Fatty acid composition of isolated cardiac sarcoplasmic reticulum from rats subjected to left ventricular hypertrophy due to volume overload.

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Abstract

Left ventricular hypertrophy (LVH) is a compensatory adaptation to an increased workload in the heart. Although beneficial at first, detrimental effects become apparent as hypertrophy progresses. One of the deleterious effects is cardiac contractility. The sarcoplasmic reticulum (SR) is an intracellular organelle which regulates the contraction-relaxation cycle in the heart by modulating intracellular Ca^{2+} concentrations. Lipids are important for the activity of membrane proteins; fatty acids constitute a major component of membrane lipids. The objective of this study was to determine the fatty acid composition of the SR in control and volume overload (VO) induced hypertrophic hearts. Heart function was analyzed by echocardiography. Volume overload was induced in male Sprague-Dawley rats by creating a shunt between the descending aorta and the inferior vena cava. Characterization of fatty acids was done in control and hypertrophied hearts by gas chromatography. A total of 29 fatty acids were observed in the SR of control and VO groups. The profile consisted of fatty acids ranging from C12:0 to C22:6n-3. Of those characterized, nine fatty acids varied between the control and VO group. At 4 and 28 weeks, a decrease was observed in heptadecenoate in the VO group compared to the control group, while docosadienoate and unidentified fatty acids “X”, and “Z” were reduced at 16 and 28 weeks in the VO groups compared to the control. At 28 weeks, decreases in trans-oleate, nonadecenoate, oleate, eicosenoate, and docosenoate were observed in the VO group. The results of this study show alterations in the SR fatty acid composition of VO hearts which could in part contribute to impairment of SR function and cardiac contractility.

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Abbreviations

AV – Aortocaval Shunt

Ca^{2+} - Calcium

ddH_20 – Double distilled water

DHA – Docosahexaenoic Acid

DHPR – Dihydropyridine receptor

EPA – Eicosapentaenoic Acid

FA – Fatty Acid

FAMES - Fatty Acid Methyl Esters

FS – Fractional Shortening

GC – Gas Chromatography

H - Homogenate

HBW – Heart-to-Body weight

IVC – Inferior Vena Cava

kDA – kilodalton

LV – Left ventricular or left ventricle

LVDD - Left ventricular end-diastolic diameter

LVH – Left Ventricular Hypertrophy

LVSD - Left ventricular end-systolic diameter.

MUFA – Monounsaturated Fatty Acid

MVR – Mitral Valve Regurgitation

PLB – Phospholamban

PUFA – Polyunsaturated Fatty Acids

RyR – Ryanodine Receptor

S - Saturated

SERCA2a – Sarco-endoplasmic Reticulum Ca^{2+} - ATPase

SR – Sarcoplasmic Reticulum

US – Unsaturated

VO – Volume Overload

List of Fatty Acids

C12:0 - Dodecanoate

C16:0 – Palmitate

C16:1 - Palmitoleate

C17:1 - Heptadecenoate

C18:0 - Stearate

C18:1 – Oleate

C18:1tΔ^{9} – Trans – oleate

C18:3n-6 – Gamma - linolenate

C19:0 – Nonadecanoate

C20:1cΔ^{5} - Eicosenoate

C22:0 – Docosanoate

C22:2 – Docosadienoate

C22:6n-3 - Docosahexaenoate

I. Introduction

Overview

Heart failure is a major cardiovascular disease affecting more than 22 million people around the world and is the major cause of mortality in the United States (Hunter and Chien 1999). Incidence of heart failure is on a rapid rise soon to reach epidemic proportions (Tamargo and Lopez-sendon 2004). Cardiac hypertrophy is one of the most common risk factors for the subsequent development of heart failure. This condition refers to the enlargement of cardiomyocytes which occurs in response to increased workload. Progressive deterioration of cardiac contractility is a central feature of cardiac hypertrophy and heart failure (Chiiariello and Perrone-Filardi 1999). The sarcoplasmic reticulum (SR) represents a specialized form of endoplasmic reticulum present in muscle which regulates the contraction-relaxation cycle by controlling Ca^{2+} concentration in the sarcoplasm. Key Ca^{2+} cycling proteins such as the sarcoplasmic reticulum Ca^{2+} ATP-ase (Serca2a) and the Ryanodine receptor (RyR), undergo alterations in expression/function due to cardiac hypertrophy (Balke and Shorofsky 1998).

The lipid bilayer membrane of cells and organelles is integral to the functioning of membrane proteins. Fatty acids (FA) are a key component of the polar lipids forming the lipid bilayer. Alteration in the FA composition of lipid membranes has been shown to affect the action of integral membrane proteins (Eze 1990). Not only is the presence of certain FA in membrane lipids essential for optimal protein activity, but various characteristics of FA such as chain length and degree of saturation can affect the functioning of associated proteins (Eze 1990; Lee 1998). This study was based on the characterization of the FA composition in isolated cardiac sarcoplasmic reticulum (SR) in

rats undergoing left ventricular hypertrophy (LVH) due to volume overload (VO). Analysis entailed characterization in addition to comparison of the FA profiles between control and volume overloaded hearts as LVH progressed. The volume overload model was induced in male Sprague-Dawley rats by creation of an aortacaval shunt and analysis of SR FA composition was carried out by means of gas-chromatography.

Cardiac Hypertrophy and Heart Failure

Heart failure is a leading cause of death that is reaching epidemic proportions (Tamargo and Lopez-Sendon 2004). Heart failure is a multifactorial syndrome wherein the heart's ability to pump sufficient blood to meet the demands of the body is compromised. Cardiac failure is always characterized by an impairment in the contractility of cardiomyocytes. As a consequence of reduced contractility, a number of compensatory mechanisms take place to counteract the reduced intrinsic performance – one of which includes cardiac hypertrophy. The development of heart failure may be caused by various diseases such as hypertension (Gradman and Alfayoumi 2006), valvular heart disease (Strozecki *et al*. 2006), ischemic heart disease (Saeedi *et al*. 2006), myocarditis (Shariff *et al*. 2004) and diabetes (Scognamiglio *et al.* 2004). Regardless of the initial stimulus, the heart attempts to counter the effects with cardiac hypertrophy – a compensatory enlargement of cardiomyocytes which occurs in response to increased workload.

One of the most remarkable and important capacities of the heart is adaptability changes in hemodynamic load. Initially, the hypertrophic response may serve to normalize wall stress and maintain normal cardiac function. However, prolonged

hypertrophy can become detrimental, resulting in cardiac dysfunction and subsequent heart failure. Hypertrophic growth accompanies many forms of heart disease, including ischemic heart disease, hypertension, heart failure and valvular heart disease. Enhanced protein synthesis and heightened organization of the sarcomere are accompanied by hypertrophy. There are two broad types of cardiac hypertrophy: concentric hypertrophy due to pressure overload and eccentric hypertrophy due to volume overload (Opie, 1998).

Eccentric hypertrophy

Ventricular remodeling constitutes structural changes in the left ventricle in response to a sustained excessive workload on the heart. For remodeling to occur, a mechanical event (hemodynamic load), must be translated into a biochemical signal for growth. If the precipitating stress is one of volume overload (VO), the ventricle responds by adding new sarcomeres in series to existing sarcomeres. This serial addition results in eccentric hypertrophy – dilation of the left ventricle chamber size without a prominent increase in wall thickness. The mechanisms leading to ventricular remodeling and the many signals involved are not fully understood and constitute an extremely active area of research (Opie *et al*. 2006).

Volume Overload

Volume overload is characterized by an increase in blood volume either delivered to or remaining in the left ventricle. The left ventricle adapts to this by dilating so that a greater volume of blood can be ejected with each contraction (Carabello 1996). Diseases

such as anemia, congenital defects, and aortic or mitral valve regurgitation result in volume overload.

Mitral Valve Regurgitation (MVR)

The mitral valve is the valve located between the left atrium and ventricle. The valve opens during ventricular diastole – filling - and closes to prevent backflow of blood during ventricular systole - emptying. In MVR, the valve becomes weakened or damaged so that it does not close properly. This causes blood to leak back (regurgitate) into the left atrium when the left ventricle contracts (Carabello and Crawford 1997). Eccentric hypertrophy allows for an increase in stroke volume (volume of blood ejected from ventricle during each contraction) to make up for the stroke volume which is lost due to backflow into the left atrium (Otto 2001).

Aortocaval (AV) Shunt

The creation of an AV shunt has been used by many researchers to create the VO model. In an AV shunt, blood from the aorta is shunted into the inferior vena cava due to the high arterial pressure of the aorta as compared to the inferior vena cava (IVC). This in turn results in an increase in venous return to the heart (Dorn *et al*. 1988). Eccentric hypertrophy allows for the heart to compensate for excess venous return while maintaining adequate blood supply to the rest of the body

Calcium Handling

It is a well established fact that Ca^{2+} ions play an important role in the excitation-contraction cycle of the heart – the process by which an action potential triggers a myocyte to contract (Sjaastad *et al*. 2002). As a result of a myocyte being depolarized by

an action potential, Ca^{2+} is able to enter the cell through the L-type voltage dependent Ca^{2+} channels (also known as the *dihydropyridine receptors*(DHPR)), located on the sarcolemma. This calcium triggers a subsequent release of calcium that is stored in the SR through the SR calcium release channels – Ryanodine Receptors (RyR). This process is termed "Ca^{2+} induced Ca^{2+} release." Calcium release by the SR increases the intracellular calcium concentration, allowing calcium to bind to troponin-c, thus enabling the interaction between actin and myosin – which leads to force generation (contraction). For relaxation to occur, Ca^{2+} must dissociate from troponin-c which occurs when the intracellular Ca^{2+} concentration declines. Cytosolic Ca^{2+} is returned to the SR through the sarcoendoplasmic reticulum Ca^{2+} -ATPase (SERCA2a) which is regulated by the inhibitory protein phospholamban (PLB).

Alterations in Calcium Handling

Reduced cardiac contractility plays a defining role in the pathogenesis of heart failure. Numerous studies have focused on alterations in key Ca^{2+} cycling proteins and their function/expression during the progression of hypertrophy and heart failure. These studies have found a decrease in expression and/or activity of SERCA2a, increased PLB to SERCA2a ratios and hyperphosphorylation of RyR (Eisner *et al*. 2004).

The Biological Membrane

Fluid Mosaic model

Cellular membranes are important both physiologically and pathophysiologically. The biological membrane has numerous functions of which include: maintaining cellular

(or organellar) isolation from its outer environment; cell signaling; and providing a selective permeable barrier (Lehninger and Cox 1993). The lipid components of the biological membrane are arranged in two layers classically known as the phospholipid bilayer. The first glimpse of the structure of the biomembrane came in 1925, when Gorter and Grendell established the bilayer architecture of membranes. This was established through analysis of an erythrocyte phospholipid monolayer on an air-water interface. Decades later the discovery of vertical "flip flop" movements of phospholipids and lateral motions of proteins embedded within and transversing the bilayer were made. In 1972, Singer and Nicholson further clarified the aforementioned discoveries and coined the term "Fluid Mosaic Model." The major components of the cell membrane include phospholipids with their fatty acid tails, proteins and cholesterol. Each of these components has a specific function and plays an identifying role within the membrane.

Fatty Acids

Fatty acids are carboxylic acids having hydrocarbon chains which are 4-36 carbons long. Fatty acids may be saturated (no double bonds) or unsaturated (may contain one or more double bonds). Fatty acids containing one double bond are termed "monounsaturated fatty acids" (MUFA) and those with multiple double bonds are termed "polyunsaturated" (PUFA). Fatty acids can exist in different physical states dependent on their properties. Fatty acids which contain double bonds result in a "kink" within the hydrocarbon chain. This prevents side-by-side alignment of fatty acids resulting in liquid fat at room temperature. Saturated fatty acids are able to align themselves in a more compact manner and therefore exist in a solid or gel state (for longer chain FAs) at room

temperature. Other properties of fatty acids include hydrocarbon chain length, branching and the presence of hydroxyl groups or three carbon ringed (cycloproprane) structures. Combined, these factors contribute to specific physical properties of individual fatty acids (including melting temperature and solubility) as well as the physical properties of the membrane (eg. liquid or gel phase) (Lehninger and Cox 1993).

Anti-arrhythmic effects of polyunsaturated fatty acids

Polyunsaturated fatty acids or PUFAs have a special importance in a variety of disease states. Clinical, epidemiological and other laboratory based studies have clearly demonstrated that dietary PUFAs are associated with a significant reduction in the incidence of fatal cardiac arrhythmias (Leifert *et al*. 2001). Dietary n-3 PUFAs are: α-linolenic acid (LA, C18:3n-3), largely derived from plants, eicosapentaenoic acid (EPA, C20:5n-3) and docosahexanoic acid (DHA, C22:6n-3), which are primarily derived from seafood. The vast amount of research in this field has focused on dietary fish oil (EPA and DHA) and their beneficial effects on the heart (Swan *et al*. 2003).

Mechanisms behind the effects of polyunsaturated fatty acids

There is strong evidence that the anti-arrhythmic action of PUFAs is mediated through the depression of surface membrane electrical excitability and the inhibition of the calcium release mechanisms of the SR. Laboratory-based studies showed reduced membrane electrical excitability by significantly increasing the threshold of depolarizing current that was required to initiate an action potential and by prolonging the refractory period following an action potential (Ferrier *et al*. 2002). EPA and DHA have been

shown to decrease electrical excitability and automaticity. This may be related to the inhibition of Na^+ and Ca^{2+} currents (attributed to the blockade of L-type Ca^{2+} current). The exact mechanism behind the inhibitory effect on Ca^{2+} by n-3 PUFAs is not known but has been the centre of an expanding field of research (Lee 1998, Swan 2003). Several mechanisms have been proposed which involve the SR membrane and certain SR calcium cycling proteins and channels. Possible mechanisms proposed include: PUFAs – as part of the membrane polar lipid structure - may interact directly and selectively with certain channels to modify function; they may change the properties of cell and organelle membranes and indirectly modify channel behaviour or they may activate intracellular pathways to produce antiarrhythmic effects. A study by Swan *et al* (2003) strongly suggested that EPA alters SR Ca^{2+} - release by modulating Ryanodine receptor (RyR) (Ca^{2+} - release channel) function. They postulated that EPA could produce an inhibitory effect on RyR by: activating intracellular enzyme pathways and indirectly modifying the gating of RyR channels, or by interacting more locally with the RyR complex itself to cause a decrease in the open probability (Po) of RyR being incorporated into phospholipid bilayers. Another postulation is that during myocardial ischemia, non-esterified fatty acids are mobilized from membrane phospholipids and released into the cell. After being released, these fatty acids may influence second messenger pathways and the subsequent release of Ca^{2+}.

Lipid-protein Interactions.

It is a well established fact that fatty acyl chains comprising the hydrophobic core of membranes are integral to the functioning of certain proteins. Protein function within

the bilayer is influenced by specific protein-lipid interactions that are dependent on the chemical and structural anatomy of fatty acids (such as alkyl chain length and degree of unsaturation) as well as the lipid polar head groups. Protein function is also influenced by the unique self-association properties of lipids that result from the collective properties of the lipids (eg: fluidity, thickness, shape, packing properties) organized into the membrane structures. Since fatty acids are responsible for determining many of these collective properties (eg: unsaturated fatty acids cause an increase in fluidity, and length of fatty acids determine thickness) it can be said that fatty acids play a key role in protein function (Vance and Vance 2002)

Influence of membrane fatty acids on Ca^{2+}-ATPase activity

Ca^{2+} -ATPase is an integral membrane protein embedded within the biomembrane which couples the hydrolysis of one molecule of ATP to the transport of two Ca^{2+} ions across the membrane. Various studies have focused on the effects of n-3 PUFAs on this calcium pump and its activity. Tang *et al* (2006) found that Ca^{2+}-ATPase activity was low when lipids were in the gel phase (attributed to structural anatomy of fatty acids). They also concluded that other lipid related factors which influence the Ca^{2+}-ATPase pump function are: bilayer thickness, phospholipid composition, fluidity, and hydrocarbon chain length. Lee (1998) purified Ca^{2+} -ATPase from skeletal SR and reconstituted it into phospholipid bilayers of defined composition. Lee concluded that the function of the Ca^{2+} -ATPase depends on the chemical structure and physical phase of the lipids surrounding it in the membrane. Lee also determined that Ca^{2+} -ATPase activity was low in bilayers with shorter (C14) or longer (C22) fatty acyl chain lengths, thus

affirming that bilayer thickness is important. Activity varied little in the C16 to C20 range which is the range found within native SR. Optimal activity was achieved with a bilayer containing C18. Protein-lipid binding is low in gel phase lipids, presumably as a result of poor van der Waals contact between rigid chains and the rough protein surface. There is no unique mechanism explaining the effects of fatty acids on the function of the ATPase. It has been proposed that a change in fatty acyl chain length can lead to the loss of a binding site for Ca^{2+} on the ATPase. Since Ca^{2+} binding sites are located in the membrane spanning region of the ATPase, Ca^{2+} binding is highly sensitive to changes in lipid structure.

Non-bilayer forming lipids and protein function

There are numerous biological processes which can be envisioned to require discontinuity in the membrane bilayer. Integration of proteins into the bilayer may require "annular lipids" (lipids found within close proximity to the protein) to interface with the more regular structure of the bilayer. Movement of proteins through the bilayer might also require such discontinuity. Non-bilayer lipids also affect the internal pressures of membranes which in turn can result in an alteration in function of proteins (Vance and Vance 2002).

Objectives

I hypothesize that the fatty acid composition of the cardiac sarcoplasmic reticulum will be altered during the progression of cardiac hypertrophy due to volume overload.

The first goal of this study was to characterize SR fatty acid composition in sham control rats and in rats subjected to left ventricular hypertrophy induced by volume overload. The next goal of the study was to compare the two groups to identify any alterations in the cardiac SR fatty acid composition.

II. Materials and Methods

Induction of Volume Overload

The volume overload model used in this study had been previously established by Cantor *et al.* (2005). The experimental protocols involving the VO animal model, had been approved by the Animal Care Committee of the University of Manitoba and were in agreement with the *Canadian Council on Animal Care Concerning the Care and Use of Experimental Animals* (Vol.1, 2nd Edition, 1993).

Male Sprague-Dawley rats weighing 75-150 g were housed in a temperature and humidity controlled room with a 12-hour light and 12-hour dark cycle one week prior to induction of VO. Rats received standard rat chow and tap water *ad libitum.* VO was induced in the rats by means of an aortocaval (AV) shunt. All rats were anaesthetized for surgery with 5% isoflurane carried at a flow rate of 2 l/min by means of oxygen, and were then maintained in an anaesthetized state with 2% isoflurane for the duration of surgery. A laparotomy (incision in abdominal wall) was performed and the abdominal aorta and vena cava were exposed by blunt dissection between the renal arteries and the iliac bifurcation. To create the shunt, an 18-gauge needle was inserted into the aorta at a 45° angle and pushed through to the vena cava. To seal the puncture, cyanoacrylate (Krazy Glue, Elmer's Product Canada, Toronto, ON) was used. In successful surgeries, oxygenated blood from the aorta could be seen mixing with deoxygenated venous blood in the vena cava. The control group consisted of sham operated rats which underwent a laparotomy without creation of a shunt. Rat housing conditions were maintained as previously described for the duration of the 28-week study.

Echocardiography

Rats were weighed and anesthetized using isoflurane as described previously. Two-dimensionally (2D)-guided M-mode echocardiography was performed using a Sonos 5500 ultrasound system (Agilent Technologies, Andover, MA). The heart was imaged in 2D at the level of the papillary muscles with a depth setting of 3 cm. M-Mode recordings were analyzed with a sweep speed of 150 mm/s with the axis of the probe aligned with the middle of the left ventricle. The percent fractional shortening (%FS) parameter was measured through the use of echocardiography as an index of cardiac contractility. The %FS was derived from the following calculation:
%FS = 100 x (LVDD – LVSD)/LVDD where LVDD is left ventricular end-diastolic diameter and LVSD is left ventricular end-systolic diameter (Kametas *et al*. 2003).

Sacrifice

Both experimental (shunt) and control (sham) rats were sacrificed at 4, 16, and 28 weeks after the induction of VO. Rats were weighed and anesthetized using a cocktail of ketamine (90 mg/kg) and xylazine (10 mg/kg), which was injected into the hind legs. An incision running along the abdominal cavity was made to remove the heart. Once removed, the heart was washed in ice-cold saline (0.9%) and weighed. Heart-to-body (HBW) ratio was used as a marker for determining the occurrence of hypertrophy. The hearts were then separated into subsequent parts (atria, ventricles and septum) that were placed in separate containers and stored at -80°C in saline.

Isolation of Sarcoplasmic Reticulum Vesicles

SR vesicles were obtained from the left ventricle using a method described previously (Netticadan *et al.* 2001). Homogenization buffer containing (in mM): 10 $NaHCO_3$, 5 NaN_3, 15 Tris-HCl (pH 6.8) and the protease inhibitors (in μM): 1 leupeptin, 1 pepstatin, and 100 phenylmethylsulphonyl fluoride were added to the left ventricle. Heart tissue kept on ice was pulverized and homogenized using a Polytron homogenizer (Brinkman, Westbury, NY) for a period of 20 seconds and 25 seconds, with a 1 min interval pause in between, at a speed of 12,000 rpm. The homogenate was centrifuged at 10, 919 g for 20 min (JA 20.0 rotor; Beckman Instruments, Inc, Palo Alto, CA). The supernatant obtained was further centrifuged at 43,666 g for 45 min. The resulting pellet was suspended in a buffer containing 20 mM Tris-HCl (pH 6.8). This was centrifuged at 43,666 g for 45 min. The resultant pellet containing the SR fraction was suspended in a buffer containing 250 mM sucrose and 10 mM histidine (pH 7.0). All steps were performed in the cold room (at 4°C) and all resultant fractions were stored at 80°C.

Western Blotting

Protein Assay

Protein concentration was measured using the method of Lowry *et al*. (1951). Duplicates were prepared for both standards and experimental tubes. Standard tubes consisted of bovine serum albumin (BSA) (1 mg/mL) and H_2O at concentrations between 0 and 1 mg/ml. Experimental tubes consisted of either SR or homogenate samples in addition to double distilled H_20 (ddH_2O). All tubes contained 2 ml of a solution

consisting of 2% Na_2CO_3 and 0.1N NaOH, 1% $CuSO_4$, and 2% $KNaC_4H_4O_6$ (10:1:1 v/v). Phenol Indicator mix (1N) was added to each tube. Samples were then transferred to spectrophotometer cuvettes for absorbance readings and placed in an *Ultrapec 2100 Pro* UV/Visible spectrophotometer with a set wavelength of 623 nm. Protein concentrations were determined from the standard curve obtained. A final protein concentration of 1 mg/mL was achieved through the addition of a solution containing 1.0 M 1,4-Dithiothreitol and Laemmli Buffer. The Laemmli Buffer consisted of 10% sodium dodecyl sulfate, glycerol, 1.2 mL Tris-HCl (pH 6.8), bromophenol blue and ddH_20. After the final concentration was obtained, samples were boiled in a hot water bath for 3 mins, allowed to cool to room temperature and then placed in the -20°C freezer.
Protein assay and normalization procedures were conducted as a prelude to running of gels.

Western Blot

Western blot analysis was carried out using an established western blot protocol (Netticadan *et al.* 2001) to determine the protein content of SERCA2a. Fifteen µg of protein from both SR and homogenate samples were loaded onto 10% polyacrylamide gels and then separated through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using the Mini-PROTEAN 3 Electrophoretic Transfer Cell (Bio-Rad). Protein separation was conducted at room temperature. Gels were electrophoretically transferred to polyvinylidene difluride (PVDF) membranes (Millipore) using the Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad). Gel transfer was conducted in the cold room at a temperature of 4°C for 2 hours. Incubation

of PVDF membranes was conducted at room temperature in 5% skim milk (skim milk powder in TBS). A 1:1000 dilution of mouse monoclonal SERCA2a antibody (Affinity Bioreagents, Golden, CO) proceeded by a 1:500 dilution of secondary anti-mouse antibody (Amersham, Arlington Heights, IL) (45 minutes) were used to probe the membrane. Finally, a 1:3000 dilution of tertiary streptavidin-conjugated horseradish peroxidase antibody (Amersham) was used to probe the membrane. Between probing with antibodies, the PVDF membranes were washed with TBST. Antigen-antibody complexes of all probed membranes were detected by the ECL Plus Western Blotting Detection System (Amersham Life Sciences, Oakville, ON). The intensity of each band was scanned by an imaging densitometer with the aid of Molecular Analyst Software Version 1.3 (BioRad, Hercules, CA). Quality One 4.5.0 software (Bio-Rad) was used to analyze the protein bands obtained.

Preparation of Fatty Acid Methyl Esters

Fatty acid methyl esters (FAMES) were prepared (Larsen *et al*. 2005) from SR lipids by a transesterification reaction. A mixture of methanol/chloroform/hydrochloric acid (10:1:1, by vol.) were mixed with the SR pellet. The mixture was vortexed and heated for 1 h at 80°C in a 16 x 125 mm screw cap test tube. It was cooled to room temperature, mixed with distilled water and vortexed. The resultant FAMES were extracted twice with a mixture of hexane/chloroform (4:1, v/v) each time (Nichols *et al*. 2000). The FAMES in the hexane layer were washed with 2% aqueous potassium bicarbonate and dried with anhydrous Na_2SO_4. The hexane layer was transferred to a new tube and then dried under N_2. The FAMES were then reconstituted in hexane.

Analysis of FAMES

FAMES were analyzed by gas-chromatography (GC) on a Hewlett Packard 5890 series II. The GC module was equipped with a JW-88 column (100 m long, 0.25 mm diam., 0.25 µm film). FAME components in the SR samples were identified using Nu-chek Prep, Inc 463 Fatty acid standard (purchased from Nu-chek, Elysian, MN). SR fatty acids were characterized using this combination of retention time in GC.

The relative mass of the particular FAME was represented by the area under each peak. The area was then converted to the mole percent (mol%) value of the fatty acid in the SR sample by the following equation (Larsen *et al*. 2005):

$$\frac{\text{FAME peak area}}{\text{FAME molar mass}} = \text{number of moles of FAME in mixture} = n$$

$$100 * \left\{\frac{n_i}{\sum n_i}\right\} = \text{mole \% of the } i\text{th fatty acid in mixture}$$

where n is the mol% of the *i*th FAME species in the mixture. The mol% of each fatty acid was then normalized according to palmitate (16:0) due to the fact that palmitate concentrations were significantly consistent throughout the samples at each time point.

Statistical Analysis

Statistical analysis was carried out through one-way analysis of variance (ANOVA) to determine the variation between control and shunt groups. A statistical significance of $P<0.05$ was used. All data values are expressed as the mean ± SE (standard error).

III. Results

General Characteristics of Hypertrophy

From Table 1, a significant increase in HBW ratios in the shunt group is seen at the initial 4 week stage in comparison to the control group. This trend continues throughout the other time points studied.

Table 1: Heart-to-Body weight (HBW) ratios for control and shunt groups at 4, 16 and 28 weeks (n=6). All values are expressed as means ± standard error.* P<0.05

	4 week		16 week		28 week	
	Control	Shunt	Control	Shunt	Control	Shunt
HBW (mg/100g)	284.3 ± 4.0	468.2 ± 5.1 *	237.6 ± 2.4	406.1 ± 17.9 *	213.7 ± 2.5	380.1 ± 13.2 *

Echocardiography

At 4 weeks, there is no significant change in %FS between the control and shunt groups (table 2). However, a decrease in %FS does occur in the shunt group as compared to the control group at 16 and 28 weeks (Table 2).

Table 2: Percent Fractional shortening (%FS) of the left ventricle in control and shunt groups at 4, 16, and 28 weeks (n=6). * P<0.05

	4 week		16 week		28 week	
	Control	Shunt	Control	Shunt	Control	Shunt
%FS	42.7 ± 0.4	42.4 ± 0.5	43.9 ± 1.2	37.7 ± 0.8 *	44.3 ± 0.9	37.1 ± 0.5 *

Western Blot

Figure 1 shows that SERCA2a levels in the SR increased by 4.8 (approximately a 5 fold increase) in comparison to homogenate samples.

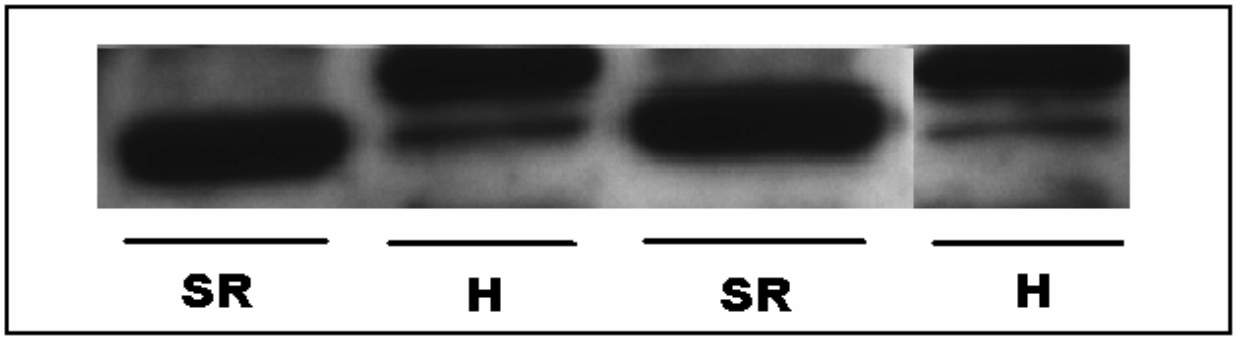

Figure 1: Western Blot analysis of the SERCA2a protein (110 kDA) in sarcoplasmic reticulum (SR) and homogenate (H) samples for 16 week VO rat hearts.

Fatty Acid Composition

The fatty acid profile created from analysis of data obtained from GC is shown in Table 3. The fatty acid range is between C12:0 and C22:6n-3 of the SR in control rats and those subjected to VO. From the profile it appears there are more unsaturated (containing one or more double bonds) fatty acids as compared to saturated (containing no double bonds).

Table 3: Fatty Acid Composition of isolated cardiac SR in control rats and rats subjected to VO (n=2-6). * P<0.05 vs. it's respective control.

	4 week				16 week				28 week			
	control		shunt		control		shunt		control		shunt	
C#	**mean**	**SE**	**mean**	**SE**	**mean**	**SE**	**mean**	**SE**	**mean**	**SE**	**mean**	**SE**
12:0	0.12	0.04	0.00	0.00	0.38	0.05	0.58	0.03	0.61	0.07	0.55	0.06
14:0	0.08	0.02	0.05	0.00	0.08	0.01	0.07	0.01	0.10	0.01	0.07	0.01
14:1	0.08	0.02	0.03	0.00	0.25	0.03	0.14	0.02	0.31	0.02	0.25	0.02
15:1	0.10	0.02	0.11	0.05	0.12	0.01	0.10	0.01	0.12	0.02	0.05	0.01
16:1	0.12	0.03	0.18	0.12	0.15	0.01	0.03	0.00	0.16	0.01	0.07	0.02
17:0	0.03	0.00	0.03	0.00	0.03	0.00	0.03	0.00	0.03	0.00	0.03	0.00
17:1	0.17	0.03	*0.10	0.02	0.17	0.01	0.17	0.01	0.20	0.02	*0.10	0.02
18:0	1.37	0.07	1.46	0.04	1.39	0.03	1.46	0.05	1.36	0.06	1.37	0.02
18:1tΔ^{9}	0.11	0.01	0.11	0.01	0.07	0.02	0.10	0.01	0.16	0.01	*0.05	0.01
18:1tΔ^{12}	0.19	0.05	0.20	0.08	0.19	0.01	0.19	0.01	0.23	0.03	0.10	0.02
18:1	0.33	0.01	0.30	0.01	0.29	0.01	0.29	0.01	0.29	0.01	0.25	0.01
18:1cΔ^{9}	0.23	0.01	0.24	0.01	0.18	0.01	0.22	0.01	0.20	0.02	*0.25	0.01
18:3n-6	1.03	0.06	1.18	0.05	1.07	0.04	1.07	0.04	0.87	0.06	1.00	0.05
19:0	0.25	0.05	0.15	0.03	0.20	0.02	0.18	0.01	0.23	0.03	*0.10	0.02
20:0	0.04	0.01	0.05	0.03	0.05	0.00	0.04	0.01	0.09	0.02	0.06	0.02
20:1cΔ^{5}	0.23	0.05	0.21	0.07	0.21	0.02	0.17	0.01	0.23	0.03	*0.09	0.02
22:0	0.23	0.06	0.20	0.06	0.20	0.03	0.16	0.02	0.24	0.02	0.09	0.02
20:3n-6	0.06	0.01	0.08	0.01	0.06	0.01	0.08	0.01	0.06	0.01	0.05	0.01
22:0	0.06	0.00	0.09	0.02	0.08	0.00	0.08	0.01	0.13	0.01	*0.09	0.01
X	0.23	0.05	0.15	0.05	0.19	0.03	*0.12	0.01	0.21	0.02	*0.08	0.02
Y	0.86	0.07	0.85	0.04	0.36	0.02	0.83	0.08	0.75	0.10	0.99	0.04
22:2	0.19	0.04	0.11	0.04	0.13	0.02	*0.08	0.01	0.15	0.00	*0.05	0.01
20:5	0.03	0.02	0.08	0.02	0.14	0.04	0.26	0.08	0.26	0.02	0.07	0.03
22:3	0.07	0.03	0.12	0.05	0.04	0.01	0.05	0.01	0.05	0.01	0.04	0.00
24:1	0.04	0.00	0.04	0.00	0.02	0.00	0.02	0.00	0.03	0.00	0.02	0.00
Z	0.36	0.06	0.38	0.07	0.36	0.02	*0.31	0.01	0.45	0.06	*0.26	0.03
22:5n-6	0.12	0.02	0.10	0.03	0.12	0.02	0.07	0.01	0.10	0.00	0.04	0.01
22:5n3	0.12	0.01	0.13	0.01	0.19	0.02	0.17	0.01	0.15	0.02	0.15	0.02
22:6n-3	0.53	0.06	0.64	0.04	0.60	0.06	0.62	0.06	0.51	0.05	0.62	0.03

For the time point of 4 weeks, the predominant peaks corresponded to palmitate (C16:0), stearate (C18:0), gamma-linolenate (C18:3n-6), fatty acid "Y" and docosahexaenoate (C22:6n-3). These peaks had the greatest abundance in both control and VO SR samples.

During 16 weeks, the predominant peaks were those for dodecanoate (C12:0), stearate, gamma-linolenate, fatty acid "Y", and docosahexaenoate. These peaks were predominant in both control and VO groups.

There is more discrepancy seen in predominant peaks occurring at 28 weeks. As in 4 and 16 weeks, dodecanoate, gamma-linolenate, fatty acid "Y", and docosahexaenoate are the predominant peaks in both control and VO groups. Marked differences here include the appearance of palmitoleate (C16:1), heptadecenoate (C17:1), oleate (C18:1), nonadecanoate (C19:0), and fatty acid "X" as a predominant peaks at 28 weeks (for both control and VO groups). Stearate was not a predominant peak at 28 weeks. An abundant peak was seen for eicosenoate (C20:1) for the VO group.

Fatty Acid Comparison between Control and VO groups as LVH progresses

Statistical analysis carried out by ANOVA showed statistically significant differences of $P<0.05$ between control and VO groups for the fatty acids listed in table 3 (denoted by *). There were relatively more unsaturated fatty acids which showed variation between the two groups as compared to saturated fatty acids. Mole fractions were normalized (as published by Larsen *et al*. 2005) according to palmitate which showed relatively consistent mole fractions during the three time points. The mean ± SE were graphed for normalized mole fractions for both control and VO groups at 4, 16 and 28 weeks.

Fatty acid comparison in regards to abundance had been grouped according to variation seen at different time points between sham and VO groups. For heptadecenoate, there was a substantial decrease in the VO groups as compared to the sham groups at 4 and 28 weeks (Fig.2). For 4 weeks, the 17:1/16:0 ratio was 0.17± 0.03 in the control group (n=4) and 0.10 ± 0.02 in the VO group (n=3). At 28 weeks the 17:1/16:0 ratio were 0.20 ± 0.02 for the sham group (n=3) and 0.10 ± 0.02 for the VO group (n=3).

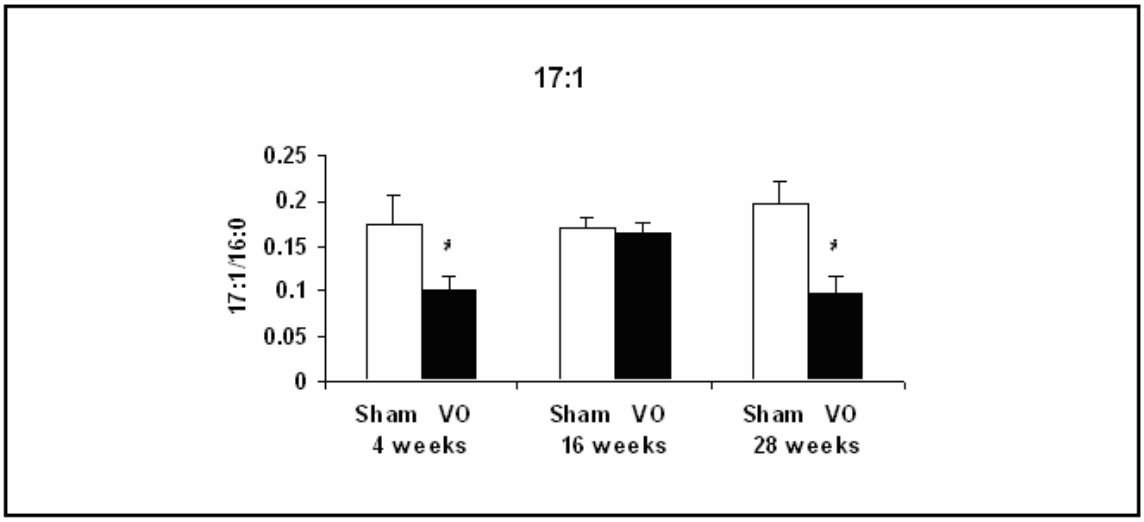

Figure 2: Normalized mole fractions for heptadecenoate (C17:1) in both control (sham) and VO groups at 4, 16, and 28 weeks. * Indicates statistically significant differences ($P<0.05$) between the two groups.

The decrease in each of the fatty acids “X”, “Z” and docosadienoate showed statistically significant differences at 16 and 28 weeks in the VO groups relative to the sham group (Fig.3). All three fatty acids decreased in the VO group compared to the sham groups at both time points. A substantial decrease in fatty acid “X” occurred in the VO group at 28 weeks. For fatty acid “X”, the X/16:0 ratios were 0.19 ± 0.03 and

0.12 ± 0.01 [sham (n=5) and VO (n=5) respectively] at 16 weeks, and 0.21 ± 0.02 and 0.08 ± 0.02 [sham (n=3) and VO (n=3) respectively] at 28 weeks. For "Z", the Z/16:0 ratios for 16 weeks are 0.36 ± 0.02 [sham (n=6)] and 0.31 ± 0.01 [VO (n=5)] and for 28 weeks the corresponding ratios are 0.45± 0.06 [sham (n=5)] and 0.26 ± 0.03 [VO (n=6)]. The 22:2/16:0 ratios at 16 weeks are 0.13± 0.02 and 0.08 ± 0.01 for sham (n=5) and VO (n=5) groups respectively. At 28 weeks the corresponding ratios for docosadienoate are 0.152 ± 0.005 for the sham group (n=5) and 0.05 ± 0.01 for the VO group (n=6)

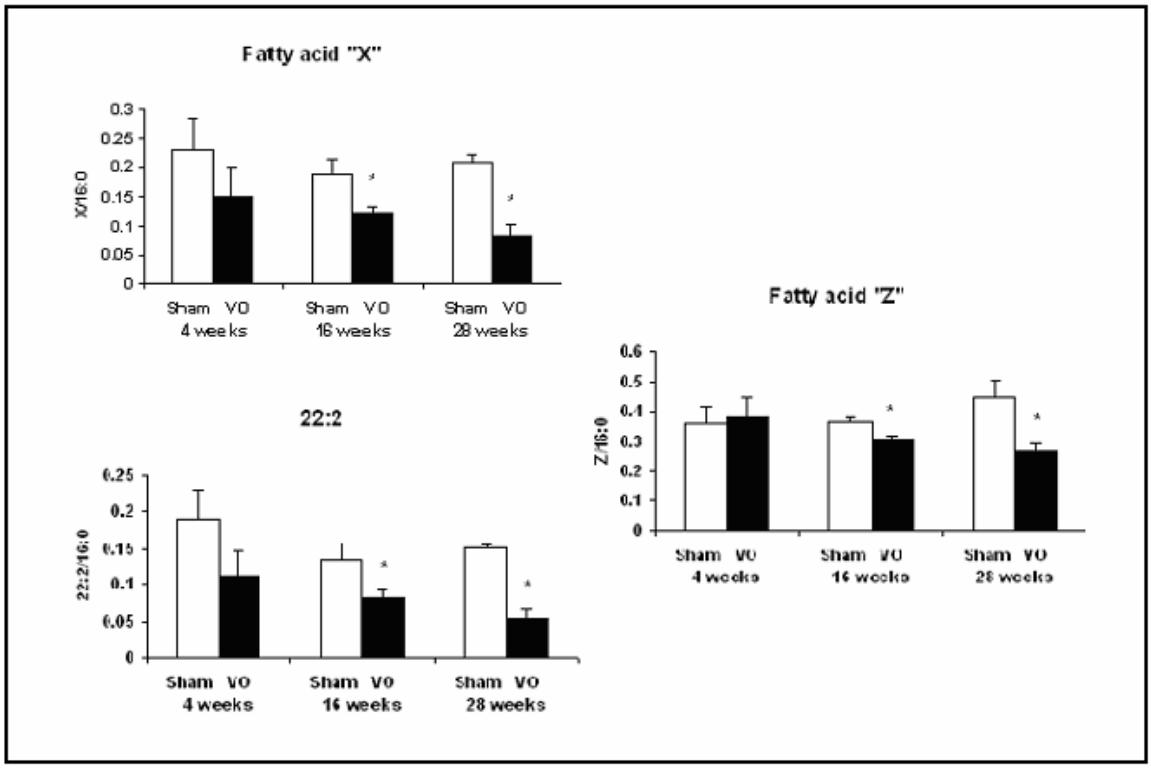

Figure 3: Normalized mole fractions for fatty acid "X", "Z" and docosadienoate (C22:2) in both control (sham) and VO at 4, 16 and 28 weeks. * Indicates statistically significant differences (P<0.05) between the two groups.

Fatty acids trans-oleate, nonadecanoate, oleate, eicosenoate, and docosanoate show significant variation between the sham and VO groups at 28 weeks with a decrease in these fatty acids in the VO group as compared to the sham (Fig.4). Substantial decreases were seen in the VO groups for trans-oleate, nonadecenoate and docosenoate The ratio {[FattyAcid]/16:0} for trans-oleate in sham (n=4) and VO (n=4) groups were 0.16 ± 0.01 and 0.05 ± 0.01 respectively; 0.2 ± 0.03 and 0.10 ± 0.02 for sham (n=3) and VO groups (n=3) for nonadecanoate; 0.29 ± 0.01 and 0.25 ± 0.01 for sham (n=3) and VO (n=3) groups for oleate. For eicosenoate the sham group (n=3) ratios were 0.23 ± 0.03 and 0.09 ± 0.02 for the VO group (n=3). The sham group (n=4) for docosanoate had a mole fraction ratio of 0.13 ± 0.01 while it was 0.09 ± 0.01 for the VO group (n=6).

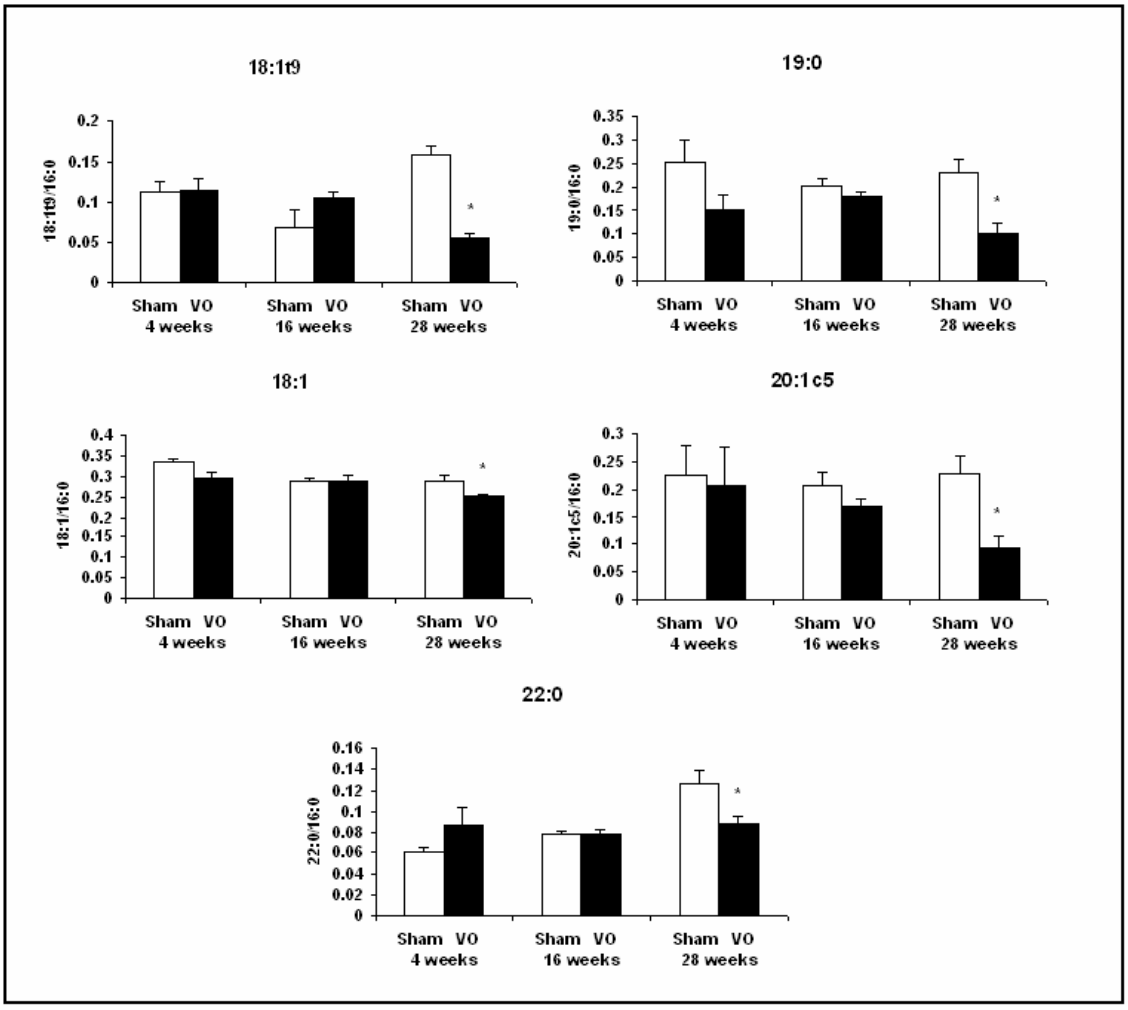

Figure 4: Normalized mole fractions for trans-oleate (C18:1tΔ^9), nonadecanoate, oleate, eicosenoate, docosanoate (C22:0) in both control (sham) and VO at 4, 16 and 28 weeks. * Indicates statistically significant differences ($P<0.05$) between the two groups.

IV. Discussion

No studies have been carried out to date pertaining to the lipid composition of the sarcoplasmic reticulum in rats subjected to volume overload induced hypertrophy. Much of the research conducted thus far has focused on alteration in function/expression of Ca^{2+} cycling proteins due to cardiac hypertrophy.

Since this study was based on determining the lipid composition of the SR in volume overloaded rat hearts, it was essential that we first determine whether VO was successfully induced. In this study an increase in HBW ratio in the experimental group as compared to a sham-control group was observed right at 4 weeks. This trend continued throughout all the time points concerned, indicating that hypertrophy occurred at 4 weeks and progressed over time. Reduced cardiac contractility is one of the deleterious effects of cardiac hypertrophy and a central factor pushing the hypertrophied heart into a state of heart failure. Serial echocardiograph analyses were carried out to measure heart function. Fractional shortening can be used as a measure of left ventricular function. At the onset of hypertrophy there was no significant compromise in cardiac contractility. However, contractility was compromised at the later stages of cardiac hypertrophy as evident from results of %FS. Trends seen in HBW ratio and %FS were consistent with findings by Cantor *et al* (2005).

The SR is a major intracellular membrane system responsible for the contraction-relaxation cycle in the heart. SERCA2a is one of the most abundant proteins of the SR. We therefore used SERCA2a as a marker for establishing the purity of our isolated membrane preparations. A 5 fold increase in SERCA2a levels was observed in the SR

samples in comparison to homogenate samples, establishing the isolated fraction as the SR. Western blot analysis carried out agree with findings of Babu *et al*. 2003.

Preliminary analysis carried out on data obtained from GC analysis showed an SR profile with a fatty acid range from C12:0 to C22:6n-3 in both control and VO groups. Most fatty acids identified in the profile were unsaturated. A study conducted by Bick *et al.* (1997) on membrane asymmetry in isolated canine cardiac sarcoplasmic reticulum characterized a fatty acid profile with a range of C14:0 to C22:6n-3 with most fatty acids being unsaturated. Within this range, Bick *et al.* characterized the following 14 fatty acids (C#): 14, 14:1, 16, 18, 18:1, 18:2, 18:3, 20, 22, 22:1, 20:4, 20:5, 22:6. In our study of rat SR (both control and VO groups) we were able to identify those fatty acids profiled by Bick *et al*. (with the exception of 18:2 and 20:4) in addition to the following (C#): 12, 15:1, 16:1, 17, 17:1, 19:1, 20:1, 20:2, 20:3, X, Y, 22:3, 24:1, and Z (the identity of each of fatty acids X,Y, and Z is yet to be established).

Fatty acids identified in the VO group were consistent with those in the control group. After analysis, it was apparent that marked differences between the control and VO groups at the various time points occurred in less than one half of the fatty acids characterized (no significant changes were observed for the rest). A general trend observed at the initial 4 week stage and at 28 weeks was a decrease in heptadecenoate (C17:1) in the VO group as compared to the control group. Fatty Acids X, Z and C22:2 showed a decrease in the VO group compared to the control group at 16 and 28 weeks. At 28 weeks, C19:0, C18:1tΔ^{9}, C18:1, C20:1c$\Delta^{5,}$ and C22:0 showed a decrease in the VO groups. Due to the preliminary nature of this study no conclusive observations can be

made on protein function/activity occurring in hypertrophy in relation to the trend seen in C17:1. However, speculative thought could be preferred. One of the trends observed was a decrease in 17:1 (an unsaturated fatty acid). This could result in a decrease in unsaturation of the membrane involved. This decrease could then result in the membrane becoming less fluid (ie more rigid). Since numerous membrane associated reactions are governed by the fluidity and physical state of the membrane involved (Eze 1990), this decrease in unsaturation may be related to alteration in protein function/activity. The aforementioned is strictly speculative thought not based on findings in this study.

Although uncertain, a reasoned thought is that the stress involved with VO causes alterations in lipid composition in the SR. Alterations in lipid composition between control and experimental groups have been studied in various diseases such as lung disease (analysis of serum fractions) (Schmidt *et al*. 2002) and type 2 diabetes (Perrassolo *et al*. 2003). The exact mechanism behind lipid alteration due to stress caused by an initial stimulus resulting in hypertrophy remains an unventured field.

Whether any changes in fatty acid composition contribute to abnormalities in the function of SR $Ca2^{+}$ cycling proteins in hypertrophied hearts remains an open question. The trends observed in this study are global changes occurring within the SR of a VO group in relation to a control group. These changes cannot be linked to specific protein activity as these integral membrane proteins are governed by their own local environment (of which fatty acids are an important part) as well as the physical state of the bulk membrane lipids involved. An educated guess would lean towards an affirmative answer

to the aforementioned statement. This is based on the extensive work done on lipids and their effect on various SR calcium cycling proteins. These include a study conducted by Swan *et al.* (2003) on the effects of EPA on cardiac SR Ca^{2+} release and ryanodine receptor function. Their research concluded that EPA inhibited SR Ca^{2+} release. Extensive research has been carried out on the integral membrane protein Ca^{2+}-ATPase and its interaction with lipids. Lee (1998) focused on the dependence of Ca^{2+}-ATPase function on the chemical and physical phase of the lipids surrounding it in the membrane. Lee found that Ca^{2+}-ATPase function was low in reconstituted bilayers consisting of shorter (C14) or longer (C22) fatty acyl chain lengths, indicating that fatty acyl chain length and thus bilayer thickness are important for protein activity. These findings in conjunction with the fact that the calcium binding sites of Ca^{2+}-ATPase are found within the membrane itself (Chen *et al.* 1996) make for a well reasoned guess that changes in lipid composition may result in alterations of protein function/activity.

V. Conclusions

(1). In this study we were able to successfully characterize the fatty acid profile of normal cardiac sarcoplasmic reticulum. The profile included 26 identified fatty acids and 3 unidentified fatty acids. Most fatty acids were unsaturated.

(2) The fatty acid profile of isolated cardiac SR in rats subjected to left ventricular hypertrophy induced by volume overload was also successfully characterized. The profile included 26 identified fatty acids and 3 unidentified fatty acids. Most fatty acids were unsaturated. This is the first study to characterize the fatty acid composition of SR during the progression of cardiac hypertrophy due to volume overload.

(3) A statistically significant decrease in heptadecenoate was observed in the volume overload group compared to the control group at 4 and 28 weeks.

(4) Fatty acids “X”, “Z”, and docosadienoate showed statistically significant decreases in their VO groups as compared to the control group at 16 and 28 weeks.

(5) A statistically significant decrease in trans-oleate, nonadecanoate, oleate, eicosenoate, and docosanoate, was observed in the volume overload group compared to the control group at 28 weeks.

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