Experimental Studies

**BAY 11-7082, a Nuclear Factor-κB Inhibitor, Reduces Inflammation and Apoptosis in a Rat Cardiac Ischemia-Reperfusion Injury Model**

Yong Sook Kim, PhD, Ji Su Kim, MD, Jin Sook Kwon, PhD, Myung Ho Jeong, MD, Jeong Gwan Cho, MD, Jung Chun Park, MD, Jung Chae Kang, MD, and Youngkeun Ahn, MD

**SUMMARY**

Despite development of therapeutic modalities, myocardial ischemia-reperfusion (I/R) injury remains an important cause of cardiac dysfunction. Multiple strategies exist experimentally, but few are clinically available. Nuclear factor kappa-B (NF-κB) is a key transcription factor in the inflammatory response and is implicated in I/R injury. We hypothesized that the NFκB inhibitor BAY 11-7082 (BAY) would decrease the extent of injury after myocardial I/R. Hypoxia-reoxygenation (H/R) was induced in rat neonatal cardiomyocytes with or without BAY pretreatment. NF-κB activation, vascular cell adhesion molecule (VCAM)-1, and monocyte chemoattractant protein (MCP)-1 were assayed by immunocytochemistry. Western blot or reverse transcriptase-polymerase chain reaction (RT-PCR) was performed. Sprague-Dawley rats were administered BAY (130 μg/kg) and I/R was induced by ligation of the left anterior descending artery (LAD) for 30 minutes followed by reperfusion. Infarct size was analyzed after 24 hours. At 2 weeks, echocardiography was performed to evaluate ventricular function and hearts were analyzed for fibrosis and apoptosis. BAY treatment inhibited NF-κB p65 activation, as well as VCAM-1 and MCP-1 expression induced by H/R in cardiomyocytes. Compared with control rats, BAY pretreated rats showed reduced infarct size. Echocardiograms demonstrated preserved systolic function as a fractional shortening in the BAY+I/R group (P < 0.05). Fibrosis was reduced in the BAY+I/R group (P < 0.05) and apoptosis was also reduced in the BAY+I/R group (P < 0.05).

In the rat myocardial I/R injury model, BAY significantly reduced the infarct size, and preserved myocardial function. These data demonstrate that a currently available and well-tolerated inhibitor of NF-κB can decrease the risk of myocardial injury associated with I/R. (Int Heart J 2010; 51: 348-353)

**Key words:** BAY 11-7082, NF-κB, Myocardial ischemia-reperfusion, Inflammation, Apoptosis

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The NF-κB family of transcription factors plays a central role in regulating the expression of a variety of inflammatory genes that have been linked to these cardiovascular pathologies. However, NF-κB controls not only inflammation, but also apoptosis in many cell types. Thus, therapeutic interventions aimed at inhibition of NF-κB signaling may adversely affect cell survival.

In this study, BAY 11-7082 (BAY), was examined to determine whether it contributes to control important factors that have roles in aggravating myocardial I/R injury.

**METHODS**

Rat neonatal cardiomyocyte (CM) culture: Cultured rat neonatal cardiomyocytes were treated with BAY 11-7082 (BAY) for 24 hours. NF-κB p65 expression was measured by Western blotting. Immunofluorescence microscopy showed that BAY treatment inhibited NF-κB activation, as well as VCAM-1 and MCP-1 expression induced by H/R in cardiomyocytes. Compared with control rats, BAY pretreated rats showed reduced infarct size. Echocardiograms demonstrated preserved systolic function as a fractional shortening in the BAY+I/R group (P < 0.05). Fibrosis was reduced in the BAY+I/R group (P < 0.05) and apoptosis was also reduced in the BAY+I/R group (P < 0.05).

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In this study, BAY 11-7082 (BAY), was examined to determine whether it contributes to control important factors that have roles in aggravating myocardial I/R injury.
CMs were prepared as previously described. The ventricles were obtained from 2-day-old Sprague-Dawley rats, and CMs were isolated by digestion with collagenase (Sigma-Aldrich, USA). The CMs pretreated with BAY (Calbiochem, USA) (10 nM) for 30 minutes were incubated within a sealed anaerobic bag for 5 hours, and this was followed by reoxygenation for 30 minutes. The CMs were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco-BRL, USA) containing 10% fetal bovine serum (FBS, Gibco-BRL, USA).

**Immunocytochemistry:** The CMs were fixed for 10 minutes with 2% paraformaldehyde at room temperature, washed 3 times with phosphate buffered saline (PBS), permeabilized for 10 minutes with 0.5% Triton X-100 in PBS, washed 3 times with PBS, and then incubated for 10 minutes in 1% bovine serum albumin (BSA, Sigma-Aldrich, USA) in PBS to block the nonspecific binding sites before labeling was carried out with NF-kB p65 antibody. The primary antibodies were applied for 1 hour at room temperature, and this was followed by incubation with Alexa Fluor 488 goat anti-rabbit (Molecular Probe, USA). The cell nuclei were counterstained with 4′-6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, USA). Fluorescent images were obtained using ImagePro software 5.0 (Media Cybernetics, Inc., USA).

**Reverse transcription polymerase-chain reaction (RT-PCR):** Total RNA of CMs was extracted with Trizol reagent (Invitrogen, USA) according to the manufacturer’s protocol. For total RNA, 1 μg was used as a template for cDNA synthesis at 65°C for 15 minutes, 25°C for 10 minutes, 42°C for 60 minutes, and 95°C for 10 minutes using SuperScript reverse transcriptase and followed by storage of the products at 4°C. First-strand cDNA was amplified by PCR with specific primers. The amplified PCR products were fractionated by 1.5% agarose gel electrophoresis, and the amplified product was then visualized by ultraviolet fluorescence after being stained with ethidium bromide. The cell culture medium was collected to be measured. The concentration of monocyte chemoattractant protein (MCP)-1 was quantified using a commercially available MCP-1 ELISA kit (R&D, USA) according to the manufacturer’s protocol.

**Mycocardial ischemia-reperfusion (I/R) injury in rats:** Rats were divided into two groups randomly; an I/R group (n = 7) and a BAY+I/R group (n = 7). BAY was injected into the BAY+I/R group intraperitoneally. Thirty minutes after BAY injection, I/R injury was induced in male Sprague-Dawley rats (170 ± 10 g) by ligation of the left anterior descending artery (LAD). Rats received 1 - 3 mL/kg of a 1:1 mixture [ketamine (50 mg/mL) and xylazine (23.3 mg/mL)] intramuscularly for anesthesia, and were ventilated artificially. The heart was exposed via left thoracotomy, and LAD was ligated using 4-0 MerSilk suture for 30 minutes. The heart was restored to the normal position and the chest was closed. The heart was frozen on day 1 to determine infarct size. At two weeks, echocardiography was performed and the heart was analyzed histologically. All animals were cared for according to the Guiding Principle in the Care and Use of Animals. All procedures were approved by the Chonnam National University Animal Care and Use Committee.

**Histological and immunohistochemical analysis:** One day after I/R injury, 1 mL of Evans blue dye (1.5% in PBS) was injected into the left ventricular cavity to measure the myocardial ischemic area at risk. The heart was removed and sectioned into 4 transverse slices. 2,3,5-Triphenyltetrazolium chloride (TTC, Sigma-Aldrich, USA) staining was used to assess myocardial tissue viability and determine myocardial infarct size. The tissue slices were incubated in 1% TTC PBS solution, pH 7.4, at 37°C for 20 minutes. Tissues were fixed in 10% PBS-buffered formalin overnight at 2°C-8°C.

The ischemic area at risk (unstained by Evans blue dye) and the infarcted area (unstained by TTC) were measured using AxioVision digital image processing software (Zeiss, Germany).

Two weeks after the surgery, the animals were sacrificed using an overdose of anaesthetic, and the hearts were removed and fixed with 10% buffered formalin for further analysis. Heart tissue was cut into transverse blocks and embedded in paraffin and 5 μm serial sections were cut and placed on microscope slides. Deparaffinization was performed by incubation in xylene for 5 minutes at room temperature followed by transfer into fresh xylene for an additional 5 minutes. The samples were rehydrated using sequential incubation in 100%, 95%, and 70% ethanol, for 5 minutes each at room temperature, followed by washing in distilled water and then in phosphate buffer saline for 5-10 minutes.

Apoptosis was determined using a terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) method according to the manufacturer’s protocol (Roche Applied Science, USA). The degree of fibrosis was evaluated by optical microscopy with Masson’s trichrome stain, and its area was calculated using NIH densitometry software.

**Echocardiographic study:** Two weeks after LAD ligation, two-dimensional (2-D) echocardiography was performed on rats using a Sonos 5500 ultrasonograph with a 15-MHz transducer (Philips, Andover, Massachusetts, USA). The rats were anaesthetized with 1-3 ml/kg of a 1:1 mixture of ketamine (50 mg/mL) and xylazine (23.3 mg/mL) intramuscularly. The chest of the rat was shaved. The rats were placed in a custom-made...
cradle on a heated platform in a supine or left lateral decubitus position to facilitate echocardiography. For quantification of left ventricular (LV) dimensions and wall thickness, we recorded 2-D clips and M-mode images in a short axis view from the mid-left ventricle at the tips of the papillary muscles.

The end-diastolic and end-systolic LV diameter and posterior wall thickness were measured on M-mode images using the leading-edge-to-leading edge convention. LV fractional shortening (FS) and mass were calculated from LV wall thickness and diameter (LVD) in the 2-D short axis view as: FS with formula \[
\frac{LVD_{\text{diastole}} - LVD_{\text{systole}}}{LVD_{\text{diastole}}} \times 100,
\]
and LV mass with formula \[
[1.04(LVID+PWT+IVST)^3 - LVID^3] \times 0.8 + 0.6,
\] where LVPW is left ventricular posterior wall thickness and IVS is interventricular septum thickness.

Statistical analysis: The data are presented as the mean ± SD. Differences were analyzed by analysis of variance (ANOVA).

Results

Hypoxia-reoxygenation (H/R)-induced expressions of vascular cell adhesion molecule (VCAM-1) and monocyte chemoattractant protein (MCP-1) were suppressed by BAY in cardiomyocytes (CMs): The effects of BAY on the H/R induced expressions of adhesion molecules and cytokines at the transcription level in the CMs were assessed by RT-PCR. The transcription levels of VCAM-1 and MCP-1 were very low in the CMs and were significantly increased in the H/R group. Pretreatment with BAY decreased the expression of VCAM-1 mRNA and MCP-1 mRNA (Figure 1A).

MCP-1 and VCAM-1 proteins released from CMs were assayed at 24 hours after H/R induction by ELISA and Western blot. CMs from the control group exhibited the basal level of MCP-1 expression. The released MCP-1 protein was significantly higher by about 7-fold in the H/R group than in the control group. Pretreatment with BAY significantly reduced the MCP-1 release by 42.9% over the H/R group (Figure 1B). VCAM-1 protein was increased in the H/R group, while it was reduced in the BAY+H/R group (Figure 1C), suggesting that BAY blocked the H/R-mediated VCAM-1 protein expression.

NF-κB translocation by hypoxia-reoxygenation (H/R) was inhibited by BAY in cardiomyocytes (CMs): NF-κB p65 in the cytosol fraction of CMs was increased in the H/R group while it was reduced in the BAY+H/R group (Figure 1D). IκB is known to bind to NF-κB to inhibit its activity, and when it is degraded, NF-κB is released to be activated and translocated to the nucleus. Reduced IκB by H/R was restored by BAY treatment in the CMs (Figure 1D). To confirm the inhibitory effect of BAY on NF-κB activation, immunocytochemistry was performed.
formed in the CMs. The CMs were insulted by H/R. As seen in Figure 1E, the NF-κB p65 was stained with green fluorescence. In the CMs, the NF-κB p65 was present in the cytosol in a diffuse manner. The NF-κB p65 translocated to the nucleus, which was also stained with DAPI in the H/R CMs, where it abided in the perinuclear cytosol in the BAY+H/R CMs (Figure 1E).

**Infarct size was reduced by BAY in ischemia-reperfusion (I/R) injured heart:** One day after I/R injury, there was no difference in the myocardial ischemic area at risk between the I/R and BAY+I/R groups. However, there was a significant reduction in the infarct area in the BAY-treated group. Compared with the I/R group, BAY reduced the area of infarct without a difference in the area at risk (Figure 2A).

**Cardiac fibrosis and apoptosis were reduced by BAY in I/R injured heart:** The area of fibrosis in each myocardial region was measured from the optical microscope picture of Masson’s Trichrome staining. Blue represents collagen deposits and quantitative image analysis revealed a marked decrease in fibrosis in the BAY+I/R group compared with the I/R group (Figure 2B).

The number of TUNEL-positive CMs was significantly reduced in the BAY-I/R group compared with the I/R group. Statistically significant differences were observed in the histopathological examination of heart tissue sections in the I/R control and I/R + BAY group (Figure 2C).

**Cardiac function after I/R injury was preserved in BAY-treated rats:** Echocardiograms were obtained after 2 weeks to determine whether the BAY group was capable of LV performance in post-MI hearts. The left ventricular diameter at end-diastole
(LVDd), left ventricular diameter at end-systole (LVDs), interventricular septum thickness (IVS), left ventricular posterior wall thickness (LVPW), and the fractional shortening (FS) were all calculated. FS was expressed as a graph in Figure 3A, and the representative M mode echocardiograms are shown in Figure 3B. The echocardiographic findings such as the IVS, LVDd, LVDs, LVPW, and FS are summarized in the Table. The IVS was lower in I/R (1.36 ± 0.15 mm) than in normal rats (1.40 ± 0.14 mm). The LVDd was greater in I/R (8.32 ± 0.58 mm) than in normal rats (7.81 ± 0.71 mm) and the LVDs was greater in I/R (6.39 ± 0.71 mm) than in normal rats (4.65 ± 0.92 mm). The LVPW was smaller in I/R (1.34 ± 0.14 mm) than in normal rats (1.45 ± 0.07 mm). Fractional shortening was significantly lower in the I/R (23.34 ± 4.04%) than in normal rats (40.65 ± 6.43%). There was no significant difference in the LVDd between the groups of rats. The results showed that there was a significant decrease in LVDs and a significant increase in LVPW in the BAY-treated I/R group. There was, however, significantly improved systolic LV function in the BAY treated group. It is readily apparent that contractile performance and LV chamber geometry are better preserved in the BAY treated I/R heart than in the I/R heart.

**Discussion**

Reperfusion injury is associated with an inflammatory cascade that perpetuates further damage to cardiac tissue after a period of ischemia. One of the central players upregulated during this process is the transcription factor nuclear factor kappa B. NF-κB regulates the expression of numerous inflammatory mediators, including interleukins, cytokines, and cell adhesion molecules. Although this has not yet been conclusively demonstrated, the central role of NF-κB makes it an appealing focus for intervention. But the inhibition of NF-κB also carries a significant risk of such adverse consequences as potentiation of apoptosis or unintentional disruption of signaling in the nontarget tissues. Many drugs commonly used in cardiovascular medicine, such as aspirin and statins, have intrinsic anti-NF-κB properties. These drugs are generally weak antagonists and inhibit central kinase in the classic NF-κB activation pathway. Concerns thus exist regarding the po-
potential of such potent anti-NF-κB therapies to cause unforeseen consequences. Not all downstream effects of inhibitory κB kinase (IKK)/β inactivation may prove cardioprotective. Previous studies suggest that a basal expression of NF-κB is required to prevent apoptosis in cardiomyocytes following ischemic insults. 9,17

In the present study, the effect of BAY was examined with two protocols. In rat neonate cardiomyocytes, BAY pretreatment was found to significantly lower the H/R induced elevation of expression of VCAM-1 and MCP-1 and NF-κB activation. In the in vivo study, LV function improved significantly in the BAY + I/R group. BAY also significantly reduced infarct size, myocardial apoptosis, and fibrosis after I/R. Apoptosis represents a potentially preventable form of cell death because of its active nature, and understanding this genetic cell death pathway may lead to therapeutic strategies for altering myocardial injury by inhibiting apoptosis.

This study has several limitations. The mechanism of BAY that prevents the expression of adhesion molecules remains to be elucidated. Taken together, BAY inhibited the expression of inflammatory genes associated with I/R injury. These data suggest the possibility that BAY could be used or developed as a useful drug for modulating cardiovascular disorder. BAY clearly attenuated the inflammatory response, apoptosis, and cardiac fibrosis. Most importantly, it reduced I/R induced FS reduction in vivo.

This study has demonstrated that BAY, a commercially available inhibitor of NF-κB, mediates its therapeutic effects possibly through suppression of the NF-κB activation pathway.

REFERENCES