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One- and three-time mild hypobaric hypoxia modifies expression of mitochondrial thioredoxin-2 in hippocampus of rat

Sergey Alexandrovich Stroev[1,2,*], Ekaterina Iosifovna Tjulkova[2], Michail Olegovich Samoilov[2] and Markku Tapio Pelto-Huikko[1]

[1]Department of Anatomy II, Tampere University Medical School and Department of Pathology, Tampere University Hospital, 33014 Tampere, Finland; [2]Laboratory of Regulation of Brain Neuron Functions, Pavlov Institute of Physiology RAS, 199034 St.Petersburg, Russia; *Email: s_stroev@hotmail.com

Our previous study demonstrated that preconditioning by 3-times repetitive mild hypoxia significantly augmented expression of mitochondrial thioredoxin-2 (Trx-2) at 3 h after subsequent acute severe hypoxia in rat hippocampus. However, it was unclear whether this augmentation was due to build up of Trx-2 by mild hypoxia before severe hypoxia or by modification of reaction to severe hypoxia itself. To answer on this question we study the expression level during and after preconditioning without subsequent severe hypoxia. Trx-2 expression was studied by immunocytochemistry 3 h and 24 h after first session and 3 h and 24 h after last session of 3-times (spaced at 24 h) mild hypobaric hypoxia (360 Torr, 2h). At 3 h after 1-time hypoxia (first session of 3-time hypoxia) the total number of Trx-2-immunoreactive cells (Nt) was significantly decreased in contrast with control in CA2, CA3 and DG. The number of cells with intensive expression of Trx-2 (Ni) was reduced in CA1 and CA3. At 24 h after the same 1-time hypoxia Nt was lower than in control and at 3 h time-point in all hippocampal areas studied (CA1, CA2, CA3 and DG); Ni was decreased only compared to control in CA1 and CA3. At 3 h after last session of 3-times hypoxia Nt and Ni were significantly down regulated in comparison with control only in CA1. At 24 h after it Nt was significantly decreased compared to control in CA1, CA2 and CA3 (in DG the decrease was not statistically significant) but in all areas was higher than at 24 h after 1-time hypoxia. Dynamics of Nt changes from 3-hours after single to 24-hours after triple moderate hypoxia had the wave phase character. These findings indicate that Trx-2 expression in most areas of hippocampus was decreased to 24 h after 3-time mild hypoxia. Thus the augmentation of Trx-2 expression in hippocampal neurons of preconditioned animals in response to subsequent severe hypoxia is caused obviously not by Trx-2 accumulation during preconditioning sessions but by modification of reaction to severe impact.

Key words: antioxidants, immunocytochemistry, hippocampus, mild hypobaric hypoxia, preconditioning, thioredoxin-2

INTRODUCTION

Thioredoxins (Trxs) are antioxidant multifunctional ubiquitous proteins with a redox-active disulfide/dithiol within the conserved active site sequence Cys-Gly-Pro-Cys (Holmgren 1985, 1989). Mammalian thioredoxin family includes in addition to the long-known and most-studied cytosolic thioredoxin-1 (Trx-1) many other members (Nakamura 2005): mitochondrial thioredoxin-2 (Trx-2; Spyrou et al. 1997), a larger thioredoxin-like protein p32TrxL (Hirota et al. 2002); thioredoxin-like proteins Txl-1 (Miranda-Vizuete et al. 1998, Jimenez et al. 2006) and Txl-2 (Sadek et al. 2003), and spermatid-specific thioredoxins Sptrx-1 (Miranda-Vizuete et al. 2001, Jimenez et al. 2002a, b), Sptrx-2 (Sadek et al. 2001, Miranda-Vizuete et al. 2003) and Sptrx-3 (Jimenez et al. 2004). Also mammalian thioredoxin superfamily include specific thioredoxins of endoplasmic reticulum ERp18 (Alanen et al. 2003) and ERdj5 (Cunnea et al. 2003), Trp-Cys-Gly-His-Cys-Lys motif-containing phospholipase C-alpha (ERp57; Hirano et al. 1994) and protein disulfide isomerases: CabP1, the rat analog of the hamster protein P5 and CabP2, the rat analog of the murine protein ERp72 (Rupp et al. 1994).

Correspondence should be addressed to: S. A. Stroev
Email: s_stroev@hotmail.com

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Trx-1 and Trx-2 are small (about 12 kDa) proteins. They are induced by hypoxia/ischemia (Tomimoto et al. 1993, Berggren et al. 1996, Stroev et al. 2004a, b) and protect cells against different kinds of oxidative stress (Hori et al. 1994, Sasada et al. 1996, Spyrou et al. 1997, Takagi et al. 1999, Chen et al. 2002, Ueda et al. 2002). In particular, in experiments with transgenic mice it was shown that overexpression of thioredoxin protects the brain cells against damage during focal ischemia (Takagi et al. 1999), and that addition of thioredoxin to cultural medium significantly reduces the damaging effects of hypoxia/reoxygenation in cell culture (Isowa et al. 2000). In contrast, inhibition of thioredoxin increases oxidative stress (Yamamoto et al. 2003).

It is known that hypoxia/ischemia induces both pathological effects (Takagi et al. 1998a, b, Simonova et al. 2003, Cai et al. 2010) and adaptive mechanisms for their compensation (Wojcik et al. 2009). Preliminary training (preconditioning) by mild hypoxia or ischemia improves resistance of organism to subsequent severe hypoxia (Vladimirov et al. 1939, Sirotinin 1939, Kreps et al. 1956, Vataeva et al. 2004a, b, Rybnikova et al. 2005a, b) including structural and functional resistance of brain neurons (Kitagawa et al. 1990, Kirino et al. 1991, Corbett and Crooks 1997, Samoilov et al. 2003, Duszczyk et al. 2006). The expression of Trxs and other antioxidants appears to provide one of the neuroprotective mechanisms activated by the preconditioning (Andoh et al. 2002, Samoilov et al. 2002, Stroev et al. 2004 a, b, Chiueh et al. 2005).

In our experimental paradigm the acute severe hypobaric hypoxia caused extensive neuronal damage in hippocampus and neocortex (about 30% cell death in CA1 region by the seventh day after severe hypoxia), but the preceding preconditioning by the three-time mild hypobaric hypoxia prevented severe hypoxia-induced neuronal death (Rybnikova et al. 2005a, b, 2006a). The preconditioning increased survival rate of rats following severe hypobaric hypoxia from 50% to 85% (Rybnikova et al. 2005a, b), shifted of the ratio of the Bcl-family pro- and anti-apoptotic proteins in the favor of antiapoptotic ones (Samoilov et al. 2005, Rybnikova et al. 2006a), reduced the hypoxia-evoked alterations in glutamatergic Ca^{2+} signaling (Semenov et al. 2008), ameliorated the memory and behavioral disturbances induced by the severe hypoxia (Vataeva et al. 2004a, b, Rybnikova et al. 2005b) and prevents development of post-stress depressions (Rybnikova et al. 2006b, Rybnikova et al. 2007a, b). We previously showed that in this experimental model severe hypobaric hypoxia increased the expression of Trx-2 (Stroev et al. 2004b) and some other antioxidants such as Trx-1 (Stroev et al. 2004a) and superoxide dismutases (Stroev et al. 2005a, b) in rat hippocampus and fronto-parietal neocortex, and that preconditioning significantly augmented this induction at early period of reoxygenation (Stroev et al. 2004a, b, 2005a, b) which is crucial for cell survival.

However, it was unclear whether the augmentation of Trx-2 content at 3 h after preconditioned severe hypoxia was due to build-up of Trx-2 by mild hypoxia before severe hypoxia or by modification of reaction to severe hypoxia itself. To answer on this question it was necessary to clarify the Trx-2 expression level during and after preconditioning, including the starting point of severe hypoxia (24 h after last session of three-time hypoxia).

METHODS

Male Wistar rats weighing 200 - 250 g and aged 80 - 90 days were subjected to 1 and to 3 sessions (once in day) of mild hypobaric hypoxia that was produced in a hypobaric chamber by maintaining the pressure at 360 Torr (equivalent to altitude 5000 m or to 10% normobaric oxygen) for 2 h. The experimental procedures were conducted in accordance with the Declaration of Helsinki under the approval of the Ethical Committee for Use of Animal Subjects at Pavlov Institute of Physiology. The Trx-2 immunoreactivity was studied in rats of 5 groups (4-6 animals per each group): 1) at 3 h following one-time hypoxia, 2) at 24 h following one-time hypoxia, 3) at 3 h following last session of three-time hypoxia, 4) at 24 h following last session of three-time hypoxia, and 5) control rats which were placed in the same chamber for same time with no hypoxia produced.

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 avidin and biotinylated horseradish peroxidase macro-

molecular complex (ABC) method. Coronal sections (11 μm) of the brain (about -2.80 mm from bregma, Paxinos and Watson 1986) were mounted onto the poly-L-lysine (Sigma) covered slides and then incubated with affinity-purified rabbit antiserum against rat Trx-2 (dilution 1:250 in PBS containing 1% BSA and 0.3% Triton X-100; Spyrou et al. 1997) at 4ºC overnight. After several washes, the sections were incubated with biotinylated goat antirabbit (Vector Labs) antibodies (dil. 1:300) and ABC for 30 min each. Diaminobenzidine was used as a chromogen to visualize the sites expressing Trx-2 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) and Image-Pro Plus (Media Cybernetics) program.

Trx-2 expression was examined in CA1, CA2, CA3 hippocampal fields and dentate gyrus (DG). The Trx-2-immunoreactive cells were quantified in the area of 500 μm in length, using Morphix program especially created by us for such analysis (Tugoy and Stroev 2006). Seven 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 to Trx-2 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-2 immunoreactivity was assayed using following criteria: the total number of immunoreactive cells shown as a percent of control (Nt) and the number of intensely-labeled cells as a percent of control (Ni). One-way ANOVA was used for statistical analysis of data and result was checked by Wilcoxon non-parametric test.

RESULTS

Immunocytochemistry revealed that mild hypoxia decreased Trx-2 expression in hippocampus

At 3 hours after one-time mild hypoxia (that is first session of three-time mild hypoxia) the total number of Trx-2 immunoreactive cells was significantly decreased compared to control in CA2 (Nt = 67%), CA3 (Nt = 80%) and DG (Nt = 63%); in CA1 (Nt = 77%) decrease

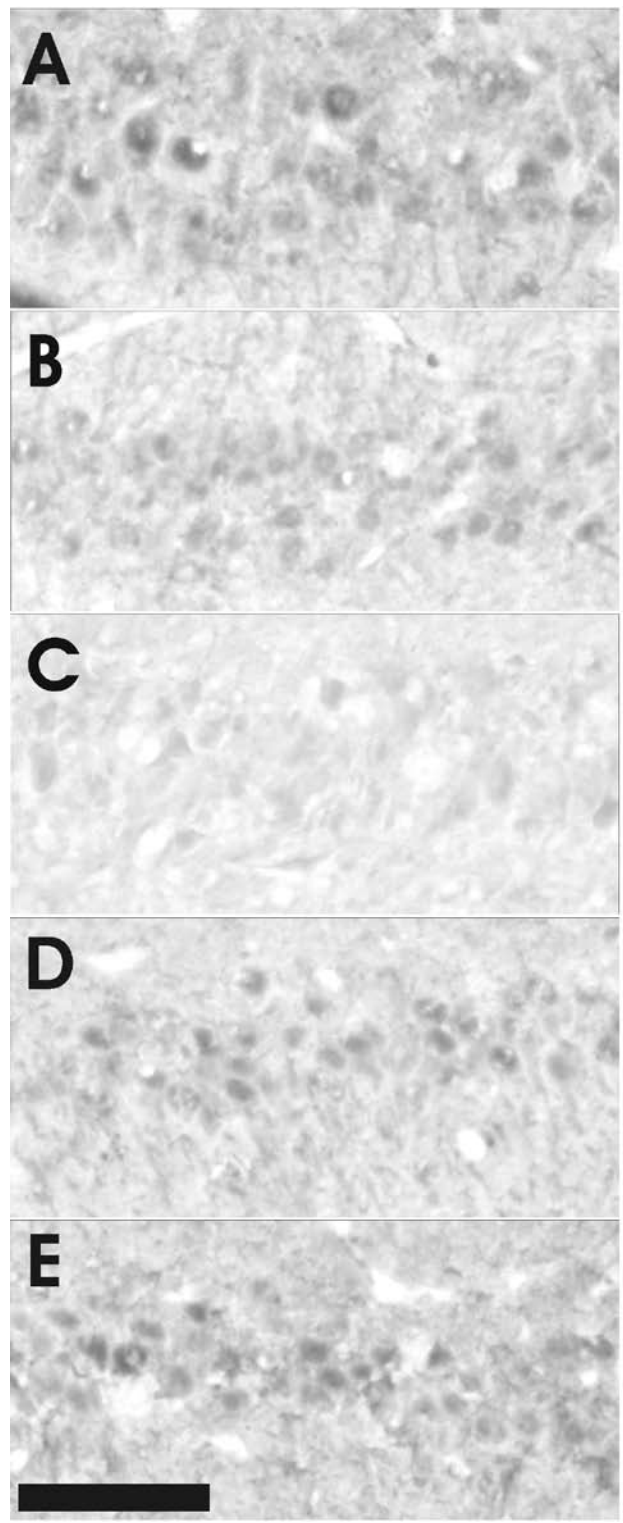

Fig. 1. Trx-2 immunoreactivity in the CA1 area of hippocampus. Photomicrographs of control hippocampal CA1 field (A), hippocampal CA1 field at 3 (B), and 24 hours (C) after one-time mild hypoxia; hippocampal CA1 field at 3 (D), and 24 hours (E) after three-time mild hypoxia. Scale bar: 50 μm.

of Nt was not significant (Fig. 1 - 2). Decrease of number of intensely expressing Trx-2 cells compared to control was significant in CA1 (Ni = 33%) and CA3 (Ni = 8%); in CA2 (Ni = 17%) and DG (Ni = 27%) this decrease was quite great but formally statistically not significant because the standard error in this cases was too large (Fig. 3).

At 24 hours after one-time mild hypoxia the total number of Trx-2 immunoreactive cells was very significantly decreased compared to control in all hippocampal fields studied (Fig. 1-2): in CA1 (Nt = 38%), CA2 (Nt = 14%), CA3 (Nt = 22%) and DG (Nt = 18%). In all fields this decrease of Nt was statistically significant also in comparison with 3 hours time-point (Fig. 1-2). Decrease of number of intensely expressing Trx-2 cells compared to control was significant in CA1 (Ni = 0%) and CA3 (Ni = 9%; Fig. 3).

At 3 hours after last session of three-time mild hypoxia the total number of Trx-2 immunoreactive cells as well as number of intensively expressed Trx-2 cells was significantly decreased compared to control only in CA1 (Nt = 74% Ni = 33%), in other hippocampal areas studied the changes of Nt and Ni in comparison with control were not significant (Fig. 1-3). Nt in CA2 at 3 hours after three-time hypoxia was significantly higher compared to 3 hours time-point after one-time hypoxia; and in all areas studied Nt at 3 hours after three-time hypoxia was significantly higher in comparison with 24 hours time-point after one-time hypoxia (Fig. 2).

At 24 hours after last session of three-time mild hypoxia the total number of Trx-2 immunoreactive cells was significantly down-regulated in comparison with control in CA1 (Nt = 59%), CA2 (Nt = 46%) and

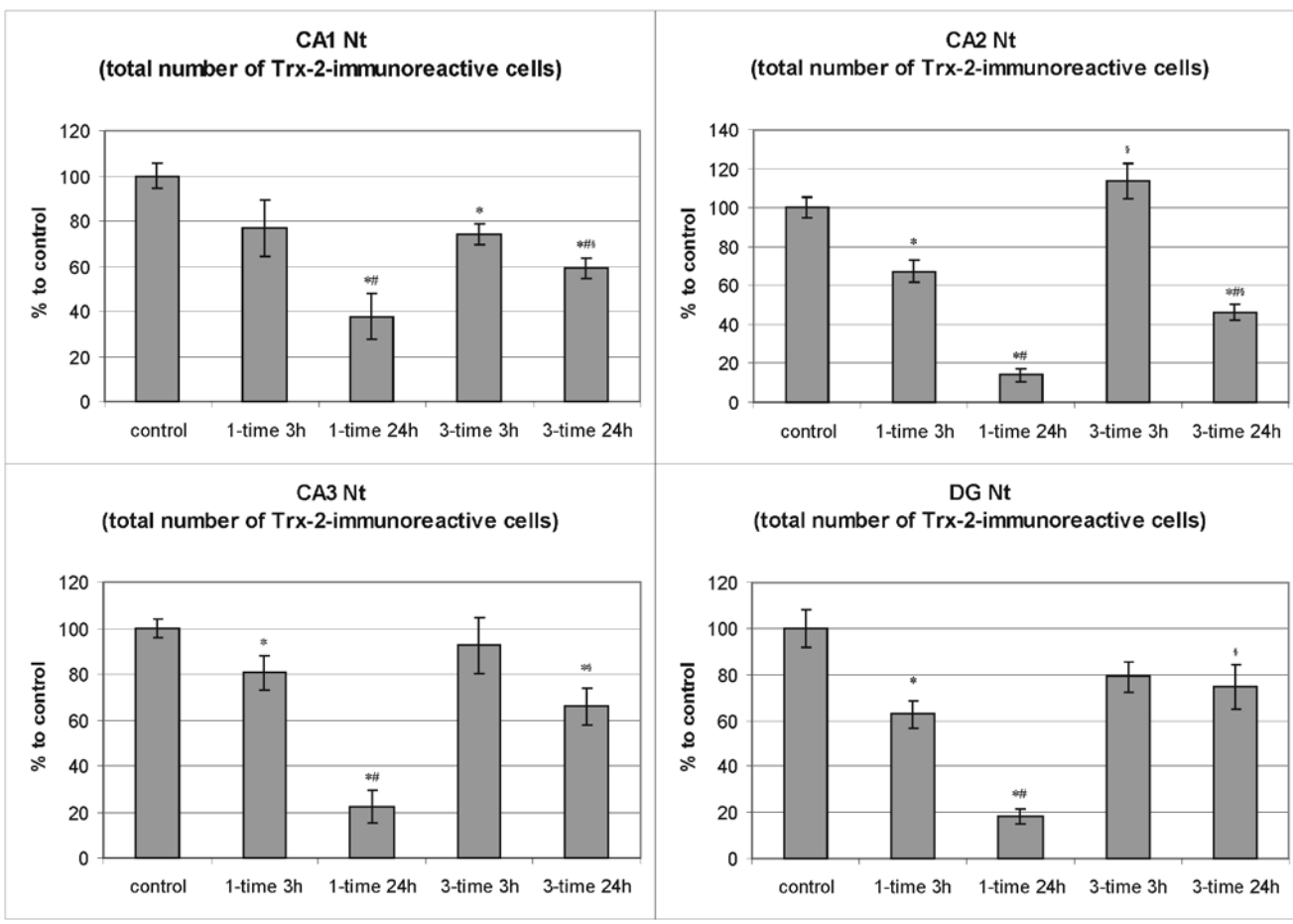

Fig. 2. Graphs showing changes in the total number of Trx-2-immunoreactive cells ±SEM expressed as a percentage of control (Nt) in different areas of rat hippocampus at 3 and 24 h after one-time and three-time mild hypoxia, as compared to control group. CA1, CA2, CA3 field of hippocampus and dentate gyrus (DG). Statistically significant (p<0.05) differences: * - as compared to control, # - 24 hours time-point as compared to 3 hours time-point, § - three-time hypoxia as compared to one-time one (at same time-point).

CA3 (Nt = 66%) (Fig. 1-2); the decrease of Ni in these areas was not significant (Fig. 3). In CA1 and CA2 the decrease of Nt at 24 hours was also significant compared to 3 hours time-point after three-time hypoxia (Fig. 2). However Nt in all areas studied and Ni in CA1 and CA3 at 24 hours after three-time hypoxia was significantly higher than at 24 hours after one-time one (Fig. 2-3).

DISCUSSION

Thioredoxins are multifunctional antioxidant proteins. Their most important function is redox status regulation. They are involved in various processes such as DNA and protein synthesis, protein structure formation and folding, regulation of gene expression and enzyme activity, apoptosis inhibition, cell growth and proliferation (Holmgren 1985, Biaglow and Miller 2005, Patenaude et al. 2005, Kondo et al. 2006). Thioredoxins regulate the activity of several transcriptional factors including NF-κB, AP-1, CREB, PEBP2/CBF, Myb and HIF-1 (Hayashi et al. 1993, Akamatsu et al. 1997, Hirota et al. 1997, 1999, 2000, Das et al. 2001, Welsh et al. 2002, 2003, Csiki et al. 2006), the p53-dependent p21 transcriptional activity and protein expression (Ueno et al. 1999), suppress the apoptosis signal-regulating kinase 1 (ASK1; Saitoh et al. 1998, Zhang et al. 2004) and p38 MAP kinase (Hashimoto et al. 1999). One of the key Trxs defense function is the buffering of reactive oxygen species (ROS; Nakamura et al. 1994, Ueda et al. 2002) and inhibition of cytochrome c release from mitochondria (Andoh et al. 2002, Chen et al. 2002, Damdimopoulos et al. 2002, Tanaka et al. 2002, Ueda et al. 2002, Nonn

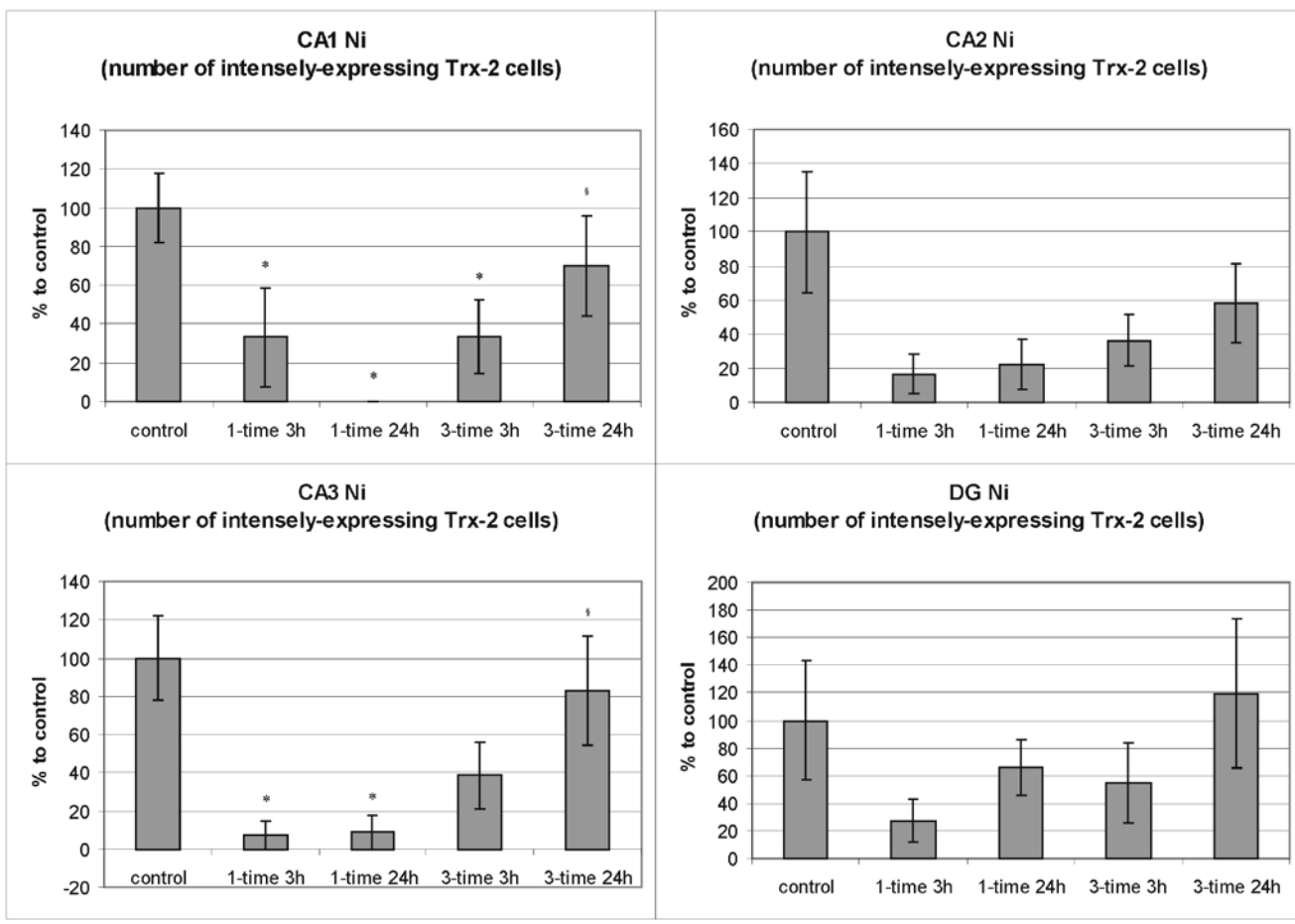

Fig. 3. Graphs showing changes in the number of intensely-expressing Trx-2 cells ±SEM expressed as a percentage of control (Ni) in different areas of rat hippocampus at 3 and 24 h after one-time and three-time mild hypoxia, as compared to control group. CA1, CA2, CA3 field of hippocampus and dentate gyrus (DG). Statistically significant (p<0.05) differences: * - as compared to control, # - 24 hours time-point as compared to 3 hours time-point, § - three-time hypoxia as compared to one-time one (at same time-point).

et al. 2003). They can also activate other antioxidant systems, e.g., Mn-superoxide dismutase (Das et al. 1997).

Thus thioredoxins play a key role in defense of cells during oxidative stress induced in particular by various hypoxic/ischemic events. Different forms of hypoxia/ischemia commonly up-regulate the Trx-1 (Tomimoto et al. 1993, Berggren et al. 1996, Stroev et al. 2004a) and Trx-2 (Stroev et al. 2004b) expression but the extremely severe hypoxic influences on the contrary can suppress it. For example Trx protein and mRNA expression was down-regulated in the ischemic core regions but up-regulated in the perifocal ischemic regions since 4 hours after focal brain ischemia (Takagi et al. 1998a, b, Hattori et al. 2002).

Based on these data it was possible to suppose that all hypoxic influences except the most extreme ones cause the defense reaction connected with up-regulation of Trxs expression. In preceding studies we have shown that severe injuring hypobaric hypoxia (180 Torr, 3h) induced the statistically significant increase of Trx-1 and Trx-2 expression in rat hippocampus (Stroev et al. 2004a, b). It was shown also that preconditioning by three-time mild hypoxia enhanced the resistance to subsequent severe hypoxia (Rybnikova et al. 2005a, b, 2006a) and essentially augmented Trx-1 and Trx-2 immunoreactivity at 3 hours after subsequent severe hypoxia (Stroev et al. 2004a, b). We supposed that mild hypoxia itself also up-regulate the expression of Trxs. However, the results presented here turned out to be unexpected. Analysis shown that at 3 hours after three-time mild hypoxia (preconditioning itself) the total number of Trx-2 immunoreactive cells and the number of intensely expressing Trx-2 cells were significantly decreased compared to control in CA1. At 24 h after three-time mild hypoxia, that was a start-point of severe hypoxia in previous experimental series (Stroev et al. 2004a, b), the total number of Trx-2 immunoreactive cells was significantly down-regulated in comparison with control in all hippocampal areas studied except DG.

Present results suggest that the augmentation of Trx-2 content at 3 h after preconditioned severe hypoxia is not caused by Trx-2 accumulation during preconditioning: at the start of severe hypoxia session (24 hours after three-time mild hypoxia) the Trx-2 level in preconditioned rats was significantly lower than in native ones. Consequently it was due to modification of reaction to severe hypoxia itself.

Analogous in principle results were recently received by us for cytosolic thiredoxin-1 (Stroev et al. 2009). It was shown that at 24 hours after three-time mild hypoxia the total number of Trx-1 immunoreactive cells as well as the number of intensively expressed Trx-1 cells were significantly decreased compared to control in all hippocampal areas studied: in CA1 (Nt = 78%, Ni = 40%), CA2 (Nt = 65%, Ni = 16%), CA3 (Nt = 50%, Ni = 7%) and DG (Nt = 75%, Ni = 34%). At 3 hours after three-time mild hypoxia the Trx-1 immunoreactivity was significantly decreased only in CA3 (Nt = 65% Ni = 44%) compared to control (Stroev et al. 2009). Inconsistency of these our results about possibility of down-regulation of Trx-1 and Trx-2 expression after mild hypoxia and literature data about up-regulation of Trxs by both hypoxia and ischemia (Tomimoto et al. 1993, Berggren et al. 1996) may be explained by difference of experimental models.

The effect of three-time mild hypoxia on expression of Cu, Zn-superoxide dismutase was also similar (Stroev et al. 2011). At 24 hours after third hypoxic session the expression of this antioxidant protein was significantly lower than in control in both dorsal hippocampal areas: CA1 (Nt = 74% Ni = 39%) and CA2 (Nt = 70% Ni = 45%). In ventral hippocampus the total number of Cu, Zn-superoxide dismutase immunoreactive cells was not differ compared to control but number of intensively expressed this antioxidant cells was at least non-significantly lower than in control: in CA3 Ni = 67% and in DG Ni = 69%.

It is interesting to note that according to our previously obtained data the expression pattern of Mn-superoxide dismutase is markedly different. At 24 hours after the last session of three-time mild hypoxia the expression of Mn-superoxide dismutase was significantly increased in CA1 and DG, but did not differ from control in CA2 and CA3 (Stroev et al. 2007). At the same time the up-regulating effect of preconditioning on the Mn-superoxide dismutase expression after subsequent severe hypoxia appears, on the contrary, in the CA2 and CA3 but not in CA1 and DG (Stroev et al. 2005b). Thus, despite the opposite directional changes in Mn-superoxide dismutase expression when compared with thioredoxins and Cu, Zn-superoxide dismutase, the result confirms the same conclusion: the neuroprotective effect of preconditioning at the early periods after severe hypoxia is associated not with the accumulation of antioxidant proteins during preconditioning, but with the modification of reaction to severe hypoxia. In contrast, in cases where preconditioning by

mild hypoxia increases the expression of antioxidant (Mn-superoxide dismutase) at the beginning of severe hypoxia, the up-regulation effect of preconditioning on its expression after severe hypoxia is absent.

One-time and three-time mild hypoxia more or less similarly influenced on total number of Trx-2 immunoreactive cells (Nt): both of them significantly decrease Nt to 24-hours time-point compared to control in all hippocampal areas studied except DG in which this reduction was statistically significant only after a one-time hypoxia (Fig. 2). However effects of one- and three-time hypoxia on number of intensely expressing Trx-2 cells (Ni) varied (Fig. 3). It is also interesting to note that the dynamics of changes in total number of Trx-2 immunoreactive cells (Nt) from 3-hours after single to 24-hours after triple moderate hypoxia has the wave phase character. It goes down from control to 3-hours (statistically significant in CA2, CA3 and DG, non-significant in CA1) and further to 24-hours time-points after first hypoxic session (statistically significant in all areas). Then it goes up from 24-hours time-point after first hypoxic session to 3-hours time-point after third hypoxic session (statistically significant in all areas); and then in all areas except DG (statistically significant in CA1 and CA2, non-significant in CA3) it goes down again from 3-hours to 24-hours time-points after third hypoxic session (Fig. 2). It is possible that the periodical recurrence of these oscillations and corresponding wave oscillations of pro- and antioxidant systems activity perhaps is the factor that takes part in formation of ameliorative effect of preconditioning and hypoxic tolerance of hippocampal neurons that connected with ability of rapid and intensive response of antioxidant systems to the subsequent severe hypoxia.

Molecular mechanisms of this functional state modification require further study. Obviously it can be caused either by accumulation of Trxs mRNAs as result of up-regulation of transcription or by down-regulation of mRNA degradation or by enhanced translation of protein from mRNA that already exists.

The last hypothesis seems most probable. In our study we showed an interesting phenomenon. In CA1 area the marked induction of Trx-2 mRNA was seen only at 24 h after non-preconditioned severe hypoxia (Samoilov et al. 2002), however the protein level was significantly increased already at 3 h (Stroev et al. 2004b). Evidently in this case the enhancement of protein synthesis takes place more rapidly than the increase in the transcription of corresponding gene. The molecular mechanism of this regulation may be connected with the translocation of translation from the ER to polysomes or with action of unknown specific translation factors. One of the factors that can have such a role may be the heterogenous ribonucleoprotein A18 (Yang et al. 2006).

It is known that the ROS mediate the preconditioning-induced rescue pathways (Marini et al. 1996, Rauca et al. 2000, Ravati et al. 2000, 2001, Rudiger et al. 2003) and exposure to exogenous antioxidants such as N-acetyl cysteine, 2-mercaptopropionyl glycine, dimethyl thiourea, N-t-butyl-alpha-phenylnitrone, 2-hydroxyoestradiol and vitamin E during preconditioning at least partially abolished its beneficial effect (Baines et al. 1997, Kaeffer et al. 1997, Vanden Hoek et al. 1998, Das et al. 1999, Rauca et al. 2000, Ravati et al. 2000, 2001, Leak et al. 2006). In our experiments the decreases of Trxs expression at 24 hours after first and third session of mild precondition hypoxia may result in increases of ROS level at these time-points. One may assume that during preconditioning the increased ROS by feedback mechanism induce changes in the expression of regulators of Trxs because it is known that ROS can induce Trx mRNA transcription (Taniguchi et al. 1996, Moon et al. 2005). Obviously later this modification of Trxs regulators expression or/and up-regulation of Trxs mRNA transcription possibly causes the augmentation of Trx-1 and Trx-2 expression after subsequent severe hypoxia.

CONCLUSIONS

One-time and three-time mild hypoxias not increase but in some hippocampal areas significantly decrease the expression of Trx-2. Thus the augmentation of Trx-2 content at 3 hours after preconditioned severe hypoxia is caused not by Trx-2 accumulation during preconditioning but modification of reaction to severe hypoxia itself.

Dynamics of changes in total number of Trx-2 immunoreactive cells from 3-hours after single to 24-hours after triple moderate hypoxia has in some cases the wave character.

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