Comparative impacts of glutathione peroxidase-1 gene knockout on oxidative stress induced by reactive oxygen and nitrogen species in mouse hepatocytes

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Selenium-dependent glutathione peroxidase-1 (GPX1) protects against reactive-oxygen-species (ROS)-induced oxidative stress *in vivo*, but its role in coping with reactive nitrogen species (RNS) is unclear. Our objective was to compare the protection of GPX1 against cytotoxicity of superoxide generator diquat (DQ), NO donor *S*-nitroso-*N*-acetyl-penicillamine (SNAP) and peroxy-nitrite generator 3-morpholinosydnonimine (SIN-1). Primary hepatocytes were isolated from GPX1-knockout (KO) and wild-type (WT) mice and cultured in complete Williams's medium E with various levels of these agents alone or in combination for up to 12 h. While the KO cells were more susceptible to cell death, DNA fragmentation and protein carbonyl formation induced by 0.25–1 mM DQ, these cells were as tolerant as the WT cells to cytotoxicity of 0.1–1 mM SNAP or 0.5–2 mM SIN-1. Treating cells with SNAP (0.1 or 0.25 mM) in addition to DQ produced synergistic cytotoxicity that minimized differences in apoptotic

cell death and oxidative injuries between the KO and WT cells. Less protein nitrotyrosine was induced by 0.05–0.5 mM DQ + 0.25 mM SNAP in the KO than in the WT cells. Total GPX activity in the WT cells was reduced by 65 and 25 % by 0.5 mM DQ + 0.1 mM SNAP and 0.5 mM DQ, respectively. Decreases in Cu,Zn-superoxide dismutase (SOD) activity and increases in Mn-SOD activity in response to DQ or DQ + SNAP were greater in the KO cells than in the WT cells. In conclusion, GPX1 was more effective in protecting hepatocytes against oxidative injuries mediated by ROS alone than by ROS and RNS together. Knockout of GPX1 did not enhance cell susceptibility to RNS-associated cytotoxicity. Instead, it attenuated protein nitration induced by DQ + SNAP.

Key words: apoptosis, diquat, nitric oxide, protein nitrotyrosine, *S*-nitroso-*N*-acetyl-penicillamine.

INTRODUCTION

Selenium is an essential trace element with important biological functions [1]. Glutathione peroxidase-1 (GPX1, EC 1.11.1.9), the first identified selenoprotein [2,3], is one of the GPX family members [4,5]. Using GPX1 knockout [GPX1^{−/−}] mice [6], we have illustrated that this selenoenzyme is not only essential, but also the major metabolic mediator of body selenium to protect mice against acute, lethal oxidative stress induced by reactive-oxygen-species (ROS) generators paraquat or diquat (DQ) [7,8]. Similar *in vivo* roles of GPX1 have also been shown by others [9] using independently developed GPX1^{−/−} mice. Moreover, the GPX1 knockout renders mice susceptible to ischaemia/reperfusion injury [10], virus-induced myocarditis [11] and neurotoxicity [12]. Despite this accumulating evidence on the protection of GPX1 against ROS, its role in coping with reactive nitrogen species (RNS), another important group of free radicals that are constantly generated under physiological and pathological conditions [13], remains to be clarified.

There are two major RNS produced in mammalian cells: NO and peroxynitrite (PN). NO is produced by both constitutive and inducible nitric oxide synthases in many types of cells [14], including hepatocytes [15]. Depending on the circumstances and availability of ROS, NO can be either protective or toxic to hepatocytes [15]. Superoxide and NO can react at a diffusion-limited rate to form PN that is highly reactive and may account in part for the NO toxicity [16,17]. The bipyridyl herbicide DQ is hepatotoxic to rodents [8,18], generating superoxide in cells via redox cycling [19]. *S*-Nitroso-*N*-acetyl-penicillamine (SNAP) is

an exogenous NO donor, and 3-morpholinosydnonimine (SIN-1) produces PN by releasing superoxide and NO simultaneously. Both SNAP and SIN-1 have been widely used to study RNS effects in cultured cells [20].

Mild oxidative stress usually induces apoptosis that can be detected by DNA fragmentation [21]. Oxidative stress also causes protein oxidation and nitration in tissues and cultured cells [22]. Among the oxidative modifications, carbonyl formation in amino acid residues is readily detectable and widely used to measure protein oxidation [23]. Protein nitrotyrosine is often used as a stable marker of protein nitration and an index of PN activity [24]. Earlier, Sies et al. [25] have reported that GPX1 can detoxify PN in a cell-free system. With a specific knockout of GPX1 [6], the GPX1^{−/−} mice provide us with GPX1-null cells to determine the metabolic role of GPX1 in coping with RNS-mediated oxidation in living cells.

Superoxide dismutases (SODs) and GPXs are two major antioxidant enzyme families that may act co-operatively in protecting cells against oxidative stress [26]. Enzymically, SOD converts superoxide to H₂O₂, which is a substrate of GPX1. Eukaryotic cells contain two types of intracellular SOD (EC 1.15.1.1): Cu,Zn-SOD and Mn-SOD [27]. Cu,Zn-SOD is located in the cytosol and constitutes the major portion of total SOD activity [28]. Mn-SOD is located in mitochondria and its expression is responsive to oxidative stress [29]. While over-expression of Cu,Zn-SOD in cells induced a compensatory response of GPX1 to oxidative stress [30], the GPX1-knockout strain exhibited an inconsistent effect on total tissue SOD activity in mice exposed to paraquat or DQ [7,8]. Thus the interaction of

Abbreviations used: GPX, glutathione peroxidase; ROS, reactive oxygen species; RNS, reactive nitrogen species; DQ, diquat; PN, peroxynitrite; SNAP, *S*-nitroso-*N*-acetyl-penicillamine; SIN-1, 3-morpholinosydnonimine; SOD, superoxide dismutase; WT, wild-type; ALT, alanine aminotransferase; DETC, diethyldithiocarbamate.

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GPX1 and intracellular SOD in coping with oxidative stress needs further clarification. Therefore, our objectives in the present study were: (i) to compare the protective role of GPX1 against oxidative stresses induced by ROS and RNS generators singularly or in combination in mouse primary hepatocytes; and (ii) to determine the co-operative response among GPX1, Cu,Zn-SOD and Mn-SOD in coping with these stresses.

EXPERIMENTAL

Culture of primary hepatocytes

The knockout mice that we used to prepare hepatocytes were generated from the 129/SVJ $\times$ C57BL/6 line [6] and were characterized by completely undetectable GPX1 mRNA and an 80–99% reduction in total GPX1 activities in various tissues compared with wild-type (WT) mice [6]. Hepatocytes were isolated from selenium-adequate GPX1^(-/-) and WT mice (8 weeks old) by collagenase D perfusion [31]. The viability was > 85% as determined by Trypan Blue exclusion. Cells were plated in 12-well plates coated with collagen at the density of 3×10^5 , and cultured at 37 °C in 5% CO₂ in William's medium E supplemented with 5% fetal bovine serum, 100 µg/ml gentamycin, 5 µg/ml insulin, 1 µg/ml glucagon, 0.5 µg/ml hydrocortisone and 10 mM Hepes, pH 7.0, for 20 h prior to various oxidative treatments. All chemicals were purchased from Sigma (St. Louis, MO, U.S.A.) unless indicated otherwise.

Induction of oxidative stress

Superoxide generator DQ (Chem Service, West Chester, PA, U.S.A.) was dissolved in PBS to prepare a 100 mM stock solution. RNS donors SNAP and SIN-1 were diluted with the culture medium to prepare 22.7 and 24.2 mM stock solutions, respectively. These three stock solutions were added at the indicated concentrations in culture medium as a bolus and mixed thoroughly. The doses of DQ, SNAP and SIN-1 and the exposure times within individual experiments were initially based on the responses of human hepatocytes to these agents [20], and selected from a series of preliminary trials. Detailed descriptions of oxidative-stress induction are given along with the results. Unless indicated otherwise, cells were treated for 12 h with various doses of DQ, SNAP, SIN-1 and their combinations or treated with 0.5 mM DQ and/or 0.1 mM SNAP for various lengths of time.

Assays for relative viability and cell integrity

After medium was removed, cells were incubated in 0.5 ml of 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in serum-free Dulbecco's modified Eagle's medium/Ham's F12 at 37 °C in 5% CO₂ for 1 h. The MTT solution was gently aspirated and 250 µl of 0.08 M HCl in isopropanol was added to each well. After vigorous shaking for 10 min at room temperature on an orbital shaker, 100 µl aliquots from each well were transferred to a 96-well plate. The final reading was conducted at 570 nm using a microplate reader (Elx15; Bio-Tek Instrument, Winooski, VT, U.S.A.) with 630 nm as reference. The net absorbance of the untreated cells was taken as the 100% viability value. The relative cell viability of the treated cells was expressed as a percentage to the untreated cells on the same protein basis. Alanine aminotransferase (ALT; EC 2.6.1.2) activity in medium was measured using a Sigma kit (ALT 10) as an indicator of hepatocyte injury.

Measure of nitrite concentration

Nitrite concentration in medium was measured by the Griess procedure [32]. Briefly, 100 µl of medium was collected after the treatments and mixed with an equal volume of Griess reagent in a 96-well plate. The subsequent absorbance was determined at 570 nm using the Elx15 microplate reader and the concentration of nitrite was calibrated by a standard curve prepared from NaNO₂.

Detection of DNA fragmentation

DNA fragmentation was detected by agarose gel electrophoresis [33]. Briefly, cells were washed twice with PBS and harvested in lysis buffer (0.5% Triton X-100, 20 mM EDTA and 5 mM Tris/HCl, pH 8.0). The lysate was treated with proteinase K, extracted twice with phenol/chloroform and once with chloroform/3-methylbutan-1-ol (24:1), and precipitated by ethanol overnight. The DNA pellet was dissolved in TE buffer (10 mM Tris/HCl, pH 7.6/1 mM EDTA/0.15 mM NaCl), treated with RNase and separated by 1.8% agarose gel. DNA fragmentation was detected by ethidium bromide staining. The λDNA digested with *Hind*III and *Eco*RI was used as a marker (Promega, Madison, WI, U.S.A.).

Preparation of cell extract

Cells were washed twice by PBS and harvested in 50 mM phosphate buffer, pH 7.8, 0.1% Triton X-100 and 1.34 mM diethylenetriaminepenta-acetic acid (DETAPAC). The lysate was sonicated and centrifuged at 12500 g for 15 min at 4 °C. The resulting supernatant was used for Western-blot and enzyme-activity analyses, and protein concentrations were measured as described by Lowry et al. [34]

Determination of protein carbonyl and nitrotyrosine

Protein carbonyl was determined as described previously [7], using anti-2,4-dinitrophenol antibody (1:1000 dilution) after the cell extract (5 µg of protein) were derivatized with 10 mM 2,4-dinitrophenylhydrazine [23]. Protein nitrotyrosine was detected by a rabbit polyclonal anti-nitrotyrosine (1 µg/ml; Upstate Biotechnology, Lake Placid, NY, U.S.A.). Cell extract (40 µg of protein) were separated by SDS/PAGE (12% gel) and transferred to a nitrocellulose membrane (Protran; Schleicher & Schuell, Keene, NH, U.S.A.) prior to immunostaining.

Assays for GPX1 protein, GPX activity and SOD activity

GPX1 protein was detected as described previously [7]. The goat anti-rabbit IgG horseradish peroxidase system (1:3000 dilution; Bio-Rad Laboratories, Hercules, CA, U.S.A.) was used to visualize the immunoreactive protein. Total GPX activity was measured by the coupled assay of NADPH oxidation using hydrogen peroxide as substrate [35]. An enzyme unit was defined as the amount needed to oxidize 1 nmol of GSH/min.

Total SOD activities were measured as described by Ukeda et al. [36]. Mn-SOD activities were determined by the same method after treating the samples with 4 mM KCN for 30 min. Cu,Zn-SOD activities were determined by subtracting the Mn-SOD activities from the total SOD activities. One unit of SOD activity was defined as the enzyme needed to inhibit 50% 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide (XTT) reduction. SOD activities were also verified by a native-gel method [37]. Diethyldithiocarbamate (DETC), a

Cu,Zn-SOD inhibitor [38], was included at 0.5 mM to determine interactions of Cu,Zn-SOD, Mn-SOD and GPX1 in response to ROS and RNS in hepatocytes.

Statistical analysis

Data analyses were conducted using the GLM (general linear model) procedure of SAS (release 6.11; SAS Institute, Cary, NC, U.S.A.). The Bonferroni *t* test was used for mean comparisons.

RESULTS

GPX1^(-/-) hepatocytes were more susceptible to DQ cytotoxicity than WT hepatocytes, but not to SNAP or SIN-1 cytotoxicity

There was a dose- and time-dependent DQ cytotoxicity in the GPX1^(-/-) hepatocytes, but not in the WT hepatocytes. After being treated with various doses of DQ for 12 h, the GPX1^(-/-) cells showed a linear decrease in viability between 0 and 0.5 mM DQ (Figure 1A). The reduction was significant ($P < 0.05$) at just 0.1 mM DQ and reached a plateau at 0.5 mM DQ. In contrast, even 1 mM DQ caused no significant reduction of viability in WT cells compared with the untreated cells. Given 0.5 mM DQ, the GPX1^(-/-) cells showed a 27.1% ($P < 0.05$) decrease in viability after only 3 h of treatment (Figure 1B). Their viability was reduced to 2.6% of the untreated controls after 12 h and the

overall decrease was linear during the 12 h period, whereas the WT cells remained virtually unaffected by 0.5 mM DQ.

However, the GPX1^(-/-) cells were not more susceptible to various doses of SNAP or SIN-1 for 12 h than the WT cells. In fact, both types of cell appeared to be resistant to relatively high concentrations of SNAP and SIN-1. GPX1^(-/-) and WT cells showed just 14 and 11% (1 mM SNAP) and 22 and 26% (2 mM SIN-1) reductions in viability ($P < 0.05$), respectively. Lower concentrations of SNAP (0.25 or 0.5 mM) or SIN-1 (1 mM) produced only marginal reductions (10–13%) in the viability of WT cells.

DQ cytotoxicity was enhanced by SNAP

Although 0.25 mM SNAP alone exerted only a slight cytotoxicity in the WT cells, it reduced viability by 14, 33, 76 and 95% in the WT cells and 80, 93, 96 and 96% in the GPX1^(-/-) cells, respectively, in the presence of 0.05, 0.1, 0.25 and 0.5 mM DQ (Figure 2A). When cells were given the combined treatment of 0.25 mM SNAP and 0.25 mM DQ, a linear decrease in viability between 0 and 9 h was observed in both types of cell (Figure 2B). This linear decrease continued to 12 h in the WT cells, but reached a plateau at 9 h in the GPX1^(-/-) cells. The WT cells had higher (between 16.1% and 3.9-fold higher; $P < 0.05$) viability than GPX1^(-/-) cells at all time points. To further characterize the synergistic cytotoxicity of SNAP and DQ cytotoxicity, we treated cells with 0.5 mM DQ and/or 0.1 mM SNAP for 12 h. The addition of 0.1 mM SNAP to the 0.5 mM DQ treatment reduced viability from 83 to 21.5% in the WT cells and from 10.1 to 2.6% in the GPX1^(-/-) cells, despite a significant difference in viability between these two groups in both cases (Table 1). The combination of DQ and SNAP also caused an increase in ALT activity from 11.9 to 47.2 units/ml of medium in the WT cells (Table 1). Probably due to the already high level of ALT activity in the medium of GPX1^(-/-) cells treated with 0.5 mM DQ, the combined treatment did not produce any further increase. Nonetheless, there was a good inverse correlation between cell viability and ALT release into the medium. Nitrite concentration in the medium was not different between the GPX1^(-/-) and the WT cells for any given treatment (Table 1), but was 24% higher ($P < 0.05$) with the combination of DQ+SNAP than with SNAP alone. Nitrite in the medium of untreated or DQ-treated hepatocytes was undetectable.

DQ induced DNA fragmentation only in GPX1^(-/-) hepatocytes

There was no detectable DNA fragmentation in the untreated GPX1^(-/-) and WT hepatocytes (Figure 3). A typical DNA ladder appeared in the GPX1^(-/-) cells treated with 0.5 mM DQ for 12 h, and became more pronounced with the addition of 0.1 mM SNAP. However, only 0.5 mM DQ+0.1 mM SNAP together, but not 0.5 mM DQ alone, caused DNA fragmentation in the WT cells. In fact, we compared DNA fragmentation of cells treated with various levels of DQ, SNAP and SIN-1 for different lengths of time (results not shown). The DQ-induced DNA fragmentation occurred 9 h after the GPX1^(-/-) cells were treated with > 0.25 mM DQ. Neither SNAP nor SIN-1 alone (up to 1 mM) produced any detectable DNA fragmentation in the GPX1^(-/-) and WT cells. However, in the presence of 0.1 mM DQ, DNA fragmentation was seen in the GPX1^(-/-) cells treated with 0.25 mM SNAP. No DNA fragmentation was developed in the WT cells treated with 0.25 mM SNAP+0.25 mM DQ for up to 12 h, whereas it appeared in the GPX1^(-/-) cells at 9 h.

Figure 1 Relative viability ($n = 3$ independent experiments) of GPX1^(-/-) and WT hepatocytes treated with DQ

(A) Treatment with 0.1–1.0 mM DQ for 12 h; (B) treatment with 0.5 mM DQ for 3–12 h. * $P < 0.05$ and ** $P < 0.001$ compared with untreated GPX1^(-/-) and the respective WT hepatocytes.

Figure 2 Relative viability ($n = 3$ independent experiments) of GPX1^{-/-} and WT hepatocytes treated with DQ + SNAP

(A) Treatment with 0.25 mM SNAP plus 0–0.5 mM DQ for 12 h; (B) treatment with 0.25 mM SNAP + 0.25 mM DQ for 3–12 h. * $P < 0.05$ and ** $P < 0.001$, lower than the respective untreated cells; # $P < 0.05$, lower than the respective WT cells.

Figure 3 DNA fragmentation of GPX1^{-/-} and WT hepatocytes treated with DQ, SNAP or both

Cells were treated with 0.5 mM DQ, 0.1 mM SNAP or both for 12 h. DNA fragmentation was analysed by 1.8% agarose gel with ethidium bromide staining; the marker was λ DNA digested with *Hind*III and *Eco*RI (Promega). The gel is representative of three independent analyses.

GPX1 protected against DQ- and SNAP-mediated protein carbonyl formation

After a 12 h exposure to various doses of DQ, the GPX1^{-/-} cells had higher levels of protein carbonyl formation than the WT cells (Figure 4A). Only the highest dose of DQ (1.0 mM) caused an apparent increase in protein carbonyl formation over the untreated control in the WT cells. At 0 mM DQ, the GPX1^{-/-} cells seemed to have a slightly higher level of protein carbonyl than the WT cells in this particular experiment. In most cases there was no such baseline difference in oxidation between the two groups of untreated cells. Whereas 0.25 mM SNAP alone did not cause any protein carbonyl formation in either type of cell, it elevated the process substantially in the presence of DQ (Figure 4B). In the GPX1^{-/-} cells, the combination of 0.25 mM

SNAP and 0.05 mM DQ triggered nearly maximal protein carbonyl formation. In the WT cells, increased protein carbonyl formation was visible with 0.25 mM DQ in the presence of 0.25 mM SNAP. Again, higher levels of protein carbonyl were shown in the GPX1^{-/-} cells than in the WT cells in all of the combined treatments.

Less protein nitrotyrosine was induced in GPX1^{-/-} hepatocytes by DQ + SNAP

Two protein bands of approx. 30–40 kDa reacted with the anti-nitrotyrosine antibody in both GPX1^{-/-} and WT cells up to 12 h after treatment with 0.25 mM SNAP and increasing doses of DQ (Figure 5A). Apparently, the intensity of the bands was DQ-dose-dependent and was stronger in the WT than in the GPX1^{-/-} cells. This rather surprising observation was verified in a following time-course study using a combination of 0.25 mM SNAP and 0.25 mM DQ. As shown in Figure 5(B), the nitrated protein bands appeared at 3 h in both types of cell and the intensity difference, particularly the upper band, between the two groups of cells was consistent across various time points. Protein nitrotyrosine was not induced by various doses of DQ or 0.25 mM SNAP alone (results not shown).

Total GPX activity and GPX1 protein in WT hepatocytes were attenuated by DQ + SNAP

Compared with the untreated controls (330.5 ± 16.2 m-units/mg of protein), total GPX activity in the WT cells at 12 h was reduced by 24.5% (258.5 ± 36.9 m-units/mg of protein, $P < 0.05$) and 64.9% (120.1 ± 48.7 m-units/mg of protein, $P < 0.01$) by DQ and DQ + SNAP, respectively. While SNAP alone had no effect on total GPX activity in the cells (317.7 ± 30.8 m-units/mg of protein), it produced a 40% greater reduction ($P < 0.05$) in activity than that by 0.5 mM DQ alone. Consistently, Western-blot analysis showed that GPX1 protein was slightly reduced by DQ, and markedly decreased by DQ + SNAP. However, it was not affected by SNAP alone (Figure 6). The GPX1^{-/-} hepatocytes had undetectable GPX1 protein and only marginal residual GPX activity (< 4.2 m-units/mg of protein), probably from other GPX enzymes.

Table 1 Cell viability, ALT activity and nitrite in medium of GPX1^(-/-) and WT hepatocytes treated with DQ and/or SNAP

Cells were treated with 0.5 mM DQ, 0.1 mM SNAP or both for 12 h. Relative cell viability was determined by MTT assay, ALT activity in the medium by ALT kit (Sigma) and nitrite concentration in the medium by Griess procedure. Data are means $\pm$ S.E.M. ($n = 3$). ND, not detectable.

	Untreated	DQ	SNAP	DQ + SNAP
Relative cell viability (% of untreated control)				
GPX1 ^(-/-)	100	10.1 $\pm$ 4.3*	98.2 $\pm$ 3.2	2.6 $\pm$ 0.6*
WT	100	83 $\pm$ 3.9	103 $\pm$ 0.9	21.5 $\pm$ 6.5†
ALT activity in medium (units/ml)				
GPX1 ^(-/-)	8.9 $\pm$ 0.1	47.9 $\pm$ 2.8*	7.1 $\pm$ 3.6	50.1 $\pm$ 2.0†
WT	9.9 $\pm$ 0.9	11.9 $\pm$ 0.7	9.6 $\pm$ 2.1	47.2 $\pm$ 7.4†
Nitrite in medium (μ M)				
GPX1 ^(-/-)	ND	ND	25.5 $\pm$ 1.0	32.0 $\pm$ 0.2‡
WT	ND	ND	25.2 $\pm$ 1.4	30.7 $\pm$ 1.6‡

* Different ($P < 0.01$) from the untreated GPX1^(-/-) and the respective WT hepatocytes.

† Different ($P < 0.01$) from the respective untreated controls.

‡ Different ($P < 0.05$) from the SNAP treatment alone.

Figure 4 Protein carbonyl formation induced by DQ or DQ + SNAP in GPX1^(-/-) and WT hepatocytes

Protein carbonyl contents were detected by Western blot with polyclonal rabbit anti-2,4-dinitrophenol antibody. (A) Treatment with 0.1–1.0 mM DQ for 12 h; (B) treatment with 0.25 mM SNAP plus 0–0.5 mM DQ for 12 h. The blots are representative of three independent analyses.

Responses of Cu,Zn-SOD and Mn-SOD to oxidant stress in hepatocytes were modulated by GPX1 expression

There was no difference in Cu,Zn-SOD or Mn-SOD activities between non-stressed GPX1^(-/-) and WT hepatocytes (Figures 7A and 7B). Compared with these untreated controls, the DQ treatment caused a 29.6% reduction of Cu,Zn-SOD activity (P

< 0.01) and an 87.3% increase in Mn-SOD activity in the GPX1^(-/-) cells. But, DQ had little effect on either type of SOD activity in the WT cells. Whereas SNAP alone did not change either SOD activity in either type of cell, the DQ+SNAP treatment decreased Cu,Zn-SOD activity by 39.9% in GPX1^(-/-) ($P < 0.01$) and 24.1% in WT cells ($P < 0.05$) compared with the controls, respectively. Notably, the double treatments of DQ and

Figure 5 Protein nitrotyrosine induced by DQ + SNAP in GPX1^(-/-) and WT hepatocytes

Protein nitrotyrosine in the cell extract was detected by Western blot with polyclonal rabbit anti-nitrotyrosine antibody. (A) Treatment with 0.25 mM SNAP plus 0–0.5 mM DQ for 12 h; (B) treatment with 0.25 mM SNAP + 0.25 mM DQ for 3–12 h. The blots are representative of three independent analyses.

Figure 6 Responses of GPX1 protein levels of GPX1^(-/-) and WT hepatocytes treated with DQ, SNAP or both

The GPX1^(-/-) and WT hepatocytes were treated with 0.5 mM DQ, 0.1 mM SNAP or both for 12 h. GPX1 protein levels were detected by Western blot with a polyclonal rabbit anti-human GPX1 antibody. The blot is representative of three independent analyses.

SNAP produced a greater increase ($P < 0.05$) in Mn-SOD activity in the GPX1 than the WT cells (107.9 versus 52.7 % over the controls). The activity responses of these two SOD enzymes to the DQ and SNAP treatments were consistent with their protein amounts shown by the native gel analysis (Figure 7C). After the GPX1^(-/-) and WT cells were treated with 0.5 mM DETC (the Cu,Zn-SOD inhibitor) for 12 h, Cu,Zn-SOD activity was reduced by approx. 60 %, whereas Mn-SOD activity was not affected in either cell type. This amount of DETC also reduced total GPX activity by 28 % in the WT cells. In the untreated or

SNAP-treated cells, the DETC treatment reduced cell viability by about 20 % (Figure 8). However, in the cells treated with DQ or DQ + SNAP, addition of DETC killed nearly all cells and minimized the viability gap between the GPX1^(-/-) and WT cells.

DISCUSSION

The present study has demonstrated different impacts of GPX1 on mouse hepatocyte susceptibility to three sources of oxidative stress. First of all, knockout of GPX1 renders these cells

Figure 7 Responses of SOD activity of GPX1^(-/-) and WT hepatocytes treated with DQ, SNAP or both

The GPX1^(-/-) and WT hepatocytes were treated with 0.5 mM DQ, 0.1 mM SNAP or both for 12 h. **(A)** Cu,Zn-SOD activity ($n = 4$); * $P < 0.05$, lower than the respective untreated hepatocytes; ** $P < 0.001$, lower than the untreated GPX1^(-/-) hepatocytes. **(B)** Mn-SOD activity ($n = 4$); * $P < 0.05$, higher than the untreated WT hepatocytes; ** $P < 0.001$, higher than the untreated GPX1^(-/-) hepatocytes; # $P < 0.01$, higher than the respective WT hepatocytes. **(C)** Cu,Zn-SOD and Mn-SOD protein bands were detected on a native gel, and the blot is representative of three independent analyses.

Figure 8 Relative viability of GPX1^(-/-) and WT hepatocytes treated with DQ, SNAP or both in the absence or presence of DETC

The GPX1^(-/-) and WT hepatocytes were treated with 0.5 mM DQ, 0.1 mM SNAP or both for 12 h in the absence or presence of 0.5 mM DETC. * $P < 0.001$, lower than the respective untreated cells; # $P < 0.001$, lower than the respective WT cells.

susceptible to cytotoxicity of DQ, indicating that GPX1 is important for protecting against ROS-mediated apoptotic death and oxidative injuries. This protection at the cellular level is also consistent with its antioxidant role at the whole-body level

[7–10,12]. In contrast, knockout of GPX1 does not seem to make hepatocytes prone to the cytotoxicity of various doses of SNAP or SIN-1, implying that GPX1 may not be essential for these cells to cope with RNS-mediated oxidative stress. However, low levels of SNAP, such as only 0.1 mM, produce a remarkable synergism on DQ cytotoxicity in both types of cell. Because the WT cells still have higher viability and less protein oxidation than the GPX1^(-/-) cells with the combination of low levels of SNAP and DQ, it is clear that GPX1 contributes to the protection against this synergistic oxidative stress of ROS and RNS. Unlike in the case of DQ alone, this protection of GPX1 is insufficient to prevent the WT cells completely from oxidative injury by relatively high levels of SNAP and DQ. As a result, WT cells show low viability, high ALT activity release and pronounced DNA fragmentation under these conditions. Thus GPX1 is less effective in protecting against the synergistic toxicity of DQ and SNAP than that of DQ alone. This may be because the superoxide ion generated by DQ [19] is converted into H₂O₂ by SOD [27]. As a physiological substrate of GPX1, H₂O₂ is reduced to a tolerable level by the enzymic action. In the presence of SNAP, certain amounts of superoxide ion generated by DQ may escape the above-described pathway and directly react with NO originating from SNAP [16]. Both result in an augmentation of DQ toxicity [39].

One of our most interesting findings in the present study is the opposite impacts of GPX1 knockout on ROS- and RNS-induced protein oxidation and nitration. In line with our earlier observations in mice injected with DQ or paraquat [7,8], protein carbonyl content was elevated by DQ in GPX1^(-/-) but not WT cells. Without any effect by itself, 0.25 mM SNAP significantly promotes DQ-mediated carbonyl formation in both types of cell.

Presumably, this accelerated protein oxidation may be caused by PN generation from the interaction of superoxide, produced by DQ, with NO, released from SNAP [16]. Likewise, the increase is still more evident in the GPX1^{-/-} than in the WT hepatocytes. Thereby, GPX1 is protective against DQ+SNAP-mediated protein carbonyl formation, despite a less complete protection than that against DQ alone. In contrast, the DQ+SNAP treatment induces more protein nitrotyrosine formation in the WT than in the GPX1^{-/-} hepatocytes, as shown in both the dose and time-course experiments. This surprising finding is also opposite to an earlier observation by Sies et al. [25] that adding GPX1 to cell lysate protected against PN-mediated protein nitration. Because PN formation depends on the availability of both superoxide ion and NO [16], and it reacts rapidly in cells with CO₂ to form reactive intermediates, such as NO₂ and CO₃, that are more effective nitrating species than either PN or NO [40], the outcome of GPX1–PN interaction in living cells could be completely different from that in the cell-lysate system. Knockout of GPX1 may affect formations of PN and its intermediates in hepatocytes, probably by altering the supply of ROS or the ratio of ROS to RNS (NO) through its enzymic role in H₂O₂ degradation. It is also possible that GPX1 knockout attenuates cell susceptibility to PN-mediated protein nitration via unknown mechanisms. As protein oxidation and nitration are involved in many diseases [22], understanding the role of GPX1 in these oxidative events may help clarify pathogenesis of these diseases.

Whereas total GPX activity in the WT cells is not affected by SNAP alone, it is reduced 25 % by DQ and 65 % by DQ+SNAP. Because these GPX activity changes concur with reductions of GPX1 protein and mRNA (results not shown), it seems that GPX1 expression responds to these two sources of oxidative stress at both the transcriptional and translational levels. As GPX1 can be inactivated by NO or PN [41,42], it will be useful to find out whether ROS and RNS generated by DQ alone or DQ+SNAP directly inactivate or destabilize GPX1 in hepatocytes.

Just as in the case of GPX1 [7–9], *in vivo* protection of SOD against oxidative damage has been illustrated by using Cu,Zn-SOD-null [43,44] and Mn-SOD-null [45,46] homozygous mice. As mentioned above, superoxide ions generated by DQ [19] require SOD to catalyse their dismutation into H₂O₂, the substrate of GPX1. A balance between cellular GPX and SOD activity [30] is crucial for cells to defend the oxidative attack by DQ and DQ+SNAP. In fact, the DQ- or DQ+SNAP-induced decrease in Cu,Zn-SOD and increase in Mn-SOD activities in mouse hepatocytes is affected by the GPX1 knockout. The induced decrease in Cu,Zn-SOD activity in GPX1^{-/-} cells is greater than that in the WT cells, and it may be viewed as a protective response to maintain an appropriate balance between GPX1 and this enzyme [7]. This greater decrease may also be caused by an accelerated inactivation of Cu,Zn-SOD, due to the elevated ROS associated with the GPX1 knockout [47]. Although Mn-SOD is protective against cell death [48], the induction of Mn-SOD in either the GPX1^{-/-} or WT cells does not help in protecting these cells from the oxidative attack of DQ or DQ+SNAP. Thus our data support the notion that Mn-SOD does not compensate for the loss of Cu,Zn-SOD [27]. In contrast, when hepatocyte Cu,Zn-SOD activity is partially inhibited by DETC (results not shown), all cells, regardless of GPX1 status, are virtually completely killed by DQ alone or DQ+SNAP. Clearly, the GPX1 protection against DQ- or DQ+SNAP-mediated oxidative stress in hepatocytes depends on the function of a full Cu,Zn-SOD activity. In addition, attenuation of Cu,Zn-SOD activity may further enhance

DQ+SNAP toxicity as the enzyme may protect cells against NO cytotoxicity [49].

In summary, our study has demonstrated that GPX1 is more protective against cell death, DNA fragmentation and protein carbonyl formation induced by DQ alone than by DQ+SNAP. Strikingly, the GPX1 knockout results in an attenuation of protein nitration mediated by DQ+SNAP. Future experiments with manipulations of cellular peroxide and selenium status may help elucidate the mechanism of this phenomenon. Both GPX1 protein and activity are reduced by DQ and DQ+SNAP in the WT hepatocytes. Responses of Cu,Zn-SOD and Mn-SOD activities to oxidative stress are opposite and affected by cellular GPX1 expression.

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