Cigarette Smoking Abolishes Ischemic Preconditioning-Induced Augmentation of Endothelium-Dependent Vasodilation

Shuji Nakamura, Masashi Kimura, Chikara Goto, Kensuke Noma, Masao Yoshizumi, Kazuaki Chayama, Yasuki Kihara, Yukihito Higashi

Abstract—We have shown recently that repetition of ischemic preconditioning stimulus augments endothelium-dependent vasodilation in forearm circulation of healthy subjects through increases in NO production and the number of circulating progenitor cells under a local condition. The purpose of this study was to evaluate the “late” effect of ischemic preconditioning on endothelial function in smokers. Ischemic preconditioning was induced by upper-limb ischemia 6 times a day for 1 month. We evaluated forearm blood flow responses to acetylcholine and sodium nitroprusside before and after ischemic preconditioning stimulus in 15 male smokers (27±7 years) and 15 male nonsmokers (26±5 years). Forearm blood flow was measured by using a strain-gauge plethysmography. The ischemic preconditioning stimulus resulted in significant increases in the circulating level of circulating progenitor cells from 1029±261 to 1232±341 mL (P=0.02), cell migration response to vascular endothelial growth factor from 38±16 to 52±17 per high-power field (P=0.02), and forearm blood flow response to acetylcholine from 25.1±5.2 to 32.4±6.6 mL/min per 100 mL of tissue (P=0.002) in nonsmokers, but these did not change in the smoker group. The forearm blood flow responses to sodium nitroprusside before and after the ischemic preconditioning stimulus were similar. Intra-arterial infusion of N\textsuperscript{G}-monomethyl-L-arginine, an NO synthase inhibitor, completely eliminated the ischemic preconditioning stimulus-induced augmentation of forearm blood flow responses to acetylcholine in nonsmokers. These findings suggest that repetition of ischemic preconditioning stimulus may be a simple, safe, and feasible therapeutic technique for endothelial protection of peripheral vessels. However, smoking abolishes ischemic preconditioning stimulus-induced augmentation of endothelium-dependent vasodilation. (Hypertension. 2009;53:00-00.)

Key Words: preconditioning | endothelial function | NO | vascular endothelial growth factor | circulating progenitor cells | smoking

Several studies have shown that prodromal angina pectoris occurring shortly before the onset of infarction reduced infarct size and improved left ventricular function.\textsuperscript{1-7} A brief ischemic period, followed by episodes of reperfusion, increases the resistance to further ischemic damage, a phenomenon known as ischemic preconditioning (IPC). IPC has been observed in the heart, liver, brain, and other organs.\textsuperscript{8-11} IPC is an important mechanism by which tissues protect themselves from impending ischemic damage. IPC has protective effects against myocardial infarction and myocardial stunning.

It is thought that IPC is a multifactorial phenomenon that includes components of endothelium-derived NO and adenosine. Endothelial function, especially NO function, plays a critical role in the development and maintenance of cardiovascular diseases.\textsuperscript{9-13} Therefore, from a clinical perspective, it is important to select an appropriate intervention that is effective in improving endothelial dysfunction in patients with cardiovascular diseases. Under the condition of hypoxia, vascular endothelial growth factor (VEGF) gene expression is upregulated by induction of hypoxia-inducible factor-1 (HIF-1), resulting in an increase in migration of endothelial progenitor cells (EPCs). Interestingly, endothelial function has been found to be associated with the number of circulating EPCs in humans.\textsuperscript{14} Recently, we have shown that repetition of IPC stimulus augments endothelial function through an increase in circulating progenitor cells.\textsuperscript{15}

Cigarette smoking is a well-established independent risk factor of cardiovascular diseases. Smoking is associated with endothelial dysfunction.\textsuperscript{16,17} It is postulated that increase in oxidative stress and decrease in EPCs contribute to vascular failure in smokers. On the other hand, several lines of evidence have shown that smoking has paradoxical beneficial effects on immediate mortality and prognosis in patients with acute myocardial infarction.\textsuperscript{18,19} However, there is no infor-
mation on the effects of smoking on IPC effects, especially IPC effect on endothelial function.

Repetition of IPC stimulus in forearm circulation is an ideal model for evaluating effects of IPC on the coronary artery, leading to protection of myocardiocytes against damage caused by severe ischemia. To determine the validity of the hypothesis that smoking diminishes the beneficial effect of “late” IPC on endothelial function, we measured forearm blood flow (FBF) responses to acetylcholine (ACh), an endothelium-dependent vasodilator, and sodium nitroprusside (SNP), an endothelium-independent vasodilator, in the presence and absence of N^G^-monomethyl-L-arginine (L-NMMA), an inhibitor of NO synthase, and we also investigated circulating levels of VEGF and the number of and function of circulating progenitor cells.

Methods

Subjects

We studied 15 young nonsmoker men (mean age: 27.5±4.5 years) and 15 young smoker men (mean age: 28.2±4.1 years). All of the subjects had no history of cardiovascular or cerebrovascular disease, hypertension, hypercholesterolemia, diabetes mellitus, liver disease, renal disease, or other diseases. The results of physical and routine laboratory examinations of the subjects were normal. None of the subjects were taking oral antioxidant vitamins or vasoactive drugs. Current smokers were defined as smokers who had smoked ≥1 pack-year. 1 pack-year being defined as 20 cigarettes per day for 1 year. All of the smokers (28.7±7.4 pack-years) had a current cigarette smoking history of ≥5 years and abstained from smoking for ≥3 hour before the measurement of vascular function. We defined nonsmokers as subjects who had never smoked. The ethical committee of Hiroshima University Graduate School of Biomedical Sciences approved the study protocol. Written informed consent for participation in the study was obtained from all of the subjects.

Study Protocol

None of the subjects received any drugs for ≥24 hours before the study. An upper-arm cuff was inflated to 200 mm Hg for 5 minutes 6 times a day for 4 weeks using a rapid cuff inflator (EC-20, Hokanson, Inc) to obtain repetition of transient ischemia as a strategy of IPC. All of the subjects underwent 4 weeks of follow-up without any lifestyle modification. Forearm vascular responses to ACh (Daichi Pharmaceutical Co) and to SNP (Maruishi Pharma Co) were evaluated before and after 4 weeks of IPC repetition. The studies began at 8:30 AM after 14 hours of the last IPC stimulus. Subjects were kept in a supine position in a quiet, dark, and air-conditioned room throughout the study. A 23-gauge catheter was inserted into the brachial artery for infusion using 1% lidocaine to record arterial pressure with an AP-641G pressure transducer (Nihon Koden Co). Another catheter was inserted into the deep antecubital vein to obtain blood samples. Total volume of the blood sample was 20 mL. After 30 minutes in the supine position, blood samples were obtained, and baseline FBF, heart rate, and arterial blood pressure were measured. Then, ACh (3.75, 7.50, and 15.00 μg/min) or SNP (0.75, 1.50, and 3.00 μg/min) was infused intra-arterially for 5 minutes at each dose with a constant-rate infusion pump (Terfusion ETG-523, Terumo Co). FBF during the final 2 minutes of each infusion was measured. The infusions of ACh and SNP were carried out in a random order. Each study proceeded after the FBF had returned to the baseline level. After a 30-minute rest period, L-NMMA (CLINALFA Co) was infused intra-arterially at a dose of 8 μmol/min for 5 minutes while the baseline FBF and arterial blood pressure were recorded. After L-NMMA infusion was complete, ACh (3.75, 7.50, and 15.00 μg/min) was administered. Please see the online data supplement for additional details.

Measurement of FBF

FBF was measured using a mercury-filled Silastic strain-gauge plethysmography (EC-5R, Hokanson, Inc), as described previously. Please see the online Data Supplement for additional details.

Measurement of the Number of Circulating Progenitor Cells

The number of circulating progenitor cells was analyzed by flow cytometry. Please see the online data supplement for additional details.

Characterization of Progenitor Cells

Mononuclear cells were isolated by Ficoll density-gradient centrifugation of human blood buffy coats from 50 mL of peripheral blood. Please see the online data supplement for additional details.

Migration Assay

Progenitor cell migration was evaluated using a modified Boyden chamber assay, as described previously. Please see the online data supplement for additional details.

Analytical Methods

Samples of venous blood were placed in tubes containing sodium EDTA (1 mg/mL) and in polystyrene tubes. Please see the online data supplement for additional details.

Statistical Methods

Values are expressed as the means±SDs. The Mann–Whitney U test was used to evaluate differences between before and after the IPC stimulus with respect to baseline parameters. Two-tailed Student’s paired t test was used to evaluate differences before and after IPC stimulus. The FBF responses to ACh and SNP before and after IPC stimulus were analyzed by 2-way ANOVA for repeated measures, followed by Scheffe’s F test. Results were considered significant at P<0.05.

Results

Clinical Characteristics

The baseline clinical characteristics of the 15 nonsmokers before (0 weeks) and after (4 weeks) IPC and the 15 smokers before (0 weeks) and after (4 weeks) IPC are summarized in the Table. Serum concentrations of interleukin 6 and hs-CRP, indices of systemic inflammation, were significantly higher in smokers than in nonsmokers. Urinary excretion of 8-OHdG was significantly higher in smokers than in nonsmokers before and after IPC. Serum concentration of malondialdehyde-modified LDL showed a tendency to be high, but not significantly, in smokers compared with that in nonsmokers before and after IPC. The plasma concentration of VEGF was increased significantly in both the nonsmoker group and the smoker group by 4 weeks of IPC. The plasma concentration of VEGF was similar in the 2 groups after the IPC stimulus. IPC stimulus did not alter systemic hemodynamics, including blood pressure, heart rate, lipid profile, inflammation markers (interleukin 6 and hs-CRP),
Effects of IPC on FBF Responses to ACh and SNP
Intra-arterial infusion of ACh and SNP increased FBF in a dose-dependent manner in all of the subjects. The response of FBF to ACh was significantly less in smokers than in nonsmokers (P<0.001; Figure 1). Vasodilatory responses to SNP were similar in the 2 groups (Figure 1). There was a significant relationship between maximal FBF response to ACh and urinary excretion of 8-OHdG (r = -0.42; P = 0.02), whereas SNP-induced vasodilation did not correlate with any parameters. There were no significant relationships among the vascular responses to ACh and SNP and serum concentrations of interleukin 6 and hs-CRP.

IPC stimulus did not alter baseline FBF in the nonsmoker group or smoker group (Table). The response of FBF to infusion of ACh was increased significantly from 25.1±5.2

<table>
<thead>
<tr>
<th>Variable</th>
<th>Nonsmoker Before (0 wk)</th>
<th>Nonsmoker After (4 wk)</th>
<th>Smoker Before (0 wk)</th>
<th>Smoker After (4 wk)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body mass index, kg/m²</td>
<td>22.8±0.9</td>
<td>22.8±0.9</td>
<td>23.0±0.8</td>
<td>23.0±0.8</td>
</tr>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>120.4±4.2</td>
<td>119.6±3.9</td>
<td>119.8±4.4</td>
<td>120.1±3.8</td>
</tr>
<tr>
<td>Diastolic blood pressure, mm Hg</td>
<td>63.5±3.0</td>
<td>62.0±2.8</td>
<td>62.9±3.1</td>
<td>63.1±2.9</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>68.2±3.2</td>
<td>67.4±2.9</td>
<td>69.1±2.8</td>
<td>68.7±2.6</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>4.86±0.22</td>
<td>4.84±0.33</td>
<td>4.79±0.26</td>
<td>4.78±0.37</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>1.37±0.19</td>
<td>1.34±0.17</td>
<td>1.36±0.28</td>
<td>1.35±0.26</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
<td>1.29±0.09</td>
<td>1.37±0.11</td>
<td>1.22±0.12</td>
<td>1.25±0.14</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/L</td>
<td>2.94±0.21</td>
<td>2.86±0.19</td>
<td>2.88±0.22</td>
<td>2.82±0.18</td>
</tr>
<tr>
<td>Glucose, mmol/L</td>
<td>3.61±0.19</td>
<td>3.86±0.29</td>
<td>3.74±0.26</td>
<td>3.69±0.24</td>
</tr>
<tr>
<td>Insulin, pmol/L</td>
<td>53.6±5.8</td>
<td>59.1±6.9</td>
<td>54.8±5.3</td>
<td>56.4±6.1</td>
</tr>
<tr>
<td>VEGF, pg/mL</td>
<td>88.2±7.3</td>
<td>118.1±11.5*</td>
<td>90.2±10.1</td>
<td>129.2±9.7*</td>
</tr>
<tr>
<td>Interleukin 6, ng/L</td>
<td>1.2±0.2</td>
<td>1.3±0.22</td>
<td>2.1±2.4†</td>
<td>2.0±2.5†</td>
</tr>
<tr>
<td>Hs-CRP, mg/L</td>
<td>1.1±1.3</td>
<td>1.0±1.4</td>
<td>1.9±2.1†</td>
<td>1.9±2.0†</td>
</tr>
<tr>
<td>MDA-LDL, μL</td>
<td>53.2±22.9</td>
<td>52.7±27.3</td>
<td>63.9±30.1</td>
<td>64.8±31.6</td>
</tr>
<tr>
<td>Urinary 8-OHdG, ng/mg of Cr</td>
<td>7.8±3.4</td>
<td>7.7±3.6</td>
<td>12.3±4.8†</td>
<td>12.1±4.2†</td>
</tr>
<tr>
<td>FBF, mL/min per 100 mL of tissue</td>
<td>4.9±0.4</td>
<td>5.2±0.6</td>
<td>5.0±0.7</td>
<td>4.9±0.6</td>
</tr>
</tbody>
</table>

HDL indicates high-density lipoprotein; CRP, C-reactive protein; MDA, malondialdehyde; Cr, creatinine. All of the results are presented as mean±SD.

*P<0.05 vs before (0 wk) in the same group.
†P<0.05 vs nonsmoker at the same follow-up period.
to 32.4±6.6 mL/min per 100 mL of tissue (P=0.002) by 4 weeks of IPC in the nonsmoker group but not altered in the 4-week follow-up period in the smoker group (Figure 1). The increases in FBF during infusion of SNP were similar at the beginning and the end of the 4-week study period in both the nonsmoker group and smoker group (Figure 1). No significant change was observed in arterial blood pressure or heart rate with intra-arterial infusion of ACh and SNP.

Intra-arterial infusion of L-NMMA significantly decreased baseline FBF from 4.9±0.4 to 4.2±0.3 mL/min per 100 mL of tissue (P<0.001) in the nonsmoker group and from 5.0±0.7 to 4.3±0.4 mL/min per 100 mL of tissue (P<0.001) in the smoker group before IPC stimulus and from 5.2±0.6 to 4.2±0.4 mL/min per 100 mL of tissue (P<0.001) in the nonsmoker group and from 4.9±0.6 to 4.1±0.3 mL/min per 100 mL of tissue (P<0.001) in the smoker group after IPC stimulus. Baseline FBF after L-NMMA infusion was similar in the 2 groups before (0 weeks) and after (4 weeks) the IPC stimulus. Intra-arterial infusion of L-NMMA decreased the response to ACh before and after the IPC stimulus in the nonsmokers and smokers (Figure 1). After L-NMMA infusion, FBF responses to ACh were similar at 0 weeks and 4 weeks in the 2 groups (Figure 1). Neither arterial blood pressure nor heart rate was significantly changed by intra-arterial infusion of ACh in the presence of L-NMMA.

Effects of IPC on Circulating Progenitor Cells
The number of circulating progenitor cells was significantly less in smokers than in nonsmokers before and after IPC (Figure 2A and 2B). IPC stimulus for 4 weeks increased the number of circulating progenitor cells from 1029±261 to 1232±341 mL (P=0.02) in nonsmokers, whereas there was no significant difference between the number of circulating progenitor cells at 0 weeks and that at 4 weeks in smokers (Figure 2B). Cells demonstrating double-positive staining lectin and 3,3′,3′-tetramethylindolo-carbocyanine perchlorate-labeled acetylated low-density lipoprotein (Dil-AcLDL) were identified to be progenitor cells (Figure 3A). Cell migration response to VEGF was significantly less in smokers than in nonsmokers before and after IPC (Figure 3B and 3C). IPC stimulus for 4 weeks increased cell migration response to

![Figure 2. A, Measurement of the number of progenitor cells by flow cytometer before ischemic preconditioning in nonsmokers and smokers. B, Comparison of the number of circulating progenitor cells at 0 weeks and 4 weeks of ischemic preconditioning in nonsmokers and smokers.](image-url)
VEGF from 38±16 to 52±17 per high-power field (P=0.02) in nonsmokers, whereas there was no significant difference between cell migration response to VEGF at 0 weeks and that at 4 weeks in smokers (Figure 3C).

Changes in maximal FBF response to ACh correlated with changes in the number of circulating progenitor cells (r=0.59; P=0.002) and changes in cell migration response to VEGF (r=0.36; P=0.04) in the nonsmoker group (Figure 4) but not in the smoker group. There was a significant relationship between changes in the number of circulating progenitor cells and changes in cell migration response to VEGF (r=0.49; P=0.01) in the nonsmoker group (Figure 4) but not in the smoker group. There were no correlations between changes in the number of circulating progenitor cells and migration of progenitor cells and increase in plasma VEGF concentration. No correlation was found between changes in maximal FBF response to ACh and changes in blood pressure, heart rate, VEGF, or other variables or between these variables and changes in maximal FBF response to SNP in the 2 groups.

**Discussion**

Four weeks of repetition of IPC stimulus augmented FBF response to ACh but not FBF response to SNP in nonsmokers, whereas repetition of IPC stimulus did not alter either FBF response to ACh or that to SNP in smokers. L-NMMA abolished the IPC stimulus-induced augmentation of endothelium-dependent vasodilation in nonsmokers. In addition, the increases in maximal FBF response to ACh correlated with the increases in the number of circulating progenitor

![Figure 3](image_url)

*Figure 3.* A, Characterization of progenitor cells by immunofluorescence for lectin binding (green), Dil-AcLDL uptake (red) and lectin/Dil-AcLDL double-positive cells (yellow; ×100). B, Measurement of the migration of cells labeled with 4',6-diamidino-2-phenylindole by fluorescence before ischemic preconditioning in nonsmokers and smokers (×200). C, Measurement of the migration of cells labeled with 4',6-diamidino-2-phenylindole by fluorescence at 0 weeks and 4 weeks of ischemic preconditioning in nonsmokers and smokers (left; ×200). Comparison of the migration of progenitor cells in nonsmokers and that in smokers at 0 weeks and 4 weeks of ischemic preconditioning (right).

![Figure 4](image_url)

*Figure 4.* Correlations between maximal FBF response to ACh and number of circulating progenitor cells (left) and migration of progenitor cells (right) at 0 weeks and 4 weeks of ischemic preconditioning in nonsmokers.
cells and migration of progenitor cells after repetition of IPC. These findings suggest that the augmentation of ACh-induced vasodilation may be related to an improvement in the function of the endothelium, not that of vascular smooth muscle, and may be because of an increase in NO production through, at least in part, an increase in circulating progenitor cells.

In the present study, to evaluate the role of smoking, per se, in IPC stimulus-induced changes in endothelial function, we selected healthy young men to avoid the possibility of alteration in endothelial function and number of circulating progenitor cells and function of progenitor cells caused by factors such as hypertension, heart failure, atherosclerosis, hypercholesterolemia, diabetes mellitus, aging, and menstrual cycle.

There are several possible explanations for the IPC stimulus-induced augmentation of endothelium-dependent vasodilation in humans. Several lines of evidence have shown that the "late" effect of IPC is mainly attributed to an increase in NO production. In the present study, L-NMMA completely abolished the IPC stimulus-induced augmentation of FBF responses to ACh. In a recent study, the nonselective NO synthase inhibitor N\textsuperscript{\textomega}-nitro-L-arginine, but not the inducible NO synthase inhibitor L-NNAME, was shown to completely abolish the IPC stimulus-induced augmentation of endothelium-dependent vasodilation in humans. Several lines of evidence have shown that the "late" effect of IPC is mainly attributed to an increase in NO production.

In the present study, repetition of IPC increased plasma VEGF levels. Increases in maximal FBF response to ACh correlated with changes in the number of circulating progenitor cells and cell migration response to VEGF after repetition of IPC. Recently, Hill et al\textsuperscript{15} also found by measurements of flow-mediated vasodilation in healthy men that the number of circulating progenitor cells is correlated with endothelial function. It has been shown that VEGF-induced and ischemia-induced mobilization of bone marrow-derived EPCs contribute to neovascularization. Increases in VEGF gene expression and circulating VEGF levels with repetition of IPC may increase the levels of circulating progenitor cells and lead to an increase in capillary density, resulting in augmentation of endothelial function through an increase in NO production. Wang et al\textsuperscript{6} reported significant increases in the number of functional capillaries and arteriole diameter in rats 24 hours after ischemic reperfusion. These findings suggest that the hypoxia-HIF-1-VEGF pathway may play an important role in IPC-induced angiogenesis in skeletal muscle. We showed a putative model of VEGF-modulating endothelial NO synthase activation by repetition of IPC (please see the online Data Supplement for additional details, Figure S2).

Although the precise mechanisms by which repetition of IPC stimulus does not induce augmentation of endothelium-dependent vasodilation in smokers remain unclear, inactivation of the VEGF-EPC pathway may contribute to failure of IPC-induced augmentation of endothelial function. In the present study, the number of circulating progenitor cells was decreased, and progenitor cell function was impaired in smokers compared with that of nonsmokers. These findings are consistent with results of previous studies.\textsuperscript{26,27} In addition, although plasma concentration of VEGF increased after 4 weeks of IPC in smokers, as well as in nonsmokers, increases in VEGF levels did not increase the number of circulating progenitor cells and did not enhance the function of progenitor cells in smokers, whereas IPC stimuli increased the number of circulating progenitor cells and enhanced the function of progenitor cells in nonsmokers. Recently, Edirisighe et al\textsuperscript{28} have shown a potential mechanism for smoking-induced endothelial dysfunction. In mouse lung and human endothelial cells in vitro, cigarette smoking downregulated VEGF receptor-2 expression, endothelial NO synthase protein levels, and VEGF-induced VEGF receptor-2 phosphorylation, leading to impaired VEGF-induced cell migration and angiogenesis. It has been suggested that systemic inflammation and oxidative stress influence the number of circulating progenitor cells and function of progenitor cells.

In the present study, urinary excretion of 8-OHdG, an oxidative stress marker, was significantly higher in smokers than in nonsmokers and was correlated with maximal FBF response to ACh. Under the condition of excess oxidative stress, depletion of VEGF-induced mobilization of progenitor cells and enhancement of progenitor cell function and inactivation of NO bioavailability may form a vicious circle, leading to a lack of IPC-induced augmentation of endothelial function in smokers.

Several lines of evidence have shown that cigarette smoking is associated with systemic inflammation.\textsuperscript{29–31} In the present study, levels of inflammation markers, interleukin 6, and hs-CRP were also significantly higher in smokers than in nonsmokers. Inflammation has a dual-sword role in EPC functions, such as mobilization, proliferation, and colony formation, a high grade of inflammation inhibits EPC functions.\textsuperscript{32,33} Interestingly, Verma et al\textsuperscript{34} have reported that C-reactive protein, per se, directly inhibits EPC differentiation, survival, and function. Inflammation-induced impairment of EPC function might lead to a lack of IPC-induced augmentation of endothelial function in smokers. Clinical studies have shown that there is an association between inflammation and endothelial dysfunction.\textsuperscript{35,36} In the present study, there was no association between vascular response to ACh and levels of inflammation markers interleukin 6 and hs-CRP, suggesting that systemic inflammation might not directly affect endothelial function in smokers. In addition, both interleukin 6 and hs-CRP were unchanged after the repetition of IPC stimulus in both groups.

Study Limitations

In the present study, we examined the effect of IPC on vascular endothelial function in the absence of a prolonged
ischemia-reperfusion stimulus. Kharbanda et al. have shown that a clinically relevant period of ischemia-reperfusion induces endothelial dysfunction in healthy subjects and that IPC attenuates endothelial dysfunction caused by ischemia reperfusion. It remains possible that IPC will prevent the attenuation of endothelium-dependent vasodilatation observed with an episode of ischemia-reperfusion injury in smokers without apparently altering the "basal state."

The present study is essentially a prospective single-arm study of IPC in 2 groups of subjects. A blinded, randomized, and crossover study design would enable a more specific conclusion concerning the role of smoking in IPC to be drawn.

Infusion of L-NMMA reduces basal endothelial NO release and FBF, confounding the interpretation of the inhibition of subsequent vasodilator responses. Coinfusion of L-NMMA with SNP to restore a steady-state NO would have improved the study design.

**Perspectives**

Repetition of IPC augmented endothelial function through an increase in NO production. Endothelial dysfunction is the initial step in the pathogenesis of atherosclerosis, resulting in cardiovascular complications. It is important to select an appropriate intervention that is effective in improving or augmenting endothelial function. Repetition of IPC may be a simple, safe, and feasible therapeutic technique for endothelial protection of peripheral vessels. Furthermore, this technique has the potential to improve endothelial function as a new treatment for cardiovascular disease associated with endothelial dysfunction. Unfortunately, IPC might not have beneficial effects in smokers. Thus, nonsmoking and smoking cessation are important to prevent myocardial damage after severe ischemia, especially in patients with angina pectoris who have brief ischemic periods. Additional studies are needed to confirm the effects of IPC in the coronary artery in smokers.

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**Disclosures**

None.

**References**

25. Asahara T, Takahashi T, Maoula H, Kalka C, Chen D, Iwaguro H, Inai Y, Silver M, Isner JM. VEGF contributes to postnatal neovascularization by...


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Cigarette Smoking Abolishes Ischemic Preconditioning-induced Augmentation of Endothelium-dependent Vasodilation

Brief title: Preconditioning and endothelial function in smokers

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Measurement of FBF
Briefly, a strain-gauge was attached to the upper part of the left arm and connected to a plethysmograph device, and placed above the level of the right atrium. A wrist cuff was inflated to a pressure 50 mm Hg above the systolic blood pressure 1 minute before each measurement and throughout the measurement of FBF to exclude the hand circulation from the measurements. The upper arm cuff was inflated to 40 mm Hg for 7 seconds during each 15-second cycle using a rapid cuff inflator (EC-20, Hokanson, Inc.) to occlude venous outflow from the arm. The FBF output signal was transmitted to a recorder (U-228, Advance Co.). FBF is expressed as mL per minute per 100 mL of forearm tissue. Four plethysmographic measurements were averaged to yield values for FBF at baseline and during the administration of drugs. FBF was calculated by two observers blinded to the experimental protocol from the linear portions of the plethysmographic recordings. The intraobserver coefficient of variation was 5.7%.

**Measurement of number of circulating progenitor cells**

Briefly, samples of venous blood were placed in tubes containing sodium EDTA (7 mg/mL) and in polystyrene tubes. The EDTA-containing tubes were chilled promptly in an ice bath. Peripheral blood mononuclear cells were immediately isolated by Ficoll density gradient centrifugation (AXIS-SHIELD, Dundee, Scotland). After thawing, $10^6$ peripheral blood mononuclear cells were incubated for 10 minutes with monoclonal antibodies against human FITC-conjugated anti-CD45 (Miltenyi Biotec, Bergisch Gladbach, Germany), PE-conjugated anti-AC133 (Miltenyi Biotec), and APC-conjugated anti-CD34 monoclonal antibody (Becton Dickinson Biosciences, Franklin Lakes, New Jersey). To assess background, isotype controls were used as negative controls based on the species and IgG subclass of each antibody. After incubation, erythrocytes were lysed, and the remaining cells were washed with phosphate-buffered saline, fixed in 2% paraformaldehyde, and analyzed on a FACS Calibur Flow Cytometer (Becton Dickinson Biosciences). Each analysis consisted of 500,000 events. To quantify the amount of $\text{CD34}^+\text{AC133}^+\text{CD45}^{\text{low}}$ cells, the mononuclear cell fraction was gated and analyzed for the expression of AC133 and CD45. Only $\text{AC133}^+\text{CD45}^{\text{low}}$ cells were finally investigated for the count of $\text{CD34}^+$ cells.

**Characterization of progenitor cells**

Then $1 \times 10^4$ mononuclear cells were plated on 6-well culture dishes coated with human
fibronectin and gelatin and maintained in endothelial cell basal medium-2 (EBM-2, CellSystem Co.) supplemented with EGM-2 microvascular single aliquots and 5% fetal bovine serum. After 3 days of culture, nonadherent cells were removed. Cytochemical analysis of adherent cells was performed on day 4. To detect the uptake of 1,1’-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine-labeled acetylated LDL (Di-AcLDL; Molecular Probes, Carlsbad, CA), cultivated cells were incubated with Di-AcLDL (10 μg/mL) at 37°C for 1 hour. Thereafter, cells were fixed with 2% paraformaldehyde for 10 minutes and incubated with fluorescein isothiocyanate (FITC)-labeled *Ulex europaeus* agglutinin I (lectin, 10 μg/mL; Sigma) for 1 hour. Cells demonstrating double-positive staining lectin and Di-AcLDL were identified to be progenitor cells. They counted 3 randomly selected high-power fields per well.

**Migration assay**

Briefly, isolated progenitor cells were detached mechanically by using a cell scraper, harvested by means of centrifugation, resuspended in 300 μL of EBM, and counted. The $2 \times 10^4$ progenitor cells were placed in the upper chamber of a modified Boyden chamber (FluroBlock, Becton Dickinson Biosciences). The chamber was placed in a 24-well culture dish containing EBM and culture medium for control and human recombinant VEGF (50 ng/mL; Sigma). After 24 hours of incubation at 37°C, the lower side of the filter was washed with PBS and fixed with 2% paraformaldehyde. For quantification of cells that had migrated, cell nuclei were stained with 4’,6-diamino-phenylidole (DAPI, Sigma). Migrated cells in the lower chamber were counted manually in the 3 random high-power fields. Each experiment was performed in triplicate.

**Analytical methods**

The EDTA-containing tubes were chilled promptly in an ice bath. Plasma was immediately separated by centrifugation at 3100g for 10 min at 4°C, and serum was separated by centrifugation at 1000g for 10 min at room temperature. Samples were stored at -80°C until the time of assay. Serum concentrations of total cholesterol, triglycerides, HDL cholesterol, LDL cholesterol, glucose, and electrolytes were determined by routine chemical methods. Plasma concentrations of VEGF were measured using ELISA kits.
(R&D Systems, Minneapolis, Minnesota). Serum concentration of hs-CRP was measured by a high sensitive nephelometry assay using a CRP kit (Dade Behring, Deerfield, Illinois). Serum concentration of interleukin-6 was measured by a high sensitivity ELISA (R&D Systems). The serum concentration of MDA-LDL was assayed by ELISA (anti-MDA-modified LDL antibody, SRL, Atsugi, Japan). The urinary excretion of 8-OHdG also was assayed by ELISA using 8-OHdG kits (Nihon Yushi, Fukuroi, Japan).

Figure S1

Figure S1 online data supplement. Infusion protocol. The infusion of acetylcholine and sodium nitroprusside were carried out in a random order.

Figure S2
Figure S2 online data supplement. Putative mechanisms of augmentation of endothelial function through vascular endothelial growth factor (VEGF)-endothelial nitric oxide synthase (eNOS) activation by repetition of ischemic preconditioning and a lack of ischemic preconditioning-induced augmentation of endothelial function in smokers. Ischemic preconditioning causes local hypoxia in skeletal muscle; hypoxia-inducible factor-1 (HIF-1) upregulates expression of the VEGF gene; VEGF activates the PI3K/Akt pathway; calcium-calmodulin together with heat shock protein 90 (HSP90) displaces eNOS from caveolin-1; the interaction of HSP90 with Akt and eNOS permits HSP90 to serve as a docking site for phosphorylation of eNOS Ser1177 by Akt; HSP90 stabilizes the binding of calmodulin and eNOS; electron flux is caused from the reductase to the oxygenase domain of eNOS; NO is released from L-arginine in endothelial cells; and VEGF induces mobilization of endothelial progenitor cells (EPCs), leading to angiogenesis and increase in NO production. VEGF directly up-regulates eNOS expression and increases subsequent NO release.