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Identification of a biologically active component in minimally oxidized low density lipoprotein (MM-LDL) responsible for aortic smooth muscle cell proliferation

Subroto Chatterjee[1], Judith A. Berliner[2], Ganesamoorthy G. Subbanagounder[2], Anil K. Bhunia[1] and Steve Koh[1]

[1] *Department of Pediatrics, Lipid Signaling and Vascular Biology Laboratory, Lipid Research-Atherosclerosis Division, Johns Hopkins University School of Medicine, Baltimore, MD, USA,* [2] *Department of Medicine, UCLA Medical Center, Los Angeles, CA, USA*

Although low concentrations (10 μg/ml) of oxidized LDL density lipoproteins (Ox-LDL) and minimally modified LDL (MM-LDL) can stimulate the proliferation of aortic smooth muscle cells the biologically active component responsible for this phenomena has not been identified. Here we report that the 1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-4-phosphocholine (m/e594.3) (POVPC) present in MM-LDL but not 1-palmitoyl-2-glutaryl-sn-glycero-3-phophochline (m/e610.2)(PGPC) can stimulate the activity of UDP-galactose:glucosylceramide (β 1→4) galactosyltransferase (GalT-2) and produce lactosyceramide (LacCer). LacCer, in turn, generated superoxide radicals ($O_2^{\cdot-}$). This is accompanied by the phosphorylation/activation of a cytosolic transcriptional factor p^{44} MAPK and the subsequent proliferation of human aortic smooth muscle cells. D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (D-PDMP), an inhibitor of GalT-2, impaired the induction of GalT-2 activity, $O_2^{\cdot-}$ generation, and cell proliferation. Thus POVPC may serve as a surrogate in MM-LDL mediated induction of aortic smooth muscle cells (A-SMC) proliferation via GalT-2 activation. The LacCer produced as a consequence of GalT-2 activation may serve as a lipid second messenger in the activation of an oxidant sensitive transcriptional pahtway that ultimately leads to cell proliferation and may contribute to the pathophysiology of atherosclerosis. *Published in 2004.*

Keywords: **proliferation, galactosyltransferase, oxidized phospholipids, minimally modified low density lipoproteins**

Abbreviations: **LDL: low density lipoprotein; MM-LDL: minimally modified LDL; Ox-LDL: oxidized LDL; A-SMC: aortic smooth muscle cells; LacCer: Lactosyl-ceramide; POVPC: 1-palmitoyl-2-(5-oxo-valeroyl)-sn-glycero-4-phosphocholine.**

Introduction

Considerable evidences suggest that the oxidative modification of low density lipoproteins (LDL) is involved in the development and progression of atherosclerosis [1,2]. Minimally modified LDL (MM-LDL) represents LDL that has undergone antioxidant depletion and oxidation of arachidonic acid-containing phospholipids, relatively less linoleic acid oxidation, and insignificant protein modification [3–6]. In highly oxidized-LDL the phospholipids and protein undergo extensive modifications with consequent changes in physical-chemical properties compared to native LDL. We have previously shown that at low concentrations (5–10 μg/ml) Ox-LDL [7–9] and/or MM-LDL [10] can induce the proliferation of cultured aortic smooth muscle cells (A-SMCs). The proliferation of A-SMC is a hallmark in the pathogenesis of atherosclerosis and in restenosis following balloon-angioplasty in experimental animals and in human subjects [11] as has been described [12]. An oxidant sensitive transcriptional pathway involving modified-LDL mediated-LDL mediated A-SMC proliferation has been described recently [12]. This involves the activation of GalT-2 [13–15] by modified LDL and the subsequent production LacCer. In turn, LacCer serves as a lipid second messenger that generates the production of superoxide ($O_2^{\cdot-}$). Ras-GTP loading, p44 MAPK activation/phosphorylation, c-fos expression and cyclin expression ultimately lead to cell proliferation [7,12,16]. The identification of the biologically active component MM-LDL initializing the

To whom correspondence should be addressed: Subroto Chatterjee, Department of Pediatrics, Johns Hopkins University, Suite 321 550-North Broadway, Baltimore, MD 21205, USA. Tel.: (410) 614-2518; Fax: (410) 614-2826; E-mail: schatte2@jhmi.edu

phenomenon above has not yet been shown, to the best of our knowledge.

During our studies with MM-LDL on monocyte-endothelial cell interactions, we have identified two active phospholipids 1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-4-phosphocholine (m/2594.3) and 1-palmitoyl-2-glutaryl-sn-glycero-3-phosphocholine (m/e610.2) [5]. Such phospholipids were present in fatty streak lesions in cholesterol-fed rabbits and were able to induce monocyte-endothelial adhesion [5,7]. In this report we have reconstituted the effects of MM-LDL by the use of oxidized phospholipids above in regard to A-SMC proliferation. Our studies suggest that POVPC is the preferred compound present in MM-LDL that mediates GalT-2 activation, $O_2^{\cdot-}$ generation, and p^{44}MAPK activation subsequently lead to A-SMC proliferation.

Materials and methods

Isotopes

[γ-^{32}P] ATP 96000 mCi/mmol) and [α-^{32}P] dCTP (3000 Ci/mmol) (uridine diphosphate-D[U-^{14}C] galactose-(327 mCi/mmol), and [6-^{3}H] thymidine 925 GBq/mmol) were purchased from Amersham Life Science Inc. [^{32}P]-orthophosphoric acid (H_3PO_4) (carrier-free) was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO).

Chemicals

All standard cell culture reagents were supplied by Gibco-BRL. Myelin Basic Protein (MBP) substrate peptide (APR TPGG RR), specific for MAP kinase, anti-MAP kinase (ERK-T) polyclonal antibody, specific for p^{44} MAPK and p^{42} MAPK (for mouse, rat and human systems) were obtained from Upstate Biotechnology Inc., Lake Placid, NY. D-PDMP was purchased from Matreya, PA. Human aortic smooth muscle cells were a gift from Dr. Cecilia Giachelli (Washington University, Seattle, WA).

Lipids

Human LDL (d 1.068 gm/dl) was prepared from plasma of normal lipidemic volunteers employing density gradient ultracentrifugation [7]. The protein content of the lipoprotein was determined. Approximately 5 mg of LDL was dialyzed against 5 μM $FeS0_4$. $7H_2O$ in phosphate buffer saline diluted 1:9 with sterile water plus 0.15 mole NaCl pH 6.8 for 48 h at room temperature. Oxidation was terminated by the addition of EDTA and BHT (100 μM and μM, respectively). The concentration of bacterial endotoxin was less than 20 pg/ml, 50 fold less than required to induce ASMC proliferation. Ox-PAPC, POVPC AND PGPC were produced, as described previously [5], using PAPC from Avanti Polar Lipids. PEIPC mixture of isomers was isolated from Ox-PAPC as described [5]. Purified oxidized phospholipids, and MM-LDL were shipped from Los Angeles to Baltimore and utilized in various experiments shortly thereafter.

Cells

Human aoric smooth cells were prepared and cultured in minimum essential medium supplemented with 10% fetal calf serum (FCS), penicillin, streptomycin (100 U/ml), and glutamine (50 μg/ml) according to the procedure of Ross [16].

Incubation of cells with lipoproteins and oxidized phospholipids

Cells ($\times 10^5$) were seeded in 100 $\times$ 15 mm^2 plastic Petri dishes in the above growth medium. Fresh medium was added every three days. On the seventh day of cell growth when cells were confluent, the medium was replaced. Cells were washed with sterile PBS. Next, 8 ml of fresh medium was added to each plate. After priming the cells for 2 h in this medium, LDL, MM-LDL, and oxidized phospholipids (Ox-PAPC, POVPC, PGPC) were added. Cells were also treated with 1-palmitoyl-2-arachidonyl-sn-glycero-5-phosphocholine (PAPC) to serve as a control. After incubation for a certain time (described separately in individual experiments) cells were washed and harvested in sterile PBS containing 1 mM sodium vanadate (Na_3VO_4) to inhibit phosphatase activity, centrifuged (500 $\times$ g, 10 min at 4°C), and the pellets stored frozen at -20°C until further analysis.

Measurement of UDP-galactose:glucosylceramide, $\beta 1 \rightarrow 4$ galactosyltransferase (GalT-2) activity

The activity of GalT-2 in cells incubated with and without LDL, MM-LDL, and various phospholipids was measured employing UDP-[^{14}C]-galactose as a nucleotide sugar donor and glucosylceramide as an acceptor as described previously [14,15]. Briefly, the GalT-2 assay mixture contained 100 μg enzyme preparation, 20 μM of cacodylate buffer (pH 6.8), 1.0 mM $Mn^{2+/}Mg^{2+}$, 0.2 mg/ml Triton X-100 cutscum (1:2 v/v), 30 nmol of GlcCer, and 0.1 nmol of UDP-galactose. Assay without exogenous GlcCer served as blanks (approximately 15–20 cpm) and were subtracted from all respective data points. The assay was terminated by the addition of 25 μmol of EDTA plus 2.5 μmol KCl. Chloroform/methanol (2:1, v/v) 500 μl and 5 μg of human kidney glycosphingolipid mixture were added, and the products were isolated and separated by Whatman SG-81 paper chromatography (VWR Scientific, San Francisco, CA) developing in chloroform/methanol water (60:17:2 by volume). Chromatogram areas corresponding in migration with standard LacCer were cut, and radioactivity was measured in a Beckman LS-38000 scintillation spectrometer using the background subtract setting and automatic quench setting, as well as a 2.00 setting for the 2 α statistical error.

Immunoprecipitation of MAP kinase

Cells were lysed in 100 μl of modified RIPA buffer containing 150 mM NaCl, 5 mM EGTA, 5 mM EDTA, 10 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM pepstatin, 25 mM Tris-HCl, pH 7.4, 1% Triton X-100, and 0.5% Nonidet P-40 described previously [13]. The lysate was centrifuged and the supernatant was incubated with 4 μg of anti-MAP kinase antibody conjugated with proteinA/agarose overnight at 4°C. The immunoprecipitates were washed twice with 25 mM Tris buffer, pH 7.4 containing 2 mM EDTA, and 150 mM NaCl. The immunocomplexes were used for MAP kinase assays.

Measurement of MAP kinase activity

MAP kinase activity was determined by the phosphorylation of the MAP kinase specific substrate MBP (peptide APRTPGGRR) as previously described [16]. The assay was performed with 2–3 μg protein in a final volume 25 μl containing 1 mg/ml MBP, 50 μM [γ-^{32}P]ATP (1800 cpm/pmol), 0.5 mM adenosine 3′-5′-cyclic monophosphate-dependent protein kinase inhibitor, and assay dilution buffer containing 30 mM β-glycerophosphate, 20 mM MOPS, pH 7.2, 20 mM $MgCl_2$, 5 mM EGTA, 1 mM dithiothreitol (DTT) and 0.5 mM Na_3VO_4. The kinase reaction was initiated upon the addition of [γ-^{32}P] ATP for 15 min a 37°C. The reaction was terminated with the addition of 10 μl of ice cold 40% trichloroacetic acid and spotted onto a 2.4 cm^2 piece of Whatman p81 phosphocellulose paper. Free [γ-^{32}P] was removed by five washes (5 min each) with 1% phosphoric acid and one wash with 95% ethanol. Radioactivity was measured by liquid scintillation counting. The activity of MAPK was expressed as pmol/min/mg protein. Protein was determined according to the method of Lowry *et al.* with the use of BSA as standard [17].

Measurement of superoxide production in intact cells

Lucigenin, an acridylium compound (Sigma, St. Louis, MO) emitting light upon reduction and interaction with $O_2^{\cdot-}$ was used to measure $O_2^{\cdot-}$ production [12]. Briefly, cultured A-SMC were harvested, and cell pellets were suspended in a balanced salt solution (130 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 35 mM phosphoric acid, and 20 mM HEPES, pH 7.4). The viability of the suspended cells as determined by the trypan blue exclusion principle was >90%. To measure $O_2^{\cdot-}$ production intact cells preincubated at room temperature were added to a 96-well plate containing dark-adapted lucigenin (500 μM) in a balanced salt solution. Next, POVPC was added to it as a stimulant, and photon emission was measured by every 20 sec for 10 min in a scintillation counter (Packard TOP counter, Groton, CT). Moreover, a vehicle (0.01% Me_2SO) served as a control in most experiments. The amount of $O_2^{\cdot-}$ produced at each time point was calculated by comparison with a standard curve generated using xanthine/xanthine oxidase [12].

Cell proliferation assay

Cells were incubated with and without MM-LDL and/or oxidized phospholipids. Another set of 96 well trays was preincubated with D-PDMP (10 μM) in cultured medium for 2 h and next the various compounds were added and incubation continued for 24 h. Next, [^{3}H] thymidine (5 μCi/ml media) was added. After incubation for 2 h, cells were washed with phosphate-buffered saline. The incorporation of [^{3}H] thymidine in cells was measured as described previously [7].

Results

MM-LDL, Ox-PAPC and POVPC stimulates the activity of GalT-2 and D-PDMP abrogates this phenomenon in A-SMC

Cells were incubated with 10 μg/ml of a variety of lipoproteins and phospholipids (1 μg/ml) for 5 min at 37°C and the activity of GalT-2 was measured. We found that MM-LDL, Ox-PAPC, and POVPC stimulated the activity of GalT-2 on the order of 2-fold , 1.5-fold, and 2.1-fold respectively as compared to the control (Figure 1). On the other hand, LDL, PAPC and oxidized phospholipids such as PGPC and PEIPC did not stimulate the activity of GalT-2. D-PDMP alone inhibited the activity of GalT-2 about 0.4-fold compared to the control. When cells

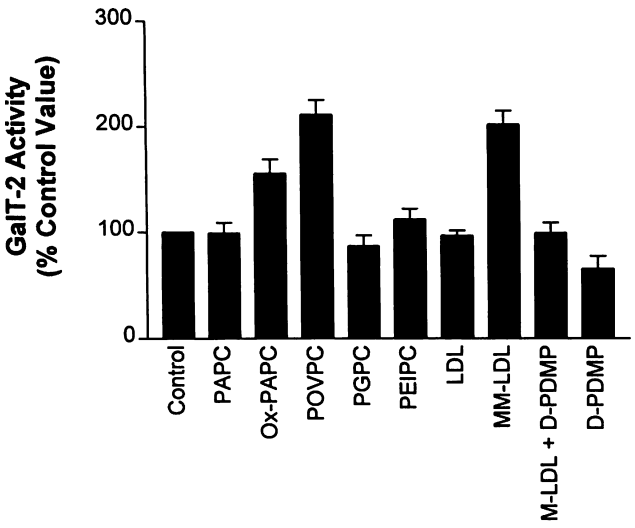

Figure 1. Effects of lipoproteins, oxidized phospholipids and D-DPMP on the activity of GalT-2 in aortic smooth muscle cells. Cells (10^5) were seeded in plastic dishes and grown to confluence. Next, medium was changed to serum free medium and lipoproteins (10 μg/ml) and oxidized phospholipids (1 μg/ml) was added and the activity of GalT-2 was measured as described in "Materials and Methods". In another set of dishes D-DPMP (10 μg/mM) was added and following incubation for 2 h MM-LDL was added. After incubation for 5 min at 37°C the cells were harvested and the activity of GalT-2 was measured. Data from three separate experiments analyzed in duplicate is presented. The activity of GalT-2 in control cells (representing 100% value) was 2.2 ± 0.3 nmole/mg protein/h.

were pre-incubated with D-PDMP (10 μM) for 2 h at 37°C and then incubated with MM-LDL, it abrogated the stimulation of GalT-2 activity.

MM-LDL and POVPC stimulates the generation of superoxide radical in A-SMC and this phenomenon was abrogated by D-PDMP

As shown in Figure 2, upon incubation of A-SMC with MM-LDL (10 μg/ml) and/or POVPC (1 μg/ml, for 10 min there was a marked stimulation of the generation of $O_2^{\cdot-}$. This was ~5-fold higher compared to the control. This phenomenon was completely abrogated by the preincubation of cells with D-PDMP. Moreover, preincubation of A-SMC for 30 min with diphenylene iodonium (DPI), an inhibitor of NADPH oxidase activity, decreased the generation of $O_2^{\cdot-}$ about 3-fold as compared to cells incubated with POVPC.

POVPC and MM-LDL stimulate p^{44} MAP kinase activity in A-SMC

Within 10 min of incubation of cells with POVPC (1 μg/ml) a 1.5-fold increase in the activity of p^{44}MAPK was observed. The activity of MAPK continued to rise up to 30 min to ~1.7-fold, compared to the control, and thereafter decreased somewhat compared to the control. The time-dependent product formation stimulation of p^{44} MAPK activity in cells incubated with MM-LDL also followed in parallel with cells incubated with POVPC (Figure 3).

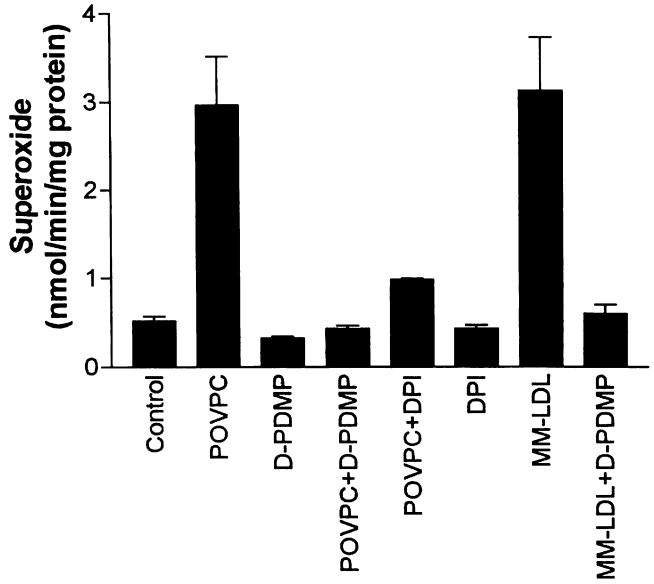

Figure 2. Effects of MM-LDL, oxidized phospholipids and D-PDMP on the generation of superoxides in aortic smooth muscle cells. Cells (10^5) were seeded and grown to confluence. Medium was removed, the monolayers washed with sterile PBS and harvested. The cell pellet was resuspended in a balanced salt solution described in "Materials and Methods". To measure $O_2^{\cdot-}$ production, the cells were taken in 96 well trays, dark adapted lucigenin and MM-LDL (10 μg/ml) oxidized phospholipids (1 μg/ml) was added to cells pre-incubated with and without D-PDMP as above. The amount of $O_2^{\cdot-}$ produced at each time point was calculated by comparison with a standard curve generated using xanthine/xanthine oxidase. The results are average values of three experiments analyzed in duplicate.

MM-LDL and POVPC stimulate the proliferation of A-SMC, and this phenomenon is abrogated by the preincubation of cells with D-PDMP

As shown in Figure 4A, the incubation of cells with MM-LDL for 24 h caused a concentration-dependent increase in the incorporation of [^{3}H]thymidine into DNA, considered as an index of cell proliferation. Thus, MM-LDL (2.5–10 μg/ml) stimulated cell proliferation ~2-fold compared to control. However, at a higher concentration (20 μg/ml) MM-LDL did not stimulate cell proliferation. D-PDMP alone exerted a concentration-dependent inhibition in cell proliferation ~0.6-fold compared to control. When 20 μM D-PDMP was employed it decreased cell proliferation 0.47-fold compared to control (Figure 4B). D-PDMP also abrogated the MM-LDL (5 μg/ml)-stimulated increase in A-SMC proliferation at all concentrations (2.0–20 μM). Like MM-LDL, POVPC (.25–1.0 μg/ml) stimulated cell proliferation ~2-fold as compared to the control (Figure 4A). When cells were incubated with POVPC (0.5 μg/ml) and increasing concentrations of D-PDMP, we observed a marked inhibition in cell proliferation as compared to cells incubated with POVPC alone (Figure 4B).

Discussion

Previous studies have shown that relatively low concentrations (5–10 μg/ml) of modified LDL can stimulate the proliferation of aortic smooth muscle cells [9–10]. The biochemical mechanisms involved in the phenomena above have been described recently [18]. These studies suggested that GalT-2 is the target for modified LDL activation in A-SMC proliferation. For example, other glycosyltranferases, in particular glucososyltranferase (GlcT-1) that leads to the synthesis of glucosylceramide was unaffected by incubation of cells with Ox-LDL [8].

Lactosylceramide produced as a consequence of enzymatic reaction serves as a lipid second messenger implicated in the activation of an oxidant-sensitive transcriptional pathway. For example, LacCer activates NADPH oxidase to produce free oxygen radicals ($O_2^{\cdot-}$) that stimulated $p21^{ras}$ GTP loading and the activation of the kinase cascade including p^{44} MAPK [9,19]. The phosphorylated form of p^{44} MAPK translocated from the cytoplasm to the nucleus where it engaged in increasing the expression of a protooncogene c-fos [9,16] and cyclin [21], ultimately leading to cell proliferation [7,22]. The focus of the present study is to define the biologically active compounds in MM-LDL that may serve as surrogates that would stimulate A-SMC proliferation via activation of the elements of the oxidant-sensitive transcriptional pathway above.

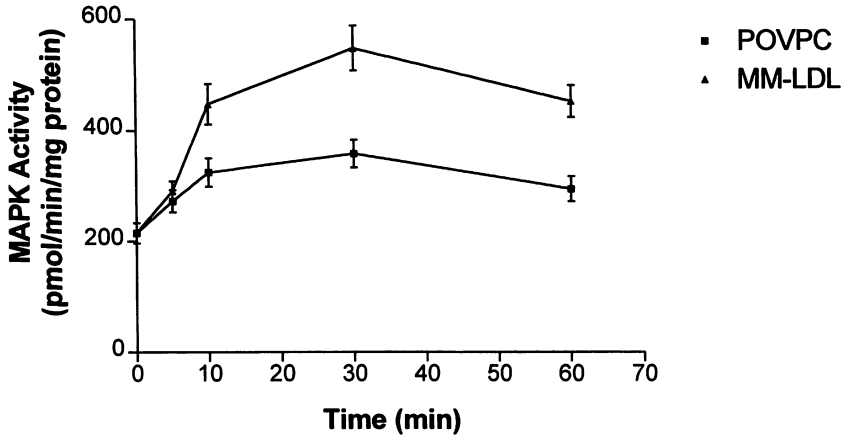

Figure 3. Effects of MM-LDL and POVPC on the activity of MAP kinase. Confluent culture of A-SMC were incubated with MM-LDL (10 μg/ml), POVPC (1 μg/ml) or vehicle for 5, 10, 30 and 60 min at 37°C. Cells were harvested and pellet lysed with RIPA buffer. MAP kinase was immunoprecipitated from the cell lysates and used as a source of enzyme for the measurement of MAPK kinase activity employing MBP (peptide APRTPGGRR) as substrate as described in "Materials and Methods". The results represent average values of three separate experiments analyzed in duplicate.

Although MM-LDL contains a variety of compounds with the potential to stimulate A-SMC proliferation, the three compounds possessing biological activity were POPVC, PGPC and PEIPC [5]. Our studies revealed that, of these compounds, only POVPC stimulated the activity of GalT-2. Previously, we have employed D-PDMP, as inhibitor of GlcT-1 and GalT-2, to abrogate Ox-LDL induced activation of GalT-2 [8,9]. In agreement with these reports, in the present study we found that D-PDMP also inhibited the activation of GalT-2 by MM-LDL. To rule out the possibility that POVPC can also affect the activity of GalT-2 in A-SMC incubated with diluted serum or albumin supplemented medium, additional experiments were performed. We found that within 5 min Ox-PAPC and POVPC stimulated the activity of GalT-2 168% and 200% as compared to control in A-SMC incubated with 1% dialyzed lipoprotein-deficient serum (comprising predominately of human albumin). We have also extended and confirmed these findings in human endothelial cells derived from the thoracic aorta in which POVPC was found to stimulate the activity of GalT-2 240%, as compared to the control. PAPC had no effect on GalT-2 activity. Thus collectively, these data suggest that POVPC exerts its stimulatory effect on GalT-2 activity irrespective of the presence or absence of lipoprotein-deficient serum. GalT-2 activation is the primary target of POVPC action that generates lactosylceramide. In turn, lactosylceramide stimulates the generation of superoxide. Therefore, we were not surprised to observe that superoxide generation occurred in cells incubated with POVPC irrespective of the presence/absence of lipoprotein-deficient serum (data not shown).

Previous studies [12] indicate that LacCer alone but not GlcCer or ceramide can induce $O_2^{\cdot-}$ generation in A-SMC. In agreement with this report, we found that MM-LDL and/or POVPC, both markedly stimulated the generation of $O_2^{\cdot-}$ in A-SMC. Since preincubation with D-PDMP abrogated POVPC induced $O_2^{\cdot-}$ generation revealing that GalT-2 activation and the consequent generation of LacCer are required for the activation of the oxidant-sensitive transcriptional pathway. Moreover, since diphenylene iodonium abrogated POVPC induced $O_2^{\cdot-}$ generation suggests that additional ROS generating enzymes may be involved in this phenomenon. Clearly, further studies are required to explore whether NADH oxidase, xanthine oxidase and nitric oxide [23,27] are implicated in the POVPC mediated phenomenon. Previous studies indicate the generation of large amount of $O_2^{\cdot-}$ in hypercholesterolemic rabbits as compared to normolipidemic rabbits [25]. Interestingly, our previous studies indicate a marked increase in the level of POVPC in hypercholesterolemic rabbits [5] and LacCer in human atherosclerotic plaques [26]. Accordingly, it is tempting to speculate that POVPC may well contribute to the generation of $O_2^{\cdot-}$ in the atherosclerotic tissue via the activation of GalT-2, LacCer generation and the consequent activation of the oxidant sensitive transcriptional pathway discussed above. The generation of ROS is accompanied by diverse biological phenomenon, such as cell proliferation, adhesion, differentiation and death [24,26]. Although the generation of large amounts of ROS is considered cytotoxic at low concentrations, ROS can stimulate cell proliferation via the activation of transcriptional factors for example, AP-1, NF-kB and MAPK [29,30]. We found that MM-LDL and/or POVPC induced GalT-2 activation and $O_2^{\cdot-}$ generation, accompanied by p^{44} MAPK activation and proliferation of A-SMC. In contrast, the PGPC and PEIPC failed to generate $O_2^{\cdot-}$, activated p^{44} MAPK and also failed to stimulate cell proliferation (data not shown). The tenet that activation of GalT-2 was an essential step in the signaling phenomenon leading to cell proliferation was supported by the finding that D-PDMP, an inhibitor of GalT-2 also abrogated POVPC induced A-SMC proliferation was supported by the finding that D-PDMP, an inhibitor of GalT-2 also abrogated

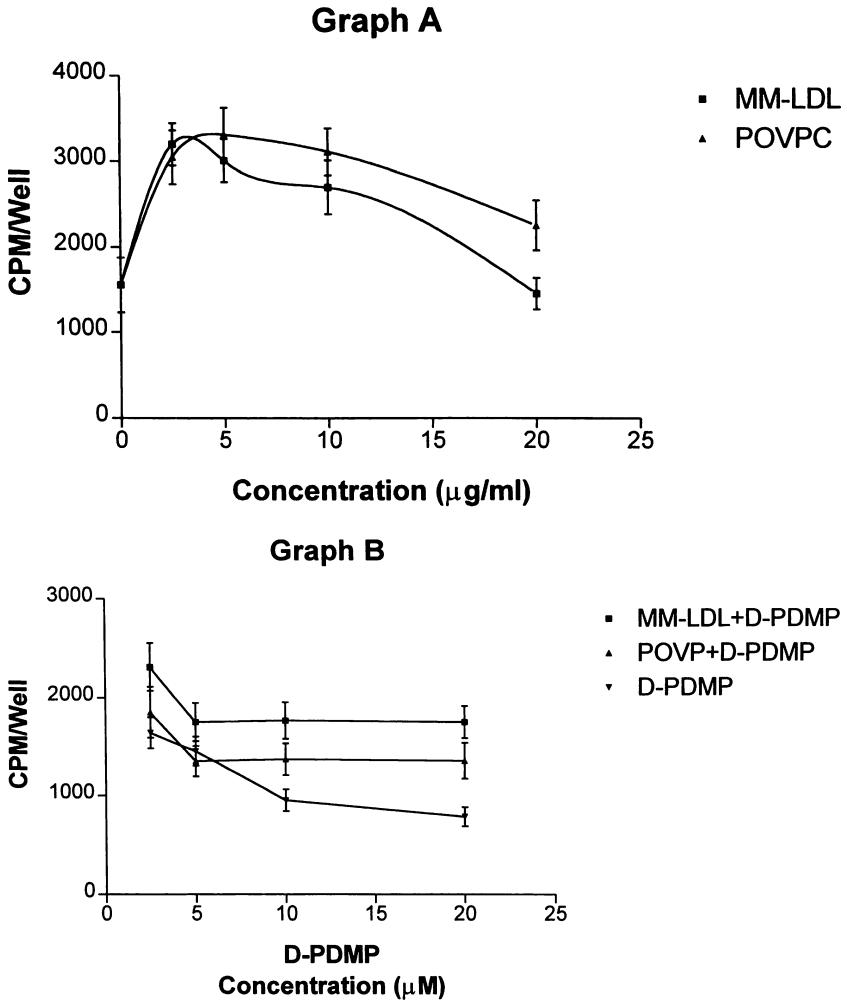

Figure 4. Effects of concentration of MM-LDL, POVPC AND D-PDMP concentration on cell proliferation. (A) Confluent culture of A-SMC grown in 96 well trays were incubated in serum free medium for 24 h with MM-LDL (10 μg/ml) and POVPC (1 μg/ml). Next, [^{3}H] thymidine (5 μgCi/ml) was added and incubation continued for an additional 2 h. The incorporation of [^{3}H]thymidine was measured by scintillation spectrometry. (B) In a companion experiment cells were incubated with D-PDMP (2.5–20 μM) alone, D-PDMP plus MM-LDL (10 μg/ml) and D-PDMP plus POVPC (1 μg/ml) for 24 h. The incorporation of [^{3}H]thymidine was measured as described above. The results are values from three separate experiments analyzed in 6 wells each.

POVPC induced A-SMC proliferation. Previously, a choline phosphoglyceride with a short chain at the *sn*-2 position having platelet activating factor (PAF) like activity was suggested to mediate A-SMC proliferation [29]. Such oxidized phospholipids were also derived from the oxidation of LDL. In that study, the stimulatory effects of Ox-LDL were abrogated by an antagonist to the PAF-receptor (WEB2086). Furthermore, previous studies with endothelial cell have shown that WEB2086 blocked the effect of POVPC on monocyte binding [32]. However, PAF had no effect on these cells which have very low levels of PAF receptor. Thus, WEB2086 may inhibit a related receptor in these cells. In the present studies, we have observed that WEB2086 had no effects on the activity of GalT-2 and it did not abrogate POVPC induced cell proliferation in A-SMC (data not shown). In another study, we found that WEB2086 also failed to abrogate LacCer induced expression of Mac-1 (CD11/CD8) in human neutrophils [30]. Therefore, we suggest that POVPC induced A-SMC proliferation is independent of PAF receptor or a related receptor but activates GalT-2 by a separate mechanism to produce LacCer. In turn, LacCer serves as a lipid second messenger that activates an oxidant sensitive transcriptional pathway that subsequently leads to cell proliferation. This pathway involves LacCer induced activation of NADPH oxidase that produces superoxide radical O_2^-. In turn O_2^- stimulates p21 Ras GTP loading, activation of a kinase cascade (Mek Raf-1, p^{44} MAPK). Next, the phosphorylated form of p^{44} MAPK enters the nucleus and increases the expression of a protoonocogene c-fos, the expression of proliferating nuclear antigen subsequently leading to cell proliferation [9,12,13,20,21].

In summary, POVPC serves as a surrogate for MM-LDL in regard to a A-SMC proliferation via activation of GalT-2 and an oxidant sensitive transcriptional pathway. Since the levels of POVPC were found to be elevated in cholesterol fed rabbits, indicates that POVPC may be especially important *in vivo* [33]. Further studies on structure function relationship in A-SMCs will elaborate the mechanisms of action of GalT-2 and its implication in the pathophysiology of vascular disease.

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