Dominant Negative Mutant of c-Jun Inhibits Cardiomyocyte Hypertrophy Induced by Endothelin 1 and Phenylephrine

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Abstract—The activator protein 1 (AP-1) transcriptional complex, containing Jun and Fos proteins, is involved in regulating many cellular processes such as proliferation and differentiation. However, little is known about a direct relationship between AP-1 activities and cardiomyocyte hypertrophy. To elucidate the roles of myocardial AP-1 activities, dominant negative mutant of c-Jun (DNJun) was overexpressed in cultured rat neonatal ventricular myocytes by adenovirus vector to abrogate endogenous AP-1 activation. Cardiomyocytes were treated with 100 nmol/L endothelin 1 (ET) and 10 µmol/L phenylephrine (PE) to induce myocardial cell hypertrophy. Both ET and PE significantly enhanced AP-1 DNA binding activities (3.4-fold by ET and 4.8-fold by PE at 3 hours, P<0.01). At 48 hours after stimulation, ET and PE significantly increased incorporation of 3H-phenylalanine (1.4-fold by ET and 1.5-fold by PE, P<0.01), cell size (2.3-fold and 2.5-fold, P<0.01), and mRNA expression of atrial natriuretic peptide (ANP; 1.9-fold and 1.8-fold, P<0.01) and brain natriuretic peptide (BNP; 1.6-fold and 1.6-fold, P<0.01). Adenovirus carrying DNJun prevented the transcriptional activation of the AP-1 by ET and PE, using AP-1 reporter enzyme firefly luciferase assay. Moreover, DNJun prevented the increase in incorporation of 3H-phenylalanine, cell size, and the mRNA expression of ANP and BNP by ET and PE. In conclusion, we provide the first evidence that DNJun inhibits cardiomyocyte hypertrophy through inhibition of AP-1 transcriptional activity. (Hypertension. 2002;39:81-86.)

Key Words: hypertrophy, cardiac ■ c-Jun ■ mutation ■ myocytes

Extracellular stimuli, including mechanical stress, growth factors, and cytokines, cause cardiac hypertrophy accompanied by myocardial cellular phenotypic changes and gene expression.1–3 The hypertrophic response in cardiomyocytes is characterized by an enlargement of myocytes, an increase in the content of contractile proteins, and expression of embryonic genes such as atrial natriuretic peptide (ANP).1 The hypertrophic response is compensatory at an early stage of various cardiac diseases, but sustained extracellular stimuli may lead to excessive cardiac remodeling and finally to heart failure.4 Therefore, an understanding of the intracellular signaling in the process of cardiac hypertrophy induced by extracellular signals is important for the design of appropriate therapy to regulate the myocardial hypertrophic response.

Transcriptional regulation is important for molecular signaling in cellular response, involving interactions between the proteins of the general transcriptional apparatus and proteins that bind gene-specific enhancer elements. One major transcription factor is the activator protein 1 (AP-1) complex, which is composed of the Jun and Fos family of DNA binding protooncoproteins. The c-Jun and c-Fos proteins form dimers that bind DNA through specific response elements to transactivate or transrepress the transcription of genes downstream from these enhancer elements. The activation of AP-1 has been shown to be involved in the regulation of a variety of cellular processes, such as proliferation, differentiation, and transformation.4–8 Moreover, recent studies have demonstrated that myocardial AP-1 DNA binding activities are significantly increased in experimental cardiac hypertrophy.9,10 These results suggest the possible implication of AP-1 in cardiac hypertrophy. However, the effects of direct inhibition of AP-1 activity on cardiac hypertrophy are still unknown. Recently, to regulate the transcriptional activities of AP-1, it has been reported that a dominant negative mutant lacking the transactivating domain of c-Jun (DNJun) inhibits AP-1 activity.11 DNJun can form homodimers as well as heterodimers with c-Jun and c-Fos and can bind specific DNA. Thus, such a mutant could inhibit wild-type AP-1 DNA binding activity through the inhibition of the function of wild-type c-Jun and c-Fos. In the present study, to clarify one of the potentially important control mechanisms of cardiomyocyte hypertrophy, DNJun was overexpressed by adenovirus transfer into cultured rat neonatal ventricular myocytes, and its effects on endothelin 1 (ET)– and phenylephrine (PE)–induced cardiomyocyte hypertrophy were ex-
examined. These results indicate a key role for c-Jun as a mediator of cardiomyocyte hypertrophy.

Methods

Cell Cultures

Primary culture of rat neonatal ventricular myocytes was prepared as previously reported. In brief, apical halves of cardiac ventricles from 1- to 2-day-old Wistar rats were separated, minced, and dispersed with 80 U/mL collagenase IV and 0.6 mg/mL pancreatin. To segregate myocytes from nonmyocytes, a discontinuous gradient of Percoll was prepared. Then the myocytes were incubated on uncoated 10-cm culture dishes for 30 minutes to remove any remaining nonmyocytes, and the nonattached viable cells were plated on gelatin-coated 6-well culture plates or 6-cm culture dishes at a density of $3 \times 10^4$ cells/cm$^2$ with DMEM supplemented with 10% FCS for 36 hours. The cells were maintained in serum-free DMEM for 24 hours. After the preconditioning period, 100 nmol/L ET (Peptide Institute) or $10^{-6}$ mol/L PE (Waco Pure Chemical Industries, Ltd) was added to the culture in serum-free DMEM.

Adenoviruses and Gene Transfer

The cDNA fragment containing the full-length coding regions of rat wild-type c-Jun was obtained from mRNA of rat vascular smooth muscle cells by the reverse transcription-polymerase chain reaction (RT-PCR) method, using a RT-PCR kit (Toyobo Co, Ltd). DNJun was constructed from wild-type c-Jun by deletion of residues 3 to 122 in the amino-terminal transcriptional activation domain. The recombinant adenovirus expressing DNJun was generated by the cosmid cassettes and adenovirus DNA-terminal protein complex (COS/TPC) method, using an Adenovirus Expression Vector Kit (Takara). Recombinant adenovirus containing bacterial beta-galactosidase gene (LacZ) was also constructed as a negative control of DNJun. For adenovirus-mediated gene transfer, cardiomyocytes were exposed to adenoviral vectors at a multiplicity of infection (MOI) of $25$ to $50$ for 1 hour, before the preconditioning with serum-free DMEM.

Electrophoretic Mobility Shift Assays

For the electrophoretic mobility shift assay, nuclear protein extracts were prepared as described previously. The sequence of the double-stranded oligonucleotide used in the present study was as follows: consensus AP-1, 5'-CGCCTTGATGACTCAGGGAA-3'; consensus nuclear factor-κB (NF-κB), 5'-AGTTGAGGC-ACCTTCCAGGC-3'; consensus specificity protein-1 (SP-1), 5'-ATTCATGGGGGCGGGCGGCCGAGC-3'; consensus cAMP responsive element binding protein (CREB), 5'-AGAGATTCGCTGAGAGAGCTA-3'.

Figure 1. ET and PE increased protein synthesis, cell size, and AP-1 DNA binding activities in cardiomyocytes. A and B, The incorporation of $^3$H-phenylalanine ($^3$H-Phe) (A) and cell size (B) in cultured cardiomyocyte treated with ET and PE for 48 hours. ET and PE significantly increased the incorporation of $^3$H-Phe and cell size (mean of size of untreated cell was $282 \pm 10$ μm$^2$). C and D, Time course of AP-1 DNA binding activity in cultured cardiomyocytes treated with ET and PE by gel mobility shift assay. C, Representative autoradiograms of AP-1 DNA binding activity at 3 hours after treatment. Each bar represents mean±SEM (n=4). The mean value of AP-1 DNA binding activity at 0 hours (control) is expressed as 1. **P<0.01 versus control. E, ET and PE did not affect NF-κB, SP-1, and CREB DNA binding activities by gel mobility shift assay in cultured cardiomyocytes treated with ET and PE for 3 hours.
To demonstrate the specificity of AP-1 DNA-protein binding, the reactions were performed in the presence of nonlabeled consensus oligonucleotide competitors. In addition, a supershift assay was carried out using rabbit polyclonal IgG against c-Fos (epitope mapping within a highly conserved domain of c-Fos p62 of human origin) or rabbit polyclonal IgG against c-Jun (epitope mapping within a highly conserved DNA binding domain of c-Jun p39 of mouse origin) (Santa Cruz Biotechnology Inc) to examine the complex containing c-Fos and c-Jun.

**Northern Blot Analysis**

Total RNA was extracted from cultured myocytes with TRIzol Reagent (Life Technologies). Northern blot analyses were performed with cDNA probes for rat ANP, rat brain natriuretic peptide (BNP), and rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as described previously. The densities of individual mRNA bands were measured by using a bioimaging analyzer (BAS-2000, Fuji Photo Film Co).

**Western Blot Analysis of c-Jun**

Protein extracts (20 μg) from myocytes were separated on a 12% SDS-polyacrylamide gel and immobilized on polyvinylidene difluoride membrane. The membrane was immunoblotted with anti-c-Jun antibody (Promega) using the enzyme-linked chemiluminescence (ECL) method as described previously. The densities of individual mRNA bands were measured by using a bioimaging analyzer (BAS-2000, Fuji Photo Film Co).

**Immunocytochemistry for α-Sarcomeric Actin**

Cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, reacted for 2 hours with anti-rat α-sarcomeric actin antibody (Sigma) to stain α-cardiac muscle actin, followed by the treatment with tetramethylrhodamine isothiocyanate (TRITC)-conjugated secondary antibody. In our culture, >99% cells were stained with the antibody. Cardiomyocyte cell size was estimated by measuring the area of α-sarcomeric actin-positive cells.

**Luciferase Assay**

The plasmid pAP1-Luc or pCREB-Luc containing the firefly luciferase reporter gene driven by a basic promoter element (TATA box) joined to tandem repeats of AP-1 or CREB binding element were from Stratagene. As an internal control, pRL-TK containing the sea pansy luciferase reporter gene driven by herpes simplex virus-thymidine kinase promotor was from Toyo Ink Co, Ltd. At 24 hours after adenovirus transfection, the cells were cotransfected with pAP1-Luc or pCREB-Luc and pRL-TK, using Lipofectamine Plus reagent (GIBCO BRL). After the preconditioning period of 24 hours, ET or PE was added to the culture for 24 hours. Both the firefly and sea pansy luciferase activities were determined in the same cell lysate using Picagene dual Sea Pansy Kit (Toyo Ink Co, Ltd). The firefly luciferase activities as AP-1 or CREB transcriptional activity were normalized for transfection efficiency by sea pansy luciferase activity.

**Protein Synthesis**

The effect of various agents on protein synthesis in cultured cardiac myocytes was evaluated by the incorporation of 3H-phenylalanine into cells. After adenovirus gene transfer and preconditioning with serum-free DMEM, the cells were incubated with 3 μCi/mL 3H-phenylalanine in serum-free DMEM for 48 hours. Then cells were washed twice with PBS, and 10% trichloroacetic acid was added to precipitate protein for 60 minutes on ice. The precipitate was washed 3 times with 10% trichloroacetic acid and then resuspended in 2% SDS. Aliquots were counted by a liquid scintillation counter.

**Statistics**

The results are expressed as means ± SE. Statistical significance was determined using ANOVA. Differences were considered statistically significant when P<0.05.

An expanded Methods section can be found in an online data supplement available at http://www.hypertensionaha.org.

**Results**

ET and PE Increased Protein Synthesis, Cell Size, and AP-1 DNA Binding Activities in Cardiomyocytes

As shown in Figure 1A, ET and PE significantly increased incorporation of 3H-phenylalanine (3H-Phe) in cultured car-
Adenovirus Transfer of LacZ and Dominant Negative Mutant of c-Jun

Figure 2A shows the evaluation of DNJun-related AP-1 complex in cardiomyocytes transfected with several concentrations of adenovirus expressing DNJun in gel mobility shift assay. The complex containing DNJun was observed at the upper portion of wild-type AP-1 complex in a dose-dependent manner. The specific expression of DNJun in cardiomyocytes analyzed by Northern blot and Western blot analyses is shown in Figures 2B and 2C. Figure 2D shows the specificity of AP-1 DNA complexes. The band of endogenous AP-1 or DNJun-related complexes was completed by nonlabeled AP-1 consensus oligonucleotide in a dose dependent manner, indicative of the specific binding. The AP-1 complex was supershifted with specific anti-c-Fos and anti-c-Jun antibody. A large amount of DNJun-related complex was observed compared with the endogenous AP-1 complex and supershifted with anti-c-Jun antibody. These results demonstrated that DNJun was overexpressed in cardiomyocytes and preferentially bound to the AP-1 site of DNA.

DNJun Inhibits the Transcriptional Activity of AP-1, Protein Synthesis, Cell Size, and mRNA Expression of ANP and BNP

As shown in Figure 3A, both ET and PE resulted in a statistically significant increase in activities of the AP-1 reporter enzyme firefly luciferase in nontransfected cardiomyocytes (2.6- and 2.9-fold, P<0.01). Figures 1C and 1D show that ET and PE enhance the AP-1 DNA binding activities in cardiomyocytes. The AP-1 DNA binding activities of ET-treated cardiomyocytes increased by 3.8-fold at 6 hours. AP-1 DNA binding activities of PE-treated cells increased by 4.8-fold at 3 hours. These results demonstrated that both ET and PE could induce cardiomyocyte hypertrophy and activate AP-1 DNA binding activities. As shown in Figure 1E, ET and PE did not change NF-kB, SP-1, and CREB DNA binding activities in cultured cardiomyocytes.

DNJun inhibited the increase in cardiomyocyte cell size induced by ET and PE. These results suggest that overexpression of DNJun inhibits cardiomyocyte hypertrophy induced by ET and PE. As shown in Figure 4, ET induced mRNA expression of ANP and BNP at 48 hours in noninfected myocytes (1.9- and 1.6-fold, P<0.01) and in myocytes transfected with adenovirus expressing LacZ (1.7- and 1.5-fold, P<0.01). PE also enhanced these mRNAs in noninfected myocytes (1.8- and 1.6-fold, P<0.01) and in myocytes expressing LacZ (1.7- and 1.5-fold, P<0.01). DNJun prevented the stimulation of ANP and BNP mRNA levels by ET and PE.

Discussion

Accumulating evidence supports the finding that mechanical stress, growth factors, and cytokines induce hypertrophic responses in cardiomyocytes.2,19,20 However, the molecular mechanism responsible for cardiomyocyte hypertrophy remains to be fully determined. In the present study, we provide the first evidence that the dominant negative mutant of c-Jun inhibits protein synthesis, cell size, and induction of ANP and BNP in ET- and PE-induced cardiomyocyte hypertrophy through the direct inhibition of transcription factor AP-1. The results of our study suggest that the activation of AP-1 is involved in cardiomyocyte hypertrophy.

As important upstream cascades of AP-1, not only ET and PE, but also many growth factors and/or mechanical stresses...
have been shown to activate p42 and p44 mitogen-activated protein kinases (MAPKs), also known as extracellular signal-regulated kinases (ERKs), through protein kinase C (PKC) and/or protein tyrosine kinase-dependent pathways. ERK phosphorylates Elk-1, a ternary complex, forms a complex with serum response factor, and binds to the promoter of numerous genes such as c-fos containing the serum response element. Therefore, phosphorylation and activation of Elk-1 by ERK leads to the increased expression of c-fos mRNA. On the other hand, it has been reported that c-Jun NH2-terminal kinase (JNK), a member of the MAPK family, is involved in the signaling of cardiomyocytes stimulated by cellular stress and cytokines. JNK activates several transcription factors, such as c-Jun, activating transcription factor-2, Elk-1, and serum response factor accessory protein. Because c-Fos and c-Jun proteins are important components of AP-1, the activation of ERK and JNK is generally thought to cause AP-1 activation. As shown previously, ET and PE activate ERK and JNK in cardiomyocytes, thereby suggesting the role of MAPK as one of the major signals upstream of AP-1 in these stimuli. Furthermore, recent work demonstrated that ERK and JNK were enhanced in the development of cardiomyocyte hypertrophy. However, the hypertrophic effect of MAPK is still controversial, and the downstream target transcription factors of MAPK for cardiac hypertrophy remain to be fully determined. Of note are the present observations that ET and PE increased the DNA-binding activity of AP-1, as shown in the results, the expression of DNJun in cardiomyocytes was rather higher than wild-type AP-1 using gene transfer with adenovirus infection at 50 MOI. These results suggest that adenoviral vectors induced a high level of transgene expression into cultured cardiomyocytes. Moreover, neither ET nor PE affected NF-kB, SP-1, or CREB DNA binding activity, suggesting the specific inhibition of AP-1 by DNJun in cardiomyocyte hypertrophy induced by ET or PE. It is reported that this DNJun can form homodimers and heterodimers with c-Jun and c-Fos and can bind specific DNA. Our findings demonstrated that DNJun could suppress the luciferase activity enhanced by ET and PE in cardiomyocytes transfected with AP1-Luc plasmid, thereby confirming the inhibiting effects of the dominant negative mutant on AP-1 activity. Moreover, DNJun inhibited the enhanced protein synthesis, cell size, and mRNA expression of ANP and BNP, which are the most useful markers of hypertrophic response in the cardiomyocyte. These results indicate that the inhibition of cardiomyocyte hypertrophy by overexpressed DNJun is possibly mediated through the inhibition of AP-1 activity.

In conclusion, we demonstrated the first evidence that DNJun directly inhibits the transcriptional activation of AP-1 in cultured rat neonatal ventricular myocytes induced by ET and PE, followed by inhibition of protein synthesis, cell size, and expression of ANP and BNP. These results suggest that the activation of AP-1 could be involved in cardiomyocyte hypertrophy. However, further work is needed to demonstrate the direct in vivo evidence of the effects of DNJun on cardiac hypertrophy.

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References


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