Role of Phasic Dynamism of p38 Mitogen-Activated Protein Kinase Activation in Ischemic Preconditioning of the Canine Heart

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Abstract—Although ischemic stress, including ischemic preconditioning (IP), activates p38 mitogen-activated protein kinase (MAPK), the relationship between p38 MAPK activation and the underlying cellular mechanisms of cardioprotection by IP is not verified in vivo. We examined the effects of the selective p38 MAPK inhibition on the cardioprotective effect of IP in the open-chest dogs. The coronary artery was occluded 4 times for 5 minutes, separated by 5 minutes of reperfusion (IP) followed by 90 minutes of occlusion and 6 hours of reperfusion. We infused SB203580 into the coronary artery during IP and 1 hour of reperfusion, during IP alone, and during sustained ischemia in the IP group. p38 MAPK activity markedly increased during IP but did not additionally increase at the onset of ischemia and was even attenuated at 15 minutes of sustained ischemia, and heat-shock protein (HSP) 27 was phosphorylated and translocated from cytosol to myofibril or nucleus without affecting total protein level at the onset of ischemia compared with the control group. SB203580 treatment (1 μmol/L) only during IP blunted the infarct size limitation by IP (37.3±6.3% versus 7.4±2.1% in the IP group, P<0.01) and attenuated either phosphorylation or translocation of HSP27 during IP. Although the SB203580 treatment throughout the preischemic and postischemic periods had no significant effect on infarct size (33.3±9.4%) in this model, treatment with SB203580 only during ischemia partially mimicked the infarct size limitation by IP (26.8±3.5%). Thus, transient p38 MAPK activation during ischemic preconditioning mainly mediates the cardioprotection followed by HSP27 phosphorylation and translocation in vivo in the canine heart. (Circ Res. 2001;88:175-180.)

Key Words: p38 mitogen-activated protein kinase ▪ heat-shock protein 27 ▪ ischemic preconditioning ▪ infarct size ▪ canine heart

Brief periods of ischemia, which precede sustained ischemia, limit infarct size markedly, a phenomenon known as ischemic preconditioning (IP).1,2 The underlying mechanisms have been studied extensively.3–5 Recent studies on the subcellular mechanisms of ischemia/reperfusion injury revealed that p38 mitogen-activated protein kinases (MAPks) are phosphorylated and activated by ischemic stress6–11 or other stresses.12,13 Importantly, p38 MAPKs transduce the extracellular stimuli to the nuclei or other compartments as well as extracellular signal-regulated kinases (ERKs) or c-Jun N-terminal kinases (JNKs). Furthermore, MAPK-activated protein kinase-2 (MAPKAPK-2), located downstream of p38 MAPK,7,14 is known to phosphorylate 27-kDa small heat-shock protein (HSP27),15–17 which plays a protective role against ischemic19 or oxidative20 stress. The translocation of HSP27 from cytosol to myofibril or nucle-11 may prevent actin fragmentation23 or microtubule degradation.24 Thus, both p38 MAPK and HSP27 might be involved in the pathophysiology in the protection against myocardial ischemia/reperfusion injury. Recently, some studies showed that p38 MAPK activation during IP mediates its beneficial effect,6–8 whereas other studies report that the p38 MAPK inhibition during ischemia and reperfusion protects myocardium.9–11 However, the cause-and-effect relationship...
between p38 MAPK activation and the infarct size–limiting effect is not verified in vivo. Therefore, we examined the effect of the selective inhibitor of p38 MAPK, SB203580 (SB), on infarct size, p38 MAPK activity, and HSP27 activation during or after IP using an in vivo infarction model of the canine heart.

Materials and Methods

All procedures were performed in careful conformance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (publication No 85-23, revised 1985).

Instrumentation

Beagle dogs (Oriental Yeast, Tokyo, Japan) weighing 9 to 14 kg were prepared as described previously. In all experiments, mean arterial blood pressure (ABP), heart rate (HR), and P02 in the systemic arterial blood in control conditions averaged 105±2.3 mm Hg, 132±2.4 beats per minute, and 106±2.6 mm Hg, respectively. Both ABP and HR were measured continuously during the experiment.

Protocol 1: Effect of p38 MAPK Inhibition on IP

We used 69 dogs in this protocol. Figure 1A indicates all details of the schedules of this protocol. IP procedure was performed by 4 cycles of 5 minutes of coronary occlusion and a subsequent 5 minutes of reperfusion by occluding the bypass tube. Then the coronary artery was occluded for 90 minutes (sustained ischemia) followed by 6 hours of reperfusion.

The dose of SB for an intracoronary infusion was 1.18 μg·kg⁻¹·min⁻¹, theoretically corresponding to 1 μmol/L at the coronary arterial blood. This concentration has been shown in previous studies to specifically inhibit p38 MAPK.9,10,18,20 We used the vehicle dimethyl sulfoxide at a final concentration of <0.15%, which does not influence infarct size.26

Protocol 2: Effect of IP on p38 MAPK Activity

We used 28 dogs in this protocol. Figures 1B and 1C indicate all details of the schedules of this protocol. We quickly sampled myocardial tissue supplied by the left anterior descending coronary artery (LAD) into liquid nitrogen and stored it at −80°C.

To immunoprecipitate p38 MAPK, 1 gram of myocardial tissue was homogenized, and in vitro kinase assay was carried out as described previously. A part of the samples were used for the measurement of MAPKAPK-2 using a commercially available immunoprecipitation-kinase assay kit (Upstate Biotechnology).

Protocol 3: Effect of IP on the Phosphorylation and Translocation of HSP27

We used 19 dogs in this protocol. With or without the IP procedure, we infused SB (1.18 μg·kg⁻¹·min⁻¹ intracoronary infusion) or saline into the LAD in the identical manner with protocol 2 (IP + SB group, n=5; IP group, n=4; SB group, n=5; and control group, n=5). We quickly sampled myocardial tissue supplied by the LAD just before the sustained ischemia and stored it at −80°C.

To evaluate the phosphorylation of HSP27, each specimen was subjected, immunoblotted, and measured as described previously, with modification, using the antibodies to either HSP27 or phosphospecific HSP27 at Ser 78 (Upstate Biotechnology).

To evaluate the intracellular translocation of HSP27, each specimen in protocol 3 was homogenized, separated into P1 (nuclear and myofibrillar), P2 (membranous), and S (cytosolic) fractions, immunoblotted, and measured as described previously.

Criteria for Exclusion

To ensure that all animals included in the data analysis of infarct size were healthy and exposed to similar extents of ischemia, exclusion criteria described previously were used.

Measurement of Infarct Size and Myocardial Collateral Blood Flow

We measured infarct size and regional myocardial blood flow as described previously, with modification. For randomization of the study, all measurements were done at the completion of protocol without knowledge of the treatment in each heart.

Statistical Analysis

Each value was expressed as mean±SEM. Statistical analysis was performed using ANOVA with Fisher’s post hoc test or ANCOVA.
between regional collateral blood flow and infarct size, as described previously, with \( P < 0.05 \) indicating a significant difference. An expanded Materials and Methods section can be found in an online data supplement available at http://www.circresaha.org.

**Results**

**Mortality and Exclusions**

Of 69 dogs that were randomly devoted to 10 groups for assessment of infarct size in protocol 1, 16 dogs developed lethal arrhythmia (ventricular tachycardia or fibrillation) at least once. Among these 16 dogs, lethal arrhythmia to match the exclusion criteria occurred in 4 dogs during 90 minutes of ischemia and 5 dogs during reperfusion after 90 minutes of ischemia. These 9 animals were excluded from assessment of infarct size. In the remaining 60 dogs, 5 were excluded from the data analysis because myocardial collateral blood flow was greater than 15 mL/100 g per min. Therefore, 55 dogs completed the protocols satisfactorily and were used for data analysis.

**Changes in Hemodynamic Parameters, Risk Area, and Collateral Blood Flow**

There were no significant differences in mean ABP and HR among all groups in each experiment. The risk area and collateral flow at 80 minutes of sustained ischemia were comparable in all groups in each experiment.

**Infarct Size**

Figure 2 shows infarct size in the 8 groups in protocol 1 (Figure 2A) and the regression plots of infarct size in protocol 1 as a percentage of the area at risk against collateral flow in all groups (Figure 2B). IP (group 6) markedly attenuated infarct size compared with the control group (group 1) (7.4±2.1% versus 38.9±4.7%, respectively, \( P < 0.01 \)). The treatment with SB, the potent inhibitor of p38 MAPK, only in preischemic phase blunted the infarct size-limiting effect of IP (37.3±6.3% in the IP+preSB group [group 8], \( P < 0.01 \) versus IP), although SB (1.18 \( \mu g \cdot kg^{-1} \cdot min^{-1} \)) during ischemia failed to affect the infarct size limitation afforded by IP (7.9±1.2% in the IP+ischemia SB [IscSB] group [group 7]). In our preliminary experiments, we observed that the intracoronary administration of SB (11.8 \( \mu g \cdot kg^{-1} \cdot min^{-1} \)) for only 5 minutes before the sustained ischemia did not change the infarct size in any of the control or IP groups (n=4 each, data not shown). Furthermore, SB during the preischemic and postischemic periods failed to protect the myocardium (33.3±9.4% in the SB group [group 4] versus 37.4±4.8% in the vehicle group [group 2]). However, the treatment with SB (1.18 \( \mu g \cdot kg^{-1} \cdot min^{-1} \)) during the sustained ischemia showed the partial but significant infarct size limitation (26.8±3.5% in the IscSB group [group 5] versus 41.6±5.8% in the ischemia vehicle [IscVehicle] group [group 3], \( P < 0.05 \)). On the other hand, treatment with the vehicle did not affect infarct size attributable to 90 minutes of ischemia followed by 6 hours of reperfusion during the sustained ischemia (IscVehicle group [group 3]) or during the preischemic or postischemic period (vehicle group [group 2]) (41.6±5.8% and 37.4±4.8%, respectively).

**p38 MAPK Activity During IP and After Sustained Ischemia**

Figure 3 shows representative cases in p38 MAPK activity (left), the substrate of activated p38 MAPK, and the MAPKAPK-2 activity (right), estimated by the amount of phosphorylated substrate protein. Immunoblotting of the representative specimens (top left for p38 MAPK and top right for MAPKAPK-2) indicated by arrows and the changes in mean value (bottom left, n=3 or 4 each for p38 MAPK and bottom right, n=3 each for MAPKAPK-2) are demonstrated. \( * P < 0.05 \) vs other group.
marked increase in p38 MAPK activity during IP. Interestingly, p38 MAPK was no longer activated at the end of IP procedure and returned to the control levels. Fifteen minutes after the onset of sustained ischemia, p38 MAPK activity increased only in the control group, which was attenuated in the IP group. Furthermore, 20 minutes after the onset of sustained ischemia, MAPKAPK-2 activation increased when SB compound was pretreated in the SB group (group 4 in Figure 1C), whereas it was attenuated when SB compound was continuously treated during sustained ischemia in the IscSB group (group 5 in Figure 1C).

Phosphorylation and Translocation of HSP27 During IP

Figure 4A shows the representative cases in Western blotting of total HSP27 in each fraction (top) and of total or phosphospecific HSP27 (bottom) for 4 groups in protocol 3. The mean values for n=4 or 5 each (B) of the total HSP27 in each fraction (left) and of total or phosphospecific HSP27 (right) are indicated in panel B. All values are shown as the relative score, representing the total amount of HSP27 in the control group for the value 1. S indicates cytosolic fraction; P1, myofibrillar and nuclear fraction; and P2, membranous fraction. *P<0.05 vs control.

Discussion

The cardioprotection caused by IP has been intensively investigated basically and clinically, because it has been confirmed that IP limits infarct size.1–3,5,7,25,26 Nevertheless, subcellular mechanisms remain unclear despite a great deal of effort by many research groups. In the present study, we clarified that IP causes the phasic increase in P38 MAPK, transient strong activation during IP, and the inhibition after sustained ischemia. Furthermore, transient activation of p38 MAPK during IP, much more than during sustained ischemia or during reperfusion, mediates the infarct size-limiting effect of IP as a trigger through the phosphorylation and translocation of HSP27 to myofibril in in vivo canine heart.

Activation of p38 MAPK During IP

Because p38 MAPK activity shows the phasic pattern during and after IP and the inhibition of p38 MAPK during sustained ischemia10,11 or in early phase of reperfusion after lethal ischemia9 mediates cardioprotection, it is likely that the transient activation of p38 MAPK triggers the cardioprotection of IP or that the deactivation of p38 MAPK after the onset of lethal ischemia mediates cardioprotection. The present study revealed that the inhibition of p38 MAPK during the IP procedure using SB completely reverses the infarct size-limiting effect and that although the intracoronary pretreatment with SB influences neither infarct size nor the infarct size limitation by IP, continuous existence of SB during sustained ischemia protects the myocardium. This observation strongly suggests the important role of the transient activation of p38 MAPK in the attenuation of ischemia/reperfusion injury after the IP procedure, as supported by previous studies.7,8,10 However, we cannot deny the possibility that the deactivation of p38 MAPK during sustained ischemia may also mediate the cardioprotection of IP in the present study, which is also supported by several investigations.9–11 In the present study, SB administration before and after 90 minutes of ischemia did not reduce infarct size as was seen in IP, but the continuous treatment with SB during sustained ischemia partially mimicked the infarct size–limiting effect of IP. This observation may clarify the disparity between the effects of preischemic treatment with SB and the continuous treatment with SB during sustained ischemia in the present study. In dogs, there is substantial collateral blood flow; therefore, the pretreated drug in the ischemic region may be washed out, thereby showing no significant effect. On the other hand, the continuous infusion of SB into the ischemic region can avoid the washout even in the canine hearts, which assures the effect of SB during sustained ischemia. Therefore, the washout of the drug attributable to collateral blood flow may account for the disparity in this model, and SB during ischemia in the present...
model may also have the infarct size–limiting effect, as strongly suggested by the difference in the present study comparing the MAPKAPK-2 activity between the SB and the IscSB groups during sustained ischemia. This may also account for the difference between our present observation and a previous study using the in vivo swine model,11 because the swine model has no collateral flow. The evidence of the activation of p38 MAPK during sustained ischemia and the limited effects of SB administered during sustained ischemia on infarct size in the present study suggests that there may be additional inhibition of p38 MAPK for the infarct size-limiting effect of IP in canine hearts during sustained ischemia. On the other hand, previous studies7,8 show that IP increases p38 MAPK activity during sustained ischemia, although Mackay et al10 and the present study showed that IP prevents prolonged activation of p38 MAPK during sustained ischemia. The differences in the activity of p38 MAPK during sustained ischemia7–11 may be attributable to the experimental models, protocols for the IP procedure, species differences, or time of myocardial ischemia.

The scenario that transient activation of p38 MAPK triggers the infarct size–limiting effect of IP may be quite similar to protein kinase C (PKC) activation, which has been largely accepted to mediate the cardioprotection of IP.25–31 PKC activation after the onset of reperfusion is no longer cardioprotective, and after this period, PKC inhibition does protect the myocardium.14 Other studies show that p38 MAPK is downstream of PKC,32 which has also yet to be clarified in our model. On the other hand, one recent study33 mentions that SB can also inhibit JNKs in a higher dose, whereas 1 μmol/L of SB is specific for the inhibition of p38 MAPK, suggesting that the modulation of cardioprotection in this study may be independent of JNKs. This is additionally supported in other studies.7,10

Involvement of the Phosphorylation and Translocation of HSP27 in IP

We also observed that IP increased the translocation of HSP27 associated with the increase in phosphorylation of HSP27, both of which were blunted by SB in this model. The observations that HSP27 is translocated by p38 MAPK activation21,22,34 or is linked with cardioprotection18–20 are described in some recent studies, although there are other candidates for pathways that can activate HSP27. Furthermore, the positive role of HSP27 phosphorylation is also stated in other systems,15–20 and the synchronicity of the phosphorylation and translocation of HSP27 is established in other types of cells.35 Taking these findings together, the phosphorylation and translocation of HSP27 may also occur sequentially in this model. In addition, the overexpression of HSP27 itself is reported to exert cardioprotection.36,37 The recent studies investigating the molecular mechanisms of HSP27-induced cell protection show that HSP27 binds to Z-bands of myofibrils21,22 or carboxyl-terminal region of the protein48 and protects against conformation changes or fragmentation in myofibril and cytoskeleton.24,37 Furthermore, a recent study shows that HSP27 negatively regulates cell death by preventing the interaction of Apaf-1 with procaspase-9 through binding with cytochrome-c, which is released from mitochondria and enhances this interaction.39

Time Point for the Activation of p38 MAPK Among Repeated Cycles of a Brief Period of Ischemia

It is important to consider how many cycles of a brief period of myocardial ischemia are necessary to activate p38 MAPK. To answer this issue, we should investigate the exact time course of p38 MAPK/HSP27 activation during 4 cycles of ischemia and reperfusion of the IP procedure in this model. The heart can be sufficiently preconditioned after first occlusion, and p38 MAPK is fully activated at the end of the first transient ischemia in IP in the rabbit.40 Although this may also be the case in the present model, this has yet to be clarified.

These findings may indicate that ischemic damages accumulate after the second occlusion of the IP procedure, although the repetition of 5 minutes of ischemia does not cause myocardial injury or impair the IP-induced cardioprotection. Sampling at more frequent time points might clarify this issue.

Clinical Implication

Because the present results suggest that the phosphorylation and translocation of HSP27 after the transient activation of p38 MAPK is beneficial, the way to translocate HSP27 may be essential for the mediation of IP. HSP27 may protect the myofibril and cytoskeleton, whereas KATP channel opening, another possible mediator of IP, inhibits calcium overload or improves energy condition at mitochondria. Therefore, we need to identify the pharmacological or molecular biological methods, including the gene transfer to activate HSP27, and we need to use this method in combination with others to open KATP channels (eg, nicorandil) in patients with ischemic heart disease.

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