Tempol Alleviates Chronic Intermittent Hypoxia-Induced Pancreatic Injury Through Repressing Inflammation and Apoptosis

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Summary
Obstructive sleep apnea (OSA) has been demonstrated to be implicated in disorder of insulin secretion and diabetes mellitus. In this study, we aimed to evaluate the protective role of tempol, a powerful antioxidant, in chronic intermittent hypoxia (IH)-induced pancreatic injury. The rat model of OSA was established by IH exposure. The pathological changes, increased blood-glucose level, and raised proinsulin/insulin ratio in pancreatic tissues of rats received IH were effectively relieved by tempol delivery. In addition, the enhanced levels of pro-inflammatory cytokines, TNF-α, IL-1β, IL-6, and inflammatory mediators, PGE2, cyclooxygenase-2 (COX-2), NO, and inducible nitric oxide synthase (iNOS) in pancreatic tissue were suppressed by tempol. Moreover, tempol inhibited IH-induced apoptosis in pancreatic tissue as evidenced by upregulated Bcl-2 level, and downregulated Bax and cleaved caspase-3 levels. Finally, the abnormal activation of mitogen-activated protein kinase (MAPK) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathways induced by IH was restrained by tempol administration. In summary, our study demonstrates that tempol relieves IH-induced pancreatic injury by inhibiting inflammatory response and apoptosis, which provides theoretical basis for tempol as an effective treatment for OSA-induced pancreatic injury.

Key words
Tempol • Intermittent hypoxia • Pancreatic injury • Inflammation response • Apoptosis

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Introduction
Obstructive sleep apnea (OSA), characterized by recurrent apnea, sleep fragmentations, and daytime sleepiness, is a rather common chronic sleep disorder (Punjabi 2008). Long-term untreated OSA may result in multiple health impairments, such as kidney injury (Abuyassin et al. 2018), cardiovascular disease (Walia et al. 2014), lung damage (Braun et al. 2017), metabolic disorders (Drager et al. 2013), which compromises the quality of life, reduces productivity, and brings psychological and economic burdens to patients.

It has been reported that OSA significantly correlates with high-risk of incident diabetes (Nagayoshi et al. 2016). Our previous results suggested that OSA could lead to insulin secretion disorder and pancreatic injury in rats exposure to chronic intermittent hypoxia (IH) rats (Wang et al. 2017). Moreover, IH promotes the production of large amounts of reactive oxygen species (ROS), which results in insulin resistance and glucose intolerance (Clarenbach et al. 2011). Although the detailed pathogenesis of OSA-induced pancreatic injury is not well understood, oxidative stress may participate in the pathogenic mechanisms. For now, antioxidant therapy
has been confirmed to be effective for treating OSA (Sadasivam et al. 2011). This may provide clues to searching potential therapeutic measures for OSA-induced pancreatic injury.

Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl) is a soluble superoxide dismutase (SOD) mimetic agent. Tempol is a powerful anti-oxidant and contributes to mopping up ROS in a variety models of oxidative stress (Wilcox 2010). Tempol suppressed IH-induced myocardial infarction aggravation (Ramond et al. 2013), sustained elevation of blood pressure (Khan et al. 2011), pharyngeal dilator muscle dysfunction (Skelly et al. 2012), and so on. Previous studies also suggested that tempol protected against pancreatic damage in pancreatitis rat model (Erbis et al. 2015, Marciniak et al. 2016). In addition, tempol treatment could protect pancreatic beta cells via inhibiting fibrosis and apoptosis in type 2 diabetes rats (Lee et al. 2011). However, the role of tempol in IH-induced pancreatic injury has not been determined.

In the present study, the rats were subjected to IH to simulate the OSA model in vivo. The beneficial effect of tempol on IH-induced pancreatic injury and its related mechanisms were elucidated.

**Materials and Methods**

**Animal model**

Twenty-four Wistar rats (body weight range of 200-220 g) were obtained from HFK Bioscience Co., LTD (Beijing, China) and randomly divided into four experimental groups: i) normal air (NA) group, ii) chronic intermittent hypoxia (IH) group, iii) IH+tempol group, iv) NA+tempol group. To determine the protective effects of tempol in IH-induced pancreatic injury, the rats in iii) & iv) groups were received equal-volume freshly prepared drinking water containing 1 mM tempol during receiving IH or NA every day. The rats in the other groups drank equal-volume tap water. The rats in IH group were persistently exposed to 5 % O₂ (60 s)/21 % O₂ (60 s) cycles in a controlled chamber every day from 9:00 to 5:00 pm for 12 weeks. The rats of NA groups were subjected to air always containing 21 % O₂. Twelve weeks after the treatment, the rats were anesthetized by pentobarbital sodium (50 mg/kg, intraperitoneal injection) and sacrificed. The arterial blood and pancreatic tissues were collected for further tests. All animal experimental procedures were approved by the Institutional Animal Care and Use Committees of the Second Affiliated Hospital of Kunming Medical University.

**Hematoxylin-eosin (HE) staining**

The pancreatic tissues of rats were fixed in 4 % formaldehyde, embedded in paraffin and then cut into 5-μm sections for routine HE staining. The morphologic alterations were observed under a light microscopy.

**Detection of insulin, proinsulin content and blood glucose level**

The insulin or proinsulin content in pancreatic tissues was assessed by Chemiluminescent Immunoassay Kit For Insulin (USCN, China) or commercially-available ELISA Kit for proinsulin (WHB, China), and the blood glucose level was detected by the Glucose Assay Kit (Nanjing Jiancheng Bioengineering Institute, China), following the manufacturer’s instructions.

**Detection of pro-inflammatory cytokines TNF-α, IL-1β and IL-6**

The levels of TNF-α, IL-1β, and IL-6 in the pancreatic tissues were assessed by commercially-available ELISA kits (Boster, China), according to the manufacturer’s instructions.

**Detection of NO and PGE2 content**

The content of NO or PGE2 in pancreatic tissues were determined by Nitric Oxide (NO) assay kit (Nanjing Jiancheng Bioengineering Institute) or Prostaglandin E2 (PGE2) ELISA Kit (USCN), following the manufacturer’s instructions.

**Western blot**

Proteins were extracted from the pancreatic tissues by RIPA lysis buffer (Beyotime, China) containing 1 % PMSF (Beyotime). Protein concentration in each sample was quantified by an Enhanced BCA Protein Assay Kit (Beyotime). Protein samples with equal amount (40 µg) were subjected to a SDS-PAGE gel and then transferred onto polyvinylidine difluoride membranes (Millipore, USA). Then the membranes were blocked with 5 % skim milk for 1 h at room temperature, incubated with primary antibodies against COX-2 (1:700, Proteintech, USA), iNOS (1:500, Proteintech, USA), iKBα (1:1,000, Cell Signaling Technology, USA), p-iKBα (1:1,000, Cell Signaling Technology, USA), NF-κB (1:1,000, Proteintech, USA), Bax (1:1,000, Proteintech, USA), Bel-2 (1:700, Abcam, UK), cleaved caspase-3.
(1:1,000, Cell Signaling Technology, USA), ERK (1:1,000, Cell Signaling Technology, USA), p-ERK (1:2,000, Cell Signaling Technology, USA), JNK (1:1,000, Cell Signaling Technology, USA), p-JNK (1:1,000, Cell Signaling Technology, USA), P38 (1:1,000, Cell Signaling Technology, USA), p-P38 (1:1,000, Cell Signaling Technology, USA), GAPDH (1:5,000, Bioss, China) and Histone H3 (1:1,000, Proteintech, USA) at 4 °C overnight, and then with corresponding secondary antibody at 37 °C for 45 min. The bands were visualized and detected by BeyoECL Plus reagent (Beyotime). The grayscale values of the bands were analyzed by Gel-Pro-Analyze software.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)

The apoptosis in pancreatic tissues was determined by the In Situ Cell Death Detection Kit (Roche, Switzerland), according to the manufacturer's instructions. In short, the fixed pancreatic tissues were embedded in paraffin and cut into 5-µm thick sections. Then, the sections were incubated with 0.1 % Triton X-100, blocked with 3 % H2O2. After washing with PBS for three times, the sections were incubated with TUNEL reaction mixture at 37 °C for 60 min. Thereafter, the sections were counter stained with hematoxylin, observed and photographed by a light microscopy at a magnification of 400×.

Electrophoretic mobility shift assay (EMSA)

The nuclear proteins were extracted from the pancreatic tissues using a Nuclear Protein Extraction Kit (Beyotime) and quantified by an Enhanced BCA Protein Assay Kit (Beyotime). A commercial EMSA kit (Viagene, China) was used to determine the DNA binding activity of NF-κB in pancreatic tissues. Briefly, equal amount of nuclear protein (25 µg) was reacted with 0.5 µl biotin end-labelled probe for 20 min at room temperature, separated on a 6.5 % polyacrylamide gel, electronically transferred onto nylon membrane. After cross-linked under the excitation of UV light for 30 min, the results were visualized by chemiluminescence.

Statistical analysis

The GraphPad Prism 5 software was used to perform statistical analysis, and experimental data were presented as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) followed by Bonferroni's Multiple Comparison Test was used to evaluate significant differences among multiple groups. P value less than 0.05 was considered to have a significant statistical difference.

Results

Effect of tempol on IH-induced pancreatic tissue injury

First, we observed the pathological changes in pancreatic tissues using HE staining assay. As shown in Figure 1A, IH exposure resulted in obvious damage in pancreatic cells with shrunk nuclei and faded cytoplasm staining. However, administration of tempol significantly alleviated the above pathological changes in pancreatic tissues. In addition, the blood glucose level was obviously increased in IH group, which could be adjusted to the normal level by tempol treatment (Fig. 1B). Furthermore, IH exposure led to distinct decrease in insulin content, while evident increases in proinsulin content and proinsulin/insulin ratio in pancreatic tissue, as compared with NA group. Whereas, tempol administration could effectively reverse the changes in insulin, proinsulin, and proinsulin/insulin ratio induced by IH (Fig. 1C-E).

Effect of tempol on IH-induced inflammatory response in pancreatic tissue

As shown in Figure 2A-C, the levels of pro-inflammatory cytokines TNF-α, IL-6, and IL-1β in pancreatic tissue were remarkably enhanced by IH exposure, which were significantly restrained by delivery of tempol. Moreover, the inflammatory mediators PGE2 and NO levels in pancreatic tissue were determined. As presented in Figure 2D-E, administration of tempol significantly suppressed IH-induced increase in levels of PGE2 and NO in pancreatic tissue. The protein levels of COX-2 and iNOS in pancreatic tissue were upregulated by IH exposure, whereas tempol delivery obviously decreased the COX-2 and iNOS levels (Fig. 2F-H).

Effect of tempol on IH-induced apoptosis in pancreatic tissue

The apoptosis in pancreatic tissue was evaluated by TUNEL staining and showed in Figure 3A. There were evident more apoptotic cells in the pancreatic tissue, as evidenced by strong brown staining in the nuclei after treatment with IH. However, administration of tempol could significantly suppress IH-induced apoptosis. In addition, the levels of a series of apoptosis-related
proteins were detected by western blot. As illustrated in Figure 3B-E, IH exposure caused decreased level of Bel-2, while increased levels of Bax and cleaved caspase-3 in the pancreatic tissue, whereas tempol administration effectively inhibited the level changes in above proteins.

Fig. 1. Tempol relieved IH-induced pancreatic tissue injury. (A) Pathological changes of pancreatic tissue were evaluated by HE staining. (B) Blood glucose level of the rats was detected. The insulin (C) and proinsulin (D) levels in pancreatic tissue were assessed by ELISA. (E) The proinsulin/insulin ratio was calculated and shown. The experimental data were expressed as mean ± SD (n=6). **P<0.01, ***P<0.001 vs. NA. #P<0.05, ##P<0.01, ###P<0.001 vs. IH.

Effect of tempol on the activation of MAPK signaling pathway

Our previous study has demonstrated that MAPK signaling pathway participated in IH-induced pancreatic injury. So we further evaluated whether tempol regulated IH-induced activation of the MAPK pathway. As presented in Figure 4A-D, tempol delivery significantly restrained IH-induced increase in levels of p-ERK, p-JNK, and p-P38 in pancreatic injury. These results indicated that inhibition of the MAPK signaling pathway activation was involved in the beneficial effect of tempol against IH-induced pancreatic injury.

Effect of tempol on the activation of NF-κB signaling pathway

NF-κB signaling pathway has been confirmed to play crucial roles in inflammatory response. So we further investigated the role of tempol in NF-κB signaling pathway. As shown in Figure 5A-D, the protein levels of p-IκBα, nuclear NF-κB were increased, while the IκBα
and cytoplasmic NF-κB levels were decreased in the pancreatic tissue after exposure to IH, which could be obviously restrained by tempol. Moreover, IH-induced enhanced DNA binding activity of NF-κB was evidently inhibited by the administration of tempol (Fig. 5E-F).

**Fig. 2.** Tempol repressed IH-induced inflammatory response in pancreatic tissue of the rats. The levels of TNF-α (A), IL-6 (B), IL-1β (C), and PGE2 (D) in pancreatic tissues were determined by ELISA. (E) The level of NO in pancreatic tissues was detected by commercial kit. (F) The protein levels of COX-2 and iNOS were assessed by western blot assay. GAPDH was used as a loading control. (G-H) Quantitative analysis of the grayscale value of the protein bands. The experimental data were expressed as mean ± SD (n=6). ** P<0.01, *** P<0.001 vs. NA. ## P<0.01, ### P<0.001 vs. IH.

**Discussion**

This is the first study that investigates the beneficial roles of tempol in pancreatic injury in a rat model of IH. Our results showed that tempol administration relieved IH-induced pathological changes in pancreatic tissue, elevated blood glucose, raised proinsulin/insulin ratio, inflammatory response, apoptosis, and abnormal activation of MAPK and NF-κB signaling pathways.

Previous studies have demonstrated that IH may lead to impaired pancreatic β-cell function, which provides evidence for OSA patients with high risk of diabetes (Wang *et al*. 2013). Our previous results also suggested that IH caused dysfunction of pancreas and insulin secretion disorder. The pancreatic trauma may further intensify the disturbance of lipid metabolism, which contributes to the course of obesity, diabetes mellitus and cardiovascular diseases. So seeking effective intervening measures to protect against IH-induced pancreatic injury has important clinical significance. In this study, we focused on the protective effect of tempol, a powerful antioxidant. As we expected, our results indicated that tempol effectively alleviated IH-induced pancreatic injury, and improved pancreas function as evidenced by adjusting blood glucose, pancreatic insulin and proinsulin to normal levels.
Fig. 3. Tempol restrained IH-induced apoptosis in pancreatic tissue. (A) TUNEL staining was performed to evaluate the apoptosis in pancreatic tissue. (B) The protein levels of Bcl-2, Bax, and cleaved caspase-3 in pancreatic tissue were detected by western blot assay. (C-E) Quantitative analysis of the grayscale value of the protein bands. The experimental data were expressed as mean ± SD (n=6). *** P<0.001 vs. NA. ## P<0.01, ### P<0.001 vs. IH.

Inflammatory response has been demonstrated to participate in IH-induced pancreatic injury, and the levels of pro-inflammatory cytokines, TNF-α, IL-6, and IL-1β, have been shown to be enhanced by IH (Wang et al. 2017). According to the present study, tempol delivery significantly downregulated the increased levels of TNF-α, IL-6, and IL-1β in pancreatic tissue induced by IH exposure. During inflammation, a series of
inflammatory mediators, such as NO and PGE2, are excessively released and further amplify the inflammation. NO is produced by iNOS-catalyzed oxidation of L-arginine. Similarly, the biosynthesis of PGE2 is catalyzed by COX-2. In normal physiological conditions, the inflammatory mediators, iNOS and COX-2, are present at low levels (Kolyada et al. 1996, Sudbo et al. 2003). While in inflammation, these two enzymes are overproduced and cause cell and tissue damage (Choy et al. 2008). Therefore, inhibiting the levels of iNOS and COX-2, and their catalyzed production of NO and PGE2 would be effective means for restraining inflammation. In this study, the increased levels of NO and PGE2, and upregulated protein levels of iNOS and COX-2 in pancreatic tissue of rats after exposure to IH were strikingly suppressed by tempol treatment, suggesting that tempol restrained IH-induced inflammatory response in pancreatic tissue.

Apoptosis has been confirmed to be involved in the underlying mechanisms of IH-induced pancreatic β-cell death (Yokoe et al. 2008, Xu et al. 2009, Fang et al. 2014). These studies suggested that apoptosis was one of important consequences of IH exposure. Thus, suppressing apoptosis may effectively relieve pancreatic injury induced by IH. Our results were as expected and showed that tempol treatment obviously inhibited apoptosis in pancreatic tissue after exposure to IH. Moreover, the detailed mechanisms that regulated apoptosis by tempol were investigated. Apoptosis is a programmed process regulated by multiple signal transduction pathways. Apoptosis is typical of activation of caspase cascades. A research by Zheng et al. (2012) indicated that caspase-3 activation was occurred in mouse insulinemia cell line 6 that suffered apoptosis following exposure to hypoxia. It is well acknowledged that Bcl-2 family proteins implicate in the regulation of apoptosis.
Bcl-2 is a well-known anti-apoptotic protein, while Bax is a pro-apoptotic protein of Bcl-2 family. Research also demonstrated that chronic IH resulted in β-cell apoptosis by downregulating Bcl-2 and upregulating Bax (Fang et al. 2014). According to our results, tempol administration restrained IH-induced upregulation of Bax and cleaved caspase-3 and downregulation of Bcl-2 in pancreatic tissue. These results suggested that inhibiting apoptosis was involved in the beneficial effect of tempol against IH-induced pancreatic injury.

**Fig. 5.** Tempol suppressed IH-induced activation of NF-κB signaling pathway. The protein level of p-IκBα and IκBα (A), cytoplasmic NF-κB and nuclear NF-κB (C) in pancreatic tissue were assessed by western blot assay. (B, D) Quantitative analysis of the grayscale value of the protein bands. (E) EMSA was performed to assess the DNA binding activity of NF-κB in pancreatic tissue. (F) Quantitative analysis of the DNA binding activity. The experimental data were expressed as mean ± SD (n=6). ***P<0.001 vs. NA. **P<0.01, ###P<0.001 vs. IH.
MAPK and NF-κB signaling pathways are confirmed to play crucial roles in inflammatory response (Chen et al. 2018, Gao et al. 2018). Suppressing the abnormal activation of MAPK and NF-κB pathways has been reported to attenuate IH-induced inflammation (Kang et al. 2017, Liu et al. 2017). Moreover, MAPK activation has also participated in IH-induced apoptosis (Wu et al. 2016). There are three members, including ERK, JNK, and P38, in MAPK family. Upon stimulation, these members are phosphorylated and then regulate downstream signaling events. Zhao et al. (2016) and our previous results indicated that ERK, JNK, and P38 were activated after exposure to IH. In this study, IH-induced hyperphosphorylation of ERK, JNK, and P38 in pancreatic tissue was restrained by tempol treatment. It is suggested that NF-κB-mediated inflammatory response is affected by p38 MAPK (Kumar et al. 2003). NF-κB is inactivated in cytoplasm by binding to IκBα under normal conditions. Whereas, IκBα is phosphorylated and degraded under inflammatory conditions, which promotes the translocation of NF-κB from cytoplasm to nucleus and the subsequent activation of proinflammatory molecules (Fang et al. 2007, Dai et al. 2011). Our results showed that the nuclear translocation and activation of NF-κB in pancreatic tissue after exposure to IH were significantly repressed by tempol. From these results, we suggested that MAPK and NF-κB pathways were involved in the regulatory mechanisms of tempol in IH-induced pancreatic injury.

Taken together, our study demonstrated the beneficial effect of tempol on hampering IH-induced pancreatic injury that could lead to dysfunction of pancreatic β-cell and promote the progression of diabetes. Therefore, tempol may be a potential treatment for OSA patients with pancreatic injury.

Conflict of Interest
There is no conflict of interest.

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References


