Effect of angiotensin II receptor 1 antisense oligodoexynucleotides on physiological and pathophysiological growth of cardiomyocytes[1]

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KEY WORDS antisense oligodeoxynucleotides; angiotensin receptors; angiotensin II; myocardium; hypertrophy; mitogen-activated protein kinases

ABSTRACT

AIM: To evaluate the role of angiotensin II receptor 1 antisense oligodexynucleotides (AT_1R-AS-ODNs) on physiological and pathophysiological growth of cardiomyocytes from normotensive rats. **METHODS:** Cardiomyocytes were transfected with AT_1R-AS-ODNs (200 nmol/L) followed by treatment with or without angiotensin II (1 μmol/L). *In situ* hybridization and Western blot were used for AT_1R mRNA and protein detection, respectively. c-Jun N-terminal protein kinase (JNK) activity was characterized by immune complex kinase assay. c-Jun protein expression was examined by immunocytochemistry. DNA content was detected by flow cytometric assay. Atrial natriuretic factor (ANF) expression was identified by radioimmunoassay. **RESULTS:** Treatment with AT_1R-AS-ODNs for 24 h resulted in 51.2 % decrease in AT_1R mRNA and 60.7 % in protein (P<0.05 *vs* control). However, the basal level of JNK activity, c-Jun protein expression, and DNA content were not altered by AT_1R-AS treatment in absence of overactive hormonal system. After treatment with angiotensin II for 30 min, both p46JNK and p54JNK were robustly activated. By 2 h, c-Jun protein expression was increased. By 24 h, angiotensin II caused a marked increase both in G_0/G_1 and G_2/M DNA content, and increased ANF expression by 1.8-fold. All these were inhibited by AT_1R-AS-ODNs pretreatment. In contrast, sense sequence was ineffective. **CONCLUSION:** Decrease of AT_1R expression by AS-ODNs did not interfere with normal growth, but protected cardiomyocytes from angiotensin II-dependent pathophysiological growth.

INTRODUCTION

Renin-angiotensin system (RAS) plays a crucial role in the initiation and development of cardiac remodeling[1]. Despite great success with traditional therapy, such as angiotensin converting enzyme inhibitors (ACEI) and angiotensin II receptor 1 (AT_1R) antagonists, there are still many patients with poorly controlled high blood pressure and target organs dysfunction due to many inherent disadvantages including compliance, side effects, and lack of complete reversal of pathophysiological aspects of this disease[2]. Recently, several studies have shown that delivery of angiotensin II type re-

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ceptor 1 antisense (AT_1R-AS) by nonviral or viral vector mediated-systems resulted in an impressive lowering of blood pressure accompanied by attenuation of some pathophysiological events observed in the target organs in hypertensive rats[3,4], but did not reduce blood pressure in normotensive individuals[5,6]. It is likely that antisense is useful for reducing overactive hormonal systems, but does not interfere with the normal physiological functions of RAS. However, the underlying cause for such differences remains obscure. Moreover, little is currently concerning the precise mechanism of AT_1R-AS on physiological and pathophysiological growth of cardiomyocytes from normotensive rats.

The present study was designed to explore whether angiotensin II receptor 1 antisense oligodexynucleotides (AT_1R-AS-ODNs) would protect cultured cardiomyocytes of normotensive rats from developing Ang II-dependent hypertrophy by suppressing AT_1R-associated signal transduction and transcription factor overexpression, and whether the transfection would interfere with the normal growth of these myocytes.

MATERIALS AND METHODS

Cell culture Cardiac myocytes were dissociated from 1-d-old neonatal Sprague-Dawley rat hearts as described before[7], and plated on either gelatin-coated glass coverslips or 6-well plates (Corning Costar) at high density (1000-2000 cells/mm^2). The cells were kept in DMEM/F12 (GIBCO, USA) medium, supplemented with 10 % fetal calf serum (GIBCO, USA), benzylpenicillin (100 kU/L), streptomycin (100 g/L) and 5-bromo-2'-deoxyuridine (1 mmol/L, BrdU, Sigma, USA) for 72 h. Before the start of each experiment, cells were incubated for 24 h with serum-free opti-MEM (Life Technology, USA), supplemented with 1% bovine serum albumin (Sigma, USA).

Cell treatment Antisense oligodexynucleotides (5'-GTCGAATTCCGAGACTCATA-3'), targeted to bases +821 to +840 of AT_1 R mRNA according to the base sequence of Ho[8], and the sense sequence (5'-TATGAGTCTCGGAATTCGAC-3') was synthesized by a DNA synthesizer (TaKaRa Biotechnology, Japan) and purified for HPLC assay. The sequence was marked by fluorescent isothiocyanate (FITC) at 5', and all the bases were phosphorothioated. Transfection was carried out according to the manufacturer's instruction when the cells reached 30 %-50 % confluence. For transfection, ODNs 200 nmol/L and liposome 8 mg/L (oligefectamine, Life Technology, USA) were used. Unless otherwise stated, the cells were incubated with the liposome-ODNs complexes for 24 h at 37 ºC in a CO_2 incubator, after that time the cells were either harvested or continuously cultured with Ang II (1 μmol/L), until indicated time.

MTT assays Cells seeded in 96 well plates with 2×10^4 cells per well were incubated with liposome-ODNs complexes for 48 h. Then, 3-(4,5-dimethylthiazol)-2,5-diphenyl tetrazolium bromide solution (MTT, 2 g/L, Sigma, USA) was added into cardiomyocytes and incubated for 4 h. After that, the cells were solubilized with Me_2SO (Sigma, USA). The purple formazane crystals were measured at 590 nm with a spectrophotometer. Cardiomyocytes cultured in a serum-free medium without transfection complex were acted as the control.

Uptake experiment After an incubation with liposome-ODNs complexes for 0 min, 30 min, 60 min, 120 min, 240 min, and 24 h, respectively, myocytes were harvested and fixed with 4 % paraformaldehyde for 30 min, then mounted on slides of fluorescent microscopy (Olympus, Japan) for the examination of fluorescence uptake. Transfection efficiency was represented by the percentage of fluorescence stained myocytes. Ten different views were selected and the average efficiency was counted.

***In situ* hybridization for AT_1R mRNA** The probes labeled with digoxigenin-UTP and *in situ* hybridization detection kit were purchased from Boster (China). The myocytes were fixed in 4 % paraformaldehyde for 10 min and digested with proteinase K (5 mg/L) in Tris-edetic acid (pH 8.0) for 60 min at 37 ºC. Pre-hybridization buffer (NaCl 0.3 mol/L, Tris 20 mmol/L, pH 8.0, edetic acid 5 mmol/L, 5×formamide, 1×Denhardt's solution) was applied for at least 2 h at 55 ºC. The myocytes were then hybridized in the denatured probe mixture (20 ng of probe, 6 mg of salmon sperm DNA, and 3 mg tRNA) overnight at 42 ºC. After the hybridization, myocytes were washed four times in laddered standard saline citrate (SSC), and once in SSC with 1 % BSA at 55 ºC for 20 min. Staining was visualized with the StrepAvilin-Biotin immunoperoxidase reaction (Histostain™-plus SP Kit) using diaminobenzidine (Fast DAB tablets, Sigma) according to the manufacturer's instructions. The image analysis was carried out with HPLAS-2000 analysis software.

Western blot analysis of AT_1R protein Cardiomyocytes were washed with PBS buffer, and then scratched off with a rubber scraper in presence of 0.5

mL buffer A (10 % glycerol, Tris-HCl 20 mmol/L, NaCl 100 mmol/L, phenylmethylsulfonyl fluoride 2 mmol/L, edetic acid 2 mmol/L, egtazic acid 2 mmol/L, leupeptin 10 mg/L, pepstatin A 10 mg/L). The lysate was incubated on ice for 1 h and centrifuged (12 000×*g*, 4 ºC) for 30 min. The protein concentration was measured according to the method of Lowry *et al*[9]. For immunoblotting, 9 % SDS-PAGE was performed, and 50 μg protein was loaded per gel. The resolved proteins were transferred onto PDVF membranes at 15 V for 20 min. The membranes were blocked in 5 % BSA in TBS-T (pH 7.6) for 1 h and then incubated with a polyclonal antibody to AT_1R (Santa Cruz, USA) at a dilution of 1:1000 for 1 h at room temperature. The membranes were washed with TBS-T and then incubated with the secondary anti-rabbit antibody for 1.5 h. After a final wash in TBS-T, the membranes were treated with LumiGLO reagent (Cell Signal Technology, USA), and chemiluminescence was detected by an exposure to Hyperfilm-ECL for 15 min. The intensity of the bands was quantified with HPLAS-2000 analysis software.

Immune complex kinase assay Cardiomyocytes lysed in lysis buffer containing Tris 25 mmol/L, pH 7.4, Na_3VO_4 1 mmol/L, NaCl 150 mmol/L, edetic acid 1 mmol/L, egtazic acid 1 mmol/L, 1 % Triton, sodium pyrophosphate 2.5 mmol/L, leupeptin 10 mg/L, and β-glycerol phosphate 1 mmol/L. Then, the protein was extracted by centrifugation (14 000×*g*, 4 ºC, 20 min). Immunoprecipitations were performed by incubating 20 μg protein extracts with 2 μg of c-Jun fusion protein beads (Cell Signaling Technology, USA) and gentle rocking overnight at 4 ºC. The precipitates were incubated with ATP 100 μmol/L for kinase reaction at 30 ºC for 30 min, and denatured in Laemmli sample buffer, then resolved in SDS-PAGE [12 % (w/v) gel], later were transferred on PVDF membranes at 20 V for 5 h. The membranes were probed with anti-phospho-c-Jun (Ser63) antibodies (1×3000 diluted, Cell Signaling Technology, USA), stained with LumiGLO at room temperature for 2 min, and exposed to X-ray film for 20 min. The intensity of bands was quantitatively scanned using HPLAS-2000 analysis software.

Immunocytochemistry Cardiomyocytes were fixed as described before. The endogenous peroxidase was quenched with 3 % H_2O_2 in methanol, and the nonspecific binding sites were blocked with 10 % normal goat serum. After that, myocytes were incubated with rabbit anti- c-Jun (1:100 diluted, Santa Cruz, USA) overnight at 4 ºC. Staining was visualized with the Strep-Avilin-Biotin immunoperoxidase reaction (Histostain™-plus SP Kit) using diaminobenzidine (Fast DAB tablets, Sigma) according to the manufacturer's instructions. The image analysis was carried out with HPLAS-2000 analysis software.

DNA synthesis assay Cells were harvested with 0.08 % trypsin (Sigma, USA), and washed twice with PBS. Prior to flow cytometric assay analysis (Beckman Coulter Epics XL) the cells were incubated with a solution containing propidium iodide (100 mg/L, Sigma, USA), RNase A (100 mg/L, Sigma, USA), Triton X-100 (0.1 %, Sigma, USA) and edetic acid (0.01 %) for 30 min at 37 ºC and then analyzed by Multi-cycle software.

Measurement of atrial natriuretic factor The culture medium was harvested, and added into 7.5 % sodium edetic acid and trasylol. Radioimmunoassay kit (DongYa Technology, China) and SN-682Bγ-radioimmunoassay counter was used to measure the content of atrial natriuretic factor (ANF).

Statistical analysis Data are expressed as mean±SD. Statistical analysis was assayed by ANOVA or *t*-test. A value of $P<0.05$ was considered statistically significant.

RESULTS

Cardiomyocyte toxicity of transfection complex After 48-h incubation with AT_1R-AS-ODNs, AT_1R-S-ODNs, or serum-free medium, cell viability was assessed by the MTT assay. No significant difference was observed among the three groups (Fig 1).

Time course of oligodeoxynucleotide-liposome complexes uptake Myocytes were incubated with fluorescein-labeled ODNs from 0 to 24 h. At time 0

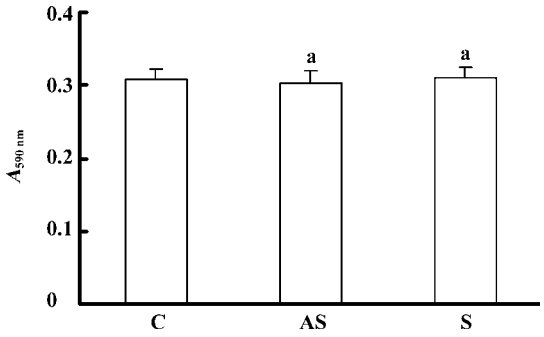

Fig 1. Cytotoxic effects of angiotensin II receptor antisense oligodeoxynucleotides (AT_1R-AS-ODNs) and AT_1R sense ODN on cardiomyocytes. *n*=12. Mean±SD. C:normal control; AS: AT_1R-AS-ODNs; S: AT_1R-S-ODNs. [a]*P*>0.05 *vs* C.

min no cellular fluorescence was detected. Partial cell surface was stained brightly at 30 min, showing the start of uptake. After 60 min incubation, ODNs rapidly migrated into myocytes and mainly accumulated into the nucleus. By 120 min, more than a half was stained yellow by fluorescein-labeled ODNs. This staining pattern did not change appreciably after an additional 2 h, except that less nucleus became positively stained with ODNs. By 24 h, ODNs still could be seen both in cytoplasms and nucleus (Fig 2).

AT_1R gene expression in cardiomyocytes Treatment with AT_1R-AS-ODNs for 24 h resulted in 51.2 % decrease in AT_1R mRNA and 60.7 % in protein (P<0.01 *vs* control), while sense sequence was ineffective. When cultured with Ang II for 24 h, both cardiomyocytes pretreated with serum-free medium and AT_1R-AS-ODNs got a 75 % decrease in AT_1R mRNA expression (P< 0.05) after a single administration. Equivalent results were seen in protein expression (Fig 3).

Effect of AT_1R-AS-ODNs on JNK pathway In the basal state, little JNK activation, but some c-Jun protein expression was detectable in cardiomyocytes. Neither sense nor antisense treatment caused a significant change in JNK activation and c-Jun expression in absence of Ang II. However, at 30 min after the addition of Ang II, p46JNK and p54JNK were markedly activated by 2.5- and 3.1-fold, respectively (P<0.01 *vs* control). After 2 h, Ang II stimulation resulted in a robustly increased c-Jun protein expression in nucleolus. Compared with Ang II group, AT_1R-AS-ODNs pretreatment significantly decreased the activity of p46JNK and p54JNK by 40.2 % and 45.2 % (P<0.05), respectively,

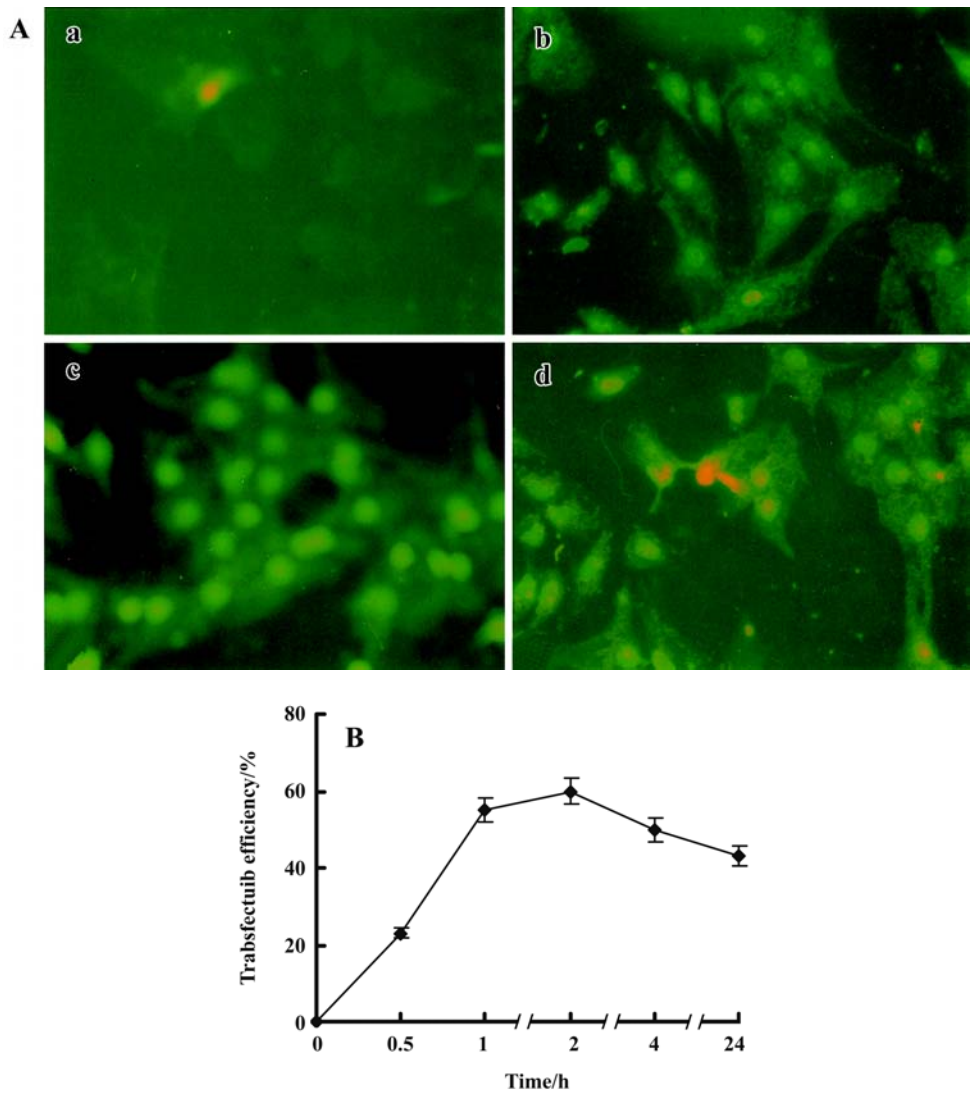

Fig 2. A) Fluorescent microcopy images (×200) obtained from myocytes incubated with FITC labeled ODNs at time 0.5 h (a), 2 h (b), 4 h (c), and 24 h (d). B) Time course of FITC labeled ODNs uptake. n=10. Mean±SD.

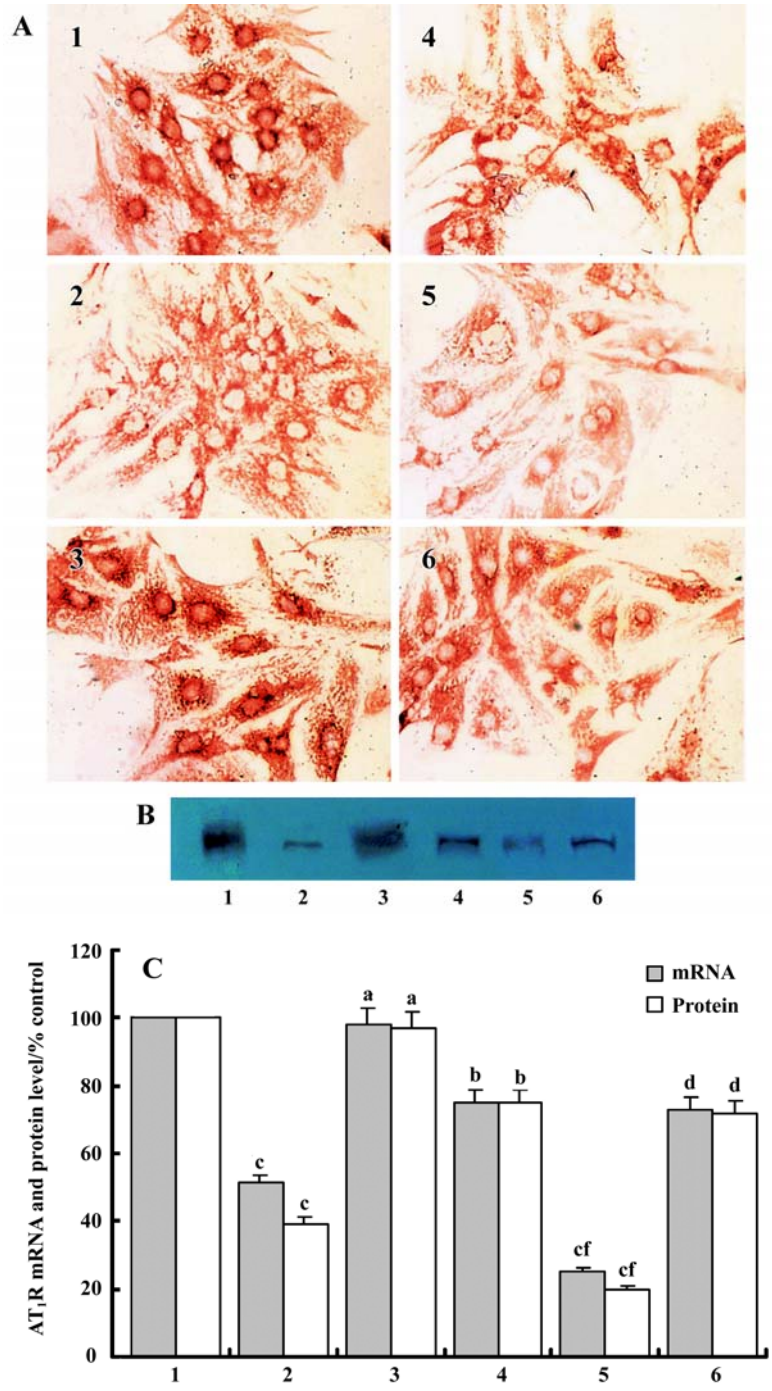

Fig 3. AT_1R mRNA expression detected by *in situ* hybridization (×200) (A). AT_1R protein expression determined by Western blot (B). Representative results of AT_1R mRNA (n=6) and protein (n=3) was expressed as % *vs* control (C). Mean±SD. Myocytes treated with serum-free medium (1), AT_1R-AS-ODNs (2), or AT_1R-S-ODNs (3) for 24 h, and then each stimulated with Ang II (4, 5, 6) for 24 h. ^aP>0.05, ^bP<0.05, ^cP<0.01 *vs* group 1. ^dP>0.05, ^fP<0.01 *vs* group 4.

and c-Jun protein level decreased by 39.3 % (P<0.05). Sense sequence was ineffective (P>0.05, *vs* Ang II group) (Fig 4, 5).

Effect of AT_1R-AS-ODNs on DNA synthesis Neither AT_1R-AS-ODNs nor sense sequence altered the amount of basal DNA content in absence of Ang II. Ang II treatment caused a marked increase both in G_0/G_1 and G_2/M DNA content (P<0.05 *vs* control), which was significantly reduced by AT_1R-AS-ODNs pretreatment by 14.2 % and 9.5 %, respectively (P<0.05 *vs* Ang II group). Sense sequence was ineffective (P>0.05 *vs* Ang II group, Tab 1).

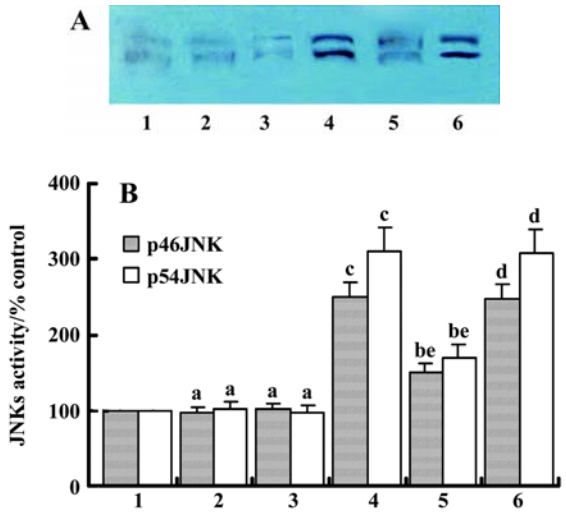

Fig 4. JNK activity was detected by immune complex kinase assay (A) and the results were expressed as % *vs* control (B). *n*=3. Mean±SD. Myocytes were treated with serum-free medium (1), AT_1R-AS-ODNs (2), or AT_1R-S-ODNs (3) for 24 h, and then each stimulated with Ang II (4, 5, 6) for 30 min. [a]*P*>0.05, [b]*P*<0.05, [c]*P*<0.01 *vs* group 1. [d]*P*>0.05, [e]*P*<0.05 *vs* group 4.

Tab 1. DNA content in cardiomyocytes treated with serum-free medium (1), AT_1R-AS-ODNs (2), or AT_1R-S-ODNs (3) for 24 h, and then each stimulated with Ang II (4, 5, 6) for 24 h. *n*=3. Mean±SD. [a]*P*>0.05, [b]*P*<0.05 *vs* 1. [d]*P*>0.05, [e]*P*<0.05 *vs* 4.

Group	DNA content/mg·kg^{-1}	
	G_0/G_1	G_2/M
1	120±4[a]	237±5[a]
2	118±4[a]	234±4[a]
3	123±6[a]	241±6[a]
4	155±7[b]	305±7[b]
5	133±6[be]	276±6[be]
6	158±4[d]	308±4[d]

Effect of AT_1R-AS-ODNs on ANF protein expression There were no significant changes in ANF protein expression following AT_1R-AS-ODNs and AT_1R-S-ODNs treatment. By comparison with control cells, ANF expression increased by 1.8-fold after 24 h treatment with Ang II. This value was decreased by 32.4 % after AT_1R-S-ODNs pretreatment (P<0.05). However the sense sequence was ineffective (P>0.05 *vs* Ang II group, Fig 6).

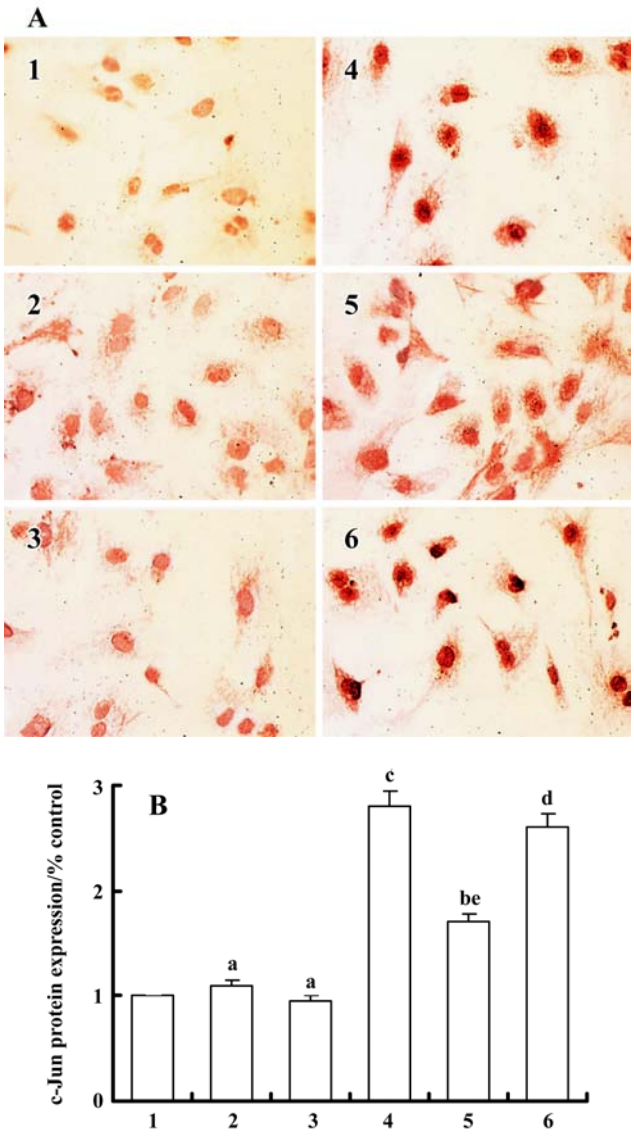

Fig 5. Light photomicrograph of c-Jun protein expression detected with immunocytochemistry (×200) in cardiomyocytes (A) and data were expressed as % *vs* control (B). *n*=6. Mean±SD. Myocytes were treated with serum-free medium (1), AT_1R-AS-ODNs (2), or AT_1R-S-ODNs (3) for 24 h, and then each stimulated with Ang II (4, 5, 6) for 2 h. [a]*P*>0.05, [b]*P*<0.05, [c]*P*<0.01 *vs* group 1. [d]*P*>0.05, [e]*P*<0.05 *vs* group 4.

DISCUSSION

The most significant finding of this study is that AS-ODNs targeting AT_1R mRNA protects cardiomyocytes from developing Ang II-dependent pathophysiological growth, but it does not interfere with normal growth.

The rationale for us choosing AT_1R as a target were of threefold: (1) AT_1R was shown to be up-regulated in

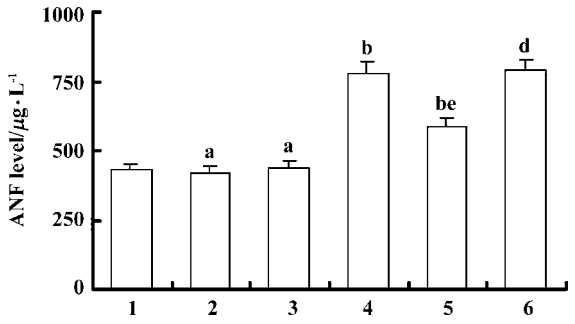

Fig 6. ANF protein expression detected by RIA. *n*=6. Mean±SD. Myocytes treated with serum-free medium (1), AT_1R-AS-ODNs (2), or AT_1R-S-ODNs (3) for 24 h, and then each stimulated with Ang II (4, 5, 6) for 24 h. [a]*P*>0.05, [b]*P*<0.05 *vs* group 1. [d]*P*>0.05, [e]*P*<0.05 *vs* group 4.

hypertrophic myocardium of several hypertensive models[10]; (2) overexpression of AT_1R in cardiomyocytes played a crucial role in the development and establishment of cardiac hypertrophy[11]; (3) AT_1R antagonist was a proved traditional pharmacological strategy for the control of hypertension[12]. Despite great success with traditional therapy, chronic treatment with such agents has been shown to induce the upregulation of AT_1R[13], which is disadvantageous to hypertrophy reversion. Therefore, interruption in the AT_1R expression by antisense becomes an available selection.

In the present study, encapsulated by liposome, AT_1R-AS-ODNs were delivered into cardiomyocytes triumphantly. Fluorescent microscopy showed rapid uptake of the AS-ODNs into cells within 120 min, where they migrated to the nucleus quickly and stayed there at least for 24 h. In order to avoid nonspecific effects and other artifacts, AS-ODNs were used at low concentrations (200 nmol/L). However, such concentration of AS-ODNs resulted in a significantly decreased AT_1R gene expression. Previous studies showed that this could occur by the hybridization of AS-ODNs with target mRNA, preventing the passage of the mRNA though the ribosome. Alternatively, DNA hybridization to RNA will stimulate the production of RNase H, which destroys the RNA and thereby releases the oligo for further hybridization. This recycling action induced by RNase H may account for the long action of AS-ODNs[14].

It is worth pointing out that, in this study, Ang II is a potent negative regulator of the AT_1R gene in cardiomyocytes. This result is consistent with Everett's[15], who proved that incubation of cardiomyocytes with Ang II resulted in a time- and dose-dependent decrease in AT_1R mRNA levels, and co-incubation with Ang II and losartan prevented the decrease in AT_1R mRNA whereas the AT_2R antagonist PD123319 was ineffective, suggesting Ang II induced AT_1R gene downregulation is mediated through the AT_1R. In contrast to losartan, AT_1R-AS-ODNs pretreatment did not block, but superinduced the downregulation of AT_1R by Ang II, whereas AT_1R-S-ODNs was ineffective. These results indicate that AS-ODNs block AT_1R expression with precise specificity based on the genetic design, but not interfere with AT_1R activity, which is extremely different from traditional AT_1R antagonists.

The mechanisms of Ang II-induced cardiac hypertrophy remain unknown. Several signal transduction pathways including PKC[16], ERKs[17], and tyrosine kinase[18], have been reported to be involved in Ang II dependent cardiac hypertrophy. However, the data here showed that JNK pathway, another major subtype of the MAPKs family, was also activated by Ang II. This was in agreement with the results observed by Murasawa[19]. Up to now, at least ten JNKs, derived from alternative splicing of three genes, have been identified. The molecular mass of these isoforms is about 46 or 54 kDa (p46JNK or p54JNK), depending on the absence or presence of a c-terminal extension[20]. It is still not clear which of the individual isoform presents and plays an important role in the myocardium growth. In this study, Ang II significantly activated both p46JNK and p54JNK, because JNK is the predominant c-Jun kinase and the potent activator of AP-1[21], we also examined c-Jun expression, and found that it was significantly increased by Ang II stimulation via AT_1R, following the activation of JNK, but preceding the onset of DNA synthesis and ANF reexpression. Interestingly, various cardiac hypertrophy related genes, such as ANF, skeletal α-actin, and even *c-jun* itself have the AP-1 consensus subsequent in their promoter regions[21]. Thus, it is postulated that Ang II triggered the activation of JNK in cardiomyocytes, which induced c-Jun gene expression and the subsequent hypertrophy. Although it is well established that ERK, another subfamily of MAPKs, plays an important role in cardiac hypertrophy induced by multiple growth factor[17], Ang II induced cardiac activation of JNK *in vivo* occurs in a more sensitive manner than that of ERK[22]. Therefore, the activity of JNK, together with the level of c-Jun expression could be taken as valuable indicators representing the reactivity of cardiomyocytes to Ang II stimulation.

In this study, AT_1R-AS-ODNs pretreatment significantly, but partially, suppressed Ang II-induced JNK

activation and subsequent pathophysiological growth. It is possible due to that not all the receptors were blocked by antisense, and these receptors remnant mediated the remaining actions of Ang II. In contrast, the sense sequence was ineffective. These results support the concept that AT_1R contributes to the JNK pathway activation during Ang II-dependent cardiomyocytes growth. These findings also suggest that interruption of AT_1R expression by AS-ODNs makes cardiomyocytes less sensitive to Ang II stimulation by suppression of AT_1R associated signal transduction pathway, such as JNK.

It is worth pointing out that, in this study, AT_1R-AS-ODNs did not suppress Ang II associated gene expression in parallel. It was possible due to that not all the non-cardiomyocytes, such as fibroblasts, were removed from cardiomyocytes during the experiment, and the extracellular matrix derived from these fibroblasts remnant interfered with the actions of Ang II. Because several studies have shown that enhanced expression of extracellular matrix components, such as collagen III, fibronectin, osteopontin, and integrin subunits correlate with advancement of cardiomyocytes hypertrophy[23].

In contrast to the prevention of Ang II-dependent pathophysiological growth, AT_1R-AS-ODNs failed to alter basal JNK activity, c-Jun expression, and DNA synthesis in myocytes in absence of overactive hormonal stimulation, despite the evidently inhibitory effect on AT_1R expression, showing that the suppression of AT_1R expression was unable to reduce the physiological growth in myocytes. Therefore, these results are consistent with previous studies *in vivo* that the expression of AT_1R-AS mediated by retroviral vector prevented SHR from developing hypertension[24], but was unable to reduce blood pressure in normotensive rats[5]. These observations are compatible with the traditional pharmacological strategy in which AT_1R antagonists express little or no effect in normal individual[25]. It further supports a long-held view that the RAS is of little relevance in the control of normal BP as the result of the existence of many other interacting physiological mechanisms[26,27]. These views led us to propose that the role of AT_1R-AS-ODNs in the normal myocytes would only come into play when the RAS was challenged.

In conclusion, we have obtained the first evidence indicating that AT_1R-AS-ODNs protects cardiomyocytes from developing Ang II-dependent pathophysiological growth by suppression of AT_1R associated signal transduction pathway, but it does not interfere with normal growth. Taken together with recent *in vivo* evidence that interruption in the expression of AT_1R prevents the SHR from developing hypertension, our present study supports the notion that antisense-targeting AT_1R is useful in the prevention of AT_1R-mediated cardiomyocytes hypertrophy.

REFERENCES

1 Flack JM, Peters R, Shafi T, Alrefai H, Nasser SA, Crook E. Prevention of hypertension and its complications: theoretical basis and guidelines for treatment. J Am Soc Nephrol 2003; 14 (7 Suppl 2): S92-S98.

2 Frances S, Raizada M, Mangi A, Melo L, Dzau VJ, Vale P, *et al.* Genetic targeting for cardiovascular therapeutics: are we near the submit or just beginning the climb? Physiol Genomics 2001; 7: 79-94.

3 Galli SM, Phillips MI. Angiotensin II AT (1A) receptor antisense lowers blood pressure in acute 2-kidney, 1-clip hypertension. Hypertension 2001; 38 (3 Pt 2): 674-8.

4 Gelband CH, Reaves PY, Evans J, Wang HW, Katovich MJ, Raizada MK. Angiotensin II type 1 receptor antisense gene therapy prevents altered renal vascular calcium homeostasis in hypertension. Hypertension 1999; 33: 360-5.

5 Pachori AS, Wang HW, Gelband CH, Ferrario CM, Katovich MJ, Raizada MK. Inability to induce hypertension in normotensive rat expressing AT1 receptor antisense. Circ Res 2000; 86: 1167-72.

6 Piegari E, Galderisi U, Berrino L, Bernardo GD, Cipollaro M, Esposito F, *et al*. *In vivo* effects of partial phosphorothioated AT1 receptor antisense oligonuleotides in spontaneously hypertensive and normotensive rats. Life Sci 2000; 66: 2091-9.

7 Goldspink PH, Russell B. Physiological role of phosphorylation of the cyclic AMP response element binding protein in rat cardiac nuclei. Cell Tissue Res 1996; 285: 379-85.

8 Ho SP, Bao YJ, Lesher T, Conklin D, Sharp D. Regulation if the angiotensin type-1 receptor by antisense oligonucleotides occurs through an RNase H-type mechanism. Mol Brain Res 1999; 65: 23-33.

9 Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951; 193: 265-75.

10 Ozono R, Matsumoto T, Shingu T, Oshima T, Teranishi Y, Kambe M, *et al*. Expression and localization of angiotensin subtype receptor proteins in the hypertensive rat heart. Am J Physiol 2000; 278: R781-R789.

11 Paradis P, Dali-Youcef N, Paradis FW, Thibault G, Nemer M. Overexpression of angiotensin II type I receptor in cardiomyocytes induces cardiac hypertrophy and remodeling. Proc Natl Acad Sci USA 2000; 97: 931-6.

12 Martina B, Dieterle T, Sigle JP, Surber C, Battegay E. Effects of telmisartan and losartan on left ventricular mass in mild-to-moderate hypertension. A randomized, double-blind

trial. Cardiology 2003; 99: 169-70.
13 Delyani JA, Rocha R, Cook CS, Tobert DS, Levin S, Roniker B, *et al*. Eplerenone: a selective aldosterone receptor antagonist (SARA). Cardiovasc Drug Rev 2001; 19: 185-200.
14 Kurreck J. Antisense technologies. Improvement through novel chemical modifications. Eur J Biochem 2003; 270: 1628-44.
15 Everett AD, Heller F, Fisher A. AT_1 receptor gene regulation in cardiac myocytes and fibroblasts. J Mol Cell Cardiol 1996; 28: 1727-36.
16 Zhang Y, Bloem LJ, Yu L, Estridge TB, Iversen PW, McDonald CE, *et al*. Protein kinase C betaII activation induces angiotensin converting enzyme expression in neonatal rat cardiomyocytes. Cardiovasc Res 2003; 57: 139-46.
17 Shih NL, Cheng TH, Loh SH, Cheng PY, Wang DL, Chen YS, *et al*. Reactive oxygen species modulate angiotensin II-induced beta-myosin heavy chain gene expression via Ras/Raf/extracellular signal-regulated kinase pathway in neonatal rat cardiomyocytes. Biochem Biophys Res Commun 2001; 283: 143-8.
18 Omura T, Yoshiyama M, Ishikura F, Kobayashi H, Takeuchi K, Beppu S, *et al*. Myocardial ischemia activates the JAK-STAT pathway through angiotensin II signaling *in vivo* myocardium of rats. J Mol Cell Cardiol 2001; 33: 307-16.
19 Murasawa S, Matsubara H, Mori Y, Masaki H, Masaki H, Tsutsumi Y, *et al*. Angiotensin II initiates tyrosine kinase pyk2-dependent signalings leading to activation of Rac1-mediated c-Jun NH_2-terminal kinase. J Biol Chem 2000; 275: 26856-63.
20 Sugden PH, Clerk A. "Stress-responsive" mitogen-activated protein kinases (c-Jun N-terminal kinases and p38 mitogen-activated protein kinases) in the myocardium. Circ Res 1998; 83: 345-52.
21 Eriksson M, Leppa S. Mitogen-activated protein kinases and activator protein 1 are required for proliferation and cardiomyocyte differentiation of P19 embryonal carcinoma cells. J Biol Chem 2002; 277: 15992-6001.
22 Yano M, Kim S, Izumi Y, Yamanaka S, Iwao H. Differential activation of cardiac c-Jun amino-terminal kinase and extracellular signal-regulater kinase in angiotensin II-mediated hypertension. Circ Res 1998; 83: 752-60.
23 Laser M, Willey CD, Jiang W, Cooper G 4th, Menick DR, Zile MR, *et al*. Integrin activation and focal complex formation in cardiac hypertrophy. J Biol Chem 2000; 275: 35624-30.
24 Reaves PY, Wang HW, Katovich MJ, Gelband CH, Raizada MK. Attenuation of hypertension by systemic delivery of retroviral vector containing type I angiotensin II receptor antisense cDNA. Methods 2000; 22: 211-8.
25 Azizi M, Chatellier G, Gnyene TT, Morieta-Geoffroy D, Menard J. Additive effects of combined angiotensin-converting enzyme inhibition and angiotensin II antagonism on blood pressure and renin release in sodium-depleted normotensives. Circulation 1995; 92: 825-34.
26 Tonyz RM. Molecular and cellular mechanisms regulating vascular function and structure: implications in the pathogenesis of hypertension. Curr J Cardiol 2000; 16: 1137-46.
27 Krieger JE. New contributions to clinical hypertension from molecular biology. Curr Opin Cardiol 1998; 13: 312-6.