Acta Physiologica Sinica, June 25, 2013, 65(3): 269–275
http://www.actaps.com.cn

Research Paper

Opioid receptors mediate enhancement of ACh-induced aorta relaxation by chronic intermittent hypobaric hypoxia

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Abstract: The present study was designed to investigate the role of opioid receptors in the vasorelaxation effect of chronic intermittent hypobaric hypoxia (CIHH) in thoracic aorta rings and the underlying mechanism in rats. Adult male Sprague-Dawley (SD) rats were randomly divided into 2 groups: CIHH treatment group and control group. The rats in CIHH group were exposed to hypoxia in a hypobaric chamber (simulated 5 000 m altitude) for 28 days, 6 h per day. The rats in control group were kept in the same environment as CIHH rats except no hypoxia exposure. The relaxation of thoracic aorta rings was recorded by organ bath perfusion technique, and expression of opioid receptors was measured by Western blot. Results are shown as follows. (1) The acetylcholine (ACh)-induced endothelium-dependent relaxation of thoracic aorta in CIHH rats was increased obviously in a concentration-dependent manner compared with that in control rats ($P < 0.05$). (2) This enhancement of ACh-induced relaxation in CIHH rats was abolished by naloxone, a non-specific opioid receptor blocker ($P < 0.05$). (3) The expressions of δ, μ and κ opioid receptors in thoracic aorta of CIHH rats were up-regulated compared with those in control rats ($P < 0.05$). (4) The enhancement of CIHH on relaxation of thoracic aorta was reversed by glibenclamide, an ATP-sensitive potassium channel (K_{ATP}) blocker ($P < 0.05$). The results suggest that opioid receptors are involved in CIHH-enhanced ACh-induced vasorelaxation of thoracic aorta through K_{ATP} channel pathways.

Key words: chronic intermittent hypobaric hypoxia; opioid receptor; vasorelaxation; ATP-sensitive potassium channel; rats

阿片受体介导慢性间歇性低压低氧对大鼠主动脉乙酰胆碱诱导舒张的增强效应

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摘 要：本研究旨在探讨阿片受体在慢性间歇性低压低氧(chronic intermittent hypobaric hypoxia, CIHH)增强大鼠胸主动脉舒张中的作用及其机制。雄性成年Sprague-Dawley (SD)大鼠随机分为2组：CIHH组和对照组。CIHH组大鼠置于低压氧舱，接受28 d模拟5 000 m高原，每天6 h的低压低氧处理。对照组大鼠不给予CIHH处理。常规制备胸主动脉环，通过离体动脉环灌流和描记方法记录动脉的舒张活动，Western blot法检测主动脉δ、μ及κ阿片受体表达。结果显示：(1) 与对照组相比，CIHH可浓度依赖性地增强乙酰胆碱诱发的大鼠胸主动脉环舒张($P < 0.05$)；(2) CIHH对胸主动脉环舒张的增强效应可被阿片受体非

Received 2012-09-01 Accepted 2012-11-18

This work was supported by the National Basic Research Development Program of China (No. 2012CB518200), the National Natural Science Foundation of China (No. 31071002), the Natural Science Foundation of Hebei Province, China (No. C2012206001), the Science and Technology Program of Hebei Province, China (No. 12276104D40, 11246125D), and the Medical Research Key Project of Health Bureau of Hebei Province, China (No. 20090319).

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特异性阻断剂纳洛酮所阻断；(3) CIHH可使胸主动脉δ、μ及κ阿片受体表达上调；(4) CIHH对胸主动脉环舒张的增强效应，可被ATP敏感钾通道(ATP-sensitive potassium channel, K_{ATP})阻断剂格列苯脲所阻断。结果提示，CIHH处理可增强大鼠胸主动脉的舒张，此作用可能通过激活阿片受体和开放K_{ATP}通道所实现。

关键词：间歇性低氧；阿片受体；血管舒张；ATP敏感钾通道；大鼠
中图分类号：R331.3

There is accumulating evidence that chronic intermittent hypobaric hypoxia (CIHH) confers a cardioprotective effect against ischemia/reperfusion (I/R) or hypoxia/reoxygenation (H/R) injury, such as promoting the recovery of cardiac function from I/R or H/R, antagonizing I/R-induced arrhythmia, and diminishing myocardial infarct area[1,2]. Epidemiology investigation reported that incidence rate of hypertension in high altitude area is lower than that in low land, which suggests the adaptation to altitude hypoxia has depression effect [3,4]. Furthermore, the CIHH has been proved to prevent hypertension and impairment of endothelium-dependent relaxation in spontaneously hypertensive rat (SHR) [5,6]. Recently our preliminary study showed that CIHH could reduce artery blood pressure in renal vascular hypertension rats (unpublished data), but the mechanism is not clear. Our previous study showed that vasocontraction of thoracic aorta rings induced by ANGII in thoracic aorta rings was weakened in CIHH treated rat, which was related to the opening of ATP-sensitive potassium channel (K_{ATP}) and the increased production of NO in aorta [7]. If the relaxation of CIHH on blood vessel *in vitro* was confirmed, it would further verify the reduction of blood pressure by CIHH. So we can reason that CIHH will increase relaxation of blood vessel.

It is well known that opioid receptors are present in cardiac cell membrane and vascular wall with κ-opioid receptor dominated, and play important roles in the regulation of vascular smooth muscle activity and cardiac function [8–10]. It is reported that stimulation of opioid receptors contributes to aortic artery dilation through activation of K_{ATP} channel in the rats [8], but it is not clear whether opioid receptor plays a role in the effect of CIHH on blood vessel, and whether K_{ATP} channel pathways are involved. This study was designed to test the hypothesis that opioid receptors mediate enhancement of vasorelaxation by CIHH. So this research on the mechanism of CIHH in reducing blood pressure will lay a solid foundation for the applications of CIHH in clinic.

1 MATERIALS AND METHODS

1.1 *Chemicals*

Phenylephrine HCl (PE), acetylcholine (ACh), and glibenclamide (GLI) were purchased from Sigma (St Louis, MO). Naloxone (NAL) was purchased from Enzo Life Sciences International. Anti-DOR, an antibody of δ-opioid receptor; Anti-MOR, an antibody of μ-opioid receptor; anti-KOR, an antibody of κ-opioid receptor; GAPDH and secondary antibodies were obtained from Santa Cruz Biotechnology, INC. Nitrocellulose membrane was obtained from Hybond-C (Amersham Life Science, UK) and the enhanced chemiluminescence (ECL) kit was obtained from Beijing Applygen Technologies.

1.2 *Animals and CIHH treatment*

All experiments were carried out in compliance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996) and was reviewed and approved by the Ethics Committee for the Use of Experimental.

Adult male Sprague-Dawley (SD) rats (weighing 300–350 g), obtained from the Animal Center of Hebei Medical University, were randomly divided into two groups: CIHH treatment group (CIHH) and control group. The rats in CIHH group were exposed to hypoxia in a hypobaric chamber (pB = 404 mmHg, pO_2 = 84 mmHg) simulated 5 000 m altitude for 28 d, 6 h per day. The control rats were kept in the same environment as CIHH except no hypoxia exposure. The physical activity of rats was monitored regularly and body weight of rats was recorded in a fixed time weekly.

1.3 *Preparation of aortic rings and measurement of vascular relaxation*

Animals were anaesthetized with pentobarbital sodium (50 mg/kg, i.p.). Thoracic aortas were dissected free, and surrounding connective tissues were carefully removed. The arterial segments were cut into 3–4 mm rings. The rings were mounted onto hooks, suspended in organ bath filled with Krebs-Henseleit (K-H) solu-

tion of the following composition (in mmol/L): NaCl 118.0, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25.0, KH_2PO_4 1.2, glucose 11.0, pH 7.4. The bath solution was constantly gassed with a mixture of 95% O_2 and 5% CO_2 and maintained at 37 °C.

Before the recording, the aortic rings were allowed to equilibrate under a resting tension of 1.0 g for 1 h, during which time K-H solution was replaced every 15 min. The aortic ring was connected to a force transducer, and the isometric force was recorded using a biological signal recording system (BL-420, Chengdu TME Technology Co., Ltd., China). To test the vasorelaxant capability of the vessel rings, the rings were maximally constricted by PE (1×10^{-6} mol/L) after 40 min of equilibration, and then were tested for the vasorelaxant responses to ACh (1×10^{-5} mol/L). After ACh testing, the rings were reequilibrated for 60 min and then again contracted submaximally as before with PE. ACh (1×10^{-9} to 1×10^{-5} mol/L) was added into the chamber cumulatively to induce vasorelaxation. The vasorelaxation is indicated as relaxed tension/PE-induced tension × 100%.

1.4 *Experimental protocols*

In the first set of experiments, cumulative relaxation responses to ACh (1×10^{-9}–1×10^{-5} mol/L) were studied in the aortic rings. When the contractile response to 1×10^{-6} mol/L PE was stabilized, the aortic rings were treated with consecutively increasing concentrations of ACh (1×10^{-9}–1×10^{-5} mol/L). The relaxation concentration-response curves of ACh in aortic rings were then generated.

The second set of experiments was designed to determine whether opioid receptor contributes to the effect of CIHH on vasorelaxation in rat aorta. Aortic rings were pretreated with NAL (1×10^{-6} mol/L), a non-specific opioid receptor blocker, 15 min before PE (1×10^{-6} mol/L) treatment in rats.

The third set of experiments was designed to test whether K_{ATP} channel was involved in the ACh-induced endothelium-dependent vasorelaxation effect of CIHH. Aortic rings were pretreated with GLI (1×10^{-5} mol/L), a K_{ATP} channel antagonist, 15 min before PE (1×10^{-6} mol/L) treatment in rats.

1.5 *Western blot analysis*

Protein expressions of δ-, μ- and κ-opioid receptors in thoracic aorta were assessed by Western blot analysis, as described previously [11]. Briefly, thoracic aortas samples were homogenized in ice-cold lysis buffer. Total proteins were extracted from the aortas; Equal amounts of protein (100 μg/lane) were loaded, subjected to electrophoresis on SDS-polyacrylamide gel and transferred onto nitrocellulose membrane. Membranes were blocked with nonfat milk and then incubated with primary antibodies anti-DOR, anti-MOR, anti-KOR (1:200 diluted in TBS-T), and anti-GAPDH (1:1 000) at 4 °C overnight. And then they were incubated with secondary antibody for 1 h at room temperature. The reaction was visualized by ECL. The films were scanned and analyzed by NIH image software. The protein contents were normalized to that of GAPDH. All experiments were repeated three times.

1.6 *Statistical analysis*

Data were expressed as mean ± SD. Protein expression was normalized to GAPDH. The logarithm of drug concentration eliciting 50% of the maximal relaxation response (ED_{50}) was calculated by regression analysis by fitting the dose-response relation for each vasorelaxant to a sigmoidal curve using Logit method. The maximum relaxant response (Rmax) was measured as the maximal response to each vasorelaxant.

Student's *t*-test was used for comparison between two groups and one-way ANOVA was used for comparison among groups. $P < 0.05$ indicates significant difference.

2 RESULTS

2.1 CIHH enhances ACh-induced vasorelaxation in rat aorta

ACh-induced relaxation of aortic rings was increased in a dose-dependent manner in CIHH rats compared with control rats. ED_{50} was ($4.55 \times 10^{-7} \pm 1.68 \times 10^{-7}$) mol/L in control rats and ($5.55 \times 10^{-8} \pm 1.72 \times 10^{-8}$) mol/L in CIHH rats. Rmax was (86.58 ± 6.94)% in control rats and (106.01 ± 5.51)% in CIHH rats ($P < 0.05$, Fig. 1 and Table 1). The concentration-response curve shifted down- and left-ward along with ACh supplement, suggesting CIHH enhances the ACh-induced endothelium-dependent vasorelaxation of thoracic aorta.

2.2 Opioid receptors mediate the effect of CIHH in aortic rings

Naloxone-pretreatment had no effect on ACh-induced relaxation of aortic rings in control rats. ED_{50} was ($4.55 \times 10^{-7} \pm 1.68 \times 10^{-7}$) mol/L in control rats and ($6.38 \times 10^{-7} \pm 1.87 \times 10^{-7}$) mol/L in control + NAL rats. Rmax was (86.58 ± 6.94)% in control rats and (85.79 ± 8.37)% in control + NAL rats ($P > 0.05$, Fig. 2 and Table 1).

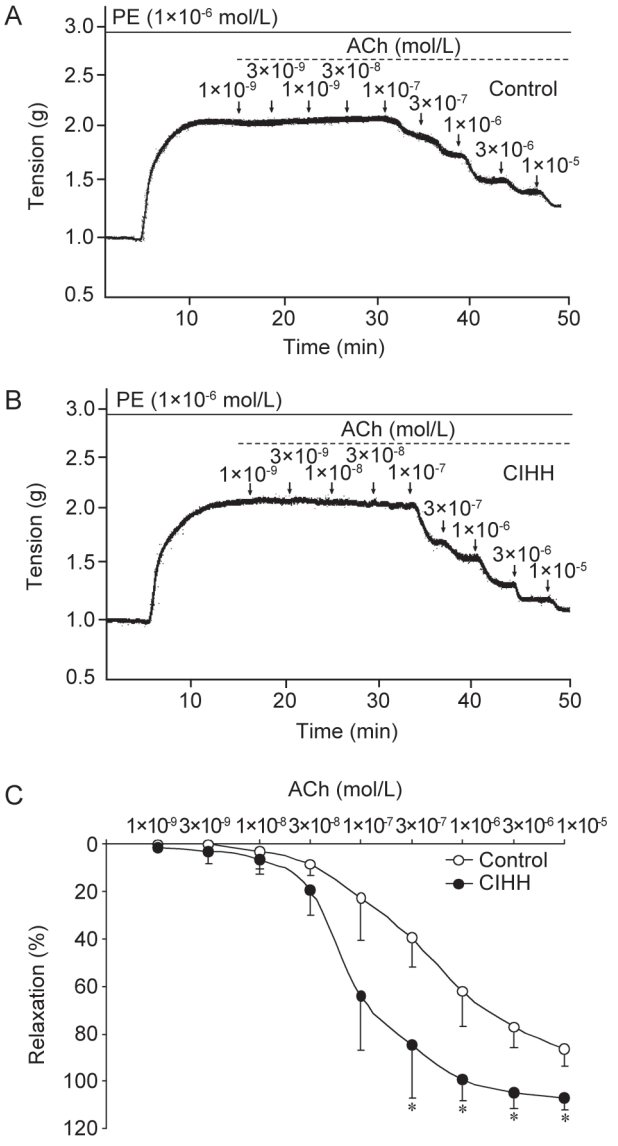

Fig. 1. ACh-induced vasorelaxation in aortic rings of rats. *A* and *B*: Representative tension curve induced by ACh in isolated thoracic aorta ring of control and CIHH rats. *C*: Statistical data for control and CIHH rats. ACh: acetylcholine; CIHH: chronic intermittent hypobaric hypoxia. All data were presented as a percentage of the contraction elicited by phenylephrine (PE) and expressed as mean ± SD, $n = 6$ for each group. $^{*}P < 0.05$ *vs* control.

However, the enhancement of ACh-induced relaxation of aortic rings in CIHH rats was disappeared in NAL-pretreated aortic rings from CIHH rats. ED_{50} was ($5.55 \times 10^{-8} \pm 1.72 \times 10^{-8}$) mol/L in CIHH rats and ($3.99 \times 10^{-7} \pm 1.39 \times 10^{-7}$) mol/L in CIHH + NAL rats ($P < 0.05$). Rmax was (106.01 ± 5.51)% in CIHH rats and (86.93 ± 5.21%) in CIHH + NAL rats ($P < 0.05$, Fig. 2 and Table 1), suggesting the enhancement of relaxation by CIHH is mediated by opioid receptors.

Table 1. Effect of CIHH on acetylcholine-induced relaxation in isolated rat aortic rings

	ED_{50} (mol/L)	Maximal relaxation (%)
Control	$4.55 \times 10^{-7} \pm 1.68 \times 10^{-7}$	86.58 ± 6.94
CIHH	$5.55 \times 10^{-8} \pm 1.72 \times 10^{-8*}$	106.01 ± 5.51*
Control + NAL	$6.38 \times 10^{-7} \pm 1.87 \times 10^{-7}$	85.79 ± 8.37
CIHH + NAL	$3.99 \times 10^{-7} \pm 1.39 \times 10^{-7\#}$	86.93 ± 5.21#
Control + GLI	$5.88 \times 10^{-7} \pm 1.94 \times 10^{-7}$	74.82 ± 12.62
CIHH + GLI	$3.90 \times 10^{-7} \pm 1.02 \times 10^{-7\#}$	79.96 ± 8.27#

ED_{50}: The logarithm of drug concentration eliciting 50% of the maximal relaxation response. Data were expressed as mean ± SD, $n = 6$ for each group. $^{*}P < 0.05$ *vs* control, $^{\#}P < 0.05$ *vs* CIHH.

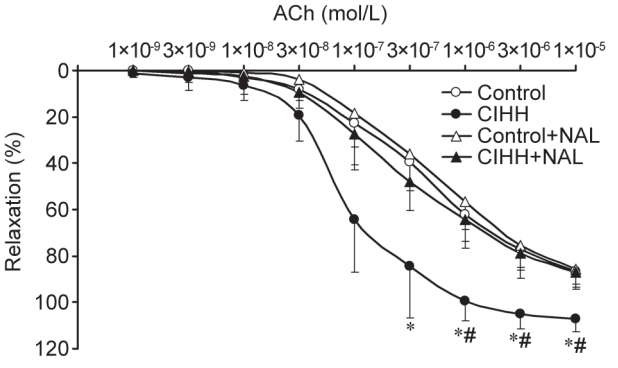

Fig. 2. ACh-induced vasorelaxation in aortic rings of rats with or without 1×10^{-6} mol/L naloxone (NAL) pretreatment. ACh: acetylcholine; CIHH: chronic intermittent hypobaric hypoxia. All data were presented as a percentage of the contraction elicited by phenylephrine and expressed as mean ± SD, $n = 6$ for each group. $^{*}P < 0.05$ *vs* control; $^{\#}P < 0.05$ *vs* CIHH + NAL.

To further determine the subtype of opioid receptors involved in the CIHH effect on aorta, Western blot method was used to assess the protein expressions of δ-, μ- and κ-opioid receptors in aorta. The results showed that protein expressions of δ-, μ- and κ-opioid receptors in CIHH treated aortic rings were up-regulated compared with those in control ($P < 0.05$, Fig. 3).

2.3 Role of K_{ATP} channel on enhancement of ACh-induced relaxation in CIHH treated aortic rings

After pretreatment with K_{ATP} channels antagonist GLI (1×10^{-5} mol/L), the enhancement of ACh-induced relaxation in aorta of CIHH treated rats were abolished, which suggests that K_{ATP} channels are involved in the enhancement of ACh-induced vasorelaxation in aortic rings of CIHH rats. ED_{50} was ($5.55 \times 10^{-8} \pm 1.72 \times 10^{-8}$) mol/L in CIHH rats and ($3.90 \times 10^{-7} \pm 1.02 \times 10^{-7}$)

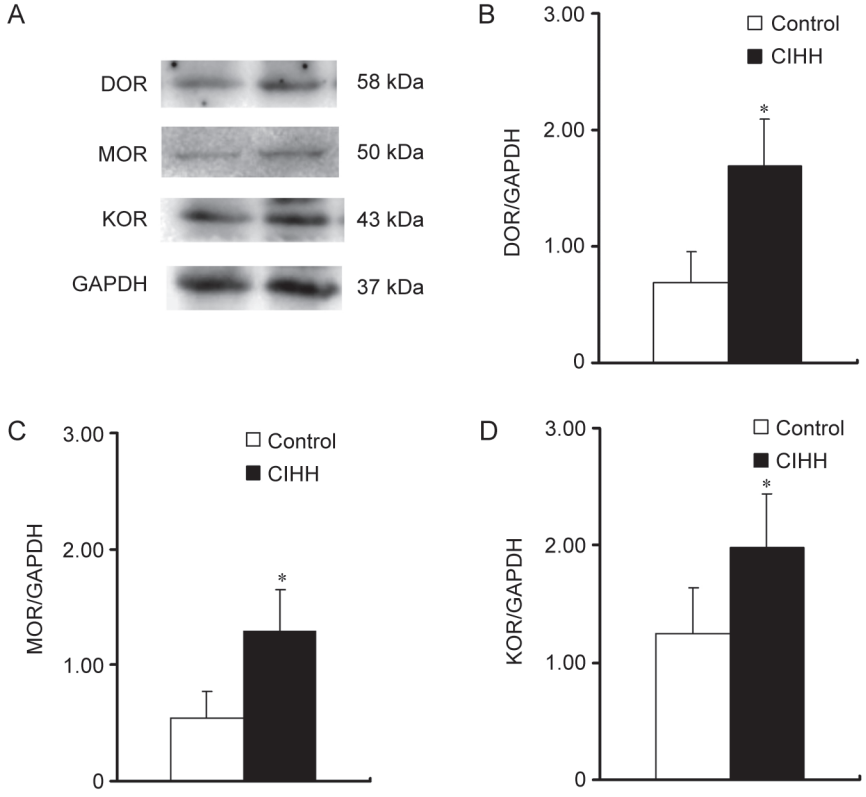

Fig. 3. Protein expression of δ-opioid receptor (DOR), μ-opioid receptor (MOR) and κ-opioid receptor (KOR) in aorta of CIHH and control rats. *A*: Representative protein expressions of opioid receptors and GAPDH; *B–D*: Quantitative analysis of protein expression of DOR, MOR and KOR. All data were expressed as mean ± SD, $n = 6$ for each group. $^{*}P < 0.05$ *vs* control.

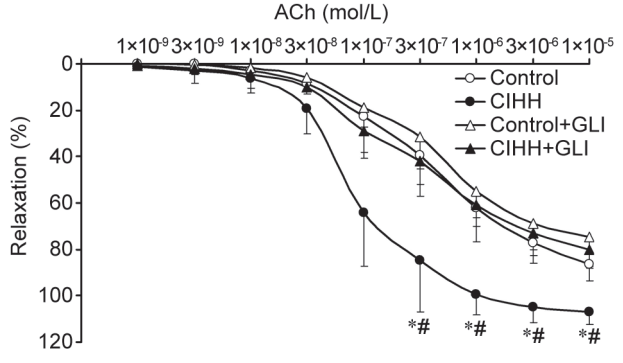

Fig. 4. ACh-induced vasorelaxation in aortic rings of rats with glibenclamide (GLI, 1×10^{-5} mol/L). ACh: acetylcholine; CIHH: chronic intermittent hypobaric hypoxia. All data were presented as a percentage of the contraction elicited by phenylephrine and expressed as mean ± SD, $n = 6$ for each group. $^{*}P < 0.05$ *vs* control; $^{\#}P < 0.05$ *vs* CIHH + GLI.

mol/L in CIHH + GLI rats ($P < 0.05$). Rmax was (106.01 ± 5.51)% in CIHH rats and (79.96 ± 8.27)% in CIHH + GLI rats ($P < 0.05$, Fig. 4 and Table 1).

3 DISCUSSION

In this study, organ bath perfusion technique was used to investigate the effect of CIHH on ACh-induced relaxation in rat aortic rings. The results showed for the first time that CIHH treatment enhances the endothelium-dependent relaxation of aorta dose-dependently. The enhancement of vasorelaxation of aorta can be reversed by NAL, a non-specific opioid receptors blocker, or GLI, a K_{ATP} channel antagonist, which suggests that CIHH enhances vasorelaxation through activation of opioid receptors and opening of K_{ATP} channel in rat thoracic aorta.

There were reports that opioid receptors extensively express in cardiovascular system, such as in the cardiac cell membrane and vascular wall, and play an important role in regulating vascular smooth muscle activity[12]. The opioid receptors can be divided into six subtypes, including μ (μ_1, μ_2), δ, κ (κ_1,κ_2), σ, ε and λ. The κ, δ and μ receptors among opioid receptors are the major opioid receptors on regulation of cardiovascular system function [13]. And it has been demonstrated by receptor

binding assay and physiological experiments that κ-opioid receptor is predominant in the heart and the peripheral vessels [14]. It was found that U50488H, a selective κ-opioid receptor agonist, could relax abdominal aorta and pulmonary artery to reduce systemic and pulmonary artery pressure accordingly [15,16]. Also μ- and δ-opioid receptors were found to mediate hypoxia-induced pial artery dilation in the newborn pig [17]. The result of present study showed that the enhancement of aorta relaxation in CIHH-treated rats was canceled by NAL, a non-selected opioid receptor antagonist. Also κ, μ and δ opioid receptors were up-regulated in aorta of CIHH-treated rats. Those results suggest that enhancement of aorta relaxation is co-mediated by κ, μ and δ opioid receptors and is realized by the up-regulation of κ, μ and δ opioid receptors.

It is known that four kinds of K^+ channels are located on vascular smooth muscles, including voltage-gated K^+ channels (K_V), Ca^{2+}-activated K^+ channels (K_{Ca}), K_{ATP} channels and inward rectifier K^+ channels (K_{IR}). They regulate the contraction and relaxation of artery by changing membrane resting potential. And it was found that K_{ATP} channels were closely related to the protective effect of opioid receptor and regulated the contraction and relaxation of artery. When K_{ATP} channels open, hyperpolarization of cell membrane takes place. The hyperpolarization in the sarcolemma of vascular smooth muscle cells prevents the entry of Ca^{2+} through the voltage-operated Ca^{2+} channels[18], inhibits the agonist-induced mobilization of Ca^{2+} from the stores[19], and reduces the sensitivity of the contractile elements to Ca^{2+}[20], leading to vasorelaxation.

Aslo there are some reports about the relationship between opioid receptor and K_{ATP} channels. For example, opioids contribute to hypoxia-induced pial artery dilation through activating K_{ATP} channel [21]. And μ-opioid receptor-dependent cAMP-PKA pathway that induces vascular relaxation by opening K_{ATP} channels[22]. And Pei *et al*. found that activation of κ-opioid receptor relaxes artery via K_{ATP} channel, resulting in reduction of blood pressure[8,9]. Consistent with previous studies, our study confirmed that K_{ATP} channel is involved in opioid receptors mediated-enhancement of vasorelaxation by CIHH.

There are reports that CIHH has anti-hypertension effect in hypertension patients and SHR [23, 24]. Vasomotion is a pivotal factor for blood pressure formation and maintaining. Vasoconstriction can cause blood pressure increase, in contrast, vasorelaxation decreases blood pressure. Recently we reported that CIHH attenuated the contraction induced by ANGII in thoracic aorta rings of rat [7], and now we provide evidence that CIHH enhances vasorelaxation in rat aorta. Both vasocontraction attenuation and vasorelaxation enhancement would help to maintain normal blood pressure during pathologic hypertension. The effect of CIHH on vasomotion might be one of the mechanisms for anti-hypertensive effect of CIHH.

There is a limitation in this study that we only investigate the big conductive artery aorta, not small artery or resistant vessel. It is well known that peripheral resistance involved in formation of artery blood pressure mainly comes from small artery [25]. The effect of CIHH on the small resistant artery like renal artery or mesenteric arteries deserves further investigation.

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