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 (AT\(_1\)) on cardiomyocytes and through growth factors released from cardiac fibroblasts. Whereas cardiomyocyte-specific AT\(_1\) receptor expression produces cardiac hypertrophy and remodeling in vivo, delineation of the signals that mediate growth to Ang II is challenging because the prevailing in vitro model (cultured neonatal cardiomyocytes) expresses low levels of AT\(_1\) receptor and responds inconsistently to Ang II. In this study, when AT\(_{1A}\) receptors were expressed using adenovirus in cultured neonatal cardiomyocytes, Ang II stimulated a robust hypertrophy that was not secondary to the release of cardiac fibroblast-derived factors, specifically endothelin-1. Hypertrophy was accompanied by the induction of the immediate-early response genes, c-fos and c-jun, and reexpression of atrial natriuretic peptide (ANP). Ang II–induced activation of an ANP promoter-reporter was inhibited by the dominant/negative mutants, Goq1 and N17Ras, indicating that hypertrophic signaling by the AT\(_{1A}\) receptor is via heterotrimeric G protein coupling and downstream Ras pathways. AT\(_{1A}\)-mediated cardiomyocyte hypertrophy and mitogen-activated protein kinase (MAPK) activation were inhibited by the MAPK kinase inhibitor, PD98059, and the epidermal growth factor (EGF) receptor kinase antagonist, AG1478, but not by PKC inhibitor, bisindolylmaleimide-1. Moreover, Ang II–induced MAPK activation was prevented by treatment with a matrix metalloproteinase inhibitor, consistent with the tyrosine phosphorylation of the EGF receptor in response to AT\(_{1A}\) receptor activation. These data unequivocally demonstrate that Ang II can directly promote cardiac myocyte growth via AT\(_{1A}\) receptors expressed on these cells and reveal for the first time the important contribution of EGF receptor–transactivated MAPK signaling to this process. (Circ Res. 2002;90:135-142.)

Key Words: AT\(_{1A}\) receptor ■ angiotensin ■ cardiomyocyte hypertrophy ■ adenovirus ■ EGF receptor transactivation

Angiotensin II (Ang II) regulates blood pressure, cardiovascular homeostasis, and cellular growth via type 1 (AT\(_1\)) angiotensin receptors, which activate G\(_{q/11}\) phospholipase C-\(\beta\)1 to generate inositol trisphosphate and diacylglycerol, and thereby increase intracellular calcium and activate protein kinase C (PKC).\(^1\) Activation of PKC and the calcium-dependent phosphatase, calcineurin, has been linked to Ang II–mediated growth.\(^2,3\) Activation of AT\(_1\) receptors also promotes tyrosine phosphorylation, stimulation of mitogen-activated protein kinases, and growth, particularly in vascular and cardiac cells.\(^1\) How AT\(_1\) 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,\(^4\) liver epithelial cells,\(^5\) and cardiac fibroblasts\(^6\) suggest that a major mechanism is the “transactivation”\(^7\) 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.\(^3,8\) Clinical and experimental evidence using inhibitors of Ang II production or antagonists of AT\(_1\) receptors suggests that inappropriate activation of AT\(_1\) receptors in the heart contributes significantly to the process of cardiac hypertrophy.\(^9-11\) This hypertrophy may involve direct activation of AT\(_1\) receptors in cardiomyocytes and/or indirect release of growth factors like endothelin-1 (ET-1), transforming growth factor-\(\beta\)1, and cytokines from cardiac fibroblasts via AT\(_1\) receptors on those cells.\(^12-15\) Recently, Paradis et al\(^11\) generated transgenic mice that express AT\(_1\) 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 AT1A 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 AT1A 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–AT1a) was subcloned into the shuttle vector, pAdTrack-CMV, with HindIII and Xbal 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 × 10^8 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 (Gaq505-359, hereafter termed Gaq509) 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 × 10^6 PFU, which infected greater than 95% of 1×10^6 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 phospho-specific 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-
tion, 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 virus (0 to 100 MOI) were unstimulated (white bars) or stimulated with Ang II (solid bars), candesartan alone (single hatch), or candesartan and Ang II (double hatch). Hypertrophy is shown as a percentage change compared with the unstimulated control (mean±SE); **P<0.001 vs control cells; §P<0.05 vs AdNHA-AT1–infected cells stimulated with Ang II (n=7). C. AdNHA-AT1–infected cardiomyocytes (20 PFU/cell; ~500 fmol receptor/mg protein) were stimulated with increasing concentrations of Ang II and total cell protein determined (mean±SE).

Ang II–mediated hypertrophy in cells infected with AdNHA-AT1 (126.5±4.8%) was comparable to PE (Figure 1A). The selective AT1 receptor 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 (~200 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 studies12–15,31 suggest that Ang II–induced cardiomyocyte hypertrophy is mediated by the release of cardiotoxic 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

Figure 1. AT1A receptor directs cardiomyocyte hypertrophy. A, Uninfected (No virus) and infected (AdNHA-AT1, MOI of 20) cardiomyocytes were stimulated with phenylephrine (PE, 25 μmol/L in the presence of 1 μmol/L propranolol) or Ang II (Ang II, 100 nmol/L), in the presence or absence of the AT1 antagonist candesartan (AT1,antag., 10 μmol/L). After 72 hours, myocytes were harvested and assayed for total protein content, which was normalized for DNA content and expressed as the average (±SE) percent change in protein content compared with control. **P<0.001 vs control cells; §P<0.05 vs AdNHA-AT1–infected cells stimulated with Ang II (n=7). B. Cardiomyocytes infected with increasing amounts of AdNHA-AT1 virus (0 to 100 MOI) were unstimulated (white bars) or stimulated with Ang II (solid bars), candesartan alone (single hatch), or candesartan and Ang II (double hatch). Hypertrophy is shown as a percentage change compared with the unstimulated control (mean±SE); **P<0.001 and *P<0.05 vs control; §P<0.05 vs AdNHA-AT1–infected cells stimulated with Ang II (n=5). C. AdNHA-AT1–infected cardiomyocytes (20 PFU/cell; ~500 fmol receptor/mg protein) were stimulated with increasing concentrations of Ang II and total cell protein determined (mean±SE).

Figure 2. AT1A-mediated cardiac hypertrophy is associated with changes in cell size and phenotype characteristic of α1-adrenergic receptor–mediated hypertrophy. Uninfected cardiomyocytes (No virus) were stimulated with vehicle (A), Ang II (C), or phenylephrine (PE; E). Cardiomyocytes infected with AdNHA-AT1 (MOI of 20) were stimulated with vehicle (B), Ang II (D), or Ang II and the AT1 antagonist candesartan (F). After 72 hours, the cells were visualized by phase or fluorescence (GFP) microscopy. G, AdNHA-AT1–infected myocytes were fixed in 4% PFA and stained with TRITC-labeled phalloidin to visualize sarcomeric organization.
significantly inhibited ET-1–mediated cardiomyocyte hypertrophy, but did not effect Ang II–mediated growth (Figure 3). Moreover, purification of the myocytes to greater than 99% purity using percol gradients had no effect on the ability of Ang II to stimulate hypertrophy of neonatal cardiomyocytes infected with 20 PFU/cell AdNHA-AT1 (data not shown). Thus, we conclude that Ang II stimulation of the AT1A receptor, expressed in cardiomyocytes, can directly couple to the hypertrophic growth, independently of the actions of cardiac fibroblasts.

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) mRNA 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 Gq and Ras

Cotransfection of a construct (GqI) expressing the carboxy-terminal peptide of the α subunit, Gqα, that inhibits Gqα25 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 Gqα 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.

AT1A receptor couples to hypertrophic growth through Gqα and Ras. Cardiomyocytes were cotransfected with the AT1A receptor (NHA-AT1A) and the ANP reporter construct (ANP) and the indicated combinations of the Gqα inhibitory construct (GqI), the dominant negative Ras expression construct (N17-Ras) or empty vector. After 24 hours, cells were stimulated with either phenylephrine (PE, 25 μmol/L) or Ang II (100 nmol/L) as indicated and levels of ANP and the control gene GAPDH were determined by RPA. Results were quantified, normalized for GAPDH expression, and presented as the average (±SE, n=4) increase in ANP mRNA levels relative to control unstimulated cells. ***P<0.0001 vs control; §P<0.0001 vs Ang II.

Ang II stimulation of the AT1A receptor, expressed in cardiomyocytes, can directly couple to hypertrophic growth, independently of the actions of cardiac fibroblasts.

Figure 3. AT1A–induced hypertrophy in AdNHA-AT1–infected cardiomyocytes is not mediated by ET-1. Uninfected and AdNHA-AT1 (MOI of 20) infected cardiomyocytes were stimulated with either phenylephrine (PE, 25 μmol/L), Endothelin-1 (ET-1, 100 nmol/L) or Ang II (100 nmol/L) in the presence or absence of the ETA receptor antagonist, bosentan (ET1antag., 10 μmol/L), or the AT1 receptor antagonist, candesartan (AT1antag., 10 μmol/L), as indicated. 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.

Figure 4. Ang II induces reexpression of ANF in AdNHA-AT1–infected cardiomyocytes. A, Uninfected and AdNHA-AT1–infected cardiomyocytes were stimulated with vehicle (Con), phenylephrine (PE, 25 μmol/L; 1 μmol/L propranolol) or Ang II (Ang II, 100 nmol/L), in the presence or absence of candesartan (AT1antag., 10 μmol/L). After 72 hours, total RNA was extracted, and levels of ANP and the control gene GAPDH were determined by RPA. B, Results were quantified, normalized for GAPDH expression, and presented as the average (±SE, n=4) increase in ANP mRNA levels relative to control unstimulated cells. ***P<0.0001 vs control; §P<0.0001 vs Ang II.

Figure 5. AT1A receptor couples to hypertrophic growth through Gqα and Ras. Cardiomyocytes were cotransfected with the AT1A receptor (NHA-AT1A) and the ANP reporter construct (ANP) and the indicated combinations of the Gqα inhibitory construct (GqI), the dominant negative Ras expression construct (N17-Ras) or empty vector. After 24 hours, cells were stimulated with either phenylephrine (PE, 25 μmol/L) or Ang II (100 nmol/L) as indicated and levels of ANP and the control gene GAPDH were determined by RPA. Results were quantified, adjusted for efficiency of transfection and presented as the percentage increase (mean±SE, n=5) in luciferase activity. **P<0.001; *P<0.01.
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 AT1 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–dependent mechanism. Interestingly, the EGFR/MAPK kinase axis appears to be tonically activated to a small degree in unstimulated cells because basal MAPK activation could be completely inhibited by PD98059 or AG1478 (Figure 6C).

PMA–mediated MAPK activation (4.3- and 4.8-fold) was completely abolished by the PKC inhibitor, BIM, (Figure 6D). In contrast, Ang II–stimulated MAPK activity (3.9-fold and 4.3-fold) was only marginally inhibited by PMA (2.9-fold and 3.1-fold) (Figure 6D), supporting the conclusion that the Ang II–mediated MAPK/growth axis is largely independent of PKC activation.

Because EGFR transactivation can be Ca2+-sensitive or -insensitive6,12,33 and may involve matrix metalloproteinase (MMP)-dependent liberation of extracellular hep-
rim bound-EGF (HB-EGF) to stimulate EGFR dimerization and autophosphorylation,7 we next investigated the effect of Ca2+ chelation and MMP inhibition on Ang II–stimulated MAPK activation (Figure 6E). Pretreatment with BAPTA-AM (10 μM/L), an intracellular Ca2+ chelator, had no effect on MAPK activation by Ang II. In contrast, pretreatment of cardiomyocytes with a selective MMP inhibitor BB94 (British Biotec), reduced Ang II–dependent MAPK activation by 75% but had no effect on MAPK activity induced by exogenously added EGF (data not shown). Thus, EGFR–dependent MAPK activation by Ang II is via increased MMP activity and subsequent “shedding” of HB-EGF, which appears to Ca2+-independent.

Discussion
We have used recombinant adenovirus to deliver modest levels of AT1a receptors into neonatal cardiomyocytes to mimic the upregulation of AT1 receptors that occurs during cardiac hypertrophy.16–20 Ang II stimulation of AT1a adenovirus–infected cardiomyocytes caused distinct phenotypic and genotypic changes, which correlated with AT1a receptor-dependent G protein coupling, Ras and MAPK activation. Ang II–induced MAPK signaling and hypertrophy was inhibited by the selective EGFR kinase inhibitor, AG1478, and 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, unfected 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 no effect on MAPK activity by Ang II. In contrast, pretreatment of cardiomyocytes with a selective MMP inhibitor BB94 (British Biotec), reduced Ang II–dependent MAPK activation by 75% but had no effect on MAPK activity induced by exogenously added EGF (data not shown). Thus, EGFR–dependent MAPK activation by Ang II is via increased MMP activity and subsequent “shedding” of HB-EGF, which appears to Ca2+-independent.

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 studies55–57 have observed growth-related signals to Ang II in this model, others have not observed hypertrophic growth.12,33 When considered with our finding that AdNHA-AT1–infected cardiomyocyte cultures exhibit hypertrophic growth proportional to the level of AT1a 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.7 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, vascular smooth muscle cells, and cardiac fibroblasts and is important for the activation of MAPK by Ang II. Interestingly, Li et al39 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 Pyk2,3,4 in conjunction with Src-kinases, or it may be Ca²⁺-independent.32,33 Alternatively, EGFR transactivation may result from a so-called “triple membrane-passing signal mechanism”7 in which GPCR stimulation induces the proteolytic processing of an extracellular pro–heparin-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 muscle41 and cardiac endothelial cells.42 In the present study, pretreatment of cardiomyocytes with the MMP inhibitor, BB94, reduced Ang II–induced MAPK activation, indicating that a significant component of Ang II–EGFR–dependent MAPK activation is mediated by increased MMP activity, presumably via shedding of HB-EGF. Treatment of cardiomyocytes with the intracellular inhibitor of Ca²⁺, BAPTA-AM, did not block Ang II–mediated 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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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 described. Myocytes, which contain less than 5% non-cardiomyocytes as determined microscopically, were purified in some experiments to > 99% homogeneity over multiple Percol gradients. Where indicated, KCl (50 mmol/L) was added to the medium to prevent spontaneous contraction characteristic of neonatal cardiomyocytes plated at high-density.

Characterization of AT1A Receptor Expression – detection, quantification and selectivity of AT1 receptors was determined by equilibrium competition binding assays using [125I]-AngII as tracer. For immunoprecipitation and Western blotting, AdNHA-AT1-infected cardiomyocyte cultures were lysed in 300 µl of RIPA buffer and AT1 receptors immunoprecipitated as described. 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 described.
RNase Protection Assays – mRNA for c-fos and c-jun and GAPDH was measured by solution hybridization/RNase protection analysis\textsuperscript{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 AT\textsubscript{1} receptors. Uninfected cultures expressed very low (10-20 fmol/mg protein) to undetectable levels of AT\textsubscript{1} receptor. AT\textsubscript{1} 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 AT\textsubscript{1} 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\textsuperscript{4}.

Competition binding assays were performed using \textsuperscript{[125I]}-AngII displaced with AngII, the non-peptide AT\textsubscript{1} receptor-selective antagonist, candesartan, or the AT\textsubscript{2} receptor-selective ligand, PD123177 (Fig. 1D). \textsuperscript{[125I]}-AngII binding to the AT\textsubscript{1A} receptor expressed on the surface of cardiomyocytes was displaced with high affinity by AngII and candesartan, but not by PD123177, identifying appropriate AT\textsubscript{1} receptor pharmacology. To confirm that the infected AT\textsubscript{1} 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 \textsuperscript{[125I]}-AngII binding\textsuperscript{4}, we observed (Fig. 1E) a rapid internalization of cell surface AT\textsubscript{1} 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 AT$_1$ receptors that display the pharmacological and functional hallmarks of wild type AT$_1$ 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 AT$_{1A}$ 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 $[^{125}\text{I}]$-AngII by AngII, the non-peptide AT$_1$ receptor-selective antagonist, candesartan, and the AT$_2$ receptor-selective ligand, PD123177. (E) AngII–induced AT$_{1A}$ receptor internalization was accompanied by a robust phosphorylation of the AT$_{1A}$ 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) vs. Virus (MOI)

C

Virus (MOI)

K_D

0 20 40 60 80 100

Phase

GFP

Supplementary Fig. 1
Supplementary Fig. 1

**D**

![Binding of \( ^{125}\text{I}-\text{AngII} \) (CPM) over time with various displacers (log M). The graph shows the binding of \( ^{125}\text{I}-\text{AngII} \) in the presence of PD123319, AngII, and Candesartan. The x-axis represents the displacer concentration (log M), while the y-axis shows the binding in CPM.](image)

**E**

![Internalization (%) of the AngII receptor over time. The graph shows the internalization percentage of the receptor at different time points (min). The x-axis represents the time in minutes, and the y-axis shows the internalization percentage.](image)
A

Supplementary Fig 2.

B

No Virus AdNHA-AT1

Con AngII

Con PE AngII

% of control

No Virus AdNHA-AT1

% of control

% of control

% of control

% of control

C-jun

C-fos

Supplementary Fig 2.