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Angiotensin II and cAMP regulate AT_1-mRNA expression in rat cardiomyocytes by transcriptional mechanism

Xi Chen*, Zhaoqiang Cui, Fujian Zhang, Wenjing Chang, Lanying Chen, Lisheng Liu

Division of Biochemistry, Cardiovascular Institute and Fu Wai Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, #167, Bei Li Shi Lu, Xi Cheng Qu, Beijing, 100037, People's Republic of China

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Abstract

Mechanisms of angiotensin II and cAMP regulating the expression of angiotensin II type 1 (AT_1) receptor mRNA were studied in neonatal rat cardiomyocytes. Angiotensin II induced a transient decrease of AT_1-mRNA expression in time- and dose-dependent manner. Maximal decrease (49.2 ± 9.5% of control) occurred at 6 h of angiotensin II (10 nmol/l) treatment. AT_1 receptor antagonists 4-ethyl-2-*n*-propyl-1-[2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl)methyl]imidazole-5-carboxylic acid (DMP811) and losartan as well as 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H-7) reversed the down-regulation of AT_1-mRNA expression. 6 h of phorbol 12-myristate 13-acetate (PMA) stimulation caused a decrease of AT_1-mRNA level. Treatment by angiotensin II plus actinomycin D for 6 h produced the same effect as actinomycin D alone. These results suggest that angiotensin II down-regulates AT_1-mRNA level of rat cardiomyocytes by inhibiting the transcription of AT_1 gene, which is mediated by AT_1 receptor and related to the activation of protein kinase C. Stimulation by forskolin plus 3-isobutyl-1-methyl-xanthine (IBMX) decreased the expression of AT_1-mRNA to 68.1 ± 21.5% of control at 6 h treatment; while increased to 207.9 ± 27.1% of control at 48 h treatment. A series of 5′-upstream deletion mutants of AT_{1A} promoter were produced and then were recombined with pGL_3 basic vector utilizing luciferase as reporter gene. Among all the constructors, p(−201/+74)Luc was of the highest luciferase activity (5.9 times higher than control) after stimulation by forskolin for 48 h. Further deletion from −201 to −61 resulted in a large decrease of activity. These results indicate that cAMP induces a time-dependent bi-directional regulation of AT_1-mRNA expression. The cAMP responsible element (CRE) *cis*-element located in the region −201/−61 of rat AT_{1A} promoter is forskolin inducible, which may mediate the up-regulation of AT_1-mRNA expression induced by cAMP long-lasting stimulation.

Keywords: Angiotensin II; Angiotensin AT_1 receptor; cAMP; Cardiomyocyte

1. Introduction

Angiotensin II, an active end product of the renin–angiotensin system, has multiple physiological effects in the cardiovascular systems (Peach, 1977). It is involved in cardiac hypertrophy that accompanies hypertension and congestive heart failure by binding to specific receptors located on the plasma membrane. Angiotensin II receptors have been classified into angiotensin type 1 (AT_1) and type 2 (AT_2) by their pharmacological characteristic and binding affinity to non-peptide antagonists losartan and (*S*)-1-{[4-(dimethylamino)3-methylphenyl]methyl}5-(diphenylacetyl)4,5,6,7-tetrahydro-1*H*-imidazo[4,5-*c*]pyridine-6-carboxylic acid (PD123319), respectively (Chiu et al., 1989). In rat, two AT_1 receptor subtypes, AT_{1A} and AT_{1B} which exhibit highly homologous sequences and similar binding and functional characteristics (Iwai and Inagami, 1992) have been identified.

It has been found that change of AT_1 receptor density of the cardiomyocytes is involved in the hypertrophy of heart (Meggs et al., 1993; Kojima et al., 1994). Besides the movement of receptor from membrane surface to the interior of cells (internalization) and rapid phosphorylation of receptor by protein kinase, one of the mechanisms for the alteration of AT_1 receptor density may be abnormal AT_1-mRNA transcription of the cardiomyocytes. The increase and decrease of AT_1-mRNA level are related positively with the development and regression of cardiac hypertrophy in spontaneously hypertensive rats (SHR) (Suzuki et al., 1993). Our experiment in vivo (Cui et al., 1999) shows that small dosage of angiotensin II with no effect on blood pressure

* Corresponding author. Tel.: +86-10-68314466x8584; fax: +86-10-68313012.

E-mail address: chenxifw@yahoo.com.cn (X. Chen).

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could induce myocardial hypertrophy together with increase of AT_1-mRNA level of rat heart, indicating close association of AT_1-mRNA expression with cell growth in the myocardium. It is, therefore, important to understand the molecular mechanisms underlying AT_1-mRNA expression control.

Many factors could alter the AT_1-mRNA levels (Dell et al., 1996; Ardaillou et al., 1999; Baumer et al., 2001), however the possible signaling pathways involved in the regulation, especially in cardiomyocytes, is as yet unclear. And, cellular AT_1-mRNA level reflects a balance between transcription of AT_1 gene and degradation of AT_1-mRNA in the cell. The influence of stimulation on AT_1-mRNA stability and the regulation of AT_1-mRNA expression by transcriptional regulatory element remain to be defined. In the present study, the effects of angiotensin II and cAMP on AT_1-mRNA level and stability in neonatal rat cardiomyocytes, and in which the role of protein kinase C were investigated. A functional CRE *cis*-element in 5′-upstream of rat AT_{1A} receptor gene was identified.

2. Materials and methods

2.1. Animals

Neonatal Sprague–Dawley rats (1–3 days old) were supplied by the Animal Center of Chinese Academy of Medical Sciences. All animals used in this study were approved by the Animal Care Committee of Cardiovascular Institute and Fu Wai Hospital, and in accordance with the Chinese Academy of Medical Sciences Guide for Care and Use of Laboratory Animals.

2.2. Main chemicals

Angiotensin II was purchased from American Peptide (USA). Fetal bovine serum, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H-7), phorbol 12-myristate 13-acetate (PMA), forskolin, 3-isobutyl-1-methyl-xanthine (IBMX), luciferase and luciferin were purchased from Sigma. Dulbecco's modified Eagle medium (DMEM), dithiothreitol, Moloney murine leukemia virus (M-MLV) reverse transcriptase and RNase inhibitor (RNasin) were the products of Gibco. Taq DNA polymerase was from Boehringer Mannheim. Trypsin was purchased from Difco. 5-Bromodeoxyuridine (Brdu) was the product of the Institute of Biochemistry of Academy Sinica (Shanghai). 4-Ethyl-2-*n*-propyl-1-[2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl)methyl]imidazole-5-carboxylic acid (DMP811) was obtained from DuPont-Merck Pharmaceutical (USA). AT_1 receptor cDNA primers and losartan were generous gifts of Professor Hideo Kanaide (Research Institute of Angiocardiology, Graduate School of Medical Sciences, Kyushu University, Japan). pGEM-T vector and klenow large fragment were Promega products. The marker of 1 kb DNA ladder was Bio-Lab product. pGL_3 basic vector was a generous gift of Professor Bingren Huang (Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing). Primers for AT_1 receptor and for 3-glyceradehyde phosphate dehydrogenase (GAPDH) were synthesized by American Genemed Biotechnologies. All other chemicals are of analytical grade and were obtained mainly from P.R. of China.

2.3. Cell culture of cardiomyocytes and 293 cell line

Rat cardiomyocytes were cultured as described by Zhou and Chen (1995). Briefly, the hearts were taken from neonatal Sprague–Dawley rats (1–3 days old) after decollation, then were minced and digested by 0.08% trypsin. Cardiomyocytes and myocardial fibroblasts were isolated by differential velocity adhesion method and then cultured in DMEM containing 10% fetal bovine serum with 0.1 mmol/l Brdu for cardiomyocytes or without Brdu for cardiac fibroblasts. Rhythmic contractions of the cells were observed by inversion microscope at the second or third day. Immunoassay by using actin monoclonal antibody demonstrated cardiomyocyte purity was over 90% and less than 10% positive cells were found in cultured cardiac fibroblasts. Sub-confluent cells were starved in DMEM containing 0.4% fetal bovine serum for 48 h prior to the experiment.

Human embryo kidney cells (HEK293 cells) were seeded in 60-mm plate and maintained in DMEM supplemented with 10% fetal bovine serum at 37 °C in a humidified atmosphere of 5% CO_2 in air.

2.4. Preparation of RNA and the determination of AT_1-mRNA level

Total RNA of the cells was prepared as described by Chomczynski and Sacchi (1987). AT_1-mRNA level in the primary culture of cardiomyocytes was determined by reverse transcription polymerase chain reaction (RT-PCR) method. First strand of cDNA was synthesized using total RNA as template by reverse transcription reaction containing 1 nmol/l antisense primers, 5′-TCGATGCTGAGACACGTGAG-3′ (374–355) for AT_1 receptor and 5′-CAAAGTTGTCATGGATGACC-3′(529–510) for GAPDH, 20 U RNasin, 10 mmol/l dithiothreitol, 0.5 mmol/l dNTP, 200 U M-MLV reverse transcriptase, 1 × PCR buffer, 2 μg total RNA, in a total volume of 20 μl. For PCR, sense and antisense primers for AT_1 receptor were 5′-GCCCTTAACTCTTCTGCTGA-3′(4–23) and 5′-TCGATGCTGAGACACGTGAG-3′(374–355), respectively, according to the sequence report by Murphy et al. (1991), while those for GAPDH were 5′-CCATGGAGAAGGCTGGG-3′(335–351) and 5′-CAAAGTTGTCATGGATGACC-3′(529–510), respectively, according to the sequence reported by Tso et al. (1985). The expected sizes of the PCR products were 371 bp for AT_1 receptor and 195 bp for GAPDH. The PCR system contained 1 nmol/l sense and antisense primers each for AT_1

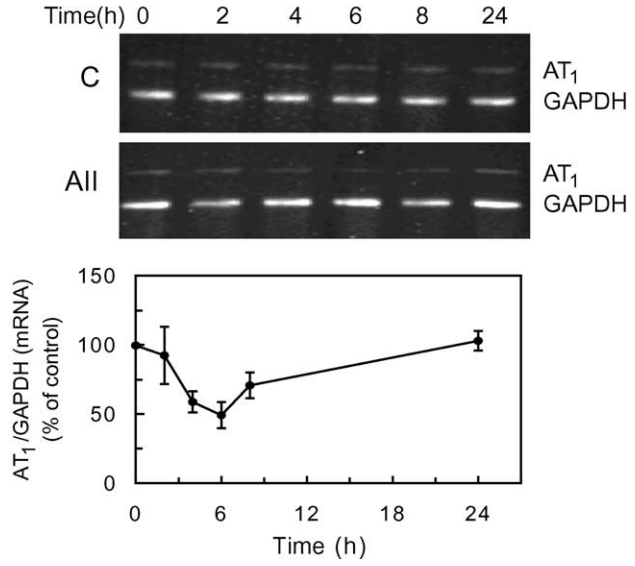

Fig. 1. Time course of effect of angiotensin II on the expression of AT_1-mRNA. Cells were incubated in DMEM containing 0.4% fetal bovine serum (C) and 10 nmol/l angiotensin II (AII) with each time period as plotted. Typical electrophoresis patterns are shown in the upper panel. In the lower panel, AT_1 receptor bands were quantified with GAPDH mRNA as internal standard using Gel Doc 1000 and imaging densitometer GS-700. Data are mean ± S.D. from five independent experiments.

receptor, 100 μmol/l dNTP, 0.5 U *Taq* DNA polymerase, 1 × PCR buffer, in a total volume of 25 μl, covered by 20 μl mineral oil. The DNA was denatured at 94 °C for 2 min first, before entering the PCR cycle. Thermal cycle profile used was: (1) denaturation for 30 s at 94 °C, (2) annealing for 40 s at 55 °C, (3) extension for 50 s at 72 °C. After 5 cycles, 1 nmol/l of sense and antisense GAPDH primers each were added into the PCR system and continued for 25 cycles as described in the above thermal cycle profile. The final step was followed by an extension for 6 min at 72 °C. A portion of the PCR products was electrophoresed on 2% agarose gel. AT_1 receptor bands were quantified with GAPDH mRNA as internal standard using Gel Doc 1000 and imaging densitometer GS-700 (Bio-Rad, USA).

2.5. The construction of recombined plasmids with 5′-flanking promoter of AT_{1A} gene

The 5′ -flanking promoter region of AT_{1A} gene corresponding to the sequences from −1222 to +74 was amplified by using PCR with the primers: 5′-TCAATGGGCTCAGGCGAGAG-3′(−1222/− 1203) and 5′-GATCGGGTGGAACAGGACTCAA3-′(+74/+53). The 1.3-kb PCR product was cloned into the pGEM-T vector to give $pTAT_{11}$ and $pTAT_{13}$ with opposite orientation. To create promoter/reporter chimeric plasmids, a series of restriction fragment deletions of the AT_{1A} receptor gene promoter region were subcloned into the pGL_3 basic vector which contained a firefly luciferase gene. To create the p(−1074/+74)Luc, $pTAT_{11}$ was digested with *Apa*I and blunted, then cut by *Sal*I. The 1.15-kb fragment (−1074/+74) was ligated into *Sma*I–*Xho*I digested pGL_3 basic vector. To subclone the 1.3-kb fragment (−1222/+74) into pGL_3 basic vector, a 250-bp fragment from $pTAT_{13}$ by using *Sac*I and *Eco*RI was ligated into a *Sac*I–*Eco*RI-cleaved p(−1074/+74)Luc. $pTAT_{11}$ was digested with *Xba*I and blunted, then cut by SalI. A recovered 640-bp fragment was ligated into the pGL_3 basic vector linearized with *Sma*I and *Xho*I, then p(−560/+74)Luc subclone was obtained. p(−783/+74)Luc subclone was generated by the ligation of 300-bp *Hin*dIII–*Xba*I fragment of $pTAT_{13}$ and a *Kpn*I–*Xba*I-digested p(−560/+74)Luc. To construct the p(−490/+74)Luc subclone, p(−1222/+74)Luc was first digested with *Sac*I and *Xho*I, and then flushed with the klenow large fragment. Finally, the larger fragment was recovered and recircularized. To create p(−201/+74)Luc chimeric plasmid, p(−560/+74)Luc linearized with *Kpn*I and *Pvu*II, then blunted and followed by recovering and recircularizing the larger fragment. The construction of p(−61/+74)Luc plasmid was performed in three steps. First, p(−1222/+74)Luc was digested with *Bgl*II and blunted, then digested with *Pst*I and the 4.8-kb fragment was recovered. Second, The 1.3-kb PCR product was digested with *Pst*I and the 140-bp fragment was recovered. Third, the 140-bp fragment and the 4.8-kb fragment were ligated. All these constructs were identified by restriction mapping analysis.

2.6. Cell transfections and luciferase assays

Cell transfections and luciferase assays were performed as described in "Short Protocols in Molecular Biology" (Ausubel et al., 1995). Briefly, optimum amount of plasmid DNA was mixed with 50 μl of 2.5 mol/l $CaCl_2$. 500 μl 2 × BES ((*N*,*N*′-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid)-buffered solution) (pH 6.95–6.98) was added and incubated for 20 min at room temperature. Calcium phosphate–DNA solution was dropped onto the plate with 293 cells while swirling the plate. The cells were incubated overnight in a 35 °C, 5% CO_2 incubator and washed with phosphate-buffered saline (PBS) (pH 7.2) before the addition of new complete medium, then were incubated for 72 h in a 37 °C, 5% CO_2 incubator. On the next day, cells were cultured without fetal

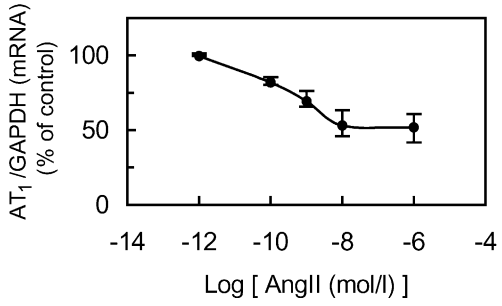

Fig. 2. Dose response of angiotensin II induced down-regulation of AT_1-mRNA expression in cardiomyocytes at 6 h in five independent experiments.

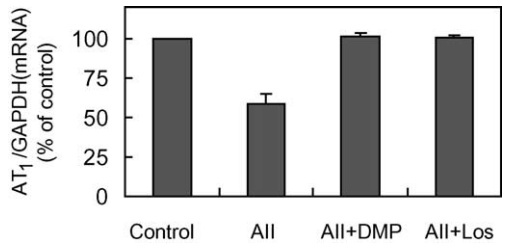

Fig. 3. Effects of AT_1 antagonists on angiotensin II induced down-regulation of the expression of AT_1-mRNA in rat cardiomyocytes. Cells were incubated for 6 h in DMEM containing 0.4% fetal bovine serum (Control); with 10 nmol/l angiotensin II (AII); 10 nmol/l angiotensin II plus 10 nmol/l DMP811 (AII + DMP); 10 nmol/l angiotensin II plus 1 μmol/l losartan (AII + Los), respectively. Data are mean ± S.D. from five independent experiments. **: $P < 0.01$ vs. control.

bovine serum and stimulated with 10 μmol/l forskolin for 48 h. Then the cells were washed three times with ice-cold PBS. Triton/glycylglycine lysis buffer (350 μl) was added to each plate. Cells were scraped with a scraper, transferred to a tube and centrifuged (5 min, 12,000 rpm, 4 °C). The supernatant (cell lysate) was transferred to a microcentrifuge tube and store on ice for assay. Luciferase activity of cells was measured by luminometer. cAMP inducibility is calculated as the ratio of luciferase activity of cells with forskolin over that without forskolin.

2.7. Statistics

Data were given as mean ± S.D. Statistical analysis was performed using Student's *t*-test. Significance was accepted at $P < 0.05$.

3. Results

3.1. Effect of angiotensin II on the expression of AT_1-mRNA in cardiomyocytes

Angiotensin II induced a transient down-regulation of AT_1-mRNA expression in a time- and dose-dependent manner. Maximum decrease (49.2 ± 9.5% of control) occurred at 6 h of angiotensin II (10 nmol/l) exposure, which was followed by a gradual return to control level (103.0 ± 7.0% of control) within 24 h (Fig. 1). The dose response curve showed that maximum decrease of AT_1-mRNA level by angiotensin II at 6 h of challenge was achieved at 10 nmol/l (Fig. 2).

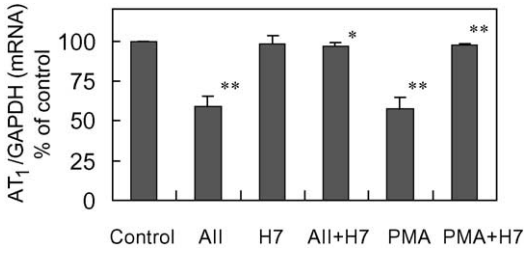

Fig. 4. Role of protein kinase C in angiotensin II induced down-regulation of the expression of AT_1-mRNA in rat cardiomyocytes in five independent experiments. Cells were incubated for 6 h in DMEM containing 0.4% fetal bovine serum (Control); with 10 nmol/l angiotensin II (AII); 30 μmol/l H-7 (H7); 10 nmol/l angiotensin II plus 30 μmol/l H-7 (AII + H7); 0.3 μmol/l PMA (PMA) and 0.3 μmol/l PMA plus 30 μmol/l H-7 (PMA + H7), respectively. *: $P < 0.05$ vs. control. **: $P < 0.01$ vs. control.

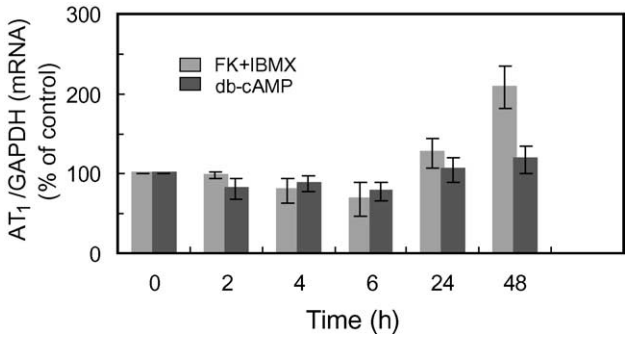

Fig. 5. Time course of effect of cAMP on the expression of AT_1-mRNA. The cells were incubated in DMEM containing 0.4% fetal bovine serum and 30 μmol/l forskolin plus 30 μmol/l IBMX or 0.5 mmol/l dibutyryl cAMP with each time period as plotted. Data are mean ± S.D. from three to five independent experiments.

3.2. Effects of AT_1 antagonists on angiotensin II-induced down-regulation of AT_1-mRNA expression

To test whether angiotensin II induced down-regulation of AT_1-mRNA is via AT_1 receptor, the cells were exposed to angiotensin II (10 nmol/l) for 6 h in the presence or absence of losartan (10 μmol/l) or DMP811 (10 nmol/l). Both losartan and DMP811 blocked the down-regulation of AT_1-mRNA induced by angiotensin II (Fig. 3).

3.3. Role of protein kinase C in angiotensin II-induced down-regulation of AT_1-mRNA expression

In order to investigate the possible role of protein kinase C in angiotensin II-induced down-regulation of AT_1-mRNA expression, H-7, a relatively specific protein kinase

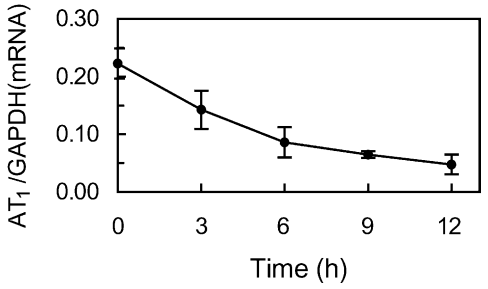

Fig. 6. Natural degradation of AT_1-mRNA of rat cardiomyocytes. Cells were incubated with actinomycin D for time periods indicated on the abscissa, prior to extraction of total RNA. Data are mean ± S.D. from four to five independent experiments.

Table 1
Effects of angiotensin II and cAMP on the stability of AT_1 mRNA

	AT_1/GAPDH (mRNA) (6 h)		AT_1/GAPDH (mRNA) (48 h)
Control	0.47 ± 0.12	Control	0.17 ± 0.01
AII	0.31 ± 0.02	FK + I	0.34 ± 0.04
Act D	0.18 ± 0.02	Act D	–[a]
AII + Act D	0.17 ± 0.04	FK + I + Act D	–[a]

Cells were incubated with 10 nmol/l angiotensin II (AII), 5 μg/ml actinomycin D (Act D), 10 nmol/l angiotensin II plus 5 μg/ml actinomycin D (AII + Act D), 30 μmol/l forskolin plus 30 μmol/l IBMX (FK + I) and 30 μmol/l forskolin plus 30 μmol/l IBMX plus 5 μg/ml actinomycin D (FK + I + Act D), respectively. Data are mean ± S.D. from three independent experiments.

[a] AT_1-mRNA level was not determined due to death of the cardiomyocytes.

C inhibitor, and PMA, a protein kinase C activator, were used. Although the treatment of cells with 10 nmol/l angiotensin II for 6 h resulted in a decrease of AT_1-mRNA expression (58.6 ± 6.6% of control), no reduction of AT_1-mRNA level (96.5 ± 2.6% of control) was found in the presence of H-7 (30 μmol/l). The incubation of 0.3 μmol/l PMA produced similar result as angiotensin II, and the fall was inhibited by H-7 (Fig. 4).

3.4. Effect of cAMP on the expression of AT_1-mRNA in cardiomyocytes

Forskolin, an activator of adenylate cyclase, and IBMX, an inhibitor of cAMP phosphodiesterase were used to increase cellular cAMP concentration. During the first 6 h, treatment of 30 μmol/l forskolin plus 30 μmol/l IBMX resulted in a decrease of AT_1-mRNA level and the nadir was 68.1 ± 21.5% of control, which was followed by a gradual increase with prolongation of incubation time. AT_1-mRNA level was elevated to 207.9 ± 27.1% of control at 48 h treatment. Membrane permeable cAMP analogue dibutyryl cAMP had similar effects as forskolin plus IBMX, i.e. short stimulation decreased AT_1-mRMA expression and long-term action increased AT_1-mRNA level in the cell (Fig. 5).

3.5. Effects of angiotensin II and cAMP on the stability of AT_1-mRNA

The expression of AT_1-mRNA determined in the above experiments reflects a balance between transcription of AT_1 gene and degradation of AT_1-mRNA in the cell. To assess whether stability of AT_1-mRNA is involved in the change of AT_1-mRNA expression induced by angiotensin II or cAMP,

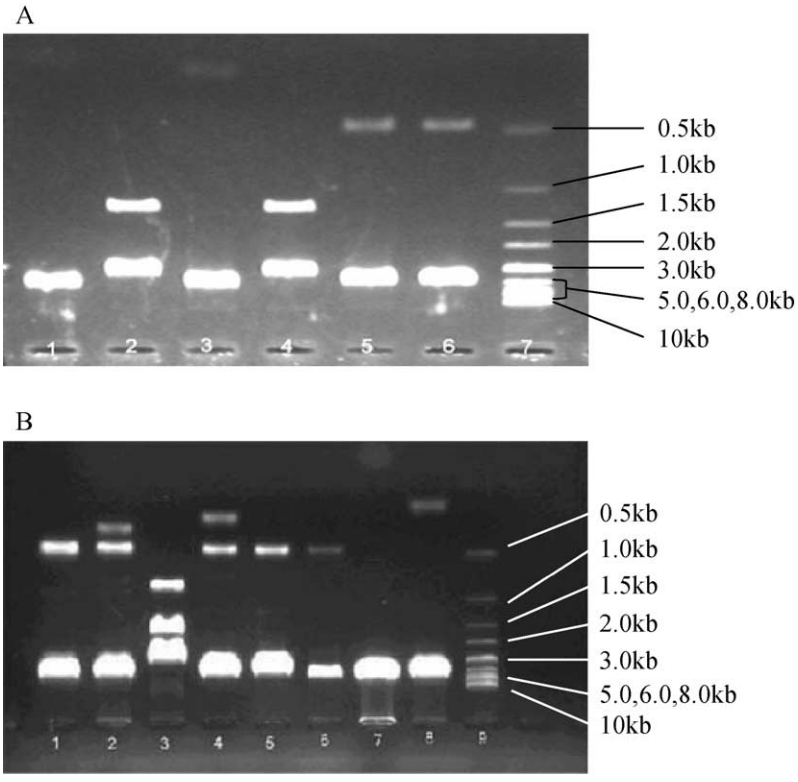

Fig. 7. Restriction analysis of TAT_{11} and TAT_{13} (A) and the mutant promoter-report chimeras (B). A1: $pTAT_{11}$/*Pst*I (130 bp + 4.2 kb), A2: $pTAT_{13}$/*Pst*I (1.2 + 3.1 kb), A3: $pTAT_{11}$/*Apa*I (150 bp + 4.15 kb), A4: $pTAT_{13}$/*Apa*I (1.2 + 3.1 kb), A5: $pTAT_{11}$/*Hin*dIII (450 bp + 3.85 kb), A6: $pTAT_{13}$/*Hin*dIII (450 bp + 3.85 kb), A7: 1 kb DNA Ladder, B1: p(−1222/ + 74)Luc/*Kpn*I + *Hin*dIII (400 bp + 450 bp + 460 bp + 4.8 kb), B2: p(−1074/ + 74)Luc/*Kpn*I + *Hin*dIII (300 bp + 400 bp + 450 bp + 4.8 kb), B3: p(−783/ + 74)Luc/*Xba*I + *Hin*dIII (650 bp + 2.4 kb + 3.3 kb), B4: p(−560/ + 74)Luc/*Kpn*I + *Hin*dIII (230 bp + 400 bp + 4.8 kb), B5: p(−490/ + 74)Luc/*Kpn*I + *Hin*dIII (400 bp + 4.8 kb), B6: p(−331/ + 74)Luc/*Kpn*I + *Hin*dIII (400 bp + 4.8 kb), B7: p(−201/ + 74)Luc/*Bgl*II (5.1 kb), B8: p(−61/ + 74)Luc/*Kpn*I + *Hin*dIII (140 bp + 4.8 kb), B9: 1 kb DNA Ladder.

actinomycin D was used to block the synthesis of new RNA. The apparent half-life of AT_1-mRNA in rat cardiomyocytes was about 4.5 h (Fig. 6). The treatment of cardiomyocytes for 6 h with angiotensin II plus actinomycin D decreased the expression of AT_1-mRNA to 40% of control which was the same as treating cells for 6 h with actinomycin D only (Table 1). Although the 48-h treatment of forskolin plus IBMX without actinomycin D increased AT_1-mRNA level to 204.1% of control, cardiomyocytes died after inhibition of the synthesis of new RNA by actinomycin D in the presence or absence of forskolin plus IBMX (Table 1).

3.6. Role of CRE in 5′-flanking region of AT_{1A} in the regulation of AT_1-mRNA expression

It has been revealed that regulation of cAMP may be related to cAMP response elements (CRE) in the 5′-flanking region of genes. According to Takeuchi et al. (1993), at least, two putative CRE sequences, −1172 to −1165 (TGTCGTCA) and −150 to −143 (CGCCGTCA) are found in rat AT_{1A} promoter region. In order to identify the presence of functional CRE in rat AT_{1A} receptor gene and to assess the role of CRE in the up-regulation of AT_1-mRNA expression by forskolin, the 1.3-kb fragment corresponding to base −1222 to +74 of AT_{1A} gene was amplified by using PCR technique. The PCR product was inserted into a T-vector. Two kinds of constructs with opposite orientation, termed TAT_{11} and TAT_{13}, were got and identified by restriction enzyme digestion. The sizes of the bands were as expected (Fig. 7A). By using restriction enzymes and gene recombination, a series of 5′ deletion mutants of 1222-bp AT_{1A} promoter with or without putative CRE were created and fused with pGL_3 basic vector containing luciferase as reporter gene (Fig. 8). The chimeras were identified by restriction analysis (Fig. 7B).

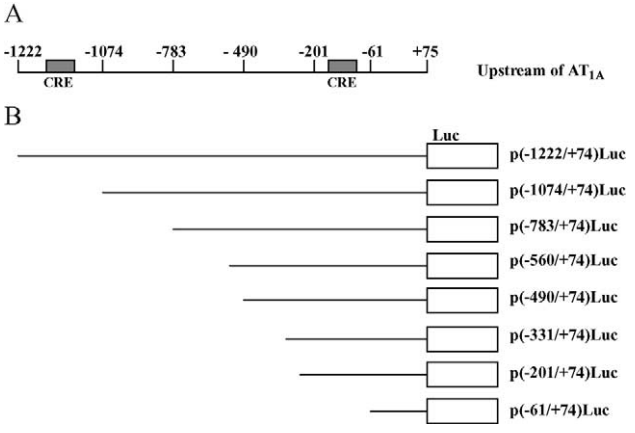

Fig. 8. Construction of 5′ deletion mutants of AT_{1A} promoter fused with pGL_3 basic vector. The 1.3-kb fragment corresponding to base −1222 to +75 of AT_{1A} gene in which two putative CRE sequences (▭) are found (A). A series of 5′ deletion mutants of AT_{1A} promoter were created and fused with pGL_3 basic vector that utilizes luciferase as reporter gene (B).

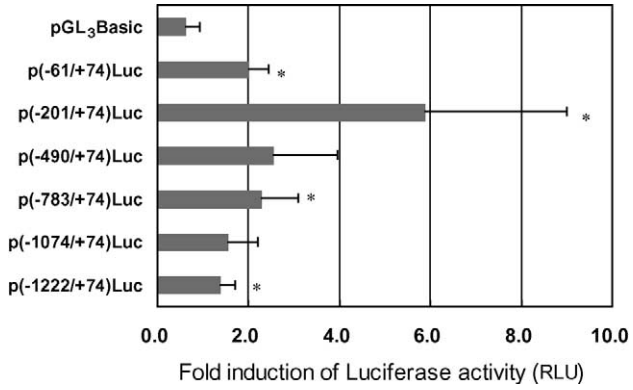

Fig. 9. Deletion analysis of the transcriptional activities of AT_{1A} promoter after forskolin stimulation. Cells were transfected with each construct as plotted and then stimulated with 10 μmol/l forskolin for 48 h. cAMP inducibility is calculated as the ratio of luciferase activity of cells in the presence over the absence of forskolin. Data are mean ± S.D. from four independent experiments. *: $P<0.05$ vs. pGL_3Basic.

A series of the above 5′ deletion mutants of 1222-bp AT_{1A} promoter were used in transient transfection assays. Promoters with 5′ termini ranging from −1222 to −61 upstream of the AT_{1A} receptor displayed in easily detectable amounts of luciferase in 293 cells after stimulation with 10 μmol/l forskolin for 48 h (Fig. 9). Construct p(−201/+74)Luc showed the highest level of inducibility (5.9 times higher than that without forskolin treatment) among the six constructs tested. Deletion of −201/−61 bp promoter region containing a CRE element reduced the action of forskolin, from 5.9 times to 2.0 times.

4. Discussion

Angiotensin II is of multi-physiological function including the involvement in cardiac hypertrophy. It exerts most of its function through AT_1 receptor. So the regulation of AT_1 receptor becomes a key point of studying the mechanism of heart remodeling induced by angiotensin II. In the present study we mainly investigated the homologous and heterologous transcriptional regulation of AT_1-mRNA by angiotensin II and cAMP, respectively.

As the concentration of AT_1 receptor in cardiomyocytes is very low, which limited, to some degree, the study of myocardial AT_1-mRNA, we used semi-quantitative RT-PCR to study the AT_1-mRNA level in cultured cardiomyocytes. Preliminary experiment showed the reliability of our experimental results as follows: RNA dilution curve and PCR cycle dynamic curve for AT_1-mRNA and GAPDH-mRNA were all linear. At 0.25–2.0 μg total RNA, both relative indexes of RNA dilution curves for AT_1-mRNA and GAPDH-mRNA were 0.9998. The relative indexes of PCR dynamic curves for AT_1-cDNA at 28–32 cycles and for GAPDH-cDNA at 23–28 cycles were 0.9998 and 0.9970, respectively.

The results that angiotensin II down-regulates AT_1-mRNA expression of cultured cardiomyocytes in a time- and dose-dependent manner are similar to those observed in glomerular mesangial cells (Makita et al., 1992) and smooth muscle cells (Chen et al., 1994). Treating cells with angiotensin II plus actinomycin D did not promote the degradation of AT_1-mRNA compared to actinomycin D alone, suggesting that down-regulation of AT_1-mRNA induced by angiotensin II is caused by the inhibition of transcription of AT_1-mRNA, but not by the acceleration of mRNA degradation.

The fact that 100 nmol/l angiotensin II induces maximum effect in smooth muscle cells (Chen et al., 1994) in comparison with 10 nmol/l angiotensin II in cardiomyocytes implies that the regulation of AT_1-mRNA expression in cardiomyocytes may be more sensitive to angiotensin II than in smooth muscle cells. The blockage of angiotensin II-induced down-regulation of AT_1-mRNA by AT_1 receptor antagonist losartan or DMP811 indicates that the down-regulation is mediated by AT_1 receptor in cardiomyocytes and it is consistent to the report in human adrenal gland cells (Naville et al., 1993).

In the present study, a down-regulation of AT_1-mRNA expression of myocardial cells appeared after the stimulation of angiotensin II for 6 h. While in the previous in vivo experiment (Cui et al., 1999), an increase of AT_1-mRNA level of rat heart was found after the infusion of angiotensin II for 7 days. Opposite results between in vitro and in vivo experiments may come from different time course of angiotensin II acting on cells. Meanwhile, in the presence of complex regulation system in vivo, angiotensin II may also activate other factors which increase the expression of AT_1-mRNA. Such as angiotensin II stimulates the release of aldosterone which could increase angiotensin II receptor number on membrane and prevent angiotensin II-induced down-regulation of angiotensin II surface receptors and angiotensin II desensitization of inositol phosphate formation (Ullian et al., 1992). It is possible that the amplitude of the increase of AT_1 receptor by aldosterone is greater than the decrease by angiotensin II. As a whole, AT_1 receptor level might increase.

Protein kinase C is a serine/threonine kinase with various functions. Activated protein kinase C is responsible for phosphorylation of many important and growth-relating proteins (Huang, 1990). It is reported that protein kinase C is implied in AT_1 signal transduction, as well as the desensitization of AT_1 receptor by receptor phosphorylation (Balmforth et al., 1997; Garcia-Caballero et al., 2001). The relationship between AT_1-mRNA expression and protein kinase C-dependent phosphorylation has also been studied widely. The activation of protein kinase C induces down-regulation of AT_1 receptor mRNA in rat vascular smooth muscle cells (Chen et al., 1994) and bovine adrenal cells (Ouali et al., 1997), while an up-regulation of AT_1 receptor mRNA in bovine vascular smooth muscle cells (Holzmeister et al., 1997) and rat neuronal cells (Lu et al., 1994). But Lassegue et al. (1995) reported that angiotensin II-induced AT_1 mRNA expression did not appear to be mediated by protein kinase C. These discrepancies may be related to the cell type and the inhibitor or activator of protein kinase C used, as well as the methods used to assess the involvement of protein kinase C. However, the role of protein kinase C in AT_1-mRNA expression in cardiomyocytes remains unknown.

In the present study, angiotensin II-induced decrease of AT_1-mRNA level of cardiomyocytes is blocked by H-7, suggesting that angiotensin II may regulate AT_1-mRNA transcription via the activation of protein kinase C. PMA, a protein kinase C activator, treatment for 6 h induces the same effect on AT_1-mRNA expression as that by angiotensin II. It is verified further that activation of protein kinase C decreases the expression of AT_1-mRNA. To our knowledge, it is the first report that activation of protein kinase C may mediate angiotensin II induced down-regulation of AT_1-mRNA expression in cardiomyocytes.

In addition to angiotensin II induced homologous regulation, we also demonstrated that cAMP heterologously regulates AT_1-mRNA expression in a time-dependent bi-directional manner, a transient down-regulation followed by a long-lasting up-regulation. It is consistent to that in rat aortic smooth muscle cells (Chen et al., 1994). Makita et al. (1992) also observed that short time (6 h) cAMP stimulation results in a decrease of AT_1-mRNA level of rat glomerular mesangial cells. Everett et al. (1996) found that long-term (24 h) stimulation of forskolin induces an increase of AT_1-mRNA level in rat cardiac cells. Cells die after inhibition of new RNA synthesis by actinomycin D treatment for 48 h in the presence or absence of forskolin plus IBMX, while 48-h treatment of forskolin plus IBMX without actinomycin D increases the AT_1-mRNA level to twice of control (Table 1). It demonstrates that the increase of AT_1-mRNA induced by long-term cAMP stimulation might be caused by increased transcription of AT_1-mRNA but not by prolonged stability of AT_1-mRNA. Thus, the effect of cAMP on the regulation of AT_1-mRNA at transcriptional level should be considered.

Thomas et al. (1992) showed that cAMP induces an up-regulation of β-3 adrenal receptor gene through CRE in 5′-upstream of the gene. Although putative CREs are found in the 5′-upstream of rat AT_{1A} receptor (Takeuchi et al., 1993) and human AT_1 receptor (Guo et al., 1994), it remains to be clarified that CREs in promoter of the gene do mediate the increase of AT_1 receptor gene transcription in response to the elevation of intracellular cAMP.

In order to identify the functional *cis*-regulatory element, a series of 5′-upstream deletion mutants of AT_{1A} promoter were produced and then were recombined with pGL_3 basic vector. The determination of luciferase activity showed that p(−201/+74)Luc is of the highest luciferase activity after a long-lasting stimulation of forskolin, further deletion from −201 to −61 results in a remarkable decrease, suggesting existence of an important *cis*-regulatory element in the −61/−201 region, where a putative CRE (−150/−143)

just lies in. As for the remarkable decrease of inducible luciferase activity of p(−490/+74)Luc might be attributed to the presence of negative *cis*-element on bases −456/−442. The above results indicate that region −201/−61 containing a CRE is functional for cAMP inducibility. Whereas another proposed CRE in −1172/−1165 is not responsible for cAMP inducibility. We believe that it is the first evidence of a functional CRE in −201/−61 region of AT_{1A} promoter mediating cAMP-induced increase of AT_1-mRNA expression.

In summary, we investigated the mechanism of AT_1-mRNA expression in rat cardiomyocytes regulated by angiotensin II and cAMP. Angiotensin II down-regulates AT_1-mRNA level by inhibiting the transcription of AT_1 receptor gene, which is mediated by AT_1 receptor and the activation of protein kinase C. The increase of cellular concentration of cAMP induces a time-dependent bi-directional regulation of AT_1-mRNA expression. A functional CRE *cis*-element locates in the −201/−61 region of rat AT_{1A} promoter is forskolin-inducible, which may mediate the up-regulation of AT_1-mRNA expression induced by cAMP long-lasting stimulation.

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