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Downregulation of cardiac lineage protein-1 confers cardioprotection through the upregulation of redox effectors

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ABSTRACT

CLP-1, the mouse homologue of human Hexim1 protein, exerts inhibitory control on transcriptional elongation factor-b of RNA transcript elongation. Previously, we have demonstrated that downregulation of cardiac lineage protein-1 (CLP-1) in CLP-1$^{+/-}$ heterozygous mice affords cardioprotection against ischemia–reperfusion injury. Our current study results show that the improvement in cardiac function in CLP-1$^{+/-}$ mice after ischemia–reperfusion injury is achieved through the potentiation of redox signaling and their molecular targets including redox effector factor-1, nuclear factor erythroid 2-related factor, and NADPH oxidase 4 and the active usage of thioredoxin-1, thioredoxin-2, glutaredoxin-1 and glutaredoxin-2. Our results suggest that drugs designed to down regulate CLP-1 could confer cardioprotection through the potentiation of redox cycling.
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1. Introduction

Cardiac lineage protein-1, a mouse homologue of human Hexim1 binds with and inhibits the positive transcription elongation factor-b (P-TEFb), which is composed of cyclin-dependent kinase 9 (Cdk9) and cyclin T1 [1–3]. P-TEFb phosphorylates the carboxyl-terminal domain (CTD) of RNA polymerase II, a major substrate of P-TEFb, and upon phosphorylation elongates nascent transcripts to form full-length messenger RNAs [2]. Hexim1 forms a protein–RNA complex composed of 7SK small nuclear RNA and
P-TEFb, and inhibits the kinase activity of CDK9, leading to the suppression of RNA polymerase II-dependent transcriptional elongation [4]. Previously we have demonstrated that CLP-1 plays an essential role in the regulation of P-TEFb activity, and the dissociation of CLP-1 from P-TEFb complex stimulates cardiac hypertrophic phenotype [1,5,6]. The CTD of human RNA polymerase II is composed of 52 repeats of a heptapeptide sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser. Cdk9 specifically phosphorylates Ser2 of the CTD of RNA polymerase II [3], whereas Cdk7 specifically phosphorylates Ser5 [7]. Both Cdk7 and Cdk9 are activated during hypertrophic stimuli, but only Cdk9 is specifically activated during acute overload by aortic banding in the myocardium [8]. Activation of Cdk9 causes cardiomyocyte enlargement and defective mitochondrial function, via diminished peroxisome proliferator-activated receptor gamma coactivator 1 (PGC-1) transcription, and confers a predisposition to heart failure [9].

In our recent study using isolated mouse heart ischemia–reperfusion model, we have demonstrated that downregulation of CLP-1 in heterozygous CLP-1$^{+/-}$ mice affords cardioprotection against ischemia–reperfusion injury [10], where the CLP-1 remains associated with P-TEFb complex in the CLP-1$^{+/-}$ mice hearts, and results in a decrease in Cdk7 and Cdk9 activities, and an enhanced level of PGC-1α and hypoxia-inducible factor1α (HIF-1α). Our present study shows that CLP-1 downregulation-mediated cardioprotection against cardiac ischemia–reperfusion injury involved the elicitation of redox active proteins such as redox effector factor-1 (Ref-1), nuclear factor erythroid 2-related factor (Nrf2), NADPH oxidase 4 (NOX4) and thioredoxin super family members.

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2. Materials and methods

2.1. Animals

All animals used in this study received humane care in compliance with the regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, NIH Publication, 1996 edition, and all the protocols were approved by Institutional Animal Care Committee.

2.2. Isolated working heart preparation and drug treatment

Wild-type CLP-1^{++} and heterozygous CLP-$1^{+/-}$ mice were anesthetized with sodium pentobarbital (80 mg/kg, i.p.) (Abbott Laboratories, North Chicago, IL, USA), and intraperitonealy heparin sodium (500 IU/kg, i.v.) (Elkins-Sinn Inc., Cherry Hill, NJ, USA) was used as an anticoagulant. After the deep anesthesia was conformed, hearts were excised, the aorta was canulated, and the hearts were perfused through the aorta in Langendorff mode at a constant (100 cm of water) perfusion pressure at 37 °C with the KHB for a 5 min washout period as described previously. The perfusion medium consisted of a modified Krebs–Henseleit bicarbonate buffer (millimolar concentration: sodium chloride 118, potassium chloride 4.7, calcium chloride 1.7, sodium bicarbonate 25, potassium dihydrogenphosphate 0.36, magnesium sulfate 1.2 and glucose 10), and after its oxygenization pH was 7.4 at 37 °C. During the washout period left atria was canulated, and the Langendorff preparation was switched to the working mode for 10 min with a left atrial filling pressure of 17 cm H_2O, aortic afterload pressure was set to 100 cm of water. At the end of 10 min, baseline cardiac function like heart rate (HR, beats/min), aortic flow (AF, ml/min), coronary flow (CF, ml/min), left ventricular developed pressure (LVDP, mmHg) and first derivative of developed pressure (LVdp/dt, mmHg/s) were recorded. After that 30 min of global ischemia was initiated by clamping the left atrial inflow and aortic outflow lines at a point close to their origins. At the end of the 30 min of ischemia, reperfusion was initiated for 120 min by unclamping the atrial inflow and aortic outflow lines. The first 10 min reperfusion was in Langendorff mode to avoid the ventricular fibrillations, after the hearts were switched to anterograde working mode.

2.3. Cardiac function assessment

Aortic pressure was measured using a Gould P23XL pressure transducer (Gould Instrument Systems Inc., Valley View, OH, USA) connected to a side arm of the aortic cannula. The signal was amplified using a Gould 6600 series signal conditioner and monitored on a CORDAT II real-time data acquisition and analysis system (Triton Technologies, San Diego, CA, USA). Heart rate, LVDP (defined as the difference of the maximum systolic and diastolic aortic pressures), and dp/dt were all derived or calculated from the continuously obtained pressure signal. Aortic flow was measured using a calibrated flow-meter (Gilmont Instrument Inc., Barrington, IL, USA) and coronary flow was measured by timed collection of the coronary effluent dripping from the heart.

2.4. Cytosolic extract preparation

About 100 mg of left ventricular tissue was homogenized in 1 ml of buffer containing 25 mM Tris, 25 mM NaCl, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 0.5 mM EDTA, 1 mM PMSF and protease inhibitors cocktail (leupeptin, aprotinin, and pepstatin). Homogenates were centrifuged at 3000 rpm for 10 min at 4 °C, and the supernatant was again centrifuged at 10 000 rpm for 20 min at 4 °C. The resultant supernatant was saved as cytosolic extract. Total protein concentration in the cytosolic extract was determined using BCA Protein Assay Kit (Pierce, Rockford, IL).

2.5. Western blot analysis

Total cell lysate from mice heart tissue was separated in SDS–PAGE and transferred to nitrocellulose filters. Filters were blocked in 5% non-fat dry milk, and probed with a primary antibody (1:1000 dilution) for overnight. Ref-1 primary antibody was obtained from Cell Signaling Technology. Nrf2, heme oxygenase-1 and NOX4 primary antibodies were obtained from Santa Cruz Biotechnology. Protein bands were identified with horseradish peroxidase conjugated secondary antibody (1:2000 dilution) and Western Blotting Luminol Reagent (Santa Cruz Biotechnology). The resulting blots were digitized, subjected to densitometric scanning using a standard NIH image program, and normalized against loading control.

2.6. Immunofluorescence staining

Heart tissue samples collected at the end of experiments were fixed in 4% buffered paraformaldehyde (pH 7.4), embedded in paraffin and sectioned. After deparaffinizing the sections the antigen retrieval treatment was performed using 10 mM sodium citrate containing 0.05% Tween 20 at 90–95 °C for 30 min. After washing with PBS, the slides were blocked with Powerblock (BioGenex, San Ramon, CA) for 10 min. Slides were washed with PBS and incubated with primary antibodies (1:25 dilution) in PBS containing 1% BSA for 2 h. After washing, the slides were incubated with fluorescein-conjugated secondary antibodies (anti-rabbit Alexa Fluor 488, green; and anti-goat Alexa Fluor 594, red; both from Molecular Probes and used at 1:1000 dilutions) in the dark for 45 min. For nuclear staining To-Pro 3 iodide (Molecular Probes; 1:1000 dilution) was used for 45 min in the dark. The slides were washed and covered with mounting medium. Confocal microscopic images were obtained using a Zeiss LSM 510 (Thornwood, NY) confocal laser scanning microscope with 40×1.3 oil immersion objective by simultaneous recording in the 488λ, 560λ, and/or 615λ channels as appropriate.

2.7. Semi-quantitative RT-PCR

Total RNA was extracted from liver tissue, using Trizol reagent (Invitrogen, CA, USA). Three micrograms of total RNA was reverse-transcribed, using a SuperScript-II First-Strand Synthesis System (Invitrogen) with oligo dT12-18, according to the manufacturer's protocol. The cDNA was amplified by PCR, using Platinum Taq-DNA polymerase (Clontech, CA, USA) following a standard semi-quantitative RT-PCR technique in which the amplified products were not saturated at the number of cycles performed. The primers used for the amplification are enlisted in Table 1. The PCR products were visualized by electrophoresis in 1% agarose gel (Bio-Rad, CA, USA) with ethidium bromide (0.5 μg/ml; Sigma, MO, USA).

2.8. Statistical analysis

All values are expressed as the means ± standard error of mean (S.E.M.). Student's *t*-test or one way analysis of variance test followed by Bonferoni's correction was first carried out to test for any differences between the mean values of all groups. The results were considered significant if $P < 0.05$.

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