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Oxidized low density lipoprotein stimulates aortic smooth muscle cell proliferation

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We have investigated the effects of oxidized low density lipoproteins (Ox-LDL) on aortic smooth muscle cell (SMC) proliferation and the biosynthesis of glycosphingolipids.

We found that Ox-LDL exerted a concentration, time, and temperature dependent alteration of cell proliferation and the biosynthesis of lactosylceramide. At low concentrations (5–10 μg/ml medium) Ox-LDL stimulated cell proliferation measured by an increase in the incorporation of [^{3}H]-thymidine in cells and the synthesis of lactosylceramide, but not glucosylceramide synthesis. Oxidized LDL exerted a threefold increase in the incorporation of [^{3}H]-galactose and [^{3}H]-serine in lactosylceramide. The activity of lactosylceramide synthetase; UDP-galactose glucosylceramide β1 → 4 galactosyltransferase (GalT-2), but not glucosylceramide synthetase (GlcT-1) was stimulated by Ox-LDL. On the other hand, LDL suppressed the activity of GalT-2 in these cells. When cells were preincubated with antibody against Ox-LDL or GalT-2 it compromised the Ox-LDL mediated stimulation in cell proliferation and GalT-2 activity. Similarly, D-PDMP an inhibitor of GalT-2 compromised the Ox-LDL mediated effects in cells. In contrast, L-PDMP further stimulated the Ox-LDL mediated cell proliferation and GalT-2 activity. However, preincubation of cells with preimmune rabbit serum IgG failed to abrogate Ox-LDL mediated stimulation in cell proliferation and GalT-2 activity.

In sum, we found that Ox-LDL stimulated aortic smooth muscle cell proliferation in culture. This effect resulted from Ox-LDL mediated activation of GalT-2 that produced lactosylceramide. Lactosylceramide in turn, contributed to cell proliferation. Such correlations are supportive of the notion that GalT-2 action mediates the signal transduction of Ox-LDL contributing to cell proliferation.

Key words: oxidized low density lipoproteins (Ox-LDL)/glucosylceramide (GlcCer)/lactosylceramide (LacCer)

Introduction

The role of Ox-LDL in atherosclerosis is becoming increasingly evident primarily because of the finding that such modified lipoproteins were found associated with the atherosclerotic plaques (Morton *et al.*, 1986). Second, when Watanabe heritable hyperlipidemic rabbits were exposed to Probucol (a potent antioxidant) it prevented the atherosclerotic process (Carew *et al.*, 1987; Kita *et al.*, 1987).

Thus, although these lipoproteins could potentially cause injury to the aortic wall, very little is known regarding its biochemical mechanism of action. Our laboratory was the first to show that Ox-LDL stimulates the proliferation of aortic smooth muscle cells (Chatterjee, 1992). In a subsequent study, we reported that exogenously supplied lactosylceramide (LacCer) may stimulate the proliferation of smooth muscle cells (Chatterjee, 1991). Accordingly, we rationalized that one of the biochemical mechanisms in Ox-LDL mediated cell proliferation may involve activation of one or more of the metabolic enzymes in LacCer metabolism. Our studies reported here reveal that Ox-LDL specifically increases the activity of GalT-2, that in turn, produces LacCer and thus may contribute to the stimulation of aortic smooth muscle cell proliferation.

Results

Effects of oxidized LDL and various antibodies on the incorporation of [^{3}H]-thymidine in aortic smooth muscle cells

Oxidized LDL exerted a concentration-dependent stimulation of thymidine incorporation in cultured cells (Figure 1A). Maximum stimulation of the incorporation of thymidine into cells was observed with about 10–20 μg/ml Ox-LDL. This data indicate that at low concentrations oxidized LDL stimulates cell proliferation. In contrast, when cells were incubated with antibody against Ox-LDL, at various dilutions, it inhibited the proliferative effects in the cultured cells (Figure 1B). However, incubation of cells with antibody against lactosylceramide did not have any effects on the oxidized LDL mediated incorporation of [^{3}H]-thymidine (Figure 1C). Finally, when the cells were incubated with antibody against GalT-2, we observed a marked inhibition in the proliferation of Ox-LDL mediated proliferation in cells (Figure 1D). In sharp contrast, preincubation of cells with preimmune rabbit serum IgG did not abrogate the proliferative effects of Ox-LDL (Figure 1B–D, solid bars). These data indicate that oxidized LDL mediated proliferation of cells may involve the activation of the enzyme, GalT-2 (see below).

Effects of inhibitors and activators of GalT-2 on oxidized LDL mediated stimulation in [^{3}H]-thymidine incorporation in smooth muscle cells

As expected, incubation of cells with Ox-LDL (20 μg/ml) stimulated the incorporation of [^{3}H]-thymidine into cells (Figure 2A,B, solid bars) compared to control 2A, B; open

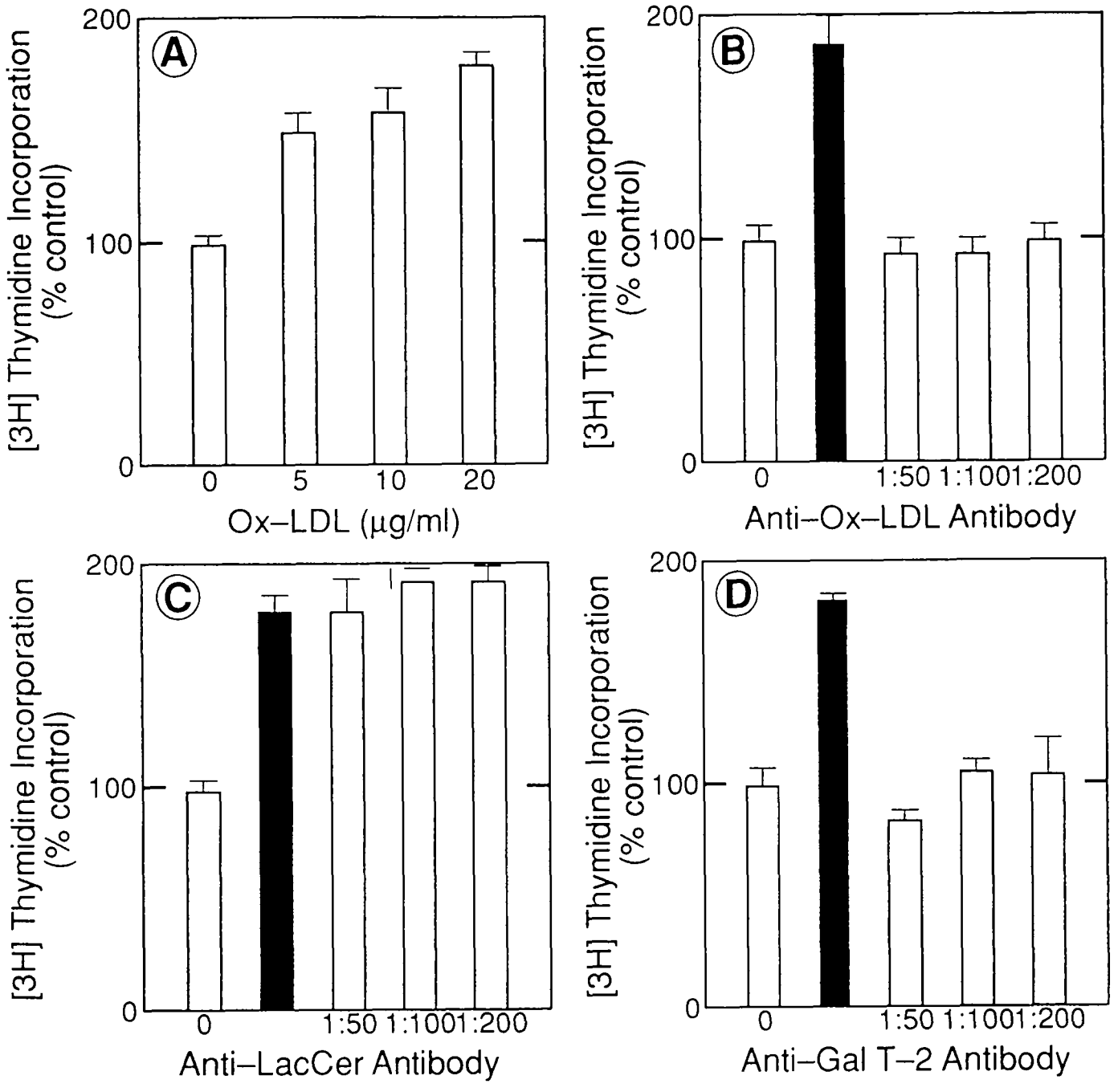

Fig. 1. Effects of Ox-LDL, anti-Ox-LDL antibody, anti-LacCer antibody, and anti-GalT-2 antibody on the incorporation of [^{3}H]-thymidine in cultured aortic smooth muscle cells. Cells ($\times 10^3$) were seeded in 96 well flat bottom trays. After 6 days of growth, fresh medium containing Ham's F-10 medium was added to one set of cultured dishes and increasing concentrations of Ox-LDL antibody was added. Cells were preincubated for about 1 h with the antibody. Then, Ox-LDL was added to cells incubated ±Ox-LDL antibody. After incubation for 22 h, [^{3}H]-thymidine (5 μCi/ml) was added to each well, incubation was continued for an additional 2 h. Subsequently, the incorporation of [^{3}H]-thymidine into cells was measured as described previously (Chatterjee, 1992). Each experiment was done in triplicate, and the data is shown from six individual wells. (**A**) Effects of increasing concentrations of Ox-LDL (0–20 μg/ml) on the incorporation of [^{3}H]-thymidine into cells. (**B**) Effects of anti-Ox-LDL antibody on the incorporation of [^{3}H]-thymidine into cells. In this experiment, antibodies at various dilutions (1:50–2:00) was added to the cultured cells. Following incubation for 1 h, Ox-LDL (10 μg/ml) was added to each well and [^{3}H]-thymidine incorporation measured as described above. (**C**) Effects of anti-LacCer antibody on the incorporation of [^{3}H]-thymidine in cultured cells. The protocol was identical to Figure 1B above. (**D**) Effects of anti-GalT-2 antibody on the incorporation of [^{3}H]-thymidine incorporation in cells. The protocol of this experiment was also identical to that described in (B) above. The solid bars in B–D represent the effects of preimmune serum IgG plus Ox-LDL (20 μg/ml) on the incorporation of [^{3}H]-thymidine in cells. The control values (100%) were 650 ± 53 × 10^3 c.p.m./mg protein.

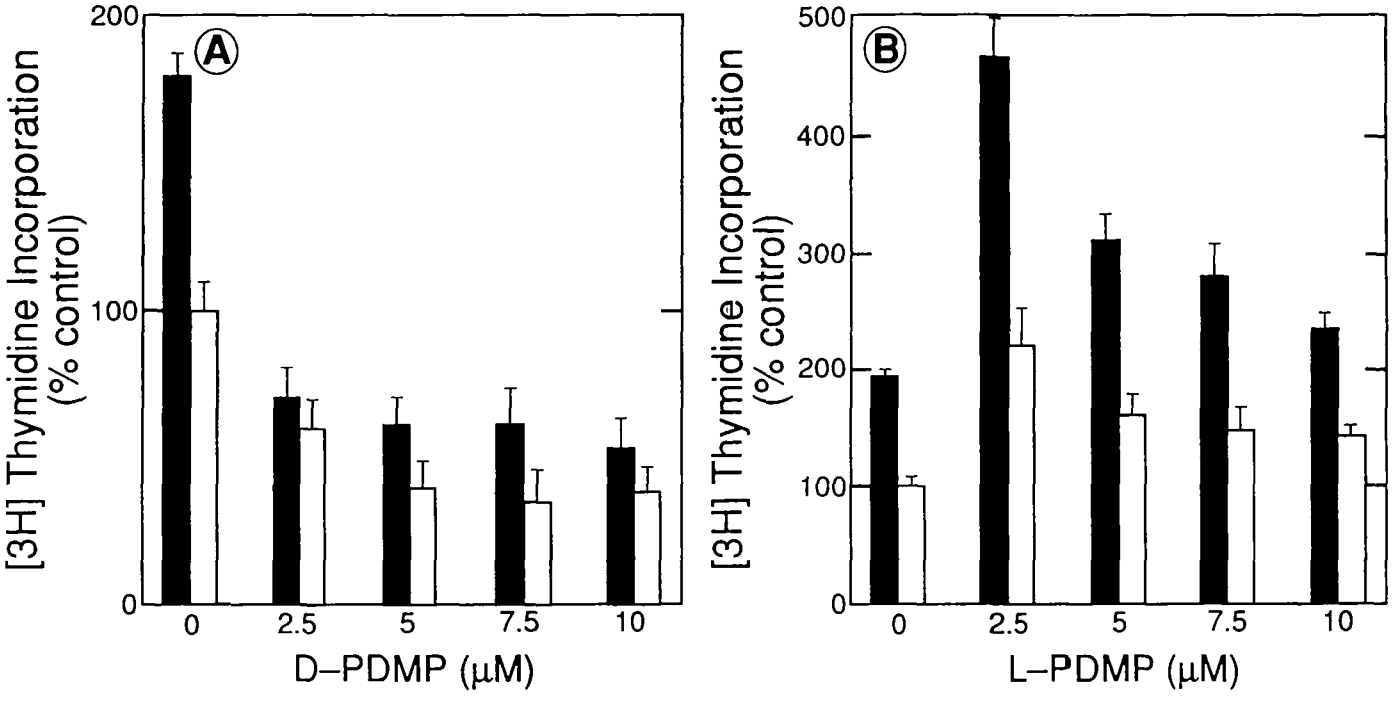

Fig. 2. Effects of D-PDMP, L-PDMP, and Ox-LDL on the incorporation of [^{3}H]-thymidine incorporation in cultured cells Cells were incubated with and without Ox-LDL (10 μg/ml) plus D-PDMP at increasing concentrations. Following incubation for 22 h, cells were further incubated with [^{3}H]-thymidine for 2 h and subsequently the incorporation of thymidine into the cells was measured as described above in Figure 1. **(A)** Effects of D-PDMP (2.5–10 μM) alone (open bars), Ox-LDL alone (solid bars; O point), and Ox-LDL ± D-PDMP (solid bars) on the incorporation of [^{3}H]-thymidine in cells. **(B)** The protocol for this experiment was identical to (A) except that the cells were incubated ± L-PDMP (2.5–10 μM, open bars) alone, Ox-LDL alone (solid bars; o point), and with increasing concentration of L-PDMP (2.5–10 μM) plus Ox-LDL (10 μg/ml; solid bars). The control values (100%) were 650 ± 53 × 10^3 c.p.m./mg protein.

bar, zero point). When cells were incubated with a fixed concentration of oxidized LDL (20 μg/ml) and increasing concentrations of D-PDMP, a concentration-dependent inhibition of [^{3}H]-thymidine incorporation was observed as compared to cells incubated with Ox-LDL alone (Figure 2A, solid bars). When cells were incubated with 2.5–10 μM of D-PDMP alone, there was an even more significant decrease in the incorporation of [^{3}H]-thymidine into cells. In that, a 50–30% decrease in the incorporation of [^{3}H]-thymidine was observed at these concentrations, respectively. In contrast, when cells were incubated with a fixed concentration of Ox-LDL and increasing concentration of L-PDMP (2.5–10 μM) under all concentrations used, we found that L-PDMP augmented the stimulatory effects of Ox-LDL in regard to the incorporation of [^{3}H]-thymidine into cells (Figure 2B, solid bars). Maximum stimulation in the incorporation (about 4.5-fold compared to control) was observed when cells were incubated with Ox-LDL (20 μg/ml) plus L-PDMP (2.5 μM). However, incubation of cells with Ox-LDL plus L-PDMP (5, 7.5, and 10 μM) stimulated the incorporation of [^{3}H]-thymidine into these cells in the order of 3-fold, 2.8-fold, and 2.5-fold compared to control (Figure 2B, solid bars). Incubation of cells with L-PDMP alone (2.5–10 μM) stimulated the incorporation of [^{3}H]-thymidine in the order of 2.25-fold to 1.5-fold (Figure 2B, open bars).

Effects of incubation of cells with Ox-LDL on the incorporation of [^{3}H]-galactose and [^{3}H]-serine into various glycosphingolipids

When cells were incubated with small concentrations of Ox-LDL (5 and 10 μg/ml), there was a marked stimulation in the incorporation of [^{3}H]-galactose into lactosylceramide. In contrast, Ox-LDL (5–10 μg/ml) did not alter the incorporation derived from [^{3}H]-galactose radioactivity into glucosylceramide. At higher concentrations of Ox-LDL (25 μg and above) there was a marked decrease in the incorporation derived from [^{3}H]-galactose radioactivity into glucosylceramide, compared to control. Interestingly, at low concentrations of Ox-LDL, we observed a threefold stimulation in the incorporation of [^{3}H]-galactose into lactosylceramide in smooth muscle cells. Even up to 25 μg of Ox-LDL in the cultured medium there was a twofold higher incorporation of [^{3}H]-galactose into lactosylceramide in these cells. At 100 μg/ml of Ox-LDL, the incorporation of [^{3}H]-galactose into lactosylceramide in smooth muscle cells was moderately inhibited.

The incorporation of [^{3}H]-serine into glucosylceramide was similarly minimally affected at low concentrations of Ox-LDL (5–25 μg/ml). It was however inhibited markedly at high concentrations (50–100 μg/ml) of Ox-LDL (Figure 3B). In contrast, the incorporation of [^{3}H]-serine into lacto-

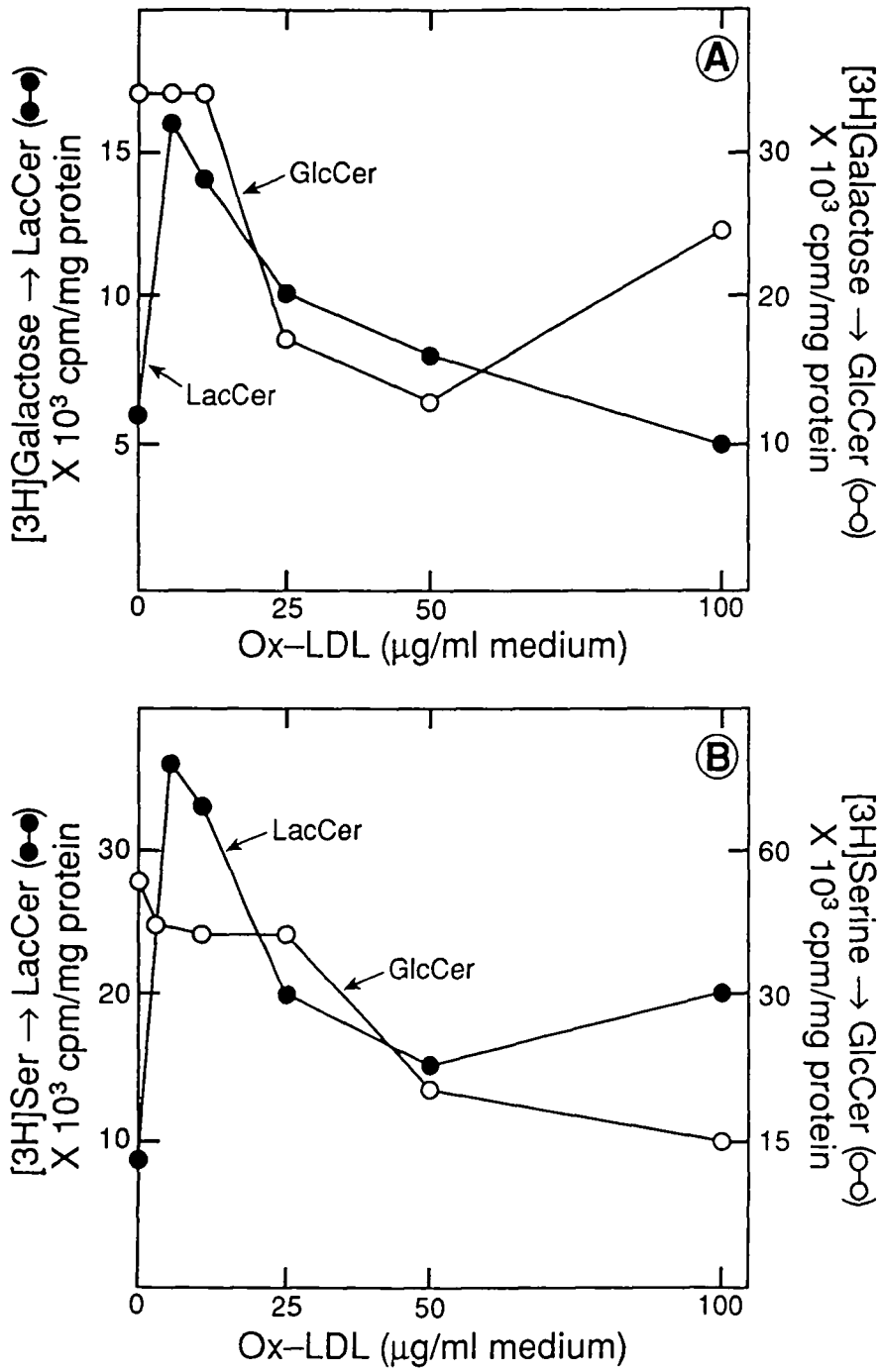

Fig. 3. Effects of Ox-LDL on the incorporation of [^{3}H]-galactose and [^{3}H]-serine into glucosylceramide and lactosylceramide in cultured aortic smooth muscle cells. Cells ($\times 10^5$) were grown in six well plastic trays for 6 days in full medium containing fetal calf serum. On the sixth day, the medium was changed and cells were incubated with Ham's F-10 medium and increasing concentrations of Ox-LDL (0–100 μg/ml) and [^{3}H]-galactose or [^{3}H]-serine (5 μCi/ml). After incubation for 24 h at 37°C, the medium was removed and cells were harvested with PBS. Subsequently, cells were extracted with organic solvents, the glycolipids were purified by silicic acid chromatography and then, they were subjected to separation by HPTLC; chloroform methanol-water 65:25:4 (v/v) was used as solvent. Gel areas corresponding to standard GlcCer and LacCer were scraped and the incorporation of radioactivity was measured. Suitable aliquots of cells were also subjected to measurements of protein according to Lowry's procedure (13). Three such experiments were performed and the data of a representative experiment is shown here. **A,** Incorporation of [^{3}H]-galactose into glucosylceramide (○) and lactosylceramide (●). **(B)** Incorporation of [^{3}H]-serine in glucosylceramide (○) and lactosylceramide (●).

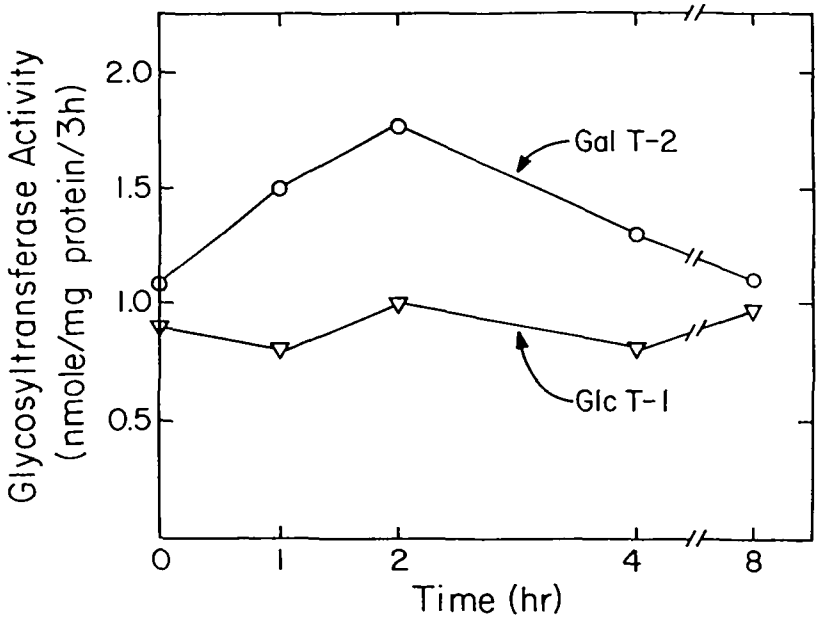

Fig. 4. Effects of time of incubation with Ox-LDL on the activity of GalT-2. Cells were grown as described in Figure 3. Next, confluent cultures were incubated with Ox-LDL (10 μg/ml). At indicated intervals (0–8 hr) cells were harvested in cacodylate buffer (6.8). Subsequently, the activity of GalT-2 and GlcT-1 was measured as described in Materials and methods. GalT-2 activity (○), and GlcT-1 activity (△) (Havel *et al.*, 1955). The results are average values of two experiments analyzed in duplicate. The activity of glycosyltransferases varied 5% among experiments.

sylceramide was stimulated in the presence of low concentrations of Ox-LDL (5–10 μg/ml) about 3.5-fold compared to control. As the concentration of Ox-LDL increased (50–100 μg/ml), there was a substantial decrease in the incorporation of [^{3}H]-serine into lactosylceramide, but at all concentrations of Ox-LDL used in this study the incorporation of [^{3}H]-serine into lactosylceramide was higher than control.

Effects of time of incubation with Ox-LDL on the activity of various glycosyltransferases and glycosidases

When cells were incubated with 10 μg of Ox-LDL/ml medium and the activity of GlcT-1 and GalT-2 was measured at various time points indicated in Figure 4, we found that Ox-LDL did not stimulate the activity of GlcT-1 enzyme activity. In contrast, within 2 h of incubation of Ox-LDL with cells, we found about a 75% increase in the activity of GalT-2. Cells incubated with Ox-LDL up to 4–8 h had only moderately higher activities of GalT-2 than control. Ox-LDL did not alter the activity of $GbOse_3Cer$ 1 → 4 galactosidase and LacCer β1 → 4 galactosidase (data not shown).

Effects of Ox-LDL and LDL concentration and temperature of incubation on the activity of GalT-2

Ox-LDL exerted a concentration-dependent stimulation in the activity of GalT-2 in these cells (Figure 5). In that, at low concentrations of Ox-LDL, we observed about a 40–60% stimulation in the activity of GalT-2. However, at high concentrations of Ox-LDL (100 μg/ml), the activity of GalT-2 was unaffected compared to control. Incubation of cells with LDL (Figure 5) did not stimulate the activity of GalT-2. In contrast, at high concentrations (25 μg/ml medium) it suppressed the activity of GalT-2 on the order of about 70% compared to control.

Incubation of cells at 4°C with Ox-LDL did not increase the activity of GalT-2. For example, the GalT-2 values obtained from cells incubated with and without Ox-LDL at 4°C were on the order of 1.06 nmol/mg protein/2 h and 1.05 nmol/mg protein/2 hr, respectively.

Effects of Ox-LDL, D-PDMP, and L-PDMP on the activity of GalT-2

As shown in Figure 6, D-PDMP exerted a concentration-dependent inhibition of the activity of GalT-2. In that, D-PDMP at 5 and 10 μM, decreased the activity of GalT-2 on the order of 50% and 40%, respectively, compared to control. Ox-LDL (10 μg/ml) stimulated the activity of GalT-2 on the order of 140%. When cells were incubated with Ox-LDL plus D-PDMP, it compromised the stimulatory effects of Ox-LDL in these cells. Incubation of cells with L-PDMP stimulated the activity of GalT-2. When Ox-LDL and L-PDMP (5 μM) were added together to cells, the effects were additive in regard to the stimulation of GalT-2 activity. The addition of Ox-LDL and higher amounts of L-PDMP (10–20 μM) sustained the stimulatory effects of GalT-2 activity in these cells (Figure 5).

Effects of various antibodies on the activity of GalT-2 in cultured aortic smooth muscle cells

In these series of experiments, cells were incubated with Ox-LDL in the presence and absence of various antibodies against Ox-LDL, lactosylceramide, and GalT-2 (Table 1).

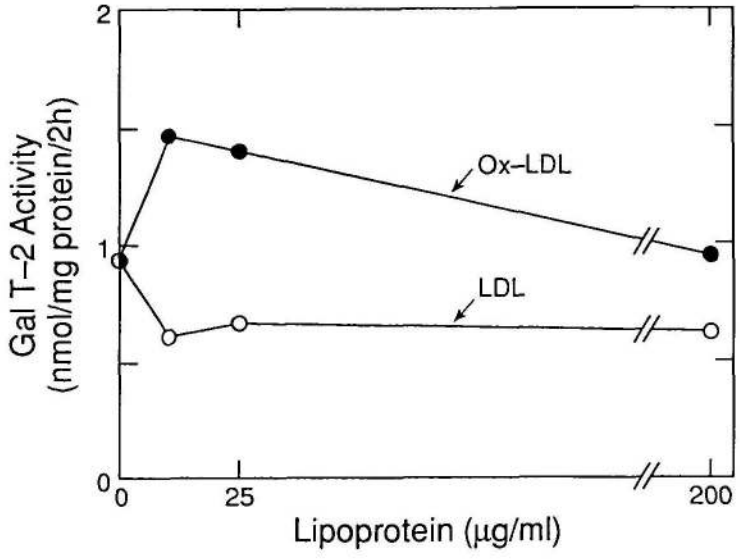

Fig. 5. Effects of concentration of LDL and Ox-LDL on the activity of GalT-2 in aortic smooth muscle cells. The protocol of this experiment was identical to the one described for Figure 4 except cells were incubated with increasing concentrations of LDL (○) or Ox-LDL (●) for 2 hr. Subsequently, the activity of GalT-2 was measured as described in Materials and methods. Two such experiments were performed and the average values are presented. The activity of GalT-2 varied 5% among experiments.

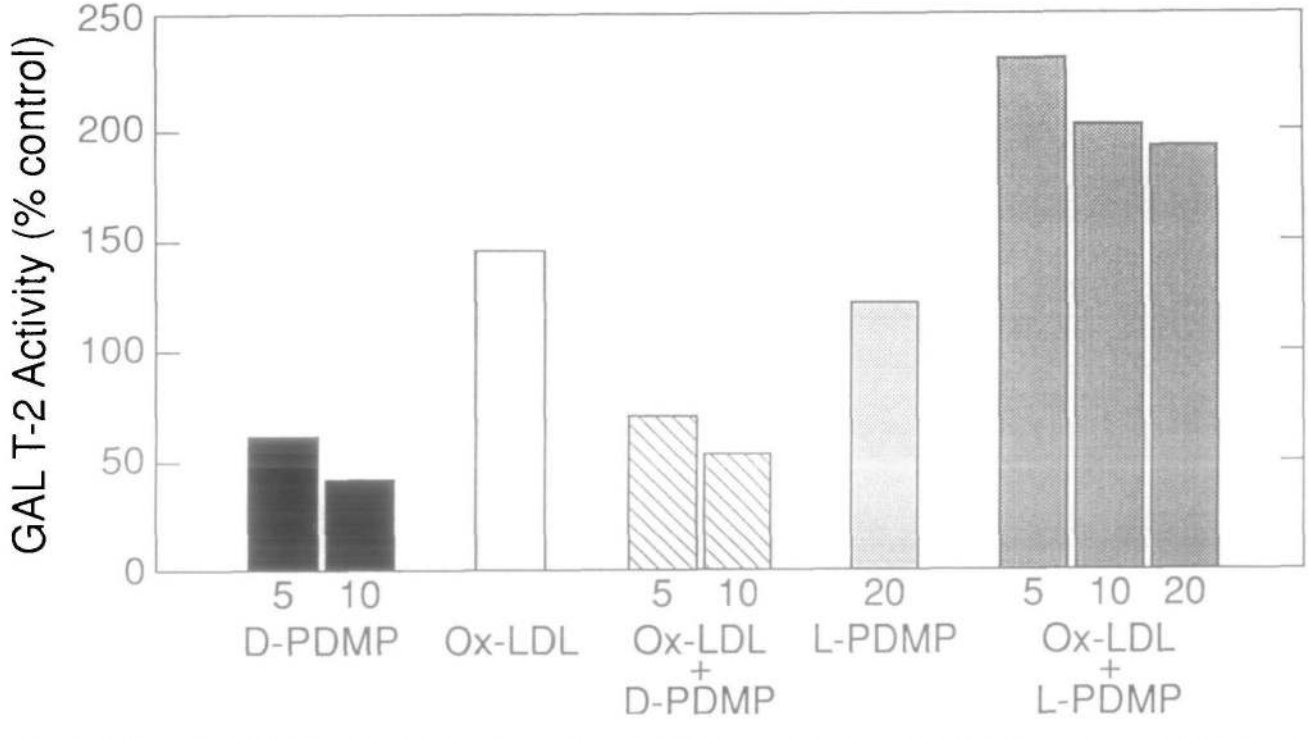

Fig. 6. Effects of D-PDMP, L-PDMP, and Ox-LDL on the activity of GalT-2 in aortic smooth muscle cells. Cells were incubated with increasing concentrations of D-PDMP, L-PDMP (5–20 μM) for 1 h at 37°C. Next, Ox-LDL (10 μg protein/ml) was added and incubation was continued for an additional 2 hr. Subsequently, medium was removed and the cells were harvested in cacodylate buffer and GalT-2 activity was measured as described in Materials and methods. The control values for GalT-2 (100%) were 2.75 nmol 12 h/mg protein.

We found that whereas Ox-LDL stimulated the activity of the enzyme, this effect was compromised when cells were preincubated with antibody against Ox-LDL. However, antibody against Ox-LDL itself did not have any effects on GalT-2. When cells were incubated with antibody against lactosylceramide and Ox-LDL we found a significant stimulation in the activity of GalT-2. In contrast, antibody against GalT-2 by itself decreased the activity of GalT-2 in these cells on the order of about 50% compared to control. This antibody when added along with Ox-LDL compromised the effects of Ox-LDL in stimulating the activity of GalT-2.

Table 1. Effects of various antibodies on the activity of GalT-2 in cultured aortic smooth muscle cells

Treatment	GalT-2 activity nmol/mg protein/2 hr	% Control value
Control	2.75 ± 0.15	100
Ox-LDL	3.85 ± 0.15	140
Anti-Ox-LDL + Ox-LDL	2.85 ± 0.25	103
Anti-Ox-LDL	2.75 ± 0 25	100
Anti-LacCer ± Ox-LDL	3.80 ± 0.20	138
Anti-GalT-2	2.70 ± 0.20	98
Anti-GalT-2 ± Ox-LDL	2.90 ± 0.20	105

Cells were incubated for 1 hr with various antibodies (dilution 1 : 100). Next, Ox-LDL (10 μg/ml) was added. After incubation for 2 hr, cells were harvested and the activity of GalT-2 was measured. The data represent standard deviation values from two separate experiments analyzed in duplicate.

Discussion

This study led to several significant findings. First, we found that Ox-LDL mediated stimulation of cell proliferation was compromised by the antibody against Ox-LDL or the antibody against the enzyme GalT-2. Second, Ox-LDL stimulated the endogenous synthesis of lactosylceramide, but not glucosylceramide from exogenously added radioactive precursors. Third, Ox-LDL specifically stimulated the activity of GalT-2 and this effect was time, concentration and temperature dependent. Fourth, Ox-LDL mediated stimulation of the activity of GalT-2 was compromised by an inhibitor of GalT-2, i.e., D-PDMP, and was stimulated by an activator of GalT-2, i.e., L-PDMP. Finally, the Ox-LDL mediated stimulation of activity of GalT-2 was compromised by antibody against GalT-2 and Ox-LDL itself, but not by antibody against lactosylceramide.

We have previously shown that Ox-LDL, particularly at low concentrations, can exert a concentration-dependent stimulation in the proliferation of aortic smooth muscle cells, (Chatterjee, 1992). Subsequent studies from our laboratory revealed that lactosylceramide alone could stimulate the proliferation of these aortic smooth muscle cells (Chatterjee, 1991). These findings are of great biological and clinical significance as Ox-LDL has been implicated in the pathogenesis of atherosclerosis. Moreover, recent studies from our laboratory reveal that both Ox-LDL and lactosylceramide are enriched in the atherosclerotic plaque in human subjects who have died of coronary artery disease (Chatterjee,S., Dey,S., Shi,W.Y., Thomas,K. and Hutchins,G., unpublished observations). Accordingly, we sought to understand biochemical mechanisms involved in the Ox-LDL mediated proliferation of aortic smooth muscle cells. Our studies reported in this manuscript reveal that indeed Ox-LDL at low concentrations can stimulate the proliferation of aortic smooth muscle cells. This phenomena (Figure 7) may involve the binding of Ox-LDL to a putative cell surface receptor. Such a receptor has been recently described by Steinberg and co-workers (unpublished observations). This tenet is also supported with the evidence that the binding of Ox-LDL at 4°C does not stimulate the activity of GalT-2. Moreover, Ox-LDL did not impart any effect on the activity of GalT-2 in cell-free extracts. Taken together, these findings imply that Ox-LDL may be taken up by the cell surface receptor for Ox-LDL and delivered to a site accessible to GalT-2. Next, the activation of GalT-2, an enzyme that has been suggested to be present in the Golgi apparatus stimulates the synthesis of lactosylceramide from glucosylceramide. Finally, lactosylceramide via stimulating the phosphorylation of mitogen activated protein kinase (p^{44} MAPK, ERK_1) (Bhunia,A.K., Snowden,A., Han,H. and Chatterjee,S., unpublished observations) stimulates proliferation of the aortic smooth muscle cells. Several lines of investigations support the notion that the enzyme GalT-2 is involved in the signal transduction process in cell proliferation. In that, antibodies against Ox-LDL blocked the proliferation process, and inhibitors of glucosylceramide synthesis, lactosylceramide synthesis as well as the GalT-2 antibody directly inhibited both the cell proliferation as well as the activity of GalT-2. How antibody against GalT-2 impairs Ox-LDL mediated induction in cell proliferation is not understood from our studies. Particularly in view of the belief that most, if not all, GalT-2 is localized in the Golgi apparatus. This would imply that GalT-2 antibody would have to have access to the enzyme and therefore, must enter the cell. Alternatively, if that pool of GalT-2 involved in the signaling pathway is present on the cell surface, then the anti-GalT-2 antibody may have access and then, compromise the effects of Ox-LDL. In this regard, we have preliminary evidence that a significant amount of GalT-2 activity is present on the cell surface. Studies are in progress to elaborate this phenomena, further.

Since smooth muscle cells do not have detectable amounts of galactosylceramide, we infer that radioactivity derived from [^{3}H]-galactose is incorporated into glucosylceramide, and subsequently into lactosylceramide as a consequence of stimulation of GalT-2 antibody by Ox-LDL. Activators of GalT-2 such as L-PDMP stimulated both cell proliferation as well as the activity of GalT-2. Whether or not this reflects an additive increase in LacCer mass in cells is not known. Previously, Radin and co-workers showed that D-PDMP exerted a marked effect on cell proliferation *in vitro* and *in vivo* (Chatterjee *et al.*, 1986; Radin *et al.*, 1993). Moreover, Inokuchi showed that L-PDMP stimulated the activity of GalT-2 in B-16 melanoma cells (Inokuchi,J.I., personal communication) Taken together, our data indicate that the activation of GalT-2 mediates the signal transduction process involved in Ox-LDL mediated proliferation of the aortic smooth muscle cells. We found that Ox-LDL mediated stimulation in the activity of GalT-2 ranged between 40–75% higher than control. This may be due batch to batch variation in Ox-LDL prepared from the LDL from different normalipidemic volunteers. In addition, this difference may be due to animal to animal variations as the primary cultures of aortic smooth muscle cells were derived from different rabbits. Nevertheless, in all of our experiments pursued over the last few years, Ox-LDL consistently stimulated the activity of GalT-2.

Recently, an epoxide derivative of glucosylceramide has been shown to specifically inhibit the endogenous synthesis of lactosylceramide (Zacharias *et al.*, 1994). Availability of such an inhibitor may be helpful in directly assessing the role of GalT-2 in the Ox-LDL mediated cell proliferation. Our laboratory is currently involved in the molecular cloning of the enzyme GalT-2. The availability of suitable

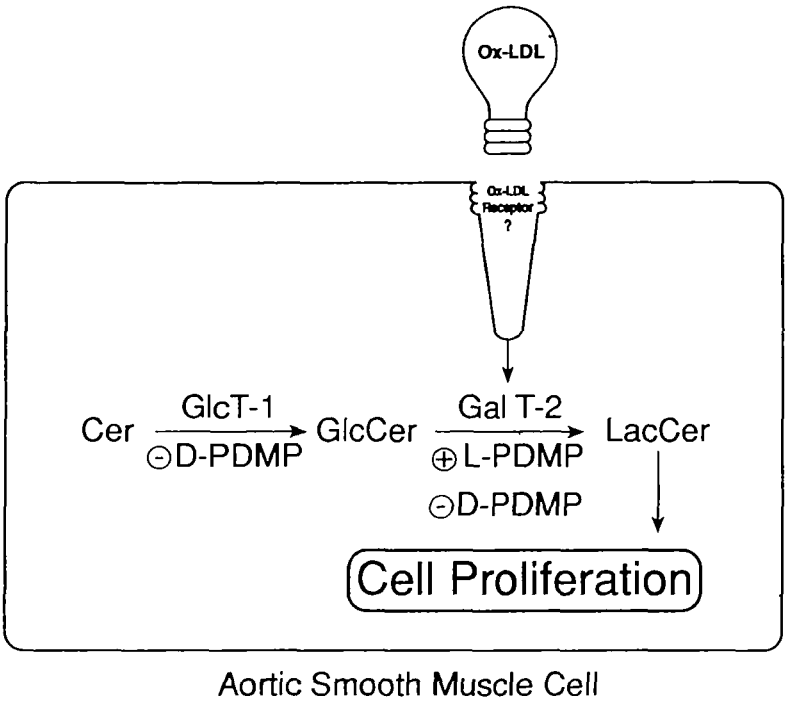

Fig. 7. Hypothetical model describing how UDP-galactose: glucosylceramide, $\beta 1 \rightarrow 4$ galactosyltransferase (GalT-2) action stimulates signal transduction of oxidized LDL (Ox-LDL) in the synthesis of lactosylceramide and stimulation of cell proliferation in the aortic smooth muscle cells.

cDNA probes for GalT-2 will help us understand the regulatory elements involved in Ox-LDL mediated activation of this enzyme and LDL mediated suppression of this enzyme in a variety of cultured cells. Such studies will hopefully lead to a better understanding of biochemical mechanisms involved in Ox-LDL mediated proliferation of aortic smooth muscle cells, a hallmark in the pathogenesis of atherosclerosis.

Materials and methods

Materials

Methyl-[^{3}H]-thymidine (specific activity 925 GBq/mmol), uridine diphosphate galactose (specific activity 12.2 GBq/mmol), d-[^{3}H]-galactose (specific activity 1.3 TBq/mmol), and l-[^{3}H]-serine (specific activity 1.07 TBq/mmol), were purchased from Amersham, Inc. D-PDMP and L-PDMP were from Matreya, Inc. All other chemicals were purchased from Sigma Chemical Co.

Aortic smooth muscle cells from New Zealand White rabbits were prepared as described previously (Ross, 1971). Large batches of primary cultures were frozen and all experiments were done within passage 2–3. Typically, cells ($\times 10^6$) were seeded in P-100 plastic petri dishes and grown in minimum essential medium containing 10% fetal calf serum, nonessential amino acids, glutamine, penicillin, and streptomycin. Medium was changed on the 3rd and 6th day of cell growth. On the 7th day of cell growth, medium was removed and cells were incubated with serum free Ham's-F-10 medium for 2 h. Then various compounds were added, incubated, and processed as described below. For cell proliferation assay, cells ($\times 10^3$) were seeded in 96-well microtiter plates.

Preparation of LDL, oxidized LDL, and ^{125}I-Ox-LDL

Human plasma low density lipoproteins were prepared employing KBr gradient centrifugation (1.019–1.063 gm/dl) as described previously (Havel, *et al.*, 1955). Ox-LDL was prepared by dialyzing LDL against 5 μM $CuSO_4$ in sterile phosphate-buffered saline. LDL and Ox-LDL were characterized using physical-chemical criteria including electrophoretic migration on agarose gels, assays for thiobarbituric acid positive substance and lysophosphatidylcholine, phosphatidylcholine levels as described (Chatterjee, 1992). ^{125}I-LDL was prepared using modification of the procedure of McFarlane as described by us previously (Chatterjee, *et al.*, 1988). ^{125}I Ox-LDL was prepared from LDL by dialyzing against a solution of 5 μM copper sulfate in phosphate buffered saline. Freshly prepared Ox-LDL was used immediately in our experiments.

Incubation of cells with Ox-LDL and LDL

Following incubation of cells with Ham's F-10 medium, freshly prepared Ox-LDL and LDL (0–100 μg protein/ml) was added and incubation carried out for 1 h. Cells were washed with PBS, harvested and stored frozen. In another set of dishes, cells were incubated with Ox-LDL (10 μg protein/ml) for 0, 5 min, 10 min, 15 min, 30 min, 45 min, 60 min, 2 h, and 4 h. Next, cells were harvested in cacodylate buffer (pH 6.8) centrifuged and the pellets stored at −20°C. Such cell pellets were used for the measurement of various enzyme activities.

Preparation of antibodies

Polyclonal antibodies against human kidney UDP-galactose: glucosylceramide, $\beta 1 \rightarrow 4$ galactosyltransferase (GalT-2), lactosylceramide and human plasma oxidized LDL were prepared against New Zealand White rabbits as described previously (Chatterjee and Ghosh, 1989; Chatterjee, 1991). Immunoglobulin G were prepared from the respective preimmune serum using ion exchange column chromatography (Econo-pac serum IgG purification kit, Bio-Rad, Richmond, CA). Next, the antibodies were immunoabsorbed against antigen and the monospecific polyclonal antibodies were eluted as described previously (Chatterjee and Ghosh, 1989).

Assay for GalT-2, other glycosyltransferases, and glycohydrolases

Cells incubated with and without Ox-LDL, LDL, and other compounds were homogenized and suitable aliquots were used to measure the following activities: UDP-glucose: ceramide $\beta 1 \rightarrow 4$ glucosyltransferase (GlcT-1), GalT-2, $GbOse_3Cer$ 1 → 4 galactosidase, and LacCer $\beta 1 \rightarrow 4$ galactosidase. The procedures used for such assays have been described previously (Chatterjee, *et al.*, 1988).

Incubation of cells with D-PDMP and L-PDMP

Stock solutions (100 μM) of D-PDMP and L-PDMP were prepared in isopropanol. Suitable aliquots (10 μl/ml) of these solutions were added to cells preincubated with Ham's F-10 medium. After incubation for 1 h, 2 h, 4 h, and 24 h, cells were harvested and the activity of GalT-2 measured.

Incubation of cells with various antibodies and Ox-LDL

Confluent monolayer of cells were incubated with Ham's F-10 medium for 2 h at 37°C. Next, GalT-2 antibody, LacCer antibody, Ox-LDL antibodies (10 μl each) alone was added to cells. After 1 h at 37°C Ox-LDL (10 μg/ml) was added to each dish. The cells were incubated further for 2 h at 37°C, harvested and subjected to GalT-2 measurements.

Measurement of cell proliferation

Cells were grown in 96-well microtiter plates for 6 d. On the sixth day medium was removed with fresh Ham's F-10 ml (100 μl) with and without Ox-LDL and antibodies. After incubation for 22 h at 37°C, [^{3}H]-thymidine (5 μCi/ml) was added and incubation continued for an additional 2 h. Next, cells were washed with PBS and the incorporation of [^{3}H]-thymidine was measured as described (Chatterjee, 1992).

Incorporation of [^{3}H]-galactose and [^{3}H]-serine into glycosphingolipids

Cells ($\times 10^5$) grown in 6-well plastic trays for 6 days were given Ham's F-10 medium with and without Ox-LDL (0–200 μg/ml) and [^{3}H]-Galactose or [^{3}H]-serine (5 μCi/ml). After incubation for 24 h, cells were harvested in PBS and centrifuged. The incorporation of radioactivity into individual glycosphingolipids was pursued following extraction of total lipids, silicic acid column chromatography, HPTLC separation, and scintillation spectrometry as described previously (Chatterjee *et al.*, 1986).

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Abbreviations

Ox-LDL, oxidized low density lipoproteins; LDL, low density lipoproteins; GlcCer, glucosylceramide; LacCer, lactosylceramide; D-PDMP, 1-phenyl-2-decanoylamino-3-morpholino-1-propanol; PBS, phosphate-buffered saline; GalT-2, UDP-galactose; GlcCer β1 → 4 galactosyltransferase.

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