Original Paper

NOD2 Deficiency Protects against Cardiac Remodeling after Myocardial Infarction in Mice

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Key Words

NLR family • Matrix metalloproteinase • Cardiac dysfunction • Inflammation

Abstract

Background/Aims: Although the pathogenesis of myocardial infarction (MI) is multifactorial, activation of innate immune system to induce inflammation has emerged as a key pathophysiological process in MI. NOD2, one member of the NOD-like receptor (NLR) family, plays an important role in the innate immune response. This study was to examine the role of NOD2 during MI. **Methods:** MI was induced by permanent ligation of the left coronary artery in wild type and NOD2^{-/-} mice and cardiac fibroblasts were isolated. **Results:** NOD2 expression was significantly increased in myocardium in post-MI mice. NOD2 deficiency improved cardiac dysfunction and remodeling after MI as evidenced by echocardiographic analysis, reduced the levels of cytokines, inflammatory cell infiltration and matrix metalloproteinase-9 (MMP-9) activity. *In vitro*, we further found that NOD2 activation induced the activation of MAPK signaling pathways, production of proinflammatory mediators and MMP-9 activity in cardiac fibroblasts. **Conclusions:** Our studies demonstrate that NOD2 is a critical component of a signal transduction pathway that links cardiac injury by exacerbation of inflammation and MMP-9 activity. Pharmacological targeting of NOD2-mediated signaling pathways may provide a novel approach to treatment of cardiovascular diseases.

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Introduction

Myocardial infarction (MI) is a leading cause of morbidity and mortality in the world. Although the pathogenesis of MI is multifactorial, an increasing number of clinical and animal model studies have implicated that the activation of innate immune system to induce inflammation has emerged as a key pathophysiological process during MI [1, 2]. The innate immune system regulates initial responses through a set of conserved pattern recognition receptors (PRRs) to recognize pathogen-associated molecular patterns (PAMPs) or dangers-associated molecular patterns (DAMPs), which stimulate the expression of multiple inflammatory mediators and growth factors, sequentially inducing the recruitment of inflammatory cells, the clearance of injured tissue, angiogenesis, and the proliferation of fibroblasts, eventually resulting in scar formation and infarct healing. Dysregulation of these responses may result in adverse cardiac remodeling, leading to functional compromise and heart failure [3, 4]. Toll-like receptors (TLRs) and Nod-like receptors (NLRs) are two major families of PRRs. TLRs have been demonstrated to play a critical role in the innate immune system by activating NF- κ B-dependent inflammatory signaling in cardiomyocytes and promote cardiac dysfunction *in vivo*, indicating that the heart possesses an intact TLR-dependent signaling machinery [2, 5]. To this regard, it has been suggested that TLR-induced inflammation contributes to the development of acute coronary syndromes in patients with coronary artery diseases [6]. Besides its role in inflammation, TLR4 stimulation in monocytes induces the production of matrix metalloproteinase 9 (MMP-9), which is considered as a marker for extracellular matrix degradation. Further studies provide direct evidence for a causal role of TLR4 in post-infarct maladaptive LV remodeling, probably via inflammatory cytokine production and matrix degradation, suggesting that TLR4 may constitute a novel target in the treatment of ischemic heart failure [7]. Recent studies have focused on determining the function of NLRs in terms of their triggers and the signaling pathways that they control in cardiovascular diseases [8]. Among NLRs, NOD2 is a well-characterized member of the NLR family and the importance of NOD2 in inflammatory homeostasis is underscored by the observation that mutations in NOD2 gene are associated with susceptibility to Crohn's disease and Blau syndrome [9]. An increasing number of studies have also revealed the association of NOD2 with several pathologies, atherosclerosis, Alzheimer disease and diabetes mellitus [10-13]. Although Shigeoka et al. were first to address the function of NOD2 in renal ischemia reperfusion injury [14], so far it is unknown the contribution of NOD2 to the pathogenesis of MI. Therefore, the present study was to examine the role of NOD2 during MI by permanent ligation of the left coronary artery and the effect of NOD2 on cardiac fibroblasts hypoxic damage. Our studies demonstrate that NOD2 is one of critical components of a signal transduction pathway that links cardiac injury to inflammation. Pharmacological targeting of NOD2-mediated signaling pathways at multiple levels may provide a novel approach to treatment of MI and other cardiovascular diseases.

Materials and Methods

Animal studies

Twelve-week-old NOD2^{-/-} and wild-type C57BL/6 (WT) mice were purchased from the Jackson laboratory (Bar Harbor, ME, USA). MI was induced by permanent ligation of the left coronary artery as described [7, 15]. In sham-operated animals, the suture was placed under the artery and removed without ligating the artery. All protocols were approved by Institutional Animal Care and Use Committee of Shandong University.

Echocardiography

Mice were lightly sedated with 1.5% isoflurane inhalation to maintain their heart rates around 500 beats/min. Echocardiography was performed with a Philips 7500 Ultrasound System (Philips Medical

Systems, Andover, MA, USA), using a 12.0-MHz transducer. Images were obtained from M-mode, two-dimensional (2-D), pulse wave (PW) Doppler and acoustic density as described [16].

Isolation and culture of cardiac fibroblasts

Adult C57BL/6 mice were anaesthetized and the hearts were extirpated and cardiac fibroblasts were isolated and cultured as described [17]. Cardiac fibroblasts were subjected to hypoxia/reoxygenation. Briefly, cardiac fibroblasts were cultured in the hypoxia chamber (Biospherix, Lacona, NY, USA) at 0.5% oxygen for 4 hours and then placed again in the normoxic incubator for 12, 24 or 48 hours, respectively. In addition, cardiac fibroblasts were also treated by endotoxin-free NOD2 ligands L18-MDP (active L-D isomer, 2 µg/ml) for 24 hours.

Detection of cytokines and chemokines

Chemokines and cytokines in myocardium and cells were measured with enzyme-linked immunoabsorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN) [18].

RNA extraction and RT-PCR

Total RNA was isolated from myocardium or cells and mRNA levels were analyzed by real-time RT-PCR using a Bio-Rad iCycler system (Bio-Rad, Hercules, CA) [19]. The mRNA level of NOD2 was normalized to the GAPDH mRNA. The specific primers for NOD2 based on the core sequence of mouse NOD2 cDNA (Accession number NM_145857) were 5'-CCTGGTACGTGCCCAAAGTAG-3' (forward) and 5'-GCCAAGTAGAAAGCGCAAA-3' (reverse).

Western blot analysis

Total cellular lysates preparation and Western blot analysis were performed as described previously [20]. Antibody to NOD2 was from ProteinTech Group (Chicago, IL); Antibodies to phospho-ERK1/2 (Thr202/Tyr204), ERK1/2, phospho-p38 MAPK (Thr180/Tyr182), p38 MAPK were from Cell Signaling Technology (Danvers, MA); Antibody to MMP-9 was from Abcam (Cambridge, MA). To document the loading controls, the membrane was reprobated with a primary antibody against housekeeping protein β -actin (ProteinTech Group).

Gelatin Zymograph

MMP-9 activity was measured by gelatin zymography as described previously [21] and 10% Zymogram Gelatin Gels (Genmed Scientifics Inc, Arlington, MA) was used in this study.

RNA interference

Small interference RNA to NOD2 (siRNA-NOD2) was synthesized and constructed into pRNAT-U6.1/Neo to get shRNA-NOD2 by Biomics Biotechnologies Co., Ltd. (Nantong, Jiangsu, China). The DNA target sequence for shRNA-NOD2 (5'-GCTCTGTATT TGCGAGATATT-3') was designed based on the core sequence of mouse NOD2 cDNA. In these experiments, shRNA transfection was performed as described [12, 22].

Statistics

Data are expressed as means $\pm$ SE. The significance of the differences in mean values between and within multiple groups was examined by one-way ANOVA followed by Duncan's multiple range test. $P < 0.05$ was considered statistically significant.

Results

NOD2 expression was significantly increased in the cardiac infarcted area in post-MI mice

As shown in Figure 1A, NOD2 mRNA levels were significantly increased in the tissue from the cardiac infarcted area in post-MI mice at different time points. The enhanced NOD2 protein levels were further confirmed by Western blot (Fig. 1B) and immunochemical (Fig. 1C) analyses in the infarcted area at 28 days following MI.

Fig. 1. NOD2 expression was significantly increased in the cardiac infarcted area in post-MI mice. A. Relative mRNA levels of NOD2 in the tissue from the infarcted area in post-MI mice at different time points. B. Representative Western blot gel documents and summarized data showing the protein levels of NOD2 in the infarcted area at 28 days following MI. C. Representative photomicrographs of NOD2 immunohistochemical staining in the infarcted area at 28 days following MI. * $P < 0.05$ vs. WT sham-operated mice ($n = 8$).

NOD2 deficiency exhibited improvements in cardiac dysfunction and remodeling after MI

Cardiac functions were examined by echocardiography at 28 days after MI. As shown in Table 1, MI significantly decreased cardiac function in WT mice as evidenced by decreases in EF%, FS%, when compared with WT basal controls, which can be recovered in *NOD2*^{-/-} mice. We also measured cardiac remodeling related parameters, which were reflected by changes in left ventricle (LV) diameter and wall thickness. As shown in Table 1, WT mice exhibited LV enlargement after MI as indicated by increases in LVIDd, LVIDs, and LVVd, respectively, compared with WT basal controls. WT mice also exhibited progressive LV wall thinning after MI as evidenced by decreases in IVSd and IVSs, respectively. In *NOD2*^{-/-} mice, the MI-induced LV enlargement and wall thinning were significantly improved. Furthermore, TUNEL staining indicated that *NOD2* deficiency protected against ischemia-induced cell death (Fig. 2A) and Masson trichrome staining indicated that cardiac fibrosis was significantly reduced in *NOD2*^{-/-} mice (Fig. 2B).

NOD2 deficiency reduced the levels of proinflammatory mediators, inflammatory cell infiltration and MMP-9 activity after MI

As shown in Figure 3A, *NOD2* deficiency significantly reduced the levels of proinflammatory cytokines and chemokines including IL-1 β , TGF- β , TNF- α and MCP-1 in

Table 1. NOD2 deficiency attenuated cardiac dysfunction and remodeling after MI. * $P < 0.05$ vs. WT sham-operated group; # $P < 0.05$ vs. WT MI group (n=8). %EF: Ejection fraction; %FS: Fractional Shortening; LVIDd: left ventricular internal diameter at diastolic phase; LVIDs: left ventricular internal diameter at systolic phase; LVVd: left ventricle end-diastolic volume; LVVs: left ventricle end-systolic volume; IVSd: interventricular septal thickness at diastolic phase; IVSs: interventricular septal thickness at systolic phase; LVPWd: left ventricular posterior wall thickness at diastolic phase; LVPWs: left ventricular posterior wall thickness at systolic phase

	Baseline		MI	
	WT	NOD2 ^{-/-}	WT	NOD2 ^{-/-}
LVIDd(mm)	2.79±0.57	2.82±0.38	3.56±0.40*	3.23±0.47*
LVIDs(mm)	1.92±0.42	1.89±0.19	3.12±0.43*	2.62±0.51*#
IVSd(mm)	0.71±0.10	0.75±0.11	0.43±0.17*	0.58±0.19*#
IVSs(mm)	0.97±0.07	1.01±0.12	0.65±0.12*	0.77±0.17*#
LVPWd(mm)	0.84±0.17	0.93±0.26	0.80±0.32	0.89±0.11
LVPWs(mm)	1.08±0.22	1.11±0.29	1.05±0.38	1.14±0.13
LVVd(μl)	32.97±5.22	30.39±6.72	65.87±5.32*	51.22±7.12*#
LVVs(μl)	10.95±3.63	12.04±2.67	27.65±7.51*	26.64±5.91*
FS (%)	34.92±5.61	33.89±4.29	25.11±3.97*	28.22±2.90*
EF (%)	66.37±4.77	65.22±6.13	41.02±5.17*	49.06±6.22*#

Fig. 2. NOD2 deficiency attenuated cardiac dysfunction and remodeling after MI. A. Representative photomicrographs of TUNEL staining indicating that NOD2 deficiency protected against ischemia-induced cell death. B. Representative photomicrographs of Masson trichrome staining indicating that cardiac fibrosis was significantly reduced in NOD2^{-/-} mice (n=8).

the cardiac infarcted area in mice after MI. Moreover, elevated MMP-9 levels (Fig. 3B) and activity (Fig. 3C) in the cardiac tissues were blocked by NOD2 deficiency after MI. Our results further confirmed that the infiltration of inflammatory cells and the expression of α -SMA was significantly reduced in NOD2^{-/-} mice after MI as shown in Figure 3D.

MDP induced activation of MAPK signaling pathways, production of proinflammatory mediators in primary cultured cardiac fibroblasts

We then detected the effect of NOD2 on MAPK signaling pathways in cardiac fibroblasts, which are important for inflammatory cytokine production and MMP expression. Therefore, we treated cardiac fibroblasts with MDP for the activation of NOD2 and found that MDP induced the activation of MAPKs as assessed by measuring the levels of phospho-specific ERK-1/2 (Fig. 4A), p38 MAPK (Fig. 4B). We also observed that MDP enhanced the production of proinflammatory mediators in primary cultured cardiac fibroblasts (Fig. 4C).

Gene silencing of NOD2 attenuated hypoxia-induced MMP-9 expression:

In primary cultured cardiac fibroblasts, we found that hypoxia/reoxygenation significantly enhanced NOD2 (Fig. 5A) and MMP-9 expression (Fig. 5B). To further investigate

Fig. 3. NOD2 deficiency reduced the levels of proinflammatory mediators, inflammatory cell infiltration and MMP activity after MI. A. Relative levels of proinflammatory mediators in the cardiac tissues from different groups of mice. B. Representative Western blot gel documents and summarized data showing the protein levels of MMP-9 in cardiac tissues. C. MI-induced MMP-9 activity was attenuated by NOD2 deficiency via Zymography analysis in lysates of cardiac tissues. D. Representative photomicrographs of CD68 and α -SMA staining, which denotes infiltrating CD68⁺ macrophages and the level of fibrosis in the infarcted area at 28 days following MI. * $P < 0.05$ vs. WT sham-operated mice, # $P < 0.05$ vs. WT MI mice ($n = 8$).

Fig. 4. MDP induced the activation of MAPK signaling pathways, production of proinflammatory mediators in primary cultured cardiac fibroblasts. A. Representative Western blot gel documents and summarized data showing the levels of phospho-ERK1/2 in isolated cardiac fibroblasts treated with MDP. B. Representative Western blot gel documents and summarized data showing the levels of phospho-p38 in cardiac fibroblasts treated with MDP. C. Relative levels of proinflammatory mediators in cardiac fibroblasts treated with MDP. * $P < 0.05$ vs. control ($n = 6$).

the role of NOD2 in cardiac fibroblasts, NOD2 gene silencing was used in this study. By real-time RT-PCR and Western blot analyses, NOD2 mRNA and protein levels were decreased by 69.3% and 65.2 % in shRNA-NOD2 transfected cardiac fibroblasts, respectively (Fig. 5C-D).

Fig. 5. Gene silencing of NOD2 attenuated hypoxia-induced MMP-9 expression. A. Representative Western blot gel documents and summarized data showing the protein levels of NOD2 expression in cardiac fibroblasts under hypoxia/reoxygenation condition. B. Representative Western blot gel documents and summarized data showing the protein levels of MMP-9 expression in cardiac fibroblasts under hypoxia/reoxygenation condition. C. Relative mRNA levels of NOD2 in cardiac fibroblasts with shRNA-NOD2 transfection. D. Western blot gel documents and summarized data showing the relative protein levels of NOD2 in cardiac fibroblasts with shRNA-NOD2 transfection. E. Representative Western blot gel documents and summarized data showing the protein levels of MMP-9 in cardiac fibroblasts with shRNA-NOD2 transfection. F. Relative levels of proinflammatory mediators in cardiac fibroblasts with shRNA-NOD2 transfection. * $P < 0.05$ vs. scramble, # $P < 0.05$ vs. vehicle of hypoxia (4 hours)/reoxygenation (24 hours) treatment ($n = 6$).

We further found that hypoxia-induced MMP-9 expression was markedly blocked by shRNA-NOD2 (Fig. 5E). Finally, we found that hypoxia-induced the production of proinflammatory mediators was attenuated by gene silencing of NOD2 in primary cultured cardiac fibroblasts (Fig. 5F).

Discussion

In this study, we identified for the first time the upregulation of NOD2 in myocardium following MI and evaluated the potential role of NOD2 in MI showing that NOD2 deficiency markedly alleviated cardiac remodeling after MI. We further demonstrated that NOD2 was associated with enhanced MMP-9 expression and activity and inflammation in myocardial fibroblasts *in vitro*.

The innate immune system is responsible for the initial response of pathogens or tissue injury and plays an essential role in the pathogenesis of many inflammatory diseases [9]. Recent studies have established a relationship between the innate immune response and cardiovascular diseases. There is a line of evidence supporting that activation of TLRs contributes to the development and progression of cardiovascular diseases including atherosclerosis, cardiac dysfunction in sepsis and congestive heart failure [23]. With respect to NLRs, only limited studies have shown the effect of these PRRs in the cardiovascular system. The NLR family currently consists of 22 members, from which can be divided into two major groups. One group includes members such as NOD1 and NOD2, mediating the

assembly of complexes that activate MAPK and NF- κ B signaling pathways. Another group includes intracellular receptors, such as NLRP1 and NLRP3, which mediate the assembly of inflammasome complexes leading to the activation of procaspase-1 and subsequent interleukin-1 β (IL-1 β) and IL-18 release [8]. NLRP3 inflammasome has been demonstrated to be required for atherogenesis and activated by cholesterol crystals [24] and responsible for mediating myocardial ischemia-reperfusion injury [17]. Studies have also indicated that NOD1 ligands induce site-specific vascular inflammation [25] and modulate cardiac fibrosis and cardiomyocyte apoptosis [26]. However, so far it is unclear the role of NOD2 in MI. In this study, we observed the upregulation of NOD2 expression in the myocardium following MI. One of the most important findings is that NOD2 deficiency protects against cardiac remodeling after MI by reduced inflammation and MMP-9 expression and activity in mice.

The extracellular matrix (ECM) is a complex entity containing a number of structural proteins, signaling molecules, and proteases. All of these ECM constituents are vulnerable to proteolytic processing by MMPs, and then alters the interactions between these different structural and biologically active interstitial molecules, thereby determining the overall structure and function of the ECM. MMPs have been identified in all forms of left ventricle remodeling as major contributory factors in the progression to heart failure [27]. Although different MMPs play diverse roles in heart failure, MMP-9 is considered as one potential biomarker for cardiac remodeling as demonstrated by both animal models and clinical studies [28, 29]. In animal MI models, MMP-9 expression is significantly increased and is linked with inflammation, extracellular matrix degradation, and cardiac dysfunction. Clinical studies have established a relationship between MMP-9 and post-MI remodeling and mortality, strongly supporting that MMP-9 is a panel member in the biomarker list to diagnose or treat the pathophysiology of post-MI ventricular remodeling and congestive heart failure [29, 30]. Therefore, in this study, we measured MMP-9 expression and activity and revealed a new function of NOD2 showing that NOD2 significantly increased MMP-9 expression and activity in MI model. An increasing number of studies have provided evidence regarding the role of TLRs in mediating MMPs. For example, MMP-9 mediates neutrophil migration into the airways in response to influenza virus-induced TLR signaling [31] and TLR4-mediated signaling induces MMP9-dependent cleavage of B cell surface CD23 [32]. However, reports about NOD2 in mediating MMPs are very limited [33]. Here, we found that MMP-9 expression and activity was significantly enhanced, which was attenuated by NOD2 deficiency in the myocardium following MI.

To further investigate the functional role of NOD2, primary isolated cardiac fibroblasts were cultured in this study. Cardiac fibroblasts are one of the most abundant cell types in the heart and serve as sentinel cells that sense injury and activate inflammatory as well as matrix regulating responses following MI [34]. Although innate immune responses have been shown to be important in response to tissue injury in the heart, very few studies have examined how cardiac fibroblasts respond to activators of innate immunity [17]. Our studies showed that myocardial fibroblasts express NOD2 and underscore a central role of cardiac fibroblasts to activate inflammation in response to MDP, the activator of NOD2. In this study, although we focus on cardiac fibroblasts and try to explore the role of NOD2 in MI, we cannot exclude that NOD2 in infiltration of immune cells also plays an important role in the development and progressions of MI. These immune cells are activated by numerous signals and then the activated immune cells act as inflammatory cells and elaborate proinflammatory cytokines [35]. In fact, we have found that enhanced CD68-positive infiltrating monocytes/macrophages in the myocardium, which are colocalized with NOD2 (data not shown). NOD2 in inflammatory cells may induce the activation of NF- κ B and trigger a cell signaling cascade to enhance the expression of cytokines and chemokines. The cardiac residential cells then respond by these signaling molecules, ultimately resulting in cardiac injury.

It should be noted that a very recent study showed that NOD2 deletion promotes cardiac hypertrophy and fibrosis induced by pressure overload [36], which differs from our current findings and by others showing the detrimental role of NODs in the regulation of cardiac functions [25, 26]. The mixed observation may result from the possible different

contribution of NOD2 in different diseased states or different experimental animal models used. Further studies are needed to confirm these findings.

In summary, the current study demonstrates that NOD2 is one of critical components of a signal transduction pathway that links cardiac injury by exacerbation of inflammation and MMP activity. Pharmacological targeting of NOD2-mediated signaling pathways at multiple levels may provide a novel approach to treatment of cardiovascular diseases.

Conflict of Interests

All the authors declared no competing interests.

Acknowledgements

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