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The effects of physiological estrogen replacement on the recovery of contractile function and energy metabolism following ischemia in the isolated working rat heart

by

Craig Alan Wilson 

A thesis submitted to the Faculty of Graduate Studies and Research in Partial fulfillment of the requirements for the degree of Master of Science

in

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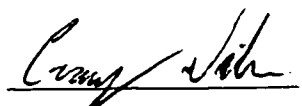

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled, *The effects of physiological estrogen replacement on the recovery of contractile function and energy metabolism following ischemia in the isolated working rat heart* by Craig Alan Wilson in partial fulfillment of the requirements for the degree of Master of Science in Medical Sciences – Oral Health Sciences.

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The journey is the reward

(Tao proverb)

Abstract

This thesis examines the effects of physiological estrogen replacement on the recovery of contractile function in the isolated working rat heart following global no-flow ischemia. Under aerobic perfusion conditions, contractile function was measured in hearts from ovariectomized rats and from ovariectomized rats given physiological estrogen replacement. Under these conditions, heart rate, aortic flow, cardiac output and cardiac work were greater in hearts from ovariectomized animals as compared to hearts from estrogen replaced rats. It was hypothesized that estrogen replacement would improve the functional recovery of isolated working rat hearts following global no-flow ischemia. It was further hypothesized that these improvements would be the result of a beneficial alteration in energy substrate metabolism. To test these hypotheses, recovery of contractile function following global no-flow ischemia was determined in hearts perfused without fatty acids as well as in hearts perfused with high concentrations of fatty acids (1.2 mmol palmitate). Preischemic and postischemic alterations in energy substrate metabolism were also determined in hearts perfused with high concentrations of fatty acids. It was concluded that, regardless of the presence or absence fatty acids, physiological estrogen replacement does not alter the recovery of contractile function or substrate energy metabolism following global no-flow ischemia.

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I gratefully recognize the thoughtful guidance provided by my supervisor Dr. D. J. Pehowich, without which my thesis would not have been so enjoyable.

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Abbreviations

ACC	Acetyl CoA carboxylase
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
H^+	Proton
BSA	Bovine serum albumin
CO_2	Carbon dioxide
CoA	Co-enzyme A
CPT I	Carnitine palmitoyl transferase I
CPT II	Carnitine palmitoyl transferase II
Cr	Creatine
CrP	Phosophocreatine
DCA	Dichloroacetate
EDTA	Ethylenediamine-tetraacetic acid
EGTA	Ethylene glycol-bis(b-aminoethyl ether)N,N,N',N'-tetaacetic acid
eNOS	Endothelial nitric oxide synthase
ER	Estrogen receptor
FADH	Flavin adenine dinucleotide
HPLC	High performance liquid chromatography
NADH	Nicotinamide adenine dinucleotide
NO	Nitric oxide
O_2	Oxygen
$O2^-$	Superoxide anion
PCA	Perchloric acid
PDC	Pyruvate dehydrogenase complex
PDH	Pyruvate dehydrogenase
PFK	Phosphofructokinase
SR	Sarcoplasmic reticulum
TCA	Tricarboxylic acid

Chapter I

Introduction

In North America, cardiovascular disease remains a leading cause of death for both men and women.[1,2,3,4] Although men suffer from an earlier onset of cardiovascular disease, women may still see a 30% reduction in the frequency of myocardial infarction after correcting for age differences.[5,6,7] Therefore, women benefit from some form of cardioprotection which is absent in men. At menopause women experience a reduction in the quantity of estrogen being produced by the ovaries. This reduction in circulating estrogen is associated with in an increased incidence of myocardial infarction which matches or exceeds that of men.[8] Clinical observations, however, have suggested that both pre-menopausal women and women receiving estrogen replacement are at a lower risk of mortality from cardiovascular disease.[8] The mechanisms by which estrogen may provide the observed cardioprotection are still unclear. Elucidation of this mechanism could potentially offer clinicians a valuable tool for both the prevention and treatment of cardiovascular disease in men and women. Furthermore, a mechanistic understanding of estrogenic cardioprotection may be especially beneficial for women who forego hormone replacement therapy because of a significant family history of breast cancer.

I) The Role of Estrogen in Cardioprotection

1.1) *Overview*

Although several mechanisms have been proposed, the exact mechanisms by which estrogen provides cardioprotection remains unclear. Although estrogen has been shown to improve the blood lipid profile by increasing the levels of circulating high density lipoproteins (HDL), it is estimated that this may only account for 20-30% of estrogen's beneficial effects.[5,9,10] It is therefore possible that other cardiovascular alterations, may account for the remaining 70-80% of estrogen's protective effects. These potential cardioprotective mechanisms of estrogen include, antioxidant actions, vascular modulation, direct myocyte alteration, and alterations of energy metabolism.

1.2) *Antioxidant Actions*

The structure of 17 β-estradiol is shown in figure I.1. Estrogen has been shown to act as an antioxidant, which may reduce damage to the vascular endothelium and cardiac tissue caused by oxygen free radicals such as $O_2^{\cdot -}$.[11,12] By directly increasing membrane stability and mimicking the membrane fluidity modulator cholesterol, estrogen may provide further vascular protection.[13] Estrogen has also been shown to prevent damage resulting from membrane lipid peroxidation.[14]

The role of estrogen *in vivo* remains even less clear. Levels of exhaled n-Pentane, a stable metabolic marker of lipid peroxidation have been reported to be lower in animals receiving estrogen replacement rather than a placebo.[15] In fact,

estrogen may even confer its antioxidant properties through the promotion of other antioxidative enzymes such as superoxide dismutase. Observations made on rats have suggested that non-estrogen replaced ovariectomy may lead to a decrease in superoxide dismutase and catalase.[16] Much work remains to be done before we more fully understand the antioxidant effects of estrogen. Furthermore, because of other estrogen mediated effects on the heart, it seem unlikely that estrogenic cardioprotection is solely the result of its antioxidant properties.

1.3) *Vascular Mediator*

Estrogen has several potential vascular effects including alterations to the nitric oxide synthase (NOS) and eicosanoid pathways. For example, vascular endothelial cells may be stimulated by the acute administration of estrogen to release the potent vasodilator, nitric oxide (NO).[17,18] Cardioprotection may be provided by the resultant relaxation of vascular smooth muscle and an increase in coronary blood flow. Furthermore, chronic administration of estrogen has been shown to result in the upregulation of endothelial nitric oxide synthase (eNOS), the enzyme catalyzes the formation of NO. [18,19] Although not directly linked to NO formation, a recent study has shown that acute estrogen administration may promote a reduction in infarct size resulting in less tissue necrosis in rabbits.[20] Because the heart is composed of a number of cell types besides vascular endothelial cells, cardioprotection stemming from estrogen is likely the result of a number of changes to a series of cell types including myocardial cells.

1.4) *Direct Effects on the Heart*

Estrogen may have direct effects on the heart. A recent study has demonstrated that the cardioprotective effect of estrogen from ischemia/reperfusion injury, may be the result of direct alterations of the cardiac myocyte which are independent from an increase in coronary flow.[21] This study further demonstrated that acute administration of estrogen resulted in no alterations in coronary flow and myocardial contractility.[21] Other studies, using acute pharmacological concentrations of estrogen, have reported contradictory effects. Using these high doses, acute administration of estrogen to Langendorff perfused rabbit hearts, has shown a decrease in contractility and coronary flow.[22] Isolated guinea-pig cardiac myocytes have shown a negative inotropic effect on cardiac contraction resulting from similar acute estrogen additions.[23] Both of these studies have suggested that estrogen may inhibit inward Ca^{2+} movement and reduce free intracellular Ca^{2+} concentrations.[22,23] The potential mechanisms by which estrogen directly influences cardiac myocytes remain unclear.

1.5) *Estrogen Receptors*

Estrogen may provide cardioprotection through transcriptional regulation of target genes. Much recent work has revolved around the identification and characterization of estrogen receptors. Estrogen receptors have been identified in cultured bovine and human endothelial cells, suggesting that estrogen may play a role in transcriptionally regulating such things as LDL metabolism and NO production.[24] Two distinct sub-types of estrogen receptors (ERα and ERβ) have recently been

identified.[25] The distribution and effects of these separate receptors throughout the body are for the most part unknown. As well, whether or not authentic estrogen receptors exist in the myocardium remains unclear. Preliminary results suggest that functional estrogen receptors do exist in the myocardium.[26] Indirect evidence for estrogen receptors also comes from estrogen mediated increases in mRNA coding for structural proteins in the rat myocardium.[27] It seems reasonable from these studies, and the ever increasing number of tissues found demonstrating estrogen receptors, that the myocardium also possesses estrogen receptors.

2) *Ischemia/Reperfusion Injury*

Cardiac ischemia occurs when there is an interruption of blood flow to the myocardium. During ischemia and immediately after, during reperfusion, cardiac muscle cells undergo several major changes. Depending on the duration of deprivation, the myocardium may fully recover, partially recover, or fail to recover normal function. This indicates that there may be a progressive transition from reversible to irreversible injury. Several mechanisms have been suggested for this transition.

Initially, as ischemia begins, cardiac muscle cells rely upon available adenosine triphosphate (ATP). As ischemia continues, cells develop a net energy deficit resulting from secondary means of energy production which are not as efficient as oxidative phosphorylation. The ATP/ADP ratio is a convenient way to express the approximate overall energy status of the cell. A decline in the ATP/ADP ratio has

been associated with several deleterious effects on numerous cellular ATP-dependent channels.[28]

After prolonged energy depletion, myocardial cells may be further damaged by reoxygenation. Recent studies suggest that resumption of energy production in the presence of high intracellular calcium can worsen cellular recovery.[29,30] This phenomenon is known as the "oxygen paradox". In myocardial cells, the sarcolema, the sarcoplasmic reticulum (SR), and mitochondria have demonstrated impaired ability to control intracellular Ca^{2+} movement in an ATP-depleted environment.[31,32,33,34] Some recently suggested methods of cardioprotection have focused on the relationship between ATP depletion and subsequent rises in intracellular Ca^{2+}.[35,36,37] A more thorough understanding of the role of physiological estrogen replacement on ATP production following ischemia is needed as it is a potential mechanism by which estrogen may confer cardioprotection.

3) *Myocardial Energy Substrate Metabolism*

3.1) *Overview*

A general overview of energy substrate metabolism is provided in Figure I.2. The adult heart is capable of fulfilling its energy requirements via several metabolic processes. Of these metabolic alternatives, fatty acid oxidation typically predominates with β oxidation supplying up to 70% of the energy used by the heart. The heart is also capable of metabolizing glucose via glycolysis and pyruvate oxidation, and lactate via pyruvate oxidation. A delicate balance and interdependence

exists between these forms of energy metabolism and profound alterations may result during pathophysiological events.

3.2) *Glycolysis*

The process of glycolysis is outlined in Figure I.3. Glycolysis is an anaerobic process of glucose metabolism which is shared by most living cells. During glycolysis, glucose is converted to 2 molecules of pyruvate through the intermediate of fructose-1,6-bisphophate. In addition, 2 ATP and 2 NADH are produced. The production of pyruvate is the result of 10 enzymatic reactions, with phosphofructokinase (PFK) acting as the rate-limiting enzyme. The fate of pyruvate is largely dependent upon the availability of oxygen, with pyruvate oxidation predominating when oxygen is readily available, and lactate production via glycolytic fermentation ensuing when oxygen is unavailable.

3.3) *Pyruvate Oxidation*

The citric acid cycle is detailed in Figure I.4. The citric acid cycle is much more complex than glycolysis, allowing several points of substrate entry and exit as well as several potential points of regulation. The citric acid cycle is oxygen dependent and reliant upon glycolysis for supply of lactate and pyruvate. Pyruvate is converted to acetyl-CoA and shuttled into the mitochondrial citric acid cycle via regulatory pyruvate dehydrogenase complex. Once in the citric acid cycle, acetyl-CoA is combined with oxaloacetate to form citrate. An ingenious series of enzymes then passes citrate through the citric acid cycle to produce 6 NADH, 2 $FADH_2$, and 2

ATP per glucose molecule, with oxaloacetate being recycled and ready for the next entering acetyl-CoA.

3.4) *β Oxidation*

β oxidation is detailed in Figure I.5. Fatty acids are converted by ATP-dependent acylation, to fatty acyl-CoA prior to being shuttled into the mitochondrial matrix by the carnitine palmitoyl transferases I and II (CTP I and CTP II). Once inside the mitochondria, fatty acids are sequentially oxidized at their β carbon to produce acetyl CoA, $FADH_2$, and NADH. The total number of acetyl CoA, $FADH_2$ and NADH produced is dependent upon the total number of carbons in the fatty acid being oxidized. For example, the oxidation of palmitate, which contains 16 carbons atoms produces 8 molecules of acetyl CoA and 7 molecules of both NADH and $FADH_2$. Ultimately, ATP may be generated with acetyl CoA entering the citric acid cycle, and NADH and $FADH_2$ donating their electrons in the electron transport chain.

3.5) *Electron Transport Chain*

The electron transport chain consists of electron acceptors and transporters which ultimately convert the energy bound in NADH and $FADH_2$ during the oxidation of glucose, fatty acid and lactate, to ATP. Electrons accepted by the electron transport chain are harnessed to drive the movement of H^+ from the matrix, across the inner mitochondrial membrane, into the intermembrane space. This creates a disequilibrium, or proton motive force, which in turn powers the movement of H^+ through the ATP-generating F_1F_0-ATPase. We must also remember that the electron

transport chain is an oxygen dependent process, as oxygen is the terminal electron acceptor.

4) *Regulation of Energy Substrate Metabolism*

The regulation of energy substrate metabolism is complex balance of energy requirements, substrate and product levels, enzyme activities, and hormone concentrations. As well as the above regulatory control, seemingly separate metabolic pathways can influence one another to add yet another layer of control. Perhaps the best way to understand some of the intricacies of metabolism is to first divide the various metabolic pathways and examine them separately.

The common first step of carbohydrate metabolism is glycolysis [see Stanley *et al* for review of carbohydrate metabolism.[39]]. As previously mentioned, under aerobic conditions glycolysis is largely controlled by the activity of PFK. In addition, PFK-2, a second isoform of PFK, has been recently described. At present, the distribution and importance of PFK-2 is not well understood. However, is has been well established that PFK is activated when AMP and ADP concentrations rise when energy demands increase, and inhibited when ATP and citrate levels rise indicating the energy demands have decreased. As well, epinephrine, norepinephrine, and glucagon regulate the concentrations of fructose-2,6-bisphosphate, which in turn can activate PFK. Elevations in intracellular Ca^{2+} concentrations is also thought to increase the activity of PFK.

Myocytes are capable of storing excess amounts of glucose as glycogen which is a multibranched polymer of glucose residues. Glycogen phosphorylase is the enzyme responsible for removing glucose residues and passing them into glycolysis, while the opposing action glycogen synthase, elongates glycogen chains. The activity of these enzymes is controlled reciprocally through phosphorylation /dephosphorylation reactions.

Pyruvate dehydrogenase (PDH) is the major regulatory enzyme mediating the oxidation of pyruvate. PDH is part of the larger pyruvate dehydrogenase complex (PDC), which is situated on the inner side of the mitochondrial membrane. In this position PDC can regulate the passage of cytosolic pyruvate in to the mitochondrial citric acid cycle as acetyl CoA. PDH is extensively regulated. In addition to substrate supply, regulation occurs via phosphorylation state, with pyruvate dehydrogenase phosphatase activating PDH through dephosphorylation and pyruvate dehydrogenase kinase inhibiting PDH by phosphorylation. PDH phophatase is also stimulated by Ca^{2+} and Mg^{2+}, while PDH kinase is stimulated by acetyl CoA and inhibited by pyruvate and ADP.

Once pyruvate enters the citric acid cycle, cycle activity is largely controlled through the enzymatic activity changes of citrate synthase, isocitrate dehydrogenase, and α-ketoglutarate dehydrogenase. These enzymes are responsive to changes in substrate availability and the feedback inhibition of cycle intermediates.

β oxidation of long chain fatty acids is the primary way in which the myocardium satisfies its energy demands [reviewed by Lopaschuk *et al.*[40]]. As previously mentioned, CPT I and CPT II are responsible for the translocation of fatty acyl CoA into the mitochondria. CPT I is primarily responsible for the translocation of long chain fatty acyl CoA and is therefore a major site for regulation of β oxidation. In turn, CPT I is regulated by malonyl CoA which is formed from acetyl CoA carboxylase (ACC) mediated carboxylation of acetyl CoA. The β oxidation pathway itself is responsive to changes in fatty acid concentrations which are subject to activity changes in triacylglycerol lipase found in adipose tissue. Through cAMP-mediated phosphorylation/dephosphorylation, triacylglycerol lipase can by stimulated by glucagon and epinephrine and inhibited by insulin.

As previously mentioned, an inter-regulation between the various metabolic pathways adds an additional layer of complexity. In the present study understanding interplay between carbohydrate and fatty acid metabolism is important (Figure I.6).[39] Following ischemia, circulating levels of fatty acids and fatty acid oxidation rates are elevated. When fatty acid concentrations exceed 0.8 mM, glucose uptake and oxidation will be inhibited. On the other hand, when concentrations of fatty acids fall below 0.3 mM glucose oxidation is stimulated. The fatty acid inhibition of glucose oxidation is the result of inactivation of PDC by elevated NADH and acetyl CoA, two high energy products of β oxidation. In turn, activation of PDC can occur when NADH and acetyl CoA concentrations are low.

5) *Uncoupling Theory of H^+ Production from Glucose Metabolism*

A schematic description of H^+ production from glucose metabolism is seen in Figure I.7. During glycolysis, the hydrolysis of ATP results in the production of $2H^+$ for each molecule of glucose metabolized. Under normal conditions, H^+ produced during glycolysis are used during the citric acid cycle, leaving a net H^+ production of zero.[41,42] However, during aerobic reperfusion following ischemia, the rate of the citric acid cycle decreases without a corresponding decrease in the rate of glycolysis, leading to excess H^+ production and intracellular acidosis.[42,43] As already described in Chapter I.4, this trend can be exacerbated when the isolated rat heart is perfused with high levels of fatty acid (>0.8 mM), resulting in inhibition of PDC and ultimately glucose oxidation.

The cell has several mechanisms by which to deal with acidosis. These typically include activation of the Na^+-H^+ exchanger, lactate-H^+ co-transporter, Na^+-HCO_3^-, and vacuolar H^+-ATPase. While addressing excess H^+ concentrations, the cell may increase its Na^+ concentrations. To reduce elevated Na^+ concentrations the cell may increase its Ca^{2+} concentration via utilization of Na^+-Ca^{2+} exchangers. Several studies have shown that significant increases in Na^+ and Ca^{2+} following ischemia can lead to impairment of contractile recovery.[44,45,46] Furthermore a disruption in either H^+ production or an impairment in the Na^+-H^+ have been shown to dramatically improve the recovery of contractile function following ischemia.[47]

6) *Study Rationale*

Cardiovascular disease is a leading cause of death in North America for both men and women. Clinical observations have suggested that pre-menopausal women and post-menopausal women receiving hormone replacement therapy have a greatly reduced mortality rate as compared to men and post-menopausal women not receiving estrogen replacement. While it has been shown that estrogen replacement may provide an improved blood lipid profile, it is believed that this may account for only 20-30% of the beneficial effects of estrogen. The remaining cardiovascular benefits of estrogen are not well understood and the mechanisms mediating the cardioprotective effects of estrogen are still unclear.

As previously discussed, substrate energy metabolism can have a profound effect on intracellular ionic homeostasis and ultimately, the recovery of myocardial contractile function following ischemia. While physiological estrogen replacement has been shown to lessen ischemic injury and improve post-ischemic contractile function, the underlying mechanism responsible remains obscured. Furthermore, while several studies have suggested that estrogen may reduce the inward Ca^{2+} current, no forthright mechanistic explanations have been offered. As well, no studies have examined the effects of physiological estrogen replacement on the isolated working heart perfused with a physiologically relevant concentration of fatty acids.

In light of these studies, I hypothesize that physiological estrogen replacement will improve the functional recovery of isolated hearts following global no-flow

ischemia. I further hypothesize that an improvement in contractile recovery will be the result of an improved substrate energy metabolism profile which reduces the overall H^+ production from glucose metabolism. If such conditions prevail, I suggest that cardiac efficiency (cardiac work/ O_2 consumed and cardiac work/TCA acetyl-CoA) will be greater in the hearts from rats receiving physiological estrogen replacement. Moreover, overall ATP concentrations and ATP/ADP ratio will be improved at the conclusion of reperfusion in hearts from estrogen replaced rats.

7) *Individual Study Outlines*

Chapter II examines the effects of physiological estrogen replacement on the functioning of isolated working rat hearts, both during aerobic conditions and during reperfusion following 20 minutes of global ischemia.

Chapter III investigates the effects of physiological estrogen replacement on energy metabolism and functional recovery of the isolated working rat heart following 25 minutes of global ischemia.

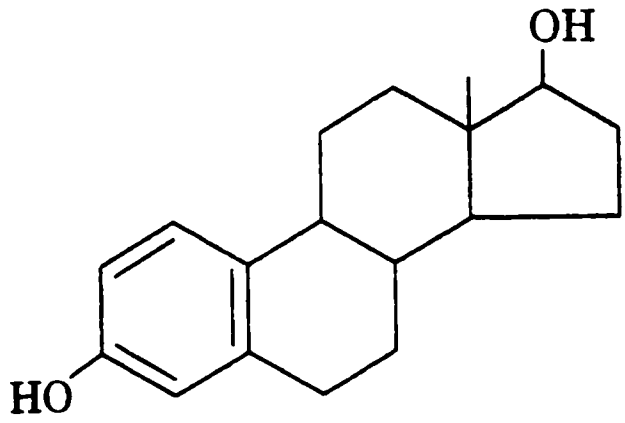

Figure I.1: Structure of 17 β-estradiol.

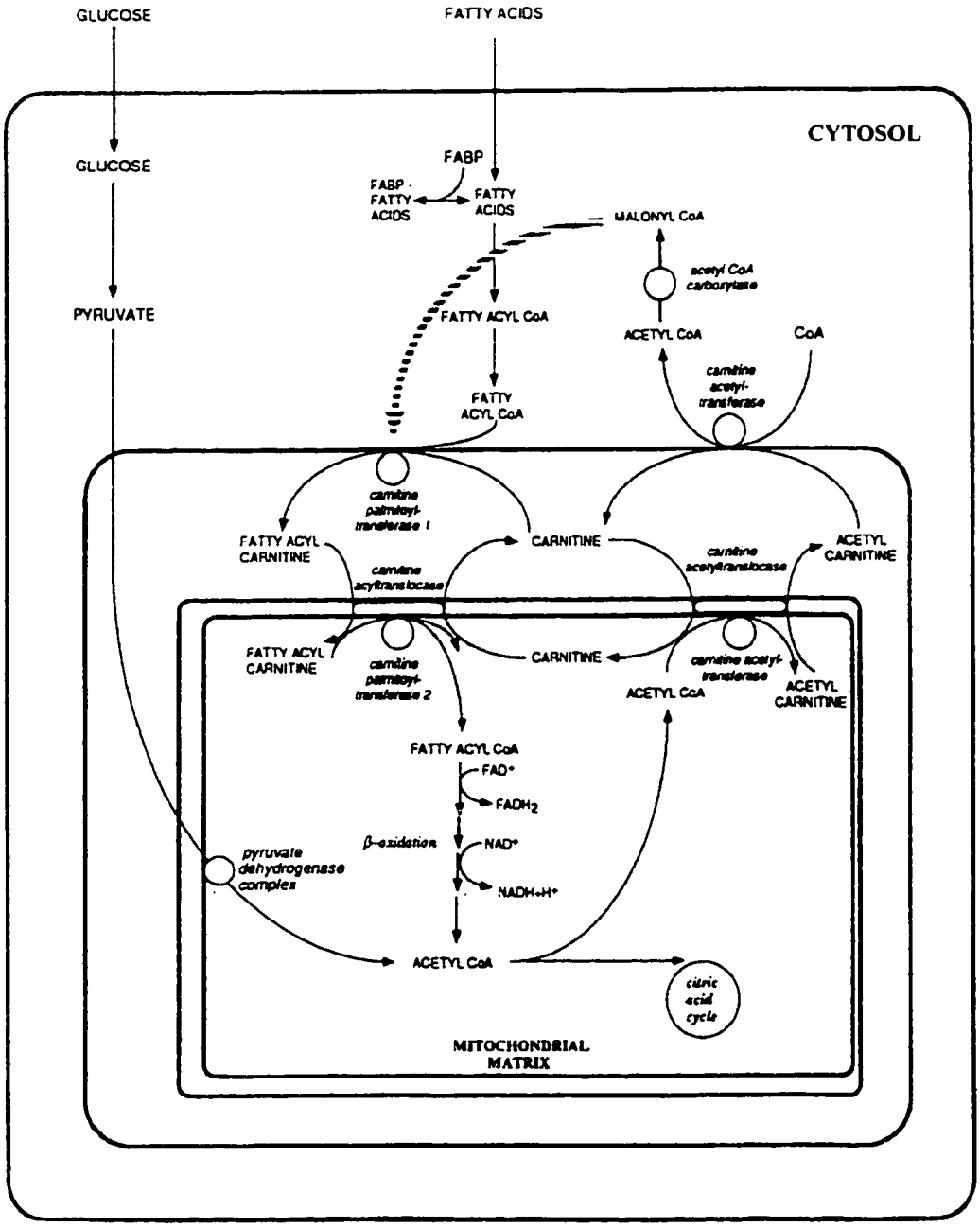

Figure I.2: Overview of cellular energy substrate metabolism.
Adapted from Lopaschuk GD, Belke DD, Gamble J, Itoi T, Schonekess BO. Regulation of fatty acid oxidation in the mammalian heart in health and disease. *Biochim Biophys Acta.* 1994;1213: 236-276.

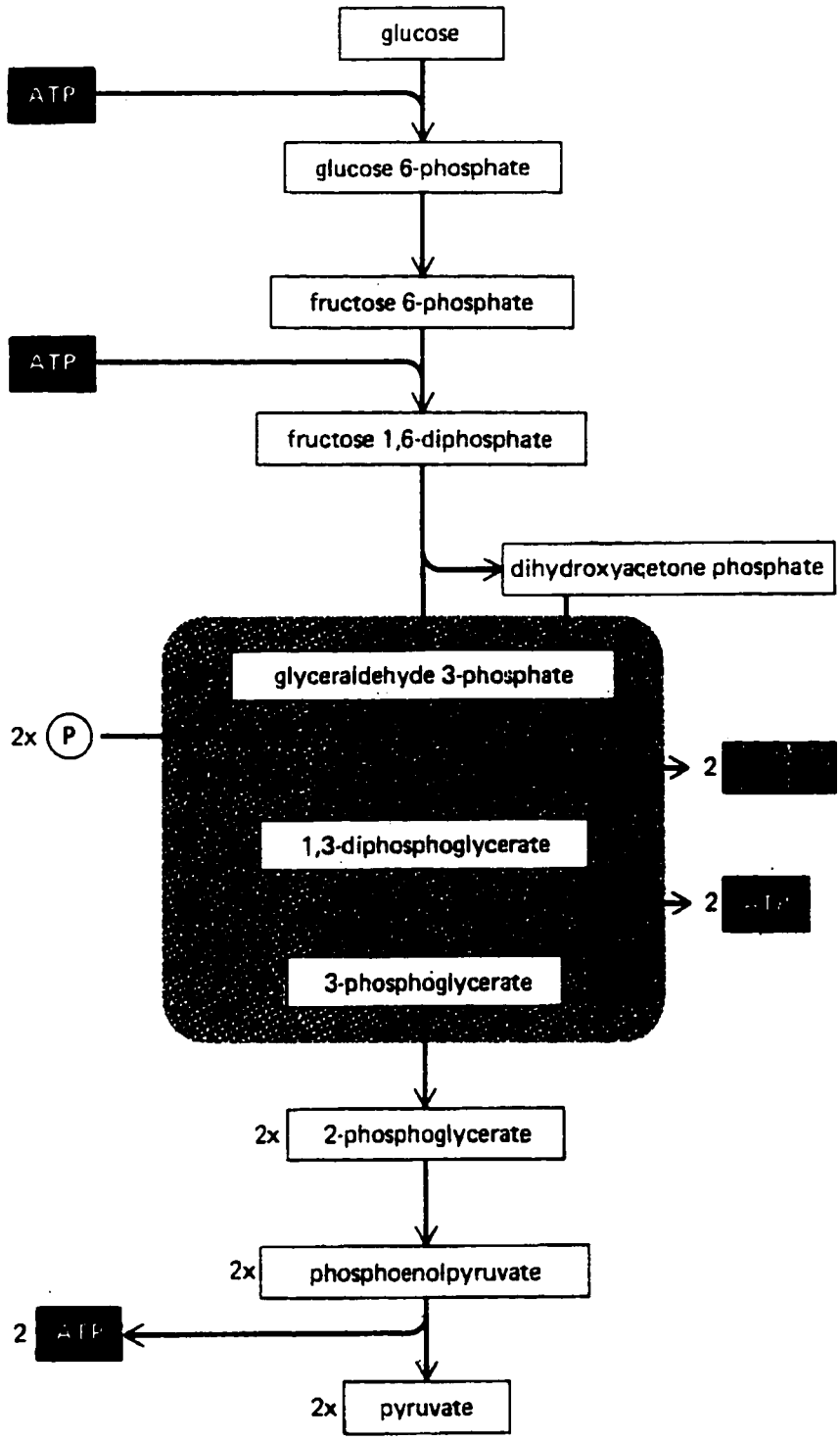

Figure I.3: Overview of glycolysis.
Adapted from Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson JD. Molecular biology of the cell, 2nd Edition. New York: Garland Publishing Inc. 1989.

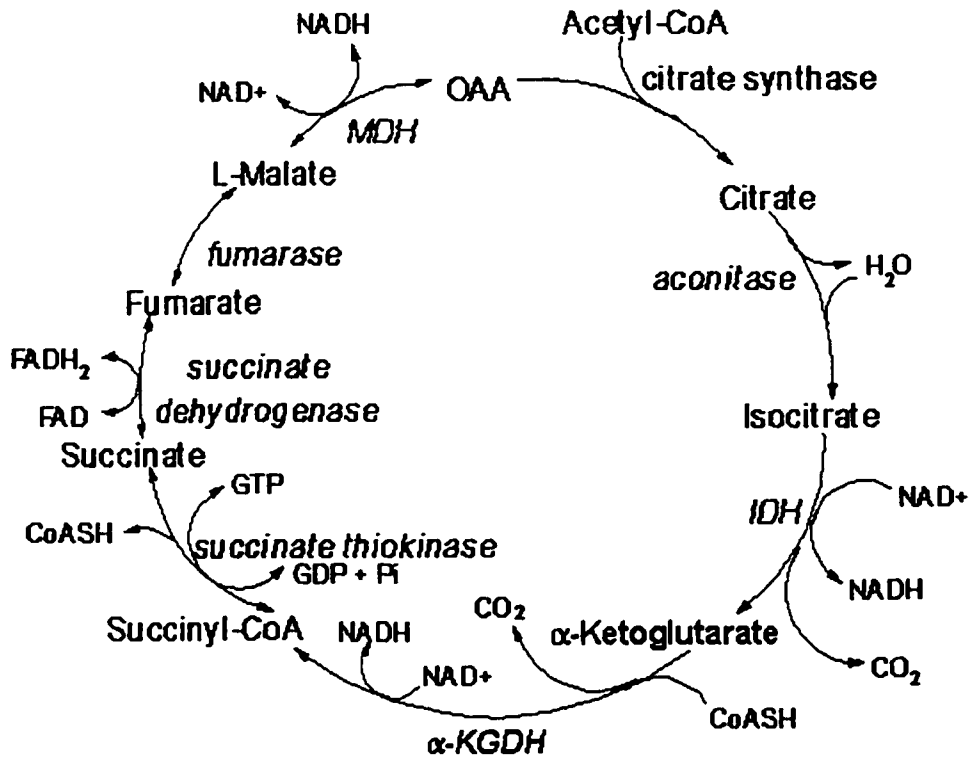

Figure I.4: Overview of the citric acid cycle.

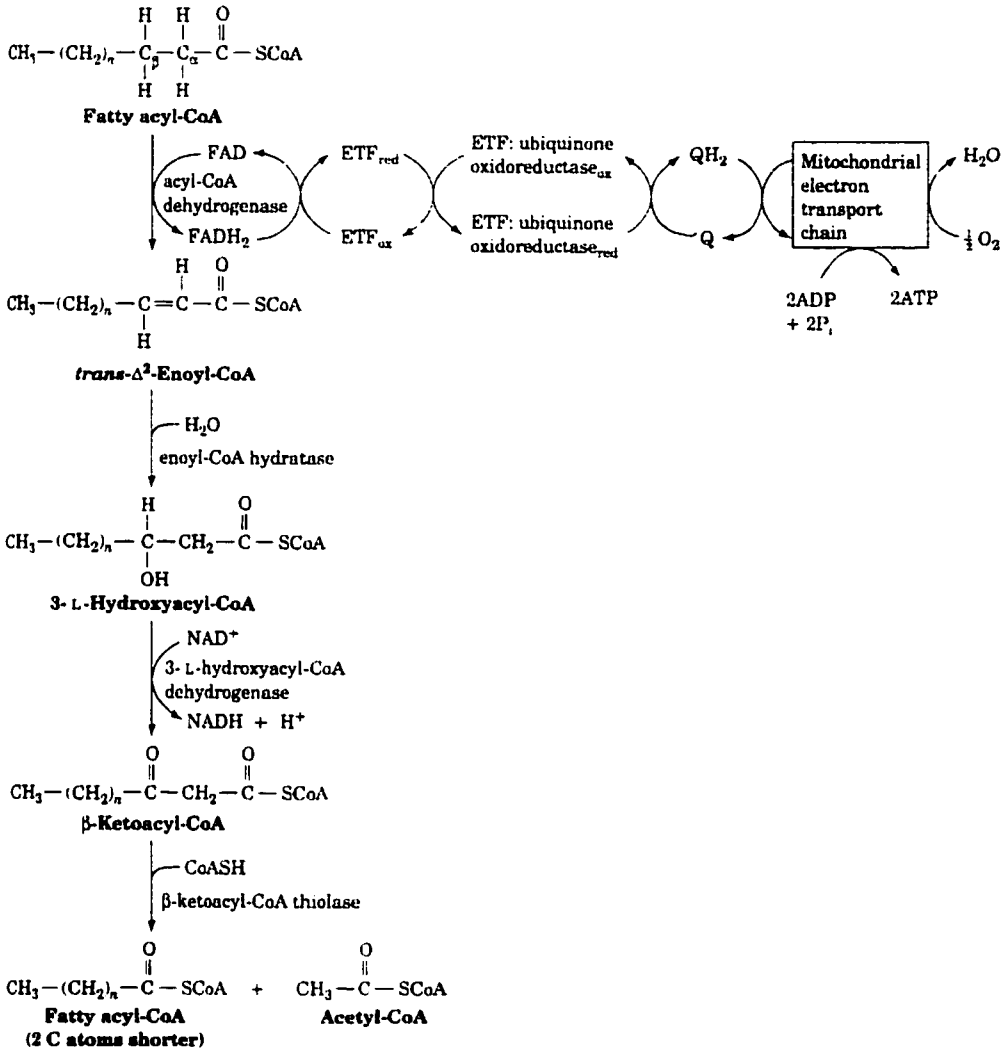

Figure I.5: Overview of βoxidation.
Adapted from Voet D & Voet JG. Biochemistry, 2nd Edition, Toronto: John Wiley & Sons, Inc. 1996.

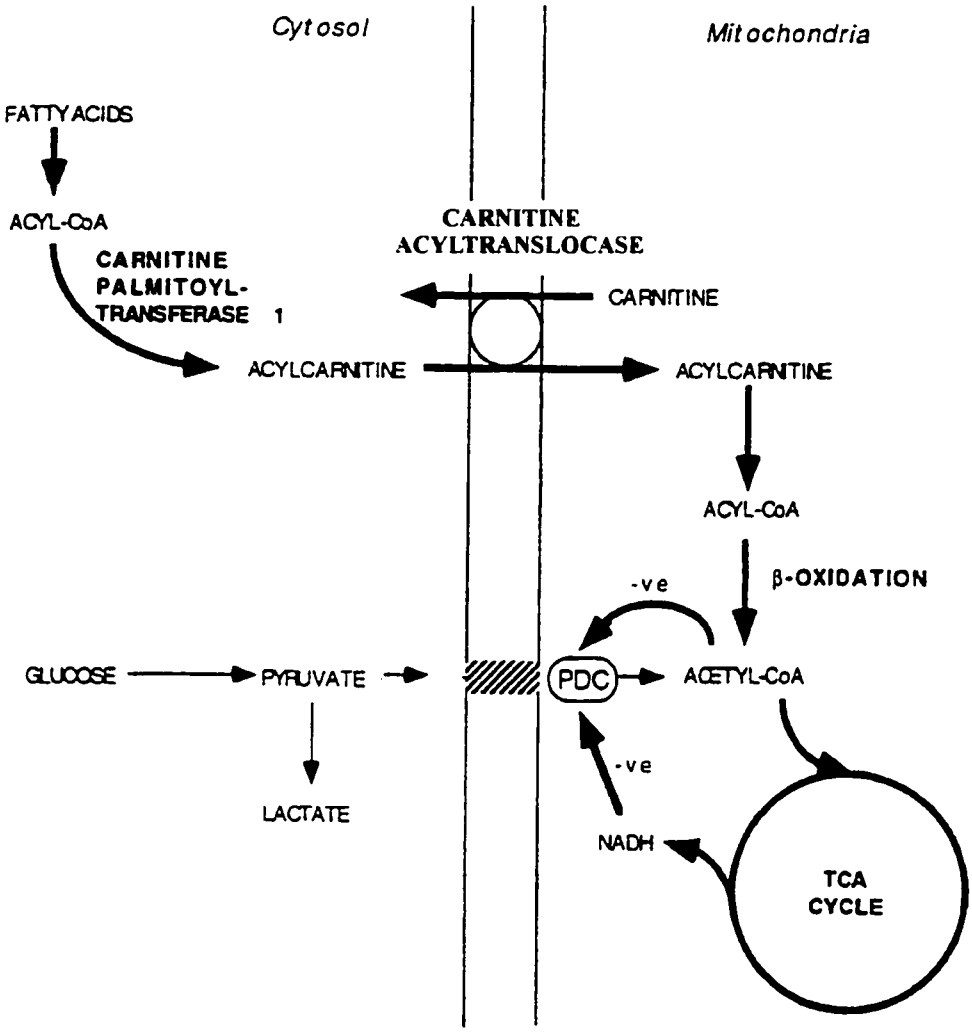

Figure I.6: Inhibition of glucose oxidation by fatty acid oxidation
Adapted from Belke DD, 1997, Hypothermia and heart metabolism

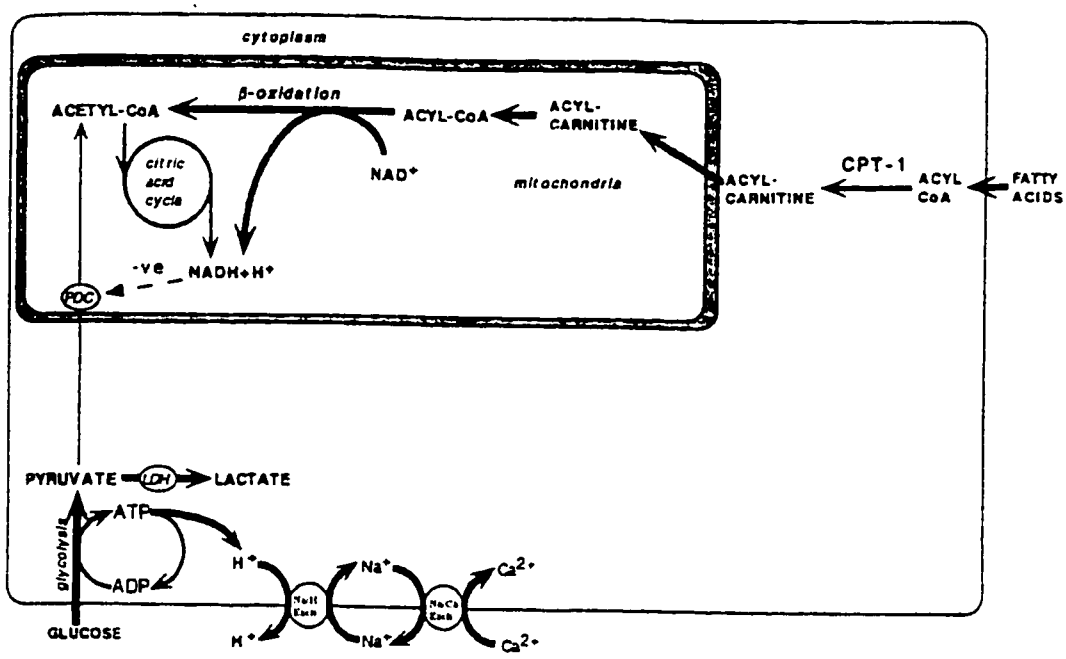

Figure I.7: H^+ production from an imbalance between glycolysis and glucose oxidation and its contribution to Ca^{2+} overload.
Adapted from Belke DD, 1997, Hypothermia and heart metabolism.

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Chapter II

Physiological Estrogen Replacement has No Effect on Functional Recovery of the Isolated Working Rat Heart Following Global Ischemia When Perfused Without Fatty Acids

Introduction

In North America, cardiovascular disease is a leading cause of death for both men and women.[1,2] Even after correcting for age differences, women may benefit from a 30% reduction in the frequency of myocardial infarction compared to men.[3] During menopause women experience a reduction in the quantity of estrogen being produced by the ovaries. This reduction has been associated with an increased incidence of myocardial infarction which matches or exceeds that of men.[4,5] Additional clinical evidence further suggests a cardioprotective role for estrogen by showing that both premenopausal women and women receiving estrogen replacement continue to be at a lower risk of heart disease.[6,7,8] While clinical evidence continues to accumulate, the underlying mechanism responsible for estrogenic cardioprotection remains unclear. Elucidation of this mechanism could potentially offer clinicians a valuable tool with which to prevent heart disease in men and an alternative to hormone replacement therapy in women with a high risk of breast cancer.

To date several estrogen mediated cardioprotective mechanisms have been proposed. Firstly, estrogen replacement therapy has been shown to have beneficial effects on the blood lipid profile by increasing the levels of circulating high density

lipoproteins (HDL).[9] These benefits however, have been speculated to account for only 20-30% of estrogen mediated effects, possibly leaving cardiovascular alterations to account for the remaining 70-80%.[10] Secondly, estrogen has been shown to act as an antioxidant, reducing the damage to the vascular endothelium caused by oxygen free radicals such as O_2^-.[11,12] Although, much less is known about the role of estrogen *in vivo*, a recent study reported a reduction in exhaled n-Pentane, a stable metabolic marker for peroxidation, in dogs receiving estrogen replacement compared to dogs receiving a placebo.[13] Given our knowledge of other estrogen mediated actions it seems unlikely that its antioxidant activity can solely account for estrogenic cardioprotection.

While the above mechanisms may occur without direct myocyte involvement, several mechanism have been proposed which propose direct estrogenic effects on the myocardium. Although current evidence for myocardial estrogen receptors is sparse, indirect evidence for authentic myocardial estrogen receptors has been demonstrated by estrogen mediated increases in mRNA coding for structural proteins in the rat myocardium.[14] While preliminary results have suggested estrogen receptors do exist in the myocardium, two distinct subtypes of estrogen receptor have been recently identified, ERα and ERβ.[15,16] The distribution and importance of ERα and ERβ in the myocardium remains largely unknown. On the basis of these studies it seems reasonable to suggest that the myocardium is a likely site for estrogen receptors and ultimately some form of estrogen mediation.

The most suggestive evidence for estrogenic cardioprotection comes from a recent study, demonstrating physiological estrogen replacement in the ovariectomized rat could protect myocardial contractile function following global ischemia.[17] Although this study reported no benefit following 15 minutes of ischemia, it did demonstrate that physiological estrogen benefited hearts which were subjected to 5 minutes of ischemic preconditioning prior to 20 minutes of global ischemia. This indicates physiological estrogen replacement may not protect against mild ischemic episodes and that it may play a role in the more complex cardioprotective actions of preconditioning.

Due to our limited understanding of the mechanism of action by which estrogen provides cardioprotection, it is reasonable to further investigate the effects of physiological estrogen replacement on functional recovery following ischemia. I hypothesize that physiological estrogen replacement will improve functional recovery in the isolated working rat heart following 20 minutes of global no-flow ischemia. In the present study, following a preliminary series of aerobic perfusions, we determined if physiological estrogen replacement was capable of protecting the myocardium following 20 minutes of global no-flow ischemia. Cardiac output, aortic flow, coronary flow, peak systolic pressure, developed pressure, and cardiac work were used as a basis of comparison. The selected metabolites of ATP, ADP, Cr, and CrP were also compared at the conclusion of reperfusion to assess potential gross metabolic alterations.

Materials and Methods

II.1a) *Animals*

Animals were cared for in accordance with the Canadian Council on Animal Care guidelines. Virgin female Sprauge-Dawley rats (250-275g) purchased from Charles River Laboratories (Montreal, Quebec, Canada) were housed under constant temperature and humidity conditions with a 12 hour light cycle. Animals were fed water and a standard rat pellet diet (Rat Chow™) *ad libitum.*

II.1b) *Materials*

21-day sustained release 17 β-estradiol pellets were purchase from Innovative Research Inc.

II.1c) *Serum Estradiol Levels*

Rats were randomly divided into two groups and bilaterally ovariectomized. At time of ovariectomy the estrogen replacement group (E_2) was implanted with a subcutaneous 21-day release pellet containing either 0.1 or 0.5 mg of 17 β-estradiol designed to maintain estrogen levels in the physiological range. A dose of 0.1 mg was used in the aerobic studies while 0.5 mg doses were used in the ischemia study The non-estrogen replaced group (OVX) received a subcutaneous placebo pellet at time of ovariectomy. Ovariectomy and pellet implantation was performed under respired Halothane™ (Halocarbon Laboratories. River Edge, New Jersey, United States of America) anesthesia at a dose of 0.075 L/min. Using a blood sample taken at time of

heart excision, serum estrogen levels were determined using a double antibody radioimmuno-assay kit (Diagnostic Products Corp., Montreal, Quebec, Canada)

II.1d) *Isolated Heart perfusion*

Two perfusion protocols were used. An initial series of aerobic perfusions were following by a separate series of ischemia/reperfusion experiments. Perfusate constituents and perfusion protocols are described in Figure II.1 (panels A and B). Briefly, 21 days after ovariectomy, rats were anesthetized with sodium pentobarbitol and hearts were rapidly removed and placed in ice-cold Krebs-Henseleit solution. Using a 37 ^{o}C oxygenated (95% O_2, 5% CO_2) Krebs-Henseleit solution, initial retrograde perfusion at a hydrostatic pressure 60 mmHg was begun following cannulation of the aorta. During this time, the buffer solution was not recirculated, allowing the heart to be cleansed of blood and excess tissue. During the initial 15 minutes of Langendorf perfusion the pulmonary artery and the left atrium were cannulated. Hearts were placed in working mode by clamping the aortic Langendorf inflow line and opening the atrial preload line. The preload line delivered oxygenated perfusate at hydrostatic pressure of 11.5 mmHg. Perfusate ejected from spontaneously beating hearts entered a compliance chamber before passing through an afterload line set at a hydrostatic pressure of 80 mmHg. All working hearts were perfused with a modified Krebs-Henseleit buffer containing 2.5 mmol/L free Ca^{2+}, 11 mmol/L glucose, and 100 μU/mL insulin.

Heart perfusion protocol for the ischemia/reperfusion study is seen in figure II.1 (Panel B). Hearts were initially aerobically perfused for 30 minutes. Global no-flow ischemia was begun by clamping both the atrial inflow and the aortic outflow lines. Following 20 minutes of global no-flow ischemia the atrial inflow and aortic outflow lines were reopened to allow for an additional 45 minutes of aeorbic reperfusion.

Hearts were allowed to beat spontaneously throughout the experiments. A Gould pressure transducer in the aortic outflow line was used to measure heart rate and aortic pressure. Timed, manual collection of perfusate exiting the pulmonary artery and afterload line were used to determine coronary flow and aortic flow respectively. Cardiac output was calculated as the sum of coronary and aortic flows.

Following reperfusion, heart ventricles were rapidly frozen using Wollenberger clamps cooled to the temperature of liquid nitrogen. Dry atrial tissue weight was determined after oven drying at 100 °C for 12 hours. Frozen ventricular tissue was powdered in a mortar and pestle cooled to the temperature of liquid nitrogen. A portion of this powdered tissue was weighed both frozen and once dried to determine the wet-to-dry ratio. Using the dry atria weight, the frozen ventricle weight, and the wet-to-dry ratio, the total dry weight of the heart was calculated

Perfusion protocol for the aerobic perfusion study, is seen in Figure II.1 (Panel A). The same perfusate was used in both the aerobic and ischemia/reperfusion

studies. Hearts were aerobically perfused for 105 minutes prior to being frozen and weighed as previously described.

II.1e) *Tissue Nucleotide Measurements*

Nucleotides were extracted from frozen ventricular tissue (100±10 mg) using the following technique. Tissue was homogenized on ice with 1.0 ml 6% PCA/0.5 mM EGTA. The homogenate was then centrifuged at 11,000 rpm for 2 minutes prior to addition of 95 µl of 0.32 DTT. Following be neutralized using 5 M K_2CO_3 to pH 6.8±0.5, samples were centrifuged at 11,000 rpm for 2 minutes. The supernatant was then collected and immediately frozen in liquid nitrogen for subsequent nucleotide determination.

As described previously, 100µl of prepared supernatant was analyzed by HPLC.[18] Briefly, nucleotides, AMP degradation products, PCr and Cr were resolved with 35mM K_2HPO_4, 6mM tetrabutyl-ammonium hydrogen sulfate buffer (pH 6.0), and a binary acetonitrile gradient on medium-bore, 250 • 3.9mm steel octadecyl-bonded (C18) columns at a flow rate of 1.5 or 1.0 ml • min^{-1}. A Beckman System Gold® software package was used to integrate peaks.

II.1f) *Statistical Analysis*

All values are mean±SEM. An unpaired Student's *t* test was used to determine differences between the single point variables of weight and the perfusion time points of preischemia and postischemia. One-way ANOVA was used to compare

differences in preischemic and postischemic values between treatment groups of OVX and E_2. Two-way ANOVA was used to compare the treatment groups of OVX and E_2 at the corresponding time of perfusion. Whenever necessary a Bonferroni post-hoc test was performed. A value of $P < .05$ was considered significant.

Results

II.2) *Preliminary aerobic study*

II.2a) *The effect of ovariectomy and physiological estrogen replacement on body weight, heart weight and serum estradiol levels during preliminary study.*

Table II.1 details the effect of physiological estrogen replacement on body weight, heart weight and serum estradiol levels during the aerobic study. Although initial body weight was similar between treatment groups, following 21 days of estrogen replacement, body weight increased 31.4±1.3% in the OVX group and 9.6±0.5% in the E_2 group. This corresponded to a 18.7±0.4% increase in the OVX over the E_2 group. Dry heart weight, determined at the conclusion of the perfusion experiments was not different between groups. Serum estradiol levels were approximately 3.5 fold greater in the E_2 group as compared to the OVX group, with values of 71.1±20 and 19.7±2.9 pg/ml respectively.

II.2b) *The effect of physiological estrogen replacement on mechanical function during aerobic perfusion*

The effect of physiological estrogen replacement on heart rate and cardiac output during the course of aerobic perfusion are seen in Figure II.2. Heart rate was greater in the OVX as group compared to the E_2 group throughout the 105 minutes of aerobic perfusion. Cardiac output was also greater in the OVX at 15, 30 and 45

minutes of perfusion even though both groups demonstrated a decreasing trend over the entire perfusion time course. Figure II.3 details the effects of physiological estrogen replacement on peak systolic pressure and developed pressure over 105 minutes of aerobic perfusion. Although no differences were seen between the OVX and E_2 treatment groups, both groups displayed a decreasing trend with the final 105 minute time point being significantly lower compared to their respective 5 minute values. Likewise, developed pressure decreased over the course of perfusion with the 105 minute time point being significantly lower compared to the 5 minute time point for both the OVX and E_2 groups. Figure II.4 shows the effect of physiological estrogen replacement on aortic flow and coronary flow during 105 minutes aerobic perfusion. Aortic flow was greater in the OVX group at the 15, 30 and 45 minute points compared to the E_2 group. No differences in coronary flow were observed between the groups during the course of aerobic perfusion. The physiological effects of estrogen replacement on cardiac work during aerobic perfusion are seen in Figure II.5. Cardiac work was greater in the OVX group at the 15, 30 and 45 minute time points as compared to the E_2 group.

II.3) *Ischemia/reperfusion study*

II.3a) *The effect of ovariectomy and physiological estrogen replacement on body weight, heart weight and serum estradiol levels*

The effects of physiological estrogen replacement on body weight, heart weight and serum estradiol levels in the ischemia/reperfusion study are seen in Table II.2 Despite similar initial body weights, following 21 days of estrogen replacement, body weight increased 37.3±0.8% and 18.9±0.5% in the OVX and E_2 groups respectively. This resulted in a 18.4±0.5% greater body weight in the OVX group as compared to the E_2 group. Heart weight was not different between treatment groups. Serum estradiol levels were lower than those reported in the aerobic study (Table II.1). However, serum estradiol levels in the E_2 group were still approximately 3 fold greater compared to the OVX group.

II.3b) *The effect of physiological estrogen replacement on the recovery of mechanical function in hearts from ovariectomized rats following 20 minutes of global no-flow ischemia*

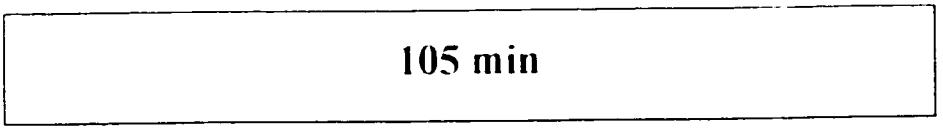
Figure II.6 details the effect of physiological estrogen replacement on the recovery of cardiac work, calculated as cardiac output x peak systolic pressure, following 20 minutes of global no-flow ischemia. Although both groups demonstrated a decrement in cardiac work following ischemia, no differences were seen between groups either before or after ischemia. The effects of physiological

estrogen replacement on the recovery of heart rate and cardiac output are seen Figure II.7. No differences were seen in heart rate between the OVX or E_2 treatment groups or between preischemic and postischemic values. Likewise no differences were seen in cardiac output between the OVX and E_2 treatment groups. However a decrement in cardiac output was seen when preischemic and postischemic OVX values were compared. A similar decrement was not seen in the E_2 group. The effect of physiological estrogen replacement on the recovery of peak systolic pressure and developed pressure are detailed in Figure II.8. No differences in peak systolic pressure were seen between the OVX and E_2 group, although the postischemic value for both groups was lower than the corresponding preischemic value. Likewise, although developed pressure was similar in both the OVX and E_2 groups, the postischemic values for both groups were lower than the corresponding preischemic values. The effect of physiological estrogen replacement on the recovery of aortic flow and coronary flow are seen in Figure II.9. Aortic flow was similar between groups, although the postischemic OVX value was lower than the preischemic value. A similar decrement in the postischemic E_2 value was not seen. No differences in coronary flow were seen either between treatment groups or between preischemic and postischemic values within corresponding treatment groups.

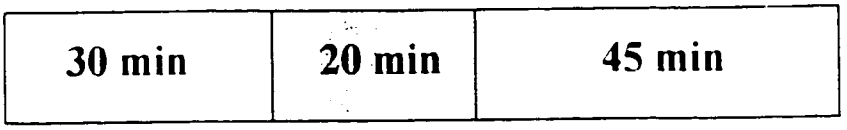
II.3c) *The effect of physiological estrogen replacement on selected metabolites at the conclusion of reperfusion following 20 minutes of global no-flow ischemia*

Table II.3 details the effects of physiological estrogen replacement on selected metabolites at the conclusion of reperfusion following 20 minutes of global no-flow ischemia. No differences were seen in levels of ATP, ADP or the ATP/ADP ratio between the OVX and E_2 treatment groups. Likewise, similar levels of AMP, CrP, and Cr were found in hearts from both OVX and E_2 treatment groups.

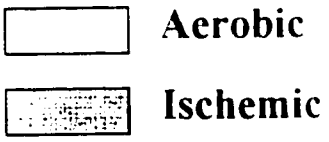
Figure II.1: Perfusion Protocols



A. Aerobic Perfusion Protocol



B. Ischemia/reperfusion Protocol



Perfusate consisted the following:

Modified Krebs-Henseleit bicarbonate buffered solution (pH 7.4) containing 2.5 mmol/L free Ca^{2+}, 11 mmol/L glucose, 100μU ml^{-1} insulin, oxygenated with 95% O_2, 5% CO_2

Table II.1

Effects of physiological estrogen replacement on body weight, heart weight and serum estradiol levels at time of perfusion for preliminary experiments.

Parameter measured		OVX (n=8)	OVX + E_2 (n=7)
Body weight (g)	**Initial**	283±8	285±11
	Final	371±11 †	312±10 §
Heart weight (mg dry wt)		294±11	277±16
Serum Estradiol (pg/ml)		19.7±2.9	71.1±20.9 §

Values are mean±SEM. Initial body weight was measured at time of ovariectomy and final body weight was measured at time of isolated heart perfusion. Dry heart weight was calculated immediately following perfusion † significantly different from initial OVX body weight (student's *t* test). § significantly different from OVX group for the corresponding parameter (one-way ANOVA).

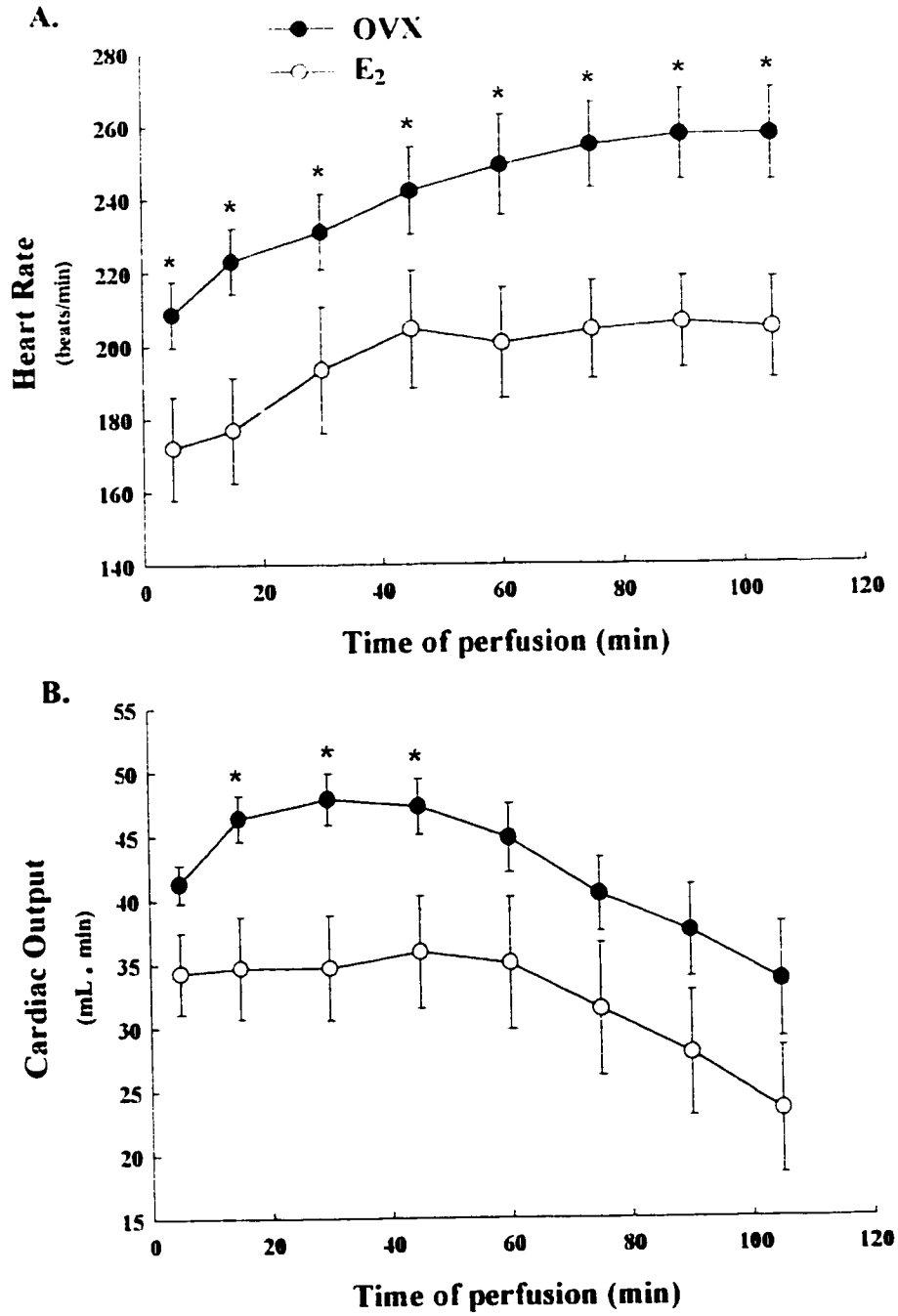


Figure II.2: Effects of physiological estrogen replacement on heart rate (A) and cardiac output (B) during preliminary aerobic perfusion. Values are mean±SEM of hearts from 8 ovariectomized rats (OVX) and 7 estrogen replaced rats (E_2).

* Significantly different from E_2 at the corresponding time of perfusion (two-way ANOVA).

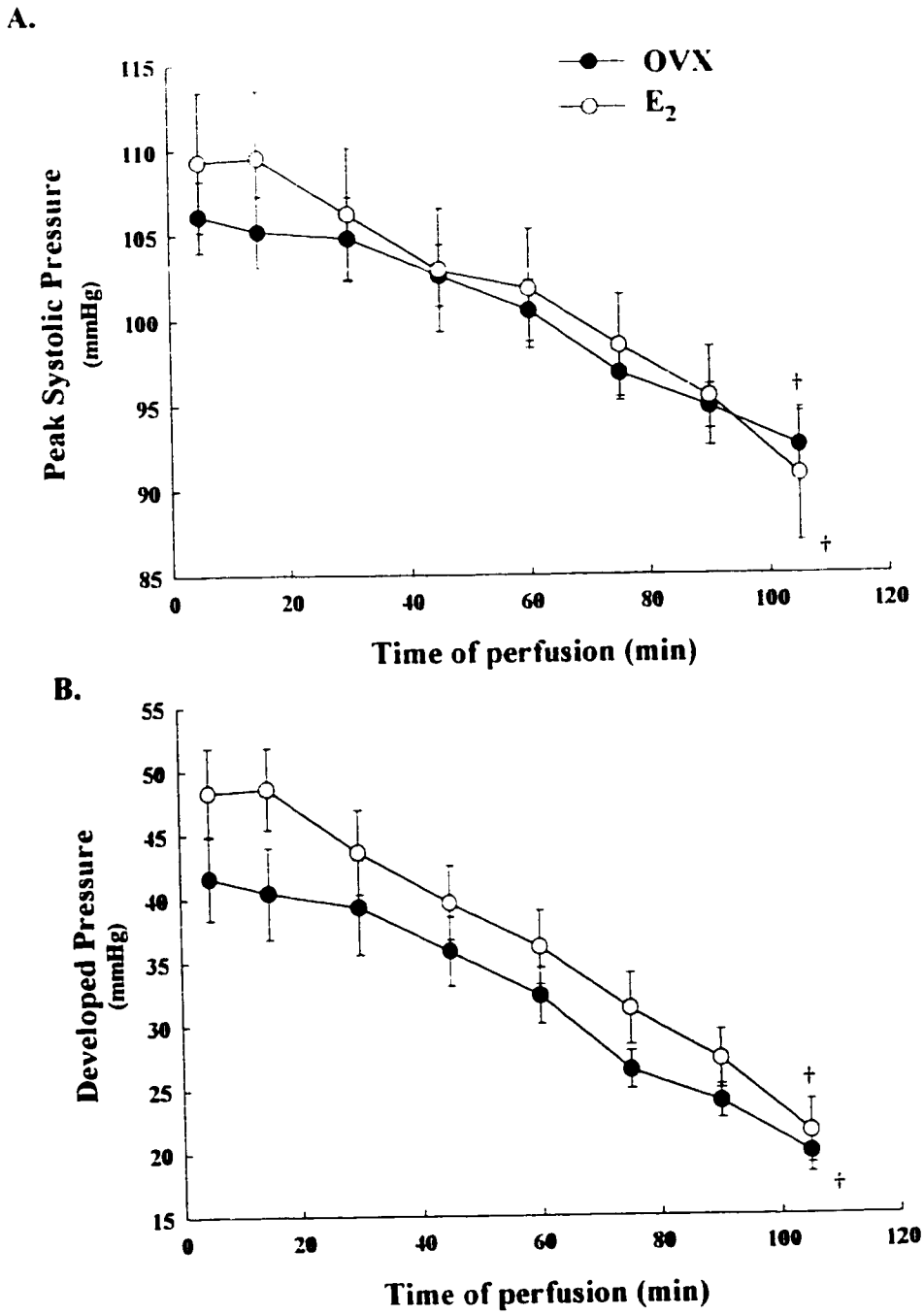


Figure II.3: Effects of physiological estrogen replacement on peak systolic pressure (A) and developed pressure (B) during preliminary aerobic perfusion. Values are mean±SEM of hearts from 8 ovariectomized rats (OVX) and 7 estrogen replaced rats (E_2). † Significantly different than the 5 minute time point within the corresponding treatment group (one-way ANOVA).

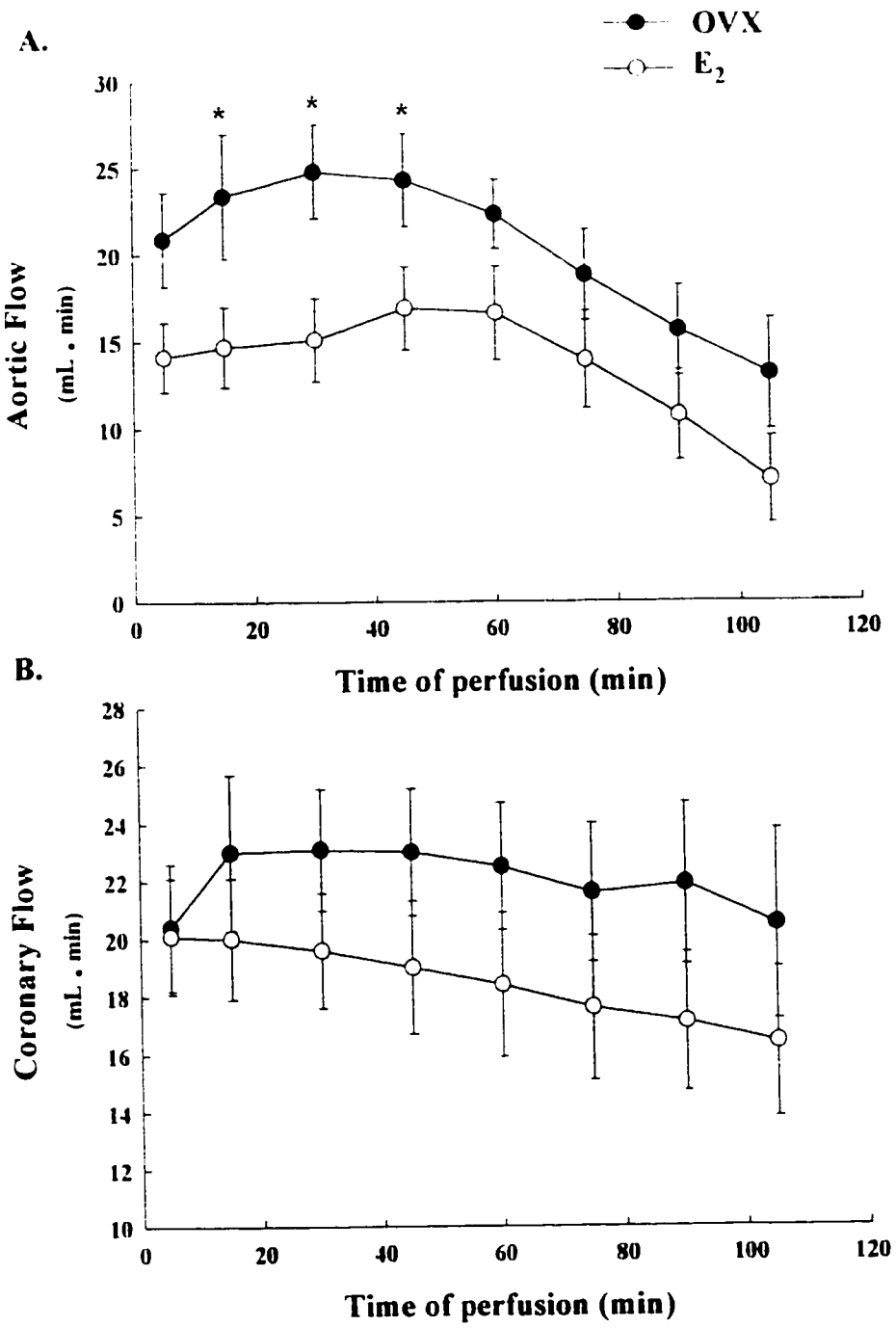


Figure II.4: Effects of physiological estrogen replacement on aortic flow (A) and coronary flow (B) during preliminary aerobic perfusion. Values are mean±SEM of hearts from 8 ovariectomized rats (OVX) and 7 estrogen replaced rats (E_2).

* Significantly different from E_2 at the corresponding time of perfusion (two-way ANOVA).

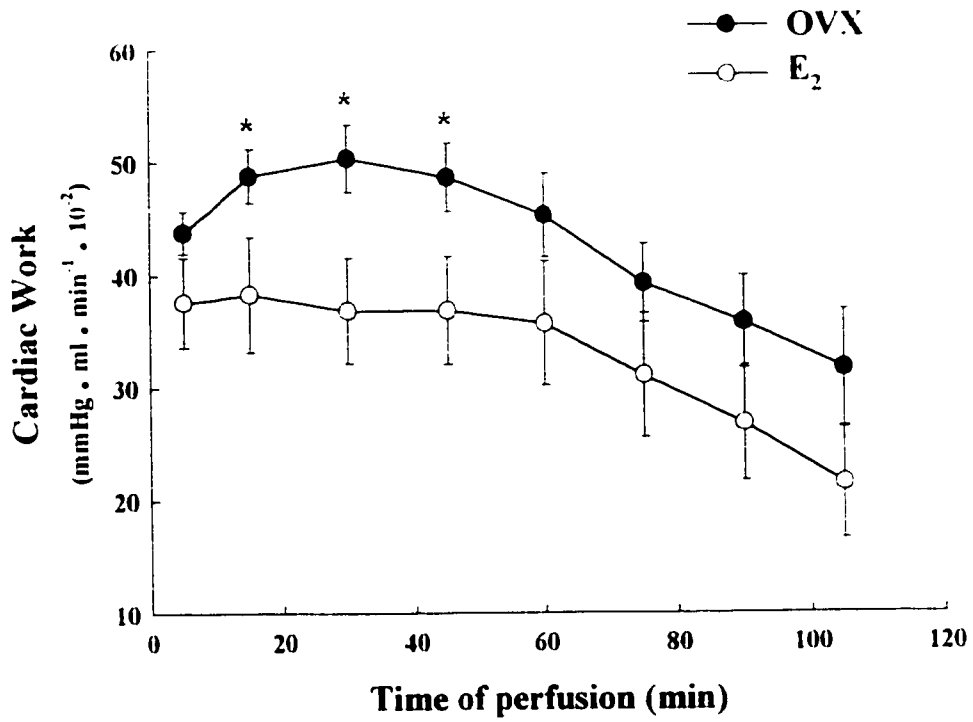


Figure II.5: Effects of physiological estrogen replacement on cardiac work during preliminary aerobic perfusion. Values are mean±SEM of hearts from 8 ovariectomized rats (OVX) and 7 estrogen replaced rats (E_2). * Significantly different than from OVX group at the corresponding time of perfusion (two-way ANOVA).

Table II.2

Effects of physiological estrogen replacement on body weight, heart weight and serum estradiol levels at time of perfusion without fatty acids.

Parameter measured		OVX (n=10)	OVX + E_2 (n=8)
Body weight (g)	**Initial**	282±2	276±6
	Final	387±8 †	328±7 †§
Heart weight (mg dry wt)		288±7	285±8
Serum Estradiol (pg/ml)		6.1±1 6	18.7±4.9 §

Values are mean±SEM. Initial body weight was measured at time of ovariectomy and final body weight was measured at time of isolated heart perfusion. Dry heart weight was calculated immediately following perfusion † Significantly different from initial OVX body weight (student's *t* test). § Significantly different from OVX group for the corresponding parameter (one-way ANOVA).

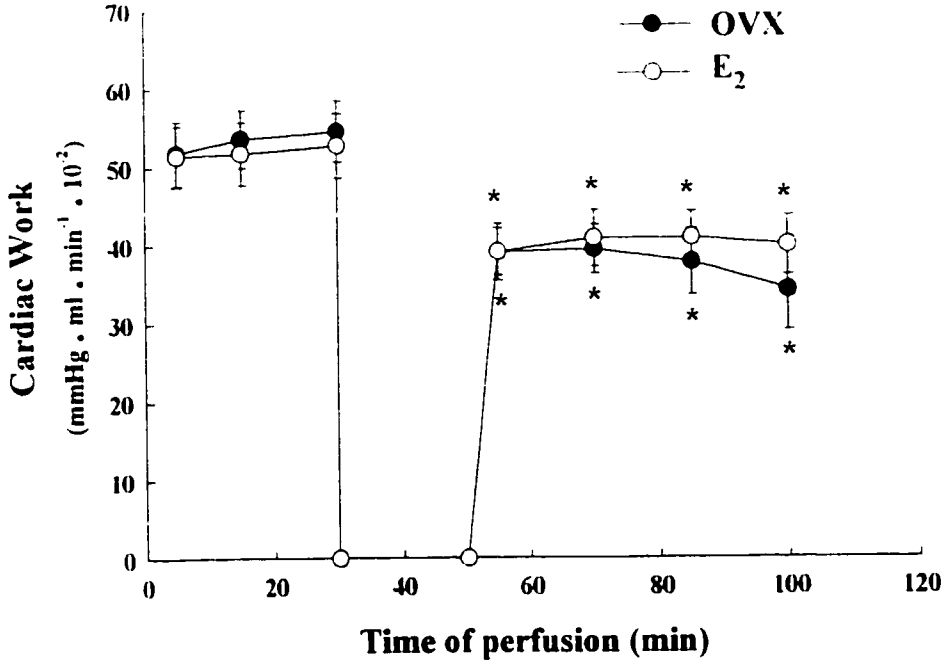


Figure II.6: Effects of physiological estrogen replacement on recovery of cardiac work following 20 minutes of global no flow ischemia. Values are mean±SEM of 10 hearts (•) from ovariectomized rats and 8 hearts (o) from estrogen replaced rats. Hearts were subjected to 30 minutes of aerobic perfusion, 20 minutes of global no-flow ischemia and 45 minutes of aerobic reperfusion. * Significantly different from value at 5 minutes of perfusion within the corresponding treatment group (one-way ANOVA).

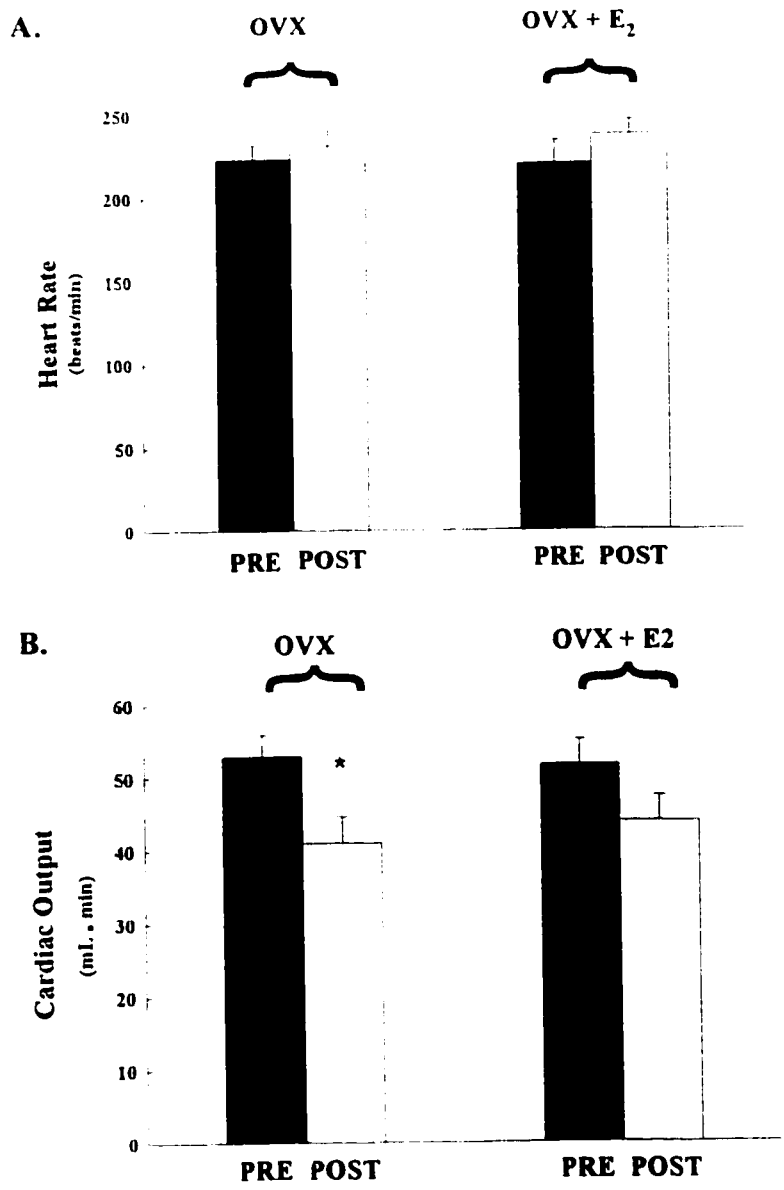


Figure II.7: Effects of physiological estrogen replacement on recovery of heart rate (A) and cardiac output (B) following 20 minutes of global no-flow ischemia. Values are mean±SEM of 10 hearts from ovariectomized rats (OVX) and 8 hearts from estrogen replaced rats (E_2). Preischemic values (PRE) were taken at 20 minutes of aerobic perfusion and postischemic values (POST) were taken after 30 minutes of reperfusion. * Significantly different from preischemic value within the treatment group (student's *t* test).

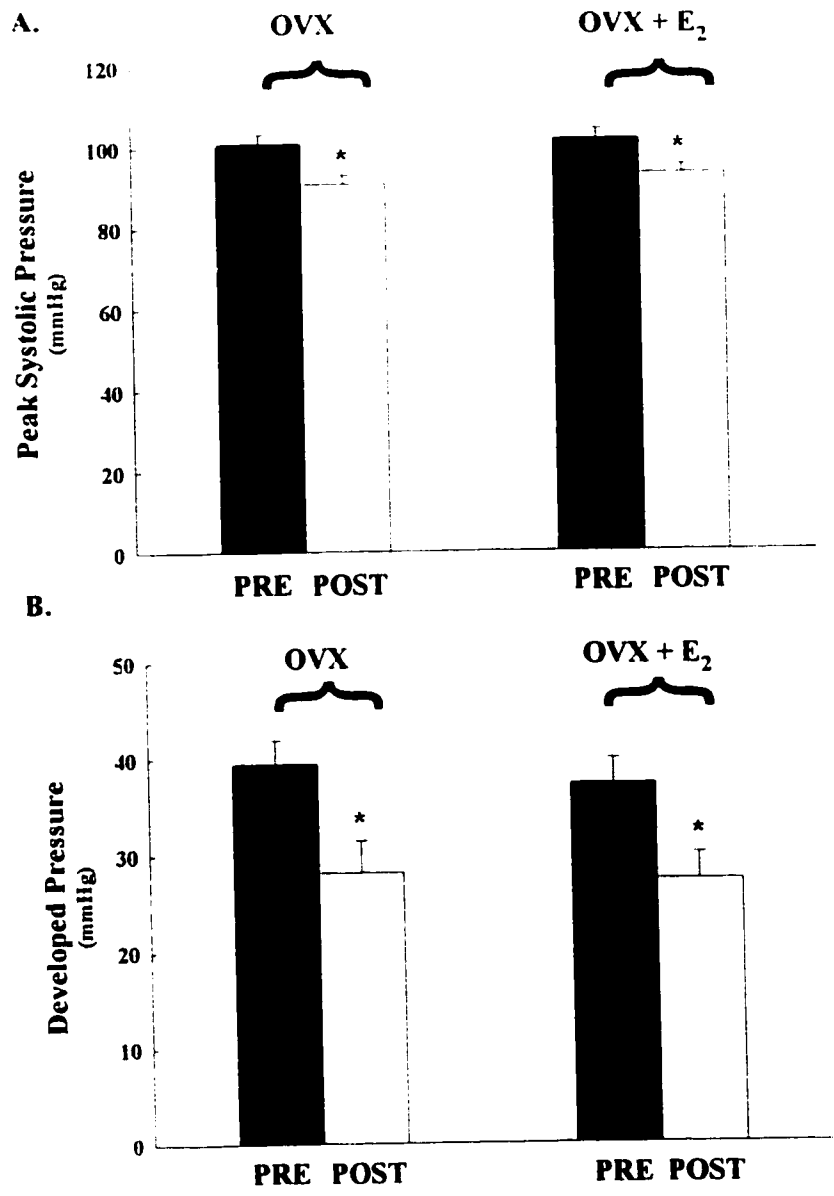


Figure II.8: Effects of physiological estrogen replacement on recovery of peak systolic pressure (A) and developed pressure (B) following 20 minutes of global no-flow ischemia. Values are mean±SEM of 10 hearts from ovariectomized rats (OVX) and 8 hearts from estrogen replaced rats (E_2). Preischemic values (PRE) were taken at 20 minutes of aerobic perfusion and postischemic values (POST) were taken after 30 minutes of reperfusion. * Significantly different from preischemic value within the corresponding treatment group (student's *t* test).

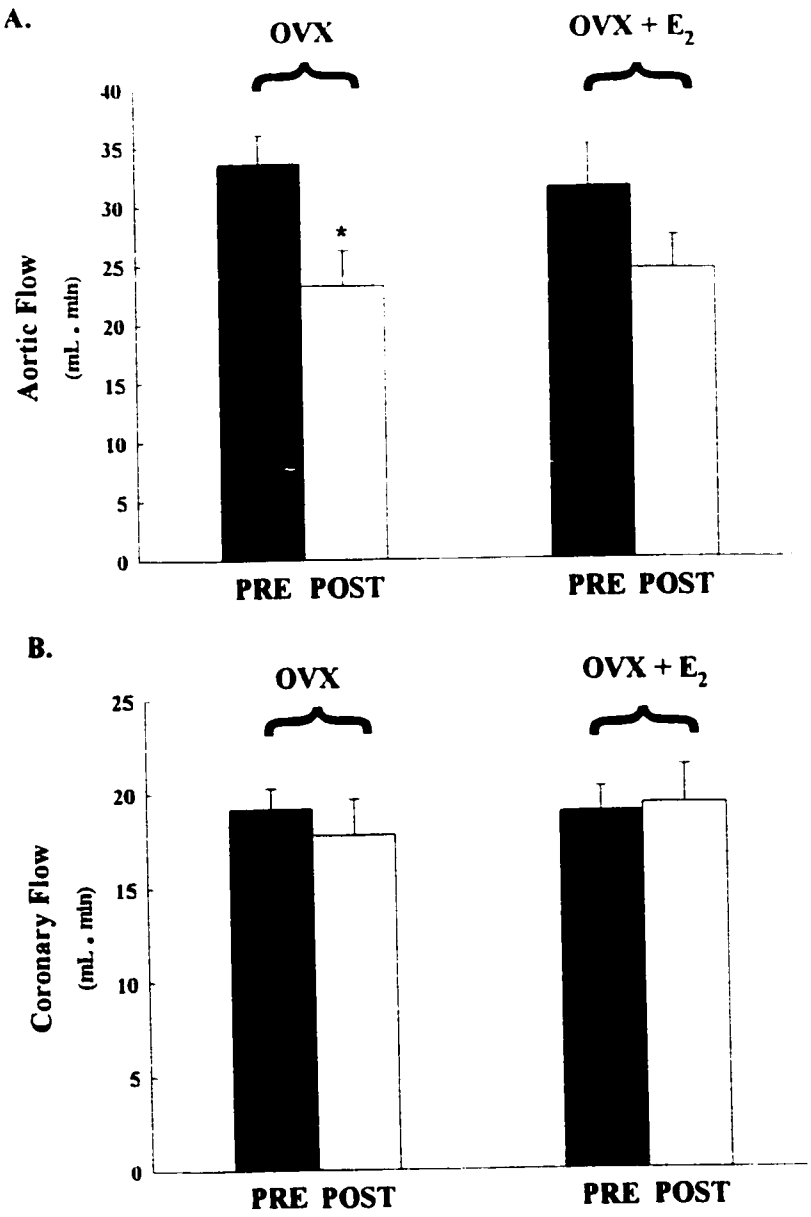


Figure II.9: Effects of physiological estrogen replacement on recovery of aortic flow (A) and coronary flow (B) following 20 minutes of global no-flow ischemia. Values are mean±SEM of 10 hearts from ovariectomized rats (OVX) and 8 hearts from estrogen replaced rats (E_2). Preischemic values (PRE) were taken at 20 minutes of aerobic perfusion and postischemic values (POST) were taken after 30 minutes of reperfusion. * Significantly different from preischemic value within the corresponding treatment group (student's *t* test).

Table II.3

Effects of physiological estrogen replacement on selected metabolites at the conclusion of reperfusion following 20 minutes of global no-flow ischemia in the isolated working rat heart

Metabolite Measured ($\mu mol \bullet g\ dry^{-1}$)	**OVX** (n=10)	**OVX + E_2** (n=8)
ATP	16.5±1.3	19.4±2.1
ADP	23.0±1.4	20.9±2.0
ATP/ADP	0.8±0.1	1.0±0.2
AMP	10.8±1.9	7.6±2.3
CrP	43.1±4.4	47.6±6.1
Cr	141.9±3.4	144.7±6.2

Values are mean±SEM of hearts from ovariectomized rats (OVX) and ovariectomized-estrogen replaced rats (OVX + E_2). Hearts were subjected to 30 minutes of aerobic perfusion, 20 minutes of global no-flow ischemia and 45 minutes of aerobic perfusion before being quick frozen for metabolic analysis.

Discussion

The serum estradiol levels found in the estrogen replaced animals in the initial aerobic study, were within the upper normal physiological range depending on estrus cycle stage (normally 30-50 pg/ml).[19] In contrast, serum estradiol levels in the ischemia/reperfusion study were at the lower end of the normal physiological range. In the initial aerobic study the estradiol levels in the OVX group were slightly elevated although they were still over 3 fold less than levels in the E_2 group. This may have been the result of inter-assay differences as separate radioimmuno-assays were used between studies. However, the estradiol levels in both studies were comparable to levels reported in a study which found physiological estrogen cardioprotective against global ischemia (approx. 44 pg/ml).[17] Similarly, in both studies gains in body weight were observed in the OVX group suggesting ovariectomy induced hyperphagia.[1]

The observed heart rate in the OVX group was within the normal range for an isolated spontaneously beating rat heart (220-240 bpm). However, in comparison to the OVX group, heart rate during the aerobic study was depressed in the E_2 group throughout perfusion period. This reduction in heart rate may be largely responsible for early decrements in aortic flow, cardiac output and cardiac work seen in the E_2 group in comparison to the OVX group. This is in contrast to previous literature which did not report depression during early aerobic perfusion prior to ischemia.[17] Reasons for estrogen induced depressions remain speculative, although alterations

appear to be largely chronotropic in nature as no group differences were seen in peak systolic pressure or developed pressure. Furthermore, coronary flows remained similar between groups, suggesting estrogen mediated changes in the vasculature were not responsible.

In contrast to the changes seen during the aerobic perfusion study, heart rate, aortic flow, cardiac output and cardiac work determined during the preischemic portion of ischemia/reperfusion study, were not different between treatment groups. Although postischemic peak systolic pressure and developed pressure were reduced in comparison to the preischemic time point, values remained comparable between treatment groups. A similar reduction was seen in the recovery of cardiac work for both treatment groups. The only differences in treatment groups were postischemic cardiac output and aortic flow, which remained depressed in the OVX as compared to the E_2 group which did not remain depressed. However no differences were seen when these postischemic values were directly compared between treatment groups.

As only relatively small decrements were seen in mechanical function following ischemia, a greater ischemic insult may be required to separate potential differences between treatment groups. While the levels of selected metabolites were comparable to values previously reported, no differences were seen in ATP, ADP, Cr and CrP levels between the E_2 and OVX groups.[21] However this previous study has also suggested that restoration of oxidative metabolism rather than overall tissue ATP is better determinant of functional recovery.[21] As well, the inclusion of fatty acids

and other oxidative metabolic substrates in the perfusion solution may produce contrary results.

In summary, physiological estrogen replacement does not improve recovery of mechanical function of the isolated working rat heart following 20 minutes of global no-flow ischemia. Furthermore, final overall levels of ATP, ADP, Cr and CrP were also unaltered with physiological estrogen replacement. Consequently additional studies to determine the physiological effects of estrogen on functional recovery and energy metabolism following longer periods of ischemia are necessary. As well, estrogen effects on oxidative metabolism could be better assessed using a perfusate solution which included the major myocardial metabolic substrate, fatty acids.

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Chapter III

Physiological estrogen replacement in the ovariectomized rat has no effect on energy metabolism and functional recovery of the fatty acid perfused isolated working rat heart following global ischemia

Introduction

Cardiovascular disease remains one of the leading causes of death in North America.[1,2] However, clinical data does suggest that premenopausal women and postmenopausal women receiving estrogen replacement therapy are at a significantly reduced risk of death from cardiovascular disease.[3,4] Although the mechanisms by which estrogen may provide the cardioprotection are still unclear, elucidation of this mechanism could potentially offer clinicians a valuable tool for both the prevention and treatment of cardiovascular disease in women and men.

A review of the current literature suggests that estrogen may act via several and potentially allied mechanisms. While estrogen has been shown to improve the blood lipid profile by increasing the levels of circulating high density lipoproteins (HDL), is estimated that this may only account for 20-30% of estrogen's beneficial effects.[4,5] Estrogen has also been shown to act as an anitoxidant in male dogs receiving estrogen replacement.[6] Acute supraphysiologic administration of estrogen in human has been shown to have direct vasodilatory effects on peripheral and coronary vasculature.[7] Furthermore, chronic administration of estrogen has been shown to result in the upregulation of endothelial nitric oxide synthase (eNOS), the enzyme which catalyzes the formation of NO.[8,9]

While these reported effects of estrogen may occur without direct myocyte involvement, several proposed mechanisms have implicated direct estrogenic action on the myocardium. While indirect evidence for myocardial targeting via estrogen receptors comes from estrogen mediated increases in mRNA coding for structural proteins in the rat myocardium, a more recent study has characterized two specific estrogen receptor subtypes, ERα and ERβ.[10,11] The distribution of these estrogen receptor subtypes and their importance in the myocardium remains largely unknown. Two previous studies have also suggested that pharmacological doses of estrogen may inhibit inward Ca^{2+} movement and reduce free intracellular Ca^{2+} concentrations.[12,13] The effect of physiological estrogen replacement on Ca^{2+} channels and free intracellular Ca^{2+} concentrations is unclear. Lastly, estrogenic activation of sarcolemal Na^{+}/K^{+}-ATPase has been demonstrated in ovariectomized dogs.[14]

An increasing body of literature has suggested that functional recovery of the myocardium from ischemic injury is largely dependent upon alterations in myocardial energy metabolism during reperfusion. During reperfusion high levels of circulating fatty acids may result in high levels of fatty acid β-oxidation, which in turn dramatically inhibits glucose oxidation.[15,16] If rates of glycolysis remain unaltered an uncoupling of glucose oxidation and glycolysis may result in the production of H^{+}.[16,17] H^{+} production from glucose is the result of hydrolysis of glycolytically derived ATP. While glycolysis contributes $2H^{+}$ for every glucose molecule metabolized, their is a net H^{+} production of zero when glycolysis is coupled to

glucose oxidation. Imbalances in intracellular H^+ may alter fluxes through the Na^+/H^+ and the Na^+/Ca^{2+} exchangers, ultimately leading to Ca^{2+} overload and cell death.[18,19] The importance of H^+ production on functional recovery of the heart has been further demonstrated by altering both the source and fate of H^+ in the isolated working rat heart.[17]

Stemming from our limited mechanistic understanding of estrogenic effects and recent data demonstrating the importance of myocardial metabolism during ischemia/reperfusion, it is not unreasonable to examine the metabolic consequences of physiological estrogen replacement. I hypothesize that physiological estrogen replacement will improve functional recovery following 25 minutes of global no-flow ischemia. I further hypothesize that these improvements will be the result of alterations in metabolism which reduce H^+ production from glucose metabolism.

In the present study we determined whether physiological estrogen replacement was capable of protecting the myocardium following global no-flow ischemia. To more accurately reproduce the metabolic conditions surrounding *in vivo* myocardial ischemia, the heart was perfused with a physiologically relevant level of fatty acids (1.2 mmol palmitate). Mechanical function, cardiac work, myocardial oxygen consumption (MVO_2), glycolysis, selected metabolite levels, and the oxidative metabolism of glucose and palmitate were used as a basis of comparison Our results demonstrate that physiological estrogen replacement does not provide myocardial protection or alter cardiac work, MVO_2, glycolysis, or oxidative rates of

glucose and palmitate metabolism in the isolated working rat heart perfused with a solution containing fatty acids. Our findings further suggest that any observed myocardial protection offered by physiological estrogen replacement during reperfusion may not be the result of an improvement in H^+ production from glucose metabolism.

Materials and Methods

III.1a) *Animals*

Animals were cared for in accordance with the Canadian Council on Animal Care guidelines. Virgin female Sprauge-Dawley rats (250-275g) purchased from Charles River Laboratories (Montreal, Quebec, Canada) were housed under constant temperature and humidity conditions with a 12 hour light cycle. Animals were fed water and a standard rat pellet diet (Rat Chow™) *ad libitum.*

III.1b) *Materials*

21-day sustained release 17 β-estradiol pellets were purchase from Innovative Research Inc. Radiolabelled substrates, ([U-^{14}C]-glucose, [U-^{3}H]-glucose and [5-^{14}C]-palmitate, were purchased from New England Nuclear. Hyamine hydroxide® (methylbenzethonium; 1M in methanol) was purchased from ICN Radiochemicals. Bovine serum albumin (fraction V) was purchased from Boehringer Mannheim.

III.1c) *Serum Estrogen Levels*

Rats were randomly divided into two groups and bilaterally ovariectomized. At time of ovariectomy the estrogen replacement group (E_2) was implanted with a subcutaneous 21-day release pellet containing 0.5 mg of 17 β-estradiol designed to maintain estrogen levels in the physiological range. The non-estrogen replaced group (OVX) received a subcutaneous placebo pellet at time of ovariectomy. Ovariectomy and pellet implantation was performed under respired Halothane™ (Halocarbon Laboratories. River Edge, New Jersey, United States of America) anesthesia at a dose

of 0.075 L/min. Using a blood sample taken at time of heart excision, serum estrogen levels were determined using a double antibody radioimmuno-assay kit (Diagnostic Products Corp., Montreal, Quebec, Canada)

III.1d) *Isolated Heart perfusion*

21 days after ovariectomy, rats were anesthetized with sodium pentobarbitol and hearts were rapidly removed and placed in ice-cold Krebs-Henseleit solution. Using a 37 °C oxygenated (95% O_2, 5% CO_2) Krebs-Henseleit solution, initial retrograde perfusion at a hydrostatic pressure 60 mmHg was begun following cannulation of the aorta. During this time, the buffer solution was not recirculated, allowing the heart to be cleansed of blood and excess tissue. During the initial 15 minutes of Langendorf perfusion the pulmonary artery and the left atrium were cannulated. Hearts were placed in working mode by clamping the aortic Langendorf inflow line and opening the atrial preload line. The preload line delivered oxygenated perfusate at hydrostatic pressure of 11.5 mmHg. Perfusate ejected from spontaneously beating hearts entered a compliance chamber before passing through an afterload line set at a hydrostatic pressure of 80 mmHg. All working hearts were perfused with a modified Krebs-Henseleit buffer containing 2.5 mmol/L free Ca^{2+}, 11 mmol/L glucose, 1.2 mmol/L palmitate, 100 μU/mL insulin, and 3% bovine serum albumin (fraction V, Boehringer Mannheim). Palmitate was bound to albumin as previously described.[20]

Heart perfusion protocol is seen in figure III.1. Hearts were initially aerobically perfused for 30 minutes. Global no-flow ischemia was begun by clamping both the atrial inflow and the aortic outflow lines. Following 25 minutes of global no-flow ischemia the atrial inflow and aortic outflow lines were reopened to allow for an additional 45 minutes of aeorbic reperfusion.

Hearts were allowed to beat spontaneously throughout the experiments. A Gould pressure transducer in the aortic outflow line was used to measure heart rate and aortic pressure. Transonic ultrasound flow probes in the preload line and after load lines were used to measure cardiac output and aortic flow respectively. Coronary flow was calculated as the difference between cardiac output and aortic flow. The oxygen content of perfusate leaving the pulmonary artery was measure using a Clark-type oxygen electrode. In accordance with the Fick principle, myocardial oxygen consumption (MVO_2) was calculated using the coronary flow rate and the perfusate atriovenous oxygen concentration difference. Cardiac work was calculated as the product of cardiac output and systolic pressure. Cardiac efficiency was calculated as both cardiac work over MVO_2 and as cardiac work over total TCA cycle activity (the total TCA cycle production of acetyl-CoA).

Following reperfusion, heart ventricles were rapidly frozen using Wollenberger clamps cooled to the temperature of liquid nitrogen. Dry atrial tissue weight was determined after oven drying at 100 °C for 12 hours. Frozen ventricular tissue was powdered in a mortar and pestle cooled to the temperature of liquid

nitrogen. A portion of this powdered tissue was weighed both frozen and once dried to determine the wet-to-dry ratio. Using the dry atria weight, the frozen ventricle weight, and the wet-to-dry ratio, the total dry weight of the heart was calculated.

III.1e) *Measurement of Glycolysis, Glucose Oxidation, and Palmitate Oxidation*

As described previously, glycolysis and glucose oxidation were measured simultaneously using perfusate containing [5-^{3}H/U-^{14}C] glucose (Figure III.2).[21] In brief, during the 30-minute initial aerobic perfusion and the 40-minute reperfusion period, total myocardial $^{3}H_2O$ and $^{14}CO_2$ production was determined at 10-minute intervals. Total $^{14}CO_2$ production, both as $^{14}CO_2$ released in the oxygenation chamber and $^{14}CO_2$ dissolved in the perfusate, was quantitatively measured to determine glucose oxidation. Hyamine hydroxide was used to trap gaseous $^{14}CO_2$ exhausted from the oxygenation chamber. $^{14}CO_2$ dissolved as $^{-}HCO_3$ in the perfusate was also trapped using hyamine hydroxide following liberation with 9N H_2SO_4 while inside a sealed 20ml stoppered test tube. Rates of glycolysis were determined by separating $^{3}H_2O$ from [^{3}H]glucose and [^{14}C]glucose in perfusate samples. This was accomplished by passing the perfusate sample through a Dowex column containing Dowex 1-X4 anion exchange resin (200 to 400 mesh) washed with H_2O following pretreatment with 0.2mol/L potassium tetraborate.

Palmitate oxidation was determined by adding a [1-^{14}C]palmitate label to the 1.2 mmol/L palmitate in the perfusate (Figure III.3). Similar to the measurement of glucose oxidation, total $^{14}CO_2$ production was measured both as $^{14}CO_2$ released in the

oxygenation chamber exhaust line and the $^{14}CO_2$ dissolved as $^{14}HCO_3^-$ in the perfusate. The same techniques were also used to trap gaseous $^{14}CO_2$ and liberate $^{14}CO_2$ dissolved as $^{14}HCO_3^-$ in the perfusate.

III.1f) *Calculation of H^+ Production from Glucose Utilization, Acetyl-CoA and ATP Production Rates.*

As previously described, calculations of H^+ production, acetyl-CoA and ATP production rates were made for each heart.[22] The overall rate of H^+ ion production from glucose utilization was calculated by subtracting the rate of glucose metabolism from the rate of glycolysis and multiplying by 2. The rate of acetyl-CoA production for TCA cycle was calculated assuming 2 acetyl-CoA from glucose oxidation and 8 acetyl-CoA from palmitate oxidation. Presented as a percentage of total ATP production, ATP production from glycolysis, glucose oxidation and palmitate were determined assuming 2 ATP are produced per glucose passing through glycolysis, 30 ATP per glucose oxidized, and 106 ATP per palmitate oxidized. Furthermore, calculations of ATP production assume negligible mitochondrial proton leak.

III.1g) *Tissue Nucleotide Measurements*

Nucleotides were extracted from frozen ventricular tissue (100±10 mg) using the following technique. Tissue was homogenized on ice with 1.0 ml 6% PCA/0.5 mM EGTA. The homogenate was then centrifuged at 11,000 rpm for 2 minutes prior to addition of 95 µl of 0.32 DTT. Following be neutralized using 5 M K_2CO_3 to pH 6.8±0.5, samples were centrifuged at 11,000 rpm for 2 minutes. The supernatant was

then collected and immediately frozen in liquid nitrogen for subsequent nucleotide determination.

As described previously, 100μl of prepared supernatant was analyzed by HPLC.[23] Briefly, nucleotides, AMP degradation products, PCr and Cr were resolved with 35mM K_2HPO_4, 6mM tetrabutyl-ammonium hydrogen sulfate buffer (pH 6.0), and a binary acetonitrile gradient on medium-bore, 250 • 3.9mm steel octadecyl-bonded (C18) columns at a flow rate of 1.5 or 1.0 ml • min^{-1}. A Beckman System Gold® software package was to integrate peaks.

III.1h) *Statistical Analysis*

All values are mean±SEM. An unpaired Student's *t* test was used to determine differences between the single point variables of weight and the perfusion time points of preischemia and postischemia. One-way ANOVA was used to compare differences in preischemic and postischemic values between treatment groups of OVX and E_2. Two-way ANOVA was used to compare the treatment groups of OVX and E_2 at the corresponding time of perfusion. Whenever necessary a Bonferroni post-hoc test was performed. A value of $P < .05$ was considered significant.

Results

III.2a) *The effect of ovariectomy and physiological estrogen replacement on body weight, heart weight and serum estradiol levels.*

Table III.1 shows the effect of physiological estrogen replacement on body weight, heart weight and serum estradiol levels in ovariectomized rats. While initial body weight was similar between groups, 21 days following ovariectomy, body weight increased 30±0.3% in the OVX group while only increasing 9.5±0.3% in the E_2 group. This resulted in the final body weight for the OVX group being 20.5±0.4% greater than the E_2 group. Following 21 days of estrogen replacement, serum estradiol levels in the E_2 group were nearly 20 fold greater than the OVX group, with values of 37.3±8.2 and 1.88±0.17 pg/ml respectively.

III.2b) *The effect of physiological estrogen replacement on the recovery of mechanical function in hearts from ovariectomized rats following 25 minutes of global no-flow ischemia*

The effect of physiological estrogen replacement on the recovery of mechanical function following 25 minutes of ischemia is shown in Table III.2. During reperfusion, both heart rate and coronary flow returned to preischemic values in both the OVX and E_2 groups. However, during reperfusion, peak systolic pressure, developed pressure, cardiac output, aortic flow and coronary flow were significantly

depressed in both groups. No significant differences were observed between the OVX and E_2 groups, either before or following 25 minutes of ischemia.

Figure III.4 (panel A) shows the effect of physiological estrogen replacement on the recovery of cardiac work following 25 minutes of ischemia. During reperfusion, cardiac work in both OVX and E_2 groups was significantly depressed throughout the entire 40 minute reperfusion period. In contrast, MVO_2 returned to preischemic values in both groups (Figure III.4, panel B). The resulting expression of cardiac efficiency (cardiac work / MVO2), was significantly depressed during reperfusion in the OVX group at the 65, 75, and 95 minute time points and in the E_2 group at the 65 minute time point (Figure III.4, panel C). To account for any differences in heart weight, cardiac work was normalized for dry heart weight (Figure III.5). This resulted in significant depressions in the E_2 group throughout reperfusion and in the OVX group at 85 and 95 minutes of perfusion.

III.2c) *The effect of physiological estrogen replacement on glycolysis, glucose oxidation and palmitate oxidation both before and following 25 minutes of global no-flow ischemia*

Table III.3 shows the effects of physiological estrogen replacement on steady state rates of glycolysis, glucose oxidation, and palmitate oxidation. Although slight variations were observed, steady state rates of glycolysis, glucose oxidation, or palmitate oxidation were not significantly different when comparing preischemic and

postischemic values within treatment groups. Likewise, no significant differences were observed between OVX and E_2 treatment groups, either before of after ischemia.

III.2d) *The effect of physiological estrogen replacement on TCA Acetyl-CoA and ATP production rates both before and following 25 minutes of global no-flow ischemia*

Rates of TCA Acetyl-CoA production were calculated from glucose and palmitate oxidation both before and after ischemia. As shown in Table III.4, total TCA Acetyl-CoA production following ischemia was significantly greater than preischemic production in both the OVX and E_2 groups. Physiological estrogen replacement also increased total postischemic TCA Acetyl-CoA production as compared to the OVX group. It should also be noted that although total TCA Acetyl-CoA production recovered or surpassed preischemic values following ischemia, cardiac work remained significantly depressed.

Table III.5 shows the effect of physiological estrogen replacement on ATP production from glycolysis, glucose oxidation, and palmitate oxidation. Following ischemia, the percent contribution of glycolysis to total ATP production was significantly lower in OVX hearts. While a similar trend was demonstrated in the E_2 group, no other significant changes in percent contribution to total ATP production were seen.

III.2e) *The effect of physiological estrogen replacement on H^+ production and the relationship between cardiac work and TCA Acetyl-CoA production both before and following ischemia*

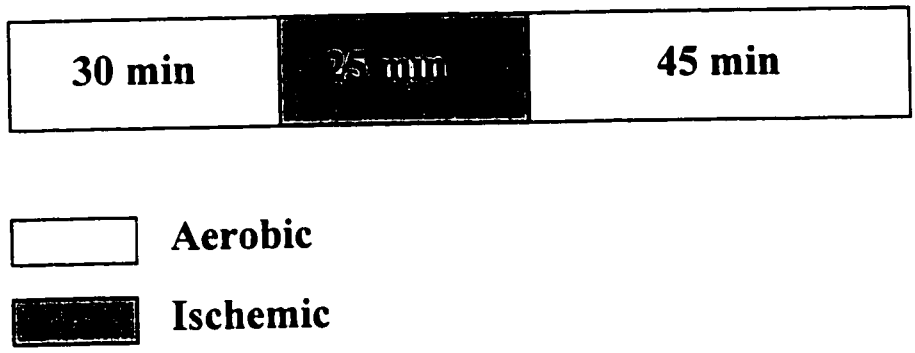
The effect of physiological estrogen replacement on cardiac efficiency, measured as cardiac work normalized for TCA Acetyl-CoA production is shown in Figure III.6. As compared with preischemic values, a significant decrease was seen in postischemic cardiac work/TCA Acetyl-CoA in both the OVX and E_2 groups. Physiological estrogen replacement did not significantly improve the recovery of cardiac work/TCA Acetyl-CoA following 25 minutes of ischemia.

Figure IV.4 shows the effects of physiological estrogen replacement on rates of H^+ production following 25 minutes of global no-flow ischemia. As compared to the preischemic and postischemic values seen OVX group, H^+ production rates were significantly greater in the E_2 group, both before and after ischemia. No significant differences were seen when preischemic and postischemic values were compared within treatment groups. Although the elevation seen pre and postischemically in the E_2 group is largely the result of an elevated glycolytic rate, as seen in Table III.3, physiological estrogen replacement did not alter H^+ production rates calculated following ischemia.

III.2f) *The effect of physiological estrogen replacement on selected metabolites at the conclusion of reperfusion following 25 minutes of global no-flow ischemia*

The effects of physiological estrogen replacement on selected metabolites at the conclusion of reperfusion following 25 minutes of global no-flow ischemia are detailed in Table III.6. No differences were seen in levels of ATP, ADP or the ATP/ADP ratio between the OVX and E_2 treatment groups. Likewise, similar levels of AMP, CrP, and Cr were found in hearts from both OVX and E_2 treatment groups.

Figure III.1: Perfusion protocol



Perfusate consisted the following:

Modified Krebs-Henseleit bicarbonate buffered solution (pH 7.4) containing 2.5 mmol/L free Ca^{2+}, 11 mmol/L glucose, 1.2 mmol/L palmitate, 3% BSA, 100μU.ml^{-1} insulin, oxygenated with 95% O_2, 5% CO_2.

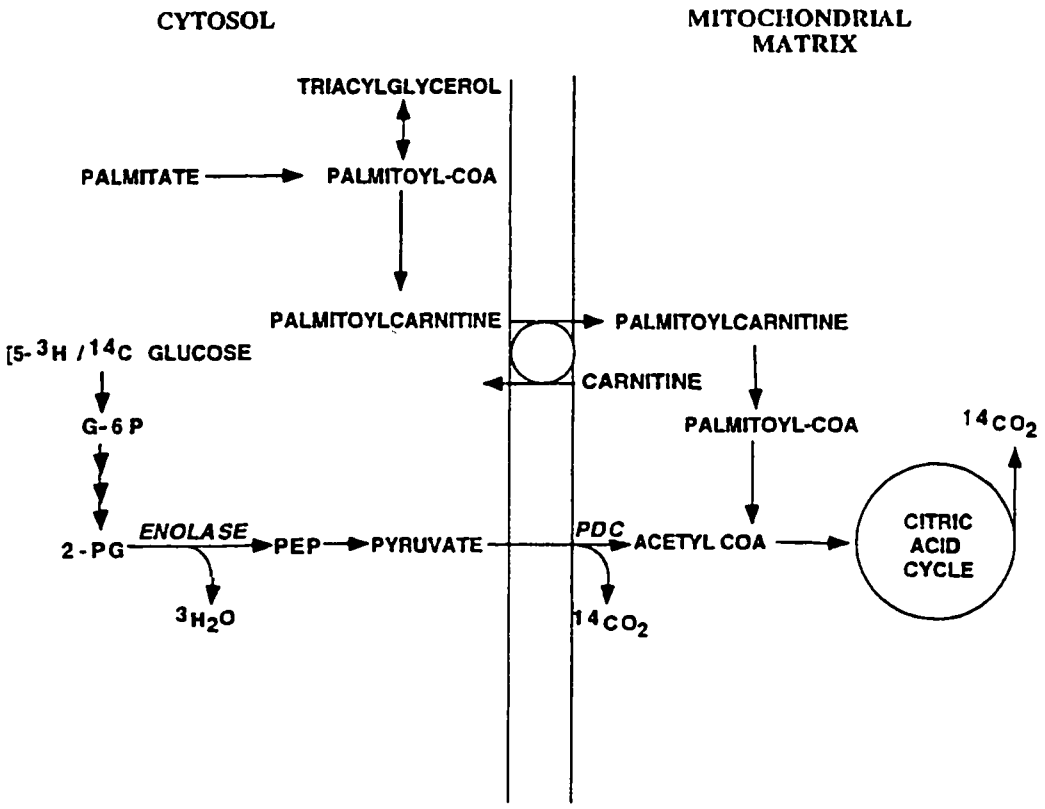


Figure III.2: Production of $^{3}H_2O$ and $^{14}CO_2$ from the metabolism of glucose. Adapted from Belke DD, 1997, Hypothermia and heart metabolism.

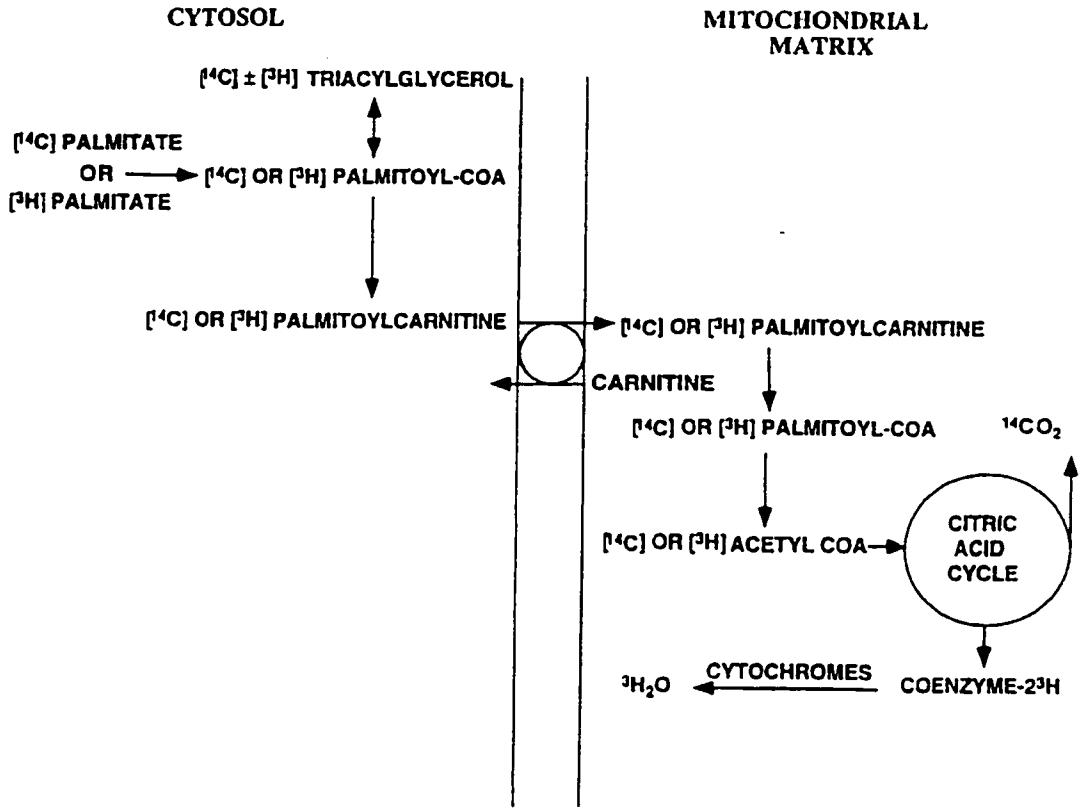


Figure III.3: Production of $^{3}H_2O$ or $^{14}CO_2$ from the oxidation of palmitate. Adapted from Belke DD, 1997, Hypothermia and heart metabolism.

Table III.1

Effects of physiological estrogen replacement on body weight, heart weight and serum estradiol levels at time of perfusion.

Parameter measured		OVX (n=14)	OVX + E_2 (n=12)
Body weight (g)	**Initial**	272±3	269±6
	Final	354±4 †	294±5 †§
Heart weight (mg dry wt)		237±6	223±5
Serum Estradiol (pg/ml)		1.88±0.17	37.3±8.2 §

Values are mean±SEM. Initial body weight was measured at time of ovariectomy and final body weight was measured at time of isolated heart perfusion. Dry heart weight was calculated immediately following perfusion † significantly different from initial value in within the corresponding treatment group (student's *t* test). § significantly different from OVX group for the corresponding parameter (one-way ANOVA).

Table III.2

Effects of physiological estrogen replacement on the recovery of mechanical function in postischemic working rat hearts.

Parameter measured		OVX (n=14)	OVX + E2 (n=12)
Heart rate (beats/min)	Preischemia	224±10	227±8
	Postischemia	219±12	217±4
Peak systolic pressure (mmHg)	Preischemia	118±3	120±3
	Postischemia	105±2†	107±3†
Developed pressure (mmHg)	Preischemia	60±4	65±4
	Postischemia	44±4†	46±4†
Cardiac output ($mL \bullet min^{-1}$)	Preischemia	35±2	36±3
	Postischemia	26±3†	27±3†
Aortic flow ($mL \bullet min^{-1}$)	Preischemia	18±2	18±2
	Postischemia	9±2†	9±2†
Coronary flow ($mL \bullet min^{-1}$)	Preischemia	17±1	18±2
	Postischemia	16±1	18±2

Values are mean±SEM. Hearts were subjected to 30 minutes of aerobic perfusion, 25 minutes of global no-flow ischemia, and 45 minutes of aerobic reperfusion. Preischemic values were determined at 20 minutes of aerobic perfusion and postischemic values were determined at 30 minutes of aerobic reperfusion to allow heart function to stabilize following ischemia. † significantly different from preischemic value in within the corresponding treatment group (student's *t* test).

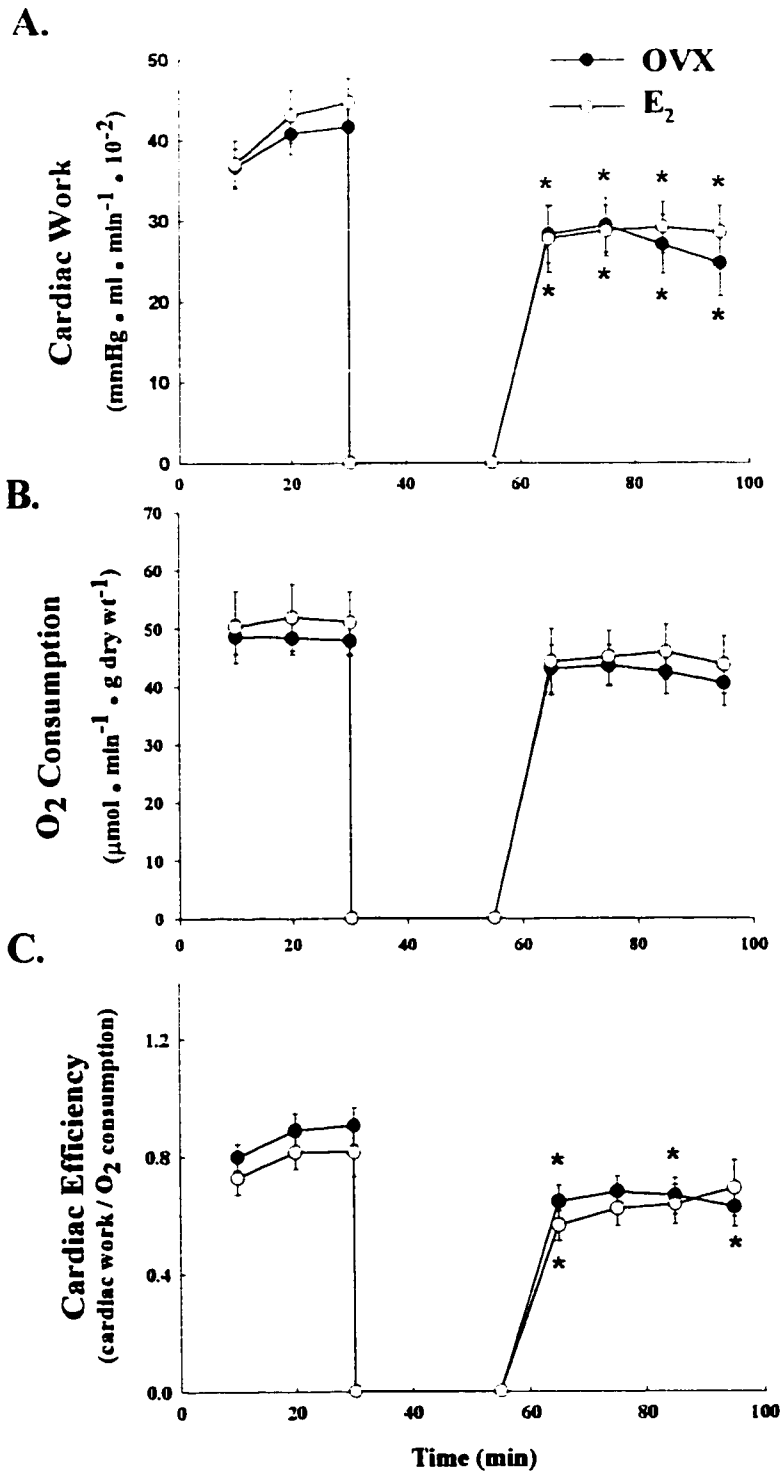


Figure III.4. Effects of physiological estrogen replacement on the recovery of cardiac work (A), O2 consumption (B), and cardiac efficiency (C) on hearts reperfused following 25 minutes of global no-flow ischemia. Values are mean±SEM of 14 hearts(•) from ovariectomized rats and 12 hearts (o) from estrogen replaced rats. * Significantly different than 20 minutes of perfusion within the corresponding treatment group (one-way ANOVA).

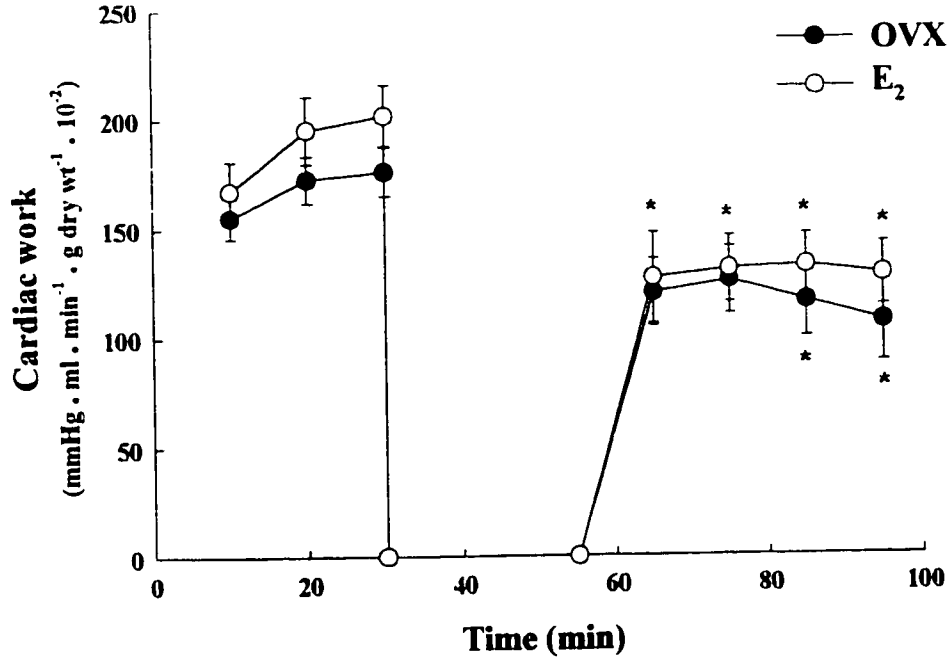


Figure III.5 Effects of physiological estrogen replacement on cardiac work normalized for heart weight. Values are mean±SEM of 14 hearts (•) from ovariectomized rats and 12 hearts (o) from estrogen replaced rats. Hearts were subjected to 30 minutes of aerobic perfusion, 25 minutes of global no-flow ischemia and 45 minutes of aerobic reperfusion. * Significantly different from 20 minutes of perfusion within the corresponding treatment group (one-way ANOVA).

Table III.3

Effects of physiological estrogen replacement on steady state rates of glycolysis, glucose oxidation, and palmitate oxidation in postischemic hearts following global no-flow ischemia.

Parameter measured		OVX	OVX + E_2
Glycolysis	Preischemia	3.74±1.28	5.01±1.66
(μmol • g dry wt^{-1} • min^{-1})	Postischemia	1.21±0.30	3.17±1.40
Glucose oxidation	Preischemia	208±52	311±74
(nmol • g dry wt^{-1} • min^{-1})	Postischemia	230±38	360±62
Palmitate oxidation	Preischemia	281±36	340±81
(nmol • g dry wt^{-1} • min^{-1})	Postischemia	386±60	571±79

Values are mean±SEM of 14 hearts from ovariectomized (OVX) rats and 12 hearts from ovariectomized, estrogen replaced (OVX + E_2) rats. Hearts were subjected to 30 minutes of aerobic perfusion, 25 minutes of global no-flow ischemia, and 45 minutes of aerobic reperfusion. Preischemic values were determined at 20 minutes of aerobic perfusion and postischemic values were determined at 30 minutes of aerobic reperfusion to allow heart function to stabilize following ischemia.

Table III.4

Effects of physiological estrogen replacement on steady state rates of TCA Acetyl-CoA production from substrate metabolism in postischemic hearts following global no-flow ischemia.

		Source of TCA Acetyl-CoA Production ($\mu mol \bullet g\ dry\ wt^{-1} \bullet min^{-1}$)		
		From Glucose Oxidation	**From Palmitate Oxidation**	**Total TCA Acetyl-CoA**
OVX	Preischemia	0.42±0.10	2.25±0.29	2.66±0.19
(n=14)	Postischemia	0.46±0.08	3.09±0.48	3.55±0.31 †
OVX + E_2	Preischemia	0.62±0.15	2.72±0.65	3.34±0.59
(n=12)	Postischemia	0.72±0.13	4.57±0.63	5.29±0.52 †§

Values are mean±SEM of 14 hearts from ovariectomized (OVX) rats and 12 hearts from ovariectomized, estrogen replaced (OVX + E_2) rats. Hearts were subjected to 30 minutes of aerobic perfusion, 25 minutes of global no-flow ischemia, and 45 minutes of aerobic reperfusion. Preischemic values were determined at 20 minutes of aerobic perfusion and postischemic values were determined at 30 minutes of aerobic reperfusion to allow heart function to stabilize following ischemia. † Significantly different from preischemic value within the corresponding treatment group (student's *t* test). § Significantly different from postischemic OVX value (one-way ANOVA).

Table III.5

Effects of physiological estrogen replacement on contributions to ATP production from substrate metabolism in postischemic hearts following global no-flow ischemia.

		Source of ATP Production (% contribution to total ATP production)		
		From Glycolysis	**From Glucose Oxidation**	**From Palmitate Oxidation**
OVX (n=14)	Preischemia	1.7±0.4	16.8±4.2	81.5±10.6
	Postischemia	0.41±0.3 †	14.2±2.3	85.4±13.3
OVX + E_2 (n=12)	Preischemia	1.8±0.7	20.0±4.8	78.3±18.7
	Postischemia	0.73±0.3	14.9±2.6	84.4±11.7

Values are mean±SEM of 14 hearts from ovariectomized (OVX) rats and 12 hearts from ovariectomized, estrogen replaced (OVX + E_2)rats. Hearts were subjected to 30 minutes of aerobic perfusion, 25 minutes of global no-flow ischemia, and 45 minutes of aerobic reperfusion. Preischemic values were determined at 20 minutes of aerobic perfusion and postischemic values were determined at 30 minutes of aerobic reperfusion to allow heart function to stabilize following ischemia. † Significantly different from preischemic value within the corresponding treatment group (student's *t* test).

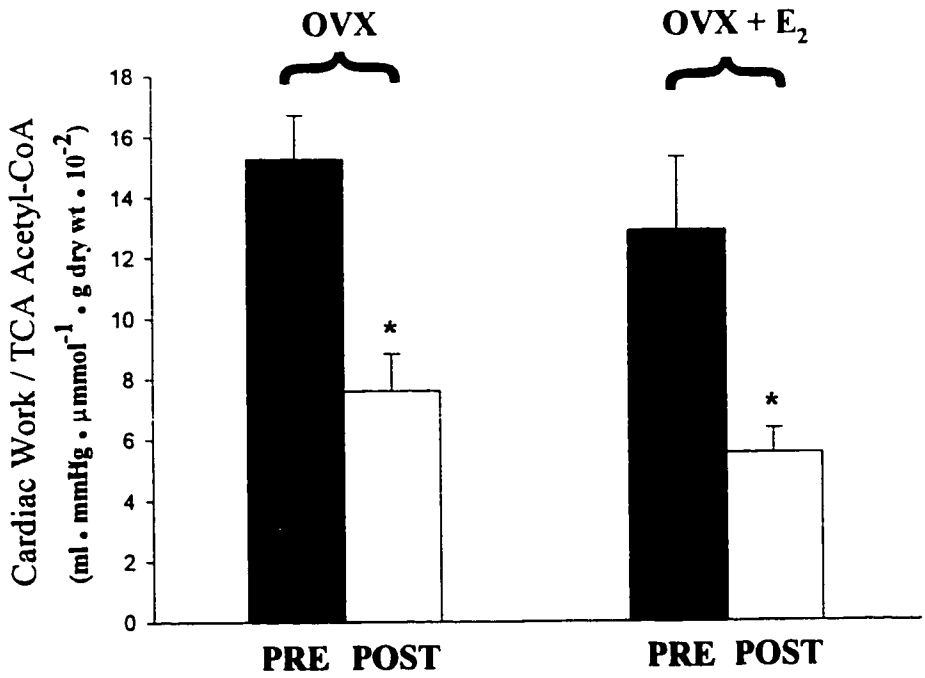


Figure III.6 Effects of physiological estrogen replacement on the recovery of cardiac efficiency expressed as cardiac work per unit TCA acetyl-CoA production. Values are mean±SEM of 14 hearts from ovariectomized (OVX) rats, and 12 hearts from ovariectomized, estrogen replaced (OVX + E_2)rats. Hearts were subjected to 30 minutes of aerobic perfusion, 25 minutes of global no-flow ischemia, and 45 minutes of aerobic reperfusion. Preischemic values (PRE) were determined at 20 minutes of aerobic perfusion and postischemic values (POST) were determined at 30 minutes of aerobic reperfusion to allow heart function to stabilize following ischemia. * Significantly different from preischemic hearts within the treatment group (student's *t* test).

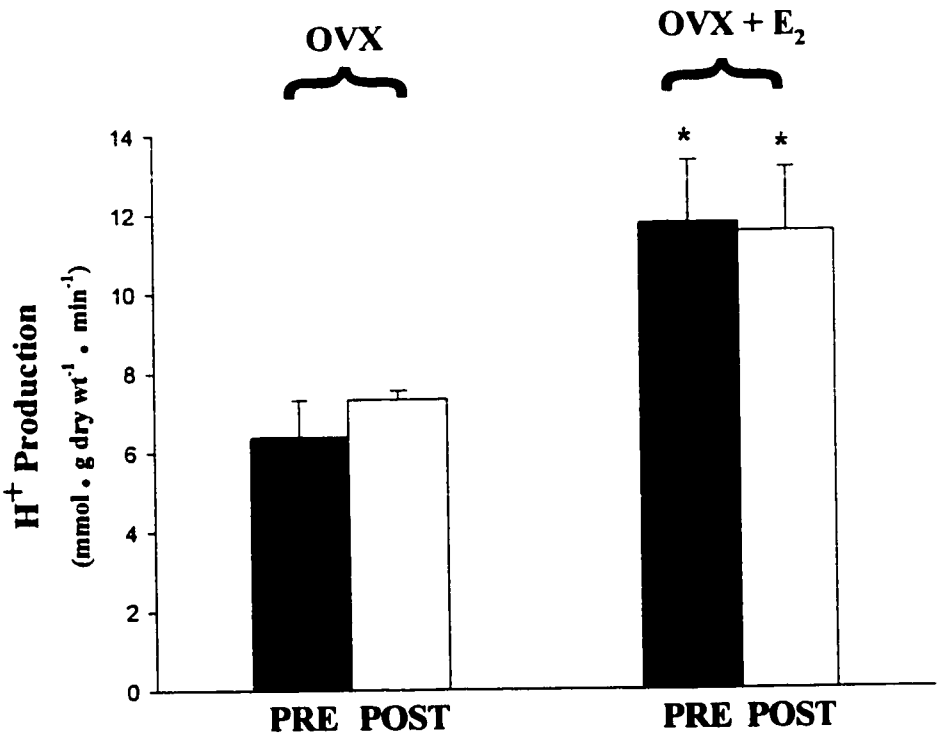


Figure III.7 Effects of physiological estrogen replacement on H^+ production before and after 25 minutes of global no-flow ischemia. Values are mean±SEM of 14 hearts from ovariectomized (OVX) rats, and 12 hearts from ovariectomized, estrogen replaced (OVX + E_2)rats. Hearts were subjected to 30 minutes of aerobic perfusion, 25 minutes of global no-flow ischemia, and 45 minutes of aerobic reperfusion. Preischemic values (PRE) were determined at 20 minutes of aerobic perfusion and postischemic values (POST) were determined at 30 minutes of aerobic reperfusion to allow heart function to stabilize following ischemia. * Significantly different from OVX group at the corresponding perfusion time (one-way ANOVA).

Table III.6

The effects of physiological estrogen replacement on selected metabolites at the conclusion of reperfusion following 25 minutes of ischemia in the isolated working rat heart

Metabolite Measured ($\mu mol \bullet g\ dry^{-1}$)	**OVX** (n=14)	**OVX + E_2** (n=11)
ATP	19.4±1.4	19.4±1.9
ADP	16.1±1.5	16.5±1.6
ATP/ADP	1.29±0.1	1.22±0.1
AMP	5.13±0.9	5.02±1.2
CrP	35.2±5.2	31.7±5.4
Cr	112.2±9.9	118.2±11.1

Values are mean±SEM of hearts from ovariectomized rats (OVX) and ovariectomized-estrogen replaced rats (OVX + E_2). Hearts were subjected to 30 minutes of aerobic perfusion, 25 minutes of global no-flow ischemia and 45 minutes of aerobic perfusion before being quick frozen for metabolic analysis.

Discussion

Previous literature reports that the serum estrogen level in the intact female rat peaks at 30-50 pg/ml during the estrus portion of the estrus cycle.[24] This suggests the serum estrogen levels reported in this study are well within the physiological range for the cycling female rat. Furthermore, serum estrogen levels in a previous study suggesting physiological estrogen replacement in combination with preconditioning protects the myocardium from ischemic injury, were approximately 40 pg/ml.[25] This suggests that physiological estrogen replacement in this study is well within a relevant dose range which may be expected to provide myocardial protection during ischemic insult.

Coronary flow was not significantly different between treatment groups either before or after ischemia. Although a previous study using acute supraphysologic doses of estrogen reported vasodilatory effects, another study using physiological estrogen replacement did not report any changes in coronary flow.[7,25] The present study also suggests that increases in coronary flow are not responsible for improvements in cardiac function following global no-flow ischemia in the physiological estrogen replacement model.

Although, MVO_2 and TCA Acetyl-CoA production rates returned to preischemic values following 25 minutes of global no-flow ischemia, a persistent and significant decrease in cardiac function was observed. This decrement was seen in both treatment groups. As well, when cardiac efficiency was expressed as cardiac

work normalized for TCA Acetyl-CoA production, a significant decrease was observed in both treatment groups during reperfusion. These observed decrements in cardiac efficiency correspond with decrements reported in isolated rat hearts perfused under similar conditions.[26,27]

Results from these previous studies also suggest that the underlying mechanism responsible for the imbalance between cardiac function and TCA Acetyl-CoA production may be the result of an uncoupling of glycolysis from glucose oxidation during ischemia. Under these conditions, H^+ production from glucose metabolism may contribute to impaired recovery of mechanical function through the reallocation of ATP required to reestablish ionic homeostasis. Although an increase in H^+ production rates were seen both pre and postischemically in the estrogen replacement group, no significant increases in H^+ production were observed during reperfusion. It appears the elevated rates of glycolysis are responsible for elevation in H^+ production both before and after ischemia. Therefore, physiological estrogen replacement did not protect the myocardium against ischemic injury or a post ischemic imbalance between cardiac work and TCA Acetyl-CoA production. Since this study did not find estrogen associated cardioprotection, it is not possible to conclude that potential estrogenic benefits are the result of an improved coupling of glycolysis and glucose oxidation.

The present study differs most dramatically from others in that it uses a high physiological concentration of the fatty acid palmitate (1.2 mmol/L), to simulate the

metabolic conditions seen during clinical ischemia. The same concentration of palmitate has been shown to stimulate β-oxidation, and thereby, dramatically reduce glucose oxidation in the hearts from male rats.[15,16,20] Although the present study does not suggest that increases in the postischemic rate of H^+ production from glucose is significantly different between OVX and E_2 groups, a fatty acid mediated impairment of glucose oxidation may have overcome any increase in estrogenic increases in glucose oxidation. Future studies which examine the effects of physiological estrogen replacement on hearts perfused with different concentration of fatty acids are needed. This methodological difference may explain some of the differences between this work and previous studies. As well, it is possible to speculate that dissimilarity between the incidence of heart disease and the clinical mortality seen in women with heart disease, may be the result of circulating levels of fatty acid. Under such conditions the beneficial effects of estrogen on the heart and vasculature may not alter clinical outcome once disease has begun.

Although the 25 minute length of ischemia was longer than a previous study reporting the no benefits of physiological estrogen replacement following a 15 minute ischemic episode, recovery of cardiac function was relatively high in both OVX and E_2 groups.[22] It is possible that the beneficial effects of physiological estrogen replacement are so slight that increasing ischemic times and reducing cardiac recovery is necessary to discern the difference between treatment groups. A previous study was only able to show the benefits of physiological estrogen replacement after a protocol of precondioning.[25] This also suggests that the underlying mechanism

responsible for clinically observed estrogen protection may be complicated and involve several other mechanisms of protection.

In summary, physiological estrogen replacement does not improve cardiac efficiency, contractile function or reduce H^+ production from glucose metabolism following global no-flow ischemia in the isolated working rat heart. This further suggests that isolated working hearts perfused with solutions containing physiologically relevant levels of fatty acids may produce contrary results to those isolated hearts perfused with glucose as the primary metabolic substrate. Furthermore, when these results are considered with the results presented in Chapter II, it is clear that more work is needed to determine the importance of ischemia duration and preconditioning in estrogenic cardioprotection.

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Chapter IV

Discussion

Thesis Objectives

The overall objective of this study was to determine the effects of physiological estrogen replacement on the contractile recovery of the isolated working rat heart following global no-flow ischemia. I hypothesized that physiological estrogen replacement would improve the postischemic recovery of contractile function by altering the profile of substrate energy metabolism in a way which reduces H^+ production from glucose metabolism. I further hypothesized that under such conditions, cardiac efficiency would return to a greater extent in hearts from estrogen replaced rats. Lastly, I hypothesized that at the conclusion of reperfusion, ATP concentrations and the ATP/ADP ratio would be improved in hearts from rats which were estrogen replaced.

To investigate this objective two studies were conducted. The first study examined the effects of physiological estrogen replacement on the recovery of contractile function in isolated working rat hearts after 20 minutes of global no-flow ischemia. The second study examined the effects of physiological estrogen replacement on the recovery contractile function, glycolysis, glucose oxidation, and palmitate oxidation in isolated working rat hearts following 25 minutes of global no flow ischemia. In addition, in contrast to the first study which used no fatty acids in

the perfusion solution, the second study examined the effects of a relatively high concentration of fatty acid (1.2 mM palmitate) to more closely replicate *in vivo* conditions.

Conclusions

Based on the objectives of this thesis, more detailed conclusions are provided in the following individual study sections. However, the primary conclusion of this thesis is that physiological estrogen replacement does not improve recovery of contractile function following global no-flow ischemia in isolated working rat hearts. As well, physiological estrogen replacement did not alter the profile of substrate energy metabolism or the overall production of H^+ from glucose metabolism. Moreover, physiological estrogen replacement did not improve cardiac efficiency or ATP concentrations following global no-flow ischemia. Based on these findings I must refute the previously stated hypothesis.

Physiological estrogen replacement has no effect on functional recovery of the isolated working rat heart following global ischemia when perfused without fatty acids

This study found that physiological estrogen replacement did not improve functional recovery of the isolated working rat heart following a 20 minute global no-flow ischemic insult. Furthermore, during a series of initial aerobic perfusion, physiological estrogen replacement, in general, did not alter cardiac function. This is in agreement with a previous study which found no improvements in the functional

recovery of hearts from physiologically estrogen replaced rats following 15 minutes of ischemia.[1] However, the same study did find that physiological estrogen replacement improved functional recovery when 20 minutes of global no-flow ischemia was preceded by 5 minutes of preconditioning global no-flow ischemia.

The disparity between our results and the results from this study may be explained on the basis of several observations. Firstly, while the estradiol level obtained in both our aerobic study and ischemia/reperfusion studies were well within the physiological range, the estradiol levels in the ischemia/reperfusion study were in the lower physiological range. It is interesting to note that the estrogen levels in our ischemia/reperfusion study were similar to estradiol levels reported in the previous 15 minute ischemia study, which also found no improvement in postischemic functional recovery.[1] Further support for the potential effects of slight differences in estradiol levels comes from a comparison of our own preliminary aerobic study and ischemia/reperfusion study. The estradiol levels in the estrogen replaced animals from the aerobic study were over 3 fold greater than estradiol levels found in the corresponding animals used in the ischemia/reperfusion. Although estrogen replacement did slightly alter functional parameters in the aerobic study, estrogen replacement did not alter functional parameters in the initial aerobic portion of perfusion in the ischemia/reperfusion study. This further suggests that even slight variations in chronic estradiol levels may significantly alter functional recovery following ischemia.

Secondly, while the importance of preconditioning cannot be directly assessed in the present study, we my gain a clearer understanding when the two studies are considered together. While Kolodgie *et al.* could only find estrogen-mediated improvement after preconditioning prior to 20 minutes of global no-flow ischemia, not data was given for 20 minutes of global no-flow ischemia without preconditioning. This leaves interpretation open as to whether an increase in ischemic insult, or addition of preconditioning is the variable responsible for revealing estrogenic cardioprotection. On the basis of the present study, I suggest that the addition of a preconditioning episode was responsible for revealing the cardioprotective effects of estrogen. This further suggests that the mechanism by which estrogen confers cardioprotection is complex and likely involves numerous mechanisms.

Although chronic administrations of estrogen seem less likely to promote rapid releases of NO than acute estrogen doses, a study using chronic estrogen administration has shown an upregulation in eNOS.[2] In addition another study reports a reduction in the area of necrosis seen in postischemic rabbit hearts following acute estrogen administration.[3] Although not directly providing evidence of myocardial blood flow changes, it does suggest that estrogen may be capable of improving myocardial blood flow following ischemia. The present study reports that following ischemia, physiological estrogen replacement resulted in no alterations in coronary blood flow following ischemia. Although our isolated rat heart model is not

completely suited to examining vascular blood flow changes, this suggests that estrogen-mediated alterations in blood flow may not be solely responsible for estrogenic cardioprotection.

Measures of ADP, ATP and the ATP/ADP ratio have been used as predictors of functional recovery of the myocardium.[4] Although these indicators provide only a limited insight into myocardial energy metabolism, they can provide another measure of comparison with which to investigate potential estrogenic effects. The metabolites of ADP, ATP, Cr, CrP and the ATP/ADP ratio are similar between the treatment groups. This further indicates that no estrogen-mediated cardioprotection occurred, although it can not rule out changes in energy metabolism. More detailed information on energy metabolism is included in Chapter III.

Physiological estrogen replacement in the ovariectomized rat has no effect on energy metabolism and functional recovery of the fatty acid perfused isolated working rat heart following global ischemia

This study found that physiological estrogen replacement did not improve functional recovery of the isolated working rat heart following a 25 minute global no-flow ischemic insult. These findings are in agreement with our previous research which reported no cardioprotective benefits from physiological estrogen replacement following 20 minutes of ischemia (Chapter II). These findings are also in agreement with a previous study which reported no estrogen-mediated benefits following 15

minutes of global no-flow ischemia.[1] Kolodgie *et al.*, did however report that physiological estrogen replacement did provide cardioprotection against a 20 minute global no-flow ischemic insult if ischemia was preceded by a 5 minute ischemic preconditioning episode. Therefore, it is difficult to determine from this previous work, if lengthening the duration of global no-flow ischemia or preconditioning is the condition responsible for revealing estrogen's cardioprotective benefits. On the basis of our two studies (Chapter 2 and Chapter 3) which used either comparable or longer lengths of ischemia, and had similar levels of estrogen replacement, I suggest that under these conditions, preconditioning may be the alteration responsible for bringing forth the protective effects of estrogen.

Although no direct measurement of estrogen-mediated alterations in vascular tone were made, our results do allow speculation. Previous studies have suggested that acute estrogen administration may cause the release of NO, a potent vasodilator.[6,7] More comparably, chronic administration of estrogen has demonstrated an upregulation of eNOS, an isoform of the NOS enzyme responsible for catalyzing the formation of NO.[5,6]. We observed no alterations in coronary flow suggestive of estrogen-mediated vasodilation. We did not however, measure NO formation or eNOS directly. Additional work is needed to reveal potential estrogen-mediated changes in vascular tone.

As well, this study found that physiological estrogen replacement did not alter energy substrate metabolism or the production of H^+ from glucose metabolism either

before or following ischemia. Because male hearts are predominantly used in metabolic studies, evaluating these findings in the light of other studies is difficult. However, although no direct comparisons are possible between this work and previous metabolic studies, useful insights may still be made when this study is compared to the male isolated working rat heart model.

Rates of glycolysis were slightly higher in female hearts as compared to glycolytic rates reported for hearts from male rats.[7,8] This elevation glycolytic rate was comparable between ovariectomized animals regardless of estrogen replacement. Although not significant, a similar decrement was seen in both the OVX and E_2 treatment groups following ischemia. As glycolytic rates in male hearts following ischemia remain constant or increase slightly, the importance of this decrease in female hearts is not full understood. Further experiments are need to determine the validity of this observation and potential importance of this phenomenon.

Because rates of glucose oxidation vary with the concentration of fatty acid in the perfusate hearts in this study could only be compared to male hearts perfused with similar levels of fatty acid (1.2 mmol palmitate). Rates of glucose oxidation determined in this study were comparable those reported for male rat hearts.[8,9] Following ischemia postischemic rates of glucose oxidation in hearts from female rats remained comparable to those of their male counterparts. Although no direct comparisons were made, it appears that a perfusate containing 1.2 mmol palmitate, results in rates of palmitate oxidation which were slightly lower in hearts from female

rats than in hearts from male rats.[8,9] Following ischemia, the palmitate oxidation rates remained constant or slightly higher, which is consistent with previous data from male hearts perfused under similar conditions.[8,9]

When the contribution of glucose oxidation and palmitate oxidation to overall TCA acetyl-CoA production is considered, both the OVX and E_2 treatment groups displayed and increased steady state rate of TCA Acetyl-CoA production. This is consistent with steady state rates of TCA acetyl-CoA reported for hearts from male rats following ischemia.[8] However, following ischemia the steady state rate of TCA acetyl-CoA production was greater in the E_2 as compared to the OVX group. This may be explained by slight, but non-significant increases in glucose and palmitate oxidation rates in the E_2 group, as compared to the OVX group. The importance of this increase remains to be adequately explained as cardiac efficiency, expressed as cardiac work/TCA acetyl-CoA, was not different in the E_2 group than the OVX group either before or following ischemia. Perhaps, increases in energy metabolism alone cannot fully predict functional recovery of the myocardium. This does however suggest that estrogen may play a role in the preservation the cellular energy substrate metabolism.

H^+ production from glucose metabolism was also calculated. Compared to previous studies on hearts from male rats, preischemic H^+ production rates appear to be elevated in both the OVX and E_2 groups.[8,9] The reason for this increase is speculative, but it appears to be largely the result of an increased glycolytic rate in

both treatment groups. The increase glycolytic rate is consistent in both groups, suggesting that this may be a phenomenon particular to hearts from females, or that both groups may have undergone some form of ischemic stress prior to perfusion. H^+ production from glucose metabolism following ischemia was greater in both groups as compared to their preischemic values. These increases are similar to those reported for hearts from male rats undergoing comparable ischemic insults.[8,9] These findings suggest that physiological estrogen replacement does not protect the heart from imbalances in glycolysis and glucose oxidation, and potential damage from H^+ formation.

The concentrations of the selected metabolites of ADP, ATP, Cr and CrP was comparable between groups. Furthermore, these values were consistent with the values reported for hearts from males.[10] The validity of these metabolites as predictors of functional recovery has been challenged.[10] Furthermore, the relationship between these metabolites and calculations of energy substrate metabolism previously discussed remains to be investigated. It is likely that determination of metabolic events occurring immediately after ischemia are better predictors and of greater importance in improving overall functional outcome, than more distant measures of ATP, ADP, Cr and CrP.[11,12]

Summary

Following physiological estrogen replacement, cardiac efficiency remains unchanged and contractile function is neither harmed or improved following global

no-flow ischemia. Likewise, after global ischemia, physiological estrogen replacement did not alter H^+ production from glucose or ATP concentrations. The lack of estrogen-mediated cardioprotection may be the result of a relatively high recovery of contractile function in both control and estrogen replaced rats and relatively few alteration in energy metabolism. Under these conditions it is possible that slight estrogen-mediated improvements in energy metabolism and contractile function are masked by a large recovery of contractile function following ischemia.

Limitations

One limitation of this thesis is the lack of numerous ischemia times. Although two lengths of ischemia were used, 20 and 25 minutes, a relatively high recovery of contractile function was seen. A series of increasingly longer ischemic events, with a corresponding decrease in functional recovery, would have allowed a better examination of potential estrogenic cardioprotection. While another study reported no differences after 15 minutes of ischemia, it did find estrogen-mediated cardioprotection following preconditioning.[1] This indicates that estrogenic cardioprotection may be a complex event, requiring specific conditions to produce observable effects. As well, inclusion of a low flow model of ischemia may allow better elucidation of estrogenic cardioprotection.

Our model of estrogen replacement is also a source of limitation. While ovariectomized rats were estrogen replaced for 21 days it is clear that clinical estrogen replacement therapy is much longer. Therefore it is possible that the present estrogen

replacement protocol misses mechanisms which take longer to develop. Furthermore, the rats used in this study are relatively young while in clinical practice, typically older women receive hormone replacement therapy. This means that estrogen replacement may have potentially different effects on vascular endothelium, vascular smooth muscle and myocardial cells, depending on the age of the individual.

Another limitation of this thesis is the lack of data on key regulatory enzymes. The observed metabolic pathways of glycolysis, glucose oxidation, and fatty acid oxidation, all have specific enzymes which contribute to their regulation. As such, the activity of these pathways is determined to a great extent by the activity and expression of these enzymes like PDC, ACC, and AMPK. Determination of expression and activity of key enzymes would help piece together a better picture of metabolism. Furthermore, any observed changes in regulatory enzymes may help identify estrogens sites of action

This thesis is also limited by our knowledge of metabolism in female rat hearts. While numerous studies have examined the effects of ischemia on the recovery of contractile function in male rat hearts, only inference could be used as a basis of comparison between male and female hearts. Consequently, an additional experimental series, designed to investigate metabolism in hearts from intact female rats would allow a more accurate comparison between hearts from intact female, ovariectomized and estrogen replaced ovariectomized rats.

Future Directions

As eluded to as a potential limitation, variations in the duration of ischemia may produce contrasting results. A greater ischemic insult may better show the benefits of physiological estrogen replacement. For example, if estrogenic cardioprotection is the result of its antioxidant activity, then a short ischemic time may not create a sufficient environment of oxidative stress for estrogen to be beneficial. It is also possible, that slight improvements in each mechanism may combine to create an observable benefit under conditions of greater stress.

Additionally, identification and localization of authentic estrogen receptors is needed. It is possible that myocardial, vascular endothelial, and vascular smooth muscle cells, contain very different distributions of ERα and ERβ. Furthermore, the potential role of theses estrogen receptors in cardioprotection is unknown. Localization of these estrogen receptors may also be valuable when interpreting evidence of estrogenic regulation of cellular processes. For example, while evidence has already suggested that estrogen is involved in the regulation of eNOS, which of the estrogen receptor subtype or subtypes responsible is unclear.[2,13,14]

Future *in vivo* studies could also contribute valuable insight into the nature of potential estrogenic benefits. Numerous studies have used snare occlusion techniques on the left anterior descending coronary artery to create ischemia. After the use of the snare the heart can be frozen, sectioned and dyed to reveal areas of local ischemia and area at risk. Such techniques could help reveal changes in blood flow and antioxidant

activities mediated by estrogen. In addition, these studies would benefit from a basal level of circulating estrogen which would be present at time of ischemia. This is something we have not been able to reproduce in the isolated working rat heart model

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