Oxidized low density lipoprotein-mediated activation of phospholipase D in smooth muscle cells: a possible role in cell proliferation and atherogenesis

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Abstract Low density lipoproteins (LDL) are risk factors in atherosclerosis and oxidative modification of LDL to oxidized LDL (OX-LDL) increases its atherogenicity. Development of atherosclerosis likely involves OX-LDL-mediated smooth muscle cell (SMC) proliferation. However, the mechanism(s) of SMC proliferation by OX-LDL is unknown. We hypothesized that OX-LDL may mediate SMC proliferation by activation of phospholipase D (PLD) through the generation of the second-messenger, phosphatidic acid (PA). To test this hypothesis, activation of PLD by OX-LDL was investigated in [^{3}H]myristic acid- or [^{32}P]orthophosphate-labeled rabbit femoral artery smooth muscle cells (RFASMC) in the presence of 0.5% ethanol or 0.05% butanol. Phospholipase D activation, as measured by labeled phosphatidylethanol (PEt) or phosphatidylbutanol (PBt) formation, was enhanced (3- to 5-fold) by OX-LDL. This activation of PLD was specific for OX-LDL, as native LDL or acetylated LDL had no effect. Further, OX-LDL-mediated [^{32}P]PEt formation was dose- and time-dependent. To determine the mechanism(s) of OX-LDL-induced PLD activation, the role of protein kinase C (PKC) and Ca^{2+} was investigated. Pretreatment of [^{32}P]orthophosphate-labeled RFASMC with known inhibitors of PKC such as staurosporine, calphostin-C, or H-7, had no effect on OX-LDL-induced PLD activation. Also, down-regulation of PKC by 12-O-tetradecanoylphorbol 13-acetate (TPA) (100 nM, 18 h) did not alter the OX-LDL-mediated [^{32}P]PEt formation. However, pretreatment of RFASMC with genistein, a putative inhibitor of tyrosine kinases, attenuated the OX-LDL-mediated [^{32}P]PEt formation. In addition, exposure of RFASMC to sodium orthovanadate, an inhibitor of phosphatases, enhanced the OX-LDL-mediated PLD activation. The effects of genistein and vanadate on PLD activation were specific for OX-LDL as these agents did not alter the TPA-induced [^{32}P]PEt formation. Treatment of quiescent RFASMC with OX-LDL increased [^{3}H]thymidine incorporation into DNA. This enhanced incorporation of [^{3}H]thymidine into DNA was also mimicked by exogenously added phosphatidic acid (PA) or lysophosphatidic acid (LPA). These findings suggest that OX-LDL is a potent activator of the PLD pathway in SMC. The activation of PLD by OX-LDL generates second-messengers like PA and/or LPA which modulate mitogenesis. Thus, these results indicate that OX-LDL, in atherosclerotic lesions, may enhance SMC proliferation through the modulation of signal transduction pathways including activation of PLD.—**Natarajan, V., W. M. Scribner, C. M. Hart, and S. Parthasarathy.** Oxidized low density lipoprotein-mediated activation of phospholipase D in smooth muscle cells: a possible role in cell proliferation and atherogenesis. *J. Lipid Res.* 1995. **36:** 2005–2016.

Supplementary key words signal transduction • rabbit femoral artery • protein kinase C • tyrosine kinases • phosphatidic acid • lysophosphatidic acid • phosphatidylalcohol • vanadate

Recent studies suggest that low density lipoproteins (LDL) are atherogenic and that oxidative modification of LDL to oxidized (OX) LDL enhances its atherogenicity (1–3). Accumulation of OX-LDL in atherosclerotic lesions from humans and rabbits indicates possible modification of LDL to OX-LDL in vivo (4, 5). Furthermore, conversion of LDL to OX-LDL occurs in vitro upon incubation of LDL with either endothelial cells (EC), smooth muscle cells (SMC), and macrophages or in the presence of metal ions such as Cu^{2+} (6).

The proliferation of vascular SMC plays an important role in atherogenesis (7). However, the mechanism(s) responsible for SMC proliferation is not clear. It is

Abbreviations: BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid; CALP-C, calphostin-C; DAG, diacylglycerol; DMEM, Dulbecco's modified Eagle's medium; EC, endothelial cell; EGF, epidermal growth factor; EGTA, ethylenebis(oxyethylenenitrilo) tetraacetic acid; LDL, low density lipoproteins; LPA, lysophosphatidic acid; OX-LDL, oxidized LDL; PA, phosphatidic acid; PAO, phenyl arsineoxide; PAF, platelet activating factor; PBt, phosphatidylbutanol; PC, phosphatidylcholine; PDGF, platelet derived growth factor; PEt, phosphatidylethanol; PKC, protein kinase C; PLC, phospholipase C; PLD, phospholipase D; RFASMC, rabbit femoral artery smooth muscle cell; SMC, smooth muscle cell; TPA, 12-O-tetradecanoylphorbol-13-acetate.

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hypothesized that OX-LDL-mediated modulation of transmembrane signalling pathways and generation of second messengers is involved in SMC proliferation and subsequent development of atherosclerosis. It has been demonstrated that OX-LDL, as compared to LDL, enhances the chemotactic, mitogenic, and adhesive properties of EC, SMC, and monocytes (1, 8–10). Exposure of vascular SMC to OX-LDL increases intracellular free calcium (11), phosphoinositide turnover (12, 13), and prostacyclin (PGI_2) production in human vascular EC (14). These data indicate that exposing cultured vascular EC and SMC to OX-LDL modulates the generation of second messengers which may alter cell functions such as proliferation, secretion, migration, and adhesion.

One possible mechanism of OX-LDL-mediated mitogenic signalling may be through the generation of phosphatidic acid (PA) and/or lysophosphatidic acid (LPA). Both PA and LPA elicit a wide variety of biological responses, including stimulation of DNA synthesis in diverse types of mammalian cells (15–18). In addition to the de novo biosynthesis, PA is also rapidly and transiently generated through the activation of phospholipase D or phospholipase C (PLC)/diacylglycerol (DAG) kinase pathways. Activation of PLD and subsequent generation of PA is recognized as an important signal transduction pathway in mammalian cells (19–21). Phosphatidic acid can then be either dephosphorylated by PA phosphatase (PA Pase) giving rise to DAG (22), or PA can be converted to LPA by phospholipase A_1 or A_2 (20). Stimulation of PLC-catalyzed hydrolysis of phosphatidylinositol 4,5 bisphosphate (PIP_2) or phosphatidylcholine (PC) generates (DAG), an endogenous activator of protein kinase C (PKC) (23). Diacylglycerol can be converted to PA by DAG kinase or to monoacylglycerol by DAG lipase (19–22).

In this study, we have examined OX-LDL-induced PLD activation and the possible role of PA and LPA in SMC proliferation. Our results show that OX-LDL is a potent activator of PLD in SMC. This activation of PLD by OX-LDL was PKC- and Ca^{2+}-independent and was sensitive to genistein and vanadate. Further, exogenous addition of PA or LPA to SMC increased [^{3}H]thymidine incorporation, suggesting that OX-LDL-induced PLD activation may represent a signal for SMC proliferation and, therefore, contribute to the development of atherosclerotic lesions.

METHODS

Materials

Staurosporine, H-7, sodium orthovanadate, genistein, 12-O-tetradecanoylphorbol 13-acetate, Medium 199, fetal calf serum, and Dulbecco's phosphate-buffered saline (PBS) were purchased from Sigma Chemical Co., St. Louis, MO. [^{32}P]orthophosphate (carrier-free) and [^{3}H]myristic acid (sp act 40 Ci/mmol) were obtained from DuPont NEN, Boston, MA. [Methyl-[^{3}H]]thymidine (sp act 64.7 Ci/mmol) was obtained from American Radiolabeled Chemicals Inc., St. Louis, MO. Phosphatidic acid, lyso phosphatidic acid, phosphatidylethanol, phosphatidylbutanol, phosphatidylcholine, and dioleoylglycerol were from Avanti Polar Lipids, Inc., Alabastar, AL. Silica gel-coated TLC plates were from Analtech, Newark, DE.

Tissue culture

Smooth muscle cells were isolated from rabbit femoral arteries. Briefly, the femoral artery was removed, cleaned under a dissecting microscope, and small pieces of media were carefully stripped from the vessel wall. Four or five small strips (2 × 2 mm dimension) were placed in 100-mm-diameter tissue culture dishes containing Media 199 supplemented with 10% fetal calf serum, 10 U/mL Penn/Strep, and 40 μg/mL gentamycin. Within 2 weeks, a large number of cells had migrated from the explants. SMC from the 100-mm dishes were subcultured in T-75 flasks, were identified according to morphological and immunological criteria (11, 24), and were used between 5th and 12th passage.

Preparation of LDL and OX-LDL

LDL was isolated from normal human plasma by sequential ultracentrifugation in the presence of 0.26 mM EDTA and stored at 4°C at a concentration of 7.6 ± 3.2 mg LDL protein/ml (6). Oxidation of LDL was performed in PBS using 100 μg/mL LDL protein and 5 μM $CuSO_4$ (6). After incubation at 37°C for 24 h, OX-LDL was reisolated by ultracentrifugation after density adjustment to 1.09 g/mL. The OX-LDL preparations usually gave over 50 nmol/mg of thiobarbituric acid products before reisolation and were stored after dialysis and filter sterilization at 4°C.

Measurement of intracellular free Ca^{2+}_i

RFASMC were incubated in culture medium containing the cell permeant fura-2 AM (acetoxymethyl ester form, 10 μM in DMSO) (Molecular Probes Eugene, OR) and studied as monolayers grown on glass cover slips. Fura-2 loading was conducted in the dark at room temperature for 45 min to minimize the translocation of the indicator to intracellular organelles (25). After washing, the coverslips were allowed to incubate in Hank's Balanced Salt Solution plus 10 mM HEPES (HBSH) at room temperature for 30 min in the dark to minimize leakage of the hydrolyzed indicator from the cells and to prevent photobleaching. Immediately prior to study, coverslips were washed with HBSS at 37°C to

remove any fura-2 that may have leaked from the cells. Next, monolayers were transferred to a 3-mL quartz cuvette containing HBSH at 37°C. The coverslips were held by a custom-made support that positions the coverslip above a star-headed stir bar at a 45° angle to the excitation beam. Coverslips were studied at 37°C in the thermostatted sample compartment of a PTI spectrofluorometer (Deltascan) controlled by an IBM-386 PC programmed to alternately excite the fura-2-loaded cells at 340 and 380 nm and monitor fluorescence at 510 nm at a rate of 10/sec. The coverslips were allowed several minutes to undergo temperature stabilization prior to initiating recordings. LDL or OX-LDL was added to the cuvette at final concentrations of 10–25 μg/mL. The ratios of fluorescence intensities at 340 and 380 nm were calculated and $[Ca^{2+}]_i$ was estimated as previously described (26). Oxidized LDL does not have any fluorescence in the specified range, and in all experiments measurements were corrected for the autofluorescence from cells not loaded with fura-2.

Radiolabeling of RFASMC

Two protocols were used for labeling phospholipids of RFASMC. Confluent RFASMC in 35-mm dishes were incubated with [^{32}P]orthophosphate (5 μCi/mL) in DMEM phosphate-free media containing 2% fetal bovine serum for 24 h at 37°C in a 5% CO_2–95% air atmosphere (27). About 1–2% of the added [^{32}P]orthophosphate was incorporated into total phospholipids and approximately 50–60% of the label was in the PC fraction. RFASMC were also labeled with [^{3}H]myristic acid (2 μCi/mL) for 24 h in medium 199 containing 20% fetal bovine serum. About 30% of the added [^{3}H]myristic acid was incorporated into total lipids and approximately 85% of the label was in the PC fraction (27).

Measurement of PLD activation

RFASMC, prelabeled as described above, were washed in serum-free RPMI 1640 medium and incubated with 1 mL of the same buffer containing LDL, OX-LDL, or indicated agonist in the presence of either 0.5% ethanol or 0.05% butanol. Incubations were carried out for various times, and the reaction was terminated by addition of 1 mL CH_3OH–HCl 100:1 (v/v) (28, 29). The cells were scraped into glass test tubes, and the 35-mm dishes were reextracted with an additional 1 mL of CH_3OH–HCl 100:1 (v/v). Lipids were extracted by addition of 2 mL of chloroform and 0.8 mL of water according to the method of Bligh and Dyer (30). The labeled lipids in the chloroform phase were evaporated to dryness under N_2 and were redissolved in 50 μL of chloroform for the determination of labeled products formed by PLD activation. PLD activity was assessed by quantifying the formation of either labeled PEt or PBt after separation by TLC on silica gel H plates containing 1% potassium oxalate (27, 28) developed with the upper phase of a mixture of ethyl acetate–2,2,4-trimethyl pentane–glacial acetic acid–water 65:10:15:50 (vol/vol). This TLC system separated labeled PA from PEt or PBt and other phospholipids. Radioactive PEt or PBt and PA were detected by autoradiography, identified with unlabeled standards under I_2 vapor and were quantified by liquid scintillation spectrometry.

[^{3}H]thymidine incorporation into DNA

About 80% confluent RFASMC were made quiescent by incubating for 48 h in RPMI-1640 medium containing 0.25% fetal bovine serum. Quiescent cells in 24-well clusters were incubated with media alone or media containing LDL, OX-LDL, PA, LPA, or other agents for 16 h. At the end of 16 h, [^{3}H]thymidine (0.5 μCi/well) was added and incubations were continued for an additional 6 h (31). The radioactive medium was aspirated, cells were washed twice in ice-cold PBS buffer followed by 5% TCA exposure for 2 h at room temperature. The TCA solution was aspirated and 0.2 mL of 1 N NaOH was added to solubilize the insoluble material. At the end of 2 h, 0.2 mL of 1 N HCl was added to each well to neutralize the NaOH and [^{3}H]thymidine incorporation was quantified by liquid scintillation counting.

Other methods

Conjugated dienes in the LDL and OX-LDL were determined by measuring the absorbance (100 μg/mL in PBS buffer) at 324 nm with a Beckman UV/VB spectrophotometer.

Statistical analysis

All results are expressed as mean ± SD. Statistical comparisons were made by analysis of variance, and pairwise comparisons were performed using Fisher's LSD. Statistical significance was accepted at $P < 0.05$.

RESULTS

Stimulation of PLD by OX-LDL

Addition of OX-LDL to [^{3}H]myristic acid-labeled RFASMC, in the absence of exogenous ethanol, resulted in the accumulation of [^{3}H]PA and [^{3}H]DAG (**Table 1**). Formation of these two products was specific to OX-LDL treatment as addition of LDL or vehicle caused no increase in [^{3}H]PA or DAG. However, the formation of [^{3}H]PA may be due to activation of the PLD pathway or activation of the phospholipase C (PLC)-catalyzed hydrolysis of PC and subsequent conversion of [^{3}H]DAG to [^{3}H]PA by DAG kinase (32). In order to elucidate the PLD versus PLC/DAG kinase pathways, the formation

TABLE 1. Stimulation of PLD activity by OX-LDL

Treatment	^{3}H-Labeled Products Formed					
	(-) Ethanol			(+ Ethanol)		
	PEt	PA	DAG	PEt	PA	DAG
	dpm/dish			*dpm/dish*		
Vehicle	148 ± 18	839 ± 86	4065 ± 158	238 ± 22	793 ± 70	3537 ± 185
LDL	157 ± 19	817 ± 52	3975 ± 124	309 ± 14	847 ± 44	3612 ± 132
OX-LDL	244 ± 48	1574 ± 47	6472 ± 269	723 ± 42	1191 ± 43	5490 ± 183

RFASMC were labeled with [^{3}H]myristic acid (2 μCi/dish) for 24 h. The cells were washed in RPMI 1640 medium and were treated with RPMI 1640 or media containing LDL (25 μg/mL) or OX-LDL (25 μg/mL) for 1 h at 37°C in the absence or presence of 0.5% ethanol. Lipids were extracted under acidic conditions. [^{3}H]PEt and [^{3}H]PA were separated by TLC on silica gel H plates containing (1%) potassium oxalate developed with the upper phase of ethyl acetate–isooctane–glacial acetic acid–H_2O 65:10:15:50 (by vol), and DAG was separated by TLC on silica gel H plates developed with hexane–diethylether–glacial acetic acid 50:50:1 (by vol). Values are mean ± SD of three independent determinations.

of phosphatidylethanol (PEt) in the presence of ethanol (0.5%), a widely accepted index of PLD activity (33), was evaluated. Addition of 0.5% ethanol in the presence of OX-LDL resulted in an increase in the [^{3}H]PEt formation (3-fold over the basal) in addition to [^{3}H]PA, consistent with PLD-catalyzed transphosphatidylation of PA formed to added ethanol (Table 1). These data suggest that OX-LDL is an activator of PLD in RFASMC and utilizes PC as one of the substrates.

In addition to OX-LDL, we also examined the effect of acetyl-LDL in the activation of PLD. As shown in **Table 2,** acetyl modification of LDL had no effect on PLD activity, and only oxidative modification of LDL to OX-LDL stimulated the PLD activity in RFASMC. In RFASMC prelabeled with [^{32}P]orthophosphate, the OX-LDL-mediated accumulation of [^{32}P]PEt was dose-dependent (**Fig. 1**), and a significant increase in [^{32}P]PEt (3- to 4-fold) was observed with 50 μg/mL of OX-LDL. Activation of PLD by OX-LDL in RFASMC was linear up to 60 min (**Fig. 2**) with the response plateauing after 60 min (data not shown).

Effect of OX-LDL on intracellular $[Ca^{2+}]_i$

OX-LDL is known to increase intracellular free calcium concentrations $[Ca^{2+}]_i$ in SMC (11), therefore, we assessed the role of OX-LDL in altering $[Ca^{2+}]_i$ in RFASMC. **Figure 3** contains representative tracings of changes in $[Ca^{2+}]_i$ in fura-2-loaded RFASMC. OX-LDL, but not LDL, transiently elevated $[Ca^{2+}]_i$ which peaked approximately 50 sec after addition of OX-LDL. Furthermore, progressive increases in $[Ca^{2+}]_i$ were observed by increasing the OX-LDL concentration from 10 μg/mL to 25 μg/mL.

TABLE 2. Effect of LDL, acetyl-LDL, and OX-LDL on PLD activation

Treatment	Concn.	[^{32}P]PEt Formed	Activity
	μg	*dpm/dish*	*% control*
Vehicle	–	304 ± 61	100
n-LDL	25	380 ± 48	125
Acetyl-LDL	25	388 ± 29	128
OX-LDL	25	940 ± 70	309

RFASMC labeled with [^{32}P]orthophosphate (5 μCi/dish, for 24 h in phosphate-free DMEM media, were treated with RPMI 1640 medium or medium containing n-LDL, acetyl-LDL, or OX-LDL for 1 h at 37°C in the presence of 0.5% ethanol. Lipids were extracted under acidic conditions and [^{32}P]PEt was separated by TLC and quantified as described in Methods. Values are mean ± SD of triplicate determinations.

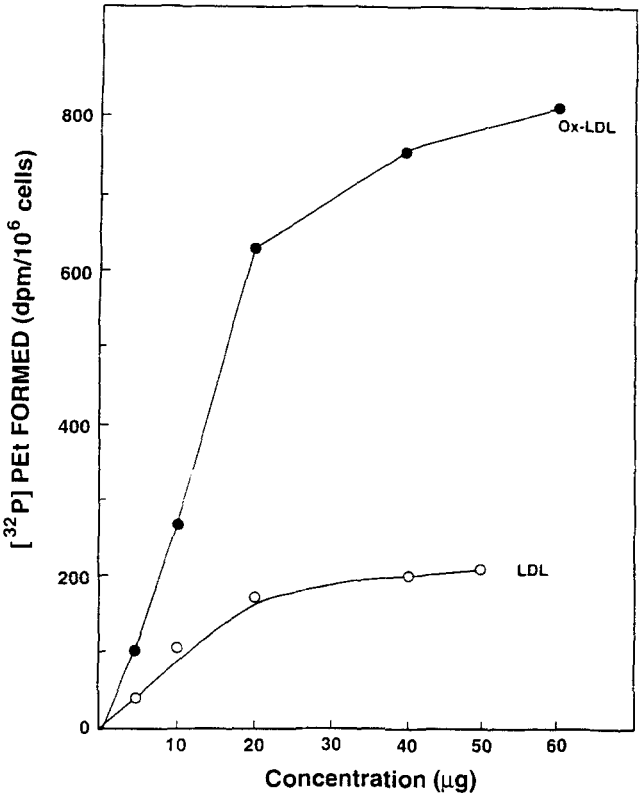

Fig. 1. Concentration dependence of OX-LDL-induced PLD activation. RFASMC were labeled with [^{32}P]orthophosphate (5 μCi/dish) for 24 h. Cells were washed in RPMI-1640 medium and were treated with either RPMI-1640 medium or medium containing varying amounts of LDL or OX-LDL in the presence of 0.5% ethanol for 30 min at 37°C. Lipids were extracted and [^{32}P]PEt was quantified as described in Methods. Values are average of two independent experiments in duplicate.

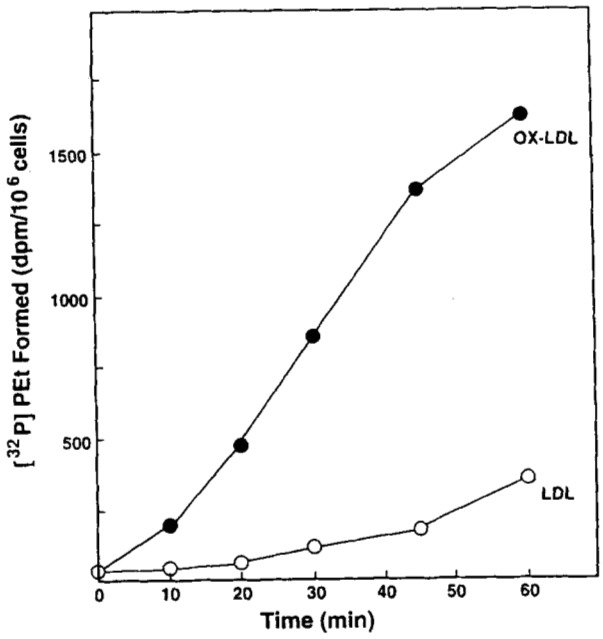

Fig. 2. Time course of OX-LDL-induced PLD activation. [^{32}P]orthophosphate-labeled RFASMC, as described in Fig. 1, were incubated with LDL (25 μg/mL) or OX-LDL (25 μg/mL) in RPMI-1640 medium containing 0.5% ethanol for indicated time periods at 37°C. Lipids were extracted and [^{32}P]PEt was quantified as described in Methods. Values are average of two independent experiments in duplicate.

Role of Ca^{2+} in OX-LDL-induced PLD activation

To investigate the role of Ca^{2+} in the regulation of PLD activation, RFASMC were challenged with OX-LDL after chelation of exogenous and endogenous Ca^{2+}. As evidenced in **Table 3,** chelation of extracellular Ca^{2+} with 5 mM EGTA did not significantly affect OX-LDL-induced [^{32}P]PEt accumulation. The role of $[Ca^{2+}]_i$ in stimulation of PLD activity in response to OX-LDL was investigated with BAPTA, a chelator of intracellular free Ca^{2+} (27). Pretreatment of RFASMC with BAPTA-AM (25 μM, 30 min) had no effect on OX-LDL-induced [^{32}P]PEt formation. However, addition of EGTA or BAPTA attenuated the ionophore A23187-mediated [^{32}P]PEt accumulation in RFASMC (data not shown). These data suggest that although OX-LDL increases $[Ca^{2+}]_i$ in RFASMC, OX-LDL-induced PLD activation is Ca^{2+}-independent.

Role of PKC in OX-LDL-mediated PLD activation

While agonist-induced PLD activation in vascular EC and SMC involves PKC activation (21, 34), recent data suggest that H_2O_2, fatty acid hydroperoxide and 4-HNE-induced PLD stimulation in EC is PKC-independent (28, 29). Hence, we examined the role of PKC in OX-LDL-mediated PLD activation in RFASMC by using PKC inhibitors and by down-regulating PKC with prolonged exposure to TPA (100 nM, 18 h). Pretreatment of [^{32}P]orthophosphate-labeled RFASMC with staurosporine (0.1 μM), H-7 (100 μM), or calphostin-C (1 μM) for 15 min did not effect the OX-LDL-induced [^{32}P]PEt formation (**Fig. 4**). However, the PKC-inhibitors staurosporine and calphostin C but not H-7 attenuated the TPA-induced [^{32}P]PEt formation in RFASMC. Down-regulation of PKC by prolonged treatment of RFASMC to 100 nM TPA (18 h) resulted in the loss of PKC activity (data not shown), but had no effect on OX-LDL-induced [^{32}P]PEt formation (**Fig. 5**). However, under similar incubation conditions, the TPA-mediated [^{32}P]PEt accumulation was abolished (Fig. 5). These data suggest that OX-

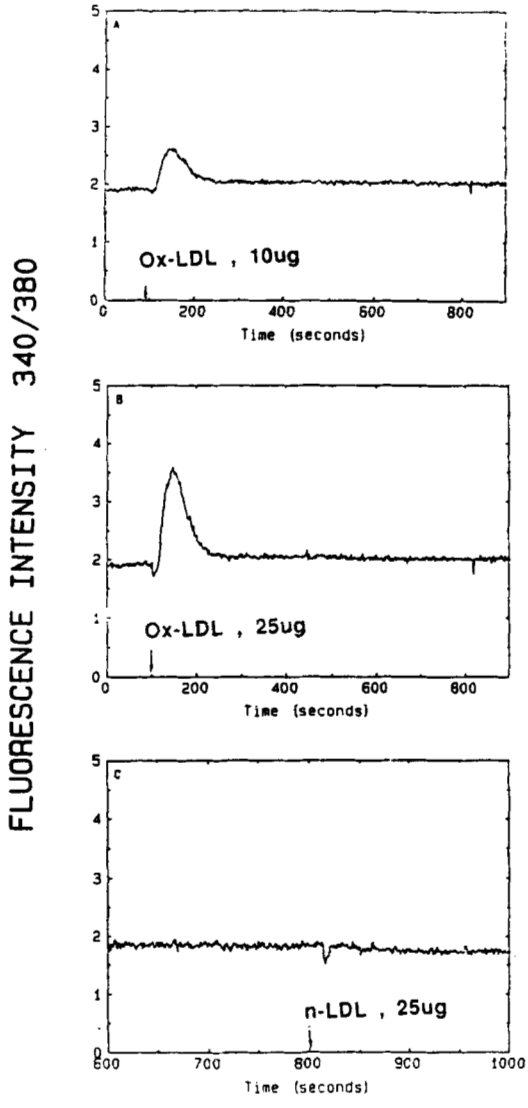

Fig. 3. Effect of OX-LDL in intracellular free Ca^{2+}_i. RFASMC grown on glass coverslips were loaded with fura-2 AM as described in Methods. After washing, the coverslips were placed in the quartz cuvette of a spectrofluorometer containing HBSH at 37°C. After monitoring the baseline fluorescence intensity, OX-LDL (10 or 25 μg/mL) or native LDL (n-LDL, 25 μg/mL) was added as indicated on the horizontal axis. The ratio of fluorescence intensity at 510 nm, produced by alternately exciting the coverslips at 340 nm or 380 nm, is proportional to $[Ca^{2+}]_i$. Representative tracings are depicted.

TABLE 3. Effect of EGTA and BAPTA on OX-LDL-induced PLD activation

	[^{32}P]PEt Formed			
	EGTA (5 mM)		BAPTA (25 μM)	
Treatment	(-)	(+)	(-)	(+)
	dpm/dish		*dpm/dish*	
Vehicle	315 ± 28	298 ± 37	295 ± 17	312 ± 24
OX-LDL	878 ± 44	960 ± 32	798 ± 26	942 ± 29

RFASMC were labeled with [^{32}P]orthophosphate (5 μCi/dish) in phosphate-free DMEM media for 24 h. Cells were washed and then treated with either RPMI 1640 medium or medium containing OX-LDL (25 μg/mL) for 1 h at 37°C. Lipids were extracted under acidic conditions and [^{32}P]PEt was quantified as described in Methods. Values are mean ± SD of triplicate determinations.

LDL–induced PLD stimulation is independent of PKC activation in RFASMC.

Role of GTP-binding proteins in OX-LDL-induced PLD activation

The possible involvement of GTP-binding proteins in OX-LDL-induced PLD activity was investigated with bacterial toxins known to modulate GTP-binding proteins (35). Preincubation of RFASMC with cholera (stimulates G_s) or pertussis toxin (inhibit G_i) (1 μg/mL for 30 min) did not significantly alter either the basal or the OX-LDL-induced PLD activity, suggesting that G_i and G_s are not involved in OX-LDL-mediated activation of PLD in RFASMC (data not shown).

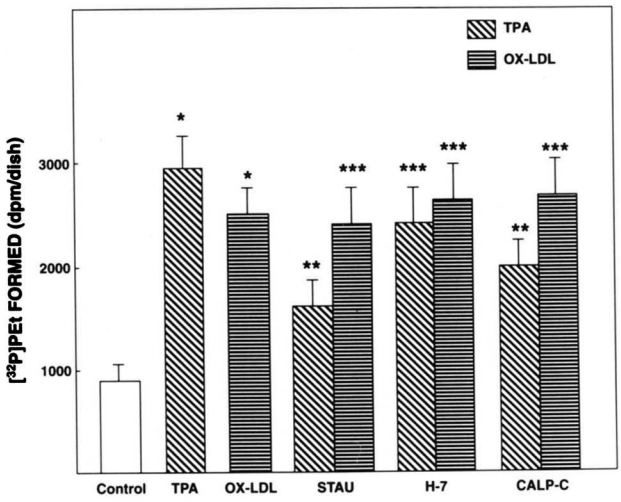

Fig. 4. Effect of PKC-inhibitors on OX-LDL-induced PLD activation. [^{32}P]orthophosphate-labeled RFASMC, as described in Fig. 1, were treated with either staurosporine (100 nM), H-7 (100 μM), or calphostin-C (1 μM) in RPMI-1640 medium for 15 min at 37°C. After the preincubation, cells were washed once in medium and then exposed to medium or medium containing OX-LDL (25 μg/mL) and 0.5% ethanol for 1 h at 37°C. Lipids were extracted and [^{32}P]PEt was quantified. Values are mean ± SD of triplicate determination. *Significantly different as compared to control ($P < 0.01$); **significantly different as compared to TPA treatment ($P < 0.05$); ***not significant as compared to TPA or OX-LDL treatment ($P > 0.05$).

Effect of tyrosine kinase and phosphatase inhibitors on OX-LDL-induced PLD activation

To further characterize the mechanism of OX-LDL-mediated PLD activation, we evaluated the effect of tyrosine kinase inhibitors on OX-LDL-induced PLD activity. Preincubation of [^{32}P]orthophosphate-labeled RFASMC with genistein (100 μM), a putative tyrosine kinase inhibitor (36), attenuated OX-LDL-mediated [^{32}P]PEt and [^{32}P]PA formation (**Fig. 6**). The effect of genistein was dose-dependent, and genistein by itself had no effect on the basal PLD activity. The inhibitory effect of genistein was specific for OX-LDL-induced PLD activation as the TPA-mediated [^{32}P]PEt and PA formation were not altered (Fig. 6). In addition to genistein, herbimycin also effectively attenuated with OX-LDL-induced [^{32}P]PEt formation (data not shown).

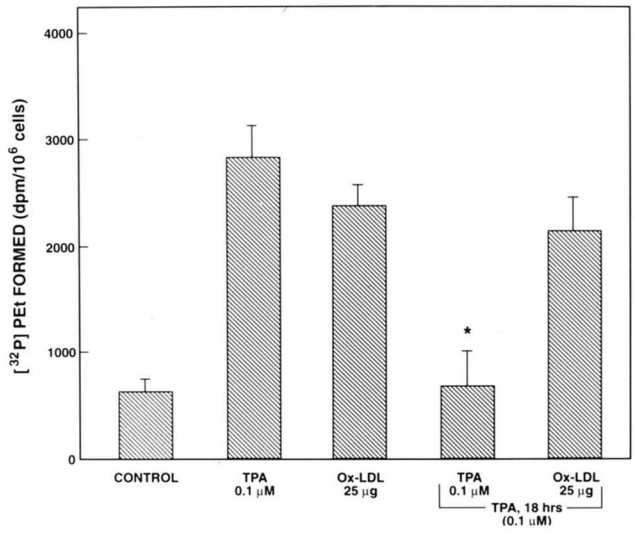

Fig. 5. Effect of PKC-down-regulation on OX-LDL-induced PLD activation. [^{32}P]orthophosphate-labeled RFASMC, as described in Fig. 1, were treated with RPMI 1640 medium or medium containing TPA (100 nM) for 18 h at 37°C. At the end of the down-regulation, cells were washed and challenged with medium, TPA (100 nM), or OX-LDL (25 μg/ml) in the presence of 0.5% ethanol for 30 min at 37°C. Lipids were extracted under acidic conditions and [^{32}P]PEt was quantified by TLC. Values are mean ± SD of triplicate determinations. *Significantly different as compared to TPA treatment ($P < 0.01$).

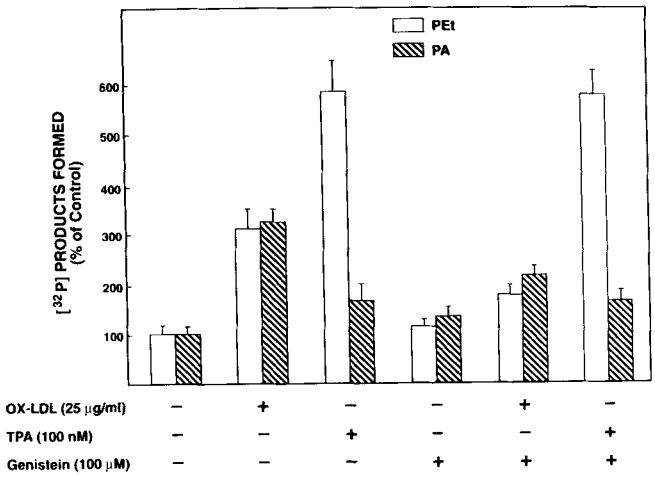

Fig. 6. Effect of genistein on OX-LDL-inducing PLD activation. [^{32}P]orthophosphate-labeled RFASMC, as described in Fig. 1, were pretreated with RPMI 1640 medium or medium containing genistein (100 μM) for 30 min at 37°C. At the end of pretreatment, TPA (100 nM) or OX-LDL (25 μg/mL) was added and incubations were continued for an additional 30 min at 37°C in the presence of 0.5% ethanol. Lipids were extracted under acidic conditions and [^{32}P]PEt and [^{32}P]PA were quantified as described under Methods. Values are mean ± SD of triplicate determinations.

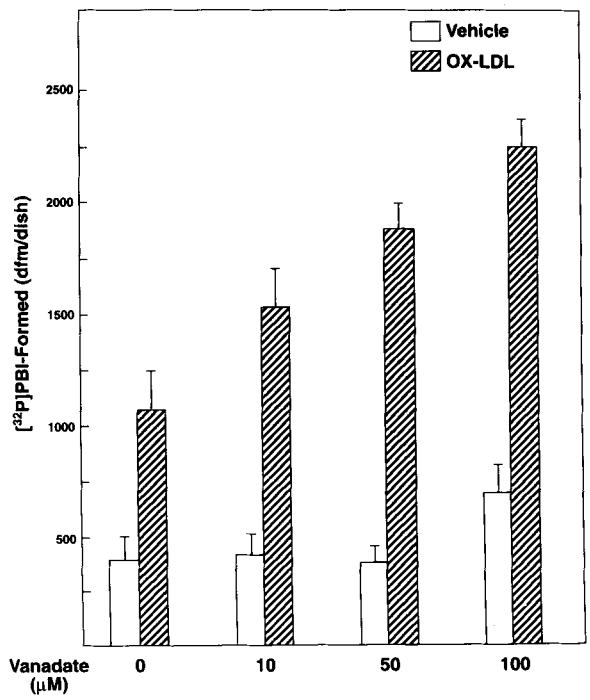

Fig. 8. Concentration dependence of vanadate on OX-LDL-induced PLD activation. RFASMC, labeled as described in Fig. 1, were treated with RPMI-1640 medium or medium containing varying concentrations of vanadate for 15 min. Cells were washed and treated with OX-LDL (25 μg/mL) for 45 min at 37°C in the presence of 0.05% butanol. Lipids were extracted and [^{32}P]PBt was quantified as described in Methods. Values are mean ± SD of triplicate determinations.

These data suggest that OX-LDL-induced PLD activation in RFASMC may involve protein tyrosine phosphorylation, mediated by tyrosine kinases. Inhibition of protein tyrosine phosphatases intrinsically up-regulates tyrosine kinase-mediated protein tyrosine phosphorylation (37). To determine whether inhibition of tyrosine phosphatase would modulate the OX-LDL-induced PLD activation, RFASMC were pretreated with sodium orthovanadate (100 μM) for 15 min. As shown in **Fig. 7,** addition of vanadate (100 μM) to RFASMC potentiated the OX-LDL-induced PLD activation by 2-fold. The potentiating effect of vanadate on OX-LDL-induced PLD activation was dose-dependent and exhibited maximal [^{32}P]PEt formation at 100 μM of vanadate (**Fig. 8**). At lower concentrations, vanadate by itself had no effect on basal PLD activity; however, at 100 μM vanadate a slight increase in the basal PLD activity was observed (Fig. 8). In addition to vanadate, phenylarsine oxide addition to cells as a phosphatase inhibitor potentiated the OX-LDL-induced [^{32}P]PBt formation (**Table 4**). However, diamide showed no effect on OX-LDL-induced [^{32}P]PBt formation. These data suggest a role for protein tyrosine phosphorylation in OX-LDL-induced PLD activation.

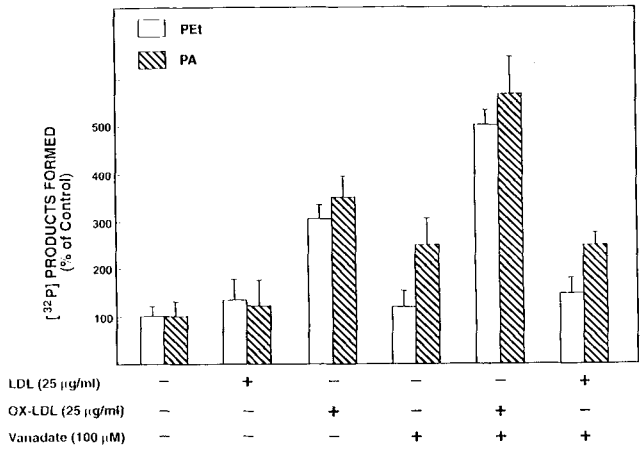

Fig. 7. Effect of vanadate on OX-LDL-induced PLD activation. RFASMC were labeled with [^{32}P]orthophosphate as described in Fig. 1. Cells were pretreated with sodium orthovanadate (100 μM) for 15 min at 37°C, were washed once, and challenged with LDL (25 μg/mL) or OX-LDL (25 μg/mL) for 30 min in the presence of .05% butanol. Lipids were extracted under acidic conditions and [^{32}P]PEt and [^{32}P]PA were separated as described in Methods. Values are mean ± SD of two independent experiments in triplicate.

Mitogenic effects of OX-LDL, PA, and lyso PA

The ability of OX-LDL to stimulate DNA synthesis in RFASMC was investigated using [^{3}H]thymidine. As shown in **Fig. 9,** OX-LDL (25 μg/ml) stimulated [^{3}H]thymidine incorporation into quiescent RFASMC DNA while LDL showed no appreciable stimulatory effect. As the products of PLD hydrolysis, namely PA and/or LPA, are known to possess mitogenic properties

TABLE 4. Effect of vanadate, phenylarsineoxide, and diamide on OX-LDL-induced PLD activation

Pretreatment (15 min)	Treatment (45 min)	[^{32}P]PBt Formed	Activity
		dpm/dish	*% control*
Vehicle	Vehicle	358 ± 54	100
Vehicle	OX-LDL	1351 ± 74	377
Vanadate	Vehicle	501 ± 116	140
Vanadate	OX-LDL	2386 ± 204	666
PAO	Vehicle	501 ± 38	140
PAO	OX-LDL	2120 ± 212	592
Diamide	Vehicle	503 ± 87	140
Diamide	OX-LDL	1353 ± 79	378

RFASMC were prelabeled with [^{32}P]orthophosphate (5 μCi/dish) in DMEM phosphate-free media containing 2% fetal calf serum for 24 h. The cells were washed in RPMI-1640 medium and were incubated in the same medium or medium containing vanadate (100 μM) or PAO (25 μM) or diamide (1 mM) for 15 min at 37°C. Cells were washed and then incubated with medium alone or medium containing OX-LDL (25 μg/ml) for 45 min in the presence of 0.05% butanol. Lipids were extracted under acidic conditions as described in Methods and [^{32}P]PBt was quantified by TLC. Values are mean ± SD of triplicate determinations.

(15–18), we studied the relative mitogenicity of PA, lyso PA, and dioleoyl PC in quiescent RFASMC. As seen in **Fig. 10,** both PA and LPA increased the basal [^{3}H]thymidine incorporation 2-fold. This increase was comparable to OX-LDL-mediated increases in [^{3}H]thymidine incorporation into DNA. Under identical incubation conditions, 2% FBS in the medium showed 8- to 10-fold increase (data not shown) while dioleoyl PC (100 μM) exhibited no mitogenic effect (Fig. 10). These data suggest that the relative mitogenic responses of PA and lyso PA are very similar, whereas LDL and PC lacked significant mitogenic activity. The mitogenic efficacy of PA and LPA were similar over a concentration range of 10–100 μM. The amount of [^{3}H]thymidine incorporated into DNA as compared to control (2338 ± 156 dpm/dish) was: at 10 μM (PA, 4265 ± 181; LPA, 4392 ± 140 dpm/dish) and at 100 μM (PA, 6672 ± 230; LPA 7123 ± 431 dpm/dish). The PA used in our experiments showed less than 5% contamination by LPA (as verified by TLC). Therefore, the mitogenic response to PA and LPA are independent events and cannot be due to cross contamination of either of the lipids.

As an alternative to adding exogenous PA to the cells, PA was generated within the plasma membrane of the cells by incubating with 1, 2, or 5 units of PLD from *S. chromofuscus* for 3 h. Treatment with exogenous PLD also resulted in an increase in [^{3}H]thymidine incorporation into DNA (**Table 5**) similar to that obtained with the exogenous addition of PA or lyso PA (Fig. 10).

DISCUSSION

In this study, we have characterized the effect of OX-LDL on the PLD signalling pathway and its possible role in atherogenesis. Our data demonstrate for the first time that oxidative modification of LDL to OX-LDL activates PLD activity in RFASMC monolayers. Activation of PLD has been observed after exposure to hormones, neurotransmitters, growth factors, cytokines, and reactive oxygen species in a variety of mammalian cells including vascular endothelial and smooth muscle cells (21, 38). Earlier studies in endothelial cells have demonstrated that PKC inhibitors almost completely abolished the agonist-induced PLD activation, thus implying an obligate role for PKC (27, 39–41). The mechanism of PKC-mediated activation of PLD is unclear, but recent studies suggest that energy-dependent protein phosphorylation may not be involved (42).

As OX-LDL-induced PLD activation was found to be independent of PKC (Figs. 4 and 5) and calcium (Table 3), we examined the role of protein tyrosine phosphorylation in the regulation of PLD activation. Specifically,

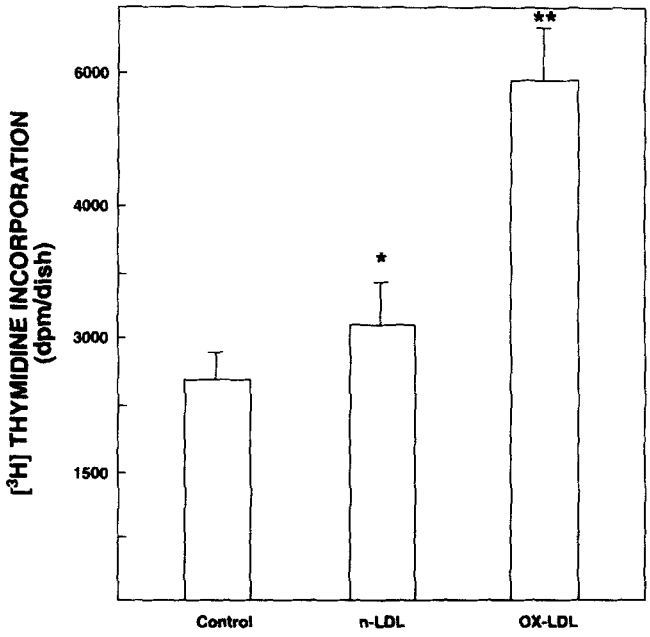

Fig. 9. Effects of LDL and OX-LDL on [^{3}H]thymidine incorporation into RFASMC DNA. RFASMC (2 × 10^5 cells) seeded in 24 well clusters were grown to 90% confluency and were made quiescent by treating with RPMI-1640 medium containing 0.25% fetal calf serum for 48 h. The cells were then treated with RPMI 1640 medium or medium containing LDL (25 μg/mL) or OX-LDL (25 μg/mL) and were incubated for 16 h at 37°C. [^{3}H]thymidine (0.5 μCi/mL) was then added to each well and incubations were continued for an additional 6 h. The incorporation of [^{3}H]thymidine into cells was measured as described in Methods. Values are mean ± SD of two independent experiments in triplicate. *Not significant as compared to control ($P < 0.05$); **significantly different as compared to control ($P < 0.01$).

treatment with putative tyrosine kinase inhibitors attenuated the OX-LDL-mediated PLD activation but not the TPA-mediated response. Further, a role for protein tyrosine phosphorylation in PLD activation was confirmed by using phosphotyrosine phosphatase inhibitors. Preincubation of RFASMC with sodium vanadate resulted in potentiation of OX-LDL-mediated PLD activation without affecting basal activity. Vanadate alone or in combination with H_2O_2 is known to mimic insulin action (43), promote insulin-receptor tyrosine phosphorylation (44), and also inhibit insulin receptor-associated phosphotyrosine phosphatase activity (45). Taken together, these data suggest that the effect of vanadate on OX-LDL-induced PLD activation could be due to either the stimulation of tyrosine kinases, inhibition of phosphotyrosine phosphatases, or a combination of both.

While the effect of OX-LDL on protein tyrosine phosphorylation in RFASMC has not been evaluated, pervanadate has been shown to phosphorylate in tyrosine several proteins including MAP kinases in human neutrophils and HL-60 cells (46). There is indirect evidence for protein tyrosine phosphorylation in EGF- and PDGF-induced PLD activation in fibroblasts (47) and platelets (48). In SMC, the PDGF-mediated PLD activation seems to involve protein tyrosine phosphorylation of PLC γ-1 and subsequent involvement of PKC (49). Similar to OX-LDL-mediated activation of PLD reported here, in neutrophils fMLP, PAF, and leukotriene B_4 stimulated PLD activity that was inhibited by tyrosine kinase inhibitors and potentiated by pervanadate, thereby suggesting a regulatory role for tyrosine kinases in the regulation of PLD (50).

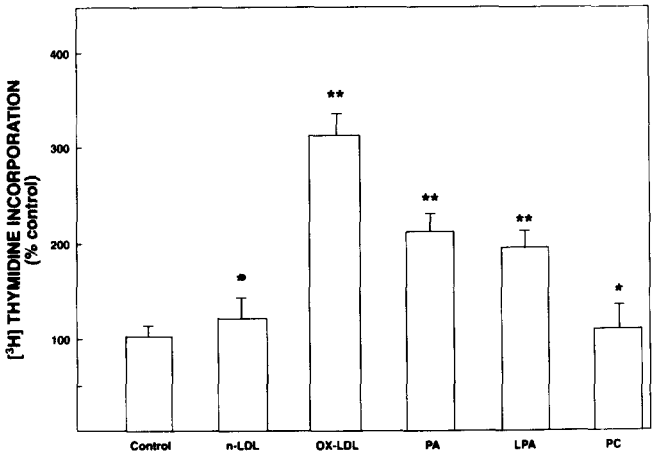

Fig. 10. Effects of LDL, OX-LDL, PA, LPA, and PC on [^{3}H]thymidine incorporation into RFASMC DNA. RFASMC in 24-well clusters were made quiescent as described in Fig. 9. Cells were incubated with RPMI 1640 medium or medium containing LDL (25 μg/mL), OX-LDL (25 μg/mL), PA (100 μM), LPA (100 μM), or PC (100 μM) for 16 h at 37°C. [^{3}H]thymidine (0.5 μCi/ml) was then added to each well and incubations were continued for an additional 6 h. The incorporation of [^{3}H]thymidine into cells was measured as described in Fig. 9. Values are mean ± SD of two independent experiments in triplicate. *Not significant as compared to control ($P > 0.05$); **significantly different as compared to control ($P < 0.01$).

TABLE 5. Effect of exogenous PLD on DNA synthesis in smooth muscle cells

PLD Added (units/mL)	Incorporation of [^{3}H]Thymidine into DNA	
	dpm/10^6 cells	Relative to control
0	799 ± 99	1.00
1	1203 ± 204	1.51 ± 0.21
2	1467 ± 900	1.84 ± 0.17
5	2056 ± 85	2.57 ± 0.15

RFASMC were made quiescent as described in Methods. To the quiescent RFASMC was added PLD from *S. chromofuscus* and cells were incubated for 3 h. The cells were washed and incubated for 16 h in serum-free RPMI 1640. [^{3}H]thymidine (0.5 μCi/dish) was added at the end of 16 h and incubations were continued for an additional 6 h. The incorporation of [^{3}H]thymidine into DNA was measured as described in Methods. Results are mean ± SD of triplicate determinations.

It is clear from the current data that oxidative modification of the LDL particle allows it to function like a signalling molecule and modulate the generation of second messengers within SMC. In cultured EC and SMC, OX-LDL stimulates eicosanoid synthesis (14), activates inositol phospholipid metabolism (12, 13) and calcium mobilization (11), enhances production of PDGF AA and PDGF-receptors (51), stimulates monocyte endothelial interactions (52), and inhibits nitric oxide synthesis (53). While native or acetylated LDL showed no stimulation of PLD activity as compared to OX-LDL in RFASMC, LDL and HDL seem to affect signal transduction and exocytosis in type II cells (54). Both LDL and HDL temporarily stimulate inositol phosphate release, Ca^{2+} mobilization, and PKC translocation from cytosol to membranes in alveolar type II cells (54). These stimulating effects of LDL in type II cells seem to be mediated through LDL-receptors, while the HDL-elicited responses were independent of LDL receptors (54).

At present, it is not clear whether OX-LDL-induced PLD activation is receptor-mediated or receptor-independent although scavenger receptors have been characterized in rabbit SMC (55), fibroblasts (55), and human EC (56). There may be several reasons why oxidized LDL and not acetyl-LDL stimulates PLD in RFASMC. First, oxidized LDL may contain peroxidized lipid components that are not present in acetyl-LDL. Peroxidized lipid components have been suggested to alter a number of processes including activation of oncogenes (57), chemotaxis (58), and expression of adhesion molecules (59) in SMC. Second, as OX-LDL used in these studies is terminally oxidized, it is likely that it possesses ligands to receptors distinct from the one that recognizes acetyl-LDL. Recently, evidence for the presence of such receptors in Kupffer cells of the liver (60, 61) and a 95-kDa protein in macrophages that specifically bind to

OX-LDL and not acetyl-LDL were reported (62). Third, Hampton, Golenbock, and Raetz (63) have shown that the property of inducing TNFα by LPS is not shared by acetyl LDL. Although oxidized acetyl-LDL was not tested, in an earlier study it was shown that LPS also binds to a 95-kDa protein (64). More recently, Lipton et al. have shown that the modified protein component of OX-LDL and not acetyl-LDL induced IL-1 release from arterial foam cells (personal communication). These studies suggest that both lipid and protein components of OX-LDL have the capacity of interacting with receptors other than acetyl-LDL receptors and some of these may be involved in signal transduction. The protein scavenger receptor in SMC has been reported by Pitas et al. (65) and this receptor has been suggested to be activated by PMA as well as by platelet recruiting products (55). So far there has not been a suggestion of a specific OX-LDL-receptor in SMC.

Although increased generation of cytokines, growth factors, and reactive oxygen species have been proposed to play a role in SMC migration and proliferation (2, 3), the biochemical mechanisms involved in the initiation and development of atherogenesis are unclear. Phosphatidic acid and LPA have been found to be mitogenic to fibroblasts and epithelial cells in culture (15–18). Recent studies suggest that the mitogenic effect of LPA may be mediated, in part, by a membrane-bound G-protein of the G_i family (16). In view of the reported mitogenic effects of PA and LPA in fibroblasts (17) and epithelial cells (18), our findings that OX-LDL increased [^{3}H]thymidine incorporation into RFASMC DNA suggest that PLD activation and subsequent generation of PA and/or LPA may be involved in the mitogenic signal. In fact, in addition to OX-LDL, exogenously added PA and LPA exhibited mitogenic activity in RFASMC, suggesting a role for the PLD signalling pathway in SMC proliferation. Furthermore, exogenous addition of the PLD enzyme preparation from *S. chromofuscus* to RFASMC in culture also increased [^{3}H]thymidine incorporation confirming a possible regulatory role for PA and/or LPA in mitogenic action. Thus, OX-LDL-induced SMC proliferation may involve generation of PA, lyso-PA, and/or other mitogenic factors that can activate protein kinases, including tyrosine kinases and MAP kinases, with subsequent modulation of nuclear transcription factors. Recent studies suggest that addition of OX-LDL to macrophages suppressed expression of the TNFα gene by modulating activation of NFkB (66). The question whether OX-LDL contains PA and LPA that could stimulate SMC proliferation needs to be further investigated. In addition to the PLD-mediated PA formation, the PLC pathway can also contribute to the accumulation of PA through the DAG kinase reaction. At present, there are no specific inhibitors available to block the PLD reaction to quantify the relative contribution of this pathway in the generation of PA.

In conclusion, these results provide evidence that OX-LDL activates PLD-mediated signalling pathways in SMC. The PLD-catalyzed generation of PA and/or LPA is potentially of biological interest as these pathways may be involved in SMC proliferation in the development of atherosclerosis. Further studies on the ability of OX-LDL to modulate mitogenesis, signalling pathways, and the regulation of transcription factors will help identify the molecular basis for the genesis of atherosclerosis.

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