Biochimica et Biophysica Acta 1802 (2010) 632–638

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

Pharmacological inhibition of c-Jun N-terminal kinase signaling prevents cardiomyopathy caused by mutation in *LMNA* gene

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ARTICLE INFO

Article history:
Received 20 January 2010
Received in revised form 24 March 2010
Accepted 5 April 2010
Available online 11 April 2010

Keywords:
Cardiomyopathy
Lamin
MAP kinase
JNK
Emery–Dreifuss muscular dystrophy

ABSTRACT

Mutations in *LMNA*, which encodes A-type nuclear lamins, cause disorders of striated muscle that have as a common feature dilated cardiomyopathy. We have demonstrated an abnormal activation of both the extracellular signal-regulated kinase (ERK) and the c-Jun N-terminal kinase (JNK) branches of the mitogen-activated protein kinase signaling cascade in hearts from $Lmna^{H222P/H222P}$ mice that develop dilated cardiomyopathy. We previously showed that pharmacological inhibition of cardiac ERK signaling in these mice delayed the development of left ventricle dilatation and deterioration in ejection fraction. In the present study, we treated $Lmna^{H222P/H222P}$ mice with SP600125, an inhibitor of JNK signalling. Systemic treatment with SP600125 inhibited JNK phosphorylation, with no detectable effect on ERK. It also blocked increased expression of RNAs encoding natriuretic peptide precursors and proteins involved in the architecture of the sarcomere that occurred in placebo-treated mice. Furthermore, treatment with SP600125 significantly delayed the development of left ventricular dilatation and prevented decreases in cardiac ejection fraction and fibrosis. These results demonstrate a role for JNK activation in the development of cardiomyopathy caused by *LMNA* mutations. They further provide proof-of-principle for JNK inhibition as a novel therapeutic option to prevent or delay the cardiomyopathy in humans with mutations in *LMNA*.

1. Introduction

Mutations in *LMNA* encoding A-type nuclear lamins are responsible for at least three severe diseases involving striated muscles: autosomal Emery–Dreifuss muscular dystrophy [1], limb girdle muscular dystrophy type 1B [2], and dilated cardiomyopathy type 1A [3]. A common feature of these disorders is dilated cardiomyopathy, which is characterized by an age of onset generally in the third decade of life and frequently associated with a progressive conduction system disease leading to implantation of defibrillators [4]. Affected individuals eventually develop heart failure, for which there is currently no curative treatment, ultimately necessitating cardiac transplantation.

Identification of *LMNA* mutations in patients with dilated cardiomyopathy raised intriguing questions about the relationship between A-type nuclear lamins and dilated cardiomyopathy, since lamins are not known to contribute in force transmission or generation in cardiomyocytes. The link between cardiomyopathy and abnormalities in A-type lamins is poorly understood and only a few hypotheses have been raised concerning pathophysiology. We have recently reported an abnormal activation of the extracellular signal-regulated kinase (ERK) and the c-Jun N-terminal kinase (JNK) branches of the mitogen-activated protein kinase (MAP kinase) signalling cascade in hearts of *Lmna* H222P knock-in mice, a model of autosomal Emery–Dreifuss muscular dystrophy [5]. Male $Lmna^{H222P/H222P}$ mice developed left ventricular (LV) dilatation and depressed contractile function starting at approximately 8–10 weeks of age and invariably developed LV dilatation and decreased cardiac contractility at 16 weeks of age with death typically occurring between 16 and 36 weeks [6]. On the basis of our observations that ERK and JNK are activated in these mice before the onset of clinically detectable cardiomyopathy, as well as our demonstration that lamin A variants that cause striated muscle disease activate both of these protein kinases when expressed in cultured cells, we hypothesized that abnormal activation of ERK and

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doi:10.1016/j.bbadis.2010.04.001

JNK plays a primary pathogenic role in the development of cardiomyopathy [5]. Recently, we showed that blocking ERK signalling in male *Lmna*$^{H222P/H222P}$ mice, using a small molecule inhibitor of extracellular signal-regulated kinase kinase (MEK) that activates ERK, induced normal LV diameters and cardiac ejection fraction (EF) at the age of 16 weeks, when placebo-treated mice had significant abnormalities in these parameters [7]. In the present study, we sought to determine if pharmacological inhibition of JNK signaling would similarly prevent or delay the development of dilated cardiomyopathy in *Lmna*$^{H222P/H222P}$ mice. To test this hypothesis, we treated *Lmna*$^{H222P/H222P}$ mice with SP600125, an inhibitor of JNK.

2. Methods

2.1. Mice

Lmna$^{H222P/H222P}$ mice were generated and genotyped as previously described [6]. Genotyping was performed by polymerase chain reaction using genomic DNA isolated from tail clippings and oligonucleotides with sequences 5′-cagccatcacctctcctttg-3′ and 5′-agcaccagggagaggacagg-3′. Mice were fed a chow diet and housed in a disease-free barrier facility with 12-h/12-h light/dark cycles. The Institutional Animal Care and Use Committee at Columbia University Medical Center approved the use of animals and the study protocol. The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.2. JNK inhibitor

The anthrapyrazolone inhibitor of JNK, SP600125 (Calbiochem), was dissolved in dimethyl sulfoxide (DMSO) (Sigma) at a concentration of 0.5 mg/ml and administered at a dose of 3 mg/kg/day for 5 days a week. The inhibitory activity of SP600125 is selective for all JNK isoforms (IC_{50} = 40 nM for JNK-1 and JNK-2 and 90 nM for JNK-3) [8]. The placebo consisted of DMSO alone and was delivered in the same volume. Placebo and SP600125 were administered by intraperitoneal injection using a 27 5/8-gauge syringe. Treatment was initiated when mice were 8 weeks of age and continued until 16 weeks of age.

2.3. Thansthoracic echocardiography

At 16 weeks of age, mice were anesthetized with 1.5% isoflurane in O_2 and placed on a heating pad (37 °C). Echocardiography was performed using a Visualsonics Vevo 770 ultrasound with a 30-MHz transducer applied to the chest wall. Cardiac ventricular dimensions and EF were measured in 2D mode and M-mode three times for the number of animals indicated. A “blinded” echocardiographer (J.S.), unaware of the genotype or treatment, performed the examinations and interpreted the results.

2.4. Histopathological analysis

Mice were sacrificed at 16 weeks of age after being examined by echocardiography and freshly removed hearts were fixed in 4% formaldehyde for 48 hours, embedded in paraffin, sectioned at 5 µm, and stained with Gomori's trichrome or hematoxylin and eosin. Representative stained sections were photographed using a Microphot SA (Nikon) light microscope attached to a Spot RT Slide camera (Diagnostic Instruments). Images were processed using Adobe Photoshop CS (Adobe Systems).

2.5. Protein extraction and immunoblotting

Heart tissue was homogenized in extraction buffer as previously described [7]. Protein samples were subjected to SDS–PAGE, transferred to nitrocellulose membranes, and blotted with primary antibodies against JNK (Santa Cruz), phosphorylated JNK (no. 9251; Cell Signaling), phosphorylated c-Jun (no. Sc-822; Santa Cruz), ERK1/2 (no. Sc-94; Santa Cruz), and phosphorylated ERK1/2 (no. 9101; Cell Signaling). Secondary antibodies were horseradish peroxidase–conjugated (Amersham). Recognized proteins were visualized by enhanced chemiluminescence (ECL; Amersham). The signal generated using antibody against GAPDH was used as an internal control to normalize the amounts of protein between immunoblots.

2.6. Quantitative real-time RT–PCR analysis

Total RNA was extracted using the RNeasy Isolation Kit (Qiagen) as previously described [7]. cDNA was synthesized using Superscript first strand synthesis system according to the manufacturer's instructions (Invitrogen) on total RNA. For each replicate in each experiment, RNA from tissue samples of different animals was used. Primers were designed correspond to mouse RNA sequences using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) for *Nppa* (forward 5′-gcttccaggccatattggag-3′, reverse 5′-ccctgcttcctcagtctgct-3′), *Nppb* (forward 5′-ggaccaaggcctcacaaaag-3′, reverse 5′-tacagcccaaacgactgacg-3′), *Myl4* (forward 5′-cccaagcctgaagagatgag-3′, reverse 5′-agacaacagctgctccacct-3′), *Myl7* (forward 5′-tcaaggaagccttcagctgc-3′, reverse 5′-cggaacacttaccctcccg-3′), *Myh7* (forward 5′-tgcagcagttcttaaccac-3′, reverse 5′-tcgaggcttctggaagttgt-3′), and *JunD* (forward 5′-atgtgcacgaaaatggaaca-3′, reverse 5′-cctgacccgaaaagtagctg-3′). The real-time RT–PCR reaction contained iQ SYBR Green Super Mix (Bio-Rad), 200 nM of each primer, and 0.2 µl of template in a 25-µl reaction volume. Amplification was carried out using the MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad) with an initial denaturation at 95 °C for 2 min followed by 50 cycles at 95 °C for 30 s and 62 °C for 30 s. Relative levels of mRNA expression were calculated using the $\Delta\Delta C_T$ method [7]. Individual expression values were normalized by comparison with *Gapdh* mRNA (forward 5′-tgcaccaccaactgcttag-3′, reverse 5′-ggatgcagggatgatgttc-3′).

2.7. Statistical analysis

Results of immunoblots, real-time RT–PCR, and fibrosis quantification were compared using the Student unpaired *t*-test. Comparisons of the echocardiographic parameters between DMSO-treated *Lmna*$^{H222P/H222P}$ and *Lmna*$^{+/+}$ mice and between SP600125-treated and DMSO-treated *Lmna*$^{H222P/H222P}$ were performed using the Student unpaired *t*-test; to validate these results, a nonparametric test (Mann–Whitney) was performed and concordance was checked. Statistical analyses were performed using GraphPad Prism software.

3. Results

3.1. Effect of SP600125 on JNK activity

Systemic administration of SP600125 to *Lmna*$^{H222P/H222P}$ mice partially blocked phosphorylation of JNK in hearts as shown by immunoblot (Fig. 1A). At 3 mg/kg/day (5 times a week), we did not detect inhibition of phosphorylation of ERK in the hearts (Fig. 1A). Quantification of the immunoblot signals for JNK showed that DMSO-treated *Lmna*$^{H222P/H222P}$ mice had a 2.5-fold increase of phosphorylated JNK expression compared to *Lmna*$^{+/+}$ mice but *Lmna*$^{H222P/H222P}$ treated with SP600125 had a significantly reduced level of phosphorylated JNK similar to *Lmna*$^{+/+}$ mice (Fig. 1A). Phosphorylation of the downstream target, c-Jun, was also significantly reduced by SP600125