Lipoxin A4 mitigates experimental autoimmune myocarditis by regulating inflammatory response, NF-κB and PI3K/Akt signaling pathway in mice


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Abstract. – OBJECTIVE: Myocarditis, an acute inflammation disease of the heart, is a potentially lethal disease and can lead to sudden death. This study aims to investigate the therapeutic effect of lipoxin A4 (LXA4) on experimental autoimmune myocarditis (EAM) and to explore the underlying mechanism.

MATERIALS AND METHODS: EAM was induced in BALB/c mice by injection of porcine cardiac myosin and LAX4 at doses of 10 or 50 μg/kg was administrated from day 1 to 21. The severity of myocarditis was evaluated by detection of heart weight/body weight (HW/BW) ratio and histopathological examination of the heart. Cardiac function and heart structure were assessed by echocardiography. Serum levels of Th1 and Th2 cytokines were determined by ELISA. Protein expression was detected by Western blot analysis.

RESULTS: The results demonstrated that LXA4 mitigated the severity of myocarditis by decreasing HW/BW ratio and reducing infiltration of inflammatory cells. Echocardiographic analysis indicated that cardiac function of LXA4-treated rats was significantly improved compared with non-treated group. LXA4 treatment significantly increased the levels of Th1 cytokines (TNF-α and IL-6) and decreased Th2 cytokines (IL-4 and IL-10). Furthermore, LXA4 administration effectively inhibited NF-κB nuclear translocation and deactivated PI3K/Akt pathway.

CONCLUSIONS: LXA4 has a protective effect against EAM by reducing the inflammatory response and inhibiting NF-κB and PI3K/Akt signaling pathway.

Key Words: Lipoxin A4, Experiment autoimmune myocarditis, Inflammation, NF-κB, PI3K/Akt.

Introduction

Myocarditis is defined as an inflammation of myocardium with consequent myocardial injury. It is a precursor of dilated cardiomyopathy (DCM) and represents the most common cause of chronic heart failure, or even sudden death1,2. Although the pathogenesis of myocarditis is not fully understood, there is considerable evidence indicating that autoimmunity may arise due to inefficient elimination of the pathogen resulting in overaggressive immune surveillance and/or due to antigenic mimicry between pathogenic epitopes and cardiac myosin3,4. In animals, infiltrating T lymphocytes and secreted cytokines have been shown to play prominent roles in the establishment of various autoimmune disease models such as experiment autoimmune myocarditis (EAM)4. EAM in rodent may be elicited by immunization of cardiac myosin, and EAM in rats mimics human fulminant myocarditis in the acute phase and human DCM in the chronic phase5,6. This animal model has been shown to be mediated by T cell and been particularly suitable for studying immunopathology of myocarditis. Up to now, there is no universally ac-
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Lipoxin A4 (LXA4) is the major physiological lipoxin during inflammation in mammalian systems. Lipoxin A4 (LXA4) is the major physiological lipoxin during inflammation in mammalian systems. Lipoxins, including LXA4, have been proven to demonstrate powerful anti-inflammatory function under many pathological conditions related to inflammation, including asthma, inflammatory pain, and arthritis. Based on these beneficial effects of LXA4, this study aims to investigate the therapeutic effect on EAM and to explore the underlying mechanisms in mice.

**Materials and Methods**

**Animals and Treatments**

Six-week old BALB/c mice were obtained from the Experimental Animal Center of Suzhou Aiermaite Technology Co. Ltd. (SPF grade, Certificate No. SCXK20140007, Suzhou, China). All mice were housed in the animal care facility at the Laboratory Animal Center, Provincial Hospital Affiliated to Shandong University and provided food and water ad lib, under standard condition. All of the animal procedures were approved by the Institutional Authority for Laboratory Animal Care and were performed in accordance with the Guidelines for Animal Experiments of Provincial Hospital Affiliated to Shandong University.

**Immunization**

EAM was induced in BALB/c mice by subcutaneous injection of 0.1 ml of porcine cardiac myosin (6 mg/ml, Sigma-Aldrich, St. Louis, MO, USA), mixed with an equal volume of Freund’s complete adjuvant (FCA) supplemented with Mycobacterium tuberculosis H37 Ra (Difco Laboratories, Detroit, MI, USA) on day 1 and 8. Control group (group C) was immunized with FCA alone. The animals were daily observed up to the end of the experiment. The day of injection was designed day 1. LXA4, from Cayman Chemical Company (Ann Arbor, MI, USA), was stored at -80°C until being diluted in phosphate buffered saline (PBS) immediately before use. Mice successfully induced with EAM were randomly assigned into three groups (each group n=10): non-treated group (group N), low-dose LXA4 group (10 μg/kg/day, group L) and high-dose LXA4 group (50 μg/kg/day, group H). LXA4 therapy started at the same time of immunization. The animals were administered intraperitoneally for 3 weeks from day 1 to day 21 after immunization. Non-treated EAM animals received physiological saline.

**Echocardiographic Studies**

Echocardiography was conducted on day 21 post-myosin injection to assess the cardiac function and heart structure of the mice. Mice were anesthetized with an intraperitoneal injection of 40 mg/kg sodium pentobarbital (Beijing propbs Biotechnology Co., Ltd, Beijing, China). A 12 MHz probe was placed at the left 4th intercostal space for M-mode imaging using 2D echocardiography (Sono 550, Philips, Amsterdam, The Netherlands). Left ventricular end-diastolic diameter (LVEDd), left ventricular end-systolic diameter (LVEDs), and left ventricular posterior wall thickness (LVPW) were determined using the leading-edge method. Left ventricular fractional shortening (LVFS) and left ventricular ejection fraction (LVEF) were calculated as previously described.

**Histopathology**

The body weight of mice was noted just before the surgical produce. After the echocardiographic analysis, the mice were sacrificed under sodium pentobarbital anesthesia, blood samples were obtained from abdominal aorta and the hearts were removed and weighed to calculate the ratio of HW/BW (mg/g). One portion heart was rinsed with phosphate buffered saline (PBS) and fixed in 4% paraformaldehyde and embedded in paraffin (Beijing Solarbio Science & Technology Co., Ltd, Beijing, China); the other portion was frozen at -80°C for protein studies. Embedded tissue was...
cut on a microtome (3 μm thickness) and stained with Haematoxylin and Eosin (H&E) (Sigma-Aldrich, St. Louis, MO, USA), and evaluated by light microscopy.

**Cytokine Measurement**

Enzyme-linked immunosorbent assay (ELISA) was performed to detected serum levels of TNF-α and IL-6 as representative Th1 cytokines and IL-4, IL-10 as representative Th2 cytokine on day 21 with ELISA kits (BioSource International, Camarillo, CA, USA) according to manufacture’s instructions.

**Western Blot Analysis**

Myocardial tissue samples were homogenized with lysis buffer. The protein concentrations were measured by the bicinchoninic acid assay. Proteins were denatured, separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis and transferred to a polyvinylidene difluoride (PVDF) membrane. After being blocked with 5% non-fat milk, blots were incubated with primary antibodies overnight at 4°C, and then exerted to incubation with the corresponding secondary antibodies at room temperature for 2 h. Membranes were developed with electrochemiluminescence (ECL) Western blot substrate (GE Healthcare, Buckinghamshire, UK) and Western blots were visualized on the Kodak Image Station (Carestream Health Inc., New York, NY, USA). Antibodies specific for p-IκBα, p-NF-κB p65, p-IKKα/β, IKKα, p-Akt, Akt, PI3K, p-PI3K and GAPDH were obtained from Cell signaling Technology (Danvers, MA, USA). Anti-IκBα and anti-NF-κB p65 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

**Statistical Analysis**

Data analyses were performed using one-way analysis of variance (ANOVA) followed by the Newman-Keuls t-test to isolate significantly different values. Results were expressed as means ± standard deviation (SD). p<0.05 was considered statistically significant.

## Results

### Heart Weight/Body Weight Ratio

There was no death during the study period in all the groups. As manifested in Table I, HW and BW were recorded and HW/BW ratios were calculated to assess cardiac hypertrophy. After immunization, HW/BW ratio was significantly increased in-group N compared with group C (p<0.01). In contrast, there was a significant reduction in the HW/BW ratio in the high-dose LXA4-treated group compared with the non-treated group (p<0.01).

### LXA4 Treatment Improved the Cardiac Function

The heart function and cardiac structure in all groups were evaluated by echocardiographic analysis on day 21 after immunization. As demonstrated in Figure 1, LVEDs and LVEDd were significantly enhanced (p<0.05), while LVFS was markedly impaired (p<0.01) in-group N compared with group C. However, LXA4 intervention decreased LVEDs and LVEDd and increased LVFS compared with group N. Also, LVEF was significantly decreased (p<0.01) in the group N compared with the group C, while treatment with LXA4 inhibited the decrease. These results indicated that the left ventricular dysfunction and severely altered heart structure were observed following EAM, and LXA4 treatment effectively suppressed the progression of left ventricular remodeling.

### Effect of LXA4 on Myocarditis-Affected Areas

Histopathologic analysis was performed on heart removed from experimental mice on 21 days post-immunization. As illustrated in Figure 2, the typical signs of myocarditis with severe in-

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**Table I.** HW, BW and HW/BW ratio in different groups.

<table>
<thead>
<tr>
<th></th>
<th>HW (mg)</th>
<th>BW (g)</th>
<th>HW/BW (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group C</td>
<td>131.1 ± 3.9</td>
<td>39.2 ± 1.7</td>
<td>3.34 ± 0.32</td>
</tr>
<tr>
<td>Group N</td>
<td>179.8 ± 9.8**</td>
<td>28.4 ± 1.8**</td>
<td>6.33 ± 0.71**</td>
</tr>
<tr>
<td>Group L</td>
<td>165.4 ± 1.7</td>
<td>29.2 ± 1.2</td>
<td>5.66 ± 0.43</td>
</tr>
<tr>
<td>Group H</td>
<td>149.9 ± 6.8**</td>
<td>32.1 ± 1.4**</td>
<td>4.70 ± 0.38**</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SD, n = 10; **p<0.01 vs. group C, *p<0.05, **p<0.01 vs. group N.
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Inflammatory lesions showed myocardial necrosis, degeneration and infiltration by inflammatory cells, which were observed in-group N. However, LXA4 treatment significantly attenuated the severity of the disease as evaluated by detecting the typical signs of myocarditis.

**LXA4 Treatment Regulated Th1/Th2 Cytokine Balance**

ELISA was performed to investigate the changes of Th1 and Th2 cytokine balance in different groups. As shown in Figure 3, the production of Th1-type cytokines TNF-α and IL-6 were significantly elevated, accompanied by a decrease in the production of Th2 cytokines IL-4 and IL-10 in group N compared with group C ($p<0.01$). However, LXA4 intervention significantly reversed those changes. The therapeutic effect of LXA4 on EAM might be due to its suppression of Th1 immune response and augmentation of Th2 response, as confirmed by a regulation of Th1/Th2 cytokine production.

**LXA4 Treatment Suppressed NF-κB Activation**

The NF-κB is reported to play an important role in regulating the induction of a variety of pro-inflammatory cytokines in cardiac inflammation. Therefore, we investigated whether LXA4 affected NF-κB signaling in EAM. As manifested in Figure 4, EAM indeed enhanced the phosphorylation of IκBα and NF-κB p65 as well as led to the degradation of IκBα and NF-κB p65 in the group N. However, LXA4 treatment effectively suppressed phosphorylation of both IκBα and NF-κB p65. We also evaluated the phosphorylation status of IKKα/β proteins, which are upstream of the IκB/NF-κB complex. As illustra-

![Figure 1. Echocardiography examination of experimental mice. Values are expressed as means ± SD, n=10; *$p<0.05$, **$p<0.01$ vs. group C, ***$p<0.05$, ****$p<0.01$ vs. group N. LVEDd: left ventricular end-diastolic diameter; LVEDs: left ventricular end-systolic diameter; LVFS: left ventricular fractional shortening; LVEF: left ventricular ejection fraction.](image-url)
ted in Figure 5, IKKα/β protein phosphorylation was dramatically increased in the group N and this phosphorylation was strongly inhibited by LXA4 treatment. These results indicated that NF-κB signaling pathway is affected by LXA4, which makes it a useful anti-inflammation therapy.

**LXA4 Treatment Inhibited PI3K/Akt Activation**

The PI3K/Akt pathway is reported to be important for NF-κB activation via modulation of IκBα phosphorylation and degradation\(^{17,18}\). To investigate whether LXA4-mediated inhibition of NF-κB activation involved the PI3K/Akt pathway, we examined the effect of LXA4 on PI3K/Akt activation in EAM. As demonstrated in Figure 6, the phosphorylation of PI3K and Akt in the myocardial tissue elevated significantly in the group N compared with the group C, while LXA4 treatment reversed these events.

**Discussion**

Acute myocarditis is a potentially lethal disease, and frequently precedes the development of dilated cardiomyopathy\(^{19,20}\). Although significant progress has been achieved recently, there is still much to be done to improve the therapy for this disease. In this study, we used a murine model of EAM, which mimics human myocarditis complications, as well as a variety of biomarker to monitor disease evolution. The results demonstrated that treatment with LXA4...
Lipoxin A4 mitigates experimental autoimmune myocarditis significantly attenuated the severity of EAM by regulating inflammatory response, NF-κB and PI3K/Akt signaling pathway. Myocarditis is associated with inflammation and cytokine modulation, and myocarditis was the state of some derangements of the immune system, such as T-cell dysfunction and the excessive production of detrimental cytokine. Overproduction of pro-inflammatory cytokine plays a pivotal role in the pathogenesis of myocarditis. Also, myocarditis was a CD4+ T-cell-mediated disease, and the imbalance between Th1 and Th2 is believed to be responsible for the initiation and mediation of myocarditis. LXA4 is known for its powerful anti-inflammatory function. LXA4 can suppress antigen-presenting cell functions and regulate cytokine-driven immune reactions towards Th2 responses. LXA4 treatment attenuates ischemia/reperfusion injury by modulating Th1/Th2 balance accompanied by up-regulating the level of the anti-inflammatory cytokines IL-4 and IL-10 and downregulating the level of IL-2 and TNF-α. Also, LXA4 alleviates acute rejection with a parallel shift from Th1 to Th2 responses in solid organ transplantation. In this study, the production of Th1-type cytokines (TNF-α and IL-6) was significantly increased, accompanied by a decrease in the production of Th2 cytokines (IL-4 and IL-10) in EAM rats. LXA4 treatment significantly down-regulated the increased serum levels of Th1 cytokine and up-regulated Th2 cytokine significantly in myocarditis mice. It is conceivable that LXA4 has immunosuppressive properties that result in a reduction of inflammation associated with the shift of Th1 cells towards Th2 cells, and ultimately in a reduction in autoimmunity–mediated myocardial injury. LXA4 finally suppressed the releasing of inflammatory cytokines associated with the shift of Th1 to Th2 cytokine balance. NF-κB signaling regulation is considered as an important therapeutic target for inflammatory...

Figure 3. Levels of cytokine of Th1 (TNF-α and IL-6) and Th2 (IL-4 and IL-10) evaluated by ELISA. Values are expressed as means ± SD, n = 10; *p<0.05, **p<0.01 vs. group C, #p<0.05, ##p<0.01 vs. group N.
diseases because inappropriately activated NF-κB signaling contributes to inflammatory disorders[27]. In unstimulated cells, NF-κB complex is localized and inactivated in the cytoplasm by binding to the inhibitory protein IκB[28]. IκB kinase (IKK) can phosphorylate IκB and lead to its degradation. NF-κB is then released to translocate to nucleus and activate transcription[29]. NF-κB activity has been proved to be essential for the production of pro-inflammatory cytokines in cardiac myocytes[30]. There is direct evidence that inhibiting NF-κB activity by NF-κB inhibitor can block the production of pro-inflammatory cytokines in cardiac tissues or myocytes, thus preventing the development of myocarditis[31,32]. In this study, we investigated the effect of LXA4 on the activity of NF-κB in EAM mice. Results demonstrated that LXA4 treatment reduced degradation and phosphorylation of IκBα and NF-κB p65, prevented nuclear translocation of NF-κB p65, and suppressed phosphorylation of the upstream signaling protein IKKα/β, indicating that LXA4 affected the IKK complex in the NF-κB signaling pathway. The PI3K/Akt pathway is implicated in the regulation of a series of physiological activities and can be activated by a variety of extracellular stimuli[33]. It is reported that PI3K/Akt pathway may be an important therapeutic target for many inflammatory diseases, including diovascular diseases[30,34]. The pathway in comprised of two main driving molecules: PI3K3Kinase (PI3K) and AKT. PI3K is a member of a subfamily of lipid kinases that phosphorylate the 3-hydroxyl group of phosphoinositides and involved in many physiological processes[35,36]. AKT, the downstream target of PI3K, is cytosolic protein whose translocation is induced by the binding to PI3K products and
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acts as a key protein mediator for a wide range of cellular processes\textsuperscript{37,38}. The PI3K/Akt pathway is reported to be important for NF-κB activation; it can influence NF-κB activity by promoting IKK activation and subsequent phosphorylation and degradation of IκBα, which activates the RelA/p65 subunit of NF-κB in some systems\textsuperscript{30,39,40}. In this study, Western blot analysis demonstrated that the phosphorylation of PI3K and Akt in the myocardial tissue significantly increased in the non-treated group, LXA4 treatment dramatically repressed these events. These results indicated that LXA4 alleviated EAM at least partly via modulating PI3K/Akt pathway.

**Conclusions**

The LXA4 administration greatly reduces the severity of EAM and the mechanism can be at least partly explained by removing of pro-inflammatory cytokines, regulating the transition of helper T-cell balance from Th1 to Th2, and inhibiting PI3/Akt and NF-κB signaling pathway. Therefore, LXA4 may be a promising agent for the clinical treatment of myocarditis and it represents a paradigm of immunosuppressive therapy on the immune-mediated disease.

**Ethical Approval**

The research was conducted in accordance with the Declaration of Helsinki and the United National Institutes of Health.

**Conflict of interest**

The authors declare no conflicts of interest.

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