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Role of Ras/PKCζ/MEK/ERK1/2 signaling pathway in angiotensin II-induced vascular smooth muscle cell proliferation

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Abstract

The role of protein kinase C (PKC) and its cross talk with extracellular signal-regulated kinase (ERK) cascade in angiotensin II (AngII)-elicited vascular smooth muscle cell (VSMC) proliferation are still unclear. In this study, the PKC pathway of AngII to activate ERK1/2 and induce cell proliferation was investigated in rat aortic smooth muscle cells. The proliferation of VSMCs was tested by [^{3}H]-thymidine incorporation assay. Phosphorylated and non-phosphorylated PKCζ, ERK1/2, Elk-1, and mitogen-activated ERK-activating kinase (MEK) were estimated by Western blot analysis. The interactions of signal molecules were examined by immunoprecipitation. AngII-induced VSMC proliferation and activation of ERK1/2 and nuclear transcription factor Elk-1 were all down-regulated by PKC non-specific inhibitor (staurosporine) and PKCζ pseudosubstrate inhibitor (PS-PKCζ). Dominant negative Ras transfection into VSMCs decreased AngII-induced PKCζ and ERK1/2 phosphorylation. AngII stimulated the association of PKCζ with Ras. AngII-induced MEK phosphorylation was inhibited by PKCζ pseudosubstrate inhibitor and the PKCζ–MEK complex was detected by immunoprecipitation. These results suggest that PKCζ isoform is involved in VSMC proliferation and Elk-1 activation. AngII can activate ERK1/2 by Ras/PKCζ/MEK pathway, which may be one of the important signal transduction pathways in AngII-induced VSMC proliferation.
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1. Introduction

Angiotensin II (AngII), the main peptide hormone of the renin–angiotensin system, is considered to contribute to the development of various cardiovascular diseases such as hypertension, atherosclerosis, and restenosis because of its growth-promoting effect on vascular smooth muscle cells (VSMCs). It is important to define the signaling pathway of AngII that mediate the growth response of VSMCs.

AngII promotes VSMC proliferation through the action of the G protein-coupled AT1 receptor. Recent studies have demonstrated that AngII can induce cell proliferation by multiple signal transduction pathways. The extracellular signal-regulated kinase (ERK) cascade is one of the important pathways. Although the mechanisms of AngII-mediated ERK activation are not fully understood, it has been established that epidermal growth factor (EGF) receptor transactivation is involved. The possible connection between the receptor tyrosine kinase and the AngII receptor may involve non-receptor tyrosine, reactive oxygen species and/or cleavage of an EGF receptor ligand such as heparin binding-EGF (HB-EGF) by a metalloproteinase [1–3]. Activated EGF receptor tyrosine kinase can phosphorylate ERK1/2 through Grb2/Sos/Ras/Raf/mitogen-activated ERK-activating kinase (MEK) pathway,

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finally resulting in altered gene expression, DNA synthesis and cell proliferation [3–5].

Protein kinase C (PKC) is a key regulatory kinase involved in the regulation and cross talk between signal transduction pathways associated with various cellular functions [3,4]. It has also been implicated in the mitogenic response and growth of a number of different cell types such as VSMCs [6–8], airway smooth muscle cells [9] and fibroblasts [10]. PKC stimulation activates ERK through different pathways depending on the cell types, both Ras-dependent and Ras-independent mechanisms are involved. In GN4 rat liver epithelial cells, AngII activated ERK via a PKC-dependent, Ras-independent pathway [11]. In bovine adrenal glomerulosa cells, AngII-induced ERK activation was mediated via both PKC and Ras [12]. In cardiac fibroblasts, activation of ERK by AngII was independent of PKC [13].

In VSMCs, the role of PKC in AngII-mediated ERK activation is still controversial. PKC was found to be involved in VSMC proliferation induced by many growth factors including AngII and platelet-derived growth factor (PDGF) [6–8]. However, Eguchi reported that ERK activation could not be inhibited by pretreatment of the PKC inhibitor GF109203X or PKC depletion by overnight exposure to a phorbol ester [14]. PKCζ was reported to mediate AngII-induced ERK activation [15]. However, the exact signal transduction pathway by which PKC mediates the proliferation of VSMCs is still not fully understood.

The present study was conducted to investigate the signal pathway of VSMC proliferation induced by AngII via PKC. The results indicate that PKCζ is involved in AngII-dependent VSMC proliferation and transcription factor Elk-1 activation. AngII can activate ERK1/2 by Ras/PKCζ/MEK pathway, which may be one of the important signal transduction pathways in AngII-induced VSMC proliferation.

2. Materials and methods

2.1. Animals and reagents

Healthy male Sprague–Dawley rats were supplied by the Experimental Animal Center of Peking University Health Science Center. All antibodies (phospho-ERK1/2 and ERK1/2, phospho-PKCζ and PKCζ, phospho-MEK and MEK, Ras) and PKCζ pseudosubstrate inhibitor (PS-PKCζ) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA), staurosporine, collagenase and elastase from Sigma (St. Louis, MO), chemiluminescence reagents from Amersham Pharmacia Biotech (Little Chalfont, UK), transfection reagents from Invitrogen (Carlsbad, CA), [^{3}H]-thymidine from Atomic Energy Institute of Chinese Academy of Sciences (Beijing, China). Dominant negative Ras and pEGFP-N1 plasmids were kindly provided by Dr. Dalong Ma (Department of Molecular Immunology, Peking University Health Science Center). The other reagents were all of analytical grade.

2.2. Cell culture of VSMCs

Primary cultures of rat VSMCs were prepared by enzymatic digestion. Briefly, rat thoracic aorta was digested in 2 g/l collagenase solution after removal of external connective tissues, then, the adventitial layer and endothelial cells were removed. The tissues were digested in fresh enzyme solution (2 g/l collagenase and 0.25 g/l elastase) for about 2 h at 37 °C. The cells were collected and resuspended in DMEM medium containing 20% fetal bovine serum and incubated at 37 °C in an incubator containing 5% CO_2. The passaged VSMCs were maintained in DMEM medium containing 10 mM sodium pyruvate and 10% fetal bovine serum. The identity of the VSMCs was confirmed by morphological examination and by staining for α-actin. VSMCs of 3rd to 10th passages were used for the experiments.

2.3. [^{3}H]-thymidine incorporation assay

For proliferation assays, VSMCs were plated at 5×10^4 cells/ml in 96-well plates, reaching about 70% confluence, cells were growth-arrested by incubating the cells in serum-free DMEM for 24 h. The cells were stimulated with 10^{-7} M AngII for 48 h and 1 μCi/ml [^{3}H]-thymidine was added to the cultures in each well during the last 12 h of incubation. When inhibitors were used, they were applied 30 min or 1 h prior to the addition of AngII. Incorporated [^{3}H]-thymidine was collected on glass fiber filters and the radioactivity was counted by β-liquid scintillation counter (Beckman. LS 6500).

2.4. Plasmid transfection

VSMCs were grown to 70% confluence in 100 mm dishes and transiently transfected with dominant negative or wild type Ras plasmids using Lipofectamine PLUS™ Reagent following a protocol recommended by the manufacturer. After 24 h transfection, cells were starved in serum-free DMEM for 24 h before treatment with AngII and inhibitors. The efficiency of transfection of VSMCs with dominant negative or wild-type Ras plasmid was estimated by cotransfection with a green fluorescent protein plasmid pEGFP-N1.

2.5. Cytosolic and nuclear extract preparation

When culture terminated, VSMCs were washed three times with PBS and lysed in lysis buffer (50 mM Tris–HCl, pH 7.2, 150 mM NaCl, 40 mM NaF, 0.1% sodium deoxycholate, 1% Triton X-100, 5 mM EDTA, 5 mM EGTA, 2.175 mM sodium orthovanadate, 0.1% SDS, 0.1% aprotinin, 1 mM PMSF) at 4 °C for 30 min. The lysate was

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