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

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

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16 **Abstract**

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

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

30

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

32 Introduction

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

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

51 In addition, OSA is associated with glucose metabolism disorder, being identified as an
52 independent risk factor of type 2 diabetes mellitus (T2DM) (Wang et al. 2013). It has been
53 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 (Xinjiang, Jiande, Zhejiang, China). After being treated with CIH for 12 weeks, all rats were fasted overnight, anesthetized

76 with 10% (w/v) chloral hydrate (3.5 ml/kg) and sacrificed. The arterial blood was collected and
77 the pancreatic tissues were isolated.

78 ***Detection of insulin and proinsulin content***

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

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

86 ***ELISA***

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

92 ***Hematoxylin-eosin (HE) staining***

93 The tissue was fixed with 4% (w/v) paraformaldehyde (Sinopharm, Beijing, China) at room
94 temperature overnight, rinsed with waster for 4 h, and dehydrated with graded concentrations of
95 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
96 hyalinized with xylene (Sinopharm) for 30 min, paraffin-embedded, and cut into sections of 5 μ m.
97 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.

105 ***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%.

119 ***Western blot***

Protein was extracted 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)

Results

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-1 β 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-1 β 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

164 were performed. As shown in the HE staining results, after CIH treatment, pancreatic cells were
165 deformed, cytoplasm staining was faded and nucleus shrank (Fig. 3A). TUNEL-positive cells rate
166 increased 2.3-fold, 4.07-fold, 6.21-fold and 8.5-fold, respectively, by graded CIH (Fig. 3B and C), as
167 compared with Normoxia group.

168 **CIH activated MAPK signaling pathway**

169 Mitogen-activated protein kinase (MAPK) is a central regulator participating in numerous
170 signaling pathways, including oxidative stress response and inflammation response, and MAPK
171 members are activated by phosphorylation. Western blot was carried out to measure the
172 expression level of several members of MAPK family, ERK, JNK and P38, and their activated forms.
173 The results showed that graded CIH increased the relative level of p-ERK/ERK by 1.65 folds, 2.12
174 folds, 2.26 folds and 3.36 folds respectively (Fig. 4A), increased the relative level of p-JNK/JNK by
175 1.62 folds, 2.13 folds, 3.82 folds and 5.57 folds respectively (Fig. 4B), and increased the relative
176 level of p-P38/P38 by 1.67 folds, 2.72 folds, 3.39 folds and 4.93 folds respectively (Fig. 4C), as
177 compared with Normoxia group.

178 **Discussion**

179 It is well-known that OSA can evoke metabolism disorders, including glucose metabolism
180 disorder and lipid metabolism disorder. CIH is the primary characteristic of OSA, which enhances
181 the production of ROS, leading to lipid peroxidation and increased blood lipid. Moreover, ROS
182 and peroxidized lipid damage multiple tissues, including pancreatic tissue. Pancreatic tissue injury
183 disturbs insulin secretion, increases blood glucose level, which in turn aggravates lipid
184 metabolism chaos, leading to obesity, diabetes and cardiovascular diseases. Obesity, OSA, T2DM
185 and cardiovascular diseases often connected with each other and influence each other in clinic.

186 In this study, we established rat varying OSA model via graded CIH, and found that CIH
187 stimulated insulin secretion, but also increased fasting glucose level, suggesting insulin resistance,
188 which is the salient character of T2DM prophase (Nathan et al. 2007). Meanwhile, the insulin
189 level was decreased and the proinsulin/insulin ratio was increased in pancreatic tissue after
190 treatment of CIH, suggesting pancreatic dysfunction. Insulin secretion disorder and pancreatic
191 dysfunction are the fundamental reasons of diabetes mellitus, since the insulin is the unique
192 hypoglycemic hormone. Moreover, pancreatic inflammation, lesions and apoptosis, and insulin
193 resistance were aggravated with the decrease of oxygen concentration, suggesting that the
194 metabolic disorders and pancreatic injury rely on the OSA severity.

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

206 In addition, we detected the expression level of HIF-1 α in pancreatic tissue and found that
207 HIF-1 α was upregulated by graded CIH also in a dose-manner (Fig. S2). HIF-1 α is a well-known

208 factor involving in hypoxia response. It has been reported that ERK1/2 activates HIF-1 α (Richard
209 et al. 1999), and the inhibitor of ERK1/2 is able to block the expression of HIF-1 α and its target
210 genes induced by nitric oxide (NO) (Kasuno et al. 2004). The inhibition of JNK specially suppresses
211 HIF-1 α -dependent transcriptional activity (Comerford et al. 2004). The phosphorylation of P38
212 contributes to the increased expression and activity of HIF-1 α in β -amyloid precursor protein
213 Swedish mutant (APP^{sw}) mice cells treated with Deferoxamine (Guo et al. 2015). These reports
214 suggest that the roles of HIF-1 α and MAPK are accordant under hypoxia, and consistent with the
215 observations in our study. However, more detailed mechanism still need to be illuminated. There
216 are many other signaling pathways involved in inflammation and oxidative stress response, such
217 as NF- κ B. However, it remains further investigation whether these signaling pathways play roles
218 in CIH-induced pancreatic injury and insulin secretion disorder.

219 **Conclusions**

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

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Draft

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292

293

294 **Figure legends**

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

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

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

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

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

308 (A) Hematoxylin-eosin (HE) staining was performed to detect the pathological changes in the
 309 pancreatic tissue. (The scale bar represented 100 μ m. The local image was observed at 400 $\times$
 310 magnification, and the arrows indicated the injury regions in pancreatic tissues.) (B) TUNEL assay
 311 was carried out to examine the apoptosis of pancreatic cells. (The scale bar represented 50 μ m.
 312 The local image was observed at 600 $\times$ magnification, and the arrows indicated TUNEL-positive
 313 cells.) (C) The cell apoptosis rate in the pancreatic tissue of rats in each group. (The data of six
 314 individual experiments were analyzed by two-tailed t test, and compared to Normoxia group.
 315 ****P<0.01, ns: no significance*)

316 **Fig. 4 CIH activated MAPK signaling pathway**

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

321 **Fig. S1 Oxygen concentration of terrariums in different groups**

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

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

326 The expression level of HIF-1 α in pancreatic tissues of rats treating with graded CIH was detected
327 by western blot. (The data of five individual experiments were analyzed by two-tailed t test, and
328 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)

figure 4

296x103mm (300 x 300 DPI)