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p38α MAPK inhibits stretch-induced JNK activation in cardiac myocytes through MKP-1

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ABSTRACT

Mechanical stretch is a major determinant that leads to heart failure, which is associated with a steady increase in myocardial angiotensinogen (Aogen) expression and formation of the biological peptide angiotensin II (Ang II). c-jun NH_2-terminal kinase (JNK) and p38α have been found to have opposing roles on stretch-induced Aogen gene expression in neonatal rat ventricular myocytes (NRVM). JNK negatively regulated Aogen expression in NRVM following acute stretch, whereas with prolonged stretch, JNK phosphorylation was downregulated and p38α was found responsible for upregulation of Aogen expression. However, the mechanisms responsible for regulation of these kinases, especially the cross-talk between p38 and JNK1/2, remain to be determined. In this study, a combination of pharmacologic and molecular approaches (adenovirus-mediated gene transfer) were used to examine the mechanisms by which p38 regulates JNK phosphorylation in NRVM under stretch and non-stretch conditions. Pharmacologic inhibition of p38 significantly increased JNK phosphorylation in NRVM at 15 min, whereas overexpression of wild-type p38α significantly decreased JNK phosphorylation. While p38α overexpression prevented stretch-induced JNK phosphorylation, pharmacologic p38 inhibition abolished the JNK dephosphorylation during 15–60 min of stretch. Expression of constitutively-active MKK3 (MKK3CA), the upstream activator of p38, abolished JNK phosphorylation in both basal and stretched NRVM. Pharmacologic inhibition of MAP kinase phosphatase-1 (MKP-1) or protein phosphatase-1 (PP1) increased JNK phosphorylation in NRVM, suggesting the involvement of these phosphatases on reversing stretch-induced JNK activation. Inhibition of MKP-1, but not PP1, reduced JNK phosphorylation in NRVM overexpressing MKK3CA under basal conditions (no-stretch). Inhibition of MKP-1 also enhanced stretch-induced JNK phosphorylation in NRVM at 15 to 60 min.

In summary, these results indicate that MKP-1 inhibits JNK phosphorylation in stretched NRVM through p38 dependent and independent mechanisms, whereas PP1 regulates JNK through a p38-independent mechanism.

1. Introduction

Mechanical stretch is the primary stimulus responsible for inducing heart failure in the hemodynamically challenged myocardium [1–3]. Although the heart is able to dynamically adapt to physiologic increases in mechanical load, chronic overload of the myocardium results in the activation of maladaptive processes, which adversely affect both the structure and function of the heart. Mechanical stress induces activation of the cardiac renin-angiotensin system [4,5], which results in increased local production Ang II [6–8] and stimulation of myocardial remodeling and hypertrophy. We have reported that the stress-activated protein kinase family members c-jun NH_2-terminal kinase (JNK) and p38, have opposing roles on stretch-induced angiotensinogen (Aogen) expression in neonatal rat ventricular myocytes (NRVM) [5]. In this report, mechanical stretch was shown to initially inhibit Aogen gene expression as a result of increased JNK activation. However, prolonged stretch restored JNK phosphorylation to basal levels, whereas p38α phosphorylation remained elevated and was responsible for upregulation of Aogen expression [5]. The diminished phosphorylation of JNK could result from decreased activity of upstream kinases mitogen-activated protein kinase kinases 4 and 7 (MKK4/7) and/or increased dephosphorylation of JNK. The serine/threonine protein phosphatase 2A (PP2A), a member of the phosphoprotein phosphatase (PPP) family has been shown to inhibit JNKs by dephosphorylating MKK4/7 [9]. MAP kinase family members can also be inactivated by simultaneous dephosphorylation of the activation loop threonine and tyrosine residues by MAP kinase phosphatases (MKPs), members of the dual specificity protein phosphatase (DUSPs) family [10]. MAPK phosphatase 1 (MKP-1), which is widely

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expressed and activated by p38, dephosphorylates the activation loop of JNK. Reciprocal regulation of JNK and p38 in cardiac myocytes following chronic stretch suggests that increased p38 might negatively regulate JNK activation. In the present study, we used primary cultures of NRVM to determine the regulatory effects of p38α on JNK activation under basal and stretch conditions. These studies revealed that JNK1/2 activation is regulated under basal and stretch conditions by MKP-1, which occurs via p38 dependent and independent mechanisms.

2. Materials and methods

2.1. Antibodies and reagents

Phospho-p38-Thr^{180}/Tyr^{182} antibody (9211), p38 antibody (5F11), phospho-SAPK/JNK-Thr^{183}/Tyr^{185} antibody (9251), JNK antibody (9252), phospho-c-Jun-Ser^{73} (D47G9), c-Jun (60A8), phospho-MAPK-activated protein kinase 2 (MK2)-Thr^{222} (3044), MK2 (3042) and horseradish peroxidase-conjugated secondary antibodies were obtained from Cell Signaling Technology, Inc. (Danvers, MA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (MAB1501) was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and desmin mouse monoclonal antibody (DE-U-10) was obtained from (Sigma Chemical Co., St. Louis, MO). DNase-free and protease-free bovine serum albumin (BSA, P-753) was obtained from Boston BioProducts (Ashland, MA). Percoll® was obtained from GE Healthcare Life Sciences (Pittsburgh, PA). Enhanced chemiluminescence (ECL) reagent (Western Lightning™) was obtained from Perkin Elmer Life Science (Boston, MA). SB203580 (SB580) was purchased from LC Laboratories (Woburn, MA). SP600125 (SP125), JNK inhibitor VIII (JI8), phosphatase inhibitors calyculin A (CCA) and 2-benzylidene-3-(cyclohexylamino)-2,3-dihydro-1H-inden-1-one (BCI) were obtained from Calbiochem (San Diego, CA). Tissue-culture grade dimethylsulfoxide (DMSO) Hybri-Max® used for diluting pharmacologic inhibitors was purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Isolation of neonatal rat ventricular myocytes

Primary cultures of NRVM were prepared from 1 to 2-day-old neonatal Sprague Dawley rats as previously described [11]. Dispersed cardiac cells were separated using a discontinuous Percoll gradient, as previously described [11]. The NRVM were plated on deformable membranes coated with collagen-IV (1 μg/cm^2) on Flexcell® Bioflex plates (Flexcell International Corp, Hillsborough, NC), at a density of 0.75×10^6 cells/well in DMEM/M199 medium and maintained at 37 °C in humid air with 5% CO_2. Cytosine arabinoside (100 μM) was added to prevent cell division of nonmyocytes (primarily fibroblasts) and culture media was changed to serum-free DMEM/M199 media (SFM) 12 h prior to initiation of experiments. The cultured myocytes were >95% pure, as revealed by microscopic observation of contractile characteristics and by flow cytometry, after staining with anti-desmin antibody. Two days after plating, the culture medium was changed to SFM and experiments performed 12 h later. For stretch experiments, NRVM were exposed to 20% equiaxial static-stretch for various times in a Flexcell® FX-3000 strain unit equipped with loading posts. This study conforms to the *Guide for the Care and Use of Laboratory Animals* (Eighth edition) revised by the National Research Council in 2011 (published by The National Academies Press). Experimental protocols were approved by the Baylor Scott and White Health Institutional Animal Care and Use Committee.

2.3. Adenovirus expansion and infection of cells

Recombinant adenoviruses expressing wild-type p38α (Ad-p38αWT), dominant-negative p38α (Ad-p38αDN), HA-tagged constitutively-active MKK3 (MKK3CA), HA-tagged constitutively-active MKK6 (MKK6CA) and green-fluorescent protein GFP (Ad-GFP) were gifts from Dr. Yibin Wang (Cardiovascular Research Laboratory, University of California, Los Angeles). Recombinant adenoviruses expressing wild-type MKP-1 (Ad-MKP-1) was a gift from Dr. Jeffery Molkentin (Molecular Cardiovascular Biology, Children's Hospital Medical Center, Cincinnati, OH). Amplification of adenoviruses were performed in transformed 293 human embryonic kidney (HEK) cells (CRL-1573, ATCC, Manassas, VA), followed by cesium chloride density-gradient purification as described [12]. The viral multiplicity of infection (MOI) was determined by dilution assay in HEK-293 cells. Titration assays were used to determine the minimal MOI that would result in at least a two-fold increase in expressed protein and/or block endogenous target protein phosphorylation in NRVM. Corresponding MOIs of adenoviruses expressing GFP were used to control for viral effects. After 24 h of plating, NRVM infected with adenoviruses expressing p38αWT (50 MOI), p38αDN (100 MOI), constitutively-active MKK3 (MKK3CA, 100 MOI), constitutively-active MKK6 (MKK6CA, 100 MOI), wild-type MKP-1 (Ad-MKP-1, 40 MOI), and GFP (Ad-GFP, 50 MOI) diluted in DMEM/M199. At these MOI, the NRVM did not display any obvious signs of cell toxicity (cell detachment, cellular vacuoles, cell rounding). Levels of expressed proteins were determined using Western blot analysis (not shown). After 24 h of transfection, the medium was replaced with virus-free and serum-free DMEM/M199, and cells were cultured for an additional 12 h prior to stretch experiments.

2.4. Preparation of cell lysates and Western blotting

Cell lysates were obtained by lifting NRVM from culture dishes using lysis buffer (Cell Signaling) containing 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM 2-(2-aminoethyl)-benzenesulfonyl fluoride, hydrochloride and 1 mM sodium orthovanadate. Insoluble material in cell extracts was removed by centrifugation at 14,000 g for 15 min, after which protein content in soluble fractions was determined using a kit (Bio-Rad DC™ Protein Assay) according to the manufacturer's recommendation (Bio-Rad, Hercules, CA). Western blot analyses were performed as previously described [12]. Briefly, equal amounts of protein (30 μg) from cell lysates were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto polyvinylidene fluoride (PVDF) transfer membranes. The membranes were blocked with 3% BSA in TBST buffer (10 mM Tris, 100 mM NaCl, 0.1% Tween 20, pH 7.4) for 2 h. Blots were incubated with the primary antibodies in 5% BSA in TBST buffer overnight at 4 °C with light agitation. Bound primary antibodies were visualized using horseradish peroxidase-labeled secondary antibodies and detected using ECL. Densities of the protein bands were measured using ImageJ software (NIH). Signals from the phosphoproteins in each sample were normalized to total protein, obtained by stripping and reprobing blots with the corresponding total antibody. Blots were again stripped and probed with GAPDH antibody to confirm equal loading.

2.5. Quantitative measurement of Aogen mRNA using real-time PCR

Total RNA was isolated from NRVM using a commercial kit (Totally RNA™, Life Technologies, Carlsbad, CA). First strand cDNA was reverse-transcribed (RT) with random hexamer primers using the High Capacity cDNA Archive kit for real-time RT-PCR (Applied Biosystems, Foster City, CA). Real-time RT-PCR was performed in a MX3005P thermocycler (Agilent Technologies, Santa Clara, CA) using TaqMan® Universal PCR Master Mix (Applied Biosystems). Absolute levels of Aogen mRNA were quantified using 21-base sense (5′-AGCA CGACTTCCTGACTTGGA-3′) and antisense primers (5′-TTGTAGGATCCC CGATTTCC-3′), which span the second intron of the genomic sequence and produce an 88 base-pair amplicon. Amplification of Aogen DNA was performed using oligonucleotide (6FAM-5′-CCGTCTGACCCTGCCG CAGC- 3′-TAMRA), as the reporter probe. Aogen primers and reporters were multiplexed with GAPDH, for a “house-keeper” RNA (proprietary