Postconditioning effect of granulocyte colony-stimulating factor is mediated through activation of risk pathway and opening of the mitochondrial $K_{ATP}$ channels

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Abstract Postconditioning effect of granulocyte colony-stimulating factor is mediated through activation of risk pathway and opening of the mitochondrial $K_{ATP}$ channels. Am J Physiol Heart Circ Physiol 299: H1174–H1182, 2010. First published August 6, 2010; doi:10.1152/ajpheart.00116.2010.—Granulocyte colony-stimulating factor (G-CSF) has been reported to improve cardiac function after myocardial infarction. However, whether postinfarct acute effect of G-CSF is mediated through the same signaling pathways as those of ischemic postconditioning is still unclear. We examined the postinfarct acute effect of G-CSF on myocardial infarct size and its precise molecular mechanism. Japanese white rabbits underwent 30 min of ischemia and 48 h of reperfusion. Rabbids were intravenously injected 10 μg/kg of G-CSF (G-CSF group) or saline (control group) immediately after reperfusion. The wortmannin + G-CSF, PD-98059 + G-CSF, $N^\omega$-nitro-$l$-arginine methyl ester (l-NAME) + G-CSF, and $N^\omega$-hydroxydecanic acid sodium salt (5-HD) + G-CSF groups were respectively injected with wortmannin (0.6 mg/kg), PD-98059 (0.3 mg/kg), l-NAME (10 mg/kg), and 5-HD (5 mg/kg) 5 min before G-CSF administration. Myocardial infarct size was calculated as a percentage of the risk area of the left ventricle. Western blot analysis was performed to examine the signals such as protein kinase B (Akt), extracellular signal-regulated protein kinase (ERK), eNOS, p70S6 kinase (p70S6K), and glycogen synthase kinase-3β (GSK3β) in the ischemic myocardium after 48 h of reperfusion. The infarct size was significantly smaller in the G-CSF group (26.7 ± 2.7%) than in the control group (42.3 ± 4.6%). The infarct size-reducing effect of G-CSF was completely blocked by wortmannin (44.7 ± 4.8%), PD-98059 (38.3 ± 3.9%), l-NAME (42.1 ± 4.2%), and 5-HD (42.5 ± 1.7%). Wortmannin, PD-98059, l-NAME, or 5-HD alone did not affect the infarct size. Western blotting showed higher myocardial expression of phospho-Akt, phospho-ERK, phospho-eNOS, phospho-p70S6K, and phospho-GSK3β at 10 min and 48 h after reperfusion in the G-CSF group than in the control group. In conclusion, postreperfusion G-CSF administration reduces myocardial infarct size via activation of phosphatidylinositol 3-kinase-Akt and ERK prosurvival signaling pathways and downstream target eNOS, p70S6 kinase, GSK3β, and mitochondrial ATP-dependent K⁺ channel.

Key words: myocardial infarction; postconditioning; infarct size; protein kinase B; extracellular signal-regulated protein kinase; endothelial nitric oxide synthase; p70S6 kinase; glycogen synthase kinase-3β; adenosine 5′-diphosphate-dependent potassium ion channels; reperfusion injury salvage kinase

Myocardial infarction is the most common cause of cardiac death and mortality, and preventing left ventricular (LV) remodeling is very important since LV remodeling causes progression to heart failure. It has been reported that granulocyte colony-stimulating factor (G-CSF) improves cardiac function and prevents LV remodeling (7, 14, 16, 17). We previously demonstrated that postinfarct treatment with G-CSF for 5 days starting 24 h after infarction accelerated the healing process and improves LV function and remodeling (14). It has also been reported that intracoronary injection of G-CSF ameliorates the progression of LV remodeling after myocardial ischemia and reperfusion in rabbits (9) and that G-CSF directly inhibits myocardial ischemia and reperfusion injury through the protein kinase B (Akt)-eNOS pathway (20). However, whether postinfarct acute effect of G-CSF on myocardial infarct size is mediated through a mechanism similar to that for ischemic postconditioning proposed by Zhao et al. (23) has not yet been fully examined. Therefore, in the present study, we investigated the postinfarct acute effect of G-CSF on myocardial infarct size and its precise molecular mechanisms in an in vivo rabbit model of myocardial infarction without collateral circulation (8).

Materials and Methods

In the present study, all rabbits received humane care in accordance with the Guide for the Care and Use of Laboratory Animals, published by the United States National Institutes of Health (NIH publication 8523, revised 1985). The study protocol was approved by the Ethical Committee of Gifu University School of Medicine, Gifu, Japan.

Chemicals used. Wortmannin, 5-hydroxydecanic acid sodium salt (5-HD), $N^\omega$-nitro-$l$-arginine methyl ester (l-NAME), and PD-98059 were purchased from Sigma Chemical (St. Louis, MO). G-CSF was purchased from Chugai Pharmaceutical (Tokyo, Japan).

Surgical preparation. Male Japanese white rabbits, each weighing 2.0–2.5 kg, were anesthetized with 30 mg/kg pentobarbital sodium and mechanically ventilated with room air. Surgical procedures were performed aseptically. A polyethylene catheter (0.9-mm lumen diameter) was inserted in the internal carotid artery and advanced ~1 cm toward the heart for blood pressure monitoring. Blood pressure was measured with a fluid-filled pressure transducer connected to the end of the cannula. Drugs and saline were administered via the ear vein. After left thoracotomy was performed in the third intercostal space, the heart was exposed, and 4–0 silk thread was placed beneath the large arterial branch coursing down the middle of the anterolateral surface of the left ventricle. Coronary arterial occlusion and reperfusion were performed by pushing or releasing the snares made from thread.

Physiological studies. Both before ischemia at baseline and 48 h after reperfusion, echocardiographic studies (SSD2000; Aloka) and measurement of arterial blood pressure, heart rate, and cardiac function such as $\ddot{d}p/dt_{max}$ using a micromanometer-tipped catheter were performed under light anesthesia with 10 mg/kg pentobarbital sodium and spontaneous respiration. A two-dimensional parasternal long-axis view of the LV was obtained. In general, the best views were obtained with the transducer lightly applied to the midst upper left anterior chest
wall. The transducer was then gently moved cephalad or caudal and angulated until desirable images were obtained. Ejection fraction (EF) and left ventricular end-diastolic dimensions (LVEDD) were obtained. EF was measured using the Teichholz method from M-mode images by echocardiography.

Arterial blood pressure and heart rate were also measured via a catheter introduced into the carotid artery. A micrometer-tipped catheter (SPR 407; Millar Instruments) was inserted in the left ventricle to record \( \frac{dp}{dt_{\text{max}}} \), representing the cardiac systolic function, as well as \( -\frac{dp}{dt_{\text{max}}} \), the indicator of cardiac diastolic function.

To examine the effect of intravenous G-CSF administration on the progression of LV remodeling at 14 days after myocardial ischemia-reperfusion, EF and fractional shortening (FS), LVEDD, and left ventricular end-systolic dimension (LVEDS) were measured by echocardiography before and 14 days after myocardial infarction. All measurements were made by two persons blinded to the treatment.

**Protocol for determination of myocardial infarct size.** To investigate the infarct size-reducing effect of G-CSF and its precise molecular mechanism, 100 Japanese white rabbits underwent 30 min of coronary occlusion followed by 48 h of reperfusion and were assigned randomly to 10 groups as shown in Fig. 1 (n = 10 in each). In the control group, saline was administered intravenously immediately after reperfusion. The G-CSF group was identical to the control group except that 10 mg/kg of G-CSF was administered intravenously instead of saline. The wortmannin + G-CSF, PD-98059 + G-CSF, l-NAME + G-CSF, and 5-HD + G-CSF groups were identical to the G-CSF group except that wortmannin [a phosphatidylinositol 3-kinase (PI 3-kinase) inhibitor, 0.6 mg/kg], PD-98059 [an extracellular signal-regulated protein kinase (ERK) inhibitor, 0.3 mg/kg], l-NAME (a NOS inhibitor, 10 mg/kg), or 5-HD [a mitochondrial ATP-dependent K\(^+\) (K\(_{ATP}\)) channel blocker, 5 mg/kg] was injected intravenously 5 min before the start of G-CSF injection in the respective groups. The wortmannin, PD-98059, l-NAME, and 5-HD groups were injected intravenously with wortmannin (0.6 mg/kg), PD-98059 (0.3 mg/kg), l-NAME (10 mg/kg), or 5-HD (5 mg/kg) 5 min before reperfusion, respectively. Hemodynamic parameters were recorded throughout the experiment until 20 min after reperfusion. Next, the chest was closed, and the rabbits were allowed to recover from anesthesia for 2 days' survival.

**Postmortem study.** The rabbits were heparinized (100 U/kg) and killed with an intravenous overdose of pentobarbital (100–200 mg/kg) at the end of the study. The heart was excised and mounted on a Langendorff apparatus. The coronary branch was reoccluded, and Evans Blue dye (0.2%; Wako Pure Chemical Industries, Osaka, Japan) was injected via the aorta at 80 mmHg. After fixation in 10% phosphate-buffered formalin for 3 days, both the atrial and right ventricular free walls were removed. The left ventricle was cut into seven transverse slices, each of which was weighed and photographed. The area at risk (area without blue dye) was identified and traced from the enlarged projection (magnification \( \times 10 \)) of the photographic slide of each ventricular slice. The area at risk and the infarct areas were calculated as a percentage of the total slice area and multiplied by a slice's weight and then summed to obtain the total tissue weight of the area at risk and infarct areas. The infarct size was obtained as a percentage of the area at risk as previously reported (13).

**Western blot analysis.** Western blotting was performed to assess levels of Akt and phospho-Akt, ERK and phospho-ERK, phospho-eNOS, p70S6 kinase (p70S6K), and glycogen synthase kinase-3β (GSK3β) in the infarct area of the myocardium following 10 min and 48 h of reperfusion. Hearts were excised, and transmural samples, each weighing \( \sim 200 \) mg, were taken from the center of the LV ischemic region and the opposite nonischemic region. The border of the ischemic region was defined by the distribution of cyanosis and marked on the epicardium in ink. The samples were frozen immediately and stored at \(-83^\circ\)C until the assays were performed. Samples were weighed, homogenized, and used for the following measurements. Proteins were separated and transferred to membranes using standard protocols, after which they were probed with antibodies against p70S6K (Cell Signaling) and GSK3β (Cell Signaling). Phosphorylation states of Akt (phospho-Akt, serine-473, Cell Signaling), ERK (Cell Signaling), and eNOS (antibodies against phospho-Akt, phospho-ERK (Santa Cruz Biotechnology), and phospho-eNOS (Becton-Dickinson). Statistical analysis. All values are presented as means ± SE. Risk and infarct size were compared among the groups by one-way ANOVA combined with Bonferroni’s post hoc test for multiple comparisons. Differences in hemodynamics over the time course between the control and the drug-treated groups were assessed by two-way repeated-measures ANOVA. Differences with P < 0.05 were considered significant.

**RESULTS**

**Physiological findings.** Table 1 shows hemodynamic parameters that might influence the infarct size. There were no significant differences in blood pressure or heart rate among the 10 groups. Figure 2A shows echocardiographic data and \( \pm \frac{dp}{dt} \) before and after infarction. There was no significant difference in LVEF before ischemia and 48 h after infarction among the 10 groups. There was no significant difference in the LVEDD among the 10 groups either before infarction among the 10 groups. There was no significant difference in LVEF before ischemia and 48 h after infarction among the 10 groups. There was no significant difference in LVEDD among the 10 groups either before or after reperfusion.
PD-98059, L-NAME, or 5-HD alone did not affect the infarct size.

Hemodynamic parameters

Among the 10 groups, there were no significant differences in systolic and diastolic blood pressure or heart rate that might have influenced the infarct size (15). Therefore, the infarct size-reducing effect of G-CSF was not caused by a decrease in oxygen consumption. G-CSF significantly improved LV function and LV remodeling. Western blot analysis demonstrated that G-CSF activates Akt, ERK, eNOS, p70S6K, and GSK3β in the infarct areas of the myocardium.

DISCUSSION

The present study demonstrated that G-CSF administered immediately after reperfusion reduces the myocardial infarct size. The infarct size-reducing effect of G-CSF was abolished by pretreatment with wortmannin, a PI-3 kinase inhibitor; PD-98059, an ERK inhibitor; l-NAME, a NOS inhibitor; and 5-HD, a mitochondrial K<sub>ATP</sub> channel blocker. G-CSF improved LV function and LV remodeling. Western blot analysis demonstrated that G-CSF activates Akt, ERK, eNOS, p70S6K, and GSK3β in the infarct areas of the myocardium.

Among the 10 groups, there were no significant differences in systolic and diastolic blood pressure or heart rate that might have influenced the infarct size (15). Therefore, the infarct size-reducing effect of G-CSF was not caused by a decrease in oxygen consumption. G-CSF significantly improved LV function at 48 h after infarction and significantly improved LV function and LV remodeling at 14 days after infarction.

In the present study, administration of G-CSF immediately after reperfusion significantly reduced the myocardial infarct size. The infarct size-reducing effect of G-CSF was abolished by pretreatment with wortmannin, a PI-3 kinase inhibitor, suggesting that the infarct size-reducing effect of G-CSF was due to the activation of PI-3 kinase. Furthermore, the infarct size-reducing effect of G-CSF was abolished by pretreatment with wortmannin.

Values are means ± SE. G-CSF, granulocyte colony-stimulating factor; 5-HD, 5-hydroxydecanoic acid sodium salt; l-NAME, N<sup>ω</sup>-nitro-l-arginine methyl ester.

Table 1. Hemodynamic parameters

<table>
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<tr>
<th></th>
<th>Before Occlusion</th>
<th>10 min</th>
<th>20 min</th>
<th>30 min</th>
<th>10 min Reperfusion</th>
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<tr>
<td>Mean blood pressure, mmHg</td>
<td></td>
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<tr>
<td>Control</td>
<td>90.9 ± 7.0</td>
<td>72.7 ± 5.2</td>
<td>63.0 ± 6.2</td>
<td>62.0 ± 3.3</td>
<td>65.7 ± 4.4</td>
</tr>
<tr>
<td>G-CSF</td>
<td>91.2 ± 4.8</td>
<td>63.8 ± 3.0</td>
<td>61.1 ± 3.2</td>
<td>67.8 ± 5.6</td>
<td>76.3 ± 4.1</td>
</tr>
<tr>
<td>G-CSF + 5-HD</td>
<td>91.2 ± 4.8</td>
<td>61.4 ± 3.5</td>
<td>63.8 ± 3.0</td>
<td>61.1 ± 3.2</td>
<td>67.8 ± 5.6</td>
</tr>
<tr>
<td>G-CSF + wortmannin</td>
<td>97.3 ± 3.0</td>
<td>74.4 ± 5.9</td>
<td>74.3 ± 6.0</td>
<td>71.9 ± 7.7</td>
<td>76.3 ± 4.1</td>
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<tr>
<td>G-CSF + l-NAME</td>
<td>90.7 ± 5.7</td>
<td>67.3 ± 2.7</td>
<td>63.5 ± 4.0</td>
<td>59.2 ± 6.4</td>
<td>64.1 ± 5.1</td>
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<tr>
<td>G-CSF + PD-98059</td>
<td>86.8 ± 5.0</td>
<td>70.1 ± 4.6</td>
<td>57.7 ± 6.2</td>
<td>61.7 ± 1.8</td>
<td>72.2 ± 4.7</td>
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<tr>
<td>5-HD</td>
<td>87.6 ± 4.9</td>
<td>68.6 ± 4.9</td>
<td>65.4 ± 5.7</td>
<td>65.5 ± 2.1</td>
<td>67.0 ± 4.7</td>
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<td>Wortmannin</td>
<td>89.2 ± 8.2</td>
<td>78.4 ± 7.8</td>
<td>73.9 ± 7.9</td>
<td>70.2 ± 5.5</td>
<td>73.5 ± 5.9</td>
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<tr>
<td>l-NAME</td>
<td>99.6 ± 3.0</td>
<td>87.6 ± 5.2</td>
<td>80.3 ± 8.3</td>
<td>79.2 ± 4.3</td>
<td>78.0 ± 4.0</td>
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<tr>
<td>PD-98059</td>
<td>87.0 ± 4.3</td>
<td>75.3 ± 3.9</td>
<td>76.7 ± 2.3</td>
<td>78.2 ± 3.5</td>
<td>77.1 ± 2.9</td>
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</table>

Heart rate, beats/min

<table>
<thead>
<tr>
<th></th>
<th>257.9 ± 18.1</th>
<th>235.5 ± 6.2</th>
<th>239.2 ± 14.7</th>
<th>271.7 ± 13.7</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>304.6 ± 7.3</td>
<td>254.8 ± 14.3</td>
<td>242.8 ± 11.3</td>
<td>236.5 ± 6.2</td>
</tr>
<tr>
<td>G-CSF</td>
<td>280.4 ± 5.8</td>
<td>242.1 ± 12.9</td>
<td>244.2 ± 11.7</td>
<td>250.8 ± 10.6</td>
</tr>
<tr>
<td>G-CSF + 5-HD</td>
<td>263.0 ± 13.5</td>
<td>243.9 ± 12.6</td>
<td>245.4 ± 9.0</td>
<td>246.5 ± 12.4</td>
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<tr>
<td>G-CSF + wortmannin</td>
<td>286.8 ± 10.3</td>
<td>261.9 ± 10.9</td>
<td>258.2 ± 8.3</td>
<td>252.5 ± 8.6</td>
</tr>
<tr>
<td>G-CSF + PD-98059</td>
<td>274.3 ± 10.6</td>
<td>237.7 ± 11.3</td>
<td>243.4 ± 8.7</td>
<td>249.4 ± 8.7</td>
</tr>
<tr>
<td>5-HD</td>
<td>265.0 ± 12.2</td>
<td>240.6 ± 13.9</td>
<td>244.4 ± 10.7</td>
<td>243.7 ± 10.4</td>
</tr>
<tr>
<td>Wortmannin</td>
<td>264.2 ± 6.6</td>
<td>237.8 ± 6.9</td>
<td>254.2 ± 12.7</td>
<td>250.8 ± 5.5</td>
</tr>
<tr>
<td>l-NAME</td>
<td>270.6 ± 12.9</td>
<td>231.6 ± 10.4</td>
<td>248.9 ± 8.6</td>
<td>242.6 ± 7.9</td>
</tr>
<tr>
<td>PD-98059</td>
<td>271.5 ± 13.8</td>
<td>266.7 ± 10.4</td>
<td>245.7 ± 10.2</td>
<td>258.1 ± 18.3</td>
</tr>
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</table>

Western blot analysis. At both 10 min after reperfusion (Fig. 4A) and 48 h after reperfusion (Fig. 4B), there were no significant differences in the expression of ERK in the infarct area between the control and G-CSF groups. However, the expression of phospho-ERK was significantly upregulated in the G-CSF group compared with that in the control group. Similarly, there were no significant differences in the expression of Akt protein in the infarct area between the control and G-CSF groups, but the expression of phospho-Akt was significantly upregulated in the infarct area in the G-CSF group compared with that in the control group. The expression of phospho-eNOS protein in the infarct area was also upregulated in the G-CSF group compared with that in the control group. Furthermore, at both 10 min after reperfusion (Fig. 5A) and 48 h after reperfusion (Fig. 5B), although there were no significant differences in the expression of p70S6K and GSK3β in the infarct area between the control and G-CSF groups, the expression of phospho-p70S6K and phospho-GSK3β was significantly upregulated in the infarct area in the G-CSF group compared with those in the control group.
Fig. 2. A: echocardiographic data and \( \frac{\Delta p}{dt} \) before and 2 days after myocardial infarction. EF, ejection fraction; EDD, end-diastolic dimension. *\( P < 0.05 \) vs. control. 
B: echocardiographic data before and 14 days after myocardial infarction. FS, fractional shortening; ESD, end-systolic dimension; LVEDD, left ventricular end-diastolic dimension; LVESD, left ventricular end-systolic dimension. *\( P < 0.05 \) vs. control.
with PD-98059, an ERK inhibitor, suggesting that the infarct size-reducing effect of G-CSF was due to the activation of ERK. In the present study, we performed Western blot analysis using cardiac tissue samples obtained from 10 min after reperfusion and 48 h after reperfusion. The reason why we performed Western blot analysis using samples obtained from 10 min after reperfusion was to show that changes in phosphorylation of the kinases reflect causes of protection rather than results of protection, and to avoid the effect of numerous inflammatory cells in the reperfused myocardium at 48 h reperfusion on the results of Western blot analysis. Western blot analysis showed greater upregulation of phospho-Akt and phospho-ERK in the infarct area of the myocardium in the G-CSF group than in the control group at both 10 min and 48 h after reperfusion. These findings suggest that treatment with G-CSF during reperfusion activates the PI 3-kinase and Akt pathway and activates the MEK-1/2-ERK-1/2 pathway in the infarct area, and both of these contribute to the infarct size-reducing effect of G-CSF. It has been reported that the activation of the PI 3-kinase and Akt pathway and activation of the MEK-1/2-ERK-1/2 pathway are both involved in ischemic postconditioning (3, 19). Therefore, because treatment with G-CSF during reperfusion reduced the infarct size through the activation of prosurvival signaling pathways such as the PI 3-kinase and Akt pathway and the MEK-1/2-ERK-1/2 pathway, the infarct size-reducing effect of G-CSF was mediated through a mechanism similar to that for ischemic postconditioning. Furthermore, the infarct size-reducing effect of G-CSF was abolished by pretreatment with L-NAME, a NOS inhibitor, suggesting that the infarct size-reducing effect of G-CSF was due to the production of NO. Interestingly, NO has been reported to mediate the infarct size-reducing effect of ischemic postconditioning in rabbits (21). In the present study, Western blot analysis showed the overexpression of phospho-eNOS in the infarct area of the myocardium in the G-CSF group compared with the control group at both 10 min and 48 h reperfusion. Furthermore, overexpression of phospho-p70S6K was observed in the infarct area of the myocardium in the G-CSF group compared with the control group at both 10 min and 48 h reperfusion. It has been reported that eNOS and p70S6K are situated downstream of PI 3-kinase-Akt activation in ischemic postconditioning (19, 24). Therefore, the infarct size-reducing effect of G-CSF is suggested to be due to the activation of PI 3-kinase and Akt and then phosphorylation of eNOS and p70S6K.

Concerning the mitochondrial KATP channels, it has been reported that ischemic preconditioning, a brief episode of ischemia and reperfusion, opens the mitochondrial KATP channels and reduces the myocardial infarct size (4, 11). It has also been reported that ischemic postconditioning reduces the myocardial infarct size by opening mitochondrial KATP channels in rabbits (22). In the present study, the infarct size-reducing effect of G-CSF was abolished by pretreatment with 5-HD, a

Fig. 3. A: area at risk as a percentage of the left ventricle (LV). There was no significant difference among the groups. AAR, area at risk. *P < 0.05 vs. control group.
mitochondrial K$_{\text{ATP}}$ channel blocker, suggesting that the infarct reduction by G-CSF was mediated by opening of the mitochondrial K$_{\text{ATP}}$ channels. Because NO has been reported to open the mitochondrial K$_{\text{ATP}}$ channels (6, 18), it is possible that G-CSF activates the PI 3-kinase-Akt and eNOS pathway, opens the mitochondrial K$_{\text{ATP}}$ channels, and reduces the myocardial infarct size.

In the present study, GSK-3$\beta$ was also upregulated in the infarct area in the G-CSF group compared with that in the control group. GSK-3$\beta$ is usually activated, and phosphorylation of GSK-3$\beta$ inhibits activation of GSK-3$\beta$. It has been reported that one of the signaling pathways of ischemic postconditioning is the activation of PI 3-kinase and Akt (19), and the downstream target of Akt is GSK-3$\beta$. In the present study, treatment with G-CSF upregulated the phosphorylation of GSK-3$\beta$ in the ischemic area of the myocardium on day 2 postmyocardial infarction. This suggests that phosphorylation of GSK3$\beta$ also contributes to the beneficial effect of G-CSF.

Fig. 4. A: Western blot analysis of myocardial protein kinase B (Akt), phospho (p)-Akt, extracellular signal-regulated protein kinase (ERK), p-ERK, and p-eNOS expression in the sham, control, and G-CSF groups at 10 min after reperfusion. *$P < 0.05$ vs. control. B: Western blot analysis of myocardial Akt, p-Akt, ERK, p-ERK, and p-eNOS expression in the sham, control, and G-CSF groups at 48 h after reperfusion. *$P < 0.05$ vs. control.
GSK3β is known to be a common target of converging cell-protective signals provoked by different trigger mechanisms. The mechanism by which phospho-GSK3β protects the myocardium remains unclear, but it is likely that suppression of mitochondrial permeability transition pores that open during reperfusion plays a significant role in protection (5, 10). Therefore, G-CSF may suppress the opening of the mitochondrial permeability transition pores by upregulating GSK3β. From the result of the present study, it is difficult to clarify the association of KATP channels with GSK3β and the mitochondrial permeability transition pore. However, there have been reports suggesting that the opening of mitochondrial K$_{\text{ATP}}$ channel inhibits the mitochondrial transition pore (1) and inhibition of GSK3β phosphorylation of GSK3β opens the mitochondrial K$_{\text{ATP}}$ channel (2).

Fig. 5. A: Western blot analysis of myocardial p70S6 kinase (70S6K), p-70S6K, glycogen synthase kinase-3β (GSK3β), and p-GSK3β expression in the sham, control, and G-CSF groups at 10 min after reperfusion. *$P < 0.05$ vs. control. B: Western blot analysis of myocardial 70S6K, p-70S6K, GSK3β, and p-GSK3β expression in the sham, control, and G-CSF groups at 48 h after reperfusion. *$P < 0.05$ vs. control.
G-CSF can be obtained. Because the infarct size-reducing effect by G-CSF was completely abolished by treatment with 5-HD, wortmannin, l-NAME, or PD-98059, the blockade of either PI 3-kinase activation, ERK activation, NO production, or opening of the mitochondrial KATP channels would prevent reaching the threshold level to reduce the infarct size. A possible explanation for these observations is that it requires the summation of many signals to reach the threshold level to reduce the infarct size.

**Clinical implications.** Cardioprotection by ischemic postconditioning is mediated by Akt and ERK signaling (3, 19). Therefore, in the clinical setting, pharmacological postconditioning can be duplicated with G-CSF as shown in the present study and would be a useful strategy for the treatment of acute myocardial infarction after treatment with percutaneous coronary intervention. To see whether signaling pathways in pharmacological preconditioning of G-CSF are similar to those in pharmacological postconditioning of G-CSF, the effects of G-CSF pretreatment before ischemia and reperfusion on the infarct size and signaling pathways were investigated in a rabbit model of 30 min of ischemia and 48 h of reperfusion. As a result, G-CSF (10 μg/kg) administered 5 min before 30 min ischemia significantly reduced the myocardial infarct size and showed phosphorylation of Akt, ERK, and eNOS but not GSK3β or p70S6K (Fig. 7). Therefore, signaling pathways in preconditioning of G-CSF are likely to be similar to that of postconditioning of G-CSF but not likely to be the same. However, we believe that the pharmacological postconditioning effect of G-CSF is the more practical strategy for the treatment of myocardial infarction because we cannot predict precisely the time when acute myocardial infarction occurs.

**Fig. 6.** A: schematic representation of signal transduction by G-CSF treatment that may lead to cardioprotection. Solid square, confirmed pharmacologically or by Western blot analysis in this study; dashed square, not confirmed in this study but a possible mechanism. RISK, reperfusion injury salvage kinase; mPTP, mitochondrial permeability transition pore; BAD, Bcl2 antagonist of cell death. B: scheme suggesting that a threshold level of signal transduction must be reached before infarct size-reducing effect of G-CSF can be obtained. The infarct size-reducing effect by G-CSF was completely abolished by treatment with 5-HD, wortmannin, l-NAME, or PD-98059 in the present study, suggesting that blockade of either phosphatidylinositol 3-kinase (PI 3-kinase) activation, ERK activation, NO production, or opening of the mitochondrial ATP-dependent K⁺ (KATP) channels would prevent that threshold from being reached.

**Fig. 7.** Effect of preischemic treatment with G-CSF on myocardial infarct size and Western blot analysis of p-Akt, p-ERK, and p-eNOS. *P < 0.05 vs. control.
In conclusion, postinfarction treatment with G-CSF is protective against ischemia reperfusion injury by a mechanism similar to that of ischemic postconditioning. Cardioprotection by postischemic G-CSF treatment is mediated through activation of the reperfusion injury salvage kinase pathway and opening of the mitochondrial K\textsubscript{ATP} channels. The findings of the present study may provide new insights into therapeutic strategies for the treatment of acute myocardial infarction.

DISCLOSURES

No conflicts of interest are declared by the authors.

REFERENCES


