Compartmentalized Production of Ceramide at the Cell Surface*

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Ceramide produced by the hydrolysis of sphingomyelin is an important cellular intermediate in hormone action. Here, we present evidence that interleukin 1β (IL-1β) binding to normal human fibroblasts initiates a lipid messenger cascade that takes place in a sphingomyelin-rich plasma membrane domain with the characteristics of caveolae. Hormone binding first stimulated the appearance of diacylglycerol (DAG) in a caveolearich membrane fraction isolated from whole cells. This was immediately followed by the loss of a resident population of sphingomyelin from the fraction and the concomitant appearance of ceramide. The ceramide produced in response to IL-1β blocked platelet-derived growth factor-stimulated DNA synthesis. IL-1β stimulated the appearance of DAG in other fractions from the same cell, but this DAG was not coupled to ceramide production. This indicates that ceramide production is highly compartmentalized at the cell surface. Since caveolae are known to be involved in membrane internalization, they may be essential for the delivery of ceramide to a site of action within the cell.

The caveola is a membrane domain that can undergo an internalization cycle. The cycle begins with membrane invagination, which leads to the formation of a plasmalemmal vesicle. These vesicles may migrate toward the center of the cell (1) or remain nearby the cell surface (2–4). Plasmalemmal vesicles do not appear to merge with other endocytic pathways as they deliver internalized molecules to either the cytoplasm (5–7) or to the endoplasmic reticulum (8) of the cell. Eventually the vesicle returns to the cell surface to complete the cycle.

The caveola internalization cycle depends on several resident molecules. Cholesterol appears to be a structural molecule that is necessary for the integrity of the caveola membrane coat (9) and the shape of the membrane (10). PKCα is a resident protein that seems to control membrane invagination (11, 12). Caveolae contain a 90-kDa protein that is a substrate for PKCα. Conditions that remove PKCα from caveolae prevent phosphorylation of this protein and block membrane invagination (12). Finally, caveolae contain a protein phosphatase that dephosphorylates the 90-kDa protein (12). The phosphatase may be a target for drugs that inhibit the return of vesicles to the membrane (1).

The molecular composition of caveolae is different from other membrane domains. The core membrane structure appears to be enriched in cholesterol (8, 13, 14), gangliosides (1, 15, 16), GPI-anchored membrane proteins (2, 17–19), and the integral membrane protein caveolin (9, 18). It may also be enriched in sphingomyelin (20). Recent biochemical and immunoelectron microscopic evidence indicates that caveolae contain high concentrations of several different signaling molecules. These include 1) GPI-anchored hormone receptors (21), 2) inositol 1,4,5-trisphosphate receptor (22), 3) protein kinase C (PKC)1 (12, 23), 4) G protein-coupled membrane receptors (3, 24, 25), 5) multiple heterotrimeric GTP binding proteins (17, 18), 6) non-receptor tyrosine kinases (18, 26), 7) an ATP-dependent Ca2+ pump (27), and the epidermal growth factor receptor (19). The presence of these molecules has prompted a search for signaling events that originate at this location.

There are many ways that the caveola internalization cycle could be harnessed for signal transduction (28). One possibility is that caveolae are used to compartmentalize the synthesis of key intermediates in a signaling cascade. Wieghmann et al. (29) have shown that the tumor necrosis factor receptor has two different domains that can stimulate ceramide production. One domain acts on a neutral sphingomyelinase and the other on an acid sphingomyelinase. To do this, tumor necrosis factor must stimulate ceramide production within different compartments of the same cell. The high concentration of sphingomyelin in that appears to be in caveolae (20) suggests that they could be one of the compartments. To investigate this possibility, we developed a human fibroblast model system for studying IL-1β-dependent ceramide production (30). We now present evidence that IL-1β stimulates the conversion of sphingomyelin to ceramide in a membrane fraction that has the biochemical characteristics of caveolae. The ceramide produced in this membrane inhibits DNA synthesis.

**EXPERIMENTAL PROCEDURES**

**Materials**

[3H]Palmitate (36 Ci/mmol), [3H]arachidonic acid (100 Ci/mmol), [3H]choline chloride (81 Ci/mmol), [3H]thymidine (86.2 Ci/mmol), and [3]Hcholine-labeled sphingomyelin (54.5 mCi/mmol) were purchased from DuPont NEN. Sphingomyelin, ceramide, and DAG standards as well as leupeptin, benzamidine, soy bean trypsin inhibitor, pepstatin A, phenylmethylsulfonyl fluoride, and sphingomyelinase were obtained from Sigma. IL-1 was from Promega. Thin layer chromatography plates were purchased from J.T. Baker Inc. Monoclonal anti-caveolin antibody was purchased from Transduction Laboratories (Lexington, KY). ECL Western blotting detection reagents were purchased from Amersham (Buckinghamshire, United Kingdom). Alkaline phosphatase substrate kit and protein assay kit were obtained from Bio-Rad. Penicillin and MEM were from Life Technologies, Inc. D609, synthetic C6-ceramide, and synthetic DAG C8:0 were from Biomol (Plymouth Meeting, PA). PDGF (BB form) was from UBI (Lake Placid, NY). Fetal bovine serum was from Hyclone (Logan, UT). PVDF membrane was from Millipore.

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1 The abbreviations used are: PKC, protein kinase C; DAG, diacylglycerol; PDGF, platelet-derived growth factor; GPI, glycosylphosphatidylinositol; IL-1, interleukin 1β; ER, endoplasmic reticulum; BSA, bovine serum albumin; MEM, minimal essential medium; PVDF, polyvinylidene difluoride; PBS, phosphate-buffered saline.
Methods

Cell Culture—Human fibroblast primary cultures were grown in medium A (MEM supplemented with penicillin and 10% of fetal bovine serum). All of the experiments were done on confluent cells. Cells were cultured from 4–7 days in the indicated dish or plate, and confluence was determined by visual inspection. All experiments were done on cells that had been passaged no more than 18 times. At confluence, cells were washed twice with PBS and incubated in serum-free MEM for 2 h at 37 °C before the indicated additions to the medium. Cells were further incubated for the indicated time at 37 °C.

Radiolabeling of Cells—Cells were labeled with arachidonate by incubating them for 24 h in medium A that contained 10 μCi/150-mm dish [3H]arachidonic acid. Sphingomyelin was labeled with choline by cultivating cells in medium A that contained 10 μCi/25-mm dish [3H]choline chloride. Cells were labeled with palmitate by cultivating them in medium A for 48 h that contained either 60 μCi/150-mm dish or 10 μCi/well [3H]palmitate.

Isolation of Caveolae—Caveolae were isolated by a modification of the method of Sargiacomo et al. (38). Three to four 150-mm dishes of confluent human fibroblast were used in each preparation. Caveolae were isolated in the following steps: 1) Cells were chilled on ice for 5 min and washed twice with 10 ml/dish buffer A (PBS plus 10 μM leupeptin, 10 μg/ml benzamidine, 10 μg/ml soy bean trypsin inhibitor, 1 μg/ml pepstatin A, and 200 μg/ml phenylmethylsulfonfluoride). 2) Cells were scrapped in 5 ml/dish buffer A, combined into a 50-ml tube and centrifuged at 1400 rpm for 5 min at 4 °C. 3) The pellet was mixed with 1 ml of ice-cold 1% Triton X-100 in buffer A. 4) The sample was bounced 20 times, mixed with 1 ml of 80% sucrose in buffer B (150 mM NaCl, 10 mM Tris-HCl, pH 7.5), and loaded on the bottom of a 12-ml ultracentrifuge tube. 5) The sample was overlaid with a 10–30% sucrose gradient in buffer B and centrifuged at 29,000 × g for 2 h at 4 °C in a SW 41 rotor. 6) 700–μl fractions were collected in Eppendorf tubes and maintained on ice until the indicated analysis.

Alkaline Phosphatase Assay—PBS-washed filter paper was placed in a Bio-Rad Bio-Dot apparatus and overlaid with PVDF membrane that had been washed sequentially once with 50 ml of methanol, three times with 100 ml of water, and once with 50 ml of PBS. The excess PBS was removed by suction, and 50 μl of sample was loaded in each well. A vacuum was applied to the apparatus to transfer proteins in the sample to the PVDF membrane. The membrane was then washed with 50 ml of PBS and developed using 50 ml of substrate from Bio-Rad alkaline phosphatase substrate kit. The reaction was stopped by washing the membrane with water. Alkaline phosphatase activity is approximately proportional to the color of the PVDF membrane.

Lipid Extraction, Separation, and Detection—The lipids were extracted by the method of Bligh and Dyer (31). An extraction mixture consisting of 720 μl of chloroform, 600 μl of methanol, and 100 μl of water was used. The mixture was vortexed, and 100 μl of the organic phase was collected and dried under nitrogen gas. The sample was redissolved in 40 μl of chloroform, and 100 μl of H 2O.

RESULTS

IL-1β-dependent Inhibition of DNA Synthesis Mediated by Ceramide—We chose the normal human fibroblast for these studies because invaginated caveolae make up 2% of the surface of these cells (9). Cytokines that stimulate ceramide production inhibit cell growth, induce cell differentiation, or stimulate apoptosis (35, 36). For this reason, we measured the effects of ceramide on PDGF-stimulated [3H]thymidine incorporation (Fig. 1). Cells were grown in the absence of serum for 30 h. PDGF (5 ng/ml) plus various concentrations of IL-1β (Fig. 1A) was added to the dish, and the cells were incubated an additional 12 h before [3H]thymidine incorporation was measured. The amount of [3H]thymidine incorporated by untreated cells is shown on the ordinate (100%, Fig. 1A). PDGF alone caused a 2-fold increase (40% on the ordinate, Fig. 1A). The addition of increasing amounts of IL-1β to the medium caused a reciprocal decline in DNA synthesis. Maximal inhibition occurred at ~25 ng/ml IL-1β.

IL-1β also caused a dose-dependent increase in the concentration of ceramide (38). Cells were incubated in the presence of [3H]palmitate to label the sphingomyelin pool and then exposed to various concentrations of IL-1β for 3 h. A basal level of [3H]ceramide occurred in the absence of IL-1β, but this increased up to 2-fold as the concentration of IL-1β was increased. The response was maximal at ~15 ng/ml IL-1β.

Concomitantly added ceramide suppressed PDGF-stimulated DNA synthesis (Fig. 2). Confluent fibroblasts cultured for 24 h in the absence of serum incorporated 7 pmol of [3H]thymidine per mg of protein (control, Fig. 2A). Incubation of these cells in the presence of PDGF for 12 h stimulated [3H]thymidine incorporation nearly 3-fold (PDGF, Fig. 2A). This increase was completely blocked by the addition of 4 μg/ml C6-ceramide to the medium (PDGF + ceramide, Fig. 2A). DNA synthesis was suppressed equally well by 20 μg/ml DAG C8:0. (PDGF + DAG, Fig. 2A), a membrane-permeable diacylglycerol (37). DAG may have this effect because in some cells it stimulates ceramide synthesis (29, 38).

To determine if DAG was an intermediate in IL-1β-stimulated ceramide production, we blocked DAG production with an inhibitor of phosphatidylinositol-specific phospholipase C (39). Fig. 2B shows that PDGF stimulated DNA synthesis 2-fold (PDGF, Fig. 2B), but the presence of IL-1β in the media completely blocked this increase (PDGF + IL-1β, Fig. 2B). The combination of the phosphatidylinositol-specific phospholipase C inhibitor D609 (20 μg/ml) and IL-1β in the media prevented IL-1β from suppressing DNA synthesis (compare PDGF with PDGF + D609 + IL-1β, Fig. 2B). D609 did not stop C6-ceramide from inhibiting DNA synthesis, indicating that the drug did not
block the signaling activity of ceramide (PDGF + D609 + ceramide, Fig. 2B). The results in Fig. 2 suggest that DAG is an obligate intermediate in IL-1β-mediated ceramide formation, but ceramide is the active signaling molecule.

IL-1β Stimulates Appearance of Ceramide in Caveola-rich Fraction—Much of the sphingomyelin in the cell is at the plasma membrane (40) associated with a Triton X-100 insoluble complex that can be separated from soluble lipids by flotation on a sucrose gradient (20). Four electron microscopic markers specific for invaginated caveolae have been used to show that this fraction contains purified caveolae: caveolin (18), cholesterol enrichment (8), PKCα (12), and GPI-anchored membrane proteins (18, 19). The material in these fractions also has the dimensions (41) and general morphology of caveolae (23), indicating it contains a specific piece of membrane. We used Triton X-100 insolubility combined with sucrose density centrifugation to prepare caveola-rich fractions from human fibroblasts (Fig. 3). Cells were cultured in the presence of [3H]arachidonate and processed as described. Equal fractions from the sucrose gradient were collected and assayed for protein content (Fig. 3A), total [3H]arachidonate-labeled lipids (Fig. 3B), the GPI-anchored membrane protein alkaline phosphatase (alkaline phosphatase, Fig. 3C), and caveolin (caveolin, Fig. 3C). Nearly all of the protein in the gradient was in the bottom four fractions that contained the soluble material (Fig. 3A). A small peak of protein (inset, Fig. 3A), corresponding to ~0.6% of the total, was detected in fractions 4–7. These same three fractions contained ~1% of all of the [3H]arachidonate-labeled lipids in the cell (inset, Fig. 3B). When equal volumes of each fraction (50 μl) were assayed for the presence of GPI-anchored alkaline phosphatase (Fig. 3C), most of the activity was in fractions 4–7. These fractions also contained all of the immunodetectable caveolin (Fig. 3C), even though we loaded half of the protein in each bottom fraction (fractions 11–15) on the gel. Therefore, the isolation procedure yields a highly selective subfraction of membrane that is enriched in caveola markers. We refer to this preparation as the caveola fraction.

One advantage of this method is that the fractionation procedure allows a comparison between the caveola fractions (fractions 4–7) and the remainder of the cell (fractions 11–15). To determine the distribution of sphingomyelin on this gradient, we cultured cells in the presence of [3H]choline for 48 h
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before fractionating the cells and measured the amount of \( ^{3}H \)choline-labeled sphingomyelin (Fig. 4). The majority of the sphingomyelin was in the caveole fractions (fractions 4–7). On average, this fraction contained 50–70% of the total cellular sphingomyelin. Nearly all of this sphingomyelin was located in the extracellular leaflet of the plasma membrane because treatment of cells with 0.1 unit/ml of neutral sphingomyelinase prior to the fractionation procedure removed the majority of the choline head groups from the sphingomyelin (Fig. 4). This suggests that the Triton X-100 insoluble sphingomyelin did not come from internal membranes of the cells.

The caveole fraction was also highly enriched in ceramide (Fig. 5). We labeled cells with \( ^{3}H \)palmitate, prepared sucrose gradient fractions, and measured the ceramide content. Approximately 50% of all the \( ^{3}H \)ceramide in the cell was in the caveole fractions (Fig. 5). The remainder was in the soluble fractions at the bottom of the gradient. A replicate set of cells that had been incubated in the presence of IL-1\( \beta \) for 1 h had \( \sim \)50% more \( ^{3}H \)ceramide in the caveole fractions than control cells (Fig. 5). By contrast, \( ^{3}H \)ceramide was not increased in the bottom fractions (compare fractions 11–14, Fig. 5). All of the IL-1\( \beta \)-stimulated increase in ceramide detected in whole cells could be accounted for by the amount that appeared in the caveole fraction (compare Fig. 6, left ordinate, with \( \triangle \), right ordinate, Fig. 5).

The sphingomyelin in the caveole fraction was the source of the IL-1\( \beta \)-stimulated increase in ceramide (Fig. 6). Fibroblasts were cultured in the presence of \( ^{3}H \)palmitate to label the sphingomyelin. The media were replaced with fresh media containing 10 ng/ml IL-1\( \beta \), and the cells were incubated for various times. The caveole fractions were prepared at the end of each incubation, and the amount of both \( ^{3}H \)sphingomyelin (Fig. 4, left ordinate, Fig. 6) and \( ^{3}H \)ceramide (Fig. 5, right ordinate, Fig. 6) was measured. With time in the presence of IL-1\( \beta \), there was a reciprocal decline in the amount of \( ^{3}H \)sphingomyelin and an increase in the amount of \( ^{3}H \)ceramide in this fraction. Nearly all of the radioactivity appearing in the ceramide lipid at each time point could be accounted for by the amount of \( ^{3}H \)sphingomyelin that was lost.

These results suggested that the caveole fraction contains sphingomyelinase activity. We prepared sucrose gradient fractions and assayed each fraction for \( Zn^{2+} \)-independent, acid sphingomyelinase activity (Fig. 7). Most of the activity was in the whole cell fractions at the bottom of the gradient (fractions 11–15). However, there was a peak of activity in the caveole fractions (fractions 4–8). Despite the low total activity, the specific activity in the peak (fraction 6) caveole fraction was comparable (25 nmol/mg protein/h) to the activity in the peak (fraction 14), whole cell fraction (42 nmol/mg protein/h). Very little neutral sphingomyelinase activity was detected in the caveole fraction (data not shown).

IL-1\( \beta \) Stimulates a Lipid Cascade—We next measured the effect of IL-1\( \beta \) on the level of diacylglycerol in the caveole fractions (Fig. 8). We cultured cells for 48 h in the presence of \( ^{3}H \)palmitate before preparing cell fractions on a sucrose gra-
Cells were grown to confluence in 150-mm dishes. During the final 48 h in culture, [(H]palmitate (60 μCi/dish) was present in the dish. Cells were washed once with 10 ml of PBS per dish and cultured in MEM containing 100 μg/ml BSA for 2 h at 37 °C. The medium was replaced with fresh medium of the same composition, and the cells were incubated for 1 h in the presence (△) or absence (□) of IL-1β (10 ng/ml). Cells were fractionated, and the total lipids in each fraction were extracted and separated by TLC. The ceramide spot was scraped and counted.

The DAG spot was scraped and counted. The ceramide spot was scraped and counted.

FIG. 6. IL-1β stimulates the loss of sphingomyelin and an increase in ceramide in caveolae fractions. Cells were grown to confluence in 150-mm dishes. During the final 48 h in culture, [(H]palmitate (60 μCi/dish) was present in the dish. Cells were washed once with 10 ml of PBS per dish and cultured in MEM containing 100 μg/ml BSA for 2 h at 37 °C. The medium was replaced with fresh medium of the same composition, and the cells were incubated for the indicated time in the presence of 10 ng/ml IL-1β. The caveolae fraction (fractions 5–8 from the sucrose gradient) was collected from sucrose gradients and pooled for lipid extraction. Lipids were separated by TLC, and the sphingomyelin and ceramide spots were scraped for counting. Each point is the mean ± S.D. (n = 3).

FIG. 7. Distribution of Zn2+-independent acid sphingomyelinas activity in sucrose density gradient fractions. Cells were grown to confluence in 150-mm dishes and fractionated on sucrose gradients. Each fraction (100 μl) was mixed with 100 μl of [14C]choline-labeled sphingomyelin prepared as substrate micelles and incubated for 15 min at 37 °C. The released [14C]choline was extracted and counted as described.

FIG. 8. IL-1β stimulates DAG production in both caveolae and whole cell fractions. Cells were grown to confluence in 150-mm dishes. During the final 48 h in culture, [(H]palmitate (60 μCi/dish) was present in the dish. Cells were washed once with 10 ml of PBS per dish and cultured in MEM containing 100 μg/ml BSA for 2 h at 37 °C. The medium was replaced with fresh medium of the same composition, and the cells were incubated for 15 min at 37 °C. The released [14C]choline was extracted and counted as described.

If the DAG that appeared in the caveolae fraction was coupled to ceramide synthesis (29), then D609 should prevent the IL-1β-dependent appearance of ceramide in this fraction (Fig. 9). When [(H]palmitate-labeled cells were incubated in the presence of IL-1β for 5 min, the level of [(H]ceramide increased in the caveolae fractions but not other fractions in the gradient (compare □ with △, Fig. 9A). The increase was more modest than seen in other experiments owing to the shorter incubation time. The IL-1β-dependent appearance of [(H]ceramide was completely blocked by D609 (compare □ with △, Fig. 9B). We also incubated a replicate set of cells in the presence of DAG C8:0 for 5 min (Fig. 9C) to see if exogenous DAG could mimic the effects of IL-1β. Surprisingly, the [(H]ceramide level was only elevated in the caveolae fractions (compare □ with △, Fig. 9C).

DISCUSSION

We have localized IL-1β-mediated ceramide production to a highly select region of membrane that has the characteristics of caveolae. Arachidonate labeling indicates that this fraction represents ~1% of the total membrane, yet it contains all of the detectable caveolin, most of the GP1-anchored alkaline phosphatase, most of the plasma membrane PKCα (12), and is
that in human fibroblasts, IL-1α neutralizes phosphatidylserine. Our results suggest that IL-1α-stimulated sphingomyelinase occurs at the cell surface and is concentrated in invaginations of the membrane (40). Triton X-100 insolubility of the lipid and GPI-anchored protein components of this membrane (42) have shown that sphingomyelin and cholesterol are most likely accounts for the Triton X-100 insolubility of the lipid and GPI-anchored protein components of this membrane (42).

Hormones that stimulate ceramide production utilize either a neutral or acidic sphingomyelinase (40). Our results suggest that in human fibroblasts, IL-1β uses an acidic enzyme to generate ceramide. First, we were able to detect an acidic-dependent sphingomyelinase in the caveolae fractions (Fig. 8). Most likely, this enzyme requires a low pH compartment to be active. Second, plasmalemmal vesicles appear to be an acidic compartment (7, 43). Finally, we found that IL-1β-stimulated ceramide production in human fibroblasts is strictly dependent on DAG formation (Fig. 9). Previous work has shown DAG to be an intermediate specific for the acid sphingomyelinase pathway (29).

Besides offering a suitable environment for ceramide production, caveolae provide a way to deliver this molecule to other compartments in the cell. Smart et al. (8) have shown that oxidation of membrane cholesterol causes caveolin to move from the plasma membrane to the Golgi apparatus by way of the ER. This appears to be a normal cycle required for shuttling sterols between the two compartments. Since caveolin is a high affinity binding site for long chain, unsaturated fatty acids (45), it may also transport fatty acids to the ER. This would explain why caveolin (47) and caveolae (48) dramatically increase during adipocyte differentiation. Therefore, IL-1β binding may be coupled to caveolae-mediated transport of the ceramide to the ER, where subsequent steps in the signal cascade take place.

The potential transport of cholesterol to the ER by caveolae also suggests a reason for why ceramide formation can stimulate cholesterol esterification (46, 49, 50). This occurs either after the addition of sphingomyelinase to the cell (49, 50) or when endogenous sphingomyelinase is activated (46). Cholesterol esters are synthesized by the ER enzyme, acyl coenzyme A cholesterol acyltransferase. Acyl coenzyme A cholesterol acyltransferase is constitutively active (44) and siphons off any excess ER cholesterol for storage in lipid droplets. Stimulation of ceramide production in caveolae may mobilize the cholesterol normally complexed with sphingomyelin. This cholesterol would then be available for transport to the ER by caveolae. When the ER cholesterol pool rises too high, acyl coenzyme A cholesterol acyltransferase diverts the excess into storage.

IL-1β-dependent ceramide production in fibroblasts is an example of a specific signaling molecule that is made in caveolae in response to a hormonal stimulus (28). All of the ceramide produced in the cell appeared in the caveolae fraction (compare Fig. 2B with Fig. 6), even though IL-1β stimulated an increase in DAG in both the caveolae and the whole cell fractions (Fig. 8). More remarkably, the addition of synthetic DAG C8:0 to the media only stimulated ceramide production in caveolae (Fig. 9C). What caveolae must do is spatially segregate sphingomyelin on the cell surface into a compartment that is optimally responsive to one type of stimulus. This ensures that the newly made ceramide will only go to a location in the cell where it can act on a specific signaling pathway. Cells that use ceramide for more than one signaling activity must have multiple pools of sphingomyelin (29).

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