P2X7 Receptor Antagonism Attenuates the Intermittent Hypoxia-induced Spatial Deficits in a Murine Model of Sleep Apnea Via Inhibiting Neuroinflammation and Oxidative Stress

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Abstract

Background: The mechanism of the neural injury caused by chronic intermittent hypoxia (CIH) that characterizes obstructive sleep apnea syndrome (OSAS) is not clearly known. The purpose of this study was to investigate whether P2X7 receptor (P2X7R) is responsible for the CIH-induced neural injury and the possible pathway it involves.

Methods: Eight-week-old male C57BL/6 mice were used. For each exposure time point, eight mice divided in room air (RA) and IH group were assigned to the study of P2X7R expression. Whereas in the 21 days-Brilliant Blue G (BBG, a selective P2X7R antagonist) study, 48 mice were randomly divided into CIH group, BBG-treated CIH group, RA group and BBG-treated RA group. The hippocampus P2X7R expression was determined by Western blotting and real-time polymerase chain reaction (PCR). The spatial learning was analyzed by Morris water maze. The nuclear factor kappa B (NFκB) and NADPH oxidase 2 (NOX2) expressions were analyzed by Western blotting. The expressions of tumor necrosis factor α, interleukin 1β (IL-1β), IL-18, and IL-6 were measured by real-time PCR. The malondialdehyde and superoxide dismutase levels were detected by colorimetric method. Cell damage was evaluated by Hematoxylin and Eosin staining and Terminal Transferase dUTP Nick-end Labeling method.

Results: The P2X7R mRNA was elevated and sustained after 3-day IH exposure and the P2X7R protein was elevated and sustained after 7-day IH exposure. In the BBG study, the CIH mice showed severer neuronal cell damage and poorer performance in the behavior test. The increased NFκB and NOX2 expressions along with the inflammation injury and oxidative stress were also observed in the CIH group. BBG alleviated CIH-induced neural injury and consequent functional deficits.

Conclusions: The P2X7R antagonism attenuates the CIH-induced neuroinflammation, oxidative stress, and spatial deficits, demonstrating that the P2X7R is an important therapeutic target in the cognition deficits accompanied OSAS.

Key words: Inflammation; Intermittent Hypoxia; Oxidative Stress; P2X7 Receptor; Sleep Apnea

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cells may release high concentrations of ATP into the pericellular space. At the same time, the proinflammatory cytokines upregulate P2X7R expression and increase its sensitivity to extracellular ATP.\cite{9} As we know, CIH during sleep may contribute to a persistent, chronic inflammatory state.\cite{10} Therefore, we hypothesized that the P2X7R signaling pathways might be involved in CIH-induced neural injury. Interestingly, activation of P2X7R may stimulates multiple cellular processes, including the synthesis and release of multiple inflammatory mediators, the translocation and DNA binding of nuclear factor kappa B (NFκB), the formations of NADPH oxidase (NOX) complex and the generation of reactive oxygen species (ROS).\cite{11} However, it is not known whether P2X7R affects CIH-induced neural injury via the regulation of inflammation or oxidative stress.

In the present study, we aimed to investigate the P2X7R levels in the CIH mice hippocampus and to test the possible pathway of P2X7R in CIH model using a selectively P2X7R antagonist (Brilliant Blue G [BBG]).\cite{12}

**Methods**

All experiments in this study were approved by the Institutional Animal Care and Use Committee of Tongji Medical College, Huazhong University of Science and Technology.

**Hypoxic exposures**

Eight-week-old male C57BL/6 mice (16–20 g; provided by the Experimental Animal Center of Tongji Hospital, Huazhong University of Science and Technology, China) were used in this study. For each exposure time point (3, 7, 14, 21 day), eight mice randomly divided into IH group and room air (RA) group were assigned to studies of P2X7R mRNA and protein. For BBG study, 48 mice were used. They were randomly divided into four groups of 12 each: a CIH group, a BBG-treated CIH group, an RA group and a BBG-treated RA group. Eight to ten mice were housed in a chamber (BioSpherix OxyClycer A84, USA) and exposed to either IH or air-air cycling for 8 h/day during the light period from 9:00 a.m. to 5:00 p.m. For CIH, oxygen levels within the animals’ cage were changed from normal (21 ± 1%) to low (6 ± 1%) for every 90 s as previously described,\cite{13,14} with some modification. The cages of RA mice were infused with RA and pure oxygen to maintain normoxia. The mice were housed on a 12-h light/dark cycle with free access to food and water. For BBG study, BBG (Sigma, USA) was intraperitoneal administered (50 mg/kg BBG diluted in phosphate-buffered saline [PBS]) per day 3 days prior and throughout the 4 weeks before CIH or air exposure, and control group were treated with PBS only. All efforts were made to minimize animal suffering and to reduce the number of animals used.

**Behavioral testing**

Spatial learning and memory were assessed in the Morris water maze as previously described.\cite{15} The maze consisted of a circular pool, 1.22 m in diameter and 0.51 m in height, filled to a level of 35 cm with water maintained at a temperature of 21 ± 1°C. Pool water was made opaque by the addition of milk. A Plexiglas platform (10 cm in diameter) was positioned 0.8 cm below the water surface. Distinctive, geometric, extra maze cues surrounding the maze were located at fixed locations on the white wall and were visible to the mice while in the maze. Maze performance was recorded by a video camera suspended above the maze and interfaced with a video tracking system (Ethovision System, The Netherlands). After 21 days exposures to IH or RA, a standard place-training reference memory task was conducted on mice in the water maze. Place learning was then assessed over 5 consecutive days, and each training session consisted of four trials. On a given daily session, each mouse was placed into the pool from 1 of 4 quasirandom start points and allowed a maximum of 60 s to escape to the platform where the mice would stay for 15 s. Mice that failed to escape were led to the platform. The position of the platform remained constant during the trials. Twenty-four hours following the final training session, the platform was removed for a probe trial.

**Tissue preparation**

Eight mice of each group were deeply anesthetized, and the hippocampus was dissected, frozen in liquid nitrogen and then stored at −80°C until use. The rest mice were transcardially perfused with saline, followed by fixation with 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.4). Brains were postfixed overnight in 4% paraformaldehyde followed by cryoprotection with 30% sucrose (pH 7.4) until brains permeated. The fixed brains were embedded with paraffin until use.

**Quantitative real-time polymerase chain reaction**

Total RNA was prepared from hippocampal tissue samples using TRIzol reagent (Invitrogen, USA). Isolated total RNAs were reverse transcribed to single-stranded cDNA using Prime Script RT Master Mix (TaKaRa, Japan). Then, cDNAs were subjected to real-time polymerase chain reaction (PCR) analysis with SYBR Premix EX Taq (TaKaRa) and 7500 real-time PCR System (Applied Biosystems, USA) following the manufacturer’s protocol. The primers (Sangon, China) for cDNA were used as follows: P2X7R 5'-AGCAGAATGAGCCACCGT-3' and 5'-CCCCAACCTCTGAGCATCT-3', tumor necrosis factor-α (TNF-α) 5'-TCTTGTCATCCGATGG-3' and 5'-GGTCTGGCCATAGAACAGA-3'; interleukin-1β (IL-1β) 5'-TGATAGAAGAGAAGCGCAGCC-3' and 5'-TCTCTCTTGG-3'; IL-12 p40 5'-ATGGTGAGTTGCACTGGGCT-3' and 5'-GGTTAGGCTGTCTTTTGCAAGC-3'; IFN-γ 5'-GGGCTGTCATTGGGCTTGCT-3' and 5'-GGATCTTTCAACG-3'; NFκB 5'-TACTGGATTGCTTGG-3' and 5'-CGTCAACTTCAAGG-3'; tumor necrosis factor-α (TNF-α) 5'-TCTTGTCATCCGATGG-3' and 5'-GGTCTGGCCATAGAACAGA-3'; interleukin-1β (IL-1β) 5'-TGATAGAAGAGAAGCGCAGCC-3' and 5'-TCTCTCTTGG-3'; IL-12 p40 5'-ATGGTGAGTTGCACTGGGCT-3' and 5'-GGTTAGGCTGTCTTTTGCAAGC-3'; IFN-γ 5'-GGGCTGTCATTGGGCTTGCT-3' and 5'-GGATCTTTCAACG-3'; NFκB 5'-TACTGGATTGCTTGG-3' and 5'-CGTCAACTTCAAGG-3'. The cycling condition consisted of one cycle at 95°C for 30 s and 40 s two-segment cycles (95°C for 5 s, 60°C for 34 s). Each sample was performed three times and averaged. The expression differences between the control and treated group

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Western blotting analysis
Hippocampal samples from different groups were lysed with lysis buffer and centrifuged at 12,000 × g/min for 5 min. Protein concentration of samples was then determined by protein assay kit (Beyotime Biotech, China). Next, the protein samples were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis then transferred onto polyvinylidene difluoride membranes (0.45 μm, Millipore). The membranes were washed with TBS-0.1% Tween buffer and then incubated with 5% nonfat dry skim milk for 30 min at room temperature. Next, they were incubated with P2RX7 (ABGENT, USA), Phospho-NFκB p65 (Cell Signaling, USA), NADPH oxidase subtype 2 (NOX2, Abcam, USA), β-actin (Santa Cruz, USA) at 4°C overnight. After three washes with TBS-0.1% Tween, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (Biology, China) for 1 h at room temperature. The membranes were then treated with an enhanced chemiluminescence detection kit (Beyotime Biotech, China), and the signals were detected by exposure to X-ray films (Kodak, USA) for the appropriate time. Western blots were performed in triplicate for accuracy. The band intensity was quantified by Image J software (National Institutes of Health, USA).

Oxidative stress
Oxidative stress is a well-established mechanism of cellular injury in the brain. Superoxide dismutase (SOD) and malondialdehyde (MDA), the commonly used indicator of oxidative stress, lipid peroxidation, and subsequent cellular injury in cells and tissues were assayed using SOD kits and MDA kits according to instructions (Nanjing “JianCheng” Institute of Biological Engineering, China). Briefly, hippocampus tissues were homogenized in 20 mmol/L phosphate buffer (pH 7.4) containing 0.5 mmol/L butylated hydroxytoluene to prevent sample oxidation. After proteins concentration measurement, the samples were then measured at 550 nm. The level of SOD and MDA were calculated with the standard curve according to the manufacturer’s instructions (Nanjing “JianCheng” Institute of Biological Engineering).

Hematoxylin and Eosin staining and terminal Transferase dUTP Nick-end Labeling method
Sagittal 5 μm thick brain sections from four mice of each group were cut and stained with Hematoxylin and Eosin. Transferase dUTP Nick-end Labeling (TUNEL) method was performed as follows: Dewaxed sections were washed three times (5 min each) in 0.01 mol/L PBS and permeabilized in proteinase K for 10 min. Endogenous peroxidase was deactivated by 0.3% hydrogen peroxide. These sections were washed three times again. Then they were incubated with TDT at 37°C for 1 h and incubated with an antibody at 37°C for 1 h. These sections were stained by 3,3′-diaminobenzidine, and after hematoxylin poststaining, were mounted and observed under the light microscope. Five slides were randomly selected from each group, and in each slide, five visual fields (>40) in the hippocampus were randomly selected. The number of damage cells was counted with about 500 cells counted per slide. The damage cells rate was calculated to equal (the number of damage cells/total cells) ×100%.

Statistical analysis
All statistical analyses were calculated using SPSS (version 19.0, International Business Machines corporation, USA) Data were expressed as mean ± standard division (SD). Two group comparisons were evaluated by unpaired t-tests. Multiple comparisons Data were generally tested by analysis of variance (ANOVA) followed by Tukey’s post-hoc test. For morris water maze, acquisition trials were generally averaged in blocks of four and plotted as block means. Repeated measures and multivariate ANOVA process of the general linear model followed by post-hoc least significant differences tests were used to analyze each trial block. For all comparisons, a P < 0.05 was considered to achieve statistical significance.

Results
Intermittent hypoxia induces a significant increased expression of P2X7 receptor mRNA and protein
To establish whether alteration of P2X7R expression in hippocampus tissue occurs after exposure to IH, P2X7 mRNA expression was measured by real-time PCR. P2X7 mRNA was increased at 3 days of IH exposure (P < 0.05) and remaining elevated until day 21 (P < 0.01; Figure 1a). P2X7R protein expression was assessed by Western blotting. IH induced marked increases in P2X7R protein expression at day 7 (P < 0.05), which were sustained throughout IH exposures (P < 0.01; Figure 1b and c).

Brilliant Blue G improves spatial learning performance of chronic intermittent hypoxia mice
Chronic intermittent hypoxia + PBS mice exposed to CIH exhibited longer latencies to locate the hidden platform when compared to RA + PBS, RA + BBG, and CIH + BBG group [Figure 2a]. Overall analysis for the entire trial blocks revealed significant changes of latency between the different treatment groups (F = 8.67, P = 0.002), indicating that CIH adversely affected task performance and BBG treatment alleviated the effects. Significant differences in latencies were observed during blocks four (F = 21.87, P < 0.001) and five (F = 71.69, P < 0.001), with no significant differences in blocks one, two and three. In the probe trial test, one-way ANOVA revealed significant effect of treatment on the numbers of passes over the previous target platform (F = 11.81, P < 0.001, Figure 2b) and the percentage of time spent in quadrant (F = 9.043, P < 0.001, Figure 2c). CIH group performed poorly on the hippocampal-dependent spatial learning task and BBG treatment during CIH demonstrated deficits resistance in the acquisition and retention of the spatial task.
P2X7 receptor, NADPH oxidase 2 and nuclear factor kappa B expression in the hippocampus

We observed that BBG did not affect the expression of P2X7R expression [Figure 3a]. The nuclear p65 activity in CIH group was significantly elevated as compared to other three groups ($F = 47.68$, $P < 0.01$, Figure 3b). Similarly, NOX2 expression was significantly elevated in the CIH + PBS group compared to the control group, whereas the change was significantly suppressed by treatment with BBG ($F = 43.40$, $P < 0.01$, Figure 3c).

Chronic intermittent hypoxia-induced inflammatory response was diminished by Brilliant Blue G in hippocampus

Tumor necrosis factor-α mRNA level was significantly increased in hippocampus of CIH mice, but it was markedly

Figure 1: P2X7 receptor (P2X7R) expression was increased after intermittent hypoxia exposure (IH). The results were respectively normalized with the house keep gene β-actin, and the mRNA and protein levels in RA3d group are presented as 100%. P2X7 mRNA was increased at 3 days of IH exposure ($P < 0.05$) and remaining elevated until Day 21 (a); IH induced marked increases in P2X7R protein expression at day 7 ($P < 0.05$), which were sustained throughout IH exposures (b and c). ($n = 4/experimental group; *P < 0.05, †P < 0.01$).

Figure 2: Brilliant Blue G (BBG) treatment improved the behavior performance in chronic intermittent hypoxia mice. (a) Mean latencies (s) to locate the target platform during place training in four groups of mice; (b) Mean passes through the target platform and (c) mean percentage time in the target quadrant during probe trial ($n = 12/experimental group; *P < 0.05, †P < 0.01$).

Figure 3: P2X7 receptor (P2X7R), nuclear factor kappa B (NFκB), and NADPH oxidase 2 (NOX2) expression in chronic intermittent hypoxia (CIH) mice. The results were, respectively, normalized with the house keep gene β-actin, and protein levels in room air + PBS group is presented as 100%. CIH but not BBG affect the expression of P2X7R (a); Significant increases in protein levels for phosphorylated NFκB (b) and NOX2 (c) were observed in CIH+PBS group, and they decreased when treated with BBG ($n = 4/experimental group, *P < 0.01$).
suppressed by BBG treatment ($F = 20.28$, $P < 0.01$, Figure 4a). There were similar change in mRNA levels of IL-1β ($F = 62.54$, $P < 0.01$, Figure 4b), IL-18 ($F = 40.35$, $P < 0.01$, Figure 4c) and IL-6 ($F = 8.56$, $P < 0.01$, Figure 4d).

**Chronic intermittent hypoxia-induced oxidative stress was attenuated by Brilliant Blue G in hippocampus**

To assess the effect of BBG treatment on oxidative stress in our model, we measured MDA and SOD production in the mice hippocampus. CIH groups were associated with significant increase in the relative MDA production in the hippocampus of mice in comparison to RA groups ($P < 0.01$); the amount of MDA production in CIH + BBG was significantly lower than CIH groups ($P < 0.05$); animals exposed to BBG-treated RA showed no significant differences in comparison to RA mice ($P = 0.71$, Figure 5a). Figure 5b showed the average SOD concentrations in the hippocampus region. CIH group showed lower SOD levels compared with RA groups; a significant increase in SOD levels was observed in BBG treatment groups in comparison to CIH groups ($P < 0.05$); no significant differences were observed between RA mice and BBG-treated RA mice ($P = 0.34$).

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**Figure 4:** Brilliant Blue G (BBG) reduced chronic intermittent hypoxia (CIH)-induced elevated mRNA levels of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-18, and IL-6 in hippocampus. The results were, respectively, normalized with the house keep gene β-actin, and mRNA levels in room air + PBS group is presented as 100%. There were significant increases in mRNA levels for (a) TNF-α; (b) IL-1β; (c) IL-18; and (d) IL-6 in CIH + PBS group and they were significantly suppressed when treated with BBG ($n = 4$/experimental group, *$P < 0.05$; †$P < 0.01$).

**Figure 5:** Brilliant Blue G (BBG) attenuated oxidative stress in chronic intermittent hypoxia (CIH) mice. BBG decreased the relative malondialdehyde (MDA) production in the hippocampus of CIH mice (a); As compared with room air (RA) mice, the MDA levels in CIH groups were increased. The amount of MDA production CIH + BBG group was significantly lower than CIH groups. BBG increased relative superoxide dismutase (SOD) production in the hippocampus of CIH mice (b). As compared with room air mice, the SOD levels in CIH groups were reduced. A significant increase in SOD levels was observed in BBG treatment groups in comparison to CIH groups ($n = 4$/experimental group, *$P < 0.05$; †$P < 0.01$).
Pathological changes of hippocampal neurons in chronic intermittent hypoxia mice were diminished by Brilliant Blue G

There were few apparently visible damage cells in RA group and RA + PBS group. Hippocampal neurons from CIH mice showed different degrees of degeneration, characterized by cell swelling or condensation, cytoplasmic vacuoles, nuclear shrinkage, and the disappearance of intracellular constituents. BBG reduced the number of damage cells in CIH group (P < 0.01, Figure 6). TUNEL method is sensitive to evaluate apoptosis in the hippocampus. The results indicated that neuron apoptosis indices in the hippocampus region were elevated in CIH mice in comparison to RA mice (P < 0.01). This elevation of apoptosis indices was attenuated in animals receiving BBG during CIH (P < 0.01) [Figure 7].

DISCUSSION

The accurate pathophysiology of OSAS-induced cognition dysfunction is still poorly understood. Neuronal loss is one of the most prominent pathophysiological characteristics of neurocognitive deficits of OSAS, especially in the hippocampus.[1,4] The hippocampus is a special structure considered to play a key role in learning and memory in the mammalian brain, because of its involvement in long-term potentiation and synaptic remodeling.[1,7] Unfortunately, compared to other brain structures, the hippocampus is particularly vulnerable and susceptible to CIH, the most characteristic pathophysiological change resulting from OSAS.[18,19] There is no doubt that the pathogenesis underlying OSAS-related neuronal damage is multifactorial; endothelial dysfunction, glutamate excitotoxicity, and neurotrophic factors’ anomalies may be involved. Current

Figure 6: Chronic intermittent hypoxia (CIH) induced neuronal damage is partly inversed by Brilliant Blue G (BBG). a-d: H&E staining, original magnification ×400. (a) Room air mice (RA); (b) BBG-treated RA mice; (c) CIH mice; (d) BBG-treated CIH mice; (e) Quantification of damage cells. The apparently visible damaged cells were pointed by black arrows. BBG promoted a reduction in number of damage cells in CIH + BBG group, compared to CIH group (n = 4/experimental group; *P < 0.01).

Figure 7: Apoptotic cells in the hippocampus of chronic intermittent hypoxia (CIH) mice were decreased by Brilliant Blue G (BBG). Apoptotic neurons appear brown stained whereas Transferase dUTP Nick-end Labeling (TUNEL)-negative nuclei appear blue. a-d: TUNEL, original magnification ×400. (a) Room air (RA) mice; (b) BBG-treated RA mice; (c) CIH mice; (d) BBG-treated CIH mice; (e) Quantification of TUNEL-positive cells (n = 4/experimental group; *P < 0.01).
Here, we pay attention to the CIH-induced inflammatory responses in the hippocampus of mice. We found that the expression of NFκB and inflammatory genes including TNF-α, IL-1β, IL-18 and IL-6 were increased in CIH mice. Selectively blocked P2X7R by BBG alleviated CIH-induced inflammatory processes and the cognition defect indicating that P2X7R may participate in the neuroinflammation caused by CIH.

It is widely known that oxidative stress is critical for evoking phenotypic adverse effects in experimental models of CIH and patients with recurrent apneas. Although ROS can be produced through various systems, recent studies have demonstrated that NOX2 is a key enzyme responsible for ROS generation for the development of pathological conditions caused by CIH. In addition, severe OSAS patients had a tendency toward higher serum levels of soluble NOX2-derived peptide. Moreover, CIH-induced NOX2 upregulation in the hippocampus contributes to oxidative stress and related neurobehavioral impairments. Consistent to previous studies, we observed that NOX2 expression and MDA levels were increased in CIH mice while SOD was decreased in CIH mice compared to RA mice. Then we found BBG treatment could rescue the oxidative stress in CIH mice, implicating the regulation of CIH-induced neural injury by P2X7R may partly come from its ability to trigger the ROS.

There are some limitations in this study. First, we did not use P2X7 knock-out mice, which may give more robust evidences to support the result. Second, activation of P2X7R can stimulate multiple other signaling processes such as iron fluxes and mitogen-activated protein kinases activation, so there might be other signaling processes activated by P2X7R in response to CIH. Further studies are needed to understand the precise function of P2X7R in sleep apnea.

Taken together, our study reveals a new role of P2X7R in the development of CIH-induced neural defects. In response to CIH exposure, increased expression of P2X7R may induce the expression of the NFκB and NOX2 in the hippocampus, resulting in elevation of inflammatory factors, oxidative stress, and neuronal damage. The P2X7R antagonism affords neuronal protection against the inflammatory processes and ROS elicited by CIH, indicating that the P2X7R can be a novel target site for new therapies for cognition deficits accompanied OSAS.

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