Comparison of the effects of docosahexaenoic acid and palmitic acid on ischemia reperfusion injury using an isolated perfused rat heart

by

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AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

Dietary docosahexaenoic acid (22:6n-3, DHA) has been shown to exert beneficial effects on coronary heart disease including the prevention of ischemia reperfusion injury. The ability to acutely infuse DHA to the heart to prevent ischemia reperfusion injury is a potentialy valuable tool in planned surgery where reperfusion and/or ischemia will take place including coronary artery bypass surgery and angioplasty. In the present study, hearts from chow-fed (AIN-93M) Sprague Dawley rats (male) 9-12 weeks of age were isolated and artificially perfused. The protocol included: 30 min stabilization period, 30 min global no flow ischemia, 15 min fatty acid infusion with reperfusion, and 75 min reperfusion in the absence of fatty acids. The fatty acid infusions included 10, 20, 40, 60, 80, 100 or 120 μM of either palmitate or DHA complexed to 3% essentially fatty acid free bovine serum albumin as well as a vehicle control. Heart functional data was recorded continuously and total heart infarct volume was determined after staining with triphenyltetrazolium chloride. DHA at 10μM significantly reduced the infarction area at the end of the reperfusion period compared to that observed in the10μM of palmitate and vehicle control conditions. Infarction areas after infusions with DHA or palmitate were similar to controls after 20-60 μM infusions and greater than controls after 80-120 μM infusions, except for the 100 μM palmitate conditions which were similar to the low and high doses. In this model of infusion, 120 μM of fatty acid was the maximum amount of DHA tolerated, as several hearts went into fibrillation and did not recover and failed to complete the reperfusion at concentrations greater than 120 μM of DHA. DHA and palmitate also exerted dose dependent effects on functional parameters. In summary, infusion of DHA and palmitate cause dose dependent effects on heart function.

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List of Abbreviations

AA	arachidonic acid	22:5n-6
ACC	acetyl-CoA carboxylase	
AGPAT	1-acylglycerol-3-phosphate O-acyltransferase	
ALA	alpha-linolenic acid	18:3n-3
AMP	adenosine monophosphate	
AMPK	5' AMP-activated protein kinase	
ANT	adenine nucleotide translocase	
ATP	adenosine-5'-triphosphate	
BSA	bovine serum albumin	
CAT	carnitine translocase	
CDP-DAG	cytidine diphospho-DAG	
CHD	coronary heart disease	
CL	cardiolipin	
COX-2	cyclooxygenase- 2	
CPT-1 and 2	palmitoyl transferase-1 and 2	
DAG	diacylglycerol	
DHA	docosahexaenoic acid	22:6n-3
DMA	dimethyl acetals	
DMSO	dimethyl sulfoxide	
EPA	eicosapentaenoic acid	20:5n-3
ER	endoplasmic retuiculum	
FA	fatty acid	
FABP	fatty acid binding protein	
FABPpm	plasma membrane associated fatty acid binding protein	
$FADH_2$	flavin adenine dinucleotide and 2 hydrogen	
FAT/CD-36	fatty acid translocase	
FATP	fatty acid transport proteins	
FO	fish oil	
GPAT	glycerol-3-phosphate acyltransferase	
HR	heart rate	
HSP	heat shock proteins	
iNOS	cytokine-inducible nitric oxide	
JNK	c-Jun N-terminal kinases	
KHB	Krebs-Henseleit bicarbonate buffer	
LA	linoleic acid	18:2n-6
LCFA	long chain fatty acid	
LCPUFA	long chain polyunsaturated fatty acid	
LDH	lactate dehydrogenase	
LPA	lysophosphatidic acid	
LPAAT	LPA acyltransferase	
LPLAT	lysophospholipid acyltransferase	
LVDevP	left ventricular developed pressure	

LVEDP	left ventricular end-diastolic pressure
MBCD	methyl-β-cyclodextrin
MI	myocardial infarction
MPTP	mitochondrial permeability transition pore
mRNA	messenger ribonucleic acid
MUFA	monounsaturated fatty acids
n-3	omega - 3
n-6	omega - 6
NADH	nicotinamide adenine dinucleotide + hydrogen
NEFAs	non-esterified fatty acids
NHE	sodium hydrogen exchanger
PA	phosphatidic acid
PC	phosphatidylcholine
PDH	pyruvate dehydrogenase
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PI	phosphatidylinositol
PI3K	phosphatidylinositol 3-kinases
PKC	protein kinase C
PL	phospholipids
PLA_2	phospholipase A_2
PS	phosphatidylserine
PUFA	polyunsaturated fatty acids
ROS	reactive oxygen species
Sys	systolic
TAG	triacylglycerols
TBARS	thiobarbituric acid
TBS-T	tris-buffered saline- tween
TCA	tricarboxylic acid cycle
TLC	thin-layer chromatography
TLE	total lipid extracts
TNF-α	tumour necrosis factor alpha
TTC	triphenyltetrazolium chloride
TTC	triphenyltetrazolium chloride
VDAC	voltage anion channel
VLCFA	very long chain fatty acid

Chapter 1
Introduction

1.1 Introduction

The cardiovascular benefits of dietary fish oil were first noted in early 1970's as the Inuit of Greenland were observed to have a very low rate (3.5%) of death from ischemic heart disease relative to Western populations despite a significantly higher intake of meat and fat and lower intakes of fruits and vegetables (Bang, Dyerberg, & Nielson, 1971). Due to the landscape of northern Greenland, the natives are mostly hunters and fishermen, eating mostly meat of whales, seals/walrus, and fish. Meat from marine origin is very high in omega-3 polyunsaturated fatty acids (PUFA), specifically the long-chain omega-3 PUFA, or omega-3 highly unsaturated fatty acids (HUFA, ≥20 carbons, ≥3 carbon-carbon double bonds). After analyzing the diet of the Greenland Inuit, it was found that their diet consisted of approximately half the amount of omega-6 fatty acids and almost 5 times the amount of omega-3 fatty acids compared with Danish controls. Their serum lipid profiles reflected this; omega-6 HUFA were replaced largely by omega-3 HUFA (Bang, Dyerberg, & Sinclair, 1980).

One of the main benefits of dietary fish oils on coronary heart disease is an antiarrhythmic effect that was first noticed in the GISSI-Prevenzione trial (Valagussa et al., 1999). This was a large, prospective, randomized controlled trial in which fish oil therapy reduced sudden cardiac death (death within 1 hour of symptoms of an acute MI attributed to sustained ventricular arrhythmias) 45% compared to controls ($P < .001$) (Valagussa et al., 1999). Risk of sudden cardiac death has also been shown to be inversely related to base-line blood levels of eicosapentaenoic acid (EPA) and docosahexaenoic Acid (DHA) in men without evidence of prior cardiovascular disease (Albert et al., 2002).

The antiarrhythmic effect of dietary fish oil has also been demonstrated in animal studies. This includes dietary intervention studies, where rats fed a diet supplemented with tuna fish oil significantly reduced the incidence and severity of arrhythmias, preventing ventricular fibrillation during both occlusion and reperfusion and that feeding rats saturated fatty acids from sheep fat (high in saturated fats) significantly increased the severity of arrhythmias (McLennan, Abeywardena, & Charnock, 1988). Acute infusion with n-3 HUFA has also produced antiarrhythmic results. In a highly reliable surgically induced ischemia model of sudden cardiac death in canines, infusion with a fish oil emulsion prevented the occlusion that typically followed arrhythmia in untreated animals or animals treated with a soybean based infusion. (Billman, Hallaq, & Leaf, 1994; Billman, Kang, & Leaf, 1997). These results were reproduced using pure infusions of EPA and DHA (Billman, Kang, & Leaf, 1999).

Ischemia reperfusion injury is a major determinant of infarct size. Infarcted myocardium causes fatal ventricular arrhythmias (Zeghichi-Hamri et al., 2010). Previous dietary studies have been conducted to examine the effect of omega 3 highly unsaturated fatty acids on protection from ischemia reperfusion injury. A few studies have used an isolated perfused (Langendorff) heart model to examine the effects of omega 3 fatty acids in the absence of any neuronal or hormonal factors and have reported conflicting results. It has been shown that diets high in n-3 HUFA (and low in saturated and n-6 fatty acids) reduce (Zeghichi-Hamri et al., 2010; Abdukeyum, Owen, & McLennan, 2008) or have no effect (Force et al., 1989) on myocardial infarct size in rats. This effect may have been caused by the reduction of other dietary fatty acids and not just the increase in omega 3 HUFA or the effect of the diets on other organs. Generally, DHA was incorporated into the hearts as phospholipids (Zeghichi-Hamri et al., 2010) which were likely released by a variable amount by phospholipases whose activities are increased during

ischemia (Ford, Hazen, Saffitz, & Gross, 1991). To the best of our knowledge no study has been conducted examining the affects of an acute infusion of DHA on ischemia reperfusion injury. Therefore, the purpose of the present study is to examine the effect of an acute infusion of DHA in a model of ischemia reperfusion. The ability to acutely infuse DHA to the heart to prevent ischemia reperfusion injury would be an extremely valuable tool in planned surgery where reperfusion and/or ischemia will take place including coronary artery bypass surgery and angioplasty.

1.2 Biochemical and Physiological Foundations

1.2.1 Anti-arrhythmic Effects of Omega-3 HUFA

Antiarrhythmic effects as well as enhanced postischemic recovery of heart function have been shown for dietary fish oil in several animal studies (see **Table 1**). The mechanism by which free HUFA slows the contractions of cardiomyocytes and prevents arrhythmias is not fully known, however it is likely a result of their effects on the electrophysiology of cardiomyocytes (Kang & Leaf, 1995). N-3 HUFA act on ion channels to hyperpolarize membrane resting potential, increase the action potential threshold, and reduce membrane electrical excitability by significantly raising the threshold of depolarizing current required to initiate an action potential. Myocytes on the border of an ischemic zone have comparatively depolarized resting membrane potentials and probably trigger fibrillation because they are easily excited (Xiao et al., 2008). In addition, n-3 HUFA prolong the refractory period and decrease action potential duration. This is accomplished by suppressing Na^+, K^+ and voltage gated Ca^{2+} currents. Activation/opening of Na^+ channels allows a rapid influx of sodium ions which leads to the initiation of action potentials. The cardiac Na^+ channel is comprised of a large α-subunit and a smaller β_1-subunit. It is

speculated that the α-subunit has a specific binding site for n-3 HUFA. EPA and DHA binding to the Na^+ channel accelerates the transition from the resting state to the inactivated state. This prolongs the recovery time from inactivation (Xiao, Sigg, & Leaf, 2005).

Voltage gated L-type Ca^{2+} current causes a Ca^{2+} induced Ca^{2+} release from the sarcoplasmic reticulum. An overload of Ca^{2+} in the cytosol can cause cardiac arrhythmias, so prevention of this overload would be antiarrhythmic. The free fatty acid form of n-3 HUFA is antiarrhythmic during Ca^{2+} overload in isolated cultured neonatal rat cardiac myocytes (Kang & Leaf, 1994). EPA prevents fibrillations when added before Ca^{2+} induction and EPA terminates fibrillations when added after induction. Treatment with bovine serum albumin (BSA), which extracts non-esterified fatty acids, re-establishes the induced contractures and fibrillations (Kang & Leaf, 1994). This finding suggests that incorporation of the fatty acids into the membrane phospholipid is not required for their action. BSA mediated extraction would not be possible if the fatty acids had been incorporated into phospholipids or if any other covalent or ionic bonding had occurred (Leaf, Xiao, Kang, & Billman, 2003). The direct action of the n-3 HUFA is the blockade of the voltage-gated L-type Ca^{2+} current which reduces Ca^{2+} entry. Only the free fatty acid form of the HUFA with a free carboxyl group was able to block cardiac Ca^{2+}, suggesting that it might be interacting with the positively charged amino acids of the channels by noncompetitive inhibition, prolonging the inactivated state (Xiao, Gomez, Morgan, Lederer, & Leaf, 1997) (Xiao, Wright, Wang, Morgan, & Leaf, 2000).

In heart, voltage-gated K^+ channels determine the resting potential, shape, and length of the action potential, thus controlling cardiac performance (Honore, Barhanin, Attali, Lesage, & Lazdunski, 1994). It has been demonstrated that DHA blocks the major voltage dependent K^+ channel (Kv1.5) in cardiac cells. Inhibition of the Kv1.5 channel activity by HUFA is similar to

that produced by class 3 antiarrhythmic agents and is also expected to be beneficial during ischemia when extracellular accumulation of K^+ occurs. Blockage of Kv1.5 resulted in inhibition of the delayed rectifier current (I_K) and the transient outward current (I_{to}), the two main outward K^+ currents (Honore et al., 1994; Xiao et al., 2005). The inward rectifying current (I_{K1}),which is the main inward K^+ current, was unchanged by n-3 HUFA (Xiao et al., 2005). MUFA's and saturated fatty acids which are not antiarrhythmic exert no effects on the major K^+ currents (Xiao et al., 2005). N-3 HUFA assist in stabilizing the increased excitability of partially depolarized cells and in doing so prevent arrhythmias (Xiao et al., 2008).

It has been demonstrated that n-3 HUFA in the phospholipid pool have no antiarrhythmic effect (Weylandt, Kang, & Leaf, 1996), but they are available to perform their antiarrhythmic action after release by phospholipases, specifically phospholipase A_2 which acts to release fatty acids (commonly n-3 HUFA) from their sn-2 position. Phospholipase A_2 is rapidly activated by catecholamines as with a myocardial infarction (MI) (by activation of the sympathetic innervation in the heart) as well as during ischemia (Ford et al., 1991). After release, the free fatty acids are available to exert their antiarrhythmic effects. During a period of increased n-3 HUFA intake, the phospholipid content of EPA and DHA increases (Charnock, Abeywardena, & McLennan, 1986). This increases the availability of DHA to be released from phospholipids to act on ion channels. In addition, the incorporation of HUFA into membranes increases membrane fluidity which may indirectly affect the activity of sarcolemmal proteins including ion channels (Spector & Yorek, 1985).

Table 1. Dietary fatty acids and ischemia reperfusion studies.

Authors	*Sex and type subjects*	*Treatment*	*Special diet (n)*	*I/R time (min)*	*Conclusions*
(McLennan et al., 1988)	Male, hooded wistar	*In vitro* occlusion of left anterior descending coronary artery	Control (11), TFO (10), SF (11), SSO (11)	15/15	Dietary tuna FO diet significantly reduced the incidence and severity of arrhythmias, preventing ventricular fibrillation during both occlusion and reperfusion.
(Demaison et al. 2003)	Male, wistar	Langendorff	8 wks (10) SSO, (10) FO	20/50	Dietary FO improves the recovery of the cardiac pump function during reperfusion.
(McLennan, 1993)	Sex not specified, SD	*In vitro* occlusion of left anterior descending coronary artery	12 wk on SSO (25), SFA (24), OO (25), FO (24)	15/10 5/10	Dietary replacement of saturated fats by n-6 and especially n-3 PUFA but not MUFAs can reduce the likelihood of an ischemic event leading to SCD.
(Abdukeyum et al., 2008)	Male, Wistar	IPC vs. diet 30 min ischemia and 120 reperfusion	6 weeks on FO, SSO, or SFA (54)	30/120	Dietary FO induces a form of preconditioning. Provides cardioprotection as powerful as IPC.
(McLennan, Bridle, Abeywardena, & Charnock, 1993)	Marmoset monkeys	*In vitro* electrophysiological testing to determine arrhythmia vulnerability.	16 week, OO (25), SFA (24), SSO (25), FO (24)	15/30	Dietary replacement of saturated fats by n-6 and especially n-3 PUFA but not MUFAs can reduce the likelihood of an ischemic event leading to SCD.
(Pepe & McLennan, 1996)	Male Hooded-Wistar Rats	Electrophysiological testing in Langendorff perfused hearts.	16 wk Low fat (20), SFA (10), FO (10)	15/10	Dietary FO prevented VF in reperfusion after ischemia and elevated the current required to elicit VF during a programmed ventricular stimulation protocol under control or ischemic conditions. When arrhythmias occurred in the hearts from fish oil-fed animals following any stimulus, the hearts always rapidly and spontaneously reverted to sinus rhythm
(Zeghichi-Hamri et al., 2010)	Male. Wistar, n=42	Occlusion of left coronary artery	8 week, SFA, n-6, and n-3 (42)	30/120	Improved myocardial resistance to ischemia-reperfusion

FO, fish oil; OO, olive oil; SSO, sunflower seed oil; SFA, saturated fatty acids; IPC, ischemic preconditioning; MUFA, monounsaturated fatty acids; SCD,sudden cardiac death; VF, ventricular fibrillation

1.2.2 Ischemia Reperfusion Injury

Ischemia is a lack of blood flow which causes an imbalance between the demand for oxygen and substrates, and their delivery through blood, leading to anaerobic metabolism and decreased contractile function (Verdouw, van den Doel, de Zeeuw, & Duncker, 1997). Ischemia causes an overload of intracellular calcium. Ion channel problems begin to occur as ATP stores become depleted. Na^+/K^+ ATPase activity is decreased and the intracellular levels of Na^+ rise. The sodium hydrogen exchanger (NHE) tries to correct the increased H^+ levels and in doing so increases the influx of Na^+ ions. High intracellular levels of Na^+ reverses the direction of the Na^+/Ca^{2+} exchanger and increases Ca^{2+} influx and subsequently Ca^{2+} intracellular levels. Calcium reuptake by sarcoplasmic reticulum Ca^{2+}-ATPase, is reduced further contributing to an overload of calcium ions due to energy depletion (Carmeliet, 1999).

Reperfusion of coronary flow can save ischemic tissue and restore overall heart function. Reperfusion can also paradoxically induce contracture, cellular injury and death (Piper, Meuter, & Schafer, 2003). While reperfusion injury and ischemic injury are distinct, they share common pathways (**Table 2**) including, the production of reactive oxygen species (ROS), intracellular calcium overload and endothelial dysfunction (Verma et al., 2002). Excessive levels of ROS lead to damage of proteins and lipids and can induce apoptosis (Diep, Intengan, & Schiffrin, 2000).

Table 2. Summary of the mechanisms of ischemia reperfusion injury.

Ischemia	*Reperfusion*
Oxidative Stress	Oxidative Stress
Intracellular calcium overload	Intracellular calcium overload
Endothelial dysfunction	Endothelial dysfunction
ATP depletion	Restoration of pH
Tissue acidosis	Opening of the MPTP

MPTP, mitochondrial permeability transition pore.

Calcium overload in cytosol is very damaging to cardiac cells and causes cardiac arrhythmias and contracture (ter Keurs et al., 2001). An overload leads to cell damage by activating phospholipases (Carmeliet, 1999) and opening of the mitochondrial permeability transition pore (MPTP). MPTP is a protein complex spanning both inner and outer mitochondrial membranes. It is made up of the voltage anion channel (VDAC) in the outer membrane, the adenine nucleotide translocase (ANT) in the inner mitochondrial membrane and cyclophilin-D in the matrix. Under physiological conditions it is closed (Lavi & Lavi, 2011). Opening of this pore occurs during high levels of intracellular Ca^{2+} as well as depletion of adenine nucleotides and allows the release mitochondrial calcium and apoptotic inducing factors including Cytochrome-C (a caspase activator) and Smac/DIABLO (a caspase co-activator) (Zamzami & Kroemer, 2001). MPTP opening is inhibited by a low pH, correction of ischemia induced acidosis during reperfusion allows MPTP to open. This causes the pore to open mainly in reperfusion and less often during ischemia (Halestrap, Clarke, & Javadov, 2004) contributing to reperfusion injury. Cytochrome c loss results in uncoupling of the mitochondrial respiratory chain and a decrease in ATP synthesis (Zamzami & Kroemer, 2001). Cytochrome C binds to the cystolic protein Apaf-1 which causes activation of caspase 3 and 9 (Adrain & Martin, 2001). Smac/DIABLO indirectly activates Caspase 3 and 9 by sequestering caspase-inhibitory proteins (Zamzami & Kroemer, 2001). Pro-apoptotic and anti-apoptotic regulating proteins such as the Bcl-2 family of proteins can affect the MPTP by promoting either its opening or closure by associating with the components of the mitochondrial pore. Bcl-2 and Bcl-xl are anti-apoptotic and conversely, Bad, Bid, Bim, and Bax are pro-apoptotic (Gross, McDonnell, & Korsmeyer, 1999).

1.2.3 Reperfusion and Omega-3 HUFA

Reperfusion injury contributes to the ventricular arrhythmia and ischemic death (Bolli & Marban, 1999) therefore prevention of this is of upmost importance. DHA appears to play a role in the prevention of reperfusion injury. Dietary DHA supplementation delays Ca^{2+} induced MPTP opening (Khairallah et al., 2010), but more studies are needed. The ability to acutely infuse DHA to the heart to prevent ischemia reperfusion injury would be an extremely valuable tool in planned surgery where reperfusion and/or ischemia will take place including coronary artery bypass surgery and angioplasty. Therefore, the purpose of the present study is to examine the effect of an acute infusion of DHA to a model of ischemia reperfusion.

1.2.4 Ischemia, Preconditioning and Cardioprotective Agents

Following periods of ischemia and subsequent reperfusion, the heart often experiences an episode of reversible prolonged postischemic contractile dysfunction known as stunning (Kloner & Jennings, 2001b). Depending on the degree and duration of the initial ischemic insult, this can last from hours to weeks (Bolli & Marban, 1999). The mechanism of stunning is complex and not fully known, however it appears that it involves oxygen derived free radicals generated during reperfusion as well as an alteration in calcium homeostasis (Bolli & Marban, 1999). Evidence for this comes from experimental studies showing that oxygen radical scavengers and calcium channel blockers enhance function of stunned myocardium (Kloner & Jennings, 2001a). Brief periods of ischemia (≤15 minutes) can contribute to prolonged left ventricular dysfunction, but they can also have a cardioprotective role. Brief episodes of ischemia followed by reperfusion have been shown to protect the heart from extensive necrosis during subsequent longer periods of ischemia, and this phenomenon is termed ischemic preconditioning. Preconditioning is the

exposure to a nonlethal stimulus activating the natural endogenous cellular stress response to protect against a subsequent more lethal injury. Ischemic preconditioning was first shown by Murry at al. in 1986 using a canine model. These authors found that 4 bouts of 5 min cycles of ischemia with reperfusion protected the heart from a subsequent episode of ischemia (40 min) and reduced infarct size up to 75%. Following these brief periods of sub lethal ischemia, the heart quickly adapted itself to a phenotype that was more resistant to infarction (Murry, Jennings, & Reimer, 1986). Ischemic preconditioning markedly reduces arrhythmias during both ischemia and reperfusion, and also completely eliminates ventricular fibrillation (Hagar, Hale, & Kloner, 1991). Preconditioning also delays the onset of necrosis and preserves levels of high-energy phosphate intermediates (Hagar et al., 1991).

Ischemic preconditioning demonstrates a biphasic pattern with an "early phase" which starts within a few minutes of the preconditioning stage and lasts up to 3 hours, and a "late phase" which begins 12-24 hours following preconditioning and lasts 2-4 days (Marber, Latchman, Walker, & Yellon, 1993). The mechanism of ischemic preconditioning is not well understood, however, it appears that adenosine, opioids, low doses of reactive oxygen species (ROS), tumour necrosis factor alpha (TNF-α), bradykinins and prostaglandins that are released during ischemia may play a role. These signals activate G-protein-coupled receptors and initiate a signaling cascade mediated largely by protein kinase C (PKC) (Das & Das, 2008). During a time of negative energy balance such as during ischemia, adenosine is free to move into the interstitial space where it can associate with adenosine receptors. Two of the four subtypes of this receptor, A_1 and A_3, have been implicated as triggers of the preconditioned state (Liu et al., 1994). This binding of adenosine to its receptors activates protein kinase C (PKC), specifically the ε isoform (PKCε), which has been implicated as the isoform responsible for ischemic preconditioning (Liu,

Cohen, Mochly-Rosen, & Downey, 1999). PKCε triggers a cascade of events that are not fully understood (Lavi & Lavi, 2011).

While the early phase is caused by quick signaling cascades using existing proteins, the late phase involves synthesis of new proteins. Ischemic preconditioning induces transcription of proteins shown to be cardioprotective mediators such as iNOS, COX-2 and heat shock proteins (HSP), and their expression coincides with the "late phase" of preconditioning (Valen, 2003). Increased HSP expression in rats through *in vivo* heat exposure has been demonstrated to provide a protective effect against ischemia ex vivo with induced ischemia with a Langendorff model (Currie, Karmazyn, Kloc, & Mailer, 1988). In particular, focus has been on HSP 70, which has two isoforms; the constitutive HSP70c (HSP73) and the inducible HSP70i (HSP72) (Latchman, 2001). HSP72 is the predominant isoform involved in protection from ischemia reperfusion injury. HSP inhibits apoptosis, decreases cytokine production and decreases activation of the pro-apoptotic JNK pathway (Latchman, 2001).

1.2.5 Preconditioning and Omega-3 HUFA

A preconditioning effect has also been shown for dietary n-3 HUFA (Abdukeyum et al., 2008). For 6 weeks, male Wistar rats were fed a diet containing either DHA and olive oil (n-3 PUFA), sunflower seed oil and olive oil (n-6 PUFA), or beef tallow and olive oil (saturated). Hearts from rats fed the n-3 PUFA diet had significantly lowered heart rate, end diastolic pressure and ischemic and reperfusion arrhythmias. Most importantly these hearts had a smaller infarct size compared to both n-6 and saturated fat dietary groups. This nutritional preconditioning effect was as powerful as the ischemic preconditioning effect seen in the n-6

PUFA and saturated fat groups. In addition, sub maximal ischemia preconditioning provided a benefit for n-6 PUFA and saturated groups, but no additional benefits for the n-3 PUFA group.

In an acute study, rabbits were pretreated for 4 days over 4 hours with a lipid emulsion containing both EPA and DHA at a dose of 5 ml/kg prior to an in situ 30 minute ischemic insult followed by 3 hours of reperfusion. This produced a 40% reduction in infarct size ($p<0.01$) compared to controls receiving 0.9% saline infusions and a 225% increase in heat shock protein 72 (HSP72) levels ($p<0.02$) (McGuinness, Neilan, Sharkasi, Bouchier-Hayes, & Redmond, 2006). HSP72 induction is used as a marker of preconditioning because a direct correlation between the amount of HSP72 induced and the degree of myocardial protection has been found (Hutter, Sievers, Barbosa, & Wolfe, 1994).

The term preconditioning is loosely defined and the difference between a preconditioning agent and a cardioprotective agent is unclear. With preconditioning, a nonlethal stimulus activates the natural endogenous cellular stress response to protect against a subsequent more lethal injury. It may be possible that n-3 HUFA act as both a preconditioning and a cardioprotective agent. Observations of increased heat shock proteins and a reduced infarct size implies that DHA infusions may provide an initial insult to the myocardium which upregulates protective preconditioning mechanisms (McGuinness et al., 2006). Alternatively, studies demonstrating a benefit of acute infusion of pure DHA and EPA (Kang & Leaf, 1994; Kang & Leaf, 1995; Kang & Leaf, 1996; Xiao et al., 2008) provide the argument that n-3 HUFA act as a direct cardioprotective agent.

1.2.6 Fatty Acid Uptake and Oxidation

Short Chain (≤6 carbons) and medium chain (8-12 carbons) fatty acids are transported via simple diffusion or "flip-flop". Long chain (14-22 carbons) and very long chain fatty acids (>22 carbons) may require additional steps in their movement across muscle plasma and mitochondrial membranes. Long chain fatty acids cross membranes with the assistance of fatty acid transporter (Mahadeva & Sauer, 1971). These fatty acid transporters include the integral membrane protein fatty acid translocase (FAT/CD-36), the peripheral membrane protein plasma membrane associated fatty acid binding protein (FABPpm), heart specific cytoplasmic fatty acid binding protein (H-FABP), and fatty acid transport proteins (FATP1 and FATP6). These transporters are all present in heart and located at the sarcolemma (Luiken, Turcotte, & Bonen, 1999; Luiken, vanNieuwenhoven, America, Vandervusse, & Glatz, 1997; Abumrad, Park, & Park, 1984; Luiken et al., 1997). The rate of uptake is governed by the rate of subsequent metabolic reactions inside the cell (Degrella & Light, 1980). A trans-sarcolemmal rate of uptake of 65 to 85 nmol/ gram of wet weight of myocardial tissue in 1 minute has been reported (Musters, Bassingthwaighte, Van Riel, & van der Vusse, 2006).

The heart is a metabolic omnivore with the capacity to oxidize fatty acids, carbohydrates, lactate, ketone bodies and even amino acids. Glucose is rapidly phosphorylated and is either incorporated into glycogen or catabolised (Schwenk, Luiken, Bonen, & Glatz, 2008). Long chain fatty acids (12-18 carbons and varying degrees of unsaturation) are a major fuel source of the heart, with fatty acid oxidation normally providing 60-70% of the hearts energy requirements (Bing, 1965; Neely & Morgan, 1974). These levels can be changed based on the availability of substrates. Because there is limited capacity for lipid storage in cardiac myocytes, the uptake and oxidation of fatty acids need to be tightly coupled (Chabowski, Gorski, Calles-Escandon,

Tandon, & Bonen, 2006). The β-oxidation of fatty acids is under complex dynamic control which depends on a number of factors, including fatty acid supply to the heart; structure and saturation of the fatty acid being supplied, endogenous supplies; the presence of competing energy substrates (glucose, lactate, ketones, amino acids); hormonal and neuronal control; energy demand of the heart; oxygen supply; fatty acid uptake and esterification; mitochondrial transport and drugs (Lopaschuk, Ussher, Folmes, Jaswal, & Stanley, 2010). Existing double bonds of unsaturated fatty acids commonly need to be relocated by an isomerase to allow for a required dehydration reaction to occur before β-oxidation can begin (Christie, 1989). Very long-chain fatty acids (>22carbons) cannot directly enter the mitochondria. Their long hydrocarbon chain must first be shortened to only 18 carbons by peroxisomal β-oxidation (Reddy & Hashimoto, 2001).

1.2.7 Oxidation during Ischemia

During periods of limited oxygen supply in the heart, both fatty acid and carbohydrate oxidation decrease and ATP production is impaired as oxidative phosphorylation is limited. In an attempt to compensate for the decrease in ATP supply, glycolysis and glycogenolysis initially increase but become uncoupled from oxidative phosphorylation. The shift from aerobic or mitochondrial metabolism to anaerobic glycolysis occurs after only 8 seconds of reduced arterial flow to the heart. The shift occurs as soon as the O_2 trapped in the tissue as oxyhemoglobin and oxymyoglobin is consumed (Jennings, Murry, Steenbergen, & Reimer, 1990). As oxygen becomes scarcer, mitochondrial electron transport must decrease. The cycling of acetyl intermediaries declines leading to a rise in the acetyl-CoA/CoA ratio. This results in a decrease in both fatty acid β-oxidation and pyruvate dehydrogenase complex activity. Levels of H^+, K^+, AMP

and lactate climb (Saddik & Lopaschuk, 1991) and fatty acids are diverted from β-oxidation into the myocardial lipids depots (Moore, Radloff, Hull, & Sweeley, 1980; Vandervusse, Roemen, Prinzen, Coumans, & Reneman, 1982). At this time cell-to-cell electrical uncoupling occurs with the onset of ischemic contracture (stiffening of the myocardium), ECG changes appear and cystolic Ca^{2+} increases significantly. Contracture, which is marked by a distinct rise in left ventricular end diastolic pressure, occurs by an absence of ATP and an overload of calcium in the myocyte causing actin-myosin crossbridges to remain in an attached state (Piper et al., 2003). Ischemic contracture makes cardiomyocytes more fragile and susceptible to mechanical damage. Late in the reversible phase of ischemia up to 80% of the ATP present at the onset of ischemia has disappeared. Reperfusion and oxidative flux may occur before permanent damage occurs to the myocardium, but the control of fatty acid and glucose oxidation remains impaired (Jennings, Schaper, Hill, Steenbergen, & Reimer, 1985).

During reperfusion of the reversibly injured heart, the predominant source of ATP production is fatty acid oxidation (Saddik & Lopaschuk, 1991). These high rates of fatty acid oxidation exert detrimental effects on functional recovery of hearts following ischemia (Lopaschuk, Wall, Olley, & Davies, 1988) and contribute to contractile dysfunction or "stunning" by markedly inhibiting glucose oxidation rates (Kantor, Dyck, & Lopaschuk, 1999; Lopaschuk, Wambolt, & Barr, 1993), and by increasing myocardial oxygen consumption (Oram, Bennetch, & Neely, 1973). This alteration in energy utilization may be partly caused by substrate supply to the heart during reperfusion. In a clinical setting, elevated plasma concentrations of fatty acids occur either after acute myocardial infarction or cardiac bypass surgery (Kurien & Oliver, 1966). In the presence of high circulating levels of fatty acids, oxidation of fatty acids increases and

accounts for almost all of the myocardial ATP production (Bing, 1965; Neely & Morgan, 1974), while concurrently suppressing glucose oxidation (Randle, Garland, Newsholme, & Hales, 1963).

Another possible cause of this shift in metabolism is the dramatic decrease in malonyl-CoA levels (Kantor et al., 1999). During ischemia AMPK is activated by an accumulation of AMP, resulting in phosphorylation and inactivation of ACC during reperfusion, causing the observed decrease in malonyl-CoA levels. This accelerates fatty acid oxidation rates by a decreased inhibition of CPT-1 (Kudo, Barr, Barr, Desai, & Lopaschuk, 1995).

1.2.8 Phospholipid Remodeling and Eicosanoid Synthesis

Glycerophospholipids are important structural and functional components of biological membranes and also play important roles as precursors of lipid mediators such as eicosanoids (Shimizu, Ohto, & Kita, 2006). Phospholipids are formed through the Kennedy pathway (*de novo* pathway) (Kennedy, 1961), and rapidly remodeled through the Land's cycle (Lands, 1958). They contain a diacylglycerol (DAG) phosphatidic acid backbone and a spectrum of potential fatty acids. EPA, DHA, arachidonic acid (AA) and other HUFA compete with each other to become incorporated in membranes at the *sn-2*-position (Shindou & Shimizu, 2009). Saturated and monounsaturated fatty acids are commonly esterfied at the *sn-1*-position (Shindou & Shimizu, 2009).

During *de novo* phospholipid synthesis, glycerol-3-phosphate (G3P) is acylated with a long-chain fatty acid from fatty acyl-CoA to form lysophosphatidic acid (LPA). LPA is then acylated (with usually an unsaturated fatty acid) to form phosphatidic acid (PA) (Shindou & Shimizu, 2009). PA is converted to DAG or cytidine diphospho-DAG (CDP-DAG) by a cytosolic phosphatase. DAG is converted into either triacylglycerols or phosphatidylcholine (PC)

and phosphatidylethanolamine (PE). Phosphatidylserine (PS) can later be synthesized from PC or synthesized from CDP-DAG along with phosphatidylglycerol (PG), diphosphatidylglycerol (CL, Cardiolipin) and phosphatidylinositol (PI) (Shindou & Shimizu, 2009).

HUFA such as DHA, are incorporated into phospholipids after *de novo* synthesis through the remodeling pathway (Lands, 2000). AA is the primary fatty acid which becomes esterfied in phospholipids with a more minor contribution by other PUFA including DHA. The proportions can be altered by the fatty acid composition of the diet (Yamashita, Sugiura, & Waku, 1997). The remodeling pathway (or Lands' cycle) uses concerted activation of phospholipase A_2 (PLA_2) and lysophospholipid acyltransferases (LPLATs) to alter the fatty acyl composition of phospholipid. PLA_2 liberate fatty acids from phospholipids creating lysophospholipids. LPLATs work in the opposite direction converting fatty acyl-CoAs and lysophospholipids to phospholipids. Phospholipase activation to release fatty acids is also critical for various cell signaling and transduction pathways. HUFA such as AA, EPA and DHA are converted to various eicosanoids and docosanoids that act as potent local hormones (Hishikawa et al., 2008).

Global ischemia dramatically increases phospholipase activity (Ford et al., 1991). The enzymes Cyclooxygenase (COX) and lipoxygenase (LOX) are able to produce n-2 series eicosanoids from AA. These n-2 series eicosanoids exert strong inflammatory and vasoconsrictive effects. When using EPA and DHA as substrates these enzymes create n-3 series prostaglandins and thromboxanes, and 5-series leukotrienes which exert less vasoconstricting and inflammatory effects (Lands et al., 1992). In addition, the LOX pathways can also create Resolvins and Protectins from DHA. These chemical mediators have potent anti inflammatory effects, which have the potential to play a role in atherosclerosis (Ariel & Serhan, 2007). These n-3 HUFA derived mediators are likely contributing to the cardioprotection by n-3 HUFA in

dietary studies. An increase of DHA and ALA in the diet is able to increase the amount of DHA in phospholipids (Lands et al., 1992). Any increases in the phospholipid content of DHA would increase the amount being released during ischemia and would therefore increase the substrate amount for the creation of n-3 series eicosanoids (Abeywardena & Head, 2001) as well as resolvins and protectins (Calder, 2007). In addition, increased DHA in the phospholipid pools would mean less n-6 HUFA for use as substrates (Calder, 2007).

The role of eicosanoids and docosanoids in myocadial ischemia reperfusion injury is unclear as they appear to exert both beneficial and harmful effects. Prostacyclin (PGI_2), a major prostaglandin produced in the heart during ischemia and reperfusion, may have protective vasodilatation effects. Thromboxanes (TXA_2), are potent vasoconstrictors, and contribute to infarction and arrhythmias during ischemia and reperfusion (Bouchard & Lamontagne, 1999). PD1, a DHA derived protectin has powerful anti-apoptotic effects (Hersberger, 2010). Despite the important role that eicosanoids and docosanoids play in ischemia reperfusion injury, studies on their effects on heart are lacking.

1.2.9 N-3 HUFA and Apoptosis

DHA can induce apoptosis through an increased production of reactive oxygen species (ROS) and caspase-3 activity (Diep et al., 2000; Feng, Chen, Lin, & Yang, 2011). In monocytic leukemia U937 cells, DHA stimulates phospholipase C (PLC) which hydrolyzes the phosphatidylinositol 4,5-bisphosphate in membranes releasing free Inositol Trisphosphate (IP_3) and diacylglycerol (DAG). Production of these second messengers results in a rapid release of Ca^{2+} from the endoplasmic reticulum. PKCγ, a Ca^{2+} and DAG dependent isoform and PKCδ, a DAG dependent isoform of protein kinase C (PKC) become activated and phosphorylate store-

operated Ca^{2+} channels at the plasma membrane facilitating an increased influx of Ca^{2+} across the plasma membrane. Increased intracellular calcium concentrations as well as the activation of PKCγ and PKCδ can lead to ROS production from the mitochondria and downstream signaling resulting in caspase-3 activation contributing to apoptosis (Aires et al., 2007). An apoptotic effect has also been shown for DHA in Hep2 human larynx tumour cells (Colquhoun, de Mello, & Curi, 1998), vascular smooth muscle cells from rats (Diep, Touyz, & Schiffrin, 2000; Diep et al., 2000) as well as human coronary artery smooth muscle cells (Feng et al., 2011)

Pro-apoptotic effects have also been shown for several fatty acids including arachidonic acid (Artwohl et al., 2009; Pilane & Labelle, 2002), EPA (Finstad et al., 1998), and palmitate (Artwohl et al., 2009; deVries et al., 1997). A study using human smooth muscle cells incubated with stearic, oleic, linoleic, α-linolenic and arachidonic acid found that cells underwent apoptosis at a rate correlating with the fatty acids chain length and level of unsaturation. In this study, co-incubation with caspase inhibitors decreased apoptosis to a level not significantly different than controls (Artwohl et al., 2009). The mechanism for this effect of fatty acids on apoptosis is unidentified. In addition to caspase 3 activation (Aires et al., 2007), some proposed mechanisms include activation of, p38 mitogen-activated protein kinase, cytochrome c, PPAR-α (Diep et al., 2000; Diep et al., 2000).

1.2.10 Negative Effects of Fish Oil

Not all studies examining omega-3 HUFA and heart function have shown beneficial effects, with some studies indicating a proarrhythmic effect of fish oil. There appears to be a heterogeneous response to fish oil in patients with heart disease. The effects of fish oils on arrhythmias was examined in patients with implanted cardiac defibrillators in three double-blind,

randomized, placebo-controlled intervention studies (Brouwer et al., 2006; Leaf et al., 2005; Raitt et al., 2005). The results were varied, antiarrhythmic, proarrhythmic or no effect at all was found. The patients included in these trials had received an implantable defibrillator because of an existing ventricular tachycardia or ventricular fibrillation. This heterogeneous effect of fish oil might be explained by the mechanism of arrhythmia.

There appears to be an association between the type of arrhythmia and the effect of fish oil. N3-HUFA appear to be protective against arrhythmias following healed infarction and heart failure (ex. GISSI trial), and inefficient in patients with acute ischemia (angina pectoris) (Coronel et al., 2007). Heart failure associated arrhythmias are based on triggered activity, in this situation decreased excitability is antiarrhythmic. Triggered activity occurs when an inward current occurs due to after-depolarizations that are large enough to reach the threshold potential for activation (Vermeulen et al., 1994). However, under conditions of re-entry as in myocardial ischemia, a decrease in myocardial excitability and action potential shortening, an effect that n-3-HUFA exert (Leaf et al., 2005), is proarrhythmic (Coronel et al., 2007).

1.3 Methodological Foundations

1.3.1 Investigation of Cardiac Metabolism

Cardiac metabolism can be investigated in a variety of ways (see **Table 3** for a summary of experimental models of ischemia). Whole animal *in vivo* studies are the most representative of heart function and metabolism, however, compensatory mechanisms and other confounding factors such as blood components and neurohormonal changes, prevent the isolation of individual aspects in this highly dynamic system. It is also difficult to control the environment of the heart and its substrates *in situ* which makes *in vivo* studies difficult and impractical. The use of

cultured cardiomyocytes is very common and is very useful for obtaining biochemical information; however, their application to the whole heart is restricted. Quiescent isolated cardiac myocytes do not represent the metabolic demand of the working heart and lack the cell to cell paracrine interactions that are critical to optimal functioning of the heart (Evans, Bennett, & Hauton, 2000). To avoid some of these issues, the *ex vivo* Langendorff heart preparation was developed.

Table 3. Experimental models of ischemia.

Cell culture models

- Cells are raised under controlled experimental conditions
- Ability to introduce pharmacological agents, cDNAs, antisense RNA and interfering peptides
- Contractile function may be measured
- Ability to induce transfection with certain genes

In vivo

- Most representative of heart function, the contribution of blood components and neurohormonal changes is considered.
- Can make ECG, and hemodynamic parameter recordings
- Possible to induce ischemia by coronary artery occlusion using a suture
- Allows the examination of acute or chronic effects of ischemia
- Many confounding factors may be involved

Isolated perfused heart

- Control of confounding factors such as; circulating neurohormonal factors and innervations
- Heart rate can be paced to maintain a constant rhythm
- Flow and pressures can be adjusted
- Perfusate composition can be varied
- Creating ischemia is easy
- Contractility can be measured by using an intraventricular balloon
- Can make ECG recordings
- Can only investigate the acute effects on ischemia and reperfusion

The isolated perfused heart was introduced by Oscar Langendorff more than a century ago and is now a predominant technique in pharmacological and physiological research. The technique allows the examination of cardiac contractile strength (inotropic effects), heart rate

(chronotropic effects) and vascular effects without the neuronal and hormonal complications of an intact animal model. The classical animals used for Langendorff preparations are small animals such as rats, rabbits, mice and guinea pigs. In the Langendorff preparation, the ascending aorta is cannulated and the heart is perfused in a retrograde fashion with constant flow or constant pressure. The perfusate is a buffered, nutrient rich, oxygenated solution (95%O_2, 5%CO_2). The perfusate commonly used is Krebs-Henseleit buffer which contains glucose and is kept at a constant temperature of 37°C and pH of 7.4. The pressure of the solution causes the aortic valve to shut and the perfusate is then forced into the ostium and into the coronary vessels during diastole. This allows the heart to beat for several hours after its removal from the animal. If measurement of force is intended, a balloon for isovolumetric measurement of force is inserted via the left atrium into the cavity of the left ventricle. Isolated heart preparations are denervated, but beat within the physiological range spontaneously. Some parameters that can be obtained include heart rate, left ventricular pressure, end-diastolic pressure and the classical ECG parameters such as duration of QRS (Dhein, Michaelis, & Mohr, 2005). This preparation has several advantages including the ability to control and adjust perfusate composition, as well as the ability to induce conditions such as ischemia and hypoxia (Deleiris, Harding, & Pestre, 1984). It also allows the researcher to infuse pharmacological agents or any other substance for any precise amount of time and concentration. In doing this confounding factors such as absorption from the gastrointestinal system and blood and absorption by other tissues can be avoided compared to an *in vivo* model.

1.3.2 Fatty Acid Delivery Vehicles

Fatty acids are insoluble in water and therefore require a delivery vehicle in an aqueous environment. There are several possible fatty acid delivery vehicles for physiological studies (**Table 4**).

Table 4. Fatty acid delivery vehicles

Vehicle	*Pro*	*Con*
BSA		
	• Commonly used in literature • Most physiological	• Shown to have antioxidant properties (Brown et al., 1989) • Controversy regarding its delivery to cells • Trace amounts exert cardioprotective effects (maintains LVEDP to pre-ischemia values) (Watts & Maiorano, 1999)
Ethanol		
	• Easy and inexpensive	• Exerts a cardioprotective effect against I/R injury. Appears to use the same signalling cascade as ischemic preconditioning (Chen, Gray, & Mochly-Rosen, 1999)
Dimethyl sulfoxide (DMSO)		
	• Dissolves polar and nonpolar compounds and is highly miscible in water	• Effect on inotropy and force generation of papillary muscles (Ogura, Kasamaki, & McDonald, 1996). Depressed contractile function of seen on diaphragm fibres by reversible dose-dependent inhibition of excitation contraction coupling (Reid & Moody, 1994).
Methyl- β –cyclodextrin (MBCD)		
	• Reported to extract less nutrients from cell and to deliver less impurities • FA dissociates from MBCD faster that from albumin	• Disintegrates lipid rafts, which appear to be essential for ischemic preconditioning (0.2mM of MBCD abolished all cardioprotection by IPC) (Das et al., 2008). • The longer the chain the more that is needed. It is unknown how much would be required to complex this with DHA.

LVEDP, left ventricular end diastolic pressure; I/R, ischemia reperfusion; IPC, ischemia preconditioning

Dimethyl sulfoxide (DMSO) and BSA have been used previously in isolated perfused heart models. Methyl-β-cyclodextrin (MBCD) has not been used in an isolated perfused rat heart but has been utilized to deliver fatty acids to cells in transport studies (Brunaldi, Huang, & Hamilton, 2010). Initial pilot work indicated that ethanol is not an appropriate fatty acid delivery vehicle for this protocol as the ethanol and fatty acid mixture was not miscible with the buffer system at a low concentration of ethanol. As such, palmitate precipitated out of solution upon mixture with the aqueous Krebs Bicarbonate buffer and the precipitate accumulated in the heart causing it to fail.

In the literature, BSA is by far the most commonly used vehicle and represents the most physiological method (**Table 5**). A study to validate the use of BSA as a fatty acid delivery vehicle to an artificially perfused rat heart was conducted (see below). A concentration of 3% BSA was chosen based on an examination of the literature. No detectable differences in heart palmitate or DHA content were found compared to control hearts not receiving an infusion of BSA. In addition, the infusion of BSA had no effect on heart functional parameters including HR ($p=0.88$), LVEDP ($p=0.17$), rate of pressure development ($p=0.11$) or infarction size ($p=0.63$). These results indicated that 3% essentially fatty acid free BSA is an appropriate vehicle for fatty acid delivery to the artificially perfused rat heart.

Table 5. Langendorff studies using 3% albumin complexed to fatty acids.

BSA used	*Authors*
3% BSA with 1.2mM palmitate	(Soltys et al., 2002)
3% BSA with 1.2 mM palmitate	(Grist, Wambolt, Bondy, English, & Allard, 2002)
3% BSA with 1.2 mM palmitate	(Lopaschuk et al., 1992)
3% BSA with 1.2 mM palmitate	(Davies, Lovlin, & Lopaschuk, 1992)
3% BSA with 0.4 or 2.4mM palmitate	(Ito et al., 2010)
3% BSA with .4 or 2.4mM palmitate	(Folmes, Sowah, Clanachan, & Lopaschuk, 2009)

BSA, bovine serum albumin

Chapter 2
Rationale and Objectives

2.1 Rationale

There appears to be sufficient evidence indicating a protective role of DHA in ischemia reperfusion injury. Studies have used the Langendorff perfused heart model to examine heart parameters in the absence of neuronal, hormonal and blood complications (Zeghichi-Hamri et al., 2010; Abdukeyum et al., 2008). However, these studies have used dietary protocols and therefore cannot conclusively determine the effect of n-3 HUFA on ischemia reperfusion injury. In dietary studies, the n-3 HUFA are incorporated into cellular membranes. One mechanism by which n-3 HUFA exert their function on ion channels is as a free fatty acid upon release from phospholipids by phospholipases which are activated during ischemia. The purpose of the present study is to examine the effect of DHA following 30 min of global no flow ischemia on heart function and infarction in the absence of previous n-3 HUFA phospholipid enrichment, and neuronal, blood, or hormonal complications. DHA will be infused immediately following ischemia to mimic the physiological infusion of n-3 HUFA caused by phospholipases. This will be paired with a similar dose response protocol for palmitate a reference saturated fatty acid.

2.2 Statement of Objectives

The main objective of this thesis is to examine a dose response curve of DHA infused immediately following 30 minutes of global no flow ischemia on ischemia reperfusion injury as measured by area of infarction. In addition, functional measurements of Left Ventricular End Diastolic Pressure (LVEDP, mmHg), Systolic (mmHg), Left Ventricular Developed Pressure (LVDevP, mmHg), Coronary Perfusuion Pressure (CPP) and the maximal Rate of Pressure

Development (+dP/dt, mmHg/s) will be made. LVEDP is an indicator of contracture and LVDevP is a measure of contractility. These results will be compared with a vehicle control and a fatty acid control consisting of the same infusion concentrations of palmitate. In order to accomplish the main objective, several preliminary objectives were necessary to establish the model. These included characterizing the fatty acid pools of the heart during the Langendorff procedure and developing a local technique to infuse specific fatty acids.

2.3 Hypotheses

The hypotheses of the present study are that;

1. DHA infusion immediately following 30 minutes of global no flow ischemia will significantly reduce infarction area.
2. Infusion of palmitate will neither significantly increase nor decrease the infarcted area.
3. DHA infusion will reduce the increase in contracture seen during reperfusion, measured by left ventricular end diastolic pressure, and maintain left ventricular developed pressure, a measure of the hearts ability to contract.
4. Infusion of palmitate will increase contracture and reduce left ventricular developed pressure.

Chapter 3
Methods

3.1 Langendorff Perfusion Procedure

Prior to experimentation, rats are anesthetised intraperitoneally with sodium pentobarbital. Hearts are excised by first making a transverse incision in the abdominal cavity with scissors. The diaphragm is transected and lateral incisions are made along both sides of the rib cage. The anterior chest wall is folded back to reveal the heart and to remove the pericardium. Next, fine tipped forceps grasp the ascending aorta as it is cut by scissors. The heart is lifted out of the thorax as remaining connective tissue and pericardium are removed and the heart is placed in ice-cold Krebs Henseleit Buffer. The aorta is cannulated and perfused in a retrograde fashion with a modified Krebs-Henseleit bicarbonate buffer containing (in mmol/l) 118 NaCl, 4.7 KCl, 1.2 KH2PO4, 1.2 MgSO4, 10 glucose, 2.5 CaCl2, and 2.5 NaHCO3, equilibrated with 95% O2-5% CO2 and warmed to 37° C at a pH of 7.4. An equation which estimates *in vivo* flow rate from heart weight is used to set flow rate for this model;

((((body weight (g)*0.0027)+0.6)^0.56)*7.43).

Ventricular pressure is measured by a balloon catheter filled with degassed water (connected to a pressure transducer) and is adjusted to a mean left ventricular end-diastolic pressure (LVEDP) of approximately 5-10 mmHg during the initial equilibration. Perfusion pressure is also measured using a pressure transducer. Heart Rate (HR), Coronary perfusion pressure, Left Ventricular Developed Pressure, Left Ventricular End Diastolic and systolic (Sys) pressure, and rate of developed pressure are continuously recorded using PowerLab software. A three-way stopcock above the aortic root will be used to stop flow to the heart and create global ischemia.

3.2 Animals and Diets

All animal procedures were approved by the University of Waterloo Animal Care Committee and were in accordance with the guidelines of the Canadian Council on Animal Care. Animal were housed in the animal housing facility of the Department of Kinesiology, University of Waterloo with temperature of 21 ± 1 °C and a reversed 12:12-h light-dark cycle. Rats were fed a standard chow diet (Harlan, Teklad AIN-93G) (16) and had access to food and water ad libitum. The fatty acid composition of the diet was confirmed by triplicate analyses in our laboratory (**Table 6**).

Table 6. Fatty acid composition of Harlan Teklad 8640 rodent diet.

Fatty Acid	*Diet Content (µg fatty acid/g of chow)*
16:0	5783 ± 447
18:0	1523 ± 166
Total saturated fatty acids	8058 ± 644
16:1	315 ± 6
18:1(n-7)	2938 ± 4
18:1 n-9	9237 ± 2
20:1(n-9)	134 ± 9
Total monounsaturates	9611 ± 1198
18:2(n-6)	21211 ± 1576
20:4(n-6)	48 ± 7
Total (n-6) polyunsaturates	21364 ± 1547
18:3(n-3)	2511 ± 143
20:5(n-3)	126 ± 1
22:5(n-3)	27 ± 1
22:6(n-3)	106 ± 7
Total (n-3) polyunsaturates	2769 ± 147
Total Fatty Acids	42759 ± 3233

Values are mean ± SD from triplicate analysis

3.3 Ischemia Reperfusion Protocol and Time Course

A 30 minute normoxic period was used to allow the hearts to adjust to cannulation and to the intraventricular balloon. This normoxic period was sufficient time to achieve constant baseline left ventricular pressures. During this time, the balloon is inflated to the desired end diastolic pressure. This requires time to allow the degassed H_2O to warm to the hearts temperature. The heart will then experience 30 minutes of ischemia. Ischemic arrhythmias are most pronounced in the first 15 minutes (McLennan et al. 1988). Pilot work found that 15 minutes of global no flow ischemia was not severe enough to elicit an obvious amount of contracture and decreased contractility so the ischemia protocol was increased to 30 minutes of global no flow ischemia which has been used previously(Abdukeyum et al., 2008). Fifteen minutes of infusion was chosen to mimic previous research which infused varying concentrations of DHA to a Langendorff perfused heart without ischemia. This time was sufficient to allow the DHA to elicit electrophysiological changes to the hearts (Dhein et al., 2005). Seventy-five minutes of reperfusion was used to allow control hearts to return to baseline levels of ventricular pressures and to allow sufficient washout time of dehydrogenases for accurate infarct size staining (Schwarz, Somoano, Hale, & Kloner, 2000). Hearts were excluded from the data if any of the exclusion criteria were met including a heart rate of less than 100 bpm or greater than 400 bpm, or a left ventricular developed pressure of less than 65 mmHG.

3.4 Functional Measurements of Recovery

Heart function was evaluated by the recovery of left ventricular developed pressure (LVDevP, systolic – diastolic pressure), left ventricular systolic pressure (LVSysP) and left ventricular end diastolic pressure (LVEDP, a negative index of myocardial performance).

LVDevP is a measure of contractility which is decreased following ischemia. LVEDP is a measure of contracture which is elevated during ischemia and persists through reperfusion. In addition, the rate of developed pressure (+dP/dt mmHg/s), another measure of contractility which is decreased following ischemia, and coronary perfusion pressure (CPP), a measure of the pressure gradient between the aorta and the right atrium, were also measured continuously.

3.5 Measurement of Ischemia

Ischemic damage will be measured as the percentage of ischemic tissue in all heart tissue using triphenyltetrazolium chloride (TTC) staining. TTC stains viable tissue dark red as the tetrazolium salts react with the dehydrogenases in the cells and the infarcted tissue stains a pale-white since they lack the enzymes with which the TTC reacts. Frozen tissue is sliced transversely into 1 mm slices, thawed and blotted dry. Fresh 1% w/v triphenyltetrazolium chloride (TTC) is made up in a phosphate buffer (88 mM Na_2HPO_4 , 1.8 mM NaH_2PO_4, pH=7.4) and poured over the heart slices. Heart slices are incubated for 10 minutes at 37°C for 10 minutes, agitated, then incubated for another 10 minutes. Slices are dabbed dry and imaged between 2 cover slides under a dissection microscope. A photograph is taken and the ischemic area in total heart area is digitally imaged using ImageJ software (NIH public domain, http://rsbweb.nih.gov/ij). The area of all hearts are summed together and converted to millimeters squared to get a ratio of total infarction area/ total area.

3.6 Analysis of Fatty Acids

Prior to lipid extraction, docosatrienoic acid (22:3 n-3) ethyl ester and 17:0 standards (Avanti Polar Lipids Inc, Alabaster, AL) were used as internal standards for total lipid extracts (TLEs), triacylglycerols (TGs), phospholipids (PLs), and non-esterified fatty acids (NEFA).

Lipids were extracted from hearts based on the method of Bligh and Dyer (Bligh & Dyer, 1959) via homogenization in methanol [containing 50μg/ml butylated hydroxyl toluene (BHT)] and subsequent addition of chloroform and sodium phosphate buffer (0.2 M, creating final solvent ratios of chloroform:methanol:phosphate buffer of 2:2:1.8), followed by collection of the organic phase. Total lipid extracts were stored at -80 °C until analysis.

For fatty acid compositional analyses of individual lipids classes, total lipid extracts were applied to thin-layer chromatography (TLC) plates (20 x 20 cm) with a 60 Å silica gel layer (Whatman Internation LTD, Maidstone, England) and placed in a resolving tank with reagents. The mobile phase reagents were heptane: diethyl ether: glacial acetic acid (60:40:2 v/v/v). Bands were visualized using 2,7-dicholroflourescein (Sigma-Aldich, Oakville, ON, Canada) under an ultraviolet lamp, and identified by comparison to a reference standard. Lipid classes of interest, triacylglycerols (TAG), nonesterified fatty acids (NEFA) and phospholipids (PL), were collected for transmethylation.

The fatty acids in the total lipid extract and NEFA, TAG, and PL fractions were transesterified by heating at 85°C in 14% BF_3 in methanol for 1 h (Morrison & Smith, 1964). The fatty acid methyl esters were collected and separated by fast gas chromatography using a Varian 3900 gas chromatograph (Varian Inc, Mississauga, ON, Canada) with a DB-FFAP capillary column with 15 m × 0.10 mm inner diameter × 0.10 μm film thickness capillary column (J & W Scientific, Agilent Technologies, Palo Alto, CA). H_2 was used as the carrier gas at a flow rate of 30 ml/min. Chromatograms were analyzed by comparing to a reference standard (Nu-Check Prep Inc), and fatty acid concentrations were determined by comparison to the peak area of the internal standards.

3.7 Immunoblotting

Heart samples were homogenized in a lysis buffer containing .25M sucrose, .01M tris-HCL, 0.01M $MGCl_2$, 2.5mM DTT, and protease inhibitor (Roche Applied Science, Laval, QC, Canada). A bicinchoninic acid procedure was used to quantify protein content. 20 µg proteins were run on a 7.5-12.5% SDS-PAGE gel and transferred to a polyvinylidene fluoride membrane (Bio-Rad Laboritories, Misssissuga, ON). Membranes were blocked with 5% BSA or milk in TBS with 0.5% Tween (TBS-T) for 1 hour, incubated with primary antibody of the protein of interest, and washed with TBS-T. Membranes were then incubated for 1 hour with horseradish peroxidase-conjugated secondary (goat anti-rabbit or rabbit anti-goat, Santa Cruz Biotechnology) and washed with TBS-T. Chemiluminesence Western Blotting Detection Reagents (GE healthcare, PQ) were used for detection. Protein was visualized on a Chemigenius2 Bioimaging system (Syngene inc., Fredrick, MD). Molecular weights of proteins were confirmed using Precision Plus Protein WesternC Standards and Precision Protein Strep-Tactin HRP Conjugate (Bio-Rad Laboratories). Luminescence was normalized to controls and expressed as mean optical density in arbitrary units. To confirm equal protein loading and adequate transfer ponceau S stain (Bioshop, 148 Burlington, ON, Canada) was used.

3.8 Fatty Acid Bovine Serum Albumin (BSA) Complex

Fatty acid - BSA complexes were made fresh immediately prior to infusion and protected from light to mimic the free fatty acid or nonesterified fatty acid pool in plasma. DHA plasma levels have been reported to range from approximately 7µM to 80 µM DHA in humans (Newens, Thompson, Jackson, Wright, & Williams, 2011). In n-3 PUFA deficient rat models, DHA can be undetectable and increase to 37 µM with fish meal based chow feeding (Bazinet, Rao, Chang,

Rapoport, & Lee, 2006). The appropriate amounts of fatty acids were dried down under nitrogen from an ethanol stock solution and 3% (w/v) essentially fatty acid free BSA (Sigma-Aldich, Oakville, ON, Canada) was added to achieve the required molarity. The fatty acid – BSA mixtures were vortexed for 20 min for 3 cycles, with the tubes being checked between cycles to make sure the vortex equipment was not damaging them. Immediately before infusion, the pH was raised to 7.4. Concentrations of DHA of 10, 20, 40, 60, 80, 100 and 120 µM DHA were chosen based on pilot work and equimolar concentrations of palmitate were repeated afterwards. Based on n=10 hearts, 100 µM of DHA is the highest concentration of DHA tolerated. Four out of 6 hearts failed during the infusion of 120 µM DHA immediately following 30 min of global no flow ischemia due to fibrillation. In addition, no hearts were able to survive any concentration of DHA greater that 120 µM.

3.9 Study Protocols

3.9.1 Effect of Perfusion on Heart Fatty Acids and Fatty Acid Transport Proteins

The effect of the Langendorff procedure on heart fatty acid pools and relevant fatty acid proteins were examined. Nine-week old female Sprague Dawley rats were sacrificed (n =12), heart tissue excised and then randomized to a baseline control (buffer rinse with no perfusion), 30 min of perfusion and 4 h of perfusion in Langendorff mode with buffer as described above with no detectable fatty acids was used as the perfusate. All hearts were immediately frozen for lipid and protein analysis upon excision (control) or after perfusion (30 min and 4 h groups). Lipids and protein were extracted from tissue in the methods described in the methods section (section 3.6).

3.9.2 Effects of Ischemia and BSA on Heart Fatty Acids and Heart Function

Ten male Sprague Dawley rats approximately 10 weeks of age were used to evaluate the effects of BSA and ischemia on heart function and fatty acid content. The rat hearts were randomized into 3 groups, an Ischemia group which was allowed to equilibrate for 30 min before being exposed to 30 min of global no flow ischemia followed by 1.5 h of reperfusion; a control group which was perfused for the same amount of time as the ischemia group but did not experience ischemia; and a BSA group which had 3% (w/v) essentially fatty acid free BSA added to the perfusion buffer for the last 15 minutes of the normoxic equilibration period before 30 minutes of global no flow ischemia and 1.5 hrs of reperfusion at the original flow rate. All hearts were perfused in a retrograde fashion in Langendorff mode with the buffer described previously throughout the experiment, except during ischemia.

3.9.3 The Effect of Post-ischemic Perfusion of DHA and Palmitate on Heart Function and Infarction

For the main experiment of the project male Sprague Dawley (SD) rats 9 weeks of age were purchased from Harlan (Mississauga, ON, Canada) and housed in the animal housing facility of the Department of Kinesiology, University of Waterloo in a reversed 12:12-h light-dark cycle and a temperature of 21 ±1 °C. All rats were allowed access to food and water ad-libitum. All hearts were perfused in Langendorff mode for 30 min with buffer (described above). During this time the hearts were cleaned of extraneous tissue. All hearts experienced 30 minutes of global no-flow ischemia. Immediately following ischemia hearts were reperfused with KHB and an infusion of either 10, 20, 40, 60, 80, 100 or 120 µM of DHA or Palmitate complexed to 3% (w/v) essentially fatty acid free BSA for 15 minutes. Control hearts received an infusion of

3% BSA alone in addition to the KHB. Hearts were then reperfused for an additional 75 minutes at the original flow rate in the absence of fatty acids (**Figure 1**). At the end of the protocol hearts were quickly removed and placed in ice cold KHB, and aorta and atrial tissue were removed. Hearts were dried, wrapped in plastic wrap and tinfoil and slowly frozen in -80°C where they were stored before TTC staining for measurement of ischemic area using the methods described above.

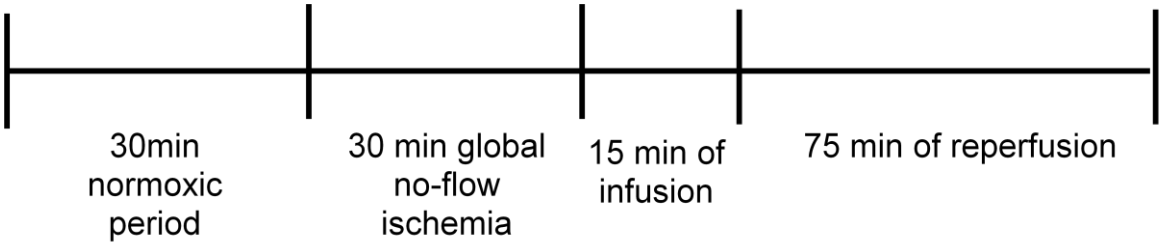

Figure 1. Langendorff protocol of ischemia reperfusion used in the present study.

3.10 Statistics

Statistical analyses were performed using SPSS for windows version 19.0. End point variables were compared using either independent t-tests (effect of BSA vehicle on heart fatty acids), 1-way ANOVA (perfusion time course on heart fatty acids and transport proteins), 2-way ANOVA (effect of dose and on infarction area) or 3-way ANOVA (effect of time, dose and infused fatty acid on heart functional variables). A priori comparisons between the fatty concentrations of DHA and palmitate with vehicle control, and between equimolar concentrations of DHA and palmitate were completed by independent t tests. To protect against an increasing error rate, a two way ANOVA for fatty acid type and concentration was performed prior to

multiple comparisons (with significant F-value set at $p<0.05$) and the significance of a priori t tests set at $p<0.025$.

Chapter 4

Results

4.1 Effect of Perfusion on Heart Fatty Acids and Fatty Acid Transport Proteins

Total nonesterified fatty acid concentrations, were decreased ($p<0.05$) after 4 hours of perfusion relative to baseline control (**Figure 2**). Total fatty acids also decreased in the triacylglycerol fraction as well, but failed to reach statistical significance due to high variability in this pool (**Figure 3**). The phospholipid fatty acid (**Figure 4)** and total lipid pool (**Figure 5**) remained relatively stable during perfusion. Immunoassays indicated that after 4hr perfusion, heart type fatty acid binding protein (H-FABP) was decreased ($p=0.002$), membrane associated fatty acid binding protein (FABPpm) was slightly but not significantly increased ($p=0.09$), and heart FAT/CD36 did not differ relative to baseline ($P=0.27$) (**Figure 6**).

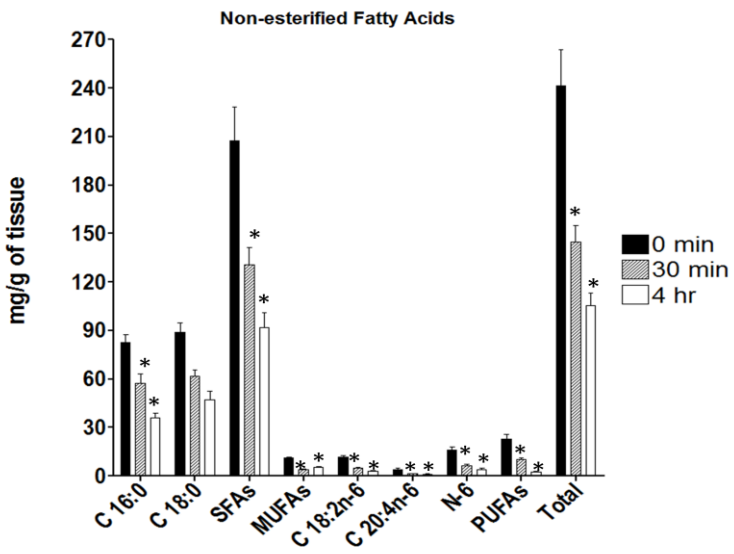

Figure 2. Fatty acid composition of the non-esterified fatty acid fraction of heart lipid extracts following artificial perfusion for 0 minutes, 30 minutes, and 4 hours. Data are expressed as means ± SEM. MUFA, monounsaturated fatty acids; SFA, saturated fatty acids; n-6, omega 6 fatty acids; PUFA, polyunsaturated fatty acids. *$p<0.05$ compared to control.

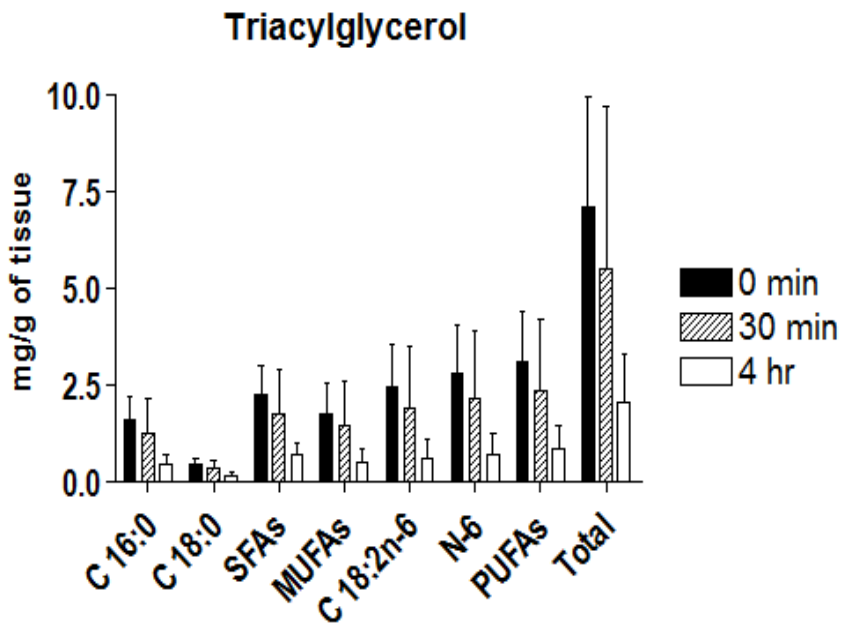

Figure 3. Fatty acid composition of the triacylglycerol fraction of heart total lipids following artificial perfusion for 0 minutes, 30 minutes, and 4 hours. Data are expressed as means ± SEM. MUFA, monounsaturated fatty acids; SFA, saturated fatty acids; n-6, omega 6 fatty acids; PUFA, polyunsaturated fatty acids

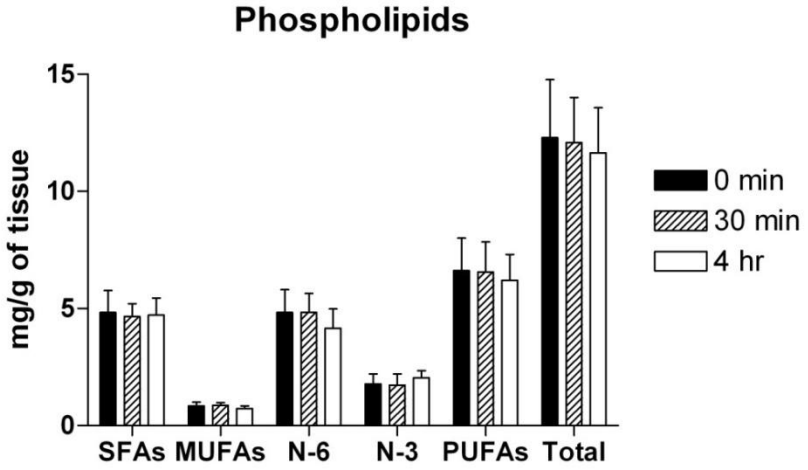

Figure 4. Fatty acid composition of the phospholipid fraction of heart total lipids following artificial perfusion for 0 minutes, 30 minutes, and 4 hours. Data are expressed as means ± SEM. MUFA, monounsaturated fatty acids; SFA, saturated fatty acids; n-6, omega 6 fatty acids; PUFA, polyunsaturated fatty acids

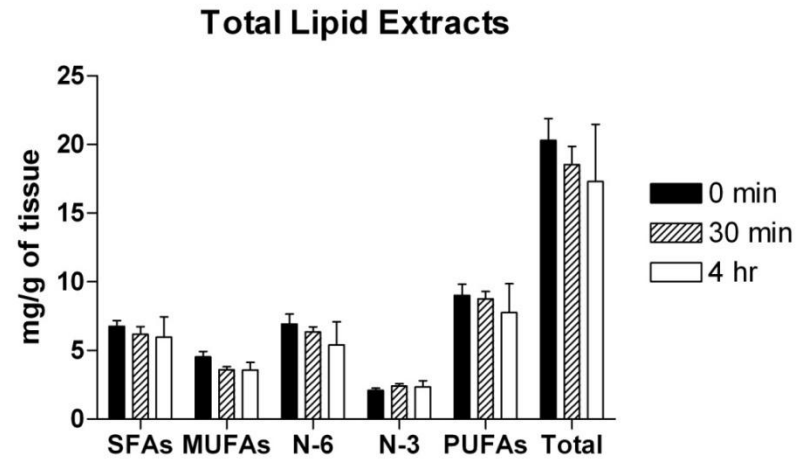

Figure 5. Fatty acid composition of total lipids of heart total lipids following artificial perfusion for 0 minutes, 30 minutes, and 4 hours. Data are expressed as means ± SEM. MUFA, monounsaturated fatty acids; SFA, saturated fatty acids; n-6, omega 6 fatty acids; PUFA, polyunsaturated fatty acids.

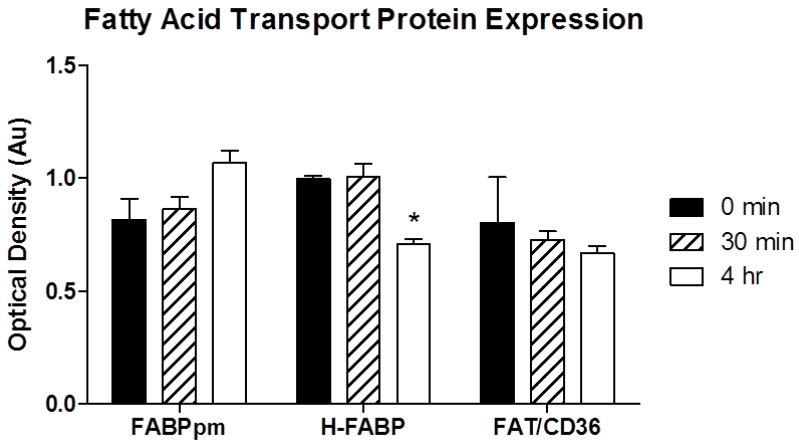

Figure 6. Heart protein expression of $FABP_{pm,}$ H-FABP, FAT/CD36 after 0 minutes, 30 minutes, and 4 hours of artificial perfusion. Data are expressed as means ± SEM. * $p<0.002$ by independent samples t-test. (4hr vs. 0min).

4.2 Effects of Ischemia and BSA on Heart Fatty Acids and Heart Function

Thirty minutes of global no flow ischemia and 1.5 hrs of reperfusion did not cause any significant decreases on heart total lipids (84.24±16.09 vs. 88.67±12.06 µM fatty acid), total TAGs (9.79 ± 7.12 vs. 7.65 ± 3.38 µM fatty acid), total PL (16.01 ± 1.55 vs. 15.58 ± 1.22 µM

fatty acid), or total heart NEFA (0.37 ± 0.22 vs. 0.45 ± 0.12 μM fatty acid) compared to controls (**Tables 7-10**).

The infusion of 3% BSA had more significant effects on heart fatty acid content. Heart total lipids did not decrease significantly compared to those hearts which received no BSA and were exposed to the same ischemia protocol (72.02 ± 6.82 vs. 84.24 ± 16.09 μM fatty acid). The most marked decreases caused by the infusion of BSA for 15 minutes prior to ischemia were seen in the dimethyl acetals (DMA). DMA are generated from plasmalogens during fatty acid extraction and derivitization to fatty acid methyl esters. During acid mediated transesterification, the vinyl ether bond of fatty acids in plasmologens generates a doubly methylated fatty acid rather than the usual fatty acid methyl ester generated from typical acyl esters in triacylglycerols and phospholipids. Compared to the ischemia alone group, 16:0 DMA (1.08 ± 0.10 vs. 1.40 ± 0.17 μM fatty acid), 18:0 DMA (0.54 ± 0.03 vs. 0.70 ± 0.06 μM fatty acid) and 18:1 DMA (0.35 ± 0.03 vs. 0.48 ± 0.03 μM fatty acid) all decreased significantly ($p<0.05$) in the total lipid fraction. Similarly, in the PL fraction 16:0 DMA ($p=0.0002$), 18:0 DMA ($p=0.003$) and 18:1 DMA ($p=0.002$) were all decreased compared to the ischemia alone group (**Table 7 and 8**).

There were no functional differences between BSA and KHB hearts at the end of infusion in HR, LVEDP, maximal rate of pressure development or infarction size. Additionally, BSA did not cause any significant differences in heart fatty acid content of DHA and palmitate which the following studies will be specifically examining. These results indicate that 3% essentially fatty acid free BSA is an appropriate fatty acid delivery vehicle for the infusion of fatty acid to an artificially perfused heart.

Table 7. Heart total lipid fatty acid composition

	Control	Ischemia-reperfusion	Bovine serum albumin
		μM of fatty acid	
C 12:0	0.05 ± 0.05	0.05 ± 0.05	0.05 ± 0.05
C 14:0	0.39 ± 0.04	0.35 ± 0.13	0.31 ± 0.09
C 16:0	12.05 ± 1.56	12.09 ± 2.81	9.75 ± 0.19
C 16:0 dma	1.50 ± 0.1	1.40 ± 0.17	1.08 ± 0.10*
C 18:0	17.89 ± 2.43	16.13 ± 1.12	14.52 ± 1.34
C 18:0 dma	0.70 ± 0.06	0.64 ± 0.03	0.54 ± 0.03*
C 20:0	0.26 ± 0.03	0.26 ± 0.03	0.22 ± 0.03
C 22:0	0.15 ± 0.03	0.18 ± 0.03	0.18 ± 0.03
C 24:0	0.03 ± 0.03	0.03 ± 0.03	0.03 ± 0.03
Total SFA	33.02 ± 4.33	31.13 ± 4.4	26.68 ± 1.89
C 12:1	0.15 ± 0.05	0.20 ± 0.05	0.20 ± 0.05
C 16:1	0.31 ± 0.08	0.35 ± 0.31	0.20 ± 0.08
C 18:1 dma	0.48 ± 0.03	0.48 ± 0.06	0.35 ± 0.03*
C 18:1n-7	3.79 ± 0.35	3.54 ± 0.53	3.26 ± 0.07
C 18:1n-9	9.77 ± 0.46	10.09 ± 3.12	8.53 ± 1.49
C 20:1n-9	0.68 ± 0.03	0.68 ± 0.13	0.61 ± 0.03
C 22:1n-9	1.77 ± 0.35	2.22 ± 0.30	2.07 ± 0.09
Total MUFA	16.95 ± 1.35	17.56 ± 4.50	15.22 ± 1.84
C 18:2n-6	14.87 ± 2.64	14.94 ± 4.03	11.73 ± 0.68
C 20:2n-6	0.19 ± 0.03	0.19 ± 0.03	0.16 ± 0.03
C 20:3n-6	0.20 ± 0.03	0.16 ± 0.03	0.16 ± 0.03
C 20:4n-6	15.08 ± 1.81	12.91 ± 1.41	12.02 ± 0.79
C 22:4n-6	0.72 ± 0.12	0.63 ± 0.21	0.54 ± 0.12
C 22:5n-6	0.64 ± 0.21	0.45 ± 0.21	0.27 ± 0.03
Total N-6	31.70 ± 4.84	29.28 ± 5.92	24.88 ± 1.68
C 18:3n-3	0.32 ± 0.07	0.40± 0.25	0.22 ± 0.11
C 22:5n-3	0.21 ± 0.02	0.21 ± 0.02	0.18 ± 0.03
C 22:6n-3	6.47 ± 1.45	5.66 ± 1.00	4.84 ± 1.27
Total N-3	7.00 ± 1.54	6.27 ± 1.27	5.24 ± 1.41
Total PUFA	45.7 ± 7.92	41.82 ± 8.46	35.36 ± 4.50
Total fatty acids	88.67 ± 12.06	84.24 ± 16.09	72.02 ± 6.82

dma, dimethyl acetal; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids. Values are means ±SEM. *, Significantly different than Ischemia-Reperfusion matched controls ($p<0.05$) by independent samples t-test.

Table 8. Heart phospholipid fatty acid composition

	Control	Ischemia-reperfusion	Bovine serum albumin
		μM fatty acid	
C 14:0	0.04 ± 0.04	0.04 ± 0.04	0.04 ± 0.04
C 16:0	2.11 ± 0.08	2.18 ± 0.08	1.91 ± 0.16
C 16:0 dma	0.28 ± 0.03	0.31 ± 0.03	0.17 ± 0.03*
C 18:0	4.08 ± 0.18	4.08 ± 0.14	3.66 ± 0.35
C 18:0 dma	0.13 ± 0.03	0.16 ± 0.03	0.10 ± 0.03*
C 20:0	0.03 ± 0.03	0.03 ± 0.03	0.03 ± 0.03
C 22:0	0.03 ± 0.03	0.03 ± 0.03	0.03 ± 0.03
Total SFA	6.70 ± 0.42	6.83 ± 0.38	5.94 ± 0.67
C 18:1 dma	0.10 ± 0.03	0.10 ± 0.03	0.06 ± 0.03*
C 18:1n-7	0.53 ± 0.04	0.57 ± 0.04	0.53 ± 0.04
C 18:1n-9	0.53 ± 0.07	0.50 ± 0.04	0.50 ± 0.07
Total MUFA	1.16 ± 0.14	1.17 ± 0.11	1.09 ± 0.14
C 18:2n-6	2.60 ± 0.18	2.82 ± 0.36	2.50 ± 0.53
C 20:2n-6	0.03 ± 0.03	0.03 ± 0.03	0.03 ± 0.03
C 20:3n-6	0.03 ± 0.03	0.03 ± 0.03	0.03 ± 0.03
C 20:4n-6	3.32 ± 0.2	3.32 ± 0.30	3.12 ± 0.07
C 22:4n-6	0.15 ± 0.03	0.15 ± 0.03	0.15 ± 0.03
C 22:5n-6	0.15 ± 0.06	0.12 ± 0.06	0.06 ± 0.03
Total N-6	6.28 ± 0.53	6.47 ± 0.81	5.89 ± 0.72
C 18:3n-3	0.04 ± 0.04	0.04 ± 0.04	0.04 ± 0.04
C 22:5n-3	0.04 ± 0.01	0.05 ± 0.01	0.04 ± 0.01
C 22:6n-3	1.36 ± 0.09	1.45 ± 0.21	1.24 ± 0.30
Total N-3	1.44 ± 0.13	1.54 ± 0.25	1.32 ± 0.35
Total PUFA	7.72 ± 0.66	8.01 ± 1.06	7.21 ± 1.07
Total FA	15.58 ± 1.22	16.01 ± 1.55	14.24 ± 1.88

dma, dimethyl acetal; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids. Values are means ±SEM. *, Significantly different than Ischemia-Reperfusion matched controls ($p<0.001$) by independent samples t-test.

Table 9. Heart triacylglycerol fatty acid composition.

	Control	Ischemia-reperfusion	Bovine serum albumin
		µM fatty acid	
C 14:0	0.13 ± 0.04	0.13 ± 0.09	0.13 ± 0.09
C 16:0	1.83 ± 0.62	2.26 ± 1.36	2.11 ± 1.05
C 18:0	0.63 ± 0.07	0.70 ± 0.32	0.95 ± 0.46
Total SFA	2.59 ± 0.73	3.09 ± 1.77	3.19 ± 1.60
C 16:1	0.16 ± 0.08	0.16 ± 0.12	0.08 ± 0.08
C 18:1n-7	0.21 ± 0.07	0.25 ± 0.18	0.25 ± 0.14
C 18:1n-9	1.66 ± 0.92	2.09 ± 1.59	1.63 ± 1.17
Total MUFA	2.03 ± 1.07	2.50 ± 1.89	1.96 ± 1.39
C 18:2n-6	2.71 ± 1.35	3.71 ± 3.07	2.39 ± 1.57
C 20:2n-6	0.03 ± 0.03	0.03 ± 0.03	0.03 ± 0.03
C 22:5n-6	0.12 ± 0.06	0.21 ± 0.15	0.36 ± 0.21
Total N-6	2.86 ± 1.44	3.95 ± 3.25	2.78 ± 1.81
C 18:3n-3	0.14 ± 0.11	0.22 ± 0.18	0.11 ± 0.07
C 22:6n-3	0.03 ± 0.03	0.03 ± 0.03	0.03 ± 0.03
Total N-3	0.17 ± 0.14	0.25 ± 0.21	0.14 ± 0.10
Total PUFA	3.03 ± 1.58	4.2 ± 3.46	2.92 ± 1.91
Total FA	7.65 ± 3.38	9.79 ± 7.12	8.07 ± 4.90

SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids. Values are means ±SEM. No significant differences were found.

Table 10. Heart nonesterified fatty acid composition.

	Control	Ischemia-reperfusion	Bovine serum albumin
		µM fatty acid	
C 18:0	0.25 ± 0.04	0.18 ± 0.07	0.21 ± 0.11
Total SFA	0.41 ± 0.08	0.3 ± 0.11	0.37 ± 0.15
C 18:1n-9	0.04 ± 0.04	0.07 ± 0.11	0.04 ± 0.04
Total MUFA	0.04 ± 0.04	0.07 ± 0.11	0.04 ± 0.04
Total FA	0.45 ± 0.12	0.37 ± 0.22	0.41 ± 0.19

SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids. Values are means ±SEM. No significant differences were found.

4.3 The Effect of Post-ischemic Perfusion of DHA and Palmitate on Heart Function and Infarction

4.3.1 Infarction Size

Increasing concentrations of both palmitate and DHA caused increased infarction area in the hearts infused with fatty acids for 15 min immediately following 30 min of global no flow ischemia (**Figure 7**) and the interaction effect between the type of fatty acid and the concentration was significant ($p=0.006$). Compared to controls which received an infusion of only 3% fatty acid free BSA, hearts receiving infusions of 80, 100 and 120 µM of DHA experienced significantly increased infarction ($p<0.005$) (**Figure 7**). In fact, 120 µM of fatty acid was the maximum amount of DHA tolerated as several hearts (4/6) failed to complete the reperfusion protocol with the 120 µM infusion of DHA. Following a dramatic increase in contracture these hearts went into fibrillation and did not recover. In addition, hearts receiving 80, 100 and 120 µM of palmitate experienced greater infarction than controls ($p<0.025$) (**Figure 7**). Both palmitate and DHA cause increasing infarction with increasing concentrations, however 10µM of DHA caused significantly less infarction than 10µM of palmitate ($p<0.025$) (**Figure 7**). The infarction area after infusion with 10µM of DHA was also significantly lower than the vehicle control ($p<0.025$). At infusion concentrations >10 µM, there was no differences in the infarction areas for equimolar concentrations of palmitate and DHA, although infarction area with DHA tended to get larger.

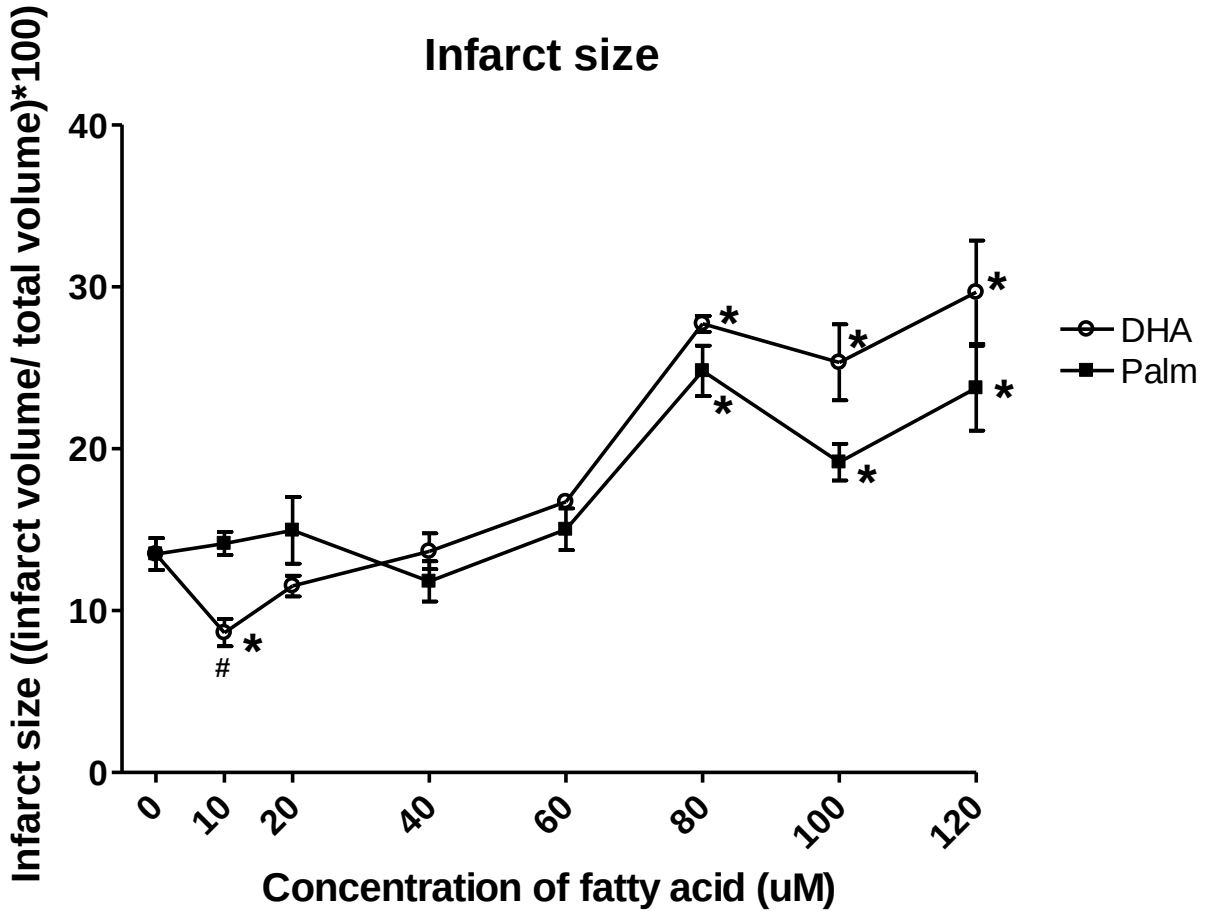

Figure 7. Myocardial Infarct Size. The effect of increasing concentrations of palmitate (Palm) and docosahexaenoic acid (DHA) on post ischemia and reperfusion infarct size (infarct volume/total heart volume). Valus are mean ± SEM. Two way ANOVA revealed a significant interaction ($p=0.006$). A priori comparisons between the fatty concentrations of DHA and palmitate with vehicle control, and between equimolar concentrations of DHA and palmitate were completed by independent t tests with significance set at $p<0.025$. *Significantly different from vehicle control. #Significantly different from equimolar palmitate.

4.3.2 Functional measurements

Baseline functional measurements of heart rate, Left Ventricular End Diastolic Pressure, Left Ventricular Systolic Pressure, Left Ventricular Developed Pressure or maximal Rate of Pressure Development did not differ between groups after an analysis of variance (**Table 11**). For

each functional variable, dose responses over time are presented for each fatty acid and the difference between equimolar concentrations of palmitate and DHA were calculated and plotted to highlight the differences between the fatty acids.

LVEDP rose gradually with ischemia and slowly returned to baseline in the control hearts (n=3). The fatty acid dose and type interaction (p=0.004) and fatty acid dose and time interaction (p=0.014) were significantly different (**Figures 8-10)**. For both fatty acids, LVEDP tended to increase with the concentration, but the pressure gain was significantly greater with palmitate at 60-100 µM. In addition, with 80 and 100 µM infusions of palmitate, there was a dramatic and rapid increase in pressure during fatty acid infusion that then partially resolved with continued reperfusion. Hearts receiving an infusion of 10 (n=3), 20 (n=3), and 40 (n=3) µM of DHA as well as 10 µM (n=4) of palmitate exhibited a very similar response as the controls.

Left Ventricular systolic pressure decreased with ischemia, and rapidly retuned to baseline levels with the onset of reperfusion and then tended to decline over the full course of reperfusion in the vehicle control hearts. The fatty acid dose and type of fatty acid interaction (p<0.001) and fatty acid dose and time interaction (p=0.027) were significantly different (**Figures 11-13)**. All fatty acid infusions had a general response similar to control, but there did not appear to be a consistent pattern between concentration and pressure. For example, for DHA infusions, 10 and 100 µM infusions tended to have the lowest reperfusion pressures, while 20 and 40 µM infusions tended to have the highest reperfusion pressures and there were no concentration similarities to the palmitate responses.

Left ventricular developed pressure (LVDevP) is a measure of contractility. A decreased pressure reflects an inability of the heart to exert pressure. LVDevP is normalized to each concentrations respective value to represent the percent change from baseline. The vehicle

control demonstrated a rapid and practically total decrease in pressure that rapidly increased with the start of reperfusion with a tendency to decline afterwards. The fatty acid dose and type of fatty acid interaction ($p<0.001$), the fatty acid dose and time interaction ($p=0.001$) and the fatty acid type and time interaction ($p=0.002$) were all significantly different (**Figures 14-16)**. Infusion of low concentrations of palmitate (10 and 20μM palmitate) tended to increase contractility as compared to higher concentrations (60 and 80 μM palmitate). Similarly to the hearts infused with palmitate, hearts infused with DHA exhibited increased LVDevP with lower concentrations (10 and 20 μM) as compared with higher concentrations of DHA (60, 80 and 100 μM). In addition, palmitate LVDevP tended to be higher for 10 and 20 μM and 80 and 100 μM, but similar for 40 and 60 μM as compared with DHA LVDevP.

Coronary perfusion pressure (CPP) is an indicator of the resistance in coronary arteries. The type of fatty acid and fatty acid dose interaction was significant ($p<0.001$) (**Figures 17-19**). In the vehicle control, ischemia caused a rapid decrease and slightly negative pressure, that was restored with and slightly increased during reperfusion. During infusion with fatty acids, the increasing concentrations tended to result in increased pressure as reperfusion progressed with the highest concentration of each fatty acid causing the greatest CPP. The higher doses of palmitate tended to causing higher pressures as compared with DHA.

Maximal rate of pressure development (+dP/dt) is normalized to baseline values to represent the percent change from baseline during reperfusion. Again, the vehicle control demonstrated a rapid and practically absolute decrease with ischemia, followed by a rapid increase with reperfusion and evidence of decline throughout the reperfusion. The relative range of the rate response was large for the various fatty acid infusions. The fatty acid dose X type of fatty acid X time interaction was significant ($p<0.001$) (**Figures 20-22)**. At the end of

reperfusion, +dP/dt was lower with the high concentrations of palmitate and DHA. The infusion of 100 µM of palmitate or DHA resulted in 46% and a 52% decrease respectively from baseline values compared to a 21% decrease in controls (**Figures 20 and 21**). The rate differences between DHA and palmitate were relatively similar for 40 µM. The rate was generally lower for DHA infusions relative to palmitate except for 60 µM, where DHA was greater.

Table 11. Heart baseline functional values.

	LVEDP	LVSysP	LVDevP	+ dP/dt	HR
		mmHg		*mmHg/s*	*bpm*
Control					
	7.8 ± 2.9	105 ± 16	97 ± 16	2446 ± 560	254 ± 21
DHA µM					
10	9.5 ± 0.8	91 ± 16	81 ± 17	2063 ± 399	290 ± 26
20	9.4 ± 3.1	115 ± 3	106 ± 4	2801 ± 618	256 ± 42
40	7.5 ± 0.7	111 ± 3	104 ± 3	3600 ± 162	242 ± 23
60	7.1 ± 1.0	121 ± 15	114 ± 15	3911 ± 530	309 ± 6
80	7.0 ± 1.5	98 ± 14	91 ± 12	2358 ± 470	211 ± 35
100	7.2 ± 1.3	99 ± 10	93 ± 9	2835 ± 364	288 ± 5
Palmitate µM					
10	7.7 ± 1.1	139 ± 5	131 ± 4	3107 ± 363	224 ± 14
20	16.3 ± 2.0	162 ± 28	146 ± 30	4614 ± 709	273 ± 40
40	12.9 ± 1.1	157 ± 15	145 ± 16	5177 ± 448	308 ± 17
60	9.0 ± 2.4	121 ± 18	112 ± 17	2765 ± 339	284 ± 15
80	12 ± 3.8	161 ± 17	149 ± 19	5368 ± 722	293 ± 15
100	9.7 ± 2.0	121 ± 17	111 ± 16	2784 ± 803	299 ± 6

Heart Baseline Functional Values. LVEDP, Left Ventricular End Diastolic Pressure; LVSysP, Left Ventricular Systolic Pressure, LVDevP, Left Ventricular Developed Pressure; + dP/dt, maximal rate of pressure development; HR, Heart Rate.

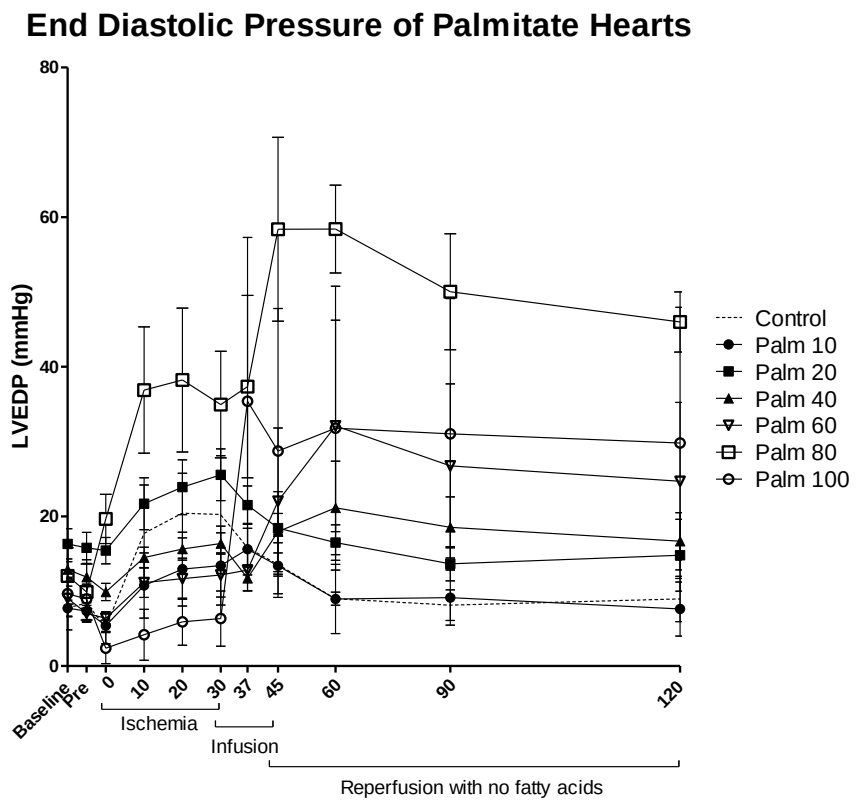

Figure 8. Left Ventricular End Diastolic Pressure (LVEDP) and contracture curve during stabilization, ischemia, infusion and reperfusion of hearts receiving infusions of 10, 20, 40, 60, 80 and 100 µM of palmitate (Palm). Values represent means ± S.E.M.

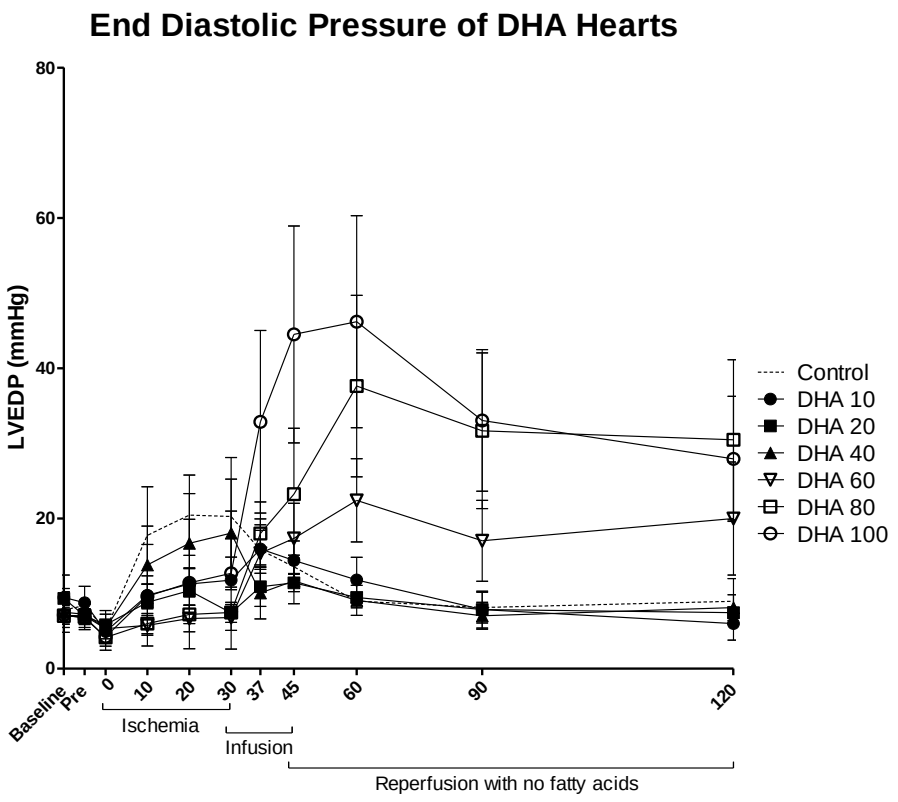

Figure 9. Left Ventricular End Diastolic Pressure (LVEDP) and contracture curve during stabilization, ischemia, infusion and reperfusion of hearts receiving infusions of10, 20, 40, 60, 80 and 100 µM of docosahexaenoic acid (DHA). Values represent means ± S.E.M.

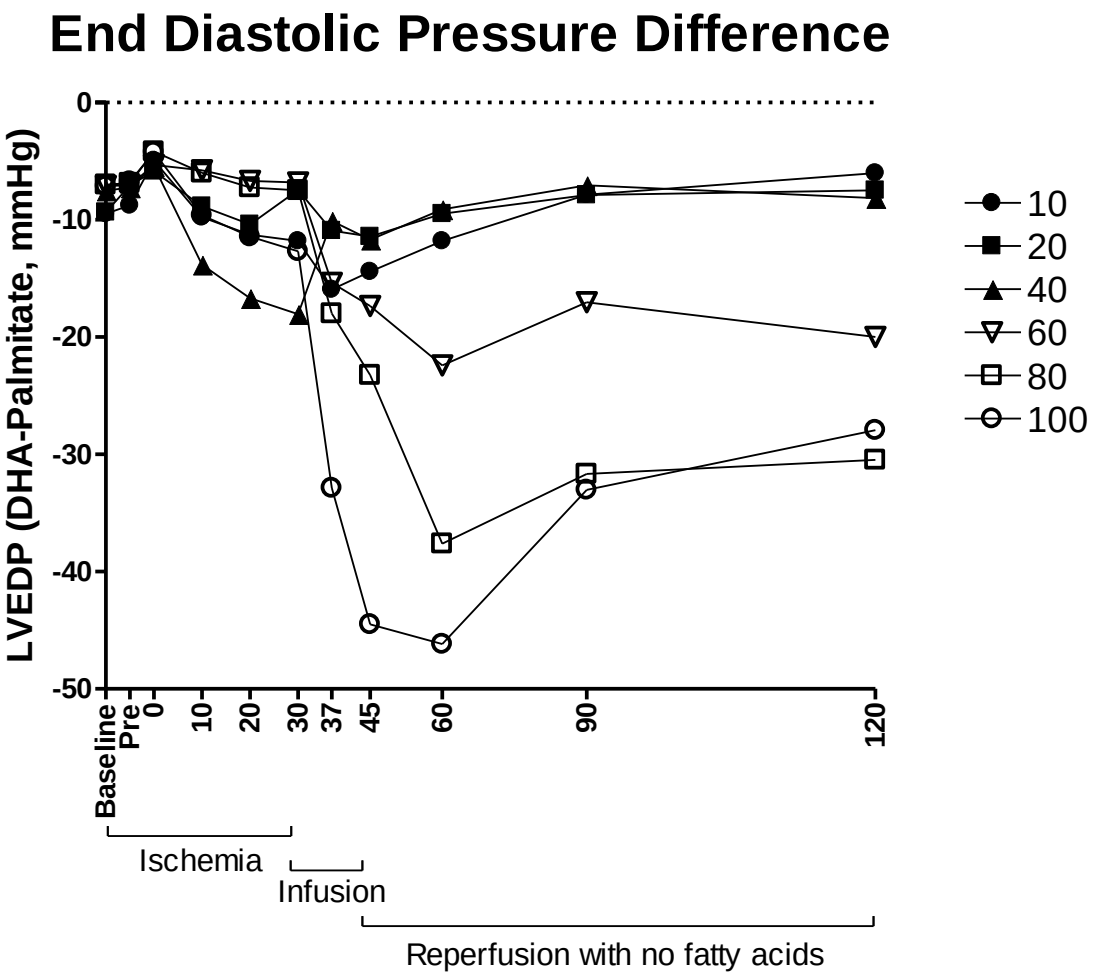

Figure 10. The average Left Ventricular End Diastolic Pressure (LVEDP) of hearts receiving an infusion of DHA subtracted by the average LVEDP of hearts receiving infusions of palmitate. Values represent means ± S.E.M.

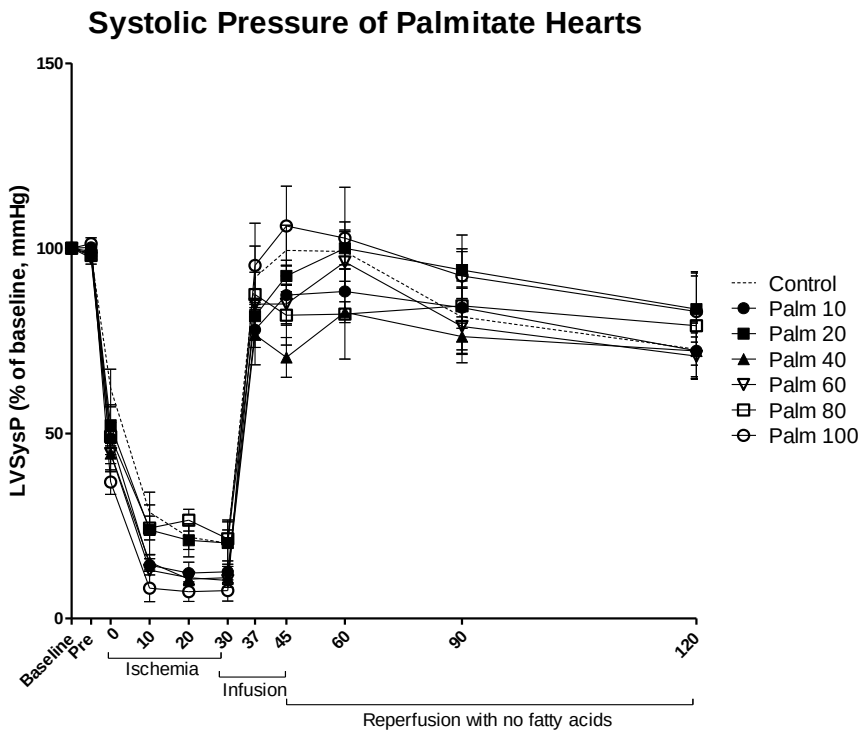

Figure 11. Left Ventricular Systolic Pressure (LVSysP) curve during stabilization, ischemia, infusion and reperfusion of hearts receiving infusions of10, 20, 40, 60, 80 and 100 µM of palmitate (Palm). Values represent means ± S.E.M.

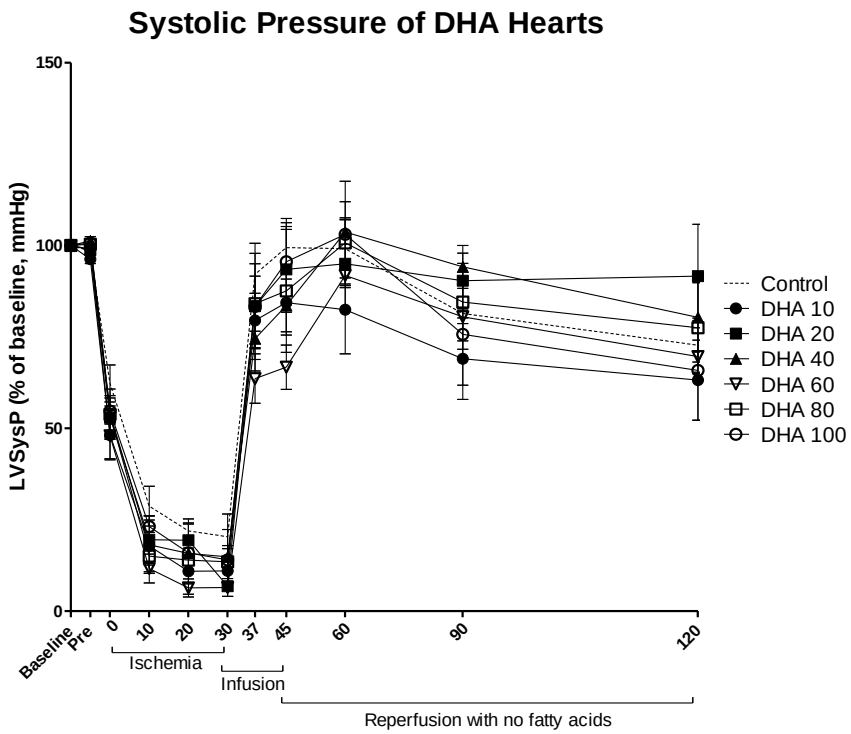

Figure 12 Left Ventricular Systolic Pressure (LVSysP) curve during stabilization, ischemia, infusion and reperfusion of hearts receiving infusions of10, 20, 40, 60, 80 and 100 µM of docosahexaenoic acid (DHA). Values represent means ± S.E.M.

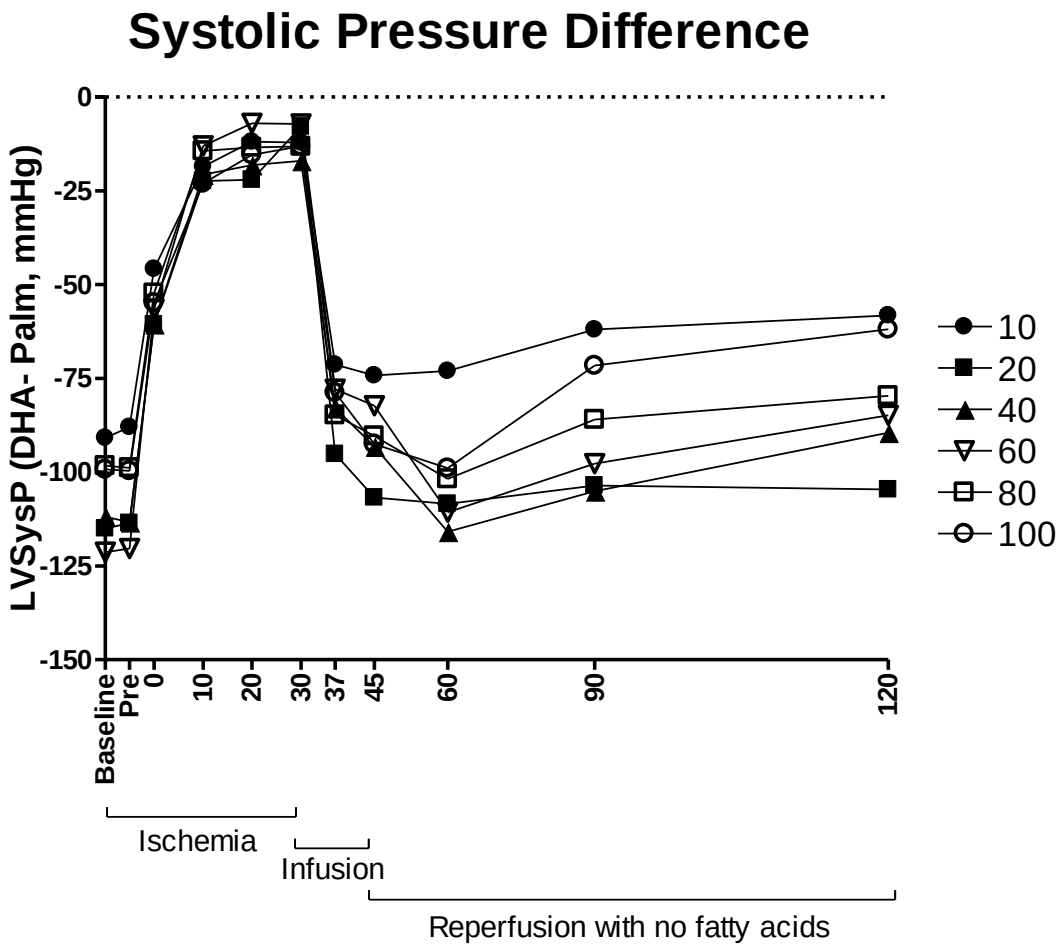

Figure 13. The average Left Ventricular Systolic Pressure (LVSysP) of hearts receiving an infusion of DHA subtracted by the average LVSysP of hearts receiving infusions of palmitate. Values represent means ± S.E.M.

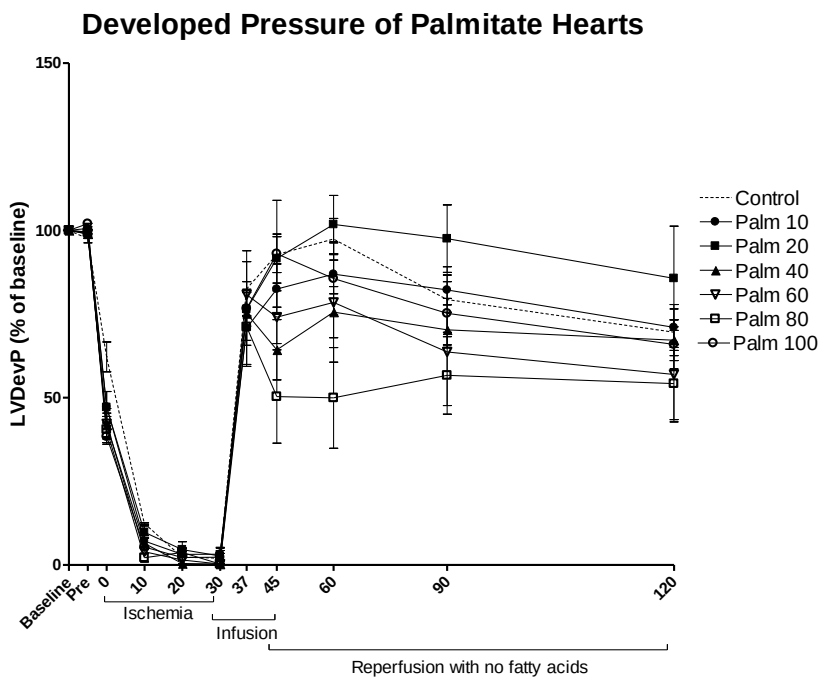

Figure 14 Left Ventricular developed pressure (LVDevP) of hearts infused with palmitate (Palm). LVDevP is the difference between systolic and diastolic pressure a measure of the hearts ability to contract. All data points are normalized to the respective baseline values. Values represent means ± S.E.M.

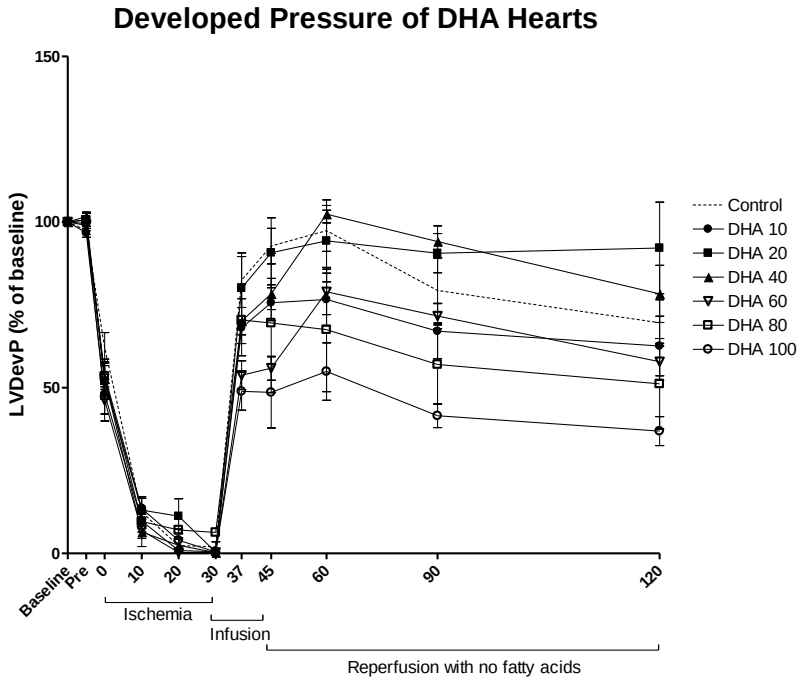

Figure 15. Left Ventricular developed pressure (LVDevP) of hearts infused with docosahexaenoic acid (DHA). LVDevP is the difference between systolic and diastolic pressure a measure of the hearts ability to contract. All data points are normalized to the respective baseline values. Values represent means ± S.E.M.

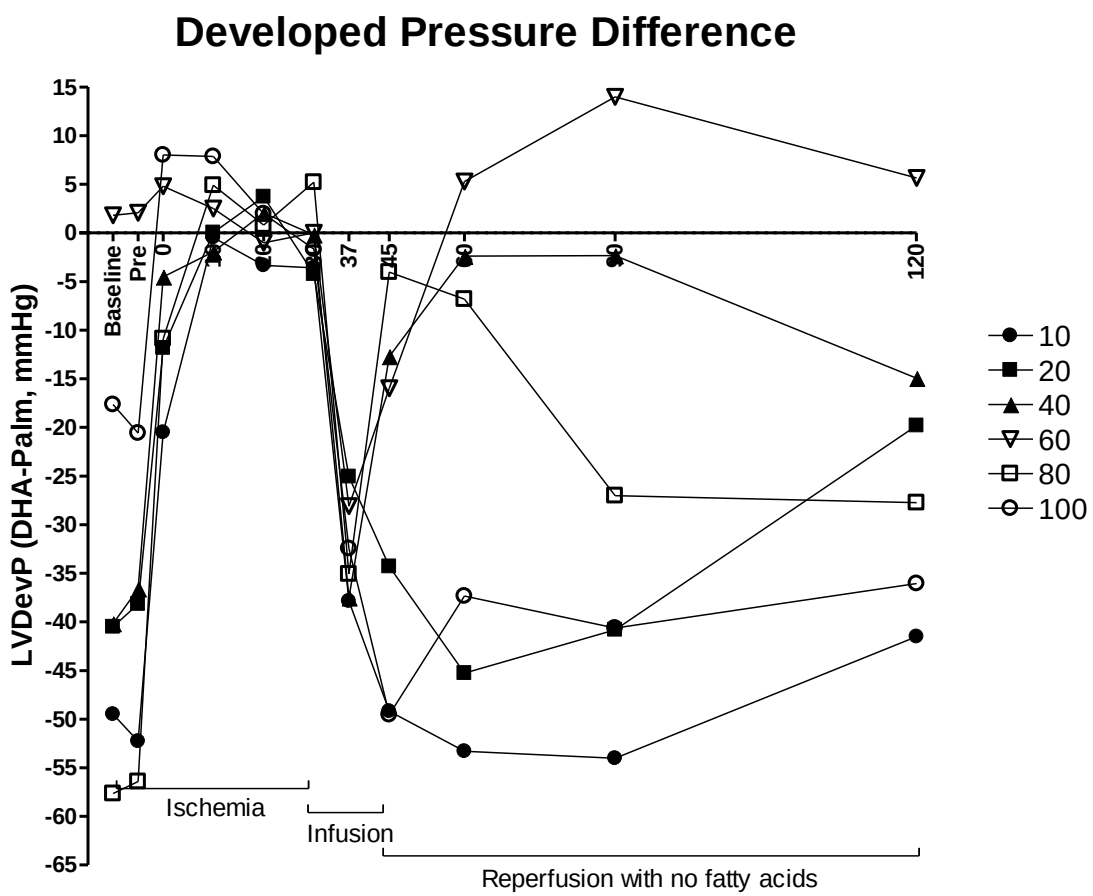

Figure 16. The average Left Ventricular Developed Pressure (LVDevP) of hearts receiving an infusion of DHA subtracted by the average LVDevP of hearts receiving infusions of palmitate. Values represent means ± S.E.M.

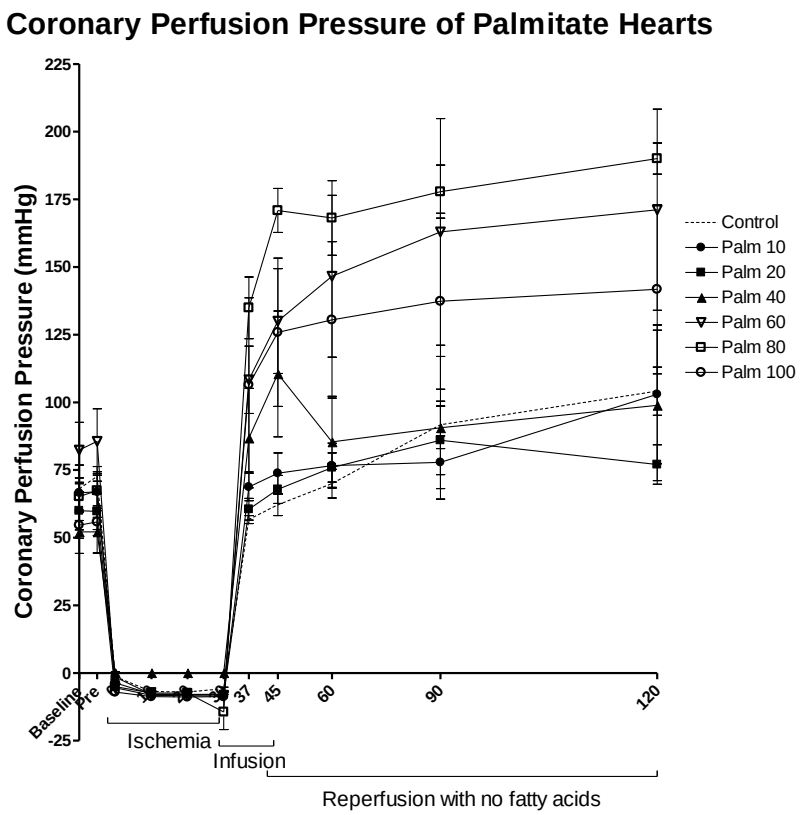

Figure 17. Coronary Perfusion Pressure (CPP) curve during stabilization, ischemia, infusion and reperfusion of hearts receiving infusions of 10, 20, 40, 60, 80 and 100 µM of palmitate (Palm). Values represent means ± S.E.M.

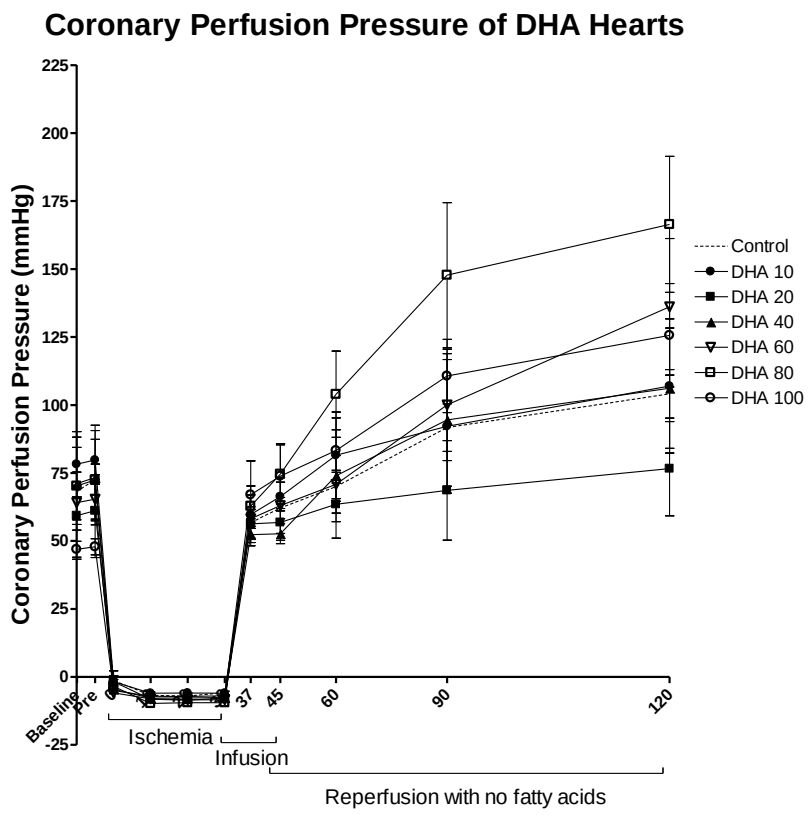

Figure 18. Coronary Perfusion Pressure (CPP) curve during stabilization, ischemia, infusion and reperfusion of hearts receiving infusions of 10, 20, 40, 60, 80 and 100 µM of docosahexaenoic acid (DHA). Values represent means ± S.E.M.

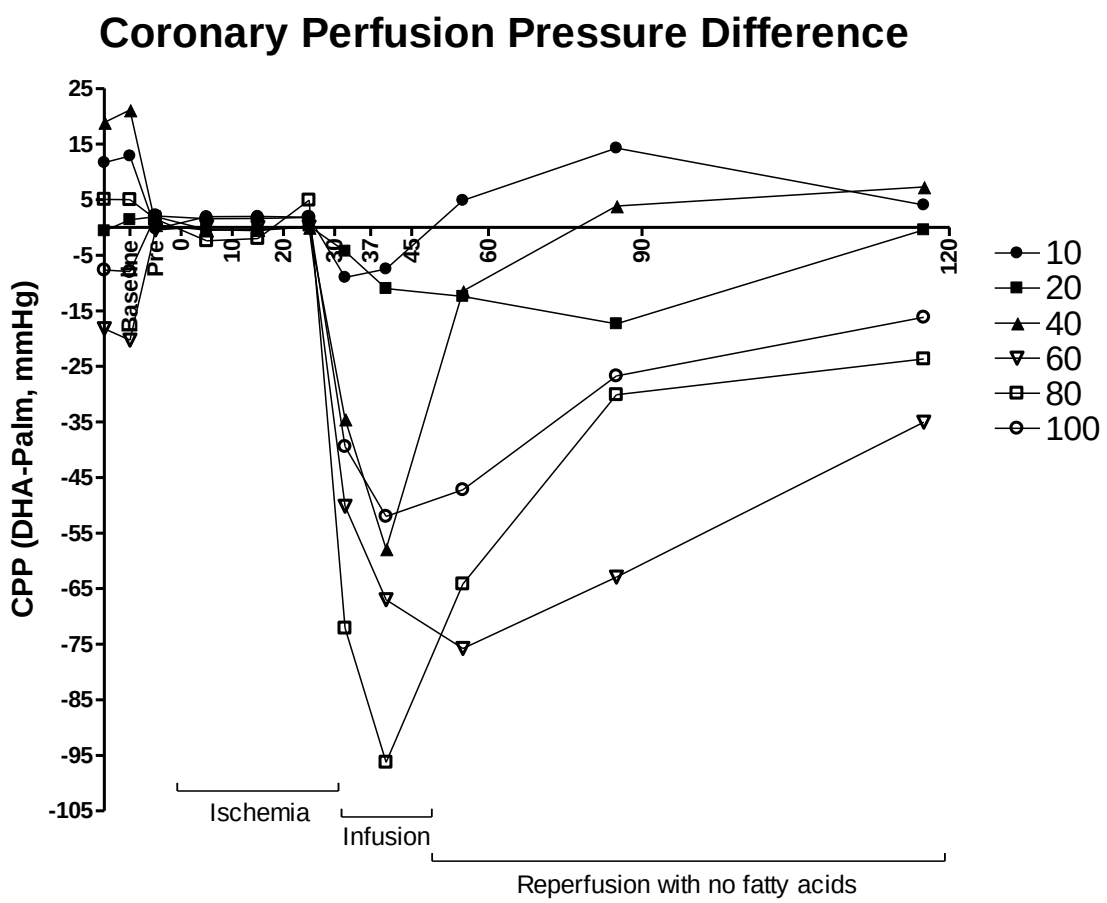

Figure 19. The average Coronary Perfusion Pressure (CPP) of hearts receiving an infusion of DHA subtracted by the average CPP of hearts receiving infusions of palmitate. Values represent means ± S.E.M.

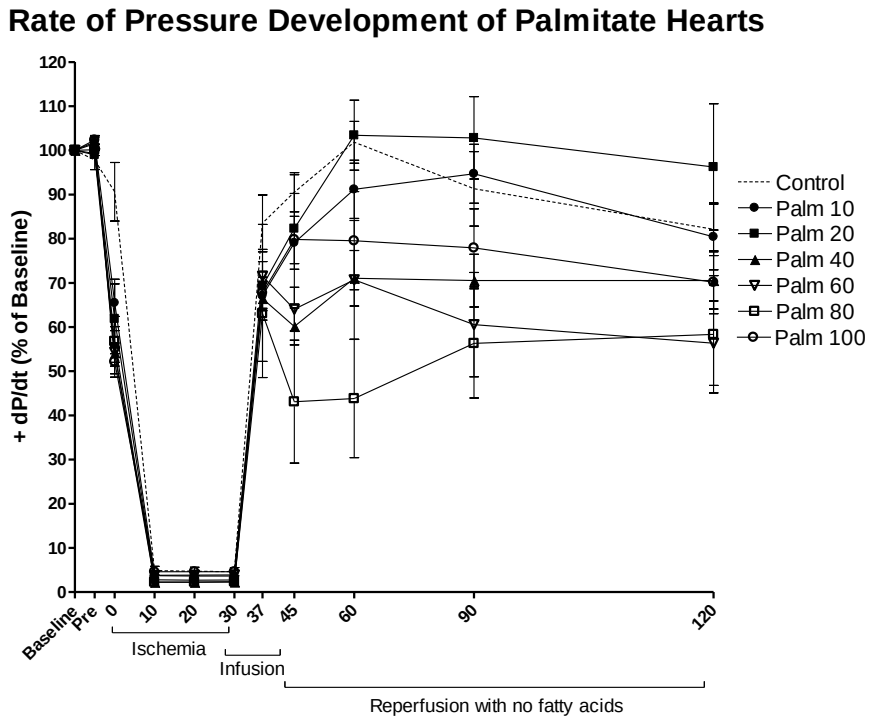

Figure 20. Maximal rate of pressure development (+dp/dt) of hearts receiving and infusion of palmitate (Palm). All data points are normalized to their respective baseline values. Values represent means±S.E.M

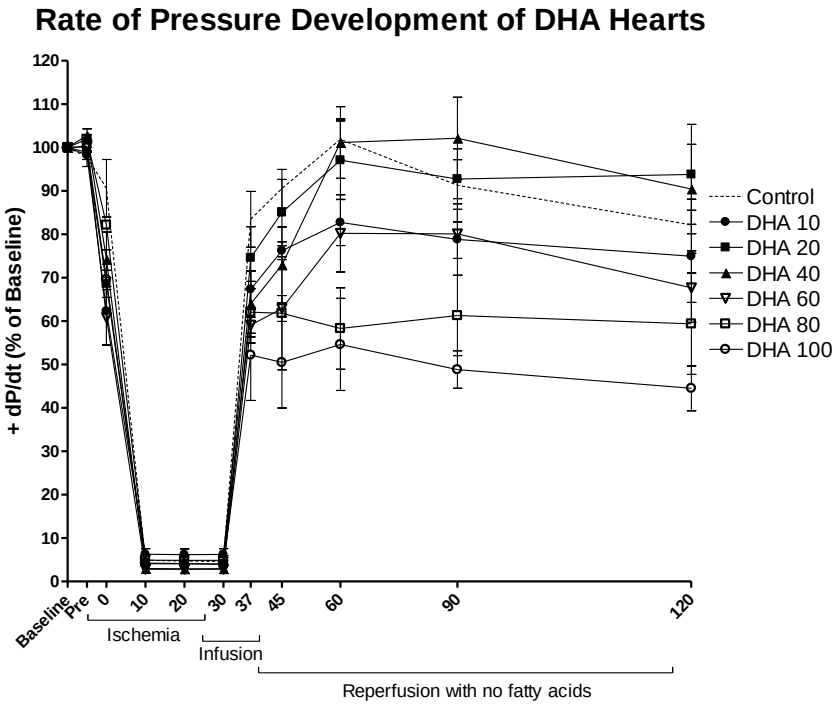

Figure 21. Maximal rate of pressure development (+dp/dt) of hearts receiving and infusion of docosahexaenoic acid (DHA). All data points are normalized to their respective baseline values. Values represent means±S.E.M

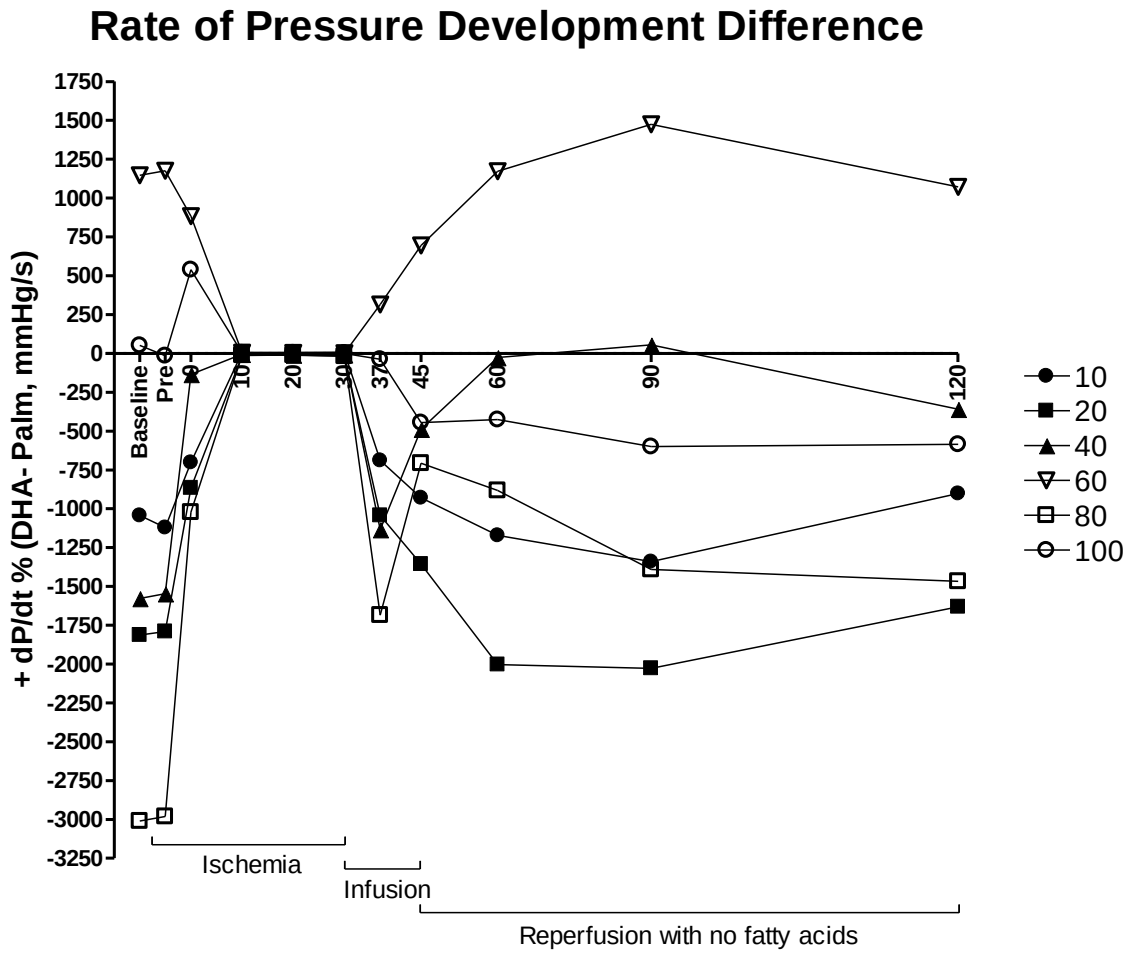

Figure 22. The average maximal Rate of Pressure Development (+dP/dt) of hearts receiving and infusion of DHA subtracted by the average +dP/dt of hearts receiving infusions of palmitate. Values represent means ± S.E.M.

Chapter 5
Discussion

5.1 Comments on Hypotheses

1. DHA infusion immediately following 30 minutes of global no flow ischemia will significantly reduce infarction area.

The infusion of DHA immediately following a bout of 30 min global no flow ischemia dose-dependently affects the infarct area in artificially perfused male Sprague Dawley rat hearts. The lowest concentration of DHA (10µM) delivered to the artificially perfused rat hearts significantly reduced the infarction area at the end of the reperfusion period. This was also significantly less than the infarction area of control hearts which only received an infusion of the fatty acid delivery vehicle 3% essentially fatty acid free bovine serum albumin ($p = 0.02$ for both by LSD post hoc after significant F-value for 2-way ANOVA). However, this significant effect was not revealed using Tukey's Honest Significance test ($p=0.46$ and $p=0.75$ compared to equimolar palmitate and controls respectively). The infarct sizes after infusion of 20, 40 and 60 µM for DHA were similar to controls. Infusions of 80, 100 and 120 µM of DHA, increased infarct sizes above that of controls and the lower dose fatty acid infusions. In fact after receiving infusions of 120 µM of DHA several hearts went into ventricular fibrillation which they could not recover from.

2. Infusion of palmitate will neither significantly increase nor decrease the infarcted area.

Hearts infused with 80 and 120 µM of palmitate experienced greater infarction than controls. The finding that 120 µM of palmitate causes increased infarction compared to controls was not expected as pilot work using 1.2mM of palmitate (n=3), i.e. 10 times the molarity of the largest concentration used in the DHA infusion study, found no significant increases compared to

controls (n=2) (data not shown). However, this was based on a small number of hearts. It has been reported that palmitic acid (16:0) and stearic acid (18:0) induce apoptosis and alterations in the phospholipid membranes of neonatal rat ventricular myocytes that can be counteracted by the co-addition of equimolar amounts of monounsaturated fatty acids (deVries et al., 1997).

3. DHA infusion will reduce the increase in contracture seen during reperfusion, measured by left ventricular end diastolic pressure, and maintain left ventricular developed pressure, a measure of the hearts ability to contract.

Contrary to our hypothesis, the infusion of DHA 15 minutes immediately following 30 minutes of global no flow ischemia caused an increase in Left Ventricular End Diastolic Pressure and contracture. Infusion of DHA also causes a decrease in Left Ventricular Developed Pressure and Rate of Pressure Development. This indicates that the hearts are no longer able to generate as much ventricular pressure.

4. Infusion of palmitate will increase contracture and reduce left ventricular developed pressure.

As hypothesized, an acute infusion of palmitate increased the level of Left Ventricular End Diastolic Pressure and contracture and decreased the hearts ability to generate force as indicated by a decrease in Left Ventricular Developed Pressure and maximal rate of pressure development.

5.2 Effect of Perfusion on Heart Fatty Acids and Fatty Acid Transport Proteins

Langendorff perfusion results in a decreased protein expression of the fatty acid transport protein H-FABP. This may be indicative of down regulation of fatty acid utilization as cellular fatty acid pools decreased. Artificial perfusion results in modest decreases in fatty acid concentrations in nonesterified and triacylglycerol fatty acid pools possibly as a result of fat oxidation. These changes may reflect the composition of the perfusate which contained no fatty acids which the heart prefers to oxidize. No effects on phospholipids or total lipid extracts were found. This makes the Langendorff heart an ideal model for the examination of the effects of fatty acid on a working heart.

5.3 Effects of Ischemia and BSA on Heart Fatty Acids and Heart Function

Thirty minutes of global no flow ischemia and 1.5 hrs of reperfusion did not cause any significant decreases in the concentration of heart total lipids, total triacylglycerols, total phospholipids, or heart total non-esterified fatty acids compared to controls. Although, phospholipase activity is dramatically increased during ischemia (Ford et al., 1991), the phospholipid pool is extremely large relative to circulating plasma free fatty acids pools.

The infusion of 3% BSA had more significant effects on heart fatty acid content. Heart total lipids did not decrease significantly compared to those hearts which received no BSA and were exposed to the same ischemia protocol. The most marked decreases caused by the infusion of BSA for 15 minutes prior to ischemia were seen in the dimethyl acetals (DMA). Compared to the ischemia alone group 16:0 DMA, 18:0 DMA and 18:1 DMA all decreased significantly ($p<0.05$). Similarly, in the PL fraction 16:0 DMA, 18:0 DMA and 18:1 DMA were all decreased compared to the ischemia alone group ($p<0.05$). Early at the onset of global ischemia (2 minutes)

membrane-associated calcium-independent plasmalogen-selective phospholipase A_2 activity has been shown to increase over 400%, to be nearly maximally activated after only 5 minutes, and to remain activated throughout the entire ischemic interval (2-60 minutes) (Ford et al., 1991). It is possible that during the long 1.5 hour of reperfusion the plasmologens, which were possibly hydrolyzed by phospholipases, were re-esterified back into plasmalogens since no decreases in DMA were seen following ischemia (**Tables 7 and 8**). The infusion of BSA might have interfered with this by extracting the fatty acids prior to re-esterification, decreasing the levels of DMA. More studies would be required to elucidate this possible effect.

Importantly, there were no effects caused by BSA on functional parameters including HR, LVEDP, Rate of Pressure Development or infarction size. There was also no effect on the fatty acid content of DHA or palmitate. These results validated the use of BSA in this model of fatty acid infusion.

5.4 The Effect of Post-ischemic Infusion of DHA and Palmitate on Heart Function and Infarction

The present study demonstrates that DHA as a free fatty acid has the potential to provide induce both beneficial and detrimental effects in the heart upon infusion during reperfusion post ischemia and that these effects are dose dependent. At 10 µM DHA appears to exert a cardioprotective effect as indicated by reduced infarction size relative to vehicle and palmitate control. This is physiologically relevant as DHA free fatty acid concentrations in plasma can be undetectable and increase to 37 µM in a fasting rat (Bazinet et al., 2006). Intermediate infusion concentrations (20-60 µM) showed no benefits or detriments on infarction relative to control, while doses above 80 µM demonstrated increased infarction. While the higher doses may not be

physiological for fasting animals, there is evidence that with feeding, postprandial levels of DHA can fall in this range in humans (Newens et al., 2011). These results indicating a negative effect of DHA at high doses contradict other infusion studies (Billman et al., 1999; Kang & Leaf, 1994; Kang & Leaf, 1995; Kang & Leaf, 1996; Xiao et al., 2005; Xiao et al., 2008) and the hypothesis of this current study. The increased infarction area, increased contracture and decreased contractility caused by the infusion of varying levels of palmitate and DHA indicate a harmful effect on ischemia reperfusion injury, at least for this specific model of ischemia reperfusion. The negative effects observed with single treatment could be beneficial over chronic exposure through the preconditioning phenomenon, but further experimentation is needed. However, it is clear that high DHA in the present model has negative effects as hearts infused with 120 μM of DHA went into fibrillation and failed. The potential use of DHA infusion directly to the heart following ischemia in humans needs to be approached with caution as DHA.

LVEDP is a measurement of contracture in the heart. Contracture, which is marked by a distinct rise in left ventricular end diastolic pressure, occurs by an absence of ATP and an overload of calcium in the myocyte causing actin-myosin crossbridges to remain in an attached state (Piper et al., 2003). The increased contracture caused by high levels of both palmitate and DHA are likely caused by excessive intracellular concentrations or possibly an inadequate amount of ATP available. It has been shown before that DHA can stimulates phospholipase C (PLC) which hydrolyzes the phosphatidylinositol 4,5-bisphosphate in membranes releasing free inositol trisphosphate (IP_3) and diacylglycerol (DAG). Production of these second messengers results in a rapid release of Ca^{2+} from the endoplasmic reticulum causing an increase in intracellular calcium. This is possibly a contributing factor leading to the increase in contracture observed at high concentrations of DHA. Increased calcium concentrations can also lead to ROS

production from the mitochondria and downstream signaling resulting in caspase-3 activation contributing to apoptosis (Aires et al., 2007). It is possible that the increase in infarction seen following DHA infusion may be caused by apoptosis.

Previous studies using isolated perfused rat hearts have reported protective effects of n-3 HUFA on infarction (Zeghichi-Hamri et al., 2010; Abdukeyum et al., 2008). These dietary studies differ from the present study in one important way. Dietary studies cannot differentiate between effects caused by increased n-3 HUFA or a decrease in other nutrients. These studies also cannot rule out the possibility that the fatty acids provided their benefit through interactions with other organs independent of the heart. In addition, in these studies the fatty acids are incorporated into heart phospholipids (Zeghichi-Hamri et al., 2010) and released by the increased activity of phospholipases during ischemia. Hearts in the present study did not experience the increase in DHA until the start of reperfusion (excluding endogenous release) which may have affected the influence of DHA on infarction.

Rats consuming a normal chow diet as in this study still contain an adequate (for normal functioning) amount of DHA in their myocardial phospholipids. DHA is obtained through the minor amounts of preformed DHA in the chow as well as through biosynthesis of its dietary essential precursor alpha-linolenic acid (ALA; 18:3n-3). It is possible that with the endogenous infusion of DHA from phospholipids by phospholipases and the infusions of high concentrations of DHA in this study the cumulative amount that the heart and ion channels are experiencing is affecting the electrophysiology of cardiomyocytes in a negative way, while the lower concentrations infused are still within a beneficial range. Future studies should attempt to

quantify the amount of DHA released from phospholipids from rats on normal chow diets, as well as from rats consuming n-3 HUFA supplemented and deficient diets.

5.5 Limitations

A limitation of the artificially perfused heart model is that it does not actually represent a physiological environment. Circulating factors, hormonal and neural contributions which all affect ischemia reperfusion injury cannot play a role in this model. In addition, the acute infusion of DHA in this study is probably not an accurate representation of the physiological infusion during ischemia reperfusion. In a physiological setting, phospholipases release fatty acids during ischemia in addition to reperfusion. These lipases are also hydrolyzing other fatty acids from the sn-2 position such as arachidonic acid which would likely be playing a competitive role against n-3 HUFA. Therefore, while the free fatty acid concentrations infused in the present study cover the physiological range for DHA, the relative range of 100% of the free fatty acids as DHA is not physiological.

Also, palmitate was used as the fatty acid control. Palmitate is a very abundant fatty acid in the circulation. It is relatively inexpensive and as such it was used for the model development. In the study, the infusion of palmitate caused a significant increase in infarction compared to controls. Palmitic acid (16:0) and stearic acid (18:0) induce apoptosis in neonatal rat ventricular myocytes and oleic acid (18:1n-9) has no affect on apoptosis (deVries et al., 1997). Oleic acid may be an inert control to use in the future. In addition, AA should be considered as a physiological control as it tends to be the dominant HUFA in the sn-2 position of phospholipids, particularly when n-3 PUFA intake is low.

5.6 Future Directions

Follow up studies should focus on quantifying apoptotic and necrotic cell death in this model of infusing fatty acids to an artificially perfused rat heart. This could be accomplished by examining the morphological features of apoptosis including; cell condensation and shrinkage, cell membrane blebbing, and cytoplasmic condensation or by examining the release of cytosolic proteins. In addition, it would also be valuable to examine any possible changes in the expression and activation of proteins involved in apoptotic signaling including but not limited to Bax, Bcl-2, p38 MAPK and Caspase-3. Measurements of intracellular calcium would assist in the understanding of the increased contracture caused by high levels of palmitate and DHA.

In the future, an examination of other reference fatty acids may be relevant. It has been shown that palmitic acid (16:0) and stearic acid (18:0) two saturated fatty acids induce apoptosis when directly applied to neonatal rat ventricular myocytes and that oleic acid (18:1n-9) has no affect on apoptosis (deVries et al., 1997). Oleic acid may be an inert control to use in the future. In addition, AA should be considered as a physiological control as it tends to be the dominant HUFA in the sn-2 position of phospholipids.

5.7 Conclusion

The results of the present study indicate that the effects of an acute infusion of DHA immediately following 30 min of global no flow ischemia are dose dependent. The lowest concentration of DHA (10μM) delivered to the artificially perfused rat hearts significantly reduced the infarction area at the end of the reperfusion period. This was also significantly less than the infarction area of control hearts which only received an infusion of the fatty acid delivery vehicle 3% essentially fatty acid free bovine serum albumin. Hearts receiving infusions

of 20, 40 and 60 µM of DHA or palmitate experienced similar areas of infarction as vehicle control. The infusion of high concentrations of palmitate and DHA (80 and 100 µM) exacerbated ischemia reperfusion injury as demonstrated by an increase in infarction area, an increase in contracture and a decrease in the hearts ability to contract and generate force and infusion of 120 µM of DHA tended to result in fibrillation and heart failure. To be able to fully understand the mechanism of the protective effect of DHA on coronary heart disease and sudden cardiac death it is crucial to understand the dose response of DHA on the heart. In addition this dose response is vital when trying to accurately set dietary recommendations for DHA consumption.

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