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Molecular mechanisms of the antagonistic action between AT_1 and AT_2 receptors

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ABSTRACT

Although angiotensin II (Ang II) binds to Ang II type 1 (AT_1) and type 2 (AT_2) receptors, AT_1 and AT_2 receptors have antagonistic actions with regard to cell signaling. The molecular mechanisms that underlie this antagonism are not well understood. We examined AT_1 and AT_2 receptor-induced signal cross-talk in the cytoplasm and the importance of the hetero-dimerization of AT_1 receptor with AT_2 receptor on the cell surface. AT_1 and AT_2 receptors showed antagonistic effects toward inositol phosphate production. AT_1 receptors mainly formed homo-dimers, rather than hetero-dimers with AT_2 receptor, on the cell surface as determined by immunoprecipitation, and subsequently induced cell signals. AT_2 receptor mainly formed homo-dimers, rather than hetero-dimers with AT_1 receptor, on the cell surface. The expression levels of homo-dimerized AT_1 receptor or AT_2 receptor on the cell surface did not change after treatment with Ang II, the AT_1 receptor antagonist telmisartan or the AT_2 receptor antagonist PD123319. Finally, AT_1 and AT_2 receptor-induced signals antagonized phospholipase C-β_3 phosphorylation. In conclusion, Ang II-induced AT_1 receptor signals may be mainly blocked by AT_2 receptor signals through their negative cross-talk in the cytoplasm rather than by the hetero-dimerization of both receptors on the cell surface. The proper balance of the expression levels of AT_1 and AT_2 receptors might be critical for the antagonistic action between these receptors.

Introduction

Angiotensin II (Ang II) plays a central role as a major regulator of blood pressure, electrolyte balance, and endocrine function related to cardiovascular disease. Two distinct subtypes of Ang II receptors, type 1 (AT_1) and type 2 (AT_2), have been identified, and both belong to the G-protein coupled receptor superfamily (GPCRs) [1]. Most of its physiological effects are mediated through the AT_1 receptor [2]. The idea that AT_1 and AT_2 receptors exert antagonistic activity is supported by the observation of Ang II-induced cell growth and anti-growth effects, respectively [3,4].

GPCRs are able to both homo-dimerize and hetero-dimerize. For example, β_2-adrenergic, muscarinic, dopamine D_2, and opioid receptors undergo homo-dimerization following agonist stimulation [5–8]. The presence of homo-dimerization suggests that it may be possible to modulate receptor function through intermolecular interactions. On the other hand, several recent reports demonstrate that GPCRs in monomeric form effectively activate G proteins [9–11], and that receptor dimerization is actually less effective. Although the dimerization of GPCRs is a highly controversial issue, a detected AT_1 and AT_2 receptor hetero-dimerization that led to AT_1 receptor signal inhibition was independent of the binding of Ang II to AT_2 receptor [12], and AT_2 receptor has constitutive activity [13,14], which suggests that the constitutive activity of AT_2 receptor itself might induce hetero-dimerization independent of Ang II stimulation. This hetero-dimerization may explain why the functions of AT_1 and AT_2 receptors are antagonistic [12]. Another possible mechanism may explain why the functions of AT_1 and AT_2 receptors are antagonistic, i.e., Ang II-induced AT_1 and AT_2 receptor signals show cross-talk in the cytoplasm. In fact, Horiuchi et al. reported that the stimulation of AT_2 receptor in AT_2-receptor-transfected vascular smooth muscle cells (SMC) inhibited AT_1 receptor-mediated tyrosine phosphorylation of signal transducers and activators of transcription (STAT)-1α/β, STAT2 and STAT3 [15]. AT_1 and AT_2 receptors regulate STAT activation by negative cross-talk.

Although the antagonistic effect of AT_1 and AT_2 receptors through their cross-talk is well-known, we determined whether their signal cross-talk mainly occur in the cytoplasm (one molecule interacts AT_1 receptor-signaling with AT_2 receptors-signaling) or on the cell surface (direct interaction between AT_1 receptor and

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AT_2 receptor by hetero-dimerization). Therefore, we hypothesized that Ang II-induced AT_1 receptor signals were blocked by AT_2 receptor signals in the cytoplasm rather than by the hetero-dimerization of both receptors on the cell surface.

Methods

Materials. The following antibodies and reagents were purchased: anti-phospholipase (PL) C-β_3, γ_1 and γ_2 antibodies (Abs), anti-phospho(p)-PLC-β_3, γ_1 and γ_2 Abs, anti-inositol 1,4,5-trisphosphate (IP_3) receptor Ab and anti-β-actin Ab (Cell Signaling Technology Japan, K.K.), anti-AT_1 and AT_2 receptor Abs (Santa Cruz Biotechnology, Inc., CA, USA), Ang II analogues (Bachem, Bubendorf, Switzerland), the AT_1 receptor antagonist telmisartan (Toronto Research Chemical, Canada) and the AT_2 receptor antagonist PD123319 (Sigma–Aldrich Inc., USA).

Cell cultures and transfection. HepG2 cells and human embryonic kidney 293 (HEK) cells (American Type Culture Collection, Rockville, MD) were cultured using Dulbecco's modified essential medium (DMEM) with 10% fetal bovine serum (FBS) (HyClone) and penicillin/streptomycin in 5% CO_2 at 37 °C. Human coronary artery SMCs were purchased from Clonetics. SMCs were cultured and used from 3 to 5 passages in media supplemented with 5% FBS, penicillin/streptomycin and SMC growth supplement (Takara Co., Osaka, Japan) in 5% CO_2 at 37 °C.

The synthetic rat AT_1 and AT_2 receptor genes were used for expression [13,16]. To express AT_1 and AT_2 receptor protein, 10 μg of purified plasmid DNA per 10^7 cells were used in transfection. The DNAs were transfected into HEK cells using the Lipofectamine 2000 liposomal reagent according to the manufacturer's instructions (Invitrogen). Cell viability was >95% by trypan blue exclusion analysis in control experiments.

Receptor binding assay. The K_d and B_{max} values of the receptor binding were determined by ^{125}I-[Sar^1, Ile^8]Ang II-binding experiments under equilibrium conditions, as previously described [16,17]. Cell membranes were prepared by the nitrogen Parr bomb disruption method in the presence of protease inhibitors. The membranes were incubated with 100 pM ^{125}I-[Sar^1, Ile^8]Ang II for 1 h at 22 °C in a volume of 125 μl. Binding was stopped by filtering the incubation mixture through Whatman GF/C glass-fiber filters, and the residues were extensively washed further with binding buffer. The bound ligand fraction was determined by the counts per minute (cpm) remaining on the membrane. Binding kinetics values were determined as previously described [16,17].

Inositol phosphate (IP) formation studies. Total soluble IP was measured by the perchloric acid extraction method, as described previously [16,17]. Brifly, semi-confluent cells in 60-mm Petri dishes were labeled for 24 h with [^{3}H]-myoinositol, specific activity 22 Ci/mmol (Amersham), at 37 °C with or without antagonist in medium containing 10% serum. On the day of the functional assay, the labeled cells were washed with Hank's Balanced Salt solution (HBSS) three times and incubated with HBSS containing 10 mM LiCl for 20 min; agonist was added and incubation continued for another 45 min at 37 °C with or without antagonist. At the end of incubation, the medium was removed, and total soluble IP was extracted from the cells by the perchloric acid extraction method.

Polyacrylamide gel electrophoresis (PAGE), Western blotting, and immunoprecipitation. Cell membrane and cytoplasmic fractions were prepared as described previously [14]. For Western blotting, equal amounts of samples on a protein basis as determined using the Bradford reagent (Bio-Rad) were resolved on nonreducing 7–10% SDS–PAGE. Cell membrane was incubated at room temperature without boiling for 30 min with loading buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris–HCl (pH 7.4), 1 mM EDTA, 0.2 mM sodium orthovanadate, 0.2 mM phenylmethylsulfonyl fluoride, 0.5% NP-40). Western blot analysis was performed with primary antibodies as specified in each case. Horseradish peroxidase-conjugated secondary antibody and the enhanced chemiluminescent substrate system (Amersham) were used for detection. The signal was independently quantified by a digital image-analysis system.

Statistical analysis. The results are expressed as the mean ± standard error of three or more independent determinations (Figs. 1–4). The significance of differences between measured values was evaluated by unpaired Student's t test and an analysis of variance with Fisher's test. Statistical significance was set at a level of $p < 0.05$.

Results

AT_1 and AT_2 receptors showed antagonistic effects toward IP production

First, we used HepG2 cells because they endogenously expressed AT_1 and AT_2 receptors (B_{max}, 7.9 ± 0.7 and 6.8 ± 1.4 fmol/mg protein,

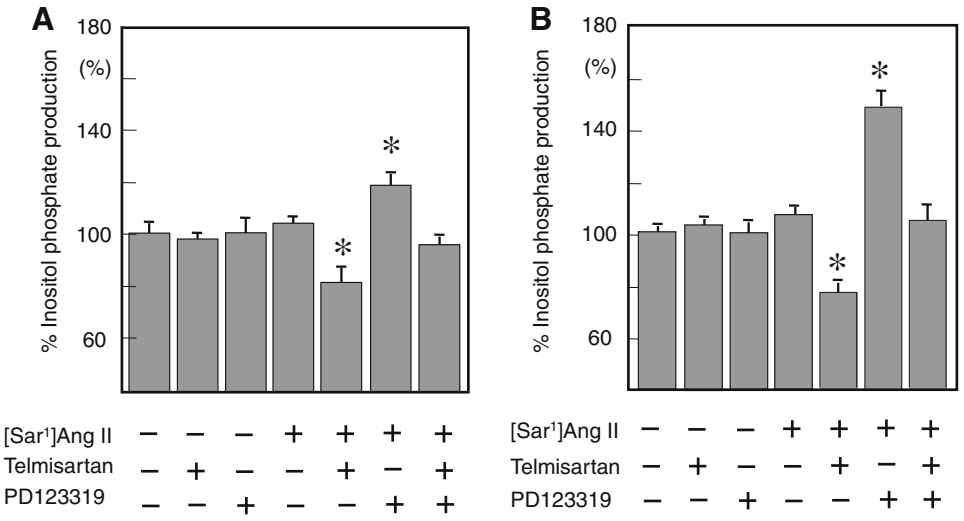

Fig. 1. % Inositol phosphate (IP) production in the presence or absence of 0.1 μM [Sar^1]Ang II, 1 μM telmisartan and 1 μM PD123319 in HepG2 cells (A) and SMCs (B). Basal IP production (A, 450 cpm; B, 1150 cpm) without treatment adjusted to 100% IP production.*$p < 0.05$ vs. no treatment.

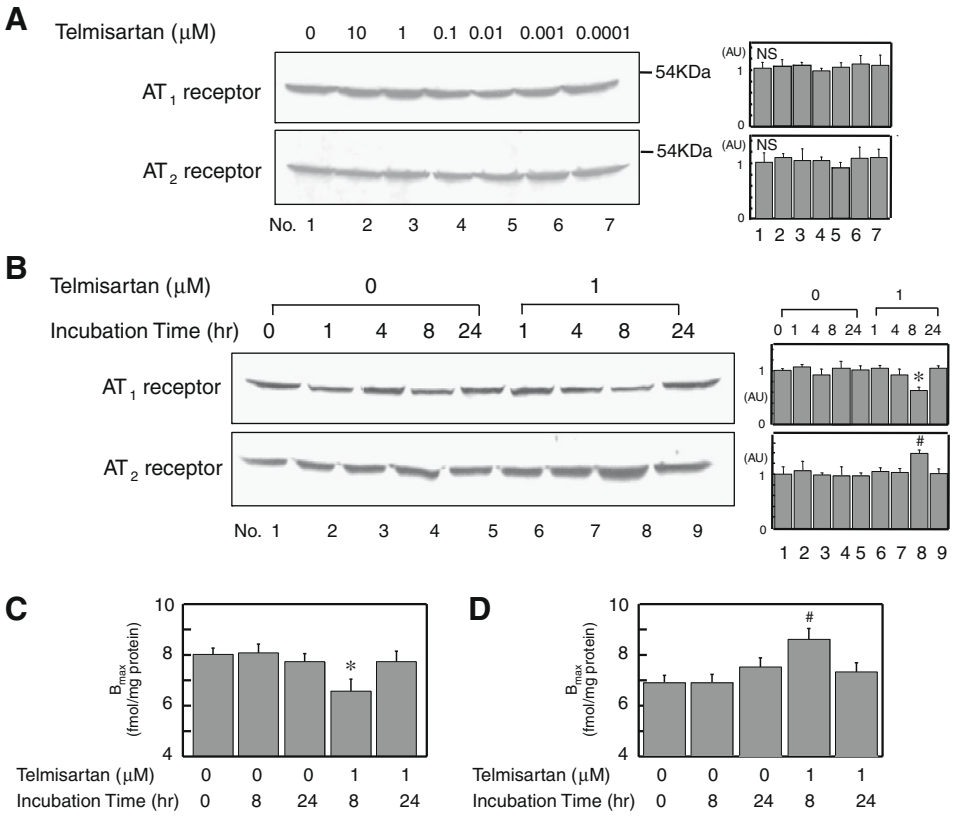

Fig. 2. Expression levels of AT_1 receptor or AT_2 receptor on the HepG2 cell surface with or without 1 μM telmisartan under the indicated concentrations (A) and incubation time (B) by immunoblotting. Proteins were resolved on 10% SDS-containing polyacrylamide gels under reducing conditions, and the receptors were detected by immunoblotting. Expression levels of AT_1 receptor (C) or AT_2 receptor (D) on the HepG2 cell surface with or without 1 μM telmisartan under the indicated concentrations and incubation time by receptor binding assay. After incubation, cell membranes were prepared by the freeze/thaw method. The cell membranes were washed twice with assay buffer and subjected to receptor binding study. $^{*}p < 0.05$ vs. no treatment. $^{\#}p < 0.05$ vs. no treatment. NS, not significant; AU, arbitrary unit.

respectively). [Sar[1]]Ang II (0.1 μM) did not induce significant IP production (Fig. 1A). In the presence of 1 μM PD123319, the same concentration of [Sar[1]]Ang II significantly stimulated IP production. On the other hand, in the presence of 1 μM telmisartan, 0.1 μM [Sar[1]]-Ang II significantly suppressed IP production. Since the clinical plasma concentration of telmisartan (C_{max}) is about 1 μM [18], the concentration of telmisartan used in the experiments was 1 μM. When both receptors were blocked by telmisartan and PD123319, [Sar[1]]Ang II did not induce significant IP production.

Since HepG2 is a perpetual cell line which was derived from the human liver tissue with a well differentiated hepatocellular carcinoma, we also used native SMCs which endogenously expressed AT_1 and AT_2 receptors (B_{max}, 19 ± 8 and 17 ± 4 fmol/mg protein, respectively). As shown in Fig. 1B, the results of IP production using SMCs showed the similar to those using HepG2 cells. In this way, AT_1 and AT_2 receptors showed antagonistic action against IP production.

Expression levels of AT_1 receptor or AT_2 receptor on the cell surface with telmisartan and PD123319

Since HepG2 cells were incubated with telmisartan and PD123319 for 24 h before the measurement of Ang II-induced IP production (Fig. 1), we analyzed the expression levels of AT_1 and AT_2 receptors on the cell surface with the indicated concentrations of telmisartan or PD123319 for the indicated incubation times. There were no differences in the expression levels of AT_1 and AT_2 receptors throughout the experimental period under the no-treatment condition using immunoblotting (Fig. 2A and B). Although 1 μM telmisartan transiently decreased the expression level of AT_1 receptor and increased the level of AT_2 receptor after 8 h of incubation, the expression levels of both receptors returned to the basal levels after 24 h. In addition, PD123319 did not alter the expression level of AT_2 receptor under different concentrations and incubation times (data not shown).

To confirm the expression levels of Ang II receptors, we also analyzed the receptors expression by receptor binding assay (Fig. 2C and D). One μM telmisartan transiently decreased the expression level of AT_1 receptor and increased the level of AT_2 receptor after 8 h of incubation, the expression levels of both receptors returned to the basal levels after 24 h.

AT_1 receptor mainly formed homo-dimers, rather than hetero-dimers with AT_2 receptor, on the cell surface

To analyze the dimerization of AT_1 receptor and AT_2 receptor on the cell surface, we performed immunoprecipitation using total cell lysate (cytoplasm and cell membrane) and cell membrane (Fig. 3). The ratio of the expression levels of homo-dimerized AT_1 receptors, homo-dimerized AT_2 receptors and hetero-dimerized AT_1 and AT_2 receptors was 4.3 ± 1.2:8.0 ± 2.0:1.0 ± 0.2 in total cell lysate and 4.1 ± 0.9:12.2 ± 2.9:1.0 ± 0.1 on the cell surface in SMCs, respectively. In addition, the ratio of the expression levels of homo-dimerized AT_1 and AT_2 receptors and hetero-dimerized receptors was 6.9 ± 0.9:13.2 ± 1.6:1.0 ± 0.1 in total cell lysate and 4.7 ± 1.2:10.7 ± 2.5:1.0 ± 0.2 on the cell surface in HepG2, respectively. Thus, AT_1 receptor mainly formed homo-dimers, rather than hetero-dimers with AT_2 receptor, on the cell surface, and AT_2 receptor mainly formed homo-dimers, rather than hetero-dimers with AT_1 receptor, on the cell surface. Next, we examined the expression levels of the

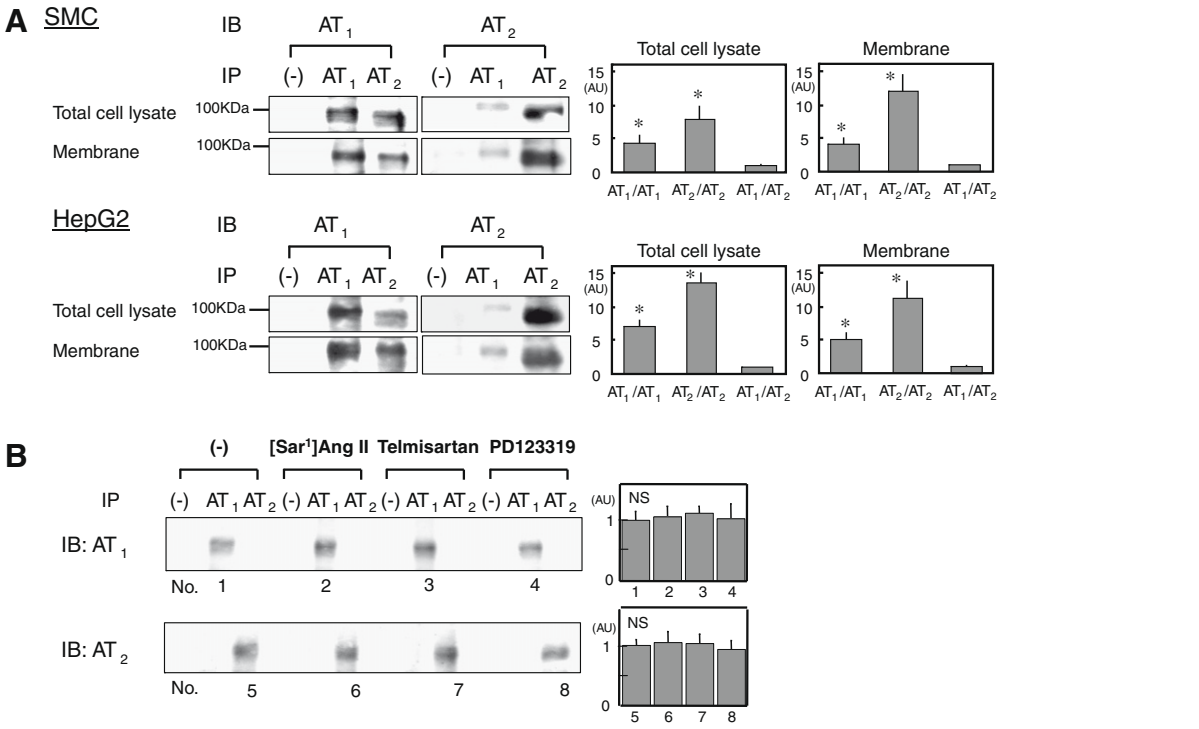

Fig. 3. Expression levels of homo-dimerized or hetero-dimerized AT_1 receptor and/or AT_2 receptor in SMCs and HepG2 cells. Samples (total cell lysate and membrane fraction) were separated on 7% SDS-containing polyacrylamide gels under nonreducing conditions after immunoprecipitation (IP), and the receptors were detected by immunoblotting (IB). (A) The ratio of the expression levels of homo-dimerized AT_1 receptors (AT_1/AT_1), homo-dimerized AT_2 receptors (AT_2/AT_2) and hetero-dimerized AT_1 and AT_2 receptors (AT_1/AT_2) were quantified by digital image-analysis. $^*p < 0.05$ vs. AT_1/AT_2. (B) Expression levels of homo-dimerized AT_1 receptor and AT_2 receptor with or without 0.1 μM [Sar^1]Ang II, 1 μM telmisartan and 1 μM PD123319 for 24 h in HepG2 cells. NS, not significant; AU, arbitrary unit.

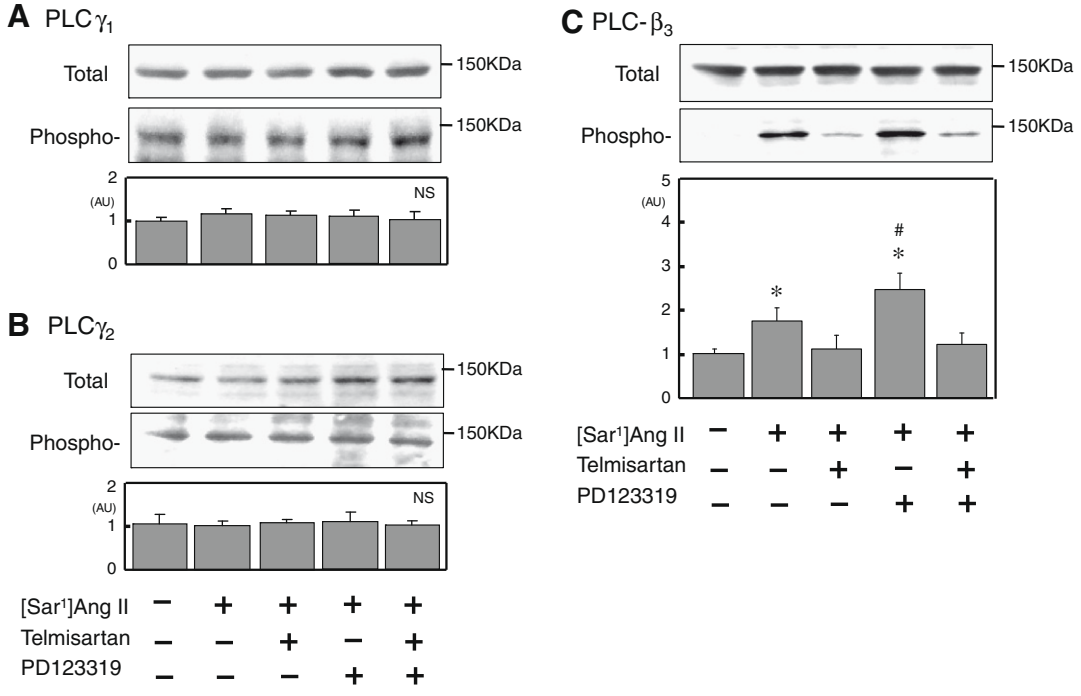

Fig. 4. Protein expression levels of PLCs [PLC-γ_1 (A), γ_2 (B) and β_3 (C)] were analyzed under stimulation with 0.1 μM [Sar^1]Ang II (1 min) after pretreatment with 1 μM telmisartan or 1 μM PD123319 for 1 h using HEK cells that had been transfected with AT_1 and AT_2 receptors. PLC activities were analyzed by anti-phospho(p)-PLC-β_3, γ_1 and γ_2 Abs. Proteins were resolved on 7% SDS-containing polyacrylamide gels under reducing conditions, and bands were detected by immunoblotting. $^*p < 0.05$ vs. no treatment. $^\#p < 0.05$ vs. [Sar^1]Ang II. NS, not significant; AU, arbitrary unit.

homo-dimerization of AT_1 or AT_2 receptor on the cell surface with 0.1 μM [Sar^1]Ang II after incubation for 40 min, and with 1 μM telmisartan and 1 μM PD123319 after incubation for 24 h. Neither [Sar^1]-Ang II, telmisartan, nor PD123319 altered the expression levels of the homo-dimerization of both receptors.

AT_1 and AT_2 receptor-induced signals antagonize PLC-β_3 phosphorylation

Since AT_1 and AT_2 receptors showed antagonistic action toward IP production and the mechanism was not the hetero-dimerization of both receptors, we analyzed the mechanism of signal cross-talk in the cytoplasm (Fig. 4). Since PLC are located immediately downstream from the receptor, we analyzed 3 isoforms (PLC-β_3, γ_1 and γ_2) which may be important for Ang II-induced IP production in AT_1 and AT_2-receptor-transfected HEK cells. Since HEK cells did not endogenously express either receptor by our receptor binding assay, we used these cells as surrogate models to link the *de novo* expression of the receptors to PLC phosphorylation. The expression levels of AT_1 and AT_2 receptors were similar (B_{max}, 8.4 ± 1.3 and 7.9 ± 2.1 pmol/mg protein, respectively). Neither [Sar^1]Ang II, telmisartan nor PD123319 significantly altered the ratio of phosphorylated to total protein levels of PLC-γ_1, γ_2 (Fig. 4A and B) and β-actin (data not shown). Although [Sar^1]Ang II induced phosphorylation of PLC-β_3, [Sar^1]Ang II induced additional phosphorylation in the presence of PD123319 (Fig. 4C). On the other hand, in the presence of telmisartan, [Sar^1]Ang II did not induce the phosphorylation of PLC-β_3. When both receptors were blocked by telmisartan and PD123319, PLC-β_3 was not phosphorylated by [Sar^1]Ang II.

Next, we examined the expression levels of IP_3 receptor with or without Ang II, telmisartan and PD123319 in AT_1 and AT_2-receptor-transfected HEK cells because the down-regulation of IP_3 receptor represents a reversible adaptive response to protect cells against the adverse effects of constitutively active AT_1-N111G mutant receptor [19]. IP_3 receptor was similarly expressed in the presence or absence of Ang II, telmisartan and PD123319 (data not shown). AT_1 and AT_2 receptor-mediated signals did not change the expression level of IP_3 receptor. Therefore, AT_1 and AT_2 receptor-induced IP production may directly regulate diverse physiological functions through regulation of the phosphorylation of PLC-β_3.

Discussion

The main finding of the present study is that AT_1 receptor mainly formed homo-dimers, rather than hetero-dimers with AT_2 receptor, on the cell surface, and AT_2 receptor also mainly formed homo-dimers, rather than hetero-dimers with AT_1 receptor, on the cell surface. Furthermore, AT_1 receptor signals may be blocked by AT_2 receptor signals through their negative cross-talk in PLC-β_3 activation, rather than by hetero-dimerization of both receptors on the cell surface.

Members of the GPCRs undergo homo-dimerization to induce cell signaling. Homo-dimers of AT_1 or AT_2 receptor are also clearly important for inducing cell signals. As for AT_1 receptor, intracellular factor XIIIA transglutaminase cross-links Ang II-induced AT_1 receptor homo-dimers through Gln^{315} in the carboxyl-terminal tail of the AT_1 receptor [20]. The elevated levels of AT_1 receptor dimers on monocytes promote atherogenesis in ApoE-deficient mice. In addition, constitutively active homo-dimerization of AT_2 receptor was localized in the cell membrane without Ang II stimulation and induced apoptosis without changes in receptor conformation [14]. The constitutive activity of AT_2 receptor itself might serve to effect hetero-dimerization independent of Ang II stimulation. AbdAlla et al. reported that the AT_2 receptor binds directly to the AT_1 receptor and thereby antagonizes the function of the AT_1 receptor. They also found that the AT_1-specific antagonism of the AT_2 receptor was independent of AT_2 receptor activation and signaling [12]. Unexpectedly, the present study indicated that Ang II-induced AT_1 receptor signals may be blocked by AT_2 receptor signals through their negative cross-talk, rather than through the hetero-dimerization of AT_1 and AT_2 receptors. The discrepancy between our data and their study may be due to the use of different types of cells and expression levels of AT_1 and AT_2 receptors. Since transglutaminase cross-links Ang II-induced AT_1 receptor homo-dimers in monocytes [20], AT_1 and AT_2 receptors also may hetero-dimerize by an unknown enzyme. It is possible that HepG2 cells, but not PC12 cells, may lack such an enzyme. In addition, HepG2 cells equally express AT_1 and AT_2 receptors. It has been reported that AT_1-receptor hetero-dimerized bradykinin (BK) type B_2 (B_2) receptor [21], dopamine receptor [22], endothelin type B receptor [23] and Mas receptor [24]. Hetero-dimerization of AT_1 receptor and B_2 receptor significantly increased in preeclampsia [21]. In this case, the hetero-dimerization in preeclampsia correlated with a 4- to 5-fold increase in B_2 receptor protein levels compared to AT_1-receptor levels. Moreover, since we also reported that constitutively active homo-dimerization, which was due to disulfide bonding between Cys^{35} in one AT_2 receptor and Cys^{290} in another AT_2 receptor [14], was localized in the cell membrane, this bonding might not be easy to disrupt. If the expression level of AT_2 receptor is much higher than that of AT_1 receptor, AT_2 receptor may more easily hetero-dimerize with AT_1 receptor and induce an antagonistic action of AT_1 receptor as a dominant negative receptor. In fact, AbdAlla et al. analyzed protein levels of the hetero-dimerization of AT_1 and AT_2 receptor on myometrial biopsies from pregnant and nonpregnant women [12]. Although the hetero-dimerized level in nonpregnant women was much higher than that in pregnant women, the expression level of AT_2 receptor in nonpregnant women was also about 10-fold higher. The proper balance of expression levels of AT_1 and AT_2 receptors may be critical for antagonistic action through hetero-dimerization between these receptors.

AT_2 receptor may have anti-proliferative and apoptotic effects. In fact, overexpression of the AT_2 receptor downregulates AT_{1a} receptor expression in rat SMC in an Ang II-independent manner that is mediated by the BK/nitric oxide pathway [25]. In our study, there were no differences in the expression levels of AT_1 and AT_2 receptors under the no-treatment condition, and the expression levels of AT_1 and AT_2 receptors with telmisartan or PD123319 returned to the basal levels after 24 h when IP production was performed. The expression levels of AT_1 and AT_2 receptors may not affect IP production in our cell system.

Finally, we analyzed the possibility that Ang II-induced AT_1 receptor signals were blocked by AT_2 receptor signals through their cross-talk in the cytoplasm. Three families of PLC isozymes, PLC-β, -γ and -δ have been described [26]. PLC phosphorylation is one of the earliest events in Ang II signaling [27]. Ang II binding to AT_1 receptor induces a rapid phosphorylation of PLC to produce IP_3. AT_1 receptors sequentially couple to PLC-β_1 via a heterotrimeric G protein and to PLC-γ via a downstream tyrosine kinase in SMC [28]. In this study, although we did not analyze PLC-β_1, PLC-β_3 but not PLC-γ_1 or -γ_2 is important for Ang II-induced IP production. Since we examined these PLC phosphorylations after 1 min of stimulation by Ang II, since IP_3 formation is markedly increased in a few seconds [27], we should also examine IP formation in an early phase. AT_1 receptor blockade decreased PLC-β_3 phosphorylation, and AT_2 receptor blockade increased phosphorylation. AT_1 receptor signals may be mainly blocked by AT_2 receptor signals through negative cross-talk in PLC-β_3 phosphorylation.

AT_1 receptor-induced signaling can counteract the function of AT_2 receptors. Although AT_1 receptor antagonists that prevent

Ang II-induced signaling are clinically available, a new drug may be discovered that resembles a dimeric ligand formed by two monovalent ligands for AT_1 and AT_2 receptors. In this study, we indicated that hetero-dimerization did not explain why the functions of AT_1 and AT_2 receptors are antagonistic. Therefore, discovery of AT_2 receptor agonist, but not a dimeric ligand for AT_1 and AT_2 receptors, may be more useful to block AT_1 receptor-induced signaling in their negative cross-talk in cytoplasm.

In conclusion, AT_1 receptor signals may be mainly blocked by AT_2 receptor signals through their negative cross-talk in PLC-β_3 phosphorylation rather than by the hetero-dimerization of both receptors on the cell surface. The balance of the expression levels of AT_1 and AT_2 receptors may be critical for the antagonistic action between these receptors.

Disclosures

None.

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