

Hyperthermia-Induced Cardioprotection Is Potentiated by Ischemic Postconditioning in Rats

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We tested the hypothesis that the protective effects of hyperthermia (HT) could be augmented by ischemic postconditioning (PostC) via enhancement of reperfusion-induced Akt phosphorylation. The role of the mitoK_{ATP} channel as an effector to protect hearts against ischemia/reperfusion injury was also investigated. In isolated perfused heart experiments using a Langendorff apparatus, 30 min of no-flow global ischemia was followed by 120 min of reperfusion. Ischemic PostC, 5 cycles of 10-sec reperfusion/10-sec ischemia, was achieved at the initial moment of reperfusion. Hyperthermia (HT, 43°C for 20 min) was applied 24 hr before ischemia onset. Ischemic PostC alone did not show significant protection, but HT did. The HT-induced protection in terms of infarct size, recovery of left ventricular performance, amount of released creatine kinase and apoptosis were enhanced by ischemic PostC. These protective effects were consistent with the levels of Akt phosphorylation 7 min after reperfusion and were completely blocked by the pretreatment with the phosphatidylinositol 3-kinase inhibitor wortmannin. HT-induced protection was also completely abolished by concomitant perfusion with 5-hydroxydecanoate (5HD, 100 μM), an inhibitor of the mitochondrial ATP-sensitive potassium (mitoK_{ATP}) channel. However, the potentiated protection by ischemic PostC remained, even in the presence of 5HD. In conclusion, ischemic PostC could potentiate the protective effects of HT possibly via enhancement of reperfusion-induced Akt phosphorylation. Although the opening of the mitoK_{ATP} channel is predominantly involved as an effector in HT-induced protection, potentiated protection by ischemic PostC may

involve mechanisms other than the mitoK_{ATP} channel. *Exp Biol Med* 234:573–581, 2009

Key words: ischemia; reperfusion; mitochondrial ATP-sensitive potassium channel

Introduction

The term “postconditioning” was first described by Na et al (1) in 1996. Subsequently, several investigators reported that brief ischemia at the initial moment of reperfusion attenuates the severity of ischemia/reperfusion injury (2–4). This strategy, “ischemic postconditioning” (PostC), has attracted much scientific and clinical interest. Ischemic PostC was reported to protect the heart by activation of phosphatidylinositol 3-kinase (PI3K)-Akt at the time of reperfusion (5, 6), albeit the precise protective mechanisms have not been fully clarified.

Heat-shock responses, including the induction of heat-shock proteins (HSPs), are endogenous cytoprotective mechanisms (7). We have shown that treatment with hyperthermia (HT) or geranylgeranylacetone (GGA) 24 hr before ischemia affords protection against ischemia/reperfusion injury via induction of HSP72 (8–11). The mitochondrial ATP-sensitive potassium (mitoK_{ATP}) channel was reported to be involved predominantly in myocardial ischemic tolerance after HT (12–14) and after GGA administration (15).

Based on these observations, we hypothesized that the protective effects of HT could be augmented by ischemic PostC via enhancement of reperfusion-induced Akt phosphorylation. The role of the mitoK_{ATP} channel as an effector to protect hearts against ischemia/reperfusion injury was also investigated.

Methods

All experimental procedures were in accordance with the guidelines of the Physiological Society of Oita

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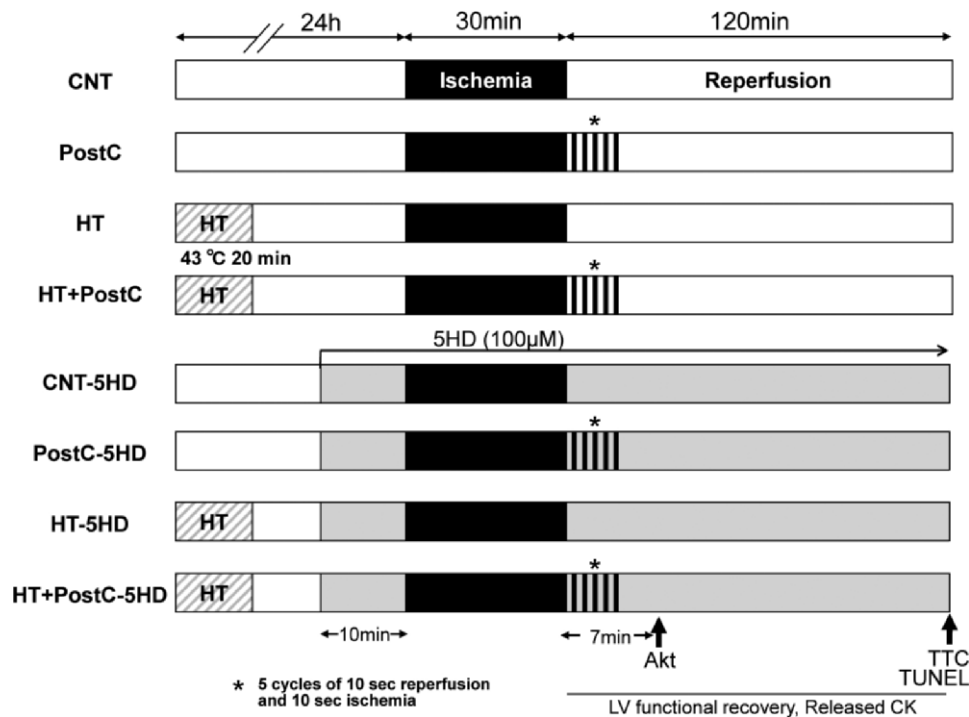


Figure 1. Experimental protocol. In isolated perfused hearts, 30 min of no-flow global ischemia was followed by 120 min of reperfusion. Control (CNT) group: there was no intervention before or after ischemia. Ischemic postconditioning (PostC) group: at the initial onset of reperfusion, ischemic PostC, 5 cycles of 10-sec reperfusion and 10-sec no-flow global ischemia, were applied. Hyperthermia (HT) group: whole-body HT was applied 24 hr before ischemia/reperfusion. HT+PostC group: whole-body HT was followed by ischemic PostC. Each heart from the four groups was perfused in the presence or absence of 5-hydroxydecanoate (5HD; 100 μmol/L). Concomitant perfusion with 5HD was initiated 10 min before the introduction of ischemia and continued to the end of the reperfusion period.

University, Japan, for the care and use of laboratory animals.

Materials. Antibodies were purchased to mouse HSP72 (Stressgen Biotechnologies Corp., Ann Arbor, MI, USA), rabbit phosphorylated Akt (p-Akt), total Akt (t-Akt) (Cell Signaling Technology, Inc., Danvers, MA, USA) and mouse actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). 5-Hydroxydecanoate (5HD) was purchased from Sigma (St. Louis, MO, USA).

Animals and Temperature Treatment. Ten-week-old male Sprague-Dawley rats ($n = 168$) were housed in a room illuminated daily from 07:00 to 19:00 hr (12:12 hr light/dark cycle) with temperature maintained at $21^{\circ} \pm 1^{\circ}\text{C}$. All animals were allowed free access to tap water and standard pellet rat chow (Clea, Japan). HT (43°C for 20 min) or normothermia (NT; 37°C for 20 min) was applied (8–11). Briefly, rats were anesthetized with pentobarbital (20 mg/kg, i.p.) and placed with their heads on a pillow to avoid aspiration of water for 20 min in a water bath set at 43°C . Rectal temperature was measured throughout the temperature treatment experiments. Application of HT at 43°C elevated the rectal temperature to 41°C within 10 min of initiation, and the temperature was maintained at $41^{\circ}\text{--}42^{\circ}\text{C}$ during HT application.

Isolated Perfused Heart Experiments. Isolated perfusion experiments using the Langendorff apparatus were done to examine reperfusion-induced left ventricular

(LV) functional recovery (8–11). Each rat was heparinized and anesthetized. The heart was isolated and perfused retrogradely with Krebs–Henseleit buffer (KHB: pH 7.4; [in mM] 118 NaCl, 4.7 KCl, 2.5 CaCl_2 , 1.2 MgSO_4 , 1.2 KH_2PO_4 , 25.0 Na_2HCO_3 , and 11.0 glucose) equilibrated with a 95% O_2 –5% CO_2 gas mixture at 36.5°C at a constant pressure of 75 mm Hg. A water-filled latex balloon was inserted through the mitral valve orifice into the LV, and the LV end-diastolic pressure (LVEDP) adjusted to 0–5 mm Hg. After the 15-min equilibration period, no-flow global ischemia was initiated for 30 min, followed by reperfusion for 120 min. The coronary effluent during the reperfusion period was collected for measurement of CK content (released CK), and the ratio of released CK to ventricular weight was calculated. LV pressure was measured using a pressure transducer to obtain the peak positive and negative first derivatives of LV pressure ($\text{dP}/\text{dt}_{\text{max}}$ and $\text{dP}/\text{dt}_{\text{min}}$). LV developed pressure (LVDP) was defined as the difference between the LV systolic and diastolic pressure. LV pressure, coronary perfusion pressure (CPP) and electrocardiogram were continuously recorded on a polygraph recorder (WS-681G, Nihon Kohden) and stored on a PCM data recorder (RD-111T, TEAC) for later analysis.

Animal Grouping. Eight groups, control (CNT), PostC, HT, HT+PostC, CNT-5HD, PostC-5HD, HT-5HD and HT+PostC-5HD, were used (Fig. 1). No intervention was applied before or after ischemia in the CNT group.

Table 1. Hemodynamic Parameters at Baseline^a

	<i>n</i>	B.W., g	LVDP, mm Hg	H.R., bpm	+dP/dt, mm Hg/s	-dP/dt, mm Hg/s	CPP, mm Hg
CNT	10	270 ± 4	85 ± 2	267 ± 7	2090 ± 34	-1510 ± 54	74 ± 0.3
PostC	10	265 ± 4	85 ± 2	261 ± 7	2040 ± 36	-1500 ± 47	75 ± 0.3
HT	10	274 ± 6	81 ± 4	248 ± 11	2080 ± 52	-1520 ± 36	75 ± 0.4
HT+PostC	10	270 ± 5	86 ± 3	257 ± 9	2040 ± 45	-1520 ± 38	74 ± 0.3
CNT-5HD	10	262 ± 5	84 ± 2	270 ± 8	2040 ± 36	-1500 ± 38	75 ± 0.3
PostC-5HD	10	261 ± 5	86 ± 3	262 ± 8	2060 ± 40	-1470 ± 42	75 ± 0.3
HT-5HD	10	273 ± 5	84 ± 3	265 ± 8	2040 ± 36	-1490 ± 38	75 ± 0.4
HT+PostC-5HD	10	270 ± 5	86 ± 3	261 ± 8	2020 ± 37	-1490 ± 40	75 ± 0.3

^aData are mean ± SEM; *n*, number of hearts; B.W., body weight; LVDP, left ventricular develop pressure; H.R., heart rate; dP/dt, maximal positive and negative value of first derivate of left ventricular pressure; CPP, coronary perfusion pressure; CNT, control; PostC, ischemic postconditioning; HT, hyperthermia; 5HD, 5-hydroxydecanoate. *n* = 10 in each group.

From the point of initial reperfusion in the PostC group, 5 cycles of 10-sec reperfusion and 10-sec no-flow global ischemia were applied (three other protocols, i.e., 3 cycles of 10-sec reperfusion/10-sec ischemia, 3 cycles of 10-sec reperfusion/30-sec reperfusion and 6 cycles of 10-sec reperfusion/10-sec reperfusion, were also carried out). In the HT group, whole-body HT was applied 24 hr before ischemia/reperfusion; in the HT+PostC group, whole-body HT was followed by ischemic PostC. In addition, some rats of the HT group and HT+PostC group (*n* = 4 for each group) were injected with wortmannin (16 µg/kg, i.v.) into a tail vein 15 min before sacrifice. Each heart from the four groups was perfused in the presence or absence of 5HD at a concentration of 100 µmol/L. Concomitant perfusion with 5HD was initiated 10 min before the introduction of ischemia and continued to the end of the reperfusion period. Infarct size, LV performance and amount of released creatine kinase (released CK, *n* = 8 or 12 for each group) and apoptosis (*n* = 4 for each group) were assessed during or at the end of 120 min of the reperfusion period.

Measurement of Infarct Size. At the end of the 120-min reperfusion period, hearts were rapidly removed from the Langendorff apparatus and sliced across the long axis of the LV, from apex to base, into 2-mm-thick transverse sections. Hearts were incubated in 1% triphenyltetrazolium chloride (TTC; Sigma Chemical, St. Louis, MO) in phosphate buffer (pH 7.4) at 37°C for 20 min (2). Infarct areas were enhanced by storage in 10% formaldehyde solution for 24 hr before final measurement. Infarct size was quantitated using Scion Image software (Scion Corporation, Frederick, MD), as previously described (16, 17).

Western Blotting. Western blotting was carried out as described previously (8–11). Seven minutes (for phosphorylated Akt, *n* = 4 for each group) or 120 min (for HSP72, *n* = 4 for each group) after the onset of reperfusion, each heart was rapidly removed from the Langendorff apparatus and frozen in liquid nitrogen. Frozen tissues were homogenized with lysis buffer (50 mM Tris-HCl at pH 7.4, 10% glycerol, 2 mM ethylenediaminetetraacetic acid [EDTA], 150 mM NaCl, 1 mM MgCl₂, 50 mM

glycerophosphate, 2 mM Na₃VO₄, 20 mM NaF, 1 mM phenylmethylsulfonylfluoride [PMSF], 10 µg/ml leupeptin, 10 µg/ml aprotinin and 1% Nonidet P-40). Samples were centrifuged, and protein concentrations were measured by the Bradford method (18). An equal amount of total protein in each fraction was electrophoresed on 10% sodium dodecyl sulfate–polyacrylamide gel (SDS–PAGE), and transferred electrophoretically onto a polyvinylidene difluoride (PVDF) membrane. After blocking with 5% non-fat milk, membranes were incubated with antibodies. After repeated washing, the membranes were incubated with secondary antibodies. Proteins were detected by enhanced chemiluminescence after exposure to Hyperfilm. The amount of protein on the immunoblots was quantified using Scion Image software.

Apoptosis Assay. At the 120-min reperfusion period, hearts were fixed by 4% polyparaformaldehyde solution in 0.1 M NaH₂PO₄ for 12 hr at room temperature, embedded in paraffin, cut into serial sections of 5-µm thickness and stained. Apoptotic cells were detected with the *in situ* terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) method using an apoptosis kit (Medical Biological Lab, Nagoya, Japan). Sections were initially treated with proteinase K, then with a mixture of terminal deoxynucleotidyl transferase, FITC-dUTP and TdT buffer II at 37°C for 1 hr. After washing these slides with phosphate-buffered solution (PBS), they were mounted with mounting medium using 4',6-diamidino-2-phenylindole (DAPI).

Statistical Analyses. Data are expressed as mean ± SEM. Serial changes in LVDP, the ratio of released CK to ventricular weight, infarct size, relative intensity of each protein and the percentage of TUNEL-positive cells were analyzed using two-way ANOVA followed by the Bonferroni-Dunn test. *P* values <0.05 were considered statistically significant.

Results

Hemodynamic Parameters. Hemodynamic parameters at baseline are shown in Table 1. There were no

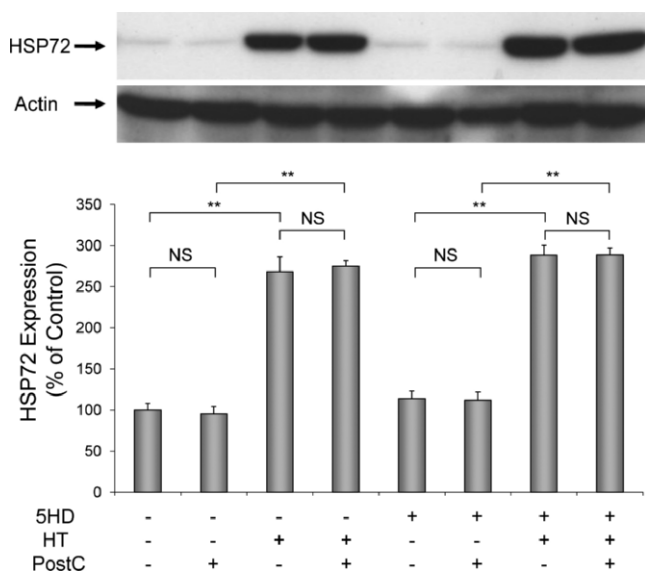


Figure 2. Cardiac heat-shock protein 72 (HSP72) expression. Above: Representative expression of HSP72 in each group. Below: Quantitative expression of HSP72 (relative density). ** $P < 0.01$, NS: not significant. $n = 4$ for each group.

significant differences in hemodynamic parameters and body weight among the eight groups.

HSP72 Expression. Minimal expression of HSP72 was observed in the CNT group and PostC group (Fig. 2). In the HT group and the HT+PostC group, HSP72 was abundantly expressed ($P < 0.01$) with no significant difference between the two groups. Perfusion with 5HD did not influence the expression levels of HSP72.

Infarct Size. Figure 3 shows the infarct size determined by TTC staining, assessed at the end of the 120-min

reperfusion period. Infarct size was $34.5 \pm 2.4\%$ in the CNT group. Ischemic PostC alone (PostC group) did not significantly reduce infarct size ($31.4 \pm 2.1\%$), but it was reduced by HT ($22.4 \pm 1.2\%$, $P < 0.01$ vs. CNT group). In the HT+PostC group, a further reduction in infarct size was obtained ($18.3 \pm 1.1\%$) when compared to the HT group ($P < 0.05$). With respect to the effects of 5HD (Fig. 3A), although it did not influence the infarct size in the CNT group ($36.6 \pm 2.9\%$) or PostC group ($38.5 \pm 3.3\%$), the reduction in infarct size observed in the HT group was eliminated by 5HD ($38.4 \pm 1.8\%$, $P < 0.05$). Thus, there was no significant difference in infarct size among CNT-5HD, PostC-5HD and HT-5HD groups. However, the value in the HT+PostC-5HD group was lower compared with that in the HT-5HD group ($25.5 \pm 3.5\%$ vs. $38.4 \pm 1.8\%$, $P < 0.01$). The reduction in infarct size of the HT group and the HT+PostC group was reversed by pretreatment with wortmannin ($36.5 \pm 3.3\%$ and $35.1 \pm 1.7\%$, respectively, Fig. 3B).

Reperfusion-Induced LV Functional Recovery.

Figure 4A shows the serial changes in LVDP during the 120-min reperfusion period. Rat hearts in the CNT group showed a gradual but weak increase in LVDP as they approached 30 min of reperfusion. Thereafter, LVDP values gradually declined. Similar changes in LVDP during the reperfusion period were observed in the PostC group. No significant difference was observed between the CNT and PostC groups. The HT-treated heart (HT group) showed a sizable increase in LVDP towards 30 min of reperfusion. Thus, reperfusion-induced LV functional recovery was greater in the HT group than in the CNT group ($P < 0.01$). In the HT+PostC group, functional recovery was further enhanced compared to that in the HT group ($P <$

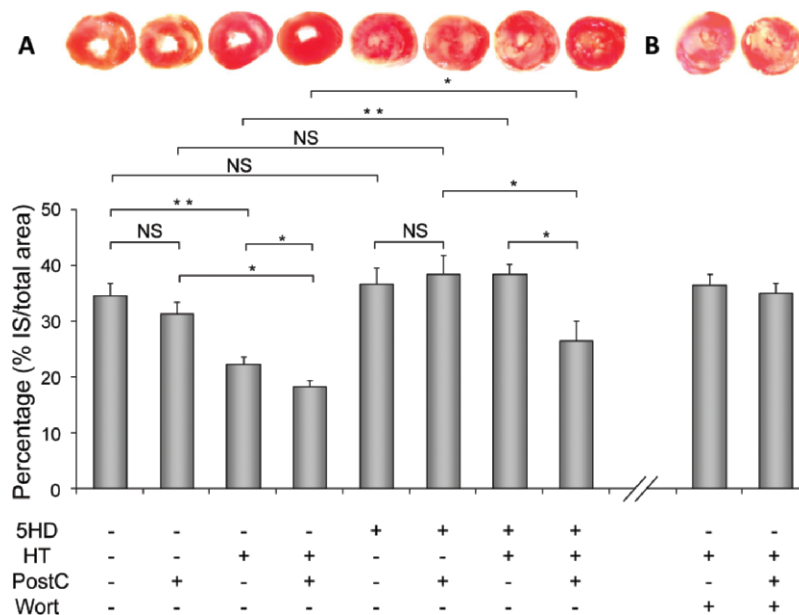


Figure 3. Infarct size. A. Representative triphenyltetrazolium chloride (TTC) staining (above) and quantitative results of TTC staining (below). * $P < 0.05$, ** $P < 0.01$, NS: not significant. $n = 8$ for each group. B. Effects of wortmannin (Wort) pretreatment on infarct size. $n = 4$ for each group.

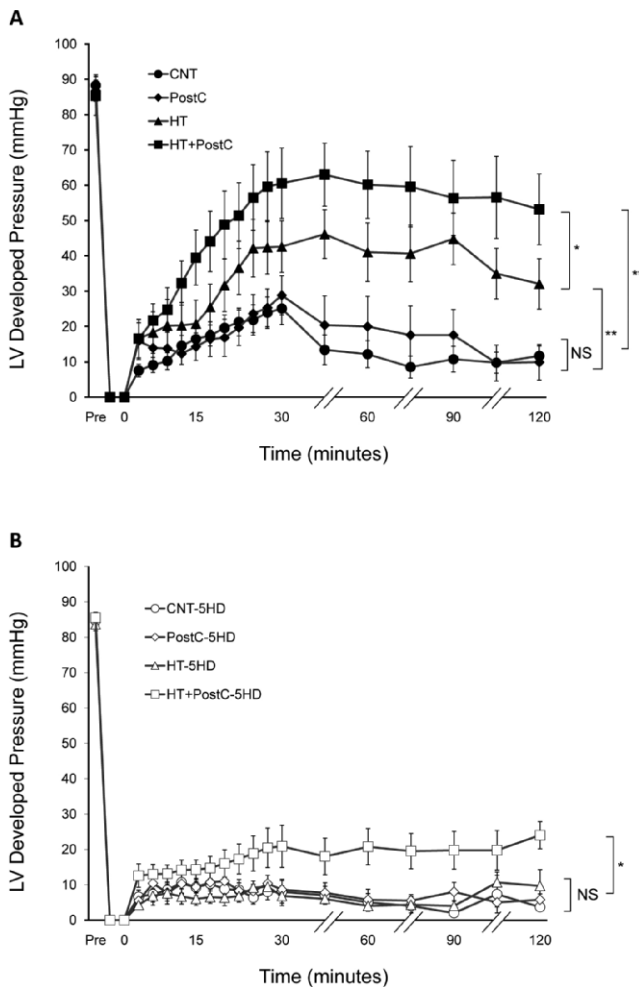


Figure 4. Serial changes in left ventricular developed pressure (LVDP) during the 120-min reperfusion period. A. Each heart was perfused in the absence of 5-hydroxydecanoate (5HD). B. Each heart was perfused in the presence of 5HD. * $P < 0.05$, ** $P < 0.01$, NS: not significant. $n = 8$ in each group.

0.05). In the CNT, PostC and HT groups, concomitant perfusion with 5HD prevented the LV functional recovery in response to reperfusion, resulting in the similar low levels of LVDP (Fig. 4B). However, the recovery was greater in the HT+PostC group when compared to HT group ($P < 0.05$), even in the presence of 5HD.

Amount of Released CK During Reperfusion. The amount of CK released during the reperfusion period in each group is shown in Figure 5. The ischemic Post C (PostC group) did not reduce the amount of released CK compared to the CNT group (64.8 ± 9.7 IU/g vs. 68.8 ± 7.0 IU/g). In the HT group, released CK was significantly reduced compared with the CNT group (45.8 ± 5.4 IU/g, $P < 0.05$). The amount of released CK in the HT+PostC group (28.8 ± 8.1 IU/g) was lower than that of the HT group ($P < 0.05$). Although the concomitant perfusion with 5HD did not influence the released CK in the CNT group (69.6 ± 3.8 IU/g) or PostC group (65.0 ± 4.7 IU/g), the reduction in the released CK observed in the HT group was

eliminated by 5HD (70.4 ± 2.4 IU/g, $P < 0.05$ between HT group and HT-5HD group). As a result, there was no significant difference in released CK among CNT-5HD, PostC-5HD and HT-5HD groups. In the HT+PostC group, the reduction in released CK was reversed by 5HD (46.1 ± 2.5 IU/g vs. 28.8 ± 8.1 IU/g, $P < 0.05$). However, the value was lower compared with that in the HT-5HD group (46.1 ± 2.5 IU/g vs. 70.4 ± 2.4 IU/g, $P < 0.05$).

Apoptosis Assays. Apoptosis assays were done using LV tissue at the end of the 120-min reperfusion period (Fig. 6). Compared with the CNT group ($9.6 \pm 0.6\%$), ischemic PostC alone (PostC group) did not reduce the number of TUNEL-positive cells ($9.9 \pm 1.2\%$), but HT treatment (HT group) did ($6.7 \pm 0.3\%$, $P < 0.05$). A further reduction in the number of TUNEL-positive cells was obtained in the HT+PostC group ($3.8 \pm 0.1\%$, $P < 0.05$ vs. HT group).

With respect to the effects of 5HD, although it did not influence the number of TUNEL-positive cells in the CNT group ($10.1 \pm 1.2\%$) or PostC group ($11.4 \pm 1.0\%$), the reduction in the apoptosis observed in the HT group was eliminated by 5HD ($10.9 \pm 1.6\%$, $P < 0.05$). Thus, there was no significant difference in the number of TUNEL-positive cells among CNT-5HD, PostC-5HD and HT-5HD groups. However, the value in the HT+PostC-5HD group was lower compared with that in the HT-5HD group ($6.3 \pm 1.2\%$ vs. $10.9 \pm 1.6\%$, $P < 0.05$).

Akt Phosphorylation During the Early Stages of Reperfusion. Our preliminary results investigating the time course of Akt phosphorylation after the initiation of reperfusion showed that 7 min is appropriate to detect the difference among the groups. Figure 7 shows Akt phosphorylation 7 min after the initiation of reperfusion. Ischemic PostC alone (PostC group) failed to enhance reperfusion-induced Akt phosphorylation, whereas HT treatment (HT group) enhanced it compared with the CNT group ($P < 0.01$). Ischemic PostC applied to HT-treated hearts (HT+PostC group) further enhanced reperfusion-induced Akt phosphorylation compared with the HT group ($P < 0.05$). Concomitant perfusion with 5HD did not influence Akt phosphorylation (data not shown).

Discussion

The core findings of the present study are as follows. 1) Ischemic PostC alone did not show significant protection, but HT did. 2) The HT-induced protection in terms of infarct size, recovery of LV performance, amount of released CK and apoptosis was enhanced by ischemic PostC. 3) These protective effects were consistent with the levels of Akt phosphorylation 7 min after reperfusion and were completely blocked by pretreatment with wortmannin. 4) HT-induced protection was also completely abolished by concomitant perfusion with 100 μ M 5HD. The potentiated protection by ischemic PostC remained, even in the presence of 5HD. Taken together, it can be postulated that

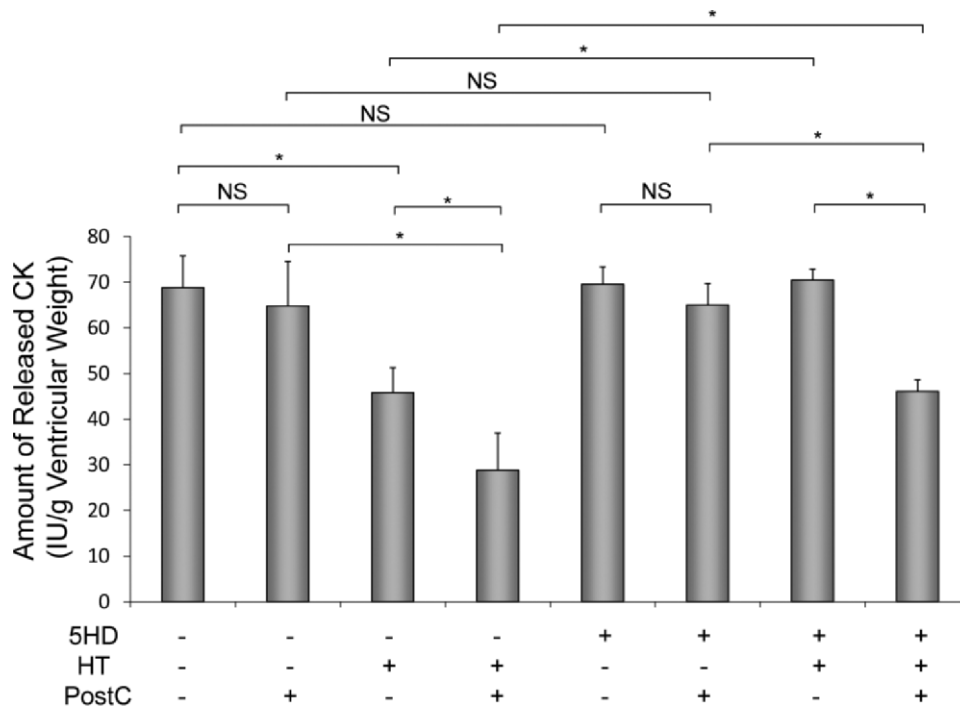


Figure 5. Amount of released creatine kinase (CK) relative to ventricular weight during the reperfusion period. * $P < 0.05$, NS: not significant. $n = 8$ in each group.

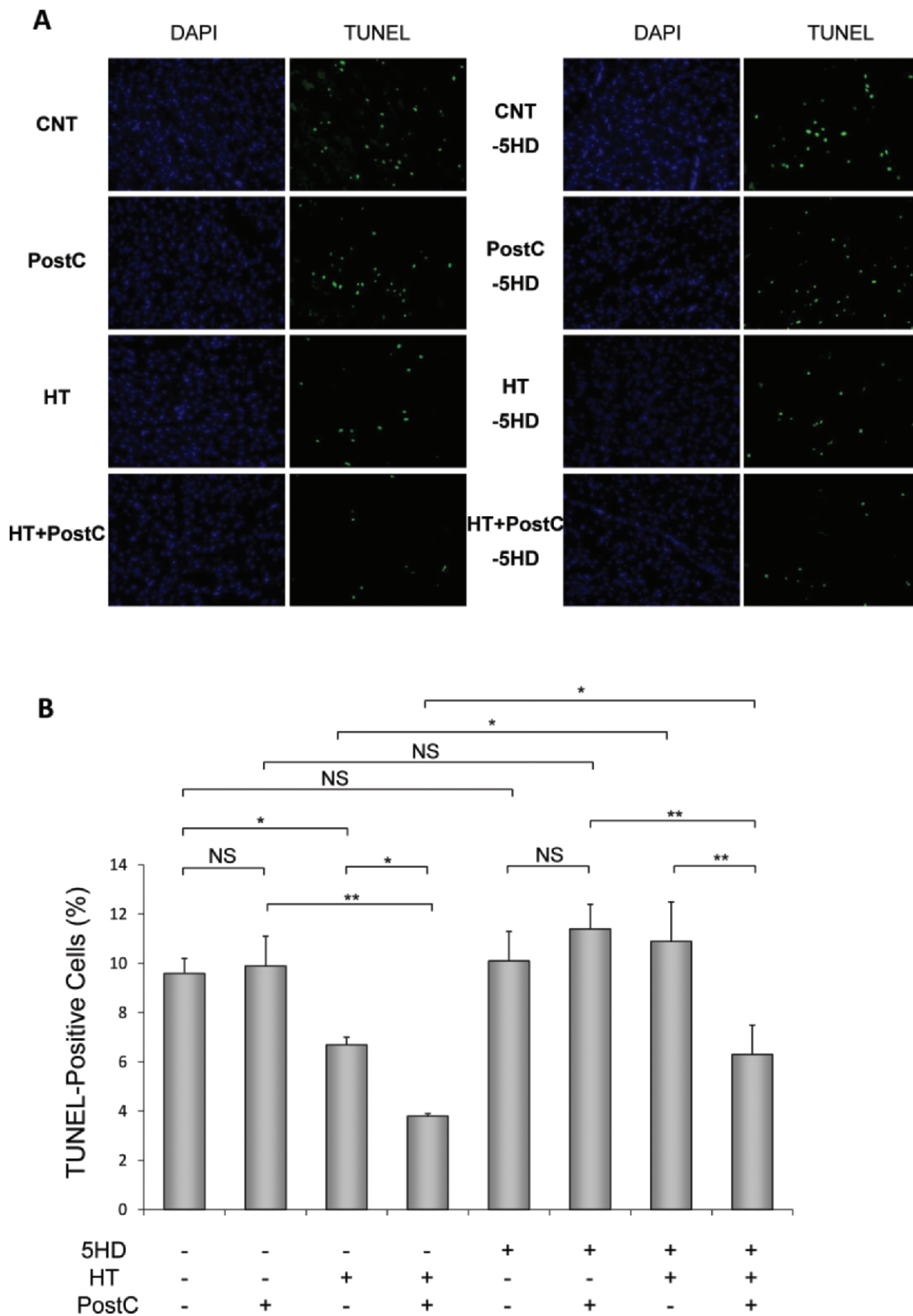
reperfusion-induced Akt phosphorylation by ischemic PostC is augmented under the condition where heat-shock responses, including HSP72, are induced, leading to the further protection. The opening of the mitoK_{ATP} channel may work as a downstream effector to protect the heart against ischemia/reperfusion injury.

It is unclear why ischemic PostC alone could not afford protection. In addition to the protocol of ischemic PostC (Fig. 1), three other protocols, i.e., 3 cycles of 10-sec reperfusion/10-sec ischemia, 3 cycles of 10-sec reperfusion/30-sec reperfusion and 6 cycles of 10-sec reperfusion/10-sec reperfusion, were carried out, resulting in the failure to afford protection (data not shown). Besides our current study, ischemic PostC has been shown to not protect hearts against ischemia/reperfusion injury (19–22). Dow and Kloner (22) reported that various PostC protocols failed to reduce myocardial infarct size in an *in vivo* regional ischemia rat model, and suggested that interspecies differences and differences of collateral blood flow may have a role. Sato et al. (19) reported that, very similar to our observations, while ischemic PostC alone did not reduce infarct size, its combination with ischemic preconditioning resulted in a robust reduction in infarct size. By assessing the cyclo-oxygenase-2 (COX-2) protein expression, COX-2 activity and the effects of the COX-2 inhibitor celecoxib, the authors attributed the additive protection to PostC-induced enhancement of cyclo-oxygenase-2 activity (19). Perfusion without blood may in part explain the ineffectiveness of ischemic PostC alone, because leukocytes and adhesion molecules, known targets for ischemic PostC, were absent

in our Krebs–Henseleit perfusion buffer (2, 3). More recently, nevertheless, in the *in vivo* rat ischemia-reperfusion model, Kocsis et al. (23) reported that ischemic PostC effectively reduced infarct size. Further studies are required to identify the precise mechanism that leads to the effectiveness of ischemic PostC.

Studies have shown that ischemic PostC protects the myocardium by activating the PI3K-Akt pathway (6). In line with the degree of infarct size, LV functional recovery, released CK and apoptosis, the level of Akt phosphorylation at the time of reperfusion was enhanced by HT alone but not by ischemic PostC, whereas ischemic PostC applied to HT-treated heart further amplified Akt phosphorylation. The protective effects observed in the HT group and the HT+PostC group were completely counteracted by pretreatment with wortmannin, suggesting the important role of the PI3K-Akt pathway. The role of Akt phosphorylation remains controversial. Schwartz and Lagranha (24) demonstrated that Akt phosphorylation induced by ischemic PostC did not correlate with reduction in infarct size. Activation of Akt alone may not be sufficient to protect the heart against ischemia/reperfusion injury.

Although the association between Akt phosphorylation and the mitoK_{ATP} channel has not been well clarified, our observations may be explained by the hypothesis that Akt phosphorylation and subsequent opening of the mitoK_{ATP} channel plays a part in the protective mechanisms when HT-treated hearts are subjected to ischemia/reperfusion. The important role of the mitoK_{ATP} channel in myocardial ischemic tolerance after HT has been reported (12–14). In



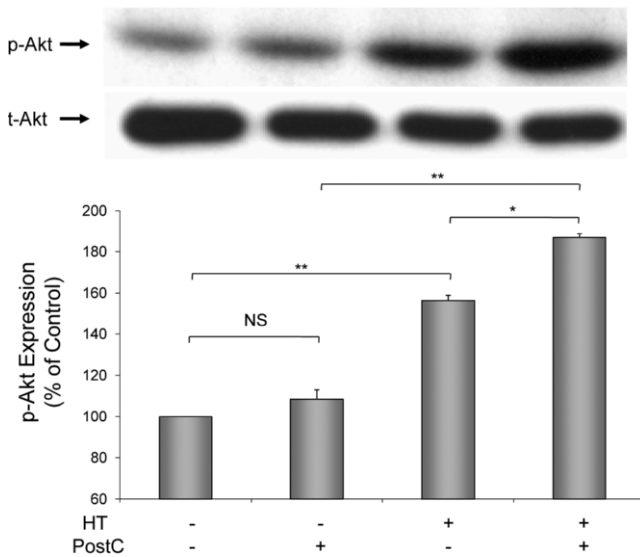


Figure 7. Akt phosphorylation 7 min after reperfusion. Above: Representative immunoblot bands of phosphorylated (p)-Akt and total (t)-Akt. Below: Quantitation of the ratio of each p-Akt to t-Akt. * $P < 0.05$, ** $P < 0.01$, NS: not significant. $n = 4$ for each group.

isolated perfused rat hearts, pre-perfusion with 100 μ M 5HD was demonstrated to appropriately inhibit the mitoK_{ATP} channel (14). In the present study, 100 μ M 5HD was also used, as in our previous report (15). This concentration completely eliminated better functional recovery observed in the HT group. However, even in the presence of 5HD, the protections remained in the HT+PostC group. These observations suggest that the cardioprotective effects afforded by HT treatment predominantly depend on the opening of the mitoK_{ATP} channel, but the potentiated protection on HT-treated hearts by ischemic PostC could involve mechanisms other than opening of the mitoK_{ATP} channel.

Our findings are interesting from a clinical viewpoint, but HT applied 24 hr before ischemia cannot be introduced in patients with acute myocardial infarction. The effects of ischemic PostC, of value as a clinically applicable intervention, could be enhanced if heat-shock responses are induced. This procedure may be applied in coronary artery bypass surgery or organ transplantation. Whether the induction of heat-shock responses in the acute phase of myocardial infarction could protect the heart against ischemia/reperfusion injury and could potentiate the protective effects of ischemic PostC should be studied further.

Several limitations should be noted in the present study. HT is known to induce broad classes of protective proteins, including others in the HSP family (7). Further studies are required to clarify which types of heat-shock responses play the most dominant parts in HT-induced cardioprotection susceptible to augmentation by subsequent application of ischemic PostC. Regarding the effects of 5HD, concomitant perfusion with 5HD at 100 μ mol/L was initiated 10 min before the introduction of ischemia. This concentration and

the timing may not be sufficient to completely block the mitoK_{ATP} channel, resulting in the failure to total abolishment of protection observed in the HT+PostC group. In addition, we could not directly evaluate the functional opening of the mitoK_{ATP} channel. Finally, ineffectiveness of ischemic PostC should be considered more carefully. As shown in Figure 3, it may be better to state that in our present model, ischemic PostC alone did not significantly reduce the infarct size. Although we consider that protective effects of HT were potentiated by ischemic PostC, a simple additive effect of HT and PostC may explain our observations.

Summary. In conclusion, ischemic PostC could potentiate the protective effects of HT, possibly via enhancement of reperfusion-induced Akt phosphorylation. Although the opening of the mitoK_{ATP} channel is predominantly involved as an effector in HT-induced protection, potentiated protection by ischemic PostC may involve mechanisms other than the mitoK_{ATP} channel.

1. Na H, Kim Y, Yoon Y, Han H, Nahm S, Hong S. Ventricular premature beat-driven intermittent restoration of coronary blood flow reduces the incidence of reperfusion-induced ventricular fibrillation in a cat model of regional ischemia. *Am Heart J* 132:78–83, 1996.
2. Zhao Z, Corvera J, Halkos M, Kerendi F, Wang N, Guyton R, Vinten-Johansen J. Inhibition of myocardial injury by ischemic postconditioning during reperfusion: comparison with ischemic preconditioning. *Am J Physiol Heart Circ Physiol* 285:H579–H588, 2003.
3. Kin H, Zhao Z, Sun H, Wang N, Corvera J, Halkos M, Kerendi F, Guyton R, Vinten-Johansen J. Postconditioning attenuates myocardial ischemia-reperfusion injury by inhibiting events in the early minutes of reperfusion. *Cardiovasc Res* 62:74–85, 2004.
4. Ferdinandy P, Schulz R, Baxter G. Interaction of cardiovascular risk factors with myocardial ischemia/reperfusion injury, preconditioning, and postconditioning. *Pharmacol Rev* 59:418–458, 2007.
5. Hausenloy D, Yellon D. New directions for protecting the heart against ischaemia-reperfusion injury: targeting the Reperfusion Injury Salvage Kinase (RISK)-pathway. *Cardiovasc Res* 61:448–460, 2004.
6. Tsang A, Hausenloy D, Mocanu M, Yellon D. Postconditioning: a form of “modified reperfusion” protects the myocardium by activating the phosphatidylinositol 3-kinase-Akt pathway. *Circ Res* 95:230–232, 2004.
7. Benjamin I, McMillan D. Stress (heat shock) proteins: molecular chaperones in cardiovascular biology and disease. *Circ Res* 83:117–132, 1998.
8. Ooie T, Takahashi N, Saikawa T, Nawata T, Arikawa M, Yamanaka K, Hara M, Shimada T, Sakata T. Single oral dose of geranylgeranyl-acetone induces heat-shock protein 72 and renders protection against ischemia/reperfusion injury in rat heart. *Circulation* 104:1837–1843, 2001.
9. Shinohara T, Takahashi N, Ooie T, Hara M, Shigematsu S, Nakagawa M, Yonemochi H, Saikawa T, Yoshimatsu H. Phosphatidylinositol 3-kinase-dependent activation of akt, an essential signal for hyperthermia-induced heat-shock protein 72, is attenuated in streptozotocin-induced diabetic heart. *Diabetes* 55:1307–1315, 2006.
10. Taniguchi Y, Ooie T, Takahashi N, Shinohara T, Nakagawa M, Yonemochi H, Hara M, Yoshimatsu H, Saikawa T. Pioglitazone but not glibenclamide improves cardiac expression of heat shock protein 72 and tolerance against ischemia/reperfusion injury in the heredity insulin-resistant rat. *Diabetes* 55:2371–2378, 2006.

11. Yamanaka K, Takahashi N, Ooie T, Kaneda K, Yoshimatsu H, Saikawa T. Role of protein kinase C in geranylgeranylacetone-induced expression of heat-shock protein 72 and cardioprotection in the rat heart. *J Mol Cell Cardiol* 35:785–794, 2003.
12. Auchampach J, Gross G. Adenosine A1 receptors, KATP channels, and ischemic preconditioning in dogs. *Am J Physiol* 264:H1327–H1336, 1993.
13. Hoag J, Qian Y, Nayeem M, D'Angelo M, Kukreja R. ATP-sensitive potassium channel mediates delayed ischemic protection by heat stress in rabbit heart. *Am J Physiol* 273:H2458–H2464, 1997.
14. Pell T, Yellon D, Goodwin R, Baxter G. Myocardial ischemic tolerance following heat stress is abolished by ATP-sensitive potassium channel blockade. *Cardiovasc Drugs Ther* 11:679–686, 1997.
15. Shinohara T, Takahashi N, Kohno H, Yamanaka K, Ooie T, Wakisaka O, Murozono Y, Taniguchi Y, Torigoe Y, Hara M, Shimada T, Saikawa T, Yoshimatsu H. Mitochondria are targets for geranylgeranylacetone-induced cardioprotection against ischemia-reperfusion in the rat heart. *Am J Physiol Heart Circ Physiol* 293:H1892–H1899, 2007.
16. Kostyak J, Hunter J, Korzick D. Acute PKCdelta inhibition limits ischaemia-reperfusion injury in the aged rat heart: role of GSK-3beta. *Cardiovasc Res* 70:325–334, 2006.
17. Korzick D, Kostyak J, Hunter J, Saupe K. Local delivery of PKCepsilon-activating peptide mimics ischemic preconditioning in aged hearts through GSK-3beta but not F1-ATPase inactivation. *Am J Physiol Heart Circ Physiol* 293:H2056–H2063, 2007.
18. Bradford M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254, 1976.
19. Sato H, Bolli R, Rokosh G, Bi Q, Dai S, Shirk G, Tang X. The cardioprotection of the late phase of ischemic preconditioning is enhanced by postconditioning via a COX-2-mediated mechanism in conscious rats. *Am J Physiol Heart Circ Physiol* 293:H2557–H2564, 2007.
20. Tang X, Sato H, Tiwari S, Dawn B, Bi Q, Li Q, Shirk G, Bolli R. Cardioprotection by postconditioning in conscious rats is limited to coronary occlusions <45 min. *Am J Physiol Heart Circ Physiol* 291:H2308–H2317, 2006.
21. Hale S, Mehra A, Leeka J, Kloner R. Postconditioning fails to improve no reflow or alter infarct size in an open-chest rabbit model of myocardial ischemia-reperfusion. *Am J Physiol Heart Circ Physiol* 294:H421–H425, 2008.
22. Dow J, Kloner R. Postconditioning does not reduce myocardial infarct size in an in vivo regional ischemia rodent model. *J Cardiovasc Pharmacol Ther* 12:153–163, 2007.
23. Kocsis G, Pipis J, Fekete V, Kovács-Simon A, Odendaal L, Molnár E, Gircz Z, Janáky T, van Rooyen J, Csont T, Ferdinandy P. Lovastatin interferes with the infarct size-limiting effect of ischemic preconditioning and postconditioning in rat hearts. *Am J Physiol Heart Circ Physiol* 294:H2406–H2409, 2008.
24. Schwartz L, Lagranha C. Ischemic postconditioning during reperfusion activates Akt and ERK without protecting against lethal myocardial ischemia-reperfusion injury in pigs. *Am J Physiol Heart Circ Physiol* 290:H1011–H1018, 2006.