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·Original Article·

Chronic cerebrovascular hypoperfusion affects global DNA methylation and histone acetylation in rat brain

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ABSTRACT

DNA methylation and histone acetylation can be modified by various pathological or physiological factors such as hypoxia, thus influencing gene expression. In this study, we investigated the changes of global DNA methylation and histone acetylation and the related enzymes in rat brain after chronic cerebrovascular hypoperfusion by bilateral common carotid occlusion (2-VO) surgery. Colorimetric and immunohistochemistry staining were used to evaluate the global DNA methylation and histone acetylation levels, respectively. The expressions of DNA methyltransferase 1/3a (DNMT1/3a), methyl-CpG binding domain protein 2 (MBD2), histone deacetylase 3 (HDAC3) and acetyltransferase (HAT) were assessed by Western blot. We found that the level of global DNA methylation was decreased to 31.7% (P <0.01) of the sham-operated group at 10 days and increased by 30% (P <0.01) compared with the sham group at 90 days after 2-VO surgery. DNMT3a expression was down-regulated to 75.7% of the sham group, while MBD2 expression was up-regulated by 95% compared with sham group at 90 days after 2-VO. The histone H3 acetylation level was markedly decreased to 75.3% of the sham group at 10 days and 73.5% at 90 days after 2-VO, while no significant change was found for histone H4 acetylation. HDAC3 expression was markedly down-regulated to 36% of the sham group, whereas cAMP-response element binding protein expression was up-regulated by 33.6% compared with the sham group at 90 days after 2-VO. These results suggest that chronic cerebrovascular hypoperfusion influences global DNA methylation and histone acetylation levels through the related enzymes, and therefore might contribute to several neurodegenerative diseases.

Keywords: cerebrovascular hypoperfusion; global DNA methylation; histone acetylation; rat

INTRODUCTION

The term “epigenetics” refers to the study of changes in gene expression in response to gene-environment interactions without changes in the underlying DNA sequence. Epigenetic modification involves DNA methylation, histone modification, genomic imprinting, and chromatin remodeling[1,2]. By influencing gene function and characteristics, epigenetic processes can regulate cell proliferation and differentiation[3]. Recently, epigenetic mechanisms have been increasingly shown to play crucial roles in the origin and development of many human diseases such as cancer[4-6] and neurodegenerative disorders[7-9]. Alzheimer disease (AD) is one of the most common neurodegenerative disorders in the elderly population. Its exact pathogenesis, especially the sporadic form, is still not clear. Recent studies reveal that AD is among a few neurodegenerative diseases that may involve low folate and B12 and high homocysteine in the blood, indicating an imbalance in the S-adenosylmethionine cycle that is responsible for epigenetic regulation through DNA

methylation[10]. These findings have highlighted the role of epigenetic mechanisms in the pathogenesis of AD.

Chronic cerebrovascular hypoperfusion is a common pathological process in clinical cases[11]. Persistent reduction in regional cerebral blood flow results in a decline of cognitive function, as well as the development of neurodegenerative disorders such as Parkinson's disease (PD) and AD[12-15]. Some data also suggest that the decline in cognitive function can be improved by correction of the chronic cerebral hypoperfusion in humans[16,17]. Moreover, recent studies indicate that the activity of hypoxia-induced transcription factors, such as hypoxia-inducible factor-1, which are basic for determining the hypoxic response, is regulated epigenetically[18-20]. Moreover, through the regulation of enzymes that modulate DNA methylation and histone modification, hypoxia itself can influence gene expression to some extent[21,22]. Since hypoxia is a direct consequence of hypoperfusion, changes in DNA methylation and histone acetylation levels may contribute to brain dysfunction under chronic cerebral hypoperfusion.

In the present study, we investigated the effects of cerebrovascular hypoperfusion on global DNA methylation and histone acetylation levels in the rat brain after bilateral carotid occlusion (two-vessel occlusion, 2-VO). Meanwhile, changes in enzymes related to DNA methylation and histone acetylation were investigated.

MATERIALS AND METHODS

Animals and Surgery

Male Sprague-Dawley rats aged 23 weeks (Vital-River, Beijing, China) were raised in the Experimental Animal Center of Capital Medical University under a 12:12 h light/dark cycle. All procedures were approved by the Ministry of Science and Technology of China and conformed to the guidelines of the Animals Care and Use Committee. The rats were randomly divided into six groups (14/group), three groups with 2-VO and the other three as sham-operated controls. The detailed procedures of 2-VO surgery were as described previously[23].

Global DNA Methylation Analysis

Genomic DNA was extracted from the parietal cortex using the Wizard® SV Genomic DNA Purification System (Promega, WI). DNA concentration and purity were determined by absorbance at 260 and 280 nm. Global DNA methylation was measured with the MethyFlash™ Methylated DNA Quantification Kit, Colorimetric (Epigentek, New York) according to the manufacturer's instructions. In this assay, DNA was bound to strip wells that were specifically treated to have a high DNA affinity. The methylated fraction of DNA was detected using capture and detection antibodies and then quantified colorimetrically by reading the absorbance in a microplate spectrophotometer. Simple calculation of the percentage of 5-methylcytosine (5-mC) in total DNA was carried out using the following formula:

$$5\text{-mC\%}=\frac{OD_{sample-blank}/100}{OD_{positive\ control-blank}\times 2/5}\times 100\%.$$

Histone Acetylation Assay

Global histone H3 and H4 acetylation was measured using the EpiQuik™ Global Histone H3/H4 Acetylation Assay Kit (Epigentek, New York) according to the manufacturer's instructions as described previously[24]. The acetylation rate was calculated as follows:

$$\text{Acetylation rate (\%)}=\frac{OD_{sample-blank}}{OD_{control-blank}}\times 100\%.$$

Western Blot Analysis

The parietal cortex was dissected out and homogenized. Routine procedures were carried out as described previously[25]. The primary antibodies used were: mouse anti-β-actin monoclonal antibody (1:500, ZSGB-BIO), rabbit anti-histone deacetylase 3 (HDAC3)/(DNA methyltransferase 1) DNMT1 monoclonal antibody and anti-DNMT3a polyclonal antibody (1:1 000, Cell Signaling, MA), mouse anti-p300/CBP monoclonal antibody (1:50, Abcam, London, UK) and rabbit anti-methyl-CpG binding domain protein 2 (MBD2) polyclonal antibody (1:1 000, Santa Cruz, CA). The densities of the bands on the membrane were scanned by a Gel-Doc Image Scanner (Bio-Rad, CA) and analyzed with an image analyzer (ImageJ, Broken Symmetry Software).

Immunohistochemistry

Section preparation and immunohistochemical staining were carried out as described previously[25]. All the sections were selected from identical sections of the brain tissue according to the Paxinos and Watson rat brain atlas. DNA

was denatured *in situ* with 4 mol/L HCl at room temperature for 15 min before serum blockade. Section were incubated with rabbit anti-5-mC primary antibody (1:300, Merck) overnight at 4°C, followed by anti-rabbit secondary antibody (1:500; Dylight 488, Invitrogen) for 2 h at room temperature. All sections were observed and photographed under a laser confocal microscope (TCS SP5, Leica).

Statistical Analysis

All the data are presented as mean ± SD. Data analyses were performed with the SPSS software version 17.0. Differences between means within experiments were evaluated with the independent samples *t*-test. P <0.05 was considered statistically significant.

RESULTS

Cerebrovascular Hypoperfusion Leads to Changes in Global DNA Methylation

As the circle of Willis allows continuing blood flow after the onset of 2-VO, the cerebral hypoperfusion is global and no distinct ischemic core and penumbra region as well as cell death were observed. Colorimetric assay was performed to determine the global DNA methylation levels in the parietal cortex. The methylation level was decreased to 31.7% of the sham-operated group after cerebrovascular hypoperfusion for 10 days, was comparable to the sham group after 30 days, and increased by ~30% compared with the sham group after 90 days (Fig. 1). To further evaluate the effect of hypoperfusion on DNA methylation, immunohistochemical staining for 5-mC was performed. The 5-mC immunoreactivity was located in nuclei in the hippocampus, and decreased after hypoperfusion for 10 and 30 days, but was greater after 90 days, compared with the sham group (Fig. 2). There was no significant difference in 5-mC expression between hippocampus and cortex (data not shown).

Cerebrovascular Hypoperfusion Induces Abnormal DNMT and MBD2 Expression

DNA methylation is completed by DNA methyltransferases (DNMTs), and MBD2, which binds to and recognizes 5-mC, is accepted to have demethylase activity. Since the global DNA methylation level was influenced under our experimental conditions, it was essential to determine whether it was associated with the expression of DNMTs and MBD2. We found that DNMT3a expression decreased to 75.7% and MBD2 increased by 95% compared with the sham-operated group. However, DNMT1 expression did not change significantly (Fig. 3).

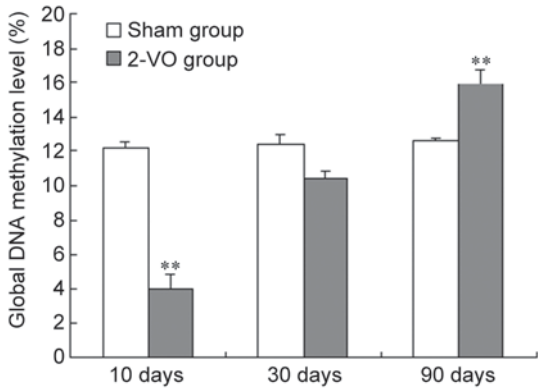

Fig. 1. Global DNA methylation levels in the parietal cortex homogenates from sham-operated and 2-vessel occlusion (2-VO) rats at 10, 30 and 90 days after surgery, as determined by colorimetric assay. Data are presented as mean ± SD (n = 6). $^{**}P$ <0.01 *vs* sham-operated group.

Decreased Histone H3 Acetylation Level Is Accompanied by Downregulation of HDAC3 and Upregulation of p300/CBP under Cerebrovascular Hypoperfusion

To further investigate the epigenetic mechanisms, histone H3/H4 acetylation assay kits were used to evaluate the global H3/H4 acetylation levels in rat brain. The global H3 acetylation level was decreased to 75.3% of the sham-operated group at 10 days and 73.5% at 90 days after 2-VO surgery (Fig. 4A), while the H4 acetylation level did not change significantly (Fig. 4B). Since histone acetylation is induced by histone acetyltransferases (HATs) (the co-activators p300 and CBP are HATs[26]) whereas deacetylation is regulated by histone deacetylases (HDACs), we next assessed the protein levels of p300/CBP and HDAC3 by Western blot. P300/CBP expression in the brain increased by 33.6% compared with sham, whereas HDAC3 expression decreased to 36% of the sham group at 90 days after 2-VO (Fig. 4C, D). All these results implied that DNA methylation and histone acetylation are involved in the pathological process of chronic cerebral hypoperfusion.

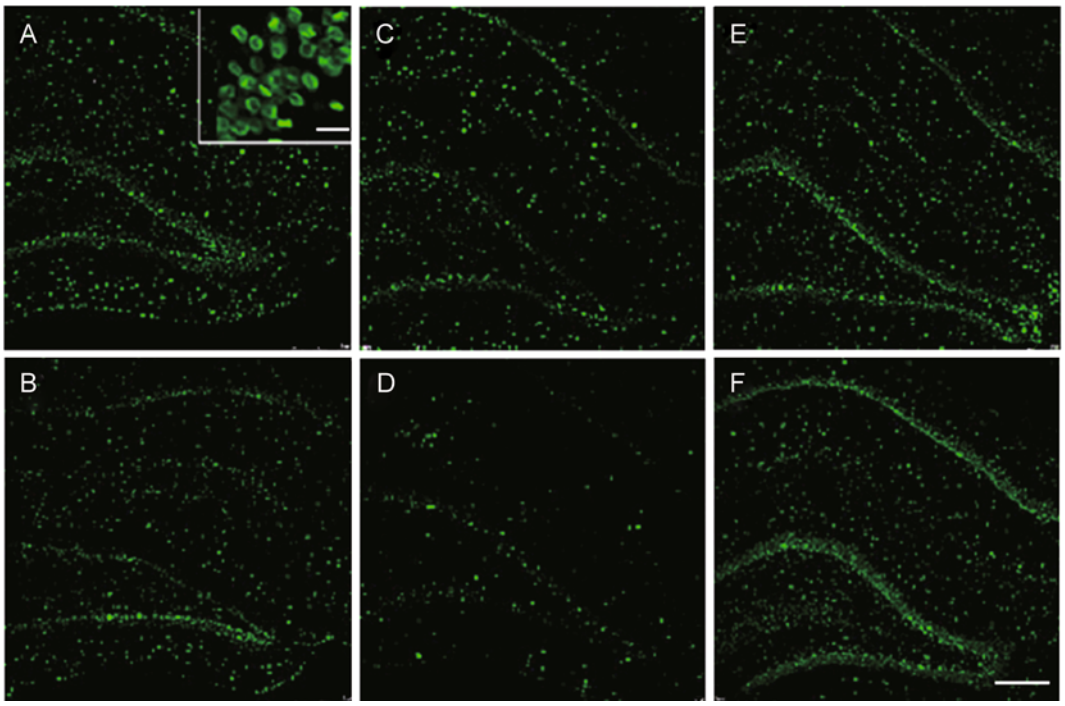

Fig. 2. Immunohistochemical staining for 5-methylcytosine in rat hippocampus after sham operation (A, 10 days; C, 30 days; E, 90 days) and after 2-vessel occlusion (B, 10 days; D, 30 days; F, 90 days after surgery). Images were obtained using a confocal microscope. Inset in A is a higher magnification image showing 5-methylcytosine immunoreactivity located in the nuclei. Scale bar for A–F, 250 μm; for the inset in A, 50 μm.

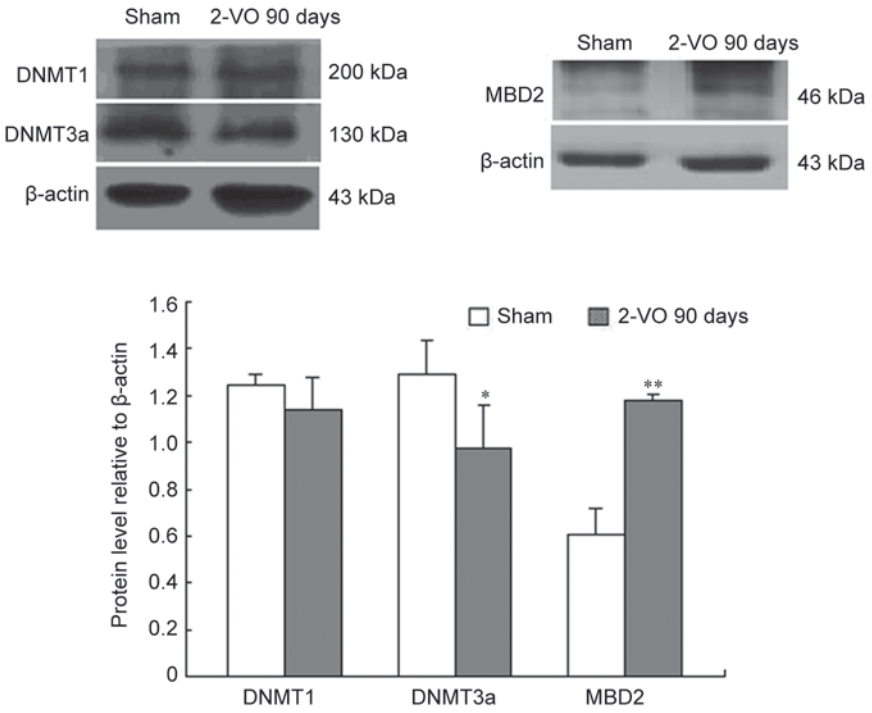

Fig. 3. Upper panels: Western blots for DNMT1/3a (left) and MBD2 (right) in rat parietal cortex at 90 days after sham operation or 2-vessel occlusion (2-VO). Lower panel: densitometric quantification of each protein. Mean ± SD (n = 6). $^{*}P$ <0.05, $^{**}P$ <0.01 *vs* sham-operated group.

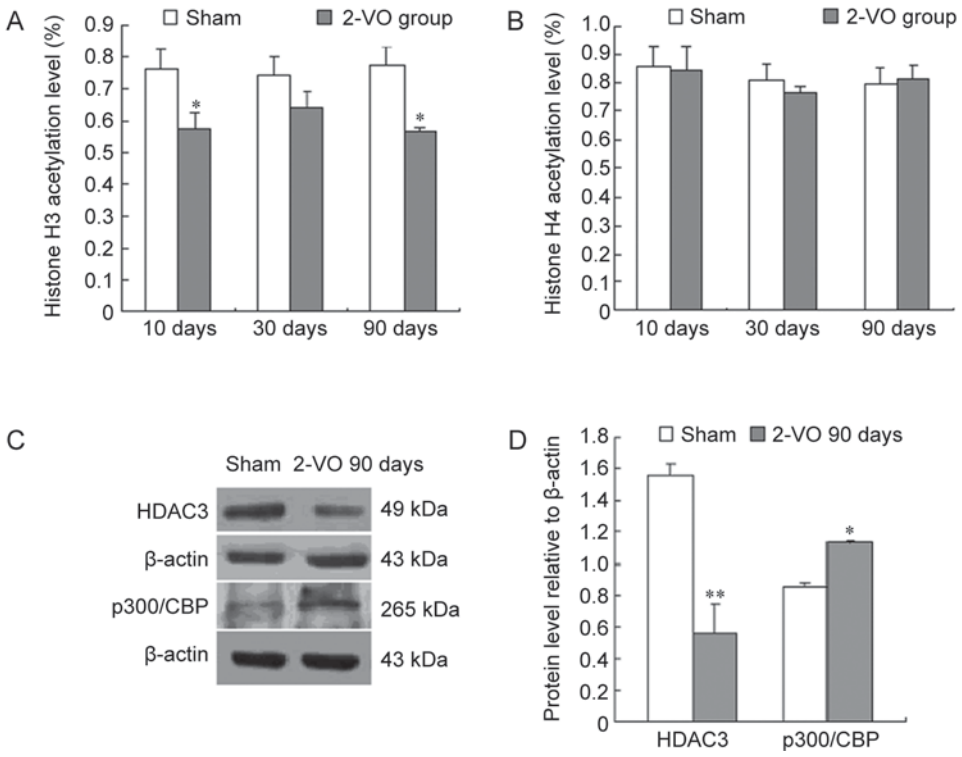

Fig. 4. Histone H3 and H4 acetylation levels and p300/CBP and HDAC3 protein expression in rat parietal cortex. Acetylation levels of histone H3 (A) and H4 (B) were calculated as the acetylation rate (%). C and D show Western blot and statistical analysis of p300/CBP and HDAC3 protein levels. Data are presented as mean ± SD (n = 6). $^{*}P$ <0.05, $^{**}P$ <0.01 *vs* sham-operated group.

DISCUSSION

Sufficient cerebral blood flow is essential for maintaining normal brain functions. Prolonged, insufficient perfusion leads to cognitive impairment[27,28]. Chronic cerebral hypoperfusion is assumed to be the pathogenic mechanism underlying several types of neurodegenerative disorders. In previous studies, we found that chronic cerebrovascular hypoperfusion results in memory impairment and β-amyloid protein (Aβ) accumulation in the brain[25,29]. Permanent, bilateral occlusion of the common carotid arteries (2-VO) in rats is widely used to investigate the effects of chronic cerebral hypoperfusion on neurodegenerative diseases[22,30]. In this model, the occlusion is permanent, and significantly reduces cerebral blood flow[12,31].

Epigenetic changes can influence gene expression. Recent studies have suggested that epigenetic mechanisms play pivotal roles in several human disorders[1,32] of which the most commonly studied are DNA methylation and histone acetylation. DNA methylation usually represses transcription[33]. The enzymes responsible for the methylation process are a family of DNA methyltransferases, including DNMT1, 2, 3a and 3b in mammals. DNMT1 is a key player in maintaining methylation in somatic cells in which it has already occurred, whereas DNMT3a mainly promotes new methylation. MBD2 is a protein that binds to methylated regions of DNA and acts as a demethylase[34,35]. In this study, we demonstrated that the level of global DNA methylation in rat brain was significantly decreased at 10 days and increased at 90 days after 2-VO. However, DNMT3a was significantly decreased and MBD2 protein was conversely increased compared to the sham-operated group. Regarding this, the results of global DNA methylation analysis to some extent disagreed with the changes in DNMT1/3a and MBD2 protein levels. The reason is not clear. Some researchers have reported that DNMT1 down-regulation is not an essential condition for

global genomic hypomethylation in the human placenta[36]. This suggests that, although DNA methylation is mainly mediated by DNA methyltransferases, the expression of DNMT and DNA methylation may not always be consistent. DNA methylation is a complex process influenced by various environmental factors[1]. Since humans have tens of thousands of genes, the global DNA methylation level cannot represent a specific gene methylation status. Much effort is needed to study the specific mechanisms in order to reveal the relationships between disease and DNA methylation.

Histone acetylation is responsible for transcriptional activation, while deacetylation is linked with transcriptional repression. Both increases and decreases in histone acetylation may occur at specific loci[4,37]. HATs and HDACs are the main enzymes in this process. HATs are known to interact with various transcription factors, such as p300 and CBP, to regulate gene transcription. HDACs, classified into four families, are sequence-specific to genetic loci leading to transcriptional repression by forming a complex of methyl-CpG binding protein (MECP) and recruited HDACs[37]. HDAC3 is one of the most highly-expressed class I HDACs throughout the brain, including the hippocampus[38]. Some studies have shown that HDAC3 is a critical negative regulator of long-term memory formation[39]. However, there are few studies on the function of HDAC3 in AD[40], in which the common clinical manifestation is memory impairment. Here, we revealed that the histone H3 acetylation level was decreased, accompanied by downregulation of HDAC3 and upregulation of p300/CBP during cerebrovascular hypoperfusion. But the H4 acetylation level did not change significantly; the reason for this is not clear.

Taken together, our data demonstrated that chronic cerebrovascular hypoperfusion modified the status of DNA methylation and histone acetylation, probably leading to changes in gene transcription. Chronic cerebrovascular hypoperfusion is involved in several neurodegenerative disorders, such as AD. It is widely accepted that epigenetics is a critical pathological mechanism in AD[41-43]. Hypomethylation in the promoter region of the amyloid-β precursor protein (APP) gene has been detected in an AD patient[44]. A previous study revealed that Aβ induces both H3 and H4 acetylation in the hippocampal CA1 region in Tg2576 mice that carry the human APPswe mutation[45]. Changes in epigenetic regulation have also been reported in PD[45].

In summary, epigenetics is a relatively new frontier in understanding the pathogenic mechanisms underlying human diseases, especially with regard to the regulation of gene expression in the brain. Further studies are needed to investigate the epigenetic changes of specific genes in different neurodegenerative disorders associated with chronic cerebrovascular hypoperfusion to provide new targets for therapeutic intervention in the treatment of these diseases.

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