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Journal

Developmental Neuroscience, 37(4-5)

ISSN

0378-5866

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Publication Date

2015

DOI

10.1159/000375369

Peer reviewed

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

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Running Title: GPx in neonatal hypoxia-ischemia

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Abstract

We have previously shown that glutathione-peroxidase overexpressing mice have reduced brain injury after neonatal hypoxia-ischemia, 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-1α (HIF-1α) and their transcription products, such as erythropoietin (EPO), in both the initial protection and subsequent reversal of protection. Glutathione peroxidase overexpressing mice and their wild-type littermates underwent the Vannucci procedure of hypoxic-ischemic brain injury at P9: left carotid artery ligation followed by exposure to 10 % oxygen for 50 min. Brain cortices and hippocampi were subsequently collected 0.5 h, 4 h and 24 h later for determination of protein expression by Western blot for GPx, HIF-1α, HIF-2α, EPO, EPO receptor, ERK1/2 and 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, GPx overexpressing mouse cortex had approximately 3 times the GPx expression as wild-type naïve. Also, GPx expression remained higher in GPx overexpressing brain than wild-type at all time points after HI (0.5h, 4h, 24h). HIF-1α was not significantly changed in hGPx-tg as a consequence of HI, but decreased in wild-type cortex 4 h after HI. HIF-2α decreased in wild-type hippocampus after HI. EPO was higher in GPx overexpressing cortex and hippocampus 30 min after HI compared to wild-type, but 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 wild-type cortex 4 h and 24 h after HI and spectrin 120 increased 24 h after HI, perhaps

reflecting greater injury in the wild-type 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.

Keywords: Glutathione peroxidase, Hydrogen peroxide, Hypoxia-ischemia, Neonatal brain injury , hypoxia inducible factor, ERK

This work was funded by NIH grant NS33997.

Introduction

The developing brain is highly susceptible to oxidative stress, more so than the adult brain [1]. As a consequence of immature anti-oxidative mechanisms, for example, the developing mouse brain accumulates more H_2O_2 after hypoxia-ischemia (HI) than does 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 over-express GPx (hGPx-tg), have less brain injury 5 days after HI and increased GPx activity 24 h after HI than do 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 (BSO) in these cultures increased cell death, abolishing the protection afforded by the increased GPx activity, 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, which 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 P7 [unpublished observations].

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-1α

(HIF-1α) and the products of its target genes were subsequently shown to be key to hypoxia preconditioning protection [9] [10] [11]. Perhaps activation of HIF-1α and its targets genes is necessary for protection in neonatal HI both in the setting of HI and pre-conditioning. In fact, we have shown that elimination of HIF1 results in loss of protection and loss of pre-conditioning [12] [13].

One of the HIF gene products, erythropoietin (EPO) has frequently been suggested as a protective agent when administered after HI [14] [15] [16]. A possible mechanism for the protective effect of EPO treatment is through increased GPx activity. In support of this idea, Kumral et al 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 [17]. It is not known if endogenous EPO has a similar affect on GPx levels [18] [19].

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

Further support for the importance of GPx in ameliorating injury comes from work with GPx deficient mice, which 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 P7 [unpublished observations].

The present study explores the role of hypoxia gene signaling in the setting of GPx over-expression in the neonatal brain through characterization of expression of GPx, HIF-

1α, as well HIF-2α, which is thought to be astrocyte specific [22]; the transcription factor EPO and its receptor, ERK1/2 and phosphorylated 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 (LARC). 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]. Mice then underwent the Vannucci procedure of hypoxia-ischemia at postnatal day 9 (P9) [24] [25] [26]. At 30 min, 4 h or 24 h after hypoxia, mice were killed by rapid decapitation, brains removed and ipsi- and contralateral hippocampi and cortices were dissected and flash frozen for Western Blot. In addition, naïve GPx-tg and WT mice were deeply anesthetized with euthasol and perfused intracardiac with 4% paraformaldehyde for immunohistochemistry.

Hypoxia-Ischemia

Under isofluorane anesthesia, the left common carotid artery of each mouse was isolated and permanently ligated. When sufficiently recovered from the anesthesia to walk, pups were returned to the dam for 1 h. They were then placed in chambers maintained at 36.5 degree 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 combined into one sample at homogenization due to small size of tissue, thus presented as n=5 for all groups) using the Nuclear and Cytoplasmic Extraction Reagents (NE-PER, Pierce Biotechnology, Rockford, IL) according to the manufacturers protocol. Protein concentrations were measured by BCA assay (Pierce). 20 ug of nuclear or 40 ug of cytoplasmic protein were loaded onto 4-12% polyacrylamide gels (Invitrogen, Carlsbad, CA) for electrophoresis. Proteins were transferred to PVDF membranes (Bio-Rad, Hercules, CA) and membranes were blocked in 5% non-fat dry milk for 1 h at room temperature and incubated in the following antibodies overnight at 4 degree C: GPx (1:1000, Epitomics, Burlingame, CA), HIF-1α (1:2000, Novus Biologicals, Littleton, CO), HIF-2α (1:200, Novus), EPO (1:500, Abcam, Cambridge, MA), EPO receptor (1:1000, Abcam), ERK1/2 (1:4,000 EMD Millipore, Billerica, MA), phospho-ERK1/2 (1:1000, Cell Signaling, Danvers MA), spectrin (1:4000, EMD Millipore). β-actin (1:2,000, Santa Cruz Biotechnology, Santa Cruz, CA) 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 um thick. Sections were incubated in rabbit anti-GPx primary antibody (1:200, Abcam) overnight at 4 deg C, goat-anti-rabbit IgG secondary antibody (1:200, Santa Cruz), for 2 h at room temperature and diaminobenzidine was used as the chromophore. Mounted sections were counterstained with cresyl violet.

Statistical Analysis

Optical densities normalized to beta-actin were analyzed with unpaired by t-test. Data are expressed as mean ± SEM normalized to wild-type naive. Statistical analyses were

performed with GraphPad Prism 6.0 (San Diego, CA). $p<0.05$ 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 (fig. 1a, $p<0.001$) and this difference between GPx-tg and WT was maintained at all time points after HI (30 min $p<0.002$, 4 h $p<0.0001$ and 24 h $p<0.0001$. Results are similar for the hippocampus. Naïve GPx-tg HC compared to WT naïve (fig. 1b $p<0.05$), GPx-tg and WT after HI (30 min and 4 h both $p<0.05$ and 24 h $p<0.03$).

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

HIF-1α protein expression

HIF-1α expression was not different between GPx-tg and WT, whether naïve or post-HI in either cortex or hippocampus (fig. 2a, b). However, there was a *decrease* in HIF-1α protein expression in the WT cortex 4 h after HI compared to WT naïve cortex (fig. 2a $p<0.04$).

HIF-2α protein expression

HIF-2α also was not different between GPx-tg and WT, whether naïve or post-HI in cortex or hippocampus (fig. 2c, d, respectively). However, there was a decrease in HIF-2α in 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 EPO receptor protein expression

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

EPO receptor, however, did not demonstrate any differences between hGPx-tg and WT in cortex or hippocampus for 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 (fig. 4a, $p<0.03$ and $p<0.05$, respectively). In the hippocampus, ERK1/2 phosphorylation occurred earlier, at 4 h, in both WT and hGPx-tg (fig. 4b, $p=0.05$ and $p<0.03$, respectively).

Spectrin expression

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

Discussion

In this study, overexpression of GPx did not alter 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 cortex and hippocampus of GPX overexpressing brain relative to WT early (30 min) after HI. This may be explained, at least in part, by the increased ERK activation in both WT and hGPx-tg hippocampus at 4 h and 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-1α in the developing rat brain after HI [21], which may indicate an alternate pathway for EPO expression.

While activation and stabilization of HIF-1α is a well-established consequence of hypoxia, reports vary for HI. We recently showed increased HIF-1α at 15 min, 4 h and 24 h in mice after hypoxia, but an increase only at 15 min, not 4 h or 24 h after HI [13]. However, Chavez-Valdez et al have shown increased HIF-1α in mice 24 h after HI but not after 3 h [28]. In rats, HIF-1α 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 upregulation 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 both 4 h 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 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, 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 cell death processes than cortical white matter. HIF-2α, thought to be astrocyte-specific, declined in WT hippocampus at all time points after HI, but was not significantly altered in WT cortex. As astrocytes are important for clearance of H_2O_2, 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 hypoxia-ischemia [31]. Previous work by us, and others has also suggested HIF-1α 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-1α knockout mice after neonatal HI [13]. When neonatal mice that have both GPx overexpression and HIF-1α reduction - hybrid offspring of the hGPx-tg and HIF-1α knockout – the outcome is worse injury than WT littermates, indicating the protective effect of increased GPx is lost in the

setting of HIF-1α knock-down, implying a role for HIF-1α independent of the anti-oxidative mechanisms of GPx in the neonatal brain [unpublished observations].

It has been suggested that an antioxidant state with reduced levels of H_2O_2 limits the activation of the HIF pathway, whereas a pro-oxidant state allowing elevated H_2O_2 levels promotes it [34]. In fact, exogenous H_2O_2 has been shown to up-regulate HIF-1α and provide preconditioning protection to neurons in vitro [35]. Perhaps activation of HIF-1α and its targets genes is necessary for protection in neonatal HI. In this case, the lack of any increase in HIF-1α in the hGPx-tg brain in response to HI seen here is not surprising, given the presumably low amounts of H_2O_2 present. In fact, it may be that ERK activation is more important in the setting of low H_2O_2 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 results from our laboratory with GPX-knockout mice indicating HI injury is the same as WT in these mice after HI [unpublished information. We have yet to determine the status of HIF in these animals.

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

Figure Legends

Fig. 1. GPx protein expression is several-fold higher in hGPx-tg mouse cortex and hippocampus compared to WT cortex and hippocampus. **a** hGPx cortex compared to WT. Naïve (**p<0.001); 30 min post-HI (**p<0.002), 4 h post-HI (***p=0.0001) and 24 h post-HI (***p=.0001). **b** hGPx-tg hippocampus compared to WT. Naïve (*p=0.05), 30 min post-HI (*p=0.05), 4h post-HI (*p<0.05) and 24 h post-HI (*p<0.03). Mean OD normalized to P9 naïve. Representative photomicrograph of immunostaining for GPx counterstained with cresyl violet in naive GPx-tg and WT brain: Naïve GPx-tg cortex (**c**) and hippocampus (d). Naïve WT cortex (e) and hippocampus (d). Scale bar shown in (c) = 100 um for all.

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

Fig. 3. EPO (**a, b**) and EPOr (**c, d**) expression in cortex and hippocampus.
a EPO expression was higher in hGPx-tg cortex 30 min after HI compared to WT cortex 30 min after HI (p<0.008). **b** EPO expression was also higher in hGPx-tg hippocampus 30 min after HI compared to WT hippocampus 30 min after HI *p<0.04). EPO receptor,

however, did not demonstrate any differences between GPx-tg and WT in cortex (**c**) or hippocampus (**d**) for naïve or any treatment group.

Fig. 4. ERK1/2 phosphorylation in cortex and hippocampus**. (a)** Phosphorylated 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. (**b)** Phosphorylated 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. 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 hGPx-tg cortex. (**a).** Spectrin 145/150 increased in the hGPx-tg hippocampus (**b**) 30 min after HI compared to WT 30 min after HI (*$p<0.04$). Spectrin 120 increased in WT cortex (**c**) 24 h after HI compared to hGPx-tg 24h after HI (*$p=0.05$) as well as compared to WT naïve ($p=0.05$). In the hippocampus (**d**), spectrin 120 is higher in WT 4h after HI compared to hGPx-tg 4h after HI (*$p=0.03$), as well as to WT naïve (*$p<0.05$).

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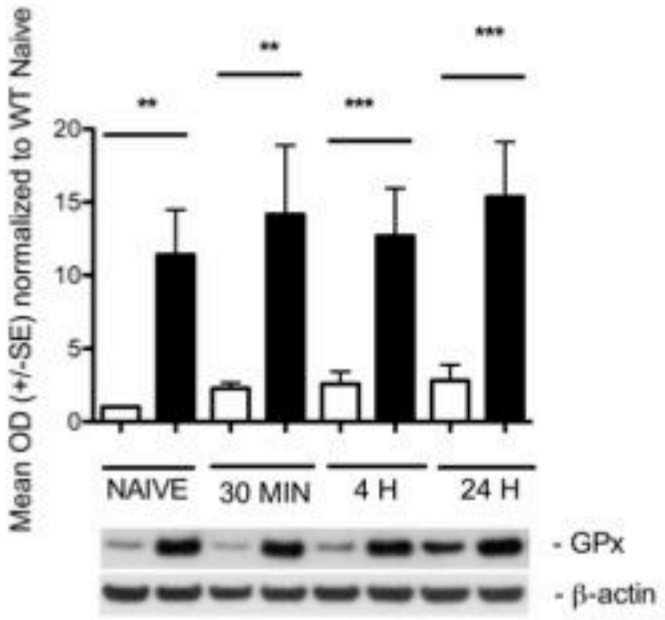
A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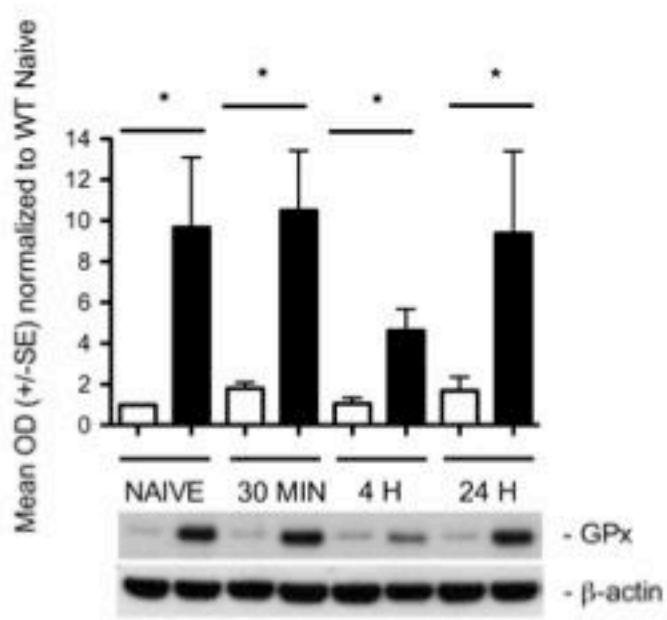
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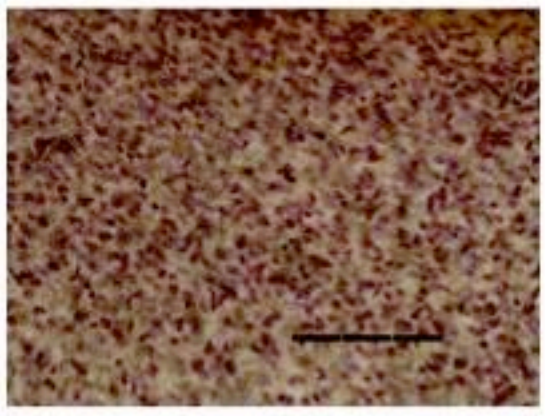
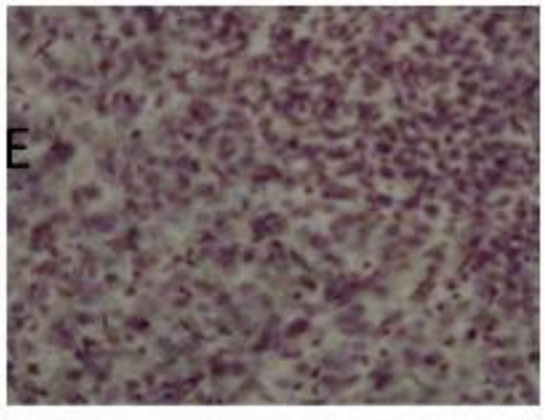
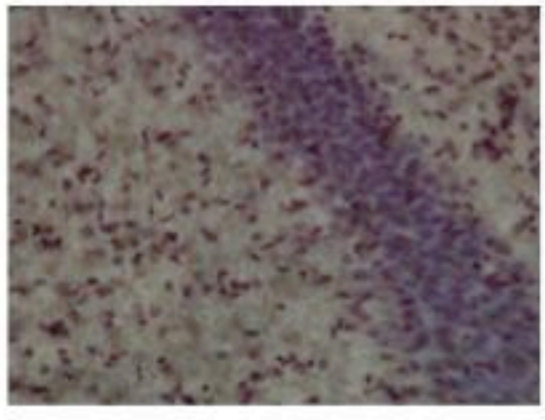


D

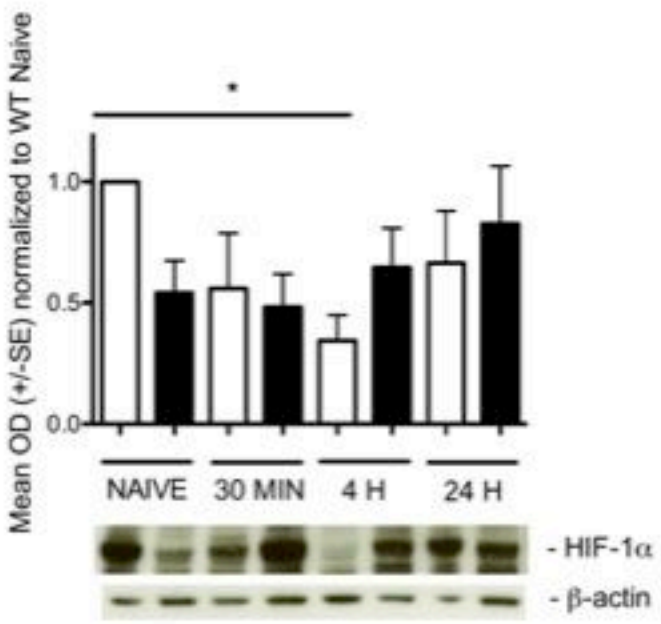
E


E

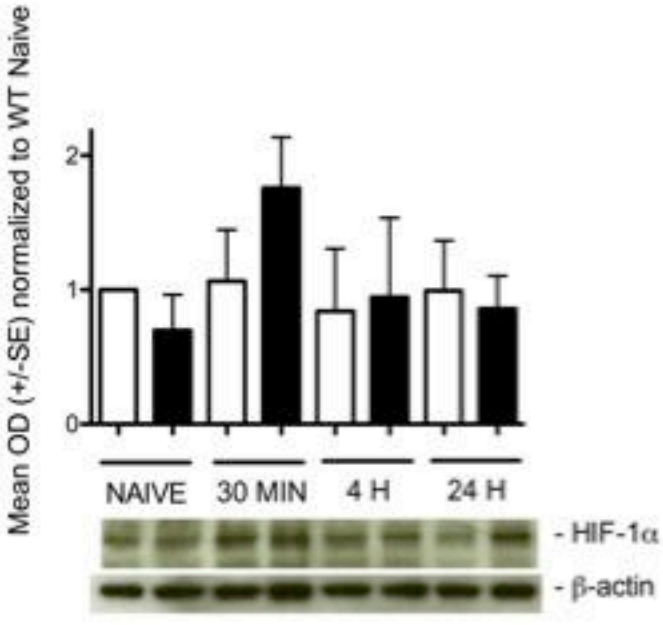
F



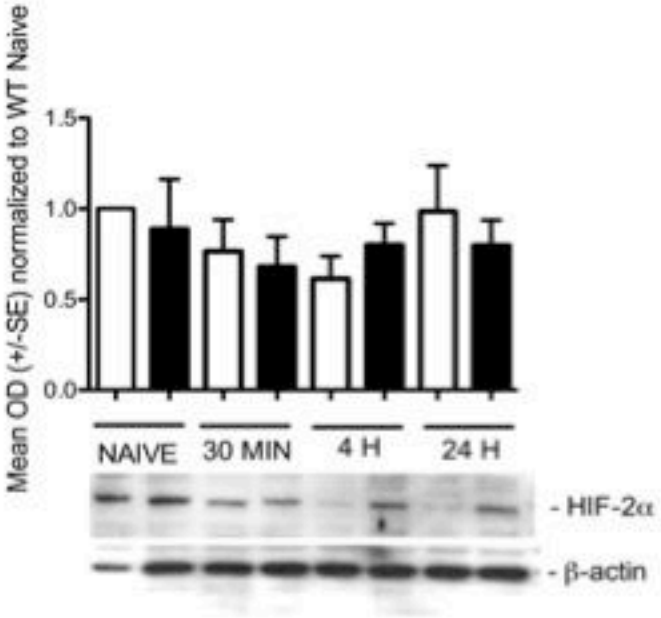
A


Mean OD (+/-SE) normalized to WT Naive
*
1.0
0.5
0.0
NAIVE
30 MIN
4 H
24 H
- HIF-1α
- β-actin

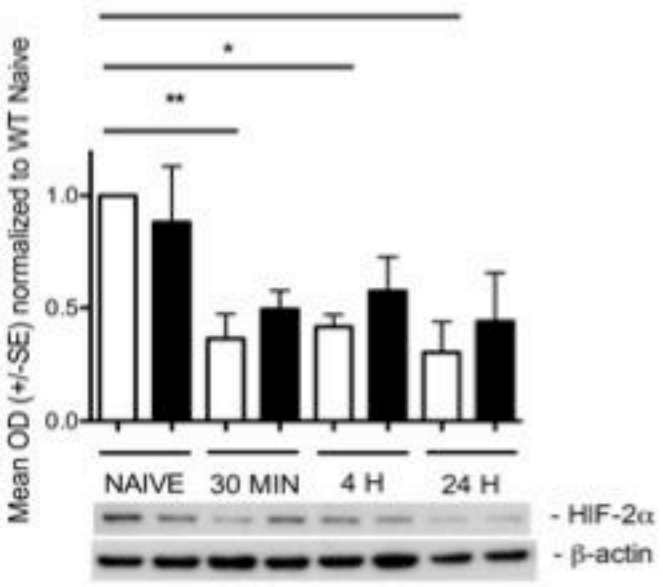
B


Mean OD (+/-SE) normalized to WT Naive
2
1
0
NAIVE
30 MIN
4 H
24 H
- HIF-1α
- β-actin

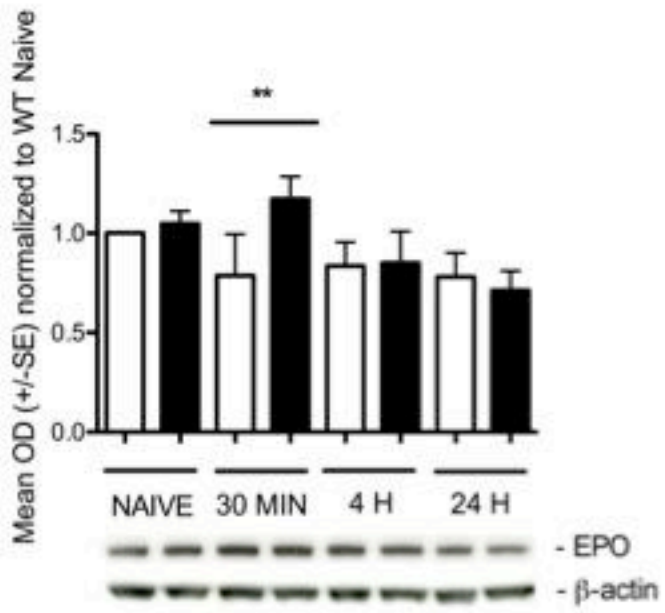
C


Mean OD (+/-SE) normalized to WT Naive
1.5
1.0
0.5
0.0
NAIVE
30 MIN
4 H
24 H
- HIF-2α
- β-actin

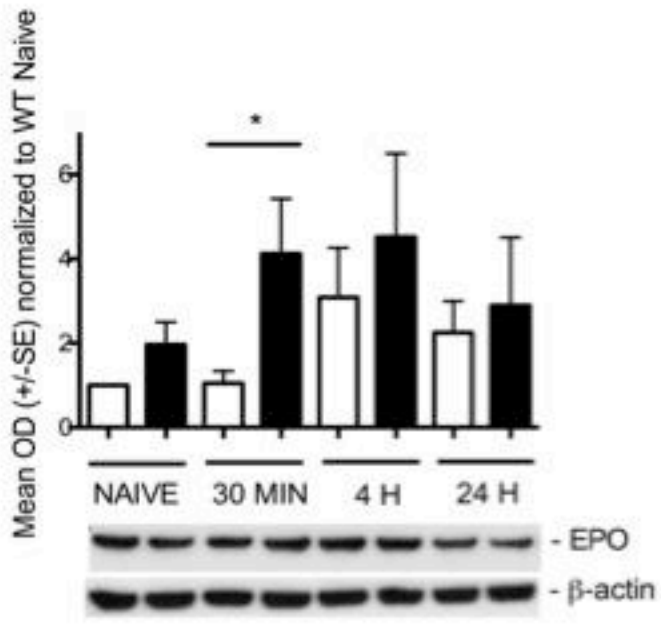
D


*
*
**
Mean OD (+/-SE) normalized to WT Naive
1.0
0.5
0.0
NAIVE
30 MIN
4 H
24 H
- HIF-2α
- β-actin

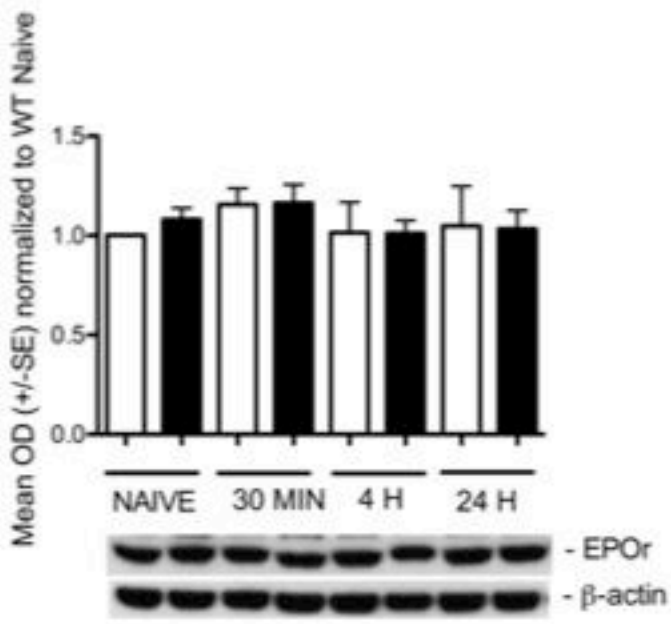
A


**
Mean OD (+/-SE) normalized to WT Naive
1.5
1.0
0.5
0.0
NAIVE
30 MIN
4 H
24 H
- EPO
- β-actin

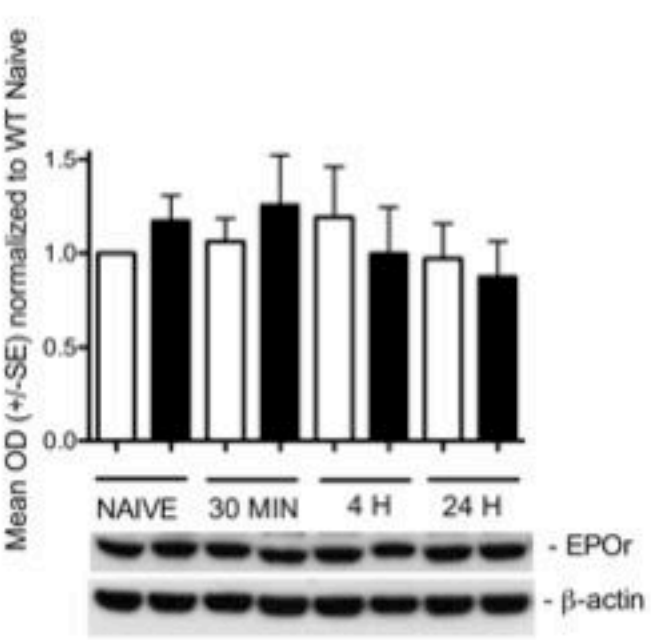
B


*
Mean OD (+/-SE) normalized to WT Naive
6
4
2
0
NAIVE
30 MIN
4 H
24 H
- EPO
- β-actin

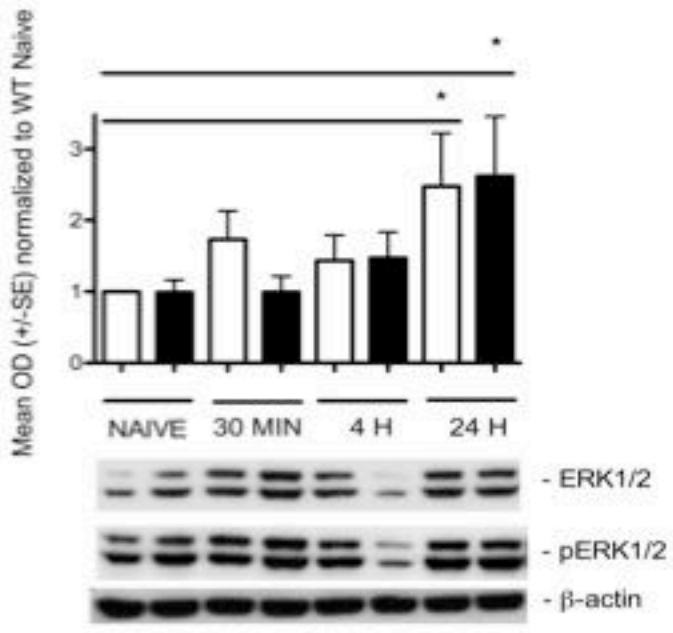
C


Mean OD (+/-SE) normalized to WT Naive
1.5
1.0
0.5
0.0
NAIVE
30 MIN
4 H
24 H
- EPOr
- β-actin

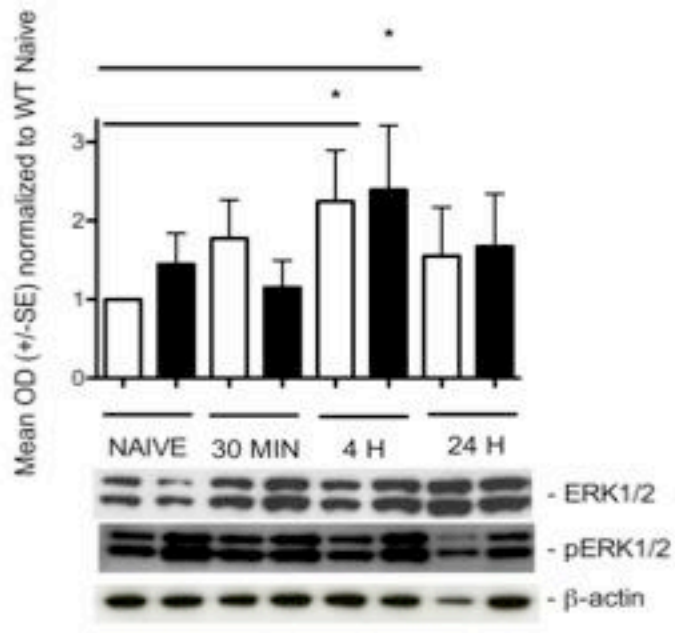
D


Mean OD (+/-SE) normalized to WT Naive
1.5
1.0
0.5
0.0
NAIVE
30 MIN
4 H
24 H
- EPOr
- β-actin

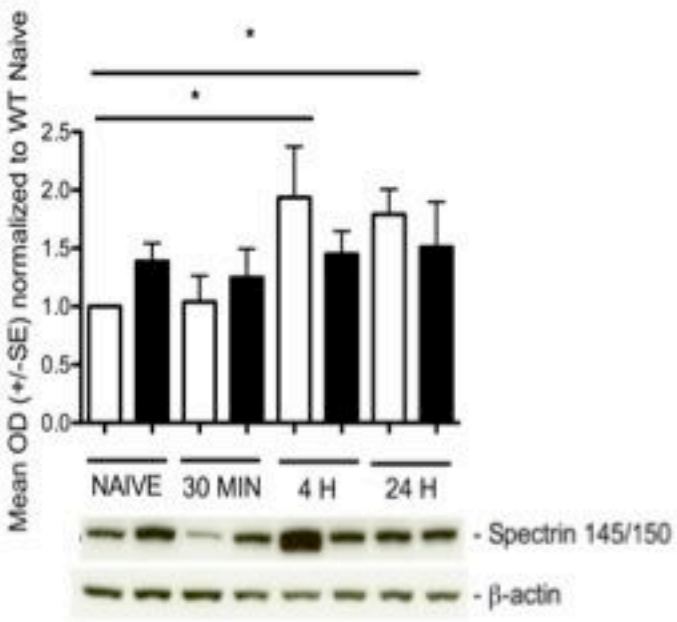
A


Mean OD (+/-SE) normalized to WT Naive
0
1
2
3
*
*
NAIVE
30 MIN
4 H
24 H
- ERK1/2
- pERK1/2
- β-actin

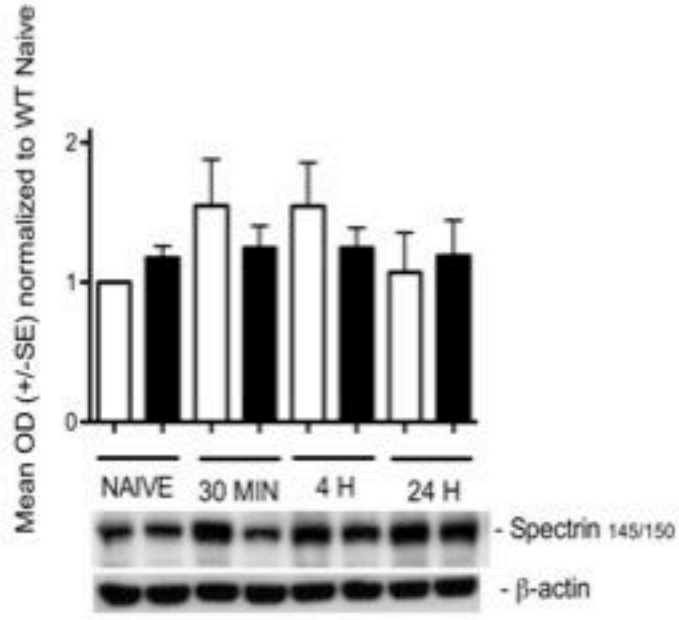
B


Mean OD (+/-SE) normalized to WT Naive
0
1
2
3
*
*
NAIVE
30 MIN
4 H
24 H
- ERK1/2
- pERK1/2
- β-actin

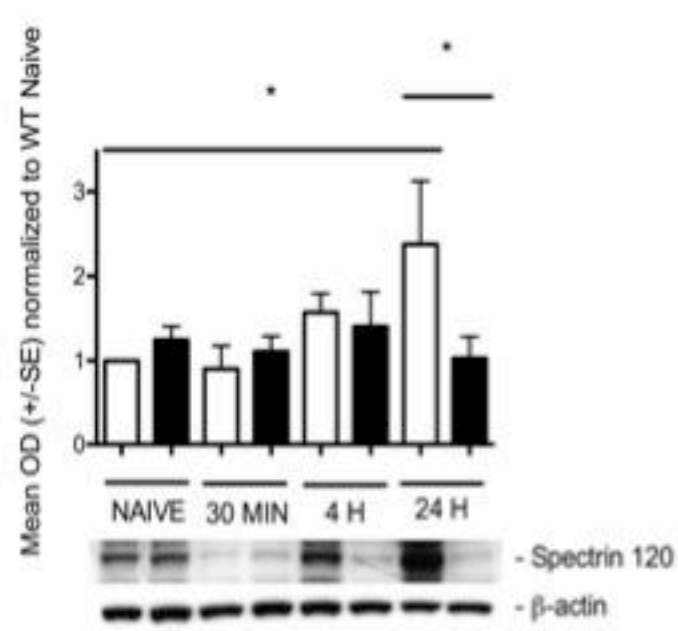
A


Mean OD (+/-SE) normalized to WT Naive
2.5
2.0
1.5
1.0
0.5
0.0
*
*
NAIVE
30 MIN
4 H
24 H
- Spectrin 145/150
- β-actin

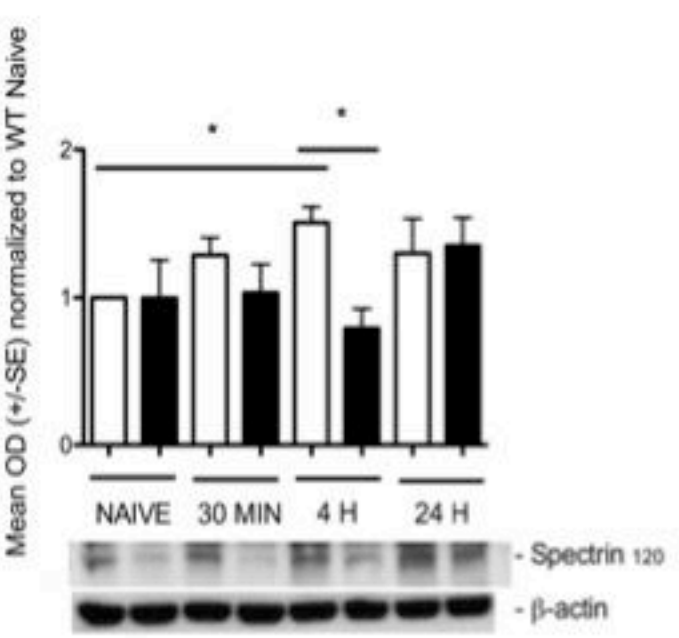
B


Mean OD (+/-SE) normalized to WT Naive
2
1
0
NAIVE
30 MIN
4 H
24 H
- Spectrin 145/150
- β-actin

C


Mean OD (+/-SE) normalized to WT Naive
3
2
1
0
*
NAIVE
30 MIN
4 H
24 H
- Spectrin 120
- β-actin

D


Mean OD (+/-SE) normalized to WT Naive
2
1
0
*
*
NAIVE
30 MIN
4 H
24 H
- Spectrin 120
- β-actin