Neuroprotection of antioxidant enzymes against transient global cerebral ischemia in gerbils

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Abstract: Experimentally transient global cerebral ischemia using animal models have been thoroughly studied and numerous reports suggest the involvement of oxidative stress in the pathogenesis of neuronal death in ischemic lesions. In animal models, during the reperfusion period after ischemia, increased oxygen supply results in the overproduction of reactive oxygen species (ROS), which are involved in the process of cell death. ROS, such as superoxide anions, hydroxyl free radicals, hydrogen peroxide and nitric oxide are produced as a consequence of metabolic reactions and central nervous system activity. These reactive species are directly involved in the oxidative damage of cellular macromolecules such as nucleic acids, lipids and proteins in ischemic tissues, which can lead to cell death. Antioxidant enzymes are believed to be among the major mechanisms by which cells counteract the deleterious effect of ROS after cerebral ischemia. Consequently, antioxidant strategies have been long suggested as a therapy for experimental ischemic stroke; however, clinical trials have not yet been able to promote the translation of this concept into patient treatment regimens. This article focuses on the contribution of oxidative stress or antioxidants to the post-ischemic neuronal death following transient global cerebral ischemia by using a gerbil model.

Key words: Mongolian gerbil, Global cerebral ischemia, Neuronal death, Reactive oxygen species, Antioxidants

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Introduction

Global cerebral ischemia occurs when the blood supply to the entire brain or a large part of the brain is disrupted, resulting in the tissue deprivation of oxygen and glucose that may give permanent brain damage. In adult humans, global cerebral ischemic injury occurs in some conditions like cardiac arrest, coronary artery bypass surgery, cardio-respiratory failure, and others leading to the drastic reduction of the blood flow to the brain [1]. Global cerebral ischemia, for a short period of time, results in selective neuro-degeneration in vulnerable brain areas, such as the CA1 region of the hippocampus [2, 3]. The underlying mechanisms of CA1 neuronal loss following global cerebral ischemia may involve excitotoxicity, reactive oxygen species (ROS), inflammation, and apoptosis [4]. However, the exact mechanisms underlying neuronal damage including delayed neuronal death after ischemia-reperfusion injury have not been elucidated yet. Among these, cerebral ischemia-reperfusion in particular are responsible for oxidative stress due to the generation of free radicals [5], which culminates into deleterious effects during pathogenesis [6]. Therefore, oxidative stress has emerged as an important underlying factor in the delayed neuronal death induced by global cerebral ischemia [6, 7].
Animal Models Inducing Global Cerebral Ischemia

Various methods have been used to induce global cerebral ischemia in experimental animal models including decapitation without recirculation [8], neck tourniquet [9], bilateral common carotid artery occlusion (2-VO) [10] and four-vessel occlusion (4-VO) [11]. Cardiac arrest achieved through ventricular fibrillation has been also used to produce global cerebral ischemia [12, 13]. One of the various animal models to produce global cerebral ischemia, experimentally induced transient cerebral ischemia has been widely induced by the occlusion of related artery or arteries. Pulsinelli and Brierley [14] developed a 4-VO model to provide a method of reversible global cerebral ischemia in rats. This model has been also utilized for morphological and metabolic studies in anesthetized rats [15, 16]. The 4-VO model involves a two-stage procedure to produce global cerebral ischemia. In the first stage, on the day before the experiment, non-traumatic claspers are placed loosely around each common carotid artery and exteriorized in the neck of the animal. The vertebral arteries are then electro-cauterized via the alar foramina of the first cervical vertebra. On the second day, the common carotid arteries are occluded while the animal is awake, and ischemia is produced. This procedure must result in a complete loss of the righting reflex for the animal to be included in the study. It can be utilized in either awake or anesthetized animals, which makes it extremely useful. However, it is not an easy model to use, and there has been much variability in results between laboratories. The success rate of the model is approximately 50% to 75%; however, the effects of ischemia are quite variable between rat strains. It is believed that this variability may be the result of variability of collateral circulation present in each strain [11]. As alternative to the 4-VO model, global cerebral ischemia has been widely induced by the occlusion of the two common carotid arteries, i.e., by 2-VO together with the induction of hypotension for a limited time period. In this forebrain ischemia model, selective injury in the CA1 of the hippocampus, the caudate putamen and neocortex is observed [17]. Mongolian gerbils have a unique and convenient vascular anatomy, thus, they have been used for global ischemia studies. The unique anatomical feature of the Mongolian gerbil is that they do not have the posterior communicating artery, which connects the carotid and vertebrobasilar arterial system [18]. Thus, the Mongolian gerbil has been used as a good 2-VO animal model to investigate the mechanisms of selective neuronal death following transient global cerebral ischemia. In this animal, bilateral common carotid arteries occlusion reduces cerebral blood flow to almost zero in most gerbils [10]. When both the common carotid arteries are transiently occluded for 5 minutes, neuronal death in the hippocampus is observed [19]. These changes are also accompanied by various behavioral impairments as spatial learning and memory deficits that vary with the degree and the duration of occlusion. The major advantages of the gerbil model are relative simplicity of the surgical procedure that allows for the study regarding transient global cerebral ischemia, and are easily suitable for long-term studies because of long survival after ischemic injury. An important feature of global cerebral ischemic damage is the vulnerability of specific neuronal populations. Especially, pyramidal neurons in the hippocampal CA1 region do not die immediately but rather survive over several days. One clear aspect is that the model can be physiologically controlled so that the resulting injury is reproducible and variability as limited as possible. In the Mongolian gerbil, prolonged global cerebral ischemia kills about 96% of the CA1 pyramidal neurons after 4 days [20]. Delayed and rapid neuronal death appears to follow a similar sequence of cellular events. The lag between an ischemic episode and neuronal death depends upon the severity and/or duration of ischemia [21]. Finally, it is important to mention the Mongolian gerbil because this animal has been widely used as a good animal model of transient global cerebral ischemia [19].

Generation of Oxygen Free Radicals

Oxygen free radicals are molecular species that contain one or more unpaired valence electrons and they are highly active with other molecules, such as DNA and lipids, pairing with their single electrons and causing oxidation of those molecules [22]. Several oxygen free radicals, including superoxide anions (O$_2^•$), hydroxyl radical (•OH), nitric oxide (NO•) and hydrogen peroxide (H$_2$O$_2$) have been implicated in the development of many neurological disorders and brain dysfunctions [23, 24]. These radicals are known to initiate lipid peroxidation and to cause protein oxidation and DNA damage in cells.

O$_2^•$ is formed when oxygen acquires an additional electron, leaving the molecule with only one unpaired electron [25]. This process is mediated by pro-oxidant enzymes such as nicotine adenine dinucleotide phosphate (NAD(P)H)
oxidase and xanthine oxidase. The major site for producing
O$_2^{•−}$ is mitochondria, which are the machinery of the cells
to produce adenosine triphosphate. Normally, electrons are
transferred through mitochondrial electron transport chain
for the reduction of oxygen to water; however, approximately
2% to 5% of electron flow is used to produce them [6]. O$_2^{•−}$ is
converted into H$_2$O$_2$ by the action of superoxide dismutases
(SODs). SODs detoxify O$_2^{•−}$ to H$_2$O$_2$ (O$_2^{•−}$ + 2 H$^+$→H$_2$O$_2$),
which is further converted to H$_2$O by catalase or glutathione
peroxidase (GPx) (2H$_2$O$_2$→2H$_2$O+O$_2$) [6]. O$_2^{•−}$ itself can also
react with H$_2$O$_2$ and generate OH$^−$ [26].

On the other hand, •OH is a neutral form of hydroxide ion
and has a high reactivity, making it a very dangerous radical
with a very short in vivo half-life [27]. Highly reactive •OH is
not produced as a by-product of any known enzymatic
reaction; however, it is produced from H$_2$O$_2$ through Fenton
reaction (H$_2$O$_2$+Fe$^{2+}$→OH$^−$+Fe$^{3+}$+•OH) and Haber-Weiss
reaction (O$_2^{•−}$+H$_2$O$_2$→•OH+HO$^+$+O$_2$) or by peroxynitrite
(ONOO$^−$) [28, 29]. Once formed, •OH reacts almost instantaneously
with many cellular components, including polyunsaturated
fatty acids of membrane lipids. The initial reaction of •OH with polyunsaturated fatty acids produces an alkyl
radical, which in turn reacts with molecular oxygen to form
a peroxyl radical (ROO$^•$). The ROO$^•$ can abstract hydrogen
from an adjacent fatty acid to produce a lipid hydroperoxide
(ROOH), propagating a chain reaction of lipid oxidation [30].

NO$^•$ is formed by the enzymatic oxidation of L-arginine to
citrulline by nitric oxide synthases and serves as an important
regulator of vascular response and neuronal signaling [31].
NO$^•$ has a half-life of only a few seconds in an aqueous
environment. NO$^•$ has greater stability in an environment
with a lower oxygen concentration. However, it readily dif-
fuses through the cytoplasm and plasma membranes, since
it is soluble in both aqueous and lipid media [32]. NO$^•$
has effects on neuronal transmission as well as on synaptic
plasticity in the central nervous system. O$_2^{•−}$ does not directly
induce lipid peroxidation; however, it can react with NO$^•$
to produce ONOO$^−$, which is a strong oxidative radical that
causes protein nitration and lipid peroxidation, which leads
to cell dysfunction [29, 33]. Lipid peroxidation is not the sole
route of cellular damage initiated by •OH and ONOO$^−$ as
these species also oxidize proteins and DNA [30].

H$_2$O$_2$ easily diffuses across the plasma membrane. H$_2$O$_2$ is
also produced by xanthine oxidase, amino acid oxidase, and
NAD(P)H oxidase [34, 35]. In the presence of chloride ion,
H$_2$O$_2$ is converted to hypochlorous acid (HOCl). The HOCl
is highly oxidative and plays an important role in killing
pathogens in the airways [36]. However, HOCl can react with
DNA, induce DNA-protein interactions, produce pyrimidine
oxidation products and add chloride to DNA bases [37]. The
accumulation of H$_2$O$_2$ is reported to impair mitochondrial
function. H$_2$O$_2$ is a longer-lasting reactive species, electrically
neutral, able to pass through cell membranes and more stable
than O$_2^{•−}$, •OH, and other ROS. Therefore, H$_2$O$_2$ may persist
for longer time after reperfusion to produce neuronal injury.

**Oxidative Stress by Global Cerebral Ischemia**

It has been well established that oxidative stress displays an
important role in the pathophysiology of cerebral ischemia
[38] and that the excessive production of ROS occurs during
cerebral ischemia and reperfusion [39]. Generally, alteration
in Ca$^{2+}$ homeostasis in the brain due to an increase in ROS
has been suggested to explain the adverse effects of cerebral
ischemia and reperfusion. The activation of N-methyl-D-
aspartate receptor by glutamate increases Ca$^{2+}$ influx as well
as the activation of neuronal nitric oxide synthase, which
generates nitric oxide (NO) from l-arginine. Under these
conditions, the oxidation of xanthine and hypoxanthine
by xanthine oxidase is accompanied with the generation of
O$_2^{•−}$ and H$_2$O$_2$ [40, 41]. NO reacts with O$_2^{•−}$ to generate the
harmful radical ONOO$^−$ that contributes to neuronal injury
deruring reperfusion. Oxidative stress and increased Ca$^{2+}$
cause the opening of mitochondrial permeability transition pore.
This allows an entry of water and solutes from the cytoplasm
resulting in mitochondrial swelling and damage. The genera-
tion of ROS also occurs in the cytoplasm through the action
of NAD(P)H oxidase. The resulting oxidative stress can out-
weigh antioxidant defenses and lead to cell death because
oxidative stress can cause a widespread damage to cellular
components such as DNA, lipids, and proteins that ultimately
promote cellular damage and death during cerebral ischemia
and reperfusion [6, 39].

**Antioxidants in Global Cerebral Ischemic Injury**

Global cerebral ischemic injury is associated with the
oxidative stress caused by the overproduction of ROS and
other free radicals [42, 43]. Excessive ROS production is
followed by dysfunctions of important redox-sensitive en-
zymes, membrane receptors and ion channels, DNA da-
mage, membrane lipid peroxidation, and cytochrome c release from mitochondria, which activate caspases that aggravate cell death after ischemia and reperfusion injury [44]. Thus, neuronal defense mechanisms against oxidative stress have focused on antioxidant systems. Antioxidant system may be classified into enzymatic and non-enzymatic antioxidants. Several enzymatic antioxidants exist that convert ROS into less noxious compounds, for example, SODs, GPx, and catalase. Collectively, these enzymes provide a first line of defense against O$_2^•^\text{−}$ and •OH. Amongst these, copper, zinc-superoxide dismutase (CuZn-SOD, also called SOD1) provides a defense system against oxidative stress by catalyzing the dismutation of O$_2^•^\text{−}$ into O$_2$ and H$_2$O$_2$ [45]. It has been reported that SOD1 overexpression prevents neuronal injury in the hippocampal CA1 region in a murine cardiac arrest model. In addition, Kim et al. [46] have reported that increased SOD1 reduces oxidative DNA damage and subsequent DNA-fragmented cell death after photothrombotic ischemia in SOD1 transgenic mice. However, some investigators have obtained various degrees in success and failure when free non-modified SOD1 is used to ameliorate ischemic brain injury [47]. The extremely short half-life of SOD1 in circulating blood and its failure in passing the blood-brain barrier make it difficult to use enzyme therapy in cerebral ischemia. To overcome this shortness, substantial progress has been made in the development of cell-penetrating peptide-based extracellular and intracellular limitation. Polyethylene glycol-conjugated SOD1 has been successfully used to reduce infarct volume in animals that have been subjected to global cerebral ischemia [48]. Liposome-entrapped SOD1 has an increased half-life (4.2 hours), blood-brain barrier permeability, and cellular uptake, and it has proved to be an effective treatment in reducing the severity of global ischemic brain injuries [49]. Especially, it is reported that that the administration of PEP-1 SOD1 fusion protein significantly inhibits neuronal death in the gerbil hippocampal CA1 region induced by transient global cerebral ischemia [50, 51]. Similarly, manganese superoxide dismutase (Mn-SOD2, called SOD2) in mitochondria converts superoxide produced in the mitochondrial matrix into H$_2$O$_2$ [52]. Increased SOD2 immunoreactivity is consistently observed with an antioxidant response to the increased superoxide formation caused by hyperglycemic cerebral ischemia [53, 54]. Increased SOD2 immunoreactivity is also demonstrated in a study by Bidmon et al. [55]; a large region with the increased SOD2 immunoreactivity is observed following a small cortical ischemic lesion, indicating a widespread response to a well-localized insult. In addition, transgenic mice that over-express SOD2 suffer a less ischemic brain damage compared with the wild-type mice [56]. Therefore, SOD2 has been demonstrated to be a key enzyme in protecting the brain from ischemia and reperfusion injury. Recently, it has been reported that increased and longer maintained SODs levels in the young hippocampal CA1 region may provide an evidence to explain more delayed and less neuronal death compared with those in the adult CA1 region after 5 min of transient cerebral ischemia [57, 58]. Furthermore, the regulation of SOD2 activity displays a neuroprotective effect against cerebral ischemic damage [59]. Thus, there is a clear need for a systematic study to determine the exact role of SODs in protection against ischemia and reperfusion injury. In consistent to this, an increasing number of investigators have demonstrated the importance of SODs in protecting the brain from global ischemia and reperfusion injury using a gerbil model [60-66].

GPx is another important enzyme contributing to H$_2$O$_2$ scavenging. In several in vitro studies, GPx confers a greater protection against oxidative stress than SOD. Antioxidant treatment should prevent the loss of GPx activity by effectively scavenging the excess ROS. Many researchers have reported that the regulation of GPx activity displays neuroprotective effects in the gerbil brain following global cerebral ischemia and it is involved in the control of cellular damage after ischemic insults [60, 62, 65, 67, 68].

On the other hand, catalase is a membrane bound enzyme that is present in peroxisomes; however, its activity has been observed in mitochondrial matrix [69]. Catalase is an important enzyme for the maintenance of intracellular concentration of reduced glutathione and has a crucial role as a free radical scavenger [70]. Decrease in the level of catalase is noted in the brain of ischemic rats. However, the role of catalase enzyme during global brain ischemia is controversial; a few studies have suggested the decrease of catalase activity in the brain induced by global brain ischemia [62, 63, 71, 72], while others have suggested the increase of catalase activity in ischemic brain tissue [73]. Recently, it has been reported that neuronal damage in the young gerbil hippocampal CA1 region induced by transient cerebral ischemia is much more delayed and less severe than that in the adult, showing that CAT immunoreactivity and its protein level are increased in the young CA1 region after ischemia and reperfusion injury [58]. Especially, the administration of PEP-1-catalase fusion
protein displays significant neuroprotection in the CA1 region of the hippocampus after transient cerebral ischemia [74]. Similarly, several studies have demonstrated that an enhanced expression of catalase significantly attenuates the injury of brain tissues after global ischemia in gerbils [60, 62, 68, 75-77].

In addition to these major enzymes, thioredoxin (Trx) and peroxiredoxins (Prxs) redox system exerts important roles in antioxidant regeneration and the regulation of intracellular ROS level [78-80]. In particular, Trx/Prx redox system is a major route for removing H₂O₂ in cellular organs [81]. Recent studies have reported that Trx/Prx redox system is a strong neuroprotective effect in cerebral ischemia [82, 83]. Trx is well known as a radical scavenger [84, 85]. A recent study has shown that Trx is a neuroprotective factor against neuronal damage subsequent to ischemia and oxidative stress [86] and that Trx is a trophic factor for neuronal homeostasis [87]. Among Trx subtypes, TrxR2 plays a key role in the mitochondrial Trx2/Prx3 redox system via a reduction of oxidized Trx2 utilizing NADPH electron to protect cells from an oxidative injury [79, 88]. Recently, it has been reported that Trx2 immunoreactivity in CA1 pyramidal neurons is increased 30 minutes after ischemia and reperfusion, decreased 6 hours after ischemia and reperfusion and increased again 1 day after ischemia and reperfusion [82]. This finding suggests that the decrease of Trx2 in the CA1 pyramidal neurons at 6 hours after ischemia and reperfusion may be associated with the depletion of antioxidants because of distinct increases in ROS in the CA1 region after ischemia and reperfusion. However, the increase of Trx2 in the CA1 pyramidal neurons at 1 day after post-ischemia may be associated with compensatory mechanisms against ischemic damage. Prxs are also considered efficient enzymes in removing low levels of H₂O₂ because they have a high affinity toward H₂O₂ [89, 90]. Prx family is divided into six subtypes: Prx1–4 have two conserved cysteine residues, whereas Prx5 and Prx6 have one of the cysteine residues involved in peroxidase activity [90, 91]. It has been reported that Prx3 has a potential to play a major role in mitochondrial dependent antioxidant effects [92, 93]. Recently, it has been reported that Prx3 immunoreactivity and protein level significantly increase in the pyramidal neurons of the hippocampal CA1 region 1 day after ischemia and reperfusion [82]. This indicates that Prx3 increases to remove ROS in the ischemic CA1 region because Prx3 can scavenge not only H₂O₂, in cooperation with thiol, but also ONOO⁻ by itself [94]. In addition, it is reported that mitochondrial Trx2 interacts closely with Prx3 in mitochondria, and the increase of interaction between Trx2 and Prx3 protects cells from oxidative stress [79]. Furthermore, it is demonstrated that the co-treatment of Prx3 and Trx2 more efficiently protects CA1 pyramidal neurons from ischemia and reperfusion injury in the gerbil hippocampus induced by transient global cerebral ischemia [82].

Conclusion

Oxidative stress can arise from the overproduction of ROS that shift the balance between oxidant/antioxidant statuses leading to potential cellular damage, and plays a central role in the initiation of neuronal damage in global cerebral ischemia. The development of novel antioxidant treatments is based on the complete understanding of the role of oxidative stress in global cerebral ischemic injury. However, little is known about the formation of ROS in brain ischemia due to innate difficulty in studies on oxidative stress; however, knowledge toward blocking the sources of ROS will be extremely useful in the design of effective therapies against global cerebral ischemia.

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