Expression of Thioredoxin Family Proteins During Perinatal Asphyxia

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Abstract: *Background:* Alterations in the gas exchange in the placenta and fetus lungs malfunction in humans can lead to perinatal asphyxia (PA). Every organ in the body is affected by PA, yet it is particularly deleterious for the central nervous system (CNS). The reoxygenation that follows a PA event produces a great quantity of ROS, which can ultimately lead to irreversible changes in the redox balance. Thioredoxins (Trxs) are a group of proteins that are closely involved in redox regulation and have been recognized as the most important regulators of the thiol redox state. In the present study, we analyze the changes in Trxs expression and distribution in hippocampus, striatum and cerebellum caused by PA, in 30 days-old rats.

Method: The model for common carotid artery ligation used in this study mimics PA in murine animals. On post-natal day seven, Sprague-Dawley rats were subjected to a ligation of the common carotid artery, followed by nitrogen exposure (HI group) or sham-operation procedures (ctrl group). Animals were euthanized, and the obtained samples were analyzed by Western blot, ELISA and immunohistochemistry.

Results and conclusions: Our data showed changes in the expression of Prx2, Trx1, Trx2, Grx2, Grx5 and TrxR1. This study shows the complexity of the expression and distribution of Trxs in the different areas of the CNS and sheds light on the importance of some of the Trxs family members in PA, and thus, as possible targets for therapeutic interventions.

Keywords: Hypoxia-ischemia, Reoxygenation, Common carotid artery occlusion, Thioredoxins, Perinatal asphyxia.

INTRODUCTION

Alterations in the gas exchange in the placenta and fetus lungs malfunction, between the 28th week and the 7th postnatal day in humans, can lead toperinatal asphyxia (PA) [1]. Severe hypoxia owing to an asphyctic event can lead to an ischemic insult due to a diminution of the cardiac function [2]. Ischemia consists in the reduction of irrigation in a tissue, causing a decrease in nutrients and the accumulation of cellular waste leading to a full metabolic failure. Every organ in the body is affected by PA, however it is particularly deletereous for the central nervous system (CNS) [3] where it has been known to generate long term changes and damages [4, 5]. The incidence of severe PA is estimated to be about 1/1000 live birth in developed countries, and 5–10/1000 live births in developing countries [6]. A number of those alterations triggered by PA in the CNS could contribute to the development of neurodegenerative disorders, some of the most common complications that can follow PA are cerebral palsy, mental retardation, and epilepsy [7-11].

The hypoxic-ischemic insult inhibits oxidative phosphorylation, which causes a rapid reduction in adenosine triphosphate (ATP) levels followed by a decrease in Sodium/Potassium (Na^+/K^+) ATPase pump activity, generating changes in cellular membrane polarization [16, 17]. These changes are associated with an increment in extra cellular concentrations of glutamate (Glut) as well as other neurotransmitters [18, 19]. The increase in Glut evokes depolarizing potentials in post-synaptic neurons generating an increase in intracellular Calcium (Ca^{2+}) that leads to several metabolic malfunctions [20, 21] that include Nitric oxide (NO) liberation and the production of reactive oxygen species (ROS) [22, 23]. The reoxygenation that follows an hypoxic-ischemic event produces a great quantity of ROS, and the quantity of these species increases greatly, particularly after the previous depletion of endogenous antioxidants [24]. Under physiological conditions, ROS are part of specific signaling processes, regulating events such as developmental processes, cell proliferation, differentiation and apoptosis [25, 26]. Nevertheless, excessive production of ROS can lead to irreversible oxidative damage to different macromolecules [22]. The low capability of the system to detoxify free radicals and ROS is usually called "oxidative stress" [24], yet new assertions describe oxidative stress as the imbalance of normally occurring redox signaling events [27]. Thioredoxins (Trxs), are a group of proteins that are closely involved in redox regulation, they have been recognized as the

*Address correspondence to this author at the Instituto de Investigaciones Cardiológicas (ININCA), UBA-CONICET, Marcelo T. de Alvear 2270, C1122AAJ, Buenos Aires, Argentina; Tel/Fax: +5411 4508 3880/8; E-mail: franciscocapani@hotmail.com; fcapani@fmed.uba.ar

most important regulators of the thiol redox state [27] and are of most significance in several cellular processes such as transcription regulation, intracellular signaling and antioxidative defense [19]. Members of the Trx family are small proteins with a cysteine (Cys)-X-X-Cys active site, crucial for electron transfer and general oxidoreductase activity [13, 28, 29]. Trxs family proteins are divided in three mayor groups, Thioredoxins (Trx), Glutaredoxins (Grx) and Peroxiredoxins (Prx) [30] and share a common structural motif known as the Trx fold, that consists of a central core of four stranded β-sheets surrounded by three or more α-helices [30]. These proteins maintain a reduced environment inside cells by reducing disulfides using NADPH as an electron donor, and they have been known as key regulators in cell responses to redox signals [28, 31]. Thus, the role of Trxs in the antioxidative defense is particularly important after an hypoxic-ischemic event due to the increase of ROS and free radicals immediately after its occurrence [30].

Mammalian cells contain different members of the Trx family that are involved in different pathways. Trx1 and its reductase, TrxR1, are primarily cytosolic proteins, while Trx2 and TrxR2 are located in the mitochondria [32-34]. Trx1 can be secreted under different circumstances [34, 35], or translocated into the nucleus upon different stimuli [36]. Trx1plays a key role regulating the activity of transcription factors that present a Cys residue in their DNA binding domains, among them NFκB and HIF-1α, and other proteins that belong to the Trxs family known as Prxs [26, 36, 37]. Several of these latter proteins are present in the mammalian cell, Prx1, Prx2, Prx3 and Prx4are typical 2-Cys Prxs (dimeric proteins that form an inter-subunit disulfide bond), while Prx5 is an atypical 2-Cys Prx (it forms an intra-subunit disulfide bond) and Prx6 is a 1-Cys Prx [38-39]. Prxs mainly reduce different peroxides and peroxynitrite [37, 40]; Prx1, Prx2 and Prx5 are mainly nuclear and cytoplasmatic proteins, whereas Prx3 and Prx5 are found in mitochondria, and Prx4 and Prx6 are localized only in the cytoplasm [13, 37]. Grxs are also amply expressed in mammalian cells, among them we can number the cytosolic Grx1, Grx2c and Grx3, the mitochondrial Grx5 and Grx2a, and the least frequent nuclear Grx2b [29, 30]. Grxs are reduced by glutathione, which in its turn is reduced by the glutathione reductase that obtains its electrons from NADPH [13].

The model for common carotid artery ligation that we use in the present study mimics PA in murine subjects, it has been developed and validated by Lopez-Aguilera *et al*., 2012 (12) and previously employed by our group (13). It is generally accepted that the rat brain at 7 days of age is histologically similar to that of a 32-34-week gestation human fetus or newborn infant (13-15). Hence, we subject 7 days old Sprague Dawley rats to a surgical procedure in order to mimic as closely as possible the PA event.

In the present study we aim to analyze the changes in Trxs expression produced in hippocampus, striatum and cerebellum by PA and the distribution of Thioredoxins in this tissue in young (30 days old) control and asphyctic animals, following the hypothesis that the early changes produced by PA could lead to long-term sustained modifications in the thiol redox state.

MATERIAL AND METHODS

Animals

Every experiment was 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). Every possible effort was made in order to reduce the number of animals employed for the experiments and to minimize their suffering.

Sprague-Dawley Pregnant rats were obtained from the central vivarium in the School of Veterinary Sciences' at the University of Buenos Aires and transferred to our local vivarium one week prior to delivery. Pregnant rats were housed individually in standard cages with *adlibitum* access to food and tap water. Male pups were subjected to the experimental procedures. 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.

Carotid Artery Ligation

Seven-days-old (P7) male Sprague–Dawley rats were anesthetized with a combination of ketamine (40 mg/kg) and xylazine (4 mg/kg) and placed on a heat plate in order to guarantee a constant body temperature of 37 °C. The right common carotid artery (CCA) was exposed by performing an incision on the right side of the neck, the artery was isolated from nerve and vein and permanently ligated with a 6–0 surgical silk thread (hypoxic- ischemic group [HI]; n = 14). The wound was rapidly sutured and the

animals were returned to their dams for recovery for 4-5 h. After this time had lapsed, the pups were placed in a stoppered 1 L glass jar and exposed to 100% nitrogen (delivered at 3 L perminute) for 3min 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 [ctrl]; n=12) the right CCA was exposed but not ligated and no nitrogen was supplied.

Brain Dissection

Animals were anesthetized as we explained above, at 30 days of age and brains were dissected as described in Chiu *et al*. [41]. Brains were isolated and dissected at 4 °C. They were cut in half in order to separate right and left hemispheres and subsequently, 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 [42]), the second at the anterior tip of the Fornix (approx. Bregma −1.0 mm based on the rat brain atlas by Paxinos and Watson [42]) and the last was situated at the 4th ventricle (~Bregma −8.0 mm based on the rat brain atlas by Paxinos and Watson [42]). The striatum was dissected between the first and second cut with the help of two Miltex Iris Tissue Forceps, and stored at −80 °C. After the second cut was made, the midbrain was removed to expose the hippocampus, the latter was dissected from the cortex using two tissue forceps, and stored at −80 °C. Lastly, the cerebellum was separated from the ponds and medulla oblongata with the help of two tissue forceps after the third cut was made, and stored at −80 °C.

Western Blotting and ELISA

Western blot analysis was carried out as previously described in Godoy *et al*. [43]. Briefly, animals were euthanized by decapitation, the hippocampus, striatum and cerebellum were dissected, homogenized in ice-cold lysis buffer (10 mM Tris/HCl, pH 7.4, 10mM NaCl, 3 mMMgCl2, 0.1% NP-40, protease inhibitors) and fast frozen in liquid nitrogen. Brain tissue was thawed on ice and centrifuged at 13,000 rpm for 15 min at 4 °C in order to obtain the lysates. The remaining supernatants were analyzed for total protein concentration using Bradford solution (Bio-Rad, Munich, Germany) and bovine serum albumin (BSA) as standard.30 μg of total protein were diluted in sample buffer (0.3 M Tris/HCl, pH 7, 50% glycerol, 5% SDS, 1mMEDTA, 0.1% bromphenol blue). Samples were then subjected to

SDS-PAGE using the Novex mini-cell (Invitrogen, Carlsbad, CA, USA) with precast 4–20% Precise gels (Pierce-Thermo Fisher). Proteins were transferred to PVDF membranes (Schleicher & Schuell, Germany) according to the manufacturer's instructions. Membranes were blocked with 5% non-fat milk powder and 1%BSA dissolved in Tris-buffered saline containing 0.05% Tween 20 and later incubated with specific primary antibodies at 4 °C overnight. Antigen–antibody complexes were stained using horseradish peroxidase (HRP)-coupled antibodies (Bio-Rad, Richmond CA, USA) and enhanced chemiluminescence solutions. Luminescence was recorded using a gel documentation system from Intas (Göttingen, Germany). The generation and validation of the antibodies (Grx1, Grx2, Grx3, Grx5, Prx1, Prx2, Prx3, Prx4, Prx5, Prx6, Trx1, Trx2, TrxR1 and TrxR2) for Western blot analysis used in this study are described in Aon-Bertolino *et al*. [39] and Godoy *et al*. [38]. Anti-GAPDH antibody (Sigma-Aldrich, St. Louis, MO, USA) was used as a loading control. Thermo sensitive sandwich ELISA was used to quantify the levels of Grx2 as described in Hanschmann *et al*. [44]; the antibodies for Grx2 were not validated for Western blot analysis.

Immunohistochemistry

Immunohistochemistry analysis was performed as described in Aon-Bertolino *et al*. [39] and Godoy *et al*. [38] with slight modifications. Briefly, animals were anesthetized with 28% (w/v) chloral hydrate, 0.1 ml/100 g of body weight, and perfused intracardially with 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) freshly prepared in 0.1 M phosphate buffer, pH 7.4. Brains were dissected and post-fixed for 2 h in the paraformaldehyde solution. Coronal brain sections (40 μm thick) were cut using an Oxford vibratome. Prior to staining, sections were incubated in 3% hydrogen peroxide for 10 min to quench endogenous peroxidases. After three washing steps in PBS, nonspecific antibody binding sites were blocked with 10% normal goat serum (Invitrogen Corporation, Camarillo, CA, USA) in PBS and sections were incubated overnight with the primary antibodies at 4 °C. The generation and validation for IHC of the antibodies against the Trx family of proteins (Grx1, Grx2, Grx3, Grx5, Prx1, Prx2, Prx3, Prx4, Prx5, Prx6, Trx1, Trx2, TrxR1 and TrxR2) used in this study were described in Aon-Bertolino *et al*. [39] and Godoy *et al*. [38]. Sections were washed three times with PBS and subsequently incubated with a biotinylated secondary antibody (Vector Laboratories Inc., Burlingame, CA, USA) for

60min at room temperature. Extravidin-Peroxidase detection system (Sigma-Aldrich, St. Louis, MO, USA) was used for antigen staining according to manufacturer's recommendations. Sections were incubated with Diaminobenzidine (Sigma-Aldrich, St. Louis, MO, USA) for 5 min at room temperature. Samples were mounted with Canada balsam (Sigma-Aldrich, St. Louis, MO, USA). Sections without incubation with the primary antibody were used as control to verify the specificity of the secondary antibody. Sections were examined by light microscopy using a Leitz Laborlux S microscope (Heidelberg, Germany) equipped with a CCD video camera (Canon). Images were analyzed and compiled using Adobe Photoshop 11.0 CS4. Note that for each protein staining, samples (ctrl and HI) were processed together in the same batch, using the same antibody dilutions and the same time for DAB development. Intensity assessment was carried out as a blind test with a 0 to +++ score used as a qualitative measurement.

Statistical analysis

Band intensities in Western blots were quantified using GelPro 3.1 and expressed as percentage of the control levels (ctrl rats). Bar diagrams depict the mean of four independent quantifications of each sample of ctrl (n = 6) and HI (n = 8) animals + SD, correlated to total protein. Two-way ANOVA and Tukey post hoc tests were employed to analyze the statistical significance of changes in protein levels. The level of significance was set up at 5%. All analyses were performed using SPSS 15.0 (Chicago, IL, USA).

RESULTS

We used western blot and ELISA techniques in order to analyze the expression pattern of 14 proteins that belong to the Thioredoxin family in different areas of the brain: cerebellum, striatum and hippocampus. At 30 days of age we could observe a significant increase in Prx2 levels in the striatum (p > 0.05) and an increase in Trx1levels in cerebellum (p > 0.01(Figure **1**). The rest of the Thioredoxins did not show any significative modifications. When we compared the western blot observations with the immunohistochemistry staining data we observed a highly correlation. In Figure **2** we can observe that at 30 days of age Trx1 immunostaining was increased in neuronal bodies of the dorsal striatumand the *cornu ammonis* area 1 (CA1) of the hippocampus in HI animals. On the other hand, we can see a higher Trx1 immunostaining in axonal projections in CA1 pyramidal cells of control

animals in comparison to the HI ones (arrows). Prx2 presented, mainly, a cytosolic distribution in both HI and ctrl animals, whereas it showed a higher immunostaining in cell body and axonal projections of Purkinje cells in animals that had been subjected to the carotid ligation (arrowheads). We could also observe a disruption of the Purkinje cell layer in the carotid group that indicates the deleterious effect of the hipoxia-ischemia procedure on the nervous tissue.
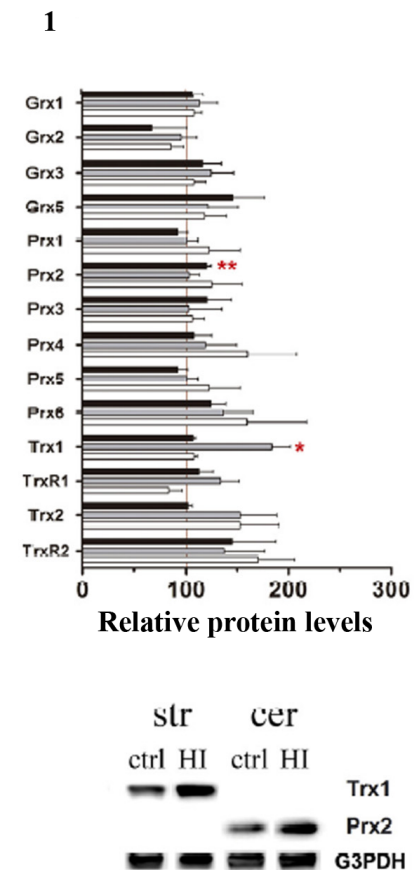

Figure 1: A-Changes in the expression of Thioredoxin family proteins after a hypoxic-ischemic event. The hippocampus (hip), striatum (str) and cerebellum (cer) are isolated and analyzed in order to determine protein expression levels by western blot or ELISA (for Grx2). The bar chart represents percentage of protein expression compared to the control. Representative Western blots, for the groups that show a significant change, are illustrated next to each graphic. Bars represent the mean + SD of 8HI rats and 6 ctrl animals for each protein. We used G3PDH as a loading control. *p ≤ 0.05, **p ≤ 0.01.

DISCUSSION AND CONCLUSIONS

To our knowledge, not much has been published regarding Thioredoxins protein family and PA. In the present work we aimed to obtain a wide picture of the expression changes that undergo many of the members of the Trx family of proteins of hypoxic-
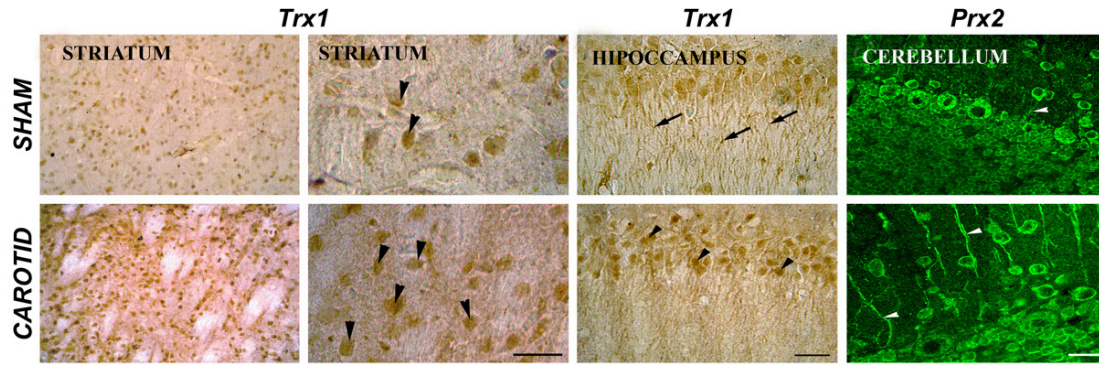
Figure 2: B-Immunohistochemical analysis of the expression pattern of Trxs family of proteins in sham and carotid animals, at 30 days of age. a) Representative images depicting Trx1 immunostaining in dorsal striatum. Arrows show strongly stained cell bodies. **b**) Representative pictures showing Trx1 immunostaining in the CA1 area of the hippocampus. Arrowheads point at highly immunopositive cell bodies; arrows point at greatly immunostained cell projections. **c**) Representative photomicrographs depicting Prx2 immunostaining in cerebellum. Arrows show strongly stained cell projections. n=6 ctrl, n=6HI.

ischemic insult. Therefore, we worked with 14 members of said family in a murine model, in order to assess changes in expression and localization in different areas of the CNS at 30 days after a PA event. The reperfusion that follows a hypoxic-ischemic event has been known to cause an increase in the production of free radicals, mainly through a disruption of the mitochondrial electron transport [45], thus generating a redox imbalance [46, 47]. This redox imbalance has been proposed as one of the most important causes of neuronal death after a PA event [22, 47-50]. Since Trxs are closely related to redox regulation we propose that their changes in expression and distribution after an asphyctic event are of most importance to understand the roles they play in this episode, and to hit upon new targets for future studies regarding Trxs and PA.

Trx1 is a ubiquitously expressed disulfide reductase, that plays a major part in redox regulation and that itself is reduced by the Trx1 reductase TrxR1 [51]. In previous works, it has been shown that Trx1 is up-regulated in different areas of the hippocampus and cerebellum after ischemic events [13, 52] while overexpression of Trx1 has been proven beneficial in a murine model of focal cerebral ischemia [53]. It has been demonstrated that Trx1 regulates cell proliferation trough activation of HIF-1α [51]. Trx1 overexpression following a hypoxic-ischemic event has been shown to go hand in hand with higher levels of hypoxia inducible factor (HIF)-1α, while a decrease in TrxR1 levels blocks HIF-1α activation [51, 54]. In the present work we could observe a higher expression of Trx1 in CA1 pyramidal cells in 30-days-old rats that had been subjected to the carotid artery ligation. This increase in Trx1 expression could be interpreted as a system

response to deal with the damage produced by the hypoxic-ischemic insult in the hippocampus. Furthermore, it has been demonstrated that the overexpression of Trx1, Grx2 and Prx2 diminishes the damage produced by an ischemic event in neurons [55-57]. In the present work we have also observed changes in the expression levels and distribution of Prx2. Previous reports have shown that Prx2 plays a crucial role in antioxidant defense while it has a clear neuroprotective effect in neurons after an ischemic event [58]. Prx2 protein levels were increased in 30-days-old rats that had been subjected to the carotid ligation. We hypothesize that the higher levels of Prx2 could be related to mitigating mechanisms that are activated after the asphyctic event [58].

As evidenced, the expression and distribution of Thioredoxins is highly complex and it varies in the different areas of the CNS. Despite the importance of these proteins in redox control, and thus in PA and its effects in the CNS, little is known about their intricate expression and its changes after such an event. Some authors have highlighted the importance of these proteins as possible therapeutic elements in the pathogenesis developed after a hypoxic-ischemic insult [58]. In our experiments we showed some selective expression in some areas that are more affected for perinatal asphyxia as CA1, striatum and cerebellum. In addition, only 2 of the 14 members of this protein family. In the case of Trx1 could be related with fact that is involved in cell differentiation. Recently we have published some data that showed and increment in the number of synapses in the attempt to rescue the system for the perinatal asphyxia damage showed a significant increment [59]. In addition, Prx-2 has been

showed to be neuroprotective modulating the neurons redox state the in different model of hypoxia ischemia. Although this data is promising we need to perform new experiments in cell culture to study more in details the mechanism involved in the proctective effect of this Trxs in nervous tissue. In addition, we should perform new experiment using both Trxs to improve the individual effect of each one. In fact some clinical trials are usng this strategies since many of them did not show any enthusiastic result.

Therefore, taken together, our results shed light on the importance of some of the Thioredoxin family members in PA and which of these proteins can be potential targets for therapeutic interventions.

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