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Oxidized low density lipoproteins stimulate galactosyltransferase activity, ras activation, p^{44} mitogen activated protein kinase and c-fos expression in aortic smooth muscle cells

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Previously, our laboratory has shown that oxidized low density lipoproteins (Ox-LDL) can exert a concentration-dependent stimulation in the proliferation of aortic smooth muscle cells, "a hallmark in the pathogenesis of atherosclerosis" (Chatterjee,S. (1992) *Mol. Cell. Biochem.*, 111, 143–147). Here we report a novel aspect of Ox-LDL-mediated signal transduction. We demonstrate that in aortic smooth muscle cells, Ox-LDL stimulates the activity of a UDP-galactose:glucosylceramide β1→4 galactosyltransferase (GalT-2) and phosphorylation/activation of p^{44} mitogen-activated protein (MAP) kinase (p^{44} MAPK). The activity of GalT-2 increased about 2-fold within 2.5–5 min of incubation of cells with Ox-LDL (10 μg/ml). After 5 min of incubation of cells with Ox-LDL, but not LDL, there was a 2-fold increase in the activity of p^{44} MAPK. Phosphoamino acid analysis employing thin layer chromatography revealed that the tyrosine and threonine moieties of p^{44} MAPK was phosphorylated by Ox-LDL. D-1-Phenyl-2-decanoylamino-3-morpholino-1-propanol (D-PDMP; a potent inhibitor of GalT-2) impaired the Ox-LDL mediated induction of p^{44} MAPK activity and the phosphorylation of tyrosine and threonine residues in p^{44} MAPK. This phenomenon was bypassed by the simultaneous addition of lactosylceramide. The upstream and downstream parameters in MAP kinase signaling pathways were investigated next. We found that Ox-LDL stimulated (9-fold) the loading of GTP on Ras. Interestingly, Ox-LDL specifically induced c-fos mRNA expression (6.5-fold) in these cells, as compared to the control. Thus, one of the biochemical mechanisms in Ox-LDL mediated induction in the proliferation in aortic smooth muscle cells may involve GalT-2 activation, lactosylceramide production, Ras GTP loading, activation of the kinase cascade, and c-fos expression.

Key words: Ox-LDL/p^{44} MAPK/signaling/lactosylceramide/GalT-2

Introduction

The role of Ox-LDL in atherosclerosis has become 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), this prevented the atherosclerotic process (Carew *et al.*, 1987; Kita *et al.*, 1987).

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 demonstrate that Ox-LDL stimulates the proliferation of aortic smooth muscle cells (Chatterjee, 1992). In a subsequent study, we reported that Ox-LDL can specifically stimulate the synthesis of lactosylceramide by activating a UDP-Gal:glucosylceramide β1→4 galactosyltransferase (GalT-2; Chatterjee and Ghosh, 1996). Moreover, large amounts of Ox-LDL and LacCer were found to be associated with aortic plaque intima from patients who died of atherosclerosis compared to unaffected intima (Chatterjee *et al.*, 1997). In other studies, we reported that exogenously supplied lactosylceramide (LacCer) stimulates the proliferation of smooth muscle cells by stimulating Ras GTP loading, kinases (MEK, Raf), p^{44} mitogen-activated protein kinase (MAPK), and c-*fos* expression (Bhunia *et al.*, 1996; Chatterjee and Ghosh, 1996). Accordingly, we rationalized that if LacCer serves as a signaling molecule in Ox-LDL mediated cell proliferation, then inhibition of GalT-2 should abrogate this signaling phenomena. In this article, we present evidence that Ox-LDL specifically activates GalT-2 and the phosphorylation of tyrosine and threonine residues in p^{44} MAPK in cultured human aortic smooth muscle cells. This phenomena is abrogated by D-PDMP, an inhibitor of GalT-2.

The best known mitogen-activated protein (MAP) kinases are p^{44} MAPK (extracellular signal regulated kinase, ERK_1) and p^{42} MAPK (ERK_2). These are a group of serine/threonine protein kinases that constitute an activation process triggered by a variety of growth stimuli (Boulton *et al.*, 1991; Rossomando *et al.*, 1992; Davis, 1994; Cano and Mahadevan, 1995). Such protein kinases have been suggested to phosphorylate and activate transcriptional factors such as c-*myc* (Seth *et al.*, 1991; Traverse *et al.*, 1992), c-*fos* (Deng and Karin, 1994), and $p62^{TCF}$ (Gille *et al.*, 1992; Marais *et al.*, 1993), which regulates the expression of genes essential for cell proliferation (Davis, 1993). In this article, we present evidence that upstream activators, such as Ras, are involved in Ox-LDL mediated activation of p^{44} MAPK. The latter, in turn, specifically stimulates c-*fos* proto-oncogene expression that eventually leads to cell proliferation.

Results

Effects of concentration of Ox-LDL, LDL and Ox-LDL antibody on the phosphorylation/activity of p^{44} MAPK

Ox-LDL exerted a concentration-dependent stimulation in the phosphorylation as shown in Western immunoblot assays (Figures 2, 3). The maximum stimulation (~3-fold) in the activity

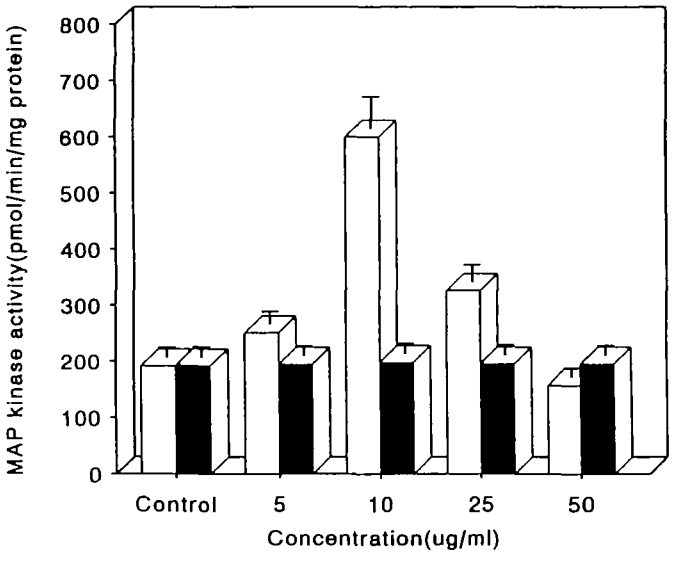

Fig. 1. Effects of Ox-LDL and LDL concentrations on MAP kinase activity in cultured human aortic smooth muscle cells. Cells were treated with various concentrations (0–50 μg/ml) of Ox-LDL and LDL for 10 min and lysed in lysis buffer. The MAP kinases were immunoprecipitated from the cell lysate with anti-MAP-kinase antibody conjugated with protein A/agarose and MAPK activity was measured in the immunoprecipitates. Each point is the mean ± SD of three individual experiments. □, Ox-LDL; ■, LDL

of MAPK occurred with 10 μg/ml Ox-LDL (Figure 1). At a higher concentration of Ox-LDL (50 μg/ml), MAPK activity was somewhat decreased, compared to the control. In contrast, MAPK Western immunoblot assays revealed that incubation of cells with similar concentrations of LDL (Figures 1, 2B,D) did not appreciably stimulate the phosphorylation of p^{44} MAPK. The antibody against Ox-LDL abrogated the Ox-LDL mediated increase in the phosphorylation of p^{44} MAPK (Figure 2B,D). Interestingly, Ox-LDL did not alter the level of nonphosphorylated form of p^{44} MAPK (middle band in Figure 2A) and nonphosphorylated p^{42} MAPK (bottom band in Figure 2), whereas the mass of phosphorylated form of p^{44} MAPK (indicated by an arrow in Figure 2) was altered.

Effects of time of incubation with Ox-LDL on the activation of p44 MAPK

We found that, within 5 min of incubation of cells with Ox-LDL (10 μg/ml), a significant increase (2-fold) in the activation of p^{44} MAPK occurred (Figure 3). Maximum stimulation (3-fold) in the activation of p^{44} MAPK, as compared to the control, occurred 10 min after the incubation of cells with Ox-LDL. Thereafter, the activation of p^{44} MAPK decreased continuously up to 60 min; at the latter time point, activation was moderately below normal levels.

Phosphoamino acid analysis of p44 MAPK

To determine which amino acids in p^{44} MAPK were phosphorylated as a consequence of incubation of cells with Ox-LDL, we pursued experiments in the presence of [^{32}P]-orthophosphate. Following immunoprecipitation with MAPK antibodies and washing, the immunoprecipitates were digested with acid, and subjected to two-dimensional TLC. We found that Ox-LDL markedly increased the phosphorylation of tyrosine and threonine residues in MAPK, but not the serine residue (Figure 4B). The ratio of phosphorylated tyrosine versus threonine residues of p^{44} MAPK in the control was ~1.6:1, whereas that of p^{44} MAPK in Ox-LDL treated cells was 2:1. Moreover, we observed a ~7.2-fold and 5.5-fold increase in the phosphorylation in tyrosine and threonine residues, respectively, in p^{44} MAPK in Ox-LDL treated cells, relative to the controls (Figure 4A,4B). The incorporation of [^{32}P] in tyrosine and threonine residues in control cells was 352 c.p.m. and 223 c.p.m., respectively. In contrast, the incorporation of [^{32}P] in tyrosine and threonine in cells incubated with Ox-LDL was on the order of 2534 c.p.m. and 1223 c.p.m., respectively.

Effects of time of incubation with Ox-LDL on the activity of UDP-galactose. Glucosylceramide, β1→4 galactosyltransferase (GalT-2).

Within 2.5 min of incubation of cells with Ox-LDL (10 (μg/ml), a 2-fold increase in the activity of GalT-2 was observed (Figure 5). GalT-2 activity increased up to 2.5-fold over a 30 min time course upon incubation of cells with Ox-LDL. In comparison, the activity of GalT-2 remained unchanged in cells incubated without exogenous Ox-LDL (control).

Effects of D-PDMP and L-PDMP on the activity of p^{44} MAPK

Preincubation of cells with D-PDMP alone (an inhibitor of GalT-2) inhibited the basal activity of p^{44} MAPK on the order of ~60%, compared to the control. Moreover, it markedly abrogated the Ox-LDL mediated increase in the MAPK activity (Figure 6). In contrast, L-PDMP alone (an activator of GalT-2) stimulated the activity of p^{44} MAPK on the order of 2.5-fold, compared to the control. Incubation of cells with L-PDMP and

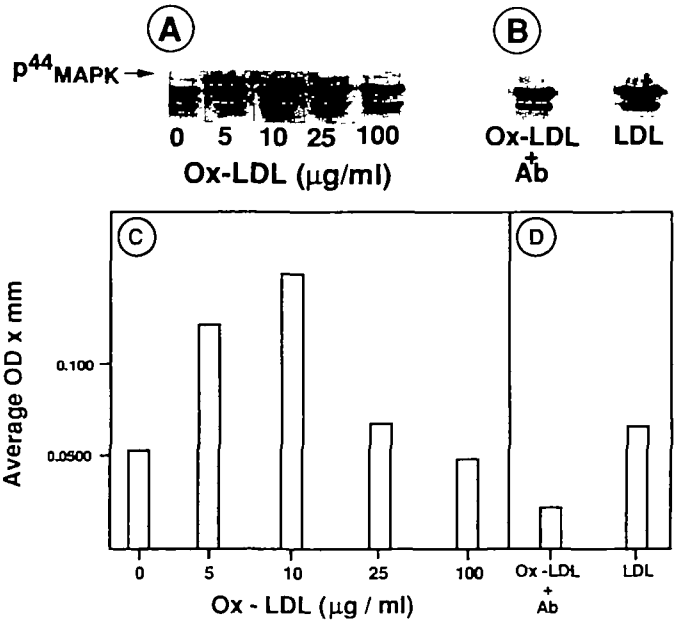

Fig. 2. Effects of Ox-LDL concentration, Ox-LDL antibody, and LDL on MAPK. Cells were incubated with the indicated concentrations of Ox-LDL (A), and Ox-LDL (10 μg/ml) plus Ox-LDL antibody; (1:200 dilution v/v), and LDL (10 μg/ml) (B). After incubation for 10 min, cells were harvested and subjected to Western immunoblot assay employing antibodies specific for p^{44} MAPK and p^{44} MAPK; arrow pointing p^{44} MAPK means phosphorylated p^{44} MAPK. (C) Following Western immunoblot assay, the gels were subjected to densitometric scanning of the phosphorylated p44 MAPK (D).

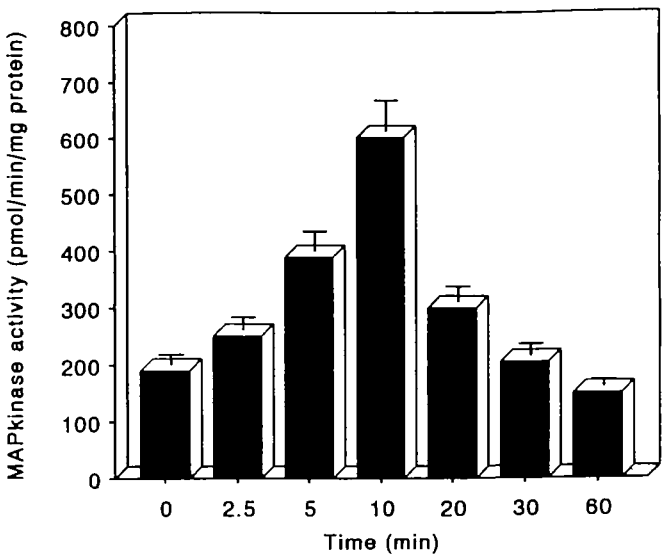

Fig. 3. Effects of time of incubation with Ox-LDL on the activity of MAP kinase in cells. Cells were treated with 10 μg/ml Ox-LDL for different time intervals. MAP kinase activity was measured after immunoprecipitation of cell lysates prepared from Ox-LDL stimulated cells as described in Figure 1. For each point, three different experiments were done.

Ox-LDL had a significant effect on the activity of p^{44} MAPK (Figure 6). We also assessed whether D-PDMP mediated suppression of Ox-LDL mediated phosphorylation of p^{44} MAPK can be reversed by LacCer. Simultaneous addition of LacCer to cells incubated with D-PDMP or D-PDMP plus Ox-LDL (Figure 6) inhibited the D-PDMP mediated suppression of activity of p^{44} MAPK. D-PDMP alone markedly inhibited the incorporation of [^{32}P] into tyrosine and threonine residues in p^{44} MAPK (Figure 4C). The incorporation of [^{32}P] into these amino acids was 58 c.p.m. and 59 c.p.m., respectively. In addition, D-PDMP suppressed the Ox-LDL mediated phosphorylation of p^{44} MAPK (Figure 4D). The incorporation of [^{32}P] into tyrosine and threonine residues in cells incubated with Ox-LDL plus D-PDMP was 345 c.p.m. and 243 c.p.m., respectively.

Effects of Ox-LDL on Ras-GTP loading

Cells prelabeled with [^{32}P] were incubated with Ox-LDL (10 μg/ml). At various time points, the cell lysates were prepared and immunoprecipitated with $p21^{ras}$ antibody. The nucleotides eluted from immunoprecipitates were subjected to TLC analysis (Figure 7A) and densitometric scanning (Figure 7B). We found that, within 2.5 min, there was a 9-fold increase in Ras GTP loading that decreased to 4-fold in 5 min, compared to the controls (Figure 7B). After 30 min of incubation of cells with Ox-LDL, a near basal level of GTP radioactivity was associated with Ras.

Effects of Ox-LDL on the expression of proto-oncogenes. c-fos, c-myc, c-jun, and MAPK phosphatase

Northern assays (Figure 8A) and densitometric scanning analysis (Figure 8B) revealed that after 1 h of incubation of cells with (10 μg/ml Ox-LDL, the level of c-*fos* was increased by 6.5-fold, compared to the control. Ox-LDL did not alter the mRNA levels of c-*myc* and c-*jun* (Figure 8A,B), or MAPK phosphatase (data not shown).

Discussion

Our studies generated several novel findings. First, we found that Ox-LDL specifically stimulated the phosphorylation of p^{44} MAPK, whereas LDL did not. Second, Ox-LDL stimulated the activity of GalT-2 in aortic smooth muscle cells (A-SMC). In contrast, D-PDMP, an inhibitor of GalT-2, markedly abrogated Ox-LDL mediated induction in the phosphorylation of p^{44} MAPK. This phenomenon was stopped by the simultaneous addition of LacCer. Third, Ox-LDL stimulated Ras GTP loading and c-*fos* proto-oncogene mRNA levels in A-SMC.

Our laboratory was the first to report that Ox-LDL exerts a concentration-dependent stimulation in the proliferation of aortic smooth muscle cells (Chatterjee, 1992; Chatterjee and Ghosh, 1996). Since then, several other laboratories have reported similar observations (Auge *et al.*, 1995; Chai *et al.*, 1995, Turquiah *et al.*, 1995). Using 10 μg Ox-LDL/ml, we have repeatedly observed a 2- to 3-fold stimulation in A-SMC proliferation. In comparison, epidermal growth factor and platelet derived growth factor (0.04 ng) stimulated A-SMC proliferation on the order of 2.5- and 6-fold, respectively (unpublished observations). Thus, the extent of cell proliferation induced by Ox-LDL is within the range of cell proliferation induced by these growth factors.

We previously reported that Ox-LDL can specifically induce the endogenous activity of lactosylceramide synthase (GalT-2), but not glucosylceramide synthase (GlcT-1) (Chatterjee and

Fig. 4. Phosphoamino acid analysis of p44 MAP kinase by two-dimensional thin layer chromatography. [^{32}P]-labeled cells were incubated + Ox-LDL (10 μg/ml) and D-PDMP + Ox-LDL, and immunoprecipitated with the use of p44 MAPK antibody. The immunoprecipitates were digested with 6N HCl. The solvents used in the first and second dimensions were: butanol: pyridine:acetic acid:water (13:10:2:8, by volume), and butanol:pyridine:acetic acid:water (15:10:3:12, by volume), respectively. Various nonradioactive phosphoamino acids were added to the digest and two-dimensional chromatographic separation of phospho amino acids was pursued. Phosphoamino acids were detected by ninhydrin staining. (**A**) Control; (**B**) Ox-LDL (10 μg/ml) treated cells; (**C**) D-PDMP (10 μM) treated cells; and (**D**) Ox-LDL (10 μg/ml) plus D-PDMP (10 μM) treated cells. PY, Phosphotyrosine; PT, phosphothreonine; and PS, phosphoserine.

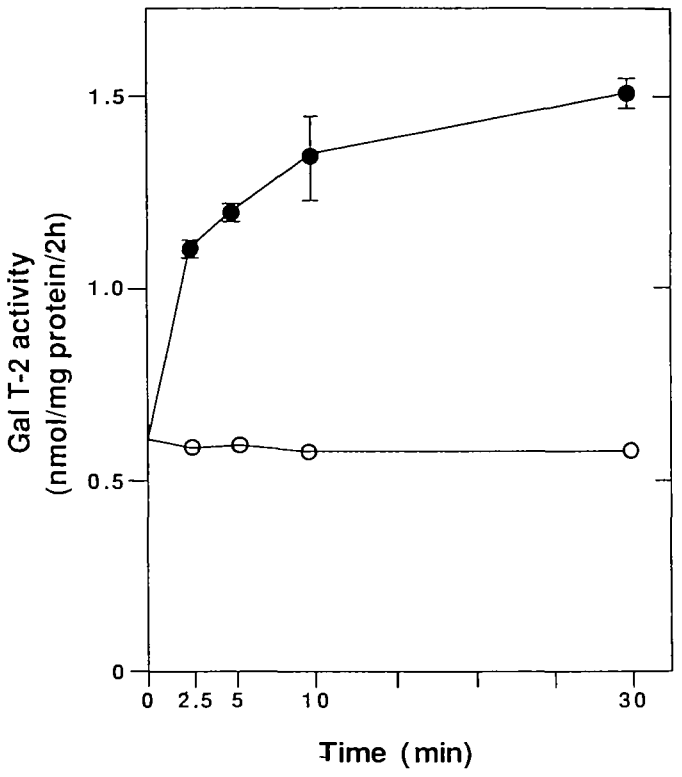

Fig. 5. Effect of Ox-LDL and LDL on the activity of UDP-galactose. glucosylceramide β1→4 galactosyltransferase (GalT-2) activity in human aortic smooth muscle cells. After incubation with Ox-LDL (10 μg/ml) cells were harvested at indicated time points, and the activity of GalT-2 was measured as described in the text. The results represent ± SD values of two experiments. ●, Ox-LDL; ○, control (without Ox-LDL).

Ghosh, 1996). In this report, we show that there is a tight correlation in the time taken to activate both GalT-2 and to phosphorylate p^{44} MAPK, i.e., about 2.5 min after the addition of Ox-LDL, compared to the control. We employed the use of endogenous inhibitors/activators of GlcT-1 and GalT-2 (Inokuchi and Radin, 1987; Chatterjee, 1991; Chatterjee and Ghosh, 1996) to investigate their effects on Ox-LDL mediated induction in p^{44} MAPK phosphorylation (Figures 4, 6). In that, we incubated cells with Ox-LDL ± D-PDMP, and Ox-LDL ± L-PDMP, respectively. D-PDMP markedly abrogated the Ox-LDL induced p^{44} MAPK activity and the phosphorylation of Thr and Tyr residues in p^{44} MAPK (Figure 4). These findings are in agreement with our previous observations that D-PDMP can impair aortic smooth muscle cell proliferation (Chatterjee, 1991; Chatterjee and Ghosh, 1996). In additional studies, we found that D-PDMP (10 μM, 4 h) decreased the cellular level of LacCer from 0.46 ± 0.4 nmol/mg protein to 0.29 ± 0.2 nmol/mg protein. Other studies in Chinese hamster ovary cells have also revealed that D-PDMP can reduce the level of glycosphingolipids markedly within 2.5 min (Rosenwald *et al.*, 1992). In contrast, L-PDMP stimulated the activity of GalT-2 (Chatterjee, 1991; Chatterjee and Ghosh, 1996), p^{44} MAPK phosphorylation, and the proliferation of aortic smooth muscle cells (Chatterjee, 1991; Chatterjee and Ghosh, 1996). We have previously shown that LacCer, but not GlcCer or ceramide, can phosphorylate p^{44} MAPK (Bhunia *et al.*, 1996). Taken together, these studies imply that the endogenous synthesis of LacCer via the activation of GalT-2 by Ox-LDL plays an important role in p^{44} MAPK phosphorylation and cell proliferation. Furthermore, GlcCer is not involved in Ox-LDL mediated induction in p^{44} MAPK phosphorylation and proliferation. However, this pathway may be bypassed by the exogenous addition of LacCer (Bhunia *et al.*, 1996). For example, preincubation of cells with LacCer, but not GlcCer, reversed D-PDMP mediated inhibition of p^{44} MAPK phosphorylation by Ox-LDL (Figure 6). A hypothetical model depicting the role of GalT-2 and LacCer in the induction of signal transduction of Ox-LDL in p^{44} MAPK activation and cell proliferation is presented in Figure 9.

Recent studies reveal that the induction of MAPKs may also be due to the inactivation of MAPK phosphatases via generation of oxygen free radicals and hydrogen peroxide in neutrophils (Frankie *et al.*, 1992; Fialkow *et al.*, 1994). To rule out this possibility, we pursued Northern blot assays in cells incubated ± Ox-LDL and found that the mRNA level of MAPK phosphatase was similar (data not shown). Thus, our findings indicate that Ox-LDL mediated induction of p^{44} MAPK is not due to the inhibition of phosphatase activity.

The proto-oncogene $p21^{ras}$ has been identified as a key molecular switch involved in regulating cell activation triggered by various mitogens (Chen *et al.*, 1994). In its resting state, $p21^{ras}$ is in a GDP-bound state. After *in vivo* activation $p21^{ras}$, it releases GDP and binds GTP (Boguski, 1993; Bokoch, 1993). We found that a substantial increase in GTP bound

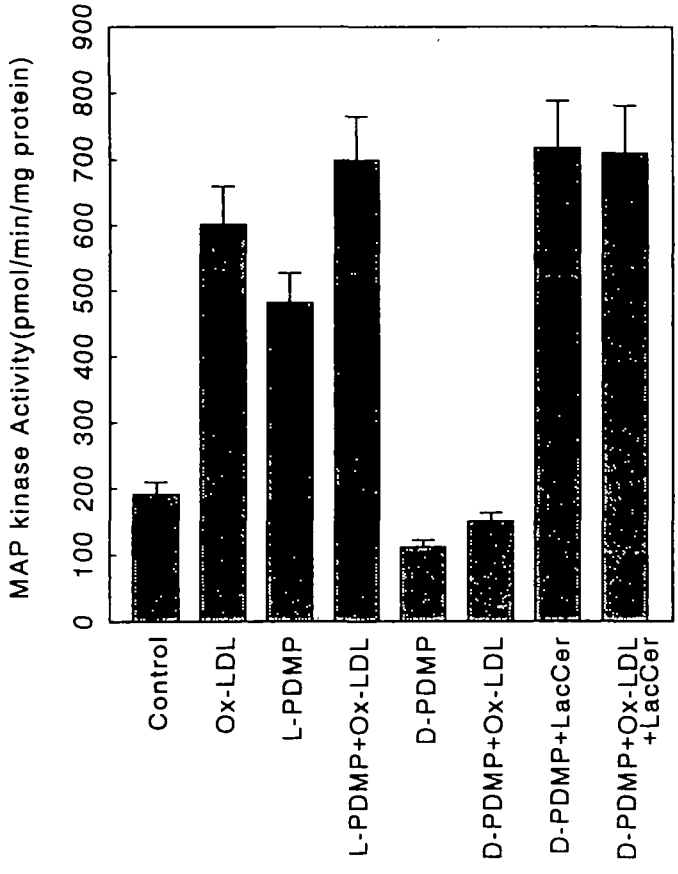

Fig. 6. Effects of D-PDMP, L-PDMP, and Ox-LDL on MAPK activity. Confluent cultures of human aortic smooth muscle cells were preincubated for 2 h with D-PDMP (10 μM) or L-PDMP (10 μM). Next, Ox-LDL (10 μg/ml) was added to the cells. After incubation for 10 min at 37°C, cells were harvested and MAP kinase activity was measured in the immunoprecipitates. The results represent +SD values of three separate experiments analyzed in duplicate.

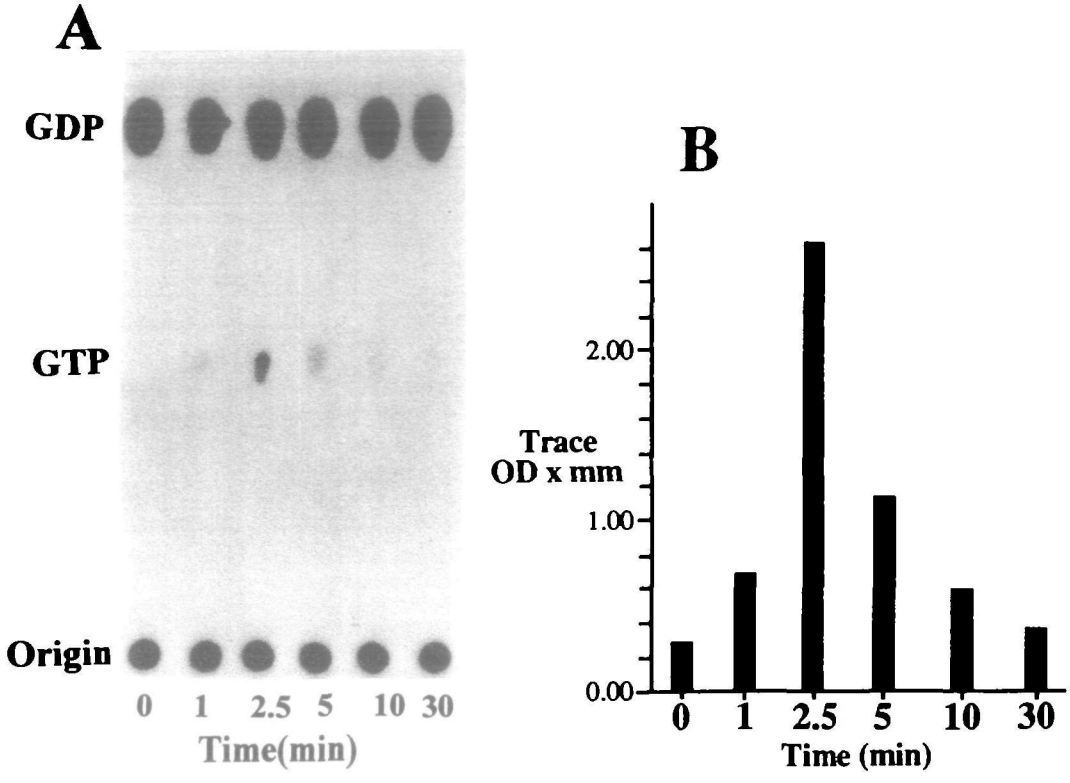

Fig. 7. Effects of Ox-LDL on $p21^{ras}$ activation in aortic smooth muscle cells. [^{32}P] labeled cells were stimulated with Ox-LDL (10 μg/ml) for different times as indicated. The cell lysate was immunoprecipitated with $p21^{ras}$ antibody. The bound nucleotides (both GTP and GDP) were eluted and separated on polyethyl-eneimine TLC plate using 0.75 M KH_2PO_4 (pH 3.4) as solvent and (**A**) subjected to autoradioagraphy, and (**B**) densitometric scanning of band representing [^{32}P] GTP.

$p21^{ras}$ occurred 2.5 min after Ox-LDL addition. Thereafter, GTP bound $p21^{ras}$ was decreased. This time-dependent increase of $p21^{ras}$ GTP loading suggests that Ox-LDL activates p^{44} MAPK via the activation of $p21^{ras}$. Recently, we found that LacCer-induced $p21^{ras}$ GTP is rather transient (1 min) (Bhunia *et al.*, 1996). These findings, taken together, may suggest that first Ox-LDL activates GalT-2, and then LacCer is produced. The latter, in turn, can activate Ras, and subsequently, can activate the kinase cascade involving Raf-1, MEK 2, and p^{44} MAPK (Figure 9; Bhunia *et al.*, 1996).

The induction in the expression of proto-oncogene c-*fos*, c-*jun*, and c-*myc* mRNA is an early response to various growth stimuli (Lau and Nathans, 1987) and encode for nuclear binding transcriptional factors that in turn, play a crucial role in mitogen induced cell proliferation (Holt *et al.*, 1986). To determine whether these proto-oncogenes are also involved in Ox-LDL induced A-SMC proliferation, subsequent to p^{44} MAPK activation, we measured mRNA levels of these proto-oncogenes. Only c-*fos* mRNA level increased after 1 h of Ox-LDL incubation, compared to the control. In contrast, the cellular levels of c-*jun* or c-*myc* mRNA in Ox-LDL treated and control cells were similar. This data suggests that Ox-LDL like LacCer can specifically induce c-*fos* proto-oncogene expression via activation of p^{44} MAPK and induces cell proliferation (Chatterjee, 1991, 1992; Bhunia *et al.*, 1996; Chatterjee and Ghosh, 1996).

Why Ox-LDL and LacCer induce the phosphorylation of p^{44} MAPK and c-*fos* mRNA expression is not clearly understood from our studies. Previously, fatty acids, e.g., linoleic acid and arachidonic acid were shown to stimulate the phosphorylation of both p^{42} MAPK and p^{44} MAPK (Rao *et al.*, 1994). Moreover, linoleic acid stimulates the mRNA levels of c-*fos*, c-*myc*, and c-*jun* in rat vascular smooth muscle cells (Rao *et al.*, 1995) and also in our studies with human A-SMC (Bhunia *et al.*, 1996). However, in another study (Scott-Burden *et al.*, 1989) in human smooth muscle cells from arterioles, the presence of LDL increased the expression of c-*fos* and c-*jun* but did not alter cell proliferation. The reasons for the discrepancy in this report and our findings are not clear. We can only speculate that the vascular cells from diverse origins may respond to LDL differently (Scott-Burden *et al.*, 1989; Rao *et al.*, 1995).

Since other studies have shown that the ERK group of MAP kinases phosphorylates Elk-1 and increases ternary complex formation (Gille *et al.*, 1992) and activation of c-*fos* downstream, it is possible that Ox-LDL, via activating GalT-2, may employ LacCer as a second messenger in mediating cell proliferation. Further work in this area is warranted to explain this phenomenon to understand the pathophysiology of Ox-LDL/LacCer mediated A-SMC proliferation in atherosclerosis.

Materials and methods

Isotopes

[γ-^{32}P] ATP (6000 mCi/mmol) and [α-^{32}P] dCTP (3000 Ci/mmol) were purchased from Amersham Life Science Inc., and [^{32}P]-orthophosphoric acid (H_3PO_4; carrier-free) was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO).

Chemicals

All standard cultured reagents were supplied by Gibco-BRL. Myelin Basic Protein (MBP) substrate peptide (APR TPGG RR), specific for MAP kinase,

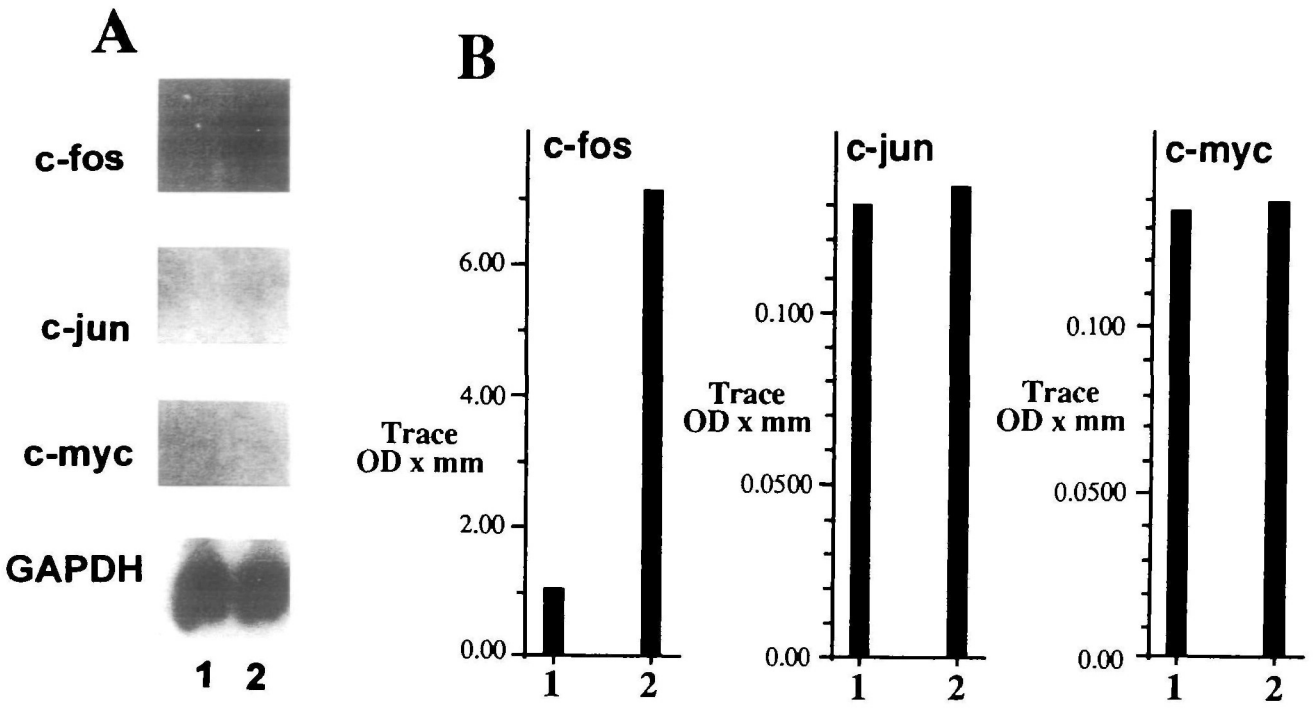

Fig. 8. Northern blot analysis of the expression of proto-oncogenes mRNAs in aortic smooth muscle cells stimulated by LacCer. Aortic smooth muscle cells were incubated with 10 μM LacCer for 1 h and total cellular RNA was isolated. (**A**) Twenty micrograms of total RNA were analyzed by Northern blotting for c-fos, c-jun, c-myc and GAPDH transcripts using respective [^{32}P]-labeled cDNA probes. Analysis of total cellular RNA for each condition from three separate experiments provided similar results. (**B**) Densitometric scanning of bands representing various proto-oncogenes.

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). cDNAs for c-*fos* and c-*jun* were a generous gift from Prof. Daniel Nathans, and c-myc was a gift from Dr. Chi Van Dang at The Johns Hopkins University. cDNA for GAPDH was a gift from Dr. D. Dewitt, Department of Biochemistry, Michigan State University. The polyethyleneimine TLC plates were purchased from E.M. Separations, Gibbstown, NJ. cDNA for MAP kinase phosphatase-I, 3CH134 was a gift from Dr. Jennifer L. Duff (University of Washington, Seattle, WA). D-PDMP and L-*threo*-PDMP were purchased from Matreya, PA. Human aortic smooth muscle cells were a gift from Dr. Cecilia Giachelli (University of Washington, Seattle, WA). Antibodies against oxidized LDL were obtained as a gift from Dr. Joseph Witzum (University of California, San Diego, CA).

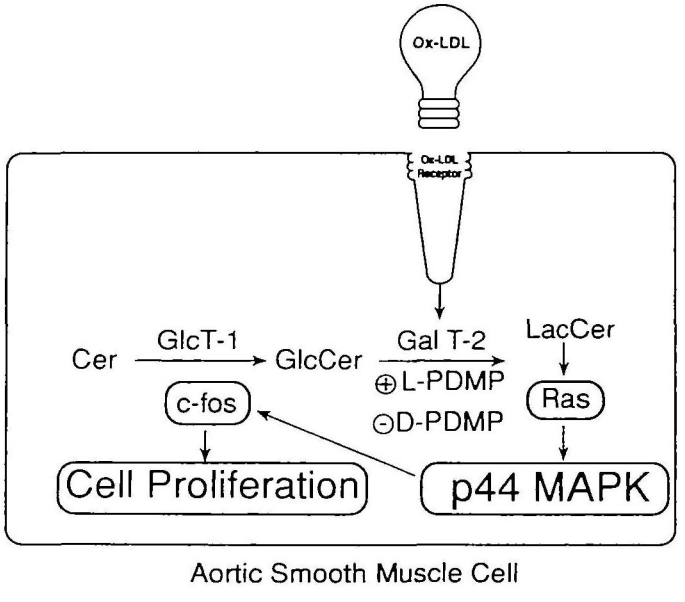

Fig. 9. Hypothetical model depicting the role of UDP-galactose: glucosylceramide β1→4 galactosyltransferase (GalT-2), and lactosylceramide in the induction of signal transduction of oxidized LDL in p^{44} mitogen-activated protein kinase and proliferation in human aortic smooth muscle cells.

Lipoproteins

Human plasma LDL (d 1.068 g/dl) and HDL (d 1.112 g/dl) were prepared from pooled plasma from normal lipidemic volunteers (Havel *et al.*, 1955). Both LDL and HDL were oxidized by dialysis against 5 μM $CuSO_4$ solution in phosphate-buffered saline and characterized as described previously (Chatterjee, 1992).

Cells

Human aortic smooth muscle cells human A-SMC (H-ASMC) 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 (1971).

Incubation of cells with lipoproteins

Cells ($\times 10^5$) were seeded in 100 × 15 mm^2 plastic petri dishes in the above growth medium. Fresh medium were added every 3 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 Ham's F-10 medium was added to each plate. After priming the cells for 2 h in this medium, LDL, Ox-LDL, and Ox-HDL were added. Vehicle alone was added to control dishes. 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 × *g*; 10 min at 4°C), and the pellets were stored frozen at −20°C until further analysis.

Immunoprecipitation of MAP kinase

Human-SMC 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 (g/ml aprotinin, 10 (g/ml leupeptin, 1 mM pepstatin, 25 mM Tris–HCl, pH 7.4, 1% Triton X-100, and 0.5% Nonidet P-40 (Laird *et al.*, 1995). The lysate was centrifuged and the supernatant was incubated with 4 μg of anti-MAP kinase antibody conjugated with protein A/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. A portion of the immunoprecipitate was boiled for 5 min in Laemmli sample buffer (Laemmli, 1970), rapidly sedimented, and the supernatant was analyzed by SDS–PAGE. Another set of 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 (Clark-Lewis *et al.*, 1991). The assay was performed with 2–3 μg protein in a final volume of 25 μl containing 1 mg/ml MBP, 50 μM [γ-^{32}P] ATP (1800 c.p.m./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 at 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 Whatmann 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 with the use of BSA as standard (Lowry *et al.*, 1951). Next, the gel was photographed and subjected to densitometric scanning with the use of Protein-DNA densitometer instrument (PDI, Boston).

Western blot analysis

Following incubation ± agonists/antagonists, human aortic smooth muscle cells were washed *in situ* with ice-cold phosphate-buffered saline containing 1 mM Na_3VO_4, and then lysed in a buffer containing 20 mM MOPS buffer, pH 7.2, 5 mM EGTA (v/v), and Nonidet P-40 for 20 min at 4°C. The detergent-insoluble material was separated by a centrifuge (10,000 × *g*, 15 min, 4°C), and the soluble supernatant fraction was used as the enzyme source. The enzyme preparation (40 μg/well protein) was subjected to electrophoresis by a 12.5% SDS–PAGE under both reducing and denaturing conditions along with low molecular weight standard markers (Bio-Rad). The protein was then transferred onto a polyvinylidine (PVDF) membrane, and probed with anti-MAPK antibody as described earlier (Chatterjee and Ghosh, 1989; Bhunia *et al.*, 1996).

Phosphoamino acid analysis of p^{44} MAPK

Human aortic smooth muscle cells were metabolically labeled with [^{32}P]-orthophosphoric acid (H_3PO_4) as described previously (Sengupta *et al.*, 1988). Briefly, cells were incubated for 90 min in phosphate-free DMEM, and subsequently labeled by incubation for 4 h with 3 mCi/ml carrier-free O-[^{32}P]-phosphate. Cell lysis and immunoprecipitation of p^{44} MAPK were performed as described above. [^{32}P]-labeled p^{44} MAPK immunoprecipitates were digested with 6 N HCl at 110°C under vacuum for 1 h. Phosphoamino acids were separated by two-dimensional thin layer cellulose chromatography together with unlabeled DL-phosphoserine, DL-O-phosphothreonine, and DL-phosphotyrosine (1 mg/ml). The solvents used for the first and second dimensions were butanol: pyridine:acetic acid:water (13:10:2:8) (v/v), and butanol: pyridine:acetic acid and water (15:10:3:12) (v/v), respectively (Boyle *et al.*, 1991). Following development, the chromatograms were stained with Ninhydrin reagent and subjected to autoradiography at −70°C for 6 days to identify the labeled phosphoamino acid.

Ras activation assay

A-SMC were labeled with [^{32}P]-orthophosphate in phosphate free media for 16 h as described and incubated with 10 μM LacCer. At various time points, cells were lysed in RIPA lysis buffer (Laird *et al.*, 1995). The whole cell lysates were immunoprecipitated with anti-human p21^{ras} antibody. Immunoprecipitates were washed with 8 × 1 ml of 50 mM HEPES (pH 7.4), 500 mM NaCl, 5 mM $MgCl_2$, 0.1% Triton X-100, and 0.005% SDS. Nucleotides (GTP and GDP) associated with Ras were eluted with 2 mM EDTA, 2 mM DTT, 0.2% SDS, 0.5 mM GTP, 0.5 mM GDP at 68°C for 20 min (Warne *et al.*, 1993). The eluted nucleotides were separated on polyethyleneimine TLC plates using 0.75 M KH_2PO_4 (pH 3.4) as a solvent and exposed to x-ray film.

*Northern blot analysis of c-*fos*, c-*jun*, and c-*myc *genes expression and MAPK phosphatase*

A-SMC were grown in p100 dish in 8 ml of complete Dulbecco's minimum essential medium (DMEM) supplemented with 10% serum and antibiotics. When the cells were sparsely confluent (80%) fresh medium containing 0.5% serum was added and incubation was continued for 72 h. Fresh F10 medium then was added, and 2 h later, cells were incubated for 1 h with and without linoleic acid (20 μM) and Ox-LDL (10 μg/ml). The total RNA was isolated by modified acid guanidinium thiocyanate-phenol-chloroform extraction method (Duff *et al.*, 1993) and 20 μg of total RNA were separated by electrophoresis on a 1% formaldehyde gel (25 mM MOPS (pH 7.8), 1 mM EDTA, 1% (wt/vol) formaldehyde), transferred to Zeta-probe blotting membrane (Bio-Rad) by alkaline blotting in transfer buffer (pH 12.0) containing 3 M NaCl, 8 mM NaOH, 2 mM sodium lauryl sarcosine) overnight. Next, 1.25 kb of HindII and EcoRI fragment of human c-*fos* cDNA, 2.6 kb of EcoRI fragment of mouse c-*jun* cDNA and 1.8 kb of EcoRI fragment of human c-myc cDNAs were labeled with [α-^{32}P]-dCTP using random labeling method according to the instructions provided by the manufacturer (Bethesda Research Laboratory). The membranes were prehybridized in prehybridization buffer (50% formamide, 5× SSC, 5× Denhardt's reagent, 50 mM sodium phosphate, 250 μg/ml salmon sperm DNA) at 42°C for 2 h and hybridized in hybridization buffer (prehybridization buffer with 10% (wt/vol) dextran sulfate and appropriate probe, 2 × 106 c.p.m./ml) at 42°C overnight. The blots were washed with wash buffer I (2 × SSC, 0.2% SDS) 2× at room temperature, 15 each time. Then the blots were washed with buffer-II (0.1 × SSC, 0.1% SDS) 2× at 37°C, 20 each time prior to autoradiography. As a control, the blot was stripped off and reprobed with labeled cDNA for GAPDH and photographed.

Measurement of UDP-galactose:glucosylceramide, β1→4 galactosyltransferase (GalT-2) activity

The activity of GalT-2 in cells incubated + Ox-LDL was measured employing UDP-[^{14}C]-galactose as nucleotide sugar donor and glucosylceramide as an acceptor as described previously (Chatterjee and Castiglione, 1987). Briefly, the GalT-2 assay mixture contained 5–100 μg of 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 mmol of UDP-galactose. Assays without exogenous GlcCer served as blanks (approximately 20–30 d.p.m.) 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, vol/vol) and 5 μg of human kidney GSL 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 (unless otherwise specified) were cut, and radioactivity was measured in a Beckman LS-3800 scintillation spectrometer using the background subtract setting and automatic quench setting, as well as a 2.00 setting for the 2 σ statistical error.

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Abbreviations

GSL, glycosphingolipid; MOPS, (3-[N-morpholino] propanesulfonic acid); ERK, extracellular signal regulated kinase; MAPK; mitogen activated protein kinase; p^{44} MAPK, phosphorylated p^{44} MAPK, p^{42} MAPK, phosphorylated p^{42} MAPK; MEK; MAPK kinase, Raf-1, Rous sarcoma associated factor-1; L-PDMP, L-1-phenyl-2-decanoylamino-3-morpholino-1-propanol; D-PDMP, D isoform of PDMP; MBP, myelin basic protein; PMSF, phenylmethylsulfonyl fluoride; GAPDH, glyceraldehyde 3-phospho dehydrogenase. Ox-LDL, oxidized low density lipoproteins; A-SMC, aortic smooth muscle cells; LacCer, lactosylceramide; GalT-2, UDP-Gal:glucosylceramide β1→4 galactosylceramide; and GlcT-1, UDP-glucose:ceramide β1→4 glucosyltransferase.

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