Long-term, regular remote ischemic preconditioning improves endothelial function in patients with coronary heart disease

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Abstract

Remote ischemic preconditioning (RIPre) can prevent myocardial injury. The purpose of this study was to assess the beneficial effects of long-term regular RIPre on human arteries. Forty patients scheduled for coronary artery bypass graft (CABG) surgery were assigned randomly to a RIPre group (n = 20) or coronary heart disease (CHD) group (n = 20). Twenty patients scheduled for mastectomy were enrolled as a control group. RIPre was achieved by occluding arterial blood flow 5 min with a mercury sphygmomanometer followed by a 5-min reperfusion period, and this was repeated 4 times. The RIPre procedure was repeated 3 times a day for 20 days. In all patients, arterial fragments discarded during surgery were collected to evaluate endothelial function by flow-mediated dilation (FMD), CD34$^+$ monocyte count, and endothelial nitric oxide synthase (eNOS expression). Phosphorylation levels of STAT-3 and Akt were also assayed to explore the underlying mechanisms. Compared with the CHD group, long-term regular RIPre significantly improved FMD after 20 days (8.5 ± 2.4 *vs* $4.9 \pm 4.2\%$, $P < 0.05$) and significantly reduced troponin after CABG surgery (0.72 ± 0.31 and 1.64 ± 0.19, $P < 0.05$). RIPre activated STAT-3 and increased CD34$^+$ endothelial progenitor cell counts found in arteries. Long-term, regular RIPre improved endothelial function in patients with CHD, possibly due to STAT-3 activation, and this may have led to an increase in endothelial progenitor cells.

Key words: Remote ischemic preconditioning; endothelial function; coronary heart disease; STAT-3

Introduction

Studies have shown that remote ischemic preconditioning (RIPre) can reduce infarct size and decrease ischemia-reperfusion injury, making it an attractive, novel strategy to prevent myocardial injury (1,2). It has been shown to be effective in reducing myocardial reperfusion injuries in both experimental and clinical settings (3). Recently, the application of RIPre for patients with acute myocardial infarction during ambulance transit was shown to reduce infarct size, but the mechanism is largely unknown (4). Signal transducer and activator of transcription-3 (STAT-3) activation is thought to play a cardioprotective role during myocardial infarction by ischemic preconditioning.

Patients with coronary heart disease (CHD) who experience myocardial infarction exhibit endothelial dysfunction (5). This contributes to the pathogeneses of atherosclerosis and its complications and is closely related to cardiovascular events. RIPre before hospital admission increases myocardial salvage and has a favorable safety profile (6); however, the direct role of RIPre in regulating human arterial function remains elusive.

The aims of the present study were twofold: to identify the beneficial effects of long-term regular RIPre in human arteries, especially with regard to endothelial function protection, and to determine its potential mechanisms.

Material and Methods

Study design

Forty patients who had significant coronary arterial stenosis and were scheduled for coronary artery bypass graft (CABG) surgery were enrolled in the study. The patients were randomly assigned to the RIPre (n = 20) or CHD group (n = 20). Twenty patients with breast cancer who were scheduled for mastectomy were included as a control group. Exclusion criteria were the presence of an acute coronary syndrome, heart failure, hypertrophic cardiomyopathy, uncontrolled hypertension, serum creatinine concentration >2 mg/dL, or anticipation of non-compliance with the study protocol. The study objectives and protocol were fully explained, and written informed consent was obtained from all patients. This study was

approved by the regional Ethics Committee in accordance with the standards of the Declaration of Helsinki.

RIPre was administered while upper arm blood pressure was monitored 3 times a day for 20 days. A blood pressure cuff was placed around the upper arm and inflated to 200 mmHg to occlude arterial blood flow for 5 min. The cuff was then deflated to allow reperfusion. The procedure was repeated 4 times at 5-min intervals. Limb ischemia was confirmed by a change in the color of the skin and a decrease in skin temperature. Following limb reperfusion, the skin color returned to pink, and the skin temperature returned to the baseline level. In the CHD group, blood pressure was routinely measured three times daily. After 20 days, all patients underwent CABG surgery, and discarded artery fragments were collected following the procedure for later analysis (7,8). Routine patient treatment for CHD was maintained during the study in both the RIPre and CHD groups. Antihypertensive treatment was titrated to achieve a target blood pressure of <130/80 or <140/90 mmHg for patients with or without diabetes, respectively, during the study period.

Measurement of flow-mediated vasodilation (FMD) response

FMD response was measured at baseline and on day 20 and used as an index of endothelial function. Color Doppler ultrasound measurement of the brachial artery was performed in the resting state end-diastolic dimension, (D0), pneumatic compression to 250-300 mmHg was maintained for 5 min, and 60-90 s after deflation, reactive hyperemia of the brachial artery diastolic diameter (D1) was determined. The color Doppler evaluation of FMD is reported as the percent change in brachial artery diameter and was calculated as follows: (D1–D0)/D0 × 100. FMD values ≥10% and <10% were considered to indicate normal vascular endothelial function and endothelial dysfunction, respectively.

CD34$^+$ monocyte count by flow cytometry

CD34$^+$ monocyte and leukocyte counts were performed in all patients at baseline and on day 20. A total of 10 µL blood was added to two tubes, and IgG antibody was added to one tube as a control. Fluorescein isothiocyanate (FITC)-conjugated CD34 and phycoerythrin (PE)-conjugated CD38 antibodies were added to the remaining patient samples and incubated for 30 min in the dark. Subsequently, 1 mL erythrocyte lysis buffer was added, and after an additional 15 min in the dark, phosphate-buffered saline was added to adjust the cell concentration for flow cytometry (FACSCAN Becton Dickinson, USA). The antibody positive rate was recorded as the percentage of CD34$^+$ leukocytes.

Arterial CD34$^+$ monocytes by immunohistochemisty

Tissue sections were stained as previously described (9). Cryosections of internal mammary artery fragments (7,8) were stained for CD34 (BD Pharmingen, USA), counterstained with FITC-fluorescence (Sigma, USA) and mounted with fluorescent mounting medium (Dako, Denmark). Sections were examined using a Nikon E600 fluorescence microscope (Japan) and the Lucia Measurement software 4.6 (India).

Endothelial nitric oxide synthase (eNOS) mRNA expression by semiquantitative RT-PCR

Total RNA was extracted from arterial tissue samples using the SV total RNA Isolation System (Promega, USA) and diluted in 30 µL RNA-free water. The purity of RNA can be confirmed by RNA electrophoresis. The RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) was used to obtain 3 µg total RNA. The reaction mixture contained 2 µL cDNA, 10 µL 2× PCR Taqmix, 1 µL upstream and downstream primers, and 5 µL RNA enzyme water, for a total volume of 20 µL. The primers used were as follows: eNOS-specific (299 bp) sense, 5′-TTCCGGGGATTCTGGCAGGAG-3′ and antisense, 5′-GCCAT-GGTAACATCGCCGCAG-3′); glyceraldehyde phosphate dehydrogenase (GAPDH)-specific (343 bp) sense, 5′-CTCTAAGGCTGTGGGCAAGGTCAT-3′ and antisense, 5′-GAGATCCACCACCCTGTTGCTGTA-3′. The 35 PCR cycles were carried out as follows: 94°C for 5 min, 94°C for 30 s, 65°C for 45 s, and 72°C for 30 s. RT-PCR products were analyzed by 2% agarose gel electrophoresis, visualized with ethidium bromide, and quantified by densitometry. The results are reported as the ratio of eNOS and GAPDH.

Western blotting

Fragments of arterial tissue obtained during CABG surgery were used to measure protein phosphorylation by immunoblotting for survival kinases (10). The excised tissue was clamped between stainless steel tongs precooled with liquid nitrogen, frozen in liquid nitrogen, and stored at –80°C. Approximately 100 mg powdered arterial tissue was used for protein extraction. Frozen tissue samples were homogenized on ice in 0.5-mL ice-cold lysis buffer containing 30 mM HEPES, 20 mM KCl, 2.5 mM EGTA, 2.5 mM EDTA, 40 mM sodium fluoride, 4 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 10% glycerol, 1% Nonidet P-40, a phosphatase inhibitor cocktail (Sigma), and a protease inhibitor cocktail (Complete Mini; Roche Applied Science, Germany). Following electrophoresis and blotting, membranes were incubated overnight at 4°C with antibodies against ^{705}Tyr-phospho-STAT-3, total STAT-3, total serine/threonine-protein kinase (Akt), and ^{473}Ser-phospho-Akt (Cell Signaling Technology, USA). Beta-actin (Sigma) was used as a loading control. After washing in Tris-buffered saline and Tween-20, the membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated anti-human IgG secondary antibody (1:2000, Santa Cruz Biotechnologies, USA), and the bound

antibody was detected using an enhanced chemilumines-cence Western blotting kit (Santa Cruz Biotechnologies). The densities of bands with appropriate molecular masses (60 kDa for Akt and 80 kDa for STAT-3) were semiquantitatively determined using a LAS-3000 mini Lumino image analyzer (Fujifilm, Japan). Blood troponin concentration was assayed in all patients after CABG surgery using a diabody one-step sandwich enzyme immunoassay (Access Immunoassay System; Beckman Coulter, USA).

Statistical analysis

Data are reported as means $\pm$ SD for continuous variables or frequency percentages for categorical vari-ables. The Kolmogorov-Smirnov test was used to determine the normality of distribution for continuous variables. Multiple comparisons were evaluated by one-way ANOVA, and the differences between two groups were analyzed by Student-Newman-Keuls post hoc test. Either chi-square or Fisher's exact tests were used to compare differences between categorical variables. Differences of continuous variables that were not normally distributed were compared with Kruskal-Wallis or Mann-Whitney U-tests. Differences were considered to be statistically significant if the P value was less than 0.05. All analyses were performed with the S-Plus statistical package (Mathsoft Inc., USA).

Results

Patient characteristics

As shown in Table 1, the control, RIPre, and CHD groups did not differ significantly in age, gender, body mass index, blood pressure, or smoking status. At the beginning of the study, the patients in RIPre and CHD groups were well matched with respect to age, gender, angina class, and cardiovascular risk factors. On echo-cardiography, left ventricle (LV) end-diastolic dimensions were similar in the two groups. In addition, there were no differences between the RIPre and CHD groups in medical treatment (Table 1). The use of beta blockers, calcium channel blockers, and statins was similar between the two groups. Coronary angiography showed serious lesions in coronary vessels in all RIPre and CHD patients, and all underwent CABG surgery (Table 2). Hematologic analysis showed that the three groups had similar white cell and mononuclear cell counts (Table 3).

FMD and eNOS mRNA expression showed that long-term regular RIPre improved arterial endothelial function

RIPre can improve arterial endothelial function in patients with CHD. At baseline, patients in both the CHD and RIPre groups had lower FMD than controls (FMD = 12.8 $\pm$ 3.5%, 4.6 $\pm$ 3.2%, and 5.5 $\pm$ 3.3% in the

control, RIPre and CHD groups, respectively; both $P < 0.05$ vs control; Table 2). FMD was significantly improved in the RIPre group compared to the CHD group 30 days after treatment (8.5 $\pm$ 2.4% vs 4.9 $\pm$ 4.2%, $P < 0.05$). eNOS mRNA levels were measured by semi-quantitative RT-PCR. Total cell RNA was isolated, amplified, and then subjected to RT-PCR with eNOS- and GAPDH-specific primers in separate reactions. As shown in Figure 1, RIPre treatment significantly enhanced eNOS mRNA expression compared with the CHD group (n = 20, $P < 0.05$).

Interestingly, troponin expression was significantly lower in the RIPre group than the CHD group after CABG surgery (0.72 $\pm$ 0.31 ng/L and 1.64 $\pm$ 0.19 ng/L, $P < 0.05$), indicating that less cardiomyocyte necrosis occurred during the procedure.

Long-term regular RIPre activated STAT-3

There were no significant differences in total Akt or STAT-3 protein levels among the study groups. As illustrated in Figures 2 and 3, phospho-Akt levels were significantly enhanced in RIPre compared with control group patients ($P < 0.05$). While RIPre treatment was more effective in increasing endothelial function than medical treatment alone in the CHD group, Akt was significantly more phosphorylated in RIPre than in CHD, suggesting that another signaling pathway might play a role in the enhanced cardioprotection afforded by this treatment. When testing the Survivor Activating Factor Enhancement (SAFE) pathway (11), phospho-STAT-3 levels were significantly increased following RIPre. Interestingly, RIPre significantly increased phospho-STAT-3 levels compared with the controls and CHD patients. ($P < 0.05$, Figure 3).

Long-term regular RIPre increased immunohistochemical staining of endothelial progenitor cells (CD34^{+}) in arteries

The arterial CD34^{+} cells of patients in the RIPre group exhibited increased immunofluorescence compared with those of patients in the CHD group. The arterial CD34^{+} cells of patients in the control and CHD groups had the highest and lowest immunofluorescence, respectively, and the immunofluorescence observed in the RIPre group was significantly higher than that in the CHD group (n = 20, $P < 0.05$; Figure 4). The number of CD34^{+} cells was significantly higher in the control group than in patients in the CHD or RIPre group. At the beginning of the study, the absolute number of CD34^{+} cells was significantly different (2.35 $\pm$ 0.72 cells/μL, 1.48 $\pm$ 0.49 cells/μL and 1.56 $\pm$ 0.37 cells/μL, $P < 0.05$) in control group patients than in patients in the CHD and RIPre groups. On the other hand, no significant difference was noted in the number of CD34^{+} cells in the RIPre and CHD groups. On day 20, the numbers of CD34^{+} cells in the RIPre and CHD groups were significantly different

Table 1. Clinical, electrocardiographic and echocardiographic data of remote ischemic preconditioning (RIPre) and coronary heart disease (CHD) patients.

Clinical data	Control	RIPre	CHD
Age (years)	64 ± 10	64 ± 10	64 ± 10
Gender (male/female)	11/9	12/8	8/12
CCS class	0	2.4 ± 0.7	2.2 ± 0.6
NYHA functional class			
II	0	10	10
III or IV	0	10	10
Hypertension	2	3/17	4/16
Diabetes	0	5/15	6/14
Hypercholesterolemia	1	13	12
BMI	23.85 ± 7.3	24.78 ± 6.8	23.95 ± 7.5
Current Smoking	7/13	8/12	6/10
Previous myocardial infarction	0	13	12
EF%	60 ± 12	60 ± 7	58 ± 10

Data are reported as means ± SD or number. CCS: Canadian Cardiovascular Society; NYHA: New York Heart Association; BMI: body mass index; EF%: ejection fraction.

(1.79 ± 0.31 cells/μL *vs* 1.34 ± 0.20 cells/μL, $P < 0.05$; Figure 5). Because the increase in endothelial progenitor cells (EPCs) and improvement in endothelial function may be correlated, we carried out a regression analysis that revealed a slight but significantly positive correlation between FMD and the number of $CD34^+$ cells ($r = 0.46$, $P < 0.05$).

Discussion

The present study showed that RIPre is effective in improving endothelial function in patients with CHD and that the mechanism may involve activation of the Akt and STAT-3 pathways. The findings also indicated that improvement in endothelial function was related to increased EPCs in CHD.

Ischemic preconditioning experiments in animal models have demonstrated significant cardioprotective effects and reductions in the size of myocardial infarcts. A recent clinical study found that administering RIPre to patients with acute myocardial infarction while still in transit in a ambulance reduced infarct size (11).

Here we demonstrated that long-term, regular RIPre had a cardioprotective effect. After CABG surgery, the

Table 2. Quantitative coronary angiographic data and medical treatment of remote ischemic preconditioning (RIPre) and coronary heart disease (CHD) patients.

Characteristics	RIPre (n = 20)	CHD (n = 20)
Extension of disease		
Single vessel disease (left main lesion)	8 (40%)	7 (35%)
Multivessel disease	12 (60%)	13 (65%)
Vessel disease		
LAD involved	14 (70%)	13 (65%)
Cx involved	11 (55%)	10 (50%)
RCA involved	13 (65%)	14 (70%)
Medication		
ACE inhibitors	16 (80%)	15 (75%)
Beta-blockers	19 (95%)	20 (100%)
Diuretics	4 (20%)	6 (30%)
Calcium antagonists	3 (15%)	5 (25%)
Statins	20 (100%)	20 (100%)

Data are reported as number and percent. LAD: left anterior descending artery; Cx: left circumflex artery; RCA: right coronary artery; ACE: angiotensin-converting enzyme. There were no significant differences between groups (chi-square test).

Table 3. Number of endothelial progenitor cells (CD34^{+}), white cells and flow-mediated dilation (FMD) in remote ischemic preconditioning (RIPre) and coronary heart disease (CHD) patients and the control group.

Features	Control	RIPre		CHD	
		Before	After	Before	After
White cells (10^3/mL)	9.86 ± 2.79	8.96 ± 2.87	7.93 ± 3.04	6.92 ± 1.67	7.34 ± 2.56
CD34^{+} (cells/μL)	2.35 ± 0.72	1.48 ± 0.49	1.79 ± 0.31*[#]	1.56 ± 0.37	1.34 ± 0.20*
FMD (%)	12.8 ± 3.5	4.6 ± 3.2	8.5 ± 2.4*[#]	5.5 ± 3.3	4.9 ± 4.2*

Data are reported as means ± SD. * P<0.05 vs control; [#]P<0.05 vs before treatment (Student-Newman-Keuls test).

mean troponin levels in the RIPre (0.72 ± 0.31 ng/L) and CHD groups (1.64 ± 0.19 ng/L) were significantly different, indicating that RIPre protected patients and reduced myocardial cell damage. This finding is consistent with previous reports (12,13). As most patients with CHD have endothelial dysfunction, we propose that the cardioprotective effect of long-term regular RIPre is related to improved endothelial function.

This study provides some insight into the relationship between regular RIPre and improved endothelial function in CHD patients. Currently, echocardiography or arterial FMD is used as a standard tool for the evaluation of endothelium-dependent vasodilatation. In this study, assessment of FMD, endothelial progenitor cells, CD34+ monocyte counts, and eNOS mRNA gene expression were carried out to evaluate endothelial function (11), and we
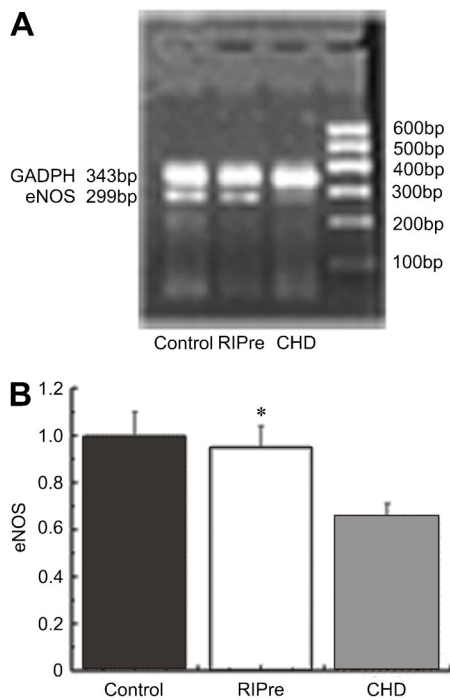

Figure 1. A, Effects of remote ischemic preconditioning (RIPre) on endothelial expression of endothelial nitric oxide synthase (eNOS) mRNA as measured by semiquantitative RT-PCR. RIPre caused an increase in eNOS mRNA expression. B, Relative expression of eNOS mRNA (density ratio, eNOS:GADPH) is reported as means ± SE for n = 20 in each group. *P<0.05 vs CHD (Student-Newman-Keuls test). Note that the example does not exactly reflect the mean value of all samples.
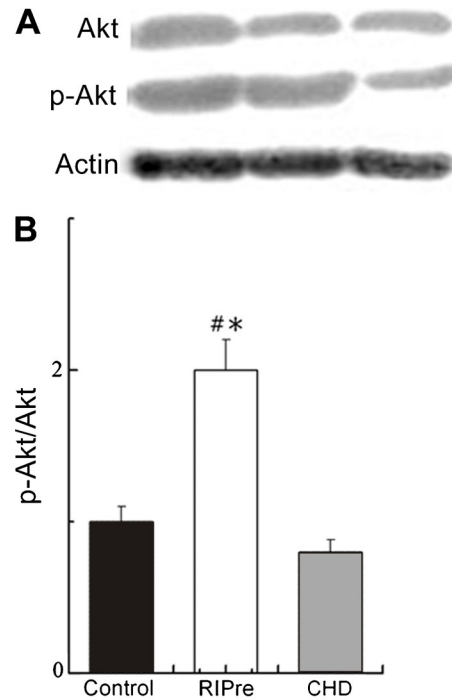

Figure 2. A, Representative Western blot showing phosphorylated serine-threonine protein kinase (p-Akt), total Akt, and actin in arterial tissues. B, Ratio of p-Akt to Akt. Data are reported as means ± SE for n = 20 in each group. RIPre: remote ischemic preconditioning; CHD: coronary heart disease. *P<0.05 vs control group; [#]P<0.05 vs CHD (Student-Newman-Keuls test). Note that the immunoblot example does not exactly reflect the mean value of all samples.
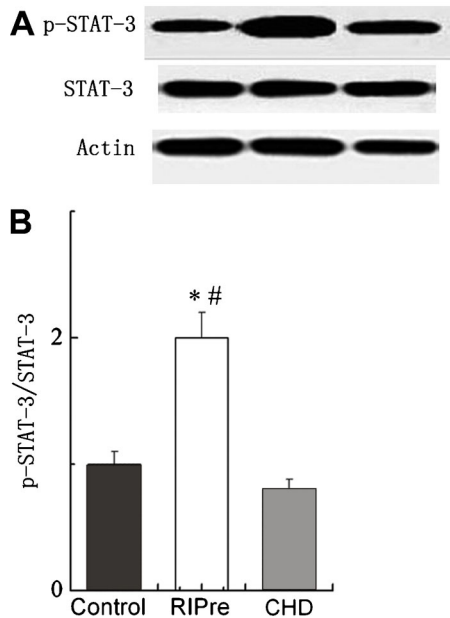
Figure 3. *A*, Representative Western blot of phosphorylated STAT-3 (p-STAT-3), total STAT-3, and actin in arterial tissue. *B*, Ratio of p-STAT-3 to STAT-3. Data are reported as means ± SE for n = 20 in each group. RIPre: remote ischemic preconditioning; CHD: coronary heart disease. *P<0.05 *vs* control group; #P<0.05 *vs* CHD (Student-Newman-Keuls test). Note that the immunoblots do not exactly reflect the mean values of all samples.

significant in the CHD group (5.5 ± 3.3% *vs* 4.9 ± 4.2%). After treatment, FMD in the RIPre patients was close to normal and significantly improved compared with the CHD group. The observed increase in FMD demonstrates that long-term regular RIPre significantly improved endothelial function. However, these methods test peripheral arteries rather than coronary vascular endothelial function. To this end, we tested samples of the internal thoracic artery obtained during surgery (7,8). eNOS mRNA gene expression in artery samples (7,8) was higher in the RIPre group than in the CHD group. These results indicate that increased eNOS mRNA gene expression resulted from improved endothelial function (15).

Recently, ischemic preconditioning studies demonstrated activation of STAT-3 as a part of the SAFE pathway (16,17). Studies in animal models have confirmed that the cardioprotective effects of RIPre are due to STAT-3 activation (18,19). The SAFE pathway is thought to have a cardioprotective effect related to RIPre in myocardial infarction (9,20), and its role has been confirmed in patients after CABG surgery in whom troponin values were significantly different between patients in RIPre and CHD groups. This indicates that in the event of myocardial infarction, long-term regular RIPre can protect myocardial cells and reduce myocardial necrosis. In this model, RIPre activates STAT-3 (SAFE pathway), increases p-Akt, and then increases eNOS mRNA gene expression, resulting in improved endothelial function and FMD. Hence, we presume that the increase in eNOS mRNA gene expression is correlated with the activation of STAT-3 (SAFE pathway). However, as this was a human experiment, blockers could not be applied to test our hypothesis.
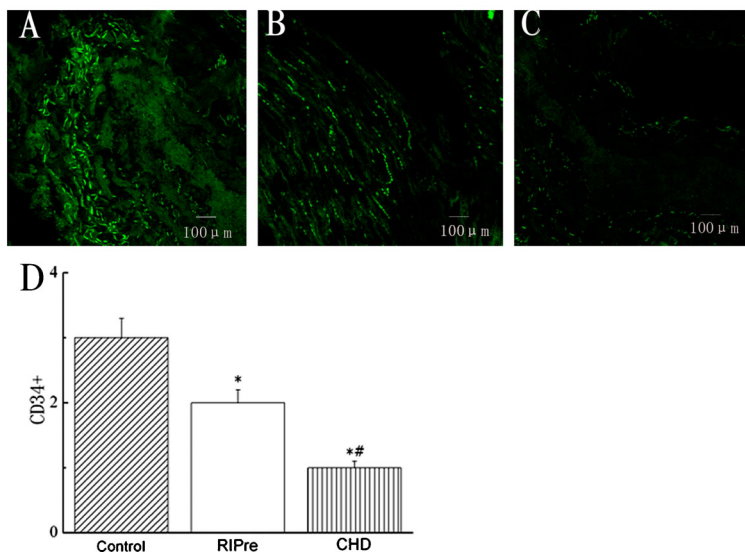
found significant improvements consistent with previously reported results (14). In the RIPre group, FMD improved from 4.6 ± 3.2% to 8.5 ± 2.4%, but this change was not

Figure 4. Representative micrographs of immunofluorescence staining of arterial CD34+ cells in the *A*, control group; *B*, remote ischemic preconditioning (RIPre) group; and *C*, coronary heart disease (CHD) group. Cells expressing hematopoietic progenitor cell antigen CD34 (CD34+) were significantly reduced in the CHD group compared with the control group but were significantly increased by RIPre (P<0.05). *D*, Relative fluorescence of EPC staining of endothelial progenitor cells (EPCs); CD34+ staining. Data are reported as means ± SE for n = 20 in each group. *P<0.05 *vs* control group; #P<0.05 *vs* RIPre group (Student-Newman-Keuls test). Note that the example does not exactly reflect the mean value of all samples.
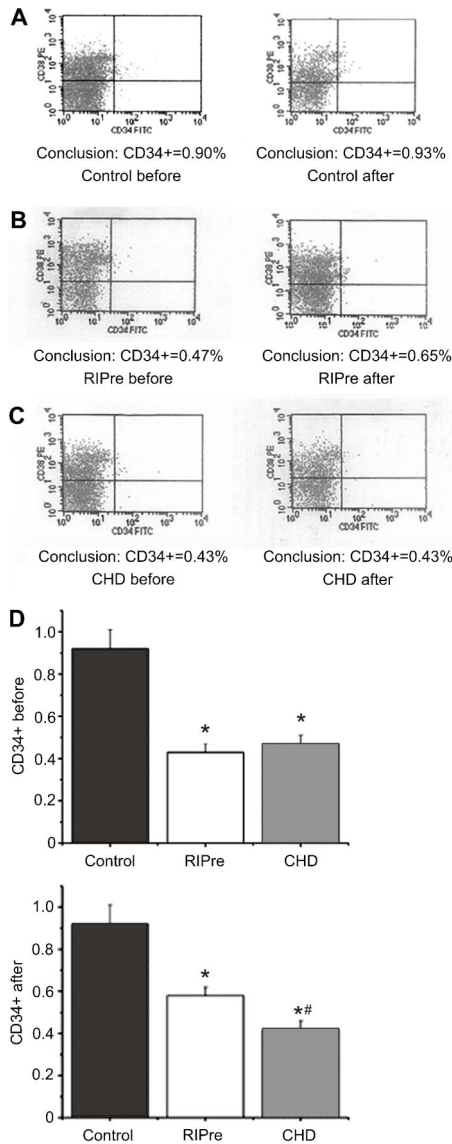
Figure 5. *A*, Representative flow cytometry measurements of CD34^{+} monocytes in blood before and after treatment in the *A*, control group; *B*, remote ischemic preconditioning (RIPre) group; and *C*, coronary heart disease (CHD) group. *D*, CD34^{+} monocytes in blood before and after treatment. Data are reported as means ± SE. *P<0.05 *vs* control group; [#]P<0.05 *vs* RIPre group (Student-Newman-Keuls test).

Stem cell research in the past decade has shown that EPCs, a population of bone marrow-derived cells, circulate in the blood and modulate new vessel formation and homeostasis in adults (21-23). In addition, low EPC

number is correlated with poor vascular function (24) and is an independent risk factor for adverse events in CHD patients (25,26). It follows that the number of circulating EPCs may determine the capacity to repair vascular damage and may be viewed as an index of vascular health. The popular concept is that circulating EPCs are protective, and their alteration may account for the progression of endothelial dysfunction and atherosclerosis that are commonly seen in LV dysfunction and CHD (27). The role of EPCs in endothelial dysfunction, however, remains a matter of debate. Due to the difficulties in their identification, limited information is available about the normal range of EPCs in CHD. In addition, earlier studies have been hampered by their retrospective nature and by the lack of uniform criteria to precisely identify EPCs. Studies have mainly focused on cells positive for the CD34 endothelial marker, which is an adhesion molecule expressed in hematopoietic stem cells (28), because it is not expressed in mature endothelium (29). Conversely, there are few data on the pathophysiological significance of other subsets of stem/progenitor cells. The levels of EPCs are decreased in coronary artery disease, and animal models have shown that a decrease in the endogenous pool of progenitor cells may accelerate the course of atherosclerosis (30,31). Similarly, early investigations showed that circulating EPCs are involved in modulating LV remodeling processes that lead to heart failure (32). It was recently reported that patients with ischemic and nonischemic cardiomyopathy have a lower number of EPCs than healthy individuals (33). In the present study, immunohistochemistry of internal mammary artery specimens (7,8) revealed that EPCs were positive for the CD34 endothelial marker and that they increased in CHD. Although this does not prove a causal relationship, we found a significant positive correlation between FMD and CD34^{+}.

Accordingly, overall increases in stem cell number and function have been proposed as a therapeutic means for improving LV function in CHD patients. Stem and progenitor cell circulation and pretreatment of cardiac homing have been shown to reduce infarct size and improve cardiac function.

In conclusion, long-term regular RIPre improved endothelial function in patients with CHD, possibly via STAT-3 activation (SAFE pathway), and may have been associated with an increase in arterial EPCs.

Acknowledgments

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