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Endothelium-independent hypoxic contraction of porcine coronary arteries may be mediated by activation of phosphoinositide 3-kinase/Akt pathway

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ABSTRACT

Phosphoinositide 3-kinase (PI3K)/Akt signaling pathway plays an essential role in the regulation of vascular tone. The present study aimed to determine its role in hypoxic coronary vasoconstriction. Isometric tension of isolated porcine coronary arteries was measured with organ chamber technique; the protein levels of phosphorylated and total MLC were examined by Western blotting; the activities of PI3K and Rho kinase were determined by the phosphorylation of their respective target protein Akt and MTPT1. Acute hypoxia induced a rapid contraction followed by a short-term relaxation and then a sustained contraction in porcine coronary arteries. The rapid but not the sustained contraction was abolished by endothelium removal. The sustained contraction was attenuated by inhibitors of PI3K (LY294002) and Akt (Akt-I). The attenuation effect caused by LY294002 was not affected by nifedipine, but was abolished by Y27632, an inhibitor of Rho kinase. The sustained hypoxic contraction was associated with altered phosphorylation of MLC and Akt, which was inhibited by LY294002. The sustained hypoxic contraction was also accompanied with increased phosphorylation of MYPT1, which was inhibited by LY294002 and Y27632. This study demonstrates that sustained hypoxia causes porcine coronary artery to contract in an endothelium-independent manner. An increased PI3K/Akt/Rho kinase signaling may be involved.

1. Introduction

Coronary artery spasm refers to an intense vasoconstriction of epicardial coronary arteries that may lead to vessel occlusion and hence acute ischemic heart disease such as angina pectoris and acute coronary syndrome [1,2]. Studies show that the coronary vasospasm can be evoked by hypoxia [3,4]. It is well known that hypoxia causes dilatation of systemic arteries but contraction of pulmonary arteries [5]. After Vanhoutte first reported that acute hypoxia caused a rapid increase in tension of canine saphenous veins [6], hypoxic vasoconstriction has been observed in a number of systemic vessel types including coronary arteries [3,4,6–12]. These studies focus on vasoconstriction which elicited acute hypoxia and demonstrate that hypoxic contraction is endothelium-dependent and dependent on the activation of soluble guanylyl cyclase by nitric oxide (NO) [7,10,11]. Karmazyn et al. found that prolonged hypoxia also caused a sustained constriction in isolated hypoxic rat heart model [3]. The underlying mechanism, however, remains unresolved.

Phosphoinositide 3-kinase (PI3K)/Akt pathway is a key modulator of vascular tone under physiologic and pathophysiologic conditions [13–16]. Activation of the pathway in endothelial cells induces vasodilatation via the formation of NO resulting from phosphorylation of endothelial nitric oxide synthase (eNOS) [17]. In vascular smooth muscle the activation of PI3K/Akt pathway causes vasoconstriction due to the stimulation of L-type calcium channels [18–21] and activation of Rho kinase [22–24] and phosphodiesterase type 5 (PDE5) [25]. PI3K/Akt signaling can be activated by hypoxia in vascular smooth muscle cells [26]. It is unclear whether or not PI3K/Akt is involved in the sustained coronary vasoconstriction caused by hypoxia.

In the present study we demonstrate that hypoxia induced a rapid contraction of porcine coronary arteries followed by relaxation

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and then a sustained contraction. The rapid contraction was dependent on the presence of endothelium as described by Vanhoutte and others [7,10,11]. The sustained hypoxic contraction was endothelium-independent and PI3K/Akt/Rho kinase signaling was critically involved.

2. Materials and methods

2.1. Porcine coronary arteries preparations

Fresh porcine hearts were collected from a local slaughterhouse. The left anterior descending arteries were carefully dissected and cut into rings (outside diameter: ~3 mm) in ice-cold modified Krebs–Ringer bicarbonate buffer [composition (in mM): NaCl, 118.3; KCl, 4.7; $CaCl_2$, 2.5; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 25.0; glucose, 11.1]. In some experiments, the endothelium was removed mechanically by inserting the tip of a small forceps through the lumen of the vessel and rolling on filter paper soaked in ice-cold buffer. The removal of the endothelium was verified by the absence of relaxant response to bradykinin (3×10^{-7} M). Animal handling and study protocols were in accordance with US National Institutes of Health guidelines. They were reviewed and approved by Animal Care and Use Review Committees of Peking University Health Science Center [27].

2.2. Vessel tension study

Rings of coronary arteries were suspended in organ chambers filled with 8 ml of the modified Krebs–Ringer bicarbonate solution maintained at 37 ± 0.1 °C and aerated with 95% O_2–5% CO_2 (pH 7.4). Two stirrups passed through the lumen suspending each ring. One stirrup was anchored to the bottom of the organ chamber, and the other was connected to a strain gauge and the isometric force was measured with a force transducer (MLT0202/D, ADInstruments Pty Ltd, Castle Hill, Australia) and recorded with an ML785 PowerLab/8sp Recording and Analysis System (ADInstruments Pty Ltd, Castle Hill, Australia).

At the beginning of the experiment, each vessel ring was stretched to its optimal resting tension. This was achieved by step-wise stretching until the active contraction of the vessel ring to 100 mM KCl reached a plateau. The optimal resting tension of porcine coronary arteries was ~2.5 g. One hour of equilibration was allowed. The effect of hypoxia (95% N_2–5%CO_2) was determined in arteries pre-constricted with U46619 or KCl in different concentrations. In some experiments the concentration–response curves of KCl and U46619 were determined after exposure of the arteries to hypoxia for 1 h. All experiments were carried out in a parallel fashion under control conditions or with different treatments [28].

2.3. Western blotting analysis

Isolated porcine coronary arteries without endothelium were incubated in Krebs–Ringer bicarbonate buffer maintained at 37 ± 0.1 °C and aerated with 95% O_2–5% CO_2 (pH 7.4) in the presence of solvent or LY294002 (10^{-5} M). The vessel rings were snappy frozen with liquid nitrogen prior to and after treatment with U46619 (3×10^{-7} M) for 20 min, and the arteries were treated with U46619 (3×10^{-7} M) for 20 min followed by hypoxia exposure for 0, 15, 30 and 60 min.

Tissue lysates were prepared from isolated porcine coronary arteries treated as aforementioned [28]. Lysate each containing 20 μg protein was subjected to SDS–PAGE, and electro-transferred to PVDF. Nonspecific binding of antibody was blocked by washing with TBS buffer containing 10% milk for 1 h. The blot was then subjected to two brief washes with TBS plus 0.5% Tween-20, incubated with the primary antibody of MLC, MLC-p(S19) (Cell Signaling Technology, MA, USA) overnight at 4°C and the secondary antibody for 1 h at room. It was developed using the chemiluminescent detection method (Amersham ECL™). Proteins of MLC, MLC-p in blots were quantified by densitometry using a Gel Doc 2000 densitometer (BIO-RAD, CA, USA) and normalized to scanning signals of actin (Calbiochem, CA, USA) [27].

2.4. PI3K and Rho kinase activity assay

The activities of PI3K and Rho kinase were evaluated by examining the phosphorylation of their respective target protein. Isolated porcine coronary arteries without endothelium were incubated in Krebs–Ringer bicarbonate buffer maintained at 37 ± 0.1 °C and aerated with 95% O_2–5% CO_2 (pH 7.4) in the presence of solvent, LY294002 (10^{-5} M) or Y27632 (10^{-5} M) for 30 min. They were then treated with U46619 (3×10^{-7} M) for 20 min followed by hypoxia exposure (95% N_2–5%CO_2) for 0, 15, 30 and 60 min before being snappy frozen with liquid nitrogen. The activity of PI3K was determined by the ratio of Akt-p(S473) over total Akt and that of Rho kinase was determined by the ratio of MYPT1-p(T853) over total MYPT1 with Western blotting. The antibodies of Akt, Akt-p(S473), MYPT1, and MYPT1-p(T853) were from Cell Signaling Technology.

2.5. Data analyses

Data are shown as means ± SEM. When mean values of two groups were compared, Student's *t* test for unpaired observations was used. Comparison of mean values of more than two groups was performed with one-way ANOVA test with Student–Newman–Keuls test for post hoc testing of multiple comparisons. Statistical significance was accepted when the *P* value (two tailed) was less than 0.05. In all experiments, *n* represents the number of animals.

2.6. Reagents

The following drugs were used (unless otherwise specified, all were obtained from Sigma, St. Louis, MO, USA): Akt-I [1,3-Dihydro-1-(1-((4-(6-phenyl-1H-imidazo(4,5-g)quinoxalin-7-yl) phenyl)methyl)-4-piperidinyl)-2H-benzimidazol-2-one trifluoroacetate salt hydrate], indomethacin, LY294002 [2-(4-Morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride], nifedipine, nitro-L-arginine, U46619 (9,11-Dideoxy-11α,9α-epoxymethanoprostaglandin F2α), and Y27632 [(R)-(+)-trans-4-(1-Aminoethyl)-N-(4-Pyridyl)cyclohexanecarboxamide dihydrochloride monohydrate].

Akt-I, LY294002, and nifedipine, were dissolved in DMSO (final concentration < 0.2%). Preliminary experiments showed that DMSO at that concentration had no effect on contraction to U46619. The other drugs were prepared with distilled water.

3. Results

Hypoxia induced a rapid contraction followed by a relaxation and then a sustained contraction in porcine coronary arteries with intact endothelium that was pre-constricted by U46619 (3×10^{-7} M; Fig. 1A). The rapid vasoconstriction was abolished by the removal of the endothelium (Fig. 1A) or treatment with nitro-L-arginine (NLA, 10^{-4} M; Fig. 1B), but not by indomethacin (3×10^{-5} M; Fig. 1B). The sustained contraction was not affected by denudation of endothelium or incubation with NLA and indomethacin (Fig. 1A and B). The rapid contraction caused by hypoxia was also observed in porcine coronary arteries constricted with KCl (20–60 mM) and U46619 at a lower concentration (3×10^{-8} M; Fig. 1C). The increases in tension induced by U46619 prior to hypoxic exposure in arteries without endothelium or in the presence of NLA were greater than those in the respective controls (Fig. 1A and B). When the control arteries were contracted to a tension level similar to those treated with NLA using different concentrations of U46619 (3×10^{-8} M and 3×10^{-8} M, respectively) hypoxia-induced rapid contraction was observed in the control group but not in the NLA group (Fig. 1D). The sustained contraction evoked by hypoxia