Olate and Linoleate Enhance the Growth-promoting Effects of Insulin-like Growth Factor-I through a Phospholipase D-dependent Pathway in Arterial Smooth Muscle Cells*

Received for publication, May 24, 2002, and in revised form June 28, 2002 Published, JBC Papers in Press, May 22, 2002, DOI 10.1074/jbc.M205112200

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Diabetes causes accelerated atherosclerosis and subsequent cardiovascular disease through mechanisms that are poorly understood. We have previously shown, using a porcine model of diabetes-accelerated atherosclerosis, that diabetes leads to an increased accumulation and proliferation of arterial smooth muscle cells in atherosclerotic lesions and that this is associated with elevated levels of plasma triglycerides. We therefore used the same model to investigate the mechanism whereby diabetes may stimulate smooth muscle cell proliferation. We show that lesions from diabetic pigs fed a cholesterol-rich diet contain abundant insulin-like growth factor-I (IGF-I), in contrast to lesions from non-diabetic pigs. Furthermore, two fatty acids common in triglycerides, olate and linoleate, enhance the growth-promoting effects of IGF-I in smooth muscle cells isolated from these animals. These fatty acids accumulate predominantly in the membrane phospholipid pool; olate accumulates preferentially in phosphatidicholine and phosphatidylethanolamine, whereas linoleate is found mainly in phosphatidylethanolamine. The growth-promoting effects of olate and linoleate depend on phospholipid hydrolysis by phospholipase D and subsequent generation of diacylglycerol. Thus, concurrent increases in levels of IGF-I and triglyceride-derived olate and linoleate in lesions may contribute to accumulation and proliferation of smooth muscle cells and lesion progression in diabetes-accelerated atherosclerosis.

Both type 1 and type 2 diabetes result in an increased risk of developing atherosclerosis and subsequent myocardial infarction and stroke (1). The mechanisms whereby diabetes accelerates atherosclerosis remain to be elucidated. A recently developed porcine model of diabetes-accelerated atherosclerosis demonstrates that the increased progression of atherosclerosis in diabetes is associated with increases in plasma triglycerides and blood glucose (2). We have shown that in this model, diabetes leads to accumulation and proliferation of arterial smooth muscle cells (SMCs) in fibroatheromas and that high glucose levels are unable to directly increase proliferation of SMCs isolated from these pigs (3). Thus, while diabetes increases the accumulation of SMCs in the atherosclerotic lesion, this appears to be independent of a direct growth-promoting effect of glucose.

Our studies therefore focused on the association between elevated plasma triglycerides and lesion SMC proliferation in diabetes. There is strong evidence that higher plasma concentrations of fatty acids, a principal component of triglycerides, are correlated with increased risk of cardiovascular disease (4) and are commonly present in humans and animals with diabetes (2, 5). The major fatty acids in plasma triglycerides are palmitic acid (16:0), linoleic acid (cis 18:2), stearic acid (18:0), oleic acid (cis 18:1), and arachidonic acid (cis 20:4).

The results of this study show that IGF-I, a SMC growth factor (6), is abundant in lesions of atherosclerosis from diabetic pigs, whereas lesions from non-diabetic pigs contain little IGF-I. Furthermore, oleic acid (OA) and linoleic acid (LA) markedly enhance the growth-promoting effects of IGF-I through a phospholipase D (PLD)-dependent pathway in SMCs isolated from these animals. We propose that the increased SMC proliferation seen in diabetes-accelerated atherosclerosis is, at least in part, caused by an increase in IGF-I-induced PLD activity and subsequent generation of diacylglycerol (DAG) fueled by the elevated levels of triglycerides containing OA and LA.

EXPERIMENTAL PROCEDURES

Materials—Sodium salts of OA, LA, palmitic, arachidonic (AA), elaidic (EA), stearic acids (SA), and conjugated LA were purchased from Nuchar Prep (Elyssia, MN). Human recombinant IGF-I was obtained from Upstate Biotechnology (Lake Placid, NY). H[3]Thymidine (6.7 Ci/mmol) was obtained from PerkinElmer Life Sciences. [14C]OA (56.0 mCi/mmol) and [14C]OA (56 mCi/mmol) were obtained from Amersham Biosciences. BSA, indomethacin, OA-CoA, LA-CoA, and a monoclonal anti-β-actin antibody were obtained from Sigma. Propranolol, atenolol,

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* These studies were supported in part by National Institutes of Health Grants HL62887 (to K. B.), HL34300 and HL25394 (to M. C.), and HL55798 (to R. G.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

** Supported by National Institutes of Health Training Grant HL07312.

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1 The abbreviations used are: SMC, smooth muscle cell; AA, arachidonic acid; BSA, bovine serum albumin; EA, elaidic acid; SA, stearic acid; DAG, diacylglycerol; PKB, extracellular signal-regulated kinase; HPLC, high pressure liquid chromatography; HODE, hydroxyoctadecadienoic acid; IGF-I, insulin-like growth factor I; LPL, lipoprotein lipase; LA, linoleic acid; OA, oleic acid; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PPI, phosphatidylcholine; PPA, peroxisome proliferator-activated receptor; PS, phosphatidylserine; p70S6K, p70 S6 kinase.
ONO-RS-082, U73312, U73345, R59022, L655238, baicaeline, Wy-14643, LY294002, and wortmannin were purchased from Biomol Research Laboratories (Plymouth Meeting, PA). 1-Butanol and 2-butanol were purchased from Fisher. All inhibitors were used at selective concentrations that did not result in cytotoxicity. Anti-phospho(S473)-PKB/AKT, anti-phospho(Thr202/Tyr204)-ERK antibodies, and anti-IGF-I receptor β-subunit antibodies were obtained from Cell Signaling Technologies (Beverly, MA). Anti-phospho antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). A rabbit polyclonal anti-ERK antibody (7854) generated against the extracellular signal-regulated kinase (ERK) subdomain-XI peptide RRITVEALHPYEQYY-DPDTDE was generously provided by Dr. Edwin G. Krebs.

The Porcine Model of Diabetes-accelerated Atherosclerosis—The porcine model of streptozotocin diabetes-accelerated atherosclerosis has been described in detail previously (2, 3).

Immunohistochemical Detection of IGF-I in Atherosclerotic Lesions—Fixed segments obtained from the area between the third and forth intercostal artery of the thoracic aorta were embedded in paraffin and cut into sections. The sections were prepared for immunohistochemistry as described previously (3). Immunoreactive IGF-I was detected using a monoclonal anti-IGF-I antibody (Upstate Biotechnology) at a final concentration of 100 μg/ml. A control antibody of the same subclass (IgG1) and concentration was used as a negative control (Zymed Laboratories Inc.). IGF-I immunoreactivity was visualized by using an alkaline phosphatase kit (9).

Isolation and Culture of Porcine SMCs—SMCs were isolated from the thoracic aorta (between the third and forth intercostal artery) of non-diabetic pigs fed a chow diet via an explant method (3). Passages 4–11 were used for experiments. Prior to all experiments, the SMCs were made quiescent by incubation in Dulbecco’s modified Eagle’s medium + 0.5% fetal bovine serum for 48 h. All experiments were performed in Dulbecco’s modified Eagle’s medium containing 5.6 mM glucose + 0.5% fatty acid-free-BSA. These conditions did not reduce cell viability or induce toxicity.

Preparation of Fatty Acid-BSA Complex—Sodium salts of fatty acids were dissolved in distilled H2O (50 mg/ml) and diluted in sterile Dulbecco’s modified Eagle’s medium + 0.5% fatty acid-free-BSA (78 μM) to a final concentration of 70 μM. This mixture was equilibrated for 1 h at 37 °C in 5% CO2 prior to addition to the cells, allowing BSA-fatty acid complexes to form. This method has been estimated to result in an effective free fatty acid concentration in the nanomolar range (7). The concentrations of fatty acid and BSA were based on a physiological ratio between fatty acid and the carrier protein (BSA) and the concentration of albumin present in the extracellular fluid of the intestinal portion of the arterial wall (8).

Measurements of DNA Synthesis and Cellular Proliferation—SMC proliferation was measured as [3H]thymidine incorporation into DNA and determination of cell number, as described previously (3).

Western Blot Analysis—Expression of the IGF-I receptor β-subunit and phosphorylation of downstream kinases were analyzed by immunoblotting. SMCs were stimulated with fatty acids and/or IGF-I for the indicated periods of time. Cell lysates were prepared as described previously (9), separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to polyvinylidene difluoride membranes. After transfer, membranes were blocked in 5% nonfat milk in Tris-buffered saline-Tween 20 overnight at 4 °C. Branes. After transfer, membranes were blocked in 5% nonfat milk in ° Tris-buffered saline-Tween 20 overnight at 4 °C. After optimal transfer and blocking, membranes were probed with the following antibodies: monoclonal anti-IGF-I antibody (Upstate Biotechnology) at a final concentration of 100 μg/ml. A control antibody of the same subclass (IgG1) and concentration was used as a negative control (Zymed Laboratories Inc.). IGF-I immunoreactivity was visualized by using an alkaline phosphatase kit (9).

Statistical Analyses—Results are expressed as mean ± S.E. of a minimum of three experiments, performed in triplicate. Data were analyzed by one-way analysis of variance, using Graph Pad Prism (GraphPad Software, San Diego, CA). Simultaneous multiple comparisons were based on post-hoc comparison tests using a Student-Newman-Keuls test. Statistical significance was established at p < 0.05.

RESULTS

IGF-I Immunoreactivity Is Increased in Atherosclerotic Lesions from Diabetic Fat-fed Pigs—IGF-I acts as a SMC growth factor and may play a role in lesion progression. We therefore determined levels of immunoreactive IGF-I in lesions from non-diabetic and diabetic pigs fed a cholesterol-rich diet. As shown in Fig. 1A, lesions from non-diabetic pigs fed a cholesterol-rich diet contained little IGF-I immunoreactivity. Lesions from diabetic pigs fed the same diet, on the other hand, were large and contained abundant IGF-I immunoreactivity that was localized mainly to the macrophage-rich region of the lesion (Fig. 1B). A control antibody resulted in no staining of an adjacent section of the tissue (Fig. 1C).

Fig. 1. IGF-I immunoreactivity is increased in atherosclerotic lesions from diabetic fat-fed pigs. Thoracic aortas from non-diabetic and diabetic pigs fed a cholesterol-rich diet were fixed and processed as described under “Experimental Procedures.” Immunoreactive IGF-I was detected using a mouse monoclonal anti-IGF-I antibody. The slides were developed by using alkaline phosphatase, which results in a red reaction product. A, low levels of IGF-I immunoreactivity in a lesion from a non-diabetic pig fed a cholesterol-rich diet. B, extensive IGF-I immunoreactivity in the macrophage (Mφ)-rich region in a lesion from a diabetic pig fed a cholesterol-rich diet. C, a control antibody gave no staining. The position of the internal elastic lamina is indicated by arroes. L, lumen. Cellular composition and proliferation, measured as proliferating cell nuclear antigen-positive cells, of these lesions have been described elsewhere (3). The sections are shown at a 100× magnification. Three different animals from each group were analyzed with similar results.

extracts were applied to unmodified Silica Gel G TLC plates and developed in a solvent system of hexane:diethyl ether:glacial acetic acid (105:45:3) for 60 min. Plates were then dried and exposed to a phosphorimaging screen for 24 h. Standards containing mono-, di-, and triglycerides (Nucheck Prep) were visualized by exposure of the plate to iodine vapor. An Amersham Biosciences Storm 860 PhosphorImager was used for detection and quantification of radioactive spots.

During the past decade, it has been shown that fat feeding is an important factor in the development of atherosclerosis, and the experimental model of porcine diabetes has been widely used to study the effects of this particular diet on atherosclerotic lesion progression. We have recently reported that in this model, IGF-I expression is markedly increased in diabetic fat-fed pigs (3). In this study, we investigated the effect of diabetes on IGF-I expression in atherosclerotic lesions and compared it with expression in non-diabetic lesions. We found that IGF-I expression was significantly higher in diabetic lesions than in non-diabetic lesions. These results are consistent with previous reports showing that IGF-I expression is increased in atherosclerotic lesions from diabetic patients and animals (4-6). The increased expression of IGF-I in diabetic lesions suggests that this factor may play a role in lesion progression. Therefore, understanding the mechanisms that regulate IGF-I expression in atherosclerotic lesions may provide insights into the underlying biology of lesion progression and potential therapeutic targets.
OA and LA Potentiate IGF-I-induced SMC Proliferation—We evaluated the role of long-chained fatty acids common in triglycerides on basal and IGF-I-stimulated [3H]thymidine incorporation in porcine SMCs. We used a concentration of IGF-I (1 nM) that results in maximal stimulation of thymidine incorporation (data not shown). OA and LA potentiated the effects of IGF-I (Fig. 2A), whereas the trans isomer of OA, EA, and the positional isomer of LA, conjugated LA (Fig. 2A), did not potentiate the effects of IGF-I. Furthermore, other long-chained fatty acids, such as AA, SA, and palmitate, did not enhance the effects of IGF-I. However, SA and palmitate exhibited cytotoxic effects at higher concentrations (>70 μM). To study the time course of OA- and LA-induced effects on IGF-I-stimulated thymidine incorporation, SMCs were pre-incubated for 0–24 h with 70 μM OA or LA and then stimulated with IGF-I for an additional 18 h. OA and LA potentiated the effects of IGF-I in a time-dependent manner, with a maximal effect observed after a 24-h preincubation with LA and a 6-h preincubation with OA (Fig. 2B and C, hatched bars). Because fatty acids require the esterification to a CoA moiety catalyzed by acyl-CoA synthases to achieve biological activity (12), we determined the effects of preincubation of the CoA esters of OA and LA on IGF-I-induced increases in thymidine incorporation. Fig. 2B and C demonstrates that the effects of OA and LA are increased with their esterification to CoA, as both OA-CoA and LA-CoA were more effective in potentiating the mitogenic effects of IGF-I, and a significant potentiation was seen earlier than that of unmodified OA and LA (Fig. 2B and C, cross-hatched bars).

To confirm these results, we analyzed the effects of OA and LA on IGF-I-mediated increases in SMC number. OA and LA significantly (p < 0.05) enhanced the effects of IGF-I after a 6-day stimulation (control: 54,970 ± 5,498 cells per well; OA: 61,830 ± 969 cells per well; LA: 68,843 ± 1,279 cells per well; IGF-I: 62,903 ± 786 cells per well; IGF-I + OA, 72,163 ± 3,307 cells per well; and IGF-I + LA, 81,270 ± 2,161 cells per well; mean ± S.E., n = 3). Thus, OA and LA enhance the growth-promoting effects of IGF-I in SMCs.

The LA-induced Mitogenic Effects Are Not Caused by Hydroxyoctadecadienoic Acid (HODE) Formation—We investigated the mechanism of action of OA- and LA-mediated enhancement of the IGF-I mitogenic effect. In some cell types, mitogenic effects of LA have been shown to be caused by the conversion of LA into its hydroxy metabolites, 7- and 13-HODEs, mostly via the action of the lipoygenase pathway (13). However, our results show that LA-induced potentiation of the growth-promoting effects of IGF-I is independent of lipoygenase activity, as pharmacological inhibition of either 5-lipoxygenase (L655238, 5 μM) or 12-lipoxygenase (baicalein, 25 μM) did not block the effect of LA (data not shown). It has also been demonstrated in other tissues that LA can serve as a substrate for cyclooxygenase (14). In the porcine SMCs, inhibition of cyclooxygenase-1/cyclooxygenase-2 with indomethacin (10 μM) had no effect (data not shown). Furthermore, HPLC analysis of the media of SMCs labeled with [14C]LA and stimulated with IGF-I confirmed the pharmacological data, as there was neither release into the media nor any esterification of LA (data not shown).

There is also evidence that conversion of LA into HODEs may lead to activation of peroxisome proliferator-activated receptors (PPARs) (15). However, it is unlikely that the effects of OA and LA on IGF-I-stimulated SMC proliferation are mediated by PPARs because other fatty acid PPAR activators, such as AA and EA did not mimic the effects. We also observed that the PPARα agonist Wy-14643 did not mimic the effects of OA and LA (data not shown). These results indicate that HODEs do not mediate the actions of LA on IGF-I-stimulated SMC proliferation.

OA and LA Do Not Enhance IGF-I-induced Signaling—Mitogenic signaling pathways induced by the IGF-I receptor in SMCs were next studied. Activation of the phosphatidylinositol 3-kinase (PI3K) pathway has been reported as a main mitogenic pathway activated by IGF-I in SMCs (16), a finding that was confirmed in our studies (data not shown). As shown in
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Fig. 3. Neither OA nor LA increases IGF-I-induced kinase phosphorylation. Purified SMCs were stimulated with LA, OA, and/or IGF-I for the indicated time periods. Total cell lysates (60 μg lane) were prepared and separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to membranes, and incubated with antibodies (final dilutions given within parentheses) specific to phospho-AKT (1:1,000), AKT (1:1,000), phospho-p42/p44 ERK (1:1,000), p42/p44 ERK (1:1,000), phospho-p70S6K (1:1,000), p70S6K (1:1,000), or the IGF-I receptor β-subunit (1:1,000). β-Actin (1:10,000) was used as a loading control. The experiment was performed three times with similar results.

OA and LA Are Not Used as a Source of Fuel but Instead Are Incorporated into Membrane Phospholipids—In mammalian cells, long-chain fatty acids undergo β-oxidation for use as an energy source, or they enter different lipid pools. However, neither OA nor LA undergoes significant β-oxidation in SMCs, as incubation of [14C]OA and [14C]LA did not lead to a detectable increase in release of 14CO2 for up to 24 h. Furthermore, expression of the IGF-I receptor β-subunit was not increased by OA or LA (Fig. 3).

OA and LA Are Not Used as a Source of Fuel but Instead Are Incorporated into Membrane Phospholipids—In mammalian cells, long-chain fatty acids undergo β-oxidation for use as an energy source, or they enter different lipid pools. However, neither OA nor LA undergoes significant β-oxidation in SMCs, as incubation of [14C]OA and [14C]LA did not lead to a detectable increase in release of 14CO2 for up to 24 h (data not shown). We therefore investigated the degree of OA and LA incorporation into different lipid pools in SMCs, using TLC. As shown in Table I, the majority (~90%) of OA and LA is incorporated into the phospholipid pool, with minor amounts in triglycerides, diglycerides, and monoglycerides. We next determined which phospholipid pools were the destination of OA and LA, by using HPLC. Fig. 4, A and B, demonstrates that following exposure of SMCs to exogenous OA and LA, OA is found mainly in membrane phosphatidylethanolamine (PE) and phosphatidylserine (PS) (Fig. 4A), whereas OA is distributed mainly into the PE, PS, and phosphatidylcholine (PC) pools (Fig. 4B). These data demonstrate that the principal destination of OA and LA is the membrane phospholipid pools, which may serve as substrates for IGF-I-induced, phospholipase-dependent signaling pathways.

OA- and LA-induced Potentiation of the Mitogenic Effects of IGF-I Depends on PLD Activity and Subsequent DAG Formation—There are three groups of phospholipases that hydrolyze phospholipids, namely phospholipase C (PLC), phospholipase A2 (PLA2), and PLD. Because IGF-I may be able to activate all of these phospholipases, we investigated the contribution of PLC, PLA2, and PLD to OA and LA-induced potentiation of the mitogenic effect of IGF-I. Although IGF-I has previously been shown to activate PLC in SMCs (17), the results show that pharmacological inhibition of PLC (U73342, 3 μM) did not affect fatty acid-induced potentiation of IGF-I-stimulated DNA synthesis, indicating that PLC activity is not required for this effect (data not shown). The fact that neither OA nor LA is incorporated into phosphatidylinositol (Fig. 4) supports the lack of PLC involvement in the mitogenic effects induced by these fatty acids.

Inhibition of PLA2 with arachidonoyltrifluoromethyl ketone (AACOF3, 10 μM) or ONO-RS-082 (10 μM) also did not affect OA- and LA-mediated potentiation (data not shown). Furthermore, cytosolic PLA2 cleaves fatty acids off the sn-2 position of phosphatidylcholine (PC) and is associated with the production of LA- and AA-derived hydroperoxy metabolites (18). Our findings that OA and LA are not incorporated into phosphatidylcholine and that their effects are not mimicked by AA further support the lack of cytosolic PLA2 involvement, as does a previous study showing that IGF-I does not activate cytosolic PLA2 in SMCs (9).

To determine the involvement of PLD, we used 1-butanol (an inhibitor of PLD action) and 2-butanol (inactive control). As shown in Fig. 5A, 1-butanol (0.2% v/v), but not 2-butanol (Fig. 5B), completely blocked the potentiating effects of OA and LA. The role of the PLD pathway was also confirmed by using propranolol (a β-adrenergic blocker that also inhibits phosphatidate phosphohydrolase (PPH) activity). PPH catalyzes the conversion of phosphatidic acid (PA) to DAG. Atenolol, another β-blocker without PPH-blocking activity, was used as a negative control. Propranolol (Fig. 5C), but not atenolol (Fig. 5D), blocked OA- and LA-induced potentiation of the growth-promoting effects of IGF-I. These results indicate that PLD activation mediates the effects of OA and LA. Because PLD cleaves membrane PC and PE to form PA, which in turn is metabolized into DAG via the activity of PFP, we hypothesized that elevation of DAG levels should mimic the effects of OA and LA. Therefore, we inhibited DAG kinase (which phosphorylates DAG to PA and thus decreases levels of DAG) by using R59022. Indeed, inhibition of DAG kinase dose-dependently increased IGF-I-induced thymidine incorporation (Fig. 6), thus mimicking the effects of OA and LA. Together, these results show that the effects of OA and LA depend on PLD activity and subsequent generation of DAG.

DISCUSSION

Physiologically Relevant Concentrations of OA and LA Selectively Enhance the Growth-promoting Effects of IGF-I in SMCs—We show that exposure of SMCs to physiological concentrations of OA and LA results in a marked enhancement of the growth-promoting effects of IGF-I, a growth factor present in lesions of atherosclerosis. Interestingly, OA and LA have previously been demonstrated to enhance the effects of other growth factors in SMCs, such as angiotensin II (19–21) and endothelin-1 (22). The results of the present study support and extend earlier findings (23) that the ability to enhance growth factor effects appears to be specific to OA and LA since SA,
palmitate, AA, the trans isomer of OA, and the positional isomer of LA (EA and conjugated LA, respectively), do not mimic the effects of OA and LA. It has also been shown that micromolar concentrations of OA and LA can induce proliferation of SMCs in the absence of growth factors when added without prior coupling to a carrier protein (13, 23). Although our results indicate that OA and LA appear to exert weak mitogenic effects by themselves when complexed to BSA, their main effect is to enhance the growth-promoting effects of IGF-I. Thus, unbound OA and LA may induce cellular responses quite different from those observed when OA and LA are complexed to a carrier protein at a physiological ratio (7).

How Do OA and LA Potentiate the Effects of IGF-I?—We show that the effects of OA and LA on IGF-I-mediated SMC proliferation are likely to depend on entry of OA and LA into the cell and subsequent generation of CoA-esterified fatty acids, since OA-CoA and LA-CoA induced potentiation of the IGF-I response more efficiently and rapidly than OA and LA. Indeed, ~90% of incorporated OA and LA were found in the membrane phospholipid pool. However, it is important to note that there was a dissimilar distribution of OA and LA into the membrane pools because OA was distributed mainly into membrane PC, PE, and PS, whereas LA was distributed mainly into PE and PS.

To investigate how OA- and LA-containing phospholipids enhance the mitogenic effects of IGF-I, we studied a number of different possibilities. Our results show that OA and LA do not result in increased expression of the IGF-I receptor β-subunit nor do they enhance the ability of IGF-I to phosphorylate downstream kinases implicated in the mitogenic effects of IGF-I. Furthermore, the effects of OA and LA are unlikely to be caused by increased formation of reactive oxygen species, corroborating a recent finding that the contribution of OA to mitochondrial oxidation is minor (24). Accordingly, pretreatment with antioxidants had no effect on OA- or LA-induced potentiation of the growth-promoting effects of IGF-I.3 Finally, under the conditions used in this study, neither activation of PPARs nor formation of HODEs appears to mediate the effects of OA and LA.

Instead, our results show that the effects of OA and LA on IGF-I mitogenic action are mediated through a PLD-dependent mechanism. It has been shown, in SMCs as well as other cell types, that growth factors such as IGF-I can induce PLD activation (25, 26) and result in the hydrolysis of PC and, to some extent, PE. This hydrolysis leads to an increase in formation of PA, which is converted to sn-1, 2-DAG via the action of PPH (see Fig. 7 and Ref. 26). Inhibition of either PLD or PPH resulted in attenuation of the OA- and LA-mediated potentiation of the growth-promoting effects of IGF-I. Thus, it is likely that increased DAG formation, not PA, mediates the effects of OA and LA. Furthermore, the fact that inhibition of DAG kinase mimics the effects of OA and LA further supports that PLD-mediated generation of DAG is responsible for our observations (Fig. 7).

Therefore, we propose that the incorporation of OA and LA into membrane PC and PE, in combination with IGF-I-stimulated PLD activity, leads to an increase in OA- and LA-containing species of DAG that may have a higher degree of activity than the “native” DAG. Indeed, it has been reported that changing the molecular species of the acyl chains of DAG (27) can modulate the stimulation of protein kinase C activity, as measured in vitro. DAG has also been shown to activate other signaling molecules with potential mitogenic effects (28). Another possibility is that more DAG may be formed as a result of changing the molecular species of DAG that may have a higher degree of activity than the “native” DAG. Indeed, it has been reported that changing the molecular species of the acyl chains of DAG (27) can modulate the stimulation of protein kinase C activity, as measured in vitro.
derived growth factor–B-chain homodimer-induced increases in DAG kinase activity in SMCs and that this attenuation of activity is associated with an increase in intracellular DAG concentrations (29). We are currently investigating these alternate pathways.

Do OA and LA Contribute to SMC Proliferation in Diabetes-accelerated Atherosclerosis?

We have previously shown, using the porcine model of diabetes-accelerated atherosclerosis, that diabetes results in a marked increase in lesion progression and SMC accumulation and proliferation (3). The present study demonstrates a possible mechanism whereby elevated triglycerides and non-esterified fatty acids seen in diabetes may contribute to SMC proliferation and lesion progression (30). Our preliminary results show that levels of immunoreactive LPL are markedly elevated.

Fig. 5. LA- and OA-induced potentiation of IGF-I-induced DNA synthesis depends on PLD-mediated generation of DAG. Porcine SMCs were pretreated for 18 h with LA or OA. 1-Butanol (1-but; 0.2%) (A), 2-butanol (2-but; 0.2%) (B), propranolol (prop; 10 μM) (C), or atenolol (aten; 10 μM) (D) was then added 30 min prior to the addition of IGF-I. DNA synthesis was measured as described in Fig. 2. Values are represented as mean ± S.E. of triplicate samples of representative experiments (n = 3). *, p < 0.01 versus IGF-I alone; **, p < 0.001 versus IGF-I alone; †, p < 0.05 versus IGF-I alone.

Fig. 6. Inhibition of DAG kinase increases the proliferative effects of IGF-I in porcine SMCs. Porcine SMCs were pretreated with R59022 at the indicated concentrations for 30 min prior to the addition of IGF-I (1 nM). DNA synthesis was measured as described in Fig. 2. Values are represented as mean ± S.E. of triplicate samples of representative experiments (n = 3). *, p < 0.01 versus IGF-I alone.

Fig. 7. Schematic of OA- and LA-induced potentiation of IGF-I-stimulated proliferation in porcine SMCs. OA and LA are transported into the cell and activated via an esterification process to a CoA ester by acyl-CoA synthase. The activated fatty acids are then incorporated in membrane phospholipids, which, upon stimulation of a PLD by a growth factor, such as IGF-I, result in the release of PA. PA, in turn, is metabolized by PPH into DAG, which may act via activation of a PKC isoform or possibly via other pathways to increase SMC proliferation.

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in the macrophage-rich area in lesions from diabetic pigs compared with non-diabetic pigs. Interestingly, high glucose levels and advanced glycation end products have been shown to increase LPL (35) expression in macrophages. In the present study, lesions from diabetic pigs were found to contain more IGF-I immunoreactivity than lesions from non-diabetic pigs. The IGF-I associated mainly with macrophage-rich areas of the lesions. We are currently investigating the possible mechanisms responsible for this increased IGF-I immunoreactivity. One possibility is that macrophages are the main source of IGF-I in the lesion and that lesions from diabetic pigs contain more macrophages than lesions from non-diabetic pigs. Other possibilities include a direct effect of factors associated with the diabetic environment on macrophage expression of IGF-I (36) or trapping of IGF-I derived from circulation, or other lesion cell types, in macrophage-rich areas. Whatever the source of IGF-I (endocrine, paracrine, or autocrine), the growth-promoting effects of IGF-I on SMCs are enhanced by OA and LA. Thus, we propose that diabetes results in increased levels of LPL and IGF-I in atheromas and that these events, in combination with the elevated levels of OA- and LA-containing triglycerides, accelerate SMC proliferation and lesion progression.

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doi: 10.1074/jbc.M205112200 originally published online July 22, 2002

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