Angiotensin II-Induced Generation of Reactive Oxygen Species Is Regulated by a Phosphatidylinositol 3-Kinase/L-Type Calcium Channel Signaling Pathway

Jin Seo Yeon, Ha Jung Min, Kim Young Whan, Lee Hye Sun and Bae Sun Sik*

MRC for Ischemic Tissue Regeneration, Medical Research Institute, Department of Pharmacology, Pusan National University School of Medicine, Yangsan 626-870, Korea

Received January 3, 2015 /Revised January 28, 2015 /Accepted February 2, 2015

Angiotensin II (AngII) is an essential hormone that affects vascular physiology. For example, stimulation of vascular smooth muscle cells (VSMCs) rapidly induces vasoconstriction and results in the up-regulation of blood pressure. Chronic stimulation of VSMCs with AngII also results in hypertrophy. In this study, we confirmed an involvement of phosphatidylinositol 3-kinase (PI3K)-dependent calcium mobilization in AngII-induced generation of reactive oxygen species (ROS). Stimulation of rat aortic smooth muscle cells (RASMCs) with AngII significantly induced the generation of ROS in a dose- and time-dependent manner. AngII-induced generation of ROS was completely abolished by pharmacological inhibition of PI3K (with LY294002), but inhibition of the ERK signaling pathway had no effect. AngII-induced calcium mobilization was completely blocked by inhibition of PI3K, whereas inhibition of the ERK signaling pathway by PD98059 was ineffective. Depletion of extracellular calcium or inhibition of the L-type calcium channel by nifedipine completely blocked AngII-induced calcium mobilization. Depletion of extracellular calcium by EGTA and incubation of RASMCs with calcium-free medium both significantly blocked AngII-induced ROS generation. Inhibition of the L-type calcium channel also significantly blocked AngII-induced ROS generation. These results suggest that AngII-induced ROS generation is regulated by calcium mobilization, which, in turn, is modulated by a PI3K/L-type calcium channel signaling pathway.

Key words: Angiotensin, L-type calcium channel, PI3K (phosphatidylinositol 3-kinase), ROS (reactive oxygen species), smooth muscle cell

Introduction

The renin-angiotensin-aldosteron (RAA) system is an important regulator of blood pressure, blood flow, and electrolyte homeostasis. Angiotensin II (AngII) which is produced by sequential proteolytic cleavage of angiotensinogen plays a crucial role in the regulation of systemic arterial blood pressure. For example, arterial blood pressure is rapidly increased by AngII-induced vasoconstriction [22-23]. In addition to this acute role of AngII in vasoconstriction, chronic stimulation of vessel walls with AngII facilitates hypertrophy and hyperplasia of vascular smooth muscle cells (VSMCs) [2].

Angiotensin receptor (ATR) comprises two distinct receptors including angiotensin type 1 receptor (AT1R) and angiotensin type 2 receptor (AT2R). These receptors are coupled with G proteins. For example, AT1R is coupled with Gq or G12/13 and subsequently activates phospholipase C (PLC) [18]. On the other hand, AT2R is coupled with Gs followed by activation of protein kinase A (PKA). Activation of AT1R by AngII results in intracellular calcium mobilization which binds to calmodulin and activates myosin light chain kinase (MLCK) [24]. On the other hand, activation of AT2R receptor by AngII results in the chronic vascular response such as proliferation, migration, and growth. Although PLC and PKA seems to be major signaling molecules for AngII, multiple signaling pathways such as tyrosine kinase, MAPKs, Akt, and redox-sensitive transcription factors are also involved in the AngII-dependent regulation of VSMC physiology [18-19].

Reactive oxygen species (ROS) are highly reactive molecules that are generated during normal physiological process as well as under various stress conditions [14]. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system is one of the major sources for ROS generation in
vasculature. It consists of a catalytic subunit (Nox1, Nox2, Nox3, Nox4, or Nox5) and regulatory subunit (p22phox, p47phox, p67phox, and Rac1) [7]. The generation of ROS, most notably superoxide and hydrogen peroxide, in VSMCs can be regulated by vasoactive hormones including AngII [8]. In addition, it has been reported that platelet-derived growth factor (PDGF) [9].

Recently, it has been reported that activation of Nox1 plays pivotal role in AngII-induced generation of ROS [8]. Since AT1R is coupled with Gq, activation of PLC and subsequent generation of inositol-trisphosphate (IP3) and diacylglycerol (DAG) is important for the generation of ROS [18]. For instance, PKC is activated by DAG and calcium which is mobilized by IP3 from endoplasmic reticulum. Activation of PKC leads to the phosphorylation of p47phox which is a key regulatory subunit for NADPH oxidase [15].

Phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway is activated by many types of cellular stimuli and regulates fundamental cellular functions such as transcription, translation, proliferation, growth, and survival [1]. Binding of growth factors to their cognate receptor tyrosine kinase (RTK) or G protein-coupled receptors (GPCR) stimulates class Ia and Ib PI3K isoforms, respectively. PI3K catalyzes the production of phosphatidylinositol-3,4,5-triphosphate (PIP3) at the cell membrane. The role of ROS in the regulation of signaling molecules has been unveiled so far. For example, generation of ROS is required for AngII-induced activation of Akt in VSMCs [18]. In addition, ROS modulates intracellular free calcium concentration which is a major determinant of AngII-dependent vascular contraction. Up-regulation of intracellular calcium concentration by ROS enhances expression of AT1R, which in turn further stimulates calcium signaling [11]. However, there is no direct evidence that shows the involvement of PI3K and calcium signaling in the generation of ROS. In the present study, we showed that PI3K-dependent mobilization of intracellular calcium plays critical role in AngII-induced generation of ROS.

Materials and Methods

Materials

Dulbecco's modified eagle medium (DMEM) culture medium, fetal bovine serum (FBS), trypsin EDTA, and antibiotics were purchased from Hyclone Laboratories, Inc. (Logan, UT, USA). Fura-2/AM was purchased from Invitrogen (Grand Island, NY, USA) and PI3K inhibitor (LY294002) and ERK inhibitor (PD98059) were obtained from Merk Millipore (Billerica, MA, USA). Calcium inhibitor (Nifedipine), DCF-DA, and AngII were purchased from Sigma-Aldrich (St Louis, MO, USA). All other high quality reagents were purchased from Sigma-Aldrich unless otherwise indicated.

Rat aortic smooth muscle cell (RASMC) preparation

Pathogen-free Sprague-Dawley rats (3 to 4 weeks old) were housed under diurnal lighting conditions and allowed unlimited access to feed and tap water. All animal experiments were performed under university animal welfare guidelines (PNU-2009-2008). Rat aortic smooth muscle cells (RASMCs) were isolated from the thoracic aorta by a tissue explanting method as described previously [5]. Briefly, sodium barbital-anesthetized rats were perfused with PBS for 5 min. Thoracic aorta was aseptically isolated and surrounding fat and connective tissues were discarded. Vessels were longitudinally cut and the lumen side was scraped with a razor blade to remove the intima. Vessels were fragmented into 3-5 mm lengths and explanted lumen side down on collagen-coated culture dishes. Tissue fragments were maintained in DMEM/10% FBS with changing of the medium every two days. After seven days of explanting, tissue fragments were discarded and sprouted RASMCs were collected and used (passage 2 to 4) for experiments.

ROS generation assay

To measure ROS generation, RASMCs were grown in 48-well plates, after being starved for 12 hr, RASMCs were incubated with 2',7'-dichlorofluorescein diacetat (DCF-DA, 20 μM) for 1 hr and then stimulated with AngII (1 μM) for 15 min under the indicated conditions. Cells were washed three times with phosphate-buffered saline (PBS). Fluorescence was detected using a fluorescence microscope at 10× magnification (Axiovert200, Carl Zeiss, Jena, Germany). Pixel intensity in a field was measured by MetaMorph software (Molecular Devices, Sunnyvale, CA, USA).

Measurement of cytosolic calcium concentration

Intracellular calcium concentration was measured using Fura-2/AM, a calcium-sensitive fluorescent dye, as described previously [3]. Briefly, a total of 1×10^5 RASMCs were incubated with 3 μM Fura-2/AM at 37°C in fresh serum-free DMEM medium with stirring for 50 min. A total of 1×10^5 cells were aliquoted into Locke’s solution (154 mM NaCl, 5.6 mM KCl, 1.2 mM MgCl₂, 5 mM HEPES pH 7.3, 10 mM glucose, 2.2 mM CaCl₂ and 0.2 mM EGTA). Fluorescence
was measured at emission wavelength of 500 nm using and excitation wavelength of 340 nm and 380 nm.

**Statistical analysis**

Data are expressed as means ± S.D. of three independent experiments (n = 3 for each experiment). The statistical differences between treated and non-treated groups were analyzed by an unpaired Student’s t-test to assess differences. P-values less than 0.05 were considered significant and indicated by ∗.

**Results**

**AngII stimulates ROS generation in RASMCs**

To ascertain capability of AngII in generation of ROS, we examined the effect of AngII on the generation of ROS in RASMCs. AngII (1 μM) significantly induced the generation of ROS in a time- and dose-dependent manner (Fig. 1). Production of ROS reached a maximum within 15 min after stimulation (Fig. 1A and 1B). AngII evoked ROS generation at concentrations between 1 nM and 1 μM (Fig. 1C and 1D). These results indicate that AngII stimulates ROS generation in RASMCs.

**AngII-induced ROS generation is regulated by PI3K**

Previously, it has been reported that PI3K is involved in the AngII signaling pathway. To examine whether PI3K is involved in the generation of ROS, kinase activity of PI3K was shunted by pharmacological inhibitor (LY294002, 10 μM). As shown in Fig. 2, inhibition of PI3K completely blocked AngII-induced generation of ROS, whereas inhibition of ERK signaling pathway by PD98059 (10 μM) had no effect. These results indicate that PI3K plays an essential role in AngII-induced generation of ROS in RASMCs.

**AngII-induced calcium mobilization through PI3K and L-type calcium channel**

Since activation of PI3K was necessary for AngII-induced generation of ROS (Fig. 2), we examined whether PI3K activity is required for the AngII-induced calcium mobilization which plays an essential role in the production of ROS in VSMCs. As shown in Fig. 3, inhibition of PI3K significantly attenuated AngII-induced calcium mobilization. However, inhibition of ERK did not affect. AngII-induced calcium mobilization was also significantly blocked by extracellular calcium chelation by EGTA. In addition, inhibition of L-type calcium channel by nifedipine (20 μM) significantly blocked AngII-induced calcium mobilization. These results indicate that AngII-induced calcium mobilization is mediated by PI3K and L-type calcium channel.

**ROS is generated by L-type calcium channel**

Since calcium mobilization of RASMCs was regulated by
**Fig. 3.** PI3K and L-type calcium channel-dependent calcium mobilization. (A) RASMCs were pretreated with each pharmacological inhibitor of PI3K (LY294002, 10 μM), ERK (PD98059, 10 μM), or L-type calcium channel (Nifedipine, 20 μM) for 20 min. In some condition, AngII-induced calcium mobilization was measured in the presence of EGTA (5 mM) in buffer. The time of AngII stimulation is indicated by an arrow head. (B) Calcium mobilization was measured as described in “Materials and Methods.” Data are means ± S.D. of three independent experiments. Asterisks indicate statistical significance (p<0.05).

L-type calcium channel, we examined the role of L-type calcium channel during AngII-induced generation of ROS. As shown in Fig. 4, chelation of extracellular calcium by EGTA (5 mM) or calcium-free buffer strongly inhibited AngII-induced ROS generation. In addition, blocking of L-type calcium channels by nifedipine (20 μM) completely abolished AngII-induced ROS generation. These results suggest that ROS is generated by calcium mobilization which is regulated by L-type calcium channel.

**Discussion**

ROS play an important role as modulators of AngII signal transduction in VSMC [16]. In the present study, we provided significant role of calcium mobilization by PI3K/L-type calcium channel during AngII-induced ROS generation. Plethora of reports suggests that AngII mainly activates p38 MAPK, ERK1/2 and Akt signaling pathways. In addition, our data support the idea that PI3K plays an essential role in the generation of ROS although the mechanism underlying activation of PI3K is still unclear.

Calcium mobilization plays critical roles in many VSMC physiologies. There are two main pathways that lead to intracellular calcium mobilization. First, activation of PLC generates IP3, which bind to calcium channel on the surface of endoplasmic reticulum. Second, subsequent activation of G protein releases Gβγ subunit which in turn binds to p110 catalytic subunit of PI3K. Activation of PI3K activates L-type calcium channel on the plasma membrane thereafter stimulates calcium uptake from extracellular fluid [12, 21]. In correlation with this, our results showed that inhibition of PI3K completely blocked AngII-induced calcium mobilization (Fig. 3). In addition, depletion of extracellular calcium or inhibition of L-type calcium channel significantly abolished AngII-induced calcium mobilization (Fig. 4). It is also noteworthy that inhibition of MAPK signaling pathway had no effect on the mobilization of calcium (Fig. 3), indicating that MAPK signaling pathway is not involved in the calcium mobilization. Therefore, PI3K/L-type calcium channel axis seems to be important pathways during AngII-induced calcium mobilization.

Several lines evidence support that calcium mobilization is critical for the ROS generation in VSMCs. For example, reciprocal regulation of calcium and ROS retains many VSMC physiologies [17]. It is noteworthy that our results showed that inhibition of L-type calcium channel or depletion of extracellular calcium abolished AngII-induced calcium mobilization (Fig. 3). In addition, blocking of L-type calcium channel and chelation of extracellular calcium completely blocked AngII-induced ROS generation (Fig. 4), suggesting that calcium mobilization is prerequisite for ROS generation.

**Fig. 4.** AngII-induced ROS generation is mediated by L-type calcium channel. (A) RASMCs were pretreated with EGTA (5 mM), nifedipine (20 μM), or incubated in calcium-free media for 1 hr, and followed by stimulation with AngII for 15 min. Images were taken under fluorescent microscope at 10× magnitude. (B) Fluorescence intensity was measured by MetaMorph software. Data are means ± S.D. of three independent experiments (n=3). Asterisks indicate statistical significance (p<0.05). Scale Bar, 200 μm.
generation. Particularly, our results demonstrated that calcium mobilization-dependent ROS generation was regulated by PI3K/L-type calcium channel pathways. Currently, we think that the source of ROS generation is NADPH oxidase since stimulation of VSMCs with nicotinamide adenine dinucleotide phosphate (NAPDH) oxidase inhibitors such as diphenylene iodonium and apocynin (data shown). Currently, we are investigating the activation mechanism of NADPH oxidase by AngII stimulation.

Role of ROS seems to be diverse. AngII is involved in the pathogenesis of cardiac hypertrophy, hypertension and restenosis. For example, it has been observed that activation of NADPH oxidase and subsequent production of ROS is major leading cause of AngII-induced hypertension [13]. The molecular mechanism underlying ROS-dependent regulation of pathophysiology are currently ambiguous, however, the function role of ROS seems to reside in the modulation of signaling cascade. For instance, AngII-dependent activation of Akt was attenuated by both NADPH oxidase inhibitor (DPI) and overexpression of catalase, suggesting that ROS plays as a potential signal transducer linking AT1R to the PI3K/Akt pathway in VSMCs [6, 18]. In addition to ROS-dependent activation of PI3K/Akt signaling pathway, activation of p38 MAPK and Jun-N-terminal kinase (JNK) requires AngII-dependent production of ROS [10, 20]. More importantly, chronic production of ROS suppresses the expression of AT1R thereby counter balance the AngII signaling pathways [11]. Therefore, ROS plays role as a positive as well as negative regulator in AngII-mediated signaling pathways. In this regard, production of ROS in VSMCs may play key roles in AngII-dependent regulation VSMC physiology such as vasoconstriction, restenosis, and vascular hyperplasia.

In summary, AngII strongly induced the production of ROS in VSMCs. AngII-dependent activation of PI3K plays key role in the mobilization of intracellular calcium. PI3K-dependent calcium mobilization is mediated by L-type calcium channel. Finally, L-type calcium channel-dependent calcium mobilization is major responsible second messenger for the production of ROS in VSMCs. Therefore, it is likely that PI3K/L-type calcium channel signaling pathway seems to be major responsible pathways that regulate ROS generation in VSMCs.

Acknowledgement

This work was supported for two years by Pusan National University Research Grant.

References

1. Downward, J. 1998. Mechanisms and consequences of activation of protein kinase B/Akt. Curr. Opin. Cell Biol. 10, 262-267.
2. Geisterfer, A. A., Peach, M. J. and Owens, G. K. 1988. Angiotensin II induces hypertrophy, not hyperplasia, of cultured rat aortic smooth muscle cells. Circ. Res. 62, 749-756.
3. Grynkiewicz, G., Poenie, M. and Tsien, R. Y. 1985. A new generation of Ca2+ indicators with greatly improved fluorescence properties. J. Biol. Chem. 260, 3440-3450.
4. Hanna, I. R., Taniyama, Y., Szöcs, K., Rocic, P. and Greindl, K. 2002. NAD(P)H oxidase-derived reactive oxygen species as mediators of angiotensin II signaling. Antioxid. Redox Signal. 4, 899-914.
5. Hall, K. L., Harding, J. W. and Hosick, H. L. 1991. Isolation and characterization of clonal vascular smooth muscle cell lines from spontaneously hypertensive and normotensive rat aortas. In Vitro Cell. Dev. Biol. 27A, 791-798.
6. Konishi, H., Matsuzaki, H., Tanaka, M., Takemura, Y., Kuroda, S., Ono, Y. and Kikkawa, U. 1997. Activation of protein kinase B (Akt/RAC-protein kinase) by cellular stress and its association with heat shock protein Hsp27. FEBS Lett. 410, 493-498.
7. Lambeth, J. D. 2004. NOX enzymes and the biology of reactive oxygen. Nat. Rev. Immunol. 4, 181-189.
8. Lassègue, B., Sorescu, D., Szöcs, K., Yin, Q., Akers, M., Zhang, Y., Grant, S. L., Lambeth, J. D. and Greindl, K. K. 2002. Novel gp91(phox) homologues in vascular smooth muscle cells: nox1 mediates angiotensin II-induced superoxide formation and redox-sensitive signaling pathways. Circ. Res. 88, 888-894.
9. Marumo, T., Schini-Kerth, V. B., Fisslthaler, B. and Busse, R. 1997. Platelet-derived growth factor-stimulated superoxide anion production modulates activation of transcription factor NF-kappaB and expression of monocyte chemotacttractant protein 1 in human aortic smooth muscle cells. Circulation 96, 2361-2367.
10. Nishida, M., Tanabe, S., Maruyama, Y., Mangmool, S., Urayama, K., Nagamatsu, Y., Takagahara, S., Turner, J. H., Kozasa, T., Kobayashi, H., Sato, Y., Kawanishi, T., Inoue, R., Nagao, T. and Kurose, H. 2005. G alpha 12/13- and receptor regulation of reactive oxygen species-dependent activation of c-Jun NH2-terminal kinase and p38 mitogen-activated protein kinase by angiotensin receptor stimulation in rat neonatal cardiomyocytes. J. Biol. Chem. 280, 18434-18441.
11. Nickenig, G., Streilow, K., Bümmer, A. T., Baudler, S., Wassmann, S., Sauer, H. and Böhm, M. 2000. Negative feedback regulation of reactive oxygen species on AT1 receptor gene expression. Br. J. Pharmacol. 131, 795-803.
초록: Angiotensin II에 의해 유도되는 활성산소발생 기전에 대한 연구

진서연 · 하정민 · 김영환 · 이해선 · 배순식*  
(부산대학교 의학전문대학원 약리학교실)

Angiotensin II (AngII)는 혈관평활근세포의 수축을 통해 혈관을 수축시키는 강력한 작용을 나타낼 뿐만 아니라 혈관세포의 성장 등에 중요한 역할을 한다. 본 연구에서는 AngII에 의해 형성되는 활성산소가 phosphatidylinositol 3-kinase (PI3K)에 유리되는 활성산소에 의해 조절된다는 것을 전층하였다. 즉의 대동맥으로부터 분리된 혈관평활근세포에서 AngII에 의해 형성된 수축은 PI3K의 억제에 의해 억제되었으나 EKR의 억제제에 의해서는 억제되지 않았을 것으로 사료된다. AngII에 의해 유리되는 함수는 L-type 칼슘이온통로 Nifedipine 또는 배양액의 칼슘에 따라 교체된 환경에서 억제될 수 있었다. 마지막으로 AngII에 의해 형성되는 활성산소는 배양액의 칼슘에 취약한 조건이나 L-type 칼슘이온통로 억제제를 전처리 하였을 경우 억제되는 것을 확인하였다. 이러한 결과들은 마탕으로 쥐의 대동맥으로부터 분리된 혈관평활근세포에서 AngII에 의한 활성산소의 형성은 PI3K/L-type 칼슘이온통로 통한 기전을 통해 조절됨을 제안한다.