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Chronic intermittent hypoxia disturbs insulin secretion and causes pancreatic injury via MAPK signaling pathway

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Abstract

Obstructive sleep apnea (OSA) is a breath disorder during sleep with a most prominent character of chronic intermittent hypoxia (CIH), inducing the generation of reactive oxygen species (ROS) to damage multiple tissues and cause metabolism disorders. In this study, we established rat varying OSA model by graded concentration of CIH (12.5% O₂, 10% O₂, 7.5%O₂ and 5% O₂) for 12 weeks, and found that CIH stimulated insulin secretion, reduced the insulin/proinsulin ratio in the pancreatic tissue, and caused pancreatic tissue lesions and cells apoptosis in a dose-dependent manner. Moreover, CIH promoted the production of tumor necrosis factor (TNF)-α, Interleukin (IL)-1β and IL-6, and activated mitogen-activated protein kinase (MAPK) family members, extracellular regulated protein kinase (ERK), c-Jun N-terminal kinase (JNK) and P38 relying on the O₂ concentration.

In summary, CIH disturbed insulin secretion, caused inflammation, lesion and cell apoptosis in pancreatic tissue via MAPK signaling pathway, which may be of great significance for clinical treatment of OSA and type 2 diabetes mellitus (T2DM).

Keywords: chronic intermittent hypoxia; insulin; MAPK; obstructive sleep apnea; pancreas
Introduction

Obstructive sleep apnea (OSA) is a common sleep disorder characterized by repetitive occlusion of upper airway during sleep, generally defined as breathing pause lasting for ≥10 s and the apnea-hypopnea index (AHI) of ≥5 events/h during sleep (Rivas et al. 2015). The morbidity of OSA is 5-14% in adults, and higher in older people (Rivas et al. 2015).

Repeated apneas cause chronic intermittent hypoxia (CIH), leading to alveolar hypoxia and hypercapnia. Subsequently, alveolar hypoxia causes pulmonary artery vasoconstriction and increased blood returning to the right heart, further results in pulmonary arterial hypertension and right ventricular overload, which later develop to arrhythmias and right heart failure (Tamisier et al. 2015). OSA causes increased platelet adhesiveness, vascular endothelial dysfunction, decreased cerebral blood flow, hypercoagulability and atherosclerosis, increasing the risk of cardiovascular events (Arzt et al. 2005). Several studies have proved that OSA is an independent risk factor for stroke (Munoz et al. 2006; Redline et al. 2010; Yaggi et al. 2005; Yazdan-Ashoori and Baranchuk 2011), and one study suggests that OSA might affect the recovery from a stroke (Mansukhani et al. 2011). A recent investigation reported that OSA may increase the risk of atrial fibrillation (AF) by 4 folds, and 32-49% of patients with AF have documented OSA (Mansukhani et al. 2013; Yazdan-Ashoori and Baranchuk 2011). Moreover, OSA can cause inflammation in lung, liver and heart (da Rosa et al. 2012; Gonchar and Mankovska 2012; Rozova and Mankovska 2012).

In addition, OSA is associated with glucose metabolism disorder, being identified as an independent risk factor of type 2 diabetes mellitus (T2DM) (Wang et al. 2013). It has been reported that CIH increases pancreatic cells apoptosis and exacerbates dysfunction in a polygenic
rodent model of diabetes (Sherwani et al. 2013). Mimicking sleep apnea through CIH exposure increases pancreatic β-cell proliferation, β-cell death and β-cell area may via reactive oxygen species (ROS) (Xu et al. 2009). So far, studies about the effects of OSA on pancreatic cells, insulin and diabetes are still few, and the molecular mechanism remains unclear. In this study, we simulated OSA by treating with CIH to explore how it affects pancreatic cells apoptosis and insulin secretion.

**Methods**

**Ethical statement**

Animals in this study were taken care of according to Guide for the Care and Use of Laboratory Animals (1996, published by National Academy Press, 2101 Constitution Ave. NW, Washington, DC 20055, USA) and Laboratory Animal Care and Use Standard of Kunming Medical University. Animal treatment and experimental procedures were approved by the Ethics Committee of Kunming Medical University.

**Animal model**

Healthy male Wistar rats of 200-220 g (Vital River, Beijing, China) were randomly divided into 5 groups: Normoxia, 12.5% (v/v) O₂, 10% O₂, 7.5% O₂ and 5% O₂ (n=6 per group). All rats were kept in a controlled environment (22-24 °C with 12 h light/12 h dark cycles; lights on at 7:00 am) on a standard Purina chow diet (4% fat) with free access to water (Savransky et al. 2007). The rats in Normoxia group were breathed with air room (21% O₂) all the time as the control, and other rats were treated with cycles of intermittent hypoxia 8 h/day from 8:30 am to 16:30 pm (Fig. S1). The oxygen concentration of terraria was measured by an oxygen meter (Xinanjiang, Jiande, Zhejiang, China). After being treated with CIH for 12 weeks, all rats were fasted overnight, anesthetized...
with 10% (w/v) chloral hydrate (3.5 ml/kg) and sacrificed. The arterial blood was collected and the pancreatic tissues were isolated.

**Detection of insulin and proinsulin content**

The blood was naturally precipitated at room temperature for 20 min and centrifuged at 2000 rpm for 20 min, and the serum in the superstratum was collected. The tissue was freeze-thawed thrice, dispersed into cell suspension with moderate PBS, and centrifuged at 12000 rpm for 10 min, and the supernatant was collected.

The insulin or proinsulin content in serum and supernatant of tissue was detected by Chemiluminescent Immunoassay kit for Insulin (Rat) (USCN, Wuhan, Hubei, China) or Rat Proinsulin ELISA kit (WHB, Shanghai, China) according to the manufacturer's instruction.

**ELISA**

The tissue was freeze-thawed thrice, and dispersed into cell suspension with a 9-fold volume of PBS. The cell suspension was centrifuged at 12000 rpm for 10 min, and the supernatant was collected as protein sample. Content of tumor necrosis factor (TNF)-α, Interleukin (IL)-1β or IL-6 in the protein sample was detected by TNF-α (R) ELISA kit (Boster, Wuhan, Hubei, China), IL-1β (Rat) ELISA kit (Boster) or IL-6 (R) ELISA kit (Boster) according to the protocol.

**Hematoxylin-eosin (HE) staining**

The tissue was fixed with 4% (w/v) paraformaldehyde (Sinopharm, Beijing, China) at room temperature overnight, rinsed with water for 4 h, and dehydrated with graded concentrations of ethanol (70% (v/v) for 2 h, 80% for 2 h, 90% for 2 h and 100% for 1 h twice). Then the tissue was hyalinized with xylene (Sinopharm) for 30 min, paraffin-embedded, and cut into sections of 5 μm. After drying on glass slide at 60 °C, the sections were dewaxed in xylene (Sinopharm) for 15 min.
twice, and rehydrated with 100%, 95%, 85% and 75% ethanol. Thereafter, the sections were
stained with hematoxylin (Solarbio, Beijing, China) for 5 min, soaked in 1% (w/v) hydrochloric
acid/ethanol for 3 s, and stained with eosin (Sinopharm) for 3 min. Afterwards, the sections were
dehydrated with ethanol of 75%, 85%, 95% for 2 min each, and 100% for 5 min twice, and
hyalinized with xylene for 10 min twice. After removing the residual liquid, the sections were
mounted with half one drop of gum, dried at room temperature, and photographed with a
microscope (Olympus, Tokyo, Japan) at 200× magnification.

**TUNEL assay**

The tissue was made to paraffin sections as previous description. The sections were dewaxed
with xylene for 15 min twice, rehydrated with ethanol of different concentrations, hyalinized with
0.1% (w/v) Triton X-100 (Beyotime, Haimen, Jiangsu, China) for 8 min, blocked with 3% (w/v)
H₂O₂ (Sinopharm) for 10 min, and labeled with TUNEL reaction solution (Roche, Basel,
Switzerland) at 37 °C for 60 min in dark. After rinsing with PBS, the sections were incubated with
Converter-POD (Roche) at 37 °C for 30 min, reacted with DAB developing buffer (Solarbio).
Thereafter, the sections were stained with hematoxylin 3 min, soaked in 1% (w/v) hydrochloric
acid/ethanol for 3 s, and washed with water for 20 min. Thereafter, the sections were dehydrated
with ethanol and xylene. After removing the residual liquid, the sections were mounted with half
one of drop of gum, dried at room temperature, and photographed with a microscope (Olympus)
at 400× magnification. The total cells and TUNEL-positive cells in six sections of each group were
counted respectively, and the apoptosis rate = TUNEL-positive cell number/total cell number ×
100%.

**Western blot**
Protein was exacted with whole cell lysis assay (Wanleibio), separated by SDS-PAGE (40 μg for each lane) and transferred to polyvinylidene fluoride (PVDF) membrane (Millipore, Boston, MA, USA). After blocking with 5% (w/v) skim milk (YILI, Hohhot, Inner Mongolia, China), the PVDF membrane was incubated with the following antibodies at 4 °C overnight: rabbit anti-extracellular regulated protein kinase (ERK) (1:500) (Bioss, Beijing, China), rabbit anti-p-ERK (1:500) (Bioss), rabbit anti-c-Jun N-terminal kinase (JNK) (1:500) (Bioss), rabbit anti-p-JNK (1:500) (Bioss), rabbit anti-P38 (1:500) (Bioss), rabbit anti-p-P38 (1:500) (Bioss) and rabbit anti-hypoxia-induced factor-α (HIF-α) (1:200) (Santa Cruz, CA, USA). After rinsing with PBS, the PVDF membrane was incubated with goat anti-rabbit IgG-HRP (1:5000) (Wanleibio) at 37 °C for 45 min, followed by signal exposure with ECL reagent (Wanleibio). After removing antibodies with stripping buffer (Wanleibio), the PVDF membrane was incubated with mouse anti-β-actin (1:1000) (Santa Cruz) and goat anti-mouse IgG-HRP (1:5000) (Wanleibio) to detect the internal control, β-actin.

Statistical analysis

All data were presented as mean ± standard deviation (SD) in this study. The data in detections of blood glucose, insulin, proinsulin, TNF-α, IL-1β, IL-6, HE staining and TUNEL assay were in six individual animal samples, and the data in western blot were in five individual animal samples. The detection in each sample was repeated in triplicate and the average value was calculated and applied. Two-tailed t test was adopted to analyze the data and identify the differences among the results of three experiments. P<0.05 was considered statistically significant. (*P<0.05, **P<0.01, ***P<0.001, ns: no significance)
CIH stimulated insulin secretion

The rat OSA models of varying degree were established via CIH of graded oxygen concentrations (12.5% O₂, 10% O₂, 7.5% O₂ and 5% O₂) for 8 h everyday to simulate the OSA patients of different severity degree. After treating for 12 weeks, the levels of fasting glucose, insulin and proinsulin in the blood were detected. As shown in Fig. 1, CIH treatment (5% O₂) increased fasting glucose level by 1.51 folds (Fig. 1A), elevated fasting insulin level by 5.19 folds (Fig. 1B), and decreased fasting proinsulin level by 60% (Fig. 1C), respectively, as compared with the Normoxia group. At the same time, the level of insulin in pancreatic tissue was reduced by dramatically (Fig. 1D), but proinsulin in pancreatic tissue raised slightly (Fig. 1E), so the proinsulin/insulin ratio in pancreatic tissue was increased by treating with CIH (Fig. 1F). In together, CIH stimulated insulin secretion, however, the level of glucose also elevated, suggesting insulin resistance.

CIH caused inflammation in pancreatic tissue

It is well known that CIH would cause generation of a large number of ROS, leading to inflammation in multiple tissues. Several common inflammatory factors, TNF-α, IL-1β and IL-6 in pancreatic tissue were detected. The ELISA results showed that graded CIH treatment increased the expression of TNF-α 1.97-fold, 2.79-fold, 4.46-fold and 15.41-fold (Fig. 2A), IL-1β 1.17-fold, 1.58-folds, 2.18-fold and 3.17-fold (Fig. 2B), IL-6 1.31-fold, 1.59-fold, 2.46-fold and 4.56-fold (Fig. 2C), respectively, as compared with Normoxia group. These results suggested inflammation in the pancreatic tissue.

CIH caused lesions and cell apoptosis in pancreatic tissue

To measure the pathological changes in the pancreatic tissue, HE staining and TUNEL assay
were performed. As shown in the HE staining results, after CIH treatment, pancreatic cells were deformed, cytoplasm staining was faded and nucleus shrunk (Fig. 3A). TUNEL-positive cells rate increased 2.3-fold, 4.07-fold, 6.21-fold and 8.5-fold, respectively, by graded CIH (Fig. 3B and C), as compared with Normoxia group.

**CIH activated MAPK signaling pathway**

Mitogen-activated protein kinase (MAPK) is a central regulator participating in numerous signaling pathways, including oxidative stress response and inflammation response, and MAPK members are activated by phosphorylation. Western blot was carried out to measure the expression level of several members of MAPK family, ERK, JNK and P38, and their activated forms.

The results showed that graded CIH increased the relative level of p-ERK/ERK by 1.65 folds, 2.12 folds, 2.26 folds and 3.36 folds respectively (Fig. 4A), increased the relative level of p-JNK/JNK by 1.62 folds, 2.13 folds, 3.82 folds and 5.57 folds respectively (Fig. 4B), and increased the relative level of p-P38/P38 by 1.67 folds, 2.72 folds, 3.39 folds and 4.93 folds respectively (Fig. 4C), as compared with Normoxia group.

**Discussion**

It is well-known that OSA can evoke metabolism disorders, including glucose metabolism disorder and lipid metabolism disorder. CIH is the primary characteristic of OSA, which enhances the production of ROS, leading to lipid peroxidation and increased blood lipid. Moreover, ROS and peroxidized lipid damage multiple tissues, including pancreatic tissue. Pancreatic tissue injury disturbs insulin secretion, increases blood glucose level, which in turn aggravates lipid metabolism chaos, leading to obesity, diabetes and cardiovascular diseases. Obesity, OSA, T2DM and cardiovascular diseases often connected with each other and influence each other in clinic.
In this study, we established rat varying OSA model via graded CIH, and found that CIH stimulated insulin secretion, but also increased fasting glucose level, suggesting insulin resistance, which is the salient character of T2DM prophase (Nathan et al. 2007). Meanwhile, the insulin level was decreased and the proinsulin/insulin ratio was increased in pancreatic tissue after treatment of CIH, suggesting pancreatic dysfunction. Insulin secretion disorder and pancreatic dysfunction are the fundamental reasons of diabetes mellitus, since the insulin is the unique hypoglycemic hormone. Moreover, pancreatic inflammation, lesions and apoptosis, and insulin resistance were aggravated with the decrease of oxygen concentration, suggesting that the metabolic disorders and pancreatic injury rely on the OSA severity.

Subsequently, the expression levels of MAPK family members, ERK, JNK and P38, were examined. MAPK cascade activation, the center of multiple signaling pathways, accepts a variety of stimuli, activates its downstream signaling pathways via cascade phosphorylation, and mediates cell proliferation, differentiation, apoptosis, inflammation and stress response to various stimuli. It has been reported that JNK is associated with insulin resistance of mice (Tuncman et al. 2006). Select blocking ERK pathway with MAPK/ERK kinase (MEK) inhibitors is beneficial for the treatment of insulin resistance and T2DM (Ozaki et al. 2016). In addition, a P38 inhibitor, SB239063, prevents free fatty acids-induced hepatic insulin resistance in Wistar (Pereira et al. 2016). In this study, we found that CIH promoted phosphorylation of ERK, JNK and P38, which maybe the mechanism CIH induced pancreatic injury and insulin secretion disorder in a dose-dependent manner.

In addition, we detected the expression level of HIF-1α in pancreatic tissue and found that HIF-1α was upregulated by graded CIH also in a dose-manner (Fig. S2). HIF-1α is a well-known
factor involving in hypoxia response. It has been reported that ERK1/2 activates HIF-1α (Richard et al. 1999), and the inhibitor of ERK1/2 is able to block the expression of HIF-1α and its target genes induced by nitric oxide (NO) (Kasuno et al. 2004). The inhibition of JNK specially suppresses HIF-1α-dependent transcriptional activity (Comerford et al. 2004). The phosphorylation of P38 contributes to the increased expression and activity of HIF-1α in β-amyloid precursor protein Swedish mutant (APPsw) mice cells treated with Deferoxamine (Guo et al. 2015). These reports suggest that the roles of HIF-1α and MAPK are accordant under hypoxia, and consistent with the observations in our study. However, more detailed mechanism still need to be illuminated. There are many other signaling pathways involved in inflammation and oxidative stress response, such as NF-κB. However, it remains further investigation whether these signaling pathways play roles in CIH-induced pancreatic injury and insulin secretion disorder.

Conclusions

We established varying OSA model via graded CIH and demonstrated that CIH disturbed the insulin secretion, caused pancreatic inflammation and lesion and cells apoptosis by activating MAPK signaling pathway in rats in a dose-dependent manner. These findings may provide research basic for clinical treatment of OSA and T2DM.
Acknowledgements

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**Figure legends**

**Fig. 1 Chronic intermittent hypoxia (CIH) stimulated insulin secretion**

(A) The blood glucose level was detected with a glucometer. (B) The blood insulin level after fasting. (C) The blood proinsulin level after fasting. (D) The insulin content in the pancreatic tissue of rats in each group. (E) The proinsulin content in the pancreatic tissue of rats in each group. (F) The relative proinsulin/insulin ratio in the pancreatic tissue of rats in each group. (The data of six individual experiments were analyzed by two-tailed t test, and compared to Normoxia group. *P<0.05, **P<0.01, ***P<0.001, ns: no significance)

**Fig. 2 CIH caused inflammation in the pancreatic tissue**

(A) The levels of tumor necrosis factor (TNF)-α in the pancreatic tissue detected by ELISA. (B) The interleukin (IL)-1β level in the pancreatic tissue detected by ELISA. (C) The IL-6 content in the pancreatic tissue detected by ELISA. (The data of six individual experiments were analyzed by two-tailed t test, and compared to Normoxia group. **P<0.01, ***P<0.001, ns: no significance)

**Fig. 3 CIH caused lesions and cell apoptosis in the pancreatic tissue**

(A) Hematoxylin-eosin (HE) staining was performed to detect the pathological changes in the pancreatic tissue. (The scale bar represented 100 μm. The local image was observed at 400× magnification, and the arrows indicated the injury regions in pancreatic tissues.) (B) TUNEL assay was carried out to examine the apoptosis of pancreatic cells. (The scale bar represented 50 μm. The local image was observed at 600× magnification, and the arrows indicated TUNEL-positive cells.) (C) The cell apoptosis rate in the pancreatic tissue of rats in each group. (The data of six individual experiments were analyzed by two-tailed t test, and compared to Normoxia group. ***P<0.01, ns: no significance)
Fig. 4 CIH activated MAPK signaling pathway

(A) The expression levels of p-ERK and ERK were detected by western blot. (B) The expression level of p-JNK and JNK were detected by western blot. (C) The expression levels of p-P38 and P38 were detected by western blot. (The data of five individual experiments were analyzed by two-tailed t test, and compared to Normoxia group. **P<0.01, ***P<0.001, ns: no significance)

Fig. S1 Oxygen concentration of terrariums in different groups

Oxygen concentration of rat terrariums in different groups was measured in real-time by an oxygen meter.

Fig. S2 The expression level of hypoxia induced factor (HIF-1α) was increased by CIH in a dose-dependent manner

The expression level of HIF-1α in pancreatic tissues of rats treating with graded CIH was detected by western blot. (The data of five individual experiments were analyzed by two-tailed t test, and compared to Normoxia group. *P<0.05, **P<0.01, ***P<0.001)
figure 1

293x160mm (300 x 300 DPI)
figure 2

101x216mm (300 x 300 DPI)
figure 3

126x231mm (300 x 300 DPI)