Determination of the Exact Molecular Requirements for Type 1 Angiotensin Receptor Epidermal Growth Factor Receptor Transactivation and Cardiomyocyte Hypertrophy


Abstract—Major interest surrounds how angiotensin II triggers cardiac hypertrophy via epidermal growth factor receptor transactivation. G protein–mediated transduction, angiotensin type 1 receptor phosphorylation at tyrosine 319, and β-arrestin–dependent scaffolding have been suggested, yet the mechanism remains controversial. We examined these pathways in the most reductionist model of cardiomyocyte growth, neonatal ventricular cardiomyocytes. Analysis with [32P]-labeled cardiomyocytes, wild-type and [Y319A] angiotensin type 1 receptor immunoprecipitation and phosphor-maging, phosphopeptide analysis, and antiphosphotyrosine blotting provided no evidence for tyrosine phosphorylation at Y319 or indeed of the receptor, and mutation of Y319 (to A/F) did not prevent either epidermal growth factor receptor transactivation in COS-7 cells or cardiomyocyte hypertrophy. Instead, we demonstrate that transactivation and cardiomyocyte hypertrophy are completely abrogated by loss of G-protein coupling, whereas a constitutively active angiotensin type 1 receptor mutant was sufficient to trigger transactivation and growth in the absence of ligand. These results were supported by the failure of the β-arrestin–biased ligand SII angiotensin II to transactivate epidermal growth factor receptor or promote hypertrophy, whereas a β-arrestin–uncoupled receptor retained these properties. We also found angiotensin II–mediated cardiomyocyte hypertrophy to be attenuated by a disintegrin and metalloprotease inhibition. Thus, G-protein coupling, and not Y319 phosphorylation or β-arrestin scaffolding, is required for epidermal growth factor receptor transactivation and cardiomyocyte hypertrophy via the angiotensin type 1 receptor. (Hypertension. 2011;57:973-980.)

Key Words: angiotensin II ■ AT1A receptor ■ cardiomyocyte hypertrophy ■ EGFR ■ transactivation ■ ADAM ■ G protein

Angiotensin II (Ang II) contributes to progression of left ventricular hypertrophy, a major independent risk factor for myocardial infarction and sudden death, yet the molecular mechanisms governing cardiac hypertrophy remain controversial. Recently, a new paradigm for Ang II hypertrophy has emerged. We and others have shown that angiotensin type 1 receptors (AT1Rs) hijack the epidermal growth factor receptor (EGFR) via a process termed the “triple membrane-passing signaling paradigm of EGFR transactivation.” More specifically, AT1R activation causes metalloprotease-dependent EGF-like ligand release and subsequent EGFR binding to initiate hypertrophic signaling cascades. The process is likely more complicated: multiple G protein–coupled receptor (GPCR) signals, a disintegrin and metalloproteases (ADAMs), EGF-like ligands, and EGFR subtypes have been implicated in this process.

A major focus of Ang II research is the mechanism by which the AT1R signals to the EGFR. Recent studies suggested a G protein–independent pathway for growth activation, despite evidence that Gαq/11, a heterotrimeric G protein that couples to the AT1R, is essential for hypertrophy. Indeed, others indicate that G-protein coupling to AT1R is required for EGFR transactivation in COS-7 and vascular smooth muscle cells. Meanwhile, evidence for β-arrestin–dependent functional selectivity of the Ang II analog sarcosine, isoleucine, isoleucine, Ang II (SII Ang II), β-arrestin–mediated EGFR transactivation by β1-adrenoceptors independent of G-protein coupling, and the very recent demonstration of β-arrestin–dependent hypertrophy resulting from mechanical stretch suggests that β-arrestin scaffolding of extracellular signal–regulated kinase (ERK) 1/2 may contribute to transactivation and/or cardiac hypertrophy.
Given the therapeutic implications of identifying the precise mechanism of Ang II–mediated EGFR transactivation, we investigated the effect of ADAM inhibition and G protein-coupling, β-arrestin scaffolding, and Y319 phosphorylation on transactivation in a cell culture model of hypertrophy. Herein, we provide evidence that G-protein coupling, but not Y319 phosphorylation or β-arrestin–dependent signaling, is obligatory for EGFR transactivation and AT1R- and ADAM-dependent cardiomyocyte hypertrophy.

Materials and Methods

cDNA and Adenovirus Constructs

AT1R mutants pRC/CMV-[Y215F]AT1,17 and pRC/CMV-[Y319A]AT118 were generated from rat N-terminal hemagglutin antigen epitope-tagged AT1 receptor (pRC/CMV-NHA-AT1)18 using QuikChange (Stratagene); pRC/CMV-[N111G]AT1 and the ANP-luc reporter are described.4,19 The reporter heparin-binding-epidermal growth-factor-alkaline phosphatase (HB-EGF-AP) was from M. Klagsbrun (Children’s Hospital Boston, Boston, MA). Adenoviruses were generated as described.4 [Y319F]AT1A adenovirus was a generous gift from J. Sadoshima (New Jersey Medical School, Newark, NY).8

Cell Culture

Ventricular cardiomyocytes from 1-day–old Sprague-Dawley rats were Percoll purified to >99% homogeneity, as described.4 Animals from the Baker Heart Research Institute Precinct Animal Centre were handled in accordance with the Australian Code of Practice for Care and Use of Animals for Scientific Purposes. COS-7 cells, maintained in DMEM with 10% FBS, were transfected using LipofectAMINE (Invitrogen) and assayed as described.20 Go probes11 inhibitor YM-254890 was a gift from Astellas (Japan).

Hypertrophy Assays

Myocytes were infected 48 hours after plating with purified adenoviruses at multiplicities of infection titrated to give modest expression within the physiological range, assessed by radioligand binding (Table S1, available in the online Data Supplement at http://hyper.ahajournals.org). Control green fluorescent protein–expressing adenovirus infected at 300 multiplicities of infection failed to cause hypertrophy (data not shown), demonstrating receptor specificity. After 48 hours, myocytes were stimulated with Ang II (50%, a single ADAM is unlikely to dominate in Ang II–mediated hypertrophy (Figure S1). We also examined dominant-negative ADAMs 10, 12, and 17 in Ang II–mediated HB-EGF release, an effect that was blocked by the broad-spectrum metalloprotease (matrix metalloproteinase) inhibitor GM6001 and was therefore matrix metalloproteinase dependent (Figure 1C).

Results

Ang II Mediates Neonatal Rat Ventricular Cardiomyocyte Hypertrophy via AT1R, EGFR, and Matrix Metalloproteinase/ADAMs

Two models were used in these studies. The first is an extensively characterized neonatal rat ventricular cardiomyocyte (NRVM) model where the AT1R is reintroduced by adenoviral infection to recapitulate in vivo levels of expression23 (the AT1R is downregulated during purification9). Isolated NRVMs have been used extensively to delineate signaling preceding cell hypertrophy,22 and we have found that Ang II–mediated NRVM hypertrophy is entirely dependent on EGFR transactivation. The second model involves COS-7 cells, which have been used extensively by other groups5,8,11,12 to examine EGFR transactivation because they express high endogenous EGFR but do not express AT1R.

In NRVMs infected to express the WT AT1R (Table S1 for expression), Ang II (100 nmol/L) stimulated ERK1/2 phosphorylation, which could be blocked by the AT1R antagonist candesartan (Figure 1A). EGFR transactivation was necessary for both ERK1/2 activation and subsequent NRVM protein accumulation indicative of hypertrophy, because the EGFR inhibitor AG1478 abrogated the effects of Ang II (Figure 1A and 1B), consistent with our previous characterization of Ang II–mediated transactivation in NRVMs.4 To assess the triple membrane–passing signaling paradigm mode of transactivation, heparin was used as a nonspecific inhibitor of HB-EGF, and it abolished ERK1/2 phosphorylation and cardiomyocyte hypertrophy (Figure 1A and 1B). In COS-7 cells, cotransfection of WT AT1R and an HB-EGF-AP enabled examination of the ability of Ang II to stimulate HB-EGF release, an effect that was blocked by the broad-spectrum metalloprotease (matrix metalloproteinase) inhibitor GM6001 and was therefore matrix metalloproteinase dependent (Figure 1C).

Although AT1R can activate and upregulate matrix metalloproteinase activity, the involvement of ADAMs in EGFR transactivation, hypertrophy, and cardiac remodeling remains unproven. In NRVMs, GM6001 and also the ADAM10/17 inhibitor tumor necrosis factor-alpha protease inhibitor-1 (TAPl-1) significantly attenuated Ang II–stimulated growth (Figure 1D). We also examined dominant-negative ADAMs 10, 12, and 17 in Ang II–mediated HB-EGF release and hypertrophic growth and found that, whereas each dominant negative inhibited Ang II effects by ~50%, a single ADAM is unlikely to dominate in Ang II–dependent EGFR transactivation and cardiomyocyte hypertrophy (Figure S1).

AT1R Is Not Tyrosine Phosphorylated

Tyrosine phosphorylation of the AT1R has been both proposed8,26–28 or refuted11–12,18,29 by various groups as a signaling mechanism for the receptor. Therefore, we sought to confirm the phosphorylation profile of the AT1R and, more specifically, phosphorylation of tyrosine 319. Y319 was mutated to alanine (Y319A), rendering it incapable of being phosphorylated. Initial studies demonstrated that [Y319A]AT1R had equivalent receptor expression, Ang II affinity, and inositol phosphates accumu-
Y319 Is Not Required for Ang II– Stimulated EGFR Transactivation or NRVM Hypertrophy

Although we found no evidence for Y319 phosphorylation, a recent study found Y319 mutation to suppress cardiac hypertrophy in vivo, despite the indication that Y319 was not required for EGFR transactivation in COS-7 or vascular smooth muscle cells. Thus, we next investigated whether Y319 mutation disrupted EGFR transactivation or NRVM hypertrophy independent of tyrosine phosphorylation. Stimulation of [Y319A]AT1R in cardiomyocytes resulted in significant activation of the growth pathway intermediate, ERK1/2, comparable to WT AT1R (Figure 3A and 3B). Ang II also stimulated robust EGFR-transactivation pathway, releasing similar levels of HB-EGF and matrix metalloproteinases/ADAMs. This was also the case when the [Y319F]AT1R-mediated hypertrophy remained EGFR dependent on transactivation. This was also the case when the EGFR was overexpressed (data not shown) to recapitulate the experimental conditions of Seta and Sadoshima. To establish whether the triple membrane-passing signaling paradigm pathway, rather than EGFR transactivation overall, was disturbed by [Y319A]AT1R mutation, COS-7 cells were cotransfected with the HB-EGF-AP reporter and either WT or [Y319A]AT1R, Rs by Western blotting (Figure 2A(ii)), despite detection of strong phosphotyrosine blotting for the positive control (EGF-stimulated A431 cell extracts, Figure 2A(iv)). To assess the level of tyrosine phosphorylation independent of the sensitivity and specificity of the antiphospho-tyrosine Western blotting, the amino acid specificity of AT1R phosphorylation was determined by partial acid hydrolysis of immunopurified AT1R from [32P]-labeled cardiomyocytes (Figure 2B(ii)). In case the glycosylation state of the receptor-affected hydrolysis, both untreated (normally glycosylated) and deglycosylated samples were analyzed. Strikingly, Ang II–mediated AT1R phosphorylation was entirely on serine residues. Critically, there is no Ang II–mediated tyrosine phosphorylation of the AT1R. Taken together, these results indicate that the AT1R is not phosphorylated at Y319 or any other tyrosine residue on Ang II stimulation in either COS-7 cells or cardiomyocytes, directly in contrast with the findings of Seta and Sadoshima.

G-Protein Coupling Is Required for EGFR Transactivation and Hypertrophy

Goq/11 is a well-established mediator of cardiac hypertrophy, so we hypothesized that G-protein coupling to the
AT1R was necessary for both EGFR transactivation and EGFR-mediated cardiomyocyte hypertrophy. In parallel studies to those with [Y319A/F]AT1R, we used a G protein–uncoupled AT1R, [Y215F]AT1, that is uncoupled from Gα11 but still internalizes, albeit to a lesser extent than WT (Figure 4A).17 [Y215F]AT1 was confirmed as a G protein–uncoupled mutant with robust expression (Figure S2 and Table S1) and, although [Y215F]AT1 was described previously with reduced radioligand affinity, we found no statistically significant difference in affinity compared with WT, and the Ang II concentrations used herein are sufficient to ensure almost complete receptor occupancy at either receptor. In COS-7 cells, Ang II failed to cause robust ERK1/2 activation or HB-EGF release via [Y215F]AT1R (Figure 4B and 4C). Furthermore, in [Y215F]AT1-analog NRVMs, Ang II failed to alter total protein or NRVM morphology (Figure 4D and 4E), despite higher expression than WT (Table S1).

We confirmed these findings with the Gα11 inhibitor YM-254890 and Ang II analog (SII Ang II) that only activates β-arrestin–dependent signaling.14,20 In isolated perfused hearts, YM-254890 abrogated Ang II–mediated protein synthesis (Figure S3), and YM-254890 pretreatment prevented robust Ang II–mediated ERK1/2 activation, hypertrophy, and ANP-luc induction in WT-expressing NRVMs (Figure 4F through 4H). Meanwhile, SII Ang II was unable to promote significant sarcomeric reorganization, NRVM protein changes, or ANP-luc (Figure 4I through 4K). Overall, β-arrestin signaling appears neither necessary nor sufficient for Ang II–mediated EGFR transactivation (data not shown) and cardiomyocyte hypertrophy.

G-Protein Coupling Is Sufficient for Cardiomyocyte Hypertrophy

A gain-of-function approach was used using [N111G]AT1,19 which is constitutively coupled to Gα11 (Figure 5A). [N111G]AT1,R-infected NRVMs displayed enhanced basal actin reorganization, NRVM protein changes, or ANP-luc (Figure 4I through 4K). In contrast, a truncated AT1R (TK325) unable to recruit β-arrestins still stimulated hypertrophy and ANP-luc (Figure 4J and 4K). Overall, β-arrestin signaling appears neither necessary nor sufficient for Ang II–mediated EGFR transactivation (data not shown) and cardiomyocyte hypertrophy.
coupling to the AT$_1$R is sufficient for cardiomyocyte growth and depends on EGFR transactivation.

**Discussion**

AT$_1$R-mediated EGFR transactivation has generated substantial interest, yet the mechanism remains equivocal. The major finding of our study is that G-protein coupling, but not $\beta$-arrestin–dependent signaling or Y319 phosphorylation, is the mechanism of Ang II–stimulated EGFR transactivation and cardiomyocyte hypertrophy. Furthermore, although our pharmacological inhibitor studies suggest that ADAMs 10, 12, and 17 are involved in NRVM growth, significant redundancy appears to exist among ADAMs in this system.

Current theories predict that GPCRs adopt multiple conformations to selectively activate signaling pathways within a cell, known as functional selectivity. Indeed, selectively G protein–uncoupled AT$_1$Rs may nevertheless activate markers of growth. These findings were exciting in that they implicated aspects of receptor function, distinct from G-protein coupling, as important growth pathway mediators and suggested that pathway-specific ligands could be developed with greater efficacy and fewer off-target effects. Although surrogate growth markers can be stimulated without G-protein coupling, we report that G-protein coupling is crucial at the cardiomyocyte level for hypertrophy, consistent with a recent study in vascular smooth muscle cells. Moreover, AT$_1$R G-protein coupling is sufficient to trigger cardiomyocyte hypertrophy, as seen with [N111G]AT$_1$AR. This observation is consistent with a recent knock-in study using N111S/329 AT$_1$R, which found that mice had significantly larger heart weight/body weight ratios and elevated brain natriuretic peptide levels, although the more profound phenotype was that of hypertension and cardiac fibrosis. Although G$_{\alpha_q}$ is the obvious candidate for mediating the G-protein–dependent effects described herein, it is important to note that other G-protein subunits can be activated by the AT$_1$R in both cardiomyocytes and vascular smooth muscle cells.

Functional selectivity also describes the ability of $\beta$-arrestins to act as ligand-regulated scaffolds. Because $\beta$-arrestin binding precludes catalytic interaction between GPCRs and G proteins, $\beta$-arrestin binding could be viewed as switching the receptor between two qualitatively, temporally, and spatially distinct signaling modes, G-protein coupled and G-protein uncoupled. Notably, several proteins recruited to GPCR-bound $\beta$-arrestins are components of growth pathways. Perhaps the best characterized example of $\beta$-arrestin–mediated signaling is SII Ang II at AT$_1$Rs. Herein, we used...
both SII Ang II and [TK325]AT,R to demonstrate that β-arrestin signaling was neither sufficient nor necessary for transactivation and hypertrophy. Clearly, much remains to be determined with regard to the effects of subcellular trafficking and regulation on signal transduction in the cardiovascular system.

Our results diametrically oppose those of Seta and Sadoshima\(^8\) in cells and Zhai et al\(^9\) in mice, because we failed to find evidence of either Y319 phosphorylation or Y319-isoleucine-dependent transactivation and cardiomyocyte hypertrophy. We cannot disregard the possibility that a very low proportion of EGFR to Y319 are the cardiomyocyte-intrinsic mechanisms of EGFR transactivation. This antibody has not been used in other published studies since the original publication and is not commercially available, so it is difficult to evaluate its selectivity and specificity. Nevertheless, their results comparing cardiac hypertrophy and function in WT and overexpressed Y319F transgenic mice are compelling\(^8\) but possibly reflect more complex interactions between myocytes and other cardiac cells in the hypertrophic process. Instead, our findings agree with two noncardiomyocyte studies: in COS-7, CHO-K1, and vascular smooth muscle cells, while heterologous expression systems: in COS-7, CHO-K1, and vascular smooth muscle cells, whereas Shah et al\(^12\) described Ang II–stimulated HB-EGF release or EGFR phosphorylation\(^11\) neither truncation nor point mutation of Y319 disrupted Ang II–mediated HB-EGF release or EGFR phosphorylation\(^11\) whereas Shah et al\(^12\) described Ang II–stimulated HB-EGF release or EGFR phosphorylation\(^11\) and overexpressed Y319F transgenic mice are compelling\(^8\) but possibly reflect more complex interactions between myocytes and other cardiac cells in the hypertrophic process. Instead, our findings agree with two noncardiomyocyte studies: in COS-7, CHO-K1, and vascular smooth muscle cells, whereas Shah et al\(^12\) described Ang II–stimulated HB-EGF release or EGFR phosphorylation\(^11\) neither truncation nor point mutation of Y319 disrupted Ang II–mediated HB-EGF release or EGFR phosphorylation\(^11\).
Pressure-induced left ventricular remodeling is known to cause AT1R-dependent metalloprotease activation in the heart. Although the responsible metalloprotease(s) is yet to be identified, the ADAM family of proteases are likely mediators, with ADAM17/tumor necrosis factor-alpha converting enzyme the favored candidate. Although our inhibitor studies using GM6001 and TAPI-1 were consistent with this hypothesis, no individual ADAM E/A construct abolished EGFR transactivation, indicating that significant redundancy exists for ADAMs 10, 12, and 17. The multifarious nature of EGFR transactivation remains a significant question.

**Perspectives**

Cardiac hypertrophy is a major independent risk factor for heart attack and sudden death and presents a significant health burden. Ang II contributes to the development of cardiac hypertrophy in vivo by transactivating the EGFR, and numerous mechanisms have been proposed as therapeutic targets. We have found that the classic pathway of G protein–mediated signal transduction enables AT1R-mediated EGFR transactivation, at least in the reductionist in vitro model of NRVMs overexpressing the AT1R. The task remains to confirm these findings in whole animals with native receptor expression and for researchers to identify how GPCRs activate ADAMs to liberate EGF-like ligands, a question that has eluded scientists for more than a decade.

**Acknowledgments**

We thank Anna Jenkins for myocyte preparations.

**Sources of Funding**

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**Disclosures**

None.

**References**

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Hypertension SUPPLEMENTAL MATERIAL

DETERMINATION OF THE EXACT MOLECULAR REQUIREMENTS FOR TYPE 1 ANGIOTENSIN RECEPTOR EGFR TRANSACTIVATION AND CARDIOMYOCYTE HYPERTROPHY

Nicola J. Smith¹*, Hsiu-Wen Chan¹,², Hongwei Qian¹, Allison M. Bourne¹,², Katherine M. Hannan³, Fiona J. Warner⁴, Rebecca H. Ritchie¹, Richard B. Pearson³, Ross D. Hannan³, Walter G. Thomas¹,²*. 
Supplemental References


### Table S1: Characteristics of AT₁R Adenoviruses

<table>
<thead>
<tr>
<th>Virus</th>
<th>PFU*/µl</th>
<th>MOI†</th>
<th>Ki (nmol/L)</th>
<th>Bmax ‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT₁R WT</td>
<td>0.64 × 10⁹</td>
<td>14</td>
<td>1.31</td>
<td>213 ± 45 (n=7)</td>
</tr>
<tr>
<td>[N111G]AT₁A</td>
<td>3.20 × 10⁸</td>
<td>20</td>
<td>1.40</td>
<td>227 ± 65 (n=7)</td>
</tr>
<tr>
<td>[Y215F]AT₁A</td>
<td>3.30 × 10⁹</td>
<td>7</td>
<td>2.83</td>
<td>1016 ± 56 (n=3)</td>
</tr>
<tr>
<td>[Y319F]AT₁A</td>
<td>1.53 × 10⁹</td>
<td>2</td>
<td>2.34</td>
<td>501 ± 153 (n=4)</td>
</tr>
</tbody>
</table>

* Plaque forming units  
† Multiplicity of Infection [(PFU × volume)/number of cells]  
‡ Expressed as fmol receptor/mg protein
Figure S1 – Effect of dominant-negative ADAMs on HB-EGF release and NRVM hypertrophy.

A. Activity of ADAMs in membrane extracts from COS-7 cells transfected with various constructs was determined using a quenched fluorescence substrate assay. Substrate: MCA-Pro-Leu-Ala-Gln-Ala-Val-Dpa-Arg-Ser-Ser-Arg-NH₂. ADAM12 wild type, E/A and myc constructs were also tested but showed no affinity for the assay substrate. Shown is a representative experiment measured in quadruplicate and expressed as mean ± S.E.M. Murine ADAM17 was from R. Black (Amgen, Washington, USA), ADAM10 from P. Saftig (Christian Albrechts Universitat, Kiel, Germany) and ADAM12 wild type and dominant-negative ADAM12 E/A (E349A) from J. White (University of Virginia, Virginia, USA). Dominant-negative ADAM10 (E385A) and ADAM17 (E406A) were cloned using QuikChange mutagenesis (Stratagene).

B. Co-transfection of COS-7 cells with HB-EGF-AP, AT₁ receptor and ADAM10, 12 and 17 dominant/negatives for measurement of HB-EGF release (n=3).

C. Induction of ANP-luc reporter activity in cardiomyocytes co-expressing the AT₁ receptor and ADAM10, 12 or 17 dominant/negatives (n=3). †p<0.001 compared to unstimulated; *p<0.05 compared to own control; #p<0.05 compared to AngII stimulated.

Figure S2: Comparison of receptor affinity and inositol phosphate signaling for wild type and mutated AT₁ receptors expressed in COS-7 cells

A. Radioligand competition binding assays reveal similar affinities for the various receptors (filled square, WT; downward triangle, Y215F; upward triangle, Y319A).

B. Levels of mutant receptor expression (Bmax) were determined by [3H]-AngII saturation binding. There was no significant different between expression levels using one-way ANOVA. Experiments were performed in triplicate; n=6. Columns represent mean ± S.E.M.

C. COS-7 cells expressing the wild type and mutated receptors were stimulated with 100 nmol/L of AngII and examined for inositol phosphate (IPx) accumulation. Data are expressed relative to AngII-stimulated IPx accumulation for the wild type receptor, n=3-7.

Figure S3 - Effect of YM-254890 on AngII-mediated hypertrophy in whole heart. The inhibitory capacity of YM-254890 was examined in an ex vivo model of hypertrophy, as described1-2. A. Experimental protocol and time-course (in minutes) for measuring whole heart hypertrophy. ([3H]-Phe is [3H]-phenylalanine). B. Effect of AngII and YM-254890 on hypertrophy, as measured by [3H]-Phe incorporation into newly synthesized protein (n = 6). C. Real-time PCR of ß-myosin heavy chain
mRNA, normalized to an 18S ribosomal RNA marker, in tissues extracted from whole hearts after experimental treatment indicated in A. n = 3. Data is presented as mean ± S.E.M.. According to one-way ANOVA and Bonferroni's Multiple Comparisons Test, *p<0.05 and **p<0.01 when compared to unstimulated control; #p<0.01 compared to YM-254890 treatment without AngII.