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Hyperglycemia-Enhanced Ischemic Brain Damage in Mutant Manganese SOD Mice Is Associated With Suppression of HIF-1 α

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Abstract

Both preischemic hyperglycemia and reduction of manganese superoxide dismutase activity are known to enhance neuronal death induced by transient cerebral ischemia. Transcriptional factor Hypoxia-inducible factor 1 (HIF-1) regulates multiple downstream genes that modulate cell metabolism, survival, death, angiogenesis, hematopoiesis, and other functions. The objectives of this study were to determine (i) whether hyperglycemia is able to increase ischemic brain damage in mutant manganese superoxide dismutase (SOD2) mice and (ii) whether reduction of SOD₂ activity has a profound effect on HIF-1 protein expression under hyperglycemic ischemic condition. Both wild type and mutant SOD deficient (SOD2^{-/+}) mice were induced to hyperglycemia 30 minutes before induction of a 30-minute transient middle cerebral artery occlusion (tMCAO). Brains were extracted after 5 and 24 hours of reperfusion for immunohistochemistry and Western blot analyses. The results showed that preischemic hyperglycemia significantly increased infarct volume in SOD2^{-/+} mice and that HIF-1 α protein levels were significantly reduced in ischemic core area at 5- and 24 hrs of reperfusion in hyperglycemic SOD2^{-/+} mice. However, the HIF-1 α protein levels were not significantly decreased in hyperglycemic wild type animals subjected to stroke. The results suggest that the increased brain damage observed in hyperglycemic SOD2^{-/+} mice is associated with HIF-1 α suppression, while hyperglycemia *per se* does not seem to exert its detrimental effects on ischemic brain via modulating HIF-1 pathway.

Keywords

Hypoxia-inducible factor; cerebral ischemia; manganese superoxide dismutase; hyperglycemia; free radicals

Transient ischemia induces neuronal cell death by activating several biochemical cascades. Among them, increased production of reactive oxygen species (ROS) plays a critical role in mediating cell death induced by ischemia and reperfusion [6]. In a normal brain, a small amount of free radicals produced primarily by mitochondrial oxidative phosphorylation is degraded by superoxide dismutases (SODs) and glutathione (GSH). Under pathological conditions such as ischemia and reperfusion, a large amount of free radicals produced overwhelms the endogenous

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antioxidant system, which causes damage to lipids, proteins, and DNA. To date, studies have provided clear evidence for the importance of free radicals in mediating ischemic brain damage. For example reduction of copper-zinc SOD (SOD1) or manganese SOD (SOD2) increases neuronal death and edema after transient focal cerebral ischemia by enhancing ROS production and by activating cell death pathways [20,22,29]. Upregulation of SOD1, or SOD2 activity decreases infarct volume after ischemia by inhibiting ROS accumulation by suppressing mitochondrial-mediator cell death pathways and by preventing activation of MMP-9 and disruption of the Blood Brain Barrier (BBB) [12,19,31].

Hyperglycemia exacerbates brain damage after cerebral ischemia and reperfusion by further enhancing free radical production [27], causing early damage to the mitochondria, activating mitochondria-initiated cell death pathways [24,28], eliciting inflammatory responses [11] and suppressing neuro-survival pathways [15]. It is not known whether hyperglycemia is able to further exacerbate brain damage in SOD2 heterozygous knockout mice (SOD2^{-/+}) after a 30-min transient middle cerebral artery occlusion (tMCAO).

Hypoxia-inducible factor 1 (HIF-1) is a transcription factor that regulates the expression of more than a hundred genes encoding proteins that regulate cell metabolism, survival, death, angiogenesis, vascular tone, hematopoiesis, and other functions [7]. HIF-1 is a heterodimeric transcription factor consisting of HIF-1 α and HIF-1 β subunits. HIF-1 α is sensitive to oxygen partial pressure change, while HIF-1 β protein is not regulated by hypoxia. Therefore, HIF-1 α is a key regulator for cell adaptation to hypoxia or ischemia [13]. Under hypoxic condition, the HIF-1 α and HIF-1 β heterodimerize to form the HIF-1 complex. The downstream genes that are regulated by HIF include vascular endothelial growth factor (VEGF) and tumor suppressor p53. Thus, upregulation of HIF-1 could result in either promotion of angiogenesis or suppression of cell survival and/or death. Previous studies have shown that HIF-1 α is expressed in both neurons and glia [5,8,9]. The expression of HIF-1 α is elevated after cerebral ischemia and reperfusion [4,24]. It is not known however, if hyperglycemia or hyperglycemia in combination with SOD2 knockout modifies the HIF-1 α protein expression in animals subjected to a transient cerebral ischemia event. The objectives of this study were to determine whether preischemic hyperglycemia increases brain damage after ischemia and reperfusion injury and whether hyperglycemia alters the protein levels of HIF-1 α in ischemic brain tissue of the wild type and SOD2^{-/+} mice.

Both wild type CD1 mice and SOD2^{-/+} mice (n=24 each) were used in the current study. The genetic background of the SOD2^{-/+} mutants (CD/SV129 background) was backcrossed with CD1 mice [26]. All animals were treated in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*, and protocols were approved by The Institutional Animal Care and Use Committee at North Carolina Central University. All animals were intraperitoneally injected with 0.3–0.4 ml of a 25% glucose solution 20–30 min prior to the induction of a 30 min tMCAO. Animals were divided into 3 subgroups for both SOD2^{-/+} and wild type mice, namely, sham control, 30 min MCAO plus 5 hrs, and 24 hrs of reperfusion. Animals used for assessment of brain damage (n=4 in each subgroup) were perfusion-fixed with 4% paraformaldehyde at pre-determined end points and their brains were sectioned on a vibrating microtome (Leica VT1000) with a thickness of 30 μ m. Sections were preserved in antifreeze solution and stored at –20°C until use. Five coronal brain sections from bregma 1.70 mm to –4.20 mm were selected and stained with propidium iodide on glass slides using anti-fade Vectashield mounting medium (Vector Laboratories Inc., Burlingame, CA) to view nuclear morphology. Areas with condensed nuclei were outlined on brain maps and the analyses of areas of infarction at each section were performed using the NIH Image J program. The volumes of infarction were calculated from all sections with corrections of intersection distance. Brains that were used for Western blot analysis (n=4 in each subgroup) were frozen in liquid nitrogen. The ipsilateral cortex and striatum were dissected out in a thermo-controlled

glove box with temperature maintained at -13°C . Both cortical and striatal tissues were homogenized and nuclear fractions were obtained by centrifugation at 800g for 10 min. Proteins were loaded (20 μg for each lane) and electrophoresis was performed. Proteins were transferred to nitrocellulose membrane and blotted with primary mouse anti HIF-1 α antibody at 1:500 dilution (H1 α 67, NB100–105, Novus, Littleton, CO, U.S.A.) overnight at 4°C and then incubated in secondary polyclonal rabbit anti-mouse IgG conjugated to HRP at a dilution of 1:1000 (NB7544, Novus, Littleton, CO, U.S.A.) in room temperature for 2 hrs. The blots were then developed using the Supersignal West Dura Extended Duration Substrate (Thermo Scientific). Membranes were stripped with stripping buffer (Western Blot Stripping Buffer, Thermo Scientific) and re-blotted with monoclonal anti β -actin (1:2000, Sigma) as an internal control. The HIF-1 α protein band intensity was measured and presented as the ratio of HIF-1 α to control β -actin. Values from wild type sham control were set as 100%. ANOVA followed by Tukey's Multiple Comparison Test was used to analyze data from the same group and Student *t* test was used to analyze data between wild type and SOD2 $^{-/+}$ mice at the identical reperfusion time points.

Propidium iodide nuclear staining revealed that hyperglycemia in wild type mice caused moderate damage at 5 hrs of recovery. At 24 hrs of recovery, damage was increased. However, a few survival neurons could still be observed in the ischemic core area (Figure 1 arrows). Compared to wild type animals, 30 min tMCAO in hyperglycemic SOD2 $^{-/+}$ mice resulted in greater severity of damage after 24 hrs of recovery. A set of representative microphotographs is given in Figure 1. Measurement of infarct volume showed that the infarct volume was not significantly different between wild type and SOD2 $^{-/+}$ mice (58 ± 8 versus 79 ± 14 mm^3 , $p > 0.05$) at 5 hrs of recovery. However, infarct volume was increased from 108 ± 40 mm^3 (mean $\pm$ SD) in wild type animals to 206 ± 58 mm^3 ($p < 0.01$) in SOD2 $^{-/+}$ mice at 24 hrs of recirculation. As shown in Figure 2A, the HIF-1 α levels in the striatum were slightly increased at 5- and 24- hrs of recovery in wild type CD1 mice; but these increases did not reach statistical significance. In SOD2 $^{-/+}$ animals, the levels of HIF-1 α decreased to less than 50% at 5 hrs and to 30% of the control level at 24 hrs ($p < 0.01$). Compared to wild type animals at 5- and 24 hrs, the protein levels of HIF-1 α were much lower in SOD2 $^{-/+}$ mice ($p < 0.001$). As shown in Figure 2B, HIF-1 α levels in the cortex were not significantly altered at 5 hrs or 24 hrs of the wild type animals. In contrast to the wild type samples, SOD2 $^{-/+}$ mice showed a significant increase in HIF-1 α levels in the cortex at 24 hrs of recovery ($p < 0.05$). Therefore, HIF-1 α levels were significantly decreased in the striatum, while a significant increase of HIF-1 α was observed in the cortex.

Our results demonstrated that HIF-1 α proteins levels were significantly decreased in SOD2 $^{-/+}$ mice compared with wild type ischemic animals in the ischemic core area. Such suppression is inversely correlated with the severity of brain tissue damage observed after 24 hrs of reperfusion. Thus, although infarct volumes were not significantly different between wild type and SOD2 $^{-/+}$ mice at 5 hrs of recovery, the infarct volume was increased in SOD2 $^{-/+}$ mice at 24 hrs of recirculation. This is consistent with the studies published by Baranova and colleagues [4] showing that neuron-specific knockdown of HIF-1 α significantly increased brain tissue damage in mice subjected to 30 min tMCAO, and that activation of HIF-1 α by pharmacological means (3,4-dihydroxybenzoic acid, deferoxamine, and 2,2'-dipyridyl) offered neuroprotection after tMCAO.

It is not clear why hyperglycemic ischemia in SOD2 $^{-/+}$ mice produced a pronounced suppression of HIF-1 in the ischemic core region. It has been previously shown that deletion of SODs led to a drastic increase of superoxide ($\text{O}_2^{\cdot -}$) accumulation in brain after ischemia [19,20]. Furthermore, preischemic hyperglycemia also significantly enhanced the production of superoxide and peroxynitride (ONOO^-) [27]. Because synthesis of ONOO^- requires reaction between $\text{O}_2^{\cdot -}$ and nitric oxide (NO), one can envisage a low level of NO in the

hyperglycemic, ischemic brain. A decrease in the steady-state concentration of NO is associated with a proportional reduction in the levels of HIF-1 α [32]. It is likely that HIF-1 regulation by NO is determined by intracellular generation of O₂⁻ [21]. In addition to hypoxia, acidosis may promote HIF-1 expression. When PC12 cells and primary cortical neurons were incubated in acidic media (pH 6.5) for 20 hrs, HIF-1 protein levels were significantly increased [35]. The present study, however, does not support the stimulating effect of acidosis on HIF-1 α since preischemic hyperglycemic, which is known to reduce intracellular pH level to <6.3 during ischemia, did not cause an increase in HIF-1 α in both ischemic core and penumbra regions of wild type and SOD2^{-/+} mice.

HIF is a major regulator of the cellular response to O₂ homeostasis. HIF upregulates the expression of many genes, including those responsible for cell growth, cell survival, tumor progression and angiogenesis [34]. Whether the effect of HIF-1 α is anti- or pro- cell survival is still a matter of debate. On the one hand, early inhibition of HIF-1 α with small interfering RNA reduced ischemic brain damage in rats via inhibition of VEGF, p53 and caspase-3 [10]; on the other hand, upregulation of HIF-1 α using desferoxamine pretreatment protected against cerebral ischemia [24] and this protective effect was significantly attenuated in neuron-specific HIF-1 deficient mice [4]. Moreover, downregulation of neuron-specific HIF-1 α increased brain damage induced by cerebral ischemia [4,16]. It seems that the discrepancy could be attributed to the type, duration, severity and phase of the insult [3]. Under mild conditions, hypoxia induces stabilization of the HIF1 complex, leading to the transcriptional activation of adaptive genes. However, under conditions of sustained hypoxia, HIF-1 α stabilization increases cellular p53 and promotes the transactivation of pathologic genes like bax [14]. In the present study, the reduction of HIF-1 α was observed after 5 hrs of reperfusion when there was no massive neuronal death observed. Such a reduction persisted for 24 hrs and was associated with increased infarct volume. These findings support the notion that HIF-1 α plays a neuroprotective role. The neuroprotective effect of HIF-1 α may be mediated, at least partially, by the induction of vascular endothelial growth factor (VEGF), erythropoietin (epo) and the receptor for advanced glycation end products (RAGE) [31], as well as by activation of phosphatidylinositol 3 kinase (PI3-K/Akt) and the mitogen-activated protein kinase/extracellular signal-regulated kinase pathways [18,25].

The present study revealed a different pattern of HIF-1 alteration after ischemia and reperfusion in different brain regions. In contrast to the ischemic core region in the SOD2^{-/+} mice that showed a significant reduction of HIF-1 α protein content, there was a detectable increase of HIF-1 α in the ischemic penumbra area of the SOD2^{-/+} mice. This difference may be related to the damage of the mitochondrial electron transport chain. Previous studies have shown that ischemia induces severe suppression of activities of the mitochondrial complexes I – IV and the degree of suppression is correlated with the severity of blood perfusion deficit [2,17,33]. Inhibition of complex I and complex II is known to block HIF-1 α DNA binding activity under hypoxic conditions, suggesting that electron transport chain activity is required for activation of HIF-1 α [1]. Hyperglycemia is known to further exacerbate mitochondrial damage by causing marked mitochondrial swelling during early reperfusion stage [23]. The significant reduction of HIF-1 α in the striatum may reflect the degree of mitochondrial damage in this region.

In summary, the present study demonstrated that preischemic hyperglycemia is capable of further enhancing brain damage induced by transient focal ischemia in SOD2 deficient animals. Such enhanced damage is associated with a significant reduction of HIF-1 α level in the ischemic core region.

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Figure 1. Microphotographs showing nuclear morphology produced by propidium iodide staining on brain sections from control, 5- and 24-hrs of reperfusion in both wild type (WT) and SOD₂^{-/+} mice. Arrows denote intact nuclei. Magnification 20X.

Figure 2.

Western blots showing the immunointensity of HIF-1α in the striatum (A) and cortex (B) collected from wild type (WT) and SOD2^{-/+} (KO) mice at 5- and 24-hrs of reperfusion. The band intensity ratio between HIF-1α and β-actin was calculated and converted to percent change. Data are expressed as Mean ± SD. *p<0.05 and **p<0.01 vs. control, #p<0.05 and ##p<0.01 vs. WT at the same reperfusion time.