THE EFFECT OF FREE FATTY ACIDS ON ENDOTHELIAL CELLS

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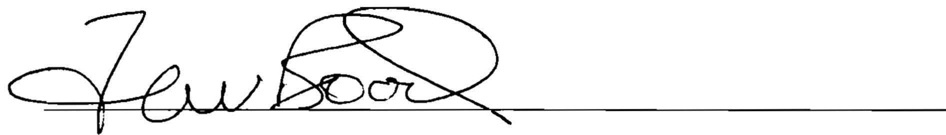

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ABSTRACT

Investigations show that lipids decrease endothelial nitric oxide synthase (eNOS) protein and that this is putatively important for how oxidized low density lipoprotein (OxLDL) contributes to vascular dysfunction. A key question that remains is if specific components of OxLDL molecules such as oxidized polyunsaturated fatty acids (PUFAs), or some other atherogenic lipid, such as saturated free fatty acids (FFA), are alone sufficient to decrease eNOS protein content in endothelial cells. The purpose of this thesis was to test the hypothesis that some types of FFA, either oxidized PUFAs or saturated FFAs, alone have the ability to decrease eNOS protein content in cultured cells cells.

Twenty hour exposure of porcine aortic endothelial cells (PAECs) with the oxidized PUFAs (0-100μM) linoleic acid (LA), eicosapentaenoic acid (EPA), and arachidonic acid (AA), DID not alter eNOS protein content within the cells. However, PAEC incubation with the saturated FFAs (0 - 0.6mM) palmitic acid (PA) and stearic acid (SA), which do not undergo oxidative modification, dose dependently decrease eNOS protein content within the cells.

The results of this study indicate that oxidized PUFAs are not sufficient to contribute to NO mediated endothelial dysfunction induced by OxLDL. However, saturated FFA may contribute to NO mediated endothelial dysfunction produced by OxLDL by decreased eNOS protein content within cultured endothelial cells.

LIST OF ABBREVIATIONS

AA	Arachidonic Acid
BAEC	Bovine Aortic Endothelial Cells
BSA	Bovine Serum Albumin
eNOS	Endothelial Nitric Oxide Synthase
EPA	Eicosapentaenoic Acid
FBS	Fetal Bovine Serum
FFA	Free Fatty Acid
GAPDH	Glyceraldehydephosphate Dehydrogenase
H_2O_2	Hydrogen Peroxide
HDL	High Density Lipoprotein
HUVEC	Human Umbilical Vein Endothelial Cells
HPODE	Hydroperoxyoctadecadienoic Acid
HODE	Hydroxyoctadecadienoic Acid
ICAM-1	Intercellular Adhesion Molecule-1
LA	Linoleic Acid
LDL	Low Density Lipoprotein
LOX-1	Lectin-Like Oxidized LDL Receptor-1
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
MCP-1	Monocyte Chemotactic Protein -1
MCSF	Macrophage Colony Stimulating Factor

$O_2^{\cdot -}$	Superoxide
OA	Oleic Acid
OxLDL	Oxidized Low Density Lipoprotein
PA	Palmitic Acid
PAEC	Porcine Aortic Endothelial Cells
PBS	Phosphate Buffered Saline Solution
PUFA	Polyunsaturated Fatty Acid
ROS	Reactive Oxygen Species
SA	Stearic Acid
SOD	Superoxide Dismutase
TBARS	Thiobarbituric Acid Reactive Substances
VCAM-1	Vascular Cell Adhesion Molecule
VLDL	Very Low Density Lipoprotein

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I. Introduction

Cardiovascular disease is an illness effecting millions of individuals worldwide and particularly a large proportion of the population in the United States. This disease is known to be associated with impairments in endothelium-dependent vascular relaxations, one also known as vascular dysfunction. Many tissue culture studies involving vascular cells and monocytes/macrophages have provided key insight into cardiovascular disease initiation and progression. It has been shown in many of these investigations that some lipids present in the blood can contribute to the onset and progression of this disease more than others. These studies have shown specific lipids and products of lipid oxidation may contribute to this process more than others through induction of vascular endothelial dysfunction. This can occur through an endothelial mediated inflammatory response and may involve monocyte recruitment and foam cell proliferation. The goal of this study was to assess how a variety of plasma free fatty acids (FFAs), which compose plasma lipoprotein alter endothelial cellular protein levels that exhibit vasoprotective properties. Where possible, this study also focuses on several types of specific free fatty acids in their oxidized states and their roles in the regulation of endothelial nitric oxide synthase (eNOS) protein content. The hypothesis tested was that specific FFAs and oxidized FFAs, all of which may be present in OxLDL molecules, alter cellular eNOS protein content differently and therefore potentially other genes important for normal endothelial function.

Oxidation of biological lipids can exhibit a plethora of effects on the endothelial cell layer of arteries (Hessler, Robertson et al. 1979; Hirata, Miki et al. 1995; Liao, Shin et al. 1995; Cominacini, Rigoni et al. 2001; Li, Chen et al. 2001). This layer provides a

means of protection from monocyte cell invasion into the intima where atherosclerotic lesions may develop. It also serves as a mechanism to inhibit a pro-thrombotic state within the vasculature brought on by presence of adhesion molecules, and platelet aggregation. The endothelium is also important in the neutralization of reactive oxygen species (ROS) through the production of nitric oxide (NO). In the vasculature, this molecule is produced by constitutive eNOS as well as inducible NOS (iNOS) and is very important in the regulation of vascular tonus and the maintenance of a healthy vasculature.

Forms of lipids and free fatty acids have been seen to attenuate the formation of NO in endothelial cells for some time. For example, Hirata et al. showed that eNOS mRNA and protein could be upregulated through exposure to low concentrations (10µg protein/mL) of oxidized low density lipoprotein (OxLDL) (Hirata, Miki et al. 1995) while others have shown treatment of cells with higher concentrations (50-100µg protein/mL) downregulate eNOS mRNA (Liao, Shin et al. 1995). Observing the effects that OxLDLs exhibit on endothelial cell function in vitro is possible through cell/ tissue cultures and functional experimental models. However, a disadvantage to studying the effects of OxLDL on the endothelium is that oxidation of lipids can yield an array of products; all of which can exert various effects and potentially act through various mechanisms to change endothelial cell function/ morphology. Another is that the constituent FFAs that comprise the LDLs may be different between experiments.

For these reasons, these experiments concentrated on specific FFAs of varying chain length and degrees of saturation in order to specifically define their roles in endothelial cell eNOS protein regulation. It was hypothesized that various plasma FFAs in their

oxidized forms will not all effect endothelial eNOS protein content in the same manner. This hypothesis was tested by applying specific biological concentrations of the lipids and their oxidized counterparts to cultured endothelial cell monolayers derived from porcine thoracic aorta tissue segments. Variations in eNOS protein content were then measured using Western blot analysis. All eNOS results were standardized using glyceraldehydephosphate dehydrogenase (GAPDH). GAPDH is a protein common to all cell types and is critical in cellular glycolysis. This protein commonly used as a standard in Western blot techniques as it is ubiquitous in all cells.

Recent studies have shown that FFAs of varying chain lengths, degrees of saturation, and steric configuration are able to exhibit varying effects upon endothelial cell physiology. For example NO production was seen to be diminished in human umbilical vein endothelial cells (HUVECs) incubated with palmitic acid (PA) (a saturated FFA with a carbon chain length of 16), but not stearic acid (SA) (a saturated FFA with a carbon chain length of 18) (Moers and Schrezenmeir 1997). In another investigation, SA, a saturated fatty acid was seen to increase the incidence of endothelial cellular apoptosis whereas oleic acid (OA), monounsaturated fatty acid, did not. The polyunsaturated fatty acids (PUFAs) linoleic and γ-linolenic and arachidonic acid (AA) induced endothelial cell apoptosis only at the highest concentration used (300μmol/L) (4.0mg/mL final albumin concentration) (FFA:albumin ratio = 6.8:1) (Artwohl, Roden et al. 2004). Bovine aortic endothelial cells (BAECs) treated for twenty four hours with oxidized linoleic acid (13-hydroperoxyoctadecadienoic acid;13-HPODE), a biologically active component of OxLDL, significantly increased eNOS protein content and enzyme activity (no supplemental albumin) (Ramasamy, Parthasarathy et al. 1998).

Because different types of FFAs have been seen to elicit different responses in vascular cells, studying them individually is beneficial in understanding their biological function. In the studies cited previously involving native, or unoxidized, free fatty acids, concentrations used varied between 10-300µM (.04 – 20mg/mL final albumin concentration). However, plasma free fatty acid levels have been reported much higher in such conditions as during short periods of fasting and for sustained periods in individuals that are obese and/or type II diabetics (Kuriki, Nagaya et al. 2003; Kashyap, Belfort et al. 2004). In this study, various types of FFAs in high physiologic concentrations were used in order to define their roles in endothelial eNOS protein regulation. Because effects reported have varied depending on the FFA of interest, it is important that this study includes various FFAs in their native and oxidized forms that differ in chain length and degree of saturation to understand their roles in vascular function.

Fatty acids of the same chain length that vary in their degree of saturation were utilized in these experiments to determine whether saturated, monounsaturated, or polyunsaturated fat characteristics determine endothelial gene regulation. The FFAs used for these experiments are listed in Table 1. The structures of these fatty acids are depicted in Figure 1.

TABLE 1
Common Biological Free Fatty Acids

Fatty Acid	Shorthand
Palmitic Acid	16:0
Stearic Acid	18:0
Oleic Acid	Δ9-18:1
Linoleic Acid	Δ9,12-18:2
Arachidonic Acid	Δ5,8,11,14-20:4
Eicosapentaenoic Acid	Δ5,8,11,14,17-20:5

[Δ denotes carbon at which double bond is located]

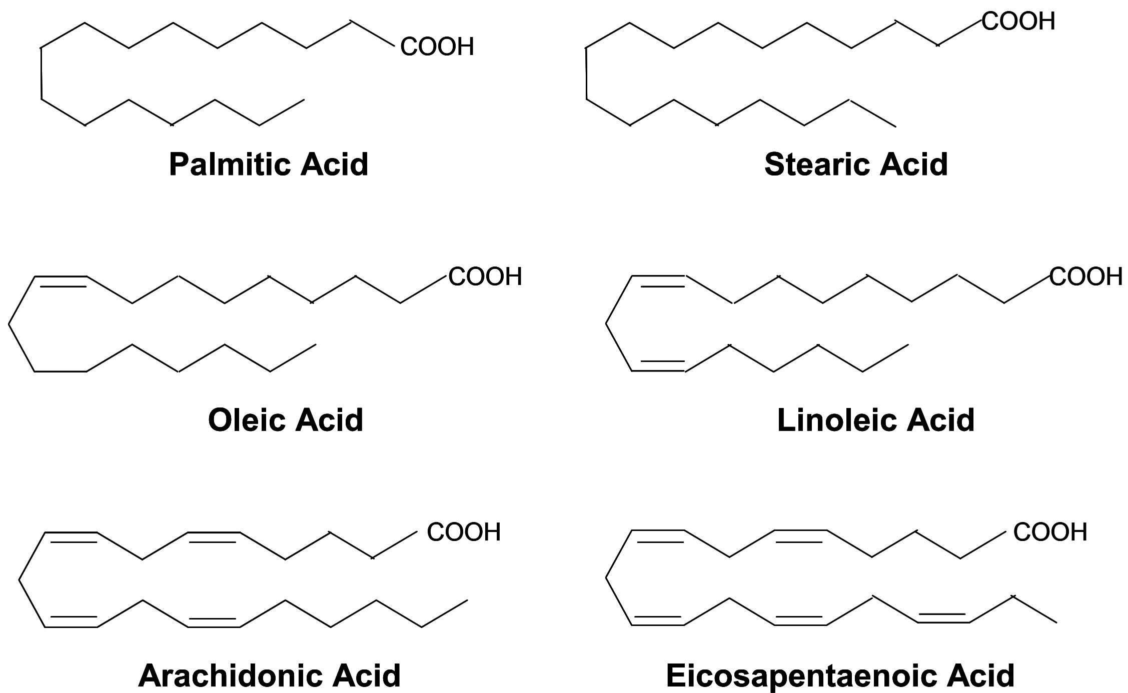


Figure 1. Common biological fatty acids.

Where possible, the fatty acids were oxidized using either enzymatic oxidation, (LA) or by induced autoxidation (AA and eicosapentaenoic acid (EPA)). The goal of these experiments was to provide a better understanding of the effects that common plasma FFAs and their oxidation may have on vascular eNOS protein regulation as a mediator of vascular health.

II. Literature Review

Atherosclerosis and Cardiovascular Disease

According to the American Heart Association, overweight, obesity, and inactivity together represent the number 2 leading cause of preventable death in the United States second only to cigarette smoking. As one consequence of a high fat diet over time, atherosclerosis is a disease that is responsible for 50% of all deaths in westernized societies and is the primary cause of heart disease and stroke within those communities. Also known as cardiovascular disease, this illness is a disorder of the large arteries characterized by accumulation of lipids and fibrous elements within those effected arteries. Atherosclerosis is progressive starting very early in childhood and progresses throughout adulthood. In humans, fatty streak lesions found within the aorta can be seen to form within the first decade of life. Within the second decade, these lesions can be seen in the coronary arteries. By the third and fourth decades of life, the lesions can be seen within the cerebral arteries (Lusis 2000).

Upon feeding of a high-fat, high-cholesterol diet, lipoprotein particles accumulate in the intimal regions of these lesions. In a short period of time, generally days or weeks following the beginning of a high fat diet, monocytes can be seen adhering to the surface of the endothelium of the vasculature. The monocytes then transmigrate across the endothelial monolayer into the intima where they proliferate and differentiate into macrophages. There they take up lipoproteins through phagocytosis and form foam cells. Through time these foam cells will apoptos and form a lipid-filled core of the lesions. Smooth muscle cells sometimes then migrate from the medial layer to the intima layer

where they can potentially proliferate. The extent to which this may occur depends heavily on the production of effector molecules that regulate smooth muscle cell proliferation. The smooth muscle cells may then produce fibrous elements contributing to the formation of fibrous plaques that develop and increase in size. The lesions grow initially toward the adventitia until they reach a certain point. After this point the lesions will expand into the lumen and continue to grow into the lumen of the artery, forming a plaque cap. Mononuclear cells in the circulation are further recruited to the site of the lesion from the blood and enter the lesion through the shoulder of the blood vessel. There cell proliferation, extracellular matrix production, and the accumulation of extracellular lipids continue to occur. Angiogenesis also occurs within the plaque formation area.

Although generally the formation of new blood vessels serves an essential role in wound repair and collateral blood flow to ischemic regions of the vasculature, angiogenesis in inflamed regions augments inflammatory destruction. In time, plaque cap thinning occurs due to a loss of structural cells and extracellular collagen production. Potentially, the plaque cap may rupture leading to thrombosis, or the formation of a blood clot within the vessel. This is the most important factor leading to the lethal consequences of coronary atherosclerosis.

In order to understand how various types of plasma lipids obtained in the diet are able to contribute to the development of atherosclerosis, it is important to understand the physical properties of the different types of lipids such as low density lipoproteins (LDL) and their constituent FFAs. Structural changes that occur to LDL either within the vascular lumen or within the atherosclerotic lesion can contribute to further vascular dysfunction potentiating the atherogenic process. Of particular interest here are the

effects of these changes in LDL chemistry and more specifically FFAs upon the vascular endothelium, which plays an integral role in the regulation of vascular function.

LDL and FFA Stucture

Free Fatty Acids

Fatty acids (FAs) exist in the body as free (unesterified) or as fatty acyl esters located in more complex molecules such as triacylglycerols. Varying levels of these FAs can be found in the plasma and in tissues located throughout the body. Generally over 90% of the FAs found in the plasma are in the form of fatty acid esters (triacyglycerol, cholestryl esters, and phospholipids). Levels are usually higher in the plasma. Free fatty acids in the plasma are bound to the plasma protein albumin and are transported from adipose tissue or from circulating lipoproteins to their point of consumption in the tissues where they are utilized for cellular energy through beta-oxidation.

The FAs consist of a hydrocarbon chain of varying length and a terminal carboxyl group. The carboxyl group (-COOH) is able to ionize becoming ($-COO^{-}$) and therefore have an affinity for water. This allows the FA to be amphiphatic (having both a hydrophobic and hydrophilic region). The FA can exist in either saturated forms (containing no double bonds in its hydrocarbon backbone) or in their unsaturated forms (having one or more double bonds in the hydrocarbon chain). Palmitic acid for example has a hydrocarbon chain length of 16 carbons and contains no double bonds within the backbone structure. Structural lipids and triacylglycerols usually contain fatty acids of this type (16 carbon length). The PUFA LA has a hydrocarbon chain length of 18 and

contains two double bonds at carbons 9 and 12. This FA is considered essential in that it is the precursor to AA, which in turn is a FA used in the synthesis of the prostaglandins.

LDLs

The plasma lipoproteins are molecular complexes of lipids and proteins called apolipoproteins. These particles are constantly being synthesized, degraded and removed from the plasma. The lipoprotein particles include the chylomicrons, very low density lipoproteins (VLDL), LDL, and high density lipoproteins (HDL). These lipoproteins serve as an efficient method of packaging and transporting lipids in the plasma to the tissues where they are metabolized. The main lipids carried by lipoproteins are triacyglycerols and cholesterol obtained in the diet or through de novo synthesis. They are composed of a neutral lipid core (containing triacyglycerols, cholesterol esters or combinations of both) surrounded by apolipoprotiens, phospholipid, and nonesterified cholesterol. These are all oriented with their polar ends extending outward from the lipid core as to maintain solubility in an aqueous solution.

Chylomicrons are the lipoprotein particles lowest in density and largest in surface area, containing the most lipid and lowest amount of protein. VLDLs and LDLs are more dense, respectively, having a higher content of protein and less lipid. HDL's are the most dense of all the plasma lipoproteins, having the highest protein and lowest lipid content. The lipid portions of the plasma lipoproteins are composed primarily of triacylglycerols, formerly referred to as glycerides, which consist of one, two, or three molecules of FA esterified to a molecule of glycerol. The FAs are esterified through their carboxyl groups. This causes the loss of a negative charge on the FA resulting in the formation of a neutrally charged lipid molecule. The glycerol phosphate molecule is the initial

acceptor of the FAs to form the triacylglycerols. The liver (also where triacyglycerols are primarily formed) and adipose tissue are the sites where glycerol phosphates are formed from the glycolytic metabolism of glucose. The FAs must be converted to their activated forms (attached to coenzyme A) before they are able to undergo triacylglycerol synthesis. This conversion is catalyzed by the enzyme Fatty acyl-CoA synthetase (thiokinase).

The FAs that are on the one glycerol molecule are usually not of the same type. The FA on carbon 1 is usually saturated, that on carbon 2 is usually unsaturated, and that on carbon 3 can be either. The presence of unsaturated fatty acid(s) decreases the melting temperature of the lipid. Fats that are rich in unsaturated FA (such as olive oil) are in liquid form at room temperature. Those that have a higher amount of saturated FAs are generally quite firm solids (like butter) at room temperature. The longer chain length FAs that compose the saturated fat, the more firm it is at room temperature.

Free fatty acids are seen to exist in the extracellular space, such as in triacyglycerol breakdown within adipocytes and subsequent release into the vasculature. These FAs can be transported in the blood via albumin to tissues where they will then be used for energy. The FAs are usually readily transported across the membrane and into the mitochondria for β-oxidation. Free fatty acids within the circulation are usually in small quantities whereas most of them are transported to tissue bound to albumin or in the form of lipoprotein. The triacylglycerols in lipoproteins are hydrolyzed to fatty acids at the inner surfaces of capillaries in the peripheral tissues. The hydrolysis involves the activation of extracellular enzyme lipoprotein lipase by apoprotein C-II. Following release of these fatty acids, they are absorbed by the nearby endothelial cells for metabolic energy production. In certain circumstances those fatty acids may be altered by

free radicals located either within or around the endothelial cell layer. There either the altered form of the free fatty acids or the free radicals themselves can cause endothelial cell dysfunction and injury.

Endothelial cell injury can be induced by superoxide ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2) leading to apoptosis (programmed cell death). Loss of endothelial cells and their protective roll within the vasculature can lead to atherogenesis and a pro-coagulative state. Apoptosis can also be induced by oxidized forms of low density lipoproteins, angiotensin II, high glucose, and TNF-α. This phenomenon can be inhibited by super oxide dismutase, catalase, NAC, and antioxidant vitamins (Dimmeler and Zeiher 2000). Another type of programmed cell death known as anoikosis, results from detachment of endothelial cells. This occurance has also been associated with reactive oxygen species, such as $O_2^{\cdot -}$ and has been seen to be inhibited by *N*–acetylcysteine and diphenylene iodonium, an inhibitor of flavin-containing enzymes such as *N*-acetycysteine NAD(P)H(Li, Ito et al. 1999).

Nitric Oxide

The role of nitric oxide in vascular physiology was first described upon its discovery by Furchgott and Zawdzki in 1980 and was defined as endothelium-derived relaxing factor (EDRF) (Furchgott and Zawadzki 1980). NO found in animal tissues has a half-life of ~6-30 seconds and is produced by the nitric oxide synthase enzymes, NOS, which metabolize L-arginine to L-citrulline. The three identified isoforms of NOS are reviewed by Guzik et al. (Guzik, Korbut et al. 2003). These include NOS found

primarily in endothelial cells, eNOS, the neuronal form, or nNOS, and the inducible iNOS. The latter has been seen in various cell types.

Within the endothelium, NO is formed by the constitutive endothelial NOS, eNOS and is responsible for maintaining low vascular tone and preventing leukocytes and platelets from adhering to the vascular wall. This form of eNOS is present in endothelial cell caveolae and the cytosol and requires additional cofactors for its activation. These include tetrahydrobiopterin (BH_4), nicotinamide adenine dinucleotide phosphate reductase (NADPH), flavin adenine dinucleotide (FAD), and flavin mononucleotide (FMN).

The activity of eNOS can be regulated by stimulation of endothelial muscarinic, purinergic, kinin, substance P, or thrombin receptors. Vasoactive agonists cause an increase in intercellular Ca^{2+} concentrations due to endoplasmic release of Ca^{2+}. The receptor initiated activity of eNOS can be bypassed by calcium ionophores (e.g. A23187) and polycations (e.g. poly-L-lysine) which cause a rise in $[Ca^{2+}]_i$. The activity of eNOS can also be regulated by shear stress, 17beta-estradiol and insulin, as reviewed by Wyatt et al. (Wyatt, Steinert et al. 2004). These cause phosphorylation of the serine/threonine protein kinase Akt/protein kinase B in a phosphoinositide 3-kinase-dependent manner. This activation of eNOS occurs at basal $[Ca^{2+}]_i$ levels.

NO that is produced by neuronal constitutive NOS (nNOS) acts as a neuromodulator or neuromediator in some central neurons and in peripheral non-adrenergic non-cholinergic nerve endings. The activity of nNOS in central neurons is regulated by glutamate binding to NMDA receptors which causes a rise in $[Ca^{2+}]_i$ concentrations due to opening of voltage gated calcium channels. Unlike eNOS and nNOS, iNOS does not require a

rise in $[Ca^{2+}]_i$ to initiate its activity. NO formed by iNOS in macrophages and other cells is involved in the inflammatory response and is known to be initiated by the cytokines IFN-γ, TNF-α, or IL-1.

NO activates soluble guanylyl cyclase, producing increased concentrations of cyclic GMP. The effects that are elicited by increased levels of cyclic GMP depend on the cell type in which this rise occurs as reviewed by Cannon (Cannon 1998). In the vascular smooth muscle cell layers, the rise in cyclic GMP activates GMP-dependent kinases resulting in a decrease in smooth muscle intracellular Ca^{2+} levels ($[Ca^{2+}]_i$), thereby resulting in vasorelaxation. Increased cyclic GMP in platelets due to NO released into the vascular lumen results in decreased platelet activation and adhesion to the endothelial monolayer. Endothelin-1, a vasoconstrictor molecule, is also inhibited by rises in cyclic GMP levels in the systemic, coronary, and pulmonary vasculature. NO is also shown to exhibit protective roles in the vasculature by inhibiting growth factors that are produced and released by various cell types in and around the blood vessel.

Reactive Oxygen Species

Free radicals have been studied greatly in cell biology since Ben Harman's discovery of their presence in biological materials fifty years ago (Droge 2002). He proposed that these molecules could be responsible for such events as cellular damage, mutagenesis, cancer, and the degenerative process of biological aging. Upon the discovery of the enzyme superoxide dismutase (SOD) by McCord and Fridovich (McCord and Fridovich 1969), it was conceived that free radicals could play a biologically valuable role.

The presence of reactive oxygen species (ROS) within the vascular endothelium has been shown to have many effects on endothelial mediated function. One benefit of low concentration of ROS is seen in vascular cells that have evolved to use ROS as signaling molecules. A very important ROS found in the vasculature is $O_2^{\cdot-}$ which is formed through the reduction of triplet-state molecular oxygen ($^3O_2$). Many enzymatic systems have been found to produce $O_2^{\cdot-}$. One such example is the formation of this anion by NAD(P)H oxidase in vascular smooth muscle cells as well as activated or dysfunctional endothelial cells. This form of oxygen can combine with endothelial derived NO to generate peroxynitrite ($ONOO^-$), a deleterious ROS. The action on O_2 by the enzyme SOD produces the more stable hydrogen peroxide (H_2O_2) which is then converted enzymatically to H_2O by catalase and glutathione peroxidase. Other non-enzymatic antioxidant compounds are those such as α-tocopherol (vitamin E), β-carotene, ascorbate (vitamin C) and glutathione. The presence of reduced transition metals (Fe^+ and Cu^+) can also give rise to the highly reactive hydroxyl radical (·OH) (Chance, Sies et al. 1979). Shortage or absence of endogenous antioxidant systems can produce a condition known as oxidative stress which is seen to be harmful to a variety of cells.

Oxidative stress is known as a biological condition in which excess reactive oxygen species overwhelm endogenous antioxidant systems, such as SOD and are able to elicit various effects. One commonly known harmful effect that ROS exhibits is through the scavenging of vasoactive nitric oxide (NO) (Wolin 2000) and therefore limiting endothelial control of vascular smooth muscle relaxation. The radical NO is produced by some organisms through the oxidation of one of the terminal guanidonitrogen atoms of L-

argenine by the enzyme nitric oxide synthase (NOS) (Griffith and Stuehr 1995). ROS have also been shown to be responsible for the adherence of leukocytes to endothelial cells (Sellak, Franzini et al. 1994). High concentratons of ROS have been seen to be a characteristic of as well as induce apoptotic cell death in various cell types (Slater, Stefan et al. 1995). These events upon the vasculature are characteristic features of disease states including atherosclerosis, diabetes and hypertension. Yet another potentially harmful characteristic of oxidative stress is that ROS can cause oxidation of lipids and proteins. The incidence of this oxidation is of particular interest in this proposal due to the effects on endothelial cell morphology and physiologic cell function that can occur when the vascular endothelium is exposed to high concentrations of oxidized LDL.

LDL Oxidation and Its Pathobiological Significance

It has been known for quite some time that low density lipoproteins are highly susceptible to oxidative damage. More recently it has been a concern that these oxidized forms of the LDL may have a role in the onset and progression of atherogenesis. Two well known observations as reviewed by Steinberg (Steinberg 1997) led to this conclusion. The first is that atherosclerotic lesions have been seen to form in subjects completely lacking an LDL receptor. The second was that the two cell types that give rise to cholesterol laden foam cells (monocyte/macrophage and smooth muscle cells) do not accumulate cholesterol in vitro even in the presence of very high concentrations of native forms of LDL. However, upon macrophage cell incubation with LDL that had previously been incubated with endothelial cells for 24 hours, it was seen that macrophage uptake of LDL was increased dramatically. Incubation of smooth muscle

cells with LDL overnight produced the same results (Henriksen, Mahoney et al. 1983). These results let a hypothesized modification of LDL molecules that increased their propensity to be taken up by vascular cells. The unknown modification that was occurring to LDL turned out to be oxidation (Morel, DiCorleto et al. 1984; Steinbrecher, Parthasarathy et al. 1984). Receptors for this form of modified LDL have been shown to exist in monocytes and macrophages giving rise to development of foam cells (Smirnova, Sawamura et al. 2004). Other deleterious effects that OxLDL has seen to exhibit are that it is a chemoattractant for monocytes (Quinn, Parthasarathy et al. 1987), and it inhibits the motility of macrophages(Quinn, Parthasarathy et al. 1985). Oxidized LDL is cytotoxic to cultured endothelial cells (Hessler, Robertson et al. 1979), it inhibits vasodilatation that is normally induced by NO(Kugiyama, Kerns et al. 1990), it is mitogenic for macrophages and smooth muscle cells(Yui, Sasaki et al. 1993; Chatterjee and Ghosh 1996), and it can stimulate the release of monocyte chemotactic protein -1 (MCP-1) and macrophage colony stimulating factor (MCSF) from endothelial cells(Cushing, Berliner et al. 1990; Rajavashisth, Andalibi et al. 1990).

Prior work had demonstrated that endothelial cells internalize and degrade oxidized LDL, potentially as a safety method, by a receptor mechanism different from that for native LDL. The cDNA for this receptor was identified by Sawamura et al. in 1997 and was designated as lectin-like oxidized LDL receptor-1, LOX-1(Sawamura, Kume et al. 1997). It is a membrane protein that belongs to a family of C-type lectin-like molecules as reviewed by Sakurai et al. (Sakurai and Sawamura 2003). Generally expression of this receptor is low in endothelial cells, however it can be induced by proinflammatory cytokines and vasoconstrictive peptides in vitro(de Rijke and van

Berkel 1994; Kume, Murase et al. 1998; Morawietz, Rueckschloss et al. 1999; Morawietz, Duerrschmidt et al. 2001) as well as its own ligand, OxLDL(Mehta and Li 1998; Aoyama, Fujiwara et al. 1999). The native form of LDL showed no effect on the regulation of LOX-1 expression (Mehta and Li 1998). LOX-1 could therefore be upregulated by the binding of oxidized LDL to LOX-1 which would therefore increase binding of oxidized LDL to its receptor and propagate endothelial dysfunction further.

Oxidized LDL decreases endothelial NO concentrations due to superoxide formation (Cominacini, Rigoni et al. 2001). Li et al. also showed that in human coronary artery endothelial cells, oxidized LDL decreases phosphorylation (activity) of PKB/Akt and downregulated eNOS activity (Li, Chen et al. 2001). This showed that oxidized LDL exhibited its effects at various stages in the formation of NO by endothelial cells. Li et al. showed that LOX-1 activation in endothelial cells induced the expression of endothelin-1, AT_1 receptor, E-selectin, P-selectins, vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1), and MCP-1(Li and Mehta 2000; Li, Chen et al. 2001).

Previous work in bovine and human derived endothelial cells has demonstrated that oxidized low density lipoprotein (LDL) can decrease eNOS protein and mRNA expression at high concentrations (50-100μg protein/ml) (no supplemental albumin)and upregulate protein and mRNA levels at lower levels (10μg protein/ml) (0.02 final albumin concentration) (Hirata, Miki et al. 1995; Liao, Shin et al. 1995) whereas oxidized linoleic acid (5,10 and 20μM) (no supplemental albumin), a constituent of biological LDLs, has been seen to increase eNOS protein and mRNA expression. One purpose of the experiments summarized herein was to discern whether specific FFA in their oxidized

forms (which comprise common biological LDLs, that vary in chain length and degree of saturation), effect eNOS protein differently. This hypothesis was tested by incubating primary porcine aortic endothelial cell cultures with specific unesterified FFA in their native and, where possible, oxidized forms. Treating the endothelial cells with single specific FFA is advantageous in understanding a single fatty acid's role in cellular function. Cellular eNOS protein expression was then assessed using Western blot analysis and quantified using densitometric analysis.

The complexity of varying chain length derivatives formed during the oxidative process complicates the study of the effects of larger lipoprotein molecules such as oxidized LDL on the vasculature. "The breakdown of these PUFAs in cholesterol esters, phospholipids, and triglycerides yield a broad array of smaller fragments, 3-9 carbons in length, including aldehydes and ketones that become conjugated to other lipids". Another example as stated by Steinberg (Steinberg 1997), is that of malondialdehyde (or other aldehydes) that can form Schiff bases* with lysine residues and can then generate cross links with other lipids or proteins.

[*] any of a class of bases of the general formula RR′C=NR″ that are obtained typically by condensation of an aldehyde or ketone with a primary amine (as aniline) with elimination of water, that usually polymerize readily if made from aliphatic aldehydes, and that are used chiefly as intermediates in organic synthesis and in some cases as dyes

Oxidation of Polyunsaturated Fatty Acids

The oxidation of low density lipoproteins has been shown to yield an array of products as reviewed by Steinberg (Steinberg 1997). LDL modification due to oxidation has been quantitated by assays measuring thiobarbituric acid reactive substances (TBARS), conjugated dienes, total lipid peroxides, enhanced electrophoretic mobility, uptake and degredation if LDL in macrophages, or high performance liquid chromatography(Schuh, Fairclough et al. 1978; Parthasarathy, Printz et al. 1986; el-Saadani, Esterbauer et al. 1989; Leake and Rankin 1990; Jessup, Darley-Usmar et al. 1991; Rankin, Parthasarathy et al. 1991; Folcik and Cathcart 1994).

Using the HPLC method, it has been shown in monocytes that the major product of LDL oxidation is esterified hydroperoxyoctadecadienoic acid (HPODE). This is the predominant oxidation product of LA, the most common PUFA in human LDL. HPODE is a form of lipid hydroperoxide. These molecules are the result of lipid oxidation of FFAs. Generally, PUFAs are characterized by the structural elements R-CH=CH-CH_2-CH=CH-R are the predominate target for this form of oxidation. The double bonds within the carbon backbone of the PUFA serve as the main site of free radical attack. Lipid hydroperoxides are recognized as a form of reactive oxygen species and in high concentrations can lead to oxidative stress within the cell.

The ability of PUFA to undergo oxidation varies linearly with their degree of saturation and increases with the number of double bonds in the fatty acid's carbon backbone (Cosgrove, Church et al. 1987; Wagner, Buettner et al. 1994). These double bonds located between allylic carbons in the FA backbone allow for electrophillic attack due to the presence of weaker π (pi) bonds. Typically three different forms of oxidation

can occur. These are photo-oxidation, lipid autoxidation, and enzymatic oxidation (Porter, Caldwell et al. 1995).

The oxidation process of PUFAs is considered to be a free radical chain reaction that consists of three steps. These key events as reviewed by Yin and Porter are initiation, propagation, and termination (Yin and Porter 2005). These reaction steps are outlined in Table 2.

TABLE 2
Chain Sequence For Free Radical Lipid Autoxidation

Initiation:	$LH + X^{\cdot} \rightarrow L^{\cdot} + XH$
Propagation:	$L^{\cdot} + O_2 \rightarrow LOO^{\cdot}$ $LOO^{\cdot} + LH \rightarrow LOOH + L^{\cdot}$
Termination:	$LOO^{\cdot} + LOO^{\cdot} \rightarrow LOOOOL$

Key:
- L – Lipid
- $L^{\cdot}$ – Lipid Radical
- O_2 – Molecular Oxygen
- H – Hydrogen
- $LOO^{\cdot}$ – Lipid Peroxyl Radical
- LOOH – Lipid Hydroperoxide
- LOOOOL – Unstable Tetraoxide Intermediate

In the initiation step the lipid radical ($L^{\cdot}$) is formed when hydrogen ($H^{\cdot}$) abstraction from L-H is performed by the initiator radical ($X^{\cdot}$). Biologically this can occur through reactive oxygen species found in and around the cell at ambient temperatures. Once this lipid radical is formed, molecular oxygen is added to form a peroxyl radical ($LOO^{\cdot}$) signaling the beginning of the propagation step. This radical is

then free to abstract a hydrogen ion from another lipid allowing for the continuation of the chain reaction. Once again, the C-H bond strength and the stability of radicals formed determine which lipid peroxyradical is formed. In the termination step, two radicals couple to form nonradical products such as a tetraoxide (LOOOOL). Peroxides and tetraoxide products are generally unstable and decompose to give aldehydes, alcohols, and many other products. The number of products increases greatly with the length and degree of saturation of the fatty acid. Figure 2 depicts common products formed from the oxidation process on biological LDL. Upon oxidation of LDL, several different isomers of HPODE are formed. Reduction of these molecules can lead to the formation of their hydroxyoctadecadienoic acids (HODEs) (Folcik and Cathcart 1994). Further decompostition of these molecules can lead to the formation of various smaller products such as the aldehydes depicted in Figure 3.

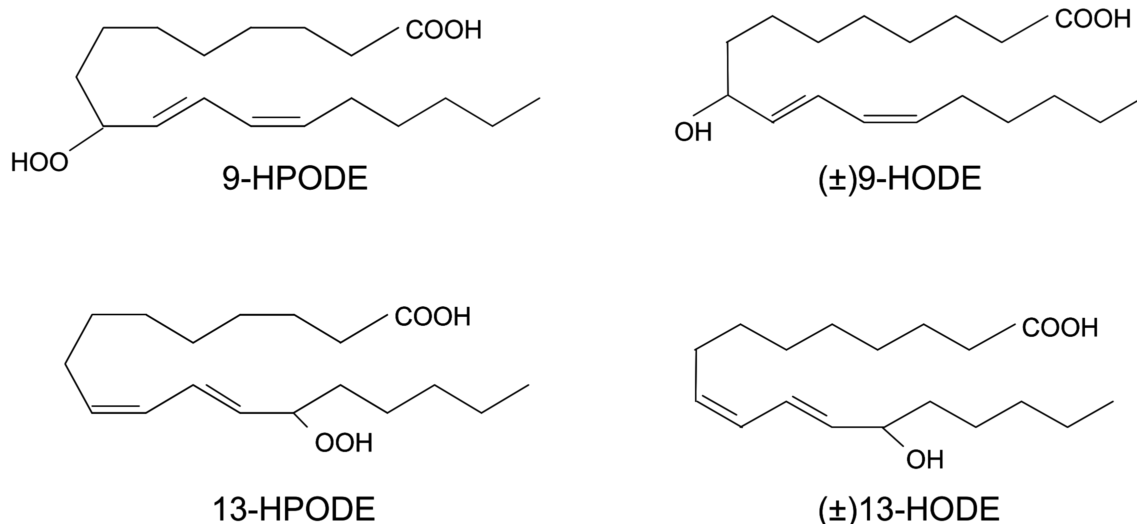


Figure 2. Examples of oxidation products formed from the autoxidation of LDL or linoleic acid. Multiple lipid oxidation products are produced upon enzymatic or autoxidation of low density lipoprotein molecules and free fatty acids such as linoleic acid. These molecules have been implicated in endothelial function and vascular health. (Folcik et. Al. 1994, Thomas et. Al. 1994)

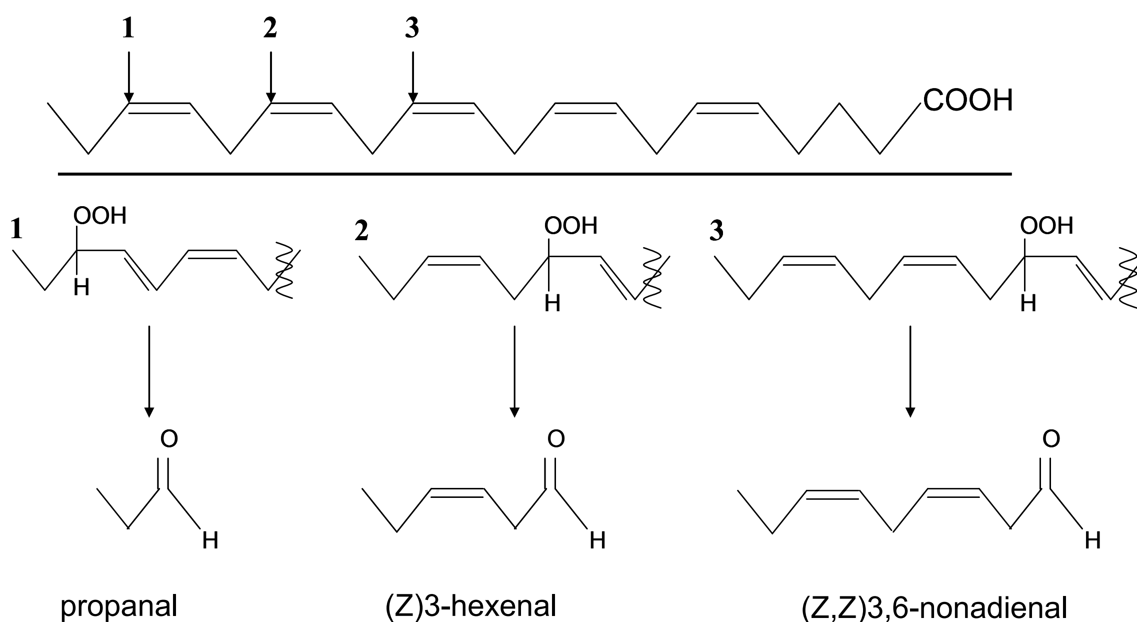


Figure 3. Example of free fatty acid autoxidation. Upon free radical attack at the numbered positions, hydroperoxides may be formed of varying chain lengths. These newly formed hydroperoxide molecules may then separate from the parent fatty acid chain resulting in a mixture of aldehydes.

III. Matericals And Methods

Cell Harvest and Culture

Thoracic aortas were harvested from the Yukatan miniature swine and immediately placed in ice cold Dulbeco's Phosphate Buffered Saline (without calcium choride and magnesium chloride) (Gibco) containing 5% Pen-Strep and stored at 4ºC. All cell harvests were completed within 5 hours of tissue collection. Before cell harvest, connective tissue was removed and the thoracic aorta was cut into segments approximately 5-6cm in length. Aortic segments were cut lengthwise and placed face down in approximately 1mL collagenase (Roche) solution (.2mg/mL) in PBS and incubated at 37ºC for 30 minutes. Endothelial cells were then removed using a cell scraper (Fisher) and rinsed into a 100mm X 20mm cell culture dish (Corning) containing complete medium. To ensure endothelial cell purity, all cultures were immunohistochemically stained using biotin label cell markers for eNOS, Factor VIII (Von-Willibrand Factor), and smooth muscle cell α-actin. Figure 4 depicts representative cell culture images that have been probed for cell markers. The complete medium consisted of EGM-2 MV-Microvascular Endothelial Cell Medium-2 Bullet Kit (Corning) and supplemented with 10% Fetal Bovine Serum (FBS) and 5% Pen-Strep. All cells were used between passages 3 and 5. For experiments, cell culture media consisted of EGM-2 cell culture media supplemented with 1% FBS and 1% essentially fatty acid free bovine serum albumin (BSA)(Sigma).

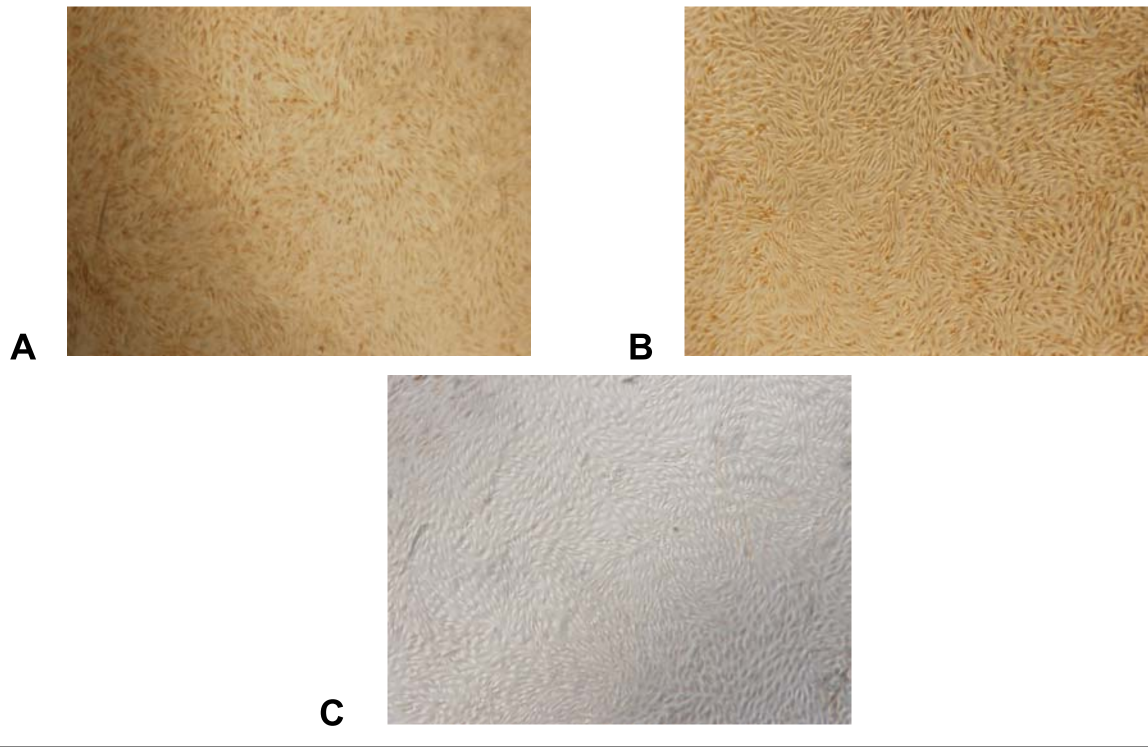


Figure 4. Cell Immunohistochemistry : To assess cell culture purity, endothelial cell cultures were stained for A. eNOS B. factor VIII, and C. smooth muscle α-actin

Preparation of Native (Unoxidized) Free Fatty Acids:

Linoleic acid (Sigma) and oleic acid (Cayman) were purchased in liquid form. The fatty acids were added to complete medium containing 1% heat inactivated FBS and 1% fatty acid free BSA. Total concentrations of FFA in BSA medium did not exceed .66mM. The sodium salt of palmitic acid was dissolved in EGM-2 media at 60ºC and 1% fatty acid free bovine serum albumin was added after medium had reached 37ºC. Stearic acid was dissolved in minimal amount of ethanol and subsequently added to complete media. Dissolution of all fatty acids into medium/BSA solution was confirmed with NEFA C kit (Wako) at initial time and after 24hours. Fatty acid to albumin ratios in experiment media were within physiologic range and did not exceed 6:1.

Preparation of 13-HPODE:

Hydroperoxyoctadecadioenoic acid was produced by an adaptation of methods described earlier by Parthasarathy et. al. (Parthasarathy, Wieland et al. 1989) and Hamberg (Hamberg and Samuelsson 1965). A 0.017% linoleic acid solution was prepared by adding 50µl of 99% pure cell culture grade LA (Sigma) to 50µl 95%v/v ethyl alcohol. The mixture was vortexed and 200mM borate buffer solution (pH 9.0 at 25ºC) was added to make total volume 50mL. 5.0mL of this solution was added to 20mL borate buffer solution and 5.0ml distilled de-ionized water. Lipoxidase enzyme (Sigma) (5,000-10,000 units) was then added and the reaction was monitored by measuring the absorbance of a 1mL aliquot of reaction mixture at 234nm. The reaction was stopped using 1N HCl. Final assay concentrations were as follows; In a 3.00ml reaction mix, the final concentrations were 178 mM boric acid, 0.011% (v/v) linoleic acid, 0.01% (v/v)

ethanol and 500-1000 units lipoxidase. This solution was diluted to yield a 2mM stock of HPODE. The concentration of HPODE was calculated using the extinction coefficient of 2.52×10^4 M^{-1}. Figure 5 depicts a graphical representation of the enzymatic oxidation of linoleic acid by lipoxidase at time points 0 through 20 minutes. The HPODE stock was then dissolved in complete medium by one of two methods. In the first, HPODE was dissolved directly in the experiment medium to yield the desired concentrations for dose response experiments. In the second method, ethanol was completely evaporated under nitrogen gas from the stock HPODE sample and then dissolved at 38°C to yield a 2mM stock solution. This method yielded a HPODE stock solution free of ethanol.

Preparation of Oxidized EPA and AA:

EPA and AA (Cayman Chemical) were oxidized using similar procedures previously described (Chaudhary, Mishra et al. 2004). EPA and AA were purchased and stored at -20ºC in .3M concentrations, respectively. For oxidation, 3.3mM concentrations of both fatty acids were prepared in PBS (pH 5,0). Approximately, 3mL of fatty acid/PBS solution was placed in a 100 mm X 20 mm cell culture dish (Corning) to allow for maximal exposure to atmospheric oxygen. Fatty acids in dishes were then oxidized in a cell culture incubator at 37º C with 5% CO_2 for 16hrs. Oxidation of fatty acids was confirmed by a HP 5988A gas chromatography mass spectrometry (GCMS) system equipped with a 12 meter HP-1 capillary column. Samples were prepared by acidification with 0.1N HCl followed by extraction with dichloromethane. Samples were then concentrated by evaporation and assessment was performed. The free fatty acid form of EPA was used in GCMS analysis of FFA oxidation, whereas for AA oxidation

assessment, the methyl ester of AA was used. This was prepared by treatment of AA with ethereal diazomethane. The unoxidized (native) samples revealed a single peak associated with the unoxidized forms of the fatty acids. The oxidized EPA samples contained little or no GCMS peaks that were associated with the native fatty acids and a number of additional peaks that were associated with the oxidized products. The oxidized AA sample contained some unoxidized free fatty acid as well as several peaks associated with products. Figures 6-9 depict representative GCMS results for the native and oxidized FFAs.

Oxidized EPA and AA samples were diluted in ethanol vehicle for delivery to cell cultures during all experiments. The highest dose of either OX FFA contained 8mM ethanol. Dilution in ethanol was unavoidable because the oxidation process took place in buffered saline solution.

Western Blot Protein Analysis:

After treatment periods, cell culture plates were exposed to 500 µL of passive lysis buffer (Promega). Cell plates were scraped with cell scrapers and cell lysate was collected and stored at -80º C until needed. Cell samples were freeze-thawed 4 times to ensure lysing of cell membranes and subsequently vortexed and spun. Total sample protein content was quantified using Bradford protein assay. Laemmli buffer: 62.5 mM Tris, pH 6.8, 6 M urea, 160mM 1,3-dithiothreitol, 2% SDS, and 0.001% bromophenol blue, was added to 5 µg protein and placed in 60º C water bath for 5 minutes. Cell lysates were subjected to SDS-PAGE under reducing conditions, and proteins were then transferred to polyvinylidene difluoride membrane (Hybond-ECL, Amersham). The

blots were incubated overnight at room temperature with primary antibody against ecNOS (1:2,000; Transduction Laboratories and GAPDH (1:20,000, Chemicon) followed by incubation for 1 hour with secondary antibody (1:5,000; horseradish peroxidase-conjugated anti-mouse).

Specific eNOS protein was detected by enhanced chemiluminescence (ECL, Amersham) and evaluated by densitometry (National Institutes of Health Image). ecNOS protein is expressed as arbitrary densitometric units after correction for differences in loading.

Statistical Analysis:

All experiment data is reported as means ± standard error measurements of at least 3 independent experiments in duplicate. Statistical significance was determined using a 1-Factor ANOVA followed by a Fisher's least significant difference post hoc test when there was a significant main effect for treatment. Values of $P < 0.05$ were considered to be statistically significant.

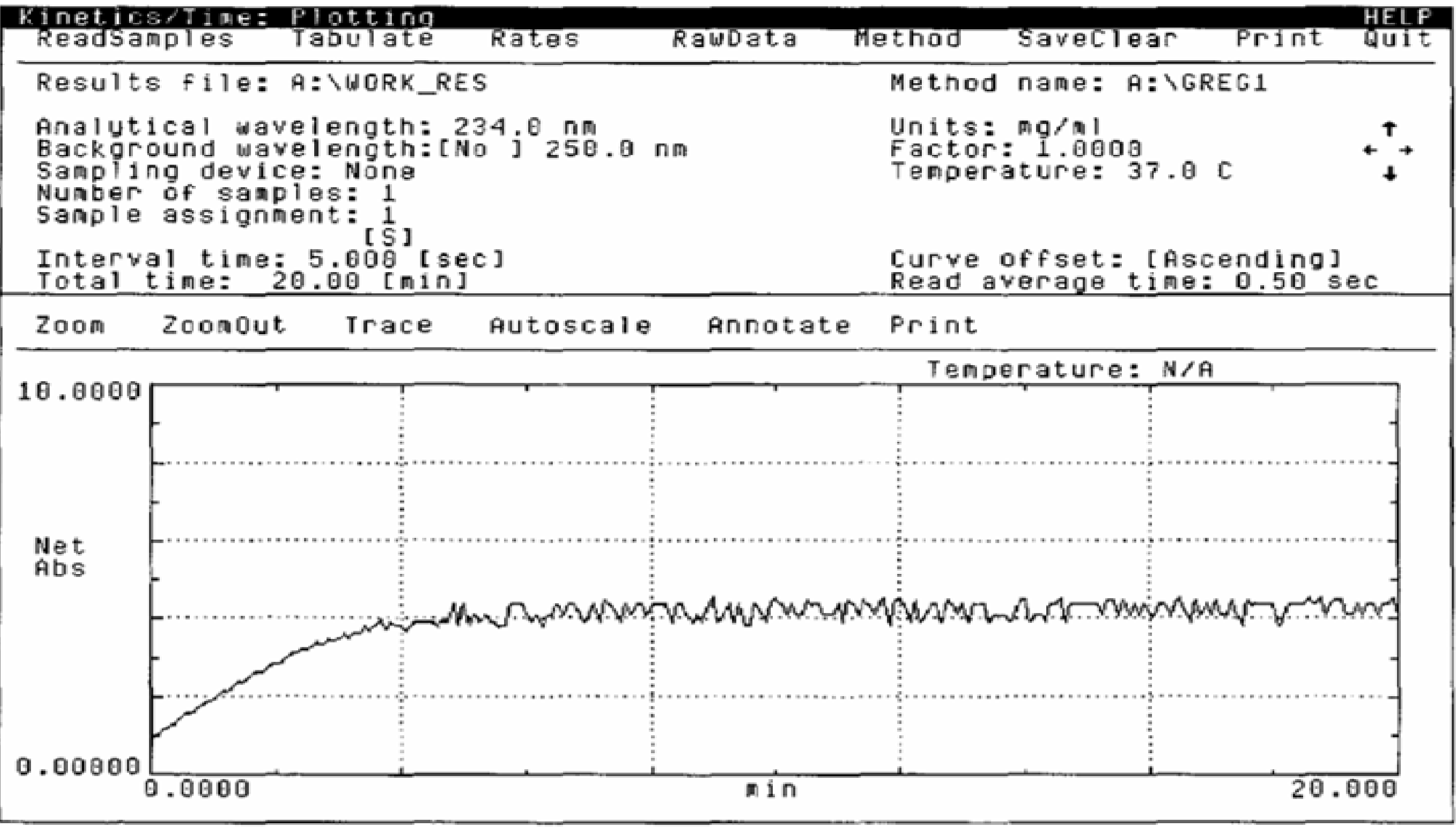


Figure 5. Visual representation of the enzymatic oxidation of linoleic acid. As the substrate FFA is converted to its oxidation products, the absorbance increases linearly until the native form of the fatty acid is exhausted. The measurements are taken at a wavelength of 234nm.

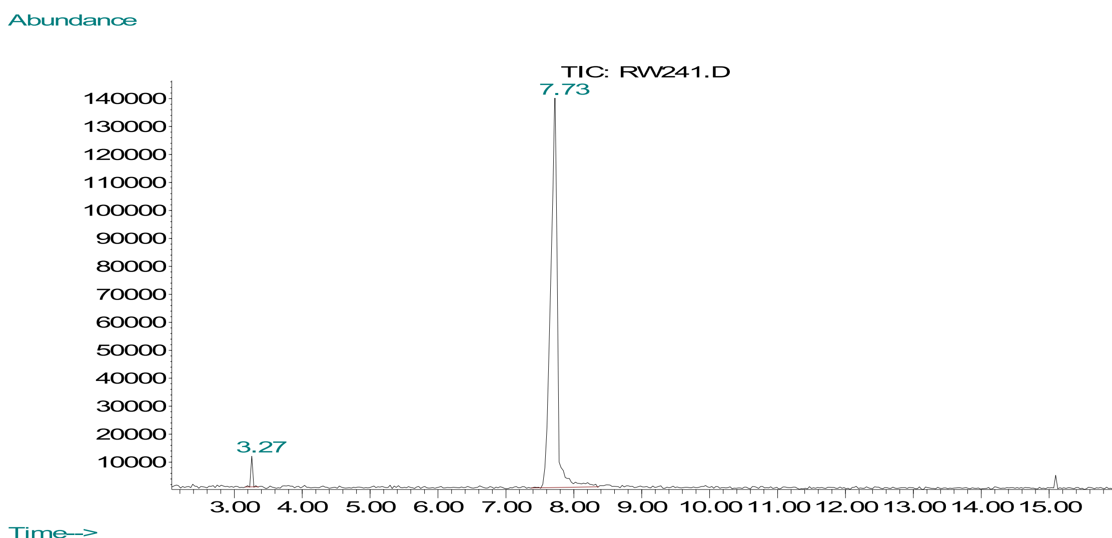


Figure 6. GCMS analysis revealed a single peak associated with the native or unoxidized form of the PUFA EPA at time point 0 hours.

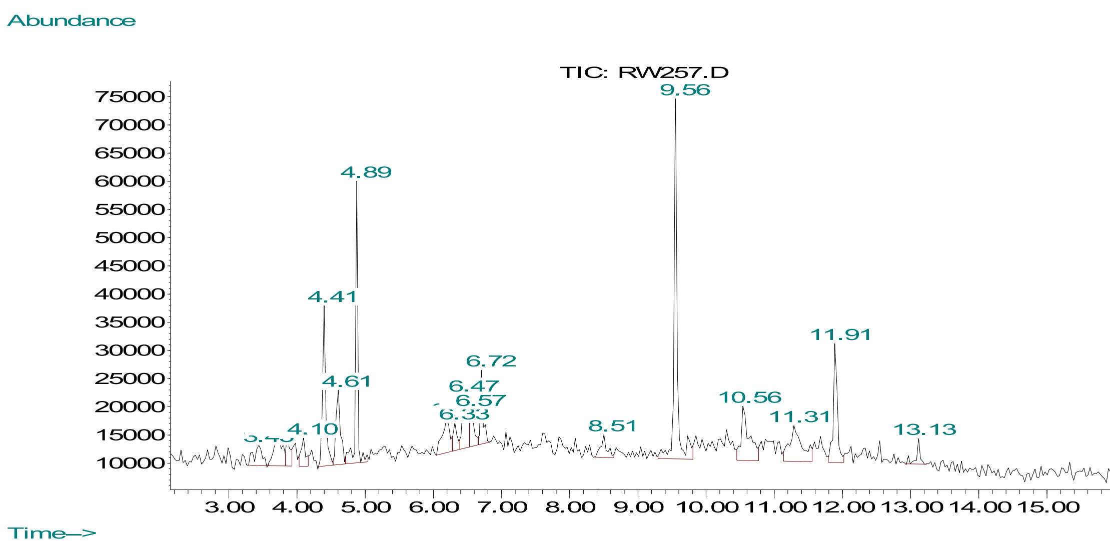


Figure 7. GCMS analysis showing resultant peaks associated with the oxidized products of the PUFA EPA at time point 16 hours.

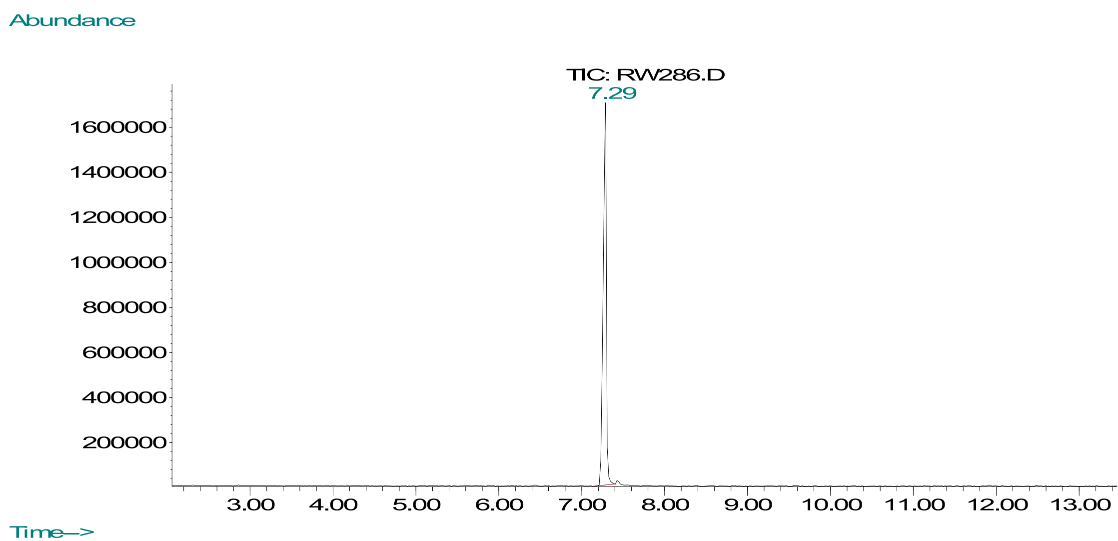


Figure 8. GCMS analysis revealed a single peak associated with the native or unoxidized form of the methyl este r form of the PUFA AA at time point 0 hours.

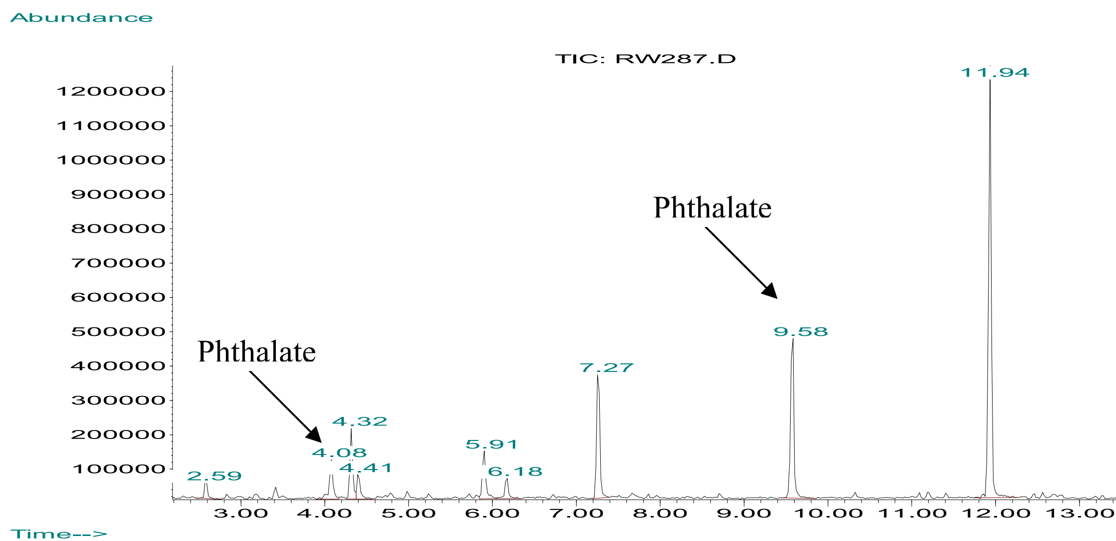


Figure 9. At time point 16 hours, GCMS showed several peaks resulting from AA autoxidation as well as some unoxidized AA.

IV. Results

Oxidized Arachidonic Acid Treatment Results.

Endothelial cells were exposed to 0, 10, 20, 40, 70, or 100µM oxidized AA for 20 hours and eNOS protein expression was measured using Western Blot analysis. Figure 10A shows representative blots of endothelial cells exposed to oxidized AA treatment. GAPDH protein was used as a loading control and as a marker of cell viability after the treatment period. Figure 10B shows that no concentrations of oxidized AA treatments resulted in a statistically significant change in eNOS protein expression when compared to either control containing no ethanol or control containing 8.00mM ethanol. Figure 10C shows graphic representation of eNOS normalized to GAPDH from 4 experiments conducted in duplicate.

Oxidized EPA Treatment Results.

Similar to AA dose response experiments, endothelial cells were exposed to 0, 10, 20, 40, 70, and 100µM oxidized EPA for 20 hours and eNOS protein expression was measured by Western Blot. Figure 11 shows that in 6 experiments completed in duplicate, oxidized EPA treatments resulted in no changes in eNOS protein expression when compared to control that contained 8.00mM ethanol. Representative Western blots are depicted in A. eNOS protein data normalized to control is depicted in B. eNOS normalized to GAPDH is depicted in C.

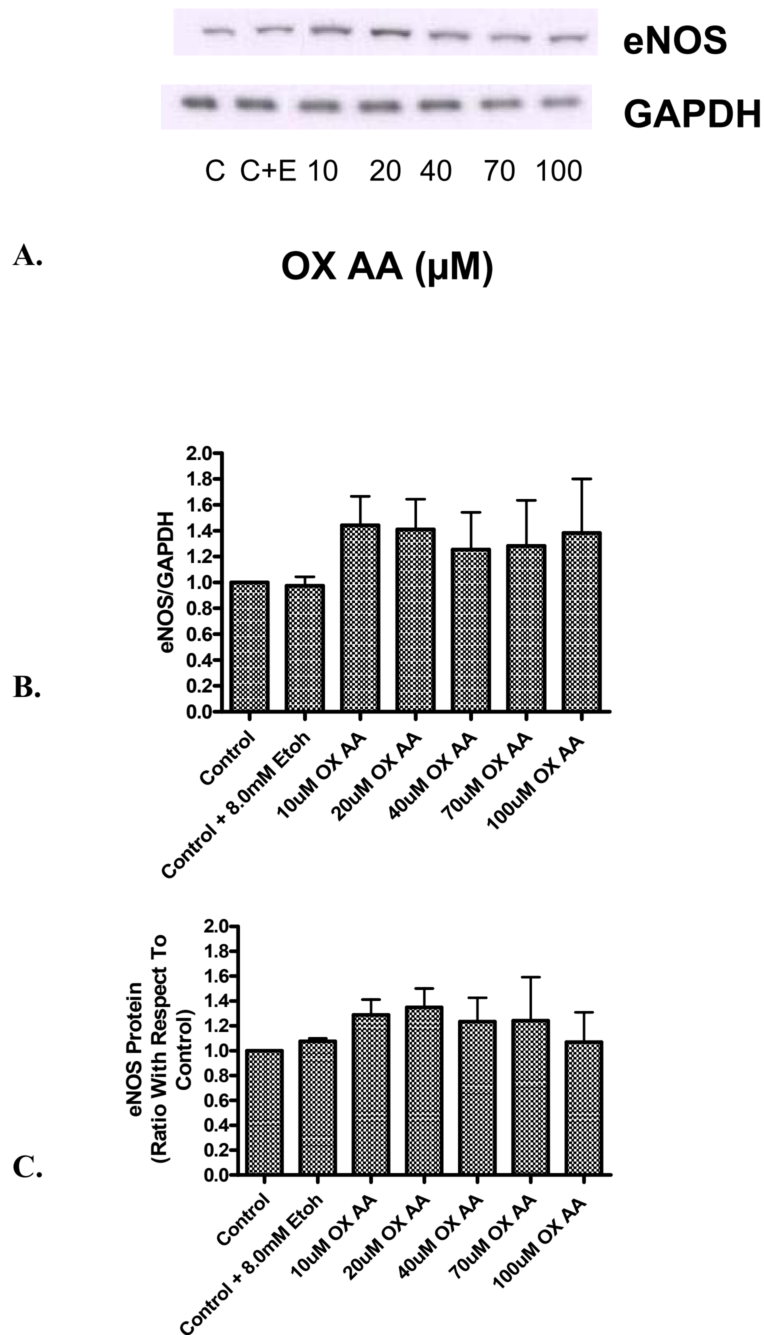
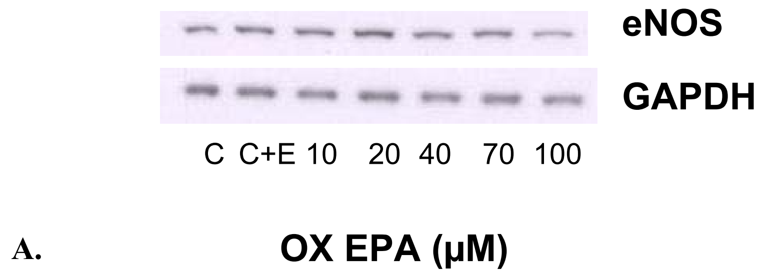
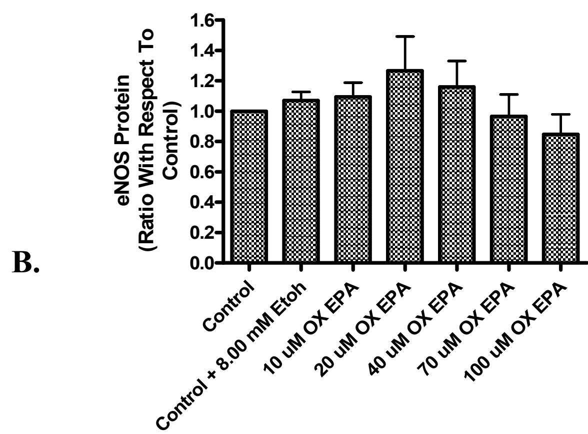


Figure 10. Arachidonic acid treatment does not effect eNOS protein content. The endothelial cells were exposed to 0, 10, 20, 40, 70 and 100µM arachidonic acid for 20hr. The representative Western blots of endothelial cells exposed to different doses of arachidonic acid are shown in panel A. The graphic representation of eNOS data relative to control is depicted in panel B. eNOS data normalized to GAPDH is depicted in panel C. Data are mean ± SEM of four separate experiments conducted in duplicate.





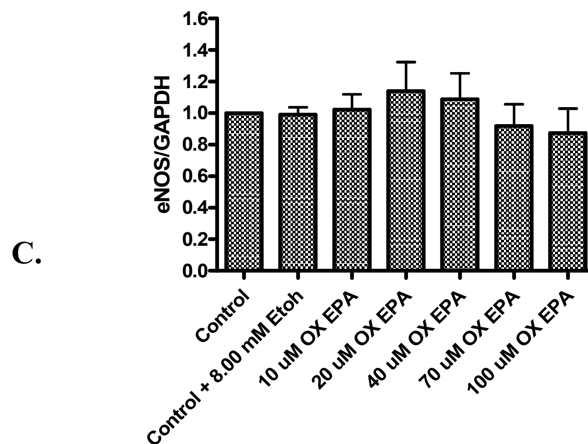


Figure 11. EPA treatment does not effect eNOS protein content. Endothelial cells were exposed to 0, 10, 20, 40, 70 and 100µM EPA for 20hr. Representative Western blots are depicted in panel A. Graphic representation of eNOS data with respect to control is depicted in B. eNOS data normalized to GAPDH is depicted in C. Data are mean ± SEM of 6 separate experiments conducted in duplicate.

HPODE With Ethanol Vehicle Treatment Results.

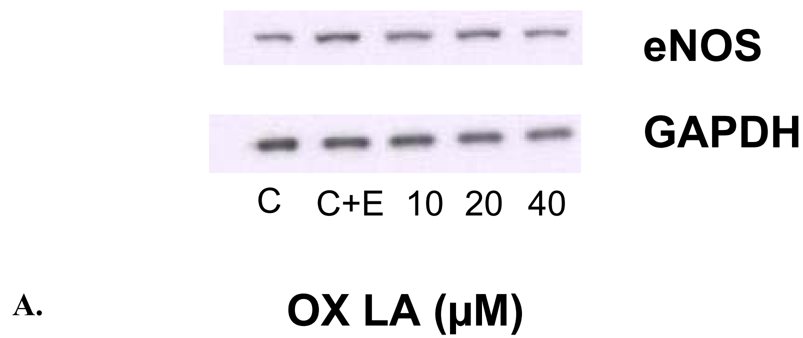
Similar to previous investigations (Ramasamy, Parthasarathy et al. 1998),endothelial cell cultures were treated with 0, 10, 20, or 40 μM 13-HPODE in presence of ethanol vehicle. Figure 12 shows that 3 experiments completed in duplicate resulted in no significant changes in eNOS by treatment with either the native form or the oxidized product of linoleic acid.

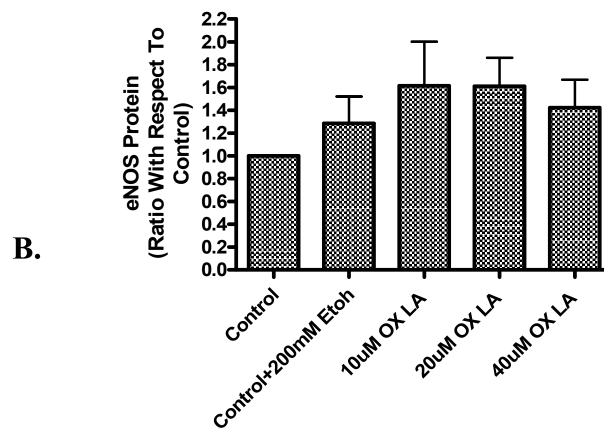
13-HPODE In Absence Of Ethanol Vehicle Treatment Results.

Experiments conducted involving 13-HPODE bound to albumin enriched cell culture media alone, in the absence of ethanol vehicle, resulted in no changes in eNOS protein content. Cells were exposed to 0, 10, 20, or 30 μM LA or similar concentration of HPODE. Figure 13 depicts graphical representation of 3 experiments conducted in duplicate.

Native Linoleic Acid Treatment Results

The results of experiments involving treatment of PAECs with LA concentrations (0-.6mM) show no significant changes in eNOS protein content. Cells were treated with .05, .1, .2, .4, or .6 mM FFA (albumin ratio = .5:1, 1:1, 2:1, 4:1, and 6:1 respectively). Figure 14A depicts representative western blots of eNOS and GAPDH proteins. Figure 14B depicts the results of eNOS normalized to control. Figure 14C depicts eNOS data normalized to GAPDH.





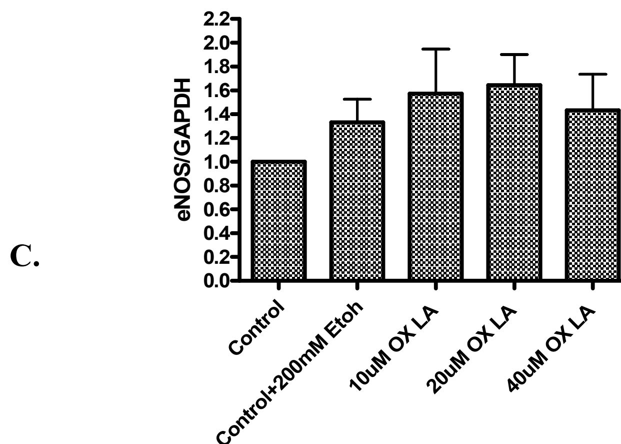
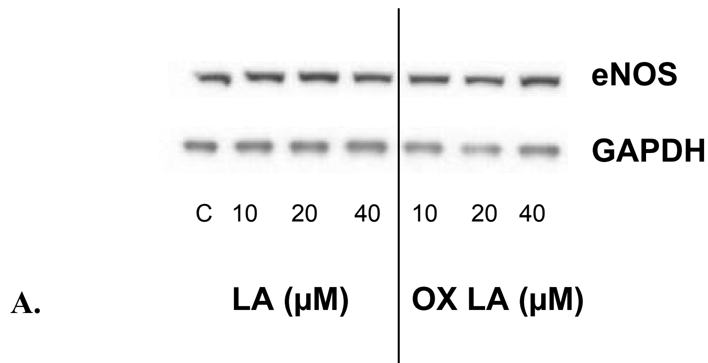
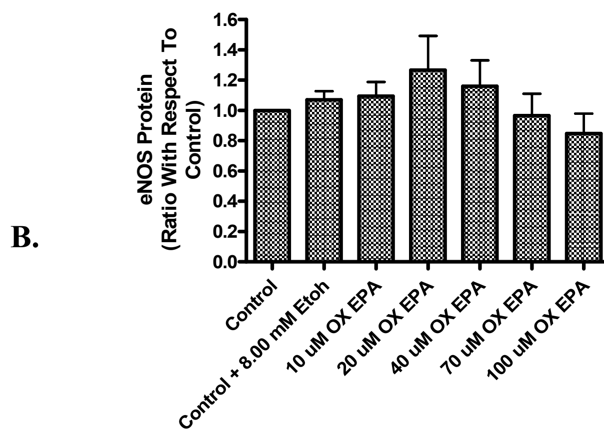


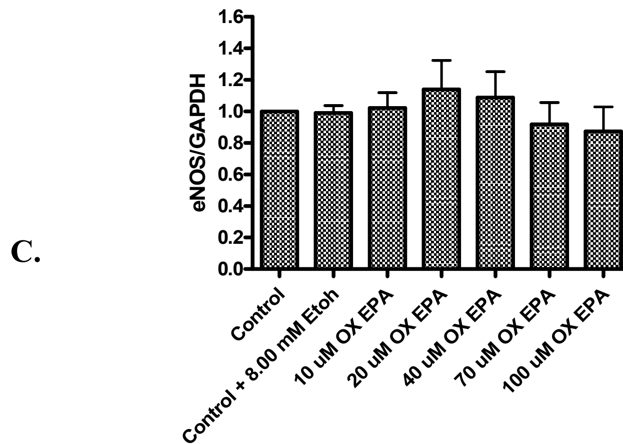
Figure 12. Representative Western blots of endothelial cell cultures treated with 10, 20, or 40µM 13-HPODE in the presence of ethanol vehicle are depicted in figure A. Graphic representation of eNOS data with respect to control is depicted in B. eNOS data normalized to GAPDH is depicted in C. Data are mean ± SEM of 3 separate experiments conducted in duplicate.



A.

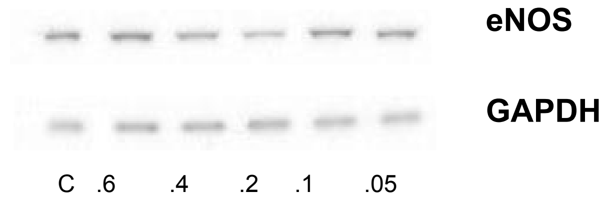


B.

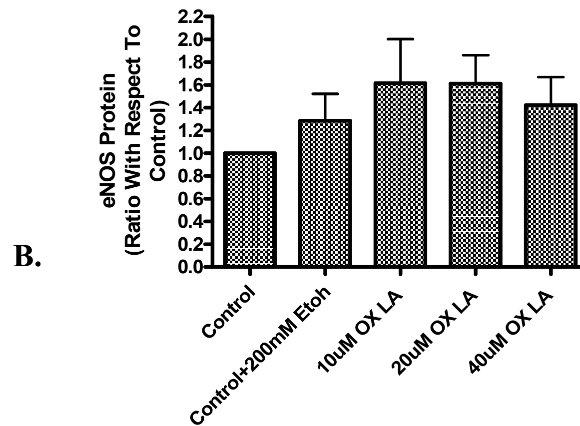


C.

Figure 13. No effect on eNOS protein content was elicited in endothelial cell cultures treated with linoleic acid or its oxidized product 13-HPODE in absence of ethanol vehicle. Representative Western blots are depicted in panel A. Graphic representation of eNOS data with respect to control is depicted in B. eNOS data normalized to GAPDH is depicted in C. Data are mean ± SEM of 3 separate experiments conducted in duplicate.



A. **Linoleic Acid (mM)**



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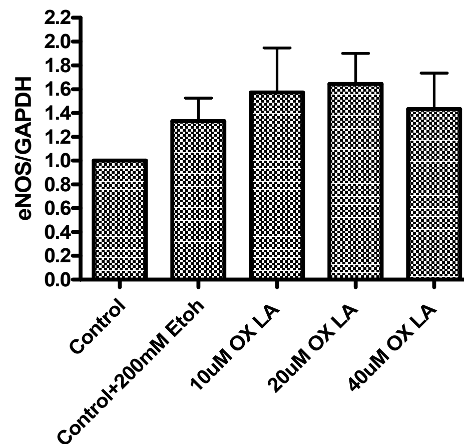
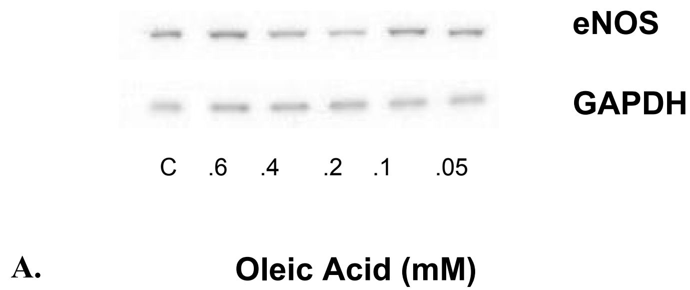
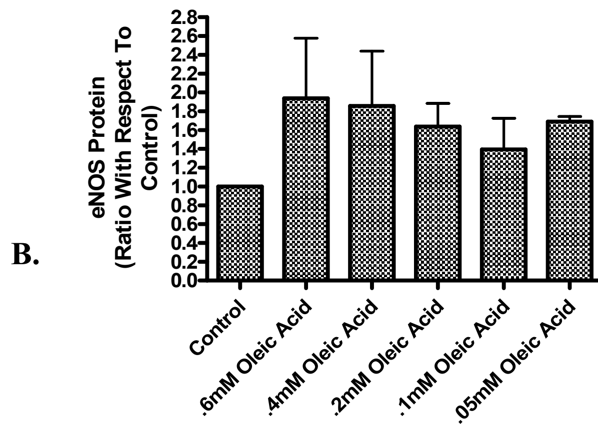


Figure14. Linoleic acid treatment did not effect eNOS protein content in PAECs treated for 20hrs. Representative Western blots of cells treated with 0, .6, .4, .2, .1, or .05mM linoleic acid are depicted in panel A. Graphic representation of eNOS data with respect to control is depicted in B. eNOS data normalized to GAPDH is depicted in C. Data are mean ± SEM of 3 separate experiments conducted in duplicate.

Oleic Acid Treatment Results

Endothelial cells were treated with 0, .05, .1, .2, .4, or .6mM OA for 20 hours and effects were analyzed using Western blot. Representative Western blots are depicted in 15A. Results of eNOS with respect to control are depicted in 15B. eNOS normalized to GAPDH data are shown in 15C.





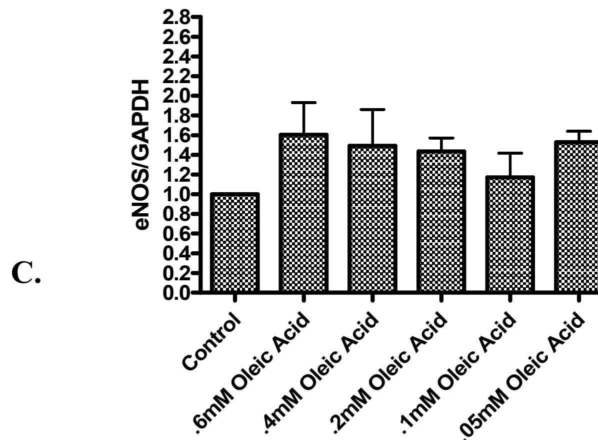


Figure15. Oleic acid elicited no significant changes in eNOS protein content. Representative Western blots of cells treated with 0, .6, .4, .2, .1, or .05mM oleic acid are depicted in A. Graphic representation of eNOS data with respect to control is depicted in B. eNOS data normalized to GAPDH is depicted in C. Data are mean ± SEM of 3 separate experiments conducted in duplicate.

Stearic Acid Dose Response Results

Endothelial cells were treated with 0, .05, .1, .2, .4, or .6mM SA for 20 hours. Fatty acid to albumin ratios were 0:1, .5:1, 1:1, 2:1, 4:1, and 6:1, respectively. Figure 16A shows representative blots of cells treated with SA. Treatment of cells resulted in a dose dependent decrease in cellular eNOS and GAPDH protein expression. Figure 16B depicts eNOS and GAPDH protein levels which were statistically different from control at concentrations of .2, .4, and .6mM. Figure 16C depicts eNOS protein normalized to GAPDH from 3 experiments completed in duplicate.

Palmitic Acid Dose Response Results

Similar to the other FFA experiments, endothelial cells were treated with 0, .05, .1, .2 , .4, or .6mM PA. Again, fatty acid to albumin ratios were 0:1, .5:1, 1:1, 2:1, 4:1, and 6:1, respectively. Figure 17A shows a representative Western blot of cells treated with PA. Treatment with palmitic acid resulted in a dose dependent decrease in eNOS and GAPDH protein levels as depicted in figure 17B. Figure 17C depicts graphical representations of the resulting eNOS data normalized to GAPDH from 3 separate experiments completed in duplicate.

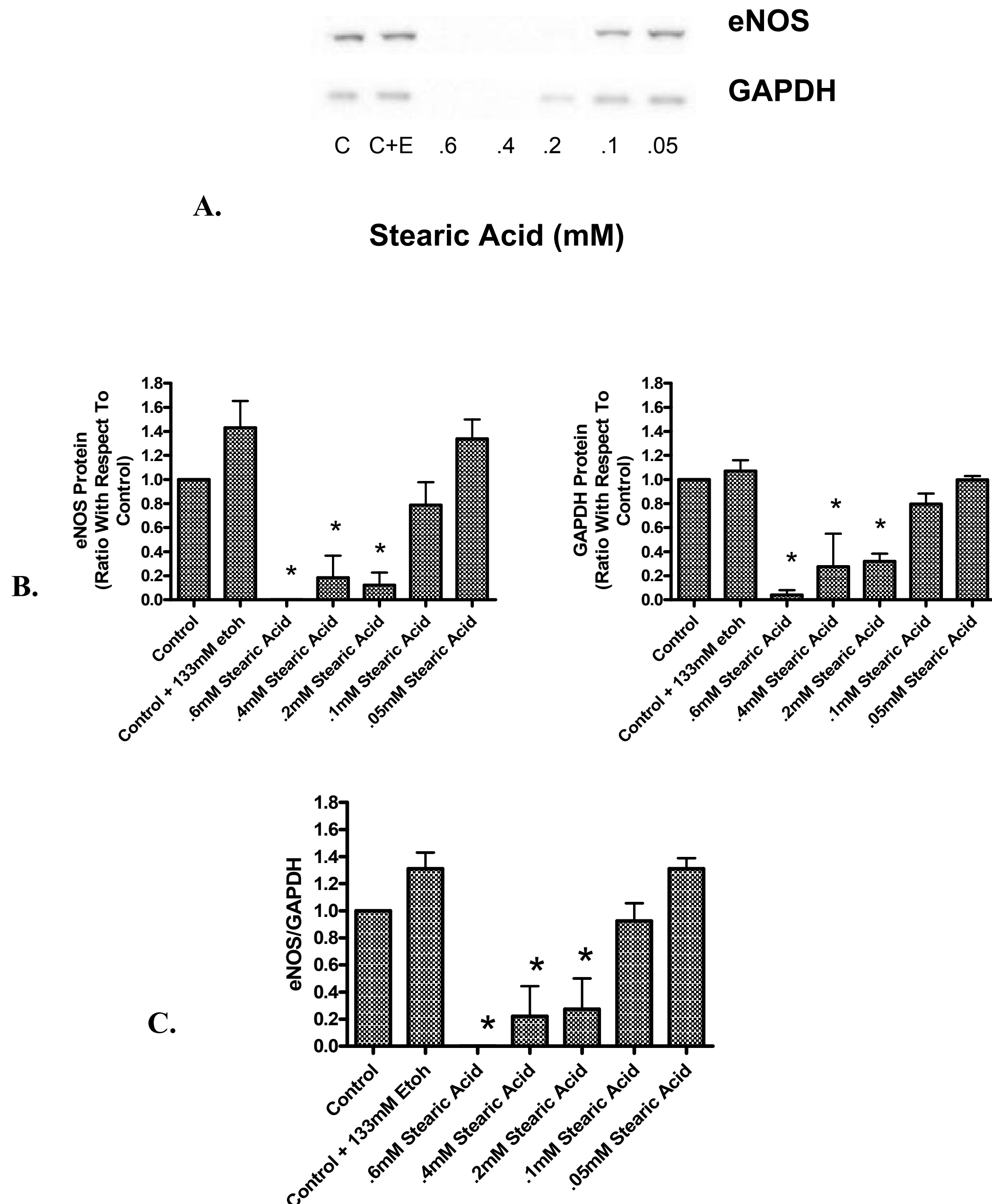


Figure 16. Stearic acid causes a dose-dependent decrease in eNOS and GAPDH protein content. The endothelial cells were exposed to 0, .6, .4, .2, .1, and .05mM SA for 20hr. Fatty acid to albumin ratios were 0:1, 6:1, 4:1, 2:1, 1:1, and .5:1, respectively. Representative Western blots of endothelial cells exposed to various concentrations of stearic acid are depicted in panel A. Cumulative results of eNOS and GAPDH blots with respect to controls are depicted in B. Cumulative data of eNOS normalized to GAPDH are depicted in C. Data are mean ± SEM of 3 separate experiments in duplicate. Results denoted by asterisks were considered significant at $P < 0.05$ level.

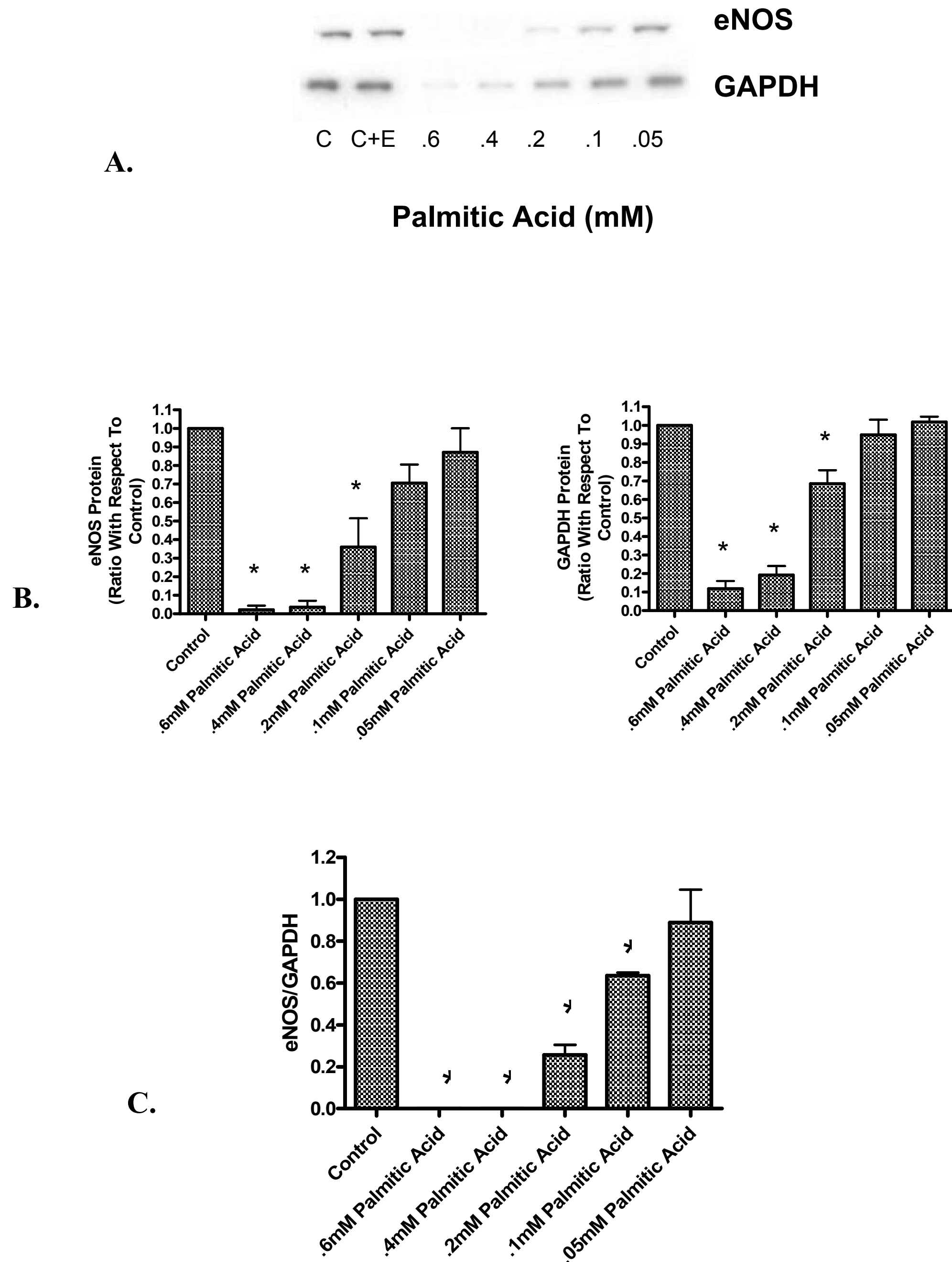


Figure 17. Palmitic acid causes a dose-dependent decrease in eNOS protein content. The endothelial cells were exposed to 0, .6, .4, .2, .1, and .05mM palmitic acid for 20hr. Fatty acid to albumin ratios were 0:1, 6:1, 4:1, 2:1, 1:1, and .5:1, respectively. Representative Western blots of endothelial cells exposed to various concentrations of stearic acid are depicted in panel A. Cumulative results of eNOS and GAPDH blots with respect to controls are depicted in B. Cumulative data of eNOS normalized to GAPDH are depicted in C. Data are mean ± SEM of 3 separate experiments in duplicate. Results denoted by asterisks were considered significant at $P < 0.05$

V. Discussion And Conclusion

Previous studies have shown that lipids decrease eNOS protein and this is putatively important for how OxLDL contributes to vascular dysfunction. For this to be true, it is key to determine if components of OxLDL molecules such as oxidized PUFAs, or some other atherogenic lipid, such as saturated fatty acids, are alone sufficient to decrease eNOS protein content in endothelial cells. The purpose of this thesis was to test the hypothesis that some types of FFA, either oxidized PUFAs or saturated FFA, are sufficient to decrease NOS protein content in endothelial cells. The results of this study indicate that oxidized FFAs, which comprise OxLDLs, alone do not alter eNOS protein content within the cells. However, the results of this investigation indicate that saturated FFAs, which do not undergo oxidative modification, dose dependently decrease eNOS protein content within the cells. Therefore, this investigation indicates that oxidized PUFAs do not alone contribute to NO mediated endothelial dysfunction, caused by decreases in eNOS protein content, that have been seen to be induced by OxLDL. However, saturated FFAs are sufficient by themselves to decrease eNOS protein levels in isolated aortic endothelial cells.

The results of the present study involving PAEC treatment with the oxidation product of the FFA linoleic acid, 13-HPODE, in absence of ethanol vehicle seem to indicate that hydorperoxide derivatives do not affect eNOS protein content within the cell. The results however, of previous studies by Ramasamy et. al. indicated that 24 hour treatment of bovine aortic endothelial cells (BAECs) with 20 μM 13-HPODE (no supplemental albumin) resulted in a more than 2 fold increase in eNOS mRNA and a 47% increase in eNOS protein content. In these earlier experiments by Ramasamy,

preparation of 13-HPODE in cell culture media utilized ethanol as an organic solvent to dissolve the oxidized fatty acid product into cell culture media. This method, however useful for solubilizing lipids in aqueous solution, may have misled interpretation of results of the effects that 13-HPODE elicit because high concentrations of ethanol have been shown to effect endothelial function. For example, 96 hour treatment of BAEC's with 100mmol/L ethanol significantly increased bradykinin-, adenosine 5'-triphosphate-, and ionomycin-stimulated nitric oxide synthase activity without affecting basal enzyme activity (Davda, Chandler et al. 1993). Contrary to experiments conducted by Ramasamy et. al., the study conducted here involving 13-HPODE and the use of ethanol vehicle in PAEC treatment resulted in no significant differences in eNOS protein content after 20 hour treatment. These results could be caused by lower concentrations of ethanol used as ethanol vehicle in the experiments presented here however, precise concentrations in previous experiments involving dissolving of 13-HPODE are unknown and therefore comparisons are not made. This hypothesis is supported by the similar results of experiments involving 13-HPODE that do not contain ethanol vehicle presented here. Since no changes were seen in eNOS protein within the endothelial cells, it would suggest that ethanol could be the cause of increased eNOS seen in the experiments by Ramasamy et Al. Another potential cause for this inconsistency with previous studies is that this investigation utilized supplemental purified bovine serum albumin (1% or 10mg/mL final albumin concentration). This alone could result in less overall concentrations of free fatty acid that could elicit effects on the vasculature.

The analysis of oxidation of the PUFAs EPA and AA used in these experiments suggest that under normal physiologic conditions, EPA and AA are susceptible to

oxidation. Gas chromatography mass spectrometry analysis of EPA showed that little or none of the native form of either highly unsaturated fatty acid remained in FFA containing samples after 16 hours. Although native AA was present in oxidized samples after 16 hours, the fatty acid underwent oxidation readily. One explanation for AA being less susceptible to autoxidation than EPA is the number of double bonds located in its carbon backbone. The readiness of these fatty acids to undergo autoxidation is potentially one explanation why studies are not in abundance involving treatment of cell cultures for a prolonged period with native forms of highly polyunsaturated fatty acids. It was for this reason that this study did not include the use of the native forms of EPA and AA as a control treatment of PAECs with the oxidized counterparts of these FFA. Responses elicited after 20 hour treatment of cells with these fatty acids could have been the result of oxidation products of the FFA and not their native forms.

Arachidonic Acid is known as one of the most important biological PUFAs located in mammalian phospholipid membranes. The FFA serves as the precursor for the eicosanoid family of hormones. Many of these molecules are known to be involved in the process of inflammation, leukocyte adhesion, nuetrophil chemoattraction and vasoresponsiveness (prostacyclin). It has been shown that not all AA undergoes the biosynthetic pathway for eicosanoid production and that this amount is susceptible to nonenzymatic attack by free radicals (Morrow, Hill et al. 1990). Under conditions involving high local concentrations of reactive oxygen species, it is possible that AA that would previously have been used in eicosanoid synthesis could undergo peroxidation, thereby changing its role from a cardioprotective to a proatherogenic one. Previous studies have shown that DNA damage and mutations can be induced by arachidonic acid

that has undergone lipid peroxidation (Lim, Sadre-Bazzaz et al. 2003). However, the specific products of oxidation where not identified in this study.

The products of arachidonic acid peroxidation are known to be different than those produced by diene lipid peroxidation as occurs with linoleic acid.(Porter, Caldwell et al. 1995) Although free radical attack may occur on analogous carbons during the oxidation process, the resulting hydroperoxide products are nevertheless longer in length due to the carbon chain length of the parent FFA. It was for these reasons, as well as arachidonic acids continual presence within cell membranes, that it was necessary to study the effects of this and other PUFA's individually and the effect of their hydroperoxides on regulators of vascular function. The results of this study indicate however, that the products of FFA peroxidation, do no effect the cells eNOS protein content any differently than do the hydroperoxides produced by the oxidation of linoleic acid.

In a study conducted by Artwohl, experiments showed that the PUFAs linoleic acid (18:2), linolenic acid (18:3), and arachidonic acid caused endothelial cell apoptosis only at the highest doses used (300μM) (.4 or 4mg/mL final albumin concentration) (Artwohl, Roden et al. 2004). One hypothesis made based on this result was that highly polyunsaturated fatty acids being highly susceptible to oxidation could contribute to the atherogenic process by causing oxidative stress to the cells thereby activating an inflammatory response. The results of the experiment conducted in this investigation do not serve to prove or refute this hypothesis in that the highly polyunsaturated fatty acid oxidation products of both EPA and AA showed no effect on eNOS or GAPDH protein content and cell viability was normal as assessed visually after oxidized FFA treatment.

The oxidation properties of linoleic acid allow for the native form of this fatty acid to be highly present in the plasma under normal physiologic condition. Previous studies have shown the linoleic acid can induce hyperpolarization and relaxation in the pig coronary artery (Pomposiello, Alva et al. 1998). The results of that study showed that this result was elicited via smooth muscle cells. The results of this investigation show that although linoleic acid, in its native form, can elicit cardioprotective roles, it does not do so by its effect on endothelial levels of eNOS protein content within PAECs.

Recent studies involving the omega-3 fatty acid eicosapentaenoic acid (EPA) common in fish oil and docosahexaenoic acid (DHA) have shown that the oxidized form of the fatty acids may serve cardiovascular protective roles. The oxidized highly polyunsaturated fatty acid (EPA) but not the unoxidized FFA was seen to inhibit cytokine-induced glomerular endothelial and mesangial cell expression of the adhesion receptors monocyte chemotactant protein-1 (MCP-1) and interlukin-8 (IL-8). The oxidized form of EPA was also seen to inhibit cytokine-induced activation of NF-κB in these cells as well as mesangial cells (Chaudhary, Mishra et al. 2004). Further studies indicated that this inhibition occurred through a PPARα-dependent mechanism. Other studies involving diets supplemented with fish oil resulted in higher plasma levels of the omega-3 oxidized products suggesting that the oils are readily oxidized in vivo. These studies suggest that the oxidized omega-3 fatty acids and not their native forms are the contributing factors to anti-atherogenic properties of marine fish oil diets. The results of this study indicate that the products of oxidation of EPA, do not affect normal processes of eNOS protein production within porcine derived endothelial cells. These findings suggest that the anti-atherogenic properties elicited by marine fish oil rich diets do not

include improved vascular responsiveness do to higher eNOS content within vascular cells.

It was the original aim of this study to examine the effects of products of lipid oxidation derived from saturated fatty acids including palmitic acid and stearic acid. However, previous studies outlining the molecular processes of free radical initiated oxidation show that for this to occur, double bonds in the carbon backbone of the fatty acid are required as serving as the site for free radical attack. For these reasons, the saturated fatty acids palmitic and stearic acid could not be induced to undergo lipid oxidation as they normally do not under physiologic conditions. Oleic acid, albeit a monounsaturated fatty acid that can be induced to undergo lipid peroxidation, does not easily under normal physiologic conditions. Previous studies have also shown that peroxidation of this fatty acid and its products are not known to induce cellular DNA or cytotoxic effects seen produced by oxidized LA and AA (de Kok, ten Vaarwerk et al. 1994; Lim, Sadre-Bazzaz et al. 2003). These properties also contribute to the body of evidence that oleic acid serves vascular protective roles. Understanding the roles that these fatty acids play in endothelial function is of great importance.

Previous studies involving oleic acid, the predominant fatty acid in olive oil and the Mediterranean diet has been shown to attenuate the development of atherosclerosis (Carluccio, Massaro et al. 1999). One method for doing so is preventing endothelial cell activation and inhibiting the inflammatory response for endothelial cell adhesion molecule expression for circulating monocytes. Artwohl et. Al. showed that concentrations ranging from 0 to 300 μM of stearic acid (.4% or 4mg/mL final albumin concentration) caused a dose dependent increase in the incidence of cell apoptosis due to

cell cycle halting. The same concentrations of oleic acid did not elicit the same effect. This finding validated the contention that a diet rich in saturated fatty acids such as stearic acid is one risk factor for developing cardiovascular disease. The results of this study indicate that the cardiovascular protective effects elicited by oleic acid do not include increased production of eNOS and therefore increased bioavailability of NO. However, since no decrease in eNOS protein content was observed, this study shows that oleic acid does not interfere with the presence of eNOS content within the cell allowing for its cardiovascular protective role through the production of NO.

The negative effects of a diet rich in saturated fatty acids such as palmitic and stearic acid are well documented. The typical American diet, containing large proportions of each of these fatty acids, gives cause for concern as stearic acid is largely found in animal fats and palmitic acid can be found in vegetable and animal fats, both of which are commonly found in the US diet. Previous studies involving low concentrations of saturated FFA showed that palmitic acid (10-100μM) but not stearic acid (10-100μM) (2% or 20mg/mL final albumin concentration) dose dependently decreased production of NO in human umbilical vein endothelial cells (HUVECs) (Moers and Schrezenmeir 1997). The results of this investigation show that the saturated FFAs elicit similar effects regarding eNOS protein content in PAECs.

The results of these experiments show that eNOS protein content within the cell decreased in a dose dependent manner when exposed to increasing doses of palmitic acid and stearic acid. In the same instance, GAPDH protein in the cells was also diminished signifying that either these treatments effected cellular GAPDH protein production or that

treatment of the cell with increased concentrations of stearic acid and palmitic acid may have caused the endothelial cells to undergo apoptosis.

The aforementioned 2004 study by Artwohl did not involve the saturated fatty acid PA. However, treatment of the cells in that study with the saturated fatty acid SA resulted in cellular apoptosis at 300µM (.4% or 4mg/mL albumin, FFA:albumin ratio = 6.8:1) and similar concentrations of palmitic acid have been seen induce apoptosis in other cell types. This deems it possible to be the cause of the results seen by higher concentrations of SA and PA (0.6 and 0.4mM) (FFA:albumin ratio = 6:1 and 4:1 respectively) since these concentrations translate to 2.4mM and 1.6mM FFA treatment respectively (human albumin concentrations = ~4%). However, SA and PA cell treatment concentrations of 0.2, 0.1, and 0.05mM (FFA:albumin ratio = 2:1, 1:1, and 0.5:1 respectively) FFA concentrations translate to .8, .4, and .2mM which are typical physiological plasma concentrations for these FFA. Further studies have to show whether the concentrations used in these experiments resulted in cellular apoptosis and thus a drop in all cell protein content.

The results of this investigation seem to concur with prior studies in that endothelial exposure to high levels of saturated fatty acids for prolonged periods results in endothelial dysfunction. This is more evident in subjects that have higher than normal levels of plasma lipids. For example, previous investigations have shown that endothelial mediated dilation is acutely impaired following a high fat meal in subjects that are mildly dyslipidemic hypertriglyceridemic (Giannattasio, Zoppo et al. 2005). This impairment however was not noted in subjects that were normotrighlyceridemic. Endothelial dependent vasodilation was also seen to be impaired in subjects after feeding of a high fat

meal consisting of deep fat frying oils previously used in fast food restaurants, but not unused cooking oils (Williams, Sutherland et al. 1999). The results of this investigation may be most analogous to individuals receiving large proportions of their daily fat intake from fast food restaurants over prolonged periods.

The reactions that occur in the oxidation of fatty acids consist of several basic chemical reaction types. First hydrogen atom transfer occurs generating a carbon radical. Oxygen then binds to the lipid substrate producing the peroxyl radicals. Fragmentation and cyclization of these molecules occurs producing a variety of products, many of which are suspected to be involved in possible cellular signaling mechanisms including the hydroperoxides and more recently aldehyde derivatives of PUFAs (Forman and Dickinson 2004). Although identification and isolation of specific products of lipid oxidation would be beneficial in determining their roles in cellular processes, this can prove to be a difficult task since literally hundreds of products may be formed from longer chain fatty acids. The aim of this study involved the investigation of FFA peroxiation and all of the resultant products as potential regulators of endothelial cell protein production. Though no single product of these reactions is investigated in this study, results produced by a particular family of hydroperoxide derivatives might give light as to a specific subset of bioactive molecules that can then be further studied.

Since the discovery of peroxisome proliferator-activated receptors (PPARs), lipids and eicosanoids have been studied as potential regulators of gene expression by serving as ligands to a number of PPAR receptors. PPARs are lipid-activated transcription factors that belong to the steroid/thyroid/retinoic acid receptor superfamily. The receptors act as transcription factors in the cell and their characterized target genes

encode proteins that participate in lipid homeostasis. Many essential and nonessential fatty acids have been identified as being ligands to one or more PPAR receptor types (Krey, Braissant et al. 1997). Recently, lipids as well as their metabolites and products of oxidation have been studied extensively and have been found to act through PPAR dependent mechanisms. Since it has been seen that some lipids can exhibit cardiovascular protective effects, such as OA, while others elicit negative cardiovascular effects, such as the saturated fatty acids palmitic and stearic acid, it has been important to study individual FFA separately and their potential roles in gene regulation and therefore cellular function.

The aim of this study has been to assess the role that many of these fatty acids and their oxidation products, have in the regulation of endothelial eNOS protein, an important marker and modulator of vascular health. The result of this investigation show that of the many FFA common in the plasma, many of which undergo oxidation under physiological conditions, palmitic acid and stearic acid, two common FFA found in the diet decrease endothelial eNOS protein content in cultured PAECs.

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VITAE

Gregory David VanVickle was born in Blytheville, Arkansas on January 19, 1978. He graduated from Sacred Heart High School in Sedalia, Missouri in 1996. He attended his first year of college in the School of Aviation at Central Missouri State University. In 1997, he then transferred to the University of Missouri-Columbia and began working in the lab of Dr. Harold Laughlin where he remained throughout his undergraduate career. He obtained his Bachelor of Science in Biological Sciences in the winter 2002 and began work on his Master of Science in the Department of Biomedical Sciences the following fall under Dr. Harold Laughlin and Dr. Marc Hamilton. He completed his M.S. in 2005 and currently lives in Surprise, Arizona.