Direct Effect of Ceramide on the Mitochondrial Electron Transport Chain Leads to Generation of Reactive Oxygen Species

ROLE OF MITOCHONDRIAL GLUTATHIONE*

(Ca 2) Ceramide production in the signaling of TNF whose effect compared with mitochondria from untreated cells.

showed an increase (2–3-fold) in the amount of ceramide chain complex IV compared with GSH-repleted mito-

 Peroxide induced by C2 was not triggered by mitochon-

toxicity is mediated by overproduction of reactive oxygen species from mitochondria, we have exam-

ined the role of ceramide in generation of oxidative stress in isolated rat liver mitochondria.

These results suggest that mitochondria are a target of exposure to ceramide resulted in a potentiated increase production of hydrogen peroxide and consequently this phenomena may account for the generation of reactive oxygen species during TNF cytotoxicity.

Tumor necrosis factor (TNF) 1 is a cytokine produced by a wide variety of cell types whose production is up-regulated in a number of stressful and pathological conditions (1–3). TNF exerts a pleiotropic mode of action on multiple cell functions including regulation of immune responses, host defense reac-

tions, and gene regulation. In addition, its role as a mediator of cytotoxicity on certain susceptible transformed cell lines has been well documented (4–7). Upon binding to its receptor subtypes, TNF evokes a complicated array of intracellular signals, including G-coupled activation of phospholipase A2, release of arachidonic acid, DAG production, and activation of protein kinase C, some of which may participate in the chain of reactions that result in cell killing (5–7). An overproduction of ROS has been proposed as an important mechanism to mediate the cytotoxic and gene regulating effects that TNF exerts on tumor cells (8, 9–12).

Ceramide has attracted considerable attention due to its role as an intracellular effector molecule that mimics some of the biological effects exerted by inflammatory cytokines such as TNF (13–15). In addition to its de novo biosynthesis, which is initiated by the condensation of serine and palmitoyl-CoA, ceramide can also be generated by sphingomyelin hydrolysis. Thus, enzymes that hydrolyze sphingomyelin such as sphingo-

myelinases stand as regulators of intracellular ceramide levels and consequently ceramide-mediated functions. These enzymes are key components of the so-called sphingomyelin pathway, an ubiquitous system that functions in transducing the signals of cytokines to the cell interior (13–15).

Sphingomyelinase is known to exist in two forms depending on their intracellular localization and pH optima (13–16). A Mg2+-dependent membrane-bound with a neutral pH optima initiates signaling by generating ceramide at or near the vicinity of the plasma membrane. In addition to the membrane-

associated enzyme, another cytosolic neutral SMase independent of Mg2+ has been identified and partially purified, which

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1 The abbreviations used are: TNF, tumor necrosis factor; AA, anti-

mycin A; BSO, buthionine-sulfoximine; C 2, N-acetylphosphosine; C 2 DH,

N-hexanoylsphingosine; DAG, 1,2-diacylglycerol; DEM, diethylmaleate; DHR, dihydrohydroxide 123; DCF, 2′-7′-dichl-

oro-fluorescein; DCFDA, 2′-7′-dichlorofluorescin diacetate; MOPS, 4-morpholinepropanesulfonic acid; NF-κB, nuclear factor κB; PC-PLC, phosphatidylincholine-dependent phospholipase C; Q, ubiquinone; ROS, reactive oxygen species; SMase, sphingomyelinase; TFFA, thonytryfluoroacetone.
appears to hydrolyze intracellular sphingomyelin stores to initiate signaling (19). The signal initiated by these enzymes is then transmitted further down in the signaling cascade by activation of ceramide-activated protein phosphatases and ceramide-dependent protein kinases (13–15). In addition to the neutral SMase forms, an acidic SMase form has also been identified, displaying an pH optima around 5, the bulk of which seems to be located at the lysosomes/endosomal compartment. Although it appears that acidic SMase plays a role in signaling, the molecular mechanism of its activation and recruitment during signaling is unclear. Indirect evidence has suggested that DAG generated by PC-PLC activates the acidic enzyme at or near the plasma membrane since inhibitors of PC-PLC prevent activation of the acidic SMase. This hypothesis, which implies a redistribution of the enzyme from the lysosomal compartment to or near the plasma membrane, requires further verification (20, 21). Despite the existence of the neutral and acidic SMases, an alkaline form of the enzyme has been described recently, although its role in signaling remains to be defined (22).

There is compelling evidence to propose ceramide as a second messenger in the sphingomyelin pathway similar to DAG in the glycerophospholipid pathway. The role that ceramide fulfills within the cell are numerous and of varied nature (13–16). It has been shown that ceramide plays a critical role in apoptosis, proliferation, cellular senescence, and gene regulation through activation of transcription factors such as NF-κB (20, 21). However, the possibility that ceramide may interact with mitochondria leading to production of ROS has not been documented to our knowledge, and constitutes the basis of the present report.

Mitochondria are one of the most important cellular sources of ROS due to its quantitative consumption of molecular oxygen. Since ceramide appears as an important mediator of the effects elicited by TNF and due to the participation of mitochondria in the TNF-induced ROS production (9–12), the purpose of the present work was to analyze the effects of ceramide and other sphingolipids, on the production of hydrogen peroxide in isolated mitochondria from rat liver. Furthermore, since reduced GSH is the only defense provided to metabolize peroxides generated from the electron transport chain through GSH redox cycle (23), we determined the role of mitochondrial GSH in modulating the production of hydrogen peroxide and its consequences upon incubation of mitochondria with ceramide. Our studies demonstrate for the first time that addition of ceramide to mitochondria results in a dose-dependent increase in hydrogen peroxide, which is prevented when complex I and II of respiration are inhibited. Furthermore, mitochondria from TNF-treated hepatocytes displayed an increased level of ceramide supporting the role of ceramide as an intermediate in the TNF-induced ROS generation from mitochondria. Depletion of matrix GSH prior to exposure to ceramide results in an additional increase of hydrogen peroxide, which peroxidizes lipids from mitochondrial resulting in loss of mitochondrial function. These results suggest that ceramide produced in the signaling of TNF is responsible, at least in part, for some of the TNF-induced cytotoxic effects.

**MATERIALS AND METHODS**

GSH, GSSG, ethacrynic acid, DAG, BSO, DEM, AA, rotenone, atracyloside, TFFA, SMase (Bacillus cereus), ceramide III, and sphenogamine were obtained from Sigma. ATP, myo-inositol, and dithiothreitol were from Boehringer Mannheim. N-acetylsphingosine (C2-ceramide), N-hexanoylsphingosine (C6-ceramide), N-palmitoylsphingosine (C16-ceramide), sphingosine, and C2-dihydroceramide were purchased from Bional (Plymouth Meeting, PA). DCFDA, DHR and cis-parinaric acid were obtained from Molecular Probes (Eugene, OR). \( \gamma^{32} \text{P} \) ATP (3000 Ci/mmol) was purchased from Amer sham. Escherichia coli dicyglycero phospoinositol kinase was from Calbiochem. Recombinant human TNF-α (specific activity 2.7 \( \times \) 10⁹ units/mg of protein) was obtained from Promega.

**Isolation and Culture of Hepatocytes—**Hepatocytes were isolated as described previously, plated on rat tail collagen, and cultured in DMEM/F12 (24, 25). Cell densities were determined using a Coulter counter, model multisizer II (Coulter Electronics) and verified by hemocytometer. Cell viability was determined by trypan blue exclusion and by the measurement in the medium of glutathione S-transferase.

**Fluorescence Microscopy—**Cultured hepatocytes in the presence or absence of TNF or ceramide were incubated with fluorescence probes DCFDA or DHR (2 μM) for 1 h, followed by washing to remove excess probe. To quantify the generation of peroxides fluorescence microscopy was used, a Nikon Diaphot 300 (Tokyo, Japan) inverted microscope equipped with a CF Fluo 40× objective was used. Fluorescence intensity was measured using a 3CCD camera (model DIX-930P, Sony, Tokyo, Japan) with an attached MPU-F100P intensifier (Sony). Images were recorded on a SR-SSS58E (JVC, Tokyo, Japan) video cassette recorder and analyzed with a PC computer equipped with 24-bit Movie Machine II graphics. The limits of the hepatocyte monolayer in the area of observation were traced by a clear field transillumination image. Thereafter, the mean fluorescence intensity of the delimited area was measured in the corresponding fluorescence image. Five random images were analyzed for every experimental condition. Values of fluorescence are the result of subtracting background fluorescence (measured in cultures in the absence of fluorescent probes) from the values obtained in the same image referred to a grayscale (0–255).

**Preparation of Mitochondria and Incubation with Ceramides—**Rat liver mitochondria were isolated by differential centrifugation (26). Enrichment of mitochondria was ascertained by the specific activity of succinic dehydrogenase found in mitochondria relative to that of homogenate. Mitochondrial integrity was determined by the acceptor control ratio of oxygen consumption in states 3 and 4 of respiration using a Clark oxygen electrode with glutamate/malate or succinate. Mitochondrial integrity was determined by the acceptor control ratio of oxygen consumption in states 3 and 4 of respiration using a Clark oxygen electrode with glutamate/malate or succinate as substrates for respiratory sites for complex I or II as described previously (25, 27).

Mitochondria were incubated in a shaking bath at 25 °C under ambient air in the presence of mediators of TNF or inhibitors of respiratory complexes for up to 1 h as detailed in the figures. When fluorescence probes were present, incubation was carried out in the dark.

Stock solutions of sphingolipids were made up in dimethyl sulfoxide and stored at −80 °C under nitrogen; when added to aqueous reaction mixtures containing mitochondria, the final concentration of the carrier solvent did not exceed 0.5%. Control mitochondria contained only carrier solvent whose presence did not affect the fluorescence of DCF.

**Flow-Cell Analysis of Mitochondria—**All measurements of mitochondrial fluorescence and side light scatter (SSC, 90° angle) were made for at least 10,000 events/test using a FACStar flow cytometer (Becton Dickinson, San Jose, CA). Data on mitochondrial fluorescence and light scatter were obtained using a 5-watt argon ion laser tuned at 488 nm and 250 milliwatts. Fluorescence of DCF from oxidation of DCFDA was measured through a 530-nm bandpass filter placed in front of emission for excitation. Slit widths of 10 and 5 nm, respectively (27). The mean intensity of the green fluorescence caused by mitochondria incubated with DCF in the presence or absence of ceramide was determined using the Cell Quest software program and expressed as fluorescence channels (scale from 0 to 10,000 arbitrary units). Graphs were plotted using the Cell Quest software program (Becton Dickinson).

**Determination of Hydrogen Peroxide and Lipid Peroxidation—**Hydrogen peroxide measurement was determined spectrophotometrically using DCFDA. Mitochondria were incubated with the fluorescent probe, 2 μM, in the absence or presence of ceramide or other electron transport inhibitors (see figure legends). Fluorescence at 509 nm for emission and 503 nm for excitation, with slit widths of 10 and 5 nm, respectively (27, 29). Fluorescence of DCF was correlated with increasing concentrations of hydrogen peroxide allowing determination of hydrogen peroxide as described (27).

Lipid peroxidation was determined by quenching of fluorescence of cis-parinaric acid as described previously (30). Mitochondria treated
were added to mitochondrial suspension at 1–5 μM in Me₂SO for 1 h and determination of fluorescence of DCF determined by autoradiography. Incorporated 32P (Whatman) using chloroform:acetone:methanol:acetic acid:water (10:4:2:2:1, v/v) as solvent and detected by autoradiography. Incorporated 32P was quantified by the diacylglycerol kinase assay as described previously (31, 32). Lipids from mitochondria were extracted and dried under nitrogen and resuspended in 100 μl of 150 μg of cardiolipin, 280 μM diethylenetriaminepentacetic acid, 51 mM octyl-β-D-glucopyranoside, 50 mM NaCl, 51 mM imidazole, 1 mM EDTA, 12.5 mM MgCl₂, 2 mM diithiothreitol, 0.7% glycerol, 70 μM β-mercaptoethanol, 1 mM ATP, 10 μM of γ-[32P]ATP, and 35 μM/ml E. coli diacylglycerol kinase at pH 6.5. After 30 min at room temperature, the reaction was stopped by extraction of lipids with 1 ml of chloroform:methanol:1 N HCl (100:100:1) and 170 μl of PBS. Major lipid products of the phosphorylation reaction, phosphatic acid (from diacylglycerol) and ceramide 1-phosphate (from ceramide) were resolved by thin-layer chromatography on Silica Gel 60 plates (Whatman) using chloroform:acetone:methanol:acetic acid:water (10:4:2:2:1, v/v) as solvent and detected by autoradiography. Incorporated 32P was quantified by liquid scintillation counting. The level of ceramide was calculated by comparison with a standard curve generated using known amounts of ceramide type III.

Statistical Analyses—Statistical analyses for comparison of mean values for multiple comparisons between mitochondrial preparations were made by one-way analysis of variance (ANOVA) followed by Fisher’s test.

RESULTS AND DISCUSSION

Ceramide Leads to Generation of Reactive Oxygen Species in Intact Cells and Isolated Mitochondria—Ceramide has drawn attention since the description of its role as a sphingolipid second messenger whose levels are increased in cells stimulated by inflammatory cytokines such as TNF. Since one of the characteristic features of the TNF-induced cytotoxicity is mediated by overproduction of ROS, we first determined the effect of direct addition of permeable ceramide analogues, such as C₂-ceramide, to cultured hepatocytes to monitor its effect on the fluorescence of probes that are sensitive to oxidative stress, such as DHR. Primary cultured hepatocytes were labeled with DHR, washed to remove excess fluorochrome, and analyzed for changes in fluorescence assessing ROS production. Fig. 1 shows a representative fluorescence microscopic photograph of hepatocytes labeled with DHR. Upon incubation of cells with C₂ (5 μM), we observed a significant increase (2–3-fold versus control) in the fluorescence of DHR. Because peroxides are the species specifically monitored by such fluorescent probe, these results indicate that in this paradigm, there was a burst of hydrogen peroxide induced by C₂ compared with control cells in the absence of the sphingolipid. Similar results were obtained when hepatocytes were incubated with DCFDA, a non-fluorescent probe, which upon oxidation, mainly by peroxides, is converted to the highly fluorescent derivative DCF (22, 24) (data not shown). Hepatocytes remained viable under these conditions, indicating that the increase in ROS was not a consequence of cell dysfunction. The fluorescence microscopic appearance of hepatocytes incubated with C₂ was reminiscent of the effect that TNF produced in hepatocytes, suggesting that ceramide reproduced in parenchymal cells the increase in ROS that TNF evokes on multiple cell types (9–12).

Thus, data from intact cells incubated with C₂ leading to overproduction of ROS suggest but do not demonstrate that such effects were either mediated by direct action of ceramide nor were they originated from mitochondria. Involvement of

FIG. 1. Generation of reactive oxygen species by ceramide. A, cultured rat hepatocytes were incubated with C₂-ceramide (5 μM) for 2 h. Cells were labeled with DHR followed by washing to remove excess probe and fluorescence microscopy determined as described under “Materials and Methods.” Original magnification, ×200. B, isolated mitochondria (1 mg/ml) were incubated with succinate to drive electron flow through succinic dehydrogenase, labeled with DCFDA (2 μM), and washed to remove excess fluorochrome. Mitochondria were then further incubated in the presence of C₂-ceramide (1 μM in Me₂SO) for 1 h and determination of fluorescence of DCF determined by flow cytometry as described under “Materials and Methods.” Control mitochondria were incubated with equal volume of Me₂SO (0.1%), which did not affect the fluorescence of DCF.
substrates downstream in the signaling of ceramide could have been the effectors of the increased production of ROS. In this regard, activation of transcription factor NF-κB by ceramide (20, 21, 33) would lead to increased expression of genes that contain κB sites in their promoter/enhancer, such as nitric oxide synthetase. Rising nitric oxide levels could contribute to generation of other potent oxidants, thus potentially participating in the generation of ROS observed in cells (34).

Evidence has recently been provided that mitochondria from cells exposed to TNF are the main source of ROS generation produced by the cytokine (9–12). Therefore, we hypothesized that ceramide may directly affect mitochondria leading to production of hydrogen peroxide. To test this hypothesis, and to demonstrate a direct effect of ceramide on mitochondria, we isolated mitochondria from rat liver and examined the effect that incubation with C2 exerts when monitoring the generation of hydrogen peroxide by flow cytometry using DCFDA as fluorogenic probe to follow its conversion to DCF (22, 24). Mitochondria were incubated with succinate to drive electron flow directly at succinic dehydrogenase complex. As shown in Fig. 1C, flow cytometric profile of mitochondria labeled with DCFDA displayed a greater fluorescence intensity of DCF upon addition of C2 (2-fold), reproducing the phenomena observed with intact cells. Similar results but of lesser magnitude were observed when NAD-linked substrates were used instead of succinate (data not shown). Oxygen consumption at states 3 and 4 of respiration (acceptor control ratio) did not differ significantly between control or ceramide-treated mitochondria, indicating that the increased fluorescence of DCF was not the result of unspecific effects due to loss of mitochondrial integrity (data not shown).

In view of the evidence that ceramide acts as a messenger in transmitting the signaling of TNF (13–18) and on the direct effect of ceramide on mitochondria leading to ROS production, we sought to determine if treatment of hepatocytes with TNF increases the level of ceramide in mitochondria. We first verified that treatment of cultured hepatocytes with TNF resulted in generation of ROS. As seen in Fig. 2, the generation of hydrogen peroxide determined by fluorescence of DCF in hepatocytes labeled with DCFDA increased upon treatment with TNF. Subsequently, mitochondria from these cells were isolated and the level of ceramide determined by the diacylglycerol kinase assay. The mitochondrial fraction was enriched in succinic dehydrogenase (3–4-fold) and de-enriched in lactic dehydrogenase relative to intact cells. Compared with mitochondria isolated from control cells, the mitochondrial fraction from cells treated with TNF revealed a significant increase (2–3-fold) in the amount of ceramide (Fig. 2B). Therefore, these data correlate the increase in ceramide in mitochondria of cells treated with TNF with its ability to overproduce ROS.

Our findings demonstrating direct effect of ceramide in mitochondria have extended previous related observations that mitochondria isolated from septic rats generated ROS to a greater extent than mitochondria from control rats mainly from FAD-linked substrates (35). However, these studies did not examine the role of TNF or identify its mediators as causal effectors for the increased hydroxyl radical generation from septic mitochondria. Taken together these data demonstrate that inflammatory cytokines such as TNF leads to increased generation of ceramide associated with mitochondria which by interacting with mitochondria may account for the increased generation of ROS in intact cells. To our knowledge, the direct effect of ceramide on mitochondria has not been previously described, and therefore we sought to further characterize such phenomena in terms of specificity and mechanism(s).

**Structural Specificity of Sphingolipids in Generating Hydrogen Peroxide from Mitochondria**—Since C2 is a permeable analogue of natural ceramides, we tested if other analogs of C2 including N-hexanoylsphingosine (C6-ceramide) also led to generation of ROS. As shown in Fig. 3, the magnitude of generation of ROS by C6 was similar to that observed by C2. Similar results were also obtained when N-palmitoylsphingosine was used as the effector lipid (data not shown). These results indicate that N-fatty acyl-sphingosine derivatives mediate the increased generation of hydrogen peroxide from mitochondria, regardless of the length of the alkyl moiety. One of the features of sphingolipids such as ceramide is the presence of a trans double bond in atom 4 of sphingosine. Ceramide is formed from dihydroceramide by the introduction of the trans-4,5-double bond (reviewed in Ref. 15). Therefore, we tested if the immediate precursor of ceramide, dihydroceramide, which lacks the trans double bond, mimics the effect of C2 regarding its ability to generate hydrogen peroxide in mitochondria. As shown in Fig. 3, the dose-dependent effect of dihydroceramide was shifted to the right compared with C2; the increased production of hydrogen peroxide by C2-dihydroceramide at 1 μM was of similar potency to that of C2. However, at lower concentrations (0.25–0.5 μM) compared with the effect elicited by C2-ceramide, dihydroceramide did not result in generation of hydrogen peroxide.

The ability of dihydroceramide to mimic the effect induced by ceramide in the generation of hydrogen peroxide is an intriguing...
Structural specificity and dose-dependent effect of ceramide in the generation of hydrogen peroxide from isolated mitochondria. A, freshly isolated mitochondria (1 mg/ml) were incubated with 1 μM C2-ceramide, C2-ceramide, or dihydro-C2 (C2DH) for 60 min in the presence of DFCDA (2 μM). Sphingosine (Sphs), sphinganine (Sphn), and sphingomyelin (Sphm) were present at same concentration as ceramides. Increasing their concentration to 5 μM did not modify fluorescence of DCF. After 60 min of incubation, an aliquot was transferred to the spectrofluorometer to determine fluorescence of DCF as described under "Materials and Methods." Control incubation was performed in the presence of Me2SO, the solvent used in the stock solution of sphingolipids. Mean fluorescence was correlated with hydrogen peroxide using known concentrations of the latter (27). B, isolated mitochondria (1 mg/ml) were incubated with increasing concentrations of C2-ceramide (open circles) and C2DH (closed circles) for 60 min and hydrogen peroxide was determined from DCF fluorescence in a spectrofluorometer as described under "Materials and Methods." The bar represents the fluorescence of control mitochondria incubated with Me2SO (0.5%). Results are mean ± S.D. of three separate observations. *, p < 0.05 versus control.

Incubation of mitochondria with sphingosine or its precursor sphinganine at 1 μM, concentration at which C2 elicited a maximal increase in hydrogen peroxide, did not result in production of hydrogen peroxide (Figs. 3A and Fig. 4). Only sphingosine at ≥10 μM induced a significant increase in hydrogen peroxide. Addition of other sphingolipids, such as sphingomyelin, did not increase fluorescence of DCF (Fig. 3A). Incubation of mitochondria with the enzyme responsible for sphingomyelin hydrolysis, SMase that leads to generation of ceramide in cells did not exert any effect in DCF-labeled mitochondria (data not shown). These results suggest that ceramide is not locally produced within mitochondria by action of SMases acting on the sphingomyelin, implicating that even the small fraction of sphingomyelin of the mitochondrial membrane is not accessible to hydrolysis by SMase or that the ceramide that would have been generated in situ had not built up to exert any significant effect on mitochondria.

Although ceramide is one of the lipid mediators that reproduce many of the effects exerted by cytokines such as IL-1 or TNF (13–16), other lipid molecules such as DAG and arachidonic acid also arise within cells in response to these cytokines. These lipid signals, DAG or arachidonic acid, accumulate in cells in response to cytokines by the action of PC-PLC and phospholipase A2, respectively. Incubation of mitochondria with short chain diacylglycerol, 1,2-diacylglycerol, over the same range of concentrations tested for C2, did not result in significant increase in hydrogen peroxide (Fig. 4). Arachidonic acid at concentration up to 10 μM failed to result in any significant change on the generation of hydrogen peroxide (Fig. 4). Nevertheless, this fatty acid at concentrations exceeding 20 μM decreased state 3 while increasing state 4 respiration (data not shown). Similarly to these results with isolated mitochondria, incubation of hepatocytes with either DAG or arachidonic acid did not rise fluorescence of DCF or DHR (data not shown).

The lack of effect of DAG in comparison with ceramide is of interest and adds as another example illustrating the divergent functional behavior of these mediators. In this regard, protein kinase C ζ isoenzyme, which is insensitive to phorbol ester or DAG, becomes activated by ceramide (14, 33). Although these differential functions described here for DAG and ceramide...

Sphingolipids contain sphingosine as the sugar backbone to which a fatty acid is linked through an amide bond at carbon 2. The effect of C2-ceramide was dose-dependent, displaying a bifunctional effect starting at 0.25 μM and reaching the maximum at 0.5–5 μM (Fig. 3B). The mechanism underlying this behavior is unclear, although similar effects have also been seen in other cell types. Hence, studies describing the regulation of FMLP-induced superoxide anion generated by neutrophils found that C2 concentrations below 1 μM potentiated the generation of this reactive species, whereas at concentrations greater than 1 μM inhibited its production (40). In addition, the ability of ceramide to activate and phosphorylate protein kinase C ζ, an atypical protein kinase C isoform, has been shown to be bifunctional (33).

Phospholipids contain sphingosine as the sugar backbone to which a fatty acid is linked through an amide bond at carbon 2.
might have been predicted based on different structures between glycerolipids (DAG) and sphingolipids (ceramide), there are examples of enzymes that recognize either lipid as substrates. Sphingomyelin synthase, a mammalian enzyme responsible for sphingomyelin synthesis, transfers the phosphocholine to ceramide, generating sphingomyelin from phosphatidylcholine. The lack of effect of TNF itself or its lipid mediators in generating ROS (Table I) strengthens the hypothesis, supported by our findings, that ceramide is an important link between TNF binding to its receptor at the plasma membrane and the distally evoked generation of ROS from mitochondria (11).

**Potentiated Effect of Ceramide and Antimycin A in the Generation of Hydrogen Peroxide and Effect of Blocking Electron Flow at Complex I and II of Respiration**—The generation of hydrogen peroxide from mitochondria arises from superoxide anion upon its dismutation catalyzed by Mn-superoxide dismutase. The production of superoxide anion originates from the ubiquinone, Q cycle, of complex III where one electron from ubiquinol is transferred directly to molecular oxygen. Reduction of Q to ubiquinol occurs at NADH dehydrogenase and succinate dehydrogenase complexes. The transfer of one electron from ubiquinol to the cytochrome bc₁ complex catalyzed by the Rieske iron-sulfur center generates ubisemiquinone. Ubisemiquinone by transferring a second electron to the ubiquinone pool of complex III is the major site of hydrogen peroxide generation by C2, as previously shown, AA led to an increased generation of hydrogen peroxide (2–3-fold) determined as fluorescence of DCF of similar magnitude to that of C2 (Fig. 5) (27). Interestingly, the addition of C2 to AA-supplemented mitochondria resulted in an additive production of hydrogen peroxide compared with either of these separately.

To further support the view that inhibition of electron flow at the ubiquinone pool of complex III is the major site of hydrogen peroxide generation by C2, we inhibited electron flow at complexes I and II with known blockers of electron transfer at these sites, i.e. rotenone and TTFA, respectively, separately or in combination. When mitochondria energized with succinate were incubated with rotenone and TTFA, respectively, the fluorescence of DCF did not increase, indicating lack of production of hydrogen peroxide. However, inhibition of electron flow at these complexes significantly prevented the increase in hydrogen peroxide resulting from incubation of mitochondria with C2. Similarly, blocking electron flow at complexes I and II did also partially prevent the increase of hydrogen peroxide resulting from C2 plus AA, compared with the combined presence of the two. This phenomena has been described also in the generation of ROS of rat liver mitochondria when incubated with AA as well as for splenic T lymphocytes committed to programmed cell death, where generation of ROS was diminished by inhibiting mitochondrial electron transport with rotenone, highlighting the role of the Q cycle as the electron source for ROS (27, 41). The additive effect of C2 and AA and the similar effect of complexes I and II blockers in preventing the effect of C2 in inducing hydrogen peroxide suggest that C2 favors the electron...
transfer to molecular oxygen at or near same center where AA acts in the Q cycle of complex III. Further evidence in favor of this site as the main generator of DCF increase came when mitochondria were incubated with myxothiazol. This compound inhibits oxidation of ubiquinol to ubisemiquinone by the Rieske iron-sulfur center of cytochrome bc₁ complex and is expected to block superoxide anion formation. Accordingly, myxothiazol prevented the increase in DCF caused by C₂-ceramide (Fig. 5), indicating that the Q cycle of complex III is a significant source of ROS produced by ceramide.

Lack of Involvement of Mitochondrial Permeability Transition in the Burst of Hydrogen Peroxide by Ceramide—Mitochondrial membrane permeability is a phenomena that has been studied for decades in isolated mitochondria. It has been proposed that such process is a critical mechanism involved in cell damage. The mitochondrial membrane permeability is characterized by a sudden increase in the permeability of the inner mitochondrial membrane to small solutes. The permeability transition occurs through the opening of a transmembrane pore in the inner mitochondrial membrane. The opening of the pore is facilitated by loading mitochondria with calcium, pH, oxidation of thiols, and by activation of the adenine nucleotide translocator. This process collapses ion gradients across the inner mitochondrial membrane, leading to mitochondrial depolarization, loss of oxidative phosphorylation, and generation of ROS (41, 42). Therefore, in further defining the mechanism(s) leading to the opening of the pore responsible for the mitochondrial permeability transition contributes to the generation of ROS. Compared with positive inducers of permeability transition such as atractyloside (Fig. 6) or tert-butylhydroperoxide (data not shown), which induce a fall in absorbance at 540 nm indicating opening of the pore, mitochondria incubated with C₂ (1-5 µM) did not reveal any significant change in optical density at 540 nm, which was maintained over time at levels similar to control mitochondria (Fig. 6). The opening of the pore induced by atractyloside was prevented by cyclosporin A, as seen by the maintenance of the optical density at 540 nm (data not shown); however, this inhibitor did not affect the absorbance recording at 540 nm of mitochondria in the presence of C₂, indicating lack of swelling of mitochondria incubated with C₂ (Fig. 6).

The time pattern of DCF fluorescence of mitochondria in the presence of C₂ did not parallel that of A₅₄₀ nm, since the fluorescence of DCF increased over time despite lack of opening of the pore, suggesting that the former was not caused by engagement of the latter. Beyond 30 min of incubation, there was a fall in absorbance in the presence of C₂ indicating swelling of mitochondria; however, the onset in the opening of the pore was preceded by the increase in DCF fluorescence.

These results suggest that the generation of hydrogen peroxide induced by C₂ is not the consequence of increased membrane permeability leading to the mitochondrial swelling and generation of ROS; in fact, our findings indicate that the overproduction of ROS induced by C₂ would result in activation of the pore as indicated by the time relationship of these two mutually regulated processes. The opposite has also been noticed, since it has been shown in lymphocytes committed to cell death that engagement of permeability transition leads to ROS overproduction (41). The control of the opening of the pore by ROS imply the existence of critical sulphhydryls that are subject to redox regulation. This constitute the basis for the opening of the pore in the presence of strong prooxidants such as tert-butylhydroperoxide. Accordingly, in view of the reciprocal regulation of the permeability transition and ROS, our results suggest the possibility that upon generation of ROS induced by ceramide engagement of the permeability transition would entail as an amplification wave-like mechanism contributing to the overproduction of ROS generated by ceramide in response to inflammatory cytokines.

Mitochondrial GSH Depletion Results in Loss of Mitochondrial Function by Oxidative Stress Induced by Ceramide—Hydrogen peroxide generated within the electron transport chain can undergo two possible fates: conversion to hydroxyl radical with the participation of transition metals in the Haber-Weiss reaction or reduction to water by the catalysis of GSH peroxidases with the required participation of reduced GSH as cofactor. Since mitochondrial GSH is the only defense to metabolize peroxides, depletion of GSH prior to exposure of these mitochondria to C₂ would be expected to result in a potentiating increase in hydrogen peroxide. Thus, we have determined the magnitude of hydrogen peroxide production by C₂ and the degree of lipid peroxidation as consequence of the oxidative stress induced by C₂ in mitochondria depleted of GSH. We have used several maneuvers to deplete GSH in mitochondria: by in vitro incubation of mitochondria with ethacrynic acid, mitochondrial GSH is depleted to about 50% of control levels (Fig. 7A). The same degree of depletion was achieved when DEM was used (data not shown). In addition to these in vitro maneuvers, depletion was achieved by in vivo administration of BSO, a selective inhibitor of the γ-glutamylcysteine synthetase, which leads to a cellular depletion of GSH including mitochondria (43) or after ethanol feeding to rats, which results in a
selective depletion of mitochondrial GSH as consequence of impaired transport of GSH from cytosol into mitochondria (28). GSH-depleted mitochondria incubated with C2 revealed greater production of hydrogen peroxide (75–80%) compared with mitochondria with repleted levels of GSH. This magnitude in the generation of hydrogen peroxide was comparable to that observed by combination of C2 and AA in GSH-repleted mitochondria (Fig. 7B).

To evaluate the consequences of increased level of hydrogen peroxide under these circumstances, we examined the fluorescence of cis-parinaric-labeled mitochondria to determine the degree of lipid peroxidation (30). In the GSH-repleted mitochondria, C2 did not significantly lead to increased loss of cis-parinaric compared with control mitochondria, indicating lack of lipid peroxidation. However, the degree of lipid peroxidation was increased 2-fold upon addition of C2 to GSH-depleted mitochondria. In these conditions, GSH depletion prior to exposure to C2 led to a significant loss of complex IV activity determined as cytochrome c oxidase as parameter subject to inactivation by ROS (44). Similar consequences in terms of generation of hydrogen peroxide, lipid peroxidation, and loss of complex IV activity by C2 were observed when mitochondria from BSO- or ethanol-treated rats were used (data not shown). The equivalent results observed between the in vitro or in vivo-induced depletion of GSH discard the possibility that the outcome obtained from ethacrynic acid-treated mitochondria were caused by unspecific effects of the toxicant.

Recent studies by Goosens et al. (11) provided indirect evidence that mitochondrial GSH was critical in scavenging the ROS generated by TNF in murine fibrosarcoma cell line L929, based on differential effects of DEM versus BSO in accelerating cytotoxicity. However, these studies did not report the level of mitochondrial GSH in L929 cells after these maneuvers. Our findings have demonstrated the critical importance of mitochondrial GSH in scavenging the ROS produced in the organelle as consequence of interference of electron transport by C2 at complex III. Similar conclusion were obtained when oxidative stress in isolated rat liver mitochondria was induced by blocking electron flow at complex III of respiration (27).

Recently, a critical role of mitochondria has been deciphered in splenic lymphocytes committed to programmed cell death induced by a variety of stimuli, including ceramide, where a loss of mitochondrial transmembrane potential and generation of ROS constitute an important feature of early apoptosis (41), although these studies did not address the effect of ceramide on isolated mitochondria. In light of our findings, it could be speculated that depletion of GSH in mitochondria prior to exposure to ceramide could accelerate or increase the degree of apoptosis. The fact that ceramide interacts with components of the complex III of the electron transport chain favoring the production of ROS highlights the pivotal role of GSH as the primary line of defense of mitochondria due to virtual lack of catalase activity. Thus, mitochondrial GSH status will be a critical modulator of mitochondrial function and cell viability and, hence, in diseases and/or tissue injury mediated by oxidative stress in mitochondria, mitochondrial GSH depletion will accentuate the adverse effects of ROS (10, 24, 25, 27, 43, 45, 46). Since mitochondrial GSH arises by the existence of an ATP-dependent carrier, which translocates cytosol GSH into the matrix, it would be critical to characterize its nature and properties at a molecular level (47).

In summary, we have determined the capability of ceramide to result in increased generation of hydrogen peroxide in iso-
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REFERENCES

1. Beutler, B., and Cerami, A. (1986) Nature 320, 584–588
2. Tracey, K. J., Wei, H, Manogue, K. B., Fong, Y., Hesse, D. G., Nguyen, H. T., Kuo, G. C., Beutler, B., Cotran, R. S., and Cerami, A. (1988) J. Exp. Med. 167, 1211–1227
3. Fiers, W. (1991) FEBS Lett. 285, 199–212
4. Brach, M. A., Gruss, H. J., Asano, Y., DeVos, S., Ludwig, W. D., Mertelsmann, R., and Herrmann, F. (1992) Cancer Res. 52, 2197–2201
5. Elbaz, O., Budel, L. M., Hoogerbrugge, H., Touw, I. P., Delwel, R., Mahmoud, L. A., and Lowenberg, B. (1991) J. Exp. Med. 173, 119–125
6. Brach, M. A., Gruss, H. J., Scott, C., and Herrmann, F. (1995) Mol. Cell. Biol. 15, 4824–4830
7. Belka, C., Wiegmann, K., Adam, D., Holland, R., Neuhold, M., Herrmann, F., Kronke, M., and Brach, M. A. (1995) EMBO J. 14, 1156–1165
8. Jones, A. L., and Selby, P. (1989) Cancer Surv. 8(4), 817–836
9. Schulze-Osthoff, K., Beyaert, R., Vandervoore, V., Haegeman, G., and Fiers, W. (1992) J. Biol. Chem. 267, 5317–5323
10. Schulze-Osthoff, K., Bakker, A. C., Vanhaesebroeck, B., Beyaert, R., Jacob, W. A., and Fiers, W. (1993) EMBO J. 12, 3095–3104
11. Goossens, V., Grooten, J., DeVos, K., and Fiers, W. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 8115–8119
12. Adamson, G. M., and Billings, R. E. (1992) Arch. Biochem. Biophys. 294, 223–229
13. Kolesnick, R., and Golde, D. W. (1994) Cell 77, 325–328
14. Kolesnick, R., and Fuchs, Z. (1995) J. Exp. Med. 181, 1949–1952
15. Hannun, Y. (1994) J. Biol. Chem. 269, 3125–3128
16. Zhang, Y., and Kolesnick, R. (1995) Endocrinology 136, 4157–4160
17. Hannun, Y. A., and Bell, R. M. (1989) Science 243, 500–507
18. Spiegel, S., and Merrill, A. H. (1996) FASEB J. 10, 188–1897
19. Okazaki, T., Bielawska, A., Domal, N., Bell, R. M., and Hannun, Y. A. (1994) J. Biol. Chem. 269, 4070–4077
20. Schütze, S., Potthoff, K., Machleidt, T., Berkovic, D., Wiegmann, K., and Kronke, M. (1992) Cell 71, 756–776
21. Wiegmann, K., Schütze, S., Machleidt, T., Witte, D., and Kronke, M. (1994) Cell 78, 1005–1015
22. Nyboe, L., Duan, R. D., Axelsson, J., and Nilsson, A. (1996) Biochim. Biophys. Acta 1300, 42–48
23. Kaplowitz, N., Aw, T. Y., and Oskhtens, M. (1985) Annu. Rev. Pharmacol. Toxicol. 25, 715–744
24. Fernández-Checa, J. C., García-Ruiz, C., Oskhtens, M., and Kaplowitz, N. (1991) J. Clin. Invest. 87, 397–405
25. García-Ruiz, C., Morales, A., Ballesta, A., Rodes, J., Kaplowitz, N., and Fernández-Checa, J. C. (1994) J. Clin. Invest. 94, 193–201
26. Schnaitman, C. A., and Greenawalt, J. W. (1968) J. Biol. Chem. 243, 158–175
27. García-Ruiz, C., Colell, A., Morales, A., Kaplowitz, N., and Fernández-Checa, J. C. (1995) Mol. Pharmacol. 48, 825–834
28. Fernández-Checa, J. C., Oskhtens, M., and Kaplowitz, N. (1989) J. Clin. Invest. 83, 1247–1252
29. Cathcart, R., Schwiers, E., and Ames, B. N. (1983) Anal. Biochem. 134, 111–116
30. Hedley, E., and Chow, S. (1992) Cytometry 13, 686–692
31. Dressler, R. A., and Kolesnick, R. N. (1990) J. Biol. Chem. 265, 14917–14921
32. Van Veldhoven, P. P., Bishop, W. R., and Bell, R. M. (1998) Anal. Biochem. 183, 177–189
33. Muller, G., Ayoub, M., Storz, P., Rennecke, J., Fabbro, D., and Pfizenmaier, K. (1995) EMBO J. 14, 1961–1969
34. Rasi, R., Rodriguez, M., Castro, L., and Tellieri, R. (1994) Arch. Biochem. Biophys. 308, 89–95
35. Taylor, D. E., Ghin, A. J., and Piantadosi, C. A. (1995) Arch. Biochem. Biophys. 316, 70–76
36. Bose, R., Verheij, M., Haimovitz-Friedman, A., Scotto, K., Fuchs, Z., and Kolesnick, R. (1995) Cell 82, 405–414
37. Chen, J., Nikolova-Karakashian, M., Merrill, A. H., Jr., and Morgan, E. T. (1995) J. Biol. Chem. 270, 25233–25238
38. Olivera, A., Zhang, H., Carlson, R. O., Mattie, M. E., Schmidt, R. R., and Spiegel, S. (1994) J. Biol. Chem. 269, 17924–17930
39. Schroeder, J. J., Crane, H. M., Xia, J., Liotta, D. C., and Merrill, A. H., Jr. (1994) J. Biol. Chem. 269, 3475–3481
40. Wang, K., Li, X.-B., and Hunchak, N. (1995) J. Biol. Chem. 270, 3056–3062
41. Zamzami, N., Marchetti, P., Castedo, M., Decaudin, D., Macho, A., Hirsch, T., Susin, S. A., Petit, P. X., Mignotte, B., and Kroemer, G. (1995) J. Exp. Med. 182, 367–377
42. Nieminen, A. L., Saylor, A. K., Tesfai, S. A., Herman, B., and Lemasters, J. J. (1995) Biochem. J. 307, 99–106
43. Martensson, J., Jain, A., Frazer, W., and Meister, A. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 5290–5300
44. Zhang, Y., Marcillat, O., Giulivi, C., Ernster, L., and Davies, K. J. A. (1990) EMBO J. 9, 4865–4873
45. Newmeyer, D. D., Parschon, D. M., and Reed, J. C. (1994) Cell 79, 353–364
46. Papu Y., Johnson, M. E., and Gores, G. J. (1995) Hepatology 20, 177–185
47. García-Ruiz, C., Morales, A., Colell, A., Rodes, J., Yi, J.-R., Kaplowitz, N., and Fernández-Checa, J. C. (1995) J. Biol. Chem. 270, 15946–15949

related mitochondria. Our results clearly demonstrate that ceramide exerts a direct effect in mitochondria describing a new functional role of sphingolipids as inducers of oxidative stress. Since ceramide is an intermediate generated intracellularly upon stimulation of cells with inflammatory cytokines, our findings demonstrate a role of ceramide in mediating the cytotoxicity of TNF by increased generation of ROS. Furthermore, the present studies identify mitochondria as a primary target of ceramide leading to generation of ROS by interacting with complex III of electron transport chain. GSH in mitochondria being the only defense to cope with deleterious effects of ROS produced within mitochondria stands as a critical preventative factor whose depletion or limitation may be of significance in amplifying the cytotoxic effect of TNF.