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Translocation of HSP27 to Sarcomere Induced by Ischemic Preconditioning in Isolated Rat Hearts

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We investigated the role of the 27-kDa heat shock protein (HSP27) in cardiac protection using Langendorff-perfused rat hearts. After preconditioning (a single episode of 5 min global ischemia followed by 5 min of reperfusion), HSP27 redistributed from the cytosol to the sarcomere and recovery of the contractile function, after 40 min of global ischemia and 50 min of reperfusion, was significantly enhanced. Both SB203580, a p38 MAP kinase inhibitor, and bisindolylmaleimide I, a protein kinase C inhibitor, prevented the effects of preconditioning. Both 2-chloro-N^6-cyclopentyladenosine (adenosine A1 agonist) and anisomycin (activator of p38 MAP kinase and c-jun N-terminal kinase) mimicked preconditioning. These results suggest that activation of protein kinase C followed by activation of p38 MAP kinase elicits translocation of HSP27 to the sarcomere, a process which may be involved in the cardioprotective mechanism afforded by ischemic preconditioning in rat heart.

A brief period of ischemia followed by reperfusion protects against subsequent ischemia and reperfusion, and this phenomenon is called ischemic preconditioning (1). Although its precise mechanism is unknown, protein kinase C (PKC) (2–5) with subsequent activation of a tyrosine kinase (6, 7) is postulated as part of the signal transduction system. p38 mitogen-activated protein kinase (MAPK), a substrate of a tyrosine kinase, is activated by ischemia-reperfusion in rat heart (8, 9). The 27-kDa heat shock protein (HSP27), phosphorylated by the p38 MAPK cascade, has unique features among heat shock proteins. This protein becomes phosphorylated in response to various stimuli, including cellular stressors (10–12). MAPK-activated protein kinases, (MAPKAPK)-2 and -3, which are kinases downstream of p38 MAP kinase, have been reported to phosphorylate HSP27 (11, 13, 14). Recently, stabilization of the microfilaments by HSP27 was reported, and it is thought responsible for the protective activity that HSP27 has against oxidative stress in the Chinese hamster lung cell line, CCL39 (15). We recently reported that HSP27 redistributed from the cytosol to the cytoskeleton by repetitive hypoxia-reoxygenation and that SB203580, a p38 MAPK inhibitor, abolished the cardioprotective effect of preconditioning and redistribution of HSP27 in rat myoblast cell line, H9c2 (16). It is of interest to examine whether activation of the p38 MAPK/HSP27 pathway is crucial for the cardioprotective effect of preconditioning in functional beating hearts.

MATERIALS AND METHODS

Rat heart preparation and experimental protocol. Langendorff perfused rat hearts were prepared as described (17) using male SD rats. The hearts were perfused retrogradely at a constant pressure of 100 cm H_2O, placed in a chamber filled with modified Krebs–Henseleit buffer solution, comprised of (in mM): NaCl 118, KCl 4.7, $CaCl_2$ 1.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, and glucose 11. The left ventricular pressure (LVP) and heart rate were monitored using a latex balloon inserted into the left ventricle connected to a pressure transducer (MPU-0.5, Toyo Baldwin, Tokyo, Japan), and the coronary flow was measured with a blood flow transducer (FF-040T, Nihon Kohden) connected to an electromagnetic flow meter (MEZ-7200, Nihon Kohden).

In hearts to be preconditioned, a single 5 min period of global ischemia (complete shutting off the perfusion flow) was applied, followed by a 5-min period of reperfusion. After preconditioning or sham perfusion, the hearts were subjected to 40 min of global ischemia and 50 min of reperfusion. Infusion of vehicle (0.1% DMSO), 1 or 10 μM SB203580, or 3 μM bisindolylmaleimide I was started 10 min prior to preconditioning or sham perfusion and maintained until the reperfusion period of preconditioning. For 2-chloro-N^6-cyclopentyladenosine (CCPA) treatment, the heart was subjected to electrical pacing so that the heart rate after drug treatment was kept to the pre-treatment value, since CCPA itself produces negative chronotropism.

Fractionation of heart muscle and immunoblot analysis. The hearts were homogenized with 50 mM Tris–HCl, 150 mM sucrose, 5 mM EDTA, 2 mM EGTA, 0.1 mM sodium orthovanadate, 50 mM sodium fluoride, 0.1 mM PMSF, and 1 mM leupeptin. Cellular fractionation via centrifuge into nuclear-myofibril (1000*g* pellet), particulate (100,000*g* pellet), and soluble (100,000*g* supernatant) fractions

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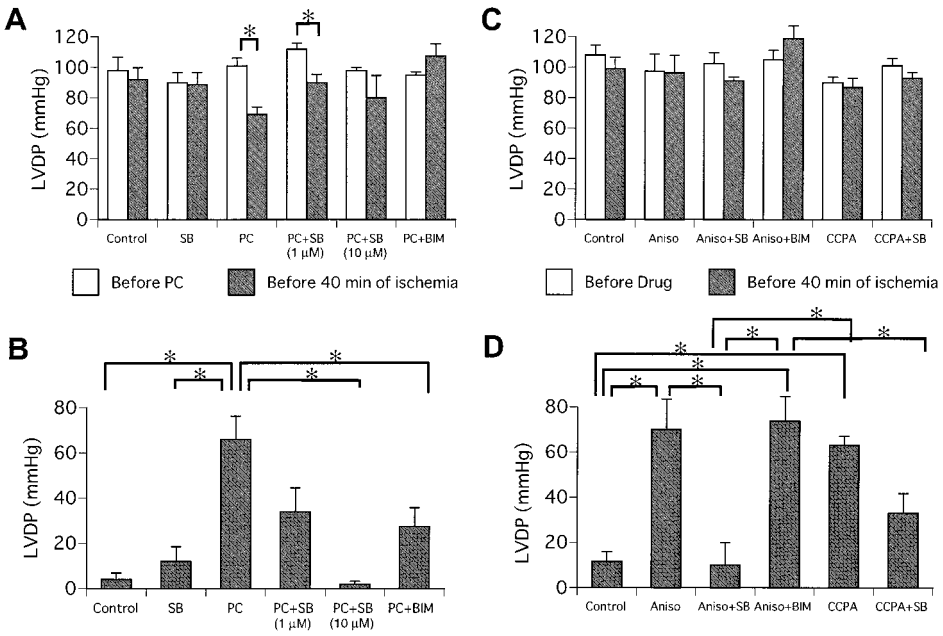

FIG. 1. Changes in left ventricular developed pressure (LVDP) in Langendorff-perfused rat hearts. (A) LVDP before preconditioning (open column) and before ischemia (filled column) are shown. (B) Recovery of LVDP at the end of reperfusion for 50 min after ischemia is shown. (C) LVDP before chemical preconditioning (open column) and before ischemia (filled column) are shown. (D) In the CCPA-treated group, the hearts were subject to electrical pacing so that the heart rate after drug treatment was kept to the pretreatment value. The data represent the mean ± SE of four or five hearts. *$P < 0.05$: compared with all.

was carried out (18) and analyzed by Western blotting with anti-HSP27 polyclonal antibody (16).

Immunohistochemical analysis. Hearts, before or after 40 min of ischemia, were also fixed with 3.7% paraformaldehyde and frozen tissues were stained with anti-HSP27 polyclonal antibody (16) or anti-α-actin monoclonal antibody (New England Biolabs, Beverly, MA) and then visualized by biotinylated anti-mouse/rabbit IgG, and peroxidase-labeled streptavidin (DAKO LSAB-2 rat system, Dako, Carpinteria, CA).

RESULTS AND DISCUSSION

SB203580 blocks contractile recovery afforded by preconditioning. Figure 1A shows the LVDP values before ischemia. Preconditioning significantly decreased LVDP during ischemia. This decrease was attenuated by treatment with SB203580, p38 MAP kinase inhibitor, or bisindolylmaleimide I, a protein kinase C inhibitor. The heart rate was not different to a significant degree in any of the groups (data not shown). Figure 1B shows recovery of LVDP after 50 min of reperfusion, an index of cardiac protection. Cardiac function in the preconditioning group was significantly higher than that of the control. This recovery was dose-dependently inhibited by pre-treatment of SB203580 or by bisindolylmaleimide I. These results suggest that both p38 MAP kinase and protein kinase C are involved in cardioprotection by preconditioning.

To test the above possibility, the effects of CCPA, an adenosine A1 agonist, and anisomycin, an activator of p38 MAP kinase and c-jun N-terminal kinase (19) used in combination with SB203580 and bisindolylmaleimide I were examined. As shown in Fig. 1C, LVDP was not significantly affected by those drugs. Figure 1D shows the recovery of cardiac function at the end of 50 min of reperfusion. The recovery of LVDP was significantly improved both in anisomycin-treated and CCPA-treated groups, showing that both agents can mimic preconditioning. The cardioprotective effect of anisomycin was abolished by concomitant treatment with SB203580, but not at all blocked by bisindolylmaleimide I. The cardioprotective effect of CCPA was partially blocked by SB203580.

These results are consistent with our previous report using H9c2 cells (16) and other reports using rabbit isolated myocytes (20) or H9c2 (21). The most significant point in this study is that involvement of p38 MAPK is strongly suggested in working hearts. Although there is no direct evidence that p38 MAPK was phosphorylated in this study, p38 MAPK and MAPKAPK-2 were reported to be activated by ischemia-reperfusion in rat hearts (8). Preconditioning was reported to increase phosphorylation of p38 MAPK in rabbit hearts *in vivo* (21). These taken together, suggest that the activation of p38 MAPK is involved in the mechanism of ischemic preconditioning in rat heart.

Activation of PKC has been reported to be an important step in cardioprotection by preconditioning (2–5). The stimulation of adenosine A1 receptor, a process

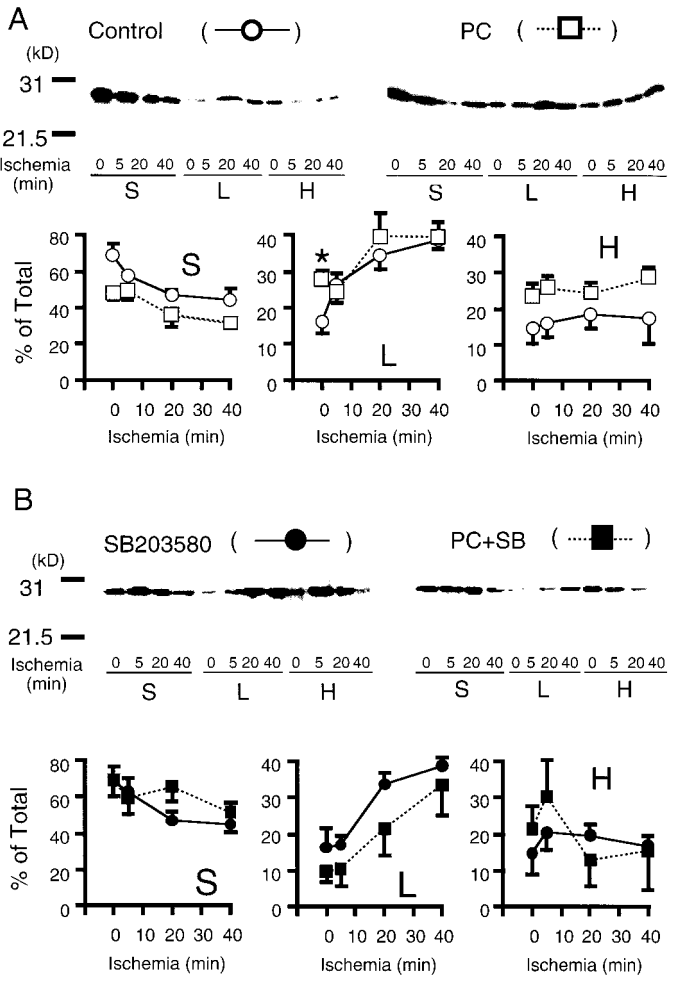

FIG. 2. Redistribution of HSP27 in Langendorff-perfused rat hearts during 40 min of ischemia. The hearts with (squares) or without (circles) preconditioning were homogenized at the indicated time and separated into the soluble (S), the nuclear-myofibril (L), and the particulate (H) fractions. Each fraction was then subject to immunoblotting with anti-HSP27 antibody. The upper panel of each shows the representative immunoblots of four independent experiments, and the lower graph shows the % of total amount of HSP27 in each fraction as determined by densitometry of the immunoblots. (A) Control hearts. (B) Hearts treated with 10 μM SB203580.

which mimics preconditioning (22, 23), is known to activate PKC (24). In the present study, SB203580 partially blocked the cardioprotective effect of CCPA and the cardioprotective effect of anisomycin was not blocked by bisindolylmaleimide I. In H9c2 cell, protection against hypoxia-reoxygenation by phorbol ester, a PKC activator, was blocked by SB203580 (20). Therefore, we propose that p38 MAPK is downstream from PKC in signal transduction of ischemic preconditioning in rat working hearts.

Redistribution of HSP27 by preconditioning. Figure 2A shows the fractional distribution of HSP27 at each time point during ischemia. In control hearts before 40 min of sustained ischemia, 70% of HSP27 was located in the soluble fraction (S in Fig. 2A) and less than 15% of the total was found in the nuclear-myofibril fraction (L in Fig. 2A). In preconditioned hearts before ischemia, the content of HSP27 in the soluble fraction decreased to 50%, whereas that in the nuclear-myofibril fraction increased to 30%. In both groups, as the ischemic period proceeded, HSP27 in the soluble fraction decreased, and that in the nuclear-myofibril fraction increased. In the membrane fraction (H in Fig. 2A) no time-dependent changes were observed, although the amount of HSP27 tended to be higher in the preconditioned group throughout ischemia.

Figure 2B shows that SB203580 abolished translocation at the onset of sustained ischemia in the preconditioning group. However, the drug failed to prevent the translocation of HSP27 from cytosol to the nuclear-myofibril fraction in association with progress of ischemia. The amount of HSP27 in the particulate fraction (H) was not changed by ischemic stress in any groups.

In this series of experiments, the redistribution of HSP27 from cytosol to the nuclear-myofibril fraction caused by preconditioning seemed to be part of the cardioprotection. However, it was also observed that control hearts showed a similar redistribution in that the amount of HSP27 in the nuclear-myofibril fraction became equivalent to that in the preconditioned heart as ischemia proceeded. To make this point clear, the next experiment was conducted.

Immunohistochemistry of HSP27. To visualize the change in the distribution of HSP27 by preconditioning, an immunohistochemical analysis was performed. In control hearts, HSP27 resided mainly in the cytosol with a little in the sarcomere before sustained ischemia (Fig. 3A). In preconditioned hearts, strong positive signals were seen in the sarcomere (Fig. 3B). The localization in the sarcomere was confirmed by immunostaining with anti-α-actin antibody (data not shown). The redistribution of HSP27 afforded by preconditioning was blocked by treatment with 10 μM SB203580 (Fig. 3C). At the end of sustained ischemia in control hearts, HSP27 was not seen in the sarcomere, but condensed to form amorphous precipitates within the cytosol (Fig. 3D). In contrast, HSP27 stayed in the sarcomere in preconditioned hearts (Fig. 3E).

These results strongly suggest that the form of HSP27 in the nuclear-myofibril fraction in Fig. 2 was different between control and preconditioned hearts. Namely, the presence of HSP27 in the nuclear-myofibril fraction in preconditioned hearts appeared to be due to the fact that it redistributed from cytosol to the sarcomere and stayed there until the end of ischemia. In the case of control hearts, redistribution of HSP27 to the nuclear-myofibril fraction appeared to result in formation of immuno-positive amorphous pre-

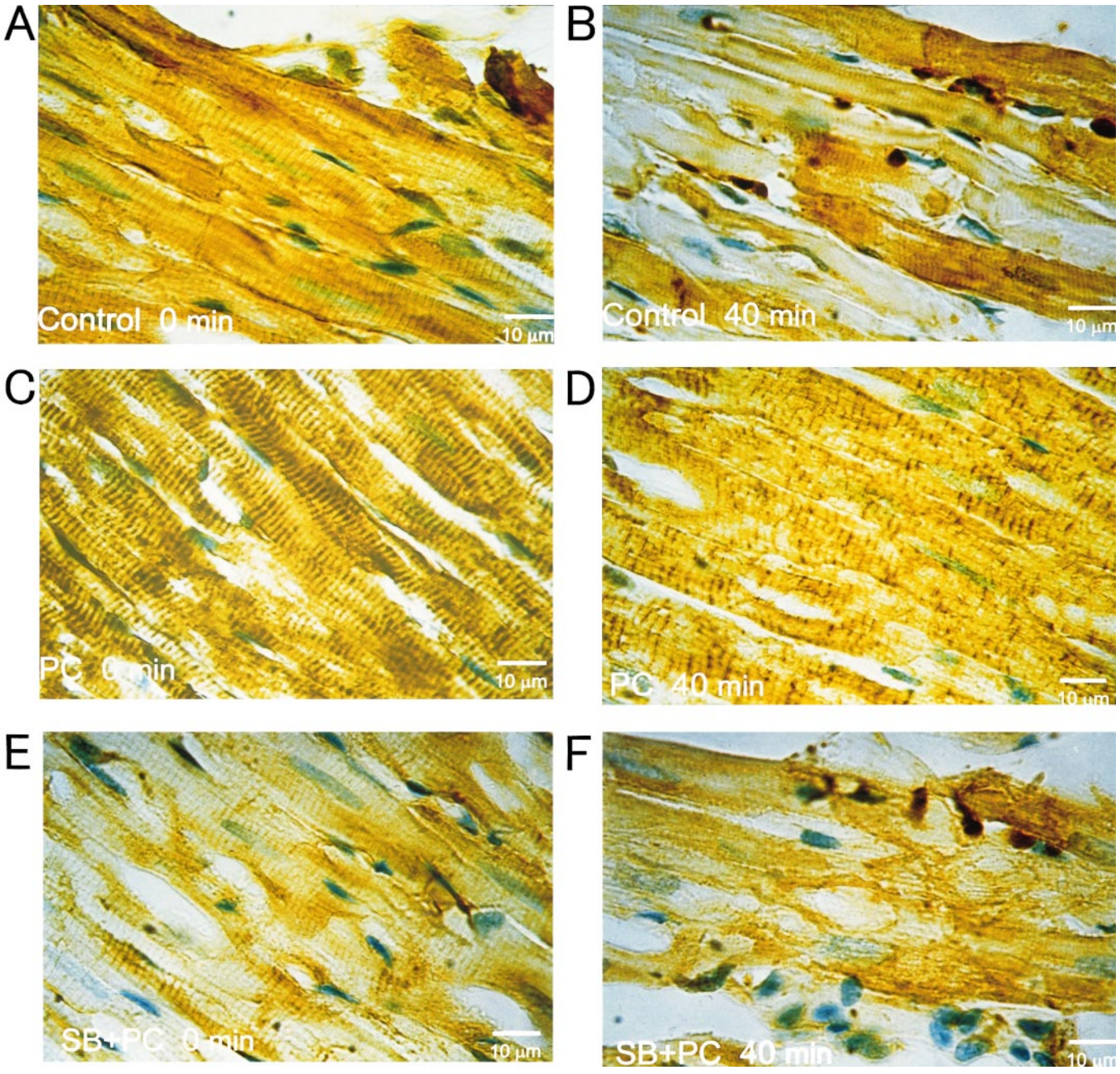

FIG. 3. Immunohistochemical localization of HSP27. Hearts with (C, D, E, and F) or without (A and B) preconditioning in the absence (A, B, C, and D) or the presence (E and F) of 10 μM SB203580 were fixed and stained with anti-HSP27 antibody. A, C, and E were hearts before 40 min of ischemia, and B, D, and F were hearts at the end of the ischemia.

cipitates in the cytosol. Although the significance of the granular HSP27 form is unclear at present, it may be that this structure indicates some damage to the cardiac myocytes, because its formation showed a good correlation with cardiac damage after reperfusion. This was further supported by the observation that pretreatment with SB203580 produced amorphous precipitates in the cytosol, making the preconditioned heart like that of the control (Fig. 3F).

Anisomycin and CCPA mimicked the effect of preconditioning on redistribution of HSP27, i.e., the positive staining on the sarcomere. The effect of anisomycin was resistant to the PKC inhibitor, bisindolylmaleimide I (data not shown).

Possible mechanism of cardiac protection by HSP27. HSP27 was shown to be phosphorylated in response to various stimuli, including cellular stressors such as

heat shock, oxidants and cytokines, as well as growth factors and PKC activators (10–12). This phosphorylation was inhibited by SB203580 (12). MAPKAPK-2 and -3, which are kinases downstream of p38 MAPK, have been reported to phosphorylate HSP27 (11, 13, 14). Moreover, these stressors produce a quick redistribution of HSP27 from the cytosol to the nuclear fraction in HeLa and human glioma cells (25–27). Recently, we have shown that HSP27 translocated from the cytoplasm to the cytoskeleton in response to repetitive hypoxia-reoxygenation in H9c2 cells, and that was blocked by SB203580 (16).

In the present study, preconditioning led to redistribution of HSP27 from the cytosol to the sarcomere, and the change in localization remained until the end of 40 min of ischemia. This change was blocked by SB203580. These observations led us to the conclusion that phosphorylation of HSP27 by the p38 MAP kinase cascade correlated with HSP27 translocation from cytosol to the sarcomere in rat hearts. αB-crystallin, which is highly homologous to HSP27, has been reported to redistribute from the cytosol to the myofibrils after ischemia–reperfusion in rat hearts (28). In the normal condition, HSP27 and αB-crystallin are known to form a complex which dissociates from cellular stresses, such as heat shock (26). Cells overexpressing HSP27 (but not mutant HSP27 which was not phosphorylated), were reported to be resistant to oxidative stress (29, 30). In the Chinese hamster lung cell line CCL39, stabilization of microfilaments by HSP27 was reported to increase protective activity against oxidative stress (15).

Based on these reports, we suggest that phosphorylated HSP27 translocates to the myofibrils, in particular to the sarcomere, in response to ischemia-reperfusion in rat hearts in a way similar to αB-crystallin. It is thus possible that cardioprotection by preconditioning is produced by a better integrity of the actin filament complex with HSP27.

The fact that the action of CCPA was partially blocked by SB203580 could indicate that other mechanisms independent of p38 MAPK may contribute to the protective effect. It has been reported that stimulation of adenosine A1 receptor activated PKCδ in rat cardiomyocytes (24). Recently, PKCδ was reported to phosphorylate HSP27 directly in rat corpus luteum (31). The translocation of HSP27 induced by CCPA, was not blocked by SB203580 (our unpublished observations). Thus, the direct phosphorylation of HSP27 by PKCδ followed by translocation of HSP27 may be involved in the mechanism of cardioprotection by CCPA.

CONCLUSION

We have demonstrated that HSP27 translocated from the cytoplasm to the sarcomere by ischemic preconditioning in isolated rat hearts. SB203580, a p38 MAPK inhibitor, abolished both translocation of HSP27 and cardioprotection by preconditioning. Anisomycin, a p38 MAPK activator, and CCPA, a selective adenosine A1 receptor agonist, both mimicked ischemic preconditioning. The cardioprotective effect of anisomycin was not blocked by bisindolylmaleimide I, a PKC blocker. SB203580 partially blocked the cardioprotective effects of CCPA. Our results suggest that p38 MAPK is located downstream from PKC in signal transduction of ischemic preconditioning, and that translocation of HSP27 from the cytosol to the sarcomere before the ischemia starts contributes to the cardioprotective effect of ischemic preconditioning in isolated rat hearts.

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