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Cell-Autonomous Autophagy Protects Against Chronic Intermittent Hypoxia Induced Sensory Nerves and Endothelial Dysfunction of the Soft Palate

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Background: Chronic intermittent hypoxia (CIH) is a key feature of obstructive sleep apnea (OSA) syndrome. The pathogenesis of CIH-induced soft palate lesion is not well understood. Understanding the mechanisms of CIH-induced soft palate damage could provide new strategies for clinical treatment.

Material/Methods: Twenty male Sprague-Dawley rats were randomized into a control group (n=10) and experimental group (n=10). The experimental group were exposed to CIH for 28 days. The control experiments were run in parallel. Morphological changes of CIH-induced soft palate were examined by hematoxylin and eosin. Peripheral nerves and vascular associated markers were analyzed by western blot and immunohistochemical staining. LC3B expression and transmission electron microscopy analysis was detected to investigate the destiny of cells in CIH-induced soft palate.

Results: Histological studies demonstrated the thicken mucosal layer, muscular changes consistent with glands hyperplasia, and loose connective tissues of the soft palate in CIH induced rat models. CIH exposure significantly decreased the expression of annexin V but did not change argin level, suggesting that sensory nerves not motor nerves were damaged when exposed to intermittent hypoxia. Moreover, in response to CIH, the vascular vessel around the nerves and muscles became enlarged and caveolin-1 was overexpressed. Autophagy occurs in response to CIH-induced neuromuscular and vascular endothelial injury.

Conclusions: Sensory nerves and endothelial dysfunction contributed to the morphological damage of soft palate under intermittent hypoxia. Autophagy as a compensatory mechanism protects against CIH-induced injury. These findings have important implications for understanding mechanisms contributing to the increased soft palate lesion in patients with OSA.

MeSH Keywords: Autophagy • Cell Hypoxia • Palate, Soft • Pharyngeal Muscles • Sleep Apnea, Obstructive

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Background

Obstructive sleep apnea (OSA) is characterized by repetitive upper airway obstruction and reductions or even cessation in airflow during sleep. This reduction in airflow could lead to intermittent hypoxia and sleep disturbances (e.g., frequent awakenings and daytimes sleepiness) [1,2]. OSA is a common yet unrecognized health problem. Based on a recent systematic review, when OSA was defined as an apnea-hypopnea index of 5 or greater per hour of sleep, the overall population prevalence ranged from 9% to 38% [3]. OSA has been related to the risk of various diseases, including cardiovascular disease [4,5], metabolic disease [6], pregnancy-related outcomes [7,8], and Alzheimer disease [9,10].

Various pathophysiological mechanisms may be involved in the development of OSA, while the collapse of the soft palate in the upper airway is one of the main causes of stopped breath. The pathophysiology for collapse of the soft palate is due to complex anatomic changes in the soft palate [11]. It has been reported that the hypertrophy of the salivary glands, congestion and dilation of the thin-walled vessels, and atrophy of the muscle bundles were observed in patients with OSA syndrome [12]. But the exact pathophysiology leading to dysfunctions of the soft plate is not well understood. The human soft palate plays an important role in respiration, swallowing, and speech. These motor activities depend on reflexes mediated by sensory nerve endings. But it has been suggested that neuromuscular injury leads to inefficient muscle function, accompanied with upregulation of neurotrophic factors of snores in sleep apnea patients [13]. Some authors have found atrophy and abnormal distribution of fiber types in the palatopharyngeal muscles, supporting a neurogenic alteration in OSA patients. Moreover, evidence has indicated that reduced palatal muscle activation and complex neurogenic changes in the soft plate might be an explanation for pathological soft plate collapse [11,14,15]. And has also been reported that the degree of sensory neuropathy in the upper airway correlates with the degree of obstructive sleep disorder [16]. In addition, except for nerve and muscle damage, snoring-induced mechanical vibration might also impair microcirculation [16,17].

Chronic intermittent hypoxia (CIH) is a key feature of the OSA. Evidence suggests that CIH can decrease upper airway stability and results in damage, weakness, and fatigue in respiratory muscles [1]. And based on CIH animal model, Skelly et al. [18] reported that the upper airway muscles were weak in male rats. Veasey and colleagues [19] found that CIH decreased excitability of the hypoglossal motor nerve which innervates dilator muscles of the pharynx.

Building upon the previous evidence, we hypothesized that the changed microenvironment of muscle and nerves in the soft palate might play an important role in the collapse of the upper airway. In the present study, we first develop a CIH rat model to mimic the clinic OSA syndrome. Then we detected the anatomic, neurogenic, and angiogenic changes in the soft palate mucosa and muscles. Finally, the cellular destiny under physiopathological conditions was investigated. Findings from this study will provide more evidence about the underlying pathophysiology of OSA.

Material and Methods

Animal models

All experiments were performed following the Guidelines of the Institutional Animal Use and Care Committee of the Second Military Medical University. Six-week-old male Sprague-Dawley rats weighting about 170 g were randomly assigned to 2 groups (n=10 per group). The rats were housed in polycarbonate cages and given standard rat chow and water.

The rats in the intermittent hypoxia group were kept in the plastic cage equipped with the intermittent hypoxia apparatus for 8 hours per day during the light cycle of 12 hours. CIH was conducted during the 12 hours of the light cycle to coincide with the animal sleep cycle. The CIH rats were given intermittent low oxygen for 28 days with a cycling program for 8 hours daily (9: 00 am to 5: 00 pm). In each cycle, the oxygen concentration inside was reduced from 21% to 8.5% over 4 minutes, followed maintaining for 2 minutes. And then oxygen concentration inside was quickly returned to 21% for 2 minutes followed maintaining for 1 minute. The animals in the control group were placed in the same chamber filled with 21% oxygen.

Histology and immunohistochemistry

Soft palate harvested from rats in both groups were fixed with paraformaldehyde, and later embedded in paraffin. Paraffin sections (4 μm thick) were then stained with hematoxylin and eosin (H & E) to examine the histological change of mucosa and muscles in soft palate. For immunohistochemistry, paraffin sections were de-paraffinized, rehydrated with xylene and ethanol, and antigen was retrieved by incubation in 0.01 M citrate buffer (pH 6.0) at 121°C/100 kpa in a pressure cooker for 3 minutes. Slides were incubated overnight with primary antibodies including anti-annexin V (1: 200, ab14196 Abcam), anti-CD31 (1: 200, ab28364 Abcam), anti-caveolin-1 (1: 100, ab2910 Abcam), and anti-LC3B (1: 200, 3868 R&D) at 4°C, then incubated with secondary antibody conjugated with fluorescent dye at 37°C for 30 minutes. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, St. Louis, MO, USA). Images were acquired with a 50i Nikon fluorescence microscope (Nikon, Melville, NY, USA) and analyzed using Adobe Photoshop CS4 software (San Jose, CA, USA).

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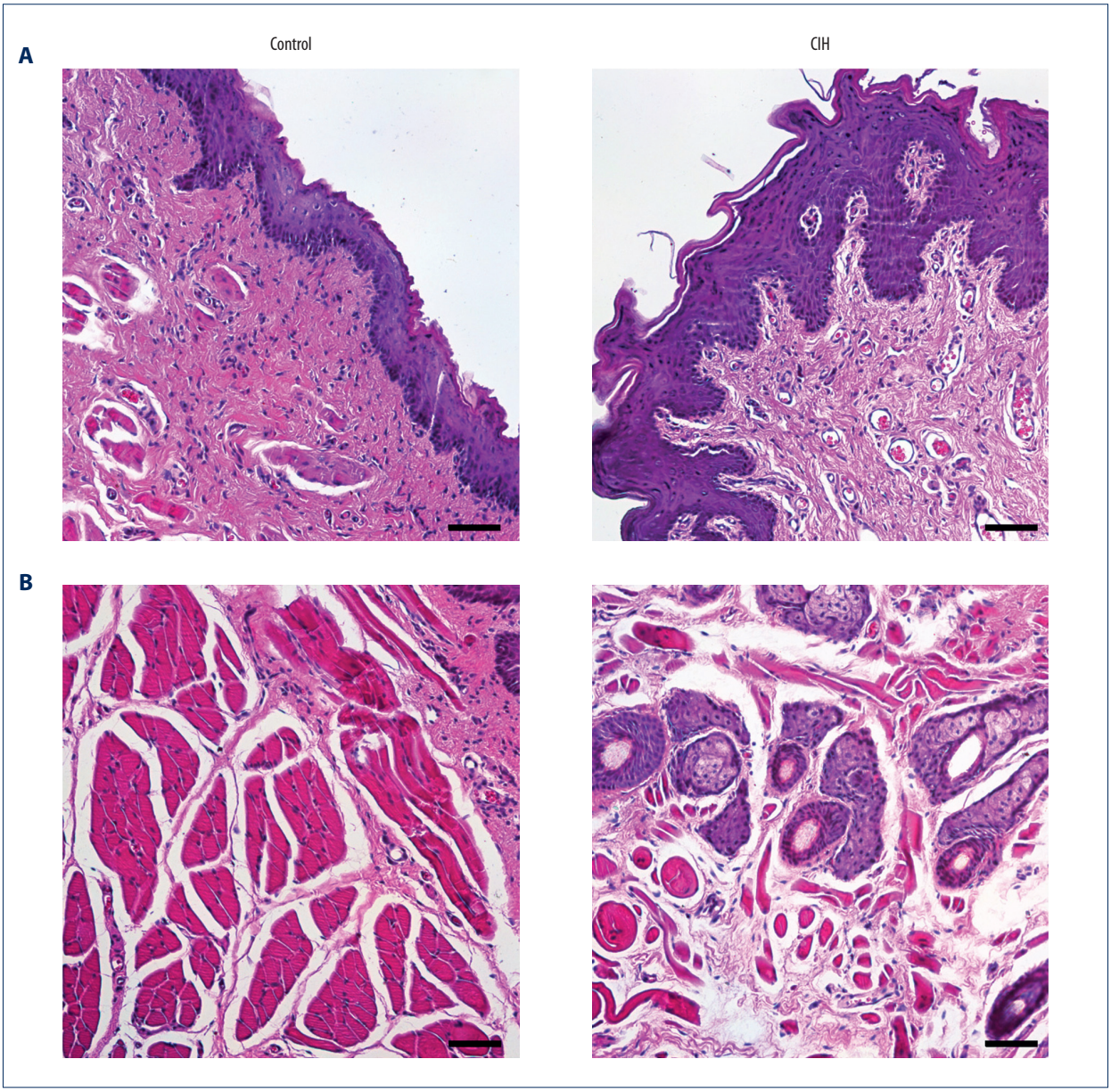

Figure 1. Morphological structure of the soft palate was changed in the CIH exposed rat model. Hematoxylin and eosin staining was performed to identify structure of (**A**) mucosa and (**B**) connective tissue in the soft palate of the control group and CIH group. (**A**) The mucosal layer became thicker when exposed to CIH, and squamous epithelial cells were enlarged and irregularly arranged. (**B**) Glands widely infiltrated into muscles, and oropharyngeal muscles obviously were destroyed in the CIH-induced soft palate. Scale bar is 100 μm.

RNA isolation, RT-PCR and quantitative PCR

Total RNA from rat tissues was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcriptase reactions were performed with SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer's protocol. Quantitative real-time polymerase chain reaction (PCR) was performed with SYBR Green master mix (Applied Biosystems) in 3 repeats of each sample on an ABI-7900 (Applied Biosystems Foster, City, CA, USA). The fold change was calculated by the $2^{-\Delta\Delta CT}$ method [20] using β-actin as the internal control.

Western blot analysis

Western blotting analysis was performed as described previously [21]. Protein samples from soft palate tissue were extracted with Total Protein Extraction Kit (Merck Millipore, Germany) according to the manufacturer's instructions. Proteins were

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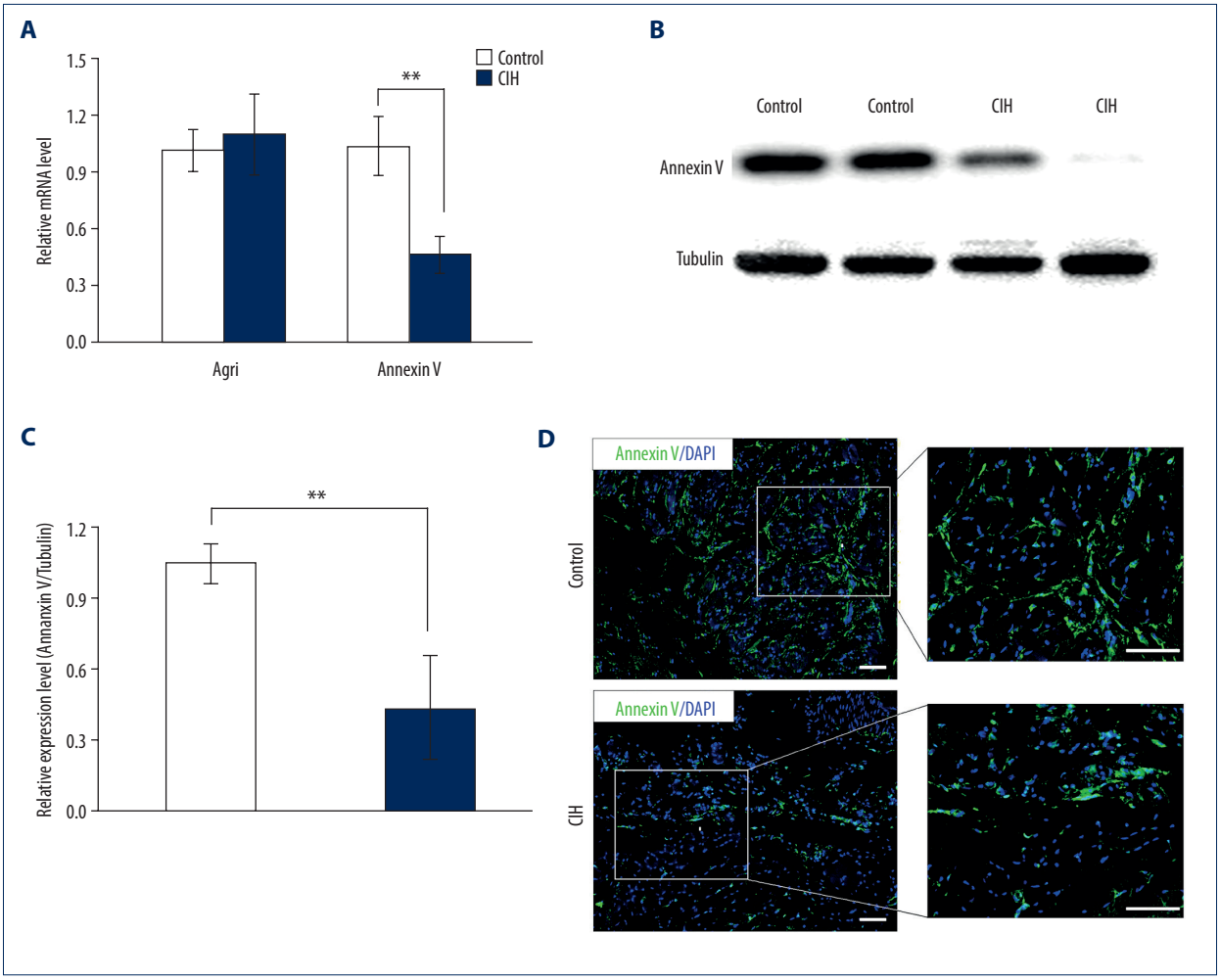

Figure 2. Sensory nerves but not motor nerves were decreased in CIH exposed soft palate. (**A**) qPCR analysis quantified the mRNA levels of agrin and annexin V in the soft palate of the control group and the CIH group. (**B**) Western blot showing expression of annexin V in the control group and the CIH group. (**C**) The bar was quantitation of protein levels. The relative protein expression was calculated based on control group which was considered equal to 1. (**D**) Immunohistochemistry staining of annexin V on the soft palate of the control group and the CIH group. The right panel is the magnification of the white box. All values are expressed as mean±standard deviation. ** Significantly different from the control group, $P<0.01$. Scale bar is 100 μm.

subjected to 10% sodium dodecyl sulfate-polyacrylamide gels before being transferred to polyvinylidene fluoride membranes (Merck Millipore). Membranes were blocked in 5% bovine serum albumin for 1 hour at room temperature. Membranes were then incubated overnight with primary antibodies at 4°C, followed by incubated with horseradish peroxidase (HRP)-conjugated secondary antibody at 37°C for 30 minutes. Protein bands were detected with a chemiluminescent substrate.

Statistical analysis

The data were presented as mean±standard deviation (SD). All experiments were performed at least 3 times. Significant differences were analyzed by the Student's *t*-test using GraphPad Prism 5.0. $P<0.05$ was considered statistically significant.

Results

CIH induced morphological damage of soft palate in a rat model

Histopathological examination of the soft palate tissues from CIH and the control groups showed that the mucosal layer became thicker in rats exposed to CIH for 28 days (Figure 1A). The thickness of the mucosal layer was 125.85±6.34 μm in the CIH group while that was only 57.26±4.67 μm in the control group. Squamous epithelial cells in mucosal layer became enlarged and were irregularly arranged. Moreover, the structure of the connective tissue became loose. In addition, as shown in Figure 1B, CIH induced the significant hyperplasia and hypertrophy of glands in soft palate. The increased glands widely

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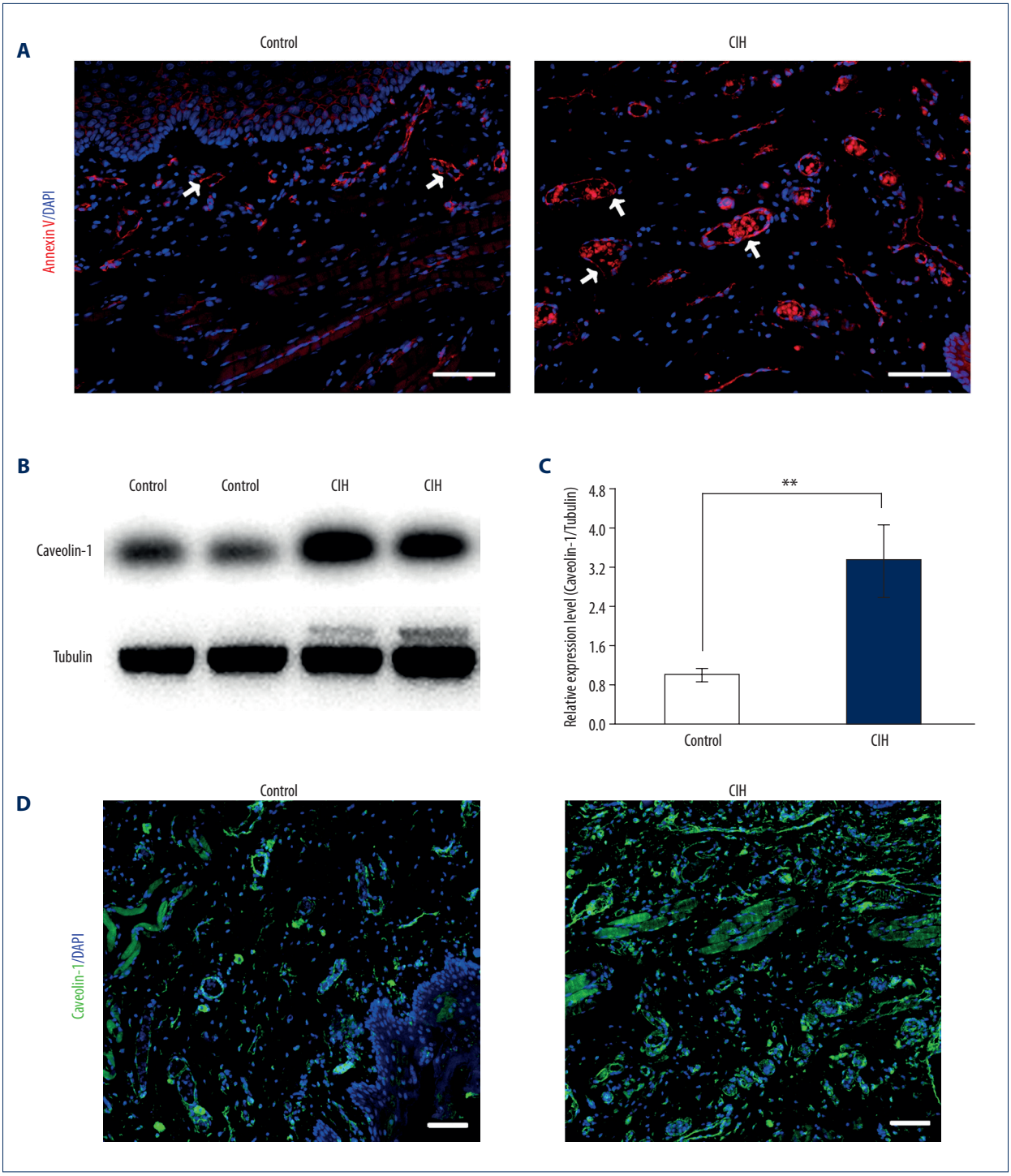

Figure 3. CIH resulted in the dysfunction of vascular endothelial cells. (**A**) Immunohistochemistry staining of CD31 (red) on the soft palate of the control group and the CIH group. The arrow shows vascular vessels in the connective tissues of the soft palate, and CIH exposure resulted in the enlarged vessels. (**B**) Western blotting demonstrates the expression of caveolin-1 in the control group and the CIH group. (**C**) The right bar showing the relative expression level. The relative protein expression was calculated based on the control group which was considered equal to 1. (**D**) Immunohistochemistry staining for caveolin-1 in the rat soft palate exposed CIH group and the control group. All values presented as mean±standard deviation. ** Significantly different from the control group, $P<0.01$. Scale bar is 100 μm.

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infiltrated into the muscles, resulting in the destroy and atrophy of the oropharyngeal muscles. These data suggested that thickening of mucosa might aggravate upper airway stenosis, and atrophy and collapse of oropharyngeal muscles may further affect the expansion of upper airway.

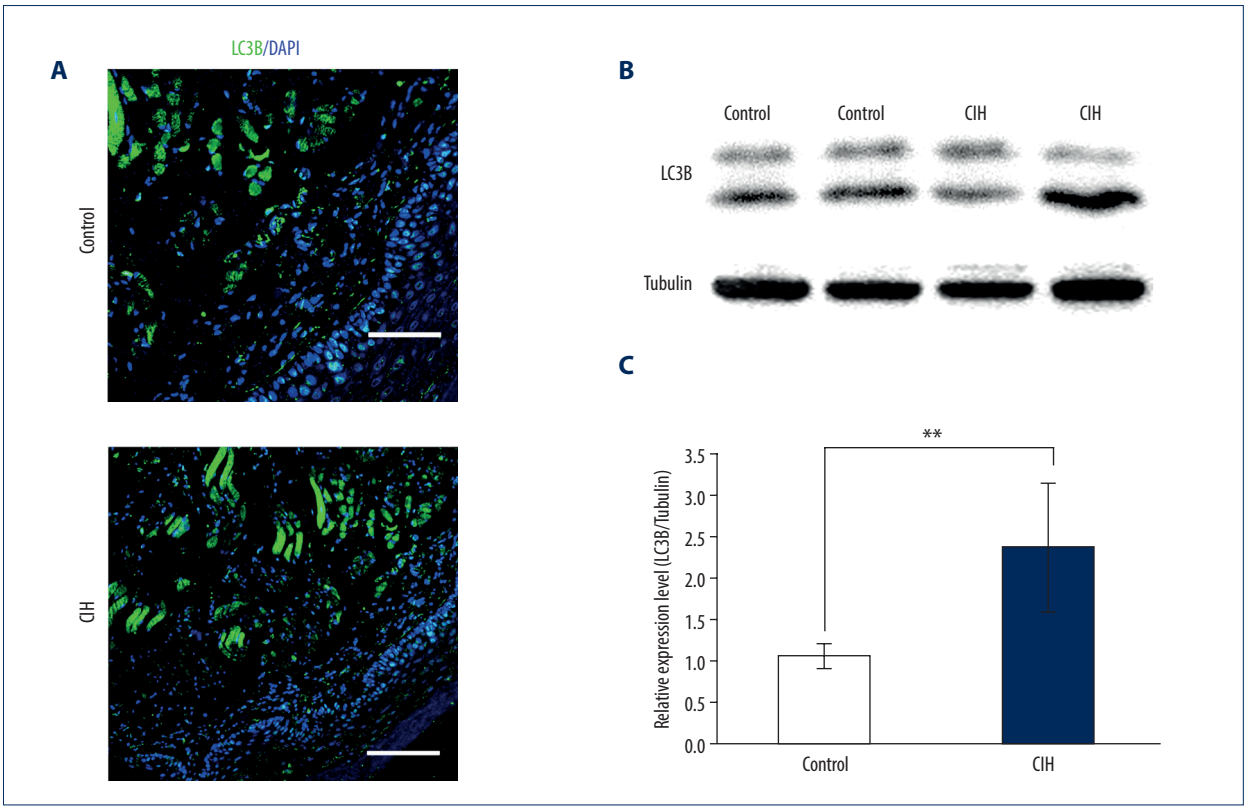
CIH decreased peripheral sensory nerves in soft palate

As oropharyngeal muscles function depending on reflexes nerves, peripheral motor and sensory nerves levels were investigated. qRT-PCR analysis showed that during intermittent hypoxia treatment, the expression of sensory nerves marker annexin V was significantly decreased compared to untreated groups (Figure 2A). But the agrin expression that is the marker of motor nerves was not difference between CIH-induced soft palate and the control groups. Western blot detection from oropharyngeal muscles confirmed that the annexin V level was about 2-fold decrease (Figure 2B, 2C). *In situ* immunohistochemical staining indicated that annexin V was located in the plasma and membrane of the sensory nerves. And the annexin V positive sensory nerves around the muscles were sharply deduced (Figure 2D). These results demonstrated that CIH could reduce the sensory nerve endings and the transmission of stimuli, suggesting its contributing to the collapse of soft palate in the upper airway innervated by the muscles.

CIH resulted in the dysfunction of vascular endothelial cells

Because intermittent hypoxia-induced angiogenesis is a crucial compensatory mechanism for providing oxygen supply to different tissues during hypoxic conditions [22], we hypothesized that long term CIH may enlarge the capillary vascular network. As shown in Figure 3A, the number of CD31 positive cells were not different between the CIH group and the normoxic control group, suggesting the vascular and capillary density was not changed by exposing the rats to intermittent hypoxia. But CIH led to markedly enlarged diameter of vascular vessels, which might be a compensatory response to hypoxia (Figure 3A).

Caveolin-1 is a membrane protein which is essential for cardiac protection in ischemia/reperfusion injury [23,24]. We examined the effect of CIH on caveolin-1 expression and showed that CIH significantly increased caveolin-1 protein level (Figure 3B, 3C). And immunohistochemical results further confirmed that caveolin-1 expression was mostly located in the membrane of vascular endothelial cells (Figure 3D). This indicated that exposing rats to intermittent hypoxia, the vascular vessels became enlarged accompanied with overexpression of caveolin-1, suggesting that endothelial dysfunction was a compensatory response to hypoxia.



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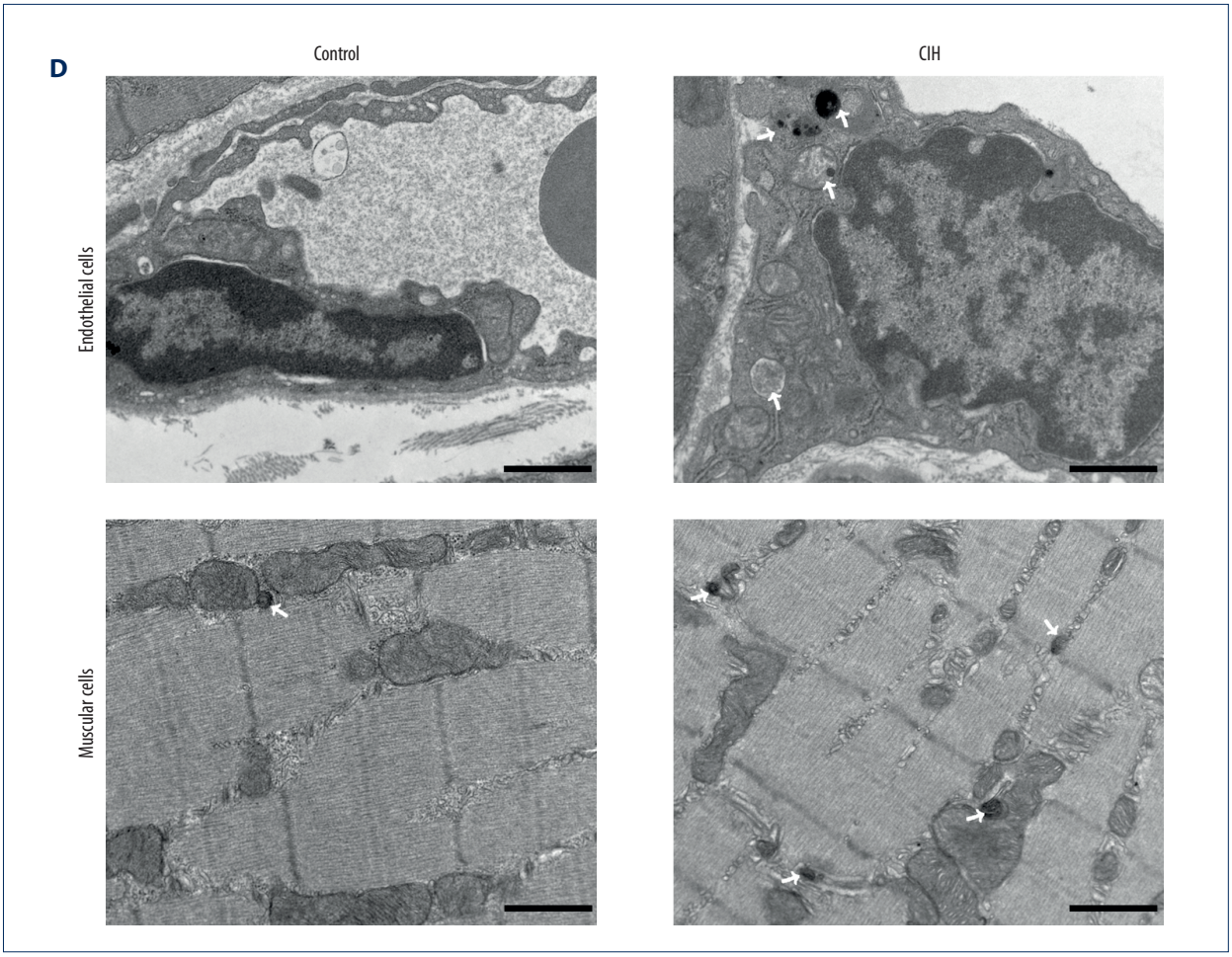

Figure 4. Autophagy was induced to protect CIH-induced dysfunction. (**A**) Immunohistochemistry staining of LC3B on the soft palate of the control group and the CIH group. The LC3B positive cells were located in the mucosa and muscle layers. Scale bar is 100 μm. (**B**) Western blotting of LC3B expression in the control group and the CIH group. (**C**) The bar showing the relative expression level. The relative protein expression was calculated based on the control group which was considered equal to 1. All values are expressed as mean±standard deviation. * $P<0.05$. (**D**) Transmission electron microscopy depicting ultras structures of autophagolysosomes in endothelial and muscular cells of the soft plate exposed to normal or chronic intermittent hypoxia. Autophagic vacuoles are highlighted by arrows. Bar scale 1 μm.

CIH increased autophagy in the soft palate

Under cell damage, autophagy may be protective. To determine whether CIH induced autophagy, immunohistochemistry and western blot were performed to quantify the levels of LC3B. Immunohistochemistry images showed that cells in the mucosa and connective tissue widely expressed LC3B (Figure 4A). The results of western blot analysis showed CIH elevated LC3B expression (Figure 4B, 4C). Transmission electron microscopy examination of soft palate structures further revealed accumulation of autophagolysosomes in vascular endothelial cells and muscular cells following CIH exposure (Figure 4D). The data indicated that autophagy was enhanced by CIH-induced cellular dysfunction.

Discussion

Obstructive sleep apnea (OSA) is characterized by recurrent nocturnal episodes of upper airway narrowing or collapse, which lead to reduced ventilation or apnea. In this study, we successfully established a rat model mimicking CIH process of OSA, in order to explore the pathology and the underlying mechanisms of soft palate contributed to upper airway collapse under chronic hypoxia condition. First, similar to previous reports [12,25], histological studies demonstrated the thicken mucosal layer, muscular atrophy consistent with glands hyperplasia, and loose connective tissues of the soft palate in CIH-induced rat models. Although muscular alteration is considered an adaptive compensation due to snoring of patients with OSA [26], the atrophy of this muscular tissue still failed

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to resist pharyngeal collapse. Palatopharyngeal muscle is important to regulate the upper airway, thus understanding the mechanism of pathological changes will help to alleviate OSA. A possible explanation for these muscular changes exposed to intermittent hypoxia condition is neuronal damage or vascular endothelial cells dysfunction of the soft palate that supports nutrition for muscle motor.

Second, peripheral motor and sensory nerve levels were investigated and found to favor progressive neurol dysfunction along the CIH exposure. We found the sensory nerves were significantly decreased in CIH-induced rat. Sensory inputs from the upper airway play an important role in initiation of various physiological reflexes and in feedback control of motor activities [27]. Clinical testing of the soft palate and pharyngeal mucosa in OSA patients by cold sensory testing showed upper airway sensory impairment [28]. Sensory nerve degeneration in soft palate mucosa and denervation of the palatal muscles have been documented in OSA [29]. These studies demonstrated that peripheral sensory nerve lesions contribute to the pathogenesis of muscles in OSA patients.

In the present study, we found that the vascular vessels became enlarged. Consistent with our data, it has been reported that intermittent hypoxia results in an increased production of vascular endothelial growth factor (VEGF) and vascularization [30]. Previous studies have demonstrated that the neurological function could be recovered by improving angiogenesis through the caveolin-1/VEGF signaling pathway [31]. Our data showed that CIH increased caveolin-1 expression in the soft palate of rats. Caveolin-1 is involved in the angiogenesis of the brain after ischemic injury [32], but our data found that upregulation of caveolin-1 level was relative to endothelial proliferation. It has been reported that increased caveolin-1 expression may promote inflammation and cell apoptosis, thereby, contributing to the development of atherosclerosis [33]. However, it is also possible that caveolin-1 is expressed in response to CIH to protect cells against the CIH insult. As capillaries are produced to provide oxygen and nutrition to muscles, we suggest that the enlarged vascular vessels and overexpression of caveolin-1 might be a compensatory response to intermittent hypoxia.

Taken together, our work demonstrated that CIH efficiently induced pathological changes of the soft palate with thicken mucosa, gland hyperplasia, and muscular atrophy. Mechanically, the explanation for atrophy of pharyngeal muscles was decreased sensory nerves and vascular endothelial dysfunction with caveolin-1 overexpression during intermittent hypoxia. Moreover, we suggest that the autophagy that occurs in CIH treated soft palate might be one of the compensatory mechanisms on intermittent hypoxia and neuromuscular and vascular endothelial injury. Our current knowledge will provide a theoretical foundation for further study of the strategy for alleviating soft palate lesions in patients with OSA.

Conclusions

Sensory nerves and endothelial dysfunction contributed to the morphological damage of the soft palate under intermittent hypoxia. Autophagy as a compensatory mechanism that protects against CIH-induced injury. We revealed the mechanisms underlying CIH-induced soft palate damage, which might provide support for new strategies of OSA syndrome treatment.

Acknowledgment

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Conflict of interests

None.

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