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Inhibition of cardiac oxidative and endoplasmic reticulum stress-mediated apoptosis by curcumin treatment contributes to protection against acute myocarditis

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Abstract

Curcumin is used anecdotally as an herb in traditional Indian and Chinese medicine. In the present study, the effects and possible mechanism of curcumin in experimental autoimmune myocarditis (EAM) rats were further investigated. They were divided randomly into a treatment and vehicle group, and orally administrated curcumin (50 mg/kg/day) and 1% gum arabic, respectively, for 3 weeks after myosin injection. The results showed that curcumin significantly suppressed the myocardial protein expression of inducible nitric oxide synthase (iNOS) and the catalytic subunit of nicotinamide adenine dinucleotide phosphate reduced (NADPH) oxidase. In addition, curcumin significantly decreased myocardial endoplasmic reticulum (ER) stress signaling proteins and improved cardiac function. Furthermore, curcumin significantly decreased the key regulators or inducers of apoptosis. In summary, our results indicate that curcumin has the potential to protect EAM by modulating cardiac oxidative and ER stress-mediated apoptosis, and provides a novel therapeutic strategy for autoimmune myocarditis.

Keywords: *Curcumin, oxidative stress, endoplasmic reticulum stress, apoptosis, autoimmune myocarditis*

Introduction

Curcumin is a natural polyphenolic compound abundant in the rhizome of the perennial herb turmeric, *curcuma longa*. It has been commonly used as a dietary spice and coloring agent in cooking, and has been used anecdotally as an herb in traditional Indian and Chinese medicine [1, 2]. Curcumin has a diverse range of molecular targets, including transcription factors, growth factors and their receptors, cytokines, enzymes, gene-regulating cell proliferation and apoptosis. As a result, curcumin has potential anti-inflammatory, anti-oxidant, anti-carcinogenic, anti-thrombotic, and cardiovascular protection effects. Its therapeutic effects have been demonstrated in many conditions, including wound healing, diabetes, neuronal diseases, pulmonary disease, arthritis, inflammatory bowel disease, cancer and cardiovascular diseases [3–5]; however, to our knowledge, no study to date has addressed the effect of curcumin on autoimmune myocarditis either in human or animal models.

Experimental autoimmune myocarditis (EAM) can be induced in rats by immunizing them with cardiac myosin together with complete Freund's adjuvant, providing a model that mimics the pathophysiology of human giant cell myocarditis [6, 7]. Histological examination of hearts with EAM demonstrates the infiltration of inflammatory cells with myocardial damage 2 weeks after immunization. Thereafter, myocarditis peaks around the 3rd week, and then gradually subsides during the 4th week. In the later stage, the 6th week, myocarditis progresses to dilated cardiomyopathy [8].

Acute myocarditis by progressive autoimmune myocardial injury is a potentially lethal disease and

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frequently precedes the development of acute and chronic heart failure (CHF) [9]. The disease is usually self-limiting, but approximately half of all patients continue to have significant left ventricular (LV) dysfunction, symptoms of CHF and a poor prognosis [10]. Recently onset CHF of nonischemic origin is mostly related to acute myocarditis or idiopathic dilated cardiomyopathy [10]. Moreover, in patients with heart failure, reactive oxygen species (ROS) have been found to be elevated in plasma [11]. Also, recent reports have indicated that myocardial injury was produced by ROS [12, 13, 14]. Moreover, recent evidence has suggested that endoplasmic reticulum (ER) stress plays an important role in the pathogenesis of heart failure [15]. Since the oxidative and ER stress process in the myocardium is closely associated with LV dysfunction leading to CHF, it is desirable to seek therapeutic agents which can stop the progression of oxidative and ER stress in myocarditis, and also improve LV dysfunction.

The purpose of this study was to investigate whether curcumin can be used as a therapeutic agent for acute myocarditis. In this report, we administered curcumin for 3 weeks orally to EAM rats after the induction of myocarditis, analyzed the therapeutic effects of curcumin on EAM rats, and elucidated the mechanism, especially focusing on its cardioprotective and inhibitory effects on oxidative and ER stress, and subsequently, cardiac apoptosis.

Materials and methods

Materials Curcumin from *curcuma longa* was purchased from Sigma-Aldrich (St. Louis, MO). Lewis rats (male, 8 weeks old) were purchased from Charles River Japan Inc. (Kanagawa, Japan).

Experimental design

All studies were carried out using 8-week-old male Lewis rats weighing about 230-250 g (Charles River Japan Inc.). Eight-week-old male Lewis rats were injected in the footpads with antigen-adjuvant emulsion according to the procedure described previously [6, 16]. In brief, porcine cardiac myosin was dissolved in phosphate-buffered saline at 5 mg/ml and emulsified with an equal volume of complete Freund's adjuvant with 11 mg/ml *Mycobacterium tuberculosis* H37RA (Difco Lab., Detroit, MI). EAM in rats was induced by immunization with 0.1 ml emulsion once by subcutaneous injection into their rear footpads (0.1 ml into each footpad). The morbidity of EAM was 100% in rats immunized by this procedure [6, 16]. Rats immunized with myosin became ill and immobile in the 2nd week. After myosin injection, rats were divided randomly into daily oral treatment with curcumin (50 mg/kg/day) (Group C50, n = 6) or vehicle (1% gum arabic) (Group V, n = 7) for 3 weeks. Age-matched Lewis rats without immunization were used as normal controls (Group N, n = 5). Rats were maintained with free access to water and chow throughout the period of study, and animals were treated in accordance with the Guidelines for Animal Experimentation of our institute. All animals were handled according to the approved protocols and animal welfare regulations of the Institutional Review Board at Niigata University of Pharmacy and Applied Life Sciences [16].

Echocardiographic and histopathological studies

Two-dimensional echocardiographic studies were performed under 0.5% halothane using an echocardiographic machine equipped with a 7.5-MHz transducer (SSD-5500; Aloka, Tokyo, Japan). M-mode tracings were recorded from the epicardial surface of the right ventricle, and the short axis view of the left ventricle was recorded to measure the LV dimension in diastole (LVDd) and LV dimension in systole (LVDs). LV fractional shortening (FS) and ejection fraction (EF) were calculated using LVDd and LVDs. The study was performed in a blinded manner. After the measurement of echocardiographic parameters, the hearts were excised and weighed immediately, and the ratio of heart weight (HW) to body weight (BW), (HW/BW) was calculated. The excised hearts were cut into about 2 mm transverse slices and fixed in 10% formalin. After being embedded in paraffin, several transverse sections were obtained from the ventricle.

Terminal deoxynucleotidyl transferase dUTP-meditated nick-end labeling assay

Frozen LV tissues embedded in OCT compound were cut into 4-μm-thick sections and fixed in 4% paraformaldehyde (pH 7.4) at room temperature. Terminal deoxynucleotidyl transferase dUTP-meditated nick-end labeling (TUNEL) apoptosis analysis was performed as specified in the in situ apoptosis detection kit (Takara Bio, Shiga, Japan), and sections were examined under fluorescence microscopy at 200-fold magnification (CIA-102; Olympus, Tokyo, Japan) [17, 18]. For each animal, five sections were scored for apoptotic nuclei.

Western blotting

LV homogenates were prepared from rats treated as described above and age-matched untreated normal control rats in the 3rd week. For the determination of protein levels, equal amounts of protein extracts (30μg) were separated by 10%, 12.5% or 15% SDS-polyacrylamide gel electrophoresis (Bio-Rad, CA, USA),

respectively, and electrophoretically transferred to nitrocellulose membranes (semidry transfer) [19]. Membranes were blocked with 5% non-fat dry milk in Tris-buffered saline (20 mM Tris, pH 7.6, 137 mM NaCl) with 0.1% Tween 20, washed, and then incubated with primary antibody. Primary antibodies employed included: goat polyclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH), anti-p67 phox, anti-gp91 phox and anti-glucose regulated protein 78 (GRP78) (Santa Cruz Biotechnology Inc., Santa Cruz, CA), rabbit polyclonal anti-inducible nitric oxide synthase (iNOS) (Santa Cruz Biotechnology Inc.), anti-caspase-12 (Bio Vision, CA 94043 USA), and anti-mitogen-activated protein kinase (MAPK)-activated protein kinase 2 (MAPKAPK-2), anti-phospho-MAPKAPK-2, anti-cJUN NH2-terminal kinase (JNK), anti-phospho-JNK, anti-caspase-7 and anti-caspase-3 (Cell Signaling Technology Inc., MA), and mouse monoclonal anti-growth arrest and DNA damage-inducible gene 153 (GADD153). After incubation with the primary antibody, the bound antibody was visualized with the respective horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz Biotechnology Inc.) and chemiluminescence developing agents (Amersham Biosciences, Buckinghamshire, UK). The level of GAPDH was estimated in every sample. Films were scanned and band densities were quantified by densitometric analysis using Scion image software (Epson GT-X700; Tokyo, Japan). Finally, Western blotting data were normalized with those for cardiac GAPDH. MAPK activation was quantified by normalizing the phospho-MAPK expression level with MAPK expression in the same sample.

Statistical analysis

All values are expressed as the mean ± SEM. Statistical analysis of differences between groups was performed by one-way analysis of variance (ANOVA), followed by Tukey or Bonferroni methods of post-hoc analysis and the two-tailed t-test when appropriate. A value of $p < 0.05$ was considered significant.

Results

HW/BW and Echocardiographic assessments

HW/BW (an index of hypertrophy) in Group V was significantly increased compared to those in Group N. Interestingly, in Group C50, HW/BW was significantly decreased compared with those in Group V (Figure 2A). Echocardiographic parameters EF and FS were significantly decreased in Group V compared to Group N, which indicated decreased LV systolic function in

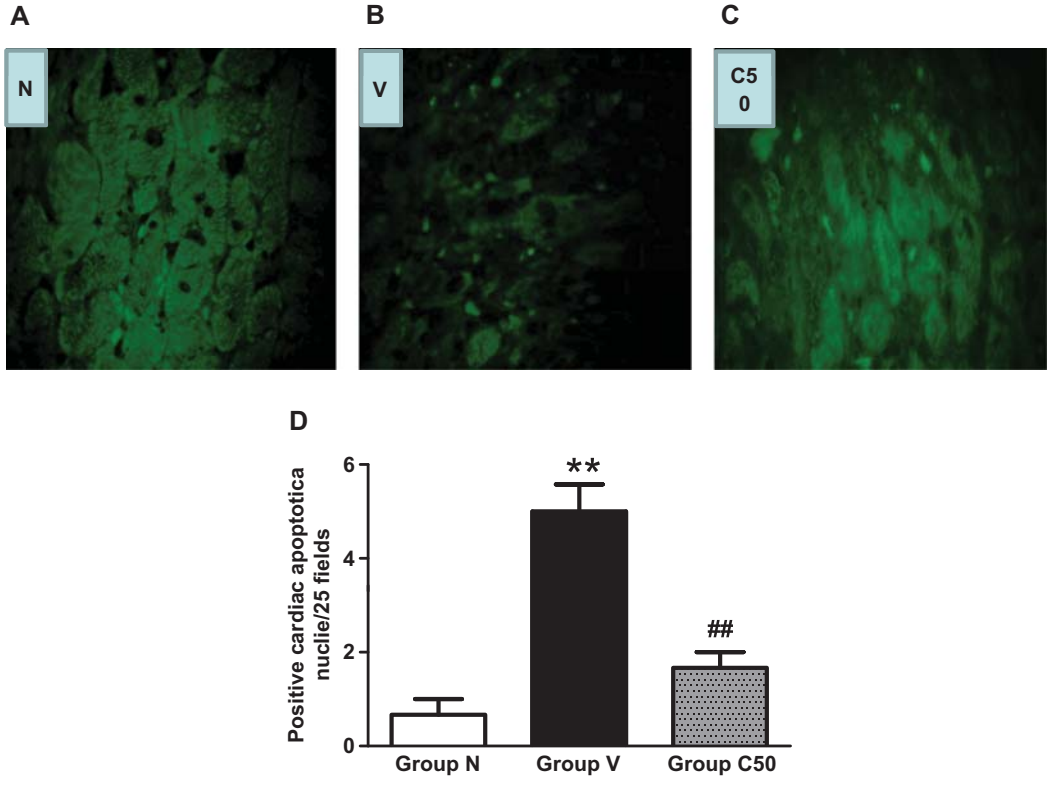

Figure 1. **(A–C)** Myocardial tissue sections terminal deoxynucleotidyl transferase dUTP-meditated nick-end labeling stained for apoptotic nuclei. Bar graph shows positive cardiac apoptotic nuclei/25 fields (D). Group N, age-matched untreated rats; Group V, rats with experimental autoimmune myocarditis treated with vehicle; Group C50, rats with experimental autoimmune myocarditis orally treated with curcumin (50 mg/kg/day). All values are expressed as the mean ± SEM. $^{**}p < 0.01$ vs Group N; $^{\#\#}p < 0.01$ vs Group V.

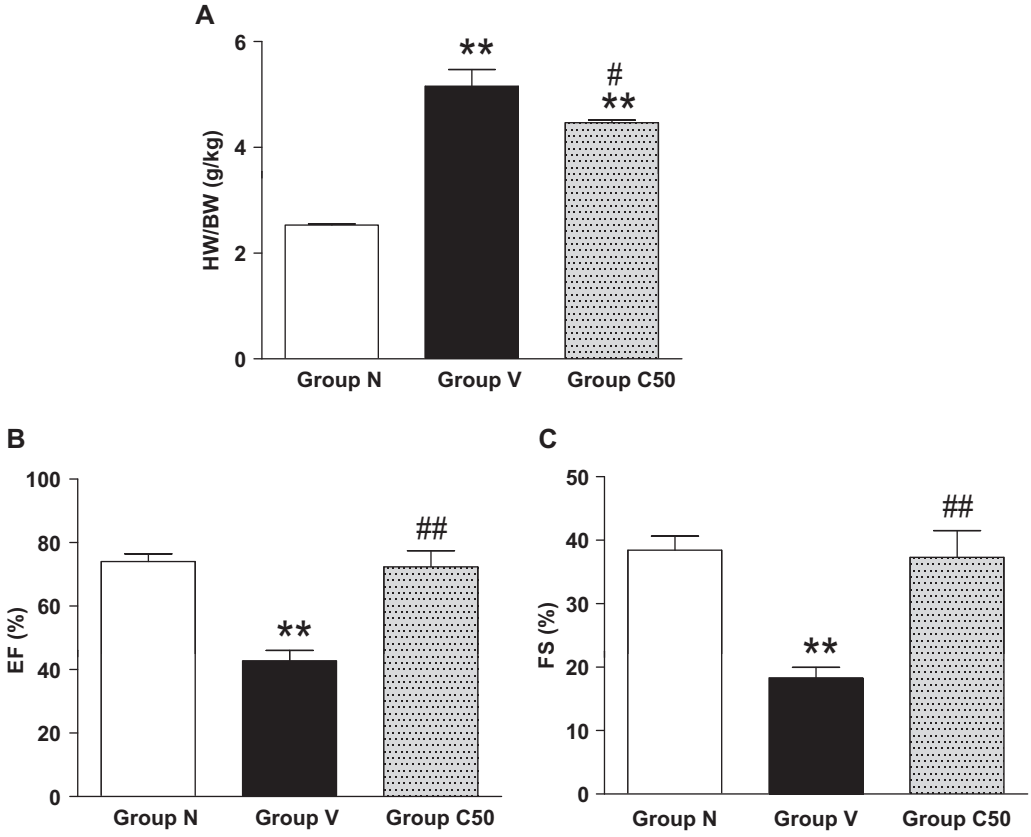

Figure 2. Echocardiographic parameters. Changes in the heart weight-to-body weight ratio (HW/BW) (A) and echocardiographic parameters, EF (B) and FS (C). Group N, age-matched untreated rats; Group V, rats with experimental autoimmune myocarditis treated with vehicle; Group C50, rats with experimental autoimmune myocarditis orally treated with curcumin (50 mg/kg/day). All values are expressed as the mean ± SEM. $^{**}p < 0.01$ vs Group N; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs Group V.

EAM rats. In addition, LV systolic function of EAM rats was improved by administration of curcumin, since EF and FS were significantly increased in Group C50 compared to Group V (Figure 2B and C).

Myocardial apoptosis assessed by TUNEL staining

TUNEL-positive nuclei were rare or absent in the hearts of normal rats, whereas the number of TUNEL positive nuclei was significantly increased in Group V compared to Group N (Figure 1B and D). Treatment with curcumin significantly decreased the number of TUNEL-positive nuclei in the myocardium compared to Group V (Figure 1C and D). A representative microphotograph of TUNEL staining is shown in Figure 1A.

Myocardial protein levels of p67 phox, g p91 phox and iNOS assessed by Western blotting

To investigate the molecular mechanism of the therapeutic effects of curcumin on EAM rats, we next analyzed the expression of several myocardial protein levels. Western blotting analysis has shown that the expressions of myocardial protein levels of p67 phox, gp91 phox and iNOS were significantly increased in Group V compared to Group N (Figure 3A-D). In Group C50, administration of curcumin significantly decreased the expression of myocardial protein levels of p67 phox, gp91 phox and iNOS compared to Group V (Figure 3A–D).

Myocardial protein levels of GRP78, cleaved-caspase-12 and GADD153 assessed by Western blotting

The expressions of myocardial protein levels of GRP78, cleaved-caspase-12 and GADD153 were also significantly increased in Group V compared to Group N (Figure 4A-D). In Group C50, treatment with curcumin significantly decreased the myocardial protein expressions of GRP78, cleaved-caspase-12 and GADD153 compared to Group V (Figure 4A–D).

Myocardial protein levels of MAPKAPK-2 and JNK assessed by Western blotting

The myocardial protein expressions of JNK were significantly increased in Group V compared to Group N (Figure 5A and C). In Group C50, treatment with curcumin significantly decreased the expression of the myocardial protein level of JNK compared to Group V (Figure 5A and C).

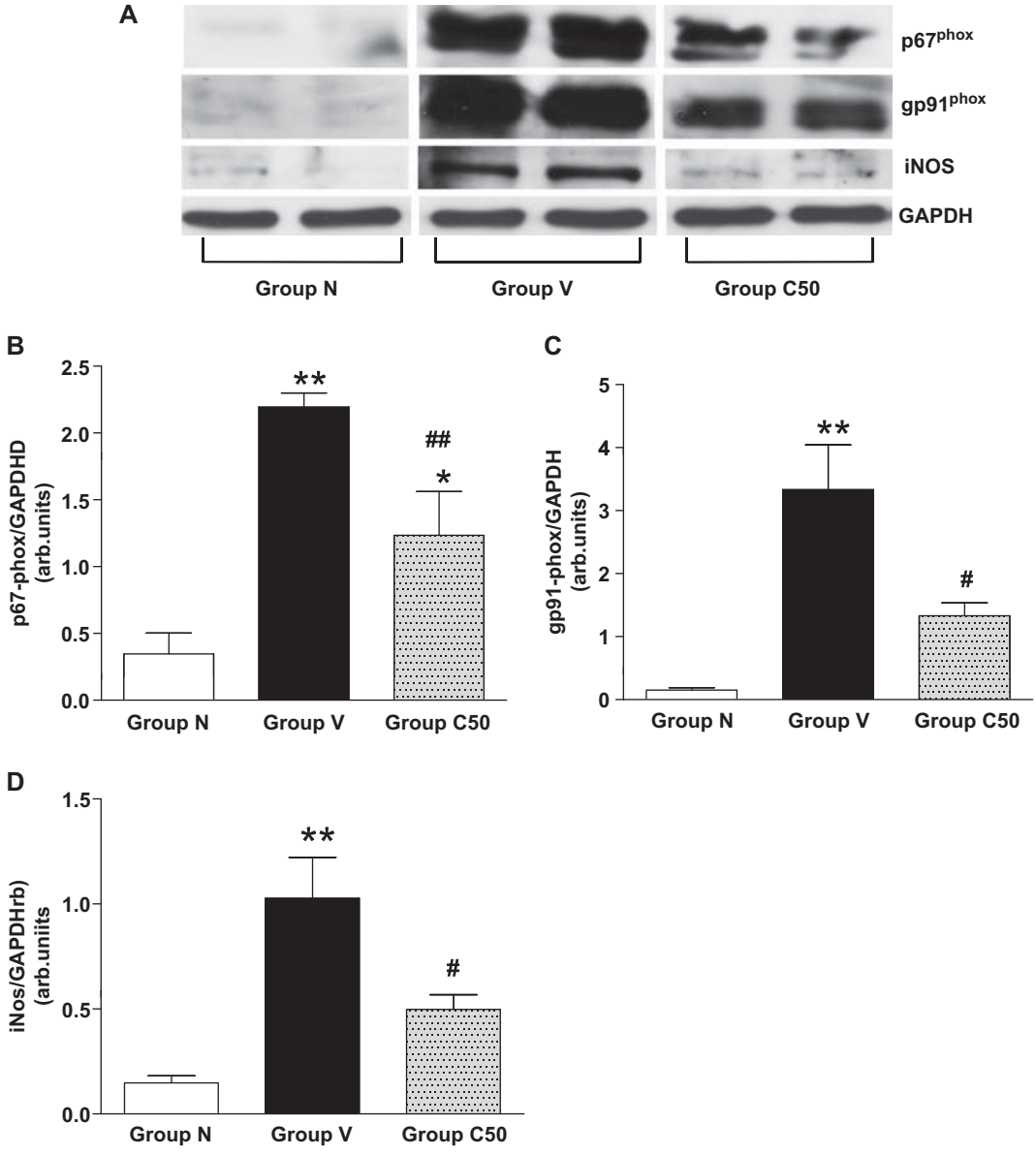

Figure 3. Myocardial expressions of p67 phox, gp91 phox and iNOS proteins. Representative Western blots showing specific bands of p67 phox, gp91 phox, iNOS and GAPDH as an internal control. An equal amount of protein sample obtained from left ventricular homogenate was applied in each lane (A). Group N, age-matched untreated rats; Group V, rats with experimental autoimmune myocarditis treated with vehicle; Group C50, rats with experimental autoimmune myocarditis orally treated with curcumin (50 mg/kg/day). Densitometric data of protein analysis. The mean density values of p67 phox (B), gp91 phox (C) and iNOS (D) are expressed as a relative ratio to that of GAPDH. All values are expressed as the mean ± SEM. $^{**}p < 0.01$ vs Group N; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs Group V.

In contrast, the expression of the myocardial protein level of MAPKAPK-2 showed no difference among the groups (Figure 5A and B).

Myocardial protein levels of caspase-7 and caspase-3 assessed by Western blotting

The myocardial protein expressions of caspase-7 and caspase-3 were significantly increased in Group V compared to Group N (Figure 6A–C). In Group C50, administration of curcumin significantly decreased the myocardial protein expressions of caspase-7 and caspase-3 compared to Group V (Figure 6A–C).

Discussion

Previously, curcumin was used as a therapeutic agent to alleviate cardiovascular disease and had a protective role in the cardiovascular system [3, 20]. Recent evidence showed that curcumin has anti-oxidant and free radical scavenger properties [21], and also triggers ER stress-induced cell death, which involves cleavage of caspases [22]; however, to the best of our knowledge, no study has addressed the role of curcumin in cardiac oxidative and ER stress-mediated progression of autoimmune myocarditis either in human or experimental animal models. In this report, reduction of the HW/BW ratio and improvement of

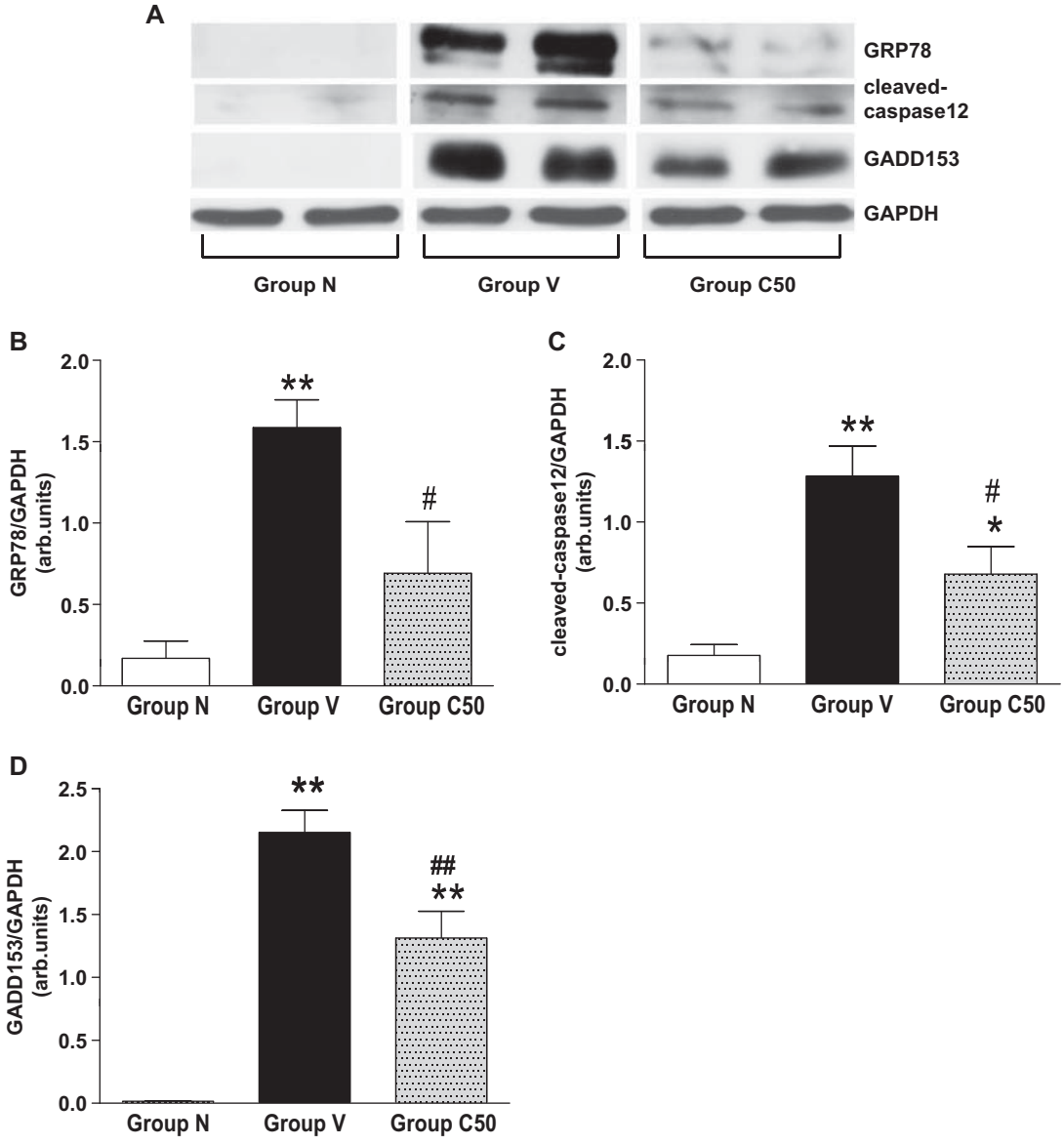

Figure 4. Myocardial expressions of GRP78, cleaved-caspase-12 and GADD153 proteins. Representative Western blots showing specific bands of GRP78, cleaved-caspase-12, GADD153 and GAPDH as an internal control. An equal amount of protein sample obtained from left ventricular homogenate was applied in each lane (A). Group N, age-matched untreated rats; Group V, rats with experimental autoimmune myocarditis treated with vehicle; Group C50, rats with experimental autoimmune myocarditis orally treated with curcumin (50 mg/kg/day). Densitometric data of protein analysis. The mean density values of GRP78 (B), cleaved-caspase-12 (C) and GADD153 (D) are expressed as a relative ratio to that of GAPDH. All values are expressed as the mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$ vs Group N; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs Group V.

echocardiographic data clearly suggested that curcumin can provide a protective effect in the acute phase in EAM rats. To reveal the precise mechanism we mainly focused on the anti-oxidant properties of curcumin, because oxidative stress has been determined to play a key role in the pathogenesis and development of cardiac diseases, such as heart failure, endothelial dysfunction, atherosclerosis, hypertension and myocardial infarction [9, 23, 24, 25].

Increased generation of ROS eventually leads to oxidative stress, which has been determined to contribute to the initiation and progression of cardiac dysfunction including cardiovascular diseases [23, 26]. ROS may also occur in pro-apoptotic signaling through the activation of MAP kinase, such as SAPK/JNK [27]. A previous report has suggested that myocardial protein oxidative damage was significantly increased in acute EAM [28]. Vascular enzymes such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidases are involved in the production of ROS [23]. It is well known that the formation of intracellular ROS is mainly catalyzed by NADPH oxidase [29]. Gp91 phox, also known as Nox2, is a critical and catalytic subunit in NADPH oxidase [25, 29]. In response to a number of factors and conditions, the increased activity of NADPH oxidase is related to the

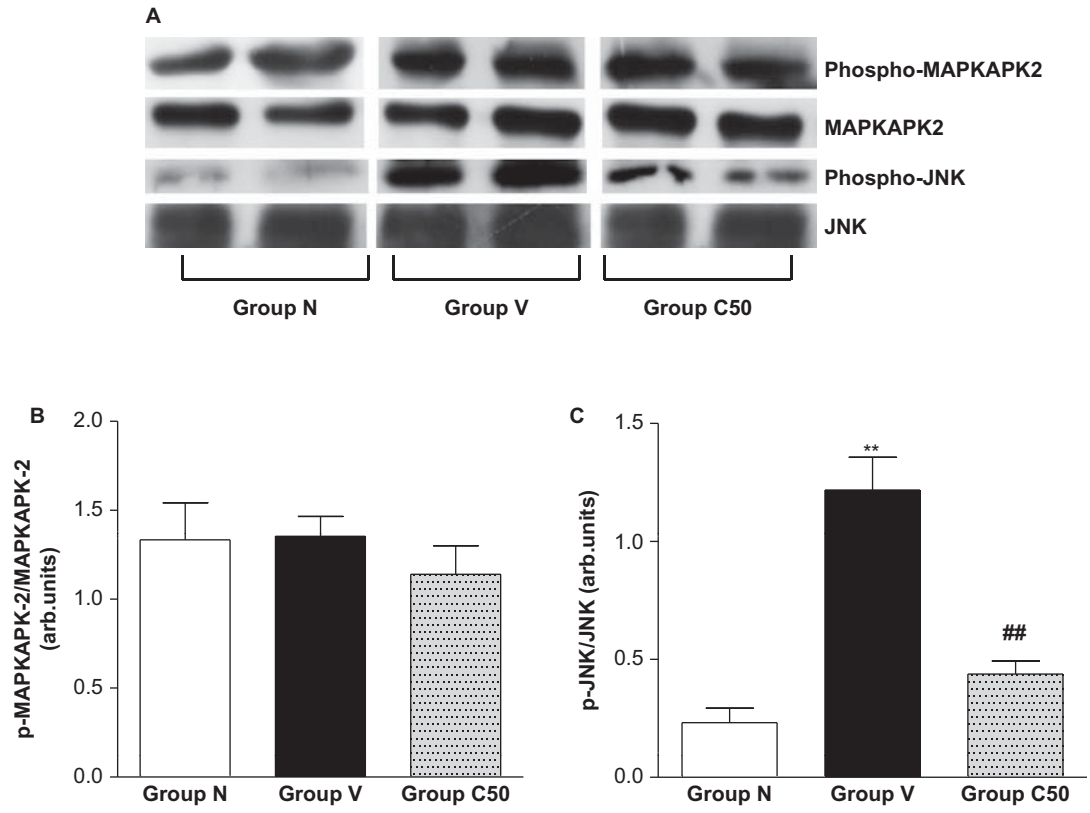

Figure 5. Myocardial expressions of MAPKAPK2, phospho-MAPKAPK2, JNK and phospho-JNK proteins. Densitometric data of protein analysis. Representative Western blots showing specific bands of MAPKAPK2, phospho-MAPKAPK2, JNK and phospho-JNK as an internal control. An equal amount of protein sample obtained from left ventricular homogenate was applied in each lane (A). Group N, age-matched untreated rats; Group V, rats with experimental autoimmune myocarditis treated with vehicle; Group C50, rats with experimental autoimmune myocarditis orally treated with curcumin (50 mg/kg/day). Densitometric data of protein analysis. The mean density values of phospho-MAPKAPK2 (B) and phospho-JNK (C) are expressed as a relative ratio to that of MAPKAPK2 and JNK. All values are expressed as the mean ± SEM. $^{**}p < 0.01$ vs Group N; $^{\#\#}p < 0.01$ vs Group V.

up-regulation of p67 phox and gp91phox expression [29, 30]. A previous report suggested that p22 phox, p47 phox and gp91 phox were up-regulated in failing hearts [25]. In this report, we have shown that the myocardial protein expression levels of p67 and gp91 were significantly increased in EAM and that curcumin treatment significantly attenuated these protein expression levels in rats with EAM.

It has been reported that increased production of ROS, which reacts with nitric oxide (NO), contributes to endothelial dysfunction in failing hearts [31]. NO is a free radical, largely synthesized by the enzyme NO synthase, activated macrophages and other cells, and increasing evidence has shown that the NO pathway plays an important role in the pathogenesis of inflammatory and immunological diseases [21, 32]. It has been reported that excessive production of NO by iNOS contributes to progressive myocardial damage in myocarditis [33] and furthermore, overexpression of cardiomyocyte iNOS in mice results in peroxynitrite generation, heart block, and sudden death [34]. Recent evidence has shown up-regulated myocardial iNOS in rats with EAM [32]. In this report, we also observed up-regulated protein expression of myocardial iNOS in rats with EAM, while curcumin treatment significantly reduced the free radical enzyme, iNOS. Our results correlate with a previous report suggesting that one of the potential ameliorating effects of curcumin by which it acts against the acute phase of EAM might be through the inhibition of oxidative stress and nutritive stress.

Recently, ER stress has been reported to be activated by various stress responses, including oxidative stress, and contributes to cardiac myocyte apoptosis during the progression of cardiac hypertrophy to failure [26]. ER stress has been shown to promote cell apoptosis through the activation of GADD153/CHOP, GRP78 caspase and JNK expression [26, 35]. Recent evidence has shown that ER stress chaperones, such as GRP78 and CHOP, were up-regulated in a hypertensive heart disease model in the heart failure phase [15].

Moreover, procaspase-12 is cleaved and activated specifically by ER stress, and its activation is caused by caspase-7 and activated caspase-12, which then cleaves and activates procaspase-9, which in turn activates its downstream caspase cascade including caspase-3 [36]. In this study, we observed increased oxidative and ER stress in EAM, which resulted in the activation of JNK, but interestingly not the

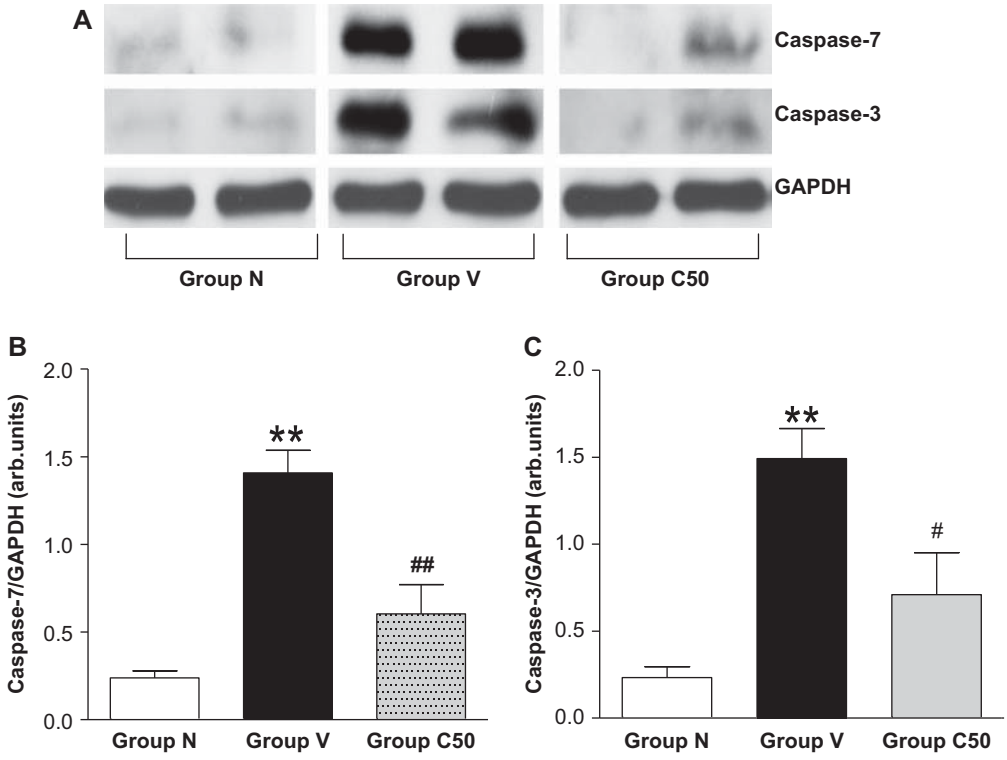

Figure 6. Myocardial expressions of caspase-7 and caspase-3. Representative Western blots showing specific bands of caspase-7, caspase-3, and GAPDH as an internal control. An equal amount of protein sample obtained from left ventricular homogenate was applied in each lane (A). Group N, age-matched untreated rats; Group V, rats with experimental autoimmune myocarditis treated with vehicle; Group C50, rats with experimental autoimmune myocarditis orally treated with curcumin (50 mg/kg/day). Densitometric data of protein analysis. The mean density values of caspase-7 (B) and caspase-3 (C) are expressed as a relative ratio to that of GAPDH. All values are expressed as the mean ± SEM. $^{**}p < 0.01$ vs Group N; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs Group V.

phosphorylation of MAPKAPK-2. Moreover, curcumin significantly decreased ER stress markers and caspase family proteins. In addition, TUNEL assay showed that the number of apoptotic cells fell significantly by curcumin treatment in EAM. Thus, another protective effect of curcumin against EAM could be mainly the inhibition of ER stress rather than p38 MAPK signaling, which eventually results in the down-regulation of myocardial apoptosis.

In conclusion, the present study indicates that curcumin has protective effects against cardiac oxidant and ER stress-induced apoptosis in EAM rats, and provides a novel therapeutic strategy for acute autoimmune myocarditis. Future application of this nontoxic dietary natural compound as a therapeutic agent for acute autoimmune myocarditis in humans would be particularly interesting.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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