Cardiac Muscle Cell Hypertrophy and Apoptosis Induced by Distinct Members of the p38 Mitogen-activated Protein Kinase Family*

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p38 mitogen-activated protein (MAP) kinase activities were significantly increased in mouse hearts after chronic transverse aortic constriction, coincident with the onset of ventricular hypertrophy. Infection of cardiomyocytes with adenoviral vectors expressing upstream activators for the p38 kinases, activated mutants of MAP kinase kinase 3b(E) (MKK3bE) and MAP kinase kinase 6b(E) (MKK6bE), elicited characteristic hypertrophic responses, including an increase in cell size, enhanced sarcomeric organization, and elevated atrial natriuretic factor expression. Overexpression of the activated MKK3bE in cardiomyocytes also led to an increase in apoptosis. The hypertrophic response was enhanced by co-infection of an adenoviral vector expressing wild type p38β, and was suppressed by the p38β dominant negative mutant. In contrast, the MKK3bE-induced cell death was increased by co-infection of an adenovirus expressing wild type p38α, and was suppressed by the dominant negative p38α mutant. This provides the first evidence in any cell system for divergent physiological functions for different members of the p38 MAP kinase family. The direct involvement of p38 pathways in cardiac hypertrophy and apoptosis suggests a significant role for p38 signaling in the pathophysiology of heart failure.

A variety of pathophysiologival stimuli, such as myocardial infarction, hypertension, valvular diseases, viral myocarditis, and dilated cardiomyopathy can lead to an increase in cardiac workload and elevated mechanical stress on cardiomyocytes. In response to hemodynamic overload, an adaptive hypertrophic response is triggered, which is characterized by an increase in the mass and volume of individual myocytes, resulting in an increase of heart weight without an increase in the number of cardiomyocytes (reviewed in Refs. 1 and 2). During the hypertrophic response, cardiomyocytes activate a distinct pattern of gene expression that eventually results in qualitative and quantitative alterations in contractile protein content and the induction of an embryonic gene program (3, 4). As hemodynamic overload persists, the stressed heart enters a critical transition from compensatory hypertrophy to decompensated heart failure. Chamber dilatation, excitation-contraction uncoupling, abnormal interstitial morphology, sarcomeric disorganization, altered energy metabolism, and the loss of viable myocytes are common features found in end-stage failing hearts (5). Signaling molecules that transduce the signals from this extracellular stress to different cellular compartments play central roles in mediating the hypertrophic process and the transition to heart failure. Accordingly, the identification and characterization of these signaling molecules have been the focus of intense study in recent years (6).

One recently identified group of signaling molecules that mediates environmental stress responses in various cell types is the family of p38 mitogen-activated protein (MAP)1 kinases. The p38 MAP kinase activity is activated by dual phosphorylation on a Thr-Gly-Tyr motif in response to endotoxin, cytokines, physical stress (such as hyperosmolarity), and chemical stress (such as hydrogen peroxide) (7–12). In non-cardiac cells, p38 MAP kinases have been implicated in gene regulation, morphological alterations, and cell survival in response to various environmental stimuli (13–20). Recently, it has been reported that in ischemia/reperfusion-treated hearts, p38 MAP kinase activities are elevated in association with the onset of hypertrophy and programmed cell death (30, 31). In addition, p38 kinase activities are also significantly induced in transgenic mouse hearts expressing activated Ha-Ras(V12), correlating with the onset of cardiac hypertrophy. However, the specific function of p38 in the development of cardiac hypertrophy and cardiac cell apoptosis have not yet been directly demonstrated.

The intracellular activation cascade for p38 MAP kinases under physiological conditions is still unclear, but several upstream MAP kinase kinases (MKKs) have been identified from in vitro analysis, including MKK3b and MKK6b (24–26). In the family of p38 MAP kinases, at least four isoforms have been identified thus far (8, 12, 27–29). Two well characterized isoforms, α and β, share extensive sequence similarity and a broad range of tissue distribution, including relatively high levels in the heart (8, 27). Although different isoforms of p38 have sim-

1 The abbreviations used are: MAP, mitogen-activated protein; TAC, transverse aortic constriction; MKK, MAP kinase kinase; ANF, atrial natriuretic factor; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH2-terminal kinase; GFP, green fluorescent protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-5-diphenyl tetrazolium bromide.

ilar kinase activities *in vitro* on a given substrate, their specific functions under *in vivo* physiological conditions are largely unknown.

The present study was designed to critically assess the potential function of the p38 pathway in the onset of features that relate to cardiac muscle cell hypertrophy and failure. The p38 MAP kinase activities are activated during hypertrophy following *in vivo* pressure overload, suggesting their potential role in signal transduction of mechanical stimuli. To dissect specific functions of the p38 MAP kinases, we utilized recombinant adenoviruses to achieve efficient expression of the p38 signaling molecules in cultured cardiomyocytes, which allowed biochemical as well as morphological analysis on entire cell populations. Forced activation of p38 MAP activity results in characteristic features of hypertrophy, whereas the activation of p38 activ-ity leads to the induction of myocyte apoptosis. The opposing effects of the p38 MAP kinase isoforms suggest that the activation of the p38 pathway may contribute to the development of hypertrophy and the transition to overt heart failure.

**EXPERIMENTAL PROCEDURES**

**Transverse Aortic Constriction Surgery**—Transverse thoracic aortic constriction was performed as described previously (32, 33) on 8-week-old adult mice (C57BL/6XSJL, Jackson Laboratories). Briefly, in the anesthetized animals, a 7–0 nylon suture ligature was tied against a 27-gauge needle at the transverse aorta to produce a 65–70% constric-tion following the removal of the needle. At 4 h or 7 days after surgery, animals from the experimental and sham-operated groups were killed and the hearts removed. Ventricular chambers were weighed and quickly frozen in liquid nitrogen for protein extraction.

**Recombinant Adenovirus Vectors**—Recombinant adenoviruses expressing activated MKK3bE, MKK6bE, wild type p38b, and their corresponding dominant negative (TGY→GAF) mutants, p38bΔdn and p38bΔjdn, driven by a cytomegalovirus promoter were gen-erated as described previously (22, 34). Similarly, recombinant adeno-viruses expressing GFP and Ha-Ras-v were generated using cDNAs from pEGFP (CLONTECH) and mutant Ha-Ras(V12) (35). All recom-binant adenoviruses were tested for transgene expression in cardiac myocytes by reverse transcriptase-polymerase chain reaction, Western blot, or kinase assays. The concentrated recombinant adenoviruses were prepared and titered as described (34).

**Cardiomyocyte Culture and Adenoviral Infection**—Neonatal car-diomycocytes were prepared using a Percoll gradient method as de-scribed previously (36). Myocytes from 1–2-day-old Sprague-Dawley rats were plated in serum-containing medium (4:1 Dulbecco’s modified Eagle’s medium:medium 199, 10% horse serum, 5% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10 μM glutamine) overnight. Subsequently, the cells were changed into low serum medium containing 1% horse serum and 0.5% fetal bovine serum, and infected with adenoviruses as a multiplicity of infection of 50–100 particles/cell for 12 h. The cells were then cultured in serum-free me-dium for an additional 36–70 h before morphological or biochemical analysis.

**MAP Kinase Assays**—Protein extracts from heart or myocytes were prepared and assayed for kinase activities, as described previously (37). Briefly, crushed frozen heart tissue or cells were harvested in lysis buffer (25 mM HEPES, pH 7.6, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 3 mM β-glycerophosphate, 100 mM Na3VO4, 1% Nonidet P-40, 1 mM dithiothreitol, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 1 mM phenylmethylsulfonyl fluoride). p38 kinases were immunoprecipitated using rabbit polyclonal anti-p38 antisum (from J. Han (Scirrps Research Institute, La Jolla, CA) and Santa Cruz Biotechnology, Inc.) conjugated to protein A-Sepharose. The kinase assays were then performed at 30 °C using γ-32P]ATP and myelin basic protein (Sigma) or glutathione S-transferase-ATF2 as a substrate. The phosphorylated substrate was separated by SDS-polyacrylamide gel electrophoresis, and visualized by autoradiography. The incorporated 32P in the substrate was quantified by radioanalytic scanning (AMBIS). A similar protocol was used to assay kinase activities of ERK1 and c-Jun N-terminal kinase (JNK) using myelin basic protein and c-Jun as substrates, as described previously (37).

**Immunohistochemical Assay**—Cells were fixed in 3.7% formaldehyde and permeabilized in 0.3% Triton X-100. The atrial natriuretic factor (ANF) protein was detected using rabbit anti-rat α-ANF polyclonal antibody (Peninsula Laboratories) and fluorescein isothiocyanate-con-

![Fig. 1. p38 MAP kinase activities in the pressure overloaded murine heart after chronic transverse aortic constriction.](http://www.jbc.org/content/doi/10.1074/jbc.M080906200.full)
animal hearts as compared with those from the sham-operated group. Activation of the p38 MAP kinase activities during the development of hypertrophy suggested a potential role for this pathway in mediating defined features of the hypertrophic response.

Expression of p38 Signaling Molecules in Cultured Cardiomyocytes by Adenoviral Vector-mediated Gene Transfer—To study the function of the p38 pathway in neonatal cardiac myocytes, we utilized recombinant adenoviruses as an efficient way to deliver signaling molecules in the p38 MAP kinase pathway to induce hypertrophy in cardiomyocytes. A constitutively active mutant of MKK3bE and MKK6bE were detected by a monoclonal anti-hemagglutinin antibody (Boehringer Mannheim) and the FLAG-tagged wild type p38 MAP kinases (26, 27).

**Activation of the p38 Pathway in Cardiomyocytes Induces Several Independent Features of the Hypertrophic Response and Cell Death**—To assess the effects of the activated p38 pathway on cardiomyocytes, a number of independent effects on cellular morphology were assayed including surface area, F-actin organization, and sarcomere organization. The expression of a marker of the hypertrophic response, ANF, was also monitored by immunohistochemistry and RNase protection assays. In comparison to uninfected cells, MKK3bE- and MKK6bE-infected cells displayed an increase in cell surface area, enhanced organization of sarcomeric units with increased nonstriated myofibrils, and induction of ANF expression (Table I and Fig. 3). Levels of ANF mRNA, as quantified by RNase protection, were elevated approximately 2.9-fold by MKK3bE and 4.42-fold by MKK6bE (Fig. 4). These are all well characterized features of myocardial cell hypertrophy in this *in vitro* assay system induced by other bona fide hypertrophic stimuli (3, 4). To determine whether the effects of MKK3bE and MKK6bE were indeed mediated by the p38 MAP kinases, cardiomyocytes were treated with SB202190, a pyridinyl imidazole compound that specifically inhibits p38 kinase activity (12, 13). When cardiomyocytes were infected with vectors expressing MKK3bE and MKK6bE, the endogenous p38 MAP kinase activities were suppressed both morphologically and biochemically, as indicated by the decrease in kinase activity and the decrease in induction of ANF expression in infected cardiac myocytes was detected at comparable levels by Western blot analysis (Fig. 2B). When cardiomyocytes were infected with vectors expressing MKK3bE and MKK6bE, the endogenous p38 MAP kinase activities were induced 12.2-fold and 3.0-fold, respectively (Fig. 2C). In contrast, the endogenous JNK activity and ERK activity were not activated by either virus (data not shown). This result was consistent with previous studies that have established MKK3bE and MKK6bE as specific upstream activators of the p38 MAP kinases (26, 27).

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Further observations at a later time point indicated that inhibition of MKK3bE also led to a significant increase in cell death (Fig. 5A). Using the MTT staining method as a quantitative cell survival assay, we estimated that the number of viable myocytes dropped to approximately 50% 72 h after infection as compared with control samples (Fig. 5C). In contrast,
increase in cell surface area that was occasionally associated in myocytes had little effect, with the exception of a modest along with recombinant adenoviruses that direct the expres-

sion of lacZ. Top panel, myocytes were infected with MKK3bE virus alone or in the presence of 20 mM SB202190 (Calbiochem, San Diego, CA), as indicated. Bottom panel, myocytes were infected with MKK6bE alone or in the presence of the same p38 inhibitor. All pictures were photographed at the same magnification. Positive staining for ANF protein was identified as a “ringlike” green fluorescent signal at the perinuclear regions of the myocytes.

MKK6bE did not appear to suppress cell death (Fig. 5B) even though its expression level was comparable with that of the MKK3bE in myocytes (Fig. 2B).

Differential Effects of p38α and p38β on Cardiac Muscle Cells—Although it has been suggested from previous in vitro studies that p38α and p38β isoforms may have preferential upstream activators and different downstream target substrates (27, 28, 29), their specific functions under physiological conditions has not yet been demonstrated. To dissect the specific roles of these two isoforms in mediating cardiac muscle cell hypertrophy and cell death, adenoviruses expressing the wild type and dominant mutants of p38α or p38β were co-infected along with recombinant adenoviruses that direct the expression of MKK3bE or MKK6bE.

Overexpression of the wild type p38α or p38β isoform alone in myocytes had little effect, with the exception of a modest increase in cell surface area that was occasionally associated with adenoviral infection (Table I).3 Overexpression of wild type p38α in MKK3bE-infected cells, however, disrupted the hypertrophic morphology (Fig. 5A), partially inhibited ANF induction from 2.9-fold to 1.8-fold (Fig. 4), reduced the cell surface area to basal levels (Table I), and led to a marked induction of cell death (Fig. 5C). Although p38α did not appear to significantly affect the size and survival of the MKK6bE-infected cardiomyocytes (Fig. 5B, Table I), the ANF mRNA induction was significantly reduced from 4.6-fold to 2.8-fold (Fig. 4). In contrast, overexpression of wild type p38β augmented the MKK3bE-induced hypertrophy (Fig. 5A), and further induced the ANF mRNA expression from 2.9-fold to 3.9-fold (Fig. 4). Forced activation of p38β also promoted cell survival in the MKK3bE-infected myocytes, as indicated by the MTT assay (Fig. 5C). In MKK6bE-infected cells, overexpression of wild type p38β also had a positive, albeit marginal, effect on the already highly elevated ANF mRNA levels, increasing its expression from 4.6-fold to 5.4-fold (Fig. 4). The surface area was also further increased from 2.67-fold over basal levels to 4.42-fold (Table I). From these results, we postulated that activation of p38β was able to induce several features of the hypertrophic response and also promoted survival of cardiac myocytes, whereas activation of p38α was able to antagonize such an effect and resulted in cell death.

To further support the distinctive roles of p38 isoforms in cardiac myocytes, dominant negative mutants (TGY → AGF) of p38α (p38αdn) and p38β (p38βdn) were also co-expressed along with MKK3bE and MKK6bE (27). Overexpression of the p38α dominant negative mutant in MKK3bE-infected cardiomyocytes did not suppress the hypertrophic response, as indicated by cell surface area (in Table I), cellular morphology (Fig. 5A), and ANF expression (Fig. 4, lane 6). Suppressing p38α activity, however, increased the survival of MKK3bE-infected myocytes (Fig. 5C), therefore confirming the role of p38α in apoptotic induction. In contrast, overexpression of the p38β dominant negative mutant in MKK3bE-infected cells abolished the characteristic features of the hypertrophic phenotype, including a decrease in cell surface area (in Table I), disruption of cellular

3 Y. Wang and K. R. Chien, unpublished observations.
morphology (Fig. 5B), and a reduction in ANF expression (Fig. 4, lane 7). Suppression of p38β activity also led to a significant increase in cell death (Fig. 5C), suggesting its role in promoting survival of myocytes. Similar effects were also observed in MKK6bE-infected cells on ANF expression (Fig. 4, lanes 12 and 13), but to a much lesser extent on cellular morphology and cell survival (see Table I and Fig. 5B). These data further support the notion that activation of p38α was able to induce cell death and suppress hypertrophy, whereas activation of p38β was able to induce hypertrophy and promote cell survival. The opposing effects of the two p38 isoforms were best demonstrated in the MKK3bE-infected cells, whereas the MKK6bE-infected cells were not affected by the different p38 isoforms to a similar extent.

p38α-mediated Cardiac Muscle Cell Death Involves Apoptotic Pathways—To determine whether the observed cell death in
myocytes involved apoptosis, a programmed genetic process, we performed DNA fragmentation assays to detect the presence of internucleosomal laddering in the genomic DNA, which is the hallmark of apoptosis (see Fig. 6). DNA fragmentation was observed in samples from myocytes that were infected with the MKK3bE vector, and the DNA laddering was significantly induced in samples from myocytes co-infected with wild type p38α or the dominant negative mutant of p38β vectors. In comparison, DNA laddering was not detected from control myocytes or myocytes infected with MKK6bE. We also analyzed the integrity of myocyte nuclei by Hoescht dye staining in myocytes. Chromosomal condensation and fragmentation of nuclei, another characteristic feature of apoptotic cells, was also observed in a high percentage of tropomyosin-positive cardiac muscle cells co-infected with MKK3bE and wild type p38α or the dominant negative mutant p38β (shown by arrows in Fig. 7). Taken together, these data suggested that cell death induced by the activation of the p38 MAP kinase pathway was an apoptotic process.

**DISCUSSION**

Cardiac hypertrophy is an adaptive process to cellular stress that involves changes in both gene expression and sarcomeric organization (1, 2). It is believed to be mediated by signaling molecules that transduce the stress signals from the environment into different cellular compartments (for a review, see Ref. 39). In this report, we documented that the p38 MAP kinase activities are induced during the onset of in vivo hypertrophy in an experimental pressure overload model. In cultured cardiomyocytes, activation of the p38 pathway induces several independent characteristic features of myocyte hypertrophy, including an increase in cell surface area, enhanced sarcomeric organization, and expression of an embryonic marker gene, ANF. In the case of MKK3bE-infected myocytes, activation of the p38 pathway is also able to induce cell death. In addition, we have been able to dissect the specific roles of the p38 family members and demonstrate that the p38β isoform mediates the hypertrophic response, whereas the p38α isoform is involved in an apoptotic process.

The distinct phenotypes of hypertrophy and cell death in MKK3bE-infected cardiac muscle cells appears to be dictated by the balance of the relative activity between two different p38 isoforms. This observation could be the result of a quantitative difference between the ability of the two isoforms to activate a common signaling pathway or a qualitative difference in the activation of divergent bona fide pathways for apoptosis and hypertrophy. In other cell types, the p38α isoform has been implicated as part of the Fas-induced apoptotic pathway involving ICE/Ced-3 proteases, suggesting a direct role for the p38 kinase in apoptotic responses (21, 22). Our data with the dominant negative mutants of p38α and p38β also supports a qualitative difference between the p38α- and p38β-mediated responses in apoptosis and hypertrophy, respectively. The final outcome of p38 activation in myocytes, either programmed cell death or hypertrophy, may be determined by the competing downstream pathways as suggested by previous studies of other MAP kinase pathways (48). Interestingly, MKK3b- and MKK6b-induced responses are differentially affected by the overexpression of p38 molecules. Both MKK3b and MKK6b are able to phosphorylate and activate different p38 kinases in vitro, although p38β has been suggested as a preferred substrate for MKK6b rather than MKK3b (25, 26). Therefore, the difference between the effects of MKK3bE and MKK6bE in cardiomyocytes may result from their different specificities to various members of the p38 MAP kinase family. This conclusion remains to be tested when isoform-specific monoclonal antibodies for p38 kinases become available.

Activation of gene expression and the change of cellular morphology in p38 activated cardiac myocytes could be mediated by several distinct downstream target molecules that have been identified in other cell types. A number of transcription factors, including ATF-2, ELK-1, CREB (13, 14), and MEF-2C, have been identified in other cell types. A number of transcription factors, including hsp25 and hsp27, through phosphorylation of MAP kinase-activated protein kinase 2/3 (11, 18, 19, 20). Interestingly, it has been...
shown in non-cardiac cells that p38-mediated activation of hsp27 can induce F-actin reorganization and vinculin recruitment to the focal adhesion complex (46). Additional studies are needed to identify the specific activators as well as downstream targeting molecules of different p38 isoforms and to dissect out the relationship among the effectors of this signaling pathway in vivo.

Two other groups of MAP kinases mediate signal transduction in parallel with the p38 pathway, including ERK and JNK pathways (25–27, 40–44). Previous studies have documented that ERK activation is not sufficient to initiate a hypertrophic response in vitro (44), whereas in vivo its activation is not associated with the hypertrophic phenotype in the Ras transgenic mouse (37). On the other hand, the JNK pathway is also activated in the Ras transgenic animals and its activation is essential for a hypertrophic response in vitro (37). It is highly likely that p38 and JNK are both required to generate a hypertrophic or an apoptotic response in overloaded hearts. Therefore, the potential interactions between the p38 pathway and the JNK or other signaling pathways in cardiac muscle cells is worthy of further investigation.

The finding that a stress-activated signaling pathway may play direct roles in inducing apoptosis has significant implications. There is an increasing body of evidence which suggests that apoptotic cells are a clear feature of heart failure in various animal models, in ischemia/reperfusion-treated hearts, as well as in human end-stage failing hearts (recently reviewed in Ref. 47). Programmed cell death may therefore serve as one of the underlined mechanisms for the transition from hypertrophy to decompensated heart failure. The implication of p38α in apoptosis of cardiomyocytes thereby provides a potential signaling pathway for such an apoptotic response. It will become of interest to determine if p38 pathways play any role in the cell survival effects mediated by cardiostatin-1 (49) via GP130-dependent pathways (50). It will also be of interest to determine if manipulation of the p38 MAP kinase pathway and their downstream target molecules in vivo would have an effect in animal models of heart failure that are associated with apoptosis.

In conclusion, activation of p38 MAP kinase activities during hypertrophy, and the opposing effects of hypertrophic and cell death mediated by the two members of p38 MAP kinase family suggest a potential role of the p38 pathway in the onset of hypertrophy and heart failure. As presented in Fig. 8, a working model can now be constructed whereby hemodynamic stress, as a result of mechanical overload or chronic ischemia, can activate the p38 MAP kinase activities, which subsequently contribute to the hypertrophic response in the initial compensatory phase. As the stress stimulus persists, the balance between hypertrophic and apoptotic signaling is disrupted and, as a consequence, cardiomyocytes lose cellular viability and structural integrity and enter the cell death pathway. The loss of contractile function and viable cells eventually places more stress on the surviving myocytes and initiates the irreversible deterioration of cardiac function, resulting in overt heart failure. The recent development of miniaturized physiological technology (51), strategies for conditional transgenesis and ventricular chamber-restricted gene targeting in the murine heart (54), and genetically based mouse models of distinct forms of concentric and asymmetric hypertrophy (52) and failure (53) should allow a rigorous assessment of the in vivo validity of this model.

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