Title: Inhibitory Kappa B Kinase Beta (IKKβ) Inhibition Attenuates Myocardial Injury and Dysfunction Following Acute Ischemia-Reperfusion Injury

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Running Head: IKKβ Inhibition and Myocardial IR

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ABSTRACT

Despite years of experimental and clinical research, myocardial ischemia-reperfusion (IR) remains an important cause of cardiac morbidity and mortality. The transcription factor, nuclear factor-kappa B (NF-κB), has been implicated as a key mediator of reperfusion injury. Activation of NF-κB is dependent upon the phosphorylation of its inhibitor, IκBα, by the specific inhibitory kappa B kinase (IKK) subunit, IKKβ. We hypothesized that specific antagonism of the NF-κB inflammatory pathway through IKKβ inhibition reduces acute myocardial damage following ischemia-reperfusion injury. C57BL/6 mice underwent left anterior descending (LAD) artery ligation and release in an experimental model of acute IR. Bay 65-1942, an ATP-competitive inhibitor that selectively targets IKKβ kinase activity, was administered intraperitoneally either prior to ischemia, at reperfusion, or 2 hours after reperfusion. Compared with untreated animals, mice treated with IKKβ inhibition had significant reduction in left ventricular infarct size. Cardiac function was also preserved following pre-treatment with IKKβ inhibition. These findings were further associated with decreased expression of phosphorylated-IκBα and phosphorylated-p65 in myocardial tissue. In addition, IKKβ inhibition decreased serum levels of TNF-α and IL-6, two prototypic downstream effectors of NF-κB activity. These results demonstrate that specific IKKβ inhibition can provide both acute and delayed cardioprotection and offers a clinically accessible target for preventing cardiac injury following ischemia-reperfusion.

Keywords
myocardial infarction, nuclear factor-κB, reperfusion injury
INTRODUCTION

Coronary heart disease is responsible for nearly one of every five deaths in the United States. Survivors of acute coronary syndromes are 15 times more prone to develop additional cardiovascular morbidity and mortality (28). These outcomes persist despite three decades of experimental and clinical research. Successful treatment strategies for acute myocardial infarction, including systemic thrombolitics or percutaneous coronary artery angioplasty, are specifically directed toward the ischemic source of myocardial damage. The associated reperfusion injury, however, has remained an elusive therapeutic target. Thousands of animal studies have been performed investigating pharmacological and procedural methods to limit myocardial injury after ischemic events, yet few have been reproducible or shown clinical promise (4). As such, a continued need exists to develop cardioprotective therapies for reperfusion injury that can be translated successfully into clinical practice.

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) (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 kappa B alpha (IκBα). Reactive oxygen species, cytokines, and shear stress, resulting from reperfusion injury, stimulate NF-κB via proximal kinase activation. The inhibitory kappa B 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 IκBα at the serine 32 and 36 residues (7, 8), and is upregulated during myocardial reperfusion (13). Once phosphorylated, IκBα is targeted for polyubiquitination and degradation by the 26S
proteasome. After IκBα 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 previously documented (11, 16), and its inhibition 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 non-specific (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 IκBα by the IKK complex (37). We investigated whether specific inhibition of the IKKβ subunit would provide cardioprotection in a murine model of acute myocardial ischemia-reperfusion 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 Albert Baldwin, PhD, University of North Carolina. Immediately prior to use, Bay 65-1942 was dissolved in a solution of 10% cremaphor in water. Previous pharmacokinetic testing of Bay 65-1942 demonstrated that dosages in mice of 2 mg/kg intravenously and 10 mg/kg orally had moderate clearance rates and desirable pharmacokinetic profiles (37). We therefore administered an intraperitoneal (ip) injection within that dosing range
of 5 mg/kg at appropriate dosing time-points. Non-treatment groups received a vehicle of 10% cremaphor in water.

*Experiment Design.* To investigate IKKβ inhibition in myocardial ischemia-reperfusion injury, mice were subjected to 30 minutes of cardiac ischemia followed by varying periods of reperfusion (Figure 1). In treatment groups, Bay 65-1942 was delivered either prior to ischemia, at the time of reperfusion, or 2 hours after reperfusion injury. Infarct size was measured 24 hours after reperfusion injury in sham, vehicle, and each treatment group. To confirm myocardial injury, serum creatine kinase-MB fraction (CK-MB) levels were measured 1 hour after reperfusion in animals pre-treated 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 was recorded 3 days after the initial ischemia-reperfusion insult. The UNC 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-hour light cycling, and food and water access ad libitum.

*Surgical Procedures.* Male C57BL/6 mice, 8 to 10 weeks 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 microliters with a rate of 125 cycles/min. A left thoracotomy was performed, the pericardium retracted, and direct visualization of the LAD artery obtained. The LAD 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. EKG changes were monitored and pallor of the left ventricle observed to document ischemia. After
the desired 30-minute 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 without placement of the PE 10 tubing. Temperature was monitored using 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 (PV) Millar catheter (PVR-1045, Millar Instruments, Houston, TX) in a closed-chest technique. Mice were anesthetized with an ip injection of ketamine-xylazine (100mg/kg/dose 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 was then analyzed with PVAN (Millar Instruments). For volume calibration, a parallel conductance coefficient \( V_p \) was calculated for each animal group following injection of 15 microliters 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. 24 hours following reperfusion injury, animals were sacrificed and their hearts excised. After removal of the left atrium, the aorta was cannulated with a blunt 22-guage needle and flushed with 1 ml of PBS. The LAD was ligated at the sight 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 freezing the hearts for at least 20 minutes, 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 minutes. After incubation with TTC, viable myocardium appears dark red while 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 (NIH, USA) analysis software. Infarct size is presented as a percentage of AAR (infarct:AAR).

**Creatine Kinase-MB Fraction Analysis.** Prior to sacrifice, animals were bled with the facial vein technique. Samples were collected in pediatric serum separator tubes (Becton Dickinson, Franklin Lakes, NJ) and centrifuged at 1300g for 10 minutes at 4°C. Serum was then stored at -20°C. Using a Vitros 250 Chemistry System (Ortho-Clinical Diagnostics, Raritan, NJ), CK-MB fraction was measured.

**Western Blot Analysis.** Left ventricular samples were homogenized in whole-cell lysis buffer (Cell Signaling Technology, Inc, Danvers, MA) with additional phosphatase and protease inhibitors. Left ventricular homogenates were then centrifuged at 18,000g for 30 minutes 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 1mmol/L EDTA, 0.5% Triton X-100, 1mmol/L phenylmethylsulfonyl fluoride, and protease inhibitors. Membranes were incubated in 5% milk, tris-buffered saline-Tween (TBST) buffer and either phosphorylated (phospho) p65 (ser 536), phospho-IκBα (ser 32/36), or beta-tubulin (Cell Signaling Technology, Inc) 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-IkBα or phospho-p65 to beta-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 is expressed as mean ± standard error. 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 to the control group. Statistical significance was accepted at the 95% confidence interval.

RESULTS

IKKβ inhibition Decreases Size of Infarction. Delivery of Bay 65-1942 prior to ischemia significantly decreased left ventricular infarct size compared to animals receiving vehicle (Figure 2A). Compared to sham animals, animals receiving vehicle had a significant increase in the infarct to AAR ratio (70.7 ± 3.4% versus 5.8 ± 3.4%, p<0.05). As shown in Figure 2B, this ratio was significantly reduced by treatment with Bay 65-1942 at each timepoint (prior to ischemia 42.7 ± 4.1%; at reperfusion 42.7 ± 7.5%; two hours of reperfusion 29.4 ± 5.2%; each group p<0.05 versus control). The differences in this ratio between those pre-treated 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 one hour following reperfusion. The CK-MB fraction was significantly elevated in the vehicle group (n=3) compared to the sham group (n=4) (30530±371.2 versus 9675±608.4 units, p<0.05). Animals pre-treated with Bay 65-1942 (n=3) had significantly attenuated CK-MB levels compared with those animals without treatment prior to IR (14170 ± 3219 units, p<0.05 versus control).

**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 minutes of LAD occlusion followed by three 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, while 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 pre-treatment groups (n=3 for each group) sacrificed 30 minutes and one hour after reperfusion were performed to observe the effects of IKKβ inhibition on the NF-κB pathway (Figure 3). Expression of phospho-IκBα, the direct
downstream product of IKKβ activation, was significantly elevated in vehicle animals compared to sham animals 30 minutes after reperfusion (p<0.05). This difference between sham and vehicle groups was statistically lost one hour following reperfusion, suggesting that initial IKKβ activation is at its height within an hour of reperfusion injury. Animals treated with Bay 65-1942 had lower levels of phospho-IκBα expression compared to the vehicle group at both 30 minutes and one hour following reperfusion (p<0.05, Bay 65-1942 versus vehicle).

Similarly, we evaluated phospho-p65 expression, the active subunit of NF-κB. Compared to sham animals, phospho-p65 was increased in the left ventricles of the vehicle group both 30 minutes and one hour after reperfusion (p<0.05). The expression of phospho-p65 in the Bay 65-1942 group was significantly decreased compared to vehicle at both timepoints (p<0.05) indicating successful suppression of NF-κB by Bay 65-1942.

**IKKβ Inhibition Decreases TNF-α and IL-6 Expression.** TNF-α and IL-6 are NF-κB dependent cytokines, activated in response to myocardial injury (7, 15, 29). One hour after reperfusion, levels of TNF-α and IL-6 were significantly elevated in the vehicle groups compared to the sham animals (TNF-α: 248.6 ± 25 versus 35.1 ± 35.1 pg/ml, p<0.05; IL-6: 5974 ± 1976 versus 433.9 ± 83.1 pg/ml, p<0.05). When administered Bay 65-1942 prior to ischemia, the amount of serum TNF-α dropped significantly compared to the vehicle group (22.4 ± 7.3 pg/ml, p<0.05 versus vehicle). IL-6 was also significantly lower in animals that received the IKKβ inhibitor compared to vehicle animals (417.8 ± 118.2 pg/ml, p<0.05 versus vehicle). There were no differences in cytokine concentrations between sham and treatment animals (Figure 4).
DISCUSSION

In the present study of acute IR, we have demonstrated that targeted NF-κB blockade by inhibiting IKKβ activity decreased myocardial injury and preserved cardiac function. These findings were associated with decreased myocardial expression of phospho-ΙκBα and phospho-p65 as well as downstream elaboration of prototypic 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 ΙκBα, 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 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). While 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. Furthermore, no sign of organ damage occurred following administration to rodents for several weeks (37). Most impressively, in comparison to IMD-0354 and other NF-κB inhibitors, Bay 65-1942 provided cardioprotection even when delivered with a single systemic dose 2 hours after reperfusion.

Our study compares favorably to and expands upon previously evaluated non-specific 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 to controls (20). Subsequent clinical trials of intracoronary adenosine infusion improved functional outcomes in patients receiving percutaneous coronary artery angioplasty within three hours of acute myocardial infarction (14). Additionally, large clinical trials such as ISIS-4 led to current guidelines that ACE inhibitors be administered to certain patients within 24 hours of acute myocardial infarction (1). Interestingly, and in comparison to our 40% reduction in infarct size, original animal studies with ACE inhibition in myocardial IR documented a decrease of only 24% (6). Furthermore, these studies utilized intracoronary drug delivery that usually started at reperfusion and continued for 1 hour. In our model, we achieved favorable results with a single systemic dose of IKKβ inhibitor, that was successful even when administered up to 2 hours 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 cardioprotective. Previous studies suggest that a basal expression of NF-κB is required to prevent apoptosis in cardiomyocytes following ischemic insults (17, 27). 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 pharmacologic 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 two hours 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.

ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Figure 1  Experimental protocol. An ischemic period of 30 minutes was produced by occlusion of the left anterior descending artery (LAD Occl.). Bay 65-1942 was administered either 30 minutes prior to ischemia (Pre), at the time of LAD release (Rep.), or after 120 minutes of reperfusion (Post).

Figure 2  IKKβ inhibition and infarct analysis. A. Two representative cross-sections of left ventricles at the level of the papillary muscle stained with 1%TTC (photomicrographs at 5x) 24 hours after ischemia-reperfusion injury. Infarcted areas remain white, while healthy myocardium is stained dark red. B. Size of infarct following IR is represented by percent infarct area relative to area at risk (Infarct:AAR). Infarct measurements were taken 24 hours after injury in sham (n=4), animals receiving vehicle (n=4), and animals treated with Bay 65-1942 30 minutes prior to ischemia (Bay-Pre; n=6) at the time of reperfusion (Bay-Rep.; n=4), and 120 minutes after reperfusion (Bay-Post; n=4). *p<0.05, sham versus vehicle; **p<0.05 vehicle versus treatment with Bay 65-1942.

Figure 3  IKKβ Inhibition and the NF-κB pathway. Left ventricular homogenates were probed by Western Blot for phosphorylated (phospho) p65 and phospho-IκBα at 30 minutes and 1 hour after reperfusion. Beta-tubulin served as a loading control.

Figure 4  IKKβ inhibition and cytokine production. Serum was obtained from mice 1 hour following reperfusion in sham, vehicle, and pre-treatment groups and assayed for TNF-α (n=3 for each group) or IL-6 (n=4 for sham, n=5 for vehicle and treatment groups). *p<0.05, sham versus control; **p<0.05 control versus Bay 65-1942.
Table 1  Hemodynamic parameters of mice after ischemia-reperfusion injury with and without IKK-β inhibition.

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<th>Baseline</th>
<th>Ischemia-Reperfusion</th>
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<tbody>
<tr>
<td></td>
<td>Vehicle (n=4)</td>
<td>Bay (n=5)</td>
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<tr>
<td>Body Weight (gr)</td>
<td>25.25 ± 0.7500</td>
<td>23.60 ± 0.4000</td>
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<tr>
<td>Heart Rate (beats/min)</td>
<td>440.8 ± 19.63</td>
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<td>-7663 ± 522.1</td>
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<tr>
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<td>14.23 ± 4.331</td>
<td>8.840 ± 1.638</td>
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<tr>
<td>LVEF (%)</td>
<td>58.40 ± 2.729</td>
<td>56.15 ± 4.509</td>
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Values are means +/- SEM. Baseline: mice without ischemia-reperfusion injury; Ischemia-Reperfusion: mice that undergo 30 minutes of LAD occlusion 3 days prior to pressure-volume analysis; Bay mice were administered Bay 65-1942 either 3 days prior to measurements (baseline) or 30 minutes prior to LAD occlusion (IR). LV dP/dt max, maximal derivative of LV pressure; LV dP/dt min, minimal derivative of LV pressure; LVEDP, LV end-diastolic pressure; LVEF, LV ejection fraction. *p<0.05, IR Vehicle vs Baseline Vehicle; †p<0.05, IR Vehicle vs Baseline Bay; ‡p<0.05 IR Bay vs IR Vehicle.
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*p<0.05, sham versus control; **p<0.05 control versus Bay 65-1942.

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