

Hypoxic Preconditioning Reverses Protection After Neonatal Hypoxia-Ischemia in Glutathione Peroxidase Transgenic Murine Brain

R. ANN SHELDON, ALEXANDRA AMINOFF, CHRISTINA L. LEE, STEPHAN CHRISTEN, AND DONNA M. FERRIERO

Departments of Neurology [R.A.S., A.A., C.L.L., D.M.F.] and Pediatrics [D.M.F.], University of California San Francisco, San Francisco, California 94143; Institute for Infectious Diseases [S.C.], CH-3010, Berne, Switzerland

ABSTRACT: The effect of hypoxic preconditioning (PC) on hypoxic-ischemic (HI) injury was explored in glutathione peroxidase (GPx)-overexpressing mice (human GPx-transgenic [hGPx-tg]) mice. Six-day-old hGPx-tg mice and wild-type (Wt) littermates were preconditioned with hypoxia for 30 min and subjected to the Vannucci procedure of HI 24 h after the PC stimulus. Histopathological injury was determined 5 d later (P12). Additional animals were killed 2 h or 24 h after HI and ipsilateral cerebral cortices assayed for GPx activity, glutathione (GSH), and hydrogen peroxide (H_2O_2). In line with previous studies, hypoxic PC reduced injury in the Wt brain. Preconditioned Wt brain had increased GPx activity, but reduced GSH, relative to naive 24 h after HI. Hypoxic PC did not reduce injury to hGPx-tg brain and even reversed the protection previously reported in the hGPx-tg. GPx activity and GSH in hGPx-tg cortices did not change. Without PC, hGPx-tg cortex had less H_2O_2 accumulation than Wt at both 2 h and 24 h. With PC, H_2O_2 remained low in hGPx-tg compared with Wt at 2 h, but at 24 h, there was no longer a difference between hGPx-tg and Wt cortices. Accumulation of H_2O_2 may be a mediator of injury, but may also induce protective mechanisms. (*Pediatr Res* 61: 666–670, 2007)

The developing brain is particularly susceptible to oxidative stress, more so than the mature brain (1). One reason for this susceptibility may be the different developmental profiles of antioxidant enzymes in the newborn brain compared with the mature brain. For example, total GPx activity increases sharply between E18 and P1, declines in the early postnatal period, then stabilizes through P21 (2). GSH levels also increase between E18 and P1, but remain lower than P21 (2). One consequence of this difference is that the developing brain accumulates H_2O_2 after HI, whereas the mature brain does not (3). H_2O_2 accumulation has also been associated with increased injury in superoxide dismutase-overexpressing neonatal murine brain (4), and greater cell death is seen when immature neurons are exposed to H_2O_2 compared with mature neurons (5). Increased H_2O_2 accumulation may be the result of relative insufficiency of the endogenous enzyme GPx. Under physiologic circumstances, the brain has efficient anti-

oxidant defense mechanisms, including GPx, which converts potentially harmful H_2O_2 to oxygen and water at the expense of reduced GSH. Under oxidative stress, in the immature brain, endogenous levels of GPx may be inadequate for converting excess H_2O_2 . Transgenic mice that overexpress GPx (hGPx-tg), when subjected to HI have less histologic brain injury than their Wt littermates (6). In addition, the cortex exhibits increased GPx enzyme activity at 24 h, whereas GPx activity remains unaltered in the Wt brain. In addition, neurons cultured from GPx-tg brain are resistant to injury from exogenously applied H_2O_2 (7). Neurons cultured from hippocampus and cortex that are transfected with genes for catalase and GPx also show protection from neurotoxic insults and a corresponding decrease in H_2O_2 accumulation (8). These findings indicate that adequate GPx activity can ameliorate injury to the immature brain from oxidative stress due to H_2O_2 .

It is well established that a previous stress to the brain can induce tolerance to subsequent injury, a phenomenon called PC. In neonatal rodents, protection against HI brain injury has been induced by preconditioning with a period of hypoxia before the induction of HI (9–11). The mechanisms of this protection have yet to be fully determined, but it has been established that a large number of genes are induced in response to hypoxia (12). Many of these genes are regulated by the transcription factor hypoxia-inducible factor 1 α (HIF-1 α), perhaps most importantly vascular endothelial growth factor (VEGF) and erythropoietin (EPO). VEGF is up-regulated after focal ischemic injury in the neonatal rat, in parallel with induction of HIF-1 α (13).

Although the production of reactive oxygen species (ROS) can activate cell death pathways leading to brain injury, ROS also appear to have a role in inducing PC protection (14,15). Our aim in the current study was to determine whether HI brain injury could be reduced by hypoxic PC and, if so, whether changes in oxidant/antioxidant status correlate with the injury. It may be possible that PC exists in the human

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Correspondence: R. Ann Sheldon, Neonatal Brain Disorders Center, University of California San Francisco, 521 Parnassus Avenue, P.O. Box 0663, San Francisco, CA 94143; e-mail: ann.sheldon@ucsf.edu

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Abbreviations: FLIP, [Fas-associated death domain like–interleukin-1 β converting enzyme]-inhibitory protein; GPx, glutathione peroxidase; GSH, reduced glutathione; hGPx-tg, human glutathione peroxidase transgenic; HI, hypoxia-ischemia; HIF-1 α , hypoxia-inducible factor-1 α ; ROS, reactive oxygen species

neonate because many fetuses have repeated challenges of oxidative stress before delivery. Therefore, a better understanding of PC mechanisms can lead to new therapies and outcomes for these affected newborns.

MATERIALS AND METHODS

Animals. All animal protocols were approved by the Institutional Animal Care and Use Committee at the University of California San Francisco and carried out with the highest standards of care and housing, according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Heterozygous transgenic mice carrying the human glutathione peroxidase (GPx1) gene (16) were bred with Wt (CD1) mice to produce mixed litters of GPx1-overexpressing transgenic pups and Wt littermates. Genotype was identified by polymerase chain reaction, as described previously (6).

PC. Hypoxic PC was induced by placing pups in interconnected chambers, two per chamber, through which 8% oxygen/balance nitrogen flowed for 30 min. One pup was monitored to ensure that rectal temperature did not exceed 37°C. Nonpreconditioned pups were in a chamber exposed to room air. Thirty minutes of hypoxia was chosen to correspond to the duration of hypoxia pups were exposed to for the full HI insult. A period of 60 min of hypoxia was also tested and found to be similar to 30 min (data not shown).

HI. Twenty-four hours after PC, at P7, pups underwent the Vannucci procedure for inducing HI, (17), or sham surgery, as previously described. A separate group of mice underwent HI 4 h after hypoxic PC to determine the best time interval between PC stimulus and injury. No protective effect was seen with this paradigm (data not shown).

Histologic analysis. Five days after HI, pups were anesthetized with pentobarbital (100 mg/kg) and brains fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2, via intracardiac perfusion. Fifty-micrometer sections were cut on a vibrating microtome, and alternate sections stained with cresyl violet or Perl's iron stain. Each brain was scored in a blinded fashion, as previously described (18). A total of 54 mice underwent HI, with or without PC, 48 of these survived to perfusion and brains were scored for degree of injury (*n* survived/*n* died: Wt HI 12/1, GPx HI 9/0, Wt PC HI 15/3, GPx PC HI 12/2). In addition, injury scores were analyzed for possible gender effects for each group (*n* female/*n* male: Wt HI 8/4, GPx HI 2/7, Wt PC HI 12/3, GPx PC HI 6/6).

GPx activity. At either 2 h or 24 h after removal from the hypoxia chambers, room air-exposed chambers or P7 and P8 for naive mice, tissue was obtained by rapid decapitation and removal of the brain onto a cold surface, and right and left cortices were dissected free and flash frozen. Selenium-dependent GPx activity was measured spectrophotometrically in a coupled test system in which reduced glutathione and tert-butyl hydroperoxide were used as substrates, and oxidized glutathione produced by GPx activity was measured by kinetically monitoring glutathione reductase-mediated reduced nicotinamide adenine dinucleotide phosphate oxidation at 340 nm as previously described (7). Protein content was determined by the Pierce BCA spectrophotometric protein assay (Pierce, Rockford, IL). A total of 88 brains were dissected for GPx assay: Wt naive (*n* = 22), hGPx-tg naive (*n* = 9), Wt PC (*n* = 5), hGPx-tg PC (*n* = 12), Wt PC HI 2 h (*n* = 7), hGPx-tg PC HI 2 h (*n* = 8), Wt PC HI 24 h (*n* = 14), hGPx-tg PC HI 24 h (*n* = 11).

Glutathione levels. At either 2 h or 24 h after HI, pups were anesthetized with pentobarbital (100 mg/kg) and perfused with ice-cold deoxygenated 0.1 M sodium phosphate-buffered saline (pH 7.2). Brains were removed, cortices dissected free on a cold pack, weighed, and nine volumes of ice-cold perchloric acid (0.6 M) with L-methionine (0.5 mM) were added to tissue and rapidly frozen on dry ice. Total glutathione (which is >99.5% GSH) was measured by a modified Tietze assay using the Bioxytech GSH/GSSG-412 kit from Oxis Health Products (Portland, OR). A total of 95 brains were dissected for GSH assay: Wt naive (*n* = 11), hGPx-tg naive (*n* = 6), Wt hypoxia (sham) 2 h (*n* = 6), hGPx-tg hypoxia (sham) 2 h (*n* = 4), Wt HI 2 h (*n* = 3), hGPx-tg HI 2 h (*n* = 6), Wt PC HI 2 h (*n* = 7), hGPx-tg PC HI 2 h (*n* = 5), Wt hypoxia (sham) 24 h (*n* = 7), hGPx-tg hypoxia (sham) 24 h (*n* = 4), Wt HI 24 h (*n* = 9), hGPx-tg HI 24 h (*n* = 8), Wt PC HI 24 h (*n* = 13), hGPx-tg PC HI 24 h (*n* = 6).

Inhibition of catalase activity by aminotriazole. H₂O₂ was measured indirectly via the inhibition of catalase by aminotriazole as previously described (3,4). Aminotriazole selectively and irreversibly inhibits catalase that is bound to H₂O₂ (compound 1); thus, the extent of catalase inhibition by aminotriazole is directly proportional to the H₂O₂ concentration at the time of aminotriazole exposure (19). Two hours before killing, mice were injected intraperitoneally with aminotriazole (200 mg/kg in normal saline) or an equivalent volume of vehicle. This time point was chosen based on previous

experiments in which a time curve of inhibition of catalase after injection of aminotriazole demonstrated 50% inhibition at 2 h (3,4). Catalase activity was then measured as described previously with slight modification (4). All values were normalized to an internal control that consisted of a pool of homogenized cortices. Data are expressed as the percentage of inhibition of catalase activity. A total of 200 brains were dissected for catalase assay (*n* aminotriazole treated/*n* of saline treated): Wt naive (9/9), hGPx-tg naive (7/9), Wt hypoxia 2 h (11/13), hGPx-tg hypoxia 2 h (10/11), Wt HI 2 h (9/7), hGPx-tg HI 2 h (8/7), Wt PC HI 2 h (3/4), hGPx-tg PC HI 2 h (4/4), Wt hypoxia 24 h (13/6), hGPx-tg hypoxia 24 h (6/6), Wt HI 24 h (7/2), hGPx-tg HI 24 h (5/4), Wt PC HI 24 h (7/6) hGPx-tg PC HI 24 h (6/7).

Tissue levels of aminotriazole in Wt (*n* = 6) and hGPx-tg (*n* = 7) were assayed by the colorimetric method of Green and Feinstein (20) using a protocol previously described in detail.

Statistical analysis. Ordinal data (histologic injury scores) were analyzed by a Mann-Whitney test. Comparisons between groups were made by one-way analysis of variance (ANOVA) followed by Bonferroni *post hoc* testing for multiple comparisons for continuous data for differences in GPx activity and GSH levels. Results are presented as the percentage of naive value. Catalase activity (units/mg) after inhibition with aminotriazole was analyzed with two-way ANOVA followed by Bonferroni *post hoc* testing. Aminotriazole concentrations were analyzed by a *t* test and expressed as mean $\mu\text{g}/\text{mg}$ protein \pm SEM. Significance was established at *p* < 0.05. All statistical analyses were performed with Prism Version 4 software (Graphpad Software, San Diego, CA).

RESULTS

Histopathological injury. Hypoxic PC 24 h before HI protects the Wt neonatal brain compared with Wt brain without PC (Fig. 1, *p* < 0.02). Confirming results previously described (6), GPx overexpression protects the neonatal brain from HI injury compared with Wt littermates (Fig. 1, *p* = 0.004). However, PC in hGPx-tg mice before HI *reverses* the protection seen without PC; hGPx-tg mice with PC have higher injury scores than hGPx-tg mice without PC (*p* < 0.03). Median injury scores were Wt = 19.5, hGPx-tg = 9, Wt with PC = 13, hGPx-tg with PC = 20.5). There was no difference between male and female in any of the groups (Wt, *p* > 0.69; hGPx-tg, *p* > 0.69; Wt with PC, *p* > 0.84; hGPx-tg with PC, *p* > 0.48).

GPx activity. We have previously shown that GPx activity in the Wt cortex 24 h after HI does not increase compared with

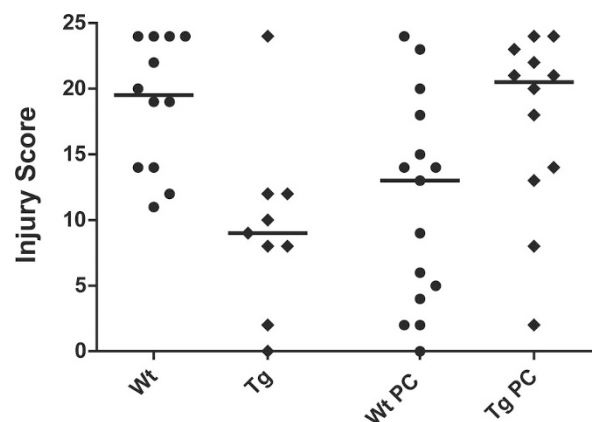


Figure 1. Histological injury scores of mouse brain 5 d after HI. All mice underwent HI and were killed 5 d later. Some mice also received hypoxic PC 24 h before initiation of HI. hGPx-tg mice have less injury than brains of Wt littermates after HI (*p* = 0.004). Wt mice that received hypoxic PC have less injury than Wt mice without PC (*p* < 0.02). However, hGPx-tg mice that underwent hypoxic PC have greater injury than brains of hGPx-tg mice without PC (*p* < 0.03). The horizontal line is the median value for each group. Analysis by Mann-Whitney test.

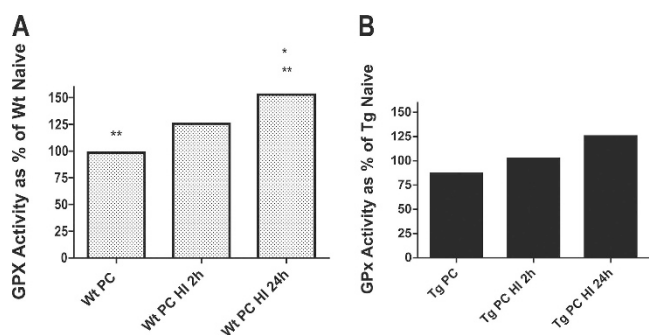


Figure 2. GPx activity. (A) Wt cortex with hypoxic PC. GPx activity is increased 24 h after HI in mice that received PC ($n = 14$), compared with naive ($n = 22$, $*p < 0.001$) and to PC alone ($n = 5$, $**p < 0.05$). (B) hGPx-tg cortex with PC. There are no differences between the treatment groups (naive, $n = 9$; PC, $n = 12$; PC HI 2 h, $n = 8$; PC HI 24 h, $n = 11$). Analysis by ANOVA. GPx activity is shown as the percentage of naive values for both A and B. PC in the left-hand column of A and B refers to mice that underwent hypoxia only 24 h before being killed.

naive (6). With PC, however, there is an increase in GPx activity in Wt cortex 24 h after HI compared with naive ($p < 0.001$) and compared with Wt with hypoxia only measured 24 h later ($p < 0.05$), but there is no difference in PC cortex 2 h after HI (Fig. 2A). In contrast, we also showed previously that GPx activity in hGPx-tg mice increases 24 h after HI compared with naive (6). Here, with PC, we show no difference between the treatment groups (Fig. 2B). Results are shown as a percentage of naive values. When GPx activity in Wt is compared with hGPx-tg for each treatment group, only the naive cortex shows a difference: hGPx-tg has higher activity than Wt, confirming overexpression (Table 1, $p < 0.05$).

GSH. In Wt cortex, GSH decreases 24 h after hypoxia alone, HI, and HI preceded by PC, compared with naive mice (Fig. 3A, $p < 0.05$, $p < 0.001$, and $p < 0.001$, respectively). There is no change in GSH 2 h after hypoxia alone, after HI, or after HI preceded by PC. In hGPx-tg mice, there is no change in GSH at 2 h or 24 h after hypoxia alone, after HI, or after HI preceded by PC, compared with naive mice (Fig. 3B). Results are shown as a percentage of naive value. When Wt is compared with hGPx-tg mice for each treatment group, Wt mice had less GSH than hGPx-tg mice 24 h after HI preceded by PC (Table 1, $p < 0.004$).

H₂O₂ accumulation. Equivalent levels of aminotriazole were achieved in Wt and hGPx-tg mouse cortex (Wt = $63.7 \pm$

Table 1. GPx activity and GSH levels (n): Wt compared with hGPx-tg

Genotype/treatment	GPx (U/mg protein)	GSH (mmol/mg protein)
Wt naive	$31.1 \pm 4.3^*$ (22)	35.0 ± 7.6 (11)
hGPx-tg naive	$38.7 \pm 11.0^*$ (9)	32.5 ± 5.0 (6)
Wt PC	30.6 ± 3.5 (5)	35.6 ± 5.0 (7)
hGPx-tg PC	33.9 ± 8.4 (12)	38.2 ± 9.1 (4)
Wt PC HI 2 h	39.1 ± 3.4 (7)	31.1 ± 2.3 (7)
hGPx-tg PC HI 2 h	38.3 ± 5.3 (8)	36.2 ± 9.1 (5)
Wt PC HI 24 h	47.6 ± 13.2 (14)	$21.3 \pm 4.7^\dagger$ (13)
hGPx-tg PC HI 24 h	47.4 ± 13.4 (11)	$29.7 \pm 4.4^\dagger$ (6)

* $\bar{x} \pm$ SD. Wt naive versus hGPx-tg naive ($p < 0.05$).

† $\bar{x} \pm$ SD. Wt PC HI 24h versus hGPx-tg PC HI 24 h ($p < 0.004$).

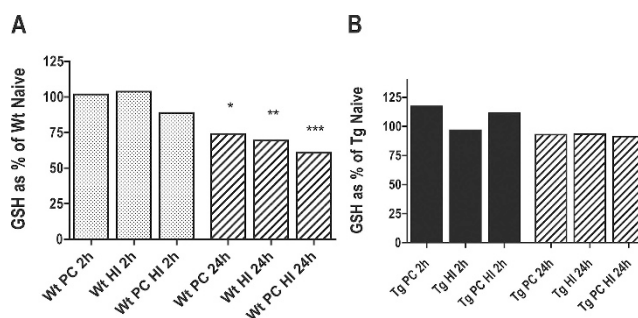


Figure 3. Glutathione levels. (A) Wt cortex, with or without PC. There is a decrease in the levels of GSH after hypoxia alone ($n = 7$, $*p < 0.05$), HI ($n = 9$, $**p < 0.001$), and in HI preceded by hypoxia (PC, $n = 13$, $***p < 0.001$) by 24 h compared with naive mice ($n = 11$). There is no change in levels of GSH at 2 h for any treatment condition ($n = 3-7$). (B) hGPx-tg cortex, with or without PC. There are no changes in levels of GSH for any treatment condition ($n = 4-8$). Analysis by ANOVA. GSH levels for (A and B) are presented as the percentage of naive littermate values.

$11.5 \mu\text{g}/\text{mg}$ protein, hGPx-tg = $57.5 \pm 11.0 \mu\text{g}/\text{mg}$ protein, $p > 0.70$), ensuring that differences in the inhibition of catalase, and hence H₂O₂ accumulation, in both groups was due to experimental procedures, as previously described (3,4).

At 2 h after HI, less H₂O₂ accumulated in the hGPx-tg cortex compared with Wt cortex (Fig. 4, $p < 0.05$), and after HI preceded by PC (Fig. 4, $p < 0.001$). At 24 h after HI, there was no longer a difference in H₂O₂ accumulation between the PC Wt and hGPx-tg cortex. However, there remained less H₂O₂ in the hGPx-tg cortex compared with Wt without PC (Fig. 4, $p < 0.01$).

DISCUSSION

It is well established that hypoxic PC protects the neonatal brain from subsequent ischemic insults (9,11,21). However, the mechanisms of this protection remain poorly understood.

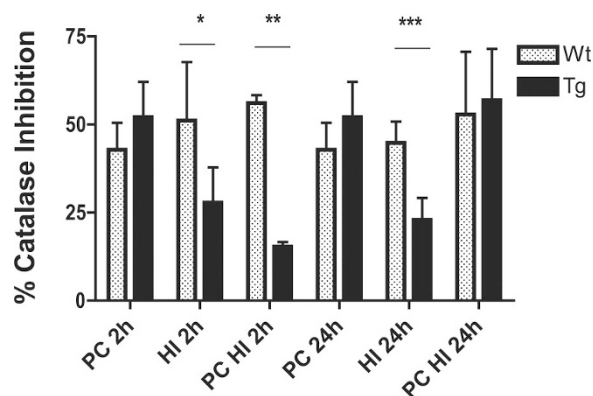


Figure 4. Accumulation of H₂O₂ in Wt and hGPx-tg cortex with and without PC. At 2 h after HI, less H₂O₂ accumulated in hGPx-tg ($n = 15$) compared with Wt cortex ($n = 16$, $*p < 0.05$) and in hGPx-tg PC cortex ($n = 8$), compared with Wt PC cortex ($n = 7$, $**p < 0.001$). By 24 h, there is no longer a difference between hGPx-tg ($n = 13$) and Wt cortex ($n = 13$) with PC, but there is a difference in hGPx-tg cortex that received HI, but not PC (both $n = 9$, $***p < 0.01$). H₂O₂ accumulation is expressed as a percentage of catalase inhibition by aminotriazole. Each bar represents a ratio of cortex from aminotriazole-treated mice to saline-treated mice, therefore expressed as the percentage of catalase inhibition.

We confirmed our previous finding that GPx overexpression reduces brain injury compared with Wt after neonatal HI (6). Here we show that PC protects the neonatal brain from HI injury, but only in Wt mice. Whereas GPx overexpression protects the brain from an HI insult, PC the hGPx-tg reverses this protection. This seemingly paradoxical situation may be explained, in part, by alterations in the brain's endogenous antioxidant defense mechanisms in response to a stressor such as hypoxia or HI, and the subsequent accumulation or elimination of ROS such as H₂O₂.

The PC Wt brain compensated by increasing GPx activity 24 h after injury, correlating with a reduction in injury. The effect of this increase in GPx activity to protect the brain is strengthened by our previous findings showing that GPx activity in the Wt cortex 24 h after HI (*without* PC and therefore severely injured) is not different from naïve cortex (6).

Adequate stores of antioxidants are necessary to protect the brain from oxidative injury. For example, depletion of neuronal GSH has been shown to exacerbate oxidative injury *in vitro* (22–24). In the current study, using an *in vivo* model of brain injury, the depletion of GSH in both the PC and non-PC Wt cortex 24 h after HI does not correlate with injury in the Wt mice because the former has reduced injury, but not the latter. Hence, to confirm, in separate experiments without PC, we repleted GSH via the administration of alpha lipoic acid and saw no reduction in injury (data not shown). Wallin *et al.* (25) found a similar reduction in total GSH levels 24 h after HI in neonatal rats, with levels still low, but beginning to increase by 72 h.

Unlike in the Wt cortex, levels of H₂O₂ in the hGPx-tg cortex are greatly reduced for several treatment conditions, both in comparison with other hGPx-tg groups and with Wt. The early relative decline (at 2 h) for both PC and non-PC cortex continues only in the non-PC cortex, not the PC cortex, later (at 24 h). This corresponds with the ultimate lack of protection in hGPx-tg from PC. It is conceivable that the relatively low, as well as constant, amount of H₂O₂ in the hGPx-tg mice *without* PC, both at 2 h and 24 h, reflects the fact that the non-PC hGPx-tg brain has less injury 5 d later. It is notable that the PC hGPx-tg cortex has the lowest amount of H₂O₂ at 2 h but the highest at 24 h. Clearly, mechanisms are active for the generation of H₂O₂ during the intervening hours, which appear to trigger increased brain injury. In support of this notion, it has been suggested that low amounts of H₂O₂ early on may stimulate survival mechanisms that protect the brain from subsequent injury. Generation of H₂O₂ during brief oxygen-glucose deprivation induces PC neuronal protection in primary cultured neurons (15). The PC Wt brain may have provided protection by preventing fluctuations in the level of H₂O₂. In fact, levels of H₂O₂ remained remarkably stable in the Wt mice after HI, being virtually the same as naive for all treatment conditions. These values may represent a baseline amount necessary for activation of mediators of PC such as HIF and its target genes. We have recently found that low-dose endogenous H₂O₂ contributes to PC protection in primary cortical neurons preconditioned with 10 min of hypoxia or exogenous H₂O₂. Both hypoxia and low-dose H₂O₂ (15 μM) PC induced neuronal protection 24 h later against a 2-h

oxygen-glucose deprivation insult. HIF-1α protein expression increased after hypoxia or low-dose H₂O₂ treatment. These preliminary results suggest that endogenous H₂O₂ might stabilize or up-regulate HIF-1α and thereby mediate PC protection (data not shown).

Susceptibility to peroxide toxicity and efficiency of peroxide removal varies depending on the type of brain cell, although all major cell types contribute to antioxidative defense (26). Astroglia may be of particular importance in protection because they are the primary reservoir of GSH and provide GSH precursors to other brain cells (27–29). Different regions of the brain also have differing degrees of hypoxia tolerance, reflected in gene activation (30). Cells in culture from different brain regions also have differing susceptibility and tolerance (8,31).

Further support for the importance of GPx comes from GPx-deficient mice, which have shown increased injury after ischemia/reperfusion in the adult brain (32). Astrocytes in culture from these mice have a greatly impaired ability to clear peroxide compared with Wt astrocytes, and GSH depletion further impaired clearance (33).

Neurodegeneration after neonatal HI is the result of activation of a Fas-mediated cell death pathway (34). ROS participate in this death pathway (35). The Fas death receptor signaling can be forced to favor cell survival if, in signaling through the death-inducing signaling complex (DISC), cleavage of caspase 8 to its active form is blocked by [Fas-associated death domain–like interleukin-1β converting enzyme]-inhibitory protein (FLIP), a dominant negative of caspase 8. H₂O₂ quickly down-regulates expression of FLIP and FLIP levels appear to be regulated by the oxidant status after HI injury. Specifically, FLIP expression is up-regulated more efficiently after HI injury in mice that are more capable of scavenging H₂O₂. Consequently, it has recently been shown in hGPx-tg mice after HI compared with Wt that the overall degree of injury seen correlates well with changes in expression of Fas death receptor signaling proteins favoring neuroprotection, *i.e.* increased FLIP expression (36). Thus, the mechanism by which antioxidant status alters FLIP levels after neonatal HI may be related to the relative abilities of the hGPx-tg mice compared with their Wt littermates to detoxify H₂O₂ produced after neonatal HI. It is not yet known how hypoxic PC affects levels of FLIP.

In summary, this study lends further support to GPx activity as a critical factor in protection. A thorough understanding of the mechanisms of hypoxia-induced ischemic tolerance may ultimately lead to the development of therapies for neonatal hypoxic-ischemic brain injury.

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