The MEKK-JNK Pathway Is Stimulated by α₁-Adrenergic Receptor and Ras Activation and Is Associated with in Vitro and in Vivo Cardiac Hypertrophy*‡

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In neonatal rat ventricular myocytes, stimulation of the α₁-adrenergic receptor (α₁-AdR) activates a program of genetic and morphological changes characterized by transcriptional activation of the atrial natriuretic factor (ANF) gene and enlargement (hypertrophy) of the cells. The low molecular weight GTPase Ras has been established as an important regulator of hypertrophy both in vitro and in vivo. Ras activates a kinase cascade involving Raf, the mitogen-activated protein kinase kinase (MEK), and the extracellular signal-regulated protein kinase (ERK). However, the extent of involvement of this pathway in regulating hypertrophic responses is controversial. We demonstrate here that both α₁-AdR stimulation and Ras can also activate the c-Jun NH₂-terminal kinase (JNK) in cardiomyocytes. The α₁-AdR effect on JNK occurs through a pathway requiring Ras and MEK kinase (MEKK). A constitutively activated mutant of MEKK that preferentially activates JNK, stimulates ANF reporter gene expression, while a dominant negative MEKK mutant inhibits ANF expression induced by PE. Furthermore, JNK activity is increased in the ventricles of mice overexpressing oncogenic Ras, whereas ERK activity is not. These results suggest that the α₁-AdR mediates ANF gene expression through a Ras-MEKK-JNK pathway and that activation of this pathway is associated with in vitro and in vivo hypertrophy.

Stimulation of the G protein-linked α₁-adrenergic receptor (α₁-AdR) in neonatal rat ventricular myocytes triggers a hypertrophic response characterized by the transcriptional activation of a number of genes, including that for atrial natriuretic factor (ANF). The hypertrophic response is further characterized by increases in cell size and organization of myofilaments into sarcomeric units (reviewed in Ref. 1). Ras, a low molecular weight GTPase known to transduce mitogenic signals from growth factor-activated tyrosine kinase receptors, has been shown to be required for α₁-AdR-mediated ANF gene expression, increases in cell size and myofilament organization (2). Evidence for the involvement of Ras comes from studies in which microinjection of a dominant-interfering Ras expression vector blocked α₁-AdR-induced increases in ANF protein and cell size (2). Furthermore expression of oncogenic Ras in transgenic mice results in the development of pathological hypertrophy (3, 4). Thus it is well-established that Ras-dependent pathways mediate hypertrophy in vivo as well as in response to α₁-AdR stimulation in vitro.

The precise signaling events that ensue subsequent to Ras activation and are important in the development of the hypertrophic phenotype are not clearly understood. Ras activates the Raf-MEK-ERK pathway in a number of cell systems. This pathway has been linked to cell proliferation and growth regulation. Studies from several laboratories have implicated Raf (5), MEK (6), and the ERKs (7–9) in the regulation of α₁-AdR-mediated hypertrophic responses. On the other hand, ERK1 and ERK2 are activated by agonists (e.g. carbachol and ATP) that fail to induce ANF expression or myofilament organization (10). In addition, expression of activated MEK, the kinase that activates the ERKs, failed to induce ANF expression and paradoxically inhibited it (11). Thus, it appears that activation of the Raf-MEK-ERK pathway alone is insufficient to transduce the α₁-AdR-induced hypertrophic responses.

Ras can also couple to and activate MEKK (12, 13). This kinase was originally described as an activator of the MEK-ERK cascade (14) but has more recently been shown to activate JNK, a dual specificity kinase that phosphorylates and activates JNK, another member of the MAP kinase superfamily (15–17). JNK, in turn, phosphorylates c-Jun which dimerizes with c-Fos to activate AP-1-dependent gene transcription (18). The role of the MEKK-JNK-JNK pathway in regulating cardiomyocyte hypertrophy has not been extensively studied. Recent studies in myocytes demonstrate that cellular stressors can activate JNK (19, 20). Additionally, transfection of myocytes with activated MEKK and JNK has been shown to increase cell size and ANF transcriptional activation (21).

We have investigated the effects of α₁-AdR stimulation on activation of the MEKK-JNK signaling cascade. Our data demonstrate that PE causes a sustained activation of JNK and that the resultant increase in c-Jun transcriptional activity is dependent on Ras and MEKK. We show that MEKK, rather than Raf, is a mediator of PE-induced ANF gene expression. In-
creased JNK activity is also detected in the ventricles of transgenic Ras mice, whereas ERK activities are not elevated above those in control mice. We propose that the maintenance of elevated JNK activity is important in the development of the hypertrophic state in vitro as well as in vivo.

EXPERIMENTAL PROCEDURES

Cell Culture—Neonatal ventricular myocytes were cultured from 1- to 3-day-old Harlan Sprague Dawley rats as described previously (22). Tricrested hearts were dissociated by treatment with collagenase II (Worthington) and pancreatin (Life Technologies, Inc.), and myocytes were purified by centrifugation through a discontinuous Percoll gradient. Cells were plated at a density of 4 × 10^5/cm² on gelatin-coated tissue culture dishes and maintained overnight in 4:1 Dulbecco’s modified Eagle’s medium/medium 199 (Life Technologies, Inc.) containing 10% horse serum, 5% fetal calf serum, and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin).

Ras Activity Assay—Myocytes plated on 100-mm dishes were serum-starved for 24 h, washed twice with phosphate-free Dulbecco’s modified Eagle’s medium, and incubated with phosphate-free Dulbecco’s modified Eagle’s medium containing 0.5 mCi/ml [32P]orthophosphate for 3 h. Cells were stimulated with 100 μM propranolol to block β-adrenergic receptors for the indicated times. GDP and GTP-bound Ras protein was immunoprecipitated from cell lysates, and the bound GDP and GTP were eluted, separated by thin layer chromatography, and visualized by autoradiography as demonstrated in the representative experiment shown in A. Data were analyzed by densitometry, and the mean ± S.E. of 3–7 determinations was plotted in B. The variation in the intensity of the GDP spots in the autoradiogram shown in A was peculiar to that experiment; on average, the GDP spots were not significantly different in PE-treated versus control samples at any of the time points assayed over 3–4 experiments.

RESULTS

α1-AdrR Stimulation Activates Ras—It is well-established that Ras mediates PE-induced ANF expression (2). To directly establish that stimulation of the α1-AdrR causes activation of Ras, myocytes were metabolically labeled with [32P]orthophosphate and treated with or without PE for short times. Ras was then isolated and the proportion of GTP-bound Ras determined. The amount of Ras in its GTP-bound (activated) state increased by 35% after 1 min of agonist stimulation (Fig. 1A). This increase, although small, was reproducible and was sustained for at least 10 min.

α1-AdrR Stimulation and Oncogenic Ras Activate JNK—Multiple signaling pathways can be activated downstream of Ras. These include the Raf-MEK-ERK cascade and the more recently characterized MEKK-JNK cascade. We have previously shown that PE stimulates ERK activity in myocytes (10). To determine if α1-AdrR stimulation also activates JNK, we examined the effects of PE on JNK activity. Myocytes were treated in the absence or presence of PE for various times, and JNK was immunoprecipitated and assayed for its ability to phosphorylate its substrate GST-c-Jun. Induction of JNK activity was associated with an increase in JNK protein levels even at long times of PE stimulation (Fig. 2C). These data indicate that PE activates existing JNK rather than increasing its expression.

The MEKK-JNK cascade is activated by PE in cardiac myocytes.
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PE-induced Stimulation of c-Jun Transcriptional Activity Is Dependent on Ras and MEKK—Since PE and Leu-61 Ras both increased JNK activity, we sought to establish whether PE activated JNK through its effect on Ras. To address this question, we employed an assay that uses c-Jun transcriptional activity as a readout for JNK activation in transiently transfected cells. c-Jun transcriptional activity is stimulated following its phosphorylation by JNK at Ser-63 and Ser-73 (29, 30). Thus the α₁-AdrR-induced increases in JNK activity should result in induction of c-Jun transcriptional activity. Phenylephrine stimulated the expression of a GAL4-luciferase reporter gene that was responsive to a GAL4-c-Jun-(1–223) fusion protein containing the c-Jun transactivation domain. This stimulation was completely inhibited by coexpression of dominant negative (Aaa-17) Ras (Table I), indicating that the effects of PE are mediated through Ras. The stimulation of c-Jun transcriptional activity by PE was dependent on c-Jun phosphorylation since PE failed to stimulate the activity of a mutant GAL4-c-Jun-(1–223;A63/73) fusion protein in which the serines normally phosphorylated by JNK were replaced by alanines (data not shown). To determine if the protein kinase cascade that leads to activation of JNKs is dependent on MEKK, we asked whether PE-induced c-Jun transcriptional activity (and hence JNK activation) was inhibited by dominant negative MEKK. Coexpression of a dominant negative mutant of MEKK along with the GAL4-luciferase reporter gene abolished PE-induced c-Jun transcriptional activity (Table I). In parallel experiments, we found that coexpression of dominant negative Raf paradoxically elevated the basal GAL4-luciferase activity; nonetheless, there was little decrease in the fold stimulation of GAL4-luciferase by PE (data not shown). These data indicate that PE stimulates JNK activity, leading to the phosphorylation and transcriptional activation of c-Jun, and that these events are dependent on Ras and MEKK but not Raf.

Both Activated MEKK and Activated Raf Stimulate ANF Reporter Gene Expression—MEKK was originally identified as an alternative to Raf in activating MEK, leading to the activation of the ERKs (14). However, it is now clear that Raf is a more specific activator of the ERK pathway and MEKK is a more specific activator of the JNK pathway (16, 17, 25). To determine the effects of activated MEKK and activated Raf on ANF expression in cardiomyocytes, we coexpressed activated MEKK or activated Raf with the ANF-luciferase reporter gene. As shown in Table II, both activated MEKK and activated Raf stimulated ANF reporter expression, although the stimulation by activated MEKK was greater than that by activated Raf even when a 10-fold lower amount of activated MEKK cDNA was utilized.

PE-mediated ANF Expression Is Mediated through MEKK Rather than Raf—The observation that activated MEKK and activated Raf both stimulate ANF expression does not indicate whether PE-induced cardiac gene expression is dependent on MEKK or Raf or both. To address this question, myocytes were transfected with empty vector or vector encoding dominant

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**FIG. 2. PE induces sustained activation of JNK.** Myocytes were mock-stimulated or stimulated with PE for the indicated times, and endogenous JNK was immunoprecipitated and assayed for activity by measuring incorporation of [γ-32P]ATP into the GST-c-Jun-(1–79) substrate. JNK activity was apparent within 20 min and was sustained for at least 48 h (A). B is an autoradiogram from one of three experiments summarized in A. A Western blot from the same experiment probed with an anti-JNK1 antibody, which recognizes both JNK1 and JNK2, shows no increase in JNK1 or JNK2 protein levels with PE treatment (C).

**FIG. 3. Activated Ras stimulates JNK activity.** Myocytes were transfected with 10 μg of SRα3 (control) or activated (Leu-61) Ras (Ras*) together with 5 μg of SRα-HA-JNK1. The HA-tagged kinase was immunoprecipitated, and activity was assayed by assessing incorporation of [γ-32P]ATP into GST-c-Jun-(1–79). Data are from two experiments performed in duplicate and are represented as the average fold stimulation ± S.E. of kinase activities over vector control-transfected samples. A representative autoradiogram from one of the experiments is shown in the inset; the two left lanes are control-transfected samples, and the two right lanes are Leu-61 Ras-transfected samples.

| Plasmid       | GAL4-luciferase expression (% maximal) |
|--------------|--------------------------------------|
|              | Control  | +PE       |
| Vector (SRα3) | 4.5 ± 1.3    | 100.0 ± 7.1 |
| dn-Ras       | 7.7 ± 2.5    | 14.6 ± 2.0  |
| Vector (SRα3) | 13.9 ± 5.2   | 100.0 ± 13.0|
| dn-MEKK      | 6.1 ± 1.5    | 7.3 ± 1.4  |

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**TABLE I**

| Plasmid       | GAL4-luciferase expression (% maximal) |
|--------------|--------------------------------------|
|              | Control  | +PE       |
| Vector (SRα3) | 4.5 ± 1.3    | 100.0 ± 7.1 |
| dn-Ras       | 7.7 ± 2.5    | 14.6 ± 2.0  |
| Vector (SRα3) | 13.9 ± 5.2   | 100.0 ± 13.0|
| dn-MEKK      | 6.1 ± 1.5    | 7.3 ± 1.4  |
interfering Raf-1 or dominant interfering MEKK along with the ANF reporter gene and subsequently stimulated with PE. Dominant interfering Raf-1 failed to block PE-mediated ANF-luciferase expression, although it was able to inhibit the response to activated (Val-12) Ras (Fig. 4, A and B). In contrast, dominant negative MEKK significantly inhibited PE- and Ras-induced ANF reporter gene expression (Fig. 4, A and B). These data suggest that PE mediates its effects on ANF gene expression, in addition to its effects on JNK (Table I), through MEKK rather than Raf.

**JNK but Not ERK Is Activated in Left Ventricles of Ras Transgenic Mice**—It has recently been shown that ventricular expression of Val-12 Ras in mice induces cardiac hypertrophy characterized by increased ratios of left ventricular weight to body weight and increased ventricular ANF expression (3). The left ventricles of 6-week-old transgenic Ras and wild-type (control) mice were rapidly isolated, frozen, homogenized, and subjected to kinase assays to determine JNK, ERK1, and ERK2 activities. Tissue from six Ras animals and four control animals were analyzed. JNK activity was consistently higher in the tissue from Ras animals; however, no difference was observed in either ERK1 or ERK2 activity (Fig. 5). These data demonstrate that constitutive activation of Ras in vivo correlates with increased JNK, but not ERK, activity.

**DISCUSSION**

Hypertrophic agents such as the α1-Adr agonist PE activate a program of genetic and morphological changes in terminally differentiated cardiac myocytes. The PE-induced hypertrophic responses have been shown to be Ras-dependent (2). Consistent with this, we find that PE increases the amount of GTP-bound Ras and hence the activity of Ras. These data confirm findings reported by Thorburn's laboratory (31) and raised the question as to the effectors of Ras involved in regulating hypertrophic responses.

Stimulation of cardiomyocytes with PE activates the ERKs (32, 33), and several studies have implicated the Raf-MEK-ERK pathway in mediating PE-induced gene expression (5–9). However, the extent of involvement of this signaling pathway remains controversial (10, 11). Our current data demonstrate that while constitutively activated Raf can induce ANF expression, a dominant negative mutant displays no inhibitory effect on PE-induced ANF expression. As a result, activation of an additional signaling pathway would appear to be necessary for the transduction of PE-evoked signals leading to hypertrophy.

The JNK family of MAP kinases can be activated via heterotrimeric G-proteins (34–38), as well as low molecular weight GTPases including Ras (24, 25, 39, 40). While JNK activation has previously been associated with stress responses such as apoptosis (41–43), it is now evident that it does not simply regulate these pathological states. In cardiac myocytes, we demonstrate that α1-AdrR stimulation increases JNK activity within 20 min of PE treatment and that JNK activity remains elevated for at least 48 h. This is in contrast to the ERKs, which have been shown to be maximally activated at 5 min, declining to basal levels thereafter (8, 10). That the kinetics of JNK...
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