Protein tyrosine kinase signaling is necessary for NO donor-induced late preconditioning against myocardial stunning

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Tang, Xian-Liang, Eitaro Kodani, Hitoshi Takano, Michael Hill, Ken Shinmura, Thomas M. Vondriska, Peipei Ping, and Roberto Bolli. Protein tyrosine kinase signaling is necessary for NO donor-induced late preconditioning against myocardial stunning. Am J Physiol Heart Circ Physiol 284: H1441–H1448, 2003. First published December 12, 2002; 10.1152/ajpheart.00789.2002.—Although protein tyrosine kinases (PTKs) signaling has been implicated in the late phase of ischemic preconditioning (PC), it is unknown whether PTK signaling is necessary for the development of nitric oxide (NO) donor-induced late PC. Thus conscious rabbits underwent a sequence of six 4-min coronary occlusion (O)/4-min reperfusion (R) cycles followed by a 5-h recovery period of reperfusion for 3 consecutive days (days 1, 2, and 3). On day 0 (24 h before the 6 O/R cycles on day 1), rabbits received no treatment (control), the NO donor diethylenetriamine (DETA)/NO (DETA/NO), the PTK inhibitor 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2), or DETA/NO plus PP2 (DETA/NO + PP2). In control rabbits (n = 6), the six O/R cycles on day 1 resulted in delayed functional recovery, indicating severe myocardial stunning. In rabbits pretreated with DETA/NO (n = 5) on day 1, myocardial stunning caused by the six O/R cycles on day 1 was markedly attenuated, with a significant reduction (~60%) in the total deficit of wall thickening (WTh) compared with controls, indicating that DETA/NO induced a late PC effect against stunning. However, in rabbits pretreated with DETA/NO + PP2 (n = 5), the total deficit of WTh was significantly greater than that in rabbits treated with DETA/NO alone and was similar to that in controls, indicating that PP2 prevented the development of DETA/NO-induced late PC. In rabbits pretreated with PP2 on day 0 (n = 4), the total deficit of WTh was similar to that in controls, indicating that PP2 does not affect myocardial stunning in itself. We conclude that a PTK-dependent signaling mechanism is necessary for the development of NO donor-induced late PC against myocardial stunning in conscious rabbits.

diethylenetriamine/nitric oxide; pp2; ischemia-reperfusion injury; rabbit

PROTEIN TYROSINE KINASES (PTKs), a diverse family of enzymes that transfer phosphate from ATP to tyrosine residues on specific cellular proteins, are known to mediate a wide variety of cellular responses (30). The nonreceptor PTKs are specifically designed for signal transduction from cell surface to intracellular enzymes and factors, usually by protein-to-protein interactions (30). Recent evidence indicates that PTKs play a role in the signaling mechanism underlying both the early (1, 27, 42) and the late (10, 19, 32) phases of ischemic preconditioning (PC).

The late phase of ischemic PC is a cardioprotective phenotypic shift whereby exposure to a brief ischemic stress increases the tolerance of the heart to stunning and infarction 24–72 h later (2, 6, 7, 26). Besides ischemia, a delayed cardioprotective effect can be elicited by pretreatment with a variety of pharmacological agents, including nitric oxide (NO) donors (16, 40), adenosine A3 or A3 receptor agonists (3, 21, 38), endotoxin or endotoxin derivatives (12, 44), and bradykinin (22). In our previous studies in conscious rabbits, administration of the NO donor diethylenetriamine/NO (DETA/NO) induced a powerful late PC effect against myocardial stunning and infarction (40). More recently, we found that NO donor-induced PC in rabbits was accompanied by an increase in PKCe-associated Src protein and activity (43), suggesting that the Src family of PTKs may play an essential role in the development of NO donor-induced late PC as well. However, it is unknown whether the increase in Src protein and activity plays a causative role in NO donor-induced late PC.

The aim of this study was to reconcile this issue. Specifically, by using the potent and selective Src family kinase inhibitor PP2 [4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine] (15, 46), we sought to determine whether the Src family of PTKs plays an essential role in the signaling mechanism underlying NO donor-induced late PC against myocardial stunning in conscious rabbits.

METHODS

The present study was performed in accordance with the guidelines of the Animal Care and Use Committee of the University of Louisville (KY) School of Medicine and with the Guide for the Care and Use of Laboratory Animals [Department of Health and Human Services, Publication No. (NIH) 86-23].

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Experimental Preparation

The experimental preparation has been described in detail previously (6, 20, 21, 25, 31, 40, 41, 45). Briefly, New Zealand White male rabbits (weight, ~2.0–2.5 kg; age, 3–4 mo) were instrumented under sterile conditions with a balloon occluder around a major branch of the left coronary artery, a 10-MHz pulsed Doppler ultrasonic crystal in the center of the region to be rendered ischemic, and bipolar ECG leads on the chest wall. Before being assigned to the experiments, the animals were allowed to recover for a minimum of 10 days after surgery. Throughout the occlusion-reperfusion protocol, rabbits were kept in a cage in a quiet, dimly-lit room. Left ventricular systolic wall thickness (WTh), range gate depth, and the ECG were continuously recorded on a thermal array chart recorder (Gould TA6000; Valley View, OH). Arterial blood pressure was monitored during drug administration.

Experimental Protocol

The experimental protocol consisted of three consecutive days of coronary artery occlusions (days 1, 2, and 3, respectively). On each day, conscious rabbits were subjected to a sequence of six 4-min coronary artery occlusion/4-min reperfusion cycles (Fig. 1). The performance of successful coronary occlusion was verified by observing the development of ST-segment elevation and changes in the QRS complex on the ECG and the appearance of paradoxical systolic Wth on the ultrasonic crystal recordings.

Rabbits were assigned to four groups. Group I (control group) underwent the 3-day coronary occlusion-reperfusion protocol without any treatment. Twenty-four hours before the first day of coronary occlusion-reperfusion (on day 0), group II (DETA/NO) received four consecutive intravenous bolus doses of the NO donor DETA/NO (0.1 mg/kg each) every 25 min (total dose, 0.4 mg/kg); group III (PP2) received two intravenous boluses of the PTK inhibitor PP2 (7 μg/kg each at a 45-min interval, total dose, 14 μg/kg), and group IV (DETA/NO + PP2) received DETA/NO (same dose as in group II) plus two boluses of PP2 (same dose as in group III; one 10 min before the first bolus of DETA/NO and one 10 min after the second bolus of DETA/NO) (Fig. 1). DETA/NO (Alexis) was dissolved in PBS (total volume infused, 4 ml). To remove oxygen from the solution, the PBS was bubbled with nitrogen for at least 30 min before dissolving DETA/NO. PP2 (Calbiochem) was dissolved in DMSO and then diluted to 14 g/ml in saline at a final concentration of 4% (vol/vol) DMSO. All solutions were freshly made and filtered through a 0.2-μm Millipore filter to ensure sterility.

Measurement of Regional Myocardial Function and Ischemic Zone Size

Regional myocardial function was assessed as systolic thickening fraction by using the pulsed Doppler probe, as previously described (6, 40, 41). The total deficit of systolic Wth (an integrative assessment of the overall severity of myocardial stunning) was calculated by measuring the area...
comprised between the systolic WTh-versus-time line and the baseline (100% line) during the 5-h recovery phase after the sixth reperfusion (6, 10, 31, 33, 40, 41, 45). In all animals, measurements were averaged from at least 10 beats at baseline and from at least 5 beats at all subsequent time points. The size of the ischemic-reperfused zone was determined by postmortem perfusion with Phthalo blue dye as previously described (31, 34, 39, 40).

Statistical Analysis

Data are reported as means ± SE. For intragroup comparisons, hemodynamic variables and WTh were analyzed by a two-way repeated-measures ANOVA (time and day) to determine whether there was a main effect of time, a main effect of day, or a day-by-time interaction. If the global tests showed a significant main effect or interaction, post hoc contrasts between different time points on the same day or between different days at the same time point were performed with Student’s t-tests for paired data, and the resulting P values were adjusted according to the Bonferroni correction. For intergroup comparisons, continuous variables were analyzed by either a one-way or a two-way repeated-measures (time and group) ANOVA, as appropriate, followed by unpaired Student’s t-tests with the Bonferroni correction.

RESULTS

Exclusions

A total of 26 rabbits was instrumented for this study. Seven rabbits were assigned to each of groups I, II, and IV and five to group III. Six rabbits were excluded because of malfunction of either the WTh probe (3, one each in groups I, III, and IV) or the balloon occluder (3, two in group II and one in group IV). Therefore, 20 rabbits (6, 5, 4, and 5, respectively, in groups I, II, III, and IV) completed the protocol.

Postmortem Analysis

The size of the occluded-reperfused vascular bed was similar in the four groups: 1.00 ± 0.13 g (19.7 ± 1.1% of LV weight), 0.99 ± 0.13 g (19.5 ± 1.2%), 0.97 ± 0.12 g (21.3 ± 2.4%), and 0.96 ± 0.09 g (22.5 ± 2.4%) in groups I, II, III, and IV, respectively. Tissue staining with triphenyltetrazolium chloride confirmed that the injury associated with the six 4-min occlusion/4-min reperfusion cycles was completely reversible. In all rabbits, the ultrasonic crystal was found to be at least 3 mm from the boundaries of the ischemic-reperfused region.

Hemodynamic Variables

There were no significant changes in heart rate, arterial blood pressure, or systolic WTh at any time during or after the administration of DETA/NO or DETA/NO + PP2 in groups II and III on day 0 (Fig. 2). These results indicate that neither DETA/NO nor PP2 at the dose selected for this study had any effects on hemodynamic variables or regional myocardial function in conscious rabbits. On days 1, 2, and 3, there were no appreciable differences in heart rate among the three groups, either during the sequence of coronary occlusion-reperfusion cycles or during the 5-h reperfusion period (Table 1).

Regional Myocardial Function

Baseline systolic thickening fraction on days 1, 2, and 3 averaged 37.0 ± 5.5%, 35.4 ± 4.4%, and 36.7 ± 4.6%, respectively, in group I; 35.8 ± 3.5%, 36.9 ± 3.6%, and 38.2 ± 4.3% in group II; 45.7 ± 5.8%, 45.5 ± 7.9%, and 48.0 ± 8.5% in group III; and 33.7 ± 3.3%, 33.0 ± 2.7%, and 33.4 ± 3.0% in group IV. There were no significant differences among groups I, II, III, and IV on the same day or among different days within the same group. Furthermore, within the same group there were no significant differences among days 1, 2, and 3 with respect to the extent of paradoxical systolic thinning during the six occlusions (Figs. 3–6).
Table 1. Heart rate during coronary occlusion and reperfusion

<table>
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<tr>
<th></th>
<th>Baseline</th>
<th>O3</th>
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<th>1 h</th>
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<tr>
<td>Day 1</td>
<td>257 ± 13</td>
<td>260 ± 11</td>
<td>263 ± 18</td>
<td>251 ± 11</td>
<td>242 ± 8</td>
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<td>Day 2</td>
<td>265 ± 13</td>
<td>255 ± 14</td>
<td>245 ± 8</td>
<td>244 ± 7</td>
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<tr>
<td>Day 3</td>
<td>262 ± 6</td>
<td>243 ± 16</td>
<td>244 ± 7</td>
<td>243 ± 6</td>
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| Group II (DETA/NO, n = 5) |
| Day 1 | 255 ± 7 | 245 ± 10 | 233 ± 13 | 232 ± 17 | 220 ± 12 | 233 ± 12 |
| Day 2 | 263 ± 12| 246 ± 13 | 233 ± 12 | 232 ± 9 | 220 ± 13 | 223 ± 13 |
| Day 3 | 253 ± 14| 240 ± 13 | 233 ± 13 | 227 ± 15 | 224 ± 11 | 233 ± 14 |

| Group III (PP2, n = 4) |
| Day 1 | 232 ± 11| 254 ± 23 | 228 ± 8  | 227 ± 4  | 211 ± 17 | 215 ± 9  |
| Day 2 | 250 ± 13| 230 ± 9  | 220 ± 10 | 205 ± 10 | 2450 ± 9 | 232 ± 5  |
| Day 3 | 239 ± 4 | 230 ± 9  | 250 ± 4  | 223 ± 7  | 230 ± 11 | 208 ± 13 |

| Group IV (DETA/NO + PP2, n = 5) |
| Day 1 | 235 ± 16| 236 ± 12 | 264 ± 23 | 244 ± 14 | 225 ± 22 | 236 ± 20 |
| Day 2 | 250 ± 12| 266 ± 15 | 240 ± 13 | 234 ± 16 | 246 ± 17 | 234 ± 16 |
| Day 3 | 239 ± 21| 242 ± 15 | 236 ± 13 | 241 ± 19 | 229 ± 14 | 234 ± 13 |

Values are means ± SE (in beats/min); n, number of rabbits. DETA, diethylenetriamine; NO, nitric oxide; PP2, 4-amino-5-(4-chlorophenyl)-7-(t-butyl) pyrazole [3,4-d]pyrimidine. All rabbits underwent a sequence of 6 cycles of 4-min coronary occlusion (O) 4-min reperfusion (R), followed by a 5-h observation period on days 1, 2, and 3. Heart rate was measured 5 min before occlusion (baseline), at 3 min into the third (O3) and the sixth (O6) occlusion, and at 1, 3, and 5 h after the sixth reperfusion.

Group I. On day 1, thickening fraction remained significantly (P < 0.05) depressed for 4 h after the sixth reperfusion and recovered by 5 h (Fig. 3), indicating that the sequence of six 4-min occlusion/4-min reperfusion cycles resulted in severe myocardial stunning that lasted, on average, 4 h. On days 2 and 3, however, the recovery of WTh after the sixth occlusion-reperfusion cycles was markedly improved compared with day 1 (Fig. 3). The total deficit of WTh after the sixth reperfusion was 54% less on both days 2 and 3 compared with day 1 (P < 0.01) (Fig. 7). Thus, as expected (6, 33, 40), myocardial stunning was attenuated markedly, and to a similar extent, on days 2 and 3 compared with day 1.

Group II. Although on day 1 the extent of paradoxical wall thinning in group II was similar to that noted in control rabbits, the recovery of WTh after the sixth reperfusion was markedly faster than in the control...
group, and this improvement was sustained throughout the entire reperfusion interval (Fig. 4). The total deficit of WTh in group II was 60% less than that observed in control rabbits on day 1 (P < 0.05) and similar to that observed in control rabbits on days 2 and 3 (Fig. 7). On days 2 and 3, there was no further improvement in either the recovery of WTh (Fig. 4) or the total deficit of WTh (Fig. 7) compared with day 1. Thus administration of DETA/NO 24 h before the sequence of six 4-min occlusion-reperfusion cycles resulted in an attenuation of myocardial stunning on day 1 that was essentially equivalent to that effected by ischemic PC.

Group III. Both the recovery of WTh (Fig. 5) and the total deficit of WTh (Fig. 7) on days 1–3 were similar to those observed in the control group, indicating that pretreatment with PP2 in itself had no effect on myocardial stunning.

Group IV. On day 1, both the recovery of WTh (Fig. 6) and the total deficit of WTh (Fig. 7) were virtually indistinguishable from those noted on day 1 in the control group, indicating the absence of PC against stunning. Thus the PC effect induced by DETA/NO was completely abrogated by the concomitant administration of PP2. A PC effect became apparent on days 2 and 3, as documented by the enhanced recovery of WTh (Fig. 6) and the reduced deficit of WTh (Fig. 7) compared with day 1.

**DISCUSSION**

The goal of the present study was to determine whether PTK signaling is necessary for the development of NO donor-induced late PC against myocardial stunning. Using conscious, chronically instrumented rabbits, we found that the NO donor DETA/NO, in the absence of ischemia, induces significant protection against myocardial stunning 24 h later, confirming our previous observations (31, 40). Our results further demonstrate that the ability of DETA/NO to induce delayed protection against stunning was completely abrogated by the concomitant administration of the Src-selective PTK inhibitor PP2, indicating that the mechanism whereby NO induces late PC involves the activation of the Src PTK family. The inhibition of late PC by PP2 cannot be ascribed to the vehicle used for PP2 (0.02 ml/kg DMSO), because previous studies have demonstrated that higher doses of DMSO (up to 0.5 ml/kg), given on day 1, do not interfere with the development of ischemia-induced late PC against stunning in this rabbit model (33).

Although previous studies have demonstrated that PTKs play a key role in the signaling mechanism underlying the late phase of ischemic PC (9, 10, 19, 32) and that NO donor-induced PC in rabbits is accompanied by an increase in PKC-associated Src protein and activity (43), a causative role of the Src family of PTKs in eliciting NO donor-induced preconditioning has not been documented. PP2 is a Src family-selective PTK inhibitor (4, 15, 46) and has been employed as a tool to investigate the molecular mechanisms of pathological events associated with activation of this family of kinases (8, 11, 13, 36). Using PP2, in the present study, we have provided evidence that the Src family of PTKs plays an essential role in NO donor-induced late PC against myocardial stunning.

**Fig. 5.** Systolic thickening fraction in the ischemic-reperfused region in group III (PP2 group) 5 min before the first occlusion (baseline), 3 min into each coronary occlusion, 3 min into each reperfusion, and at selected times during the 5-h reperfusion interval following the sixth occlusion. To facilitate comparisons, the data pertaining to day 1 of group I (control) are also shown (n = 6). Thickening fraction is expressed as a percentage of baseline values. Data are means ± SE; n = 4 for all 3 days.

**Fig. 6.** Systolic thickening fraction in the ischemic-reperfused region in group IV (DETA/NO + PP2 group) 5 min before the first occlusion (baseline), 3 min into each coronary occlusion, 3 min into each reperfusion, and at selected times during the 5-h reperfusion interval following the sixth occlusion. To facilitate comparisons, the data pertaining to day 1 of group I (control) are also shown (n = 6). Thickening fraction is expressed as a percentage of baseline values. Data are means ± SE; n = 5 for all 3 days.
Several PTK inhibitors are available, including lavendustin A, genistein, and staurosporine. The rationale for using PP2 instead of lavendustin A as a PTK inhibitor was that the former has a much greater range of selectivity for the Src family of PTKs than the latter and thus enabled us to specifically interrogate this particular subset of PTKs. Indeed, PP2 is the most potent and selective inhibitor of the Src family of PTKs reported to date (15, 46). For example, its IC₅₀ for Lck and Fyn is 4–5 nM, considerably less than that for other PTKs [e.g., >100 μM for ZAP-70, >50 μM for JAK2, 0.5 μM for EGF receptor PTK (15)]. It has no activity on PKA or PKC (15). By comparison, lavendustin A is 100-fold less potent for Src PTKs (IC₅₀ = 500 nM vs. 5 nM with PP2) and exhibits much higher affinity for EGF receptor PTK (IC₅₀ = 11 nM) than for Src PTKs (IC₅₀ = 500 nM) (28, 29). Other widely used PTK inhibitors, such as genistein and staurosporine, are general inhibitors of PTKs with little specificity for individual subgroups (15, 23, 46). To minimize the possibility that PP2 inhibited kinases other than Src PTKs, we used a dose that produced estimated serum levels of PP2 of ~40 nM, i.e., 10 times higher than the IC₅₀ for the Src family of PTKs (~4–5 nM) but much lower than the IC₅₀ for the epidermal growth factor receptor kinase, JAK2, and ZAP-70, as indicated above (15, 35). Because this dose had no discernible hemodynamic effects (Fig. 2), the possibility that DETA/NO could elicit PC by causing myocardial ischemia as a result of hypotension and/or tachycardia can be excluded.

The present study was performed in conscious animals in an effort to rigorously test the potential cardioprotective action of NO donors under conditions that are as physiological as possible. Open-chest animal preparations are associated with a number of factors, such as anesthesia, surgical trauma, fluctuations in temperature, elevated catecholamine levels, abnormal hemodynamics, exaggerated formation of reactive oxygen species (ROS), etc., which may interfere with myocardial stunning (5, 24) and/or with ischemic PC (14, 37). Moreover, because the focus of this study was to examine the role of NO in triggering late PC, we felt it was important to eliminate experimental conditions that may modulate the activity of NO synthase (NOS). For example, the trauma and the inflammatory reaction associated with a thoracotomy may promote the release of cytokines, which in turn could induce inducible NOS (iNOS) activity. In this regard, Hoshida et al. (17) found in dogs that the myocardial content of manganese superoxide dismutase in the nonischemic (control) region increased significantly 24 h after a thoracotomy, possibly as a result of the release of cytokines in the initial hours following surgery. Also, surgical exposure of the brain has been found to induce iNOS in cerebral tissue even in the absence of ischemia (18). Because transcription of the iNOS gene is controlled in part by antioxidant-sensitive transcription factors (e.g., nuclear factor-κB), the excessive formation of ROS in open-chest animals (24) could also contribute to artifactual iNOS induction in these models.

In conclusion, this study indicates that PTK signaling is necessary for the initiation of NO donor-induced late PC against myocardial stunning in conscious rabbits. The data expand our previous findings (10, 32) that Src PTK activity is required for the initiation of ischemia-induced late PC against stunning (10) [but not against infarction (9)] in this same rabbit model. Together with these previous data, the present study supports a central function of Src PTKs in the genesis of the late PC phenotype.

Fig. 7. Total deficit of WTh after the sixth reperfusion on days 1, 2, and 3 in the control (n = 6), DETA/NO (n = 5), PP2 (n = 4), and DETA/NO + PP2 (n = 5) groups (groups I, II, III, and IV, respectively). Left, values of total deficit of WTh in individual rabbits; right, mean ± SE values of total deficit of WTh. Total deficit of WTh was measured in arbitrary units, as described in the text.

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REFERENCES


37. Schwartz LM, Jennings RB, and Reimer KA. Premedication with the opioid analgesic butorphanol raises the threshold for

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