Adenoviral-Directed Expression of the Type 1A Angiotensin Receptor Promotes Cardiomyocyte Hypertrophy via Transactivation of the Epidermal Growth Factor Receptor

Walter G. Thomas, Yves Brandenburger, Dominic J. Autelitano, Thao Pham, Hongwei Qian, Ross D. Hannan

Abstract—Angiotensin II (Ang II) may cause cardiac hypertrophy via type 1 Ang II receptors (AT1) on cardiomyocytes and through growth factors released from cardiac fibroblasts. Whereas cardiomyocyte-specific AT1 receptor expression produces cardiac hypertrophy and remodeling in vivo, delineation of the signals that mediate growth to Ang II is still not clear, but recent evidence from vascular smooth muscle cells, liver epithelial cells, and cardiac fibroblasts suggests that a major mechanism is the “transactivation” of the EGF receptor (EGFR).

Angiotensin II (Ang II) regulates blood pressure, cardiovascular homeostasis, and cellular growth via type 1 (AT1) angiotensin receptors, which activate Gαq/11 phospholipase C-β1 to generate inositol trisphosphate and diacylglycerol, and thereby increase intracellular calcium and activate protein kinase C (PKC). Activation of PKC and the calcium-dependent phosphatase, calcineurin, has been linked to Ang II–mediated growth. Activation of AT1 receptors also promotes tyrosine phosphorylation, stimulation of mitogen-activated protein kinases, and growth, particularly in vascular and cardiac cells. How AT1 receptors, which lack intrinsic tyrosine kinase activity, induce tyrosine phosphorylation, MAP kinase, and growth factor signaling pathways is not clear, but recent evidence from vascular smooth muscle cells, liver epithelial cells, and cardiac fibroblasts suggest that a major mechanism is the “transactivation” of the EGF receptor (EGFR).

Cardiac hypertrophy involves cardiomyocyte growth as well as extracellular matrix accumulation and although initially a beneficial adaptive response to increased cardiovascular demand, prolonged hypertrophy results in dilated cardiomyopathy, heart failure, and death. Clinical and experimental evidence using inhibitors of Ang II production or antagonists of AT1 receptors suggests that inappropriate activation of AT1 receptors in the heart contributes significantly to the process of cardiac hypertrophy. This hypertrophy may involve direct activation of AT1 receptors in cardiomyocytes and/or indirect release of growth factors like endothelin-1 (ET-1), transforming growth factor-β1, and cytokines from cardiac fibroblasts via AT1 receptors on those cells. Recently, Paradis et al generated transgenic mice that express AT1 receptors exclusively in cardiomyocytes. These mice, despite normal blood pressure and heart rate, spontaneously develop cardiac hypertrophy and remodeling.
display increased cardiac atrial natriuretic peptide (ANP) and interstitial collagen deposition, and die prematurely of congestive heart failure. This implicates the AT₁ receptor in the pathogenesis of cardiac hypertrophy and failure and provides compelling evidence that direct activation of cardiomyocytes by Ang II is sufficient for this process.

In the Paradis study, the cardiac AT₁ receptor expression in the transgenic animals was very high (>50 000 fmol/mg protein); for comparison, normal heart tissue expresses 10 to 50 fmol AT₁ receptor/mg protein, although levels can increase during disease states. Moreover, such in vivo models do not allow a molecular dissection of the cellular signaling pathways that control cardiac hypertrophy. To this end, many groups have used cultured rat neonatal cardiomyocytes as an in vitro model, but unfortunately, attempts to recapitulate Ang II–induced hypertrophy in these cardiomyocytes have yielded inconsistent results, ostensibly due to low and variable AT₁ receptor expression. In our hands, cardiomyocyte cultures produce undetectable/low (∼10 fmol/mg protein) levels of endogenous AT₁ receptors, and fail to hypertrophy in response to Ang II stimulation.

To circumvent these problems, we used adenoviruses to efficiently deliver AT₁ receptors into neonatal rat cardiomyocytes. These receptors functionally couple Ang II stimulation to cellular signals and phenotypic/genotypic processes that are the hallmarks of cardiac hypertrophy and allow, for the first time, the identification of an essential contribution of EGFR transactivation to this process.

Materials and Methods

cDNA and Adenovirus Constructs

The pAd-Easy1 virus and shuttle plasmid, pAd-TrackCMV, were obtained from Dr B. Vogelstein (Johns Hopkins Oncology Center, Baltimore, Md). A rat AT₁A receptor bearing a N-terminal HA epitope tag (NHA-AT₁ ) was subcloned into the shuttle vector, pAdTrack-CMV, with HindIII and XbaI linkers, to yield pAdTrackNHA-AT₁. Recombinant adenovirus (AdNHA-AT₁) was generated by bacterial homologous recombination between pAdTrackNHA-AT₁ and pAdEasy-1. Large-scale amplification and purification of AdNHA-AT₁ virus was performed as described. The titer of AdNHA-AT₁ viral stock used was 1×10⁹ PFU/µL. Dominant/negative Ras (pCMV5-N17Ras) was obtained from Dr N. Dhanasekaran (Temple University School of Medicine, Philadelphia, Pa.). A construct expressing the Gaq inhibitor peptide (G903–319 otherwise termed G90I) was from Dr W. Koch (Duke University Medical Center, Durham, NC). The reporter construct for atrial natriuretic peptide promoter (ANP328-Luc) was from Dr M. Nemer (Institut de recherches cliniques de Montreal, Quebec, Canada). Animals were supplied by the Baker Medical Research Institute Animal House and were handled according to protocols approved by the Australian Code of Practice for Care and Use of Animals for Scientific Purposes.

Cell Culture

Cardiomyocytes were isolated from the ventricles of 1-day-old Sprague-Dawley rat pups and plated at high (1250 cells/mm²) or low density (330 cells/mm²) as described. Myocytes, which contain less than 5% non-cardiomyocytes as determined microscopically, were purified in some experiments to >99% homogeneity over multiple Percoll gradients. Where indicated, KCl (50 mmol/L) was added to the medium to prevent spontaneous contraction characteristic of neonatal cardiomyocytes plated at high-density.

Adenoviral Infection of Cardiomyocytes

Twenty-four hours after plating, cardiomyocytes were infected with purified viruses at a multiplicity of infection (MOI) of 0.1 to 100, as indicated. A MOI of 100 corresponds to 1×10⁹ PFU, which infected greater than 95% of 1×10⁶ myocytes as defined by green fluorescent protein (GFP) fluorescence.

Induction of Myocyte Hypertrophy

One day after infection with adenovirus, AT₁ receptor–mediated hypertrophy was initiated by adding Ang II (100 nmol/L). After 72 hours of stimulation, cells were harvested and hypertrophy defined as a significant increase in protein content (Lowry assay) in the absence of any significant change in DNA content (Burton assay).

Phalloidin Staining of Actin Filaments

Control and hypertrophic cardiomyocytes were fixed in 4% phosphate buffered paraformaldehyde and stained with TRITC–labeled phalloidin prior to visualization by fluorescence microscopy.

MAPK Assays

Western blots of cardiomyocyte extracts were probed with a monoclonal antibody (NEB BioLabs, E10) to phosphosphoric p44/42 MAPK (T202/Y204) and a polyclonal antibody (Santa Cruz, C-16). Active and total MAPK were quantified using ScionImage (www.scioncorp.com) and active MAPK was normalized for total MAPK loading on the gel and expressed as a fold-increase from unstimulated levels.

Reporter Assays

Cardiomyocytes were transfected with equal amounts of plasmid constructs using lipofectamine. Hypertrophy was initiated 24 hours after transfection, and cells were harvested 72 hours later and assayed for chloramphenicol transferase (CAT), luciferase, or β-galactosidase (βGal) activity. The results of the assays were normalized to βGal expression.

RNase Protection Assays

mRNA for ANP, c-fos, c-jun, and GAPDH was measured by solution hybridization/RNase protection analysis.

EGFR Phosphorylation

Cardiomyocytes were infected for 3 days with AdNHA-AT₁ (MOI, 20), stimulated with Ang II (3 minutes, 100 nmol/L), and the EGFR immunoprecipitated from cell lysates using anti-EGFR antibody (Santa Cruz, 1005). Western blots were probed sequentially with an anti-phosphotyrosine antibody (PY20, Santa Cruz) and the anti-EGFR antibody.

Statistical Analysis

For all multi-group comparisons, data were analyzed by 1-way ANOVA followed by Fisher’s protected least significant difference for post hoc comparisons. Student’s t test was used for comparisons between 2 groups. All data are presented as mean±SE, and a value of P<0.05 was considered to be significantly different.

An expanded Materials and Methods section can be found in the online data supplement available at http://www.circresaha.org.

Results

AT₁ Receptor-Directed Cardiomyocyte Hypertrophy

Ang II stimulation of uninfected neonatal cardiomyocytes failed to elicit a hypertrophic response (Figure 1A). In contrast, activation of α₁-adrenergic receptors resulted in significant hypertrophy (129.5±1.2%), demonstrating that the myocytes were capable of responding to hypertrophic stimuli. To determine if the lack of response to Ang II stimulation was due to low levels of AT₁A receptor expres-
We examined the capacity of Ang II to stimulate cardiac growth in cells infected with an adenovirus (AdNHA-AT1) that directs expression of the AT1A receptor. Cardiomyocytes infected with increasing amounts of AdNHA-AT1 expressed functional, high affinity AT1 receptors with appropriate selectivity for non-peptide antagonists and a capacity for robust phosphorylation and internalization (see online data supplement).

Ang II–mediated hypertrophy in cells infected with AdNHA-AT1 (126.5±4.8%) was comparable to PE (Figure 1A). The selective AT1 antagonist, candesartan, prevented Ang II–stimulated hypertrophic growth, whereas treatment with vehicle or antagonist alone failed to induce hypertrophy (Figure 1A). Significant hypertrophy (113.1±1.1%) was observed at a MOI of 10 PFU/cell (~500 fmol receptor/mg protein), whereas maximal growth (141.0±1.9%) occurred at 50 PFU/cell (~1000 fmol receptor/mg protein) (Figure 1B). An Ang II–induced growth curve in AdNHA-AT1–infected cardiomyocytes (20 PFU/cell; ~500 fmol receptor/mg protein) demonstrated that the EC50 for the hypertrophic effect was 6.8 nmol/L Ang II (Figure 1C).

The hypertrophic effect of Ang II on AdNHA-AT1–infected cardiomyocytes was confirmed by phase and fluorescence microscopy (Figures 2A through 2G). Cardiomyocytes (330 cells/mm²) displayed a morphology typified by small, irregularly-shaped cells, which were poorly attached to the substrate (Figure 2A) and not altered by stimulation of uninfected cells with 100 nmol/L Ang II (Figure 2C) or by infection with AdNHA-AT1 (Figure 2B). However, Ang II stimulation of AdNHA-AT1–infected cardiomyocytes resulted in both an increase in cell size (Figure 2D) and hypertrophic phenotype, similar to that observed for PE (Figure 2E) and exemplified by increased organization of sarcomeric units (Figure 2G). These phenotypic changes were blocked by coincubation with the specific AT1 receptor antagonist, candesartan (Figure 2F).

Cardiac Fibroblasts Are Not Required for the AT1A-Mediated Cardiomyocyte Hypertrophic Response

Recent studies suggest that Ang II–induced cardiomyocyte hypertrophy is mediated by the release of cardiotrophic factors, including ET-1, from adjacent fibroblasts. Hence, we tested the capacity of the selective ET1 receptor antagonist, bosentan, to block Ang II–mediated hypertrophic growth in AdNHA-AT1–infected cardiomyocyte cultures. Bosentan...
AdNHA-AT1 but not in uninfected cells after Ang II stimulation. Hypertrophy (±SE, n=4) was measured 72 hours after stimulation as described above. ***P<0.001 vs control; ¶P<0.001 vs ET-1; §P<0.05 vs Ang II; and #P=NS vs Ang II.

Ang II Induces Reexpression of Phenotypic Markers of Cardiac Hypertrophy in AdNHA-AT1-Infected Cardiomyocytes

RNAse protection analysis (RPA) was used to assess the effect of Ang II on expression of the hypertrophy-induced marker gene ANP (Figures 4A and 4B). In control cardiomyocytes, Ang II did not elevate ANP mRNA levels. In contrast, after infection with AdNHA-AT1, Ang II caused a 5-fold induction of ANP mRNA that was blocked by an AT1 receptor antagonist (Figure 5). Similarly, a robust upregulation of c-fos (960% of control) and c-jun (150% of control) mRNAs was observed in cardiomyocyte cultures infected with AdNHA-AT1 but not in uninfected cells after Ang II stimulation (see online data supplement).

AT1A Couples to Hypertrophic Signaling Through Gαq and Ras

Cotransfection of a construct (GαqI) expressing the carboxy-terminal peptide of the α subunit, Gαq, that inhibits Gαq with the AT1A receptor and an ANP promoter reporter, inhibited the ability of Ang II to activate ANP expression (Figure 5). Similarly, coexpression of a dominant/negative mutant of the small molecular weight GTPase, Ras (N17-Ras), resulted in a significant inhibition of the Ang II-mediated ANP response (Figure 5). Thus, the AT1A receptor couples to prototypic markers of hypertrophy via Gαq and Ras.

Ang II–Mediated Cardiomyocyte Hypertrophy Involves MAPK Activation and Transactivation of the EGFR

Hypertrophy of AdNHA-AT1–infected cells in response to Ang II (128.9±2.4% relative to control) could be returned to unstimulated levels by pretreating with the MAPK inhibitor, PD98059 (97.1±5.1%), or the EGFR inhibitor, AG1478 (92.9±5.2%) (Figure 6A). Thus, MAPK signaling, predominantly via the EGFR, is a major mediator of Ang II–induced hypertrophy in AdNHA-AT1–infected cardiomyocytes.
cardiomyocyte growth. Growth responses to PE (139.8±13.1%) were partially inhibited by PD98059 (125.9±11.1%) and AG1478 (125.9±12.1%) (data not shown), indicating the specificity of EGFR transactivation and MAPK signaling for Ang II–mediated hypertrophic growth. PD98059 and AG1478 did not significantly effect cellular protein levels in unstimulated myocytes (91.7±4.3% and 86.7±7.1%, respectively), suggesting that EGFR transactivation and MAPK signaling does not contribute significantly to the maintenance of basal growth.

Ang II–mediated hypertrophy was not inhibited by pretreatment with the PKC inhibitor bisindolylmaleimide (BIM) (129.10±4.6%) (Figure 6A). In contrast, hypertrophy induced by PMA (126.7±3.9%), an activator of PKC, was blocked by BIM (104.9±4.6%). Thus, PKC activation is sufficient to initiate cardiomyocyte hypertrophy, but this kinase family does not mediate hypertrophy initiated by Ang II.

To examine whether the AT1A receptor transactivates the EGFR, noninfected and AdNHA-AT1–infected cardiomyocytes were stimulated with Ang II and the EGFR was subsequently immunoprecipitated, Western blotted, and probed with anti-phosphotyrosine antibody (Figure 6B). In infected cells, but not uninfected cells, Ang II stimulation promoted tyrosine phosphorylation of the EGFR, confirming transactivation.

Western blotting and quantitative analysis for phosphorylated MAPK42/44 (Figure 6C) revealed that maximal Ang II stimulation of uninfected cardiomyocytes lead to a weak, but consistent, activation of MAPK (1.9-fold control), relative to the strong activation (5.0-fold) with EGF stimulation. MAPK activation in response to EGF was completely inhibited by pretreatment with AG1478 (0.14-fold). In contrast, Ang II stimulation of AdNHA-AT1–infected cardiomyocytes lead to a robust activation of MAPK (4.7-fold), similar to that observed with EGF. This activation was significantly inhibited by PD98059 and AG1478 pretreatment (reduced to 1.5-fold and 1.6-fold, respectively), but remained above basal and comparable to the level observed in uninfected cells stimulated by Ang II. Thus, a small proportion of Ang II–induced MAPK activity is via a MEK1- and EGFR-mediated, MAPK/growth axis is largely independent of PKC activation.

Because EGFR transactivation can be Ca2+-sensitive or -insensitive and may involve matrix metalloproteinase (MMP)-dependent liberation of extracellular hepa-

Figure 6. Ang II–mediated cardiomyocyte hypertrophy involves MAPK activation and transactivation of the EGFR. A, Uninfected and AdNHA-AT1–infected cardiomyocytes were stimulated with Ang II (100 nmol/L, 4 minutes) or PMA (0.1 μmol/L) in the presence or absence of the MAPK kinase inhibitor (PD98059; 20 μmol/L), the specific inhibitor of the EGFR tyrosine kinase (AG1478; 5 μmol/L) or the PKC inhibitor bisindolylmaleimide-1 (BIM, 1 μmol/L). Hypertrophy was measured 72 hours after stimulation as described. *P<0.001 vs control; ‡P<0.05 vs AdNHA-AT1–infected cells stimulated with Ang II; #P<0.001 vs cells stimulated with PMA; and ΔP=NS vs control cells. B, Ang II transactivates the EGFR. Uninfected (No virus) and infected (AdNHA-AT1; MOI of 20) cardiomyocytes were stimulated with EGF (10 nmol/L, 4 minutes) or Ang II (100 nmol/L, 4 minutes) in the presence and absence of the inhibitors PD98059 or AG1478. Western blots of cell extracts were probed for activated and total p44/42 MAPK (ERK1/2) activity relative to nonstimulated cells. Representative blots are shown from 3 experiments.
robust hypertrophy to Ang II. Third, we found that the ETA receptor antagonist, bosentan, although completely inhibiting Ang II−mediated cardiomyocyte growth. Our MAPK data point to AT1A receptor activation promoted the tyrosine phosphorylation of the EGFR, implicating EGFR transactivation in Ang II−mediated cardiomyocyte growth. Our MAPK data point to an MMP activity in this transactivation, which was independent of intracellular Ca2+.

Direct Ang II−mediated cardiomyocyte growth has remained controversial because of compelling evidence that factors like ET-1, cytokines, and growth factors, which are released from AT1-expressing cardiac fibroblasts in response to Ang II, can promote growth of adjoining cardiomyocytes,12−15,31 Conversely, observations from the present study and others strongly support a significant direct component to Ang II−mediated cardiomyocyte growth. First, uninfected cultures did not hypertrophy after Ang II stimulation, suggesting that any contaminating fibroblasts were not capable of marshaling a growth response under these conditions. Second, highly purified cardiomyocyte cultures (less than 0.1% fibroblasts), infected with AdNHA−AT1, displayed robust hypertrophy to Ang II. Third, we found that the ETα receptor antagonist, bosentan, although completely inhibiting the hypertrophic effects of ET-1, had no effect on Ang II−mediated growth. This contrasts with evidence supporting a role for ET-1 in Ang II−mediated growth.12−14 Finally, persuasive in vivo evidence for the direct action of Ang II on cardiomyocyte AT1 receptors has come from transgenic models of cardiomyocyte-specific overexpression of the AT1 receptor.12,34

Although AT1α mRNA can be detected in neonatal cardiomyocyte cultures by sensitive techniques such as RNase protection assay, AT1 receptor expression is very low, as measured by radio-ligand binding assays31 (and this study). Nevertheless, uninfected cardiomyocyte cultures, which fail to exhibit Ang II−induced hypertrophy, were capable of displaying small increases in MAPK and c-fos expression. This observation confirms that there are low levels of coupled AT1 receptors in these cultures, but that the low-grade MAPK and c-fos responses, detected by highly sensitive/amplified assays, are incapable of mobilizing the full hypertrophic cellular program. Moreover, complete inhibition of the low levels of activated MAPK observed in basal cells did not lead to a concomitant reduction in growth, demonstrating that MAPK activity contributes where MAPK activity must be elevated above a critical level to stimulate hypertrophic pathways.

In our opinion, the paradox of weak signal activation (in the absence of growth to Ang II) has hampered attempts to delineate the bone fide signals that drive Ang II−mediated cardiac hypertrophy. Indeed, although previous studies35−37 have observed growth-related signals to Ang II in this model, others have not observed hypertrophic growth.13,31 When considered with our finding that AdNHA−AT1−infected cardiomyocyte cultures exhibit hypertrophic growth proportional to the level of AT1α receptor expression, we conclude that the density of AT1 receptor in cardiomyocytes and their capacity to transactivate the EGFR and stimulate MAPK activity above a threshold level are the most critical determinants for Ang II−induced growth. This conclusion supports the hypothesis that upregulation of AT1 receptors in heart muscle cells after cardiac trauma16−20 is a major causative factor for the subsequent progression of cardiac hypertrophy. These data are not necessarily in conflict with the concept that Ang II also mediates myocyte growth through paracrine factors from surrounding non-cardiomyocyte cells; rather, the data demonstrate that a direct effect is possible and that most probably both mechanisms exist in vivo.

The sensitivity of MAPK activation and AT1-dependent hypertrophy to AG1478 implicates EGFR transactivation as a major signaling mechanism for Ang II−mediated growth. Such transactivation of the EGFR by ligands for GPCRs was first reported by Daub et al38 in rat-1 fibroblasts and subsequently observed in additional cell types and for other GPCR ligands.3 This process, which results in the rapid tyrosine phosphorylation of the EGFR, appears crucial for cellular growth, induction of c-fos transcription, and for activation of the Ras-MAPK pathway by GPCRs. For Ang II, EGFR transactivation was first reported in rat GN4 liver epithelial cells,3 vascular smooth muscle cells,7 and cardiac fibroblasts8 and is important for the activation of MAPK by Ang II. Interestingly, Li et al9 demonstrated that MAPK (ERK1/2) activation could be both Ras-dependent (via EGFR transactivation) or Ras-independent. In agreement, we observed that AG1478 treatment markedly, but not completely, inhibited Ang II−mediated ERK1/2 activation. In contrast, AG1478 completely blocked EGF-mediated and basal ERK1/2 phosphorylation. Our interpretation is that the EGFR-mediated component of Ang II−induced MAPK activation is predominant and crucial for cardiomyocyte growth in response to Ang II.

In addition to MAPK signaling, activation of the PKC family has been linked to GPCR−mediated MAPK signaling and cardiomyocyte hypertrophy.2,37,39 However, in the current experiments, a PKC inhibitor had no effect on Ang II−stimulated hypertrophy and only marginally reduced EGFR-dependent MAPK activity in myocytes infected with...
AdNHA-AT1. Thus, our results indicate that although activation of PKC by PMA is sufficient to promote cardiac growth, PKC does not contribute to cardiomyocyte hypertrophy and MAPK signaling induced by Ang II–mediated transactivation of the EGFR. This finding is in agreement with previous studies, which demonstrated that Ang II–induced EGFR transactivation in smooth muscle cells occurred via a PKC-independent mechanism.4,5

The mechanism of EGFR transactivation is unclear and probably depends on the cell type and the environment.7 For Ang II, it may involve the activation of an intracellular Ca2+-sensitive proline-rich tyrosine kinase, termed Pyk2, in conjunction with Src-kinases, or it may be Ca2+-independent.22,33 Alternatively, EGFR transactivation may result from a so-called “triple membrane-passing signal mechanism” in which GPCR stimulation induces the proteolytic processing of an extracellular pro–hepin-binding-EGF (pro–HB-EGF) that liberates free HB-EGF to stimulate EGFR dimerization and autophosphorylation. The latter mechanism has been reported for Ang II–mediated EGFR transactivation in vascular smooth muscle cells41 and cardiac endothelial cells.42 In the present study, pretreatment of cardiomyocytes with the MMP inhibitor, BB94, reduced Ang II–induced MAPK activation, implying a Ca2+-independent EGFR transactivation in these cells.

In summary, we have quantitatively transferred AT1A receptors into neonatal cardiomyocytes to determine the molecular mechanisms by which Ang II regulates cardiac hypertrophic growth. These receptors functionally couple Ang II stimulation to cellular signals and phenotypic/genotypic processes that are the hallmarks of cardiac hypertrophy and have allowed for the first time the identification of an essential contribution of EGFR transactivation and MAPK signaling to this process.

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References


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Supplementary Data

Adenoviral-Directed Expression of the Type 1A Angiotensin Receptor Promotes Cardiomyocyte Hypertrophy via Transactivation of the Epidermal Growth Factor Receptor.

by

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Short running title: AT₁A-induced cardiomyocyte hypertrophy
INTRODUCTION

Our manuscript details the use of an adenoviral construct (AdNHA-AT1) that directs the expression of the angiotensin AT1A receptor in cardiomyocyte cultures. An important component of this work was to functionally characterize the expressed receptors. As detailed below, we confirmed that cardiomyocytes infected with increasing amounts of AdNHA-AT1 (MOI, 0-100) expressed functional, high affinity AT1 receptors with appropriate selectivity for non-peptide antagonists, capacity for robust phosphorylation and internalization and coupling to re-expression of phenotypic markers of hypertrophy.

MATERIALS AND METHODS

Cell culture – cardiomyocytes were isolated from the ventricles of 1-day-old Sprague-Dawley rat pups and plated at high (1250 cells/mm²) or low density (330 cells/mm²) as described1. Myocytes, which contain less than 5% non-cardiomyocytes as determined microscopically, were purified in some experiments to >99% homogeneity over multiple Percoll gradients2. Where indicated, KCl (50 mmol/L) was added to the medium to prevent spontaneous contraction characteristic of neonatal cardiomyocytes plated at high-density2,3.

Characterization of AT1A Receptor Expression – detection, quantification and selectivity of AT1 receptors was determined by equilibrium competition binding assays using [125I]-AngII as tracer4. For immunoprecipitation and Western blotting, AdNHA-AT1-infected cardiomyocyte cultures were lysed in 300 µl of RIPA buffer and AT1 receptors immunoprecipitated as described4. The immunoprecipitates were Western blotted with monoclonal anti-HA antibody (3F10; 1:1000) using chemiluminescence. AT1A receptor internalization and phosphorylation in response to AngII stimulation was performed as described4.
RNase Protection Assays – mRNA for *c-fos*, *c-jun* and GAPDH was measured by solution hybridization/RNase protection analysis^5^.

RESULTS

Characterization of AdNHA-AT1-infected Cardiomyocytes – Cardiomyocytes were infected with increasing amounts of AdNHA-AT1 (MOI, 0-100) as described in Methods. Shown in Supplement Figure 1A are phase contrast and fluorescent images of AdNHA-AT1 infected cardiomyocytes. At 100-200 PFU/cell, >95% of cells displayed green fluorescence protein (GFP) fluorescence (Fig.1A) and radio-ligand binding assays demonstrated high-level expression (>2000 fmol/mg protein) of AT₁ receptors. Uninfected cultures expressed very low (10-20 fmol/mg protein) to undetectable levels of AT₁ receptor. AT₁ receptor expression could be titrated with increasing amounts of AdNHA-AT1 (Fig. 1B); receptor expression was linear up to approximately 1000 fmol receptor per mg protein and 50 PFU/cell of virus. Western blotting of immunoprecipitated AT₁ receptors from cardiomyocytes revealed that receptor protein increased linearly with virus (Fig. 1C). The receptor resolved into both non-glycosylated (~40 KDa) and glycosylated (50-150 KDa) forms as previously described^4^.

Competition binding assays were performed using [¹²⁵I]-AngII displaced with AngII, the non-peptide AT₁ receptor-selective antagonist, candesartan, or the AT₂ receptor-selective ligand, PD123177 (Fig. 1D). [¹²⁵I]-AngII binding to the AT₁, receptor expressed on the surface of cardiomyocytes was displaced with high affinity by AngII and candesartan, but not by PD123177, identifying appropriate AT₁ receptor pharmacology. To confirm that the infected AT₁ receptors retained appropriate regulatory processes, the capacity of AngII to promote receptor phosphorylation and endocytosis was determined. Using an established internalization assay, based on acid-insensitive [¹²⁵I]-AngII binding^4^, we observed (Fig. 1E) a rapid internalization of cell surface AT₁ receptors in response to AngII stimulation with about
60% of receptors internalized by 20 min. This internalization was accompanied by a robust phosphorylation of the receptor in response to AngII stimulation (Fig. 1E, inset).

Together, these data confirm that infection of cardiomyocytes with AdNHA-AT1 results in expression of recombinant AT₁ receptors that display the pharmacological and functional hallmarks of wild type AT₁ receptors.

**AngII induces re-expression of phenotypic markers of cardiac hypertrophy in AdNHA-AT1 infected cardiomyocytes** – Acute stimulation with PE (45 min), led to significant upregulation of both c-fos (960% of control) and c-jun (150% of control) mRNA transcripts in cardiomyocyte cultures (Fig 2A and B) using solution hybridization/RNase protection analysis. Unlike PE, AngII had no significant effect on c-jun gene expression and caused a very weak, yet significant, increase in c-fos mRNA levels in uninfected cardiomyocytes. The response to AngII was enhanced in cells infected with AdNHA-AT1, with AngII causing significant up-regulation of both c-fos and c-jun gene expression (Fig. 2B).

**FIGURE LEGENDS**

**Figure 1 Characterization of adenovirus-induced AT₁A receptors.** (A) Cardiomyocytes were infected at a MOI of 200 with AdNHA-AT1 and cells visualized under phase contrast (left panel) or fluorescence (right panel). Cardiomyocytes were infected with increasing amounts of AdNHA-AT1 (MOI, 0-100) and receptor expression determined by radio-ligand binding assays (B) or by receptor immunoprecipitation and Western blot analysis (C). (D) Competition displacement curves of [¹²⁵I]-AngII by AngII, the non-peptide AT₁ receptor-selective antagonist, candesartan, and the AT₂ receptor-selective ligand, PD123177. (E) AngII–induced AT₁A receptor internalization was accompanied by a robust phosphorylation of the AT₁A receptor in response to AngII stimulation (100 nmol/L, 10 min; inset).
Figure 2 AngII induces expression of fos/jun in AdNHA-AT1 infected cardiomyocytes.

(A) Contraction-arrested uninfected (No virus) and infected (AdNHA-AT1; MOI of 20) cardiomyocytes were treated with either vehicle (Con), phenylephrine (PE, 25 µmol/L; 1 µmol/L propranolol), or AngII (AngII, 100 nmol/L). Total RNA was extracted and the levels of c-jun and c-fos were simultaneously determined by solution hybridization/RPA analysis.

(B) The results from four experiments were quantified and presented as the average (± S.E.) increase in c-jun (upper panel) or c-fos (lower panel) mRNA levels relative to control unstimulated cells. ***, P<0.0001 vs control; *, P= 0.002 vs control (t-test).

REFERENCES


Supplementary Fig. 1

A

Phase

GFP

B

Receptor expression (fmol/mg protein)

Virus (MOI)

C

Virus (MOI)

K_D

Supplementary Fig. 1
**Supplementary Fig. 1**

**D**

![Graph D](image)

**E**

![Graph E](image)
Supplementary Fig 2.

A

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**c-jun**

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<th>AngII</th>
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**c-fos**

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