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Neocortical pCREB and BDNF expression under different modes of hypobaric hypoxia: Role in brain hypoxic tolerance in rats

Michail Samoilov[a], Anna Churilova[a], Tatjana Gluschenko[a], Elena Rybnikova[b,*]

[a] Laboratory of Regulation of Brain Neuron Functions, Pavlov Institute of Physiology, Russian Academy of Sciences, Makarova 6, 199034 St. Petersburg, Russian Federation

[b] Laboratory of Neuroendocrinology, Pavlov Institute of Physiology, Russian Academy of Sciences, Makarova 6, 199034 St. Petersburg, Russian Federation

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ABSTRACT

Preconditioning with repetitive mild hypobaric hypoxia is known to increase tolerance of susceptible brain neurons to severe hypoxia, whereas a single trial of mild hypoxia has been ineffective. In the present study, the effects of three-trial and one-trial hypobaric preconditioning on the expression of the protective transcription factor phosphorylated CREB (pCREB) and neurotrophin BDNF, before and after severe hypobaric hypoxia, have been comparatively studied in the neocortex of rats. As revealed by quantitative immunocytochemistry, the severe hypobaric hypoxia (180 Torr, 3 h) substantially down-regulated the levels of pCREB and BDNF in cortical neurons assessed 24 h after the treatment. One trial of mild hypoxia (360 Torr, 2 h) also reduced by half the number of BDNF-expressing cells, but had no effect on pCREB expression in the neocortex. In contrast, the exposure to three trials of mild hypoxia at 24 h intervals considerably up-regulated pCREB and BDNF levels in the neocortex of rats. Only preconditioning by three trials of mild hypoxia (360 Torr, 2 h, 24 h intervals), but not a single trial preconditioning, was neuroprotective significantly enhancing the pCREB and BDNF neuronal expression in response to severe hypoxic challenge. The results of the present study indicate that development of the neuronal hypoxic tolerance induced by the three-trial mild hypoxic preconditioning is apparently associated with activation of CREB and BDNF expression.

Introduction

CREB (cAMP response element-binding protein) is a central transcription factor that mediates cyclic AMP (cAMP) and calcium-dependent gene expression through the cAMP response element (CRE) (Lonze and Ginty, 2002). Members of the CREB family share the basic leucine zipper (bZIP) domain responsible for DNA binding and dimerization. CREB activation depends on the intracellular calcium Ca^{2+} increase mediated through NMDA, AMPA and mGLU receptors (Choe and Wang, 2001; Mabuchi et al., 2001; Kitagawa, 2007). The active form of CREB is formed through phosphorylation of the Ser-133 residue by protein kinase A (PKA), extracellular signal-regulated protein kinase (MAPK/ERK), and calcium-calmodulin-dependent protein kinases (CaMKII, IV) (Finkbeiner, 2000; Mabuchi et al., 2001; Meller et al., 2005). CREB is critical for neuronal plasticity and for the survival of brain neurons (Walton and Dragunow, 2000; Kandel, 2001; Mabuchi et al., 2001; Lonze and Ginty, 2002; Kitagawa, 2007; Tan et al., 2012). It also plays one of the major roles in regulation of adaptive behavior, learning and memory (Silva et al., 1998; Kandel, 2001; Kida et al., 2002; Gruart et al., 2012). Active CREB, phosphorylated CREB (pCREB), is known to exert its action through regulation of a diverse array of genes, which include immediate early genes c-fos, zif 268 (Hata et al., 1998), activity-regulated inhibitor of death (AID) genes (Tan et al., 2012), genes of peptide antioxidants (Trx1) (Chiueh et al., 2005), anti-apoptotic factor bcl-2 (Sugiura et al., 2004; Chiueh et al., 2005; Meller et al., 2005), and neurotrophins such as BDNF (brain-derived neurotrophic factor) (Shieh et al., 1998; Tao et al., 1998; Sugiura et al., 2004).

BDNF belongs to a family of the neurotrophic factors that also includes nerve growth factor and neurotrophins 3 and 4. An induction of BDNF transcription has been observed after training of contextual learning, physical exercise, exposure to novel environment, chronic exposure to drugs of abuse, as well as kainic acid-induced seizures (for review see Zheng et al., 2012). BDNF

Abbreviations: CREB, cAMP response element-binding protein; BDNF, brain-derived neurotrophic factor; SH, severe hypoxia; PC, preconditioning.

* Correspondence author at: Pavlov Institute, of Physiology, Russian Academy of Sciences, 199034 St. Petersburg, Russia.
E-mail addresses: rybnikova1@rambler.ru, annch05@mail.ru (E. Rybnikova).

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transcription is substantially activated by NMDAR-mediated Ca^{2+} (Tao et al., 1998; Zheng et al., 2012) and neural activity. The initiation of BDNF mRNA expression requires transcriptionally active factor NF-kB (Lipsky et al., 2001). BDNF plays an important role in maintaining cell survival as well as regulation of synaptic functions (Segal and Greenberg, 1996; Huang and Reichardt, 2001; Gómez-Palacio-Schjetnan and Escobar, 2013; Gray et al., 2013). The biological activity of BDNF depends on its interaction with the tropomyosin-receptor-kinase (Trk) receptor B. BDNF binding to TrkB triggers intracellular signaling cascades through phospholipase Cγ (PLCγ), mitogen-activated protein/extracellular signal-regulated kinase (MAPK/ERK), and phosphoinositide 3-kinase (PI3K)/Akt pathways (Kaplan and Miller, 2000; Chao, 2003; Reichardt, 2006). The activation of the latter results in up-regulation of genes, including the *bdnf* gene itself, genes of pro-survival proteins, in particular Bcl-2 and antioxidant enzymes (Mattson et al., 1995), as well as other plasticity-related genes (Koponen et al., 2004).

The increase in pCREB immunoreactivity was observed in the hypoxia-resistant granule nerve cells after ischemia (Hu et al., 1999). Activation of CREB is known to prevent ischemic neuronal damage and induce ischemic tolerance (Tanaka et al., 2000; Hara et al., 2003). Several studies have demonstrated that ischemic preconditioning can facilitate activation of CREB (Nakajima et al., 2002; Hara et al., 2003; Meller et al., 2005). On the other hand, there are data suggesting a direct link between neuronal survival and BDNF levels. In particular, neuronal activity-dependent up-regulation of BDNF signaling may mediate the increased survival of newly generated neurons in the hippocampus (Lee et al., 2002; Rossi et al., 2006). Decreased BDNF expression was observed in response to ischemia (Kokaia et al., 1995). On the contrary, up-regulation of BDNF expression was seen in the penumbral region after ischemia (Kokaia et al., 1995). Close interaction between CREB and BDNF appears to contribute significantly to the above processes, since *in vivo* and *in vitro* neuroprotective effects of BDNF were dependent on CREB (Tao et al., 1998; Lee et al., 2009).

Although there is accumulating evidence supporting involvement of CREB and BDNF in the mechanisms of neuroplasticity and brain tolerance against harmful exposure, their precise role in the signal pathways mediating the neuroprotective action of ischemic/hypoxic preconditioning (PC), which provides an efficient and promising tool for increasing brain resistance (Kitagawa et al., 1990; Kirino et al., 1991; Dirnagl et al., 2003; Samoilov et al., 2003; Rybnikova et al., 2005a,b; Steiger and Hangii, 2007; Stenzel-Poore et al., 2007), remains largely unexplored.

The experimental paradigm of hypobaric hypoxia has been used for several years in our studies devoted to the elucidation of the mechanisms of brain hypoxic injury and neuroprotection. It was shown that severe hypobaric hypoxia (SH) resulted in extensive neuronal damage in the vulnerable brain areas (hippocampus, neocortex) whereas triple sessions of mild hypoxia used as a PC-stimulus prevented injury and loss of brain neurons, as well as the impairment of adaptive behavior, learning and memory caused by SH (Rybnikova et al., 2005a,b, 2006). Recently we reported that one-trial PC, in contrast to the three-trial treatment, was ineffective in preventing structural and functional damage of neurons in these brain regions following SH (Churilova et al., 2013). Diverse neuroprotective efficacy of these two PC protocols in our paradigm provides a good experimental model to explore the role of CREB and BDNF in the mechanisms of brain tolerance. For this reason the aim of the present study was to perform a comparative analysis of the effects of various regimes of hypobaric hypoxia, differed in their injurious/protective performance, on the expression of pCREB and BDNF in the neocortical neurons in rats.

Materials and methods

Subjects and hypobaric hypoxia paradigm

Experiments were carried out on 3-month old male Wistar rats weighing 200–220 g. Experimental procedures were conducted in accordance with The Guidelines for Reporting Animal Research (Kilkenny et al., 2010) under the approval of the Ethical Committee for Use of Animal Subjects at the Pavlov Institute of Physiology.

Severe hypoxia was produced in a hypobaric chamber by maintaining the pressure at 180 Torr (equivalent to 5% normobaric oxygen) for 3 h. The severe hypoxia (SH) produced in such a paradigm caused extensive neuronal damage in the hippocampus and neocortex (Rybnikova et al., 2005a, 2006). To achieve preconditioning (PC) rats were exposed to single or triple trials of mild hypoxia (360 Torr equivalent to 10% normobaric oxygen, each trial lasted 2 h, for multiple sessions the trials were spaced at 24 h intervals).

The experimental animals were divided into 6 groups: (1) control animals (no hypoxia applied), (2) rats subjected to one trial of preconditioning by mild hypoxia (PC); (3) rats subjected to three trials of PC; (4) rats subjected to SH; (5) rats subjected to severe hypoxia (SH) with one trial of PC; (6) rats subjected to SH with three trials of PC. Each experimental group consisted of 6–8 rats.

Immunocytochemistry

Changes in the pCREB and BDNF expression in the neocortex were detected by quantitative immunocytochemistry and the computer image analysis 24 h after the exposure of rats to mild (groups 2, 3) or severe hypoxia (groups 4, 5, 6) compared to animals kept in the hypobaric chamber with no hypoxia applied (group 1). For immunocytochemical analysis the rats were sacrificed, the brains were rapidly excised, and the coronal pieces of the brain containing the neocortical region were fixed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS; pH 7.3) for 24 h. The samples were then immersed in paraffin wax according to a routine histological protocol and sectioned. The coronal sections (7 μm) of the brain about 2.80 mm from the bregma (Paxinos and Watson, 1986) were deparaffinized and incubated overnight with polyclonal rabbit antibodies against rat phosphorylated CREB (pCREB; Santa Cruz Biotechnology, Santa Cruz, CA, USA; dilution 1:100) and BDNF (Santa Cruz; dilution 1:100) at +4 °C overnight. The sections were further processed using rabbit ABC Staining System kit according to the standard protocol (Santa Cruz). Diaminobenzidine (DAB) was used as a chromogenic substrate to visualize the immunoreactive cells. Negative controls lacking the primary antibody produced no immunostaining.

The stained sections were assayed with an image analysis system consisted of light microscope (Carl Zeiss, Germany), digital camera Baumer 12CX05c (Baumer Optronic, Germany), IBM-PC computer, and Videotest Master Morphology software (version 5.2, VideoTest Ltd., St. Petersburg, Russia). pCREB and BDNF immunoreactivity was examined in the frontoparietal neocortex, in particular, in its upper (II and III) and lower (V) pyramidal layers which, as is well known, differ significantly in their cellular structure. While the upper layers include small pyramidal cells and interneurons, which are implemented in the associative connections, the lower layer V includes the large pyramids, which represent the main projection of the neurons of the neocortex and are involved in cortical-subcortical circuits. The immunoreactive cells were quantified in a square of 400 μm × 400 μm, using Videotest Morphology software. All immunopositive cells were divided into two classes: lightly labeled class 2, which includes the cells with staining intensity within the range from 0.02 to 0.11 of the gray level conventional units above the background, and intensely labeled class 1,