M KK6 Activates Myocardial Cell NF-κB and Inhibits Apoptosis in a p38 Mitogen-activated Protein Kinase-dependent Manner*

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In cardiac myocytes the stimulation of p38 mitogen-activated protein kinase activates a hypertrophic growth program and the induction of the cardiac-specific genes associated with this program. This study focused on determining whether these novel growth-promoting effects are accompanied by the p38-mediated inhibition of apoptosis, and if so, what signaling pathways might be responsible. Primary neonatal rat ventricular myocytes were driven into apoptosis by treatments known to induce apoptosis in other cell types, e.g. incubation with anisomycin or overexpression constitutively active MEKK-1 (MEKK-1COOH), a protein that strongly activates extracellular signal-regulated kinase and N-terminal c-Jun kinase, but not p38. Overexpression of constitutively active MKK6, MKK6 (Glu), which selectively activates p38 in cardiac myocytes, protected cells from either anisomycin- or MEKK-1COOH-induced apoptosis. This protection was blocked by SB 203580, a selective p38 inhibitor. MKK6 (Glu) also activated transcription mediated by NF-κB, a factor which protects other cell types from apoptosis. The activation of NF-κB and the protection from apoptosis mediated by MKK6 (Glu) were both blocked by SB 203580. Interestingly, overexpression of a mutant form of IκBα, which inhibits nuclear translocation of NF-κB, completely blocked MKK6 (Glu)-activated NF-κB but had little effect on MKK6s anti-apoptotic effects. These findings suggest that, in part, the overexpression of MKK6 (Glu) may foster growth and survival of cardiac myocytes by protecting them from apoptosis in a p38-dependent manner. Additionally, while NF-κB is activated in myocardial cells by p38, this does not appear to be the major mechanism by which MKK6 (Glu) exerts its anti-apoptotic effects in this cell type, suggesting a novel pathway for p38-mediated protection from apoptosis.

The proper growth, development, and function of most tissues requires the appropriate balance between cell growth and death (1). Although a great deal is known about the molecular aspects of cell growth, the nuances of the mechanisms used by cells to regulate death have only recently begun to be appreciated. Among the most widely studied forms of cell death is apoptosis, a form of programmed cell death, or cell “suicide” that is believed to be responsible for the deletion of unwanted cells during organ and tissue development, as well as pathologically induced tissue damage. Apoptosis is an energy-requiring molecular suicide program characterized by cytoplasmic shrinkage, nuclear condensation, and DNA fragmentation into 200-base pair fragments (2–6). Another form of cell death, necrosis, is usually the result of massive cell injury characterized by decreased mitochondrial function and ATP depletion, cytoplasmic swelling, membrane permeabilization, and DNA fragmentation into random sizes. In addition to being activated during development-related cell reduction, apoptosis can be triggered in many cell types by various stresses, including ionizing radiation, osmotic stress, expression of viral proteins such as E1A, and exposure to certain cytokines (7).

Myocardial cell apoptosis is believed to contribute to cardiac dysfunction, including ischemia/reperfusion injury, vascular wall remodeling, heart failure, and myocardial infarction (8). For example, in humans the cardiomyopathy resulting from intractable congestive heart failure has been attributed in part to the loss of myocytes through apoptosis (9). Mimicking cardiac overload by stretching cultured myocardial cells initially activates myocyte growth (10, 11) but later induces myocyte apoptosis (12). Accordingly, it is believed that although the initial response of the myocardium to overload may be cardiac myocyte hypertrophy, prolonged overload results in a remodeling of the myocardium that includes increases in fibroblast growth and collagen deposition, accompanied by decreased numbers of cardiac myocytes, a result of apoptosis (13).

Myocardial cell apoptosis may also serve important developmental roles (14). For example, in the postnatal heart there is a decrease in right ventricular muscle mass that takes place as an adaptation to the decreased load in that chamber just after birth; this selective decrease in tissue mass, which takes place while the left ventricle continues to grow, is the result of chamber-specific apoptosis of cardiac myocytes (12, 15). Apoptosis has also been shown to account for cell death in the conotruncal cushions; this programmed cell death mediates remodeling of the bulbus cordis during early development (16, 17). Also, a cardiac-specific growth factor, cardiotrophin 1, which is believed to play important roles during cardiac development, is believed to protect cardiac myocytes from apoptosis (18). Accordingly, a better understanding of apoptosis in the myocardium is required to establish a clearer picture of cardiac pathology as well as normal growth and development of the heart.

Studies of signaling mechanisms have revealed that the mitogen-activated protein kinases (MAP1 kinases) may play im-

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1 The abbreviations used are: MAP, mitogen-activated protein; MAP-KAP, MAP kinase-activated protein kinase; ERK, extracellular signal-

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In NIH 3T3 and REF52 fibroblasts, overexpression of MEKK1COOH, a constitutively active form of MEKK1 and a well characterized upstream activator of JNK, leads to apoptosis, which can be attenuated by ERK activation (19, 20). In PC-12 cells and sympathetic neurons, nerve growth factor-mediated ERK activation is crucial for cell survival and neurite outgrowth. The removal of nerve growth factor from PC-12 cells leads to apoptosis (21, 22) as well as the activation of JNK and p38 and the inhibition of ERK (19, 23). In PC-12 cells the activation of JNK and p38 and the inhibition of ERK are critical for induction of apoptosis. Thus, PC-12 cell survival, neurite extension, and differentiation require the proper balance between the activities of ERK, JNK, and p38 MAP kinases.

Like differentiated neurons, cardiac myocytes are post-mitotic, electrically excitable cells that respond to growth factor treatment with an augmentation of the differentiated phenotype. In response to growth factors, primary cardiac myocytes undergo a hypertrophic growth program typified by an increase in cell size, enhanced sarcomeric organization, activation of spontaneous contractile activity, and the up-regulation of a group of cardiac genes that are generally expressed during early embryonic development (e.g. ANF, brain natriuretic peptide, α-skeletal actin), or at very low levels in the adult myocardium (e.g. β-myosin heavy chain, myosin light chain-2) (e.g. Ref. 24). This growth program not only recapitulates cardiac development, but probably also represents some of the cellular processes that contribute to the maintenance of a properly functioning myocardium in the adult.

Given this background it seems possible that myocardial cell ERK activation might promote cell growth, while the activation of JNK and p38 might result in apoptosis. However, recent observations suggest that in cultured myocardial cells, p38 activation leads to hypertrophic growth (25) and might, therefore, protect against apoptosis, while ERK activation, although required for growth, is not in itself sufficient to support the hypertrophic phenotype (26). Accordingly, the present study was undertaken to address the hypothesis that p38 activation can protect primary myocardial cells from apoptosis.

MATERIALS AND METHODS

Cell Culture—Primary ventricular myocytes were prepared from 1–4 day-old neonatal rats as described previously (27, 28). Following the enzymatic dissociation of ventricular tissue the cells were plated onto uncoated plastic dishes in DMEM/F-12 (1:1) (Life Technologies, Inc.) containing 10% fetal bovine serum for 1 h, during which time most of the fibroblasts adhered to the dish. The recovered cells were then either transfected (see below) and then plated or, in some cases, plated directly without transfection. Cultures were plated onto fibronectin-coated glass slides for TUNEL analyses, 100-mm fibronectin-coated plastic culture dishes for DNA fragmentation analyses, or onto fibronectin-coated 16- or 24-mm plastic dishes for reporter enzyme analyses. After 18 h in DMEM/F-12 (1:1), 10% fetal bovine serum, the cultures were washed transfected (see below) and then reseeded with serum-free DMEM/F-12 (1:1), which contained phenylephrine (10 μM), 1 μM propranolol, transferrin (5 μg/ml), insulin (1 μg/ml), selenium (0.1 μg/ml), and triiodothyronine (0.5 ng/ml), unless otherwise stated. After 24 h, various test agents (e.g. anisomycin or sphingosine) were then added, and the cultures were maintained for an additional 48 to 96 h in the test agents followed by TUNEL analysis or DNA extraction. For reporter enzyme assays cells were maintained in DMEM/F-12 (1:1) for 48 h after transfection (see below).

Transfections.—After preplating (see above), myocardial cells were usually resuspended at a density of 30 million cells/ml of minimum medium (DMEM/F-12 containing 1 mg/ml bovine serum albumin) and transfections were carried out as described previously (27, 28). Briefly, for each transfection, 300 μl, between 5 and 9 million cells, were mixed with 1.5–45 μg of test construct (see below) and 9 μg of CMV-β-galactosidase to normalize for transfection efficiency or to serve as a transfection marker in the TUNEL analyses (see below). The levels of plasmid transfectants in each culture within an experiment were equalized using empty vector DNA, such as pCEP. Each 300-μl aliquot was then electroporated in a Bio-Rad gene pulser at 500 V, 25 microfarads, 100Ω in a 0.2-cm gap cuvette, a protocol that allows for the selective transfection of only cardiac myocytes (25, 27). Under these conditions only cardiac myocytes are transected, and the resultant viability is approximately 30% (27); accordingly, the 1–3 million viable cells were plated into four-chamber Lab Tek chamber slides at 0.15–0.9 × 10^6 cells/2-cm^2 well. In two experiments (DNA ladder, Fig. 1B; ERK, JNK, and p38 kinase assays, Fig. 1C) cultures were plated into 100-mm fibronectin-coated culture dishes, and in one experiment, cultures were also transfected with NF-κB-Luc (29) and plated onto 35-mm fibronectin-coated plastic culture dishes (Fig. 5A).

Expression Constructs.—To assess the effects of various signaling proteins, the following constructs were used: RSV-Raf-1 BBX (codes for activated Raf-1 kinase; from U. Rapp and U. Wurzburg, Wurzburg, Germany), pCMV5 MEKK1COOH (codes for activated MEKK1; from G. Johnson, University of Colorado, Denver, CO), pCDNA3 MKK6 (Glu) (codes for activated MKK6 or p38/MPARKK; from R. Davis, University of Massachusetts, Worcester, MA), pCDNA3 MKK6 K52A (codes for a kinase-dead form of MKK6; from R. Davis, University of Massachusetts), pDNAS MKK6b (Glu) (codes for activated MKK6b); from J. Han, The Scripps Research Institute, La Jolla, CA), pCDNA3 MKK3b (Glu) (codes for activated MKK3b; from J. Han, The Scripps Research Institute), pSRa3 JNKK K116R (codes for dominant-negative JNK kinase; from G. Johnson, University of Colorado, p2XNF-b-Luc (codes for a luciferase reporter driven by 2 NF-κB consensus sites; from M. Karin, University of California, San Diego, CA), pCMX-IKeBαM (codes for IkBaS32/A36; from I Verma, The Salk Institute, San Diego, CA), pSRa-IkBoA32/36 (codes for IkBaA32A36; from M. Karin, University of California, San Diego). TUNEL—TUNEL of fragmented DNA was performed on transfected myocardial cells plated on fibronectin-coated glass slides essentially as described previously (30) according to the manufacturer's protocol (Boehringer Mannheim), with the exception that either an ANF or a β-galactosidase monoclonal antibody was added to the TUNEL incubation mixture. This allowed for the co-staining of cells for ANF, which allowed the positive identification of cardiac myocytes, and/or β-galactosidase, which allowed the identification of transfected cells, and DNA strand breaks. In previous studies we have carried out double staining experiments on cultures transfected with luciferase and β-galactosidase-expressing reporters and shown that the co-transfections efficiency is 85% (25). This provided positive confirmation that the observed TUNEL-positive cells were cardiac myocytes (ANF-positive) and were transfected with the appropriate test constructs (β-galactosidase-positive).

In each experiment 100 β-galactosidase or ANF-positive cells were assessed for nuclear staining using the TUNEL assay.

DNA Fragmentation.—To assess the effects of anisomycin or sphingosine on the structural integrity of DNA, myocardial cells were plated at 2 × 10^6 cells/100-mm plastic culture dish, treated as described under the "Cell Culture" section of "Materials and Methods," above, and then lysed by suspending the cells in 10 mM Tris (pH 8), 100 mM NaCl, 25 mM EDTA, 0.5% SDS, and 0.5 mg/ml DNase-free proteinase K and incubated for 5 h at 55°C. Following two phenol extractions and one chloroform extraction, the DNA was precipitated with isopropanol alcohol and incubated with RNase (10 units of DNase-free RNase) for 1 h at 37°C. Following a final ethanol precipitation, 5 μg of DNA derived from each treatment were fractionated on 2% agarose gel, and the DNA was viewed following staining of the gel with ethidium bromide.

ERK, JNK, and p38 Assays.—The effects of anisomycin or sphingosine on ERK, JNK, and p38 were assessed using cardiac cell cultures (1.5 × 10^6 cells/35-mm well). Cultures that had been maintained for 24 h in serum-free medium, as described above, were treated for 30 min with anisomycin (0.20 μM) or sphingosine (10 μM). For ERK and JNK assays cultures were extracted in a buffer containing 10 mM Tris (pH 7.6), 1% Triton X-100, 0.05 mM NaCl, 5 mM EDTA, 2 mM sodium o-ovanadate, and 20 μg/ml aprotinin. After brief centrifugation, extracts were analyzed by Western blotting.
Effects of anisomycin or sphingosine on DNA integrity and ERK, JNK, and p38 activities in myocardial cells.

A, primary neonatal rat ventricular myocytes were prepared and maintained for 48 h as test agent as described under the "Cell Culture" section of "Materials and Methods." TUNEL analyses were carried out as described previously (30) and under "Materials and Methods." The TUNEL procedure was carried out following the instructions for the in situ fluorescein cell death kit (Boehringer Mannheim) with the exception that a mouse anti-ANF antibody (32) was added to the TUNEL reaction mixture. Upon viewing using a rhodamine-compatible fluorescence filter, cardiac myocytes were identified as ANF-positive cells. 100 ANF-positive cells were scored for apoptosis using the TUNEL assay. The percentage of ANF-positive cells that were TUNEL-positive is shown as the mean of three identical cultures ± S.E.; the anisomycin results are shown using the left-hand y axis, and the sphingosine results are shown using the right-hand y axis. **, p < 0.01; *, p < 0.05 compared with control (no anisomycin or sphingosine).

B, DNA integrity was evaluated as described previously (30) and under "Materials and Methods." Shown are the results of an agarose gel on which a sample of DNA comprised of fragments differing in size by 100-base pair increments is shown (Ladder), while total p38 levels were determined using a non-phospho-specific p38 antibody (shown as p38) (see "Materials and Methods"). The D, endogenous p38 activity levels were evaluated as described previously (25) and under "Materials and Methods." Cultures plated identically to those described in B received either no test agent (Con), 0.20 µM anisomycin (An), or 10 µM sphingosine (Sph) are viewed. For comparison a sample of DNA derived from cells receiving either no test agent (Con), 0.20 µM anisomycin (An), or 10 µM sphingosine (Sph) are viewed. For comparison a sample of DNA derived from cells receiving either no test agent (Con), 0.20 µM anisomycin (An), or 10 µM sphingosine (Sph) are viewed.

C, endogenous ERK and JNK activity levels were evaluated as described previously (25) and under "Materials and Methods." Cultures plated identically to those described in B received either no test agent (Con), 0.20 µM anisomycin (An), or 10 µM sphingosine (Sph), three cultures per treatment. Following either 30-min exposures to anisomycin or sphingosine, previously determined to be maximal exposure times for each of these kinases, cultures were extracted and ERK or JNK activity levels were determined using immune complex assays and either myelin basic protein or GST-c-Jun for the substrates. Shown are the phosphorimager analyses of the gels for ERK (myelin basic protein) and JNK (GST-c-Jun); lanes 1–3 are control; lanes 4–6 are anisomycin, and lanes 7–9 are sphingosine. The bar graphs represent the densitometric conversions of the phosphorimager results and are plotted as the mean kinase values of three cultures per treatment ± S.E. These results are representative of at least three experiments. **, p < 0.01 compared with control (no anisomycin or sphingosine).

D, endogenous p38 activity levels were evaluated as described previously (25) and under "Materials and Methods." Cultures plated identically to those described in B received either no test agent (Con), 0.20 µM anisomycin (An), or 10 µM sphingosine (Sph), three cultures per treatment. Following either 30-min exposures to anisomycin or sphingosine, p38 activity levels were determined by Western blotting using a phospho-p38-specific antibody (shown as P-p38), while total p38 levels were determined using a non-phospho-specific p38 antibody (shown as p38) (see "Materials and Methods"). The relative densities of these blots were determined using a phosphorimager, and the P-p38 values were divided by the total p38 values to obtain the level of p38 Thr/Tyr phosphorylation and thus activation. The results of the phosphorimager analyses are shown as the bar graph depicting the mean kinase values of three cultures per treatment ± S.E. These results are representative of at least three experiments. **, p < 0.01 compared with control (no anisomycin or sphingosine).

were incubated for 2 h at 4°C with anti-ERK (raised against the C-terminal 16 amino acids of ERK-1; Santa Cruz SC-983) or anti-JNK (raised against the C-terminal 17 amino acids of JNK-1; Santa Cruz SC-474) bound to protein A-Sepharose (Amersham Pharmacia Biotech), and immune complex kinase assays were carried out using the appropriate substrates, as described previously (26, 31). Briefly, reactions were initiated by the addition of 1 µg of myelin basic protein for ERK, GST-c-Jun for JNK, and 6 µM γ-[32P]ATP (5000 Ci/mmol) in a final volume of 30 µl of kinase buffer (20 mM HEPES (pH 7.4), 20 mM MgCl2, 20 mM γ-glycerophosphate, 2 mM dithiothreitol, 20 µM ATP). After 30 min at 25°C, the reactions were terminated by the addition of Laemmli sample buffer, and the phosphorylation level of substrate proteins was evaluated by SDS-polyacrylamide gel electrophoresis followed by autoradiography and phosphorimage analyses. p38 assays were carried out as described previously (25). Briefly, myocardial cells (1.5 × 106 cells/35-mm culture well) were extracted in 80 µl of Laemmli buffer containing 1 mM p-nitrophenyl phosphate, 100 µM sodium o-vanadate, 10 µg/ml aprotinin, 10 µg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. Sixty-µl aliquots of each extract were fractionated on a 10% SDS-polyacrylamide gel followed by Western blotting using a p38 antibody specific for the phosphorylated/activated form of the kinase (specific for phosphothreonine 180/phosphotyrosine 182; New England Biolabs, Inc., catalog number: NE 92115). In each experiment three identically treated cultures (1.5 × 106 cells/35-mm dish) were used for each treatment, and following densitometric analyses of the exposed phosphorimage plates, values for each treatment were averaged.

Reporter Enzyme Assays—To test the effects of various test constructs on NF-κB activation, each culture of approximately 3 × 106 myocardial cells was transfected with 10 µg of NF-κB/Luc and 9 µg of CMV-β-galactosidase and 10 µg of the appropriate test construct and then plated into a 35-mm fibronectin-coated plastic culture dish. Following a 48-h incubation in serum-free DMEM/F-12 (1:1), the cultures were extracted and luciferase and β-galactosidase assays were performed as described (27). Luciferase activity was measured for 30 s on
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**Fig. 2. Effect of Raf BXB, MEKK1COOH, or MKK6 (Glu) overexpression on myocardial cell apoptosis.** Myocardial cells were transfected with CMV-β-galactosidase and an empty vector control plasmid (pCEP) or a plasmid encoding constitutively active forms Raf (BXB), MEKK1, or MKK6. Following maintenance for 72 h, as described under the “Cell Culture” section of “Materials and Methods,” the cells were fixed and submitted to the TUNEL procedure following the instructions for the in situ fluorescein cell death kit (Boehringer Mannheim), with the exception that a mouse anti-β-galactosidase antibody (Promega) was added to the TUNEL reaction mixture, and β-galactosidase expression was detected using a Texas Red-conjugated anti-mouse IgG. Cells were then examined by fluorescence microscopy using a rhodamine-compatible filter (A and C), which allowed them to be scored for β-galactosidase production (i.e., transfected), and the same cells were then examined using a fluorescein-compatible filter (B and D), which allowed them to be scored for DNA strand breaks indicative of apoptosis. In E cells were transfected with the test constructs shown and after 72 h, between 50 and 100 β-galactosidase-positive cells were scored for positive nuclei assessed using the TUNEL assay. In F myocardial cells were transfected with CMV-β-galactosidase and either an empty vector control (pCEP) or a plasmid encoding a constitutively active form of MKK6. Following maintenance for 24 h ± anisomycin, ± SB 203580 (10 μM), as described under the “Cell Culture” section of “Materials and Methods,” the cells were fixed and submitted to the TUNEL procedure and stained for β-galactosidase expression as described in the legend for Fig. 2. The percentage of the transfected cells that were TUNEL-positive is shown as the mean of three identical cultures ± S.E. E and F, these results are representative of at least three experiments. **, p < 0.01 compared with all other treatments.

To establish a model for studying the role of the p38 pathway on myocardial cell apoptosis, experiments were carried out to evaluate the effects of several known apoptotic agents on primary neonatal rat ventricular myocytes. Cultures were treated with either anisomycin or sphingosine and following fixation they were stained for ANF expression using an ANF monoclonal antibody (32) and for DNA fragmentation using the TUNEL assay (30, 33, 34). Only the cells that stained positively for ANF were evaluated for DNA fragmentation, thus ensuring the evaluation of apoptosis in cardiac myocytes only.

Both anisomycin and sphingosine displayed dose-dependent abilities to induce myocardial cell apoptosis (Fig. 1A). Within 48 h of treatment with 0.2 μM anisomycin, approximately 20% of the transfected cells scored positive for apoptosis compared with about 1% of the untreated cells, representing a 20-fold induction of apoptosis. In other experiments levels of anisomycin greater than 0.2 μM did not significantly increase the percentage of cells driven into apoptosis (not shown). As expected (30), sphingosine also strongly induced apoptosis within the same time frame, with greater than 80% of the cells exposed to 10 μM sphingosine scoring positive for apoptosis compared with about 1% of the untreated cells.

Although anisomycin was a less powerful apoptotic agent of these two compounds, we found that it produced more consistent results than sphingosine. In contrast, the effects of sphingosine were more variable, resulting sometimes in such severe cell death, that by the end of 48–72 h, most of the cells had detached from the dish. This detachment made it impossible to score them for ANF expression, transfection, or TUNEL staining. Accordingly, in most of the experiments shown in later figures, the soluble apoptotic agent of choice was anisomycin.

As further characterization of the apoptotic response, DNA was isolated from cells treated with 0.20 μM anisomycin or 10 μM sphingosine and analyzed on an agarose gel. The 200-base
pair fragments characteristic of apoptotic cells (35) were evident in the anisomycin- and sphingosine-treated cells, but not in the control cells (Fig. 1B). Also, consistent with the relative potencies of the compounds in the TUNEL analysis, the fragmentation pattern was much stronger in the sphingosine-treated cultures than in those treated with anisomycin.

To begin addressing how MAP kinases might be involved in apoptosis in cardiac myocytes in response to anisomycin or sphingosine, the activity levels of ERK, JNK, and p38 were assessed. Anisomycin activated myocardial cell JNK by 10-fold and p38 by 11-fold; however, it had no effect on ERK, while sphingosine activated all three MAP kinases by about 5–10-fold (Fig. 1, C and D). Thus, it did not seem likely that ERK could be involved in apoptosis mediated by both of these compounds; however, either JNK or p38 might be involved.

To assess the effects of each of the MAPK pathways on myocardial cell apoptosis, cultures were transfected with plasmids encoding constitutively active forms of Raf-1 kinase, MEKK1 or MKK6, which preferentially activate ERK, JNK, or p38, respectively (25). Only the myocytes that overexpressed constitutively active MEKK1 (MEKK1COOH) displayed significant increases in the number of TUNEL-positive cells (Fig. 2, A–D), which amounted to approximately 30-fold over cells transfected with a control plasmid (Fig. 2E). Moreover, the TUNEL-positive nuclei in the MEKK1COOH-transfected cells were pyknotic (Fig. 2D), consistent with the double strand DNA breaks characteristic of apoptosis (34). Since MEKK1COOH serves as such a strong activator of JNK in myocardial cells, while BXB and MKK6 activate mainly ERK and p38 (25), these results were consistent with the hypothesis that activation of components of the JNK pathway could foster apoptosis in cardiac myocytes.

Given the recent finding that MKK6-activated p38 contributes to myocardial cell hypertrophic growth (25), and since MKK6 overexpression did not appear to induce apoptosis (Fig. 2E), we investigated whether the overexpression of constitutively active MKK6, MKK6 (Glu), could protect against apoptosis. As expected, compared with untreated controls, cultures transfected with the empty vector control, pCEP, displayed about 15-fold more TUNEL-positive cells in response to anisomycin treatment (Fig. 2F). However, the overexpression of MKK6 (Glu) decreased anisomycin-induced apoptosis to near control levels, supporting the view that in contrast to PC-12 cells, p38 is anti-apoptotic in cardiac myocytes. To further assess the anti-apoptotic effects of the p38 pathway, the p38-specific inhibitor, SB 203580 (36), was employed. SB 203580 completely blocked the anti-apoptotic effects of MKK6 (Glu) overexpression (Fig. 2F). Since MKK6 (Glu) is a selective p38 activator in cardiac myocytes (25), and since SB 203580 also displays p38 specificity in cardiac myocytes, these results strongly suggest that it is through p38 that MKK6 confers this protection against anisomycin-mediated programmed cell death.

*Even though SB 203580 is selective for the inhibition of p38 in other cell types (74), we carried out control experiments to establish the p38 specificity of SB 203580 in cardiac myocytes. In one experiment SB 203580 was shown to completely block MKK6 (Glu)-stimulated MEF2C transactivation in cardiac myocytes (25); this mode of transactivation activation has previously shown to be mediated by p38 and not JNK or ERK (73). In another experiment we transfected primary myocardial cells with ΔMEKK4 (75), a constitutively active selective stimulator of JNK. The cultures were incubated ≥ 10 μM SB 203580 for 48 h, and after extraction, a JNK assay was performed; the cultures incubated with SB 203580 displayed the same level of JNK as those incubated without SB 203580. Additionally, in one experiment 10 μM SB 203580 was added to the immunoprecipitated JNK derived from ΔMEKK4-transfected myocardial cells, and there was no effect of the compound on JNK enzyme activity.

We next evaluated whether MKK6 (Glu) overexpression could effect protection against MEKK1COOH-induced apoptosis. As expected, the TUNEL-positive, MEKK1COOH-transfected cells frequently displayed the pyknotic nuclei that are typical of cells undergoing apoptosis (Fig. 3D), while most of the cells transfected with MKK6 (Glu) alone (Fig. 3F) and many of the cells co-transfected with MKK6 (Glu) and MEKK1COOH (Fig. 3H) did not score positive in the TUNEL assay. The assessment of 100 cells from each transfection revealed that MKK6 (Glu)
MKK6, a well characterized p38 MAP kinase kinases (47, 48), could decrease the numbers of TUNEL-positive cells in MEKK1COOH-transfected cells from about 6-fold over control to about 2–3-fold over control (Fig. 3I). The partial inhibition of MEKK1COOH-induced apoptosis by MKK6 (Glu), which amounted to about 50%, suggests that the apoptotic effects of MEKK1COOH may be mediated by a number of pathways, only a subset of which is functionally interrupted by MKK6. This is consistent with recent reports demonstrating the MEKK1 is a large, membrane-associated protein with various functional domains that appear to confer apoptosis via several different pathways (37–39).

One possible mechanism by which MKK6 (Glu) could protect myocardial cells from apoptosis could be through the activation of NF-κB, a transcription factor shown to protect several other cell types from cytokine-induced apoptosis (29, 40–44). In support of this hypothesis is a study showing that p38-specific inhibitors block tumor necrosis factor-mediated NF-κB activation in L929 cells (45) and another report that p38 and NF-κB are often activated by the same stimuli in 293 cells (46).

Accordingly, experiments were carried out to determine whether overexpression of MKK6 (Glu) can activate NF-κB in primary myocardial cells. Cultures were co-transfected with a luciferase reporter construct possessing two copies of a consensus NF-κB binding site and with various test constructs. Two different known activators of p38, MKK6 (Glu) (47), and MKK6b (Glu) (48) were able to activate NF-κB-mediated transcription by about 10-fold in an SB 203580-sensitive manner (Fig. 4A). A kinase-dead form of MKK6, MKK6 (K82R) (47), was unable to activate NF-κB-mediated transcription. Additionally, overexpression of a form of I-κB, that cannot dissociate from NF-κB (I-κBα), thus rendering it inactive in the cytosol (42, 49), was a potent blocker of MKK6 (Glu)-activated NF-κB-mediated transcription. These results clearly showed that using this assessment of NF-κB activation, the p38 pathway is able to activate NF-κB in primary myocardial cells.

Further experiments were carried out to test whether it is through NF-κB that MKK6 (Glu) protects cardiac myocytes from apoptosis. Anisomycin-induced apoptosis was blocked by MKK6 (Glu) in an SB 203580-sensitive manner; however, transfecting cells with I-κBα, which inhibits NF-κB, had no effect on MKK6 (Glu)-mediated protection from anisomycin-induced apoptosis (Fig. 4B). This suggested that the protective effects of MKK6 (Glu) were not due to its ability to activate NF-κB. Interestingly, SB 203580 treatment of cells that had been transfected with both MKK6 (Glu) and I-κBαM augmented anisomycin-induced apoptosis. This finding suggests that when p38 is blocked, NF-κB can serve to protect against apoptosis. To further test this hypothesis, apoptosis was assessed in myocardial cells in the absence of anisomycin. Under these conditions, SB 203580 or I-κBαM alone had no statistically significant effect on the number of apoptotic cells; however, SB 203580 and I-κBαM together increased apoptosis by about 5-fold (Fig. 4C). These results support the view that NF-κB can protect cardiac myocytes against apoptosis, but only upon inhibition of p38.

Taken together, the results of the present study indicate that MKK6, a well characterized p38 MAP kinase kinases (47, 48).
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phosph 1 protects cells from apoptosis, but through a pathway that appears to require ERK (18). Thus, it appears that in myocardial cells ERK and p38 are anti-apoptotic, and JNK may promote apoptosis. Future studies aimed at determining the molecular mechanism by which p38 exerts anti-apoptotic, growth-promoting effects in cardiac myocytes will likely reveal important new aspects of this stress kinase pathway in the regulation of the delicate balance between cell growth and cell death.

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REFERENCES

1. Jacobson, M. D., Weil, M., and Raff, M. C. (1997) Cell 88, 347–354
2. Kerr, J. F. R., Wyllie, A. H., and Currie, S. (1972) Br. J. Cancer 26, 239–257
3. Clarke, P. O., and Clarke, S. (1995) Nature 378, 230
4. Cohen, J. J. (1993) Immunol. Today 14, 126–130
5. Peitsch, M. C., Mannherz, H. G., and Tschopp, J. (1994) Trends Cell Biol. 4, 22–41
6. Chinnaiyan, A. M., and Dixit, V. M. (1996) Curr. Biol. 6, 555–562
7. Nagata, S. (1997) Cell 88, 355–365
8. Olivetti, G., Abbi, R., Quaini, F., Kajstura, J., Cheng, W., Nitahara, J. A., and Quaini, F., Loveto, C. C., Belin, C. A., Krajeski, S., Reed, J. C., and Anversa, P. (1997) N Engl. J. Med. 336, 1311–1314
9. Yamaizuti, K., Komuro, I., and Yazaki, Y. (1995) J. Mol. Cell. Cardiol. 1, 133–140
10. Sadoshima, J., and Isomu S (1997) Annu. Rev. Physiol. 59, 551–571
11. Cheng, W., Li, B., Rajaltet, J., Li, P., Wollin, M. S., Sonnenblick, E. H., Hintze, T. H., and Olivetti, G. (1995) J. Clin. Invest. 96, 2247–2259
12. Rajaltet, J., Mannukkahi, M., Cheng, W., Reiss, K., Krajeski, S., Reed, J. C., Quaini, F., Sonnenblick, E. H., and Anversa, P. (1995) Exp. Cell Res. 219, 110–121
13. Teiger, E., Dam, T.-V., Richard, L., Wisnewsky, C., Tea, B.-S., Gaboury, L., Tremblay, J., Schwartz, K., and Hamet, P. (1996) J. Clin. Invest. 97, 2891–2897
14. MacLellan, W. R., and Schneider, M. D. (1997) Curr. Res. 81, 137–144
15. James, T. N. M. D. (1994) Curr. Biol. 950, 556–573
16. Krstic, R., and Pesieder, T. (1973) Z. Anat. Entwicklungsgesch 140, 337–350
17. Takada, K., Yu, Z. X., Nishikawa, T., Tanaka, M., Hobb, S., Ferrans, V. J., and Kasugaijinaga, T. (1996) J. Mol. Cell. Cardiol. 28, 209–215
18. Sheng, Z., Knowlton, K., Chen, J., Hoshijima, M., Brown, J. H., and Chien, K. R. (1997) J. Biol. Chem. 272, 5783–5791
19. Xia, Z., Dickens, M., Raingreb, J., Davis, R. J., and Greenberg, M. E. (1995) Science 270, 1326–1331
20. Johnson, N. L., Gardner, A. M., Diener, K. R., Lane-Carter, C. A., Gleavys, J., Jarpe, M. B., Minden, A., Karin, M., Zou, L., and Johnson, G. L. (1996) J. Biol. Chem. 271, 3229–3237
21. Batistatou, A., and Greene, L. A. (1993) J. Cell Biol. 122, 532–533
22. Pittman, R. N., Wang, S., Dibenedetto, A. J., and Mills, J. (1993) J. Neurosci. 13, 3669–3680
23. Park, D. S., Stefanis, L., Yan, C. Y. I., Garinelli, S. E., and Greene, L. A. (1996) J. Biol. Chem. 271, 21898–21905
24. Chien, R. K., and Grace, D. (1994) Heart Disease: A Textbook of Cardiovascular Medicine (Brunswald, E., ed) 5th Ed., pp. 1626–1649, W. B. Saunders Co., Philadelphia, PA
25. Zecevic, D., Thuerau, D. J., and Greenberg, M. E. (1988) Science 239, 115–127
26. Post, G. R., Goldstein, D., Thuerau, D. J., Glebontski, C. C., and Brown, J. H. (1990) J. Biol. Chem. 265, 8452–8457
27. Spence, A. B., Murray, S. P., and Glebontski, C. C. (1995) Curr. Res. 77, 1060–1069
28. Thuerau, D. J., and Glebontski, C. C. (1997) J. Biol. Chem. 272, 7464–7472
29. Liu, Z.-G., Hsu, H., Greed, D. V., and Karin, M. (1996) Cell 87, 565–578
30. Krown, K. A., Page, M. T., Nguyen, C., Zecevic, Z., Gutierrez, V., Comstock, K. L., Glebontski, C. C., Quintana, P. J. E., and Sabadini, R. A. (1996) J. Clin. Invest. 98, 2854–2865
31. Derijard, B., Hibi, M., Wu, I., Barrett, T., Su, B., Deng, T., Karin, M., and Davis, R. J. (1994) Cell 76, 1025–1037
32. Glebontski, C. C., Oronzi, M. E., Li, X., Shields, P. R., Johnston, J. H., Kallen, R. G., and Gibson, T. R. (1997) Endocrinology 128, 843–852
33. Gavriel, Y., Sherman, Y., and Ben-Sasson, S. A. (1992) J. Cell Biol. 119, 493–501
34. Charron-Marlangue, C., and Ben-Ari, Y. (1995) Neuroreport 7, 61–64
35. Wadner, P. R., and Sikora, M. (1994) Biochem. Cell Biol. 72, 615–625
36. Young, P., McDonnel, P., Dunnington, D., Hand, A., Laydon, J., and Lee, J. (1993) Agents Action 39, C67–69
37. Xu, S., Robbins, D. J., Christensen, L. B., English, J. M., Vanderbilt, C. A., and Cobb, M. H. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 5291–5295
38. Cardone, M. H., Salvesen, G. S., Widmann, C., Johnson, G., and Frisch, S. M. (1997) Cell 90, 315–323
39. Lee, F. S., Hagler, J., Chen, Z., and Maniatis, T. (1997) Cell 88, 213–222
40. Beg, A. A., and Baltimore, D. (1996) Science 274, 782–784
41. Sonnenshein, G. E. (1997) Semin. Cancer Biol. 8, 113–119
42. Van Antwerp, D. J., Martin, S. J., Kafri, T., Green, D. R., and Verma, I. M. (1996) Science 274, 785–789
43. Wu, M., Lee, H., Bellas, R. E., Schauer, S. L., Aruura, M., Katz, D., FitzGeral, M. J., Rosthein, T. L., Sher, D. H., and Sonnenshein, G. E. (1996) EMBO J.

2 While this paper was under review, a study was published that also indicated that p38-δ may be anti-apoptotic in cardiac myocytes and that p38-α may be conducive to apoptosis (76).
44. Wang, C.-Y., Mayo, M. W., and Baldwin, A. S. (1996) Science 274, 784–787
45. Beyaert, R., Cuenda, A., Berge, W. M., Plaisance, S., Lee, J. C., Haegeman, G., Cohen, P., and Fiers, W. (1996) EMBO J. 15, 1914–1923
46. Wasselborg, S., Bauer, M. K. A., Vogt, M., Schmitz, M. L., and Shulze-Osthoff, K. (1997) J. Biol. Chem. 272, 12422–12429
47. Raingeaud, J., Whitmarsh, A. J., Barrett, T., Derijard, B., and Davis, R. J. (1996) Mol. Cell. Biol. 16, 1247–1255
48. Han, J., Lee, J.-D., Li, Z., Feng, L., and Ulevitch, R. J. (1996) J. Biol. Chem. 271, 2886–2891
49. DiDonato, J. A., Mercurio, F., and Karin, M. (1995) Mol. Cell. Biol. 15, 1302–1311
50. Ludwig, S., Engel, K., Hoffmeyer, A., Sithanandam, G., Neufeld, B., Palm, D., Marber, M. S., Latchman, D. S., and Yellon, D. M. (1993) Circulation 88, 1264–1272
51. Sharma, H. S., Stahl, J., Weisensee, D., and Low-Friedrich, I. (1996) Mol. Cell. Biochem. 160–161, 217–224
52. Gerwins, P., Blank, J. L., and Johnson, G. L. (1997) J. Biol. Chem. 272, 4261–4267
53. Chin, J. H., Okazaki, M., Hu, Z. W., Miller, J. W., and Hoffman, B. B. (1996) J. Biol. Chem. 271, 2360–2371
54. Whitmarsh, A. J., Yang, S. H., Su, M. S. S., Sharrocks, A. D., and Davis, R. J. (1997) Mol. Cell. Biol. 17, 2360–2371
55. Gerwins, P., Blank, J. L., and Johnson, G. L. (1997) J. Biol. Chem. 272, 8288–8295
56. Wang, Y., Huang, S., Sah, V. P., Ross, J., Brown, J. H., Han, J., and Chien, K. R. (1998) J. Biol. Chem. 273, 2162–2168

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