Pre-Clinical Research Report

Journal of INTERNATIONAL MEDICAL RESEARCH

Journal of International Medical Research
48(5) 1–11

DOI: 10.1177/0300060520926065
journals.sagepub.com/home/imr
SAGE

Ghrelin suppresses migration of macrophages via inhibition of ROCK2 under chronic intermittent hypoxia

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Abstract

Objectives: Migration of macrophages and atherosclerosis result in various diseases, including coronary heart disease. This study aimed to clarify the roles that ghrelin and Rho-associated coiled-coil-containing protein kinase 2 (ROCK2) play in migration of macrophages under chronic intermittent hypoxia (CIH).

Methods: A rat model of CIH was constructed and changes in ghrelin and ROCK2 protein expression were measured by western blot assay. The migratory ability of macrophages was determined by the transwell assay. Hematoxylin and eosin staining was applied to detect the changes in intima-media thickness.

Results: We found that CIH enhanced migration of macrophages, and this effect was attenuated by exogenous ghrelin. Additionally, the facilitative effect of CIH on migration of macrophages was strengthened or decreased by upregulation or downregulation of ROCK2, respectively. This phenomenon indicated that ROCK2 was involved in CIH-induced migration in macrophages. Furthermore, western blot and transwell assays showed that ghrelin inhibited CIH-induced migration via ROCK2 suppression in macrophages.

Conclusions: In summary, the present study shows that ghrelin inhibits CIH-induced migration via ROCK2 suppression in macrophages. Our research may help lead to identifying a new molecular mechanism for targeted therapy of atherosclerosis and its associated coronary artery diseases under intermittent hypoxia.

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Keywords
Coronary heart disease, atherosclerosis, chronic intermittent hypoxia, ghrelin, ROCK2, macrophage migration, obstructive sleep apnea

Date received: 27 November 2019; accepted: 22 April 2020

Introduction

Coronary heart disease (CHD), which is commonly caused by atherosclerosis (AS), is the main reason for heart-related mortality in older people.[1] CHD is the leading cause of death in this population, and more than 27% of total deaths in men and 32% in women are attributed to CHD worldwide.[2] Chronic intermittent hypoxia (CIH) in patients with obstructive sleep apnea (OSA) is equivalent to ischemia–reperfusion injury, which accelerates formation of AS lesions through oxidative stress.[3–6] The prevalence of CHD in patients with OSA is approximately 20% to 30%, and the 5-year mortality of patients with CHD combined with OSA is 62%.[7,8] However, the detailed mechanism of CHD induced by OSA remains unclear.

Accumulation of macrophages is a critical step during development of chronic inflammation, which initiates progression of many devastating diseases, including AS. Macrophages migrate to the endothelial cell space, and absorb cholesterol and convert it into lipid streaks or plaques, which are an important part of the chronic inflammatory response of the AS vascular wall.[9,10] Aziz et al. reported that upregulation of CD11d promoted retention of macrophages in vascular lesions and development of AS.[11] Therefore, inhibition of migration of macrophages is important for preventing and treating AS.

Ghrelin is an endogenous ligand of growth hormone secretion-promoting receptor (GHS-R), which has a protective effect on vascular endothelium.[12,13] Proghrelin, a hormone precursor, produces two important brain–intestinal hormones, ghrelin and obestatin, through different splicing and modifications. Recent studies have shown that mRNA expression encoding ghrelin/obestatin and its receptor is present in the hypothalamus, myocardium, blood vessels, macrophages, and other sites. These sites are antagonistic to each other and jointly involved in regulating dietary intake, blood lipids, and arterial inflammation.[14–16]

The present study aimed to determine the relationships between protein expression of ghrelin and Rho-associated coiled-coil-containing protein kinase 2 (ROCK2) and migration of macrophages or AS caused by CIH in a rat model. The findings of our research might provide new directions for protection and treatment of AS.

Materials and methods

Rat model

Twenty-four male Wistar rats (body weight: 140–160 g) were purchased from Liaoning Changsheng Biotechnology Co., Ltd. (License No.: SCXK [Liaoning] 2015-0001; Benxi, China). The rats were randomly divided into three groups (eight in each group) as follows: control group (normal oxygen inhalation), CIH group (CIH stimulation with hypoxia chambers; BioSpherix, Lacona, NY, USA), and ghrelin group (CIH stimulation and intraperitoneal injection of 100 ug/kg/day of ghrelin). All rats were fed with a high-fat diet. In CIH stimulation, the oxygen concentration was adjusted according to the principle of

nitrogen dilution. Each cycle lasted for 90 s, in which the concentration of 5% oxygen was for 45 s and the concentration of 21% oxygen was for 45 s. This type of CIH intervention lasted for 8 hours a day for 35 days. A total of 35 days later, all rats were sacrificed and the carotid artery was harvested. All animal experiments conformed with the Regulation of Animal Care Management of the Ministry of Public Health, People's Republic of China. All procedures were performed under the permission of the Institute Research Medical Ethics Committee of the Central Hospital Affiliated to Shenyang Medical College.

Cell culture and intermittent hypoxic stimulation

The macrophage cell line RAW264.7 was provided by the Tissue Engineering Laboratory of China Medical University (Shenyang, China), and was cultured in RPMI-1640 medium (Gibco, El Paso, TX, USA) supplemented with 10% fetal bovine serum (Gibco), 100 U/mL penicillin G (Gibco), 100 mg/L streptomycin (Gibco), and 1% glutamine (Gibco). All cells were incubated in an incubator at 37°C with a humidified atmosphere containing 5% carbon dioxide. For intermittent hypoxic stimulation, RAW264.7 cells were seeded on a self-made cell culture chamber and exposed to intermittent hypoxia as previously reported.[17]

Migration of macrophages to the vascular endothelium is an important reason for formation of lipid plaques and is closely related to AS.[12] ROCK2 is a protein related to the cytoskeleton and is involved in a variety of cell types in migration, including macrophages.[19] To further examine the role of ghrelin and ROCK2 in CIH-mediated AS, we applied a macrophage CIH model at the cellular level. Macrophages were cultured under the condition of CIH, while 10^{-7} mmol/L of ghrelin and 10^{-7} mmol/L of obestatin were supplemented to CIH macrophages individually.

Western blot analysis

Total protein from tissue samples and RAW264.7 cells were obtained by using RIPA lysis buffer (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Samples were fractionated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were transferred onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). Membranes were blocked for 1 hour and were then incubated with ghrelin-, ROCK2-, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific antibodies at 4°C overnight as follows: anti-ghrelin antibody (1: 250; ab129383; Abcam, Cambridge, MA, USA), anti-ROCK2 antibody (1 μg/mL; ab71598; Abcam), and anti-GAPDH antibody (1/10000; a181602; Abcam). The next day, membranes were incubated with secondary antibodies (Abcam; dilution of 1: 2000) at 25°C for 1 hour. Protein bands were detected on X-ray film using an enhanced chemiluminescence detection system (ECL Western Blotting Substrate Kit; Abcam).

Hematoxylin and eosin staining

Carotid arteries were fixed with 4% formaldehyde. Paraffin sections were dewaxed by xylene I and II for 5 minutes each, placed in 100%, 95%, 90%, 80%, and 70% alcohol solution for 3 to 5 minutes, and then placed in hematoxylin stain for 10 to 15 minutes after distilled water for 3 minutes. The sections were immersed in 1% hydrochloric acid in ethanol until the sections turned red. The sections were then dehydrated in 70% and 80% ethanol for 3 to 5 minutes, counterstained in 1% Yihong for 10 s, and washed quickly with distilled water. The sections were placed in 95%

ethanol (30 s to 1 minute) to remove the excess red stain after 70%, 80%, and 90% washing in alcohol solution. The sections were then placed in absolute ethanol for 3 to 5 minutes and xylene for 3 to 5 minutes for two times. The sections were photographed with an optical microscope after being sealed by neutral gum. The intima-media thickness (IMT) was measured.

Transwell chamber migration assay

The transwell chamber migration assay was performed as previously described.[18,19] Macrophages were seeded on upper chambers (BD Biosciences, San Jose, CA, USA). Culture medium with and without 10% fetal bovine serum was supplemented into the lower and upper wells, respectively, and incubated for 24 hours under intermittent hypoxic exposure. The next day, non-migrated cells were wiped out. The filters were then fixed in 90% ethanol and followed by crystal violet staining. Five random fields were counted per chamber by using an inverted microscope (Olympus, Tokyo, Japan).

Statistical analysis

All experiments were repeated in triplicate, and all data from three independent experiments are expressed as mean ± standard deviation. GraphPad Prism V5.0 software (GraphPad Software, Inc., La Jolla, CA, USA) and SPSS version 21.0 (IBM Corp., Armonk, NY, USA) statistical software were used for statistical analysis. The correlation between ghrelin and ROCK2 expression was analyzed by using two-tailed Pearson's correlation analysis. Differences between two groups were analyzed by the Student's t-test or by one-way ANOVA. Differences were considered as significant if P was <0.05

Results

Ghrelin expression is decreased and ROCK2 expression is increased in the AS model under CIH

We first measured vascular endothelial changes in the rat models. IMT was significantly higher in the CIH group than in the control and ghrelin groups (both $P < 0.01$) (Figure 1a). However, the effect of CIH was remarkably weakened by intraperitoneal injection of ghrelin ($P < 0.01$, ghrelin group vs CIH group). Additionally, we determined ghrelin and ROCK2 protein expression by western blotting. Exposure to CIH significantly downregulated ghrelin protein expression ($P < 0.01$ vs control group), and this was alleviated by ghrelin injection ($P < 0.01$, ghrelin group vs CIH group) (Figure 1b). Furthermore, exposure to CIH significantly upregulated ROCK2 protein expression ($P < 0.01$ vs control group), but this upregulation was significantly reduced by ghrelin injection ($P < 0.01$, ghrelin group vs CIH group) (Figure 1c). We analyzed the relationship between ghrelin and ROCK2 expression. A significant inverse correlation was found between ghrelin and ROCK2 expression as shown by Spearman's correlation analysis ($r = -0.7518$, $P = 0.0315$) (Figure 1d).

Ghrelin/ROCK2 axis is closely involved in CIH-induced migration in macrophages

The pressure of oxygen and carbon dioxide, and pH values in the CIH and control groups are shown in Table 1. Ghrelin expression in macrophages in each group was detected by western blot assay. Exposure to CIH significantly suppressed ghrelin protein expression in macrophages ($P < 0.01$ vs control group), and this suppressive effect was reversed by injection of ghrelin ($P < 0.01$, ghrelin group vs CIH group) (Figure 2a). Additionally, ghrelin

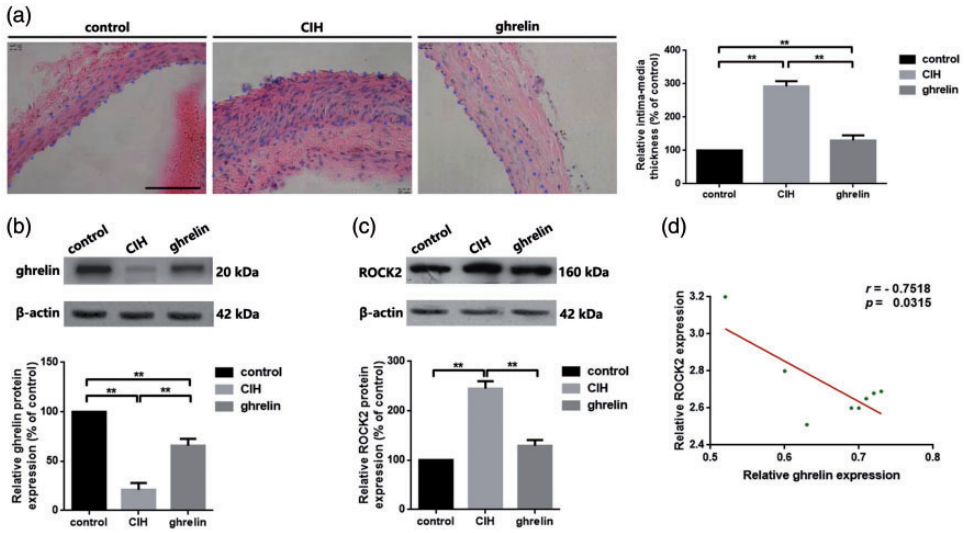

Figure 1. Decreased ghrelin, but increased ROCK2, protein expression in the atherosclerosis model under CIH. (a) Representative photographs of vascular endothelial changes in each group as shown by hematoxylin and eosin staining. Scale bar, 50 μm; magnification, ×40. Intima-media thickness was significantly higher in the CIH group compared with the control group. (b, c) Ghrelin (b) and ROCK2 (c) protein expression in each group as measured by western blot assays. (d) Ghrelin expression was significantly negatively correlated with ROCK2 expression (Spearman's correlation analysis). $^{**}P<0.01$. Data were normalized to the control group and are shown as mean ± standard deviation from three independent experiments. ROCK2: Rho-associated coiled-coil-containing protein kinase 2; CIH: chronic intermittent hypoxia.

Table 1. PO_2, PCO_2, and pH values in intermittent hypoxic culture of macrophages and controls.

	After hypoxia			After reoxygenation		
	PO_2 (mmHg)	PCO_2 (mmHg)	pH	PO_2 (mmHg)	PCO_2 (mmHg)	pH
CIH	50.22 ± 3.78	38.99 ± 1.82	7.40 ± 0.01	79.87 ± 6.33	38.33 ± 0.46	7.39 ± 0.01
Control	79.37 ± 3.33	38.39 ± 0.82	7.35 ± 0.01	–	–	–

Data are mean ± standard deviation. CIH: chronic intermittent hypoxia.

protein expression was inhibited by the selective ghrelin blocker obestatin. We also measured the changes in ROCK2 protein expression in macrophages in each group. CIH significantly promoted ROCK2 protein expression in macrophages ($P < 0.01$ vs control group). This facilitative effect was significantly attenuated by ghrelin injection ($P < 0.01$, ghrelin group vs CIH group), while inhibition of ghrelin promoted ROCK2 protein expression ($P < 0.01$, obestatin group vs ghrelin and CIH groups) (Figure 2b).

We used the transwell assay to evaluate the role of ghrelin in migration of macrophages. In the CIH group, the migratory ability of macrophages was significantly enhanced compared with the control group ($P < 0.001$, Figure 2c). However, this facilitative effect was remarkably weakened by injection of ghrelin ($P < 0.001$, ghrelin group vs CIH group).

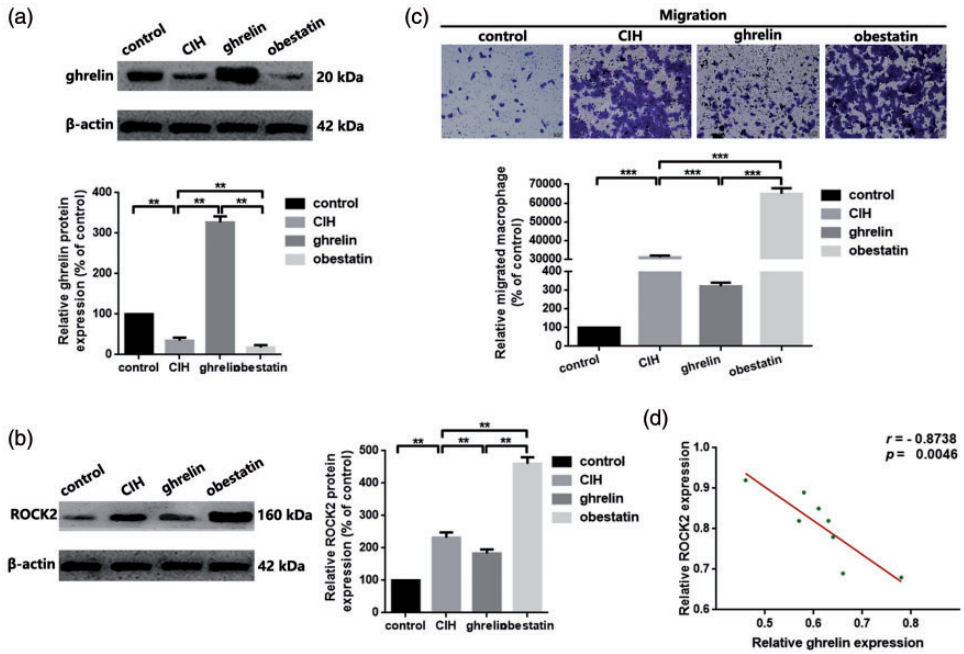

Figure 2. The ghrelin/ROCK2 axis is closely involved in CIH-induced migration in macrophages. (a) Ghrelin protein expression was measured by western blotting. (b) ROCK2 protein expression was evaluated by western blotting. (c) The changes in migratory ability of macrophages were determined by a transwell assay. Scale bar, 50 μm; magnification, ×200. (d) Ghrelin expression was negatively correlated with ROCK2 expression (Spearman's correlation analysis). $^{**}P < 0.01$, $^{***}P < 0.001$. Data were normalized to the control group and are shown as mean ± standard deviation from three independent experiments. ROCK2: Rho-associated coiled-coil-containing protein kinase 2; CIH: chronic intermittent hypoxia.

Additionally, the migratory ability of macrophages was restored by a decrease in ghrelin ($P < 0.001$, ghrelin group vs the obestatin group). These findings strongly indicated that ghrelin was a major regulator in CIH-induced migration in macrophages. Finally, we observed a significant negative correlation between ghrelin and ROCK2 expression at the cellular level ($r = -0.8738$, $P = 0.0046$) (Figure 2d).

ROCK2 is involved in CIH-induced migration in macrophages

As mentioned above, we found that ghrelin and ROCK2 were involved in CIH-induced migration in macrophages. We also found that up- and downregulation of ghrelin negatively affected ROCK2 expression. Therefore, we speculated that ROCK2 was the downstream target of ghrelin. We applied arachidonic acid (a specific ROCK2 activator) and fasudil (a selective ROCK2 blocker) to increase and decrease ROCK2 expression in CIH-treated macrophages, respectively. Western blot analysis (Figure 3a) showed that ROCK2 protein expression was significantly up- and downregulated by arachidonic acid and fasudil, respectively (both $P < 0.01$, vs the CIH group). We further examined the changes in migratory ability of macrophages. The transwell assay showed significantly higher and lower rates of migration of macrophages with addition of arachidonic acid and fasudil, respectively, compared with

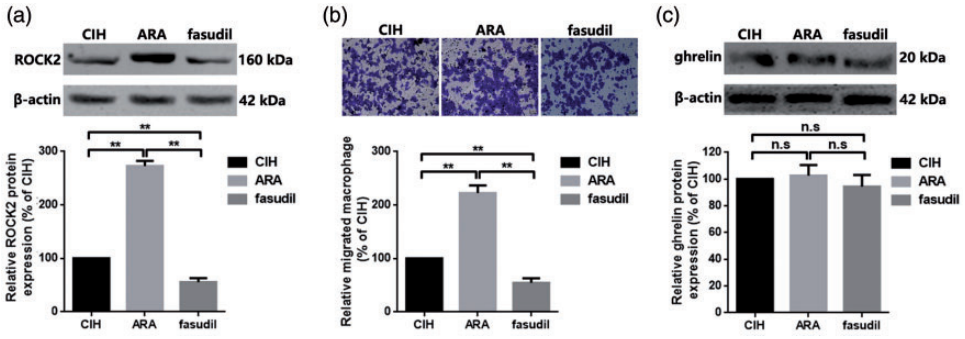

Figure 3. ROCK2 is involved in CIH-induced migration in macrophages. (a) ROCK2 protein expression was assessed by western blotting. (b) A transwell assay was performed to evaluate the changes in migratory ability of macrophages after up- and downregulation of ROCK2. Scale bar, 50 μm; magnification, ×200. (c) Ghrelin protein expression was assessed by western blotting. **$P < 0.01$. Data were normalized to the CIH group and are shown as mean ± standard deviation from three independent experiments. ROCK2: Rho-associated coiled-coil-containing protein kinase 2; CIH: chronic intermittent hypoxia; ARA: arachidonic acid; n.s: not significant.

CIH-induced migration (both $P < 0.01$, Figure 3b). Finally, we measured ghrelin protein expression after addition of arachidonic acid and fasudil. We found that up- and downregulation of ROCK2 had no significant effect on ghrelin protein expression (Figure 3c). These findings suggested that ROCK2 was a downstream target of ghrelin and mediated migration of macrophages after CIH.

Ghrelin inhibits CIH-induced migration via ROCK2 suppression in macrophages

We showed above that the ghrelin/ROCK2 axis was involved in CIH-induced migration in macrophages. We then examined whether the suppressive role of ghrelin on migration of macrophages was achieved through ROCK2. We first applied ghrelin and obestatin to increase and decrease ghrelin expression in macrophages. Arachidonic acid and fasudil were then added to macrophages and they up- and downregulated ROCK2 expression, respectively. Western blotting showed that up- and downregulation of ROCK2 did not affect ghrelin protein expression (Figure 4a and 4d). We further use the transwell assay to evaluate the changes in migratory ability of macrophages. We found that arachidonic acid significantly enhanced migration of macrophages and fasudil suppressed migration of macrophages (all $P < 0.01$, Figure 4b and 4e). Finally, western blotting analysis showed ROCK2 protein expression in each group (Figure 4c and 4f). In brief, our findings indicated that ROCK2 was a major downstream regulator of ghrelin-mediated suppression of migration under CIH.

Discussion

CIH is a unique pathological mechanism of OSA, and the experimental animal model of CIH has been widely used in studying OSA.[20] Although the pathogenesis of occurrence and development of AS induced by OSA has not been fully determined, involvement of OSA as an independent risk factor in development and progress of diseases, such as CHD or AS, has been studied extensively.[21] In this study, we mainly focused on the mechanism of AS induced by CIH and regulation of ghrelin. In our study, we found that CIH exposure

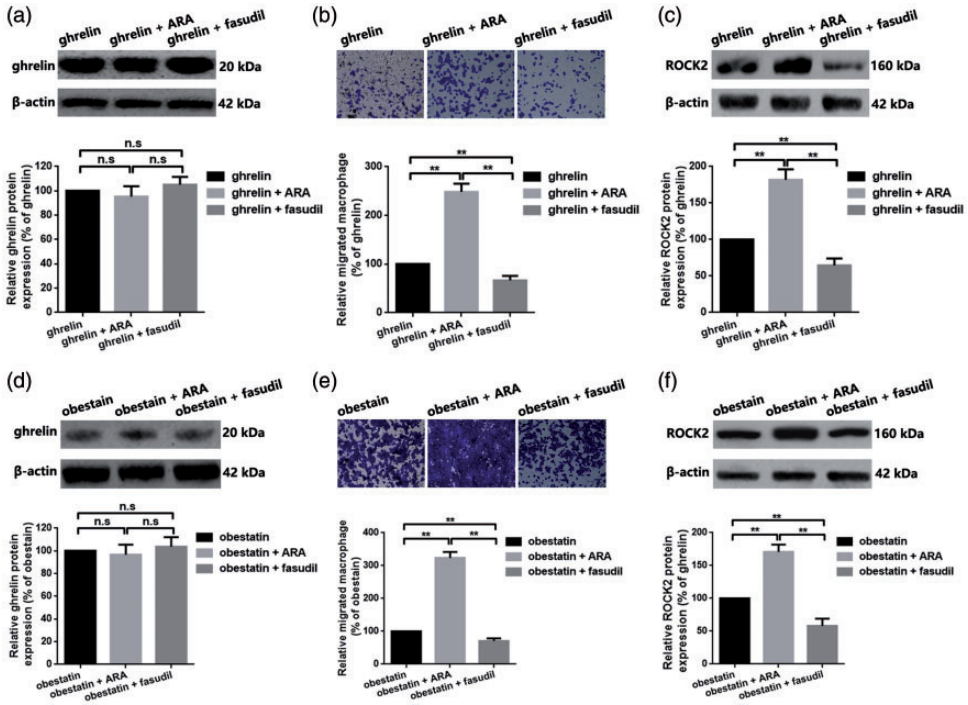

Figure 4. Ghrelin inhibits CIH-induced migration via ROCK2 suppression in macrophages. (a and d) Ghrelin protein expression was assessed by western blotting. (b and e) A transwell assay was performed to evaluate the changes in migratory ability of macrophages after up- and downregulation of ROCK2. Scale bar, 50 μm; magnification, ×200. (c and f) ROCK2 protein expression was assessed by western blotting. $^{**}P < 0.01$. Data were normalized to the CIH group and are shown as mean ± standard deviation from three independent experiments. ROCK2: Rho-associated coiled-coil-containing protein kinase 2; CIH: chronic intermittent hypoxia; ARA: arachidonic acid; n.s: not significant.

increased IMT, and this suggested that CIH promoted progression of AS. We also showed that ghrelin expression was decreased, but ROCK2 protein expression was increased, in the AS model.

Ghrelin was isolated and purified from gastric mucosal cells of rats by Kojima in 1999.[22] Increasing evidence has shown that ghrelin has a wide range of physiological functions in the body and is closely related to the pathophysiological processes of various diseases, such as obesity, cardiovascular disease, and diabetes.[23–26] Ghrelin not only participates in appetite regulation of the body, but also has a variety of cardiovascular protective effects. These effects include increased myocardial contractility, vasodilation, protection of endothelial cells, ischemia–reperfusion injury and heart failure after myocardial infarction, regulation of immunity and the inflammatory response, and improvement of myocardial energy metabolism.[27,28] In the present study, we found that CIH suppressed ghrelin expression at the cellular level. We also found that upregulation of ghrelin alleviated the facilitative effect of CIH on migration of macrophages.

The current study focused on the relationship between ghrelin and ROCK2 expression. ROCK2 belongs to the Rho kinase family, and is one of the most

characteristic members of the Ras protein superfamily (Rho). ROCK2 is a type of serine/threonine kinase, and it is expressed in the heart and blood vessels.[29,30] ROCK2 plays important roles in regulating a variety of biological behaviors via promoting stress fiber formation and refactoring the cytoskeleton, which participates in cell shrinkage, migration, and morphological changes.[31] In this study, we examined the relationships between protein expression of ghrelin and ROCK2 and migration of macrophages or AS caused by intermittent hypoxia in a rat model and in macrophages. We found that ghrelin protein expression was decreased and ROCK2 protein expression was increased. Additionally, there was a negative correlation between ghrelin and ROCK2 expression during intermittent hypoxia in the AS rat model and in macrophages. Furthermore, ROCK2 was the downstream target of ghrelin, and ghrelin was able to negatively regulate ROCK2 expression and slow down the AS process caused by migration of macrophages under CIH. These findings will hopefully provide new directions for the protection and treatment of AS.

Arachidonic acid activates ROCK directly without Rho.[32,33] Fasudil is a specific antagonist of ROCK.[34,35] Upregulation of ghrelin can reduce ROCK2 expression and prevent progress of AS in rats under CIH. Furthermore, migration of macrophages is the underlying cause of vascular plaque formation or AS. In this study, we observed that migration of macrophages was increased under the CIH environment and upregulating ghrelin inhibited migration of macrophages induced by CIH. Our study indicates that CIH may induce migration of macrophages to participate in the AS process through the ghrelin/ROCK2 inflammatory signaling pathway, and that ghrelin is a protective factor for AS induced by CIH or vascular endothelial inflammation.

In this study, we found that ROCK2 protein expression was increased in AS model rats and it induced the migratory ability of macrophages under CIH. Additionally, upregulation of ghrelin inhibited the progress of AS in OSA model rats, migration of macrophages induced by CIH, and ROCK2 expression in rats or macrophages. ROCK2 was the target of ghrelin, but upregulation of ROCK2 levels could not change ghrelin expression in macrophages under the CIH condition. Our study showed that application of ROCK2 worsened the effect of migration of macrophages compared with the effect of ghrelin. This finding indicated that there was an opposite effect of ROCK2 and ghrelin during regulation of progress of AS or arterial inflammation induced by CIH. Additionally, ROCK2 was downstream of ghrelin in the process of AS caused by migration of macrophages. Through acquisition and deletion function experiments of macrophages, we were able to clarify the regulatory characteristics of the ghrelin/ROCK2 signaling pathway in intervening in migration of macrophages under CIH exposure. In conclusion, our study showed that ghrelin delayed the process of AS caused by migration of macrophages in CIH because of negative regulation of ROCK2.

AS is a complex biological process involving multiple factors, and the inflammatory factor ghrelin and its downstream site ROCK are only a small branch of a complicated network. Our study findings may provide a new direction for intervention in AS.

Authors' contributions

Hong Chen wrote the first draft of the manuscript. Jianfeng Du and Siying Zhang acquired the data, and analyzed and interpreted the data. Hao Tong acquired the data and performed randomization and monitored quality control of data acquisition. Man Zhang designed the

study, secured funding, and interpreted the data of the manuscript. All listed authors meet authorship criteria and no others meeting the criteria have been omitted.

Availability of data and material

The datasets from the current study are available from the corresponding author on reasonable request.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

Funding

This work was supported by the Technological Innovation Fund of Shenyang Technology Division (Nos. 17-230-9-32 and 17-230-9-70) and the Natural Science Foundation of Liaoning Province of China (Nos. 201705040869 and 201705040881).

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