Published in final edited form as:

Neurosci Lett. 2008 August 15; 441(1): 134–138.

E xogenous low dose hydrogen peroxide increases hypoxia-inducible factor-1 α protein expression and induces preconditioning protection against ischemia in primary cortical neurons

Shengjun Chang, Ph.D. *

University of California, San Francisco, Neurology

Xiangning Jiang, MD., Ph.D. *

University of California, San Francisco, Neurology

Chong Zhao,

University of California, San Francisco, Neurology

Christina Lee, and

University of California, San Francisco, Neurology

Donna M. Ferriero, MD.

University of California, San Francisco, Neonatal Brain Disorder Center; University of California, San Francisco, Neurology, Pediatrics

Neuroprotection against neonatal ischemic brain injury may be achieved by hypoxic or ischemic preconditioning, a phenomenon that occurs when a brief exposure to sublethal hypoxia or ischemia renders the brain less vulnerable to subsequent (24-48 h later) prolonged and injurious ischemia [13,17,20]. The molecular mechanisms for this ischemic tolerance are not fully understood. We and others have shown that hypoxia-inducible factor 1 (HIF-1), a transcription factor sensitive to regulation by hypoxia/ischemia (H/I), plays a pivotal role in H/I preconditioning protection in the neonatal brain [5,6,24]. HIF-1, a heterodimeric complex of HIF-1 α and HIF-1 β , is a key regulator for induction of genes that increase blood flow, stimulate glucose and lactate delivery to cells, enhance metabolism and promote growth and repair in neurons and blood vessels [36]. These genes include erythropoietin [38], which increases the proliferation of erythrocytes; tyrosine hydroxylase, which is necessary for the synthesis of the neurotransmitter dopamine in the carotid bodies; the angiogenic factor vascular endothelial growth factor, which stimulates growth of new capillaries [11,25,38] and endothelial nitric oxide synthase and heme oxygenase-1 (HO-1) [27], which are potent vasodilatory substances that augment perfusion of hypoxic tissue. At the cellular level, hypoxia and HIF-1 induce the expression of virtually all glycolytic enzymes, including phosphoglycerate kinase-1, enolase 1, and lactate dehydrogenase-1 [14,37]. Both HIF-1 α and HIF-1 β are constitutively expressed in cells and tissues, but the α subunit protein is rapidly degraded by prolyl hydroxylase-dependent proteasomal system under normoxic conditions

Xiangning Jiang, MD. Ph.D. Department of Neurology, Box 0663, University of California, San Francisco, 521 Parnassus Ave, San Francisco, CA 94143-0663, Tel: 415-502-5822, Fax: 415-502-5821, Email: xiangning.jiang@ucsf.edu.

*These authors contributed equally to this work.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

[22]. The half-life of HIF-1 α is very short, only about 5 min [32]. How cells sense low oxygen levels to elicit HIF-1 α induction and stabilization and how HIF-1 α is involved in H/I preconditioning protection is not clear.

Recent studies suggest that generation of mitochondrial reactive oxygen species (ROS), especially H₂O₂, is required for hypoxic HIF-1 activation and stabilization [12,18,29]. In human lung epithelial A549 cells, overexpression of antioxidant enzymes that scavenge H₂O₂, such as catalase or glutathione peroxidase 1 (GPx 1) prevents hypoxic stabilization of the HIF-1 α protein. However, overexpression of superoxide dismutase 1 or 2, which detoxifies superoxide to H₂O₂, does not alter hypoxic HIF-1 stabilization [8]. Exogenous H₂O₂ activates the HIF-1 α promoter via a functional NF κ B site in human pulmonary artery smooth muscle cells [7]. In the setting of H/I preconditioning, there is complete loss of ischemic preconditioning-induced cardioprotection in mice heterozygous for deficiency of HIF-1 α , which is associated with a 50% decrease in H₂O₂ production in these mice [9]. In neurons, we previously showed that hypoxic preconditioning reverses protection after neonatal H/I in GPx 1 transgenic murine brain, indicating the importance of H₂O₂ in mechanisms of hypoxic preconditioning [39]. Furthermore, generation of H₂O₂, but not superoxide, during brief oxygen-glucose deprivation (OGD, an in vitro model of ischemia) induces preconditioning neuronal protection in mature primary cortical neurons [15]. Whether this protection is mediated by H₂O₂-dependent HIF-1 α activation is unknown.

The aim of this study was to investigate whether brief ischemia (OGD) or low dose exogenous H₂O₂ could trigger HIF-1 α expression and thereby, contribute to H/I preconditioning protection in the immature brain. To test this hypothesis, we used 6-8 days in vitro (DIV) primary cortical neurons from embryonic day 16 (E16) CD1 mouse brains and preconditioned them with either 10 min of OGD or exogenous H₂O₂ at doses from 5-50 μ M. HIF-1 α protein levels were assessed at different time points after preconditioning. Cells were exposed to 2 h of OGD 24 h after preconditioning, and cell viability was determined 24 h thereafter.

All animal experiments were approved by the Institutional Animal Care and Use Committee at the University of California San Francisco. Cortical tissue was dissected from the brains of E16 CD1 mice (Charles River Laboratories Inc., Wilmington, MA) and maintained in MEM Eagle's HBSS medium (UCSF Cell Culture Facility) at 4°C during dissection. After dissociation with trypsin (1 mg/ml) and DNase (10 μ g/ml) and centrifugation, the cells were resuspended in neurobasal (NB) medium (Invitrogen Corporation, Carlsbad, CA) supplemented with 2% B-27 (Invitrogen), 0.5mM GlutaMax I (Invitrogen) and 100 U/ml penicillin and 100 μ g/ml streptomycin. Neurons were plated onto 24-well/6-well plates or 35mm dishes coated with poly-D-lysine (25 μ g/ml, Sigma, St. Louis, MO) at a density of $2.0 \times 10^5/\text{cm}^2$. The cultures were kept at 37°C in a humidified incubator with a 5% CO₂ atmosphere. Because B27 supplement contains 5 antioxidants (Vitamin E, Vitamin E acetate, Superoxide dismutase, Catalase and Glutathione) that would interfere with studying free-radical damage to neurons, the medium was replaced half on DIV 2 and completely on DIV 6 with B27 minus antioxidants-supplemented NB medium (Invitrogen). Purity of neuronal cultures was assessed by immunocytochemistry using the Vectastain avidin-biotin-peroxidase system. Neurons were identified by neuron-specific enolase immunoreactivity and astrocytes were identified by glial fibrillary acidic protein immunoreactivity. Astroglial contamination was confirmed less than 5% in all experiments (data not shown) [23].

For OGD preconditioning (as a positive control), on DIV6-8, the medium was removed and stored in a 37°C incubator. Cells were washed once with OGD medium (NB medium without glucose, supplemented with B27 minus antioxidants, 0.5mM GlutaMax I and 100 U/ml of penicillin and 100 μ g/ml of streptomycin). OGD medium was added to cells and cells were transferred to a humidified Modular Incubator Chamber and flushed by a gas mixture consisting

of 95% N₂ / 5% CO₂ for 5 minutes. The chamber was then sealed and kept in a 37°C incubator for 10 minutes. Oxygen concentration was maintained at 0.3-1.0% monitored by an oxygen analyzer throughout the experiment. OGD was ended by removing the medium, washing once with NB medium, adding half fresh NB medium with glucose supplemented with B27 minus antioxidants and half stored old medium (in order to increase cell viability) and cells were returned back to normoxic incubator.

For H₂O₂ preconditioning, on DIV 6-8, the medium was removed and stored in a 37°C incubator and cells were washed once with NB medium. Then NB medium supplemented with B27 minus antioxidants was added to the cultures. The cells were treated with H₂O₂ at different concentrations (5μM, 15μM, 30μM or 50μM) and kept in a 37°C incubator for 10 minutes. H₂O₂ treatments were ended by removing the medium, washing twice with NB medium, adding half fresh NB medium supplemented with B27 minus antioxidants and half stored old medium and returning the cells back to normoxic incubator.

Cells at 24 h after preconditioning were subjected to 2 h OGD and cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay 24 h after 2 h OGD. MTT was added to the cultures at a final concentration of 375 μg/ml. The insoluble purple formazan product was dissolved with buffer (20% SDS and 50% N,N-Dimethylformamide (DMF)) after 2-4 hours of incubation with MTT. Optical density was read at 580 nm, and the percentage of cell survival was calculated relative to control cells without any preconditioning or OGD.

For immunoblot analysis, after preconditioning, cells were lysed at different time points (4 h, 8 h, 18 h, 32 h, 48 h and 72 h) with RIPA buffer (1× PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) with mixed protease inhibitors (Complete Mini cocktail, Roche, Indianapolis, IN). Protein concentration was determined by the Bradford method (BioRad, Richmond, CA) using bovine serum albumin (BSA) as the standard. Equal amount of protein (30-60μg) was separated on 4-12% SDS polyacrylamide gels (Invitrogen), transferred to PVDF membranes, blocked 2 h in 5% nonfat dry milk TBS buffer with Tween 20. The membranes were then incubated with rabbit HIF-1α polyclonal antibody (1:200, Santa Cruz Biotechnology, Santa Cruz, CA) at room temperature for 1 h [34]. Mouse anti-β-actin monoclonal antibody (1:2000, Sigma) was detected on immunoblots as a loading control for protein quantization. Following washes, the membranes were incubated with secondary antibodies (1:2000, Santa Cruz). Signal of bound antibodies was developed by enhanced chemiluminescence (Amersham Life Science, Arlington Heights, IL). NIH image was used to measure the densities of the protein signals on X-ray films after scanning.

Statistical significance was tested by analysis of variance (ANOVA) for repeated measures with Fisher post-hoc analysis. Statistical significance was set at $p < 0.05$.

DIV6-8 primary cortical neurons from E16 CD1 mouse embryos were preconditioned with 10 min of OGD or exogenous H₂O₂ at doses from 5-50 μM. Preconditioning with OGD or doses of 5-50 μM H₂O₂ did not produce cell toxicity as measured by MTT assay 24 h later (cell death was 10% with 10 min of OGD and 5-12% with different doses of H₂O₂ preconditioning, not different from control cells). We observed significant cytotoxicity with 100 μM H₂O₂ treatment [1,33]. Both OGD and low dose H₂O₂ (15μM) preconditioning provided neuronal protection 24 h later against a severe 2 h OGD insult. Cell survival was 34.9±1.8% and 35.8±3.8% with OGD and H₂O₂ preconditioning respectively vs 20.0±0.4% without preconditioning ($P<0.01$) (Fig. 1). Neither lower (5 μM) nor higher (50 μM) doses of H₂O₂ preconditioning provided neuroprotection. We measured HIF-1α protein at different time points following preconditioning. We found that for OGD preconditioning, HIF-1α protein expression level started to increase at 4 h and peaked at 8 h, then declined at 18 h and increased again to reach

another peak at 32 h (Fig. 2), while HIF-1 α expression level following H₂O₂ preconditioning at dose of 15 μ M H₂O₂ started to increase at 8 h and reached a peak at 32 h (Fig. 2). For both preconditioning paradigms, HIF-1 α protein declined to baseline by 72 h (Fig. 2). When HIF-1 α levels were compared at 32 h after preconditioning, we found a marked increase in HIF-1 α expression by IPC, and by preconditioning with different doses of H₂O₂ with a maximum induction at 15 μ M (Fig. 3). Lower (5 μ M) or higher (30 μ M) doses of H₂O₂ resulted in higher HIF-1 α expression than control cells, but failed to provide preconditioning protection, suggesting that a threshold effect of HIF-1 α may exist in the mediation of H/I preconditioning.

Our study shows that brief OGD or low dose exogenous H₂O₂ provides neuroprotection against severe OGD 24 h later. H₂O₂ induces HIF-1 α protein expression in a dose-dependent manner with a maximal induction at 15 μ M, and at dose of 15 μ M, H₂O₂ resulted in a time-dependent increase in HIF-1 α expression with maximal levels at 32 h after preconditioning. Oxidative stress and ROS have been shown to be critical mediators of the injury response after H/I in the neonatal brain and this response is unique and at times paradoxical to that seen in the mature brain [31]. Evidence suggests that H₂O₂ toxicity might be related to cell death with greater sensitivity of immature cortical neurons to H₂O₂ than more mature cells [33] and greater H₂O₂ accumulation in immature brains in regions of damage over adult [26]. However, a growing literature suggests that H₂O₂ is also a signaling molecule in the brain [2], and has been implicated in a variety of homeostatic functions including preconditioning. Preconditioning the neonatal brain with H/I before a severe H/I insult results in preservation of neurons and brain volume [16,40]. Recent studies suggest that preconditioning protection in vitro and in vivo is dependent on H₂O₂ production [15,39]. From this study, preconditioning protection appears to be tightly correlated to the levels of H₂O₂ present in the neurons at the time of preconditioning, suggesting that H₂O₂ may regulate whether downstream regulators will be pro or anti-apoptotic (i.e., high levels are necrotic and pro-apoptotic, whereas low levels induce an anti-apoptotic program). Previous studies, including our own, strongly suggest that preconditioning protection depends on gene expression induced by activation of the transcription factor hypoxia-inducible factor-1 (HIF-1) [5,6,24]. There is emerging evidence that not only hypoxia (through inhibition of prolyl hydroxylase-dependent degradation of HIF-1 α), but also H₂O₂ is a critical factor in the activation of HIF-1 and regulation of its activity [3,4,10,12,19,28,35]. This regulation seems to be dose and cell type-dependent, since high dose of H₂O₂ (1 mM) pretreatment has been reported to block hypoxia-induced HIF-1 α protein accumulation in human Hep3B cells and Hela cells [21]. Non-toxic levels of H₂O₂ generated by the enzyme glucose oxidase showed no effect on HIF-1 α stabilization in HT1080 human fibrosarcoma cells [42]. Severity of hypoxia is also important, because functional mitochondria are not required for HIF-1 α stabilization under near anoxic conditions [41,43], but is necessary at more moderate levels of hypoxia [8,18,30].

Our result of HIF-1 α induction by low dose H₂O₂ is in general agreement with other studies [15], suggesting mitochondria-derived ROS are sufficient to initiate HIF-1 α activation during hypoxia. Our data showed that after 10 min OGD or low dose H₂O₂ preconditioning, HIF-1 α protein expression in primary cortical neurons could be maintained at a high level for more than 24 h (Fig.2,3). Preconditioning protection falls in that period, suggesting that a series of downstream genes initiated by HIF-1 α might contribute to the preconditioning protection. Furuichi et al. measured increased intracellular levels of H₂O₂ in primary cultured cortex neurons on DIV 10 from rat embryos preconditioned with 10 min of OGD and produced H₂O₂ was responsible for preconditioning protection [15]. In this study, we observed that low dose of exogenous H₂O₂ not only conferred cells a tolerance to subsequent lethal insult, but also alone upregulated HIF-1 α protein expression at a significant level, suggesting that H₂O₂ produced during OGD preconditioning may stabilize and upregulate HIF-1 α . HIF-1 α stabilized by endogenous hydrogen peroxide induced by H/I preconditioning might mediate H/I preconditioning protection. We also noticed that HIF-1 α protein expression induced by

H₂O₂ alone is lower than by OGD (Fig. 2, 3). Given the complex responses to H/I, other factors may also contribute to induce HIF-1 α during OGD. However, HIF-1 α induced by H₂O₂ alone may be enough to initiate up-regulation of protective downstream genes.

Acknowledgements

The authors gratefully acknowledge Joel Faustino for cell culture assistance and Dezhi Mu for helpful suggestions. Support was provided by the National Institutes of Health: NS033997-10 (DMF) and REAC (SC) from UCSF.

References

1. Almlil, LM.; Hamrick, SE.; Koshy, AA.; Tauber, MG.; Ferriero, DM. Multiple pathways of neuroprotection against oxidative stress and excitotoxic injury in immature primary hippocampal neurons; *Brain Res Dev Brain Res*. 2001. p. 121-9.
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=11744116
2. Avshalumov, MV.; Chen, BT.; Marshall, SP.; Pena, DM.; Rice, ME. Glutamate-dependent inhibition of dopamine release in striatum is mediated by a new diffusible messenger, H₂O₂; *J Neurosci*. 2003. p. 2744-50.
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12684460
3. Bell, EL.; Klimova, TA.; Eisenbart, J.; Moraes, CT.; Murphy, MP.; Budinger, GR.; Chandel, NS. The Qo site of the mitochondrial complex III is required for the transduction of hypoxic signaling via reactive oxygen species production; *J Cell Biol*. 2007. p. 1029-36.
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17562787
4. Bell, EL.; Klimova, TA.; Eisenbart, J.; Schumacker, PT.; Chandel, NS. Mitochondrial reactive oxygen species trigger hypoxia-inducible factor-dependent extension of the replicative life span during hypoxia; *Mol Cell Biol*. 2007. p. 5737-45.
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17562866
5. Bergeron, M.; Gidday, JM.; Yu, AY.; Semenza, GL.; Ferriero, DM.; Sharp, FR. Role of hypoxia-inducible factor-1 in hypoxia-induced ischemic tolerance in neonatal rat brain; *Ann Neurol*. 2000. p. 285-96.
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=10976634
6. Bernaudin, M.; Tang, Y.; Reilly, M.; Petit, E.; Sharp, FR. Brain genomic response following hypoxia and re-oxygenation in the neonatal rat. Identification of genes that might contribute to hypoxia-induced ischemic tolerance; *J Biol Chem*. 2002. p. 39728-38.
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12145288
7. Bonello, S.; Zahringer, C.; BelAiba, RS.; Djordjevic, T.; Hess, J.; Michiels, C.; Kietzmann, T.; Grolach, A. Reactive oxygen species activate the HIF-1 α promoter via a functional NF κ B site; *Arterioscler Thromb Vasc Biol*. 2007. p. 755-61.
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17272744
8. Brunelle, JK.; Bell, EL.; Quesada, NM.; Vercauteren, K.; Tiranti, V.; Zeviani, M.; Scarpulla, RC.; Chandel, NS. Oxygen sensing requires mitochondrial ROS but not oxidative phosphorylation; *Cell Metab*. 2005. p. 409-14.
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16054090
9. Cai, Z.; Zhong, H.; Bosch-Marce, M.; Fox-Talbot, K.; Wang, L.; Wei, C.; Trush, MA.; Semenza, GL. Complete loss of ischaemic preconditioning-induced cardioprotection in mice with partial deficiency of HIF-1 α ; *Cardiovasc Res*. 2008. p. 463-70.
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=18006459

10. Chandel, NS.; McClintock, DS.; Feliciano, CE.; Wood, TM.; Melendez, JA.; Rodriguez, AM.; Schumacker, PT. Reactive oxygen species generated at mitochondrial complex III stabilize hypoxia-inducible factor-1 α during hypoxia: a mechanism of O₂ sensing; *J Biol Chem.* 2000. p. 25130-8.
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=10833514
11. Czyzyk-Krzeska, MF.; Furnari, BA.; Lawson, EE.; Millhorn, DE. Hypoxia increases rate of transcription and stability of tyrosine hydroxylase mRNA in pheochromocytoma (PC12) cells; *J Biol Chem.* 1994. p. 760-4.
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=7903970
12. Emerling, BM.; Platanias, LC.; Black, E.; Nebreda, AR.; Davis, RJ.; Chandel, NS. Mitochondrial reactive oxygen species activation of p38 mitogen-activated protein kinase is required for hypoxia signaling; *Mol Cell Biol.* 2005. p. 4853-62.
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15923604
13. Ferriero, DM. Protecting neurons; *Epilepsia.* 2005. p. 45-51.
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16201995
14. Firth, JD.; Ebert, BL.; Pugh, CW.; Ratcliffe, PJ. Oxygen-regulated control elements in the phosphoglycerate kinase 1 and lactate dehydrogenase A genes: similarities with the erythropoietin 3' enhancer; *Proc Natl Acad Sci U S A.* 1994. p. 6496-500.
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=8022811
15. Furuichi, T.; Liu, W.; Shi, H.; Miyake, M.; Liu, KJ. Generation of hydrogen peroxide during brief oxygen-glucose deprivation induces preconditioning neuronal protection in primary cultured neurons; *J Neurosci Res.* 2005. p. 816-24.
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15668910
16. Gidday, JM.; Kim, YB.; Shah, AR.; Gonzales, ER.; Park, TS. Adenosine transport inhibition ameliorates postischemic hypoperfusion in pigs; *Brain Res.* 1996. p. 261-8.
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=8896833
17. Gustavsson, M.; Wilson, MA.; Mallard, C.; Rousset, C.; Johnston, MV.; Hagberg, H. Global gene expression in the developing rat brain after hypoxic preconditioning: involvement of apoptotic mechanisms?; *Pediatr Res.* 2007. p. 444-50.
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17515869
18. Guzy, RD.; Hoyos, B.; Robin, E.; Chen, H.; Liu, L.; Mansfield, KD.; Simon, MC.; Hammerling, U.; Schumacker, PT. Mitochondrial complex III is required for hypoxia-induced ROS production and cellular oxygen sensing; *Cell Metab.* 2005. p. 401-8.
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16054089
19. Guzy, RD.; Sharma, B.; Bell, E.; Chandel, NS.; Schumacker, PT. Loss of the SdhB, but Not the SdhA, subunit of complex II triggers reactive oxygen species-dependent hypoxia-inducible factor activation and tumorigenesis; *Mol Cell Biol.* 2008. p. 718-31.
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17967865
20. Hagberg, H.; Dammann, O.; Mallard, C.; Leviton, A. Preconditioning and the developing brain; *Semin Perinatol.* 2004. p. 389-95.
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15693395
21. Huang, LE.; Arany, Z.; Livingston, DM.; Bunn, HF. Activation of hypoxia-inducible transcription factor depends primarily upon redox-sensitive stabilization of its α subunit; *J Biol Chem.* 1996. p.

- 32253-9.
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=8943284
22. Huang, LE.; Gu, J.; Schau, M.; Bunn, HF. Regulation of hypoxia-inducible factor 1alpha is mediated by an O2-dependent degradation domain via the ubiquitin-proteasome pathway; *Proc Natl Acad Sci U S A*. 1998. p. 7987-92.
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=9653127
 23. Jiang, X.; Mu, D.; Manabat, C.; Koshy, AA.; Christen, S.; Tauber, MG.; Vexler, ZS.; Ferriero, DM. Differential vulnerability of immature murine neurons to oxygen-glucose deprivation; *Exp Neurol*. 2004. p. 224-32.
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15473995
 24. Jones, NM.; Bergeron, M. Hypoxic preconditioning induces changes in HIF-1 target genes in neonatal rat brain; *J Cereb Blood Flow Metab*. 2001. p. 1105-14.
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=11524615
 25. Kuroki, M.; Voest, EE.; Amano, S.; Beerepoot, LV.; Takashima, S.; Tolentino, M.; Kim, RY.; Rohan, RM.; Colby, KA.; Yeo, KT.; Adamis, AP. Reactive oxygen intermediates increase vascular endothelial growth factor expression in vitro and in vivo; *J Clin Invest*. 1996. p. 1667-75.
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=8833917
 26. Lafemina, MJ.; Sheldon, RA.; Ferriero, DM. Acute hypoxia-ischemia results in hydrogen peroxide accumulation in neonatal but not adult mouse brain; *Pediatr Res*. 2006. p. 680-3.
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16627881
 27. Lee, PJ.; Jiang, BH.; Chin, BY.; Iyer, NV.; Alam, J.; Semenza, GL.; Choi, AM. Hypoxia-inducible factor-1 mediates transcriptional activation of the heme oxygenase-1 gene in response to hypoxia; *J Biol Chem*. 1997. p. 5375-81.
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=9038135
 28. Lin, X.; David, CA.; Donnelly, JB.; Michaelides, M.; Chandel, NS.; Huang, X.; Warrior, U.; Weinberg, F.; Tormos, KV.; Fesik, SW.; Shen, Y. A chemical genomics screen highlights the essential role of mitochondria in HIF-1 regulation; *Proc Natl Acad Sci U S A*. 2008. p. 174-9.
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=18172210
 29. Liu, J.; Narasimhan, P.; Yu, F.; Chan, PH. Neuroprotection by hypoxic preconditioning involves oxidative stress-mediated expression of hypoxia-inducible factor and erythropoietin; *Stroke*. 2005. p. 1264-9.
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15890996
 30. Mansfield, KD.; Guzy, RD.; Pan, Y.; Young, RM.; Cash, TP.; Schumacker, PT.; Simon, MC. Mitochondrial dysfunction resulting from loss of cytochrome c impairs cellular oxygen sensing and hypoxic HIF-alpha activation; *Cell Metab*. 2005. p. 393-9.
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16054088
 31. McLean, C.; Ferriero, D. Mechanisms of hypoxic-ischemic injury in the term infant; *Semin Perinatol*. 2004. p. 425-32.
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15693399
 32. Meloni, BP.; Majda, BT.; Knuckey, NW. Evaluation of preconditioning treatments to protect near-pure cortical neuronal cultures from in vitro ischemia induced acute and delayed neuronal death; *Brain Res*. 2002. p. 69-75.
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=11844473

33. Mischel, RE.; Kim, YS.; Sheldon, RA.; Ferriero, DM. Hydrogen peroxide is selectively toxic to immature murine neurons in vitro; *Neurosci Lett.* 1997. p. 17-20.
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=9280157
34. Mu, D.; Jiang, X.; Sheldon, RA.; Fox, CK.; Hamrick, SE.; Vexler, ZS.; Ferriero, DM. Regulation of hypoxia-inducible factor 1alpha and induction of vascular endothelial growth factor in a rat neonatal stroke model; *Neurobiol Dis.* 2003. p. 524-34.
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=14678768
35. Page, EL.; Chan, DA.; Giaccia, AJ.; Levine, M.; Richard, DE. Hypoxia-inducible Factor-1 {alpha} Stabilization in Nonhypoxic Conditions: Role of Oxidation and Intracellular Ascorbate Depletion; *Mol Biol Cell.* 2008. p. 86-94.
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17942596
36. Ran, R.; Xu, H.; Lu, A.; Bernaudin, M.; Sharp, FR. Hypoxia preconditioning in the brain; *Dev Neurosci.* 2005. p. 87-92.
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16046841
37. Semenza, GL.; Jiang, BH.; Leung, SW.; Passantino, R.; Concordet, JP.; Maire, P.; Giallongo, A. Hypoxia response elements in the aldolase A, enolase 1, and lactate dehydrogenase A gene promoters contain essential binding sites for hypoxia-inducible factor 1; *J Biol Chem.* 1996. p. 32529-37.
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=8955077
38. Semenza, GL.; Wang, GL. A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation; *Mol Cell Biol.* 1992. p. 5447-54.
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=1448077
39. Sheldon, RA.; Aminoff, A.; Lee, CL.; Christen, S.; Ferriero, DM. Hypoxic preconditioning reverses protection after neonatal hypoxia-ischemia in glutathione peroxidase transgenic murine brain; *Pediatr Res.* 2007. p. 666-70.
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17426643
40. Sheldon, RA.; Jiang, X.; Francisco, C.; Christen, S.; Vexler, ZS.; Tauber, MG.; Ferriero, DM. Manipulation of antioxidant pathways in neonatal murine brain; *Pediatr Res.* 2004. p. 656-62.
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15295091
41. Srinivas, V.; Leshchinsky, I.; Sang, N.; King, MP.; Minchenko, A.; Caro, J. Oxygen sensing and HIF-1 activation does not require an active mitochondrial respiratory chain electron-transfer pathway; *J Biol Chem.* 2001. p. 21995-8.
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=11342528
42. Tuttle, SW.; Maity, A.; Oprysko, PR.; Kachur, AV.; Ayene, IS.; Biaglow, JE.; Koch, CJ. Detection of reactive oxygen species via endogenous oxidative pentose phosphate cycle activity in response to oxygen concentration: implications for the mechanism of HIF-1alpha stabilization under moderate hypoxia; *J Biol Chem.* 2007. p. 36790-6.
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17666400
43. Vaux, EC.; Metzen, E.; Yeates, KM.; Ratcliffe, PJ. Regulation of hypoxia-inducible factor is preserved in the absence of a functioning mitochondrial respiratory chain; *Blood.* 2001. p. 296-302.
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=11435296

Fig 1.

Comparison of neuronal protective effect of exogenous hydrogen peroxide and 10 min OGD preconditioning. Neurons were treated with OGD or hydrogen peroxide for 10 min, and then subjected to 2 h OGD 24 h later. Neuronal survival rates were determined after 24 h reoxygenation. Data are presented as means $\pm$ SE of five individual experiments. * P < 0.01 compared to OGD group.

Fig 2. HIF-1α expression level normalized to β-actin at different time points following 10 min OGD or 15 μM H₂O₂ preconditioning. Horizontal axis represents time following OGD/H₂O₂ preconditioning and vertical axis represents relative HIF-1α expression level normalized to β-actin.

Fig 3.

Effect of 10 min OGD or hydrogen peroxide treatments on HIF-1 α expression at 32 h after preconditioning. (A) Equal amount of total protein from cell lysates (60 μg) were analyzed by Western blotting with mouse HIF-1 α polyclonal antibody. One band at 120 kDa corresponding to HIF-1 α was induced in OGD-or hydrogen peroxide-treated mouse primary cortical neurons at 32 h after preconditioning. β -actin was used to document equal protein loading. (B) Relative optical density of the blots normalized to β -actin is shown. Data are presented as means $\pm$ SE of three individual experiments. ** P<0.01; * P<0.05 compared to control.