Alteration in Downstream Hypoxia Gene Signaling in Neonatal Glutathione Peroxidase Overexpressing Mouse Brain after Hypoxia-Ischemia

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Key Words

Glutathione peroxidase · Hydrogen peroxide · Hypoxia-ischemia · Neonatal brain injury · Hypoxia-inducible factor · ERK

Abstract

We have previously shown that glutathione peroxidase (GPx) overexpressing mice (hGPx-tg) have reduced brain injury after neonatal hypoxia-ischemia (HI) as a consequence of reduced hydrogen peroxide accumulation. However, this protection is reversed with hypoxia preconditioning, raising the question of the roles of the genes regulated by hypoxia-inducible factor-1 α (HIF-1 α) and their transcription products, such as erythropoietin (EPO), in both the initial protection and subsequent reversal of protection. hGPx-tg and their wild-type (WT) littermates underwent the Vannucci procedure of HI brain injury at postnatal day 9 – left carotid artery ligation followed by exposure to 10% oxygen for 50 min. Brain cortices and hippocampi were subsequently collected 0.5, 4 and 24 h later for the determination of protein expression by Western blot for GPx, HIF-1 α , HIF-2 α , EPO, EPO receptor, ERK1/2, phospho-ERK1/2, spectrin 145/150 (as a marker of calpain-specific necrotic cell death), and spectrin

120 (as a marker of apoptotic cell death mediated via caspase-3). As expected, the GPx overexpressing mouse cortex had approximately 3 times the GPx expression as WT naïve. Also, GPx expression remained higher in the GPx overexpressing brain than WT at all time points after HI (0.5, 4, 24 h). HIF-1 α was not significantly changed in hGPx-tg as a consequence of HI but decreased in the WT cortex 4 h after HI. HIF-2 α decreased in the WT hippocampus after HI. EPO was higher in the GPx overexpressing cortex and hippocampus 30 min after HI compared to WT, but the EPO receptor was unchanged by HI. ERK1/2 phosphorylation increased in the hippocampus at 4 h after HI and in the cortex at 24 h after HI in both WT and hGPx-tg. Spectrin 145/150 was increased in the WT cortex at 4 and 24 h after HI, and spectrin 120 increased 24 h after HI, perhaps reflecting greater injury in the WT brain, especially at 24 h when brain injury is more evident. The effect of GPx overexpression does not appear to upregulate the HIF pathway, yet EPO was upregulated, perhaps via ERK. This might explain, in part, why cell death takes a necrotic or apoptotic path. This may also be an explanation for why the GPx overexpressing brain cannot be preconditioned. This information may prove valuable in the development of therapies for neonatal HI brain injury.

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Introduction

The developing brain is highly susceptible to oxidative stress, more so than the adult brain [1]. As a consequence of immature antioxidative mechanisms, for example, the developing mouse brain accumulates more H_2O_2 after hypoxia-ischemia (HI) than the adult [2]. This disparity is presumably due to lower levels of glutathione peroxidase (GPx) in the immature brain [3]. Under normal physiological circumstances, H_2O_2 is produced as a result of the conversion of superoxide by superoxide dismutase. GPx then reduces H_2O_2 by converting it to oxygen and water. After HI, endogenous levels of GPx are inadequate for the excess H_2O_2 generated in the neonatal brain, but mice that overexpress GPx (hGPx-tg) have less brain injury 5 days after HI and increased GPx activity 24 h after HI than wild-type (WT) littermates [4]. There is also protection against exogenous H_2O_2 in primary neuronal cultures in hGPx-tg. Specific depletion of GSH with buthionine sulfoximine in these cultures increased cell death, abolishing the protection afforded by the increased GPx activity and therefore implicating the availability of reducing equivalents in neuroprotection [5]. Further support for the importance of GPx in ameliorating injury comes from work with GPx-deficient mice that have shown increased injury after ischemia/reperfusion in the adult brain [6]. We have also seen increased injury in these GPx-deficient mice when subjected to HI at postnatal day 7 (P7) [unpubl. data].

This protective effect of increased GPx against oxidative stress is lost, however, when the hGPx-tg mice are exposed to hypoxia preconditioning prior to HI [7]. Hypoxia preconditioning has been shown to reduce subsequent injury [8] and, indeed, the WT littermates in this study had reduced injury. Accordingly, hypoxia-inducible factor-1 α (HIF-1 α) and the products of its target genes were subsequently shown to be key to hypoxia preconditioning protection [9–11]. Perhaps the activation of HIF-1 α and its target genes is necessary for protection in neonatal HI both in the setting of HI and preconditioning. In fact, we have shown that the elimination of HIF-1 results in loss of protection and loss of preconditioning [12, 13].

One of the HIF gene products, erythropoietin (EPO), has frequently been suggested as a protective agent when administered after HI [14–16]. A possible mechanism for the protective effect of EPO treatment is through increased GPx activity. In support of this idea, Kumral et al. [17] found increased GPx activity after EPO treatment of

neonatal rats subjected to HI, suggesting that the neuroprotective effects of EPO are due, at least in part, to modulation of GPx. It is not known whether endogenous EPO has a similar effect on GPx levels [18, 19].

Previously, we showed HIF-1 α to be elevated equally in both the WT and hGPx-tg cortex immediately after hypoxia alone. However, ERK1/2 phosphorylation was elevated only in the WT (at 30 min after hypoxia) [20]. After HI in neonatal rats, both HIF and p-ERK are upregulated at 4–8 h after the injury and the induction of HIF is blocked by a specific inhibitor of ERK1/2 [21].

The present study explores the role of hypoxia gene signaling in the setting of GPx overexpression in the neonatal brain through characterization of the expression of GPx, HIF-1 α , HIF-2 α (which is thought to be astrocyte specific [22]), the transcription factor EPO, the EPO receptor (EPOR), ERK1/2, phospho-ERK1/2, and the cell death markers spectrin 145/150 (for evidence of calpain-specific necrosis) and spectrin 120 (for evidence of caspase-3 based apoptosis).

Materials and Methods

Mice

hGPx1-tg mice were bred and maintained at the UCSF laboratory animal research center. Experiments were approved by the Institutional Animal Care and Use Committee at UCSF, in accordance with NIH guidelines for the Care and Use of Laboratory Animals. Male mice heterozygous for hGPx1 were bred with female WT CD1 mice and genotype of the resulting litters was determined by standard methods, as previously described [4, 23]. The mice then underwent the Vannucci procedure of HI at P9 [24–26]. At 30 min, 4 h or 24 h after hypoxia, the mice were killed by rapid decapitation, brains removed, and ipsi- and contralateral hippocampi and cortices dissected and flash frozen for Western blot. In addition, naïve GPx-tg and WT mice were deeply anesthetized with Euthasol and perfused intracardially with 4% paraformaldehyde for immunohistochemistry.

Hypoxia-Ischemia

Under isoflurane anesthesia, the left common carotid artery of each mouse was isolated and permanently ligated. When sufficiently recovered from the anesthesia to walk, the pups were returned to the dam for 1 h. They were then placed in chambers maintained at 36.5°C and exposed to 10% oxygen for 50 min.

Western Blots for Protein Expression

Nuclear and cytoplasmic fractions were prepared from the cortices (n = 8 for all groups) and hippocampi (n = 10; however, 2 were combined into 1 sample at homogenization due to the small size of tissue, thus presenting as n = 5 for all groups) using the nuclear and cytoplasmic extraction reagents (NE-PER; Pierce Biotechnol-

ogy, Rockford, Ill., USA), according to the manufacturers protocol. Protein concentrations were measured by BCA assay (Pierce). 20 µg of nuclear or 40 µg of cytoplasmic protein were loaded onto 4–12% polyacrylamide gels (Invitrogen, Carlsbad, Calif., USA) for electrophoresis. Proteins were transferred to PVDF membranes (Bio-Rad, Hercules, Calif., USA), and membranes were blocked in 5% nonfat dry milk for 1 h at room temperature and incubated in the following antibodies overnight at 4°C: GPx (1:1,000; Epitomics, Burlingame, Calif., USA), HIF-1α (1:2,000; Novus Biologicals, Littleton, Co., USA), HIF-2α (1:200; Novus), EPO (1:500; Abcam, Cambridge, Mass., USA), EPOr (1:1,000; Abcam), ERK1/2 (1:4,000; EMD Millipore, Billerica, Mass., USA), phospho-ERK1/2 (1:1,000; Cell Signaling, Danvers Mass., USA), and spectrin (1:4,000; EMD Millipore). β-Actin (1:2,000; Santa Cruz Biotechnology, Santa Cruz, Calif., USA) was used as a loading control.

Immunohistochemistry for GPx

Naïve paraformaldehyde-fixed brains of hGPx-tg and WT P9 mice were cut on a vibratome 50 µm thick. Sections were incubated in rabbit anti-GPx primary antibody (1:200; Abcam) overnight at 4°C and goat anti-rabbit IgG secondary antibody (1:200; Santa Cruz Biotechnology) for 2 h at room temperature, and diaminobenzidine was used as the chromophore. Mounted sections were counterstained with cresyl violet.

Statistical Analysis

Optical densities (OD) normalized to β-actin were analyzed with the unpaired t test. Data are expressed as mean ± SEM normalized to WT naïve. Statistical analyses were performed with GraphPad Prism 6.0 (San Diego, Calif., USA). $p < 0.05$ was considered statistically significant.

Results

GPx Overexpression Is Demonstrated by Western Blot and Is Visible by Immunohistochemistry

The naïve GPx-tg mouse cortex had several-fold higher GPx protein expression compared to WT naïve ($p < 0.001$; fig. 1a), and this difference between GPx-tg and WT was maintained at all time points after HI (30 min, $p < 0.002$; 4 h, $p < 0.0001$; 24 h, $p < 0.0001$). Results were similar for the hippocampus. Naïve GPx-tg hippocampus compared to WT naïve ($p < 0.05$; fig. 1b), GPx-tg and WT after HI (30 min and 4 h, both $p < 0.05$; 24 h, $p < 0.03$).

In addition, immunohistochemistry demonstrated abundant GPx-positive cells in the naïve hGPx-tg cortex and hippocampus (fig. 1c, d) compared to the WT cortex and hippocampus (fig. 1e, f).

HIF-1α Protein Expression

HIF-1α expression was not different between GPx-tg and WT, whether naïve or after HI in either the cortex or hippocampus (fig. 2a, b). However, there was a de-

crease in HIF-1α protein expression in the WT cortex 4 h after HI compared to the WT naïve cortex ($p < 0.04$; fig. 2a).

HIF-2α Protein Expression

HIF-2α was also not different between GPx-tg and WT, whether naïve or after HI in the cortex or hippocampus (fig. 2c, d). However, there was a decrease in HIF-2α in the hippocampus after HI compared to WT naïve at 30 min ($p < 0.006$), 4 h ($p < 0.04$) and 24 h ($p < 0.03$; fig. 2d).

EPO and EPOr Protein Expression

EPO expression was higher in the GPx-tg cortex 30 min after HI compared to the WT cortex ($p < 0.008$; fig. 3a) as well as the hippocampus ($p < 0.05$; fig. 3b).

EPOr, however, did not demonstrate any differences between hGPx-tg and WT in the cortex or hippocampus for the naïve or any treatment group (fig. 3c, d).

ERK1/2 Expression and ERK1/2 Phosphorylation

ERK1/2 phosphorylation was observed in both the WT and hGPx-tg cortex 24 h after HI ($p < 0.03$ and $p < 0.05$, respectively; fig. 4a). In the hippocampus, ERK1/2 phosphorylation occurred earlier, at 4 h, in both WT and hGPx-tg ($p = 0.05$ and $p < 0.03$, respectively; fig. 4b).

Spectrin Expression

Spectrin 145/150 protein expression was not different between the GPx-tg and WT cortex for any treatment group. However, spectrin 145/150 was increased in the WT cortex at 4 and 24 h after HI compared to WT naïve (both $p < 0.05$; fig. 5a). Spectrin 145/150 protein expression was not different between the GPx-tg and WT hippocampus for any treatment group (fig. 5b). However, spectrin 120 was increased in the WT cortex compared to hGPx-tg (24 h after HI; $p = 0.05$) and WT naïve ($p = 0.05$; fig. 5c). Also, spectrin 120 was increased in the WT hippocampus compared to hGPx-tg (4 h after HI; $p = 0.03$) and WT naïve ($p = 0.05$; fig. 5d).

Discussion

In this study, the overexpression of GPx did not alter the expression of HIF-1α after neonatal HI. In the WT cortex, HIF-1α declined at 4 h relative to naïve. Despite the lack of HIF accumulation, EPO was upregulated in the cortex and hippocampus of the GPx overexpressing brain relative to WT early after HI (30 min). This may be explained, at least in part, by the increased ERK

Fig. 1. GPx protein expression is several-fold higher in the hGPx-tg mouse cortex and hippocampus compared to the WT cortex and hippocampus. **a** hGPx cortex compared to WT: naïve (** $p < 0.001$), 30 min after HI (** $p < 0.002$), 4 h after HI (***) $p = 0.0001$), and 24 h after HI (***) $p = 0.0001$). **b** hGPx-tg hippocampus compared to WT: naïve (* $p = 0.05$), 30 min after HI (* $p = 0.05$), 4 h

after HI (* $p < 0.05$), and 24 h after HI (* $p < 0.03$). Mean OD ($\pm$ SEM) normalized to P9 naïve. **c–f** Representative photomicrograph of immunostaining for GPx counterstained with cresyl violet in naïve GPx-tg and WT brain: naïve GPx-tg cortex (**c**) and hippocampus (**d**); naïve WT cortex (**e**) and hippocampus (**f**). Scale bar (shown in **c**) = 100 μ m for all.

Fig. 2. HIF-1 α (**a**, **b**) and HIF-2 α (**c**, **d**) protein expression in the cortex and hippocampus. **a** HIF-1 α was decreased in the WT cortex 4 h after HI compared to WT naïve (* $p < 0.04$). **b** HIF-1 α expression was not changed in the hippocampus. **c** HIF-2 α expres-

sion was not changed in the cortex. **d** HIF-2 α decreased in the WT hippocampus after HI compared to WT naïve at 30 min (** $p < 0.006$), 4 h (* $p < 0.04$) and 24 h (* $p < 0.03$).

activation in both the WT and hGPx-tg hippocampus at 4 h and in the cortex at 24 h. The ERK1/2 pathway has been shown to be part of potentially protective mechanisms in neonatal HI [27], and ERK1/2 phosphorylation has been shown to peak prior to the peak expression of HIF-1 α in the developing rat brain after HI [21], which may indicate an alternate pathway for EPO expression.

While activation and stabilization of HIF-1 α is a well-established consequence of hypoxia, reports vary for HI. We recently showed increased HIF-1 α at 15 min and 4 and 24 h in mice after hypoxia but an increase only at 15 min (not at 4 or 24 h) after HI [13]. However, Chavez-Valdez et al. [28] have shown increased HIF-1 α in mice 24 h after HI but not after 3 h. In rats, HIF-1 α has been

shown to be upregulated, beginning at 4 h and peaking at 8 h after HI [21, 29].

A possible explanation for the paradoxical EPO up-regulation in the hGPx-tg brain relative to WT is differing degrees of injury, differing cell death mechanisms (depending on time after HI) and regional variation between the cortex and hippocampus. Indeed, in the cortex, spectrin 145/150 was increased in WT at both 4 and 24 h after HI relative to WT naïve, suggesting early and prolonged necrotic cell death in the WT cortex. Spectrin 120, on the other hand, increased only at 24 h in the WT cortex compared to both WT naïve and hGPx-tg after HI, indicating apoptotic mechanisms at this stage. In the hippocampus, there were no differences in spectrin 145/150, indicating a low amount of necrosis in this region. Spectrin 120,

Fig. 3. EPO (a, b) and EPOr (c, d) expression in the cortex and hippocampus. **a** EPO expression was higher in the hGPx-tg cortex 30 min after HI compared to the WT cortex (** $p < 0.008$). **b** EPO expression was also higher in the hGPx-tg hippocampus 30 min

after HI compared to the WT hippocampus (* $p < 0.04$). EPOr, however, did not demonstrate any differences between GPx-tg and WT in the cortex (c) or hippocampus (d) for the naïve or any treatment group.

Fig. 4. ERK1/2 phosphorylation in the cortex and hippocampus. **a** Phospho-ERK1/2 is increased in the cortex of both WT (* $p < 0.03$) and hGPx-tg (* $p < 0.05$) 24 h after HI compared to WT naïve.

ive. **b** Phospho-ERK1/2 is increased in the hippocampus of both WT (* $p = 0.05$) and hGPx-tg (* $p < 0.03$) 4 h after HI compared to WT naïve.

Fig. 5. Spectrin 145/150 and spectrin 120 indicate cell death mechanisms. **a** Spectrin 145/150 increased in the WT cortex 4 h (* $p < 0.04$) and 24 h (* $p = 0.05$) after HI compared to WT naïve. There were no changes in spectrin 145/150 in the hGPx-tg cortex. **b** Spectrin 145/150 increased in the hGPx-tg hippocampus 30 min after HI compared to WT 30 min after HI (* $p < 0.04$). **c** Spectrin 120

increased in WT cortex 24 h after HI compared to hGPx-tg 24 h after HI (* $p = 0.05$), as well as compared to WT naïve (* $p = 0.05$). **d** In the hippocampus, spectrin 120 was higher in WT 4 h after HI compared to hGPx-tg (* $p = 0.03$), as well as to WT naïve (* $p < 0.05$).

however, increased at 4 h in the hippocampus. Thus, the WT brain experiences ongoing necrotic injury by 4 h in the cortex, which is exacerbated by apoptosis by 24 h. Cell death in the WT hippocampus, however, is largely – if not entirely – apoptotic, beginning by 4 h. These results may have relevance to the observation that the hippocampus is particularly vulnerable to HI injury in the mouse, and hippocampal neurons are more susceptible to oxidative stress *in vitro* [30]. The hippocampal white matter may also be more susceptible to cell death processes than cortical white matter. HIF-2 α , thought to be astrocyte specific, declined in the WT hippocampus at all time points after HI but was not significantly altered in the WT cortex. As astrocytes are important for the clearance of H₂O₂, this may also be a reflection of increased injury in the WT,

particularly the hippocampus. The hGPx-tg brain displayed neither spectrin 145/150 nor spectrin 120 upregulation, reflecting the resistance to HI injury we have previously seen in these mice [4].

GPx is a relatively neglected enzyme in the study of oxidative stress, despite its critical role in the protection of the brain against oxidative stress and inflammation in a number of conditions such as stroke, neurodegenerative disorders, traumatic brain injury, and HI [31]. Previous work by our group and others has also suggested that HIF-1 α is involved in the mechanisms of protection from HI [12, 29, 32, 33]. Indeed, like the hGPx-tg mice, hypoxia preconditioning protection is lost in HIF-1 α knockout mice after neonatal HI [13]. When neonatal mice that have both GPx overexpression and HIF-1 α reduction –

hybrid offspring of the hGPx-tg and HIF-1 α knockout – the outcome is worse injury than WT littermates, indicating that the protective effect of increased GPx is lost in the setting of HIF-1 α knockdown, implying a role for HIF-1 α independent of the antioxidative mechanisms of GPx in the neonatal brain [unpubl. data].

It has been suggested that an antioxidant state with reduced levels of H₂O₂ limits the activation of the HIF pathway, whereas a prooxidant state allowing elevated H₂O₂ levels promotes it [34]. In fact, exogenous H₂O₂ has been shown to upregulate HIF-1 α and provide preconditioning protection to neurons in vitro [35]. Perhaps the activation of HIF-1 α and its target genes is necessary for protection in neonatal HI. In this case, the lack of any increase in HIF-1 α in the hGPx-tg brain in response to HI seen here is not surprising given the presumably low amounts of H₂O₂ present. In fact, it may be that ERK activation is more important in the setting of low H₂O₂ and is affected by GPx1 overexpression. We have previously shown that GPx1 overexpression prevents both the global and nuclear increase in activated

ERK at 0.5 h after hypoxic preconditioning (HPC) and causes a significant decrease in phospho-ERK/ERK levels at 24 h after HPC, thus explaining the lack of HPC in these GPx brains [20].

Further confirmation of the importance of GPx in protection from neonatal HI are seen in results from our laboratory with GPx knockout mice, indicating that HI injury is the same as WT in these mice after HI [unpubl. data]. We have yet to determine the status of HIF in these animals.

In summary, HIF-1 α may have a more complex role in neonatal HI than previously believed. The antioxidant status of the brain may influence some of the mechanisms regulated by HIF- α . A better understanding of these mechanisms may lead to improved therapy for human neonates with HI encephalopathy.

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