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Curcumin reduces the cardiac ischemia–reperfusion injury: involvement of the toll-like receptor 2 in cardiomyocytes

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Abstract

Curcumin, a polyphenolic compound derived from turmeric, has protective effects on myocardial injury through attenuation of oxidative stress and inflammation. Toll-like receptor 2 (TLR2), a key mediator of the innate immune system, is involved in myocardial infarction and examined if controlled by curcumin. Rat cardiomyocytes (CMs) were stimulated with tumor necrosis factor (TNF)-α, peptidoglycan (PGN) or hypoxia/reoxygenation (H/R) with or without curcumin pretreatment. Sprague–Dawley rats were fed curcumin (300 mg/kg/day) 1 week before cardiac ischemia/reperfusion (I/R) injury. The expression level of TLR2 and cardiac function were assessed. Both mRNA and protein of TLR2 were up-regulated in infarcted myocardium, while TLR4 remained unchanged. In CMs, TLR2 and monocyte chemoattractant protein (MCP)-1 mRNAs were increased by TNF-α, PGN or H/R, whereas they were blunted by curcumin. Immunofluorescence staining of CMs also showed that TLR2 and MCP-1 were increased after H/R, whereas curcumin-pretreated CMs were not. In animal study, 2 weeks after I/R, TLR2 was increased in the infarct zone, whereas it stayed unchanged in the Cur+I/R group. Macrophage infiltration (CD68), high-mobility group box 1 and fibrosis were increased in the I/R group, whereas they were decreased in the Cur+I/R group. Connexin 43 was reduced in the I/R group, while it recovered significantly in the Cur+I/R group. Cardiac contractility in the Cur+I/R group was also improved compared with that in the I/R group (max dp/dt in Cur+I/R group: 9660±612 vs. I/R group: 8119±366, $P<.05$). These results suggest that selective inhibition of TLR2 by curcumin could be preventive and therapeutic for myocardial infarction.

Keywords: Curcumin; Toll-like receptor 2; Cardiac ischemia–reperfusion injury; Cardiomyocytes; Inflammation

1. Introduction

Ischemic heart disease and consequent heart failure remain the leading cause of morbidity and mortality worldwide [1]. Myocardial infarction (MI) is associated with an inflammatory response, and the inflammatory phase contributes to cardiac remodeling and eventual host outcome. Cytokine release is a hallmark of the early inflammatory phase [2,3].

Curcumin [1,7-bis-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione], an active ingredient of *Curcumin longa*, has received a great interest in their pleiotropic effects such as antioxidation, anti-inflammation, antitumorigenicity and cardioprotective potential [4–8]. Inhibition of nuclear factor-κB (NF-κB) is demonstrated to be the major action of curcumin [4,9]. NF-κB is a pivotal transcription factor involved in the regulation of the genes responsible for the inflammatory and immune reactions in both cytoprotective and cell death pathways [10,11].

Toll-like receptors (TLRs) have been identified as crucial signaling receptors mediating the innate immune recognition [12]. There are more than 10 TLRs in humans, and they bind to pathogenic agonists such as lipopeptide, double-strand RNA, lipopolysaccharides, flagellin and CpGDNA [13].

Several studies have shown that the heart possesses a functionally intact innate immune system that becomes activated nonspecifically in response to ischemia–reperfusion (I/R) injury [14]. TLRs also recognize endogenous host signaling mediators during cellular injury or death [15]. Among the TLRs, TLR2 and TLR4 are the most studied in the heart [16,17]. TLR2, known to recognize lipoproteins and peptidoglycan (PGN) of microorganisms [18], can activate NF-κB pathway and make a cardiomyocyte (CM) contractile dysfunction [19,20]. Signaling through TLR2 was increased in CMs by hydrogen-peroxide-induced oxidative stress which was prevented by anti-TLR2 antibody [21]. In TLR2 knockout mice, the infarct size, infiltrated neutrophil, reactive oxygen species generation and cytokine release were reduced after cardiac I/R injury [22].

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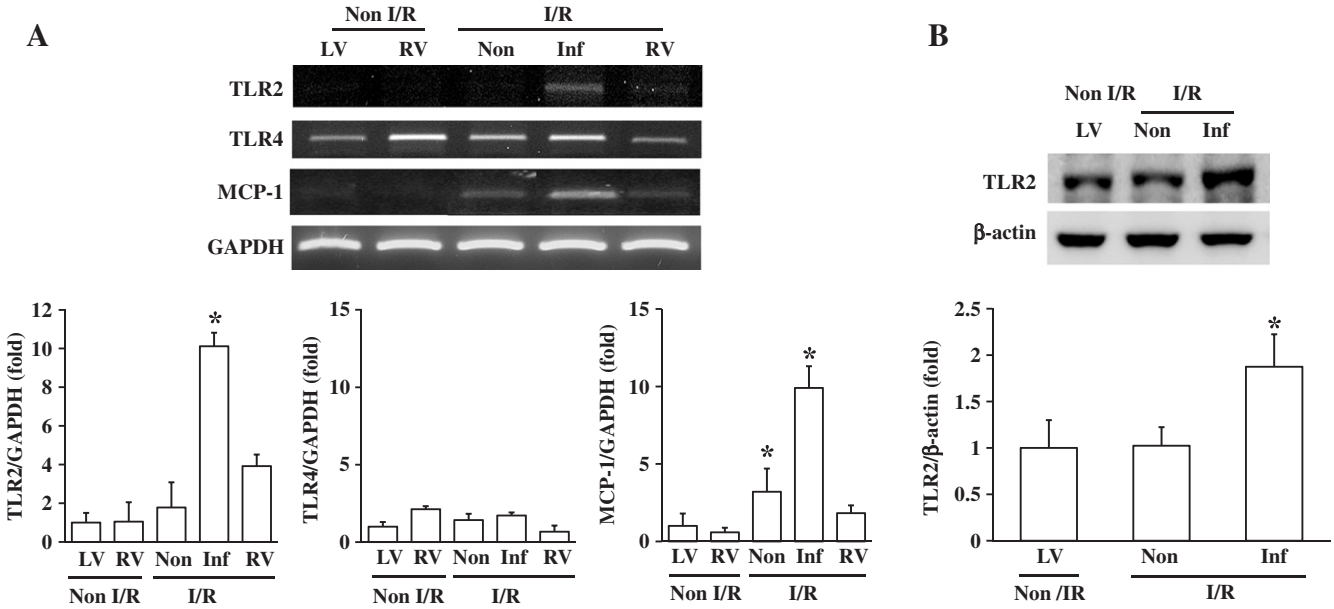


Fig. 1. TLR2 expression is induced in I/R injured rat myocardium. Cardiac I/R injury was induced in rats, and heart tissue was isolated after 2 weeks. mRNA and protein were extracted to evaluate level of TLR2. TLR2 was increased in infarcted myocardium in protein levels. RV=right ventricle, Inf=infarct, *P<.05 vs. non-I/R. (A) The expression of mRNA of TLR2, TLR4 and MCP-1 in I/R injured heart tissue was assessed by RT-PCR. GAPDH was used as a loading control. Densities were calculated and expressed as graphs. (B) TLR2 protein levels were assessed by Western blot analysis. β-actin was blotted as a loading control. Densities were calculated and expressed as graphs.

The purpose of this study was to examine the involvement of TLR2 in the cardioprotective effects of curcumin, and the study was designed to better understand the beneficial effects of curcumin on cardiac I/R model.

2. Materials and methods

2.1. Isolation of neonatal rat CMs and induction of hypoxia/reoxygenation

Primary CMs were isolated from the left ventricles (LVs) of 2-day-old Sprague–Dawley rat pups as described previously [23] and then were plated on eight-well glass chamber slides (Nunc, USA) in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL, USA) supplemented with 10% fetal bovine serum (Gibco-BRL, USA). After 2 days, CMs were stimulated with tumor necrosis factor (TNF)-α (10 ng/ml, R&D Systems, USA) or the TLR2 agonist PGN (10 μg/ml, Sigma, USA) with or without curcumin pretreatment (10 μM) for 3 h.

The hypoxic condition was generated using a hypoxia chamber according to the manufacturer's instructions. Briefly, CMs were subjected to hypoxia in serum-free DMEM by sealing the humidified hypoxic chambers (Billups-Rothenberg Inc.), flushing the chamber with a gas mixture consisting of 5% CO_2 and 95% N_2 for 15 min, and incubating the hypoxic chamber in a modular incubator at 37°C for 6 h. Following hypoxia, the media were changed to growth media, and CMs were reoxygenated by further incubation for 18 h in 5% CO_2.

2.2. Reverse transcriptase–polymerase chain reaction (RT-PCR)

To compare mRNA expression levels in cell injected and control rats, myocardial specimens were pulverized and homogenized in Trizol solution (Invitrogen, USA) according to manufacturer's instructions. cDNA was synthesized to perform RT-PCR. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an loading control, and primers used were as follow: TLR-2, forward: 5′-TCTGAGAATGATGTGGGCGT, reverse: 5′-GGGCCACTCCAGGTATGTCT, TLR-4, forward: 5′-GGAGAAGTCCTTGCTGAGGC, reverse: 5′-TGCTACTTCCTTGTGCCCTG, monocyte chemoattractant protein-1 (MCP-1), forward: 5′-TTCACAGTTGCTGCCTGTAGC, reverse: 5′-TCTGATCTCACTTGGTTCTGG, and GAPDH, forward: 5′-GGCCAAGGTCATCCATGA, reverse: 5′-TCAGTGAGCCCAGGATG.

2.3. Western blot

Cells were washed with ice-cold phosphate-buffered saline (PBS), resuspended in lysis buffer (20 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 mg/ml leupeptin, 1 mM Na_3VO_4) and sonicated briefly. After centrifugation at 10 000g force for 10 min, the supernatant was prepared as a protein extract. Equal concentrations of proteins were fractionated by electrophoresis on 8% or 10% acrylamide gels and were transferred onto a polyvinylidene fluoride (Millipore, USA) membrane followed by blotting with antibodies against TLR2 (Santa Cruz, USA) and β-actin (Sigma-Aldrich, USA). Protein levels were determined using Western Breeze reagents (Santa Cruz, USA) and Image Reader (LAS-3000 Imaging System, Fuji Photo Film, Japan).

2.4. Immunofluorescence staining of CMs

CMs were fixed with 4% paraformaldehyde or ice-cold methanol for 10 min and were washed with PBS three times. After permeabilization by 0.1% Triton X-100 for 10 min and blocking with 5% bovine serum albumin for 1 h, primary antibodies were incubated overnight at 4°C followed by sequential incubation with secondary antibody conjugated with Alexa Fluor 488 or Alexa Fluor 594 (Molecular Probe, USA, 1:300). Primary antibodies used for immunofluorescence included TLR2 (Abcam, USA, 1:100) and MCP-1 (Santa Cruz, USA, 1:100). Actin fiber was stained with phalloidin-tetramethylrhodamine B isothiocyanate (Sigma, USA). After washing with PBS, the slides were mounted with medium

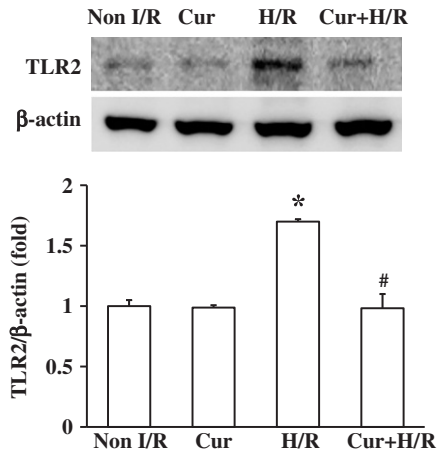


Fig. 2. Curcumin attenuates the induction of TLR2 in CMs. CMs were pretreated with curcumin (10 μM) for 18 h and then stimulated with H/R. H/R insult was induced by incubation of CMs in an anaerobic bag for 6 h followed by further incubation for 18 h in 5% CO_2. TLR2 protein levels were assessed in CMs by Western blot. β-actin was blotted as a loading control. Densities were calculated and expressed as graphs. Cur=Curcumin.

containing diaminophenylindole (Invitrogen, USA) and were observed under confocal microscopy. Immunofluorescence was detected by using a Carl-Zeiss confocal microscope. Images were obtained by using Zeiss LSM version 3.2 SP2 software.

2.5. Curcumin administration to cardiac I/R rat model

The study was reviewed and approved by the Chonnam National University Institutional Animal Care and Use Committee. Male Sprague–Dawley rats (weighing 200–230 g, Jung Ang Animals, Korea) were randomly grouped and orally received curcumin in distilled water (300 mg/kg/day) or vehicle alone once a day for 7 days before and for 14 days after I/R surgery.

IR injury was produced in both groups: IR group and curcumin+IR (Cur+I/R) group. For I/R injury, rats were anesthetized with an intramuscular injection of ketamine (50 mg/kg) and xylazine (5 mg/kg), intubated and mechanically ventilated. The proximal left anterior descending coronary artery (LAD) was ligated for 30 min and released. Finally, the heart was repositioned in the chest, and the chest was closed. The animals remained in a supervised setting until becoming fully conscious. Curcumin or PBS feeding was maintained for 2 weeks.

2.6. Assessment of area at risk

After I/R (30 min/30 min), the chest was opened. The LAD was reoccluded using thread left in place, and 1 ml of 1% Evans blue was injected through the apex into the left ventricular cavity to delineate the ischemic area. The heart was excised, and 2-mm-thick slices were cut and fixed in 10% neutralized formalin overnight. Fixed slices were scanned into digitalized images. The ischemic area (area at risk) was distinguished from the area not at risk by Evans blue dye staining. The area at risk of the LV was quantified by use of Image J software (NIH, USA).

2.7. Immunohistochemical staining

At the end of the experiment, the rats were killed, and the hearts were rapidly removed. For measurement of cardiac fibrosis, the hearts were fixed with 4% paraformaldehyde, embedded in wax and cut into 10-μm sections. Histological examinations were performed by Masson's trichrome staining.

For immunohistochemical analysis, slides were treated with 3% hydrogen peroxide in PBS for 10 min at room temperature to block endogenous peroxidase activity. After blocking nonspecific binding with 5% normal goat serum (Sigma-Aldrich, USA), they were incubated with primary antibodies against TLR2 (Abcam, USA, 1:50), CD68 (Biomedicals, Switzerland, 1:100), high-mobility group box 1 (HMGB1) (Santa Cruz, USA, 1:100) or connexin 43 (Sigma-Aldrich, USA; 1:100) for 18 h at 4°C. Sections were washed with PBS three times and then incubated for 1 h with Alexa Fluor 488 or 594 secondary antibodies. Macrophage infiltration, inflammation and cell–cell interaction were determined by the expression of CD68, HMGB1 and connexin43, respectively. The reaction product was visualized by using ABC reagent (Vector Labs Inc., USA), and the sections were counterstained with hematoxylin. After washing, slides were

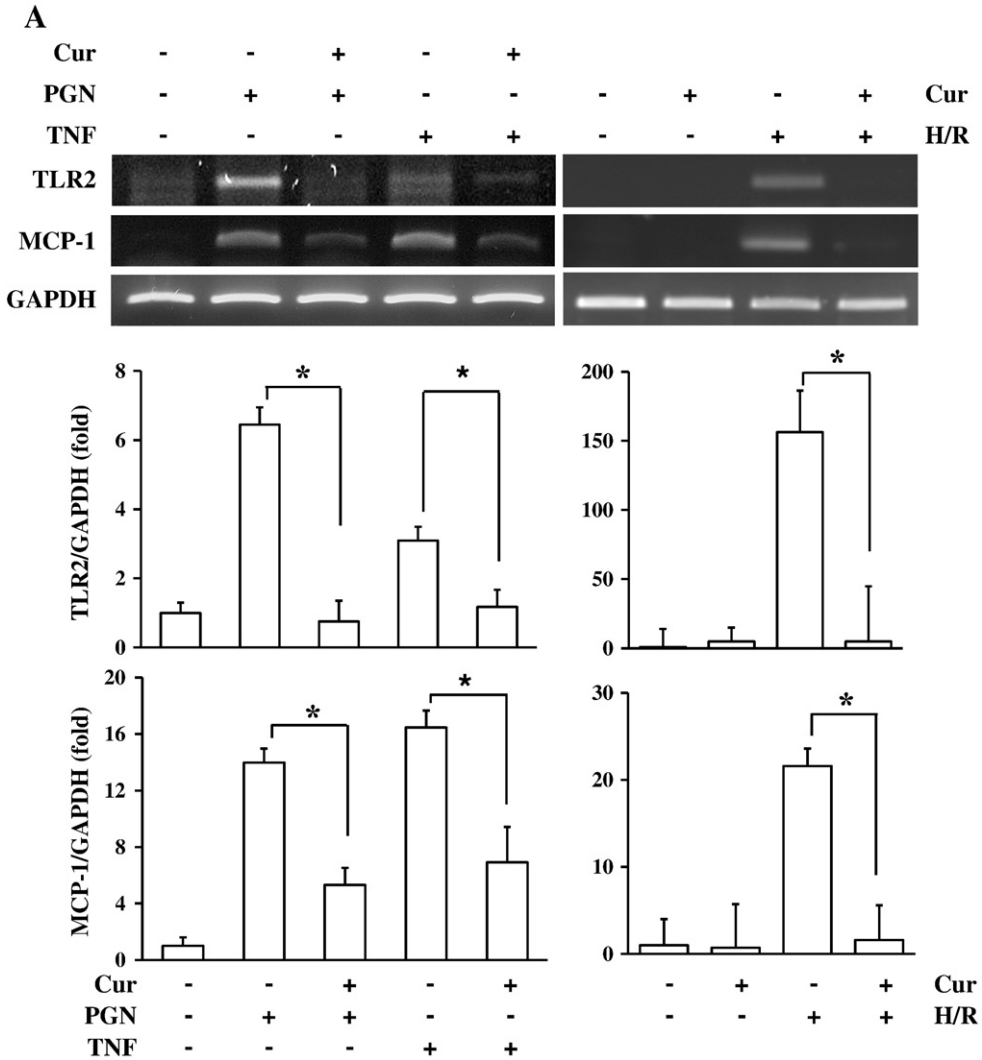


Fig. 3. Curcumin inhibits the induction of TLR2 in H/R injured CMs. CMs were pretreated with curcumin (10 μM) for 18 h and then stimulated with TNF-α (50 ng/ml), PGN (10 μg/ml) or H/R. H/R insult was induced by incubation of CMs in an anaerobic bag for 6 h followed by further incubation for 18 h in 5% CO_2. (A) The expression of mRNA of TLR2 in H/R injured CMs was assessed by RT-PCR. GAPDH was used as a loading control. Densities were calculated and expressed as graphs. (B) The protein level of TLR2 in CMs was assessed by immunofluorescence staining. CMs were fixed with 4% paraformaldehyde to be fluorescently stained with anti-TLR2 or anti-MCP-1 antibodies. Fiber actin was stained with phalloidin, and cell nucleus was counterstained with DAPI. Representative confocal images were shown. NX=normal, H/R=hypoxia/reoxygenation, TLR2 (upper panel, green) or MCP-1 (lower panel, green), phalloidin (red), DAPI (blue).

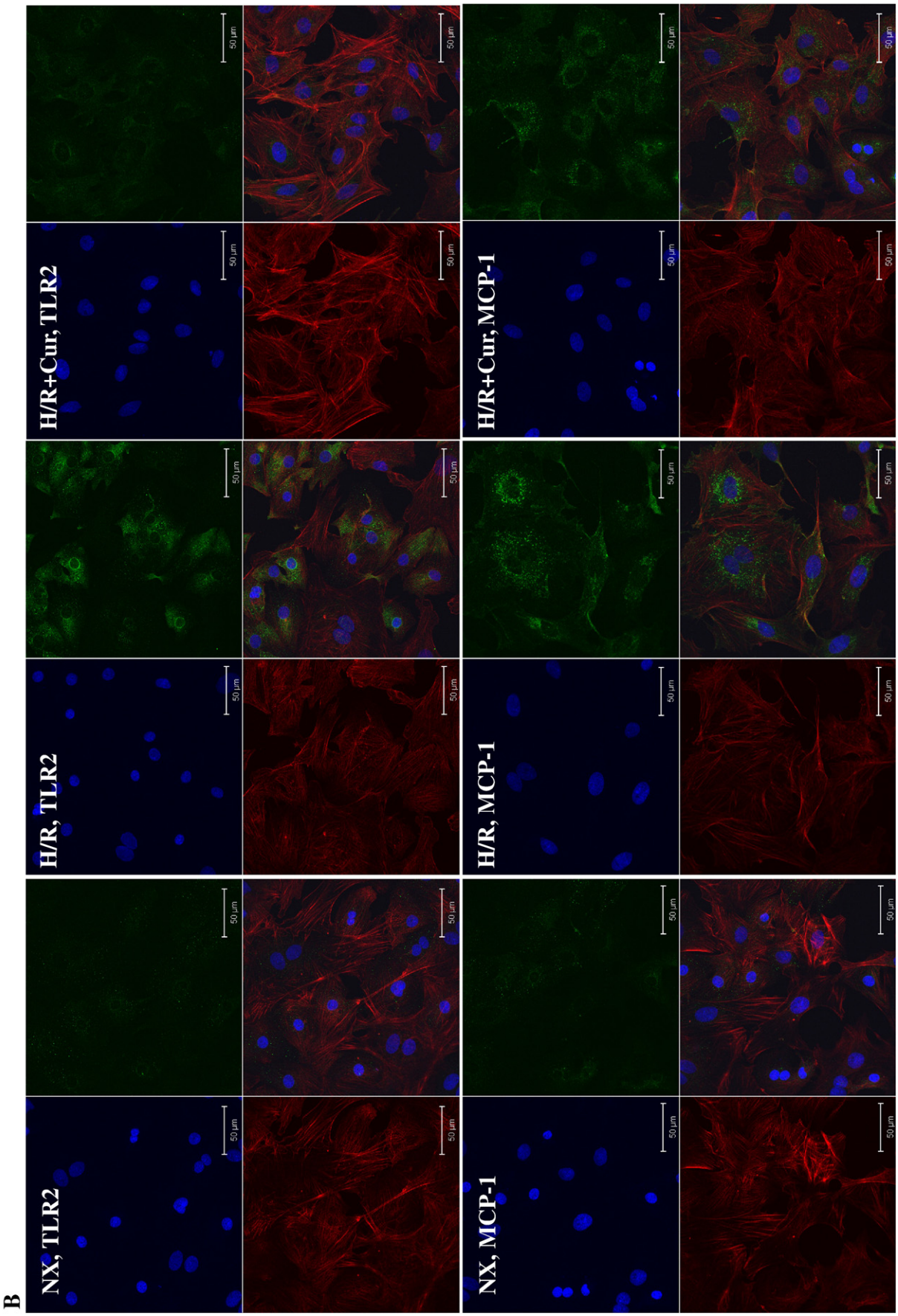


Fig. 3 (*continued*).

Table 1
Hemodynamic changes in I/R injury rat model

	Non-I/R	I/R	Cur+I/R
1 week, *n*	5	6	5
HR	315±21.21	314±3.46	280±24.98
LVEDP	5.46±1.93	11.18±2.36	7.52±2.21 *
SBP	133.67±7.23	140.27±3.31	140.24±7.76
DBP	115.00±4.36	109.41±7.17	105.99±7.76
Max dp/dt	11685.1±193.23	8919.81±122.28	10590.8±575.951 *
Min dp/dt	7184.45±96.6433	4710.82±210.652	6180.3±429.75 *
2 weeks, *n*	5	7	8
HR	273±3.00	323.25±8.89	330.6±6.15
LVEDP	5.86±0.74	14.74±2.23	10.37±3.02 *
SBP	128.60±1.60	139.80±6.14	125.00±2.26 *
DBP	114.03±2.09	117.84±5.79	107.65±3.10 *
Max dp/dt	10712.03±165.10	8120.0±365.91	9660.13±612.88 *
Min dp/dt	8342.56±115.80	4588.61±390.39	6014.24±222.18 *

Administration of curcumin or vehicle was started 7 days before I/R injury and continued for a further 2 weeks.
Cur, curcumin; HR, heart rate; SBP, systolic blood pressure; DBP, diastolic blood pressure; Max dp/dt, maximum rate of pressure change in the ventricle; Min dp/dt, minimum rate of pressure change in the ventricle.
* $P<.05$ vs. I/R group.

coverslipped with mounting medium (VectaMount mounting medium, Vector Labs Inc., USA). Images were obtained and digitized on a computer by using an Olympus CX31 microscope (Olympus, Japan) equipped with an Infinity 1 camera (Lumenera Scientific, Ottawa, Canada).

2.8. Hemodynamic measurement

After 1 and 2 weeks, the animals were again anesthetized, intubated and mechanically ventilated to study hemodynamic variables with a 1.4-Fr micronanometer-tipped catheter (Millar Instruments, Houston, USA). The catheter was inserted into the right carotid artery and advanced into the LV to measure pressures, which were analyzed with the Chart V5 analysis program (Millar Instruments, USA).

Table 2
Echocardiograph in I/R injury rat model

	Non-I/R	I/R	Cur+I/R
3 days, *n*	3	8	6
LVAWs (mm)	1.98±0.06	1.50±0.22	1.87±0.37 *
LVEDd (mm)	7.71±0.10	8.12±0.56	7.85±0.57
LVEDs (mm)	5.37±0.06	7.45±0.80	6.39±0.98 *
FS (%)	26.76±0.09	13.28±3.45	22.74±6.52 *
EF (%)	49.81±1.58	32.95±5.62	44.60±10.27 *
1 week, *n*	3	6	4
LVAWs (mm)	1.84±0.06	1.64±0.12	1.68±0.26
LVEDd (mm)	7.9±0.01	9.24±0.36	8.76±0.42
LVEDs (mm)	6.36±0.09	8.15±0.27	7.69±0.12 *
FS (%)	23.33±0.92	12.76±3.77	16.47±1.50
EF (%)	50.57±6.51	29.66±6.41	36.22±3.62
2 weeks, *n*	3	4	3
LVAWs (mm)	1.72±0.24	1.44±0.80	1.64±0.06 *
LVEDd (mm)	8.82±0.37	10.11±0.44	8.94±0.04 *
LVEDs (mm)	7.18±0.67	8.99±0.62	7.40±0.16 *
FS (%)	19.52±4.41	11.60±2.08	19.22±2.12 *
EF (%)	45.95±5.03	25.9±6.34	41.71±0.51 *

Administration of curcumin or vehicle was started 7 days before I/R injury and continued for a further 2 weeks. Echocardiography was performed at days 3, 7 and 14 after I/R injury.
LVAWs, end-systolic left ventricular anterior wall diameter.
* $P<.05$ vs. I/R group.

2.9. Echocardiogram

Three days, 1 week and 4 weeks after I/R surgery, echocardiography was performed to measure the cardiac function and wall thickness. Echocardiographic studies were performed with a 15-MHz linear array transducer system (iE33 system, Philips Medical Systems) by an expert who was not noticed about experimental conditions to exclude bias. Two-dimensional guided M-mode of the LV was obtained from the parasternal view. LV (left ventricular) cavity dimension and LV free and IVS (interventricular septum) wall thickness were measured, and percent change in LV

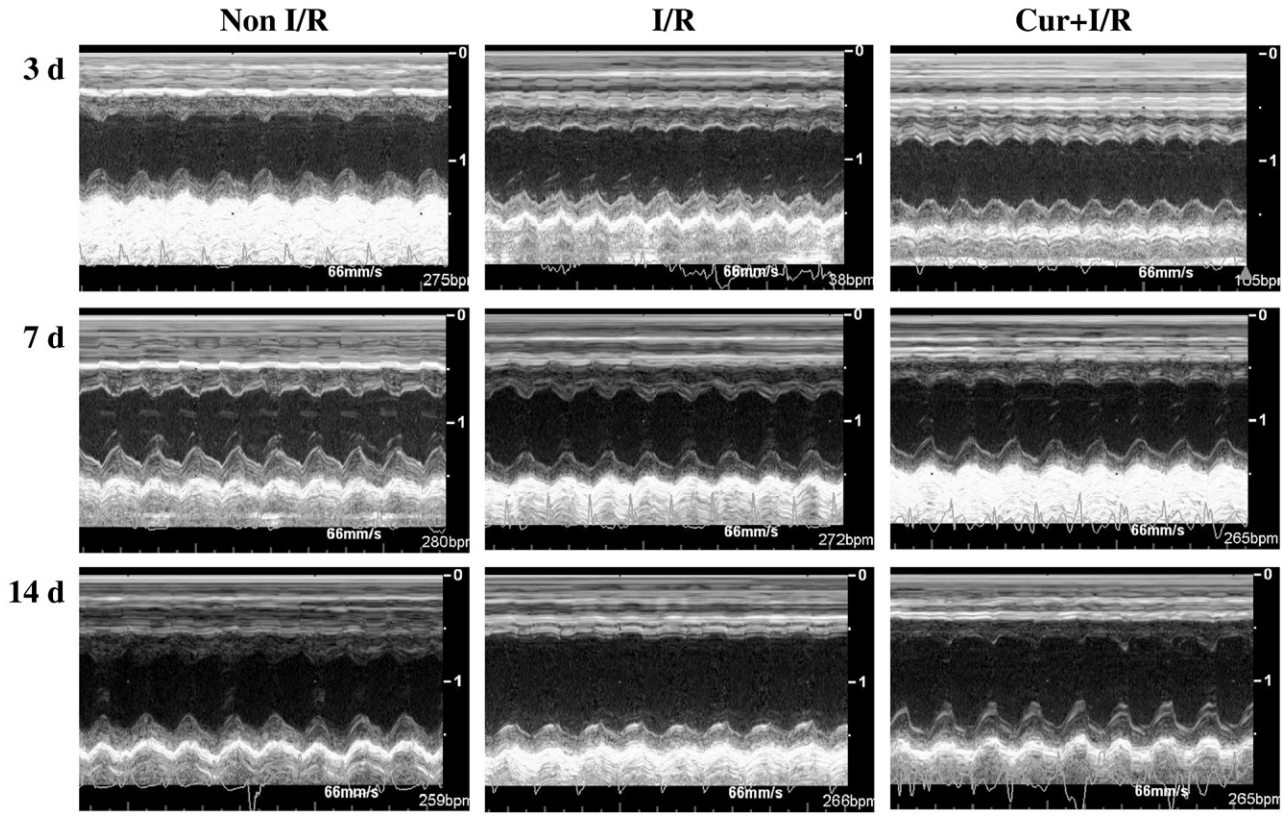


Fig. 4. Echocardiographic evaluations were performed 3, 7 and 14 days after I/R injury. Representative M-mode echocardiograms from non-I/R, I/R and Cur+I/R groups were shown.

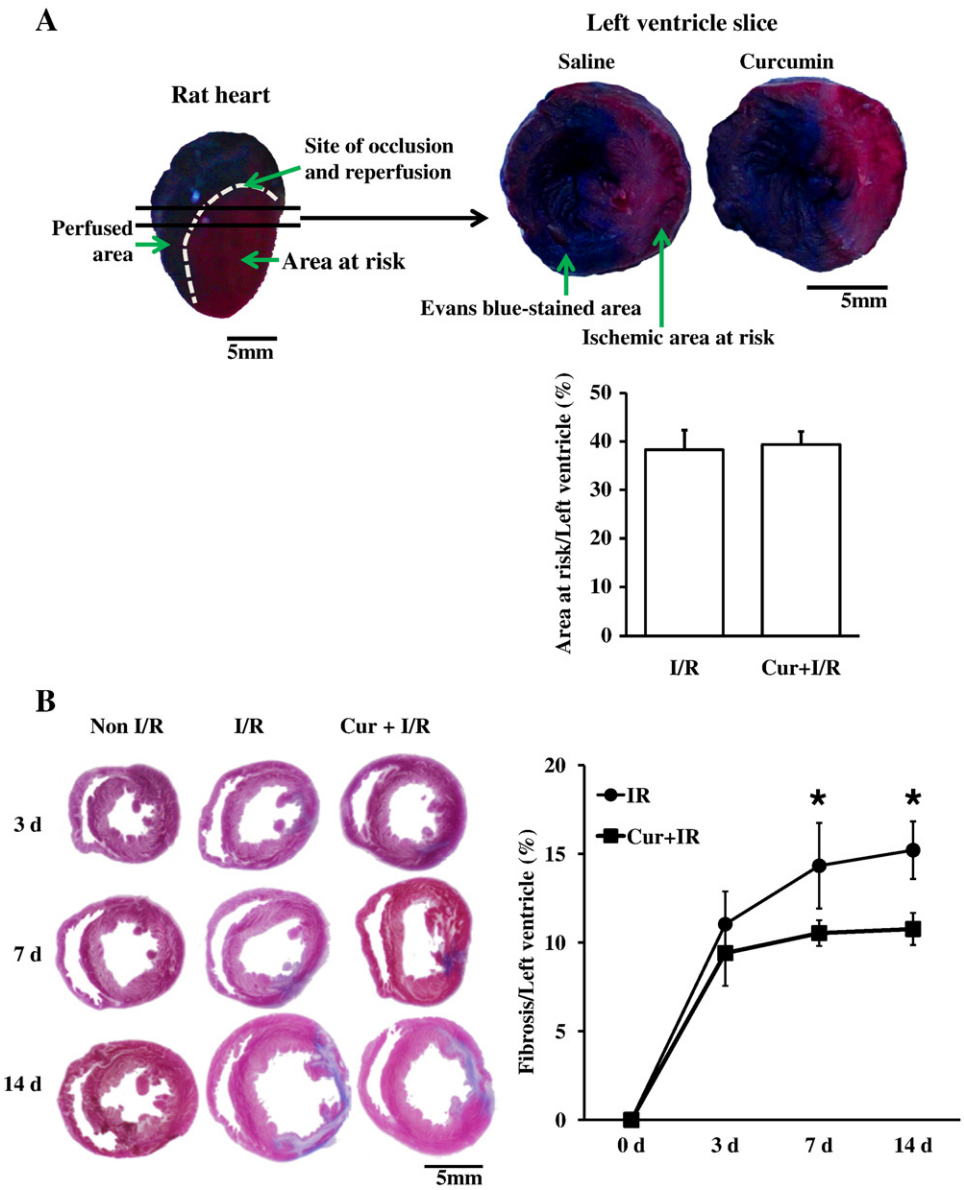


Fig. 5. Determination of area at risk and cardiac fibrosis. (A) Myocardial ischemic area in rats subjected to 30 min of ischemia followed by 30 min of reperfusion. The blue-stained areas represent nonischemic tissue, and red area represents the area at risk. (B) Cardiac fibrosis was assessed by Masson's trichrome stain, and representative images were shown. Images are representative of heart sections from each group at days 3, 7 and 14 after I/R injury. Fibrosis was detected by blue staining and quantified to express as a graph.

dimension [fractional shortening (FS), LV%FS] was calculated as: LV%FS=[(LVDd-LVDs)/LVDd]×100, where LVDd is LV dimension at end-diastole and LVDs is LV dimension at end-systole. LV% ejection fraction (EF) was calculated as: LV%EF=[(EDV-ESV)/EDV]×100, where EDV is LV volume at end-diastole and ESV is LV volume at end-systole. LV volume was estimated by area-length method. The LV Tei index was calculated as a myocardial performance index: LV Tei index=(LV isovolumic contraction time+LV isovolumic relaxation time)/LV ejection time.

2.10. Statistical analysis

Each experiment was performed at least three times. The data are presented as means±S.D. Differences were analyzed by Student's *t* test and were considered statistically significant when *P* value<.05.

3. Results

3.1. TLR2 is increased in infarcted myocardium

To examine the expression level of TLR2 in the infarcted heart tissue, rat heart was extracted 2 weeks after IR injury. TLR2 mRNA was not detected in normal LV and right ventricle, whereas was increased in infarct lesion significantly (Fig. 1A). On the other hand, TLR4 mRNA in infarcted heart tissue was not significantly changed. MCP-1, an inflammatory cytokine, mRNA was induced in IR injured heart, especially in infarcted region. Transcriptional changes of TLR2, TLR4 and MCP-1 were quantified and expressed as graphs.

To confirm the up-regulation of TLR2 in infarct lesion, protein level of TLR2 was determined by Western blot. As shown in Fig. 1B, TLR2 protein was increased in infarcted heart tissue with significance. From these results, TLR2 was demonstrated to be involved in cardiac IR injury.

3.2. Curcumin inhibited the TLR2 increase in CMs

To confirm the possibility of inhibitory effect of curcumin on TLR2 induction, TLR2 expression was examined in CMs. CMs were isolated from neonatal rat LV and insulted by hypoxia/reoxygenation (H/R,

6 h/18 h) with or without pretreatment with curcumin for 3 h. Pretreatment of CMs with curcumin attenuated the TLR2 up-regulation induced by H/R (Fig. 2).

Next, to examine whether curcumin can inhibit the TLR2 induced by other stimuli, CMs were isolated from neonatal rat LV and insulted by TNF-α (50 ng/ml), PGN (10 μg/ml) and H/R (6 h/18 h) with or without pretreatment with curcumin for 3 h. Pretreatment of CMs with curcumin attenuated the TLR2 mRNA up-regulation induced by TNF-α, PGN and H/R. In addition to TLR2, MCP-1 was measured. The mRNA expression of MCP-1 in CMs was induced by TNF-α, PGN and H/R and attenuated by curcumin pretreatment (Fig. 3A).

To evaluate of the protein expressions of TLR2 and MCP-1, CMs were stained with specific antibodies against TLR2 or MCP-1. In immunofluorescence staining, TLR2 and MCP-1 was increased in H/R CMs. In curcumin-pretreated CMs, up-regulation of TLR2 and MCP-1 was inhibited significantly (Fig. 3B).

3.3. Curcumin exerted cardioprotective effects

Cardiac function was assessed by measurement of blood pressure and contractility after 1 week and 2 weeks (Table 1). Heart rate was not significantly different between three groups: non-I/R, I/R and Cur+I/R. Deteriorated cardiac functions such as left ventricular end-diastolic pressure (LVEDP), maximum dp/dt, and minimum dp/dt by I/R injury were observed at day 7 and were restored in Cur+I/R group with statistical significance at days 7 and 14. Subsequent to protection by curcumin, cardiac function also preserved after I/R injury.

Cardiac function and morphometric changes were traced by echocardiography at days 3, 7 and 14 after I/R injury (Table 2), and representative echocardiograms were shown in Fig. 4. In Cur+I/R group, the deteriorations of left ventricular end-diastolic dimension (LVEDd), FS and EF were significantly attenuated at day 3 and preserved until day 14. The LV anterior wall, containing an occluded LAD, became thin, and LV chamber dimensions were significantly increased at day 3 in I/R group. On the other hand, anterior wall thickness and left ventricular end-systolic dimension (LVEDs) were conserved in Cur+I/R group. Taken together, these findings suggested that curcumin contributes to protection from the post-I/R remodeling, at least in part, through conservation of wall thickness and LV chamber dimension.

Next, fibrotic changes of damaged myocardium was assessed. To confirm whether I/R surgery induced similar ischemia in all animals, area at risk was assessed in I/R group and Cur+I/R group. After administration of vehicle or curcumin for 7 days, I/R injury was induced by ligation of LAD for 30 min. After reperfusion for 30 min, the area at risk was measured and quantified. This result showed that the I/R injury was similar in both groups (38.31%±4.00% in I/R group vs. 39.35%±2.70% in Cur+I/R group, $P>.1$, Fig. 5A), and the possibility of the different cardiac ischemia injury in both groups was ruled out.

The cardiac fibrosis was evaluated at days 3, 7 and 14 by Masson's trichrome staining and quantified (Fig. 5B). The fibrotic changes were not different at day 3 (11.04%±1.84% in I/R group vs. 9.41%±1.85% in Cur+I/R group, $P>.05$), whereas these were reduced significantly after day 7 (14.33%±2.42% in I/R group vs. 10.53%±0.72% in Cur+I/R group, $P<.05$) and day 14 (15.21%±1.62% in I/R group vs. 10.77%±0.90% in Cur+I/R group, $P<.05$) in Cur+I/R group.

Histological analysis was performed to examine whether TLR2 was involved in curcumin action. Peri-infarcted regions from four groups were stained with anti-TLR2 antibody. TLR2 appeared on day 7 and became abundant in I/R group, whereas it was hardly detected in Cur+I/R group (Fig. 6A). Infiltration of macrophage (Fig. 6B) was detected as staining with anti-CD68 antibody. Macrophages were exceedingly recruited into infarcted lesion at day 3 and then started to decrease at day 7 in both groups. On the other hand, macrophages that remained at day 14 were observed less in Cur+I/R group.

MCP-1, HMGB1 and connexin 43 (Fig. 6C) were stained in the peri-infarct region. They were increased in I/R group and were significantly reduced in Cur+IR group. Reduced connexin 43 in I/R group was restored and organized in Cur+I/R group.

4. Discussion

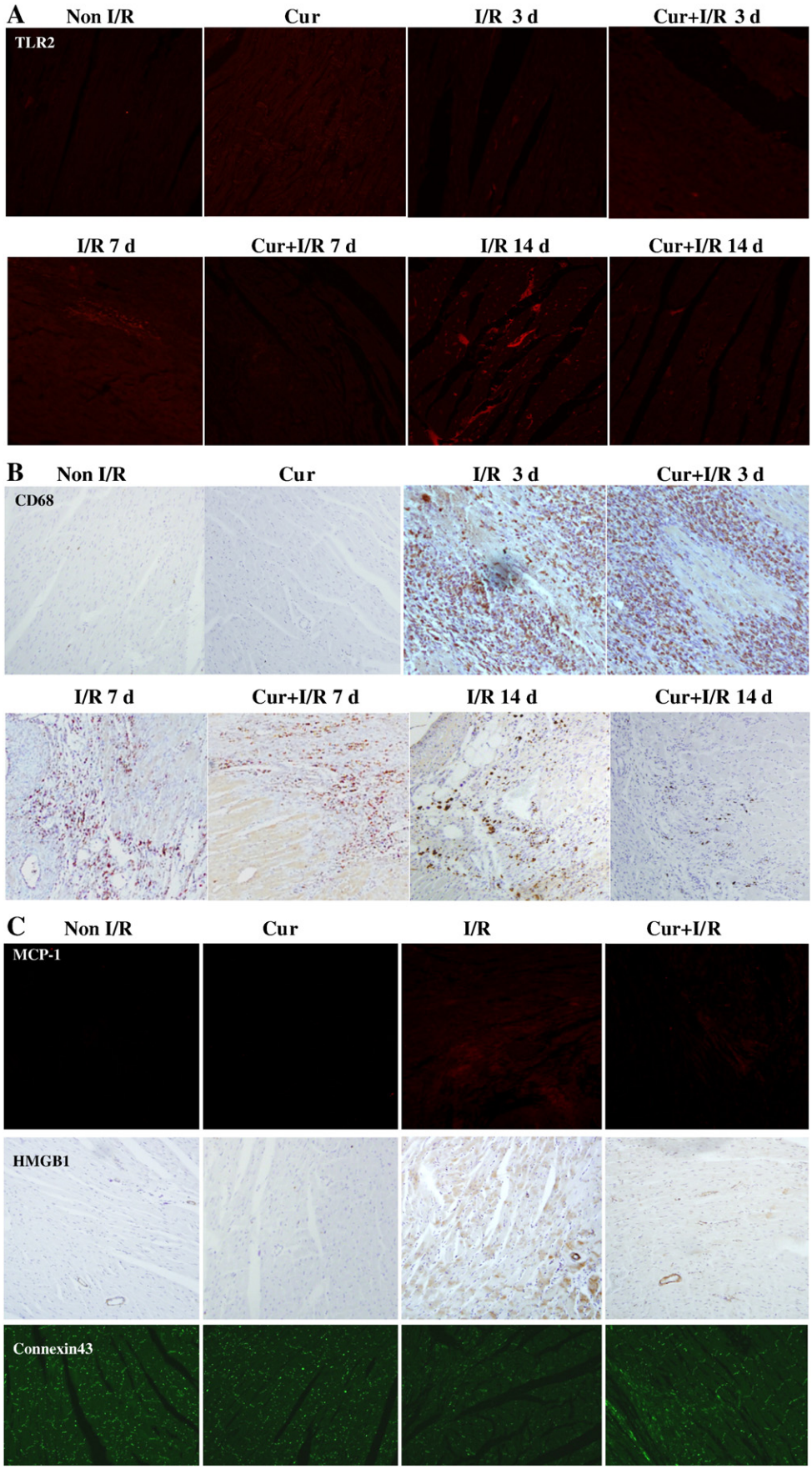
This study was designed to investigate whether TLR2 contributed to the cardioprotective role of curcumin in cardiac IR-induced cardiac inflammation and left ventricular dysfunction.

Previous studies have provided the evidence of involvement of TLR2 in the myocardial injury. Several studies showed that TLR2 contributed to the development of IR-induced left ventricular dysfunction in the adult heart [17,22,24].

TLRs are a family of signal transduction molecules which are critical for the induction of immunity and in host defense [13,15]. Activation of TLR2 recruits an adaptor protein called myeloid differentiation factor-88 (MyD88). MyD88 recruits IL-1 receptor associated receptor kinases and results in nuclear translocation of NF-κB, which then initiates transcription of genes associated with innate immune responses and inflammation [10,21]. Since TLR-mediated signaling triggers and regulates immune/inflammatory responses, which play a critical role in cell death after I/R, TLRs have been reported to play a role in the pathological process of cardiac I/R injury [22]. TLR2 and TLR4 are evidently responsible in cardiac injury [16,24]; however, our data showed that TLR2 is significantly involved in the curcumin-mediated inhibition of reperfusion injury responses in CMs.

Ligands for TLR2 and TLR4 include HMGB1, heat shock protein (HSP) 60, HSP70, endotoxin, hyaluronan, advanced glycation end (AGE) products and extracellular matrix components [25]. HMGB1, a late proinflammatory mediator, mediated a response to tissue damage and infection. HMGB1 is released from necrotic or damaged cells and serves as a signal for inflammation. During I/R injury, endogenous HMGB1 is released by necrotic and inflammatory cells [26,27], and induces activation of intracellular signaling pathways via TLR2 [28], TLR4 [26], TLR9 [29] and the receptor for AGE [27]. MyD88 is a key adaptor protein that is critical for transducing signals from TLR2 [30]. Overexpression of dominant negative MyD88 led to a reduction in infarct size and apoptosis in a rat I/R model [31], and reduced neutrophil numbers, cytokine release and infarct size were also observed after I/R in MyD88-deficient mice [32]. TLR-deficient mice showed less left ventricular remodeling and improved left ventricular function after permanent coronary artery ligation model [24]. HMGB1 can bind to TLR-2 and TLR-4 to mediate

Fig. 6. Immunohistochemical analysis showed curcumin attenuated the increase of TLR2, macrophage infiltration, MCP-1, HMGB1 and connexin 43 disruption in infarcted myocardium. Curcumin (300 mg/kg/d in distilled water) or vehicle (distilled water) was orally administered for 7 days before I/R injury and continued to the end of experiments. Two weeks after I/R injury, the heart tissues were isolated to be analyzed. (A) TLR2 was immunofluorescently stained in peri-infarct region at days 3, 7 and 14 after I/R injury. Significant increase of TLR2 in I/R group was attenuated in Cur+I/R group. Images are representative of heart sections from each group (×200 magnification). (B) Infiltrated macrophage (CD68, brown color) was evaluated by staining with CD68 at days 3, 7 and 14 after I/R injury. Intense recruitment of macrophages into infarcted myocardium was observed at days 3 and 7 in both groups. At day 14, less macrophages remained in Cur+I/R group (×100 magnification). (C) MCP-1 (red fluorescence), HMGB1 (brown color) and connexin 43 (green fluorescence) were stained in the peri-infarct region. Both macrophage and HMGB1 increased in I/R group and were significantly reduced in Cur+I/R group. Images are representative of heart sections from each group (×100 magnification). Reduced connexin 43 was restored and organized in Cur+I/R group.


A
Non I/R
Cur
I/R 3 d
Cur+I/R 3 d
TLR2
I/R 7 d
Cur+I/R 7 d
I/R 14 d
Cur+I/R 14 d
B
Non I/R
Cur
I/R 3 d
Cur+I/R 3 d
CD68
I/R 7 d
Cur+I/R 7 d
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C
Non I/R
Cur
I/R
Cur+I/R
MCP-1
HMGB1
Connexin43

cellular responses including chemotactic cell movement and release of proinflammatory cytokines [29]. HMGB1 was reported to respond to tissue damage as a late proinflammatory mediator and induced activation of intracellular signaling pathways via TLR2, TLR4 and the receptor for advanced glycation end-products (RAGE). A recent study demonstrated that endogenous HMGB1 is released by necrotic and inflammatory cells during reperfusion tissue injury and activated inflammatory cells to induce proinflammatory mediators.

Curcumin-mediated inhibition of TLR2 was also documented by reduced expression of HMGB1, one of the ligands of TLRs (Fig. 6C). HMGB1 binds to RAGE in early IR injury resulting in the activation of proinflammatory pathways and enhanced myocardial injury [33].

While curcumin significantly reduced cardiac damages such as inflammation and connexin 43 disarray, the reduction of fibrosis was minimal in this IR model. A recent study showed that genetic deficiency of TLR2 significantly abrogates the proinflammatory state of type I diabetic mellitus and suggested TLR2 as a therapeutic target in inflammatory complications [34].

We showed that curcumin inhibited TNF-α- and reperfusion-induced up-regulation of TLR2 and its ligand HMGB1. Myocardial cell injury is associated with systolic and diastolic dysfunction of the heart. The present study demonstrated that in cardiac IR model, curcumin prevented deterioration of cardiac contractility with reduction in cardiac fibrosis (Fig. 5B, Tables 1 and 2). A limitation of this study is the lack of a mechanism that curcumin directly inhibits TLR2 expression. Overall, our data suggest that curcumin down-regulates TLR2 involved in cardiac I/R injury in CMs.

In addition to its inhibitory action on TLR2 induction, curcumin inhibits transcriptional activation by NF-κB, a pivotal intracellular mediator of the inflammatory response [4,35]. Inhibitory effect of curcumin on NF-κB [4] may be important in inflammatory responses in cardiovascular disease, in which inflammatory process plays a key role in the development of chronic heart diseases.

Cardiac hypertrophic signal was modified by curcumin. Histone acetyl transferase p300 acetylates histone and hypertrophy-responsive transcription factors such as serum response factor, myocyte enhancer factor-2 and GATA5. Cardiac p300 activity is increased in common types of heart failure in which pathological CM overgrowth occurs in response to hemodynamic overload. Inhibited acetylation of histones and GATA4 by curcumin mediated the repression of myocardial cell hypertrophy [36].

Several clinical trials (www.clinicaltrials.gov) for evaluation of curcumin on healthy subjects or various diseases including neoplasms, Alzheimer's diseases, diabetes and rheumatoid arthritis are now ongoing or completed, mainly targeting colon cancer [37]. The human clinical trials have been designed for oral administration of curcumin at doses up to 12 g/d. This dosage exceeds the dosage used in this study (300 mg/kg/d).

Curcumin acts as a free radical scavenger and antioxidant, inhibiting lipid peroxidation and oxidative DNA damage. These multiple actions of curcumin may be beneficial when clinically applying to heart failure patients.

We believe that the use of curcumin, which targets nuclear signaling pathways in CMs, will provide a novel therapeutic strategy against cardiac failure. Future application of this nontoxic dietary natural compound as a therapeutic agent for prevention or treatment of cardiac diseases in humans would be particularly interesting.

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