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The role of Na^+, K^+-ATPase in the hypoxic vasoconstriction in isolated rat basilar artery

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ABSTRACT

Hypoxia-induced cerebrovascular dysfunction is a key factor in the occurrence and the development of cerebral ischemia. Na^+, K^+-ATPase affects the regulation of intracellular Ca^{2+} concentration and plays an important role in vascular smooth muscle function. However, the potential role of Na^+, K^+-ATPase in hypoxia-induced cerebrovascular dysfunction is unknown. In this study, we found that the KCl-induced contraction under hypoxia in rat endothelium-intact basilar arteries is similar to that of denuded arteries, suggesting that hypoxia may cause smooth muscle cell (SMC)-dependent vasoconstriction in the basilar artery. The Na^+, K^+–ATPase activity of the isolated basilar artery with or without endothelium significantly reduced with prolonged hypoxia. Blocking the Na^+–Ca^{2+} exchanger with Ni^{2+} (10^{-3} M) or the L-type Ca^{2+} channel with nimodipine (10^{-8} M) dramatically attenuated KCl-induced contraction under hypoxia. Furthermore, prolonged hypoxia significantly reduced Na^+, K^+-ATPase activity and increased $[Ca^{2+}]_i$ in cultured rat basilar artery SMCs. Hypoxia reduced the protein and mRNA expression of the α_2 isoform of Na^+, K^+-ATPase in SMCs *in vitro*. We used a low concentration of the Na^+, K^+-ATPase inhibitor ouabain, which possesses a high affinity for the α_2 isoform. The contractile response in the rat basilar artery under hypoxia was partly inhibited by ouabain pretreatment. The decreased Na^+, K^+-ATPase activity in isolated basilar artery and the increased $[Ca^{2+}]_i$ in SMCs induced by hypoxia were partly inhibited by pretreatment with a low concentration of ouabain. These results suggest that hypoxia may educe Na^+, K^+-ATPase activity in SMCs through the α_2 isoform contributing to vasoconstriction in the rat basilar artery.

1. Introduction

Cerebral ischemia is a condition where the brain or parts of the brain do not receive enough blood flow to maintain normal neurological function. Through blood flow, the cerebral vasculature supplies the oxygen and glucose required to maintain the normal brain metabolism. It has been demonstrated that chronic hypoxia influences vascular tone and alters both vasoconstriction and vasodilation responses in isolated cerebral vessels [2,14,25]. For example, middle cerebral arteries from hypoxic guinea pigs had greater contractile sensitivity to the vasoconstrictor U-46619 compared with those of normoxic guinea pigs ($-\log EC_{50} = 7.86 +/- 0.11$ *vs.* $7.62 +/- 0.06$, respectively, $p < 0.05$) [25]. Therefore, hypoxia-induced vascular dysfunction is a critical factor in the occurrence and development of cerebral ischemia.

Hypoxia affects all systems of the body, and causes both vasoconstriction and vasodilation. For instance, hypoxia causes vasodilation in coronary arteries, increasing blood flow and oxygen delivery to hypoxic tissues, which is an important protective response [11,24]. Hypoxia also causes pulmonary hypertension *via* vasoconstriction of the pulmonary arteries [13]. However, whether hypoxia causes vasoconstriction in the basilar artery has been unexplored.

Na^+, K^+-ATPase is a plasma membrane-embedded transport ATPase that establishes and maintains the low cytosolic Na^+ and high cytosolic K^+ concentrations in animal cells. It transports three Na^+ ions out of the cell and two K^+ ions into the cell with the hydrolysis of an ATP molecule. By maintaining the electrochemical gradient, the Na^+, K^+-ATPase coupled to the Na^+–Ca^{2+} exchanger can affect intracellular Ca^{2+} levels, and play an important role in the regulation of vascular tone [7,18].

Hypoxia was shown to alter Na^+, K^+-ATPase activity in various cell types originating from the heart, brain, and lungs by inducing severe ATP depletion [10,20,26,28]. In SV40 ATII cells cultured under hypoxic conditions for 12 h, a time-dependent decrease in ouabain-sensitive rubidium (osRb) influx was induced, an indication that hypoxia impairs Na^+, K^+-ATPase activity [20]. This hypoxia-dependent inhibition of Na^+, K^+-ATPase may result in increased pulmonary vascular tone [27]. These data imply that the Na^+, K^+-ATPase is associated with hypoxia, however, the potential role of Na^+, K^+-ATPase in hypoxia-induced cerebrovascular dysfunction is still unclear.

The first objective of the present study was to examine the effect of hypoxia on the function of isolated rat basilar arteries. The second

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objective was to explore the role of Na^+, K^+-ATPase in this process. Understanding the role of Na^+, K^+-ATPase in hypoxia-induced cerebrovascular dysfunction would contribute to the development of clinical treatments for cerebrovascular disease.

2. Materials and methods

2.1. Experimental animals

The Animal Care and Ethical Committee of Hebei Medical University (Shijiazhuang, China) approved the use of animals in this study. All the Sprague–Dawley rats, weighing 250–300 g were bred in-house under 12:12 h light–dark cycle.

2.2. Preparation of rat arterial rings

Rats were anesthetized with diethyl ether, the entire brain was removed quickly, and the basilar artery was isolated from the brain. Arterial rings of 2–3 mm were placed in a chamber containing physiological saline solution (PSS) at 37 ± 0.5 °C and aerated with 95% O_2, 5% CO_2. Two tungsten wires (40 μm) were passed through the lumen of the arterial rings. To record the isometric tension, one end of each wire was connected to an isometric transducer (Multi Myograph system-610, Danish Myo Technology) and the other was attached to the holder. The endothelium was removed by gently rubbing the inner surface.

The arterial rings were loaded with optimal tension and allowed to equilibrate for 60 min with PSS washes at 15 min intervals. Prior to experimentation, the arterial rings were stimulated three times with 1 μM norepinephrine (Sigma, St. Louis, MO) to test their functional integrity and reproducibility of responses. Endothelium removal was confirmed by the lack of relaxation in response to 1 μM acetylcholine (Ach; Sigma, St. Louis, MO) in arterial rings pre-contracted with 1 μM norepinephrine. Arterial rings were considered to have an intact functional endothelium, when 1 μM Ach produced over 80% relaxation.

For hypoxia experiments, the procedure was performed following Becker S′s method with some modification [1]. The gas mixture was changed from 95% O_2, 5% CO_2 to 95% N_2, 5% CO_2. When indicated, an inhibitor was added to the bath 30 min before application of agonist.

2.3. Cell isolation

Smooth muscle cells (SMCs) were isolated from basilar arteries and subjected to enzymatic digestion for 1 h at 37 °C in the following buffer: 130 mM NaCl, 5 mM KCl, 0.2 mM $CaCl_2$, 1.3 mM $MgCl_2$, 5 mM glucose, 10 mM HEPES, 0.5 g/l type II collagenase, and 0.1 g/l deoxyribonuclease I. Cells were grown in DMEM/F12 media supplemented with 10% fetal bovine serum, 2 mM glutamine, 10,000 U/ml penicillin, and 5 mg/ml streptomycin at 5% CO_2, 37 °C.

2.4. Hypoxic exposure

The hypoxic procedure was performed following Planès C′s method with some modifications [20]. To achieve hypoxic conditions, cells were cultured in a humidified incubator with 5% CO_2, 95% N_2 at 37 °C for 3, 6, 12, 24, or 48 h. The control normoxic cells were cultured in a humidified incubator with 5% CO_2, 95% air for the same period.

2.5. Measurement of Na^+, K^+-ATPase activity

Na^+, K^+-ATPase activity was defined as the difference between inorganic phosphates (Pi) released in the absence and presence of 1 mM ouabain corrected for spontaneous non-enzymatic ATP breakdown. After hypoxic exposure for 3, 6, 12, 24, or 48 h, cells were harvested, homogenized manually on ice, and centrifuged for 10 min at 3000 rpm. The pellets were resuspended in the same buffer and used immediately. Briefly, 10 μl samples were preincubated for 5 min at 37 °C in 1 ml buffer containing 50 mM Tris–HCl, pH 7.5, 140 mM NaCl, 14 mM KCl, 5 mM $MgCl_2$, 0.5 mM EDTA, and 1 mM ouabain. The reaction mixture contained ouabain for the Mg^{2+}-ATPase assay, but not for the total ATPase assay. Reactions were initiated by addition of 3 mM ATP solution at 37 °C. After 1 h, the reaction was stopped by adding 0.1 ml of ice-cold 5% sodium dodecyl sulfate, and the color was developed with 3 ml of acidic ammonium molybdate and 0.1 ml of ANSA reagent (25 mg 1-amino-2 naphthol-4-sulfonic acid, 1.2 g sodium metabisulfite, 120 mg sodium sulfite). The Pi in the reaction mixture was assayed according to the Yohatalou method [30], and total protein was estimated by Lowry's method. Results were expressed in micromoles of Pi per milligram of protein per hour. To determine the Na^+, K^+-ATPase activity in isolated basilar arteries, basilar artery rings were homogenized in saline (10% *w*/*v*) on ice, centrifuged at 1000 rpm for 5 min, and the supernatant was used for measuring the Na^+, K^+-ATPase activity.

2.6. RT-PCR

Total RNA was isolated using an RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The total RNA mixture was reverse transcribed to cDNA in a 25 μl reaction mixture containing 2 μg total RNA, 0.5 μl Oligo DT (0.5 μg/ml), 0.5 μl RNase inhibitor (30 U/μl), 2.5 μl dNTPs (10 mM), 5× RT buffer, and 0.4 μl AMV (5 U/μl, Promega, Beijing, China). The expression of Na^+, K^+-ATPase α_1 and α_2 isoform mRNAs was detected by semi-quantitative RT-PCR using the PCR Master Mix System (Promega). The sequences of primers used were as follows: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), forward: 5′-ACCACAGTCCATGCCATCAC-3′, reverse: 5′-TCCACCACCCTGTTGCTGTA-3′, α_1 isoform forward: 5′-AAGGACGC CTTTCAGAATGCCT-3′, reverse: 5′-TGACCATGATGACCTTAATCC-3′ and α_2 isoform forward: 5′-CACCTACTTTGTAATACTGGC-3′, reverse: 5′-ATCAGGATCTTGTTCTTCATGCC-3′. Images were quantified using densitometric analysis software (BIO-1D), and the expression of α_1 and α_2 mRNAs was normalized against the endogenous GAPDH from the same sample.

2.7. Western blot analysis

The protein expression of the α-isoforms in SMCs during 48 h hypoxia was detected by western blot. The cells were lysed in buffer (50 mM Tris–HCl, 150 mM NaCl, 5 mM Na_2EDTA, 0.5% Nonidet P-40, 0.5% Triton X-100, 0.1% SDS, 10% glycerol). The cell lysates were centrifuged at 12,000 rpm for 30 min, and the proteins in the supernatant were separated by SDS-PAGE at 120 V for 2 h and transferred to polyvinylidene difluoride (PVDF) membrane at 100 V for 3 h at 4 °C. Non-specific proteins were blocked with 1% blocking reagent in Tris-buffered saline with 0.05% Tween-20 (TBST) for 1 h at RT, followed by overnight incubation with an anti-α_1 isoform of Na^+, K^+-ATPase (1:200) and anti-α_2 (1:500) antibodies at 4 °C. Peroxidase-conjugated goat anti-rabbit (Calbiochem, La Jolla, CA) secondary antibodies were used, and the protein signals were developed using an enhanced chemiluminescence (ECL) system. The images were quantified using Snygene-Image Systems' densitometric analysis software, and the results are presented as the mean band intensity, relative to GADPH control band intensity. The mean p65 band intensity is presented as relative to histone band intensity.

2.8. Measurement of intracellular Ca^{2+}

To determine the effect of hypoxia on intracellular Ca^{2+} concentrations in SMC, we recorded the absolute levels of intracellular Ca^{2+} concentrations. A fluorometric method with Fluo-4/AM as the Ca^{2+} indicator was used to measure the cytoplasmic Ca^{2+} concentration. Cells were incubated with 1 μM Fluo-4/AM and pluronic F-127 (0.1%)