Loss of Myocardial Ischemic Postconditioning in Adenosine A₁ and Bradykinin B₂ Receptors Gene Knockout Mice

Lei Xi, MD; Anindita Das, PhD; Zhi-Qing Zhao, MD, PhD; Vanessa F. Merino, PhD; Michael Bader, PhD; Rakesh C. Kukreja, PhD

Background—Ischemic postconditioning (PostC) is a recently described cardioprotective modality against reperfusion injury, through series of brief reflow interruptions applied at the very onset of reperfusion. It is proposed that PostC can activate a complex cellular signaling cascade, in which cell membrane receptors could serve as the upstream triggers of PostC. However, the exact subtypes of such receptors remain controversial or uninvestigated. To this context, the purpose of present study was to determine the definitive role of adenosine A₁ and bradykinin B₁ and B₂ receptors in PostC.

Methods and Results—The hearts isolated from adult male C57BL/6J wild-type mice or the mice lacking adenosine A₁, or bradykinin B₁ or B₂ receptors subjected to zero-flow global ischemia and reperfusion in a Langendorff model. PostC, consisting of 6 cycles of 10 seconds of reperfusion and 10 seconds of ischemia, demonstrated significantly reduced myocardial infarct size (22.8±3.1%, mean±SEM) as compared with the non-PostC wild-type controls (35.1±2.8%, P<0.05). The infarct-limiting protection of PostC was absent in adenosine A₁ receptor knockout mice (34.9±2.7%) or bradykinin B₂ receptor knockout mice (33.3±1.7%) and was partially attenuated in bradykinin B₁ receptor–deficient mice (25.6±2.9%; P>0.05). On the other hand, PostC did not significantly alter posts ischemic cardiac contractile function and coronary flow.

Conclusions—With the use of three distinctive strains of gene knockout mice, the current study has provided the first conclusive evidence showing PostC-induced infarct-limiting cardioprotection could be triggered by activation of multiple types of cell membrane receptors, which include adenosine A₁ and bradykinin B₂ receptors. (Circulation. 2008; 118[suppl 1]:S32–S37.)

Key Words: adenosine ■ bradykinin ■ infarction ■ receptors ■ reperfusion

Ischemic postconditioning (PostC) is a series of brief mechanical interruptions of reperfusion applied at the very onset of reperfusion. Since the original description of PostC in an in vivo dog model in 2003, this cardioprotective phenomenon has been confirmed by a number of research groups in several mammalian species including rat, mouse, rabbit, dog, and human. This novel cardioprotective strategy has received considerable interest, mainly because of its potential clinical applicability as a posts ischemic intervention to reduce the cellular damages caused by the pathological factors during the initial minutes of reperfusion. In terms of the timing of intervention, PostC has an advantage over ischemic preconditioning, which has to be applied before a sustained ischemic event whose occurrence is usually not easy to predict precisely. In contrast, PostC apparently does not have the same pretreatment timing restraint for preconditioning and therefore it can be used during the routine interventional reperfusion procedures in the patients suffering acute myocardial infarction.

It is now widely accepted that PostC has interrelated protective mechanisms. During the PostC maneuvers, the washout of intracoronary release of adenosine (possibly bradykinin as well) was significantly delayed. Furthermore, the PostC-induced intermittent accumulation/release of adenosine and bradykinin could also facilitate the activation of their corresponding receptors on cardiac cell membranes, which in turn triggers the signaling cascade of PostC. Nevertheless, the exact subtypes of adenosine and bradykinin receptors involved in triggering PostC remain controversial or unknown. To resolve this issue, the present study was undertaken to determine the efficacy of PostC in reducing myocardial infarct size in strains of mice lacking adenosine A₁ and bradykinin B₁ and B₂ receptors.

Materials and Methods

Animals

Adult male C57BL/6J wild-type (C57-WT) mice and homozygous (−/−) bradykinin B₂ receptor knockout (B2-KO) mice were purchased from The Jackson Laboratory (Bar Harbor, Me). Adult male adenosine A₁ receptor knockout (A1-KO) mice were bred in the Virginia Commonwealth University (VCU) animal facility using the
breeding pairs of homozygous (−/−) A1-KO mice, a generous gift from Dr Jurgen Schnerrmann (NIDDK, Bethesda, Md). Adult male homozygous (−/−) bradykinin B1 receptor knockout (B1-KO) mice were generated at the Max-Delbrück-Center for Molecular Medicine, Berlin-Buch, Germany according to the published methods. The B1-KO mice at age of 6 weeks were transferred to VCU animal facility. Because all of the gene-knockout mice used in this study were generated from C57BL/6J background, C57-WT mice were used as the common control group for A1-KO, B1-KO, and B2-KO groups. The animal care and experiments were conducted in conformity with the guidelines on humane use and care of laboratory animals for biomedical research published by NIH (No. 85-23, revised 1996), and the rodent experiment protocol was approved by the Institutional Animal Care and Use Committee of VCU.

Model of Global Ischemia-Reperfusion in Langendorff Isolated Mouse Heart
The methodology of Langendorff isolated buffer-perfused mouse heart preparation was previously described in details. In brief, the mouse was anesthetized with pentobarbital sodium (100 mg/kg, 33 IU heparin; i.p.) and the heart was quickly removed from the thorax and placed in ice-cold buffer. The aortic opening was rapidly cannulated (time delay <3 minutes) and tied on a 20-gauge blunt needle connected to Langendorff perfusion system. The heart was retrogradely perfused at constant pressure of 55 mm Hg with Krebs-Henseleit buffer containing (in mmol/L): 118 NaCl, 24 NaHCO3, 2.5 CaCl2, 4.7 KCl, 1.2 KH2PO4, 1.2 MgSO4, 1 glucose, and 0.5 EDTA. The buffer was continuously gassed with 95% O2 + 5% CO2 (pH ~7.4) and warmed by a heating bath/circulator. The heart temperature was continuously monitored and maintained at 37±0.5°C. Ventricular function was measured by a force-displacement transducer (model FT03, Grass) attached to the apex with surgical thread and metal hook. The resting tension was adjusted to ~0.30 g. The contractile force was continuously recorded with a PowerLab 8SP computerized data acquisition system connected to the force transducer. Coronary flow rate was calculated by timed collection of the outflow perfusate. No electric pacing was applied to the heart.

Experiment Groups and Protocol
As illustrated in Figure 1, C57-WT control mice and the three strains of receptor-deficient mice (ie, A1-KO, B1-KO, B2-KO) were randomized into the following 8 experiment groups. Group 1: C57-WT (n=8), C57-WT subjected to no-flow global ischemia and 10 minutes of reperfusion; Group 2: C57-WT+PostC (n=8), C57-WT subjected to ischemia and PostC intervention (6 cycles of 10 seconds of reperfusion and 10 seconds of ischemia) during the initial 2 minutes of reperfusion; Group 3: A1-KO (n=8), A1-KO subjected to ischemia and reperfusion; Group 4: A1-KO+PostC (n=7), A1-KO subjected to ischemia and reperfusion; Group 5: B1-KO (n=9), B1-KO subjected to ischemia and postconditioning; Group 6: B1-KO+PostC (n=9), B1-KO subjected to ischemia and reperfusion; and Group 8: B2-KO+PostC (n=7), B2-KO subjected to ischemia and PostC.

Measurement of Infarct Size
At the end of experiment, the heart was immediately removed from Langendorff apparatus, weighed, and frozen at ~20°C. The frozen heart was manually cut into 7 to 8 transverse slices (~1 mm thickness), which were incubated in 10% TTC for 30 minutes at room temperature. TTC was then replaced with 10% formaldehyde for 3 to 4 hours of fixation before infarct size was measured using a computerized morphometry system (Bioquant 98). The risk area was calculated as total ventricular area minus cavities. Infarct size was calculated as % of risk area.

Data Analysis and Statistics
The experimental data were presented as the group means and standard errors (SEM). The difference among experimental groups was analyzed with 2-way ANOVA followed by Bonferroni post hoc test for pair-wise comparison. This analysis examines the effects of two variables (ie, Factor 1: PostC intervention and Factor 2: animal genetic background), both individually and together, on the experimental response. P<0.05 was considered as statistically significant.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results
Animal Exclusion Record and Morphometric Characteristics
Of 66 mice originally used in the present study, 3 mice (ie, 4.5% of the total) were excluded because of persistent arrhythmia or poor preischemic function. The body weight and heart weight were significantly higher in A1-KO and B1-KO mice as compared with the non-PostC C57-WT mice, indicating a genetic background related variation in body/heart mass (Table 1).

Myocardial Infarct Size
As shown in Figures 2 and 3, PostC resulted in reduction of infarct size following ischemia-reperfusion in C57-WT mice (22.8±3.1%), as compared with the wild-type control mice without PostC (35.1±2.8%; P<0.05). Infarct size in the non-PostC A1-KO and B2-KO was not different from the C57-WT controls (P=0.05). However, the infarct-limiting protection of PostC was completely abolished in mice lacking either adenosine A1 receptors or bradykinin B2 receptors (Figure 3; P>0.05 versus the corresponding non-PostC A1-KO or B2-KO). Genetic deletion of bradykinin B1 receptors also did not alter myocardial infarct size (ie, 30.9±3.6% in B1-KO versus 35.1±2.8% in C57-WT; P>0.05). PostC was able to marginally reduce infarct size to 25.6±2.9% in B1-KO (Figure 3; P>0.05 versus non-PostC B1-KO group), indicating PostC was partially attenuated in the mice deficient of bradykinin B1 receptors.
Baseline and Postischemic Cardiac Contractile Function

There was no significant difference in the preischemia baseline levels of ventricular developed force and rate-force product among the eight experimental groups (Table 1; \( P > 0.05 \)), despite the baseline contractile function tended to be lower in B2-KO groups. After the global ischemia-reperfusion, the developed force and rate-force product were remarkably depressed in all of the 8 experimental groups, whereas heart rate remained at a constant level similar to the preischemic values at both early (5 minutes) and late (30 minutes) time points of reperfusion (Tables 1 and 2; Figure 4). Interestingly, PostC did not improve the postischemic cardiac contractile function at either 5 minutes or 30 minutes of reperfusion in any strain of the wild-type and gene knockout mice (Table 2 and Figure 4; \( P > 0.05 \)). It is notable that the early postischemic functional recovery was remarkably improved in B1-KO group (ie, 89.8\( \pm \)10.6\% of preischemic baseline for rate-force product versus 58.9\( \pm \)12.6\% in C57-WT group; see Figure 4B), although it failed to reach the statistical significance mainly because of the high intragroup variability for the contractile function parameters (\( P > 0.05 \) with 2-way ANOVA). Furthermore, this trend of functional improvement in B1-KO mice disappeared at the end of 30 minutes reperfusion (Figure 4D).

Baseline and Postischemic Coronary Flow

Genetic deletion of adenosine A\(_1\), or bradykinin B\(_1\) or B\(_2\) receptors did not alter the preischemic and postischemic coronary flow as compared with the corresponding non-PostC groups (\( P > 0.05 \); Figure 5). There was only a slight trend toward higher postischemic coronary flow in A1-KO mice (Figure 5). PostC also had no effect on the postischemic coronary flow as compared with the corresponding non-PostC groups (\( P > 0.05 \); Figure 5).

Discussion

The present study has demonstrated the existence of PostC against myocardial ischemia-reperfusion injury in C57BL/6J wild-type mice. The 6 cycles of 10 seconds of reperfusion and 10 seconds of ischemia PostC maneuvers applied at the very onset of reperfusion significantly reduced infarct size in the
globally ischemic-reperfused mouse hearts (Figures 2 and 3). The degree of infarct size reduction of PostC was approximately 35% from the non-PostC controls, which appears to be less potent than the 42% reduction observed in our previous ischemic preconditioning study in ICR outbred mice performed in Langendorff perfused hearts with similar duration of ischemia-reperfusion. These results are comparable to the previous studies demonstrating efficacy of PostC in mice with 31% reduction in infarct size and 37% reduction in cardiac troponin I release—a specific marker for cardiac cell necrosis. These results also support the concept that protective efficacy of PostC is somewhat less robust as compared to ischemic preconditioning based on the findings in conscious rats and anesthetized rabbits. Furthermore, the infarct-limiting effect of PostC was not associated with any improvement in ventricular contractile function at 5 minutes or 30

Table 2. Postischemic Cardiac Function

<table>
<thead>
<tr>
<th></th>
<th>CS7-WT (n=8)</th>
<th>CS7-WT + PostC (n=8)</th>
<th>A1-KO (n=6)</th>
<th>A1-KO + PostC (n=7)</th>
<th>B1-KO (n=9)</th>
<th>B1-KO + PostC (n=9)</th>
<th>B2-KO (n=7)</th>
<th>B2-KO + PostC (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>At 5 minutes of reperfusion</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>368±25</td>
<td>339±18</td>
<td>336±18</td>
<td>362±29</td>
<td>360±23</td>
<td>308±22</td>
<td>341±22</td>
<td>347±27</td>
</tr>
<tr>
<td>Developed force, g</td>
<td>0.48±0.13</td>
<td>0.47±0.14</td>
<td>0.53±0.19</td>
<td>0.43±0.16</td>
<td>0.61±0.15</td>
<td>0.58±0.18</td>
<td>0.27±0.10</td>
<td>0.41±0.14</td>
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<tr>
<td>Rate-force product, g×bpm</td>
<td>182±46</td>
<td>168±53</td>
<td>178±62</td>
<td>168±66</td>
<td>216±53</td>
<td>195±69</td>
<td>85±25</td>
<td>158±61</td>
</tr>
<tr>
<td>At 30 minutes of reperfusion</td>
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</tr>
<tr>
<td>Heart rate, bpm</td>
<td>345±26</td>
<td>309±17</td>
<td>324±19</td>
<td>321±26</td>
<td>288±24</td>
<td>301±32</td>
<td>354±24</td>
<td>334±24</td>
</tr>
<tr>
<td>Developed force, g</td>
<td>0.53±0.15</td>
<td>0.48±0.13</td>
<td>0.55±0.20</td>
<td>0.42±0.16</td>
<td>0.55±0.14</td>
<td>0.59±0.19</td>
<td>0.27±0.14</td>
<td>0.28±0.09</td>
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<tr>
<td>Rate-force product, g×bpm</td>
<td>190±53</td>
<td>151±45</td>
<td>172±59</td>
<td>143±55</td>
<td>148±36</td>
<td>164±62</td>
<td>85±36</td>
<td>103±37</td>
</tr>
</tbody>
</table>

Values are Mean±SEM. bpm indicates beats per minute. No significant difference was found between the groups for any of the functional parameters (2-way ANOVA with Bonferroni post-hoc test).
minutes of reperfusion (Table 2 and Figure 4). This dissociation of infarct size and ventricular function was previously observed by our laboratory and others in various settings of preconditioning\textsuperscript{17,19} and PostC.\textsuperscript{9,19} Some previous studies did report significant improvement in cardiac function after adenosine A2A receptors\textsuperscript{7} were used.

Our knowledge, there were only 2 previous PostC studies in mice lacking A2A.\textsuperscript{7} However, another in vivo rabbit study showed that pharmacological inhibition of B2 receptors by 8-(13-chlorostyryl)caffeine.\textsuperscript{11} In contrast, the present study also yielded a blurry picture on the role of B1 receptors in PostC. We found that PostC produced a marginally smaller infarct size in B1-KO mice (25.6±2.9\% versus 31.3±3.6\%; \textit{P}>0.05; Figure 3). These results suggest that PostC was only partially attenuated in the absence of B1 receptors. Therefore, we believe that bradykinin B1 receptors do not play an important role in triggering PostC.

**Bradykinin B1 Receptors and PostC**

Contrary to the above-mentioned controversies concerning the role of adenosine A1 and bradykinin B1 receptors in myocardial reperfusion injury and cardioprotection, there is a unanimous agreement on the crucial importance of bradykinin B2 receptors in myocardial protection, such as ischemic and pharmacological preconditioning.\textsuperscript{24,25} The present study further supports the notion that bradykinin B2 receptors are also indispensable in PostC, because the infarct-limiting effect of PostC observed in C57-WT mice was completely lost in B2-KO mice (Figure 3). Our results also showed that genetic deficiency of bradykinin B2 receptors did not significantly modify myocardial tolerance to ischemia-reperfusion injury, indicated by the similar infarct size (ie, 35.1±2.8\% in C57-WT versus 31.3±3.0\% in B2-KO; \textit{P}>0.05; Figure 3). Similar results were observed after in vivo ischemia-reperfusion in B2-KO mice.\textsuperscript{23,25} Furthermore, a recent study showed that pharmacological inhibition of B2 receptors by administration of HOE140 or WIN64338 blocked the infarct size reduction afforded by PostC or intermittent bradykinin infusion at the onset of reperfusion.\textsuperscript{26} These results further suggest that the intact presence of bradykinin B2 receptors at the early onset of reperfusion is critical for transmitting the cell survival signals of PostC.

**Role of Other G Protein–Coupled Receptors in PostC**

It is notable that other types of G protein–coupled receptors could also involve with the signaling cascades of PostC. As we previously discussed, in addition to the involvement of adenosine A1 demonstrated by our present study and others,\textsuperscript{19} other subtypes of adenosine receptors were also shown to be indispensable for PostC by several research groups. These G protein–coupled receptors include adenosine A2A,\textsuperscript{3,7} A3,\textsuperscript{11} and A\textsubscript{1b}.\textsuperscript{21}
and A3.3. Furthermore, a few recent studies suggested that opioid receptors are the likely participants in PostC signaling, which essentially confirmed the concept originally proposed by Gross and colleagues that pharmacological activation of opioid receptors at the early phase of reperfusion is as protective as preconditioning with the opioid receptor agonists in the rat heart. However, it remains unclear how these G protein–coupled receptors cross-talk with each other, which leads to loss of PostC-induced cardioprotection in the absence of one of these receptors.

Conclusion
The present study using 3 distinctive strains of gene knockout mice has provided conclusive evidence for the essential role of both adenosine A1 and bradykinin B2 receptors on infarct-limiting protection of PostC in globally ischemic-reperfused mouse hearts. Future studies are necessary to elucidate the exact signaling cascades after the PostC-induced activation of each of these membrane receptor subtypes that ultimately lead to the cytoprotective phenotype against cardiac reperfusion injury.

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Disclosures
None.

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