Opposite Roles of Selenium-dependent Glutathione Peroxidase-1 in Superoxide Generator Diquat- and Peroxynitrite-induced Apoptosis and Signaling*

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Oxidative injuries including apoptosis can be induced by reactive oxygen species (ROS) and reactive nitrogen species (RNS) in aerobic metabolism. We determined impacts of a selenium-dependent glutathione peroxidase-1 (GPX1) on apoptosis induced by diquat (DQ), a ROS (superoxide) generator, and peroxynitrite (PN), a potent RNS. Hepatocytes were isolated from GPX1 knockout (GPX1−/−) or wild-type (WT) mice, and treated with 0.5 mM DQ or 0.1–0.8 mM PN for up to 12 h. Loss of cell viability, high levels of apoptotic cells, and severe DNA fragmentation were produced by DQ in only GPX1−/− cells and by PN in only WT cells. These two groups of cells shared similar cytochrome c release, caspase-3 activation, and p21WAF1/CIP1 cleavage. Higher levels of protein nitration were induced by PN in WT than GPX1−/− cells. Much less and/or slower cellular GSH depletion was caused by DQ or PN in GPX1−/− than in WT cells, and corresponding GSSG accumulation occurred only in the latter. In conclusion, it is most striking that, although GPX1 protects against apoptosis induced by superoxide-generator DQ, the enzyme actually promotes apoptosis induced by PN in murine hepatocytes. Indeed, GSH is a physiological substrate for GPX1 in coping with ROS in these cells.

Reactive oxygen species (ROS)† and reactive nitrogen species (RNS) are constantly generated in aerobic metabolism and involved in pathogenesis of many diseases (1, 2). Pro-oxidants such as diquat (DQ) also induce cellular production of ROS such as superoxide anion (O2•−), hydrogen peroxide (H2O2), and hydroxyl radical (OH•) (3). Peroxynitrite (PN), a potent RNS, may be formed by O2•− and nitric oxide (NO) at a diffusion-limited rate (4). As PN nitrates a variety of biomolecules (5), formation of nitrotyrosine in proteins is often used to assess its nitration (6). As PN nitrates a variety of biomolecules (5), formation of nitrotyrosine in proteins is often used to assess its nitration (6). In addition, nitration of protein tyrosine residues may impair the tyrosine phosphorylation-related signaling and function (21), their finding has physiological relevance. However, the metabolic role of GPX1 in intact cells in coping with PN might be different from that in cell lysates, because of a strong reactivity between PN and CO2 to form more active intermediates such as NO2− or CO3− (22), a possible inactivation of GPX1 by PN in oxidative state (23), and modulations of cellular ROS on cytoxicity (24).

Because apoptosis is induced by moderate levels of ROS in many types of cells (25), and by PN in HL-60 (26), PC12 (27), and human endothelial cells (28), it can be used to assess oxidative injury. Two key events in the induced apoptosis include cytochrome c release from mitochondria and activation of caspase-3 (29). During the early stage of apoptosis, the activated caspase-3 cleaves p21WAF1/CIP1, a cyclin-dependent kinase inhibitor that protects cells from apoptosis (30), at a specific aspartate residue (Asp-112) and causes the loss of its localization and function in nuclei (31). c-Jun NH2-terminal protein kinase (JNK) and p38 kinase, two mitogen-activated protein kinases (MAPK), are also activated in apoptosis induced by diverse stimuli (32). It is unknown how GPX1 affects the DQ- and PN-induced apoptosis and related signaling.

Intracellular GSH may play three roles in metabolism: as an independent antioxidant, as a presumed physiological substrate of GPX1 to be oxidized to GSSG and regenerated by NADPH-dependent glutathione reductase (EC 1.6.4.2) reaction (33, 34), and as a regulator of apoptosis (35). It is fascinating to find out how GPX1 knockout affects the responses of cellular GSH/GSSG to ROS and RNS. Therefore, our objective was to dissect the metabolic role of GPX1 in cell death, apoptotic signaling, protein nitration, and GSH/GSSG responses induced by the ROS generator DQ and RNS donor PN in primary hepatocytes isolated from the GPX1−/− and the WT mice. Most strikingly, we found that GPX1 knockout did not attenuate, but enhanced hepatocyte resistance to the PN-mediated
apoptosis, which was completely opposite to its impact on the DQ-mediated apoptosis or our expectation.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Antibodies**—All chemicals were purchased from Sigma unless indicated otherwise. We obtained antibodies against phospho-p38 MAPK (Thr-180/Tyr-182), p38 MAPK, and phosphorylated stress-activated protein kinase/JNK (Thr-183/Tyr-185) from New England Biolabs (Beverly, MA); against JNK2 (D-2) and p21 (C-19) from Santa Cruz Biotechnology (Santa Cruz, CA); against cytochrome c from PharMingen (San Diego, CA); against caspase-3 from Transduction Laboratories (Lexington, KY); and against nitrotyrosine from Upstate Biotechnology (Lake Placid, NY).

**Culture of Primary Hepatocytes and ROS/RNS Generation**—Hepatocytes were prepared from 8-week old GPX1−/− and WT mice (16) by collagenase D perfusion (36), and plated in 8- or 12-well collagen-coated plates (at the density of 6 or 3 × 10^4). In all experiments, viability of the isolated cells, as determined by trypan blue exclusion, was >85%. Cells were grown at 37°C in 5% CO₂ in William’s medium E supplemented with 5% fetal bovine serum, 100 µg of gentamicin/ml, 5 µg of insulin/ml, 1 µg of glucagon/ml, 0.5 µg of hydrocortisone/ml, and 10 mM HEPES, pH 7.0. After 20 h of culture, cells were incubated with superoxide generator DQ (diquat dibromide monohydrate, Chem Service, West Chester, PA; 0.5 mM dissolved in saline) or PN (0.1–0.8 mM in 4.7% NaOH, Calbiochem, La Jolla, CA) for different lengths of time. Both DQ and PN were added as a bolus into the media and mixed thoroughly for 30 s.

**Cell Viability, DNA Fragmentation, and Apoptosis**—Cell viability was assessed by the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to formazan. Actual values were read at 570 nm in a Microplate Reader (Elix15, Bio-Tek, Winooski, VT) and expressed as percentage of the untreated controls. DNA fragmentation was detected by ethidium bromide staining after the cellular DNA was extracted with phenol/chloroform and separated in 1.8% agarose gel. Apoptosis was quantified by terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) assay kit (Roche Molecular Biochemicals) according to the manufacturer’s instruction. Positive stained nuclei were counted in ~80 cells from each of four random fields using a fluorescence microscope (Olympus, Seattle, WA).

**Western Blot Analyses of Whole Cell, Cytosolic, and Nucleic Extracts**—Cells were washed twice with saline and harvested in lysis buffer (50 mM phosphate buffer, pH 7.8, 0.1% Triton X-100, 1.34 mM diethylenetriaminepentaacetic acid, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml each of leupeptin, aprotinin, and pepstatin A). After the lysis was sonicated and centrifuged at 14,000 × g for 15 min at 4°C, supernatant was used for nitrotyrosine and GPX activity analyses. For the detection of p38 MAPK and JNK activations, whole cell lysates were prepared in modified radiomune precipitation buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 10% glycerol, 1.5 mM MgCl₂, 1 mM EGTA, 1 mM sodium vanadate, 10 mM sodium pyrophosphate, 10 mM NaF, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 10 µg of leupeptin/ml, and 10 µg of aprotinin/ml). For the detection of cytochrome c, caspase-3, and p21WAF1/CIP1, cytosolic and nucelic extracts were prepared as described by Bassy-Wetzel et al. (37). Protein concentrations were measured by Bradford protein assay (Bio-Rad). Western blot analyses were conducted as described previously (17), and immunoreactive proteins were visualized with SuperSignal West Pico chemiluminescent substrate system (Pierce).

**Glutathione and GPX Activity**—Total (GSH + GSSG) and oxidized (GSSG) glutathione were measured as described by Anderson (38) and expressed as nanomoles/mg of protein. Total GPX activity was measured by the coupled assay of reduced NADPH oxidation using H₂O₂ as substrate (39). The enzyme unit was defined as 1 nmol of GSH oxidized/min.

**Statistics**—Data were analyzed using the GLM procedure of SAS (release 6.11, SAS Institute, Cary, NC). The Bonferroni t test was used for mean comparisons.

**RESULTS**

**GPX1 Knockout Renders Mouse Hepatocytes Susceptible to DQ-induced, but Resistant to PN-induced, Apoptotic Death**—Compared with the untreated controls, viability of GPX1−/− hepatocytes was decreased by 0.5 mM DQ from 82% at 3 h to 4.9% at 12 h, whereas that of WT remained >84% throughout (Fig. 1A). In contrast, over 85% of GPX1−/− cells were viable after being treated with 0.2–0.8 mM PN for 12 h, whereas viability of WT cells was reduced to 30 and 10% by 0.4 and 0.8 mM PN, respectively (Fig. 1B). Similarly, 0.4 mM PN caused only <16% reduction in viability of GPX1−/− cells at various time points, but it decreased viability of WT cells to 50% at 3 h and further to 12% at 12 h (Fig. 1C). The PN vehicle alone, 4.7% NaOH, did not affect viability of either type of cells (data not shown). DNA fragmentation was produced by 0.5 mM DQ in only GPX1−/− cells and by 0.4 mM PN in only WT cells at 9 h, and the DNA ladder became pronounced at 12 h in these two groups (Fig. 2A). TUNEL assay showed 53.8 and 43.6% apoptotic cells at 9 h in the DQ-treated GPX1−/− and the DQ-treated WT hepatocytes, respectively (Fig. 2B). However, there was no detectable DNA fragmentation and only <3.5% apoptotic cells in the untreated, the DQ-treated WT, or the PN-treated GPX1−/− hepatocytes.

**Similar Apoptotic Signaling Occurs in the DQ-treated GPX1−/− and the PN-treated WT Cells**—Cytochrome c release (Fig. 3A) and cleavage of caspase-3 (Fig. 3B) was initially detected in the DQ-treated GPX1−/− at 6 h and in the PN-treated WT hepatocytes at 3 h. At the following time points, cytochrome c was accumulated in the cytosolic fraction and...
caspase-3 activation was also observed in the whole cell extracts of the DQ-treated WT and the PN-treated WT cells. After cells were treated with DQ or PN for 0, 3, 6, 9, or 12 h, nuclei fraction was prepared to detect p21<sub>WAF1/CIP1</sub> protein as described in Fig. 4A. In contrast, it was decreased to approximately the base line at 6 h and remained at a low level at 9 and 12 h in the DQ-treated GPX1<sup>−/−</sup> and the PN-treated WT cells. The up-regulation of p21<sub>WAF1/CIP1</sub> protein expression was also observed in the whole cell extracts of the DQ-treated WT and the PN-treated GPX1<sup>−/−</sup> hepatocytes (Fig. 4B). However, there was no such initial increase in p21<sub>WAF1/CIP1</sub> in the DQ-treated GPX1<sup>−/−</sup> or the PN-treated WT hepatocytes. Instead, the protein showed significant decreases in these two groups at 6 and 3 h, respectively, and remained low thereafter. Although total p38 MAPK or JNK protein was unaffected by GPX1 knockout, DQ, or PN, both kinases were activated at 30 min in both types of cell by DQ and PN (Fig. 5). However, PN seemed to be a stronger stimulus than DQ and produced a greater level of p38 MAPK phosphorylation in WT than in GPX1<sup>−/−</sup> cells.

Intracellular GSH and GSSG in GPX1<sup>−/−</sup> Hepatocytes Respond to DQ and PN Differently from That in WT Cells—A much more abrupt time-dependent decline in intracellular GSH was produced by 0.5 mM DQ in WT than in GPX1<sup>−/−</sup> hepatocytes (Fig. 6A). Compared with the untreated controls, the decrease was 31 and 88% in WT cells, but only 7.5 and 46% in GPX1<sup>−/−</sup> cells at 30 min and 3 h, respectively. Thus, intracellular GSH was higher (<i>p</i> &lt; 0.05) in GPX1<sup>−/−</sup> than in WT cells at these time points. Although DQ produced no change in intracellular GSSG in GPX1<sup>−/−</sup> hepatocytes at all, it resulted in a 15.5-fold increase over the untreated controls at 30 min in WT cells (Fig. 6B). That increase peaked at 1 h (18.2-fold), and declined to 9.3-fold at 6 h. In the PN-treated WT hepatocytes, intracellular GSH was decreased by 40.2% at only 5 min of the treatment (Fig. 6C). The decrease progressed linearly to 66.7% at 30 min and reached 82.3% at 6 h with a slight rise at 1 h. In contrast, the only significant decrease of GSH (46.2%, <i>p</i> &lt; 0.05) caused by PN in GPX1<sup>−/−</sup> cells was seen at 3 h, along with a nearly complete restoration to the untreated cell level at 6 h. Likewise, PN did not affect intracellular GSSG in GPX1<sup>−/−</sup> cells, but caused a 5.4-fold increase over the untreated controls at 5 min in WT hepatocytes (Fig. 6D). That increase was progressively attenuated to 4.8-, 4.4-, and 1.9-fold at 10, 20, and 30 min, respectively, with a total disappearance at 1 h. In both DQ- and PN-treated GPX1<sup>−/−</sup> cells, ratios of intracellular GSH/GSSG were much higher than those in WT cells throughout or at most of the time points.

***PN Induces More Protein Nitration in the WT than in GPX1<sup>−/−</sup> Hepatocytes—Despite a PN-dose dependent protein nitration

WT and the PN-treated GPX1<sup>−/−</sup> cells continued to rise or was maintained at a fairly constant high level at 9 and 12 h (Fig. 4A). In contrast, it was decreased to approximately the base line at 6 h and remained at a low level at 9 and 12 h in the DQ-treated GPX1<sup>−/−</sup> and the PN-treated WT cells. After an initial increase at 3 h over the base line, p21<sub>WAF1/CIP1</sub> protein in the nucleic fraction of the DQ-treated

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...cleavage of caspase-3 progressed further in these cells. There was no cytochrome c release or caspase-3 cleavage in the DQ-treated WT or the PN-treated GPX1<sup>−/−</sup> hepatocytes at any time point. After an initial increase at 3 h over the base line, p21<sub>WAF1/CIP1</sub> protein in the nucleic fraction of the DQ-treated...
nitrotyrosine formation in both types of cells at 12 h, the total band intensity was 64 and 76% greater in WT than GPX1−/− cells treated with 0.2 and 0.4 mM PN, respectively (Fig. 7). Treating WT hepatocytes with 0.4 mM PN for 12 h decreased total GPX activity by 34% compared with the untreated controls (207 versus 316 units/mg of protein, *p* < 0.05). However, DQ alone did not induce protein nitration in either type of cells or significant reduction of GPX activity in WT cells (data not shown).

**DISCUSSION**

It is remarkable that GPX1 knockout exerted completely opposite impacts on susceptibility of mouse hepatocyte to DQ and PN-induced apoptotic death. Because high levels of H2O2 could be produced by DQ (3), the substantial loss of cellular defense against DQ-induced apoptosis in GPX1−/− over WT cells is consistent with the whole body responses of the GPX1−/− mice challenged with ROS generators (18, 40, 41). However, the positive impact of GPX1 knockout on hepatocyte resistance to PN cytotoxicity is rather striking and does not agree with the notion that selenoproteins such as GPX1 (20) and selenoprotein P (42) may protect against PN-induced oxidative stress in vivo. Although PN is highly reactive with a short half-life, our results are reproducible and physiologically relevant. This is because we demonstrated a PN-dose dependent response of cell viability and nitrotyrosine formation, a reliable indicator of PN activity in the cell (6). Our selected PN dose (0.4 mM), similar to that used by others (20, 43), was the minimal level that distinguished GPX1−/− from WT cells. Comparable results were obtained by using different sources or manipulations of PN treatment (data not shown). The enhanced nitrotyrosine formation in WT cells over that in GPX1−/− cells treated with 0.2 or 0.4 mM PN reflects a promoting role of GPX1, similar to that of other peroxidases (44), in the PN-mediated protein nitration.

A fundamental question is how GPX1 affects the PN-mediated apoptosis and protein nitration. In cell lysate, extrinsic GPX1 was able to reduce PN to nitrite using GSH in a two-electron catalysis (20). However, we did not see a difference in PN reduction or medium nitrite level between GPX1−/− and WT cells treated with 0–0.8 mM PN for 12 h (data not shown). Because of the strong reactivity between PN and CO2 (22), GPX1 in the cultured cells, unlike in the cell lysates (20), might be encountered with not only authentic PN, but also more reactive PN intermediates such as NO2 or CO3. As PN inactivated a good portion of GPX1 (23) in WT cells, the projected enzymatic reduction of PN by GPX1 (20) might not be as...
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Relative intensity: 1.0 1.9 2.2 2.9 1.2 2.4 3.6 5.1

**FIG. 7.** Protein nitration in hepatocytes induced by 0 to 0.4 mM PN. Cells were treated with 0.1, 0.2, or 0.4 mM PN for 12 h. Whole cell lysate (10 μg) was separated by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with anti-nitrotyrosine antibody. The relative intensity of the total bands was measured using the NIH Image Program (version 1.61). The blot is a representative of three independent experiments.

effective in competing against thiols or CO₂ as for direct reactions with PN (42) in these cells. However, this PN-mediated inactivation of GPX1 could not explain the enhanced sensitivity with PN (42) in these cells. More likely, GPX1 exerted its role by affecting H₂O₂ removal and thus cellular balances of ROS and RNS that could modulate PN toxicity (24, 45, 46).

The DQ-treated GPX1/— and the PN-treated WT cells clearly underwent apoptosis and exhibited similar apoptotic signaling, because cytochrome c release and pro-caspase-3 cleavages preceded the appearance of apoptotic cells and severe DNA fragmentation in these cells. As a critical step in stress-induced apoptosis, cytochrome c release from mitochondria enables it to bind to Apaf-1 and caspase-9, leading to the activation of caspase-9 that in turn activates caspase-3 (29). Caspase-3 is an executioner of apoptosis with many target proteins, including p21<sup>WAF1/CIP1</sup> (31) that protects against apoptosis (30). Cleavage of p21<sup>WAF1/CIP1</sup> mediated by caspase-3 and the consequent activation of cyclin A/Cdk2 have been shown as prerequisite for the execution of apoptosis in human hepatoma cells SK-HEP-1 induced by ginsenoside Rg2 (47). In the present study, the initial up-regulation of p21<sup>WAF1/CIP1</sup> protein expression at 3 h over the base line was maintained later only in the DQ-treated WT cells and the PN-treated GPX1/— cells that showed no induced apoptosis. In contrast, the DQ-treated GPX1/— and the PN-treated WT cells exhibited significant decreases of p21<sup>WAF1/CIP1</sup> at 6 and 9 h over the levels at 0 or 3 h. Both p38 MAPK and JNK, two kinases involved in stress-induced apoptosis (32, 48), were activated by DQ or PN at 30 min, but their responses were not consistent with the changes of the three assayed apoptotic signal molecules. Seemingly, GPX1 exerted its role in the PN- or DQ-induced apoptotic events downstream or independent of activation of these two kinases. Inhibition of the PN-induced activity of p38 MAPK and JNK by selenite in the cultured rat liver epithelial cells has been suggested to be through selenium-containing proteins, including GPX (49). In our study, activation of p38 MAPK was slightly stronger by DQ and much so by PN in WT than GPX1/— cells. Thus, GPX1 promoted its activation mediated by ROS or RNS in mouse hepatocytes, indicating a possible cell-specific or GPX1-independent effect of selenite on these kinases.

Distinct differences in the DQ-induced cellular GSH/GSSG changes between the GPX1/— and WT cells in the present study support the idea that GSH is a physiological substrate of GPX1 in metabolism (33, 34). In the presence of GPX1, WT cells displayed a sharp decrease in GSH, along with an abrupt rise of GSSG, within 60 min after the DQ treatment. Although this GSH depletion attenuated after 60 min, probably because of the decrease in ROS production and/or an accelerated regeneration of GSH from GSSG by glutathione reductase, GSH was indeed oxidized to GSSG by GPX1 to reduce the DQ-generated H₂O₂ and other hydroperoxides at a very high rate initially. In contrast, GPX1/— cells responded to DQ or PN with much less and slower depletion of GSH than WT cells, without any GSSG accumulation at all. Clearly, lack of GPX1 spared the oxidation of GSH to GSSG and left it for direct and/or GPX1-independent protections (33, 34, 50). In the PN-treated WT cells, GSH seemed to act as a substrate of GPX1 initially and then became more like a GPX1-independent protector because a sharp rise in GSSG along with the GSH depletion was not seen after 60 min. In comparison with these distinct roles of GSH in functioning as a GPX1 substrate and a major antioxidant, the suggested necessity of certain amount of cellular GSH for cells to undergo apoptosis instead of necrosis (35) was not fully shown in our study. Although apoptotic events occurred in the DQ-treated GPX1/— cells in which cellular GSH was indeed greater than in WT cells, these events were also exhibited in the PN-treated WT cells in which cellular GSH was depleted to a very low level initially. Thus, cellular GSH alteration alone may not be sufficient to regulate apoptosis.

Elucidating the opposite role of GPX1 in DQ- and PN-induced oxidative injury has broad implications. It teaches us that antioxidant protection for a given enzyme or protein such as GPX1 may not be a general property, but depends on the specific nature of oxidants. Although pro-oxidant properties of high levels of vitamin E or C (51, 52) and overexpression of Cu,Zn-superoxide dismutase (53) have been reported previously, our study provides the first evidence to show the “double-edged sword” function of an “antioxidant” enzyme at its physiological expression level in metabolically normal primary cells. The potent role of GPX1 in turning off the DQ-induced and in switching on the PN-induced apoptosis will help us in elucidating mechanisms of ROS/RNS in regulating cell death and related signaling (54), and in developing novel therapeutic strategies for the ROS and RNS involved diseases (55). Our findings also caution the public that blind antioxidant supplementation in clinic or nutrition may not always be desirable. In line of our view, knockout of GPX1 enhanced mouse brain resistance to the kainic acid-induced epileptic seizure (56), whereas overexpressing GPX1 promoted acetaminophen toxicity to mice (57) and tumorigenesis (58). Likewise, vitamin C was able to induce decomposition of lipid hydroperoxides to endogenous genotoxins (59).

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