Altered Wnt Signaling Pathway in Cognitive Impairment Caused by Chronic Intermittent Hypoxia: Focus on Glycogen Synthase Kinase-3β and β-catenin

Yue-Ying Pan, Yan Deng, Sheng Xie, Zhi-Hua Wang, Yu Wang, Jie Ren, Hui-Guo Liu
Department of Respiratory and Critical Care Medicine, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Key Laboratory of Respiratory Disease of the Ministry of Health, Wuhan, Hubei 430030, China

Abstract

Background: Cognitive impairment is a severe complication caused by obstructive sleep apnea (OSA). The mechanisms of causation are still unclear. The Wnt/β-catenin signaling pathway is involved in cognition, and abnormalities in it are implicated in neurological disorders. Here, we explored the Wnt/β-catenin signaling pathway abnormalities caused by chronic intermittent hypoxia (CIH), the most characteristic pathophysiological component of OSA.

Methods: We divided 32 4-week-old male C57/BL mice into four groups of eight each: a CIH + normal saline (NS) group, CIH + LiCl group, sham CIH + NS group, and a sham CIH + LiCl group. The spatial learning performance of each group was assessed by using the Morris water maze (MWM). Protein expressions of glycogen synthase kinase-3β (GSK-3β) and β-catenin in the hippocampus were examined using the Western blotting test. EdU labeling and terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling staining methods were used, respectively, to determine the proliferation and apoptosis of neurons in the hippocampal dentate gyrus region.

Results: Mice exposed to CIH showed impaired spatial learning performance in the MWM, including increased mean escape latencies to reach the target platform, decreased mean times passing through the target platform and mean duration in the target quadrant. The GSK-3β activity increased, and expression of β-catenin decreased significantly in the hippocampus of the CIH-exposed mice. Besides, CIH significantly increased hippocampal neuronal apoptosis, with an elevated apoptosis index. Meanwhile, LiCl decreased the activity of GSK-3β and increased the expression of β-catenin and partially reversed the spatial memory deficits in MWM and the apoptosis caused by CIH.

Conclusions: Wnt/β-catenin signaling pathway abnormalities possibly play an important role in the development of cognitive deficits among mice exposed to CIH and that LiCl might attenuate CIH-induced cognitive impairment via Wnt/β-catenin signaling pathway.

Key words: β-catenin; Chronic Intermittent Hypoxia; Cognition; Glycogen Synthase kinase-3β; Hippocampus; Obstructive Sleep Apnea

Introduction

Obstructive sleep apnea (OSA) is characterized by repeated pharyngeal collapse during sleep, leading to nocturnal intermittent hypoxemia and microarousals. It is a growing health concern, with a prevalence of 4% among middle-aged men. The long-term consequences of untreated OSA include daytime sleepiness, cardiovascular disease, and neurocognitive impairments, which can reduce social functioning and quality of life. The mechanism of multorgan damage in patients with OSA is still unclear. However, chronic intermittent hypoxia (CIH) plays a major role, as it causes serious damage to multiple organs, especially the brain. Besides, our previous and many other studies suggest that neurocognitive deficits are closely associated with
regional brain damage, particularly in the hippocampus.[6-8] The most notable changes are observed in the dentate gyrus region, which plays a vital role in hippocampal learning.[9]

The underlying mechanisms of CIH-induced cognitive impairment are still unclear. In fact, a large number of factors are proposed to be involved. These include apoptosis, oxidative stress, inflammation, endothelial dysfunction, increased reactive oxygen species production, excitotoxicity, decreased cAMP-responsive element-binding protein phosphorylation, nitric oxide production, and reduced brain-derived neurotrophic factor production.[10]

The canonical Wnt/β-catenin pathway initiates a signaling cascade that is crucial during both embryonic development and throughout the life of the organism, in virtually every tissue and organ system (Xu, 2015 #19016).[11] In the absence of Wnt ligands, β-catenin is targeted for degradation, by a complex composed of glycogen synthase kinase-3β (GSK-3β), axin, and adenomatous polyposis coli in the cytoplasm. Phosphorylation of β-catenin by GSK-3β provides a binding site for the E3 ubiquitin ligase subunit that targets the ubiquitination of β-catenin and its degradation in the proteasome.[12] In the presence of Wnt ligands, β-catenin degradation is inhibited. Therefore, it accumulates in the cytoplasm, translocates to the nucleus and activates Wnt-responsive genes.[13]

Kaminska et al.[14] reported that sleep disturbances were frequent in Parkinson’s disease (PD). These include insomnia, hypersomnia, sleep architecture, and circadian abnormalities. OSA is reported to occur in 20–60% of PD subjects.[15-17] Piano et al.[18] found that patients with Huntington’s disease (HD) showed severe sleep disruption and that 26% of the patients had a high risk of OSA. OSA is common among patients with Alzheimer’s disease (AD), with a prevalence of over 40%.[19] Numerous data suggests that the Wnt/β-catenin signaling pathway plays an important role in neurodegenerative diseases such as PD, HD, and AD.[20-23] Lithium treatment demonstrated neuroprotective effects in in vivo models of PD, HD, and AD via GSK-3β inhibition.[24-25] Strong pathophysiological similarities exist between OSA and neurodegenerative diseases such as apoptosis, oxidative stress, and neuroinflammation. The complex interactions between OSA and neurodegenerative diseases suggest that Wnt/β-catenin pathway plays an important role in the development of cognitive deficits among patients with OSA.

To our knowledge, there is no report on the Wnt/β-catenin pathway in CIH. Here, we investigated the Wnt/β-catenin pathway dysfunction in a mouse model of CIH-induced cognitive impairment and explore potential treatment strategies.

**Methods**

**Animals**

Four-week-old male C57/BL mice (20–26 g, provided by the Experimental Animal Center of Tongji Hospital, Huazhong University of Science and Technology, China) were used in the study. The mice were randomly divided into four groups (n = 8 for each group): CIH + normal saline (NS), CIH + LiCl, sham CIH + NS, and sham CIH + LiCl. The animal use protocol listed below had been reviewed and approved of by the Institutional Animal Care and Use Committee, Tongji Medical College, Huazhong University of Science and Technology.

**Chronic intermittent hypoxia**

The CIH protocol details have been previously described,[26] with some modifications. The mice were placed in a chamber (BioSpherix OxyCycler A84, USA) and exposed to CIH or air-air cycling for 8 h from 10:00 to 18:00 for 5 weeks. The CIH protocol was as follows: oxygen level was reduced from 21% to 8% over a period of 120 s, held at 8% for 120 s, returned to 21% over a period of 50 s, and held at 21% for 300 s. The mice were treated daily with LiCl (4 mmol·kg⁻¹·d⁻¹, 0.2 mol/L in NS) or NS via intraperitoneal injection, while following the same schedule for 5 weeks. The mice were housed with unlimited access to laboratory feed and water at 22–25°C.

**Behavioral testing-Morris water maze**

The Morris water maze (MWM) test was performed after 5 weeks of CIH/sham CIH exposure. Spatial learning was assessed in the MWM, as previously described in platform and probe trials,[6,27] with some modifications. The MWM consisted of a black circular pool, 70 cm in diameter and 40 cm in height, filled to a depth of 25 cm, with water maintained at a temperature of 28°C ± 1°C. Water was made opaque by the addition of 200 g of milk powder. An escape platform, 8 cm in diameter, was hidden, submerged 1 cm below the water surface. Distinctive, geometric, extra-maze cues were affixed to the white wall surrounding the pool and were visible to the mice while in the maze. The MWM performance was recorded with a video camera suspended above the maze and interfaced with a video tracking system (EthoVision System, Noldus Information Technology, The Netherlands). During the platform trial days, the mice were placed in the pool from quasi-random start points (North, South, East, or West) and allowed a maximum of 60 s to find the escape platform. They were allowed to remain there for 30 s. Mice that failed to locate the platform at the end of 60 s were manually guided to the platform and allowed to remain there for 30 s. The position of the platform remained constant all the time. Platform trials were conducted for 4 days, and mean escape latencies were analyzed. On the 5th day, a 30 s probe trial was performed, in which the platform was removed, and the percentage of time spent in the target quadrant and the times taken for passing over the previous location of the missing target platform during the 30 s test were analyzed.

**Western blotting**

Twenty-four hours after completing the MWM, the mice were anesthetized with chloral hydrate (10%, 0.01 ml/g), and then executed by heart perfusion with NS. The brains were removed and medisected. The hippocampus of the...
one-half brain was isolated at 4°C and kept at −80°C until the immunoblot analysis. The other half of brain was fixed in 4% paraformaldehyde for histological analysis.

GSK-3β and β-catenin protein expression were determined by using the Western blot test, which was performed according to a routine procedure. Briefly, after tissue homogenization, proteins were extracted from the hippocampal samples using RIPA lysis buffer (P0013B, Beyotime, Jiangsu, China), which contained a protease inhibitor cocktail to prevent protein degradation. Protein concentration was determined using a Bradford protein assay kit (Bio-Rad, Hercules, CA, USA). Proteins (40 μg/band) were separated via 10% sodium dodecyl sulfate-Polyacrylamide gel electrophoresis and transferred to a 0.2 μm polyvinylidene difluoride membrane. The membranes were blocked in 5% nonfat dry milk in TBS + 0.05% Tween-20, for 1 h at room temperature. The membranes were incubated with rabbit β-catenin antibody (1:1000, #51067-2-AP, Proteintech Group, Inc., USA), rabbit GSK antibody (1:1000, #22104-1-AP, Proteintech Group, Inc., USA), rabbit P-GSK antibody (1:1000, #9323, Cell Signaling Technology, Inc., USA), and mouse β-actin antibody (1:8000, #KM9001, Tianjin Sungene Biotech Co., Ltd., China), overnight at 4°C. After this, the membranes were incubated with a secondary antibody conjugated with horseradish peroxidase at room temperature, for 2 h. The reactive proteins were analyzed with an ECL (Beyotime Institute of Biotechnology, China) Western blot detection system. The relative signal intensity was quantified by densitometry using ImageJ software (National Institutes of Health, USA).

**EdU labeling and detection**

A commercially available EdU labeling kit (C10314-3, RiboBio, China) was used according to the manufacturer’s instructions, to detect hippocampal neuronal proliferation. EdU was administered to mice via intraperitoneal injection (5 mg/kg, dissolved at 1 mg/ml in phosphate-buffered solution), 24 h before their execution. After EdU labeling, Hoechst 33342 was used to label cell nuclei. All the images were collected on an Ultraview microscope (Olympus BX51TF, Japan), using standard excitation and emission filters for Hoechst 33342 and FITC (Alexa Fluor-488). For quantification, ×200 magnification fields were collected for each region of interest in the hippocampus, and for each coronal brain section. EdU positive cells were counted manually, viewing each image in Adobe Photoshop CS5.

**Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling staining**

We used terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) staining for apoptotic cells in the hippocampus, to evaluate hippocampal impairment. A commercially available TUNEL staining kit (Roche Inc., Swiss) was used to detect apoptosis according to the manufacturer’s instructions. The numbers of apoptotic cells and total hippocampal cells were determined in five visual fields (>400). The apoptosis index was the mean value of the number of apoptotic cells/total hippocampal cells in the five visual fields.

**Statistical analysis**

All the statistical analysis was conducted using SPSS (version 19.0, IBM Corp., USA). All data were expressed as a mean ± standard deviation. Multiple comparison data were tested by analysis of variance, followed by Tukey’s post hoc test. A P < 0.05 was considered statistically significant.

**Results**

**Treatment with LiCl alleviated spatial memory deficits caused by chronic intermittent hypoxia**

The results of MWM are summarized in Figure 1. Increased mean escape latencies to the target platform during platform trials, decreased mean times passing through the target platform and mean duration in the target quadrant during probe trials, were observed to be statistically significant in the CIH-exposed mice (P < 0.05). Treatment with LiCl could partially reverse these changes (P < 0.05). The mice exposed to CIH had a much longer mean escape latency (50.01 ± 8.66) when compared with the control group (12.26 ± 4.33) (P < 0.05). However, the latency time was significantly shortened in the LiCl-treated group (32.18 ± 7.73) (P < 0.05) [Figure 1a]. The mean crossover times in the CIH + NS group (1.2 ± 1.6) were less than those in the sham CIH + NS (5.4 ± 3.1) (P < 0.05) and the CIH + LiCl group (4.0 ± 1.2) [Figure 1b]. The mean time in the target quadrant for the CIH-exposed mice (7.82 ± 3.60) was shorter when compared with the control group (22.98 ± 6.48) (P < 0.05). Further, LiCl-treated mice (16.8 ± 4.2) (P < 0.05) performed better in this assessment [Figure 1c].

**Chronic intermittent hypoxia increased glycogen synthase kinase-3β activity and decreased β-catenin expression significantly, while LiCl partially reversed the changes**

The results of the Western blot test are summarized in Figure 2. Phosphorylation at serine 9 inactivates GSK-3β. That is, the higher the density ratio of “S9/GSK-3β,” the lower the GSK-3β activity. GSK-3β activity and expression of β-catenin decreased significantly in the hippocampus of CIH-exposed mice, when compared with the sham CIH group (P < 0.05) [Figure 2a and 2b]. We found that LiCl partially reversed the changes (P < 0.05) [Figure 2c and 2d]. To further elaborate, the mean density ratio of p-GSK-3β/GSK-3β in the hippocampus was decreased among the CIH + NS group (0.086 ± 0.021) when compared with the sham CIH + NS (0.1300 ± 0.0029) (P < 0.05) and the CIH + LiCl groups (0.1100 ± 0.0097) (P < 0.05) [Figure 2b]. Whereas, the mean density ratio of β-catenin/β-actin in the hippocampus was decreased in the CIH + NS group (0.3800 ± 0.012), when compared with the sham CIH + NS (0.4800 ± 0.0095) (P < 0.05) and the CIH + LiCl groups (0.4300 ± 0.0044) (P < 0.05) [Figure 2d].
Figure 1: Treatment with LiCl improved cognitive deficits caused by CIH. (a) The mean escape latencies to locate the target platform during platform trials in mice exposed to sham CIH (+NS/+LiCl) and CIH (+NS/+LiCl); (b) The mean times passing through target platform during probe trials in mice exposed to sham CIH (+NS/+LiCl) and CIH (+NS/+LiCl); (c) The mean duration in target quadrant during probe trials in mice exposed to sham CIH (+NS/+LiCl) and CIH (+NS/+LiCl). *P < 0.05 compared to sham CIH + NS, †P < 0.05 compared to CIH + LiCl. CIH: Chronic intermittent hypoxia; NS: Normal saline.

Figure 2: GSK-3β activity increased and β-catenin expression decreased significantly in CIH mice, and LiCl partially reversed the changes. (a) The image of Western blotting for p-GSK-3β, GSK-3β, and β-actin in hippocampus from mice exposed to sham CIH (+NS/+LiCl) and CIH (+NS/+LiCl); (b) Mean ratios of p-GSK-3β to GSK-3β in hippocampus from mice exposed to sham CIH (+NS/+LiCl) and CIH (+NS/+LiCl); (c) The image of Western blotting for β-catenin and β-actin in hippocampus from mice exposed to sham CIH (+NS/+LiCl) and CIH (+NS/+LiCl); (d) Mean ratios of β-catenin to β-actin in hippocampus from mice exposed to sham CIH (+NS/+LiCl) and CIH (+NS/+LiCl). *P < 0.05 compared to sham CIH + NS, †P < 0.05 compared to CIH + LiCl. GSK-3β: Glycogen synthase kinase-3β; CIH: Chronic intermittent hypoxia; NS: Normal saline.
Chronic intermittent hypoxia and LiCl did not affect the proliferation of hippocampal neurons

The results of neuronal proliferation in the hippocampus by EdU labeling are summarized in Figure 3. As shown in Figure 3a and 3b, little neuronal proliferation was found in the hippocampus of all groups. The number of EdU positive neurons did not differ significantly between the CIH + NS (6.0 ± 1.4) and sham CIH + NS (6.67 ± 2.31) groups (P > 0.05). No significant differences were observed between the CIH + LiCl (7.0 ± 2.6) and CIH + NS (6.0 ± 1.4) groups (P > 0.05) [Figure 3b].

Chronic intermittent hypoxia-induced hippocampal neuronal apoptosis while LiCl could alleviate it

The results of hippocampal neuronal apoptosis demonstrated in TUNEL staining are shown in Figure 4. CIH significantly increased hippocampal neuronal apoptosis (P < 0.05), and treatment with LiCl effectively attenuated CIH-induced neuronal apoptosis in the mice hippocampi (P < 0.05). The apoptosis index of CIH + NS group (0.38 ± 0.12) was increased, when compared with the sham CIH + NS (0.071 ± 0.016) (P < 0.05) and the CIH + LiCl groups (0.27 ± 0.12) (P < 0.05) [Figure 4b].

Discussion

OSA is the most common form of sleep-disordered breathing and is a major public health problem because of the severe
complications, especially the cognitive impairment. Both adult and child patients with OSA might suffer from cognitive deficits with functional effects on work and school efficiency.\textsuperscript{[20]} OSA is characterized by CIH, which is associated with neurodegenerative changes in several brain regions and which causes cognitive dysfunction.\textsuperscript{[29]}

The Wnt/\(\beta\)-catenin pathway is involved in brain region development and regulates neural patterning, axonal remodeling, and synaptic plasticity in the adult hippocampus via synapse formation, dendritic morphogenesis, and long-term potentiation.\textsuperscript{[130-133]} The main biological effect of the canonical Wnt/\(\beta\)-catenin pathway is to regulate the degradation of \(\beta\)-catenin through GSK-3\(\beta\).\textsuperscript{[13]} Active GSK-3\(\beta\) promotes the degradation of \(\beta\)-catenin, which then prevents \(\beta\)-catenin from entering the nucleus, and inhibits the regulation of downstream target genes.

Neurodegenerative diseases are common causes of cognitive dysfunction and cause severe public health problems worldwide. Recent studies suggest that the Wnt/\(\beta\)-catenin pathway contributes to hippocampal learning and memory, and that hippocampal Wnt/\(\beta\)-catenin signaling is dysregulated in neurodegenerative diseases such as PD, HD, and AD.\textsuperscript{[20-22]} OSA has a relatively high prevalence among patients with neurodegenerative diseases.\textsuperscript{[14,18,19]} Many studies report down-regulation of Wnt signaling in the pathogenesis of PD, and also highlight the importance of this pathway in neuroprotection.\textsuperscript{[20]} Godin \textit{et al.}\textsuperscript{[21]} reported that accumulation of \(\beta\)-catenin was toxic to striatal neurons and contributed to the pathogenesis of HD. The level of \(\beta\)-catenin is reduced in the brain of AD patients, and an increase in its level might contribute to cognitive improvement.\textsuperscript{[64]}

In this study, we investigated whether the Wnt/\(\beta\)-catenin pathway was involved in the cognitive deficits caused by CIH and whether lithium treatment could reverse the impairment. The CIH mice model was adopted to mimic the cognitive impairment observed in patients with OSA. CIH caused spatial memory deficits in MWM, including increased mean escape latencies for the target platform, decreased mean times taken to pass through the target platform, and mean duration in the target quadrant [Figure 1]. Besides, the hippocampal neuronal apoptosis index increased [Figure 4] in mice exposed to CIH. The results were consistent with our previous and other studies.\textsuperscript{[35,36]}

Furthermore, CIH increased the activity of GSK-3\(\beta\) and then decreased \(\beta\)-catenin expression in mice hippocampi [Figure 2]. This correlated positively with the spatial memory deficits seen in MWM, and with increased hippocampal neuronal apoptosis. This implied that GSK-3\(\beta\) and \(\beta\)-catenin played a role in cognitive deficits among the CIH-exposed mice. Our result was in partial accordance with a recent study,\textsuperscript{[37]} which indicated that exposure to mild continuous hypoxia (10% oxygen for 6–72 h) stimulated the activation of the Wnt/\(\beta\)-catenin signaling pathway in the hippocampus, and promoted cell proliferation and neurogenesis in the subgranular zone (SGZ) of adult mice. It implied that exposure to intermittent hypoxia for a long time (5 weeks), or mild continuous hypoxia for a short duration (10% oxygen for 6–72 h), could stimulate the Wnt/\(\beta\)-catenin signaling pathway in the hippocampi of adult mice. However, significant neurogenesis was not observed in the hippocampi of mice exposed to CIH [Figure 3], which was different from the results observed in the SGZ of adult mice. This might suggest the differential effects of CIH and mild continuous hypoxia for short durations, on the proliferation of neurons in the mice hippocampi.

The inhibition of GSK-3\(\beta\) activity prevented degradation of \(\beta\)-catenin. The FDA-approved mood stabilizing agent lithium, activated Wnt/\(\beta\)-catenin signaling, reversed and prevented neurodegeneration, and memory impairment in adult rat hippocampi.\textsuperscript{[38]} Here, the application of LiCl, a typical GSK-3\(\beta\) inhibitor, decreased the activity of GSK-3\(\beta\), increased \(\beta\)-catenin expression in mice hippocampi [Figure 2], attenuated the memory deficits [Figure 1], and hippocampal neuronal apoptosis in CIH-exposed mice [Figure 4]. This could provide a new avenue to treat the cognitive impairment caused by CIH.

Some recent studies suggested that CIH-affected neuronal proliferation and differentiation. However, it was still unclear whether CIH could increase or decrease neuronal proliferation.\textsuperscript{[39,40]} Few EdU positive neurons were observed in all study groups [Figure 3]. This implied that CIH and LiCl did not affect the hippocampal neuronal proliferation. The dose of EdU was determined in a previous study,\textsuperscript{[41]} but the time from EdU injection to execution varied in different studies. Here, we adopted 24 h, which was the median time of these studies. In future study, we would try to prolong the time from EdU injection to execution, which could facilitate EdU passing through the blood brain barrier, and could better indicate possible neuronal proliferation increase.

In summary, CIH caused impaired spatial learning and memory, and increased apoptosis of hippocampal neurons, possibly via deactivating the Wnt/\(\beta\)-catenin signaling pathway. LiCl attenuated the cognitive deficits in CIH-exposed mice and the apoptosis of hippocampal neurons, probably by inhibiting the activity of GSK-3\(\beta\). Further research should study the whole pathway to better understand the role of Wnt/\(\beta\)-catenin in the spatial learning and memory deficits caused by CIH.

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Conflicts of interest
There are no conflicts of interest.

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