Metoprolol Inhibits Cardiac Apoptosis and Fibrosis in a Canine Model of Chronic Obstructive Sleep Apnea

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Key Words
Obstructive sleep apnea • Apoptosis • Fibrosis • Metoprolol • Canine

Abstract

\textbf{Aims:} Emerging evidence suggested that obstructive sleep apnea (OSA) was independently associated with the development of heart failure. In this study, we explored the influence of chronic OSA on left ventricular structural remodeling in canines, and the potential therapeutical role of metoprolol.

\textbf{Methods:} Chronic OSA model was established by stopping the ventilator and closing the airway for 4 h/day apnea-ventilation cycles every other day for 12 weeks while metoprolol (5 mg·kg\textsuperscript{-1}·day\textsuperscript{-1}) were administered continuously. Norepinephrine concentration was measured by Enzyme Linked Immunosorbent Assay. Transmission electron microscopy, Hematoxylin and eosin, TUNEL and Masson trichrome staining were employed to detect the morphology, apoptosis and fibrosis of cardiomyocytes. Protein expression of apoptosis and fibrosis-related factors including apoptosis-inducing factor (AIF), caspase 3, Bcl-2, Bax, \(\alpha\)-smooth muscle actin (\(\alpha\)-SMA), transforming growth factor-\(\beta\)1 (TGF-\(\beta\)1), and p38 mitogen-activated protein kinase (MAPK) were examined by Western blotting.

\textbf{Results:} Norepinephrine concentration was markedly increased in chronic OSA dogs and reduced by metoprolol. Both the apoptotic ratio and collagen volume fraction were significantly increased in left ventricular myocytes of chronic OSA dogs, and was reversed by metoprolol. Moreover, chronic OSA-induced upregulation of AIF, cleaved caspase 3, Bax, \(\alpha\)-SMA, and TGF-\(\beta\)1 as well as downregulation of Bcl-2 was markedly recovered by metoprolol, which was mediated by p38 MAPK.

\textbf{Conclusion:} Metoprolol protects against chronic OSA-induced cardiac apoptosis and fibrosis in left ventricular myocytes of canines, which may provide new potential strategy for drug therapy of OSA.
Introduction

Obstructive sleep apnea (OSA) is a common breathing disorder characterized by recurrent episodes of airway collapse that affects at least 5% of the adult population [1]. However, only about 10% of OSA patients are diagnosed and treated, which has direct impacts on public health because of the high financial costs of untreated OSA [2]. Both clinical and experimental studies demonstrates that OSA is associated with increased risk of hypertension, coronary heart disease, atrial and ventricular arrhythmias [3]. The pathophysiologic mechanisms of OSA include sympathetic hyperactivation, impairment of vasomotor reactivity, vascular inflammation, oxidative stress, endothelial dysfunction and metabolic disorders, which are closely related to the function of left heart [4]. Meanwhile, previous studies have revealed atrial remodeling including electrical, structural and autonomic remodeling in both OSA animal models and patients [5, 6]. Lately, left ventricular concentric geometry and systolic dysfunction has been found independently associated with moderate and severe OSA and nighttime blood pressure levels in patients with resistant hypertension [7]. However, the correlation between OSA and ventricular remodeling are not well described. Furthermore, effective intervention measurements to inhibit OSA-induced cardiac remodeling have been paid more and more attention.

Nowadays, it has been widely accepted that OSA-induced cardiovascular disorders could be ameliorated by continuous positive airway pressure (CPAP) treatment [8]. However, approximately 23% of patients abandon the treatment within 5 years because of the unpleasant side effects from the mask, noise, air leaks, and patient’s nasal anatomy of CPAP based on previous study, although very long-term compliance with CPAP increased of follow-up in patients with OSA [9]. Moreover, there is still no evidence that CPAP could reverse ventricular structural remodeling of chronic OSA. As a result, alternative treatments are desired for those patients suffered from chronic OSA.

Metoprolol, a selective β1-adrenergic receptor antagonist, has been demonstrated capable of reversing cardiac remodeling in the post-infarction rat heart [10]. Previous studies also reported that metoprolol increased left ventricular ejection fraction (LVEF) and a reversal of left ventricular global, structural and biochemical remodeling in dogs [11]. Moreover, Ablad et al. [12] found that metoprolol reduced the inducibility of ventricular fibrillation. Nevertheless, the potential role of metoprolol in chronic OSA-induced left ventricular structural remodeling remains unclear. Therefore, the present study was designed to elucidate the potential effect and mechanism of metoprolol on chronic OSA-induced cardiac apoptosis and interstitial fibrosis in a canine model.

Materials and Methods

Animals

All experiments in this study were approved by the ethic committees of Harbin Medical University, and performed in compliance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (8th edition, 2011). Fifteen male mongrel dogs (weight 20–25 kg, from Experimental Animal Center of the First Affiliated Hospital of Harbin Medical University) were housed in cages under the standard laboratory conditions. Dogs were anaesthetized with ketamine (5.3 mg/kg, iv), diazepam (0.25 mg/kg, iv), and xylazine (1 mg/kg, iv). Adequacy of anesthesia was monitored from the disappearance of the corneal reflex and jaw tone. All dogs were randomly divided into three groups as follows: sham, OSA and metoprolol-treated group. Among them, five dogs underwent sham operation (anesthesia and tracheal intubation only) and the remaining dogs were suffered from chronic OSA with or without 5 mg·kg⁻¹·day⁻¹ metoprolol succinate administration continuously.

OSA simulation

Tracheal intubation was done in dogs after anesthesia. Then, the tube in the trachea was clamped to imitate apnea at the end of the exhalation. The protocol of chronic OSA was according to our previous study.
[13] with minor change. Briefly, the apnea hypopnea index (AHI) was set as 6 for the duration of trachea blockage was 1 min and ventilation was 9 min in the first week. Then, the duration of ventilation was 1 min shorter progressively than the previous week in the next three weeks. At the last eight weeks, the duration of trachea ventilation was 5 min and AHI was 10. The ventilation and blockage was exchanged for 4 h every other day in the duration of 12 weeks.

**Norepinephrine concentrations assay**

Norepinephrine concentration in the left ventricle were detected using commercially available Enzyme Linked Immunosorbent Assay (ELISA) kit (BlueGene, Shanghai, China) according to the manufacturer’s protocol.

**Echocardiography**

Transthoracic and transesophageal echocardiography was performed with a phased-array system (Sonos7500, Philips Ultrasound) using a 2.3 MHz probe in next day of last obstruction to measure the following parameters: LV end-systolic diameter (LVESD), left ventricular end-diastolic diameter (LVEDD), left ventricular percent fractional shortening (LVFS), LVEF, interventricular septum diameter (IVSD) and LV posterior wall diameter (LVPWD).

**Measurement of femoral and pulmonary artery pressures**

Femoral and pulmonary artery pressures were measured according to previous study [14]. Briefly, to record femoral artery pressure, the 6F sheath was introduced percutaneously into the femoral artery. To record pulmonary artery pressure, the sheath was introduced into pulmonary artery from femoral vein. The sheath was connected to a tubing system and a pressure transducer to detect simultaneously the values of systolic arterial pressure (SAP), diastolic arterial pressure (DAP), mean arterial pressure (MAP), pulmonary artery systolic pressure (PASP), pulmonary artery diastolic pressure (PADP) and pulmonary artery mean pressure (PAMP).

**Transmission electron microscopy**

Left ventricles were fixed in 2.5% glutaraldehyde in 0.1 M phosphate-buffered saline (PBS) (pH=7.35) and subsequently rinsed in buffer, post-fixed in PBS 1% OsO4 for 2 h, stained with 1% uranyl acetate, dehydrated in graded ethanol and embedded in epoxy resin according to previous studies [15]. The sections were electron-stained and examined under a JEM-1200 electron microscope (JEOL Ltd., Tokyo, Japan).

**HE and Masson trichrome staining**

The samples were embedded in paraffin, cut into 5-μm thick sections and stained with Hematoxylin and eosin (HE) and Masson trichrome for histological and collagen analysis. The interstitial fibrotic areas were calculated with image analysis software (Image-pro plus 6.0, Meida Cybernetics LP). Collagen volume fraction (CVF) was calculated as collagen area/total area×100% [16, 17].

**TUNEL assay**

Effect of metoprolol on cardiomyocyte apoptosis were determined by Terminal deoxynucleotidyl transferase mediated dUTP nick end-labeling (TUNEL) assay according to the manufacturer’s instructions (Roche, Indianapolis, IN, USA). TUNEL-positive cells showed dark buffy nuclei staining while TUNEL-negative cells exhibited blue nuclei under an Olympus BX-60 microscope (Olympus, Tokyo, Japan). The number of apoptotic cardiomyocytes and overall cell number were counted, and the myocardial apoptotic rate was calculated as the number of apoptotic cells/total number of cells×100% [16].

**Western blotting**

Total protein samples were extracted from left ventricles with the procedures as previously described [13, 18, 19]. The samples were subjected to electrophoresed in 10% SDS-PAGE and the separated proteins were transferred to PVDF membrane (Millipore, Bedford, MA, USA). The membranes were blocked by 5% nonfat dry milk in PBS and incubated overnight at 4°C with different primary antibodies: anti-AIF (Abcam, 1:1000), anti-active caspase 3 (Abcam, 1:1000), anti-Bcl-2 (Abcam, 1:750), anti-Bax (Abcam, 1:750), anti-α-SMA (Abcam, 1:1000), anti-TGF-β1 (Abcam, 1:300), anti-p38, anti-phospho-p38 (Cell Signaling, 1:1000)
and anti-GAPDH (Kangcheng, China, 1:1000). Then, the membranes were rinsed and incubated with horseradish peroxidase-conjugated secondary antibody (Higene, China) for 1 h at 37°C. Band intensity was quantified by Quantity One Software (Bio-Rad, Hercules, CA, USA).

Statistical analysis
All data were expressed as mean ± standard deviation (SD). Multiple comparisons were carried out by one-way ANOVA. p<0.05 was considered statistically significant. Figures were constructed by GraphPad Prism 5.0 software.

Results

Norepinephrine concentration in chronic OSA and metoprolol-treated dogs
To elucidate the alteration of sympathetic neurotransmitter in chronic OSA dogs, we firstly examine the level of norepinephrine. As shown in Fig. 1, we found that norepinephrine concentration in left ventricles was significantly increased in chronic OSA dogs while reduced by metoprolol.

Morphological changes of ventricular myocytes in chronic OSA and metoprolol-treated dogs
To examine the morphological alteration of ventricular myocytes in chronic OSA and metoprolol-treated dogs, we conducted transmission electron microscopy and HE staining. As shown in Fig. 2A, the cardiac muscle fibers of the sham dogs showed a clear regular structure under transmission electron microscope. In contrast, deranged myofibers and swollen mitochondria were shown in cardiomyocytes of chronic OSA dogs (Fig. 2B), which was markedly ameliorated after metoprolol treatment (Fig. 2C). Meanwhile, similar results were found in HE staining results. Fig. 2D indicated that normal cardiomyocytes contained compactly arranged fibres without inter-cellular space in the sham dog. However, myocardial fibers were disordered, cardiomyocytes were hypertrophic and oedematous, and the nuclei appeared distorted and varied in size in the OSA dog (Fig. 2E). The changes in chronic OSA dogs were significantly inhibited by metoprolol (Fig. 2F). Thus, these data uncovered that metoprolol alleviated chronic OSA-induced morphological changes in ventricular myocytes.

Metoprolol inhibits cardiac apoptosis and fibrosis in chronic OSA dogs
We further detected structural remodeling in metoprolol-treated chronic OSA dog. On one hand, TUNEL assay was employed to examine the effect of metoprolol on chronic OSA-induced cardiac apoptosis. The percentage of labeled TUNEL-positive cells in myocardium was significantly higher in the chronic OSA dogs than sham group (52.34 ± 0.07% vs. 21.62 ± 0.02%, p<0.001, n=5, Fig. 3D), which was reversed by metoprolol (35.17 ± 0.05%, p<0.001, n=5, Fig. 3D). On the other hand, Masson staining was used to observe fibrosis in metoprolol-treated chronic OSA dog. The ventricles of sham dogs appeared grossly normal under light microscopy. In contrast, extensive interstitial fibrosis was observed in OSA dogs. Bundles of

Fig. 1. The concentrations of norepinephrine detected by ELISA assay in left ventricles of chronic OSA and metoprolol-treated dogs. n=5 in each group.
myofibers were packed less tightly in chronic OSA dogs than sham dogs and were separated by thick layers of fibrous tissue, which was reversed by metoprolol (Fig. 3E-G). Statistically, chronic OSA increased CVF which was repressed by metoprolol (Fig. 3H). 

**Protein expression of apoptosis and fibrosis-related factors in metoprolol-treated chronic OSA dogs**

To elucidate the molecular mechanisms in chronic OSA-induced ventricular structural remodeling, we also detected the following apoptosis and fibrosis-related proteins including AIF, caspase 3, Bcl-2, Bax, α-SMA, TGF-β1. We found that protein expression of AIF, caspase 3, Bax was all increased while Bcl-2 was decreased in chronic OSA, which was recovered by
metoprolol (Fig. 4A-H). In addition, ratio of Bcl-2/Bax was decreased in chronic OSA and was recovered by metoprolol (Fig. 4I). Thus, these data suggested that metoprolol inhibited cardiac apoptosis in chronic OSA dog, which is consistent with the above TUNEL results (Fig. 3D). At the same time, protein expression of both α-SMA and TGF-β1 was found increased in chronic OSA dogs and was reduced by metoprolol (Fig. 5), which is consistent with Masson staining data (Fig. 3H). Importantly, it was also found that phospho-p38 MAPK was upregulated in chronic OSA dogs and downregulated by metoprolol (Fig. 6). Taken together, these data supported that chronic OSA induced structural remodeling (apoptosis and...
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Cardiac function after apnea for 12 weeks. Metoprolol significantly inhibited OSA-induced cardiac apoptosis and fibrosis partly by p38 MAPK signaling pathway.

**Left ventricular function in metoprolol-treated chronic OSA dogs**

Echocardiography showed that no significant changes were detected in LVESD, LVEDD, LVFS, LVEF, IVSD and LVPWD in OSA and metoprolol-treated dogs compared with sham dogs (Fig. 7). These data suggested that chronic OSA did not affect the left ventricular physiology in dogs. Besides, we also examined systemic blood pressure in OSA and metoprolol-treated dogs. We found that neither femoral nor pulmonary artery pressure significantly increased in chronic OSA dogs compared with sham dogs (Fig. 8).

**Discussion**

In the present study, we have firstly demonstrated that chronic OSA for 12 weeks could induce significant ventricular structural remodeling (cardiac apoptosis and interstitial fibrosis) by upregulating AIF, Bax, caspase 3, TGF-β1 and α-SMA as well as downregulating...
Bcl-2. Moreover, metoprolol could inhibit cardiac apoptosis and fibrosis caused by chronic OSA by inhibiting p38 MAPK in canines.

Many clinical studies support the notion that chronic OSA has important effects on left ventricular function. For example, left ventricular diastolic dysfunction and LV hypertrophy occur in patients with OSA even before the development of hypertension and other cardiovascular diseases [20]. Furthermore, in a rat chronic intermittent hypoxia model, there was a significant elevation in both LVESD and left ventricular end-systolic volume along with the decrease in both LVEF and LVFS [21]. However, the underlying molecular mechanisms remain poorly understood. In the present study, we detected obvious cardiac apoptosis and fibrosis in ventricles of chronic OSA dogs, with upregulated protein expression of apoptosis and fibrosis factors. Interestingly, no significant change of LV function was found in both our previous study [13] and this study. As shown in Fig. 7, it was suggested that there were no significant differences in LVEDD, LVESD, IVSD and LVPWD among sham, chronic OSA and metoprolol-treated dogs. Additionally, there were no significant differences in both LVFS and LVEF among those three groups. Considering the duration of OSA was 12 weeks in our study, we inferred that longer period may cause damage in cardiac function.

In our previous study, we observed sympathetic nerves hyperinnervation in chronic OSA dogs [13]. Moreover, in this study, the norepinephrine concentration in the left ventricles of chronic OSA canines was significantly enhanced. It implied that sympathetic nerve may participate in the structural alterations. So it is interesting to explore effective drugs to reverse structural remodelings induced by chronic OSA. Cardiomyocyte apoptosis has been recognized to be involved in cardiovascular diseases, which was thought as one of the important predictors of cardiac diseases [22, 23]. Previous studies have revealed ventricular myocyte apoptosis in other intermittent hypoxia models. For instance, Lai et al [24] found that chronic intermittent hypoxia (CIH)-induced apoptosis in mice hearts through Fas-dependent and mitochondria-dependent pathway. In another rat model, CIH-induced left ventricular dysfunction and associated myocardial apoptosis by inhibition of ROS-dependent ER stress [21].

It has been reported that metoprolol normalized myocardial oxygen consumption, decreased myocardial damage, augmented cardiomyocyte survival, improved cardiac...
function, reduced the incidence of arrhythmia, thus decreasing the occurrence of cardiac events [25]. Additionally, our previous study has demonstrated that metoprolol prevented the cardiotoxicity of ketamine probably by reducing myocardial apoptosis [16]. In human trials, it has been observed that metoprolol led to a reverse in LV remodeling potentially through its anti-apoptotic effects with reduced the plasma concentrations of the apoptotic mediators soluble Fas and soluble Fas ligand in patients with CHF [26].

On the other hand, ventricular interstitial fibrosis has been considered to be the mechanism for constructing the ventricular remodeling substrate by destroying cell-to-cell electrophysiological coupling. Several studies have shown that cardiac damage was evident with increased cardiac fibrosis in the IH-exposed rat model [27, 28]. In the present study, we have documented the enhanced expression of intercellular fibrosis in chronic OSA. And ventricular fibrosis was markedly suppressed by metoprolol. Meanwhile, metoprolol could decreased the upregulated pro-fibrotic growth cytokine TGF-β1 and α-SMA induced by chronic OSA. Similarly, anti-fibrotic effect of metoprolol has been found in other studies. For example, metoprolol reduced myocyte hypertrophy and collagen deposition in a rat model of renovascular hypertension-induced cardiac hypertrophy [29]. Moreover, Serpi et al [30] suggested that metoprolol treatment attenuated adverse remodeling via decreased apoptosis and fibrosis in the peri-infarct region in a model of coronary ligation in rats. Thus, based on our study, it was worth noting that metoprolol could reverse chronic OSA-induced structural remodeling. It is well known that p38 MAPK signaling pathway play an important role in the apoptosis and fibrosis in cardiovascular diseases. In this study, protein expression of p38 MAPK was increased in chronic OSA dog and was restored by metoprolol. Thus anti-apoptosis and anti-fibrosis effect of metoprolol was mediated at least partially by p38 MAPK. Because chronic OSA did not induce systemic hypertension in dogs in this study, the protective role of metoprolol against left ventricular remodeling was not result from controlling of blood pressure. Instead, the effect of metoprolol may associate with the inhibition of sympathetic nerves.

It has been reported that β-blockers improve function of the failing LV, prevent or reverse progressive LV dilation, hypertrophy, and consequently positively impact cardiac remodeling [31, 32]. But the administration of β-blockers is disputed in patients suffering of chronic airway obstructive diseases, owing to the result that single-dose administration of non-selective β-blockers may cause airway narrowing in these patients [33]. However, results from meta-analysis suggest that using of cardioselective β-blockers seems to be safe in patients with chronic airway obstruction [34]. Salpeter et al examined a large number of clinical studies in patients with reversible airway disease and COPD exposed to single dose or continued treatment with cardioselective β-blockers. They found that acute β-blockers treatment resulted in a small reduction in forced expiratory volume in one second (FEV1). Furthermore, chronic treatment produced no change in FEV1, symptoms, or inhaler use [34]. Of course, more studies are needed to validate the efficiency and safety of metoprolol in OSA patients. It should be cautious and taken into attention when extrapolating from our animal data to human.

Although our study demonstrated that metoprolol reduced cardiac apoptosis and fibrosis in the left ventricles of OSA dogs, the concrete mechanism of left ventricular remodeling in chronic OSA dogs remain unclear. Moreover, it has been reported that OSA could induce hypertension in previous studies [35]. Interestingly, both systemic blood pressure and echocardiographic parameters of left ventricles were unchanged in canine model of chronic OSA in the present study. Nevertheless, our previous work uncovered that sympathetic nerves play an important role in the left ventricular remodeling [16]. Therefore, we believe that sympathetic activation may participate in the process of left ventricular remodeling. Besides, it should be noted that like all animal models of human disease, the model we established here may not a perfect counterpart of OSA condition in human. More work is needed before pursuing a drug therapy. Collectively, our study demonstrated that metoprolol could reverse chronic OSA-induced ventricular apoptosis and fibrosis in dogs.
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Disclosure Statement

None declared.

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