Redox-regulated Signaling by Lactosylceramide in the Proliferation of Human Aortic Smooth Muscle Cells*

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Previously, our laboratory reported that lactosylceramide (LacCer) stimulated human aortic smooth muscle cell proliferation via specific activation of p44 mitogen-activated protein kinase (MAPK) in the p21raw/Raf-1/MEK2 pathway and induced expression of the transcription factor c-fos downstream to the p44 MAPK signaling cascade (Bhunia A. K., Han, H., Snowden, A., and Chatterjee S. (1996) J. Biol. Chem. 271, 10660–10666). In the present study, we explored the role of free oxygen radicals in LacCer-mediated induction of cell proliferation. Superoxide levels were measured by the lucigenin chemiluminescence method, MAPK activity was measured by immunocomplex kinase assays, and Western blot analysis and c-fos expression were measured by Northern blot assay. We found that LacCer (10 μM) stimulates endogenous superoxide production (7-fold compared with control) in human aortic smooth muscle cells specifically by activating membrane-associated NADPH oxidase, but not NADH or xanthine oxidase. This process was inhibited by an inhibitor of NADPH oxidase, diphenylene iodonium (DPI), and by antioxidant, N-acetyl-l-cysteine (NAC) or pyrrolidine dithiocarbamate. NAC and DPI both abrogated individual steps in the signaling pathway leading to cell proliferation. For example, the p21raw-GTP loading, p44 MAPK activity, and induction of transcription factor c-fos all were inhibited by NAC and DPI as well as an antioxidant pyrrolidine dithiocarbamate or reduced glutathione (GSH). In contrast, depletion of GSH by l-buthionine (S,R)-sulfoximine up-regulated the above described signaling cascade.

In sum, LacCer, by virtue of activating NADPH oxidase, produces superoxide (a redox stress signaling molecule), which mediates cell proliferation via activation of the kinase cascade. Our findings may explain the potential role of LacCer in the pathogenesis of atherosclerosis involving the proliferation of aortic smooth muscle cells.

Glycosphingolipid (GSL)† and its metabolic products have been shown to play critical roles as bioregulators of a variety of processes such as cell proliferation (1, 2), cell mobility (3), and programmed cell death (apoptosis) (4). Lactosylceramide (LacCer), a ubiquitous GSL, has been implicated in diverse biological functions (5). For example, we have found that LacCer exhibits a time- and concentration-dependent proliferation of aortic smooth muscle cells (ASMC) (1). Because proliferation of smooth muscle cells is considered a hallmark in the pathogenesis of atherosclerosis, we previously measured the levels of LacCer and other GSLs in human subjects who had this disease. We found that the levels of LacCer and glucosylceramide were markedly higher in the plaque and calcified plaque than in unaffected aorta from patients who died from atherosclerosis at The Johns Hopkins Hospital (6). Recently, we observed that LacCer stimulated the activation of p44 mitogen-activated protein kinase (p44 MAPK) and the expression of the transcription factor c-fos (7), which perhaps regulates the genes essential for cell proliferation. Moreover, upstream activators of p44 MAPK, p21raw, Raf, and MEK2, but not MEK1, are involved in the activation of p44 MAPK by LacCer. Although our findings suggested that LacCer stimulated this kinase cascade, it is unclear whether LacCer itself or second messengers generated by LacCer are responsible for the activation of this signaling cascade.

A class of highly diffusible and ubiquitous molecules, termed reactive oxygen species, has recently been recognized to act as signaling intermediates for cytokines including interleukin-1 and tumor necrosis factor-α (8, 9). The reactive oxygen species (ROS) encompass species such as superoxide (O2·−), hydrogen peroxide, nitric oxide, and hydroxyl radicals (10). Oxidative stress, which is an excess production of ROS, plays a role in different pathological conditions such as atherosclerosis and cancer (11, 12). In addition, O2·− has numerous effects on cell function including induction of growth, regulation of kinase activity, and inactivation of endothelial derived relaxation factor, nitric oxide (13, 14). Thus superoxide and its metabolites can function as intracellular and intercellular second messengers, transducing receptor stimulation into biochemical response. Because they are very small, rapidly diffusible, and highly reactive, free radical and redox stress are now thought to participate in cellular signaling (13, 15, 16). Current evidence indicate that the different stimuli use reactive oxygen species as signaling messengers to activate transcription factors and induce gene expression (17, 18). The functional role of GSLs, particularly LacCer, in generating free oxygen radicals has not been reported to the best of our knowledge. In the present study, we found that LacCer stimulated the generation of O2·− via activation of NADPH oxidase in H-ASMC. Alteration in the redox status by the LacCer-dependent production of O2·−

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The abbreviations used are: GSL, glycosphingolipid; LacCer, lactosylceramide; MOPS, 3-(N-morpholino)propanesulfonic acid; NAC, N-acetyl-l-cysteine; SOD, superoxide dismutase; ROS, reactive oxygen species; BSO, l-buthionine (S,R)-sulfoximine; DPI, diphenylene iodonium; ASMC, aortic smooth muscle cell(s); H-ASMC, human ASMC(s);

GSE, GSH monoester; Raf, Rous sarcoma-associated factor; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; PMA, phorbol 12-myristate 13-acetate; PDTC, pyrrolidine dithiocarbamate.
stimulated the loading of GTP to Ras, p44 MAPK activation, c-fos expression, and cell proliferation.

MATERIALS AND METHODS
Isotopes and Chemicals—[γ-32P]ATP (6000 mCi/mmol), [α-32P]dCTP (3000 Ci/mmol), and [32P]orthophosphoric acid (H3PO4) (carrier-free) and [3H]thymidine were purchased from Amersham Life Science Inc. Diphenylene iodonium (DPI) obtained from LC Laboratories. Glycosphingolipids and all other chemicals were purchased from Sigma. Bovine erythrocyte membrane-derived LacCer was a gift from Prof. T. Taki (Department of Biochemistry, Tokyo Medical and Dental University, Japan). Human plaque intima-derived LacCer was prepared in our laboratories. The purity of glycosphingolipids (>99%) was assessed by high pressure liquid chromatography and/or high pressure thin layer chromatography. Anti-p21" antagonist and anti-MAPK antibody (specific for p44 MAPK and p42 MAPK) were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). cDNAs for c-fos and glyceraldehyde-3-phosphate dehydrogenase were a generous gift from Prof. Daniel Nathans (The Johns Hopkins University) and Dr. D. Dewitt (Department of Biochemistry, Michigan State University), respectively. The polyethyleneimine TLC plates were purchased from E. M. Separations (Gibbstown, NJ).

Confluent H-ASMC were prepared and cultured in minimum essential medium supplemented with 10% fetal calf serum; penicillin (100 U/ml), streptomycin (100 μg/ml), and glutamine (50 μg/ml) according to the procedure of Ross (19).

Isolation of LacCer from Human Atherosclerotic Plaque—Isolation, purification, characterization, and quantitation of LacCer from human atherosclerotic plaque intima was performed according to the published protocol in our laboratory (6).

Vehicle for Glycosphingolipids—Stock solution of LacCer were prepared in chloroform-methanol (1:2, v/v), dried under a stream of nitrogen, dissolved in dimethyl sulfoxide (Me2SO) and added to culture medium to give the desired concentrations of LacCer. Cells incubated with 0.01% Me2SO served as a control. PMA, staurosporine, or DPI stock solutions were prepared in Me2SO and stored at 20 °C until use. Aqueous solutions of NAC and allopurinol were prepared either in medium or in buffer.

Measurement of Superoxide Production in Intact Cells—Lucigenin, an acridinium compound (Sigma) that emits light upon reduction and interaction with O2•-, was used to measure O2•- production (20). Briefly, cultured H-ASMC were harvested, and cell pellets were suspended in a balanced salt solution (130 mM NaCl, 5 mM KCl, 1 mM MgCl2, 1 mM CaCl2, 55 mM phosphoric acid, and 20 mM HEPES, pH 7.4). Viability of the suspended cells as determined by the trypan blue exclusion principle was >90%. To measure O2•- production, intact cells preincubated at room temperature were added to a 96-well plate containing 10 μl of ice-cold 40% trichloroacetic acid and spotted onto p81 phosphocellulose paper. Free [γ-32P]ATP was removed by five washes (5 min each) with 1% phosphoric acid. Radioactivity was measured by liquid scintillation counting.

Western Blot Analysis—Immunocomplexes, prepared as described above, were subjected to electrophoresis in 12.5% SDS-polyacrylamide gel electrophoresis. The protein was then transferred onto a polyvinylidene membrane and blotted with anti-MAP kinase antibody as described previously (7).

Ras-GTP Loading Assay—H-ASMC were metabolically labeled with [32P]orthophosphate in phosphate-free media for 16 h as described previously (7) and incubated with 10 μM LacCer with or without antagonists. In different time points, cells were lysed, immunoprecipitation lysis buffer and cell lysates were directly used for the reaction mixture containing 50 mM phosphate buffer (pH 7.0), 1 mM EGTA, 150 mM sucrose, and 500 μM lucigenin as the electron acceptor and either 100 μM NADPH or 100 μM NADH as an electron donor. The reaction was initiated by the addition of membrane homogenate (150–200 μg of protein). Luminescence was measured as described above. In some experiments, NADPH oxidase activity was measured in MAP kinase preparations in the presence of 1 mM KCN (mitochondrial poison). Protein content was measured by the method of Lowry et al. (23) with bovine serum albumin serving as a standard.

Immunoprecipitation and MAPK Activity Assay—LacCer were lysed in 100 μl of radioimmune precipitation lysis buffer containing 50 mM NaCl, 7.5 mM EGTA, 10 mM sodium fluoride, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM pepstatin, 25 mM Tris-HCl (pH 7.4), 1% Triton X-100, and Nonidet P-40. The lysate was centrifuged and immunoprecipitated with anti-MAP kinase antibody conjugated with protein A-agarose as described earlier (7). Part of the immunocomplex was directly used for the MAP kinase activity assay (24). Briefly, 25 μl of total reaction mixture contained 1 mg/ml myelin basic protein (peptide APRTGGGRG), 50 μM [γ-32P]ATP (1800 cpm/ml), 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 MgCl2, 5 mM EGTA, 1 mM dithiothreitol, 0.5 mM Na3VO4, and 2–3 μg of immunoprecipitated protein. The reaction was initiated upon the addition of [32P]ATP for 15 min at 30 °C and terminated by the addition of 10 μl of ice-cold 40% trichloroacetic acid and spotted onto p81 phosphocellulose paper. Free [γ-32P]ATP was removed by five washes (5 min each) with 1% phosphoric acid. Radioactivity was measured by liquid scintillation counting.

Northern Blot Analysis—Immunocomplexes, prepared as described above, were subjected to electrophoresis in 12.5% SDS-polyacrylamide gel electrophoresis. The protein was then transferred onto a polyvinylidene membrane and blotted with anti-MAP kinase antibody as described previously (7).

RESULTS
Activation of Superoxide (O2•-) Production by LacCer—Intact H-ASMC were incubated with different GSLs and their constituent sugars as indicated. Only LacCer induced the generation of O2•- (Fig. 1). Other GSLs, e.g. cerebroside sulfide, and N-acetyl glucosamine have no effect on the O2•- generation in ASMC (Fig. 1) up to a concentration of 200 μM (data not shown). LacCer stimulated O2•- production in a concentration-dependent manner (Fig. 2A) as measured by the lucigenin chemiluminescence assay. Maximal generation of O2•- (7-fold increase as compared with control) was observed with 10 μM LacCer. Kinetic analysis revealed that the lucigenin reaction mixture contained 50 mM phosphate buffer (pH 7.0), 1 mM EGTA, 150 mM sucrose, and 500 μM lucigenin as the electron acceptor and either 100 μM NADPH or 100 μM NADH as an electron donor.
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**Fig. 1. Effect of different glycosphingolipids and sugars on O$_2^-$ generation in H-ASMC.** When 80–90% confluent, H-ASMC were harvested and suspended in balanced salt solution. Intact cell suspensions were placed in a 96-well black microtiter plate (Packard) for the measurement of O$_2^-$ generation by the lucigenin chemiluminescence method. In a final volume of 200 µl, the assay mixture contained a 10 µM concentration of the indicated glycosphingolipids and constituents as stimulants and 500 µM lucigenin, and after 2.5 min the rate of generation of O$_2^-$ was measured as described under “Materials and Methods.” Glucose and lactose dissolved in balanced salt solution and comparison glycosphingolipids were solubilized in Me$_2$SO. The final concentration of Me$_2$SO exposed to cells was 0.01%. Control cells of each glycosphingolipid experiment were incubated with 0.01% Me$_2$SO. Glucose; Lac, lactose; Cer, ceramide; GlcCer, glucosylceramide; LacCer, stearoyl LacCer; LacCer(P), bovine erythrocyte membrane-derived LacCer; LacCer(E), LacCer isolated from human atherosclerotic plaque. Data presented here are the average ± S.D. for six individual experiments.

Chemiluminescence signal was maximal (7-fold) at 2.5 min and then decreased to about 4.5-fold at 30 min (Fig. 2B). To examine the specific effect of LacCer in the stimulation of O$_2^-$ generation, we incubated H-ASMC with two additional sources of LacCer. Bovine erythrocyte membrane LacCer (LacCer(E)) or LacCer derived from human atherosclerotic plaque intima (LacCer(P)) (Fig. 1) both exerted a concentration- (Fig. 2C) and time- (Fig. 2D) dependent stimulation of O$_2^-$ production with similar kinetics to stearoyl LacCer (Fig. 2, A and B). To test whether the membrane integrity of cells was lost upon incubation of cells with LacCer, we measured LDH activity in the culture medium. No increase of LDH activity was observed in culture medium up to 50 µM LacCer in the incubation mixture (Fig. 2E). These data indicate that membrane integrity was intact during LacCer- (10 µM) induced O$_2^-$ generation.

**Effect of Superoxide Dismutase (SOD) and Antioxidants on LacCer-induced Superoxide Generation**—The addition of SOD (100 units/ml) to intact cells did not inhibit LacCer-mediated generation of O$_2^-$ (Fig. 3A). But NAC or PDTC, both cell-permeable antioxidants (28), abrogated lucigenin signals (O$_2^-$ production) in control cells and in cells incubated with LacCer (Fig. 3A). The addition of LacCer (10 µM) to cell homogenate (before subcellular fractionation) also generated O$_2^-$ (Fig. 3B). This phenomenon was blocked by a 10-fold excess of 100 µM of other glycosphingolipids (Fig. 3B) or 15 µM NAC or 100 µM PDTC (Fig. 3C).

**Effect of LacCer and 1-Buthionine (S,R)-Sulfoximine on the Intracellular Level of GSH in H-ASMC**—Intracellular level of GSH was decreased in a time-dependent manner by LacCer (Fig. 4A). A low level of GSH was observed upon incubation of cells with BSO (100 µg/ml) and also with BSO plus LacCer (Fig. 4A). The level of GSH was also decreased in a concentration-dependent manner in cells incubated with BSO only (Fig. 4B).

Concomitantly, the basal level of O$_2^-$ was high (1.1 nmol/mg of protein) (Fig. 4C) in cells preincubated with 100 µg/ml BSO, an inhibitor of de novo GSH synthesis, (15, 29) for 24 h. Moreover, chemiluminescence signals (O$_2^-$ production) were further increased (about 8-fold compared with control) upon incubation of BSO-preincubated cell with LacCer (Fig. 4C).

**LacCer Activates NADPH Oxidase**—At various time points, following stimulation of H-ASMC with LacCer, NADPH oxidase activity was measured in a plasma membrane preparation with NADPH as a co-factor. NADPH oxidase activity in control cells (vehicle only) was 2.9 ± 0.03 nmol/min/mg of protein but was increased 4-fold within 2.5 min, as compared with control (Fig. 5A). NADPH oxidase activity was 3-fold higher in cells incubated with LacCer at 10 min compared with control. The mitochondrial poison KCN did not inhibit LacCer-induced NADPH oxidase activity (data not shown). Preincubation of LacCer-stimulated plasma membrane preparations with 5 µM DPI, an inhibitor of NADPH oxidase (30, 31), completely abrogated LacCer stimulated NADPH oxidase activity (Fig. 5A). No NADPH oxidase activity was observed in cytosolic fraction (Fig. 5B).
due to the activation of NADPH oxidase but independent of PKC or xanthine oxidase.

Effect of LacCer on MAPK Activity/Phosphorylation in Cells Preincubated with NAC, GSE, BSO, and DPI—Western blot assay employing antibody against p42 MAPK and p44 MAPK protein kinase C in LacCer-mediated O2\textsuperscript{\textbullet} generation (Fig. 6A). The addition of staurosporine (STP), a potent inhibitor of protein kinase C, or depletion of protein kinase C by treatment of cells with PMA (100 nM) for 24 h (32, 33) failed to impair the LacCer-mediated stimulation of O2\textsuperscript{\textbullet} using the lucigenin chemiluminescence method. Cells incubated with medium only served as a control. A, NADPH oxidase activity in plasma membrane fraction of both nonstimulated (vehicle only) and LacCer-stimulated cells. In another experiment, NADPH oxidase activity was measured in both membrane fractions after preincubation with 5 mM DPI for 5 min. Con, control (NADPH oxidase activity in medium-incubated cell cytosol), †, vehicle-incubated cell membrane fraction with DPI; ‡, LacCer-stimulated membrane fraction with DPI. B, NADPH oxidase activity in cytosolic fraction. †, vehicle-incubated cell cytosol; ‡, LacCer-stimulated cell cytosol. C, NADPH oxidase activity in membrane and cytosolic fraction (●) in LacCer-stimulated cells. Con, control (NADPH oxidase activity in medium-incubated cells). NADPH oxidase activity (both membrane fraction and cytosolic fraction) of MeS\textsuperscript{SO}-(0.01%) incubated cells remained unchanged (data not shown). Each point represents the mean ± S.D. of five individual experiments.

Followed by densitometric scan (scan data not shown) revealed that LacCer specifically stimulated the phosphorylation of p44 MAPK approximately 3.5-fold as compared with control (Fig. 7A). H-ASMC pretreated with 15 mM NAC for 30 min or 100 μM

5B). No stimulation of NADH oxidase activity was observed in LacCer-stimulated cytosol or in membrane preparation (Fig. 5C). An addition of exogenous LacCer after the isolation of plasma membrane preparations of nonstimulated cells did not alter NADPH oxidase activity (data not shown). These findings suggest that LacCer induced generation of O2\textsuperscript{\textbullet} due to the activation of NADPH oxidase.

Contribution of NADH Oxidase, Xanthine Oxidase, and Protein Kinase C in LacCer-mediated O2\textsuperscript{\textbullet} Generation—To ascertain that LacCer-mediated O2\textsuperscript{\textbullet} production is due to the activation of NADPH oxidase and not NADH oxidase, we measured O2\textsuperscript{\textbullet} generation in intact cells preincubated with DPI (5 μM) prior to stimulation with LacCer. DPI completely inhibited LacCer-mediated O2\textsuperscript{\textbullet} generation (Fig. 6A). A moderate inhibition of O2\textsuperscript{\textbullet} production also occurred in control cells incubated with DPI (Fig. 6A). The addition of staurosporine (STP), a potent inhibitor of protein kinase C, or depletion of protein kinase C by treatment of cells with PMA (100 nM) for 24 h (32, 33) failed to impair the LacCer-mediated stimulation of O2\textsuperscript{\textbullet} production (Fig. 6B). The contribution of xanthine oxidase in O2\textsuperscript{\textbullet} generation in LacCer-stimulated H-ASMC was examined next. Incubation of cells with 100–200 μM allopurinol, a specific inhibitor of xanthine oxidase (34, 35), did not inhibit O2\textsuperscript{\textbullet} production by LacCer (Fig. 6C). These observations suggested that LacCer-induced O2\textsuperscript{\textbullet} generation was dependent on NADPH oxidase but independent of PKC or xanthine oxidase.

FIG. 3. Effect of SOD and antioxidants on LacCer-induced superoxide generation. A, rate of generation of O2\textsuperscript{\textbullet} in intact cells at different time intervals as indicated. Intact cell suspension was prepared as described in the legend to Fig. 1. ○, vehicle; ●, LacCer (10 μM); △, the addition of SOD (100 units/ml) to cells; ▽, incubation of cells with 10 μM LacCer and 100 units/ml SOD; ▼, preincubation of cells with 15 mM NAC for 30 min; ◀, preincubation of cells with 15 mM NAC for 30 min followed by the addition of 10 μM LacCer; ○, preincubation of cells with PDTC (100 μM) for 1 h; ●, preincubation of cells with PDTC (100 μM) for 1 h followed by the addition of 10 μM LacCer; △, generation of O2\textsuperscript{\textbullet} in cell homogenate following incubation with vehicle; ○, 10 μM LacCer (●), 100 units/ml SOD (△), or 100 units/ml SOD with 10 μM LacCer (▲). C, generation of O2\textsuperscript{\textbullet} in cell homogenate following incubation with vehicle (○), 10 μM LacCer (●), preincubation with 15 mM NAC for 30 min (△), preincubation with 15 mM NAC for 30 min followed by 10 μM LacCer addition (▲), preincubation with PDTC (100 μM) for 1 h (○), or preincubation with PDTC (100 μM) for 1 h followed by the addition of 10 μM LacCer (●). Each point represents the mean ± S.D. of four individual experiments.

FIG. 4. Effect of LacCer and BSO on the intracellular level of GSH in H-ASMC. A, cells were incubated with 10 μM LacCer, and at different time intervals the intracellular level of GSH was measured fluorometrically as described under "Materials and Methods." In another experiment, cells were preincubated with 100 μg/ml BSO for 24 h. Next, intracellular levels of GSH were measured at different time intervals following incubation with/without 10 μM LacCer. ○, control; ●, 10 μM LacCer; △, BSO; ▼, BSO and LacCer. B, cells were incubated with different concentrations of BSO, as indicated, for 24 h followed by the measurement of intracellular GSH as demonstrated above. ○, control; ●, BSO; △, the rate of generation of superoxide was measured in intact cells following incubation with vehicle (○), 10 μM LacCer (●), preincubation of BSO (100 μg/ml) for 24 h (△), or preincubation of BSO (100 μg/ml) for 24 h with 10 μM LacCer (▼). Intact cell suspensions were prepared as described in the legend to Fig. 1. Each point represents the mean ± S.D. of four individual experiments.
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**Fig. 6.** Effect of DPI, staurosporine (STP), PMA, and allopurinol on LacCer-dependent \( \Delta \psi \) generation in intact H-ASMC. **A**, cells were preincubated with or without 5 \( \mu \)M DPI for 30 min, cell suspensions were prepared as described in the legend to Fig. 1, and generation of \( \Delta \psi \) was measured by the addition of 10 \( \mu \)M LacCer. C, vehicle; \( \bigcirc \), LacCer; \( \triangle \), DPI; \( \square \), DPI with LacCer. **B**, H-ASMC were preincubated with 1 \( \mu \)g/ml STP for 1 h and 100 \( \mu \)M PMA for 24 h. C, allopurinol (100–200 \( \mu \)M) for 24 h. ■, allopurinol; ●, allopurinol and LacCer. \( \Delta \psi \) generation was measured by the addition of 10 \( \mu \)M LacCer for 2.5 min. Each point represents the mean ± S.D. of five individual experiments.

**Fig. 7.** Effect of GSH, GSE, NAC, DPI, BSO and PDTC in LacCer-induced activation/phosphorylation of p44 MAPK in H-ASMC. **A**, Western blot analysis of p44 MAPK phosphorylation. Cells were preincubated with 15 \( \mu \)M NAC for 30 min, 5 \( \mu \)M DPI for 30 min, 15 \( \mu \)M GSE for 4 h, 100 \( \mu \)M PDTC for 1 h, and 100 \( \mu \)g/ml BSO for 24 h, followed by stimulation with 10 \( \mu \)M LacCer for 5 min. Next, cells were harvested, lysed, immunoprecipitated, run on 12.5% SDS-polyacrylamide gel electrophoresis, and blotted against MAPK antibody specific for p44 and p42 MAPK as described under “Materials and Methods.” **B**, activity assay of immunoprecipitated p44 MAPK of cells incubated with agonists and antagonists (above). Each point is the mean ± S.D. of three individual experiments.

**Fig. 8.** Effect of NAC, DPI, and BSO in LacCer-induced p21\(^{\text{ras}}\) activation in H-ASMC. **A**, \( ^{32} \)P-labeled cells were stimulated with 10 \( \mu \)M LacCer (A), preincubated with 15 \( \mu \)M NAC for 30 min and then incubated with 10 \( \mu \)M LacCer (B), preincubated with 5 \( \mu \)M DPI for 30 min and then with 10 \( \mu \)M LacCer (C), preincubated without (−) or with 100 \( \mu \)g/ml BSO only for 24 h (D), or preincubated with 100 \( \mu \)g/ml BSO for 24 h followed by stimulation with 10 \( \mu \)M LacCer (without incubation with LacCer, serving as BSO control) (E). At different time points, as indicated, cells were harvested, lysed, and immunoprecipitated with p21\(^{\text{ras}}\) antibody. Bound \( ^{32} \)P-labeled nucleotides were eluted and separated onto polyethyleneimine TLC plates.

**DISCUSSION**

LacCer, a ubiquitous GSL, plays a pivotal role in the biosynthesis of complex GSL (5). However, its biological function is not well understood. Our laboratory has reported a close relationship between increased levels of LacCer and hyperproliferation in diverse human diseases. For example, in human atherosclerotic plaque (6), familial hypercholesterolemia (36, 37), and human polycystic kidney disease, an increased cellular/tissue level of LacCer was accompanied by cell hyperproliferation (38). Among several GSLs investigated, we found that...
LacCer exerts the highest stimulation in H-ASMC proliferation (1), and LacCer from human atherosclerotic plaque tissue was significantly more effective in stimulating H-ASMC proliferation than LacCer from the unaffected aorta (6). Such studies indicated that one of the biological functions of LacCer may involve cell proliferation. Next, we showed that LacCer specifically stimulated the activation of Ras-GTP loading, Raf-1, and MEK2 upstream to p44 MAPK, and the expression of growth response early gene c-fos, downstream to the p44 MAPK signaling pathway (7). However, these studies did not elucidate whether LacCer itself or second messengers generated by LacCer regulated this pathway. In this report we provide evidence that NADPH oxidase dependent O$_2^-$ generation was increased in H-ASMC upon LacCer addition in a time- and concentration-dependent manner. This, in turn, activated the p21$^{ras}$-GTP loading, activation of the kinase cascade, and induction of c-fos mRNA that finally led to cell proliferation. Our hypothetical model depicting LacCer-mediated redox signaling leading to the proliferation of aortic smooth muscle cells is summarized in Fig. 11.

The generation of O$_2^-$ in our study was measured by the lucigenin chemiluminescence method. Lucigenin chemiluminescence is sensitive to detect both intracellular and extracellular O$_2^-$ because diacridinium nitrate (lucigenin) can enter into the cells and upon reaction with O$_2^-$ emits light. This light was detected using a single photon counter (Packard Top counter). To determine the specificity of LacCer-mediated stimulation of O$_2^-$ generation, we incubated cells with three different sources of LacCer; stearoyl LacCer, bovine erythrocyte membrane-derived LacCer, and LacCer-derived from human atherosclerotic plaque intima and catabolic products of LacCer. Only LacCer stimulated the generation of O$_2^-$ with similar kinetics (Fig. 2, A–D). The integrity of membrane was not compromised in cells incubated with LacCer as evidenced by the absence of leakage of cytosolic lactate dehydrogenase in the medium.

The following observations support the tenet that O$_2^-$ was generated intracellularly. First, preincubation of cells with SOD (a scavenger of superoxide radicals) did not abrogate LacCer-induced O$_2^-$ production. This observation may be due to the inability of SOD to penetrate the cell membrane. Second, NAC and PDTC (both are membrane-permeable molecules) inhibited LacCer-induced O$_2^-$ generation. We were concerned that the hydrolysis product of NAC, acetate, might contribute to the NAC-mediated inhibition of LacCer-induced O$_2^-$ generation. Accordingly, the effects of acetate in LacCer-mediated O$_2^-$ generation were measured. We found that sodium acetate (15 mM) did not inhibit O$_2^-$ generation in control cells (vehicle only) or in LacCer-incubated cells (data not shown). Taken together, our data suggest that LacCer-mediated O$_2^-$ generation in intact cells is endogenous.

To determine which reactive oxygen species-generating enzymes are involved in LacCer-induced O$_2^-$ generation, we took advantage of inhibitors known specifically to inhibit NADPH oxidase/NADH oxidase and xanthine oxidase. We found that LacCer specifically stimulated NADPH oxidase activity in a time-dependent manner, but not NADH oxidase. Moreover, O$_2^-$ production in LacCer-stimulated intact cells was completely blocked by DPI, a flavoprotein containing NADPH oxidase inhibitor. Previously, DPI has been used to demonstrate its specific effect on the inhibition of NADPH oxidase (30, 31). The nonspecific effects of DPI on other flavoproteins by direct binding have also appeared. Since mitochondrial poison KCN did not inhibit LacCer-induced NADPH oxidase activity (data not shown) it appears that the LacCer-induced O$_2^-$ production was due to plasma membrane-bound NADPH oxidase. Allopurinol has been shown to specifically inhibit xanthine oxidase (34, 35). We found that allopurinol did not inhibit LacCer-induced O$_2^-$ production. Staurosporine is a well known potent inhibitor of PKC (32, 33). Similarly, preincubation of cells with PMA (100 nM) for 24 h depletes PKC activity (32, 33). Under these conditions, LacCer-mediated O$_2^-$ generation was not impaired. Such studies indicate that PKC may not be involved in LacCer-induced O$_2^-$ production. This finding confirms our previous studies in which we showed that staurosporine failed to
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impair the p44 MAPK activation and cell proliferation by LacCer (7). Taken together, our data suggest that LacCer-induced O$_2^-$ production in H-ASMC occurs predominantly due to PKC-independent activation of NADPH oxidase. Previously, high concentrations of O$_2^-$ were shown to have a growth-inhibitory effect that may lead to apoptosis (39). By contrast, in our study, LacCer induced the production of a relatively small amount of O$_2^-$ (3.5 nmol/mg of protein) in H-ASMC via the activation of NADPH oxidase. This small amount of O$_2^-$ generated by LacCer may be responsible for the growth-promoting effect of LacCer. Thus, O$_2^-$ may serve as integral signaling molecules that exert a concentration-dependent effect on cell proliferation and apoptosis.

To prevent oxidative damage and allow survival in an oxygen environment, mammalian cells have developed an elaborate antioxidant defense system that includes enzymatic and nonenzymatic antioxidants. The major nonprotein thiol and potent antioxidant in the cells is GSH, which principally buffers the intracellular redox state. We found that LacCer decreased the intracellular GSH level. GSH, an inhibitor of de novo GSH-synthesizing enzyme γ-glutamyl cysteine synthetase, decreased basal GSH level. GSH level was further decreased upon the addition of LacCer to cells preincubated with BSO. The decrease in intracellular GSH may lead to alterations in the activity of redox-sensitive enzymes, including protein-tyrosine kinases, p21ras, and MAP kinases (15, 41). Moreover, it was reported earlier that the generation of O$_2^-$ produced an increase in intracellular pH (40). Since p21ras is one of the redox-sensing proteins (41), alteration of redox status by LacCer via production of endogenous O$_2^-$ caused the activation of p21ras by loading GTP. We have previously shown that activation of p21ras transmits its activation signal to p44 MAPK via the Raf and MEK2 pathway (7). Moreover, it is evident from our study that depletion of GSH increased the susceptibility of p21ras to oxidative stress generated by LacCer, whereas inhibition of O$_2^-$ production with DPI and reduction of O$_2^-$ level by antioxidant PDTC (data not shown) or NAC abrogated LacCer-induced p21ras-GTP loading. This observation is consistent with a previous report that O$_2^-$ caused activation of p21ras in Jurkat cell lines (41).

Previously, we reported that p44 MAPK activation was the target of LacCer-mediated signal transduction (7). In the present study, phosphorylation/activation of p44 MAPK was abrogated by PDTC, NAC, and DPI. Therefore, it is possible that LacCer-mediated O$_2^-$ production via the activation of NADPH oxidase specifically phosphorylates p44 MAPK but not p42 MAPK. We were interested in whether GSH supplementation caused inhibition of p44 MAPK phosphorylation and activation. Since GSH is not transported across the membrane, we incubated cells with GSE, a readily transported derivative of GSH (42). Preincubation of GSE blocked the p44 MAPK phosphorylation/activation by LacCer. In contrast, in vivo depletion of GSH by BSO increased phosphorylation/activation of p44 MAPK by LacCer as compared with control. Thus, decreased levels of GSH potentiate the sensitivity of cells to LacCer and enhance mitogenic signaling via activation of p44 MAPK. In contrast, phosphorylation/activation of p44 MAPK but not p42 MAPK was abrogated by inhibitors of superoxide-generating enzyme DPI and antioxidant PDTC or NAC. Although the precise MAPK-dependent cellular alterations engendering a modified response to oxidants remain to be defined, the present study provides strong support for a crucial role for the p44 MAPK signaling pathway in regulating cell proliferation in response to oxidative stress induced by LacCer.

The protooncogene c-fos functions as an inducible transcription factor in signal transduction processes (43). Elevated expression of the c-fos gene via alteration of redox state has been previously shown to accompany cell proliferation. Also, in our previous study, LacCer specifically induced the expression of the c-fos transcriptional factor (7, 43) downstream to p44 MAPK. As expected, DPI and NAC both abrogated LacCer-induced c-fos mRNA expression. In contrast, BSO stimulated c-fos mRNA expression. These results support the notion that oxidative stress generated by LacCer induced expression of c-fos mRNA as well as cell proliferation.

Exogenously added GSLs, e.g. LacCer, to the culture medium may be incorporated into the plasma membrane, the lipophilic ceramide moiety being inserted into the lipid bilayer and thus increasing the proportion of this lipid in cell membrane (44). An increased level of LacCer also occurs in patients with hypercholesterolemia (36), perhaps contributing to an increase in the production of O$_2^-$ in endothelial cells (45). In fact, recently, we have observed that LacCer can stimulate O$_2^-$ production in human arterial endothelial cells via the activation of NADPH oxidase.2 How LacCer activates NADPH oxidase is not clear from our study and requires further investigation. In summary, LacCer-mediated generation of low levels of superoxide may constitute a novel biochemical signaling pathway in H-ASMC proliferation. Our findings may explain the potential role of LacCer in the pathogenesis of atherosclerosis involving the proliferation of aortic smooth muscle cells.

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