Inhibition of NF-κB activation by a novel IKK inhibitor reduces the severity of experimental autoimmune myocarditis via suppression of T-cell activation

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Submitted 25 February 2013; accepted in final form 22 September 2013

Watanabe R, Azuma RW, Suzuki J, Ogawa M, Itai A, Hirata Y, Komuro I, Isobe M. Inhibition of NF-κB activation by a novel IKK inhibitor reduces the severity of experimental autoimmune myocarditis via suppression of T-cell activation. Am J Physiol Heart Circ Physiol 305: H1761–H1771, 2013. First published October 4, 2013; doi:10.1152/ajpheart.00159.2013.—NF-κB, which is regulated by NF-κB, is the main factor in the development of myocarditis; inflammation, inhibition of its activation may be an effective therapy for myocarditis from the standpoint of preventing inflammation.

Recently, we developed IMD-0354, a novel inhibitor of IKK (19, 34, 41). This drug is a selective IKK-β inhibitor, blocks IkBα phosphorylation, and prevents NF-κB p65 nuclear translocation. Some previous studies reported the beneficial effects of IMD-0354 and its prodrug on inflammation-related cardiovascular diseases (15, 34, 35, 42). Therefore, regulation of NF-κB activation by the IKK inhibitor might have a potent effect on the treatment of myocarditis. However, the effect of IKK inhibitor treatment on the progression of myocarditis is unknown. Thus we assessed the hypothesis that IKK inhibitor treatment attenuates cardiac inflammation in the progression of myocarditis. EAM in a rat model is characterized by severe myocardial damage and multinucleated giant cell infiltration. This has been used as a disease model for human acute giant cell myocarditis (8). We examined the effect of IKK inhibition on EAM.

METHODS

Induction of EAM. Male Lewis rats (6-wk-old; body weights 150 to 200 g) were purchased from Charles River Laboratories Japan. They were fed a standard diet and water and were maintained in compliance with animal welfare guidelines of the Institute of Experimental Animals, Tokyo Medical and Dental University. Also, protocols were approved by the Institutional Animal Care and Use Committee of the Tokyo Medical and Dental University. Purified porcine cardiac myosin (Sigma Chemical, St. Louis, MO) was emulsified with an equal volume of complete Freund’s adjuvant (Difco, Sparks, MD) supplemented with Mycobacterium tuberculosis H37RA (Disco) at a concentration of 10 mg/ml. On day 0, rats were injected in the footpads subcutaneously with 0.2 ml of emulsion, yielding an immunizing dose of 1.0 mg/body of cardiac myosin per rat (9) anesthetized by intraperitoneal administration of pentobarbital sodium (25 mg/kg; Daimihon Chemical, Osaka, Japan). We also used unimmunized (normal)
rats parallel with the diseased protocol. Unimmunized rats were injected saline instead of emulsion of myosin on day 0.

IKK inhibitor administration. The IKK inhibitor IMD-0354 was provided by the Institute of Medicinal Molecular Design. The drug was dissolved in 0.5% carboxy methyl cellulose (CMC) solution immediately before use. In the EAM phase, cardiac inflammation starts on approximately day 14, and the peak of inflammation is expected to occur on day 21 (25). For this reason, we administered the IKK inhibitor either from day 1 or from day 14, and harvested hearts and spleens on day 21. Rats were assigned randomly to five groups: 1) daily CMC intraperitoneal injection to normal rats from day 1 to day 20 [normal + vehicle group; n = 6], 2) daily IMD-0354 intraperitoneal injection (15 mg·kg⁻¹·day⁻¹) to normal rats from day 1 to day 20 [normal + IKK(i-1–20); n = 5], 3) daily CMC intraperitoneal injection to EAM rats from day 1 to day 20 [EAM + vehicle; n = 18], 4) daily IMD-0354 intraperitoneal injection (15 mg·kg⁻¹·day⁻¹) to EAM rats from day 1 to day 20 [EAM + IKK(i-1–20); n = 13], and 5) daily IMD-0354 intraperitoneal injection (15 mg·kg⁻¹·day⁻¹) to EAM rats from day 14 to day 20 [EAM + IKK(i-14–20); n = 10]. Administration dose of the IKK inhibitor was calculated from that of previous articles (17, 19, 31, 34, 35).

Echocardiogram. Transthoracic echocardiography was performed on animals anesthetized by intraperitoneal administration of pentobarbital sodium (25 mg/kg) on day 21. An echocardiography machine with a 7.5-MHz transducer (Nemio; Toshiba, Tokyo, Japan) was used for M-mode left ventricular (LV) echocardiographic recording. A two-dimensional targeted M-mode echocardiogram was obtained with a 7.5-MHz transducer (Nemio; Toshiba, Tokyo, Japan) by 10.220.33.5 on October 15, 2017 http://ajpheart.physiology.org/ Downloaded from

Histopathological examination. Hearts and spleens were harvested immediately after all rats were killed by the cutting of the abdominal aorta under anesthesia after the echocardiographic examination on day 21. After heart weights were measured, hearts were divided into apex, midventricular, and basal level slices. Apex level slice was frozen by frozen sections were incubated with primary antibodies against CD4, CD8, and CD68 in randomly selected five random

- Table 1. Echocardiographic parameters

<table>
<thead>
<tr>
<th>n</th>
<th>LV Diameter at Diastole, mm</th>
<th>LV Diameter at Systole, mm</th>
<th>Ejection Fraction, %</th>
<th>Interventricular Septal Thickness at Diastole, mm</th>
<th>LV Posterior Wall Thickness at Diastole, mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal + vehicle</td>
<td>6</td>
<td>6.3 ± 0.26</td>
<td>3.0 ± 0.17</td>
<td>89 ± 0.5</td>
<td>1.8 ± 0.16</td>
</tr>
<tr>
<td>Normal + IKK(i-1–20)</td>
<td>5</td>
<td>6.5 ± 0.21</td>
<td>3.4 ± 0.09</td>
<td>85 ± 1.4</td>
<td>1.6 ± 0.09</td>
</tr>
<tr>
<td>EAM + vehicle</td>
<td>11</td>
<td>6.0 ± 0.28</td>
<td>3.6 ± 0.33</td>
<td>75 ± 4.7</td>
<td>2.5 ± 0.20</td>
</tr>
<tr>
<td>EAM + IKK(i-1–20)</td>
<td>12</td>
<td>6.6 ± 0.15</td>
<td>4.0 ± 0.27</td>
<td>76 ± 3.9</td>
<td>1.7 ± 0.11</td>
</tr>
<tr>
<td>EAM + IKK(i-14–20)</td>
<td>7</td>
<td>6.1 ± 0.11</td>
<td>3.8 ± 0.16</td>
<td>74 ± 2.2</td>
<td>1.6 ± 0.09</td>
</tr>
</tbody>
</table>

Values are means ± SE. Early IKK inhibitor treatment [experimental autoimmune myocarditis (EAM) + IKK(i-1–20)] suppressed an increase of interventricular septal thickness at diastole and left ventricular (LV) posterior wall thickness at diastole values, whereas late IKK inhibitor treatment [EAM + IKK(i-14–20)] only suppressed an increase of interventricular septal thickness at diastole value compared with vehicle treatment [EAM + vehicle] on day 21. 

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CT (ΔΔCt) method, and the mRNA expression was normalized by normal rat hearts (28, 32).

T-cell proliferation assay. Cells were isolated from spleen in the EAM + vehicle group on day 21. Cells (5 × 10⁶/well) were cultured in 96-well culture plates with 50 μg/ml purified porcine heart myosin. The IKK inhibitor was added to each well at various concentrations. In vitro dosage of the IKK inhibitor was determined according to that in previous articles (34, 35). Cultures were incubated at 37°C under 5% CO₂ for 3 days. Cells were centrifuged at 1,200 rpm for 5 min, and the supernatants were stored at −80°C until before use. T-cell proliferation was assessed with the Cell Counting Kit-8 (Dojindo, Tokyo, Japan) with the use of cells of the precipitation. Cell proliferation was expressed as the optical density (9).

Enzyme-linked immunosorbent assay. We performed enzyme-linked immunosorbent assay (ELISA) to examine the production of Th1 cytokines from T cells using the supernatants of cell culture in previous articles (34, 35). Cultures were incubated at 37°C under 5% CO₂ for 3 days. Cells were centrifuged at 1,200 rpm for 5 min, and the supernatants were stored at 80°C until before use. T-cell proliferation assay.

Cell proliferation was assessed with the Cell Counting Kit-8 (Dojindo, Tokyo, Japan) with the use of cells of the precipitation. Cell proliferation was expressed as the optical density (9).

Statistical analysis. All data are expressed as means ± SE. Statistical analyses were performed with statistical software (Stat View; SAS Institute). Student’s t-test was used to compare data between the two groups. Data differences between multiple groups were subjected to ANOVA followed by a Bonferroni-Dunn test. Differences were considered statistically significant at a value of P < 0.05.

RESULTS

Echocardiographic parameters. On day 21, the values of LV diameter at diastole, LV diameter at systole, and EF did not show any statistical difference among all groups. Regarding IVSTd and LVPWd values, no significant difference was observed between the normal + vehicle group and the normal + IKKi(1–20) group. IVSTd values significantly increased in the EAM + vehicle group compared with the normal + vehicle group. IVSTd and LVPWd values in the EAM + IKKi(1–20) group were reduced compared with those of the EAM + vehicle group. Additionally, IVSTd value was reduced in the EAM + IKKi(14–20) group compared with that of the EAM + vehicle group, although LVPWd value was not changed. There was no significant difference of this ratio between the EAM + IKKi(14–20) group and the EAM + IKKi(1–20) group (Fig. 2).

The IKK inhibitor reduced myocardial damage characterized by cell infiltration and fibrosis in EAM. Histopathologically, the heart of the EAM + vehicle group showed severe myocarditis lesions that were composed of extensive inflammatory cell infiltration and myocardial fibrosis on day 21. However, the cell infiltration area ratios in the EAM + IKKi(1–20) group (12.9 ± 3.6%; P < 0.05) and the EAM + IKKi(14–20) group (22.5 ± 2.9%; P < 0.05) were significantly fewer than those in the EAM + vehicle group (36.5 ± 3.4%) (Fig. 3, A–C). Similarly, the fibrosis area ratios in the EAM + IKKi(1–20) group (15.8 ± 4.0%; P < 0.05) and the EAM + IKKi(14–20) group (21.4 ±...
Fig. 3. Histopathological findings. Representative low-power (magnification, ×10; scale bars = 1 millimeter; A) and high-power (magnification, ×400; scale bars = 100 μm; B) photomicrographs of hematoxylin and eosin-stained cross-sections are shown. C: quantitative data of cell infiltration area. IKK inhibitor treatment significantly suppressed cell infiltration into myocardium on day 21 [normal + vehicle, n = 5; normal + IKKi(1–20), n = 5; EAM + vehicle, n = 14; EAM + IKKi(1–20), n = 12; EAM + IKKi(14–20), n = 10]. *P < 0.05. D: representative photomicrographs (magnification, ×200; scale bars = 100 μm) of Mallory-stained cross-sections. E: quantitative data of fibrosis area. IKK inhibitor treatment significantly suppressed cardiac fibrosis on day 21 [normal + vehicle, n = 5; normal + IKKi(1–20), n = 5; EAM + vehicle, n = 14; EAM + IKKi(1–20), n = 12; EAM + IKKi(14–20), n = 10]. *P < 0.05.
The IKK inhibitor attenuated inflammatory cell infiltration in EAM hearts. After histopathological examination, we analyzed the effect of IKK inhibitor treatment on each inflammatory infiltrating cell. Hearts from the EAM + vehicle group showed massive infiltrations of CD4-positive T cells and CD68-positive macrophages, and moderate infiltration of CD8-positive T cells on day 21. However, the EAM + IKKi(1–20) group showed significant reductions of CD4-, CD8-, and CD68-positive cell numbers compared with the EAM + vehicle group (Fig. 4, A–D).

Localization of NF-κB activation in EAM. To investigate NF-κB activity in EAM, we performed immunohistochemical analysis. On day 21, NF-κB p65 was expressed in the cytoplasm of cardiac tissues from the normal + vehicle group. In contrast, strong expression of NF-κB p65 was observed mainly in the nucleus of the infiltrating cells in the myocardium as shown by arrows in the hearts from the EAM + vehicle group (Fig. 5A). On this occasion, NF-κB p65 phosphorylation (as a marker of NF-κB activation) was detected in CD4-positive T cells that infiltrated the myocardium in the EAM + vehicle group (Fig. 5B). CD4-positive T cells play a critical role in the development of EAM. The spleen is a major source of immune cells, including CD4-positive T cells. Immune cells derived from the spleen migrate to the inflammatory affected area through the bloodstream (37). Hence, we examined NF-κB activation in the spleen during the development of EAM. In the spleen obtained from the EAM + vehicle group, we observed enhanced colocalization of phospho-NF-κB p65 and CD4-positive T cells compared with that of the normal + vehicle group (Fig. 5C).

IKK inhibitor suppressed NF-κB activation in EAM hearts. To examine the effect of IKK inhibitor treatment on NF-κB activity in the heart of EAM, we analyzed nuclear NF-κB p65 protein expression, NF-κB p65 phosphorylation level, and cytosolic phospho-IκBα-to-IκB ratio (35). The EAM + vehicle group showed enhanced nuclear NF-κB p65 protein expression compared with the normal + vehicle group. However, nuclear NF-κB p65 protein expression was significantly reduced in the EAM + IKKi(1–20) group compared with the EAM + vehicle group on day 21 (Fig. 6A and B). Phosphorylation level of NF-κB p65 (as shown by phospho-NF-κB-to-total NF-κB ratio) was reduced by IKK inhibitor administration (Fig. 6C and D). Moreover, the level of cytosolic phospho-IκBα-to-IκBα

Fig. 4. Infiltrations of inflammation-related cells on day 21. A: representative CD4 staining, CD8 staining, and CD68 staining photomicrographs (magnification, ×200; scale bars = 100 μm). Quantitative results of immunohistochemistry for CD4-positive T cell [EAM + vehicle, n = 8; EAM + IKKi(1–20), n = 7; B], CD8-positive T cell [EAM + vehicle, n = 7; EAM + IKKi(1–20), n = 8; C], and CD68-positive macrophage [EAM + vehicle, n = 7; EAM + IKKi(1–20), n = 6; D] are shown. IKK inhibitor treatment reduced numbers of CD4-, CD8-, and CD68-positive infiltrating cells in EAM hearts on day 21. *P < 0.05.
IKK INHIBITOR SUPPRESSES MYOCARDITIS

A  Normal + vehicle  EAM + vehicle

B  DAPI  CD4  P-NF-kappa B p65  Merged

C  Normal + vehicle DAPI  CD4  P-NF-kappa B p65  Merged

EAM + vehicle DAPI  CD4  P-NF-kappa B p65  Merged
ratio in EAM hearts increased in the EAM + vehicle group compared with the normal + vehicle group. However, it decreased by IKK inhibitor administration (Fig. 6, E and F).

Altered inflammation-related gene expression by IKK inhibition in EAM hearts. Th1 cytokines and chemokines are known to promote myocardial inflammation during EAM (7, 11). Therefore, we examined the effect of IKK inhibitor treatment on mRNA expression of a chemokine and Th1 cytokines in hearts on day 21 using real-time RT-PCR. In the EAM + vehicle group, mRNA expressions of IFN-γ, IL-2, and MCP-1 were enhanced compared with the normal + vehicle group. However, mRNA expressions of these genes were significantly suppressed in the EAM +...
IKK(1–20) group compared with the EAM + vehicle group (Fig. 7, A–C).

Suppression of antigen-induced T-cell proliferation by IKK inhibitor treatment. Our present study revealed that NF-κB activation was observed in CD4-positive T cells in the heart and spleen during the development of EAM. Additionally, previous studies reported that EAM is transferable to other individuals via T cells derived from the spleen of EAM (21, 46). T cells activated by antigen mediate the development of EAM. Therefore, we performed a T-cell proliferation assay to examine the effect of IKK inhibitor on antigen-induced T-cell proliferation using splenocytes of EAM. As a result, T-cell proliferation was significantly increased by myosin restimulation. IKK inhibitor treatment suppressed myosin-induced T-cell proliferation in a dose-dependent manner (Fig. 8).

Effect of IKK inhibitor on the production of Th1 cytokines in vitro. It is known that Th1 cytokines released from CD4-positive T cells mediate the progression of EAM (33). Thus, to examine the effects of IKK inhibitor on the production of Th1 cytokines from T cells activated by antigen, we performed ELISA using supernatants collected from T-cell proliferation assays. In consequence, productions of IFN-γ and IL-2 were significantly increased by myosin restimulation. However, IKK inhibitor treatment suppressed the production of these Th1 cytokines in a dose-dependent manner (Fig. 9, A and B).

DISCUSSION

NF-κB is a key factor for the progression of inflammation (35, 47). Our previous articles showed that inhibition of NF-κB activation by the IKK inhibitor significantly suppressed pro-inflammatory mediators in myocardial ischemia model (34, 45). Inflammation is an essential pathological feature of acute myocarditis. Although the effectiveness of the IKK inhibitor on cardiac diseases associated with inflammation was shown, the effect of the IKK inhibitor on myocarditis remains unknown.

IKK inhibitor treatment did not change the value of EF compared with vehicle treatment on day 21. However, the EAM + IKKi(1–20) group exhibited an antihypertrophic effect, as shown by suppression of increases of LVPWd and IVSTd values. This effect was consistent with the result that IKK inhibitor treatment suppressed heart weight gain. The increased wall thickness is considered to be owing to interstitial edema associated with progression of myocardial inflammation. In contrast, the EAM + IKKi(14–20) group suppressed the increase of IVSTd value; however, it did not affect LVPWd value and heart weight gain. Earlier IKK inhibitor treatment may be more effective. The severity of histological myocardial damage characterized by cell infiltration and fibrosis was significantly reduced in both the EAM + IKKi(14–20) group and the EAM + IKKi(1–20) group compared with the EAM + vehicle group. Additionally, it should be noted that there was no significant difference regarding this effect between the EAM + IKKi(14–20) group and the EAM +
IKK inhibitor (1–20) group. We revealed that even when IKK inhibitor administration began from day 14, IKK inhibitor treatment suppressed myocardial damage and it was not inferior to the early IKK inhibitor treatment group [EAM + IKKi(1–20)]. Thus our results suggest that the treatment effect of IMD-0354 was due to the suppression of the inflammatory reaction that was caused around day 14. In a clinical setting, time lag occurs before patients are diagnosed with myocarditis and receive medical treatment. Therefore, IKK inhibitor treatment from day 14 to day 21 [EAM + IKKi(14–20)] was a trial to inspect utility as a therapeutic medicine of myocarditis. Meanwhile, there was no significant difference in echocardiographic parameters and histopathological findings between the normal + vehicle group and the normal + IKKi(1–20) group. These results suggest that the IKK inhibitor IMD-0354 does not affect normal hearts.

We observed massive infiltrations of CD4-positive T cells and CD68-positive macrophages, and moderate infiltration of CD8-positive T cells in the EAM + vehicle group hearts tissue. Infiltration cells into myocardium consist of mainly macrophages and CD4-positive T cells in EAM, and this result is consistent with those of previous studies (14, 22). These inflammatory cell infiltrations were suppressed by IKK inhibitor administration. NF-κB induces activation of inflammatory cells such as T cells and macrophages (2, 18). During the inflammatory phase of EAM, Th1 cytokines are enhanced and they mediate interaction between CD4-positive T cells and macrophages, which lead to cardiac damages (8, 14, 33). In particular, CD4-positive T cells proliferate oneself by releasing IL-2 and activate macrophages by releasing IFN-γ. Subsequently, activating macrophages damage myocardium (33). MCP-1 is known as a chemotactic factor and plays essential role in the inflammatory reaction involved in the progress of myocarditis by having an influence on the recruitment of inflammatory cells (11). Many inflammation-related genes including IFN-γ, IL-2, and MCP-1 are expressed by NF-κB (5, 43, 44), and its suppression prevents the development of EAM (11, 27, 36). Our in vivo studies revealed that mRNA expression of IFN-γ, IL-2, and MCP-1 were suppressed by IKK inhibitor treatment through inhibition of NF-κB activation in EAM heart tissues. Therefore, inhibition of NF-κB activation by IKK inhibitor treatment to suppress expression of inflammation-related genes may prevent harmful inflammatory cell infiltration in myocarditis.

In the process of EAM, CD4-positive T cells were activated by cardiac myosin and then recruited to the target organ (heart) (39). It is known that this cell play a particularly significant role in the pathophysiology of EAM. We demonstrated that NF-κB activation was observed mainly in CD4-positive T cells infiltrating the myocardium in EAM. A major source of inflammatory cells, including CD4-positive T cells, is the spleen (37). Therefore, we examined NF-κB activity in CD4-positive T cells of the spleen during EAM. We also detected NF-κB activation of CD4-positive T cells in spleens in EAM. A recent study reported that immune cells derived from the spleen reach the myocardium via the blood stream and induced inflammatory reaction during inflammation-related cardiac disease (24). Moreover, some previous studies reported that splenocytes play a role of the pathogen and transferred myocarditis (21, 26, 46). Therefore, we investigated the direct effects of the IKK inhibitor on T cells, which were activated by antigen and derived from the spleen. Our in vitro study revealed that IKK inhibitor treatment suppressed myosin induced T-cell proliferation and production of Th1 cytokines. NF-κB activation is necessary for T-cell activation, differentiation, and proliferation (4). Activated T cells secrete various cytokines and chemokines via NF-κB activation, which activate other inflammatory cells or cardiomyocytes in the myocardium and cause further recruitment of inflammatory cells (34, 48). Antigen-induced T-cell proliferation and subsequent production of Th1 cytokines from T cells are an essential response for the development of EAM (8). This evidence demonstrates that the IKK inhibitor suppressed the activation of T cells and attenuated the development of EAM. Therefore, our results suggested that IMD-0354 attenuated the severity of EAM by targeting NF-κB of T cells during the inflammatory phase in EAM.

The present study showed for the first time that the IKK inhibitor IMD-0354 reduces the severity of EAM. This effect is associated with the reduction of inflammatory-related gene expression by inhibition of NF-κB activation. Some previous studies reported that administration of pharmacological agents indirectly inhibited NF-κB activation and suppressed the progression of EAM (1, 14, 40). Therefore, the IKK inhibitor IMD-0354, which directly inhibits NF-κB activation, may be promising as a therapeutic drug for the treatment of myocarditis. It is noteworthy that the IKK inhibitor will be clinically available in the near future. IMD-1041, which is a prodrug of IMD-0354, also inhibits IKK-β in vivo and in vitro (6).
Because this compound is an investigational drug, it is not yet on the market. To prove the clinical effect of IMD-1041, we started an interventional, randomized, placebo control and double blind clinical trial. Thus the IKK inhibitor will be used to treat myocarditis and other cardiovascular diseases in clinical settings in the future. Our data clearly indicate that IKK is critical for EAM development and its inhibition has significant effects for treating myocardial inflammation. In conclusion, IKK regulation is promising in the treatment of clinical acute myocarditis.

ACKNOWLEDGMENTS

We thank Noriko Tamura and Yasuko Matsuda for excellent technical assistance.

GRANTS

This study was supported by Takeda Science Foundation, Suzuken Memorial Foundation, a grant from the Research Foundation for Pharmaceutical Sciences, and the Japan Society for the Promotion of Science through its Funding Program for World-Leading Innovative R & D on Science and Technology (FIRST Program).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: R.W., R.W.A., J.-i.S., and M.O. performed experiments; R.W. and R.W.A. analyzed data; R.W., R.W.A., J.-i.S., M.O., and M.I. interpreted results of experiments; R.W. prepared figures; R.W. drafted manuscript; R.W., J.-i.S., M.O., and M.I. edited and revised manuscript; R.W., J.-i.S., M.O., A.I., Y.H., I.K., and M.I. approved final version of manuscript.

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