The role of ADAM protease in the tyrosine kinase-mediated trigger mechanism of ischemic preconditioning

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Abstract

Objective: The aim of this study was to determine the role of an adisintegrin and metalloprotease (ADAM) in tyrosine kinase-mediated mechanisms of ischemic preconditioning (PC). Methods and results: In isolated rabbit hearts, PC was performed with two cycles of 5 min ischemia/5 min reperfusion and infarction was induced by 30 min global ischemia/2 h reperfusion. Translocation of protein kinase C-ε (PKC-ε) and tyrosine phosphorylation in the tissue and TNF-α in coronary effluent were determined by immunoblotting. PC reduced infarct size from 55.1 ± 6.8% of the left ventricle to 24.4 ± 5.2%, and this protection was mimicked by pretreatment with 100 nM angiotensin II. Both the PC effect and angiotensin II-induced protection were abolished by genistein and by 10 μM KB-R7785 (KBR), an inhibitor of ADAM12/17, but not by a lower ADAM12-selective dose (1 μM) of KBR. AG1478, an inhibitor of EGF receptor tyrosine kinase, did not inhibit protection afforded by PC. PC provoked release of TNF-α into the coronary effluent, which was abolished by 10 μM KBR but not by 1 μM of KBR or calphostin C, a PKC inhibitor. PKC-ε translocation by PC was not affected by KBR. PC induced tyrosine phosphorylation of 60 and 90 kDa proteins, and this phosphorylation was abolished by 10 μM KBR but not by calphostin C. Pretreatment with TNF-α limited infarct size to 16.7 ± 3.7% and induced tyrosine phosphorylation of a 60 kDa protein. Conclusions: The results support the hypothesis that an ADAM contributes to the triggering of a tyrosine kinase-mediated and PKC-independent pathway of PC. The ADAM responsible for this tyrosine kinase-mediated pathway is likely to be ADAM17, which sheds TNF-α.

Keywords: Matrix metalloproteinases; Infarction; Preconditioning; Signal transduction

1. Introduction

Exposure of the myocardium to a brief period of sublethal ischemia markedly enhances its tolerance to subsequent ischemic injury. This adaptive response is called ischemic preconditioning (PC). It has been established that the mechanism of PC is triggered by activation of Gq/Gi protein-coupled receptors (GPCRs), including adenosine A1/3 and bradykinin B2 receptors [1]. An important step in PC downstream of stimulation of these receptors is activation of protein kinase C (PKC) [1–3]. In addition to PKC, involvement of tyrosine kinases in a distinct pathway in PC has been suggested by earlier studies, including ours, using tyrosine kinase inhibitors [4–6]. However, the role of tyrosine kinase in signal transduction in PC and its relationship with GPCRs remain unclear.

In the present study, we hypothesized that stimulation of GPCRs by PC induces activation of an adisintegrin and metalloprotease (ADAM) [7,8], which in turn activates a tyrosine kinase-mediated trigger mechanism of PC. The rationale for this hypothesis is 2-fold. Firstly, it has been shown that stimulation of GPCRs in cardiac and non-cardiac tissues activates ADAMs, resulting in shedding of membrane-anchored receptor ligands, including heparin-binding epidermal growth factor (HB-EGF) and tumor necrosis factor-α (TNF-α) [8–11]. Secondly, the tyrosine kinase-mediated protective mechanism is likely to be provoked either by the EGF receptor, a receptor

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tyrosine kinase, or by the TNF-α receptor, which couples with nonreceptor tyrosine kinases [12,13]. A study by Krieg et al. [6] demonstrated that GPCR activated EGF receptors in the heart, and LeCour et al. [14] reported that preischemic stimulation of the TNF-α receptor mimicked PC. To examine our hypothesis, we used KB-R7785, a hydroxamic acid-based inhibitor of ADAMs [15], and assessed its effects on PC-induced PKC activation, tyrosine phosphorylation, TNF-α release, and infarct size limitation. Two doses of KB-R7785 were examined to differentiate roles of ADAM12 and ADAM17 (TNF-α-converting enzyme). In addition, effects of exogenous TNF-α on infarct size and tyrosine phosphorylation were determined. Not only genuine ischemic PC but also pharmacological PC with AT1 receptor stimulation was used to analyze links between GPCRs and ADAMs in PC.

2. Methods

This study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996).

2.1. Experiment 1: effects of KB-R7785, an inhibitor of ADAMs, on infarct size limitation by PC

2.1.1. Preparation

Isolated rabbit hearts were prepared as previously described [2,16]. In brief, hearts were excised from male albino rabbits (Japanese White) that had been anesthetized and mechanically ventilated. Each heart was quickly mounted on a Langendorff apparatus and perfused at a pressure of 75 mm Hg with nonrecirculating modified Krebs–Henseleit buffer (mmol/l: NaCl 118.5, KCl 4.7, MgSO4 1.2, KH2PO4 1.2, NaHCO3 24.8, CaCl2 2.5, and glucose 10). The buffer was gassed with 95% O2/5% CO2, resulting in a pH of 7.4–7.5, and the temperature of the perfusate was maintained at 38 °C. A fluid-filled latex balloon with a PE-160 tube was inserted into the left ventricle (LV) to monitor LV pressure, and baseline LV end-diastolic pressure was adjusted to <5 mm Hg. Atrial pacing was performed at 210 bpm if the spontaneous rate was lower. Coronary flow was measured by timed collection of coronary effluent. The heart was excluded from the study if LV systolic pressure was <70 mm Hg or if arrhythmias persisted after a 20-min stabilization period.

2.1.2. Experimental protocols

2.1.2.1. Protocol 1. After a stabilization period, all hearts were subjected to 30 min global ischemia and 2 h reperfusion. Before global ischemia, each heart was subjected to one of the following treatments, as shown in Fig. 1: no pretreatment (control), PC with two cycles of 5 min global ischemia/5 min reperfusion, infusion of 100 nM angiotensin II (Ang II) as a PC mimic, and infusion of 50 μM genistein, a nonselective tyrosine kinase inhibitor, genistein plus PC, or genistein plus Ang II. Ang II was infused for 10 min commencing 15 min before the global ischemia. KB-R7785 infusion and genistein infusion were started 25 min before ischemia and continued for 25 min.

2.1.2.2. Protocol 2. In this protocol, the roles of ADAM12 and EGF receptors in PC were examined by using low doses of KB-R7785 and AG1478, respectively. Based on previous reports [10,15], 1 μM KB-R7785 was selected as a dose to inhibit ADAM12 without affecting ADAM17. AG1478 was used at a dose of 5 μM to selectively inhibit EGF receptor tyrosine kinase [17]. Isolated hearts were subjected to no pretreatment (control), PC, 1 μM KB-R7785 plus PC, AG1478, or AG1478 plus PC. KB-R7785 and AG1478 were infused for 25 min as shown in Fig. 1. All hearts underwent 30 min global ischemia/2 h reperfusion as in Protocol 1.

2.1.2.3. Protocol 3. Hearts were subjected to no pretreatment (control), infusion of recombinant human TNF-α (0.5 ng/ml), or KB-R7785 (10 μM) plus TNF-α before 30 min global ischemia and 2 h reperfusion. TNF-α was infused for 10 min commencing 15 min before the onset of 30-min ischemia, and KB-R7785 was infused as in Protocol 1 (Fig. 1).
2.1.3. Determination of infarct size
After 2 h of reperfusion, hearts were weighed, frozen, and cut into 2-mm-thick sections from apex to base. Infarcts in the heart slices were visualized by tetrazolium staining, and sizes of infarct and the LV were measured by computer-assisted planimetry as previously reported [2,16].

2.2. Experiment 2: TNF-α release by PC

2.2.1. Preparation and protocol
Rabbit hearts were isolated, perfused, and subjected to 30 min global ischemia and 2 min reperfusion.

2.2.1.1. Protocol 1. Before the ischemia, hearts received one of four pretreatments: no pretreatment (untreated control), PC, infusion of 10 μM KB-R7785 plus PC, and infusion of calphostin C plus PC. PC and infusion of KB-R7785 (10 μM) were performed as in Experiment 1, and infusion of calphostin C (200 nM) was commenced at 25 min before the sustained ischemia. The dose of calphostin C was selected on the basis of results of our previous studies [18], in which the same dose of this PKC inhibitor abolished PC-induced translocation of PKC-ε.

2.2.1.2. Protocol 2. Hearts were subjected to PC or infusion of 1 μM KB-R7785 plus PC. The protocol of PC and timing of KB-R7785 infusion were the same as those in Protocol 1.

2.2.2. Preparation of samples for TNF-α assay
Coronary effluent was collected for 2 min under the baseline condition, immediately after PC ischemia or its time control period, and after reperfusion following the 30-min ischemia. Eighty milliliters of each effluent sample was concentrated to 1 ml by using Centricon Plus-80 (Millipore, Bedford, MA, USA), a filter device with a molecular cutoff of 10,000. The concentrated samples were stored at −70 °C until use for assays. To obtain a positive control of rabbit TNF-α, 100 μg/kg of lipopolysaccharide (LPS) generated from E. coli (SIGMA) was intravenously injected into a rabbit, and plasma was sampled 90 min after the LPS injection.

2.2.3. TNF-α assay
The samples and a positive control were run on a 15/25% polyacrylamide gradient gel and then electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane. After the transfer, the membrane was blocked with 5% donkey serum in a buffer containing 100 mM NaCl, 10 mM Tris–HCl (pH 7.4), and 0.1% Tween 20 for 1 h and then incubated overnight with 10,000-fold diluted goat anti-rabbit TNF-α antibody (PharMingen, San Diego, CA, USA) at 4 °C overnight and then with 100,000-fold diluted peroxidase-conjugated donkey anti-goat IgG (Jackson ImmunoResearch Laboratory, West Grove, PA, USA) at room temperature for 1 h. The immunocomplexes were detected by an ECL Advanced Western Blotting Detection Kit (Amersham, Buckinghamshire, UK). In a separate series of experiments, 10 μl buffer samples containing 0, 0.1, 0.5, and 1.0 pg/ml of rabbit TNF-α (BD Biosciences Pharmingen, USA) were loaded on the gel together with 1 μl of the positive control serum, and the regression equation for the relationship between the known amount of TNF-α and TNF-α signals in the immunoblots was calculated (v = 140 + 1842x, r = 0.98, p < 0.05). Using this equation, the level of TNF-α in the control serum and TNF-α levels in the effluent samples were determined. TNF-α signals in the immunoblots were quantified by using SigmaGel, gel analysis software (SPSS, Chicago, IL, USA).

2.3. Experiment 3: effects of ADAM inhibition on PKC translocation by PC

2.3.1. Tissue preparation
Rabbit hearts were isolated, perfused, and subjected to 10 min global ischemia. Prior to the sustained ischemia, each heart received one of the following three pretreatments: no treatment (control), PC, and infusion of KB-R7785 (10 μM) were performed as in Experiment 1. LV biopsy samples (0.5–1.0 g) were taken at three time points: after stabilization, immediately before the sustained ischemia, and at 10 min after the onset of ischemia. Immediately after biopsy, the tissue samples were frozen in liquid nitrogen and stored at −70°C until use for biochemical analyses.

2.3.2. Immunoblotting
Cytosolic and particulate fractions were prepared, and the PKC-ε level in each fraction was determined by Western blotting as previously reported [2,16]. In brief, frozen heart samples were homogenized in cold buffer containing 50 mM Tris–HCl (pH 7.4), 5 mM EDTA, 10 mM EGTA, 50 mM NaF, 50 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 0.3% β-mercaptoethanol. The homogenate was centrifuged at 10000 × g for 10 min, and then the supernatant was centrifuged at 100,000 × g for 60 min. The 100,000 × g supernatant was used as the cytosolic fraction. The 100,000 × g pellet was treated with 0.3% Triton X-100 and centrifuged at 10,000 × g for 10 min to obtain the particulate fraction as supernatant. Cytosolic and particulate samples (30 μg protein for each) were loaded on a 12.5% polyacrylamide gel, electrophoresed, and Western blotting was performed by using anti-PKC-ε antibodies [2,16]. PKC-ε was the only isoform analyzed in this study, because it has been established that this isoform is responsible for PC in rabbit hearts [1–3].
2.4. Experiment 4: effects of inhibition of ADAM and PKC on PC-induced tyrosine phosphorylation

2.4.1. Tissue preparation

Rabbit hearts were perfused and subjected to 10 min global ischemia as in Experiment 3. Before the global ischemia, each heart received one of the following six pretreatments: no pretreatment (control), PC, KB-R7785 plus PC, PP1 plus PC, calphostin C plus PC, and TNF-α. PC and KB-R7785 (10 μM) infusion were performed as in Experiment 1. PP1 (4-amino-5-(4-methylphenyl)-7-(t-buty1)-pyrazolo-3,4-D-pyrimidine; 5 μM), a Src kinase inhibitor [19], and calphostin C (200 nM) were infused from 5 min before PC until the onset of reperfusion. TNF-α was infused at a dose of 0.5 ng/ml as in Experiment 1.

2.4.2. Immunoblotting

Heart samples were homogenized in ice-cold buffer containing 20 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 μg/ml leupeptin and a protease inhibitor cocktail (Complete, Roche Molecular Biochemicals, Mannheim, Germany). The samples were centrifuged for 15 min at 13,000 × g, and the supernatant was used for Western blotting. Samples were electrophoresed on 7.5% polyacrylamide gel and electroblotted onto PVDF membranes. The blots were blocked with 1% bovine serum albumin in a buffer containing 100 mM NaCl, 10 mM Tris–HCl (pH 7.4), and 0.1% Tween 20 for 1 h. For detection of tyrosine phosphorylation, 1000-fold diluted antibodies against phosphoryrosine (Transduction Laboratories; Lexington, KY, USA) were used. We normalized each phosphoryrosine signal in immunoblots by protein level in the corresponding band in the gels stained with Coomassie Brilliant Blue after electrophoresis; protein levels in the stained bands were determined by densitometry.

2.4.3. Chemicals

Ang II, genistein and AG1478 were obtained from SIGMA (St. Louis, MO, USA). PP1 was purchased from Tocris (Ellisville, MO, USA). KB-R7785 was kindly provided by Nippon Organon KK (Osaka, Japan). Recombinant human TNF-α was obtained from Pepro Tech EC (London, UK).

2.4.4. Statistics

All data are presented as means ± SEM. Inter-group differences in body weight, heart weight, and infarct size were examined by one-way analysis of variance (ANOVA) combined with the Student–Newman–Keuls post hoc test. Differences in hemodynamic parameters and TNF-α levels between study groups were tested by two-way repeated-measures ANOVA. One-way repeated-measures ANOVA

<table>
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<td>CF (ml/min)</td>
<td>LVDP (mm Hg)</td>
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<td>KBR (10 μM)+TNF-α</td>
<td>223 ± 8</td>
<td>71 ± 5</td>
<td>99 ± 4</td>
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</table>

HR, heart rate; CF, coronary flow; LVDP, left ventricular developed pressure; KBR, KB-R7785; Ang II, angiotensin II; Gen, genistein. Measurements were made under baseline condition (Baseline), 1 min before global ischemia (Treatment) and 120 min after the onset of reperfusion (Reperfusion).

*P<0.05 vs. Control in each protocol. Values are means ± SEM.
was used to test differences in levels of PKC and tyrosine-phosphorylated proteins during experiments in each study group. These statistical analyses were performed using SigmaStat (SPSS). The difference was considered significant if the *P* value was < 0.05.

### 3. Results

#### 3.1. Exclusion of hearts

One hundred and sixty-seven hearts were used in the present study, and seven were excluded according to the exclusion criteria: two hearts showed arrhythmias and five hearts failed to develop LV pressure >70 mm Hg.

#### 3.2. Experiment 1

##### 3.2.1. Hemodynamic parameters

Hemodynamic parameters in Protocols 1, 2 and 3 are summarized in Table 1. There were no significant differences in baseline heart rate, LV developed pressure (LVDP), and coronary flow among the study groups. PC slightly reduced LVDP before the 30-min ischemia. Administration of genistein and AG1478 had little effect on heart rate and LVDP but significantly increased coronary flow. Ang II infusion increased LVDP by 20% and reduced coronary flow by 15%, but these effects were cancelled by co-infusion of genistein. Post-ischemic recovery of LVDP was improved in the PC and Ang II-treated groups. These beneficial effects were abolished by 10 μM KB-R7785 and genistein (Protocol 1) but not by 1 μM of KB-R7785 and AG1478 (Protocol 2). TNF-α tended to improve LVDP after reperfusion regardless of co-infusion with KB-R7785.

##### 3.2.2. Infarct size data

Heart weights and LV volumes in the study groups were comparable (data not shown). In Protocol 1 (Fig. 2), PC and Ang II significantly reduced infarct size as a percentage of LV (%IS/LV) from 55.1 ± 6.8% to 24.4 ± 5.2% and 23.7 ± 4.7%, respectively. Both PC- and Ang II-induced protection were abolished by 10 μM KB-R7785 (%IS/LV = 54.5 ± 4.6% and 58.7 ± 6.2%, respectively) and also by genistein (%IS/LV = 50.1 ± 4.6% and 53.6 ± 2.9%, respectively), although 10 μM KB-R7785 alone and genistein alone did not modify %IS/LV. In Protocol 2 (Fig. 3), PC reduced %IS/LV from 51.9 ± 7.0% to 20.5 ± 4.8% as in Protocol 1. Neither 1 μM of KB-R7785 nor AG1478 inhibited this infarct size-limiting effect of PC (%IS/LV = 26.9 ± 4.9% and 19.0 ± 5.1%, respectively). In Protocol 3 (Fig. 4), infusion of TNF-α reduced %IS/LV from 49.6 ± 7.8% to 16.7 ± 3.7%, and this PC-like effect of TNF-α was not inhibited by 1 μM of KB-R7785 (%IS/LV = 21.8 ± 2.9%).

![Fig. 2. Infarct size normalized as a percentage of the left ventricle in Protocol 1. Open circles represent individual data, and means ± SEM are also shown for each group. *P* < 0.05 vs. control. KBR = KB-R7785, Gen = 50 μM genistein.](image1)

![Fig. 3. Infarct size normalized as a percentage of the left ventricle in Protocol 2. Open circles represent individual data, and means ± SEM are also shown for each group. *P* < 0.05 vs. control. KBR = KB-R7785, AG1478 = 5 μM AG1478.](image2)

![Fig. 4. Infarct size normalized as a percentage of the left ventricle in Protocol 3. Open circles represent individual data, and means ± SEM are also shown for each group. *P* < 0.05 vs. control. KBR = KB-R7785.](image3)
3.3. Experiment 2

Although TNF-α was not detectable in the coronary effluent before ischemia, there was significant TNF-α release after 30 min ischemia (Fig. 5). PC alone induced TNF-α release and attenuated the TNF-α release after the sustained ischemia. TNF-α release both after PC and after the sustained ischemia was suppressed by 10 μM KB-R7785. In contrast, 1 μM KB-R7785 failed to inhibit TNF-α release after PC. Calphostin C did not affect TNF-
α release after PC but eliminated PC-induced suppression of TNF-α release after sustained ischemia.

3.4. Experiment 3

The percentage of the particulate fraction in the total (i.e., particulate fraction/cytosolic fraction plus particulate fraction; %PF) of PKC-ε under the baseline condition was 54.3 ± 2.8%, and it was increased to 64.9 ± 1.7% after 10 min of ischemia (Fig. 6). PC significantly increased the %PF of PKC-ε from 50.8 ± 3.1% at baseline to 59.1 ± 3.3%, and it was further increased to 68.9 ± 3.0% after 10 min of ischemia. Infusion of KB-R7785 did not inhibit the increase in %PF of PKC-ε after PC and that after subsequent ischemia (61.9 ± 3.7% and 73.4 ± 5.3%, respectively, both p < 0.05 vs. 51.6 ± 4.0% at baseline).

3.5. Experiment 4

As shown in Fig. 7, tyrosine phosphorylation of 60 and 90 kDa proteins was enhanced by 1.5-fold and 1.7-fold, respectively, at 10 min after ischemia in untreated controls. PC enhanced tyrosine phosphorylation of the two proteins with the same molecular weight and suppressed the phosphorylation during the subsequent 10 min of ischemia. KB-R7785 completely inhibited the effects of PC on tyrosine phosphorylation. PP1 also prevented PC-induced tyrosine phosphorylation of 60 and 90 kDa proteins. In contrast, calphostin C did not inhibit phosphorylation of the proteins with these molecular weights by PC. Preischemic infusion of TNF-α mimicked the effect of PC on tyrosine phosphorylation of 60 kDa proteins but not that on 90 kDa proteins.

4. Discussion

In the present study, we hypothesized that ADAM12 or ADAM17 contribute to PC, since activation of these ADAMs induces shedding of HB-EGF and TNF-α, respectively, which would provoke tyrosine kinase-mediated signals. If ADAM12 is involved in PC, 1 μM of KB-R7785 should be sufficient to abolish PC against infarction, because KB-R7785 is ∼ 470-fold more selective to ADAM12 than to ADAM17, and IC₅₀ for ADAM12 is approximately 0.2 μM [10]. However, this lower dose of KB-R7785 failed to abolish cardioprotection of PC (Fig. 3). Furthermore, PC protection was not affected by AG1478, which should have blocked activation of the EGF receptor by HB-EGF. These results indicate that ADAM12 is not crucially involved in PC. Contribution of other matrix metalloproteinases such as gelatinase and stromelysin, for which IC₅₀ values of KB-7785 are even lower (1.9–3.9 nM) [15], to PC is also highly unlikely. In contrast, 10 μM of KB-R7785, which is sufficient to inhibit ADAM17 (TNF-α-converting enzyme), successfully abrogated PC-induced anti-infarct tolerance. Conversely, TNF-α infusion before ischemia mimicked infarct size limitation by PC, which confirmed the finding by Lecour et al. [14] in rat hearts. These results are consistent with the hypothesis of involvement of ADAM17 in PC against infarction.

To confirm the contribution of ADAM17 to PC, we examined whether PC indeed induces TNF-α production in the heart. Although Belosjorow et al. [20] have shown that PC of the rabbit heart in situ increased the TNF-α level in peripheral blood, the origin of TNF-α has not been identified. In the present study, PC induced significant release of TNF-α into the coronary effluent, which was
inhibited by 10 μM KB-R7785 but not by 1 μM KB-R7785 (Fig. 5). The level of TNF-α in the coronary effluent after PC (3–5 pg/ml) was much lower than the dose of TNF-α (0.5 ng/ml) that was shown to mimic PC (Fig. 4). However, the level of TNF-α in the cardiac interstitium at the time of ischemic insult is crucial for anti-infarct tolerance[1–3]. KB-R7785 did not affect the PC-induced PKC-ε translocation (Fig. 6), indicating that this agent lacks non-specific inhibitory effects on PKC activation. In non-cardiac cells, PKC-δ has been shown to participate in regulation of the activity of ADAMs [28]. However, such a regulation by PKC is unlikely to be important in activation of ADAM17 by PC in rabbit hearts because calphostin C failed to mimic KB-R7785 in terms of inhibition of PC-induced release of TNF-α (Fig. 5) and tyrosine phosphorylation (Fig. 7).

The importance of tyrosine kinases downstream of GPCR in PC was confirmed by the present findings that genistein abolished infarct size-limiting effects of PC and Ang II (Fig. 2). Furthermore, KB-R7785 inhibited tyrosine phosphorylation of 60 and 90 kDa proteins by PC. Infusion of TNF-α in place of PC induced tyrosine phosphorylation of 60 kDa proteins but not 90 kDa proteins. The difference between the patterns of tyrosine phosphorylation in PC- and TNF-α-treated hearts could be due to the difference in tissue levels of TNF-α and/or signal input from activation of an ADAM that is different from ADAM17 in ischemic PC. Although the tyrosine kinases in the ADAM-mediated pathway remain unclear, three lines of evidence suggest that Src tyrosine kinase is involved. First, Src kinase activation is necessary for PC to be cardioprotective[29,30]. Second, stimulation of TNF-α receptors has been shown to activate Src tyrosine kinase[12]. Third, PP1, a Src-selective inhibitor, abolished PC-induced tyrosine phosphorylation as did KB-R7785 in the present study (Fig. 7). However, possible involvement of other types of tyrosine kinases and identities of the proteins that were tyrosine-phosphorylated by PC remain to be determined.

Recently, Krieg et al. [6] found the role of Src kinase in activation of the PI3 kinase/Akt pathway after pharmacological PC. In isolated rabbit hearts, stimulation of muscarinic receptors by acetylcholine induced Src-dependent transactivation of EGF receptor tyrosine kinase and subsequent phosphorylation of Akt. However, this signaling mechanism is unlikely to be predominant in ischemic PC in our model. First, acetylcholine is not an endogenous trigger in ischemic PC [6,31]. Second, Krieg et al. [6] observed that adenosine, a primary trigger substance of ischemic PC in our preparation [16], induced a much lower level of Akt phosphorylation. Third, AG1478 failed to abolish PC-induced protection (Fig. 3). It is notable that anti-infarct tolerance afforded by acetylcholine was also resistant to AG1478 [6], indicating the existence of an EGF receptor-independent mechanism.

The present study provided pharmacological evidence suggesting the involvement of an ADAM in the tyrosine-mediated mechanism of PC. However, alteration of ADAM activities by PC and effects of KB-R7785 on their activities were not directly determined. Also, tyrosine kinases activated by PC and their substrates remain to be identified. These are limitations in the present study and they warrant further investigation.

In conclusion, ADAM may play a crucial role in the trigger mechanism of PC via activation of tyrosine kinase-
mediated pathways. The ADAM relevant to PC is likely to be ADAM17, which sheds TNF-α as a signaling molecule, although the possibility of contribution of other ADAMs cannot be excluded.

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