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Disruption of a single copy of the p38α MAP kinase gene leads to cardioprotection against ischemia–reperfusion

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Abstract

The p38 mitogen-activated protein kinase (p38) is activated in the heart during ischemia–reperfusion. However, it is not clear whether the activation of p38 is the protective response or the kinase mediates the cellular damage by ischemia–reperfusion. We examined the role of p38α in ischemia–reperfusion injury by studying $p38\alpha^{+/-}$ mice. The p38α protein level in the $p38\alpha^{+/-}$ heart was $50 \pm 8.7\%$ compared with that in the $p38\alpha^{+/+}$ heart. Upon reperfusion following ischemia for 25 min, p38α activity was transiently increased. The maximum level of p38 activity in $p38\alpha^{+/-}$ was $60 \pm 10.5\%$ compared with that in $p38\alpha^{+/+}$. In the $p38\alpha^{+/+}$ heart, 25 min ischemia and 2 h reperfusion resulted in necrotic injury ($37.1 \pm 2.7\%$ of the area at risk), whereas infarct size was drastically reduced to $7.2 \pm 0.7\%$ in the $p38\alpha^{+/-}$ heart. These suggested that p38α plays a pivotal role in the signal transduction pathway mediating myocardial cell death caused by ischemia–reperfusion.

Keywords: Ischemia–reperfusion; p38 mitogen-activated protein kinase; Heart; Cardioprotection; Necrosis

Reperfusion of the ischemic myocardium has become a mainstay of optimal therapeutic intervention for myocardial infarction. Paradoxically, reperfusion also results in cell death and scar formation. The signal transduction mechanism, by which ischemia–reperfusion leads to cell death, has not yet been clearly identified.

Recent studies suggest an important role of mitogen-activated protein (MAP) kinase family, particularly p38 MAP kinase (p38), in ischemia–reperfusion in hearts. The p38 is activated in the heart following ischemia–reperfusion [1–5]. The reported role of p38 in sustained ischemia and protective effect of ischemic preconditioning, acquisition of tolerance against lethal ischemia by brief sublethal ischemia [6], is somewhat enigmatic, however [7]. It has been reported that ischemic preconditioning activates p38 [8,9] as well as MAPKAPK2 [10], a downstream substrate of p38. Inhibition of p38 with SB203580, a specific inhibitor of p38, completely blocked the infarct size limiting effect of ischemic preconditioning in isolated rat [11,12] and rabbit [13] hearts. SB203580 had no effect on injury in non-preconditioned myocardium. These suggest that activation of p38 is essential for the mediation of cardioprotection by ischemic preconditioning. In contrast, Ma et al. [4] reported that addition of SB203580 before global ischemia reduced necrosis, apoptosis, and postischemic contractile function in a perfused rabbit heart. Schneider et al. [14] noted that in rat hearts, SB202190, another specific inhibitors of p38, reduced ischemic injury and did not block protective effects of preconditioning. In support of these observations, SB203580 also reduced lactate dehydrogenase release and delayed apoptosis

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measured after 7–9 h of simulated ischemia [15]. These data suggest that inhibition of p38 is protective.

At the center of the controversy are data obtained using the specific p38 inhibitors, SB203580 and SB202190. It has been reported that the actions of the SB compounds are not confined to inhibition of p38, but can inhibit JNK [16] and other enzymes such as thromboxane synthetase and cyclooxygenases-1 and -2 [17]. This may contribute to the inconsistent findings in the literature [7]. Four isoforms (p38α–δ) of p38 have been identified so far [18]. SB compounds act as inhibitors of the α- and β-isoforms but not δ- and γ-isoforms of p38. Studies in cultured cells have shown that individual isoforms of p38 may possess distinct biological functions [19]. Thus, a second plausible explanation for the controversy may be the different actions of individual isoforms of p38. Recently, using adenovirus encoding a mutated α-isoform of p38 lacking SB203580 binding site, Martin et al. [20] reported that anti-ischemic effects of SB203580 in isolated cardiac myocytes are mediated through the inhibition of p38α isoforms, but not through p38β or other SB-sensitive enzymes.

To clarify the role of p38α in ischemia–reperfusion in vivo, we attempted to use p38α knockout mice in this study. The $p38\alpha^{-/-}$ mice die during embryonic development [21–23]. Thus, we used $p38\alpha^{+/-}$ to examine the in vivo role of p38α in ischemia–reperfusion injury. We investigated infarct size, the only clinically relevant endpoint for an anti-infarct intervention. We found that p38α heterozygotes undergo drastically decreased ischemia–reperfusion injury in heart compared with wild-type mice.

Materials and methods

This study was carried out under the supervision of the Animal Research Committee in accordance with the Guidelines for Animal Experiments of Osaka University and the Animal Protection and Management Law of Japan (No. 25).

Ischemia–reperfusion studies. The p38α-deficient mice were described previously [21]. All experiments were performed on 8–10 week-old $p38\alpha^{+/-}$ and age-matched $p38\alpha^{+/+}$ littermate mice. Hemodynamic measurements were performed as previously described [24]. Ischemia–reperfusion studies were performed as described previously [25]. Silk thread (7-0 type) was passed around the left coronary artery (LCA) about 1 mm distal to the LCA origin and made into an occlusive snare. Following a 25-min ligation of the LCA, the snare was released for 2 h. Then, the infarct size was evaluated by the double staining using Evans blue dye and triphenyltetrazolium chloride (TTC) [25]. The arterial blood pressure, heart rate, and ECG changes were monitored continuously [25]. The area at risk was defined as the ratio of the area of the ischemic region to the left ventricular (LV) area and the infarct size as the ratio of the area of the infarct region to that of the ischemic region.

Immunoblotting. Extracts (20 μg) from hearts were subjected to Western blot analysis by using the polyclonal antibody against p38α or p38β (Santa Cruz Biotechnology) [26]. To measure p38α activity, extracts (200 μg) were incubated with protein A-conjugated p38α antibody and then with activating transcription factor-2 (ATF-2) protein in the presence of ATP using p38 MAP Kinase Assay Kit (Cell Signaling). Phosphorylation of ATF-2 was estimated using a phospho-ATF-2 antibody with an ECL Western Blotting Detection Kit (Amersham Pharmacia Biotech).

Statistical analysis. Data are expressed as means ± SEM. The significance of the differences between groups was assessed by unpaired t tests or 1- or 2-way ANOVA with Bonferroni's post hoc test for multiple comparisons. A level of $P < 0.05$ was considered statistically significant.

Results

Physiological characterization of the experimental model

In previous studies, the $p38\alpha^{-/-}$ mice die during embryonic development [21–23]. The $p38\alpha^{+/-}$ mice appeared phenotypically normal when compared to $p38\alpha^{+/+}$. Body and heart weights were not significantly different for those of control and $p38\alpha^{+/-}$ mice (Table 1). Histological analysis by hematoxylin and eosin staining indicated that the hearts of $p38\alpha^{+/-}$ mice were normal (results not shown). The baseline functional parameters for hearts from $p38\alpha^{+/+}$ and $p38\alpha^{+/-}$ were established (Table 1). The maximum and minimum first derivatives of the LV pressure during both contraction and relaxation were essentially the same in the two groups. Heart rates, systolic and diastolic blood pressures, LV systolic pressures, and left ventricular end-diastolic pressures were similar in both groups.

Table 1
Baseline parameters of $p38\alpha^{+/+}$ and $p38\alpha^{+/-}$

	$p38\alpha^{+/+}$ ($n = 9$)	$p38\alpha^{+/-}$ ($n = 9$)
Body weight (g)	26.3 ± 0.8	25.6 ± 0.7
Heart weight (mg)	125 ± 7	123 ± 5
Heart rate (beats/min)	222 ± 19	211 ± 11
Systolic blood pressure (mmHg)	124.1 ± 4.7	113.2 ± 11.1
Diastolic blood pressure (mmHg)	94.3 ± 6.3	86.8 ± 10.1
LV systolic pressure (mmHg)	104.2 ± 8.4	103.2 ± 6.3
LV dp/dt max (mmHg/s)	4603 ± 140	4437 ± 139
LV dp/dt min (mmHg/s)	−3936 ± 155	−3514 ± 173
LVEDP (mmHg)	3.8 ± 0.3	3.7 ± 0.3

LV, left ventricle; LVEDP, left ventricular end-diastolic pressure. LV dp/dt max and LV dp/dt min are the maximum rates of pressure development during contraction and relaxation, respectively. Data are expressed as means ± SEM.

The protein level and activity of p38 in p38α$^{+/-}$ and p38α$^{+/+}$ mouse hearts

Western blot analysis indicated that the p38α protein levels in p38α$^{+/-}$ hearts were $50 \pm 8.7\%$ ($n = 5$) compared to those in p38α$^{+/+}$ hearts (Fig. 1). The p38β protein levels showed no significant difference between the groups. Significant p38α activity, measured by immune complex kinase assay using ATF-2 as a substrate, was present in hearts from both p38α$^{+/-}$ and p38α$^{+/+}$. The p38α activity in p38α$^{+/-}$ was $49 \pm 8.5\%$ ($n = 5$) compared to those in p38α$^{+/+}$ hearts. Mouse hearts were first subjected to ischemia for 25 min and then reperfused for 10–30 min. The protein levels of p38α and β remained constant throughout the ischemia–reperfusion (data not shown). p38α activity was significantly increased in hearts from both p38α$^{+/-}$ and p38α$^{+/+}$ subjected to 25 min of ischemia. Upon reperfusion following ischemia, p38α activity was drastically increased at 10 min and returned to the basal level within 30 min. The maximum level of p38α activity in p38α$^{+/-}$ was $60 \pm 10.5\%$ ($n = 5$) compared to that in p38α$^{+/+}$ (Fig. 1B).

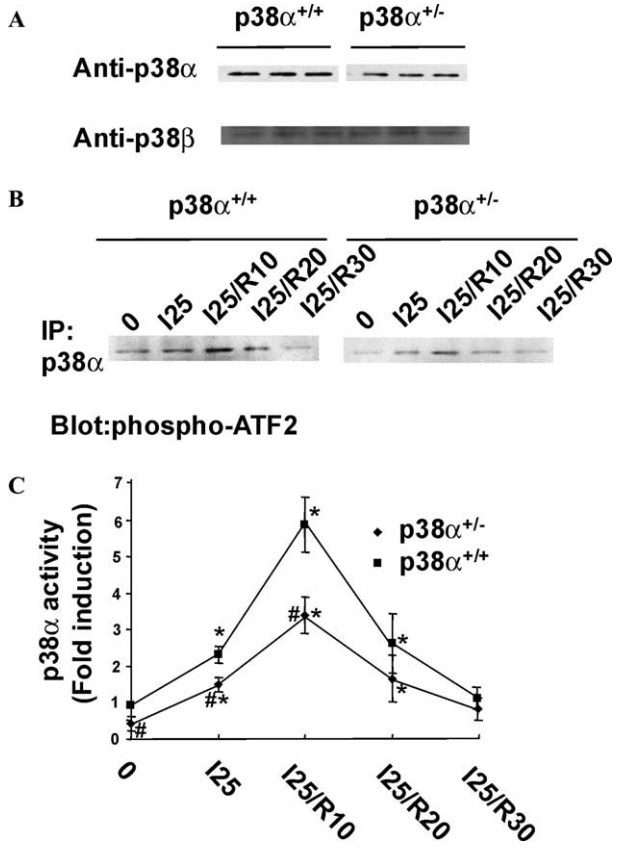

Fig. 1. Western blot analysis on p38 in ischemic-reperfused p38α$^{+/+}$ and p38α$^{+/-}$ hearts. (A) Western blot analysis on the protein levels of p38α and β in extracts from p38α$^{+/+}$ and p38α$^{+/-}$. (B) Western blot analysis of ATF-2 phosphorylation by p38α using antibodies against phospho-ATF2. I25, ischemia for 25 min. I25/R10, ischemia for 25 min and reperfusion for 10 min, I25/R20, ischemia for 25 min and reperfusion for 20 min. I25/R30, ischemia for 25 min and reperfusion for 30 min. (C) Densitometric analysis on ATF-2 phosphorylation by p38α. ATF-2 phosphorylation was expressed as fold stimulation relative to p38α$^{+/+}$ at time zero. *$P < 0.05$ vs time zero, #$P < 0.05$ vs p38$^{+/+}$ at the corresponding time points.

Effect of ischemia–reperfusion on infarct size

To examine the effect of reduced p38α activity on ischemia–reperfusion injury, mice were subjected to 25 min of LCA occlusion followed by 2 h of reperfusion. No significant differences were observed in the rate-pressure product or in the rectal temperature during the infarct protocol among the groups before ischemia or at the end of the ischemic period (data not shown). The area at risk, identified by the lack of Evans blue stain, in the p38α$^{+/-}$ and p38α$^{+/+}$ hearts ($47.1 \pm 2.2\%$ vs $46.2 \pm 2.7\%$) was not significantly different (Fig. 2). In p38α$^{+/+}$ hearts, ischemia–reperfusion resulted in significant necrotic injury, as evidenced by a large area of negative TTC staining ($37.1 \pm 2.7\%$ of the area at risk). In p38α$^{+/-}$, the infarct was limited to the region close to

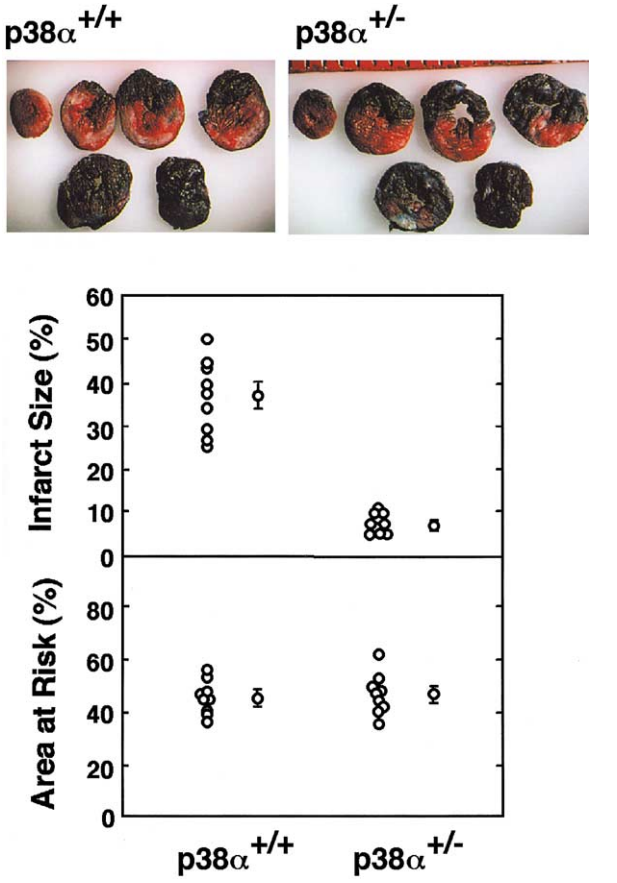

Fig. 2. The size of the myocardial infarct in p38α$^{+/+}$ and p38α$^{+/-}$ subjected to ischemia–reperfusion. The upper panel shows representative images of heart slices double-stained with Evans blue and TTC. The lower panel shows the size of the infarct and ischemic area at risk in mice hearts subjected to ischemia–reperfusion.

the LCA origin where the silk thread was passed around the LCA and infarct size was drastically reduced to $7.2 \pm 0.7\%$ ($P < 0.05$) (Fig. 2).

Discussion

By using the p38 specific inhibitors SB202190 and SB203580, p38 has been shown to be involved in both cell survival and death induced by ischemia–reperfusion. The p38 isoforms may differ in their physiological function [19]. Recent studies in neonatal cardiomyocytes support the concept of a role for p38α in mediating cell death [19,27]. However, these were in vitro studies and used experimental designs that require overexpression of the proteins involved. To identify the exact and physiological roles of p38α in ischemia–reperfusion injury, we employed genetically engineered p38$\alpha^{+/-}$ mice for this study. For this purpose we chose p38$\alpha^{+/-}$ mice with infarct size as an end-point. The results indicated that disruption of a single copy of the p38α MAP kinase gene leads to cardioprotection against ischemia–reperfusion and confirmed the in vitro findings that the strong activation of p38α by reperfusion plays a significant role in subsequent myocardial injury. However, it is possible that compensatory changes in the expression of p38β may lead to the acquisition of tolerance against ischemia–reperfusion. No obvious change in the relative expression level of p38β was observed in either p38$\alpha^{+/-}$ or p38$\alpha^{+/+}$ in hearts in agreement with a previous report examined in isolated embryonic fibroblasts [21]. We failed to estimate p38β activity in heart, because p38β-specific antibody, which is good enough to immunoprecipitate the protein in tissues, is not available. The maximal level of total p38 phosphorylation by ischemia–reperfusion in p38$\alpha^{+/-}$ was about 42% compared to that in control mice (data not shown), suggesting marked compensatory activation of p38β was not likely to occur in p38$\alpha^{+/-}$. These might exclude a possibility that compensatory changes in the expression of p38β may lead to the acquisition of tolerance against ischemia–reperfusion in p38$\alpha^{+/-}$. Thus, the results indicated that the activation of p38α is detrimental in ischemia–reperfusion injury. This conclusion is in agreement with some of the previous reports using the p38 inhibitors. Ma et al. [4] reported that SB203580 administered before global ischemia and continuing in reperfusion decreased infarct size. Schneider et al. [14] noted that SB202190 protected ischemic injury in non-preconditioned in rat hearts. However, this finding contrasts with several reports. Nakano et al. [13] and Mocanu et al. [12] reported that SB203580 does not affect infarct size on its own, but selectively blocks anti-infarct effect of preconditioning in the intact hearts. We have no clear explanations for differences among these reports. However, our results clearly show that the activation of p38α during ischemia–reperfusion is detrimental in our mouse model.

A partial reduction in p38α expression and its accompanying activation resulted in almost complete protection for the heart against ischemia–reperfusion injury. This suggests the existence of specific threshold for p38α activation to promote necrotic cell death induced by lethal stress.

In conclusion, p38$\alpha^{+/-}$ mice attain tolerance of the heart to lethal ischemia–reperfusion insult, which suggests a role for p38α in mediating cell death. Since previous studies [4,5,15] using the SB compounds showed only limited beneficial effects of p38 inhibition on cell death, an isoform-specific inhibition of p38α may be the best target for prevention of ischemia–reperfusion injury.

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