EFFECTS OF TRANS FATS IN HUMAN MACROPHAGES

by

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ABSTRACT

The mechanism by which trans fats contribute to atherosclerosis, an important public health issue, is unclear. Trans fats may influence cell membrane stability, inflammatory responses, and signaling. Cellular metabolism of oleate (C18:1Δ9-10 cis), elaidate (C18:Δ9-10 trans), and stearate (C18:0) were compared in adherent peripheral human macrophages, the first responders in atherosclerosis. Metabolism was monitored by acylcarnitine measurement in supernatants by MS/MS, determination of whole cell fatty acid content by GC/MS, and β-oxidation evaluation using radiolabeled fatty acids. Macrophages incubated in elaidate for 44 h accumulated more unsaturated fatty acids, both longer- and shorter-chain, and had reduced C18:0 relative to incubation with oleate or stearate. Cell supernatants exposed to trans fats accumulated both C12:1- and C18:1-carnitines, suggesting inhibited β-oxidation proximal to the trans bond. Next, competitive β-oxidation assays with [9,10-3H]oleate showed that tritium release rates decreased when elaidate replaced unlabeled oleate. Yet, when [1-14C]oleate was compared to [1-14C]elaidate β-oxidation, initial elaidate degradation rates were comparable to oleate, supporting inhibition of double bond isomerization by elaidate. An expression array comparing human macrophages incubated with 30 μM oleate or elaidate showed eight genes associated with zinc homeostasis. Changes in metallothioneins 1X and 2A and SLC39A10 expression were confirmed by qPCR. Parallel qPCR experiments with saturated fatty acids showed elevated metallothionein expression at 44 h, but at 15 h elaidate, stearate, and palmitate have comparable metallothionein expression lower than oleate. Next we investigated these effects on intracellular zinc. Expression changes paralleled intracellular zinc at both
time points confirmed quantification in elaidate-, stearate-, and palmitate-treated cells. Elaidate, stearate, and palmitate increased labile zinc at 15 h, but only elaidate-treated remained elevated at 44 h. To determine whether zinc changes corresponded to inflammation, proportional nuclear localization of nuclear factor-κB (NF-κB) was determined. A parallel experiment was conducted with the addition of 5 μM zinc chelator, TPEN. Elaidate, stearate, and palmitate caused the most NF-κB nuclear localization. Addition of TPEN nullified the treatment effect; all conditions, even controls, caused similar effects. These data show the similar initial effects of elaidate, stearate, and palmitate on macrophage zinc homeostasis and NF-κB activation, but the elaidate zinc effect is persistent.
Dedicated to my parents
Scott and Amy Zacherl
and to my boyfriend
Michael Ingram
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1.0 INTRODUCTION

1.1 DIETARY FATTY ACIDS: SATURATED, MONOUNSATURATED, NATURAL, AND ARTIFICIAL

Elaidate ((E)-octadec-Δ9-enoic acid) is an 18-carbon monounsaturated trans fatty acid with the trans bond at the C9-C10 position. Trans fatty acids, specifically elaidate, account for from 4-10% of the average individual’s daily fatty acid intake (1). Elaidate is not a naturally occurring fatty acid but is produced through industrial hydrogenation. Unsaturated fatty acids can be converted to saturated fatty acids by bubbling hydrogen through them at high temperatures in a closed container with a catalyst. Hydrogenation was discovered by a French chemist in the late 1800s (2,3). Elaidate is the predominant of many trans isomers that may be manufactured during hydrogenation. Double bonds in the carbon chain may be moved to make various isomers with double bonds at various positions between C4 and C14 (3),(4). The purpose of trans fatty acids is to replace saturated fatty acids in foods, such as baked goods, since most trans fatty acids have similar characteristics to saturated fatty acids (5). Widely used partially hydrogenated soy oil contains 15% saturated fatty acids, and <1% trans fatty acids (6). The most prevalent saturated fatty acids humans consume include palmitate (C16:0), stearate (C18:0), myristate (C14:0), and laurate (C12:0). Ninety percent of saturated fatty acids consumed consist of palmitate and
stearate (7). Saturated and trans fatty acids improve shelf-life of foods and preserve taste and textures in food staples of the typical human diet.

However, there is an increasing amount of evidence asserting that both trans and saturated fatty acids (long chain in particular) have damaging effects on the health of humans, predominantly with regards to obesity, diabetes, and heart disease (8). According to Kummerow, et al. 2009, diets comprising of high amounts of long-chain saturated fatty acids and/or small amounts of trans fatty acids can be damaging to arterial walls possibly causing atherosclerotic lesions to develop (9). However, not all trans fatty acids are manufactured. Naturally occurring trans fatty acids include conjugated linoleic acid (CLA) (C18:2) and vaccenic acid (C18:1 ω-7). CLA and vaccenic acid are produced in ruminants, and therefore, consumed by humans through meat and dairy of ruminants. Natural trans fatty acids are handled by the body differently from industrial trans fatty acids. A 2004 study of two litters of piglets whose mothers were fed a diet containing either ruminant trans fatty acids in the form of butterfat or corn oil (2-4% trans fat) or industrial trans fatty acids in the form of highly hydrogenated soy oil (30.3% trans fat) showed ruminant trans fatty acids to be nearly harmless. Unfortunately, the piglets whose mother consumed hydrogenated soy oil showed increased fatty streaks in their arteries and a build-up of intermediates from the conversion of linoleic acid to arachidonic acid, a polyunsaturated fatty acid prominent in cell membranes. This metabolic stall allowed linoleic and arachidonic acid intermediates to accumulate, and 3% more trans fatty acids were assimilated into phospholipid membranes in the arteries (10).

At this point, according to Piotrowski, et al., 1996, the inner arterial lining or vascular intima amasses caseous lipid debris known as plaque in advanced stages of atherosclerotic disease (11). There is none of this accumulation in healthy vascular intima (12). In studies
focused on the composition of arterial plaques, researchers have found that they not only contain caseous lipid debris, but also cholesterol and oxidized phospholipids, fat-engorged monocytes which have differentiated to macrophages (13), and proliferating smooth muscle cells (12,14-16). The caseous lipid debris includes dead cells, specifically monocyte derived macrophages. Trans fatty acid ingestion could be responsible for some cell death since it has been shown that phospholipids originating from trans fatty acids are more likely to be incorporated into membranes than their cis counterparts, resulting in reduced fluidity and organization (10,17-19). Membrane fluidity is an important functional characteristic in human macrophages. Rigid membranes have reduced ability to handle cholesterol and they have a negative impact on cell signaling and viability (20).

1.2 VICTIOUS CIRCLE OF MACROPHAGES, FOAM CELLS AND ATHEROSCLEROTIC DEVELOPMENT

Macrophages are inflammatory, phagocytic cells whose role is to ingest and remove toxins, cholesterol, and cellular debris from the plaque site (12,21). In atherosclerotic lesions, the macrophages are attempting to remove fatty cellular debris but become engorged with lipids and very often die. These remnant macrophages are termed “foam cells” (12).

Epidemiological works have also come to the conclusion that trans fatty acid consumption increases the threat of atherosclerosis. In 2009, the World Health Organization collected human clinical and observational research studies and determined that trans fatty acid consumption is a major cardiovascular disease risk factor (22). Also in 2009, Mozaffarian, et al. conducted a meta-analysis of clinical information regarding human trans fatty acid ingestion,
and the same conclusion was reached. *trans* fatty acid consumption increased total serum cholesterol while decreasing beneficial high density lipoprotein-cholesterol (HDL-c), a combination that has been shown to increase the risk of cardiovascular disease (23,24). The valuable function of HDL is that it accepts and binds the cholesterol efflux from cells in the vascular intima, thereby reducing inflammation and the magnitude of the plaque (25). Low density lipoprotein (LDL) is a cholesterol transport molecule predominantly for transport from the liver to peripheral tissues of cholesterol. LDL receptors are synthesized by the cell and transport LDL-cholesterol across the membrane (26). Upon consumption of *trans* fatty acids, plasma triglyceride and LDL concentrations increase indicating that cells, especially macrophages, are endocytosing cholesterol and becoming foam cells (27-29).

### 1.3 CONSEQUENCES OF DIETARY TRANS FATTY ACID: SHOULD WE CONSUME SATURATED INSTEAD?

The assertion that *trans* fatty acids have major health consequences was previously demonstrated in rat heart and liver tissue by Guzman, et al. (30) and Dorfman, et al. (31). The Dorfman group fed Sprague-Dawley rats two different diets for eight weeks: 1. A control chow diet with 10% of energy as fat nearly half saturated, no *trans* fat, or 2. A diet with 10% of energy from fat where 4% of the fat was elaidate. The animals were monitored periodically by *in vivo* magnetic resonance spectroscopy to determine intra-myocellular lipid content and intra-hepatic lipid content. After the eight weeks, visceral fat and liver lipid levels increased in the second group compared to the control group. Overall glycogen synthesis was also increased in the second group. This led the researchers to believe that not only are responses to *trans* fatty acids
conducted by a separate signaling pathway than saturated fatty acids but that \textit{trans} fatty acids may be more detrimental to health than saturated fatty acids \cite{31}.

Since \textit{trans} and saturated fatty acids are industrial substitutes for each other, Judd, et al. \cite{32} conducted a study to determine which was more toxic. Fifty-eight healthy men and women were given four different regulated diets with 40\% energy from fat. The control diet contained 16.7\% \textit{cis} fat in the form of oleate (\textit{cis-\textDelta}^9\text{-octadecenoic acid}). There were two diets containing \textit{trans} fatty acids: a moderate level with 3.8\% energy as \textit{trans} fatty acid and a higher level with 6.6\% energy as \textit{trans} fatty acid. The last diet contained 16.2\% of energy as saturated fatty acids. When compared to the control diet subjects, subjects on all other diets displayed increased LDL-cholesterol, and for those on the saturated fatty acid diet, HDL-cholesterol increased compared to control, as well. HDL-cholesterol was unchanged with the moderate \textit{trans} fatty acid diet but decreased slightly with the higher level \textit{trans} fatty acid diet. The variance in HDL-cholesterol response to saturated fatty acids and \textit{trans} fatty acids suggest a major difference in the handling of the two types of fatty acids. \cite{32}

1.4 MACROPHAGE INFLAMMATORY RESPONSE TO \textit{TRANS} AND SATURATED FATTY ACID

Studies have attempted to elucidate the difference between saturated and \textit{trans} fatty acids handling by examining mechanisms and signaling pathways. A major response to ingestion of saturated and/or \textit{trans} fatty acids is rapid, acute inflammation. The cellular response to saturated fatty acids is better understood than that of \textit{trans} fatty acids. Palmitate (C16:0) exposure has been shown to elicit a stress response in many different cell types. This stress response can come in
the form of endoplasmic reticulum stress, ceramide production, and/or oxidative stress, which is a known precursor for apoptosis (33). Saturated fatty acid exposure causes mouse and human macrophages to release many pro-inflammatory cytokines, signaling an immune response (7,34). Inflammatory macrophages are known as M1 macrophages. All other types of macrophages are termed M2 macrophages. M1 macrophages have a hurried, intense reaction to bacterial infection and to endotoxic stressors like arterial lesions. Since they are rapid responders, these macrophages produce energy through the more immediate pathway of glycolysis as opposed to the less damaging, more efficient process of oxidative phosphorylation, similar to the transition undergone by tumor cells as described in the Warburg hypothesis (35). Glycolysis can be damaging since by-products include reactive oxygen species (ROS) (36-38). Elevated production of ROS is considered a hallmark of M1 macrophages (39). The acute M1 reaction also includes release of proinflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) which are known to be prominent in the plasma of patients with diabetes and cardiovascular disease (39-41). Interleukin-8 (IL-8) and interleukin-6 (IL-6) are two more crucial proinflammatory cytokines in the development of atherosclerosis (21,42). Another cytokine released is cyclooxygenase-2 (COX-2), an enzyme that catalyzes the conversion of arachidonic acid to inflammatory prostaglandins (43). Cytokine-feed-forward loop signaling eventually leads to a chronic inflammatory response to protect surrounding cells from the threat. It is this chronic response that actually elevates the rate at which atherosclerosis develops by mounting a feed-forward process recruiting more macrophages which release more cytokines. As the macrophages engulf the oxidized phospholipids, they become foam cells and die, adding to the plaque (44-46).
Palmitate and stearate metabolism intermediates and by-products have been implicated in the macrophage signaling pathway for production of cytokines COX-2, TNF-α, IL-1β, and IL-8. Other saturated fatty acids of carbon length C4-C14 have not been associated with apoptosis signaling (47,48). This cytokine signaling pathway is mediated, in major part, by Toll-like receptor 4 (TLR4) in human macrophages (49,50). TLR4 is a pattern recognition receptor of innate immunity and is activated by signals from saturated and trans fatty acids (51,52).

1.5 CELL DEATH BY LIPOTOXICITY: THE ROLE OF TLR4

In 2001, Listenberger, et al. (53) piloted a group of experiments using Chinese hamster ovary (CHO) cells treated with 100-500 μM palmitate (C16:0) or oleate (C18:1 cis) to induce cell death by lipotoxicity. Cell death was measured by Annexin V binding and caspase 3 activity fluorescent stains, as well as gel electrophoresis of nuclear material to determine sizes of DNA fragments. In the oleate-treated cells, none of the three measurements showed a significant amount of cell death after five hours. The palmitate-treated cells, however, showed a statistically significant increase in cell death even in the lowest concentration. A typical reaction of cells to exposure of palmitate is increased ceramide production. Ceramide is a fatty metabolite synthesized by the condensation of the amino acid serine and long-chain saturated fatty acids, particularly palmitate. This reaction is catalyzed by serine palmitoyl-transferase in the endoplasmic reticulum (ER) which is why treatment with palmitate is also known to cause ER stress (54). Ceramide is a signaling molecule for actions such as differentiation, cell growth, and programmed cell death. This ceramide effect is common in conditions such as obesity, cancer, cardiovascular disease, and chronic inflammation (55), but the actual mechanism has not been
clearly deduced (56). Armed with this knowledge, the Listenberger group hypothesized that ceramide overproduction could cause cell death and added ceramide production inhibitors to CHO cell media to determine if ceramide production was necessary for death. They found only a slight reduction in apoptosis due to the inhibitors and concluded that ceramide was not required for cell death. In contrast, their addition of a fluorescent probe for oxidant products showed that palmitate-treated cells had increased 3.5-fold in ROS, leading the researchers to conclude that cell death was due to ROS. This hypothesis was conformed when an antioxidant treatment greatly reduced cell death(53).

Recently, Schilling, et al., (57) conducted a set of experiments using primary human macrophages and transformed mouse RAW 264.7 cells in which they purposely caused cell stress and death by stimulating the cells with a mixture of 500 μM palmitate and lipopolysaccharide (LPS). LPS is a lipid-sugar endotoxin embedded in the outer membrane of gram negative bacteria which elicits strong immune responses in mammals by forming an activating complex with TLR4 (58,59). As expected, cell death was elevated in the treated cells compared to controls. As mentioned before, a typical reaction to exposure to palmitate-treatment is ceramide overproduction (54,55), but inhibitors revealed that ceramide overproduction was not responsible for apoptosis. After conducting similar experiments to the Listenberger group, the Schilling group discovered that cell death was also ROS-independent. Since the researchers could not classically define the death pathway involved, they focused on the TLR4 signaling pathway. They obtained wild type (WT) mouse macrophages and knockout (KO) macrophages of TLR4 (TLR4-null), TIR domain-containing adapter (TRIF) (TRIF-null), MyD88, which is a protein infection-fighting target of TLR4 (60), (MyD88-null), and a double KO of TRIF and MyD88. With the TLR4-null cells, there was no cell death following LPS and palmitate
treatment, while the WT (TLR4 expressing) cells died. TRIF-nulls had markedly decreased cell death, while MyD88-nulls had cell death levels statistically the same as WT. The double KO had almost no cell death. Consequently, they concluded that TLR4 signaling to TRIF is required for this cell death pathway. In addition, they found that the final downstream cause of death was depletion of lysosomal contents and membrane damage. Upon further examination of the lysosomal consequences, they determined that TRIF downstream signaling eventually led to lysosomal biogenesis transcription factor EB. Transcription factor EB is associated with excess autophagy, a mechanism utilizing lysosomal tools to break down superfluous cell constituents to conserve energy for more dire purposes (61,62). This study confirmed that there are differences between Chinese hamster and human/murine inflammatory responses to fatty acids. Also, they defined an important programmed cell death pathway for saturated fatty acid lipotoxicity and hypothesized a possible drug target in lysosomal transcription factor EB (57).

1.6 THE METABOLISM OF DETRIMENTAL FATTY ACIDS AND INTERMEDIATE ACCUMULATION

Even though saturated and trans fatty acids serve similar functions in the food industry (5), they are handled differently in the body. Not nearly as much information is known about trans fatty acid utilization (19,63) and much comes from rodent models. A distinguishing characteristic of trans fatty acid metabolism is that intermediates reach higher concentrations. β-Oxidation, the process by which fatty acids are broken down in the mitochondria, is slowed during trans fatty acid metabolism, leading to a back-up in multiple steps of the pathway in rats. Previous reports have made the point that no intermediates accumulate during saturated fatty acid β-oxidation, but
according to conclusions from a 1966 study by Willebrands, et al. (64), *trans* fatty acid treated rat tissues do accumulate fatty acid intermediates. Liang, et al.(65), many years later, found a similar conclusion in that elaidate causes “leaky” β-oxidation where intermediates build up. The term “leaky β-oxidation” was originally coined by Yu, et al. in 2004 (66). This group of researchers collected oleate-, elaidate-, or stearate-treated rat heart and liver mitochondria. The mitochondria successfully metabolized all three fatty acids, but in the elaidate-treated mitochondria, the oxidation rate was 50% lower than that of oleate and 33% lower than that of stearate. Also, the elaidate-originating intermediate 5-*trans*-tetradecenoyl-CoA was present in the matrix in 10x higher concentrations than parallel intermediates from the other two fatty acids, as ascertained by HPLC and structure profiling studies. Long-chain acyl-CoA dehydrogenase (LCAD) was kinetically analyzed, and the researchers determined that LCAD had a lower affinity for 5-*trans*-tetradecenoyl-CoA than 5-*cis*-tetradecenoyl-CoA. LCAD had a much higher affinity for the saturated intermediate than either of the two from monounsaturated fatty acids. The lack of affinity of LCAD for 5-*trans*-tetradecenoyl-CoA caused an accumulation of this intermediate in the mitochondrial matrix in rats(66).

Another issue in studying *trans* fatty acid β-oxidation is that the murine and human relevant enzymes, their expression levels, and their substrate affinities differ. In 2009, Chegary, et al. (67) performed experiments comparing murine WT fibroblasts, murine LCAD KO fibroblasts, and very long-chain acyl-CoA dehydrogenase (VLCAD) KO fibroblasts. Human control fibroblasts were compared to those from mice along with fibroblasts from VLCAD-deficient patients. Molecular and biochemical methods were used to deduce that in mice, LCAD and VLCAD have intersecting roles with common fatty acids, and LCAD compensates for a deficiency in VLCAD and vice versa. LCAD is more essential for β-oxidation in mice, and
LCAD-deficient mice exhibit symptoms similar, although markedly milder, to VLCAD-deficient humans (68). This was confirmed by the Chegary group through acylcarnitine analysis of both sets of unsaturated fatty acid-treated fibroblasts. In humans, the two enzyme functions do not overlap as researchers found LCAD mRNA expression in fibroblasts to be scarce at best, which agrees with previous research (69). In contrast to the highly functional LCAD and VLCAD in mice, only VLCAD is responsible for nearly all human β-oxidation of fatty acids over 14 carbons in length, accounting for the more detrimental phenotype in deficient patients (67).

### 1.7 A BRIEF DISCUSSION OF LIPID RAFTS

So far, it has been demonstrated that both saturated fatty acids and industrial *trans* fatty acids activate inflammatory pathways, negatively affect endothelial cell function, and increase total blood cholesterol levels (27,70). And in the case of *trans* fatty acids, intermediates of their β-oxidation accumulate with unknown consequences (67). The pro-atherogenic destruction caused by saturated fatty acids and industrial *trans* fatty acids signal acute activation of the M1 macrophage mentioned above (36-38). Many M1 acute responses are regulated by TLR4 (50-52). On the outer cell membrane the saturated acyl chains of sphingolipids and cholesterol align to form a nanoscale (<200 μm) (71) “lipid raft” that contains TLR4 (72). Precise physical properties of the lipid raft remain arguable since imaging techniques cannot accurately capture a subject that is not thermodynamically stable (73). Even so, researchers are intrigued at the lateral interactions that could be going on at the edges of lipid rafts, but examination methods have yet to be generated. At present, fluorescence microscopy is used to study synthetic membrane lipid rafts (74). Besides the outer plasma membrane, the membranes of the Golgi apparatus and
lysosomes have been reported to contain lipid rafts occasionally (73). All lipid rafts may move fluidly throughout the phospholipid bilayer and act as aid vessels for functional processes such as organization of signal transduction (75), ligand-receptor interactions (76), and fluidity of the membrane (77-80). It has been demonstrated in mouse and human LPS-activated macrophages that when cellular cholesterol influx rate increases, the size of lipid rafts increases to prepare for the coming elevation of TLR4 activity. Increase in size of the dense membrane-lipid rafts can have major effects on membrane fluidity. Cholesterol content and phospholipid structure are the major factors affecting the function of lipid rafts (81-84).

1.8 DISPERSION OF CHOLESTEROL

Macrophages require a method of removal for excess lipids and cholesterol unneeded by lipid rafts. Multiple ATP-binding cassette proteins transport cholesterol from the cell to apolipoproteins for dispersion. Synthesis of specific apolipoproteins A1 (ApoA1) and E (ApoE) in human HDL is a response to increasing expression of ATP-binding cassette A1 (ABCA1) (85). ABCA1 transports cholesterol from the macrophage through lipid raft signaling to acceptor Apo-A1 and ApoE, eventually forming the high-density lipoprotein (HDL) mentioned above (86). ABCG1 is another important cholesterol transport protein with a function very similar to ABCA1, although the exact function of ABCG1 is still unclear (87). To discern the functions of the two, an experiment was conducted in human macrophages with antisense oligonucleotides for ABCG1 mRNA that had no effect on ABCA1 function, and then proceeded to measure remaining cholesterol and lipid efflux. Cholesterol efflux was reduced by 32% and phospholipid efflux was reduced by 25%. (85,87). Upon deeper examination of patients with Tangier’s
disease, which is a condition associated with a deficiency of ABCA1 (88-90), it was determined that ABCG1 is more involved in intracellular organization and mobilization of cholesterol and lipids, as well as extracellular transport, while ABCA1 is primarily involved in extracellular HDL-targeted transport. Both proteins have been identified in atherosclerotic foam cells by RT-PCR (real-time polymerase chain reaction) (91). A separate study of ABCA1-null murine macrophages resulted in a hyper-sensitive reaction to LPS activation of TLR4, boosting downstream processes like inflammatory cytokine production, enabling chronic inflammation to develop. The loss of capability to remove lipids and cholesterol in the ABCA1-null macrophages caused excess cholesterol to collect in the lipid rafts and oxidized phospholipids to accumulate inside the cell. This experiment proved that ABCA1 has anti-atherogenic functions in human macrophages. (92). ABCA1 may also remove LPS from the TLR4, making its importance even more obvious (93). Nearly the same reaction occurs when another ATP-binding cassette protein is knocked down. A study was conducted by Yvan-Charvet, et al. (94) where four different genotypes of peritoneal macrophages were engineered and assessed for expression levels of known inflammatory genes. The genotypes assessed were WT, ABCA1-null, ABCG1-null, and the double null of both. As expected, the double null showed the largest increase in inflammatory gene expression. There was a similar median response between the ABCA1-null and the ABCG1-null, but the stress response was even more distinct in the latter, establishing the importance of ABCG1 in inflammatory regulation (94).

Another group examining ATP-binding cassettes took this line of investigation a step further and conducted expression studies of ABCA1 and G1 in murine and human macrophages that had been incubated for 34 hours in media containing 70 μM industrial trans fatty acid (largely elaidate), naturally occurring trans fatty acid (vaccenate), saturated fatty acid
(palmitate), or a control media. ABCA1 and G1 expression of the vaccenic-treated murine macrophages remained similar to controls. Both remaining treatments of cells showed only 77% of the control ABCA1 expression, and ABCG1 expression did not change. The elaidate result was the same in the human cells, although not nearly as evident (89% of control). The researchers next added a cholesterol load to both cell types. In murine macrophages, cholesterol-loading did not alter the previous result. In the human macrophages, ABCA1 expression in the elaidic acid-treated cells was 36% lower than controls and other treatments. The investigators surmised that the decrease in ABCA1 expression after elaidate incorporation could result from lowered cell membrane fluidity. The cellular free cholesterol to phospholipid molar ratio (FC:PL) is an easily measured determinant of the integrity of membrane fluidity. In the murine macrophages, there were no significant changes in FC:PL from treatment to control. In human cells, both FC and PL increased, resulting in an unchanged ratio but an altered cell membrane. Elaidate was incorporated into the phospholipids of the cell membrane in greater quantity than any other treatment fatty acid, rendering the membrane more rigid. This increase in rigidity could explain the deficit in ABCA1 mediated cholesterol efflux in murine macrophages treated with elaidate (20). As mentioned before, trans fatty acid assimilates into the cell membrane more easily than other fatty acids, resulting in unwanted effects, which is why its consumption comes with warning (10,17-19).

1.9 THE ROLE OF ZINC ACTIVITY AND METALLOTHIONEIN

The demonstration that elaidic acid decreases functionality of the cell membrane should be taken into consideration during discussions of membrane-bound protein signaling function. The LPS-
TLR4 complex is a dynamic and promiscuous lipid raft-bound receptor that reacts to many different stimuli, and manages many different forms of macrophage stress and immune responses (75). As M1 macrophages fight to control atherosclerotic damage turning to foam cells in the process, inflammatory signaling downstream of TLR4 is high throughout the bloodstream. Each proinflammatory cytokine signals a specific receptor(s) feeding forward the process (44-46). It is obvious how inflammation can become chronic and out of control. The metal ion, zinc\(^{2+}\) (Zn\(^{2+}\)), may flow freely in circulation and suppress cytokine secretion, particularly in those reactions dependent on tyrosine phosphorylation (95,96), a process by which ATP adds a phosphate group to the amino acid tyrosine (97), and Zn\(^{2+}\) is required for the active LPS/TLR4 complex to form (98). TNF-\(\alpha\), IL-1, and IL-6 are cytokines produced by macrophages that may be regulated by Zn\(^{2+}\) (96,99). Conversely, in a study by Wellinghausen, et al. (100), human peripheral blood mononuclear cells were treated with LPS followed by labile zinc (active zinc), which is zinc that is attached lightly to carriers and can be dispersed easily (95). Using fluorescence and infrared spectroscopy measurements, the researchers determined that zinc decreased the fluidity of the hydrocarbon chains of LPS. The rigidity of LPS chains induced rather than suppressed cytokine production after binding TLR4 (100). Consequently, it is now thought that labile zinc may be pro- or anti-inflammatory depending on its circulating concentration (95,96).

The most common regulator of [Zn\(^{2+}\)] is metallothionein (MT), a small protein high in cysteine. The oxidation of this cysteine allows mobility, while the thiol group chelates up to seven metal ions (101,102). There are four families of MTs, and all are synthesized in the liver and kidneys at a rate depending on changes in metal ion concentration and availability of cysteine (103). An important secondary function of metallothionein is to capture oxidant radicals that may be damaging the cell. A cysteine from the metallothionein is oxidized during
sequestration of the oxidant radical, and the offending oxidant is transported away from the stressed area (104).

A set of experiments conducted by Kelly, et al. (105) and Lazo, et al. (106), utilized mice with null alleles of MT I and MT II. Zinc and cadmium ion concentrations and observational properties of various organs were assessed. The mice with null alleles had more difficulty managing both zinc toxicity and deficiency, concluding that MT I and II are required for maintaining an optimum Zn$^{2+}$ concentration in the bloodstream (105,106).

1.10 SPECIFIC AIMS

Our focus after reviewing the literature was first defining whether the stall in catabolism of trans fatty acids in rat liver (107) and heart (64) also occurred in human macrophages. Accumulations of fatty acids and their metabolites can result in lipotoxicity with unknown consequences for cell signaling, viability, and proliferation.

Our major aim was to determine the effect of cis and trans unsaturated fats versus saturated fatty acids on macrophage lipid metabolism. After we established that trans fatty acids block fatty acid β-oxidation, the laboratory performed an exploratory gene expression array to illuminate the effects of the cis fatty acid oleate versus its trans isomer, elaidate. A striking finding in the expression array was the alterations in genes with zinc homeostatic products. This led to the question of whether these changes would be reflected in a functional change in labile zinc homeostasis. Since both lipid signaling (108) and zinc metabolism (95) are associated with inflammation, the final studies focused on correlating the zinc and lipid effects on NF-κB activation (108).
2.0 A REVIEW: WHEN MACROPHAGES CANNOT DEGRADE CELL MEMBRANE LIPIDS, ATHEROMAS FORM: THE ROLE OF DIETARY FATS

Submitted for publication.

2.1 ABSTRACT

Membranes of somatic cells, such as red blood cells, incorporate fatty acids proportionally to dietary intake. When excessive saturated and trans-unsaturated fats are incorporated, membrane fluidity is restored by fatty acid desaturases acting on the saturated fats. In health, when cells die macrophages recycle completely the cell components including membranes. Cell membrane debris is exported as cholesterol or cholesterol esters for disposal by the liver or as triglycerides or phospholipids for lipid storage or re-use in membranes. When macrophages cannot fully recycle cell membranes, semi-liquid masses of partially oxidized fatty acids and cholesterol, foamy macrophages, and proliferating stromal cells accumulate in arterial walls, resulting in atherosclerosis. From ancient times, dietary excess has been known to cause atherosclerosis. The dramatic increase of atherosclerotic disease in developed countries since 1940 reflects in large part superabundant nutrition and altered dietary composition. Dietary changes include increased saturated and artificial trans unsaturated fat intake. The biochemical basis of this epidemic of
atherosclerosis reflects limited reverse transport capacity and a partial metabolic block in β-oxidation caused by certain trans fatty acid intermediates.

2.2 INTRODUCTION

The ability of the human body to digest and transform a wide variety of foods is remarkable. Most people can maintain their health deriving their calories from a broad spectrum of sources along with small quantities of some specific required nutrients, including essential amino acids, vitamins, minerals, and two essential fatty acids, α-linolenic and linoleic (109). The essential fatty acids have double bonds three (ω-3) or six (ω-6) carbons from the end distal to the carboxyl and cannot be synthesized by humans (110). Modern human diets contain large amounts of fatty acids (1) may challenge cell membrane maintenance and recycling.

There is remarkably little selectivity as to which dietary fatty acids are distributed to phospholipids in peripheral cells. For example, a diet rich in the artificial C18:1 trans fatty acid elaidic acid, ((E)-octadec-Δ9-enoic acid), results, within days, in newly synthesized red blood cell (RBC) membranes containing elaidic acid in proportion to the dietary intake (19). Indeed, it is suspected that elaidic acid may assimilate into new cell membranes more readily than other unusual dietary fatty acids, and so its consumption poses a particular problem (9,17-19).

When diets rich in saturated fat or containing trans fat are ingested, the ratio of long-chain unsaturated and polyunsaturated fatty acids typically decreases relative to C18:0 (18,111,112). Populations with increased saturated fat diets or consuming significant amounts of trans fat show, in numerous studies, increased risk for atherosclerotic disease. It isn't solely what one eats but also how much. Sedentary lifestyle or smoking may add to risk; these secondary risk
factors, as well as inborn errors in genes with products that contribute to lipid accumulation, are outside of our focus. We are concentrating on the relationship of major dietary lipids, with emphasis on trans fatty acids to atherosclerosis. Diets rich in saturated fat or artificial trans fats reduce cell membrane fluidity and activate compensatory changes to increase the unsaturated fat proportion in the cell membranes (18). We will emphasize evidence that membrane lipid constituent alterations are important factors promoting the development of atherosclerosis.

2.3 FATTY ACIDS, SATURATED, CIS AND TRANS UNSATURATED, NATURAL AND ARTIFICIAL

The most prevalent saturated fatty acids humans consume are palmitic acid (C16:0), stearic acid (C18:0), with smaller quantities of myristic acid (C14:0), and lauric acid (C12:0), in that order. About 90% of saturated fatty acids consumed are palmitic acid and stearic acid, with typically two thirds of this being palmitic (7). Diets with large quantities of animal fat have about 50% saturated fat and 40% natural singly cis-unsaturated fat and are unhealthy, increasing the risk of coronary artery atherosclerosis (113).

Artificial trans fatty acids, specifically elaidic acid, accounted for 4-10% of the average individual’s daily fatty acid intake in the USA around the turn of the 21st century (1). Why this component has been a major problem will be examined from a biochemical point of view. It is important to understand that while elaidic acid is the abundant trans fatty acid in partially hydrogenated oils, there are ruminant-derived trans fatty acids that comprise less than 1% of fat intake and are not toxic, at least in small quantities. Natural trans bonds also occur between the 2,3 carbons during β-oxidation (Fig 2.1B) and occur also at the 2,3 position relative to a
Figure 2.1 Fatty Acids and Degradation by Beta-Oxidation

A. Unsaturated, cis and trans, and saturated fatty acids. Trans bonds occur in some natural fatty acid derivatives but in central regions of long chain fatty acids they are not natural and usually are the result of partial hydrogenation. Animal fats contain typically 50% saturated fatty acids, with ~12% C18:0 (stearate, illustrated) and about twice as much C16:0 (palmitic acid) as C18:0. Unsaturated cis fatty acids have lower melting points than their saturated counterparts and increase membrane flexibility; oleic acid (top) is a liquid. Unsaturated fatty acids with trans bonds are stiff and have higher melting points, e.g. elaidic acid (middle) is a solid at room temperature.

B. Degradation of unsaturated fatty acids requires enoyl Co-A isomerase (EC1) action. The degradation of unsaturated fatty acids adds special steps which add to the complexity of degrading the fatty acids. Both natural cis-unsaturated and trans-unsaturated fatty acids can, when present in large quantities, reduce the efficiency of β-oxidation, but the effect of trans fat is much larger than that of natural unsaturated fats15. Inefficient processing of trans-unsaturated fatty acids by ECIs lead to accumulation of trans C12- and trans C-14 intermediates, which are poor substrates for fatty acid β-oxidation and may inhibit β-oxidation by competing for rate limiting enzymes [Yu, 2004].

Unsaturated fatty acids can be converted to saturated fatty acids by exposure to hydrogen at high temperatures while excluding oxygen. Direct hydrogenation was developed by the French chemist Paul Sabatier in the late 1800s (2,3). Sabatier was awarded the Nobel Prize in chemistry in 1912 (114) for this invention, including the use of catalysts for efficiency and its application...
for converting food oils into solids. The intended purpose of hydrogenation is to adjust the texture of fats for use in foods, particularly baked goods. Oils may be converted into solids that are excellent substitutes for animal fat (from the standpoint of texture), since trans fatty acid-containing modified vegetable fats have similar properties to animal fats containing mainly singly unsaturated fatty acids and saturated fatty acids with smaller amounts of polyunsaturates (5). Partially hydrogenated oils also have a very long shelf life. Elaidic acid is one of many trans isomers that occur during partial hydrogenation, where double bonds re-form in the lowest energy state, which is the trans isomer, rather than the uniform cis isomer made by enzymatic desaturation by the stearoyl CoA desaturase-1, an enzyme that regulates membrane fluidity. Double bonds in the carbon chain may migrate to make isomers with double bonds at various positions between C4 and C14 (3,4). Hydrogenated soy oil, depending on manufacture, can contain trans fatty acid loads approaching 50% or, with complete hydrogenation, no trans fatty acid at all. Unfortunately, elaidic acid is, by far, the most abundant species in partially hydrogenated oils, presumably due to its high stability.

Not all trans fatty acids are manufactured nor are they dangerous to health: natural trans fatty acids include conjugated linoleic acid (C18:2, several isomers) and vaccenic acid (C18:1 ω-7). Conjugated linoleic acid and vaccenic acid are produced in ruminants and are consumed in small quantities in meat and dairy products. Trans bonds occur as conjugated double bonds, which are interconverted by isomerases. But if a single trans bond occurs an odd number of carbons from the acid group, such as in elaidic acid (Fig 2.1B, right side), after four rounds of β-oxidation, the odd numbered trans unsaturated bond inhibits subsequent β-oxidation because the 2,3 acyl-CoA delta isomerase does not efficiently process the trans double bond (115).
Subsequently, references to *trans* fatty acids refer exclusively to the products of partial hydrogenation, mainly C18:1 ω9 *trans* (elaidic acid).

### 2.4 DEVELOPMENT OF ATHEROSCLEROSIS AND EFFECTS OF DIETARY FATS

There is evidence that *both trans* fatty acids as well as high fat diet including saturated fatty acids have damaging effects on the health of humans, predominantly in regards to obesity, diabetes, and heart disease (8). According to Kummerow *et al*, diets comprised of high amounts of long-chain saturated fatty acids or small amounts of *trans* fatty acids can be damaging to arterial walls, possibly causing atherosclerotic lesions to develop (9).

The inner arterial lining or vascular intima normally contains no fat stores. In an early stage of atherosclerosis called lipid streaking, fat-laden foam cells derived from macrophages (12) accumulate and are visible on the inner arterial surface (116). From a systematic point of view, atherosclerosis proceeds with proliferation of fibroblast-like and smooth muscle cells, death of phagocytes, and ultimately, the intima amasses semi-liquid lipid debris known as plaque with surrounding fibrous tissue and reactive foamy macrophages (Figure 2.2) (11). None of these alterations are found at any age in healthy vascular intima (12).
Normally the intima is a thin layer of connective tissue between the media and the endothelial lining. In atherosclerosis, a partially liquid oxidized membrane lipids and cholesterol from the cell membranes of dead cells forms as an acellular mass. When these lipids are hydrolysed and analyzed on GC-MS, the analysis is very close to that of cellular lipids. The plaque is surrounded by foamy macrophages (not visible here) and proliferating fibromuscular cells. The plaque may grow to the point that it compromises the lumen, or rupture releasing material that would cause a shower of debris downstream. The coronary arteries are common sites for plaque formation, where they cause angina and myocardial infarctions.

The lipid of arterial plaques consists of cholesterol, oxidized cholesterol, phospholipids, and oxidized phospholipids in proportions similar to those of the dead cell membranes from which this material derives. Fat-engorged foamy macrophages are derived from blood macrophages (13), while proliferating fibroblasts and smooth muscle cells derive ultimately from mesenchymal stem cells present in the intima (12,15,16,117).

From the cellular standpoint, the effect of dietary fatty acids on atheroma development is not adequately studied, but there are important related findings, particularly regarding trans fatty acids. These include that elaidic acid, the predominant artificial fatty acid, impairs the ability of macrophages to export cholesterol (20). The basis for this effect is probably complex and may reflect effects of elaidate on β-oxidation of fatty acids (115), acetyl CoA from β-oxidation being the material from which cholesterol for export is synthesized. Trans fat may also impair the
ability of cells to regulate their polyunsaturated fatty acids in cell membranes (10). The evidence for this is limited, but this is an attractive hypothesis in that polyunsaturates maintain membrane fluidity, and cell survival might be compromised by inability to sufficiently regulate membrane function. In subsequent sections, the biochemical effects of dietary fats will be considered in specific contexts.

2.5 EPIDEMIOLOGY OF ATHEROSCLEROSIS

Prior to the period of World War II deaths from coronary artery disease were unusual in the developed world, as is seen in longitudinal studies of autopsy deaths from records at St Barts, London (118), where the fraction of deaths from ischemic heart disease in adults over age 40 has been evaluated for about 150 years. The rate changed from about 1% before 1920 to about 10% after 1960. The major deflection began in 1929-1935 prior to antibiotic use or significant changes in longevity related to that factor. Before 1900 the median percentage of autopsies over age 40 was about 60%, while by 1980 it approached 90%, which does create a bias in the data. Nonetheless, the ~10-fold change in rate of death from coronary atherosclerosis is dramatic and represents a change obviously much too large to attribute to longevity, with the midpoint of the inflection being just about 1940.
2.6 NEGATIVE EFFECTS OF ANIMAL FATS

It is difficult to isolate saturated fat as a risk factor for atherosclerosis from confounding effects including obesity, excessive total caloric intake, and smoking (which also increased greatly between 1930 and 1950). However, it remains that large amounts of animal fat increase the risk of developing atherosclerosis (119). The venerable Framingham study correlated development of atherosclerotic heart disease with total dietary fat and with monounsaturated and saturated fat (found together in animal fat) in middle aged subjects entered in the study at 40-50 years old and followed subsequently for sixteen years (113).

2.7 POSITIVE EFFECTS OF UNSATURATED FATTY ACIDS

In spite of the Framingham findings, there is a large literature indicating that there are important health benefits from selected food oils, such as olive oil, that contain large proportions of monounsaturated ω-9 fatty acids. Interpretation of this literature is complicated by the presence of non-lipid compounds in these oils that may be beneficial (120). In addition, the ω-3 group of polyunsaturated fatty acids (121) has shown cardiovascular benefits. With the ω-6 polyunsaturated fatty acids, the situation is more controversial. Although both ω-3 and ω-6 polyunsaturated fatty acids are essential nutrients, a high ratio of ω-6:ω-3 appeared to be pro-atherogenic (122) in some studies. Among the polyunsaturated data is an interesting study showing that northern indigenous people eating fish high in ω-3 fatty acids with high overall dietary fat intake did not have high rates of atherosclerotic disease (123). For brevity, ω-3 and ω-6 fatty acids will not be reviewed in detail. However, it is worth noting that in recent work,
adding relatively small amounts of olive oil and nuts to the diet substantively reduced progression of atherosclerotic plaque within three years, arguing that major stabilization might result from relatively moderate, and late, dietary intervention (124).

2.8 FACTORS THAT WORK TO PROMOTE PLAQUE PROGRESSION INCLUDE

OXIDATION OF LIPIDS IN PLAQUE

In most cases, once significant atherosclerotic lesions have formed, dietary interventions to cause regression have been disappointing. However, animal and human studies have shown that atherosclerosis can under some conditions be at least partially reduced (125). Plasma carriers that remove lipid debris have a limited cellular capacity for reverse transport of plaque components and are likely a limiting factor (126). Nonetheless, it is clear that losing weight, exercising appropriately, stopping smoking, limiting red meat intake, and eliminating trans fats (127) improve cardiovascular outcomes.

However, importantly, plaque develops over time when certain LDL components are not efficiently degraded by macrophages. These poorly metabolized components include trans fatty acids, considered in the next section, but also oxidized phospholipids, which have complex negative physiological effects.

Specifically, once atherosclerotic cell debris gets ahead of the capacity of macrophages to remove it, easily oxidized phospholipids, particularly those containing arachidonic acid (with four double bonds, the precursor of prostaglandins), form a bewildering variety of ketone and hydroxide derivatives, which do not have good pathways for uptake and metabolism. While the
specific biochemistry remains in most cases to be worked out, it is clear that these are major contributors to lipid toxicity and hence progression of atherosclerosis (128).

2.9 A BRIEF DISCUSSION OF STATIN EFFECTS

A large proportion of adults with atherosclerosis are treated with statins. There is no doubt that, in patients who have suffered coronary events and in selected patients with very high LDL cholesterol, statins improve outcomes. Statins reduced recurrence of coronary artery blockage from 13.2 to 10.2% of all patients, at 5 years, relative to controls not statin treated, in patients with mean LDL cholesterol averaging 209 mg/dl (129). But the tendency to regard statins as a panacea for atherosclerosis and to push their use in almost all adults, in place of proven dietary and lifestyle interventions, raises complex issues.

Statins are hydroxymethylglutaryl coenzyme-A reductase (HMGCR) inhibitors. They reduce cholesterol synthesis in the liver, and hence reduce outbound transport of cholesterol mainly via LDL, eventually providing cholesterol for cell membrane synthesis. Since reduced cholesterol synthesis leads to up-regulation of SREBP2 and the LDL-receptor (130), statins also increase LDL-cholesterol uptake from the circulation. It is important to note that LDL does not dump its cholesterol into plaque, as is sometimes assumed. Plaque originates from cell membranes. Further, statins, with the possible exception of the most hydrophilic statins, lower HMGCR activity in all cells, including macrophages, which synthesize cholesterol from membrane lipids for transport, largely via HDL, for elimination by the liver. Under some circumstances, this might further impair ability of macrophages to process lipids. For example, inhibition of cholesterol synthesis with simvastatin leads to up-regulation of SREBP2 and miR-
33, which decreases ABCA1 expression and cholesterol efflux (131). In young healthy volunteers, not on statins, given large doses of elaidic acid as artificial margarine, HDL decreased and LDL cholesterol increased within three weeks (19), suggesting that the trans fat itself impairs cholesterol synthesis for transport back to the liver; it is not known whether statins might have an additive effect.

Outcomes-based evidence for lifelong and increasingly high-dose statin use relative to dietary intervention is not available. A large scale review (132) suggests that statins for prevention should be used only where there is good evidence that they benefit patients; we suggest that relatively modest dietary intervention might be more productive in patients where clear indications for statins are not present (124). But in any case, it does no harm to advise adding olive oil and nuts to the diet, with or without statins.

2.10 EXPERIMENTAL EVIDENCE ON TRANS FATS AND LIPID ACCUMULATION

When significant quantities of trans fatty acids are included in the plaque burden, the ability of macrophages to export cholesterol is limited (20,133). This blockage is important because typically 30% of plaque is cholesterol. In addition, trans fatty acids directly inhibit β-oxidation in human macrophages (115), further compromising the ability of macrophages to potentially clear existing plaque. Judd, et al. (32) conducted a study to compare trans fatty acids versus saturated fat supplemented diets in fifty-eight healthy men and women. The control diet was supplemented with cis monounsaturated oleic acid (cis-Δ9-octadecenoic acid) as 16.7% of energy. Two diets contained trans fatty acids with 3.8% or 6.6% of energy as trans fatty acid elaidate, with the rest of the supplement as oleate. A fourth diet contained replaced the 6.2% of
the oleate supplement with saturated fatty acids. Compared to the control diet, all other diets increased LDL-cholesterol. On the saturated fatty acid diet, HDL-cholesterol increased compared to control. HDL-cholesterol was unchanged in the moderate trans fatty acid diet, but decreased in the high level trans fatty acid diet. The variance in HDL-cholesterol response to saturated fatty acids and trans fatty acids suggest a major difference in the handling of the two types of fatty acids (32). Later prospective human studies (19) strongly supported the findings.

Dietary variability is a major reason that outcomes of long-term studies of progression of atherosclerotic disease in humans are variable. Given this, animal models are important since they can be better controlled. However, animal lipid handling often differs significantly from that of humans (134). Nonetheless, well controlled animal studies are highly suggestive of mechanisms that are likely to be important in humans. That trans fatty acids have consequences on lipid accumulation in rats has been studied in heart and liver (30,31). Findings included that in rats fed 8 week diets with 10% of energy as fat, half saturated, with or without 4% trans fat as elaidic acid, visceral and liver fat increased in the trans fat group. Overall glycogen synthesis also increased with trans fat. Conclusions included that trans fatty acids affect signaling pathways differently than saturated fatty acids, and that trans fatty acids are more detrimental than saturated fatty acids.
2.11 INFLAMMATORY RESPONSE TO SATURATED AND TRANS FATTY ACIDS IN MACROPHAGES AND OTHER CELLS

A major response to ingestion of saturated or trans fatty acids is rapid activation of acute inflammatory pathways. Mouse or human macrophages incubated with saturated fatty acids release proinflammatory cytokines, which provoke an immune response (7,34). Inflammatory macrophages are also called M1; other types of macrophages are M2 or subtypes of M2(135). M1 macrophages react rapidly and intensely to bacterial infection or endotoxic stressors. They produce energy through glycolysis and thus are associated with metabolic acid, rather than the more energy sparing oxidative phosphorylation, paralleling the situation in tumor cells (35). Glycolysis by-products can include reactive oxygen species (ROS) (36-38); thus, elevated production of ROS is a hallmark of M1 macrophages (39). The acute reaction of M1 cells to fatty acids includes proinflammatory cytokines, interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α), which are prominent in the plasma of patients with diabetes and cardiovascular disease (39-41). Cyclooxygenase-2 (COX-2), an enzyme that catalyzes the conversion of arachidonic acid to inflammatory prostaglandins, is also produced (43). Cytokine signaling promotes a chronic inflammatory response, ideally healing the damaged tissue. This response coupled with continued presence of debris actually elevates the rate at which atherosclerosis develops. It recruits more macrophages, which produce more cytokines, and the cells become overloaded with lipids and die, adding to the plaque (44-46).

Palmitic acid (C16:0) elicits a stress response in many cell types including macrophages. Consequently, palmitic acid, the most abundant dietary fatty acid, mouse or human macrophages is often used to study the response to saturated fatty acids. When lipid content exceeds the capacity to process or store fatty acids, a lipotoxic response occurs, and includes changes in gene
expression, destruction of organelle membranes, and apoptosis (33). Metabolites of palmitic acid or stearic acid (C18:0) are implicated in macrophage production of COX-2 and the cytokines TNF-α, IL-1β, and IL-8. Other less abundant saturated fatty acids of carbon length C4-C14 are not generally associated with this response or apoptosis (47,48). This cytokine pathway is mediated, in part, by Toll-like receptor 4 (TLR4) mainly expressed in human macrophages and dendritic cells (49,50). TLR4 is an innate-immunity pattern-recognition receptor; it is activated by saturated fatty acids and trans fatty acids (51,52) as well as by foreign molecules including bacterial lipopolysaccharide, and it signals via pathways including MAP kinases and NF-kB that are also activated by TNF-α.

Palmitic acid, 100-500 µM, induces lipotoxicity and apoptosis within five hours in Chinese hamster ovary (CHO) cells (53), while in similarly treated oleic acid controls (C18:1 cis), no significant cell death occurred. Palmitic acid treatment also drives ceramide production. Ceramide is synthesized by the condensation of serine and long-chain saturated fatty acids, particularly palmitic acid in the endoplasmic reticulum (ER); thus, palmitic acid is classified as an ER stressor (54). Ceramide is a secondary signal modulating differentiation, cell growth, and apoptosis and is commonly active in obesity, cancer, cardiovascular disease, and chronic inflammation (55). With palmitic acid incubation, added ceramide synthesis inhibitors caused only minor reductions in apoptosis, while reactive oxygen species increased 3.5 fold. In contrast, antioxidant treatment greatly reduced cell death, suggesting a major role for ROS and not ceramide in palmitic acid induced apoptosis (53).

Apoptosis has also been studied in primary human macrophages or transformed murine RAW 264.7 cells stimulated with 500 µM palmitic acid and lipopolysaccharide (LPS) (57). Cell death was elevated in the treated macrophages compared to controls, and the combination was
synergistic, resulting in more death than that found with palmitate or LPS alone. LPS forms an activating complex with TLR4 (58,59). In this study, apoptosis was ROS-independent and not related to ceramide production.

To further specify the role of the TLR4 pathway in lipotoxicity, wild type mouse macrophages and macrophages without TLR4 (TLR4-Null) were studied, in combination with mice lacking the TIR domain-containing adapter (TRIF-Null) or MyD88 (MyD88-Null), key intracellular adaptor proteins that complex with activated TLR4 (60) to mediate cell signaling. The TLR4-Null showed that cell death after treatment with LPS and palmitic acid required TLR4. TRIF null showed markedly decreased cell death, while MyD88-NULLs showed cell death rates similar to WT. Double TRIF/MyD88-Nulls had minimal cell death. Thus, in murine macrophages TLR4 signaling via TRIF is a key pathway mediating lipotoxicity-related apoptosis. TRIF downstream signaling caused transcription of lysosomal biogenesis transcription factor EB (TFEB) which increases autophagy, a mechanism using lysosomes to degrade cellular components; this may promote destruction of pathogens, or may support autoimmune destruction (61,62).
Even though saturated fatty acids and trans fatty acids are used interchangeably in the food industry (5), they are handled differently in the body. A distinguishing characteristic of trans fatty acid catabolism is that intermediates accumulate in human macrophages (115) and in rat hearts (64). During β-oxidation in human macrophages, trans fatty acid degradation is slowed at the 3,4 to 2,3-double bond isomerase step (Fig 3.1C, right side). In contrast, no intermediates accumulate during saturated fatty acid β-oxidation (65). The term “leaky β-oxidation” for elaidic acid oxidation in rat liver was coined by Yu, et al. in 2004 (66) for defective oxidation of trans fatty acids. Rat heart or liver mitochondria metabolized oleic, elaidic, or stearic acids, but elaidic acid oxidation rate was significantly slower than that of oleic acid or stearic acid. The intermediate 5--tetradecenoyl-CoA accumulated at ten times higher concentrations with the trans fatty acid, elaidic acid, as substrate. Long-chain acyl-CoA dehydrogenase (LCAD) has a lower affinity for 5-trans-tetradecenoyl-CoA than for 5-cis-tetradecenoyl-CoA, which in turn was much lower than affinity of LCAD for tetradecanoyl-CoA than for either cis or trans monounsaturated intermediates. And 5-trans-tetradecenoyl-CoA accumulates in the mitochondrial matrix (66).
2.13 DIFFERENCES IN MOUSE AND HUMAN FATTY ACID METABOLISM

There are major differences in human and mouse lipid metabolism, so that studies in mice always should be confirmed with human cells to avoid pitfalls due to species differences. In a relevant example, murine and human fatty acid β-oxidation enzymes differ in their substrate specificities. Murine wild type fibroblasts, long-chain acyl-CoA dehydrogenase (LCAD) null fibroblasts, and very long-chain acyl-CoA dehydrogenase (VLCAD) null fibroblasts have been compared to human normal fibroblasts or fibroblasts from VLCAD-deficient patients (67). In mice, LCAD and VLCAD both are highly expressed and have overlapping functions in straight chain β-oxidation. LCAD is much less expressed in humans and does not contribute significantly to straight chain β-oxidation (68). Human VLCAD is responsible for nearly all β-oxidation of straight chain fatty acids over 14 carbons, and VLCAD deficient patients have a severe phenotype (67).

2.14 CELLULAR RESPONSE TO LIPIDS IN MURINE AND HUMAN CELLS: LIPID RAFTS AND LIPID-TRANSPORTING RECEPTORS

On the outer cell membrane, the saturated acyl chains of sphingolipids and cholesterol align to form nanoscale (<200 nm) lipid rafts that contain TLR4 (72). Precise physical properties of the lipid raft are arguable; imaging techniques cannot accurately capture the subject in situ, because it is not thermodynamically stable (73). Lipid rafts may move fluidly throughout the phospholipid bilayer and act as aid vessels for functional processes such as organization of signal transduction (75), ligand-receptor interactions (76), and of course to modify the fluidity of the
membrane for special functions (77,78,80,117). In mice and human LPS-activated macrophages, as cholesterol influx increases, lipid raft size also increases, which enables the subsequent elevation of TLR4 activity (81-84). Similarly, caveolae also have roles in macrophage lipid metabolism, especially cholesterol (136). Cholesterol content and phospholipid structure are the major factors affecting the function of lipid rafts.

Macrophages require a method for removal of excess lipids including cholesterol, in part to modulate lipid raft activity. Multiple ATP-binding cassette (ABC) proteins transport cholesterol from the cell to apolipoproteins for dispersion. Synthesis of specific apolipoproteins A1 (ApoA1) and E (ApoE) in human macrophages is a response to increasing expression of ATP-binding cassette A1 (ABCA1) (85). ABCA1 transports cholesterol from the cell through lipid raft signaling to acceptor Apo-A1 and ApoE, eventually forming the high-density lipoprotein (HDL) discussed above (86).

ABCG1 is another cholesterol transport protein with a function similar to ABCA1, but its exact function is unclear (87). To discriminate the functions of these lipoproteins, antisense oligonucleotides for ABCG1 mRNA were used. Knocking down ABCG1 had no effect on ABCA1 function. However, cholesterol and phospholipid efflux were reduced by ~30% (85,87). In patients with Tangier’s disease, with a deficiency of ABCA1 (88-90), ABCG1 was shown to mediate intracellular organization and mobilization of cholesterol and lipids, as well as extracellular transport. ABCA1 primarily mediates extracellular HDL-targeted transport. Both proteins occur in atherosclerotic foam cells (91).

Murine macrophages null for ABCA1 have hypersensitive reaction to LPS and activation of TLR4, with enhanced inflammatory cytokine production (92). The loss of capacity to remove lipids and cholesterol in the ABCA1-null caused cholesterol deposition in the lipid rafts and
oxidized phospholipid accumulation inside the cell. This experiment suggests that ABCA1 may also be important in limiting atherogenesis in humans. In another study in which four different lipid-handling ABC transporter genotypes (WT, ABCA1-null, ABCG1-null, and double-null) were generated from peritoneal macrophages, expression levels of inflammatory genes were compared (94). Inflammatory gene expression was most increased in the double null cells, with a similar but lower response in ABCA1 or ABCG1 null cells. However, in the single null cells, the identity of immune-enhanced genes differed, suggesting that both transporters have specific roles in preventing inflammation, most likely associated with their substrates.

Expression of ABCA1 and ABCG1 has also been studied in murine and human macrophages after incubation for 34 hours in media with 70 µM of elaidic acid, the naturally-occurring \(\omega-7\) \textit{trans} fatty acid vaccenic acid, the C16 saturated fatty acid palmitic acid, or control medium (20). ABCA1 and ABCG1 expression in vaccenic-treated murine macrophages were similar to controls. Elaidic acid or palmitic acid treatments reduced ABCA1 expression to 77% of the control, while ABCG1 expression did not change. The elaidic acid result was similar in human cells, but reduction from control was less (89% of control). In murine macrophages, cholesterol-loading did not change the result. In the human macrophages, ABCA1 expression in elaidic acid-treated cells was 36% lower than controls or other treatments when cholesterol loading was done. It was hypothesized that elaidic acid incorporation affected cell membrane fluidity and indirectly affected receptor expression. The cellular free cholesterol to phospholipid ratio (FC:PL) did not change with fatty acid treatment. In human cells, both FC and PL increased, resulting in an unchanged ratio but altered membrane properties. Elaidic acid was incorporated into the phospholipids of the cell membrane in greater quantity than any other treatment fatty acid, rendering the membrane more rigid. This increase in rigidity might explain
the deficit in ABCA1 mediated cholesterol efflux in murine macrophages treated with elaidic acid.

2.15 REACTIVE OXYGEN SPECIES (ROS), METALS, AND EPIGENETIC MODIFICATION BY FATTY ACID SIGNALING PATHWAYS

In addition to lipid modifying and transport enzymes, lipid receptors including TLR4 and downstream inflammatory-type molecules, another key system activated during atherogenesis is ROS production. Two key mechanisms involved in regulation of the ROS system in atherogenesis are metal signals, particularly zinc and related changes in gene expression by epigenetic mechanisms (137).

It is noteworthy that zinc is a key modulator of the ROS and inflammatory-type signaling system (138). Zinc signaling has many parallels to calcium signaling (139) but is a more recent discovery; zinc particularly is important in modulating inflammatory signals. The active zinc ion, Zn$^{+2}$, occurs in circulation and suppresses cytokine secretion, particularly tyrosine phosphorylation (95-97). Tyrosine phosphorylation is required for LPS-TLR4 signaling (98). Production of TNF-α, IL-1, and IL-6 by macrophages is regulated, at least in part, by Zn$^{+2}$ (96,99). Zinc has both extracellular and intracellular effects, with extracellular including reduced LPS binding to TLR4, thus inhibiting cytokine production (100). However, labile zinc donors may be pro- or anti-inflammatory depending on the context (95,96).

The major regulator of labile Zn is metallothionein (MT), a small cysteine-rich protein. Oxidation of these cysteines mobilizes Zn$^{+2}$ or other bound ions; MT chelates up to seven metal ions including Zn, Cu, Cd, or Se (101,102). Four isoforms of MT are synthesized in the liver and
kidney at rates dependent on metal ion concentrations and cysteine availability (103). MT also
captures oxidant radicals. A cysteine from the MT is oxidized, and the offending oxidant
neutralized (104). Mice with MT I or II absent (105,106), when stressed with zinc, had
developed zinc toxicity. How zinc and metallothioneins contribute to atherosclerosis progression
is not well studied, but given the known involvement of oxidation in production of catabolism-
resistant oxidized lipids (128), this pathway deserves further study.

2.16 SUMMARY

It might be possible to eliminate almost all atherosclerotic disease by promoting a Mediterranean
diet, limiting animal fat, outlawing partially hydrogenated oils and tobacco, promoting exercise,
and preventing obesity. This scenario is unlikely to happen, although some parts may be
achieved. In particular, outlawing partially hydrogenated oils is proposed, and if physicians
promote use of olive oil and ω-3 sources such as nuts with the tenacity that has been used in
prescribing statins, a major positive effect is likely (124).

The pro-atherogenic effect of a diet with excess animal fat and, even more so, with
artificial trans fatty acids involves activation of M1 type macrophages with formation of foam
cells and production of inflammatory factors that can cause a vicious cycle. This cycle includes
that oxidation of membrane phospholipids within atheromatous debris (128), as well as inclusion
of trans fatty acid components, which together reduces the ability of macrophages to degrade the
debris and restore health. Many of these macrophage responses are regulated by TLR4. Human
macrophages also are directly damaged by trans fatty acids. These accumulate in cell membranes
with consequences that affect cell signaling, viability, and proliferation. These effects may last
for years after cessation of *trans* fat feeding, with extracellular debris containing the offending materials lying in wait to produce generations of foam cells that will promote atheroma growth.

Of all the lifestyle changes that can be made to reduce atherosclerosis, removal of partially hydrogenated oils, the source of *trans* fats, from the food supply is the simplest and one of the most potentially efficacious changes. We are hopeful that our government will be successful in removing this unnecessary fat from the diet.
3.0 ELAIDATE, AN 18-CARBON TRANS-MONOENOIC FATTY ACID, INHIBITS B-OXIDATION IN HUMAN PERIPHERAL BLOOD MACROPHAGES

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3.1 ABSTRACT

Consumption of trans unsaturated fatty acids promotes atherosclerosis, but whether degradation of fats in macrophages is altered by trans unsaturated fatty acids is unknown. We compared the metabolism of oleate (C18:1Δ9-10 cis; (Z)-octadec-9-enoate), elaidate (C18:Δ9-10 trans; (E)-octadec-9-enoate), and stearate (C18:0, octadecanoate) in adherent peripheral human macrophages. Metabolism was followed by measurement of acylcarnitines in cell supernatants by MS/MS, determination of cellular fatty acid content by GC/MS, and assessment of β-oxidation rates using radiolabeled fatty acids. Cells incubated for 44 hours in 100 µM elaidate accumulated more unsaturated fatty acids, including both longer- and shorter-chain and had reduced C18:0 relative to those incubated with oleate or stearate. Both C12:1 and C18:1 acylcarnitines accumulated in supernatants of macrophages exposed to trans fats. These results suggested β-oxidation inhibition one reaction proximal to the trans bond. Comparison of [1-14C]oleate to [1-14C]elaidate catabolism showed that elaidate completed the first round of fatty
acid β-oxidation at rates comparable to oleate. Yet, in competitive β-oxidation assays with [9, 10-3H]oleate, tritium release rate decreased when unlabeled oleate was replaced by the same quantity of elaidate. These data show specific inhibition of monoenoic fat catabolism by elaidate that is not shared by other atherogenic fats.

### 3.2 INTRODUCTION

A 2009 World Health Organization study summarizing human clinical and observational studies of trans fatty acids concluded that these fats contribute significantly to cardiovascular risk (22). Atherosclerotic disease is promoted by diets containing large amounts of long-chain saturated fat or by relatively small amounts of fat containing artificial trans-monounsaturates (9,140). In either case, advanced atherosclerotic disease is characterized by vascular intima accumulation of semi-liquid cellular debris, consisting, in large part, of oxidized membrane phospholipids and cholesterol (11,141). The surrounding tissue is a mixture of proliferating smooth muscle, fibroblast-like cells, and “foam cells,” a type of macrophage containing large amounts of lipid. In contrast, healthy vascular intima does not contain cell debris, proliferating cells, or lipid-laden macrophages. Under normal conditions, macrophages remove and recycle damaged cells to prevent the accumulation of toxic products. While it is known that macrophages in the atherosclerotic environment have altered metabolic and phenotypic features (12), whether macrophages contribute to atherogenesis and, in particular, whether trans-unsaturated fatty acids have a direct role in these changes is unclear.

Studies of trans fatty acid metabolism using whole animals and liver cells established their adverse effects on overall fat, cholesterol, and lipoprotein production. In Sprague–Dawley
rats fed a diet with 10% of energy as fat and 4% of the fat as elaidate (C18:Δ9-10 trans; (E)-octadec-9-enoate) for 8 weeks, visceral fat and liver lipid content increased compared to rats fed a 10% fat control diet (no trans-fat). This result led the investigators to speculate that the liver, muscle, and adipose tissue responses to trans fatty acids are different from that of saturated fats with respect to its handling of glycogen, glucose, and other nutrients (31). Other studies showed that trans-monounsaturated fatty acid feeding increased cholesterol and total triacylglycerol synthesis in rat liver, while HDL cholesterol concentration declined (30). Further, trans-fatty acids are incorporated into cell membranes to a degree quantitatively similar to their occurrence in the diet (18,19). A meta-analysis of human clinical studies showed that trans-monounsaturated fatty acid ingestion increased cholesterol and total triacylglycerol synthesis, while HDL cholesterol concentration declined (23). Although these metabolic consequences of trans fat consumption are consistent with known risk factors for cardiovascular disease, we hypothesized that chemically derived trans-fatty acids have unique biochemical effects for encouraging the accumulation of cellular debris in peripheral tissues, specifically in tissue macrophages.

Our purpose here is to identify whether high concentrations of lipids containing trans fats, such as may be found in semi-liquid atheromatous debris, are toxic via their effects on the tissue macrophages that normally remove and recycle dead cells. As a first step, we focused on how human macrophages degrade trans fats in β-oxidation. No previous work has specifically addressed the degradation of trans fats in humans, but studies in rats showed that the β-oxidation of elaidate, the major artificially generated trans fatty acid, was incomplete, resulting in the accumulation of 5-trans-tetradecenate in perfused rat hearts and liver mitochondria, respectively (64,66). β-oxidation of fatty acids usually goes to completion without any specific intermediates accumulating (63,65). Yu et al. 2004 (66) proposed that this
intermediate accumulation in rodents occurred because elaidate is a poor substrate for the acyl-CoA dehydrogenases involved in the early steps of β-oxidation (Fig. 3.1A). However, in humans, these same acyl-CoA dehydrogenases have very different substrate specificities and levels and patterns of expression (67), suggesting that the catabolism of elaidate in humans may differ, with potentially important consequences, from that in rats. Furthermore, a poorly metabolized substrate entering β-oxidation can affect the flow of all substrates through the pathway and, thus, can affect both the quantity and quality of fatty acids available for signaling and membrane synthesis.
To test the hypothesis that human macrophages are defective in their ability to degrade *trans* fats, we derived fresh macrophages from peripheral blood monocytes (142) and investigated how these cells process large quantities of elaidate, the major *trans*-C18-monounsaturated fatty acid, as compared to oleate, its *cis*-monounsaturated fatty acid isomer and
to stearate, the 18-carbon saturated fatty acid (Fig. 3.1B). Additionally, after feeding the cells matched cis- or trans-octadecenoic acids or unprocessed and trans-processed dietary fat, we used MS/MS to assess fatty acid β-oxidation intermediates as acylcarnitines in cell supernatants. We determined fatty acid composition of cells after similar treatments using GC/MS. Also, we compared the effect of accompanying cis- or trans-octadecenoic acids or stearate on tritiated water release from [9,10-3H]oleate. We show that elaidate, trans-octadec-9-enoate, causes a previously uncharacterized disruption of monoenoic fat catabolism in primary human macrophages.

3.3 MATERIALS AND METHODS

3.3.1 Cell Cultures

Human peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats as described (142) with the approval of The Institutional Review Board. The PBMCs were transferred to DMEM with 10% FBS, penicillin, streptomycin, 1 µg/ml carnitine (Sigma, St. Louis, MO), and 20 µg/ml human macrophage CSF-1 (Peprotech, Rocky Hill, NJ). For all experiments, 8 × 10^6 cells were plated on 10 cm² plates. After 2–3 days of incubation, the medium was replaced and all non-adherent cells were discarded. The PBMCs were used 4–7 days after plating. Hepatocytes (from human livers not suitable for transplantation), also obtained by protocols approved by the Institutional Review Board, were cultured in DMEM supplemented with 10% FBS, insulin, gentamicin, amphotericin B, and dexamethasone (HMM SingleQuots, Lonza, Basel, Switzerland). Two to three days after plating, dexamethasone was removed from
the cultures in two steps over 48 h. In all fatty acid treatment assays, 10% FBS was replaced with treatment fatty acid. Assays were performed 24 h after dexamethasone removal. Unless specified, media were obtained from Thermo-Fisher (Waltham, MA) and chemicals were from Sigma–Aldrich.

### 3.3.2 Cell Protein Determination

Cell protein was quantified by bicinchoninic acid dye binding after hydrolysis of cells overnight at 4°C in 1 N NaOH followed by neutralization with HCl. For 14C assays, proteins were determined using duplicate cell cultures.

### 3.3.3 Acylcarnitine Analysis by MS/MS

Macrophage cultures were incubated with fatty acids in DMEM with penicillin/streptomycin, 1 µg/ml carnitine, and 20 µg/ml of human CSF-1 for 7 days at 37°C in 5% CO2. Fatty acids were from Grace Davison (Deerfield, IL) and were prepared as stock solutions of 3.5 mM fatty acids in 9% (1.4 mM) defatted albumin (BSA Fraction V, Sigma). They were added to DMEM at final concentrations of 100 µM in 400 µM albumin or as 400 µM albumin alone. For feeding with soy oil or partially hydrogenated soy oil, FBS was saturated with soy oil or partially hydrogenated soy oil containing 7% trans-octadecenoic acids. To saturate, FBS was incubated with 2 mg/ml of either oil for 20 min at 50°C, with vortexing, followed by filtration to remove fat not adsorbed to proteins. Cultures used 10% FBS saturated with soy oil or partially hydrogenated soy oil with untreated FBS as their controls. After the 7 days of fatty acid or fat feeding, 35 µl samples of cell supernatant were spotted onto Schleicher & Schuell Grade 903 filter paper, dried at room
temperature, and stored at −20°C. Four wells of each treatment were assayed; each assay was performed using two 5 mm punches from the filter paper. Samples were processed for acylcarnitine MS/MS analysis essentially as described (143,144). Briefly, punched paper with about 14 µl of dried tissue culture supernatant was reconstituted in 300 µl of methanol containing deuterated carnitine and acylcarnitines (free, C2-, C3-, C4, C5-, C8-, C14-, and C16-carnitine) as internal standards. Solvent was dried under nitrogen and then incubated in 3 N HCl in n-butanol at 65°C for 15 min to form butyl carnitine derivatives and dried again. Then samples were dissolved in 1:1 acetonitrile: 0.2% formic acid and injected into the electrospray ion source of the MS/MS (API Sciex 3000, Foster City, CA), scanning to record precursors with m/z 200–500 containing the butyl signature, m/z of 85.

3.3.4 Fatty Acid Composition of Cells by GC/MS

Fatty acid incubation media were prepared as above (acylcarnitine analysis), using 100 µM final concentration of free fatty acids on BSA 1:4 M/M for 2 days. After the 2 days of fatty acid loading, cells were washed three times and harvested in 300 µl of PBS by scraping. Heptadecanoic acid (C17:0, 40 ng) was added as an internal standard. Lipids were extracted, de-esterified, and converted to methyl esters in a 3:1 methanol to methylene chloride solution with acetyl chloride catalyst at 75°C for 90 min. The resulting mixture was neutralized with 7% potassium carbonate, and esterified acids were extracted with hexane as described (18), except that the samples were not derivatized with tetramethylsilane since the conversion to methyl esters was essentially quantitative (18). Extracts were dried under nitrogen and lipids were reconstituted in 50 µl of hexane; a 2 µl fraction was injected for separation by GC with identification of components by MS in a Hewlett-Packard 6890 instrument (Agilent
Technologies, Santa Clara, CA). Chromatography was run in helium and used a 60 m nonpolar dimethylpolysiloxane column, 250 μm inside diameter, 0.02 μm film (DB-1MS, Agilent). The chromatography was started with 1 min at 50°C, followed by a 25°C/min ramp to 175°C. The column was held at 175°C for 10 min, followed by a 1°C/min ramp to 192°C, with a final increase at 10°C/min to 230°C. All results were normalized to the C17 internal standards.

3.3.5 Competitive Tritium Release β-Oxidation Assay

Tritium-release assays were performed after the method of Bennett (145). Specifically, PBMCs, prepared as described above in 10 cm² wells, were washed once in PBS and then incubated with 0.34 μCi [9,10-³H]oleate ((Z)-octadec-9-enoate) (45.5 Ci/mMole; Perkin–Elmer, Waltham, MA) (Fig. 3.1) and 50 nmol fatty acids in 0.5 ml PBS with 1 μg/ml carnitine for 60 min at 37°C. The unlabeled fatty acids consisted of 25 nmol of oleate with 25 nmol of elaidate, stearate, or oleate. Fatty acid solutions were solubilized in α-cyclodextrin in PBS as described (146). For ³H₂O collection, a column was prepared for each sample containing 750 μl of anion exchange resin in water (AG 1 × 8 acetate, 100–200 Mesh, BioRad, Richmond, CA). After incubation, the medium from each well was applied to the resin to bind the labeled fatty acids while the tritium released by β-oxidation of the fatty acids flowed through the column. The flow-through from the incubation and three 1 ml deionized water washes were collected and mixed with 10 ml of scintillation fluid (Ultima Gold, Sigma), followed by counting tritium released with a Packard Tri-CARB scintillation counter. Assays were performed in triplicate with triplicate blanks (incubation step omitted) for each sample. Standards contained a 500 μl aliquot of the incubation mix with 3 ml of deionized water and 10 ml of scintillation fluid. Hepatocytes were assayed identically except that the incubation time was reduced to 30 min. For experiments utilizing (+)-
etomoxir sodium salt and antimycin A, the final quantities were 100 μM and 50 ng/sample, respectively.

3.3.6 $^{14}$C-Water Soluble Product β-Oxidation Assay

Elaidate ((E)-octadec-9-enoate), [1-$^{14}$C], and oleate, [1-$^{14}$C], both 55 mCi/mMole, were from American Radiolabeled Chemicals (St. Louis, MO). For each incubation, 0.1 μCi of [1-$^{14}$C]fatty acid was mixed with its free fatty acid to make 30 nmol total. Fatty acids were solubilized in α-cyclodextrin as above except that the final volume per 10 cm$^2$ well was 300 μl. Cells were incubated at 37°C for 60 min. Reactions were stopped by adding 120 μl of 18% perchloric acid to each well, followed by incubation for 15 min at 4°C. The soluble fraction was transferred next to glass tubes, and water-soluble reaction products were separated from the chloroform–methanol layer containing the lipids after Folch et al. (147). Water-soluble $^{14}$C was then determined by scintillation counting.

3.3.7 Messenger RNA Expression Studies

The PBMCs were purified and plated as described above except that before plating, the final preparation was further purified by CD14 magnetic bead purification (142) as described. Cells were incubated for 44 h in 30 μM fatty acids as above and, after washing, they were trypsinized and scraped into PBS and frozen at −80°C as a pellet. Messenger RNA was isolated using a RNeasy Mini kit (Qiagen, Valencia, CA), and first strand cDNA synthesis was performed by reverse transcription using random primers and Superscript III reverse transcriptase (Invitrogen, Grand Island, NY). Quantitative PCR was conducted using brilliant SYBR green fluorescent
DNA intercalating master mix at 55°C for 40 cycles (Stratagene, La Jolla, CA) and the QuantiTect Primer Assays for human enoyl-CoA δ-isomerase 1 (ECI1) and enoyl-CoA δ-isomerase 2 (ECI2) (Qiagen). Substrate concentrations and incubation times were chosen to optimize for primary cell health and to investigate the substrate under a steady state.

3.4 RESULTS

3.4.1 Acylcarnitine Profiling

To address whether, in human macrophages, trans fats are metabolized differently from naturally occurring dietary fats, we started with acylcarnitine profiling of media from cells cultured with fatty acids (144), a well-characterized method to detect blocks in the β-oxidation pathway (Fig. 3.1A). Lipid catabolic anomalies result in the accumulation of characteristic patterns of acylcarnitines. Specifically, in cultured cells, if inhibition occurs at any point of fatty acid catabolism, the cells will accumulate the reaction-specific acyl-CoAs. The cultured cells subsequently eliminate these excess acyl-CoAs by transesterifying them to form acylcarnitines that are transported out of the cell into the medium. When PBMCs were fed elaidate, they accumulated C12:1- and C18:1-carnitines in their media with p<0.01 and p<0.05 relative to oleate, respectively (Fig. 3.2A) with larger differences (p<0.01 in all cases) relative to C12:1- and C18:1-carnitines for the albumin-only control or stearate-fed cells. The oleate-fed cells accumulated some of these same two species, C12:1- and C18:1-carnitines, relative to stearate fed cells (p<0.05), but the effect of oleate feeding was quantitatively much less than the effect of elaidate feeding.
Accumulation of acylcarnitines in supernatants after 44 h lipid incubation. Acylcarnitines accumulate in supernatants of human macrophage cultures fed unsaturated fatty acids. Determination by MS/MS, N = 4, mean ± SD is indicated. A: Effect of 100 µM fatty acids in albumin or albumin alone (left) on supernatant monounsaturated acylcarnitines at 44 h. **P < 0.01 relative to all other conditions, *P < 0.05 relative to oleate and P < 0.01 relative to albumin control or stearate feeding, †P < 0.05 relative to stearate-fed cells or albumin controls. B: Comparison of saturating 10% FBS with soy oil or with partially hydrogenated soy oil containing 7% trans-octadecenoic acids relative to untreated FBS. *P < 0.05 relative to untreated FBS, **P < 0.05 relative to soy oil. C10:1 groups serum versus serum with partially hydrogenated soy oil approached significance at P = 0.07.
To determine whether these findings could be reproduced when dietary triglycerides, rather than the more toxic free fatty acids, were the source of the trans-unsaturated fatty acids, we compared acylcarnitine profiles of macrophages incubated for 1 week in 10% fetal bovine serum (FBS) to those incubated with FBS saturated with soy oil or with partially hydrogenated soy oil containing 7% elaidate with little residual other unsaturates (determined by GC/MS, see Methods Section) (Fig. 3.2B). In this experiment, the FBS saturated with soy oil caused a threefold increase in C12:1-carnitine relative to control FBS (p<0.05), while FBS saturated with partially hydrogenated soy oil increased C12:1-carnitines eightfold relative to the control FBS (p<0.01) and over twofold relative to the untreated soy oil (p<0.05). The partially hydrogenated soy oil also increased C14:1-carnitine twofold relative to both of the other groups (p<0.01). There was also a trend toward accumulation of C10:1-carnitine with the partially hydrogenated soy oil medium (p<0.07) relative to the FBS alone.

3.4.2 Fatty Acid Composition of Cells by GC/MS

Subsequently, we used GC/MS analysis to determine how treating macrophages with the different fatty acids on albumin affected total cellular fatty acid composition. Again, PBMCs were incubated for 44 h with 100 µM oleate, elaidic, or stearate, to assess steady state effects. Typical GC fatty acid separations are presented in Figure 3.3.
Figure 3.3 GC/MS Chromatograms

Percent of fatty acid residues found in macrophages. Typical chromatograms of fatty acids isolated from whole macrophage cultures after feeding for 44 h with 100 µM fatty acids on albumin. The top chromatogram is from an oleate-fed culture, and the bottom is from an elaidate-fed culture. Key fatty acids identified by GC/MS are in shaded boxes labeled at the top. Quantitative data from several samples from all groups are presented in Table 3.1.

When these data were compiled as percentile of each fatty acid for each treatment (Table 3.1), quantifiable amounts of many more fatty acid species were found with elaidate rather than either oleate or stearate incubation. Unique species included chain-shortened products, such as both cis and trans C16:1, as well as the elongated fatty acids, C20:1, C20:2 and the polyunsaturated product C18:2. In addition, the proportion of C18:0 was greatly reduced in elaidate fed cells (by 80–90% relative to oleate or stearate, p < 0.01), with a lesser reduction of C16:0 (25–33%) relative to oleate feeding that did not reach significance. In the elaidate-fed macrophages, this saturated fraction was replaced by an increase in the total monounsaturated fraction, p < 0.01. Not surprisingly, the cells also accumulated large quantities of whatever fatty acid they had been fed.
### Table 3.1 The Effect of Elaidate Feeding on Distribution of Fatty Acids in Macrophages

<table>
<thead>
<tr>
<th>% of Each Species</th>
<th>Oleate (C18:1 cis)</th>
<th>Elaidate (C18:1 trans)</th>
<th>Stearate (C18:0)</th>
<th>Media only</th>
</tr>
</thead>
<tbody>
<tr>
<td>C16:1 (cis)</td>
<td>1.1+/-2.2</td>
<td>2.2+/-0.4</td>
<td>0</td>
<td>1.8+/-1.6</td>
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<tr>
<td>C16:1 (trans)</td>
<td>0</td>
<td>1.5+/-0.9</td>
<td>0</td>
<td></td>
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<tr>
<td>C18:1 (cis)</td>
<td>45.2+/-5.9</td>
<td>13.4**+/-7.9</td>
<td>33.5+/-9.7</td>
<td>27.6+/-4.8</td>
</tr>
<tr>
<td>C18:1 (trans)</td>
<td>3.5+/-4.2</td>
<td>49.2**+/-10.5</td>
<td>7.9+/-1.6</td>
<td>2.6+/-4.5</td>
</tr>
<tr>
<td>C20:1</td>
<td>1.9+/-1.3</td>
<td>49.8</td>
<td>68.2 **</td>
<td>41.4</td>
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</table>

**Total unsaturated fraction (%)**

<table>
<thead>
<tr>
<th></th>
<th>Oleate (C18:1 cis)</th>
<th>Elaidate (C18:1 trans)</th>
<th>Stearate (C18:0)</th>
<th>Media only</th>
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<tbody>
<tr>
<td>C14:0</td>
<td>2.5+/-0.6</td>
<td>1.8+/-1.7</td>
<td>2.2+/-3.0</td>
<td>2.2+/-1.9</td>
</tr>
<tr>
<td>C16:0</td>
<td>26.2+/-2.0</td>
<td>17.3+/-9.7</td>
<td>23.3+/-8.0</td>
<td>30.8+/-3.7</td>
</tr>
<tr>
<td>C18:0</td>
<td>17.4+/-2.0</td>
<td>3.6**+/-3.0</td>
<td>29.2**+/-1.1</td>
<td>24.3+/-1.8</td>
</tr>
</tbody>
</table>

**Ratio % C18:1(cis)/%C18:0**

<table>
<thead>
<tr>
<th></th>
<th>Oleate (C18:1 cis)</th>
<th>Elaidate (C18:1 trans)</th>
<th>Stearate (C18:0)</th>
<th>Media only</th>
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<tbody>
<tr>
<td>C18:2</td>
<td>0</td>
<td>3.2+/-0.8</td>
<td>0</td>
<td>2.6+/-3.8</td>
</tr>
<tr>
<td>C20:2</td>
<td>0</td>
<td>0.9+/-1.1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>C20:4</td>
<td>3.2+/-0.7</td>
<td>4.7+/-1.2</td>
<td>3.8+/-1.7</td>
<td>6.7+/-6.7</td>
</tr>
<tr>
<td>C22:4</td>
<td>0.8+/-0.6</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

Methyl esters of fatty acids were prepared from cell lysates as indicated in Methods Section, separated by GC and identified by MS as shown in Figure 2.3. Cells were treated for 44 h with 100 μM fatty acids. Total fatty acids were normalized to 100%. Blanks indicate that average values are <1%. Significance of differences was determined only for products present at over 3% in one or more groups. A significant difference relative to both other groups, \( P < 0.01 \), is indicated **n = 3 for oleate, elaidate, and media only and n = 2 for stearate. Mean ± SD is indicated.

### 3.4.3 Competitive Tritium Release β-Oxidation Assay

The findings that with elaidate incubation, both cis and trans intermediates accumulate in the cells (Table I) and that a C12:1-carnitine intermediate accumulates (Fig. 3.2) are consistent with elaidate causing a delay at the enoyl-CoA \( \delta \)-isomerization (ECI) step of the β-oxidation of C18:1 (\( \omega-9 \)) (Fig. 3.1). To test this more specifically, we utilized a tritium release assay using [9,10-\( ^3 \)H]oleate, a substrate that must pass through ECI, hypothetically the block point, before the tritium can be released. In addition, the experimental media included 50 μM unlabeled oleate.
with an additional 50 µM oleate, elaidate, or stearate. In macrophages, the added elaidate slowed the catabolism of [9,10-^3H]oleate by almost 70% relative to addition of oleate, while [9,10-^3H]oleate degradation increased when the accompanying fatty acid was stearate (Fig. 3.4A).

**Figure 3.4 Comparison of β-oxidation rates**

In each case, cells were incubated in 100 µM total fatty acid and results are reported as nmol/mg protein/h. Means ± SD are shown. Matched cell cultures were used for all assays. A. In macrophages, tritium released from [9,10-^3H]oleate (see Fig. 2.1B) in 50 µM oleate with an additional 50 µM of oleate (left bar), elaidate (middle bar), or stearate (right bar) added as competitors, n = 4. Elaidate reduced the rate of oleate β-oxidation, **P < 0.01. 50 µM Stearate increased the rate of oleate degradation, *P < 0.05. B. An identical experiment using primary hepatocytes, n = 3. Stearate also increased the rate of oleate degradation, **P < 0.01. C: Comparison of oleate β-oxidation rates when macrophages were treated with mitochondrial inhibitors. N = 5 for antimycin A (50 ng/sample) experiments, n = 3 for etomoxir (100 µM) using the same conditions as Figure 2.4A studies. D: Relative rate of the first round of β-oxidation for elaidate versus oleate in macrophages. Activity was measured using [1−^14C]-labeled fatty acids. P = 0.16, n = 4.

When we performed the same experiment with isolated human hepatocytes (Fig. 3.4B), replacement of unlabeled oleate or elaidate with stearate again increased significantly the rate of
oleate β-oxidation across the cis-double bond, as in macrophages. However, replacement with elaidate did not reduce the β-oxidation of oleate in hepatocytes.

The next studies (Fig. 3.4C) addressed whether the inhibition of β-oxidation by elaidate is specific to mitochondria or peroxisomes. The addition of the mitochondrial oxidative phosphorylation Complex 3 inhibitor Antimycin A (148) reduced β-oxidation activity by 93%, 73%, and 88% with oleate, elaidate, and stearate competition, respectively. The CPT1 inhibitor etomoxir (148) was slightly less potent, 83%, 77%, and 91%, respectively, in reducing β-oxidation activity. There was no significant difference in the quantity of residual β-oxidation activity when mitochondrial inhibitors were included.

3.4.4 $^{14}$C-Water Soluble Product β-Oxidation Assay

To determine whether the effect of elaidate on oleate degradation in macrophages reflected a preferential initiation of β-oxidation on one isomer, we compared the β-oxidation rate of carbon 1 using [1-$^{14}$C]oleate and [1-$^{14}$C]elaidate (Fig. 2.4D). Here, we found that elaidate entry into the first round of β-oxidation was at least as rapid as that of oleate, with a trend toward increased rates $p = 0.16$, $n = 3$.

3.4.5 Messenger RNA Expression Studies of Enoyl-CoA Isomerase

Finally, to address the discrepancy that oleate tritium release was inhibited by elaidate in PBMCs, but not in hepatocytes, we hypothesized that expression of ECI differs between the two cell types. Mitochondria express two ECIs, ECI1 and ECI2. Quantitative PCR (Fig. 3.5) showed that hepatocyte ECI2 mRNA was threefold more highly expressed relative to that in
macrophages after oleate ($p < 0.05$) or elaidate ($p < 0.01$) incubation. Human hepatocytes to test the point further were not available.

Figure 3.5 Comparison of ECI1 and ECI2 expression by quantitative PCR

Cells ($n = 5$ for macrophages and $n = 2$ for hepatocytes) were treated for 44 h with 100 μM fatty acid as described. Fatty acid treatments did not affect expression significantly compared to each other. For ECI2, the expression differences in macrophages and hepatocytes reached significance of $P < 0.05$ for oleate treated and $P < 0.01$ for elaidate treated. For hepatocytes, range was used to test for significance instead of standard deviation.
3.5 DISCUSSION

Our study shows that in human macrophages, elaidate or \textit{trans}-octadec-9-enoate, the major \textit{trans}-unsaturated fat in artificially modified lipids, is poorly metabolized, resulting in its incomplete $\beta$-oxidation with C12:1-carnitine accumulation (Fig. 3.2). This intermediate forms where enoyl-CoA $\delta$-isomerase (ECI) moves the double bond into a favorable position to complete unsaturated fatty acid $\beta$-oxidation (Fig. 3.1A). The tritium release studies in macrophages with labeled oleate verified that elaidate is a more potent inhibitor of $\beta$-oxidation than an equal quantity of oleate (Fig. 3.4A). Furthermore, replacing half of the fat with stearate enhanced oleate $\beta$-oxidation, supporting the conclusion that the block occurs during the isomerization of the double bond (Fig. 3.4 A-B). Finally, both cis and \textit{trans} C16:1 accumulated in macrophages in response to elaidate feeding (Table 3.1). We conclude that elaidate interferes with $\beta$-oxidation of all unsaturates at the isomerase step and this extra step may be rate limiting. Furthermore, inhibitor studies suggest that this block occurs in mitochondria.

Mitochondria contain two ECIs, ECI1, and ECI2. When mice null for ECI1 were fed a high oleate diet, cis-3-C12:1-carnitine accumulated (149). When ECI2 expression was reduced in ECI1 null fibroblasts, even more cis-3-C12:1-carnitine accumulated, verifying that C12:1-carnitine accumulates when ECI activity is blocked. Since our acylcarnitine analysis does not separate the cis and \textit{trans} isomers, we could not identify the isomers. The high oleate-fed ECI1 null mice also accumulated small quantities of C18:1-carnitine (149), as did our macrophages fed elaidate (Fig. 3.1), suggesting that even substrate entry into $\beta$-oxidation can back up if ECI activity is inhibited. Rat liver also contains two ECIs that will process the cis and \textit{trans} products of the C18:1 fatty acids (107), but this study is the first to show C12:1 accumulation from a $\beta$-
oxidation limitation in primary human cells. We are also the first to show that in human cells excess elaidate can interfere with unsaturated fatty acid β-oxidation.

With elaidate as substrate, rat liver mitochondria also accumulated chain-shortened intermediates, but here C14:1-carnitine was identified rather than C12:1-carnitine (66). The authors postulated that this product appeared because the long-chain acyl-CoA dehydrogenase (ACADL) had a low preference for the elaidate intermediate 5-trans-tetradecenoyl-CoA, relative to that for oleate intermediates. Similarly, the high oleate-fed ECI1 null mice accumulated some C14:1-carnitine, as well as C12:1-carnitine. Thus, it appears that in rodents β-oxidation of unsaturated fatty acids slows one round of β-oxidation prior to block identified in human macrophages. It is not surprising that the elaidate β-oxidation differs in humans, since null mouse models for the long chain acyl-CoA dehydrogenases do not fully recapitulate the human disorders (67). At least three mitochondrial acyl-CoA dehydrogenases are active in long chain fatty acid β-oxidation (150), and a review comparing the two species has clearly shown that the mouse and human enzymes differ in both substrate specificities and tissue distributions (67). In humans, C14:1-carnitine accumulates in very long chain acyl-CoA dehydrogenase deficiency (144).

However, the rodent studies and our human studies of elaidate β-oxidation share a central finding. Elaidate causes “leaky” β-oxidation, where intermediates accumulate as acylcarnitines, whereas β-oxidation normally occurs to completion (65). Some acylcarnitine intermediate accumulation is also associated with adult Type 2 diabetes and obesity (143). However, that accumulation is limited, involves several acylcarnitine species, and is thought to be associated with oxidative phosphorylation deficiencies. This single species C12:1-carnitine accumulation is consistent with a specific block in β-oxidation.
To support the hypothesis that the enzymatic block with elaidate is at the enoyl-CoA δ-isomerase (ECI) step, we used [9,10-3H]oleate, a substrate that must pass through ECI before its label can be processed, to compare the effect of an equal quantity of elaidate to oleate on β-oxidation rates. We reasoned further that if we replaced half of the unlabeled oleate with stearate, the rate of oleate β-oxidation should increase relative to oleate alone. The results validated both these expectations. We also found, using [1-14C]-labeled substrates, that elaidate goes through the first round of β-oxidation at least as rapidly as oleate, in agreement with an earlier study in hepatocytes (30).

The same tritium competition studies using primary human hepatocytes (Fig. 3.4B) resulted in a similar increase in tritium release when stearate replaced half of the oleate but no corresponding alteration in β-oxidation rate when elaidate replaced it. Searching for possible explanations for these differences in hepatocytes, we found that ECI2 was more highly expressed here than in macrophages (Fig 3.5). Thus, macrophages are more susceptible than liver cells to the deleterious effects of trans fats, while liver may have inherently more capacity to mediate toxic fatty acids. Primary human hepatocytes for this work have been difficult to obtain, hence, the n = 2 for qPCR. Available hepatocytes were limited because those from fatty livers were excluded.

In experiments where we replaced the free fatty acids with mixed triglyceride-based human dietary fats, the C12:1-carnitine was still the primary intermediate when trans fats were used, reinforcing the original data and eliminating the possibility of an effect from the use of large quantities of elaidate in its free fatty acid form (Fig. 3.2). Because partial hydrogenation produces a mixture of trans isomers at different carbons with elaidate still dominating, there was a less distinct pattern of accumulating intermediates. Curiously, controls fed serum saturated
with regular soy oil also accumulated C12:1-carnitines, albeit to a much lesser extent than that caused by partially hydrogenated soy oil. Soy oil contains about 15% saturated fatty acids, 25% monounsaturates, and 60% polyunsaturates, with <1% trans-unsaturated fatty acids (6). Apparently, large quantities of cis-polyunsaturated and monounsaturated fatty acids can challenge the capacity of the ECIs to degrade them, suggesting that in macrophages the isomerase step may be rate limiting for unsaturates. Consequently, these results show that triglycerides containing small quantities (7%) of trans-unsaturated fatty acids can still inhibit unsaturated β-oxidation.

To characterize further how macrophages accommodate the unnatural fat elaidate, we analyzed whole cell composition. While our acylcarnitine profiling suggested a β-oxidation block at C12:1, the shortest unsaturated product accumulating at detectable concentrations was C16:1. Both cis and trans products accumulated, again consistent with a rate limitation in the isomerization of the double bond in odd-chained unsaturates. This chain length discordance between whole cells and their excreted acylcarnitines was reminiscent of ACADL null mice, which accumulate C14:1-carnitine on a high fat diet (151). When these mice were fed labeled cis-C14:1(ω-9), it was elongated to C18:1 before incorporation into diglycerides. We found similarly that the elaidate-fed cells had a higher percentage of total C18:1 than the oleate-fed cells. However, these changes were balanced by a small reduction in C16:0 and an even greater reduction in C18:0. This alteration in C18:0 with an increase in C18:1 can also result from activation of stearoyl-CoA desaturase, an enzyme involved in enhancing membrane fluidity (152). A similar reduction in saturated fatty acid concentration with a high trans fatty acid oral intake was reported in a recent large in vivo human study (153). Overall, this altered fatty acid pattern in the elaidate-fed animals suggests that the cells may be responding by both elongating
and desaturating the pool of elaidate fatty acid products prior to their incorporation into cells. An incidental finding in fatty acid-cultured PBMCs was a low percentage of C18:2 (Table 3.1). Even the PBMC cultured in our basic 10% FBS media (Table 3.1) had low C18:2 levels, suggesting that our incubation with large quantities of other fatty acids probably reduced the proportion of C18:2 to the limits of our detection.

The difficulties in processing elaidate by macrophages are important because trans-unsaturated fatty acids are incorporated into new cell membranes rapidly after they are consumed (19). Thus, when lesions occur in vascular intima after exposure to diets containing significant amounts of trans fat, macrophages would be faced with the extra challenge of degrading and removing cell membranes and related debris containing these poorly metabolized artificial fatty acids or their products. Removal of cellular debris is the central factor in progression of atherosclerosis, and macrophages are the primary responders in the prevention of this process. When lipid-rich debris cannot be removed rapidly, oxidized low density lipoprotein bound with phospholipids is hypothesized to contribute to the progression of lesions by overloading macrophages with lipid debris (14-16).

Overall, this study, which addresses the altered handling of trans fatty acids by macrophages, gives us a platform for investigating the metabolic effects of elaidic acid. We identified three specific effects of elaidate on human macrophage fatty acid metabolism. First, elaidate causes a specific block in β-oxidation of monounsaturates including oleate. This block is also associated with the accumulation of many unusual fatty acid intermediates. Finally, the fatty acid species in elaidate incubated cells differ from that of cells grown in normal medium or even from cells grown in medium highly enriched in oleate. Particularly notable is a reduction in C18:0 in elaidate-treated cells with a compensatory increase in unsaturated long-chain fatty acids.
(Table 3.1). These lipid alterations will be useful in further characterization of the effects of trans fats on overall human macrophage physiology. We are particularly interested in the effects of accumulating products of elaidate metabolism on signaling pathways and on membrane function and composition. We recognize that trans fats may alter cellular properties by multiple pathways, including by increasing membrane rigidity. This alteration, in turn, affects cholesterol transport by macrophages (20). However, trans fats also inhibit β-oxidation with accumulation of unusual intermediates and changes in cell composition. Consequently, elaidate and its metabolites may affect cell metabolism and signaling in other unknown and unexpected ways.
4.0 ELAIDIC ACID CAUSES A PERSISTENTLY ELEVATED ZINC CHANGE IN HUMAN PERIPHERAL BLOOD MACROPHAGES

Will submit for publication.

4.1 ABSTRACT

The role of trans fatty acids in atherosclerosis is not well characterized. The idea that zinc homeostasis can be altered by these fatty acids has not been considered. In a gene expression array comparing human macrophages incubated with 30 μM of cis fat oleate (OL) or the trans fat elaidate (EL), eight genes associated with zinc homeostasis were altered. Changes in metallothioneins 1X and 2A and in SLC39A10 expression were confirmed by qPCR. SLC39A10 was significantly elevated in EL-treated cells. Parallel qPCR experiments with SFAs showed elevated metallothionein expression at 44 h, but at 15 h EL, ST, and PA have comparable metallothionein expression lower than OL. Next we investigated these effects on intracellular zinc. Expression changes paralleled intracellular zinc at both time points confirmed by FluoZin-3 labile zinc quantification in EL-, ST-, and PA-treated cells. EL, ST, and PA increased labile zinc at 15 h, but only EL-treated remained elevated at 44 h. To determine whether zinc changes corresponded to inflammation, proportional nuclear localization of nuclear factor-κB (NF-κB) was determined. A parallel experiment was conducted with the addition of 5 μM zinc chelator,
TPEN, EL, ST, and PA caused the most NF-κB nuclear localization. Addition of TPEN nullified the treatment effect; all conditions, even controls, caused similar effects. These data show the similar initial effects of EL, ST, and PA on macrophages zinc homeostasis and NF-κB activation, but the EL zinc effect is persistent.

4.2 INTRODUCTION

Free fatty acids are capable of eliciting highly varied physiological reactions in the human body. Small quantities of artificial trans-mono-unsaturates and large quantities of long chain saturated fats in the diet have been shown to promote atherosclerotic disease and other inflammatory conditions (9,140). The disturbance in signaling by free fatty acids, especially trans and saturated may influence inflammatory conditions, allergies, metal ion balances, and cancer through signaling (108,154,155). Because of the interconnectivity among lipid signaling pathways, there has been difficulty elucidating exact downstream networks. Most experiments study a pathway in isolation for sake of simplicity, even though many have interactions with one another (154).

In humans, many inflammatory pathways are induced indirectly by upstream free fatty acid signaling (108). Recently, free fatty acid signaling to Toll-like receptors (TLRs), specifically TLR4 in peripheral blood macrophage membranes, was found to operate through free fatty acids bound to the glycoprotein fetuin-A (156), resulting in an increase in pro-inflammatory cytokines and oxidative stress. The canonical pathway of TLR4 activation requires lipopolysaccharide (LPS) and occurs more quickly. A common inflammatory pathway directed by TLR4 relies on the activation of nuclear factor-Kappa B (NF-κB) through the IκB kinase (IKK) complex in macrophages (12,157,158). NF-κB is important in the induction of
adhesion during smooth muscle cell proliferation surrounding atherosclerotic sites (159), and it also regulates signaling pathways from TLR4, the receptor responsible for initiating the innate immune response in atherosclerosis (160). Human macrophages protect the vascular endothelium by removing oxidized phospholipids and cellular debris from inflamed vascular intima, thereby preventing atherosclerotic lesions. Atherosclerotic development is enhanced by high levels of certain free fatty acids, typically saturated and \textit{trans} monounsaturated free fatty acids (15,16). Through binding with TLRs and fetuin-A, free fatty acids may act as a catalyst for an inflammatory storm. Fortunately, the human body has mechanisms to reverse the inflammation, but many of these processes are not completely effective or well understood.

Because of the lack of information regarding mechanisms, we chose to search for fresh information by conducting an exploratory gene array comparing elaidate (EL) (C18:A9-10 \textit{trans}; (E)-octadec-9-enoate)- and oleate (OL) (C18:A9-10 \textit{cis}; (Z)-octadec-9-enoate)-stimulated human macrophages after 44 hours of exposure. An interesting expression difference was found in the metallothionein (MT) family of genes. The primary function of the MT families of proteins is intracellular zinc storage. Zinc is essential to many biological processes and interacts with more than 2800 proteins (161). Although MT-null mice subsist, they cannot store as much zinc as wild type mice, even with zinc-loading diets, and zinc supplementation is required for their survival (162,163). Labile zinc inhibits various enzymes in vitro (95), and has been shown to influence over 1000 genes coding for cytokine manufacture and innate immunity in the monocyte cell line THP-1 (164).

Since our expression array results suggested that MTs were involved in free fatty acid signaling in macrophages, we chose to determine whether these alterations, in turn, would affect labile zinc in macrophages. In this study, we incubated human macrophages with the following...
fatty acids: oleate (OL), elaidate (EL), stearate (ST), and palmitate (PA). Here we show that saturated and trans-monounsaturated free fatty acids have differing effects on the availability of labile zinc but both fat types cause increased nuclear localization of NF-κB. We also displayed evidence for the role of zinc in activating of NF-κB.

4.3 EXPERIMENTAL PROCEDURES

4.3.1 Cell Culture

Human peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats as described (142). The PBMCs were transferred to AIM-V medium containing 20 μg/ml human CSF-1 (Peprotech, Rocky Hill, NJ). For quantitative PCR and expression array analysis, PBMCs were CD14 purified with magnetic beads from Miltenyi Biotec (San Diego, CA) as described (142). Three days after isolation, CD14 purified cells or PBMCs were treated with 30 μM OL, EL, ST, and PA, all in fat-free BSA (Sigma Aldrich, St. Louis, MO), with 20 μg/ml human CSF-1 in DMEM. Controls included a 10% FBS and a BSA only incubation. Fatty acid solutions were solubilized in BSA in PBS as described (146). For the expression arrays, qPCR and zinc quantification studies, macrophages were treated for 15 or 44 hours with fatty acids. For the NF-κB quantification studies, macrophages were treated with fatty acids for 1 hour.
4.3.2 Expression Array Studies

PBMCs were purified and plated as described above including CD14 magnetic bead purification (142). After a 44 hour incubation with either 30 μm elaidate or 30 μm oleate, the media was removed and the cells were washed twice with PBS. Macrophages were treated with 0.25% trypsin EDTA at 37 °C to loosen cells. Trypsin action was stopped with an equal volume of DMEM with 10% FBS. Cells were scraped from the plate and washed twice with PBS before freezing at -80 °C. All further expression analysis was performed at University of Pittsburgh Department of Pathology using the Affymetrix human U133 Plus 2.0 array per the manufacturer’s directions.

4.3.3 Messenger RNA Expression Studies

PBMCs were purified and plated as described above including CD14 magnetic bead purification (142). Cells were incubated for 15 or 44 h in 30 μM fatty acids as above. The rest of the protocol was followed as described (115). QuantiTect Primer Assays were used for human metallothionein-1X (MT1X), metallothionein-2A (MT2A), and solute carrier family 39, member 10 (SLC39A10) as directed (Qiagen, Venlo, Netherlands). Results were normalized as a percentage of GAPDH (Life Technologies, Grand Island, NY) expression.

4.3.4 Quantification of Labile Zinc by FluoZin-3, AM Fluorescence Microscopy

Human primary macrophages were cultured as described on 35 mm MatTek (Ashland, MD) dishes containing a 10 mm glass microwell. Cultures included the following fatty acid treatments
at 30 μM: OL, EL, ST, and PA, and two controls, 10% FBS and BSA alone, in DMEM with 20 μg/ml human CSF-1. Cultures were incubated with all six combinations for 15 or 44 hours. Cells were then rinsed with fat-free DMEM, and 5 μl of 5 mM FluoZin-3, AM (Life Technologies, Grand Island, NY) in 1 ml of DMEM was added. After incubation for 30 minutes, the FlouZin-3 was removed by rinsing with fat-free media. Cells were photographed immediately in fresh fat-free media using an AndorZyla VSC-00073 camera with 40x oil DIC H N2 optics with a 200 ms exposure and a mono 16 bit image readout. To quantify the intensity of signal, 11-21 images were measured per condition, with approximately 100 cells in each image.

4.3.5 Quantification of Nuclear Localization of Nuclear factor Kappa B +/- TPEN

Human primary macrophages were cultured as described on 35 mm MatTek (Ashland, MD) culture dishes with a 10 mm glass microwell. For studies, cells were treated for one hour +/- 5 μM N,N,N,N’-Tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) at 37 °C with all treatments and controls described above. All of the subsequent steps were performed at room temperature. Cultures were rinsed with PBS containing 0.2 mM EDTA and fixed in 3% formaldehyde for 15 minutes, followed by another rinse. Cells were blocked with 1% BSA for 30 minutes. Then, they were incubated with 500 μL of 1:250 NF-κB antibody from Sigma (St. Louis, MO). After another rinse, the cultures were incubated with 1:50 Alexa Fluor 594 donkey anti-rabbit for one hour (Life Technologies, Grand Island, NY) and rinsed. Cultures were finally incubated in Hoescht fluor (Life Technologies, Grand Island, NY) for one minute. The cells were rinsed and cultures were stored in PBS with EDTA at -20°C until ready for photography. Cell nuclei were defined by the Hoescht fluor, and the nuclear proportion of NF-κB was determined. Images were obtained on a Nikon TE2000 inverted phase-fluorescence microscope using a 12-bit 1600×1200
pixel monochrome charge-coupled device with an RGB filter wheel for color photographs (Spot Instruments, Sterling Heights, MI). For red fluorescence, excitation was at 536–556 nm with a 580 nm dichroic mirror and a 590 nm barrier. For blue fluorescence, excitation was at 380–425 nm with a 430 nm dichroic filter and a 450 nm barrier. Fluorescent signal was photographed using 1.3 NA 40× or 100× oil objectives.

4.4 RESULTS

4.4.1 Gene Expression Array

The gene expression array showed that macrophages incubated for 44 hours in 30 μM elaidate had decreased expression of six MT genes (with ten different MT probes) relative to those incubated with its cis isomer, oleate (Table 1). In addition, the gene for the zinc transporter, SLC39A10 had enhanced expression. DDX42 is a metallothionein-related nuclear protein.

<table>
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4.4.2 Messenger RNA Expression Studies

We assayed MT1X, MT2A, and SLC39A10 expression in CD14 purified macrophages by quantitative polymerase chain reaction (qPCR) after 44 hours of incubation (Figure 4.1A) to confirm the expression array results. EL-treated cells had the lowest expression for both MTs (p<0.01 compared to OL and p<0.001 compared to ST and PA). The OL-treated cells were intermediate in expression (p<0.01 compared to all other treatments), and the saturated fatty acid-treated cells, ST and PA, had the highest MT expression (p<0.001 compared to all other treatments). Also, the EL-treated cells showed the highest levels of SLC39A10 expression (p<0.05 compared to PA, and p<0.01 compared to OL and ST) of the fatty acid treated cells, while OL-, ST-, and PA-treated all had less SLC39A10 expression than the controls (p<0.05). At 44 hours, saturated fatty acids caused the highest expression of MTs, while EL-treated cells had the highest expression of the zinc transporter, SLC39A10.
Figure 4.1 Fatty Acid Treatment Effects on Two Metallothioneins and a Zinc Transporter

Macrophages (n = 3-5) were treated for 15 or 44 hours with 30 μM fatty acid as described. A. Quantitative PCR measurement of mRNA expression as % GAPDH after 44 hours of fatty acid incubation. With both MTs, incubation with the saturated fatty acids ST and PA, showed higher MT expression compared with EL, OL, and the two controls, BSA and FBS. EL-treated cells had the highest expression of the zinc transporter, SLC39A10, with both saturated fatty acids having the lowest. (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). B. Quantitative PCR measurement of mRNA expression after 15 hours of fatty acid incubation. With both MTs, highest expression was with the OL and FBS treatments. The zinc transporter, SLC39A10, had the significantly highest expression in control treated cells, with all other treatments lower in expression. (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). Changes over time are indicated by the lines and arrows between graphs (**p<0.01, ***p<0.001). Only OL and EL treatments caused a significant change over time.

To determine whether gene expression differences were constant over time, we performed a 15 hour time point with the same expression experiments (Figure 4.1B). Expression of both MTs in saturated fatty acid-treated cells barely changed, while their expression in OL-treated and controls was significantly higher (p< 0.05 compared to PA-treated and p< 0.01 compared to ST and EL). EL-treated cells had more MT expression, similar to that of ST and PA. The expression of SLC39A10 was highest in controls, but only significantly lower in OL-
treated cells (p<0.06). Thus, we confirmed that MT expression is affected by fatty acid type and that the MT expression changes over time. The zinc transporter expression changes later.

The change in expression of the genes in the macrophages by treatment from 15 to 44 hours is represented by the dashed lines between Figure 4.1A-B. OL and EL treatment caused significant decreases (p<0.001, p< 0.01, respectively) in MT expression over time. Saturated fatty acids, ST and PA, did not affect MT expression over time. None of the treatments caused a significant change in the zinc transporter, SLC39A10, from 15 to 44 hours.

4.4.3 Labile Zinc Quantification by FluoZin-3, AM

To confirm that the MT and SLC39A10 expression alterations modified the intracellular labile zinc concentration, immunofluorescence was conducted on PBMCs treated as described above using FluoZin-3, AM (Figure 4.2). FluoZin-3 binds only Zn^{+2} ions and is the most specific marker for low concentrations of intracellular Zn^{+2}. At the 44 hour time point, the EL-treated cells had the highest quantity of labile zinc (p< 0.001 compared to all other treatments), in contrast to the lowest MT and elevated SLC39A10 expression at this time. However, low expression levels of MTs and in OL-and control-treated cells did not correspond to the minimal zinc levels quantified. However, the low expression of SLC39A10 in OL- and control-treated cells does correlate to the lack of zinc quantified. A higher level of zinc would be the contrasting quantity in OL-treated cells based on the low expression of the MTs. ST and PA-treated cells showed a contrasting intracellular zinc level to the expression results, even though SLC39A10 expression was low. At the 15 hour time point, the values for each treatment contrasted with the expression levels of the MTs, as would be expected and zinc quantities significantly differ from each other (Figure 4.2B). EL-, ST-, and PA-treated cells had the most labile zinc (EL: p< 0.001
compared to controls, p<0.01 compared to OL; ST: p<0.001 compared to OL and controls; PA: p<0.001 compared to OL, and controls). Although EL, ST, and PA have more and similar labile zinc levels at the 15 hour time point, at 44 hours, there is relatively less labile zinc in saturated fatty acid-treated macrophages. The indicators between graphs signify the change over time (Figure 4.2). In EL-treated cells, the measured amount of labile zinc increased (p<0.05) from 15 to 44 hours, While in ST- and PA-treated cells, the amount of measured labile zinc decreased over time (p<0.001 and p< 0.05 respectively), while the amount of labile zinc remained unchanged in OL-treated macrophages.
Intracellular zinc activity after fatty acid incubation as measured by FluoZin-3, AM. Macrophages were treated with 30 μM fatty acids for 44 or 15 hours, and FluoZin-3, AM was applied for 30 minutes. Cells were photographed in untreated media. A. Images of cells at the 44 hour time point. Note that the EL-treated cells had the most zinc signal with PA-treated cells showing the least. B. Quantified intracellular labile zinc after treatment with fatty acid for 44 hours. EL-treated cells significantly had the most labile zinc, while the PA-treated cells had the least. (*p<0.01, **p<0.001, ***p<0.0001) C. Quantified intracellular labile zinc after treatment with fatty acid for 15 hours. EL-, ST-, and PA-treated cells had significantly higher labile zinc levels than the other three conditions (*p<0.01, **p<0.001, ***p<0.0001). From 15 to 44 hours, labile zinc levels increased only for EL-treated cells (p<0.01), while decreasing or not changing significantly for all other treatments. Labile zinc levels decreased with ST- (p<0.0001), with PA-incubation (p<0.01), and with FBS (p<0.0001), while OL and BSA did not significantly change labile zinc levels from 15 to 44 hours.

4.4.4 Nuclear Factor-κB Quantification by Immunofluorescence Microscopy

Because we hypothesized that the zinc response to fatty acids might be associated with their role in inflammation, we wanted to determine whether fatty acids utilize zinc in activating NF-κB.
Since NF-κB translocation is rapid (165), fatty acids were incubated for 1 hour. After treatment, EL, ST, and PA-treated macrophages showed the greatest proportion of intranuclear NF-κB (EL, ST, and PA: p< 0.0001 compared to OL and controls). OL-treated cells showed the lowest NF-κB nuclear localization at 22% similar to controls. The increased intranuclear proportion of NF-κB in the trans- (65% NF-κB nuclear localization) and saturated fatty acid-treated macrophages (57% NF-κB nuclear localization) indicates probable downstream signaling similarities and is consistent with our previous results at the 15 hour time point.

To determine whether labile zinc played a role in the NF-κB pathway response, 5 μM of the zinc chelator, TPEN, was added to the incubation media. After TPEN treatment, all conditions had similar nuclear NF-κB proportions with an average of 30% for all treatments. Relative to the original incubations, the proportion of nuclear NF-κB decreased in the EL-, ST-, and PA- treated cells (EL, ST, and PA: p<0.0001) and increased in OL-treated and controls (OL, FBS: p< 0.05, BSA: p<0.0001). (See arrows between 3A and 3B). This experiment provides evidence that labile zinc plays a role in activation of NF-κB.
Macrophages were treated with 30 μM fatty acids for 1 hour with and without the addition of 5 μM TPEN. Immunofluorescence was conducted on the fixed cells and photographed. A. Images of cells from both extremes of response. OL-treated cells show almost no nuclear localization of NF-κB. Dark holes can be seen where the nuclei should be. In the EL-treated cells, nuclear localization of NF-κB is obvious in the brightest areas of the cells. B. Graph showing the proportion of NF-κB localized to the nucleus with each treatment. EL-, ST-, and PA- treated cells show significantly more nuclear localization than do the OL- and control-treated cells (*p<0.01, **p<0.001, ***p<0.0001). C. This graph shows the same experiment after the addition of 5 μM TPEN to the incubation medium. The addition of TPEN neutralized the effect that treatment had on the localization of NF-κB as treatment had no effect on NF-κB localization. Interestingly, all treatments that showed an increased NF-κB in graph 3B showed a significant decrease in NF-κB localization with the addition of TPEN, while all other treatments showed a significant increase in NF-κB localization with the addition of TPEN (*p<0.01, **p<0.001, ***p<0.0001).

4.5 DISCUSSION

Initially, we performed an expression array analysis to identify mechanisms involved in EL toxicity in human macrophages. When incubations of macrophages with EL were compared to
those with OL, eight genes with products that affect zinc homeostasis were identified as altered. When we validated these gene alterations by qPCR, we expanded the study to include a comparison to incubation with saturated fatty acids, since these compounds are also associated with causing atherosclerosis. Our study shows that although EL, ST, and PA increase intracellular zinc levels at shorter time points, EL causes a sustained increase in labile zinc that lasts longer than that with saturated fatty acids, as evidenced by the FluoZin-3 quantification of labile zinc at 44 hours (Figure 4.2). The increase in expression of zinc transporter, SLC39A10 cells treated with EL coincides with elevated zinc concentration (Figure 4.1).

In our previous work (115), EL was shown to stall fatty acid metabolism and cause accumulation of intermediates. We hypothesize that EL is difficult for macrophages to degrade, leading to an increase in labile zinc whereas all other treatments caused a decrease or no change over time. Labile zinc is also a key modulator of NF-κB activation (Figure 4.3), as evidenced by the neutralization of NF-κB activity by the labile zinc chelator, TPEN. Zinc homeostasis in macrophages is altered by ST and PA as evidenced by the increased labile zinc measured at the 15 hour time point (Figure 4.2C) and the high proportion of NF-κB localized to the macrophage nucleus in both treatments, compared to OL-treated cells and controls (Figure 4.3B). The lack of zinc elevation at 44 hours in OL and controls suggests that the reduction of expression of MTs does not always negatively correlate with zinc release. Obviously, MTs and SLC39A10 follow a different mechanism in OL- and control-treated cells than threatening treatments, and further experimentation would be required for explanation. MTs comprise a 10-isoform family with four major subdivisions (166) of small proteins that are localized to the Golgi apparatus in macrophages and many other cell types (167). MT is distinctive in that it has no aromatic compounds in its structure and is composed of approximately 30% cysteine residues which
enable it to loosely bind heavy metal ions, such as zinc, nickel, copper, mercury, and silver (167,168). Pro-inflammatory cytokines and oxidative stress induce the synthesis of the MT protein. Because little is known about the zinc transporter SLC39A10, we cannot speculate as to its contribution to labile zinc levels. The increased levels of intracellular zinc in \textit{trans} and saturated fatty acid-treated cells corresponded to the low level of MT expression at the 15 hour time point but only continued in the 44 hour time point for \textit{trans}-treated macrophages. Thus we conclude that although EL, ST, and PA are all toxic to cells, the response to EL is more persistent and could lead to further downstream issues.

We have demonstrated that certain fatty acids alter intracellular zinc levels which in turn can have downstream consequences to NF-κB activation (Figure 4.4), but the mechanism by which zinc directly modulates NF-κB is unclear. In our macrophages, the addition of zinc chelator, TPEN, to the culture medium neutralized the response of NF-κB. Without labile zinc, fatty acid treatment had no effect on NF-κB and held around 30% nuclear localization for all treatments. According to the literature, many experiments with various cell types and parameters have been conducted on the relationship between zinc and NF-κB, but the exact mechanism is still unclear (169-172). Furthermore, zinc has been shown to positively and negatively affect toll-like receptors, as well as NF-κB (171,173,174). Zinc and NF-κB have been widely studied, but no previous experiments have assessed the consequences of free fatty acids on zinc and NF-κB together.
Figure 4.4 Effects of fatty acids on zinc metabolism in human macrophages

Fetuin-A binds free fatty acids in the cytoplasm which aids in their interaction with TLR4. In this way, free fatty acids signal through TLR4. TLR4 activates cytokine and ROS production, which allows NF-κB liberation from the IKK complex and entrance to the nucleus. NF-κB continues to up-regulate proinflammatory cytokines. Metallothioneins (MTs) begin to release Zn^{2+} ions in order to counteract the inflammatory storm. Also, when MT is not bound to zinc, it can act as an antioxidant neutralizing free radicals. When the inflammation is under control, metal-responsive element-binding factor 1 (MTF-1) signals to MT to begin collecting zinc ions again. Zinc homeostasis is crucial to control inflammation while allowing enough inflammation to prevent the injury or infection from spreading. Zinc ions can only travel across membranes with the use of transporters. The SLC30 family functions to bring zinc out of the cytoplasm into organelles or out of the cell completely. The SLC39 family functions to bring zinc into the cytoplasm from outside or from the organelles.

It is well documented that saturated and trans-fatty acids cause atherosclerotic build-up (14-16), but their mechanism for causing this problem, especially regarding zinc signaling, is unknown. Labile zinc must be actively transported across membranes, and transporters occur as two families: the Zip family and the Znt family. Both families are located in organelle and cell

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membranes. The Zip family of transporters increases the concentration of labile zinc in the cytosol, while the Znt family reduces its cytosolic concentration (175). Expression of the Zip family member SLC39A10 was significantly increased when macrophages incubated with EL were compared with those incubated with OL in the expression array (Table 1).

A main relationship of zinc with NF-κB is the balance of inflammation during injury and/or infection (173). Inflammation activated through NF-κB prevents the spread of injury and infection but damages the cell, while zinc reduces inflammation by inhibiting NF-κB activation, modulating proteins and interacting with cytokines (99,176). Because of the delicate balance between the need for inflammation and the need to suppress it, the level of zinc required to maintain homeostasis is difficult to predict (174). In addition, all cell types have a critical limit of zinc where it becomes toxic.

In a study conducted in LPS-stimulated RAW 264.7 mouse macrophages, the thiol-reactive metals gold, zinc, and copper, blocked the increase in NF-κB activation from the LPS stimulation by preventing the activation of the IKK complex (176). Although these results are counter to ours, several recent studies have shown that LPS stimulates by a different mechanism than fatty acids such as palmitate (177). This study also mentions that before the stimulation by LPS, there was little IKK activation. Since there was no significant difference in the treatment effect on NF-κB nuclear localization with TPEN, it is probable that the chelation of zinc has an effect on or upstream of the IKK complex. Zinc has the ability to directly affect TLRs (174), and LPS activation (100), which are positioned between fatty acid stimulation and the IKK complex on the pathway of interest.

Although TPEN is frequently used in experiments to chelate labile zinc, TPEN can affect bound zinc within the cell. The zinc finger transcription factor, Zn3-Sp1 was influenced by TPEN.
at 100 μM concentrations in U87 mg glioblastoma cells, which contain a large amount of MT that is unsaturated with zinc (apo-MT). Without TPEN, Zn₃-Sp1 maintains its zinc levels even in the presence of apo-MT, which has a moderate affinity for zinc. Yet, the addition of a more powerful zinc chelator like TPEN causes the loss of Zn₃-Sp1 structural integrity in about 30% its total protein (178). Since our study limited TPEN to 5 μM, the chelation may not have been as extensive. However, this study demonstrates that TPEN can influence zinc-finger motifs. One example of a zinc-finger containing component of the NF-κB pathway is IKKγ or nuclear factor-κB essential modulator (NEMO), which is a subunit of the IKK complex (Figure 4.4). The proper functioning of NEMO is essential for NF-κB activation (179). It is possible that the addition of TPEN interfered with NEMO’s function, and we plan to address this question in the future.

Studies of NEMO have confirmed that it must associate with zinc for proper NF-κB activation, even though zinc has been found to inhibit the activation of NF-κB. In fetal mouse fibroblasts, zinc inhibits NF-κB by binding with IKKβ, another subunit of the IKK complex (169). When researchers grew fibroblasts in vitro with a zinc deficient medium, they found that NF-κB nuclear localization greatly increased. NF-κB was also increased with the addition of TPEN. In the same study, IKKβ (-/-) cultured mouse embryonic fibroblasts (MEFs) showed reduced NF-κB activation when stimulated by the cytokine IL-1β compared to controls, even in the presence of intact IKKα, which suggested that IKKβ and not IKKα was the site of zinc inhibition of NF-κB. Confocal microscopy studies with overexpressed DS-red tagged IKKβ further supported the premise that IKKβ may indeed bind labile zinc under LPS stimulation. Co-localization between tagged IKKβ and labile zinc occurred in the cytosol of the cells. This same study also included a set of experiments with NEMO (-/-) MEFs and concluded that zinc-
regulated effects were not observable because the IL-1β did not stimulate these cells (169). Damage to NEMO’s ability to recognize threats would support our data because all treatments resulted in the same percentage of NF-κB nuclear localization. NEMO was also rendered null in MEFs in an experiment by Rudolph, et al. 2000. Even after stimulation by LPS, IL-1, and other inducers, detectable NF-κB DNA binding did not occur. These researchers concluded similarly that functioning NEMO is required for full NF-κB activation and translocation to the nucleus, hence, it is required for threat recognition. However, this study is conducted in fetal mouse fibroblasts which do not express TLR4. The NF-κB mechanism must function through another path.

Based on our results and the previous studies, we hypothesize that the incubation of primary human macrophages with trans and saturated fatty acids results in the activation and translocation of NF-κB through zinc signaling in IKKβ with threat recognition by NEMO (Figure 4.3A), with significantly more response than is found with cis-fatty acid-treated cells or controls. With the addition of TPEN, we showed that zinc has a significant effect on NF-κB activation (Figure 4.3B), presumably by TPEN altering the structural integrity of NEMO, as well as lack of zinc for the binding sites of IKKβ. Since only 5μM TPEN was used for a one hour incubation time in our experiments, it is deduced that the low concentration caused minimal damage to NEMO. In the provoking treatments (EL, ST, PA), the proportion of NF-κB nuclear localization was reduced by about half with the addition of TPEN (Figure 4.3B), while NF-κB nuclear localization actually increased slightly in the OL- and control-treated cells. Furthermore, our NF-κB findings show another route for zinc metabolism to influence inflammation. Because NF-κB is crucial in the innate immune response to atherosclerosis (159,160), it is important to fully elucidate the ways in which NF-κB can be rendered dysfunctional. According to the World
Health Organization, zinc deficiency accounts for 1.4% of deaths globally (180), so it is important to understand labile zinc interactions with NF-κB activation, as well as the molecular structural issues that take place in the human body. The direct relation of dietary intake to atherosclerosis generation also provokes the thought that dietary fatty acids may influence many other novel pathways.
5.0 OTHER WORK

5.1 OTHER QUANTITATIVE PCR

After inspection of the gene expression array conducted on human macrophages treated for 44 hours with oleate (OL) or elaidate (EL), there were many obvious groupings to which the data pointed: A. Lipotoxicity genes, B. inflammatory genes, C. EGF family genes, D. macrophage differentiation genes in FBS and BSA conditions as well, and genes involved with zinc metabolism (Figure 4.1). Figure 5.1D shows that macrophages used in all of our experiments were M1 activated macrophages. This information will be used in a later publication. All graphs apart from D are measured in fold change from OL to EL. Graph D is measured in % GAPDH since there are more than two conditions shown. n=3-5 for all mRNAs measured. Genes are discussed in Appendix A.
5.2 LIPID INCLUSION QUANTIFICATION

In the project to explore lipotoxicity, I conducted multiple fluorescence stains on treated cells to identify and quantify lipid droplets in different FA conditions. Stains and fluors used were Oil Red O, Bodipy, and LipidTOX. After multiple attempts, this protocol was abandoned because of unreliable results.
5.3 CYTOKINE DETECTION

I collected supernatants from treated cells to be examined for cytokine production in various treatments. This data was successful and will be published at a later date.
5.4 HISTOLOGY

I embedded and cut frozen sections for two different projects. One was for a colleague in Blair lab, and the other was for Dionysios Papachristou MD, PhD in Greece. This data was successful and will be published at a later date.
6.0 CONCLUSION

As a continuation of studies already completed in the lab, this dissertation initially aimed to further understand the toxicity of the trans-fatty acid, elaidate, primarily by comparing macrophage responses to lipotoxic situations. According to previous data from acylcarnitine profiling of cell supernatants and GC/MS of whole cells both treated with OL, EL, ST, or albumin alone, fatty acid intermediates accumulated that were both longer and shorter carbon chains than the original fatty acid in the EL-treated cells, leading us to believe that the enzyme step in question is reversible. Also, the main intermediate accumulating was C12:1-carnitine. This intermediate would be formed right before the enoyl-CoA-isomerase step of β-oxidation. Fatty acids are consumed in the diet in mixtures, so we utilized competitive tritium release to determine what effect the trans blockage had on β-oxidation of other fatty acids. Since human macrophages are the cells that remove of lipid waste at pre-atherosclerotic sites, they were ideal to use in a lipid-handling experiment. Radiolabeled oleate was used in the macrophage media as a β-oxidation competitor in three situations: mixed 1:1 with cold oleate as a control, mixed 1:1 with cold elaidate, and mixed 1:1 with cold stearate. We observed that the macrophages β-oxidation of oleate was partially blocked by the presence of elaidate compared to the control situation, and oleate was preferred to stearate. Using 1-C\textsuperscript{14} labeled oleate and elaidate, we found that macrophages perform the initial round of β-oxidation of oleate and elaidate at equal rates, statistically, with a trend toward more rapid elaidate metabolism. Therefore, it was concluded
that elaidate inhibits the metabolism of oleate. This suggests that macrophages have difficulty negotiating the *trans* bond of elaidate, which accounts for the stall in β-oxidation shown by the radiolabeled oleate experiment.

Others have demonstrated the ability of fatty acids to directly signal to pathways of inflammation and distress, but many are not well understood. The interconnectivity of so many pathways makes it difficult to define one individual pathway and determine its significance. In order to further discriminate fatty acid effects on signaling pathways, we conducted a gene expression microarray analysis on primary human macrophages treated with 30 μM oleate or elaidate for 44 hours. The expression array revealed that elaidate affects the expression of many zinc metabolism genes, particularly metallothioneins (MT). In cells treated with elaidate, MT expression was greatly reduced, and zinc transporter expression of those genes coding for an increase of zinc into the cytosol was significantly increased compared to cells treated with oleate. Since the main function of MT is to bind heavy metals, particularly zinc, we assumed that the lowering of the expression of MT in elaidate-treated cells signified a release of labile zinc ions to be in the cell. Quantitative PCR confirmed the reduction in MT expression at the 44 hour time point, but a question of how these changes compare to those found with saturated fatty acids remained. Because of the expense of expression microarrays, saturated fatty acid information was only collected using qPCR. We found that in cells treated with both stearate and palmitate MT expression was increased relative to cells treated with either oleate or elaidate. Finding this odd, we decided to reduce our incubation time to see if MT expression was greater or lesser at 15 hours. This hunch was correct as MT expression in elaidate-treated cells was about 200% higher at 15 hours, and oleate-treated cells showed a 300% increase of expression at 15 hours.
Interestingly, MT expression was not appreciably changed in saturated fatty acid-treated cells (Figure 4.1). To assess the functionality of MTs, labile zinc had to be quantified.

Changes in available zinc were measured by FluoZin-3, AM within the macrophages at all treatments and both time points. After 15 hours of fatty acid incubation, elaidate-, stearate-, and palmitate-treated cells all showed high intracellular concentrations of zinc compared to oleate-treated cells and control cells. At 44 hours, oleate and controls remained about the same, while stearate- and palmitate-treated cells showed a decrease in intracellular zinc concentration. Elaidate-treated cells were the only treated cells to show an increase in intracellular zinc at the 44 hour time point. These results support the conclusion that while elaidate, stearate, and palmitate all cause an initial zinc elevation, this response is persistent in the elaidate treatment. The reduction in MT expression and the high amount of labile zinc quantified in the elaidate-treated cells at both time points lead us to believe that the cells are respond to the trans threat with zinc release at 15 hours, and that the response lasts until the 44 hour time point. Even though stearate and palmitate caused zinc elevation at 15 hours, it seems to be over by the 44 hour time point. It also coordinates with the idea that oleate- and FBS-treated cells showing a reduction in MTs over time do not necessarily equate with zinc release. Reduction in MT alone does not prove distress, but must coordinate with a release in zinc ions as well.

To prove that labile zinc (formally, zinc activity) was actually acting as a counterbalance to the threats that are saturated and trans-fatty acids, we decided to single out a common inflammatory factor, NF-κB. Because fatty acids are known to signal to macrophages through the membrane bound Toll-like Receptor 4 (TLR4) and the IKK complex, we thought that NF-κB, a downstream responder to TLR4 activation whose activation requires zinc, would be a logical place that this labile zinc would act. The IKK complex is made up of three subunits, IKKα,
IKKβ, and IKKγ or NEMO. Zinc has been shown to directly modify IKKα and IKKβ, but not NEMO. However, the structural stability of NEMO depends on a zinc finger motif (181).

Immunofluorescence was conducted on cells treated for 1 hour with all six fatty acid incubation conditions. To verify the involvement of zinc, we also conducted a parallel experiment adding a zinc chelator, TPEN, to the incubation media. TPEN has been shown to chelate free (active) zinc and disrupt zinc finger structural motifs in some intracellular proteins (179). The proportion of NF-κB activated was determined by nuclear localization. Elaidate-, stearate-, and palmitate-treated cells had the highest NF-κB nuclear localization at 0.5, while oleate-, FBS-, and BSA-treated cells were only around 0.2. When the zinc chelator, TPEN was added, the effects of the fatty acids on NF-κB localization to the nucleus were completely neutralized. The more NF-κB activated cells showed a decrease, while the less threatened cells showed an increase in NF-κB nuclear localization. All six treatments showed a proportion of approximately 0.3. Our suspicions are that zinc is required for conventional activation of NF-κB, and that TPEN removes zinc and disrupts the process. Since zinc supplementation has been shown to interact with IKKα and IKKβ to deactivate NF-κB, we were initially confused that the NF-κB in our cells was not much higher in all treatments. Upon further reading we discovered that TPEN may also damage zinc fingers rendering proteins containing them damaged. NEMO is required for NF-κB activation and may be damaged by the addition of TPEN. TPEN was only included at 5 μM for a one hour incubation. Therefore, we hypothesize that TPEN may have damaged NEMO in some cells reducing the zinc-deficient activation of NF-κB, regardless of treatment.

Overall, these studies have shown that the trans-fatty acid, elaidate causes a partial blockage in β-oxidation leading to the stall of metabolism of oleate. The resulting trans-fatty
acid signaling affects metallothioneins which release zinc in the cell, presumably to counteract inflammation. The zinc response is more intense and prolonged than the response to saturated fatty acids stearate and palmitate.

There are limitations to the validity of these studies. One weakness is the lack of activation of our cells with LPS or fetuin-A. Because oleate was used as a control, this weakness does not void the results, but a greater reaction may have been seen in activated cells. Another limitation is that primary macrophages were used. Although using primary human cells may be more physiologically relevant, primary cells are sometimes difficult to manipulate and their viability is less than transformed cells.

In the future, these results may be utilized to increase the understanding of how dietary fatty acids affect signaling. Also, the role of zinc in inflammation and heart disease may be further investigated to determine a proper zinc dosage for optimum health. The field of public health may be greatly influenced by these studies since heart disease is the number one cause of death in the United States. An active effort to remove trans fats from the diet would increase public health, as would a program to ensure appropriate zinc supplementation for the population.
APPENDIX: GENE ABBREVIATIONS

Lipid Synthesis and Fat Transport

- **SREBP1** – sterol regulatory element binding transcription factor 1, transcription factor that binds to the sterol regulatory element-1 (SRE1), which is a decamer flanking the low density lipoprotein receptor gene and some genes involved in sterol biosynthesis. The protein is synthesized as a precursor that is attached to the nuclear membrane and endoplasmic reticulum. Following cleavage, the mature protein translocates to the nucleus and activates transcription by binding to the SRE1. Sterols inhibit the cleavage of the precursor.

- **SCD1** – stearoyl-CoA desaturase (delta-9-desaturase), catalyzes rate limiting step in unsaturated fatty acid synthesis. (Main product is oleic acid)

- **LPIN1** - Plays important roles in controlling the metabolism of fatty acids at different levels, Acts also as a nuclear transcriptional coactivator for PPARGC1A/PPARA to modulate lipid metabolism gene expression

- **DHCR7** – 7-dehydrocholesterol reductase, catalyzes the final step in cholesterol synthesis.

- **LDLR** – Low Density Lipoprotein Receptor, used for anterograde cholesterol transport (from Liver to extrahepatic cells).
• ABCA1 and G1 – ATP-binding cassette, sub-family G (WHITE) member 1, Molecule Transporter. Macrophage cholesterol and phospholipids transport, and may regulate cellular lipid homeostasis in other cell types.

EGF Family
• TGFBR1 – transforming growth factor beta receptor 1, protein kinase for signal transduction of TGF.
• HBEGF – heparin-binding EGF-like growth factor, involved in macrophage-mediated cellular proliferation
• EREG – Ligand for EGFR (Epidermal Growth Factor Receptor)
• AREG – amphiregulin, member of the epidermal growth factor family, related to epidermal growth factor (EGF) and transforming growth factor alpha (TGF-alpha). This protein interacts with the EGF/TGF-alpha receptor to promote the growth of normal epithelial cells
• SOCS3 – Suppressor of cytokine signaling 3, negative regulator of cytokine signaling

Immune Response
• IL-1B – Interleukin-1B, Innate immune inflammatory molecule.
• PTGS2 – Cox-2, Inflammatory mediator in the arachidonic acid pathway.
• NR4A2 – nuclear receptor subfamily 4, group A, member 2, Modulates DA metabolism. Also modulates fatty acid metabolism in some diseases such as colorectal cancer.
Macrophage Activation

- PFKFB1 - 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 1
- PFKFB3 - 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3

Zinc Regulation

- MT-2A and MT-1x – metallothioneins, contain high cysteine content to bind various heavy metals, transcriptionally regulated by both heavy metals and glucocorticoids
- SLC39A10 – Transporter of zinc into the cytosol


