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Endothelin-1 enhanced carotid body chemosensory activity in chronic intermittent hypoxia through PLC, PKC and p38MAPK signaling pathways

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ABSTRACT

Endothelin-1 (ET-1), as it functions as a neuromodulator, has been associated with hypertension in chronic intermittent hypoxia (CIH) which attribute to enhanced carotid body sensibility to hypoxia. However, the molecular mechanism of ET-1 on carotid body sensibility in CIH is still not clear. Here, effect of ET-1 on carotid body chemosensory stimulation in rats exposed to either CIH or room air (Normoxia) was explored. Furthermore, Phospholipase C (PLC), Protein kinase C (PKC) or p38 MAPK antagonists were adopted to clarify the signalling pathways involved. Results showed that ET-1 induced a higher increase of carotid sinus nerve activity (CSNA) in animals exposed to CIH. Both ETA and ETB receptor expression were up-regulated by CIH exposure, but only ETA is responsible for ET-1 induced CSNA increase. Additional, the increase was inhibited by PLC, PKC, p38 MAPK antagonists and calcium channel blocker. Our findings support that ETA receptor mediates ET-1-induced CSNA increase through PLC, PKC and p38 MAPK signalling pathways in chronic intermittent hypoxia. Also, our study indicated that calcium influx was necessary for enhancing effect of ET-1 on CSNA.

1. Introduction

Obstructive sleep apnoea (OSA) is a sleep-breathing disorder that results in periodic decreases in O_2 levels or chronic intermittent hypoxia (CIH) in arterial blood in patients worldwide (Del Rio et al., 2014; Moya et al., 2016). Peripheral chemoreceptors, also known as glomus cells, continuously monitor the levels of oxygen in the arterial blood and elicit cardiorespiratory changes to ensure an adequate oxygen supply under low oxygen conditions (Kumar and Prabhakar, 2012). In mammals, these oxygen-sensitive cells are mainly located in the carotid and aortic bodies. In response to hypoxia, the afferent nerve is activated and conveys the chemosensory information to the nucleus of the solitary tract (NTS) in the brainstem, which engages in autonomic and respiratory control, evoking responses of enhanced respiratory drive, sympathetic activation and parasympathetic stimulation (Zoccal, 2015). The most accepted model for chemoreception proposes that hypoxia closes K^+ channels, leading to glomus cell depolarization. This step is followed by Ca^{2+} entry and excitatory transmitters (ACh and ATP) release, which in turn increases the discharge in the nerve endings of chemosensory neurons (Iturriaga et al., 2014).

Increased pressor and ventilatory responses to hypoxia and development of the hypertension suggest that the peripheral chemoreflex is enhanced by CIH (Bosc et al., 2010; Prabhakar et al., 2012). In the last decade, substantial evidence supports the hypothesis that the carotid body is necessary for the progression of the CIH-induced hypertension (Dempsey et al., 2010; Garvey et al., 2009; Iturriaga et al., 2009). According to the results from both in vivo and in vitro experiments, exposure to CIH increased basal carotid body chemosensory discharges and enhanced the chemosensory response to hypoxia (Bosc et al., 2010; Del Rio et al., 2010; Del Rio et al., 2012). However, the mechanism by which CIH enhances carotid body chemosensory reactivity to hypoxia is not entirely clear (Iturriaga et al., 2009).

Carotid bodies (CB) express a variety of neurotransmitters or modulators, including biogenic amines, peptidergic modulators and

Abbreviations: ET-1, endothelin-1; CIH, chronic intermittent hypoxia; PLC, phospholipase C; PKC, protein kinase C; CSNA, carotid sinus nerve activity; OSA, obstructive sleep apnoea; NTS, nucleus of the solitary tract; CB, carotid bodies; DAG, diacylglycerol; MAPKs, mitogen-activated protein kinases; IP_3, inositol trisphosphate

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gasotransmitters, which play important roles in the chemosensory potentiation induced by CIH (Kumar, 2011; Kumar and Prabhakar, 2012). Endothelin system was postulated to be associated with the CIH-induced potentiation of the carotid body chemosensory discharge (Iturriaga, 2013; Peng et al., 2013; Zoccal, 2015). Exposure to CIH increases ET-1 expression in the carotid body, and an ET-1 receptor antagonist prevents CIH-induced sensitization of the chemosensory response to hypoxia (Peng et al., 2013; Rey et al., 2008). Based on these results, a local increase in ET-1 expression in the carotid body might contribute to enhance the carotid body chemosensory tone induced by CIH (Prabhakar et al., 2015). The two G protein-coupled receptors to which ET-1 binds, the ET_A and ET_B receptors, mediate phospholipase C signalling and the subsequent production of IP3 and diacylglycerol (DAG). The subsequent activation of protein kinase C (PKC) and p47phox induces these proteins to form a complex, thereby facilitating the signalling cascade for ROS. Moreover, in addition to IP3-dependent Ca^{2+} release, DAG directly activates several types of non-selective cation channels to increase Ca^{2+} influx. In return, the increased intercellular calcium concentration further increases PKC activation.

First, we aimed to clarify the effects of an infusion of ET-1 into the carotid artery on carotid body chemosensory activity in rats exposed to normoxia or CIH. Then, the expression of ET_A/ET_B receptors, and phospholipase C (PLC), PKC and p38 MAPK in the carotid body was measured to assess which signalling pathways were involved in CIH-induced carotid body sensitization. Finally, the role of calcium influx in enhancing the effect of ET-1 on carotid sinus nerve activity (CSNA) was also determined.

2. Methods

2.1. Ethical approval

All experiments were performed on male Sprague-Dawley rats purchased from the Hebei Experimental Animal Center (Shijiazhuang, China) that weighed approximately 200 g at entry into the protocol. Rats were housed in standard rat cages and were exposed to a 12 h light:12 h dark cycle. Food and water were provided ad libitum. All surgical procedures and experimental protocols were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee of Medical Ethics of Hebei University of Chinese Medicine (SYXK 2017-005). The authors of this study understand the ethical principles of the journal and made every attempt to ensure that this study complied with the animal ethics checklist.

2.2. Cyclic hypoxia exposure

Hypoxia exposure was designed to mimic the cyclic hypoxia experienced by patients with obstructive sleep apnoea. Animals were exposed to hypoxia during their normal rest period (light). On each day, animals were placed in commercial hypoxic chambers (Oxycycler model A84XOV, BioSpherix, Lacona, NY, USA). These chambers were flushed with 100% nitrogen to a fraction of inspired O_2 (FIO_2) nadir of 9% for 1.5 min and the FIO_2 was gradually allowed to return to 21% over the remainder of each cycle. The exposure cycle was repeated every 3 min for 8 h/day, 7 days/week for 3 weeks. Control animals were subjected to identical handling and exposure conditions, but chambers were flushed with room air rather than N_2. Exposed animals were selected randomly for either physiological recordings or for molecular analyses of the carotid bodies.

2.3. Carotid sinus nerve dissection and recording

After rats were anaesthetized with pentobarbital sodium (50 mg/kg, ip), the nerve to the baroreceptor was first cut to record sensory activity in the carotid sinus nerve. The left carotid sinus nerve was dissected at the site where it joins the glossopharyngeal nerve. The nerve was prepared for recording by placing it on a bipolar platinum electrode connected to a bioelectrical amplifier (CP 511, Grass Astro). The carotid sinus nerve and surrounding structures were immersed in mineral oil mixed with Vaseline to maintain tissue integrity. The output of the Grass amplifier was amplified with a bandwidth of 30–1000 Hz and was fed to a polygraph (AD Instruments PowerLab 8SP). The raw signal was recorded and was simultaneously integrated using a low-pass filter with a time constant of 5 s. The integral of carotid sinus nerve activity (CSNA) was obtained and measured. Prompt augmentation of sensory discharge in response to 9% O_2 and a prompt decrease in response to 100% O_2 were assessed to assess carotid body afferent activity. Further confirmation of carotid body afferent activity was obtained from the nerve response to arterial occlusion. A reduction in the pressure in the carotid sinus by occluding the common carotid artery for 10 s caused no change or an increase in sinus nerve activity, but never a decrease, indicating that the sensory activity was from the carotid body rather than a baroreceptor.

2.4. Perfusion of the isolated left carotid sinus

We made an incision just under the chin down the midline to the sternal notch and then cut the sternomastoid and omohyoid muscles to expose the bifurcation of the common carotid artery into the external and internal carotid arteries. Carotid body areas were fully exposed by rostrally turning the trachea and oesophagus. The external and internal carotid, occipital, and carotid body arteries were identified under a surgical operating microscope (SMOIF, China). The bilateral aortic nerves, right carotid sinus nerve, cervical sympathetic nerves and recurrent laryngeal nerves were all sectioned. With the exception of the carotid body artery, smaller arteries originating from the common, external and internal carotid arteries were exposed and ligated, while carefully leaving the left carotid sinus nerve undisturbed. Ligation of the occipital artery at its origin from the external carotid artery excluded baroreceptor nerves from the carotid sinus to the isolated carotid body, thereby preventing a secondary effect on baroreceptor activation. The common carotid artery was ligated at the proximal portion, and the distal portion of the external carotid artery was ligated. Plastic catheters were inserted into the left common carotid artery in the anterograde direction (served as an inlet tube) and in the external carotid artery in the retrograde direction (served as an outlet tube). The internal carotid artery was ligated at its beginning. The carotid body was then perfused with warm (37 °C) oxygenated modified Krebs-Henseleit (K—H) solution (mmol/L: NaCl 118.0, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.6, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 5.6, pH 7.35–7.45) bubbled with 95% O_2 and 5% CO_2.

2.5. Experimental protocol

One hour elapsed between the surgical procedures and initiation of the protocols, while the preparation was maintained in a 21% O_2/balance N_2 atmosphere. After this period of stabilization, baseline CSNA was recorded in room air for approximately 30 min. Using in vivo recordings of the carotid sinus nerve as described above, we examined the effect of CIH exposure on carotid activity after the administration of ET-1(200 pM, ENZO, ALX-155-001-PC01), a neuromodulator known to enhance carotid body afferent activity. Rats that had been exposed to CIH for 3 weeks were compared with appropriate normoxia-exposed animals. Then, we separately examined effect of an ET_A receptor antagonist (BQ123, 10 μM, ENZO, ALX-155-004-P001), ET_B receptor antagonist (BQ788, 10 μM, ENZO, ALX-155-020-M001), PLC antagonist (U73122, 10 μM, ENZO, BML-ST391-0005), PKC antagonist (chelerythrine chloride, 1 μM, SIGMA, PHL89896) and p38 MAPK inhibitor (SB203580, 10 μM, SIGMA, S8307) on ET-1-induced CSNA.

The Ca^{2+}-free bath solution was modified by removing 2.5 mM $CaCl_2$, adding 0.1 mM EGTA and increasing the concentration of $MgSO_4$

(3.6 mM) to ascertain the role of calcium influx in ET-1-induced CSNA. Additionally, the selective L-type calcium channel blocker nifedipine (10 μM, SIGMA, N7634) was used to provide further evidence.

Doses of all agents were determined after preliminary studies in which dose-response testing was performed to determine doses producing maximum carotid sinus nerve responses and minimum systemic haemodynamic effects. The vehicle had no effect on CSNA in any protocol.

2.6. Western blotting

Carotid bodies were homogenized and lysed in RIPA lysis buffer containing a mixture of protease inhibitors. The homogenates were centrifuged at 14,000*g* for 10 min at 4 °C. Supernatants were collected and stored in a − 80 °C freezer. Total protein concentration was determined by BCA assay (CWBIO). Equivalent amounts of the proteins (20 μg) were electrophoresed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred onto Immobilon transfer membranes (Millipore). Gel concentrations and blotting times depended on the molecular weight of the protein being examined. Nonspecific binding sites on the membrane were blocked with 5% (*w/v*) non-fat dry milk or bovine serum albumin in PBS-T buffer (0.15 M NaCl, 100 mM sodium phosphate buffer, pH 7.4, and 0.1% (*v/v*) Tween100) for 1 h at room temperature. The membranes were incubated with proper dilutions of the primary antibodies overnight at 4 °C and then washed in PBS-T buffer three times for 10 min at room temperature. Subsequently, the membranes were incubated with horseradish peroxidase-conjugated secondary anti-rabbit (1:2000 dilutions in PBS-T) or anti-mouse (1:4000 dilutions in PBS-T) antibodies (Amersham Biosciences) for 1 h at room temperature. After three washes with PBS-T buffer, the immunoreactive bands were visualized using the SuperSignal West Pico Chemiluminescent Substrate detection system (Pierce), according to the manufacturer's instructions. The following primary antibodies were used: rabbit anti-endothelin-1 polyclonal antibody (Biogenesis, 1:1000); rabbit anti-endothelin A receptor polyclonal antibody (Abcam, 1:1000); rabbit anti-endothelin B receptor polyclonal antibody (Sigma, 1:500); mouse anti-phosphorylated p38 monoclonal antibody (Santa Cruz Biotechnology, sc-7973, 1:500); mouse anti-p38 monoclonal antibody (Santa Cruz Biotechnology, sc-136,210, 1:500); mouse anti-PKC monoclonal antibody (Santa Cruz Biotechnology, sc-8393, 1:500), mouse anti-p-PKC monoclonal antibody (Abnova, MAB5593,1:200), and mouse anti-PLC monoclonal antibody (Santa Cruz Biotechnology, sc-5291, 1:500).

2.7. Statistical analysis

Baseline CSNA was compared by calculating the absolute value of integrated activity. Data are presented as the means ± SD, and the statistical analyses were performed using SPSS software. Student's *t*-test was used to determine the statistical significance between the Normoxia and CIH groups, and two-way ANOVA was conducted to compare data from three or more groups, followed by a comparison of individual differences using the Student-Newman-Keuls test. $P < .05$ was considered to indicate statistical significance.

3. Results

3.1. Effects of an infusion of ET-1 on CSNA

We examined the effect of a CB infusion of ET-1 on CSNA in rats exposed to normoxia or CIH for 3 weeks. The infusion of ET-1 (200 pmol/L) into the external carotid artery of rats for 10 s resulted in a rapid increase in basal carotid sinus nerve discharge in the CIH and Normoxia groups, and basal carotid sinus nerve discharge was significantly increased as the ET-1 perfusion dose increased (Fig. 1A, $*p < .05$ vs Normoxia). However, compared with the Normoxia group,

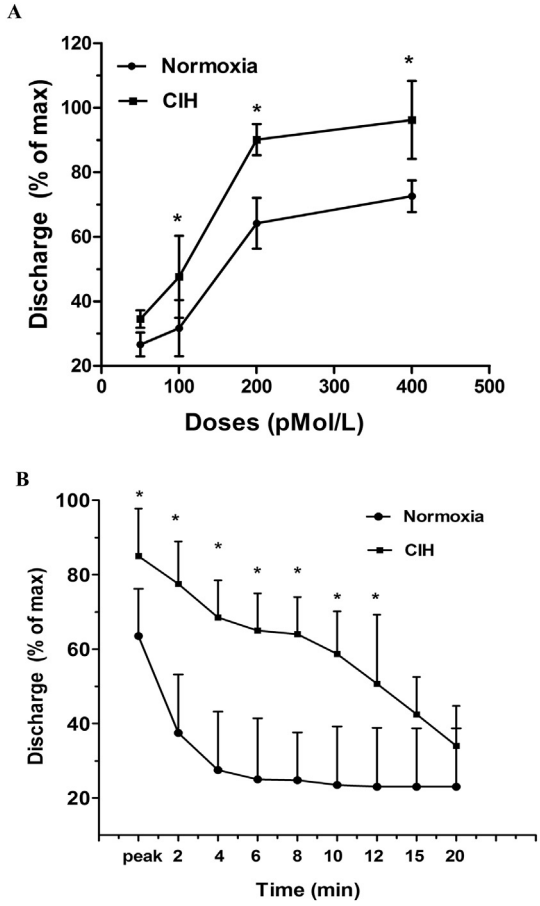

Fig. 1. Effects of the ET-1 infusion on CSNA. A: Dose-response curves representing CSNA as a percentage of the maximal response elicited by stop flow. The CIH group exhibited a more robust response at all doses. B: CSNA as a percentage of the maximal activity recorded at different time points after a 10 min infusion of ET-1 (200 pmol/L) in the CIH group and Normoxia group. The response of the CIH group is greater at all time points, except 15 min and 20 min. The results are presented as the means ± SD ($n = 6$). $*p < .05$ compared to the Normoxia group.

ET-1 significantly increased CSNA in the CIH group. Surprisingly, this increase in CSNA persisted for a long time after the termination of the infusion. Greater CSNA was observed in the CIH group after a 10 s infusion of ET-1 (200 pmol/L) than in the Normoxia group (except for CSNA recorded at 15 min and 20 min) (Fig. 1B, *p < .05 vs Normoxia). The peak response was greater and the duration of elevated CSNA was longer in CIH-exposed rats than in normoxia-exposed rats.

3.2. Roles of ET receptors in ET-1-induced CSNA

We assessed levels of the ET receptor proteins by Western blotting carotid bodies from Normoxia-exposed and CIH-exposed rats to further assess whether hypoxia exposure regulated the expression of ET receptor subunits in the carotid body. As shown in Fig. 2A ($*p < .05$ vs Normoxia), levels of both the ET_A and ET_B receptors were increased in the rat carotid body after CIH exposure compared with the Normoxia group.

As noted above, an infusion of ET-1 produced sustained activation of the carotid sinus nerve, with augmented activity persisting substantially beyond the duration of the infusion. To our surprise, the administration of BQ123, a specific ET_A receptor blocker, before the ET-1 infusion substantially reduced ET-1-induced CSNA (Fig. 2B, $*p < .05$ vs baseline, #p < .05 vs ET-1 administration). However, BQ788, a selective ET_B receptor blocker, had no effect on the increase in CSNA

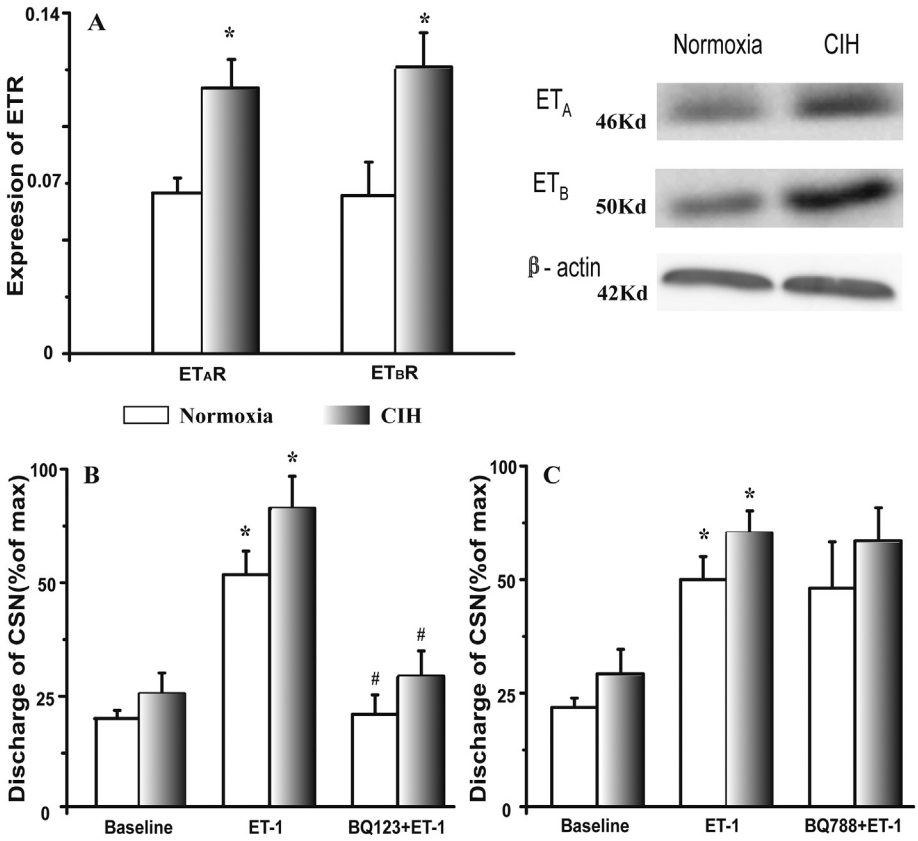

Fig. 2. Roles of ET receptors in ET-1-induced CSNA. A: Expression of ET_A and ET_B receptors in carotid bodies. CIH increased levels of the ETA and ETB proteins in the carotid body. B: Effects of the selective ETA blocker BQ123 (10 μmol/L) on ET-1-induced CSNA in the CIH group and Normoxia group. C: Effects of the selective ETB blocker BQ788 (10 μmol/L) on ET-1-induced CSNA in the CIH group and Normoxia group. CSNA as a percentage of baseline activity over time after the infusion of ET-1 (200 pmol/L), with and without previous administration of the selective receptor blocker. Results are presented as the means ± SD (n = 6). *p < .05 compared to baseline, #p < .05 compared to ET-1 administration.

induced by ET-1 (Fig. 2C, *p < .05 vs baseline). Based on these findings, the ET_A receptor might be important in ET-1-induced CSNA.

3.3. Effect of PLC on ET-1-induced CSNA

We tested the response of CIH animals to ET-1 after pre-treatment with U73122. Results showed that the ET-1 response was significantly reduced by the U73122 pre-treatment (Fig. 3A–B, *p < .05 vs the first ET-1-induced CSNA, #p < .05 vs Normoxia). Additional, we found that there is no significant difference of PLC levels between Normoxia and CIH group (Fig. 3C). This indicated that PLC activity may be potentially involved in the actions of ET-1 on CSNA.

3.4. Effect of PKC on ET-1-induced CSNA

We examined levels of the p-PKC protein in the carotid bodies from Normoxia- and CIH-exposed rats by Western blotting. As shown in Fig. 4A (*p < .05 vs Normoxia), levels of the p-PKC protein were increased in the carotid body of CIH-exposed rats compared with normoxia-exposed rats.

We tested the response of CIH animals to ET-1 (200 pmol/L) after pre-treatment with chelerythrine chloride, and the ET-1 response was significantly reduced by the prior administration of chelerythrine chloride (Fig. 4B, *p < .05 vs Baseline, #p < .05 vs the first ET-1-induced CSNA). Thus, PKC proteins were functional in the carotid bodies of CIH-exposed rats, and PKC might mediate the actions of ET-1 on CSNA.

3.5. Effect of p38 MAPK on ET-1-induced CSNA

We tested levels of the p38 MAPK and p-p38 MAPK proteins in the carotid bodies of Normoxia-and CIH-exposed rats by Western blotting. As shown in Fig. 5A (*p < .05 vs Normoxia), levels of the p38 MAPK/−p38 MAPK were increased in the rat carotid body after CIH exposure compared with the Normoxia-exposed rats.

We tested the response of CIH animals to ET-1 (200 pmol/L) after the administration of SB203580, and the ET-1 response was significantly reduced by the SB203580 pre-treatment (Fig. 5B, *p < .05 vs Baseline, #p < .05 vs the first ET-1-induced CSNA). Based on these findings, p38 MAPK might mediate the actions of ET-1 on CSNA.

3.6. Role of calcium influx in ET-1-induced CSNA

Calcium-free perfusion buffer was employed to confirm that Ca^{2+} mediated ET-1-induced CSNA. As shown in Fig. 6A–B (**p < .01 vs the first ET-1-induced CSNA, ##p < .01 vs Normoxia), CSNA was significantly reduced in calcium-free perfusion buffer, particularly in the CIH group. Furthermore, the selective L-type calcium channel blocker nifedipine (10 μM) also inhibited the increase in CSNA in both the Normoxia and CIH groups, but the decrease was more substantial in the CIH group (Fig. 6C–D, **p < .01 vs the first ET-1-induced CSNA, ##p < .01 vs Normoxia).

4. Discussion

As shown in the present study, CIH increased carotid body chemosensory sensitivity through a mechanism mediated by the ET-1 receptor signalling pathway. This alteration in carotid sinus nerve discharges was accompanied by over-expression of ET_A receptor and ET_B receptor, and increased levels of p38 MAPK and PKC proteins in the carotid body. Thus, the results supported the hypothesis that the enhanced CSNA induced by CIH was attributed to ET_A receptor-mediated PLC, PKC and p38 MAPK signalling pathways. In addition, Ca^{2+} influx also contributed to the increase in carotid body chemosensory sensitivity in this experimental model.

Carotid bodies are regarded as the peripheral chemoreceptors that are responsible for sensing O_2 level and the chemoreflex regulating cardiorespiratory functions (Prabhakar et al., 2015). CB receives

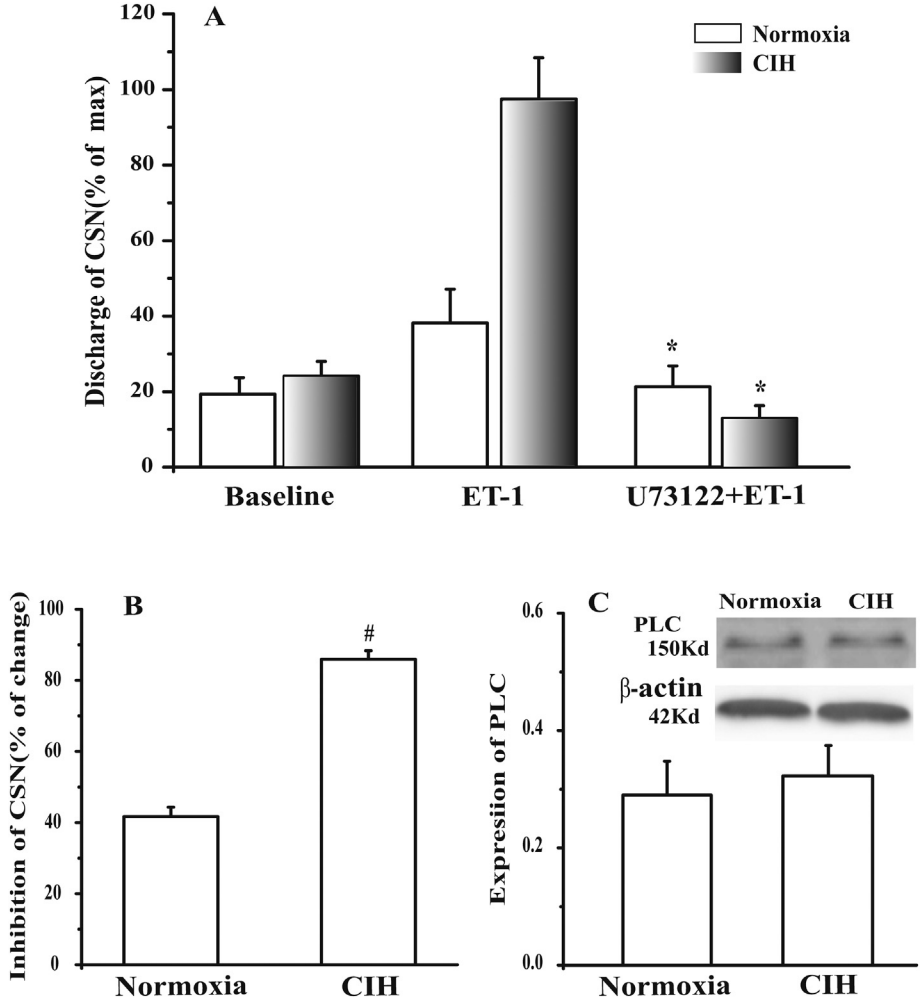

Fig. 3. Effects of the selective PLC blocker U73122 (10 μmol/L) on ET-1-induced CSNA in the CIH group and Normoxia group. A: CSNA as a percentage of maximal activity after the perfusion of ET-1 (200 pmol/L), with and without previous administration of the U73122. B: Change in the percentage after the administration U73122. C: Expression of PLC in rat carotid bodies; no significant difference was observed between the Normoxia and CIH groups. The results are presented as the means ± SD (n = 6). *p < .05 compared to the first ET-1-induced CSNA. #p < .05 compared to the Normoxia group.

sensory innervation from the carotid sinus nerve. Hypoxia increases the CSNS, which in turn stimulates breathing, sympathetic nerve activity and increased blood pressure. Hypoxia, as the primary stimulus to the carotid body, could be continuous or intermittent. Recently, the carotid body chemoreflex has been shown to contribute to the development of enhanced sympathetic activity and hypertension following intermittent hypoxia (Dempsey et al., 2010; Moya et al., 2016). Rodents exposed to CIH show increased carotid body chemosensory discharges following exposure to normoxia and hypoxia (Del Rio et al., 2010; Del Rio et al., 2012), and a long-lasting increase in baseline carotid body neural activity is known as sensory long-term facilitation (Peng et al., 2014). The present study showed a significantly greater basal discharge of the

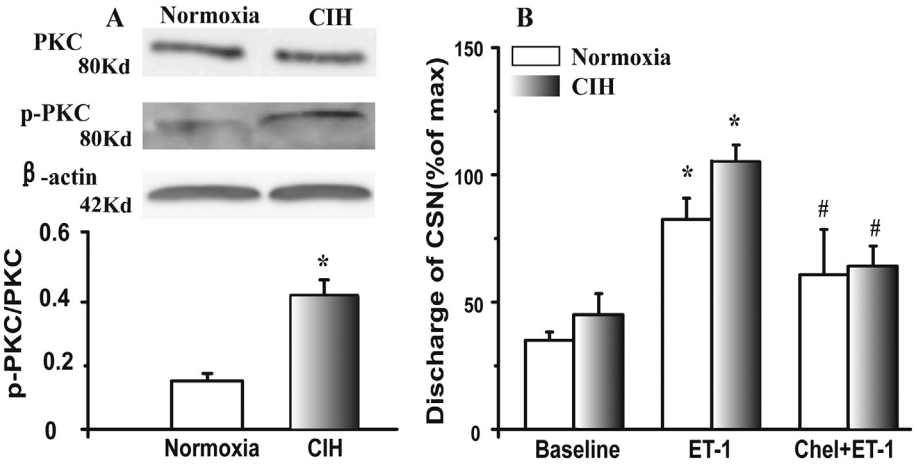

Fig. 4. Effects of the selective PKC blocker chelerythrine chloride (1 μmol/L) on ET-1-induced CSNA in the CIH group and Normoxia group. A: Levels of p-PKC in rat carotid bodies. B: CSNA as a of maximal activity after the perfusion of ET-1 (200 pmol/L), with and without previous administration of chelerythrine chloride. CSNA as a percentage of the maximal activity after the perfusion of ET-1 (200 pmol/L), with and without previous administration of chelerythrine chloride. The results are presented as the means ± SD (n = 6). *p < .05 compared to the Normoxia group, #p < .05 compared to the first ET-1-induced CSNA.

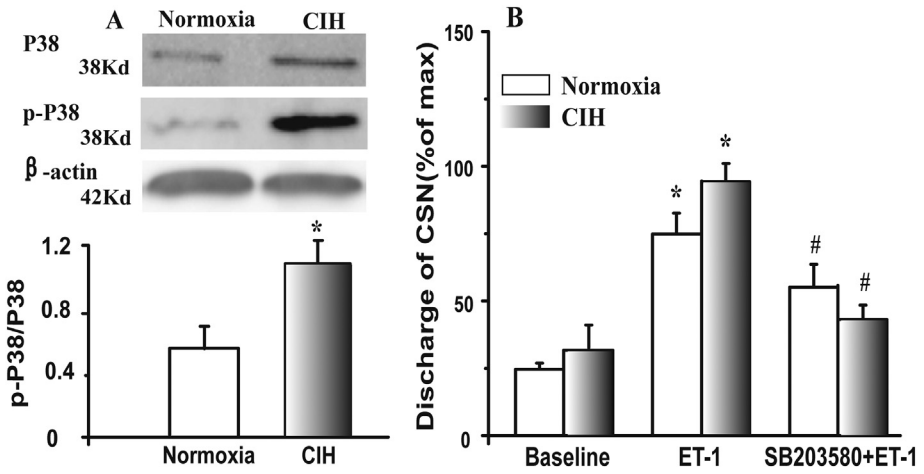

Fig. 5. Effects of the selective p38 MAPK blocker SB203580 (10 μmol/L) on ET-1-induced CSNA in the CIH group and Normoxia group. A: Levels of p38MAPK and p-p38MAPK in rat carotid bodies. B: CSNA as a percentage of the maximal activity after the perfusion of ET-1 (200 pmol/L), with and without previous administration of SB203580. Results are presented as the means ± SD (n = 6). *p < .05 compared to the Normoxia group, #p < .05 compared to the first ET-1-induced CSNA.

carotid sinus nerve in the CIH group than in the Normoxia group. Based on this finding, CIH induced a significant increase in the carotid body sensory nerve activity.

ET-1 is an effective peptide that modulates the hypoxic sensory response. Exogenous administration of ET-1 increases the hypoxic sensory response by acting directly on the chemoreceptor tissue (Chen et al., 2000). In our study, a 10 s infusion of ET-1 into the external carotid artery of rats resulted in a rapid increase in CSNA, and the strengthened carotid sinus nerve discharge was associated with the concentration of ET-1. Surprisingly, this increase persisted long after the termination of the infusion. The peak response was greater and the duration of elevated CSNA was longer in CIH-exposed rats than in normoxia-exposed rats.

The effects of ET-1 were mediated by the G-protein coupled receptors ET_A and ET_B. Some studies using an ET_A receptor blocker showed that ET-1 modulated the chemoreceptor activity of the carotid body via the ET_A receptor (Chen et al., 2002a; Chen et al., 2002b). Chronic hypoxia markedly increases the expression of ET-1 and ET_A receptors in the carotid body (Chen et al., 2007). Furthermore, the increased activity of the chemoreceptor within the carotid body corresponds to the increased expression of ET-1 and ET_A (Chen et al., 2002a). More importantly, selective blockade of ET_A receptors inhibits the enhanced hypoxic chemosensory responses of these carotid bodies in animals exposed to chronic hypoxia (Chen et al., 2000; Chen et al., 2007). According to recent evidence, ET-1 also plays an important role in increasing the carotid body chemosensitivity after CIH exposure. In

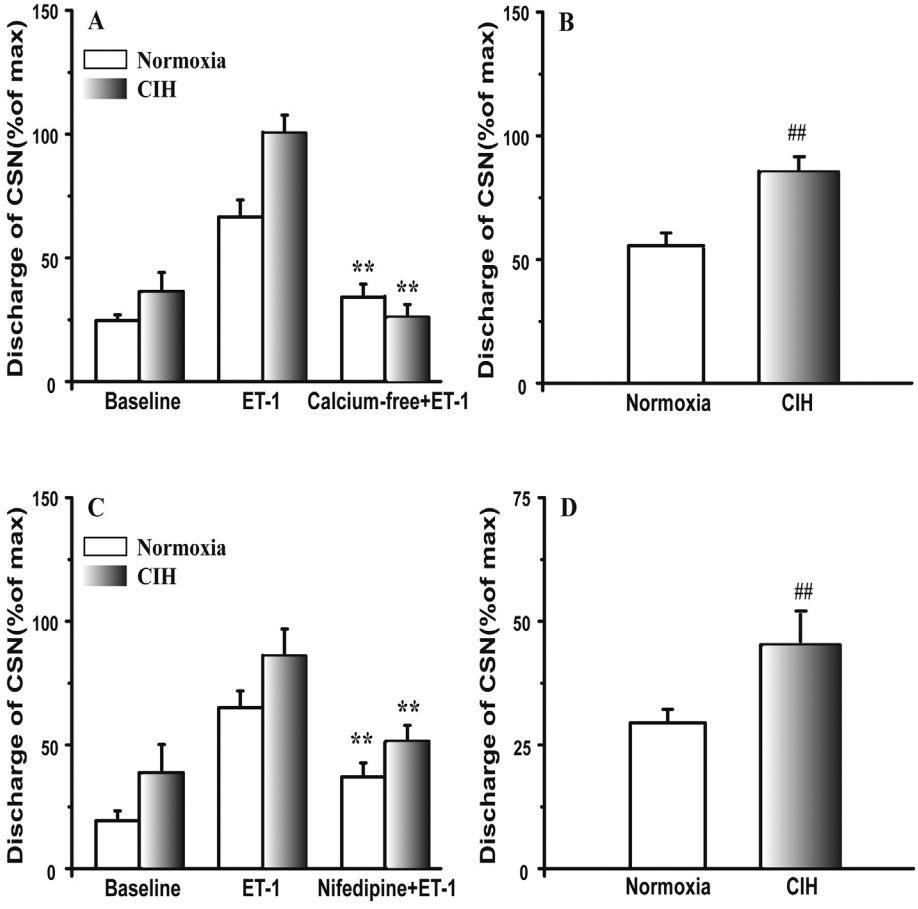

Fig. 6. Effect of calcium influx on ET-1-induced CSNA. A: CSNA was significantly reduced in calcium-free perfusion buffer. B: Change in the percentage after CIH exposure. C: The selective L-type calcium channel blocker nifedipine (10 μM) also inhibited the increase in Ca^{2+} concentrations. D: Change in the percentage after CIH exposure. The results are presented as the means ± SD (n = 6). **p < .01 compared to the first ET-1-induced CSNA, ##p < .01 compared to the Normoxia group.

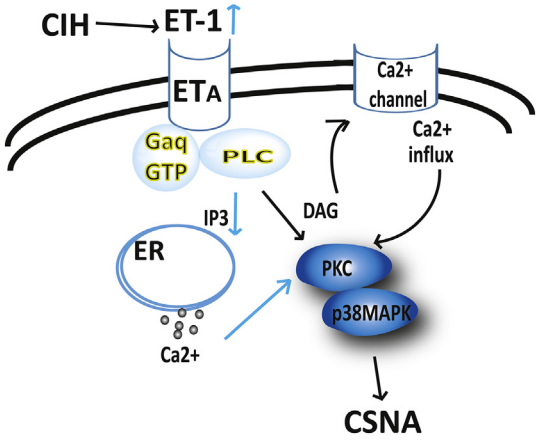

Fig. 7. Roles of the PLC, PKC and p38MAPK signalling pathways in the mechanism by which ET-1 increases CSNA in rats exposed to CIH. In rats exposed to CIH, ETA receptor was up-regulated in carotid body that mediated the effect of ET-1 on PLC activation. Then, in one hand, diacylglycerol (DAG) can activate protein kinase C (PKC)-p38MRAK, which is related to CSNA augmentation. In the other hand, DAG is contributed to calcium influx through calcium channel, which could also activate PKC signalling pathway.

cats exposed to CIH for four days, ET-1 expression was significantly increased in the carotid bodies, and a mixed ET_A/ET_B receptor antagonist bosentan prevented the increases in basal and hypoxic chemosensory responses induced by CIH exposure (Rey et al., 2006). In the present study, we observed an increase of CSNA in isolated carotid bodies in response to exogenous ET-1 after CIH. The expression of both the ET_A and ET_B receptors in carotid body was up-regulated after CIH exposure, but only the ET_A receptor antagonist reversed the inhibitory effect of ET-1 on CSNA. This finding was consistent with previous studies (Chen et al., 2000; Chen et al., 2002a) showing that the ET_A receptor is responsible for the ET-1-induced increase in the sensitization of the hypoxic sensory response to CIH.

The mitogen-activated protein kinases (MAPKs) comprise a family of ubiquitous proline-directed protein-serine/threonine kinases that are required for the sequential transduction of biological signals from the cell membrane to the nucleus (L et al., 2011). In mammalian cells, three well-defined sub-groups of MAPKs have been identified: extracellular signal-regulated kinases, the c-Jun N-terminal kinases and the p38MAPKs. The p38 MAPK protein is activated by a variety of stimuli, and in turn, the activated MAPKs might typically phosphorylate a number of downstream substrates, which regulate cell functions and survival (Roux and Blenis, 2004; VA et al., 2003). Sustained cellular hypoxia is associated with the activation of a MAPK pathway (Kiec-Wilk et al., 2010). Study has shown that hypoxia induced activation of p38 mitogen-activated protein kinase (MAPK) is activated by VEGFR-1 and 2 receptors (Maugeri et al., 2017b). Also, the breakdown of the blood-retinal barrier (BRB) is mediated through activation of MAPK signal pathway (Maugeri et al., 2017a). Additionally, MAPK signalling pathways are activated in the hippocampus under intermittent hypoxic conditions (Zhao et al., 2016). In the present study, we assayed the tissues for potential candidate downstream signalling pathways induced by endothelin-1, such as p38 MAPK and phosphorylated p38 MAPK. Higher levels of p38 MAPK and p-p38 MAPK were observed in the carotid body from the CIH group than the Normoxia group. After a 10 min infusion of ET-1 into the rat carotid body preparation, the increase in CSNA in the CIH group was significantly inhibited by the p38 MAPK inhibitor (SB203580). Based on these findings, we concluded that p38 MAPK activation might be related to the ET-1-induced increase in CSNA in response to CIH exposure.

PKC is a member of family of serine/threonine kinases that belong to the AGC superfamily of protein kinases (Rosse et al., 2010; Ryu et al., 2010). PKC is a lipid-dependent kinase that is activated by the lipid second messenger diacylglycerol (DAG) and calcium. Additionally, the engagement of growth factor or cytokine receptors activates PLC, which cleaves phosphatidylinositol 4,5-bisphosphate to generate DAG and the soluble second messenger inositol trisphosphate (IP_3). The production of DAG recruits classical and novel PKCs to the plasma membrane, where they undergo a conformational change resulting in full activation. Previous study showed that, CIH can directly affect the secretory capacity of chromaffin cells via ROS-mediated activation of PKC pathway (Kuri et al., 2007). The present study also investigated the role of the PLC/PKC pathway in ET-1-induced, CIH-mediated increases in CSNA. Higher levels of p-PKC expression were detected in the CB from the CIH group than the Normoxia group. The selective PLC antagonist U73122 and PKC antagonist chelerythrine chloride blocked ET-1-induced increases in CSNA, suggesting that the PLC/PKC pathway contributed to the ability of ET-1 to increase CSNA. Thus, PKC or p38 MAPK only partially inhibited the effect of ET-1 on increasing CSNA. The expression of the p47Phox subunit of NADPH oxidase was increased following IH exposure, suggesting that NADPH oxidase may represent another possible potential pathway involved in this mechanism.

Furthermore, ET-1-induced CSNA was significantly reduced in calcium-free perfusion buffer, and the L-type calcium channel blocker nifedipine also inhibited increased Ca^{2+} concentrations, suggesting that Ca^{2+} influx from the extracellular space was involved in ET-1-induced increase in CSNA.

5. Conclusions

In conclusion, PLC, PKC and p38MAPK signalling pathways were involved in ET-1-induced increases in CSNA in rats exposed to CIH. The contribution of calcium influx to ET-1-induced CSNA under CIH conditions was also confirmed (Fig. 7).

Competing interests

No conflicts of interest, financial or otherwise, are declared by the authors.

Author contributions

J. Woodrow Weiss and En-Sheng Ji conceived and designed the experiments and wrote the paper; Jieru Li and Shengchang Yang performed experiments; Fuyang Yu analyzed data.

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