Direct Inhibition of Mitochondrial Respiratory Chain Complex III by Cell-permeable Ceramide*

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Ceramide is a lipid second messenger that mediates the effects of tumor necrosis factor-α and other agents on cell growth and differentiation. Ceramide is believed to act via activation of protein phosphatase, proline-directed protein kinase, or protein kinase C. Tumor necrosis factor-α-induced common pathway of apoptosis is associated with an early impairment of mitochondria. Herein, we demonstrate that ceramide can directly inhibit mitochondrial respiratory chain function. In isolated mitochondria, a rapid decline of mitochondrial oxidative phosphorylation occurs in the presence of N-acetyl-sn-sphingosine (C2-ceramide), a synthetic cell-permeable ceramide analog. An investigation of the site of ceramide action revealed that the activity of respiratory chain complex III is reduced by C2-ceramide with half-maximum effect at 5–7 μM. In contrast, N-acetylphospho-N-6-ceramide, which lacks a functionally critical double bond and is ineffective in cells, did not alter mitochondrial respiration or complex III activity. We suggest that these in vitro observations may set the stage for identifying a novel mechanism of regulation of mitochondrial function in vivo.

The pro-inflammatory cytokine tumor necrosis factor-α (TNF-α) elicits a wide variety of cellular responses including profound alterations in transcriptional programs, perturbation of mitochondrial function, and apoptosis in a number of cell types (1, 2). Strong evidence supports a pivotal role for TNF-α in the genesis of septic shock (2), and it also has been implicated in ischemia reperfusion injury of heart (3). A recent report demonstrates that a physiologically relevant concentration of TNF-α induced apoptosis in rat cardiomyocytes as quantified by single cell microgel electrophoresis of nuclei and in situ 3’ nick end labeling assay (4). Mitochondria are considered an early target in TNF-α-induced cytotoxicity because they appear swollen with a reduced number of cristae, in association with profound inhibition of mitochondrial respiration (1, 5, 6). A growing body of evidence suggests that treatment of cells with TNF-α results in an electron transport inhibition at the level of complex III (1, 6) followed by an increased generation of oxygen radicals in mitochondria (7–9). However, the mechanism of TNF-α-induced inhibition of mitochondrial respiration has not been elucidated.

The sphingomyelin pathway has been implicated as a major signaling mechanism mediating the action of a number of extracellular agents (such as TNF-α, Fas ligands, and chemotherapeutic agents) causing the activation of sphingomyelinases that cleave membrane sphingomyelin resulting in the formation of ceramide (10, 11). Synthetic cell-permeable ceramide analogs have been shown to mimic many TNF-α-induced cell responses (10–13). In malignant and nonmalignant cell lines, ceramides specifically induce apoptosis that involves activation of interleukin-1β-converting enzyme-like proteases, whereas closely related dihydro-analogues are inactive (10). The intracellular targets for ceramide are poorly understood. Some of the cellular activities of ceramide appear to be mediated by ceramide-activated protein phosphatase (CAPP), proline-directed protein kinase, and protein kinase C (10, 14, 15).

Given the multiplicity of TNF-α cellular effects that involve the activation of several signal transduction pathways, the impairment of mitochondrial respiration could have causative links with the sphingomyelin signaling pathway. Alternatively, it could occur downstream of ceramide, for example, via the phosphorylation/dephosphorylation mechanism driven by CAPP. We propose that mitochondrial dysfunction results from the direct interaction of ceramide with mitochondrial respiratory chain. It is noteworthy that physiologically relevant and direct targets of ceramide are affected by ceramide in vitro, and only they could mediate the most proximal effects of ceramide in cells (10). To gain an insight into the mechanism of mitochondrial damage in TNF-α-treated cells, we tested the hypothesis that mitochondrial function is directly modulated by ceramide using isolated mitochondria.

MATERIALS AND METHODS

Reagents—N-Acetylsphingosine (C1-ceramide), N-acetylphospho-N-6-ceramide (C2-dihydroceramide), and N-hexanoylsphingosine (C6-ceramide) were obtained from Matreya. Decylubiquinone, cytochrome c, myelin basic protein, okadaic acid, and H7 were obtained from Sigma. Other chemicals were obtained from commercial sources and were reagent grade or better.

Preparation of Mitochondria—Rat heart mitochondria were isolated as described previously (16), except that a modified Chappell-Perry buffer (Buffer A: 100 mM KCl, 50 mM MOPS, 1 mM EGTA, 5 mM MgSO4, 1 mM ATP, pH 7.4) was used for mitochondrial isolation. Briefly, cardiac tissue was finely minced, placed in buffer A supplemented with 0.2% bovine serum albumin (BSA), and homogenized with a Polytron tissue processor (Brinkman Instruments Inc.) for 2.5 s at a reostat setting of 6.0. The polytron homogenate was centrifuged at 500 × g to separate the subsarcolemmal mitochondria (SSM) from myofibrils. The supernatant containing the SSM was saved, and the pellet was washed by resuspension in the buffer A supplemented with 0.2% BSA and recentrifuged at 500 × g. The combined supernatants were centrifuged at 3000 × g for 10 min to sediment SSM, washed twice, and then sus-
pended in a small volume of buffer B (100 mM KCl, 50 mM MOPS, 0.5 mM EGTA, pH 7.4). The myofibrillar pellet containing the interfibrillar mitochondria (IFM) was resuspended in buffer A, nagarse was added to a final concentration of 5 mg/g weight of tissue, and the suspension was immediately homogenized with Potter-Elvehjem homogenizer. Termination of the action of nagarse was accomplished by the addition of buffer A containing 0.2% BSA, followed by centrifugation at 5000 × g. The pellet was resuspended in buffer A containing 0.2% BSA and centrifuged at 5000 × g for 10 min to pellet IFM. IFM were washed twice and suspended in a small volume of buffer B. Protein concentration was assayed by Biuret method with BSA as the standard.

Rat skeletal muscle mitochondria were isolated essentially as described (16) for interfibrillar subpopulation of heart mitochondria. Rat liver mitochondria were isolated as described (17). HL-60 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with a nutrient mixture of Ham’s F-12 (Sigma) containing 10% fetal calf serum plus an antibiotic/antimicrobial mixture (Life Technologies, Inc.) and incubated at 37 °C with 5% CO2.

Mitochondrial Respiration—Oxygen consumption by intact mitochondria was measured in a chamber equipped with a Clark-type oxygen electrode (Yellow Springs Instrument Co.) at 30 °C. The incubations contained 0.5 mg/ml mitochondrial protein in 80 mM KCl, 50 mM MOPS, 5 mM KH2PO4, 1 mM EGTA, 0.1% BSA, pH 7.4. After depletion of endogenous substrates by the addition of ADP, the substrate was added to the incubation. Then state 3 respiration was initiated by the addition of ADP or the uncoupled respiration rate was determined in the presence of the uncoupler, 2,4-dinitrophenol.

Mitochondrial Oxidase Activities—Mitochondrial oxidase activities were determined with freeze-thawed mitochondria as described (18) and were performed at 30 °C using an oxygen electrode. Incubations contained 20 mM KH2PO4, 0.1 mM EDTA, 0.32 mM oxidized cytochrome c, mitochondrial protein, and substrates, which were added last. Activities were determined in the absence and in the presence of specific inhibitors. NADH oxidase activity was measured with 2.8 mM NADH and 7.5 μM rotenone as a coupler. Durohydroquinol oxidase was measured with 2 mM durohydroquinol and 18 μM antimycin A as an inhibitor. Cytochrome c oxidase was measured in the presence of 10 mM ascorbate and 1 mM N,N,N′,N′-tetratetramethyl-p-phenylenediamine, with and without 2 mM sodium azide as inhibitor.

Complex III Activity—Complex III (decylubiquinol:ferricytochrome c oxidoreductase) activity was measured using a diode array spectrophotometer by following the increase in reduced cytochrome c absorbance as described (19). Mitochondria (1 mg/ml) were solubilized with 2% sodium cholate in 25 mM KH2PO4, pH 7.4, diluted 10-fold with 25 mM KH2PO4, pH 7.4, and this diluted solution was used for the measurement of complex III activity. The assay mixture contained 0.1% bovine serum albumin, 0.1 mM EDTA, 60 μM cytochrome c, 3 mM sodium azide, and 50 mM KH2PO4, pH 7.4. The mitochondrial sample (2 μg of mitochondrial protein) or 0.07 μg of purified complex III or 60 μg of cellular protein was added last into 1 ml of the assay mixture followed by the addition of cytochrome c as substrates. After a 2-min equilibration period, the reaction was started by the addition of 100 μM decylubiquinol, and the increase in absorbance at 550 nm was monitored for 1 min. The initial rate was linear during the first 60 s. The activity was measured with and without 18 μM antimycin A in parallel cuvettes. The antimycin A-insensitive component, which represents nonenzymatic reduction of cytochrome c, was less than 20% of the total activity. The activity was calculated using an extinction coefficient of 19.1 μM−1 cm−1 for reduced cytochrome c and was expressed as the antimycin A-sensitive rate.

RESULTS AND DISCUSSION

Treatment of isolated heart mitochondria oxidizing glutamate in the presence of ADP (state 3) with C2-ceramide resulted in immediate inhibition of ATP synthesis coupled respiration (Fig. 1). Preincubation of mitochondria with C2-ceramide for 1–5 min did not increase the effect (data not shown). There was no change in the state 2 (only substrate for respiration is present) and state 4 (ADP-limiting) respiration rates in mitochondria treated with C2-ceramide at a concentration of 1–50 μM, compared with control (data not shown). Therefore, the permeability of the mitochondrial inner membrane was not affected by C2-ceramide under this condition.

The sensitivity of mitochondrial oxidative phosphorylation (state 3 respiration) to ceramide inhibition varied depending upon the tissue. Liver mitochondria were most sensitive (IC50 = 20 μM), followed by subsarcolemmal and interfibrillar heart mitochondria (IC50 = 28–31 μM). Mitochondria isolated from skeletal muscle were the least sensitive to ceramide with IC50 = 42 μM.

In our experiments, glutamate was used as the substrate for mitochondrial respiration. Glutamate enters mitochondria via a specific transporter and is metabolized in the mitochondrial matrix by glutamate dehydrogenase generating NADH that in turn is oxidized by respiratory chain enzymes resulting in formation of ATP. The C2-ceramide-induced decrease in the state 3 respiration rate could be due to inhibition of substrate transporter, glutamate dehydrogenase activity, inhibition of respiratory chain enzyme activity(s), and/or inhibition of ATP synthesis machinery (ATP synthase, adenine nucleotide translocase, and phosphate transporter). To exclude the ATP synthesis machinery as a potential site of ceramide action, we treated mitochondria with C2-ceramide in the presence of uncoupler (2,4-dinitrophenol). The dose response of uncoupled mitochondria in the presence of C2-ceramide was almost identical to that seen with phosphorylating mitochondria (Fig. 2). These results suggest that the substrate transporter, glutamate dehydrogenase, or the respiratory chain enzymes could be the sites of C2-ceramide attack.

To rule out the possibility that C2-ceramide affects substrate transporter and glutamate dehydrogenase, we used mitochondria that had been subjected to a freeze-thawing cycle that makes the mitochondria permeable to NADH. Thus, in permeabilized mitochondria, NADH can be used as a substrate and is readily oxidized by respiratory chain enzymes. NADH oxida-
Ceramide Inhibition of Complex III

Mitochondrial oxidase activities in the presence of C2-ceramide. Frozen-thawed rat heart interfibrillar mitochondria were incubated as described under "Materials and Methods." Activity was expressed as inhibitor-sensitive rate. Specific activities in control were as follows: NADH oxidase, 1680 nM/min/mg protein; duroquinol (DHQ) oxidase, 2510 nM/min/mg protein; cytochrome c oxidase, 4310 nM/min/mg protein. The results are representative of three independent experiments.

Inhibition of mitochondrial complex III activity by C6- and C2-ceramide. Rat heart interfibrillar mitochondria were incubated as described under “Materials and Methods.” The activity was expressed as the antimycin A-sensitive rate. The values are the means ± S.D. pooled from four mitochondrial preparations. Specific activities in control were 4640 ± 480 millunits/mg protein.

isolated from the beef heart mitochondria and purified to homogeneity (Fig. 5). The activity of the purified complex III was decreased in concentration-dependent fashion up to 93% in the presence of 20 μM C6-ceramide (Fig. 5, curve 2) with half-maximum effect at 7 μM.

Next, it was important to establish specificity of ceramide inhibition of mitochondrial complex III. Low concentrations of C6-ceramide (1–5 μM), another synthetic cell-permeable ceramide analog, were as effective as C2-ceramide (Fig. 4). However, greater concentrations of C6-ceramide did not produce further reduction of complex III activity. The reason for such an interaction of C6-ceramide with the complex III is unclear and is currently under investigation in this laboratory. A close structural analog of C2-ceramide, C2-dihydroceramide, lacks the 4,5-trans-double bond in the sphingoid backbone, which has been shown to be critical for imparting the biological activity of ceramide (12). C2-dihydroceramide (5–20 μM) did not affect the complex III activity (Fig. 4 and Fig. 5, curve 1) nor oxidative phosphorylation (data not shown). Therefore, the ability of C2-ceramide to inhibit complex III activity is due to specific

**Fig. 2.** Effect of C2-ceramide on respiration of phosphorylating and uncoupled mitochondria. Oxygen consumption was measured in intact rat heart interfibrillar mitochondria as described under “Materials and Methods” with 10 mM glutamate as a substrate. 1, mitochondria respiring in the presence of 2 mM ADP; 2, mitochondria uncoupled by 150 μM of DNP. The values are the means ± S.D. from three separate mitochondrial preparations. Rates of oxygen consumption in control were 388 ± 26 nM/min/mg of protein.

**Fig. 3.** Mitochondrial oxidase activities in the presence of C2-ceramide. Frozen-thawed rat heart interfibrillar mitochondria were incubated as described under “Materials and Methods”. Activity was expressed as inhibitor-sensitive rate. Specific activities in control were as follows: NADH oxidase, 1680 nM/min/mg protein; duroquinol (DHQ) oxidase, 2510 nM/min/mg protein; cytochrome c oxidase, 4310 nM/min/mg protein. The results are representative of three independent experiments.

**Fig. 4.** Inhibition of mitochondrial complex III activity by C6- and C2-ceramide. Rat heart interfibrillar mitochondria were incubated as described under “Materials and Methods.” The activity was expressed as the antimycin A-sensitive rate. The values are the means ± S.D. pooled from four mitochondrial preparations. Specific activities in control were 4640 ± 480 millunits/mg protein.
FIG. 5. C2-ceramide inhibits the activity of purified complex III. Complex III isolated from beef heart mitochondria was incubated as described under “Materials and Methods.” The activity was expressed as the antimycin A-sensitive rate. I, in the presence of C2-dihydroceramide; 2, in the presence of C2-ceramide. The values are the means ± S.D. from three measurements. Similar results were obtained in two independent experiments.

structural requirements as opposed to nonspecific hydrophobic interactions.

Evidence has recently been provided that ceramide contents in mitochondria isolated from the cells exposed to TNF-α are greatly elevated (21). The mechanism underlying the accumulation of ceramide in mitochondria is unclear, although the data indicate that ceramide is not locally produced. Having demonstrated the direct inhibition of complex III activity by C2-ceramide in isolated mitochondria, we sought to determine if treatment of the cells with TNF-α or C2-ceramide inhibits mitochondrial complex III activity. The HL-60 cells were incubated in Dulbecco’s modified Eagle’s medium (without serum) with 15 μM C2-ceramide or 80 ng/ml of TNF-α at 37 °C for 3 h and then washed with phosphate-buffered saline and collected in 25 mM potassium phosphate buffer, pH 7.4, containing 2% sodium cholate. The activity of complex III was 35 and 45% lower in the cells treated with C2-ceramide and TNF-α, respectively, compared with control. Taken together these data suggest that the inflammatory cytokines such as TNF-α leads to increased generation of ceramide followed by ceramide trafficking to mitochondria where ceramide interacts directly with complex III.

This study further supports the notion that ceramide is a new modulator of respiratory chain function in mitochondria. An appreciation of the mechanisms by which mitochondria are integrated in cellular metabolism has evolved considerably in several decades of intensive investigation. But, the signal transduction pathways regulating mitochondrial function are just being explored. It has been established that Ca2+ and cAMP are the second messengers that control mitochondrial enzyme activities such as matrix dehydrogenases and oxidative phosphorylation (22, 23). Our data indicate that sphingomyelin-dependent signal transduction pathway may participate in regulation of vital mitochondrial function.

The data presented here suggest that mitochondrial respiratory complex III is a novel direct target of ceramide in cells. Our findings demonstrating the inhibition of complex III by ceramide in mitochondria have extended previous observations that mitochondrial respiration is impaired in the cells treated with TNF-α (1, 5, 6) and have also defined the mechanism of TNF-α effect on mitochondria. A rapid response of isolated mitochondria and purified complex III to ceramide treatment seems to exclude the involvement of intermediates. The concentrations of C2-ceramide that were required for complex III inhibition are similar to those employed in studies of the effects of ceramide in vivo (Refs. 13, 24, and 25; reviewed in Ref. 10). The profound inhibition of complex III activity by C2-ceramide but not by C2-dihydroceramide, which also is active in cells, implicates complex III as a potential mediator of proximal ceramide effects in cells.

Whereas the specific mechanism whereby mitochondrial complex III can transduce the effects of ceramide is not known, there is a possibility that generation of free radical plays a role. Mammalian complex III is an 11-subunit protein complex that links proton translocation to electron transfer from ubiquinol to cytochrome c by the proton motive Q cycle mechanism (26, 27). Complex III has been established as an important source of oxygen radical production in the mitochondrial respiratory chain, the radicals being formed at the level of ubiquinone (28). Inhibitors of cytochrome b reoxidation via center i in the Q-cycle (antimycin A, for example) potentiate autooxidation of unstable ubisemiquinone resulting in an increased oxygen radical generation (29). Whether ceramide affects cytochrome b reoxidation in the Q-cycle with subsequent generation of oxygen radicals certainly requires further investigation. This is in line with the recent study by Garcia-Ruiz et al. (21) where C2-ceramide treatment of isolated mitochondria led to the generation of hydrogen peroxide, which was dependent on the electron transport in the respiratory chain. In TNF-α- and C2-ceramide-treated cells, increased mitochondrial oxygen radical formation was also detected (7, 9, 21).

Additionally, a recent report has described the opening of mitochondrial permeability transition pore (PTP) as an essential feature of the TNF-α cytotoxicity in L929 fibroblasts. Ceramide was able to replace TNF-α both in inducing PTP and in killing the cells (30). Thus, these data support the role of ceramide in the mitochondrial effects of TNF-α. In isolated mitochondria, the opening of PTP results in dissipation of an inner mitochondrial membrane potential, uncoupling of oxidative phosphorylation (increase in respiratory state 4 rate), loss of low molecular solutes from mitochondrial matrix, and swelling of mitochondria (31). In our experiments, the uncoupling of oxidative phosphorylation that is suggestive of PTP opening was not detected, whereas complex III activity was reduced following ceramide treatment of mitochondria. This observation raises the possibility that mitochondrial PTP opening is proximal to ceramide binding to complex III. It is noteworthy that this pore can develop in isolated mitochondria and cells under the oxidative stress condition associated with increased free radical generation (32). Also, antioxidants can prevent the PTP opening (33). Certainly, it remains to be determined whether ceramide binding to complex III causes excessive free radical generation that favors the subsequent PTP opening or ceramide interacts with multiple sites in mitochondria via different mechanisms. A clearer understanding of the mechanisms responsible for modulation of mitochondrial function by ceramide may prove critical to the study of signal transduction pathways involved in growth suppression and apoptosis.

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