n = 12 | Vehicle n = 6 | Control n = 6 | P value |
|---|---|---|---|---|
| Week 0 Wt | *‡672 ± 66 | †‡631 ± 60 | *†652 ± 82 | *† <.001, ‡<.0001 |
| Week 1 Wt | †667 ± 64 | *†641 ± 58 | *669 ± 84 | *† <.0001 |
| Week 2 Wt | †684 ± 63 | *†669 ± 54 | *685 ± 87 | *† <.001 |
| Week 3 Wt | *689 ± 63 | †684 ± 46 | *†700 ± 92 | * <.05, † <.01 |
| Week 4 Wt | 717 ± 58 | 712 ± 45 | 710 ± 83 | NS |
| Week 0 HR | *371 ± 24 | 383 ± 33 | *397 ± 24 | * <.05 |
| Week 1 HR | 368 ± 30 | 364 ± 22 | 368 ± 31 | NS |
| Week 2 HR | 358 ± 32 | *342 ± 38 | *371 ± 31 | * <.05 |
| Week 3 HR | 368 ± 35 | 360 ± 32 | 367 ± 25 | NS |
| Week 4 HR | *361 ± 26 | †350 ± 29 | *†384 ± 23 | * <.05, † <.01 |
| Week 0 FS | .42 ± .04 | .44 ± .04 | .45 ± .03 | NS |
| Week 1 FS | .47 ± .04 | .44 ± .04 | .44 ± .03 | NS |
| Week 2 FS | .46 ± .04 | .46 ± .04 | .45 ± .05 | NS |
| Week 3 FS | .45 ± .03 | .45 ± .05 | .43 ± .05 | NS |
| Week 4 FS | .45 ± .03 | .46 ± .07 | .46 ± .03 | NS |
| Week 0 E/A | 1.5 ± 0.2 | 1.7 ± 0.1 | 1.4 ± 0.3 | NS |
| Week 1 E/A | 1.5 ± 0.2 | 1.4 ± 0.2 | 1.5 ± 0.2 | NS |
| Week 2 E/A | *1.4 ± 0.3 | *1.8 ± 0.3 | 1.6 ± 0.3 | * <.01 |
| Week 3 E/A | *†1.6 ± 0.2 | †1.9 ± 0.4 | *2.0 ± 0.1 | *† <.01 |
| Week 4 E/A | 1.4 ± 0.2 | 1.6 ± 0.3 | 1.6 ± 0.4 | NS |
| Week 0 DT | .05 ± .01 | .05 ± .01 | .04 ± .00 | NS |
| Week 1 DT | .04 ± .00 | .04 ± .00 | .04 ± .01 | NS |
| Week 2 DT | †.05 ± .01 | *†.05 ± .01 | *.05 ± .01 | * <.05, † <.01 |
| Week 3 DT | .05 ± .01 | *.05 ± .01 | *.04 ± .00 | * <.01 |
| Week 4 DT | .05 ± .01 | .05 ± .00 | .04 ± .01 | NS |

Each reading represents an average of 3 measurements, where possible in most cases.
Wt = Weight (g), HR = Heart rate (beats per min), FS = Fractional shortening, E/A = Ratio of maximum filling velocity (E velocity) to the atrial velocity (A velocity) of LV diastolic filling.

**Table 4. Summary of Serial Echocardiographic Data (Part 1 of 2).**

### *4.3.3 Left Ventricular Structure*

Left ventricular systolic and diastolic dimensions, as well as the thickness of the septum and of the posterior wall, were compared among the 3 groups (Table 5). Even though statistical significance was detected in some of the comparisons, the functional significance was unclear as the differences did not seem to point to any unifying, consistent pattern of structural abnormalities. A more likely explanation would be variability introduced as a result of technical challenges in achieving the ideal position during image acquisition, such as in achieving the exact same plane at the level of the papillary muscles in the mid-LV on serial measurements. These challenges arose primarily due to difficulties in positioning the obese rat in the presence of excess, soft adipose tissues and a subcutaneously-implanted pump on its dorsal side within the permitted anesthesia time frame of approximately 15 minutes (which included preparation prior to image acquisition). Similarly, the differences seen in the comparison of the calculated LV mass may possibly be explained by the above, as the formula for LV mass calculation includes the above measurements (386,406), assuming spherical LV geometry (407): LV mass = $1.04 \times [(LVEDD + PW + ST)^3 - LVEDD^3]$, where 1.04 g/ml = specific gravity of muscle, LVEDD = LV end-diastolic dimension (in cm), PW = end-diastolic posterior wall thickness (in cm), ST = septal thickness (in cm).

| *Parameter over 4 weeks (mean±SD) | SEA0400 n = 12 | Vehicle n = 6 | Control n = 6 | P value |
|---|---|---|---|---|
| Week 0 ST | .166 ± .015 | .160 ± .011 | .166 ± .014 | NS |
| Week 1 ST | .160 ± .012 | .163 ± .010 | .163 ± .014 | NS |
| Week 2 ST | .167 ± .014 | .161 ± .009 | .164 ± .011 | NS |
| Week 3 ST | *.167 ± .012 | *.159 ± .014 | .165 ± .006 | *<.05 |
| Week 4 ST | .170 ± .011 | .168 ± .018 | .171 ± .016 | NS |
| Week 0 PW | †.169 ± .012 | *†.161 ± .011 | * .171 ± .009 | *†<.05 |
| Week 1 PW | .167 ± .008 | .164 ± .011 | .166 ± .013 | NS |
| Week 2 PW | *.172 ± .012 | *.161 ± .008 | .167 ± .011 | *<.01 |
| Week 3 PW | *.170 ± .008 | *.162 ± .005 | .169 ± .007 | *<.05 |
| Week 4 PW | .170 ± .007 | .174 ± .014 | .171 ± .007 | NS |
| Week 0 LVESD | .391 ± .043 | .380 ± .030 | .369 ± .035 | NS |
| Week 1 LVESD | *.357 ± .049 | .377 ± .064 | *.400 ± .022 | *<.05 |
| Week 2 LVESD | *.359 ± .036 | .360 ± .036 | *.393 ± .059 | *<.05 |
| Week 3 LVESD | .380 ± .035 | .396 ± .027 | .409 ± .058 | NS |
| Week 4 LVESD | .372 ± .037 | .403 ± .052 | .391 ± .035 | NS |
| Week 0 LVEDD | .678 ± .046 | .680 ± .026 | .674 ± .034 | NS |
| Week 1 LVEDD | *.667 ± .057 | .677 ± .078 | *.717 ± .030 | *<.05 |
| Week 2 LVEDD | *.663 ± .034 | .668 ± .032 | *.707 ± .049 | *<.05 |
| Week 3 LVEDD | .688 ± .037 | .718 ± .027 | .713 ± .052 | NS |
| Week 4 LVEDD | *†.671 ± .042 | †.740 ± .030 | *.722 ± .049 | *†<.05 |
| Week 0 LVM | .760 ± .105 | .720 ± .097 | .760 ± .090 | NS |
| Week 1 LVM | *.720 ± .124 | .737 ± .150 | *.809 ± .091 | *<.05 |
| Week 2 LVM | .746 ± .100 | *.699 ± .075 | *.803 ± .143 | *<.05 |
| Week 3 LVM | .788 ± .103 | .786 ± .092 | .824 ± .111 | NS |
| Week 4 LVM | *†.761 ± .062 | †.895 ± .104 | *.864 ± .100 | *<.01 †<.001 |

Each reading represents an average of 3 measurements, where possible in most cases.
ST = Septal thickness (cm), PW = Posterior Wall (cm), LVESD = Left ventricular end-systolic dimensions (cm), LVEDD = LV end-diastolic dimensions (cm), LVM = Left ventricular mass (g).

**Table 5. Summary of Serial Echocardiographic Data (Part 2 of 2).**

### 4.3.4 *Left Ventricular Systolic and Diastolic Function*

While the above may have posed some limitations to the measurement of wall thicknesses and ventricular dimensions, the evaluation of left ventricular systolic and diastolic function, which was the primary goal of *in vivo* evaluation of SEA0400 in this study would likely be affected to a lesser extent, since ratios (as opposed to absolute values) were involved in the assessment of fractional shortening (LVEDD - LVESD)/LVEDD and E/A ratio [ratio of the early maximum filling velocity (E velocity) to the atrial velocity (A velocity) of left ventricular diastolic filling]. As shown in Table 4, the fractional shortening of all groups appeared quite similar, ranging from 0.42 to 0.47. There were no statistically significant differences detected in the comparison of fractional shortening between groups at all time points of serial echocardiography. With regard to diastolic function assessment, statistically significant differences were shown in some of the comparisons between groups at weeks 2 and 3, but the differences were no longer present at week 4. The functional significance of these findings, if any, was unclear, especially given the very small numeric differences, in the deceleration time. Moreover, the use of mitral inflow parameters for the assessment of diastolic function has a number of limitations, which will be detailed under 'DISCUSSION'.

### 4.3.5 *Other Findings*

Delayed re-growth of hair following shaving for osmotic pump implantation was observed in obese rats in both the SEA0400-treated group and the vehicle-treated group, up to 4 weeks after the surgical procedure (Figure 23).

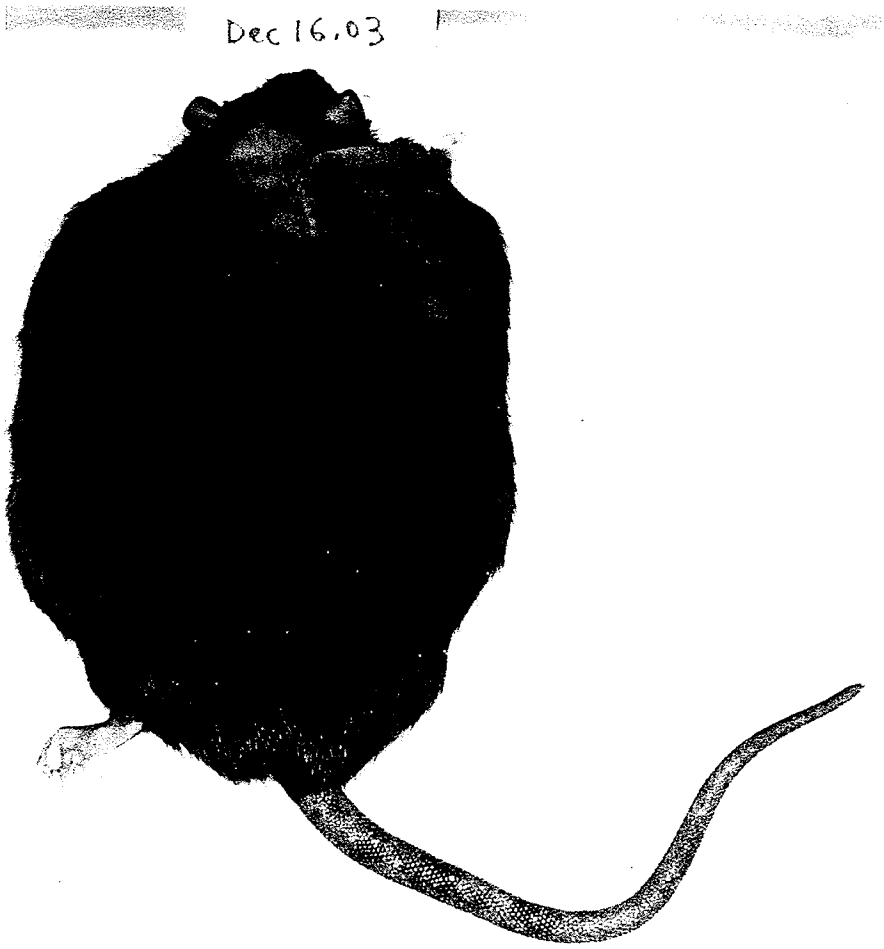



**Figure 23. Example of Delayed Regrowth of Hair at 4 Weeks after Osmotic Pump Implantation in a *cp/cp* Rat.**

## 4.4 Sensitivity to Ischemia as Assessed in the Langendorff-Perfused Isolated Hearts (Senescent, Obese JCR:LA-*cp* Rats *vs* Lean Controls)

Obese and lean JCR:LA-*cp* rats were subjected to global ischemia of 30, 40, and 50 minutes followed by 90-minute reperfusion. The main focus of this comparison was to compare the myocardial response of obese rats and lean rats subjected to 30 minutes of ischemia, as this duration was also employed in the evaluation of SEA0400 in obese rats (section 4.6) – based on results of preliminary experiments showing approximately 40% spontaneous recovery of LVdevP. However, comparisons were also made for longer durations of ischemia (40 min and 50 min) with a goal toward substantiating findings by increasing the severity of the ischemic insult.

As blinding was not possible in this part of the study, time intervals for manual recording of data were pre-defined to be every 10 minutes throughout the experiments to minimize bias. Of note, it was observed on many occasions that an undulating baseline appeared during reperfusion (Figure 24, lower panel). The source of the undulation, based on its frequency, was determined to originate from the roller pump. Since this undulation was compressed when the tracings were displayed on a compressed time scale (upper panel) and could not be automatically distinguished from the true LV developed pressure by the computer program, instantaneous pressure readings at the peaks of the undulations at pre-specified time points were used in all data collection to minimize variation introduced by this artifact. As illustrated in the example in Figure 24, where the % recovery of LV developed pressure at the end of the experiment was calculated using both methods, an overestimation of the parameter

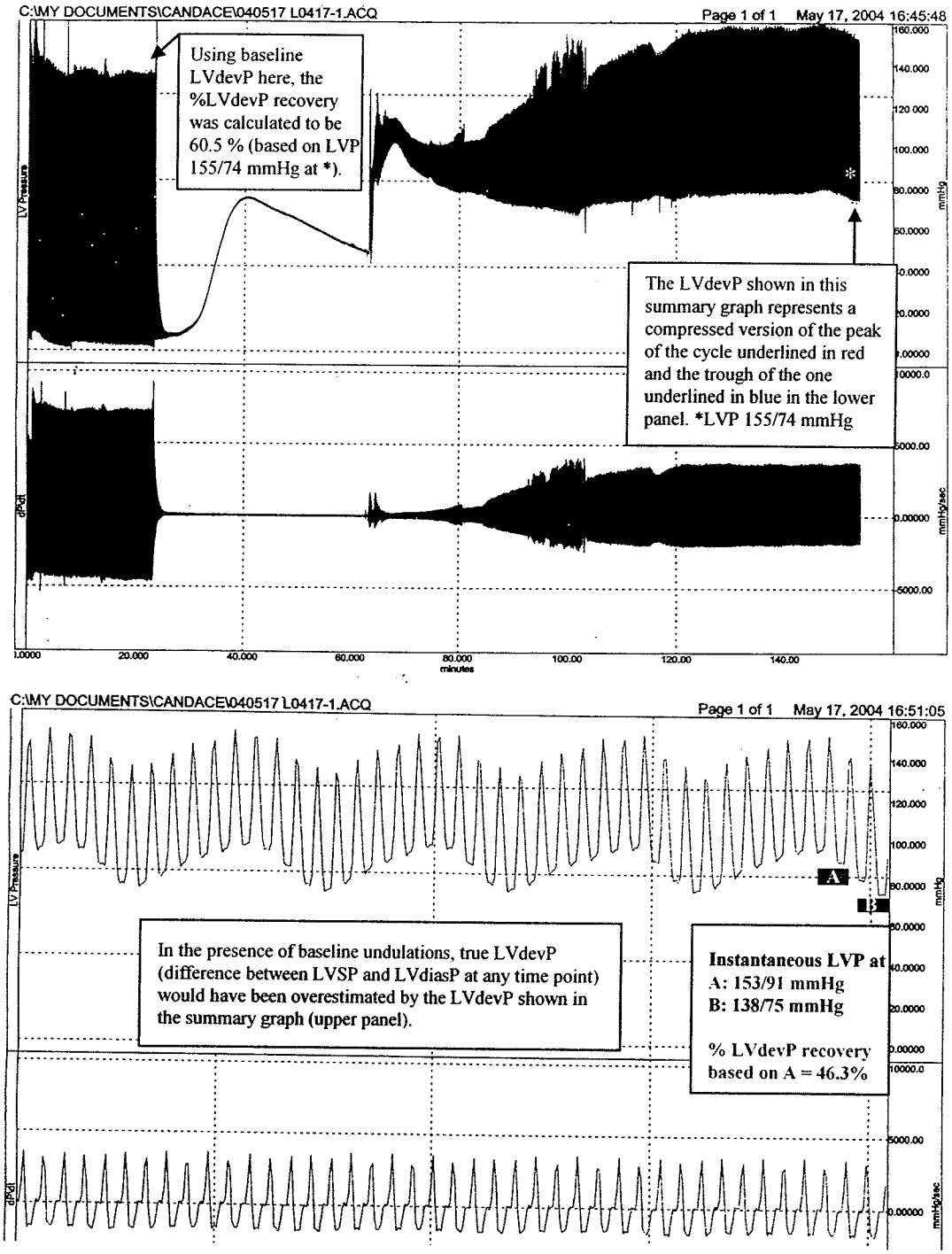



**Fig. 24. Example of Baseline Undulations Observed During Reperfusion.**
Upper Panel: Summary Chart (from top to bottom: LVP, dP/dt).
Lower Panel: Expanded Time Scale (last 8 sec of the experiment).

occurred when the LV pressure readings from the summary graph (upper panel) was used (60.5% recovery of LV developed pressure) as opposed to 46.8%, using the instantaneous pressure readings at the last peak of the undulating tracing (point A, lower panel) of the entire experiment.

### *4.4.1 Thirty Minutes of Global Ischemia*

Data on the cardiac performance of isolated hearts subjected to 30 minutes of global ischemia, as well as the statistical analysis at pre-defined time points (pre-ischemia, end-ischemia; 30, 60, and 90 minutes of reperfusion), are presented in Table 6. The corresponding % pre-ischemia of the hemodynamic data are also presented in Table 6. Data at other time points are plotted graphically in Figures 25 - 28. As shown in Table 6, parameters of cardiac performance (mean ± SEM, O *vs* L) were similar between the 2 groups both at baseline (LVdiasP 5.2 ± 0.7 *vs* 5.1 ± 1.0 mmHg, LVdevP 100.6 ± 8.3 *vs* 118.3 ± 10.8 mmHg, $dP/dt_{max}$ 5562.6 ± 555.8 *vs* 6788.3 ± 536.3 mmHg/s, and $dP/dt_{min}$ -3570.4 ± 297.5 *vs* -2774.4 ± 1216.1 mmHg/s) and at the end of 30 minutes of ischemia (LVdiasP 74.2 ± 6.4 *vs* 59.3 ± 2.0 mmHg, LVdevP 1.3 ± 0.2 *vs* 1.8 ± 0.7 mmHg, $dP/dt_{max}$ 59.4 ± 11.4 *vs* 133.0 ± 74.2 mmHg/s, and $dP/dt_{min}$ -60.8 ± 16.2 *vs* -129.6 ± 72.4 mmHg/s). After 30 minutes of reperfusion, all parameters were significantly worse in the obese group (O *vs* L, $p < 0.01$, except for $dP/dt_{max}$ where $p < 0.05$): LVdiasP 96.0 ± 4.7 *vs* 60.4 ± 7.7 mmHg, LVdevP 21.2 ± 6.2 *vs* 65.8 ± 11.6 mmHg, $dP/dt_{max}$ 1127.0 ± 386.4 *vs* 3517.7 ± 673.4 mmHg/s, $dP/dt_{min}$ -786.1 ± 219.1 *vs* -2405.0 ± 355.6 mmHg/s. The difference remained significant after 60 and 90 minutes of reperfusion ($p < 0.05$ for all parameters, except for LVdiasP, where $p < 0.01$): at 60

| | LVdiasP (mmHg) | % pre-I | LVdevP (mmHg) | % pre-I | dP/dt $_{max}$ (mmHg/s) | % pre-I | dP/dt $_{min}$ (mmHg/s) | % pre-I |
|---|---|---|---|---|---|---|---|---|
| **Pre-I** | | | | | | | | |
| Ob | 5.2 ± 0.7 | 100.0 ± 0 | 100.6 ± 8.3 | 100.0 ± 0 | 5562.6 ± 555.8 | 100.0 ± 0 | -3570.4 ± 297.5 | 100.0 ± 0 |
| Ln | 5.1 ± 1.0 | 100.0 ± 0 | 118.3 ± 10.8 | 100.0 ± 0 | 6788.3 ± 536.3 | 100.0 ± 0 | -2774.4 ± 1216.1 | 100.0 ± 0 |
| **End-I** | | | | | | | | |
| Ob | 74.2 ± 6.4 | 1604.1 ± 335.0 | 1.3 ± 0.2 | 1.4 ± 0.2 | 59.4 ± 11.4 | 1.1 ± 0.2 | -60.8 ± 16.2 | 1.8 ± 0.5 |
| Ln | 59.3 ± 2.0 | 1451.2 ± 299.6 | 1.8 ± 0.7 | 2.0 ± 1.0 | 133.0 ± 74.2 | 2.6 ± 1.8 | -129.6 ± 72.4 | 3.7± 2.3 |
| **30' R** | | | | | | | | |
| Ob | †96.0 ± 4.7 | 2038.0 ± 386.1 | †21.2 ± 6.2 | 22.3 ± 6.6 | *1127.0 ± 386.4 | 22.0 ± 7.0 | †-786.1 ± 219.1 | 23.8 ± 7.3 |
| Ln | †60.4 ± 7.7 | 1399.8 ± 257.0 | †65.8 ± 11.6 | 58.6 ± 12.1 | *3517.7 ± 673.4 | 54.7 ± 12.5 | †-2405.0 ± 355.6 | 62.7 ± 9.4 |
| **60' R** | | | | | | | | |
| Ob | †97.6 ± 8.8 | 2069.4 ± 405.4 | *31.7 ± 10.7 | 33.0 ± 11.5 | *1846.4 ± 688.1 | 35.4 ± 12.5 | *-1127.8 ± 358.5 | 34.2 ± 12.0 |
| Ln | †57.8 ± 8.0 | 1304.9 ± 212.7 | *72.4 ± 8.5 | 64.5 ± 10.7 | *3962.4 ± 518.9 | 61.0 ± 10.4 | *-2599.6 ± 297.2 | 67.3 ± 6.7 |
| **90' R** | | | | | | | | |
| Ob | †101.8 ± 9.1 | 2158.0 ± 422.4 | *32.8 ± 8.5 | 34.0 ± 9.2 | *1856.0 ± 526.7 | 36.3 ± 10.9 | *-1117.1 ± 311.9 | 33.5 ± 9.9 |
| Ln | †60.7 ± 5.5 | 1444.4 ± 290.9 | *64.3 ± 4.7 | 58.2 ± 9.8 | *3658.4 ± 259.4 | 57.0 ± 8.9 | *-2210.9 ± 132.5 | 58.0 ± 4.4 |

All values shown are mean ± SEM. n = 5 for obese (Ob), n = 6 for lean (Ln) rats. Two-tailed, independent t-test was performed on absolute values (in mmHg or mmHg/s), * $p < 0.05$, † $p < 0.01$ (Ob vs. Ln). Pre-I = pre-ischemia; end-I = end-ischemia; 30'R = 30 min of reperfusion

**Table 6. Cardiac Performance of Isolated Hearts Subjected to 30 Minutes of Ischemia (Obese *vs* Lean).**

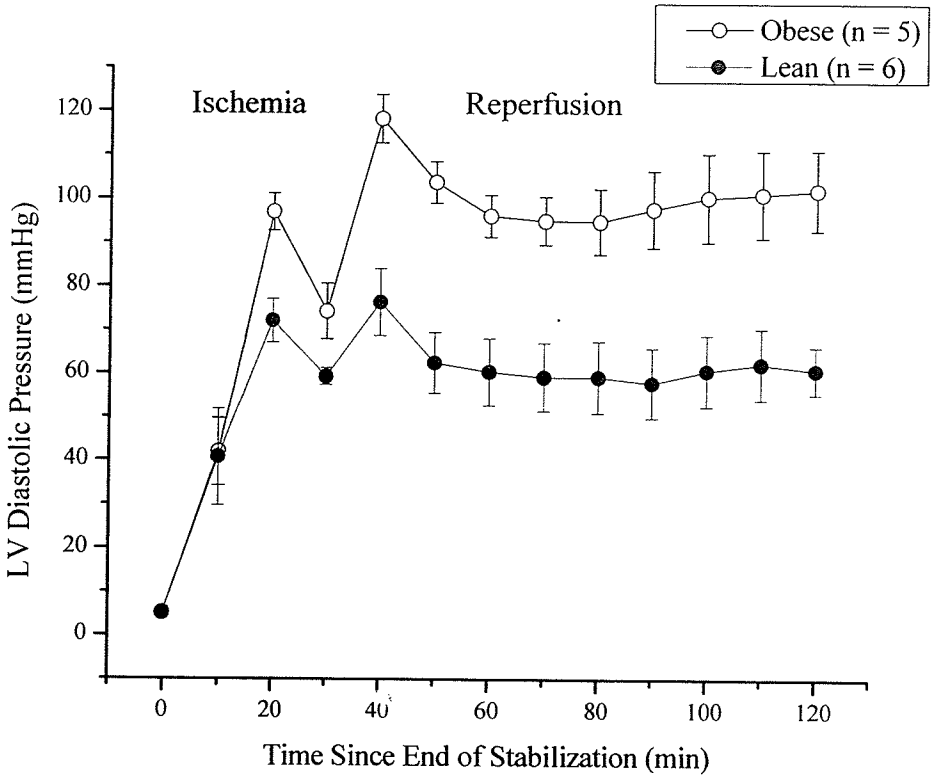



**Fig. 25. Left Ventricular Diastolic Pressure in Isolated Hearts from Obese and Lean JCR:LA-*cp* Rats Subjected to 30 Minutes of Global Ischemia.** Values shown are means ± SEM.

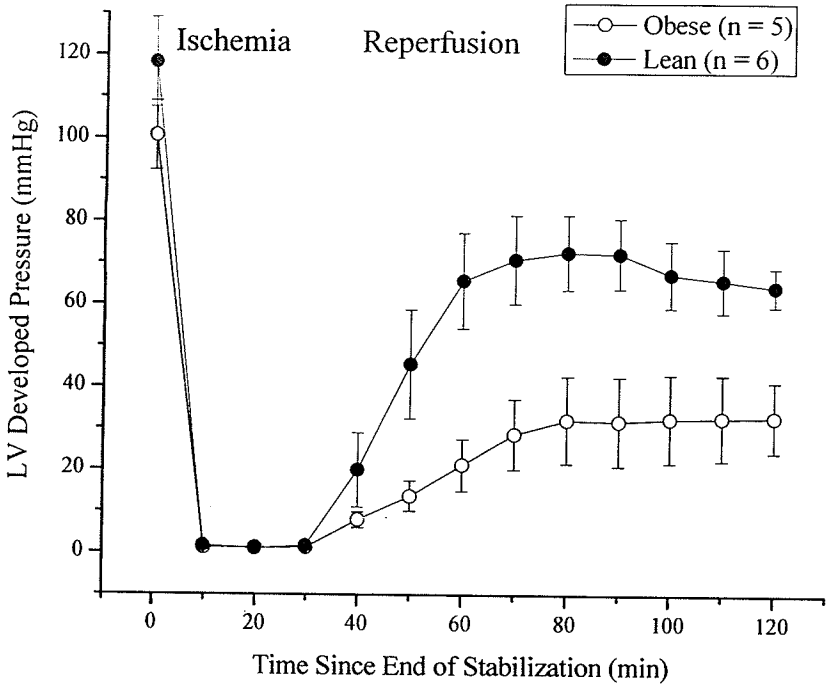



**Fig. 26. Left Ventricular Developed Pressure in Isolated Hearts from Obese and Lean JCR:LA-*cp* Rats Subjected to 30 Minutes of Global Ischemia.**
Values shown are means ± SEM.

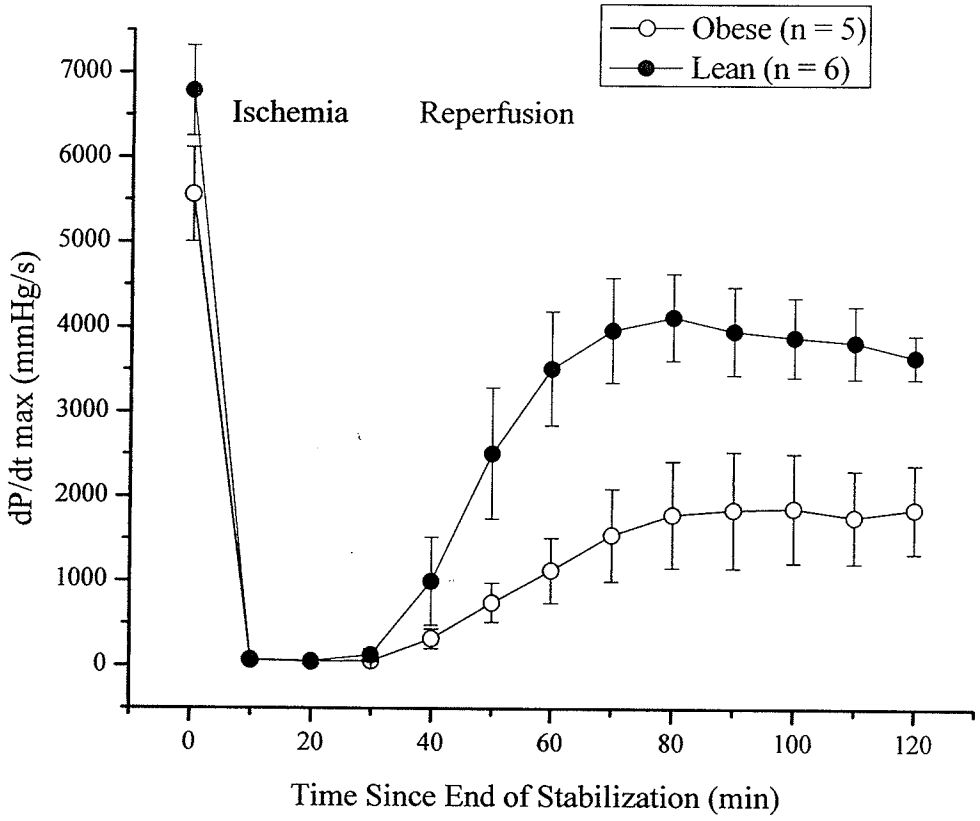



**Fig. 27. Maximum Rate of LV Pressure Development ($dP/dt_{max}$) in Isolated Hearts from Obese and Lean JCR:LA-*cp* Rats Subjected to 30 Minutes of Global Ischemia.** Values shown are means ± SEM.

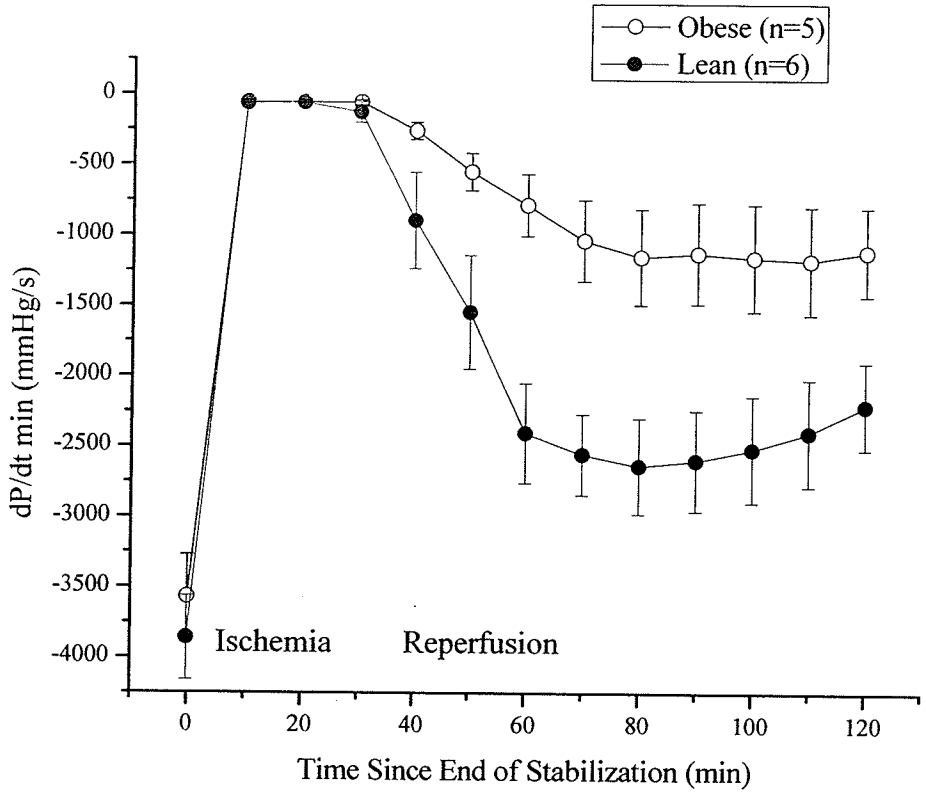



**Fig. 28. Maximum Rate of LV Pressure Decline ($dP/dt_{min}$) in Isolated Hearts from Obese and Lean JCR:LA-*cp* Rats Subjected to 30 Minutes of Global Ischemia.** Values shown are means ± SEM.

minutes of reperfusion LVdiasP 97.6 ± 8.8 *vs* 57.8 ± 8.0 mmHg, LVdevP 31.7 ± 10.7 *vs* 72.4 ± 8.5 mmHg, $dP/dt_{max}$ 1846.4 ± 688.1 *vs* 3962.4 ± 518.9 mmHg/s, $dP/dt_{min}$ -1127.8 ± 358.5 *vs* -2599.6 ± 297.2 mmHg/s); and at 90 minutes of reperfusion (LVdiasP 101.8 ± 9.1 *vs* 60.7 ± 5.5 mmHg, LVdevP 32.8 ± 8.5 *vs* 64.3 ± 4.7 mmHg, $dP/dt_{max}$ 1856.0 ± 526.7 *vs* 3658.4 ± 259.4 mmHg/s, $dP/dt_{min}$ -1117.1 ± 311.9 *vs* -2210.9 ± 132.5 mmHg/s ).

Moreover, significantly more lactate dehydrogenase (mean ± SEM, $p < 0.05$) was released during early reperfusion from isolated hearts of obese rats 1.25 ± 0.19 units *vs* those of the lean rats 0.67 ± 0.08, suggesting a greater extent of myocardial injury.

### *4.4.2 Forty Minutes of Global Ischemia*

Data on the cardiac performance of isolated hearts subjected to 40 minutes of global ischemia, as well as the statistical analysis at pre-defined time points (pre-ischemia, end-ischemia; 30, 60, and 90 minutes of reperfusion), are presented in Table 7. In addition, the corresponding % pre-ischemia of the data are presented in Table 7. Data at other time points are plotted graphically in Figures 29 - 32. As shown in Table 7, parameters of cardiac performance were similar between the 2 groups at baseline (LVdiasP 6.7 ± 2.5 *vs* 6.3 ± 0.6 mmHg, LVdevP 102.2 ± 12.9 *vs* 92.2 ± 12.9 mmHg, $dP/dt_{max}$ 5835.7 ± 588.4 *vs* 5039.3 ± 787.4 mmHg/s, and $dP/dt_{min}$ -3128.0 ± 386.1 *vs* -3295.8 ± 451.0 mmHg/s). At the end of 30 minutes of ischemia, the only statistical difference detected was in LVdiasP 67.1 ± 4.3 vs 49.3 ± 3.7 mmHg ($p < 0.05$). Similarly, after 30 and 60 minutes of reperfusion, LVdiasP remained the only parameter which was found to be significantly different ($p < 0.05$ for both comparisons) between the 2 groups (Table 7). Specifically, mean LVdiasP (O *vs* L) was 119.2 ± 9.5 *vs* 85.9 ± 5.0 mmHg after 30 minutes of reperfusion and was 122.4 ± 11.6 *vs* 81.6 ± 3.9 mmHg after 60 minutes of reperfusion. However, after 90 minutes of reperfusion, all parameters were significantly worse in the obese group (O vs L, $p < 0.05$ for all comparisons): LVdiasP 130.5 ± 6.6 *vs* 80.8 ± 5.8 mmHg, LVdevP 5.0 ± 0.7 *vs* 34.5 ± 6.6 mmHg, $dP/dt_{max}$ 202.6 ± 96.8 vs 2141.1 ± 466.4 mmHg/s, $dP/dt_{min}$ -151.8 ± 37.4 *vs* -1421.2 ± 291.9 mmHg/s. A summary of the recovery of LV developed pressure (% pre-ischemia) after 30 min and 40 min of ischemia in obese rats and lean rats is presented graphically in Figure 33.

| | LVdiasP (mmHg) | % pre-I | LVdevP (mmHg) | % pre-I | dP/dt $_{max}$ (mmHg/s) | % pre-I | dP/dt $_{min}$ (mmHg/s) | % pre-I |
|---|---|---|---|---|---|---|---|---|
| **Pre-I** | | | | | | | | |
| Obese | 6.7 ± 2.5 | 100.0 ± 0 | 102.2 ± 12.9 | 100.0 ± 0 | 5835.7 ± 588.4 | 100.0 ± 0 | -3128.0 ± 386.1 | 100.0 ± 0 |
| Lean | 6.3 ± 0.6 | 100.0 ± 0 | 92.2 ± 12.9 | 100.0 ± 0 | 5039.3 ± 787 4 | 100.0 ± 0 | -3295.8 ± 451.0 | 100.0 ± 0 |
| **End-I** | | | | | | | | |
| Obese | *67.1 ± 4.3 | 1392.3 ± 426.2 | 0.8 ± 0.1 | 0.9 ± 0.1 | 47.5 ± 5.3 | 0.9 ± 0.2 | -42.9 ± 6.1 | 1.5 ± 0.5 |
| Lean | *49.3 ± 3.7 | 805.5 ± 91.4 | 1.0 ± 0.1 | 1.1 ± 0.1 | 56.2 ± 9.0 | 1.2 ± 0.3 | -43.1 ± 10.7 | 1.4 ± 0.3 |
| **30' R** | | | | | | | | |
| Obese | *119.2 ± 9.5 | 2562.7 ± 804.6 | 6.7 ± 5.2 | 9.6 ± 8.3 | 370.8 ± 302.8 | 8.6 ± 7.6 | -229.5 ± 178.6 | 10.6 ± 9.2 |
| Lean | *85.9 ± 5.0 | 1421.1 ± 180.9 | 17.2 ± 3.8 | 20.3 ± 6.5 | 890.9 ± 256.1 | 18.7 ± 6.9 | -692.4 ± 180.7 | 21.9 ± 7.0 |
| **60' R** | | | | | | | | |
| Obese | *122.4± 11.6 | 2662.8 ± 857.2 | 16.2 ± 13.8 | 23.9 ± 21.8 | 969.9 ± 874.3 | 23.1 ± 21.6 | -485.7 ± 437.9 | 23.4 ± 22.1 |
| Lean | *81.6 ± 3.9 | 1346.4 ± 160.9 | 33.2 ± 7.2 | 35.5 ± 4.1 | 1943.7 ± 502.0 | 37.4 ± 4.3 | -1270.1 ± 278.1 | 37.9 ± 4.9 |
| **90' R** | | | | | | | | |
| Obese | *130.5 ± 6.6 | 2740.7 ± 818.2 | *5.0 ± 0.7 | 5.5 ± 1.6 | *202.6 ± 96.8 | 4.3 ± 2.6 | *-151.8 ± 37.4 | 5.7 ± 2.4 |
| Lean | *80.8 ± 5.8 | 1340.7 ± 182.3 | *34.5 ± 6.6 | 37.2 ± 3.5 | *2141.1 ± 466.4 | 42.2 ± 4.5 | *-1421.2 ± 291.9 | 42.8 ± 5.1 |

All values shown are mean ± S.E.M. n = 4 for both obese (Ob) and lean (Ln) rats. Two-tailed, independent t-test was performed on absolute values (in mmHg or mmHg/s), * $p < 0.05$ (Ob vs. Ln). Pre-I = pre-ischemia; end-I = end-ischemia; 30'R = 30 min of reperfusion

**Table 7. Cardiac Performance of Isolated Hearts Subjected to 40 Minutes of Ischemia (Obese *vs* Lean).**

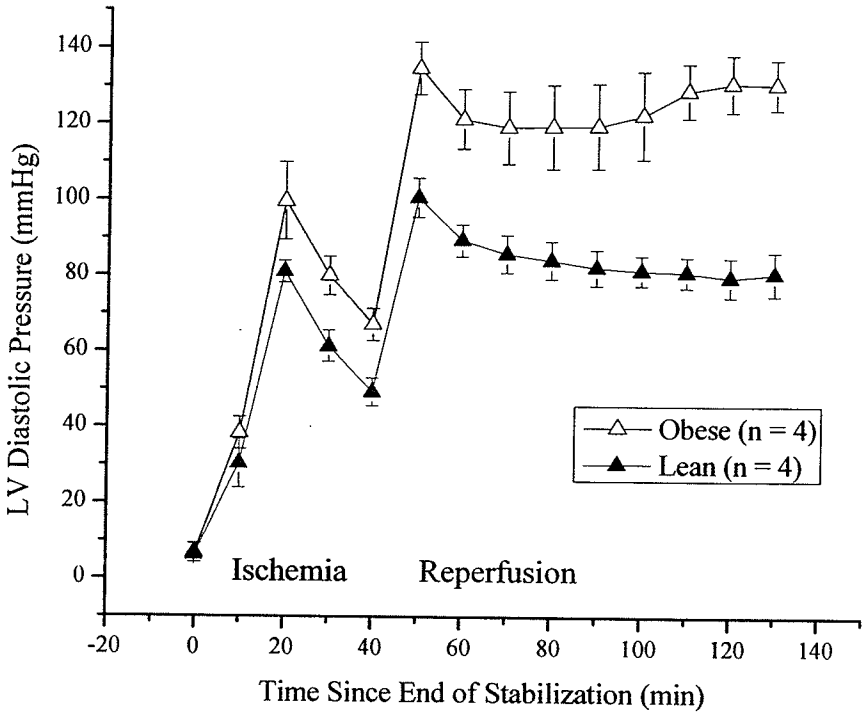



**Fig. 29. Left Ventricular Diastolic Pressure in Isolated Hearts from Obese and Lean JCR:LA-*cp* Rats Subjected to 40 Minutes of Global Ischemia.** Values shown are means ± SEM.

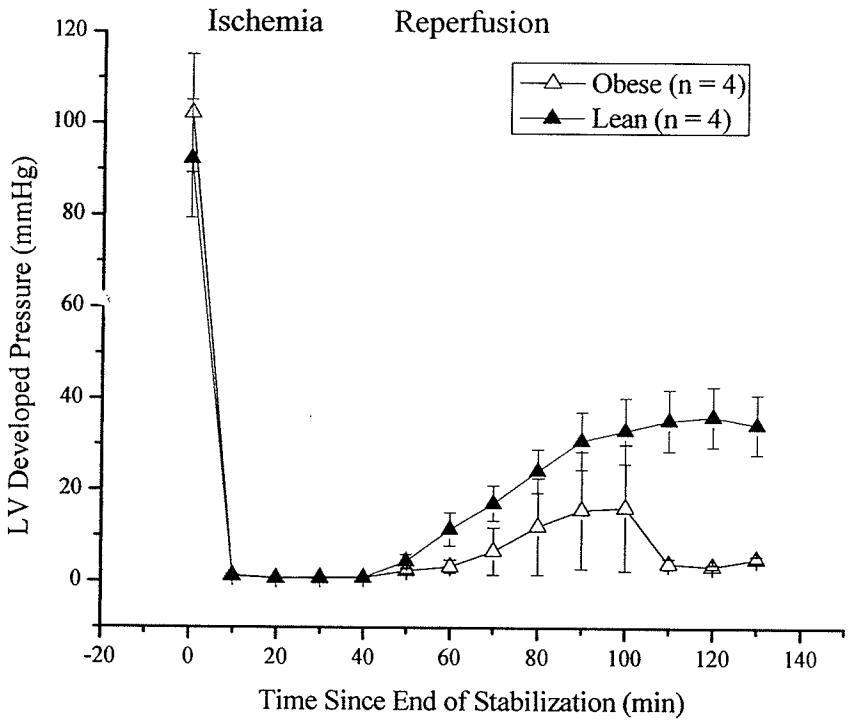



**Fig. 30. Left Ventricular Developed Pressure in Isolated Hearts from Obese and Lean JCR:LA-*cp* Rats Subjected to 40 Minutes of Global Ischemia.** Values shown are means ± SEM.

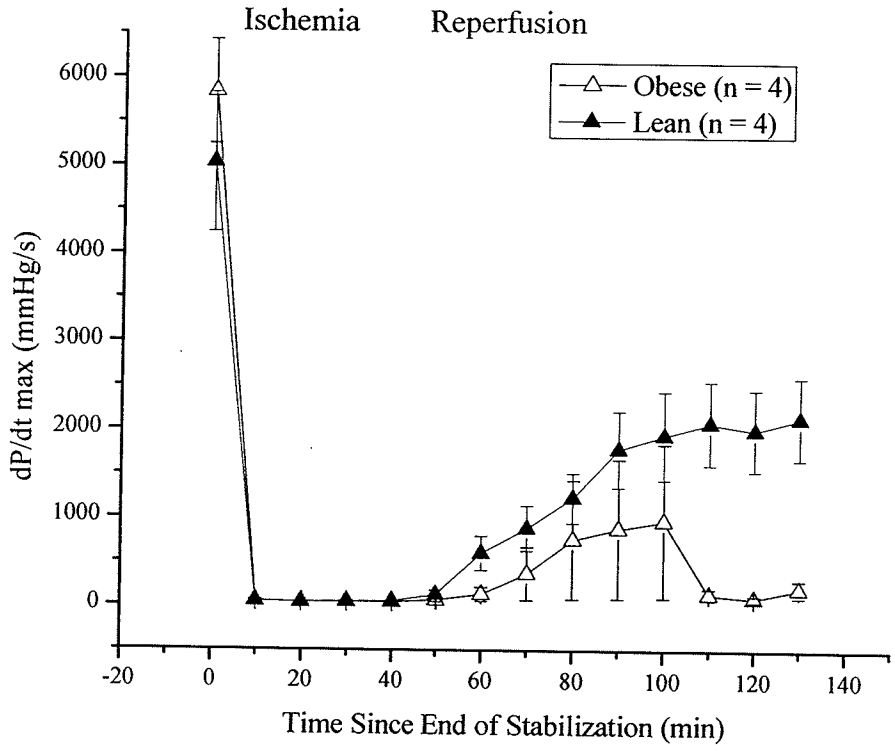



**Fig. 31. Maximum Rate of LV Pressure Development ($dP/dt_{max}$) in Isolated Hearts from Obese and Lean JCR:LA-*cp* Rats Subjected to 40 Minutes of Global Ischemia.** Values shown are means ± SEM.

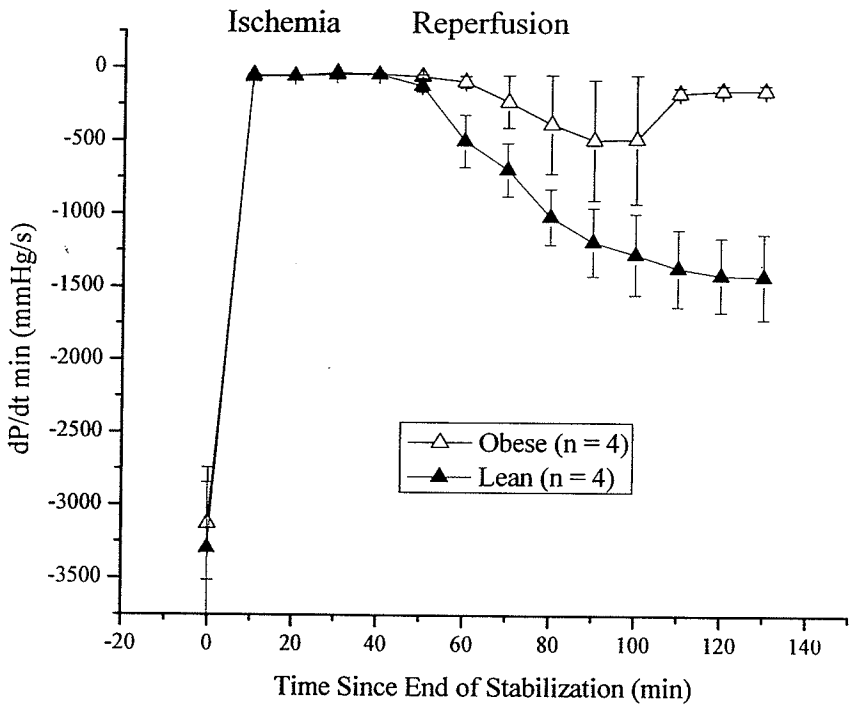



**Fig. 32. Maximum Rate of LV Pressure Decline ($dP/dt_{min}$) in Isolated Hearts from Obese and Lean JCR:LA-*cp* Rats Subjected to 40 Minutes of Global Ischemia.** Values shown are means ± SEM.

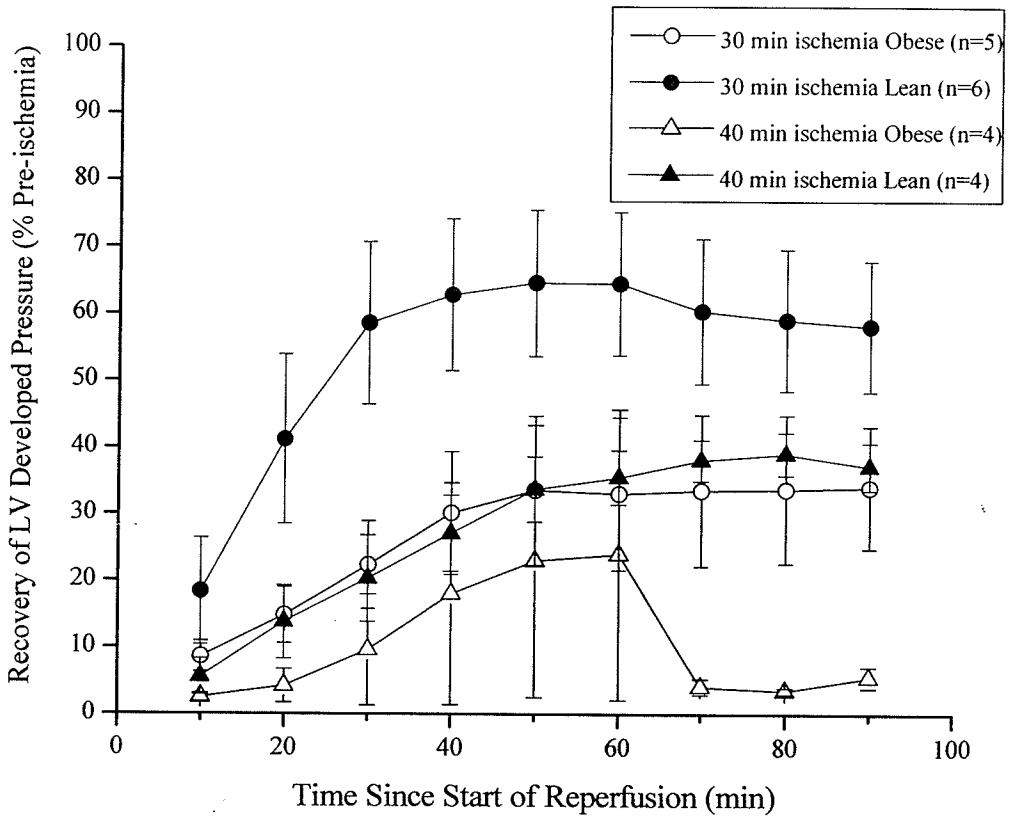



**Fig. 33. Percent Recovery of LV Developed Pressure in Isolated Hearts from Obese and Lean JCR:LA-*cp* Rats Subjected to 30 and 40 Minutes of Global Ischemia.** Values shown are means ± SEM.

Moreover, more lactate dehydrogenase (mean ± SEM, p = 0.12) was released during early reperfusion from isolated hearts of obese rats 1.24 ± 0.21 units *vs* those of the lean rats 0.83 ± 0.09, although the difference did not achieve statistical significance.

### *4.4.3 Fifty Minutes of Global Ischemia*

Data on the cardiac performance of isolated hearts subjected to 50 minutes of global ischemia, as well as the statistical analysis at pre-defined time points (pre-ischemia, end-ischemia; 30, 60, and 90 minutes of reperfusion), are presented in Table 8. The corresponding % pre-ischemia of the data are also presented in Table 8. Data at other time points are plotted graphically in Figures 34 - 37. As shown in Table 7, except for baseline differences in LVdevP and $dP/dt_{min}$, comparisons of all hemodynamic parameters of isolated hearts from obese *vs* lean rats (n = 3 in each group) at all the pre-defined time points did not show any statistical significance.

There was also no significant difference in the amount of lactate dehydrogenase released during early reperfusion (mean ± SEM, O *vs* L, p = 0.48): 1.20 ± 0.18 *vs* 1.03 ± 0.13 units.

An overall summary of the recovery of LV developed pressure (% pre-ischemia) after 30, 40, and 50 min of ischemia in obese rats and lean rats is presented graphically in Figure 38, and the corresponding summary of the LDH data is shown in Figure 39.

| | LVdiasP (mmHg) | % pre-I | LVdevP (mmHg) | % pre-I | $dP/dt_{max}$ (mmHg/s) | % pre-I | $dP/dt_{min}$ (mmHg/s) | % pre-I |
|---|---|---|---|---|---|---|---|---|
| **Pre-I** | | | | | | | | |
| Obese | 6.4 ± 0.2 | 100.0 ± 0 | *114.4 ± 1.9 | 100.0 ± 0 | 6577.0 ± 60.0 | 100.0 ± 0 | *-3555.8 ± 117.7 | 100.0 ± 0 |
| Lean | 6.2 ± 1.7 | 100.0 ± 0 | *133.9 ± 2.6 | 100.0 ± 0 | 7820.3 ± 711.1 | 100.0 ± 0 | *-4785.8 ± 155.1 | 100.0 ± 0 |
| **End-I** | | | | | | | | |
| Obese | 52.9 ± 10.5 | 844.0 ± 189.6 | 1.2 ± 0.3 | 1.0 ± 0.2 | 50.2 ± 8.6 | 0.8 ± 0.1 | -37.3 ± 4.3 | 1.1 ± 0.1 |
| Lean | 50.3 ± 2.1 | 951.8 ± 291.2 | 1.5 ± 0.9 | 1.2 ± 0.7 | 75.1 ± 29.9 | 1.0 ± 0.5 | -68.2 ± 18.6 | 1.4 ± 0.4 |
| **30' R** | | | | | | | | |
| Obese | 116.5 ± 19.9 | 1849.0 ± 364.1 | 9.9 ± 5.0 | 8.8 ± 3.9 | 493.4 ± 276.9 | 7.5 ± 4.2 | -362.7 ± 184.2 | 10.3 ± 5.4 |
| Lean | 74.2 ± 12.0 | 1498.6 ± 617.7 | 51.7 ± 21.3 | 39.1 ± 16.3 | 2535.7 ± 967.1 | 32.0 ± 11.2 | -1994.5 ± 740.6 | 41.1 ± 14.5 |
| **60' R** | | | | | | | | |
| Obese | 115.9 ± 17.7 | 1837.5 ± 329.8 | 17.4 ± 8.8 | 15.5 ± 7.9 | 931.1 ± 545.4 | 14.1 ± 8.3 | -573.8 ± 297.3 | 16.3 ± 8.8 |
| Lean | 73.3 ± 10.8 | 1477.5 ± 590.3 | 54.1 ± 16.0 | 40.8 ± 12.4 | 2902.7 ± 806.7 | 36.9 ± 9.2 | -2076.5 ± 569.1 | 43.1 ± 11.3 |
| **90' R** | | | | | | | | |
| Obese | 118.8 ± 15.8 | 1883.3 ± 301.9 | 23.5 ± 10.5 | 20.9 ± 9.4 | 1348.8 ± 667.7 | 20.4 ± 10.2 | -716.0 ± 329.7 | 20.3 ± 9.7 |
| Lean | 75.0 ± 9.4 | 1481.4 ± 574.9 | 48.0 ± 10.6 | 36.1 ± 8.4 | 2919.3 ± 809.0 | 37.0 ± 8.9 | -1978.4 ± 480.6 | 41.2 ± 9.7 |

All values shown are mean ± S.E.M. n = 3 for both obese (Ob) and lean (Ln) rats. Two-tailed, independent t-test was performed on absolute values (in mmHg or mmHg/s), * $p < 0.05$ (Ob vs. Ln). Pre-I = pre-ischemia; end-I = end-ischemia; 30'R = 30 min of reperfusion

**Table 8. Cardiac Performance of Isolated Hearts Subjected to 50 Minutes of Ischemia (Obese *vs* Lean).**

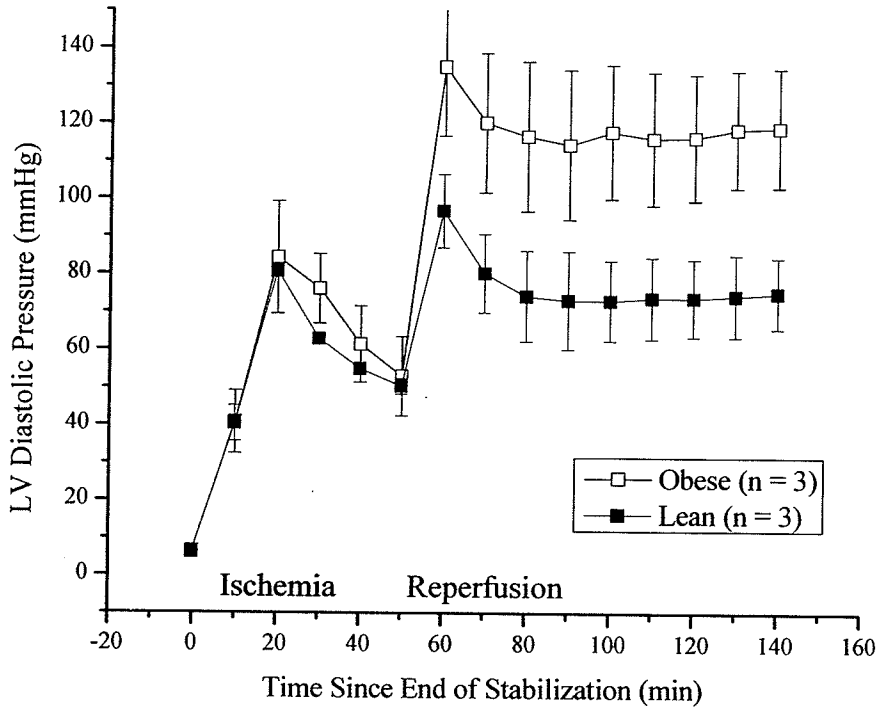



**Fig. 34. Left Ventricular Diastolic Pressure in Isolated Hearts from Obese and Lean JCR:LA-*cp* Rats Subjected to 50 Minutes of Global Ischemia.** Values shown are means ± SEM.

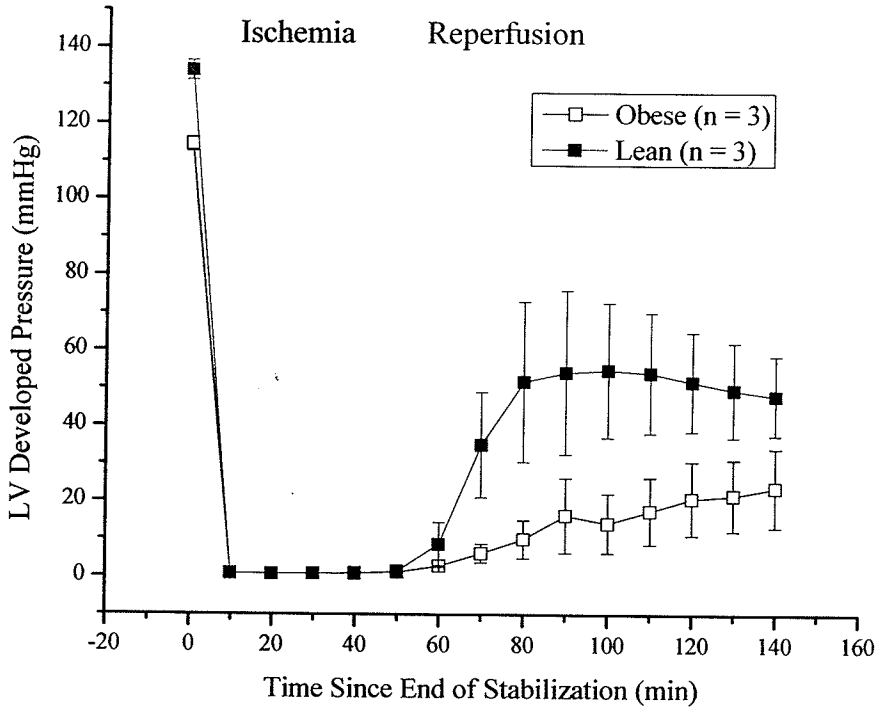



**Fig. 35. Left Ventricular Developed Pressure in Isolated Hearts from Obese and Lean JCR:LA-*cp* Rats Subjected to 50 Minutes of Global Ischemia.** Values shown are means ± SEM.

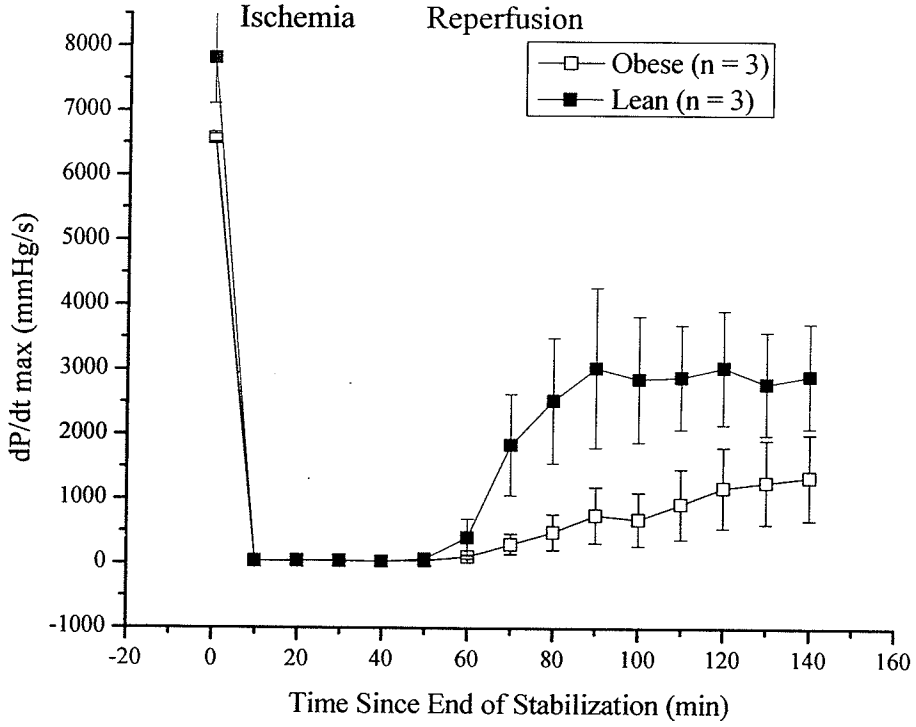



**Fig. 36. Maximum Rate of LV Pressure Development ($dP/dt_{max}$) in Isolated Hearts from Obese and Lean JCR:LA-*cp* Rats Subjected to 50 Minutes of Global Ischemia.** Values shown are means ± SEM.

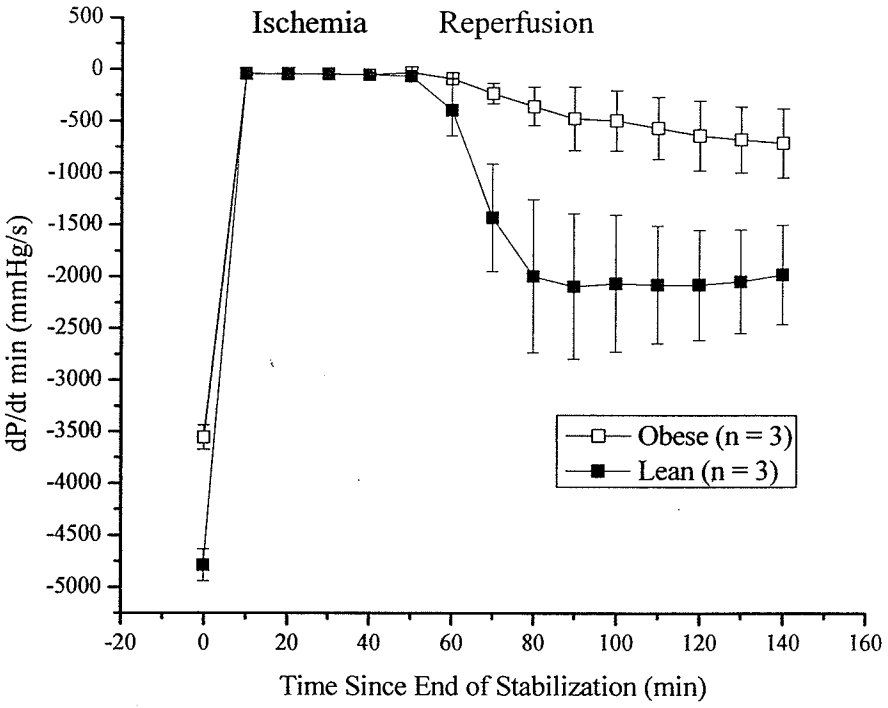



**Fig. 37. Maximum Rate of LV Pressure Decline ($dP/dt_{min}$) in Isolated Hearts from Obese and Lean JCR:LA-*cp* Rats Subjected to 50 Minutes of Global Ischemia.** Values shown are means ± SEM.

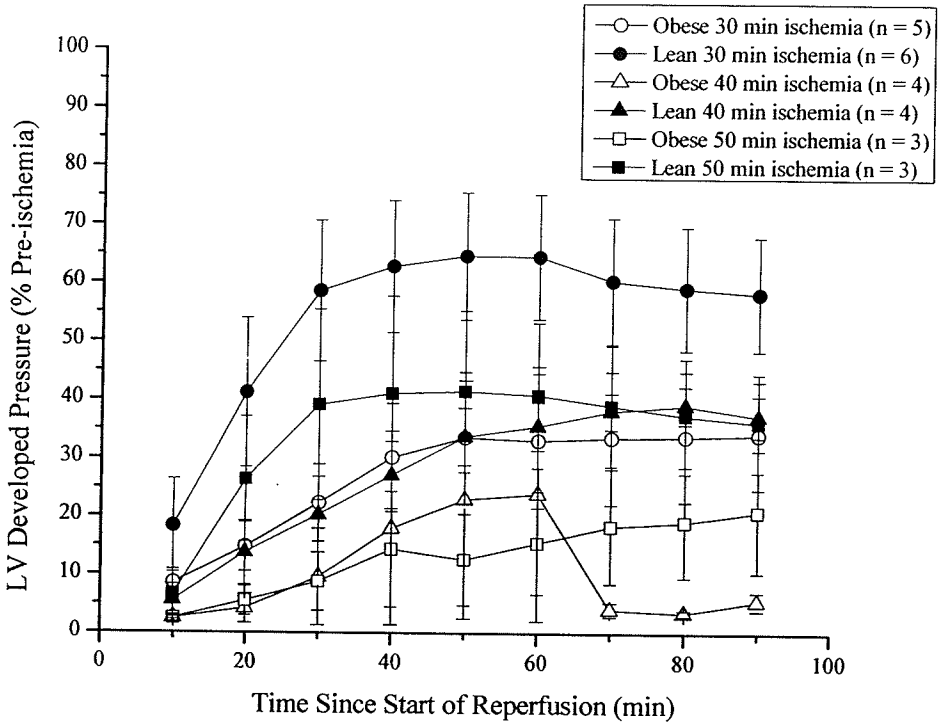



**Fig. 38. Percent Recovery of LV Developed Pressure in Isolated Hearts from Obese and Lean JCR:LA-*cp* Rats Subjected to 30, 40, and 50 Minutes of Global Ischemia.** Values shown are means ± SEM.

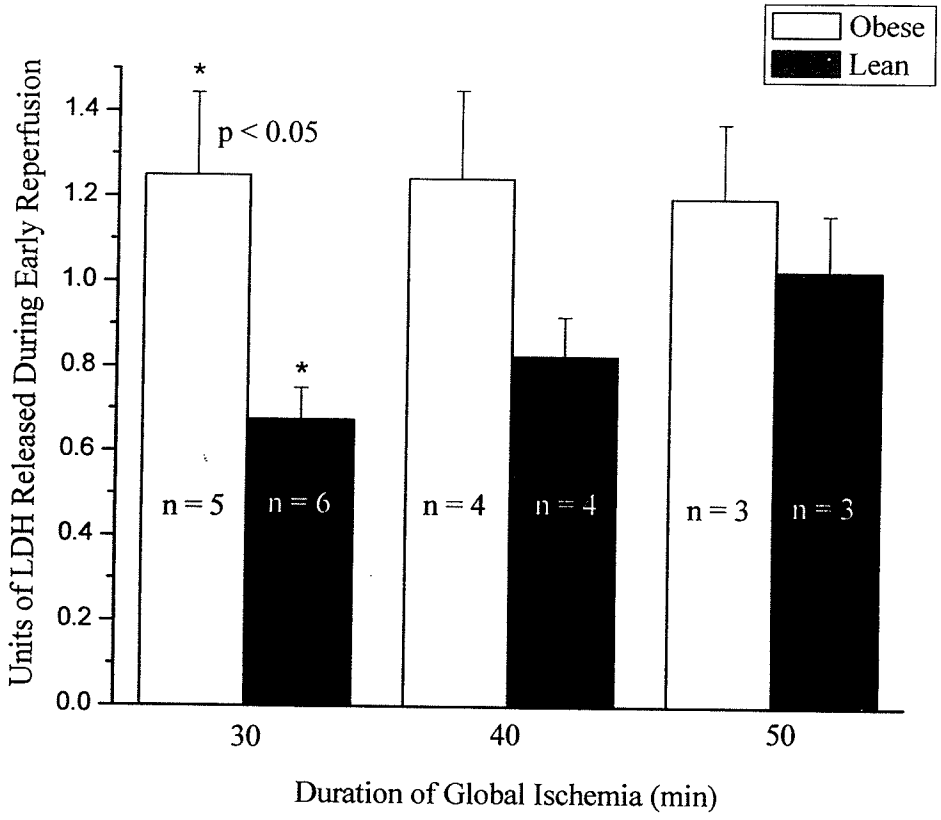



**Fig. 39. Release of Lactate Dehydrogenase During Early Reperfusion from Isolated Hearts of Obese and Lean JCR:LA-*cp* Rats Subjected to 30, 40, and 50 Minutes of Global Ischemia.**
Values shown are means ± SEM.

## 4.5 Effects of SEA0400 on Baseline Cardiac Performance

Data on cardiac performance before and after 10-minute infusion of SEA0400 immediately prior to global ischemia are presented in Table 9. At 100 nM, no significant change in cardiac performance was observed before *vs* after pre-ischemic infusion of SEA0400. At both 300 nM and 1 μM concentrations, significant differences in LVdiasP, LVdevP, and $dP/dt_{min}$ were detected. Specifically, the above parameters of cardiac performance before and after infusion of SEA0400 at 300 nM were 7.0 ± 1.4 *vs* 4.9 ± 1.1 mmHg, 113.7 ± 9.1 *vs* 129.6 ± 11.7 mmHg, and -3889.9 ± 355.2 *vs* -4402.9 ± 415.1 mmHg/s, respectively ($p < 0.05$). The corresponding data for SEA0400 at 1μM were 5.3 ± 0.9 *vs* 3.7 ± 0.6 mmHg, 123.6 ± 10.7 *vs* 132.9 ± 8.9 mmHg, and -3983.8 ± 386.8 *vs* -4699.4 ± 309.9 mmHg/s, respectively ($p < 0.05$).

| Dose and time | LVdiasP (mmHg) | LVdevP (mmHg) | $dP/dt_{max}$ (mmHg/s) | $dP/dt_{min}$ (mmHg/s) | P value |
|---|---|---|---|---|---|
| **SEA0400 100 nM** | | | | | |
| -10 min | 5.1 ± 1.3 | 115.0 ± 7.1 | 5855.4 ± 719.1 | -3938.9 ± 255.7 | NS |
| 0 min | 5.5 ± 1.9 | 114.0 ± 5.4 | 5870.5 ± 658.5 | -4275.5 ± 311.9 | |
| **SEA0400 300 nM** | | | | | |
| -10 min | *7.0 ± 1.4 | †113.7 ± 9.1 | 5905.0 ± 567.6 | ‡-3889.9 ± 355.2 | *p = 0.02 |
| 0 min | *4.9 ± 1.1 | †129.6 ± 11.7 | 6954.1 ± 916.4 | ‡-4402.9 ± 415.1 | †p = 0.04<br>‡p = 0.04 |
| **SEA0400 1 μM** | | | | | |
| -10 min | *5.3 ± 0.9 | †123.6 ± 10.7 | 6811.4 ± 601.7 | ‡-3983.8 ± 386.8 | *p = 0.03 |
| 0 min | *3.7 ± 0.6 | †132.9 ± 8.9 | 7186.4 ± 657.4 | ‡-4699.4 ± 309.9 | †p = 0.01<br>‡p = 0.02 |

All values shown are mean ± S.E.M. (n = 5 per group). Comparisons were made by 2-tailed paired t-test.
'-10 min' = 10 min before ischemia. '0 min' = immediately before onset of ischemia

**Table 9. Cardiac Performance of Isolated Hearts Before and At the End of Infusion of SEA0400 for 10 Minutes Prior to Ischemia.**

## 4.6 Effects of SEA0400 in the Langendorff-Perfused Isolated Rat Heart Subjected to Global, Zero-Flow Ischemia

### *4.6.1 Cardiac Performance*

Isolated hearts from obese rats were randomly assigned to treatment with one of three concentrations of SEA0400, 100 nM, 300 nM and 1 μM, for 10 minutes before and again for 10 minutes after 30-minute global ischemia, followed by 90 minutes of reperfusion. For control, isolated hearts from obese rats were treated with an equivalent amount of DMSO used for dissolving SEA0400.

As shown in Table 10 and 11, there were no significant differences in all parameters between groups at all pre-specified time points for statistical analyses, *i.e.*, at baseline, at the end of ischemia, and at 30, 60, and 90 minutes of reperfusion. The corresponding % pre-ischemia of the data are also presented in Tables 10 and 11. Data at other time points are shown graphically in Figures 40 – 43, 44 – 47, and 48 – 51 for SEA0400 100 nM, 300 nM, and 1 μM, respectively.

A summary of the recovery of LV developed pressure (% pre-ischemia) of all the SEA0400-treated groups and the controls is presented in Figure 52.

### *4.6.2 Release of Lactate Dehydrogenase*

The amount of lactate dehydrogenase release during early reperfusion did not differ significantly between groups, as shown in Figure 53.

| | LVdiasP (mmHg) | % pre-I | LVdevP (mmHg) | % pre-I | dP/dt $_{max}$ (mmHg/s) | % pre-I | dP/dt $_{min}$ (mmHg) | % pre-I |
|---|---|---|---|---|---|---|---|---|
| **Pre-I** | | | | | | | | |
| Control | 5.2 ± 0.7 | 100.0 ± 0 | 100.6 ± 8.3 | 100.0 ± 0 | 5562.6 ± 555.8 | 100.0 ± 0 | -3570.4 ± 297.5 | 100.0 ± 0 |
| **SEA0400** | | | | | | | | |
| 100nM | 5.5 ± 1.9 | 100.0 ± 0 | 114.0 ± 5.4 | 100.0 ± 0 | 5870.5 ± 658.5 | 100.0 ± 0 | -4275.5 ± 311.9 | 100.0 ± 0 |
| 300nM | 4.9 ± 1.1 | 100.0 ± 0 | 129.6 ± 11.7 | 100.0 ± 0 | 6954.1 ± 916.4 | 100.0 ± 0 | -4402.9 ± 415.1 | 100.0 ± 0 |
| 1 μM | 3.7 ± 0.6 | 100.0 ± 0 | 132.9 ± 8.9 | 100.0 ± 0 | 7186.4 ± 657.4 | 100.0 ± 0 | -4699.4 ± 309.9 | 100.0 ± 0 |
| **End-I** | | | | | | | | |
| Control | 74.2 ± 6.4 | 1604.1 ± 335.0 | 1.3 ± 0.2 | 1.4 ± 0.2 | 59.4 ± 11.4 | 1.1 ± 0.2 | -60.8 ± 16.2 | 1.8 ± 0.5 |
| **SEA0400** | | | | | | | | |
| 100nM | 77.7 ± 2.3 | 2678.9 ± 1018.8 | 2.3 ± 0.8 | 2.0 ± 0.7 | 99.8 ± 31.3 | 2.0 ± 0.8 | -98.0 ± 23.5 | 2.4 ± 0.6 |
| 300nM | 83.3 ± 2.5 | 2377.1 ± 839.5 | 1.6 ± 0.6 | 1.2 ± 0.4 | 67.0 ± 16.0 | 1.1 ± 0.3 | -67.4 ± 15.4 | 1.5 ± 0.2 |
| 1μM | 76.7 ± 3.4 | 2327.5 ± 424.5 | 1.4 ± 0.2 | 1.0 ± 0.1 | 57.1 ± 5.9 | 0.8 ± 0.2 | -58.9 ± 4.4 | 1.3 ± 0.2 |

All values shown are mean ± S.E.M. (n = 5 per group). No significant differences were detected by analysis of variance (ANOVA).
Pre-I = pre-ischemia; end-I = end-ischemia.

**Table 10. Cardiac Performance of Isolated Hearts at Baseline and at the End of Ischemia (Control *vs* SEA0400).**

| | LVdiasP (mmHg) | % pre-I | LVdevP (mmHg) | % pre-I | dP/dt $_{max}$ (mmHg/s) | % pre-I | dP/dt $_{min}$ (mmHg/s) | % pre-I |
|---|---|---|---|---|---|---|---|---|
| **30' R** | | | | | | | | |
| Control | 96.0 ± 4.7 | 2038.0 ± 386.1 | 21.2 ± 6.2 | 22.3 ± 6.6 | 1127.0 ± 386.4 | 22.0 ± 7.0 | -786.1 ± 219.1 | 23.8 ± 7.3 |
| **SEA0400** | | | | | | | | |
| 100nM | 92.2 ± 9.4 | 2892.3 ± 991.2 | 18.1 ± 11.7 | 17.9 ± 12.5 | 884.4 ± 593.6 | 18.7 ± 13.7 | -706.6 ± 459.8 | 19.6 ± 13.7 |
| 300nM | 100.4 ± 7.3 | 3024.2 ± 1182.8 | 18.8 ± 8.9 | 16.6 ± 8.5 | 757.7 ± 366.3 | 13.9 ± 7.5 | -761.2 ± 385.0 | 18.4 ± 9.6 |
| 1 μM | 92.0 ± 8.7 | 2703.3 ± 410.8 | 10.9 ± 4.6 | 9.2 ± 4.2 | 411.1 ± 182.6 | 6.8 ± 3.4 | -358.2 ± 152.2 | 8.2 ± 3.5 |
| **60' R** | | | | | | | | |
| Control | 97.6 ± 8.8 | 2069.4 ± 405.4 | 31.7 ± 10.7 | 33.0 ± 11.5 | 1846.4 ± 688.1 | 35.4 ± 12.5 | -1127.8 ± 358.5 | 34.2 ± 12.0 |
| **SEA0400** | | | | | | | | |
| 100nM | 92.5 ± 10.0 | 2875.4 ± 989.2 | 29.1 ± 12.4 | 27.8 ± 13.2 | 1412.4 ± 590.8 | 26.9 ± 13.5 | -1015.2 ± 465.9 | 26.8 ± 14.1 |
| 300nM | 96.4 ± 10.2 | 2956.8 ± 1201.5 | 34.0 ± 15.9 | 29.5 ± 14.7 | 1482.4 ± 696.0 | 27.0 ± 14.2 | -1294.7 ± 651.9 | 30.5 ± 15.0 |
| 1 μM | 93.1 ± 9.3 | 2719.4 ± 390.5 | 14.6 ± 4.6 | 12.2 ± 4.4 | 729.7 ± 313.0 | 11.9 ± 5.5 | -540.9 ± 177.3 | 12.6 ± 4.7 |
| **90' R** | | | | | | | | |
| Control | 101.8 ± 9.1 | 2158.0 ± 422.4 | 32.8 ± 8.5 | 34.0 ± 9.2 | 1856.0 ± 526.7 | 36.3 ± 10.9 | -1117.1 ± 311.9 | 33.5 ± 9.9 |
| **SEA0400** | | | | | | | | |
| 100nM | 95.8 ± 9.3 | 2980.8 ± 995.9 | 28.3 ± 10.9 | 26.9 ± 11.8 | 1504.3 ± 610.0 | 28.6 ± 14.2 | -890.0 ± 465.4 | 23.6 ± 13.7 |
| 300nM | 96.1 ± 7.7 | 2958.5 ± 1225.4 | 34.7 ± 15.3 | 29.7 ± 14.1 | 1546.9 ± 651.2 | 27.4 ± 13.5 | -1271.7 ± 641.9 | 29.3 ±14.1 |
| 1 μM | 95.8 ± 10.4 | 2804.4 ± 435.5 | 17.0 ± 4.2 | 13.8 ± 4.0 | 784.4 ± 238.4 | 12.2 ± 4.2 | -579.6 ± 166.4 | 13.3 ± 4.4 |

All values shown are mean ± S.E.M. (n = 5 per group). No significant differences were detected by analysis of variance (ANOVA).
30'R = 30 min of reperfusion.

**Table 11. Contractile Parameters of Isolated Hearts During Reperfusion (Control *vs* SEA0400).**

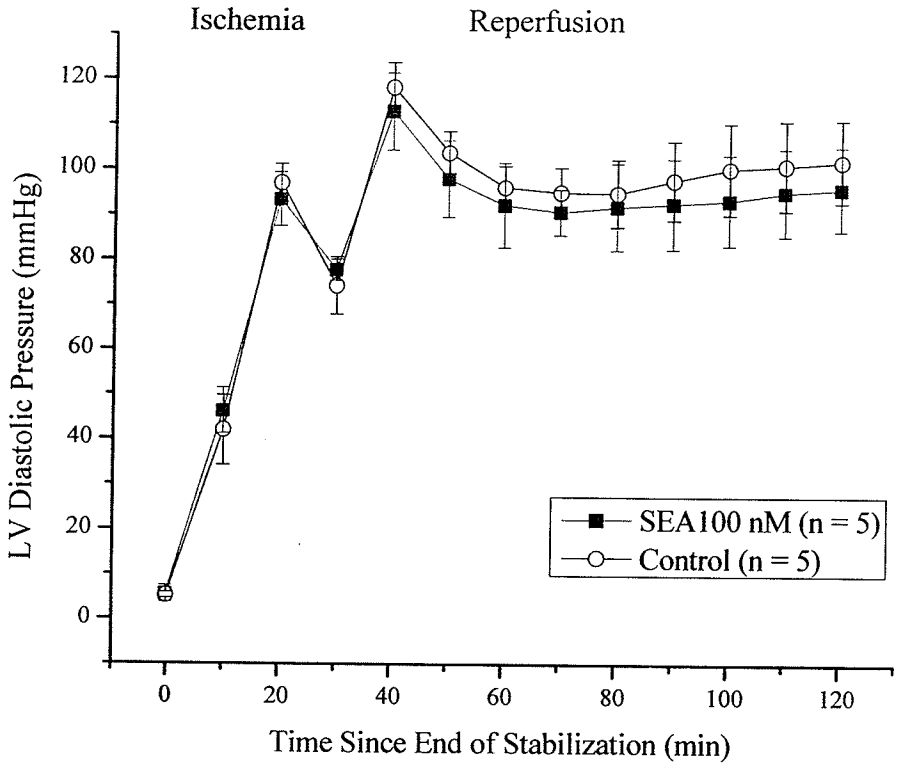



**Fig. 40. LV Diastolic Pressure in Isolated Hearts from Obese JCR:LA-*cp* Rats Treated with 100 nM SEA0400 (SEA) *vs* Controls.**
Values shown are means ± SEM.

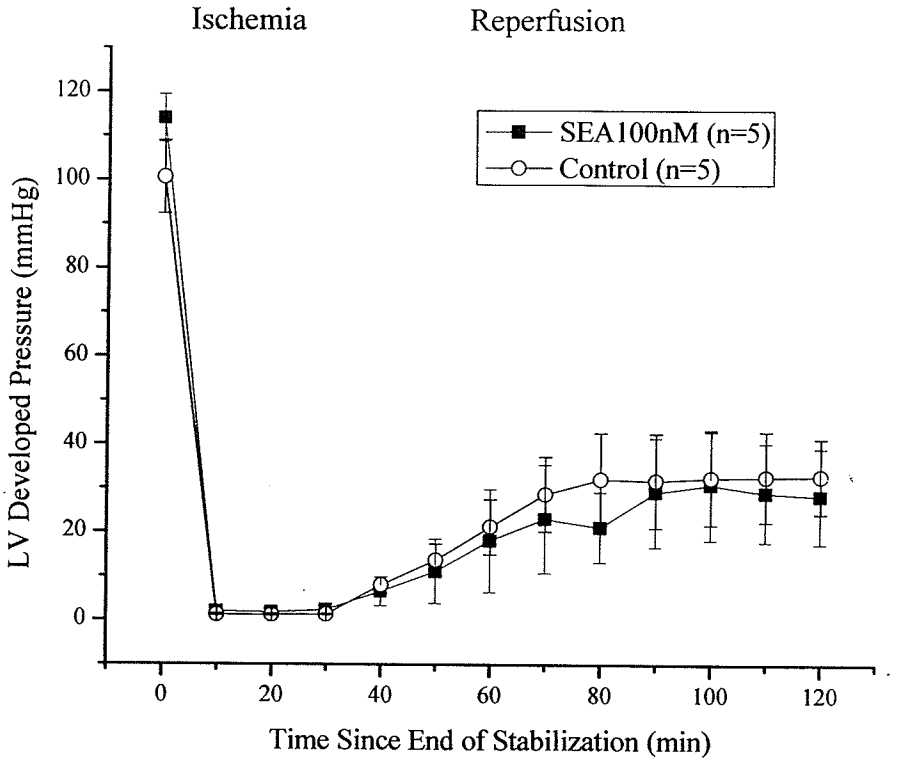



**Fig. 41. LV Developed Pressure in Isolated Hearts from Obese JCR:LA-*cp* Rats Treated with 100 nM SEA0400 *vs* Controls.**
Values shown are means ± SEM.

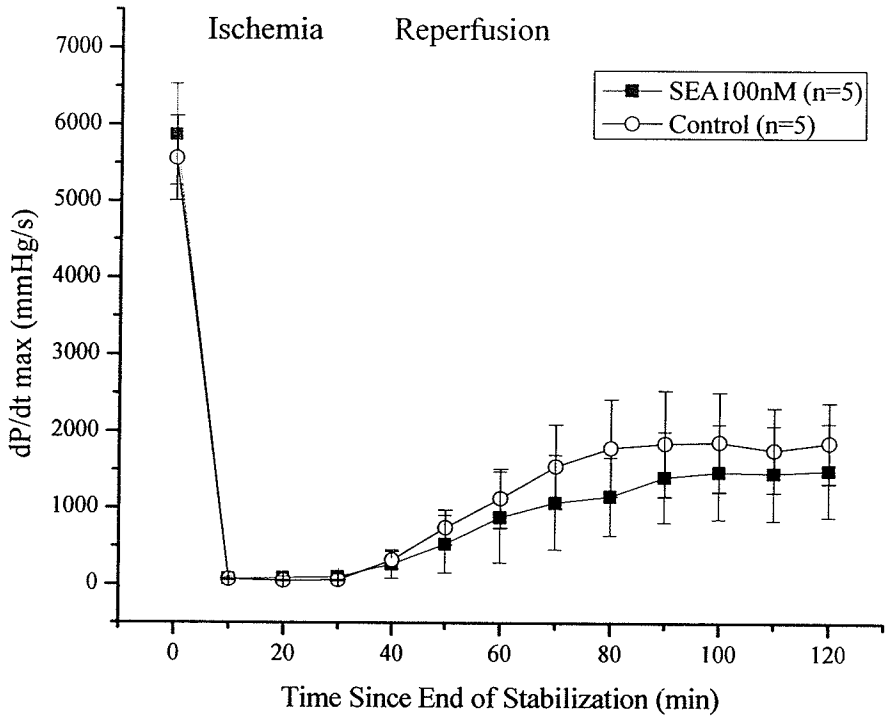



**Fig. 42. Maximum Rate of LV Pressure Development in Isolated Hearts from Obese JCR:LA-*cp* Rats Treated with 100 nM SEA0400 *vs* Control.** Values shown are means ± SEM.

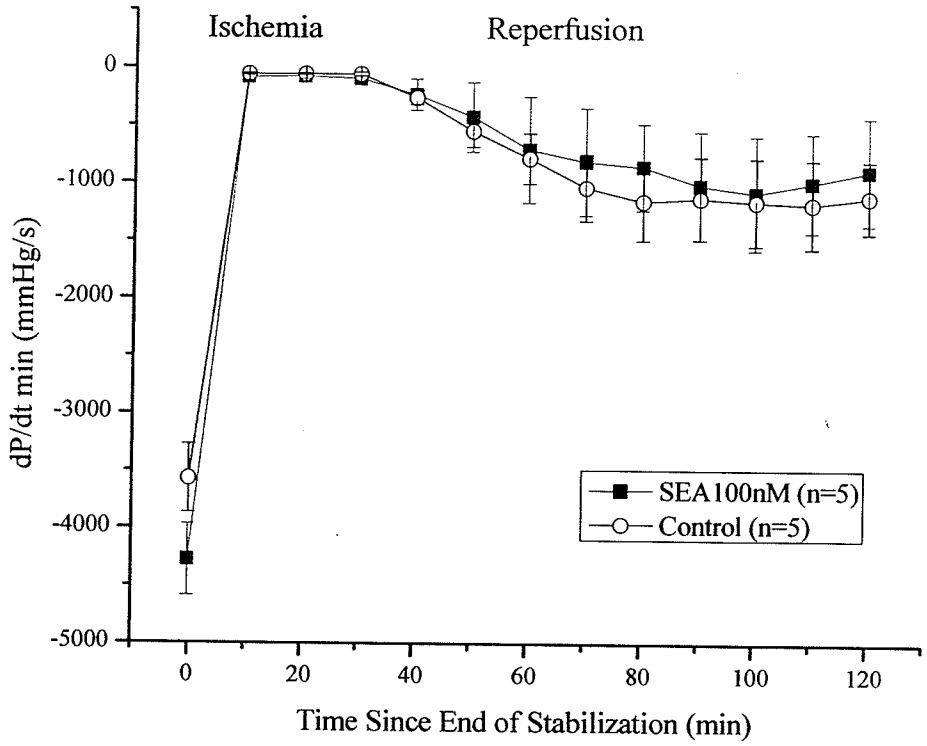



**Fig. 43. Maximum Rate of LV Pressure Decline in Isolated Hearts from Obese JCR:LA-*cp* Rats Treated with 100 nM SEA0400 *vs* Control.** Values shown are means ± SEM.

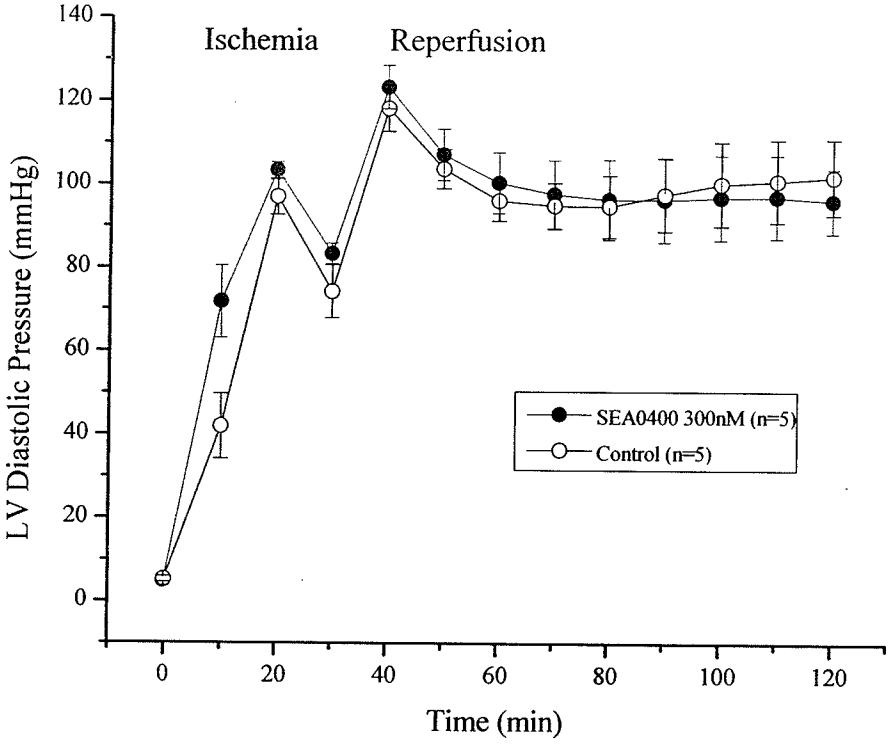



**Fig. 44. LV Diastolic Pressure in Isolated Hearts from Obese JCR:LA-*cp* Rats Treated with 300 nM SEA0400 *vs* Controls.**
Values shown are means ± SEM.

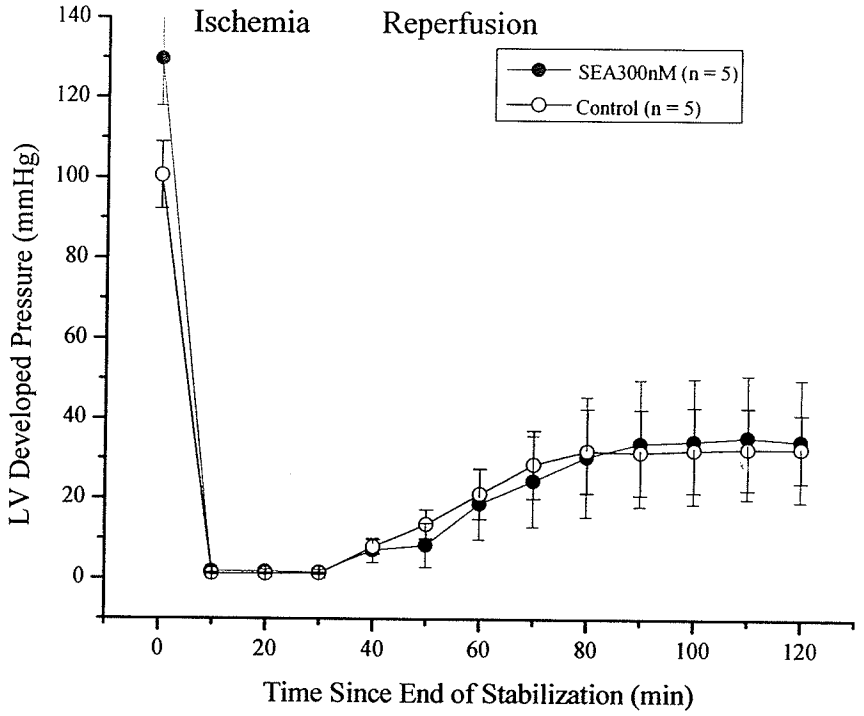



**Fig. 45. LV Developed Pressure in Isolated Hearts from Obese JCR:LA-*cp* Rats Treated with 300 nM SEA0400 *vs* Controls.**
Values shown are means ± SEM.

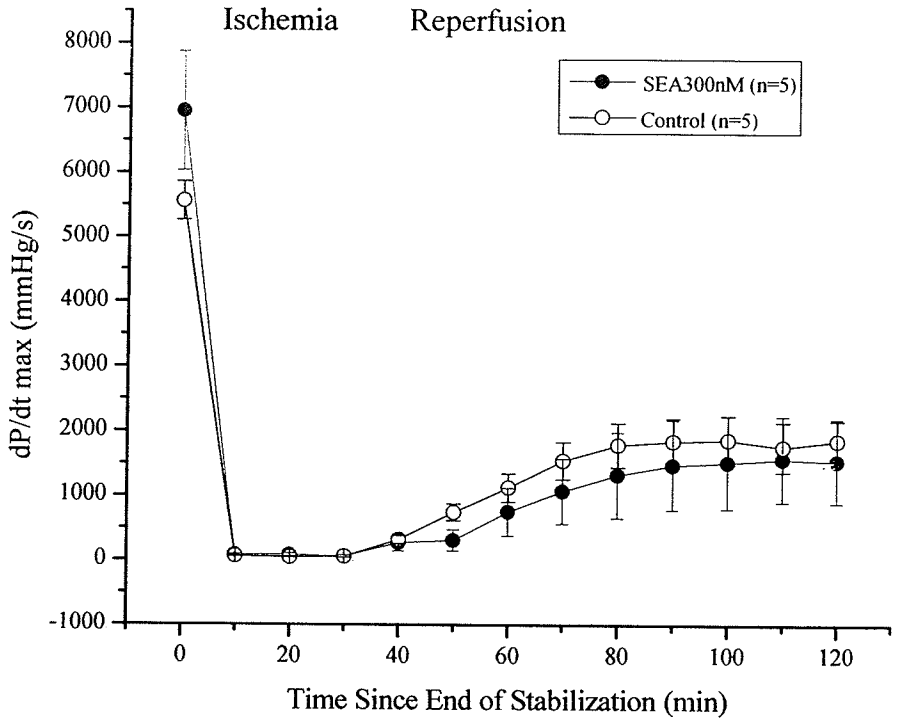



**Fig. 46. Maximum Rate of LV Pressure Development in Isolated Hearts from Obese JCR:LA-*cp* Rats Treated with 300 nM SEA0400 *vs* Controls.** Values shown are means ± SEM.

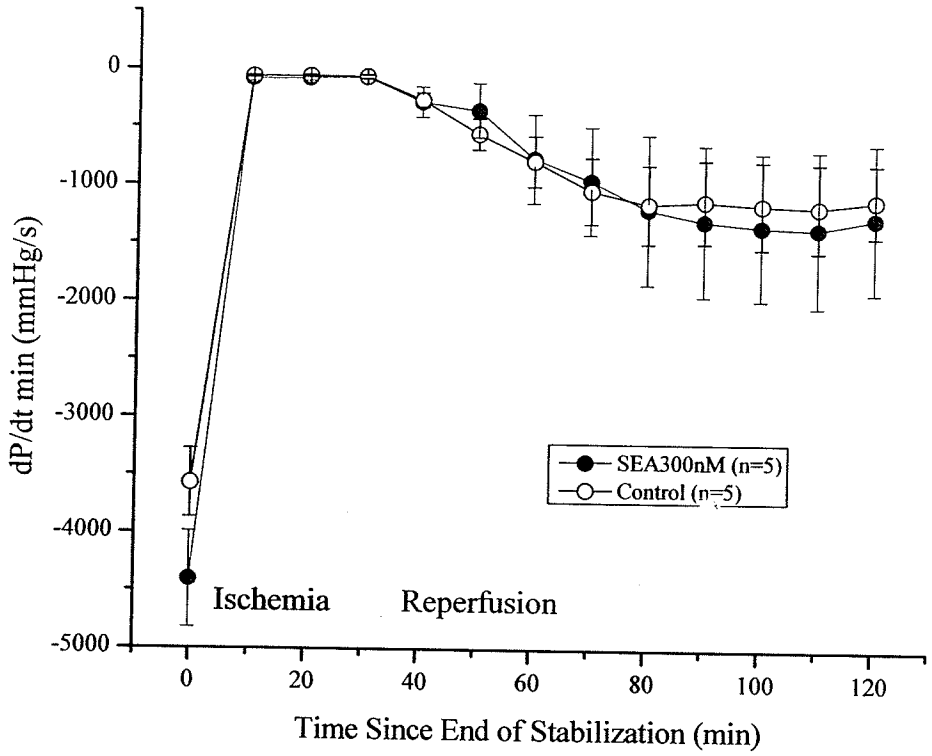



**Fig. 47. Maximum Rate of LV Pressure Decline in Isolated Hearts from Obese JCR:LA-*cp* Rats Treated with 300 nM SEA0400 *vs* Controls.** Values shown are means ± SEM.

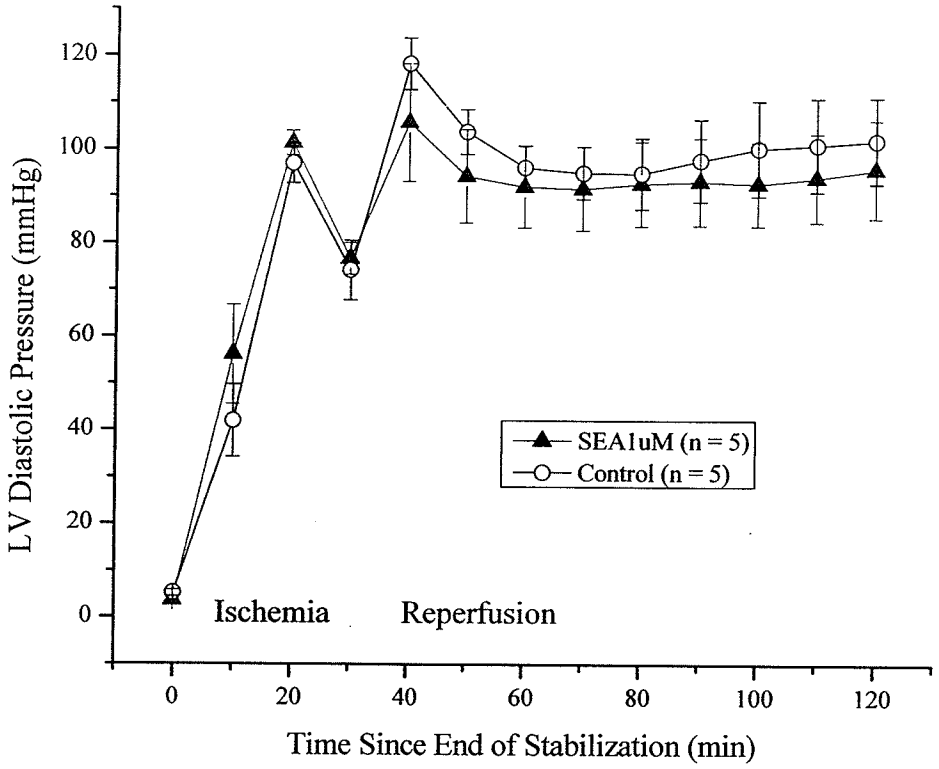



**Fig. 48. LV Diastolic Pressure in Isolated Hearts from Obese JCR:LA-*cp* Rats Treated with 1 μM SEA0400 *vs* Controls.**
Values shown are means ± SEM.

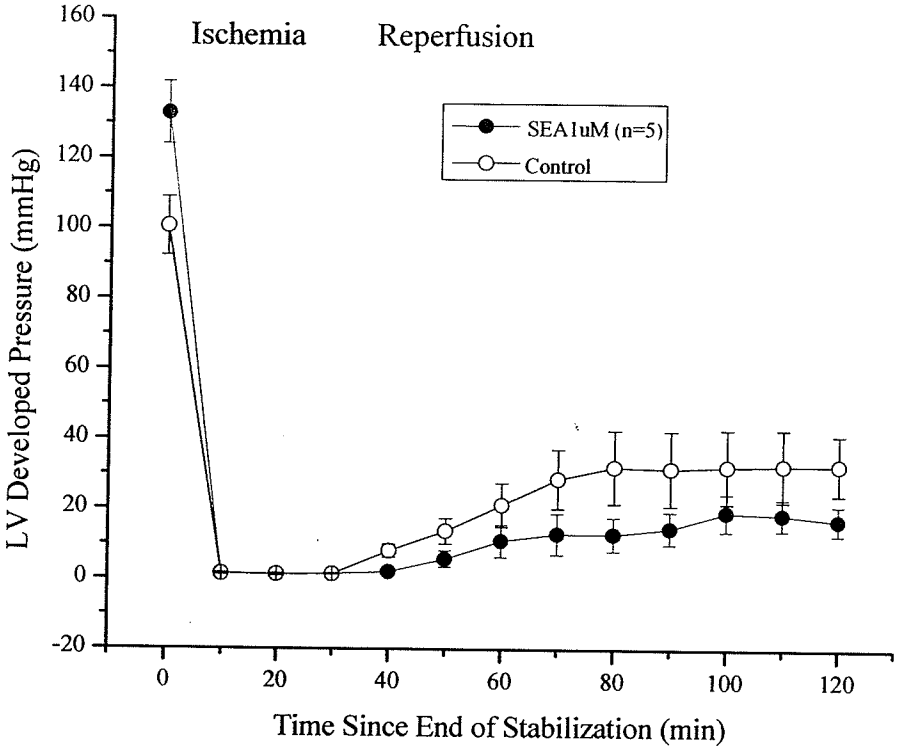



**Fig. 49. LV Developed Pressure in Isolated Hearts from Obese JCR:LA-*cp* Rats Treated with 1 μM SEA0400 *vs* Controls.**
Values shown are means ± SEM.

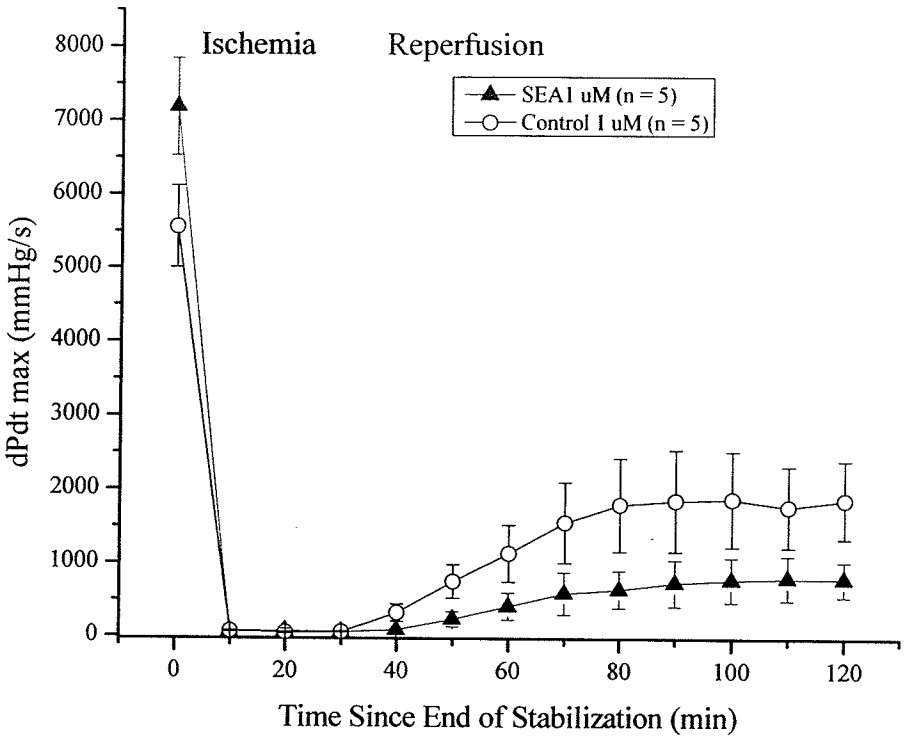



**Fig. 50. Maximum Rate of LV Pressure Development in Isolated Hearts from Obese JCR:LA-*cp* Rats Treated with 1 μM SEA0400 *vs* Controls.**
Values shown are means ± SEM.

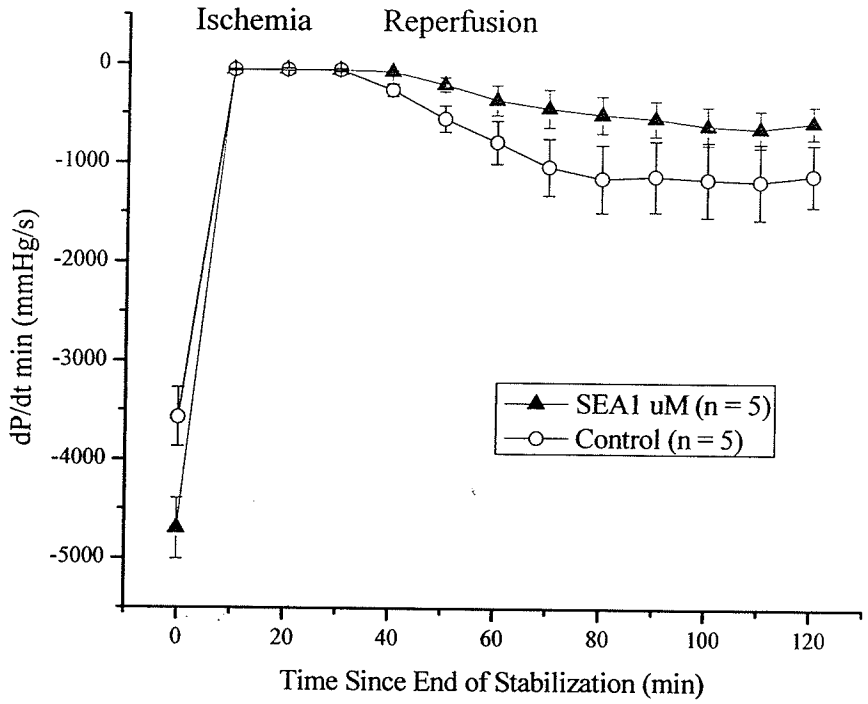



**Fig.51. Maximum Rate of LV Pressure Decline in Isolated Hearts from Obese JCR:LA-*cp* Rats Treated with 1 μM SEA0400 *vs* Controls.** Values shown are means ± SEM.

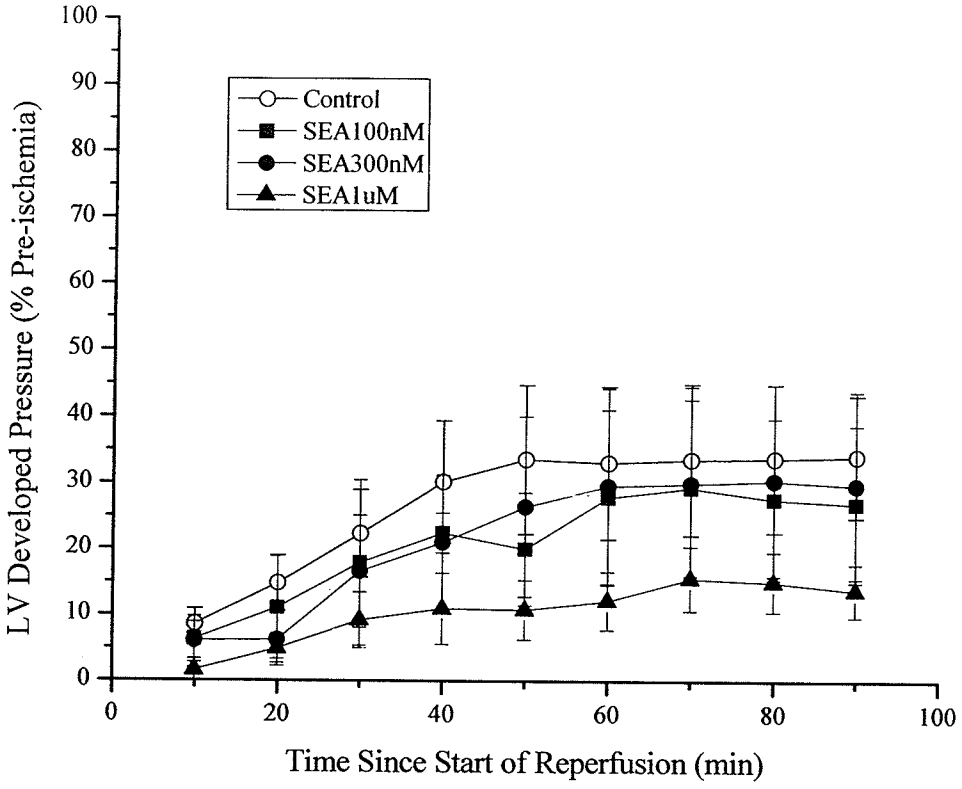



**Fig. 52. Percent Recovery of LV Developed Pressure in Isolated Hearts from Obese JCR:LA-*cp* Rats Treated with SEA0400 (100 nM, 300 nM, 1 μM) *vs* Controls Following 30 Minutes of Global Ischemia and 90 Minutes of Reperfusion.** Values shown are means ± SEM.

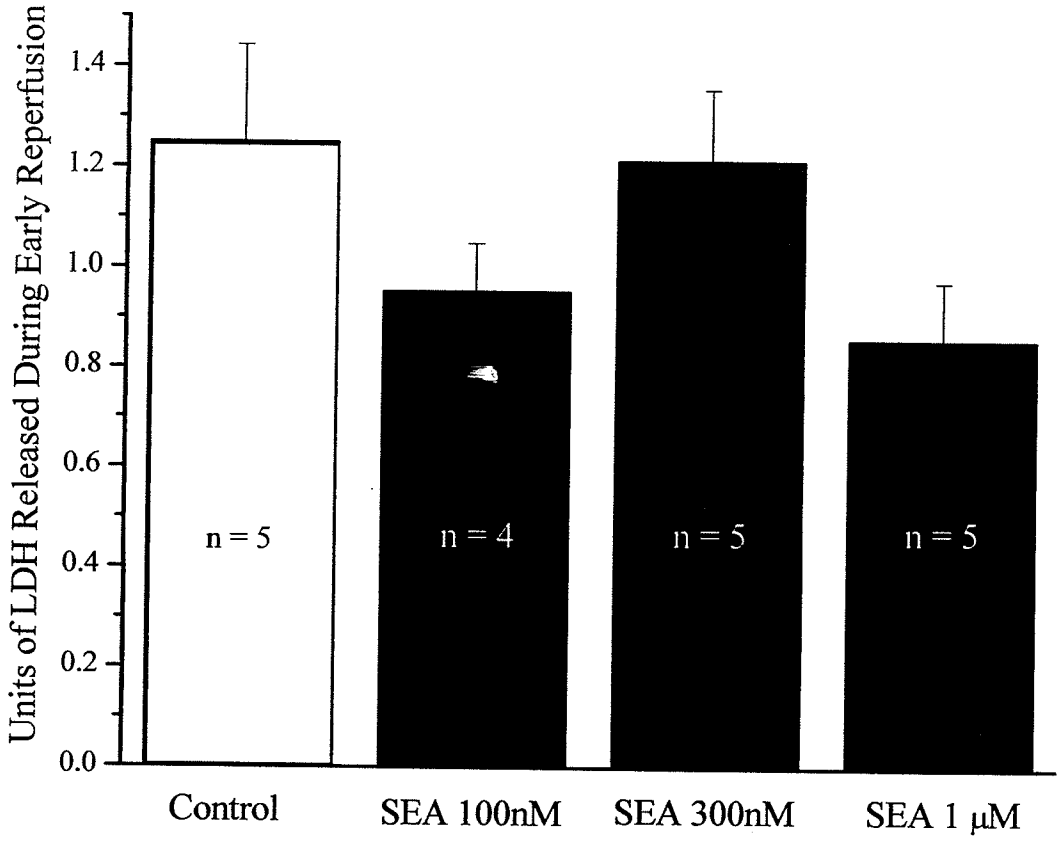



**Fig. 53. Release of Lactate Dehydrogenase During Early Reperfusion from Isolated Hearts of Obese Rats Treated with SEA0400 (100 nM, 300 nM, 1 μM) *vs* Controls.** Values shown are means ± SEM. No significant difference was detected by analysis of variance (ANOVA).

### *4.6.3 Timing of Drug Administration and Method of Drug Delivery*

Preliminary experiments were performed to test a regimen of pre-ischemic administration alone. However, on cessation of the infusion of SEA0400 immediately before global zero-flow ischemia, residual SEA0400 remained in the tubing connecting the drug chamber and the heart chamber (Figure 14, upper panel). On reperfusion, at a constant flow rate of 10 ml/min, it required about 2 minutes for the residual volume to be replaced, in other words, there was an obligatory infusion of SEA0400 for 2 minutes on reperfusion. Because of this reason, pre-ischemic administration of SEA0400 could not be precisely delivered. Moreover, preliminary experiments showed poor recovery of cardiac function and no obvious attenuation in LDH release, this pre-ischemic regimen was not further pursued. Similarly, a post-ischemic regimen could not be precisely tested, due to an obligatory delay of about 2 minutes for the transit of SEA0400 from the drug chamber to the heart chamber, at a constant flow rate of 10 ml/min.

In view of the above, an attempt was made to modify the method of delivery of SEA0400, specifically by means of an infusion pump connected to the side-port in close proximity to the isolated heart (Figure 14, lower panel, orange arrow). However, preliminary experiments showed minimal recovery of LV function. One potential explanation was that drug delivery by the infusion pump necessitated the use of a relatively high concentration of SEA0400 [2.6 μM, in comparison with its solubility limit of approximately 3 μM (231)] within a short distance from the isolated heart, thus introducing the possibility of inadequate mixing between the concentrated drug solution with the perfusion solution flowing at only 10 ml/min.

# 5. DISCUSSION

This is the first study that has evaluated the novel NCX inhibitor, SEA0400, in a polygenetic model of the metabolic syndrome and cardiovascular disease. The following findings are demonstrated for the first time:

a) the feasibility of transthoracic echocardiography in the JCR:LA-*cp* rats;

b) echocardiographic features of the obese JCR:LA-*cp* rats and the lean controls;

c) the feasibility of prolonged *in vivo* administration of SEA0400 in rats under physiological conditions and the absence of significant effects of SEA0400 on systolic and diastolic function as assessed by serial echocardiography;

d) an increased sensitivity to myocardial ischemia in the obese JCR:LA-*cp* rat at 10 to 13 months of age as compared with lean controls;

e) the absence of significant cardioprotection of SEA0400 in Langendorff-perfused isolated hearts from senescent, obese, JCR:LA-*cp* rats under our experimental conditions;

f) quantification of baseline C-reactive protein in the obese and lean JCR:LA-*cp* rats;

g) quantification of abdominal girth as a baseline characteristic in this strain.

The JCR:LA-*cp* rat was utilized for the evaluation of SEA0400 in this study, in light of its severe insulin resistance and other atherogenic metabolic risk factors, as well as its unique cardiovascular complications, that closely mimic those in humans (354,422). Insulin resistance, hyperleptinemia, obesity, hypertriglyceridemia, glucose intolerance, increased plasminogen activator inhibitor-1 of the *cp/cp* rat (352,375) are

commonly found in the metabolic syndrome (277,314,423), of which polygenetic animal models are most valuable (424).

## 5.1 Baseline Characteristics, Including C-Reactive Protein

### *5.1.1 Weight and Abdominal Girth*

The significant differences in body weight between obese rats and lean rats were consistent with previous findings (352).

The significant differences in abdominal girth between the 2 groups were as anticipated, given the obese phenotype of the *cp/cp* rats. Based on the correlation coefficients (r) for abdominal girth *vs* body weight of 0.72 (obese rats) and 0.53 (lean rats), the corresponding values for $r^2$, which measures the strength of the relationships (425), were 0.52 and 0.28, respectively. In other words, in obese rats, body weight and abdominal girth had 52% of their variation in common, whereas in lean rats, the 2 variables only had 28% in common (425).

### *5.1.2 Non-fasting Glucose*

With regard to non-fasting glucose, the lack of significant difference between obese rats and lean rats (13.4 *vs* 14.0 mmol/L, respectively) was consistent with previous findings by Czubryt *et al* (426) in 9-month-old, male, obese and lean JCR:LA-*cp* rats (224 and 152 mg/dl, respectively, p = NS). [For comparison, the corresponding values in the International System of Units were 12.4 mmol/L and 8.4 mmol/L,

respectively, using a conversion factor of 0.05551 (427).] In the study by O'Brien *et al,* the non-fasting glucose levels of obese and lean JCR:LA-*cp* rats at 6 months of age were found to be 147 and 192 mg/dl, respectively, as measured by a glucose oxidase procedure (428). [The corresponding values in the International System of Units were 8.16 mmol/L and 10.66 mmol/L, respectively, using a conversion factor of 0.05551 (427)]. The investigators indicated that plasma glucose in this strain 'tend to be variable' (428), thus providing a potential explanation for the higher non-fasting glucose observed in the lean rats.

### *5.1.3 C-Reactive Protein*

The lack of significant difference in baseline CRP levels between obese rats and lean rats was intuitively not in keeping with findings from human investigations. With the advent of high-sensitivity assays, which are in clinical use, C-reactive protein is considered the best candidate as a biomarker of inflammation and is an independent predictor of coronary risk in human (< 1, 1 – 3, and > 3 mg/L for low, average, and high risk categories, respectively) (316). However, some differences exist between human CRP and rat CRP. First, human CRP is low at baseline and can increase up to 1000-fold in the response to infection or tissue injury (429), whereas rat CRP is not considered an acute-phase protein (430) and its basal level is more than 100 times higher than that in human (429,431,432) – basal plasma level of rat CRP has been reported to be in the range of 300 – 500 mg/L (429). In comparison, CRP in mice has been found to be at low levels both at baseline and in the setting of an acute-phase response (329). Second,

despite considerable homology between the structure of rat CRP, which is made up of 5 monomers (430), and those of human, rabbit and mouse, rat CRP is unique among mammalian CRP in being a glycoprotein and in having covalently linked dimer in the pentameric structure (430).

Alternative explanations for the lack of significant difference in the baseline CRP levels between the obese and the lean JCR:LA-*cp* rats have been considered. For instance, it is conceivable that a superimposed process (such as an occult infection) might have brought any pre-existing differences in CRP levels between the 2 groups to an equivalent level. However, in view of the CRP levels of the JCR:LA-*cp* rats in this study, in the ~ 200 μg/mL (or mg/L) range, being below the reported range for rats in the literature (300 – 500 mg/L) (429), this explanation seems unlikely. Nevertheless, this possibility cannot be ruled out, as CRP levels may not be comparable between rats of different strains, or in the presence *vs* absence of cardiovascular and metabolic abnormalities. In addition, technical aspect of blood collection (immediately on excision of heart) being a confounding factor was considered. However, the time lapse between excision of the heart and collection of blood sample (performed consistently by 2 designated individuals – a team member and myself) was typically in the range of 20 - 30 seconds, which is a relatively brief period as compared with the time course of CRP elevation reported in the literature (433). Specifically, a previous study evaluated the time course of elevation of acute phase proteins in response to injection of an inflammatory stimulus (turpentine) in rats and found that there was a 9-hour delay between the injection and elevation of CRP, which peaked at 48 hours, presumably due to the time required for some intermediate steps leading to response (433). Even though

change in CRP level is reflective of change in its production by hepatocytes (431), regulation of CRP secretion is also important in that CRP is retained in the endoplasmic reticulum of hepatocytes under the basal state (through binding to carboxylesterases) and upon stimulations, there is a decrease in binding as well as a reduction in time for secretion (329).

A previous study in human evaluated the rate of increase in acute phase proteins in response to major and minor surgery and showed that CRP increased at 6 to 8 hours after the initial incision and peaked at 48 hours (434). Moreover, the severity of injury did not appear to influence the time course of change in CRP (434). In another study assessing patients undergoing major operations, CRP was found to peak at 48 to 96 hours after the operation (435). In the setting of acute myocardial infarction, CRP has been shown to increase within 1 to 2 hours after the onset of chest pain and the duration of rising CRP levels was significantly longer in patients with extensive infarction (reaching a peak > 3 days after the onset of pain) as compared with those with mild infarction (corresponding time of 2 days) (436). The calculated mean initial rates of increase in CRP levels were 0.116 μg/mL/hr and 0.270 μg/mL/hr for mild and extensive infarction, respectively (436). The authors commented that they had noted comparable findings in the clinical settings of various surgical procedures (436), as well as in rabbits subjected to intravenous injection of endotoxins (437). Based on the above descriptions in experimental and clinical studies, it is speculated that collection of exsanguinated blood from the JCR:LA-*cp* rats within 30 seconds of excision of the heart would be an unlikely mechanism for a sudden rise in CRP to the extent of equilibrating any potential pre-existing significant difference in CRP between the obese rats and the lean rats.

## 5.2 Characterization of Echocardiographic Features of the JCR:LA-*cp* Rats

Echocardiography was employed as a tool for *in vivo* drug evaluation in this study. The procedure was feasible and uneventful in all the JCR:LA-*cp* rats. Precautions were taken to minimize stress during handling of the rats and anesthesia with documented safety in this strain was used (352). Care was taken to avoid applying excessive pressure on the thorax of the rats during image acquisition with the transducer, as hypotension (438), extreme bradycardia and cardiac arrest could occur (400).

The rationale for performing echocardiography in younger rats was to minimize confounding factors introduced by coronary artery disease in the assessment of left ventricular function by 2-D guided M-mode echocardiography, as atherosclerotic lesions have been shown to increase with age in this strain and, by 9 months of age, advanced intimal lesions of the aortic arch were observed in essentially all *cp/cp* male rats (352). The above was balanced against the logistical issue of housing a large number of JCR:LA-*cp* rats in our facility until 10 months of age for Langendorff-perfusion experiments. As a result, 4-5 months were chosen as the age range for echocardiography, recognizing that atherosclerosis is a continuous process and had been previously documented in this strain of rats by 12 weeks of age (352). For the logistical reasons as detailed under 'RESULTS', the final age range of the obese rats was extended to 3 - 6 months, whereas the lean rats were all of 4 months of age.

The major findings of the 26 obese JCR:LA-*cp* rats (3 - 6 months), as compared with the 12 lean controls (4 months), were increased septal and posterior wall thickness and increased LV mass, consistent with left ventricular hypertrophy (LVH).

There were also suggestions of mild systolic and diastolic dysfunction, as evidenced by decreased fractional shortening and reduced E/A ratio, respectively.

In clinical echocardiography, the E/A ratio is a ratio of the early maximum filling velocity (E velocity) to the atrial velocity (A velocity) of left ventricular diastolic filling (2), which are recorded using pulsed-wave Doppler echocardiography by placing the sample volume between the tips of the mitral leaflets during diastole (408). Normally, early diastolic filling of the LV constitutes approximately 80% of the diastolic filling (439) and the E velocity coincides with the maximum pressure gradient between the left atrium (LA) and the LV (2). This is then followed by flow deceleration and the 'deceleration time' is defined as the time interval between the peak of the E wave and the point where the deceleration slope intersects with the zero baseline (2). After the period of diastasis (when minimal flow occurs), atrial contraction contributes 15 – 20 % of the late diastolic filling (439) and forms the second velocity peak (or A velocity) (2). A reduced E/A ratio and prolonged deceleration time are findings of impaired relaxation (439).

In this study, subsequent analysis focusing on 10 obese rats (3 - 4 months) *vs* 12 lean rats was performed to rule out the possibility that the relatively larger age range might have contributed to the observed differences in echocardiographic features. The findings of increased wall thicknesses and LV mass were still present, although the differences in fractional shortening and in the decreased E/A ratio were no longer statistically significant, suggesting that additional factors or pre-existing factors that progressed with time might have contributed to the systolic and diastolic dysfunction

observed in the overall analysis. In addition, potential effects of anesthesia on cardiac function cannot be excluded.

The echocardiographic finding of the LVH in this study is in keeping with morphometric characteristics of the whole hearts of JCR:LA-*cp* rats previously reported by Misra *et al* (377). Specifically, in 3-month-old *cp/cp* rats and age-matched controls, a significant difference in heart weight was observed: 0.98 g and 0.77 g, respectively ($p < 0.05$). The heart weight/body weight ratio was also significantly different (O *vs* L, $p < 0.05$): 1.98 mg/g *vs* 2.35 mg/g, respectively (377). In comparison, the corresponding calculated values in the present study, based on echocardiographic measurements of the left ventricle of obese rats (3 - 4 months) vs lean rats (4 months) were: LV mass 0.70 *vs* 0.60 g ($p < 0.01$), and LV mass/body weight 1.21 *vs* 1.67 mg/g ($p < 0.0001$), respectively (Table 3). The reversed direction of difference after indexing for body weight could be explained by the presence of morbid obesity, as body weight constituted the denominator in the calculation, as similarly observed in *db/db* mice (440).

In the study by Barouch *et al* (440), *db/db* mice, which were analogous to the *cp/cp* rats in having a leptin receptor defect (441), were demonstrated to have LVH at 6 months of age (*db/db* vs *db* control): wall thickness 0.80 *vs* 0.58 cm, LV mass 105 *vs* 77 mg, LV mass/body weight 1.69 *vs* 2.11 mg/g, respectively ($p < 0.01$ for all 3 comparisons), without any significant difference in end-systolic or end-diastolic dimensions. Hypertrophy of the cardiac myocytes were observed under light microscopy (440). In that study, LVH was also demonstrated in *ob/ob* mice, which lacked leptin [the *ob* gene product (371)], and the increased wall thickness was reversed after leptin infusion, suggesting an anti-hypertrophic effect of leptin (440,442). In

contrast, other studies have shown direct hypertrophic effects of leptin in cardiac myocytes (443,444) and the reason for the difference is unclear. Notably, leptin receptors are also present in the heart (442,445) and a detailed study of the leptin system in various areas of the heart has been reported recently (445). It is quite possible that the precise role of leptin in cardiac hypertrophy has yet to be fully explored.

It is speculated that other possible contributors to LVH in the obese JCR:LA-*cp* rats may also include obesity, insulin resistance, and other metabolic abnormalities, based on studies in other strains of rats (446,447) and in human (448-452). As the JCR:LA-*cp* rats, at 12 weeks of age, have been reported not to have hypertension as measured by inserting a needle (attached to a pressure analyzer) into the femoral artery (453), hypertension is not likely to be a contributing factor in the LVH observed in the present study.

There are a number of potential limitations in this study pertaining to the application of M-mode and pulsed-wave Doppler echocardiography, as listed as follows:

(a) Anesthetic agents might affect cardiac function during echocardiography (454). Nevertheless, isoflurane, which was used in this study, has been shown to have less cardiovascular depressive effects, as compared with halothane, in rodents (455). In addition, it was associated with the most reproducible results in fractional shortening in serial murine echocardiography, as compared with several other anesthetic regimens, in one study (454).

(b) Gating with electrocardiography was attempted in the JCR:LA-*cp* rat but the signal was not reliable. In the absence of ECG gating, diastolic

measurements were made at the time of maximal LV diastolic dimension on M-mode images, whereas end-systolic measurements were made at the time of minimal LV dimension (438).

(c) Despite the advantage of M-mode echocardiography in providing excellent temporal resolution [due to its high sampling rate at 1800 frames per second (401)], the image represents a one-dimensional view of the heart displayed over time (388,389,401). The implications are that non-perpendicular orientation (401) to the cardiac structure during measurement would potentially reduce accuracy. In addition, M-mode echocardiography would be best utilized only in the presence of uniform ventricular geometry and wall motion (389). To maximize accuracy in the present study, 2D images in two orthogonal planes were used in guiding M-mode measurements (401) and younger rats were used to minimize the possibility of concomitant presence of significant coronary heart disease.

(d) Previous investigators have reported difficulty with identifying the leading edge of the anterior wall (or septum) on M-mode images of rat hearts (400,402). Every effort was made in this study to take multiple images to facilitate selection of best quality images for data analysis.

(e) In the application of the pulsed-wave Doppler technique for measuring velocities of blood flow, the accuracy of this measurement depends on a parallel orientation between the ultrasound waves and the direction of blood flow (408). To maximize accuracy, 2D imaging was used to guide positioning of the sample volume (2).

(f) Mitral inflow parameters are preload-dependent and may also be altered in the presence of concomitant cardiovascular pathologies (2). In addition, being measures of LV filling dynamics, abnormal mitral inflow parameters do not necessarily imply dysfunction of intrinsic diastolic properties of the LV (456).

(g) Fusion of the E and A waves in the presence of high heart rates, which are of particular relevance to echocardiography in rats and mice (388), may preclude measurement of mitral inflow parameters.

## 5.3 *In Vivo* Administration of SEA0400

These experiments were designed to test the hypothesis that prolonged *in vivo* administration of SEA0400 would be feasible and innocuous to global ventricular function, as there were no data on this subject at the inception of this study and the concept of chronic prophylactic therapy with an NCX inhibitor has never been tested. A formal evaluation of the above was warranted as a study on SEA0400 by Tanaka *et al* (219) in 2002 showed equivalent inhibitory potency of this compound toward the forward mode and the reverse mode of NCX in guinea pig ventricular myocytes. In another study, Reuter *et al* (220) evaluated SEA0400 in embryonic heart tubes of NCX1 knock-out mice and demonstrated depression of $Ca^{2+}$ transients by SEA0400, suggesting a non-specific action of this compound. Since NCX has an established role in maintaining $Ca^{2+}$ homeostasis on a beat-to-beat basis under physiological conditions, it is postulated that the following would be important attributes of NCX inhibitors for chronic prophylactic use:

(a) no significant inhibitory effects on ion channels and transporters other than NCX,

(b) no significant inhibitory effects on the forward mode of NCX,

(c) selective inhibition of the reverse mode of NCX,

(d) no adverse effects on overall systolic and diastolic function,

(e) no significant adverse effects on other organ systems.

The present study was conducted to test the hypothesis that prolonged use of an NCX inhibitor would not significantly affect systolic and diastolic function, as the role of the reverse mode of NCX under physiological conditions is negligible and that

SEA0400 is selective for the reverse mode of NCX, particularly under conditions when intracellular $Na^+$ is elevated, such as during ischemia/reperfusion. As it is anticipated that the potential use of an NCX inhibitor on a chronic basis for cardiac protection would be best suited for individuals with cardiac risk factors, an animal model of the metabolic syndrome, the JCR:LA-*cp* rat, was chosen.

As SEA0400 was available only as an intravenous drug at the inception of this study and very limited information was available on this drug at that time, the osmotic pump was chosen as a method of drug delivery to ensure bioavailability (397). The dose of SEA0400 used, 1 mg/kg/day as a continuous infusion for 4 weeks, was based on the study by Takahashi *et al* (97) using SEA0400 1 mg/kg in acute IR experiments in anesthetized rats, as that was the only available *in vivo* cardiac study in rats at that time.

### *5.3.1 Feasibility of Osmotic Pump Implantation*

In this part of the project, 26 rats were randomized to 1 of 3 groups: osmotic pump implantation with SEA0400, osmotic pump implantation with vehicle, no intervention. The purpose of the latter was to assess any potential effects of the surgical procedure itself on ventricular function in these stress-prone rats. As detailed under 'RESULTS', two rats had to be euthanized within the first week after osmotic pump implantation. Since one of these rats was from the SEA0400-treated group and the other one was from the vehicle-treated group, it is likely that these adverse events were not related to the active component of SEA0400.

### 5.3.2 *Systolic and Diastolic Function*

Serial echocardiographic data of the remaining 24 rats showed stable systolic function (as assessed by fractional shortening) and stable diastolic function (as assessed by the E/A ratio and the deceleration time) with no significant differences at the end of 4 weeks.

### 5.3.3 *Delayed Hair Re-growth*

During echocardiographic follow-up, delayed re-growth of hair following shaving for osmotic pump implantation was noted (even at the end of week 4) in some of the obese rats, both from the SEA0400-treated group and the vehicle-treated group. Since impaired wound healing has been recently reported in obese JCR:LA-*cp* rats, as compared with lean controls (457), it is possible that our observations may be attributed to this reason. Of note, delay in regrowth of hair following shaving has been reported in mice, which was observed increasingly with advancing age of the mice, across the age groups of 20, 66, 188, and 312 days (458). In that study, biochemical analysis of skin with and without re-growth of hair showed differences in certain enzyme levels although the precise mechanism remained unclear (458). Overall, as delayed regrowth of hair occurred in both the SEA0400-treated group and the vehicle-treated group, it is not likely to be related to the active component of the drug.

### 5.3.4 *Differences in Body Weights*

Notably, statistically significant differences in body weights were detected for all comparisons between groups at baseline. Potential contributing factors are as follows: a) the scarcity of the *cp/cp* rats and the associated difficulties in achieving a tight range of age might have contributed to imbalances in body weights at baseline; b) logistical difficulties in coordinating surgical, anesthesia, and echocardiographic schedules resulted in delays and re-scheduling of experiments, resulting in a widening of the age range of the rats (from 4 - 5 months to 3 - 6 months), which might have impacted on the comparison of body weights at baseline). At weeks 1, 2, and 3, some of the above significant differences persisted, as shown in Table 4. However, at week 4, no significant differences were detected in any of the comparisons.

In review of the age of the rats on the day of osmotic pump implantation, it was noted that more of the rats randomized to SEA0400 treatment were closer to the upper end of the age range (*i.e.*, 6 months). Since these SEA0400-treated rats had a significantly higher mean baseline weight than the other 2 groups and this difference was no longer present at the end of the 4 weeks, one interpretation would be a relative paucity of weight gain in this group, which may or may not drug-related. The following observation would suggest a non-drug-related reason: in Figure 16, there seemed to be a part of the growth curve, around 5 - 6 months, where the rate of weight gain was relatively attenuated. (It is recognized that the small 'n' at 5 months in Figure 16 might be a potential limitation of the growth curve.) In addition, in review of a figure published by others (352), a gradual slowing of weight gain also appeared to be present approximately between 12 to 18 weeks.

The baseline weight of the control group being in the middle among the 3 groups would be consistent with this hypothesis, as their age comprised both ends of the range of 3-6 months.

On the other hand, an alternative explanation would be a potential inhibitory effect of SEA0400 on weight gain (or growth) and an enhancing effect on weight gain by the vehicle, which seemed unlikely but cannot be completely excluded.

It is recognized that these experiments mainly serve the purpose of proof of principle in a clinically relevant animal model with intrinsic cardiac and metabolic abnormalities, since the appropriate dose and duration for chronic administration of SEA0400 were both unknown. In addition, the presence of significant obesity, in general, would affect pharmacokinetics (459). A previous study suggested that the age and the strain of rats might influence the dose requirements for KB-R7943 (196). At the time of this study, such information is not available for SEA0400. Thus, findings from the JCR:LA-*cp* rats in this study may not be applicable to other strains of rats.

It has been established that the proportion of $Ca^{2+}$ efflux through NCX is lower in the rat (7% via NCX, 92% via SR $Ca^{2+}$-ATPase, 1% via sarcolemmal $Ca^{2+}$-ATPase and mitochondrial uniport), as compared with the rabbit (with corresponding values being 28%, 70%, and 2%, respectively) (9). As the balance of $Ca^{2+}$ fluxes in human is closer to that in rabbit rather than in rat (9), the findings from the JCR:LA-*cp* rats may not be generalized to their human counterparts. Nevertheless, these findings may provide the basis for further evaluation of any potential effects of chronic NCX inhibition on cardiac function in larger animal models (such as the rabbit and the dog).

## 5.4 Sensitivity of Senescent JCR:LA-*cp* Rats to Ischemia/Reperfusion

In this part of the study, the myocardial sensitivity of the senescent JCR:LA-*cp* rat to global ischemia followed by reperfusion (to simulate the setting of cardiac surgery) was evaluated. The age range of 10 – 13 months was chosen based on the following reasons: (a) the senescent JCR:LA-*cp* rat may serve as a model of the metabolic syndrome in the elderly, as the prevalence of the metabolic syndrome (302) and that of ischemic heart disease (460) increase with age; (b) such information on the senescent rat was not available in the literature.

The main focus was on the protocol of 30 minutes of global ischemia followed by 90 minutes of reperfusion. Additional experiments were performed using 40 and 50 minutes of ischemia (both followed by 90 minutes of reperfusion) to further evaluate ischemic response.

The key finding in this part of the project is the increased sensitivity to 30 minutes of global ischemia in the senescent, obese JCR:LA-*cp* rats *vs* lean controls, as evidenced by the significantly worse post-ischemic contractile function of the obese rats at 30, 60, and 90 minutes of reperfusion, and the significantly larger amount of LDH released during early reperfusion. Data from 40 minutes of ischemia are also in favour of these findings, although the statistical comparisons are not as significant, possibly due to the onset of irreversible injury in both the obese rats and the lean rats (to a differential extent) after 40 minutes of ischemia. A previous report by Palmer *et al* (461) documentating a transition to irreversible injury at approximately 20 minutes of normothermic global ischemia in isolated hearts from Sprague-Dawley rats would be in keeping with this hypothesis. The data on 50 minutes of ischemia are limited by the

small numbers and the relatively large variability in the data, although the trends of the plots (Figures 34 - 37) are still in keeping with the results from 30 minutes of ischemia.

Overall, the above findings are consistent with those reported by Maddaford *et al* (361), who evaluated the obese rats and the lean rats at 3 – 9 months of age and demonstrated increased sensitivity to ischemia in the obese rats at 6 and 9 months of age, as compared with lean controls.

It is speculated that the unexpected finding of an undulating baseline (Figure 24) during reperfusion and the exceedingly high diastolic pressures observed in this study were both manifestations of IRI, either directly or indirectly. Despite restoration of epicardial coronary blood flow, IR-induced microcirculatory dysfunction was probably present, due to a potential combination of endothelial dysfunction, myocyte necrosis, tissue edema, injury from oxygen free radicals, and obstruction of capillaries by microemboli (462) – the latter could have originated from atherosclerotic lesions in the ascending aorta at the time of excision of the heart (this hypothesis will be further discussed under section 5.5.2). Swelling and contracture of the endothelial cells might have resulted in increased permeability of the microvascular endothelium (due to the formation of gaps between endothelial cells) (54). The formation of tissue edema (462) could have led to extravascular compression and compromise of microvascular blood flow (54,463). It has been previously demonstrated by Gaasch and Bernard (464) in anesthetized dogs that (a) LV end-diastolic wall thickness (as measured by echocardiography) decreased during coronary ligation and increased above baseline on reperfusion (reactive hyperemia), (b) prevention of reactive hyperemia by controlled release of the ligature also prevented the overshoot in wall thickness during early

reperfusion. Such acute increase in wall thickness on reperfusion has also been demonstrated in a porcine model using percutaneous intracoronary balloon inflation to induce transmural infarction (465). In that study (465), an acute increase in end-diastolic wall thickness (as monitored by echocardiography) was observed which increased 'logarithmically' during 60 minutes of reperfusion, with subsequent histological demonstration of massive extracellular edema. The end-diastolic cavity size decreased immediately on reperfusion with no further changes afterwards (465). In the context of the present study, it is postulated that the undulating baseline represented the exaggerated motion of the roller pump transmitted to the transducer as it pumped against markedly increased coronary resistance on reperfusion, due to extravascular compression resulting from massive myocardial edema and/or capillary damage and obstruction (466), in the presence of a fixed flow rate set by the roller pump. This potential limitation of constant flow perfusion has been previously pointed out by Sutherland and Hearse (467) in that 'autoregulatory mechanisms are overridden' and, in the setting of ischemia/reperfusion, a fixed volume of perfusate would be pumped through a diminished vascular bed. Since no obvious rhythmic motion of the heart could be observed during the experiment, it is speculated that this exaggerated motion was transmitted to the balloon inside the left ventricle through the edematous endocardium abutting against the balloon (especially if an acute reduction in end-diastolic cavity size also occurred). Conceivably, the exceedingly high diastolic pressure might also have been partially contributed by active compression from the edematous endocardium, in addition to severe diastolic dysfunction in the presence of a globally edematous, non-complaint ventricle with pre-existing hypertrophy, as well as

probable vascular dysfunction and coronary artery disease (352,468). These abnormalities were probably further aggravated by an *in vitro* phenomenon that could be likened to persistent reactive hyperemia during the entire reperfusion period (it was 'persistent' due to the constant flow rate employed in the experimental protocol).

The excess ventricular arrhythmias observed during the early course of our study was likely due to a combination of the relatively higher stimulation rate (469), at 300 beats per minute, as compared with 200 beats per minutes employed by Maddaford *et al* (361), along with the relatively high $Ca^{2+}$ concentration (1.8 mM) in the perfusion solution. Notably, in a model of isolated working heart, the myocardium of the *cp/cp* rats has documented to be sensitive to $Ca^{2+}$ concentration in the perfusate (470). During the early course of the experiments, trauma to the aorta during dissection or cannulation would have accounted for some of the arrhythmias but this problem resolved with time and experience. Moreover, the possibility of an intrinsic predisposition to ventricular arrhythmias was also contemplated, although the prompt correction of this problem with lowering of $Ca^{2+}$ concentration would favour the first explanation as the most likely reason in this situation.

In this study, there are limitations pertaining to the isolated heart technique (411,467). First, the absence of blood components in the crystalloid solution precluded evaluation of contribution of platelets and leukocytes in the simulated IR, which are known to play key roles in the pathophysiology (467). Second, isoflurane has been reported to confer cardioprotection in a number of studies (471) (which will be reviewed in section 5.5.1) and could have been a potential confounding factor. Third, the senescent rats likely had pre-existing coronary artery disease. It is speculated that any

coronary lesion, even if mild, in the setting of massive myocardial edema on reperfusion, might have become hemodynamically significant.

## 5.5 Effects of SEA0400 in the Langendorff-Perfused Isolated Hearts Subjected to Global, Zero-Flow Ischemia

In this study, no significant benefits of SEA0400 could be demonstrated in attenuating post-ischemic contractile dysfunction or LDH release during early reperfusion. The 3 concentrations of SEA0400 tested (100 nM, 300 nM, and 1 μM) were based on the available publications in the literature evaluating SEA0400 in the isolated hearts of rats (97) and rabbits (98) during the course of this project. Even though cardioprotective effects of post-ischemic administration of SEA0400 were demonstrated in both of these studies [one of which also tested pre-ischemic drug initiation and showed similar benefit to post-ischemic drug initiation (98)], important differences exist between these studies and the present study. First, young, healthy rats and rabbits were evaluated the above studies, as compared with the older rats with pre-existing severe metabolic and cardiovascular abnormalities assessed in this study. Second, the protocols for induction of ischemia differed in duration, anatomic extent, and severity in that 30 minutes of regional ischemia was employed in rabbits by Magee *et al* (98) and 60 minutes of hypoperfusion (induced by lowering perfusion pressure from 65 mmHg to 7 cm $H_2O$ in Wistar rats) was used by Takahashi *et al* (97), as compared with the 30-minute, global, zero-flow ischemia protocol utilized in this study. Notably, protocols of regional ischemia and global hypoperfusion have likely made the testing of post-ischemic administration feasible in that SEA0400 could be delivered into, or in close proximity to, the isolated heart at the end of the ischemic period for immediately use at the moment of reperfusion, whereas timely drug delivery on reperfusion was challenging in this study using global zero-flow ischemia, as detailed

elsewhere in this thesis. Third, the duration of drug administration differed in that SEA0400 was initiated either at 30 minutes before regional ischemia or at 1 minute before reperfusion and was continued for the entire experiment (including 120 min of reperfusion) in rabbits (98), for 10 min immediately upon return to normal perfusion at 65 mmHg following hypoperfusion in Wistar rats (*i.e.*, without pre-ischemic administration) (97); and, in this study, for 10 minutes before global ischemia and again for 10 min on reperfusion. The 10-minute post-ischemic administration in this study was chosen based on the protocol of Takahashi *et al*, and the rationale for pre-ischemic administration was based on a combination of logistical reasons and previous findings in the NHE literature (52). Due to the above differences in experimental protocols, direct comparison between these studies and the present investigation would not be possible.

It is speculated that the most likely explanation of the findings in the JCR:LA-*cp* rats in this part of the study is a lack of efficacy of SEA0400 in this pathological animal model (with extreme metabolic abnormalities, cardiovascular sequelae, and increased susceptibility to IRI), in the setting of a severe ischemic insult induced by global, zero-flow ischemia of a substantial duration. This lack of efficacy is in distinct contrast to the encouraging results demonstrated in otherwise healthy animals subjected to IR in the literature (97,98,232-234), as well as findings from preliminary experiments on 6 Sprague-Dawley rats (male, 545 – 820 g, 3 control and 3 treated with SEA0400 of different concentrations) in our laboratory (not shown). Notably, there has also been a canine study, by Nagasawa *et al* (239), in which SEA0400 did not demonstrate any efficacy against IR-induced ventricular arrhythmias. Specifically, using an *in vivo* canine model of regional ischemia (induced by ligation of the left anterior descending

artery for 30 minutes followed by reperfusion), the investigators demonstrated that neither pre-ischemic administration of SEA0400 (1.0 mg/kg at 10 minutes prior to coronary ligation) nor post-ischemic administration (0.3, 1.0, 3.0 mg/kg at 1 minute before coronary reperfusion) resulted in a significant reduction in the incidence of ventricular fibrillation (239). Notably, previous studies that had evaluated KB-R7943 as a potential cardioprotective agent also documented some negative findings in that KB-R7943 did not significantly attenuate IR-induced arrhythmias in dogs (202), and did not improve energy metabolism or reduce 'LV stiffness' in diabetic rat hearts subjected to hypoperfusion/reperfusion, with or without exacerbation by norepinephrine (224). On the contrary, in the latter abstract (224), KB-R7943 tended to increase the injury exaggerated by norepinephrine. Based on the above findings, it is speculated that while inhibition of the reverse mode of NCX with SEA0400 (and KB-R7943) appeared promising as a cardioprotective strategy against IRI in healthy animals, whether it would eventually reach the stage of clinical investigation would likely depend on the reproducibility of the cardioprotective effects in animal models with pre-existing pathologies and ultimately in their human counterparts. Indeed, it has been pointed out that 'almost all studies' of cardioprotection and cardiac arrest in the experimental settings have resulted in findings that were inconsistent and/or non-reproducible, thus representing major challenges in the translation of cardioprotective therapy from the research bench to the bedside (172).

### 5.5.1 *Isoflurane*

Even though a lack of efficacy of SEA0400 is the most likely explanation for the results of these experiments, other possibilities must also be considered as detailed below. Specifically, experimental data in the literature to date have largely supported the cardioprotective properties of isoflurane in various models across over a range of doses (471-481), with few exceptions (482-484). Some clinical studies have also evaluated pre-operative or intra-operative administration (prior to aortic cross-clamping) of isoflurane as a cardioprotective strategy and demonstrated certain favourable effects on post-operative hemodynamics (485,486) and reduction in cardiac enzyme release (487), although neutral findings have also been shown (488) and long-term data on clinical outcomes are lacking (489). Potential mechanisms of isoflurane in cardioprotection may include preservation of adenosine-triphosphate (ATP) (490,491); decrease in $Ca^{2+}$ influx (471,492); inhibition of neutrophil activities (480,493); and, importantly, preconditioning (471) – the latter may involve multiple signaling pathways (492,494-496), release of reactive oxygen species (477,497), activation of ATP-sensitive potassium channels of the mitochondria (495,498) or the sarcolemma (474,499,500). Neuroprotective effects of isoflurane have also been demonstrated in several experimental studies (501-504). Even though the role of isoflurane as a confounding factor in this study remains speculative (as information on the exact duration of exposure to isoflurane of each rat, as administered by the staff on duty of the day, would not be available to supplement our data analysis), it may partially explain the wide variation in myocardial response to ischemia/reperfusion observed in this study (including preliminary data that are not reported in this thesis), as any variation in the

concentration and duration of exposure could be likened to concomitant administration of a variable dose of another cardioprotective agent. The above may also have ramifications for clinical evaluation of cardioprotective agents in surgical settings, since some of the anesthetic agents in clinical use, including volatile anesthetics, have cardioprotective properties (471,489).

### 5.5.2 *Potential Impact of Pre-existing Cardiovascular Disease on the Isolated Heart Experiments*

It is likely that the obese rats would have developed pre-existing coronary lesions to a variable degree, by 10 to 13 months of age (362), thus potentially introducing variability to the extent of reperfusion. In addition, the possibility of embolization of atherosclerotic debris to the coronary arteries upon cannulation of the aorta was contemplated. As it has been documented that by 9 months of age, 'essentially all' male, obese JCR:LA-*cp* rats had 'advanced intimal lesions' of the aortic arch (including thrombi demonstrated by scanning electron microscopy), similar to atherosclerotic changes in human vasculature (352). It is conceivable that excision of the heart at the level of the ascending aorta, followed by cannulation of the aorta and retrograde perfusion (from the aorta) into the coronary arteries might have resulted in

embolization of atherosclerotic debris into the coronary vasculature – a situation analogous to embolization to the cerebral circulation following cross-clamping of severely atherosclerotic aortae at the time of cardiac surgery (505-507) (with dislodgement of atherosclerotic debris in the antegrade direction from the aorta to the brain, as a potential cause of embolic stroke), or following catheter-based procedures (507,508).

If this proposed mechanism indeed occurred in the Langendorff-perfused isolated heart experiments, such microemboli would possibly have compromised microvascular perfusion and introduced variability into the experiments, as the pattern of microembolization in the coronary vasculature would be unpredictable. While it is likely that any potential emboli introduced would have been small, given the stable baseline hemodynamics and the virtual absence of LDH in baseline measurements, these findings could not be regarded as absolute reassurance due to the following reasons: (i) global ventricular function might not have been significantly affected by localized occlusion of the microvasculature (509), (ii) any LDH released during the early part of the stabilization period would not have been captured, since baseline samples of coronary effluent were collected routinely at the end of the stabilization period.

In summary, while SEA0400 may have no cardioprotective effects in this rat model of extreme metabolic abnormalities with spontaneous cardiovascular sequelae, alternative explanations of our findings would potentially include intrinsic variabilities due to pre-existing coronary artery disease and the use of volatile anesthesia.

## 5.6 Effects of SEA0400 on Baseline Cardiac Performance

The effect of SEA0400 on basal cardiac function was assessed during pre-ischemic administration. Overall, no negative effects on cardiac function were observed (Table 9). Statistically significant differences were present in some of the comparisons of cardiac function, although these findings would also be compatible with changes that commonly occurred during the stabilization period, especially since the time lapse between cannulation of the aorta and initiation of SEA0400 was typically about 15 minutes (once the parameters of cardiac performance appeared stable).

In review of the literature, 3 studies have not identified a significant inotropic effect associated with SEA0400 administration. This includes isolated rabbit (98) and rat (97) hearts subjected to retrograde perfusion according to Langendorff, an *in vivo* canine model of IR-induced arrhythmias, anesthetized dogs subjected to transient occlusion of the left anterior descending artery followed by reperfusion to induce myocardial stunning (233). In particular, in the latter *in vivo* study (233), it was noted that SEA0400 did not significantly affect the product of heart rate and LV systolic pressure (an index of myocardial oxygen demand), when comparisons were made either between the groups receiving vehicle *vs* different doses of SEA0400 or within the groups themselves throughout the experiments (233). However, in an abstract report (245), SEA0400 increased contractile force in the guinea pig, rat and mouse ventricle (along with increased amplitude of $Ca^{2+}$ transients) and suppressed ouabain induced positive inotropy in the guinea pig in a concentration-dependent manner.

The absence of negative effects of SEA0400 on contractile function in isolated rabbit hearts was reported by Magee *et al* (98). Significant improvement in the

development and the decline of LV pressure (± dP/dt) post-ischemia were observed in hearts treated with SEA0400 (1 μM) (98). However, evaluation of higher concentrations of SEA0400 was precluded by its limited solubility (98). In the study by Takahashi *et al* (97), SEA0400 (≤ 1 μM) did not have any significant effects on heart rate and coronary flow in the ischemia/reperfusion model of Langendorff-perfused isolated rat hearts; and did not exert any significant effects on mean blood pressure and heart rate in the IR-arrhythmia model of anesthetized rats (≤ 1 mg/kg, administered i.v. at 1 minute before reperfusion). Similarly, in the study by Iwamoto *et al* (238), infusion of SEA0400 in normal rats and dogs did not affect arterial blood pressure.

The absence of negative hemodynamic effects of SEA0400 have also been demonstrated in dogs by Takahashi *et al* (233). In this canine model of myocardial stunning, electrocardiographic and hemodynamic parameters, as well as coronary blood flow after reperfusion, were unaffected after *in vivo* administration of SEA0400 (0.3 mg/kg or 1.0 mg/kg) as an i.v. bolus at 1 minute before reperfusion (233). Moreover, no significant change in segmental shortening was observed in the non-ischemic myocardial after administration of SEA0400, supporting the notion that SEA0400 does not exert significant effects on contractility (233). In another *in vivo* canine study, Nagasawa *et al (239)* reported no significant change in baseline heart rate or blood pressure (as monitored through right femoral arterial catheter) following pre-ischemic administration of SEA0400 1.0 mg/kg i.v. (at 10 minutes prior to coronary artery ligation). No change in the heart rate, blood pressure, or ECG parameters was observed after treatment with vehicle (239). At 20 minutes after administration of SEA0400 i.v. bolus in anesthetized dogs with digitalis-induced arrhythmias, the mean blood pressure

was noted to have decreased, as compared with that prior to SEA0400 administration, and this decrease persisted until the end of the 60-minute observation period (239) – approximately 155, 175 (baseline), and 115 mmHg, respectively, based on plotted data (239). As details of the above were not available, the mechanism and the significance of this finding remain to be clarified.

## 5.7 Future Directions

A unique challenge in utilizing NCX as a therapeutic target is that both the forward ($Ca^{2+}$-efflux) and the reverse ($Ca^{2+}$-influx) mode of NCX have to be taken into consideration when an inhibitor is applied. As the forward mode is a primary mechanism for removing $Ca^{2+}$ that enters the myocyte through the L-type $Ca^{2+}$ channels on a beat-to-beat basis (27), the ideal drug would be either (a) one that inhibits only the reverse mode of NCX (hence preventing $Ca^{2+}$ overload) and has no effects against the forward mode of NCX, other ion channels and transporters; or (b) a drug that has the intrinsic property of inhibiting both modes of transport (differentially or equally) and such inhibition would only occur under pathophysiological conditions (such as during ischemia/reperfusion when intracellular $Na^{+}$ is elevated). Experimental data to date on SEA0400 suggest the latter as the likely explanation for its efficacy (230,231), although the mechanism of action of novel NCX inhibitors is still an area under active investigation. An important caveat in studying the pharmacology of NCX inhibitors is that actions other than NCX inhibition may exist (220). Nevertheless, research and development in the recently discovered compounds, such as KB-R7943 and SEA0400, must be considered important progress in this field (12) and will undoubtedly continue to be one of the priorities for research in the field of NCX. While NCX inhibition appears promising against cardiac, cerebral, and renal IRI, its effects on the brain require further delineation and clarification, as there have also been reports suggesting deleterious effects of NCX inhibition in experimental settings of IR in the brain (510-512) (although the use of non-selective NCX inhibitors was a limitation in those studies), as well as the preliminary report of increase in stroke observed in the EXPEDITION

study (6) (where NHE inhibition with cariporide *vs* placebo was tested in high-risk cardiac surgical patients). In addition, while the cardioprotective effects of post-ischemic administration of SEA0400 appear impressive in animal models, as demonstrated in rats (97), rabbits (98), dogs (233), confirmation of the efficacy of post-ischemic administration of NCX inhibitors requires large-scale prospective clinical trials, especially in light of previous experience from the NHE literature (154). In the interim, the anticipated final report of the recently completed CASTEMI trial, as well as future findings from another phase II clinical trial (in progress) evaluating the safety, tolerability, and efficacy of MCC-135 in patients with STEMI undergoing PCI (264), would be valuable in guiding future research on the potential use of NCX inhibitors for cardioprotection.

Recent advancements in experimental techniques in the use of microbubbles (513-515), ultrasound enhancement (514-516), specialized catheter systems (517) and surgical protocols, such as, retrograde delivery (of cells) through the coronary venous system in the presence of arterial occlusion (518), appear promising as potential novel strategies for facilitating drug delivery during acute coronary thrombosis. Whether these techniques would have potential applications for the delivery of SEA0400 or other novel inhibitors of NCX remains to be investigated. Notably, ultrasound-enhanced fibrinolysis has been successfully applied in humans in the setting of acute ischemic stroke (519). Similar experimental data pertaining to the coronary territory also appear promising (520,521).

Despite recent advances in pharmacological and catheter-based reperfusion in the management of the acute coronary syndromes and in cardioprotection during cardiac

surgery, there is still a need for therapies that specifically target the pathophysiology of IRI (168,381,522,523). In fact, it has been suggested that progress in further reducing mortality in patients with ACS, beyond timely reperfusion, might be limited by injuries induced by IR (524), for which there is currently no clinical therapy (172). While it would be ideal to have one single agent with an established safety profile that could be easily administered in a timely manner to protect the myocardium against IRI regardless of mechanism within the complex pathophysiology of IRI, this goal may not be attainable. Alternatively, combination therapy may theoretically afford greater cardioprotective benefits (68), although, conceivably, the challenge would be to identify the best agents (and in the right proportions) to be used in combination with NCX inhibitors so as to achieve the most favourable benefit to risk ratio for the individual patient. There will likely be vast potential in the evaluation of combination therapies, since the progress made in the field of NCX inhibitors has been relatively recent [largely as a result of the dearth of selective NCX inhibitors in the past (12)], as compared with the overall experience accumulated in the evaluation of various cardioprotective compounds or strategies over the past 3 decades (172). Moreover, potential combination of non-pharmacological therapies with NCX inhibitors also appears to be an attractive strategy. In particular, 'post-conditioning', which consists of 3 cycles of 30-second reperfusion, each followed by 30-second reocclusion, at the start of reperfusion (525), has been shown to protect the myocardium against IRI (525-527). The precise mechanisms of cardioprotection are under active investigation, although investigations to date suggest the involvement of $K_{ATP}$ channels (527), nitric oxide (527), reperfusion salvage kinase pathway (527,528), adenosine receptors and other signaling

pathways implicated in pre-conditioning (529). As experimental data on SEA0400 to date support the efficacy of post-ischemic administration for cardioprotection (97,98,233), it is speculated that the combination of SEA0400 and post-conditioning may potentially provide incremental cardioprotective effects through different mechanisms. Similarly, whether there is any therapeutic advantage in combining preconditioning with NCX inhibition (applied before or/and after ischemia) remains to be investigated.

In addition, the use of animal models with pre-existing cardiovascular pathologies may be particularly valuable in providing insights into human disease (382). Specifically, in the context of evaluation of SEA0400, models mimicking increased risk for CAD in humans, such as models of hypercholesterolemia, diabetes or the metabolic syndrome, hypertension, and aging; as well as those of cardiac hypertrophy and heart failure would be of particular clinical relevance. Despite the lack of cardioprotective effects of SEA0400 demonstrated in the JCR:LA-*cp* rats under our experimental conditions, further investigations using other animal models of the metabolic syndrome, or using the same model at earlier stage of life [for example, at 6 months when both insulin resistance (352) and sensitivity to myocardial ischemia (361) have developed] in combination with a standardized anesthesia protocol may reduce potential variabilities due to pre-existing coronary artery disease and variable exposure to anesthesia, respectively. Even with these precautions, the possibility of underestimating any cardioprotective effects of the compound under investigation cannot be excluded (especially if volatile anesthesia is used in the standardized protocol). On the other hand, the above may also be viewed as a unique opportunity for testing a combined

cardioprotective strategy with clinically established anesthetic agents, which may be of potential relevance to cardiac surgical patients or non-cardiac surgical patients with pre-existing coronary artery disease. Notably, coronary revascularization (PCI or CABG) prior to major elective vascular surgery in patients with stable coronary disease in a recent randomized study (530) did not result in improved outcomes at 30 days (in terms of death, myocardial infarction, or length of hospitalization) or any long-term reduction in mortality (at 2.7 years after randomization). Yet with the post-operative myocardial infarction rate being 12% and 14% (within 30 days of surgery, as defined by an increase in troponin) in the revascularization and non-revascularization groups, respectively (530), there seems to be opportunities for further improving current pharmacotherapies the peri-operative settings in this subset of patients with stable CAD prior to surgery. While the combination therapy of an NCX inhibitor with anesthetic agents has not been studied (in the experimental or clinical settings), similar strategies with NHE inhibitors have been evaluated by Mathur *et al* (473,531) in isolated rat hearts subjected to global ischemia. Specifically, the combinations of cariporide with isoflurane (473), sevoflurane (473), or propofol (531) all afforded additive and superior cardioprotection.

Conceivably, the use of combination therapy with agents that act by different mechanism may enable the use of a lower dose of some or all of the agents to achieve similar cardioprotective effects, yet minimizing potential dose-related side effects. It is tempting to conjecture that there may be a role for combining NHE and NCX inhibitors, possibly at reduced doses, in myocardial protection. Nasagawa *et al* (239) have recently demonstrated in an *in vivo* canine model of IR-induced ventricular arrhythmias that SEA0400 had no effects on these arrhythmias, in contrast to the results of Takahashi *et*

*al* (97) which showed the reduction of ventricular fibrillation and mortality by SEA0400 in anesthetized rats. Apart from potential differences between experimental models, the investigators speculated that NCX inhibitors might not as effective as NHE inhibitors in treating IR-induced arrhythmias (239), based on their previous investigations on NHE inhibition (133,532-535), as well as mixed reports in the literature on the anti-arrhythmic potential of KB-R7943 – with some studies demonstrating benefit (194-196,199,201), but not in others (58,202). In addition, these investigators pointed out that as $Na^+$ overload is not prevented with inhibition of the reverse mode of NCX, deleterious effects of $Na^+$ overload, such as those on the mitochondria (69,536), may contribute to IRI (239). On the other hand, Matsumoto *et al* (198) suggested that inhibition of the reverse mode of NCX might be a more 'efficient' strategy in protecting the myocardium than NHE inhibition as the former would also antagonize the effects of $Na^+$ from other sources of influx in promoting NCX-mediated $Ca^{2+}$ influx during IR, such as the $Na^+$ channels and the NBC (20). Based on the above, it is conceivable that combination therapy consisting of a blocker of $Na^+$ influx (such as an NHE inhibitor) and an NCX inhibitor would offer the following possible advantages: (a) attenuation of $Na^+$ overload and its associated deleterious effects, such as on the mitochondria, through NHE inhibition; (b) $Na^+$ accumulation as a result of $Na^+$ influx from pathways other than NHE would facilitate the actions of the newer NCX inhibitors, such as SEA0400 (230,231) and SN-6 (191), in preferentially blocking the reverse mode of NCX; (c) the concomitant presence of an NCX inhibitor might enable the use of a lower dose of the NHE inhibitor and minimize any potential dose-related side effects; (d) since evidence to date supports the efficacy of either pre-ischemic or post-ischemic administration of

NCX inhibitors (97,98,233), whereas pre-ischemic administration of NHE inhibitors seems to confer more consistent benefits (154,537), combination therapy might extend the time window for potential therapeutic benefits; (e) the anti-arrhythmic effects of NHE might compensate for the relative lack (239) of anti-arrhythmic effects of NCX inhibitors against IR-induced arrhythmias (pending confirmation of the latter in future studies). At the point of writing, such combination therapy has not been evaluated in the setting of IRI to date. Mechanistic studies on the effects on $[Na^+]_i$, $[Ca^{2+}]_i$ and pH recovery in response to IR would be most valuable and, if proved beneficial, may guide future investigation with regard to the optimal dosing, timing, and route of administration, as well as potential adverse effects, of NHE and NCX inhibitors in the setting of myocardial IRI.

Based on the available evidence in the literature on NHE and NCX, it seems reasonable to speculate that NCX inhibitors may have therapeutic potential in the following clinical scenarios of ischemia/reperfusion: (a) cardiac surgery; (b) major non-cardiac surgery in the patient with significant coronary artery disease; (c) cardiac resuscitation, where protective effects in other organs, such as the brain and the kidney, become important attributes [although its anti-arrhythmic potential requires further evaluation, in light of the mixed findings (97,239) in the literature]; (d) acute coronary syndromes (as an adjunctive therapy to be administered at the earliest available opportunity prior to percutaneous or pharmacological reperfusion, such as in the patient who is awaiting transfer to a hospital with such capabilities), (e) for cardiac allograft preservation in cardiac transplantation, (f) as chronic prophylactic therapy in the patient with established coronary artery disease, especially if another indication is present, such

as salt-sensitive hypertension. It is conceivable that this strategy may avoid delays in drug administration in the acute coronary syndromes and may extend the therapeutic window for myocardial salvage. A recent study suggested that inhibition of the reverse mode of NCX may have potential application in the management of pulmonary arterial hypertension, as it was demonstrated that the reverse mode of NCX was involved in both vasoconstriction secondary to $Ca^{2+}$ entry into the cytosol of cultured smooth muscle cells of human pulmonary artery and the proliferation of these cells in the presence of serum and growth factors (538). The NCX inhibitor, KB-R7943, was shown to attenuate the increase in cytosolic $Ca^{2+}$ and the smooth muscle cell proliferation (538).

Novel strategies of drug design and delivery may also have potential applications in the field of NCX. A recent report by Palandoken *et al* (539) that described the design of an inactive 'prodrug' of amiloride appears most intriguing. It is anticipated that this 'prodrug' could be chronically administered on a prophylactic basis and, in the event of acute cerebral ischemia, be immediately activated by endopeptidases released during ischemia (539). Whether similar strategies could be applied to NCX inhibitors remain to be explored. Moreover, whether there is any role in incorporating an NCX inhibitor as an adjunctive drug in drug-eluting coronary stents for localized drug delivery has never been tested in the experimental setting to my knowledge. Such a strategy might offer potential additional advantages, at least on a theoretical basis, of attenuating IRI and of inhibiting the reverse mode of NCX of platelets (540-542), although the efficacy and safety, in combination with other established anti-thrombotic therapies in PCI (543), would require thorough evaluation. It is conceivable that such a strategy would also require administration of concomitant cardioprotective therapy

through another route (*e.g.*, intravenous or oral, such as at the time when aspirin would be taken, assuming no adverse drug interactions) to bridge the time between presentation to hospital and eventual placement of the stent. Moreover, whether NCX inhibition would have potential applications in newer reperfusion strategies, such as pharmacoinvasive therapy [combining pharmacological and catheter-based strategies (522,544)] and hybrid procedures [combining PCI and surgery (545,546)] in selected subgroups of patients, along with other clinical scenarios of myocardial ischemia/reperfusion (Figure 54) remain to be explored.

As a potential chronic therapy, NCX inhibition appears promising as a novel strategy in the management of salt-sensitive hypertension, given the recently identified role of the reverse mode of NCX in the pathophysiology, as depicted in Figure 55. Elevated cardiotonic steroids in the salt-sensitive hypertensive state inhibit $Na^+/K^+$-ATPase in smooth muscle cells and increase $[Na^+]_i$, which reduces $Ca^{2+}$ efflux through the forward mode of NCX and favours $Ca^{2+}$ influx through the reverse mode, resulting in elevation of $[Ca^{2+}]_i$, vasoconstriction, and systemic hypertension (238,251). If left untreated, hypertension is known to cause target organ damage, such as left ventricular hypertrophy, which increases the risk of myocardial ischemia, arrhythmias, and ventricular dysfunction (547,548). While most anti-hypertensive agents have been shown to produce regression of LVH (547), the prognostic importance of LV mass reduction, independent of any blood pressure lowering, has only been recently established (549,550). In light of this new evidence, further research directed toward demonstration of LV mass reduction in experimental models using NCX inhibitors would likely provide clinically relevant information for future human investigation.

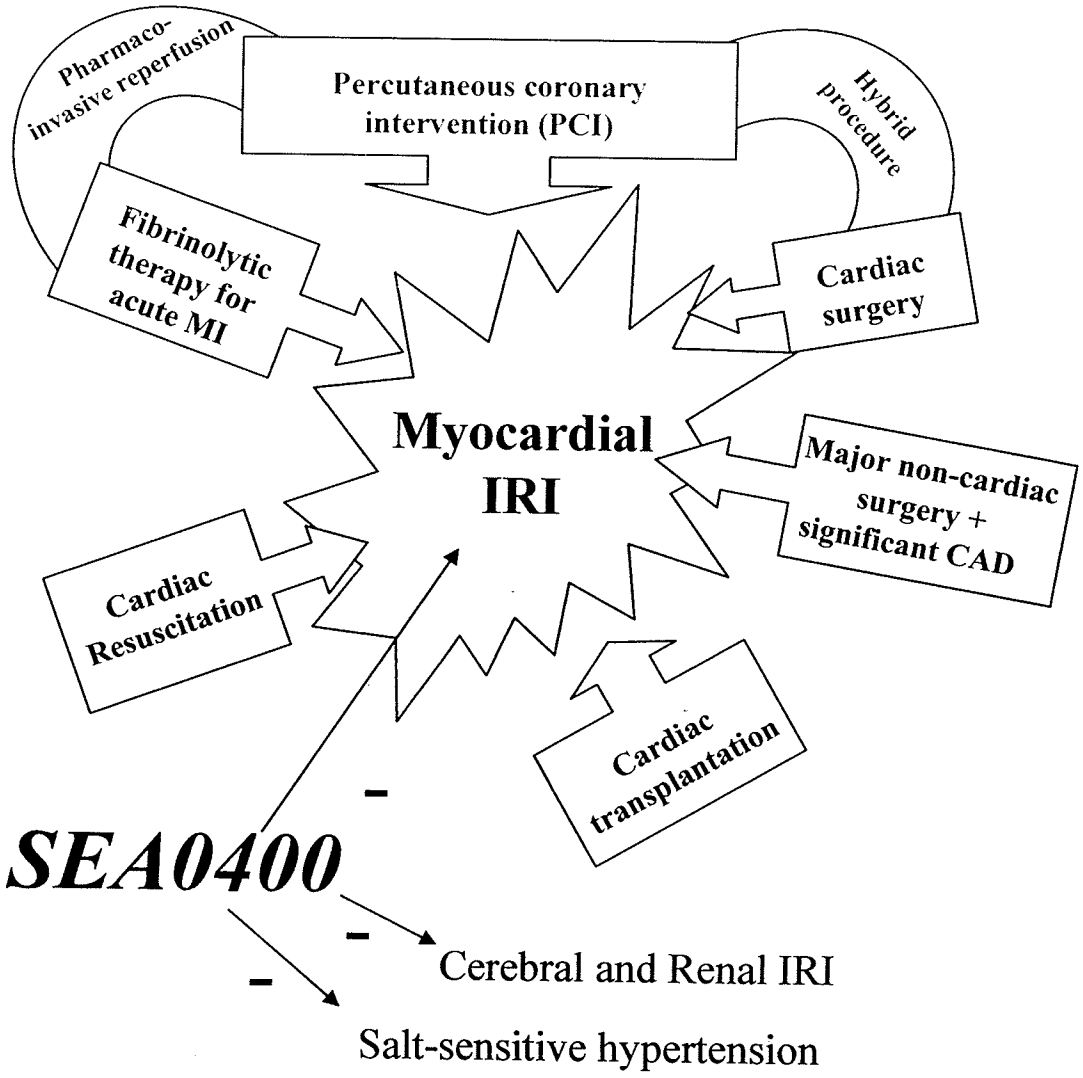



**Figure 54. Clinical Scenarios Where SEA0400 May Have Potential Therapeutic Applications.**

'-' denotes inhibition.

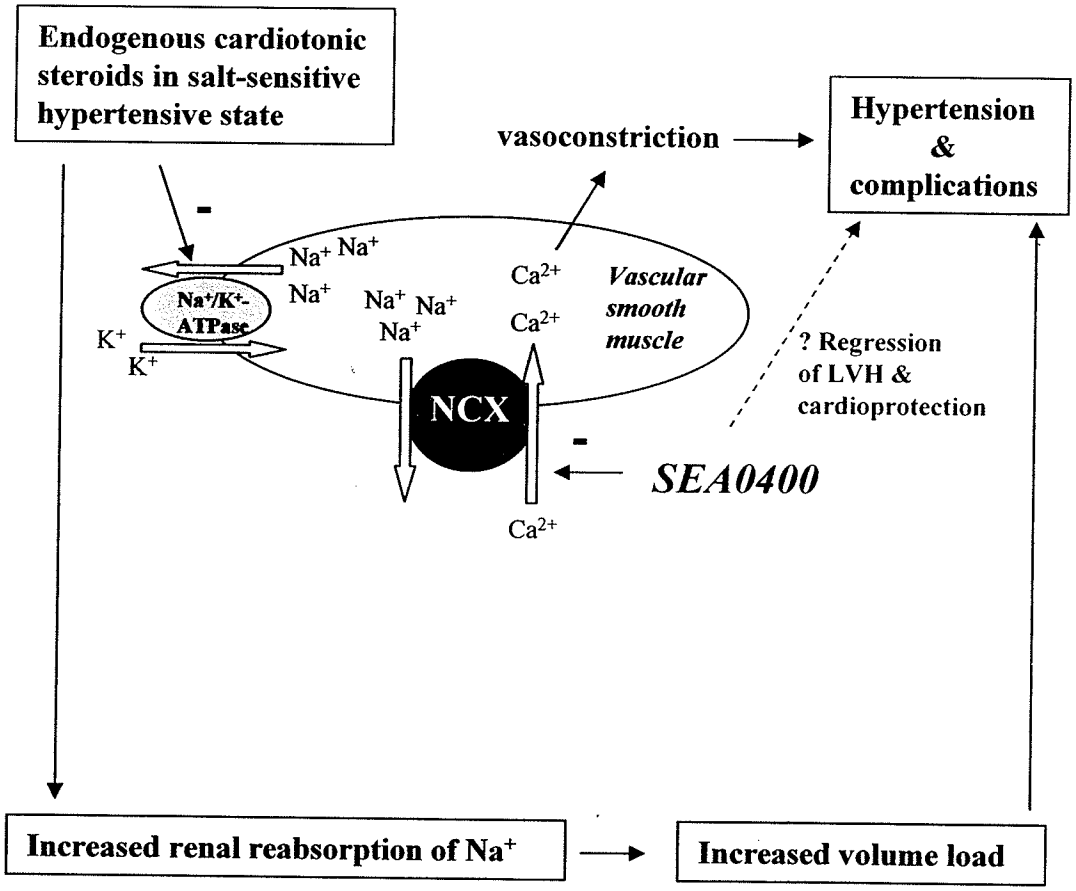



**Figure 55. Role of Vascular NCX in Salt-Sensitive Hypertension.**
(Based on ref. #238 & 251.) '-' denotes inhibition.

In the recent study by Iwamoto *et al*, SEA0400 was shown to lower arterial pressure in deoxycorticosterone acetate (DOCA)-salt hypertensive rats in a dose-dependently manner, after a single oral dose (238). Prolonged administration of SEA0400 over 3 weeks prevented the development of hypertension, vascular hypertrophy, and renal dysfunction in this model (238). Moreover, the therapeutic response to SEA0400 appears promising as a potential diagnostic tool for delineation of the mechanism of hypertension (238,251). Whether chronic NCX inhibition with SEA0400 would reduce LV mass in established salt-sensitive hypertension and protect the hypertrophied myocardium against ischemia/reperfusion injury on a prophylactic basis is unknown. The latter would also represent a potential strategy to circumvent delays in drug administration in the event of acute ischemia. The recent demonstration, by Romero *et al* (551), of the involvement of reverse-mode NCX in signal transduction leading to fibrogenesis may offer additional opportunities for therapeutic intervention. On the other hand, as the reverse mode of NCX has also been shown to play a role in endothelium-dependent relaxation (552), the therapeutic potential and the safety of chronic NCX inhibition remain to be investigated.

# 6. CONCLUSIONS

In conclusion, the senescent JCR:LA-*cp* obese rat is more susceptible to myocardial ischemia/reperfusion injury as compared with the lean control, consistent with previous findings at an earlier stage of life in this strain. The selective sodium-calcium exchange inhibitor, SEA0400, did not confer significant cardioprotection against IRI in the JCR:LA-*cp* obese rats under the experimental conditions of this study. Prolonged administration of SEA0400 was feasible and did not significantly affect left ventricular systolic and diastolic function as assessed by serial echocardiography. Future discovery and development of NCX inhibitors with increased potency and selectivity holds the promise of enhancing our ability to pharmacologically manipulate this complex yet attractive therapeutic target of NCX toward potential application in the clinical setting.

## 7. REFERENCES


(1) Tzivoni D. Reduction of infarct size and improved left ventricular function with IV caldaret [MCC-135] in patients with ST elevation myocardial infarction undergoing primary PCI. Available at http://www acc04online org/daily/news/print asp?stid=4&progid=1 2004. Accessed on November 26, 2004.

(2) Otto CM. Ventricular diastolic filling and function. Textbook of Clinical Echocardiography. Philadelphia: W.B. Saunders, 2004: 166-195.

(3) Bourne HR, von Zastrow M. Drug receptors and pharmacodynamics. In: Katzung BG, editor. Basic and Clinical Pharmacology. New York: Lange Medical Books/McGraw-Hill, 2004: 9-34.

(4) Nill K. Glossary of Biotechnology Terms. 3rd ed. Boca Raton, FL: CRC Press, 2002.

(5) Zeymer U, Suryapranata H, Monassier JP, Opolski G, Davies J, Rasmanis G, Linssen G, Tebbe U, Schroder R, Tiemann R, Machnig T, Neuhaus K-L, ESCAMI Investigators. The $Na^+/H^+$ exchange inhibitor eniporide as an adjunct to early reperfusion therapy for acute myocardial infarction. Results of the Evaluation of the Safety and Cardioprotective Effects of Eniporide in Acute Myocardial Infarction (ESCAMI) trial. J Am Coll Cardiol 2001;38:1644-1650.

(6) Mentzer RM, Jr., on behalf of the Expedition Study Investigators. Effect of $Na^+/H^+$ exchange inhibition by cariporide on death and nonfatal myocardial infarction in patients undergoing coronary artery bypass graft surgery: The Expedition Study. Circulation 2003;108:3-4. [abstract].

(7) Théroux P, Chaitman BR, Danchin N, Erhardt L, Meinertz T, Schroeder JS, Tognoni G, White HD, Willerson JT, Jessel A, for the GUARd During Ischemia Against Necrosis (GUARDIAN) Investigators. Inhibition of the sodium-hydrogen exchanger with cariporide to prevent myocardial infarction in high-risk ischemic situations. Main results of the GUARDIAN trial. Circulation 2000;102:3032-3038.

(8) Blaustein MP, Lederer WJ. Sodium/calcium exchange: its physiological implications. Physiol Rev 1999;79:763-854.

(9) Bers DM. Cardiac excitation-contraction coupling. Nature 2002;415:198-205.

(10) Quednau BD, Nicoll DA, Philipson KD. The sodium/calcium exchanger family-SLC8. Pflugers Arch 2004;447:543-548.

(11) Hryshko LV. Membrane pumps and exchangers. In: Zipes DP, Jalife J, editors. Cardiac Electrophysiology: From Cell to Bedside. Philadelphia, PA: W.B. Saunders, 2004: 42-50.

(12) Hryshko LV. The cardiac $Na^+$-$Ca^{2+}$ exchanger. In: Page E, Fozzard HA, Solaro RJ, editors. Handbook of Physiology. Section 2: The Cardiovascular System. V. 1: The Heart. Oxford: Oxford University Press, 2002: 388-419.

(13) Iwamoto T. Forefront of $Na^+/Ca^{2+}$ exchanger studies: molecular pharmacology of $Na^+/Ca^{2+}$ exchange inhibitors. J Pharmacol Sci 2004;96:27-32.

(14) Reuter H, Seitz N. The dependence of calcium efflux from cardiac muscle on temperature and external ion composition. J Physiol 1968;195:451-470.

(15) Nicoll DA, Longoni S, Philipson KD. Molecular cloning and functional expression of the cardiac sarcolemmal $Na^+$-$Ca^{2+}$ exchanger. Science 1990;250:562-565.

(16) Iwamoto T, Nakamura TY, Pan Y, Uehara A, Imanaga I, Shigekawa M. Unique topology of the internal repeats in the cardiac $Na^+/Ca^{2+}$ exchanger. FEBS Lett 1999;446:264-268.

(17) Philipson KD, Nicoll DA. Sodium-calcium exchange: a molecular perspective. Annu Rev Physiol 2000;62:111-133.

(18) Nicoll DA, Ottolia M, Philipson KD. Toward a topological model of the NCX1 exchanger. Ann N Y Acad Sci 2002;976:11-18.

(19) Nicoll DA, Ottolia M, Lu L, Lu Y, Philipson KD. A new topological model of the cardiac sarcolemmal $Na^+$-$Ca^{2+}$ exchanger. J Biol Chem 1999;274:910-917.

(20) Bers DM, Barry WH, Despa S. Intracellular $Na^+$ regulation in cardiac myocytes. Cardiovasc Res 2003;57:897-912.

(21) Nicoll DA, Hryshko LV, Matsuoka S, Frank JS, Philipson KD. Mutation of amino acid residues in the putative transmembrane segments of the cardiac sarcolemmal $Na^+$-$Ca^{2+}$ exchanger. J Biol Chem 1996;271:13385-13391.

(22) Ottolia M, Nicoll DA, Philipson KD. Mutational analysis of the alpha -1 repeat of the cardiac $Na^+$-$Ca^{2+}$ exchanger. J Biol Chem 2005;280:1061-1069.

(23) Li Z, Nicoll DA, Collins A, Hilgemann DW, Filoteo AG, Penniston JT, Weiss JN, Tomich JM, Philipson KD. Identification of a peptide inhibitor of the cardiac sarcolemmal $Na^+$- $Ca^{2+}$ exchanger. J Biol Chem 1991;266:1014-1020.

(24) Matsuoka S, Nicoll DA, He Z, Philipson KD. Regulation of cardiac $Na^+$-$Ca^{2+}$ exchanger by the endogenous XIP region. J Gen Physiol 1997;109:273-286.

(25) Levitsky DO, Nicoll DA, Philipson KD. Identification of the high affinity $Ca^{2+}$-binding domain of the cardiac $Na^{+}$-$Ca^{2+}$ exchanger. J Biol Chem 1994;269:22847-22852.

(26) Matsuoka S, Nicoll DA, Hryshko LV, Levitsky DO, Weiss JN, Philipson KD. Regulation of the cardiac $Na^{+}$-$Ca^{2+}$ exchanger by $Ca^{2+}$. Mutational analysis of the $Ca^{2+}$-binding domain. J Gen Physiol 1995;105:403-420.

(27) Bridge JH, Smolley JR, Spitzer KW. The relationship between charge movements associated with $I_{Ca}$ and $I_{Na-Ca}$ in cardiac myocytes. Science 1990;248:376-378.

(28) Hinata M, Yamamura H, Li L, Watanabe Y, Watano T, Imaizumi Y, Kimura J. Stoichiometry of $Na^{+}$-$Ca^{2+}$ exchange is 3:1 in guinea-pig ventricular myocytes. J Physiol 2002;545.2:453-461.

(29) Henderson SA, Goldhaber JI, So JM, Han T, Motter C, Ngo A, Chantawansri C, Ritter MR, Friedlander M, Nicoll DA, Frank JS, Jordan MC, Roos KP, Ross RS, Philipson KD. Functional adult myocardium in the absence of $Na^{+}$-$Ca^{2+}$ exchange: cardiac-specific knockout of NCX1. Circ Res 2004;95:604-611.

(30) Eisner DA, Sipido KR. Sodium calcium exchange in the heart: necessity or luxury? Circ Res 2004;95:549-551.

(31) Hurtado C. Role of sodium-calcium exchange in cardiac physiology and pathophysiology [dissertation]. Winnipeg, MB: University of Manitoba, 2005.

(32) Hurtado C, Maddaford TG, Pierce GN. Strong inhibition of $Na^{+}$-$Ca^{2+}$ exchanger expression by RNAi does not prevent cardiac contraction. J Mol Cell Cardiol 2004;230-230 [abstract].

(33) Bers D. Na/Ca exchange and the sarcolemmal Ca-pump. Excitation-Contraction Coupling and Cardiac Contractile Force. Boston: Kluwer Academic Publishers, 2001: 133-160.

(34) Lazdunski M, Frelin C, Vigne P. The sodium/hydrogen exchange system in cardiac cells: its biochemical and pharmacological properties and its role in regulating internal concentrations of sodium and internal pH. J Mol Cell Cardiol 1985;17:1029-1042.

(35) Murphy E, Cross H, Steenbergen C. Sodium regulation during ischemia versus reperfusion and its role in injury. Circ Res 1999;84:1469-1470.

(36) Kimura J. Cardiac $Na^{+}$-$Ca^{2+}$ exchanger: pathophysiology and pharmacology. In: Sperelakis N, Kurachi Y, Terzic A, Cohen MV, editors. Heart Physiology and Pathophysiology. San Diego, CA: Academic Press, 2001: 417-425.

(37) Pogwizd SM, Sipido KR, Verdonck F, Bers DM. Intracellular $Na^+$ in animal models of hypertrophy and heart failure: contractile function and arrhythmogenesis. Cardiovasc Res 2003;57:887-896.

(38) Satoh H, Mukai M, Urushida T, Katoh H, Terada H, Hayashi H. Importance of $Ca^{2+}$ influx by $Na^+/Ca^{2+}$ exchange under normal and sodium-loaded conditions in mammalian ventricles. Mol Cell Biochem 2003;242:11-17.

(39) Hilgemann DW. Regulation and deregulation of cardiac $Na^+$-$Ca^{2+}$ exchange in giant excised sarcolemmal membrane patches. Nature 1990;344:242-245.

(40) Hilgemann DW, Matsuoka S, Nagel GA, Collins A. Steady-state and dynamic properties of cardiac sodium-calcium exchange. Sodium-dependent inactivation. J Gen Physiol 1992;100:905-932.

(41) Shattock MJ, Bers DM. Rat vs. rabbit ventricle: Ca flux and intracellular Na assessed by ion-selective microelectrodes. Am J Physiol Cell Physiol 1989;256:C813-C822.

(42) Donoso P, Mill JG, O'Neill SC, Eisner DA. Fluorescence measurements of cytoplasmic and mitochondrial sodium concentration in rat ventricular myocytes. J Physiol 1992;448:493-509.

(43) Despa S, Islam MA, Pogwizd SM, Bers DM. Intracellular $[Na^+]$ and $Na^+$ pump rate in rat and rabbit ventricular myocytes. J Physiol 2002;539:133-143.

(44) Yao A, Su Z, Nonaka A, Zubair I, Lu L, Philipson KD, Bridge JHB, Barry WH. Effects of overexpression of the $Na^+$-$Ca^{2+}$ exchanger on $[Ca^{2+}]_i$ transients in murine ventricular myocytes. Circ Res 1998;82:657-665.

(45) Verdonck F, Volders PGA, Vos MA, Sipido KR. Intracellular $Na^+$ and altered $Na^+$ transport mechanisms in cardiac hypertrophy and failure. J Mol Cell Cardiol 2003;35:5-25.

(46) Bers DM. Cardiac Na/Ca exchange function in rabbit, mouse and man: what's the difference? J Mol Cell Cardiol 2002;34:369-373.

(47) Opie LH. Channels, pumps, and exchangers. In: Opie LH, editor. Heart Physiology: From Cell to Circulation. Philadelphia, PA: Lippincott Williams & Wilkins, 2004: 73-118.

(48) Orlowski J, Grinstein S. Diversity of the mammalian sodium/proton exchanger SLC9 gene family. Pflugers Arch 2004;447:549-565.

(49) Avkiran M. Basic biology and pharmacology of the cardiac sarcolemmal sodium/hydrogen exchanger. J Card Surg 2003;18 Suppl 1:3-12.

(50) Fliegel L. The $Na^+/H^+$ exchanger isoform 1. Int J Biochem Cell Biol 2005;37:33-37.

(51) Karmazyn M. Mechanisms underlying NHE-1 involvement in myocardial ischemic and reperfusion injury. In: Karmazyn M, Avkiran M, Fliegel L, editors. The Sodium-Hydrogen Exchanger. From Molecule to its Role in Disease. Boston: Kluwer Academic Publishers, 2003: 221-237.

(52) Karmazyn M. Amiloride enhances postischemic ventricular recovery: possible role of $Na^+$-$H^+$ exchange. Am J Physiol Heart Circ Physiol 1988;255:H608-H615.

(53) Tani M, Neely JR. Role of intracellular $Na^+$ in $Ca^{2+}$ overload and depressed recovery of ventricular function of reperfused ischemic rat hearts. Possible involvement of $H^+$-$Na^+$ and $Na^+$-$Ca^{2+}$ exchange. Circ Res 1989;65:1045-1056.

(54) Garcia-Dorado D. Myocardial cell protection in acute coronary syndromes. In: Théroux P, editor. Acute Coronary Syndromes. A Companion to Braunwald's Heart Disease. Philadelphia, PA: Saunders, 2003: 453-472.

(55) Dhalla NS, Temsah RM, Netticadan T, Sandhu MS. Calcium overload in ischemia/reperfusion injury. In: Sperelakis N, Kurachi Y, Terzic A, Cohen MV, editors. Heart Physiology and Pathophysiology. San Diego: Academic Press, 2001: 949-965.

(56) Eng S, Maddaford TG, Kardami E, Pierce GN. Protection against myocardial ischemic/reperfusion injury by inhibitors of two separate pathways of $Na^+$ entry. J Mol Cell Cardiol 1998;30:829-835.

(57) Eigel BN, Hadley RW. Contribution of the $Na^+$ channel and $Na^+/H^+$ exchanger to the anoxic rise of $[Na^+]$ in ventricular myocytes. Am J Physiol Heart Circ Physiol 1999;277:H1817-H1822.

(58) Lu HR, Yang P, Remeysen P, Saels A, Dai DZ, De Clerck F. Ischemia/reperfusion-induced arrhythmias in anaesthetized rats: a role of $Na^+$ and $Ca^{2+}$ influx. Eur J Pharmacol 1999;365:233-239.

(59) Schafer C, Ladilov YV, Siegmund B, Piper HM. Importance of bicarbonate transport for protection of cardiomyocytes against reoxygenation injury. Am J Physiol Heart Circ Physiol 2000;278:H1457-H1463.

(60) Ten Hove M, Nederhoff MGJ, Van Echteld CJA. Relative contributions of $Na^+/H^+$ exchange and $Na^+/HCO^{3-}$ co-transport to ischemic $Na^+_i$ overload in isolated rat hearts. Am J Physiol Heart Circ Physiol 2005;288:H287-H292.

(61) Gan XT, Chakrabarti S, Karmazyn M. Modulation of $Na^+/H^+$ exchange isoform 1 mRNA expression in isolated rat hearts. Am J Physiol 1999;277:H993-H998.

(62) Piper HM, Garcia-Dorado D, Ovize M. A fresh look at reperfusion injury. Cardiovasc Res 1998;38:291-300.

(63) Garcia-Dorado D, Oliveras J. Myocardial oedema: a preventable cause of reperfusion injury? Cardiovasc Res 1993;27:1555-1563.

(64) Zhang M, Sallam T, Xu Y-J, Dhalla NS. Modification of ischemia-reperfusion-induced injury by cardioprotective interventions. In: Salerno TA, Ricci M, editors. Myocardial Protection. Elmsford, NY: Futura/Blackwell Publishing, 2004: 18-32.

(65) Mitani A, Shattock MJ. Role of Na-activated K channel, Na-K-Cl cotransport, and Na-K pump in [K]e changes during ischemia in rat heart. Am J Physiol Heart Circ Physiol 1992;263:H333-H340.

(66) Bright CM, Ellis D. Intracellular pH changes induced by hypoxia and anoxia in isolated sheep heart Purkinje fibres. Exp Physiol 1992;77:165-175.

(67) Shivkumar K, Deutsch NA, Lamp ST, Khuu K, Goldhaber JI, Weiss JN. Mechanism of hypoxic K loss in rabbit ventricle. J Clin Invest 1997;100:1782-1788.

(68) Hearse DJ. Myocardial protection during ischemia and reperfusion. Mol Cell Biochem 1998;186:177-184.

(69) Takeo S, Iwai T, Tanonaka K. Sodium overload and mitochondrial damage in ischemic/reperfused heart. In: Dhalla NS, Takeda N, Singh M, Lukas A, editors. Myocardial Ischemia and Preconditioning. Boston, MA: Kluwer Academic Publishers, 2003: 35-60.

(70) Golden AL, Bright JM, Pohost GM, Pike MM. Ischemic dysfunction and impaired recovery in hypertensive hypertrophied hearts is associated with exaggerated intracellular sodium accumulation. Am J Hypertens 1994;7:745-754.

(71) Tani M, Suganuma Y, Hasegawa H, Shinmura K, Ebihara Y, Hayashi Y, Guo X, Takayama M. Decrease in ischemic tolerance with aging in isolated perfused Fischer 344 rat hearts: relation to increases in intracellular $Na^+$ after ischemia. J Mol Cell Cardiol 1997;29:3081-3089.

(72) Imahashi K, London RE, Steenbergen C, Murphy E. Male/female differences in intracellular $Na^+$ regulation during ischemia/reperfusion in mouse heart. J Mol Cell Cardiol 2004;37:747-753.

(73) Murphy E, Perlman M, London RE, Steenbergen C. Amiloride delays the ischemia-induced rise in cytosolic free calcium. Circ Res 1991;68:1250-1258.

(74) Ladilov Y, Haffner S, Balser-Schäfer C, Maxeiner H, Piper HM. Cardioprotective effects of KB-R7943: a novel inhibitor of the reverse mode of $Na^+/Ca^{2+}$ exchanger. Am J Physiol Heart Circ Physiol 1999;276:H1868-H1876.

(75) Eigel BN, Hadley RW. Antisense inhibition of $Na^+/Ca^{2+}$ exchange during anoxia/reoxygenation in ventricular myocytes. Am J Physiol Heart Circ Physiol 2001;281:H2184-H2190.

(76) Seki S, Taniguchi M, Takeda H, Nagai M, Taniguchi I, Mochizuki S. Inhibition by KB-R7943 of the reverse mode of the $Na^+/Ca^{2+}$ exchanger reduces $Ca^{2+}$ overload in ischemic-reperfused rat hearts. Circ J 2002;66:390-396.

(77) Inserte J, Garcia-Dorado D, Ruiz-Meana M, Padilla F, Barrabés JA, Pina P, Agulló L, Piper HM, Soler-Soler J. Effect of inhibition of $Na^+/Ca^{2+}$ exchanger at the time of myocardial reperfusion on hypercontracture and cell death. Cardiovasc Res 2002;55:739-748.

(78) Pogwizd SM. Clinical potential of sodium-calcium exchanger inhibitors as antiarrhythmic agents. Drugs 2003;63:439-452.

(79) Piper HM, Abdallah Y, Schafer C. The first minutes of reperfusion: a window of opportunity for cardioprotection. Cardiovasc Res 2004;61:365-371.

(80) Dhalla NS, Golfman L, Takeda S, Takeda N, Nagano M. Evidence for the role of oxidative stress in acute ischemic heart disease: A brief review. Can J Cardiol 1999;15:587-593.

(81) Singh R, Dhalla NS. Mechanisms of cardioprotection against ischemia-reperfusion injury. In: Dhalla NS, Rupp H, Angel A, Pierce GN, editors. Pathophysiology of Cardiovascular Disease. Boston: Kluwer Academic Publishers, 2004: 303-326.

(82) Eigel BN, Gursahani H, Hadley RW. ROS are required for rapid reactivation of $Na^+/Ca^{2+}$ exchanger in hypoxic reoxygenated guinea pig ventricular myocytes. Am J Physiol 2004;286:H955-H963.

(83) Eigel BN, Gursahani H, Hadley RW. The sodium-calcium exchanger plays a key role in inducing apoptosis following prolonged hypoxia in cultured guinea pig ventricular myocytes. Am J Physiol Heart Circ Physiol 2004;287:H1466-H1475.

(84) Menown IB, Adgey AA. Cardioprotective therapy and sodium-hydrogen exchange inhibition: current concepts and future goals. J Am Coll Cardiol 2001;38:1651-1653.

(85) Logue SE, Gustafsson ÅB, Samali A, Gottlieb RA. Ischemia/reperfusion injury at the intersection with cell death. J Mol Cell Cardiol 2005;38:21-33.

(86) Garcia-Dorado D. Myocardial reperfusion injury: a new view. Cardiovasc Res 2004;61:363-364.

(87) Ruiz-Meana M, Garcia-Dorado D, Hofstaetter B, Piper HM, Soler-Soler J. Propagation of cardiomyocyte hypercontracture by passage of $Na^+$ through gap junctions. Circ Res 1999;85:280-287.

(88) Garcia-Dorado D, Rodriguez-Sinovas A, Ruiz-Meana M. Gap junction-mediated spread of cell injury and death during myocardial ischemia-reperfusion. Cardiovasc Res 2004;61:386-401.

(89) Ambrosio G, Tritto I. Reperfusion injury: experimental evidence and clinical implications. Am Heart J 1999;138:S69-S75.

(90) Collard CD, Gelman S. Pathophysiology, clinical manifestations, and prevention of ischemia-reperfusion injury. Anesthesiology 2001;94:1133-1138.

(91) Verma S, Fedak PWM, Weisel RD, Butany J, Rao V, Maitland A, Li RK, Dhillon B, Yau TM. Fundamentals of reperfusion injury for the clinical cardiologist. Circulation 2002;105:2332-2336.

(92) Haigney MC, Miyata H, Lakatta EG, Stern MD, Silverman HS. Dependence of hypoxic cellular calcium loading on $Na^+$-$Ca^{2+}$ exchange. Circ Res 1992;71:547-557.

(93) Ziegelstein RC, Zweier JL, Mellits ED, Younes A, Lakatta EG, Stern MD, Silverman HS. Dimethylthiourea, an oxygen radical scavenger, protects isolated cardiac myocytes from hypoxic injury by inhibition of $Na^+$-$Ca^{2+}$ exchange and not by its antioxidant effects. Circ Res 1992;70:804-811.

(94) Cross HR, Lu L, Steenbergen C, Philipson KD, Murphy E. Overexpression of the cardiac $Na^+/Ca^{2+}$ exchanger increases susceptibility to ischemia/reperfusion injury in male, but not female, transgenic mice. Circ Res 1998;83:1215-1223.

(95) Ohtsuka M, Takano H, Suzuki M, Zou Y, Akazawa H, Tamagawa M, Wakimoto K, Nakaya H, Komuro I. Role of $Na^+$-$Ca^{2+}$ exchanger in myocardial ischemia/reperfusion injury: evaluation using a heterozygous $Na^+$-$Ca^{2+}$ exchanger knockout mouse model. Biochem Biophys Res Commun 2004;314:849-853.

(96) Schäfer C, Ladilov Y, Inserte J, Schäfer M, Haffner S, Garcia-Dorado D, Piper HM. Role of the reverse mode of the $Na^+/Ca^{2+}$ exchanger in reoxygenation-induced cardiomyocyte injury. Cardiovasc Res 2001;51:241-250.

(97) Takahashi K, Takahashi T, Suzuki T, Onishi M, Tanaka Y, Hamano-Takahashi A, Ota T, Kameo K, Matsuda T, Baba A. Protective effects of SEA0400, a novel and selective inhibitor of the $Na^+/Ca^{2+}$ exchanger, on myocardial ischemia-reperfusion injuries. Eur J Pharmacol 2003;458:155-162.

(98) Magee WP, Deshmukh G, DeNinno MP, Sutt JC, Chapman JG, Tracey WR. Differing cardioprotective efficacy of the $Na^+/Ca^{2+}$ exchanger inhibitors SEA0400 and KB-R7943. Am J Physiol Heart Circ Physiol 2003;284:H903-H910.

(99) Satoh H, Katoh H, Terada H, Hayashi H. Involvement of $Na^+/Ca^{2+}$ exchange in normal cardiac excitation-contraction coupling and in $Ca^{2+}$ overload during ischemia and reperfusion. In: Dhalla NS, Takeda N, Singh M, Lukas A, editors. Myocardial ischemia and preconditioning. Boston: Kluwer Academic Publishers, 2003: 481-503.

(100) Avkiran M. Rational basis for use of sodium-hydrogen exchange inhibitors in myocardial ischemia. Am J Cardiol 1999;83:10G-17G.

(101) Karmazyn M, Sostaric JV, Gan XT. The myocardial $Na^+/H^+$ exchanger: a potential therapeutic target for the prevention of myocardial ischaemic and reperfusion injury and attenuation of postinfarction heart failure. Drugs 2001;61:375-389.

(102) Allen DG, Xiao XH. Role of the cardiac $Na^+/H^+$ exchanger during ischemia and reperfusion. Cardiovasc Res 2003;57:934-941.

(103) Linz WJ, Busch AE. NHE-1 inhibition: from protection during acute ischaemia/reperfusion to prevention/reversal of myocardial remodelling. Naunyn Schmiedebergs Arch Pharmacol 2003;368:239-246.

(104) Karmazyn M. The sodium-hydrogen exchange system in the heart: a target for the protection of the ischemic and reperfused heart and inhibition of heart failure. In: Pugsley MK, editor. Cardiac Drug Development Guide. Totowa,NJ: Humana Press, 2003: 123-139.

(105) Meng H, Pierce GN. Involvement of sodium in the protective effect of 5-(N,N-dimethyl)-amiloride on ischemia-reperfusion injury in isolated rat ventricular wall. J Pharmacol Exp Ther 1991;256:1094-1100.

(106) Duff HJ, Brown CE, Cragoe EJ, Rahmberg M. Antiarrhythmic activity of amiloride: mechanisms. J Cardiovasc Pharmacol 1991;17:879-888.

(107) Scholz W, Albus U, Linz W, Martorana P, Lang HJ, Scholkens BA. Effects of $Na^+/H^+$ exchange inhibitors in cardiac ischemia. J Mol Cell Cardiol 1992;24:731-739.

(108) Meng HP, Maddaford TG, Pierce GN. Effect of amiloride and selected analogues on postischemic recovery of cardiac contractile function. Am J Physiol 1993;264:H1831-H1835.

(109) Anderson SE, Murphy E, Steenbergen C, London RE, Cala PM. Na-H exchange in myocardium: effects of hypoxia and acidification on Na and Ca. Am J Physiol Cell Physiol 1990;259:C940-C948.

(110) An J, Varadarajan SG, Camara A, Chen Q, Novalija E, Gross GJ, Stowe DF. Blocking $Na^+/H^+$ exchange reduces $[Na^+]_i$ and $[Ca^{2+}]_i$ load after ischemia and improves function in intact hearts. Am J Physiol Heart Circ Physiol 2001;281:H2398-H2409.

(111) Strömer H, de Groot MC, Horn M, Faul C, Leupold A, Morgan JP, Scholz W, Neubauer S. $Na^+/H^+$ exchange inhibition with HOE642 improves postischemic recovery due to attenuation of $Ca^{2+}$ overload and prolonged acidosis on reperfusion. Circulation 2000;101:2749-2755.

(112) Varadarajan SG, An J, Novalija E, Smart SC, Stowe DF. Changes in $[Na^+]_i$, compartmental $[Ca^{2+}]$, and NADH with dysfunction after global ischemia in intact hearts. Am J Physiol 2001;280:H280-H293.

(113) Masereel B, Pochet L, Laeckmann D. An overview of inhibitors of $Na^+/H^+$ exchanger. Eur J Med Chem 2003;38:547-554.

(114) Garcia-Dorado D, Gonzalez MA, Barrabes JA, Ruiz-Meana M, Solares J, Lidon RM, Blanco J, Puigfel Y, Piper HM, Soler-Soler J. Prevention of ischemic rigor contracture during coronary occlusion by inhibition of $Na^+$-$H^+$ exchange. Cardiovasc Res 1997;35:80-89.

(115) Ruiz-Meana M, Garcia-Dorado D, Julia M, Inserte J, Siegmund B, Ladilov Y, Piper M, Tritto FP, Gonzalez MA, Soler-Soler J. Protective effect of HOE642, a selective blocker of $Na^+$-$H^+$ exchange, against the development of rigor contracture in rat ventricular myocytes. Exp Physiol 2000;85:17-25.

(116) Pike MM, Luo CS, Clark MD, Kirk KA, Kitakaze M, Madden MC, Cragoe EJ, Jr., Pohost GM. NMR measurements of $Na^+$ and cellular energy in ischemic rat heart: role of $Na^+$-$H^+$ exchange. Am J Physiol Heart Circ Physiol 1993;265:H2017-H2026.

(117) Myers ML, Mathur S, Li GH, Karmazyn M. Sodium-hydrogen exchange inhibitors improve postischemic recovery of function in the perfused rabbit heart. Cardiovasc Res 1995;29:209-214.

(118) Tani M, Shinmura K, Hasegawa H, Nakamura Y. Effect of methylisobutyl amiloride on $[Na^+]_i$, reperfusion arrhythmias, and function in ischemic rat hearts. J Cardiovasc Pharmacol 1996;27:794-801.

(119) Docherty JC, Yang L, Pierce GN, Deslauriers R. $Na^+$-$H^+$ exchange inhibition at reperfusion is cardioprotective during myocardial ischemia-reperfusion; $^{31}P$ NMR studies. Mol Cell Biochem 1997;176:257-264.

(120) Choy IO, Schepkin VD, Budinger TF, Obayashi DY, Young JN, DeCampli WM. Effects of specific sodium/hydrogen exchange inhibitor during cardioplegic arrest. Ann Thorac Surg 1997;64:94-99.

(121) Iwai T, Tanonaka K, Inoue R, Kasahara S, Kamo N, Takeo S. Mitochondrial damage during ischemia determines post-ischemic contractile dysfunction in perfused rat heart. J Mol Cell Cardiol 2002;34:725-738.

(122) Scholz W, Albus U, Counillon L, Gogelein H, Hans J, Linz W, Weichert A, Scholkens BA. Protective effects of HOE642, a selective sodium-hydrogen exchange subtype 1 inhibitor, on cardiac ischaemia and reperfusion. Cardiovasc Res 1995;29:260-268.

(123) Gumina RJ, Auchampach J, Wang R, Buerger E, Eickmeier C, Moore J, Daemmgen J, Gross GJ. $Na^+/H^+$ exchange inhibition-induced cardioprotection in dogs: effects on neutrophils versus cardiomyocytes. Am J Physiol Heart Circ Physiol 2000;279:H1563-H1570.

(124) Rodriguez-Sinovas A, Garcia-Dorado D, Padilla F, Inserte J, Barrabes JA, Ruiz-Meana M, Agullo L, Soler-Soler J. Pre-treatment with the $Na^+/H^+$ exchange inhibitor cariporide delays cell-to-cell electrical uncoupling during myocardial ischemia. Cardiovasc Res 2003;58:109-117.

(125) Castella M, Buckberg GD, Saleh S, Tan Z, Ignarro LJ. A new role for cardioplegic buffering: should acidosis or calcium accumulation be counteracted to salvage jeopardized hearts? J Thorac Cardiovasc Surg 2003;126:1442-1448.

(126) Klein HH, Pich S, Bohle RM, Lindert-Heimberg S, Nebendahl K. $Na^+/H^+$ exchange inhibitor cariporide attenuates cell injury predominantly during ischemia and not at onset of reperfusion in porcine hearts with low residual blood flow. Circulation 2000;102:1977-1982.

(127) Knight DR, Smith AH, Flynn DM, MacAndrew JT, Ellery SS, Kong JX, Marala RB, Wester RT, Guzman-Perez A, Hill RJ, Magee WP, Tracey WR. A novel sodium-hydrogen exchanger isoform-1 inhibitor, zoniporide, reduces ischemic myocardial injury in vitro and in vivo. J Pharmacol Exp Ther 2001;297:254-259.

(128) Gumina RJ, Moore J, Schelling P, Beier N, Gross GJ. $Na^+/H^+$ exchange inhibition prevents endothelial dysfunction after I/R injury. Am J Physiol Heart Circ Physiol 2001;281:H1260-H1266.

(129) Reffelmann T, Kloner RA. Is microvascular protection by cariporide and ischemic preconditioning causally linked to myocardial salvage? Am J Physiol Heart Circ Physiol 2003;284:H1134-H1141.

(130) Klass O, Fischer UM, Perez E, Easo J, Bosse M, Fischer JH, Tossios P, Mehlhorn U. Effect of the $Na^+/H^+$ exchange inhibitor eniporide on cardiac performance and myocardial high energy phosphates in pigs subjected to cardioplegic arrest. Ann Thorac Surg 2004;77:658-663.

(131) Jung O, Albus U, Lang HJ, Busch AE, Linz W. Effects of acute and chronic treatment with the sodium hydrogen exchanger 1 (NHE-1) inhibitor cariporide on myocardial infarct mass in rabbits with hypercholesterolaemia. Basic Clin Pharmacol Toxicol 2004;95:24-30.

(132) Yasutake M, Ibuki C, Hearse DJ, Avkiran M. $Na^+/H^+$ exchange and reperfusion arrhythmias: protection by intracoronary infusion of a novel inhibitor. Am J Physiol Heart Circ Physiol 1994;267:H2430-H2440.

(133) Sugiyama A, Aye NN, Sawada N, Hashimoto K. Cariporide, a highly selective $Na^+/H^+$ exchange inhibitor, suppresses the reperfusion-induced lethal arrhythmias and "overshoot" phenomenon of creatine phosphate in situ rat heart. J Cardiovasc Pharmacol 1999;33:116-121.

(134) Hashimoto K, Nagasawa E, Zhu B-M. Na/H exchange and arrhythmia. In: Dhalla N, Takeda N, Singh M, Lukas A, editors. Myocardial Ischemia and Preconditioning. Boston: Kluwer Academic Publishers, 2003: 389-398.

(135) Muraki S, Morris CD, Budde JM, Zhao ZQ, Guyton RA, Vinten-Johansen J. Blood cardioplegia supplementation with the sodium-hydrogen ion exchange inhibitor cariporide to attenuate infarct size and coronary artery endothelial dysfunction after severe regional ischemia in a canine model. J Thorac Cardiovasc Surg 2003;125:155-164.

(136) Chakrabarti S, Hoque AN, Karmazyn M. A rapid ischemia-induced apoptosis in isolated rat hearts and its attenuation by the sodium-hydrogen exchange inhibitor HOE 642 (cariporide). J Mol Cell Cardiol 1997;29:3169-3174.

(137) Portman MA, Panos AL, Xiao Y, Anderson DL, Ning XH. HOE-642 (cariporide) alters $pH_i$ and diastolic function after ischemia during reperfusion in pig hearts in situ. Am J Physiol Heart Circ Physiol 2001;280:H830-H834.

(138) Ruiz-Meana M, Garcia-Dorado D, Pina P, Inserte J, Agullo L, Soler-Soler J. Cariporide preserves mitochondrial proton gradient and delays ATP depletion in cardiomyocytes during ischemic conditions. Am J Physiol 2003;285:H999-H1006.

(139) Tracey WR, Allen MC, Frazier DE, Fossa AA, Johnson CG, Marala RB, Knight DR, Guzman-Perez A. Zoniporide: a potent and selective inhibitor of the human sodium-hydrogen exchanger isoform 1 (NHE-1). Cardiovasc Drug Rev 2003;21:17-32.

(140) Gawaz M. Role of platelets in coronary thrombosis and reperfusion of ischemic myocardium. Cardiovasc Res 2004;61:498-511.

(141) Vinten-Johansen J. Involvement of neutrophils in the pathogenesis of lethal myocardial reperfusion injury. Cardiovasc Res 2004;61:481-497.

(142) Besse S, Tanguy S, Boucher F, Le PC, Rozenberg S, Riou B, Leiris J, Swynghedauw B. Cardioprotection with cariporide, a sodium-proton exchanger inhibitor, after prolonged ischemia and reperfusion in senescent rats. Exp Gerontol 2004;39:1307-1314.

(143) Ryan JB, Hicks M, Cropper JR, Garlick SR, Kesteven SH, Wilson MK, Fenley MP, MacDonald PS. Cariporide (HOE-642) improves cardiac allograft preservation in a porcine model of orthotopic heart transplantation. Transplantation 2003;75:625-631.

(144) Ryan JB, Hicks M, Cropper JR, Garlick SR, Kesteven SH, Wilson MK, Feneley MP, MacDonald PS. Sodium-hydrogen exchanger inhibition, pharmacologic ischemic preconditioning, or both for extended cardiac allograft preservation. Transplantation 2003;76:766-771.

(145) Cropper JR, Hicks M, Ryan JB, MacDonald PS. Cardioprotection by cariporide after prolonged hypothermic storage of the isolated working rat heart. J Heart Lung Transplant 2003;22:929-936.

(146) Humphreys RA, Haist JV, Chakrabarti S, Feng Q, Arnold JM, Karmazyn M. Orally administered NHE1 inhibitor cariporide reduces acute responses to coronary occlusion and reperfusion. Am J Physiol Heart Circ Physiol 1999;276:H749-H757.

(147) Pierce GN, Ramjiawan B, Dhalla NS, Ferrari R. $Na^+$-$H^+$ exchange in cardiac sarcolemmal vesicles isolated from diabetic rats. Am J Physiol 1990;258:H255-H261.

(148) Goel DP, Pierce GN. Role of the sodium-hydrogen exchanger in ischemia-reperfusion injury in diabetes. J Thromb Thrombolysis 1999;8:45-52.

(149) Khandoudi N, Bernard M, Cozzone P, Feuvray D. Intracellular pH and role of $Na^+/H^+$ exchange during ischaemia and reperfusion of normal and diabetic rat hearts. Cardiovasc Res 1990;24:873-878.

(150) Imahashi K, Hashimoto K, Yamaguchi H, Nishimura T, Kusuoka H. Alteration of intracellular $Na^+$ during ischemia in diabetic rat hearts: the role of reduced activity in $Na^+/H^+$ exchange against stunning. J Mol Cell Cardiol 1998;30:509-517.

(151) Ramasamy R, Schaefer S. Inhibition of $Na^+$-$H^+$ exchanger protects diabetic and non-diabetic hearts from ischemic injury: insight into altered susceptibility of diabetic hearts to ischemic injury. J Mol Cell Cardiol 1999;31:785-797.

(152) Kevelaitis E, Qureshi AA, Mouas C, Marotte F, Kevelaitiene S, Avkiran M, Menasché P. $Na^+/H^+$ exchange inhibition in hypertrophied myocardium subjected to cardioplegic arrest: an effective cardioprotective approach. Eur J Cardiothorac Surg 2005;27:111-116.

(153) Hurtado C, Pierce GN. Sodium-hydrogen exchange inhibition: pre-versus post-ischemic treatment. Basic Res Cardiol 2001;96:312-317.

(154) Avkiran M, Marber MS. $Na^+/H^+$ exchange inhibitors for cardioprotective therapy: progress, problems and prospects. J Am Coll Cardiol 2002;39:747-753.

(155) Mentzer RM, Jr., Lasley RD, Jessel A, Karmazyn M. Intracellular sodium hydrogen exchange inhibition and clinical myocardial protection. Ann Thorac Surg 2003;75:S700-S708.

(156) Scholz W, Beier N. Development of NHE inhibitors for cardiovascular therapeutics. In: Karmazyn M, Avkiran M, Fliegel L, editors. The Sodium-Hydrogen Exchanger. From Molecule To Its Role In Disease. Boston: Kluwer Academic Publishers, 2003: 255-264.

(157) Murphy E, Cross HR, Steenbergen C. Is Na/Ca exchange during ischemia and reperfusion beneficial or detrimental? Ann N Y Acad Sci 2002;976:421-430.

(158) Meng HP, Lonsberry BB, Pierce GN. Influence of perfusate pH on the postischemic recovery of cardiac contractile function: involvement of sodium-hydrogen exchange. J Pharmacol Exp Ther 1991;258:772-777.

(159) Matsuda N, Kuroda H, Ashida Y, Okada M, Mori T. Possible involvement of $Na^+$-$H^+$ exchange in the early phase of reperfusion in myocardial stunning. J Surg Res 1992;53:529-534.

(160) Linz W, Albus U, Crause P, Jung W, Weichert A, Scholkens BA, Scholz W. Dose-dependent reduction of myocardial infarct mass in rabbits by the NHE-1 inhibitor cariporide (HOE 642). Clin Exp Hypertens 1998;20:733-749.

(161) Karmazyn M. The role of the myocardial sodium-hydrogen exchanger in mediating ischemic and reperfusion injury. From amiloride to cariporide. Ann N Y Acad Sci 1999;874:326-334.

(162) Mentzer RM, Jr., Lasley RD, Jessel A, Karmazyn M. Intracellular sodium hydrogen exchange inhibition and clinical myocardial protection. Ann Thorac Surg 2003;75:S700-S708.

(163) Clements-Jewery H, Sutherland FJ, Allen MC, Tracey WR, Avkiran M. Cardioprotective efficacy of zoniporide, a potent and selective inhibitor of $Na^+/H^+$ exchanger isoform 1, in an experimental model of cardiopulmonary bypass. Br J Pharmacol 2004;142:57-66.

(164) Rupprecht H-J, vom Dahl J, Terres W, Seyfarth KM, Richardt G, Schultheiß H-P, Buerke M, Sheehan FH, Drexler H. Cardioprotective effects of the $Na^+/H^+$ exchange inhibitor cariporide in patients with acute anterior myocardial infarction undergoing direct PTCA. Circulation 2000;101:2902-2908.

(165) Boyce SW, Bartels C, Bolli R, Chaitman B, Chen JC, Chi E, Jessel A, Kereiakes D, Knight J, Thulin L, Theroux P. Impact of sodium-hydrogen exchange inhibition by cariporide on death or myocardial infarction in high-risk CABG surgery patients: Results of the CABG surgery cohort of the GUARDIAN study. J Thorac Cardiovasc Surg 2003;126:420-427.

(166) Bolli R. The role of sodium-hydrogen ion exchange in patients undergoing coronary artery bypass grafting. J Card Surg 2003;18:21-26.

(167) Mentzer RM, Jr. Clinical trials with NHE inhibitors: where are we now and where are we going? J Mol Cell Cardiol 2004;37:359-359 [abstract].

(168) Kloner RA, Rezkalla SH. Cardiac protection during acute myocardial infarction: Where do we stand in 2004? J Am Coll Cardiol 2004;44:276-286.

(169) Weber W, Harnisch L, Jessel A, for the GUARDIAN Trial Investigators. Lessons learned from a phase III population pharmacokinetic study of cariporide in coronary artery bypass graft surgery. Clin Pharmacol Ther 2002;71:457-467.

(170) Ten Hove M, Van Echteld CJA. Limited effects of post-ischemic NHE blockade on $[Na^+]_i$ and $pH_i$ in rat hearts explain its lack of cardioprotection. Cardiovasc Res 2004;61:522-529.

(171) Schafer C, Ladilov YV, Siegmund B, Piper HM. Importance of bicarbonate transport for protection of cardiomyocytes against reoxygenation injury. Am J Physiol Heart Circ Physiol 2000;278:H1457-H1463.

(172) Bolli R, Becker L, Gross G, Mentzer R, Jr., Balshaw D, Lathrop DA. Myocardial protection at a crossroads: the need for translation into clinical therapy. Circ Res 2004;95:125-134.

(173) Gazmuri RJ, Ayoub IM, Hoffner E, Kolarova JD. Successful ventricular defibrillation by the selective sodium-hydrogen exchanger isoform-1 inhibitor cariporide. Circulation 2001;104:234-239.

(174) Gazmuri RJ, Hoffner E, Kalcheim J, Ho H, Patel M, Ayoub IM, Epstein M, Kingston S, Han Y. Myocardial protection during ventricular fibrillation by reduction of proton-driven sarcolemmal sodium influx. J Lab Clin Med 2001;137:43-55.

(175) Gazmuri RJ, Ayoub IM, Kolarova JD, Karmazyn M. Myocardial protection during ventricular fibrillation by inhibition of the sodium-hydrogen exchanger isoform-1. Crit Care Med 2002;30:S166-S171.

(176) Ayoub IM, Kolarova JD, Sehgal MA, Sanders R, Sheth O, Gazmuri RJ. Sodium-hydrogen exchange inhibition minimizes adverse effects of epinephrine during cardiac resuscitation. Circulation 2003;108:IV-420 [abstract].

(177) Ayoub IM, Kolarova J, Yi Z, Trevedi A, Deshmukh H, Lubell DL, Franz MR, Maldonado FA, Gazmuri RJ. Sodium-hydrogen exchange inhibition during ventricular fibrillation: beneficial effects on ischemic contracture, action potential duration, reperfusion arrhythmias, myocardial function, and resuscitability. Circulation 2003;107:1804-1809.

(178) Vidyarthi V, Trivedi A, Gopalakrishnan PP, Ayoub IM, Kolarova JD, Sehgal MA, Deshmukh H, Gazmuri RJ. Cariporide but not epinephrine prevents ischemic contracture during closed-chest resuscitation. J Am Coll Cardiol 2004;43:300A [abstract].

(179) Cingolani HE, Ennis IL, Mosca SM. NHE-1 and NHE-6 activities: ischemic and reperfusion injury. Circ Res 2003;93:694-696.

(180) Kolarova JD, Ayoub IM, Gazmuri RJ. Cariporide enables hemodynamically more effective chest compression by leftward shift of the flow-depth relationship. Am J Physiol Heart Circ Physiol 2005;Published online February 11, 2005. Accessed February 15, 2005.

(181) Ayoub IM, Kolarova JD, Kantola R, Sanders R, Sheth O, Gazmuri RJ. Cariporide minimizes adverse myocardial effects of epinephrine during resuscitation from ventricular fibrillation. Circulation 2004;110:III-531 [abstract].

(182) Rabkin DG, Cabreriza SE, LaCorte JC, Weinberg AD, Coku L, Walsh R, Mosca R, Spotnitz HM. Sodium-hydrogen exchange inhibition preserves ventricular function after ventricular fibrillation in the intact swine heart. J Thorac Cardiovasc Surg 2003;125:1499-1509.

(183) Iwamoto T, Satomi K. Development and application of $Na^+/Ca^{2+}$ exchange inhibitors. Mol Cell Biochem 2004;259:157-161.

(184) Hobai IA, O'Rourke B. The potential of $Na^+/Ca^{2+}$ exchange blockers in the treatment of cardiac disease. Expert Opin Investig Drugs 2004;13:653-664.

(185) Akabas MH. $Na^+/Ca^{2+}$ exchange inhibitors: potential drugs to mitigate the severity of ischemic injury. Mol Pharmacol 2004;66:8-10.

(186) Shigekawa M, Iwamoto T. Cardiac $Na^+$-$Ca^{2+}$ exchange: molecular and pharmacological aspects. Circ Res 2001;88:864-876.

(187) Iwamoto T, Watano T, Shigekawa M. A novel isothiourea derivative selectively inhibits the reverse mode of $Na^+/Ca^{2+}$ exchange in cells expressing NCX1. J Biol Chem 1996;271:22391-22397.

(188) Watano T, Kimura J, Morita T, Nakanishi H. A novel antagonist, No. 7943, of the $Na^+/Ca^{2+}$ exchange current in guinea-pig cardiac ventricular cells. Br J Pharmacol 1996;119:555-563.

(189) Matsuda T, Arakawa N, Takuma K, Kishida Y, Kawasaki Y, Sakaue M, Takahashi K, Takahashi T, Suzuki T, Ota T, Hamano-Takahashi A, Onishi M, Tanaka Y, Kameo K, Baba A. SEA0400, a novel and selective inhibitor of the $Na^+$-$Ca^{2+}$ exchanger, attenuates reperfusion injury in the in vitro and in vivo cerebral ischemic models. J Pharmacol Exp Ther 2001;298:249-256.

(190) Hasegawa H, Muraoka M, Matsui K, Kojima A. Discovery of a novel potent $Na^+/Ca^{2+}$ exchanger inhibitor: design, synthesis and structure-activity relationships of 3,4-dihydro-2(1*H*)-quinazolinone derivatives. Bioorg Med Chem Lett 2003;13:3471-3475.

(191) Iwamoto T, Inoue Y, Ito K, Sakaue T, Kita S, Katsuragi T. The exchanger inhibitory peptide region-dependent inhibition of $Na^+/Ca^{2+}$ exchange by SN-6 [2-[4-(4-nitrobenzyloxy)benzyl]thiazolidine-4-carboxylic acid ethyl ester], a novel benzyloxyphenyl derivative. Mol Pharmacol 2004;66:45-55.

(192) Anderson SE, Cala PM, Steenbergen C, London RE, Murphy E. Effects of hypoxia and acidification on myocardial Na and Ca. Role of Na-H and Na-Ca exchange. Ann N Y Acad Sci 1991;639:453-455.

(193) Pogwizd SM, Bers DM. Na/Ca exchange in heart failure: contractile dysfunction and arrhythmogenesis. Ann N Y Acad Sci 2002;976:454-465.

(194) Nakamura A, Harada K, Sugimoto H, Nakajima F, Nishimura N. Effects of KB-R7943, a novel $Na^+/Ca^{2+}$ exchange inhibitor, on myocardial ischemia/ reperfusion injury. Nippon Yakurigaku Zasshi 1998;111:105-115.

(195) Mukai M, Terada H, Sugiyama S, Satoh H, Hayashi H. Effects of a selective inhibitor of $Na^+/Ca^{2+}$ exchange, KB-R7943, on reoxygenation-induced injuries in guinea pig papillary muscles. J Cardiovasc Pharmacol 2000;35:121-128.

(196) Yamamura K, Tani M, Hasegawa H, Gen W. Very low dose of the $Na^+/Ca^{2+}$ exchange inhibitor, KB-R7943, protects ischemic reperfused aged Fischer 344 rat hearts: considerable strain difference in the sensitivity to KB-R7943. Cardiovasc Res 2001;52:397-406.

(197) Schafer C, Ladilov Y, Inserte J, Schafer M, Haffner S, Garcia-Dorado D, Piper HM. Role of the reverse mode of the $Na^+/Ca^{2+}$ exchanger in reoxygenation-induced cardiomyocyte injury. Cardiovasc Res 2001;51:241-250.

(198) Matsumoto T, Miura T, Miki T, Genda S, Shimamoto K. Blockade of the $Na^+$-$Ca^{2+}$ exchanger is more efficient than blockade of the $Na^+$-$H^+$ exchanger for protection of the myocardium from lethal reperfusion injury. Cardiovasc Drugs Ther 2002;16:295-301.

(199) Elias CL, Lukas A, Shurraw S, Scott J, Omelchenko A, Gross GJ, Hnatowich M, Hryshko LV. Inhibition of $Na^+/Ca^{2+}$ exchange by KB-R7943: transport mode selectivity and antiarrhythmic consequences. Am J Physiol Heart Circ Physiol 2001;281:H1334-H1345.

(200) Van Wagoner DR, Bond M. Reperfusion arrhythmias: new insights into the role of the $Na^+/Ca^{2+}$ exchanger. J Mol Cell Cardiol 2001;33:2071-2074.

(201) Woodcock EA, Arthur JF, Harrison SN, Gao X-M, Du X-J. Reperfusion-induced ins(1,4,5)$P_3$ generation and arrhythmogenesis require activation of the $Na^+/Ca^{2+}$ exchanger. J Mol Cell Cardiol 2001;33:1861-1869.

(202) Miyamoto S, Zhu BM, Kamiya K, Nagasawa Y, Hashimoto K. KB-R7943, a $Na^+/Ca^{2+}$ exchange inhibitor, does not suppress ischemia/reperfusion arrhythmias nor digitalis arrhythmias in dogs. Jpn J Pharmacol 2002;90:229-235.

(203) Higuchi M, Tanaka C, Nishi K. Effects of KB-R7943, a selective NCX inhibitor, on underperfusion injury in diabetic rat hearts. J Pharmacol Sci 2003;91:154P [abstract].

(204) Hagihara H, Yoshikawa Y, Ohga Y, Takenaka C, Murata Ky, Taniguchi S, Takaki M. $Na^+/Ca^{2+}$ exchange inhibition protects the rat heart from ischemic-reperfusion injury by blocking energy-wasting processes. Am J Physiol Heart Circ Physiol 2005;288:H1699-H1707.

(205) Gazmuri RJ, Hoffner E, Katakam R, Thompson KM, Ayoub IM. Sarcolemmal $Na^+$-$Ca^{2+}$ exchanger inhibition ameliorates post-resuscitation diastolic dysfunction. Crit Care Med 1999;27:A35 [abstract].

(206) Sehgal MA, Kolarova JD, Ayoub IM, Sheth O, Gazmuri RJ. Reverse mode inhibition of the $Na^+$-$Ca^+$ exchanger protects the myocardium during resuscitation from ventricular fibrillation. Circulation 2003;108:IV-380 [abstract].

(207) Amran MS, Homma N, Hashimoto K. Pharmacology of KB-R7943: a $Na^+$-$Ca^{2+}$ exchange inhibitor. Cardiovasc Drug Rev 2003;21:255-276.

(208) Maczewski M, Beresewicz A. Role of nitric oxide and free radicals in cardioprotection by blocking $Na^+/H^+$ and $Na^+/Ca^{2+}$ exchange in rat heart. Eur J Pharmacol 2003;461:139-147.

(209) Schröder UH, Breder J, Sabelhaus CF, Reymann KG. The novel $Na^+/Ca^{2+}$ exchange inhibitor KB-R7943 protects CA1 neurons in rat hippocampal slices against hypoxic/hypoglycemic injury. Neuropharmacology 1999;38:319-321.

(210) Li S, Jiang Q, Stys PK. Important role of reverse $Na^+$-$Ca^{2+}$ exchange in spinal cord white matter injury at physiological temperature. J Neurophysiol 2000;84:1116-1119.

(211) Pilitsis JG, Diaz FG, O'Regan MH, Phillis JW. Inhibition of $Na^+/Ca^{2+}$ exchange by KB-R7943, a novel selective antagonist, attenuates phosphoethanolamine and free fatty acid efflux in rat cerebral cortex during ischemia-reperfusion injury. Brain Res 2001;916:192-198.

(212) Bondarenko A, Svichar N, Chesler M. Role of $Na^+$-$H^+$ and $Na^+$-$Ca^{2+}$ exchange in hypoxia-related acute astrocyte death. Glia 2005;49:143-152.

(213) Kuro T, Kobayashi Y, Takaoka M, Matsumura Y. Protective effect of KB-R7943, a novel $Na^+/Ca^{2+}$ exchange inhibitor, on ischemic acute renal failure in rats. Jpn J Pharmacol 1999;81:247-251.

(214) Yamashita J, Ogata M, Takaoka M, Matsumura Y. KB-R7943, a selective $Na^+/Ca^{2+}$ exchange inhibitor, protects against ischemic acute renal failure in mice by inhibiting renal endothelin-1 overproduction. J Cardiovasc Pharmacol 2001;37:271-279.

(215) Yamashita J, Itoh M, Kuro T, Kobayashi Y, Ogata M, Takaoka M, Matsumura Y. Pre- or post-ischemic treatment with a novel $Na^+/Ca^{2+}$ exchange inhibitor, KB-R7943, shows renal protective effects in rats with ischemic acute renal failure. J Pharmacol Exp Ther 2001;296:412-419.

(216) Sobolevsky AI, Khodorov BI. Blockade of NMDA channels in acutely isolated rat hippocampal neurons by the $Na^+/Ca^{2+}$ exchange inhibitor KB-R7943. Neuropharmacology 1999;38:1235-1242.

(217) Pintado AJ, Herrero CJ, García AG, Montiel C. The novel $Na^+/Ca^{2+}$ exchange inhibitor KB-R7943 also blocks native and expressed neuronal nicotinic receptors. Br J Pharmacol 2000;130:1893-1902.

(218) Arakawa N, Sakaue M, Yokoyama I, Hashimoto H, Koyama Y, Baba A, Matsuda T. KB-R7943 inhibits store-operated $Ca^{2+}$ entry in cultured neurons and astrocytes. Biochem Biophys Res Commun 2000;279:354-357.

(219) Tanaka H, Nishimaru K, Aikawa T, Hirayama W, Tanaka Y, Shigenobu K. Effect of SEA0400, a novel inhibitor of sodium-calcium exchanger, on myocardial ionic currents. Br J Pharmacol 2002;135:1096-1100.

(220) Reuter H, Henderson SA, Han T, Matsuda T, Baba A, Ross RS, Goldhaber JI, Philipson KD. Knockout mice for pharmacological screening: testing the specificity of $Na^+$-$Ca^{2+}$ exchange inhibitors. Circ Res 2002;91:90-92.

(221) MacDonald AC, Howlett SE, Ferrier GR. Protective and adverse effects of the $Na^+/Ca^{2+}$ exchange inhibitor KB-R7943 in a single cell model of ischemia and reperfusion. Biophys J 2004;86:65a. [abstract].

(222) Kurogouchi F, Furukawa Y, Zhao D, Hirose M, Nakajima K, Tsuboi M, Chiba S. A $Na^+/Ca^{2+}$ exchanger inhibitor, KB-R7943, caused negative inotropic responses and negative followed by positive chronotropic responses in isolated, blood-perfused dog heart preparations. Jpn J Pharmacol 2000;82:155-163.

(223) Miyata A, Zipes DP, Hall S, Rubart M. KB-R7943 prevents acute, atrial fibrillation-induced shortening of atrial refractoriness in anesthetized dogs. Circulation 2002;106:1410-1419.

(224) Higuchi M, Kouzuma N. Effects of NCX inhibitor on underperfusion injury in diabetic rat hearts. J Mol Cell Cardiol 2004;37:232 [abstract].

(225) Satoh H, Ginsburg KS, Qing K, Terada H, Hayashi H, Bers DM. KB-R7943 block of $Ca^{2+}$ influx via $Na^+/Ca^{2+}$ exchange does not alter twitches or glycoside inotropy but prevents $Ca^{2+}$ overload in rat ventricular myocytes. Circulation 2000;101:1441-1446.

(226) Kimura J, Watano T, Kawahara M, Sakai E, Yatabe J. Direction-independent block of bi-directional $Na^+/Ca^{2+}$ exchange current by KB-R7943 in guinea-pig cardiac myocytes. Br J Pharmacol 1999;128:969-974.

(227) Hotta Y, Lu XL, Yajima M, et al. Protective effect of SN-6, a selective $Na^+$-$Ca^{2+}$ exchange inhibitor, on ischemia-reperfusion-injured hearts. Jpn J Pharmacol 2002;88 suppl 1:P784.

(228) Iwamoto T, Kita S, Shigekawa M. Functional analysis of $Na^+/Ca^{2+}$ exchanger using novel drugs and genetically engineered mice. Nippon Yakurigaku Zasshi 2002;120:91-93.

(229) Iwamoto T, Shigekawa M. Differential inhibition of $Na^+/Ca^{2+}$ exchanger isoforms by divalent cations and isothiourea derivative. Am J Physiol Cell Physiol 1998;275:C423-C430.

(230) Bouchard R, Omelchenko A, Le HD, Choptiany P, Matsuda T, Baba A, Takahashi K, Nicoll DA, Philipson KD, Hnatowich M, Hryshko LV. Effects of SEA0400 on mutant NCX1.1 $Na^+$-$Ca^{2+}$ exchangers with altered ionic regulation. Mol Pharmacol 2004;65:802-810.

(231) Lee C, Visen N, Dhalla NS, Le HD, Isaac M, Choptiany P, Gross G, Omelchenko A, Matsuda T, Baba A, Takahashi K, Hnatowich M, Hryshko LV. Inhibitory profile of SEA0400 [2-[4-[(2,5-Difluorophenyl)methoxy]phenoxy]-5-ethoxyaniline] assessed on the cardiac $Na^+$-$Ca^{2+}$ exchanger, NCX1.1. J Pharmacol Exp Ther 2004;311:748-757.

(232) Yoshiyama M, Nakamura Y, Omura T, Hayashi T, Takagi Y, Hasegawa T, Nishioka H, Takeuchi K, Iwao H, Yoshikawa J. Cardioprotective effect of SEA0400, a selective inhibitor of the $Na^+/Ca^{2+}$ exchanger, on myocardial ischemia-reperfusion injury in rats. J Pharmacol Sci 2004;95:196-202.

(233) Takahashi T, Takahashi K, Onishi M, Suzuki T, Tanaka Y, Ota T, Yoshida S, Nakaike S, Matsuda T, Baba A. Effects of SEA0400, a novel inhibitor of the $Na^+/Ca^{2+}$ exchanger, on myocardial stunning in anesthetized dogs. Eur J Pharmacol 2004;505:163-168.

(234) Tanaka H, Namekata I, Nakamura H, Smimada H, Shigenobu K. Cardioprotection without cardiosuppression by SEA0400 during ischemia and reperfusion in guinea-pig myocardium. J Mol Cell Cardiol 2004;37:1079 [abstract].

(235) Lee C, Hryshko LV. SEA0400: a novel sodium-calcium exchange inhibitor with cardioprotective properties. Cardiovasc Drug Rev 2004;22:334-347.

(236) Ogata M, Iwamoto T, Tazawa N, Nishikawa M, Yamashita J, Takaoka M, Matsumura Y. A novel and selective $Na^+/Ca^{2+}$ exchange inhibitor, SEA0400, improves ischemia/reperfusion-induced renal injury. Eur J Pharmacol 2003;478:187-198.

(237) Amran MS, Hashimoto K, Homma N. Effects of sodium-calcium exchange inhibitors, KB-R7943 and SEA0400, on aconitine-induced arrhythmias in guinea pigs in vivo, in vitro and in computer simulation studies. J Pharmacol Exp Ther 2004;310:83-89.

(238) Iwamoto T, Kita S, Zhang J, Blaustein MP, Arai Y, Yoshida S, Wakimoto K, Komuro I, Katsuragi T. Salt-sensitive hypertension is triggered by $Ca^{2+}$ entry via $Na^+/Ca^{2+}$ exchanger type-1 in vascular smooth muscle. Nat Med 2004;10:1193-1199.

(239) Nagasawa Y, Zhu B-M, Chen J, Kamiya K, Miyamoto S, Hashimoto K. Effects of SEA0400, a $Na^+/Ca^{2+}$ exchange inhibitor, on ventricular arrhythmias in the in vivo dogs. Eur J Pharmacol 2005;506:249-255.

(240) Iwamoto T, Kita S, Uehara A, Imanaga I, Matsuda T, Baba A, Katsuragi T. Molecular determinants of $Na^+/Ca^{2+}$ exchange (NCX1) inhibition by SEA0400. J Biol Chem 2004;279:7544-7553.

(241) Nagano T, Kawasaki Y, Baba A, Takemura M, Matsuda T. Up-regulation of $Na^+$-$Ca^{2+}$ exchange activity by interferon-gamma in cultured rat microglia. J Neurochem 2004;90:784-791.

(242) Nagy ZA, Virag L, Toth A, Biliczki P, Acsai K, Banyasz T, Nanasi P, Papp JG, Varro A. Selective inhibition of sodium-calcium exchanger by SEA-0400 decreases early and delayed afterdepolarization in canine heart. Br J Pharmacol 2004;143:827-831.

(243) Amran M, Homma N, Hashimoto K. Effects of sodium-calcium exchange inhibitors, KB-R7943 and SEA0400, on aconitine-induced arrhythmias in guinea pigs in vivo, in vitro and computer simulation studies. J Pharmacol Exp Ther 2004;310:83-89.

(244) Noguchi K, Hiyoshi T, Takahashi T, Yoshida S, Nakaike S, Matsuda T, Baba A. The effects of SEA0400, a novel and selective inhibitor of the $Na^+/Ca^{2+}$ exchanger, on the glutamate receptor/channel-mediated currents. Jpn J Pharmacol 2002;88:152P [abstract].

(245) Aikawa T, Hirayama W, Kawanura T, Masuda H, Nishimaru K, Masumiya H, Tanaka H, Kawanishi T, Shigenobu K. Effects of SEA0400, a novel inhibitor of sodium-calcium exchanger, on electrical and mechanical activities of myocardia. J Pharmacol Sci 2003;91:151P. [abstract].

(246) Zhang J, Lee MY, Lingrel JB, Philipson KD, Iwamoto T, Ferrari P, Bianchi G, Wier WG, Blaustein MP. Ouabain antagonists and Na/Ca exchange inhibitor block ouabain-induced increase in myogenic tone in mouse small arteries. Circulation 2004;110:III-166 [abstract].

(247) Iwamoto T, Kita S, Zhang J, Blaustein MPAY, Yoshida S, Wakimoto K, Komuro I, Katsuragi T. The reverse mode of vascular $Na^+/Ca^{2+}$ exchanger type-1 is essential for the development of salt-sensitive hypertension. J Mol Cell Cardiol 2004;37:230. [abstract].

(248) Houchi H, Nishisako H, Ishizawa K, Ozawa Y, Ikehara T, Minakuchi K. Effects of sodium-calcium exchanger inhibitors on catecholamine secretion from cultured bovine adrenal chromaffin cells. J Neurochem 2003;87:46 [abstract].

(249) Saesue P, Horiuchi T, Goto T, Tanaka Y, Hongo K. Functional role of the $Na^+/H^+$ exchanger in the regulation of cerebral arteriolar tone in rats. J Neurosurg 2004;101:330-335.

(250) Beaugé L, DiPolo R. SEA0400, a potent inhibitor of the $Na^+/Ca^{2+}$ exchange, as a tool to study the exchanger ionic and metabolic regulations. Am J Physiol Cell Physiol 2005;doi:10.1152/ajpcell.00492.2004. Available online February 9, 2005. Accessed February 10, 2005.

(251) Danilczyk U, Penninger J. Hypertension with a grain of salt. Nat Med 2004;10:1163-1164.

(252) Matsuda T, Takuma K, Arakawa N, Takahashi K, Koyama Y, Baba A. Anti-apoptotic effect of SEA0400, a novel and selective inhibitor of the $Na^+$-$Ca^{2+}$ exchanger, in an in vitro astrocyte injury model. Jpn J Pharmacol 2002;88:244P. [abstract].

(253) Matsuda T, Koyama Y, Baba A. Functional proteins involved in regulation of intracellular $Ca^{2+}$ for drug development: pharmacology of SEA0400, a specific inhibitor of the $Na^+$-$Ca^{2+}$ exchanger. J Pharmacol Sci 2005;97:339-343.

(254) Nagano T, Osakada M, Ago Y, Koyama Y, Baba A, Maeda S, Takemura M, Matsuda T. SEA0400, a specific inhibitor of the $Na^+$-$Ca^{2+}$ exchanger, attenuates sodium nitroprusside-induced apoptosis in cultured rat microglia. Br J Pharmacol 2005;144:669-679.

(255) Koyama Y, Matsui S, Itoh S, Osakada M, Baba A, Matsuda T. The selective $Na^+$-$Ca^{2+}$ exchange inhibitor attenuates brain edema after radiofrequency lesion in rats. Eur J Pharmacol 2004;489:193-196.

(256) Hotta Y, Lu X, Yajima M, Ishikawa N, Ito K, Inoue Y, Sakaue T. Protective effect of SN-6, a selective $Na^+$-$Ca^{2+}$ exchange inhibitor, on ischemia-reperfusion-injured hearts. Jpn J Pharmacol 2002;88 suppl 1:257 [abstract].

(257) Inoue Y, Ito K, Sakaue T, Iwamoto T, Kita S. Characterization of SN-6, a novel exchange inhibitor and its renal protective effect. Clin Calcium 2004;14:1262-1269.

(258) Kuramochi T, Kakefuda A, Yamada H, Sato I, Taguchi T, Sakamoto S. Synthesis and structure-activity relationships of phenoxypyridine derivatives as novel inhibitors of the sodium-calcium exchanger. Bioorg Med Chem 2004;12:5039-5056.

(259) Kuramochi T, Kakefuda A, Sato I, Tsukamoto I, Taguchi T, Sakamoto S. Synthesis and structure-activity relationships of 6-4-[(3-fluorobenzyl)oxy]phenoxy nicotinamide derivatives as a novel class of NCX inhibitors: a QSAR study. Bioorg Med Chem 2005;13:717-724.

(260) Kuramochi T, Kakefuda A, Yamada H, Ogiyama T, Taguchi T, Sakamoto S. Synthesis and structure-activity relationships of benzyloxyphenyl derivatives as a novel class of NCX inhibitors: effects on heart failure. Bioorg Med Chem 2005;13:725-734.

(261) Ohta T, Nakanishi M, Aibe I, Taguchi M, Tomisawa K. JP Patent 11092454, 1999.

(262) Satoh N, Sato T, Shimada M, Yamada K, Kitada Y. Lusitropic effect of MCC-135 is associated with improvement of sarcoplasmic reticulum function in ventricular muscles of rats with diabetic cardiomyopathy. J Pharmacol Exp Ther 2001;298:1161-1166.

(263) Satoh N, Kitada Y. Cardioprotective effect of MCC-135 is associated with inhibition of $Ca^{2+}$ overload in ischemic/reperfused hearts. Eur J Pharmacol 2004;499:179-187.

(264) Mitsubishi Pharma Corporation. MCC-135 as adjunct therapy to primary percutaneous coronary intervention in ST-segment elevation acute myocardial infarction patients. In: ClinicalTrials gov [Internet] Bethesda (MD): National Library of Medicine; 2000- Available from: http://clinicaltrials gov/show/NCT00078013 NLM Identifier: NCT00078013 Accessed on November 28, 2004.

(265) Zile M, Gaasch W, Little W, Francis G, Tavazzi L, Cleland J, Davies M. A phase II, double-blind, randomized, placebo-controlled, dose comparative study of the efficacy, tolerability, and safety of MCC-135 in subjects with chronic heart failure, NYHA class II/III (MCC-135-GO1 study): rationale and design. J Card Fail 2004;10:193-199.

(266) The Thrombolysis in Myocardial Infarction (TIMI) trial. Phase I findings. TIMI Study Group. N Engl J Med 1985;312:932-936.

(267) Antman EM. ST-elevation myocardial infarction: management. In: Zipes DP, Libby P, Bonow RO, Braunwald E, editors. Braunwald's Heart Disease: A Textbook of Cardiovascular Medicine. Philadelphia,PA: Elsevier Saunders, 2005: 1167-1226.

(268) Hibberd M, Tzivoni D, Bar F, Brachmann J, Reiber H, Krucoff M, Davies M, Tatsuno J, on behalf of the (CASTEMI) Study Group. Caldaret (MCC-135) lowers the frequency of severe systolic left ventricular dysfunction in STEMI patients undergoing PCI. J Am Coll Cardiol 2005;45 Suppl 1:242A [abstract].

(269) Reaven GM. Banting lecture 1988. Role of insulin resistance in human disease. Diabetes 1988;37:1595-1607.

(270) Reaven G. Metabolic syndrome: pathophysiology and implications for management of cardiovascular disease. Circulation 2002;106:286-288.

(271) Reaven GM. Insulin resistance and its consequences: type 2 diabetes mellitus and the insulin resistance syndrome. In: LeRoith DL, Taylor SI, Olefsky JM, editors. Diabetes Mellitus: A Fundamental And Clinical Text. Philadelphia, PA: Lippincott Williams & Wilkins, 2004: 899-915.

(272) Salmenniemi U, Ruotsalainen E, Pihlajamaki J, Vauhkonen I, Kainulainen S, Punnonen K, Vanninen E, Laakso M. Multiple abnormalities in glucose and energy metabolism and coordinated changes in levels of adiponectin, cytokines, and adhesion molecules in subjects with metabolic syndrome. Circulation 2004;110:3842-3848.

(273) Alberti KG, Zimmet PZ. Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. Diabet Med 1998;15:539-553.

(274) Canadian Diabetes Association Clinical Practice Guidelines Expert Committee. Canadian Diabetes Association 2003 clinical practice guidelines for the prevention and management of diabetes in Canada. Can J Diabetes 2003;27:S1-S152.

(275) Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) Final Report. Circulation 2002;106:3143-3421.

(276) Einhorn D, Reaven GM, Cobin RH, Ford E, Ganda OP, Handelsman Y, Hellman R, Jellinger PS, Kendall D, Krauss RM, Neufeld ND, Petak SM, Rodbard HW, Seibel JA, Smith DA, Wilson PW. American College of Endocrinology position statement on the insulin resistance syndrome. Endocr Pract 2003;9:237-252.

(277) Grundy SM, Brewer HB, Jr., Cleeman JI, Smith SC, Jr., Lenfant C, for the Conference Participants. Definition of metabolic syndrome: report of the National Heart, Lung, and Blood Institute/American Heart Association Conference on scientific issues related to definition. Circulation 2004;109:433-438.

(278) Reaven G. The metabolic syndrome or the insulin resistance syndrome? Different names, different concepts, and different goals. Endocrinol Metab Clin North Am 2004;33:283-303.

(279) Cameron AJ, Shaw JE, Zimmet PZ. The metabolic syndrome: prevalence in worldwide populations. Endocrinol Metab Clin North Am 2004;33:351-375.

(280) Cheal KL, Abbasi F, Lamendola C, McLaughlin T, Reaven GM, Ford ES. Relationship to insulin resistance of the Adult Treatment Panel III diagnostic criteria for identification of the metabolic syndrome. Diabetes 2004;53:1195-1200.

(281) Grundy SM. Metabolic syndrome: Part I. Endocrinol Metab Clin North Am 2004;33:IX-XI.

(282) Haffner SM, Mykkanen L, Festa A, Burke JP, Stern MP. Insulin-resistant prediabetic subjects have more atherogenic risk factors than insulin-sensitive prediabetic subjects: implications for preventing coronary heart disease during the prediabetic state. Circulation 2000;101:975-980.

(283) Isomaa B, Almgren P, Tuomi T, Forsen B, Lahti K, Nissen M, Taskinen MR, Groop L. Cardiovascular morbidity and mortality associated with the metabolic syndrome. Diabetes Care 2001;24:683-689.

(284) Lakka HM, Laaksonen DE, Lakka TA, Niskanen LK, Kumpusalo E, Tuomilehto J, Salonen JT. The metabolic syndrome and total and cardiovascular disease mortality in middle-aged men. JAMA 2002;288:2709-2716.

(285) Alexander CM, Landsman PB, Teutsch SM, Haffner SM. NCEP-defined metabolic syndrome, diabetes, and prevalence of coronary heart disease among NHANES III participants age 50 years and older. Diabetes 2003;52:1210-1214.

(286) Lorenzo C, Okoloise M, Williams K, Stern MP, Haffner SM. The metabolic syndrome as predictor of type 2 diabetes: the San Antonio Heart Study. Diabetes Care 2003;26:3153-3159.

(287) Sattar N, Gaw A, Scherbakova O, Ford I, O'Reilly DS, Haffner SM, Isles C, Macfarlane PW, Packard CJ, Cobbe SM, Shepherd J. Metabolic syndrome with and without C-reactive protein as a predictor of coronary heart disease and diabetes in the West of Scotland Coronary Prevention Study. Circulation 2003;108:414-419.

(288) Mensah GA, Mokdad AH, Ford E, Narayan KM, Giles WH, Vinicor F, Deedwania PC. Obesity, metabolic syndrome, and type 2 diabetes: emerging epidemics and their cardiovascular implications. Cardiol Clin 2004;22:485-504.

(289) Ninomiya JK, L'Italien G, Criqui MH, Whyte JL, Gamst A, Chen RS. Association of the metabolic syndrome with history of myocardial infarction and stroke in the Third National Health and Nutrition Examination Survey. Circulation 2004;109:42-46.

(290) Malik S, Wong ND, Franklin SS, Kamath TV, Italien GJ, Pio JR, Williams GR. Impact of the metabolic syndrome on mortality from coronary heart disease, cardiovascular disease, and all causes in United States adults. Circulation 2004;110:1245-1250.

(291) Rutter MK, Meigs JB, Sullivan LM, D'Agostino RB, Sr., Wilson PWF. C-reactive protein, the metabolic syndrome, and prediction of cardiovascular events in the Framingham Offspring Study. Circulation 2004;110:380-385.

(292) American Heart Association. Heart Disease and Stroke Statistics - 2005 Update. Dallas, TX: American Heart Association, 2005: 44-45. Available at: americanheart.org/statistics. Accessed on January 3, 2005.

(293) Olijhoek JK, van der Graaf Y, Banga JD, Algra A, Rabelink TJ, Visseren FLJ. The metabolic syndrome is associated with advanced vascular damage in patients with coronary heart disease, stroke, peripheral arterial disease or abdominal aortic aneurysm. Eur Heart J 2004;25:342-348.

(294) Howard BV, Rodriguez BL, Bennett PH, Harris MI, Hamman R, Kuller LH, Pearson TA, Wylie-Rosett J. Prevention Conference VI: diabetes and cardiovascular disease: writing group I: epidemiology. Circulation 2002;105:132e-137e.

(295) Bonora E, Targher G, Formentini G, Calcaterra F, Lombardi S, Marini F, Zenari L, Saggiani F, Poli M, Perbellini S, Raffaelli A, Gemma L, Santi L, Bonadonna RC, Muggeo M. The metabolic syndrome is an independent predictor of cardiovascular disease in type 2 diabetic subjects. Prospective data from the Verona Diabetes Complications Study. Diabet Med 2004;21:52-58.

(296) Bruno G, Merletti F, Biggeri A, Bargero G, Ferrero S, Runzo C, Prina Cerai S, Pagano G, Cavallo-Perin P. Metabolic syndrome as a predictor of all-cause and cardiovascular mortality in type 2 diabetes: the Casale Monferrato Study. Diabetes Care 2004;27:2689-2694.

(297) Anand SS, Yi Q, Gerstein H, Lonn E, Jacobs R, Vuksan V, Teo K, Davis B, Montague P, Yusuf S. Relationship of metabolic syndrome and fibrinolytic dysfunction to cardiovascular disease. Circulation 2003;108:420-425.

(298) De S CJL, Title LM, on behalf of the ICONS investigators. The metabolic syndrome leads to worse long-term outcomes after percutaneous coronary intervention. Can J Cardiol 2004;20:172D [abstract].

(299) Solymoss BC, Bourassa MG, Lesperance J, Levesque S, Marcil M, Varga S, Campeau L. Incidence and clinical characteristics of the metabolic syndrome in patients with coronary artery disease. Coron Artery Dis 2003;14:207-212.

(300) McNeill AM, Rosamond WD, Girman CJ, Heiss G, Golden SH, Duncan BB, East HE, Ballantyne C. Prevalence of coronary heart disease and carotid arterial thickening in patients with the metabolic syndrome (The ARIC Study). Am J Cardiol 2004;94:1249-1254.

(301) Turhan H, Erbay AR, Yasar AS, Bicer A, Sasmaz H, Yetkin E. Impaired coronary blood flow in patients with metabolic syndrome: documented by Thrombolysis In Myocardial Infarction (TIMI) frame count method. Am Heart J 2004;148:789-794.

(302) Ford ES, Giles WH, Dietz WH. Prevalence of the metabolic syndrome among US adults: findings from the Third National Health and Nutrition Examination Survey. JAMA 2002;287:356-359.

(303) Eckel RH, York DA, Rossner S, Hubbard V, Caterson I, Jeor ST, Hayman LL, Mullis RM, Blair SN. Prevention Conference VII: obesity, a worldwide epidemic related to heart disease and stroke: executive summary. Circulation 2004;110:2968-2975.

(304) Grundy SM. What is the contribution of obesity to the metabolic syndrome? Endocrinol Metab Clin North Am 2004;33:267-282.

(305) Ginsberg HN. Insulin resistance and cardiovascular disease. J Clin Invest 2000;106:453-458.

(306) Lemieux I, Pascot A, Couillard C, Lamarche B, Tchernof A, Almeras N, Bergeron J, Gaudet D, Tremblay G, Prud'homme D, Nadeau A, Despres JP. Hypertriglyceridemic waist: a marker of the atherogenic metabolic triad (hyperinsulinemia; hyperapolipoprotein B; small, dense LDL) in men? Circulation 2000;102:179-184.

(307) Lemieux I, Almeras N, Mauriege P, Blanchet C, Dewailly E, Bergeron J, Despres JP. Prevalence of 'hypertriglyceridemic waist' in men who participated in the Quebec Health Survey: association with atherogenic and diabetogenic metabolic risk factors. Can J Cardiol 2002;18:725-732.

(308) Carr MC, Brunzell JD. Abdominal obesity and dyslipidemia in the metabolic syndrome: importance of type 2 diabetes and familial combined hyperlipidemia in coronary artery disease risk. J Clin Endocrinol Metab 2004;89:2601-2607.

(309) Yusuf PS, Hawken S, Ounpuu S, Dans T, Avezum A, Lanas F, McQueen M, Budaj A, Pais P, Varigos J, Lisheng L. Effect of potentially modifiable risk factors associated with myocardial infarction in 52 countries (the INTERHEART study): case-control study. The Lancet 2004;364:937-952.

(310) Reeder BA, Senthilselvan A, Despres JP, Angel A, Liu L, Wang H, Rabkin SW. The association of cardiovascular disease risk factors with abdominal obesity in Canada. Canadian Heart Health Surveys Research Group. CMAJ 1997;157 Suppl 1:S39-S45.

(311) Office of Nutrition Policy and Promotion. Canadian guidelines for body weight classification in adults. Ottawa: Health Canada 2003;available at http://www.hc-sc.gc.ca/hpfb-dgpsa/onpp-bppn/weight_book_tc_e.html; accessed on November 28, 2004.

(312) Carr DB, Utzschneider KM, Hull RL, Kodama K, Retzlaff BM, Brunzell JD, Shofer JB, Fish BE, Knopp RH, Kahn SE. Intra-abdominal fat is a major determinant of the National Cholesterol Education Program Adult Treatment Panel III criteria for the metabolic syndrome. Diabetes 2004;53:2087-2094.

(313) Dobbelsteyn CJ, Joffres MR, MacLean DR, Flowerdew G. A comparative evaluation of waist circumference, waist-to-hip ratio and body mass index as indicators of cardiovascular risk factors. The Canadian Heart Health Surveys. Int J Obes Relat Metab Disord 2001;25:652-661.

(314) Ridker PM, Brown NJ, Vaughan DE, Harrison DG, Mehta JL. Established and emerging plasma biomarkers in the prediction of first atherothrombotic events. Circulation 2004;109:IV-6-IV-19.

(315) Morange PE, Alessi MC, Juhan-Vague I. Relations between hemostatic variables, insulin resistance and inflammation. Hematol J 2004;5 Suppl 3:S15-S19.

(316) Pearson TA, Mensah GA, Alexander RW, Anderson JL, Cannon RO, III, Criqui M, Fadl YY, Fortmann SP, Hong Y, Myers GL, Rifai N, Smith SC, Jr., Taubert K, Tracy RP, Vinicor F. Markers of inflammation and cardiovascular disease: application to clinical and public health practice: a statement for healthcare professionals from the Centers for Disease Control and Prevention and the American Heart Association. Circulation 2003;107:499-511.

(317) Pai JK, Pischon T, Ma J, Manson JE, Hankinson SE, Joshipura K, Curhan GC, Rifai N, Cannuscio CC, Stampfer MJ, Rimm EB. Inflammatory markers and the risk of coronary heart disease in men and women. N Engl J Med 2004;351:2599-2610.

(318) Feuerstein GZ, Firestein R, Mann DL, Libby P. A biomarker of inflamation in cardiovascular disease. In: Feuerstein GZ, Libby P, Mann DL, editors. Inflammation and Cardiac Diseases. Basel, Switzerland: Birkhäuser Verlag, 2003: 9-20.

(319) Willerson JT, Ridker PM. Inflammation as a cardiovascular risk factor. Circulation 2004;109:II-2-II-10.

(320) Libby P. Prevention and treatment of atherosclerosis. In: Kasper DL, Braunwald E, Fauci AS, Hauser SL, Longo DL, Jameson JL, editors. Harrison's Principles of Internal Medicine. New York: McGraw-Hill, 2005: 1430-1433.

(321) Ridker PM, Cannon CP, Morrow D, Rifai N, Rose LM, McCabe CH, Pfeffer MA, Braunwald E, the Pravastatin or Atorvastatin Evaluation and Infection Therapy-Thrombolysis in Myocardial Infarction. C-reactive protein levels and outcomes after statin therapy. New Engl J Med 2005;352:20-28.

(322) Nissen SE, Tuzcu EM, Schoenhagen P, Crowe T, Sasiela WJ, Tsai J, Orazem J, Magorien RD, O'Shaughnessy C, Ganz P, the Reversal of Atherosclerosis with Aggressive Lipid Lowering (REVERSAL) Investigators. Statin therapy, LDL cholesterol, C-reactive protein, and coronary artery disease. New Engl J Med 2005;352:29-38.

(323) Ridker PM, Wilson PWF, Grundy SM. Should C-reactive protein be added to metabolic syndrome and to assessment of global cardiovascular risk? Circulation 2004;109:2818-2825.

(324) Lemieux I, Pascot A, Prud'homme D, Almeras N, Bogaty P, Nadeau A, Bergeron J, Despres JP. Elevated C-reactive protein : another component of the atherothrombotic profile of abdominal obesity. Arterioscler Thromb Vasc Biol 2001;21:961-967.

(325) Festa A, D'Agostino R, Jr., Howard G, Mykkanen L, Tracy RP, Haffner SM. Chronic subclinical inflammation as part of the insulin resistance syndrome: the Insulin Resistance Atherosclerosis Study (IRAS). Circulation 2000;102:42-47.

(326) Tillett WS, Francis T, Jr. Serological reaction in pneumonia with a non-protein somatic fraction of pneumococcus. J Exp Med 1930;52:561-571.

(327) MacLeod CM, Avery OT. The occurrence during acute infections of a protein not normally present in the blood. II. Isolation and properties of the reactive protein. J Exp Med 1943;73:183-191.

(328) Ledue TB, Rifai N. Preanalytic and analytic sources of variations in C-reactive protein measurement: implications for cardiovascular disease risk assessment. Clin Chem 2003;49:1258-1271.

(329) Du Clos TW, Mold C. C-reactive protein: an activator of innate immunity and a modulator of adaptive immunity. Immunol Res 2004;30:261-278.

(330) Ridker PM, Brown NJ, Vaughan DE, Harrison DG, Mehta JL. Established and emerging plasma biomarkers in the prediction of first atherothrombotic events. Circulation 2004;109:IV6-IV19.

(331) Black S, Kushner I, Samols D. C-reactive protein. J Biol Chem 2004;279:48487-48490.

(332) Jabs WJ, Theissing E, Nitschke M, Bechtel JFM, Duchrow M, Mohamed S, Jahrbeck B, Sievers HH, Steinhoff J, Bartels C. Local generation of C-reactive protein in diseased coronary artery venous bypass grafts and normal vascular tissue. Circulation 2003;108:1428-1431.

(333) Pearson TA, Mensah GA, Hong Y, Smith SC, Jr. CDC/AHA Workshop on Markers of Inflammation and Cardiovascular Disease: application to clinical and public health practice: overview. Circulation 2004;110:e543-e544.

(334) Ridker PM, Wilson PW, Grundy SM. Should C-reactive protein be added to metabolic syndrome and to assessment of global cardiovascular risk? Circulation 2004;109:2818-2825.

(335) Ridker PM, Cushman M, Stampfer MJ, Tracy RP, Hennekens CH. Inflammation, aspirin, and the risk of cardiovascular disease in apparently healthy men. N Engl J Med 1997;336:973-979.

(336) Ridker PM, Rifai N, Rose L, Buring JE, Cook NR. Comparison of C-reactive protein and low-density lipoprotein cholesterol levels in the prediction of first cardiovascular events. New Engl J Med 2002;347:1557-1565.

(337) Ridker PM. Clinical application of C-reactive protein for cardiovascular disease detection and prevention. Circulation 2003;107:363-369.

(338) Pasceri V, Willerson JT, Yeh ETH. Direct proinflammatory effect of C-reactive protein on human endothelial cells. Circulation 2000;102:2165-2168.

(339) Verma S, Wang CH, Li SH, Dumont AS, Fedak PW, Badiwala MV, Dhillon B, Weisel RD, Li RK, Mickle DA, Stewart DJ. A self-fulfilling prophecy: C-reactive protein attenuates nitric oxide production and inhibits angiogenesis. Circulation 2002;106:913-919.

(340) Verma S, Li SH, Badiwala MV, Weisel RD, Fedak PW, Li RK, Dhillon B, Mickle DA. Endothelin antagonism and interleukin-6 inhibition attenuate the proatherogenic effects of C-reactive protein. Circulation 2002;105:1890-1896.

(341) Verma S, Badiwala MV, Weisel RD, Li SH, Wang CH, Fedak PW, Li RK, Mickle DA. C-reactive protein activates the nuclear factor-kappaB signal transduction pathway in saphenous vein endothelial cells: implications for atherosclerosis and restenosis. J Thorac Cardiovasc Surg 2003;126:1886-1891.

(342) Verma S, Kuliszewski MA, Li SH, Szmitko PE, Zucco L, Wang CH, Badiwala MV, Mickle DA, Weisel RD, Fedak PW, Stewart DJ, Kutryk MJ. C-reactive protein attenuates endothelial progenitor cell survival, differentiation, and function: further evidence of a mechanistic link between C-reactive protein and cardiovascular disease. Circulation 2004;109:2058-2067.

(343) Yeh ETH. CRP as a mediator of disease. Circulation 2004;109:II-11-II-14.

(344) Ford ES. The metabolic syndrome and C-reactive protein, fibrinogen, and leukocyte count: findings from the Third National Health and Nutrition Examination Survey. Atherosclerosis 2003;168:351-358.

(345) Ridker PM, Buring JE, Cook NR, Rifai N. C-reactive protein, the metabolic syndrome, and risk of incident cardiovascular events: an 8-year follow-up of 14 719 initially healthy American women. Circulation 2003;107:391-397.

(346) Haffner SM, Hanley AJ, Festa A, Wagenknecht LE, D'Agostino RB, Jr. Prediction of type 2 diabetes (DM) using alternate definitions of the metabolic syndrome (MetS): how useful is the addition of C-reacive protein (CRP)? Diabetes 2004;53:A244 [abstract].

(347) Moreno PR, Fuster V. New aspects in the pathogenesis of diabetic atherothrombosis. J Am Coll Cardiol 2004;44:2293-2300.

(348) Grundy SM, Hansen B, Smith SC, Jr., Cleeman JI, Kahn RA, for conference participants. Clinical management of metabolic syndrome: report of the American Heart Association/National Heart, Lung, and Blood Institute/American Diabetes Association conference on scientific issues related to management. Circulation 2004;109:551-556.

(349) Prabhakaran D, Anand SS. The metabolic syndrome: an emerging risk state for cardiovascular disease. Vasc Med 2004;9:55-68.

(350) Unger RH. Lipotoxicity. In: LeRoith DL, Taylor SI, Olefsky JM, editors. Diabetes Mellitus: A Fundamental And Clinical Text. Philadelphia, PA: Lippincott Williams & Wilkins, 2004: 141-149.

(351) Pierce GN, Maddaford TG, Russell JC. Cardiovascular dysfunction in insulin-dependent and non-insulin-dependent animal models of diabetes mellitus. Can J Physiol Pharmacol 1997;75:343-350.

(352) Russell JC, Graham SE. The JCR:LA-cp rat: an animal model of obesity and insulin resistance with spontaneous cardiovascular disease. In: Sima A, Shafrir E, editors. Animal Models of Diabetes. A Primer. Amsterdam: Harwood Academic Publishers, 2001: 227-246.

(353) Brindley DN, Russell JC. Animal models of insulin resistance and cardiovascular disease: some therapeutic approaches using JCR:LA-cp rat. Diabetes Obes Metab 2002;4:1-10.

(354) Russell JC. Reduction and prevention of the cardiovascular sequelae of the insulin resistance syndrome. Curr Drug Targets Cardiovasc Haematol Disord 2001;1:107-120.

(355) Shafrir E. Contribution of pathophysiology of animal diabetes to the understanding of human diabetes. In: Raz I, Skyler J, Shafrir E, editors. London: Martin Dunitz, 2003: 511-545.

(356) Reitman ML. Rodent genetic models of obesity and type 2 diabetes mellitus. In: LeRoith DL, Taylor SI, Olefsky JM, editors. Diabetes Mellitus: A Fundamental And Clinical Text. Philadelphia, PA: Lippincott Williams & Wilkins, 2004: 1041-1058.

(357) Russell JC, Koeslag DG, Amy RM, Dolphin PJ. Myocardial disease and catecholamine metabolism in JCR:LA-corpulent rat. Exp Mol Pathol 1991;54:31-40.

(358) Russell JC, Koeslag DG, Amy RM, Dolphin PJ. Independence of myocardial disease in the JCR:LA-corpulent rat on plasma cholesterol concentration. Clin Invest Med 1991;14:288-295.

(359) Russell JC, Amy RM, Graham SE, Dolphin PJ, Wood GO, Bar-Tana J. Inhibition of atherosclerosis and myocardial lesions in the JCR:LA-cp rat by beta, beta'-tetramethylhexadecanedioic acid (MEDICA 16). Arterioscler Thromb Vasc Biol 1995;15:918-923.

(360) Russell JC, Dolphin PJ, Graham SE, Amy RM. Cardioprotective and hypolipidemic effects of nisoldipine in the JCR:LA-cp rat. J Cardiovasc Pharmacol 1997;29:586-592.

(361) Maddaford TG, Russell JC, Pierce GN. Postischemic cardiac performance in the insulin-resistant JCR:LA-cp rat. Am J Physiol 1997;273:H1187-H1192.

(362) Russell JC, Graham SE, Richardson M. Cardiovascular disease in the JCR:LA-cp rat. Mol Cell Biochem 1998;188:113-126.

(363) Russell JC, Graham SE, Amy RM, Dolphin PJ. Cardioprotective effect of probucol in the atherosclerosis-prone JCR:LA-cp rat. Eur J Pharmacol 1998;350:203-210.

(364) Russell JC, Graham SE, Amy RM, Dolphin PJ. Inhibition of myocardial lesions in the JCR:LA-corpulent rat by captopril. J Cardiovasc Pharmacol 1998;31:971-977.

(365) O'Brien SF, Russell JC, Davidge ST. Vascular wall dysfunction in JCR:LA-cp rats: effects of age and insulin resistance. Am J Physiol 1999;277:C987-C993.

(366) Brunner F, Russell JC, Wslkart G, Wascher TC. Microvascular dysfunction and myocardial contractility in the JCR : LA-corpulent rat. Circulation 1999;100:554.

(367) Koletsky S. Animal model: obese hypertensive rat. Am J Pathol 1975;81:463-466.

(368) Hansen CT. Two new cogenic rat strains for nutrition and obesity research. Fed Proc 1983;42:537.

(369) Wu-Peng XS, Chua SC, Jr., Okada N, Liu SM, Nicolson M, Leibel RL. Phenotype of the obese Koletsky (*f*) rat due to Tyr763Stop mutation in the extracellular domain of the leptin receptor (*Lepr*): evidence for deficient plasma-to-CSF transport of leptin in both the Zucker and Koletsky obese rat. Diabetes 1997;46:513-518.

(370) Ahima RS, Osei SY. Leptin signaling. Physiol Behav 2004;81:223-241

(371) Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM. Positional cloning of the mouse obese gene and its human homologue. Nature 1994;372:425-432.

(372) Ren J. Leptin and hyperleptinemia - from friend to foe for cardiovascular function. J Endocrinol 2004;181:1-10.

(373) Schwartz MW, Seeley RJ, Campfield LA, Burn P, Baskin DG. Identification of targets of leptin action in rat hypothalamus. J Clin Invest 1996;98:1101-1106.

(374) McArthur MD, Graham SE, Russell JC, Brindley DN. Exaggerated stress-induced release of nonesterified fatty acids in JCR:LA-corpulent rats. Metabolism 1998;47:1383-1390.

(375) Schneider DJ, Absher PM, Neimane D, Russell JC, Sobel BE. Fibrinolysis and atherogenesis in the JCR:LA-cp rat in relation to insulin and triglyceride concentrations in blood. Diabetologia 1998;41:141-147.

(376) Dolphin PJ, Amy RM, Russell JC. Effect of age on serum lipids and lipoproteins of male and female JCR:LA-corpulent rats. Biochim Biophys Acta 1990;1042:99-106.

(377) Misra T, Gilchrist JS, Russell JC, Pierce GN. Cardiac myofibrillar and sarcoplasmic reticulum function are not depressed in insulin-resistant JCR:LA-*cp* rats. Am J Physiol 1999;276:H1811-H1817.

(378) Misra T, Russell JC, Clark TA, Pierce GN. $Mg^{2+}$-dependent ATPase activity in cardiac myofibrils from the insulin-resistant JCR:LA-*cp* rat. In: Angel A, Dhalla N, Pierce G, Singal P, editors. Diabetes and Cardiovascular Disease: Etiology, Treatment, and Outcomes. New York, NY: Kluwer Academic/Plenum Publishers, 2001: 247-252.

(379) Solymoss BC, Bourassa MG, Lesperance J, Levesque S, Marcil M, Varga S, Campeau L. Incidence and clinical characteristics of the metabolic syndrome in patients with coronary artery disease. Coron Artery Dis 2003;14:207-212.

(380) Gaziano JM, Manson JE, Ridker PM. Primary and secondary prevention of coronary heart disease. In: Zipes D, Libby P, Bonow RO, Braunwald E, editors. Braunwald's Heart Disease. Philadelphia: W. B. Saunders, 2005: 1057-1084.

(381) Antman EM, Anbe DT, Armstrong PW, Bates ER, Green LA, Hand M, Hochman JS, Krumholz HM, Kushner FG, Lamas GA, Mullany CJ, Ornato JP, Pearle DL, Sloan MA, Smith SC, Jr. ACC/AHA guidelines for the management of patients with ST-elevation myocardial infarction: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines. (Committee to Revise the 1999 Guidelines for the Management of Patients With Acute Myocardial Infarction) 2004;Available at www.acc.org/clinical/guidelines/stemi/index.pdf. Accessed on November 29, 2004.

(382) Hearse DJ, Sutherland FJ. Experimental models for the study of cardiovascular function and disease. Pharmacol Res 2000;41:597-603.

(383) Gad SC. Principles of screening and drug design. Safety Pharmacology in Pharmaceutical Development and Approval. Boca Raton, FL: CRC Press, 2004: 23-42.

(384) Taylor RP, Starnes JW. Age, cell signalling and cardioprotection. Acta Physiol Scand 2003;178:107-116.

(385) Hurtado C, Pierce GN. Sodium-hydrogen exchange inhibition: pre-versus post-ischemic treatment. Basic Res Cardiol 2001;96:312-317.

(386) Litwin SE, Katz SE, Morgan JP, Douglas PS. Serial echocardiographic assessment of left ventricular geometry and function after large myocardial infarction in the rat. Circulation 1994;89:345-354.

(387) Schwarz ER, Pollick C, Meehan WP, Kloner RA. Evaluation of cardiac structures and function in small experimental animals: transthoracic, transesophageal, and intraventricular echocardiography to assess contractile function in rat heart. Basic Res Cardiol 1998;93:477-486.

(388) Coatney RW. Ultrasound imaging: principles and applications in rodent research. ILAR J 2001;42:233-247.

(389) Hoit BD. Echocardiographic assessment of the mouse heart and aorta. In: Hoit BD, Walsh RA, editors. Cardiovascular Physiology in the Genetically Engineered Mouse. Kluwer Academic Publishers, 2002: 177-190.

(390) Wu JC, Nasseri BA, Bloch KD, Picard MH, Scherrer-Crosbie M. Influence of sex on ventricular remodeling after myocardial infarction in mice. J Am Soc Echocardiogr 2003;16:1158-1162.

(391) Otto CM. Left and right ventricular systolic function. Textbook of Clinical Echocardiography. Philadelphia: W.B. Saunders, 2004: 131-165.

(392) Gottdiener JS, Bednarz J, Devereux R, Gardin J, Klein A, Manning WJ, Morehead A, Kitzman D, Oh J, Quinones M, Schiller NB, Stein JH, Weissman NJ. American Society of Echocardiography recommendations for use of echocardiography in clinical trials. J Am Soc Echocardiogr 2004;17:1086-1119.

(393) Oh JK, Seward JB, Tajik AJ. The Echo Manual. Philadelphia, PA: Lippincott-Raven, 1999.

(394) Amy RM, Dolphin PJ, Pederson RA, Russell JC. Atherogenesis in two strains of obese rats. The fatty Zucker and LA/N-corpulent. Atherosclerosis 1988;69:199-209.

(395) Shafrir E. Diabetes in animals: contribution to the understanding of diabetes by study of its etiopathology in animal models. In: Porte DJ, Sherwin RS, Baron A, editors. Ellenberg & Rifkini's Diabetes Mellitus. New York: McGraw-Hill, 2003: 231-255.

(396) Material Safety Data Sheet for SEA0400. Taisho Pharmaceutical Co ,Ltd 2001.

(397) Perkins L, Peer C, Murphy-Hackley P. The use of mini-osmotic pumps in contiuous infusion studies. In: Healing G, Smith D, editors. Handbook of Preclinical Continuous Intravenous Infusion. London: Taylor & Francis, 2003: 265-284.

(398) ALZET® osmotic pump - overview. http://www alzet com/products/overview php 2004; accessed on December 12, 2004.

(399) Watson LE, Sheth M, Denyer RF, Dostal DE. Baseline echocardiographic values for adult male rats. J Am Soc Echocardiogr 2004;17:161-167.

(400) de Simone G, Wallerson DC, Volpe M, Devereux RB. Echocardiographic measurement of left ventricular mass and volume in normotensive and hypertensive rats. Necropsy validation. Am J Hypertens 1990;3:688-696.

(401) Otto CM. Transthoracic views, normal anatomy, and flow patterns. Textbook of Clinical Echocardiography. Philadelphia: Elsevier Saunders, 2004: 30-67.

(402) Litwin SE, Katz SE, Weinberg EO, Lorell BH, Aurigemma GP, Douglas PS. Serial echocardiographic-Doppler assessment of left ventricular geometry and function in rats with pressure-overload hypertrophy. Chronic angiotensin-converting enzyme inhibition attenuates the transition to heart failure. Circulation 1995;91:2642-2654.

(403) Scherrer-Crosbie M, Steudel W, Ullrich R, Hunziker PR, Liel-Cohen N, Newell J, Zaroff J, Zapol WM, Picard MH. Echocardiographic determination of risk area size in a murine model of myocardial ischemia. Am J Physiol 1999;277:H986-H992.

(404) Sahn DJ, DeMaria A, Kisslo J, Weyman A. Recommendations regarding quantitation in M-mode echocardiography: results of a survey of echocardiographic measurements. Circulation 1978;58:1072-1083.

(405) Schiller NB, Shah PM, Crawford M, DeMaria A, Devereux R, Feigenbaum H, Gutgesell H, Reichek.N, Sahn D, Schnittger I, Silverman NH, Tajik J. Recommendations for quantitation of the left ventricle by two-dimensional echocardiography. J Am Soc Echocardiogr 1989;2:358-367.

(406) de Simone G, Wallerson DC, Volpe M, Devereux RB. Echocardiographic measurement of left ventricular mass and volume in normotensive and hypertensive rats. Necropsy validation. Am J Hypertens 1990;3:688-696.

(407) Derumeaux G, Mulder P, Richard V, Chagraoui A, Nafeh C, Bauer F, Henry JP, Thuillez C. Tissue Doppler imaging differentiates physiological from pathological pressure-overload left ventricular hypertrophy in rats. Circulation 2002;105:1602-1608.

(408) Quinones MA, Otto CM, Stoddard M, Waggoner A, Zoghbi WA. Recommendations for quantification of Doppler echocardiography: a report from the Doppler quantification task force of the nomenclature and standards committee of the American Society of Echocardiography. J Am Soc Echocardiogr 2002;15:167-184.

(409) Hassard TH. Designing an experimental study. Understanding Biostatistics. St. Louis, Missouri: Mosby Year Book, 1991: 132-166.

(410) Langendorff O. Untersuchungen am uberlebenden Saugethierherzen. Pflugers Arch fur die Gesamte Physiologie Menschen der Tiere 1895;61:291-332.

(411) Ytrehus K. The ischemic heart--experimental models. Pharmacol Res 2000;42:193-203.

(412) Singh RB, Chohan PK, Dhalla NS, Netticadan T. The sarcoplasmic reticulum proteins are targets for calpain action in the ischemic-reperfused heart. J Mol Cell Cardiol 2004;37:101-110.

(413) Schafer C, Ladilov Y, Inserte J, Schafer M, Haffner S, Garcia-Dorado D, Piper HM. Role of the reverse mode of the $Na^+/Ca^{2+}$ exchanger in reoxygenation-induced cardiomyocyte injury. Cardiovasc Res 2001;51:241-250.

(414) Colorimetric assay for cytotoxicity. Product No. LK 100. Oxford, Michigan: 2001.

(415) Nagpurkar A, Mookerjea S. A novel phosphorylcholine-binding protein from rat serum and its effect on heparin-lipoprotein complex formation in the presence of calcium. J Biol Chem 1981;256:7440-7446.

(416) Technical data sheet: rat C-reactive protein (CRP) ELISA kit. BD Biosciences 2003;Canada.

(417) Hassard TH. Comparing two groups. Understanding Biostatistics. St. Louis, Missouri: Mosby Year Book, 1991: 52-74.

(418) Bolton S, Bon C. Pharmaceutical Statistics. Practical and Clinical Applications. 4th ed. New York, NY: Marcel Dekker, Inc, 2004.

(419) Hassard TH. Analysis of variance. Understanding Biostatistics. St. Louis, Missouri: Mosby Year Book, 1991: 75-97.

(420) OriginLab Corporation. Version 7.5 Getting Started Manual. ORIGIN® Scientific Graphing and Analysis Software. Northampton,MA: 2003.

(421) d'Uscio LV, Kilo J, Lüscher TF, Gassmann M. Circulation. In: Krinke GJ, editor. The Laboratory Rat. San Diego, CA: Academic Press, 2000: 345-357.

(422) Clark TA, Pierce GN. Cardiovascular complications of non-insulin-dependent diabetes: the JCR:LA-cp rat. J Pharmacol Toxicol Methods 2000;43:1-10.

(423) Wallace AM, McMahon AD, Packard CJ, Kelly A, Shepherd J, Gaw A, Sattar N. Plasma leptin and the risk of cardiovascular disease in the West of Scotland Coronary Prevention Study (WOSCOPS). Circulation 2001;104:3052-3056.

(424) Russell JC. Of mice and men, rats and atherosclerosis. Cardiovasc Res 2003;59:810-811.

(425) Hassard TH. Relationship between continuous measurements. Understanding Biostatistics. St. Louis, Missouri: Mosby Year Book, 1991: 121-151.

(426) Czubryt MP, Russell JC, Sarantopoulos J, Gilchrist JS, Pierce GN. Age- and sex-related differences in nuclear lipid content and nucleoside triphosphatase activity in the JCR:LA-cp corpulent rat. Mol Cell Biochem 1997;176:327-335.

(427) Fontanarosa, P.B. Units of measure. In: Iverson CC, editor. American Medical Association Manual of Style: A Guide for Authors and Editors. Baltimore: Williams & Wilkins, 1998: 479-509.

(428) O'Brien SF, Davidge ST, Zhang Y, Russell JC. Protection of vascular wall function in insulin-resistant rats from copper oxidative stress. Br J Pharmacol 2001;133:477-484.

(429) Diaz PN, Bleeker WK, Lubbers Y, Rigter GM, Van Mierlo GJ, Daha MR, Hack CE. Rat C-reactive protein activates the autologous complement system. Immunology 2003;109:564-571.

(430) Rassouli M, Sambasivam H, Azadi P, Dell A, Morris HR, Nagpurkar A, Mookerjea S, Murray RK. Derivation of the amino acid sequence of rat C-reactive protein from cDNA cloning with additional studies on the nature of its dimeric component. J Biol Chem 1992;267:2947-2954.

(431) Gabay C, Kushner I. Acute-phase proteins and other systemic responses to inflammation. New Engl J Med 1999;340:448-454.

(432) Ford ES, Giles WH, Myers GL, Mannino DM. Population distribution of high-sensitivity C-reactive protein among US men: findings from National Health and Nutrition Examination Survey 1999-2000. Clin Chem 2003;49:686-690.

(433) Myers MA, Fleck A. Observations on the delay in onset of the acute phase protein response. Br J Exp Pathol 1988;69:169-176.

(434) Colley CM, Fleck A, Goode AW, Muller BR, Myers MA. Early time course of the acute phase protein response in man. J Clin Pathol 1983;36:203-207.

(435) Kragsbjerg P, Holmberg H, Vikerfors T. Serum concentrations of interleukin-6, tumour necrosis factor-alpha, and C-reactive protein in patients undergoing major operations. Eur J Surg 1995;161:17-22.

(436) Kushner I, Broder ML, Karp D. Control of the acute phase response. Serum C-reactive protein kinetics after acute myocardial infarction. J Clin Invest 1978;61:235-242.

(437) Yen-Watson B, Kushner I. Rabbit CRP response to endotoxin administration: dose-response relationship and kinetics. Proc Soc Exp Biol Med 1974;146:1132-1136.

(438) Collins KA, Korcarz CE, Lang RM. Use of echocardiography for the phenotypic assessment of genetically altered mice. Physiol Genomics 2003;13:227-239.

(439) Oh JK, Appleton CP, Hatle LK, Nishimura RA, Seward JB, Tajik AJ. The noninvasive assessment of left ventricular diastolic function with two-dimensional and Doppler echocardiography. J Am Soc Echocardiogr 1997;10:246-270.

(440) Barouch LA, Berkowitz DE, Harrison RW, O'Donnell CP, Hare JM. Disruption of leptin signaling contributes to cardiac hypertrophy independently of body weight in mice. Circulation 2003;108:754-759.

(441) Pedersen OESM. Genetics of human obesity. In: LeRoith DL, Taylor SI, Olefsky JM, editors. Diabetes Mellitus: A Fundamental And Clinical Text. Philadelphia, PA: Lippincott Williams & Wilkins, 2004: 827-838.

(442) Sader S, Nian M, Liu P. Leptin: a novel link between obesity, diabetes, cardiovascular risk, and ventricular hypertrophy. Circulation 2003;108:644-646.

(443) Rajapurohitam V, Gan XT, Kirshenbaum LA, Karmazyn M. The obesity-associated peptide leptin induces hypertrophy in neonatal rat ventricular myocytes. Circ Res 2003;93:277-279.

(444) Xu FP, Chen MS, Wang YZ, Yi Q, Lin SB, Chen AF, Luo JD. Leptin induces hypertrophy via endothelin-1-reactive oxygen species pathway in cultured neonatal rat cardiomyocytes. Circulation 2004;110:1269-1275.

(445) Purdham DM, Zou MX, Rajapurohitam V, Karmazyn M. The rat heart is a site of leptin production and action. Am J Physiol Heart Circ Physiol 2004;287:H2877-H2884.

(446) Mizushige K, Yao L, Noma T, Kiyomoto H, Yu Y, Hosomi N, Ohmori K, Matsuo H. Alteration in left ventricular diastolic filling and accumulation of myocardial collagen at insulin-resistant prediabetic stage of a type II diabetic rat model. Circulation 2000;101:899-907.

(447) Fredersdorf S, Thumann C, Ulucan C, Griese DP, Luchner A, Riegger GAJ, Kromer EP, Weil J. Myocardial hypertrophy and enhanced left ventricular contractility in Zucker diabetic fatty rats. Cardiovascular Pathology 2004;13:11-19.

(448) Sasson Z, Rasooly Y, Bhesania T, Rasooly I. Insulin resistance is an important determinant of left ventricular mass in the obese. Circulation 1993;88:1431-1436.

(449) Lorell BH, Carabello BA. Left ventricular hypertrophy: pathogenesis, detection, and prognosis. Circulation 2000;102:470-479.

(450) Iacobellis G, Ribaudo MC, Zappaterreno A, Vecci E, Tiberti C, Di MU, Leonetti F. Relationship of insulin sensitivity and left ventricular mass in uncomplicated obesity. Obes Res 2003;11:518-524.

(451) Chinali M, Devereux RB, Howard BV, Roman MJ, Bella JN, Liu JE, Resnick HE, Lee ET, Best LG, de SG. Comparison of cardiac structure and function in American Indians with and without the metabolic syndrome (the Strong Heart Study). Am J Cardiol 2004;93:40-44.

(452) Wong CY, Moore-Sullivan T, Leano R, Byrne N, Beller E, Marwick TH. Alterations of left ventricular myocardial characteristics associated with obesity. Circulation 2004;110:3081-3087.

(453) McKendrick JD, Salas E, Dube GP, Murat J, Russell JC, Radomski MW. Inhibition of nitric oxide generation unmasks vascular dysfunction in insulin-resistant, obese JCR:LA-cp rats. Br J Pharmacol 1998;124:361-369.

(454) Roth DM, Swaney JS, Dalton ND, Gilpin EA, Ross J, Jr. Impact of anesthesia on cardiac function during echocardiography in mice. Am J Physiol Heart Circ Physiol 2002;282:H2134-H2140.

(455) Shibutani M. Anesthesia, artificial ventilation and perfusion fixation. In: Krinke GJ, editor. The Laboratory Rat. San Diego: Academic Press, 2000: 511-521.

(456) Maurer MS, Spevack D, Burkhoff D, Kronzon I. Diastolic dysfunction: can it be diagnosed by Doppler echocardiography? J Am Coll Cardiol 2004;44:1543-1549.

(457) Bauer BS, Ghahary A, Scott PG, Iwashina T, Demare J, Russell JC, Tredget EE. The JCR:LA-cp rat: a novel model for impaired wound healing. Wound Repair Regen 2004;12:86-92.

(458) Ogawa H, Hattori M. Regulation mechanisms of hair growth. Curr Probl Dermatol 1983;11:159-170.

(459) Blouin RA, Warren GW. Pharmacokinetic considerations in obesity. J Pharm Sci 1999;88:1-7.

(460) Ford ES, Giles WH, Mokdad AH. The distribution of 10-year risk for coronary heart disease among U.S. adults: findings from the National Health and Nutrition Examination Survey III. J Am Coll Cardiol 2004;43:1791-1796.

(461) Palmer BS, Hadziahmetovic M, Veci T, Angelos MG. Global ischemic duration and reperfusion function in the isolated perfused rat heart. Resuscitation 2004;62:97-106.

(462) Yen MH, Topol EJ. Microcirculatory dysfunction in acute myocardial infarction: evaluation, management, and treatment. In: Topol EJ, editor. Acute Coronary Syndromes. New York,NY: Marcel Dekker, 2005: 615-633.

(463) Braunwald E, Kloner RA. Myocardial reperfusion: a double-edged sword? J Clin Invest 1985;76:1713-1719.

(464) Gaasch WH, Bernard SA. The effect of acute changes in coronary blood flow on left ventricular end-diastolic wall thickness. An echocardiographic study. Circulation 1977;56:593-598.

(465) Turschner O, D'hooge J, Dommke C, Claus P, Verbeken E, De Scheerder I, Bijnens B, Sutherland GR. The sequential changes in myocardial thickness and thickening which occur during acute transmural infarction, infarct reperfusion and the resultant expression of reperfusion injury. Eur Heart J 2004;25:794-803.

(466) Kaul S, Ito H. Microvasculature in acute myocardial ischemia: part II: evolving concepts in pathophysiology, diagnosis, and treatment. Circulation 2004;109:310-315.

(467) Sutherland FJ, Hearse DJ. The isolated blood and perfusion fluid perfused heart. Pharmacol Res 2000;41:613-627.

(468) Richardson M, Schmidt AM, Graham SE, Achen B, DeReske M, Russell JC. Vasculopathy and insulin resistance in the JCR:LA-cp rat. Atherosclerosis 1998;138:135-146.

(469) Curtis MJ. Characterisation, utilisation and clinical relevance of isolated perfused heart models of ischaemia-induced ventricular fibrillation. Cardiovasc Res 1998;39:194-215.

(470) Lopaschuk GD, Russell JC. Myocardial function and energy substrate metabolism in the insulin-resistant JCR:LA corpulent rat. J Appl Physiol 1991;71:1302-1308.

(471) Kato R, Foex P. Myocardial protection by anesthetic agents against ischemia-reperfusion injury: an update for anesthesiologists. Can J Anaesth 2002;49:777-791.

(472) Cope DK, Impastato WK, Cohen MV, Downey JM. Volatile anesthetics protect the ischemic rabbit myocardium from infarction. Anesthesiology 1997;86:699-709.

(473) Mathur S, Karmazyn M. Interaction between anesthetics and the sodium-hydrogen exchange inhibitor HOE 642 (cariporide) in ischemic and reperfused rat hearts. Anesthesiology 1997;87:1460-1469.

(474) Kersten JR, Schmeling TJ, Pagel PS, Gross GJ, Warltier DC. Isoflurane mimics ischemic preconditioning via activation of $K_{ATP}$ channels: reduction of myocardial infarct size with an acute memory phase. Anesthesiology 1997;87:361-370.

(475) Roscoe AK, Christensen JD, Lynch C, III. Isoflurane, but not halothane, induces protection of human myocardium via adenosine A1 receptors and adenosine triphosphate-sensitive potassium channels. Anesthesiology 2000;92:1692-1701.

(476) Li F, Hayes JK, Wong KC, Szakacs J. Administration of sevoflurane and isoflurane prior to prolonged global ischemia improves heart function in isolated rat heart. Acta Anaesthesiol Sin 2000;38:113-121.

(477) Tanaka K, Weihrauch D, Kehl F, Ludwig LM, LaDisa JF, Jr., Kersten JR, Pagel PS, Warltier DC. Mechanism of preconditioning by isoflurane in rabbits: a direct role for reactive oxygen species. Anesthesiology 2002;97:1485-1490.

(478) Shi Y, Hutchins WC, Su J, Siker D, Hogg N, Pritchard KAJ, Keszler A, Tweddell JS, Baker JE. Delayed cardioprotection with isoflurane: role of reactive oxygen and nitrogen. Am J Physiol Heart Circ Physiol 2004;288:H175-H184.

(479) Obal D, Weber NC, Zacharowski K, Toma O, Dettwiler S, Wolter JI, Kratz M, Mullenheim J, Preckel B, Schlack W. Role of protein kinase C-ε (PKCε) in isoflurane-induced cardioprotection. Br J Anaesth 2005;94:166-173.

(480) Hu G, Salem MR, Crystal GJ. Isoflurane and sevoflurane precondition against neutrophil-induced contractile dysfunction in isolated rat hearts. Anesthesiology 2004;100:489-497.

(481) Chiari PC, Bienengraeber MW, Pagel PS, Krolikowski JG, Kersten JR, Warltier DC. Isoflurane protects against myocardial infarction during early reperfusion by activation of phosphatidylinositol-3-kinase signal transduction: evidence for anesthetic-induced postconditioning in rabbits. Anesthesiology 2005;102:102-109.

(482) Martini N, Preckel B, Thamer V, Schlack W. Can isoflurane mimic ischaemic preconditioning in isolated rat heart? Br J Anaesth 2001;86:269-271.

(483) Dworschak M, Breukelmann D, Hannon JD. Isoflurane applied during ischemia enhances intracellular calcium accumulation in ventricular myocytes in part by reactive oxygen species. Acta Anaesthesiol Scand 2004;48:716-721.

(484) Dworschak M, Breukelmann D, Hannon JD. The impact of isoflurane during simulated ischemia/reoxygenation on intracellular calcium, contractile function, and arrhythmia in ventricular myocytes. Anesth Analg 2004;99:1302-1307.

(485) Haroun-Bizri S, Khoury SS, Chehab IR, Kassas CM, Baraka A. Does isoflurane optimize myocardial protection during cardiopulmonary bypass? J Cardiothorac Vasc Anesth 2001;15:418-421.

(486) Wang X, Jarvinen O, Kuukasjarvi P, Laurikka J, Wei M, Rinne T, Honkonen EL, Tarkka M. Isoflurane produces only minor preconditioning in coronary artery bypass grafting. Scand Cardiovasc J 2004;38:287-292.

(487) Belhomme D, Peynet J, Louzy M, Launay JM, Kitakaze M, Menasche P. Evidence for preconditioning by isoflurane in coronary artery bypass graft surgery. Circulation 1999;100:II340-II344.

(488) Fellahi JL, Gue X, Philippe E, Riou B, Gerard JL. Isoflurane may not influence postoperative cardiac troponin I release and clinical outcome in adult cardiac surgery. Eur J Anaesthesiol 2004;21:688-693.

(489) Riess ML, Stowe DF, Warltier DC. Cardiac pharmacological preconditioning with volatile anesthetics: from bench to bedside? Am J Physiol Heart Circ Physiol 2004;286:H1603-H1607.

(490) Freedman BM, Hamm DP, Everson CT, Wechsler AS, Christian CM. Enflurane enhances postischemic functional recovery in the isolated rat heart. Anesthesiology 1985;62:29-33.

(491) Boutros A, Wang J, Capuano C. Isoflurane and halothane increase adenosine triphosphate preservation, but do not provide additive recovery of function after ischemia, in preconditioned rat hearts. Anesthesiology 1997;86:109-117.

(492) Stowe DF, Kevin LG. Cardiac preconditioning by volatile anesthetic agents: a defining role for altered mitochondrial bioenergetics. Antioxid Redox Signal 2004;6:439-448.

(493) Hu G, Vinten-Johansen J, Salem MR, Zhao ZQ, Crystal GJ. Isoflurane inhibits neutrophil-endothelium interactions in the coronary circulation: lack of a role for adenosine triphosphate-sensitive potassium channels. Anesth Analg 2002;94:849-856.

(494) Ludwig LM, Weihrauch D, Kersten JR, Pagel PS, Warltier DC. Protein kinase C translocation and Src protein tyrosine kinase activation mediate isoflurane-induced preconditioning in vivo: potential downstream targets of mitochondrial adenosine triphosphate-sensitive potassium channels and reactive oxygen species. Anesthesiology 2004;100:532-539.

(495) Zaugg M, Lucchinetti E, Spahn DR, Pasch T, Schaub MC. Volatile anesthetics mimic cardiac preconditioning by priming the activation of mitochondrial $K_{ATP}$ channels via multiple signaling pathways. Anesthesiology 2002;97:4-14.

(496) Xu P, Wang J, Kodavatiganti R, Zeng Y, Kass IS. Activation of protein kinase C contributes to the isoflurane-induced improvement of functional and metabolic recovery in isolated ischemic rat hearts. Anesth Analg 2004;99:993-1000.

(497) Ludwig LM, Tanaka K, Eells JT, Weihrauch D, Pagel PS, Kersten JR, Warltier DC. Preconditioning by isoflurane is mediated by reactive oxygen species generated from mitochondrial electron transport chain complex III. Anesth Analg 2004;99:1308-1315.

(498) Nakae Y, Kwok WM, Bosnjak ZJ, Jiang MT. Isoflurane activates rat mitochondrial ATP-sensitive $K^+$ channels reconstituted in lipid bilayers. Am J Physiol Heart Circ Physiol 2003;284:H1865-H1871.

(499) Fujimoto K, Bosnjak ZJ, Kwok WM. Isoflurane-induced facilitation of the cardiac sarcolemmal $K_{ATP}$ channel. Anesthesiology 2002;97:57-65.

(500) Stadnicka A, Bosnjak ZJ. Isoflurane decreases ATP sensitivity of guinea pig cardiac sarcolemmal $K_{ATP}$ channel at reduced intracellular pH. Anesthesiology 2003;98:396-403.

(501) Blanck TJ, Haile M, Xu F, Zhang J, Heerdt P, Veselis RA, Beckman J, Kang R, Adamo A, Hemmings H. Isoflurane pretreatment ameliorates postischemic neurologic dysfunction and preserves hippocampal $Ca^{2+}$/calmodulin-dependent protein kinase in a canine cardiac arrest model. Anesthesiology 2000;93:1285-1293.

(502) Kapinya KJ, Lowl D, Futterer C, Maurer M, Waschke KF, Isaev NK, Dirnagl U. Tolerance against ischemic neuronal injury can be induced by volatile anesthetics and is inducible NO synthase dependent. Stroke 2002;33:1889-1898.

(503) Xiong L, Zheng Y, Wu M, Hou L, Zhu Z, Zhang X, Lu Z. Preconditioning with isoflurane produces dose-dependent neuroprotection via activation of adenosine triphosphate-regulated potassium channels after focal cerebral ischemia in rats. Anesth Analg 2003;96:233-237.

(504) Zheng S, Zuo Z. Isoflurane preconditioning induces neuroprotection against ischemia via activation of P38 mitogen-activated protein kinases. Mol Pharmacol 2004;65:1172-1180.

(505) Katz ES, Tunick PA, Rusinek H, Ribakove G, Spencer FC, Kronzon I. Protruding aortic atheromas predict stroke in elderly patients undergoing cardiopulmonary bypass: experience with intraoperative transesophageal echocardiography. J Am Coll Cardiol 1992;20:70-77.

(506) Davila-Roman VG, Barzilai B, Wareing TH, Murphy SF, Schechtman KB, Kouchoukos NT. Atherosclerosis of the ascending aorta. Prevalence and role as an independent predictor of cerebrovascular events in cardiac patients. Stroke 1994;25:2010-2016.

(507) Kronzon I, Tunick PA. Atheromatous disease of the thoracic aorta: pathologic and clinical implications. Ann Intern Med 1997;126:629-637.

(508) Tunick PA, Kronzon I. Protruding atherosclerotic plaque in the aortic arch of patients with systemic embolization: a new finding seen by transesophageal echocardiography. Am Heart J 1990;120:658-660.

(509) He KL, Dickstein M, Sabbah HN, Yi GH, Gu A, Maurer M, Wei CM, Wang J, Burkhoff D. Mechanisms of heart failure with well preserved ejection fraction in dogs following limited coronary microembolization. Cardiovasc Res 2004;64:72-83.

(510) Pignataro G, Tortiglione A, Scorziello A, Giaccio L, Secondo A, Severino B, Santagada V, Caliendo G, Amoroso S, Di Renzo G, Annunziato L. Evidence for a protective role played by the $Na^+/Ca^{2+}$ exchanger in cerebral ischemia induced by middle cerebral artery occlusion in male rats. Neuropharmacology 2004;46:439-448.

(511) Pignataro G, Gala R, Cuomo O, Tortiglione A, Giaccio L, Castaldo P, Sirabella R, Matrone C, Canitano A, Amoroso S, Di RG, Annunziato L. Two sodium/calcium exchanger gene products, NCX1 and NCX3, play a major role in the development of permanent focal cerebral ischemia. Stroke 2004;35:2566-2570.

(512) Annunziato L, Pignataro G, Di Renzo GF. Pharmacology of brain $Na^+/Ca^{2+}$ exchanger: from molecular biology to therapeutic perspectives. Pharmacol Rev 2004;56:633-654.

(513) Feinstein SB. The powerful microbubble: from bench to bedside, from intravascular indicator to therapeutic delivery system, and beyond. Am J Physiol Heart Circ Physiol 2004;287:H450-H457.

(514) Tsutsui JM, Grayburn PA, Xie F, Porter TR. Drug and gene delivery and enhancement of thrombolysis using ultrasound and microbubbles. Cardiol Clin 2004;22:299-312.

(515) Bekeredjian R, Grayburn PA, Shohet RV. Use of ultrasound contrast agents for gene or drug delivery in cardiovascular medicine. J Am Coll Cardiol 2005;45:329-335.

(516) Dijkmans PA, Juffermans LJ, Musters RJ, Van WA, Ten Cate FJ, Van GW, Visser CA, De JN, Kamp O. Microbubbles and ultrasound: from diagnosis to therapy. Eur J Echocardiogr 2004;5:245-256.

(517) Beeri R, Guerrero JL, Supple G, Sullivan S, Levine RA, Hajjar RJ. New efficient catheter-based system for myocardial gene delivery. Circulation 2002;106:1756-1759.

(518) Suzuki K, Murtuza B, Fukushima S, Smolenski RT, Varela-Carver A, Coppen SR, Yacoub MH. Targeted cell delivery into infarcted rat hearts by retrograde intracoronary infusion: distribution, dynamics, and influence on cardiac function. Circulation 2004;110:II-225.

(519) Alexandrov AV, Molina CA, Grotta JC, Garami Z, Ford SR, varez-Sabin J, Montaner J, Saqqur M, Demchuk AM, Moye LA, Hill MD, Wojner AW, the C, I. Ultrasound-enhanced systemic thrombolysis for acute ischemic stroke. N Engl J Med 2004;351:2170-2178.

(520) Luo H, Steffen W, Cercek B, Arunasalam S, Maurer G, Siegel RJ. Enhancement of thrombolysis by external ultrasound. Am Heart J 1993;125:1564-1569.

(521) Francis CW. Ultrasound-enhanced thrombolysis. Echocardiography 2001;18:239-246.

(522) Antman EM, Van de Werf F. Pharmacoinvasive therapy: the future of treatment for ST-elevation myocardial infarction. Circulation 2004;109:2480-2486.

(523) Mentzer RM, Jr., Jahania MS, Lasley RD. Myocardial protection. In: Cohn LH, Edmunds LH, Jr., editors. Cardiac Surgery in the Adult. New York: McGraw-Hill Education, 2003: 413-438.

(524) Gumina RJ, Jahangir A, Gross GJ, Terzic A. Cardioprotection: emerging pharmacotherapy. Expert Opin Pharmacother 2001;2:739-752.

(525) Zhao ZQ, Corvera JS, Halkos ME, Kerendi F, Wang NP, Guyton RA, Vinten-Johansen J. Inhibition of myocardial injury by ischemic postconditioning during reperfusion: comparison with ischemic preconditioning. Am J Physiol Heart Circ Physiol 2003;285:H579-H588.

(526) Kin H, Zhao ZQ, Sun HY, Wang NP, Corvera JS, Halkos ME, Kerendi F, Guyton RA, Vinten-Johansen J. Postconditioning attenuates myocardial ischemia-reperfusion injury by inhibiting events in the early minutes of reperfusion. Cardiovasc Res 2004;62:74-85.

(527) Yang XM, Proctor JB, Cui L, Krieg T, Downey JM, Cohen MV. Multiple, brief coronary occlusions during early reperfusion protect rabbit hearts by targeting cell signaling pathways. J Am Coll Cardiol 2004;44:1103-1110.

(528) Tsang A, Hausenloy DJ, Mocanu MM, Yellon DM. Postconditioning: a form of "modified reperfusion" protects the myocardium by activating the phosphatidylinositol 3-kinase-Akt pathway. Circ Res 2004;95:230-232.

(529) Yang XM, Philipp S, Downey JM, Cohen MV. Postconditioning's protection is not dependent on circulatin blood factors or cells but involves adenosine receptors and requires PI3-kinase and guanylyl cyclase activation. Basic Res Cardiol 2005;100:57-63.

(530) McFalls EO, Ward HB, Moritz TE, Goldman S, Krupski WC, Littooy F, Pierpont G, Santilli S, Rapp J, Hattler B, Shunk K, Jaenicke C, Thottapurathu L, Ellis N, Reda DJ, Henderson WG. Coronary-artery revascularization before elective major vascular surgery. New Engl J Med 2004;351:2795-2804.

(531) Mathur S, Farhangkhgoee P, Karmazyn M. Cardioprotective effects of propofol and sevoflurane in ischemic and reperfused rat hearts: role of $K_{ATP}$ channels and interaction with the sodium-hydrogen exchange inhibitor HOE 642 (cariporide). Anesthesiology 1999;91:1349-1360.

(532) Aye NN, Xue YX, Hashimoto K. Antiarrhythmic effects of cariporide, a novel $Na^+$-$H^+$ exchange inhibitor, on reperfusion ventricular arrhythmias in rat hearts. Eur J Pharmacol 1997;339:121-127.

(533) Aye NN, Komori S, Hashimoto K. Effects and interaction, of cariporide and preconditioning on cardiac arrhythmias and infarction in rat in vivo. Br J Pharmacol 1999;127:1048-1055.

(534) Xue YX, Aye NN, Hashimoto K. Antiarrhythmic effects of HOE642, a novel $Na^+$-$H^+$ exchange inhibitor, on ventricular arrhythmias in animal hearts. Eur J Pharmacol 1996;317:309-316.

(535) Zhu BM, Miyamoto S, Kamiya K, Komori S, Hashimoto K. Inhibitory effects of pre-ischemic and post-ischemic treatment with FR 168888, a $Na^+/H^+$ exchange inhibitor, on reperfusion-induced ventricular arrhythmias in anesthetized rat. Jpn J Pharmacol 2002;88:93-99.

(536) Iwai T, Tanonaka K, Inoue R, Kasahara S, Motegi K, Nagaya S, Takeo S. Sodium accumulation during ischemia induces mitochondrial damage in perfused rat hearts. Cardiovasc Res 2002;55:141-149.

(537) Hurtado C, Pierce GN. Sodium-hydrogen exchange inhibition: pre- versus post-ischemic treatment. Basic Res Cardiol 2001;96:312-317.

(538) Zhang S, Yuan JX, Barrett KE, Dong H. Role of $Na^+$-$Ca^{2+}$ exchange in regulating cytosolic $Ca^{2+}$ in cultured human pulmonary artery smooth muscle cells. Am J Physiol Cell Physiol 2005;288:C245-C252.

(539) Palandoken H, By K, Hegde M, Harley WR, Gorin FA, Nantz MH. Amiloride peptide conjugates: prodrugs for sodium-proton exchange inhibition. J Pharmacol Exp Ther 2005;312:961-967.

(540) Takano S, Kimura J, Ono T. Inhibition of aggregation of rabbit and human platelets induced by adrenaline and 5-hydroxytryptamine by KB-R7943, a $Na^+/Ca^{2+}$ exchange inhibitor. Br J Pharmacol 2001;132:1383-1388.

(541) Roberts DE, Bose R. Reverse mode $Na^+/Ca^{2+}$ exchange in the collagen activation of human platelets. Ann N Y Acad Sci 2002;976:345-349.

(542) Roberts DE, McNicol A, Bose R. Mechanism of collagen activation in human platelets. J Biol Chem 2004;279:19421-19430.

(543) Popma JJ, Berger P, Ohman EM, Harrington RA, Grines C, Weitz JI. Antithrombotic therapy during percutaneous coronary intervention: the Seventh ACCP Conference on Antithrombotic and Thrombolytic Therapy. Chest 2004;126:576S-599S.

(544) Dauerman HL, Sobel BE. Synergistic treatment of ST-segment elevation myocardial infarction with pharmacoinvasive recanalization. J Am Coll Cardiol 2003;42:646-651.

(545) Murphy GJ, Bryan AJ, Angelini GD. Hybrid coronary revascularization in the era of drug-eluting stents. Ann Thorac Surg 2004;78:1861-1867.

(546) Aggarwal K, Gupta V, Rajeev AG. Hybrid revascularization: another step forward in coronary revascularization. J Invasive Cardiol 2004;16:426-427.

(547) Chobanian AV, Bakris GL, Black HR, Cushman WC, Green LA, Izzo JL, Jr., Jones DW, Materson BJ, Oparil S, Wright JT, Jr., Roccella EJ. The Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure: the JNC 7 report. JAMA 2003;289:2560-2572.

(548) Kaplan NM. Systemic hypertension: mechanisms and diagnosis. In: Zipes D, Libby P, Bonow RO, Braunwald E, editors. Braunwald's Heart Disease: A Textbook of Cardiovascular Medicine. Philadelphia: W. B. Saunders, 2005: 959-987.

(549) Devereux RB, Wachtell K, Gerdts E, Boman K, Nieminen MS, Papademetriou V, Rokkedal J, Harris K, Aurup P, Dahlof B. Prognostic significance of left ventricular mass change during treatment of hypertension. JAMA 2004;292:2350-2356.

(550) Okin PM, Devereux RB, Jern S, Kjeldsen SE, Julius S, Nieminen MS, Snapinn S, Harris KE, Aurup P, Edelman JM, Wedel H, Lindholm LH, Dahlof B, for the LIFE Study Investigators. Regression of electrocardiographic left ventricular hypertrophy during antihypertensive treatment and the prediction of major cardiovascular events. JAMA 2004;292:2343-2349.

(551) Romero JR, Rivera A, Lanca V, Bicho MDP, Conlin PR, Ricupero DA. $Na^+/Ca^{2+}$ exchanger activity modulates CTGF mRNA expression in TGF-beta 1- and Des-$arg^{10}$-kallidin-stimulated myofibroblasts. J Biol Chem 2005;10.1074/jbc.M410052200. Published online February 9, 2005. Accessed February 28, 2005.

(552) Schneider JC, El Kebir D, Chereau C, Mercier JC, l'Ava-Santucci J, nh-Xuan AT. Involvement of $Na^+/Ca^{2+}$ exchanger in endothelial NO production and endothelium-dependent relaxation. Am J Physiol Heart Circ Physiol 2002;283:H837-H844.