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Pharmacologic inhibition of the NLRP3 inflammasome preserves cardiac function after ischemic and non-ischemic injury in the mouse

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Abstract

Background—Sterile inflammation resulting from myocardial injury activates the NLRP3 inflammasome and amplifies the inflammatory response mediating further damage.

Methods—We used two experimental models of ischemic injury (acute myocardial infarction [AMI] with and without reperfusion) and a model of non-ischemic injury due to doxorubicin 10 mg/Kg, to determine whether the NLRP3 inflammasome preserved cardiac function after injury.

Results—Treatment with the NLRP3 inflammasome inhibitor in the reperfused AMI model caused a significant reduction in infarct size measured at pathology or as serum cardiac troponin I level (–56% and –82% respectively, both $p < 0.001$), and preserved LV fractional shortening (LVFS, 31 ± 2 vs vehicle $26 \pm 1\%$, $p = 0.003$). In the non-reperfused AMI model treatment with the NLRP3 inhibitor significantly limited LV systolic dysfunction at 7 days (LVFS of 20 ± 2 vs $14 \pm 1\%$, $p = 0.002$), without a significant effect on infarct size. In the DOX model, a significant increase in myocardial interstitial fibrosis and a decline in systolic function were seen in vehicle-treated mice, whereas treatment with the NLRP3 inhibitor significantly reduced fibrosis (–80%, $p = 0.001$) and preserved systolic function (LVFS 35 ± 2 vs vehicle $27 \pm 2\%$, $p = 0.017$).

Conclusion—Pharmacological inhibition of the NLRP3 inflammasome limits cell death and LV systolic dysfunction following ischemic and non-ischemic injury in the mouse.

Keywords

acute myocardial infarction; inflammation; inflammasome; caspase-1; NLRP3

INTRODUCTION

NLRP3 (NOD-like receptor family, pyrin domain containing 3), also known as NALP3 or Cryopyrin, is one of the sensors of the inflammasome, a macromolecular structure involved in interleukin-1 β (IL-1 β) and interleukin-18 (IL-18) processing.¹ NLRP3 senses intracellular danger during intracellular infections (i.e. bacterial and viral proteins)^{2, 3} or tissue injury (i.e. ischemia)⁴. NLRP3 activation leads to recruitment of ASC (apoptosis-associated speck-like protein containing carboxy-terminal caspase recruitment domain) and caspase-1 leading to inflammasome formation and ultimately, cell death.²

Growing evidence suggests a central role of the inflammasome in adverse cardiac remodeling following acute myocardial infarction (AMI)^{4–7} leading to further dysfunction and heart failure (HF). Cardiac remodeling and HF, however, are not complications limited to AMI, and may occur also following non-ischemic injury.

Much interest has been devoted to cardiac injury following therapy with antineoplastic chemotherapeutic agents. Doxorubicin (Dox) is an anthracycline used to effectively treat several forms of cancer. Unfortunately, the use of Dox is limited due to its cardiovascular complications that are manifested as acute and chronic cardiotoxicity^{8,9} which appear to be, at least in part, mediated by the NLRP3 inflammasome and IL-1 β .¹⁰ To date, there are no available therapies to inhibit the NLRP3 inflammasome in patients.

Recently, we discovered a new NLRP3 inflammasome inhibitor, a small molecule referred to as 16673-34-0, which is an intermediate in the synthesis of glyburide.¹¹ This molecule prevents the formation of the NLRP3 inflammasome *in vitro* and limits infarct size after myocardial ischemia/reperfusion without affecting glucose metabolism in mouse.¹¹

In the present study, we tested the effects of this novel NLRP3 inflammasome inhibitor on cardiac function in two models of ischemic myocardial injury by way of left coronary artery ligation (transient and permanent) and in a non-ischemic model of doxorubicin cardiotoxicity.

METHODS

The NLRP3 inflammasome inhibitor

The description of the synthesis of the inhibitor is included in the Supplemental Material and in a prior publication.¹¹ In order to determine absorption and plasma distribution of the inhibitor, high-performance liquid chromatography with tandem mass spectrometric (LC/MS/MS) was used to measure levels of NLRP3 inflammasome inhibitor in the plasma collected at 1, 4, and 24 hours after a single injection of 100 mg/kg. Briefly, plasma (30 μ L) from NLRP3 inhibitor-treated mice (N=5) was diluted with 250 μ L of 1% formic acid. Samples were centrifuged at 3000 RPM for 5 min and the supernatant was collected onto a

collection plate using Tomtec vacuum manifold (Tomtec Inc, Hamden, CT). Samples were evaporated to dryness using spin vacuum, reconstituted with 100 μ L of 0.5% formic acid in acetonitrile and 25 μ L were analyzed. The LC/MS/MS method employed positive electrospray ionization (ESI) with multiple reactions monitoring (MRM) mode. Chromatographic separation was achieved using a Shimadzu HPLC (Columbia, MD), with a reversed phase column (Aquasil C18 column 50 $\times$ 2.1 mm, 3.0 μ m, Thermo Scientific, Waltham, MA). Linear gradient conditions were used using mobile phase A (95:5 H₂O/ACN in 0.5% formic acid) and mobile phase B (ACN in 0.5% formic acid) with a flow rate of 0.3 ml/min at any given time with specified concentration. The total run time was 6.5 minutes. Results were processed using MassLynx V4.1 software.

Experimental AMI model

All animal experiments were conducted under the guidelines of the “Guide for the care and use of laboratory animals” published by National Institutes of Health (revised 2011).

To test the effect of the NLRP3 inflammasome inhibitor on cardiac function during AMI, we used two different models of ischemia. Adult male ICR mice (8–12 weeks old), supplied by Harlan Laboratories (Charles River, MA) underwent experimental myocardial ischemia/reperfusion (I/R) or permanent ischemia by coronary artery occlusion. Briefly, mice were anesthetized using pentobarbital (50–70 mg/kg, Sigma-Aldrich, St. Louis, MO) followed by orotracheal intubation. After placing them in the right lateral decubitus position, the mice were subjected to left thoracotomy, pericardiectomy, and the proximal left coronary artery was ligated for 30 minutes and then released (I/R model), or ligated permanently (ischemia without reperfusion model). Different groups of mice were treated with the inhibitor (100 mg/kg in 0.1 ml) or a matching volume of vehicle (0.1ml) (N=4–6 in each group).

Mice in Group 1 underwent 30 minutes of ischemia and were treated with the inhibitor or vehicle at reperfusion, and then sacrificed after 24 hours of reperfusion for the assessment of infarct size (Group 1a), or allowed to recover, and then sacrificed on day 7 for pathology after undergoing echocardiography (Group 1b).

In Group 2, mice underwent permanent coronary artery ligation surgery without reperfusion and received treatment with the inhibitor or vehicle after ligation and then daily thereafter. At day 7, mice underwent echocardiography followed by sacrifice for pathology. A subgroup of mice in each experiment (N=4–6) underwent sham surgery. Moreover, for additional comparison, a group of mice undergoing I/R was treated with glyburide (Enzo Life Sciences Inc., Farmingdale, NY) (see Supplemental Material).

Myocardial damage was determined measuring serum troponin I level at 24 hours after surgery and with pathology assessment of viability. Briefly, mice were anesthetized and the blood was drawn from the inferior vena cava and collected in Vacutainer tubes (BD Vacutainer, Franklin Lakes, NJ) for serum isolation. Mouse troponin I levels were determined by ELISA (Life Diagnostic Inc., West Chester, PA).

Infarct size was measured at 24 hours after induction of ischemia using triphenyl tetrazolium chloride (TTC) (Sigma-Aldrich) to stain viable myocardium (see Supplemental Material).

Infarct scar size on day 7 was measured at pathology. The hearts were explanted and fixed in formalin 10% for at least 48 hours. Slides (5 µm) of the transverse heart's section were collected after inclusion in paraffin followed by staining with Masson's Trichrome (Sigma-Aldrich). The areas of fibrosis and the whole left ventricle were determined by computer morphometry using the Image Pro Plus 6.0 software.¹²

Doppler echocardiography

All mice underwent light anesthesia using pentobarbital (30–50 mg/kg) and echocardiography was performed (see Supplemental Material).

Doxorubicin-induced toxicity

A single dose of Doxorubicin hydrochloride (10mg/kg) (Tocris, Bristol UK) was used to induce cardiotoxicity in ICR male mice. Mice were randomly assigned to treatment with the NLRP3 inflammasome inhibitor (100 mg/kg in 0.1 ml) or a matching volume of the vehicle (N=6–8 in each group) administered daily intraperitoneally for 10 days. Echocardiography was performed at baseline and at 10 days to measure left ventricular dimension and function, as described above.

Interstitial fibrosis

Interstitial LV fibrosis was measured in sham and doxorubicin-treated groups using Masson's staining as described above.

Effects of the NLRP3 inflammasome inhibitor *in vitro*

We have previously described the characteristics of the inhibitor *in vitro*.¹¹ We now expand the description by analyzing the effects of the inhibitor on primary adult rat cardiomyocytes stimulated for the formation of the NLRP3 inflammasome *in vitro* to validate the findings seen in HL-1 cultured cardiomyocytes¹¹ (see Supplemental Material). We also induced formation of the NLRP3 inflammasome using monosodium urate crystals (MSU) and cholesterol crystals (CC) in cultured murine macrophages to determine if the effects of the inhibitor were stimulus dependent (see Supplemental Material). Furthermore, we used bone marrow-derived macrophages from a mutant mouse displaying constitutively active NLRP3¹³ to determine if the inhibitor acted upstream or downstream of NLRP3 activation (see Supplemental Material).

Statistical analysis

Differences between the groups were analyzed using the Student's T test for unpaired data (2 groups) or one-way ANOVA (3 or more groups). Interval changes in repeated measures in echocardiographic data were analyzed comparing the individual percent changes in each group. $p < 0.05$ were considered significant throughout. SPSS (IBM) version 21.0 for Mac was used.

RESULTS

Pharmacokinetics of the NLRP3 inflammasome inhibitor

To evaluate the pharmacokinetic properties of the NLRP3 inhibitor, mice were injected with a single dose of 100 mg/kg and blood samples were obtained at baseline, and at 1, 4, and 24 hours. Following injection with the NLRP3 inhibitor, plasma concentrations were maintained above 1000 ng/mL between 1 hour ($1,363 \pm 194$ ng/mL) and 4 hours ($1,091 \pm 180$ ng/mL) and declined to near baseline values at 24 hours (136 ± 23 ng/mL), suggesting a terminal half-life between 6 and 10 hours that is similar to reported values of glyburide in human patients [DiaBeta prescribing information, Version 2009, Sanofi-Aventis US LLC (Bridgewater, NJ)].

Inhibition of the NLRP3 inflammasome reduces infarct size after myocardial ischemia-reperfusion

The NLRP3 inflammasome inhibitor was administered at reperfusion in mice following transient ligation of the proximal left coronary artery to test the infarct-sparing effect of the inhibitor. The NLRP3 inflammasome inhibitor significantly reduced infarct size as well as troponin I serum levels (Figure 1). These data demonstrate that a single dose of the drug is sufficient to inhibit the NLRP3 inflammasome and protect the heart against I/R injury. Glyburide did not show any protective effect in this model (Supplemental Material and Supplemental Figure 1).

Inhibition of the NLRP3 inflammasome ameliorates cardiac function following ischemia-reperfusion injury

To evaluate the effects of NLRP3 inflammasome inhibition on cardiac remodeling and function, mice underwent I/R and were followed up to 7 days. Mice were treated with a single dose of the inhibitor or vehicle given at reperfusion. As shown in Figure 1, treatment with the inhibitor significantly reduced the degree of LV systolic dysfunction following I/R injury as reflected by preserved LVFS (LVFS = 31.3 ± 1.6 vs vehicle 26.2 ± 0.7 $p=0.003$).

Effect of the NLRP3 inhibitor on cardiac function in a non-reperfused myocardial infarction model

To test the effect of the NLRP3 inflammasome inhibitor in a more severe ischemic injury, mice underwent permanent ligation of the descending left coronary artery, and left ventricular dimensions and function were measured by echocardiography 7 days later. As shown in Figure 2, coronary ligation led to a marked LV dilatation (significant increase in LVEDD and LVESD) and systolic dysfunction (significant decrease in LVFS). Treatment with the inhibitor once daily for 7 days significantly limited the increase in LVEDD and LVESD, and the decrease in LVFS (Figure 2). These results occurred without affecting infarct scar size measured at 7 days after surgery (Figure 2), illustrating that the benefits of this inhibitor are independent of infarct reduction in this model.

Effect of NLRP3 inflammasome inhibition on doxorubicin-induced cardiomyopathy

A single intraperitoneal administration of doxorubicin (10mg/kg) induced a significant increase in myocardial interstitial fibrosis and LV systolic dysfunction (reduced LVFS [P<0.05, Figure 3], reduced systolic thickness of the posterior wall [P<0.05, Supplemental Table 1], and a trend toward reduced systolic thickness of the anterior wall [Supplemental table 1]) at 10 days.

In this model, treatment with the NLRP3 inflammasome inhibitor significantly reduced the amount of fibrosis in the LV and preserved systolic function (Figure 3), thus showing prevention of doxorubicin-induced cardiomyopathy. The effects of the doxorubicin treatment on the LV dimensions are reported in Supplemental Table 1.

Effects of the NLRP3 inflammasome inhibitor *in vitro*

The effects of the inhibitor on HL-1 cultured cardiomyocytes *in vitro* have been already partially characterized.¹¹ Here, for the first time, we show that the inhibitor also prevents the formation of the NLRP3 inflammasome in primary adult rat cardiomyocytes (see Supplemental Material), confirming our previous results in HL-1 cells. We also show that the effects of the inhibitor on NLRP3 are not stimulus-specific as it also inhibited the effects of MSU and CC in murine macrophages (see Supplemental Material). Finally, we show that the inhibitor acts downstream of NLRP3 activation as it was also active in bone marrow-derived macrophages from a mutant mouse displaying constitutively active NLRP3 (see Supplemental Material).

Discussion

Activation of the NLRP3 inflammasome following tissue injury promotes further injury.⁴ Our results show that in a model of myocardial injury due to ischemia-reperfusion, severe ischemia, or doxorubicin, inhibition of the NLRP3 inflammasome ameliorated cardiac remodeling and attenuated LV systolic dysfunction (Figure 4).

AMI is a major cause of mortality and morbidity^{14, 15} and results from the sudden onset of myocardial ischemia. Timely reperfusion still represents the best modality to attenuate injury and improve outcomes. However, reperfusion injury still plagues this technique and efforts aimed at preventing reperfusion injury are constantly being investigated. In a mouse model of ischemia followed by reperfusion (I/R), reperfusion therapy with a single administration of the NLRP3 inflammasome inhibitor reduced infarct size (a direct measure of cardiac injury)¹⁶ and preserved systolic function (an independent predictor of long-term outcome).¹⁷

Although successful reperfusion, by variable degrees, can be attained in the majority of patients after the initial ischemic injury, patients with poor, delayed, or no reperfusion at all are the ones associated with worse prognoses.¹⁸ In a mouse model of large anterior wall non-reperused AMI, we show that treatment with the NLRP3 inflammasome inhibitor, initiated after the onset of ischemia, significantly limits adverse cardiac remodeling and systolic dysfunction. In this model, the benefit appears to be, at least in part, independent of infarct scar size and more directly related to attenuation of ischemic cardiomyopathy.⁷ This

is in agreement with the literature regarding the beneficial effects of IL-1 β blockers in the mouse,^{19–22} and preliminary data from pilot clinical trials.^{6, 23, 24}

Nonetheless, myocardial injury also occurs in the absence of ischemia. Doxorubicin is one of the most common chemotherapeutic drugs and is associated with cardiotoxicity.^{8, 9} The NLRP3 inflammasome inhibitor reduced interstitial myocardial fibrosis and preserved LV systolic function in our mouse model of doxorubicin-induced cardiomyopathy. This further highlights the central role of the NLRP3 inflammasome in mediating cardiac injury in response to different causes and the importance of developing safe and effective NLRP3 inflammasome inhibitors.

The inhibitor prevents the formation of NLRP3 inflammasome formation in HL-1 cultured murine cardiomyocytes and macrophages¹¹ as well as primary adult rat cardiomyocytes. While the exact mechanism of action of this drug is not entirely clear, its effects are specific for the NLRP3 inflammasome¹¹ are independent of the stimulus activating NLRP3; and demonstrate that it likely acts downstream of NLRP3 activation as shown by its activity on bone-marrow derived macrophages from genetically modified mice expressing constitutively active NLRP3. The inhibitor has no effects on glucose levels, as it does not affect insulin release, and is also tolerated when given at repeated doses.¹¹ Inflammasomes other than NLRP3 are unaffected by the inhibitor¹¹ thus potentially limiting off-target effects. To our knowledge, this compound represents the first available *in vivo* NLRP3 inflammasome inhibitor.

The beneficial effects seen in this study are consistent with the reported role of the NLRP3 inflammasome in AMI⁴ and doxorubicin toxicity.¹⁰ Mice with genetic deletion or modulation of the NLRP3 inflammasome are indeed protected in models of myocardial I/R^{25, 26} and non-reperfused AMI.⁴ Several studies have also confirmed a central role of the NLRP3 inflammasome and IL-1 β in adverse cardiac remodeling after AMI.⁶ The NLRP3 inflammasome is also required for IL-1 β production in murine bone marrow-derived macrophages following doxorubicin stimulation.¹⁰ Furthermore, increased levels of IL-1 β are seen in a model of doxorubicin-induced cardiotoxicity *in vivo*.²⁷ Accordingly, we have shown that the NLRP3 inflammasome inhibitor limits doxorubicin-induced systolic dysfunction in the mouse. The NLRP3 knockout mouse is also protected in an inflammatory cardiomyopathy mouse model^{28, 29} showing that the activation of the NLRP3 inflammasome is independent of the nature of the insult. The NLRP3 inflammasome is involved in a variety of clinical syndromes ranging from rare genetic diseases to common degenerative conditions such as gout.³⁰ The mechanisms by which the inhibitor of the NLRP3 inflammasome protects the heart from injury are not explored in this study, although it has been shown that activation of the NLRP3 inflammasome induced cell death in primary and cardiomyocytes cell line^{4, 11, 31, 32} and consequently, its inhibition in the acute setting reduced infarct size.

It has been reported that formation of the active NLRP3 inflammasome in fibroblasts promotes collagen deposition.^{5, 33} The release of IL-1 β and IL-18 from the active inflammasome enhances injury, dysfunction, and fibrosis.^{6, 12, 34–37} Accordingly, we have

shown that the NLRP3 inflammasome inhibitor reduced fibrosis and limited systolic dysfunction in the mouse.

This study, as any preclinical study, presents several limitations. First, the use of a single animal breed, the mouse, which has obvious differences with the human. Second, despite our attempt to simulate clinical scenario (i.e. treatment at reperfusion), the conditions seen in patients with AMI or cancer cannot be thoroughly reproduced in the experimental animal. Third, the use of echocardiogram to measure LV dimensions and function at 7–10 days has subjective variability, limiting its external validity. Fourth, the choice of the time point was arbitrary, with longer time point having potential of showing different effects.

Conclusion

Pharmacological inhibition of the NLRP3 inflammasome preserves cardiac systolic function following ischemic and non-ischemic injury *in vivo*, and protects primary and cultured cardiomyocytes against the detrimental effects of inflammasome activation. Further translational studies are needed in order to determine whether this inhibitory strategy is feasible in patients with cardiac injury or heart failure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. The NLRP3 inflammasome inhibitor reduces infarct size following ischemia/reperfusion (I/R) injury

(A) Schematic representation of the experimental design; (B) Triphenyl tetrazolium chloride (TTC) stain of a mid-ventricular heart section from mouse from the respective groups of treatment; (C) Mean±SEM percent of left ventricle (LV) at risk of infarction (area at risk) following I/R event; (D) Mean± SEM percent of LV infarct size 24 hours following I/R event evaluated by TTC stain, * $p < 0.001$ vs vehicle-treated mice; (E) Mean±SEM of serum cardiac troponin I levels 24 hours after ischemia/reperfusion; # $p < 0.001$ vs sham, * $p < 0.001$

vs *vehicle-treated mice*; (F) Mean $\pm$ SEM of LV fractional shortening (LVFS) at 7 days following the I/R event; # $p<0.001$ vs *sham*, * $p=0.003$ vs *vehicle-treated mice*. N= 4–6 per group.

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Figure 2. NLRP3 inhibitor limits left ventricle (LV) dilatation and systolic dysfunction after ischemia without reperfusion

(A) Schematic representation of the experimental design; (B–C) Representative images of heart sections stained with Masson's Trichrome from mouse treated with vehicle (B) or NLRP3 inhibitor (C) 7 days after AMI; (D) Mean $\pm$ SEM LV end-diastolic diameter (LVEDD), * $p=0.006$ vs vehicle-treated mice; (E) Mean $\pm$ SEM LV end-systolic diameter (LVESD), * $p=0.001$ vs vehicle-treated mice; (F) Mean $\pm$ SEM LV fractional shortening (LVFS), * $p=0.002$ vs vehicle-treated mice; (G) Mean $\pm$ SEM percent of LV infarct size at 7

days following permanent coronary artery ligation evaluated by Masson's Trichrome stain, $p=0.4$ vs *vehicle-treated mice*. N= 4–6 per group.

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Figure 3. Effects of the NLRP3 inhibitor in the doxorubicin-induced toxicity model

(A) Schematic representation of the experimental design; (B) Representative images of heart sections stained with Masson's Trichrome from mouse treated with vehicle (upper panel) or NLRP3 inhibitor (bottom panel) 10 days after doxorubicin; (C) Mean $\pm$ SEM percent of interstitial area of fibrosis in the left ventricle (LV) of mice 10 days after doxorubicin (% of LV), $\#p=0.002$ vs saline, $*p=0.001$ vs vehicle-treated mice; (D) Mean $\pm$ SEM LV fractional shortening (LVFS), $\#p<0.02$ vs saline, $*p=0.017$ vs vehicle-treated mice. N= 6–8 per group.

Figure 4. Central role of the NLRP3 inflammasome in the inflammatory response to ischemic and non-ischemic cardiac injury

Activation of NLRP3 inflammasome amplifies the inflammatory response to tissue injury and mediates further damage. Pharmacological inhibition of the NLRP3 inflammasome, using a novel inhibitor, preserves cardiac function following ischemic and non-ischemic injury.