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Full paper

Acetylcholine protects mesenteric arteries against hypoxia/reoxygenation injury via inhibiting calcium-sensing receptor

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ABSTRACT

The Ca^{2+}-sensing receptor (CaSR) plays an important role in regulating vascular tone. In the present study, we investigated the positive effects of the vagal neurotransmitter acetylcholine by suppressing CaSR activation in mesenteric arteries exposed to hypoxia/reoxygenation (H/R). The artery rings were exposed to a modified 'ischemia mimetic' solution and an anaerobic environment to simulate an H/R model. Our results showed that acetylcholine (10^{-6} mol/L) significantly reduced the contractions induced by KCl and phenylephrine and enhanced the endothelium-dependent relaxation induced by acetylcholine. Additionally, acetylcholine reduced CaSR mRNA expression and activity when the rings were subjected to 4 h of hypoxia and 12 h of reoxygenation. Notably, the CaSR antagonist NPS2143 significantly reduced the contractions but did not improve the endothelium-dependent relaxation. When a contractile response was achieved with extracellular Ca^{2+}, both acetylcholine and NPS2143 reversed the H/R-induced abnormal vascular vasoconstriction, and acetylcholine reversed the calcimimetic R568-induced abnormal vascular vasoconstriction in the artery rings. In conclusion, this study suggests that acetylcholine ameliorates the dysfunctional vasoconstriction of the arteries after H/R, most likely by decreasing CaSR expression and activity, thereby inhibiting the increase in intracellular calcium concentration. Our findings may be indicative of a novel mechanism underlying ACh-induced vascular protection.

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1. Introduction

In experimental models and clinical cases, blood vessels are very vulnerable to the deleterious consequences of cardiovascular disease, particularly ischemia/reperfusion (I/R). I/R affects endothelial function and vasomotricity, including the production of nitric oxide, relaxation/contraction of vascular smooth muscle cells, and expression of inflammatory factors (1, 2). The intracellular calcium concentration ($[Ca^{2+}]_i$) plays a key role in vasomotricity, and it can be increased via activation of the specific inositol 1,4,5-trisphosphate receptor (IP_3R) when the calcium-sensing receptor (CaSR) is activated (3). CaSR belongs to the G protein-coupled receptor family that was first identified by Brown et al. (4) CaSR is widely expressed in the cardiovascular system and plays important roles in cardiac hypertrophy, vascular calcification, and hypertension (5,6). Gou et al. reported that acute myocardial infarction and atherosclerosis can activate CaSR in the heart (7). Li et al. reported that CaSR was related to the regulation of pulmonary artery tension by increasing $[Ca^{2+}]_i$ via the G-protein/phospholipase C/IP_3R pathway (8). However, it is unknown whether CaSR is involved in I/R-induced vasomotor dysfunction.

The parasympathetic system and the sympathetic system are sub-divisions of the autonomic nervous system. The action of the parasympathetic system has been well studied. Normally, the activities of the sympathetic and parasympathetic systems are in dynamic balance. However, in disease states, particularly in cardiovascular disease, increased sympathetic nervous system activity and decreased parasympathetic activity are common (9). Vagal stimulation and the vagal neurotransmitter acetylcholine (ACh) are used for the prevention and treatment of ventricular fibrillation, ventricular tachycardia, and vascular endothelial dysfunction in

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cardiovascular disease (10–12). However, the underlying protective mechanism of the vagus nerve has not been fully defined. Additionally, little information is available regarding the relationship between ACh and CaSR.

Therefore, the aims of the present study were to examine the effects of ACh on mesenteric arteries exposed to hypoxia/reoxygenation (H/R) and to determine the possible relationship between ACh and CaSR using a suitable and reliable *in vitro* model of vascular H/R.

2. Materials and methods

2.1. Animal care

The adult male Sprague–Dawley rats (180–200 g) used in these experiments were provided by the Experimental Animal Center of Xi'an Jiaotong University. The experimental methods were approved by the Animal Ethics Committee of Xi'an Jiaotong University. The rats were kept in a pathogen-free animal breeding room maintained at 24 °C with a 12-h day/night cycle and given *ad libitum* access to water and standard rat chow.

2.2. Chemicals and agents

ACh, phenylephrine (PE) and sodium nitroprusside (SNP) were obtained from Sigma. The LDH and CK assay kits were obtained from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The following antibodies were used in the Western blot analysis: rabbit polyclonal anti-CaSR and rabbit polyclonal anti-p-CaSR$^{(Thr888)}$ (Signalway Antibody, Inc., Pearland, TX, USA). Calcimimetic R568 hydrochloride and the CaSR antagonist NPS2143 hydrochloride were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The drug concentrations are expressed as the final molar concentration within the organ chamber.

2.3. Experimental protocol

The mesenteric artery rings were randomly assigned to the following experimental groups (n = 8 per group): the control group, in which rings were incubated in a normoxic incubator at 37 °C with DMEM; the ACh group, in which ACh (10^{-6} mol/L) was administered 15 min before normoxic incubation; the H/R group, in which rings underwent 4 h of hypoxic culture with modified 'ischemia mimetic' solution and 12 h of normoxic culture with DMEM; the H/R + ACh group, in which ACh pretreatment was started 15 min before hypoxia and continued for 4 h of hypoxia and 12 h of reoxygenation; and the antagonist/agonist groups, in which rings were treated with the CaSR activator R568 (1 μmol/L) or the CaSR inhibitor NPS2143 (10 μmol/L) 30 min before initiation of the H/R protocol (Fig. 1).

2.4. In vitro vascular H/R

The mesenteric artery rings were prepared as 1–2-mm-long cylindrical segments and were quickly moved to a dish filled with oxygenated Krebs solution (in mmol/L: NaCl: 119; KCl: 4.7; $MgCl_2$: 1; $CaCl_2$: 2.5; $NaHCO_3$: 25; KH_2PO_4: 1.2; D-glucose: 11). The detailed method for this procedure was described previously (13). The rings were assigned to receive 2, 4, 8, or 12 h of ischemia using a modified 'ischemia mimetic' solution (in mmol/L: NaCl: 135; KCl: 8; $MgCl_2$: 0.5; $CaCl_2$: 1.8; NaH_2PO_4: 0.33; HEPES: 5.0; Na^+-lactate: 20; pH 6.80; bubbled with 100% N_2 for more than 45 min before the experiment was started to reduce the oxygen tension by 75%) in a hypoxic environment. Using an anaerobic incubation bag, the rings were incubated in 24-well, flat-bottom culture plates (one sample per well) with the modified 'ischemia mimetic' solution. The bag was flushed with humidified 93% N_2: 5% CO_2: 2% O_2 at 37 °C. After incubation under ischemic conditions, the medium was replaced with Dulbecco's modified Eagle's medium (DMEM) containing L-glutamine (584 mg/L) and supplemented with penicillin (100 U/mL) and streptomycin (100 mg/mL). Then, the rings were moved to a normoxic incubator (95% air: 5% CO_2), followed by 2, 6, 12, 18, or 24 h of reperfusion.

2.5. Vascular functional studies

The isometric tension of the prepared artery rings was continuously recorded using a Tai Meng BL-420F biotic signal collection and analysis system (Chengdu, China). The complete experimental details can be found in our previously published paper (13). The contractile capacity of the artery rings was tested by exposure to a high-K^+ Krebs solution (60 mmol/L KCl) prepared by substituting NaCl with an equimolar amount of KCl.

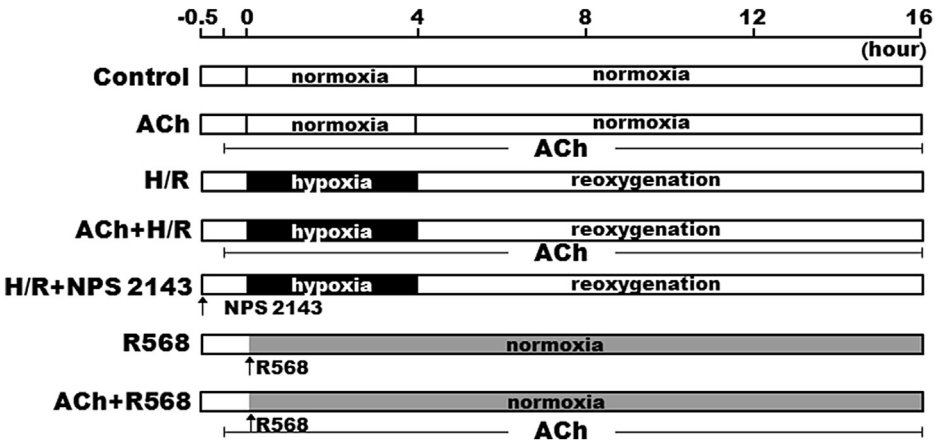

Fig. 1. Experimental protocol. Black boxes indicate periods of hypoxia induced by a modified 'ischemia mimetic' solution and a hypoxic environment, open boxes indicate periods of reoxygenation, and gray boxes indicate periods of R568 intervention. The H/R protocol consisted of 4 h of hypoxic culture with the modified 'ischemia mimetic' solution and 12 h of normoxic culture with DMEM; the ACh + H/R protocol consisted of a 15-min pretreatment period with ACh (10^{-6} mol/L) before hypoxia and continued with 4 h of hypoxia and 12 h of reoxygenation; the control and ACh protocols consisted of routine culture at 37 °C with ACh or medium; and the antagonist/agonist protocol consisted of an intervention 30 min before initiation of the H/R protocol.

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