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

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1. Introduction

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 (MAP kinase) signalling cascade in hearts from Lmna H222P/H222P mice that develop dilated cardiomyopathy. We previously showed that pharmacological inhibition of cardiac ERK signalling 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.
JNK plays a primary pathogenic role in the development of cardiomyopathy [5]. Recently, we showed that blocking ERK signalling in male Lmna<sup>H222P/H222P</sup> 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 signalling would similarly prevent or delay the development of dilated cardiomyopathy in Lmna<sup>H222P/H222P</sup> mice. To test this hypothesis, we treated Lmna<sup>H222P/H222P</sup> mice with SP600125, an inhibitor of JNK.

2. Methods

2.1. Mice

Lmna<sup>H222P/H222P</sup> 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′-cagccatactctctttg-3′ and 5′-acacgaggagagccagc-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 antrypyravalone 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<sub>50</sub> = 40 nM for JNK-1 and JNK-2 and 90 nM for JNK-3) [7]. The placebo consisted of DMSO alone and was delivered in the same volume. Placebo and SP600125 were administered by intraperitoneal injection using a 27-gauge syringe. Treatment was initiated when mice were 8 weeks of age and continued until 16 weeks of age.

2.3. Thanshoracic echocardiography

At 16 weeks of age, mice were anesthetized with 1.5% isoflurane in O<sub>2</sub> 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 function were measured using a Visualsonics Vevo 770 ultrasound with a 30-MHz transducer applied to the chest wall. Cardiac ventricular dimensions and function were measured using a Visualsonics Vevo 770 ultrasound with a 30-MHz transducer applied to the chest wall. Cardiac ventricular dimensions and function were measured using a Visualsonics Vevo 770 ultrasound with a 30-MHz transducer applied to the chest wall. 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).

2.4. 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′-ccttcgaggcatatgag-3′, reverse 5′-cctccgctctctacctgc-3′), Nppb (forward 5′-ggaccaaggctccaaag-3′, reverse 5′-tacagcaaccaagctgc-3′), Myl4 (forward 5′-ccagcgtgagagatag-3′, reverse 5′-acagacaagctctgcc-3′), Myl7 (forward 5′-taagggagctcctgcc-3′, reverse 5′-ggaacactttcctcc-3′), Myh7 (forward 5′-tcagcagtttcaac-3′, reverse 5′-tgcagttctggagttt-3′), and Gnatd (forward 5′-tgcacccgaggaagagtgc-3′, reverse 5′-tgatgcagggtagttgc-3′). The real-time RT–PCR reaction contained 1× 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 ΔΔC<sub>T</sub> method [7]. Individual expression values were normalized by comparison with Gapdh mRNA (forward 5′-tgacccagaaatgtggga-3′, reverse 5′-ggtagcagggtagttgc-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<sup>H222P/H222P</sup> and Lmna<sup>+/-</sup> mice and between SP600125–treated and DMSO-treated Lmna<sup>H222P/H222P</sup> 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<sup>H222P/H222P</sup> 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<sup>H222P/H222P</sup> mice had a 2.5-fold increase of phosphorylated JNK expression compared to Lmna<sup>+/-</sup> mice but Lmna<sup>H222P/H222P</sup> treated with SP600125 had a significantly reduced level of phosphorylated JNK similar to Lmna<sup>+/-</sup> mice (Fig. 1A). Phosphorylation of the downstream target, c-Jun, was also significantly reduced by SP600125.
as well as the expression of JunD mRNA (Fig. 1C), confirming the efficacy of the small molecule inhibitor in the heart at the given dose.

3.2. Effect of SP600125 on cardiac expression of natriuretic peptides and myosin

A feature of dilated cardiomyopathy is the up-regulation of natriuretic peptides [9]. The up-regulation of genes involved in sarcomere organization also occurs in dilated cardiomyopathy [5]. In hearts from male LmnaH222P/H222P mice, the expressions of NppA and NppB mRNAs encoding natriuretic peptides precursors were significantly increased compared to Lmna+/-/+ mice (Fig. 2A). Similarly, the expression of Myl7 and Myh4 mRNAs encoding myosin light chain and the expression of Myh7 mRNA encoding myosin heavy chain were significantly increased compared to Lmna+/-/+ mice (Fig. 2B). SP600125-treated LmnaH222P/H222P mice had a significantly decreased cardiac expression of Myl7, Myh4, Myh7, NppA, and NppB compared to DMSO-treated LmnaH222P/H222P mice (Figs. 2A and B).

3.3. Effect of JNK inhibition on cardiac function

Histopathological analysis of hearts at 16 weeks of age showed that DMSO-treated LmnaH222P/H222P mice had an increase in fibrotic tissue compared to Lmna+/-/+ mice (P<0.0005; Fig. 3A). As assessed by quantification of collagen staining, SP600125-treated LmnaH222P/H222P mice had a statistically significant decrease of fibrosis compared to DMSO-treated LmnaH222P/H222P (P<0.05; Fig. 3A). Hence, treatment with SP600125 prevents cardiac fibrosis in LmnaH222P/H222P mice. To confirm the degree of fibrosis, we further determined the expression of Col1a1 and Col1a2 encoding type I collagen by quantitative real-time RT–PCR. At 16 weeks of age, DMSO-treated LmnaH222P/H222P mice had a significantly decreased cardiac expression of Myl7, Myh4, Myh7, NppA, and NppB compared to DMSO-treated Lmna+/-/+ mice (Fig. 2A). SP600125-treated LmnaH222P/H222P mice had a significantly decreased cardiac expression of Myl7, Myh4, Myh7, NppA, and NppB compared to DMSO-treated LmnaH222P/H222P mice (Figs. 2A and B).
had a significantly increased expression of both genes in the heart compared to Lmna+/+ mice (Fig. 3B). In DMSO-treated LmnaH222P/H222P mice, Col1a1 and Col1a2 mRNAs were increased by 3-fold compared to Lmna+/+ mice (Fig. 3B). Treatment with SP600125 significantly lowered the expression of both Col1a1 and Col1a2 mRNAs in the heart of LmnaH222P/H222P mice compared to the DMSO-treated LmnaH222P/H222P mice (Fig. 3B).

We recently reported alterations in nuclear morphology, including abnormal elongation of nuclei, in cardiomyocytes of LmnaH222P/H222P mice [7]. At 16 weeks of age, DMSO-treated LmnaH222P/H222P mice had a significant increase in nuclear length, compared to Lmna+/+ mice (Fig. 4A). Cardiomyocyte nuclei in SP600125-treated LmnaH222P/H222P mice had an overall length that was similar to those in Lmna+/+ mice (Fig. 4A). Mean lengths of cardiomyocyte nuclei in placebo-treated LmnaH222P/H222P mice were significantly longer than in Lmna+/+ mice and SP600125-treated LmnaH222P/H222P mice (Fig. 4B).

Compared with Lmna+/+ mice, LmnaH222P/H222P mice treated with DMSO had significant increases in LV end-diastolic and end-systolic...
diameters and decreases in interventricular septal diameter, EF, and fractional shortening (Table 1), consistent with what has been described in previous studies [6,7]. We then analyzed the effects of the JNK inhibitor compared to the placebo on echocardiographic parameters. The analysis was performed with heart rates similar between the two groups (358.47 ± 81.75 beats/min for DMSO-treated Lmna H222P/H222P mice and 355 ± 80.77 beats/min for SP600125-treated Lmna H222P/H222P mice). When treated with SP600125, Lmna H222P/H222P mice had approximately 5% smaller mean LV end-diastolic diameter, although the difference compared to placebo treatment mice did not reach statistical significance. When treated with SP600125, Lmna H222P/H222P mice had a 15% smaller mean LV end-systolic diameter compared to DMSO-treated Lmna H222P/H222P mice (P < 0.05). Systemic treatment with SP600125 had a positive effect on cardiac function leading to an EF approximately 20% higher than in DMSO-treated Lmna H222P/H222P mice (P < 0.005). Hence, treatment with SP600125 for 8 weeks prevented or delayed the development of significant cardiac contractile dysfunction in Lmna H222P/H222P mice.

4. Discussion

Our results show that abnormal activation of the stress-induced JNK signalling pathway contributes to the pathogenesis of cardiomyopathy caused by mutations in LMNA encoding A-type lamins. It remains unclear how A-type lamins with amino acid substitutions activate JNK signalling. Some investigators have hypothesized that alterations in response to stress may underlie the development of cardiac disease caused by LMNA mutation [10]. Abnormal responses to stress in cardiomyocytes with abnormalities in A-type lamins could therefore potentially have an impact on activation of JNK. This hypothesis remains to be tested.
We have demonstrated that partial pharmacological inhibition of JNK in vivo, using SP600125, prevents significant cardiomyopathy in male Lmna<sup>H222P/H222P</sup> mice at an age when placebo-treated controls have detectable cardiac dysfunction. We recently reported that the partial pharmacological blockade of ERK signalling for the same duration in Lmna<sup>H222P/H222P</sup> mice of the same age similarly prevents cardiomyopathy [7]. Data in our previous study [7] and our current results show that inhibiting either the ERK or the JNK branches of the MAP kinase signalling cascade prevents the re-expression of fetal genes such as those encoding myosins, the up-regulation in expression of natriuretic peptides, LV dilatation, and decreased cardiac contractility. We have also shown that the JNK inhibitor prevents onset of cardiac fibrosis in 16-week-old Lmna<sup>H222P/H222P</sup> mice.

This preclinical study in mice assessed primary (LV dilatation, EF) and secondary (expression of natriuretic factors) end points that are used in many human clinical heart failure trials. The measurements of LV function we used are strictly correlated to prognosis, and in many human clinical trials, their behaviour parallels changes in mortality with treatment [11]. For example, LV end-systolic volume is the major determinant of survival in human subjects after recovery from myocardial infarction and after coronary artery bypass grafting for impaired LV function [12,13]. While mortality is a reasonable end point in phase III clinical trials for advanced heart failure, it is rarely, if ever, used in the initial drug assessment phase or in treatment of subjects with early heart disease [11], as were both the case in our study. The effect of JNK inhibition at later treatment onset, after the development of decreased cardiac ejection fraction, is currently being evaluated in our laboratory, as many patients will be diagnosed at an advance stage. Nonetheless, our previous [7] and current results clearly provide proof-of-principle that specific inhibitors that target both JNK and ERK signalling could prevent or delay the onset of cardiomyopathy caused by LMNA mutations and indicate that additional studies are warranted. Future studies of the effects of ERK and JNK signalling pathway inhibitors on cardiac conduction defects could also be interesting, given that early conduction abnormalities usually occur in human subjects with LMNA mutations.

JNK has been shown to play a central role in tissue remodelling through its ability to interact to AP-1-mediated transcription [14,15]. AP-1 function is regulated both through changes in the abundance of its Jun and Fos components and posttranslational modification by phosphorylation [16]. Of interest, AP-1 modulates the regulation of type I collagen [17,18]. This is consistent with our observation that SP600125 decreased the expression of JunD mRNA as well as Col1a1 and Col1a2 mRNAs in hearts of Lmna<sup>H222P/H222P</sup> mice. We also showed that there was a markedly decreased amount of myocardial fibrosis in hearts of 16-week-old Lmna<sup>H222P/H222P</sup> mice treated with the JNK inhibitor. This antifibrotic action of SP600125 could be secondary to the beneficial effect on the cardiac structure and function.

Additional preclinical research should be performed before initiating clinical trials of ERK and JNK inhibition in human subjects with cardiomyopathy caused by LMNA mutations. To resolve the possible but unlikely issue that off-target effects are providing benefits on cardiomyopathy in Lmna<sup>H222P/H222P</sup> mice, these animals should be treated with other drugs in these classes, such as JNK inhibitors of different structure that recognize a different interaction site [19]. For ERK signalling, several compounds of different structures that act at various sites in the pathway are currently in clinical development and several have already been used in human subjects [20]. The effects of longer-term ERK and JNK inhibition on various tissues also need to be examined in experimental animals, especially on skeletal muscle that is often simultaneously affected in individuals with LMNA mutations that cause cardiomyopathy [1,2].

Conflict of interest

H.J.W. and A.M. are inventors on a pending PCT patent application on methods for treating and/or preventing cardiomyopathies by ERK and JNK inhibition filed by the Trustees of Columbia University in the City of New York.
Echocardiographic data at 16 weeks of age for Lmna+/– mice and LmnaH222P/H222P mice treated with DMSO or SP600125.

<table>
<thead>
<tr>
<th>Genotype (treatment)</th>
<th>n</th>
<th>LVEDD (mm)</th>
<th>LVESD (mm)</th>
<th>IVSD (mm)</th>
<th>EF (%)</th>
<th>FS (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lmna+/– (no treatment)</td>
<td>13</td>
<td>3.35 ± 0.09</td>
<td>2.00 ± 0.09</td>
<td>0.72 ± 0.02</td>
<td>76.82 ± 2.01</td>
<td>44.21 ± 1.77</td>
</tr>
<tr>
<td>LmnaH222P/H222P (DMSO)</td>
<td>15</td>
<td>3.59 ± 0.09*</td>
<td>2.68 ± 0.14***</td>
<td>0.65 ± 0.03*</td>
<td>56.89 ± 2.87***</td>
<td>29.70 ± 1.87***</td>
</tr>
<tr>
<td>LmnaH222P/H222P (SP600125)</td>
<td>13</td>
<td>3.46 ± 0.07</td>
<td>2.30 ± 0.12†</td>
<td>0.72 ± 0.02*</td>
<td>69.24 ± 2.66H</td>
<td>38.70 ± 2.05H</td>
</tr>
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LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; IVSD, interventricular septum diameter; EF, ejection fraction; FS, fractional shortening.

Values are means ± standard errors.

Comparison between DMSO-treated LmnaH222P/H222P and Lmna+/– mice was performed using the Student unpaired t-test, *P < 0.05, **P < 0.005, ***P < 0.0005.

Comparison between SP600125-treated and DMSO-treated LmnaH222P/H222P was performed using the Student unpaired t-test and results validated by a nonparametric test (Mann–Whitney), †P < 0.05, ‡P < 0.005.

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References


