Ceramide Interaction with the Respiratory Chain of Heart Mitochondria†

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ABSTRACT: A study is presented on the interaction of ceramide with the respiratory chain of rat heart mitochondria, and a comparison is made between the effects elicited by short- and long-chain ceramides. *N*-Acetylsphingosine (C_2-ceramide) and *N*-palmitoylsphingosine (C_{16}-ceramide) inhibited to the same extent the pyruvate+malate-dependent oxygen consumption. Succinate-supported respiration was also inhibited by ceramides, but this activity was substantially restored upon the addition of cytochrome *c*, which, on the contrary, was ineffective toward the ceramide-inhibited NADH-linked substrate oxidation. Direct measurements showed that short- and long-chain ceramides caused a large release of cytochrome *c* from mitochondria. The ceramide-dependent inhibition of pyruvate+malate and succinate oxidation caused reactive oxygen species to be produced at the level of either complex I or complex III. The activity of the cytochrome *c* oxidase, measured as ascorbate/TMPD oxidase activity, was significantly stimulated and inhibited by C_2- and C_{16}-ceramide, respectively. Similar effects were observed on the activity of the individual respiratory complexes isolated from bovine heart. Short- and long-chain ceramides had definitely different effects on the mitochondrial membrane potential. C_2-ceramide caused an almost complete collapse of the respiration-dependent membrane potential, whereas C_{16}-ceramide had a negligible effect. Similar results were obtained when the potential was generated in liposome-reconstituted complex III respiring at the steady-state. Furthermore, C_2-ceramide caused a drop of the membrane potential generated by ATP hydrolysis instead of respiration, whereas C_{16}-ceramide did not. Finally, only short-chain ceramides inhibited markedly the reactive oxygen species generation associated with membrane potential-dependent reverse electron flow from succinate to complex I. The emerging indication is that the short-chain ceramide-dependent collapse of membrane potential is a consequence of their ability to perturb the membrane structure, leading to an unspecific increase of its permeability.

Ceramide is a sphingolipid molecule commonly considered a second lipid messenger, mediating the action of inflammatory cytokines or growth factors such as tumor necrosis factor-α (TNF-α),[1] interleukin-1β, γ-interferon, and nerve growth factor (see refs *1* and *2* for reviews). Ceramide is generated within the cell by de novo synthesis or upon activation of several sphingomyelinases which catalyze the cleavage of sphingomyelin to ceramide and phosphorylcholine. Ceramide can in turn be hydrolyzed to sphingosine or, alternatively, utilized by sphingomyelin synthase, thus completing the sphingomyelin cycle (*3*).

The various roles displayed by ceramide, ranging from apoptosis to proliferation and gene regulation, depend on the cell type, the various receptors involved, and variations in the actual concentration of ceramide, as a consequence of the activity of ceramide-utilizing enzymes (*4*). In apoptosis, the deregulation of the sphingomyelin cycle causes a net ceramide accumulation (*1*).

A role of mitochondria in the commitment phase of apoptosis is well-defined (*5*−*7*). During apoptosis, cytochrome *c* is released from mitochondria to interact with apoptotic protease activating factor-1 (Apaf-1) and procaspase-9. The activation of the latter leads to activation of other caspases which drive the biochemical execution of the apoptotic process (*7*, *8*). Mitochondria may also function to generate reactive oxygen species (ROS) as a consequence of cytochrome *c* release (*9*) or interaction with the respiratory chain of various poisons. The consequent inhibition of electron transfer and the accumulation of reduced respiratory carriers may direct one-electron transfer reaction to oxygen to form superoxide and hydrogen peroxide (*10*−*12*). These species, together with the highly reactive derived hydroxyl radical, are directly responsible for oxidative damage of cell constituents (*13*).

The interaction of ceramide with mitochondria is investigated in various laboratories. It has been found that mitochondria isolated from TNF-α-treated hepatocytes showed higher content of ceramide, compared to mitochondria from

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[1] Abbreviations: TNF-α, tumor necrosis factor-α; ROS, reactive oxygen species; C_2-ceramide, *N*-acetylsphingosine; C_6-ceramide, *N*-hexanoylsphingosine; C_{16}-ceramide, *N*-palmitoylsphingosine; DHC, C_2-dihydroceramide; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; DCFH, 2′,7′-dichlorofluorescin; HRP, horseradish peroxidase; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; DBH, decylubiquinone; $\Delta\Psi$, transmembrane electrical potential gradient.

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control cells (*14*). Furthermore, addition of *N*-acetylsphingosine (C_2-ceramide) to mitochondria from untreated cells caused an increase of ROS production (*14*). However, it has to be noted that under the experimental conditions used by these authors, ceramide did not affect either state 3 or state 4 respiration. Later papers suggested that ceramide directly inhibits the mitochondrial respiratory chain, by lowering the activity of the ubiquinol−cytochrome *c* reductase (complex III) (*15*). However, findings by several groups indicate that TNF-α causes, in cultured normal (*16*) and malignant cell types (*17*, *18*), a marked inhibition of complex I and NADH-linked substrate respiration.

Another open question concerns the type of ceramide used. Since naturally occurring ceramides do not enter the cell, most of the experiments with cells and isolated mitochondria have been carried out using short-chain permeable ceramides, in which an acetyl or hexanoyl group replaces the natural long-chain fatty acid. However, as underlined by Hofmann and Dixit (*19*), short-chain ceramides may cause perturbation of membrane structure and unspecific increase of (mitochondrial) membrane permeability, due to their physicochemical properties.

Here we present experiments on the effect of short- and long-chain ceramides on mitochondrial functions. It is shown that all ceramides tested inhibited the activity of complex I and the oxidation of NADH-linked substrates. Mitochondrial succinate oxidase activity was also inhibited by ceramides, but substantially recovered by the addition of exogenous cytochrome *c* which, on the contrary, was ineffective to release the inhibition of NADH-linked substrate oxidation. Consistent with the two inhibition sites, we show that ROS production was enhanced by ceramides at both phosphorylation sites I and II. Mitochondrial membrane potential measurements showed that short-chain ceramide caused a drop of the respiration-dependent membrane potential, either in mitochondria or in complex III-reconstituted liposomal vesicles, whereas long-chain ceramide did not. Similar results were obtained when the potential was supported by the ATP hydrolysis, instead of respiration, in mitochondria. We conclude that (i) the membrane potential collapsing effect of short-chain ceramide has to be attributed to its physicochemical properties and (ii) the inhibition of the electron-transfer rate brought about by the interaction of natural ceramide with mitochondria did not cause substantial changes in membrane potential.

MATERIALS AND METHODS

Chemicals. *N*-Acetylsphingosine (C_2-ceramide), *N*-acetylsphinganine (C_2-dihydroceramide), *N*-hexanoylsphingosine (C_6-ceramide), and *N*-palmitoylsphingosine (C_{16}-ceramide) were purchased from Calbiochem (La Jolla, CA). 2′,7′-Dichlorofluorescin diacetate (DCFH-DA) was obtained from Eastmann Kodak (Rochester, NY). Antimycin A, myxothiazol, rotenone, phenylmethylsulfonyl fluoride (PMSF), safranin-O, decylubiquinone (DBH), horse-heart cytochrome *c* (type VI), and soybean phospholipids (type II) were from Sigma Chemical Co. (St. Louis, MO). Durohydroquinone was purchased from K & K Laboratories (Plainview, NY). Horseradish peroxidase (HRP) was obtained from Boehringer, Mannheim. Anti-cytochrome *c* antibody (7H8-2C12) was purchased from Pharmingen (San Diego, CA). All other reagents were of the highest purity grade commercially available.

Preparation of Mitochondria. Rat heart mitochondria were isolated by differential centrifugation of tissue homogenate. Briefly, hearts from male Wistar rats (200−250 g) were quickly excised, and ventricles, carefully devoided of fat and connective tissue, were then finely minced and homogenized in 10 volumes of isolation medium containing 0.25 M sucrose, 10 mM Tris-HCl, pH 7.4, 1 mM EGTA, 0.25 mM PMSF. The homogenate was centrifuged at 1200*g* for 10 min. The resulting supernatant was centrifuged at 9500*g*, and the pellet, resuspended in the same buffer, was centrifuged at 14000*g* for 10 min. The pellet was washed gently to remove any light or loosely packed damaged mitochondria, resuspended in the isolation buffer, and centrifuged again as above. The final pellet was resuspended in the isolation medium at a protein concentration of 50−60 mg/mL as determined by the Biuret method. All the centrifugation steps were carried out at 0−4 °C. The isolated mitochondria exhibited respiratory control ratios routinely higher than 7 (with NADH-linked substrate), and this value was not significantly enhanced by the presence of bovine serum albumin in the isolation medium.

Determination of mitochondrial cytochrome content (nanomoles per milligram of protein) was carried out as described in (*20*).

Isolation of Respiratory Complexes. NADH−ubiquinone oxidoreductase (complex I) and ubiquinol−cytochrome *c* oxidoreductase (complex III) were isolated from bovine heart mitochondria as described in (*21*, *22*) and stored at −80 °C in TSH buffer (50 mM Tris-HCl, pH 8.0, 0.67 M sucrose, 1 mM histidine). Cytochrome *c* oxidase (complex IV) was isolated from bovine heart mitochondria as described in (*23*) and stored at −80 °C in 0.25 M sucrose, pH 7.4.

Preparation of Complex III Vesicles. Reconstitution of purified complex III into phospholipid vesicles was performed by the cholate-dialysis method (*24*) as described in (*25*, *26*). Acetone-washed soybean phospholipids (30 mg) were sonicated in 1 mL of a medium containing 100 mM K-HEPES buffer, pH 7.4, 56 mM KCl, and 2% potassium cholate. Purified complex III (2 mg of protein) was then added and the mixture dialyzed 4 h against 125 mL of 100 mM K-HEPES buffer, pH 7.4, 56 mM KCl. The second (overnight) dialysis step was performed against 125 mL of 10 mM K-HEPES buffer, pH 7.4, 96 mM KCl. The final 2 h dialysis was performed against 1 mM K-HEPES buffer, pH 7.4, 99.6 mM KCl.

Measurement of Oxygen Consumption Rate. The respiratory activity of freshly prepared mitochondria was measured polarographically with a Clark-type electrode (Yellow Spring Instruments, Yellow Spring, OH), in an all-glass reaction chamber at 25 °C; 0.1 mg of protein was suspended in a medium containing 75 mM sucrose, 50 mM KCl, 30 mM Tris-HCl, pH 7.4, 5 mM KH_2PO_4, 0.5 mM EDTA, 5 mM $MgCl_2$ (final volume 1.6 mL). State 4 respiration was started on the addition of pyruvate (3.5 mM) + malate (1.7 mM) or succinate (7 mM) in the presence of 1 μg/mL rotenone, or 1.4 mM ascorbate/0.4 mM TMPD in the presence of 1.2 μM antimycin A. State 3 and uncoupled respiration were obtained by adding 1 mM ADP and 0.3 μM FCCP, respectively.

Detection of Cytochrome c Release. Freshly isolated rat heart mitochondria were incubated at 25 °C under the same conditions described for oxygen consumption experiments, in the presence of 1 μg/mL rotenone and 7 mM succinate. Then 20 μM ceramides (vehicle in the control) was added, and after 10 min incubation, mitochondria were spun down at 14000*g* for 10 min, at 4 °C. The resulting supernatant was centrifuged at 100000*g* for 15 min. The supernatant of the second centrifugation was concentrated using a Millipore ultrafree-4 centrifugal filter and used for the detection of cytochrome *c*, spectrophotometrically at 550 nm ($\Delta\epsilon_{Red.-Ox.}$ = 21 $mM^{-1}\cdot cm^{-1}$) or by gel electrophoresis according to (*27*). After electrophoresis, the gel was blotted onto a nitrocellulose membrane as described in (*28*) and probed by a mouse monoclonal anti-cytochrome *c* antibody. Immunoblot analysis was performed with HRP-conjugated anti-mouse antibody using enhanced chemiluminescence Western blotting reagents (NEN). Relative optical densities and areas of bands were quantified using a Camag TLC scanner II densitometer equipped with a D-2000 Cromato-integrator (Merck-Hitachi).

Measurement of Redox Activities of the Respiratory Enzymes in Rat Heart Mitochondrial Particles. Enzyme activities were measured by suspending 0.1 mg of mitochondrial particles, prepared by freezing and thawing (3 times) of mitochondria isolated from rat heart, in a medium containing 50 mM potassium phosphate buffer, pH 7.4, 25 μM EDTA (final volume 1.6 mL) at 25 °C.

NADH−CoQ oxidoreductase (complex I), NADH−cytochrome *c* oxidoreductase (complex I+III), succinate−cytochrome *c* oxidoreductase (complex II+III), and ubiquinol−cytochrome *c* oxidoreductase (complex III) activities were determined spectrophotometrically with a double-beam, dual-wavelength spectrophotometer (Johnson Research Foundation, Philadelphia, PA) as reported previously in details (*12*).

NADH oxidase activity was measured as rotenone-sensitive oxygen consumption elicited by the addition of 170 μM NADH.

Succinate oxidase and quinol oxidase activities were determined by following the antimycin-sensitive oxygen consumption initiated by the addition of 7 mM succinate and 175 μM durohydroquinone (or DBH), respectively. The reaction medium also contained 1 μg/mL rotenone.

Cytochrome *c* oxidase (complex IV) activity was measured as cyanide-sensitive ascorbate/TMPD oxidase activity. Oxygen consumption was started upon addition of 1.4 mM ascorbate/0.4 mM TMPD (in the presence of 1.2 μM antimycin A). In all the above polarographic determinations of oxygen consumption rates, the mitochondrial particle suspension was supplemented with 0.2 μM cytochrome *c*.

Measurement of Redox Activities of the Respiratory Enzymes Isolated from Bovine Heart Mitochondria. The redox activity of purified complex I was measured spectrophotometrically by following the rotenone-sensitive initial rate of NADH oxidation at 360−374 nm ($\Delta\epsilon$ = 2.3 $mM^{-1}\cdot cm^{-1}$). Purified enzyme (25 μg) was suspended in a reaction mixture (final volume 1.6 mL) consisting of 10 mM potassium phosphate buffer, pH 8.0, 0.15 mg/mL sonicated soybean phospholipids, 100 μM decylubiquinone, and 1 mM KCN, at 30 °C. The reaction was started by the addition of 60 μM NADH.

Complex III activity was determined by measuring, at 550−540 nm ($\Delta\epsilon$ = 19.1 $mM^{-1}\cdot cm^{-1}$), the antimycin-sensitive initial rate of ferricytochrome *c* reduction upon addition of 30 μM reduced decylubiquinone. The reduced quinone was obtained from the oxidized form following the procedure reported in (*29*). Five micrograms of purified complex III, either soluble or reconstituted into liposomes, was suspended in 1.6 mL of a medium containing 100 mM KCl, 1 mM K-HEPES buffer, pH 7.4, 8 μM ferricytochrome *c*, and 1 mM KCN, at 25 °C. When the soluble enzyme activity was assayed, the reaction mixture also contained 0.05% TWEEN-80 or 40 μg/mL sonicated soybean phospholipids. For the measurement of complex III vesicle activity, the reaction mixture was supplemented with 0.05 μg of both valinomycin and nigericin.

Cytochrome *c* oxidase activity was measured polarographically by following the cyanide-sensitive rate of oxygen consumption. Purified enzyme (90 μg) was suspended in a medium consisting of 40 mM KCl, 10 mM K-HEPES buffer, pH 7.0, 100 μM EDTA, 2 μM antimycin A, and 2 μM ferricytochrome *c* (final volume 1.6 mL, 25 °C), and the oxygen consumption was initiated by the addition of 10 mM ascorbate/0.4 mM TMPD.

Measurement of Membrane Potential. The membrane potential, either in intact rat heart mitochondria or in complex III vesicles, was measured following the safranin fluorescence quenching at 525 nm (excitation), 575 nm (emission) with a Perkin-Elmer 650 fluorescence detector.

Freshly prepared mitochondria were suspended in 2.5 mL of the same medium described for oxygen consumption experiments, supplemented with 8 μM safranin and 1 μg of nigericin, at 25 °C. The transmembrane potential was generated by the addition of respiratory substrates or 1.5 mM ATP.

For the determination of membrane potential in complex III vesicles, reconstituted enzyme was suspended (20 μg of protein/mL) in a medium containing 100 mM KCl, 1 mM K-HEPES buffer, pH 7.4, 300 μM durohydroquinone, 0.12 μM soluble cytochrome *c* oxidase, 0.2 μg of nigericin, and 2 μM safranin. The respiration-dependent membrane potential was generated by the addition of 0.2 μM ferricytochrome *c*.

Fluorometric Determination of Mitochondrial H_2O_2 Production. The rate of mitochondrial hydrogen peroxide production was estimated by measuring the linear fluorescence increase (excitation at 475 nm, emission at 525 nm) caused by the H_2O_2-dependent oxidation of dichlorofluorescin (DCFH) to the fluorescent compound dichlorofluorescein in the presence of horseradish peroxidase (HRP) (*30*). Immediately prior to determinations, DCFH was obtained from the stable reagent dichlorofluorescin diacetate (DCFH-DA) by alkaline treatment (*30*).

Rat heart mitochondria were suspended in 2.5 mL of the same medium described for oxygen consumption assays, supplemented with 0.4 μM HRP, 5 μM DCFH. Pyruvate + malate or succinate were used as substrate. Conversion of fluorescence units to nanomoles of H_2O_2 produced was performed by measuring the fluorescence changes upon addition of known amounts of H_2O_2.

RESULTS

Effect of Ceramide on the Respiratory Chain Redox Reactions. The effect of C_2-ceramide on oxygen consumption

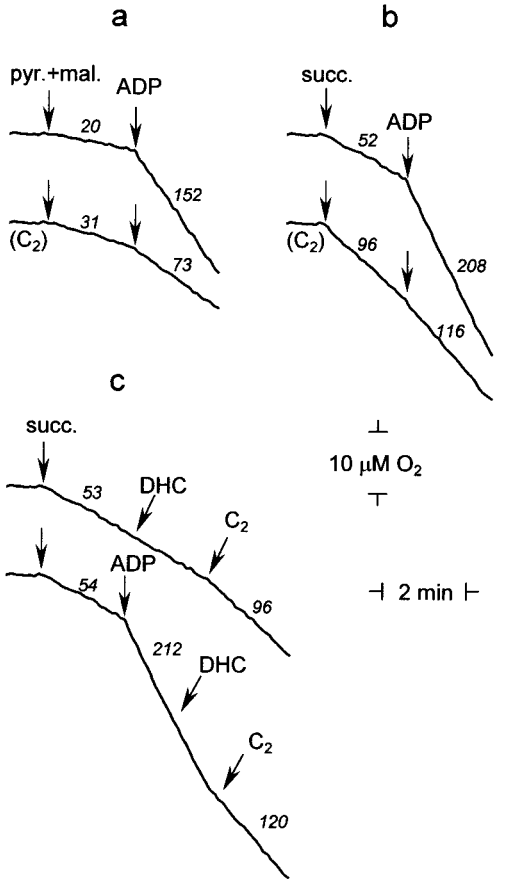

FIGURE 1: Effect of C_2-ceramide on mitochondrial oxygen consumption. Rat heart mitochondria were suspended in the reaction medium described under Materials and Methods. Oxygen consumption was initiated by the addition of the substrate pyruvate + malate (a) and succinate (b). After 2 min incubation with 20 μM C_2-ceramide (lower traces, vehicle in the control), state 4 respiration was started, followed by the addition of ADP. The effect of 20 μM DHC on succinate-supported respiration in both state 4 (c, upper trace) and state 3 (c, lower trace) is also shown. Numbers on the traces refer to the rate of oxygen consumption as nmol of $O_2 \cdot min^{-1} \cdot (mg\ of\ protein)^{-1}$.

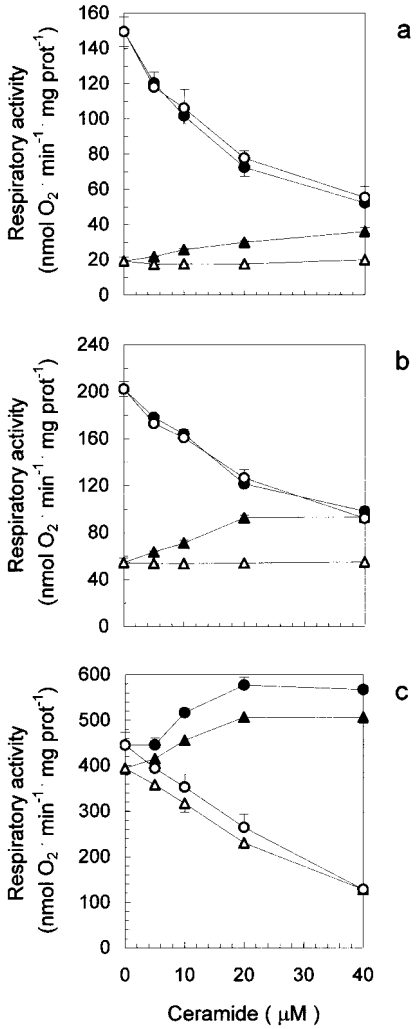

FIGURE 2: Effect of short- and long-chain ceramide on the respiratory activity of rat heart mitochondria. The respiratory activity of mitochondria was measured as described under Materials and Methods. Pyruvate + malate (a), succinate (b), and ascorbate/TMPD (c) were used as substrates. C_2-ceramide (filled symbols) and C_{16}-ceramide (open symbols) were incubated 2 min before state 4 respiration (triangles) was started. State 3 respiration (circles) was obtained by the addition of ADP. The values are the means ± SD from three to six different experiments.

by rat heart mitochondria was examined using pyruvate + malate or succinate as substrates. With both substrates, C_2-ceramide caused a dual effect: an uncoupling effect, as revealed by a net stimulation of state 4 respiration, and inhibition of ADP-stimulated (state 3) respiration (Figure 1a,b). A similar inhibitory effect was observed under uncoupler-stimulated respiration (not shown). As a result of these effects, C_2-ceramide caused a marked drop of the respiratory control ratio (RCR), i.e., state 3 vs state 4 respiratory rate. Figure 1c also shows that C_2-dihydroceramide (DHC), a closely related structural analogue of C_2-ceramide, lacking the 4,5-trans double bond in the sphingoid backbone and serving usually as negative control (*31*), did not cause any effect on both state 4 and state 3 respiration. Subsequent addition of C_2-ceramide gave rise to the same effects observed in the absence of DHC.

In the experiments summarized in Figure 2, a comparison is made of the effects elicited by short-chain, C_2, and long-chain, C_{16}-ceramide (*N*-palmitoylsphingosine), on the respiratory activity of mitochondria. The effects observed were as follows: (i) the inhibition by ceramides of coupled ADP-stimulated respiration, with both pyruvate + malate and succinate, exhibited the same concentration dependence (Figure 2a,b); (ii) C_{16}-ceramide, contrary to C_2, did not cause any stimulation under state 4 respiration with both substrates; (iii) C_2-ceramide, under the present experimental conditions, definitely stimulated the ascorbate/TMPD oxidase activity (cf. *32*), whereas C_{16}-ceramide caused a progressive inhibition (Figure 2c). State 3 and state 4 respiration are here similarly affected because of the very poor RCR measurable with ascorbate/TMPD (*33*). C_6-ceramide (*N*-hexanoylsphingosine) was also tested, and the results obtained closely reproduced those displayed by C_2-ceramide.

Work carried out to assess whether C_2-ceramide causes cytochrome *c* release from rat liver mitochondria has produced contrasting results (*32*, *34*). Figure 3 shows that the interaction of either short- or long-chain ceramides with rat heart mitochondria caused a large release of cytochrome *c*. Measurements by absorption spectroscopy of the amount of ceramide-dependent cytochrome *c* released gave an estimation of around 60% of its total mitochondrial content.

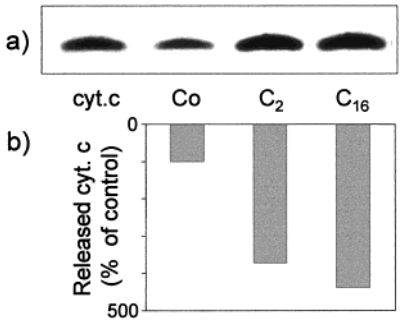

FIGURE 3: Ceramide-induced cytochrome *c* release. Rat heart mitochondria were treated with vehicle (Co), C_2-ceramide (C_2), and C_{16}-ceramide (C_{16}) as described under Materials and Methods. (a) Western blot analysis; the left lane (cyt.c) shows pure cytochrome *c*, as standard. (b) Semiquantitative analysis by densitometry of the bands. The values are reported as percent of the control band (mean of two experiments).

This average value, which refers to the whole mitochondrial population, is, most likely, representing an almost complete depletion process within a subfraction of the mitochondria being tested.

The experiments which follow were aimed at analyzing the effect of the addition of exogenous cytochrome *c* on ceramide-inhibited mitochondrial respiration. This approach is commonly used to demonstrate the release of cytochrome *c* which may have occurred and to evaluate the consequences of the process (*32*, *35*). The results obtained concerning the use of C_2-, C_6-, and C_{16}-ceramides are reported in Figure 4. It is shown that: (i) ceramide-inhibited succinate oxidase activity was substantially recovered by the addition of cytochrome *c* (Figure 4b), while the NADH-linked substrate oxidation was not (Figure 4a); (ii) as shown above, short-chain and long-chain ceramides show opposite effects on the ascorbate/TMPD oxidase activity. However, the C_{16}-ceramide-dependent inhibition of the oxidase activity could be partly released by added cytochrome *c* (Figure 4c,d).

Figure 5 reports experiments carried out using particles prepared by freezing and thawing of rat heart mitochondria. While succinate−cytochrome *c* reductase (complex II+III) and ubiquinol−cytochrome *c* reductase (complex III) were unaffected by ceramides (C_2 and C_{16}), the activities of both NADH−ubiquinone reductase (complex I) and NADH−cytochrome *c* reductase (complex I+III) were inhibited, consistent with the observed decrease of pyruvate+malate-supported respiration in intact mitochondria. Oxygen consumption measurements with mitochondrial particles showed that 20 μM samples of both C_2- and C_{16}-ceramide caused around 50% and 10−15% inhibition of NADH oxidase and durohydroquinone (or DBH) oxidase activities, respectively. The succinate oxidase activity, on the other hand, was almost unaffected (not shown). Complex IV activity, measured under these conditions, was slightly inhibited only by C_{16}-ceramide, consistent with the extent of inhibition remaining in intact mitochondria after the addition of exogenous cytochrome *c* (Figure 4c,d). These experiments also rule out the possibility that other mechanisms, including substrate transport and/or primary dehydrogenases, would be involved in the inhibition of complex I activity by ceramide.

The effect of ceramide on the activity of respiratory redox complexes isolated from bovine heart is shown in Figure 6. The activity of complex I (Figure 6a) was equally inhibited

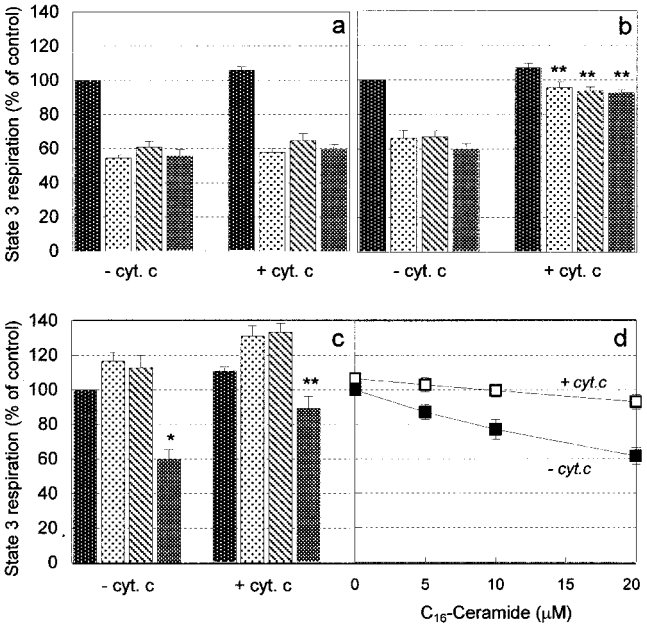

FIGURE 4: Effect of exogenous cytochrome *c* addition on ceramide-inhibited mitochondrial respiration. Oxygen consumption in rat heart mitochondria was measured with pyruvate + malate (a), succinate (b), and ascorbate/TMPD (c, d) as substrates. The effect of 20 μM C_2- (stippled bars), C_6- (cross-hatched bars), and C_{16}-ceramide (hatched bars) on state 3 respiration (vehicle in the control, white dots on black bars) was tested in the absence and in the presence of cytochrome *c*. Exogenous cytochrome *c* (0.2 μM) was added after 2 min incubation in the absence or in the presence of ceramide. The values reported are the means ± SD from three to five separate experiments. *, $p < 0.05$ versus control; **, $p < 0.05$ versus the corresponding values in the absence of added cytochrome *c*. In (d), the effect of cytochrome *c* on ascorbate/TMPD oxidase activity measured in the presence of various C_{16}-ceramide concentrations is reported (the values are the means ± SD of four determinations from two different mitochondrial preparations).

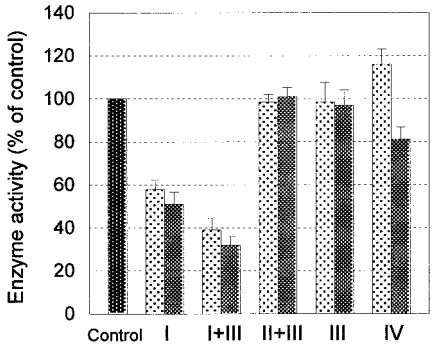

FIGURE 5: Effect of ceramide on the activity of respiratory enzymes in rat heart mitochondrial particles. The measurements of enzyme activities were performed as reported under Materials and Methods. The suspension was incubated 2 min with ethanol (control) or 20 μM ceramide (stippled bars, C_2; hatched bars, C_{16}). The values are the means ± SD from three different mitochondrial preparations. Specific activities [nmol·min^{-1}·(mg of protein)$^{-1}$] in the controls were 268.6 ± 8.7 for complex I, 212.5 ± 12.4 for complex I+III, 335.9 ± 6.9 for complex II+III, 1196 ± 42.3 for complex III, and 304.1 ± 6.5 for complex IV.

by either C_2- or C_{16}-ceramide. The purified complex I appears less sensitive to ceramide than the complex in the native membrane. This is probably due to the presence of sonicated soybean phospholipids (0.15 mg/mL), whose presence in the reaction medium was reported to be needed to obtain high activity and rotenone sensitivity (*21*). On the cytochrome *c*

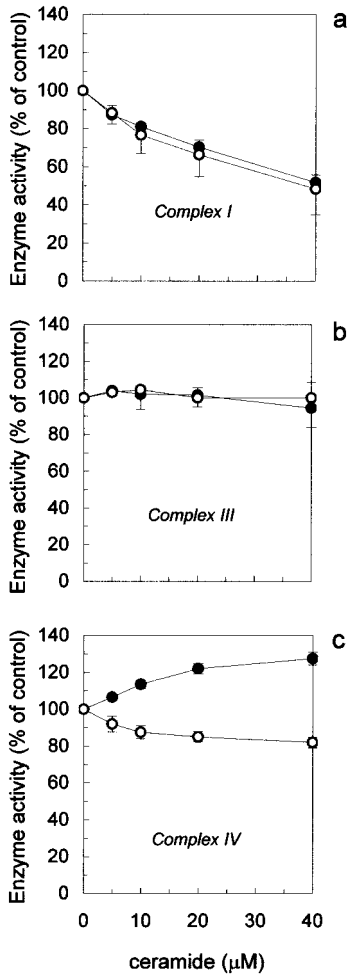

FIGURE 6: Effect of ceramide on redox activity of respiratory complexes isolated from bovine heart mitochondria. Enzyme suspension was incubated 2 min with the indicated concentrations of C_2-ceramide (filled circles) and C_{16}-ceramide (open circles) before the reaction was started. The values are the means ± SD from three different experiments. Specific activities [μmol·min^{-1}·(mg of protein)$^{-1}$] in the controls were 0.69 ± 0.1 for complex I, 13.2 ± 0.5 for complex III, and 2.83 ± 0.2 for complex IV.

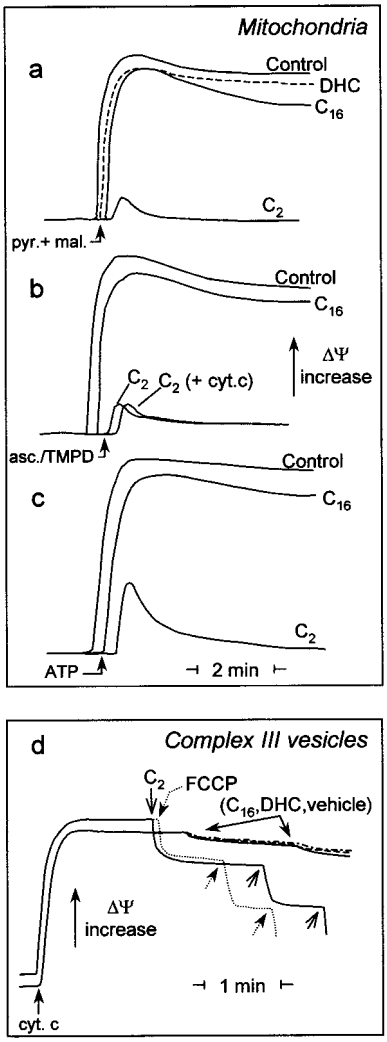

FIGURE 7: Effect of ceramide on transmembrane potential in rat heart mitochondria and complex III vesicles. Transmembrane potential of mitochondria was set up by the addition of pyruvate+ malate (a) or ascorbate/TMPD (b), under state 3 conditions, or by the addition of 1.5 mM ATP (c). The mitochondrial suspension was incubated 2 min with 20 μM C_2-ceramide, C_{16}-ceramide, or DHC (ethanol in the control) before the reaction was started. Where indicated, 0.2 μM cyt *c* was also added, as described in the legend to Figure 3. Measurement of transmembrane potential of complex III vesicles (d) was performed as described under Materials and Methods. Where indicated, ethanol (vehicle), 20 μM C_2-ceramide, C_{16}-ceramide, and DHC, or 0.8 nM FCCP were added.

oxidase activity (Figure 6c), opposite effects were produced by C_2-ceramide, which stimulated, and C_{16}-ceramide, which inhibited the enzyme activity.

As far as the bovine purified complex III is concerned, this enzyme was reported to be inhibited by C_2-ceramide (*15*). We have carried out these experiments and noted that, under the experimental conditions used in (*15*), there is a dose-dependent interaction between ceramide and cytochrome *c*, used as electron acceptor in the reaction, resulting in a hindrance of its reducibility even by sodium dithionite, as revealed by a smaller absorption increase in the α- and γ-regions of the spectrum. This may have been erroneously interpreted as ceramide inhibition of the activity of complex III. The lower the concentration of the enzyme used in the assay, the higher appears to be the contribution of the artifact. It has to be noted, however, that this subtle ceramide effect, also reported by others (*32*), could not be observed when cytochrome *c* was added to the mitochondrial suspension. Similarly, cytochrome *c* was completely dithionite-reducible when the isolated complex III suspending mixture was supplemented with a limited amount of detergent (0.05% Tween 80) or with sonicated soybean phospholipids (40 μg/mL of reaction mixture) or when the purified enzyme was reconstituted into phospholipid vesicles. When tested under these conditions, the ceramides (C_2 and C_{16}) did not display an appreciable inhibitory effect on purified complex III activity (Figure 6b).

Influence of Ceramide on Mitochondrial Membrane Potential. On the basis of the results reported above, it can be expected that the interaction of ceramide with mitochondria might affect the transmembrane potential ($\Delta\Psi$) by several mechanisms including: (i) inhibition of the activity of complex I, (ii) inhibitory effect by C_{16}-ceramide on the cytochrome *c* oxidase activity, (iii) ceramide-dependent release of cytochrome *c*, and (iv) the physicochemical properties of ceramide that might alter the membrane permeability.

Figure 7a shows traces of safranine fluorescence quenching ensuing upon the addition of pyruvate + malate to mito-

chondria preincubated in the absence or in the presence of either C_2- and C_{16}-ceramide. The presence of 20 μM C_2-ceramide in the reaction medium induced an almost complete collapse of the respiration-dependent transmembrane potential difference, while C_{16}-ceramide caused a much lower effect. C_6-ceramide caused an effect similar to that of C_2-ceramide (not shown). Considering that the short- and long-chain ceramides caused the same extent of inhibition of the NADH-linked substrate oxidation as well as of the activity of complex I (Figures 2a, 4a, 5, and 6a), it can be argued that the drop of $\Delta\Psi$ caused by C_2-ceramide is not related to the inhibition of electron flow. On the other hand, a partial inhibition of the rate of the electron transfer is not expected to cause necessarily a drop of $\Delta\Psi$, as indicated by several reports demonstrating that respiration can be substantially inhibited with only a small drop in the protonmotive force (see *36* for a review). Similar results were obtained with succinate (+rotenone) as substrate. Two more aspects are to be noted: (i) the presence of cytochrome *c* added exogenously did not reverse the C_2-ceramide-dependent $\Delta\Psi$ collapse (not shown); (ii) DHC, instead of C_2-ceramide, did not display any appreciable effect. The experiment shown in Figure 7b further supports this point. Here the effect of ceramide on ascorbate/TMPD oxidation-dependent membrane potential in antimycin-inhibited mitochondria is shown. C_2-ceramide, while stimulating the cytochrome *c* oxidase activity (Figures 2c, 4c, 5, and 6c), caused a drop of $\Delta\Psi$; C_{16}-ceramide, which on the contrary induced a partial inhibition of the enzyme redox activity, had a negligible effect. Again, externally added cytochrome *c* did not change, under these conditions, the effects observed (cf. *32*). Figure 7c represents an experiment in which $\Delta\Psi$ was generated by hydrolysis of ATP in mitochondria. The ATP-driven $\Delta\Psi$ was collapsed by C_2-ceramide, whereas C_{16}-ceramide had a negligible effect. In the experiment reported in Figure 7d, liposome-reconstituted complex III was suspended in the presence of purified cytochrome *c* oxidase and 300 μM durohydroquinone (more water soluble than DBH). Upon addition of cytochrome *c*, a membrane potential was generated, which attained a steady level. The addition of C_2-ceramide caused an FCCP-like decrease of the steady-state $\Delta\Psi$, whereas C_{16}-ceramide caused an effect similar to that elicited by DHC or by the vehicle. It should be noted that the liposomal system is definitely more enriched with lipids than the mitochondrial system (about 6 times in the present experimental conditions); thus, the effect of C_2-ceramide is consequently lower.

Reactive Oxygen Species Generation. Treatment of intact cells (*37*) as well as mitochondria isolated from rat liver (*14*) with permeant ceramides was shown to cause hydrogen peroxide production. The experiments which follow were then aimed at assessing the site-specificity of ROS production.

Addition of succinate to aerobic mitochondria in the nonphosphorylating state caused a large $\Delta\Psi$-dependent H_2O_2 production (Figure 8a), which was, in fact, uncoupler-sensitive and greatly decreased by the addition of ADP (Figure 8a, traces 1 and 5), as previously shown (*38*). The fact that this H_2O_2 production is also strongly inhibited by rotenone (trace 2) (see also *39*, *40*) has led to the conclusion that it is mainly coupled to the membrane potential-supported reverse electron transfer from succinate to complex I (*40*).

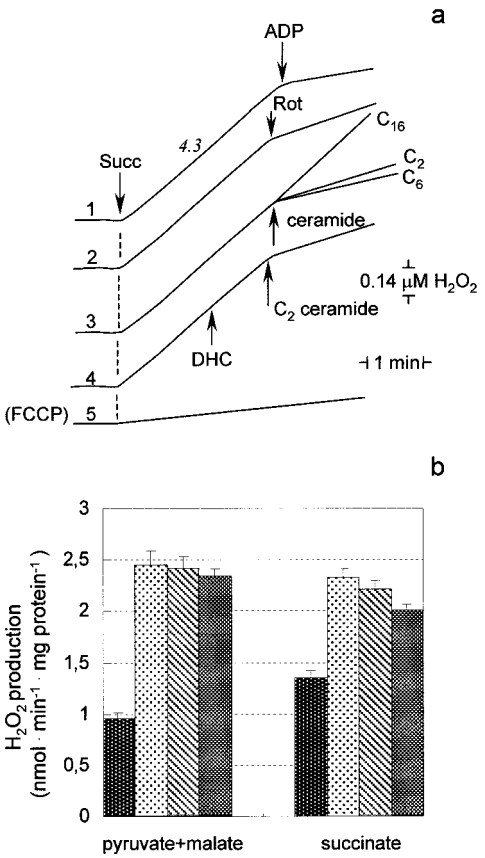

FIGURE 8: Effect of ceramide on H_2O_2 production in respiring rat heart mitochondria. Measurement of H_2O_2 production by mitochondria at the steady-state respiration was performed as described under Materials and Methods. (a) Succinate (7 mM) was used as substrate. Where indicated, 1 mM ADP (trace 1), 0.4 μg of rotenone (trace 2), 20 μM C_2-, C_6-, or C_{16}-ceramide (trace 3), and 20 μM DHC, followed by 20 μM C_2-ceramide (trace 4), 0.3 μM FCCP (trace 5) were added in the reaction mixture. The value on trace 1 represents the rate of H_2O_2 production as nmol·min^{-1}·(mg of protein)$^{-1}$. (b) The mitochondrial suspension supplemented with 1 mM ADP was incubated 2 min with 20 μM ceramide. Pyruvate + malate and succinate (in the presence of rotenone) were used as substrate. (White dots on black bars) control; (stippled bars) C_2-; (cross-hatched bars) C_6-; (hatched bars) C_{16}-ceramide.

C_2- and C_6-ceramides inhibited the H_2O_2 production, whereas C_{16}-ceramide even stimulated slightly the reaction (trace 3). Considering that all the above ceramides inhibit the activity of complex I to roughly the same extent, it is therefore obvious that the C_2-ceramide-dependent decrease of H_2O_2 production, measured under these conditions, has to be attributed to a drop of membrane potential.

ROS production associated with the forward oxidation of NADH-linked substrates is definitely lower (Figure 8b) (see also *39*, *40*). Short-chain as well as long-chain ceramides caused a substantial increase of ROS production, consistent with the inhibition they exerted on pyruvate + malate oxidation activity. The rate of H_2O_2 generation was enhanced by myxothiazol (not shown), this being considered diagnostic for ROS production at the level of complex I. Taking as the maximal potential rate of H_2O_2 production by complex I that measured after the addition of rotenone to pyruvate+malate-respiring mitochondria [which under our conditions was around 4.0 nmol of H_2O_2·min^{-1}·(mg of protein)$^{-1}$], then 20 μM ceramide can be evaluated to promote H_2O_2 production at a rate of more than 50% the maximal one.

Similarly, the ROS production associated with the forward electron flow supported by succinate (+rotenone) oxidation is stimulated by ceramides to almost the same extent (Figure 8 b). As also reported by others (*14*), here the reaction was potentiated by antimycin and decreased by myxothiazol (not shown), which is considered diagnostic for H_2O_2 production at the second site. DHC, when used instead of ceramide, did not cause any stimulation of ROS production, with both substrates (not shown).

DISCUSSION

In this paper the effect of ceramide on some mitochondrial functions is described, and a comparison is made of short- vs long-chain ceramide effects. Several independent observations presented here indicate that short-chain ceramides (C_2 and C_6) induce perturbation of membrane structure, leading to an increase of inner mitochondrial membrane permeability. The net increase by C_2-ceramide (and not by C_{16}) of state 4 respiration, observed with both pyruvate + malate or succinate as substrate (Figures 1 and 2), has to be interpreted accordingly.

In our experiments transmembrane $\Delta\Psi$ was set up under three different conditions: (i) by delivering electrons specifically to the three coupling sites of the respiratory chain in intact mitochondria; (ii) by ATP hydrolysis in mitochondria; and (iii) by feeding electrons to liposome-reconstituted complex III isolated from bovine heart. Altogether the results obtained indicate that the C_2-ceramide-dependent collapse of mitochondrial membrane potential is not a consequence of the inhibition of the electron-transfer activity; neither is it related to the release of cytochrome *c*. Rather, it appears to be a consequence of short-chain ceramide-dependent increase of the inner membrane permeability. Long-chain ceramide exhibited a much lower, if any, effect, at least in the time course of our experiments. Further support for this view can be derived from the effect on ROS production associated with $\Delta\Psi$-dependent reverse electron flow from succinate to complex I, which was strongly inhibited only by short-chain ceramides (Figure 8a). Therefore, the question raised by Hofmann and Dixit (*19*) on possible membrane structure perturbation by short-chain analogues of ceramide appears to be backed by these results. This aspect is of particular relevance when a function of short-chain ceramide in the induction of apoptosis is investigated, considering that an increase of mitochondrial membrane permeability is involved in the triggering of this process (*41*).

An interesting aspect emerging from these experiments concerns the use of DHC. This compound not only appears to lack the biochemical and biological activity of ceramide (see also *31*), but also does not share the physicochemical properties of ceramide, even in the artificial phospholipid system (Figure 7). It therefore appears as an inert molecule and, as such, cannot be considered straightforwardly as an internal negative control.

The interaction of ceramide with the respiratory chain components occurs at various levels. Short-chain as well as long-chain ceramide causes inhibition of pyruvate+malate-supported oxygen consumption in phosphorylating mitochondria (Figures 1 and 2a), inhibition of complex I and I+III activities in the native membrane (Figure 5), and inhibition of complex I isolated from bovine heart (Figure 6a). Succinate respiration activity of intact phosphorylating or uncoupled mitochondria was also inhibited (Figures 1 and 2b). This was not accompanied by a comparable inhibition of complex II+III and complex III activities in the native membrane of rat heart mitochondrial particles (Figure 5) or of complex III isolated from bovine heart mitochondria (Figure 6b). These results, together with the observation that the ceramide-inhibited succinate oxidase activity was almost completely restored upon addition of exogenous cytochrome *c* (Figure 4b), suggest that the inhibition of succinate-supported respiration has to be ascribed to ceramide-dependent cytochrome *c* release from mitochondria (Figure 3). This process, on the other hand, appears to be irrelevant on NADH-linked substrate oxidation (Figure 4a), whose rate is inhibited owing to the direct inhibitory effect of ceramide on complex I activity (Figures 5 and 6a).

The effect of ceramide on the activity of the cytochrome *c* oxidase varies with the chain length. C_2-ceramide caused a significant stimulation of the activity of the enzyme either in the membrane (Figures 2, 4c, and 5) or isolated from bovine heart (Figure 6c), whereas C_{16}-ceramide caused inhibition. The C_{16}-ceramide-dependent cytochrome *c* oxidase inhibition in mitochondria was partially curtailed by the addition of exogenous cytochrome *c* (Figure 4c,d). Our interpretation of these results is as follows: the C_2-ceramide-dependent release of cytochrome *c*, while limiting the succinate-supported oxygen consumption, does not influence the ascorbate/TMPD respiration, whose rate is even stimulated as a consequence of a direct stimulatory effect of the enzyme by C_2-ceramide. The C_{16}-ceramide-dependent cytochrome *c* release appears, on the other hand, to limit both the succinate respiration and, to lesser extent, the ascorbate/TMPD oxidase activity (Figure 4), the latter also being affected by a direct inhibitory effect of C_{16}-ceramide on the cytochrome *c* oxidase (Figures 5 and 6c).

The opposite effects of short- and long-chain ceramides on cytochrome *c* oxidase activity are currently under study in this laboratory.

Although the inhibition of complex I and cytochrome *c* release are likely the most relevant ceramide effects, the inhibition of cytochrome *c* oxidase by C_{16}-ceramide must not be disregarded, on the basis of reported experiments which show that in digitonin-permeabilized cells the oxidase activity is nearly limiting for ADP-stimulated glutamate + malate respiration (*33*).

Increased production of reactive oxygen species represents the other aspect of ceramide-dependent mitochondrial dysfunction. We have shown here for the first time that the direct interaction of naturally occurring ceramide with mitochondria causes an increase of ROS generation. In addition to those produced at the second site, potentiated by antimycin and abolished by myxothiazol, an even larger increase of ROS production is caused by ceramide-dependent inhibition of complex I (Figure 8b). As discussed in (*14*), ROS cause in turn depletion of the glutathione matrix pool, lipid peroxidation, and, at a longer time, a significant loss of cytochrome *c* oxidase activity. This subsequent inhibition, added to the ceramide-dependent direct inhibition of the cytochrome *c* oxidase and complex I, thus worsens the impairment of the mitochondrial respiratory function.

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