IKKβ inhibition attenuates myocardial injury and dysfunction following acute ischemia-reperfusion injury

Nancy C. Moss, William E. Stansfield, Monte S. Willis, Ru-Hang Tang, and Craig H. Selzman

Departments of Surgery and Pathology and Laboratory Medicine, University of North Carolina, Chapel Hill, North Carolina

Submitted 5 July 2007; accepted in final form 2 August 2007

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-κB (NF-κB) (19, 32). NF-κB regulates the expression of numerous inflammatory mediators, including interleukins, cytokines, and cell adhesion molecules (2). NF-κB exists in its heterodimer state of the Rel protein family subunits p65 and p50. It remains inactive in the cell cytoplasm while bound to its repressor, inhibitory κBα (IkBα). Reactive oxygen species, cytokines, and shear stress, resulting from reperfusion injury, stimulate NF-κB via proximal kinase activation. The IkB kinase (IKK) complex is composed of three subunits: IKKγ, IKKα, and IKKβ. In the classical pathway, the IKKβ subunit is primarily responsible for the phosphorylation of IkBα at the Ser52 and Ser56 residues (7, 8) and is upregulated during myocardial reperfusion (13). Once phosphorylated, IkBα is targeted for polyubiquitination and degradation by the 26S proteasome. After IkBα is degraded, NF-κB is released, translocates to the nucleus, and stimulates transcription of its inflammatory gene targets (32).

The activation of NF-κB following myocardial ischemia-reperfusion (IR) has been documented previously (11, 16), and its inhibition has been shown to be cardioprotective (5, 18, 26). Unfortunately, the clinical applicability of many NF-κB inhibitors remains unresolved. Several inhibitors, such as adenosine and N-acetylcysteine, are nonspecific (10, 12). Others involve myocardial injections or overexpression vectors (3, 30), the delivery of which would be difficult in the clinical setting of acute coronary syndrome. In this study, we examined the role of IKKβ inhibition in acute myocardial IR injury with the novel compound Bay 65-1942. Through competitive inhibition of ATP at the IKKβ subunit, Bay 65-1942 prevents the phosphorylation of IkBα by the IKK complex (37). We investigated whether specific inhibition of the IKKβ subunit would provide cardioprotection in a murine model of acute myocardial IR injury. Additionally, we examined the effect of delayed IKKβ inhibition on infarct size in this acute IR model, a question directly applicable to the clinical setting of reperfusion injury in acute coronary syndrome.

MATERIALS AND METHODS

Reagents. The IKKβ inhibitor Bay 65-1942 [7-[2-(cyclopropylmethoxy)-6-hydroxyphenyl]-5-[(3S)-3-piperidinyl]-1,4-dihydro-2H-pyrido[2,3-d][1,3]oxazin-2-one hydrochloride] was generously provided by Dr. Albert Baldwin, University of North Carolina. Immediately prior to use, Bay 65-1942 was dissolved in a solution of 10% methoxy)-(6-hydroxyphenyl)-5-[3-(cyclopropylmethoxy)-6-hydroxyphenyl]-5-[(3S)-3-piperidinyl]-1,4-dihydro-2H-pyrido[2,3-d][1,3]oxazin-2-one hydrochloride.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
cremaphor in water. Previous pharmacokinetic testing of Bay 65-1942 (37) demonstrated that dosages in mice of 2 mg/kg intravenously and 10 mg/kg orally had moderate clearance rates and desirable pharmacokinetic profiles. Therefore, we administered an intraperitoneal injection within that dosing range of 5 mg/kg at appropriate dosing time points. Nontreatment groups received a vehicle of 10% cremaphor in water.

**Experiment design.** To investigate IKKβ inhibition in myocardial IR injury, mice were subjected to 30 min of cardiac ischemia followed by varying periods of reperfusion (Fig. 1). In treatment groups, Bay 65-1942 was delivered either prior to ischemia, at the time of reperfusion, or 2 h after reperfusion injury. Infarct size was measured 24 h after reperfusion injury in sham, vehicle, and each treatment group. To confirm myocardial injury, serum creatine kinase-muscle-brain fraction (CK-MB) levels were measured 1 h after reperfusion in animals pretreated with Bay 65-1942. To elucidate the effect of IKKβ inhibition on the NF-κB pathway, ELISA and Western blot analysis were performed on NF-κB-associated proteins. Hemodynamic data were recorded 3 days after the initial IR insult. The University of North Carolina Institutional Animal Care and Use Committee approved all animal protocols. All animals were acclimated to the environment at least 7 days prior to experimentation in a Division of Laboratory Animal Medicine approved facility, which included 12-h light cycling and food and water access ad libitum.

**Surgical procedures.** Male C57BL/6 mice, 8–10 wk of age (Charles River Laboratories, Wilmington, MA), were anesthetized with a mixture of ketamine-xylazine (50 mg/kg dose and 1.5 mg/kg dose) and 1% inhaled isoflurane. Mice were intubated and maintained on a Harvard rodent volume-cycled ventilator at a volume of 300 μl, with a rate of 125 cycles/min. A left thoracotomy was performed, the pericardium retracted, and direct visualization of the left anterior descending (LAD) artery obtained. The LAD artery was occluded 1–2 mm below the left atrium using an 8-0 prolene suture tied over a 2-mm piece of polyethylene (PE)-10 tubing. Electrocardiogram changes were monitored and pallor of the left ventricle observed to document ischemia. After the desired 30-min ischemic period, the PE tubing was removed to allow for reperfusion. The chest was closed and the mouse extubated once awakened from anesthesia. Sham animals underwent placement of an 8-0 prolene stitch around the LAD artery without placement of the PE-10 tubing. Temperature was monitored with a rectal probe and maintained at 37°C with a heating pad and heat lamp.

Hemodynamic parameters were obtained 3 days following IR using a 1-Fr pressure-volume Millar catheter (PVR-1045; Millar Instruments, Houston, TX) in a closed-chest technique. Mice were anesthetized with an ip injection of ketamine-xylazine (100 and 1.5 mg/kg dose). The mice were intubated to control for respiratory variation during pressure-volume analysis. Bilateral vagotomy was performed to decrease parasympathetic innervation to the heart, avoiding episodes of extreme bradycardia. Following right carotid artery cannulation, the catheter was advanced into the left ventricle. Animal temperature was monitored and maintained at 37°C while pressure-volume loops were recorded with Labview 7.1 software (National Instruments, Austin, TX). Pressure-volume data were then analyzed with PVAN (Millar Instruments). For volume calibration, a parallel conductance coefficient was calculated for each animal group following injection of 15 μl of hypertonic saline into the left external jugular vein. Ex vivo cuvette calibration was also performed with heparinized murine blood samples for system calibration (9).

**Infarct size and area at risk assessment.** Twenty-four hours following reperfusion injury, animals were killed and their hearts excised. After removal of the left atrium, the aorta was cannulated with a blunt 22-gauge needle and flushed with 1 ml of PBS. The LAD artery was ligated at the site of prior occlusion. A saline solution of 0.25% fluorescent polymer microspheres was then perfused through the aorta and into the coronary arteries (35). After the hearts were frozen for ≥20 min, left ventricles were sectioned into 1-mm slices along the short axis from the site of LAD occlusion to the apex. Cross-sections were then incubated in 1% triphenyltetrazolium chloride (TTC) for 20 min. After incubation with TTC, viable myocardium appears dark red whereas infarcted myocardium is white. A 450-nm UV lamp was used to illuminate the fluorescent beads, outlining the area of LAD distribution, defined as the area at risk (AAR). Infarct area, left ventricular area, and AAR were then measured and analyzed using Image J (National Institutes of Health) analysis software. Infarct size is presented as a percentage of AAR (infarct:AAR).

**Western blot analysis.** Left ventricular samples were homogenized in whole cell lysis buffer (Cell Signaling Technology, Danvers, MA) with additional phosphatase and protease inhibitors. Left ventricular homogenates were then centrifuged at 18,000 g for 30 min at 4°C. Protein concentration was measured in the supernatants using the Bradford assay (Bio-Rad Laboratories, Hercules, CA). Samples were transferred to polyvinylidene fluoride membranes following separation with SDS-PAGE in phosphate-buffered saline containing 1 mmol/l EDTA, 0.5% Triton X-100, 1 mmol/l phenylmethylsulfonyl fluoride, and protease inhibitors. Membranes were incubated in 5% milk, Tris-buffered saline-Tween buffer, and either phosphorylated (phospho) p65 (Ser536), phospho-IκBα (Ser276), or β-tubulin (Cell Signaling Technology) primary antibodies. Goat anti-rabbit IgG secondary antibody was used to identify binding of the primary antibody. An Invitrogen kit (Invitrogen, Carlsbad, CA) was used to visualize protein bands. Densitometry was performed using Image J software. To standardize densitometry measurements between individual samples, the ratios of phospho-IκBα or phospho-p65 to β-tubulin were calculated for statistical analysis.

**Serum TNF-α and IL-6 levels.** Cytokines, TNF-α, and IL-6, were measured in serum using respective ELISA kits (R&D Systems, Minneapolis, MN). Plates were read with a spectrophotometer (Wallac 1420 VICTOR; PerkinElmer, Waltham, MA) at 560-nm and 450-nm absorbance. Using kit standards, the standard curve for each cytokine was calculated according to R&D protocol and concentration of TNF-α and IL-6 determined in pg/ml of serum.

**Statistical analysis.** Statistical comparisons were performed using an unpaired, two-tailed t-test with Welch’s correction with the statistical package Prism 4 (GraphPad, San Diego, CA). Data are expressed as means ± SE. To determine statistical significance of Western blot densitometry, a one-way ANOVA test was performed with a subsequent Dunnett’s test, comparing individual treatment and sham groups with the control group. Statistical significance was accepted at the 95% confidence interval.
IKKβ inhibition decreases size of infarction. Delivery of Bay 65-1942 prior to ischemia significantly decreased left ventricular infarct size compared with animals receiving vehicle (Fig. 2A). Compared with sham animals, animals receiving vehicle had a significant increase in the infarct-to-AAR ratio (70.7 ± 3.4 vs. 58.3 ± 3.4%, P < 0.05). As shown in Fig. 2B, this ratio was significantly reduced by treatment with Bay 65-1942 at each time point (prior to ischemia 42.7 ± 4.1%, at reperfusion 42.7 ± 7.5%, 2 h of reperfusion 29.4 ± 5.2%; each group P < 0.05 vs. vehicle). The differences in this ratio between those pretreated with the IKKβ inhibitor and those that received the inhibitor in a delayed fashion were not significant. No significant differences in AAR existed between sham, vehicle, and treatment groups (data not shown).

IKKβ inhibition attenuates myocardial injury. Myocardial injury was assessed with the measurement of serum CK-MB levels 1 h following reperfusion. The CK-MB fraction was significantly elevated in the vehicle group (n = 3) compared with the sham group (n = 4) (30,530 ± 371.2 vs. 9,675 ± 608.4 units, P < 0.05). Animals pretreated with Bay 65-1942 (n = 3) had significantly attenuated CK-MB levels compared with those animals without treatment prior to IR (14,170 ± 3,219 units, P < 0.05 vs. vehicle).

IKKβ inhibition preserves cardiac function. We assessed cardiac function by comparing pressure-volume recordings in mice at baseline and following IR with or without Bay 65-1942 (Table 1). Bay 65-1942 administration alone did not improve myocardial function above baseline hemodynamic parameters. Ejection fraction (EF) and dP/dt (the first derivative of left ventricular pressure) were significantly lower in the mice that underwent 30 min of LAD occlusion followed by 3 days of reperfusion when compared with the baseline group and the group administered Bay 65-1942 without surgery. The treatment group had a significantly improved EF and dP/dt from the IR with vehicle group, whereas no difference existed when compared with the baseline groups.

IKKβ inhibition decreases NF-κB-associated protein expression. Western blots on left ventricular homogenates from sham, vehicle, and pretreatment groups (n = 3 for each group) killed 30 min and 1 h after reperfusion were performed to observe the effects of IKKβ inhibition on the NF-κB pathway (Fig. 3). Expression of phospho-IκBα, the direct downstream product of IKKβ activation, was significantly elevated in vehicle animals compared with sham animals 30 min after reperfusion (P < 0.05). This difference between sham and vehicle groups was statistically lost 1 h following reperfusion, suggesting that IKKβ activation is at its height within 1 h of reperfusion injury. Animals treated with Bay 65-1942 had lower levels of phospho-IκBα expression compared with the vehicle group at both 30 min and 1 h following reperfusion (P < 0.05, Bay 65-1942 vs. vehicle).

Similarly, we evaluated phospho-p65 expression, the active subunit of NF-κB. Compared with sham animals, phospho-p65 was increased in the left ventricles of the vehicle group both 30

Table 1. Hemodynamic parameters of mice after IR injury with and without IKKβ inhibition

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>IR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle (n = 4)</td>
<td>Bay (n = 5)</td>
</tr>
<tr>
<td>Body Weight, g</td>
<td>25.25 ± 0.7500</td>
<td>23.60 ± 0.4000</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>440.8 ± 19.65</td>
<td>406.7 ± 26.89</td>
</tr>
<tr>
<td>LV dP/dt max, mmHg/s</td>
<td>8,396 ± 634.3</td>
<td>9,095 ± 781.5</td>
</tr>
<tr>
<td>LV dP/dt min, mmHg/s</td>
<td>-7,006 ± 523.6</td>
<td>-7,663 ± 522.1</td>
</tr>
<tr>
<td>LV EDP, mmHg</td>
<td>14.23 ± 4.331</td>
<td>8.840 ± 1.638</td>
</tr>
<tr>
<td>LVEF, %</td>
<td>58.40 ± 2.729</td>
<td>56.15 ± 4.509</td>
</tr>
</tbody>
</table>

Values are means ± SE. IR, ischemia-reperfusion; LV dP/dt max, maximal derivative of left ventricular pressure; LV dP/dt min, minimal derivative of left ventricular pressure; LV EDP, LV end-diastolic pressure; LVEF, LV ejection fraction. Baseline: mice without IR injury; IR: mice that underwent 30 min of left anterior descending occlusion 3 days prior to pressure-volume analysis; Bay mice were administered Bay 65-1942 either 3 days prior to measurements (baseline) or 30 min prior to LAD occlusion (IR). *P < 0.05, IR vehicle versus baseline vehicle; †P < 0.05, IR vehicle versus baseline Bay; ‡P < 0.05, IR Bay versus IR vehicle.
NF-κB activity and are nonspecific inhibitors. The cardiac proteasome, for example, has multiple roles in the cardiomyocyte involving the regulation of proteins in several pathways (25). In this study, therefore, we used a very targeted approach to blocking NF-κB activity.

Our results corroborate and expand upon existing studies of NF-κB inhibition and myocardial IR. Only one other group has evaluated IKKβ inhibition and cardiac IR, using the inhibitor IMD-0354 (21). Although efficacious when delivered during ischemia, IMD-0354 failed to decrease infarct size with daily delivery following myocardial infarction (22). The affinity of Bay 65-1942 for IKKβ over IKKα is greater than 50-fold despite the 50% sequence homology between the two IKK subunits. As such, it has a significant advantage over other NF-κB inhibitors in its specific suppression of one kinase critical to the classical activation of the NF-κB pathway. Bay 65-1942 has previously been shown to inhibit the release of LPS-induced TNF-α in mice and rats as well as decrease the amount of migrating eosinophils and neutrophils in a rat model of asthma (37). Furthermore, no sign of organ damage occurred following administration to rodents for several weeks (37). Most impressively, compared with IMD-0354 and other

**DISCUSSION**

In the present study of acute IR, we have demonstrated that targeted NF-κB blockade by inhibiting IKKβ decreased myocardial injury and preserved cardiac function. These findings were associated with decreased myocardial expression of phospho-IκBα and phospho-p65 as well as downstream elaboration of prototypical cytokines. In addition to showing the effects of a novel and specific NF-κB inhibitor on IR, we demonstrate for the first time that NF-κB antagonism can successfully limit myocardial injury even when delivered after reperfusion.

Multiple studies have examined the cardioprotective properties of NF-κB inhibition, targeting various portions of the NF-κB pathway. Intramyocardial injections of peptides PR-39 and PR-11, gene transfer of IκBα, and neutrophil depletion have all limited NF-κB activation following cardiac reperfusion injury (3, 10, 30). Similarly, we have previously shown that proteasome inhibition with the compound PS-519 blocks NF-κB activation and reduces infarct size in our murine model of IR (31). However, these strategies indirectly influence
NF-κB inhibitors, Bay 65-1942 provided cardioprotection even when delivered with a single systemic dose 2 h after reperfusion.

Our study compares favorably with previously evaluated nonspecific NF-κB inhibitors that have shown translational promise and proven clinical efficacy. For example, successful adenosine infusions in canine IR models decreased the infarct-to-AAR ratio compared with controls (20). Subsequent clinical trials of intracoronary adenosine infusion (14) improved functional outcomes in patients receiving percutaneous coronary artery angioplasty within 3 h of acute myocardial infarction. Additionally, large clinical trials such as the Fourth International Study of Infarct Survival (1) led to current guidelines that angiotensin-converting enzyme (ACE) inhibitors be administered to certain patients within 24 h of acute myocardial infarction. Interestingly, and compared with our 40% reduction in infarct size, original animal studies with ACE inhibition in myocardial IR (6) documented a decrease of only 24%. Furthermore, these studies utilized intracoronary drug delivery that usually started at reperfusion and continued for 1 h. In our model, we achieved favorable results with a single systemic dose of IKKβ inhibitor that was successful even when administered up to 2 h after the reperfusion event.

Many drugs commonly used in cardiovascular medicine, such as aspirin and statins, have intrinsic anti-NF-κB properties (24, 34). These drugs are generally weak antagonists and are highly nonspecific. Bay 65-1942 is a strong inhibitor of the central kinase in the classic NF-κB activation pathway. Concerns thus exist regarding the potential of such potent anti-NF-κB therapies to cause unforeseen consequences. Not all downstream effects of IKKβ inactivation may prove cardio-protective. Previous studies (17, 27) suggest that a basal expression of NF-κB is required to prevent apoptosis in cardiomyocytes following ischemic insults. Although IKKβ inhibition with Bay 65-1942 has yet to demonstrate significant experimental toxicity, we acknowledge the risk of blocking this component of NF-κB activation. At the doses used, Bay 65-1942 does not suppress NF-κB activation completely but quite possibly blocks excessive disregulation that occurs with disease. Future investigation of possible apoptotic pathways affected by pharmacological IKKβ inhibition in myocardial IR will be revealing, especially as it pertains to models of chronic left ventricular remodeling following infarction.

To further support the clinical relevance of IKKβ inhibition with Bay 65-1942, delivery of Bay 65-1942 up to 2 h after reperfusion significantly reduced infarct size. Although delayed delivery of NF-κB inhibitors has been well studied in stroke models (23, 33, 36), the benefit of delayed NF-κB inhibitors in myocardial reperfusion injury is unknown. Acute coronary events are unpredictable. In the clinical realm, patients present either during or after the acute ischemic insult, and the exact time of reperfusion is not always predictable. This point is particularly critical in successfully translating basic science into promising, clinically relevant therapy (4). Delivery of NF-κB inhibition prior to the insult of IR may elucidate the mechanism of action; however, an inhibitor with a therapeutic window beyond the time of injury is especially applicable in the treatment of acute coronary syndrome.

ACKNOWLEDGMENTS

We are appreciative of the assistance provided by Margaret Cloud and Dr. Mauricio Rojas.

GRANTS

This study was supported by grants from the University of North Carolina and the American College of Surgeons.

REFERENCES


