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Inhibitory effect of MAP kinase antisense oligonucleotide on angiotensin Ⅱ-induced c-*myc* gene expression and proliferation of rat cardiac fibroblast

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KEY WORDS Ca^{2+}-calmodulin dependent protein kinases; angiotensin Ⅱ; antisense oligonucleotides; proto-oncogenes; *myc* genes; fibroblasts; cell culture; cell division

ABSTRACT

AIM: To investigate the inhibitory effect of down-regulating mitogen activated protein kinase (MAPK) on c-*myc* gene expression and further on cardiac fibroblast proliferation. **METHODS**: Cultured neonatal rat cardiac fibroblasts was pretreated with a phosphorothioate-protected 17-mer antisense MAPK oligodeoxynucleotide (ODN) directed against the initiation of translation sites of the p42 and p44 MAPK isoforms by liposomal transfection. A 17-mer sense and mismatch sequence MAPK ODN were used as controls. After liposomal transfecting, cells were exposed to angiotensin Ⅱ (Ang Ⅱ) 10 nmol·L^{-1} for 5 min and then harvested in lysis buffer. MAPK activity was measured by Western blot and P-81 phosphocellulose filter paper method by using [γ-^{32}P]ATP and myelin basic protein as substrates. c-*myc* mRNA expression stimulated by Ang Ⅱ for 30 min was measured by Northern blot. DNA synthesis and collagen protein synthesis induced by Ang Ⅱ for 24 h were measured by [^{3}H]thymidine incorporation and [^{3}H]Proline incorporation, respectively. **RESULTS**: antisense ODN 0.2 μmol·L^{-1} reduced Ang Ⅱ-induced MAPK activities by 72 %, MAPK protein expression by 80 %, and suppressed c-*myc* mRNA expression by 97 %, respectively. [^{3}H]thymidine incorporation and [^{3}H]proline incorporation in Ang Ⅱ-induced cardiac fibroblast were inhibited by 59 % and 58 %, respectively. **CONCLUSION**: A 17-mer MAPK antisense oligonucleotide directed againsts the initiation of translation sites of MAPK could specifically inhibit Ang Ⅱ-stimulated cultured neonatal rat cardiac fibroblast proliferation through down-regulating MAPK activity and further depleting c-*myc* mRNA expression.

INTRODUCTION

Cardiac interstitium fibrosis and hypertrophy of cardiac myocytes were the two important pathologic reasons for pressure overload cardiac hypertrophy. However cardiac fibroblasts over-proliferation as well as secretion of large amounts of collagen protein were the mainly pathologic event which could result in cardiac fibrosis, cardiac remodification and further heart failure[1], in which angiotensin Ⅱ (Ang Ⅱ) has been considered as an important inducement for overhyperplasia of cardiac fibroblasts and hypertrophy of cardiac myocytes through autocrine or paracrine. Although the effect of Ang Ⅱ on cardiac fibrosis has been widely paid attention to, the mechanism is little known. Ang Ⅱ not only rapidly increases introcellular calcium and activates protein kinase C but also stimulates many of the same signal transduction events as growth factor does, including protein-tyrosine phosphorylation[2], stimulation of c-*fos*, c-*myc*[3] and mitogen-activated protein kinase (MAPK)[4,5]. MAPK are the most important component of transferring message in cellular growth[6]. MAPK are a family of serine/theronine kinase, which has two isoforms: p42 and p44 activated as an early response to a variety of stimuli involved in cellular growth, transformation, and differentiation. It could be activated by growth stimuli in quiescent cells requires phosphorylation of a dual specific protein kinase, MEK, which is itself regulated by MEK kinase and/or Raf kinase, and thereby to activate its

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substrates-nuclear transcription factors and upregulate the expression of those factors to perform their effects on biologicical regulation on cellular growth[7]. The evidences have shown that activation of MAPK occurred in cardiac fibroblast induced by Ang Ⅱ[4] and cardiac myocytes induced by phenylephrine[8] and hypoxia/reoxygenation[9], but little is reported about the effect of MAPK on proto-oncogene and collagen protein synthesis in cardiac fibroblasts.

To elucidate whether Ang Ⅱ-mediated MAPK activity promotes cardiac fibroblast proliferation by stimulation of c-*myc* gene expression, an antisense strategies was used in the present study. Antisense strategies was considered as a high scientific biological technology. Recently, a most clinical data have shown that obvious curative effects have being obtained for many serious diseases due to cellular overhyperplasia[10]. Recent evidences indicated that antisense ODN targeting nuclear transcription factor gene (c-*myb*, c-*fos*, c-*jun*, *etc*) or cell cycle gene (cycline) had significantly inhibitory effects on VSMCs proliferation resulted from pathobiology of vascular injury and repair[11]. However the using of antisense ODN in cardiac fibrosis was rarely reported. Antisense oligonucleotide targeting a special mRNA could abolish its translation and deplete biological effects of the corresponding protein[12]. We have also investigated the inhibitory effect of MAPK antisense ODN on cultured vascular smooth muscle cell (VSMc) proliferation[13]. But it has kept unknown if MAPK antisense ODN could inhibit proto-oncogene or nuclear transcription factors such as c-*myc*, c-*fos*, c-*jun*, *etc*, thereby to affect cardiac fibroblast proliferation and the collagen protein synthesis through depleting MAPK activity. On the basis of those findings, we hypothesized that inhibition of MAPK activity by its special antisense oligonucleotide might suppress cardiac fibroblast proliferation through inhibiting c-*myc* mRNA expression. The purpose of this study therefore is to examine the inhibitory effect of the translation of MAPK mRNA on c-*myc* mRNA and further on cardiac fibroblast proliferation and collagen protein synthesis.

MATERIALS AND METHODS

Chemicals [Sar^1] angiotensin Ⅱ, anti-phospho-MAP kinase, lipofectin, myelin basic protein, protein kinase inhibitor (TTT AAP IAS GAT GAA AAI HA), leupeptin, sephadex G-25 were purchased from Sigma Co. P-81 filter paper and glass-fiber filter were purchased from Whatman Co. Western blot chemiluminescence reagent plus, [γ-^{32}P]ATP were purchased from NEN™ Life Science Products. RNAgents Total RNA Isolation system kit, T_4 polynucleotide kinase kit were purchased from Promega.

Synthesis of oligonucleotides Phosphorothioate-protected oligonucleotides were used to inhibit proliferation of Ang Ⅱ-stimulated cultured neonatal rat cardiac fibroblast. The sequences of the phosphorothioate-protected oligodeoxynucleotides were 17-mer rat antisense MAPK (5′-GCC GCC GCC GCC GCC AT-3′) directed against the initiation of translation site of rat p42 and p44 MAPK mRNA. This ODN has been used successfully to downregulate both isoforms of MAPK in 3T3 cells[14] and rat cardiac myocytes[8]. 17-mer rat sense sequence MAPK (5′-AT GGC GGC GGC GGC GGC-3′) and 17-mer mismatch sequence (5′-CGC GCG CTC GCG CAC CC-3′) were used as controls. 60-mer rat c-*myc* probe sequence (5′-TGA TAG AAA TTC TCT TCC TCG TCG CAG ATG AAA TAG GGC TGC ACC GAG TCG TAG TCG AGG-3′) was used for Northern blot. 18 S rRNA probe sequence (5′-ACG GTA TCT GAT CGT CTT CGA ACC-3′) was used for inner marker. ODN were all synthesized and purified at the University of Cincinnati DNA Core.

Cell culture[4] Cardiac fibroblasts were isolated from heart ventricles of 1 – 3 d neonatal rats (Supplied by the Animal Center of Hunan Medical University, grade Ⅱ, Certificate 20-009) and maintained in M199 supplemented with 10 % fetal calf serum. The fibroblastic nature of cells were determined by immunohistochemistry staining with anti-human factor Ⅷ for endothelial cells (the positive rate 3 %), anti-desmin for muscle cells (the positive rate 2 %) and anti-vimentin for fibroblasts (the positive rate 95 %). Cells used in experiments were passages 2.

Liposomal transfection[8] ODN 0.8 μmol·L^{-1} in antibiotic- and serum-free DMEM/M199 (4:1) were vortex-mixed for 30 s, then mixed with equal volume of DMEM/M199 containing lipofectin 80 mg·L^{-1} and incubated at room temperature for 20 min. Cardiac fibroblasts (grown to 80 % confluence) were washed gently 3 times in serum and antibiotic free DMEM. ODN/lipofectin mix 200 μL was added for each well of 12-well plates, or 75 μL for each well of

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24-well plates, with equal volume of serum and antibiotic free DMEM/M199. Cardiac fibroblasts were incubated at 37 ℃ in 95 % O_2 - 5 % CO_2 for 10 h, agitating the plates gently every 2 h. Medium was then replaced with the same volume of liposome-free DMEM/M199 containing the same concentration of ODN. Cells were incubated for another 14 h, before adding Ang Ⅱ.

Measurement of DNA synthesis[5] Cell proliferation in terms of DNA synthesis was determined by measuring [^{3}H] thymidine incorporation. Cardiac fibroblasts were plated in 24-well plates at 0.75×10^5 cells·cm^{-2}. After incubation in serum-free medium for 24 h, the cells were transfected with antisense, sense and mismatch ODN for 10 h in DMEM/M199 and contained another 14 h in the same concentration of ODN without lipofectin. Cells stimulated with Ang Ⅱ for 18 h, labeled with [^{3}H] thymidine 74 kBq/well for 6 h, were washed with cold PBS(-), trypsinized, resuspended in 10 % trichloroacetic acid (TCA), and vortexed vigorously to lyze the cells. The cell lysate was vacuum-filtered through a glass-fiber filter. After washing with cold 10 % TCA followed by 95 % ethanol, the filter was dried. The radioactivity of incorporated [^{3}H] thymidine was measured in a liquid scintillation counter (Beckman). Experiments were performed 5 times in duplicate.

Measurement of collagen protein synthesis[3] The treatment of cells like that of DNA synthesis assayed, but the cells labeled with [^{3}H] proline 185 kBq/well began at same time with Ang Ⅱ stimulated and lasted for 24 h. In addition, after being trypsinized, cells must be stated in 4 ℃ 10 % TCA for 60 min to precipitate protein before being harvested onto the glass-fiber filter. The radioactivity of incorporated [^{3}H] proline was measured in a liquid scintillation counter.

Preparation of cell lysates[15] After treatment with ODN, cells were washed with PBS(-), and 0.1 mL of lysis buffer containing in mmol·L^{-1}: 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 pH 7.4, along with 0.1 % Triton X-100 and leupeptin 10 mg·L^{-1} was added. Cell lysates freezing on ice, scraping, and sonicating, were centrifuged at 18 000 × g for 15 min (4 ℃), protein concentration was estimated by the Bradford method[16].

Western blot[8] SDS sample buffer [tris-HCl 0.33 mol·L^{-1}, SDS 10 % (wt/vol), glycerol 40 % (vol/vol), and dithiothreitol 20 % (vol/vol) containing bromophenol blue 0.4 % (wt/vol)] of 1/4 volume were added to cell lysates. The extracted protein 10 μg was subjected to SDS-PAGE in a 10 % SDS gel, and the protein were then transferred to PVDF membrane, which was then blocked for 1 h with 5 % BSA in PBST (Na_2HPO_4 80 mmol·L^{-1}, NaH_2PO_4 20 mmol·L^{-1}, NaCl 100 mmol·L^{-1} containing 0.05 % Tween-20). The blots were incubated for 1 h at room temperature with the primary monoclonal antibodies of p44 and p42 MAPK at a 1:1 000 dilution, followed by incubation for 1 h with secondary antibody (horseradish peroxidase conjugated) at a 1:10 000 dilution. Immunoreactive bands of p42 and p44 MAPK were visualized by using enhanced chemiluminescence reagents. Quantification of p42 and p44 MAPK activity was made by laser scanning densitometry (Pharmacia LKB) of autoradiographs.

P-81 filter paper kinase assay[17] The cell lysates 50 μL was mixed with 10 μL of 6 × assay buffer (mmol·L^{-1}) [HEPES 120, $MgCl_2$ 60, $MnCl_2$ 12, DTT 12, Na_3VO_4 3, PH 7.2, protein kinase inhibitor (TTT AAP IAS GAT GAA AAI HA) 12, and BSA 3.6 g·L^{-1}, MBP 1 g·L^{-1}, [γ-^{32}P] ATP 74 kBq 50 μmol·L^{-1}] which is six times of the final concentration, then incubated at 30 ℃ for 15 min. The reactions, terminated by spotting 40 μL of the reaction mixture onto p-81 papers, which were immediately immersed into ice-cold H_3PO_4 75 mmol·L^{-1} solution and washed 10 min for 6 times in H_3PO_4 solution and counted. The reaction blank was a mixture containing all of the reagents but without cell lysate.

Northern blot[18] Cardiac fibroblasts were seeded on the 100-mm dishes and treated with ODN as described above before being stimulated by Ang Ⅱ for 30 min. Total RNA was extracted by using RNAgents Total RNA Isolation System kit, and 20 μg per line of total RNA was loaded on a 1 % agarose gel containing formaldehyde for gel electrophoresis. Blots were transferred to nylon membrane (Hybond^{TM}-N) and baked at 80 ℃ for 1.5 h. The transferred membrane was prehybridized at 42 ℃ with prehybridization solution supplemented with sonicated salmon sperm DNA 100 μg·ml^{-1} for 6 h. Rat c-*myc* and 18 S rRNA oligo-probes were radiolabled with [γ-^{32}P] ATP incorporation catalyzed by T_4 polynucleotide kinase to a

specific activity of 0.11 GBq · μg^{-1}, purified on sephadex G-25 spin columns, and then added to the hybridization solution for 18 h. The blots were then washed in 2 × standard saline citrate (SSC)/0.1 % sodium dodecyl sulfate (SDS) solution at room temperature (RT) and then changed with 1 × SSC/0.1 % SDS solution at RT, visualized by autoradiography, and quantified by laser scanning densitometry.

Statistical analysis Values were expressed as $\bar{x} \pm s$ and assessed by ANOVA and t-test.

RESULTS

Effect of oligonucleotides on cardiac fibroblasts DNA, collagen protein synthesis and cell number Pretreatment of cultured cardiac fibroblast with antisense ODN 0.2 μmol · L^{-1} significantly reduced Ang Ⅱ-induced [^{3}H] thymidine incorporation, [^{3}H] proline incorporation and cell number by 59 %, 58 %, and 38 %, respectively, but neither sense nor random ODN did (Tab 1).

Tab 1. Effect of MAPK ODN on [^{3}H] thymidine and [^{3}H] proline incorporation in rat cardiac fibroblast induced by Ang Ⅱ. $\bar{x} \pm s$. $n = 5$ independent experiments in cultured rat cardiac fibroblasts. $^aP > 0.05$, $^bP < 0.05$ *vs* Ang Ⅱ + lipofectin group. $^cP < 0.05$ *vs* control.

Treatment	Incorporation (Bq/well) [^{3}H]TdR	[^{3}H] Proline	Cell $\times 10^{-3}$
Control	350 ± 48	40 ± 9	284 ± 33
Ang Ⅱ	809 ± 42^c	103 ± 23^c	389 ± 28^c
Ang Ⅱ + lipofectin	744 ± 60	101 ± 18	373 ± 29
Ang Ⅱ + antisense	298 ± 52^b	42 ± 14^b	231 ± 27^b
Ang Ⅱ + sense	698 ± 53^a	88 ± 14^a	368 ± 17^a
Ang Ⅱ + mismatch	741 ± 71^a	96 ± 21^a	362 ± 26^a

Western blot analysis The monoclonal antibodies were directed against MAPK protein identified the 42- and 44-kDa bands in extracts of rat cardiac fibroblasts. Antisense ODN 0.2 μmol · L^{-1} reduced MAPK activity by 80 % after liposomal transfection. Cardiac fibroblasts exposed to lipofectin in the absence of ODN had slight inhibitory effect on phospho-MAPK protein compared with Ang Ⅱ-stimulated cardiac fibroblasts alone. Sense and mismatch sequence ODN had no inhibitory effect compared with Ang Ⅱ + lipofectin (Fig 1, Tab 2).

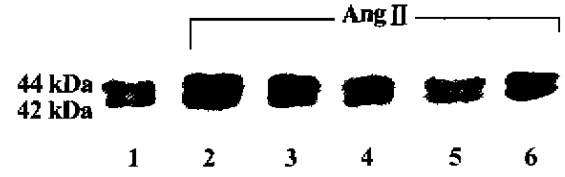

Fig 1. Effect of MAP kinase antisense ODN on the expression of cultured rat cardiac fibroblast phospho-MAPK protein by Western blot. 1) control. 2) Ang Ⅱ-stimulated. 3) Ang Ⅱ + sense. 4) Ang Ⅱ + lipofectin. 5) Ang Ⅱ + antisense. 6) Ang Ⅱ + mismatch.

Tab 2. Effect of antisense ODN on MAPK activity and MAPK Western blot in cardiac fibroblast induced by Ang Ⅱ. $\bar{x} \pm s$. $n = 5$ independent experiments in cultured rat cardiac fibroblasts. $^aP > 0.05$, $^bP < 0.05$ *vs* Ang Ⅱ + lipofectin group. $^cP < 0.05$ *vs* control.

Treatment	[γ-^{32}P]ATP incorporation/ nmol · min^{-1} · g^{-1} · $protein^{-1}$	Peak AU/cm^2
Control	14 ± 3	53 ± 5
Ang Ⅱ	65 ± 12^c	308 ± 16^c
Ang Ⅱ + lipofectin	60 ± 10	230 ± 25
Ang Ⅱ + antisense	17 ± 6^b	45 ± 9^b
Ang Ⅱ + sense	56 ± 8^a	193 ± 39^a
Ang Ⅱ + mismatch	64 ± 11^a	234 ± 37^a

P-81 filter paper kinase assays Pretreatment with antisense ODN 0.2 μmol · L^{-1} significantly reduced Ang Ⅱ-stimulated phosphorylation activity of MAPK by 72 %. Neither sense nor mismatch ODN had significantly effect on the MAPK activity (Tab 2).

Northern blot analysis The oligo probes which directed against c-*myc* mRNA sequence 4201-4260 identified the c-*myc* mRNA band in total RNA extracts of cardiac fibroblasts. 18 S rRNA was used as inner marker. Antisense MAPK 0.2 μmol · L^{-1} reduced c-*myc* mRNA expression by 97 % after liposomal transfection. Lipofectin and sense ODN had slight inhibitory effect on c-*myc* mRNA expression. But not for mismatch. (Fig 2, Tab 3).

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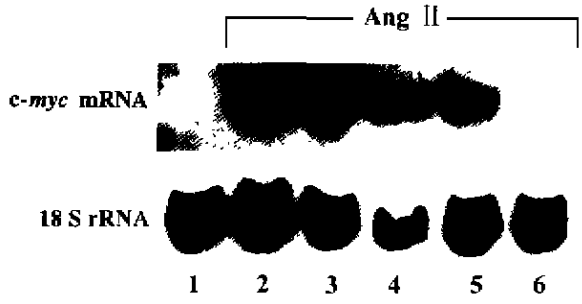

Fig 2. Effect of MAP kinase antisense ODN on the expression of cultured rat cardiac fibroblast c-*myc* mRNA by Northern blot. 1) control. 2) Ang Ⅱ + lipofectin. 3) Ang Ⅱ·stimulated. 4) Ang Ⅱ + mismatch. 5) Ang Ⅱ + sense. 6) Ang Ⅱ + antisense.

Tab 3. Effect of MAPK antisense ODN on c-*myc* mRNA expression in cardiac fibroblast induced by Ang Ⅱ. $\bar{x} \pm s$, $n = 3$ independent experiments of Northern blot in cultured rat cardiac fibroblasts. $^aP > 0.05$, $^bP < 0.05$ *vs* Ang Ⅱ + lipofectin group. $^cP < 0.05$ *vs* control.

Treatment	c-*myc* mRNA AU/cm^2	18 S rRNA AU/cm^2	c-*myc*/18 S
Control	0	576 ± 31	0
Ang Ⅱ	878 ± 50	521 ± 76	1.66 ± 0.26^c
Ang Ⅱ + lipofectin	773 ± 22	679 ± 21	1.14 ± 0.13
Ang Ⅱ + antisense	14 ± 5	466 ± 18	0.03 ± 0.01^b
Ang Ⅱ + sense	311 ± 46	520 ± 37	0.60 ± 0.05^b
Ang Ⅱ + mismatch	362 ± 23	309 ± 39	1.09 ± 0.21^a

DISCUSSION

It is well known that cardiac fibroblasts play an important role in producing and maitaining the extracellular matrix (ECM) of the heart. However, many activated factors such as vascular activated peptides, growth factors could stimulate cardiac fibroblasts proliferation and produce ECM protein[19]. The present study showed that DNA synthesis and cell number in Ang Ⅱ-induced cardiac fibroblast were increased significantly. This indicated that Ang Ⅱ was a stronger mitogen to cardiac fibroblast[20]. Meanwhile Ang Ⅱ is also an inducement of collagen protein synthesis. All the effects were inhibited evidently by MAPK antisense ODN. Neither mismatch nor sense ODN had the effects. The result showed that the inhibitory effect of MAPK antisense ODN on cardiac fibroblast proliferation is sequence specially. To investigate if the inhibitory effects of antisense ODN is resulted from down-regulating MAPK activity specifically, MAPK activity was measured by [γ-^{32}P] ATP incorporation combining with Western blot simultaneously. From the result to see that Ang Ⅱ-induced MAPK activity and Western blot were all increased markedly. When transfected with antisense ODN, Ang Ⅱ-induced MAPK activity and Western blot in cardiac fibroblast were suppressed significantly, but not for sense and mismatch ODN. This indicated that the inhibitory effect of MAPK antisense on cardiac fibroblast proliferation was due to down-regulating MAPK activity specifically. MAPK could integrate messages transferred from several systems of response such as receptor of tyrosine kinase, receptor of coupling with G protein, and play an important role in the end common pathway or confluence of transduction of several growth messengers[21]. Antisense ODN just down-regulated MAPK protein expression thereby suppressing MAPK activity. The inhibition of MAPK activity led to blocking of Ang Ⅱ-induced growth signals into nucleus, and cardiac fibroblast proliferation was prevented. MAPK was activated within very short time and arrived peak at 5 min[4], but the cell proliferation was demanded a long-time course. There must exist a middle biologic signal magnification. Whether proto-oncogene or immediate early gene serves as an important role in the course, c-*myc* gene was selected as downstream signal event in the present study. It is not difficult to find that the expression of c-*myc* gene occurred within 30 min stimulated by Ang Ⅱ in quiescent cultured rat cardiac fibroblasts. Down-regulation of MAPK activity by antisense ODN inhibited c-*myc* gene expression significantly. Mismatch ODN had not evidently inhibitory effect. Sense ODN had slight inhibitory effect on MAPK expression and c-*myc* mRNA expression. This may be a very complicated "unantisense effect", and liposomal itself has a certain inhibitory effect on cell proliferation in addition to its enhancing cells to take up ODN and protects them from cleaving of nucleases[22]. The results indicated that the expression of c-*myc* gene was required for the activation of MAPK. Therefore the activation of MAPK just triggerred off c-*myc* gene

expression which involved in Ang Ⅱ-induced rat cardiac fibroblast proliferation. There were evidences showing that c-*myc* antisense ODN could inhibit VSMc proliferation [23], and the data from our laboratory suggested that c-*myc* antisense ODN 12.8 $\mu mol \cdot L^{-1}$ decreased Ang Ⅱ-induced DNA synthesis in cardiac fibroblast by 55 % (unpublished). Therefore c-*myc* gene involved directly in cardiac fibroblast proliferation. As stated above, the inhibitory effect of MAPK antisense ODN on Ang Ⅱ-induced cardiac fibroblast proliferation and collagen protein synthesis was through interfering translation of MAPK at the level of mRNA to down regulate MAPK activity thereby prevent c-*myc* gene expression. All these implied that down-regulating MAPK activity could prevent transduction of upstream Ang Ⅱ-induced growth signals into nucleus to activate nuclear transcription factors to make cardiac fibroblast startup into dividing cycle.

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丝裂素活化蛋白激酶反义寡核苷酸对血管紧张素Ⅱ诱导的大鼠心肌成纤维细胞 c-*myc* 基因的表达及细胞增殖的抑制效应

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关键词 Ca^{2+}-钙调蛋白依赖性蛋白激酶；血管紧张素Ⅱ；反义寡核苷酸；原癌基因；*myc* 基因；成纤维细胞；细胞培养；细胞分裂

目的：探讨丝裂素活化蛋白激酶（MAPK）反义寡核苷酸（ODN）对血管紧张素Ⅱ（Ang Ⅱ）诱导的心肌成纤维细胞 c-*myc* 基因及其细胞增殖的抑制效应．**方法**：MAPK 反义 ODN 转染培养新生大鼠心肌成纤维细胞，Western Blot 法结合 p-81 滤纸法测定 MAPK 活性；Northern Blot 法检测 c-*myc* mRNA 的表达；[^{3}H]TdR 掺入和[^{3}H]脯氨酸掺入测定细胞 DNA 和胶原蛋白的合成．**结果**：MAPK 反义 ODN 显著抑制 Ang Ⅱ 诱导的 MAPK 蛋白表达及其活性；显著抑制 c-*myc* 基因的表达以及细胞 DNA 和胶原蛋白的合成．**结论**：MAPK 反义 ODN 特异性下调 MAPK 的活性，有效抑制了 Ang Ⅱ 诱导的 c-*myc* 基因的表达以及心肌成纤维细胞的增殖和胶原蛋白的合成．

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