Emodin, a Naturally Occurring Anthraquinone, Ameliorates Experimental Autoimmune Myocarditis in Rats

Zhan-Chun Song,1,2 Zhan-Sheng Wang,1 Jing-Hui Bai,1,3 Zhao Li1 and Jian Hu1

1Department of Cardiology, The First Hospital of China Medical University, China Medical University, Shenyang, P.R. China
2Department of Cardiology, Fushun Central Hospital, Fushun, P.R. China
3Intensive care unit, Liaoning Cancer Hospital, Shenyang, P.R. China

Myocarditis is an inflammatory disease of the heart and a major cause of dilated cardiomyopathy that can lead to heart failure and sudden death in young adults. Giant cell myocarditis is a severe heart disease of unknown causes and is defined histopathologically as diffuse myocardial necrosis with multinucleated giant cells in the absence of sarcoid-like granulomata. Giant cell myocarditis is often studied using a model of experimental autoimmune myocarditis (EAM) in rats. Emodin is an important component of traditional Chinese herb rhubarb, and has well-documented anti-inflammatory effect. The current study determined the potential efficacy of emodin using a rat model of EAM. Male Lewis rats (6 weeks of age) were immunized on days 0 and 7 with a porcine cardiac myosin at both footpads to induce EAM. Simultaneously with the immunization, rats received emodin (50 mg/kg/day) or distilled water by intragastric administration for 3 weeks (8 animals/group). Likewise, eight animals were immunized with adjuvant alone and treated with distilled water. The immunization significantly enlarged the hearts due to inflammatory lesions. Emodin treatment significantly improved left ventricular (LV) function and reduced the severity of myocarditis, as reflected by echocardiographic and histopathological examination. Emodin treatment decreased the serum levels of proinflammatory cytokines tumor necrosis factor (TNF)-α and interleukin (IL)-1β. Nuclear factor-κBp65 (NF-κBp65), a rapid-response transcription factor that regulates proinflammatory cytokines, in the myocardial tissue was also suppressed in the treated rats. In conclusion, emodin could ameliorate EAM, at least in part, by decreasing the production of proinflammatory cytokines TNF-α and IL-1β.

Keywords: emodin; experimental autoimmune myocarditis; heart failure; inflammation; myocarditis

Liu et al. 2009). In this study, we investigated the potential role of emodin in inhibiting the inflammatory response and alleviating EAM in rats.

### Materials and Methods

#### Experimental animals and immunization

Male Lewis rats (n = 24, 6 weeks of age, 160 to 180 g) were purchased from the Beijing Vital River Laboratory Animal Technology (Beijing, China), and were housed in a specific-pathogen-free animal facility. All animal experiment protocols were approved by the Animal Care and Use Committee of China Medical University.

Purified porcine cardiac myosin (Sigma Co., USA) was dissolved in 0.01 M phosphate-buffered saline (PBS) at a concentration of 10 mg/ml. To induce EAM, on days 0 and 7, rats were immunized with 0.2 ml of an emulsion containing cardiac myosin with an equal volume of Freund’s complete adjuvant (FCA) supplemented with Mycobacterium tuberculosis H37Ra (Sigma Co., USA) by a single subcutaneous injection in both footpads (Kodama et al. 1990). The control rats were injected with 0.01 M PBS mixed with an equal volume of FCA. No rat died prior to day 21.

#### Treatment with emodin

Emodin (purity > 98%) was a product from the Chinese National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Male Lewis rats were randomly assigned into three groups (8 animals/group): EAM group, Emodin group and Control group. EAM group and Emodin group were immunized with myosin/FCA, and the Control group was immunized with FCA alone. After the immunization, Emodin group was immediately treated with intragastric administration of 50 mg/kg per day of emodin for 3 weeks, while EAM group and Control group were given distilled water. The treatment was started on day 0.

#### Echocardiography

A trans-thoracic echocardiographic analysis was performed on day 21 using a ProSound IU22 (PHILIPS, Netherlands) with a 7.5-MHz imaging transducer. Briefly, rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (20-35 mg/kg). A M-mode echocardiography was performed at the papillary muscle level under spontaneous ventilation. The left ventricular end-diastolic dimension (LVEDd), and end-systolic dimension (LVEDs) were measured. The percentage of left ventricular fractional shortening (LVFS) was using the formula: LVFS (%) = [LVEDd - LVEDs]/LVEDd × 100. All echocardiographic measurements were averaged from at least 5 separate cardiac cycles.

#### Heart weight/body weight and histopathology

All rats were sacrificed under ether anesthesia on day 21 after immunization. Blood samples were obtained from the inferior vena cava. Hearts were removed and weighed, macroscopic findings were evaluated, and then the ratio of heart weight/body weight (Hw/Bw) was calculated. Hearts were rinsed with PBS, and fixed in 10% buffered formalin for 24 hours prior to embedding in paraffin. Tissue was sectioned, and stained with hematoxylin and eosin for examination under a light microscopy.

Macroscopic findings were classified into five grades: 0, no inflammation; 1, presence of a small discolored focus; 2, presence of multiple small discolored foci; 3, diffuse discolored areas not exceeding a total of one-third of the cardiac surface; and 4, diffuse discolored areas totaling more than one-third of the cardiac surface (Okura et al. 1998).

Cardiac sections stained with hematoxylin and eosin were examined for evidence of mononuclear and polymorphonuclear cellular infiltration, necrosis and mineralization, and were given a histologic score ranging from 0 (no involvement) to 4 (100% involvement), with 1, 2 and 3 representing 25%, 50% and 75% involvement of the histologic section, respectively (Daniels et al. 2008; Yuan et al. 2010).

#### Western blot analysis

Myocardial tissue samples were homogenized in a lysis buffer. Protein concentration was measured with a bicinchoninic acid method. For Western blots, samples (40 μg protein) were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and then transferred electrophoretically onto a nitrocellulose membrane. The membrane was incubated with a primary antibody against nuclear factor-kappaBp65 (NF-κBp65) (1: 1,000; Santa Cruz Biotechnology, Santa Cruz, CA). After extensive washes, the blot was incubated with a secondary antibody (1: 5,000; Amersham Biosciences, Piscataway, NJ) and developed with an ECL reagent (Amersham Biosciences, Piscataway, NJ). Chemiluminescence was detected with a LAS-1000 luminescent image analyzer (Fujifilm, Tokyo, Japan). β-actin was used as an internal control.

#### Measurement of serum TNF-α and IL-1β

Blood samples were collected on day 21. Serum concentration of TNF-α, IL-1β was measured with ELISA kits (BioSource International, Camarillo, CA) according to the manufacturer’s instructions.

#### Statistical analysis

All data are expressed as mean ± s.d. Statistical significance was calculated using Student’s t-tests or an one-way analysis of variance (ANOVA). A P-value < 0.05 was considered significant.

### Results

#### Echocardiographic analysis

We first examined the effect of emodin on cardiac function 21 days after myosin immunization and EAM induction using echocardiography in live rats. As shown in Table 1, Left ventricular (LV) function indices including LVEDs, LVEDd and LVFS were measured and calculated (For details, refer to Materials and Methods). All the indices in Control group were statistically significantly different compared with EAM group and Emodin group. LVEDs and LVEDd, markers of LV remodeling, were significantly decreased in Emodin group (LVEDs: 3.36 ± 0.39 mm; LVEDd: 5.80 ± 0.50 mm) compared with EAM group (LVEDs: 5.00 ± 0.46 mm, P < 0.05; LVEDd: 6.50 ± 0.43 mm, P < 0.05), and LVFS in Emodin group was significantly improved compared with EAM group (42.17 ± 3.06% vs. 23.20 ± 3.57%, P < 0.05). These results show that emodin treatment attenuated LV remodeling and preserved cardiac function of EAM rats.

#### Heart weight/body weight and histopathology

The hearts of EAM group were enlarged and contained...
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There was no statistically significant difference between the Control group and Emodin group (750.38 ± 20.74 mg vs. 779.25 ± 25.33 mg, \(P = 0.05\)) in heart weight (Hw) (Table 1). The HW and Hw/Bw ratio of Emodin group (Hw: 779.25 ± 25.33 mg; Hw/Bw: 3.46 ± 0.25 mg/g) were significantly lower than those of EAM group (Hw: 853.25 ± 35.22 mg, \(P < 0.05\); Hw/Bw: 4.93 ± 0.37 mg/g, \(P < 0.05\)) (Table 1). Thus, emodin significantly suppressed the increase in heart weight of EAM rats due to inflammation.

Macroscopic scores were significantly lower in Emodin group compared with EAM group (1.31 ± 0.46 vs. 3.25 ± 0.53, \(P < 0.05\)) (Table 2). Microscopically, the heart tissue from the Control group showed no noticeable infiltration with inflammatory cells (Fig. 1A). Inflammatory lesions with marked infiltration of mononuclear cells, polymorphonuclear neutrophils, and multinucleated giant cells were observed in EAM group (Fig. 1B). Emodin treatment significantly decreased the inflammatory cell infiltration (Fig. 1C). All cardiac sections were scored for myocardial inflammation (For details, refer to Materials and Methods), and the microscopic scores were significantly lower in

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**Table 1. Effects of emodin treatment on LV function indices and Hw/Bw.**

<table>
<thead>
<tr>
<th></th>
<th>Control group ((n = 8))</th>
<th>EAM group ((n = 8))</th>
<th>Emodin group ((n = 8))</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVEDs (mm)</td>
<td>2.91 ± 0.27</td>
<td>5.00 ± 0.46*</td>
<td>3.36 ± 0.39**</td>
</tr>
<tr>
<td>LVEDd (mm)</td>
<td>5.51 ± 0.46</td>
<td>6.50 ± 0.43*</td>
<td>5.80 ± 0.50**</td>
</tr>
<tr>
<td>LVFS (%)</td>
<td>47.09 ± 4.49</td>
<td>23.20 ± 3.57*</td>
<td>42.17 ± 3.06**</td>
</tr>
<tr>
<td>BW (g)</td>
<td>254.25 ± 10.55</td>
<td>173.38 ± 9.13*</td>
<td>225.75 ± 12.61**</td>
</tr>
<tr>
<td>HW (mg)</td>
<td>750.38 ± 20.74</td>
<td>853.25 ± 35.22*</td>
<td>779.25 ± 25.33</td>
</tr>
<tr>
<td>Hw/Bw (mg/g)</td>
<td>2.96 ± 0.16</td>
<td>4.93 ± 0.37*</td>
<td>3.46 ± 0.25*</td>
</tr>
</tbody>
</table>

LVEDd, left ventricular end-diastolic dimension; LVEDs, left ventricular end-systolic dimension; LVFS, the percentage of left ventricular fractional shortening; Bw, Body Weight; Hw, Heart Weight.

Data are mean ± s.d. *\(P < 0.05\) vs. Control group, **\(P < 0.05\) vs. EAM group.

**Table 2. Effects of emodin treatment on heart inflammation scores.**

<table>
<thead>
<tr>
<th></th>
<th>Control group ((n = 8))</th>
<th>EAM group ((n = 8))</th>
<th>Emodin group ((n = 8))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macroscopic score</td>
<td>0</td>
<td>3.25 ± 0.53*</td>
<td>1.31 ± 0.46**</td>
</tr>
<tr>
<td>Microscopic score</td>
<td>0</td>
<td>2.80 ± 0.35*</td>
<td>1.44 ± 0.44**</td>
</tr>
</tbody>
</table>

Data are mean ± s.d. *\(P < 0.05\) vs. Control group, **\(P < 0.05\) vs. EAM group.

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**Fig. 1. Effects of emodin treatment on histology of hearts.**

Cardiac inflammatory cells infiltration were assessed by staining with hematoxylin and eosin and given inflammation scores as described in Materials and Methods. (A) Representative histopathology in Control group (inflammation scores: 0). (B) Representative histopathology in EAM group. The myocardial lesions were showed infiltration by mononuclear cells, polymorphonuclear neutrophils, and multinucleated giant cells. The myocardial inflammation was diffused and exceeding 75% of histological section (inflammation scores: 4). (C) Representative histopathology in Emodin group. The myocardial lesions were showed a small focus of cellular infiltration (inflammation scores: 1). Infiltration of inflammatory cells was reduced significantly by emodin treatment compared with EAM group.
Emodin group (1.44 ± 0.44) compared with EAM group (2.80 ± 0.35) (P < 0.05) (Table 2). Overall, emodin attenuated the inflammation of the myocardium of EAM rats.

Effect of emodin on myocardial NF-κBp65 expression

To investigate the molecular mechanism of the therapeutic effects of emodin on EAM rats, we analyzed myocardial protein levels of NF-κBp65. Western blot analysis showed that myocardial NF-κBp65 levels were significantly increased in EAM group and Emodin group compared to Control group (P < 0.05). In Emodin group, administration of emodin significantly decreased myocardial protein levels of NF-κBp65 compared to EAM group (P < 0.05) (Fig. 2). These results suggest that myocardial protection by emodin might involve the NF-κBp65 pathway.

Effect of emodin on serum proinflammatory cytokines

Proinflammatory cytokines play a key role in the pathogenesis of EAM. Next we investigated the possibility that emodin could exert its cardiac protection effect by suppressing proinflammatory cytokine IL-1β and TNF-α production. Serum IL-1β and TNF-α on day 21 in all three groups were measured by ELISA. As shown in Fig. 3, serum IL-1β was markedly increased in EAM group (25.30 ± 1.48 pg/ml) compared with control group (7.22 ± 0.89 pg/ml) (P < 0.05). In emodin group, serum IL-1β (15.68 ± 1.18 pg/ml) was significantly reduced compared with EAM group not treated with emodin (P < 0.05). Similarly, serum TNF-α was also significantly reduced in EAM rats treated with emodin (60.29 ± 6.20 pg/ml) compared with those untreated (142.39 ± 18.50 pg/ml) (P < 0.05) (Fig. 3). Taken together, our data suggest that emodin can suppress inflammation in EAM rats by inhibiting the production of proinflammatory cytokines IL-1β and TNF-α.

Discussion

Acute myocarditis is an inflammatory disease of the heart muscle, and may progress to dilated cardiomyopathy and chronic heart failure. Evidence from studies using rat models suggests that proinflammatory cytokines play a crucial role in the induction and progression of myocardial injury in this disease.

Proinflammatory cytokines, including the TNF family and IL-1 family, also contribute to the progression of heart failure (Mann 2002). Circulating proinflammatory cytokines TNF-α and IL-1β are elevated in patients with myocarditis (Matsumori et al. 1994), and the degree of expression closely correlates with the severity of disease (Kishimoto et al. 2001). Furthermore, TNF-α and IL-1β have been reported to be important mediators in the devel-
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NF-κB is a rapid-response transcription factor that regulates expression of genes that encode cytokines, chemokines, and adhesion molecules (Barnes and Karin 1997). NF-κB exists in the cytoplasm as a heterodimer of 50-kDa (p50) and 65-kDa (p65) subunits, and is associated with an inhibitory protein of the IkB family. NF-κB plays an important role in regulating the induction and resolution of inflammation. Upon stimulation, NF-κB is released and translocated into the nucleus, where it induces transcriptional activation of target genes, thus leading to inflammation (Sanz et al. 2010). In the cytokine network, it has been found that proinflammatory cytokines such as IL-1β and TNF-α both activate and are activated by NF-κB (Barnes and Karin 1997; Opal and DePalo 2000; Van Miert 2002; Pulai et al. 2005). NF-κB plays a pivotal role in the regulation of myocardial damage in EAM, since NF-κB blockade by infusion of an NF-κB decoy partly protects rats from the disease (Yokoseki et al. 2001). Moreover, previous studies have revealed that cardioprotective effect in EAM rats is associated with reduced proinflammatory cytokines, such as IL-1β and TNF-α, possibly through inhibition of NF-κB activation (Azuma et al. 2004; Mito et al. 2011). Furthermore, blocking NF-κB could inhibit activation of T cells (Barnes and Karin 1997), implicating a potential role of NF-κB in the development of myocarditis.

Emodin has long been used as an anti-inflammatory agent. Many studies have demonstrated that emodin could significantly decrease TNF-α (Li et al. 2005; Wu et al. 2007; Wang et al. 2010; Ha et al. 2011) and IL-1β (Meng et al. 2010; Wang et al. 2010) in inflammatory diseases. It has been suggested that suppression of NF-κB activity is involved (Li et al. 2005; Kitano et al. 2007; Wu et al. 2007; Meng et al. 2010; Wang et al. 2010). The results of the current study support the involvement of NF-κB.

Specifically, emodin significantly improved the cardiac function, decreased the Hw/Bw ratio, reduced the myocardial pathology, and suppressed the increase of IL-1β and TNF-α in EAM rats. NF-κBp65 was highly expressed in myocardial cells, and was decreased by emodin treatment. Thus, it is reasonable to speculate that the emodin exerts its anti-inflammatory effects in the EAM by inhibiting the activation of NF-κB and the subsequent activation of proinflammatory cytokines.

In conclusion, emodin significantly reduced the severity of experimental autoimmune myocarditis. The cardio-protective effects could be partially attributed to the suppression of proinflammatory cytokines TNF-α and IL-1β due to NF-κB activity inhibition. The results also suggest that emodin is a promising candidate in the treatment of acute myocarditis, and possibly other inflammation-related cardiovascular diseases.

Conflict of Interest

We declare no conflict of interest.

References


