Unconventional Homologous Internalization of the Angiotensin II Type-1 Receptor Induced by G-Protein–Independent Signals

Ying-Hong Feng, Yaxian Ding, Shuo Ren, Lingyin Zhou, Chuan Xu, Sadashiva S. Karnik

Abstract

Internalization of a G-protein–coupled receptor (GPCR) is essential to the desensitization, endocytosis, and signal transduction of the receptor. It has been the general view that conventional homologous internalization of a GPCR requires activation of the G-protein(s) coupled to the receptor. However, whether and how GPCR-mediated G-protein–independent signals trigger receptor internalization remains unknown, although G-protein–independent internalization has been reported. Here we show that an angiotensin II (Ang II) type-1 (AT₁) receptor mutant incapable of activating any G-protein still undergoes normal internalization. Substitution of Asp¹²⁵ with Ala and Arg¹²⁶ with Leu at the highly conserved DRY motif of the AT₁ receptor disabled the ability of the receptor to activate G-proteins, as shown by various Ang II binding studies, GDP–GTP exchange, and inositol phosphate production assays. Surprisingly, the mutant internalized normally in the presence of Ang II and transactivated the epidermal growth factor receptor (EGFR). Similar to the wild-type receptor, overexpression of a dominant-negative K220R mutant GRK2 diminished the internalization of D125A-R126L but not the transactivation of EGFR. These data indicate that G-protein–independent specific signals may also trigger homologous internalizations of the AT₁ receptor through /arrestin–dependent and –independent pathways, suggesting a possible mechanism for G-protein–independent activation of G-protein–coupled receptor kinases (GRKs). This may represent a general mechanism for triggering GPCR internalization. (Hypertension. 2005;46:419-425.)

Key Words: receptors, angiotensin II | G-protein | angiotensin II

Agonist binding to a G-protein–coupled receptor (GPCR) induces conformational changes in the receptor, leading to activation of Gaβγ heterotrimers. One function of the activated G-proteins is to activate G-protein–coupled receptor kinases (GRKs) that in turn phosphorylate the specific receptor for desensitization. Subsequently, /arrestins bind to the GRK-phosphorylated motifs of the receptor and induce the receptor internalization. This homologous GPCR desensitization/internalization is agonist specific and GRK dependent. This type of feedback regulation is conventional because it requires activation of classic G-proteins.¹–³ Homologous internalization of GPCRs can also take place through /arrestin–independent pathway. However, the underlying mechanism and especially the role of G-protein activation in this mode of internalization remain poorly understood.⁴–⁶ The second messenger-stimulated kinases protein kinase A (PKA) and protein kinase C (PKC) also phosphorylate the receptor and induce heterologous desensitization/internalization that is not agonist specific.¹–³ It is known that a GPCR activates not only multiple G-protein pathways but also non–G-protein pathways. However, it remains unknown whether activation of non–G-protein signal pathways also exert homologous feedback regulations that are agonist specific.

GPCRs share common structural features, such as 7 transmembrane spanning regions (TMs), and preserve highly conserved consensus motifs, such as DRY motif at the boundary of TM3 and the second cytoplasmic loop. As a result, the GPCRs are thought to use similar mechanisms for G-protein coupling and activation.⁷,⁸ For example, the DRY motif is thought to play pivotal roles in all G-protein coupling and activation. A salt bridge formed between the Asp and Arg of the motif may serve as a switch for GDP–GTP exchange, an essential process required for any G-protein activation. Mutation of this motif in rhodopsin, especially the conserved Arg residue, has been shown to totally inhibit the receptor from G-protein coupling and activation.⁹

Angiotensin II (Ang II) type-1 (AT₁) receptors exert complex and diverse physiological actions associated with many diseases or disorders such as hypertension, hypertrophy, fibrosis, thrombosis, and atherosclerosis. In addition to activation of multiple G-proteins including G₂₁₁, Gα₁, and
G_{13},^{10–15} AT_1 receptors activate the Jak2/STAT pathway and transactivate epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) receptors.^{14–18} The AT_1 receptors even activate signal pathways in a more complex fashion by forming homodimerization or heterodimerization with the AT_2,^{19–22} AT_3,^{19} bradykinin B_2,^{23} β_2-adrenergic,^{24} and dopamine D_1,^{25,26} receptors. In the case of these complex activations, whether and how the AT_1-initiated discrete signals regulate the homologous internalization of the receptor, what signals trigger the homologous internalization and whether G-protein–independent signals are also capable of initiating receptor internalization and whether the AT_1-initiated discrete signals trigger the homologous internalization of the receptor, what G-protein–independent signals are also capable of initiating homologous internalization remain to be addressed. It has been reported that mutations of a highly conserved Asp_74 residue in TM2 of the AT_1 receptor impaired inositol phosphate-3 (IP3) production but not agonist-induced internalization. However, the Asp_74 mutants still produced a minimal amount of IP3^{27,28} and remained sensitive to GTPγS.^{29} In this report, we show that double mutations of Asp_220 to Ala and Arg_126 to Leu (D125A-R126L) at the highly conserved DRY motif of the AT_1 receptor induced uncoupling of the receptor from all G-proteins but had little effect on the receptor internalization and the EGF receptor (EGFR) transactivation.

**Materials and Methods**

**Materials**

Oligonucleotides were synthesized by Sigma-Genosys or MWG Biotech. Ang II and [Sar^1]Ang II were purchased from Bachem. Other peptide analogues of [Sar^1]Ang II were synthesized by GeneMed Synthesis. Losartan, EXP3174, and candesartan were gifts from DuPont Merck Co. (Wilmington, Del.). [Sar^1,ile^2]Ang II (2200 Ci/mmol) was purchased from The Peptide Radioidination Center of Washington State University. [3H]myo-inositol (22 mCi/mL) was purchased from Amersham Biosciences. The monoclonal antibody 1D4 was purchased from the Cell Culture Center, the AG1478 from Calbiochem (EMD Biosciences, Inc.), and the antibodies for extracellular signal-regulated kinase (ERK) and phospho-ERK from Cell Signaling Technology, GTPγS from Sigma, and [35S]GTPγS from PerkinElmer Life Science Products.

**GTPγS and GDP Exchange Assay**

[35S]GTPγS binding and immunoprecipitation were performed as described previously.^{31,32} Briefly, each tube contained 0.5 μmol/L GDP, 0.1 μmol/L [35S]GTPγS, 50 μg membrane protein, and various concentrations of Ang II in a final volume of 250 μL. The binding assay was performed in an assay buffer consisting of 50 mMol/L HEPES, 100 mMol/L NaCl, and 10 mMol/L MgCl_2, pH 7.4. Nonspecific binding of [35S]GTPγS was determined in the presence of 10 μmol/L of unlabeled GTPγS. Tubes were assembled on ice and the reaction started by incubation of tubes at 30°C. After 20 minutes of incubation, the reaction was terminated by the addition of 750 μL of ice-cold assay buffer. The reaction mixture was pelleted by centrifugation at 20000g for 5 minutes at 4°C. Pellets were resuspended in 100 μL of solubilization buffer (50 mMol/L Tris/HCl, pH 7.4, 150 mMol/L NaCl, 2 mMol/L EDTA, 1.25% (v/v) Nonidet P-40 and 0.2% (v/v) sodium dodecyl sulfate (SDS)), containing protease inhibitors. The solubilized pellets were diluted further, with 100 μL of solubilization buffer minus SDS, and precleared by the addition of 1.3% (v/v) rabbit serum and 30 μL of protein A Sepharose for 1 hour at 4°C. Samples were centrifuged at 20000g, 4°C, for 5 minutes. The supernatant (100 μL) was removed and added to a mixture of anti-G_αs, anti-G_αi, and anti-G_αq antibodies (Santa Cruz Biotechnolog). Samples were incubated at 4°C overnight before addition of 50 μL of protein A Sepharose. After a further incubation at 4°C for 2 hours, the Sepharose beads were washed 3× with 0.5 mL of solubilization buffer minus SDS. Finally, the solubilization buffer was removed and scintillation fluid added before quantification by liquid scintillation counting.

**Production of Total IPs**

The COS-1 cells, cultured in 60-mm Petri dishes, were labeled for 24 hours with [3H]myo-inositol (1 μCi/mL) at 37°C in inositol-free DMEM containing 10% bovine calf serum 24 hours after transfection. For the IP assay (ie, 48 hours after transfection), labeled cells were washed 3× with serum-free medium and incubated with DMEM containing 10 mMol/L LiCl for 20 minutes. Then medium alone or ligands were added to the cells. After incubation for 45 minutes at 37°C, the medium was removed, and total soluble IP was extracted from the cells by a perchloric acid extraction method as described previously.^{30} The amount of [3H]-IP eluted from the column was counted and a concentration–response curve generated using iterative nonlinear regression analysis.

**Measurement of AT_1 Receptor Internalization**

Internalization of the AT_1 receptors was measured by a method described previously.^{33} Briefly, COS-1 cells, transiently transfected with AT_1 receptors in 12-well plates, were stimulated with and without Ang II (0.03 and 100 mMol/L) for 10 minutes at 37°C. Surface-bound ligands were removed by a gentle acid wash (50 mMol/L sodium citrate, 0.2 mMol/L sodium phosphate, 90 mMol/L NaCl, and 0.1% BSA, pH 5.0) for 10 minutes at 4°C, which did not affect subsequent receptor binding. Then a radioligand binding assay was performed (5 hours at 4°C) to quantify receptors remaining at the cell surface. Internalized receptors are expressed as a percentage loss of cell surface binding compared with cells not exposed to Ang II.

**Immunoblots**

Cells that were serum deprived overnight were treated with Ang II in the presence and absence of 100 mMol/L of the EGF-specific inhibitor AG1478 for 20 minutes. After this, cells were washed twice with ice-cold Dulbecco’s PBS. Cells were then lysed on ice with ice-cold lysis buffer (50 mMol/L Tris, pH 7.2, 1% [vol/vol] Triton X-100, 1 mMol/L Na_3VO_4, 1 mMol/L EGTA, 0.2 mMol/L phenylmethylsulfonyl fluoride, 25 μg/mL leupeptin, and 10 μg/mL aprotinin). Samples were then centrifuged at 14 000g for 10 minutes. Protein content in the supernatants was determined by the BCA assay according to manufacturer instructions (Pierce). A total of 15 μg of total cell lysate protein was subjected to SDS-PAGE and then transferred to a polyvinylidene difluoride membrane by electroblotting at 200 mA for 1.5 hours. The membrane was immunoblotted according to a standard Western blot protocol as furnished by the manufacturers, and the immunoreactive proteins were detected by enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech Inc.). An autoradiograph of the blot was analyzed by an OS-Scan Image Analysis System to obtain the densitometry data.

**Statistical Analysis**

Results are expressed as the mean±SEM of 3 to 5 independent determinations. The significance in measured values was evaluated using an unpaired Student’s t test.

**Results**

**AT_1 Receptor Mutant: Characterization and Ligand Binding**

To abolish all G-protein activation activity of the AT_1 receptor, double mutations were introduced into an AT_1 receptor in which the highly conserved Asp_220 and Arg_126 residues were substituted with Ala_220 and Leu_126 as illustrated in Figure 1. The expression of the wild-type and mutant receptors in COS-1 cells was confirmed by ligand binding and Western blots with 1D4 monoclonal antibody (data not
Recombinant expression in transiently transfected COS-1 cells was used for characterization of the AT1 receptors as described previously.30,34 Expression in each case was measured by immunoblotting with a C-terminal epitope-directed monoclonal antibody 1D4, followed by 125I-[Sar1,Ile8]Ang II saturation binding and competition binding to the AT1 receptor ligands, along with IP production to measure function. In the total membrane fraction, the high-affinity binding of the exogenous ligands, along with IP production to measure function. In the total membrane fraction, the high-affinity binding of the expressed wild-type and mutant AT1 receptors was 0.50±0.07 and 0.53±0.09 nmol/L, respectively, for the cold peptide antagonist 125I-[Sar1,Ile8]Ang II, and 11.8±1.7 and 11.2±1.4 nmol/L, respectively, for AT1 receptor–selective nonpeptide antagonist losartan. Similar to the wild-type AT1 receptor, the Kd values of the mutant receptor estimated from competition binding for the agonist [Sar1]Ang II and the native hormone Ang II were 0.46±0.06 and 1.51±0.11 nmol/L, respectively. These values were generated with EDTA-washed membranes and therefore represent the intrinsic affinity of the receptor in the absence of G-protein coupling. The Bmax values, estimated for the 2 receptors, were close in value (≈4 pmol/mg membrane protein), consistent with the levels of receptor expression estimated by immunoblot analysis. Scatchard plot analysis of the 125I-[Sar1,Ile8]Ang II saturation binding indicated a single affinity class for both receptors (Kd 0.49±0.07 and 0.51±0.08 for wild-type and the mutant, respectively). Competition binding studies using Ang II, [Sar1]Ang II, and losartan demonstrated that the receptors expressed in COS-1 cells preserve the selectivity and affinity profile described previously for native tissue receptors and recombinant-expressed receptors.

**Mutant D125A-R126L Fails to Induce GTP–GDP Exchange**

The ability of the AT1 receptor to activate IP production in COS-1 cells has been demonstrated previously in this laboratory.34 In sharp contrast to the wild-type AT1 receptors, mutant D125A-R126L failed to produce IP in the presence of Ang II at various concentrations (Figure 2). Moreover, the basal IP production was negligible, even when the mutant was overexpressed in the COS-1 cells, whereas the wild-type receptor—expressing COS-1 cells produced 2.6% basal IP activity in the absence of any exogenous agonist (Figure 2). These IP results were the net IP production after deduction of IP values (=2.5% or 1000 cpm/10^6 cells) from mock-transfected COS-1 cells.

The complete inability of D125A-R126L in activating the Gαi pathway may not preclude the possibility that the defective mutant receptor is capable of activating other G-proteins, directly or indirectly, through other mechanisms. To examine this possibility, a series of binding experiments were performed. Figure 3 shows that the mutant receptor was completely insensitive to Mg2+ (Figure 3B) and GTPγS (Figure 3C), whereas the wild-type receptor displayed an increased binding affinity for the agonist Ang II (Figure 3A) in the presence of Mg2+ and a decreased binding affinity for the agonist 125I-Ang II in the presence of GTPγS (Figure 3C). This result indicates that the mutant receptor did not directly couple to any G-proteins.

To examine whether the mutant receptors directly or indirectly induced GTP–GDP exchange, GTPγS and GDP exchange assays were performed. Consistent with the above binding assays, stimulation of the mutant receptor with Ang II failed to induce any GTP–GDP exchange mediated by G-proteins, suggesting no activation of any Gαi proteins. In contrast, the wild-type receptor elicited an apparent GTP–GDP exchange, indicating activation of G-proteins (Figure 4).

**Mutant D125A-R126L Still Internalizes**

In view of the apparent difference in G-protein coupling and activation between mutant D125A-R126L and the wild-type AT1,
receptors, we examined the capacity of the mutant to undergo homologous internalization. Treatment of the wild-type and D125A-R126L mutant with 100 nmol/L Ang II for 10 minutes induced 51.6% and 45.8% receptor internalization, respectively (Figure 5A). The kinetics of the internalization of the mutant was similar to the wild type, although the level of the internalized mutant receptors was 5.8% lower \((P<0.05)\). Similar internalization profiles were observed for wild-type and the mutant receptors when Ang II at low level (0.03 nmol/L) was used (data not shown). The inactive Ang II analog \([\text{Sar}^1,\text{Ile}^4,\text{Ile}^8]\)Ang II failed to induce internalization of either receptor (Figure 5A), consistent with a previous report\(^{33}\). Because GRK2 is known to play an important role in initiation of homologous internalization of AT\(_1\) receptors, overexpression of a dominant-negative R220K mutant GRK2 was used in the study and reduced the internalization to 24.2% and 18.3% for the wild-type and mutant receptors, respectively (Figure 5). These reductions reflected 53.1% and 60% decreases in the capacity of receptor internalization. Interestingly, the magnitude of the reduction in the mutant receptor was 6.9% greater compared with the wild type \((P<0.05)\). These changes in magnitude are also apparent when the kinetics of receptor internalization is plotted as shown in Figure 5B. However, no changes in the time course of internalization were observed between the wild-type and mutant receptors in the presence and absence of R220K expression. Also AG1478, an EGFR kinase–specific inhibitor, failed to affect internalization of either receptor.

In the absence of Ang II, the heterologous internalization of the wild-type and mutant D125A-R126L was negligible under the experimental conditions, as detected by saturation binding assays with \([\text{Sar}^1,\text{Ile}^4,\text{Ile}^8]\)Ang II.

**Mutant D125A-R126L Receptor Transactivates EGFRs**

Because D125A-R126L still underwent almost normal homologous internalization, it must have initiated certain specific signals that preceded the internalization. To identify the specific D125A-R126L–initiated signals, transactivation of EGFR was examined. In the presence or absence of EGFR

![Figure 3](image-url)

**Figure 3.** Ligand binding property of the AT\(_1\) receptors. A and B, Competition binding of \([\text{Sar}^1,\text{Ile}^4,\text{Ile}^8]\)Ang II with agonist Ang II in the presence and absence of 10 mmol/L Mg\(^{2+}\). C, Binding of agonist \([\text{Sar}^1,\text{Ile}^4,\text{Ile}^8]\)Ang II in the presence of GTP\(_\gamma\)S. The membrane preparations used for binding assays with 10 mmol/L Mg\(^{2+}\) and GTP\(_\gamma\)S were prepared in the presence of Mg\(^{2+}\) without EDTA wash. Values are means±SEM of \(\approx3\) independent experiments in duplicate.

![Figure 4](image-url)

**Figure 4.** AT\(_1\) receptor–mediated activation of G\(_\alpha\) proteins detected by \([35S]\)GTP\(_\gamma\)S and GDP exchange assay. A, \([35S]\)GTP\(_\gamma\)S (0.1 nmol/L) binding in the presence of increasing amounts of Ang II for 20 minutes. B, Time-dependent \([35S]\)GTP\(_\gamma\)S binding in the presence of 100 nmol/L Ang II. C, Saturation binding of \([35S]\)GTP\(_\gamma\)S in the presence of 100 nmol/L Ang II for 20 minutes. The membrane proteins used for GTP\(_\gamma\)S and GDP exchange assay were prepared in the presence of Mg\(^{2+}\) without EDTA wash. Values are means±SEM of \(\approx3\) independent experiments.
kinase–specific inhibitor AG1478, the wild-type and mutant receptors displayed no difference in transactivation of EGFR on [Sar1]Ang II or Ang II stimulation (Figure 6). This suggests that the AT1 receptor–mediated transactivation of EGFR is independent of G-protein activation. Overexpression of the dominant-negative R220K mutant GRK2 did not diminish the transactivation of EGFR by either receptor (data not shown). The inactive Ang II analog [Sar1,Ile4,Ile8]Ang II also failed to induce the transactivation of EGFR in COS-1 cells expressing the wild-type and mutant AT1 receptors (data not shown).

**Discussion**

Internalization plays an important role in receptor desensitization, endocytosis, and signal transduction. Heterologous internalization of a GPCR is a passive process that does not require a specific agonist binding to the receptor. However, homologous internalization of a GPCR is an active process that requires the following: (1) specific ligand binding, (2) a conformational change of the receptor, (3) GRK-mediated phosphorylation of the receptor, and (4) signal transductions initiated by the activated receptor. It is generally believed that conventional homologous internalization of a GPCR depends on the activation of G-proteins because activation of GRKs requires preactivation of G-proteins (Figure 7). To determine whether non–G-protein signals, also activated by a GPCR, might also trigger homologous internalization of the receptor, a mutant receptor that would still interact with the agonist but fail to activate G-proteins was required. The AT1 mutant receptor D125A-R126L was constructed to serve this purpose (Figure 1). This mutant receptor showed unimpaired agonist binding capacity but failed to activate any G-protein. Therefore, it would be anticipated that the mutant receptor would no longer be able to undergo conventional homologous internalization because it no longer activated G-proteins or GRKs. To our surprise, the mutant receptor showed almost normal homologous internalization in the presence of agonist Ang II at high (100 nmol/L) and low (0.03 nmol/L) levels. The finding at low level of Ang II is consistent with previous observations made by Gaborik et al with a similar double-mutant DRY/AAY.35,36 This result indicates that homologous internalization of the AT1 receptor is also inducible through mechanisms distinct from G-protein activation. The type of homologous internalization induced through G-protein activation–independent mechanisms is therefore “unconventional” (Figure 7).

Phosphorylation of an activated GPCR by GRKs is a tightly regulated process. Activation of these GRKs often requires activated G-proteins. For example, activation of GRK2 requires double binding of the activated $G_{\alpha}$ and $G_{\beta\gamma}$ dimer to the N-terminal regulator of G-protein signaling (RGS)–like domain and the C-terminal pH domain of GRK2.37–39 The K220R mutant GRK2 is deficient only in its protein kinase activity. Its binding capacity to proteins such as $G_{\alpha}$ and $G_{\beta\gamma}$ remains intact. It is known that GRK2, GRK3, and GRK5 phosphorylate activated AT1 receptors, and expression of the dominant-negative K220R mutant GRK2 causes ~50% inhibition of the wild-type AT1 internalization.40 However, our results suggest that GRKs still played an important role in inducing internalization of the mutant D125A-R126L. This suggests that the GRKs could be activated through G-protein–independent mechanisms because the dominant-negative K220R mutant GRK2 also diminished “unconventional” homologous internalization of the mutant D125A-R126L. This calls for further study of the mechanisms of GRK activation. Indeed, a recent report has shown that the N-terminal RGS-like domain of the GRK2 also binds to the metabotropic glutamate receptor.
Other kinases may also be involved in the initiation of homologous internalizations of the AT1 receptor. Transactivation of EGFR is not only independent of AT1 receptor internalization but also independent of G-protein activation.

ed.36,44 It is also unknown whether altered or activated conformation alone of the AT1 receptor can trigger homologous internalization by recruiting β-arrestins or other molecules such as caveolin or GRKs. Our results suggest that the G-protein–independent homologous internalization of the mutant D125A-R126L could be initiated by G-protein–independent signals that transactivate EGFR. However, in the present study, we were unable to identify the exact signal that might have triggered the receptor internalization because the detailed mechanism leading to EGFR transactivation remains largely unknown. The fact that the EGFR-specific inhibitor AG1478 failed to block the homologous internalization of the mutant AT1 receptor suggests that the internalization-triggering signal is upstream of the transactivation of EGFR.

Figure 7. Internalization-triggering signals and internalization of the AT1 receptors. a, Mechanism of heterologous internalization initiated by PKA and PKC; b, mechanism of conventional homologous internalization initiated by an activated G-proteins; c, mechanism of unconventional homologous internalization initiated by G-protein–independent signals; d, β-arrestin–dependent internalization (binding of β-arrestins to a GPCR is essential); e, β-arrestin–independent internalization (binding of other non-β-arrestin molecules such as caveolin or GRK2 is essential). Here, R*, dR*, and iR* means activated, desensitized, and internalized AT1 receptors, respectively. In this illustration, GRKs play an important role in G-protein–dependent conventional homologous internalization and G-protein–independent unconventional homologous internalization.

Perspectives
This study showed that β-arrestin–dependent and –independent homologous internalizations could take place independently of G-protein activation. The G-protein–independent signals initiated by AT1 receptors may also trigger homologous internalization. This may represent a general mechanism for triggering GPCR internalization. Our results also suggest that GRKs might be activated through G-protein–independent mechanisms, and the AT1-mediated transactivation of EGFR is not only G-protein independent but also internalization independent.

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References


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