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Manganese Superoxide Dismutase Deficiency Exacerbates Ischemic Brain Damage Under Hyperglycemic Conditions by Altering Autophagy

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Abstract

Both preischemic hyperglycemia and suppression of SOD2 activity aggravate ischemic brain damage. This study was undertaken to assess the effect of SOD2 mutation on ischemic brain damage and its relation to the factors involved in autophagy regulation in hyperglycemic wild-type (WT) and heterozygous SOD2 knockout ($SOD2^{-/+}$) mice subjected to 30-min transient focal

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ischemia. The brain samples were analyzed at 5 and 24 h after recirculation for ischemic lesion volume, superoxide production, and oxidative DNA damage and protein levels of Beclin 1, damage-regulated autophagy modulator (DRAM), and microtubule-associated protein 1 light chain 3 (LC3). The results revealed a significant increase in infarct volume in hyperglycemic $SOD2^{-/+}$ mice, and this was accompanied with an early (5 h) significant rise in superoxide production and reduced SOD2 activity in $SOD2^{-/+}$ mice as compared to WT mice. The superoxide production is associated with oxidative DNA damage as indicated by colocalization of the dihydroethidium (DHE) signal with 8-OHdG fluorescence in $SOD2^{-/+}$ mice. In addition, while ischemia in WT hyperglycemics increased the levels of autophagy markers Beclin 1, DRAM, and LC3, ischemia in hyperglycemic, SOD2-deficient mice suppressed the levels of autophagy stimulators. These results suggest that SOD2 knockdown exacerbates ischemic brain damage under hyperglycemic conditions via increased oxidative stress and DNA oxidation. Such effect is associated with suppression of autophagy regulators.

Keywords

Cerebral ischemia; SOD2; Hyperglycemia; Oxidative stress; Autophagy

Introduction

Hyperglycemia (HG) increases the extent of brain damage after transient cerebral ischemia (for review, see Ref. [1]). The underlying mechanism for this phenomenon is not fully understood. It has been suggested that oxidative stress produced by generation of excessive reactive oxygen species (ROS) in hyperglycemic conditions is associated with the progression of neuronal damage [2, 3]. Mitochondrial superoxide anion (O_2^-) generated by leakage of electrons from the electron transport chain (ETC) is one of the major source of ROS in the brain [4]. The mitochondrial overproduction of superoxide, which normally is scavenged by the antioxidant enzyme manganese superoxide dismutase (MnSOD or SOD2), is deleterious to neurons, and its production is reported to be increased by HG and/or ischemia [5]. Various reports suggest that heterozygous SOD2 knockout ($SOD2^{-/+}$) mice showed exacerbated cerebral infarction after permanent focal cerebral ischemia [6].

The production of mitochondrial ROS is dependent on factors such as mitochondrial mass and integrity of the ETC. However, the increased ROS production predisposes the brain to accelerated neuronal injury [4] through oxidative damage to nuclear and mitochondrial DNA (mtDNA). The damage to mtDNA may interfere directly or indirectly with processes such as energy metabolism and metabolite synthesis, which are critical for normal mitochondrial functioning and, ultimately, cell survival. Simultaneously, it is evident that the aged/dysfunctional/damaged mitochondria and other unnecessary cellular organelles are removed or recycled by autophagy-dependent mechanisms in order to maintain the functional status of the cell [6, 7]. Autophagy plays an important role in response to oxidative stress, and it also determines the mass and integrity of mitochondria [8]. The protein levels of autophagy regulators Bcl-2 interacting protein (Beclin 1), microtubule-associated protein 1 light chain 3 (LC3), and damage-regulated autophagy modulator (DRAM) are reported to be up-regulated during cerebral ischemia [9].

Although it is established that HG aggravates stroke damage, it is not clear whether suppression of SOD2 affects autophagy following stroke under hyperglycemic conditions. Moreover, the effect of SOD2 suppression on ischemic brain damage under normoglycemic conditions has been intensively studied; its effect under hyperglycemic conditions has not been shown. In addition, the molecular mechanisms regulating autophagy and their involvement in mediating neuronal damage under hyperglycemic conditions are largely

unknown. Therefore, the present study was conducted to address whether SOD2 suppression further aggravates the ischemic brain damage in hyperglycemic mice. The association of ischemic brain damage with autophagy regulator levels were studied in hyperglycemic wild-type (WT) and $SOD2^{-/+}$ mice that were subjected to a 30-min transient middle cerebral artery occlusion (MCAO).

Materials and Methods

Experimental Animals

Male $SOD2^{-/+}$ mice (n=32) and genetic background-matched CD1 WT mice (N=32, Charles River), weighing 24 to 26 g, were used for the experiments. The animals were fasted overnight with free access to water. Anesthesia was induced by isoflurane with N_2O/O_2 (70/30) during the operation. All animals received intraperitoneal injection of 25% glucose solution 30 min before induction of cerebral ischemia. The blood glucose level was measured by a One-Touch glucometer. The animals with glucose levels between 14 and 20 mM were included in this study. The operative animal procedures were approved by the Institutional Animal Care and Use Committee at North Carolina Central University, Durham, NC.

Ischemic Model

Transient MCAO was induced by the intraluminal filament technique as previously described [10]. In brief, the right common carotid artery (CCA), internal carotid artery (ICA), and external carotid artery were exposed through a midline incision. A filament coated with silicon rubber (diameter of 0.21±0.02 mm) was inserted into the ICA through the CCA occluding the origin of the middle cerebral artery (MCA). Reperfusion was achieved by removal of the occluder after 30 min of MCAO. The rectal temperature was maintained at 37°C by a heating pad. After the occlusion, the mice were examined, and only animals with neurological signs of diminished resistance to lateral push, walking to the left after being pulled backwards by the tail, or with spontaneous contralateral circling were included in the study. Upon predetermined end points, animals were euthanized and the brains were either perfusion fixed with 4% paraformaldehyde for immunohistochemistry (IHC) or frozen in liquid nitrogen for biochemical analyses.

Experimental Groups

Both $SOD2^{-/+}$ and WT mice were subjected to a 30-min MCAO under preischemic hyperglycemic condition. Animals were divided into sham-operated, 30-min MCAO plus 5 and 24 h of reperfusion. Experimental groups and numbers of animals are given in Table 1.

Measurements of Infarct Volume

Vibratome brain sections (30 μm) were preserved in antifreeze solution and stored at −20°C until use. Five coronal brain sections from bregma 1.70 to −4.20 mm were selected and stained with propidium iodide (PI) (Vector Laboratories Inc., Burlingame, CA) to view nuclear morphology. Neuronal morphology was also confirmed by IHC using anti-NeuN antibody (1:300, mouse monoclonal; Chemicon, Temecula, CA). Images were captured, areas with condensed nuclei were outlined, and infarct volume was calculated using the NIH Image J program. We chose to use histomorphological studies to detect ischemic brain damage instead of 2,3,5-triphenyltetrazolium chloride (TTC) staining for the following two reasons. First, histological study is superior to TTC staining. While PI and NeuN staining allow investigators to observe alterations at cellular level, TTC stain can only outline an approximate damage area at tissue level. Second, an additional advantage to use vibratome section for determination of brain damage is that the consecutive brain sections obtained

from the same animal can be used for other studies such as IHC. In contrast, a different set of animal has to be produced for biochemical studies if TTC method is employed. It will not only increase number of animals used in each experiments, but also, the results obtained from biochemical studies will not be able to directly link to histological finding.

Manganese Superoxide Dismutase (SOD2) Activity Assay

Mitochondrial SOD enzyme activity was estimated in mitochondrial samples isolated from half of brain hemisphere in nonischemic control animals using the xanthine/xanthine oxidase and ferricytochrome c method [11]. Residual cytosolic SOD was blocked with 2 mM potassium cyanide (KCN). One unit of SOD was defined as the quantity of enzyme necessary to produce 50% inhibition of the rate of reduction of the ferricytochrome c. SOD2 enzyme activity was expressed in U/mg soluble protein [12].

Detection of In vivo Superoxide (O_2^-) Production and Oxidative DNA Damage

Animals were injected with 200 μl (1 mg/kg) of dihydroethidine (DHE, Molecular Probes In. Eugene, OR) intravenously 30 min before MCAO. Animals were perfusion fixed after 5 and 24 h of recirculation, and the brains were sectioned using the Leica vibratome (30-μm thickness). Colocalization of DHE and anti-8-hydroxy-2-deoxyguanosine IHC (anti-8-OHdG rabbit polyclonal antibody, 1:100 dilution; Abcam, Cambridge, MA) was also analyzed on these sections. Three microscopic fields at the 400× magnification from the cortical and striatal regions of each hemisphere were captured with a Nikon confocal laser-scanning microscope (Nikon Instruments Inc. Melville, NY). Fluorescence intensity of the oxidized DHE was measured using NIS Elements software (Nikon Instruments Inc. Melville, NY).

Western Blot Analysis

Ischemic striatum and overlaying cortex were dissected and homogenized. Nuclear and cytosolic fractions were extracted by a series of centrifugations [13]. Protein concentration was determined by the Bradford method (Bio-Rad Laboratories Inc. Hercules, CA), and 20-μg protein of each sample was loaded and run in 10% NuPAGE gel (Invitrogen, Carlsbad, CA). Following electrophoresis and transfer, the membranes were incubated with primary antibodies against Beclin 1 (1:500, sc-11427; Santa Cruz Biotechnology, Santa Cruz, CA), DRAM (0.5 μg/ml, 4033; ProSci Incorporated, Poway, CA), LC3-I/II (1μg/ml), or β-actin (1:1000, A1978, Sigma, St. Louis, MO) overnight. The membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h. The immunoblots were analyzed using the Super-signal West Dura Extended Duration Substrate (Thermo Scientific, Rockford, IL). The β-actin bands were used as an internal loading control, and the ratio of measured proteins and β-actin was presented.

Statistics

One-way analysis of variance followed by Tukey's test was used to analyze data in Figs. 2, 3, 4, 5. Data are presented as mean ± standard deviation (SD), $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ compared with controls and $^{\#}p<0.05$ and $^{\#}p<0.01$ compared between $SOD^{-/+}$ and WT at the identical reperfusion time points.

Results

Animal Physiological Parameters

Physiological variables were measured and maintained constant. Body temperature was kept at 36.7 to 37.4°C. Preischemic plasma glucose levels were 13.8±1.6, 14.4± 1.5, and 15.4±3.3 mM for control, 5 and 24 h, in WT and 15.3±3.9, 15.0±3.4, and 14.2±3.2 mM for

control, 5 and 24 h, respectively, in $SOD2^{-/+}$ mice with no significant variations among the groups.

SOD2 Suppression Aggravated Ischemic Brain Damage in Hyperglycemic Knockout Mice

Middle cerebral artery occlusion of 30 min induced neuronal death in the affected MCA territory of WT and $SOD2^{-/+}$ mice. Neuronal damage was analyzed with PI staining for damage and cellular density (Bullock et al., 2009). As shown in Fig. 1a, the infarct area is readily distinguishable from the healthy neighboring tissue as the severely condensed nuclei are displayed in the infarct area contrasting to smooth round nuclear staining in the non-damaged area. This morphological alteration was further confirmed with NeuN staining for neuronal density and neurodegeneration (Fig. 1b). The results showed well-developed cellular alteration of karyopyknosis, as well as cavitations in some areas in WT mice. The cellular density was lower in the ischemic side of the brain as compared to the contralateral nonischemic side. These changes were further enhanced in the $SOD2^{-/+}$ mice after recirculation. Affected regions showed morphological phenotypes such as karyopyknosis or condensed neuronal perikarya, which are representative of either damaged or dead cells. Moreover, NeuN staining revealed that the neurons were greatly affected morphologically and spatially, and the number of neurons was markedly reduced in $SOD2^{-/+}$ mice as compared to WT under hyperglycemic condition (Fig. 1b). Measurements of infarct volume using anti-NeuN stained sections reveal that 30 min of MCAO is associated with moderate brain damage at 5 h of recovery, and the damage progressed at 24 h. Thus, the infarct volume increased from 58±8 mm^3 (mean ± SD) at 5 h to 108±40 mm^3 at 24 h of recirculation in WT mice. The infarct volumes were significantly greater in $SOD2^{-/+}$ mice. The infarct volume increased from 79±14 mm^3 at 5 h to 206±58 mm^3 at 24 h of recovery in $SOD2^{-/+}$ mice, which is almost doubled ($p<0.05$) the volume observed in WT mice at 24 h of recovery (Fig. 2). These results clearly indicate that SOD2 suppression combined with HG plays an important role in ischemic neurodegeneration.

Increased Superoxide Production and Decreased SOD2 Activity

To determine the effect of SOD2 knockdown on ROS production and its association with HG, we measured superoxide production using a DHE fluorescent probe. The DHE, when oxidized, intercalates within the cell's DNA, staining its nucleus a bright fluorescent red (Fig. 1c). The quantitative analysis revealed that superoxide production gradually and significantly increased in the ischemic cortex and striatum of WT mice at 24 h as compared to control animals (Fig. 1c, *upper panel*). In contrast, the superoxide level rose abruptly ($p<0.05$) at 5 h of recirculation and declined thereafter in both the ischemic cortex and striatum of $SOD2^{-/+}$ mice (Fig. 1c, *lower panel*). The measurements of DHE fluorescent intensity in the ipsilateral cortex and striatum in WT and $SOD2^{-/+}$ mice were given in Fig. 3. The increase at 5 h in $SOD2^{-/+}$ mice was comparable to 24 h of recirculation in WT mice, indicating that superoxide production elevated to a maximum level early during recirculation in $SOD2^{-/+}$ group. These results suggest that hyperglycemic $SOD2^{-/+}$ animals are prone to oxidative stress-induced increase in cerebral ischemic damage.

To further verify whether enhanced superoxide production is related to the SOD2 enzyme activity, we analyzed the SOD2 enzyme activity in mitochondria. The result revealed that SOD2 enzyme activity was significantly ($*p<0.05$) reduced, nearly to half, in cortex and striatum of $SOD2^{-/+}$ animals as compared to WT control mice (Fig. 4), suggesting the apparent relationship between SOD2 enzyme and superoxide production.

Superoxide Radical is Associated With Oxidative DNA Damage

The role of superoxide production in oxidative DNA damage was evaluated to further substantiate the sensitivity of hyperglycemic $SOD2^{-/+}$ mice to ischemic damage. The DNA

oxidation marker, 8-OHdG, distinctly stained the ischemic side as compared to the nonischemic side (Fig. 1d). The number of 8-OHdG positively stained cells increased in WT samples and further increased in $SOD2^{-/+}$ animals, reflecting the role of oxidative stress in increased ischemic brain damage in hyperglycemic $SOD2^{-/+}$ mice. Colocalization studies revealed overlapping DHE signals and 8-OHdG fluorescence in many cells in the ischemic side of $SOD2^{-/+}$ animals, which clearly indicates the involvement of superoxide radicals in oxidative DNA damage (Fig. 1e).

Autophagy Suppressed in Hyperglycemic $SOD2^{-/+}$ Mice

The removal of damaged/aged mitochondria is a continuous process regulated by autophagy. Therefore, we investigated whether SOD2 suppression modulates autophagy regulators Beclin 1, DRAM, and LC3 in hyperglycemic ischemic brains (Fig. 5). Beclin 1, which is a part of class III PI3K complex that participates in autophagosome formation and localization of other autophagy proteins to the preautophagosomal membrane, is significantly up-regulated in WT cortex at 24 h of recirculation as compared to control (Fig. 5a). Unlike the cortex, Beclin 1 levels in the striatum decreased at 5 h recovery and returned to control levels at 24 h in WT group. In $SOD2^{-/+}$ animals, Beclin 1 levels in the striatum decreased at 5 h and remained at this lower level at 24 h as compared to control (Fig. 5b).

Similarly, DRAM, which is a lysosomal protein responsible for autophagy activation, showed a pattern similar to Beclin 1 in WT and $SOD2^{-/+}$. In WT animals, DRAM levels were unchanged at 5 h but significantly elevated at 24 h in both cortex and striatum. In contrast, suppression of SOD2 either abolished the increase after reperfusion in the cortex or even decreased the DRAM level at 5 h of recovery in the striatum (Fig. 5c and d).

LC3 is a sensitive marker for distinguishing autophagy when converted to LC3-II (membranous) from LC3-I (cytoplasmic). The results showed that LC3 levels significantly increased at 5 h in WT cortex (Fig. 5e). In contrast, there was no such transient elevation of LC3 protein observed in the $SOD2^{-/+}$ cortex after recirculation. The LC3-II/I ratio decreased but did not reach statistic significance in WT striatum. In $SOD2^{-/+}$ animals, however, LC3-II/I ratio decreased significantly at 5 h and remained low at 24 h of recirculation (Fig. 5f). These results indicate that suppression of SOD2 inhibits autophagy.

Discussion

When accompanied by high glucose, stroke is linked to poor clinical outcomes. In the present study, we have shown that SOD2 suppression increases the severity of ischemia under hyperglycemic conditions. It increases oxidative stress and DNA oxidation, alters the levels of factors responsible for autophagy. Taken together, these factors significantly aggravate the ischemic brain damage in hyperglycemic $SOD2^{-/+}$ mice.

Previous studies have shown that SOD2 suppression increases ischemic brain damage after transient and permanent cerebral ischemia under normoglycemic conditions [5, 14, 15]. Likewise, HG increases infarct volume after 30 min of MCAO by increasing ROS production, without compromising capillary patency [2, 10, 16]. However, it was not known if SOD2 suppression combined with HG further increases ischemic brain damage. The present study showed that SOD2 suppression increased the ischemic infarct volume under hyperglycemic conditions as compared to WT mice. This was clearly recognized by the distinct reduction in relative neuronal density (Fig. 1a, b and Fig. 2) as indicated by condensed nuclear morphology in the PI staining and by reduced or absence of NeuN staining in the ischemic brain hemisphere of $SOD2^{-/+}$ animals. The questions raised by these observations are as follows: (1) whether the increased damage in hyperglycemic $SOD2^{-/+}$ mice is related to ROS production and/or decreased SOD2 enzyme activity, 2)

whether the impaired mitochondrial SOD function affects autophagy processes. To answer these questions, we measured ROS production in vivo, determined SOD2 enzyme activity, and detected the protein levels of autophagy stimulators (Beclin 1, DRAM and LC3). The results showed that superoxide production elevated abruptly early during recirculation in the cortex and striatum of $SOD2^{-/+}$ as compared to a delayed increase in WT animals. The SOD2 enzyme activity was decreased in $SOD2^{-/+}$ mice. These results are consistent with the reports showing that deficiency of SOD2 activity increases superoxide production after permanent MCAO in normoglycemic $SOD2^{-/+}$ mice, whereas overexpression of SOD2 prevents HG-induced DNA strand breaks and ischemic brain damage by reducing apoptosis [5, 15, 17, 18]. Likewise, the superoxide production observed in the WT may be due to the slow evolution of infarction in these animals. This highlights the importance of SOD2 in reducing mitochondrial originated superoxide levels during cerebral ischemia and indicates oxidative stress as a primary cause to determine the functional status and ischemic susceptibility of affected areas in hyperglycemic $SOD2^{-/+}$ animals.

The superoxide generation produces DNA lesions by oxidizing deoxyguanosine to 8-OHdG and is an important step in the induction of cell death in the acute postischemia/reperfusion period [19]. The specific accumulation of 8-OHdG was observed in affected ischemic areas differentially and particularly stained the damaged DNA. The number of cells containing damaged DNA was markedly higher in $SOD2^{-/+}$ animals. The immunoreactivity of 8-OHdG was confined to the nuclei and also colocalized with the oxidized DHE (Fig. 1e), indicating the significant contribution of superoxide radicals in DNA lesions and subsequent cell damage.

The synthesis and removal of damaged/aged mitochondria are continuous processes regulated by autophagy. The inhibition of basal autophagy by knocking out the *Atg5* (autophagy-related 5) gene in mice is associated with progressive deficits in motor function and cellular accumulation of ubiquitin-positive inclusion bodies, thereby resulting in neuronal degeneration [6]. This can adversely influence the outcome of stroke. On the other hand, excessive activation of autophagy exacerbates the cell self-cannibalism and cell death [7]. In the present study, we observed increased levels of autophagy markers Beclin 1, DRAM, and LC3 in the cortex of WT animals (Fig. 5). This is in agreement with the reports that suggest the up-regulation of Beclin 1 and related changes in LC3 following temporary MCAO in the ischemic penumbra [9]. Increased levels of these markers reflect an enhanced autophagy either as a mechanism to recycle injured cells or to reduce damage [9]. In contrast, autophagy regulators either are maintained at a basal level (DRAM and LC3) or moderately decreased (Beclin 1) in the cortex of $SOD2^{-/+}$ mice. The autophagy factors are generally suppressed in the striatum of the WT animals, with the exception of DRAM, which showed an increase at 24 h. This suppressed level may be correlated to the increased damage in the striatum as compared with the damage to the cortex. Interestingly, all three markers were significantly suppressed in the striatum of hyperglycemic $SOD2^{-/+}$ mice, with the most pronounced suppression observed in LC3. Therefore, the postischemic levels of LC3 in $SOD2^{-/+}$are less than one fourth of the levels in WT counterparts. Again, such severe suppression of autophagy activity in the $SOD2^{-/+}$ mice impede the clearance of damaged subcellular organelles, thereby increasing the burden of extra stress to already stressed cells, which would unfavorably influence the stroke outcome.

The overall results of the present investigation indicate that SOD2 suppression combined with HG severely affects the ischemic brain damage through oxidative stress in knockout as compared to WT animals. Moreover, the increased damage in hyperglycemic $SOD2^{-/+}$ mice is linked to suppression of autophagy processes.

Acknowledgments

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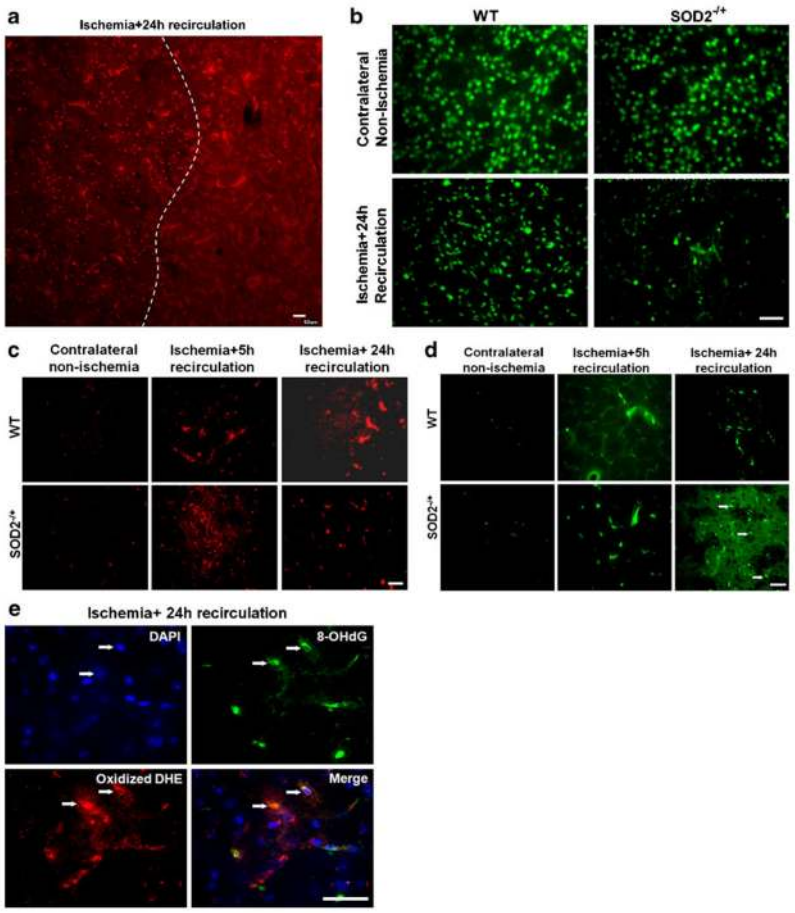

Fig. 1.

SOD2 deficiency accelerated infarct development in hyperglycemic knockout mice after 30 min of cerebral ischemia followed by recirculation. **a** Propidium iodide nuclear staining depicts the damaged area in contrast to the intact neighboring tissue. **b** *Upper panel*, Microphotographs of NeuN immunostaining shows the neuronal population in nonischemic contralateral hemisphere. *Lower panel*, NeuN staining in ischemic ipsilateral hemisphere at 24 h of recovery. Hyperglycemia caused moderate neuronal degeneration at 24 h of recirculation in WT mice, whereas NeuN immunoreactivity displayed an abnormal distribution and high degree of neuronal degeneration in $SOD2^{-/+}$ mice as compared to WT. Final magnification = ×200. **c** Microphotographs showing the cellular localization of oxidized DHE in cortical area of WT (upper panel) and $SOD2^{-/+}$ mice (lower panel). Dihydroethidium signals are detected in nuclear area as well as diffusely scattered around the nuclei. **d** Oxidative DNA damage indicated by immunofluorescence staining for 8-OHdG in the cortical area of hyperglycemic WT and $SOD2^{-/+}$ mice at 5 and 24 h of recirculation. Micrograph shows marked increase in 8-OHdG fluorescence signal at 24 h in $SOD2^{-/+}$ mice (lower panel) as compared to WT (upper panel) at the same time point. **e** Double staining of 8-OHdG and DHE in the ipsilateral ischemic cortical area of $SOD2^{-/+}$ mice at 24 h of recirculation. 8-OHdG fluorescence signal colocalized with the oxidized DHE fluorescence, suggesting the involvement of oxidative stress in hyperglycemic ischemic brain damage. Final magnification = ×400. Scale=50 μm

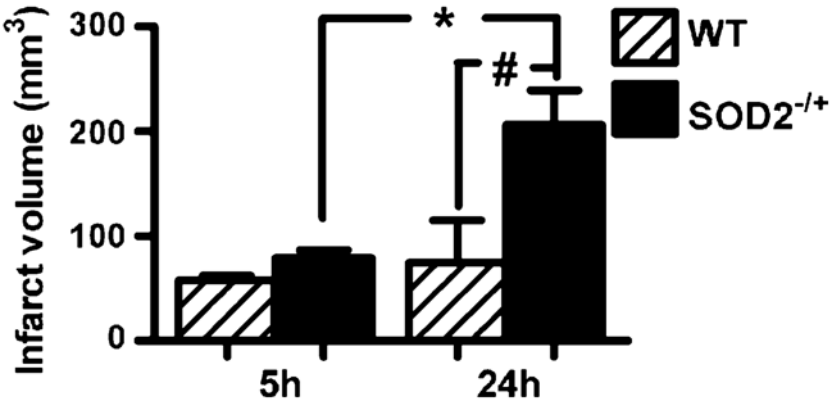

Fig. 2.

Bar graph showing infarct volume measured at 5 and 24 h of recirculation in both WT and $SOD2^{-/+}$ groups. Infarct volume increased significantly ($p<0.05$) at 24 h of recirculation in $SOD2^{-/+}$ mice

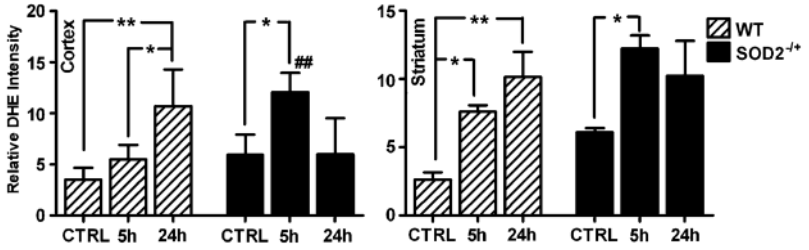

Fig. 3.

Quantitative assay of oxidized DHE fluorescent signal in the ischemic cortex and striatum of hyperglycemic WT and $SOD2^{-/+}$ mice after 30 min of occlusion followed by 5 and 24 h of recirculation. Data represented as mean ± SD. Superoxide elevated at 24 h in WT and 5 h in $SOD2^{-/+}$ mice. $^{*}p<0.05$ and $^{**}p<0.01$ compared with controls and $^{\#\#}p<0.05$ compared with the identical reperfusion group in WT and $SOD2^{-/+}$ animals

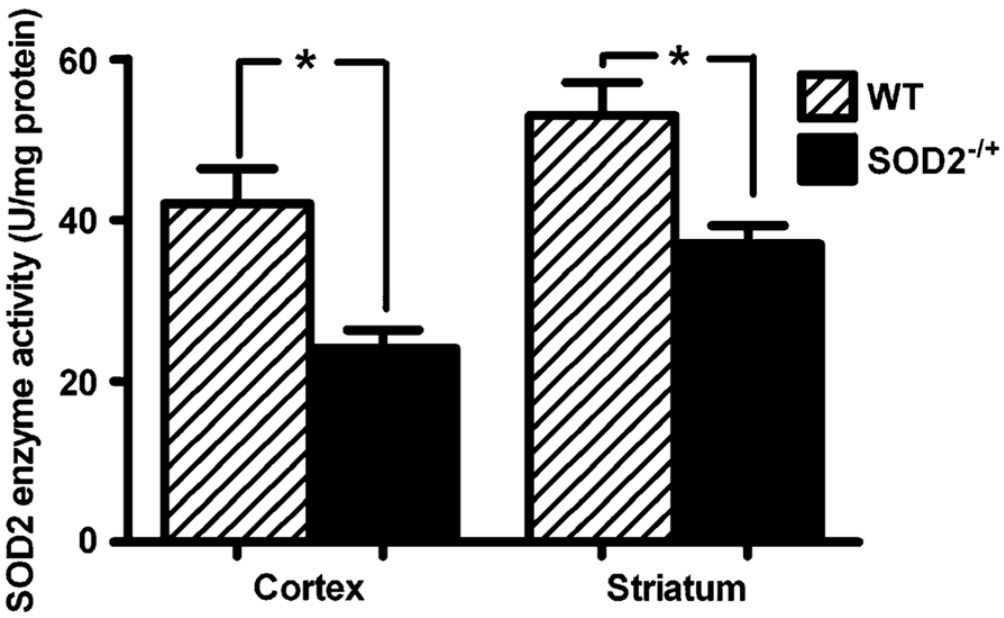

Fig. 4.

SOD2 enzyme activity measured in brain samples collected from WT and $SOD2^{-/+}$ mice demonstrates that SOD2 enzyme activity reduced almost to half in $SOD2^{-/+}$ as compared to WT control mice. *p<0.05

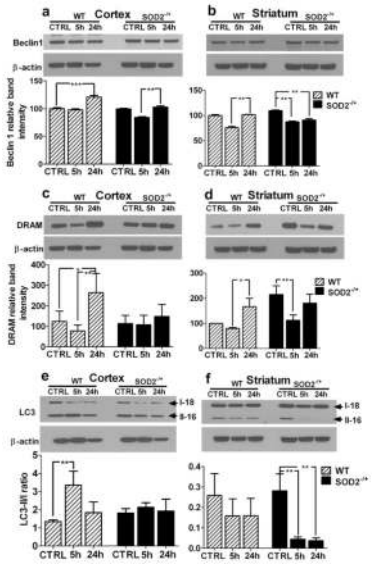

Fig. 5.

Immunoblot and quantitative analysis of Beclin 1 (**a**, b), DRAM (**c**, **d**), and LC3 (**e**, **f**) levels in the ipsilateral ischemic cortex and striatum of hyperglycemic WT and SOD2$^{-/+}$ mice at different time points after recirculation. Note that levels of these proteins either remained unchanged (cortex) or are significantly suppressed (striatum) in SOD2$^{-/+}$ mice. Data are expressed as mean ± SD, and the statistical significance is represented as increase. $^{**}p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$ compared with controls

Table 1

Experimental groups

	Sham	30 min+5 h recirculation	30 min+24 h recirculation
Morphological studies			
WT	4	8	8
$SOD_2^{-/+}$	4	8	8
Protein and enzyme studies			
WT	4	4	4
$SOD_2^{-/+}$	4	4	4