Lactosylceramide Stimulates Ras-GTP Loading, Kinases (MEK, Raf), p44 Mitogen-activated Protein Kinase, and c-fos Expression in Human Aortic Smooth Muscle Cells*

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Previously, our laboratory has shown that lactosylceramide (LacCer) can serve as a mitogenic agent in the proliferation of aortic smooth muscle cells “a hallmark in the pathogenesis of atherosclerosis” (Chatterjee) Biochem. Biophys. Res. Commun. 181, 554–561. Here we report a novel aspect of LacCer-mediated signal transduction. We demonstrate that LacCer (10 μM) can stimulate the phosphorylation of mitogen-activated protein (MAP) kinase p44MAPK to phosphorylated p44MAPK in aortic smooth muscle cells from rabbit or human origin. Western immunoblot assays and direct measurement of activity in immunoprecipitated MAP kinase revealed that within 5 min of incubation of cells with LacCer there was a 3.5-fold increase in the activity of p44MAPK. This continued up to 10 min of incubation; thereafter, the MAP kinase activity decreased in these cells. Phosphoamino acid analysis revealed that the tyrosine and threonine moieties of p44MAPK was phosphorylated by LacCer. Incubation of cells with ceramide and glucosylceramide did not significantly stimulate p44MAPK activity. Preincubation with tyrphostin (20 μM; a potent and specific inhibitor of tyrosine kinase) markedly inhibited the LacCer mediated stimulation in p44MAPK activity. Next we investigated the upstream and downstream parameters in MAP kinase signaling pathways. We found that lactosylceramide stimulated (7-fold) the loading of GTP on Ras. Concomitantly, LacCer stimulated the phosphorylation of MAP kinase kinases (MEK) and Raf within 2.5 min. Lactosylceramide specifically induced c-fos mRNA expression (3-fold) in these cells as compared to control. In summary, one of the biochemical mechanisms in LacCer mediated induction in the proliferation of aortic smooth muscle cells may involve Ras-GTP loading, activation of the kinase cascade (MEK, Raf, p44MAPK), and c-fos expression.

Glycosphingolipids are minor, yet integral, constituents of cell membranes. Glycosphingolipids and its metabolic products have been recently shown to play critical roles as bioregulators of a variety of processes such as cell proliferation (2, 3), cell mobility (4), and programmed cell death (apoptosis) (5). Previously a ganglioside, GM3, was shown to inhibit the phosphorylation of epidermal growth factor. That may in part, contribute to the inhibition of cell proliferation by this compound (3). We found that lactosylceramide (LacCer) exhibited a time and concentration-dependent proliferation of aortic smooth muscle cells (2). Since proliferation of smooth muscle cells is considered a hallmark in the pathogenesis of atherosclerosis, we measured the level of LacCer and other glycosphingolipids in human subjects who had this disease. We found that the level of glucosylceramide (GlcCer) and LacCer were markedly elevated in the plaque and calcified plaque compared to unaffected aorta from patients who died from atherosclerosis at The Johns Hopkins Hospital. Moreover, LacCer derived from plaque was at least 2-fold more potent in stimulating the proliferation of smooth muscle cells compared to LacCer from unaffected aorta tissues. Although our findings may suggest an important role for LacCer in cell proliferation, the mitogenic signaling events predicted by this lipid is not clear.

The best known mitogen activated protein (MAP) kinases are: p44MAPK (extra cellular signal regulated kinase, ERK1), and p42MAPK (ERK2). These are a group of serine/threonine protein kinases that constitute an activation process triggered by a variety of growth stimuli (6–10). Such protein kinases have been shown to phosphorylate and activate transcription factors such as c-myc (11–13), c-fos (14), and p62TCF (15, 16), which regulates the expression of genes essential for cell proliferation (17). MAP kinase kinase (MAPK-K) identified as a dual-specificity kinase, are involved in the activation and phosphorylation of the tyrosine and threonine residues in ERKs (MAP kinases) within the TEF motif in the conserved domain VIII (18). MAPK-K is a substrate for Rous sarcoma associated factor (Raf-1), a serine-threonine kinase (19). The latter has been shown to integrate the signaling of various receptor tyrosine kinases (9): G-protein coupled receptors (20, 21), upstream serine-threonine kinase, for example, protein kinase C, and other kinases (9).

In this paper, we present evidence that LacCer specifically activates the phosphorylation of p44MAPK in cultured human aortic smooth muscle cells. Upstream activators, Ras/Raf/MEK, are involved in this signal cascade. Concomitantly, LacCer specifically stimulated c-fos proto-oncogene expression. Such a combination of biochemical pathways may delineate signaling events involved in LacCer mediated induction in aortic smooth muscle cell proliferation.

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1The abbreviations used are: GMm, monosialoganglioside; LacCer, lactosylceramide; GlcCer, glucosylceramide; Raf, Rous sarcoma associated factor; MOPS, 3-(N-morpholino)propanesulfonic acid; ERK, extracellular signal regulated kinase; MAPK, mitogen-activated protein kinase; p44MAPK, phosphorylated p44MAPK; ERK2, p42MAPK, phosphorylated p42MAPK; GAPDH, glyceraldehyde-3-phosphodehydrogenase; A-SMC, aortic smooth cells; PAGE, polyacrylamide gel electrophoresis.
2S. Chatterjee, S. Dey, W. Y. Shi, K. Thomas, and G. Hutchins, personal communication.
MATERIALS AND METHODS

Isotopes—[γ-32P]ATP (6000 Ci/mmol) and [α-32P]CTP (3000 Ci/mmol) were purchased from Amersham Life Science Inc. and [32P]orthophosphoric acid (H3PO4) (carrier-free) was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO).

Chemicals—All standard culture reagents were supplied by Life Technologies, Inc. Tyrophostin AG-126 was obtained from Calbiochem (San Diego, CA). Myelin basic protein substrate peptide (APR TPGG RR), specific for MAP kinase, anti-MAP kinase (ERK-T) polyclonal (for mouse, rat, and human systems), anti-Raf-1, anti-MEK, and anti-p21ras2 antibody were obtained from Upstate Biotechnology Inc. (Lake Placid, NY). Glycophosphoinositol and other standard reagents were obtained from Sigma. 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. The polyethyleneimine TLC plates were purchased from E.M. Separations, Gibbstown, N.J.

Cells—Human aortic smooth muscle cells (A-SCM) were prepared and cultured in minimum essential medium supplemented with 10% fetal calf serum/penicillin/streptomycin, 100 units/ml, and 0.2% L-glutamine. Free antibody, specific for p44MAPK and p42MAPK (for mouse, rat, and human), were purchased from Transduction Laboratories. The membranes were prehybridized in prehybridization solution (50 mM sodium phosphate, 250 mM sodium chloride, 5 mM MgCl2, 0.1% sodium dodecyl sulfate, and 0.1% SDS) twice at room temperature, 15 min each time. Then the blots were washed with buffer II (0.1 times ISCC, 0.1% SDS) twice at 65°C, 20 min each time prior to autoradiography. As a control, the

centrifugation (10,000 × g, 15 min, 4°C), and the soluble supernatant fraction was used as enzyme source. The enzyme preparation (40 µg) was subjected to 12.5% SDS-PAGE. The protein was then transferred electrophoretically onto a polyvinylidine difluoride membrane for 1 h at 50 V and 52 mA/gel at room temperature (26). Next, the membrane was blocked for 1 h with 3% non-fat dry milk in TBS-T (10 mM Tris-HCl and 0.1% Tween 20, pH 8.0) and then incubated overnight at 4°C with the appropriate primary antibody. Next, the membrane was incubated for 2 h with the secondary antibody (alkaline phosphatase-conjugated anti-rabbit IgG) after washing four times with TBS-T. Membrane was then washed and incubated in the dark for 3–6 min with the substrate 5-bromo-4-chloro-3-indolyphosphate (100 µM) and nitroblue tetrazolium. The reaction was terminated by rinsing the membrane with distilled water.

Phosphatase Activity Analysis of p44MAPK—A-SCM were metabolically labeled with [32P]orthophosphoric acid (H3PO4) as described previously (27). Briefly, cells were incubated for 90 min in phosphate-free Dulbecco’s minimal essential medium, and subsequently labeled by incubation for 16 h with 3 µCi/ml carrier-free [32P]orthophosphoric acid. The labeled cells were stimulated by incubation with LacCer (10 µM) for 5 min. Cell lysis and immunoprecipitation of p44MAPK were performed as described above. [32P]-Labeled p44MAPK 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 p44MAPK, α-phospho-32P-Threonine, and α-phospho-32P-tyrosine. 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/water (15:10:3:12) (v/v), respectively (28). 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.

Raf-1 and MEK Assay—A-SCM were incubated with 10 µM LacCer and harvested at different time periods. Whole cell lysates were prepared as described above. Forty micrograms of total cell protein were separated by 10% SDS-PAGE and electrophoretically transferred onto polyvinylidine difluoride membrane. Next, the membrane was incubated with Raf-1 antibody overnight at 4°C. For the MEK assay, total cell proteins were separated by 12.5% SDS-PAGE and blotted with MEK antibody overnight at 4°C.

Ras Activation Assay—A-SCM were labeled with [3H]phosphatidate for 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 (23). The whole cell lysates were immunoprecipitated with anti-p21ras antibody. Immunoprecipitates were separated on polyethyleneimine TLC plates using 0.75M KH2PO4 (pH 3.4) as a solvent and exposed to x-ray film.

Northern Blot Analysis—c-fos, c-jun, and c-myc Gene Expression—A-SCM were grown in a p100 × 20-mm dish in 8 ml of complete Dulbecco’s minimum essential medium supplemented with 10% serum with antibiotics. When the cells were sparsely confluent (~80%) fresh medium containing 0.5% serum was added and incubation continued for 72 h. Then the fresh F-10 medium was added and 2 h later cells were incubated for 1 h with and without linoleic acid (20 µM) and LacCer (10 µM). The total RNA was isolated by the modified acid guanidinium thiocyanate-phenol-chloroform extraction method (29) and 40 µg of total RNA were separated by electrophoresis on 1% formaldehyde gel. The gels were exposed to x-ray film.
RESULTS

Effects of Concentration of LacCer, GlcCer, and Ceramide on the Activity of p44MAPK—

LacCer exerted a concentration-dependent stimulation in the phosphorylation/activation of p44MAPK (Fig. 1). Maximum stimulation; 3.5-fold in the activity of MAPK occurred with 10 μM LacCer. At a higher concentration of LacCer (50 μM) the induction of phosphorylation of MAPK was on the order of 2-fold compared to control. In contrast, incubation of cells with similar concentrations of either GlcCer or Cer did not appreciably stimulate the phosphorylation of p44MAPK (Fig. 1).

Effects of Time of Incubation with LacCer on MAPKs—

Western immunoblot assays (Fig. 2A) followed by densitometric analysis (Fig. 2B) revealed that incubation of cells with LacCer (10 μM) exerted a nearly 3-fold increase in the phosphorylation of p44MAPK. In contrast, LacCer did not phosphorylate p42MAPK. Immunocomplex kinase assay showed that within 2.5 min of incubation of cells with LacCer (10 μM) a significant increase (2.5-fold) in the activation of p44MAPK occurred (Fig. 2C). Maximum stimulation, 3.5-fold compared to control, occurred 5 min after the incubation of cells with LacCer. Thereafter, phosphorylation decreased continually up to 60 min when it was near normal levels.

Phosphoamino Acid Analysis of p44MAPK—To determine which amino acids in p44MAPK were phosphorylated as a consequence of incubation with cells with LacCer, we pursued the experiments in the presence of [32P]orthophosphate. Following immunoprecipitation with MAPK antibodies and washing, the immunoprecipitates were digested with acid, neutralized, and subjected to two-dimensional TLC. We found that LacCer markedly increased the phosphorylation of tyrosine and threonine residues in p44MAPK, but not the serine residue (Fig. 3). The ratio of phosphorylation of tyrosine versus threonine residues in p44MAPK in control was 1.6:1. Whereas, the labeling ratio of tyrosine versus threonine residues in p44MAPK in LacCer treated cells was on the order of 1.8:1. Moreover, we observed a 8.5-fold and 7.5-fold increase in the phosphorylation in tyrosine and threonine residues, respectively, in p44MAPK in LacCer-treated cells as compared to control.

Effects of LacCer on MAPK Phosphatase—To ascertain that the increase in the phosphorylation of p44MAPK in cells incubated with LacCer was not due to the inhibition of MAPK phosphatase, we pursued Northern assays in cells incubated with and without LacCer. The level of mRNA for MAPK phosphatase remained unchanged in cells incubated with or without LacCer (data not shown).

Effects of Tyrphostin on MAPK Activity—When cells were incubated with tyrphostin, a potent inhibitor of tyrosine kinase upstream of MAPK, it inhibited the LacCer (10–20 μM) induced phosphorylation of MAPK (Fig. 4). Such findings suggest that tyrosine kinase is involved in LacCer-mediated signal transduction cascade.

Effects of LacCer on Ras-GTP Loading—Cells prelabeled with 32P were incubated with LacCer as described earlier. At various time points, the cell lysates were prepared and immu-
noprecipitated with p21ras antibody. The nucleotides eluted from immunoprecipitates were subjected to TLC analysis (Fig. 5A). We found that within 1 min there was a 6-fold increase in Ras-GTP loading that decreased to 3-fold in 2.5 min as compared to control (Fig. 5B). After 30 min of incubation of cells with LacCer, significant GTP radioactivity was still associated with Ras.

Effects of LacCer on the Phosphorylation of Raf—The effects of time of incubation of cells, with LacCer on the phosphorylation of Raf-1 was assessed by Western immunoblot assay. We observed that 2.5 min after incubation of cells with LacCer, there was an appearance of a band with reduced mobility (presumably the phosphorylated form of Raf) compared to Raf (Fig. 6). A similar observation was made at 5 min after incubation of cells with LacCer thereafter, the phosphorylated form of Raf was not observed.

Effects of LacCer on the Phosphorylation of MEK—Western immunoblot assays employing MEK2 antibody revealed that LacCer exerted a time-dependent increase in the phosphorylation of MEK2 (Fig. 7). This was evidenced by a shift in the mobility of a MEK2 antibody recognizible band (Fig. 7A). Densitometric analysis of the gel revealed that after 2.5 min of incubation of cells with LacCer an increase in MEK2 phospho-tyrosine was observed 3-fold occurred. Maximum increase in MEK2 (4-fold) occurred 5 min after incubation of cells with LacCer as compared to control (Fig. 7B). Ten minutes after incubation of cells with LacCer there was ~2-fold higher activity of MEK kinase as compared to control.

Effects of LacCer on the Expression of Proto-oncogenes: c-fos, c-myc, and c-jun—Northern assays revealed that after 1 h of incubation of cells with LacCer for 10-μM LacCer for 5 min. The level of c-fos, c-myc, and c-jun was increased by 3-fold (Fig. 8) as compared to control. In contrast, preincubation of cells with linoleic acid (20 μM) increased the level of c-fos, c-myc, and c-jun on the order of 6-fold, 4-fold, and 2-fold, respectively, as compared to control. The level of GAPDH remained unchanged upon incubation of cells with LacCer or linoleic acid.

DISCUSSION

Our studies generated several novel findings. First, we found that LacCer specifically stimulated the phosphorylation of p44MAPK, whereas GlcCer and Cer did not. Second, tyrphostin (tyrosine kinase inhibitor) markedly abrogated LacCer medi-
ated induction in the phosphorylation of p44\textsuperscript{MAPK}. Third, LacCer stimulated Ras-GTP loading and the phosphorylation of MEK2 and Raf. Fourth, LacCer specifically stimulated the mRNA level of c-fos proto-oncogene in A-SMC.

To delineate the signal transduction events in LacCer-mediated proliferation in human A-SMC, we examined its effects on various parameters in the signal transduction cascade under carefully controlled yet, varying conditions. The cells were grown to confluence, washed, and incubated with Ham's F-10 medium without serum for 2 h. Next, various agonists and antagonists were added. We were concerned that contamination of any reagents or cell cultures with bacteria or lipopolysaccharide may also affect the phosphorylation of MAPK (31). Accordingly, appropriate control experiments were pursued to demonstrate that lipopolysaccharide (10 \( \mu \text{g/ml} \)) did not alter the phosphorylation of either p44\textsuperscript{MAPK} or p42\textsuperscript{MAPK} in cultured human A-SMC (data not shown). Next, we analyzed the effects of LacCer and other glycosphingolipids simultaneously employing two methods, these were: 1) the measurement of MAPK activity of immunoprecipitated MAPK employing myelin basic protein fragment (APRTPGGRR) as substrate and \([32P]\text{ATP as the phosphate donor; and 2) Western immunoblot assay followed by densitometric scanning of individual gel bands. As shown in various figures, both of these approaches yielded similar results confirming the validity of our experimental techniques and the interpretation of results.}

Our studies clearly revealed that LacCer induced the phosphorylation of tyrosine and threonine residues in p44\textsuperscript{MAPK}. This phenomenon was abrogated by an inhibitor of tyrosine kinase, e.g. tyrphostin (32). However, staurosporine, an inhibitor of protein kinase C, failed to impair this process (data not shown). These findings reveal that LacCer mediated p44\textsuperscript{MAPK} phosphorylation and cell proliferation are independent of protein kinase C. This is in contrast to a previous study in which GlcCer mediated proliferation in a continuous renal cell line, Madin-Darby canine kidney, was shown to be abrogated by inhibitors of protein kinase C (33).

Recent studies from several laboratories reveal that the induction of MAPKs may also be due to inactivation of MAPK phosphatases (34). In particular, generation of free oxygen radicals and hydrogen peroxide were shown to inhibit MAPK phosphatase and consequently increase the level of p44\textsuperscript{MAPK} in neutrophils (35). To rule out this possibility, we pursued Northern blot assays in cells incubated with LacCer and found that the mRNA level of MAPK phosphatase was similar (data not shown). Thus, our findings indicate that LacCer mediated induction of p44\textsuperscript{MAPK} is not due to the inhibition of phosphatase activity.

The catabolic products of LacCer are GlcCer, Cer, sphingosine, and stearic acid. In order to determine the specificity of LacCer mediated stimulation of p44\textsuperscript{MAPK} phosphorylation, we also investigated the effects of GlcCer and Cer that are highly enriched in atherosclerotic plaque intima and media, but not in unaffected intima and media.\textsuperscript{2} We found that GlcCer and Cer did not alter p44\textsuperscript{MAPK} phosphorylation.

\( ^{3} \) S. Chatterjee and A. Snowden, unpublished observations.
The effects of LacCer on upstream regulators, for example, Ras, Raf, and MEK of MAPK, were important. The proto-oncogene p21ras has been identified as a key molecular switch involved in regulating cell activation triggered by various mitogens (29, 36, 37). In its resting state, p21ras is in a GDP-bound state. Upon in vivo activation p21ras releases GDP and binds GTP (38, 39). Immunoprecipitation of p21ras from stimulated and nonstimulated cells followed by nucleotide (both GTP and GDP) elution revealed that a substantial increase in GTP bound p21ras occurred upon LacCer treatment. The maximal effect was observed at 1 min after LacCer addition. Thereafter, GTP bound p21ras was decreased. This time dependent increase of p21ras GTP loading suggests that LacCer activates p44MAPK via the activation of p21ras. We speculate that the relatively short duration of LacCer induced p21ras GTP loading may be due to the interaction of the latter with its effector or GTPase activating protein. An investigation is required whether the LacCer mediated activation occurs by direct interaction with p21ras or indirectly through secondary factors.

Raf-1, the product of the c-Raf 1 proto-oncogene is pivotal in transmitting signals from Ras in the plasma membrane to cytosolic nuclear compartments of the cells (40, 41). The stimulation of Raf-1 activity depends on the activation of the small G-protein p21ras (42). Active GTP-ras binds to the NH2-terminal domain of Raf-1 and recruits Raf-1 to the plasma membranes, which is sufficient to cause its activation (40, 41, 43). We investigated whether Raf-1 is phosphorylated/activated after stimulation of p21ras (measured by p21ras GTP loading) by LacCer. Significant phosphorylation of Raf-1 was observed within 2.5–5 min of incubation of cells with LacCer. The activation of Raf-1 by LacCer was transient in A-SMC. Since Raf-1 activity is known to participate in the G2/G1 transition of cells (42), we may speculate that LacCer mediated Raf-1 activation may similarly help in the transition of A-SMC from the G0 phase to the G1 phase of the cells. Next, we measured the phosphorylation of MEK by LacCer to assess whether the phosphorylation of MEK1, that in turn induces the activation of p44MAPK (48). In addition, linoleic acid stimulated the mRNA levels of c-fos, c-myc, and c-jun in rat vascular smooth muscle cells (49) and also in our studies with human A-SMC. Furthermore, recent studies have shown that tumor necrosis factor-α specifically stimulate MEK1, that in turn induce the activation of p44MAPK (50). Our preliminary studies indicate that LacCer did not stimulate MEK1, instead LacCer stimulated MEK2. Thus there may be a clear dichotomy in regard to agonist specific induction of MEK2 and activation/phosphorylation of p44MAPK (ERK1). Since other studies have shown that the ERK group of MAP kinases phosphorylate Elk-1 and increases ternary complex formation (15, 16) and activation of c-fos downstream in the signaling pathway, it is possible that LacCer, like other growth factors, specifically mediates this process. This may ultimately increase transcriptional activity and DNA synthesis, subsequently cell proliferation. Further work in this area is warranted to explain this phenomenon to understand the pathophysiology of LacCer mediated A-SMC proliferation in atherosclerosis.

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