Ceramide Induces Cytochrome c Release from Isolated Mitochondria

IMPORTANCE OF MITOCHONDRIAL REDOX STATE*

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In the present study we show that N-acetylphosphoglycol (C2-ceramide), N-hexanoylphosphoglycol (C6-ceramide), and, to a much lesser extent, C2-dihydroceramide induce cytochrome c (cyto c) release from isolated rat liver mitochondria. Ceramide-induced cyto c release is prevented by preincubation of mitochondria with a low concentration (40 nM) of Bcl-2. The release takes place when cyto c is oxidized but not when it is reduced. Upon cyto c loss, mitochondrial oxygen consumption, mitochondrial transmembrane potential (ΔΨ), and Ca2+ retention are diminished. Incubation with Bcl-2 prevents, and addition of cyto c reverses the alteration of these mitochondrial functions. In ATP-energized mitochondria, ceramides do not alter ΔΨ, neither when cyto c is oxidized nor when it is reduced, ruling out a non-specific disturbance by ceramides of mitochondrial membrane integrity. Furthermore, ceramides decrease the reducibility of cyto c. We conclude that the apoptogenic properties of ceramides are in part mediated via their interaction with mitochondrial cyto c followed by its release and that the redox state of cyto c influences its detachment by ceramide from the inner mitochondrial membrane.

Recently, the importance of ceramide in cell metabolism has been broadly investigated. It is now evident that ceramide is involved as a second messenger in what has become known as the sphingomyelin cycle (1), apoptosis, and differentiation in many cell types (2). The mechanisms by which ceramide mediates apoptosis have not yet been fully addressed, however, it is known that mitochondria are targets of ceramide. Thus, direct inhibition of complex III of the mitochondrial respiratory chain by ceramide (3), ceramide-induced generation of reactive oxygen species in intact mitochondria (4) and in cells (5, 6), and ceramide-induced cell death via disruption of mitochondrial functions (7) are lines of evidence of the strong influence of ceramide on mitochondria.

Cytochrome c (cyto c)1 plays a dual role in cell homeostasis. As a part of the respiratory chain, it is needed for cell life, and as one of the triggers of apoptosis, it is needed for cell death. It is now well accepted that many apoptotic factors induce cell death via mitochondrial cyto c release (8). The released cytochrome switches on the death machinery, for example, by activation of caspases (9, 10). The anti-apoptotic protein, Bcl-2, was shown to prevent apoptosis both upstream (8) and downstream (11) of cyto c release.

In the present study we show that 1) C2-ceramide (N-acetylphosphoglycol), C6-ceramide (N-hexanoylphosphoglycol), and, to a much lesser extent, DHC (C2-dihydroceramide) release cyto c from isolated mitochondria, 2) ceramide-induced cyto c release occurs when cyto c is oxidized but not when it is reduced, 3) this release is prevented by Bcl-2, 4) cyto c release causes a decrease in mitochondrial oxygen consumption, transmembrane potential (ΔΨ), and Ca2+ retention, all of which are prevented by preincubation of mitochondria with Bcl-2 and reversed by addition of cyto c, and 5) ceramide interacts with cyto c and changes its reducibility.

EXPERIMENTAL PROCEDURES

C2- and C6-ceramide were obtained from Alexis Biochemicals (Lauffeléngen, Switzerland), DHC from Calbiochem (Lauffeléngen, Switzerland), horse heart cytochrome c from Sigma, mouse monoclonal cyto c antibody from RDI (Flanders, NJ), anti-mouse Ig and horseradish peroxidase from Amersham and His6-human Bcl-2 from Novartis (Basel, Switzerland). Ceramide stock solutions were prepared at a 500 times concentration in ethanol (containing 1% Me2SO) and kept at −20 °C. The vehicle always served as control.

Mitochondrial Preparation—Isolation of rat liver mitochondria was performed by differential centrifugation as described (12). The protein content of mitochondria and the mitochondrial supernatants were determined by the Biuret method with bovine serum albumin as standard.

Detection of Cytochrome c Release—Freshly isolated mitochondria (10 mg protein/ml) were incubated at room temperature in 0.1 M HEPES buffer, pH 7.0, containing aprotinin, pepstatin A, and leupeptin (1.5 μg/ml each). To investigate the effect of ceramide when cyto c is oxidized, mitochondrial respiratory chain complex III was blocked by 50 μM antimycin A (AA), and after 1 min, ceramide (20 μM) or the vehicle was added. Mitochondria were incubated for 2 min and then energized with 1 mM ascorbate (Asc) plus 0.4 mM tetramethyl-1,4-phenylenediamine (TMPD) (Asc/TMPD). The effect of ceramide on mitochondria when cyto c is reduced was studied by addition of ceramide 1 min after Asc/TMPD. Bcl-2 (40 nM) was added 5 min before AA. After 10 min of incubation at room temperature, mitochondria were spun at 12,000 × g for 10 min at 4 °C, and the resulting supernatant was spun at 100,000 × g for 15 min at 4 °C. The supernatant of the second centrifugation was used for the detection of cyto c either spectrophotometrically or by gel electrophoresis. Spectrophotometric measurements were done in a Varian Cray spectrophotometer. As the blank sample, 10 μg of mitochondrial protein was diluted in 1 ml of the buffer, mixed gently, and PAGE, polyacrylamide gel electrophoresis.

mide, N-acetylphosphoglycol; C2-ceramide, N-hexanoylphosphoglycol; DHC, C2-dihydroceramide; ΔΨ, mitochondrial transmembrane potential; PAGE, polyacrylamide gel electrophoresis.
Ceramide releases cytochrome c

RESULTS

Ceramide-induced mitochondrial cytochrome c release was determined by SDS-PAGE (Fig. 1A), by Western blotting (Fig. 1B), and spectrophotometrically (Fig. 1C). In these experiments, ceramide was added when cytochrome c was mainly oxidized, i.e., after blocking the complex III. Addition of ceramide to mitochondria when cytochrome c was mainly reduced, i.e., in the presence of Ascorbate/TMPD, did not increase the released cytochrome c, compared to the control (Fig. 1D). Preincubation of mitochondria with 40 μM Bcl-2 (4 pmol of Bcl-2/mg of mitochondrial protein) fully prevented the release of cytochrome c induced by ceramide (see Fig. 1B). When equal volumes (20 μl) of the supernatants of mitochondria incubated with ceramides were separated by SDS-PAGE, an increase in the total protein amount released into the supernatant was detected (Fig. 1A). When equal amounts (20 μg) of the released proteins were analyzed by Western blot, a specific increase in the cytochrome c was found (Fig. 1B).

To investigate the consequences of cytochrome c release on mitochondrial functions, we measured mitochondrial oxygen consumption, ΔΨ, and Ca^{2+} homeostasis. Fig. 2A shows that addition of ceramide to mitochondria when cytochrome c was oxidized decreased the oxygen consumption supported by Ascorbate/TMPD. Conversely, ceramide added to mitochondria when cytochrome c was reduced did not change the oxygen consumption (Fig. 2B). The decreased oxygen consumption caused by ceramide was prevented by preincubation of mitochondria with 40 μM Bcl-2 (Fig. 2C) and was reversed by the addition of 200 nM exogenous cytochrome c (200 pmol of cytochrome c/mg of mitochondrial protein) (Fig. 2D).

A decrease in ΔΨ is considered important when cells commit suicide (16). Fig. 3A shows that addition of ceramide to mitochondria when cytochrome c was oxidized caused a decrease in ΔΨ. This figure also shows that addition of 200 nM exogenous cytochrome c resulted in a full gain of ΔΨ. Incubation of mitochondria with 40 μM Bcl-2 prevented the loss of ΔΨ caused by ceramide (not shown). Fig. 3B shows that ceramide did not alter Δψ when cytochrome c was mainly reduced. When ΔΨ was built up as a consequence of ATP hydrolysis instead of respiration, ceramide did not change ΔΨ, neither when cytochrome c was oxidized nor when it was reduced (not shown).

Mitochondria are important calcium buffers in eukaryotic cells, and mitochondrial calcium release is involved in apoptosis (17). Fig. 4 shows that C2-ceramide, added to mitochondria when cytochrome c was mainly oxidized, caused a decrease in Ca^{2+} retention by mitochondria, in a Bcl-2 sensitive manner. The same results were obtained with C2-ceramide and, to a minor extent, with DHC (not shown). Addition of ceramide to mitochondria when cytochrome c was reduced did not change the mitochondrial Ca^{2+} homeostasis (not shown).

Reduction of cytochrome c can be followed photometrically. Fig. 5 shows that in the presence of C2-ceramide, the reduction of cytochrome c was fully abrogated by incubation of mitochondria with 5 μM DHC (for details, see under “Experimental Procedures”). The left lane of A and D shows 5 μg of pure cytochrome c (arrowhead).

Fig. 1. Ceramide-induced cytochrome c release from rat liver mitochondria. Cytochrome c release was assayed by Coomassie Blue staining (A), Western blotting (B), and spectrophotometry (C). In these experiments, ceramide was added when cytochrome c was oxidized. In D (Coomassie Blue staining), ceramide was added when cytochrome c was reduced. Mitochondria were treated with vehicle (Cont), C2-ceramide (C2), C6-ceramide (C6), or DHC (for details, see under “Experimental Procedures”). The left lane of A and D shows 5 μg of pure cytochrome c (arrowhead).
by Asc was hampered, as evidenced by the smaller increase in the optical densities of the \( \gamma \) - and \( \alpha \)-regions. Again, \( C_2 \)-ceramide was most effective, followed by \( C_6 \)-ceramide and DHC (not shown).

**DISCUSSION**

The present study shows that ceramide induces cyto c release from isolated mitochondria, an event strongly influenced by the redox state of cyto c, and that incubation of mitochondria with Bcl-2 prevents the cyto c release. Release of cyto c decreases mitochondrial oxygen consumption, \( \Delta \Psi \), and the Ca\(^{2+} \) buffering capacity of mitochondria, all of which are reversed by addition of exogenous cyto c. For all parameters measured, the observed rank order of potency is \( C_2 > C_6 > \text{DHC} \). This study also provides evidence for a possible direct interaction of ceramide and cyto c.

There is growing evidence that mitochondria are involved in apoptosis (16). The release of cyto c from mitochondria triggers apoptosis (18), and \( \Delta \Psi \) decreases during apoptosis (16, 19–21). Accordingly, prevention of cyto c release (22) as well as stabilization of \( \Delta \Psi \) (23) prevent apoptosis. Furthermore, mitochondria carry the pro- and antiapoptotic proteins, cyto c and Bcl-2. The presence of procaspase-3 in mitochondria was also shown recently (24).

Several mediators, pathways, and factors are involved in apoptosis (25, 26). Among them, ceramide has been shown to directly target mitochondria. Zhang et al. (27) showed that in Molt-4 leukemic cells, 6 h of incubation with \( C_2 \)-ceramide increased the cytosolic concentration of cyto c, which was preventable by overexpression of Bcl-2. In a study by Amarante-Mendes et al. (28) 6 h of incubation with \( C_2 \)-ceramide caused the cytosolic accumulation of cyto c in control but not in Bcr-Abl-overexpressing HL-60 cells. In the present study, we show that ceramide directly causes the release of cyto c from isolated mitochondria and accordingly hypothesize that cyto c is the

**FIG. 2. Mitochondrial oxygen consumption in the presence of ceramide.** 10 \( \mu \)M ceramide was added when cyto c was oxidized (A) or reduced (B). C, as for A except that mitochondria were preincubated for 5 min with 40 nM Bcl-2. D, as A except that 200 nM cyto c was added at the point marked by the asterisk. Mitochondria were treated with vehicle (Cont), \( C_2 \)-ceramide (C2), \( C_6 \)-ceramide (C6), or DHC (for details, see under “Experimental Procedures”). \( \nabla \), addition of AA; \( \triangleleft \), addition of Asc/TMPD.

**FIG. 3. Transmembrane potential of mitochondria exposed to ceramide.** Ceramide was added (arrows) when cyto c was oxidized (A) or reduced (B). Addition of 200 nM cyto c is indicated by asterisks (A). Mitochondria were treated with vehicle (Cont), \( C_2 \)-ceramide (C2), \( C_6 \)-ceramide (C6), or DHC (for details, see under “Experimental Procedures”).
prime mitochondrial target of ceramide. The fact that incubation of mitochondria with a low concentration of Bcl-2, used in this study, prevents ceramide-induced cyto c release and its consequences suggests a specific function of the oncoprotein in ceramide-mediated apoptotic signals.

To investigate a possible direct interaction of ceramide with cyto c, we primed Sepharose columns with the biologically active d-C2-ceramide or biologically inactive l-C2-ceramide.2 We observed that d-C2-ceramide but not l-C2-ceramide columns selectively retained cyto c and that the retained protein could be eluted with d-C2-ceramide. We also observed that reduced cyto c had a much lower affinity to d-C2-ceramide column, compared to the oxidized cyto c. Because of that observation together with the fact that ceramide affects the reducibility of cyto c (Fig. 5), we hypothesize that ceramide may directly interact with cyto c, with a higher affinity for the oxidized protein, and that this interaction changes the physicochemical properties of cyto c, leading to its rejection from mitochondria. The fact that cyto c has multiple lipid binding sites and that the lipid-bound cyto c shows a lower affinity for attachment to the artificial membranes (29) strengthens this hypothesis.

Under our experimental conditions, prevention of mitochondrial cyto c reduction is paralleled by the disappearance of ∆Ψ. To distinguish which of these two is decisive for ceramide-induced cyto c release, we investigated by Western blot analysis whether cyto c is released because of ∆Ψ collapse. We found that the absence of ∆Ψ, achieved by blocking respiration and uncoupling of mitochondria, does not result in cyto c release, indicating that binding of cyto c to the outer side of the inner mitochondrial membrane does not require ∆Ψ. These results also indicate that the release of cyto c by ceramide is not a consequence of a nonspecific solubilization of mitochondrial membranes due to the lipophilicity of ceramide, but rather is a specific event.

The release of other mitochondrial proteins apart from cyto c is also increased upon treatment with ceramide (Fig. 1A). We argue that this apparently nonspecific protein release by ceramide is due to a general weakening of mitochondria upon cyto c release. Fig. 1D supports this argument by showing that when ceramide does not cause cyto c loss, other mitochondrial proteins are also not released. This notion, together with the fact that addition of exogenous cyto c reverses all the altered mitochondrial functions, leads us to conclude that mitochondrial cyto c is a prime target for ceramide. This conclusion does not rule out other possibilities, for example, the modification of Bcl-2 by ceramide.

In contrast to the finding that binding of cyto c to the inner mitochondrial membrane is not a function of ∆Ψ, the formation and the maintenance of ∆Ψ is dependent on the presence of a functional cyto c (cf. Fig. 3A). Disruption of ∆Ψ was shown to be involved in ceramide-induced apoptosis (30). According to our study, cyto c has a critical role in ∆Ψ formation, in that stabilization of cyto c stabilizes ∆Ψ. Bcl-2 is located at the outer mitochondrial membrane (31) and has been shown to stabilize ∆Ψ (23). It is known that Bcl-xL, another oncoprotein with mitochondrial location, binds cyto c (32). Based on the present results and the close proximity of Bcl-2 to mitochondrial cyto c, it may be speculated that Bcl-2 stabilizes ∆Ψ by preventing cyto c loss.

Nonspecific solute transport across the inner mitochondrial membrane, operated via a Bcl-2-sensitive megachannel (the “permeability transition pore”) is considered to be the reason for the collapse of ∆Ψ and many other features of apoptosis (reviewed in Ref. 33). It was also reported that operation of such a pore causes cyto c release, and thus it was concluded that cyto c release occurs downstream to pore formation and ∆Ψ collapse (34). Because addition of cyto c reverses the ceramide-induced decrease in mitochondrial oxygen consumption, ∆Ψ, and Ca2+-maintaining capacity (see Figs. 2–4), we argue that ceramide-induced apoptosis is not mediated via opening a nonspecific megachannel or pore. The fact that ceramide does not affect ∆Ψ when it is supported by ATP gives further support to this argument.

It was reported by Gudz et al. (3) that ceramide decreases the
activity of complex III of the mitochondrial respiratory chain, as deduced from the measurement of mitochondrial oxygen consumption supported by Ase7TMPD. Cyto c shuttles electrons between complex III and IV, and oxygen is consumed at the level of complex IV. Figs. 2D and 3A show that the addition of cyto c to mitochondria recovered the decreased respiration and the reduced ΔΨ. Therefore, we conclude that parts of the ceramide-induced reduction of complex III activity is due to cyto c loss.

Garcia-Ruiz et al. (4) showed that ceramide induces superoxide formation in isolated rat liver mitochondria. From the elegant recent study by Cai and Jones (35), it is evident that superoxide formation by mitochondria is a consequence, and not the cause, of cyto c loss. Release of cyto c from mitochondria produces a gap in the hierarchically arranged mitochondrial respiratory complexes III and IV, and therefore a site for electron leakage. Present finding explains the ceramide-induced superoxide formation, reported by Garcia-Ruiz et al. (4), and supports the hypothesis that cyto c releases in mitochondria is a consequence, not the cause, of cyto c loss. Release of cyto c from mitochondria is accompanied by increased reactive oxygen species formation in mitochondria and that both events are prevented by ceramide-induced reduction of complex III activity, which is due to cyto c release.

We reported (23) that TNF-α added to cells induces a drop in ΔΨ that is accompanied by increased reactive oxygen species formation in mitochondria and that both events are prevented by ceramide-induced reduction of complex III activity, which is due to cyto c release. It was reported by Hampton et al. (42) that cyto c does not necessarily need to be reduced to activate caspases. The investigators acknowledged, however, that they were unable to keep cyto c oxidized in the presence of cytosolic extracts. In the present study, we show that oxidized cyto c is released by ceramide (Fig. 1, A–C) and that ceramide decreases the reducibility of oxidized cyto c (Fig. 5). Regarding the fact that both ceramide and cyto c are strong mediators of caspase-induced apoptosis (43), the possible interaction of ceramide and cyto c may thus amplify the progression of the apoptotic cascade.

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