Angiogenic Function of Prostacyclin Biosynthesis in Human Endothelial Progenitor Cells

Tongrong He, Tong Lu, Livius V. d’Uscio, Chen-Fuh Lam, Hon-Chi Lee, Zvonimir S. Katusic

Abstract—The role of prostaglandin production in the control of regenerative function of endothelial progenitor cells (EPCs) has not been studied. We hypothesized that activation of cyclooxygenase (COX) enzymatic activity and the subsequent production of prostacyclin (PGI2) is an important mechanism responsible for the regenerative function of EPCs. In the present study, we detected high levels of COX-1 protein expression and PGI2 biosynthesis in human EPCs outgrown from blood mononuclear cells. Expression of COX-2 protein was almost undetectable under basal conditions but significantly elevated after treatment with tumor necrosis factor-α. Condition medium derived from EPCs hyperpolarized human coronary artery smooth muscle cells, similar to the effect of the PGI2 analog iloprost. The proliferation and in vitro tube formation by EPCs were inhibited by the COX inhibitor indomethacin or by genetic inactivation of COX-1 or PGI2 synthase with small interfering (si)RNA. Impaired tube formation and cell proliferation induced by inactivation of COX-1 were rescued by the treatment with iloprost or the selective peroxisome proliferator–activated receptor (PPAR)δ agonist GW501516 but not by the selective PGI2 receptor agonist cicaprost. Downregulation of PPARδ by siRNA also reduced angiogenic capacity of EPCs. Iloprost failed to reverse PPARδ siRNA-induced impairment of angiogenesis. Furthermore, transfection of PGI2 synthase siRNA, COX-1 siRNA, or PPARδ siRNA into EPCs decreased the capillary formation in vivo after transplantation of human EPCs into the nude mice. These results suggest that activation of COX-1/PGI2/PPARδ pathway is an important mechanism underlying proangiogenic function of EPCs. (Circ Res. 2008;102:80-88.)

Key Words: adult stem cells ▪ angiogenesis ▪ prostaglandins ▪ peroxisome proliferator–activated receptor ▪ cyclooxygenase

Evidence continues to accumulate on the existence of circulating endothelial progenitor cells (EPCs) capable of stimulating angiogenesis and repair of injured endothelium.1-11 However, the mechanisms underlying the reported therapeutic effects of EPCs are poorly understood, thus limiting successful translation of EPC-based therapies into the clinical arena. Arachidonic acid metabolism via cyclooxygenase (COX)-1 and/or COX-2 in mature endothelium is of major importance in cardiovascular homeostasis.12,13 Prostacyclin (PGI2) is a key vasoactive substance released from the endothelium after activation of COX(s) by chemical or physical stimuli.12,13 Most importantly, PGI2 is known to have a wide range of vasoprotective and therapeutic effects.14 Recently, it has been recognized that PGI2 also has stimulatory effects on angiogenesis.15-19 Despite the fact that a substantial amount of literature is available on functional and therapeutic significance of COX(s) and PGI2 in the vasculature, the role of arachidonic acid metabolism in the regenerative function of EPCs has not been examined. In the present study, we hypothesized that activation of COX isoforms and high production of PGI2 are important mechanisms responsible for the regenerative function of EPCs. We provide compelling evidence that the proangiogenic effects of human EPCs are in part dependent on the biosynthesis and release of PGI2, and subsequent activation of peroxisome proliferator–activated receptor (PPAR)δ.

Materials and Methods
An expanded Materials and Methods section is available in online data supplement at http://circres.ahajournals.org.

Isolation, Culturing, and Phenotyping of EPCs
The protocol for collection and use of human blood samples was approved by the Institutional Review Board at the Mayo Clinic. EPCs (late outgrowth) were outgrown 2 to 3 weeks after culturing of mononuclear cells isolated from the peripheral blood of 15 healthy male subjects (45±4 years old) as previously described.9 Both EPCs and human coronary artery endothelial cells (CAECs) (Clonetics; from 4 male donors [28±2 years old]) were cultured in endothelial growth medium-2 (EGM-2) (Clonetics). Human coronary artery smooth muscle cells (CSMCs) (Clonetics) were cultured in SmGM-2 SingleQuots (Clonetics). All experiments were performed using cells cultured from passages 4 to 8.

Morphological appearance and fluorescence-activated cell sorting were used to define endothelial cell phenotype of EPCs as previously described.14

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NO Synthase Enzyme Activity
The total (including calcium-dependent and -independent) NO synthase (NOS) enzyme activity of EPCs and CAECs was determined by measuring l-citrulline synthesis from l-arginine, as previously described.20

Prostaglandins and Thromboxane Measurement
Subconfluent cells were incubated in EBM-2 (8 mL/100 mm dish) for 24 hours. The supernatant (conditioned medium [CM]) was collected and immediately mixed with 40 μL of 0.2 mol/L EDTA/ PBS and stored at −80°C. Prostaglandin E2 (PGE2) and 6-keto prostaglandin F1α (6-keto PGF1α), the degradation product of PGI2, and thromboxane B2 (TXB2, a breakdown product of TXA2) were assayed using ELISA kits (Cayman Chemical Co).21

Western Blot Analysis
Western blotting was performed as previously described.22 Goat anti-COX-1, rabbit anti-PGI2 synthase (PGIS), rabbit anti-PPARγ, and goat anti-actin antibodies were obtained from Santa Cruz Biotechnology. Rabbit anti-COX-2, PGH-PGE isomerase (PGEI), and TXA2 synthase (TXAS) antibodies were purchased from R&D Systems. Mouse anti-endothelial (e)NOS and rabbit anti-inducible NOS antibodies were purchased from BD Transduction Laboratory. Protein expression was normalized to actin.

Recording of Smooth Muscle Cell Membrane Potentials
Membrane potentials were recorded continuously at room temperature (22°C) on cultured CSMCs using patch clamp techniques as previously described.23

PGIS and COX-1 Knockdown by Small Interfering RNA
Small interfering (si)RNA against human PGIS, COX-1, or PPARγ (PGIS siRNA, COX-1 siRNA, or PPARγ siRNA, respectively), and control (Ct) siRNA were obtained from Santa Cruz Biotechnology. The target sequences are listed in the online data supplement. EPCs at 50% confluence were transfected with 30 nmol/L (optimized concentration) PGIS siRNA or COX-1 siRNA (using 30 nmol/L Ct siRNA as a control) or 100 nmol/L PPARγ siRNA (using 100 nmol/L Ct siRNA as a control), by use of Lipofectamine 2000 (Invitrogen) in serum-free medium (EBM-2), according to the protocol of the manufacturer. Fresh EGM-2 was added 6.5 hours after transfection, and the cells were analyzed 48 hours after transfection.

In Vitro Tube Formation Assay
Endothelial tube formation was assessed using Matrigel assay (BD Biosciences) as described.9

Bromo-2’-Deoxyuridine Incorporation Assay
After EPCs were transfected with PGIS siRNA, COX-1 siRNA, PPARγ siRNA, or Ct siRNA, they were subjected to bromo-2’-deoxyuridine (BrdU) incorporation assay.

Transplantation of EPCs and In Vivo Capillary Assay
All of the experimental protocols were approved by the Institutional Animal Care and Use Committee of the Mayo Clinic. EPCs were transfected with PGIS siRNA, COX-1 siRNA, PPARγ siRNA, or Ct siRNA for 48 hours. Cells (5×10^5) were mixed with 200 μL Matrigel, 30 μL EGM-2, and 20 μL FCS and were then subcutaneously injected into the flanks of anesthetized 8 to 12 weeks old athymic nude mice (B6 Cg Foxn1, male; Jackson Laboratory [Bar Harbor, Maine]) (1 gel injection per side of flank, 2 gel injections per mouse). Two weeks later, mice were euthanized and the grafts were excised for histological evaluation.

Immunohistochemistry
Formalin-fixed, paraffin-embedded tissue sections were immunostained with mouse anti-human vascular endothelial growth factor receptor (VEGFR)-2, mouse anti-eNOS, or mouse IgG (as a control).

Statistical Analysis
Data are presented as means±SEM. Differences between mean values of multiple groups were analyzed using ANOVA followed by Tukey test (SigmaStat 2.03 for Windows). Comparison between 2 groups was made using Student t test. P<0.05 was considered statistically significant.

Results
Characterization of EPCs
Numerous studies on the morphological, functional, and biochemical characteristics of EPCs isolated from circulating blood established 2 distinct populations of cells: early EPCs and late EPCs (blood outgrowth EPCs).1,3–10 In the present study, we focused on blood outgrowth EPCs,7,9 which have been shown to accelerate angiogenesis and reendothelialization.3,5 Outgrown colonies appeared 2 weeks after culturing of mononuclear cells in EGM2 (Figure 1A). Confluent cells grew into a monolayer with cobblestone appearance (Figure 1B). Fluorescence-activated cell sorting analysis revealed that EPCs were positive for endothelial cell surface antigens (VEGFR-2, CD31, and VE-cadherin). However, they did not express the myelomonocytic cell marker CD14. Only a small portion of the population expressed the hematopoietic progenitor marker CD34 (Figure 1C).

Cultured EPCs had significantly lower total NOS enzymatic activity compared to CAECs (Figure 1D). The protein levels of eNOS were also significantly lower in EPCs (Figure 1E). Because tumor necrosis factor (TNF)-α is among the major proinflammatory cytokines released during cellular infiltration after ischemia,24 we examined the eNOS expression in response to TNF-α. Treatment with TNF-α reduced eNOS expression in both EPCs and CAECs. The effect of TNF-α was more pronounced in EPCs (Figure 1E), consistent with our previous report.9 Inducible NOS protein was undetectable in both cell types (data not shown).

Profile of Prostaglandin-Producing Enzymes and Production of Prostaglandins
In contrast to eNOS, Western blotting demonstrated that protein level of COX-1 was significantly higher in EPCs than in CAECs (Figure 2A). Moreover, the expression of TXAS protein was significantly lower in EPCs (Figure 2C), whereas CAECs and EPCs expressed similar levels of PGIS and PGEI proteins (Figure 2B and 2D). Under basal conditions, the expression of COX-2 was almost undetectable in both cell types (Figure 2E). TNF-α treatment induced COX-2 expression in both EPCs and CAECs (Figure 2E) but did not change protein levels of COX-1, PGIS, TXAS, and PGEI (data not shown).

Strikingly, under basal conditions EPCs released a 4-fold higher amount of PGI2 (determined by measuring 6-keto PGF1α) compared with that released from CAECs (Figure 3A). SC560 (0.1 μmol/L, a selective COX-1 inhibitor) or COX-1 siRNA significantly reduced PGI2 production in EPCs (Figure 3E and 3F). The production of PGI2 in EPCs...
was significantly increased by treatment with TNF-α. However, TNF-α had only a mild stimulatory effect on PGI₂ production in CAECs (Figure 3A). The productions of TXA₂ (measured as TXB₂) and PGE₂ were not significantly different between these 2 cell types under basal conditions (Figure 3B and 3C). Treatment by TNF-α increased TXA₂ and PGE₂ production in EPCs (Figure 3B and 3C). The ratio of PGI₂/TXA₂ was significantly higher in EPCs under basal conditions and in the presence of TNF-α (Figure 3D).

Hyperpolarization of Human CSMCs by CM of EPCs

To determine biological activity of prostaglandins released by EPCs, we measured the effects of EPCs CM on the membrane potentials of human CSMCs. The membrane potential at baseline (EBM-2) was $-40.8 \pm 2.9$ mV (room temperature). CM obtained from CAECs produced a mild hyperpolarization of CSMCs ($-43.1 \pm 3.5$ mV). However, CM of EPCs produced a significantly stronger hyperpolarizing effect on CSMCs ($-50.1 \pm 4.5$ mV) (Figure 4B). Incubation of EPCs with COX inhibitor indomethacin abolished the effect of CM derived from EPCs on membrane potential (Figure 4), suggesting that these effects were mediated by the products of arachidonic acid metabolism via COX pathway. The hyperpolarization of CSMCs by CM of EPCs was blocked by 0.1 μmol/L iberiotoxin (a selective BK channel blocker; data not shown), suggesting that BK channels are involved in the hyperpolarization of the membrane potential. The effect of CM of EPCs was mimicked by 1 μmol/L iloprost (a stable PGI₂ analog), which hyperpolarized membrane potential from $-39.3 \pm 6.2$ mV at baseline to $-50.4 \pm 5.7$ mV (n=3, P<0.05), demonstrating that activation of PGI₂ receptors was indeed coupled with hyperpolarization. These results were consistent with previous observation that iloprost activated BK channel in rat CSMCs. Because PGI₂ was the most abundant prostaglandin present in the CM of EPCs (Figure 3), our findings suggested that the hyperpolarizing effect of CM is most likely mediated by PGI₂.

Cell Proliferation

In the next series of experiments, we examined the role of PGI₂ in the regenerative function of EPCs. EPCs proliferation was inhibited by 10 μmol/L indomethacin (Figure 5A), and this effect was reversed by iloprost (3 μmol/L). COX-1 inhibitor SC560 (1 or 5 μmol/L) inhibited EPCs growth in a concentration-dependent manner, whereas the same concentrations of COX-2 inhibitor NS398 had no significant effect on EPCs proliferation (Figure 5B). Most importantly, PGIS siRNA or COX-1 siRNA also significantly decreased EPC growth and BrdU incorporation (Figure 6A through 6E). Iloprost rescued the decreased cell proliferation induced by PGIS siRNA or COX-1 siRNA (Figure 6C through 6F). Because iloprost and PGI₂ activate PGI₂ receptor and PPARs, we further investigated the mechanisms of PGI₂-dependent mitogenesis. It has been shown that PPARδ mediates PGI₂-induced angiogenesis and endothelial survival. Therefore, we examined the role of PPARδ in the PGI₂-mediated angiogenesis in EPCs. COX-1 siRNA transfected EPCs were treated with 3 μmol/L GW501516 (a selective PPARδ agonist) or 1 μmol/L cicaprost (a selective PGI₂ receptor agonist with almost no binding activity for PPARs). GW501516 and iloprost rescued cell proliferation in EPCs transfected with COX-1 siRNA, whereas cicaprost did not have any effect (Figure 6D and 6F).
both cicaprost (1 μmol/L) and iloprost (3 μmol/L) significantly increased cAMP in the EPCs by 2.3 ± 0.8- and 2.7 ± 0.5-fold, respectively (data not shown), demonstrating a similar stimulating effect of these compounds on PGI2 receptor. Thus, these results point to an essential role of PPAR in the PGI2-dependent and iloprost-induced mitogenesis. Furthermore, downregulation of PPARδ siRNA decreased cell proliferation (Figure 6G and 6H). The rescue effect of iloprost was abolished in the cells treated with PPARδ siRNA (Figure 6H), thereby confirming that PPARδ is the major PPAR isoform responsible for the mitogenic effect of PGI2 and iloprost.

In Vitro Angiogenesis

The in vitro tube formation by EPCs was reduced by indomethacin. This inhibitory effect was reversed by 1 μmol/L iloprost (Figure 7A and 7B). Genetic inactivation of PGIS and COX-1 by siRNA also significantly impaired the tube formation by EPCs (Figure 7C and 7D). Iloprost and GW501516, but not cicaprost, reduced the impairment of in vitro angiogenesis induced by COX-1 siRNA (Figure 7D). Furthermore, genetic inactivation of PPARδ caused a decrease in the tube formation by EPCs, which was not reversed by iloprost or GW501516 (Figure 7E). These results indicated that angiogenic effect of PGI2 and iloprost is primarily mediated by activation of PPARδ.

In Vivo Angiogenesis

To further examine the role of PGI2 in the angiogenic capacity of EPCs, we transplanted EPCs treated with COX-1 siRNA, PGIS siRNA, or Ct siRNA, into the nude mice. Two weeks later, capillaries positive for human VEGFR-2 were formed in the gel plugs, many of them containing red blood cells (Figure 8A through 8D). Transfection of PGIS siRNA or COX-1 siRNA significantly reduced the in vivo capillary formation in these preparations (Figure 8C through 8E). Downregulation of PPARδ expression also impaired the in vivo angiogenesis (Figure 8F), supporting the concept that COX-1/PGI2/PPARδ pathway in human EPCs plays an important role in angiogenesis.

Discussion

The results of the present study demonstrate that the proangiogenic function of human EPCs is critically dependent on arachidonic acid metabolism and biosynthesis of PGI2. We report several novel findings: (1) EPCs release high levels of PGI2, and this is associated with intrinsically high levels of COX-1 expression; (2) production of TXA2 and PGE2 is low and similar between EPCs and CAECs; (3) the in vitro and in vivo angiogenic capacity of EPCs is dependent on the endogenous production of PGI2 in EPCs; (4) the PGI2-dependent angiogenic function of EPCs is mediated by activation of PPARδ rather than PGI2 receptor. These results suggest that COX-1/PGI2/PPARδ pathway in human EPCs plays an important role in angiogenesis.
cells. Hyperpolarization of membrane potential is among the key mechanisms that produce smooth muscle relaxation. Our results thus suggest that EPCs may cause vasodilation and increase local blood flow by paracrine-induced hyperpolarization.

Several lines of evidence suggest that under our experimental conditions, the majority of PGI₂ was generated by activation of COX-1: (1) under basal conditions, EPCs expressed high levels of COX-1, whereas COX-2 protein was almost undetectable; (2) inhibition of COX-1 reduced EPCs proliferation, whereas COX-2 inhibitor did not affect cell

Figure 3. Production of prostanoids in EPCs and CAECs. Cells were cultured in EBM-2 (A through D) in the presence or absence of 0.5 ng/mL TNF-α for 24 hours. CMs were collected and assayed for 6-keto PGF₁α (A), TXB₂ (B), and PGE₂ (C) (n=3). *P<0.05. D, Ratio of 6-keto PGF₁α/TXB₂ under basal EBM-2 and TNF-α conditions (n=3). *P<0.05. E, EPCs were incubated with EBM-2 (control) or EBM-2 plus 0.1 μmol/L SC-560 for 24 hours. CMs were collected for measuring of 6-keto PGF₁α. Data are present as percentages of control (n=5). *P<0.05. F, EPCs were transfected with COX-1 siRNA or Ct siRNA for 48 hours. Cells were then incubated with EGM-2 (2 mL/60-mm dish) for 2 hours. The supernatants were collected for measurement of 6-keto PGF₁α (n=5). *P<0.05. Open columns represent EPCs; hatched columns, CAECs.

Figure 4. Effects of CM of EPCs and CAECs on the resting membrane potentials of CSMCs. A, A representative tracing of membrane potentials was recorded continuously from CSMCs showing the effects of CMs derived from CAECs, EPCs, or EPCs plus indomethacin (Indo) (10 μmol/L). B, Change in membrane potential (ΔmV) after treatment with CMs derived from CAECs, EPCs, or EPCs plus Indo is shown in the bar graph. Treatment with CM of EPCs significantly hyperpolarized the membrane potential by 9.29±1.95 mV, which was reversed in the presence of 10 μmol/L Indo (n=5 to 6). *P<0.05 compared with EBM-2, CM-CAECs, or CM-EPCs plus Indo.

Figure 5. Role of COX-1 in EPC proliferation. A, EPCs (20 000/well of 24-well plate, seeded in triplicate) were cultured in EGM-2 in the absence (control) or presence of indomethacin (Indo) or Indo plus iloprost (ilopr) for 3 days. The number of attached cells in each well was counted in a hemocytometer (n=7). *P<0.05 compared with the other 2 groups; **P<0.05 compared with control. B, EPCs were treated with the indicated concentrations of SC560 or NS398 for 3 days (n=3). *P<0.05 compared with EGM-2 alone (control); **P<0.05 compared with other 4 groups.
Figure 6. Role of PPARδ in PGI₂-mediated EPC proliferation. EPCs were transfected with 30 nmol/L Ct siRNA or PGIS siRNA (A) or COX-1 siRNA (B) for 48 hours. Protein samples were collected for Western blotting (n=10 [A], *P<0.05 compared with Ct siRNA; n=3 [B], *P<0.05 compared with Ct siRNA). C, After EPCs were treated with PGIS siRNA or Ct siRNA for 24 hours, cells were seeded on 24-well plates (20 000/well, in triplicate) and cultured in EGM-2 in the absence or presence of 3 μmol/L iloprost for 48 hours. Number of attached cells was counted (n=4 to 6). **P<0.05 compared with 2 other groups; *P<0.05 compared with Ct siRNA. D, After EPCs were transfected with COX-1 siRNA or Ct siRNA for 24 hours, cells were seeded in 24-well plates and treated with 3 μmol/L iloprost or GW501516 or 1 μmol/L cicaprost for 48 hours (n=5 to 12). *P<0.05 compared with COX-1 siRNA, COX-1 siRNA+cicaprost, or Ct siRNA; **P<0.05 compared with COX-1 siRNA or Ct siRNA for 48 hours, then seeded on 96-well plates and assayed for BrdU incorporation (n=3). *P<0.05 compared with control (EGM-2 alone) or Ct siRNA. E, EPCs were transfected with PGIS siRNA, COX-1 siRNA, or Ct siRNA for 48 hours, then seeded on 96-well plates and assayed for BrdU incorporation (n=3). *P<0.05 compared with control (EGM-2 alone) or Ct siRNA. F, After EPCs were transfected with COX-1 siRNA for 30 hours, cells were seeded on 96-well plates and incubated in the absence (EGM-2 alone) or presence of indicated treatments for 14 hours. Cells were then labeled with BrdU in the same incubations as before for 24 hours (n=5). *P<0.05 compared with Ct siRNA in EGM-2 alone or COX-1 siRNA+cicaprost. G, EPCs were transfected with 100 nmol/L PPARδ siRNA or Ct siRNA for 48, and protein samples were assayed for Western blotting. Quantification of 4 independent experiments is presented under the representative blot. *P<0.05 compared with Ct siRNA. H, EPCs were transfected with PPARδ siRNA or Ct siRNA for 30 hours; cells were then seeded in 96-well plates and cultured in the absence (EGM-2 alone) or presence of 3 μmol/L iloprost for 14 hours. Cells were then labeled with BrdU in the same incubations as before for 24 hours (n=5). *P<0.05 compared with Ct siRNA.
proliferation; (3) the inhibitory effect of PGIS siRNA on angiogenic response was not statistically different from the inhibition of angiogenesis induced by siRNA designed to inactivate COX-1; and (4) production of PGI2 was significantly reduced by a COX-1 selective inhibitor, SC560, or COX-1 siRNA. However, we wish to point out that at the present time, the relative degree of COX-1/PGI2 pathway contribution (as compared with COX-2 or other paracrine mechanisms) to angiogenic function of EPCs is difficult to determine. Based on our findings with selective pharmacological or genetic inhibition of COX-1 or PGIS, it appears likely that mechanisms other than COX-1/PGI2 pathway are also contributors to the ability of EPCs to stimulate angiogenesis.

There are 2 major signaling pathways responsible for the vascular effects of PGI2. The classic PGI2 signaling is mediated via a G protein–coupled cell membrane receptor, leading to an activation of adenylyl cyclase and an increase in cAMP. PGI2 may also stimulate angiogenesis by activation of PPARs. Recent studies suggest that activation of PPARδ by PGI2 is responsible for regulation of angiogenesis and apoptosis in endothelial cells. The colocalization of COX/PGIS at the nuclear membrane is consistent with the ability of endogenous PGI2 to activate nuclear receptors.
We also detected the perinuclear distribution of PGIS in EPCs (T.H. and Z.S.K., unpublished observation, 2006). In the present study, we found that the impairment of angiogenesis by inactivation of COX-1 was reversed by a selective agonist of PPARδ, GW501516. This effect was similar to the effect of iloprost, suggesting that activation of PPARδ is a major mechanism underlying the effect of iloprost. In contrast, cicaprost (a PGI₂ analog that does not activate PPARδ) did not rescue the COX-1 siRNA phenotype, supporting the concept that PGI₂ receptor plays a minor role in PGI₂-induced angiogenesis. Furthermore, downregulation of PPARδ also inhibited angiogenic function of EPCs. Iloprost failed to rescue the impairment of angiogenesis induced by PPARδ siRNA, strongly suggesting that PPARδ is the major mediator responsible for PGI₂-dependent angiogenesis. GW501516 also failed to correct the inhibitory effect of PPARδ siRNA, confirming the effectiveness of this siRNA. Most importantly, the results of in vivo experiments reinforced our conclusion that COX-1/PGI₂/PPARδ pathway is an important signaling mechanism in the angiogenic function of EPCs.

Interaction between transplanted human EPCs and endogenous mouse endothelium has not been fully characterized in our in vivo experiments. Two mechanisms may account for the reduced angiogenesis by genetically manipulated human EPCs, impaired angiogenic capacity of transplanted EPCs per se, and/or reduced ability of EPCs to stimulate angiogenic function of existing mouse endothelium. The present literature suggests that EPCs produce and release well established angiogenic molecules including VEGF, thereby supporting the concept that paracrine stimulation of existing endothelium is an important mechanism of EPC-induced angiogenesis. However, with regard to in vivo angiogenic effect of human EPCs, our results do not allow any conclusion regarding the relative contribution of transplanted EPCs per se, versus angiogenic stimulation of existing mouse endothelium by paracrine effects of EPCs.

TNF-α is among the most important proinflammatory cytokines present in the ischemic tissues. TFN-α treatment increases PGIS expression in bovine endothelial cells. In the present study, however, we did not detect the induction of PGIS by TNF-α in human EPCs or human CAECs. The reason for this discrepancy between bovine and human endothelial cells is not immediately apparent but could be attributable to the species differences. Interestingly, TNF-α-stimulated COX-2 expression in both EPCs and CAECs by a similar magnitude, however, TNF-α had significantly more pronounced stimulatory effects on PGIS production in EPCs. It is, therefore, likely that COX-2 activity is an important component of angiogenic activity of EPCs exposed to proinflammatory environment. The exact mechanisms underlying the high production of PGIS in EPCs activated by TNF-α are unclear but could be explained by an elevated expression of TNF-α receptors, increased mobilization of arachidonic acid, or high antioxidant capacity of EPCs protecting PGIS from inactivation by peroxynitrite. Indeed, previous studies demonstrated that human EPCs had a high level of manganese superoxide dismutase expression and low intracellular concentration of reactive oxygen species. These findings coupled with detected low enzymatic activity of NOS suggest that EPCs may be able to minimize production of peroxynitrite, which is generated by a chemical reaction between superoxide anion and NO in ischemic tissue. This could enhance the ability of EPCs to robustly increase PGI₂ production in response to TNF-α, thus securing preservation of strong vascular protective and proangiogenic effects of EPCs under the conditions of oxidative stress.

This study is the first to demonstrate the importance of arachidonic acid metabolism and biosynthesis of PGI₂ in the mediation of proangiogenic and vasodilator effect of human EPCs. The results provide a novel insight into the mechanism of PGI₂-dependent angiogenesis in EPCs. Our observations suggest that adverse cardiovascular effects of COX(s) inhibitors may involve interference of these compounds with regenerative program of EPCs.

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Disclosures

None.

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Angiogenic function of prostacyclin biosynthesis in human endothelial progenitor cells

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Expanded Materials and Methods

Isolation, Culturing, and Phenotyping of EPCs:

The protocol for collection and use of human blood samples was approved by the Institutional Review Board at the Mayo Clinic. EPCs (late outgrowth) were outgrown 2-3 weeks after culturing of mononuclear cells isolated from the peripheral blood of 15 healthy male subjects (45±4 years old) as previously described (1). Cells were cultured in endothelial growth medium-2 (EGM-2, Clonetics), which was composed of endothelial cell basal medium-2 (EBM-2, a serum- and growth factor-free medium), 2% fetal bovine serum (FCS), fibroblast growth factor, vascular endothelial growth factor (VEGF), insulin-like growth factor, epidermal growth factor, ascorbic acid, hydrocortisone, and heparin. In parallel, human coronary artery endothelial cells (CAECs; Clonetics) from 4 male donors (28±2 years old) were cultured in EGM-2. Human coronary artery smooth muscle cells (CSMCs, Clonetics) were cultured in SmGM-2 SingleQuots (Clonetics). All experiments were performed using cells cultured from passages 4 to 8.

For FACS analysis, collected cells were labeled with PE-mouse anti-human CD34 or CD14 (BD Pharmigen, dilution 1: 67) on ice for 1 h. An isotype-matched mouse IgG was used as negative control. For indirect staining, cells were incubated with primary antibodies [mouse anti-human CD31 (DAKO), vascular endothelial growth factor receptor-2 (VEGFR-2, Sigma), VE-Cadherin (VE-Cad, Santa Cruz) or mouse IgG (control, Sigma)] at a dilution of 1:67, on ice for 1 h, and washed with 1% bovine albumin/PBS once. Cells were then incubated with Alexa
Fluor 488-conjugated anti-mouse IgG (dilution 1:100) on ice for 1 h, washed with 1% bovine albumin/ PBS, and subjected to FACS analysis. The fluorescence intensity of stained cells was gated according to established methods (2).

**NOS Enzyme Activity:**

Cells were harvested in lysis buffer (pH 7.5) containing 50 mmol/L Tris-HCl, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 0.1% SDS, 0.1% deoxycholate, 1% IGEPAL, and mammalian protease inhibitor cocktail (all from Sigma). Cell lysates were sonicated 30 seconds, and centrifuged to remove insoluble matter. Total protein was determined using the BioRad DC Protein assay kit (BioRad, Hercules, CA). L-[14C]-Citrulline formation was measured. Fifty µg protein was added to reaction cocktails containing 1 mmol/L NADPH, 100 nmol/L calmodulin, 30 µM BH4, 2.5 mmol/L CaCl2 and 40 µM L-[U-14C]arginine (348 mCi/mmol, Amersham Life Science). To determine NOS activity, samples were incubated for 20 min in the presence and absence of 1 mM NG-nitro-L-arginine methyl ester. The reaction was terminated by the addition of 1 ml of cold stop buffer (20 mM HEPES, 2 mM EDTA, and 2 mM EGTA, pH 5.5), and the reaction mix was applied to a Dowex AG 50WX-8 resin column. Radiolabeled counts per minute of L-citrulline generation were measured and used to determine L-NAME-inhibited NOS activity.

**Recording of Smooth Muscle Cell Membrane Potentials:**

Membrane potentials were recorded continuously at room temperature (22 °C) on cultured CSMCs using patch clamp techniques. After baseline measurements were obtained in EBM-2, the medium was replaced by CM added directly into the recording chamber (1 ml volume) with an exchange time of 4~5 s. The pipette solution contained (in mmol/L): KCl 140, MgCl₂ 0.5, Na₂ATP 5, Na₂GTP 0.5, HEPES 10, EGTA 1, and CaCl₂ 0.465 (~200 nmol/L free Ca²⁺). For
preparation of CM, cells \((10^6/100\,\text{mm dish})\) were incubated in EBM-2 for 12 h, and then treated with 5 ml of EBM-2 in presence or absence of 10 \(\mu\text{mol/L}\) indomethacin for 1 h. CM was collected and immediately added to the recording chamber. Membrane potentials recorded with EBM-2 containing 1 \(\mu\text{mol/L}\) iloprost (Cayman Chemical Co.) served as positive controls.

**PGIS and COX-1 Knockdown by Small Interfering RNA (siRNA):**

All siRNAs were obtained from Santa Cruz Biotechnology, Inc. PGI\(_2\) Synthase siRNA(h) (PGIS-siRNA), a pool of 3 target-specific siRNA duplex oligonucleotides against human PGIS was used for PGIS knockdown. The target sequences for the PGIS-siRNA were bases 1294-1312 of NM_000961 (5’GGATCAGAGAAGAAGACT3’), bases 1839-1857 of NM_000961 (5’CCACAAATGCTATTCAGAT3’), and bases 2334-2352 of NM_000961 (5’GACTCCTTAGACTGATCAA3’). siRNA against human PPAR\(_\delta\) (PPAR\(_\delta\)-siRNA) is also a pool of 3 target-specific siRNA duplex oligonucleotides. The target sequences were bases 1143-1161 of NM_006238 (5’GGTTACCCTTCTCAAGTAT), bases 1277-1295 (5’CCTTCAGTGATATCATTGA), bases 2121-2139 (5’CTCCTGTCTTCAGAGCAAA). The target sequence of siRNA against human COX-1 (COX-1-siRNA) was bases 1665-1683 of NM_000962 (5’GTGCCATCCAAACTCTATC3’) (3). The sequence of the negative control siRNA duplex oligonucleotides (Ct-siRNA) was 5’ TTCTCCGAACGTGTCACGT3’. EPCs at 50% confluence were transfected with 30 nmol/L (optimized concentration) of PGIS-siRNA or COX-1-siRNA (using 30 nmol/ Ct-siRNA as a control), or 100 nmol/L of PPAR\(_\delta\)-siRNA (using 100 nmol/L of Ct-siRNA as a control), by use of Lipofectamine 2000 (Invitrogen) in serum-free medium (EBM-2), according to manufacture’s protocol. Fresh EGM-2 was added 6.5 h after transfection, and the cells were analyzed 48 h after transfection.
**In Vitro Tube Formation Assay:**

Endothelial tube formation was assessed using Matrigel™ assay (BD Biosciences). After treatment with 5 or 10 \(\mu\)mol/L indomethacin, or 10 \(\mu\)mol/L indomethacin + 1 \(\mu\)mol/L iloprost for 18 h, cells were seeded at a density of 7\(\times\)10^4/well on 24-well plates (2 wells for each condition) coated with 250 \(\mu\)l growth factor-reduced Matrigel™. Cells were incubated in EBM-2 alone (control), or in the previous treatment, for 4 h at 37\(^\circ\)C. Tube formation was examined with a phase-contrast microscope by taking pictures of 5 random chosen fields (\(\times\)10 magnification) of each well; tube circles in each picture were then counted. In some experiments, after EPCs were transfected with PGIS-siRNA, COX-1-siRNA, or PPAR\(\delta\)-siRNA for 48 h, cells were treated with iloprost, GW501516 (Alexis Biochemicals), or Cicaprost (Schering AG, Germany) for 18 h. Cells were then assayed for tube formation in the absence (EBM-2 alone) or the presence of the treatments same as before.

**Bromo-2'-deoxyuridine (BrdU) Incorporation Assay:**

Forty-eight h after transfection with PGIS-siRNA, COX-1-siRNA, or Ct-siRNA, EPCs (5,000 cells/well) were seeded on 96-well plates (experiments were performed in triplicate). Six h after seeding, EPCs were incubated with EGM-2 (100 \(\mu\)l/well) + 10\(\mu\)mol/L BrdU labeling solution (Cell Proliferation ELISA kit, Roche Applied Science) for 24 h. In some experiments, after EPCs were transfected with COX-1-siRNA or PPAR\(\delta\)-siRNA for 30 h, cells were seeded in 96-well plate for 2 h, and then treated with iloprost (Cayman Chemical), GW501516 (Alexis Biochemical), or cicaprost (generous gift of Schering AG, Germany) for 14 h, then cells were labeled with BrdU in the presence of iloprost, GW501516, or cicaprost for 24 h. Cells were fixed and exposed to anti-BrdU and substrate solutions. The reaction product was quantified by measuring absorbance with a microplate reader (Model 680XR, Bio-Rad).
Transplantation of EPCs and In Vivo Capillary Assay:

All of the experimental protocols were approved by the Institutional Animal Care and Use Committee of the Mayo Clinic. EPCs were transfected with PGIS-siRNA, COX-1-siRNA, PPARδ-siRNA, or Ct-siRNA for 48h. Cells (5×10^5) were mixed with 200 µl Matrigel, 30 µl EGM-2, and 20 µl FCS, and were then subcutaneously injected into the flanks of anesthetized 8-12 weeks old athymic nude mice [B6 Cg Foxn1, male; Jackson Laboratory (Bar Harbor, Maine)] (one gel injection per side of flank, two gel injections per mouse). Two weeks later, mice were sacrificed and the grafts were excised for histological evaluation. The gel plugs were fixed with formalin, embedded in paraffin, and cut into 4 µm sections at 100 µm intervals. All sections of each plug (one plug represented one sample) were counter-stained with hematoxylin. The capillaries in each tissue section were counted under a phase-contrast microscope (10X) in a blind fashion. The capillaries were identified by their positive immunostaining for VEGFR-2 and eNOS. The sum of capillaries in all sections from one plug was divided by the total area (mm^2, measured by using program Image J, NIH) of all sections of the plug. Data were presented as percentage of Ct-siRNA.

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue sections were deparaffinized and immersed in 1 mmol/L EDTA solution (pH 8) for 30 min at 95°C to 99°C. Slides were incubated with mouse anti–human vascular endothelial growth factor receptor-2 (VEGFR-2), mouse anti-eNOS, or mouse IgG (as a control) for 12 h at 4°C, followed by incubations with biotinlated antibody for 1 h at room temperature and with ABC reagent (Vector Laboratories) for 30 min. Slides were then developed with DAB solution (Vector Laboratories) for 1-2 min, counter-stained with hematoxylin, and analyzed by visual inspection at 40X magnification.
References:


Legends:

**Online Figure 1.** To demonstrate the time course of effect of siRNA in EPCs, we performed Western blot on samples of EPCs treated with PGIS-siRNA (A), COX-1-siRNA (B), or PPARδ-siRNA (C) for indicated periods. The inhibitory effect of siRNA lasted at least 10 days in cells cultured with EGM-2.
Online Figure I

A

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