Protein Kinase A as Another Mediator of Ischemic Preconditioning Independent of Protein Kinase C

Shoji Sanada, MD, PhD; Hiroshi Asanuma, MD, PhD; Osamu Tsukamoto, MD; Tetsuo Minamino, MD, PhD; Koichi Node, MD, PhD; Seiji Takashima, MD, PhD; Tomi Fukushima, PhD; Akiko Ogai, PhD; Yoshiro Shinozaki, MD, PhD; Masashi Fujita, MD; Akio Hirata, MD; Hiroko Okuda, MD; Hiroaki Shimokawa, MD, PhD; Hitonobu Tomoike, MD, PhD; Masatsugu Hori, MD, PhD; Masafumi Kitakaze, MD, PhD

Background—We and others have reported that transient accumulation of cyclic AMP (cAMP) in the myocardium during ischemic preconditioning (IP) limits infarct size independent of protein kinase C (PKC). Accumulation of cAMP activates protein kinase A (PKA), which has been demonstrated to cause reversible inhibition of RhoA and Rho-kinase. We investigated the involvement of PKA and Rho-kinase in the infarct limitation by IP.

Methods and Results—Dogs were subjected to 90-minute ischemia and 6-hour reperfusion. We examined the effect on Rho-kinase activity during sustained ischemia and infarct size of (1) preischemic transient coronary occlusion (IP), (2) preischemic activation of PKA/PKC, (3) inhibition of PKA/PKC during IP, and (4) inhibition of Rho-kinase or actin cytoskeletal deactivation during myocardial ischemia. Either IP or dibutyryl-cAMP treatment activated PKA, which was dose-dependently inhibited by 2 PKA inhibitors (H89 and Rp-cAMP). IP and preischemic PKA activation substantially reduced infarct size, which was blunted by preischemic PKA inhibition. IP and preischemic PKA activation, but not PKC activation, caused a substantial decrease of Rho-kinase activation during sustained ischemia. These changes were cancelled by preischemic inhibition of PKA but not PKC. Furthermore, either Rho-kinase inhibition (hydroxyfasudil or Y27632) or actin cytoskeletal deactivation (cytochalasin-D) during sustained ischemia achieved the same infarct limitation as preischemic PKA activation without affecting systemic hemodynamic parameters, the area at risk, or collateral blood flow.

Conclusions—Transient preischemic activation of PKA reduces infarct size through Rho-kinase inhibition and actin cytoskeletal deactivation during sustained ischemia, implicating a novel mechanism for cardioprotection by ischemic preconditioning independent of PKC and a potential new therapeutic target. (Circulation. 2004;110:51-57.)

Key Words: ischemia ■ infarction ■ proteins

The potent cardioprotection induced by brief periods of ischemia before sustained ischemia is termed ischemic preconditioning (IP). Previous studies have exhibited some possible mediators of IP-derived cardioprotection, suggested to link with protein kinase C (PKC). On the other hand, a brief preconditioning ischemia also induces a synchronized increase of the myocardial cyclic AMP (cAMP) level. Furthermore, we and others have reported that brief preischemic exposure to β-agonists, an adenylate cyclase activator, phosphodiesterase type III inhibitors, or a cell-permeable cAMP analogue, all of which cause rapid activation of protein kinase A (PKA), also protects the myocardium in vivo independent of PKC. However, little is known about the direct contribution of PKA to infarct limitation by IP and the associated subcellular mechanisms. Some recent studies have demonstrated that an increase of cAMP followed by activation of PKA causes temporary inhibition of small GTPase RhoA and its downstream Rho-kinase, playing major roles in enhancement of actin cytoskeleton formation followed by chemotactic migration, platelet activation, and cytokinesis. Therefore, we hypothesized that transient preischemic activation of PKA may also cause IP-induced infarct limitation through the inhibition of Rho-kinase after the onset of sustained ischemia. This study was designed to test this hypothesis in vivo.
Methods
All procedures were performed in conformity with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, 1996 revision) and were approved by the Osaka University Committee for Laboratory Animal Use.

Instrumentation
As described previously, 176 beagle dogs (Oriental Yeast; Osaka, Japan) weighing 9 to 14 kg were anesthetized with sodium pentobarbital (30 mg/kg IV) and connected to an extracorporeal bypass tube. In all experiments, the average control values of mean aortic blood pressure (ABP), heart rate (HR), and arterial blood pO2 were 101/110 mm Hg, 127/12.4/minute, and 108/13.8 mm Hg, respectively. Both ABP and HR were measured continuously during the experiment.

Experimental Protocols

Protocol 1: Infarct Size After Preconditioning
Figure 1 shows the study protocol. After hemodynamic stabilization, 176 dogs underwent 90 minutes of ischemia and 6 hours of reperfusion with or without 1 or more of the following interventions: (1) 5-minute coronary occlusion followed by 25-minute reperfusion just before sustained ischemia (IP), (2) intracoronary administration of the potent PKC activator phorbol 12-myristate 13-acetate (PMA, Sigma; 0.62 ng/kg per min), or (3) an intravenous administration of the cell-permeable cAMP analogue dibutyryl-cAMP (db-cAMP, Sigma; 5 mg/kg over 5 minutes), in combination with (4) intracoronary injection of a selective PKC inhibitor, GF109203X (Calbiochem; 40 μg/kg per min) or 1 of 2 selective PKA inhibitors (H89 [Sigma; 1.35 μg/kg per min] or the Rp-isomer of cAMP 12 [Rp-cAMP, Calbiochem; 45 μg/kg per min]) or (5) infusion of vehicle (a small volume of DMSO, which does not affect infarct size in the same model) at a rate of 0.0167 mL/kg per min (control, vehicle, IP, IP + H89, IP + Rp-cAMP, PMA, PMA + H89, PMA + Rp-cAMP, DB-cAMP, DB-cAMP + H89, DB-cAMP + Rp-cAMP, DB-cAMP + GF109203X [GFX], PMA + DB-cAMP, H89, Rp-cAMP, and GFX groups, respectively; n = 8 to 13 each).

We used the same PMA or db-cAMP regimens that were used in our previous studies, which demonstrated potent infarct limitation in this model. The regimen of GF109203X infusion was the same as that used in our previous study to blunt infarct limitation by IP and PMA.

Protocol 2: Infarct Size in the Inhibition Protocol During Ischemia
The lower part of Figure 1 shows the protocol. Sixty-six dogs underwent the same protocol for sustained ischemia-reperfusion with or without intracoronary administration of (1) a selective Rho-kinase inhibitor (hydroxyfasudil [Asahi-Kasei; 2.4 and 12 μg/kg per min] or Y27632 [Welfide; 0.7 and 3.5 μg/kg per min]), (2) an actin cytoskeleton disruptor cytochalasin-D 15 (Calbiochem; 5.1 μg/kg per min), or (3) vehicle (a small volume of DMSO, which does not affect infarct size in the same model) between the onset and 60 minutes of sustained ischemia (hydroxyfasudil-lower dose [LD], hydroxyfasudil, Y27632-LD, Y27632, cytochalasin-D, and ischemia-vehicle groups, respectively; n = 10 to 12 each).

The higher doses of hydroxyfasudil and Y-27632 for intracoronary infusion were the highest doses that could not influence systemic hemodynamics during our preliminary study in this model (data not shown).

Protocol 3: Tissue Kinase Assay
Figure 2 shows the protocol. Ninety dogs were subjected to sustained myocardial ischemia according the same method as in protocol 1 with or without (1) IP, (2) preischemic administration of db-cAMP or PMA, (3) preischemic administration of H89, Rp-cAMP, or GF109203X, or (4) administration of hydroxyfasudil or Y27632 during ischemia (sham, control, PMA, DB-cAMP, ischemia-vehicle, hydroxyfasudil-LD, hydroxyfasudil, Y27632-LD, Y27632, DB-cAMP + Rp-cAMP, IP + vehicle, IP + H89, IP + Rp-cAMP, and IP + GF109203X groups, respectively; n = 4 or 5 each).

We quickly sampled the myocardium in the target region (1) at the end of IP, PMA infusion, or db-cAMP infusion to assay PKA and (2) after 60-minute ischemia to assay Rho-kinase activity (Figure 2). The sampled tissues were rapidly frozen in liquid nitrogen and stored at −80°C.

Measurements of Collateral Blood Flow, Risk Area, and Infarct Size
In protocols 1 and 2, we measured the myocardial collateral blood flow after 60 minutes of ischemia by a nonradioactive microsphere method and evaluated both the area at risk and the infarct size by dual staining, as described previously.
Exclusion Criteria
To ensure that all of the animals included in the data analysis were healthy and were exposed to a similar extent of ischemia, the exclusion criteria described previously regarding hemodynamism, excessive collateral flow, and lethal arrhythmia were used.

PKA and Rho-Kinase Assay
We assayed PKA and Rho-kinase activity as described previously with some modifications, using specific antibodies for a substrate of PKA (phospho-CREB [Upstate Biology]) or Rho-kinase (myosin phosphatase targeting subunit [MYPT]-1 [Upstate Biotechnology]) and phospho-MYPT-1 [Thr696; Upstate Biotechnology]) as the primary antibodies. Rho-kinase activity was determined as the phosphorylated ratio of MYPT.

Statistical Analysis
Results are expressed as mean ± SEM. Statistical analysis was performed by ANOVA with modified Bonferroni’s post hoc test, and significance was defined at P<0.05.

Results
Mortality and Exclusions, Hemodynamic Parameters, Risk Area, and Collateral Blood Flow
Among the 242 dogs used in protocols 1 and 2, 55 dogs met the exclusion criteria of ventricular fibrillation or excessive myocardial collateral blood flow (>15 mL/100 g per min). Therefore, 187 dogs completed these protocols satisfactorily and were included in the data analysis (Table). The changes of ABP and HR were comparable among the 23 groups throughout the experiment (data not shown), and the area at risk and collateral blood flow were also comparable (Table).

Infarct Size
Figure 3 shows the infarct size for each group in protocol 1 (left) and protocol 2 (right). In protocol 1, IP (11.9±2.1% in the IP group and 14.8±2.1% in the IP+vehicle group) as well as preischemic treatment with PMA and db-cAMP in combination (14.0±2.6% in the PMA+db-cAMP group) markedly exhibited infarct limitation, which were more potent than either PMA (17.3±2.5%) or db-cAMP (20.1±2.2%) alone but did not reach a significant difference. Infarct size in these 4 groups was significantly smaller (P<0.05 each) than that in either the control (40.1±3.8%) or vehicle (40.6±3.6%) group. Treatment with either H89 or Rp-cAMP during preconditioning similarly blunted the infarct limitation by IP (33.4±3.8% and 34.1±4.1%, respectively; both P<0.05 versus control) and db-cAMP (37.4±3.6% and 39.1±3.9%, respectively; both P<0.05 versus the db-cAMP group), whereas these agents did not affect PMA-induced infarct limitation (19.2±3.0% and 18.5±2.7%, respectively; both P<0.05 versus control). Furthermore, infarct limitation by db-cAMP was not affected by the effective dose of GF109203X (20.8±3.1%, P<0.05 each versus control) in this model. H89, Rp-cAMP, and GF109203X alone did not affect infarct size (42.7±4.2%, 38.6±4.6%, and 41.9±4.5%, respectively) (Figure 3). In protocol 2 (Figure 3, right), administration of the vehicle during ischemia did not influence infarct size (43.4±4.4%), the area at risk, or collateral blood flow (Table 1) compared with the control group. Administration of either hydroxyfasudil or Y27632 during sustained ischemia caused dose-dependent infarct limitation like that attributable to preische-
mic PKA activation (30.5±5.3% and 22.0±4.1% in the hydroxyfasudil-LD and hydroxyfasudil groups, respectively; 30.0±5.3% and 21.7±3.9% in the Y27632-LD and Y27632 groups, respectively). Only the higher dose of each agent achieved significant (both P<0.05) infarct limitation, showing that these were the minimum doses to exert a sufficient effect in this model. Cytochalasin-D also similarly reduced the infarct size (22.9±4.4%, P<0.05 versus control).

**PKA Activity During Preconditioning**

In protocol 3, IP and db-cAMP activated PKA (267±22% and 288±34% of baseline, respectively; both P<0.05 versus sham). IP-derived PKA activation was inhibited by coadministration of H89 or Rp-cAMP (112±23% and 99±17%, respectively; both P<0.05 versus IP+vehicle). We also confirmed that Db-cAMP–derived PKA activation was cancelled by Rp-cAMP (212±22%, P<0.05 versus IP+vehicle). Furthermore, we observed that 1 to 5 doses of H89 and Rp-cAMP only caused partial (189±29% and 175±29%, respectively) inhibition of PKA (Figure 4), showing that the dose levels we used in this study were the minimum effective doses, which should eliminate the possibility of a nonspecific action in this model.

**Rho-Kinase Activity During Ischemia**

A 60-minute period of ischemia caused Rho-kinase activation (307±25% of baseline in the control group; P<0.05 versus sham), which was attenuated by IP or preischemic administration of db-cAMP (145±21% and 151±26%, respectively; P<0.05 versus control) but not by PMA (253±30%, P<0.05 versus sham). The IP-induced suppression of Rho-kinase activation was cancelled by Rp-cAMP (247±39%, P<0.05 versus IP) but not by GFX (161±34%, P<0.05 versus control) at the same dose as in protocol 1 (Figure 5; left). Furthermore, administration of either hydroxyfasudil or Y27632 during sustained ischemia caused a dose-dependent decrease of Rho-kinase activation (199±36% and 205±33% in the hydroxyfasudil-LD and Y27632-LD groups, respectively; 134±21% and 135±20% in the hydroxyfasudil and Y27632 groups, respectively), which was significant (P<0.05 versus the ischemia-vehicle group) at the higher doses (Figure 5; right).

**Discussion**

**Triggers of IP: Involvements of cAMP, PKA, and β-Adrenoceptors**

Single or repeated IP as well as repeated treatment with forskolin is reported to rapidly and transiently increase both myocardial cAMP level and tissue PKA activity. We observed in this model that the intracoronary coadministration of different types of selective PKA antagonists blunted infarct...
limitation of IP, suggesting the involvement of PKA in the IP-derived cardioprotection. However, it remains unclear whether the β-adrenoceptor is involved in this response, despite the prominent role of the α₁-adrenoceptor in PKC-mediated cardioprotection. Although a single exposure to brief ischemia or a β-agonist limits infarct size through β-adrenoceptor activation and repeated preischemic activation of the cAMP-PKA-dependent pathway is also modulated by β-adrenoceptor expression, the contribution of this receptor in cardioprotection by repeated exposures remains unclear because of PKA-induced rapid desensitization. It is reported in other systems that the repeated IP might lead to cAMP accumulation and direct PKA activation independently of the β-adrenoceptor through the inhibition of phosphodiesterase or direct sensitization of adenylate cyclase. However, we have documented preliminarily in the present model that the intracoronary coadministration of the selective ultra-short-acting β₁-adrenoceptor blocker landiolol around the preconditioning ischemia (as in protocol 1) blunted the infarct limitation by IP used in this study (35.0 ± 4.4%, n = 7) as well as PKA inhibitors during IP, additionally suggesting the essential contribution of β₁-adrenoceptor activation to cause IP-induced PKA-dependent cardioprotection in this model. However, more studies might be expected to clarify these issues.

On the other hand, IP leads to decreased cAMP accumulation during sustained ischemia, but it is controversial whether this explains the cardioprotection afforded by preischemic activation of PKA. Enhancement of cAMP accumulation during sustained ischemia fails to block cardioprotection by IP. Moreover, overexpression of β-adrenergic receptor kinase-1, which causes functional uncoupling of β-adrenoceptors, impairs ischemia-reperfusion injury, and this impairment is reversed by co-overexpression of β-adrenergic receptor kinase-1 inhibitor.

Role of PKA and PKC
Manganello et al demonstrated that PKA primarily phosphorylates the switch-I region of G-α₁₃ (an essential G-protein for signaling to RhoA stimulated by G-protein–coupled receptors) and inhibits its binding with G-β-γ, which subsequently leads to the inhibition of G-α₁₃ turnover and inactivation of RhoA.

Although PKC or its downstream kinase has been reported to induce actin assembly through activation of PKC-potentiated phosphatase inhibitor and Rho-kinase, another study found that both PKC and the downstream Src tyrosine kinase rapidly deactivate RhoA through p190 and cause actin disassembly. The PKC-Src/Lck pathway was reported to play a role in cardioprotection by IP. However, preischemic PKC activation had little influence on IP-induced Rho-kinase inhibition in this study, showing the limited contribution of PKC to this pathway. Furthermore, we have reported that Src/Lck tyrosine kinase is not involved in the infarct limitation by IP in this model. Although it did not
reach statistical significance, our present data additionally imply that (1) there are multiple pathways in parallel to confer cardioprotection of IP, PKC-induced Rho-kinase–independent and PKA-induced Rho-kinase–dependent ones, or (2) PKC-induced pathway exerts stronger effects than the PKA-induced one to cause cardioprotection of IP. Given that repeated IP only promotes transient, not sustained, activation of PKC,28 it is highly possible that transient activation of both PKA and PKC is independently but synergistically responsible for mediating a variety of cardioprotective pathways triggered by IP.

**Cardioprotection by Rho-Kinase Inhibition**

Because inhibition of Rho-kinase directly relaxes vascular smooth muscle,9,13,29 it may increase regional myocardial blood flow at sites of major coronary artery stenosis (without any inotropic or chronotropic effect) by dilating the abnormal artery.29 However, this study showed that Rho-kinase inhibition during sustained ischemia exerted cardioprotection without altering hemodynamics, even after all of the dogs with excessive collateral flow were excluded from analysis. Therefore, the infarct-limiting mechanism that involves Rho-kinase inhibition could be independent of either a change in systemic hemodynamics or the recruitment of collateral blood flow.

Rho-kinase has multiple effects on the cardiovascular system, which dominantly represent those of RhoA,9 because of inhibition of myosin phosphatase and activation of the ERM family (ezrin, radixin, or moesin) or adducin.9 Importantly, Rho-kinase plays a major role in stress fiber formation, focal adhesion, migration, and cytokinesis through activation of the ERM family and thus can enhance cardiac damage in acute ischemia. The potent infarct limitation by cytochalasin-D that mimicked PKA–Rho-kinase–mediated cardioprotection suggests that deactivation of stress fiber polymerization is a major part of this cardioprotective mechanism.

In fact, the effect of changes to the actin cytoskeleton on infarct size has been controversial, because a previous study revealed that targeted deletion of the internal actin disruptor caused the exacerbation of ischemic damage and was rescued by cytochalasin-D,30 whereas another study revealed that cytochalasin-D abolished the infarct limitation by IP.31 However, inhibition of Rho and Rho-kinase has also been reported to activate endothelial NO synthase,15 KATP channels,32 and 5'-nucleotidase,33 all of which have been reported to protect the myocardium against ischemia-reperfusion injury. Additional studies are needed to clarify such issues, because these controversies are likely to be attributable to the differences in experimental design and in the critical time window for the contribution of each factor.

Inhibition of Rho-kinase has also been shown to attenuate the production of superoxide, reduce the generation of monocyte chemoattractant protein-1 or plasminogen activator inhibitor-1, and inhibit the activation of macrophages, neutrophils, and platelets, all of which finally lead to the inhibition of stress-induced regional inflammatory responses and diminished myocardial ischemia-reperfusion injury.9 IP has been reported to modulate most of these factors,1,12 additionally supporting our present results. Accordingly, our preliminary data in the same model indicate that the intracoronary infusion of Y27632 (3 μg/kg per min) for 30 minutes just after reperfusion also reduced infarct size (22.3±4.8%, n=7) as expected. However, the underlying mechanism and its association with the IP-induced preschemic PKA activation should be further elucidated.

In conclusion, although additional studies will be needed before these can be used for the development of novel, safe, and effective therapies, inhibition of Rho-kinase during ischemia, long-term inhibition of Rho-kinase pharmacologically or genetically, or repeated short-term activation of β-adrenoceptors or PKA may also be useful.

**Acknowledgments**

This study was supported by Grants on Human Genome, Tissue Engineering and Food Biotechnology (H13-Genome-11), Grants on Comprehensive Research on Aging and Health (H13-21seiki[seikatsu]-23), Health and Labor Sciences Research from the Ministry of Health, Labor and Welfare, and a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan and in part by Mitsubishi Pharma Research Foundation, the Japan Heart Foundation, and a Grant-in-Aid for JSPS fellows from the Japan Society for the Promotion of Science.

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*Circulation*. 2004;110:51-57; originally published online June 21, 2004;
doi: 10.1161/01.CIR.0000133390.12306.C7
*Circulation* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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