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The augmentation of brain thioredoxin-1 expression after severe hypobaric hypoxia by the preconditioning in rats

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Abstract

Induction of endogenous antioxidants is one of the key molecular mechanisms of cell resistance to hypoxia/ischemia. The effect of severe hypoxia on the expression of cytosolic antioxidant thioredoxin-1 (Trx) in hippocampus and neocortex was studied in preconditioned and non-preconditioned rats. The preconditioning consisted of three trials of mild hypobaric hypoxia (360 Torr, 2 h) spaced at 24 h. Twenty-four hours after the last trial rats were subjected to severe hypobaric hypoxia (180 Torr, 3 h). Trx expression was studied by immunocytochemistry. In hippocampus severe hypobaric hypoxia rapidly induced Trx expression, which remained elevated still at 24 h. In neocortex the enhanced expression appeared only at 24 h. The preconditioning significantly augmented severe hypoxia-induced Trx-immunoreactivity at 3 h but not at 24 h. These findings point out that Trx contributes to mechanisms of brain tolerance to hypobaric hypoxia, especially in early periods after the exposure.

Keywords: Hypobaric hypoxia; Preconditioning; Antioxidants; Thioredoxin-1; Immunocytochemistry

Severe hypoxia/ischemia can induce apoptotic and necrotic neuronal cell death [3,27,61]. The oxidative stress caused by redox balance disruption and overproduction of reactive oxygen species (ROS) is an important mechanism of cell damage produced by hypoxia/ischemia [7,8,46]. Oxidative stress and redox balance impairment are followed by a dysfunction of important redox-sensitive enzymes, membrane receptors and ion channels [32,55], DNA damage [5,6,18,44], membrane lipid peroxidation [56,59] and cytochrome *c* release from mitochondria, which activate the caspases that result in cell death [28,54].

The thioredoxin and glutathione systems control the cellular redox state. Thioredoxin-1 (Trx) is a small (about 12 kDa) multifunctional ubiquitous protein with a redox-active disulfide/dithiol within the conserved active site sequence –Cys–Gly–Pro–Cys– [22,23]. The thioredoxin reductase reduces the oxidized form using NADPH [38]. Trx is induced by hypoxia/ischemia [4,50] and protects cells against different kinds of oxidative stress [24,42,48].

Mild hypoxic/ischemic preconditioning increases the neuronal resistance to subsequent severe hypoxia/ischemia [29,41]. The 2-min ischemic preconditioning suppresses the cytochrome *c* release from mitochondria induced by severe 5-min ischemia in gerbil hippocampus [36]. The expression of Trx and other antioxidants appears to provide one of the neuroprotective mechanisms activated by the preconditioning [2].

We previously showed that hypobaric hypoxia increased the expression of mitochondrial Trx-2 in different rat brain areas including hippocampus and sensory-motor neocortex. The preconditioning significantly augmented this induction

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[45]. The aim of present research is to investigate the cytosolic Trx expression in rat neocortex and hippocampus in identical experimental model.

Male Wistar rats weighing 200–250 g were subjected to hypobaric hypoxia. Severe and repetitive mild preconditioning hypoxias were produced in a hypobaric chamber by maintaining the pressure at 160–180 Torr (equivalent to 5% normobaric oxygen) for 3 h, and 360 Torr (equivalent to 10% normobaric oxygen**)** for 2 h daily for 3 days, respectively. The severe hypoxia produced in such a paradigm caused extensive neuronal damage in hippocampus and neocortex, but the preliminary preconditioning prevented severe hypoxia-induced neuronal damage [39]. All the animals were divided into three groups (four to six rats per group): (i) rats subjected to severe hypoxia; (ii) rats subjected to preconditioning hypoxia 24 h prior to the severe hypoxia; (iii) control rats placed in the chamber for 3 h with no hypoxia produced. The Trx immunoreactivity was studied 3 and 24 h following severe hypoxia.

For immunocytochemistry the rats were anaesthetized and perfused transcardially first with 100 ml of saline followed by 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS; pH 7.3) for 4–5 min. After perfusion the brains were excised and subsequently fixed by immersion in the same solution for 60 min. The samples were cryoprotected with 15% sucrose in PBS and stored at +4 °C until sectioning in the cryostat. Immunocytochemistry was performed using ABC-method. Coronal sections (11 μm) of the brain (about −2.80 mm from bregma [37]) were mounted onto the poly-L-lysine (Sigma) covered slides and then incubated with affinity-purified rabbit antiserum against mouse cytosolic Trx [48] (dil. 1:500 in PBS containing 1% BSA and 0.3% Triton X-100) at +4 °C overnight. After several washes, the sections were incubated with biotinylated goat antirabbit (Vector Labs) antibodies (dil. 1:300) and ABC complex for 30 min each. Diaminobenzidine was used as a chromogen to visualize the sites expressing Trx immunoreactivity. The sections were dehydrated, mounted and assayed with image analysis system consisting of IBM PC, Nikon Microphot-FXA microscope, SensiCam digital camera (PCO Computer Optics GmbH), Image-Pro Plus (Media Cybernetics) program.

Trx expression was examined in fronto-parietal cortex, CA1, CA2, CA3 hippocampal fields and dentate gyrus. The Trx-immunoreactive cells were quantified in the area of 500 μm in length (in hippocampus) or in square 300 μm × 300 μm (in neocortex), using Videotest Morphology program. Six sections were analyzed from each brain; one field of each brain area studied was measured per each slice. The intensity of staining was expressed as conventional value of optical density scale from 0 (absolute white) to 100 (absolute black). Immunoreactive cells were divided in 2 relative classes: slightly-labeled (staining intensity was at 1–10 conventional units above the background) and intensely-labeled (more than 10 units above the background). Trx immunoreactivity was assayed using following criteria: the total number of immunoreactive cells shown as a percent of control (N_+)

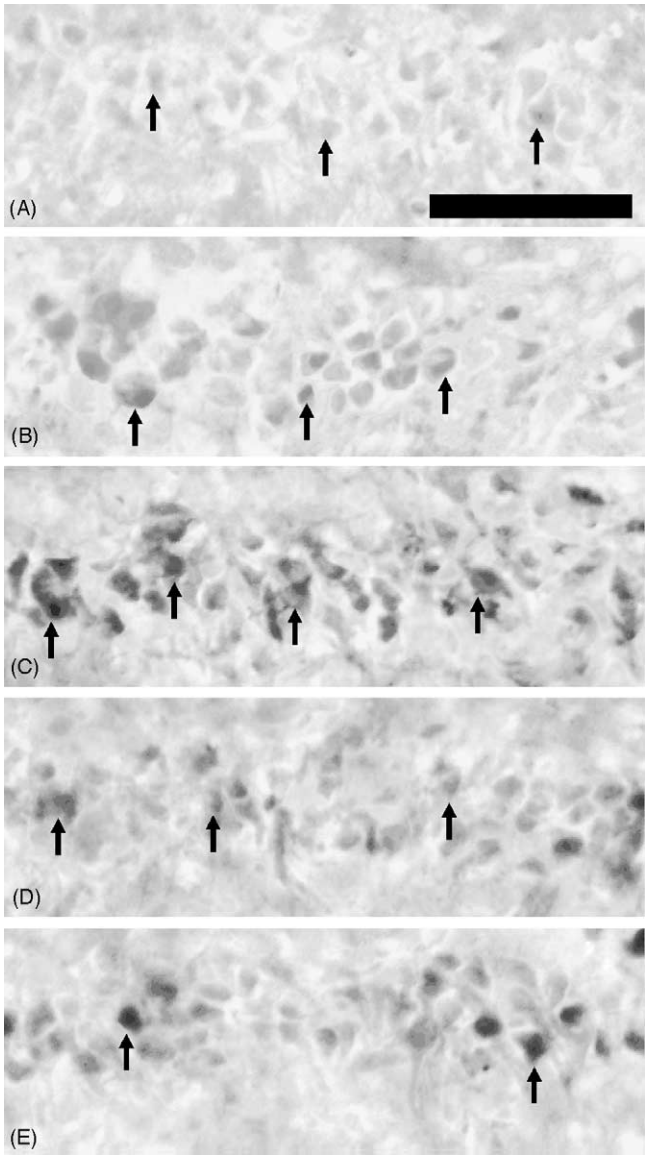

Fig. 1. Trx-immunoreactivity in the CA1 area of hippocampus. Photomicrographs of control hippocampal CA1 field (arrows point non-labeled neurons) (A), hippocampal CA1 field at 3 (arrows show lightly labeled neurons) (B), and 24 h (few neurons are moderately labeled) (D), after severe hypobaric hypoxia and after precondioned severe hypoxia at 3 (most of the neurons are strongly stained) (C), and 24 h (some neurons are strongly labeled) (E). Scale bar: 50 μm.

and the number of intensely-labeled cells as a percent of control (N_i). One-way ANOVA was used for statistical analysis of data.

Immunocytochemistry revealed that Trx expression in hippocampus and neocortex was affected by severe hypoxia and preconditioning. A notable increase in Trx immunoreactivity in all hippocampal areas examined but not in neocortex was detected 3 h after severe hypoxia (Figs. 1–3). The exposure to severe hypoxia significantly increased N_+ in CA1 (129%) and CA2 (145%) (Fig. 2). The number of intensely-labeled cells (N_i) was essentially elevated in CA1 (238%), CA2 (776%), CA3 (469%), and DG (259%) (Fig. 3).

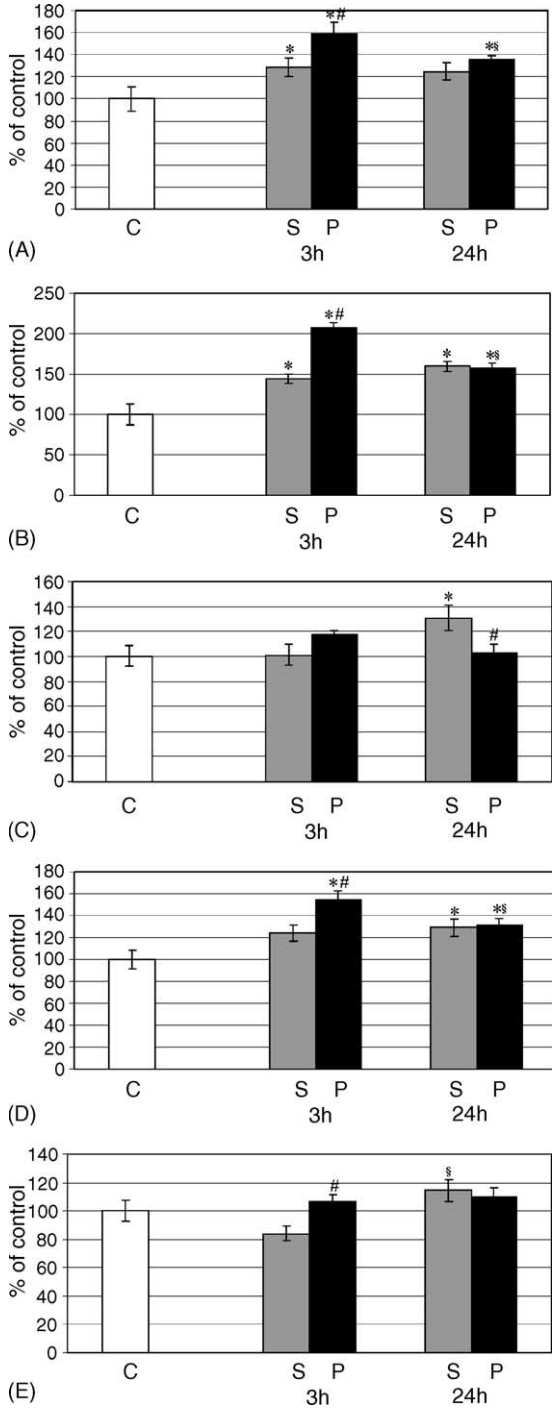

Fig. 2. Graphs showing changes in the total number of Trx-immunoreactive cells ± S.E.M. expressed as a percentage of control (N_+) in different rat brain areas at 3 and 24 h after severe hypobaric hypoxia (S) (n = 4) and preconditioned severe hypoxia (P) (n = 5), as compared to control group (C) (n = 6). CA1 field of hippocampus (A), CA2 field of hippocampus (B), CA3 field of hippocampus (C), dentate gyrus (D) and neocortex (E). Statistically significant ($P < 0.05$) differences: (*) as compared to control, (#) between non- and preconditioned animals, (§) between 3 and 24 h time-point.

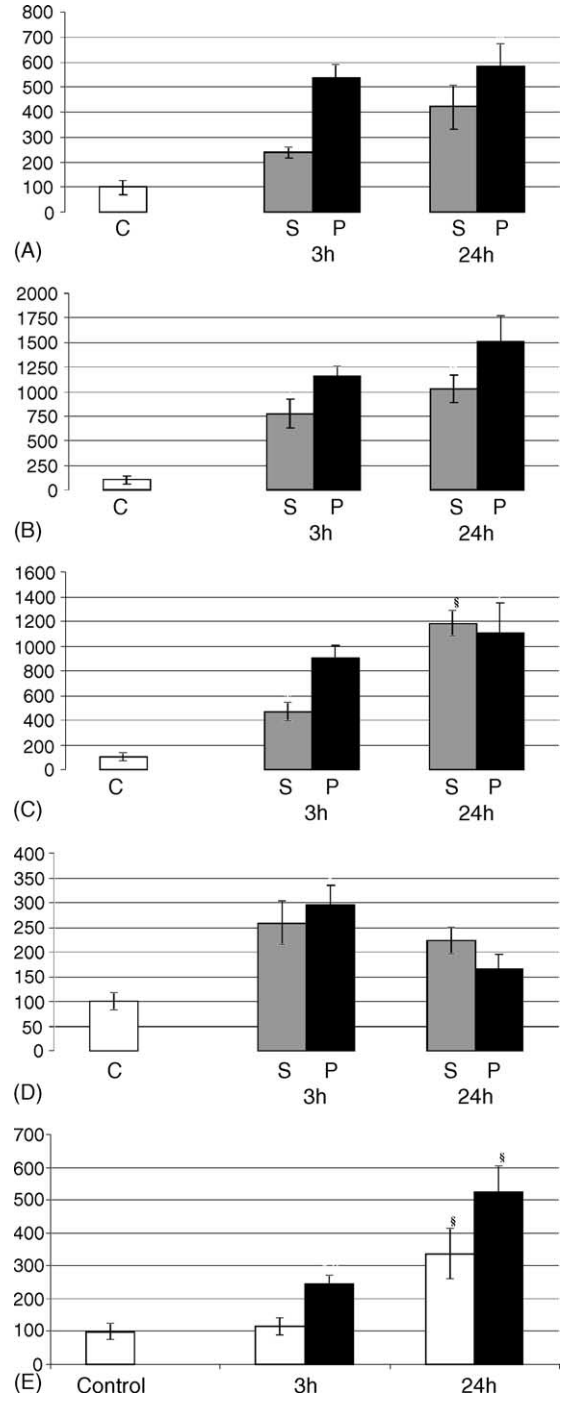

Fig. 3. Graphs showing changes in the number of intensely-labeled cells ± S.E.M. expressed as a percentage of control (N_i) in different rat brain areas at 3 and 24 h after severe hypobaric hypoxia (S) (n = 4) and preconditioned severe hypoxia (P) (n = 5), as compared to control group (C) (n = 6). CA1 field of hippocampus (A), CA2 field of hippocampus (B), CA3 field of hippocampus (C), dentate gyrus (D) and neocortex (E). Statistically significant ($P < 0.05$) differences: (*) as compared to control, (#) between non- and preconditioned animals, (§) between 3 and 24 h time-point.

Preconditioning with mild repetitive hypoxia markedly augmented severe hypoxia-induced Trx expression in all brain areas studied at 3 h time-point (Figs. 1–3). The increase in the number of immunoreactive cells (N_+) was considerably higher in preconditioned rats then in non-preconditioned ones in CA1 (159% from control), CA2 (207%), DG (154%), and neocortex (107%) (Fig. 2). The increase in N_i was obviously higher in preconditioned rats then in non-preconditioned ones in CA1 (536%), CA2 (1158%), CA3 (898%), and neocortex (244%) (Fig. 3).

At 24 h after severe hypoxia, Trx immunoreactivity remained enhanced in all hippocampal areas studied (Figs. 1–3). N_+ was markedly increased in CA2 (159%), CA3 (131%), and DG (129%) as compared to control. N_i was increased in all brain areas studied: CA1 (421%), CA2 (1027%), CA3 (1185%), DG (223%) and neocortex (337%). When compared to 3 h time-point, Trx immunoreactivity at 24 h was significantly elevated only in CA3 (N_i but not N_+) and in neocortex (N_+ as well as N_i) (Figs. 2 and 3).

At 24 h after severe hypoxia there was no remarkable difference in the Trx immunoreactivity (N_+ as well as N_i) between pre- and non-preconditioned animals (Figs. 1–3) except in CA3 where N_+ was found to be higher in non-preconditioned ones. When compared to 3 h time-point, a decrease of the immunoreactivity in hippocampal fields of preconditioned rats was apparent at 24 h time-point: the changes of N_i were not significant but N_+ noticeably decreased in CA1 (136% from control), CA2 (157%), and DG (132%). On the contrary, in neocortex Trx immunoreactivity (N_i but not N_+) of preconditioned rats was substantially elevated at 24 h as compared to 3 h time-point (Fig. 3).

Trx provides an important defense of brain neurons during various hypoxic/ischemic events. Trx protein and mRNA expression was down-regulated in the ischemic core regions but up-regulated in the perifocal ischemic regions since 4 h after focal brain ischemia [15,47,49]; the induced Trx was translocated into the nucleus after ischemia and ischemia-reperfusion. It is important that changes in Trx expression were observed in the earliest period after the insult because the first 2–4 h after the exposure to severe hypoxia are supposed to be crucial for cytochrome *c* release [11]. Transient global ischemia induced Trx in glial cells of the gerbil hippocampus [50]. Overexpression of the Trx in transgenic mice attenuates focal ischemic brain damage [48], on the contrary, its inhibition increases oxidative stress [60]. In addition, Trx reduces hypoxia-reoxygenation injury in cell culture in vitro [26].

In present study we for the first time showed that cytosolic Trx involved in neuronal responses to hypobaric hypoxia. The expression of Trx in the brain of preconditioned and non-preconditioned animals at 3 and 24 h following severe hypoxia was studied by immunocytochemistry. Severe hypoxia up-regulated the Trx expression in hippocampus at 3 h time-point; this induction appears to represent an adaptive neuronal response to oxidative stress. Preconditioning greatly promotes this protective reaction in hippocampus and induce it in neocortex. The augmentation of Trx expression at early period of reoxygenation critical for apoptosis initiation provides one possible mechanism of hypoxic/ischemic tolerance produced by the preconditioning.

In neocortex hypoxia without preconditioning induced Trx expression only at 24 h time-point. In hippocampus at this period the Trx expression remained enhanced. Hence the response in hippocampus is faster then in neocortex. In the hippocampus of preconditioned rats the Trx expression induction ceased by 24 h whereas in neocortex it continued to increase.

Thiol redox status is one of the key factors of the apoptosis regulation [43]. The protective functions of Trx during oxidative stress are diverse. One of the key Trx defense function is the buffering of ROS [35,51] and inhibition of cytochrome *c* release from mitochondria [2]. By this way Trx can inhibit the apoptosis triggering. On the other hand Trx can switch necrosis to apoptosis by the regulation of redox-sensitive caspase activity [51,52]. In addition, Trx appears to function as a potent activator of other antioxidant systems, e.g. Mn-superoxide dismutase [10].

Trx is translocated from cytoplasm to nucleus upon stress [33] and activates the transcriptional factors by enhancing their binding activity to the target DNA: NF-κB, AP-1, CREB, PEBP2/CBF, Myb, and HIF-1 [1,9,16,19–21,57,58], estrogen [17] and glucocorticoid [30,31] receptors. Trx also augmented the DNA binding activity of p53 [53]. Oxidative stress induced p53 [51] can in its turn activate a G1 cyclin-dependent kinase inhibitor $p21^{Cip1/WAF1}$ that cause the cell-cycle arrest, presumable to allow an opportunity for DNA repair [12,51]. But p53 also can induce apoptosis [3] by an activation of proapoptotic protein Bax, resulting in cytochrome *c* release [34]. Trx augments the p53-dependent p21 transcriptional activity and protein expression and thereby switch apoptosis triggering to DNA reparation way [53].

Trx is a negative regulator of apoptosis signal-regulating kinase 1 (ASK1) [40]. ASK1 was identified as one of the mitogen-activated protein (MAP) kinase kinase kinases, which activates the *c*-Jun N-terminal kinase (JNK) and p38 MAP kinase and induces stress-mediated apoptosis signaling [25]. ASK1 stimulates cytochrome *c* release and executes apoptosis mainly by mitochondria-dependent caspase activation [14]. The negative regulation of ASK1 appears to be one of the Trx cytoprotective effects. Trx also negatively regulates TNF-induced activation of p38 MAP kinase [13] activated by oxidative stress [25].

In conclusion, brain expression of thioredoxin-1 is enhanced after severe hypobaric hypoxia; the hypoxic preconditioning considerably up-regulates this enhancement. Present findings suggest a possible role for cytosolic antioxidant thioredoxin-1 in the induction of brain hypoxic tolerance.

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