Iranian Journal of Basic Medical Sciences

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Intermittent hypoxia reduces microglia proliferation and induces DNA damage *in vitro*

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ARTICLE INFO

Article type:
Original article

Article history:
Received: Sep 11, 2015
Accepted: Mar 3, 2016

Keywords:
Inflammatory cytokine
Intermittent hypoxia
Microglia
P53 signaling pathways

ABSTRACT

Objective(s): Intermittent hypoxia (IH), caused by obstructive sleep apnea (OSA), could cause hippocampus or neuron damage through multiple signaling pathways, while the underlying mechanisms are still unclear. Thus, the present study aimed to explore the effect of IH on the biological functions of microglia cells.

Materials and Methods: Cell proliferation of BV2 cells after exposure to IH were observed by MTT assay and then DNA damage was detected by comet assay. RNA-sequencing assay was performed in cells under IH condition and normal conditions to find out the differentially expressed genes, which were further confirmed by reverse transcriptase polymerase chain reaction (RT-PCR) and Western blot assay.

Results: As results, IH inhibited the proliferation of BV2 cells, as well as caused DNA damage. RNA-sequencing assay revealed 4 differentially expressed genes (p21, Cyclin D1, Cyclin E2, and Gadd45α) which were associated with the network of P53 signaling pathways in BV2 cells, among which, p21 and Gadd45α were dramatically increased while Cyclin D1 and Cyclin E2 were both decreased significantly. Moreover, inflammatory factors including IL-6, TNF-α and iNOS were significantly up-regulated in microglia cells under IH conditions for 8 hr.

Conclusion: Our results indicated that IH could inhibit cyclin D1 and cyclin E2 expression via initiating multiple P53 pathways, which further blocked cell cycle transition and attenuated proliferative capability of BV2 cells. Meanwhile, IH activated inflammation reactions in BV2 cells. Present study elaborate the effects of IH on biological functions of microglia and provide theoretical foundation for further study on new therapy methods for OSA.

► *Please cite this article as:*
Liu S, Wang Zh, Xu B, Chen K, Sun J, Ren L. Intermittent hypoxia reduces microglia proliferation and induces DNA damage *in vitro*. Iran J Basic Med Sci 2016; 19:497-502.

Introduction

Obstructive sleep apnea (OSA) is a common sleep disorder characterized by recurrent episodes of complete or partial collapse of the upper airway during sleep, resulting in oxygen desaturation, arousal from sleep (1) and excessive daytime sleepiness, further ultimately lead to substantial neurocognitive and behavioral deficits (2). In adults, the major neurocognitive manifestation of OSA include impaired cognition, and mood disturbances (3). Children with OSA were reported to have neurobehavioral disturbances, as well as an increased incidence of hyperactivity and retarded growth (4). Furthermore, both in children and adults, OSA is deleterious to the central nervous system (CNS) through causing structural neuron damage and dysfunction (5). Intermittent hypoxia (IH), one of the consequences of OSA, is traditionally defined as the presence of recurring events associated with low blood oxygen levels and followed by subsequent reoxygenation (5). It has been reported that IH could lead to the dramatically decreased expression of sclerite in cerebral hippocampus and prefrontal cortex, which was closely associated to the memory and cognition ability (6). A number of studies revealed that revealed that the IH caused hippocampus or neuron damage through multiple signaling pathways, including ion-channel alterations, glutamate excitotoxicity, oxidative stress, mitochondrial dysfunction, up-regulation of proinflammatory mediators, altered regulation of pro- and anti-

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apoptotic gene cascades, diminished apolipoprotein E, and nitric oxide (NO) over-production (1), while the process and underlying mechanisms in detail are complex and still unclear.

The CNS is composed of two major cell types: neuronal cells and glial cells. Glial cells consist of astrocytes, oligodendrocytes, and microglia. Microglia are the key elements in CNS cularization, inflammation, and neuroprotection (6). They are resident immune cells in CNS (7), which was worked for immunosurveillance as the roles of phagocytic cell in other systems, as well as trigger cascade reaction when CNS was damaged (8). Multiple kinds of stimulant (such as LPS, Aβ, environmental toxins, neurotoxin, endogenous pathogenic proteins, and damaged neuron) could activate microglia. The moderate activation of microglia is benefits to neural protection, and when it was excessive activated, inflammatory reaction would be emerged (9). Then it will undergo dramatic morphological alternations upon activation, changing from resting ramified microglia into activated amoeboid microglia. Once microglia were activated, their surface molecules such as NO, ROS, proinflammatory receptors, and glutamate will be unregulated simultaneously, which further caused neuron damage and in turn activated microglia. Thus, it will form a vicious circle, which lead to nerve inflammation related diseases through degenerative changes or death of neuron (10). Recent researches demonstrated that activated microglia induce cell apoptosis of neuron in hippocampus region, which resulting in the dysfunction of hippocampus area (11).

All of results from these study indicated that microglia play crucial roles in CNS protection. However, it is still unclear how microglia involved in the CNS damage induced by IH? Thus, in this study, we chose BV2 as experimental subject, which was applied multiple times for researches focused on inflammatory reaction and defense mechanisms in CNS damage. We detected the biological functions of BV2 cells after exposure to IH. After that, RNA-sequencing was adopted to detect the differentially expressed genes between normal person and OSA patients, aimed to explore the potential pathways associated with the pathogenesis of OSA. The results of this study could help to understand the neurocognitive function of OSA, and to provide theoretical foundation for further study on new therapy methods for OSA.

Materials and Methods

Cell culture and IH treatments

BV2 microglia cells were gifts from Professor Zengqiang Yuan (State Key Laboratory of Brain and Cognitive Science, Institute of Biophysics, Chinese Academy of Sciences). The cells were plated onto 6-well cell culture plates in Dulbecco's Modified Eagle Medium (DMEM, purchased from Thermo Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (Gibco, Grand Island, NY, USA) and 1% penicillin and streptomycin (Invitrogen, Carlsbad, CA, USA) in an incubator with 5% CO_2 at 37 °C. After 24 hr incubation, cells were fed with fresh medium for subsequent treatment.

Cultured BV2 cells were exposed to IH cell culture box, which is a controlled gas chamber regulated the flow of nitrogen and oxygen. In the chamber, the O_2 levels were alternated between 1% and 21% for 400 sec/cycle. Cells in the control group were maintained in normoxic conditions (Normal: 21% O_2). After 8 hr of the treatments, cells were collected for future assays.

MTT assay

The proliferation of BV2 cells was detected via MTT kits (purchased from Sigma-Aldrich, St. Louis, MO, USA). The procedures were followed the instruction. Briefly, BV2 cells after IH exposure and control group were plated at 4×10^6 per well, followed by incubated for 8 hr, and then 10 µl MTT reagent was added. Afterwards, incubation of the cells were continued for 2-4 hr until purple precipitate was visible. Then, 100 µl detergent reagent was added. Leave the mixed liquor at room temperature in the dark for 2 hr, then recorded the absorbance at 570 nm.

Comet assay

Genomic DNA damage was measured by using comet assay. Briefly, BV2 cells after IH exposure were embedded in 0.6% (w/v) low melting point agarose and then lysed in 2.5 mol NaCl, 10 mmol Tris-HCl (pH 10), 100 mmol EDTA, 1% (v/v) Triton X-100 at 4 °C overnight. Denaturation (20 min) followed by electrophoresis (20 min at 25 V and 300 mA) was performed in a solution of 0.3 mol NaOH, 1 mmol EDTA. Then the slides were washed, stained with SYBR Green (Thermo Fisher Scientific, Inc. Lafayette, CO, USA) and observed using a fluorescent microscope (Olympus Optical Co., Ltd. Tokyo, JP). Slides were analysed using the Komet 5 analysis software (Kinetic Imaging, Liverpool, UK).

RNA sequencing

Total RNA was isolated using an 'RNeasy' kit (Qiagen, Hilden, Germany). Total RNA was treated with DNase I (Qiagen, Hilden, Germany) during RNA isolation according to the instructions. Polyadenylated RNA was purified from DNase I–treated total RNA using the MACS mRNA Isolation kit (Miltenyi Biotec, Auburn, CA). RNA was purified from total RNA isolated from cell lines and used for cDNA synthesis followed by fragmentation into 190–210 bp fragments. Double-stranded cDNA was synthesized from purified poly(A) + RNA using a superscript double-stranded cDNA synthesis kit

(Invitrogen Inc. Carlsbad, CA, USA) and random hexamer primers (Invitrogen Inc. Carlsbad, CA, USA) at a concentration of 5 μM. The DNA fraction was excised, eluted overnight at 4 °C in 300 μl of LoTE buffer (3 mM Tris-CL and 0.2 mM EDTA) with 7.5 M ammonium acetate, 5:1) and purified using a QIAquick purification kit (Qiagen, Hilden,Germany). PCR products were purified on QIAquick MinElute columns (Qiagen, Hilden, Germany), assessed and quantified using a DNA 1000 series II assay (Agilent Technologies Inc., Folsom, CA, USA) and Qubit fluorometer (Invitrogen Inc. Carlsbad, CA, USA), respectively. The resulting libraries were sequenced on an Illumina Genome Analyzer II following the manufacturer's instructions. Differential expression (DE) values were determined as the log2 difference in normalized average coverage values between 5-FU–sensitive and –resistant cells for all feature types. Features were considered to be differentially expressed if their expression values differed by a factor of two and their corrected *P*-value was less than 0.05 (by Fisher's exact test).

Western blot

BV2 cells in each group were homogenized in lysis buffer and the homogenate was centrifuged at 12,000 ×g for 15 min at 4˚C. Then protein concentrations were analyzed by bicinchoninic acid technique. Proteins were resolved by polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF) membrane (Amersham Life Science, West Chester, PA, USA) for electrophoresis. The PVDF membranes were blocked at room temperature incubation for 2 hr in the TPBS containing 5% skimmed milk powder. They were then incubated with primary antibody for 2-4 hr at room temperature. Afterwards, the membranes were washed (3 times, 5 min every time) and incubated with the secondary antibody for 1 hr at room temperature, followed by detection with Western blot reagent. To ensure that equal quantities were loaded in each lane, the membranes were blotted with anti β-actin antibody (Cell Signaling Technology, Inc., Beverly, MA, USA).

RT-PCR

Total RNA was isolated by Trizol kit, and complementary DNA (cDNA) was synthesized using Takara RNA PCR kit (Japan) according to the manufacturer's instructions. Fluorescence real-time PCR was performed with the double-stranded DNA dye SYBR green PCR core reagents using the ABI ViiA7 system (Perkin-Elmer). Thermal cycler conditions were 95 ˚C for 30 sec, and 40 cycles of 95 ˚C for 5 sec and 60 ˚C for 30 sec. Relative gene expression was determined by delta delta CT method (ABI, Applied Biosystems) with β-actin as the endogenous control. All data were analyzed using GraghPad Prism 5 software. The primer sequence are as follows: P21-F: 3'-CCTGGTGATGTCCGACCTG-5'; P21-R: 3'-CCATGAGCGCATCGCAATC-5', Cyclin D1-F: 3'-GGGGACAACTCTTAAGTCTCAC-5'; Cyclin D1-R: 3'-CCAATAAAAGACCAATCTCTC-5', Gadd45a –F: 3'-CCTGCACTGTGTGCTGGTGA-5'; Gadd45a –R: 3'-CCACTGATCCATGTAGCGACTTTC-5', Cyclin E2-F: 3'-ATGTCAAGACGCAGCCGTTTA-5'; Cyclin E2-R: 3'- GC-TGATTCCTCCAGACAGTACA-5', P53-F: 3'-TGCAT-GGACGATCTGTTGCT-5'; P53-R: 3'-CTGTCCCAGA-CTGCAGGAAG-5'.

Statistical analysis

Comparison between 2 groups was analyzed by software SPSS 11.0 using t-test. The statistical significance was defined as $P<0.05$. Data were shown as mean ± SD.

Results

IH preconditioning reduced cell proliferation of BV2 cell lines

By applying MTT assay, the relative cell number of BV2 cells in control group and IH treated groups were detected. As shown in Figure 1, 8 hr after the treatment (normoxic conditions or IH exposure condition), the number of BV2 cells in IH treated groups ($7.11\pm1.42\times10^6$) were significantly decreased compared to control group ($11.80\pm1.59\times10^6$), which indicated that IH preconditioning reduced the cell proliferation ability of BV2 cells.

IH preconditioning induced DNA damage in BV2 cell lines

Cells exposed to IH conditions and normoxic conditions were used for DNA damage detection via comet assay. As shown in Figure 2, the results of comet assay demonstrated that 8 hr IH preconditioning caused about 2.5 fold DNA damage in BV2 cells compared to cells under normal conditions, indicating that IH condition could significantly damage the DNA in BV2 cells.

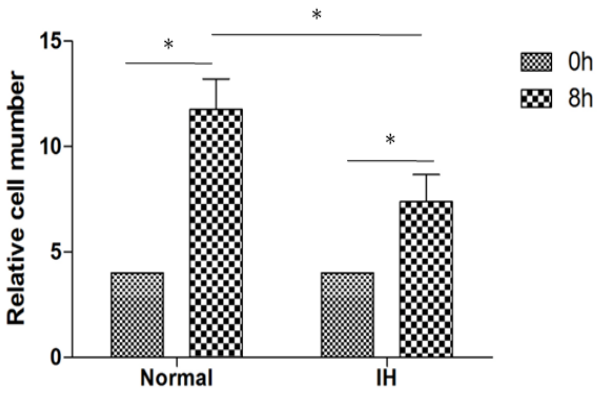

Figure 1. Relative cell number of BV2 cells in normal conditions and intermittent hypoxia exposure conditions. Before and 8 hrs after the treatment, the cell numbers of BV2 in two groups were detected via MTT assay. BV2 cells were plated at 4×10^6 per well, and the absorbance was at 570 nm. IH, intermittent hypoxia conditions. Data were presented as mean ± SD. *, $P<0.05$

Table 1. Network of p53 signaling pathways is associated with intermittent hypoxia. Total RNA was isolated from peripheral blood of normal person and obstructive sleep apnea patients, then RNA sequencing was applied

Gene ID	symbol	log2 ratio	Probability	Description
12575	Cdkn1a	2.445	0.9364	cyclin-dependent kinase inhibitor 1A (P21)
12443	Ccnd1	-1.199	0.8725	cyclin D1
13197	Gadd45α	2.332	0.9114	growth arrest and DNA-damage-inducible 45 alpha
12448	Ccne2	-1.378	0.86910907	cyclin E2

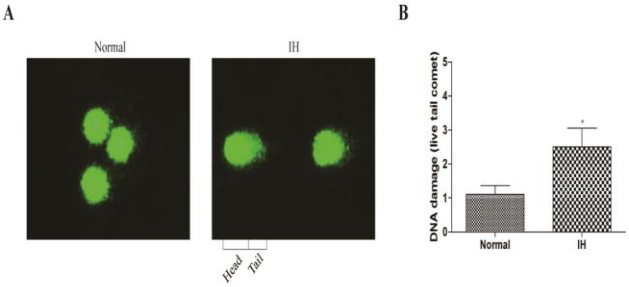

Figure 2. Genomic DNA damage detected by comet assay. BV2 cells after IH exposure were embedded and then lysed. Denaturation (20 min) followed by electrophoresis was performed, then the slides were washed, stained with SYBR Green and photographed. Slides were analysed using the Komet 5 analysis software. A, Images of comets, stained with DAPI. B, the relative DNA damage in IH groups compared to normal groups. IH, intermittent hypoxia conditions. *, $P<0.05$

P53 mediates the IH-associated preconditioning effects

In our study, by applying RNA-sequencing analysis, 4 genes were found differentially expressed between cells under normal condition and IH condition by applying RNA-sequencing (As shown in Table 1). As results, the effect of IH was gene-specific: P21 and Gadd45a were up-regulated in cells under IH condition, while cyclin D1 and cyclin E2 were down-regulated.

Validation of P53 signaling pathways in BV2 cells under IH conditions

The results of RT-PCR (Figure 3A) and Western blot (Figure 4) confirmed that, except for P53 itself, the other several key genes in P53 signaling pathways were all differentially expressed both in mRNA and protein levels. The mRNA expression of P21, Cyclin D1, Cyclin E2 and Gadd45a in cells under IH condition were changed into 1.70 ± 0.25, 0.58 ± 0.12, 0.53 ± 0.14 and 2.00 ± 0.13 folds compared to that in cells in normal condition, and all of the expression differences were significant ($P<0.05$).

Up-regulated expression of inflammatory factors in BV2 cells under IH conditions

In this study, inflammatory factors including TNF-α, IL-6 and iNOS were significantly up-regulated in BV2 cells under IH conditions when compared that to cells in normal groups (relatively increased 2.06 ±

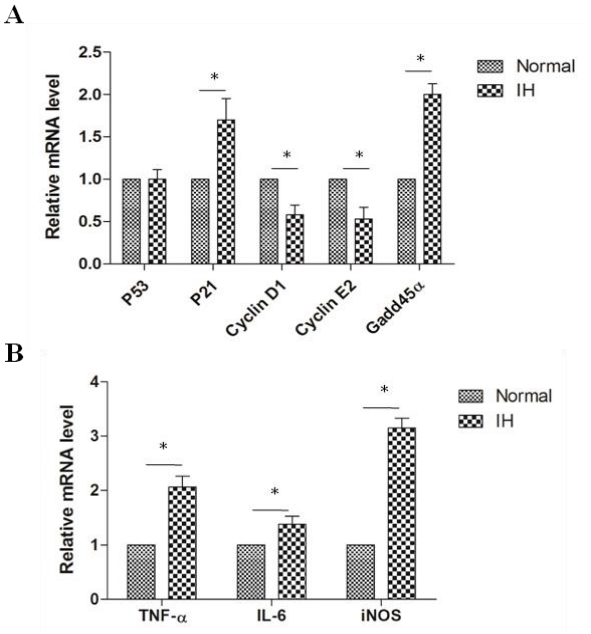

Figure 3. Relative mRNA levels of network of P53 signaling pathway (A) and inflammatory factors (B) in BV2 cells under normal conditions and intermittent hypoxia conditions for 8 hrs. Data were presented as mean±SD. After 8 hrs of the treatments in two groups, Total RNA was isolated and cDNA was synthesized. Fluorescence real-time PCR was performed with β-actin as the endogenous control. IH, intermittent hypoxia conditions. *, $P<0.05$

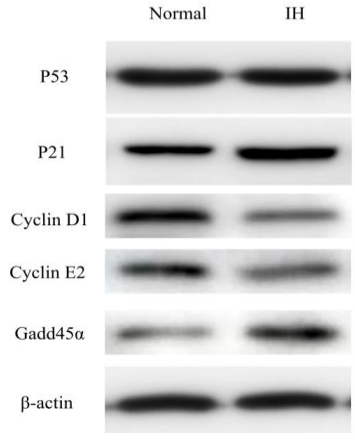

Figure 4. Expression of key factors in p53 signaling pathway in BV2 cells under normal conditions and intermittent hypoxia conditions for 8 hrs. BV2 cells in each group were homogenized and then protein concentrations were analyzed by bicinchoninic acid technique. Western blotting was adopted to detect the expression of aim proteins. β-actin was used as a loading control. IH, intermittent hypoxia conditions

0.20, 1.38 ± 0.15, and 3.15 ± 0.18 folds), indicating the activated inflammatory reaction in BV2 cells under IH condition (Figure 3B).

Discussion

Microglia, as CNS resident immune cells, were considered to be contributors to IH-induced neuroinflammation (5). In fact, microglia are very important elements in CNS development, repair, and neurotransmission. However, their activities have never directly been tested in the model of IH (12). There was also no researches focus on the effect of IH on the proliferation of microglia cells. In 2011, Zhou *et al* revealed the biological functions of primary cultured microglia cell in different stages during anoxia, in which, the microglia cells showed attenuated proliferative capability in the early time and decreased persistently following prolongation of the hypoxia time (13). However, the underlying mechanisms and potential pathways were remains unclear. Thus, it is crucial for us to find out the details of the effect of IH on biological functions of microglia and the further influence on CNS.

The results of comet assays in present study showed that the proliferative capability was attenuated mainly through the damage of DNA in BV2 cells. In this study, the expression of P21 was also up-regulated in BV2 cells in IH. IH also induced the expression of the growth arrest and DNA damage-inducible gene 45α (Gadd45α) which is associated with repair of DNA damage (14) in BV2 cells, indicating the DNA damage occurred in BV2 cells under IH conditions. Moreover, cyclinD1 and cyclin E2 were all down-regulated in BV2 cells under IH conditions in this study. It was well known that, when DNA damage was occurred, P53 would be activated, which will cause the overexpression of P21 (15). P21 is the inhibitor of cyclin-dependent kinase (CDK), so the overexpression of P21 could further arrest the cell cycle via inducing down-regulation of cyclin D1 and cyclin E2 (16). Cyclin D1 and cyclin E2 are proteins which can initiate the cell cycle and prompt the formation of DNA, and inhibit the expression of cyclin D1 and cyclin E2 would block the cell cycle at G1/S phase (17). The above findings indicated that IH could block cell cycle transition via inhibiting cyclin D1 and cyclin E2 expression through P53-P21 pathway, which further attenuated the proliferative capability of BV2 cells.

Our RNA-sequencing assay also showed that the overexpression of Gadd45α in microglia cells was associated with IH preconditioning. Gadd45α encodes an inducible nuclear protein which_plays a critical role in the checkpoint control of cells in response to a wide spectrum of DNA damaging or stress signals (18). Gadd45α was originally identified as a gene whose expression is rapidly induced in response to unreplicated DNA or DNA damage in a P53-dependent manner (19). P53, as a tumor suppressor protein, plays crucial roles in maintaining genetic stability through DNA repair and chromosome recombination. P53 is often activated in response to oxidative stress, which was reported could result in increased transcription of GAdd45α (20). Oxidative stress, which was reported as the results of IH (10), might be the explanation of the overexpression of GAdd45α and the inducement of DNA damage in this study. It was reported that the P53-GAdd45α pathway could trigger cell apoptotic by inducing the dysregulated expression or activation of proteins such as bax/bcl2, Fas/Apol, and IGF-BP3, *et al* (21). Whether the IH could induce the cell apoptosis of BV2 and the relationship between the overexpression of GAdd45α and cell apoptosis in BV2 cells needs our further researches. Nevertheless, P53 was also revealed could combine with P21 and Gadd45α to forming complexes, which can play important roles in DNA repair (22). From the above, P53, as a crucial role in DNA repair and cell cycle transition, is closely connected with IH condition, and the detailed underlying mechanism needs our continuous exploration.

Many mechanisms are thought to contribute to neuronal death following chronic exposure to IH including oxidative stress (23) and inflammation (24). It has been reported that IH promote early and long-lasting increases in IL-1β and IL-6 gene expression in medullary microglia of IH exposure rat model (25), as well as decreased TNF-α expression in cortical microglia after 1 day of IH. This is the first study to evaluate microglial phenotype directly following IH exposure, and to reveal differences in microglial responses based on CNS region and time of exposure. It was reported that, once at the site of lesion in multiple sclerosis, microglia increase inducible nitric oxide synthase (iNOS), which is required for the production of NO (26) expression. Consistent to previous researches, in present study, inflammatory factors including IL-6, TNF-α and iNOS were significantly up-regulated in microglia cells under IH conditions for 8 hr. Further researches were needed to explore the roles of these inflammatory factors in microglia cells exposure to IH.

Conclusion

Our results indicated that IH could inhibit cyclin D1 and cyclinE2 expression via initiating multiple P53 pathways, which further blocked cell cycle transition and attenuated the proliferative capability of BV2 cells. Meanwhile, IH activated inflammation reactions in BV2 cells. The damage on microglia cells induced by IH might further cause neuroinflammation and CNS damage. Thus, this study elaborated mechanisms of the effect of IH on microglia and lay the foundation for further researches on the influence of IH on CNS damage.

Acknowledgment

This work was supported by the Science Foundation of Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine (no. yjrc-004). In this study, the intermittent hypoxia cell culture chamber and intermittent hypoxia control system have been supported by Professor Qingyun Li, the Ruijin hospital affiliated to Shanghai Jiao Tong University School of Medicine.

Conflict of interest

The authors declare there are no conflicts of interest regarding the publication of this paper.

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