β_2-Adrenergic Receptor Stimulation Improves Endothelial Progenitor Cell–Mediated Ischemic Neoangiogenesis

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Rationale: **Endothelial progenitor cells (EPCs) are present in the systemic circulation and home to sites of ischemic injury where they promote neoangiogenesis. β_2-Adrenergic receptor (β_2AR) plays a critical role in vascular tone regulation and neoangiogenesis.**

Objective: **We aimed to evaluate the role of β_2AR on EPCs' function.**

Methods and Results: **We firstly performed in vitro analysis showing the expression of β_2AR on EPCs. Stimulation of wild-type EPCs with β-agonist isoproterenol induced a significant increase of Flk-1 expression on EPCs as assessed by fluorescence-activated cell sorter. Moreover, β_2AR stimulation induced a significant increase of cell proliferation, improved the EPCs migratory activity, and enhanced the EPCs' ability to promote endothelial cell network formation in vitro. Then, we performed in vivo studies in animals model of hindlimb ischemia. Consistent with our in vitro results, in vivo EPCs' treatment resulted in an improvement of impaired angiogenic phenotype in β_2AR KO mice after induction of ischemia, whereas no significant amelioration was observed when β_2AR knock out (KO) EPCs were injected. Indeed, wild-type–derived EPCs' injection resulted in a significantly higher blood flow restoration in ischemic hindlimb and higher capillaries density at histological analysis as compared with not treated or β_2AR KO EPC-treated mice.**

Conclusions: **The present study provides the first evidence that EPCs express a functional β_2AR. Moreover, β_2AR stimulation results in EPCs proliferation, migration, and differentiation, enhancing their angiogenic ability, both in vitro and in vivo, leading to an improved response to ischemic injury in animal models of hindlimb ischemia. (*Circ Res*. 2013;112:1026-1034.)**

Key Words: β_2-adrenergic receptor ■ angiogenesis ■ endothelial progenitor cells

The endothelium controls several vascular functions, including vascular tone and permeability, thrombosis, hemostasis, and angiogenesis.[1] It is noteworthy that all these functions can be regulated by activation of receptors, and the same receptor can activate multiple endothelial functions.[2] The adrenergic system is the major regulator of cardiac and vascular function, and, in particular, it controls endothelial vasodilatation through β_2- and α-adrenergic receptors (ARs). In this regard, we previously demonstrated that β_2AR plays a critical role in adult ECs proliferation and function, providing evidence on the role of β_2AR in regulating neoangiogenesis in response to ischemia.[3] Recent studies supported the thesis that regeneration of the injured endothelium involves the participation of cells from adult bone marrow. Indeed, bone marrow–derived endothelial progenitor cells (EPCs) are present in the systemic circulation and they home to sites of ischemic injury where they function to promote neovascularization.[4–7] These characteristics open a large spectrum of potential therapeutic options using EPCs for repairing injured vessel wall and neovascularization of ischemic tissues. Nevertheless, these strategies seem to be limited by the extremely low number of EPCs, which represent <0.02% of circulating cell in the peripheral blood.[8] Moreover, EPCs' number and activity inversely correlate with age and cardiovascular risk factors, such as diabetes mellitus, hypercholesterolemia, hypertension, smoking habit, and family history of coronary artery disease.[9,10] Therefore, molecular approaches aimed to improve EPCs' number and activity are pivotal in the development of this novel therapeutic strategy and, as consequence, the knowledge of the molecular mechanisms that are behind EPCs' proangiogenic properties is needful. Because we demonstrated the crucial role of β_2AR to modulate proangiogenic responses of adult

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Nonstandard Abbreviations and Acronyms	
AR	adrenergic receptor
ECs	endothelial cells
EPCs	endothelial progenitor cells
FACS	fluorescence-activated cell sorters
HUVECs	human umbilical vein endothelial cells
ISO	isoproterenol
WB	Western blot

ECs, both in vivo and in vitro,[2] we hypothesized that these receptors could be relevant also to the angiogenic function of circulating EPCs. Furthermore, it can represent a potential target to enhance EPCs' ability to promote neovascularization. Therefore, we first evaluated the expression of β_2AR on EPCs, then analyzed in vitro the effect of β_2AR stimulation on EPCs' differentiation and function. Finally, we analyzed the effect of β_2AR stimulation on EPCs, performing in vivo studies in rat and mouse models of hindlimb ischemia.

Methods

Animals

Homozygous β_2AR KO male mice 5 backcrosses[11] in a C57/BL6 background used as referral strain (wild type) at 8 to 16 weeks of age[11] and Normotensive Wistar Kyoto male rats (Charles Rivers Laboratory International) at 12 weeks of age were used for this study. All animal procedures were carried out in observance with Federico II University guidelines.

EPCs' Isolation and Characterization

Circulating EPCs were harvested 7 days after surgery and cultured on fibronectin-coated plates.[8,12,13] After 7 days of culturing, attached cells were further characterized with immunostaining and investigated the ability to express endothelial markers both by immunofluorescence and fluorescence-activated cell sorter (FACS), as reported in Online Figure I. Finally, cells were tested for the ability to form tubules in vitro when cocultured with human umbilical vein ECs. Thus, cells expressing all the characteristics reported in Table 1 are defined through this article as EPCs (see OnlineData Supplement Methods for further details).

β-AR Binding Assay

Membrane fractions were used for β-AR radioligand binding assay using the nonselective β-AR antagonist [^{125}I]-Cyanopindolol (^{125}I-CYP), as previously reported[3] (see OnlineData Supplement Methods for further details).

Table 1. Endothelial Progenitor Cells' Characterization

Ac low-density lipoprotein binding	+
Ulex europaeus agglutinin 1 plant leptin binding	+
Phenotypic appearance at fluorescence-activated cell sorters:	
Vascular endothelial cadherin	+
FLK-1	+
CD34	+
CD31	+
e-NOS	+
CD45	–
CD14	–
In vitro tube formation	+

Western Immunoblot Analysis

Western blots (WB) were performed to assess the cellular expression of β_2AR and phosphorylated isophorm of protein kinase B (AKT) and retinoblastoma protein on EPCs (see OnlineData Supplement Methods for further details).

Immunofluorescence

Cells were grown in 4-chamber slides, fibronectin-coated, and immunofluorescence was performed as previously reported[14] (see OnlineData Supplement Methods for further details).

Real Time-Polymerase Chain Reaction Analysis

β_2 AR mRNA level was determined by quantitative real-time polymerase chain reaction (Step-One, Applied Biosystems, Milano, Italy; see OnlineData Supplement Methods for further details).

Flow Cytometry

FACS analysis was used to detect the cell surface expression of the endothelial and to exclude expression of leukocyte or myeloid cell surface markers (see OnlineData Supplement Methods for further details).

Vascular Endothelial Growth Factor Quantification

ELISA was performed to quantify vascular endothelial growth factor (VEGF) production in cultured medium (see OnlineData Supplement Methods for further details).

EPCs' Migration Assay

As previously described,[8] EPCs' migration assays were performed using a modified Boyden chamber assay. To further confirm the defined EPCs' phenotype, cells migrating in the lower chamber were tested by FACS for all the cell surface markers reported in Table 1 (see OnlineData Supplement Methods for further details).

Matrigel Assay

To detect vascular network formation in vitro, Matrigel assay was performed on commercially available 96-well multidishes coated with growth factor-reduced Matrigel by Biocoat Angiogenesis system for Endothelial Cell Tube Formation (Becton Dickinson) according to the manufacturer's instructions (see OnlineData Supplement Methods for further details).

Adenoviral Constructs

Adenovirus vectors encoding for the β_2AR (Adβ_2AR) or for an empty vector were a kind gift from Professor Walter J. Koch (Temple University, Philadelphia, PA).[15]

Animal Models of Ischemia

Mice or rats were subjected to unilateral hindlimb femoral artery removal[3] (see OnlineDataSupplement Methods for further details).

Blood-Flow Analysis

Blood flow was evaluated by dyed bead perfusion and digital angiography analysis (see OnlineData Supplement Methods for further details).

Histological Analysis

Capillary density within tibialis anterior muscle was used for histological study (see OnlineData Supplement Methods for further details).

Results

Expression of β_2AR on EPCs

To investigate the expression of the β_2AR on cells cultured and characterized as above described, we first performed an immunoblot analysis that revealed the baseline expression of β_2AR on EPCs. β_2AR expression on EPCs was not increased by stimulation with the β_2AR agonist isoproterenol

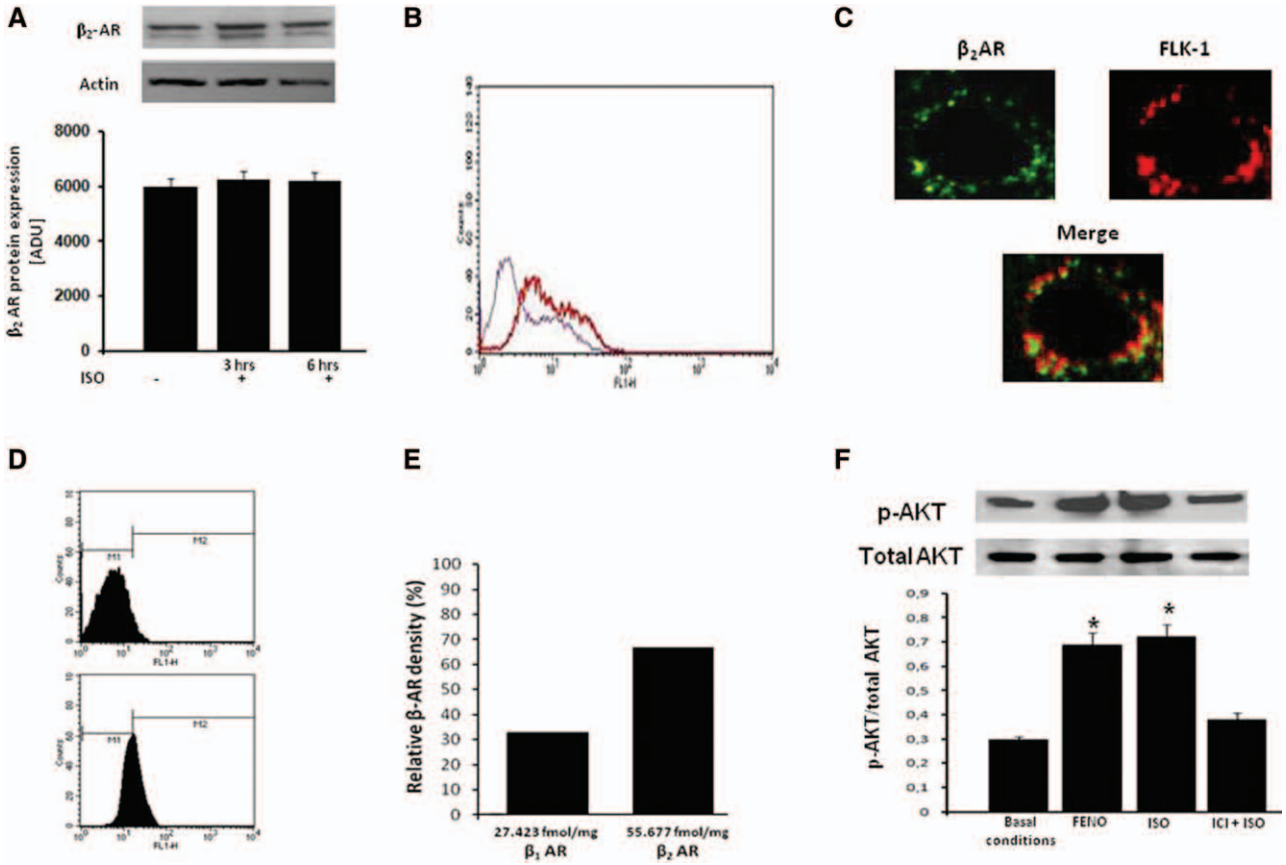

Figure 1. Expression of β_2 adrenergic receptor (β_2AR) on endothelial progenitor cells (EPCs). To investigate the expression of β_2AR, Western blot (WB) was performed on EPCs harvested from Wistar Kyoto male rats and cultured on fibronectin-coated wells. **A**, After 7 days of culturing, EPCs were stimulated with isoproterenol (ISO), a potent β receptor agonist, at different timelines, and WB analysis was performed demonstrating the expression of β_2AR on these cells. Densitometric analysis (bar graph) showed that β_2AR stimulation by ISO (10^{-8} mol/L) did not affect β_2AR expression. Equal amount of proteins were confirmed by immunoblotting for actin. Representative blots are presented in the inset. **B**, The expression of β_2AR on EPCs surface was further confirmed by fluorescence-activated cell sorters analysis. Gray line The cell basal isotype; red line cells positive for β_2AR after probing with specific β_2AR fluorescein isothiocyanate-conjugated antibody. **C**, To further investigate this issue, we performed immunocytochemistry for colocalization of β_2AR and EPCs marker Flk-1. Fluorescence microscopy revealed that EPCs express on their surface β_2AR (green fluorescence) and, as expected, Flk-1 (red fluorescence); superimposed image demonstrated the colocalization of both receptors on EPCs surface (merged image). Representative images are presented in the inset. **D**, Colocalization of β_2AR and Flk-1 was further confirmed by fluorescence-activated cell sorters analysis that showed that 60% of these cells expressed on their cells surface both markers. Flk-1-positive cells are underlying the M1 line, whereas M2 indicates cells positive for both Flk-1 and β_2AR. **E**, Finally, a β-receptor binding on EPCs was performed using the β_1AR selective blocker CGP 20712a (CGP). With this compound, we found that roughly 30% of βAR are β1, confirming the expression of βAR on these cells, with prevalence of β_2AR (70%). Value are expressed as fmol/mg on *x* axis and as relative percentages on *y* axis. **F**, To confirm that the β_2AR represents the most important subtype in response to β_2AR stimulation in EPCs, we observed AKT activation by WB before and after stimulation with ISO and we found that ISO treatment led to an increased activity of β_2AR target AKT, with a significant increase of p-AKT/total AKT ratio. Noteworthy, the stimulation with the more selective β_2AR agonist fenoterol (FENO) led to very similar increase of p-AKT as compared with ISO. Reciprocally, ISO stimulation of EPCs after pretreatment with of β_2AR-blocker ICI prevents AKT activation. The quantification of p-AKT/total AKT ratio is reported in densitometric analysis (bar graph). *P<0.05; n=3 in duplicate.

(ISO; Figure 1A). Equal amount of proteins were confirmed by immunoblot for actin. The expression of β_2AR on EPCs was further confirmed by FACS analysis (Figure 2B). The cell surface expression of the ECs' antigen Flk-1 (VEGF-receptor 2) is a typical feature of ECs and is a key for angiogenesis. Thus, we performed an immunocytochemistry analysis to demonstrate the colocalization of β_2AR and FLK-1 on EPCs' surface. As depicted in the Figure 1C, fluorescence microscopy confirmed that EPCs express on their surface β_2AR and, as expected, FLK-1. The colocalization of β_2AR with the panoply cell surface marker used to describe EPCs was further confirmed by FACS analysis, as indicated in Figure 1D. Finally, an EPC I-CYP ligand competition curve with the highly selective β1-CGP 20712a was conducted to quantify the percentage of β_2AR on these cells. As reported in the Figure 1E, using this compound we found that, on these cells, ≈70% of β-AR are β_2. The Scatchard plot of the I-CYP ligand binding data is reported in the Online Figure II. To confirm that the β_2ARs represent the most important subtype in the response to β_2AR stimulation in EPCs, we observed AKT activation by WB before and after stimulation with ISO. Cells were cultured for 7 days on fibronectin-coated wells and then stimulated with ISO, at constant concentration of 10^{-8} mol/L. WB revealed that ISO treatment led to an increased activity of β_2AR target AKT, with a significant increase of p-AKT/total AKT ratio, as previously

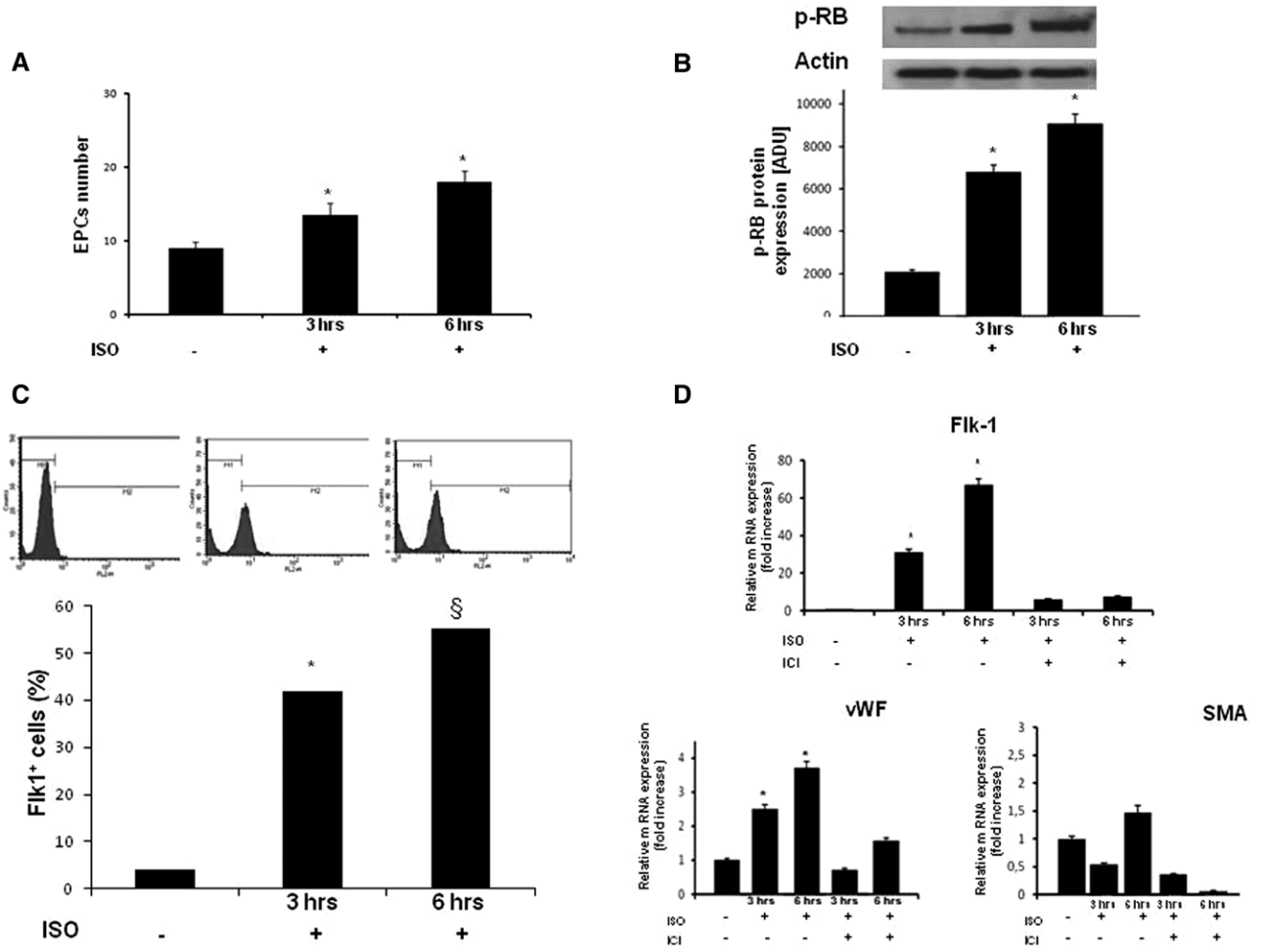

Figure 2. Effect of β_2AR on endothelial progenitor cells (EPCs). After demonstrating that EPCs express a functional β_2AR, we investigated the role of this receptor on EPCs proliferation and differentiation. **A**, EPCs' number was significantly increased by treatment with isoproterenol (ISO) after both 3 and 6 h of stimulation, with a maximal effect at 6 h. **B**, To explore the effect of ISO stimulation on EPCs' number, we investigated the expression of phosphorylated form of retinoblastoma protein (p-RB), a marker of cell proliferation, in these cells. The western blot revealed a significant increase of p-RB in EPCs when these cells were stimulated with ISO both at 3 and at 6 h as compared with basal conditions, indicating a significant increase of proliferation rate in EPCs. **C**, Then, to explore the effect of β_2AR stimulation on EPCs differentiation, we investigated Flk-1 expression on cells, cultured as above described, performing a fluorescence-activated cell sorter analysis before and after ISO stimulation. We found that ISO stimulation led to a significant increase in the expression of Flk-1, both at 3 and at 6 h, compared with the absence of stimulation, suggesting that β_2AR stimulation acts promoting EPCs differentiation. Quantitative analysis of Flk-1 expression assessed by fluorescence-activated cell sorter is showed in bar graph. **D**, Finally, to further investigate our findings, we investigated the mRNA levels of endothelial markers Flk-1 and von Willebrand factor (vWF), and of smooth muscle actin (SMA), a smooth muscle cell marker identified as indicator of endothelial-smooth muscle transition in cultured endothelial cells. reverse transcriptase-polymerase chain reaction showed significant increase of endothelial markers FLK-1 and von Willebrand factor after stimulation with ISO, at both 3 and 6 h, whereas no significant difference in expression levels of these markers was noted when cells were pretreated with ICI before being stimulated with ISO, further confirming that β_2AR signaling is involved in the differentiation of EPCs. No significant change in smooth muscle actin levels was noted between basal conditions and after the different stimulations, suggesting that this marker is independent of β_2AR. **P*<0.05 vs absence of ISO; §*P*<0.01 vs absence of ISO; n=3 in duplicate.

reported in the adult ECs. Noteworthy, the stimulation with the more selective β_2AR agonist Fenoterol led to very similar increase of p-AKT as compared with ISO. Reciprocally, ISO stimulation of EPCs after pretreatment with of β_2AR-blocker ICI 118 551 (ICI) prevents AKT activation (Figure 1F). Finally, as reported in the Online Figure III, ISO stimulation of Adβ_2AR EPC results in a further increase of downstream signaling, as demonstrated by a significant increase of p-AKT, compared with the ISO-stimulated EPCs. Taken together, these data suggest that EPCs express functionally relevant β_2AR.

Effects of β_2AR Stimulation on EPCs' Proliferation and Differentiation In Vitro

Having demonstrated that EPCs express a functional β_2AR, we investigated the role of this receptor on EPCs' proliferation and differentiation. After completion of 7-day culturing on fibronectin-coated wells, cells were stimulated with ISO 10^{-8} mol/L for 3 or 6 hours. As reported in Figure 2A, the number of EPCs, as determined by DiI-acetylated low-density lipoprotein/lectin double-positive cells, was significantly increased by ISO stimulation both at 3 and at 6 hours as compared with cells cultured in the absence of ISO. These data

were further confirmed by FACS analysis for all markers used to describe EPCs (data not showed). To explore the cause of increased EPCs' number after stimulation of β_2AR, we performed a WB on phosphorylated form of retinoblastoma protein, a marker of cell proliferation, that showed a significant increase of this protein after ISO stimulation (Figure 2B), indicating a significant increase of proliferation rate in EPCs. Then, to explore the effect of β_2AR stimulation on differentiation, we investigated Flk-1 expression on cells, cultured as described above, performing a FACS analysis before and after ISO stimulation. We found that ISO stimulation led to a significant increase in the expression of this EC maker protein, both at 3 and at 6 hours (Figure 2C), compared with basal conditions, suggesting that β_2AR stimulation acts on the EPCs' fraction of circulating mononuclear cells to promote differentiation. Moreover, ISO stimulation of Adβ_2AR EPCs results in further increase of both p-retinoblastoma protein and Flk-1 expression on these cells, as reported in the Online Figure IIIB and IIIC. To corroborate our findings, we performed a reverse transcriptase-polymerase chain reaction for Flk-1 and von Willebrand factor, as endothelial markers, and for smooth muscle actin, a smooth muscle cell marker identified as indicator of endothelial-smooth muscle transition in cultured ECs.[16] As reported in Figure 2D, we found a significant increase of endothelial markers FLK-1 and von Willebrand factor after stimulation with ISO, whereas no significant difference in expression levels of these markers was noted when cells were pretreated with ICI before being stimulated with ISO. Taken together, these data suggest that β_2AR signaling contributes to the differentiation of EPCs. Finally, no change in mRNA levels for smooth muscle actin was observed after ISO stimulation or when EPCs where treated with ISO and ICI, suggesting that this marker was independent from β_2AR.

Effects of β_2AR Stimulation on the Angiogenesis In Vitro

Cellular migration is required for vascular network formation. Therefore, we examined the effect of β_2AR stimulation with ISO on unidirectional migration of DiI-acetylated low-density lipoprotein–labeled EPCs using a modified Boyden chamber assay. As shown in Figure 3A, ISO stimulation of EPCs led to a significant increase in migratory activity compared with control. EPCs exhibit the ability to promote the formation of vascular networks in vitro.[9] Coculture of human umbilical vein endothelial cells and DiI-Ac–labeled EPCs on a Matrigel matrix revealed that ISO treatment promoted EPCs incorporation into network structures in a dose–response manner. As reported in Figure 3B through 3D both EPCs' incorporation into the network formations and the total number of tubules per microscopic field were significantly higher when EPCs were stimulated with ISO. To gain more insight on the effect of β_2AR on EPCs angiogenic properties, we performed migration and vascular formation assays using EPCs harvested from β_2AR KO mice. As depicted in Figure 3A through 3D, β_2AR KO EPCs showed a significant reduction in the in vitro migratory activity and vascular network formation when compared with wild-type EPCs, and this effect was not improved by treatment with ISO. Finally, we analyzed the VEGF production of EPCs to explore their ability to produce cytokines that improve angiogenesis. Thus, we performed an ELISA for VEGF protein production on supernatant medium collected from EPCs after 7 days of culturing on fibronectin-coated well, at basal condition and after ISO stimulation. As reported in the Figure 3E, stimulation of β_2AR by ISO on EPCs led to a significant increased level of supernatant VEGF, whereas this effect was not detected in EPCs harvested by KO mice. These data suggest that the stimulation of the β_2AR increases the EPCs angiogenic capacity in vitro by improving both their migratory and vascular network formation activity and VEGF secretion. Conversely, β_2AR KO EPCs are functionally impaired in their ability to promote neovascularization in vitro.

Effects of β_2AR Stimulation on the Angiogenesis In Vivo

Finally, we investigated the effect of β_2AR stimulation on EPC-mediated angiogenesis in vivo using a previously described model of unilateral hindlimb ischemia.[3,17] All animals survived surgical procedure. As expected, after resection of femoral artery in KO mice, there was a dramatic impairment of hindlimb perfusion, resulting in an elevated rate of blistering, necrosis, or self-inflicted amputation of the ischemic paw. This loss of perfusion was investigated by blood-flow evaluation with dyed microsphere dilution analysis and by histological analysis. In KO mice treated with wild-type EPCs, we observed a partial rescue of the phenotype, with reduced occurrence of blistering (data not shown) and ameliorated hemodynamic and histological parameters (Figure 4A–4C). However, in KO mice treated with KO EPCs, we did not observe any significant improvement as confirmed by rate of blistering, dye elution, and histological data at 28th day after the surgical induction of ischemia. These findings corroborated our in vitro data, confirming the functional impairment of β_2AR KO EPCs in promoting neovascularization and suggest that EPCs' angiogenic capacity in vivo is owing, at least in part, to activation of β_2AR pathway on these cells. Finally, we investigated the effect of β_2AR stimulation on EPC-mediated angiogenesis in vivo using the rat model of hindlimb ischemia.[3] Digital angiographies were performed before and up 4 weeks after surgery. Digital angiographies at 28th day after the surgical induction of ischemia showed that arterial infusion of cultured EPCs improved the hindlimb reperfusion, as indicated by a significant lower thrombolysis in myocardial infarction frame count. This increase was even improved when Adβ_2AR EPCs were injected (Figure 4D and 4E).

Discussion

This is the first report showing the presence of β_2AR on EPCs. Our data indicate that β_2AR stimulation results in EPCs proliferation, migration, differentiation, and VEGF production, enhancing their angiogenic ability, both in vitro and in vivo, leading to an improved response to ischemic injury in animal models of hindlimb ischemia. Moreover, the overexpression of β_2AR on EPCs results in a further increase of EPCs angiogenic ability, whereas the lack of β_2AR on these cells highlights a functional-impaired EPCs phenotype resulting in a worse tolerance to the ischemia in vivo. EPCs' discovery has dramatically altered our vision of postnatal revascularization, indicating that adult vessels could be repaired

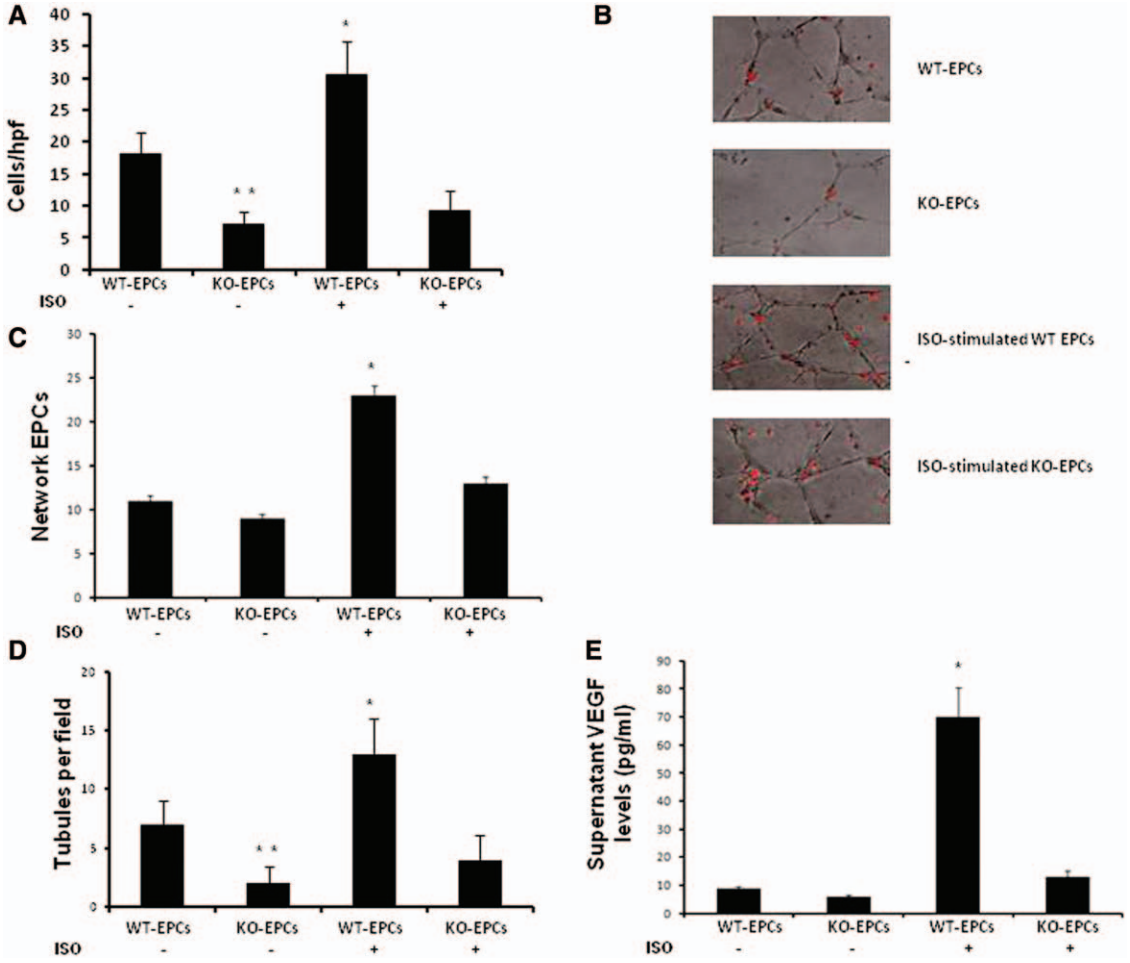

Figure 3. Vascular network formation and mobilization assay in vitro. We investigated the effect of β_2AR on endothelial progenitor cells (EPCs) angiogenic properties in vitro. **A**, EPCs' migration assay was performed using a modified Boyden chamber using vascular endothelial growth factor (VEGF) in the lower chamber. Cell migration was performed by manually counting cells in 5 random high-power field. Isoproterenol (ISO) stimulation (6 h) of EPCs led to a significant increase in migratory activity compared with the absence of ISO. To further explore the effects of β_2AR on EPCs angiogenic properties, experiment was repeated using EPCs harvested from β_2AR KO mice. EPCs lacking β_2AR revealed a significant reduction of migratory activity compared with wild-type (WT) EPCs that were not rescued by ISO treatment. **B**, Representative superimposed phase contrast and fluorescence microscopy fields are shown of human umbilical vein endothelial cells cocultured with WT or β_2AR KO EPCs at basal condition and after stimulation with ISO for 6 h. EPCs were tagged with DiI-Ac-low density lipoprotein to be recognizable at fluorescence microscopy. **C**, Quantitative analysis of the number of EPCs incorporated into human umbilical vein endothelial cells on Matrigel. Fluorescence microscopy revealed that the number of EPCs associated with human umbilical vein endothelial cells on Matrigel was significantly higher after ISO stimulation as compared with the absence of stimulation; incorporated β_2AR KO EPCs' number was lower with respect to WT EPCs, although the difference did not reach statistical significance and was not significantly increased by stimulation with ISO. **D**, Quantitative analysis of network projections formed on Matrigel for each experimental group after 24-h coculture. The total number of network projections per microscopic field after 24 h incubation was significantly higher for ISO-stimulated WT EPCs with respect to nonstimulated WT EPCs; a significant lower tubule formation was noted when β_2AR KO EPCs were used and was not significantly ameliorated by stimulation with ISO. **E**, Then, we explored the capability of EPCs to produce VEGF, a cytokine that is a key regulator of angiogenesis. The supernatant level of VEGF was assessed by ELISA assay after culturing cells harvested from WT and KO mice. Stimulation of β_2AR by ISO on WT EPCs led to a significant increased level of supernatant VEGF, whereas this effect was not detected in EPCs harvested from KO mice. **P*<0.05 vs absence of ISO; ***P*<0.05 vs WT EPCs.

not exclusively by proliferation, migration, and remodeling of neighboring mature and terminally differentiated ECs[18] but also by incorporation of bone marrow–derived EPCs in sprouting new blood vessel.[4] Several studies demonstrated that circulating EPCs are mobilized in response to ischemic stimuli and localized at the site of vascular damage where they proliferate, differentiate, and adhere to the vessel wall promoting reendothelialization of damaged vessels and inducing angiogenesis in the ischemic areas.[5,19–23] The potential therapeutic role of EPCs in repairing of injured vessel wall and neovascularization of ischemic tissue seems to be limited by the extremely low number of these cells that is still impaired in the presence of older age or other cardiovascular risk factors.[10,24] Therefore, molecular approaches aimed to improve EPCs' number and activity are pivotal in the development of this novel therapeutic strategy and, as consequence, the knowledge of the molecular mechanisms that are behind EPCs' proangiogenic properties is needful. Vascular functions and angiogenesis are thoroughly regulated by the adrenergic system, which modulates in vivo blood vessel growth. On this

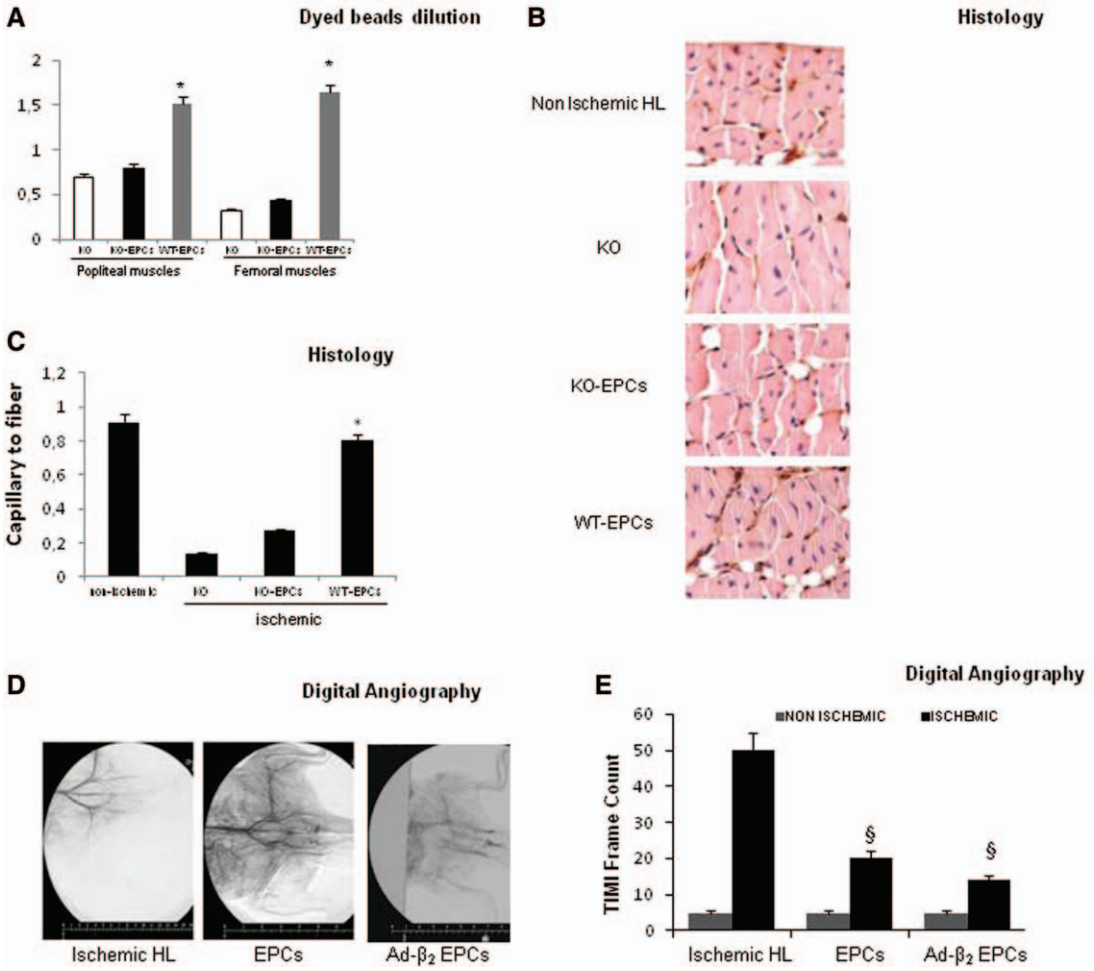

Figure 4. Increased neoangiogenic responses by β_2AR stimulation during chronic ischemia in vivo. Finally, we investigated the effect of β_2AR stimulation on endothelial progenitor cells (EPCs)–mediated angiogenesis in vivo using animal models of unilateral hindlimb ischemia and we found that in response to an ischemic stimulus, the impairment in limb reperfusion was rescued by intravenous injection of wild-type (WT) EPCs but not of β_2AR KO EPCs. **A**, Dyed bead dilution analysis is reported as the ischemic:nonischemic ratio of dyed beads content per milligram of hindlimb muscle tissue. β_2AR KO mice showed reduced blood flow under control conditions compared with β_2AR KO mice treated by WT mice–derived EPCs. Dyed bead content in muscle of β_2AR KO EPC-treated mice was similar to that of nontreated mice and significantly lower than WT EPC-treated mice. **B**, **C**, CD31 immunostaining of capillaries in the mouse hindlimb. Chronic ischemia in β_2AR KO mice produced a rarefaction on the capillary density of anterior tibial muscle evaluated as number of capillary corrected for number of muscle fibers. β_2AR KO EPCs' treatment did not produce any significant effect, whereas WT EPCs' treatment significantly enhanced the capillary density. **D**, In a rat model of limb ischemia, infusion of cultured EPCs significantly improved the hindlimb reperfusion that was even ameliorated by infusion of Ad-β_2AR-infected EPCs. **E**, Thrombolysis in myocardial infarction frame count quantification showed a significant ameliorated hindlimb perfusion after EPCs and Ad-β_2–infected EPCs infusion. *P<0.05 vs KO; §P<0.05 vs ischemic control rats; n=10 for each group. HL indicates hindlimb.

regard, we have previously provided evidence that β_2AR is involved in the control of ECs' biology with implications in neoangiogenesis in response to ischemia.[3] Thus, to explore the potential therapeutic role of enhancing adrenergic function on EPCs, we explored the effect of β_2AR on EPCs' biology.

EPCs Express a Functional β_2AR

We first demonstrated that culturing mononuclear cells for 7 days resulted in an adherent population of acetylated low-density lipoprotein/lectin-positive cells that were also positive for expression of the endothelial transcripts VEGF receptor-2 (Flk-1), endothelial nitric oxide synthase, vascular endothelial cadherin, CD34, and CD31consistent with an EPCs phenotype.[8] WB analysis performed on EPCs demonstrated the baseline expression of the β_2AR. FACS analysis further confirmed this results, and reverse transcriptase-polymerase chain reaction showed the expression of β_2AR mRNA. Then, immunochemistry analysis revealed the colocalization of β_2AR and FLK-1, which was corroborated by FACS analysis, demonstrating the colocalization of β_2AR with the panoply cell surface markers used to describe EPCs. This result was finally confirmed by the β_2AR radioligand binding assay showing that β_2AR represents the majority βAR subtype expressed on these cells. After showing that β_2AR is expressed on cultured EPCs, we found that β_2AR stimulation with ISO activates the β_2AR downstream target AKT. We have previously reported that in adult ECs, β_2ARs activate AKT, resulting in prevention of apoptosis.[25] Furthermore, we reported that, in ECs, β_2AR can also stimulate apoptosis by activation of p38/mitogen-activated protein

kinase pathway and that the net effect of proapoptotic and antiapoptotic signaling could result in an increase in cell number in response to β_2AR stimulation in the short time and in a loss of cell when β_2AR is chronically activated.[3] In addition, different studies have demonstrated that in ECs, AKT acts as integrator of multiple signal transduction pathways and regulates many critical steps in angiogenesis, such as cell migration and vascular network formation, cardiovascular homeostasis by controlling nitric oxide synthesis,[26] and promotes cell survival by inhibiting apoptosis.[27]

Effect of β_2AR Stimulation on EPCs

After demonstrating that β_2AR is expressed and functional on EPCs, we investigated the effects of this receptor on EPCs proliferation, differentiation, and angiogenic properties. We observed that β_2AR stimulation induced significant increase of cell proliferation, as shown by significant increase of DiI-acetylated low-density lipoprotein/lectin double-positive cells in ISO treatment after 7 days of culturing and by a significant increase of cellular levels of the proliferation marker p-retinoblastoma protein. Moreover, β_2AR overexpression in EPCs results in further increasing proliferation and angiogenic differentiation of EPCs in vitro. β_2AR-induced cell proliferation has also been reported in other tissues, including adult ECs as we previously demonstrated.[3] Furthermore, it seems that β_2AR stimulation with ISO can directly act on cultured circulating mononuclear cells to promote Flk-1 and von Willebrand factor expression. In addition, ISO treatment improves the EPCs' migratory activity in vitro and improves the EPCs' ability to enhance ECs' networks and these effects are lost in EPCs harvested from β_2AR KO mice. Furthermore, stimulation of β_2AR by ISO on EPCs led to a significant increased levels of supernatant VEGF, a pivotal cytokine involved in both physiological and pathological angiogenesis processes, whereas this effect was not detected in EPCs harvested by KO mice. Taken together, these data suggest that the stimulation of the β_2AR increase the EPCs angiogenic capacity in vitro by improving both their migratory and vascular network formation activity and increasing VEGF secretion. In contrast, the absence of β_2AR results in an impaired EPCs ability to promote neovascularization in vitro. Consistently, in vivo EPCs treatment resulted in an improvement of impaired angiogenic phenotype in β_2AR KO mice after induction of ischemia, whereas no significant amelioration was noted when β_2AR KO EPCs were injected. Taken together, our data suggest that β_2AR stimulation on EPCs enhances the proangiogenic properties of these cells resulting, in vivo, in a more potent neovascularization and improved tolerance to ischemia and this effect can even be improved when β_2AR density on EPCs is increased by means of a transgenic mechanism. Conversely, we found that EPCs harvested from β_2AR KO mice are ineffective in promoting neovascularization in ischemic tissue when administered systemically. We have recently shown that ECs can produce their own catecholamines during ischemia, which in turn stimulate, in a paracrine manner (β_2AR).[28] Although we have not demonstrated that EPCs possess the machinery to produce catecholamines like other myeloid cell types do, such as monocytes and ECs, it is possible to hypothesize that β_2AR KO EPCs lack this mechanism and, therefore, are dysfunctional. Thus, the impaired reperfusion in mice lacking β_2AR may be related to EPCs dysfunction and their inability to augment the neovascularization process. Consistent with this hypothesis, we found that β_2AR KO EPCs, in vitro, are impaired in their abilities to migrate and stimulate network formation on Matrigel. Moreover, our data also suggest that the reduced angiogenic capacity in β_2AR KO EPCs may also be related to a reduction in VEGF secretion by these cells. These findings are consistent with our previous observation that EPCs enhance angiogenesis by delivering software, in a paracrine manner, that facilitates the neovascularization process.[8] In conclusion, our results showed not only a novel angiogenic role for β_2AR, extending the findings in adult ECs, but also indicate a novel strategy to improve EPCs' number, differentiation, and function and, thereby, provide a potential therapeutic challenge for the use of EPCs to promote neovascularization in patients with ischemic vascular disease.

Disclosures

None.

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Novelty and Significance

What Is Known?

- Endothelial progenitor cells (EPCs) are present in the systemic circulation and home to sites of ischemia where they promote neovascularization.
- β_2-Adrenergic receptor plays a key role in the regulation of adult endothelial cells, proliferation, and function.

What New Information Does This Article Contribute?

- EPCs express a functional β_2-adrenergic receptor.
- β_2AR stimulation results in enhanced EPCs proliferation and differentiation and, moreover, improves EPCs proangiogenic ability both in vitro and in vivo.

EPCs are bone marrow–derived circulating cells that are mobilized in response to ischemic stimuli. EPCs home to sites of ischemia where they proliferate, differentiate, and adhere to the vessel wall promoting reendothelialization of damaged vessels and inducing angiogenesis. However, the therapeutic use of EPCs is limited by their low abundance in peripheral blood and by the fact that several cardiovascular risk factors further reduce circulating EPCs' levels. New strategies are, therefore, required to enhance EPCs' number and function. In this study, we show that EPCs express a functional β_2AR. We found that β_2AR stimulation enhanced EPCs' proliferation and differentiation. Moreover, β_2AR stimulation significantly enhanced EPCs' angiogenic properties, inducing vessel formation in vitro and neovascularization in animal models of ischemia. These findings not only demonstrate a novel angiogenic role for β_2AR on EPCs but also indicate a new strategy to improve EPCs' number, differentiation, and function that could assist in the development of pharmacological strategies to promote neovascularization of ischemic tissue.