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J Immunol 2004; 173:3783-3790; doi: 10.4049/jimmunol.173.6.3783

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Sphingosine Contributes to Glucocorticoid-Induced Apoptosis of Thymocytes Independently of the Mitochondrial Pathway

Sandrine Lépine,* Boris Lakatos,‡ Marie-Pierre Courageot,* Hervé Le Stunff,‡ Jean-Claude Sulpice,* and Françoise Giraud**

During the selection process in the thymus, most thymocytes are eliminated by apoptosis through signaling via TCR or glucocorticoids. The involvement of ceramide (Cer) and sphingosine (SP), important apoptotic mediators, remains poorly defined in glucocorticoid-induced apoptosis. We report that, in mouse thymocytes, apoptosis triggered by 10^{-6} M dexamethasone (DX) was preceded by a caspase-dependent Cer and SP generation, together with activation of acidic and neutral ceramidases. Apoptosis was drastically reduced by blocking either sphingolipid production (by acid sphingomyelinase inhibitor) or SP production (by ceramide inhibitors), but not by inhibition of de novo Cer synthesis. Thus, SP generated through acid sphingomyelinase and ceramidase activity would contribute to the apoptotic effect of DX. Consistent with this hypothesis, SP addition or inhibition of SP kinase induced thymocyte apoptosis. DX induced a proteasome-dependent loss of mitochondrial membrane potential (ΔΨm) and caspase-8, -3, and -9 processing. Apoptosis was abolished by inhibition of ΔΨm loss or caspase-8 or -3, but not caspase-9. ΔΨm loss was independent of SP production and caspase-8, -3, and -9 activation. However, inhibition of SP production reduced caspase-8 and -3, but not caspase-9 processing. Proteasome inhibition impaired activation of the three caspases, whereas inhibition of ΔΨm loss solely blocked caspase-9 activation. These data indicate that DX-induced apoptosis is mediated in part by SP, which contributes, together with proteasome activity, to caspase-8-3 processing independently of mitochondria, and in part by the proteasome/mitochondria pathway, although independently of caspase-9 activation. The Journal of Immunology, 2004, 173: 3783–3790.

In the thymus, positive and negative selection steps prevent nonfunctional or harmful T cells from reaching the periphery. glucocorticoid-induced apoptosis contributes to the elimination of thymocytes during clonal selection and to the creation of a fully functional immune system (1). The glucocorticoid receptor, an essential and widely expressed transcriptional regulator, is triggered by hormone binding to translocate to the nucleus, activating or repressing transcription depending on the cell and gene context (2).

Sphingolipid metabolites, ceramide (Cer) and sphingosine (SP), are now recognized as key regulators of apoptosis, cell proliferation, differentiation, or inflammation (3). Apoptosis induced by multiple factors including TNF-α, CD95, glucocorticoids, ionizing radiation, UV light, heat shock, or serum withdrawal has been linked to an elevation of Cer level (4–8). Cer can be generated by two different pathways, either sphingomyelin hydrolysis catalyzed by acid sphingomyelinase (aSMase) and neutral sphingomyelinase (nSMase) or de novo biosynthesis. Cer activates a number of enzymes involved in stress signaling cascades including protein kinases and phosphatases (9). In fact, Cer seems to redirect cellular signaling pathways by promoting proapoptotic stress-activated protein kinase/JNK at the expense of MAPK cascades. SP, produced by deacylation of Cer by ceramidases (Cerases), is rapidly generated during apoptosis mediated by TNF-α, CD95 ligation, irradiation, or doxorubicin (10–13). SP suppresses basal ERK activity and stimulates stress-activated protein kinase/JNK activity and p38 MAPK (14). Furthermore, SP down-regulates the expression or the activity of the antiapoptotic protein Bcl-2 and activates various caspases (14). In contrast to a proposed apoptotic role of SP, in some studies, SP has been reported to increase cell proliferation, an effect that may be due to its rapid conversion by SP kinase into sphingosine-1-P, which promotes cell survival in response to various apoptotic stimuli (15).

The actual contribution of sphingolipid metabolism to the apoptotic pathways induced by dexamethasone (DX), a synthetic glucocorticoid, is poorly documented. In thymocytes, DX-induced apoptosis is preceded by Cer generation through aSMase activation, itself resulting from Src-mediated phosphatidylinositol phospholipase C activation (6, 16). Among these studies, aSMase activation is caspase independent (6), in contrast to aSMase activation induced by CD95 ligation in other cells, which requires upstream activation of caspase-8 through Fas-associated death domain protein (FADD)/procaspase-8 complexes (5, 17, 18). However, in thymocytes, DX, as well as CD95 ligation, induces the formation of these complexes (16, 19). Moreover, Cer generated...
upon DX stimulation was proposed to mediate caspase-8 activation upstream of mitochondria resulting, on one hand, in cytochrome c release and caspase-9-3 activation and, in contrast, in direct activation of caspase-3 (16). Furthermore, mitochondria and proteasomes are involved in thymocyte cell death in response to DX. Indeed, apoptosis is blocked by Bcl-2 and Bcl-x, overexpression and by inhibition of the mitochondrial inner membrane potential (ΔΨm) loss and of cytochrome c release (20–24). Inhibition of proteasome activity abolishes ΔΨm loss, cytochrome c release, and caspase-3 activity, and protects thymocytes against a number of apoptosis inducers including DX, suggesting that proteasomes exert their regulatory role at a premitochondrial step during thymocyte apoptosis (22, 25, 26).

In the present study, we investigated the relationships between sphingolipid metabolism, proteasome-mitochondria pathway, and caspase processing in DX-induced apoptosis of murine thymocytes. We show that both Cer and SP are apoptotic mediators in these cells. In response to DX, SP was produced through the sequential activation of aSMAse and Cerases in a caspase-dependent manner and mediated cell death by contributing to caspase-8-3 processing through a mitochondria-independent pathway.

**Materials and Methods**

**Reagents**

Adenosine, aprotinin, bongkrekic acid (BA), β-chloroalanine (β-Cl), l-cyclosine (CS), DX, 4-deoxyriodoxin, dioleoyl phosphatidylglycerol, fumonisin B1 (FB1), leupeptin, N-oleoyl ethanolamine (NOE), PMA, aSMAse from *Staphylococcus aureus*, aSMAse from human placenta, and peroxidase-conjugated goat anti-rabbit IgG were obtained from Sigma-Aldrich (St. Louis, MO). Diacylglycerol (DAG) kinase and (1S,2R)-o-erythroy-2-(N-methylstyrlylamino)-1-phenyl-1-propanol (o-MAPP) were from Calbiochem (San Diego, CA). o-Erythro-sphingosine (SP), N,N-dimethyl sphingosine (DMS), and MG132 were from Alexis (Lausen, Switzerland). C12-NBD-Cer (NBD-C12:0, d18:1) (NBD-Cer) was obtained from Avanti Lipids (Alabaster, AL). The protein assay kit was from Bio-Rad (Hercules, CA), and [γ-32P]ATP was purchased from PerkinElmer Life Sciences (Wellesley, MA). 3,3’-Dihexylxacyclobacarynic iodide (DiOOC(3)) was obtained from Fluka (Buchs, Switzerland), and caspase inhibitors N-benzoyl-oxycarboxyln-Val-Ala-Asp- (Ile-Glu-Thr-Asp- (Leu-Glu-His-Asp- (Asp-Glu-Val-Asp)-fluoromethylethone (Z-VAD; Z-DEVD; Z-DEVD) were from R&D Systems (Minneapolis, MN). Rabbit anti-caspase-3, -8, and -9 were provided by Santa Cruz Biotechnology (Santa Cruz, CA). [(2-Isopropyl-1-4-[3-N-methyl-N-(3,4-dimethoxy-β- phenethyl)amino]propoxy]l benzenesulfonfonyl)indolizine (SR33557) was kindly supplied by Sanofi-Synthelabo Recherche (Paris, France).

**Animals and cell culture**

Thymocytes from male 3- to 6-wk-old BALB/c mice (2.5 or 5 × 10⁸/ml) were cultured at 37 °C in 5% CO₂ in HEPES-RPMI 1640 medium containing 15% heat-inactivated FCS. When required, cells were incubated with inhibitors for 30 min before the addition of the inducer. Cell viability was estimated by using the trypan blue exclusion method.

**Apoptosis assays**

Following the exposure period, thymocyte suspensions were incubated for 10 min with the DNA-binding fluorochrome bisbenzimide (Hoescht 33342; 10 µg/ml) and a marker of cell membrane integrity, propidium iodide (50 µg/ml). After centrifugation, cells were examined by flow cytometry (21). After incubation, thymocytes (10⁷ cells) were loaded with 40 nM DiOC₆(3) and incubated for 10 min at 37°C. The stained cells were analyzed by FACS (BD Biosciences, Mountain View, CA).

**Western blot analysis**

After incubation, thymocytes (4 × 10⁶ cells) were washed three times in ice-cold buffer A, lysed for 30 min on ice in 250 mM sucrose, 1 mM EDTA, 1 mM PMFS, 10 µg/ml leupeptin and aprotinin, 0.05% Triton X-100, 1 mM DTT, and 20 mM Tris-HCl (pH 7.4), and centrifuged at 4°C (15 min; 14,000 × g). The proteins of the supernatant (cytosol) was collected and kept frozen at −80°C. After incubation, thymocytes (10⁵ cells) were washed in ice-cold buffer A and centrifuged at 4°C (5 min; 200 × g). The pellet was lysed as described above and extracted with 2 ml of chloroform/methanol (2:1). The aqueous phase was dried and quantified by PhosphorImager (Storm; Molecular Dynamics, Sunnyvale, CA). Cer levels were normalized to total lipid phosphate. SP kinase activity was determined as described previously (30), on thymocyte cytosol (50 µg of protein) from the phosphorylation of added SP (50 µM).

**Cerase assay**

After incubation, thymocytes (2.5 × 10⁵ cells) were washed in ice-cold buffer A and sedimented by centrifugation at 4°C (5 min; 200 × g). Cell pellets were lysed for 10 min on ice in 0.2% Triton X-100 and 10 mM Tris-HCl (pH 7.4), and centrifuged at 4°C (15 min; 14,000 × g). Supernatants (cytosol) were homogenized with three passes through a 25-gauge needle. Cerase activity was determined using NBD-Cer, added from a 300 µM ethanolic solution to a final concentration of 1.5 µM, and incubated for 10 min at 4°C. Aliquots (150 µl, containing ~40 µg of protein) were mixed with 150 µl of buffers containing 0.2% Triton X-100 and 0.5 M Na acetate (pH 4.5) for acidic Cerase, 10 mM Tris-HCl (pH 7.4) for neutral Cerase, or 10 mM NaOH-HEPES (pH 9.5) for alkaline Cerase, and incubated for 1 h at 37°C. After addition of 300 µl of 0.2 N HCl, followed by 2.4 ml of chloroform/methanol (1:1) and 1.2 ml of H₂O, the organic phase was dried and solubilized in chloroform/methanol (2:1) for lipid analysis by TLC in chloroform/methanol/ammonia (20%) (90:20:0.8). The fluorescence of NBD-dodecanoic acid was detected at 450/530-nm emission wavelengths, quantified by a phosphor imager, and used to estimate the enzymatic activity.

**Assay for mitochondrial transmembrane potential**

ΔΨm was measured by accumulation of DiOOC(3) (3) to the mitochondria matrix and flow cytometry (22). After incubation, thymocytes (10⁵ cells) were loaded with 40 nM DiOOC(3) and incubated for 20 min at 37°C. The stained cells were analyzed by FACS (BD Biosciences, Mountain View, CA).

**Colorimetric enzyme activity assays**

Recombinant human caspase-3 (100 U) was prepared, and the enzyme activity was assayed by utilizing the colorimetric assay described by the supplier (5). The assay was performed in a 96-well plate. The reaction mixture contained 20 mM Tris-HCl (pH 7.4), 1.5 mM N-acetyl-L-aspartyl-L-glutamyl-L-valyl-L-aspartyl-L-aspartic acid (Ac-LEHD-Asp), and 150 µM Ac-LEHD-Asp. The reaction was initiated by adding the enzyme and was stopped after 30 min by the addition of 50 µl of 0.2 N HCl, followed by 2.4 ml of chloroform/methanol (1:1) and 1.2 ml of H₂O. The organic phase was dried and solubilized in chloroform/methanol (2:1) for lipid analysis by TLC in chloroform/methanol/ammonia (20%) (90:20:0.8). The fluorescence of NBD-dodecanoic acid was detected at 450/530-nm excitation/ emission wavelengths, quantified by a phosphor imager, and used to estimate the enzymatic activity.

**SP measurement and SP kinase assay**

Liver cytosol was used as a source of SP kinase. Mouse livers were rinsed in 150 mM NaCl and 20 mM Tris-HCl (pH 7.4), and pulled to pieces (3 × 3 mm). Pieces were crushed in a Dounce homogenizer in 6 ml (for one liver) of homogenization buffer (20 mM NaH₂PO₄, 80 mM K₂HPO₄ (pH 7.4), 20% glycerol, 20 µM ZnCl₂, 15 mM NaF, 1 mM NaVO₃, 10 µg/ml leupeptin and aprotinin, 0.5 mM PMFS, 2 mM DTT, and 0.5 mM 4-deoxyriodoxin) and centrifuged at 4°C (40 min; 100,000 × g). The supernatant (cytosol) was collected and kept frozen at −80°C. After incubation, thymocytes (10⁷ cells) were washed in ice-cold buffer A and centrifuged at 4°C (5 min; 200 × g). The pellet was lysed as described above and extracted with 2 ml of chloroform/methanol (2:1). The aqueous phase was extracted once more, and the organic phases were pooled, dried, and used for SP and phosphatase measurements. SP levels were determined essentially as described previously (29). Sphingosine-1,2-3P was detected and quantified by a phosphor imager. SP levels were normalized to total lipid phosphate. SP kinase activity was determined as described previously (30), on thymocyte cytosol (50 µg of protein) from the phosphorylation of added SP (50 µM).
Student of thymocytes with 10^{-8}M DX was detectable after 2.5-h treatment (data not shown). Incubation of thymocytes with 10^{-8} M DX or 100 nM PMA, without (control) or with 50 μM FB1, 300 μM CS, 5 mM β-Cl, or 20 μM SR33557 (SR); apoptosis was evaluated from the percentage of cells with apoptotic nuclei (means ± SE of three to five experiments). Statistical analysis

Data are expressed as means ± SEM. Significance was assessed by the Student’s t test for paired samples. Values of p < 0.05 were considered significant.

Results

DX-induced apoptosis is preceded by an early generation of Cer resulting from caspase-dependent sMase activation

The effect of DX on thymocyte apoptosis, after 5-h exposure, was dose dependent, whereas a weak spontaneous apoptosis was observed in untreated cells (Fig. 1A). At 10^{-6} M DX, apoptosis became detectable after 2.5-h treatment (data not shown). Incubation of thymocytes with 10^{-8} M DX induced in <5 min, an increase in Cer (1.8-fold), which returned to basal level after 60 min (Fig. 1B). In contrast, 10^{-6} M DX promoted a smaller and more transient increase in Cer (1.4-fold), culminating at 5 min, and decreasing below the basal level after 15 min (Fig. 1C). To determine whether DX-induced apoptosis could involve Cer generated from de novo synthesis and/or sphingomyelin hydrolysis, we have used different inhibitors of these pathways. Apoptosis induced by 5-h stimulation with 10^{-6} M DX was unaffected by FB1 (50 μM), an inhibitor of dihydro-Cer/Cer synthase (31), or by CS or β-Cl, two distinct inhibitors of serine palmitoyl-CoA transferase (32, 33) (Fig. 1D). Treatment for 20 h with up to 100 μM FB1 also failed to alter apoptosis induced by 10^{-9} M DX (data not shown). In contrast, these inhibitors efficiently prevented PMA-induced apoptosis (Fig. 1D), which has been shown to be mediated by the de novo Cer synthesis pathway in LNCap cells (34). These data permit the exclusion of a role for de novo synthesis of Cer, even at late times, in DX-mediated apoptosis. Remarkably, SR33557, a potent inhibitor of aSMase (35), slightly increased apoptosis in the absence of DX and reduced apoptosis induced by 10^{-6} M DX (Fig. 1D).

Interestingly, in the absence of DX, SR33557 induced Cer generation (Table I), presumably by increasing nSMase activity as already reported (35), which could explain its apoptotic effect. However, there was apparently no effect of SR33557 on Cer level in cells treated for 5 min with 10^{-8} M DX (Table I), suggesting that, whereas SR33557 induced Cer generation by activating nSMase, it could block DX-induced Cer production by inhibiting aSMase. This hypothesis would account for the incomplete apoptosis inhibition of SR33557 upon DX treatment and is consistent with previous results demonstrating the involvement of aSMase activation in the early generation of Cer induced by DX (6). Additionally, the broad-spectrum caspase inhibitor Z-VAD abolished Cer generation in response to DX (Table I). These results indicate that DX-induced Cer production was mediated through a caspase-dependent aSMase activation and did not involve the de novo pathway.

Table I. Effects of Z-VAD, MG132, or SR33557 on DX-induced sphingolipid production

<table>
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<td>100</td>
</tr>
<tr>
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<td>9</td>
</tr>
<tr>
<td>SR33557</td>
<td>134</td>
<td>14</td>
</tr>
<tr>
<td>DX</td>
<td>128</td>
<td>8</td>
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<tr>
<td>+SR33557</td>
<td>128</td>
<td>8</td>
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* For Cer determination, thymocytes were incubated for 5 min with 10^{-8} M DX, without or with 10 μM Z-VAD, 30 μM MG132, or 20 μM SR33557. For SP deter- mination, thymocytes were incubated for 15 min with 10^{-8} M DX, without or with 10 μM Z-VAD, Cer and SP levels were measured by DAG kinase and SP kinase assays, respectively, and expressed as percentages of basal levels (time 0) (means ± SE of three to four experiments).

* * , Significantly different from untreated cells.

** * , Significantly different from DX-treated cells.

FIGURE 1. DX-induced apoptosis is preceded by Cer and SP generation and does not involve de novo Cer synthesis. A, Thymocytes were incubated for 5 h with different concentrations of DX; apoptosis was evaluated from the percentage of cells with apoptotic nuclei (means ± SE of three to five experiments). B and C, Thymocytes were incubated for the indicated times with either 10^{-6} (B) or 10^{-8} M DX (C); Cer levels were measured by the DAG kinase assay and expressed as percentage of the basal level (time 0) (means ± SE of four to six experiments). D, Thymocytes were incubated for 5 h with 10^{-6} M DX or 100 nM PMA, without (control) or with 50 μM FB1, 300 μM CS, 5 mM β-Cl, or 20 μM SR33557 (SR); apoptosis was evaluated from the percentage of cells with apoptotic nuclei (means ± SE of three to five experiments). Statistical significance was assessed by the Student’s t test for paired samples. Values of p < 0.05 were considered significant.
SP generated through Cerase activation is involved in DX-induced apoptosis

In contrast to the persistent Cer accumulation observed at 10^{-6} M DX, the transient peak of Cer production detected at 10^{-6} M DX suggests that this lipid is more efficiently metabolized in the latter case. Indeed, 10^{-6} M DX triggered a modest increase in SP (maximum, 1.5-fold) detectable between 5 and 30 min (Fig. 1E), whereas 10^{-6} M DX promoted a larger and more sustained elevation in SP (2- to 2.5-fold) between 10 and 60 min (Fig. 1F). Similarly to Cer generation, SP production induced by DX was abolished by Z-VAD (Table I). Furthermore, treatment with 10^{-6} M DX induced a transient activation of both acid and neutral Cerases reaching 50 and 25%, respectively, between 5 and 30 min (Fig. 2, A and B), whereas the activity of alkaline Cerase was not significantly modified (data not shown). In contrast, stimulation with 10^{-6} M DX induced a weaker activation of acid Cerase than 10^{-6} M (Fig. 2C), without affecting the neutral or alkaline enzymes (data not shown). These data indicate that the accumulation of Cer induced by DX was prevented by its rapid conversion into SP by Cerases, especially when the concentration of DX was >10^{-8} M. To determine whether SP was involved in DX-induced apoptosis, we have used t-MAPP and NOE, two reported Cerase inhibitors (36, 37). Activation of acid and neutral Cerases, induced by 10^{-6} M DX in thymocytes, was totally prevented by either t-MAPP or NOE (10 \mu M) (Fig. 3A). t-MAPP and NOE potentiated Cer accumulation induced by 10^{-6} M DX for 5 min and completely suppressed SP production induced by 10^{-6} M DX for 15 min (Fig. 3B). Interestingly, after 5-h incubation, t-MAPP or NOE had no effect on thymocyte cell death, but reduced apoptosis triggered by 10^{-8} or 10^{-6} M DX (Fig. 3C). The fact that t-MAPP or NOE prevented SP generation and reduced apoptosis in response to DX suggests that SP mediated in part the apoptotic effect of DX in thymocytes.

Increasing cellular levels of SP or Cer induces apoptosis in the absence of DX

Exposure of thymocytes to exogenous SP for 3 h induced apoptosis in a dose-dependent manner (Fig. 4A). FB1 did not suppress SP-induced apoptosis (Fig. 4B), indicating that the effect of SP on apoptosis seems to be caused by itself rather than by its acylation into Cer. Similarly, DMS (5 \mu M), a competitive inhibitor of SP kinase (38), which was able to inhibit thymocyte SP kinase activity after 30-min treatment (data not shown), induced apoptosis after 3-h incubation (Fig. 4A). Treatment of thymocytes with exogenous aSMase promoted a significant increase in both Cer and SP levels, whereas exogenous nSMase triggered a strong increase in Cer without affecting SP level (Fig. 4C). Interestingly, both SMases induced apoptosis of ~30% of cells after 4-h incubation (Fig. 4D). DNA ladder analysis revealed that SP, DMS, and both SMases induced thymocyte apoptosis after 5-h treatment (Fig. 4, E and F). However, only apoptosis induced by aSMase was significantly inhibited by t-MAPP or NOE (Fig. 4D), suggesting that aSMase promoted apoptosis, at least in part, through SP generation, whereas nSMase mediated its effect through Cer production. Together, these data show that the increase in SP level, induced either by exogenous addition or indirectly by SP kinase inhibition or exogenous aSMase, resulted in thymocyte apoptosis.

The proteasome/mitochondrial pathway is not involved in sphingolipid-mediated apoptosis in response to DX

In DX-stimulated thymocytes, apoptosis is mediated, at least in part, by proteasome activity required for mitochondrial alterations.
and involves caspase activation (22, 24, 26). In agreement with previous reports (21, 22), we found that MG132 (30 μM), a proteasome inhibitor, and BA (50 μM), an inhibitor of the permeability transition pore (PTP), which stabilizes the Δψm and prevents the postmitochondrial manifestations of apoptosis, reduced DX-induced apoptosis by ~70% (data not shown). In contrast, Z-VAD (10 μM) totally abolished DX-induced apoptosis (see Fig. 6A). MG132 by itself had no effect on Cer level and did not block Cer production induced by DX (Table 1), indicating that proteasome activity was not required for Cer generation. As already reported (22, 23), DX stimulation of thymocytes led to a gradual disruption of Δψm, affecting 65% of cells after 5 h (Fig. 5A) and was abrogated by BA (Fig. 5B). Δψm dissipation was also reduced by MG132, as expected for a role of proteasome activity upstream from mitochondrial changes (21, 22). In contrast, neither Z-VAD nor any of the specific caspase inhibitors tested (Z-IETD (caspase-8), Z-DEVD (caspase-3), Z-LEHD (caspase-9)) were able to affect Δψm loss induced by DX, indicating that the processing of caspases, and especially of caspase-8, -3, and -9, occurred downstream and/or independently of mitochondrial damage. SR33557 alone induced a loss of Δψm in ~60% of cells, an extent similar to that triggered by DX, which prevented us from determining whether it could inhibit the effect of DX on Δψm loss. Nevertheless, Z-MAPP or NOE were unable to affect DX-induced Δψm loss (Fig. 5B). Together, these data show that the production of sphingolipids did not depend on the proteasome-mitochondrial pathway, and that SP up-regulated DX-induced apoptosis independently of this pathway.

Caspase activation involved in DX-mediated apoptosis is dependent on SP generation, proteasome activity, and mitochondrial alterations

In agreement with a previous study using 10−7 M DX for 18 h (16), we found that apoptosis induced by 10−6 M DX for 5 h was inhibited dose dependently by Z-IETD and Z-DEVD, whereas Z-LEHD had almost no effect, indicating that caspase-9 activation is dispensable for apoptosis (Fig. 6A). Processing of caspase-8, -3, and -9, analyzed by immunoblotting, became detectable after 2- to 3-h stimulation with 10−6 M DX, without any striking difference in the activation kinetic of each caspase (data not shown). SR33557, as well as Z-MAPP or NOE, decreased DX-mediated processing of caspase-8 (Fig. 6B), indicating that SP mediates, at least in part, caspase-8 activation. SR33557, Z-MAPP, or NOE also reduced caspase-3 processing, consistent with direct activation of caspase-3 by caspase-8 (39). Activation of caspase-8 and -3 was attenuated by MG132 (Fig. 6B), indicating that, in addition to SP, the proteasome activity also contributes to the activation of the caspase-8-3 cascade. However, the processing of caspase-8-3 was independent of mitochondrial alterations, because it was unaffected by BA. Furthermore, MG132 and BA abolished caspase-3 processing, supporting a role of the proteasome/mitochondria pathway in the activation of caspase-9. In contrast, SR33557, Z-MAPP, or NOE did not alter caspase-9 processing, showing that SP is not
involved in the proteasome/mitochondria/caspase-9 pathway. Our data suggest that DX-induced apoptosis depends on caspase-8-3 activation mediated by both SP and proteasome activity, but independently of mitochondria. In addition, the proteasome/mitochondria contributes to DX-mediated apoptosis independently of caspase-9 processing.

Discussion

A role for Cer in apoptosis has been proposed in several apoptotic models, including DX-mediated thymocyte death (6). In contrast, our data strongly suggest that SP, a Cer metabolite, is involved in signaling apoptosis in response to DX. Indeed, 10^{-6} M DX induced a weak and transient production of Cer, whereas the generation of SP was robust and sustained, concomitant to the activation of acid and neutral Cerases, and preceded the appearance of nuclear condensation. In contrast, at 10^{-8} M DX, the accumulation of Cer was larger and followed by a smaller conversion into SP. Interestingly, the apoptotic effect of DX was weaker in the latter conditions, suggesting that DX mediates thymocyte death through SP generation rather than Cer accumulation. t-MAPP and NOE, Cerase inhibitors, prevented the generation of SP in response to DX, and although they increased Cer accumulation, they drastically reduced apoptosis, again arguing in favor of a role of SP, rather than Cer, in DX-mediated cell death. Furthermore, exogenous SP, or inhibition of SP kinase by DMS, induced thymocyte apoptosis, as reported in some cell types (11, 14). Additionally, whereas both exogenous aSMase and nSMase triggered apoptosis and the generation of Cer, only aSMase was able to increase SP level and aSMase-mediated apoptosis was strongly reduced by Cerase inhibition. Thus, it is likely that aSMase mediates apoptosis, at least in part, through the generation of Cer in a subcellular compartment not accessible to Cerases. Consequently, Cer and SP appear to be independent apoptotic messengers in thymocytes, as previously reported in cancer cells of different origins (40). However, our data reveal an unappreciated role of SP in glucocorticoid-induced thymocyte apoptosis.

DX-induced Cer and SP production appeared to be dependent on caspase activation. Early caspase-8 activation, via FADD-procaspase-8 complexes, has been involved in aSMase activation in T cells after CD95 ligation (5, 17, 18, 41). Furthermore, CD95 clustering and recruitment of FADD-procaspase-8 complexes, independently of CD95 ligation, have been reported following anticancer drug treatment or hypoxia/serum deprivation (42, 43). Interestingly, in thymocytes, glucocorticoids promote the formation of FADD-procaspase-8 complexes (16). However, this mechanism could not account for caspase-8 activation upstream of aSMase, because the formation of FADD-procaspase-8 complexes, as well as caspase-8 activation, occurred later than the lipid...
generation (Refs. 6 and 16), and present data). Moreover, disruption of raft with methyl-β-cycloexetrin, which strongly inhibits CD95 clustering and CD95-mediating thymocyte death (19), only marginally reduced DX-induced apoptosis (S. Lépine and F. Giraud, unpublished data). At present, the identity and the activation mechanism of the caspase responsible for aSMase activation upon DX stimulation remain unknown. However, recent studies suggest a role for the Src kinase, p56<sup>ck</sup>, in caspase activation and apoptosis (44, 45). Interestingly, in thymocytes, DX-induced apoptosis is mediated through the activation of an unknown Src kinase (16). Therefore, it could be possible that DX induced early caspase activation through the stimulation of p56<sup>ck</sup>. The target of this caspase could be aSMase itself, one of its upstream regulators (Src kinase, phospholipase C (16)) or a protein that negatively couples to one of these enzymes. In fact, it is not known whether the Src kinase acts upstream or downstream of the early caspase.

The present study provides evidence that SP is mediating the activation of caspase-8/3 through a mitochondrial-independent pathway in DX-stimulated thymocytes. Indeed, blockade of SP production with Cerase inhibitors resulted in the impairment of caspase-8/3 processing, without affecting Δψ<sub>m</sub> loss or caspase-9 activation. A role for caspase-8 upstream of mitochondria, in DX-stimulated thymocytes, was proposed recently, because cytochrome <em>c</em> release was shown to be inhibited by a caspase-8 inhibitor, Z-IETD (16). However, cytochrome <em>c</em> release can be achieved in a caspase-dependent, via caspase-8 cleavage of Bid in CD95 (type II cells) and TNF-initiated cell death, or caspase-independent manner with other death-promoting stimuli (39). As Bid is not translocated to mitochondria after DX treatment of thymocytes (23), it seems unlikely that caspase-8 could mediate mitochondrial damage. Consistent with this latter conclusion, we show that Z-IETD was unable to inhibit DX-induced Δψ<sub>m</sub> loss. Z-DEVD and Z-LEHD, inhibitors of caspase-3 and -9, respectively, did not alter DX-induced Δψ<sub>m</sub> loss either, indicating that caspase-8,-3, and -9 are activated downstream and/or independently of mitochondria.

In thymocytes stimulated with the glucocorticoid, inhibition of Δψ<sub>m</sub> loss, by BA or by blocking proteasome activity, was shown to prevent cytochrome <em>c</em> release and to abolish or to reduce caspase-3 activation (23, 24, 26). In the present study, we show that blocking Δψ<sub>m</sub> loss through inhibition of either PTP by BA, or proteasome activity by MG132, abolished only caspase-9 processing, indicating that the proteasome/mitochondria pathway is responsible for caspase-9 activation, presumably through the apoptosome complex, initiated by cytochrome <em>c</em> release from mitochondria (46). However, in contrast to the results of Yoshino et al. (23), we have not observed—with thymocytes from a different mouse strain and a lower BA concentration—any inhibition of caspase-3 by BA, although caspase-9 was inhibited. Our data suggest that activation of caspase-3 did not result from upstream activation of caspase-9, but rather of caspase-8. In support of this conclusion, we found that caspase-8 and -3 activation was absolutely required for thymocyte apoptosis induced by DX, whereas caspase-9 was dispensable. The fact that BA inhibited DX-induced apoptosis could result from the blockade of mitochondrial release of apoptogenic proteins, other than cytochrome <em>c</em>, mediating activation of an unknown factor downstream of caspase-3 (47). In contrast to BA, MG132 reduced caspase-8 and -3 processing, indicating that proteasome activity is involved, as well as SP, in caspase-8/3 activation independently of mitochondrial alterations. FLIP has been identified as a blocker of apoptosis induced by TNF family death receptors (47). FLIP binds to CD95-FADD complex, inhibiting the recruitment and activation of caspase-8 (formerly known as FLICE). Interestingly, down-regulation of FLIP was shown to be mediated through ubiquitin/proteasome pathway (48), raising the possibility that DX-induced thymocyte apoptosis could be mediated through a proteasome-dependent caspase-8 activation. Additionally, proteasome activity could indirectly control caspase-9 and -3 processing, independently of mitochondria, through members of the inhibitor of apoptosis (IAP) family (XIAP and cIAP1), which are direct inhibitors of caspase-9 and -3 (49), capable of autoubiquitination and undergoing proteasome-dependent degradation in DX-treated thymocytes (50). Because DX-induced sphingolipid production is not dependent on proteasome activity, it appears that both SP and proteasome activity are controlling the caspase-8/3 cascade. This interpretation is consistent with the finding that apoptosis was partially reduced by inhibitors of either sphingolipid metabolism or proteasome activity. In contrast, inhibitors of caspase-8 and -3 totally abolished apoptosis, confirming that these caspases are activated downstream from sphingolipid and proteasome in the apoptotic cascade.

In conclusion, our study indicates that DX induces thymocyte apoptosis through different pathways (Fig. 7). One, mediated by caspase-dependent SP generation through the sequential activation of aSMase and Cerases, contributes to the activation of the caspase-8/3 cascade independently of the proteasome/mitochondria pathway. In parallel, independently of SP production, the activation of the caspase-8/3 cascade also depends on proteasome activity. A third pathway mediating apoptosis involves proteasome-dependent mitochondrial alterations, although by a mechanism independent of caspase-9 processing.

**Acknowledgments**

We thank Monique Sauvage and Pierre Mazière for their assistance in the initial experiments of this study.

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