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PKC plays an important mediated effect in arginine vasopressin induced restoration of vascular responsiveness and calcium sensitization following hemorrhagic shock in rats

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ABSTRACT

The present study investigated the mediated effect of protein kinase C (PKC) in arginine vasopressin (AVP)-induced restoration of vascular responsiveness and calcium sensitization following hemorrhagic shock. Using both isolated superior mesenteric artery from hemorrhagic shock rats and hypoxia-treated vascular smooth muscle cell (VSMC), we investigated the roles of PKC-α, δ and ε isoforms in AVP-induced restoration of vascular reactivity and calcium sensitivity. Meanwhile, effects of their specific inhibitors on the activity of myosin light chain phosphatase (MLCP), myosin light chain kinase (MLCK), and the phosphorylation of myosin light chain (MLC_{20}) in VSMC were observed. The results indicated that AVP improved the reactivity of superior mesenteric artery and VSMC to norepinephrine and calcium following hemorrhagic shock and hypoxia. PKC-α inhibitor and PKC-ε inhibitory peptide antagonized these effects of AVP, while PKC-δ inhibitor only partially antagonized these effects of AVP. AVP up-regulated the expression of PKC-α and ε in the particulate fractions of hypoxia-treated VSMC with the decrease of the activity of MLCP and the increase of the phosphorylation of MLC_{20}. These effects of AVP were inhibited by PKC-α inhibitor and PKC-ε inhibitory peptide, but not by the PKC-δ inhibitor. The results suggested that PKC plays an important role in AVP-induced restoration of vascular reactivity and calcium sensitivity following hemorrhagic shock. PKC-α and ε may be the main isoforms involved in this process and play effect via MLC_{20} phosphorylation dependent mechanism, while PKC-δ may be partially involved in AVP action by other mechanisms.

1. Introduction

Previous studies, in our laboratory and others, have demonstrated that after severe trauma or shock, vascular response to vasoconstrictors is reduced (Hasan and McDonough, 1997; Liu et al., 2003). This reduced vascular reactivity (vascular hyporesponsiveness) plays an important role in the development and the outcome of shock. Our previous studies have demonstrated that hemorrhagic shock caused calcium desensitization of blood vessels, which played an important role in the occurrence of vascular hyporeactivity following hemorrhagic shock (Liu et al., 2003; Xu and Liu, 2005). Arginine vasopressin (AVP) is a neurohypophysial hormone that has multiple physiological effects. A large body of research showed that AVP had good effect on vasodilatory or septic shock that was closely associated with its potent vasopressor effects (Parrillo, 2008; Russell et al., 2008). Recent observations from our laboratory showed that small doses of AVP had beneficial effects on hemorrhagic shock, which may be related to AVP-induced increase of vascular reactivity and calcium sensitivity of vascular smooth muscle via the activation of Rho-kinase (Yang et al., 2006a). However, the Rho-kinase antagonist, Y-27632 only partially blocked this effect of AVP (Yang et al., 2006a), which suggested that there may be other mechanisms involved.

Our previous study showed that protein kinase C (PKC) took part in the regulation of calcium sensitivity of vascular smooth muscle after hemorrhagic shock (Xu and Liu, 2005; Yang et al., 2008). PKC is comprised of three classes, and 12 isoforms (Kitazawa et al., 2000). Multiple PKC isoforms, such as α, β, γ, δ, ε and ζ are co-expressed in VSMC, but the main subtypes of PKC distributed in blood vessels are PKC-α, δ and ε. However, under the shock state, it is unknown which isoforms of PKC are involved in the protective effects of AVP on vascular reactivity and calcium sensitivity and how they participated.

Basic research shows that myosin light chain phosphatase (MLCP) plays a pivotal role in the regulation of calcium sensitivity of vascular smooth muscle cell (VSMC) through myosin light chain (MLC_{20}) phosphorylation (Kolosova et al., 2004; Salamanca and Khalil, 2005). PKC can regulate the calcium sensitivity of VSMC by inhibiting the

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activity of MLCP (Seko et al., 2003; Swärd et al., 2003). Based on the literature and our previous findings, we hypothesized that AVP could improve vascular calcium sensitivity through PKC-MLCP-MLC_{20} pathway to reverse shock-induced vascular hyporeactivity. To confirm this hypothesis, with superior mesenteric artery from hemorrhagic shock rats and hypoxia-treated VSMC, effects of PKC-α, δ and ε isoforms in AVP-induced restoration of vascular reactivity and calcium sensitivity following shock and effect of their specific inhibitor on the activity of MLCP, myosin light chain kinase (MLCK), and the phosphorylation of MLC_{20} in VSMC were investigated.

2. Material and methods

2.1. Animal and VSMC preparation

2.1.1. Animal model

This study was approved by the Research Council and Animal Care and Use Committee of Research Institute of Surgery, Daping Hospital, the Third Military Medical University. The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

One hundred and twenty Wistar rats, weighing 200–250 g, both male and female, were fasted for 12 h but allowed water *ad libitum* before the experiment. After anesthesia and the completion of the surgical procedure using routine method as described before in our lab (Li et al., 2008), rats were hemorrhaged and the mean arterial pressure was maintained at 30 mmHg for 2 h. At the end of shock, a laparotomy was performed and superior mesenteric artery were isolated and its endothelium was denuded to exclude the effect of endothelium on vascular reactivity in the present experiment by gently rubbing the intimal surface with a thin stainless steel tube. The diameter of stainless steel tubes was about 80–90% of the vessel lumen diameter.

2.1.2. Culture of VSMCs and treatment with hypoxia

Primary cultured vascular smooth muscle cells were obtained from the mesenteric artery of normal Wistar rats by an explant technique (Leik et al., 2004). The third to fifth passage of cells were used in the present study. The cells were serum starved for 24 h before the experiments. For hypoxic challenges, VSMCs were transferred into a hypoxia chamber, bubbled with 95% N_2 and 5% CO_2 at 10 L/min for 10 min, and then equilibrated for 5 min. This procedure was repeated thrice until the O_2 concentration in the chamber was less than 0.2% (Webster et al., 1993). Hypoxia-treated VSMCs was used for subsequent experiments.

2.2. Experimental protocol

2.2.1. Role of PKC-α, δ and ε in AVP-induced restoration of vascular reactivity and calcium sensitivity after hemorrhagic shock

2.2.1.1. Reactivity of isolated artery rings. With endothelium-denuded superior mesenteric artery rings, the role of PKC-α, δ and ε isoforms on AVP-induced increase of vascular reactivity and calcium sensitivity after hemorrhagic shock was investigated. Seventy two superior mesenteric arteries from sham-operated and hemorrhagic shock rats were randomized into nine groups ($n=8$/group): normal control (sham-operated) group, shock control group, shock + AVP (5×10^{-10} mol/l, Sigma, St. Louis, MO, USA) group, shock + PKC-α inhibitor, 12-(2-Cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo(2,3-a) pyrrolo(3,4-c)-carbazole (Gö6976, 5×10^{-6} mol/l, Merck, Darmstadt, Germany) group, shock + Gö6976 + AVP group, shock + PKC-δ inhibitor, 1-[6-[(3-Acetyl-2,4,6-trihydroxy-5-methylphenyl)methyl]-5,7-dihydroxy-2,2-dimethyl-2H-1-benzopyran-8-yl]-3-phenyl-2-propen-1-one (Rottlerin, 10^{-5} mol/l, Sigma) group, shock + Rottlerin + AVP group, shock + PKC-ε inhibitory peptide (10^{-5} mol/l, Merck) group, and shock + PKC-ε inhibitory peptide + AVP group. Each superior mesenteric artery was cut into two rings of 2–3 mm long. One was used for the measurement of vascular reactivity, and the other was used for the measurement of calcium sensitivity.

The vascular reactivity and calcium sensitivity of superior mesenteric artery were observed by measuring the contraction initiated by increasing doses of norepinephrine and calcium under depolarizing condition (120 mmol/l K^+) using an isolated organ perfusion system (Scientific Instruments, Barcelona, Spain) as described previously (Yang et al., 2006b; Zhou et al., 2005). Before measurement, artery rings in shock + AVP group, shock + Gö6976 group, shock + Rottlerin group and shock + PKC-ε inhibitory peptide group were incubated with AVP (5×10^{-10} mol/l), Gö6976 (5×10^{-6} mol/l), Rottlerin (10^{-5} mol/l) and PKC-ε inhibitory peptide (10^{-5} mol/l) respectively for 20 min, artery rings in shock + Gö6976 + AVP group, shock + Rottlerin + AVP group and shock + PKC-ε inhibitory peptide + AVP group were incubated with Gö6976 (5×10^{-6} mol/l), Rottlerin (10^{-5} mol/l) and PKC-ε inhibitory peptide (10^{-5} mol/l) respectively for 20 min, followed by AVP (5×10^{-10} mol/l) for 20 min, then the reactivity of artery rings to norepinephrine and calcium was determined. The cumulative concentration–response curves of superior mesenteric artery to norepinephrine and calcium were constructed. The maximal contraction and pD_2 ($-\log$[Fifty percent effective concentration, EC_{50}] of agonists) were obtained from the concentration–response curves and used to compare the vascular reactivity, calcium sensitivity and the affinity of agonists to their receptors.

Before measuring the reactivity of artery rings to norepinephrine or calcium, endothelium denudation was verified with acetylcholine. Endothelium intact superior mesenteric artery served as controls. Artery rings were precontracted with norepinephrine at a concentration of 10^{-5} mol/l, and 5 min later the relaxing effect of acetylcholine (10^{-5} mol/l) with or without endothelium was observed. The maximal relaxation of artery rings after acetylcholine administration was used to determine whether the endothelium of artery rings was denuded, the calculation formula for the maximal relaxation was: $(E_{\text{norepinephrine}}-E_{\text{acetylcholine}})/E_{\text{norepinephrine}}\times100\%$ ($E_{\text{norepinephrine}}$: maximal tension of artery rings after norepinephrine administration; $E_{\text{acetylcholine}}$: tension of artery rings after acetylcholine administration).

2.2.1.2. Reactivity of cultured VSMCs. The third to fifth passage of VSMCs were plated on the collagen-coated polyethylene trephtalate cell culture inserts of each transwell (3 μm pore size, Corning, USA), which were set into 24-well culture plates. The lower compartment of the transwell was filled with 600 μl medium and cultured for 48 h. They were divided into six groups ($n=6$/gp): normal control group, 90-min hypoxia group, 90-min hypoxia + AVP group, 90-min hypoxia + Gö6976 + AVP group, 90-min hypoxia + Rottlerin + AVP group and 90-min hypoxia + PKC-ε inhibitory peptide + AVP group.

After 90 min hypoxia, VSMCs in hypoxia + AVP group were incubated with the AVP (5×10^{-10} mol/l) for 20 min, VSMCs in hypoxia + Gö6976 + AVP group, hypoxia + Rottlerin + AVP group and hypoxia + PKC-ε inhibitory peptide + AVP group were incubated with Gö6976 (5×10^{-6} mol/l), Rottlerin (10^{-5} mol/l) and PKC-ε inhibitory peptide (10^{-5} mol/l) respectively for 20 min, followed by AVP (5×10^{-10} mol/l) for 20 min. The contractile response of VSMC to norepinephrine was determined by measuring the infiltration ratio of fluorescein isothiocyanate-conjugated bovine serum albumin (FITC-BSA, Sigma) (Essler et al., 1998; Li et al., 2008).

2.2.1.3. Expression of PKC-α, δ and ε isoforms. Similarly, the third to fifth passage of VSMCs were divided into normal control group, 90-min hypoxia group and 90-min hypoxia + AVP group. After treatment with hypoxia and/or AVP as described above, the cells were scraped with ice-cold lysis buffer (mmol/l: 20 Tris–HCl, pH 6.8, 2 EDTA, 1 EGTA, 1 Na_3VO_4, 10 NaF, 0.1% β-mercaptoethanol, protease inhibitor cocktail) and suspended on ice for 60 min. The particulate (membrane) and