

Pioglitazone restores the homocysteine-impaired function of endothelial progenitor cells via the inhibition of the protein kinase C/NADPH oxidase pathway

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Abstract. Homocysteine (Hcy) has been shown to impair the migratory and adhesive activity of endothelial progenitor cells (EPCs). As a peroxisome proliferator-activated receptor γ agonist, pioglitazone (PIO) has been predicted to regulate angiogenesis, and cell adhesion, migration and survival. The aim of the present study was to determine whether PIO could inhibit Hcy-induced EPC dysfunctions such as impairments of cell migration and adhesion. EPC migration and adhesion were assayed using 8.0- μ m pore size Transwell membranes and fibronectin-coated culture dishes, respectively. Hcy at a concentration of 200 μ M was observed to markedly impair cell migration and adhesiveness, and PIO at a concentration of 10 μ M attenuated the Hcy-mediated inhibition of EPC migration and adhesion. The mechanism of these effects may be through the inhibition of protein kinase C (PKC) and reactive oxygen species production. The expression levels of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase subunits, NADPH oxidase 2 (Nox2) and p67phox, were upregulated by Hcy, with a peak in levels following treatment with a concentration of 200 μ M. PIO downregulated the expression levels of Nox2 and p67phox via the PKC signaling pathway. Furthermore, the mechanism of PIO associated with downregulating the p67phox and Nox2 subunits of NADPH oxidase was verified. Thus, PKC and NADPH oxidase may serve a major role in the protective effects of PIO in EPCs under conditions of high Hcy concentrations.

Introduction

Endothelial progenitor cells (EPCs) enhance new vessel formation and maintain homeostasis of the endothelium due to their protective role in the cardiovascular system (1). Previous studies have shown that homocysteine (Hcy) reduces the number of EPCs and impairs their functional activities, including proliferation, migration, adhesion and *in vitro* vasculogenesis capacity (2,3). Furthermore, Hcy increases the levels of EPC apoptosis by stimulating the production of reactive oxygen species (ROS) within EPCs through the activation of NADPH oxidase (4). Analyze detection platforms such as luminescence probe could be used in studying EPC function (5,6).

Pioglitazone (PIO), which has been used for the treatment of type 2 diabetes for many years, has substantially more potential beneficial effects than previously expected. PIO has been shown to exert favorable cardiovascular effects by slowing the progression of atherosclerosis progression (7), and may reduce the risk of myocardial infarction, stroke and premature death in high-risk patients with diabetes. PIO has been shown to exert beneficial effects *in vitro* on EPCs isolated from patients with diabetes mellitus and coronary artery disease (8), and prevent apoptosis of EPCs and promote *in vivo* neoangiogenesis in mice (9). In addition, PIO ameliorates Ang II-induced senescence of EPCs (10). However, to the best of our knowledge, no previous study has investigated the role of PIO in the regulation of EPC dysfunctions and its related potential mechanisms under high levels of Hcy.

PKC activation has been demonstrated to be a common signaling pathway through which Hcy exerts its pathogenic functions in the vasculature. High levels of Hcy impair endothelial function primarily through PKC activation (11). In monocytes, Hcy stimulates phosphorylation of the NADPH oxidase subunits p47phox and p67phox via activation (12). PKC activation and NADPH oxidase phosphorylation may possibly be the signaling pathways involved in Hcy-induced EPC dysfunctions. In our previous study, we found that PIO mitigated Hcy-induced downregulation of vascular endothelial growth factor (VEGF) and interleukin (IL)-8 expression and secretion in EPCs via PKC and NADPH oxidase (13).

On the basis of these findings, we speculated that Hcy may impair the function of EPCs by initiating the activation of PKC

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and NADPH oxidase, and the underlying protective effects of PIO on the migration and adhesion of EPCs may result from the reduction of oxidative stress produced by NADPH oxidase, and the inactivation of PKC in EPCs.

Materials and methods

Materials. Peripheral blood mononuclear cells (PBMNCs) were obtained from healthy volunteers. Written informed consent was obtained from all volunteers, and the present study was approved by the Ethics Review Board of Sir Run Run Shaw Hospital, College of Medicine, Zhejiang University (Zhejiang, China). Volunteers participated in this study are up to 10, but because of contamination, EPCs were mainly collected from the blood of 8 volunteers. During this study, the doctors obeyed the ethical principles and respected the volunteers' rights. All protocols are in accordance with the Helsinki Declaration. Endothelial Lympholyte[®]-H Cell Separation Media was acquired from Cedarlane (Burlington, VT, USA). Cell Growth Medium-2 (EGM-2) was purchased from Lonza (Walkersville, MD, USA). Fetal bovine serum (FBS) was obtained from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Hcy and Diphenyleneiodoniumchloride (DPI) were procured from Sigma Chemical (St. Louis, MO, USA). PIO was generously provided by Huadong Medicine Co. Ltd (Hangzhou, China). The reagent 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H₂DCFDA) was obtained from Invitrogen (Thermo Fisher Scientific, Inc.). Lucigenin and NADPH tetrasodium salt were purchased from Enzo Life Sciences, Inc. (Farmingdale, NY, USA). Bisindolylmaleimide I (GF 109203x) was acquired from Calbiochem (Darmstadt, Germany). Anti-gp91 (NOX2) was purchased from Merck Millipore (Darmstadt, Germany). Anti-p67phox was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Anti-phospho-PKC α/β II was obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). HRP-conjugated monoclonal mouse anti-GAPDH antibody was purchased from Kangchen (Shanghai, China). PIO, DPI and GF109203x were dissolved in 0.1% dimethyl sulfoxide (DMSO) and the vehicle (0.1% DMSO) was added to the control samples.

Isolation and cultivation of EPCs. EPCs were isolated, cultured and characterized according to previously described techniques (3). PBMNCs were isolated from healthy volunteers using Ficoll density gradient centrifugation, and then the cells were cultured on human fibronectin-coated dishes in EGM-2, which contained 20% FBS, VEGF, fibroblast growth factor-2 (FGF-2), epidermal growth factor (EGF), insulin-like growth factor (IGF) and ascorbic acid. Non-adherent cells were removed by washing with phosphate-buffered saline (PBS) after 3 days in culture, and adherent cells were maintained in new medium for a further 4 days.

Intracellular fluorescence measurement of ROS. Intracellular ROS levels were measured by flow cytometry using the fluorescent probe H₂DCFDA. EPCs were cultured for 7 days with EGM-2. The cells were treated with various drugs and then loaded with 5 μ M H₂DCFDA in serum-free medium excluding interference of phenol red at 37°C for 30 min. After washing twice with PBS, cells were immediately monitored using a

flow cytometer (FACSCalibur; BD Biosciences Inc., Brea, CA, USA) at an excitation wavelength of 488 nm and an emission wavelength of 525 nm. The levels of ROS were determined by comparing the changes in the fluorescence intensity with that of the control.

Determination of NADPH oxidase activity and anti-oxidase activity. To determine the activity of NADPH oxidase and anti-oxidase, the lucigenin-derived enhanced chemiluminescence assay was used. Briefly, quiescent cells were treated as indicated and harvested. Following low-spin centrifugation, the pellet was resuspended in ice-cold buffer (pH 7.0), containing 1 mmol/l ethylene glycol tetraacetic acid (EGTA), 150 mmol/l sucrose and protease inhibitor cocktail (Merck Millipore). Subsequently, the cells were homogenized. The total protein concentration was determined using a Bradford assay and adjusted to 1 mg/ml. Protein samples (200 μ l), including 500 μ mol/l lucigenin as the electron acceptor and 100 μ mol/l NADPH as the substrate, were measured over 6 min in quadruplicate with a luminometer counter (Centro LB 960 Microplate Luminometer; Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany). Data were collected at 2 min intervals in order to measure relative changes in the levels of NADPH oxidase activity.

Western blot analysis. After treatment, cells were washed three times with ice-cold PBS and lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X 100, 0.5% sodium deoxycholate, 1 mM EDTA and 1 mM EGTA) supplemented with protease inhibitor cocktail (Merck Millipore), 1 mM PMSF, 1 mM Na₃VO₄ and 10 mM NaF.

The concentration of protein was determined by the Bradford method. After denaturing at 95°C for 5 min, a total of 30 μ g protein was loaded in each lane and subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein was then transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membranes were blocked in 5% nonfat milk and then incubated overnight with primary antibody at the appropriate dilution, before incubation for 1 h with a secondary antibody conjugated to horseradish peroxidase (1:10,000). After the reaction with the enhanced chemiluminescence reagent (Amersham, Haemek, Israel), the images were captured using the Image Reader LAS-4000 system (Fujifilm, Tokyo, Japan).

Transwell migration assay. Following the indicated treatments, the culture medium was removed and replaced with EBM-2 without any supplement. After 12 h, EPC migration was evaluated by using a Transwell migration assay. Briefly, 3x10⁴ cells were suspended in 100 μ l of EBM-2 supplemented with 0.1% BSA and placed in the upper chamber of 8.0- μ m pore size Transwell inserts (Merck Millipore). VEGF in serum-free EBM-2 was placed in the lower compartment of the chamber. After incubation for 24 h at 37°C in 5% CO₂, the cells that had not migrated were removed from the upper surface of the filters using cotton swabs and those that had migrated to the lower surface of the filters were fixed in 4% paraformaldehyde and stained with 4',6-diamidino-2-phenylindole (Roche Applied Science, Indianapolis, IN, USA). Cells that had migrated into

the lower chamber were counted manually in five random microscopic fields at a magnification, x100.

Cell adhesion assay. After 24-h incubation with Hcy, EPCs were washed with PBS and gently detached using 0.25% trypsin. Following centrifugation and resuspension in EGM-2, identical cell numbers were re-plated onto fibronectin-coated culture dishes and incubated for 30 min at 37°C. Adherent cells were counted by independent, blinded investigators.

Small interference (si)RNA transfection. p67phox sense siRNA sequence is 5'-CAGGGAACAUGUCUUGUdT T-3' and the anti-sense siRNA sequence is 5'-ACAAAGACA AUGUCCCCUGdT T-3'. The Nox2 sense siRNA sequence is 5'-CUCUGCGAUUCACACCAUdT T-3' and the anti-sense siRNA sequence is 5'-AAUGGUGUGAAUCGCAGAGdT T-3'. The negative control (NC) sense siRNA sequence was 5'-UUC UCCGAACGUGUCACGUdT T-3' and the NC anti-sense siRNA sequence was 5'-ACGUGACACGUUCGGAGA AdT T-3'. siRNA duplexes were synthesized by GenePharma (Shanghai, China). siRNA transfection into the EPCs was performed using Hiperfect Transfection Reagent (Qiagen AB, Sollentuna, Sweden) according to the manufacturer's instructions. Briefly, 150 pmol of siRNA against p67phox, Nox2 or NC siRNA was diluted in the appropriate volume of serum-free EBM-2 to give a final volume of 500 µl. For complex formation, 15 µl Hiperfect Transfection Reagent was added to the diluted siRNA and then incubated for 10 min at room temperature. Cells were incubated with the transfection complexes for 5 h under normal growth conditions. After incubation, 1 ml fresh culture medium containing serum was added to each well for further culture. Verification of siRNA efficacy was achieved by western blotting.

Statistical analysis. Data from at least three independent experiments are expressed as the mean ± standard error of the mean. Data were analyzed by unpaired Student's t-test for comparisons between two groups or one-way analysis of variance with the Student-Newman-Keuls post hoc test for multiple comparisons. Statistical analysis was performed using SPSS 19.0 software (IBM Corp., Armonk, NY, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

PIO inhibits Hcy-induced PKC activation. PKC acts as a major signaling system in response to extracellular signals. In our previous study, we investigated the phosphorylation levels of PKC in EPCs treated with Hcy and PIO (13). Cells were treated with Hcy at a concentration of 200 µM for 7.5, 15, 30, 60 and 120 min. The results of the western blot analysis showed that the Hcy treatments significantly increased the phosphorylation levels of PKC. Pre-treatment with PIO (10 µM) inhibited phosphorylation of PKC in EPCs.

PIO ameliorates Hcy-induced oxidative stress possibly by inactivation of PKC. Intracellular ROS levels were measured by flow cytometry using the fluorescent probe H2DCFH-DA. The levels of NADPH oxidase activity were measured with lucigenin-enhanced chemiluminescence in parallel. We

also observed the effects of the PKC inhibitor GF109203X (GF, 5 µM) and an NADPH oxidase inhibitor (DPI, 5 µM). Pretreatment with PIO and GF profoundly repressed Hcy-induced ROS production and NADPH oxidase activation, which was consistent with the outcomes of pretreatment with DPI. The data are also shown in our previous article (13).

PIO may inhibit Hcy-induced reduction of EPC migration and adhesiveness through inactivation of the PKC pathway and reduction of oxidative stress. The effects of PIO, GF and DPI on EPC migration and adhesion were assayed using 8.0-µm pore size Transwell membranes and fibronectin-coated culture dishes, respectively. Hcy at a concentration of 200 µM profoundly impaired cell migration and adhesiveness in accordance with the results of our previous study. PIO, GF and DPI suppressed the migration and adhesiveness impairment induced by Hcy (Fig. 1).

The results revealed that PIO attenuates Hcy-induced EPC dysfunctions such as migration and adhesiveness possibly by inhibiting PKC activation and promoting antioxidant properties.

PIO inhibits Hcy upregulation of the NADPH subunits Nox2 and p67phox. To verify whether the expression levels of NADPH subunits were affected by Hcy, cells were treated with 0, 10, 50, 100 and 200 µM Hcy for 24 h. The western blot analysis showed that the levels of Nox2 and p67phox were upregulated by Hcy, with a peak in the levels after treatment with a concentration of 200 µM. Pretreatment of EPCs with PIO (10 µM), GF (5 µM) and DPI (5 µM) for 30 min reduced Hcy-dependent Nox2 and p67phox expression (Fig. 2). The data suggests that PIO may downregulate the levels of Nox2 and p67phox via the PKC pathway.

Knockdown of Nox2 and p67phox inhibits Hcy-induced dysfunction of EPCs. To further investigate the potential mechanisms by which PIO restored Hcy-induced EPC dysfunction, Nox2-siRNA and p67phox-siRNA transfections were applied to downregulate the expression levels of these two NADPH subunits. Knockdown of the expression levels was confirmed by Western blotting as compared with the levels following treatment with the NC. The western blotting data (protein level) to confirm the knock down of Nox2-siRNA and p67phox-siRNA were shown in our previous article (13). Our results showed that EPCs transfected with Nox2 and p67phox siRNA exhibited significantly higher levels of cell migration and adhesion compared with cells transfected with control siRNA under stimulation with Hcy (Fig. 3).

Discussion

The present study demonstrated that PKC and NADPH oxidase play a major role in the protective effects of PIO against EPC dysfunction induced by high concentrations of Hcy (HHcy).

Hyperhomocysteinemia has been demonstrated to be an important pathological factor in vascular diseases, including coronary artery, cerebrovascular and peripheral arterial occlusive diseases. Hcy has been shown to inhibit the proliferation, adhesion and migration of human CD34(+) endothelial colony-forming cells (ECFCs) isolated from peripheral blood in a dose-dependent manner (14). Hcy

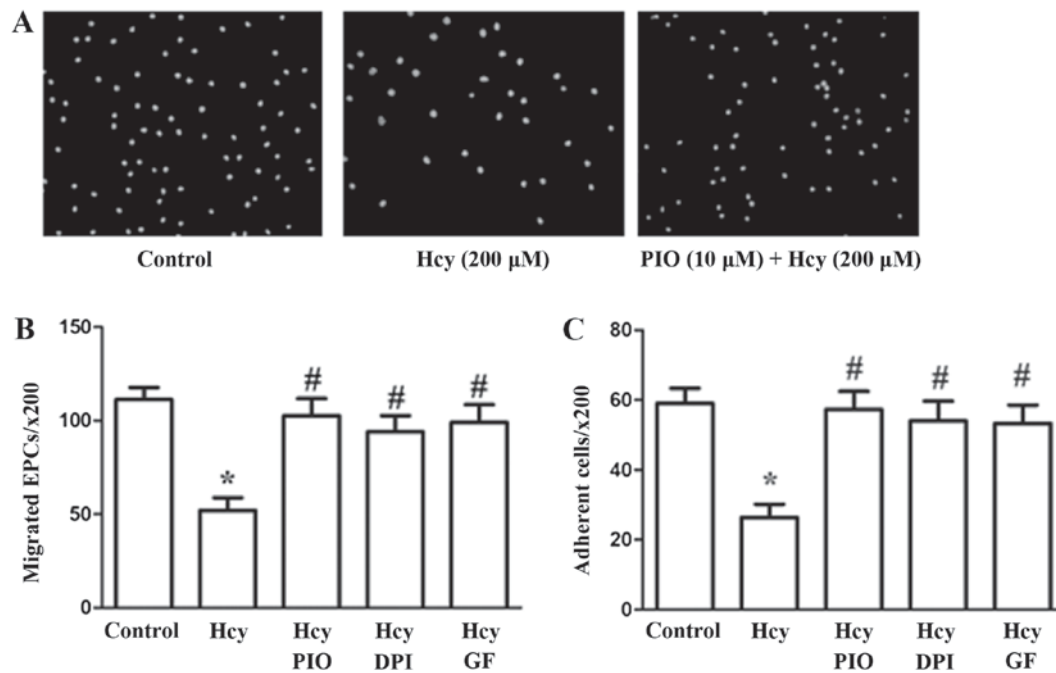


Figure 1. PIO attenuates Hcy-impaired EPC migration and adhesiveness via the inhibition of nicotinamide adenine dinucleotide phosphate oxidase and protein kinase C. (A) EPCs were pre-incubated with DPI (5 μ M) or GF (5 μ M) for 30 min prior to the addition of 200 μ M Hcy. 4',6-Diamidino-2-phenylindole staining was performed to determine the number of migrated EPCs (magnification, x200). Pretreatment with PIO, DPI and GF reversed the Hcy-induced reduction in the (B) migratory and (C) adhesive capacity of EPCs. Data are presented as the mean \pm standard error of the mean from three independent experiments. * P <0.05 vs. untreated cells; # P <0.05 vs. Hcy-treated cells. Hcy, homocysteine; EPCs, endothelial progenitor cells; PIO, pioglitazone; DPI, diphenyleneiodonium chloride.

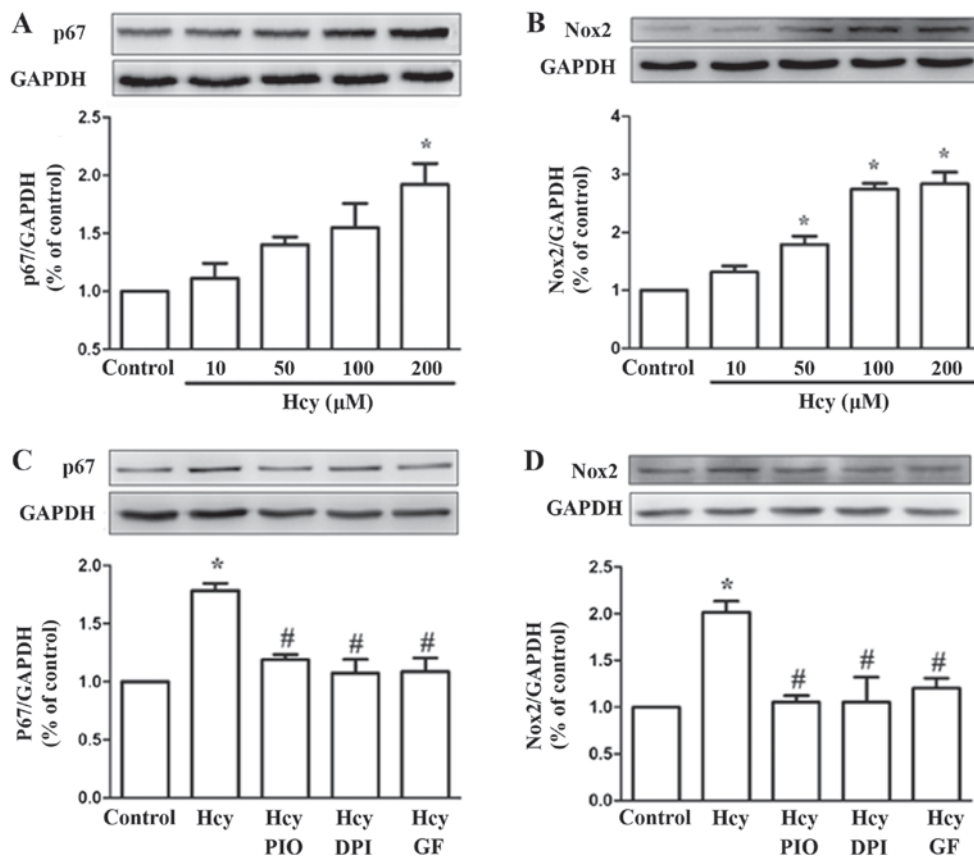


Figure 2. PIO decreases the expression levels of the NADPH oxidase subunits, p67phox and Nox2, via the protein kinase C signaling pathway. Incubation of the endothelial progenitor cells in Hcy upregulated the expression levels of (A) p67phox and (B) Nox2 in a dose-dependent manner. Pretreatment with PIO, DPI and GF inhibited the Hcy-stimulated enhancement of the expression levels of (C) p67phox and (D) Nox2. The data are presented as the mean \pm standard error of the mean from three independent experiments. * P <0.05 vs. untreated cells; # P <0.05 vs. Hcy-treated cells. Hcy, homocysteine; EPCs, endothelial progenitor cells; PIO, pioglitazone; DPI, diphenyleneiodonium chloride; NADPH, nicotinamide adenine dinucleotide phosphate; Nox2, NADPH oxidase 2.

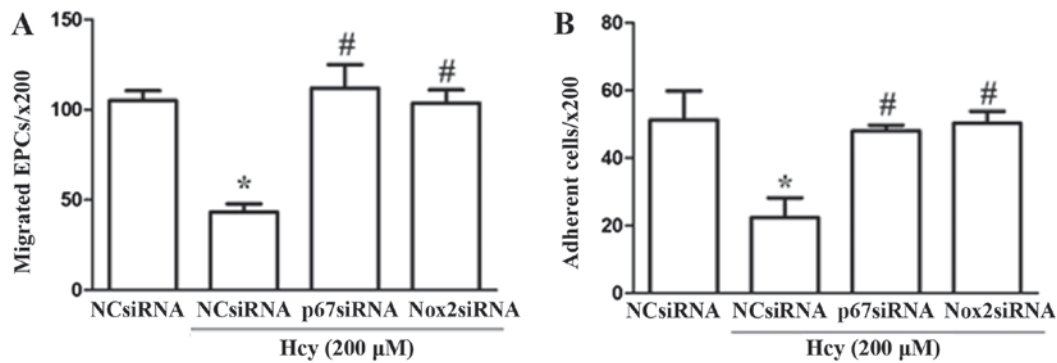


Figure 3. NADPH oxidase subunits p67phox and Nox2 serve an important role in the Hcy-induced dysfunction of EPC migration and adhesion. EPCs transfected with p67phox- and Nox2-siRNA resisted the detrimental effects of Hcy on their (A) migratory and (B) adhesive activity. * $P < 0.05$ vs. NC siRNA transfection; # $P < 0.05$ vs. NC siRNA transfection plus Hcy treatment (200 μ M for 24 h). Hcy, homocysteine; EPC, endothelial progenitor cell; siRNA, small interfering RNA; NC, negative control; NADPH, nicotinamide adenine dinucleotide phosphate; Nox2, NADPH oxidase 2.

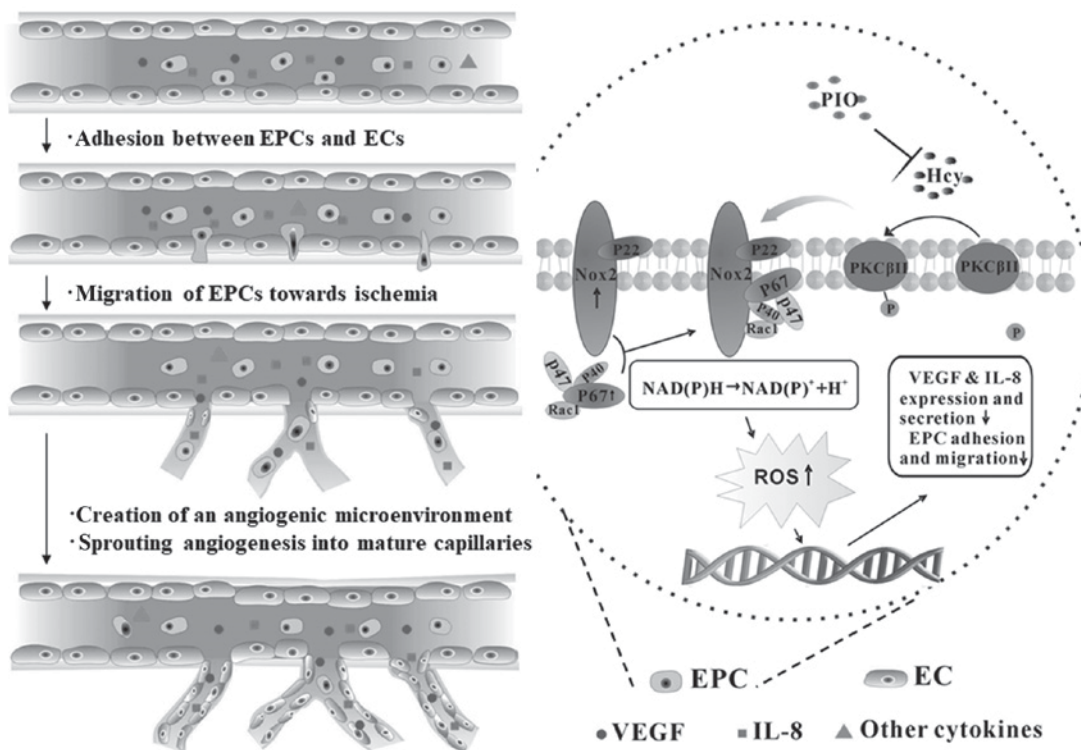


Figure 4. Mechanisms underlying PIO attenuation of Hcy-induced EPC dysfunction. Pretreatment of PIO restored EPC migratory and adhesive activity. EPC-enhanced angiogenesis in sites of ischemia via the following steps: i) EPCs adhere to the endothelium; ii) transendothelial migration and degradation of the endothelial basement membrane; iii) tunneling of EPCs create a temporary capillary-like scaffold for the neovasculature; and iv) EPCs continue to secrete multiple cytokines. In this process, VEGF and IL-8 are key; however, other biomarkers are required for further research. Hcy, homocysteine; EPC, endothelial progenitor cell; PIO, pioglitazone; NADPH, nicotinamide adenine dinucleotide phosphate; Nox2, NADPH oxidase 2; VEGF, vascular endothelial growth factor; IL, interleukin; PKC, protein kinase C; ROS, reactive oxygen species; EC, endothelial cells.

dose-dependently impairs the proliferation, migration and *in vitro* vasculogenesis capacity of EPCs (15). Moreover, PIO is convinced to its use as an insulin sensitizer, PIO is believed to have 'pleiotropic effects', including anti-apoptosis and anti-senescence (16). In our previous study, we confirmed that Hcy induced downregulation of VEGF and IL-8 expression levels and their secretion was normalized by PIO treatment. However, whether PIO exerts the same protective effect on migration and adhesion required further research.

Our previous data showed that treatment with Hcy increased the phosphorylation levels of PKC in a time-dependent

manner. We found that PIO inhibited the PKC activation induced by Hcy and suppressed Hcy-mediated ROS generation via the PKC/NADPH oxidase signaling pathway. The results of the present study show that PIO reverses the HHCy-induced inhibition of EPC migration and adhesion. To explore the mechanism of this effect, the PKC inhibitor GF and the NADPH oxidase inhibitor DPI were added prior to treatment of the cells with Hcy. As a result, the Hcy-mediated reduction in the levels of migration and adhesion was reversed, which suggests that PIO attenuates Hcy-induced EPC dysfunction by inhibition of PKC and NADPH oxidase. Additional signaling

pathways leading to Hcy-induced EPC dysfunction may be elucidated in further studies.

Hcy seems to promote the formation of ROS primarily through biochemical mechanism involving NADPH oxidase (Nox), endothelial nitric oxide synthase (eNOS) and endothelial lipid peroxidation (17). An increase of ROS including hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-) was produced by activation of the above *in-vivo* metabolism. At higher levels, O_2^- will react with NO to form a cytotoxic peroxynitrite (ONOO $^-$) and to decrease NO that plays a critical role in endothelial cell damage (18). NADPH oxidases have emerged as major enzymes responsible for the production of ROS in the blood vessel wall during cardiovascular disease progression (19). Hcy promotes the formation of ROS primarily via a biochemical mechanism involving NADPH oxidase. The family of NADPH oxidases comprises seven members, each based on a distinct core catalytic subunit. Nox2 (also known as gp91phox oxidase) was the first NADPH oxidase to be identified, and p67phox is one of its cytosolic components (20). The present study shows that Hcy dose-dependently increased the expression levels of p67phox and Nox2 and that PIO could inhibit overexpression of p67phox and Nox2. We further demonstrated that p67phox siRNA and Nox2 siRNA suppressed Hcy-impaired EPC function. This data suggests that PIO attenuated Hcy-induced EPC dysfunction possibly by inhibition of NADPH oxidase.

In the present study, we observed that PIO restored Hcy-impaired EPC migratory and adhesive capacity via inhibition of PKC and NADPH oxidase. Our previous study demonstrated that PIO also normalized the production of VEGF and IL-8 in EPCs that had been impaired by treatment with Hcy. These beneficial effects of PIO make it a potential therapeutic strategy in EPC-based cytotherapy for ischemic cardiovascular diseases such as hindlimb ischemia in patients with diabetes and myocardial infarction. EPC-driven neovascularization includes the migration of EPCs through the bloodstream, the adhesion between EPCs and the endothelium, and subsequent matrix degradation and migration of EPCs towards sites of ischemia, where EPCs create an angiogenic microenvironment through the secretion of cytokines and growth factors and induce sprouting angiogenesis by the surrounding endothelium (21). PIO may enhance the angiogenesis inhibited by Hcy via restoration of the migratory, adhesive and paracrine activity of EPCs (Fig. 4).

In conclusion, the present study demonstrated that PIO attenuates HHcy-induced EPC dysfunction, such as impaired migratory and adhesive capacity. The mechanism of its protective role on the migration and adhesion of EPCs was mediated by inactivation of the PKC pathway and inhibition of the production of intracellular oxidative products from NADPH oxidase. We further confirmed the mechanism via knockdown of p67phox and Nox2. The findings of the present study indicate the potential therapeutic role of PIO in EPC dysfunction under HHcy stimulation and present a possible EPC-based cytotherapy for patients with ischemic vascular diseases.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JZ and SX conceived and designed the study. JZ, MW and QL performed the experiments. JZ and YZ collected the data and wrote the paper. LY and YZ analyzed the data, and LY interpreted the data. SX reviewed and edited the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Written informed consent was obtained from all participants. The present study was approved by the Ethics Review Board of Sir Run Run Shaw Hospital, College of Medicine, Zhejiang University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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