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Acetylation of histones in neocortex and hippocampus of rats exposed to different modes of hypobaric hypoxia: Implications for brain hypoxic injury and tolerance

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ABSTRACT

Acetylation of nucleosome histones results in relaxation of DNA and its availability for the transcriptional regulators, and is generally associated with the enhancement of gene expression. Although it is well known that activation of a variety of pro-adaptive genes represents a key event in the development of brain hypoxic/ischemic tolerance, the role of epigenetic mechanisms, in particular histone acetylation, in this process is still unexplored. The aim of the present study was to investigate changes in acetylation of histones in vulnerable brain neurons using original well-standardized model of hypobaric hypoxia and preconditioning-induced tolerance of the brain. Using quantitative immunohistochemistry and Western blot, effects of severe injurious hypobaric hypoxia (SH, 180 mm Hg, 3 h) and neuroprotective preconditioning mode (three episodes of 360 mm Hg for 2 h spaced at 24 h) on the levels of the acetylated proteins and acetylated H3 Lys24 (H3K24ac) in the neocortex and hippocampus of rats were studied. SH caused global repression of the acetylation processes in the neocortex (layers II–III, V) and hippocampus (CA1, CA3) by 3–24 h, and this effect was prevented by the preconditioning. Moreover, hypoxic preconditioning remarkably increased the acetylation of H3K24 in response to SH in the brain areas examined. The preconditioning hypoxia without subsequent SH also stimulated acetylation processes in the neocortex and hippocampus. The moderately enhanced expression of the acetylated proteins in the preconditioned rats was maintained for 24 h, whereas acetylation of H3K24 was intense but transient, peaked at 3 h. The novel data obtained in the present study indicate that large activation of the acetylation processes, in particular acetylation of histones might be essential for the development of brain hypoxic tolerance.

1. Introduction

In recent years, epigenetic mechanisms of gene regulation in brain neurons have been intensely studied under various physiological and pathological states of the organism (Gräff et al., 2011; Rudenko and Tsai, 2014; Stankiewicz et al., 2013). Epigenetic modifications are carried out by attaching specific chemical groups both to the bases of DNA (DNA methylation) or to the amino acid residues at the N-terminal tails of nucleosome histones (acetylation, phosphorylation, methylation, ubiquitylation, sumoylation, *etc.*) and cause changes in chromatin structure (Bannister and Kouzarides, 2011). The combination of these modifications associated to either transcriptional activation or repression determines general transcriptional status of the brain neurons and contributes to the regulation of genome functional state.

The involvement of epigenetic mechanisms in cellular responses to hypoxia/ischemia, in particular in the brain, became a subject of plural recent studies (Johnson and Barton, 2007; Melvin and

Abbreviations: SH, severe hypoxia; HDACs, histone deacetylases; HATs, histone acetyltransferases; DNMTs, DNA methyltransferases; GR, glucocorticoid receptor; MR, mineralocorticoid receptor; pan-ac, pan-acetyl proteins; H3K24ac, acetylated histone H3 (Lys24).

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Rocha, 2012; Perez-Perri et al., 2011; Schweizer et al., 2013; Tsai and Wu, 2014; Watson et al., 2010; Wu et al., 2013). Considerable attention is paid on studying the epigenetic modifications in response to injurious events. Most of such studies are focused on effects of hypoxia/ischemia on the activity of epigenetic enzymes, such as histone deacetylases (HDACs), histone acetyltransferases (HATs), DNA methyltransferases (DNMTs), and based on using of their inhibitors (Faraco et al., 2006; Johnson and Barton, 2007; Ren et al., 2004; Wu et al., 2014, *etc.*). The reports on effects of hypoxia/ischemia upon levels of acetylated and methylated histones in various brain regions are still very scanty. Moreover, effects of protective preconditioning hypoxia which results in development of brain hypoxic/ischemic tolerance have not been explored so far. On the other hand, an important role of gene-dependent processes in mechanisms of long-lasting brain tolerance to severe hypoxia/ischemia has been well-documented by many researchers (for review see Kitagawa, 2007; Marini et al., 2007; Steiger and Hangii, 2007; Stetler et al., 2009). The expression of pro-adaptive genes in brain neurons is activated by the up-stream transcription factors which bind to specific response elements in gene promoters, but an access to the promoters is controlled by epigenetic mechanisms. In our recent studies it has been shown that severe injurious hypoxia and preconditioning mild hypoxia has opposite action on the expression of inducible transcriptional factors c-Fos, NGFI-A, HIF-1α, activation of CREB and NF-kB, as well as expression of glucocorticoid (GR) and mineralocorticoid (MR) receptors which in turn regulate transcription of multitude of steroid-responsive genes (Rybnikova et al., 2011, 2009, 2008, 2005). Severe hypoxia suppressed the activity of these transcriptional factors and reduced their expression, whereas mild hypoxic preconditioning leading to development of neuronal hypoxic tolerance considerably upregulated them. In addition to the transcriptional factors, the neuroprotective action of preconditioning has been associated to an overexpression of the target genes, in particular of antiapoptotic proteins (Bcl-2, Bcl-xL), neurotrophins (BDNF), antioxidants (MnSOD, Cu, ZnSOD, Trx1,2) (Rybnikova et al., 2006; Samoilov et al., 2014; Stroev et al., 2005, 2004). Taken together, these facts allow to suggest that necessary mechanism which links preconditioning-induced active transcriptional factors to mediation of their effects on target genes is provided by epigenetic modifications, in particular acetylation of histones resulting in relaxation of DNA and its availability for the transcriptional regulators. The present study has been designed to establish a missing link and reveal the role of histone acetylation in the hypoxic injury and tolerance using original well-standardized model of hypobaric hypoxia and hypoxic preconditioning. The effects of severe injurious, mild preconditioning hypoxia and their combination on the levels of total histone acetylation and levels of acetylated histone H3 Lys24 (H3K24ac) have been examined in vulnerable neurons of rat hippocampus and neocortex.

2. Materials and methods

2.1. Subjects and a model of hypobaric hypoxia

Experiments were carried out on the adult male rats (Wistar, 220–240 g). Animal procedures followed the guidelines of the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were approved by the Ethical Committee for Use of Animal Subjects at the Pavlov Institute of Physiology RAS. Hypobaric hypoxia was simulated in a flowing hypobaric chamber by maintaining the pressure at 180 mm Hg (equivalent to 5% normobaric oxygen and an altitude of 11,000 m) for 3 h (severe injurious hypoxia, SH), and 360 mm Hg (equivalent to 10% normobaric oxygen and an altitude of 5000 m) for 2 h (mild hypoxia). Three experimental protocols of hypoxia were applied: 1 – SH; 2 – three successive episodes of mild hypoxia spaced at 24 h and 3 – a preconditioning with three episodes of mild hypoxia followed by the single exposure to injurious SH in 24 h. The experimental animals were divided into 7 groups: group 1 – control animals (no hypoxia applied), groups 2, 3 – rats subjected to SH and sacrificed 3 and 24 h later, respectively; groups 4, 5 – rats subjected to three episodes of mild hypoxia and sacrificed at 3 and 24 h after the last episode of mild hypoxia; groups 6, 7 – rats preconditioned with three episodes of mild hypoxia and subjected to SH in 24 h, sacrificed 3 h and 24 h later. Each animal group consisted of six-eight rats.

2.2. Immunocytochemistry

For immunocytochemical analysis the brains of sacrificed rats were rapidly excised, and the coronal pieces containing the neocortex and hippocampus were fixed in formalin-free fixative FineFix (Milestone, Italy) for 24 h. The samples were then embedded into paraffin according to a standard 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 antibody against human acetylated (Lys 24) histone H3 (Santa Cruz biotechnology, Inc, dil. 1:100) (Ac-Histone H3 (Lys 24): sc-34262; applications: Ac-Histone H3 (Lys 24) is recommended for detection of Lysine 24 acetylated Histone H3 of broad species origin by Western Blotting, immunoprecipitation, immunofluorescence) or rabbit polyclonal pan-acetyl antibody (C4) (Santa Cruz biotechnology, Inc, dil. 1:100) (pan-Acetyl (C4)-R: sc-8663-R; applications: pan-Acetyl (C4)-R is recommended for detection of pan-Acetyl of broad species origin by Western Blotting, immunoprecipitation, immunofluorescence) at +4 °C for overnight. The sections were further processed using rabbit Vectastain ABC Staining System kit (Vector Laboratories, USA) (Catalog No. BA-1300; Lot N V1219) and diaminobenzidine as a chromogen to visualize the immunopositive cells.

2.3. Negative antibody controls

Two common types of negative control were performed. First, the primary antibodies were not added to the dilution buffer poured on the samples (no primary antibody control). This resulted in no staining both for acetylated H3 antibody and pan-acetyl antibody that indicates no non-specific binding or false positives due to non-specific binding of the secondary antibody and other reagents.

Second, to estimate specificity of binding of H3K24ac antibody to acetylated H3, as well as a proportion of acetylated histones in staining observed with pan-acetyl (C4) antibody, an immunizing peptide blocking experiment was performed according to the Abcam protocol (http://www.abcam.com/protocols/blocking-with-immunizing-peptide-protocol-peptide-competition). Briefly, before proceeding with the staining protocol, the antibodies were incubated with an excess of acetylated H3 histone or total acetylated histones used as the acetylated proteins. Acetylated histones for immunizing peptide blocking experiment were extracted from nuclear fraction of hippocampus that was isolated according to the classical method published by Drews and Wagner (1970), and using inhibitors of proteases, phosphatases and histone deacetylases (Santa-Cruz Biotechnology, USA). For extraction of acetylated H3 or total acetylated histones, the protocol by Shechter et al. (2007) was applied. The acetylated histones were then precipitated by acetone, dried in centrifugal vacuum concentrator CentriVap Concentrator (Labcono, USA), and dissolved in the deionized water for concentration quantitation. For the blocking experiment, the corresponding acetylated histones were dissolved in PBS to a final concentration of 1 μg/ml and incubated for 30 min at room temperature. The standard staining protocol described above was then followed.