Diverse Mechanisms of Myocardial p38 Mitogen-Activated Protein Kinase Activation

Evidence for MKK-Independent Activation by a TAB1-Associated Mechanism Contributing to Injury During Myocardial Ischemia

Masaya Tanno, Rekha Bassi, Diana A. Gorog, Adrian T. Saurin, Jie Jiang, Richard J. Heads, Jody L. Martin, Roger J. Davis, Richard A. Flavell, Michael S. Marber

Abstract—The ischemic activation of p38α mitogen-activated protein kinase (p38α-MAPK) is thought to contribute to myocardial injury. Under other circumstances, activation is through dual phosphorylation by MAPK kinase 3 (MKK3). Therefore, the mkk3−/− murine heart should be protected during ischemia. In retrogradely perfused mkk3−/− and mkk3+/+ mouse hearts subjected to 30 minutes of global ischemia and 120 minutes of reperfusion, infarction/risk volume was similar (50±5 versus 51±4, P=0.93, respectively), as was intraschismic p38-MAPK phosphorylation (10 minutes ischemia as percent basal, 608±224 versus 384±104, P=0.43, respectively). This occurred despite undetectable activation of MKK3/6 in mkk3−/− hearts. However, tumor necrosis factor (TNF)-induced p38-MAPK phosphorylation was markedly diminished in mkk3−/− vs mkk3+/+ hearts (percent basal, 127±23 versus 540±267, respectively, P=0.04), suggesting an MKK-independent activation mechanism by ischemia. Hence, we examined p38-MAPK activation by TAB1-associated autophosphorylation. In wild-type mice and mkk3−/− mice, the p38-MAPK catalytic site inhibitor SB203580 (1 μmol/L) diminished phosphorylation during ischemia versus control (10 minutes ischemia as percent basal, 143±2 versus 436±96, P=0.003, and 122±25 versus 623±176, P=0.05, respectively) and reduced infarction volume (infarction/risk volume, 57±5 versus 36±3, P<0.001, and 50±5 versus 29±3, P=0.003, respectively) but did not alter TNF-induced activation, although in homogenates of ischemic hearts but not TNF-exposed hearts, p38-MAPK was associated with TAB1. Furthermore, adenovirally expressed wild-type and drug-resistant p38α-MAPK, lacking the SB203580 binding site, was phosphorylated when H9c2 myoblasts were subjected to simulated ischemia. However, SB203580 (1 μmol/L) did not prevent the phosphorylation of resistant p38α-MAPK. These findings suggest the ischemic activation of p38-MAPK contributing to myocardial injury is by TAB1-associated autophosphorylation. (Circ Res. 2003;93:254-261.)

Key Words: p38 mitogen-activated protein kinase ● myocardial infarction ● TAB1 ● ischemic preconditioning ● mitogen-activated protein kinase kinase 3

Recent attention has focused on myocardial p38 mitogen-activated protein kinase (p38-MAPK) as a critical determinant of myocardial injury during ischemia and reperfusion.1 In the absence of preconditioning, most investigators find that the activation of p38-MAPK that accompanies true and simulated ischemia/reperfusion contributes to injury.2-9 Previously, using a variety of approaches, we have shown that the p38α-MAPK isoform becomes activated and increases cell death during simulated ischemia in cell lines and in adult and neonatal primary cardiocytes.1,3 Using one of these models, Wang et al10 have shown that the upstream kinase, mitogen-activated protein kinase kinase 3 (MKK3), preferentially activates p38α-MAPK by dual phosphorylation and increases cell death. This observation is in keeping with mutational analyses of MKK3 that have mapped the regions within its primary sequence responsible for this preference11 and with the resolved structure of p38α-MAPK bound to a peptide fragment of MKK3.12 In addition, using an organ-specific conditional transgenic approach, Wang and colleagues13 have shown that MKK3 activates p38-MAPK in murine myocardium where p38α-MAPK is the only isoform detected. Furthermore, even in the absence of ischemia, such activation leads to myocyte death and cardiomyopathy.13 Thus, p38α-MAPK is activated by MKK3, and its activation contributes to injury.
Based on these observations, we hypothesized that ischemia/reperfusion injury would be attenuated in murine hearts lacking MKK3. In this study we show that this hypothesis is untenable, because phosphorylation of the key Thr180 and Tyr182 residues within the activation lip are not diminished in MKK3-deficient hearts during ischemia. We show instead that under these circumstances the phosphorylation of these residues is inhibited by the p38-MAPK catalytic site inhibitor SB203580, suggesting autophosphorylation. This suggestion is consistent with the inability of SB203580 to inhibit the ischemia-induced phosphorylation of p38α-MAPK by tumor necrosis factor (TNF), which is MKK3-dependent but SB203580- and TAB1-independent.

**Materials and Methods**

All experiments were performed in accordance with United Kingdom Home Office Guidance on the Operation of Animals (Scientific Procedures) Act 1986, published by Her Majesty’s Stationary Office, London.

**M KK3-Deficient Mice**

The targeting strategy used to generate the mkk3−/− mice has been described previously.14 A colony was rederived from this source by crossing an mkk3+/− male to an outbred C57B/6 female. For the purposes of this study, to reduce background genetic variability, we used male mkk3−/− and mkk3+/+ progeny of heterozygote matings, which whenever possible were littermates.

**Perfusion of Isolated Murine Hearts**

Male mice were anesthetized with pentobarbital (300 mg/kg with 150 IU heparin, IP). Hearts were rapidly excised, placed in ice-cold modified Krebs-Henseleit (K-H) buffer containing (in mmol/L) NaCl 118.5, NaHCO3 25.0, KCl 4.75, KH2PO4 1.18, MgSO4 1.19, D-glucose 11.0, and CaCl2 1.41. The aorta was cannulated and retrogradely perfused at a constant pressure of 80 mm Hg with K-H buffer equilibrated with 95% O2 and 5% CO2. After perfusion, the hearts were then thawed, placed in 2.5% glutaraldehyde for 1 minute, and set in 5% agarose. The agarose heart blocks were then sectioned from apex to base in 0.7-mm slices using a vibratome (Agar Scientific). After sectioning, slices were placed overnight in 10% formaldehyde at room temperature before transferring into PBS for an additional day at 4°C. Sections were then compressed between Perspex plates (0.57 mm apart) and imaged using a TK-1280E digital camera (JVC). After magnification (×25), planimetry was carried out using image analysis software (NIH image v1.61 and Adobe Photoshop 4.0) and surface area of the whole, and TTC-negative, left ventricular myocardium was transformed to volume by multiplication with tissue thickness. Within each heart, after summation of individual slices, TTC-negative infarction volume was expressed as a percentage of, or plotted against, left ventricular volume.

**Western Blot Analysis**

Samples were obtained from the mouse hearts at 5, 10, or 20 minutes of ischemia and after perfusion with 5 ng/mL of TNF (Sigma) for 7 or 15 minutes, 25 μmol/L of peroxynitrite (Calbiochem) for 15 minutes, 20 μmol/L of anisomycin (Sigma) for 15 minutes, or 0.1 mol/L of sorbitol (Sigma) for 15 minutes. Samples were also harvested from H9c2 monolayers after 3 hours of simulated ischemia (see below). Samples were then homogenized in electrophoresis sample buffer (250 mmol/L Tris-HCl, 4% SDS, 10% glycerol, and 2% β-mercaptoethanol, pH 6.8). After transfer, equal loading on nitrocellulose membranes was confirmed by staining with Ponceau-S. After blocking with 5% nonfat milk, nitrocellulose membranes were exposed to the following primary antibodies: anti-dual phospho-p38 MAP kinase (Thr180 and Tyr182) (Sigma) at 1:1000, anti-p38 at 1:1000, anti-HSP27 at 1:1000, anti-MKK3/6 at 1:1000, anti-phospho MKK3/6(Ser189/Ser207) at 1:500 (all from Santa Cruz Biotechnology), anti-TAB1 at 1:500 (Active Motif or Santa Cruz Biotechnology) and anti-phospho-HSP27(Ser78) at 1:400, before appropriate secondary antibody and ECL detection (Amersham). Autoradiographic images of the Western blots were scanned and then quantified using NIH image analysis software.

**Adenoviral Construction and Transfection of H9c2 Myoblasts**

Our rationale, methods of adenovirus construction, amplification, and infection have been described previously.1,3 The culture and simulation of ischemia are also as described previously.1

**Immunoprecipitation**

Hearts were homogenized in 0.75 mL of iced immunoprecipitation buffer (20 mmol/L HEPES, 1 mmol/L EDTA, 150 mmol/L NaCl, 1 mmol/L DTT, 5 mmol/L NaF, 1 mmol/L Na3 VO4, one protease inhibitor tablet per 50 mL [Complete, Roche, Germany], 1% Triton X-100) per 100 mg of wet tissue. Homogenized tissue was centrifuged (10 000 g, 15 minutes, 4°C), and supernatant (200 μL) was incubated with 15 μL of protein G slurry (Protein G PLUS-Agarose, Santa Cruz Biotechnology) for 1 hour at 4°C to preclear the sample. After additional centrifugation for 10 minutes at 4°C, supernatants were incubated with 30 μL of agarose-conjugated anti-p38 MAPK antibody (Santa Cruz Biotechnology) and agitated for 2 hours at 4°C. Samples were then centrifuged (10 000g, 5 minutes, 4°C) and the supernatant stored for additional analysis (lysisate). Pellets were washed and resuspended three times in immunoprecipitation buffer and then finally resuspended in 30 μL of SDS sample buffer and boiled for 3 minutes. The samples were centrifuged (10 000g, 5 minutes, 4°C), and the supernatants were kept for Western blotting (immunoprecipitate).
Figure 1. Effects of MKK3 and SB203580 on myocardial infarction after 30 minutes of global ischemia and 120 minutes of reperfusion. Infarction has been measured as infarction volume as a percentage of total myocardial volume (A) and, to minimize the possibility of a type II statistical error introduced by the nonzero intercept in the infarction volume, heart volume relationship by linear regression (B). Both methods demonstrate that sensitivity to infarction is unaltered by genotype (mkk3−/− [●], n=9, vs mkk3+/− [□], n=10, P=NS) but is altered by SB203580 (SB) (1 μmol/L) immediately before and during ischemia, irrespective of genotype (c57B/6 [●], n=14, vs c57B/6+SB [□], n=14, tP<0.001, and mkk3−/− [●], n=10, vs mkk3−/−+SB [□], n=9, tP<0.003 by unpaired t test and ANCOVA).

Statistical Analysis
All data are presented as mean±SEM. Hemodynamic variables were compared between groups by 2-way repeated-measures ANOVA with a multivariate linear model to correct for the differing group sizes. An unpaired t test was used to test for differences in normalized infarct volume between two groups. Linear regression was carried out using the SigmaStat statistical package, whereas infarction volume with respect to total myocardial volume was compared by ANCOVA using an Excel plug-in (Ferris State University). P<0.05 was considered significant.

Results
mkk3−/− Hearts
The mkk3−/− mice did not differ obviously from the mkk3+/+ mice. Specifically, their body mass, heart mass, and baseline and postischemic left ventricular developed pressure and coronary flows were similar (see the online Table, available in the online data supplement at http://www.circresaha.org). We have found that 30 minutes of global ischemia results in infarction of 30% to 65% of the left ventricular volume. Our expectation was that this distribution of infarction volume would differ in mkk3−/− versus mkk3+/+ hearts. In Figure 1A, it is apparent that the normalized infarction volume does not differ by genotype. To be certain that this is not a function of the nonzero intercept between infarction and ventricular volumes,15 these are plotted separately (Figure 1B), demonstrating similar lines of identity for each genotype.

There are two possible explanations for this counterintuitive finding. The first is that intrinsichem p38-MAPK activation is deficient in mkk3−/− hearts but that this had no impact on sensitivity to ischemia. The second is that p38-MAPK activation is unaltered by the absence of MKK3. We therefore measured p38-MAPK activation by probing the phosphorylation status of the key tyrosine and threonine residues within the activation lip of p38-MAPK (p38). This shows that p38-MAPK is activated maximally at 10 minutes of ischemia at ~4-fold over basal (B). However, activation is unaltered by genotype. The total MKK3/6 antibody confirms the absence of MKK3/6 (K) and mkk3−/− (W) hearts. A, Data derived from one series of experiments. B, Quantitative data from 4 separate series of experiments. Total p38-MAPK is unaltered by genotype. Protein samples were also probed with a phospho-specific antibody recognizing the dual *T180-G181-*Y182 phosphorylation motif within the activation lip of p38-MAPK (p38). This shows that p38-MAPK is activated maximally at 10 minutes of ischemia at ~4-fold over basal (B). However, activation is unaltered by genotype. The total MKK3/6 antibody confirms the absence of MKK3 (lower band) in the mkk3−/− hearts without a compensatory increase in MKK6. Although the phospho-Ser 189/Ser207 signal of MKK3/6 is weak, it does become detectable at 10 and 20 minutes of global ischemia and is absent in the mkk3−/− hearts, suggesting it is comprised mainly of MKK3 (see phospho-MKK3/6). The activation of p38-MAPK is reflected in the phosphorylation of HSP27. NA indicates normalization not appropriate.
MAPK activation during ischemia, this is not blunted in the mkk3^{−/−} hearts (see Figure 2B). Furthermore, this preservation in activation is not the result of a compensatory increase of the other MKK upstream of p38-MAPK, MKK6. The MKKs are in turn activated by phosphorylation by upstream MKKKs such as ASK1.16 Although we probed for one of these sites of activation (Ser189/Ser207) within MKK3/6, we were never able to detect a strong signal (see phospho-MKK3/6 lane in Figures 2 and 3). Nonetheless it seems that MKK3 is preferentially activated over and above MKK6 by ischemia, with no detectable phospho-MKK3/6 signal in mkk3^{−/−} hearts. This is surprising, because although MKK4 can activate p38-MAPK in vitro, MKK3 and MKK6 are thought to be the only physiologically relevant activators of p38-MAPK in the intact cell.17 Thus, the dual phosphorylation of p38-MAPK seems unaltered in the mkk3^{−/−} hearts despite the absence of an active upstream MKK.

Effects of SB203580
The phosphorylation of p38-MAPK in the absence of detectable activation of an upstream MKK could be the result of autophosphorylation.18 Figure 3 shows the pattern of intraschematic p38 activation in C57B/6 hearts in the presence and absence of preschematic and intraschematic exposure to the p38-MAPK catalytic site inhibitor, SB203580 (1 μmol/L). Trapping SB203580 in the myocardium during zero-flow ischemia abolishes p38-MAPK activation and downstream HSP27 phosphorylation. In contrast to MKK3 deficiency, this intervention is associated with a reduction in normalized infarction volume and a downward shift in the relationship between infarction and total left ventricular volume (Figures 1A and 1B, right) The MKK3 independence of this effect is confirmed by the fact SB203580 similarly reduces infarction on the mkk3^{−/−} background (Figures 1A and 1B, left). In addition, although the ischemic activation of p38-MAPK occurs in MKK3-deficient hearts, it is still abolished by SB203580 (Figure 4C).

Thus, in contrast to the absence of detectable MKK3/6 activation seen in the mkk3^{−/−} hearts, SB203580 diminishes both p38-MAPK activation and infarction volume. The question is whether this mechanism of MKK-independent activation is common to other archetypal activators of myocardial p38-MAPK.

Mechanism of TNF-Induced p38-MAPK Activation
TNF is known to activate p38-MAPK in numerous cell types.19 Within the heart, this cytokine is thought to be involved in an amplifying autocrine/paracrine loop that leads to cachexia and worsening cardiac performance in chronic heart failure.20 We therefore examined the ability of intracoronary TNF at a concentration of 5 ng/mL to activate myocardial p38-MAPK.

At 15 minutes but not 7 minutes of exposure, there was robust activation of p38-MAPK. Figure 4A shows that the activation at 15 minutes is unaltered by coincident SB203580 at a concentration that abolishes ischemia-induced activation of p38-MAPK (compare with Figure 4B) and inhibits catalytic activity, because downstream HSP27 phosphorylation is inhibited. Moreover, in the MKK3-deficient hearts, this TNF-induced activation is absent (Figure 4A). Thus, the pattern of TNF-induced activation is SB-independent but MKK3-dependent, whereas that during ischemia is SB-independent but MKK3-dependent.

Confirming Sensitivity to SB203580 as an Index of Autophosphorylation
SB203580 is known to inhibit a variety of kinases in addition to p38-MAPK,21–24 Thus, it is possible that our observations are the result of SB203580 inhibiting a kinase upstream of p38-MAPK that is activated by ischemia but not TNF. To exclude this possibility, we expressed wild-type and mutant forms of p38α-MAPK in H9c2 cells. The mutation, where Thr106, His107, and Leu108 have been changed to Met, Pro, and Phe, respectively (T106M, H107P, and L108F), has been
constructed, studied, and shown to have effects in vivo and in vitro that are indistinguishable from wild-type p38α-MAPK. However, in contrast to wild-type p38α-MAPK, the triple mutant is inherently resistant to SB203580 in the same manner as the p38γ-MAPK and p38γ-MAPK isofoms, which share the 106M, 107P, and 108F consensus. We have previously shown that p38α-MAPK is activated in response to ischemia in H9c2 cells and that this contributes to injury.1

In Figure 5, we confirm that wild-type p38α-MAPK is activated during simulated ischemia, and this is associated with phosphorylation of HSP27; both of these changes are usually inhibited by SB203580. In contrast, although these ischemia-induced changes occur with the SB203580-resistant form of p38α-MAPK (DR), they can no longer be inhibited. These findings confirm the action of SB203580 is dependent on its ability to bind to the catalytic domain of p38α-MAPK.

**Association of p38-MAPK With TAB1**

TAB1 is a scaffold protein that binds components of a transforming growth factor-β-initiated signal transduction cascade, including p38-MAPK, where its binding promotes p38-MAPK autophosphorylation. Homogenates were prepared from hearts subjected to 10 minutes of global ischemia or 15 minutes of TNF, conditions causing p38-MAPK activation (Figure 4). Under both of these circumstances, a significant portion of immunoprecipitated total p38-MAPK was dual-phosphorylated, but the association with TAB1 was restricted to ischemia (see Figure 6 and legend for fuller explanation).

Our data, in keeping with those of Ge et al, suggest that the mechanisms leading to phosphorylation of p38 MAP kinase can differ according to stimulus. However, Ge et al found that p38-MAPK phosphorylation in HEK293 cells exposed to TNF was SB203580-sensitive. Accordingly, we examined whether other differences existed in the mode of p38-MAPK activation between HEK293 cells and the murine heart. We studied the archetypal p38-MAPK activating stresses adopted by Ge et al and examined the sensitivity to SB203580 and association with TAB1. Infusion of peroxynitrite and anisomycin resulted in marked, sorbitol resulted in less marked, phosphorylation of p38-MAPK. This phosphorylation was completely, partially, and negligibly inhibi-
Discussed with SB203580, respectively (Figure 7A). TAB1 was only strongly associated with p38 MAP kinase in peroxynitrite- and anisomycin-exposed hearts (Figure 7B).

**Discussion**

Our findings suggest that during global ischemia p38-MAPK becomes activated and that this contributes to necrosis of the isolated murine heart. Surprisingly, the mode of p38-MAPK activation seems independent of upstream MKKs but depends instead on an SB203580-sensitive mechanism of activation, which is associated with binding to TAB1. This pattern contrasts with the mode of activation by TNF and ischemia increase the proportion of dual phosphorylated p38-MAPK. Although the positive control has not been subjected to immunoprecipitation, transfection with TAB1 did cause p38-MAPK activation (data not shown). TAB1 only associates with p38-MAPK in hearts subjected to global ischemia, although similar p38-MAPK activation occurred within the precipitate from TNF-exposed hearts. The presence of excess TAB1 is confirmed in all samples by immunoblotting of the lysate. The larger apparent molecular weight of TAB1 in the positive control is the result of species differences confirmed by immunoblotting of crude mouse heart homogenates and solubilized protein from a human cell line.

**The Likely p38-MAPK Isoform and Mechanisms of Activation**

It is probable that in the present study the major endogenous SB-sensitive p38-MAPK isoform is p38α, because in rodent myocardium it is likely that the p38α isoform dominates. For example, Wang and colleagues were unable to detect the β isotype in murine myocardium, and we were unable to detect it in rat myocardium.

The traditional mechanism of p38-MAPK activation is by transphosphorylation of the activation lip by upstream MKKs. Because SB203580 occupies the ATP-binding pocket of p38-MAPK, it should only inhibit phosphorylation events downstream of p38-MAPK and not the phosphorylation of p38-MAPK itself. This view was challenged by the recent observations by Ge et al., who draw attention to previously published reports where SB203580 does inhibit p38-MAPK phosphorylation, and show this is probably the result of circumstance-specific autophosphorylation of p38α-MAPK. In the cardiac literature, it is also possible to find similar instances of SB203580-sensitive phosphorylation of p38-MAPK, including during myocardial ischemia (lane 4 of Figure 2 in the study by Maulik et al.). However, these observations may also result from the nonspecific profile of SB203580 causing the inhibition of a kinase upstream of p38-MAPK but not direct MKK inhibition and thereby reducing its transphosphorylation rather than autophosphorylation. However, Ge et al. show that phosphorylation under these circumstances is dependent on intramolecular kinase.
activity excluding transphosphorylation by another kinase or p38α-MAPK molecule. Moreover, this form of activation is related to an association with the scaffold protein TAB1. Similarly, in this study we show that the reduction in activation loop phosphorylation caused by SB203580 is unlikely to be attributable to nonspecific inhibition of another kinase, because it is dependent on its ability to bind p38α-MAPK. However, this reduction may be cell-type specific, because Ge et al.18 found p38-MAPK activation in response to TNF was SB-sensitive whereas we did not. However, the findings with peroxynitrite, anisomycin, and sorbitol exposure are more consistent across the studies. Our findings with TNF are limited to the whole heart, whereas the findings by Ge et al.18 were confined to HEK 293 cells. Furthermore, our findings are consistent with those of Wysk et al.,11 who also found TNF-induced p38-MAPK activation was MKK3-dependent in murine fibroblasts.

Consequence of p38 Activation by Transphosphorylation or Autophosphorylation

At present, it is not clear how the signals immediately upstream of p38-MAPK diverge to enable different mechanisms of activation. It is possible for example that an alternative p38-MAPK kinase other than MKK3 is responsible for the activation during ischemia. Furthermore, our observations in the whole heart could be explained if this unidentified kinase were sensitive to SB203580 and TAB1-associated. To counter this possibility, we present in vitro data in a myoblast line with adenovirus-driven expression of SB-sensitive and SB-resistant forms of p38α-MAPK. Although this system may not faithfully recapitulate the mechanisms of p38-MAPK activation in the ischemic heart, the different patterns of sensitivity to SB203580 and the absence of MKK3 must indicate divergence of upstream signals. Although speculative, it is likely that these different mechanisms of activation exist, because their downstream consequences differ. Thus, although TNF and ischemia activate p38-MAPK, signaling specificity maybe conferred by the circumstances of activation.30 Such subtypes may partly explain the diverse consequences of p38-MAPK activation reported that depend on model, ischemic conditions, and species.2 Furthermore, they may allow additional dissection of the pleiotropic responses to TNF that are frustrating clinical development of anti-TNF therapies for heart failure.30,31

In conclusion, we show that different mechanisms contribute to myocardial p38-MAPK activation during ischemia and TNF exposure. Our findings suggest that during ischemia, autophosphorylation is responsible for a form of activation that has detrimental consequences. At present, it is unclear whether the nature of activation occurring with TNF has similar consequences.32,33

Acknowledgments

This study was supported by grants from the Wellcome Trust (055696 and 064547) and the British Heart Foundation (PG/02/105/14432).

References

30. Borsch-Haubold AG, Pasquet S, Watson SP. Direct inhibition of cyclo-oxygenase-1 and -2 by the kinase inhibitors SB 203580 and PD 98059-SB


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Circ Res. 2003;93:254-261; originally published online June 26, 2003;
doi: 10.1161/01.RES.000083490.43943.85

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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### Table 1. Morphometric characteristics and hemodynamic parameters of isolated perfused hearts.

Values are mean±SEM. Baseline left ventricular developed pressure (LVDP) and coronary flow were determined after 30 minutes of stabilization.

<table>
<thead>
<tr>
<th>Experimental Groups</th>
<th>MKK3+/+</th>
<th>MKK3-/-</th>
<th>MKK3-/-+SB</th>
<th>C57B/6</th>
<th>C57B/6 + SB</th>
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</thead>
<tbody>
<tr>
<td>Number of Observations</td>
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<td>10</td>
<td>9</td>
<td>14</td>
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<td><strong>Morphological characteristics</strong></td>
<td></td>
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<tr>
<td>Body Weight (g)</td>
<td>29.9 ± 0.9</td>
<td>31.3 ± 0.6</td>
<td>30.7 ± 0.8</td>
<td>29.2 ± 0.8</td>
<td>28.1 ± 0.8</td>
</tr>
<tr>
<td>Wet Heart Weight (mg)</td>
<td>144.3 ± 9.1</td>
<td>150.3 ± 6.5</td>
<td>152.7 ± 8.5</td>
<td>129.0 ± 5.7</td>
<td>122.5 ± 4.7</td>
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<tr>
<td>Heart Volume (mm³)</td>
<td>109.8 ± 5.8</td>
<td>125.4 ± 5.5</td>
<td>124.9 ± 6.2</td>
<td>119.8 ± 4.9</td>
<td>114.4 ± 4.5</td>
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<tr>
<td><strong>Hemodynamic parameters</strong></td>
<td></td>
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<td></td>
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<tr>
<td>Coronary Flow (mL/min)</td>
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<td></td>
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<tr>
<td>Baseline</td>
<td>3.4 ± 0.2</td>
<td>3.2 ± 0.2</td>
<td>3.3 ± 0.3</td>
<td>2.9 ± 0.2</td>
<td>3.2 ± 0.2</td>
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<tr>
<td>Reperfusion 60 min</td>
<td>1.8 ± 0.2</td>
<td>1.6 ± 0.2</td>
<td>2.1 ± 0.2</td>
<td>1.7 ± 0.2</td>
<td>2.5 ± 0.2 †</td>
</tr>
<tr>
<td>Reperfusion 120 min</td>
<td>1.4 ± 0.2</td>
<td>1.5 ± 0.1</td>
<td>2.1 ± 0.2 *</td>
<td>1.5 ± 0.2</td>
<td>2.2 ± 0.2 †</td>
</tr>
</tbody>
</table>

| LVDP (mmHg) |         |         |           |        |            |
| Baseline    | 65 ± 2  | 63 ± 3  | 59 ± 2    | 60 ± 2 | 64 ± 3     |
| Reperfusion 60 min | 12 ± 2 | 13 ± 1  | 22 ± 3 *  | 12 ± 2 | 22 ± 2 †   |
| Reperfusion 120 min | 13 ± 2 | 14 ± 1  | 25 ± 2 *  | 13 ± 2 | 24 ± 3 †   |

| Diastolic pressure (mmHg) |         |         |           |        |            |
| Baseline                 | 4 ± 0   | 5 ± 0   | 5 ± 1     | 6 ± 1  | 5 ± 1      |
| Reperfusion 60 min        | 46 ± 5  | 51 ± 6  | 44 ± 6    | 47 ± 5 | 36 ± 3     |
| Reperfusion 120 min       | 40 ± 5  | 46 ± 2  | 34 ± 5 *  | 40 ± 4 | 27 ± 3 †   |

† p<0.05 vs. C57B/6,  * p<0.05 vs. MKK3-/-