Low levels of glutathione peroxidase 1 activity in selenium-deficient mouse liver affect c-Jun N-terminal kinase activation and p53 phosphorylation on Ser-15 in pro-oxidant-induced aponecrosis

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Low levels of hepatic selenium (Se)-dependent glutathione peroxidase 1 (GPX1) activity have been shown to protect against oxidative liver injury in Se-deficient mice. The objective of the present study was to determine if the GPX1 protection was associated with phosphorylations of c-Jun N-terminal kinase (JNK) and p53 on Ser-15, two key signalling events in oxidative-stress-mediated cell death. Both Se-deficient GPX1 knockout (GPX1$^{-/-}$) and wild-type (WT) mice ($n = 64$) were pretreated with an intraperitoneal injection of Se (as sodium selenite, 50 μg/kg body weight) 6 h before an intraperitoneal injection of paraquat (12.5 mg/kg). Liver aponecrosis, a mixed form of cell death sharing apoptosis and necrosis, was induced by paraquat in both groups of mice. However, its appearance was remarkably delayed and the severity was decreased by the repletion of hepatic GPX1 activity to < 4 % of the normal level by the Se injection in the WT mice, compared with that in the GPX1$^{-/-}$ mice. Consistently, the WT mice had lower levels of hepatic phospho-JNK, p53 and phospho-p53 (Ser-15) when compared with the GPX1$^{-/-}$ mice at 1–10 h after paraquat injection. Incubating liver homogenates with antibodies raised against JNK or phospho-JNK resulted in co-immunoprecipitation of phospho-p53 (Ser-15), and the amounts of the precipitated phospho-p53 were greater in the GPX1$^{-/-}$ mice when compared with that in the WT mice. The co-precipitated complex by the anti-phospho-JNK antibody was capable of phosphorylating intrinsic or extrinsic p53 on Ser-15. In conclusion, phospho-JNK may catalyse phosphorylation of p53 on Ser-15 in Se-deficient mouse liver under moderate oxidative stress, and attenuation of that cascade by low levels of GPX1 activity is associated with its protection against the pro-oxidant-induced liver aponecrosis.

Key words: apoptosis, oxidative stress, p38, paraquat, signalling.

INTRODUCTION

Selenium (Se) is an essential antioxidant nutrient that has potential in preventing cancer, viral infection, chronic disease and male infertility. Challenging Se-deficient rodents, after an acute body Se repletion by Se injections, with reactive oxygen species (ROS)-generator paraquat has been effectively used to study antioxidant roles of Se and selenoproteins [1,2]. Cellular glutathione peroxidase 1 (GPX1) is the first identified seleno-enzyme [3,4] that represents 60 % of total liver Se [5]. Using the GPX1 knockout (GPX1$^{-/-}$) mice, we have demonstrated a strong dependence on body Se status for the physiological importance of GPX1 protection against moderate oxidative stress *in vivo*. In Se-adequate mice with full expression of other selenoproteins, GPX1 was apparently not essential for the body to defend against oxidative stress induced by an intraperitoneal injection of a low level of paraquat (12.5 mg/kg) [6]. In contrast, a residual hepatic GPX1 activity in Se-deficient wild-type (WT) mice or a repletion of low levels of the enzyme by a single intraperitoneal injection of Se (50 μg/kg) in these animals exhibited a potent protection against the paraquat-induced liver injuries and the related mortality [6a]. Notably, this striking role of GPX1 is different from the views of Burk et al. [1,2] based on their studies using conventional Se-deficient rat. To elucidate the molecular mechanism of the GPX1 protection, we studied the impact of low levels of hepatic GPX1 activity on the pro-oxidant-induced death of liver cells, including necrosis, apoptosis or aponecrosis, a combined process of apoptosis and necrosis in a single cell [7].

Activation of p53, the key tumour suppressor protein, is considered as a critical cell-injury response that leads to cell-cycle arrest and apoptosis [8,9]. Phosphorylation is a major pathway for the post-transcriptional activation of p53 [10], and Ser-15 in human p53 (conserved as Ser-18 in mouse p53 [11], but described as Ser-15 herein for simplicity) is sensitive to oxidative-stress-induced phosphorylation [12]. This modification has been postulated as a key step in the p53-mediated responses to stress because it stabilizes p53 by preventing its N-terminus from binding to MDM2, a protein that promotes the ubiquitin-mediated p53 degradation [13]. Earlier studies have shown that DNA-PK, ATR, ATM and p38 can phosphorylate human p53 on Ser-15 in cells exposed to radiation or UV that induce ROS formation [14–17]. Although JNK, a member of the serine/threonine kinase family and a central transducer of extracellular signals [18], catalyses the phosphorylation of p53 at several other sites [19,20], it remains unclear if it phosphorylates p53 on Ser-15 in the ROS-induced cell death [16,21,22]. Therefore the objective of the present study was to determine if JNK activation and phosphorylation of p53 on Ser-15 were involved in the protection by low levels of hepatic GPX1 activity against paraquat-mediated cell injury and cell death in the Se-deficient mouse liver.

Table 1   Liver GPX1 activity (A; m-units/mg of protein) and aponecrosis score (B) in the Se-deficient GPX1⁻/⁻ and WT mice injected with Se and paraquat

Paraquat (12.5 mg/kg body weight) was injected 6 h after injection of Se (50 μg/kg body weight as Na_2SeO_3), and mice were killed at the indicated time points. Procedures to determine GPX1 activity (**A**) and aponecrosis scores (**B**) are described in the Experimental section. Data represent means $\pm$ S.E.M. (for the GPX1 assay, $n = 4$; for aponecrosis score, $n = 3$).

A

Mouse type	Treatment …	Saline control	Hours after paraquat injection					Se control
			0	1	3	6	10	
GPX1⁻/⁻		3.3 $\pm$ 0.2*	5.0 $\pm$ 0.7*	4.8 $\pm$ 0.4*	4.7 $\pm$ 0.5*	5.1 $\pm$ 0.8*	6.5 $\pm$ 0.1*	6.8 $\pm$ 2.3*
WT		16.5 $\pm$ 3.1	36.3 $\pm$ 2.4†	41.5 $\pm$ 3.4†	40.3 $\pm$ 2.0†	32.8 $\pm$ 1.3†	31.4 $\pm$ 3.1†	38.7 $\pm$ 2.9†

B

Mouse type	Treatment …	Saline control	Hours after paraquat injection					Se control
			0	1	3	6	10	
GPX1⁻/⁻		0	0	0	1	2	4	0
WT		0	0	0	0	0	2	0

* Different ($P < 0.05$) from the WT mice.
† Different ($P < 0.05$) from the saline-injected controls.

EXPERIMENTAL

Mice and reagents

Our experiments were approved by the Institutional Animal Care and Use Committee at Cornell University and conducted in accordance with the NIH guidelines for animal care. The weanling (3-week-old) Se-deficient GPX1⁻/⁻ and WT mice ($n = 64$) [23] were produced by feeding an Se-deficient (0.02 mg/kg) Torula-yeast basal diet [24] for 5 weeks. The moderate oxidative stress was induced by an intraperitoneal injection of paraquat (12.5 mg/kg body weight) [6]. Low levels of GPX1 activity repletion were achieved by an intraperitoneal injection of Se (as Na_2SeO_3) to the Se-deficient mice at 50 μg/kg body weight, a dose approximately equivalent to their daily need. Responses to Se injection were measured by GPX1 and GPX4 activities [5]. All chemicals were purchased from Sigma (St. Louis, MO, U.S.A.) unless indicated otherwise. Antibody raised against JNK2 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.), phospho-p53 (Ser-15), phospho-JNK (Thr-183/Tyr-185), phospho-p38 and p38 were from Cell Signaling (Beverly, MA, U.S.A.), p53 was from PharMingen (San Diego, CA, U.S.A.), rabbit IgG from Bio-Rad Laboratories (Hercules, CA, U.S.A.) and mouse IgG was from Pierce (Rockford, IL, U.S.A.). Notably, the phospho-JNK antibody recognizes both phosphorylated JNK1 and JNK2.

DNA strand breaks

Liver sections (10 μm) were formalin-fixed and paraffin-embedded [6] to assay for DNA strand breaks, using the ApopTag Kit (Oncor Inc., Gaithersburg, MD, U.S.A.) according to the manufacturer's instructions. Because the affected cells were distributed in specific areas of the liver and the staining appeared not only in nuclei but also in cytoplasm due to swollen cells with the release of oligonucleosomal fragments into cytoplasm, we developed a scoring system based on the area of the stained liver parenchyma using the TUNEL (terminal transferase deoxytidyl uridine end labelling) method. A score of 0 was assigned to liver sections where no DNA strand breaks were seen, a score of 1 when a small area (1–9%) was stained but without cytoplasmic staining, and scores 2, 3 and 4 when 1–9%, 10–24% and $\geqslant 25\%$ of the liver section showed staining in both cytoplasm and nucleus respectively.

Western-blot analysis, immunoprecipitation and GPX1 activity

Liver samples were homogenized (Polytron PT3100; Brinkmann Instruments, Littau, Switzerland) either in buffer A [100 mM Tris (pH 7.4); 250 mM sucrose; inhibitor mixture: 1 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 μM microcystin, 1 mM PMSF and 10 mM sodium fluoride] for Western-blot analyses or in buffer B [50 mM Hepes (pH 7.6), 100 mM sodium chloride, 1% Triton X-100, 5 mM EDTA and inhibitor mixture as in buffer A] for immunoprecipitation on ice. The homogenates were centrifuged at 14000 $\textbf{\textit{g}}$ for 30 min at 4 °C. Protein concentrations were estimated using the Bradford method (Bio-Rad Laboratories). The supernatants (20 μg of protein) were subjected to Western-blot analyses as described previously [25], except for using the chemiluminescent method (SuperSignal® West Pico Kit from Pierce) for detection. Relative densities of protein bands in individual blots were quantified using an IS-1000 Digital Imaging System (Alpha Innotech Co., San Leandro, CA, U.S.A.). In Figures 2 and 3, the values were normalized with the respective controls (vinculin, JNK2 or p38 MAPK) and expressed as ratios to those in the WT mice at -6 h. In Figures 4–6, the values were expressed as ratios to those in the ($+$) control, 2 μg of glutathione S-transferase (GST)-p53 or WT mice (without [γ-³²P]ATP). Supernatants (1.5 mg of protein) of liver homogenate for immunoprecipitation were precleared by 5 μl of rabbit serum and 30 μl of Protein A–Sepharose beads (50% slurry) for 30 min at 4 °C. After centrifuging at 3000 $\textbf{\textit{g}}$ for 1 min, the supernatants were incubated with the rabbit polyclonal antibodies raised against phospho-JNK or JNK overnight at 4 °C with rotation, and were then incubated with 50 μl of the Protein A–beads for 30 min at 4 °C with rotation. The beads were washed four times with 1 ml of buffer B on ice. Thereafter, 20 μl of buffer B and 5 μl of 5 × SDS buffer were added to the washed beads before Western-blot analyses of phospho-p53 (Ser-15) and/or p53. Because of the established interaction of p53 and phospho-JNK
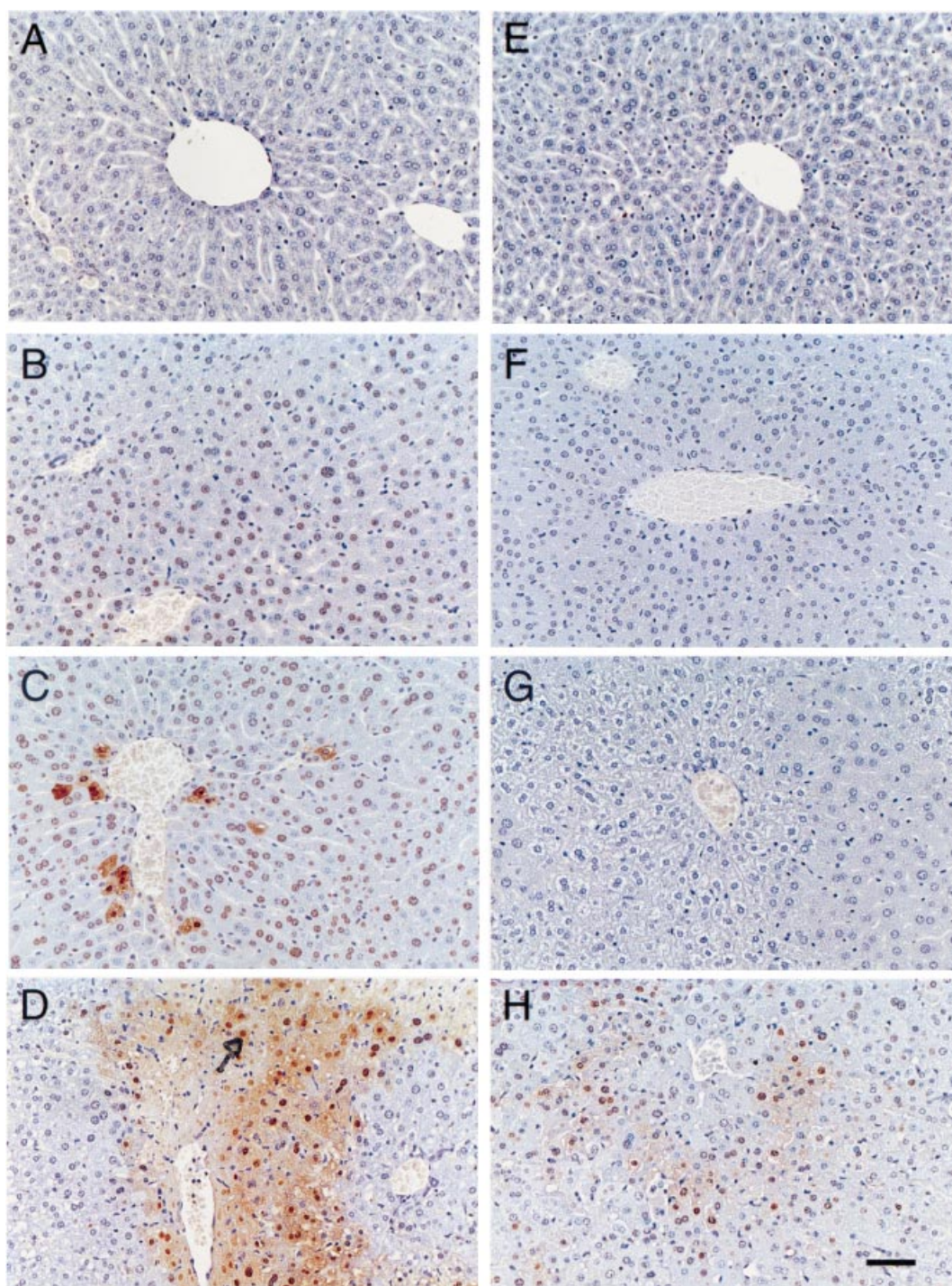
Figure 1   A representative slide ($n = 3$) to show the effects of low levels of hepatic-GPX1-activity repletion by a single injection of Se in the Se-deficient WT mice on the development and characteristics of liver aponecrosis induced by the injection of 12.5 mg of paraquat/kg body weight

Se-deficient GPX1$^{-/-}$ and WT mice (8-week-old) were pretreated with an intraperitoneal injection of Se (50 μg/kg body weight as Na$_2$SeO$_3$) 6 h before paraquat challenge. Liver samples were collected from the GPX1$^{-/-}$ mice (**A–D**) and the WT mice (**E–H**) at 0 h (**A**, **E**), 3 h (**B**, **F**), 6 h (**C**, **G**) or 10 h (**D**, **H**) after the paraquat challenge, and they were formalin-fixed, paraffin-embedded and sectioned to 10 μm. DNA strand breaks were detected using an ApopTag plus kit (Oncor Inc.) and the morphological characteristics of aponecrosis were visualized under light microscopy ($\times$10 magnification). The brown staining shows a positive response, and the arrows in (**D**) show a tendency of nuclear consolidation. Scale bar $= 123$ μm.

[19,20], other consistent data and limited mouse liver samples, we did not conduct the reciprocal co-immunoprecipitation experiment. Liver GPX1 activity was measured by the coupled assay of NADPH oxidation using H$_2$O$_2$ as a substrate, as described previously [5]. One enzyme unit was defined as the amount needed to oxidize 1 μmol of GSH/min.

Kinase assay

The anti-phospho-JNK immunoprecipitates were washed once in buffer B with 1.5 M NaCl, four times with buffer B and once with kinase buffer [20 mM Hepes (pH 7.2)/20 mM MgCl$_2$/10 mM β-glycerophosphate/10 mM p-nitrophenyl-phosphate/0.1 mM Na$_3$VO$_4$/2 mM dithiothreitol] [26] before the addition of GST-p53 (Santa Cruz Biotechnology). The immunoprecipitates were resuspended in 30 μl of kinase buffer with 50 μM ATP and 20 μCi of [γ-^{32}P]ATP and incubated for 30 min at 30 °C. After the reaction was stopped by adding 3 × SDS buffer, the mixture was resolved by SDS/PAGE (10 % gel), followed by autoradiography. After satisfactory exposures, the membranes were used for Western-blot analyses to detect p53 phosphorylation as described above.

RESULTS

Repletion of hepatic GPX1 activity as low as 4 % of the normal level in the Se-deficient mice protects against paraquat-induced liver aponecrosis

As shown in Table 1, the Se-deficient WT mice had higher ($P < 0.05$) liver GPX1 activity when compared with that of the GPX1$^{-/-}$ mice before the Se injection (16.5 versus 3.3 m-units/mg of protein). The injection of Se at 50 μg/kg increased this activity to 31.4–41.5 m-units/mg of protein in the WT mice and 4.7–6.5 m-units/mg of protein in the GPX1$^{-/-}$ mice during the time course. As we used hydrogen peroxide to measure GPX1 activity, the residual activity and the increase in the GPX1$^{-/-}$ mice by Se injection were mainly attributed to other seleno-peroxidases, such as GPX4, which was not different between the two groups (results not shown). Compared with the Se-adequate WT mice [5], hepatic GPX1 activity in the Se-deficient WT mice injected with Se represented only 4 % of the normal activity (1065.5 m-units/mg of protein, $n = 6$). Changes in liver GPX1 protein levels after the two injections were consistent with the liver GPX1 activity described above (results not shown).

Figure 1 demonstrates that more severe liver aponecrosis was induced by paraquat in the GPX1$^{-/-}$ mice when compared with that in the WT mice. Apoptotic features of this mixed type of cell death were seen in the TUNEL-stained positive hepatocytes (brown colour) that appeared in the GPX1$^{-/-}$ mice at 3 h, but not

until 10 h in the WT mice. The intensity increased with time in the former and the relative average scores ($n = 3$) at 0, 1, 3, 6 and 10 h after paraquat injection were 0, 0, 1, 2 and 4 respectively. The scores were 0, 0, 0, 0 and 2 respectively for the WT mice (Table 1). Signs of necrosis in both groups of mice included compressed sinusoids due to cellular swelling and centrilobular macrovesicular fatty change. However, the affected hepatocytes in either group of mice did not develop simple necrosis, since at no time was there an inflammatory response or phagocytosis. Overall, the first events in the WT cells appeared to involve leaking of plasma membrane without enzymic degradation of DNA, a process that was more reminiscent of necrosis. In contrast, the affected GPX1$^{-/-}$ cells seemed to encounter initially enzymic degradation of DNA, leading to nuclear consolidation (a sign of apoptosis shown by arrows in Figure 1D). Thus even very low levels of hepatic GPX1 activity in the Se-deficient WT mice were capable of not only attenuating the induced aponecrosis, but also diverting this mixed type of cell death into necrosis by suppressing the apoptotic process.

The impact of the low levels of GPX1 activity on liver aponecrosis is associated with phosphorylations of JNK and p53 on Ser-15

The paraquat injection increased liver p53 protein at 1 h in both the GPX1$^{-/-}$ and the WT mice. The induction was sustained in the former that had 1.7- to 11-fold higher ($P < 0.05$) p53 protein when compared with that in the latter over the time course (Figure 2). There was no baseline difference in total p53 protein between the two groups, although the Se injection caused an increase in the GPX1$^{-/-}$ mice, but not in the WT mice. Phosphorylation of p53 on Ser-15 was induced by paraquat at 1 h and the level was 7- and 2-fold greater ($P < 0.05$) in the GPX1$^{-/-}$ when compared with that in the WT mice at 1 and 3 h respectively. At 6 h, phospho-p53 was detected only in the GPX1$^{-/-}$ mice. Similar levels of vinculin, a cytoskeleton protein, across all lanes indicated an equal protein loading and transfer in the Western-blot analysis.

There was no difference in total JNK2 or p38 MAPK (Figure 3) between the GPX1$^{-/-}$ and the WT mice at any time point. However, phospho-JNK was detected at 1 and 3 h after paraquat injection, and the levels were 93 and 60 % higher ($P < 0.05$) in
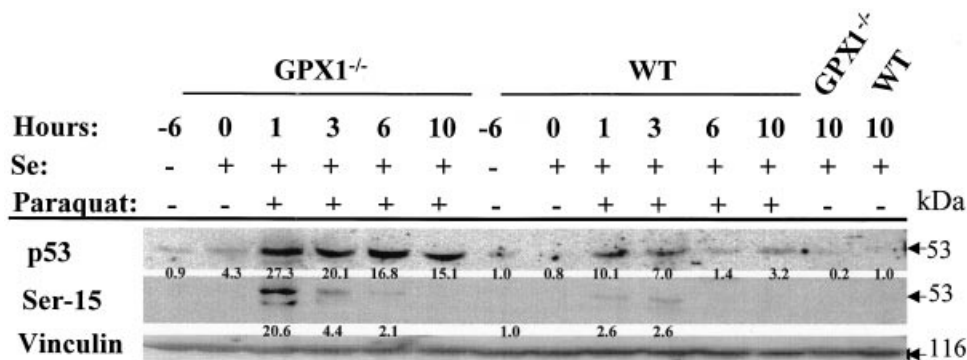

Figure 2   A representative blot ($n = 3$) to show effects of low levels of hepatic-GPX1-activity repletion by a single injection of Se in the Se-deficient WT mice on responses of liver p53 and phospho-p53 (Ser-15) protein levels to an intraperitoneal injection of 12.5 mg of paraquat/kg body weight

Se-deficient GPX1$^{-/-}$ and WT mice (8-week-old) were pretreated with an intraperitoneal injection of Se (50 μg/kg body weight as Na$_2$SeO$_3$) 6 h before the paraquat challenge, and they were killed 0, 1, 3, 6 or 10 h after the paraquat injection. Mice injected with only saline (-6 h) or only Se (the last two lanes) were used as controls to monitor the effects of injection or Se. Liver supernatants were loaded at 20 μg of protein/lane and detected using the respective mouse monoclonal antibodies against individual proteins.
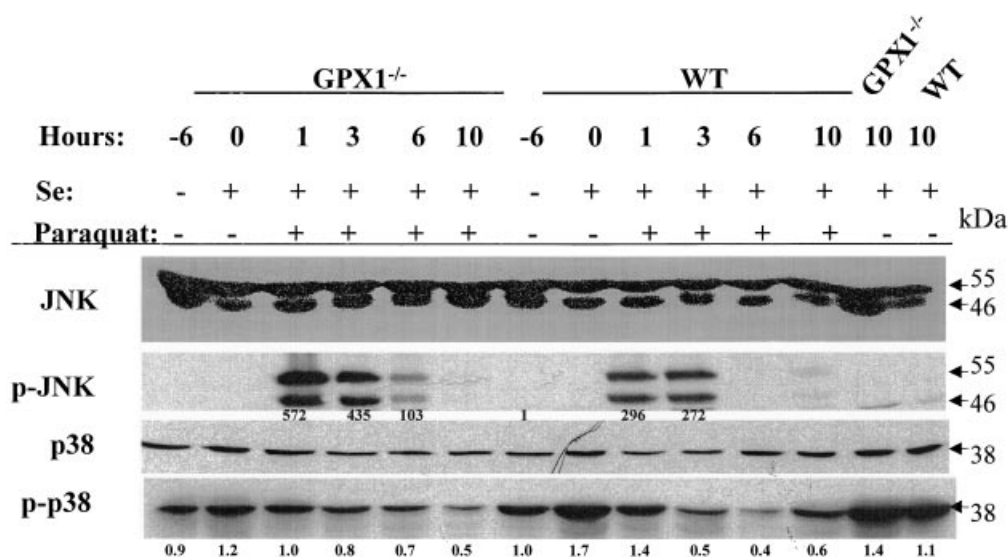
Figure 3   A representative blot (*n* = 3) to show effects of low levels of hepatic-GPX1-activity repletion by a single injection of Se in the Se-deficient WT mice on responses of liver p38, JNK2, phospho-p38 and phospho-JNK to an intraperitoneal injection of 12.5 mg of paraquat/kg body weight

Experimental procedure and labelling were the same as described in the legend of Figure 2. The individual proteins were detected using a mouse monoclonal antibody (JNK) or rabbit polyclonal antibodies (p-JNK, p38 and p-p38).
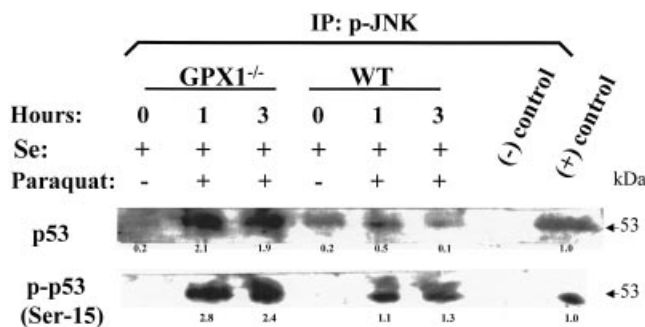

Figure 4   A representative blot (*n* = 3) to show effects of low levels of hepatic-GPX1-activity repletion by a single injection of Se in the Se-deficient WT mice on co-immunoprecipitation of phospho-JNK with p53 and phospho-p53 (Ser-15) in liver of mice intraperitoneally injected with paraquat at a dose of 12.5 mg/kg body weight

The precleared liver supernatants (1.5 mg) were prepared from the Se-injected (-6 h, 50 μg/kg body weight as Na$_2$SeO$_3$) GPX1$^{-/-}$ and WT mice killed 0, 1 or 3 h after the paraquat injection, and were immunoprecipitated (IP) with a rabbit anti-phospho-JNK polyclonal antibody, washed and subjected to Western-blot analysis using mouse monoclonal antibodies against p53 (PharMingen) or phospho-p53 (Ser-15) (Cell Signaling). The negative control contained lysis buffer only. Liver supernatant (20 μg) was loaded as positive control.

the GPX1$^{-/-}$ when compared with the WT mice respectively. Similar to the case of Ser-15 of p53, this protein was still detectable at 6 h in the GPX1$^{-/-}$ mice, but not in the WT mice. Responses of phospho-p38 to the injections of Se and paraquat were different from those of phospho-JNK, but there was no difference between the GPX1$^{-/-}$ and the WT mice.

Phospho-JNK co-immunoprecipitates p53 and phosphorylates it on Ser-15

Owing to the parallel changes in phospho-JNK and phospho-p53 (Ser-15) in response to the paraquat-induced liver apo-
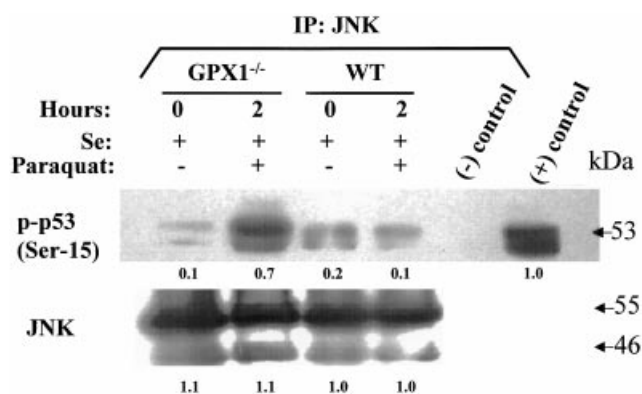

Figure 5   A representative blot (*n* = 3) to show effects of low levels of hepatic-GPX1-activity repletion by a single injection of Se in the Se-deficient WT mice on co-immunoprecipitation of JNK with phospho-p53 (Ser-15) in liver of mice intraperitoneally injected with paraquat at a dose of 12.5 mg/kg body weight

The precleared liver supernatants (1.5 mg) were prepared from the Se-injected (-6 h, 50 μg/kg body weight as Na$_2$SeO$_3$) GPX1$^{-/-}$ and WT mice killed at 0 or 2 h after the paraquat injection. They were immunoprecipitated (IP) with a rabbit polyclonal anti-JNK antibody, washed and subjected to Western-blot analysis using mouse monoclonal antibodies against phospho-p53 (Ser-15) (Cell Signaling). The negative control contained lysis buffer only, and 20 μg of liver supernatant from the paraquat-injected GPX1$^{-/-}$ was loaded as positive control. Total JNK protein in the samples used for the immunoprecipitation was detected by Western-blot analysis using the mouse monoclonal antibody against JNK2 (Santa Cruz Biotechnology).

necrosis, we examined whether these two proteins were physically associated and functionally linked. Both p53 and phospho-p53 (Ser-15) were co-immunoprecipitated with phospho-JNK from liver homogenates by the rabbit polyclonal antibody against phospho-JNK, and the precipitated protein levels were 1–18-fold greater ($P < 0.05$) in the GPX1$^{-/-}$ when compared with that in the
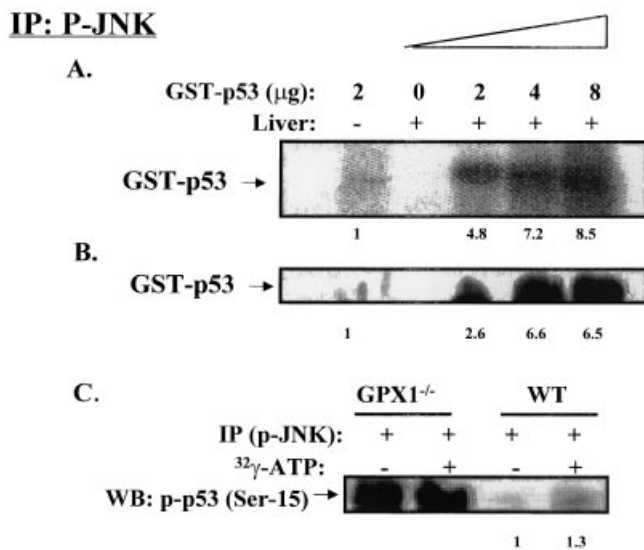
IP: P-JNK

A.

GST-p53 (μg):   2   0   2   4   8
Liver: -   +   +   +   +

GST-p53 →

1   4.8 7.2 8.5

B.

GST-p53 →

1   2.6 6.6 6.5

C.

| | GPX1⁻/⁻ | | WT | |
IP (p-JNK):   +   +   +   +
$^{32}\gamma$-ATP: -   +   -   +
WB: p-p53 (Ser-15) →

1   1.3

Figure 6   Phosphorylation of p53 on Ser-15 by phospho-JNKs

(**A**) Washed anti-phospho-JNK immunocomplex isolated from the GPX1⁻/⁻ mice at 3 h was incubated in kinase buffer before the addition of GST-p53 (Santa Cruz Biotechnology), separated and transferred to a PVDF membrane, followed by autoradiography. (**B**) After satisfactory exposures, the membrane was used for Western-blot analyses to detect p53 phosphorylation (Ser-15) as described in the legend of Figure 3. (**C**) The immunoprecipitated (IP) phospho-JNK was incubated in the kinase buffer without (−) or with (+) [γ-^{32}P]ATP, followed by Western-blot analysis to detect phospho-p53 (Ser-15) using the mouse monoclonal antibody (Cell Signaling).

WT mice (Figure 4). To confirm this association, an anti-JNK rabbit polyclonal antibody was incubated with liver homogenates of the Se-deficient GPX1⁻/⁻ and WT mice treated with Se and paraquat as described above, but from an independent animal experiment. Consistently, phospho-p53 (Ser-15) was detected in the anti-JNK immunoprecipitates from both groups of mice. The amount was 6-fold greater ($P < 0.05$) in the GPX1⁻/⁻ mice when compared with that in the WT mice at 2 h after paraquat injection (Figure 5). Meanwhile, all samples used for the immunoprecipitation had similar levels of total JNK protein by Western-blot analysis.

Because only p53, but not phospho-p53 (Ser-15), was co-precipitated with phospho-JNK at 0 h after paraquat injection, we conducted kinase assays to determine if the isolated phospho-JNK was capable of phosphorylating p53 on Ser-15. Apparently, the isolated kinase (from the GPX1⁻/⁻ mouse liver) was capable of phosphorylating the extrinsic sources of GST-p53 (Figure 6A). Subsequently, phospho-GST-p53 (Ser-15) was detected by a rabbit polyclonal antibody raised against phospho-p53 (Ser-15) (Figure 6B). This phosphorylation seemed to reach saturation at the substrate level of 8 μg. Meanwhile, the isolated phospho-JNK could further phosphorylate the intrinsic p53 (on Ser-15) precipitated from the WT mouse liver (low background), but not that of the GPX1⁻/⁻ mouse, probably due to a saturated baseline (Figure 6C). Clearly, the anti-phospho-JNK immunoprecipitates from mouse liver were capable of phosphorylating both sources of p53 on Ser-15.

DISCUSSION

Our most significant finding from the present study is the correlation among the distinct impacts of low levels of hepatic GPX1 activity (4% of normal level) on the paraquat-induced liver aponecrosis, JNK activation and p53 phosphorylation on Ser-15 in the Se-deficient mice. The co-immunoprecipitation of phospho-p53 (Ser-15) and (or) with phospho-JNK and JNK, along with their parallel temporal responses to the paraquat injection and the kinase assay results, reflects a strong functional link among these molecules in vivo. Meanwhile, their differential changes between the GPX1⁻/⁻ and WT mice after paraquat injection corresponded very well to the onset, development and severity of the induced liver aponecrosis. Overall, these results suggest that phospho-JNK may phosphorylate p53 on Ser-15 in the paraquat-mediated oxidative stress, and low levels of hepatic GPX1 activity in the Se-deficient mice attenuate this cascade event to protect against the induced liver aponecrosis. Although stress-induced phosphorylation of p53 by JNK at other sites has been shown previously [20], to the best of our knowledge, our results provide the first evidence for the capacity of phospho-JNK to phosphorylate p53 on Ser-15 under physiological conditions. These findings may help in explaining why taxol-induced p53 accumulation in MCF7 cells can be prevented by expressing a JNK2 with mutated Thr-183 and Tyr-185 residues [27] and why fibroblasts from jnk1 and jnk2 double-knockout mice or neurons from jnk3 knockout mice are resistant to UV-induced cell death [21,22]. As phosphorylation of p53 on Ser-15 represents one of the most important modifications of p53 in mediating cell-cycle arrest or cell death [9,10], our results unveil a novel mechanism for the protection by GPX1 against oxidative cell death under the current condition. In comparison with the documented inhibitory or potentiating effects of selenite and Se-containing compounds on JNK activation in various types of cells [28–31], the present study has illustrated the role of the most abundant selenoenzyme, GPX1, on the oxidative-stress-mediated JNK activation and the subsequent signalling in the Se-deficient mice. As an in vivo antioxidant enzyme [6,25], GPX1 may attenuate initially JNK activation and then p53 phosphorylation on Ser-15 by suppressing the paraquat-mediated ROS generation. Similarly, physiological GPX activity from GST α is capable of decomposing H_2O_2 and inhibiting the induction of JNK and apoptosis [32].

Subsequently, our results raise a relevant question: whether the observed impact of GPX1 activity, at its low expression level in the Se-deficient mice, on the low-dose paraquat-induced JNK activation holds true at its full expression in the Se-adequate mice. In fact, we determined responses of liver phospho-JNK between 0 and 60 min after the injection of paraquat (12.5 mg/kg) in the Se-adequate GPX1⁻/⁻ and WT mice, and we found that this signal protein appeared in the former, but not in the latter, 30 min after the injection (results not shown). Although full GPX1 activity expression in these Se-adequate mice also inhibited this low-dose paraquat-mediated JNK activation, downstream impacts on p53 phosphorylation, similar to that in the Se-deficient animals, might not exist in this case. Presumably, GPX1 might inhibit oxidative cell death via other routes such as the caspase 8 pathway [33] in Se-adequate organisms. Practically, GPX1 was not required to defend against the moderate oxidative stress induced by the low dose of paraquat (12.5 mg/kg) used in the present study. Because this insult did not cause liver aponecrosis or any injuries in the Se-adequate mice [6], regardless of their GPX1 status, other forms of Se-dependent proteins or factors were clearly responsible and sufficient for the body's defence against the stress. Selenoproteins, other than GPX1, have been shown to inhibit cell death induced by the JNK activator UV [34] or to affect the mitochondria-mediated anti-apoptotic pathways [35]. However, GPX1 is the mediator of body Se in the Se-adequate animals to protect against severe oxidative stress induced by high doses of paraquat [6]. It remains

to be tested whether the protection of the full expression of GPX1 in these Se-adequate animals also relates to the p53 pathway.

Another novel finding from the present study is the link of p53 phosphorylation on Ser-15 with the development of liver aponecrosis, in comparison with its suggested involvement in typical necrosis and apoptosis [36,37]. As mentioned above, the inhibition of liver aponecrosis by hepatic GPX1 paralleled its effects on the appearance of phospho-JNK and phospho-p53 (Ser-15). Probably, the phosphorylation of p53 on Ser-15 helped in stabilizing p53 in the GPX1$^{-/-}$ mice (see Figure 2 for the general trend), because this modification prevents the MDM2-dependent, ubiquitin-mediated p53 degradation [13], which corresponds well to the temporal changes between these two p53 forms. The sustained p53 accumulation in the late time course that appeared only in the GPX1$^{-/-}$ mice may be partially related to the p53 phosphorylation on Ser-6 [38], as we detected this p53 phosphorylation only in the GPX1$^{-/-}$ mice, but not in WT mice, 6 h after paraquat injection (results not shown). An increase in p53 may accelerate intracellular ATP depletion by stimulating ATPase [39] and transactivate the proteins that lead to mitochondria-dependent apoptosis and caspase activation [40,41]. In a parallel study, we have seen that the low levels of hepatic GPX1 activity inhibited or delayed the paraquat-induced appearance of activated caspase 3 and Bcl-Xs [6a]. Because the fate of a cell between apoptosis and necrosis may be influenced by intracellular ATP depletion and caspase activation [42], the dual roles of p53 in these two events allow the development of the mixed form of cell death (aponecrosis). The presence of low hepatic GPX1 activity in the WT mice may favour necrosis by allowing depletion of intracellular ATP, whereas its absence in the GPX1$^{-/-}$ mice may favour apoptosis by potentiating the activation of caspase 3. Treating cells with caspase inhibitors shifts the Bax-induced cell death from apoptosis to necrosis [43]. The inhibition of the paraquat-induced p53 and phospho-p53 (Ser-15) appearance by low GPX1 activity, contrary to the effects of high dose of selenite or its metabolites on p53 and apoptosis [44], might also be associated with the attenuated DNA damages. As rapid increase in p53 and its phosphorylation is stimulated by various forms of DNA damage [9,10], lower levels of the DNA damage-inducible p53 phosphorylation on Ser-6 [45] or GADD45 (Figure 6A) in the WT mice when compared with that in the GPX1$^{-/-}$ mice may reflect an *in vivo* suppression of the paraquat-mediated DNA damage by GPX1.

In summary, we have shown that the moderate oxidative stress mediated by a low level of paraquat induced liver aponecrosis in the Se-deficient mice. Repletion of a low level of hepatic GPX1 activity by Se injection in these mice decreased the severity of this mixed form of cell death and altered its characteristics. This protection by GPX1 was associated with the attenuation of the paraquat-induced p53 phosphorylation on Ser-15, a reaction that might be catalysed by phospho-JNK in mouse liver. Overall, our results illustrate a novel pathway for functional interactions among GPX1, p53 and JNK in response to oxidative stress under the Se-deficient or antioxidant-compromised conditions. As certain populations may still experience Se deficiency or anti-oxidant insufficiency and Se may be used in cancer prevention [46], elucidating these functional interactions will have clinical relevance [47,48]. These findings may help in developing novel therapeutic strategies for using GPX1-like antioxidants at low doses to modulate JNK function and treat ROS-related syndromes. In addition, the distinct protection by 4 % of normal GPX1 activity in the Se-deficient mice against liver aponecrosis adds a new explanation to the physiological need for the redundancy of mammalian systems. It implies that metabolic

essentiality of any given component of such systems should be tested under not only normal but also compromised conditions.

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