The Roles of Lipoproteins, Diet, and Peripheral Macrophages in Atherosclerotic Disease

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Abstract

We consider lipid accumulation in atherosclerosis, with emphasis on mechanisms mediating atheroma growth at peripheral sites. Macrophages normally recycle all dead cell components, including membranes. Membrane lipids are exported, as cholesterol or cholesterol esters, by lipoproteins for disposal by the liver or, as triglycerides or phospholipids, for lipid storage or re-use. Membranes of somatic cells, such as red blood cells, incorporate fatty acids that reflect dietary intake. When excessive saturated and trans-unsaturated fats are incorporated in cells, and the cells die, macrophages cannot fully recycle the membrane lipids, setting up a vicious cycle of lipid overload, cell death, recruitment of macrophages, and cell proliferation. Semi-liquid masses of partially oxidized fatty acids and cholesterol, foamy macrophages, and proliferating stromal cells accumulate in arterial walls. The dramatic increase of atherosclerotic disease since 1920 reflects superabundant nutrition and altered dietary composition, along with reduced exercise and smoking. The dietary changes included increased saturated and trans-unsaturated fats. The biochemical basis of epidemic atherosclerosis includes a partial metabolic block in β-oxidation caused by unsaturated fatty acid intermediates. Responses of cells to un-degraded saturated and trans-fatty acids include production of inflammatory cytokines, alteration of macrophage signaling pathways, and altered lipid-handling enzymes. Prevention of disease is an ideal approach, but pharmacological inhibitors including statins, PCSK9 inhibitors, or limiting macrophage catabolism of lipids by reducing carnitine availability, may limit progression of atherosclerosis.

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Lipoproteins and receptors

The discovery of the low density lipoprotien (LDL) receptor [1] was a pivotal event in study of atherosclerotic disease. Subsequent work created a complex web of clinical and experimental work focusing largely on plasma lipid components including high density lipoproteins (HDL), LDL, and triglycerides, with an expanding armamentarium of lipid-modifying drugs [2]. The use of plasma lipid profiles to monitor risk of atherosclerois risk is well reviewed elsewhere, and here we focus instead largely on the peripheral sites where atherosclerosis develops. Plasma lipid carriers are used by many different organs. This includes LDL and very low density lipoprotein (VLDL) receptors in skeletal and heart muscle, as transporters for lipids used in metabolism [3]. There are also specialized roles for lipoproteins; e.g., cortisol is made from cholesterol delivered to the adrenal via the HDL receptor Scarb1 -- absence of which causes greatly increased ACTH, but normal cortisol [4]. A surprising variety of lipoprotein defects have consequences on skeletal mass or joint disease [4,5]. Effects on the central metabolic pathways that regulate lipids have unexpected consequences such as deficiency of apolipoprotein E protecting mice from obesity and nonalcoholic fatty liver disease [6]. There are too many examples to enumerate. That said, although heart attacks and strokes account, together, for nearly 40% of deaths in “first world countries”, people do not have hereditary lipoprotein, or lipoprotein receptor, defects. Development of atherosclerotic disease reflects in major part environmental factors, diet, exercise, and smoking, and the pathology occurs entirely at peripheral sites and centers on macrophage metabolism. We consider here diet and macrophage function in atherosclerosis. We review the biochemical mechanisms affected by dietary fat that determine how and where atherosclerotic lesions accumulate. A central concept is that lipids carried on lipoproteins are not transferred into atherosclerotic lesions. Lipids are carried by lipoproteins; they are fuel for cell growth and metabolism, and in some cases, such as oxidized phospholipids at peripheral sites, are toxic to cells, typically macrophages, that receive these modified lipids. This reflects that lipoproteins are the acceptors of lipids; in atherosclerotic lesions they are processed by macrophages.

Historical perspective

Prior to World War II, atherosclerosis as a cause of death was uncommon. It was known in antiquity [7]. But, for most of history, it was a problem largely of rich individuals with rich diets, and a few unfortunates with genetic predilections, then unknown. As an illustration, deaths, in the over age 40 population group, attributed to ischemic heart disease from 1870-1980 are shown (Figure 1), from a century of autopsies at St Barts, London [8]. Many consistent reports noted the same disturbing trend around 1950. The median age of patients with myocardial infarction, depending on diagnostic criteria and population, is in the mid to late 60s, with two-thirds 45-74 years [9,10]. Since the turn of the 21st century, there are encouraging signs that atherosclerotic disease is declining with smoking decreased by half to two thirds since 1965, improvements in diet, and metabolic inhibitors to normalize blood pressure and control LDL cholesterol.

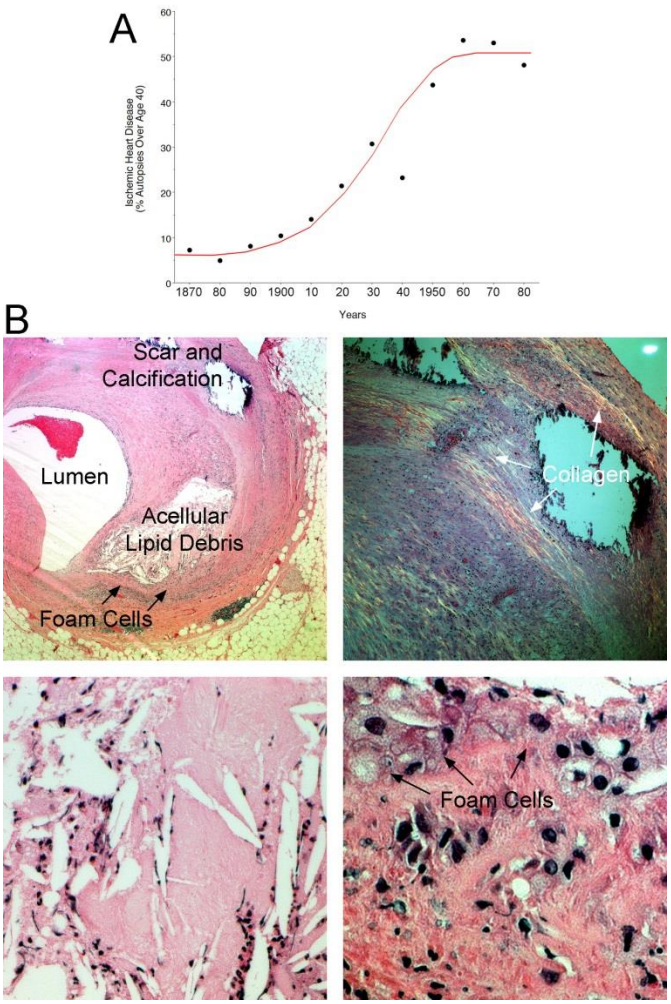

Figure 1. Atherosclerosis incidence and histological features.

A. Atherosclerosis as common pathology expanded rapidly around the time of world war II. Deaths from ischemic heart disease, of autopsies at St Bartholomew's Hospital, London, 1870-1980. Data from Table 4 of Finlayson, 1985 [8], are used with permission. Numbers of autopsies attributed to ischemic heart disease divided by the number of autopsies, in patients over age 40; data average the decade beginning with the date on the abscissa. In 1870 46% of deaths were over age 40, this increased to 70% at 1935, and then abruptly to 80-90% after world war two. A drop in the percent of ischemic deaths during world war two years (1940s) is ignored in the curve fit, but it might represent wartime rationing and related effects.

B. Features of an atherosclerotic lesion.

Top left. A low power image, 3.5 mm across of a coronary artery ~75% narrowed by atherosclerosis. The intima is expanded; in the lower portion of the lesion is caseous debris.

Bottom left. A high power image of the cell debris (300 µm across) showing clear areas where lipid was removed in processing and cell debris without nuclei or preserved cell membranes.

Top right. Medium power image (0.6 mm across) in polarized light to show the dense collagen (bright) around a mineral nodule in the scarred, inactive region of this lesion.

Bottom right. High power (image 150 µm across) shows the vacuolated, or physaliferous, macrophages "foam cells" at the border of the acellular debris.

Dietary fatty acids: Saturated and unsaturated, natural and artificial

Fatty acids have a carboxyl group at one end and a tail of carbons, with a variable number of double bonds, in cell membranes mostly 16 to 20 carbons overall; C18:2 indicates an 18 carbon fatty acid with two double bonds; C18:1 Δ9 indicates that a double bond occurs between carbons 9 and 10. Double bonds in cis configuration (Figure 1A) are Z isomers in formal IUPAC nomenclature; trans-bonds are E isomers. Fatty acid unsaturation in natural membrane

phospholipids fatty acids is always cis, double bonds are created at 3-carbon increments (that is, not conjugated). Exceptions include trans bonds between carbons 2 and 3 from the parent carboxyl chain in specialized derivatives including sphingosine, an important amino-alcohol synthesized from serine and palmitoyl CoA, and 2,3 trans bonds used during catabolism of double bonds at odd-numbered carbons, which are out of phase with 2,3 reductases (Figure 1). Linoleic acid, C18:2 Δ9,12 cis, cis, is an essential nutrient. Linoleic acid is required for synthesis of arachidonic acid; arachidonic acid makes up 10 to 20% of red cell membrane fatty acids, and arachidonic acid is the substrate for synthesis of prostaglandins. This 20 carbon fatty acid formally is 5Z,8Z,11Z,14Z-5,8,11,14-eicosatetraenoic acid. An alternate ω, or omega, nomenclature counts double bonds from the distal end of the fatty acid. It is used to designate fatty acids with double bonds near the tail, 3 or 6 carbons from the end, that cannot be synthesized by mammals but which occur in vegetable and fish oils; linoleic acid is thus, ω6, ω9-octadecadienoic acid. Simplified names, giving acids as stearate "-ate" rather than stearic acid "-ic acid", are used subsequently.

Sources of fatty acids: Differences in length and saturation

Animal fats contain ~ 50% saturated fatty acids (Figure 2A, top), of which ~ 25% is C18:0, stearate, and roughly twice as much C16:0, palmitate, as stearate. Fatty acids from vegetables, e.g., soy oil, contain mostly unsaturated fatty acids with a large proportion of polyunsaturates, including variable amounts of ω-3 and ω-6 unsaturates. Trans bonds occur in some natural fats, but in central regions of long chain fatty acids they are not natural. Usually they result from partial hydrogenation. Unsaturated fatty acids with trans-bonds are stiff and have high melting points; the singly-unsaturated cis-fatty acid oleate (Figure 2A, middle) is a liquid; its tran-isomer, elaidate, is a solid (Figure 2A, bottom). Elaidate ((E)-octadec-Δ9-enoic acid) is the key 18-carbon monounsaturated trans fatty acid, with the trans bond at the C9-10 position, Δ9. Artificial trans-fatty acids accounted for ~2.6% of individual energy intake, and over 7% of fatty acid calories, in the US at the turn of the 21st century [11]. Unsaturated fatty acids can be converted to saturated fatty acids by bubbling hydrogen through them at high temperatures in a closed container. Industrial hydrogenation was developed by the noted French chemist Paul Sabatier in the late 1800s; he received the Nobel Prize for direct hydrogenation in 1912 [12].

In vegetable oils, typically 90% of fatty acids are C18, with most unsaturated and a high proportion doubly and triply unsaturated, all of the unsaturation cis. Hydrogenation reduces double bonds; partial hydrogenation reduces some double bonds and converts others from natural cis-unsaturated bonds to trans-unsaturated bonds: Trans isomers have lower free energy, and thus are strongly favored. Elaidate is one of many trans isomers that occur with partial hydrogenation. Double bonds in the carbon chain may be moved to make various isomers with double bonds at various positions between C4 and C14; when eaten they are used in cell membrane phospholipids in a similar distribution to the unsaturated fatty acids in food [13]. But the quantitatively predominant trans fatty acid in partially hydrogenated oils is elaidate, with the double bond smack in the middle [14], the most stable isoform (Figure 2A, bottom).

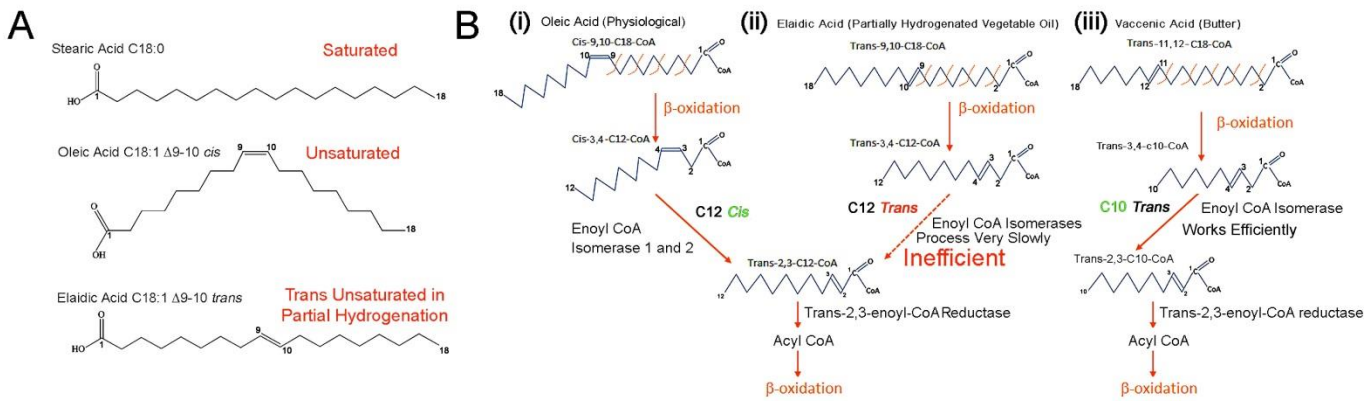

Figure 2. Unsaturated, cis and trans, and saturated fatty acids, and idiosyncratic substrate specificiites of enzymes that process double bonds during catabolism.

A. The fatty acids. Animal fats contain typically 50% saturated fatty acids, of which ~ 25% is C18:0 (stearate, illustrated) and ~ twice as much C16:0 (palmitate). Unsaturated cis fatty acids have lower melting points than their saturated counterparts, and increase membrane flexibility; oleate (middle) is a liquid. Unsaturated fatty acids with trans bonds are stiff and have higher melting points; elaidate (bottom), the major trans-fatty acid in partially hydrogenated vegetable oils, is solid at 37 °C.

B. Degradation of unsaturated fatty acids of odd, but not even, numbered double bonds requires enoyl Co-A isomerase (ECI), and ECI processes trans-bonds inefficiently. The degradation of unsaturated fatty acids at odd carbons from the terminal acid group adds to the complexity of β-oxidation. When a double bond occurs in an odd numbered position, this out of phase with trans-2,3 CoA reductase. This is adjusted by enoyl CoA reductases (two isoforms) that move the double bond from 3,4 to 2,3-trans. Fatty acids with cis double bonds at even positions are degraded similarly but by cis-2,3 CoA reductase (not shown). The enoyl CoA reductases are NADPH requiring enzymes in mitochondria.

Both cis- and trans-double bonds of odd numbered carbons are out of phase with the reductase. These fatty acids, when present in large quantities, reduce the efficiency of β-oxidation, but the effect varies idiosyncratically with the type of double bond, cis or trans, and with its position in the fatty acid. Inefficient processing of Δ9 trans C18 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. Oleate at 30 μM measurably suppresses β-oxidation, but only by ~ 10% the quantitative effect of elaidate at the same concentration [33]; vaccenic acid, with a C10 rather than C12 intermediate, is much less toxic to β-oxidation than elaidate [60].

The purpose of hydrogenated oils in food is to replace saturated fat "shortening", such as in baked goods. Saturated fatty acids and trans fatty acids improve shelf-life of foods and preserve taste and textures. Hydrogenated oils have similar food characteristics to saturated fatty acids [15], and were for a long time seen as a preferable substitute for saturated fat. With metabolic study and the advent of lipid profile screening, this conclusion was reversed. In

addition to the abnormal stiffness of membranes that include artificial trans-fatty acids, the enzymes that reduce the double bonds during catabolism have idiosyncratic substrate specificities, causing a back-up of degradation intermediates, discussed below.

The prevalent saturated fatty acids from animal fats are palmitate (C16:0) and stearate (C18:0), 90% of saturated fatty acids consumed, typically two-thirds palmitate [16]. A large majority, ~80%, of tissue membrane fatty acids is palmitate [17]. There is increasing evidence asserting that both trans fatty acids and saturated fatty acids in excess (long chain in particular) have damaging effects on the health of humans, including obesity, diabetes, and heart disease [18]. Diets including of high amounts of long-chain saturated fatty acids or small amounts of trans fatty acids promote atherosclerotic lesions [16].

Not all trans-fatty acids are manufactured, and not all fats with trans double bonds are toxic. Naturally occurring trans fatty acids include an unusual conjugated fatty acid, conjugated linoleate (C18:2, Δ9 cis, 11 trans) and vaccenic acid (C18:1 Δ11 trans (or ω-7 trans, counting from the non-carboxy end of the fatty acid)). Conjugated linoleic acid and vaccenic acid are produced in ruminant cattle, occuring in meat and dairy products. These natural trans fatty acids are handled by the same catabolic machinery as any fatty acids, but they cause fewer problems than elaidic acid (Figure 2B (iii)), reflecting that the enzymes idiosyncratic activity on different chain-length substrates, and also that quantitative loads differ. These differences are experimentally confirmed *in vivo*; in pigs, comparing diets with butterfat and corn oil (2-4% natural trans fat) to partially hydrogenated soy oil (30% trans fat, mainly elaidate) showed that the butterfat was harmless while the hydrogenated soy oil caused fatty streaks in arteries and increased unsaturation of membrane fatty acids [19]. Studies of oxidation of these and other fatty acids *in vitro* are considered below.

Progressive atherosclerosis: A vicious cycle of necrosis, reactive macrophages and intimal expansion

The vascular intima amasses caseous lipid-rich cellular debris known as plaque, with the plaque surrounded by reactive macrophages, many with lipid inclusions, foam cells, surrounded by proliferating stromal cells (Figure 1B). Healthy vascular intima has no cellular debris, no reactive macrophages, and stable stromal composition. In studies of the composition of plaque, researchers found that the caseous lipid debris is a mixture of cholesterol and oxidized phospholipids, with fat-engorged macrophages, and proliferating smooth muscle cells; there are complex biochemical interactions of macrophage and smooth muscle components [20,21,22]. The caseous debris includes dead cells, macrophages and stromal cells. The normal role of the macrophage is phagocytosis and processing of cellular debris, including lipids; the completeness and speed of this process determines whether the vessel wall is restored to normal or plaque will form. Ignoring partial oxidation effects, the profile of lipids in an atherosclerotic vascular sections and in red blood cells from the same subject is essentially the same, in fatty acid chain length, saturation, and percent cholesterol (30-40%). This reflects that membranes of dead cells are the source of the un-degraded lipids. In long-standing plaque, typically some regions are converted to scars with dense collagen and mineral nodules, while regions around caseous debris remain to show active, progressive disease (Figure 1B).

Effect on atherosclerosis of trans or excessive saturated fat, and exacerbation by immune mechanisms

Once cell membrane lipids accumulate in a vascular wall, how atheroma progression can be avoided is a key question that has no satisfactory answer. Parts of the problem include diet, excess dietary saturated fat, and modified dietary lipids, particularly artificial trans fat. A complicating factor is antibody-receptor mediated inflammation, particularly vis Fcγ, which will briefly be discussed. Other major modifiers of disease are the role of exercise in maintaining healthy circulation, exacerbation of atherosclerosis by smoking, and inborn errors of lipid metabolism affecting a small part of the population. These are important, but outside of our scope.

Although lipoproteins are not unidirectional conduits, in large part low density lipoprotein (LDL) moves cholesterol from the diet or produced in the liver to peripheral tissues; high-density lipoprotein (HDL) is used by peripheral sites to transport cholesterol for central disposal or to deliver lipids and cholesterol to other organs including the adrenal gland [23] and for bone synthesis [24].

Epidemiological work shows that artificial trans fat consumption increases the risk of atherosclerosis [25]. There are many studies supporting this view; trans fatty acid consumption increases total serum cholesterol and low density lipoprotein cholesterol, and decreases high density lipoprotein-cholesterol [26]. There are also many studies showing that consuming a large portion of dietary calories as saturated fat is unhealthy. These studies point to mechanisms that differ significantly from trans-fat consumption. In a landmark study comparing directly trans fat and saturated fat in young healthy volunteers [26], the saturated fat largely increased low density lipoprotein-cholesterol, while trans fat also reduced high-density lipoprotein cholesterol. This work also showed that dietary fatty acids appear rapidly in red blood cell membranes in essentially the same proportion as in the diet consumed. Many other studies have produced consistent results [27].

Macrophages have receptors for HDL and LDL, and take up cholesterol, oxidized or native, from lipoproteins. This process involves several binding and transport proteins [28]. When macrophages are presented with more cholesterol or lipid than can be processed and exported, lipid laden "foam cells" result (Figure 1B). This transformation is fostered not only by too much lipid, but by response via antibody-mediated processes via the Fcγ receptor, particularly isoform III [29]. While the mechanisms are complex, the Fcγ isoform signaling definitely contributes to cytokine and cellular pathways that drive macrophage overload and contribute to the vicious cycle of atheroma growth. In particular, people who have immune-related diseases and develop antibodies to oxidized lipids that form complexes with LDL are associated with acceleration of atherosclerotic disease [30]. The mechanism includes induction of inflammatory cytokines and growth factors.

Mechanism-specific effects of different fatty acids on macrophage lipid processing

Artificial trans fats, particularly elaidate, are metabolic inhibitors even when elaidate comprises only a few percent of dietary fat (Figures 2, 3B). It was demonstrated 10 years ago, in rat models, that elaidate stalls β-oxidation of fatty acids [31]. There are many differences between rodents and humans, but it a similar incomplete block in elaidic acid β-oxidation occurs in humans. The intermediate that

accumulates in humans predominately is the 12 carbon trans fatty acid [32] (Figure 3B) rather than the 14 carbon intermediate in rat liver [31]. When human macrophages are exposed to elaidate, together with accumulation of intermediates from incomplete β-oxidation (Figure 3B) [32], there are profound changes in metabolism that include increases in the ATP-binding cassette sub-family G-1, ABCG1, steroyl CoA desaturase-1 (SCD), and hydroxymethylglutaryl CoA desaturase-1 (HMGCS1) [33]. ABCG1 is a key mediator of cholesterol and phospholipid transport, and regulator of cellular lipid homeostasis. ABCG1 is a strong candidate for regulation of inflammation and atherosclerosis [34]. The protein encoded by the SCD gene desaturates stearate, 18:0 [35]. It it is linked to downstream increases in doubly unsaturated, 18:2, relative to 18:1 fatty acids, 18:1/18:2 being a marker of atherosclerotic disease progression (Figure 3A) [35,36]. The HMG reductase, one step distal to this synthase, is also highly expressed in macrophages. It is the statin target, the rate-limiting cholesterol synthesis enzyme [37]. Both SCD and HMGCS1 are implicated in regulation of inflammation and in lipid-related metabolism and pathology [34,35]. How these lipid pathway proteins are induced by trans-fat exposure [33] is not established. In addition, exposure of macrophages to elaidate uniquely down-regulates zinc binding proteins and promotes a zinc importer, resulting in a two-fold increase in intracellular zinc activity [33]. This would be expected directly to potentiate pathways, including activation of NF-κB, that depend on zinc as cofactors.

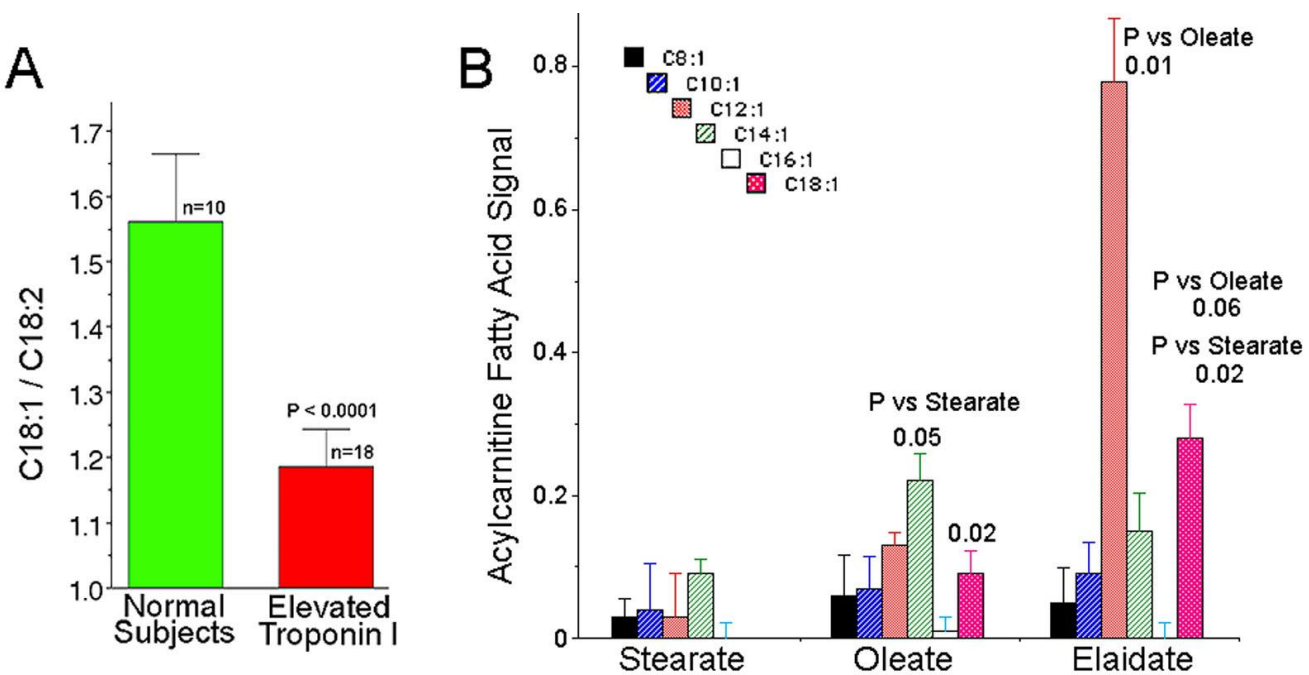

Figure 3. Key fatty acid cellular pathology: Membrane fatty acids change with atherosclerosis, and the effect of 18:1 fatty acids in macrophages on accumulation of carnitine intermediates. The graphs use data published in Sepulveda et al. [36] and Zacherl et al. [32], with permission.

A. The ratio of 18:1 to 18:2 red cell membrane fatty acids in normal and troponin-positive subjects. Analysis of human red cell membranes by GC/MS after hydrolysis and methyl esterification [36]. There is a large and significant increase in 18:2 in the troponin-positive group (right bar). This

correlates with increased stearoyl CoA desaturase in vitro in macrophages exposed to elaidate [32]. Data are mean ± SEM for n=10 (normal subjects) and n=18 (positive troponin subjects).

B. Acylcarnitine intermediates accumulating in supernatants of macrophages at five days in elaidate, oleate, or stearate, at 30 µM. Carnitine is the small-molecule carrier used in the cytoplasm and serum for free fatty acids; analysis was by direct MS/MS as described [32]. The fatty acids were added on albumin carriers, at 30 µM to cultures of human macrophages for five days, and the supernatants harvested. Note that significant increases occur with oleate (middle panel) relative to the unsaturated (stearate) control, although elaidate causes a dramatically and significantly larger quantitative effect on accumulation of C12:1 and C18:1 intermediates relative to oleate or stearate. The C18 carnitine peaks may reflect reversal of the pathway to re-synthesize C18:1. N=4, mean ± SEM.

In contrast, exposure of macrophages to large amounts of saturated fatty acids results in stimulation of cells by mechanisms including toll-like receptor 4 (TLR4), with downstream effects including production of TNF [33] and macrophage chemotactic protein-1 (MCP-1) [38], both of which contribute to the vicious cycle of proliferation and cell recruitment in atherosclerosis. There is a substantial related literature including a role of cytokines in excess lipid accumulation and apoptosis, independent of the NF-κB pathway [39,40]. The effect of saturated fatty acids is partly counteracted by ω-3 fatty acids. These effects are linked to peroxisome proliferator-activated receptor-γ (PPARγ) expression induced by saturated fatty acids [41]. It should be noted that these pathways are exacerbated by mechanisms such as Fcγ receptor mediated activation [29,30].

While the inter-relationships of the pathways are complex, the antibody-mediated pro-atherogenic effect is definitely, at least in large part, attributable to antibodies to oxidized lipids on LDL [42,43]. This association that supports the drive to reduce LDL availability (by suppressing LDL cholesterol synthesis), thus to reduce atherosclerotic progression. In saying this, it should be noted that LDL and HDL are cholesterol and lipid transporters, and both are essential, in some quantities, to normal lipid trafficking, despite the problems that occur due to increased fats in the diet, decreased exercise, as well as smoking and other factors (Figure 1A).

Thus, the available data support mechanisms promoting macrophage lipid accumulation and apoptosis due to artificial trans fats or excess saturated fatty acids which are, at least in part, independent (Figure 4). This model includes associations with unknown mechanisms, but the outcomes are consistent with clinical and experimental studies. Some studies of mechanisms including the association of stearoyl CoA desaturase with saturated fat diet have shown negative correlations [44], in contrast to the effect of trans-fatty acids [33].

Additional pathways altered by fatty acid exposure in toxic concentrations

An effect of saturated or trans fatty acids in macrophages includes acute inflammation, and although simple pathways provide important insights (Figure 4), it is important to keep in mind that the physiology is complex, that additional downstream pathways are involved, and that cells other than macrophages are affected. For example, palmitate (C16:0) or oleate (C18:1 cis) at 30 µM elicits stress responses in many different cell types including cardiac myocytes, where increased intracellular calcium at pre-

apoptotic levels occurs [45]. Cardiac myocytes regulate metabolism including of fatty acids during pathological changes including cardiac hypertrophy, with enzymes induced including HMGCoA reductase [46], highlighting the physiologically integrated nature of fatty acid response in cells other than macrophages [32,33].

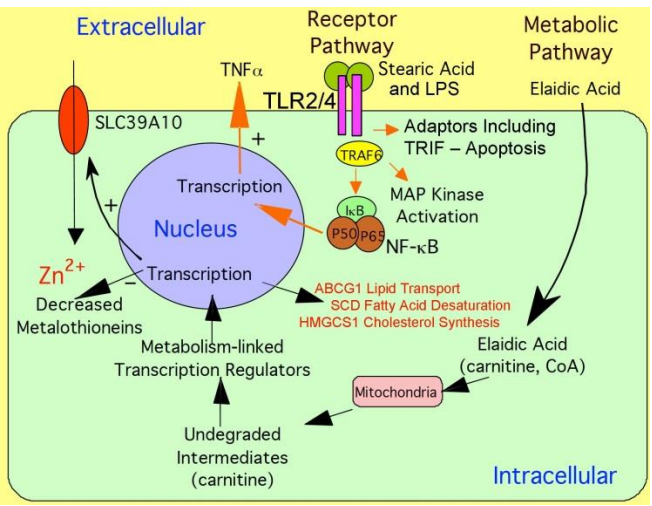

Figure 4. Trans-fatty acid and unsaturated fatty acid macrophage activation pathways.

Incomplete metabolism of the artificial trans-fatty acid elaidate [28] changes expression of many metabolic regulatory enzymes [29]. Many changes secondary to elaidate (C18:1 Δ9 trans) and its metabolites occur by undefined intermediate steps. These include increased expression of HMGCoA synthase, the rate-limiting enzyme for cholesterol synthesis, stearoyl-CoA desaturase, which unsaturates membrane fatty acids, and ABCG1, which transports phospholipids and cholesterol. Elaidate decreases synthesis of metallothioneins, reducing Zn^{+2} binding capacity, and increases expression of SLC39A10, increasing Zn^{+2} importation. Zinc homeostasis is crucial to regulation of the inflammatory response, allowing limited response while preventing cellular injury. Receptor mediated effects of fatty acids, including stearate and elaidate, or substances including lipopolysaccharide, activate TLR2/4 and related receptors. This is normally self-limited in a few hours. However, some fatty acids, particularly stearate, escape down-regulation and chronically stimulate receptor-mediated pathways with production of TNFα continuing at least to 44 hours. This correlates with maintenance of TLR4, which is downregulated in other fatty acid treatments, and maintenance of downstream signals. This mechanism may be reinforced by antibodies to oxidized LDL complexes, and thus sensitive to availability of LDL. Alternate pathways may be activated including apoptosis [53,54]. Trans-fatty acid metabolic changes and uncontrolled inflammatory signals by long-chain saturated fatty acids and immune complexes may all contribute to the accumulation of undegraded cell membrane debris in atherosclerosis, with the combined mechanisms creating more rapid and severe damage than any pathway alone.

Saturated fatty acids cause the release pro-inflammatory cytokines in serum in addition to TNF and MCP-1[33,38], including C-reactive protein, IL-6 [47] and IL-1, which implies the involvement of the liver, since the liver makes C-reactive protein and other acute phase reactants [48]. Additional serum factors, including chemokines, are reviewed elsewhere [49].

Thus, macrophages are primary, but not the sole, cellular agents responding to membrane fatty acids in catabolic pathways. In macrophages, in addition to the pathways above, response linked to TLR4 includes cyclooxygenase activation, an effect specific for the long-chain saturated fatty acids [50], such as TNF production after 44 hours incubation [33]. An underlying concept is that macrophages responding to prolonged activation

by the innate immunity-related, TLR pathway convert to "M1 macrophages". Other macrophages are "M2 macrophages". M1 macrophages mediate intense reactions to bacterial infection and to tissue damage including arterial lesions. These macrophages, in damaged tissue and with triggering of the innate immune system, activate glycolysis for anaerobic energy production [51]. Prolonged glycolysis byproducts include reactive oxygen species, a process that can be driven by increased glucose transporter activity [52].

Lipotoxic cell death

One of the difficulties in mechanisms of atheroma growth is what proportion of the effect is due to direct cell death. There definitely are high concentrations of oxidized phospholipids in contact with macrophages. The atheroma is a semiliquid mass mainly of cholesterol and partially oxidized phospholipids, around which foam cells and cell ghosts abound. With macrophages *in vitro*, long term survival is possible with 30 μM fatty acids on albumin carriers, while 100 μM fatty acids, particularly stearate, cause cell death [32,33]. *In vivo*, the situation is more complex with oxidized phospholipids interacting with LDL and antibodies [30], so it is impractical to determine toxic concentrations of components. Many modified lipids contribute to lipotoxicity acylglycerols and ceramides [53]. Ceramide is the sphingosine-fatty acid cell membrane component. It accumulates in large quantities in atherosclerotic lesions [54], indicating that processing ceramide may be important in progressive atherosclerosis. Ceramide is also a signaling molecule for apoptosis; it is important in cycles of cell death and atheroma growth [55]. It is widely regarded as important but difficult to separate physiologically from other mechanisms. The ceramide pathway amplifies the toxic effect of fatty acids via TLR4 [56].

The central role of the TLR signaling pathway for cell death by the lipopolysaccharide-saturated fatty acid pathway has been shown using macrophages null for TLR2 and the fatty acid scavenger receptor CD36 [57]. Other work has focused on TLR4, which clearly is important in foam cell production, but may be of secondary importance in cell death. Alternative work using TLR4 knockouts [58], in contrast, found that lipopolysaccharide and fatty acids caused macrophage apoptosis by a TLR4 pathway that was independent of the NF-κB signaling which mediates many other cellular effects, as discussed above. Since all of this work is supported by definitive knockout mouse models, a logical conclusion is that multiple TLR pathways, including TLR2 and TLR4, can mediate macrophage cell death with lipotoxic signals, depending on the cellular context, and that pro-apoptotic adaptor proteins [59] are involved in the macrophage cell death pathways.

Accumulation of intermediates of toxic fatty acids, and correlation with clinical disease

A distinguishing characteristic of trans fat metabolism is that intermediates reach high concentrations. The process by which fatty acids are broken down in the mitochondria, β-oxidation, is slowed during elaidate metabolism, as was demonstrated in study of rat liver mitochondria [32]. Other work showed that for vaccenic acid, C18:1 Δ11 trans (the double bond between carbons 11 and 12), where serial acetyl CoA removal creates a C10:1 intermediate, does not hinder further oxidation markedly. Whereas elaidate, C18:1 Δ9 trans (the double bond between carbons 9 and 10), where serial acetyl-CoA removal creates a C12:1 intermediate (Figure

2B), results in a significant metabolic block [60] (Figure 3B). In this case there are secondary changes that include induction of HMGCoA desaturase, the rate limiting enzyme for cholesterol synthesis. In considering this pathway, it should be noted that there are substantive differences in fatty acid oxidation between rodents and humans, making it essential that human cells are used to confirm results in rodents [61]. Further, there are large differences between central metabolism in the liver and peripheral metabolism, such as in macrophages.

Direct analysis of lipids in human atherosclerotic plaque confirm completely the importance of elaidate in atheromata [62]. In human macrophages, exposure of cells to mixed triglycerides loaded on serum, inclusion of 7% elaidate as partially hydrogenated soy oil resulted in a several fold increase in 12:1 carnitine intermediates in supernatants [32]. Analysis of cell membrane fatty acids after exposure to 30 µM elaidate resulted in increased stearoyl CoA desaturase activity with increased 18:2 membrane fatty acids, a change that mirrors that seen in patients with myocardial infarctions [36]. In human macrophages, radiolabeling at C1 or C9,10 unsaturated fatty acids showed that elaidate (C18:1 Δ9 trans) enters β-oxidation at rates at least as great as that of oleate (C18:1 Δ9 cis), but has greatly slowed rates of oxidation at the trans-bond [32]. However, this did not occur in hepatocytes, a difference attributed to greatly increased expression of enoyl CoA isomerases in the liver cells [32]. Overall, this work implicates elaidate as a metabolic inhibitor with serious consequences for lipid intermediate accumulation by mechanisms that are different from those occurring with large amounts of long chain fatty acids including stearate (C18:0). Similar changes occur in arterial smooth muscle [63].

Clinical studies, pre-dating the macrophage biochemical work, showed correlations of elaidate in red cell membranes and atherosclerotic progression, or implicated the burden of membrane trans-fats as a risk factor for atherosclerotic disease [64,65,66,67]. Interpreting this work is complicated by considerations including total dietary fat and the proportion of saturated and unsaturated fat, including ω3 and ω6 fatty acids. It is clear that elaidate should be avoided, without significant contrary opinions, and that diets including very large quantities of long chain fatty acids are unhealthy, in keeping with the studies of peripheral fat metabolism.

Fatty acid and metabolic pathology are linked, in part, by specialized membrane structures

Additional data on cell signaling that is important to place fatty acid receptor response in perspective relates to specialized cell membrane regions where saturated acyl chains of sphingolipids and cholesterol align to form "lipid rafts" with sizes on the order of 100-200 µm, which accumulate TLRs [68]. These complexes, at least in part, promote activation of the TLRs as dimers when reactive oxygen species are present [69], which supports for the synergistic effects of reactive oxygen species (ROS) and fatty acid/ lipopolysaccharide complexes. The mechanism involved, largely specific for saturated fatty acids (Figure 4) can include activation of TLR heterodimers including components other than TLR2/4 [70]. There is strong evidence that the lipid raft is as an integrator of response of metabolic and inflammatory diseases with TLR-ligand signaling [71].

Outward transport of lipid degradation products from macrophages

It is important to consider that in health macrophages may remove excess lipid, in major part as cholesterol, thus interrupting the process of atheroma growth. In brief, the key apolipoproteins involved in reverse transport of cholesterol are apolipoprotein E, largely found in intermediate density lipoproteins, which is produced by macrophages and liver cells [72] and apolipoprotein A1, the key component of high density lipoproteins "good cholesterol" carriers, overexpression of which has been shown to disperse lipid from foam cells *in vivo* [73]. Macrophage ATP-binding cassette proteins ABCA1 and ABCG1 are key receptors involved in cholesterol export by macrophages [74]. The role of β-oxidation and cholesterol synthesis, blocking of β-oxidation by elaidate, and secondary cell responses including increased synthesis of unsaturated long chain fatty acids [32,33], discussed above.

Additional metabolic changes in macrophages that may affect lipid handling

As macrophages fight to control atherosclerotic damage and convert to foam cells, inflammatory signals downstream of TLRs are prominent in the serum. An unexpected component that may promote synergistic pathology from saturated and toxic trans-fatty acids is an increase in macrophages of zinc activity. This was discovered when genome-wide screening of mRNAs in macrophages exposed to elaidate relative to oleate for two days showed a dramatic decrease in expression of several zinc-chelating metallothioneine proteins together with increased expression of a specific zinc importer, SLC39A10 [75], which correlate with an increase in elaidate exposed macrophages of zinc activity, "free zinc", by a factor of two [33]. Intracellular zinc physiology is complex, and most work has focused on low serum and intracellular zinc as requirements for normal cell signaling including by the NF-κB mechanism [76]. However, high intracellular zinc can promote apoptosis [77] by a mechanism involving Bax activation [78]; in macrophages, this might promote cell death and atherosclerosis. In contrast, in macrophages high concentrations of zinc produce inflammatory cytokines, possibly by enhancing NF-κB activation [79]. However, TNF production was not increased in macrophages with increased intracellular zinc exposed to 30 μM elaidate for two days [33], and it is possible that changes in intracellular zinc might reflect, also, cellular defense mechanisms against fatty acid toxicity.

Peripheral macrophage-related effects of pharmacological Inhibitors of LDLR, HMGCoA reductase, and mitochondrial fatty-acid uptake

Notwithstanding our hopeful outlook on preventative measures to reduce the incidence of progressive atherosclerosis, diet and exercise are frequently inadequate or not practical to control the problem. A new class of LDL cholesterol-lowering drugs is in phase III testing in humans is based on inhibiting PCSK9, the proprotein convertase subtilisin/kexin type 9. PCSK9 binds an LDLR EGF-like-repeat domain, the effect being to prevent LDLR from recycling to the cell surface, targeting its degradation. In the liver this has the effect of lowering LDL cholesterol production by greatly reducing LDLR-dependent cholesterol transport [80]. In cell models of atherosclerosis, secreted PCSK9 has been shown to reduce LDLR in macrophages, reducing processing of oxidized lipids, and hence limiting inflammation and cell death [81]. Statins, HMGCoA synthase inhibitors,

are usually regarded as a means to reduce cholesterol production for LDL loading. In addition, they play specific roles in peripheral macrophages. Statins reduce proliferation of macrophages [81], and reduce ABCG1-mediated cholesterol efflux [82]. Additionally, statins suppress Fcγ-antibody-mediated phagocytosis [83], which may lessen the effect of antibodies to oxidized phospholipids, discussed above. A promising additional tool to disrupt the vicious cycle of plaque formation is to limit β-oxidation by limiting the supply of carnitine. This new approach might intervene before macrophage overloading can occur. In a mouse model it shows promising effects on atherosclerosis progression [85].

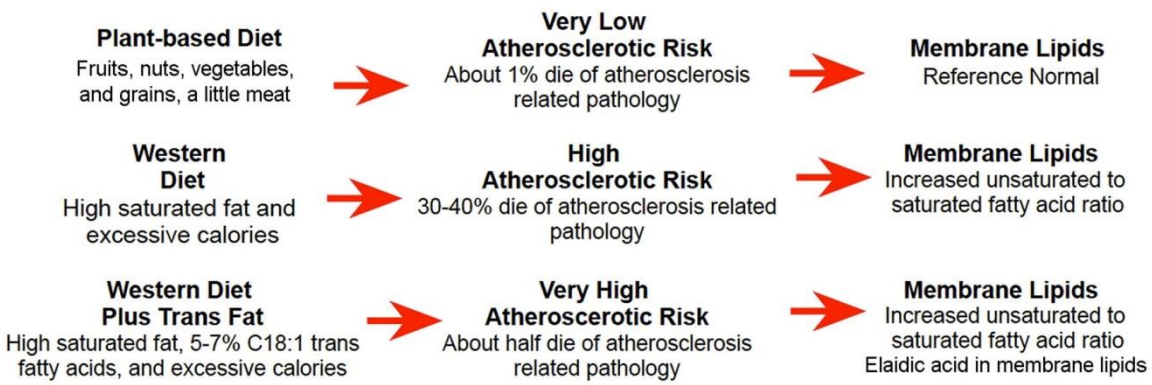

Figure 5. Dietary fats, cell membranes, and atherosclerosis risk.

Traditional diets, typically fruits and vegetables with limited meat, contain high proportions of poly-unsaturated linolenic and α-linoleic acids, low saturated fatty acids, and no artificial trans fatty acids. This diet, with normal calorie intake and physical activity, does not support atherosclerosis. Diets with excessive saturated fat increase LDL cholesterol and mediate lipid toxicity by complex mechanisms that include inflammatory growth factors and antibodies to oxidized LDL. Diets with the artificial trans fatty acid elaidate accelerate atherosclerosis by mechanisms that are at least in part different, but complementary. Both saturated fat and trans fat mechanisms are associated with higher risk of atherosclerotic disease. The estimates shown are the authors', and include components from other risk factors than diet alone.

Conclusion

Large amounts of saturated fat, or relatively modest amounts of the trans-fatty acid elaidate contribute to the development and progression of atheromata. Independently, inborn errors of metabolism contribute to atherosclerosis. The effects of excessive overall dietary fat, smoking, and infrequent exercise, are the common associations. The adverse macrophage cellular metabolic effects of C16 and C18 fatty acids largely are mediated by TLR pathways, while effects of C18:1 Δ9 trans, elaidate, appear at least in large part to reflect inability of the macrophage to degrade this fatty acid at a normal rate. Development of atherosclerosis overall reflects the inability of macrophage degradation of fatty acids, cholesterol synthesis, and back-transport to keep up with accumulation of cell membrane debris. This pathology is complicated by inflammatory cytokine production by atheroma-associated macrophages, by response to oxidized phospholipids by mechanisms including Fcγ activation, and by trans-fat metabolite associated changes in macrophage protein expression. A

conservative approach to the problem (Figure 5) includes encouraging a diet with modest amounts of animal fat and other saturated fat, elimination of modified fats containing elaidate, and use of metabolic inhibitors to control atherosclerosis progression when conservative approaches are inadequate.

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