DIRECT EFFECTS OF LONG-CHAIN NON-ESTERIFIED FATTY ACIDS ON VASCULAR CELLS AND THEIR RELEVANCE TO MACROVASCULAR COMPLICATIONS OF DIABETES

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TABLE OF CONTENTS

1. ABSTRACT

Diabetes leads to a marked increase in cardiovascular disease caused by atherosclerosis. Cardiovascular complications of diabetes are associated with lipid abnormalities, mainly manifested as elevated levels of triglycerides. Hydrolysis of triglycerides by lipases in the arterial wall is believed to cause increased levels of non-esterified fatty acids (NEFAs) in lesions of atherosclerosis. Recent research has shown that long-chain NEFAs have a multitude of direct effects on cell types involved in atherogenesis. Thus, some of the most common long-chained fatty acids present in triglycerides, oleic acid and linoleic acid, have been shown to induce adhesion molecule expression, cytokine expression and apoptosis in endothelial cells, to increase cholesterol uptake and reduce cholesterol efflux in macrophages, and to increase arterial smooth muscle cell proliferation and migration. Certain NEFAs liberated from triglycerides may therefore play an important role in accelerating atherosclerosis caused by diabetes by directly affecting the key cell types involved in atherogenesis.

2. INTRODUCTION

2.1. Cardiovascular disease and its association with diabetes and elevated triglycerides

Atherosclerosis is the major cause of morbidity and mortality in diabetes (1, 2). Despite intensive study for many years, the reason for the increased diabetes-associated cardiovascular disease is unknown. There is a large body of evidence suggesting that the dyslipidemia accompanying diabetes and insulin resistance contributes to this enhanced atherogenesis (3, 4). Thus, even though there is a correlation between glycemic control and cardiovascular disease (5), reducing hyperglycemia does not significantly lower cardiovascular events in people with Type 1 or Type 2 diabetes (6, 7). The three major components of dyslipidemia associated with diabetes are increased levels of triglyceride-rich lipoproteins, decreased high-density lipoprotein (HDL), and increased small, dense low-density lipoprotein (LDL). The increased concentration of triglyceride-rich lipoproteins is the central lipoprotein abnormality of the metabolic syndrome associated with Type 2 diabetes (8). Hypertriglyceridemia is a risk factor for the development of atherosclerosis in people with and without diabetes (9), and increased level of triglycerides is an independent risk factor for atherosclerotic heart disease (10). Levels of triglycerides are often elevated in patients with Type 2 diabetes and cardiovascular disease (11), in poorly controlled patients with Type 1 diabetes (3, 12), and in animal models of diabetes-accelerated atherosclerosis (13, 14).

In addition to the elevated levels of fatty acid-containing triglycerides associated with diabetes,

circulating levels of free fatty acids (NEFAs), are elevated in subjects with Type 2 diabetes (11, 15, 16). NEFAs have a variety of effects on cellular processes and may actually contribute to the insulin resistance in skeletal muscle cells (17). Conversion of saturated to unsaturated NEFAs may also be influenced by the diabetic state (18). Fatty acids are divided into different groups depending on their number of carbon atoms: short-chain fatty acids (<8 carbons), medium-chain fatty acids (8-12 carbons), long-chain fatty acids (13-22 carbons) and very long-chain fatty acids (>22 carbons). The most common fatty acids in triglycerides belong to the long-chain group, for example palmitate (16:0), stearate (18:0), oleate (cis 18:1) and linoleate (cis 18:2).

Is fatty acid composition in triglycerides affected by diabetes? A recent study on diabetic swine shows it is not affected to a great extent. In this study, fatty acid composition in plasma triglycerides from non-diabetic and alloxan-diabetic swine fed a lipid-rich diet for 20 weeks was analyzed (14). Oleate, linoleate and palmitate were the most abundant fatty acids in both groups. However, as in humans, there were quantitative differences between diabetic and non-diabetic animals; levels of plasma triglycerides were more than 3-fold higher in diabetic fat-fed swine compared to non-diabetic swine fed the same diet (14). These findings are consistent with human studies (19). Thus, oleate is the principal fatty acid in triglycerides within the arterial wall of people with and without diabetes (20). Although no direct measurements have been done to date, these studies suggest that the arterial wall is exposed to elevated levels of oleate and other long-chain fatty acids in people with diabetes.

2.2. Lipases localized in the arterial wall hydrolyze triglycerides and liberate fatty acids

Levels of NEFAs within the atherosclerotic lesion are most likely regulated to a great extent by the activity of lipases, e.g. lipoprotein lipase (21), secreted phospholipase A_2 (22) and endothelial lipase (23) present in the lesion. Lipoprotein lipase (LpL) is the rate-limiting enzyme for hydrolysis of lipoprotein triglycerides (24, 25, 26), and is secreted primarily from muscle and adipose tissue. Circulating LpL secreted from these tissues binds to the vascular endothelium via cell surface proteoglycans (21). In lesions of atherosclerosis, LpL is also synthesized by macrophages (27). Interestingly, LpL-deficiency targeted to macrophages has recently been shown to lead to reduced atherosclerosis in LDL receptor (LDLR)-deficient mice and apolipoprotein E (apoE)-deficient mice (21, 28, 29, 30). A role for secreted phospholipase A_2 activity in atherogenesis has also recently been confirmed in mice overexpressing this enzyme (31).

How does the diabetic state affect activity of these lipases? To date, it is not known if secreted phospholipase A2 or endothelial lipase are affected by diabetes. However, LpL expression has been shown to be increased in macrophages from people with Type 2 diabetes (32), and in isolated macrophages exposed to elevated levels of glucose (33), linoleate, palmitate or stearate (21, 34). Together, these results suggest that the elevated levels of plasma triglycerides

observed in diabetic animals and humans can be hydrolyzed by the abundant LpL in lesions of atherosclerosis. However, LpL has also been shown to exert effects independent of its triglyceride-hydrolyzing activity. For example, LpL can retain lipoproteins by direct bridging between lipoproteins and the extracellular matrix (21), and catalytically inactive LpL has recently been shown to mediate cellular cholesterol ester uptake in vivo (35).

This review focuses on direct effects of common long-chain NEFAs on the principal cell types involved in initiation and progression of lesions of atherosclerosis, namely endothelial cells, monocytes/macrophages, smooth muscle cells and lymphocytes.

2.3. Cellular uptake of NEFAs

NEFAs are bound to carrier proteins, primarily albumin, in circulation and in the extracellular space. They are therefore likely to exist in a protein-bound state when presented to the endothelium and other cell types in lesions of atherosclerosis. Albumin has been estimated to have 3-5 high affinity NEFA binding sites per molecule, and an albumin/NEFA ratio of 1:5 or less is therefore considered physiologically relevant (36, 37). Using a 1:3 ratio of albumin/oleate it has been calculated that the concentration of free oleate in a water phase is < 50 nmol/l (38). This fraction of free NEFA mediates the biological activity (39).

The mechanism(s) whereby fatty acids enter living cells are not yet fully understood. It appears that long-chain fatty acids, e.g. oleate, cross cellular membranes through a flip-flop mechanism. This mechanism is extremely rapid (within seconds) and occurs at low concentrations of fatty acid (40). In addition, it is becoming increasingly clear that proteins that act as fatty acid transporters (41) mediate at least some of the uptake. Five putative fatty acid transporter proteins have been identified. These are the 43-kDa fatty acid-binding protein FABPpm present in plasma membranes (42, 43), the 56-kDa renal FABP (44), the 22-kDa plasma membrane protein calveolin (45), the fatty acid transport protein, FATP (46) and the 78-88-kDa fatty acid translocase CD36 (47, 48). Vascular cells express a number of these putative fatty acid transporters. For example, CD36 is expressed by monocytes/macrophages, smooth muscle cells (49) and endothelial cells. It has recently been shown that uptake of exogenous arachidonate is mediated mainly by CD36 in human umbilical vein endothelial cells (50).

In addition to direct deliver of exogenous free fatty acids, some arterial cells are capable of internalizing and degrading triglyceride-rich lipoproteins through receptor-mediated mechanisms. This is particularly true for macrophages, which contain receptors that recognize apolipoprotein B48, a component of chylomicrons, very low density lipoproteins (VLDL), and their partially metabolized remnant particles (51). Receptor-mediated uptake of these particles promotes accumulation of both cholesteryl esters and triglycerides in cultured macrophages (51).

2.4. Methodological considerations

Before reviewing the effects of NEFAs on the different cell types involved in initiation and progression of

lesions of atherosclerosis it may be relevant to consider some methodological issues. For example, the total concentration of NEFA added in a given in vitro experiment is largely irrelevant without knowledge of the carrier protein/NEFA ratio. Indeed, it has been shown that fibroblasts accumulate triacylglycerol in proportion to the molar ratio of free oleate to albumin in the medium (39). Many studies in which NEFAs are added to cultured cells use fatty acid-free bovine serum albumin (BSA) as a carrier protein. Some studies use NEFAs in the absence of carrier proteins, and these conditions are likely to result in very high levels of NEFAs within the membrane compartments of the cell (36). Furthermore, pre-equilibration of the carrier protein and fatty acid can alter the biological effects of the fatty acid. Thus, when oleate is equilibrated with fatty acid-free BSA for 1 h at a ratio of less than 1:3 prior to addition to cells, oleate has a certain biological effect. If the same concentrations of oleate and BSA are added to the cells without prior equilibration, oleate causes markedly larger effects (52). This phenomenon can most likely be explained by the fact that a portion of the free oleate is rapidly taken up by the cells, possibly by a flip-flop mechanism, before it has been complexed to BSA in the cell culture medium. Finally, fatty acid-free BSA may have effects on cells that are due to sequestration of cellular components, such as lysophosphatidic acid.

3. DIRECT EFFECTS OF NEFAs ON ENDOTHELIAL CELLS

The endothelium lines the blood vessel and plays a major role in both initiation and progression of atherosclerosis. One of the first steps in lesion initiation is believed to be an increased expression of adhesion molecules on the endothelium, initiated by e.g. modified LDL or inflammatory cytokines (53, 54). This increased expression of adhesion molecules leads to adherence of monocytes and subsequent migration of these cells into the subendothelial space. The integrity of the endothelium is also likely to play an important role in progression of early lesions into more advanced lesions.

3.1. Apoptosis

Long-chain NEFAs have been shown to induce apoptosis in endothelial cells. In microvascular endothelial cells, palmitate inhibits DNA synthesis and induces apoptotic cell death (55). These effects of palmitate were completely prevented by an antioxidant, N-acetylcysteine, indicating that palmitate-induced formation of reactive oxygen species may be responsible for the apoptotic death. Linoleate has been found to potentiate tumor-necrosis factor-alpha (TNF-alpha)-mediated oxidative stress and apoptosis in endothelial cells (56, 57). On the other hand, oleate does not appear to induce apoptosis of cultured human umbilical vein endothelial cells (58). The results suggest that palmitate and linoleate may contribute to endothelial death under conditions in which levels of triglycerides and/or NEFAs are elevated.

3.2. Regulation of pro-inflammatory and pro-atherogenic molecules

Several studies have investigated the effects of long-chain fatty acids on expression of pro-inflammatory molecules expressed by endothelial cells, such as TNF-alpha, monocyte chemoattractant protein 1 (MCP-1),

vascular cell adhesion molecule 1 (VCAM-1), and intercellular adhesion molecule 1 (ICAM-1). Linoleate enhances messenger RNA levels of TNF-alpha, MCP-1, VCAM-1, and ICAM-1 in cultured endothelial cells (59, 60). Oleate and linoleate also increase interleukin-8 (IL-8) production in endothelial cells (61). This is of particular interest because IL-8 is crucial for firm adherence and transmigration of circulating monocytes into the subendothelial space (62).

On the other hand, cytokine-stimulated VCAM-1 expression is inhibited by unsaturated long-chain fatty acids, but not saturated fatty acids, such as palmitate and stearate. The ability of unsaturated fatty acids to inhibit cytokine-induced VCAM-1 expression correlates with the number of double bonds. Thus, docosahexaenoate (22:6n-3), which contain 6 double bonds, is a potent inhibitor of cytokine-stimulated expression of VCAM-1, ICAM-1, E-selectin and various cytokines in endothelial cells (63, 64), whereas the monounsaturated oleate is approximately 6 times less potent (65, 66, 67).

In addition, oleate and linoleate have been shown to result in a significant increase in plasminogen activator inhibitor 1 secretion from human umbilical vein endothelial cells (68). This may contribute to the decreased fibrinolysis associated with diabetes (69, 70, 71, 72).

4. DIRECT EFFECTS OF NEFAs ON MACROPHAGES

It is generally believed that accumulation of lipid-loaded macrophages in the arterial wall initiates formation of atherosclerotic lesions. In humans, accumulation of these macrophages is often seen in areas with intimal thickening (smooth muscle cell mass) that have formed in the subendothelial space due to adaptations to local mechanical forces (73). In many small animal models, infiltration of monocytes and subsequent activation and differentiation of these cells into lipid-loaded macrophages is often seen in areas without preexisting intimal thickening. These initial events are followed by an increased accumulation of lipid-laden macrophages and extracellular lipid, which leads to formation of a lipid core and a lesion defined by the American Heart Association as an atheroma (73). A hallmark of the developing atherosclerotic lesion is the appearance of cholesterol-laden macrophage foam cells in the intima of the artery wall (74). These fatty streak lesions are believed to progress into unstable lipid-rich cores that are vulnerable to plaque rupture and thrombosis (75). Thus, therapeutic interventions designed to minimize cholesterol accumulation in arterial cells have become major strategies for preventing cardiovascular disease.

The accumulation of cholesterol in arterial macrophages is caused by an imbalance between influx and efflux of cholesterol. Most of the influx is derived from plasma LDL, which carries about two-thirds of our plasma cholesterol. As their plasma levels increase, LDL particles infiltrate the sub-endothelial space of the vascular wall, where they become trapped and modified by oxidative and enzymatic processes (76, 77). These modified particles are
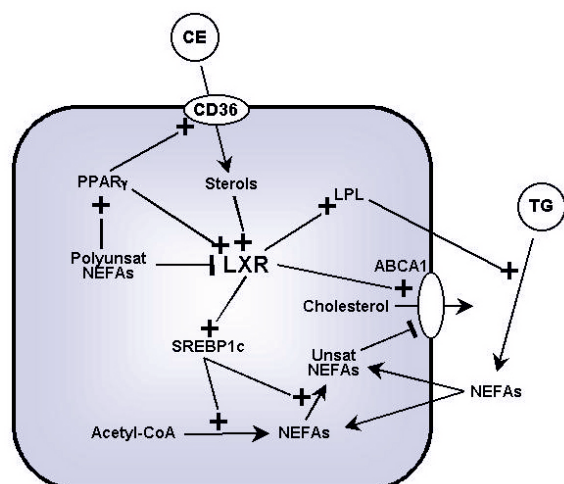
Figure 1. Interactions of the LXR system and NEFAs in macrophages. There are several points of interaction between LXRs and NEFAs in macrophages. LXRs induce several macrophage proteins involved in secreting excess cholesterol. These include ATP-binding cassette transporter A1 (ABCA1), which mediates the export of cholesterol and phospholipids from cells. LXRs also activate SREBP-1c, which induces enzymes of fatty acid synthesis and desaturation, and enhance production of macrophage lipoprotein lipase (LPL), which can hydrolyze extracellular triglycerides (TG). Thus, LXR ligands can increase the supply of unsaturated NEFAs in macrophages. Conversely, unsaturated NEFAs can dampen the effects of LXRs on cholesterol secretion from macrophages. For example, polyunsaturated NEFAs, such as arachidonate, can antagonize activation of LXR by endogenous sterols. This antagonism also can suppress cholesterol secretion from cells by reducing synthesis of ABCA1 and apolipoproteins. Furthermore, monounsaturated (e.g. palmitoleate and oleate), diunsaturated (e.g. linoleate), and polyunsaturated NEFAs (e.g. arachidonate) decrease ABCA1 levels in cells by destabilizing the protein. By inducing the desaturating enzyme SCD through activation of SREBP-1c, LXR agonists can also perturb the membrane lipid environment and impair ABCA1-mediated cholesterol transport from macrophages. Polyunsaturated NEFAs and eicosanoids, as well as pharmaceutical agonists such as thiazolidinediones, can activate PPAR gamma, which induces multiple genes involved in cholesterol and NEFA metabolism, including the lipoprotein scavenger receptor CD36 and LXR.

inflammatory agents that recruit macrophage-precursor monocytes into the arterial intima (78). Through receptor-mediated endocytosis and phagocytosis, these macrophages take up and degrade the modified lipoproteins in lysosomes, releasing free cholesterol into the cytoplasm (79). Because cells cannot degrade this excess cholesterol, they must either store it as cholesteryl ester-rich lipid droplets in the cytoplasm or secrete it. Macrophages become lipid-laden foam-cell precursors of atherosclerotic plaques when they ingest more cholesterol than they can secrete.

NEFAs modulate several steps in the cellular cholesterol synthesis, delivery, and secretion pathways.

This is because of a close regulatory link between the fatty acid and cholesterol metabolic pathways. Three families of transcriptional regulators as well as post-translational processing are involved in modulating the activities of both of these pathways. The reason for this close association between these diverse lipid pathways is unclear, but it may reflect homeostatic mechanisms important for membrane integrity. In arterial macrophages, however, NEFAs influence on cholesterol trafficking can have pathological consequences. The following sections address the interplay between the fatty acid and cholesterol pathways in macrophages, how this may underlie metabolic abnormalities associated with diabetes and cardiovascular disease, and how this relates to strategies for therapeutic interventions.

4.1. Sterol regulatory element binding proteins (SREBPs)

SREBPs are a family of transcription factors that activate expression of more than 30 genes involved in metabolism and transport of cholesterol, fatty acids, triglycerides and phospholipids (80). The human genome encodes three SREBP isoforms designated SREBP-1a, SREBP-1c, and SREBP-2. The 1a and 1c isoforms are from the same gene but are transcribed from alternative start sites (80). SREBP-1a is a potent activator of both fatty acid and cholesterol synthesis, whereas SREBP-1c and SREBP-2 predominantly activate fatty acid and sterol synthesis, respectively.

When the endoplasmic reticulum (ER) contains a normal cholesterol content, SREBPs bind to ER membranes in association with a protein complex. Depletion of membrane cholesterol releases SREBP and an escort protein that transport SREBP to the Golgi for proteolytic cleavage (81, 82). The cleaved product then translocates to the nucleus where it activates lipolytic genes (81).

Because the activation process occurs when cells become depleted of cholesterol, it does not play a role in macrophage foam cell formation. As discussed below, however, transcription of SREBP-1c, which drives fatty acid synthesis and desaturation, is activated when cells over-accumulate cholesterol. One of the enzymes induced by SREBP-1c is stearoyl CoA desaturase (SCD) (80), which converts stearoyl-CoA to oleoyl-CoA, the preferred fatty acid substrate for the cholesterol esters that accumulate in macrophage foam cells. Moreover, accumulation of cholesterol in cells requires an increased phospholipid synthesis and reacylation to incorporate the excess cholesterol into membranes. Increased delivery or production of unsaturated fatty acids in macrophages can also impair cholesterol secretion from cells (see below). Thus, in macrophages, the interplay between the fatty acid and cholesterol pathways may enhance atherogenesis.

4.2. Liver X Receptors

Liver X receptors (LXRs) alpha and beta are sterol-activated nuclear receptors that modulate gene transcription of multiple proteins involved in cholesterol trafficking and NEFA metabolism (83) (Figure 1). LXRs

form obligate heterodimers with retinoid X receptors (RXR) that preferentially bind to response elements within gene promoters (84). LXRs and RXRs bind to, and are activated by, oxysterols and retinoic acid, respectively. Binding of either one or both ligands can activate transcription, but binding of both ligands has marked synergistic effects (84). The LXR alpha gene promoter in human macrophages contains a LXR response element, indicating that LXR alpha can autoregulate its own expression (85, 86). This would serve to amplify the effects of oxysterols on gene transcription.

Since uptake of non-oxidized cholesterol by cells activates LXRs (Figure 1), cholesterol must be converted to oxysterols before inducing genes. Oxysterols therefore act like second messengers for signaling a build up of cellular cholesterol. The most potent LXR ligands contain a single stereoselective oxygen on the side chain that functions as a hydrogen bond acceptor (87). Many of these oxysterols are generated by cytochrome P450 enzymes that are particularly prevalent in the liver and play a role in bile acid metabolism. One of these enzymes, sterol 27-hydroxylase (Cyp27), is broadly distributed in various tissues and cell types, including macrophages (88), suggesting that 27-hydroxycholesterol is the major LXR ligand in macrophages and other peripheral cells.

As expected for sterol sensors, LXRs induce several macrophage proteins involved in secreting excess cholesterol. These include ATP-binding cassette transporter A1 (ABCA1) (89, 90) (Figure 1), which mediates the transport of cholesterol and phospholipids from cells to lipid-depleted apolipoprotein that are the precursors for high-density lipoprotein (HDL). LXRs also modulate macrophage expression of apolipoproteins E, CI, CII, and CIV (83), which can act as acceptors for cholesterol and phospholipids secreted from cells by the ABCA1 pathway. Studies of human disease and animal models have shown that an impaired ABCA1 pathway is associated with low plasma HDL levels and increased atherosclerosis (30, 91, 92, 93, 94, 95). LXRs activate transcription of another macrophage ABC transporter (ABCG1) that is presumed to play some as yet unidentified role in cholesterol transport (83).

Consistent with the link between sterol and fatty acid metabolism, LXRs also activate SREBP-1c (96), which induces enzymes of fatty acid synthesis and desaturation, and enhance production of macrophage LpL (97), which can hydrolyze extracellular triglycerides (Figure 1). Thus, LXR ligands can increase the supply of unsaturated NEFAs in macrophages.

The effects of LXRs on cholesterol transport from macrophages have made them attractive targets for pharmaceutical agents designed to protect against cardiovascular disease. Indeed, mouse models have suggested that activation of LXRs suppresses atherosclerosis and ablation of LXR genes is atherogenic (83). A problem with current LXR agonists, however, is that they also increase fatty acid production and plasma triglycerides (96, 98, 99). Unsaturated NEFAs can actually

dampen the effects of LXRs on cholesterol secretion from macrophages by independent mechanisms (Figure 1). First, polyunsaturated NEFAs such as arachidonate can antagonize activation of LXR by endogenous sterols. Although this acts as a feedback mechanism for controlling SREBP-generated NEFAs, this antagonism also can suppress cholesterol secretion from cells by reducing synthesis of ABCA1 and apolipoproteins. Second, monounsaturated (e.g. palmitoleate and oleate), diunsaturated (e.g. linoleate), and polyunsaturated NEFAs (e.g. arachidonate) decrease ABCA1 levels in cells by destabilizing the protein (100). Third, by inducing the desaturating enzyme SCD through activation of SREBP-1c, LXR agonists can perturb the membrane lipid environment and impair ABCA1-mediated cholesterol transport from cells (101).

These secondary effects of NEFAs on ABCA1 stability and activity may contribute to the altered plasma lipoproteins and increased atherosclerosis associated with diabetes. With Type 2 diabetes and insulin resistance, plasma HDL levels are below normal (1), consistent with a decreased flux of tissue cholesterol into the HDL pathway. It would also be expected that the fatty acid supply in the artery wall would be elevated in diabetes, impairing the secretion of excess cholesterol from arterial macrophages.

4.3. Peroxisome proliferator-activated receptors

Peroxisome proliferator-activated receptors (PPARs) are a family of nuclear receptors that regulate transcription of multiple genes involved in cell differentiation and lipid metabolism. Like LXRs, PPARs form heterodimers with RXR that bind to PPAR response elements within the regulatory regions of targeted genes (102). Unsaturated fatty acids and their metabolic products, such as eicosanoids, are ligands for PPARs (103, 104). However, it is unclear if high enough concentrations of fatty acids and eicosanoids are present in intact cells to mediate PPAR activation. For example, it has recently been shown that 15-deoxy-delta^{12,14}-prostaglandin J_2, which has commonly been believed act as an endogenous PPAR ligand, does not act as an endogenous PPAR gamma ligand in intact cells (105, 106). Synthetic agonists for PPARs (thiazolidinediones and fibrates) are used for treating metabolic diseases, such as Type 2 diabetes and dyslipidemias (107).

PPARs have several modulating effects on cholesterol homeostasis in macrophages. Cholesterol loading of macrophages induces PPAR gamma, which stimulates transcription of a scavenger receptor (CD36) that promotes uptake of oxidized lipoproteins (108, 109) (Figure 1). PPAR alpha and PPAR gamma activators suppress expression of a receptor that promotes uptake of triglyceride- and cholesterol-rich lipoproteins (110). Thus, these nuclear receptors appear to have opposing effects on the influx of lipoprotein-derived cholesterol into macrophages. Importantly, however, PPAR gamma stimulates cholesterol efflux from cells by activating transcription of the LXR alpha gene, which in turn induces ABCA1 expression (111, 112) (Figure 1). PPARs also induce expression of scavenger receptor B1 (113), which promotes cholesterol efflux from cells to HDL particles.

Animal model studies have suggested that the net effect of PPAR activation is atheroprotective. Treatment of mice with agonists for PPAR alpha and PPAR gamma reduces progression of atherosclerotic lesions and decreases cholesterol accumulation in arterial macrophages (114, 115). Moreover, transplantation of PPAR gamma null bone marrow into atherosclerosis-susceptible mice further increases atherosclerosis (111). These results suggest that, in addition to improving insulin resistance and dyslipidemia, PPAR agonists can reduce the cardiovascular disease that often accompanies these disorders through their direct effects on arterial macrophage cholesterol homeostasis.

5. DIRECT EFFECTS OF NEFAs ON ARTERIAL SMOOTH MUSCLE CELLS

Following accumulation of lipid-loaded macrophages in the subendothelial space, the next step in the sequence of progression of the lesion is increased accumulation of smooth muscle cells (SMCs) in the intima and formation of a fibroatheroma. Finally, the lesion is destabilized, possibly by thinning of the SMC-rich fibrous cap and/or increased macrophage death, events that can lead to plaque rupture, thrombosis, and the acute clinical manifestations of atherosclerosis (54, 116). The arterial SMC is thought to play important roles in the two latter stages of lesion progression.

The accumulation of SMCs in atheromas is generally believed to be due to increased migration of SMCs from the underlying media of the artery into the intima, accompanied by increased proliferation and possibly decreased death/apoptosis. However, when atheromas are formed in areas with a preexisting mass of intimal SMCs, migration may be a minor contributor to the subsequent SMC accumulation. SMC proliferation appears to play a significant role in formation of fibroatheromas (117).

Much research has recently focused on the processes that drive formation of vulnerable lesions that are likely to rupture and cause acute clinical symptoms. A thin fibrous cap and a low number of SMCs versus macrophages are often seen in ruptured lesions. Several studies have shown an increased SMC death/apoptosis in these advanced lesions (118, 119, 120), but macrophages also account for a significant part of cell death in such lesions (121). Loss of SMCs may lead to plaque instability because most of the interstitial collagen fibers, which are important for the strength of the fibrous cap, are produced by SMCs (119, 122). Furthermore, increased SMC apoptosis in the arterial wall has recently been shown to induce secretion of pro-inflammatory cytokines and to increase invasion of monocytes, which would contribute to a decreased ratio of SMCs vs. macrophages (123). However, despite the correlation between loss of SMCs in the fibrous cap and plaque rupture, there is not yet direct evidence that SMC death indeed causes plaque rupture.

Taken together, it is not clear if SMC accumulation should be viewed as "good" or "bad", and it is quite likely that "good" or "bad" depends on the stage of the lesion (124). What is clearer is that accumulation of SMCs in the neointima is an integral part of lesion progression that likely converts a reversible fatty streak into a non-reversible fibroatheroma. Proliferation of SMCs appears to be stimulated by diabetes (125).

5.1. Proliferation

Oleate and linoleate increase the growth-promoting actions of several growth factors in arterial SMCs. Thus, oleate enhances the effects of angiotensin II in rat SMCs (126, 127, 128) and oleate and linoleate enhance the mitogenic effect of endothelin-1 (129). Furthermore, oleate and linoleate have recently been shown to enhance the growth-promoting effects of insulin-like growth factor I (IGF-I) in porcine arterial SMCs (130) and of platelet-derived growth factor-BB (PDGF-BB) in human SMCs (131). The ability of oleate to enhance PDGF-BB-induced human SMC proliferation is due to an increased entry of oleate-stimulated cells into S-phase of the cell cycle (131). In contrast, stearate, palmitate and arachidonate, as well as stereoisomers of oleate (elaidic acid) and linoleate (conjugated linoleic acid) do not induce proliferation (126, 130), suggesting that the mechanism of oleate and linoleate-enhanced growth of SMCs is due to specific processes.

Exogenous long-chain fatty acids, such as oleate, are minor contributors to substrate oxidation (approximately 5%) in SMCs compared to other mitochondrial substrates, such as glucose and acetate, which account for approximately 80% of the substrates oxidized by SMCs (130, 132). These observations indicate that the growth-promoting effects of oleate and linoleate are not due to a non-specific increase in the metabolic rate. Instead, the molecular mechanisms of oleate-induced proliferation in rat SMCs have been suggested to be mediated by protein kinase C; PKC (126), mitogen-activated protein kinase; MAPK/ERK (127) and increased formation of reactive oxygen species (128). However, in these studies, oleate was not pre-complexed to a carrier protein, and the concentration of exogenous oleate in cellular membranes may have been high. The molecular mechanisms of unbound linoleate-induced proliferation of rat SMCs have been attributed to the conversion of linoleate into the bioactive metabolites hydroperoxyoctadecadienoic acids (HPODEs) and monohydroxyoctadecadienoic acids (HODEs) by lipoxygenases. 13-HPODE has been shown to stimulate proliferation of rat SMCs (133), although its mitogenic effects appear to be species-dependent, as porcine SMCs do not proliferate in response to 13-HPODE (134). Linoleate has also been shown to lead to activation of PPAR alpha and PPAR gamma (for review see 135), although there is no evidence that linoleate exerts its mitogenic effects through PPARs in SMCs.

Studies of SMCs derived from humans or pigs suggest other mechanisms of action of the growth-promoting effects of oleate and linoleate. Oleate-induced stimulation of the growth-promoting effects of IGF-I ad PDGF-BB occurs when the fatty acids are added to SMCs
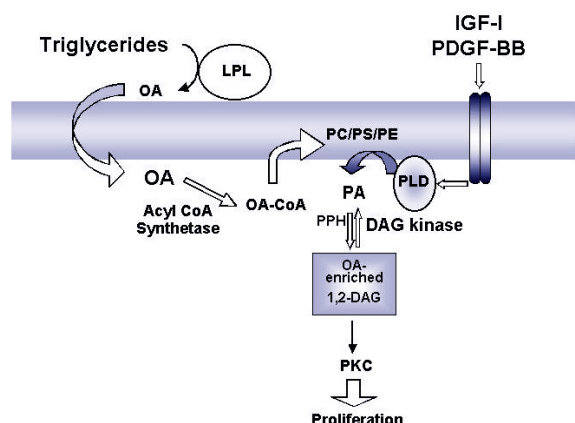
Figure 2. Model of the mitogenic effects of oleate in human SMCs. OA is transported into the cell by a flip-flop mechanism and/or by fatty acid transporters, and a large fraction is then esterified to form oleoyl-CoA (OA-CoA). OA-CoA contributes to de novo synthesis of DAG, triglycerides and phospholipids by a pathway that involves glycerol 3-phosphate (glycerol 3-P), lysophosphatidic acid (LPA), and phosphatidic acid (PA). Exposure of human SMCs to carrier protein-bound OA results increased levels of free, unesterified OA and total levels of triglycerides. OA is distributed mainly into the phospholipid pool (phosphatidylcholine, PC), but also into neutral lipids. In SMCs exposed to OA, PDGF-BB increases levels of OA-containing 1,2-DAG, most likely due to activation of phospholipase D (PLD). OA may enhance the mitogenic effects of PDGF-BB by two mechanisms involving generation of OA-enriched 1,2-DAG. First, in human SMCs exposed to OA, PDGF-BB-induced activation of PLD gives rise to increased levels of OA-enriched 1,2-DAG, which is believed to be a more efficient activator of classical PKCs than DAG containing saturated acyl chains. Second, OA may inhibit the activation of DAG kinase (DGK) mediated by PDGF-BB, thereby further increasing levels of OA-enriched 1,2-DAG. Modified from (130). PPH, phosphatidate phosphohydrolase.

pre-coupled to BSA at a ratio below 3:1 (130, 131). Under these conditions, oleate does not activate the ERK pathway (136) or enhance the effects of IGF-I on ERK activation (130). It does, however, result in a dramatic increase of levels of triglycerides (131, 136, 137), an effect that most likely does not explain the mitogenic effects of oleate (131). Instead, oleate stimulates increased formation of diacylglycerol (DAG) in the presence of PDGF-BB, and probably other growth factors (131, 138), as shown in Figure 2. Oleate has been shown to inhibit growth-factor-induced DAG kinase alpha activation in porcine SMCs (138) and since DAG kinase phosphorylates DAG to form phosphatidic acid (Figure 2), this process may explain the increased DAG levels. Furthermore, we have recently shown that inhibition of PLD activity prevents oleate- and linoleate-induced potentiation of the mitogenic effects of IGF-I, and that a DAG kinase inhibitor (R59022) enhances IGF-I-stimulated DNA synthesis in SMCs, similar to the effects of oleate and linoleate (130). These findings suggest that PLD, through increased intracellular levels of DAG, rather than the ERK pathway, may mediate the

growth-promoting effects of low concentrations of oleate and linoleate in porcine SMCs (Figure 2). However, saturated long-chain fatty acids that do not induce SMC proliferation also increase DAG formation. Thus, palmitate and stearate bound to albumin at a physiological ratio increase DAG concentrations in porcine SMCs (136, 137). This raises the interesting possibility that the fatty acid chain composition of DAG is important for its ability to promote SMC proliferation. Indeed, this hypothesis is supported by recent findings that exposure of human SMCs to oleate results in an increased oleate content in DAG (Figure 2), and a reduced content of saturated fatty acids (131). It is possible that DAGs with different acyl chains have different biological effects, and it has been suggested that DAG species enriched in monounsaturated or unsaturated fatty acids act as better activators of protein kinase C alpha than do DAG species enriched in saturated fatty acids (139). Together, these results indicate that increased levels of oleate may be important in driving SMC proliferation and accumulation by enhancing the effects of growth factors in atheromas in diabetes.

5.2. Necrosis

In SMCs derived from rat aorta, free fatty acids in the absence of albumin were found to induce marked cell death with EC_{50} values for oleic, linoleic, myristic and palmitic acid of 5, 2, 116 and 287 μmol/l, respectively (140). The effects of fatty acids on SMC death did not appear to be due to activation of apoptotic signaling pathways, and were not significantly blocked by the addition of equimolar concentrations of albumin (140). The mechanism mediating this effect of fatty acids on rat SMCs is unclear, as no significant death has been observed in SMCs derived from humans or pigs (130, 131, 141).

5.3. Migration

Oleate has been shown to stimulate basal migration of rat SMCs, and to have additive effects to those of angiotensin II (142). In these studies, SMCs were allowed to migrate in a gradient of oleate. Porcine SMCs preincubated with oleate or linoleate complexed to fatty acid-free BSA at a 1:1 molar ratio for 20 h, however, did not show a higher basal migratory potential, nor did they show an enhanced migratory response to IGF-I or PDGF-BB (Sherrid, Askari & Bornfeldt, unpublished results). Under these conditions, oleate markedly enhanced the growth-promoting effects of IGF-I and PDGF-BB (130, 131). Thus, the ability of oleate to stimulate SMC proliferation appears more consistent than its ability to affect SMC migration.

5.4. Other effects

Human SMCs express more core protein of the proteoglycans versican, decorin, and syndecan 4 when exposed to albumin-bound linoleate (141). Interestingly, the extracellular matrix produced by SMCs in the presence of linoleate bound LDL more tightly than that produced in the absence of linoleate (141). These results suggest that NEFAs can alter the extracellular matrix produced by SMCs in a manner that would lead to increased accumulation of LDL in the arterial wall. Accumulation of

proteoglycans has been proposed to contribute to atherosclerosis by retaining lipoproteins (143).

6. DIRECT EFFECTS OF NEFAs ON T-CELLS

There is now ample evidence that adaptive immunity, with its T-cells, antibodies, and immunoregulatory cytokines, regulates the process of atherogenesis (144). There is also increasing evidence that immune processes play a role in diabetes-accelerated atherosclerosis. Little is known about direct effects of long-chain fatty acids on non-transformed T-cells. The results that exist suggest that oleate and other fatty acids may inhibit T-cell responses (145, 146, 147, 148). This is an area that requires further research.

7. CONCLUSIONS AND PERSPECTIVE

Do NEFAs play a direct role in atherogenesis in the diabetic setting? A large body of evidence from in vitro studies, as reviewed in the present article, supports this concept. The hypothesis is more difficult to test in animal models and humans. Perhaps the strongest piece of support, to date, comes from studies in which mouse macrophages were designed to lack LpL expression. These mice had less atherosclerosis than mice with LpL-containing macrophages (28, 29, 30). As for humans, further clinical trials of triglyceride-lowering drugs on cardiovascular disease in patients with Type 1 and Type 2 diabetes are urgently needed. Although result of such trials will not tell us much about the mechanism of action of triglycerides in atherogenesis, they will reveal if triglyceride-lowering strategies lower the frequency of cardiovascular complications of diabetes. Finding such drugs must be our ultimate goal.

8. ACKNOWLEDGMENTS

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