Implication of Mitochondrial Hydrogen Peroxide Generation in Ceramide-induced Apoptosis*

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The key events implicated in ceramide-triggered apoptosis remain unknown. In this study we show that 25 μM C6-ceramide induced significant H₂O₂ production within 60 min, which increased up to 180 min in human myeloid leukemia U937 cells. Inactive analogue dihydro-C6-ceramide had no effect. Furthermore, no H₂O₂ production was observed in C6-ceramide-treated U937 ρ⁺ cells, which are mitochondrial respiration-deficient. We also present evidence that ceramide-induced activation of the transcription factors NF-κB and AP-1 is mediated by mitochondrial derived reactive oxygen species. Both H₂O₂ production, transcription factor activation as well as apoptosis could be inhibited by rotenone and thenoyl-trifluoroacetone (specific mitochondrial complexes I and II inhibitors) and antioxidants, N-acetylcysteine and pyrrolidine dithiocarbamate. These effects could be potentiated by antymycin A (specific complex III mitochondrial inhibitor). H₂O₂ production was also inhibited by ruthenium red, suggesting a role of mitochondrial calcium homeostasis alterations in ceramide-induced oxidative stress. Finally, C6-ceramide had no influence on mitochondrial membrane potential within the first 6 h. Altogether, our study points to reactive oxygen species, generated at the ubiquinone site of the mitochondrial respiratory chain, as an early major mediator in ceramide-induced apoptosis.

Ceramide has emerged as a potentially important mediator of a number of natural or pharmacological agents that affect cell growth, viability, and differentiation (1, 2). This lipid second messenger is the breakdown product of sphingomyelin (SPM) generated by the activation of a neutral and/or an acidic sphingomyelinase. Agonists of the SPM-ceramide pathway include: cytokines or growth factors such as tumor necrosis factor-α (TNF-α) (3), interleukin-1β (4), γ-interferon (5), nerve growth factor (6); antibodies directed against functional molecules such as Fas/APO-1 (7) or CD28 (8) proteins; as well as stress-inducing agents such as UV (9) and ionizing radiation (10, 11); antileukemic agents (12, 13); and H₂O₂ (9). The observation that cell-permeant ceramides or natural ceramide (generated by treating cells with bacterial sphingomyelinase) could mimic the biological effects of most SPM-ceramide cycle agonists has provided significant weight as to the role of ceramide in signal transduction. Finally, ceramide has been shown to exert a wide range of biological effects, depending on the cellular model, including cell activation, mitogenic signaling, survival promoting effect, growth inhibition, and apoptosis. As an example, ceramide has been described to induce growth inhibition in Molt-4 cells (14), proliferation in fibroblasts (15), and apoptosis in many other cellular models such as U937 (3) and lymphoblastoid (16) and endothelial (10) cells.

The multiplicity of biological activities of ceramide suggests that it possesses several downstream targets, which, in turn, mediate distinct intracellular pathways. Among these targets there may be a serine-threonine phosphatase termed ceramide-activated protein phosphatase (17) and/or a proline-directed protein kinase termed ceramide-activated protein kinase (18), which, in turn, could activate the mitogen-activated protein kinase cascade in U937 cells (19). Ceramide has also been described to activate stress-activated protein kinases (SAPK/c-Jun kinase), which may be more closely related to ceramide-induced apoptosis in U937 and in endothelial cells (9). In addition, ceramide has been shown to activate transcription factors such as NF-κB (20) and AP-1, which could also play an important role since, for example, inhibitors of AP-1 activation or antisense oligonucleotides for c-jun prevented ceramide-induced apoptosis of HL-60 cells (21). Nevertheless, to what extent these various signaling proteins are directly or indirectly involved within the SPM-ceramide pathway remains elusive.

Despite the characterization of various biochemical changes associated with the SPM-ceramide signaling, such as apoptosis, a consensus on the sequence of cellular events has not been reached. However, there is mounting evidence that radical oxygen species (ROS) may be central (22). Indeed, ROS are involved in apoptosis induced by agents such as TNF-α (23), UV light (24), ionizing radiation (25), and anthracyclines (26), which have been all documented to activate the sphingomyelin cycle. Furthermore, ROS themselves, such as low doses of H₂O₂, or prooxidant conditions, such as UV or γ-irradiation, activate signaling pathways and transcription factors that have been involved in ceramide-induced apoptosis such as mitogen-activated protein kinase (27) or stress-activated protein kinases cascades (28) as well as AP-1 and NF-κB activation (for review, see Ref. 29). Moreover, oxidant production has been shown to be accompanied by cell death triggered by a cell permeant ceramide (C2-ceramide) in lymphoid B cells (30). Altogether, these observations suggest ROS as a common mediator in ceramide-induced apoptosis.

Here we show that C6-ceramide induces intracellular H₂O₂ production.
production followed by DNA fragmentation in U937 cells whereas the inactive analogue (dihydro-C6 ceramide) was ineffective. Ceramide-induced apoptosis was inhibited by ROS scavengers such as dithiocarbamates (PDTC) and N-acetylcysteine, a thiol antioxidant and a glutathione (GSH) precursor. In this study, we provide evidence that H$_2$O$_2$ is produced at the ubiquinone site of the mitochondrial respiratory chain with mitochondrial Ca$^{2+}$ homeostasis alterations followed by dysregulation of mitochondrial membrane potential ($\Delta$Vm). Finally, we show that C6-ceramide could activate the transcription factor NF-κB and AP-1 via mitochondrial ROS production.

EXPERIMENTAL PROCEDURES

Drugs and Reagents—The following reagents were purchased from Sigma: N-hexanoyl-d-phenylalanine (C6-ceramide), carbonyl cyanide m-chlorophenyl hydrazone, N-acetylcysteine, PDTC, rotenone, thenoyltrifluoroacetone (TTFA), antimycin A, ruthenium red (RR). Dihydro-C6-ceramide was a generous gift from Dr. Salem Chouaib (CJF INSERM U119, Marseille, France). Stock solutions of the reagents were routinely prepared in phosphate-buffered saline (PBS), dimethyl sulfoxide or ethanol as appropriate. [methyl-3H]Thymidine (79 Ci/mmol) was purchased from Amersham (Les Ulis, France). TNF-α was purchased from PeproTech (Le Perray en Yvelines, France), and daunorubicin (Cerubidine®) was from Laboratoire Roger Bellon (Neuilly-sur-Seine, France).

Cell Culture—The human leukemia cell line U937 (monocytic) was obtained from the ATCC (Rockville, MD) and grown in RPMI 1640 at 37 °C in 5% CO$_2$. Culture medium was supplemented with 10% heat-inactivated fetal calf serum, L-glutamine, and antibiotics. Cell stocks were screened routinely for serum, L-glutamine, and antibiotics. Cell stocks were screened routinely for 

Determination of ROS—Production of ROS was monitored by using MitoTracker Green FM (Molecular Probes). Cells were incubated with MitoTracker Green for 2 h (4°C) before the addition of C6-ceramide. Within 60 min, significant H$_2$O$_2$ was detected, and this level increased up to 180 min. At low doses (5 μM) of C6 ceramide-treated cells relative to untreated cells. The mean fluorescence increased as a function of time, reflecting H$_2$O$_2$ generation in U937 cells induced by C6-ceramide. The fluorescence was analyzed by spectrophotometry using an excitation wavelength of 340 nm and an emission wavelength of 490 nm (33).

Assessment of $\Delta$Vm—To evaluate $\Delta$Vm, exponentially growing cells (5 × 10$^5$ cells/ml) were incubated with C6-ceramide for 6- or 24-h periods of time before the end of incubation, cells were cultured with DiOC$_6$ (40 nM in PBS) at 37 °C (34). After washing, cells were analyzed by flow cytometry. Control experiments were performed in the presence of carbonyl cyanide m-chlorophenyl hydrazone, an uncoupling agent that abolishes the $\Delta$Vm, at 50 μM for 15 min at 37 °C.

Nuclear Extract Preparation—Extracts were prepared as described previously (19). Cells (5 × 10$^5$) were incubated with or without C6-ceramide in the presence or absence of different inhibitors. Cells were then washed twice with ice-cold PBS and resuspended in 10 mM HEPES, pH 7.8, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 2 μM peptatin A, 0.6 μM leupeptin, 1 μg/ml aprotinin, and 0.6% Nonidet P-40. After 15 min on ice, the nuclear pellet was recovered after centrifugation at 1200 × g and resuspended in 20 mM HEPES, pH 7.9, 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA. Aliquots were then incubated at 4 °C for 30 min and centrifuged at 21,000 × g, and supernatants containing nuclear proteins were collected. Protein concentrations were determined according to Smith et al. (35) using bicinchoninic acid (Sigma).

Electrophoretic Mobility Shift Assays—Labeling of NF-κB (5′-AGTGGAGGGACCTTCGCCAGG-3′) and AP-1 (5′-CGCTTATAGCTGATCAGCTGAGAGTTGATGAGTGAGTTTCTTCT-3′) consensus oligonucleotides (binding sites are underlined) was performed using T4 polynucleotide kinase and [γ-32P]ATP (specific activity, 5000 Ci/mmol, Amersham, Les Ulis, France). Binding reactions were carried out in 2 × 10$^4$ HEPES (pH 7.5), 50 mM NaCl, 0.5 mM EDTA, 1 mM MgCl$_2$, 2% glycerol, 0.5 mM dithiothreitol, 1 μg poly(dI-dC), and 2 μg of bovine serum albumin. Typical reactions contained 50,000 cpm of end-labeled NF-κB or AP-1 consensus oligonucleotide (Promega, Madison, WI) with or without nuclear extract. After 20 min of incubation, the gel was electrophoresed through a low ionic strength 4% polyacrylamide gel (acrylamide: bisacrylamide ratio 80:1) containing 6.7 mM Tris-HCl (pH 7.9), 3.3 mM sodium acetate, 2 mM EDTA. The gel was preelectrophoresed for 90 min at 10 V/cm. Electrophoresis was carried out at the same voltage for 5 h at room temperature with buffer recirculation. The gel was then dried and autoradiographed with intensifying screens at −70 °C. Quantification of bands was performed by densitometry and by radioactivity counting of excised bands. Band specificity was determined by competition experiments using 100-fold excess unlabeled NF-κB or AP-1 consensus oligonucleotide, as well as supershift assays (data not shown) for NF-κB using p65, p50, and c-Rel-specific antibodies generously provided by Dr. J. Imbert (INSERM U119, Marseille).

RESULTS

H$_2$O$_2$ Production by C6-Ceramide—The fluorescence distribution of the C2938 dye, revealing the presence of hydrogen peroxide, was measured by flow cytometry in the viable cell population. Fig. 1A shows the increase in the mean C2938 fluorescence in 25 μM C6-ceramide-treated cells relative to untreated cells. The mean fluorescence increased as a function of time, reflecting H$_2$O$_2$ generation in U937 cells induced by C6-ceramide. Within 60 min, significant H$_2$O$_2$ was detected, and this level increased up to 180 min. At low doses (5 μM and
we observed only a low level of H$_2$O$_2$ generation at 180 min (Fig. 1A). In comparison, Fig. 1B presents the H$_2$O$_2$ produced, after 60 min, by SPM-ceramide agonists daunorubicin and TNF-$\alpha$. Treatment of U937 cells by TNF-$\alpha$ generated comparable H$_2$O$_2$ production, whereas daunorubicin induced significantly higher H$_2$O$_2$ generation. The mean fluorescence emitted by C2838 in cells treated with exogenous H$_2$O$_2$ is presented, as well as dihydro-C6-ceramide, an inactive ceramide analogue, which had no significant effect. In addition, glucose deprivation and addition of deoxyglucose (2.5 mM) did not change H$_2$O$_2$ production in U937 cells treated with C6-ceramide (data not shown). These results suggest that, in this model, H$_2$O$_2$ production is independent of the pentose cycle.

**Role of Mitochondria in H$_2$O$_2$ Production Induced by C6-Ceramide**—To further investigate the role of mitochondria in the generation of H$_2$O$_2$ induced by C6-ceramide, we tested several classic specific mitochondrial respiratory chain inhibitors (36–39). Two sites of the mitochondrial respiratory chain have been identified as sources of ROS. One depends on the autooxidation of complex I, whereas the other is dependent on the autooxidation of complex III (ubiquinone site) (40). When U937 cells were pretreated with rotenone (a specific inhibitor of complex I that interferes with the electron flow from NADH-linked substrates and NADH dehydrogenase to the ubiquinone pool), or TTFA (a specific inhibitor of complex II that interferes with the electron transport flow from succinate dehydrogenase to the ubiquinone pool) followed by C6-ceramide treatment (25 $\mu$M) during 1 h continuous exposure. Results represent the mean (± S.D.) of four separate experiments. *p < 0.05 as compared with C6-ceramide alone and evaluated by Student's t test.

**FIG. 1. Flow cytometry analysis of ROS production.** A, kinetics of ROS production induced by C6-ceramide in U937 cells; (△) 5 $\mu$M, (■) 10 $\mu$M, (●) 25 $\mu$M C6-ceramide. Results represent the mean (± S.D.) of four separate experiments. B, comparison of ROS production induced by different agents at 1 h: C6-ceramide (25 $\mu$M), dihydro-C6-ceramide (25 $\mu$M), daunorubicin (DNR) (1 $\mu$M), TNF-α (20 ng/ml), H$_2$O$_2$ (15 $\mu$M). Results represent the mean of four separate experiments. Bars, S.D. *p < 0.05 as compared with C6-ceramide alone and evaluated by Student's t test.

**FIG. 2. Effects of mitochondrial inhibitors on ROS production induced by C6-ceramide.** U937 cells were pretreated for 30 min with rotenone (5 $\mu$M), TTFA (50 $\mu$M), or antimycin A (AA) (10 $\mu$M), or 1 h with RR (25 $\mu$M), followed by C6-ceramide treatment (25 $\mu$M) during 1 h continuous exposure. Results represent the mean (± S.D.) of four separate experiments. *p < 0.01 as compared with C6-ceramide alone and evaluated by Student's t test. Inset, ROS production in 25 $\mu$M C6-ceramide treated U937 $\rho^+$ cells. a.u., arbitrary units.
mately 2-fold $H_2O_2$ production induced by C6-ceramide (Fig. 2). Furthermore, we tested $H_2O_2$ production in U937 $\rho^0$ cells. These cells have been selected in the presence of ethidium bromide and present mitochondrial DNA depletion and a reduced complex III activity in the mitochondrial respiratory chain (31). We did not observe significant $H_2O_2$ production in U937 $\rho^0$ cells treated for 1 h with 25 $\mu M$ C6-ceramide (Fig. 2, inset). These results strongly support the role of complex III in C6-ceramide-induced $H_2O_2$ production.

In addition, since calcium has been shown to play a role in apoptosis in certain experimental systems (41), we tested the contribution of calcium to the production of $H_2O_2$ induced by C6-ceramide. U937 cells were pretreated with ruthenium red (an inhibitor of the mitochondrial calcium uptake) (42) and then analyzed for the production of $H_2O_2$ generated by C6-ceramide. As shown in Fig. 2, ruthenium red inhibited C6-ceramide induced $H_2O_2$ generation by 60–70%.

C6-Ceramide-induced Apoptosis, Implication of ROS—We tested the relationship between $H_2O_2$ production and apoptosis induced by C6-ceramide in U937 cells. U937 cells were treated in kinetics experiments with different doses of C6-ceramide and analyzed for typical morphological features of apoptosis (Fig. 3A) as well as DNA fragmentation using the $[^{3}H]$thymidine release assay. At 6 h, approximately 10% of U937 cells treated by 25 $\mu M$ C6-ceramide presented typical apoptotic features. This population grew to approximately 30% at 24 h (Fig. 3B). Analysis of DNA fragmentation also reflected a similar effect (8% at 6 h; 37% at 24 h) and we observed that C6-ceramide dose-dependent DNA fragmentation correlated closely with $H_2O_2$ production (data not shown). In addition, cleavage of ICE-substrate (32) was observed as early as 4 h after 25 $\mu M$ C6-ceramide treatment (Fig. 3C).

We tested the influence of different ROS scavengers on apoptosis and DNA fragmentation induced by C6-ceramide. U937 cells were pretreated with different concentrations of N-acetylcysteine, the thiol antioxidant and GSH precursor (22), or PDTC, the oxygen radical scavenger and iron chelator (43), in combination with 6 h (data not shown) or 24 h of continuous exposure to 25 $\mu M$ C6-ceramide. C6-Ceramide induced-apoptosis (data not shown) and DNA fragmentation were inhibited by both N-acetylcysteine (Fig. 4A) and PDTC (Fig. 4B) in a dose-dependent manner. While confirming that C6-ceramide is able to induce apoptosis in U937 cells, these results implicated ROS within this process, since PDTC was also able to inhibit ROS production induced by C6-ceramide (Fig. 4B, inset). To further substantiate the role of ROS in ceramide-mediated apoptosis, we evaluated the impact of both rotenone and TTFA on this cell death process. Both mitochondrial respiratory chain inhibitors significantly inhibited C6-ceramide-triggered apoptosis (not shown) and DNA fragmentation (Fig. 5). In addition, pretreatment of U937 cells with antimycin A greatly increased the apoptotic population induced by C6-ceramide (not shown) as well as DNA fragmentation (Fig. 5). The effective nontoxic concentrations for 6 h of continuous exposure of these inhibitors were determined by dose-effect studies (data not shown).

Alteration of $\Delta \Psi m$ by C6-Ceramide—It has been described that a reduction in $\Delta \Psi m$ precedes apoptosis and may represent an early signaling event (34). Using the DiOC6 fluorescent
probe, we analyzed the ΔΨm of U937 treated with C6-ceramide. Cells were exposed to DiOC6 probe at 40 nM, 15 min before the end of incubation with C6-ceramide. As described previously, under these conditions, only mitochondria are labeled as confirmed by confocal microscopy experiments (data not shown). In addition, treatment with carbonyl cyanide m-chlorophenyl hydrazone (an uncoupling agent that abolishes the ΔΨm) and DiOC6 showed a drastic reduction of ΔΨm, whereas incubation in the presence of high concentration of KCl (120 mM) showed no difference, confirming that, under these conditions, only mitochondrial membrane potential is measured (data not shown). In our hands, C6-ceramide had no significant influence on mitochondrial membrane potential at 2 h, and only 5% of cells showed a ΔΨm reduction at 6 h. However, after 20 h of incubation with C6-ceramide, 20% of cells showed a drastic reduction of the ΔΨm (Fig. 6), suggesting that reduction of mitochondrial transmembrane potential is a distal event in DNA fragmentation triggered by C6-ceramide.

Activation of Transcriptional Factors NF-κB and AP-1 by C6-Ceramide, Implication of Mitochondria—In light of the fact that ceramide and many other conditions which induce an oxidative stress result in increased activation of NF-κB and AP-1 (21) (for review, see Ref. 44), we tested the effect of the different respiratory chain inhibitors or antioxidants on NF-κB and AP-1 activation induced by C6-ceramide. As shown in Fig. 7, A and B, C6-ceramide induced a 2–3-fold activation of both NF-κB and AP-1 within 1 h. The activation of both NF-κB and AP-1 induced by C6-ceramide was inhibited by a 30-min preincubation with rotenone (>60 and >25%, respectively), TTFA (>60 and >25%, respectively), a 2-h preincubation with N-
Mitochondrial H$_2$O$_2$ Generation in Ceramide-induced Apoptosis

Mitochondria and oxidative stress have been discovered; most reports suggest that oxidative stress may be involved in ceramide-induced mitochondrial Ca\(^{2+}\) release and oxidative stress has been discovered; most reports suggest that oxidative stress may be involved in ceramide-induced mitochondrial Ca\(^{2+}\) release. Indeed, ROS are produced at a high rate as a by-product of aerobic metabolism in mitochondria, which, in fact, constitute the greatest source of ROS as the mitochondrial electron transport system consumes 85–90% of the oxygen utilized by the cell (46). Intrinsic to this process is the generation of ROS from specific segments of the electron transport chain, mainly at the ubiquinone site in complex III. This site catalyzes the conversion of O$_2$ to O$_2^-$ and, in turn, can lead to the formation of other potent oxygen-derived free radicals (47). In fact, our study shows that inhibition of the electron transport at complex I by rotenone resulted in a reduction in ceramide-induced H$_2$O$_2$ accumulation. This suggests that ceramide-induced H$_2$O$_2$ production was distal to complex I. Inhibition at complex II by TTFA also decreased ceramide-induced H$_2$O$_2$ production, suggesting that ceramide did not interfere with complex II function. However, when electron flow was inhibited distal to the ubiquinone pool, by the addition of antimycin A, a marked potentiation of both H$_2$O$_2$ production and DNA fragmentation was observed. Moreover, the observation that C6-ceramide did not lead to H$_2$O$_2$ generation in U937 cells. This suggests that ceramide-induced H$_2$O$_2$ production is centered at the ubiquinone site of the mitochondrial respiratory chain.

Ubiquinone (also known as coenzyme Q) is the only nonprotein component of the electron transport chain that can capture one or two electrons, thereby forming ubiquinol (hydroquinone). Since the ubiquinone molecule is not tightly bound to proteins, it can play a strategic role as a mobile carrier of electrons. The function of ubiquinone as a H$_2$O$_2$-producing site is not surprising, since it has previously been shown that the mitochondrial respiratory chain generates superoxide anion secondary to the interruption of electron flow between the rotenone- and antimycin-sensitive sites through one-electron reduction (36–39).

Another respiratory linked function of the mitochondrion is Ca\(^{2+}\) accumulation that has been described to be mediated by a RR-sensitive unipporter (48). In the last few years a correlation between transmembrane mitochondrial Ca\(^{2+}\) exchange and oxidative stress has been discovered; most reports suggest that oxidative stress may induce the release of mitochondrial Ca\(^{2+}\) via a Na\(^+\)-independent pathway (41). Conversely, other studies have suggested that alterations in Ca\(^{2+}\) homeostasis may initiate oxidative stress. Thus, it has been reported that an increase in intracellular Ca\(^{2+}\) concentration in isolated hepatocytes results in the depletion of mitochondrial GSH, probably as a result of mitochondrial Ca\(^{2+}\) cycling. More recently, RR attenuated doxorubicin-induced ROS in isolated heart mitochondria, suggesting that alteration in mitochondrial Ca\(^{2+}\) transport may be involved in the release of ROS (49). Our study shows that RR inhibited H$_2$O$_2$ production in ceramide-treated cells, suggesting that ceramide induced mitochondrial Ca\(^{2+}\) homeostasis alterations which, in turn, could be involved in ceramide-induced oxidative stress. Our experiments using flow cytometry and the calcium-specific INDO-1
AM fluorescent probe (Molecular Probes, Eugene, OR) (50), in C6-ceramide-treated U937 cells (not shown) and two earlier reports in fibroblasts and T cells have shown that permeant ceramides had no effect on whole cellular Ca^{2+} content (51, 52).

In our study, we attempted to determine if ROS were important in ceramide signal transduction. We observed that N-acetylcysteine or PDTC, two potent antioxidants, significantly decreased in parallel both H_{2}O_{2} production and ceramide-induced apoptosis and DNA fragmentation. Moreover, rotenone decreased, whereas antimycin A increased ceramide-induced apoptosis and DNA fragmentation. Therefore it appears that mitochondrial ROS production is involved in these processes. However, it should be noted that N-acetylcysteine, PDTC, and rotenone significantly decreased but did not abrogate ceramide-induced DNA fragmentation. This observation can be related to the inability of antioxidants to totally block ceramide-induced H_{2}O_{2} production. Alternatively, it is conceivable that ROS mediated some but not all the multiple downstream signaling cascades activated by ceramide and that other mediators also play an important role in ceramide-induced apoptosis. Further studies are needed to evaluate the effects of ROS produced by ceramide on ceramide-activated protein kinase and ceramide-activated protein phosphatase activities as well as their role in the activation of downstream signaling events that have been involved in ceramide-induced apoptosis such as mitogen-activated protein kinase/stress-activated protein kinases cascade activation.

By altering the generation of ROS through inhibition of the electron transport chain and Ca^{2+} homeostasis, our study demonstrates a potentially significant role of ROS in triggering downstream signaling cascades leading to apoptosis in U937 cells. It has recently been reported that apoptosis is preceded by a sustained deregulation of mitochondrial functions, characterized by an initial reduction of ΔΨm followed by a rotenone- and RR-sensitive ROS production in U937 cells treated with TNF-α (54). In our study we found that ceramide-induced H_{2}O_{2} production (1 h) largely preceded reduction of ΔΨm, which in fact occurred mainly at 20 h. This suggests that the initial ROS generation produced a ripple effect consisting in the loss of ΔΨm of mitochondria, which, in turn, may accelerate the ongoing cell death process through self-amplification of ROS production and Ca^{2+} homeostasis disturbances. Altogether our study suggests that mitochondrial ROS production may represent a crucial event for ceramide-induced signaling and apoptosis.

ROS have largely been described as potent activators of transcription factors, such as NF-κB and AP-1 (21, 44, 53, 54), two transcription factors activated in ceramide-induced apoptosis (20, 21). It has also recently been reported that inhibition of mitochondrial complex III by antimycin A led to a 2–3-fold decrease in parallel both H_{2}O_{2} production and ceramide-induced apoptosis and DNA fragmentation. Moreover, rotenone decreased, whereas antimycin A increased ceramide-induced apoptosis and DNA fragmentation. Therefore it appears that mitochondrial ROS production is involved in these processes.

In conclusion, our study shows that C6-ceramide could induce mitochondrial disturbances such as H_{2}O_{2} production, alteration of calcium homeostasis followed by alteration of mitochondrial transmembrane potential. In light of these findings, it is conceivable that increased antioxidative defenses through detoxifying enzymes, such as glutathione peroxidase, or radical scavengers can decrease the apoptotic effect of ceramide in U937 cells, and that of anti-tumor agents which are SPM-ceramide agonists. In addition, the role of mitochondrial ROS production in ceramide-induced apoptosis could explain the protective effect of Bcl-2 (56–58) and Bcl-x_{L} (30, 58) against ceramide-induced apoptosis since this protein, which has been described as an antioxidant, is preferentially located in the mitochondrial inner membrane (59). Finally, two very recent studies have proposed a central role for mitochondrial cytochrome c release within the apoptotic signaling pathway (60, 61). Therefore, it would be of great interest to determine whether ceramide could directly trigger cytochrome c release and if this process is closely related to the electron-transport chain.

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Note Added in proof—While this manuscript was being reviewed, another paper also demonstrated the mitochondrial complex III origin of hydrogen peroxide generated by ceramide (62). In concert, both studies underline the novel role of ceramide as an inducer of oxidative stress or perhaps more precisely of a reactive oxygen species-mediated signaling pathway.

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