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Inhibitory effect of antisense oligodeoxynucleotide to p44/p42 MAPK on angiotensin II-induced hypertrophic response in cultured neonatal rat cardiac myocyte[1]

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

ABSTRACT

AIM: To explore the inhibitory effect of antisense oligonucleotide (ODN) to mitogen activated protein kinase (MAPK) on cardiomyocyte hypertrophy induced by angiotensin II (Ang II). **METHODS:** A 17-mer phosphorothioate-protected antisense ODN directed against the initiation of translation sites of the p42 and p44 MAPK isoforms by liposomal transfection was applied to inhibit the translation of p44/p42 MAPK mRNA. The sense and random ODNs to p44/p42MAPK were used as sequence controls. Neonatal cardiac myocytes were exposed to Ang II (10 nmol/L) for 5 min and then harvested in lysis buffer for the measurement of the activity and the phosphorylated protein content of p44/p42MAPK that were tested by P-81 phosphocellulose filter paper method and Western blotting, respectively. The rate of protein synthesis by [^{3}H]leucine incorporation and the diameter of cell were measured after exposure to Ang II for 24 h and 72 h, respectively. **RESULTS:** In cardiac myocyte Ang II increased p44/p42MAPK activity and phosphorylated protein content by 140 % and 699 %, and also increased [^{3}H]leucine incorporation and cell diameter by 40 % and 27 %. c-*fos* and c-*myc* mRNAs were induced significantly after exposure to Ang II. Antisense ODN to p44/p42MAPK (0.2 μmol/L) reduced Ang II-induced MAPK activity by 30 %, and phophorylated MAPK protein expression by 59 % in cardiac myocyte, and inhibited c-*fos* and c-*myc* mRNA expression induced by Ang II by 44 % and 43 %, respectively. The diameter and the rate of protein synthesis of cardiac myocyte induced by Ang II were decreased by 16 % and 22 % after pretreatment with antisense ODN to p44/p42MAPK. **CONCLUSION:** Antisense ODN to p44/p42 MAPK inhibited the increase of rate of protein synthesis, and the augmentation of cell diameter and expression of c-*fos* and c-*myc* mRNA induced by Ang II in cultured cardiac myocytes. p44/p42 MAPK played a critical role in the hypertrophic response induced by Ang II in cultured neonatal rat cardiac myocytes.

INTRODUCTION

Angiotensin II (Ang II) has been known to cause hypertrophic response in cardiac myocytes. This process includes an increase in protein synthesis and cell size (hypertrophy), alteration of muscle phenotype, and activation of expression of proto-oncogenes and fetal genes, but without an increase in DNA synthesis and cell number[1-4]. Ang II stimulates protein synthesis and hypertrophy of neonatal cardiac myocytes in culture[5]. But the signal transduction pathway and precise mechanism in Ang II-induced cardiac hypertrophy have not been well known. MAPK is a family of protein Ser/Thr

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Received 2002-10-15 Accepted 2003-03-04

kinases. p44/p42 MAPK, one of three major isoforms of MAPK, have been shown to play a critical role in the cellular processes such as proliferation, differentiation and development, and are the few signal proteins that could transmit extracellular signal into nucleus through their translocation from cytoplasm into nucleus[6]. The activation of p44/p42 MAPK requires phosphorylation on both a threonine and a tyrosine residue by an upstream kinase known as MEK. p44/p42MAPK are activated when cardiac myocytes were exposed to a variety of neurohormones (endothelin-1, Ang II, PE, *etc*) and mechanical loading[7-9], and may integrate signals from multiple receptor systems and a common distal signaling pathway leading to cardiac hypertrophy[4]. Several studies have suggested that Ang II may be an essential factor in mediating cardiac hypertrophy *in vivo* and *in vitro*, and rapidly increases activity of MAPK and their downstream kinase, RSK[1,2]. Glennon and his colleagues revealed that depletion of p44/p42 MAPK with antisense oligodeoxynucleotide (ODN) reduced the activity of p44/p42 MAPK and hypertrophic responses induced by PE in cultured neonatal cardiac myocytes[7]. To study the role of p44/p42 MAPK in hypertrophic responses induced by Ang II in cardiac myocytes, we also used an antisense approach to deplete p42 and p44 MAPK in cultured neonatal cardiac myocytes. Antisense ODN that used in the present study was designed to direct against the initial translation sites of the p42 and p44 MAPK isoform mRNA. Antisense ODN targeting a special mRNA could abolish the translation and biological effects of the corresponding protein, and its high specificity is different from ordinary kinase inhibitors for their different mechanisms. In the present study, we will discuss the precise mechanism of pathway of hypertrophic responses induced by Ang II in cultured neonatal rat cardiac myocytes.

MATERIALS AND METHODS

Chemicals [Sar^1]angiotensin II, lipofectin, myelin basic protein, protein kinase inhibitor (TTT AAP IAS GAT GAA AAI HA), leupeptin, DDT, M199, anti-phosphorylated p44/p42 MAPK monoclonal antibody were purchased from Sigma Co. P-81 filter, PVDF membrane and glass-fiber filter were purchased from Whatman Co. [^{32}P]ATP, [^{3}H]leucine and enhanced chemiluminescence reagents were purchased from NEN^{TM} Life Science Products.

Cell culture Neonatal cardiac myocytes were prepared from hearts of 1-3-d-old Wistar rats (Supplied by the Animal Center of Xiang Ya School of Medicine) as described previously[5]. Cells were plated at 1.25×10^5/cm^2 in M199 containing 10 % fetal bovine serum for 48 h. BrdU (0.1 mmol/L) was added to the medium to prevent proliferation of nonmyocytes. All experiments were performed in the serum-free condition 24 h after exposure to the serum-free medium.

Synthesis of oligodeoxynucleotides The antisense ODN was a 17-mer (5'-GCCGCCGCCGC-CGCCAT-3') phosphorothioate-protected ODN to p44/p42 MAPK directed against the initiation of translation site of rat p42 and p44 MAPK mRNA.The 17-mer sense (5'-ATGGCGGCGGCGGCGGC-3') and random (5'-CGCGCGCTC GCGCACCC-3') were used as sequence controls. ODN were all synthesized and purified at DNA Core of University of Cincinnati (USA).

Liposomal transfection ODN 0.8 μmol/L in nonantibiotic and serum free DMEM/M199 (4:1) was vortex-mixed with equal volume of DMEM/M199 containing lipofectin 80 mg/L for 30 s, and then incubated at room temperature for 30 min. Having been cultured in serum free medium for 24 h, cardiac myocytes were washed gently 3 times with DMEM/M199, and the ODN/lipofectin mixture was added. An equal volume of DMEM/M199 was then immediately added. The cells were incubated at 37 ºC for 10 h with gentle agitation of plates every 2 h. Medium was then replaced with the same volume of lipofectin-free DMEM/M199 containing the same concentration of ODN. The cells were incubated for another 14 h before stimulation.

Measurements of the rate of protein synthesis (incorporation of [^{3}H]leucine) and cell diameter The cells were stimulated with Ang II (10 nmol/L) for 24 h in the presence of [^{3}H]leucine, washed with PBS and precipitated with 10 % trichloroacetic acid (TCA) for 1 h at 4 ºC. The precipitate was solubilized in NaOH 0.15 mol/L. The cell lysate was vacuum-filtrated onto the glass-fiber filter. Having been washed with 10 % TCA and 100 % ethanol, the filter was dried. The radioactivity of incorporated [^{3}H]leucine was measured with a scintillation counter. The diameter of cardiac myocytes in 10 random fields in each well was measured with an ocular micrometer.

P-81 filter paper kinase assay The cell lysate was prepared as described previously[10]. After the cell lysae was washed with PBS, the lysis buffer (mmol/L: NaCl 50, NaF 50, sodium pyrophosphate 50, egtazic acid 5, edetic acid 5, Na_3VO_4 2, phenylmethylsulfonyl

fluoride 0.5, and HEPES 10 at pH7.4, along with 0.1 % Triton X-100 and leupeptin 10 mg/L)was added and then collected. After sonication, the cell lysates were centrifuged at 18 000×*g* for 15 min at 4 ºC. Protein concentration was measured by the Bradford method[11]. Cell lysate 50 μL was mixed with 10 L of 6×assay buffer [mmol/L: HEPES 120, $MgCl_2$ 12, DTT 12, Na_3VO_4 3, protein kinase inhibitor [TTT AAP IAS GAT GAA AAI HA) 12, and BSA 3.6 g/L, MBP 1g/L, γ-[^{32}P]ATP (74 kBq, 50 μmol/L], and then incubated at 30 ºC for 15 min. The reaction mixture was spotted onto P-81paper, then the paper was immediately immersed into ice-cold H_3PO_4 (75 mmol/L), and washed for 10 min for 6 times with H_3PO_4 solution and the radioactivity of γ-[^{32}P]ATP was counted. The blank reaction was a mixture containing all of the reagent but without cell lysate.

Western blot The cells were lysated in SDS sample buffer [Tris/HCl 0.33 mol/L, SDS 10 % (wt/vol), glycerol 40 % (vol/vol), and DTT 50 mmol/L containing bromophenol blue 0.4 % (wt/vol)]. After sonication and denatured by boiling for 5 min, 10 μg protein was resolved on 10 % SDS-polyacrylamide gel and then transferred to PVDF membrane. Nonspecific binding sites on the membrane were blocked with 5 % BSA in PBS containing 0.1 % Tween-20 (PBST) for 1 h. The membrane was incubated with primary monoclonal antibody of phosphorylated p42/p44 MAPK diluted 1:1000 overnight at 4 ºC, and then with the second antibody (horseradish peroxidase conjugated) diluted 1:2000. ECL method was used for detection. Quantification of phosphorylated protein of p42/p44 MAPK was determined by laser scanning densitometry (LKB).

Northern blot Total RNA was extracted using TRIzol total RNA isolation kit and resolved on 1 % agarose, and then transferred to nylon membrane. The membrane was prehybridized at 42 ºC with prehybridization solution with salmon sperm DNA and then hybridized with [^{32}P]ATP radiolabled oligo-probes of c-*fos*, c-*myc* and 18S rRNA (a 50-mer oligonucleotide probe complementary to nucleotides 270-319 of rat c-*fos*[12], a 60-mer oligonucleotide probe complementary to nucleotides 4201-4260 of rat c-*myc*[13], and a 24-mer oligonucleotide probe sequenced as 5'-ACGGTATCTGATC-GTCTTCGAACC-3' of 18S rRNA were used. All of above oligonucleotides were synthesized and purified at DNA Core of University of Cincinnati). 18S rRNA was used as internal control. The relative amount of a specific mRNA was quantified by laser scanning densitometry of the corresponding autoradiograms of the x-ray films.

Statistical analysis Data are expressed as mean±SD and assessed by ANOVA and *t*-test.

RESULTS

Ang II induced the hypertrophic responses and increased p44/p42 MAPK activity in cultured cardiac myocyte Exposure of cardiac myocytes to Ang II 10 nmol/L increased the incorporation of [^{3}H]leucine and cell diameter by 40 % and 27 % and increased the activity and the protein content of phophorylated p44/p42MAPK by 140 % and 699 %, respectively (Tab 1, Fig 1). There is no measurable expression of c-*fos* and c-*myc* mRNA in nonactivated cardiac myocyte, but the expression increased significantly after exposure to Ang II 10 nmol/L (Tab 2, Fig 2, 3).

Effect of antisense ODN to p44/p42 MAPK on Ang II-induced phosphorylated p44/p42 MAPK protein content and MAPK activity in cultured cardiac myocyte Pretreatment of cardiac myocytes with antisense ODN 0.2 μmol/L significantly reduced Ang II 10 nmol/L-stimulated phosphorylation activity and phosphorylated protein of p44/p42MAPK by 30 % and 59 %, respectively, compared with Ang II plus lipofectin group. Lipofectin alone had no effect on protein content and activity of p44/p42MAPK (data not shown). The decrease in phosphorylated protein of p44/p42MAPK was dose-dependent of antisense ODN to p44/p42MAPK (Tab 1, Fig 1). Both sense and random ODNs had no effect.

Effect of antisense ODN to p44/p42 MAPK on Ang II-induced hypertrophic responses in cultured cardiac myocyte Pretreatment of cardiac myocytes with antisense ODN 0.2 μmol/L significantly attenuated the increase in incorporation of [^{3}H]leucine and cell diameter in response to Ang II 10 nmol/L by 22 % and 16 %, respectively, compared with Ang II plus lipofectin group (Tab 1). Both sense and random ODNs had no effect.

Effect of antisense ODN to p44/p42 MAPK on Ang II-induced c-*fos* and c-*myc* mRNA expression in cultured cardiac myocyte Pretreatment of cardiac myocytes with antisense ODN 0.2 μmol/L to p44/p42 MAPK significantly reduced Ang II 10 nmol/L-stimulated c-*fos* and c-*myc* mRNA expression by 44 % and 43 % compared with Ang II plus lipofectin group (Tab 2, Fig 2,3). Both sense and random ODNs had no effect.

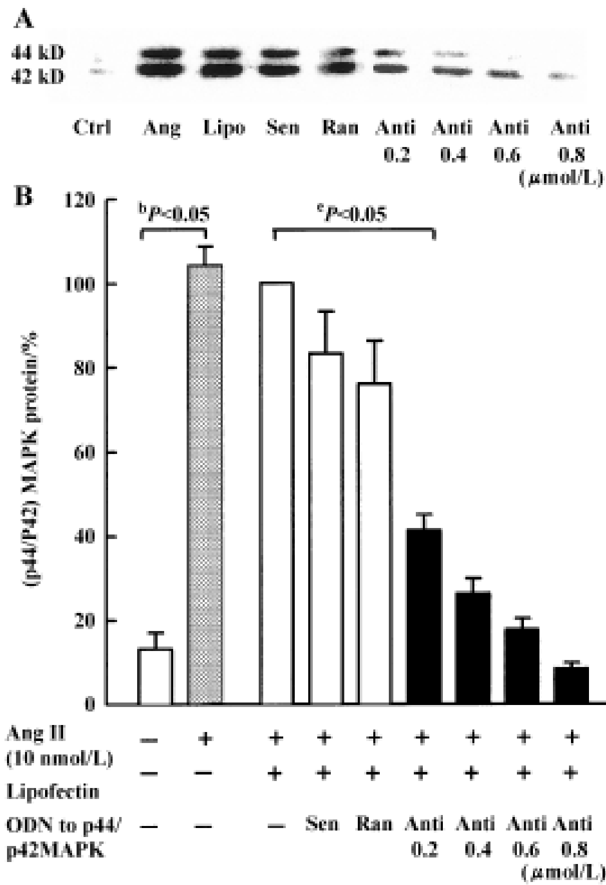

Fig 1. **The inhibitory effect of antisense ODN to p44/p42 MAPK on phosphorylated p44/p42 MAPK protein content induced by Ang II (10 nmol/L, 5 min) in cultured rat cardiac neonatal myocyte. A) Representative Western blotting of phosphorylated p44/p42MAPK. B) Quantification of immunoblots of phosphorylated p44/p42 MAPK. *n*=3. Mean ±SD. ^{b}*P*<0.05 *vs* control. ^{e}*P*<0.05 *vs* Lipofectin+Ang II. Ctrl: Control; Ang: angiotensin II; Lipo: Lipofectin+Ang II; Sen: Sense+Ang II; Ran: Random+Ang II; Anti: Antisense+Ang II.**

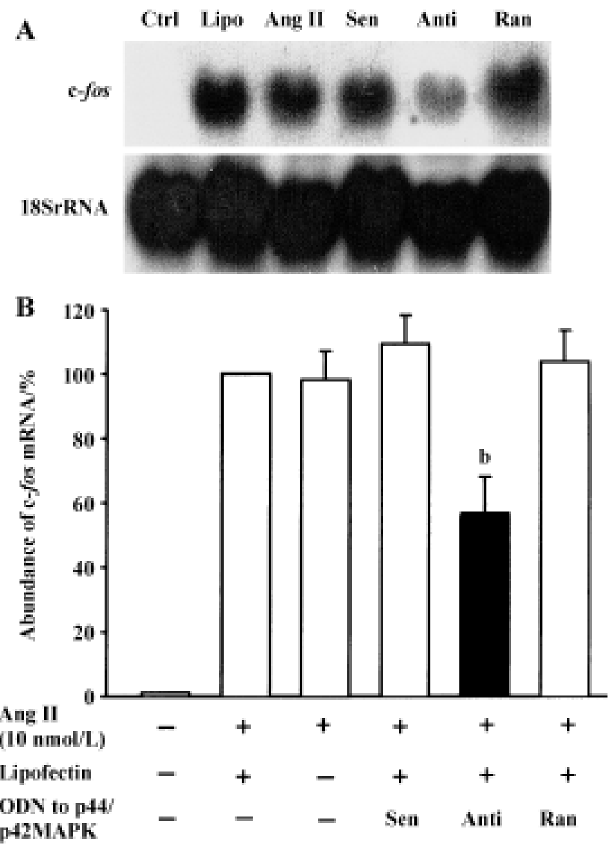

Fig 2. Inhibitory effect of antisense ODN to p44/p42 MAPK on the expression of c-*fos* mRNA induced by Ang II (10 nmol/L, 45 min) in cultured neonatal rat cardiac myocyte. A) Representative Northern blotting of c-*fos* mRNA. B) Quantification of Northern blotting of c-*fos* mRNA. *n*=3. Mean ±SD. ^{b}*P*<0.05 *vs* Lipo. Ctrl: Control; Ang II: angiotensin II; Lipo: Lipofectin+Ang II; Sen: Sense+Ang II; Ran: Random+Ang II; Anti: Antisense+Ang II.

Tab 1. Inhibitory effect of antisense ODN to p44/p42MAPK on the activation of MAPK activity and phosphorylated p44/p42 MAPK protein content and the hypertrophy response of the cultured neonatal rat cardiomyocytes induced by Ang II (10 nmol/L, 24 h and 72 h for the measurements of incorporation of [^{3}H]-leucine and cell diameter, respectively). *n*=4 or 3 in triplicate. Mean±SD. ^{b}*P*<0.05 *vs* control. ^{e}*P*<0.05 *vs* Sense group.

	Control	Ang II	Ang II+ Lipofectin			
			Lipofectin	Sense	Antisense	Random
[^{3}H]Leu incorporation (cpm/well)	1886±129	2641±216^b	2381±199	2266±64	1853±55^e	2518±98
Cell diameter/μm	19.5±0.7	24.90±0.26^b	24.9±0.9	23.8±1.4	20.8±0.9^e	24.8±0.7
MAPK activity/nmol·min^{-1}·g^{-1} (protein)	8.4±1.5	20.2±1.6^b	18.1±1.3	18.8±1.3	12.7±1.4^e	17.1±1
Phosphorylated p44/p42 MAPK protein (% *vs* Ang II+Lipo)	13±4	104±5^b	100	83±10	41±4^e	76±10

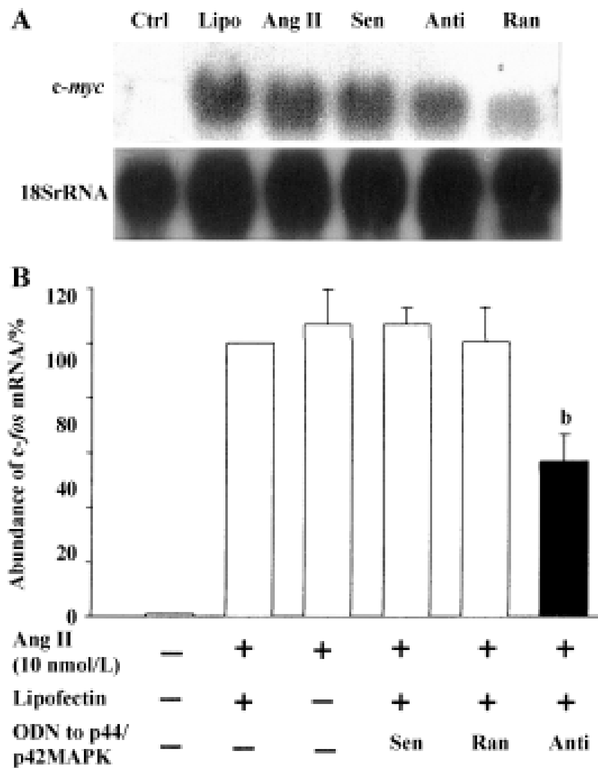

Fig 3. Inhibitory effect of antisense ODN to p44/p42 MAPK on the expression of c-*myc* mRNA induced by Ang II (10 nmol/L, 45 min) in cultured neonatal rat cardiac myocyte. A) Representative Northern blotting of c-*myc* mRNA. B) Quantification of Northern blotting of c-*myc* mRNA. *n*=3. Mean±SD. $^bP<0.05$ *vs* Lipo. Ctrl: Control; Ang II: angiotensin II; Lipo: Lipofectin+Ang II; Sen: Sense+Ang II; Ran: Random+Ang II; Anti: Antisense+Ang II.

DISCUSSION

At the molecular level, cardiac hypertrophy is characterized by an increase in contractile protein content, the induction of the immediate-early genes (c-*myc*, c-*fos*, c-*jun*, *jun*-B, *Egr*-1), and the re-expression of cardiac embryonic genes (ANF, SR Ca^{2+}-ATPase, β-MHC, skeletal α-actin). These changes are dependent on the activation of transcription of corresponding genes. Activation of one or more of intracellular kinase signaling pathways was involved in these genes transcription changes. MAPK cascade is one of the important intracellular pathways that participates in cardiac hypertrophic responses induced by humoral stimuli. p44/p42 MAPK, one of the three main isoforms of MAPK, are thought to play a central role in cardiac hypertrophy[4]. Phosphorylated p44/p42 MAPK (phosphorylated at specific sites of threosine and tyrosine residues) is the activated form. P81 filter paper assay (to detect the ability of kinase to activate the downstream) associated with content of phosphorylated protein were used together to reflect the activation of p44/p42MAPK in this paper. In the present study, we demonstrated that antisense ODN to p44/p42MAPK could significantly decrease the activation and phosphorylated protein content of p44/p42 MAPK in a dose-dependent manner. The antisense ODN to p44/p42MAPK we used could successfully inhibit the translation of p44/p42MAPK, so it could decrease the content of phosphorylated protein thereby inhibiting the activation of p44/p42MAPK in a cardiac myocyte[7] and cardiac fibroblast[10]. Sadoshima and Izumo[3] demonstrated that Ang II caused a rapid induction of immediate-early genes (c-*fos*, c-*myc*, c-*jun*, *jun*-B and *Egr*-1) and AT1 antagonist fully blocked this response. Our present study showed that depletion of p44/p42MAPK by antisense ODN attenuated the expression of c-*fos* and c-*myc* mRNA induced by Ang II in cardiac myocyte. These suggested that p44/p42 MAPK were involved in the pathway of activation of expression of c-*fos* and c-*myc* induced by Ang II in cardiac myocytes. Babu *et al*[14] also demonstrated that pretreatment of cardiac myocyte with PD98059, a

Tab 2. Inhibitory effect of antisense ODN to p44/p42MAPK on the expression of c-*fos* mRNA and c-*myc* mRNA of the cultured neonatal rat cardiomyocytes induced by Ang II (10 nmol/L, 45 min). *n*=3. Mean±SD. $^bP<0.05$ *vs* control. $^eP<0.05$ *vs* Sense group.

	Control	Ang II	Ang II+Lipofectin			
			Lipofectin	Sense	Antisense	Random
Abundance of c-*fos* mRNA (% *vs* Lipofectin + AngII)	0	98±9[b]	100	109±9	56±12[e]	104±10
Abundance of *c-myc* mRNA (% *vs* Lipofectin + AngII)	0	107±13[b]	100	107±6	57±10[e]	100±13

specific inhibitor of MEK, abolished the PE-induced c-*fos* promotor activity. Izumo[2] and our previous work[5] independently showed that Ang II could increase the protein synthesis and cell diameter in cultured neonatal rat cardiac myocyte. The present study demonstrated that pretreament of cardiac myocyte with antisense ODN to p44/p42MAPK could significantly down-regulate [^{3}H]leucine incorporation rate, augment of cell diameter induced by Ang II. These indicated that p44/p42MAPK were involved in the pathway of Ang II-induced hypertrophic response. Glennon *et al*[7] also showed that using antisense ODN could attenuate the increase in cardiac myocyte area and sarcomerogenesis, and inhibit the induction of ANF mRNA in response to PE. Antisense ODN has been well used as a pharmacological tool to inhibit the effect of corresponding protein. Antisense therapy studies were carried out as the treatment for HIV, hypertension, cardiovascular disease, leukemia[15,16]. In conclusion, the present study showed that p44/p42 MAPK played a critical role in Ang II-induced hypertrophic responses in cultured neonatal rat cardiac myocytes.

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