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ORIGINAL PAPER

Delineation of the effects of angiotensin type 1 and 2 receptors on HL-1 cardiomyocyte apoptosis

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Abstract Angiotensin II (Ang II) exerts its effects by activating its receptors, primarily type 1 (AT1R) and type 2 (AT2R). While the role of AT1R activation in cardiomyocyte physiology is well known, the role of AT2R in cardiomyocyte apoptosis remains controversial. To define the precise role of AT1R and AT2R in this process, we transfected HL-1 cardiomyocytes with AT1R or AT2R cDNA, and examined markers of apoptosis. We found that AT1R overexpression was associated with upregulation of endogenous AT2R expression, but AT2R overexpression did not affect endogenous AT1R expression. Caspase-3 staining indicated that overexpression of AT1R as well as AT2R resulted in cardiomyocyte apoptosis with appropriate alterations in annexin V, Bax and Bcl2 expression. Overexpression of AT1R and AT2R markedly increased IL-1β (AT2R>AT1R), iNOS (AT2R>AT1R) and eNOS expression. AT2R-induced cell apoptosis could be blocked by the iNOS selective inhibitor 1,400 W, and did not require exogenous Ang II. These findings suggest that AT2R overexpression induces cardiomyocyte apoptosis, most likely via iNOS upregulation. AT1R-mediated cardiomyocyte apoptosis may be partially mediated by upregulation of endogenous AT2R.

Keywords AT1R overexpression · AT2R overexpression · iNOS · Cell apoptosis

Introduction

Renin–angiotensin system (RAS) plays an important role in the pathogenesis of cardiovascular disease states, such as hypertension, myocardial ischemia and atherosclerosis via effector hormone angiotensin II (Ang II) [1]. Ang II exerts its effects on cardiovascular system by activating a number of receptors, among them perhaps the most important is type 1 receptor (AT1R). Recently, there has been much interest in the role of Ang II type 2 receptor (AT2R) in the genesis of myocardial ischemia and atherosclerosis [2].

Generally, AT2R activation is considered to oppose the effects of AT1R activation. Some investigators have even suggested that AT1R blockers exert their effect by redirecting Ang II towards AT2R [3]. In contrast to AT1R, the role of AT2R activation in cardiovascular pathophysiology is not well understood. For example, the effect of AT2R activation on the induction of cardiomyocyte apoptosis remains controversial. Qi et al. [4] reported that AT2R overexpression induced apoptosis in neonatal cardiomyocytes, but Moudgil et al. [5] could not show the pro-apoptotic effect of AT2R on cardiomyocytes. Kong and Rabkin [6] even suggested that Ang II does not affect cardiomyocyte apoptosis. Therefore, further work needs to be done to ascertain the specific role of AT2R in regulating cardiomyocyte apoptosis.

HL-1 cardiomyocytes are derived from atrial cardiomyocyte tumor lineage derived from AT-1 mouse, which maintains the differentiated adult cardiac phenotype and indefinite proliferation ability in vitro [7]. These cells are a useful model for investigating cardiomyocyte biology.

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In the present study, we transfected HL-1 cells with exogenous AT1R or AT2R cDNA and studied the differential effects of AT1R and AT2R overexpression on cardiomyocyte apoptosis.

Materials and methods

Cell culture and transfection

HL-1 cardiomyocytes were seeded in T25 flasks or multiwell plates pre-coated with 0.02 % gelatin (Becton-Dickinson, Sparks, MD) and 5 μg/ml fibronectin (Sigma-Aldrich, St. Louis, MO), and cultured in Claycomb medium (SAFC Biosciences, Erie, PA) supplemented with 10 % fetal bovine serum, 2 mM L-glutamine (Invitrogen, Carlsbad, CA) and 0.1 mM norepinephrine (Sigma-Aldrich) at 37 °C under 5 % CO_2. When cells reached 80 % confluence, they were transfected with PCMV-SPORT6 plasmid with AT1R or AT2R cDNAs (Invitrogen). The ratio of cells expressing GFP to all cells was used to determine transfection efficiency. Cells transfected with empty PCMV-SPORT6 plasmid were used as the controls.

Cell apoptosis assay

Cardiomyocyte apoptosis was detected by caspase-3 staining using a polycaspase FLICA apoptosis kit (Immunochemistry Technologies, Bloomington, MN). In brief, HL-1 cells were grown on 10 mm round cover slips pre-coated with gelatin and fibronectin. 36 h later after the transfection, cells were then exposed to 1× FLICA reagent prepared from 150× solution and incubated at 37 °C for 60 min. The cells were then washed in PBS and mounted on slides using a ProLong Gold antifade reagent with DAPI (Invitrogen) for analysis under fluorescent microscope.

RT-PCR

Total RNA was isolated from HL-1 cells using RNeasy Mini-Kits (Invitrogen) according to the manufacturer's instructions. Before using, RNA were treated with DNase I. 1 μg RNA was applied to synthesize cDNA with SuperScript II 1st Strand DNA Synthesis Kit (Invitrogen). PCR was performed using a 20 μL reaction volume containing 100 ng cDNA, 10 μL 2× PCR mixture (Sigma-Aldrich) and primers. The sequences of primers used for PCR are shown in Table 1.

Western blotting

Total proteins were extracted from HL-1 cardiomyocytes after transfection. The samples were loaded and separated by SDS-PAGE on 12 % gels, and then transferred to the PVDF membranes (Bio-Rad, Hercules, CA). The membranes were blocked with 5 % non-fat milk or 5 % BSA in Tris-buffered saline/0.1 % tween (TBS-T), and then washed three times with TBS-T, and incubated with Bcl2, Bax and β-actin antibodies (Santa Cruz, Santa Cruz, CA), and AT1R, AT2R, caspase-3, annexin V and IL-1β antibodies (ABcam, Cambridge, MA) and inducible NO synthase (iNOS) and endothelial NO synthase (eNOS) antibodies (Cell Signaling, Danvers, MA) (1:1,000) at 4 °C overnight. The blots were incubated HRP-conjugated second antibody (1:10,000) for 1 h at room temperature. The immunoreactive bands were visualized by incubation with ECL Western-blotting substrate (Thermo scientific, Rockford, IL).

Table 1 Primers for RT-PCR

Primer	Sense	Sequence	Product size (bp)
AT1R	Forward	5′-ATGGCCCTTAACTCTTCTACTGAAG-3′	1,078
	Reverse	5′-TCACTCCACCTCAGAACAAGACGC-3′	
AT2R	Forward	5′-ATGAAGGACAACTTCAGTTTTGCTG-3′	1,092
	Reverse	5′-TTAAGACACAAAGGTGTCCATTTCTC-3′	
Bcl2	Forward	5′-ATCTTCTCCTTCCAGCCTGA-3′	386
	Reverse	5′-TCAGTCATCCACAGGGCGAT-3′	
Bax	Forward	5′-CGAGTGTCTCCGGCGAATTG-3′	381
	Reverse	5′-ATGGTGAGCGAGGCGGTGAG-3′	
iNOS	Forward	5′-AATGGCAACATCAGGTCGGCCATCACTG-3′	454
	Reverse	5′-GCTGTGTGTCACAGAAGTCTCGAACTC-3′	
eNOS	Forward	5′-TTCCGGCTGCCACCTGATCCTAA-3′	341
	Reverse	5′-AACATATGTCCTTGCTCAAGGCA-3′	
β-Actin	Forward	5′-TTCTTTGCAGCTCCTTCGTTGCCG-3′	458
	Reverse	5′-TGGATGGCTACGTACATGGCTGGG-3′	

Statistical analysis

Data are presented as mean ± standard deviation (SD) from 3 to 5 independent experiments. Statistical analysis was performed with SPSS 11.5 software. All data were analyzed by a one-way ANOVA with a Newman–Student–Keul *t* test. $p < 0.05$ was considered statistically significant.

Results

Interaction between AT1R and AT2R

Figure 1 shows AT1R and AT2R expression after transfection with AT1R cDNA and AT2R cDNA. In the basal state, AT1R or AT2R mRNA was undetectable at a cycle of 32 in HL-1 cardiomyocytes (no transfection) and cells transfected with empty plasmids.

After transfection with exogenous cDNAs, AT1R (Fig. 1a, 1,078 bp) and AT2R transgenes (Fig. 1b, 1,092 bp) were expressed at a high level.

Next we examined if AT1R overexpression affects endogenous AT2R mRNA, or AT2R overexpression affects endogenous AT1R mRNA expression. AT2R mRNA expression could be seen at a cycle of 34 under AT1R overexpression with further accentuation of the signal at a cycle of 40. It is of note that AT2R signal was not seen in cells transfected with empty plasmids, even at a cycle of 40 (Fig. 1c). This suggests that AT1R overexpression can induce endogenous AT2R mRNA expression. On the other hand, AT2R overexpression had no effect on AT1R signal at 34 or 40 cycles (Fig. 1d).

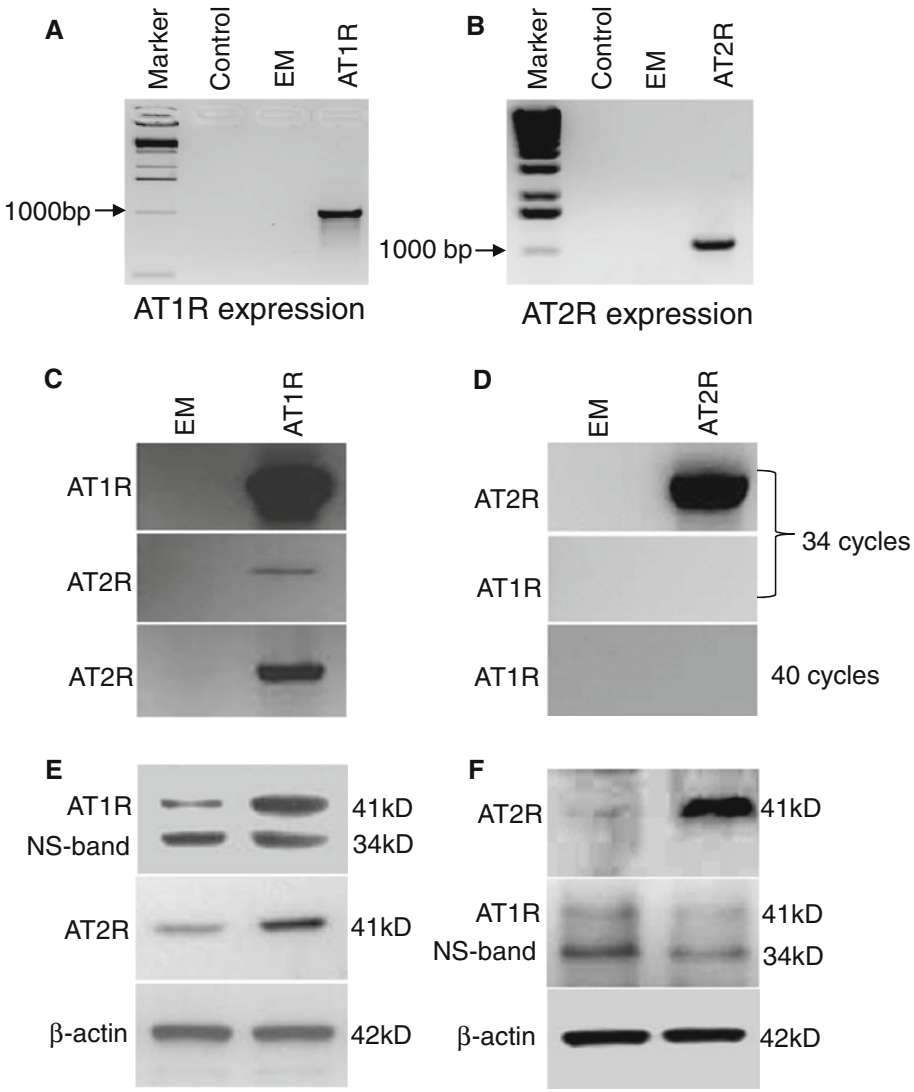

Fig. 1 AT1R and AT2R mRNA expression after transfection. **a** AT1R mRNA (1,078 bp) expression in the control cells (no transfection), in cells transfected with empty plasmids (EM) or the plasmids with AT1R cDNA (AT1R) (32 cycles); **b** AT2R mRNA (1,092 bp) expression in HL-1 control cells (no transfection), in cells transfected with empty plasmids or plasmids with AT2R cDNA (AT2R) (32 cycles); **c** Effect of AT1R overexpression on AT2R mRNA expression (34 and 40 cycles); **d** Absence of effect of AT2R overexpression on AT1R mRNA expression (34 and 40 cycles). **e** Western-blotting shows AT1R and AT2R protein expression after exogenous AT1R cDNA transfection; **f** AT2R and AT1R protein expression after exogenous AT2R cDNA transfection (*NS-band* non-specific band)

The PCR results were further confirmed by Western-blotting assay (Fig. 1e, f).

Both AT1R and AT2R induce cell apoptosis

Polycaspase-3 staining indicated that overexpression of AT1R or AT2R increased HL-1 cell apoptosis ($p < 0.05$; Fig. 2a ,b). The increase in the number of apoptotic cells was similar with AT1R or AT2R overexpression. In agreement with polycaspase staining data, there was an increase in caspase-3 and annexin V expression (Western analysis) ($p < 0.05$; Fig. 2c). RT-PCR as well as Western analysis showed that Bax expression was up-regulated and Bcl2 expression was down-regulated in both AT1R-transfected cells and AT2R-transfected cells ($p < 0.05$; Fig. 2d, e).

Both AT1R and AT2R increase IL-1β and NOS expression

Since NOS activation has been associated with cell apoptosis, and IL-1β has been reported to induce iNOS expression, we measured the expression of IL-1β and iNOS as well as eNOS in our study in HL-1 cardiomyocytes transfected with AT1R and AT2R cDNA [8, 9]. Overexpression of AT1R as well as AT2R enhanced the expression of Pro-IL-1β, iNOS and eNOS (Fig. 3; $p < 0.05$). The increase in Pro-IL-1β and iNOS mRNA was almost twice as much with AT2R overexpression as with AT1R overexpression (Fig. 3a, c). On the other hand, the increase in eNOS was similar with AT1R and AT2R overexpression.

iNOS blocker inhibited AT2R-induced apoptosis

Polycaspase-3 staining indicated AT2R-induced cell apoptosis could be inhibited by iNOS selective inhibitor 1,400 W (10 μM/L) (Fig. 4a, b), and the inhibition of apoptosis was confirmed by Western blotting for caspase-3 and annexin V and RT-PCR analysis of Bax and Bcl2 (Fig. 4c, d). Treatment with 1,400 W of AT1R cDNA-transfected cells showed modest inhibition of apoptosis. These observations suggest that HL-1 cell apoptosis induced by AT1R or AT2R overexpression-induced is at least in part iNOS-dependent.

We wondered if treatment of cells with exogenous Ang II would enhance AT2R-induced apoptosis. As shown in Fig. 5, treatment with Ang II (1 μM/L) did not alter AT2R overexpression-mediated HL-1 cell apoptosis. Further. AT2R blocker PD123319 did not affect AT2R overexpression-mediated apoptosis (data not shown).

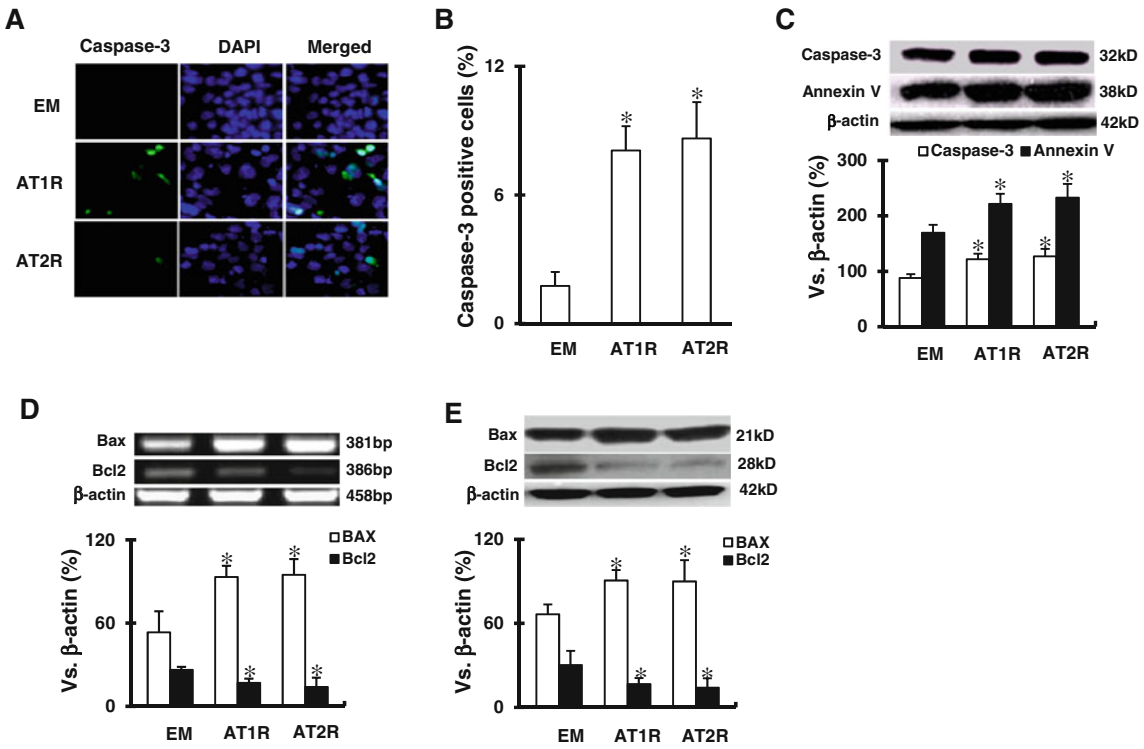

Fig. 2 Effect of AT1R and AT2R overexpression on cardiomyocyte apoptosis. **a** Caspase-3, DAPI staining and merged pictures; **b** Summary of data on caspase-3 positive cells; **c** Caspase-3 and annexin V protein expression; **d** Bax and Bcl2 mRNA expression; **e** Bax and Bcl2 protein expression. Values are mean ± SD ($n = 3$ per group); $^{*}p < 0.05$, compared with cells transfected with empty plasmids (EM)

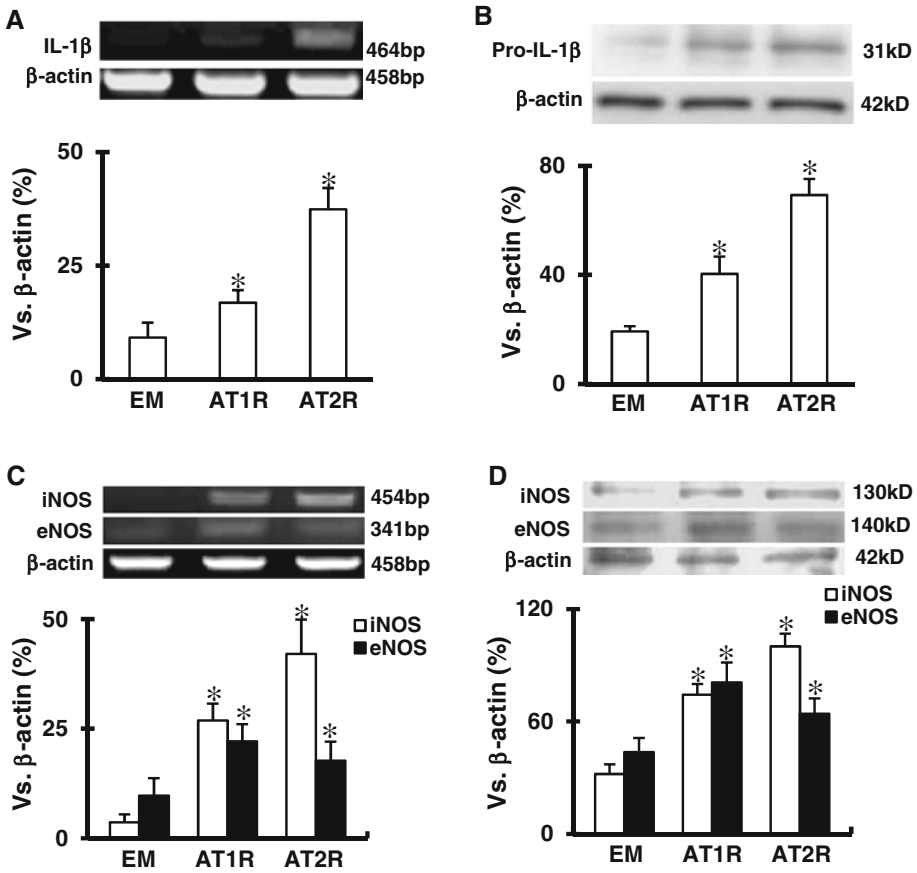

Fig. 3 Effect of AT1R and AT2R overexpression on IL-1 β, iNOS and eNOS expression. **a** IL-1β mRNA expression; **b** Pro-IL-1β expression; **c** iNOS and eNOS mRNA expression level; **d** iNOS and eNOS protein expression. Values are mean $\pm$ SD ($n = 3$ per group); $^{*}p < 0.05$, compared with cells transfected with empty plasmids (EM)

Discussion

Cardiomyocyte apoptosis is a major determinant of cardiac remodeling process in disease states, such as hypertension and myocardial ischemia. There is marked upregulation of RAS as well as of AT1R and AT2R in these disease states. Since pharmacotherapy directed at modulation of RAS is often used in these disease states, it is important to study the differential effects of AT1R and AT2R on cardiomyocyte apoptosis.

Although many studies have previously examined the pro-apoptotic and pro-inflammatory effects of Ang II in the heart, we believe this is the first study to directly assess cardiomyocyte apoptosis under the influence of selective AT1R and AT2R upregulation.

We describe several novel observations in this study. First, we observed that forced overexpression of AT1R transgene resulted in a modest but significant increase in endogenous AT2R mRNA. Second, it is the AT1R apoptosis that mainly induces apoptosis in HL-1 cells. Although there are no reports in the literature supporting or conflicting this observation, it is known that pathologic states, such as atherosclerosis and myocardial ischemia, that are associated with AT1R overexpression also show upregulated AT2R expression [10, 11]. Whether AT2R upregulation is a direct response to AT1R overexpression, or represents an unrelated phenomenon cannot be discerned from the present studies. Nonetheless, it may be postulated that AT1R overexpression-mediated increase in iNOS and apoptosis in HL-1 cells relates to increase in endogenous AT2R overexpression.

It is of note that the direct AT2R transfection had no effect on AT1R mRNA in our studies. The role of AT2R in modulating AT1R expression is currently controversial. For example, Zhu et al. reported that AT2R overexpression at lower titers of AAV (40 and 80 MOIs) had no effect on endogenous AT1R expression, but at higher titers (160 MOI) AT2R overexpression increased endogenous AT1R expression; however, this may have been a non-specific response to high titers of AAV [12]. Our observations support the earlier work by Metcalfe et al. and Hu et al. who induced AT2R in mice via AAV-mediated gene transfer and failed to observe any increase in endogenous AT1R expression [10, 13]. Matavelli et al. also found that AT2R activation did not affect AT1R transcription [14].

There are several reports suggesting AT1R activation causing cardiomyocyte apoptosis. There are also reports that AT2R activation may promote apoptosis in several cell types, such as fibroblasts, neurons, SMCs, endothelial cells, renal tubular cells and some cancer cells [15, 16].

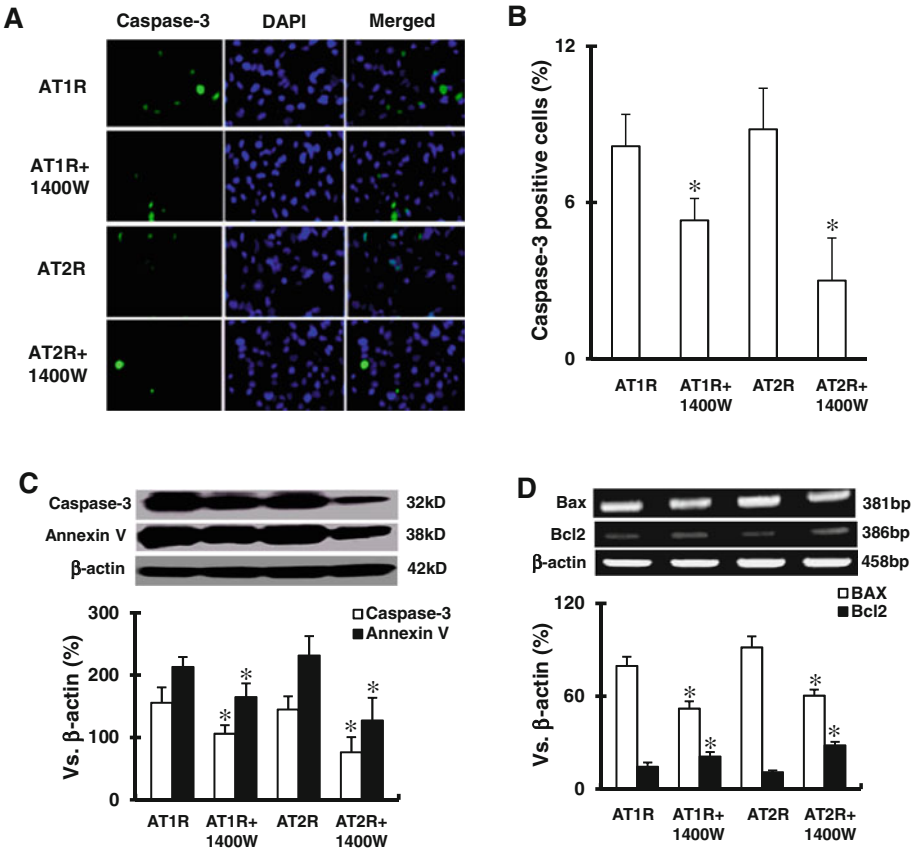

Fig. 4 Inhibition of apoptosis in cells transfected with AT1R and AT2R cDNAs by the iNOS inhibitor 1,400 W (10 mM/L). **a** Representative experiments showing caspase-3 positivity after exposure to 1,400 W; **b** Summary of data on caspase-3 positivity after exposure to 1,400 W; **c** Caspase-3 and annexin V protein expression; **d** Bax and Bcl2 mRNA expression. Values are mean ± SD ($n = 3$ per group); $^{*}p < 0.05$, compared with cells transfected with plasmids with AT1R cDNA (AT1R) or AT2R cDNA (AT2R)

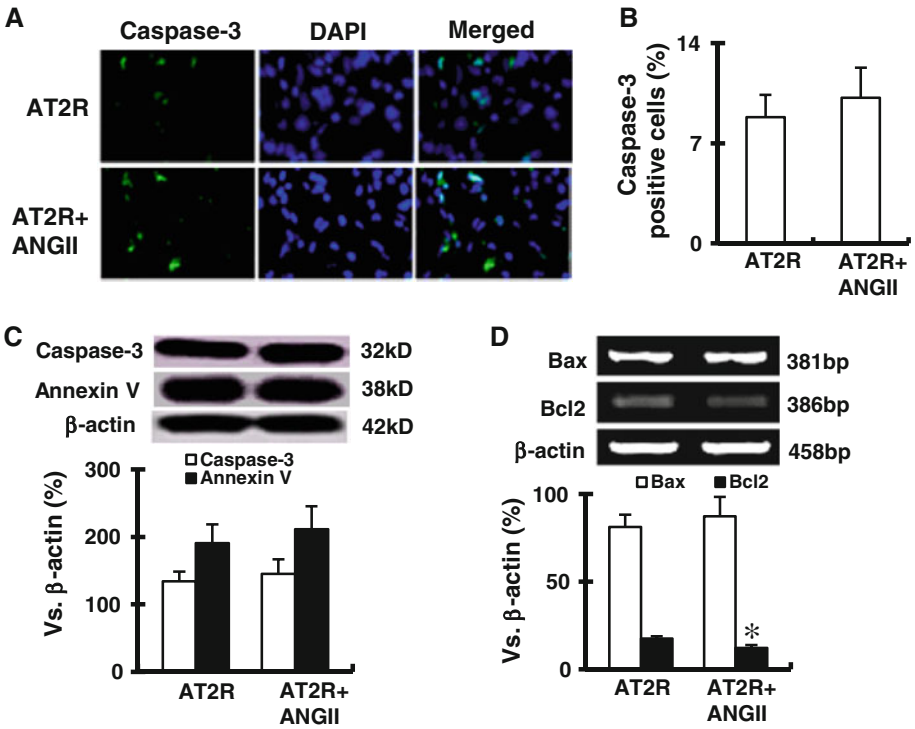

Fig. 5 AT2R-induced HL-1 cell apoptosis is not dependent on exogenous Ang II. **a** Caspase staining showing caspase-3 positive cells after exposure to Ang II; **b** Summary of data on caspase-3 positive cells after treatment with Ang II; C. Caspase-3 and annexin V protein expression; D. Bcl2 and Bax mRNA expression. Values are mean ± SD ($n = 3$ per group); $^{*}p < 0.05$, compared with cells transfected with plasmids with AT2R cDNA (AT2R)

However, direct pro-apoptotic effects of AT2R in cardiomyocytes have not been rigorously studied. We observed that overexpression of AT1R as well as AT2R increased HL-1 cardiomyocyte apoptosis in vitro. This was confirmed by polycaspase staining and results of caspase-3 and Annexin V protein measurement. Incidentally, an increase

in SMC apoptosis by AT2R overexpression was recently shown by Hu et al. [10] in the LDLR null mice given high cholesterol diet. At least two other independent investigators have also suggested that AT2R may be involved in apoptosis of cardiomyocytes, yet a direct evidence for the pro-apoptotic effect of AT2R until now has not been shown [4].

The signaling mechanisms associated with AT1R have been defined and include NADPH oxidase stimulation followed by activation of redox-sensitive transcription factors [17]. The AT2R signaling leading to apoptosis, however, is not as well studied, although Siragy and Carey suggested that iNOS and increased production of nitric oxide is relevant in the effects of AT2R [18, 19]. Recently, Li et al. showed AT2R-induced apoptosis of is partially dependent on the activation of p38MAPK in human prostate cancer cells, but did not examine the role of iNOS in this process [15]. In our experiments, we noted that AT2R overexpression enhanced the transcription of IL-1β, well known stimuli for iNOS expression [8, 20]. Further, iNOS is well known to activate caspase-dependent cell apoptosis [21]. Ing et al. [22] showed that cytokine-mediated induction of apoptosis in neonatal rat cardiac myocytes could be attributed entirely to IL-1β. Sagoo et al. [23] showed that the pro-apoptotic effect of IL-1ß could be blocked by the inhibitors of iNOS. We confirmed this observation in HL-1 cardiomyocytes, and suggest that iNOS upregulation following forced AT2R expression may well be the basis for increase in cardiomyocyte apoptosis. It is of note that AT1R overexpression increased endogenous AT2R, and then enhanced iNOS; this pathway may have contributed, at least in part, to AT1R-induced HL-1 cell apoptosis. Importantly, AT2R-induced increase in iNOS expression was twice as much as with AT1R overexpression, but the increase in eNOS transcription was similar with AT1R and AT2R overexpression. In addition, AT2R-induced apoptosis did not require Ang II, which was in consistent with the previous observation in other cancer cell lines by Li et al. [15].

The cross-talk between AT1R and AT2R described in this study is novel and may have a bearing on the effect of AT1R and AT2R activation. AT1R overexpression upregulated endogenous AT2R, but AT2R overexpression had no effect on AT1R expression. The precise implication of this phenomenon is not clear, but it is possible that AT1R overexpression induced upregulation of AT2R partially contributes AT1R-mediated HL-1 cell apoptosis.

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