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Original Article

Intermittent hypoxia induced the renal mitochondria-dependent apoptotic signals in rats

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Abstract: Objective: Obstructive sleep apnoea (OSA) is associated with the progression of chronic kidney disease. Intermittent hypoxia (IH), a critical feature of OSA, induces oxidative stress and leads to injuries to kidney. The objective of this study was to examine the pro-apoptotic effect of IH on the kidney and the interventional role of the antioxidant tempol *in vivo*. Methods: Wistar rats were divided into three groups (n = 8 each): control group, IH group, and IH and tempol group (exposure to IH and administration of tempol). We analyzed the caspase-3 cleavage and the mitochondrial related apoptotic proteins (bcl-2, bax, released cytochrome *c*, cleaved caspase-9) by Western blotting and determined the $p22^{phox}$ and c-fos mRNA expressions through RT-PCR in the kidney tissues. Results: A significant enhancement in cleaved caspase-3, bax, cytochrome *c* release, and cleaved caspase-9 levels, and a significant reduction in the bcl-2 protein level and bcl-2/bax ratio in the kidney from intermittent hypoxia-exposed rats were observed compared to control ($P < 0.01$). Moreover, the mRNA expressions of renal $p22^{phox}$ and c-fos were also elevated significantly ($P < 0.01$). The pro-apoptotic action triggered by IH was alleviated by tempol treatment. Conclusions: Intermittent hypoxia induced the renal mitochondria-dependent apoptotic signals *in vivo*, which might involve elevated $p22^{phox}$ and c-fos mRNA levels.

Keywords: Intermittent hypoxia, oxidative stress, renal apoptosis, cytochrome *c*

Introduction

Obstructive sleep apnea (OSA) is a common disorder occurring in 5-20% of general population [1, 2]. OSA may contribute to accelerate atherosclerosis and promote atherosclerotic disease, which led to increased cardiovascular morbidity in patients with untreated OSA [3, 4]. Recent research has revealed that OSA is prevalent in patients with chronic kidney disease (CKD) and has been proposed as a risk factor for loss of kidney function [5, 6]. OSA may result in development and progression of CKD through many potential pathological pathways, including endothelial dysfunction, inflammation and obesity [7]. Intermittent hypoxia (IH) plays a critical role in the pathology of OSA. It has been reported that long-term chronic IH exposure led to renal damage by oxidative stress, inflammation, apoptotic cell death and fibrosis in mice [8, 9]. However, the pathway of IH-induced renal apoptosis remains largely unknown.

The effects of antioxidants on attenuating the progression of kidney disease were reported in several experimental animal models [10, 11]. Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine*N*-oxyl), a membrane-permeable superoxide dismutase mimetic agent, has been found to ameliorate oxidant stress-mediated renal dysfunction, injury and apoptosis *in vivo* [12-14]. The effect of tempol on IH-induced renal apoptosis remains to be clarified yet. We hypothesized that mitochondrial-dependent pathway may be involved in IH-induced renal apoptosis and tempol may prevent IH-induced apoptotic effect in the rat kidney, since IH was associated with increased oxidative stress [15]. Herein, the apoptotic pathway of IH-induced renal apoptosis and the biological role of tempol were described in this work.

Material and methods

Animals

This research was approved by the Tianjin Medical University Animal Care and Use Committee. 24 male Wistar rats (eight weeks old, 160-200 g), obtained from Model Animal

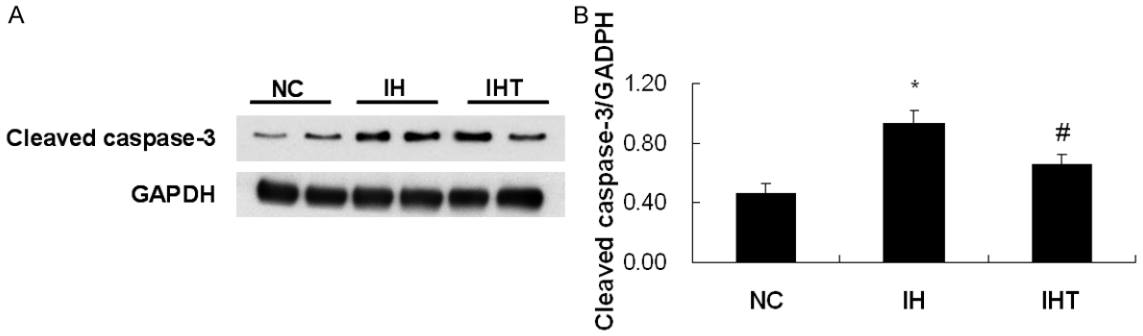

Figure 1. Representative western blot (A) and densitometric analysis (B) of cleaved caspase-3 in each group. Data are expressed as the mean ± SD, $^{*}P < 0.01$, *vs*. control group; $^{\#}P < 0.01$, *vs*. intermittent hypoxia group.

Center of Hygiene and Environmental Medicine Research Institute, Chinese Academy of Medical Science, Tianjin, China, were randomly assigned to three groups (n = 8 each): normal oxygen control group (NC), 5% (v/v) IH group for successive 6 weeks (IH), and 5% IH group plus administration of tempol (Sigma Company) for successive 6 weeks (IHT).

Intermittent hypoxia treatment

Rat models of IH were performed as described previously [16]. Briefly, rats were exposed to IH between 9 am and 5 pm every day for 6 successive weeks. Each cycle of IH lasted for 120 s, with IH being 30 s and reoxygenation 90 s. In the IHT group, the rats were treated with 1 mL 10% (w/v) tempol per kg body weight under IH environment. The kidneys were harvested individually from each animal in different group after 6 weeks.

Western blotting

The total protein from the kidney tissues of different groups was extracted as previously described [17]. Western blot analysis of caspase-3, bcl-2, bax, cytochrome *c* and caspase-9 was performed according to the protocol in our previous report [16]. The protein samples were separated on SDS-polyacrylamide gradient gel and subjected to Western blotting analysis. The blots were incubated with primary antibodies caspase-3, bcl-2, bax, cytochrome *c*, caspase-9 and GAPDH (1:400, 1:300, 1:300, 1:400, 1:400, and 1:5000 dilution, respectively; Santa Cruz Biotechnology, CA, USA) overnight at 4°C. HRP conjugated sheep anti-mouse-IgG (1:5000 dilutions, Beijing Zhongshan Biotechnology Inc, Beijing, China) was used as the secondary antibody.

RNA isolation and reverse transcriptase PCR

The regular real time PCR was carried out as described [16]. Briefly, mRNA levels of $p22^{phox}$ and c-fos were measured by the real-time RT-PCR. Primer sequences for amplification of the above genes are: $p22^{phox}$, sense: 5'-TCATCCAGCCTTCTCTCCAT-3' and antisense: 5'-AGCCTCACCATGACT ACCTT-3'; c-fos, sense 5'-TACTACCATTCCCCAGCCGA-3' and antisense 5'-GCTGTCACCGTGGGGATAAA-3'. Glyceraldehyde 3-phosphate dehydrogenase servedas an internal reference. Data acquisition was carried out on a LightCycler (Roche Diagnostics, Indianapolis, IN, USA). The relative quantification of the detected genes was calculated as $2^{-\Delta Ct}$.

Statistical analysis

The values are expressed as the mean ± standard deviation. Comparisons for the difference between groups were preformed using repeated one-way ANOVA. Statistical analyses were carried out through SPSS version 16.0 software (SPSS Inc., Chicago, IL) and P values < 0.05 were regarded statistically significant.

Results

Effect of intermittent hypoxia on renal caspase-3 activation in rats

To investigate whether IH induced the activation of caspase-3 in rats, the protein level of cleaved caspase-3 was detected by Western blot analysis. Compared with control, IH signifi-

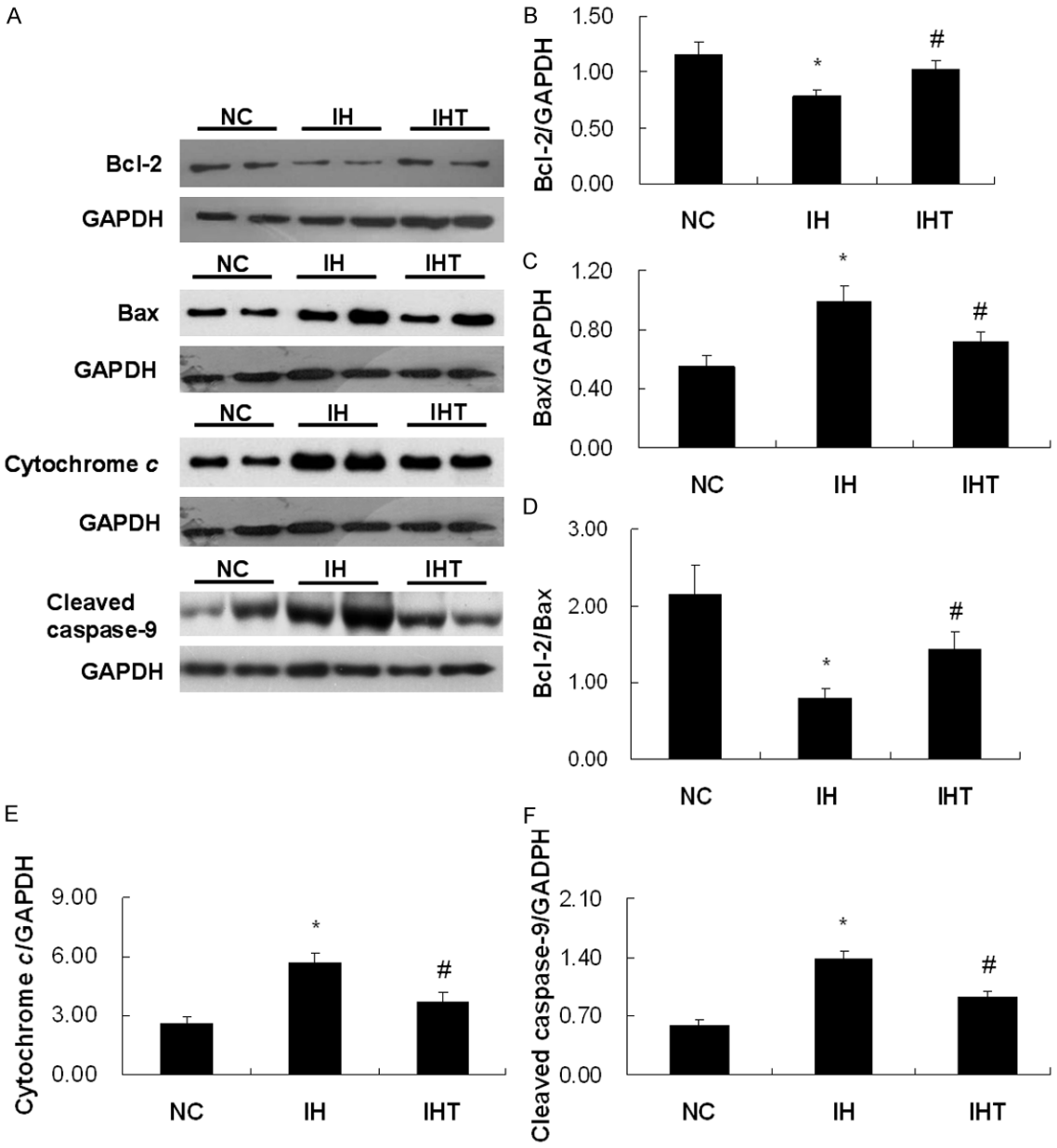

Figure 2. Representative western blots (A) and densitometric analysis of bcl-2 (B), bax (C), bcl-2/bax ratio (D), cytosolic cytochrome *c* (E) and cleaved caspase-9 (F) in each group. Data are expressed as the mean ± SD, $^{*}P < 0.01$, *vs*. control group; $^{\#}P < 0.01$, *vs*. intermittent hypoxia group.

cantly increased the cleaved caspase-3 level in the kidney tissues, indicating a pro-apoptotic potential (**Figure 1**). In addition, tempol significantly ameliorated caspase-3 cleavage in rat kidney tissues triggered by IH.

Effect of intermittent hypoxia on renal mitochondrial dysfunctions

To clarify whether IH induced the renal apoptosis through the mitochondria-dependent pathway, we investigated the protein levels of bcl-2, bax, cytochrome *c* release and cleaved caspase-9 in the three groups by Western blotting. As shown in **Figure 2**, the mitochondrial related anti-apoptotic protein bcl-2 and the bcl-2/bax ratio were significantly reduced in the IH group, while pro-apoptotic protein bax was significantly increased compared with the control group. Moreover, the amounts of cytosolic cytochrome *c* and cleaved caspase-9 were significantly

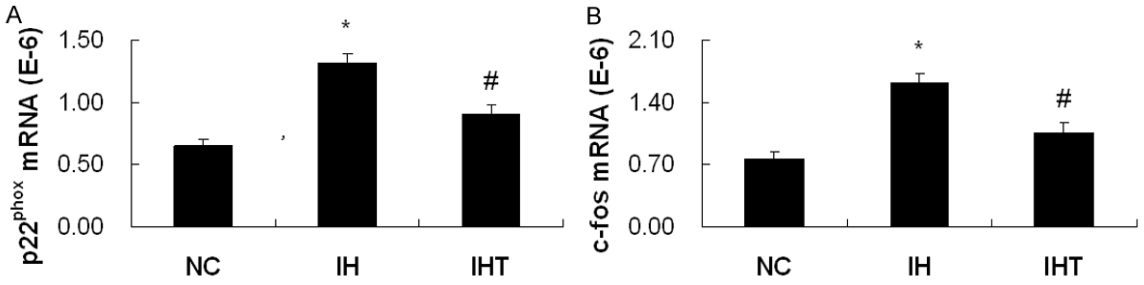

Figure 3. Intermittent hypoxia induced p22^{phox} subunit (A) and c-fos (B) mRNA expression in the kidney as assessed by real time PCR. Data are expressed as the mean ± SD, $^{*}P < 0.01$, *vs*. control group; $^{\#}P < 0.01$, *vs*. intermittent hypoxia group.

higher than the control group. Treatment with tempol effectively suppressed the IH-induced alteration of mitochondrial related proteins.

Effect of intermittent hypoxia on renal NADPH oxidase subunit p22^{phox} and c-fos mRNA expressions

To reveal preliminarily the possible mechanisms of the increased apoptotic signals in kidney tissues, NADPH oxidase subunit p22^{phox} and c-fos mRNA expression levels were detected using real time PCR. As shown in **Figure 3**, renal p22^{phox} and c-fos mRNA expression level were increased compared to control. The upregulation of p22^{phox} and c-fos was partially restored by tempol.

Discussion

Our study revealed the renal mitochondria-dependent apoptotic pathway and the protective effect of tempol treatment on renal IH-mediated apoptosis *in invo*. Furthermore, upregulated mRNA expressions of p22^{phox} and c-fos might contribute to the increase of renal apoptotic signals.

Some studies suggested a bidirectional association between CKD and OSA with both being possible risk factors for each other [18]. Indeed, Chou *et al*. have reported that there existed a high prevalence of chronic kidney disease in OSA patients even in the absence of hypertension or diabetes [19]. Glomerular hyperfiltration and severe proteinuria were both demonstrated in OSA patients [20, 21]. Therefore, it has been proposed that OSA can also accelerate loss of kidney function through potential mechanisms, including OSA-associated increased oxidative stress and inflammation [7, 18]. Wu *et al*. have shown that IH induced renal injury by oxidative stress, inflammation and fibrosis [8]. Furthermore, Sun et al. clearly demonstrated that IH promoted renal apoptotic cell death *via* oxidative and inflammatory pathways [9], indicating a possible mechanism of IH-induced kidney injury and loss of kidney function through renal cell apoptosis, since apoptosis has been recognized as one mechanism leading to tubular atrophy and kidney cell loss in CKD [22, 23].

Lai *et al*. has showed that mitochondrion was one of the subcellular targets of IH for cardiac apoptosis in mice [24], but the mitochondrial role in IH-induced renal cell apoptosis remains unclear and is a relatively new point of interest. Our study demonstrated that IH induced caspase-3 activation in rats, which supported the previous observations that 8 week IH induced caspase-3 cleavage in the mice kidney [9]. We also observed the alterations in the bax and bcl-2 protein levels and the bcl-2/bax ratio towards pro-apoptotic potential in the rat kidney. Bax interacted with the mitochondrial membrane and promoted cytochrome *c* release into cytosol, resulting in the activation of caspase-9, which was one downstream component of renal mitochondria-dependent apoptosis. To the best of our knowledge, it is demonstrate for the first time that IH induced renal apoptosis, at least in part, through mitochondrial dysfunction.

IH-associated oxidative stress plays a key role in renal damage under various pathophysiological conditions, including diabetic nephropathy, chronic renal failure and ischemia-reperfusion [25]. NADPH oxidase subunit p22^{phox} was found to be one major source for ROS production in the kidney and could play a role in pathological

conditions [26]. Modlinger *et al*. showed that siRNA targeted to renal $p22^{phox}$ led to reduce renal NADPH oxidase activity and ROS formation [27]. In addition, the increased expression of renal $p22^{phox}$ was associated with ROS-induced renal damage in a rodent model of type 2 diabetes [28]. Therefore, we examined the mRNA expression of renal $p22^{phox}$. Compared with the control group, renal $p22^{phox}$ mRNA expression was significantly upregulated, possibly implying overexpression of NADPH oxidase and overproduction of ROS. Thus, NADPH oxidase may be involved in renal mitochondria-dependent apoptosis induced by IH. Besides, c-fos was one protein member of the inflammatory transcription factor AP-1. The current investigation showed that renal c-fos mRNA expression was also significantly upregulated in the IH group, indicating a possible greater inflammatory status. Our results are consistent with the previous findings that increased renal inflammation, as demonstrated by ICAM-1, was involved in the IH-induced renal apoptosis [8, 9].

Tempol contributes to scavenging ROS by promoting SOD activity [29]. Tempol could reduce oxidative stress and apoptosis in the renal cortex in rats exposed to losartan during lactation [30]. Recently, Ahmed *et al*. reported that tempol ameliorated mitochondrial dysfunction and inhibited renal apoptosis in the rat kidney induced by cisplatin [31]. In this study, tempol inhibited the IH-induced pro-apoptotic effect in the rat kidney through alleviation of mitochondrial dysfunction and downstream caspase activation *via* reduced NADPH and c-fos mRNA levels.

In summary, this present study has demonstrated that IH triggered apoptotic signals through mitochondrial dysfunction and activation of caspase cascades in rat kidney, which might involve elevated $p22^{phox}$ and c-fos mRNA expressions. Furthermore the adverse alteration could be attenuated by the antioxidant tempol. These observations further clarify the nephrotoxic mechanisms of IH and provide preliminary evidence to suggest that IH-induced renal apoptosis might be one mechanism of OSA-associated CKD.

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Disclosure of conflict of interest

None.

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