Tyrosine Kinase Activation by the Angiotensin II Receptor in the Absence of Calcium Signaling*

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The angiotensin II type 1 (AT1) receptor signals via heterotrimeric G-proteins and intracellular tyrosine kinases. Here, we investigate a modified AT1 receptor, termed M5, where the last five tyrosines (residues 292, 302, 312, 319, and 339) within the intracellular carboxyl tail have been mutated to phenylalanine. This receptor did not elevate cytosolic free calcium or inositol phosphate production in response to angiotensin II, suggesting an uncoupling of the receptor from G-protein activation. Despite this, the M5 receptor still activated tyrosine kinases, induced STAT1 tyrosine phosphorylation, and stimulated cell proliferation. We also studied another AT1 mutant receptor, D74E, stably expressed in Chinese hamster ovarian cells and a fibroblast cell line from mice with a genetic inactivation of Gq/11. Both cell lines have a defect in calcium signaling and in G-protein activation, and yet in both cell lines, angiotensin II induced the time-dependent tyrosine phosphorylation of STAT1. These studies are the first to show the ability of a seven-transmembrane receptor to activate intracellular tyrosine kinase pathways in the absence of a G-protein-coupled rise in intracellular calcium.

The AT1 receptor is a seven-transmembrane receptor that signals via heterotrimeric G-proteins (1, 2). Ligand binding activates Gq, leading to the generation of inositol 1,4,5-trisphosphate (IP3) and a rise in cytosolic free calcium. This, in turn, affects cell contractility, secretion, gene transcription, and cell proliferation.

More recently, the AT1 receptor has also been shown to signal by activating nonmembrane tyrosine kinases including Src, Fyn, and Pyk2 (3). In 1995, our group reported that the binding of angiotensin II to the AT1 receptor activated the intracellular tyrosine kinase Jak2 and that this led to the tyrosine phosphorylation and nuclear translocation of the transcription factor STAT1 (4). Although the activation of tyrosine kinases by G-protein-coupled receptor has now been established in several systems including vascular smooth muscle cells (5, 6), mesangial cells (7), zona glomerulosa cells (8), and cardiac cells (9, 10), the interplay between G-protein activation and receptor-mediated kinase activation is less clear. Structure-function studies of the AT1 receptor have defined amino acids critical for coupling the receptor to G-proteins. For example, mutation of either Asp74 or Tyr209 results in the loss of G-protein coupling and blocks the ligand-dependent production of IP3 (11, 12). However, the effect of these mutations on AT1 receptor-dependent tyrosine kinase signaling is not known. In fact, dissecting the importance of angiotensin II-dependent tyrosine phosphorylation signaling from G-protein activation is difficult because of multiple conversion points. For example, both the G-protein and tyrosine kinase pathways activate mitogen-activated protein kinase (9, 10), and both also give rise to an increase in cytosolic free calcium (5, 6, 7).

To separate the effects of AT1 receptor activation of G-proteins from that of tyrosine kinases, we studied a modified AT1 receptor termed M5. The M5 mutant varies from the wild-type AT1 receptor in that the last five tyrosines (positions 292, 302, 312, 319, and 339) within the intracellular carboxyl tail of the AT1 receptor were mutated to phenylalanine. We found that the M5 receptor cannot induce a ligand-mediated calcium signal, suggesting an uncoupling of the mutant receptor from G-protein activation. In contrast, the M5 receptor retained its ability to activate the Jak-STAT pathway. We also studied two other cell lines with defects in calcium signaling. One is a Chinese hamster ovarian cell (CHO) cell line stably transfected with an AT1 receptor containing a D74E mutation. This modified AT1 receptor was previously shown to be uncoupled from G-protein activation (11). In addition, we investigated angiotensin II signaling in fibroblasts derived from mice with a genetic inactivation of Gq/11 (13, 14). In both cell lines, angiotensin II stimulated the tyrosine phosphorylation of STAT1. These studies are the first to show the ability of a seven-transmembrane receptor to activate an intracellular tyrosine kinase pathway in the absence of a G-protein-coupled rise in intracellular calcium.

MATERIALS AND METHODS

Chinese Hamster Ovarian Cell Clones—The M5 construct was made using dut ung mutagenesis (Bio-Rad) and verified by DNA sequencing. Native CHO were maintained in F-12 media (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Summit Biotechnology), 0.1 units/ml penicillin-streptomycin, 2 mM l-glutamine, and 1 mM sodium pyruvate. For the generation of AT1 and M5 CHO clones, the cells were stably transfected with either wild-type rat AT1a or M5 plasmid DNA under the control of the SV40 promoter (vector pZeosSV; Invitrogen, Carlsbad, CA) using Lipofectin (Life Technologies, Inc.). The transfected cells were selected with 250 μg/ml of zeocin until individual clones were prevalent. The cells were ring-cloned and screened for binding of 125I-sarcoy1, Ile⁸-angiotensin II (125I-Sar-Ile). The cells were maintained in 125 μg/ml of zeocin for all subsequent cultures. CHO cells stably expressing the AT1 D74E mutant were a kind gift of Dr. Eric Clauser, INSERM, Paris, France (11). Fibroblasts derived from knockout mice with a targeted mutation inactivating Gq/11 were a kind...
Tyrosine Kinase Activation without Calcium Signaling

20955

gift of Dr. Melvin Simon, California Institute of Technology, Pasadena, CA (13, 14).

125I-Sar-Ile Binding Assay—Ligand binding was measured as described previously (4). Briefly, cells were seeded onto 24-well plates overnight. The cells were washed twice with physiological saline (in mM): 120 NaCl, 5 KCl, 2 CaCl₂, 10 glucose, 1 MgCl₂, and 10 Hepes, pH 7.4. Varying concentrations of 125I-Sar-Ile were then added in the absence or presence of 1 μM angiotensin II for 1 h at room temperature. The plates were placed on ice and washed four times with physiological saline without bovine serum albumin. NaOH (0.5 M) was added to each well, and the lysed cells were counted with a Bio-Rad Dc protein assay kit (Bio-Rad).

Calcium Measurements—Cells were harvested using 0.05% trypsin/EDTA (Life Technologies, Inc.). The cells were washed with Hepes buffer (in mM): 140 NaCl, 5 KCl, 2 CaCl₂, 10 glucose, 1 MgCl₂, and 10 Hepes, pH 7.4. The cells were loaded with 20 μM fura-2/AM (Molecular Probes) for 30 min at 37 °C and washed for 30 min in the absence of fura-2/AM. Cells were resuspended in 2 ml Hepes buffer, and fluorescence measurements were taken on a time-based scale with an SLM Aminco spectrofluorometer. The fluorophore was excited at 340 and 380 nm. The emission was collected at 510 nm. The relative maximum and relative minimum ratio (340/380) values were acquired in the presence of 0.1% Triton X-100 and 12.5 mM KCl.

Inositol-1,4,5 Triphosphate Production—IP₃ production was measured as described previously (5). Briefly, cells were serum-deprived for 12–16 h and then stimulated with 100 nM angiotensin II for various times in 5 ml F-12 media. Trichloroacetic acid was added to stop the reaction. The cells were scraped, sonicated, and centrifuged at 10,000 × g for 10 min at 4 °C. The supernatant was collected, and protein concentration was determined using the Bio-Rad Dc protein assay kit (Bio-Rad).

Immunoprecipitation and Western Blot—Cells were serum-deprived for 16–20 h and stimulated with 100 nM angiotensin II for various durations. The cells were washed twice with cold phosphate-buffered saline and lysed with 1 ml of RIPA (20 mM Tris, pH 7.5, 10% glycerol, 1% Triton X-100, 1% deoxycholic acid, 0.1% SDS, 2.5 mM EDTA, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM NaN₃, and 10 μg/ml aprotinin). The cells were scraped, sonicated, and centrifuged at 10,000 × g for 10 min at 4 °C. The supernatant was collected, and protein concentration was determined using the Bio-Rad Dc protein assay kit. Lysate was incubated with 1 μg of antibody and 20 μl of a 50% slurry of protein A/G plus Sepharose beads (Santa Cruz Biotechnology, Santa Cruz, CA) for 2–24 h at 4 °C. The immunoprecipitant was washed two-four times with wash buffer (25 mM Tris, pH 7.5, 150 mM NaCl, and 0.1% Triton X-100) and resuspended in SDS loading buffer. Proteins were separated on 8% SDS-PAGE and transferred onto nitrocellulose membranes (Schleicher & Schuell). For Cos-7 cells, the cells were lysed by sonication with 0.5 μg of pBSOS/AT1 (gift from Dr. D. M. Wojchowski, Pennsylvania State University) and 10 μg of either pZEO/AT1 or of pZEO/M5 using Lipofectin (Life Technologies, Inc.).

The nitrocellulose membranes were blocked with 5% dry skim milk in TBST (20 mM Tris, pH 7.2, 150 mM NaCl, and 0.05% Tween 20) for 1 h at room temperature. The membrane was incubated with 1 μg/ml of monoclonal anti-phosphotyrosine (PY99, Santa Cruz Biotechnology, Santa Cruz, CA), 1:1000 polyclonal anti-phosphotyrosine (PY99), or 1:1000 monoclonal anti-STAT1 (Transduction Laboratories, Lexington, KY) for 2 h. The membrane was washed three times for 30 min and subsequently incubated with 1:4000 secondary anti-mouse IgG (Amersham Pharmacia Biotech) for 1 h. The membrane was then washed and exposed for enhanced chemiluminescence (PerkinElmer Life Sciences) on X-Omat Blue film (Eastman Kodak Co., Rochester, NY). Unless otherwise stated, all membranes were stripped after blotting with anti-phosphotyrosine and reblotted with anti-Jak2 or anti-STAT1 to ensure equal loading of the immunoprecipitated protein.

Cellular Proliferation Assay—CHO cells were plated onto 96-well plates at a density of 5 × 10³ cells/well with F-12 media containing no serum. The cells were then treated with vehicle or 125I-Sar-Ile for 4 h. MTS tetrazolium (Owen’s reagent) (CellTiter 96® Aqueous; Promega, Madison, WI) was added at a ratio of 1:5 relative to the volume of the media. The reaction proceeded for 4 h at 37 °C and was terminated by the addition of 2% SDS. The absorbance was recorded at 490 nm. For each condition, the samples were done in triplicate and averaged for each experiment.

All data were presented as representative trace or mean ± S.E.
Tyrosine Kinase Activation without Calcium Signaling

Fig. 2. IP₃ production. A, CHO AT₁ (○) or M5 (■)-expressing cells were stimulated with 100 nM angiotensin for 0, 0.25, 0.5, 3, or 5 min. IP₃ production was measured by radioreceptor assay. The data are from one experiment that is representative of three independent experiments. B, the average-fold change of IP₃ generation in AT₁ or M5 cells when stimulated with 100 nM angiotensin II for 15 s (n = 3; * depicts p < 0.05).

Response but had no effect on the ATP-evoked calcium response (data not shown). In contrast to the response of the wild-type AT₁ receptor, CHO cells expressing the M5 mutant showed no increase in cytosolic free calcium in response to angiotensin II (Fig. 1C). This is not because of an intrinsic defect in calcium signaling per se, because these cells were responsive to ATP. We also evaluated the signaling of a second CHO clone expressing the M5 receptor (clone 8). Despite a nearly 10-fold greater expression of M5 receptor levels than observed with clone 5 cells, clone 8 still showed no rise of intracellular calcium in response to angiotensin II (data not shown). These data suggested that mutation of the carboxyl-terminal five tyrosines of the AT₁ receptor disabled receptor activation of Gq.

Angiotensin II-mediated calcium signaling is dependent on IP₃ formation. Therefore, we investigated whether the M5 cells were deficient in IP₃ production. As shown in Fig. 2A, CHO cells expressing the wild-type AT₁ receptor responded to angiotensin II with a rapid increase in IP₃ levels within 15 s of stimulation. Quantitation showed a 4.0 ± 1.9-fold increase over basal levels of IP₃ after 15 s (Fig. 2B). In contrast, cells expressing the M5 receptor showed no significant change (1.1 ± 0.1-fold) in IP₃ production after angiotensin II addition. These data are consistent with the lack of a ligand-mediated calcium response; taken together, they suggest a loss of Gq activation by the M5 receptor.

In addition to IP₃ and calcium signaling, the AT₁ receptor also activates nonmembrane tyrosine kinases, such as Jak2. Initially, we chose to investigate M5 activation of the Jak-STAT pathway in transiently transfected Cos-7 cells. When these cells were transiently transfected with constructs encoding both the wild-type AT₁ receptor and Jak2, angiotensin II induced a marked increase of Jak2 tyrosine phosphorylation within 3 min of ligand addition (Fig. 3A). Surprisingly, the M5 receptor also responded to angiotensin II with an increase of Jak2 tyrosine phosphorylation. In fact, repetition of this protocol showed that the time course and magnitude of Jak2 activation were similar for both the wild-type AT₁ and M5 receptors.

A consequence of Jak2 activation is the tyrosine phosphorylation of the transcription factor STAT1. When Cos-7 cells were transiently transfected with Jak2 and either the wild-type AT₁ receptor or the M5 receptor, STAT1 was phosphorylated in an equivalent time-dependent fashion upon ligand addition. The phosphorylation of STAT1 by angiotensin II was inhibited when cells expressing either receptor type were pretreated with 10 µM valsartan, a specific AT₁ receptor antagonist. Thus, the experiments in Fig. 3 show that the M5 receptor, despite its inability to signal via calcium stimulation, remains fully capable of activating the Jak-STAT system in response to angiotensin II.

In addition to Cos-7 cells, angiotensin II also stimulates STAT1 tyrosine phosphorylation in CHO cells stably expressing the AT₁ receptor (17). Therefore, we asked whether a CHO clone expressing the M5 receptor would induce STAT1 phosphorylation. Stimulation of native CHO cells with angiotensin II did not show a time-dependent change in STAT1 tyrosine phosphorylation (Fig. 4A). In contrast, both AT₁- and M5-expressing cell clones showed an angiotensin II-mediated increase of STAT1 tyrosine phosphorylation that peaked at about 30 min after ligand addition. The difference in the time course of STAT1 tyrosine phosphorylation in transiently transfected Cos-7 cells (peak phosphorylation at 5–10 min) and stably transfected CHO cells (peak phosphorylation at ~30 min) was equivalent to that found in previously published work (4, 17).

In summary, these experiments and those in Fig. 3 show that the M5 receptor remains capable of activating tyrosine kinases despite the absence of calcium signaling.

The exact mechanism of angiotensin II-mediated STAT1 phosphorylation in CHO cells appears to be somewhat different from that observed in the transiently transfected Cos-7 cells. In CHO cells stably expressing the wild-type AT₁ or the mutant M5 receptors, we were unable to document a ligand-mediated increase of Jak2 tyrosine phosphorylation (data not shown). In addition, the Jak-specific inhibitor AG490 was not effective in
inhibiting angiotensin II-mediated STAT1 phosphorylation in CHO cells but did block STAT1 phosphorylation in smooth muscle cells, a system in which angiotensin II-mediated stimulation of Jak2 has been well established (data not shown). In contrast to the negative results with AG490, pretreatment of CHO AT1, or M5 cells with the broad spectrum tyrosine kinase inhibitor genistein did block angiotensin II-mediated STAT1 tyrosine phosphorylation (Fig. 5). Thus, although the exact mechanism for STAT1 tyrosine phosphorylation in CHO cells is not fully understood, these data establish that, despite an absence of calcium signaling, the M5 receptor can stimulate intracellular tyrosine kinases in response to angiotensin II.

To further investigate the relationship between heterotrimeric G-proteins and angiotensin II-dependent activation of tyrosine kinases, we studied two additional lines of cells. Previously, it was reported that a CHO cell line stably transfected with an AT1 receptor bearing a D74E point mutation does not couple to heterotrimeric G-proteins and, as a consequence, does not mobilize intracellular calcium in response to angiotensin II (11). When the CHO D74E cell line was stimulated with angiotensin II and studied for the time-dependent tyrosine phosphorylation of STAT1, we found this receptor was similar to the M5 receptor in that tyrosine kinase activity was preserved in the absence of calcium signaling (Fig. 6A).

We also investigated angiotensin II-dependent tyrosine phosphorylation of STAT1 using fibroblasts isolated from Gaq/11-deficient mice (13, 14). These mice were prepared using embryonic stem cell targeting and bear a mutation in both Gaq and G11 rendering these proteins functionally inactive. Again, angiotensin II resulted in the tyrosine phosphorylation of STAT1 in a time-dependent fashion (Fig. 6B). The time course of STAT1 phosphorylation was more rapid than that observed with CHO cells but was similar to the time course observed in Cos-7 cells (Fig. 3) and in rat aortic smooth muscle cells (4). As anticipated, the effect of angiotensin II on STAT1 phosphorylation in Gaq/11-deficient fibroblast was markedly blunted by the AT1 receptor inhibitor valsartan (data not shown).

A known effect of angiotensin II is the rapid stimulation of early response genes (19) and the onset of cellular proliferation
AT1 receptor, Y292F, that hindered the production of total inositol phosphates. The native CHO cells did not show any significant differences in cell proliferation with or without angiotensin II (data not presented). These data suggest that the activation of tyrosine kinases by angiotensin II appears necessary for the induction of CHO cell proliferation by this ligand. Furthermore, because proliferation is observed with the M5 mutant, G-protein-mediated calcium signaling appears not to be necessary for the proliferation initiated by angiotensin II.

DISCUSSION

The classical signaling pathway for the AT1 receptor is to couple and activate heterotrimeric G-proteins. Recently, experiments established that this receptor can also activate non-membrane tyrosine kinases including the Jak-STAT pathway (4, 18). A problem in defining the functional role of angiotensin II-mediated tyrosine kinase activation is the difficulty of differentiating the effects mediated by heterotrimeric G-proteins and calcium elevation from the effects predominantly due to the kinase activation. To gain insight into this question, we studied the M5 angiotensin II receptor. This molecule contains five discrete point mutations converting the carboxyl five tyrosine residues to phenylalanine. In response to ligand, the M5 receptor showed no significant elevation of IP3 and cytosolic free calcium. This observation was consistent with the work of Marie et al. (12) who identified a single point mutation of the AT1 receptor, Y292F, that hindered the production of total inositol phosphates. Marie et al. (12) concluded that this mutant receptor was uncoupled from its endogenous G-proteins. In our hands, the M5 mutation is not functionally identical to the Y292F mutation; we find that an AT1 receptor with a single Y292F mutation showed a partial calcium signal in response to angiotensin II (data not shown). Thus, we believe that the four additional Tyr to Phe mutations present in the M5 receptor are necessary for full elimination of G-protein-coupled signaling.

Despite the inability of the M5 receptor to stimulate an increase of intracellular calcium, the receptor is still able to activate tyrosine kinases including Jak2. Previously, our laboratory identified the AT1 motif YIPP as critical in angiotensin II-mediated activation of Jak2 (18). This work suggested that it was the proline residues within the YIPP motif that were critical for Jak2 activation. Because the M5 mutant contains a Y319F mutation, the ability of the M5 mutant to stimulate Jak2 reemphasizes that mechanisms apart from AT1 receptor phosphotyrosine residues are critical in the activation process. At present, the exact biochemical pathway by which the AT1 receptor stimulates intracellular tyrosine kinases is unknown.

Other groups have investigated the structural basis for AT1 receptor activation of heterotrimeric proteins. In particular, Bihoreau et al. (11) found that an AT1 receptor with a D74E mutation cannot induce a calcium transient. Using CHO cells stably transfected with the D74E receptor by Bihoreau et al. (11), we independently confirmed that the AT1 D74E receptor does not stimulate calcium but does stimulate STAT1 tyrosine phosphorylation, similar to our experience with M5. Finally, our studies of cells with a genetic inactivation of Goq and Go11 showed that the AT1 receptor can activate tyrosine phosphorylation in the absence of these G-proteins. Signaling by the AT1 receptor in the absence of Go11 is similar to M5 receptor signaling in that both appear to represent examples of tyrosine kinase activation in the absence of G-protein coupling.

Normally, angiotensin II-mediated signaling is complex with many conversion points between signaling pathways stimulated by elevated intracellular calcium and pathways downstream of kinase activation. Our work now establishes that in CHO cells, the onset of cell proliferation observed with angiotensin II can be attributed in large measure to an activation of tyrosine kinase pathways. The creation of mutant receptors lacking the ability to stimulate specific intracellular pathways is a powerful approach toward establishing the functional cause and effects of individual signaling pathways.

Acknowledgments—We thank Dr. Laurie Dirksen for help in conducting the M5 receptor. Dr. Eric Clauser provided the CHO-AT1 D74E clone. Dr. Melvin Simon provided the fibroblasts isolated from mice expressing the inactivated Go11 mutation. Dr. D. M. Wojcikowski provided the pBOS/Jak2 construct. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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J. Biol. Chem. 2001, 276:20954-20958.
doi: 10.1074/jbc.C100199200 originally published online April 23, 2001

Access the most updated version of this article at doi: 10.1074/jbc.C100199200

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