Liver Protection from Apoptosis Requires Both Blockage of Initiator Caspase Activities and Inhibition of ASK1/JNK Pathway via Glutathione S-Transferase Regulation*

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Control of apoptosis is essential to development and homeostasis in mammals. Progression of the apoptotic signal can be inhibited at different levels depending on death inducers and cell types. Thus, endogenous caspase inhibitors such as cellular FLICE-like inhibitory protein (c-FLIP) (1) have been shown to prevent apoptotic transduction through inhibition of the initiator caspase-8 maturation. Bcl-2 and Bcl-XI (2, 3) are also capable of blocking apoptosis, but this time at mitochondrion level by preventing cytochrome c release (4) and hence the downstream maturation of caspase-9. In addition, inactivation of caspases might even occur after their cleavage through AKT-dependent phosphorylation of caspase-9 (5) or by inhibitors of apoptosis proteins (IAPs)† that bind to cleaved caspase-3, -7, and -9 (6). Moreover, nitric oxide (NO), generated by NO donors or NO synthases, has been reported to block activity of several members of the caspase family via S-nitrosylation of their catalytic site (7, 8). All these mechanisms are aimed at preventing inappropriate precaspase maturation or inhibiting activity of matured caspases.

In TNFα-induced apoptosis, upon TNFα binding, oligomerization of TNF receptors occurs and results in aggregation of death domain-containing proteins, allowing recruitment of TRADD (TNF receptor I-associated death domain protein). TRADD binds FADD (Fas-associated death domain-containing protein) and TRAF-2 (TNF receptor I-associated protein 2) proteins, which in turn lead to activation of precaspase-8 and ASK1 (apoptosis signal-regulating kinase 1), respectively (9). ASK1 is a MAP kinase kinase kinase (MAPKKK), ubiquitously expressed, that activates the MKKK4/MKK7-JNK (c-Jun N-terminal kinase) and MKK3/MKK6-p38 signaling cascades (10). JNK and p38 MAP kinase are preferentially activated by stress agents such as UV radiation, osmotic shock, and proinflammatory cytokines, including TNFα. The disruption of the ASK1 gene in mouse (11) strongly reduces cell death induced by these activators indicating that ASK1 is a key element in cytokine- and stress-induced apoptosis (9). In addition, overexpression of wild-type or constitutively active form of ASK1 induces apoptosis in various cell types (10, 12, 13), whereas the kinase-inactive mutant of ASK1 inhibits it (10, 12, 14). ASK1 activity is negatively regulated through phosphorylation by AKT (15) or dephosphorylation by CDC25 (16) or protein phosphatase 5 (17), or through sequestration by proteins such as thioredoxin (12, 18), 14-3-3 (19), or Raf-1 (20). Intriguingly, a recent report shows that mouse glutathione S-transferase mu1 (GST M1) (21) can also bind ASK1.

GST M1 is a member of the GST protein family that catalyzes the conjugation of reduced glutathione (GSH) to a variety of electrophiles (22). In addition to this role, these proteins

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Hepatoprotection mediated by free radical scavenging molecules such as dimethyl sulfoxide (Me2SO) arose the question as to whether this effect involved one or several anti-apoptotic signals. Here, using primary cultures of rat hepatocytes and in vivo thioacetamide-induced liver failure, we showed that Me2SO failed to prevent any cleavage of initiator caspase-8 and -9 but constantly inhibited precaspase-3 maturation and apoptosis execution, pointing to an efficient inhibition of cleaved initiator caspase activities. Evidence was recently provided that apoptosis might require both caspase and ASK1/JNK-p38 activities. We demonstrated that this kinase pathway was strongly inhibited in the presence of Me2SO whereas overexpression of ASK1 was able to restore caspase-3 activity and apoptosis. Interestingly, we also found that GST M1/2 and GST A1/2 dropped under apoptotic conditions; furthermore transfection of GST M1, A1, or P1 to cells overexpressing ASK1, abolished caspase-3 activity and restored viability. This role of GSTs was further assessed by showing that their high expression level was tightly associated with inhibition of ASK1 activity in Me2SO-protected hepatocytes. Together, these results demonstrate that Me2SO-mediated hepatoprotection involves a dual inhibition of cleaved initiator caspase and ASK1/JNK-p38 activities. Furthermore, in highlighting the control of apoptosis by GSTs, these data provide new insights for analyzing the complex mechanisms of hepatoprotection.

The abbreviations used are: IAP, inhibitor of apoptosis protein; cFLIP, cellular FLICE-like inhibitory protein; Me2SO, dimethyl sulfoxide; THA, thioacetamide; PARP, poly(ADP-ribose)/polymerase; DEVD-AMC, Asp-Glu-Val-Asp-7-amino-4-methylcoumarin; ASK1, apoptosis signal-regulating kinase; JNK, c-Jun N-terminal kinase; GST, glutathione S-transferase; TRX, thioredoxin; DTT, dithiothreitol; TNF, tumor necrosis factor.
have also been shown to serve as nonenzymatic binding proteins interacting with various lipophilic compounds that include steroid and thyroid hormones (23). GSTs have been grouped into eight classes, with the most abundant ones being the alpha, mu, and pi classes (22). In rat hepatocytes, GSTP1 have been associated to cell proliferation and decreased level of differentiation in contrast to GST A1/2, which is related to high level of differentiation. GST M1/2 seemed to be expressed under both situations (24, 25). In addition, GST M1 and P1 appear to act as direct inhibitors of ASK1 and JNK, respectively, independently of their catalytic detoxication activity and of cellular GSH level (21, 26). This strongly suggests that GSTs might be involved in cell protection against apoptotic signals, such as osmotic stress, TNFα, or UV radiation, through both detoxication and inhibition of the stress-signaling cascade ASK1-JNK. However, the contribution of GSTs, proteins highly expressed in liver parenchymal cells, to apoptosis of these cells has never been investigated.

Liver injuries can be induced by a variety of apoptotic factors such as TNFα or Fas ligand (27, 28), as well as hepatotoxins such as chloroform (29), acetaminophen (30), and thioacetamide (THA) (31, 32). However the mechanisms by which they induce cell damage are poorly documented. In addition, numerous physiological factors such as IL-6 (33) or soluble TNFα receptors (34) and free radical scavengers such as Me2SO and dimethylthiourea (31), have been reported to be able to protect the liver against injuries induced by apoptotic signals.

Me2SO protects, in vivo, hepatocytes from cell death in acute hepatitis induced by THA (31) or acetaminophen (30). In vitro, Me2SO is used as a powerful inducer for long term survival and differentiation in primary hepatocyte cultures (35). Moreover, TNFα is known to induce apoptosis in Me2SO-treated hepatocyte cultures, but only after Me2SO removal (36). It has then been postulated that Me2SO might protect liver from hepatotoxic by scavenging hydroxyl radicals (31) but the mechanism by which Me2SO would inhibit apoptosis pathway in hepatocytes has not yet been elucidated.

In this study, we have investigated the mechanism by which liver protection is controlled using Me2SO as hepatoprotector by focusing our attention on the apoptotic transduction inhibition. In vivo, THA-induced liver failure known to induce hepatic apoptosis was chosen, and cultures of hepatocytes undergoing apoptosis were designed as an in vitro model. We provide evidence that hepatoprotection may result from both inhibition of caspase cascade, through an efficient inactivation of cleaved initiator caspases and prevention of ASK1-JNK activities via GST regulation.

EXPERIMENTAL PROCEDURES

Animals and Treatments—Male Lewis rats and BALB/c mice (Elevage Janvier) were injected intraperitoneally with a single dose of thioacetamide (THA, Sigma), 100 mg/kg. In some experiments, Me2SO (2 ml/kg, Sigma) was also injected 1 h prior to THA, then at 12 and 24 h after the THA injection according to the protocol of Bruck et al. (31). Animals were sacrificed at different times after THA injection (for animals receiving only THA) and 30 h after THA administration when comparing animals treated with both THA and Me2SO; this latter time corresponded to maximal DEVD-AMC caspase activity in THA-treated animals.

Reagents—Rabbit anti-GST mu1/2, alpha1/2 and pi1 antibodies were from Biotrin International (Ireland). Anti-ASK1 (DAV) was previously described (12). Anti-caspase-3, -MKP1 and -JNK were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-caspase-8 (APP-108) and anti-caspase-9 were from StressGen Biotechnologies Corp. (Tebu, France), and anti-p-JNK, -P-p38, and -p38MAPK from Cell Signaling Technology (Tebu, France). Secondary antibodies conjugated to horseradish peroxidase were from DAKO (DAKO SA, France). Fluorogenic substrates were from BACHEM (BACHEM) and prepared at 100 nm in the recommended solvent. The Caspstatin™ reagent, a specific inhibitor of caspases-3 and -7, corresponding to an Escherichia coli-expressed recombinant fusion protein, which comprised BIRs (baculovirus inhibitor of apoptosis protein) Repet1 and 2 from human XIAP (X-linked inhibitor of apoptosis protein), was purchased from Biomol (Tebu, France). Transforming growth factor β1 (TGF β1, R&D Systems) and TNFα (PromoCell, Heidelberg, Germany) were prepared according to the manufacturer's instructions.

Isolation and Primary Culture of Hepatocytes—Hepatocytes were isolated and purified from male Sprague-Dawley rats (Elevage Janvier, France) as described previously (37). Hepatocytes were seeded at 7 × 10⁶ cells/cm² on plastic dishes in a mixture of 75% minimum essential medium and 25% medium 199, supplemented with 10% fetal calf serum, and per ml: 100 IU of penicillin, 100 μg of streptomycin, 1 mg of bovine serum albumin, 2 μmol of L-glutamine, and 5 μg of bovine insulin. Four hours after plating, the medium was removed, and cultures were maintained in different media: 1) basal medium, corresponding to plating medium, deprived in fetal calf serum and supplemented with 1.4 × 10⁻⁷ M hydrocortisone hemisuccinate (Roussel-UCLAF), 2) Me2SO medium, corresponding to basal medium supplemented with 2% Me2SO, 3) TGFβ1 or TNFα medium, TGFβ1 (1 or 2.5 ng/ml) or TNFα (20 ng/ml) was added to basal medium at 24 h after cell plating. Treatment was then performed for 2 days. Appropriate media were renewed every day.

Immunoblotting Analysis—Freshly isolated hepatocytes and cultured cells were harvested, washed with phosphate-buffered saline, and stored as pellets at −80 °C. Hepatocytes were lysed in a Western blot lysis buffer, and protein content was measured as previously described (38). 100 μg of proteins were resolved on 7.5–12.5% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (PVDF, Bio-Rad). Subsequently, nonspecific binding sites were blocked with Tris-buffered saline (TBS) containing 4% bovine serum albumin, for 1 h at room temperature. Then, filters were incubated overnight at 4 °C with primary antibody in TBS containing 4% bovine serum albumin. Filters were washed three times with TBS and incubated with appropriate secondary antibody conjugated to horseradish peroxidase, for 1 h at room temperature. Following 4–5 washes with TBS, the proteins of interest were visualized with Supersignal™ (Pierce Chemical Co.).

Extraction and Agarose Gel Electrophoresis—Affinity and non-adherent hepatocytes from 60-mm dishes were harvested and centrifuged at 2000 rpm for 2 min at 4 °C. DNA was isolated from cultured cells or fresh biopsies with High Pure PCR template preparation kit (Roche Diagnostics). After purification, samples were analyzed by electrophoresis on a 1% agarose gel and observed under UV light.

Caspase Activity Assay—Hepatocytes and liver biopsies were lysed in the caspase activity buffer (39). 100 μg of crude cell lysate were incubated with 80 μM substrate-AMC for 1 h at 37 °C. Caspase-mediated cleavage of peptide-AMC was measured by spectrophotometry (Molecular Devices) at the excitation/emission wavelength pair 403/440 nm. The caspase activity was given in arbitrary units of fluorescence (per 100 μg of total protein).

Detection of S-Nitrosylation—Caspase activity was measured after preincubation of cysteine with or without 10 mM DTT for 10 min at room temperature. In these experiments, caspase activity buffer did not contain DTT.

Transfection—24 h after Me2SO addition, hepatocytes were transected using JBGM as previously described (40). Per dish, a total of 4 μg of DNA plasmid was mixed with 10 μg of JBGM. Human GST M1, A1, and P1 cDNAs were obtained by PCR from liver tissue RNA and subcloned in pClneo (Promega). pcDNA3.1 HA-ASK1 and HA-ASK1 (Km, kinase mutant) were previously described (21).

RESULTS

Massive Apoptosis Involving Caspase-9, -8, and -3 Occurs in Basal Primary Culture of Hepatocytes—To investigate the mechanism by which free radical scavengers like Me2SO modulate the apoptotic process in hepatocytes, a study of such a process in basal primary culture of adult hepatocytes was first performed. A minimal medium composed of a mixture of MEM/ M199 medium supplemented with insulin and a low concentration of glucocorticoids (10⁻⁷ M) was chosen, limiting hepatic protease activity and allowing for a few days.

Apoptotic process undergone by hepatocytes under this condition was characterized (Fig. 1) and compared with that in...
duced in the presence of TGFβ1 used as a potent apoptotic inducer in hepatocytes (41).

Cell viability loss, measured by the decrease in protein content per dish as early as 3–4 days of culture (Fig. 1A) paralleled by a clear degradation of DNA into oligonucleosomes (Fig. 1B). TGFβ1 treatment induced more rapid and extensive cell damages.

Western blot analysis was next performed to look for expression of pro- and cleaved forms of the caspase-9, -8, and -3, and PARP (C) in normal liver (NL), freshly isolated (T0), and cultured hepatocytes, 4 h after plating (4h), and at different days of culture. M, molecular weight markers. DEVD- and IETD-AMC (D) caspase activities in freshly isolated hepatocytes (T0), and cultured hepatocytes at 4 h (4h) and during 6 days of culture in basal medium. DEVD-AMC caspase activity (inset) measured in cells maintained in basal medium (basal) or treated with TGFβ1 (2.5 ng/ml). DEVD- and IETD-AMC (E) caspase activities in cell extracts in 3-day-old cultured hepatocytes, in the presence or absence of CasputinTM, a peptidic inhibitor of executioner caspases. Caspase activities were expressed in arbitrary units (A.U.) of fluorescence.

Fluorescent substrates were used to confirm that cleaved caspases were active, assays using fluorescent substrates were carried out. They were based on the differential efficiency of each caspase at processing three fluorescent tetrapeptide substrates: 1) DEVD-AMC known to be essentially cleaved by caspase-3 and to a lesser extent by caspase-7, 2) LEHD- and IETD-AMC, two main substrates of the initiator caspases, mainly caspase-9 and -8, respectively.

DEVD-AMC caspase activity was very low in freshly isolated cells and increased thereafter, displaying a biphasic induction, moderate at 1, and sharp at 3 days after plating (Fig. 1D). IETD- (Fig. 1D) and LEHD-AMC (data not shown) caspase activity values displayed a similar biphasic profile but always remained lower than DEVD-AMC caspase activities. Addition of TGFβ1 led to a dramatic induction of activity with both substrates, 24 and 48 h after treatment, reaching in the DEVD-AMC assay a 4–5-fold higher level than that measured in cells maintained in basal medium (Fig. 1D, inset).

Moreover, we have verified that the DEVD-AMC activity, in 3-day-old cultured cells, was strongly inhibited in the presence of the CasputinTM reagent, a recombinant protein of the endog-
enous caspase inhibitor XIAP, which specifically binds to and inhibits cleaved caspase-3 and -7 with negligible effects on other caspases (6). This indicated that the cleavage of DEVD-AMC was essentially due to caspase-3 and/or -7 (Fig. 1E) in contrast to IETD-AMC (Fig. 1E).

These results demonstrated that the apoptotic process of hepatocytes cultured under basal conditions involved maturation and activities of caspase-9, -8, and -3.

Addition of Me2SO to Basal Hepatocyte Culture Constantly Prevents Maturation of Executioner Caspase-3 and Blocks Late Stage of Apoptosis—To determine the mechanism involved in the inhibition of apoptosis by Me2SO, 2% Me2SO was added to the basal medium 24 h after plating according to Isom et al. (35); then, cell morphology, DNA degradation, and expression and activation of the three caspases were studied (Fig. 2).

Typical differentiated hepatocyte morphology was maintained in Me2SO-treated hepatocytes (Fig. 2B), and no DNA ladder was observed under this condition (Fig. 2D) compared with hepatocytes exhibiting characteristics of apoptosis under basal conditions (Fig. 2, A and C).

DEVD-AMC caspase activity strongly decreased at day 2 and then remained very low for at least 6 days (Fig. 2C) while no cleaved fragment of caspase-3 was detected by Western blotting (Fig. 2E). Surprisingly, the expression of the cleaved caspase-8 became detectable from 72 h and gradually accumulated from days 4–6 of culture (Fig. 2F). Similarly, the cleaved form of caspase-9 was detected as early as 24 h and its level increased with time.

These results indicated that Me2SO prevented hepatocyte apoptosis occurrence by inhibiting the caspase-3 maturation, indispensable to cell death although it failed to inhibit maturation of caspase-8 and -9.

Inhibition of Cleaved Initiator Caspases Involves Mechanism(s) Distinct from Nitrosylation, AKT-dependent Phosphorylation, or XIAP Binding—As observed above, maturation of procaspase-3 was constantly inhibited despite the presence of cleaved caspase-8 and -9. This prompted us to evaluate whether these cleaved caspase-8 and -9 were active. Caspase-8 and -9 activities (Fig. 3A) were thus measured from cell lysates in Me2SO-treated hepatocytes or not, 4 days after plating. For each activity, Me2SO-treated hepatocytes exhibited a weak activity in contrast to control cells, indicating that, in the presence of Me2SO, the cleaved caspase-8 and -9 were poorly active.

To address the question as to whether the inhibition by Me2SO of cleaved initiator caspase activities and maturation of executioner caspases was a reversible phenomenon, hepatocytes were first cultured in the presence of Me2SO between days 1 and 3 after plating and then without Me2SO for the 2 following days. IETD-AMC caspase activity was measured at days 3 and 4 (Fig. 3B). This activity was very low in hepatocytes constantly stimulated by Me2SO but strongly increased at day 4 after Me2SO removal.

We also tested whether or not Me2SO, per se, was capable of inhibiting active mature caspases in a cell extract. To this aim, both IETD- (data not shown) and LEHD-AMC (Fig. 3C) caspase activities were estimated in hepatocyte extracts of 4-day-old cultures maintained under basal conditions, i.e. exhibiting high caspase activities, to which increasing concentrations (0.5–4%)
of Me$_2$SO were added. Caspase activities were not affected by the addition of Me$_2$SO to the caspase activity assays, thus demonstrating that this molecule was not directly responsible for the inactivation of the enzymatic activity of active initiator caspases in cell extracts.

To identify the mechanism underlying inhibition by Me$_2$SO of initiator caspase activity, we determined whether initiator caspases were S-nitrosylated (42), phosphorylated by AKT (5), and/or associated with active fragment(s) of IAP (6), which corresponded to the three previously identified mechanisms known to inhibit cleaved caspases.

To estimate the level of caspase nitrosylation, DEVD- (Fig. 3D) and IETD-AMC (Fig. 3E) caspase activities were measured in the presence or absence of DTT, a reducing agent used to evidence S-nitrosylation of proteins (8, 42). Preincubation of 100 μg of cell lysates of 4-day-old hepatocytes cultured under basal condition with DTT led to an increase of DEVD- and IETD-AMC caspase activities (Fig. 3, D and E) thus indicating that hepatocytes...
undergoing apoptosis in culture contained caspases in both S-nitrosylated and non-nitrosylated forms. In Me2SO-treated cells, the DEVD-AMC activity was slightly increased in the presence of DTT but remained much lower than under basal conditions. Moreover, the IETD-AMC caspase activity was not affected by the denitrosylation agent DTT (Fig. 3E), leading us to conclude that the mature initiator caspases were not nitrosylated in the presence of Me2SO and that nitrosylation was not responsible for their low activities.

Possible inhibition of cleaved caspase-9 through its phosphorylation by AKT (5) was also investigated. The specific inhibitor, Ly294002, of the upstream Akt activator PI3K (43) was added to culture medium to test whether inhibition of this survival pathway could restore DEVD- and LEHD-AMC caspase activities in the presence of Me2SO (Fig. 3F). Both activities were similar in hepatocyte extracts from cultures maintained in the presence or absence of Ly294002 indicating that PI3K/AKT survival pathway was not essential for apoptosis inhibition by Me2SO.

Then, we examined if caspase inhibition was based on a direct binding of IAP fragment to the caspase-9 catalytic site (6). By Western blotting, we investigated if XIAP was processed and able to counteract cleaved caspase-9 in Me2SO-treated hepatocytes or maintained under the TGFβ1 condition (Fig. 3G). Cleaved fragments of XIAP were easily detectable under TGFβ1-treated cells in contrast to hepatocytes cultured in the presence of Me2SO, suggesting that mature initiator caspase-9 was not inhibited by binding to XIAP fragments.

Altogether, these results indicated that inhibition by Me2SO of cleaved initiator caspase activities and of maturation of executioner caspase-3 is mediated by one or several anti-apoptotic mechanism(s) distinct from caspase nitrosylation, XIAP binding, or AKT-dependent phosphorylation.

Me2SO Prevents Apoptosis Through Inhibition of ASK1 Transduction Pathway—Meanwhile, evidence was provided that inhibition of caspase activities would not be sufficient to allow cell survival (9), suggesting that other apoptotic transduction pathway(s) might play important roles in the cell de-
Me₂SO for two more days (Fig. 4A).

Western blotting of caspase-3, -9, and -8 (B) in normal liver (NL) and livers of rats 30 h after treatments with THA or THA and Me₂SO (THA+DMSO). T+,

hepatocytes cultured in basal medium over 4 days. Kinetics of DNA fragmentation (C) in liver extracts of rats at different times after THA injection and level after 30 h of co-treatment with THA and Me₂SO (THA+DMSO). LEHD-AMC caspase activity (D) in normal liver (NL) and in liver of rats 30 h after treatments with THA or THA plus Me₂SO. Caspase activities were expressed in arbitrary units (A.U.) of fluorescence. Caspase activities are the results of three independent experiments.

To further assess the role of ASK1/JNK pathway in the apoptotic inhibition mediated by Me₂SO, hepatocytes cultured in the presence of Me₂SO were transfected with wild type ASK1 (WT) or an inactive kinase mutant ASK1(KM) form. Twenty-four hours after transfection, apoptosis was evaluated by DEVD-AMC caspase activity measurement (Fig. 4E) and Hoechst staining. ASK1 (WT) strongly induced DEVD-AMC caspase activity (Fig. 4E) and DNA fragmentation (Fig. 4E, insets), while ASK1 (KM) and the control plasmid LacZ did not. Together, these results pointed to the ability of ASK1 to overcome inhibition of apoptosis and executioner caspase maturation mediated by Me₂SO, thus strongly suggesting that ASK1 might play a key role in apoptotic transduction in hepatocytes. This led us to analyze regulation of its activity.

Because the activities of ASK1/JNK protein kinases are inhibited by GST M1 and P1 isoforms, we investigated if Me₂SO-mediated inhibition of ASK1 might involve GST. First, GST M1/2, P1, and A1/2 expression was analyzed in the presence or absence of Me₂SO (Fig. 4D). GST M1/2 and A1/2 level rapidly decreased under basal conditions as compared with normal liver and Me₂SO-treated cells. GST P1 was detected only in hepatocytes cultured under basal conditions (Fig. 4D).

Second, we tested whether GSTs were capable of inhibiting ASK1-dependent apoptotic pathway. Me₂SO-treated hepatocytes were transiently transfected (Fig. 4E) with ASK1 in combination with GSTM1, A1, or P1. Surprisingly, GSTs M1, P1, as well as GST A1, inhibited ASK1-induced DEVD-AMC caspase activity. This demonstrated that the different GSTs expressed in hepatocytes could contribute to the ASK1/MKK4-7/JNK pathway regulation. These results indicate that Me₂SO might efficiently prevent ASK1 activity by maintaining a high level of GST proteins in hepatocytes.

Me₂SO Prevents THA-mediated Liver Damage through Inhibition of Caspases and ASK1-JNK Activities—To confirm that Me₂SO was also able to prevent apoptosis and liver failure in vivo through inhibition of both caspase activities and ASK1-JNK pathway, intraperitoneal injection of THA in mice and rats, known to induce a severe hepatocyte apoptosis and fulminating hepatitis (30, 31), was used as a model of liver injury.
DNA fragmentation and expression of caspase-3, -8, and -9 were analyzed in liver of rats treated with THA injected or not with Me₂SO prior to THA (Fig. 5). Degradation of DNA in oligonucleosomes was evidenced as early as 12 h after THA injection and strongly increased at 24 h before disappearance at 36 h (Fig. 5A). Histological examination evidenced a massive centrilobular cell death in livers of THA-treated animals (data not shown) while co-treatment with Me₂SO prevented DNA degradation (Fig. 5A) as well as histological lesions in accordance with previous reports (31).

In liver extracts obtained 30 h postinjection, expression of pro- and mature caspase-3, -8, and -9 was examined (Fig. 5B). As expected, the cleaved form of caspase-3 was found in liver of rats injected with THA but not in normal and THA plus Me₂SO-treated animals. The level of pro-caspase-8 was affected neither by THA nor by THA plus Me₂SO treatments compared with normal liver, and the cleaved caspase-8 was undetectable. In contrast, the pro-caspase-9 was strongly decreased under THA and THA plus Me₂SO conditions, compared with untreated animals, and cleaved forms of caspase-9 were clearly evidenced in both conditions (Fig. 5B).

To demonstrate that different cleaved caspases detected by Western blotting were active in THA-injured livers, caspase activities were monitored using different fluorogenic tetrapeptide substrates (Fig. 5, C and D). DEVD-AMC caspase activities were measured in liver extracts at different times after THA administration to the animals (Fig. 5C). A slight increase was first detected within 16 h postinjection, followed by a strong induction between 20 and 36 h peaking at 30 h concomitantly with DNA degradation (Fig. 5A). Co-treatment with Me₂SO totally prevented induction of DEVD-AMC caspase activity measured at 30 h (Fig. 5C). In addition, we showed that THA strongly induced LEHD-AMC caspase activity (Fig. 5D) while under the THA plus Me₂SO condition, it remained at a similar level as in normal liver, indicating the absence of caspase-9 activity in the presence of Me₂SO.

Interestingly, as previously observed in vitro under basal conditions, we showed that the expression of GST M1 and A1 (Fig. 6A) was strongly decreased in THA-treated animals, while Me₂SO prevented this down-regulation. This decrease in GST M1/2 and A1/2 expression was correlated with a sharp increase of ASK1 activity (Fig. 6B) and phosphorylated form of JNK (Fig. 6A), which demonstrated an activation of the ASK1/JNK pathway during THA-induced apoptosis in liver. Importantly, in animals co-injected with THA and Me₂SO, the level of phospho-JNK was significantly reduced and, as expected, the activity of ASK1 was barely detectable (Fig. 6B). Moreover, the increase of ASK1 and JNK activities in THA-treated animals was not due to variations of the JNK phosphatase (MKP1) or ASK1 protein levels (Fig. 6A).

Altogether, these results demonstrate that THA-induced apoptosis is dependent upon activation of caspase-9 and -3 but not of caspase-8, and is accompanied by the induction of the ASK1/JNK pathway and a strong decrease in GST A1/2 and M1/2 expression. In addition, Me₂SO inhibits THA-mediated apoptosis by both preventing the down-regulation of GSTs and decreasing activities of cleaved caspase-9 and ASK1.

**DISCUSSION**

Hepatoprotection remains one of the major challenges of clinical therapy to limit liver injuries such as chronic hepatitis and cholestasis. Many molecules have been described as liver protectors including the modified bile acid, ursodeoxycholic acid (UDCA) (45), currently used in primary treatment of cholestasis, free radical scavengers such as Me₂SO and dimethylthiourea (31), and cytokines such as IL-6 (33). However, the precise mechanisms controlling liver protection remain poorly documented. The previous data demonstrating the inhibition by Me₂SO of cell death occurring in liver during acute hepatitis induced by THA (31), supported the view that the protective effect of this agent was related to its free radical scavenging property. Nevertheless, the molecular mechanisms responsible for this scavenging protection remain to be investigated. In addition, whether this Me₂SO-protective effect requires one or several complementary mechanisms has to be elucidated.

In this report, we have attempted to highlight the key mechanisms involved in inhibition of apoptosis by Me₂SO using both hepatocytes undergoing apoptosis in primary culture and THA-induced liver failure as an in vitro model. We provide evidence that Me₂SO prevented hepatocyte apoptosis occurrence, in vitro, by inhibiting the caspase-3 maturation, but failed to inhibit maturation of caspase-8 and -9. In addition, these Me₂SO-treated hepatocytes exhibited weak initiator caspase activities in contrast to apoptotic cells, indicating that in the presence of Me₂SO the cleaved caspase-8 and -9 were poorly active. In vivo, Me₂SO totally protected against liver failure induced by THA injection as demonstrated by: 1) absence of histological lesions, 2) inhibition of pro-caspase-3 maturation and activity, and 3) absence of DNA fragmentation. In addition, cleaved caspase-9 was also detected in livers of both THA and
THA plus Me₂SO-treated animals but its activity in the presence of Me₂SO was very low.

It is noteworthy that Me₂SO is able to inhibit spontaneous as well as TGFβ1- or TNFα-induced apoptosis in primary cultures of hepatocytes, suggesting that Me₂SO may protect from cell death regardless of the nature of apoptotic stimulus. This emphasizes the protective role of Me₂SO against caspase pathway. However, the signal involved in this inhibition remains to be specified.

Among possible mechanisms of caspase inhibition, induction or activation of endogenous proteins such as heat shock proteins (HSP) (46), NO synthases (NOS) (7, 47), AKT (5), or IAPs (6), have been described. The involvement of HSP27, 70, and/or 90, known to prevent maturation of caspase-9 or -3 (46) was excluded since caspase-9 was cleaved in our in vivo and in vitro models. Moreover, Western blot analysis confirmed the absence of HSP27 induction in Me₂SO-treated hepatocytes (data not shown). Considering the three other inhibitory mechanisms, they were unlikely to be responsible for inhibition of apical caspase activities under our conditions (see Fig. 3). Since treatments with actinomycin D or cycloheximide overrode this apoptosis blockage (data not shown), the induction of one or several gene products exhibiting anti-apoptotic effects is strongly suggested.

K. Tobiume et al. (11) recently reported that efficient TNFα-induced apoptosis blockage required a dual inhibition of caspase and kinase activities (9). In this context, a Me₂SO-mediated inhibition of the ASK1/JNK pathway was therefore postulated. The observations that Me₂SO inhibits ASK1 activity and that overexpression of ASK1 restores apoptosis in Me₂SO-treated hepatocytes, provide strong evidence that ASK1 plays a key role in apoptotic transduction in hepatocytes.

An important issue to be elucidated is then the relationship between the ASK1/JNK cascade and the caspase pathway. Indeed, it is currently unclear whether the ASK1/JNK cascade is required for caspase activation and/or activity, or vice versa. K. Tobiume et al. (11) previously reported that caspase-8-like activity was present in TNFα-treated MEFs ASK1+/−/+ mice in a similar manner as ASK1−/−, whereas MEFs ASK1−/− mice did not undergo cell death. In addition, H. Nishitoh et al. (48) hypothesized that ASK1 may lie upstream of caspase-12 in ER stress-induced apoptosis and neurotoxicity by amyloid-β proteins (49). These data together with our present results led us to hypothesize that ASK1-JNK might be indispensable for maturation of executioner caspases in our model of protection by Me₂SO. They also suggest that a direct regulation of caspases by the ASK1/JNK pathway might occur. However, complexity is increased by the fact that MEFs ASK1−/− mice died in response to Fas ligand (11), indicating that two cascades might act independently and in parallel (11).

The most intriguing results concern the potent role of GSTs on apoptosis. Interesting reports show that the activities of ASK1 and JNK protein kinases are inhibited by GST M1 and P1 isoforms, respectively (21, 26). Since Me₂SO is known to maintain the expression of liver-specific genes including GSTs, we investigated if Me₂SO was protecting hepatocytes through the modulation of the expression of GSTs M1/2, A1/2, and P1. We demonstrated that endogenous inhibitor of ASK1, namely GST M1, was constantly maintained in Me₂SO-treated hepatocytes despite apoptotic signals such as THA, TNFα, and TGFβ1, and correlated with hepatocyte survival both in vitro and in vivo. Furthermore, transfection of GSTs M1, P1 as well as GST A1, counteracted ASK1-induced DEVD-AMC caspase activity, confirming the ability of these GSTs to regulate the activity of the ASK1/MKK4–7/JNK pathway and demonstrating the key role of GSTs in controlling hepatocyte apoptosis.

Our results are in agreement with those by S. G. Cho et al. (21), who recently described that ASK1 was associated with GST M1 in normal liver, preventing its oligomerization and autophosphorylation (18, 50).

S. Dorion et al. (51) recently suggested that both TRX and GST M1, two endogenous inhibitors of ASK1, may compete for the same N-terminal region. They demonstrated the release of GST M1 from ASK1, which correlates with its subsequent activation. They hypothesized (51) that during heat shock, small lipophilic molecules might be released or produced thus favoring the release of GST M1 from ASK1. In our hepatic cell models, we demonstrated that ASK1 activation was strictly correlated with a decrease of GSTs M1/2 and A1/2. In this context, it is tempting to postulate that Me₂SO may ensure hepatoprotection by preventing the decrease of ASK1 inhibitors such as GST M1.

In normal tissue, GST would represent two percent of cytosolic proteins (22). The liver constantly exposed to endogenous toxins and xenobiotics, expresses the largest panel of GSTs, a major class of detoxifying enzymes. The GST P expression differs in liver tissues between rodent species. In normal quiescent hepatocytes, two isoforms of the GST P class are found in mouse while they are absent in rat. In contrast, mouse and rat hepatocytes constantly expressed GST Ms in this status (22, 52). Since ASK1 and JNK are inhibited by GST M1 and GST P1, respectively, it is tempting to postulate that the inhibition of ASK/JNK pathway might occur at distinct levels in function of the isoforms expressed in rodent species. However, we cannot exclude an isoform compensation in rat.

Moreover, it has been shown that mutation and/or overexpression of GSTs (53), frequently occur upon transformation to malignancy and, consequently, cells acquire resistance to electrophilic anticancer drugs (54). V. Adler (26) also suggested that tumor cells overexpressing GST may escape from apoptosis through high intrinsic JNK inhibitory activity.

In conclusion, besides its free radicals scavenging properties known to afford hepatoprotection, Me₂SO is mediating apoptosis inhibition through a dual blockage of cleaved caspase and ASK1/JNK activities. Evidence was provided that GSTs should play a key role in ASK1 regulation and thus on apoptosis. These data raise the question of the controlling mechanisms that determine the reciprocal GST pools involved in detoxification or apoptosis transduction processes for reaching efficient hepatoprotection.

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