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Identification of Phosphoproteins Induced by AT1 Receptor Blocker Losartan

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The angiotensin II receptor (AT_1R) antagonists are effective in treating patients with hypertension and showed beneficial effects in diabetes and other metabolic diseases. The beneficial effects of AT_1R antagonists are mainly considered to be from inhibition of Ang II-AT_1R signaling pathway such as the activation of NADPH oxidase and the generation of reactive oxygen species. In this study, we examined whether antagonist of the AT_1R could account for phosphorylation of proteins in cells using antibody array. We have selected 6 proteins with Ser/Thr-phosphorylation sites and 12 proteins with Tyr-phosphorylation sites based on literature search. Upon AT_1R antagonist losartan treatment to serum-starved COS-1 cells, there was ~20% increase of Ser phosphorylation in small GTPase RhoA. RhoA is known to be responsible for cytoskeleton rearrangement and is down-regulated upon Ser phosphorylation *in vivo*. Our finding provides a new insight into the mechanism and signaling pathway of the AT_1R antagonist in cells.

Key words : Angiotensin II (AT1) receptor, antibody array, phosphorylation, RhoA

Introduction

The angiotensin II receptor (AT_1R) belongs to the family of G protein-coupled receptors (GPCRs) and plays an important role in blood vessel constriction and water-electrolyte balance [1]. Upon the octapeptide hormone Ang II binding to the AT_1R, a conformational change in the receptor occurs and transduces hormonal signals to the cytoplasm through signaling molecules such as Gq and Janus kinase (JAK) [1]. β-Arrestins, originally thought to be involved in the deactivation of GPCRs, not only desensitize activated GPCRs phosphorylated by G protein-coupled receptor kinases, but also mediate intracellular signals such as mitogen-activated protein kinases (MAPK) as a scaffolding protein [2]. Multiple active-state conformations of GPCRs exist depending upon which type of ligand, such as agonist, antagonist, or inverse agonist, binds to the receptor on the cell surface [3]. Recently cellular signals provoked by antagonist or inverse agonist, which were previously considered as competing with endogenous hormone or inhibiting the constitutive activity of receptor, have been reported. For example, inverse agonists of the β_2-adrenergic receptor, ICI118,551 and propranolol, which reduce Gs-coupled adenylyl cyclase activity, induce activation of ERK 1/2 [4]. Another β_2-adrenergic receptor inverse agonist, carvedilol, although uncoupled from G protein, Gs, leads to the recruitment of β-arrestin from the cytoplasm, receptor internalization, and activation of ERK 1/2 through β-arrestin [5]. However, the mechanisms of ERK 1/2 activation by the inverse agonists of the β_2-adrenergic receptor are largely unknown.

The AT_1R antagonists, commonly called as the AT_1R blockers (ARBs), were shown to have beneficial effects on the progression of atherosclerosis and other metabolic diseases such as diabetes [6]. The Effects of ARBs on atherosclerosis are mainly considered to be from inhibition of Ang II-induced signaling pathways such as the activation of NADPH oxidase and the generation of reactive oxygen species, which induces many proinflammatory cytokines [1,7]. Clinical studies have shown that ARBs relive the symptoms of diabetic patients as well as delaying the onset of diabetes [6]. The AT_1R and the insulin receptor share downstream signaling pathways such that they activate similar intracellular signaling proteins such as JAK-2, IRS-1, and PI3K [7]. Thus blockade of the AT_1R signaling by ARBs enhances insulin sensitivity.

We have shown that the AT_1R blocker losartan upon binding to the AT_1R induces site-specific cleavage of β-arrestin 1 after Pro^{276} in COS-1 and rat aortic smooth muscle cells, whereas Ang II induces a different cleavage site in the C-terminal region of β-arrestin 1, after Phe^{388} [8]. Our finding suggests that β-arrestin proteolysis could be a signal in itself since β-arrestin provides scaffolds for a number of signaling molecules in MAPK cascades and non-receptor Tyr kinases

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[9]. Furthermore, ARBs are widely used in clinical practice to treat patients with hypertension, but the potential signaling pathways by antagonists remain largely unknown. In the present study, we sought to elucidate losartan-induced intracellular signals, especially signals mediated by phospho-Ser/Thr or phospho-Tyr, in COS-1 cells expressing the AT_1R using antibody array.

Materials and Methods

Materials

Losartan was a gift from DuPont Merck Co. (Wilmington, DE). Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, CA). Monoclonal antibodies to phospho-Ser and phospho-Tyr (4G10) were purchased from Upstate (Lake Placid, NY). Antibody-spotted nitrocellulose membrane was purchased from Hypromatrix (Worcester, MA). We have selected antibodies to 6 phospho-Ser/Thr proteins and 12 phospho-Tyr proteins from literature search on Ang II-AT_1R signaling pathway as shown in Table 1 and 2. Sheep anti-mouse IgG secondary antibody was purchased from GE Healthcare (Piscataway, NJ). COS-1 cells are from American Type Culture Collection (Rockville, MD). All other reagents unless stated otherwise were from Sigma (St. Louis, MO).

Table 1. Proteins phosphorylated at Ser or Thr residues

Ser/Thr-phosphorylated	Residues	References
Akt 1/2	Thr^{308}/Ser^{473}	[17,18]
RhoA	Ser^{188}	[19]
STAT 1	Ser^{727}	[20]
c-Jun	$Ser^{63/73}$ $Thr^{91/93}$	[21,22]
PKC	$Thr^{500/641}$ Ser^{660}	[23]
PKA	$Ser^{114/338}$	[24]

Table 2. Proteins phosphorylated at Tyr residues

Tyr-phosphorylated	Residues	References
EGFR	$Tyr^{845/992/1045}$ $Tyr^{1068/1148/1173}$	[25]
ERK 1/2	Tyr^{204}	[26]
P38	Tyr^{182}	[27]
FAK	$Tyr^{407/576/577}$ $Tyr^{871/925}$	[28]
JAK 1	$Tyr^{1022/1023}$	[29]
JAK 2	$Tyr^{1007/1008}$	[30]
JAK 3	Tyr^{980}	[31]
JNK 1,2,3	$Tyr^{185/223}$	[32]
Pyk2	$Tyr^{402/580}$	[1]
TGFβ-R	$Tyr^{259/336/424}$	[33]

Cell culture and expression of the AT1R

The synthetic rat AT_1 receptor gene, cloned in the shuttle expression vector pMT3, was used for expression. To express the AT_1R protein, 60-65% confluent COS-1 cells grown in 10-cm petri dish cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum were transfected with 10 μg of purified plasmid DNA using Lipofectamine 2000 according to the manufacturer's instructions.

Western Blotting using antibody array

Basically, the antibody array utilizes antibody-spotted nitrocellulose membrane. Cell extracts are applied onto the antibody array and proteins of interest are captured by the antibody on the nitrocellulose membrane. Another antibody (for example, antibody to phospho-Ser or phospho-Tyr in this experiment) is added to the antibody array followed by incubation with horseradish-conjugated secondary antibody and the detection by enhanced chemiluminescence method. The following protocol was used for the detection of phosphoproteins shown in Fig. 1 and 2. Transfected cells cultured for 48 hr were serum starved for 16 hr. Cells were treated with 1 μM losartan for 10 min and then washed with cold PBS. Cells were scraped and suspended in lysis buffer (25 mM HEPES, pH 7.2, 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 1% Triton X-100, 1 mM PMSF, 2 mM AEBSF, 130 μM bestatin, 14 μM E-64, 1 μM leupeptin, 0.3 μM aprotinin, 10 mM sodium pyrophosphate, 10 mM sodium fluoride, 1 mM sodium orthovanadate, 10% glycerol) for 30 min on ice. Before incubation with cell lysates the nitrocellulose membrane was blocked for 1 hr at room temperature in 5% non-fat dry milk and 0.1% Tween-20 in PBS (pH 7.4). Cell lysates were centrifuged at 13,000 rpm for 10 min at 4°C to remove cell debris and applied onto the antibody array overnight at 4°C. Following washes with PBS, incubation with antibody to phospho-Ser or phospho-Tyr antibody (1:1,000) was carried out for 1 hr at room temperature. Following washes with PBS, horseradish-conjugated secondary antibody (1:10,000) was added. The detection was made with enhanced chemiluminescence (GE Healthcare). Phosphoprotein was quantified by scanning densitometry of

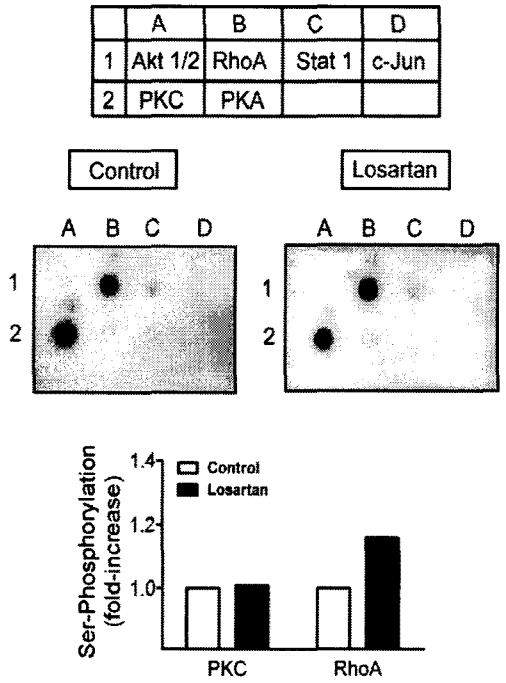

Fig. 1. Ser-phosphorylation of RhoA by activation of losartan-AT_1R. Serum-starved COS-1 cells expressing the AT_1R were treated with losartan for 10 min. Cell lysates were incubated with nitrocellulose membrane spotted with antibodies to 6 proteins with Ser/Thr phosphorylation sites as shown in Table 1. Antibody to phospho-Ser (1:1,000) was added onto the nitrocellulose membrane followed by incubation with HRP-conjugated sheep anti-mouse IgG secondary antibody (1:10,000). Experiments were carried out in duplicate. A representative blot is shown. Bar graph shows the average of duplicate experiments. Changes in protein phosphorylation were expressed as a fold-increase over levels of phosphorylation in non-stimulated cells.

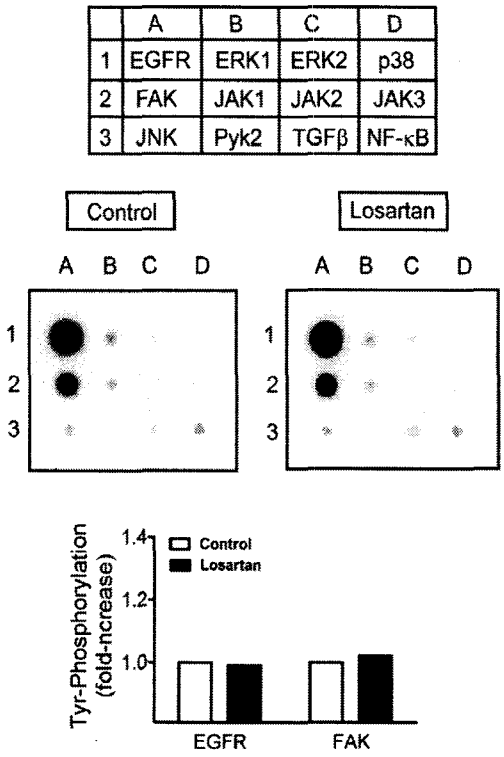

Fig. 2. Tyr-phosphorylation of proteins upon losartan treatment. Serum-starved COS-1 cells expressing the AT_1R were treated with losartan for 10 min. Cell lysates were incubated with nitrocellulose membrane spotted with antibodies to 12 proteins with Tyr phosphorylation sites as shown in Table 2. Antibody to phospho-Tyr (4G10) was added (1:1,000) to the nitrocellulose membrane followed by incubation with sheep anti-mouse IgG secondary antibody (1:10,000). Tyr-phosphorylation in control and losartan-treated sample were similar in our experiment. Experiments were carried out in duplicate. A representative blot is shown. Bar graphs are shown for EGFR and FAK.

immunoblots using Fuji Multiguage V3.0 Software (Fuji Film). Data analysis was performed using GraphPad Prism 4 (GraphPad Software). Experiments were carried out in duplicate and a representative blot is shown in the results.

Results

Phosphorylation of proteins with Ser residues

Serum-starved COS-1 cells expressing the AT_1R was incubated with losartan for 10 min. Cell lysates were incubated overnight with nitrocellulose membrane on which monoclonal antibodies to 6 proteins containing Ser/Thr-phosphorylation sites are spotted; we have selected Akt 1/2, RhoA, Stat1, c-Jun, PKC, and PKA as target molecules from their role in Ang II-AT_1R signaling pathway as shown in Table 1. The membrane was incubated with monoclonal antibody to phospho-Ser followed by incubation with HRP-conjugated secondary antibody after washing the membrane with PBS. Identification of phosphorylated proteins was made by position and signal density following enhanced chemiluminescence reaction as shown in Fig. 1. Two proteins, PKC and RhoA, showed elevated phosphorylation on Ser residues in basal condition. The level of PKC phosphorylation was similar before and after losartan treatment, but to our surprise, there was ~20% increase of Ser phosphorylation in RhoA upon losartan treatment. A dephosphorylated RhoA was shown to stimulate vascular tone by increasing smooth muscle Ca^{2+} sensitivity [10]. Our finding is consistent with the observation by Ellerbroek *et al.* that Ser phosphorylation negatively regulates RhoA *in vivo* [11]. The phosphorylation levels of Akt 1/2, Stat 1, c-Jun, and PKA

were similar.

Phosphorylation of proteins with Tyr residues

For the detection of losartan-induced phospho-Tyr proteins, we have selected EGFR, ERK 1/2, p38, FAK, JAK 1/2/3, JNK, Pyk2, TGFβ-R, and NF-κB as target molecules from their role in Ang II-AT_1R signaling pathway. Serum-starved COS-1 cells expressing the AT_1R was incubated with losartan for 10 min. Cell lysates were incubated overnight with nitrocellulose membrane on which monoclonal antibodies to 12 proteins containing Tyr-phosphorylation sites are spotted. Antibody to phospho-Tyr (4G10) was added followed by incubation with HRP-conjugated secondary antibody after washing the membrane with PBS. Identification of phosphorylated proteins was made by position and signal density as shown in Fig. 2. Among 12 phospho-Tyr proteins, EGFR and FAK showed elevated level of Tyr phosphorylation in basal condition. Contrary to the identification of RhoA among Ser phosphorylated proteins induced by losartan, we were not able to distinguish losartan-induced Tyr phosphorylated proteins compared to proteins in non-stimulated cells. Our data, however, provides evidence that in COS-1 cells, EGFR and FAK maintain higher level of Tyr phosphorylation followed by other phospho-Tyr proteins such as ERK 1/2, JAK 1/2/3, JNK 1/2/3, and NF-κB.

Discussion

We utilized antibody array as an alternative to expensive ProteinChip array to elucidate phosphoproteins induced by the AT_1 receptor antagonist losartan in COS-1 cells expressing the AT_1 receptor. We selected monoclonal antibodies to 6 Ser/Thr phosphorylated proteins and 12 Tyr phosphorylated proteins based on literature search on Ang II-AT_1R signaling pathways. We have observed that PKC and RhoA, which are Ser/Thr phosphorylated, and EGFR and FAK, which are Tyr phosphorylated, maintain high level of phosphorylation in COS-1 cells in basal condition. We found that losartan increases Ser phosphorylation of RhoA ~20% compared to non-stimulated cells. It is shown that the small GTPase RhoA is responsible for stress fiber formation and cell rounding following the AT_1R activation [12]. In HEK293 cells, β-Arrestin 1 was shown to be responsible for activation of RhoA through Gq, but it alone was not sufficient, and required heterotrimeric G protein Gq for the maximal activation of RhoA [13]. β-Arrestin provides scaffolds for a number of signaling molecules in MAPK cascades and non-receptor Tyr kinases [9]. Recently we have shown that the β-arrestin 1 bound to the AT_1R undergoes site-specific proteolysis in COS-1 and rat aortic smooth muscle cells [8]. The cleavage is specific to Ang II binding to the AT_1R and required stable interactions with the AT_1R and β-arrestin [8,14]. Surprisingly, we observed that β-arrestin is cleaved off after Pro^{276} upon losartan treatment, whereas Ang II induced the cleavage of β-arrestin 1 at a different site, after Phe^{388} [8]. In this regard, the cleavage of β-arrestin 1 could be a signal in itself and we interpret this finding as the conformation of ligand-receptor complex is transmitted to β-arrestin 1 leading to the buried cleavage site in β-arrestin 1 accessible to protease(s) in the cell, allowing changes in the scaffolding function of β-arrestin 1. The cleavage of β-arrestin 1 by losartan treatment raises an intriguing question whether the N-terminal or C-terminal fragment of cleaved β-arrestin 1 is responsible for the deactivation of RhoA through phosphorylation at Ser^{188} residue. The mechanism and signaling pathway of losartan-induced β-arrestin cleavage are being investigated.

The concept of antagonist or inverse agonist-induced GPCR signaling has emerged in recent years as our understanding of multiple active conformations of receptor has enlarged [3]. Since the first description of the constitutive activity of α_1-adrenergic receptor [15], a number of constitutively active mutations (CAM) were reported in several GPCRs such as the LH receptor and the AT_1 receptor [16]. Ligands, which reduce the elevated basal activities of CAM receptor such as cAMP or IP_3, were categorized as inverse agonist, but the experimental system, mainly dependent upon secondary messenger accumulation in the cell, hindered the discovery of inverse agonist-induced cellular signaling. Evidence shows that inverse agonists of the β_2-adrenergic receptor, though uncoupled from Gs, induce activation of ERK 1/2 through β-arrestin [4,5]. Our finding is consistent with the observations of Azzi *et al.* [4] and Wisler *et al.* [5] that antagonist or inverse agonist could account for activation of cellular signals other than cAMP or IP_3. In this aspect, each ligand-receptor complex renders a distinct conformation to signaling molecules such as G protein and β-arrestin, thus inverse agonist for a certain conformation of receptor could provoke anther signaling pathway.

The AT_1R blockers including losartan and candesartan are commonly used in practice to treat patients with hypertension. Unlike angiotensin converting enzyme inhibitors which generally cause dry cough as side effect, the

ARBs are considered having lesser side effects and also exert certain beneficial effects in diabetes and other metabolic diseases. Considering that the AT_1R activates multiple signaling pathways through Gq, JAK/STAT, and β-arrestin including activation of MAPK cascade, trans-activation of EGFR and generation of reactive oxygen species, we can envisage the activation of signaling pathways by ARBs other than inhibition of Ang II-induced vasoconstriction. Thus, the data in this study suggests that a distinct conformation of losartan-AT_1R leads to phosphorylation of Ser in small GTPase RhoA via yet unknown mechanism. Our finding provides a new insight into the molecular mechanism and signaling pathway of ARBs, hitherto unknown in the cell. Other effects of ARBs on the AT_1R signaling need to be further elucidated.

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초록 : 항고혈압 치료제 로사탄에 의해 인산화 되는 단백질 발굴

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안지오텐신II 수용체(AT1 수용체)는 혈관수축과 체내 전해질이온 조절에 중요한 역할을 한다. AT1 수용체 길항제(ARB)는 고혈압 치료에 이용되며 최근에는 당뇨병을 포함한 대사질환에 효능이 있음이 알려져 있다. 이 연구에서는 ARB 처리 후 세포 내 인산화단백질에 인산화가 일어나는지를 antibody array를 이용하여 실험하였다. 아미노산 세린 및 트레오닌에 인산화되는 단백질 6개, 티로신에 인산화되는 단백질 12개에 대한 항체를 선정하여 nitrocellulose membrane에 부착시켰다. AT1 수용체를 발현한 COS-1 세포에 로사탄(losartan)을 처리하였을 때 small GTPase인 RhoA의 세린 잔기에 인산화가 20% 증가함을 관찰하였다. RhoA는 세포골격의 재배열에 중요한 역할을 하며 세린 잔기에 인산화가 되면 활성이 억제된다. 본 연구결과로부터 ARB가 AT1 수용체에 의한 혈관수축을 억제할 뿐만 아니라 새로운 세포 신호를 생성함을 알 수 있다.