Double Null of Selenium–Glutathione Peroxidase-1 and Copper, Zinc–Superoxide Dismutase Enhances Resistance of Mouse Primary Hepatocytes to Acetaminophen Toxicity

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This study was conducted to determine the impact of knockout of selenium (Se)-dependent glutathione peroxidase-1 (GPX1 $^{-/-}$) or double knockout of GPX1 and copper, zinc (Cu,Zn)–superoxide dismutase (SOD1) on cell death induced by acetaminophen (APAP) and its major toxic metabolite N-acetyl-P-benzoquinoneimine (NAPQI). Primary hepatocytes were isolated from GPX1 $^{-/-}$, double knockout of GPX1 and SOD1 (DKO), and their wild-type (WT) mice and were treated with 5 m*M* APAP or 100 μM NAPQI for 0, 6, and 12 hrs. Compared with the WT cells, the GPX1 $^{-/-}$ and DKO hepatocytes were more resistant ($P < 0.05$) to the APAP-induced cell death but less resistant to the NAPQI-induced cell death. The APAP-mediated glutathione (GSH) depletion was greater ($P < 0.05$) at 6 hrs in the WT cells than in the GPX1 $^{-/-}$ and DKO cells, whereas there was no genotype effect on the NAPQI-mediated GSH depletion. The DKO cells had lower ($P < 0.05$) microsomal cytochrome P450 2E1 activities, but higher ($P < 0.05$) glutathione reductase and thioredoxin reductase activities than the WT cells at 0 hrs, and they responded differently to the APAP and NAPQI treatments. Glutathione-S-transferase activity was not affected by genotypes or treatments. Neither APAP nor NAPQI induced nitric oxide production or protein nitration in cells of any genotype. However, the GPX1 $^{-/-}$ and DKO cells were more resistant to peroxynitrite-mediated protein nitration than were the WT cells. In conclusion, double null of GPX1 and SOD1 enhanced the resistance of mouse primary hepatocytes to APAP toxicity by affecting events prior to or at NAPQI formation. While the double knockout attenuated the peroxynitrite-mediated protein nitration in hepatocytes, no protein nitration was detected in these cells treated with APAP or NAPQI. Exp Biol Med 231:545–552, 2006

Key words: acetaminophen; cell death; protein nitration; peroxynitrite; glutathione peroxidase; superoxide dismutase

Introduction

Hepatotoxicity, including liver failure, often occurs in clinics when acetaminophen (APAP) is overdosed as an analgesic and antipyretic drug (1). The ingested APAP is excreted largely *via* the conjugation with glucuronic acid or sulfate (2), whereas a small portion of the drug is metabolized by cytochrome P450 into the reactive intermediate N-acetyl-P-benzoquinoneimine (NAPQI) (3). Although NAPQI may be detoxified *via* the conjugation with glutathione (GSH), a reaction catalyzed by glutathione-S-transferase (4), excessive NAPQI generated from the APAP overdose depletes hepatic GSH and covalently binds to cellular macromolecules, resulting in cytotoxicity (5, 6). Thus, cellular GSH depletion in liver has been generally accepted as the critical initial event in the APAP-induced hepatotoxicity (6).

Copper, zinc (Cu,Zn)–superoxide dismutase (SOD1) and selenium (Se)-dependent cellular glutathione peroxidase-1 (GPX1) are two primary intracellular antioxidant enzymes. Because SOD1 converts superoxide anions into hydrogen peroxide that is in turn reduced to water by GPX1, their protections against reactive oxygen species (ROS)–related oxidative stress have been clearly shown in both *in vitro* and *in vivo* (7–10). However, their impacts on the oxidative stress related to the APAP-induced hepatotoxicity have been controversial. Mice overexpressing SOD1 (11) or treated with SOD1 mimic (12) exhibited an enhanced resistance, whereas mice overexpressing GPX1 (11) showed a decreased resistance to APAP hepatotoxicity. Knockout of GPX1 offered a partial protection against the APAP-induced increases in plasma alanine aminotransferase activity (13). Recently, we have demonstrated a striking protection by the double knockout of GPX1 and SOD1 against the APAP-

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induced lethality and hepatic GSH depletion.[1] Our initial metabolic proofing indicated that the protection was associated with a suppressed conversion of APAP into NAPQI as a result of the reduction of cytochrome P450 2E1 (CYP2E1). Because NAPQI has an extremely short half-life (14) that is too reactive to be administrated in animals, it is difficult, if not impossible, for us to verify that indicated mechanism in the whole-animal models. Instead, primary hepatocytes have been used to study APAP toxicity (15, 16) and are a good alternative for us to determine if the knockouts of GPX1 and SOD1 protect against cytotoxicities mediated by APAP, but not by NAPQI.

Peroxynitrite formation and the subsequently induced nitrotyrosine formation in liver have been suggested as a mediator of the APAP-induced hepatotoxicity (13). In addition, several *in vitro* experiments have shown promotions or involvements of GPX1 (17) and SOD1 (18–21) in formations of peroxynitrite and nitrotyrosine. However, animal experiments could not sort out if hepatocytes are the actual action site for the impacts of the knockout of GPX1 and SOD1 on the APAP-mediated protein nitration. This is because the formation of peroxynitrite by the spontaneous reactions between nitric oxide and superoxide anion (22) is the key factor in the APAP-induced protein nitration (13), and several sources of nitric oxide other than from hepatocytes, such as endothelial cells and macrophages (23, 24), may contribute to the formation of peroxynitrite. Therefore, we isolated primary hepatocytes from wild-type (WT) mice, GPX1 knockout mice ($GPX1^{-/-}$), and GPX1 and SOD1 double knockout mice (DKO) and treated these cells with APAP and NAPQI. Our objectives were (i) to compare the impacts of these knockouts on the APAP- and NAPQI-induced cell death, GSH depletion, activity changes of CYP2E1, and several GSH-relating enzymes, including glutathione-S-transferase, glutathione reductase, and thioredoxin reductase; and (ii) to determine if APAP or NAPQI induced nitric oxide productions and protein nitration in the cultured hepatocytes.

Materials and Methods

Preparation, Culture, and Treatment of Primary Hepatocytes. The $GPX1^{-/-}$ and WT mice were provided by Y. S. Ho of Wayne State University (Detroit, Michigan) and were generated from 129SVJ × C57BL/6 mice (25). The DKO mice were generated from the lines of $GPX1^{-/-}$ and $SOD1^{-/-}$ mice (9) (also generated by Y. S. Ho using the same lines of mice as for $GPX1^{-/-}$) in our laboratory. The three genotypes of mice shared the same genetic background, and their respective genotypes were verified by tail DNA analysis using a polymerase chain reaction method. Primary hepatocytes were isolated from the three genotypes (2 months old) by collagenase D perfusion followed by centrifugation (17). The cells were plated in collagen-coated 12-well plates at a density of 3×10^5 and were grown at 37°C with 5% CO_2 in William's medium E supplemented with 5% fetal bovine serum, 0.5 μg of hydrocortisone/ml, 5 μg of insulin/ml, 1 μg of glucagon/ml, 100 μg of gentamycin/ml, and 10 m*M* HEPES, pH 7.0. After 20 hrs of incubation, media were changed and cells were treated with 5 m*M* APAP or 100 μ*M* NAPQI (generously provided by McNeil Consumer Health Care, Fort Washington, PA) for 6 and 12 hrs. The selected doses and exposure times of APAP and NAPQI were based on results from a series of preliminary experiments. Both APAP and NAPQI were added as a bolus into media and mixed thoroughly. All chemicals or reagents were purchased from Sigma Chemical Co. (St. Louis, MO), unless otherwise indicated.

Cell Viability, Preparation of Cell Extracts, and GSH Assay. Cell viability was measured by a colorimetric assay described by Mosmann (26), using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide. For cell extract preparation, cells were washed twice with 1 ml of phosphate-buffered saline and harvested in 50 m*M* potassium phosphate buffer (pH 7.8), 0.1% Triton X-100, 1.34 m*M* diethylenetriaminepentaacetic acid, 1 m*M* phenylmethylsulfonylfluoride, 10 μg peptstain A/ml, 10 μg leupeptin/ml, and 10 μg aprotinin/ml. The lysates were sonicated and centrifuged at 12,500 *g* for 15 mins at 4°C. Protein concentration was measured by Lowry's method (27). For the determination of GSH, the cell extracts were mixed with an equal volume of 10% 5-sulfosalicylic acid. After centrifugation at 14,000 *g* for 20 mins at 4°C, the total GSH (reduced and oxidized form) in supernatant was determined by the glutathione recycling assay (28) and was expressed as nmol/mg of protein. Briefly, 20 μl of supernatant was mixed with 280 μl of freshly prepared assay cocktail containing 0.6 m*M* 5,5′-dithiobis(2-nitrobenzoic acid), 2 U/ml glutathione reductase, and 4.5 m*M* EDTA in 100 m*M* sodium phosphate buffer, pH 7.5. The GSH concentration was determined by measuring absorbance at 412 nm in 6 mins with 30-sec intervals.

Activities of Enzymes. GPX1 activity was measured by the coupled assay of NADPH oxidation using hydrogen peroxide as substrate (29). The enzyme activity was expressed as nmol of GSH oxidized per minute per milligram of protein. Total SOD activity was measured as described by Ukeda *et al.* (30). Enzyme unit was defined as the activity needed to inhibit 50% 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt reduction. Thioredoxin reductase activity was determined using the NADPH-dependent reduction of 5,5′-dithiobis-(2-nitrobenzoic acid) method (31). Enzyme unit was defined as nmol of 5′-thionitrobenzoic acid formed per minute. Glutathione-S-transferase activity was determined by the method of Habig *et al.* (32). Enzyme unit was defined as nmol of S-2,4-dinitrophenylglutathione formed per minute with 1-chloro-2,4-dinitrobenzene as substrate. Glutathione reductase activity was measured as described by

[1] Lei, McClung, Zhu. Unpublished data.

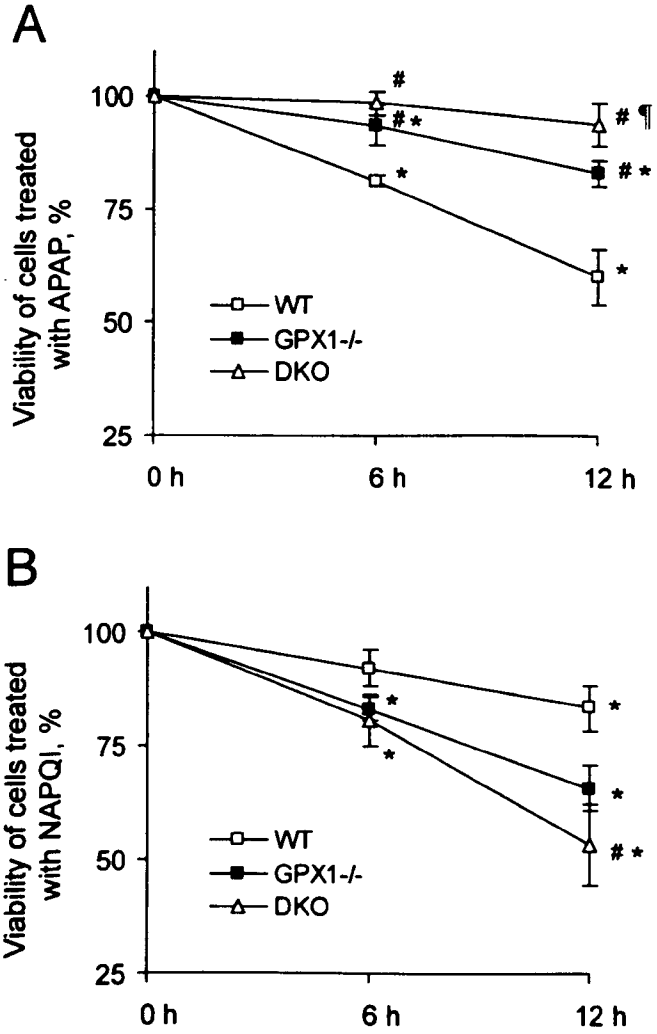

Figure 1. Cell viability of hepatocytes of different genotypes treated with 5 m*M* APAP (A) or 100 μ*M* NAPQI (B) for 0, 6, and 12 hrs. Values are means ± SEM of three independent experiments. * $P < 0.05$ vs. 0 hrs within genotypes; # $P < 0.05$ vs. WT within the same time; ¶ $P < 0.05$ vs. GPX1$^{-/-}$ within the same time.

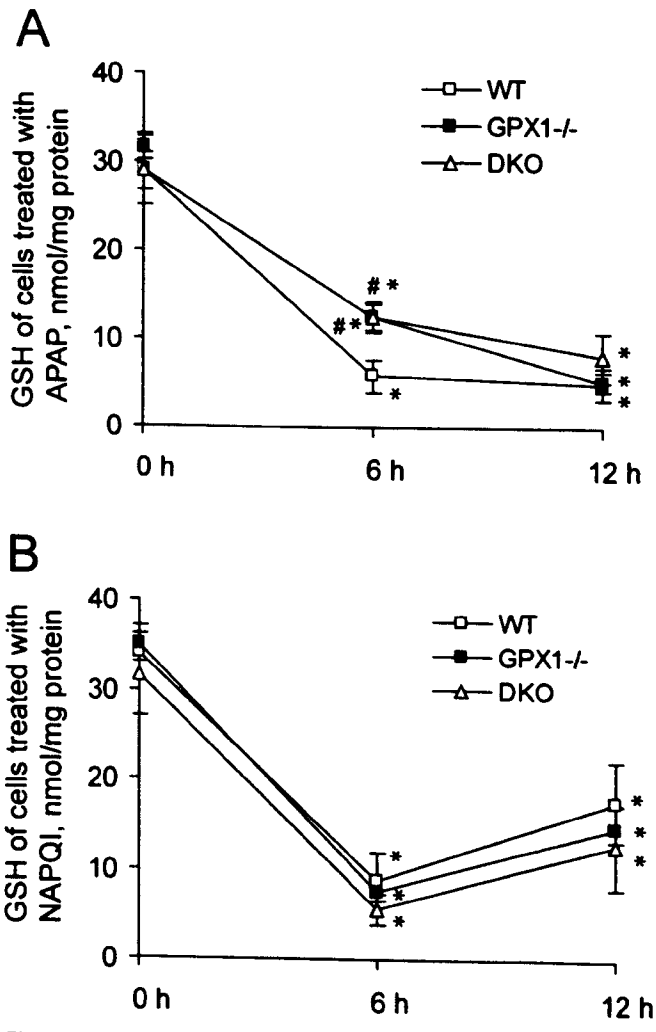

Figure 2. Changes in intracellular total GSH concentration in hepatocytes of different genotypes treated with 5 m*M* APAP (A) or 100 μ*M* NAPQI (B) for 0, 6, and 12 hrs. Values are means ± SEM ($n = 3$). * $P < 0.05$ vs. 0 hrs within genotypes; # $P < 0.05$ vs. WT within the same time.

Massey *et al.* (33). Enzyme unit was defined as nmol of the oxidized GSH form reduced per minute, using a molar extinction coefficient of 6.22×10^3 for NADPH.

For determination of microsomal CYP2E1 activity, cells were washed twice with phosphate-buffered saline, harvested in 10 m*M* Tris-HCl (pH 7.4) with 0.25 *M* sucrose, 0.1 m*M* phenylmethylsulfonylfluoride, and 1 m*M* EDTA, broken by pipetting with a microsyringe (34) and centrifuged at 12,000 *g* for 20 mins. Then the supernatants were collected and centrifuged at 100,000 *g* for 60 mins at 4°C. The pellets were resuspended in 50 μl of 50 m*M* potassium phosphate (pH 7.4), and assayed for CYP2E1 activity (35, 36).

Western Blot Analysis of Nitrotyrosine Formation. The mouse anti-nitrotyrosine antibody was purchased from Upstate Biotechnology (Lake Placid, NY). The rabbit anti-α actin antibody was from Sigma Chemical. Cell extract (15 μg of protein per lane) was loaded to 12% sodium dodecyl sulfate–polyacrylamide gel, and the subsequent procedures were conducted according to the manufacturer's instructions. The WT, GPX1$^{-/-}$, and DKO cells were treated with 0.8 m*M* peroxynitrite for 12 hrs (17) to serve as positive controls of nitrotyrosine formation. The bands were detected using a SuperSignal West Pico Kit (Pierce Chemical Co., Rockford, IL).

Determination of Nitric Oxide Generation. Medium nitrate and nitrite was measured as an indicator of nitric oxide production (37). In brief, the nitrate in 50 μl of samples was reduced to nitrite by the addition of 100 mU of nitrate reductase/ml, 6 μ*M* flavine adenine dinucleotide, and 120 μ*M* NADPH in 60 m*M* HEPES buffer, pH 7.4. After incubation at 37°C for 30 mins, the excess NADPH in the mixture was oxidized by lactate dehydrogenase (15 U/ml) in the presence of 10 m*M* sodium pyruvate, with 10-min incubation at 37°C. Thereafter, 200 μl of the supernatant was mixed with 360 μl of 40 mg/ml of Griess reagent and incubated at room

Table 1. Effect of APAP or NAPQI on GPX1, Total SOD, and Glutathione-S-Transferase Activities in Hepatocytes of Different Genotypes[a]

		APAP		NAPQI	
	0 hrs	6 hrs	12 hrs	6 hrs	12 hrs
Cellular GPX1 (nmol of glutathione oxidized/min/mg of protein)					
WT	258 ± 20	140 ± 22*	124 ± 8.6*	190 ± 23*	184 ± 14*
GPX1$^{-/-}$	3.9 ± 1.6**	4.1 ± 2.0**	2.3 ± 1.2**	2.6 ± 1.1**	2.6 ± 0.8**
DKO	3.1 ± 1.9**	3.3 ± 1.1**	2.1 ± 0.6**	2.5 ± 0.8**	3.7 ± 0.9**
Total SOD (50% formazan dye formation rate inhibition/mg of protein)					
WT	346 ± 14	315 ± 8.6	267 ± 3.5*	262 ± 8.9*	290 ± 5.7*
GPX1$^{-/-}$	340 ± 17	293 ± 50	273 ± 45	240 ± 15*	256 ± 20*
DKO	4.6 ± 2.3**	3.5 ± 1.7**	3.8 ± 0.7**	3.3 ± 0.1**	4.1 ± 2.0**
Glutathione-S-transferase (nmol of S-2,4-dinitrophenylglutathione formed/min/mg of protein)					
WT	667 ± 20	649 ± 36	636 ± 44	626 ± 74	597 ± 79
GPX1$^{-/-}$	699 ± 41	677 ± 33	652 ± 21	655 ± 34	650 ± 35
DKO	633 ± 23	600 ± 68	626 ± 11	559 ± 74	542 ± 83

[a] Hepatocytes were treated with 5 m*M* APAP or 100 μ*M* NAPQI for 0, 6, or 12 hrs. Values are means ± SEM ($n = 3$–4). GPX1, glutathione peroxidase; SOD, superoxide dismutase.
* $P < 0.05$ vs. 0 hrs within genotypes.
** $P < 0.05$ vs. WT within the same time.

temperature for 10 mins. Absorbance was read at 570 nm. Nitrate and nitrite concentration was calculated from a standard curve prepared from sodium nitrate.

Statistical Analysis. Data were analyzed using the general linear model procedure in SAS (release 6.11; SAS Institute, Inc., Cary, NC) as a one-way analysis of variance with time-repeated measurements. The Bonferroni t test was used for mean comparisons. Significance was defined as $P < 0.05$.

Results

Double Knockout of GPX1 and SOD1 Enhanced Hepatocyte Resistance to Cytotoxicity Induced by APAP, but Not by NAPQI. After being treated with 5 m*M* APAP, the WT cells showed a 19% and 40% decrease in viability ($P < 0.05$) at 6 and 12 hrs, respectively, compared with that observed at 0 hrs (Fig. 1A). The same treatment caused less cell death in the GPX1$^{-/-}$ cells and no significant decrease in the DKO cells. Thus, viabilities of the DKO and GPX1$^{-/-}$ cells were 12%–34% higher ($P < 0.05$) than those of WT cells at 6 and 12 hrs. The NAPQI-treated DKO and GPX1$^{-/-}$ cells exhibited 17%–47% ($P < 0.05$) decreases in viability at both 6 and 12 hrs compared with their initial values (0 hrs), whereas the reduction in the NAPQI-treated WT cells was 17% at only 12 hrs ($P < 0.05$) (Fig. 1B). The viability of DKO cells was 30% lower ($P < 0.05$) than that of the WT cells at 12 hrs.

Double Knockout of GPX1 and SOD1 Affected GSH Depletion Mediated by APAP, but Not by NAPQI. As shown in Figure 2A, the APAP treatment caused time-dependent decreases ($P < 0.05$) of total cellular GSH in all three genotypes between 0 and 12 hrs. The WT cells showed a much sharper decline than the other two groups of cells within the first 6 hrs of treatment. This resulted in a total GSH concentration that was twice as high in the DKO and GPX1$^{-/-}$ cells ($P < 0.05$) compared with the WT cells at the time point. The NAPQI-induced decreases ($P < 0.05$) in total cellular GSH at 6 and 12 hrs were not affected by genotypes (Fig. 2B). However, total GSH increased at 12 hrs following a sharp decline at 6 hrs in all three genotypes. There was no genotype difference in the NAPQI-mediated GSH changes at any time point.

Double Knockout of GPX1 and SOD1 Reduced CYP2E1 Activities and Responses of GSH-Related Enzymes to APAP or NAPQI. As expected, there was little GPX1 activity detected in GPX1$^{-/-}$ and DKO cells or total SOD activity in DKO cells (Table 1). GPX1 activities in the WT cells were reduced ($P < 0.05$) by the APAP treatment by 46% and 52% and by the NAPQI treatment by 26% and 29% at 6 and 12 hrs, respectively. The APAP-induced decrease in total SOD activities was 23% at 12 hrs ($P < 0.05$) in the WT cells and was not significant in the GPX1$^{-/-}$ cells. The NAPQI treatment reduced ($P < 0.05$) total SOD activity by 24% at 6 hrs and by 16% at 12 hrs in the WT cells and reduced total SOD activity by 29% at 6 hrs and by 25% at 12 hrs in the GPX1$^{-/-}$ cells, respectively. In any genotype, glutathione-S-transferase activity was not significantly affected by either APAP or NAPQI treatment.

Microsomal CYP2E1 activities in hepatocytes of the three genotypes at 0 hrs ranged from 120 ± 9.1 to 161 ± 4.4 pmol/min/mg protein, without significant responses to the APAP or NAPQI treatment at 6 hrs. The CYP2E1 activity was 26% and 28% lower ($P < 0.05$) in the phosphate-buffered saline– and APAP-treated DKO cells than in the WT cells, respectively, but this activity was not significantly different between the WT and GPX1$^{-/-}$ cells

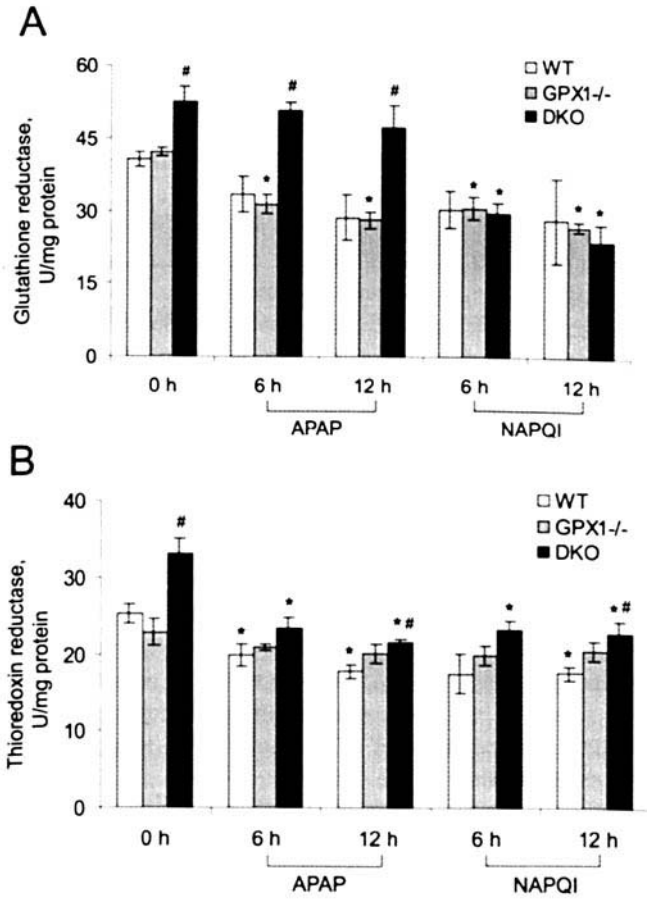

Figure 3. Changes in glutathione reductase (A) and thioredoxin reductase (B) activity in hepatocytes of different genotypes treated with 5 m*M* APAP or 100 μ*M* NAPQI for 0, 6, or 12 hrs. Values are means ± SEM ($n = 3$). * $P < 0.05$ vs. 0 hrs within genotypes; # $P < 0.05$ vs. WT within the same time.

(data not shown). Baseline activities (at 0 hrs) of glutathione reductase (Fig. 3A) and thioredoxin reductase (Fig. 3B) in the DKO cells were higher ($P < 0.05$) than those in the WT cells. While the APAP treatment caused 25% and 33% decreases ($P < 0.05$) in glutathione reductase activity in the GPX1$^{-/-}$ cells at 6 and 12 hrs, the NAPQI treatment resulted in 27%–54% activity reductions ($P < 0.05$) of the enzyme in both GPX1 $^{-/-}$ and DKO cells (Fig. 3A). The APAP and NAPQI treatments caused 22%–35% activity reductions ($P < 0.05$) of thioredoxin reductase in both WT and DKO cells at 6 and/or 12 hrs, but they did not affect the enzyme activities in the GPX1 $^{-/-}$ cells (Fig. 3B).

Double Knockout of GPX1 and SOD1 Attenuated the Peroxynitrite-Mediated Protein Nitration. Neither APAP nor NAPQI induced the formation of protein nitration in WT, GPX1 $^{-/-}$, or DKO cells (data not shown). In contrast, nitrotyrosine was detected in the cells treated with 0.8 m*M* peroxynitrite (Fig. 4). Based on three independent experiments, the intensity of the peroxynitrite-induced protein nitration, relative to the WT cells (100 ± 4.3), was lower ($P < 0.05$) in the GPX1 $^{-/-}$ (65 ± 8.4) and DKO cells (54 ± 5.8). Total nitrate/nitrite concentrations in the media ranged from 1.6 ± 0.6 to 2.5 ± 0.5 μ*M* and were not affected by APAP, NAPQI, or genotypes. Meanwhile, neither APAP nor NAPQI induced the expression of inducible nitric oxide synthase protein in cells of any genotype (data not shown).

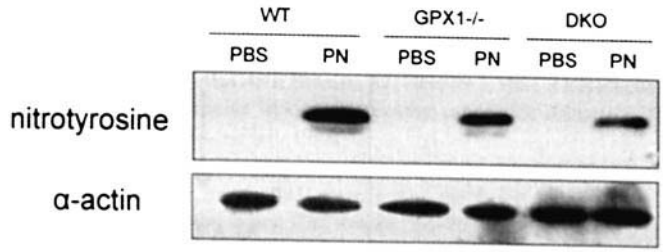

Figure 4. Western blot analysis of nitrotyrosine formation in hepatocytes of different genotypes treated with 0.8 m*M* peroxynitrite for 12 hrs. The blot is representative of three independent analyses, and α-actin was used as a loading control. PBS, phosphate-buffered saline; PN, peroxynitrite.

Discussion

One of our main findings from the present study is that knockout of GPX1 or double knockout of GPX1 and SOD1 protected primary hepatocytes against cell death induced by APAP, but not by its reactive toxic metabolite NAPQI. In coping with the APAP-induced cell death, DKO hepatocytes showed the highest resistance, followed by GPX1$^{-/-}$ hepatocytes, and WT cells were the most sensitive. In contrast, resistances to the NAPQI-induced cell death were in a reversed order. Therefore, these results support our notion, derived from the animal study, that the protection by knockout of GPX1 and SOD1 against APAP toxicity was associated with the suppressed conversion of APAP into NAPQI.

The metabolism of APAP into NAPQI is catalyzed predominately by CYP2E1 (38) and has been considered the primary event at the early phase of APAP toxicity (3, 6). Because CYP2E1 contributes to the production of ROS, a negative feedback of ROS on the enzyme has been suggested through oxidative modification and subsequent degradation (39). Therefore, the lower CYP2E1 activity in the DKO cells might result from the potential change in cellular oxidative stress due to the GPX1 and SOD1 deficiency. Decreased CYP2E1 activity could reduce the need of GSH for detoxifying NAPQI (6) in the DKO cells. Indeed, the APAP-treated DKO cells displayed less GSH depletion than did the WT cells at 6 hrs. However, the GPX1$^{-/-}$ cells had CYP2E1 activities similar to the WT cells and thus might have a different mechanism to inhibit the production of NAPQI- or APAP-induced GSH depletion.

As the conjugation between GSH and NAPQI is catalyzed by glutathione-S-transferase, an increase of glutathione-S-transferase activity in Se-deficiency has been considered a protective mechanism against APAP toxicity (40). However, glutathione-S-transferase activities in pri-

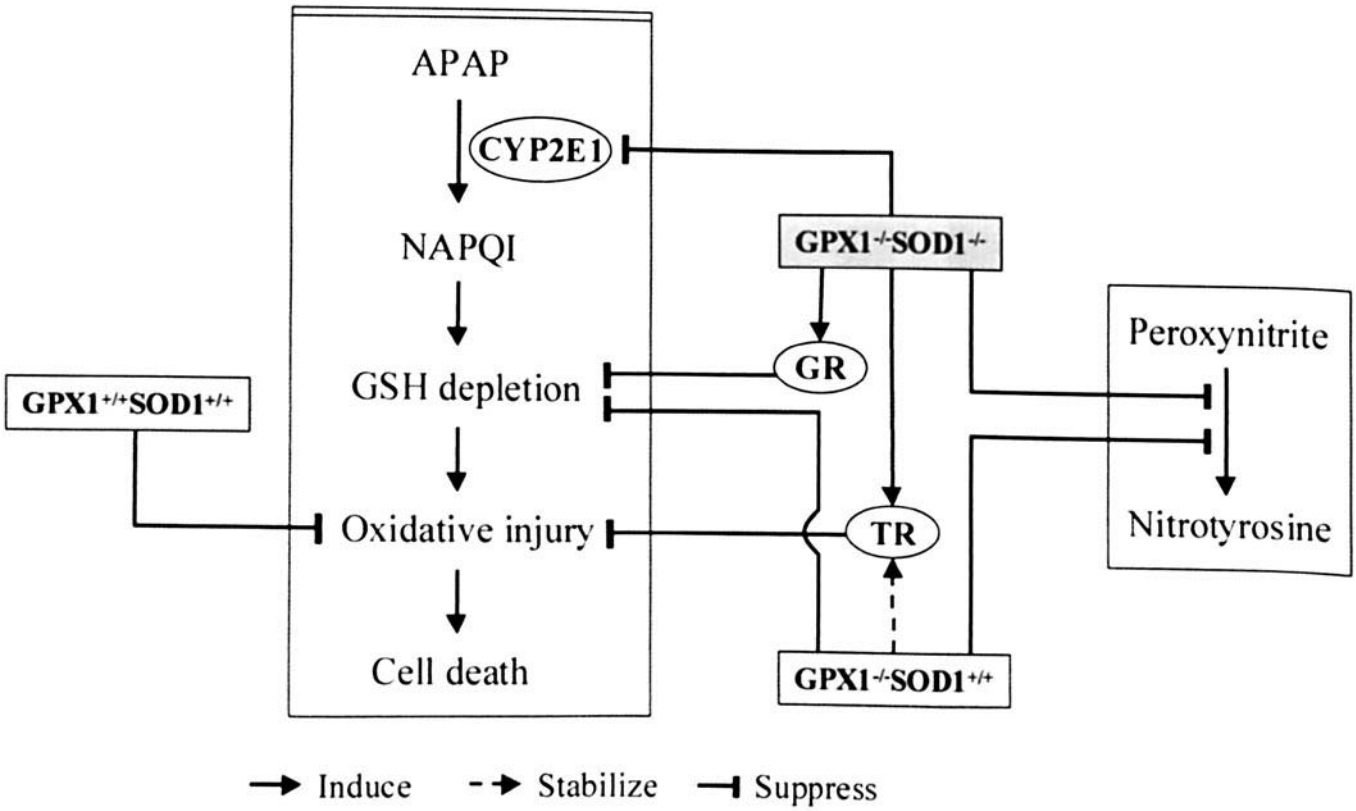

Figure 5. Schematic illustration of impacts of singular or double knockouts of GPX1 and SOD1 on APAP or NAPQI-induced cell death and peroxynitrite-mediated nitrotyrosine formation. CYP2E1, cytochrome P450 2E1; GR, glutathione reductase; and TR, thioredoxin reductase.

mary hepatocytes in the present study were not affected by genotypes or the treatments of APAP or NAPQI. Because glutathione reductase reduces oxidized GSH and thioredoxin reductase plays an important role in the redox regulation of multiple intracellular events (41), the upregulated activities of these two enzymes in the DKO cells helped in protecting against the APAP toxicity. A possible greater intracellular ROS concentration in the DKO hepatocytes than in the GPX1$^{-/-}$ hepatocytes might explain the activation of the two enzymes only in the former (42). It is interesting to note that thioredoxin reductase activities in GPX1$^{-/-}$ cells were not significantly affected by APAP or NAPQI treatment. In addition, the GPX1 activity was reduced more and earlier by the APAP treatment than by SOD activity in the WT cells, implying a differential susceptibility of these two enzymes to the APAP-mediated oxidative stress (43). However, effects of NAPQI on activities of these two enzymes were similar in the WT and/or GPX1$^{-/-}$ cells.

Despite viability differences at 12 hrs after APAP treatment, cells of all three genotypes had similar GSH levels at this point, indicating that the depletion of GSH preceded the occurrence of cell death. While the NAPQI-induced GSH depletion was not different among the three genotypes, the WT cells had higher cell viability than did the GPX1$^{-/-}$ or DKO cells at 12 hrs after treatment. It has been demonstrated that knockout of GPX1 increased sensitivity to ROS-induced cell death (7, 8), and knockout of SOD1 renders mice susceptible to oxidative stress induced by acute paraquat toxicity and myocardial ischemia/reperfusion injury (9, 10). Since NAPQI leads to the increase of oxidative stress *via* the covalent binding of cellular protein and mitochondrial dysfunction (5, 15, 16), the presence of GPX1 and SOD1 enzymes in the WT cells helps in combating the induced secondary oxidative stress. This also helps explain the paradoxical roles of GPX1 and SOD1 in APAP toxicity reported previously (11, 12) and in the present study. Upregulating SOD1 (11) had no effect on the early event of APAP toxicity, but protected against the late-phase oxidation, such as lipid peroxidation. Administering SOD mimic was protective even after the onset of the toxicity (12).

Our second interesting finding is that the APAP or NAPQI treatments caused no increase in nitric oxide production or protein nitration in primary hepatocytes. Because authentic peroxynitrite induced protein nitration in all types of cells, the negative results were not due to a technical limitation of our Western analysis of nitrotyrosine. Lack of nitric oxide synthesis and inducible nitric oxide synthase upregulation after the APAP or NAPQI treatment indicate that primary hepatocytes might not produce nitric oxide under these circumstances. No upregulated expression of inducible nitric oxide synthase by APAP in murine hepatocytes has been reported previously (44). Thus, the nitric oxide or peroxynitrite associated with hepatic protein

nitration in the APAP-treated animals might be derived largely from cells other than hepatocytes (23, 24).

As reported previously (17), GPX1 knockout enhanced mouse hepatocyte resistance to the peroxynitrite-mediated protein nitration. Strikingly, the DKO cells were also or even more resistant to the insult. This indicates that SOD1 and GPX1 may function synergistically or collectively in the process. In line with our results, SOD1 has been shown to be able to facilitate the formation of protein nitration associated with amyotrophic lateral sclerosis (20, 21). Active research is underway to find out if the attenuated peroxynitrite-mediated protein nitration by knockout of GPX1 and/or SOD1 contributes to the protection against APAP toxicity.

Taken together, our study clearly illustrates that knockout of GPX1 and SOD1 enhanced the resistance of primary hepatocytes to cytotoxicity induced by APAP, but not by NAPQI. The differential susceptibilities of DKO cells to APAP and NAPQI toxicity were primarily attributed to their reduced CYP2E1 activity, a key enzyme in converting APAP into NAPQI (Fig. 5). This helps clarify that inhibition of NAPQI formation was the key mediator for the protection by the double knockout of GPX1 and SOD1 against APAP overdose in mice. Knockout of GPX1 and SOD1 was detrimental for the cells to cope with NAPQI toxicity but caused upregulation of other antioxidant enzymes that contribute to cellular defenses against the secondary oxidative stress. It is very interesting to show that knockout of GPX1 and SOD1 suppressed the peroxynitrite-induced protein nitration. As APAP and NAPQI failed to induce protein nitration or NO production in the primary hepatocytes of any genotype, future research is needed to carefully identify the source of nitric oxide and peroxynitrite for the hepatic protein nitration detected in animals overdosed with APAP.

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