Role of 14-3-3–Mediated p38 Mitogen-Activated Protein Kinase Inhibition in Cardiac Myocyte Survival

Shaosong Zhang, Jie Ren, Cindy E. Zhang, Ilya Treskov, Yibin Wang, Anthony J. Muslin

14-3-3 family members are dimeric phosphoserine-binding proteins that regulate signal transduction, apoptotic, and checkpoint control pathways. Targeted expression of dominant-negative 14-3-3ζ (DN-14-3-3ζ) to murine postnatal cardiac tissue potentiates Ask1, c-jun N-terminal kinase (JNK), and p38 mitogen-activated protein kinase (MAPK) activation. DN-14-3-3ζ mice are unable to compensate for pressure overload, which results in increased mortality, dilated cardiomyopathy, and cardiac myocyte apoptosis. To evaluate the relative role of p38 MAPK activity in the DN-14-3-3ζ phenotype, we inhibited cardiac p38 MAPK activity by pharmacological and genetic methods. Intrapерitoneal injection of SB202190, an inhibitor of p38α and p38β MAPK activity, markedly increased the ability of DN-14-3-3ζ mice to compensate for pressure overload, with decreased mortality. DN-14-3-3ζ mice were bred with transgenic mice in which dominant-negative p38α (DN-p38α) or dominant-negative p38β (DN-p38β) MAPK expression was targeted to the heart. Compound transgenic DN-14-3-3ζ/DN-p38β mice, and to a lesser extent compound transgenic DN-14-3-3ζ/DN-p38α mice, exhibited reduced mortality and cardiac myocyte apoptosis in response to pressure overload, demonstrating that DN-14-3-3ζ promotes cardiac apoptosis due to stimulation of p38 MAPK activity.

4-3-3 proteins are intracellular phosphoserine-binding adapter molecules.1,2 14-3-3 proteins modulate several important signal transduction, apoptotic, and checkpoint control pathways by binding to many signaling proteins, including the protein kinases Ask13 and Raf-1.4 Ask1 is a mitogen-activated protein kinase kinase kinase (MAPKKK) that participates in the c-jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) pathways.5,6 14-3-3 binding to Ask1 inhibits its enzymatic activity.5,6

Previously, we investigated the biological effects of 14-3-3 proteins in cultured cells and in transgenic animals by use of dominant-negative forms of 14-3-3 (DN14-3-3).6 DN-14-3-3–transfected fibroblasts exhibited increased Ask1, p38 MAPK, and JNK activities. Inhibition of 14-3-3 activity also caused cultured cells to become sensitized to proapoptotic stimuli, including UV irradiation and serum deprivation. The proapoptotic effect of DN-14-3-3 in fibroblasts was blocked by treatment with the p38 MAPK inhibitor SB202190.

Transgenic mice were generated with cardiac-specific overexpression of DN-14-3-3ζ.6 These mice appeared normal at baseline but were unable to compensate for pressure overload induced by transverse aortic constriction (TAC), a mild stimulus of cardiac myocyte apoptosis. DN-14-3-3ζ mice tolerated TAC poorly and most animals died from overwhelming cardiac dysfunction. TUNEL studies revealed that there was massive cardiac myocyte apoptosis in DN-14-3-3ζ ventricular tissue but not in nontransgenic cardiac tissue after TAC. To evaluate the importance of p38 MAPK activation in the DN-14-3-3ζ phenotype, we inhibited cardiac p38 MAPK activity by pharmacological and genetic methods in the present work.

Materials and Methods

Transgenic mice with cardiac-specific expression of DN-14-3-3ζ were evaluated in this work.6 DN-14-3-3ζ mice were subjected to pressure overload by TAC in the presence or absence of intraperitoneal administration of SB202190, 5 mg · kg⁻¹ · d⁻¹, a chemical inhibitor of p38α and p38β MAPK. Next, DN-14-3-3ζ mice were bred with transgenic mice in the same strain that had cardiac-specific expression of dominant-negative forms of p38α MAPK (DN-p38α) or p38β MAPK (DN-p38β) to generate compound transgenic DN-14-3-3ζ/DN-p38α or DN-14-3-3ζ/DN-p38β mice.7 Compound transgenic mice were also subjected to pressure overload by TAC, and survival, MAPK activation, and apoptosis were assayed by conventional methods.

An expanded Materials and Methods section can be found in the online data supplement available at http://www.circresaha.org.

Results

An experimental protocol to administer SB202190, a specific inhibitor of p38α and p38β MAPK, was established in DN-14-3-3ζ transgenic mice. In this method, SB202190 was delivered daily by intraperitoneal injection, beginning 3 days before TAC. After 10 days of administration (7 days after TAC), ventricular tissue was isolated and cytosolic protein lysates were generated. Anti-p38 MAPK immunoprecipitates from these lysates were produced with an antibody that recognizes both p38α and p38β MAPK. Immunoprecipitations were assayed for p38 MAPK enzymatic activity by in vitro kinase assay with recombinant ATF-2 protein used as a substrate. In vitro kinase assays demonstrated that daily administration of 5 mg/kg of SB202190 inhibited p38 MAPK activity in cardiac tissue (Figure 1A). In addition, analysis of protein lysates by anti-phospho-HSP27 immunoblotting demonstrated that SB202190 treatment blocked cardiac phosphorylation of HSP27, a well-defined substrate of p38 MAPK (data not shown).

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To determine whether inhibition of p38 MAPK activity could block the DN-14-3-3 phenotype, we performed a series of TAC experiments. DN-14-3-3 mice that were injected with control buffer did not tolerate TAC and only 21% of animals survived (6 of 29) for 7 days after the surgical procedure. In contrast, 82% of animals (9 of 11) treated with SB202190 for 3 days before TAC survived for at least 7 days after surgery (Figure 1B). The difference in survival observed in compound transgenic DN-14-3-3 mice was significant by analysis with the Kaplan-Meier log-rank test ($\chi^2=11.0$, $P=0.0009$).

To specifically determine which p38 MAPK isoform was important for the abnormal response of DN-14-3-3 mice to TAC, transgenic mice with cardiac-specific expression of DN-p38α and DN-p38β were bred with DN-14-3-3 mice.7 DN-14-3-3 protein levels were not altered in compound transgenic DN-14-3-3/DN-p38α and DN-14-3-3/DN-p38β compared with single transgenic DN-14-3-3 mice (Figure 2). We previously demonstrated that DN-p38α mice exhibit reduced p38α MAPK activation and that DN-p38β transgenic mice exhibit reduced p38β MAPK activation in cardiac tissue.7

TAC experiments were performed on transgenic mice and, in each case, compound transgenic mice were compared with single transgenic DN-14-3-3 littermates and nontransgenic littermates. As expected, single transgenic DN-14-3-3 mice were intolerant to pressure overload and only 6% of mice (1 of 17) survived for 7 days after TAC. In contrast, DN-14-3-3/DN-p38β mice were completely resistant to TAC-induced death and 100% of animals (10 of 10) survived. The difference in survival observed in compound transgenic DN-14-3-3/DN-p38β mice compared with single transgenic DN-14-3-3 mice was significant by the log-rank test ($\chi^2=16.6$, $P<0.0001$). DN-14-3-3/DN-p38α mice were partially resistant to TAC-induced death and 60% of animals (9 of 15) mice survived (Figure 2). The difference in survival observed in compound transgenic DN-14-3-3/DN-p38α mice compared with DN-14-3-3 mice was significant by analysis with the log-rank test ($\chi^2=7.1$, $P=0.008$).

p38 MAPK promotes apoptosis in certain cell types and this may be due to direct phosphorylation of p53 or other effectors. In response to pressure overload, we previously demonstrated that DN-14-3-3 mice develop overwhelming cardiac myocyte death.8 Apoptosis in cardiac tissue was assessed in the present study by caspase-3 cleavage assays that confirmed that both DN-14-3-3/DN-p38β and DN-14-3-3/DN-p38α cardiac tissue had reduced apoptosis 7 days after TAC compared with DN-14-3-3 single transgenic mice (Figure 3). The percentage of left ventricular cardiac myocytes that were immunoreactive for cleaved caspase-3 7 days after TAC was 4.2±0.93% for DN-14-3-3 mice, 0.049±0.012% for DN-14-3-3/DN-p38β mice, and 0.19±0.067% for DN-14-3-3/DN-p38α mice, and 0.045±0.015% for nontransgenic mice.
The specific targets of p38 MAPK that are important for a proapoptotic response are not completely known but may include the transcription factor p53. It is not clear whether p38 MAPK–mediated p53 phosphorylation is important in cardiac myocyte apoptosis, but our results suggest that inhibition of p38 MAPK in human patients may be a useful method to ameliorate cardiac myocyte death in response to provocative stimulation such as ischemia or pressure overload.

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References

Key Words: signal transduction - apoptosis - cardiac - 14-3-3 protein - p38 mitogen-activated protein kinase

Discussion
14-3-3 proteins bind to a variety of signal transduction, checkpoint control, and apoptotic pathway proteins.1,2 14-3-3 binding to BAD and FKHL1 inhibits the proapoptotic activity of these proteins, thereby promoting cell survival.8,9 14-3-3 proteins also bind to and inhibit the activity of Ask1, a MAPKKK in the p38 MAPK and JNK pathways.3 We previously demonstrated that inhibition of 14-3-3 activity in cultured fibroblasts promoted apoptosis in a p38 MAPK-dependent fashion.8 We also showed that dominant-negative 14-3-3 increased the sensitivity of cardiac tissue to proapoptotic stimuli.6

In the present study, we addressed the role of p38 MAPK in the phenotype observed in DN-14-3-3 transgenic mice by both pharmacological and genetic means. Our results show that 14-3-3–mediated p38 MAPK inhibition is a critical factor to promote cardiac myocyte survival and suggest that p38β MAPK plays a more important role than p38α MAPK in the apoptosis response. But, we cannot rule out the possibility that each DN-p38 MAPK protein inhibits the other isoform to some degree. Several other studies demonstrated that p38 MAPK activity leads to cardiac myocyte apoptosis. For example, one group found that doxorubicin- and tumor necrosis factor-α (TNF-α)–dependent murine cardiac myocyte apoptosis was dependent on p38 MAPK activation.10,11 Another group showed that hypoxia–, angiotensin–II–, and norepinephrine-mediated canine cardiac myocyte apoptosis is dependent on p38 MAPK activity.
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Materials and methods (online data supplement)

Protein analysis and antibodies

Murine ventricular cytosolic lysates were obtained as previously described. For Western blot experiments, proteins were separated by SDS-protein acrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to nitrocellulose filters. Filters were blocked with either 5% nonfat dried milk or 5% Bovine Serum Albumin (BSA) in TBST (10 mmol/L Tris [pH 7.5], 150 mmol/L NaCl, 0.1% Tween 20). Anti-phospho-HSP27 and anti-total-HSP27 rabbit polyclonal antibodies were used at a dilution of 1:1000 (Cell Signaling, Beverly, MA). Anti-14-3-3β (anti-pan-14-3-3) and anti-cMyc rabbit polyclonal antibodies (Santa Cruz Biotech, Santa Cruz, California) were used at a dilution of 1:500. Anti-cleaved caspase-3 rabbit polyclonal antibody (Cell Signaling, Beverly, MA) was used at a 1:1000 dilution. After incubation in primary antibody, bound antibody was visualized with alkaline phosphatase or horseradish peroxidase-coupled secondary antibody and color-developing agents (Promega, Madison WI) or chemiluminescence-developing agents (ECL, Amersham, Piscataway, NJ).

In vitro protein kinase assays

In vitro p38 MAPK assays were performed with a kit from Cell Signaling Inc. (Beverly, MA) that was used in accordance with the manufacturer’s instructions. In brief, anti-phospho-p38 MAPK immunoprecipitates were derived
from ventricular cytosolic lysates obtained 7 days after TAC. The specific substrate protein ATF-2, 200 μmol/L ATP and kinase reaction buffer were added to the immunoprecipitates. Kinase reactions were terminated after a 30-minute incubation period, and proteins were separated by SDS-PAGE and then analyzed by immunoblotting with a specific anti-phospho-ATF-2 antibody.

SB202190 treatment

In order to determine an effective dose for intraperitoneal injection of SB202190, a range of doses was tested, including 0.1, 0.5, 1, 5 and 10 mg/kg/day. After 3 days of administration, cardiac lysates were used for p38 MAPK in vitro kinase assays. Maximal inhibition of cardiac p38 MAPK activity was observed after administration of 5 or 10 mg/kg/day. The dosage used in this study was 5 mg/kg/day.

For TAC experiments, SB202190 compound was administered daily by intraperitoneal injection that was started 3 days before TAC. After 10 days of administration (7 days after TAC), ventricular tissue was isolated and cytosolic protein lysates were generated.

The ability of SB202190 to block p38 MAPK activity measured by in vitro kinase assay is surprising because SB202190 is unlikely to remain bound to the kinase pocket after immunoprecipitation. We suspect that SB202190-mediated inhibition of p38 MAPK kinase activity in vivo blocks an autophosphorylation loop that normally potentiates the activation state of p38 MAPK.
Generation of DN-14-3-3/DN-p38α and DN-14-3-3/DN-p38β compound transgenic mice

DN-14-3-3 transgenic mice were generated as previously described. In brief, the coding region of the human DN(R56A and R60A)-14-3-3η cDNA with a 5'-myc-1-epitope tag was subcloned into a vector containing the α-myosin heavy chain (α-MHC) promoter and an SV40 polyadenylation site. Linearized DNA was injected into the pronuclei of one-cell C57BL/6 x SJL embryos at the Neuroscience Transgenic Facility at Washington University School of Medicine. Progeny were analyzed by PCR to detect transgene integration. Progeny were backcrossed into the C57BL/6 genetic background for 6 generations.

DN-p38α and DN-p38β transgenic mice were generated. In brief, the coding regions of human DN-p38α MAPK or DN-p38β MAPK cDNA were subcloned into a vector containing the α-MHC promoter and an SV40 polyadenylation site. Linearized DNA was injected into the pronuclei of one-cell Black Swiss embryos. Progeny were analyzed by PCR to detect transgene integration. Progeny were backcrossed into the C57BL/6 genetic background for 5-6 generations.

Hemizygous transgenic DN-14-3-3 mice were bred to hemizygous DN-p38α or DN-p38β mice to generate compound transgenic mice. Progeny were analyzed by PCR to detect transgene integration.

Transverse aortic constriction (TAC)
TAC was performed largely as previously described. In brief, 12 week-old mice were anesthetized with a mixture of xylazine (16 mg/kg) and ketamine (80 mg/kg). The chest was opened and following blunt dissection through the intercostal muscles the thoracic aorta was identified. A 7-0 silk suture was placed around the transverse aorta and tied around a 26 gauge blunt needle that was subsequently removed. The chest was closed with a purse-string suture. At the end of the procedure, the incision was closed in two layers with an interrupted suture pattern. The mouse was kept on a heating pad until responsive to stimuli. The surgeon was blinded to the transgenic status of the mice. Sham-operated animals underwent the identical surgical procedure except that the aortic constriction was not placed. After 7 days, surviving animals were killed and hearts were dissected out.

**Immunohistochemical staining of cleaved caspase-3**

Animals were euthanized, the heart was excised, fixed overnight at room temperature in 10% formalin in PBS, embedded in paraffin, and sectioned with a microtome. Heart tissue sections were incubated with anti-cleaved caspase-3 rabbit polyclonal antibody (Cell signaling, Beverly, MA) at a 1:100 dilution for overnight at 4°C. After 30 minutes incubation with secondary anti-rabbit antibody (Cell signaling, Beverly, MA), sections were incubated with ABC reagent (Vector Laboratories, Inc Burlingame, CA) and then added diaminobenzidine tetrahydrochloride (DAB) to develop color. Sections were mounted on coverslips and Cleaved caspase-3 positive cardiac myocytes evaluated by microscopy.
References of online data supplement
