

# Regulation of cardiac hypertrophy in vivo by the stress-activated protein kinases/c-Jun NH<sub>2</sub>-terminal kinases

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Cardiac hypertrophy often presages the development of heart failure. Numerous cytosolic signaling pathways have been implicated in the hypertrophic response in cardiomyocytes in culture, but their roles in the hypertrophic response to physiologically relevant stimuli in vivo is unclear. We previously reported that adenovirus-mediated gene transfer of SEK-1(KR), a dominant inhibitory mutant of the immediate upstream activator of the stress-activated protein kinases (SAPKs), abrogates the hypertrophic response of neonatal rat cardiomyocytes to endothelin-1 in culture. We now report that gene transfer of SEK-1(KR) to the adult rat heart blocks SAPK activation by pressure overload, demonstrating that the activity of cytosolic signaling pathways can be inhibited by gene transfer of loss-of-function mutants in vivo. Furthermore, gene transfer of SEK-1(KR) inhibited pressure overload-induced cardiac hypertrophy, as determined by echocardiography and several postmortem measures including left ventricular (LV) wall thickness, the ratio of LV weight to body weight, cardiomyocyte diameter, and inhibition of atrial natriuretic factor expression. Our data suggest that the SAPKs are critical regulators of cardiac hypertrophy in vivo, and therefore may serve as novel drug targets in the treatment of hypertrophy and heart failure.

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## Introduction

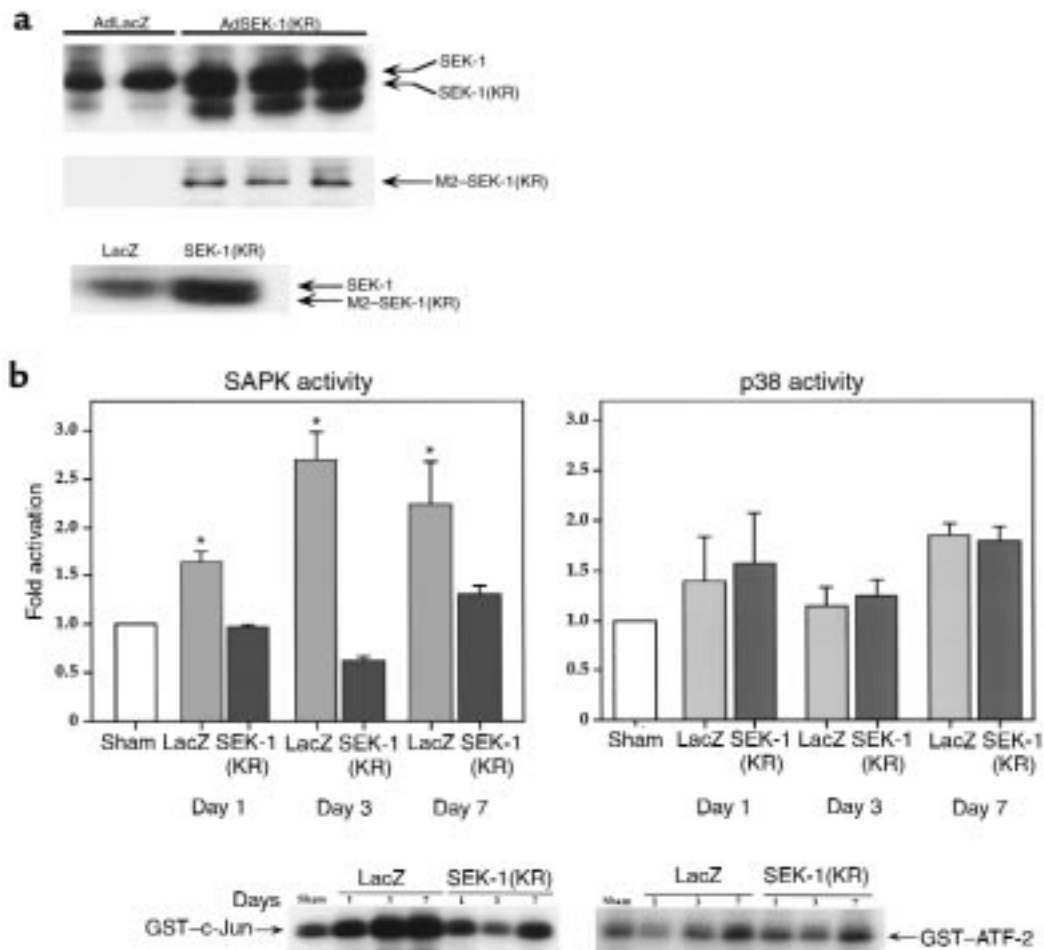
Cardiac hypertrophy is a major risk factor for the development of congestive heart failure and death. Although hypertrophy is a compensatory response to increased wall stress in its earliest stages, the hypertrophic heart often undergoes a transition to heart failure, even if the stimulus to hypertrophy is removed. Expression of constitutively active mutants of any of several signaling molecules — including the small GTP-binding protein Ras (1), the  $\alpha$  subunit of the heterotrimeric G protein Gq (2–4), protein kinase C $\beta$  (5), and the serine phosphatase calcineurin and one of its targets, a nuclear factor of activated T cells 3 (NF-AT3) (6) — can induce hypertrophic responses in the hearts of transgenic mice. Although studies using these mutants can identify a constitutively activated pathway as sufficient to induce a hypertrophic response, they cannot determine whether a pathway is necessary, or what role the pathway might play in the response to hypertrophic stimuli. Only 2 signaling molecules, G $\alpha$ q (7) and calcineurin (8, 9), have been implicated in the development of hypertrophy in response to a physiologically relevant stimulus — pressure overload — in vivo. Other studies have questioned the importance of the calcineurin/NF-AT3 pathway (10–15), and have suggested that there must be pathways parallel to calcineurin that signal hypertrophy in vivo (16–18). We postulated that one additional pathway might be the stress-activated protein kinase (SAPK), or c-Jun NH<sub>2</sub>-terminal kinase (JNK), pathway because we had demonstrated that blocking SAPK activation abro-

gated the hypertrophic response of neonatal cardiomyocytes to endothelin-1 (ET-1) (19).

To determine whether the SAPKs were important in the hypertrophic response in the intact animal, we used a protocol of adenovirus-mediated gene transfer. We previously showed that this protocol allowed the expression of transgenes throughout the myocardium of the adult rat at sufficiently high levels to alter contractile function of the heart (20). The gene we expressed in this study was M2 epitope-tagged SEK-1(KR). SEK-1(KR) is a kinase-inactive mutant of SEK-1 (also termed MKK4), the immediate upstream activator of the SAPKs (21). SEK-1(KR) functions as a specific dominant negative inhibitor of SAPK activation when expressed in cardiomyocytes (19). In this study, we determined the effect of SEK-1(KR) expression on the hypertrophic response to pressure overload induced by supraaortic aortic banding.

## Methods

*Construction of a recombinant adenoviral vector carrying SEK-1(KR) cDNA.* SEK-1(KR) was produced by PCR using primers designed to produce a lysine→arginine substitution at lysine 129 in the ATP binding site of SEK-1. Methionine 33, an alternative start site (21), was used in the creation of SEK-1(KR) to produce a protein that was smaller than the endogenous kinase. This allowed us to determine relative levels of expression of SEK-1 and SEK-1(KR). AdSEK-1(KR), the recombinant adenovirus encoding SEK-1(KR), was prepared as described (19). The recombinant adenovirus carrying the *Escherichia coli*



**Figure 1** Inhibition of pressure overload-induced SAPK activation by gene transfer of SEK-1(KR). (a) Adenovirus-mediated gene transfer of SEK-1(KR) in the rat heart. Top: Three rats were transduced with AdLacZ and 3 with AdSEK-1(KR), as described in Methods and in ref. 20. After 72 hours, myocardial lysates were prepared and subjected to SDS-PAGE and immunoblotting with anti-SEK-1 antibody. Arrows identify endogenous SEK-1 and SEK-1(KR). Middle: The lysates from the rats injected with AdLacZ or AdSEK-1(KR) (above) were also blotted with anti-M2 mAb to confirm expression of M2-SEK-1(KR). Bottom: An additional 2 rats were sacrificed 24 hours after injection of AdLacZ or AdSEK-1(KR), and lysates were blotted with anti-SEK-1 antibody to confirm expression of the transgene at this earlier time point. (b) Inhibition of SAPK activation by SEK-1(KR). Animals were transduced with AdLacZ or AdSEK-1(KR), subjected to aortic banding, and sacrificed at 1, 3, or 7 days after banding. Immune-complex SAPK assays (left) or p38 assays (right) were performed. A representative experiment is shown below each graph. In the graph, kinase activity is expressed as fold increase in activity over that of control animals that were injected with vehicle and subjected to sham surgery. For each group,  $n = 4$ . Data are presented as mean  $\pm$  SD. \* $P < 0.05$  by Student's  $t$  test for animals transduced with AdLacZ vs. sham control, and for AdLacZ vs. AdSEK-1(KR).

LacZ gene encoding  $\beta$ -galactosidase (AdLacZ) was kindly provided by David Dichek (Gladstone Institute of Cardiovascular Disease, San Francisco, California, USA)(22).

**Adenoviral delivery in vivo and pressure overload model of hypertrophy.** The method of adenoviral delivery was as described previously (20). Briefly, male Sprague-Dawley rats (weighing 280–320 g) were anesthetized with intraperitoneal pentobarbital and placed on a ventilator. A 22-gauge catheter containing 200  $\mu$ L of an adenovirus solution containing approximately  $10^{10}$  plaque-forming units of either AdLacZ or AdSEK-1(KR) was advanced from the apex of the left ventricle to the aortic root. A single clamp was placed on the aorta and the pulmonary artery at a site distal to the tip of the catheter in the aorta, and the solution was injected. The clamp was held for 10 seconds while the heart pumped against a closed system (isovolumically), and then released. The rats were

then subjected to supravalvular aortic banding with a 0.58-mm (internal diameter) tantalum clip. The clip was adjusted to achieve a left ventricular (LV) systolic pressure of approximately 170 mmHg. Control rats underwent a sham operation.

**Immune-complex kinase assays.** Assays for activity of endogenous SAPK and the stress-response mitogen-activated protein kinase p38 were performed as described (19, 23). Hearts were rapidly excised, and the left ventricle was freed and immediately immersed in liquid nitrogen, pulverized, and homogenized with a Polytron (Brinkman Instruments, Westbury, New York, USA). Supernatants from heart lysates were matched for protein concentration and then incubated with either anti-SAPK antiserum (produced as described; ref. 24) or anti-p38 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, California, USA). Kinase assays were performed with glutathione-S-

transferase-c-Jun(1-135) [GST-c-Jun(1-135)] for SAPKs, or GST-activating transcription factor-2(8-94) [GST-ATF-2(8-94)] for p38, and 100  $\mu$ M [ $\gamma$ - $^{32}$ P]ATP (3,000–5,000 cpm/pmol). The proteins were separated by SDS-PAGE, and the GST-c-Jun or GST-ATF-2 bands were excised from the gel. The incorporated radioactivity was determined by scintillation counting.

**Western blotting.** Lysates were matched for protein, separated by SDS-PAGE, and transferred to PVDF membranes (Milipore Corp., Bedford, Massachusetts, USA). The membranes were probed with anti-SEK-1, anti-SAPK, anti-p38, anti-ERK-1, anti-ERK-2 (Santa Cruz Biotechnology Inc.), anti-phospho-ERK (New England Biolabs Inc., Beverly, Massachusetts, USA), anti-M2 mAb (Sigma Chemical Co., St. Louis, Missouri, USA), or anti-rat atrial natriuretic factor (ANF) (Peninsula Laboratories Inc., Belmont, California, USA). Antibody binding was detected with peroxidase-conjugated goat anti-rabbit or anti-mouse IgG and chemiluminescence.

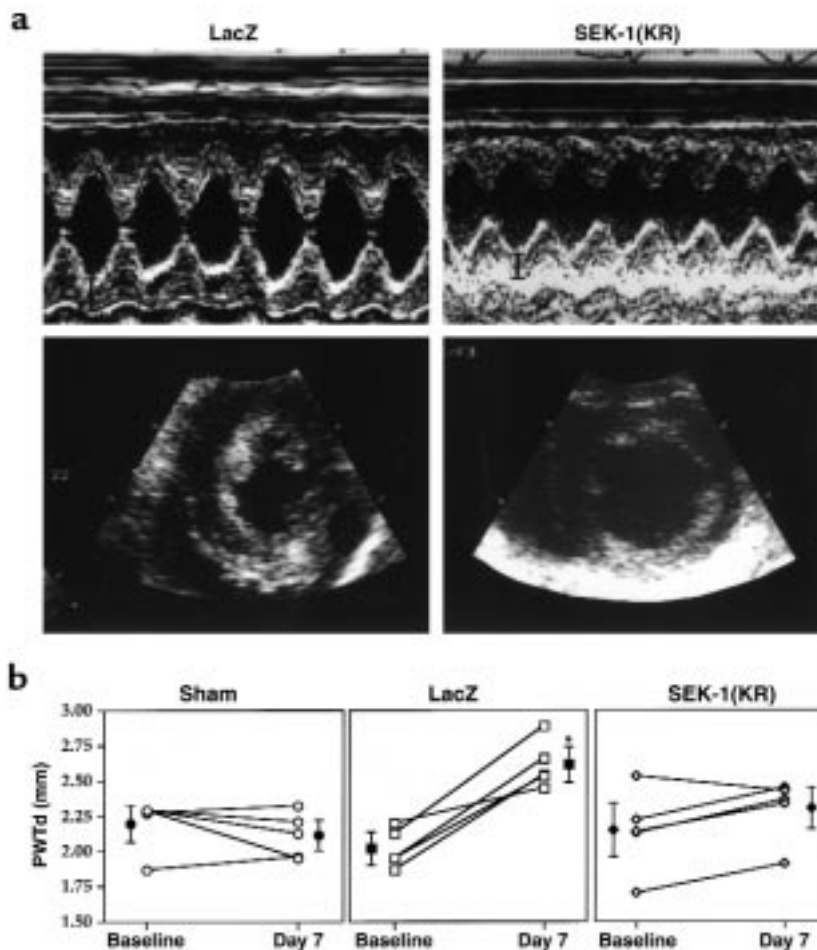
**Echocardiographic studies.** Animals were anesthetized with intraperitoneally injected pentobarbital (50 mg/kg) and mechanically ventilated; then the anterior chest was shaved. Transthoracic M-mode and 2-dimensional (2D) echocardiography was performed with a Sonos 5500 imaging system (Hewlett-Packard, Andover, Massachusetts, USA) using a 12-MHz broadband transducer. A mid-papillary-level LV short-axis view was obtained, and all images were recorded at the lowest allowable depth setting and sector width to achieve maximum frame rate. Images were digitally recorded on a magneto-optical disk and simultaneously recorded onto half-inch VHS videotape. Measurements of posterior wall thickness and LV dimension at end-diastole and end-systole were performed off-line from the digitally acquired M-mode frames by an experienced echocardiographer blinded to the experimental

protocol. Fractional shortening [(end-diastolic dimension – end-systolic dimension)/end-diastolic dimension] and percent systolic thickening of the posterior wall [(end-systolic thickness – end-diastolic thickness)/end-diastolic thickness] were then calculated. Systolic wall stress was calculated from the following formula: LV systolic wall stress =  $1.36(\text{LV systolic pressure} \times \text{LV end-systolic diameter}) / (2 \times \text{systolic wall thickness})$  (9).

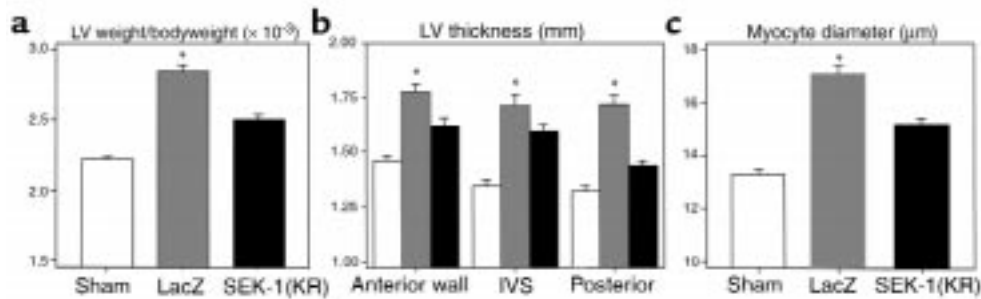
**Determination of LV wall thickness and LV weight/body weight ratio.** Animals were sacrificed 7 days after aortic banding or sham surgery. Hearts were arrested at end-diastole, and the LV free wall and the interventricular septum were freed from the right ventricle and weighed. A cross-section was obtained just below the plane of the mitral valve, and thickness of the anterior wall, posterior wall, and interventricular septum were determined.

**Determination of cardiomyocyte diameter.** Hearts were retrograde-perfused through the aorta at a perfusion pressure of 80–100 mmHg with fixative solution containing 4% paraformaldehyde for 5 minutes. Transverse mid-ventricular slices (10  $\mu$ m) of the hearts were embedded in paraffin, stained with hematoxylin and eosin, and examined by light microscopy ( $\times 100$ ). Mean myocyte diameter was determined by measurement of transnuclear widths of random, longitudinally oriented myocytes by 2 observers, as described (25).

**Statistical analysis.** Data are expressed and presented in the figures as mean  $\pm$  SD. A Student's *t* test was used to



**Figure 2**  
Inhibition of pressure overload-induced cardiac hypertrophy by gene transfer of SEK-1(KR): echocardiographic measurements. Adult rats were transduced with AdSEK-1(KR) or AdLacZ, followed by aortic banding. Control animals underwent a sham operation. (a) Echocardiographic images. Representative M-mode (top) and 2D (bottom) echocardiographic images at 7 days after aortic banding in an animal transduced with AdLacZ (left) or AdSEK-1(KR) (right). For the M-mode studies, the bar delineates the posterior wall thickness at end-diastole, the primary echocardiographic end point. (b) Quantitation of posterior wall thickness at end-diastole (PWTd) at baseline and 7 days after aortic banding. Aortic banding produced a statistically significant (32%) increase in PWT in the animals transduced with AdLacZ compared with those transduced with AdSEK-1(KR) (7% increase). For each group,  $n = 5$  animals.  $*P < 0.01$  for AdLacZ at 7 days after banding vs. baseline;  $P < 0.05$  for AdLacZ at 7 days vs. AdSEK-1(KR) at 7 days after banding. Data are presented as mean  $\pm$  SD.



**Figure 3**

Inhibition of pressure overload-induced cardiac hypertrophy by gene transfer of SEK-1(KR): postmortem measurements. (a and b) Ratio of LV weight to body weight and quantitation of wall thickness. Animals ( $n = 7$  in each group) were sacrificed at 7 days after aortic banding; hearts were removed; and the left ventricles were isolated. The left ventricles were weighed, and the ratios of LV weight to body weight were determined (a). The hearts were also sectioned, and wall thicknesses for the anterior wall, the posterior wall, and the interventricular septum (IVS) were determined (b). Open bars, sham control; gray bars, AdLacZ; black bars, AdSEK-1(KR).  $*P < 0.01$  for AdLacZ vs. AdSEK-1(KR), except for thickness of the IVS, where  $P < 0.05$ . (c) Cardiomyocyte diameter. Cardiomyocyte diameter was determined in control animals, and in animals transduced with either AdLacZ or AdSEK-1(KR) ( $n = 3$  in each group). The diameters of 60 myocytes were measured for each animal.  $*P < 0.01$  for AdLacZ vs. AdSEK-1(KR). Data are presented as mean  $\pm$  SD.

compare the means of normally distributed continuous variables. A value of  $P < 0.05$  was chosen as the limit of statistical significance.

### Results

**Expression of SEK-1(KR) in the rat heart.** To determine whether we could effectively express SEK-1(KR) in vivo by adenovirus-mediated gene transfer, we transduced rat hearts with recombinant adenoviruses encoding either  $\beta$ -galactosidase (AdLacZ) or SEK-1(KR) [AdSEK-1(KR)], and analyzed expression in myocardial lysates with immunoblotting. Immunoblotting with a polyclonal antibody to SEK-1 revealed a single band, corresponding to the endogenous protein, in animals transduced with AdLacZ, and a doublet in animals transduced with AdSEK-1(KR) (Figure 1a, top). The more rapidly migrating band of the doublet comigrated with the band recognized by the M2 mAb (Figure 1a, middle), consistent with this band being SEK-1(KR). SEK-1(KR) is approximately 3 kDa smaller than wild-type SEK-1, despite the M2 epitope tag, because we used an alternative start site in the creation of the vector encoding the mutant (21). The more slowly migrating band corresponded to endogenous SEK-1. SEK-1(KR) expression was at least as great as that of the endogenous kinase. These data were from animals that had been transduced 3 days before sacrifice, but SEK-1(KR) expression was also easily detectable at 1 day after transduction (Figure 1a, bottom).

**Effect of expression of SEK-1(KR) on SAPK activation by pressure overload.** To determine whether we could effectively block activation of SAPKs in response to pressure overload in the intact animal by adenovirus-mediated gene transfer of SEK-1(KR), we transduced rat hearts in vivo with AdLacZ or AdSEK-1(KR) and then subjected them to aortic banding. Animals were sacrificed at 1, 3, or 7 days after banding, and SAPK activity was determined in myocardial lysates.

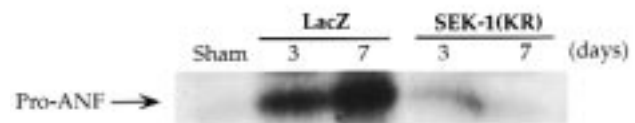
SAPKs were persistently activated 1.7- to 2.7-fold by aortic banding in animals transduced with AdLacZ, compared with sham controls (Figure 1b). This degree of activation was the same as that in animals that underwent aortic banding without prior adenoviral transduc-

tion (data not shown), demonstrating that pressure overload leads to sustained activation of the SAPK pathway.

Expression of SEK-1(KR) significantly inhibited this persistent activation in response to pressure overload. Even at 7 days after banding, when transgene expression declined somewhat, SAPK activation was inhibited by 76% (Figure 1b, left). The differences in kinase activity between groups were determined to be due to differences in specific activity, because levels of kinase expression were not different, as determined by Western blotting with anti-SAPK (not shown). These data confirmed that we could inhibit pressure overload-induced SAPK activation with gene transfer of SEK-1(KR).

Although SEK-1 is not a physiologically relevant p38 kinase, SEK-1(KR) can minimally inhibit p38 activity when markedly overexpressed in some cells (26, 27). Therefore, to confirm the specificity of SEK-1(KR) for the SAPK pathway, we assayed p38 activity in animals transduced with AdLacZ or AdSEK-1(KR) and then subjected to aortic banding (Figure 1b, right). There was modest activation of p38 (~1.5-fold) by pressure overload; this activation was not inhibited by gene transfer of SEK-1(KR).

SEK-1(KR), even when grossly overexpressed, does not block extracellular signal-regulated kinase (ERK) activation (21, 26). To confirm this in our model, we immunoblotted heart lysates with the dual phospho-specific ERK antibody that recognizes activated ERKs. We



**Figure 4**

Inhibition of pressure overload-induced ANF expression by gene transfer of SEK-1(KR). Animals were transduced with AdLacZ or AdSEK-1(KR), subjected to aortic banding, and then sacrificed at 3 days or 7 days after banding. Myocardial lysates from these animals and from a sham control were probed with anti-ANF antibody. The band recognized migrates at an  $M_r$  of 17 kDa, compatible with the  $M_r$  of pro-ANF.

found no significant phosphorylation of the ERKs in response to pressure overload at 1, 3, or 7 days compared with the sham control. Expression of SEK-1(KR) had no effect on basal ERK phosphorylation (data not shown). These data confirm that SEK-1(KR) specifically inhibited the SAPK pathway after gene transfer in vivo.

**Effect of expression of SEK-1(KR) on pressure overload-induced hypertrophy.** To determine whether expression of SEK-1(KR) inhibited pressure overload-induced hypertrophy, we compared uninfected, sham-operated rats with rats that were transduced with AdSEK-1(KR) or AdLacZ and then subjected to aortic banding. To assess ventricular wall thickness in vivo, we performed 2D echocardiography and 2D-directed M-mode echocardiography before viral injection and 7 days after aortic banding, immediately before sacrifice. Pressure overload induced significantly more LV hypertrophy in the animals transduced with AdLacZ than in those transduced with AdSEK-1(KR) (Figure 2a). The 2D-directed M-mode echocardiograms showed that the animals transduced with AdLacZ developed marked LV hypertrophy, characterized by a 32% increase in the thickness of the posterior wall at end-diastole (Figure 2b). Hypertrophy was significantly less marked in animals transduced with AdSEK-1(KR). In these animals, posterior wall thickness increased by only 7% (Figure 2b). Gene transfer of SEK-1(KR) or LacZ, in the absence of aortic banding, had no effect on LV wall thickness (data not shown).

The difference in the degree of hypertrophy was not due to a difference between the groups in the load placed on the heart by aortic banding, because at the time of placement, the aortic band was adjusted to achieve an LV systolic pressure (measured with a high-fidelity pressure transducer in the LV cavity) of approximately 170 mmHg. Animals transduced with AdSEK-1(KR) or AdLacZ demonstrated no significant difference in either transaortic pressure gradient ( $69 \pm 5$  vs.  $66 \pm 8$  mmHg, respectively;  $P = \text{NS}$ ) or LV systolic pressure ( $171 \pm 14$  vs.  $168 \pm 10$  mmHg, respectively;  $P = \text{NS}$ ).

Postmortem measurements also demonstrated that expression of SEK-1(KR) inhibited pressure overload-induced cardiac hypertrophy. The pressure overload-induced increases in the LV weight/body weight ratio (Figure 3a), and in the thickness of the anterior wall, the posterior wall, and the interventricular septum (Figure 3b), were significantly less in the rats transduced with AdSEK-1(KR) compared with those transduced with AdLacZ. The ratio of LV weight to body weight was 28.5% greater than the sham control in animals transduced with AdLacZ, but was only 12.7% greater than the control in the animals transduced with AdSEK-1(KR) (Figure 3a).

Because differences between the groups in the extent of hypertrophy could, in theory, be due to effects on cells other than cardiomyocytes, we also examined the myocyte-specific end point of changes in the diameter of LV myocytes in the 3 groups. Expression of SEK-1(KR) significantly inhibited (by 50%) the pressure overload-induced increase in myocyte diameter compared with myocytes from animals transduced with AdLacZ (Figure 3c). These data confirm that the inhibition of cardiac hypertrophy by expression of SEK-1(KR) was due to inhibition of cardiomyocyte hypertrophy.

**Table 1**

Echocardiographic parameters at 7 days after aortic banding

	POH		
	Sham	LacZ	SEK-1(KR)
PWTd (mm)	2.15 ± 0.15	2.61 ± 0.17	2.28 ± 0.22 <sup>A</sup>
PWTs (mm)	3.24 ± 0.58	3.61 ± 0.31	3.26 ± 0.30 <sup>A</sup>
% T	0.59 ± 0.22	0.38 ± 0.10	0.43 ± 0.11
LVs (mm)	2.24 ± 0.70	2.44 ± 0.45	2.43 ± 0.41
LVd (mm)	5.45 ± 0.47	4.95 ± 0.50	6.06 ± 0.31 <sup>A</sup>
LVsWS (g/cm <sup>2</sup> )	47.3 ± 21.3	77.5 ± 16.5 <sup>B</sup>	88.0 ± 22.6 <sup>B</sup>
FS	0.58 ± 0.16	0.51 ± 0.08	0.59 ± 0.05

Data are presented as mean ± SD. <sup>A</sup> $P < 0.05$  SEK-1(KR) vs. LacZ, <sup>B</sup> $P < 0.05$  LacZ or SEK-1(KR) vs. sham. PWTd, posterior wall thickness at end-diastole; PWTs, posterior wall thickness at end-systole; % T, percent systolic wall thickening; LVs, left ventricular end-systolic dimension; LVd, left ventricular end-diastolic dimension; LVsWS, left ventricular systolic wall stress; FS, fractional shortening.

**Effect of SEK-1(KR) on pressure overload-induced ANF expression.** Cardiac hypertrophy is also associated with a renewal of expression of several fetal genes not normally expressed in the adult left ventricle. These genes serve as markers of the hypertrophic response. One such gene is ANF. ANF was highly expressed at 3 days and 7 days after banding in the left ventricles of animals transduced with AdLacZ compared with sham controls (Figure 4). Gene transfer of SEK-1(KR) markedly reduced ANF expression at both time points.

**Effect of inhibiting hypertrophy on LV function.** Initially, cardiac hypertrophy is a physiologic response to the stress of an increase in the load placed on the heart. Inhibiting that response would be predicted to have deleterious consequences on LV function (9). As expected, systolic wall stress was greater, although not significantly so, in the animals transduced with AdSEK-1(KR) compared with those transduced with AdLacZ (Table 1). The difference in wall stress between the groups was almost entirely due to the decrease in hypertrophy in the AdSEK-1(KR) group. Despite the greater wall stress, we found that inhibiting the development of cardiac hypertrophy was not associated with any deterioration in cardiac contractility, as measured by echocardiographic fractional shortening or systolic thickening. However, LV end-diastolic dimension was significantly greater in the AdSEK-1(KR) group compared with the AdLacZ group, suggesting LV dilatation as a possible early harmful consequence of inhibiting hypertrophy.

## Discussion

Studies of the role of the mitogen-activated protein kinase family (which consists of the ERKs, the SAPKs, and the p38 family) in the hypertrophic response of cardiomyocytes in culture have produced confusing and sometimes contradictory results (reviewed in ref. 28). All 3 pathways have been shown to be sufficient, if constitutively activated, to induce hypertrophic responses in neonatal cardiomyocytes (29–31), but there has been disagreement over which, if any, of the pathways is necessary for expression of various components of the hypertrophic response to physiologically relevant stimuli. For example, 2 groups reported that p38 was central to at least some components of cardiomyocyte response in

culture (32, 33); however, these studies used concentrations of p38 inhibitors that are now known to block activity of SAPK isoforms, including cardiac SAPKs (34, 35). Therefore, while it is likely that p38 is involved in the hypertrophic response, its exact role remains unclear, and its role *in vivo* has not been examined (33). In studies exploring the function of the SAPKs in the hypertrophic response of neonatal cardiomyocytes, 2 studies have suggested that activation of the SAPK pathway was important for activation of an ANF reporter in response to the  $\alpha$ -adrenergic agent phenylephrine (36, 37). However, another study reported that inhibition of the SAPK pathway enhanced activation of that reporter (38). These disparate findings may be due in part to the lack of adequate pharmacologic inhibitors of the SAPKs, which forces reliance upon transfection and consequently surrogates for hypertrophy. These surrogates, such as the activity of ANF reporter constructs, are subject to artifact (39), and may or may not adequately reflect the hypertrophic response (40).

In the absence of pharmacologic inhibitors, and to sidestep the limitations of transfection, we used adenovirus-mediated gene transfer of SEK-1(KR) to inhibit SAPK signaling in neonatal rat cardiomyocytes. We found that expression of SEK-1(KR) blocked ET-1-induced hypertrophy, as determined by the 3 characteristic markers of hypertrophy in these cells: increased protein synthesis, induction of ANF mRNA expression, and enhanced organization of sarcomeres (19).

Because of the potent inhibition of ET-1-induced hypertrophy *in vitro*, we examined the role of the SAPKs *in vivo* in response to a physiologically relevant stress — pressure overload. We found that the SAPKs are persistently activated by pressure overload in the rat heart. To block SAPK signaling, we used a technique of adenovirus-mediated gene transfer developed by Hajjar et al. that allows diffuse expression of transgenes throughout the myocardium of the adult rat (20). Using this technique, we have demonstrated that gene transfer of phospholamban, the negative regulator of the sarcoplasmic reticulum calcium ATPase SERCA2a, results in profound physiologic effects on cardiac function (20). In this study, we demonstrate that we can specifically inhibit activation of the SAPK cascade with gene transfer of SEK-1(KR), a dominant inhibitory mutant of that pathway. Although *in vivo* gene transfer of gain-of-function mutants of a signaling pathway's components could be predicted to effectively activate that pathway, it was not clear whether sufficient levels of expression of dominant inhibitory mutants could be achieved to block signal transmission down a cytosolic signaling pathway. Our data confirm that this is possible, and suggest that this approach could be used to identify the role of signaling molecules in various pathologic responses *in vivo*, even in the absence of specific pharmacologic inhibitors. Determining which putative mediators of a response actually play a role in that response *in vivo* should allow better targeting of searches for small molecular weight inhibitors.

Most importantly, our data demonstrate that the development of pressure overload-induced cardiac hypertrophy depends, at least in part, on a functioning SAPK pathway, because inhibiting pressure overload-induced SAPK

activation significantly inhibited hypertrophy, as determined by echocardiography, by several postmortem measures, and by inhibition of ANF expression. We believe that our data can be reconciled with studies implicating other signaling molecules, such as  $G\alpha_q$ , Rac-1, and Ras in the hypertrophic response (1–4, 7, 41), because activated  $\alpha_q$ , Rac-1, and Ras activate the SAPKs in cells in culture, and SAPK activity is elevated in the hearts of transgenic mice expressing activated Ras (36, 42–46). Therefore, the SAPKs might be downstream effectors in the pathway signaling hypertrophy from these receptors and molecules.

Tremendous controversy has surrounded the question of whether the calcineurin inhibitor cyclosporin A can prevent cardiac hypertrophy *in vivo*. At present, the role of calcineurin in cardiac hypertrophy is unclear. We believe that the best way to reconcile the disparate findings of the various studies using calcineurin inhibitors, and our findings implicating the SAPKs, is to postulate multiple parallel pathways signaling hypertrophy (18, 47). Any 1 pathway, if constitutively activated, may be able to drive the hypertrophic response, but all pathways may cooperatively interact to produce the response to physiologically relevant stimuli. Given the dramatic reprogramming of gene expression that takes place in the heart in response to hypertrophic stress (47, 48), it is unlikely that any 1 pathway could regulate all facets of the response.

There is precedence for cooperative interactions of parallel signaling pathways in the costimulatory pathway of T cells, which requires signals from both calcineurin and the SAPKs for full expression of a number of immune-response genes (49–51). Signals from the 2 pathways are integrated at the promoters of relevant genes by cooperative interactions of NF-ATs and AP-1 (a heterodimer of c-Fos and the SAPK target c-Jun) (51, 52). Similar costimulatory pathways may be operative in the hypertrophic response (18).

Recently, concerns have arisen over possible harmful consequences of inhibiting hypertrophy, because hypertrophy is the primary means by which the heart normalizes systolic wall stress (9). While our data do not demonstrate any deterioration in LV systolic function in the animals transduced with AdSEK-1(KR), the LV end-diastolic dimension is increased in these animals compared with the AdLacZ group. This raises the possibility that LV dilatation is a compensatory mechanism for maintaining systolic function in the face of increased wall stress. Although this finding is of concern, it must be understood that the model we used induces extreme increases in wall stress. This model has been used extensively and is excellent for identifying mediators of hypertrophy; but in order to determine whether inhibiting the hypertrophic response to more physiologic stresses (e.g., chronic hypertension) has deleterious effects, chronic models with less severe stress will need to be used. Specific, nontoxic chemical inhibitors of the pathway, or gene-transfer vectors that allow long-term expression, will be required.

Our approach has certain limitations. First, SEK-1(KR) was expressed from a viral promoter, allowing expression of the transgene in organs other than the heart. This raises the possibility that the effects of SEK-1(KR) on the hypertrophic response could have been mediated by effects on the production or action of paracrine factors,

including angiotensin II, ET-1, and various cytokines. However, because most of these factors activate the ERKs and/or p38, and there were no differences in ERK or p38 activity between the AdLacZ and AdSEK-1(KR) groups, it is unlikely that AdSEK-1(KR) blocked the production or action of these factors. These data, taken together with previous reports that activation of the SAPK pathway is both necessary and sufficient for the hypertrophic response of cardiomyocytes *in vitro* (19, 31), suggest that the primary effect of SEK-1(KR) in blocking pressure overload hypertrophy is a direct effect on the myocytes. The use of cardiac-specific promoters would resolve this issue, but at present it is not clear that sufficient levels of expression of dominant inhibitory transgenes can be achieved with these promoters to block cytosolic signaling pathways. A second limitation is that gene transfer *in vivo* produces a mild inflammatory cell infiltrate in the heart (20). However, there is no disruption of normal myocardial architecture or collagen deposition, and in this study, the degree of infiltrate was not different between the AdLacZ and the AdSEK-1(KR) groups.

In summary, our data demonstrate that adenovirus-mediated gene transfer of dominant inhibitory mutants can be used to block activation of a cytosolic signaling pathway in the heart. Furthermore, our findings identify the SAPKs as important mediators of the hypertrophic response *in vivo*, and establish them as potential targets for the development of novel strategies for the treatment of cardiac hypertrophy and heart failure.

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