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Thioredoxin 1 and glutaredoxin 2 contribute to maintain the phenotype and integrity of neurons following perinatal asphyxia

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ABSTRACT

Background: Thioredoxin (Trx) family proteins are crucial mediators of cell functions via regulation of the thiol redox state of various key proteins and the levels of the intracellular second messenger hydrogen peroxide. Their expression, localization and functions are altered in various pathologies. Here, we have analyzed the impact of Trx family proteins in neuronal development and recovery, following hypoxia/ischemia and reperfusion.
Methods: We have analyzed the regulation and potential functions of Trx family proteins during hypoxia/ischemia and reoxygenation of the developing brain in both an animal and a cellular model of perinatal asphyxia. We have analyzed the distribution of 14 Trx family and related proteins in the cerebellum, striatum, and hippocampus, three areas of the rat brain that are especially susceptible to hypoxia. Using SH-SY5Y cells subjected to hypoxia and reoxygenation, we have analyzed the functions of some redoxins suggested by the animal experiment.
Results and conclusions: We have described/discovered a complex, cell-type and tissue-specific expression pattern following the hypoxia/ischemia and reoxygenation. Particularly, Grx2 and Trx1 showed distinct changes during tissue recovery following hypoxia/ischemia and reoxygenation. Silencing of these proteins in SH-SY5Y cells subjected to hypoxia-reoxygenation confirmed that these proteins are required to maintain the normal neuronal phenotype.
General significance: These findings demonstrate the significance of redox signaling in cellular pathways. Grx2 and Trx1 contribute significantly to neuronal integrity and could be clinically relevant in neuronal damage following perinatal asphyxia and other neuronal disorders.

1. Introduction

Brain damage resulting from an ischemic event in the fetus or newborn infant (also known as perinatal asphyxia, PA) remains a major cause of neonatal death and neurological deficits in children. Cerebral palsy, mental retardation, and epilepsy are among the most common complications of PA [1–4]. The incidence of severe PA is estimated to be about 1/1000 live births in developed countries, and 5–10/1000 live births in developing countries [5]. This clinical picture has been extensively reproduced in murine models of PA consisting of the ligation of the right common carotid artery followed by an exposure to an oxygen deprived environment at postnatal day 7 [3,6–10]. The model for common carotid artery ligation used in the present study has been previously developed and validated by Lopez-Aguilera et al., 2012 [7]. It has been extensively investigated and is generally accepted that the rat brain at the age of 7 days is histologically similar to that of a 32–34 week gestation human fetus or newborn infant, i.e., the cerebral cortical neuronal layering is complete, the germinal matrix is involuting, and white matter has undergone some myelination [11–13].

Damage caused by the effects of reactive oxygen species (ROS) has been proposed as an important cause of neuronal death and consequently brain damage after hypoxia-ischemia [14,15]. In aerobic cells, ROS are produced within the cytoplasm and mitochondria [16]. Under physiological conditions, they are part of

Abbreviations: CCA, Common carotid artery; cer, Cerebellum; CNS, Central nervous system; ELISA, Enzyme-linked immunosorbent assay; Grx, Glutaredoxins; hip, Hippocampus; P7, Postnatal day 7; PA, Perinatal asphyxia; PBS, Phosphate buffer saline; Prx, Peroxiredoxins; ROS, Reactive oxygen species; SEM, Standard error of the mean; str, Striatum; Trx, Thioredoxins; TrxR, Thioredoxin reductase

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specific signaling processes, regulating for instance developmental processes, cell proliferation, differentiation and apoptosis [16–18]. They are locally produced by specific enzymes and are rapidly degraded by others, affecting specific target molecules and signaling pathways [16,18]. However, excessive production of specific ROS, e.g. hydroxyl radicals, can lead to oxidative and irreversible damage to macromolecules and has been linked to various pathological conditions, including hypoxia/ischemia and reoxygenation [14,19,20]. Several therapeutic approaches to counteract the effects induced by ROS during hypoxia/ischemia and especially reoxygenation have been proposed. However, no neuroprotective agent has been proven safe and effective in the protection of neonates from neurological sequels following an ischemic insult beside hypothermia in some specific cases [21]. Therefore, it is essential to gain more insights into biochemical and cellular mechanisms of neuronal injury induced by PA to identify potential therapeutic targets, compounds and strategies [22].

The members of the thioredoxin (Trx) family are small proteins that present the characteristic Trx fold, and the cysteine(s)-containing active site motif, which is crucial for the transfer of electrons and the general oxidoreductase activity [23,24]. The Trx superfamily of proteins includes thioredoxins (Trxs) glutaredoxins (Grxs) and peroxiredoxins (Prxs) [25]. These proteins share a common structural motif, the Trx fold, consisting of a central core of four-stranded β-sheets surrounded by three or more α-helices. The thiol-disulfide oxidoreductases Trx and Grx also share the conserved active site Cys-X-X-Cys that enables them to catalyze thiol-disulfide exchange reactions [25]. Peroxiredoxins are thiol-dependent peroxidases [23,24]. Trx was first described as a hydrogen donor for ribonucleotide reductase from *Escherichia coli* [26]. Later on, these proteins were recognized as key regulators in the cell response to redox signals [24]. Mammals possess two principal Trx isoforms, the cytosolic Trx1 and the mitochondrial Trx2. These proteins are reduced by thioredoxin reductases [27,28], the cytosolic TrxR1 and the mitochondrial TrxR2, respectively. TrxRs are homodimeric flavo- and seleno-enzymes which are able to reduce Trxs, as well as peroxides and other compounds [29–31]. Mammalian genomes encode various Grxs, among them the cytosolic Grx1 and Grx3, the mitochondrial Grx5 and Grx2a, and the least frequent nuclear/cytosolic isoforms of Grx2 (Grx2b and Grx2c, respectively) [24,32]. Grxs are reduced by glutathione, with electrons from glutathione reductase and NADPH [32]. Peroxiredoxins (Prxs) reduce different peroxides and peroxynitrite [33]. Prx1 to Prx4 belong to the typical 2-Cys Prx class, Prx5 is an atypical 2-Cys Prx, Prx6 is a 1-Cys Prx [34,35]. Prx1, Prx2 and Prx5 can be found in the nucleus and cytoplasm, whereas Prx3 and Prx5 are localized in mitochondria. Prx4 and Prx6 can be found in the cytoplasm. In addition, Prx1, Prx2 and Prx4 were shown to be secreted from the cell [34,36].

Here, we have analyzed the regulation and potential functions of Trx family proteins on the effects of hypoxia/ischemia and reoxygenation on the developing brain both *in vivo* and in a neurological cell culture model.

2. Experimental procedures

2.1. Animals

All experiments were conducted according to the principles of the Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996), and approved by the Institutional Animal Care and Use Committee at the University of Buenos Aires (School of Medicine). All efforts were made to reduce the number of animals used and to minimize suffering. Pregnant rats were obtained from the School of Veterinary Sciences' central *vivarium* at the University of Buenos Aires. All animals were kept in a temperature (21 ± 2 °C) and humidity (65 ± 5%) controlled environment on a 12 h light/dark cycle. Animals had *ad libitum* access to food (Purina chow) and tap water.

2.2. Model for common carotid artery ligation

The model for common carotid artery ligation used in this study, has been previously developed and validated by Lopez-Aguilera et al. [7]. Seven days of age (P7) male Sprague–Dawley rats were anesthetized with a combination of ketamine (40 mg/kg) and xylazine (4 mg/kg). The animals were placed on a heat plate ensuring a constant body temperature of 37 °C. An incision on the right side of the neck was performed exposing the right common carotid artery (CCA), which was then isolated from nerve and vein and permanently ligated with a 6–0 surgical silk (carotid group n = 14). The wound was then sutured and the animals were returned to their dams for recovery for 4–5 h. Subsequently, pups were placed in a stoppered 1 L glass jar and exposed to 100% nitrogen (delivered at 3 L per minute) for 3 min to induce anoxia. The jar was partially submerged in a 37 °C water bath to maintain a constant thermal environment. In sham operated rats (sham group n = 12) the right CCA was exposed but not ligated and no nitrogen was supplied. At 21 days of age (14 days post-surgery), when the synaptic connections are well established in the rat [37], animals were sacrificed.

2.3. Brain dissection

Brains were dissected as previously described in Chiu et al. [38]. After the animals were sacrificed at 21 days of age, brains were isolated and dissected at 4 °C. Brains were cut in half into right and left hemisphere. Three cuts were performed in each hemisphere. The first cut was situated at the *Genu* of the *Corpus callosum* (~Bregma + 1.0 mm based on the rat brain atlas by Paxinos and Watson [39]), the second cut was situated at the anterior tip of the *Fornix* (approx. Bregma − 1.0 mm based on the rat brain atlas by Paxinos and Watson [39]). The third cut was situated at the 4th ventricle (~Bregma − 8.0 mm based on the rat brain atlas by Paxinos and Watson [39]). The striatum was dissected between the first and second cut with the help of two Miltex Iris Tissue Forceps, and stored at − 80 °C. The hippocampus was dissected after the second cut. The midbrain was removed to expose the hippocampus, which was then dissected from the cortex using two tissue forceps, and stored at − 80 °C. Finally, the cerebellum was dissected after the third cut, that was separated from the ponds and medulla oblongata with the help of two tissue forceps, and stored at − 80 °C.

2.4. Cell culture

SH-SY5Y cells were cultivated in MEM (PAA), supplemented with 10% FCS good (PAN), 2 mM L-glutamine, 100 units/ml penicillin and 0.1 mg/ml streptomycin at 37 °C in a 90% humidified atmosphere containing 5% CO_2. SH-SY5Y cells were transiently transfected with 15 μg specific, custom-made siRNA (Eurogentech) for Trx1 (sense: GUA GAU GUG GAU GAC UGU C, antisense: GAC AGU CAU CCA CAU CUA C) and Grx2 (sense: GGU GCA ACU GAC ACU CAU; antisense: UAU GAG UGU CAG UUG CAC). Unspecific control (scr)bled siRNA (sense: CAU UCA CUC AGG UCA UCA, antisense: CUG AUG ACC UGA GUG AAU) was used as control. Five million SH-SY5Y cells were resuspended in electroporation buffer (21 mM HEPES, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM D-glucose, pH 7.15), mixed with siRNA and were electroporated in a total volume of 550 μl at 230 V, 1050 microfarads and 500 ohm. FCS was immediately added to the transfected cells and they were seeded out in 1:5 conditioned medium (1 part old and 4 parts fresh medium). To sufficiently knock-down Trx1 and Grx2, cells were transfected a second time after 3 days.

Twenty-four hours following the second transfection, cells were incubated under 1% O_2 and 5% CO_2 at 37 °C (hypoxia) in a CO_2 incubator (binder) for 24 h followed by a reoxygenation period of 24 or 48 h in an atmosphere containing 20% O_2 and 5% CO_2 at 37 °C. Cells were detached by trypsin treatment, washed with PBS, lysed in lysis buffer (10 mM