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Reactive Oxygen as Modulator of TNF and Fas Receptor-Mediated Apoptosis *In Vivo*: Studies with Glutathione Peroxidase-Deficient Mice

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ABSTRACT

Reactive oxygen species (ROS) can directly induce or enhance tumor necrosis factor (TNF)-mediated apoptosis in a number of different cell lines. To test the relevance of intracellular ROS in modulating apoptotic signaling *in vivo*, we evaluated hepatocellular apoptosis mediated by the TNF or Fas receptor in wild-type and glutathione peroxidase-1 (Gpx1−/−)-deficient mice (129SV/B6 background). Apoptosis developed in livers of wild-type animals 4–6 h after intraperitoneal administration of 700 mg/kg galactosamine/100 μg/kg endotoxin. Apoptosis was indicated by processing of procaspases-3 (assessed by western blotting), a fivefold increase in caspase-3 activity (DEVD-AMC as substrate), and a 44-fold increase in DNA fragmentation (ELISA). The time course and magnitude of apoptosis were the same in Gpx1−/− mice. In contrast, Gpx1−/−mice had higher plasma alanine aminotransferase (ALT) levels and more severe hemorrhage compared to wild-type animals at 6 h. Treatment of wild-type mice with the anti-Fas antibody Jo-2 (0.6 mg/kg i.v.) resulted in processing of procaspase-3 and a sevenfold increase in caspase-3 activity in both wild-type and Gpx1−/− mice. However, higher plasma ALT values in Gpx1−/− mice at 3 h may reflect a trend to develop more rapidly secondary necrosis. These data suggest that, under our experimental conditions, intracellular ROS did not modulate the death receptor-initiated apoptotic signaling cascade in hepatocytes. As Gpx1 is located in the cytosol and in mitochondria, which are the main cellular compartments involved in apoptotic signaling, our findings indicate that the oxidant stress *in vivo* was insufficient to modulate these signaling pathways. However, Gpx1 deficiency enhances the susceptibility for secondary necrosis or neutrophil-induced cell injury. *Antioxid. Redox Signal.* 4: 733–740.

INTRODUCTION

APOPTOTIC CELL DEATH has been implicated in a number of liver diseases, such as alcoholic hepatitis, cholestasis, Wilson's disease, and viral hepatitis (21, 26, 37). In most cases, apoptosis is initiated by ligation of the Fas or tumor necrosis factor (TNF) receptor type I. The signaling mechanism of both receptors starts by assembly of the death initiating signaling complex (DISC) at the cytoplasmic tail (29). This leads to the autocatalytic activation of procaspase-8. The active caspase-8 enzyme cleaves BID, a member of the Bcl-2 family of proteins, which inserts into the outer mitochondrial membrane and signals the release of cytochrome *c* and other proapoptotic components from the mitochondria (8). The mitochondrial release of these mediators results in the assembly of the apoptosome, a complex of procaspase-9, apoptosis activating factor-1 (Apaf-1), cytochrome *c*, and dATP in the cytosol. The resulting activation of caspase-9 causes the processing of caspase-3 (25). The terminal caspase-3 can cleave substrates such as ICAD, an inhibitor

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protein of endonucleases, which leads to activation of endonucleases (33). However, this enzyme also participates in a positive amplification mechanism by cleaving more procaspase-8 (2). Pancaspase inhibitors are highly effective against liver cell apoptosis induced by both receptors *in vivo* (16, 18, 23, 32). However, inhibitors of caspase-3 or –8 proved to be considerably more protective against Fas receptor- compared with TNF receptor-mediated apoptosis *in vivo* (2, 3). Moreover, the caspase-3 inhibitor DEVD-CHO (Asp-Glu-Val-Asp-aldehyde) prevented processing of procaspase-3 in Fas- but not in TNF-induced apoptosis, suggesting that TNF receptor signaling may depend less on the mitochondrial amplification cycle than the Fas receptor (3).

Reactive oxygen species (ROS) have been suggested to induce or modulate apoptotic cell death in a number of cell types (5, 10). A moderate oxidant stress can induce apoptosis by activation of caspases (9). On the other hand, excessive reactive oxygen formation can oxidize essential sulfhydryl groups of caspases and therefore prevent apoptosis (9). In cultured hepatocytes, exogenously added ROS caused apoptotic cell death (19, 20). Furthermore, microcystin (6), transforming growth factor-β (34), TNF-α (30), or cold storage (31) induced intracellular reactive oxygen formation and apoptosis in isolated hepatocytes. Antioxidant interventions protected these cells at least in part. These data suggest that oxidant stress induced or modulated apoptosis in hepatocytes. However, all of these studies were done in primary hepatocytes or hepatocyte cell lines. Evidence for a role of reactive oxygen in hepatocellular apoptosis *in vivo* is indirect, *i.e.,* based on the up-regulation of antioxidant enzymes (28) or on the protective effect of antioxidants (36). However, the interpretation of pharmacological intervention studies *in vivo* may not always be straightforward. Reactive oxygen can modulate gene transcription of cytokines, chemokines, and adhesion molecules, in addition to the direct effect on cell injury mechanisms (14). Therefore, to address the question whether ROS can modulate receptor-mediated apoptotic cell death *in vivo*, we used animals deficient in glutathione peroxidase-1 (Gpx1) in two well established experimental models of hepatocellular apoptosis, *i.e.,* the galactosamine (Gal)/endotoxin (ET) (24) and the anti-Fas antibody (27) models of acute liver failure. Glutathione peroxidase is located in mitochondria and the cytosol (11) and is quantitatively most important for the detoxification of hydrogen peroxide in these cellular compartments (13, 15). Thus, we expected that if any relevant intracellular oxidant stress, which may affect the pathophysiology, is present in these hepatocytes, animals deficient in glutathione peroxidase should show an accelerated and/or enhanced apoptotic cell death in the liver.

MATERIALS AND METHODS

Animals

Construction of the *Gpx1*-gene knockout (Gpx1−/−) mice (129SV/B6 background) has been described (11). The animals lack mRNA for Gpx1 as assessed by northern blotting and, compared with wild-type animals, have <0.5% of the Gpx1 activity in cytosol and no detectable Gpx1 activity in mitochondria (7, 11). Ten- to 24-week-old male and female mice were used in these studies. Mice were given food (certified rodent diet no. 8640, Harlan Teklad, Indianapolis, IN, U.S.A.) and water *ad libitum*. The experimental protocols followed the criteria of University of Arkansas for Medical Sciences and the National Research Council for the care and use of laboratory animals in research. Animals were treated intraperitoneally with 700 mg/kg D-galactosamine (D-Gal; Sigma Chemical Co., St. Louis, MO, U.S.A.) and 100 μg/kg *Salmonella abortus equi* endotoxin (ET; Sigma Chemical Co.) dissolved in sterile phosphate-buffered saline (PBS), pH 7.0. Other animals were treated intravenously with 600 μg/kg of the anti-mouse Fas antibody Jo-2 (PharMingen, San Diego, CA, U.S.A.) (27). Control animals received PBS only.

Experimental protocol

Groups of animals were killed by cervical dislocation between 1.5 and 7 h after Gal/ET administration or 1 and 3 h after Fas antibody injection. Blood was collected from the vena cava into a heparinized syringe. The blood was then briefly centrifuged, and the plasma was used for determination of alanine aminotransferase (ALT) activity using test kit DG 159-UV (Sigma Chemical Co.) according to the manufacturer's instructions. Livers were sectioned transversely across the midportion of each lobe. Pieces of the liver were either immediately homogenized for caspase-3 activity measurements/western blots or frozen in liquid nitrogen and stored at −80°C for DNA fragmentation analysis.

Caspase-3 activity

Freshly excised liver was homogenized in 25 m*M* HEPES buffer, pH 7.5, containing 5 m*M* EDTA, 2 m*M* dithiothreitol, 1% CHAPS, 1 μg/ml pepstatin, leupeptin, and aprotinin. Homogenates were centrifuged at 14,000 *g* for 20 min at 4°C, and diluted supernatants were then evaluated for caspase-3 activity using the synthetic fluorogenic substrates, Ac-DEVD-MCA (acetyl-Asp-Glu-Val-Asp-4-methylcoumaryl-7-amide) (Peptide Institute, Inc., Osaka, Japan) at a final concentration of 45 μ*M*. The kinetics of the proteolytic cleavage of the substrate was monitored in a fluorescence microplate reader (Cytofluor 2350, Millipore, Bedford, MA, U.S.A.) using an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Caspase activity was calculated from the slope of the recorder trace and expressed in ΔF/min/mg of protein. Protein concentrations in the supernatants were determined using the bicinchoninic acid kit (Sigma Chemical Co.) according to the manufacturer's instructions. Densitometric analysis of the gels was performed with a GS170 Calibrated Imaging Densitometer (Bio-Rad, Hercules, CA, U.S.A.) using Quantity One 4.0.3 software (Bio-Rad).

Western blotting

Caspase-3 processing was determined by western blot analysis as described in detail (2, 23). In brief, freshly excised liver was homogenized in 25 m*M* HEPES buffer as described above. Homogenates were centrifuged at 14,000 *g* for 20 min at 4°C. Protein concentrations in the cytosolic extracts were determined using the bicinchoninic acid kit. Cytosolic ex-

tracts (50 µg per lane) were resolved by 4–20% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. Separated proteins were transferred to polyvinylidene difluoride membranes (PVDF; Immobilin-P, Millipore) which were then blocked with 5% milk in Tris-buffered saline (TBS; 20 m*M* Tris, 0.15 *M* NaCl, pH 7.4) containing 0.1% Tween 20, and 0.1% bovine serum albumin, overnight at 4°C. After washing with TBS, membranes were then incubated with goat anti-caspase-3 polyclonal IgG (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) for 2 h at room temperature. The membranes were then washed again and incubated with the secondary antibody horseradish peroxidase-coupled anti-goat IgG (Santa Cruz Biotechnology) for 1 h at room temperature. Proteins were visualized by enhanced chemiluminescence (ECL) (Amersham Pharmacia Biotech. Inc., Piscataway, NJ, U.S.A.) according to the manufacturer's instructions.

DNA fragmentation

For DNA fragmentation analysis, the Cell Death Detection ELISA (enzyme-linked immunosorbent assay) kit (Boehringer Mannheim, Indianapolis, IN, U.S.A.) was used (16, 23, 24). In brief, a 20% liver homogenate in 50 m*M* sodium-phosphate buffer (120 m*M* NaCl, 10 m*M* EDTA, pH 7.0) was prepared. Homogenates were centrifuged at 14,000 *g* for 20 min at 4°C, and diluted supernatant was used for the ELISA. In this procedure, the kinetics of product generation (v_{max}) is a measure of DNA fragmentation. The v_{max} values obtained for untreated controls (100%) are compared with those in treated groups. The assay allows the specific quantitation of cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes).

Histology

Formalin-fixed portions of the liver were embedded in paraffin and cut at 5-µm thickness. Sections were then stained with hematoxylin, phloxine, and eosin for histopathological evaluation of liver damage (2, 3). The degree of liver damage (necrosis and hemorrhage) was graded on a score of 1–5: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked, 5 = severe. The pathologist (S.L.V.) performing the histological evaluation was blinded as to the experimental treatment of the animals.

Statistics

Data are given as means ± SE. Comparisons between multiple groups were performed with one-way ANOVA followed by Bonferroni *t* test. If the data were not normally distributed, the Kruskal–Wallis test (nonparametric ANOVA) followed by Dunn's multiple comparisons test was performed. $p < 0.05$ was considered significant.

RESULTS

Gal/ET Shock Model

Intraperitoneal injection of 100 µg/kg ET into Gal-sensitized wild-type mice resulted in a fivefold increase of hepatic caspase-3 activity at 6 h (Fig. 1A). There was no further change of caspase-3 activity at 7 h. Gpx1−/−mice had slightly higher baseline values of caspase-3 activity and showed a minor increase of caspase-3 activity at 4 h after Gal/ET treatment (Fig. 1A). Similar to wild-type animals, caspase-3 activity increased considerably at 6 h. All Gpx1−/−animals of the 7-h group died shortly after 6 h. The enzyme activity measurements were confirmed by western blots, which demonstrated the processing of the proenzyme to the active enzyme (Fig. 1B). In Gpx1−/−animals, only a

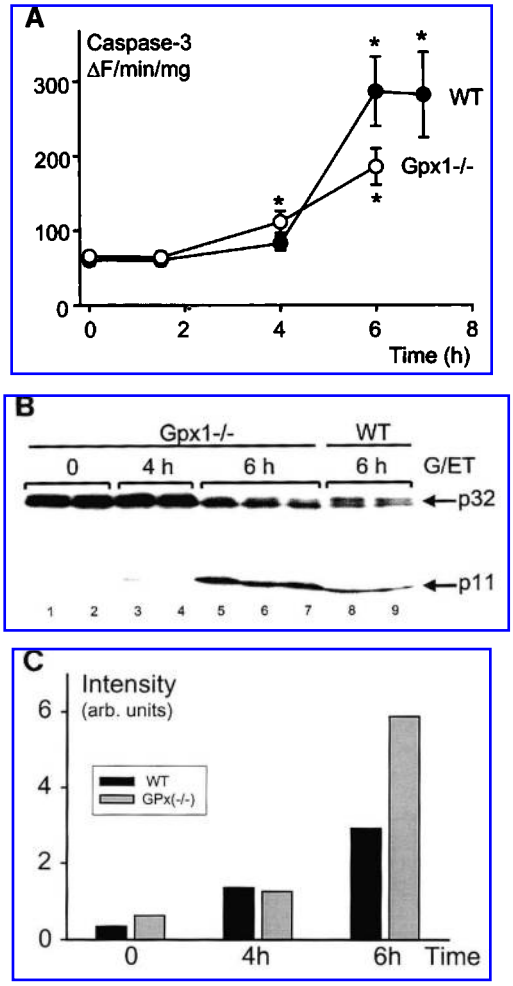

FIG. 1. Time course of Gal/ET (G/ET)-mediated activation of caspase-3 in wild-type (WT) and Gpx1−/− mice. Liver samples were taken from untreated controls ($t = 0$) and from mice treated with 700 mg/kg Gal/100 µg/kg ET. Cytosolic extracts were prepared for assessment of caspase-3 activity (**A**) using the synthetic fluorogenic substrate Ac-DEVD-MCA. Data represent means ± SE of five to 10 animals per group. Caspase-3 processing was evaluated by western blots (**B**). Cytosolic extracts (50 µg per lane) were resolved by SDS-PAGE under reducing conditions. After transfer to a PVDF membrane, procaspase-3 (p32) and the active p11 subunit of caspase-3 were detected with goat anti-caspase-3 polyclonal IgG and visualized by ECL. Two or three representative samples are shown per group. Densitometric analysis of the western blots is shown in **C.** Data represent the means of two or three samples. *$p < 0.05$, compared with $t = 0$.

minor increase of the active p11 fragment was observed at 4 h. On the other hand, a reduction of the proenzyme (p32) levels and a substantial increase of the p11 fragment were observed at 6 h (Fig. 1B). The time course of procaspase-3 processing was similar in wild-type and Gpx1−/− animals (Fig. 1C). The lower caspase-3 activity (Fig. 1A), but high processing of procaspase-3 (Fig. 1B), may reflect the more severe injury in Gpx1−/− mice with loss of cellular contents, as well as fluorescence quenching due to high hemoglobin levels in these samples. In addition to caspase-3 activation, apoptotic cell death was indicated by a 44-fold increase of DNA fragmentation at 6 h after Gal/ET (Fig. 2). Consistent with the caspase-3 data, there was no significant difference between wild-type and Gpx1−/− animals. Liver injury was evaluated by hepatic enzyme release (plasma ALT levels) and histology. There was only a moderate increase in plasma ALT values in wild-type animals at 6 h (Fig. 3). At that time, apoptosis predominates and the neutrophil-induced injury is in its early phase (16). Because of the continued neutrophil cytotoxicity, plasma ALT values further increased at 7 h (Fig. 3). In Gpx1−/− animals, plasma ALT values were significantly higher than in wild-type animals at 6 h. Blinded histological assessment of cell death (necrosis) showed marked (4.0) necrosis in wild-type animals with minimal hemorrhage (1.3) (Table 1). In Gpx1−/− mice, liver injury was assessed as marked to severe (4.8) with marked to severe hemorrhage (4.6) at 6 h. All animals of the designated 7-h Gpx1−/− group died shortly after 6 h. To verify the results, animals were treated with a lower dose of ET (700 mg/kg Gal/10 µg/kg ET). Apoptosis and liver injury were measured at 6 h, *i.e.,* before the onset of neutrophil-induced injury. Livers of both wild-type and Gpx1−/− mice showed a substantial increase in caspase-3 activity and in DNA fragmentation with only a minor increase in plasma ALT levels (Table 2). There was no significant difference in apoptosis or overall injury between wild-type and Gpx1−/− mice. In contrast, at 7 h with the attack of neutrophils, injury in Gpx1−/− (ALT, 3,130 ± 1,490 U/L; $n = 5$) was much more severe than in wild-type animals (ALT, 350 ± 180 U/L; $n = 4$; $p < 0.05$).

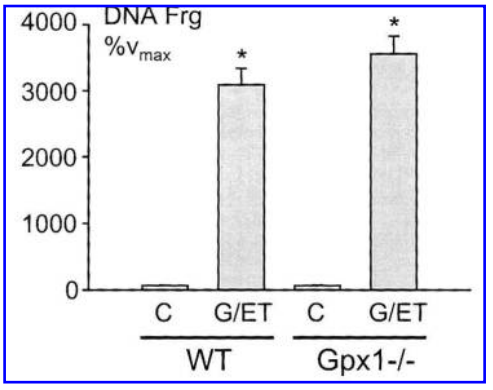

FIG. 2. Hepatocellular DNA fragmentation was assessed in controls (C) or 6 h after treatment with 700 mg/kg Gal/100 µg/kg ET (G/ET). Cytosolic extracts were prepared from livers of wild-type (WT) and Gpx1−/− mice, and DNA fragmentation (DNA Frg) was determined by ELISA as described in Materials and Methods. Data represent means ± SE of five to 10 animals per group and are expressed as % v_{max} of controls. *$p < 0.05$, compared with C.

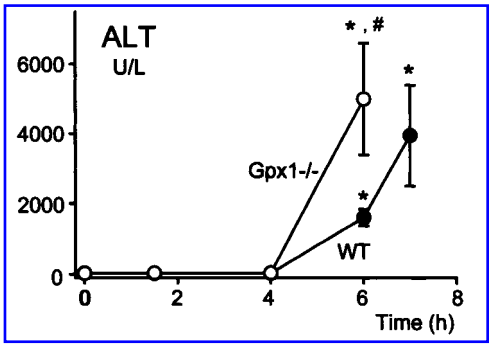

FIG. 3. Time-dependent increase of plasma ALT activities in wild-type (WT) and Gpx1−/− mice after treatment with 700 mg/kg Gal/100 µg/kg ET (G/ET). Data represent means ± SE of five to 10 animals per group. *$p < 0.05$, compared with $t = 0$; #$p < 0.05$, compared with WT.

Fas receptor-induced liver failure

To investigate if the results obtained in the Gal/ET model also apply to Fas receptor-induced apoptosis, animals were treated with 0.6 mg/kg of the anti-Fas antibody Jo-2. Intravenous administration of Jo-2 in wild-type mice showed a time-dependent increase in hepatic caspase-3 activity (Fig. 4A). Although the Gpx1−/− mice had slightly elevated baseline levels of caspase-3 activities, the time course and magnitude of the increase after Jo-2 injection was similar to those in wild-type animals (Fig. 4A). Processing of procaspase-3 followed the time course of the enzyme activities in wild-type and Gpx1−/−mice (Fig. 4B). Again, there was no significant difference between the two groups of mice (Fig. 4C). In contrast, DNA fragmentation showed a 28-fold increase in wild-type animals compared with a 57-fold increase ($p < 0.05$) in Gpx1−/− mice (Fig. 5). In addition, plasma

TABLE 1. HISTOLOGICAL EVALUATION OF LIVER INJURY IN WILD-TYPE AND GPX1-DEFICIENT MICE

	Necrosis (%)	*Hemorrhage score*
Controls WT	0	0
Controls Gpx1−/−	0	0
Gal/ET WT	4.0 ± 0.1*	1.3 ± 0.7
Gal/ET Gpx1−/−	4.8 ± 0.2*,†	4.6 ± 0.2*,†
Jo-2 WT	2.8 ± 0.9*	2.5 ± 1.0*
Jo-2 Gpx1−/−	4.0 ± 0.6*	4.3 ± 0.7*

Liver sections were evaluated from untreated animals [wild-type (W/T) and Gpx1−/− mice], 6 h after administration of 700 mg/kg Gal/100 µg/kg ET (Gal/ET) or 3 h after injection of the anti-Fas antibody Jo-2 (0.6 mg/kg). The severity of necrosis and hemorrhage was evaluated in a blinded fashion by the pathologist (scale 0–5). Data represent means ± SE of $n = 3$–5 animals per group.

*$p < 0.05$, compared with respective controls.

†$p < 0.05$, compared with respective WT groups.

TABLE 2. LOW DOSE ENDOTOXIN-INDUCED APOPTOSIS IN WILD-TYPE AND GPX1-DEFICIENT MICE

	ALT (U/L)	*Caspase-3 activity (ΔF/min/mg of protein)*	*DNA fragmentation (% V_{max})*
Controls WT	29 ± 6	78.2 ± 4.3	100 ± 7
Controls Gpx1−/−	23 ± 4	74.2 ± 5.1	100 ± 12
Gal/ET WT	174 ± 67*	409.4 ± 150.7*	879 ± 124*
Gal/ET Gpx1−/−	229 ± 34*	191.0 ± 33.4*	609 ± 45*

Liver injury (plasma ALT), hepatic caspase-3 activity and DNA fragmentation were evaluated in control animals [wild-type (WT) and Gpx1−/− mice] and 6 h after administration of 700 mg/kg Gal/10 μg/kg ET (Gal/ET). Data represent means ± SE of n = 3–5 animals per group.

*$p < 0.05$, compared with respective controls.

ALT levels were not significantly increased in wild-type animals during the 3-h observation period (Fig. 6). In contrast, the ALT activities were significantly elevated in Gpx1−/− mice at this point. Blinded histological assessment of necrosis and hemorrhage indicated a trend to higher injury, which did not reach statistical significance, in Gpx1−/− mice (Table 1).

DISCUSSION

The major objective of this investigation was to evaluate if a potential intracellular oxidant stress during endotoxemia or Fas antibody treatment may modulate apoptotic or necrotic signaling pathways in hepatocytes *in vivo*. The experimental approach was to compare wild-type animals with mice deficient in Gpx1 (11). These animals have Gpx1 activities of <0.5% compared with wild-type mice in mitochondria and the cytosol (7). We showed previously that Gpx1−/− mice produce more TNF-α than wild-type animals in response to ET (17). This indicated the redox sensitivity of TNF-α gene transcription *in vivo* presumably due to the redox sensitivity of nuclear factor-κB activation. Furthermore, hepatocytes from Gpx1−/− mice are more susceptible to neutrophil-induced injury, which suggests that these leukocytes kill target cells mainly by oxidant stress (17). In the Gal/ET model, the injury after 5–6 h consists mainly of parenchymal cell apoptosis, which causes neutrophil transmigration and a severe aggravation of the injury by these phagocytes at 7 h (16, 22). As a result of the severe cell injury and hemorrhage in the liver, most animals die from hypovolemic shock within the next few hours (18). The pathophysiology in wild-type animals followed this general scheme. At 6 h, caspase-3 processing and enzyme activities were substantially increased, but plasma ALT activities were only moderately elevated. Furthermore, there was substantial DNA fragmentation. These results are consistent with a predominantly apoptotic injury at that time and a limited neutrophil-mediated damage. In terms of apoptosis, Gpx1−/− animals behaved similarly to wild-type animals after high- or low-dose Gal/ET treatment. However, Gpx1−/− mice showed a higher necrotic injury as indicated by the increased plasma ALT values, a more severe necrosis score, and, in particular, a much higher hemorrhage score. All Gpx1−/− animals exposed to the high dose of Gal/ET died between 6 and 7 h because of hypovolemic shock induced by hemorrhage with pooling of blood in the liver. As Gal/ET induces apoptosis selectively in hepatocytes (16), the endothelial cell damage leading to hemorrhage is caused by neutrophil cytotoxicity (16–18). Thus, our data suggest that liver cells from Gpx1−/− mice are more susceptible to neutrophil-induced oxidant stress and cell injury. On the other hand, we found no relevant difference in TNF-induced apoptotic signaling in hepatocytes of wild-type versus Gpx1−/− mice (Fig. 1). These data are consistent with the documented intracellular oxidant stress in liver cells during the neutrophil-mediated injury but not at earlier time points (17). Thus, if TNF exposure triggers a limited formation of ROS in hepatocytes, as was demonstrated *in vitro* (1), our data suggest that this oxidant stress was insufficient to modulate apoptotic signaling under our experimental conditions *in vivo*.

In contrast to TNF-induced apoptosis, there is very limited information on reactive oxygen formation during Fas receptor-mediated apoptosis in different cell lines *in vitro*. It was reported that Fas ligation induces ROS formation, which appeared to promote apoptosis (35). On the other hand, superoxide formation inhibited Fas-mediated apoptosis (4). Furthermore, various antioxidants did not affect anti-Fas antibody-induced apoptosis in murine fibroblasts (13). Thus, there is no consensus if ROS are involved in Fas receptor signaling. Our *in vivo* data do not show any difference in the time course or magnitude of hepatocellular apoptosis between wild-type and Gpx1−/− mice. These results suggest that there is no relevant intracellular oxidant stress, which could affect signaling pathways during Fas-mediated apoptosis in hepatocytes *in vivo*. However, there was an accelerated release of ALT in Gpx1−/− mice at 3 h. Pathological evaluation of the tissue indicated a trend to higher injury in Gpx1−/− mice. In addition, more DNA fragmentation was observed in these animals. In contrast to the Gal/ET model, hemorrhage in the anti-Fas antibody model is caused by apoptosis of the sinusoidal lining cells (2) and not by neutrophils (22). These results suggest that hepatocytes and endothelial cells of Gpx1−/− may be more susceptible to the secondary necrosis (cell contents release) that occurs in this model rather than the initial apoptotic signaling (caspase activation). A potential reason for this increased susceptibility could be an oxidant stress during the late stage of apoptosis. However, the confirmation of ROS formation and the identification of the intracellular source require further investigations.

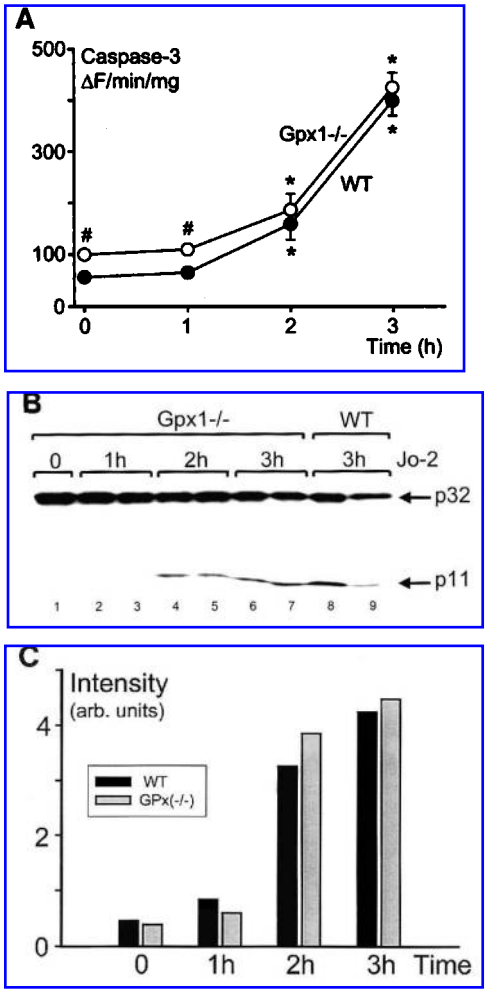

FIG. 4. Time course of caspase-3 activation in wild-type (WT) and Gpx1−/− mice after treatment with the anti-murine Fas antibody Jo-2 (0.6 mg/kg). Cytosolic extracts were prepared for assessment of caspase-3 activity (**A**) using the synthetic fluorogenic substrate Ac-DEVD-MCA. Data represent means ± SE of five animals per group. Caspase-3 processing was evaluated by western blots (**B**). Cytosolic extracts (50 μg per lane) were resolved by SDS-PAGE under reducing conditions. After transfer to a PVDF membrane, procaspase-3 (p32) and the active p11 subunit of caspase-3 were detected with goat anti-caspase-3 polyclonal IgG and visualized by ECL. One or two representative samples are shown per group. Densitometric analysis of the western blots is shown in panel **C.** Data represent one or the mean of two samples. $^{*}p < 0.05$, compared with $t = 0$, $^{\#}p < 0.05$, WT compared with Gpx1−/−.

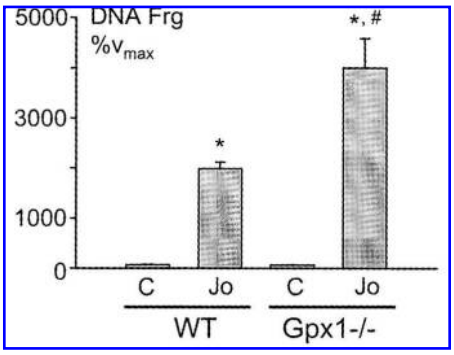

FIG. 5. Hepatocellular DNA fragmentation was assessed in controls (C) or 3 h after treatment with the anti-murine Fas antibody Jo-2 (0.6 mg/kg). Cytosolic extracts were prepared from livers of wild-type (WT) and Gpx1−/− mice, and DNA fragmentation (DNA Frg) was determined by ELISA as described in Materials and Methods. Data represent means ± SE of five animals per group and are expressed as % v_{max} of controls. $^{*}p < 0.05$, compared with C; $^{\#}p < 0.05$, WT compared with Gpx1−/−.

How can we reconcile our *in vivo* data with several, mostly opposing results obtained with different cell lines? One possible explanation for the higher incidence of ROS formation *in vitro* may be the more severe insult under culture conditions compared with the *in vivo* experiments. In fact, in most cell culture experiments, 60–80% of cells undergo apoptosis (13), whereas only 10–15% or 20–30% of hepatocytes are apoptotic during TNF- or Fas-receptor ligation, respectively (16, 23). Moreover, many of the immortalized cell lines may generate more ROS than hepatocytes *in vivo* after a similar stimulus. This conclusion is based on the observation that ROS formation in cultured cells is extensively described (1, 35). However, there is little evidence for a relevant oxidant stress during TNF-induced hepatocellular apoptosis *in vivo* (17). In addition, the antioxidant defense systems against ROS are highly effective in the intact liver (13, 15).

In summary, our data demonstrated that deficiency of the antioxidant enzyme Gpx1 had no effect on the time course or magnitude of TNF- or Fas receptor-induced apoptosis in the liver *in vivo*. These data suggest that under our experimental conditions, intracellular ROS did not modulate the death receptor-initiated apoptotic signaling cascade in hepatocytes. As Gpx1 is located in the cytosol and in mitochondria, which are the main cellular compartments involved in apoptotic signaling, our findings indicate that the oxidant stress *in vivo* was insufficient to modulate these signaling pathways. On the other hand, in the Gal/ET model and to some degree in the

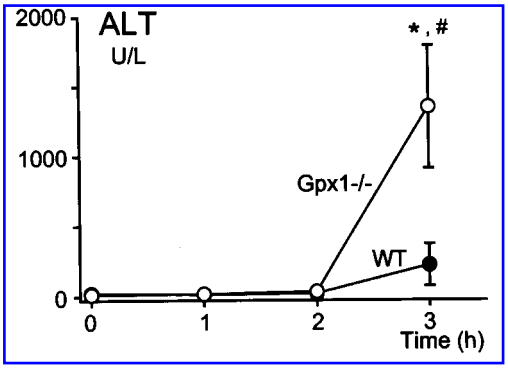

FIG. 6. Time-dependent increase of plasma ALT activities in wild-type (WT) and Gpx1−/− mice after treatment with the anti-murine Fas antibody Jo-2 (0.6 mg/kg). Data represent means ± SE of five animals per group. $^{*}p < 0.05$, compared with $t = 0$; $^{\#}p < 0.05$, WT compared with Gpx1−/−.

Fas antibody model, the overall cell necrosis and hemorrhage were higher in Gpx1−/− mice compared with wild-type animals. During TNF-induced apoptosis, this may reflect a higher susceptibility to a neutrophil-induced oxidant stress, and in the Fas antibody model it may indicate an accelerated progression to secondary necrosis.

ACKNOWLEDGMENTS

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ABBREVIATIONS

ac-DEVD-MCA, acetyl-Asp-Glu-Val-Asp-4-methyl-coumaryl- 7-amide; ALT, alanine aminotransferase; ECL, enhanced chemiluminescence; ELISA, enzyme-linked immunoabsorbent assay; ET, endotoxin; Gal, galactosamine; Gpx1, glutathione peroxidase-1; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PVDF, polyvinylidene difluoride; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; TBS, tris-buffered saline; TNF, tumor necrosis factor.

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