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Inhibition of the NLRP3 inflammasome limits the inflammatory injury following myocardial ischemia–reperfusion in the mouse

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ABSTRACT

Background: Successful reperfusion is the most effective strategy to reduce ischemic injury in acute myocardial infarction (AMI). Ischemic injury, however, also triggers a secondary ischemia–independent injury, known as reperfusion injury, contributing to the overall infarct size. We hypothesize that inhibition of the Nod-like Receptor Protein-3 (NLRP3) inflammasome limits infarct size following myocardial ischemia/reperfusion (I/R), by inhibiting the inflammatory component of the reperfusion injury.
Methods: CD-1 male mice underwent transient ligation of the left anterior descending coronary artery for 30 or 75 min followed by reperfusion. Infarct size was measured at 1, 3 and 24 h. A NLRP3 inflammasome inhibitor (NLRP3inh) or vehicle was administrated immediately at time of reperfusion or with a delay of 1 or 3 h of reperfusion.
Results: A time-dependent increase in infarct size was measured at 1, 3, and 24 h after reperfusion (11 ± 2%, 30 ± 5% and 43 ± 4% of the area at risk respectively; $P < 0.001$ for trend). NLRP3 myocardial expression was significantly increased at 24 h and 6 h vs 3 h ($P < 0.01$). Administration of the NLRP3inh at reperfusion did not reduce infarct size at 3 h, while it significantly reduced infarct size at 24 h (−56% vs vehicle, $P < 0.01$). The NLRP3inh given 1 h after reperfusion also significantly decreased caspase-1 activity and infarct size measured at 24 h, whereas the NLRP3inh did not when given with a delay of 3 h.
Conclusions: Pharmacological inhibition of the NLRP3 inflammasome within 1 h of reperfusion limits the secondary inflammatory injury and infarct size following myocardial ischemia–reperfusion in the mouse.

1. Introduction

Acute myocardial infarction (AMI) refers to a clinical syndrome in which heart muscle dies due to ischemia, an imbalance between oxygen supply and demand, most commonly due to an acute atherothrombotic obstruction of an epicardial coronary artery. The ischemic injury progresses from the endocardium to the epicardium in a '*wavefront phenomenon*' [1]. Prompt reperfusion is the most effective strategy to reduce ischemic injury and infarct size, and improve clinical outcome in patients with AMI [2]. Final infarct size, however, is influenced not only by the duration of ischemia but also by the further injury occurring at reperfusion, referred to as '*reperfusion injury*' [2]. Within minutes of reperfusion, a surge of reactive oxygen species and release of pro-apoptotic proteins from the matrix occur as a result of mitochondrial injury [2], and treatments 'protecting' the mitochondria administered during this 'window', further reduce infarct size compared with reperfusion alone [2]. Ischemic and reperfusion injury, however, also induce the activation of the Nod-like Receptor Protein-3 (NLRP3) inflammasome, leading to a secondary wave of inflammatory injury occurring minutes-to-hours following reperfusion [3,4].

We hypothesized that inhibition of the NLRP3 inflammasome using a pharmacologic inhibitor (NLRP3inh) would provide a novel strategy to specifically limit the inflammatory component of ischemia–reperfusion injury, and thus reduce infarct size following myocardial ischemia/reperfusion compared with reperfusion alone.

2. Methods

2.1. Experimental acute myocardial infarction (AMI)

Adult out-bred male ICR mice (8–12 weeks of age) were supplied by Harlan Sprague Dawley (Indianapolis, IN). All animal experiments were

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conducted under the guidelines of the "Guide for the care and use of laboratory animals" published by National Institutes of Health (revised 2011). The study protocol was approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee. Experimental AMI was induced by transient myocardial ischemia for 30 min followed by reperfusion as described previously [3–5]. Briefly, the mice were orotracheally intubated under anesthesia (pentobarbital 70 mg/kg), subjected to left thoracotomy, pericardiectomy and transient ligation of the proximal left coronary artery for 30 min before closure of the thorax followed by 1, 3, 6 or 24 h of reperfusion. An additional group of mice underwent coronary artery ligation surgery for 75 min followed by 24 h of reperfusion to assess the influence of ischemia duration. A sham surgery was performed without the final ligation of the coronary artery. The animals were euthanized using pentobarbital overdose (100 mg/kg) followed by cervical dislocation.

2.2. Infarct Size

Infarct size (IS) was measured by triphenyl tetrazolium chloride (TTC) staining at 1, 3 and 24 h as previously described [3–5]. Briefly, mice were sacrificed and the hearts were quickly removed and mounted on a Langendorff apparatus. The coronary arteries were perfused with 0.9% NaCl containing 2.5 mM $CaCl_2$. After the blood was washed out, the ligature was tied again and 1% Evans blue dye (Sigma Aldrich) was injected as a bolus into the aorta until most of the heart turned blue. Finally, the hearts were frozen and cut into 6–8 transverse slices of equal thickness (approximately 1 mm) from apex to base and incubated in a 10% TTC isotonic phosphate buffer (pH 7.4) at room temperature for 30 min. The areas of infarcted tissue (white), the non-risk zone (blue), the risk zone (non-blue), and the whole left ventricle were determined by computer morphometric analysis using Image Pro Plus 6.0 software (Media Cybernetics, Silver Spring, MD). The investigators performing the measurements were blinded to the treatment allocation.

2.3. Real-time PCR

RNA was extracted from hearts using affinity columns (ReliaPrep™ RNA Tissue Miniprep, Promega, Madison, WI), and converted to cDNA using the High Capacity cDNA Reverse Transcription kit (Life Technologies, Grand Island, NY, USA). Real-time PCR was performed using SYBR Green PCR master mix (Life Technologies) with a Roche 480 Thermocycler (Roche, Indianapolis, IN, USA).

2.4. Western blot

Tissue samples were digested in RIPA buffer (Sigma Aldrich, St Louis, MO, USA) supplemented with protease inhibitor cocktail (Sigma Aldrich) and homogenized on ice. Following SDS-PAGE, nitrocellulose membranes were blocked with 5% skim milk, and immunoblotting was performed using antibodies for NLRP3 (Santa Cruz Biotechnology, Dallas, TX, USA), and β-actin (Sigma Aldrich). The protein bands were quantified using Image J (National Institute of Health).

2.5. Caspase-1 activity

Caspase-1 activity was measured in protein extracts as previously described [3,6]. Briefly, proteins were extracted from frozen hearts using RIPA buffer (Sigma Aldrich, St Louis, MO, USA) and were diluted in caspase-1 assay buffer (31% sucrose, 3.1% HEPES, and 0.31% CHAPS; Sigma Aldrich). The activity was measured using a fluorometric substrate A2452 (Sigma-Aldrich), after subtraction of background signal in the presence of the caspase-1 inhibitor YVAD-CHO (Enzo Life Sciences, Farmingdale, NY, USA). Fluorescence was read using a Glomax fluorimeter (Promega Corporation, Fitchburg, WI, USA) and a UV filter (emission/excitation 360 nm/410–450 nm).

2.6. Plasma levels of cardiac Troponin I

Immediately before sacrifice, blood was drawn from the inferior vena cava and collected in Vacutainer tubes (BD Vacutainer, Franklin Lakes, NJ) for serum isolation. Mouse cardiac troponin I (cTnI) level was determined by an enzyme-linked immunosorbent assay (Life Diagnostic Inc., West Chester, PA).

2.7. NLRP3 inflammasome inhibitor

The NLRP3 inhibitor (NLRP3inh) was synthesized as described elsewhere [3]. The NLRP3inh (100 mg/kg) or a matching volume of vehicle were administrated intraperitoneally at time of reperfusion or with a delay of 1 or 3 h, or it was given by oral gavage immediately before surgery. The regimen was based on prior pharmacokinetic and pharmacodynamics studies [3,7].

2.8. Statistical analysis

Animals were randomly assigned between treatment groups with the operator measuring infarct size being unaware of allocation. All data are presented as mean and standard error of the mean. Differences between the three or more groups were analyzed using one-way ANOVA followed by Bonferroni corrected Student t-test or assessment for trend. Calculations were completed using the SPSS 15.0 package for Windows (SPSS, Chicago, IL).

3. Results

3.1. Infarct expansion following reperfusion

Infarct size was significantly larger at 24 h (43 ± 4% of the area at risk) vs 3 h (30 ± 5%) and 1 h (11 ± 2%, $p < 0.001$ for trend), showing an expansion of the infarct size over time after reperfusion (Fig. 1). No significant differences were measured in the area at risk (AAR) between the different groups (all $P > 0.05$).

3.2. Formation of the NLRP3 inflammasome following ischemia/reperfusion

NLRP3 expression was measured in the heart of mice after 30 min of ischemia and after 3, 6 and 24 h of reperfusion. Myocardial NLRP3 expression was increased at 6 h (927 ± 380% of sham) and 24 h (722 ± 89%) vs 3 h (111 ± 15%, $P < 0.001$ for both time points) (Fig. 2). Protein quantification using western blotting analysis showed increased NLRP3 level at 24 h of reperfusion (179 ± 19% of sham) when compared to 3 h of reperfusion (98 ± 8% of sham, $P < 0.01$)(Fig. 2). To determine the activity of the NLRP3 inflammasome during ischemia/reperfusion injury, caspase-1 activity was measured at 3, and 24 h of reperfusion: caspase-1 activity was significantly increased at 24 h (346 ± 32% of sham) ($P = 0.020$) (Fig. 2).

3.3. Effects of the NLRP3 inflammasome inhibitor on early and late infarct size

When compared to the vehicle, the NLRP3inh given i.p. at time of reperfusion showed no measureable effects on early infarct size at 3 h (30 ± 5% of the AAR vs 32 ± 3.6% in vehicle, $P = 0.70$) (Supplemental Fig. 1). When measured later in the course, at 24 h, however, the NLRP3inh given i.p. at time of reperfusion significantly reduced caspase-1 activity and infarct size (Fig. 3).

3.4. Effects of delayed NLRP3 inflammasome inhibitor treatment on infarct size

To determine the effects of the inhibition of the NLRP3 inflammasome later in the course of reperfusion, we administered the NLRP3inh i.p. 1