Differential Participation of Angiotensin II Type 1 and 2 Receptors in the Regulation of Cardiac Cell Death Triggered by Angiotensin II

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BACKGROUND
The Angiotensin II (Ang II) type 1 (AT_1R) and type 2 (AT_2R) receptors are increased in the heart following myocardial infarction and dilated cardiomyopathy, yet their contribution at a cellular level to compensation and/or failure remains controversial.

METHODS
We ectopically expressed AT_1R and AT_2R in cultured adult rat cardiomyocytes and cardiac fibroblasts to investigate Ang II-mediated cardiomyocyte hypertrophy and cardiac cell viability.

RESULTS
In adult rat cardiomyocytes, Ang II did not induce hypertrophy via the AT_1R, and no effect of Ang II on cell viability was observed following AT_1R or AT_2R expression. In adult rat cardiac fibroblasts, Ang II stimulated cell death by apoptosis via the AT_1R (but not the AT_2R), which required the presence of extracellular calcium, and induced a rapid dissipation of mitochondrial membrane potential, which was significant from 8 h.

CONCLUSIONS
We conclude that Ang II/AT_1R triggers apoptosis in adult rat cardiac fibroblasts, which is dependent on Ca^{2+} influx.

Angiotensin II (Ang II) is an active peptide that controls systolic blood pressure and also exerts long-term effects on cardiovascular tissue structure, including cardiac hypertrophy and fibrosis.[1] Two major receptors exist for Ang II termed type 1 and type 2 receptors (AT_1R and AT_2R, respectively).[2] AT_1R are predominantly coupled to Gq/11 and signal through phospholipases A, C, D, inositol phosphates, calcium channels, and a variety of serine/threonine and tyrosine kinases. Many AT_1-induced growth responses are mediated by transactivation of growth factor receptors.[2] The signaling pathways of AT_2R include serine and tyrosine phosphatases, phospholipase A2, nitric oxide, and cyclic guanosine monophosphate. The AT_2R counteracts several of the growth responses initiated by the AT1 and growth factor receptors.[2] AT_1R mediates the established actions of Ang II, including vasoconstriction, aldosterone and vasopressin release, renal sodium reabsorption, increased collagen deposition, cell proliferation, and, importantly, cardiomyocyte hypertrophy.[1,2] Meanwhile AT_2R function is less clear, but current theories support a role in opposing the AT_1R actions.[3] AT_2R is highly expressed in the fetus; however, after birth, its expression decreases.[2] Both AT_1R and AT_2R are expressed in adult human and rat heart, and they are upregulated in several cardiac pathologies.[4,5]

The effects of Ang II in the heart are cell type-specific. Cardiomyocyte function is tightly regulated by Ang II, which acts in promoting cardiac hypertrophy.[6] However, Ang II also triggers apoptosis in neonatal cardiomyocytes.[7,8] Cardiac fibroblasts play a central role in the maintenance and remodeling of extracellular matrix (ECM) in the normal heart and injured heart.[9] Cultured cardiac fibroblasts undergo AT_1R-dependent proliferation in response to Ang II,[6] and promotes net accumulation of fibrillar collagen and cardiac fibrosis *in vivo*, and their expression in cardiac fibroblasts far exceeds that in myocytes.[10] AT_2R have been shown to lead to stimulation, inhibition, or not affect cardiac fibrosis.[11–13]

Whether the up-regulation of AT_1R or AT_2R stimulates Ang II–dependent adult cardiac cell death remains unsolved. To study this problem, we ectopically expressed AT_1R and AT_2R in cultured adult cardiac fibroblasts (ACFs) and adult cardiomyocytes (ACMs), and we investigated the effect of Ang II on cardiac cell death and hypertrophy. Our data show that Ang II stimulated AT_1R-dependent apoptosis in cultured ACFs,

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which was dependent on the influx of external Ca^{2+}, while no effect was observed in viability or hypertrophy in cardiomyocyte expressing AT_1R or AT_2R.

METHODS

Adult cardiac cell isolation. Animal handling conforms to the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23). Male Sprague-Dawley rats (250 g) were anesthetized with ketamine-xylazine (66 mg/kg and 1.6 mg/kg i.p., respectively). ACMs and ACFs were isolated by cardiac retrograde aortic perfusion as described previously with a few modifications.[14] Briefly, the heart was digested with a collagenase-hyaluronidase (1:1) containing solution and cells centrifuged at 500 rpm for 1 min. The pellet, containing mainly ACMs, was gently suspended in Gerard medium (mmol/l) NaCl 128, KCl 4.0, $MgSO_4 \cdot 7H_2O$ 1.39, NaH_2PO_4 0.19, Na_2HPO_4 1.01, HEPES 10.0, glucose 5.5 and pyruvic acid 2.0 at 37 °C, pH 7.4 containing 10 mmol/l 2,3-butanedione monoxime and seeded onto laminin-coated culture dishes or round glass coverslips. In these conditions, 2×10^6 cardiomyocytes per heart (90% rod shaped) with a purity >95% were obtained (see **Supplementary Figure S4** online). The supernatant, containing mainly ACFs, was centrifuged at 1,000 rpm for 10 min and then resuspended in M199 plus 10% fetal bovine serum and seeded in nontreated culture dishes for 2 h. Then, the cells were washed with phosphate buffer saline in order to eliminate debris and nonadherent cells. ACFs were used at passage 2 and seeded on plastic dishes at density of 2×10^4 cell/cm^2 (see **Supplementary Figure S4** online). ACMs were seeded on plastic dished covered with laminin at density of 1×10^4 cells/cm^2. Cells were seeded on 35 or 60 mm plastic dishes with 2 or 4 ml, respectively.

Adenoviral transduction of cardiac cells. Adenovirus encoding AT_1R (AdNHA-AT_1R) and AT_2R (AdNHA-AT_2R) are bicistronic vectors that coexpress both the angiotensin receptors and the green fluorescent protein (GFP).[14,15] ACMs and ACFs were transduced with adenovirus 24 h after plating with a multiplicity of infection (MOI) of 1, 7-10, and 50-300, respectively. With the MOI used in ACFs (300) or ACMs (10), more than 95% of cells are positive for GFP protein, an indirect method to evaluate the expression level of AT_1R.

[^{125}I]-Sar-Ile-AngII binding assay. [^{125}I]Sar-Ile-AngII binding assays were performed on membranes of AdNHA-AT_1R or AdNHA-AT_2R, transduced ACMs and ACFs, as described previously.[16] Nonlabeled Ang II (1 µmol/l, Sigma, St. Louis, MO), the AT_1R antagonist losartan (1 µmol/l, Merck Sharp & Dohme, Whitehouse Station, NJ), and the AT_2R antagonist PD123319 (10 µmol/l, Sigma) were used to confirm the identity of the angiotensin receptor.

Western blot analysis. Cell extracts were prepared using 10 mmol/l Tris-HCl pH 7.5, 10 mmol/l ethylenediaminetetraacetic acid, 0.4% deoxycholate, 1% NP-40, 1 mmol/l phenylmethylsulfonyl fluoride, and 0.1% sodium dodecyl sulphate. Aliquots were resolved on 10% SDS-PAGE. Proteins were transferred onto a nitrocellulose membrane and then incubated with the primary antibodies that recognize Hemagglutinin (Roche Diagnostics, Mannhein, Germany), GFP (Abcam, Cambridge, MA), and cleaved caspase-3 (Cell Signaling) at 4 °C overnight. Bound antibodies were detected by a secondary antibody conjugated to horseradish peroxidase and visualized by enhanced chemiluminesence reagent plus (NEN Life Science Products, Boston, MA).

Cell viability assays and mitochondrial membrane potential (Δψm) analysis. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT, Sigma) assay was used to measure cell viability. Δψm analysis was performed in stimulated cells that were incubated for 1 h with tetramethylrhodamine methyl ester (TMR) (10 µg/ml), washed with phosphate buffer saline, trypsinized, re-suspended in 200 µl of Dulbecco's modified Eagle's medium 10% fetal bovine serum, and analyzed by flow cytometry in a Becton Dickinson FACSort. Results were analyzed with WinMDI software. Carbonyl cyanide m-chlorophenylhydrazone (CCCP) was used as control.

Apoptosis markers. The levels of caspase 3 and procaspase 3 were determined by western blot.[17] Caspase-3 activation was calculated by the ratio of caspase 3/procaspase 3. DNA laddering was determined as previously described.[17]

Cardiomyocyte hypertrophy. Cell dimensions (length, width, and area) of 100 to 200 binucleated myocytes were measured after 72 h of Ang II 100 nmol/l, with a computerized image analysis system. Cell volumes were derived from these geometric parameters assuming that cultured cells have a cross-sectional area that resembles a flattened ellipse, with a major axis that is equivalent to cell width and a minor axis that is computed from the measured ratios.[18]

Incorporation of phenylalanine into cells was determined by exposing cultures to [^{3}H] phenylalanine (0.1 µCi/ml) for 24 h and assessing the incorporation of radioactivity into acid-insoluble cell mass.[19] Nonradioactive phenylalanine (0.3 mmol/l) was added to the medium to minimize variations in the specific activity of the precursor pool responsible for protein synthesis.

Statistical analysis. Data are presented as mean ± s.e.m. of at least 3 independent experiments. Student t-test for comparisons between 2 groups and one-way analysis of variance followed by a Tukey's *post hoc* test, for multigroup comparisons, were used. Significance was accepted at $P < 0.05$.

RESULTS

Characterization of AT_1R and AT_2R on adenoviral transduced ACFs

Transduction of ACFs with AdNHA-AT_1R or AdNHA-AT_2R was verified by GFP expression, detected by epifluorescent microscopy (**Figure 1a**) and western blot for GFP (data not shown). Adenovirus overexpressing green fluorescence protein

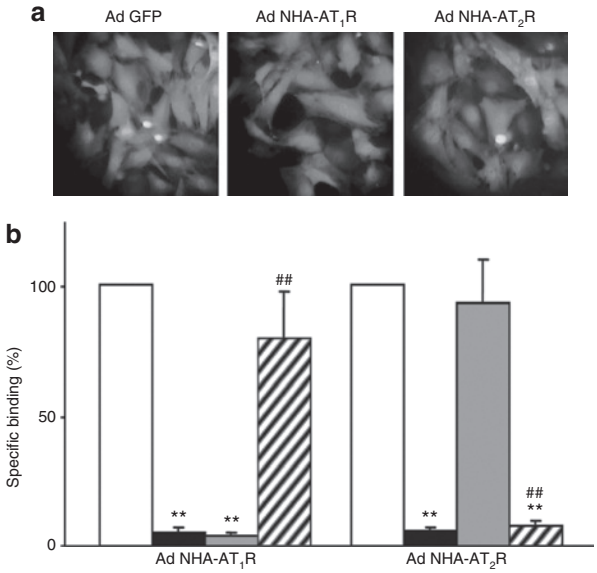

Figure 1 | Expression of AT_1R and AT_2R in cultured adult cardiac fibroblasts (ACFs). (**a**) ACFs were transduced with adenovirus overexpressing green fluorescence protein (AdGFP), AT_1R (AdNHA-AT_1R), or AT_2R (AdNHA-AT_2R) at a multiplicity of infection (MOI) of 300. The transduction was evaluated by epifluorescence microscopy for GFP (original magnification, 400x). (**b**) Competition binding for the AT_1R and AT_2R using [^{125}I]Sar-Ile-Ang II (9 nmol/l, white bar) displaced with Ang II (1 µmol/l, black bar), losartan (1 µmol/l, gray bar) and PD123319 (10 µmol/l, hatched bar), respectively. The assays were done as described in Methods. Results shown are mean ± s.e.m. of 3 separate experiments. $^{**}P < 0.01$ vs. control and $^{\#\#}P < 0.01$ vs. losartan. GFP, green fluorescent protein.

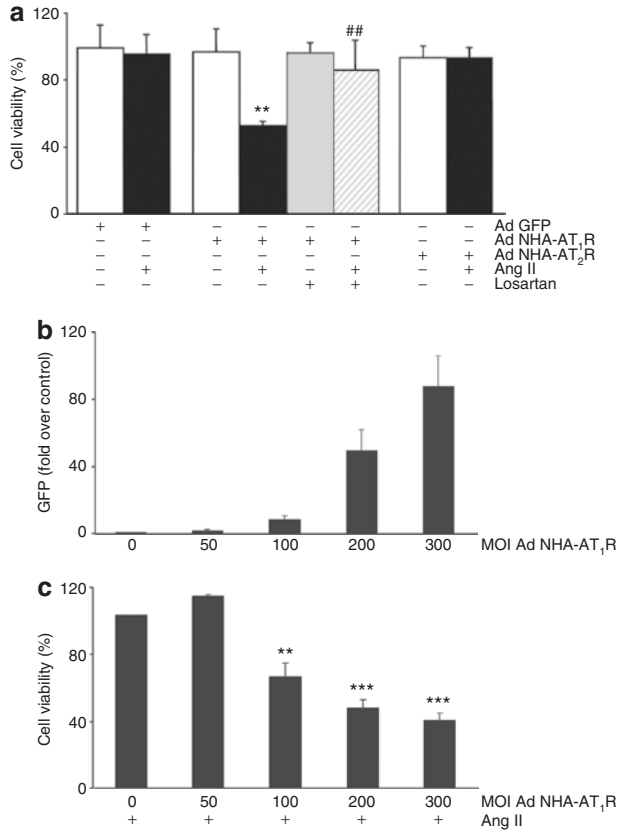

Figure 2 | Decrease in adult cardiac fibroblast viability through AT_1R is Ang II–dependent. (**a**) ACFs transduced with adenovirus overexpressing green fluorescence protein (AdGFP), AdNHA-AT_1R, or AdNHA-AT_2R were incubated without (white bars) or with Ang II 100 nmol/l (black bars) for 24 h. AdNHA-AT_1R cells were preincubated 1 h with losartan (10 µmol/l) and Ang II plus losartan for 24 h. Cell viability was determined by MTT assay. Results shown are mean ± s.e.m. of 3 separate experiments. $^{**}P < 0.01$ vs. AdGFP + Ang II and $^{\#\#}P < 0.01$ vs. AdNHA-AT_1R + Ang II. (**b–c**) ACFs were transduced with different MOI of AdNHA-AT_1R (50 to 300), stimulated with Ang II 100 nmol/l for 24 h, and GFP expression was determined by western blot (**b**), and cell viability was determined by MTT assay (**c**). Results shown are mean ± s.e.m. of 3 separate experiments. $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. nontransduced ACF (MOI = 0) + Ang II. ACF, adult cardiac fibroblasts; GFP, green fluorescent protein; multiplicity of infection; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide.

was used as control. The expression of AT_1R and AT_2R on cardiac fibroblasts was confirmed by radioligand studies. Binding assays with [^{125}I]Sar-Ile-AngII performed in membrane fractions from normal ACFs indicate that these cells had a dissociation constant of 0.8 ± 0.7 nM with a density near 16.7 ± 5.8 fmol/mg protein. Specific binding studies indicate that ACFs transduced with AT_1R and AT_2R had a dissociation constant of 1.6 ± 1.2 and 1.0 ± 1.4 nM with a density of 88 ± 34 and 460 ± 63 fmol/mg protein, respectively (see **Supplementary Figure S1** online). Binding assays with [^{125}I]Sar-Ile-Ang II performed in membrane fractions from ACFs transduced with AdNHA-AT_1R showed that radioactivity was specifically displaced by Ang II and the AT_1R-specific antagonist losartan but not the AT_2R-specific antagonist PD123319 (**Figure 1b**). In contrast, ACFs expressing AT_2R radioactivity was specifically displaced by Ang II and PD123319 but not by losartan. These results confirm the specificity of the adenoviral expression of AT_1R and AT_2R in ACFs.

Ang II triggers apoptosis in AdNHA-AT_1R-transduced fibroblasts

Stimulation with Ang II decreased the viability of AdNHA-AT_1R transduced ACFs (**Figure 2a**), which was prevented by losartan. This effect of Ang II was specific for AT_1R since it was not observed on AdNHA-AT_2R transduced ACFs. These results show that Ang II induces death by activating AT_1R in a cell-specific manner. As shown in **Figure 2b,c**, the increased receptor expression, indirectly determined by GFP levels in AdNHA-AT_1R transduced ACFs, correlated with an increase in Ang II–induced ACF death. **Figure 3a,b** depicts that Ang II induced caspase-3 activation (378 ± 46% respect to control) and increased internucleosomal DNA fragmentation (2.5 ± 0.7 fold over control) in AdNHA-AT_1R transduced ACFs at 8 and 24 h, respectively.

Participation of extracellular Ca^{2+} on Ang II–dependent cardiac fibroblast death

To investigate the role of extracellular Ca^{2+} on Ang II–dependent cardiac fibroblast death, AdNHA-AT_1R transduced ACFs were cultured in a Ca^{2+} (2 mM) containing medium or in a Ca^{2+}-free medium. As depicted in **Figure 4a**, Ang II induced cell death of AdNHA-AT_1R transduced ACFs

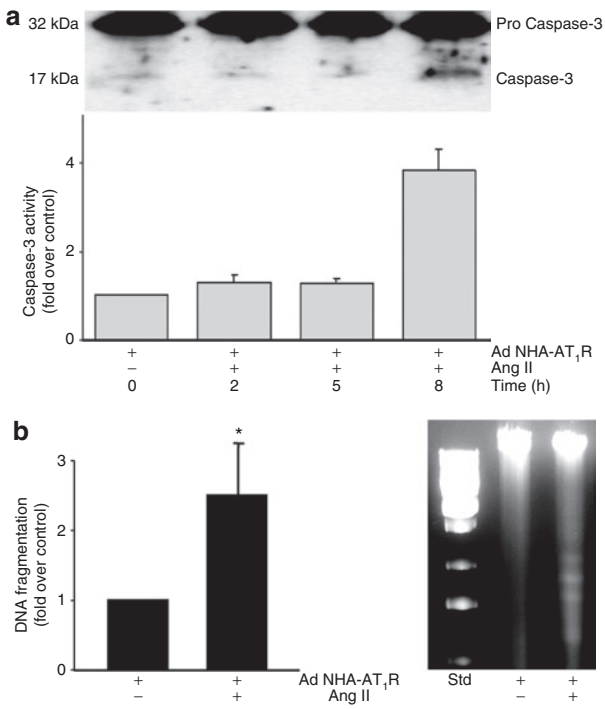

Figure 3 | Increased expression of AT_1R results in Ang II–dependent adult cardiac fibroblast apoptosis. ACFs transduced with AdNHA-AT_1R were stimulated with Ang II 100 (nmol/l) for 8 h. (**a**) Caspase-3 activity was determined by the ratio (Pro casp-3) and caspase-3 (Casp-3) levels, determined by western blot. (**b**) DNA fragmentation stimulated with Ang II for 24 h was determined as described in Methods. *$P < 0.05$ vs. control. Results shown are mean ± s.e.m. of 3 independent experiments. ACF, adult cardiac fibroblasts.

in culture media with Ca^{2+}. However, in the absence of extracellular Ca^{2+}, Ang II did not stimulate cardiac fibroblast death. Gadolinium, but not nifedipine, partially prevented Ang II–induced cell death. These results collectively suggest that extracellular Ca^{2+} participates in Ang II–dependent ACFs death, but L-type Ca^{2+} channel was not involved.

To determine the involvement of extracellular Ca^{2+} on the mitochondria-mediated pathway in Ang II–induced apoptotic cell death, we measured changes in $\Delta\psi m$. No changes by Ang II were observed in Ad-GFP cells. Ang II (100 nM) treatment of AdNHA-AT1R transduced cells induced a rapid dissipation of $\Delta\psi m$, which was significant from 8 h; Carbonyl cyanide m-chlorophenylhydrazone was used as positive control (**Figure 4b**). Gadolinium, but not nifedipine, partially prevented Ang II–induced dissipation of $\Delta\psi m$. However, in the absence of extracellular Ca^{2+} (Gerard medium), Ang II did not triggered $\Delta\psi m$ dissipation.

Effect of Ang II on AdNHA-AT_1R or AdNHA-AT_2R transduced ACMs

To investigate the effect of Ang II on cardiomyocytes, ACMs were transduced with AdNHA-AT_1R or AdNHA-AT_2R. Both AT_1R and AT_2R levels were increased in ACMs as shown in **Figure 5a–c**. Both receptors exhibited similar characteristics as described before for ACFs. Stimulation with Ang II for 24 h did not decrease cell viability in AdNHA-AT_1R or AdNHA-AT_2R transduced ACMs (**Figure 5c**). As shown in **Figure 5d,e**, the different AdNHA-AT_1R expression level in ACMs, indirectly determined by GFP levels, was not a determinant factor in the effect of Ang II on ACMs death. However, a significant Ang II-independent ACM death was observed at higher MOIs (data not shown). Collectively, these results indicate that Ang II did not trigger ACM death. In order to investigate whether AT_1R overexpression participates in ACM hypertrophy, we evaluated the effect of Ang II on cell volume (72 h) and protein synthesis (24 h). As depicted in **Figure 5f,g**, Ang II did not increase ACM volume, nor protein synthesis measured as $[^3H]$ phenylalanine incorporation.

DISCUSSION

Our main finding was that Ang II triggered cardiac fibroblast death by apoptosis through AT_1R activation, which was associated with an influx of extracellular Ca^{2+}. This effect was specific for ACFs and AT_1R since no effects were observed in cultured ACMs transduced with AdNHA-AT_1R or AdNHA-AT_2R, or ACF transduced AdNHA-AT_2R.

We controlled AT_1R and AT_2R expression in cultured ACFs and ACMs using adenoviral transduction. Under our experimental conditions, >95% of the cells were transduced with the adenovirus (assessed by the expression of GFP). The upregulation of AT_1R or AT_2R in the transduced cells was monitored by Hemagglutinin epitope and radioligand binding of $[^{125}I]$ Sar-Ile-Ang II to the cellular membrane. These last results also showed that AT_1R or AT_2R was specific, saturable, and reversible. Losartan and PD 123319, specific antagonists of AT_1R and AT_2R, respectively, blocked $[^{125}I]$ Sar-Ile-Ang II binding to the transduced ACFs or ACMs. In cells transduced with AdNHA-AT1R, the number of AT_1R was almost fivefold over GFP-transduced cells (88 vs. 17 fmol/mg protein, respectively), and the dissociation constant was doubled (1.6 vs. 0.8 nM). Schorb *et al.* showed that cardiac fibroblasts had a single high affinity (IC50, 1.0 nM) Ang II binding site (Bmax, 778 fmol/mg protein) that were coupled with proliferative growth.[20] Our results show that a significant increase of AT_1R number is linked to cell death rather than proliferation.

Changes in the expression of AT_1R have been reported in different pathophysiological conditions,[4] especially after myocardial infarction.[21,22] Moreover, AT_2R expression is upregulated in failing hearts, and fibroblasts present in the interstitial regions are the major cell type responsible for its expression.[23] Transgenic mice with heart-specific overexpression of AT_1R showed cardiac hypertrophy and remodeling, whereas the expression of AT_2R causes dilated cardiomyopathy and heart failure.[24,25] However, heart-specific expression of AT_1R and AT_2R was obtained by utilizing mouse α-myosin heavy chain and myosin light-chain promoters, respectively. Both promoters are active in cardiomyocytes but not in cardiac fibroblasts.[24,25] Since changes in Ang II receptors are mainly detected in fibroblasts instead of cardiomyocytes during a cardiovascular disease,[21–23] data obtained with Ang II receptor expressing transgenic models should be considered

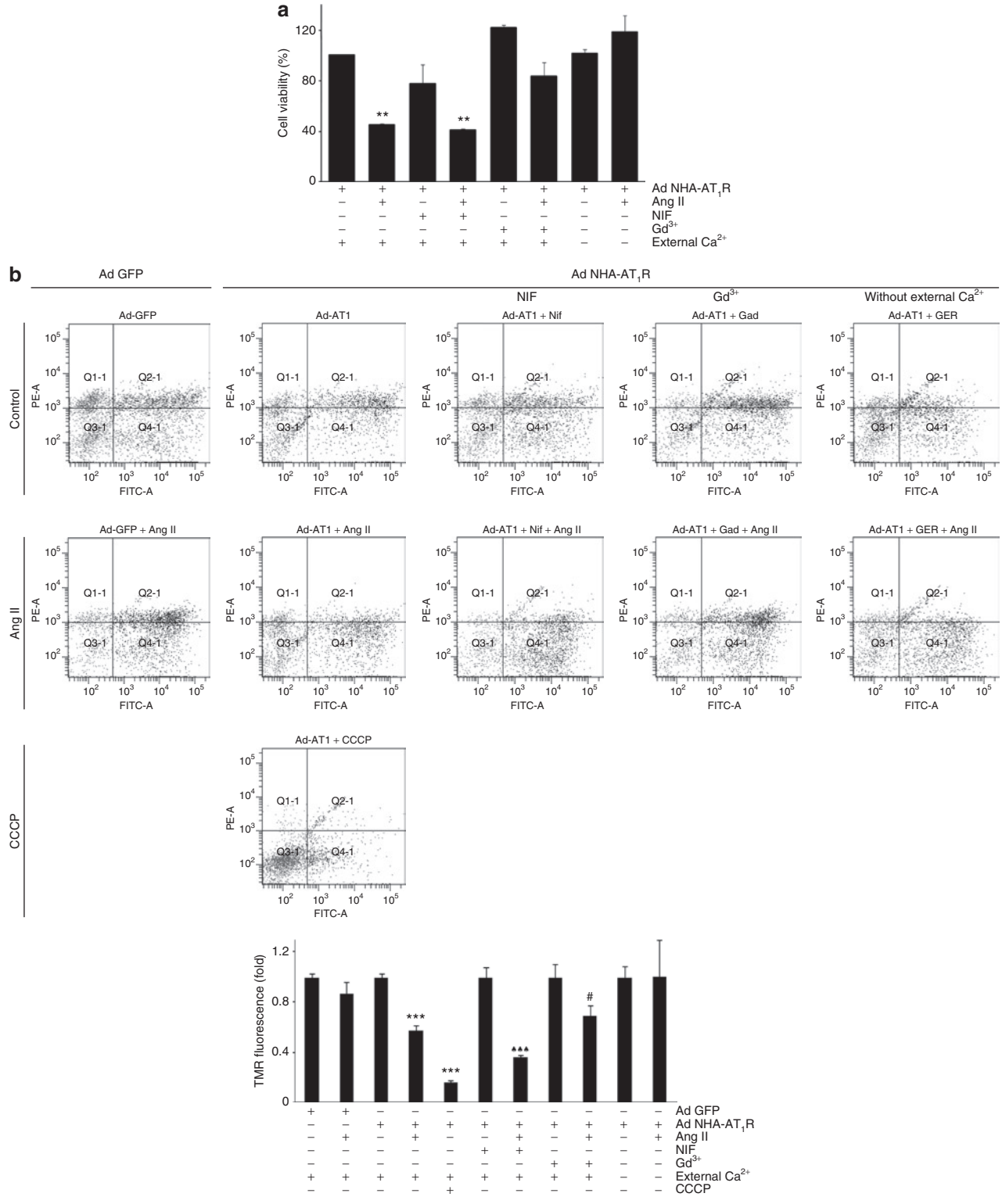

Figure 4 | Participation of external Ca^{2+} on adult cardiac fibroblast (ACFs) death triggered by Ang II. (**a**) Cell viability by MTT assay of cultured ACFs transduced with AdNHA-AT_1R stimulated with or without Ang II (100 nmol/l) for 24 h in the presence of nifedipine (10 µmol/l) or Gd^{3+} (5 µmol/l) both with external Ca^{2+} or in presence or absence of external Ca^{2+}. Results shown are mean ± s.e.m. of 3 separate experiments. ** $P < 0.01$ vs. control. (**b**) Mitochondrial membrane potential of cultured ACFs transduced with AdNHA-AT_1R stimulated with or without Ang II (100 nmol/l) for 8 h in the presence of nifedipine (10 µmol/l), Gd^{3+} (5 µmol/l) both with external Ca^{2+} or in presence or absence of external Ca^{2+} measured by TMR staining. Carbonyl cyanide m-chlorophenylhydrazone (40 nM) was used as mitochondrial uncoupler. On the bottom, the FACS images at 8 h are showed. Results are mean ± s.e.m. of 3 separate experiments. *** $P < 0.001$ vs. Ad-GFP + Ang II. ♠♠♠ $P < 0.001$ vs. AdNHA-AT1 + NIF, # $P < 0.05$ vs. AdNHA-AT1 +Gd^{+3}. MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; TMR, tetramethylrhodamine methyl ester.

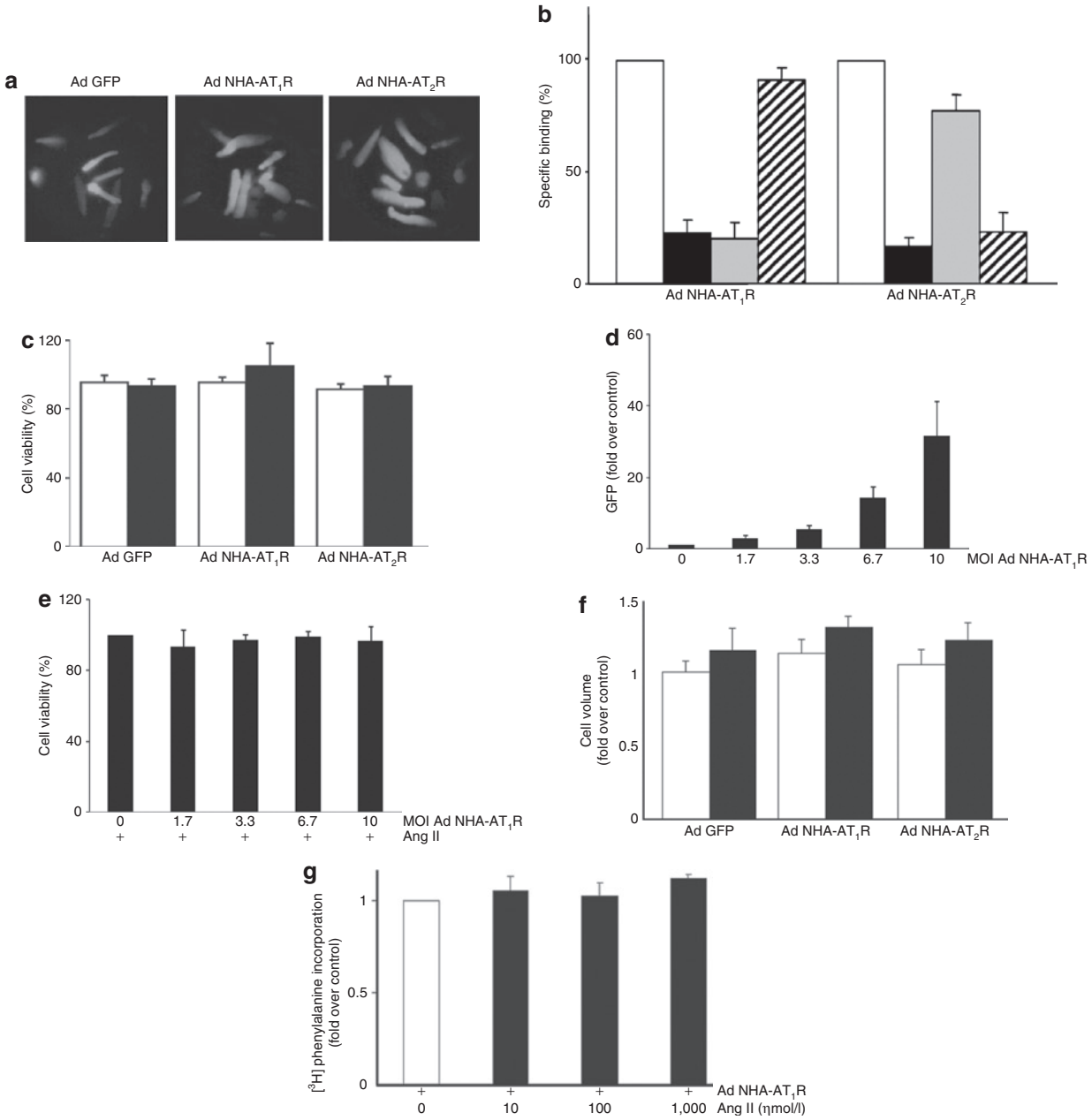

Figure 5 | Effects of Ang II on adult rat cardiomyocytes (ACMs) viability and hypertrophy. ACMs were transduced with adenovirus overexpressing green fluorescence protein (AdGFP), AdNHA-AT_1R, or AdNHA-AT_2R at a multiplicity of infection (MOI) of 10. (**a**) Adenoviral transduction was evaluated by epifluorescence microscopy for GFP (original magnification, 400x). (**b**) Competition binding for the AT_1R and AT_2R using [^{125}I]Sar-Ile-Ang II (9 nmol/l, white bar) displaced with Ang II (1 µmol/l, black bar), losartan (1 µmol/l, gray bar), and PD123319 (10 µmol/l, hatched bar), respectively. Results are mean ± s.e.m. of 3 separate experiments. (**c**) Cell viability analysis of ACMs transduced with AdGFP, AdNHA-AT_1R, or AdNHA-AT_2R stimulated without (white bar) or with Ang II (100 nmol/l, black bar) for 24 h. Results are mean ± s.e.m. of 3 separate experiments. (**d–e**) ACMs were transduced with different MOI of AdNHA-AT_1R (1, 7 to 10), stimulated with Ang II (100 nmol/l) for 24 h and GFP expression was determined by western blot (**d**), and cell viability was determined by MTT assay (**e**). Results are mean ± s.e.m. of 3 separate experiments. (**f**) Estimated cell volume analysis of ACMs transduced with AdGFP, AdNHA-AT_1R, or AdNHA-AT_2R stimulated without (white bar) or with Ang II 100 nmol/l (black bar) for 72 h. Results are mean ± s.e.m. of 3 separate experiments. (**g**) [^{3}H] phenylalanine incorporation of ACMs transduced with AdNHA-AT_1R stimulated without (white bar) or with Ang II (10, 100, or 1,000 nmol/l, black bar) for 24 h. The assays were done as described in Methods. Results are mean ± s.e.m. of 3 separate experiments. GFP, green fluorescent protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide.

with caution. Therefore, our studies of isolated fibroblasts with enhanced expression of AT_1R and AT_2R could help to clarify the contribution of this cell type in different cardiac diseases. In addition, our results show that overexpression of AT1R leads to an increase intracellular signaling. The **Supplementary Figure S2a** online shows a greater activation of the ERK 1/2 pathways in cardiac fibroblasts transduced with AT1R and treated with or without angiotensin II.

Previous works have shown that, in neonatal cardiomyocytes, adenoviral expression of AT_1R and AT_2R induced

hypertrophy and constitutive growth, respectively.[15,16] However, our results show that AT_1R and AT_2R expression in ACMs, using the same adenoviruses, does not induce hypertrophy, cell growth, or cell death. We also observed that the effect of Ang II on viability is dependent on cell type. Ang II can rescue cardiomyocytes and smooth muscle cells from apoptosis,[26,27] whereas in endothelial and epithelial cells it can induce apoptosis.[28,29] Our results regarding the role of Ang II/AT_1R on ACF apoptosis were unexpected, and they differ from those reported by other previous findings.[6,20,30,31] Some explanations are as follows: (i) difference in the number of receptors: our model increased AT_1R levels fivefold over control, whereas in the others the number was not modified. Indeed, in our control cultures (which express AT_1R) no cell death was observed to Ang II. (ii) Phospholipase C/IP_3/Ca^{2+} signaling has been involved in distinct effects of Ang II. Our results show that external Ca^{2+} was necessary to trigger death. However, future work should clarify whether different patterns on intracellular second messengers can explain the dual effects of Ang II on death or proliferation. (iii) Developmental stage of the cell: most of these reports have been in neonate cardiac fibroblasts, except Crabos *et al.*[32] Cardiac fibroblasts from adult or neonatal origin have different cell growth response to Ang II.[31] Ang II-induced ACFs death was observed in a MOI-dependent manner. These results indicate that AT_1R levels are critical to trigger different signaling pathways leading to distinct cell responses, ranging from survival and synthesis of ECM components to death. Recently and using the same experimental approach, we shown that Ang II induce apoptosis in neonatal rat cardiac fibroblasts overexpressing AT_1R.[33] Thus, a higher AT_1R expression in cardiac fibroblasts could trigger cell death by apoptosis to avoid an excessive ECM deposition, which may lead to cardiac fibrosis. The role of Ang II/AT_2R has also been associated to smooth muscle cell death.[34] However, our results showed that Ang II/AT_2R did not activate ACF death and provided the first evidence showing that Ang II, through AT_1R activation, induces apoptosis on cultured ACFs.

The molecular mechanisms involved in ACF death are complex. Interestingly, Ang II only stimulated a strong $\Delta\psi m$ dissipation and cell death in AdNHA-AT_1R transduced ACFs, which were cultured in media with Ca^{2+}. These effects were partially prevented by gadolinium (a potent but nonselective antagonist of nonselective cation current), but not by nifedipine, indicating the participation of Ca^{2+} influx, but not through the L-type Ca^{2+} channel. Recently, new findings have shown that cardiac fibroblasts express TRP channels, and gadolinium inhibited the cANF-activated current (a selective agonist for the natriuretic peptide C receptor).[35] Satoh *et al.* found that TRP channel could act as a Ca^{2+} channel activated by AT_1R, leading to myocardial apoptosis.[36] These last findings and our results could suggest that in cardiac fibroblasts overexpressing AdNHA-AT_1R, a TRP channel could be involved in cell death triggered by Ang II/AT_1R. Excessive Ca^{2+} influx has been implicated in the activation of cell death pathways. Increased intracellular Ca^{2+} levels induce $\Delta\psi m$ depolarization, which leads to the opening of the mitochondrial transition pore and the subsequent release of cytochrome c.[37] In cultured cardiac fibroblasts, Brilla *et al*. also showed that Ang II induces a biphasic increase of intracellular Ca^{2+}, with an initial transient Ca^{2+} peak depending on intracellular Ca^{2+} stores, followed by a plateau phase involving an external Ca^{2+} influx.[38] Thus, from our results we can suggest that a strong entry of extracellular Ca^{2+} induces a sharp decline in $\Delta\psi m$ in cells AdNHA-AT1R, causing cell death by apoptosis.

Our results show that effects of Ang II on ACM viability are cell specific. Our results did not show Ang II effects on cardiomyocyte hypertrophy or cell death as detected in ACFs (see **Supplementary Figure S3** online). Sil and Sen proposed that the effects of Ang II on cultured cardiomyocytes were due to the presence of cardiac fibroblast contamination.[39] Ang II could act on cardiac fibroblasts releasing growth factors that in paracrine manner stimulate cardiomyocyte growth. We have previously shown that AT_1R expression in neonatal rat cardiomyocytes induces Ang II–dependent hypertrophy, whereas AT_2R expression induces basal cardiomyocyte growth.[15] These data suggest that several of the controversial effects of Ang II on ACM and ACF could be due to the low and variable number of AT_1R and AT_2R in these cells; in this regard, Fareh *et al.* reported that Ang II–specific binding was very low on isolated ventricular cardiomyocytes, suggesting the presence of only few receptors in control conditions.[40] Finally, the lack of response to Ang II in cultured adult cardiac myocytes could be an indirect effect of other stimulus only present *in vivo* but absent *in vitro*.[41–43]

Perspectives

Our data could have ambiguous consequences for cardiac pathologies in which the AT_1R is up-regulated: (i) with positive effects avoiding cardiomyocyte apoptosis and increased cardiac fibroblast proliferation—this effect could maintain correct heart function and balanced ECM deposition impeding cardiac fibrosis—and (ii) with negative effects on cardiac wound healing where a rapid and efficient scar is necessary. Thus, the results suggest that AT1R level and its activation have a predominant role on cardiac fibroblasts than cardiac myocytes. The consequences of this activation are determinant in the cell viability and ECM turnover, two highlighted characteristics of adverse cardiac remodeling.

Limitations

The low and variable number of AT_1R and AT_2R on adult cardiac myocytes could be an important factor in the effects of Ang II. We did not quantify the densities and affinities of AT1R and AT2R on adult rat cardiac myocytes, those receptors were characterized previously in cardiac myocytes from neonatal rats, and we use similar MOI in transduced cells from adult and neonate cardiac myocytes, in which we had >95% of GFP positive cells, which correlates with receptor expression level.

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