SAPKs regulation of ischemic preconditioning

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Sato, Motoaki, Gerald A. Cordis, Nilanjana Maulik, and Dipak K. Das. SAPKs regulation of ischemic preconditioning. Am J Physiol Heart Circ Physiol 279: H901–H907, 2000.—The role of stress-activated protein kinases (SAPKs), c-Jun NH₂-terminal kinase (JNK) and p38 mitogen-activated protein (MAP) kinase, in preconditioning (PC) was examined with the use of isolated rat hearts subjected to four cyclic episodes of 5-min ischemia and 10-min reperfusion followed by 30-min ischemia and 2-h reperfusion (I/R). A group of hearts was preperfused with 100 μM curcumin, a c-Jun and JNK1 inhibitor, or 5 μM SB 203580, a p38 MAP kinase inhibitor. Another group of hearts was preperfused with 20 μM anisomycin, a stimulator for both JNK and p38 MAP kinases. I/R increased the protein levels of JNK1, c-Jun, and p38 MAP kinase. PC also enhanced the induction of these kinases, but subsequent I/R-mediated increase was blocked by PC. Curcumin blocked I/R- and PC-mediated increase in JNK1 and c-Jun protein levels, whereas it had no effects on p38 MAP kinase. SB 203580, on the other hand, was equally effective in reducing the p38 MAP kinase activation but exerted no effects on JNK1 and c-Jun induction. I/R-mediated increased myocardial infarction was reduced by any of the following compounds: anisomycin, curcumin, and SB 203580. The cardioprotective effects of PC were abolished by either curcumin or SB 203580. The results demonstrate that PC is mediated by a signal-transduction pathway involving both JNK1 and p38 MAP kinase. Activation of SAPKs, although transient, is obligatory for PC.

MATERIALS AND METHODS

ISOLATED PERFUSED HEART PREPARATION

All animals received care in compliance with the principles of laboratory animal care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals published by the National Research Council. Eighty Sprague-Dawley rats weighing ~300 g were anesthetized with pentobarbital (80 mg/kg ip). After intravenous administration of heparin (500 IU/kg), the chests were opened, and the hearts were rapidly excised and mounted on a non-recirculating Langendorff perfusion apparatus (6). Retrograde perfusion was established at a pressure of 100 cmH₂O with an oxygenated normothermic Krebs-Henseleit bicarbonate (KHB) buffer with the following ion concentrations (in mM): 118.0 NaCl, 24.0 NaHCO₃, 4.7 KCl, 1.2
KH₂PO₄, 1.2 MgSO₄, 1.7 CaCl₂, and 10.0 glucose. The KHB buffer had been previously equilibrated with 95% O₂-5% CO₂, pH 7.4, at 37°C. The hearts, perfused via working mode (6), were randomly divided into two groups: ischemia-reperfusion (I/R) study group and preconditioning (PC) study group. Each group was subdivided into five groups. The I/R study group was subdivided as follows: isolated hearts 1) perfused with KHB buffer for 2 h and 45 min (control), 2) perfused with KHB buffer for 15 min followed by 30 min of global ischemia and 2 h of reperfusion, 3) perfused with 20 μM anisomycin for 15 min followed by 30 min of ischemia and 2 h of reperfusion, 4) perfused with 100 μM of curcumin followed by 30 min of ischemia and 2 h of reperfusion, and 5) perfused with 5 μM SB 203580 followed by 2 h of reperfusion. The PC study group was subdivided as follows: isolated hearts 1) perfused with KHB buffer for 3 h and 45 min (control); 2) perfused with KHB buffer for 75 min followed by 30 min of ischemia and 2 h of reperfusion; 3) perfused with KHB buffer for 15 min and then subjected to PC protocol by induction of four cycles of 5-min ischemia, each followed by 10 min of reperfusion, and further subjected to 30 min of ischemia followed by 2 h of reperfusion; 4) hearts perfused for 15 min with KHB buffer containing 100 μM curcumin followed by PC, ischemia, and reperfusion as described for group 3; and 5) hearts perfused for 15 min with KHB buffer containing 5 μM SB 203580 followed by PC, ischemia, and reperfusion as described for group 3. The protocol of the experiment is depicted in Fig. 1. Experiments were terminated at various points, and hearts were processed to evaluate the abundance of JNK1, p38 MAP kinase, and c-Jun by Western blot analysis.

The aortic flow rate was measured by a calibrated rotameter, and the coronary flow rate was measured by timed collection of the coronary effluent. Continuous cardiac pressure measurements were recorded. All measurements were analyzed in real time with the use of a data acquisition, analysis, and presentation system. Direct measurements of heart rate, developed pressure (DP; defined as the aortic systolic minus end-systolic pressure), and the first derivative of the maximal aortic pressure (dP/dt max) were made at each time point.

Infarct Size Estimation

Hearts to be used for infarct size calculations (n = 7 for each group) were taken on termination of the experiment and immersed in 1% triphenyltetrazolium solution in phosphate buffer (88 mM Na₂HPO₄, 1.8 mM NaH₂PO₄) for 20 min at 37°C; they were then stored at −70°C for later processing (19). Frozen hearts (including only ventricular tissue) were sliced transversely in a plane perpendicular to the apico-basal axis into ∼1-mm-thick sections, blotted dry, placed in between microscope slides, and scanned on a Hewlett-Packard Scanjet 5p single-pass flatbed scanner (Hewlett-Packard, Palo Alto, CA). With the use of NIH Image 1.6.1 image...

Fig. 1. Experimental protocol. KHB, Krebs-Henseleit bicarbonate buffer; S, stabilization; PC, preconditioned; A, anisomycin; C, curcumin; SB, SB 203580; I, ischemia; R, reperfusion; 30I, 30 min of ischemia.

Fig. 2. Myocardial abundance of phosphorylated c-Jun NH₂-terminal kinase (JNK)-1 (A), c-Jun (B), and p38 mitogen-activated protein (MAP) kinase (C). Tissue proteins were prepared from hearts at the end of each experiment, separated on 10% SDS-PAGE, and transferred to Immobilon-P membranes. The phosphorylated JNK1 was detected by Western blotting with the use of specific antibodies as described in MATERIALS AND METHODS. Results are representative of 3 similar experiments/group. Lane 1, control; lane 2, 30 min of ischemia and 2 h of reperfusion (I/R); lane 3, anisomycin; lane 4, curcumin; lane 5, SB 203580. Densitometric scanning for each blot (means ± SE of 6 different experiments/blot) is shown above Western blots. *P < 0.05 compared with control. †P < 0.05 compared with PC hearts.
processing software, each digitized image was subjected to background subtraction, contrast enhancement, and grayscale conversion for improved clarity and distinctness. Risk as well as infarct zones of each slice were traced, and the respective areas were calculated in terms of pixels. The weight of each slice was then recorded to facilitate the expression of total and infarct masses of each slice in grams. The risk and infarct volumes (in ml) of each slice were then calculated on the basis of slice weight to remove the introduction of any errors due to nonuniformity of heart slice thickness. The risk volumes and infarct volumes of each slice were summed to obtain the risk and infarct volumes for the whole heart. Infarct size was taken to be the percent infarct thickness. The risk volumes and infarct volumes of each slice were then compared with the use of Student’s t-test for each group.

Western Blot Analysis

To quantify the abundance of JNK1, p38 MAP kinase, and c-Jun protein, heart tissues were homogenized and suspended (5 mg/ml) in sample buffer (10 mM HEPES, pH 7.3, 11.5% sucrose, 1 mM EDTA, 1 mM EGTA, 1 mM diisopropyl fluorophosphate, 0.7 mg/ml pepstatin A, 10 mg/ml leupeptin, and 2 mg/ml aprotinin). Proteins were then solubilized with the addition of the same amount of 2× Laemmli solution [9% (wt/vol) SDS, 6% (vol/vol) β-mercaptoethanol, 10% (vol/vol) glycerol, and a trace amount of bromophenol blue dye in 0.196 M Tris·HCl (pH 6.7)]. The cellular proteins (50-μl samples) were electrophoresed through 10% SDS-PAGE and then transferred to Immobilon-P membranes (Millipore) by use of a semi-dry transfer system (Bio-Rad). Prestained protein standards (Bio-Rad) were run in each gel. The blots were blocked in Tris-buffered saline-Tween 20 (containing 20 mM Tris base, pH 7.6, 137 mM NaCl, and 0.1% Tween 20) supplemented with 5% BSA for 1 h; incubated with a 1:1,000 dilution of primary rabbit antibodies, specifically against either Tyr-182-phosphorylated p38 MAP kinase (NEB) or JNK1 (Santa Cruz Biotech) for 2 h; and then incubated with 1:10,000 diluted secondary antibodies of horseradish peroxidase-conjugated anti-rabbit IgG (Boehringer Mannheim) for 1 h at room temperature. After three washes of 5 min each, blots were treated with enhanced chemiluminescence (ECL from Amersham) reagents, and the phosphorylated MAP kinase, JNK1, and c-Jun were detected by autoradiography for variable lengths of time (15 s to 3 min) with Kodak X-Omat film. Each blot was scanned with a scanning densitometer, and the results are shown as means ± SE of six different blots per group.

Statistical Analysis

For statistical analysis, a two-way ANOVA followed by Scheffé’s test was first carried out with the use of PRIMER computer program (McGraw-Hill) to test for any differences between groups. If differences were established, the values were compared with the use of Student’s t-test for paired data. The values were expressed as means ± SE. The results were considered significant at P < 0.05.

RESULTS

Studies with I/R Group

Effects of I/R on JNK1 and p38 MAP kinase expression. Thirty minutes of ischemia followed by 2 h of reperfusion significantly enhanced the amount of protein levels of JNK1 (Fig. 2A). Preperfusion of the hearts with anisomycin also significantly enhanced the

Table 1. Effects of ischemia-reperfusion, anisomycin, curcumin, and SB 203580 on postischemic cardiac function

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Ischemia-Reperfusion</th>
<th>Anisomycin</th>
<th>Curcumin</th>
<th>SB 203580</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B</td>
<td>P</td>
<td>B</td>
<td>I/R</td>
<td>B</td>
</tr>
<tr>
<td>Heart rate,</td>
<td></td>
<td></td>
<td>B</td>
<td>I/R</td>
<td></td>
</tr>
<tr>
<td>beats/min</td>
<td>285±5.5</td>
<td>282±3.8</td>
<td>290±4.8</td>
<td>278±5.5</td>
<td>283±4.0</td>
</tr>
<tr>
<td>Developed</td>
<td></td>
<td></td>
<td>B</td>
<td>I/R</td>
<td></td>
</tr>
<tr>
<td>pressure, mmHg</td>
<td>95.4±3.4</td>
<td>98.0±2.9</td>
<td>101.0±5.1</td>
<td>42.4±3.5*</td>
<td>94.5±4.2</td>
</tr>
<tr>
<td>dP/dt_{max}, mmHg/s</td>
<td>3.8±2.5</td>
<td>3.7±2.2</td>
<td>3.9±2.1</td>
<td>3.2±2.2</td>
<td>3.7±5.0</td>
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<tr>
<td>Coronary flow,</td>
<td></td>
<td></td>
<td>B</td>
<td>I/R</td>
<td></td>
</tr>
<tr>
<td>ml/min</td>
<td>19.5±0.8</td>
<td>18.8±0.9</td>
<td>20.3±1.0</td>
<td>18.1±1.4</td>
<td>18.0±0.7</td>
</tr>
</tbody>
</table>

Values are means ± SE. Control hearts were perfused (P) for 2 h and 45 min with Krebs-Henseleit bicarbonate (KHB) buffer. Ischemia-reperfusion hearts were subjected to 30 min of ischemia followed by 2 h of reperfusion (I/R). Anisomycin hearts were preperfused for 15 min with anisomycin for preconditioning. Curcumin hearts were preperfused for 15 min with curcumin followed by I/R. SB 203580 hearts were preperfused for 15 min with SB 203580 followed by I/R. dP/dt_{max}, first derivative of maximal aortic pressure. Results are shown at baseline level (B) and at the end of I/R. *P < 0.05 compared with control. †P < 0.05 compared with I/R.
amount of JNK1. However, anisomycin treatment significantly blocked the subsequent further enhancement of JNK1 due to I/R (data not shown). Curcumin blocked the expression of JNK1, whereas SB 203580 had no effects on JNK1 protein levels.

Similar to JNK1, I/R stimulated the induction of p38 MAP kinase protein (Fig. 2C). Again, anisomycin activated p38 MAP kinase phosphorylation after 15 min of perfusion (data not shown) but depressed further enhancement of I/R-mediated activation of p38 MAP kinase phosphorylation. I/R-induced p38 MAP kinase-phosphorylated protein level was blocked by SB 203580, whereas protein level remained unchanged after curcumin treatment.

**Effects of I/R on c-Jun expression.** Two hours of reperfusion after 30 min of global ischemia increased the abundance of the protein level of c-Jun (Fig. 2B). The I/R-mediated enhancement of c-Jun protein level was blocked by curcumin, whereas SB 203580 had no effect. Anisomycin induced the amount of c-Jun in the preischemic myocardium (data not shown) but failed to further increase the protein level after subsequent ischemia and reperfusion (Fig. 2B).

**Effects of I/R, anisomycin, curcumin, and SB 203580 on myocardial infarct size.** Thirty minutes of ischemia followed by 2 h of reperfusion produced a significant amount of myocardial infarction (Fig. 3). For example, at the end of reperfusion, the infarct size (expressed as the percentage of area at risk) was 36.3 ± 4.7 (P < 0.05) compared with only 3.2 ± 2.5. Infarct size was significantly reduced, compared with control perfused hearts, by pretreatment of the hearts with either anisomycin (24.3 ± 2.2) or curcumin (20.5 ± 2.3) or SB 203580 (19.0 ± 2.1) (P < 0.05 each, compared with I/R).

**Effects of I/R, anisomycin, curcumin, and SB 203580 on cardiac function.** As shown in Table 1, cardiac function remained unaltered after perfusion of the heart for 2 h and 45 min with the KHB buffer. Thirty minutes of ischemia followed by 2 h of reperfusion lowered the values for DP, dP/dt max, and aortic flow (42.4 ± 3.5 mmHg, 1,589 ± 122 mmHg/s, and 17.8 ± 2.0 ml/min, respectively) compared with the corresponding controls (98.0 ± 2.9 mmHg, 3,723 ± 112 mmHg/s, and 39.4 ± 1.8 ml/min, respectively). Anisomycin exerted PC stimulus as evidenced by its ability to improve I/R-induced lowered DP, dP/dt max, and aortic flow to 68.7 ± 2.8 mmHg, 2,332 ± 57 mmHg/s, and 25.0 ± 0.8 ml/min, respectively. Preperfusion of the hearts with either curcumin or SB 203580 before I/R improved cardiac function compared with the I/R group (Table 1). Coronary flow and heart rate remained unaltered by any treatment protocol.

**Studies with PC Group**

**Effects of PC on JNK1 and p38 MAP kinase.** PC of the heart resulted in the increased induction of JNK1 protein levels compared with control perfused group (Fig. 4A). However, PC-induced increase in the JNK1 level was significantly reduced during subsequent ischemia and reperfusion. Curcumin inhibited PC-mediated increased abundance of JNK1 protein level, whereas SB 203580 had no effect on JNK1 protein level. p38 MAP kinase was also induced by PC (Fig. 4C), which was reduced slightly after subsequent I/R. SB 203580, but not curcumin, inhibited the PC-mediated increase in p38 MAP kinase protein level.

**Effects of PC on c-Jun.** PC also enhanced the synthesis of c-Jun protein level; the abundance of c-Jun was significantly reduced during subsequent I/R (Fig. 4B). The PC-induced enhancement of c-Jun was blocked by curcumin, whereas SB 203580 had no effect on the amount of c-Jun induced by PC.
Effects of PC, curcumin, and SB 203580 on myocardial infarct size. PC of the heart with cyclic episodes of ischemia and reperfusion reduced the amount of infarct size significantly (19.5 ± 1.4) (P < 0.5 compared with I/R) compared with that for I/R (39.2 ± 4.3) (Fig. 5). The infarct size-lowering ability of PC was almost completely abolished by pretreatment of the hearts with either curcumin (31.4 ± 2.8) or SB 203580 (33.7 ± 2.7) (P < 0.05 compared with PC).

Effects of I/R, PC, curcumin, and SB 203580 on cardiac function. As shown in Table 2, cardiac function remained practically unaltered after perfusion of the heart for 3 h and 45 min with the KHB buffer. Sixty minutes of perfusion with KHB buffer followed by 30 min of ischemia followed by 2 h of reperfusion lowered the values for DP, dP/dt, and aortic flow (37.0 ± 2.8 mmHg, 1,320 ± 78 mmHg/s, and 13.5 ± 1.2 ml/min, respectively) compared with the corresponding controls (94.0 ± 3.2 mmHg, 3,496 ± 89 mmHg/s, and 37.4 ± 1.1 ml/min, respectively). PC of the hearts by four cyclic episodes of 5-min ischemia and 10-min reperfusion improved I/R-induced lowered DP, dP/dt, and aortic flow to 64.4 ± 3.3 mmHg, 2,235 ± 116 mmHg/s, and 28.0 ± 0.5 ml/min, respectively. Preperfusion of the hearts with either curcumin or SB 203580 before PC partially abolished the cardioprotective effects of PC (Table 2). Again, heart rate and coronary flow were not affected by any of the above treatments.

DISCUSSION

The results of our study document that 30 min of ischemia followed by 2 h of reperfusion increased the induction of JNK1, c-JUN, and p38 MAP kinase proteins. Ischemic preconditioning also enhanced these kinases compared with control. However, the subsequent I/R-mediated increase in JNK1, p38 MAP kinase, and c-Jun was blocked by preconditioning. Fifteen minutes of perfusion with anisomycin increased the amount of both JNK1 and p38 MAP kinase as well as c-Jun in the heart, all of which were decreased in amount after subsequent I/R. Curcumin blocked the I/R- and preconditioning-mediated increase in JNK1 and c-Jun, whereas it had no effect on p38 MAP kinase. SB 203580, on the other hand, was equally effective in reducing the amount of p38 MAP kinase but exerted no effects on JNK1 and c-Jun. I/R-mediated increased myocardial infarction was reduced by treatment of the hearts with anisomycin, curcumin, or SB 203580. The cardioprotective effects of preconditioning were abolished by either curcumin or SB 203580.

A number of previous studies demonstrated that JNKs are activated during the reperfusion of ischemic heart.
myocardium (12, 17). There are two members of the JNK family, JNK1 (46 kDa) and JNK2 (55 kDa), both of which can phosphorylate c-Jun on specific NH₂-terminal serine residues (13). Existing reports support the role of both JNK1 and JNK2 in myocardial I/R injury (1). The results of the present study document that JNK1 is activated by both I/R and preconditioning. However, activation of JNK1 during preconditioning appears to be transient, because the amount of JNK1 protein levels came down to near baseline values after subsequent ischemia and reperfusion. Consistent with these findings, we also noticed induction of the expression of c-Jun in the I/R and preconditioned myocardium. Induction of the expression of c-Jun mRNA during I/R and preconditioning was reported previously (4). JNK1 not only possesses high affinity for the transcription factor c-Jun, but it also stimulates the expression of genes containing c-Jun-responsive promoter elements (20).

Although preconditioning-mediated activation of JNK1 is transient, such activation appears to be obligatory for preconditioning, because inhibition of JNK1 activation by curcumin blocked the cardioprotective effects of preconditioning. Curcumin is a dietary pigment that has been shown to inhibit JNK (2) and c-Jun (11). Transient activation of JNK1 by anisomycin mimicking the preconditioning effects on heart further supports an essential role of JNK1 in preconditioning.

The results of the present study also demonstrated activation of p38 MAP kinase during I/R and preconditioning, supporting a number of previous reports (26). Interestingly, preconditioning-mediated increased p38 MAP kinase was also lowered slightly during subsequent I/R. I/R-induced p38 MAP kinase activation was significantly higher compared with that found in the preconditioned myocardium. Although the activation of p38 MAP kinase requires dual phosphorylation like other members of the MAP kinase family, the substrate specificity of p38 MAP kinase is quite different from that of the JNK subgroup of MAP kinases. Thus, unlike other MAP kinases, p38 MAP kinase activates the MAPKAP kinase 2 (21). It is speculated that P38 MAP kinase signaling has a distinct function in the cell, and this was supported by the recent findings that proinflammatory cytokines lead to the activation of p38 MAP kinase, which, in turn, results in the phosphorylation of heat stress protein (HSP)-27 (18). Recently, two MAP kinase kinases (MKK3 and MKK4) have been discovered, the former being specific for p38 MAP kinase whereas the latter can activate both p38 and JNK MAP kinases (5).

Similar to JNK1, activation of p38 MAP kinase also appears to be an essential step for preconditioning. Activation of p38 MAP kinase with anisomycin mimicked the infarct size-lowering effects of preconditioning, whereas inhibition of p38 MAP kinase with SB 203580 abolished the cardioprotective effects of preconditioning. A previous report from this laboratory demonstrated that SB 203580 also inhibits the preconditioning-mediated activation of MAPKAP kinase 2, a specific downstream target for p38 MAP kinase (15).

In summary, our results demonstrate for the first time that preconditioning-mediated activation is transient, occurring immediately on preconditioning. Such activation is obligatory, i.e., preconditioning cannot confer cardioprotection unless p38 MAP kinase and JNK1 are not activated. Our results further indicate that both p38 MAP kinase and JNK1 regulate the preconditioning-mediated cardioprotection. Thus, inhibition of one of them only partially abolsihed the cardioprotective effects of preconditioning. This was confirmed by pretreatment of the hearts with anisomycin, which activates both p38 MAP kinase and JNK. On the other hand, such activation is transient; the expression is lowered during subsequent I/R. Interestingly, I/R by itself activates both p38 MAP kinase and JNK1, which adversely affects cardiac function and contributes to I/R injury. Inhibition of the I/R-induced activation of p38 MAP kinase and JNK confers cardioprotection. It is speculated that JNK1 and p38 MAP kinase play a pivotal role in transmembrane signaling that is obligatory for preconditioning.

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