AFos Dissociates Cardiac Myocyte Hypertrophy and Expression of the Pathological Gene Program

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**Background**—Although induction of activator protein-1 (AP-1) transcription factor activity has been observed in cardiac hypertrophy, a direct role for AP-1 in myocardial growth and gene expression remains obscure.

**Methods and Results**—Hypertrophy was induced in cultured neonatal rat cardiomyocytes with phenylephrine or overexpression of a constitutively active MAP3K, MKK6. In both treatment groups, induction of the pathological gene profile was observed, ie, expression of β-myosin heavy chain (βMHC), atrial/brain natriuretic peptides (ANP/BNP), and skeletal α-actin (sACT) was increased, whereas expression for α-myosin heavy chain (αMHC) and the sarcoplasmic reticulum Ca2⁺-ATPase (SERCA) genes was repressed. The role of AP-1 in the hypertrophic phenotype was evaluated with the use of an adenoviral construct expressing a dominant negative mutant of the c-Fos proto-oncogene (AdAFos). Although AFos did not change the myocyte growth response, it abrogated the gene profile to both agonists, including the upregulation of both αMHC and SERCA expression.

**Conclusions**—Although c-Fos/AP-1 is necessary for induction of the pathological/fetal gene program, it does not appear to be critical for cardiomyocyte hypertrophy. (*Circulation. 2005;111:1645-1651.)*

**Key Words:** hypertrophy ▪ signal transduction ▪ myocytes ▪ molecular biology

Pathological myocardial hypertrophy is characterized by an increase in cardiomyocyte protein and the expression of a gene profile reminiscent of early embryonic development. Specifically, expression of β-myosin heavy chain (βMHC), skeletal α-actin (sACT), and both atrial and brain natriuretic peptides (ANP and BNP, respectively) increases, whereas that of the adult cardiac muscle–specific genes, α-myosin heavy chain (αMHC) and sarcoplasmic reticulum Ca²⁺-ATPase (SERCA), decreases. Physiological growth, on the other hand, exhibits a unique gene profile in which αMHC and SERCA increase without substantive changes in sACT, βMHC, ANP, or BNP. Experimentally, both exercise and tri-iodothyronine induce similar changes in gene expression and cardiac morphology; however, the most clinically significant difference between pathological and physiological growth is the ultimate decompensation of heart function seen with pathological hypertrophy. Although the mechanisms responsible for each type of hypertrophy have been the subject of intense research, many aspects remain elusive.

Mammalian activator protein-1 (AP-1), composed of homo- or heterodimers of proto-oncogenes c-Fos and c-Jun, plays a variety of cellular roles including proliferation, differentiation, and apoptosis. Both are bZIP DNA binding proteins, possessing leucine zipper and basic regions. The former is essential for dimer formation, whereas the latter facilitates DNA binding. Whereas c-Jun homodimerizes and activates transcription, c-Fos requires heterodimerization with c-Jun to influence gene transcription. Notably, Fos/Jun heterodimers are more potent because of an increase in stability and higher levels of DNA binding activity. Although c-Jun is constitutively expressed at low levels, c-Fos expression is only seen in response to external stimuli (reviewed by Angel and Karin). Despite a wealth of data indicating that the myocardial expression of both c-Jun and c-Fos increases in response to pathological stimuli, a cause–effect relationship for AP-1 in cardiac hypertrophy has not been confirmed.

To evaluate this question, we used a dominant negative mutant of c-Fos (AFos) in which an N-terminal acidic sequence renders DNA binding ineffective and stabilizes the AFos/c-Jun interaction ~3000-fold relative to endogenous homodimer. Using this reagent, we found that inhibition of AP-1 transcriptional activity in neonatal rat myocytes has profound effects on the myocyte gene program but little effect on the degree of hypertrophy induced by 2 well-characterized hypertrophic stimuli, phenylephrine and the constitutively activated mitogen-activated protein kinase kinase 6 (MKK6CA). The former is a well-characterized growth agonist that acts at the cell surface via global activation of the G protein–coupled receptor cascade. Con-
stittuitively activated MKK6 was used as a robust hypertrophic stimulus that is also associated with dramatic cell growth as well as the pathological gene program in the neonatal system but targets the downstream effector for just 1 arm of the mitogen-activated protein kinase (MAPK) signaling cascade.\textsuperscript{14–16} Our observation indicates that the processes of pathological gene expression and hypertrophy are independent and suggests the possibility that pathological hypertrophy may be manipulated toward that characteristic of physiological growth.

Methods

Cell Culture

Day-old neonatal rat myocytes were obtained as described previously.\textsuperscript{17} Cultures were studied under serum-free MEM supplemented with transferrin, insulin, bovine serum albumen, BrdU, and PB12.

Adenoviral Constructs

Adenoviral constructs for hemagglutinin (HA)-tagged AFos (AdAFos), \( \beta \)-galactosidase (Ad\( \beta \)Gal), and HA-tagged AdMKK6CA have been described previously.\textsuperscript{15,18} Adenoviral infection was done at a multiplicity of infection (MOI) of 5 to 10 on culture day 1 and incubated for 72 hours.

Determination of Cardiomyocyte Hypertrophy and Gene Program

Ad\( \beta \)Gal- or AdAFos-infected myocyte cultures were treated with phenylephrine (20 \( \mu \)M/L) or its vehicle on day 3 and incubated in serum-free medium containing \([\text{H}^4\text{C}]\text{phenylalanine for 24 hours.}\) The incorporation of \([\text{H}^4\text{C}]\text{phenylalanine into newly synthesized protein allows for quantification of hypertrophy as described previously.}\textsuperscript{17} AdMKK6CA was also used as a hypertrophic agent.\textsuperscript{14–16} Infection was done on culture day 1 simultaneously with AdAFos or Ad\( \beta \)Gal, and coinfected myocytes were incubated for 72 hours. \([\text{H}^4\text{C}]\text{Phenylalanine was introduced on culture day 3 and incubated for 24 hours.}\)

Changes in myocyte surface area were also measured in response to the same experimental groups. For these determinations, 8 cells from 5 high-power fields were identified, and their cell borders were traced and analyzed with the use of ImagePro. Eight individual cell culture wells from 3 independent experiments were performed for a total of 960 cells per treatment group.

RNA was extracted from myocytes with TRIzol (GIBCO), and total RNA was quantified as a third independent measure of cellular growth. For gene program, RNA was hybridized with \([\text{P}^3\text{P}]\)-labeled anti-sense probes for \( \alpha/\beta \)-MHC, SERCA, ANP, BNP, sACT, and GAPDH and subjected to RNase protection assay.\textsuperscript{3} Signals were analyzed by densitometry and corrected for RNA loading (GAPDH).

Analysis of AFos Expression

Total cell protein extract from equivalent cell numbers was used for Western analysis of AFos expression.\textsuperscript{3} HA antibody (Santa Cruz Biotechnology) was used at a 1:1000 dilution. AFos overexpression was also visualized by immunostaining with the use of FITC anti-HA antibody (Santa Cruz Biotechnology) was used at a 1:1000 dilution. AFos overexpression was also visualized by immunostaining with the use of FITC anti-HA antibody (Santa Cruz Biotechnology).

Nuclear fractions from AdAFos- and Ad\( \beta \)Gal-infected cells were prepared\textsuperscript{19} and resuspended in equivalent volume for comparison of relative AFos expression. \([\text{P}^3\text{P}]\)-labeled AP-1 consensus sequence (sc-2501; 5'-\text{CGC TTG ATG ACT CAG CGG AAG-3'; Santa Cruz}) was used as the probe. AP-1 oligonucleotide and c-Jun antibody were used to confirm specificity, and a nuclear factor-\( \kappa \)B oligonucleotide was used as nonspecific competitor (sc-2505; Santa Cruz).

Transfection

Myocytes were cotransfected as described\textsuperscript{19} with the use of calcium phosphate coprecipitation with a plasmid containing the human collagenase I promoter driving the chloramphenicol acetyltransferase (CAT) reporter (gift of Dr Paul Webb, University of California, San Francisco) along with AdAFos and Ad\( \beta \)Gal. In separate experiments, plasmids for \( \alpha/\beta \)-MHC, SERCA, and sACT promoters driving the CAT gene were also cotransfected with RSV-c-Jun and RSV\( \beta \)Gal expression plasmids, and relative promoter activity was determined. Transfection efficiency with the use of this system is routinely \( \approx 4\% \) to \( 5\%.\textsuperscript{20} \)

Data Analysis and Statistics

Mean\( \pm \)SE values are shown and were compared by 1-way ANOVA and the Newman-Keuls posttest. Probability values of \(< 0.05 \) were considered significant.

Results

Validation of AFos Overexpression/Activity in Neonatal Rat Myocytes

Increasing the multiplicity of infection of AdAFos results in a dose-dependent increase in protein expression (Figure 1A). Immunostaining confirmed that infected cells contain a clear nuclear-predominant pattern of fluorescence (Figure 1B).

Myocytes treated with phenylephrine or AdMKK6CA show an increase in AP-1–dependent promoter activity that is abrogated with AFos overexpression (Figure 2A). Notably, AFos expression alone is sufficient to inhibit AP-1 promoter activity compared with \( \beta \)Gal control.

Expression of AFos also inhibits gel shift of a consensus AP-1 element, indicating that AFos interacts specifically with AP-1 constituents (Figure 2B, lanes 2, 4, and 5).

Agonist-Induced Myocyte Hypertrophy Is Unaffected by AFos Expression

Because AdAFos infection inhibits AP-1–dependent gene activation, we next investigated its effects on phenylephrine- or MKK6CA-induced myocyte growth. Both agonists in-
and BNP transcripts was decreased. Interestingly, Afos overexpression alone was sufficient to downregulate βMHC and BNP expression below baseline and upregulate αMHC and SERCA.

**Overexpression of c-Jun Mimics the Fetal Gene Program**

Additional experiments were performed in which a c-Jun expression vector was cotransfected with promoter:reporter constructs for genes exhibiting significant reversal in the Afos studies. As shown in Figure 5, βMHC and sACT promoter activities were increased, consistent with a substantive role for AP-1 in the upregulation of these 2 fetal genes. In contrast, both αMHC and SERCA promoter activities were repressed by c-Jun, suggesting that the AP-1 complex plays a repressive role for these 2 genes. Unfortunately, the low transfection efficiency typical for cardiac myocytes in primary culture20 precludes an analysis of the effect of transfected c-Jun on myocyte size.

**Discussion**

The major conclusion drawn from the investigations described here is that cardiac myocyte hypertrophy can be disassociated from expression of the pathological myocyte gene program. To our knowledge, this is the first time that these phenotypic end points have been conclusively shown to be parallel rather than obligate processes. This observation suggests that hypertrophy, in and of itself, may not be inextricably tied to myocardial decompensation. Indeed, the growth of surviving myocytes after injury, mandatory for maintaining forward output, is functionally adaptive; however, the initially adaptive hypertrophic response often leads to decompensated failure. Although the consensus suggests that the transition to decompensation is the result of both pathological growth and associated gene program, several recent reports have challenged the obligatory nature of these 2 processes. It seems likely that a hierarchy of gene effects on contractile function exists and should be evaluated if the genes themselves are targets for intervention. Specifically, dysregulation of genes involved with calcium handling and force generation is likely deleterious to myocardial function, whereas others (ie, sACT) have less impact. The former genes would logically represent better therapeutic targets, a concept supported by reports in which their manipulation (SERCA overexpression and phospholamban inhibition) was associated with a more functional hypertrophic phenotype.21–24 Our findings with Afos make it tempting to speculate that interruption of AP-1 could also be associated with a more adaptive/physiological growth program.

Given its position at the convergence of several signaling cascades, it rationally follows that AP-1 may play a pivotal role in certain aspects of the hypertrophic phenotype. There is, in fact, an abundance of research implicating all 3 arms of the classic MAPK family (ERK, JNK, and p38) in both the growth response and pathological gene program, and each has been reported to increase abundance, activity, or both of AP-1 constituents (reviewed in Karin25). Specifically, c-Jun (obligate member of the AP-1 dimer) is a downstream target of both JNK and p38 signaling cascades, and c-Fos is increased

**AP-1 Is Necessary for Expression of the Fetal Gene Program**

With few exceptions, hypertrophy has been associated with the expression of a characteristic gene profile. Treatment of control (AdβGal) myocytes with phenylephrine or AdMKK6CA induced this gene program, ie, an increase in βMHC, sACT, ANP, and BNP but a decrease in the adult-specific genes αMHC and SERCA (Figure 4). Conversely to overexpressing Afos, however, induction of the pathological gene program by these 2 hypertrophic agonists was largely inhibited. Specifically, expression of both αMHC and SERCA was increased, whereas that for βMHC, sACT, ANP,
in response to ERK activation. Furthermore, both c-Jun and c-Fos and MAPK pathways are known to be activated by virtually all hypertrophic stimuli, and AP-1 activity is increased in human subjects with end-stage heart failure. Although the induction and activation of c-Jun and c-Fos in cardiac myocytes by growth-promoting agonists and MAPK pathways are well established, their role in growth has remained elusive. The prevailing view suggests that they lie at the terminal end of the signaling cascade linking gene expression/myocardial hypertrophy to the initiating stimuli.

Our observations with AFos suggest that although AP-1 is essential for the pathological gene profile, it is not required for maintaining the hypertrophic phenotype. Indeed, expression of the JNK interacting protein (JIP-1) in the cardiac myocyte context failed to affect the growth response to endothelin-1 and only inhibited that in response to phenylephrine by 10%. Perhaps of more clinical relevance in light of their effects on contractile efficiency and force generation is the upregulation of MHC and SERCA with AFos expression, genes normally repressed in the pathological program. Several candidate sequences with partial homology to the consensus AP-1 site are, in fact, present. A plausible explanation for our findings may be that these candidate 12-O-tetradecanoylphorbol-13-acetate (TPA)-response element TRE[AP-1] sequences are negative regulatory elements, similar to the negative thyroid response element found in the βMHC promoter. Indeed, cotransfection of neonatal myocytes with αMHC and SERCA promoter constructs and a c-Jun expression vector decreases promoter activity (Bishoprict et al and Figure 5).

In contrast to our observations, several recent reports using dominant negative mutants of c-Jun have suggested that the AP-1 complex plays a more integral role in the process of cardiac myocyte growth. This apparent discrepancy with our results may be due to the targeting of c-Jun rather than c-Fos. In support of a “hierarchy” for these 2 proteins in the abrogation of AP-1 activity, c-Fos mice display impaired bone development but remain viable, whereas both c-Jun and JunB knockouts are embryonically lethal. It is also important to consider that, in contrast to c-Fos, c-Jun has a basal level of transcription that likely represents its function in basal cellular homeostasis.

In summary, myocardial hypertrophy in the compensated state is a necessary adaptive process to accommodate the increased workload resulting from several clinically relevant conditions. For millions of cardiomyopathy patients, however, hypertrophy is insufficient and progresses to decompensated failure. These patients exhibit enlarged hearts as well as the pathological gene profile. It is intriguing to ask what the consequence of altering the gene program toward a more physiological profile would be in the absence of changing overall myocyte protein content. The conclusion from our investigations is that hypertrophy and gene expression are not obligate processes in the response to injury. The implication of this observation is that myocardial function in the presence of ongoing hypertrophy could be altered by “switching” the gene program to a more physiological one. Would cardiac mass ultimately decrease as a secondary function of the change in myocardial function, rather as an obligate first step? Some experimental evidence would certainly suggest that function can be improved with a more physiological gene program without a primary change in heart size. Investigations with the targeted overexpression of the SERCA gene
Figure 4. AP-1 transcriptional factor is crucial for phenylephrine (PE)- and MKK6- induced pathological gene expression. A, Myocytes infected with AdβGal (10 MOI) or AdAFos (10 MOI) were exposed to vehicle (control [C]), phenylephrine (20 μmol/L), or AdMKK6CA (10 MOI) for 72 hours. RNA was harvested with the use of TRIzol and subjected to RNase protection for αβMHC, SERCA, σACT, ANP, BNP, and GAPDH. Protected fragments were separated by denaturing polyacrylamide electrophoresis. B and C, Summary data for RNase protection assays from 5 to 6 separate experiments corrected for GAPDH.
product rescued the failing phenotype in several models of decompensation.\textsuperscript{22,24,45} and thyroid hormone treatment also resulted in a physiological gene program and improved cardiac function in a pressure-overload model of cardiac hypertrophy and failure.\textsuperscript{4} One obvious next step that would establish a clinically relevant connection between these investigations and the targeting of AP-1 in patients with heart failure would be to assess the functional effects of conditionally expressing A\textsc{f}os in an animal model of myocardial hypertrophy/failure. With the use of this approach, the physiological gene program could be turned “on” and “off” in the setting of the failure phenotype, and the functional effects could be assessed noninvasively. The results of such a study would give more information on the functionality of a specific gene program in the hypertrophic heart and could offer a new therapeutic target for patients with heart failure.

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References


