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Desensitization of the Permeability Transition Pore by Cyclosporin A Prevents Activation of the Mitochondrial Apoptotic Pathway and Liver Damage by Tumor Necrosis Factor-α*

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We studied the effects of cyclosporin A (CsA) administration 1) on the properties of the permeability transition pore (PTP) in mitochondria isolated from the liver and 2) on the susceptibility to hepatotoxicity induced by lipopolysaccharide of *Escherichia coli* (LPS) plus D-galactosamine (D-GalN) in rats. CsA exerted a marked PTP inhibition *ex vivo*, with an effect that peaked between 2 and 9 h of drug treatment and decayed with an apparent half-time of about 13 h. Administration of LPS plus D-GalN to naive rats caused the expected increased serum levels of tumor necrosis factor (TNF)-α, liver inflammation with BID cleavage, activation of caspase 3, appearance of terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling-positive nuclei, and release of alanine aminotransferase and aspartate aminotransferase into the bloodstream. Treatment with CsA before or within 5 h of the administration of LPS plus D-GalN protected rats from hepatotoxicity despite the normal increase of serum TNF-α and BID cleavage. These results indicate that CsA prevents the hepatotoxic effects of TNF-α by blocking the mitochondrial proapoptotic pathway through inhibition of the PTP and provides a viable strategy for the treatment of liver diseases that depend on increased production and/or liver sensitization to TNF-α.

Key advances are being made in understanding the role of mitochondria in pathophysiology, in particular as mediators of the amplification and execution phases of apoptosis (1). These advances may in turn yield novel drugs and treatments for high-prevalence conditions such as cancer and degenerative diseases (2). Mitochondria release proteins that cause cell death through both caspase-dependent and -independent mechanisms. The release of these apoptogenic factors may be triggered by the engagement of surface receptors such as the TNF-α[1] receptor (3–5). The ensuing caspase 8 activation causes cleavage of BID, the truncated form of which, tBID, targets mitochondria and causes the release of cytochrome *c* (6–8) in a process modulated by proteins of the BCL-2 family. Indeed, antiapoptotic members inhibit (9, 10), whereas proapoptotic members favor the release process (11–15). Release of cytochrome *c* and other apoptogenic factors can also be caused by opening of the PTP, an inner membrane channel that is modulated by a variety of proapoptotic signals (15–21). The two mechanisms are not mutually exclusive, because the PTP participates in the release of proapoptotic proteins through at least two mechanisms: swelling-dependent rupture of the outer mitochondrial membrane and remodeling of cristae with increased availability of cytochrome *c* for release through the specific pathways activated by outer membrane tBID insertion (19, 20).

The importance of PTP regulation in pathophysiology *in vivo* has been recently highlighted by two studies from our laboratory. We were able to cure dystrophic *Col6a1*$^{-/-}$ mice, a model of Bethlem myopathy (22), with the PTP inhibitor cyclosporin A (CsA) through a demonstrably mitochondrial effect (23), and we found that up-regulation of mitochondrial BCL-2 caused resistance to apoptosis through desensitization of the PTP during tumor promotion by the hepatocarcinogen 2-acetylaminofluorene (24). The latter observation is of specific relevance to hepatotoxic diseases that depend on liver sensitization to TNF-α (25, 26) because feeding with 2-acetylaminofluorene confers resistance to the hepatotoxicity otherwise caused by treatment with LPS plus D-GalN (24).

TNF-α triggers other well-characterized proapoptotic pathways whose effector signals may converge on mitochondria. Activation of sphingomyelinases (27) generates ceramide (28) and GD3 ganglioside (29, 30), which directly affect mitochondrial function by increasing the PTP open time (31–35). Generation of ceramide-1-phosphate by ceramide kinase activates cytosolic phospholipase A2, resulting in release of arachidonic acid (36), which in turn provides a key amplification loop through further stimulation of sphingomyelin breakdown (37) and PTP opening (18, 21). An additional mechanism specifically linked to activation of acidic sphingomyelinase is down-regulation of liver methionine adenosyltransferase 1A, which leads to depletion of glutathione and sensitizes hepatocytes to TNF-α (38).

The present study was undertaken to test whether the mitochondrial PTP is the effector system of TNF-α-triggered hepatotoxicity by evaluating the protective effects of CsA on 1) PTP

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[1] Abbreviations used are: TNF, tumor necrosis factor; tBID, truncated BID; CsA, cyclosporin A; LPS, *E. coli* lipopolysaccharide; D-GalN, D-galactosamine; PTP, permeability transition pore; CRC, calcium retention capacity; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling; MOPS, 3-(*N*-morpholino)propanesulfonic acid; CyP, cyclophylin.

opening *ex vivo* and 2) occurrence of liver damage after treatment of rats with LPS plus D-GalN. We show that treatment with 5 mg of CsA/kg of body weight immediately before or within 5 h of the administration of LPS plus D-GalN protected rats from hepatotoxicity despite a normal increase of serum TNF-α and onset of BID cleavage. Protection matched PTP desensitization, indicating that the final common pathway for the hepatotoxic effects of TNF-α is the PTP-dependent activation of the mitochondrial proapoptotic pathway. These findings provide a viable strategy for the treatment of liver diseases that depend on increased production and/or liver sensitization to TNF-α.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—LPS from *Escherichia coli* serotype O111:B4 and tetraglycol (vehicle) were from Sigma. CsA and D-GalN were from Fluka (Milano, Italy). Calcium Green-5N was purchased from Molecular Probes (Eugene, OR). The antibody against cleaved caspase 3 was from Cell Signaling (Milano, Italy), and the antibody against actin was from Sigma. The 50 mg/ml CsA stock solution (Sandimmune) used for the *in vivo* experiments was from Novartis Pharma (Basel, Switzerland). Horseradish peroxidase-conjugated goat anti-rabbit IgG and anti-mouse Ig were from Southern Biotechnology Associates (Birmingham, AL). ECL detection reagents were from Amersham Biosciences.

In Vivo Treatments—Male Albino Wistar rats (250–300 g) had free access to a standard diet and were kept under controlled conditions of temperature and humidity on a 12-h light/12-h dark cycle. Animals were anesthetized by inhalation of 1.5–3% isofluorane/O_2 when required during the *in vivo* experiments and were sacrificed by cervical dislocation. Rats received the specified doses of CsA (Sandimmune diluted in olive oil) and/or 20 μg LPS plus 700 mg of D-GalN/kg of body weight or vehicle (tetraglycol diluted in olive oil) by intraperitoneal injection. For further details, see the figure legends. All *in vivo* experiments were approved by the competent Authority of the University of Padova and authorized by the Italian Ministry of Health.

Preparation of Mitochondria and CRC Test—Liver mitochondria were prepared from liver homogenates by differential centrifugation exactly as described previously (39). The CRC of mitochondrial preparations was assessed fluorimetrically in the presence of the Ca^{2+} indicator Calcium Green-5N (1 μM; excitation, 505 nm; emission, 535 nm) with a PerkinElmer LS50B spectrofluorimeter equipped with magnetic stirring and thermostatic control. The incubation conditions are specified in the legend to Fig. 1.

Measurement of Serum TNF-α, ALT, and AST levels—At the times specified in the figure legends blood samples were withdrawn from the tail vein. The blood was drawn into sterile test tubes, which were stored for 2 h at room temperature to allow clotting, and sera were prepared by centrifugation at 2,000 $\times$ *g* for 20 min. An aliquot of serum was used to determine the concentration of TNF-α; the remainder was stored at -80 °C until determination of serum enzymes was performed. TNF-α was determined using a commercial immunoassay (Quantikine M; R&D Systems, Inc.). Serum levels of ALT and AST were kindly determined by Dr. Martina Zaninotto (Department of Laboratory Medicine, Padova University Hospital) according to standard procedures.

TUNEL and Hematoxylin & Eosin Staining—Animals were sacrificed by cervical dislocation, and individual livers were sectioned to obtain samples for hematoxylin-eosin staining, for the TUNEL reaction, and for Western blot analysis. Hematoxylin-eosin staining was carried out by standard procedures on 8-μm sections after sample fixation, dehydration, and embedding in paraffin. The TUNEL reaction was carried out on 8-μm paraffin-embedded liver sections with an *in situ* cell detection kit, which includes a fluorescein-DNA labeling system and an anti-fluorescein antibody conjugated with horseradish peroxidase (Roche Molecular Biochemicals). Sections preincubated for 30 min with 5 μg/ml DNase I (Invitrogen) served as positive controls. Slides were analyzed with the use of a Leica DMR optical microscope.

Western Blot Analysis—Liver homogenates were extracted with 1% Nonidet P-40 in 0.3 mannitol, 5 mM Tris-MOPS, pH 7.4, 4 mM KH_2PO_4, 1 mM EGTA, 2 mM phenylmethylsulfonyl fluoride, and 10 μg/ml aprotinin for 30 min at 4 °C, and the lysates were centrifuged at 90,000 $\times$ *g* at 4 °C in a Beckman Coulter L7-55 ultracentrifuge. The extracts were diluted with 2$\times$ Laemmli gel sample buffer and boiled for 3 min. Identical protein amounts were separated by SDS-PAGE in 12% acrylamide-0.8% bisacrylamide slab minigels, electroblotted onto nitrocellulose membranes (for caspase 3 and actin) or Immobilon-P polyvinylidene difluoride membranes (for tBID and actin) (Millipore), and sequentially immunoblotted with antibodies against BID, cleaved caspase 3, and actin. Signals were visualized with ECL reagents.

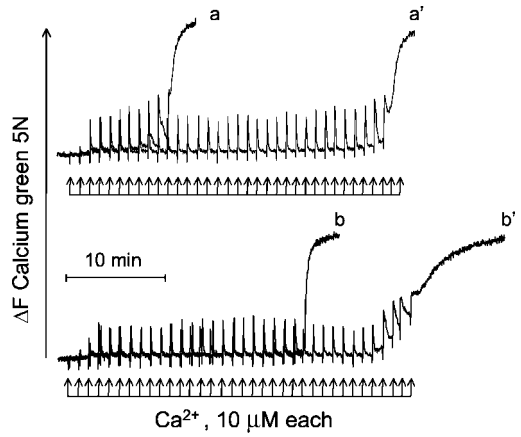

FIG. 1. **Effect of treatment with CsA *in vivo* on the CRC of isolated liver mitochondria.** Liver mitochondria were isolated 2 h after treatment of rats with vehicle (*traces a* and *a′*) or with 5 mg/kg of body weight of CsA (*traces b* and *b′*). The incubation medium contained 0.2 M sucrose, 10 mM Tris-MOPS, 5 mM succinate-Tris, 1 mM Pi-Tris, 10 μM EGTA-Tris, 2 μM rotenone, and 1 μM calcium green-5N. Final volume was 2 ml, pH 7.4, 25 °C. All the experiments were started with the addition of 0.5 mg/ml of mitochondria (not shown). Where indicated (*arrows*), pulses of 10 μM Ca^{2+} were added. In *traces a′* and *b′*, the medium was supplemented with 1 μM CsA. The experiments are representative of four replicates for each condition.

RESULTS

Properties of the PTP in Isolated Liver Mitochondria after Treatment of Rats with CsA—To assess whether the PTP was actually inhibited after administration of CsA to living animals, we treated rats with vehicle or with CsA and isolated liver mitochondria by differential centrifugation 2 h later. The occurrence of PTP inhibition was then estimated by comparing the CRC of mitochondria isolated from vehicle-treated animals with that of CsA-treated ones. The CRC is a sensitive and quantitative measure of the propensity of mitochondria to open the PTP after Ca^{2+} uptake (40). The experiments reported in Fig. 1, *trace a*, show that mitochondria isolated from the liver of a vehicle-treated rat accumulated a train of about 10 pulses of 10 μM Ca^{2+} before the precipitous Ca^{2+} release that marks onset of PTP opening. As expected, the addition of 1 μM CsA to the incubation medium caused an increase of >3-fold in the CRC (*trace a′*), which is consistent with desensitization of the PTP to Ca^{2+} (40). Mitochondria prepared from the liver of rats treated with 5 mg of CsA/kg of body weight for 2 h displayed a striking increase of the CRC, which was more than 2-fold higher than that of vehicle-treated mitochondria (compare *trace b* with *trace a*). Addition of 1 μM CsA to the assay cuvette increased the CRC further (*trace b′*), yet the maximum CRC obtained in the presence of 1 μM CsA was the same for mitochondria prepared from CsA- and vehicle-treated rats (compare *trace b′* with *trace a′*). We next determined the dependence of PTP inhibition on the dose of CsA in protocols identical to those described in Fig. 1. Fig. 2*A* shows that measurable PTP inhibition could be detected already at 2.5 mg of CsA/kg, that maximum inhibition was achieved at 5 mg/kg, and that inhibition decreased as the CsA dose was raised further to 10 mg/kg. At 5 mg of CsA/kg, PTP inhibition was maximal between 2 and 9 h of treatment, with a decay curve indicating a half-life of about 13 h for the inhibitory effect (Fig. 2, *B*). PTP inhibition could be maintained at the peak level for 24 h by a second administration of 5 mg of CsA/kg 10 h after the first dose (results not shown).

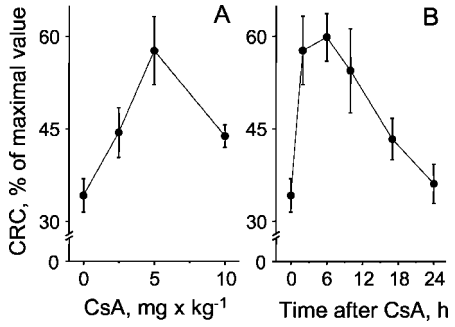

FIG. 2. **Dose dependence and time course of the effects of treatment with CsA *in vivo* on the CRC of isolated mitochondria.** *A*, liver mitochondria were prepared 2 h after treatment of rats with vehicle or with the doses of CsA indicated in the *abscissa*, and the CRC of mitochondria was determined as shown in Fig. 1. Values on the *ordinate* refer to the CRC ± S.D. relative to the maximum obtained in the presence of 1 μM CsA in the assay cuvette ($n = 4$ for each condition). *B*, liver mitochondria were prepared at the times indicated on the *abscissa* after treatment of rats with 5 mg/kg CsA. Values on the *ordinate* have the same meaning as in *A* ($n = 4$ for each condition).

Protection from LPS Plus D-GalN Hepatotoxicity by CsA—Having established the kinetics of PTP inhibition with CsA *ex vivo*, we tested whether rats could be protected with the same time course from the short-term hepatotoxic effects of the combination of 20 μg of LPS (which stimulates TNF-α production by the macrophages) plus 700 mg of D-GalN/kg of body weight (which greatly sensitizes the liver to the cytotoxic effects of TNF-α through a transcriptional block) (25, 26). Treatment of rats with LPS plus D-GalN caused the expected liver damage, which could be easily appreciated from the hematoxylin-eosin stained slides. Most hepatocytes displayed nuclear alterations ranging between chromatolysis (the pale nuclei with a rim of residual chromatin at the periphery) and pyknosis to karyolysis and cytoplasmic vacuolization; inflammatory cells could be detected within the liver parenchyma (Fig. 3*A*, *a*). The occurrence of apoptosis was confirmed by the TUNEL reaction (Fig. 3*A*, *a′*) and by cleavage of caspase 3 (Fig. 3*B*, *lanes 2–4*); the occurrence of necrosis was documented by release of AST and ALT into the bloodstream (Fig. 3*C*, *open bars*). Treatment of rats with 5 mg/kg CsA 1 h before LPS + D-GalN fully protected rats from liver damage as assessed from hematoxylin-eosin liver staining (Fig. 3*A*, *b*), from the absence of TUNEL-positive nuclei (Fig. 3*A*, *b′*), from lack of caspase 3 cleavage (Fig. 3*B*, *lanes 5–7*), and from the marginal increase in serum AST and ALT levels (Fig. 3*C*, *closed bars*). It must be mentioned that the levels of TNF-α increased to comparable levels after treatment with LPS plus D-GalN, peaking after 1–2 h at values of 11.8 ± 3.3-fold and 12.7 ± 3.6-fold over the basal level in vehicle- and CsA-treated animals, respectively ($n = 4$) and that BID underwent cleavage (not shown, but see Fig. 4), consistent with activation of apical caspases.

CsA Hepatoprotection before and after the TNF-α Increase—A question of great relevance is whether hepatoprotection by CsA requires pretreatment of the animals or can also be obtained after the hepatotoxic insult. We therefore studied the effect of the time of administration of CsA relative to that of LPS plus D-GalN on liver BID cleavage and caspase 3 activation, and on release of AST and ALT into the circulation. Fig. 4*A* demonstrates that treatment with CsA (*lanes 2–10*) did not prevent cleavage of BID. On the other hand, Fig. 4*B* shows that pretreatment with CsA (*lane 2*), treatment at the same time as LPS plus D-GalN (*lanes 3* and *4*), or treatment 1.5 h (*lanes 5* and *6*) or 3 h (*lanes 7* and *8*) after the addition of LPS plus D-GalN prevented caspase 3 activation, whereas treatment after 8 h was ineffective (*lane 9*). These results are fully consistent with inhibition of transaminase release when CsA was added up to 5 h after LPS plus D-GalN (Fig. 4*C*).

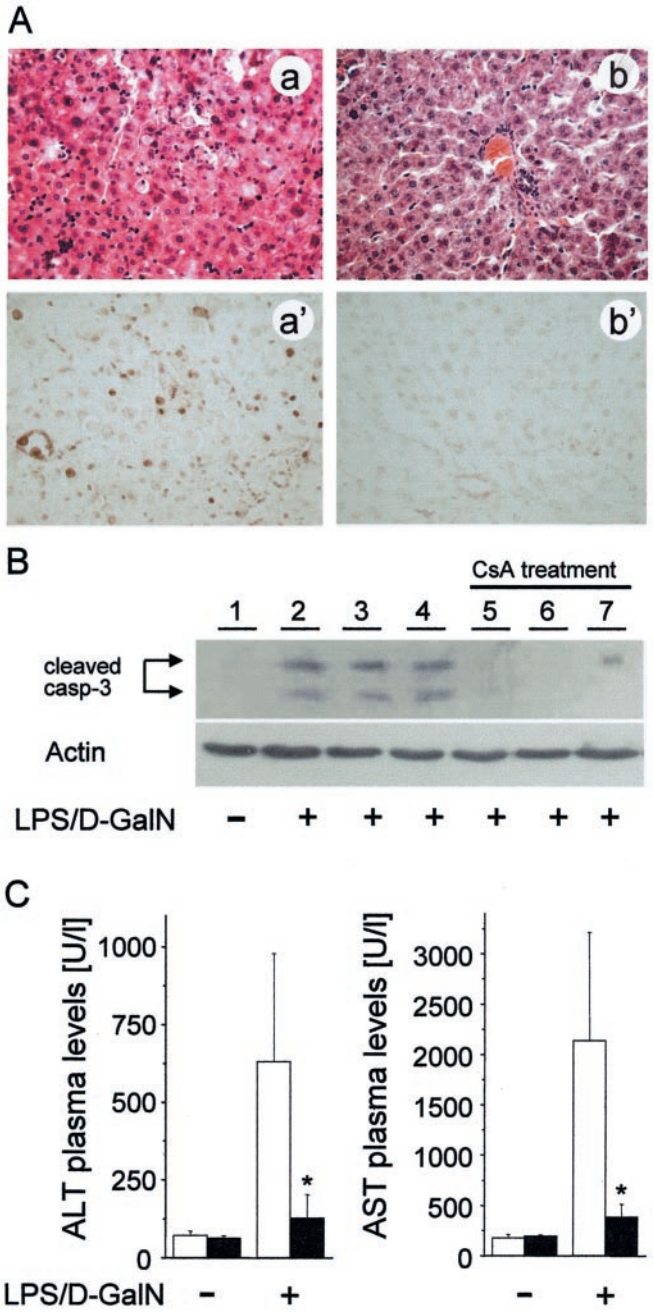

FIG. 3. **Protective effect of CsA on liver damage caused by *in vivo* treatment with LPS plus D-GalN.** *A*, paraffin-embedded liver sections were stained with hematoxylin-eosin (*a* and *b*) or processed for the TUNEL reaction (*a′* and *b′*) 8 h after treatment with 20 μg of LPS plus 700 mg of D-GalN/kg of body weight. Rats had been pretreated for 1 h with either vehicle (*a* and *a′*) or with 5 mg of CsA/kg of body weight (*b* and *b′*). *B*, liver homogenates were prepared from vehicle-treated rats (*lane 1*) and from rats treated with 20 μg of LPS plus 700 mg of D-GalN/kg for 8 h (*lanes 2–7*) and processed for detection of caspase 3 cleavage and of actin by Western blot as described under "Experimental Procedures." Rats had been pretreated for 1 h with vehicle (*lanes 1–4*) or with 5 mg/kg CsA (*lanes 5–7*). *C*, serum samples were prepared from vehicle-treated rats and from rats treated with 20 μg of LPS plus 700 mg of D-GalN/kg for 8 h where denoted by the − and + signs, respectively. Rats had been pretreated for 1 h with vehicle (*open bars*) or with 5 mg/kg CsA (*closed bars*). Values on the *ordinate* report the average concentration of serum AST and ALT ± S.D. ($n = 4$). *Asterisks* denote $p < 0.05$ relative to animals treated with CsA and LPS plus D-GalN.

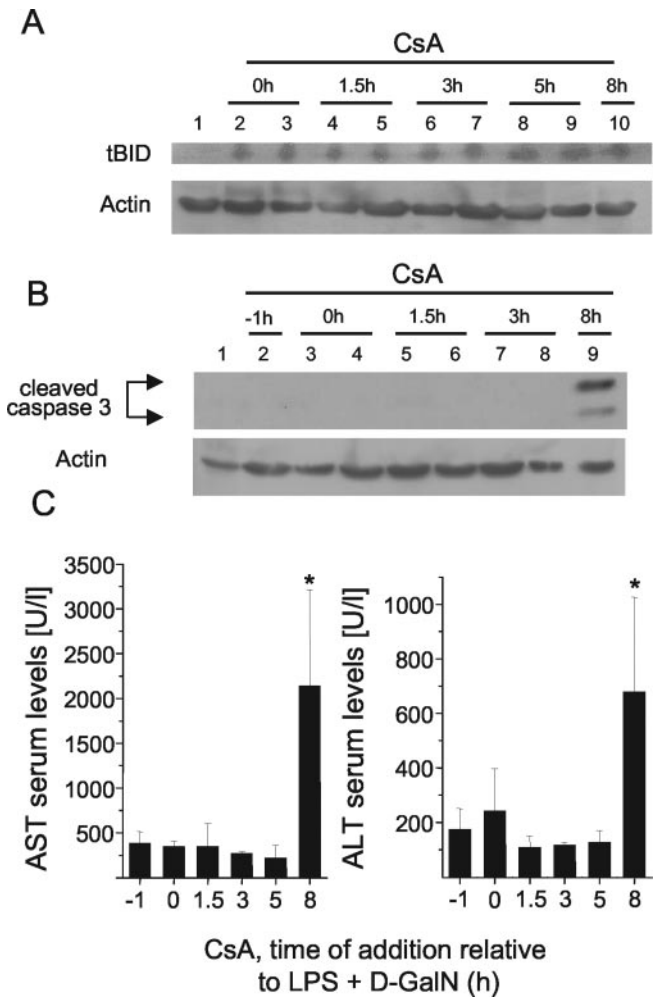

FIG. 4. **Effect of the time of CsA addition relative to LPS plus D-GalN on hepatoprotection.** *A*, liver homogenates were prepared from vehicle-treated rats (*lane 1*) and from rats treated with 20 μg of LPS plus 700 mg of D-GalN/kg (*lanes 2–10*) and processed for detection of tBID formation and of actin by Western blot as described under "Experimental Procedures." Rats had been treated with 5 mg/kg CsA at the same time of injection of LPS plus D-GalN (*lanes 2* and *3*) or 1.5 h (*lanes 4* and *5*), 3 h (*lanes 6* and *7*), 5 h (*lanes 8* and *9*), or 8 h (*lane 10*) after LPS plus D-GalN. *B,* liver homogenates were prepared from vehicle-treated rats (*lane 1*) and from rats treated with 20 μg of LPS plus 700 mg of D-GalN/kg for 8 h (*lanes 2–9*), and processed for detection of caspase 3 cleavage by Western blot as described under "Experimental Procedures." Rats had been treated with 5 mg/kg CsA 1 h before LPS plus D-GalN (*lane 2*), at the same time as LPS plus D-GalN (*lanes 3* and *4*), or 1.5 h (*lanes 5* and *6*), 3 h (*lanes 7* and *8*), or 8 h (*lane 9*) after LPS plus D-GalN. *C*, serum samples were prepared from rats treated with 20 μg of LPS plus 700 mg of D-GalN/kg. Rats had been treated with 5 mg/kg CsA at the times indicated on the *abscissa* (times are relative to the addition of LPS plus D-GalN). Values on the ordinate refer to the average concentration of serum AST and ALT ± S.D. ($n = 4$). Asterisks denote a $p < 0.05$ relative to the values displayed before treatment with LPS plus D-GalN.

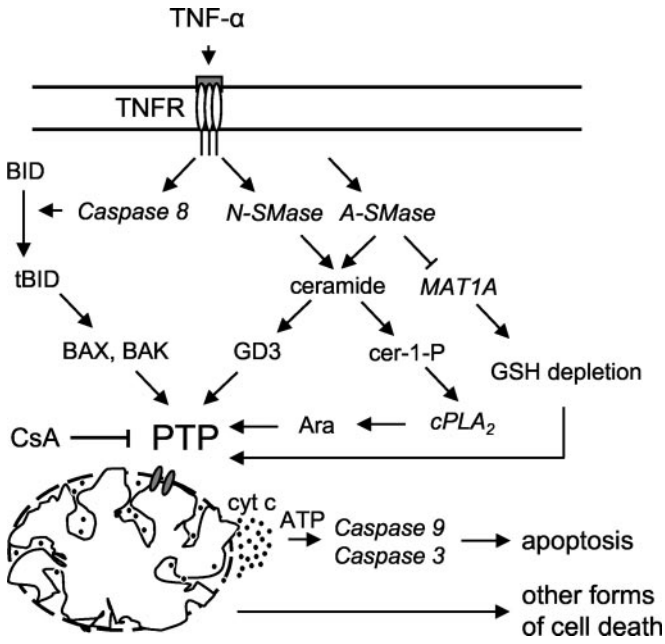

FIG. 5. **Pathways to cell death after engagement of the TNF-α receptor.** The scheme illustrates how engagement of the CD120a TNF-α receptor (*TNFR*) by TNF-α may trigger several signaling pathways converging on the PTP. Caspase 8 activation is followed by BID cleavage and BAX-BAK translocation to mitochondria, which could directly increase the PTP open time. Activation of neutral sphingomyelinase (*N-SMase*) and acidic sphingomyelinase (*A-SMase*) generates ceramide, which can be further transformed into the PTP-inducing GD3 ganglioside and into ceramide-1-phosphate (*cer-1-P*), activating in turn cytosolic phospholipase A2 ($cPLA_2$) with production of the potent PTP agonist arachidonic acid (*Ara*). An additional mechanism of PTP induction would be depletion of GSH after down-regulation of the liver-specific methionine adenosyltransferase type I (*MAT1A*) transcript. PTP opening results in cytochrome (*cyt*) *c* release and, in the presence of sufficient amounts of ATP, in caspase 9 activation followed by activation of caspase 3 and execution of the apoptotic program. If ATP levels are not adequate, caspase 9 cannot be activated despite release of cytochrome *c*. In this case, mitochondrial dysfunction (caused by PTP-dependent depolarization and by the respiratory inhibition that follows pyridine nucleotide depletion) may trigger other forms of caspase-independent cell death (*e.g.* through Ca^{2+} deregulation and overproduction of reactive oxygen species). Irrespective of the mode of cell death, treatment with proper doses of CsA desensitizes the PTP to the mediators triggered by *TNFR* engagement and prevents hepatocellular damage. For further explanation, see text.

DISCUSSION

In this study, we have shown that administration of CsA to living animals leads to *in situ* inhibition of the PTP, which can still be detected in mitochondria subsequently isolated from the liver of treated animals. By studying the CRC of isolated mitochondria, we have established the dose-dependence and the kinetics of PTP inhibition by CsA *in vivo*, and we have shown that CsA suppresses the TNF-α-dependent mitochondrial liver proapoptotic pathway with the same time course. Indeed, treatment with CsA conferred full resistance to the short-term effects of LPS plus D-GalN at doses of the latter that would otherwise cause extensive liver damage. These results integrate previous studies on death receptor signaling to mitochondria *in vivo* (38, 41–43), document the role of the PTP as the final common pathway for TNF-α liver toxicity *in vivo*, and provide a strong rationale for the use of CsA in conditions under which hepatic damage is caused by increased production and/or sensitization to the proapoptotic effects of TNF-α.

Cell Death Pathways in TNFα-dependent Hepatotoxicity—Fig. 5 summarizes the pathways involved in liver apoptotic signaling triggered by engagement of the TNF-α CD120a receptor. The involvement of all these pathways in the onset of liver damage after treatment with LPS plus D-GalN or with Fas is clearly documented by the protective effects observed after genetic ablation of BID (41, 43), of the adapter protein FAN (factor associated with neutral sphingomyelinase activation), which is essential for activation of neutral sphingomyelinase (42, 44, 45), and of acidic sphingomyelinase (46). Taken together, these findings indicate that there is no redundancy between the effector mechanisms triggered by activation of caspase 8, of neutral sphingomyelinase, and of acidic sphingomyelinase and suggest that specific, permissive signals arising from each pathway must be present for the death program to proceed. This pattern of activation is remarkably similar to

that established for the PTP, which responds to a wide variety of signals that act in a combinatorial fashion at discrete sites to determine the overall open probability (47).

Fig. 5 also illustrates how the signaling molecules generated by BID cleavage and by activation of sphingomyelinases may converge on the PTP, which, based on the protective effects of CsA, must be downstream of these pathways. It should be noted that the prevailing mode of cell death caused by PTP opening (apoptosis with caspase 9 and 3 activation *versus* other forms of cell death, necrosis being the extreme end of a continuum (48)) probably depends on the PTP open time (49). Indeed, openings of short duration could cause intramitochondrial cytochrome *c* redistribution, which stimulates cytochrome *c* release through the tBID/BAX-dependent pathway without outer membrane rupture and loss of pyridine nucleotides (19, 20). The maintained ATP levels would allow formation of an active apoptosome leading to cleavage of caspase 9 and then caspase 3, eventually resulting in apoptosis. On the other hand, openings of long duration would cause matrix swelling with outer membrane rupture, depletion of pyridine nucleotides with respiratory inhibition, irreversible depolarization, and mitochondrial hydrolysis of ATP (49). The resulting ATP depletion would prevent activation of caspase 9 and switch cell death to a different subroutine (50, 51), where caspase-independent events may predominate (48). Thus, a continuum of modes of cell death is possible that is consistent with the spectrum of morphological changes recorded by histology.

Inhibition of the PTP after CsA Administration in Vivo—CsA exerts all of its known biological effects after binding to CyPs, a family of proteins possessing peptidyl-prolyl-*cis-trans*-isomerase activity that is inhibited by CsA (52, 53). The mitochondrial effects of CsA (54, 55) are mediated by its binding to CyP-D, a unique CyP located in the mitochondrial matrix (56–58). Because of the high-affinity CsA·CyP-D interaction (the concentration of CsA required for 50% inhibition is about 100 nM), the propensity of the PTP to open is decreased through an effect that does not require calcineurin inhibition (57). It must be stressed that PTP opening in the presence of CsA is still possible (*e.g.* by increasing the load of matrix Ca^{2+} (Fig. 1) or by the effect of additional inducing factors such as oxidants and thiol reagents (see Ref. 47 for a review). The effect of CsA on the PTP is thus best described as "desensitization", an effect that can be quantitated with the CRC test (Fig. 1). Remarkable findings of our work are 1) that the mitochondrial effects of CsA display an optimum at a dose of 5 mg of CsA/kg of body weight, whereas higher concentrations are less effective; and 2) that the maximum effect is observed between 2 and 9 h of administration, with return to the basal level within 24 h of treatment. The first observation is probably caused by the overlapping effect of CsA toxicity, which is mediated by overproduction of reactive oxygen species (59, 60) that may counterbalance the desensitizing effects of CsA on the PTP (47); the second observation rules out the possibility that immunosuppression, which requires several days to develop, may be involved in the short-term hepatoprotective effects of CsA. Both observations help clarify outstanding questions about the effector mechanisms of hepatotoxicity by TNF-α and of hepatoprotection by CsA in various experimental models.

Protection against Liver Damage by CsA—The hepatoprotective effects of CsA have been tested in three *in vivo* animal models of toxicity (*i.e.* treatment of rats with LPS after liver sensitization with heat-inactivated *Propionibacterium acnes* (61) or D-GalN (62), treatment of cats with LPS alone (63), and treatment of mice with an anti-Fas antibody (64)) and in one cohort of cases of fulminant viral hepatitis in combination with interferon-β (65). In the animal models, the dose of CsA ranged between 6 (63), 10 (61, 62) and 100 mg/kg of body weight (64), which conferred variable degrees of protection; the time of administration relative to the hepatotoxic treatment ranged from pretreatment with a single dose (61, 63, 64) to repeated administrations of the same dose at different time intervals (62). This latter study revealed that the time of CsA administration was a critical factor, in that maximum protection was achieved by treatment with CsA at the same time as the hepatotoxic treatment with LPS plus D-GalN, followed by repeated CsA treatments. It is remarkable that pretreatment with CsA for 24 h and then 2 h before LPS plus D-GalN made the damage worse (62), suggesting that toxicity may become a problem as the overall dose of CsA is increased above an optimum (see Fig. 2A). Our results offer unique clues on the basis for these variable effects of CsA and provide a rationale for its use in a clinical setting. Indeed, the protective effects of CsA *in vivo* were strictly correlated with inhibition of the PTP, and determination of the time course of PTP inhibition by CsA provided a strong rationale to optimize the CsA dose and time of treatment to achieve maximum protection from hepatic damage. If it is permissible to extrapolate our results to other experimental settings, it should be possible to maximize liver protection by treatment with 5 mg of CsA/kg of body weight within 5 h of the hepatotoxic insult, followed by the same maintenance dose every 12 h during the acute phase. Very encouraging results were obtained with an initial dose of 3 mg of CsA/kg by continuous drip infusion for 2 days, followed by a maintenance dose of 1 mg/kg in a clinical trial on 13 patients with fulminant viral hepatitis. The simultaneous use of interferon-β prevents an assessment of whether the protective effects depended on CsA alone (65). Based on the present results, however, we believe that CsA should be considered a potential hepatoprotective drug in diseases that depend on liver sensitization to apoptosis triggered by TNF-α.

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