β₂-Adrenergic Receptor Stimulation Improves Endothelial Progenitor Cell–Mediated Ischemic Neoangiogenesis

Gennaro Galasso, Roberta De Rosa, Michele Ciccarelli, Daniela Sorrentino, Carmine Del Giudice, Teresa Strisciuglio, Chiara De Biase, Rossella Luciano, Raffaele Piccolo, Adele Pierrì, Giuseppe Di Gioia, Nella Prevete, Bruno Trimarco, Federico Piscione, Guido Iaccarino

Rationale: Endothelial progenitor cells (EPCs) are present in the systemic circulation and home to sites of ischemic injury where they promote neoangiogenesis. β₂-Adrenergic receptor (β₂AR) plays a critical role in vascular tone regulation and neoangiogenesis.

Objective: We aimed to evaluate the role of β₂AR on EPCs’ function.

Methods and Results: We firstly performed in vitro analysis showing the expression of β₂AR 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, β₂AR 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 β₂AR KO mice after induction of ischemia, whereas no significant amelioration was observed when β₂AR 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 β₂AR KO EPC-treated mice.

Conclusions: The present study provides the first evidence that EPCs express a functional β₂AR. Moreover, β₂AR 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: β₂-adrenergic receptor ■ angiogenesis ■ endothelial progenitor cells
ECs, both in vivo and in vitro,\textsuperscript{3} 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 $\beta_2$AR on EPCs, then analyzed in vitro the effect of $\beta_2$AR stimulation on EPCs’ differentiation and function. Finally, we analyzed the effect of $\beta_2$AR stimulation on EPCs, performing in vivo studies in rat and mouse models of hindlimb ischemia.

**Methods**

**Animals**
Homozygous $\beta_2$AR KO male mice 5 backcrosses\textsuperscript{41} in a C57/BL6 background used as referral strain (wild type) at 8 to 16 weeks of age\textsuperscript{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.\textsuperscript{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 Data Supplement Methods, for further details.

**$\beta_2$AR Binding Assay**
Membrane fractions were used for $\beta_2$AR radioligand binding assay using the nonselective $\beta_2$AR antagonist $\textsuperscript{[125I]}$Cyanopindolol ($\textsuperscript{[125I]}$-CYP), as previously reported\textsuperscript{3} (see Online Data Supplement Methods for further details).

**Table 1. Endothelial Progenitor Cells’ Characterization**

<table>
<thead>
<tr>
<th>Phenotypic appearance at fluorescence-activated cell sorters:</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vascular endothelial cadherin</td>
<td>+</td>
</tr>
<tr>
<td>FLK-1</td>
<td>+</td>
</tr>
<tr>
<td>CD34</td>
<td>+</td>
</tr>
<tr>
<td>CD31</td>
<td>+</td>
</tr>
<tr>
<td>e-NOS</td>
<td>+</td>
</tr>
<tr>
<td>CD45</td>
<td>-</td>
</tr>
<tr>
<td>CD14</td>
<td>-</td>
</tr>
<tr>
<td>In vitro tube formation</td>
<td>+</td>
</tr>
</tbody>
</table>

**Western Immunoblot Analysis**
Western blots (WB) were performed to assess the cellular expression of $\beta_2$AR and phosphorylated isofrom of protein kinase B (AKT) and retinoblastoma protein on EPCs (see Online Data Supplement Methods for further details).

**Immunofluorescence**
Cells were grown in 4-chamber slides, fibronectin-coated, and immunofluorescence was performed as previously reported\textsuperscript{14} (see Online Data Supplement Methods for further details).

**Real Time-Polymerase Chain Reaction Analysis**
$\beta_2$AR mRNA level was determined by quantitative real-time polymerase chain reaction (Step-One, Applied Biosystems, Milano, Italy; see Online Data 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 Online Data 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 Online Data Supplement Methods for further details).

**EPCs’ Migration Assay**
As previously described,\textsuperscript{9} 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 Online Data 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 Online Data Supplement Methods for further details).

**Adenoviral Constructs**
Adenovirus vectors encoding for the $\beta_2$AR (Ad$\beta_2$AR) or for an empty vector were a kind gift from Professor Walter J. Koch (Temple University, Philadelphia, PA).\textsuperscript{15}

**Animal Models of Ischemia**
Mice or rats were subjected to unilateral hindlimb femoral artery removal\textsuperscript{9} (see Online Data Supplement Methods for further details).

**Blood-Flow Analysis**
Blood flow was evaluated by dyed bead perfusion and digital angiography analysis (see Online Data Supplement Methods for further details).

**Histological Analysis**
Capillary density within tibialis anterior muscle was used for histological study (see Online Data Supplement Methods for further details).

**Results**
To investigate the expression of $\beta_2$AR on cells cultured and characterized as above described, we first performed an immunoblot analysis that revealed the baseline expression of $\beta_2$AR on EPCs. $\beta_2$AR expression on EPCs was not increased by stimulation with the $\beta_2$AR agonist isoproterenol
Equal amount of proteins were confirmed by immunoblot for actin. The expression of β2AR 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 β2AR and FLK-1 on EPCs’ surface. As depicted in the Figure 1C, fluorescence microscopy revealed that EPCs express on their surface β2AR (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. Colocalization of β2AR 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 β2AR. Finally, a β-receptor binding on EPCs was performed using the β2AR 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 β2AR (70%). Value are expressed as fmol/mg on x axis and as relative percentages on y axis. To confirm that the β2AR represents the most important subtype in response to β2AR 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 β2AR target AKT, with a significant increase of p-AKT/total AKT ratio. Noteworthy, the stimulation with the more selective β2AR agonist fenoterol (FENO) led to very similar increase of p-AKT as compared with ISO. Reciprocally, ISO stimulation of EPCs after pretreatment with of β2AR-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.
reported in the adult ECs. Noteworthy, the stimulation with the more selective \( \beta_2 \)AR agonist Fenoterol led to very similar increase of p-AKT as compared with ISO. Reciprocally, ISO stimulation of EPCs after pretreatment with \( \beta_2 \)AR-blocker ICI 118,551 (ICI) prevents AKT activation (Figure 1F). Finally, as reported in the Online Figure III, ISO stimulation of Ad\( \beta_2 \)AR 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 \( \beta_2 \)AR.

**Effects of \( \beta_2 \)AR Stimulation on EPCs’ Proliferation and Differentiation In Vitro**

Having demonstrated that EPCs express a functional \( \beta_2 \)AR, 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 6 hours as compared with cells cultured in the absence of ISO. These data suggest that EPCs express functionally relevant \( \beta_2 \)AR.
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 β₂AR, 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 β₂AR 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 β₂AR stimulation acts on the EPCs’ fraction of circulating mononuclear cells to promote differentiation. Moreover, ISO stimulation of Adβ₂AR EPCs results in further increase of both p-retinoblastoma protein and Flk-1 expression on these cells, as reported in the Online Figure IIIIB 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 β₂AR 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 β₂AR.

### Effects of β₂AR Stimulation on the Angiogenesis In Vitro

Cellular migration is required for vascular network formation. Therefore, we examined the effect of β₂AR stimulation with ISO on unidirectional migration of Dil-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 Dil-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 β₂AR on EPCs angiogenic properties, we performed migration and vascular formation assays using EPCs harvested from β₂AR KO mice. As depicted in Figure 3A through 3D, β₂AR 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 β₂AR 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 β₂AR increases the EPCs angiogenic capacity in vitro by improving both their migratory and vascular network formation activity and VEGF secretion. Conversely, β₂AR KO EPCs are functionally impaired in their ability to promote neovascularization in vitro.

### Effects of β₂AR Stimulation on the Angiogenesis In Vivo

Finally, we investigated the effect of β₂AR 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 β₂AR KO EPCs in promoting neovascularization and suggest that EPCs’ angiogenic capacity in vivo is owing, at least in part, to activation of β₂AR pathway on these cells. Finally, we investigated the effect of β₂AR 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 Adβ₂AR EPCs were injected (Figure 4D and 4E).

### Discussion

This is the first report showing the presence of β₂AR on EPCs. Our data indicate that β₂AR 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 β₂AR on EPCs results in a further increase of EPCs angiogenic ability, whereas the lack of β₂AR 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.
not exclusively by proliferation, migration, and remodeling of neighboring mature and terminally differentiated ECs but also by incorporation of bone marrow–derived EPCs in sprouting new blood vessel. 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. 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. 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
regard, we have previously provided evidence that \( \beta_2 \)AR is involved in the control of ECs’ biology with implications in neoangiogenesis in response to ischemia. Thus, to explore the potential therapeutic role of enhancing adrenergic function on EPCs, we explored the effect of \( \beta_2 \)AR on EPCs’ biology.

**EPCs Express a Functional \( \beta_2 \)AR**

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 CD31 consistent with an EPCs phenotype. WB analysis performed on EPCs demonstrated the baseline expression of the \( \beta_2 \)AR. FACS analysis further confirmed this results, and reverse transcriptase-polymerase chain reaction showed the expression of \( \beta_2 \)AR mRNA. Then, immunohistochemistry analysis revealed the colocalization of \( \beta_2 \)AR and FLK-1, which was corroborated by FACS analysis, demonstrating the colocalization of \( \beta_2 \)AR with the panoply cell surface markers used to describe EPCs. This result was finally confirmed by the \( \beta_2 \)AR radioligand binding assay showing that \( \beta_2 \)AR represents the majority \( \beta \)AR subtype expressed on these cells. After showing that \( \beta_2 \)AR is expressed on cultured EPCs, we found that \( \beta_2 \)AR stimulation with ISO activates the \( \beta_2 \)AR downstream target AKT. We have previously reported that in adult ECs, \( \beta_2 \)ARs activate AKT, resulting in prevention of apoptosis.

**Figure 4. Increased neoangiogenic responses by \( \beta_2 \)AR stimulation during chronic ischemia in vivo.** Finally, we investigated the effect of \( \beta_2 \)AR 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 \( \beta_2 \)AR KO EPCs. A, Dyed bead dilution analysis is reported as the ischemic:nonischemic ratio of dyed beads content per milligram of hindlimb muscle tissue. \( \beta_2 \)AR KO mice showed reduced blood flow under control conditions compared with \( \beta_2 \)AR KO mice treated by WT mice–derived EPCs. Dyed bead content in muscle of \( \beta_2 \)AR 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 \( \beta_2 \)AR KO mice produced a rarefaction on the capillary density of anterior tibial muscle evaluated as number of capillary corrected for number of muscle fibers. \( \beta_2 \)AR 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-\( \beta_2 \)AR-infected EPCs. E, Thrombolysis in myocardial infarction frame count quantification showed a significant ameliorated hindlimb perfusion after EPCs and Ad-\( \beta_2 \)AR-infected EPCs infusion. *\( P<0.05 \) vs KO; §\( P<0.05 \) vs ischemic control rats; \( n=10 \) for each group. HL indicates hindlimb.
kinase pathway and that the net effect of proapoptotic and antiapoptotic signaling could result in an increase in cell number in response to \( \beta_2 \)AR stimulation in the short time and in a loss of cell when \( \beta_2 \)AR is chronically activated. 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, and promotes cell survival by inhibiting apoptosis.

**Effect of \( \beta_2 \)AR Stimulation on EPCs**

After demonstrating that \( \beta_2 \)AR is expressed and functional on EPCs, we investigated the effects of this receptor on EPCs proliferation, differentiation, and angiogenic properties. We observed that \( \beta_2 \)AR stimulation induced significant increase of cell proliferation, as shown by significant increase of Dil-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, \( \beta_2 \)AR overexpression in EPCs results in further increasing proliferation and angiogenic differentiation of EPCs in vitro. \( \beta_2 \)AR-induced cell proliferation has also been reported in other tissues, including adult ECs as we previously demonstrated. Furthermore, it seems that \( \beta_2 \)AR stimulation with ISO can directly act on cultured circulating mononuclear cells to promote Flik-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 \( \beta_2 \)AR KO mice. Furthermore, stimulation of \( \beta_2 \)AR 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 \( \beta_2 \)AR 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 \( \beta_2 \)AR 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 \( \beta_2 \)AR KO mice after induction of ischemia, whereas no significant amelioration was noted when \( \beta_2 \)AR KO EPCs were injected. Taken together, our data suggest that \( \beta_2 \)AR 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 \( \beta_2 \)AR density on EPCs is increased by means of a transgenic mechanism. Conversely, we found that EPCs harvested from \( \beta_2 \)AR 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 (\( \beta_2 \)AR). 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 \( \beta_2 \)AR KO EPCs lack this mechanism and, therefore, are dysfunctional. Thus, the impaired reperfusion in mice lacking \( \beta_2 \)AR may be related to EPCs dysfunction and their inability to augment the neovascularization process. Consistent with this hypothesis, we found that \( \beta_2 \)AR 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 \( \beta_2 \)AR 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. In conclusion, our results showed not only a novel angiogenic role for \( \beta_2 \)AR, 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.

**References**


β2-Adrenergic Receptor Stimulation Improves Endothelial Progenitor Cell–Mediated Ischemic Neoangiogenesis

Gennaro Galasso, Roberta De Rosa, Michele Ciccarelli, Daniela Sorriento, Carmine Del Giudice, Teresa Strisciuglio, Chiara De Biase, Rossella Luciano, Raffaele Piccolo, Adele Pierri, Giuseppe Di Gioia, Nella Prevete, Bruno Trimarco, Federico Piscione and Guido Iaccarino

*Circ Res.* 2013;112:1026-1034; originally published online February 15, 2013;
doi: 10.1161/CIRCRESAHA.111.300152

*Circulation Research* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2013 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:

http://circres.ahajournals.org/content/112/7/1026

Data Supplement (unedited) at:

http://circres.ahajournals.org/content/suppl/2013/02/15/CIRCRESAHA.111.300152.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Circulation Research* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to *Circulation Research* is online at:
http://circres.ahajournals.org//subscriptions/
Supplemental Material.

Expanded Methods Section.

Animals.

Homozigous β2 Adrenergic Receptor (β2AR) KO male mice 5 backcrosses in a C57/BL6 background used as referral strain (Wild Type-WT) at 8-16 weeks of age and normotensive Wistar Kyoto (WKY) male rats (Charles Rivers Laboratory International) at 12 weeks of age were used for this study. All animals 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 as previously reported. Mononuclear cells (MNCs) from 2 ml whole blood, obtained by cardiac puncture, were isolated by Histopaque-1077 density gradient centrifugation at 400g for 30 min. The mononuclear fraction was collected, washed in Dulbecco’s phosphate-buffered saline and red cell were lysed through ammonium chloride solution; after purification with 3 washing steps, 2.5 x 10^4 cells/mm² MNCs were seeded on fibronectin coated plates. Cells were cultured in endothelial basal medium-2 (Lonza) supplemented with EGM-2 Bullet Kit (Lonza) and 20% FBS. After 7 days culture, the supernatant including the suspending cells was removed and the adherent cells were washed twice. EPCs characterization was performed according to AHA suggestions. Thus, attached cells, isolated and cultured as above described, were tested to the ability to stain with 1, 1’-dioctadecyl-3, 3’, 3’, 3’-tetramethylindocarbocyanine-labeled acetylated low-density lipoprotein (DiI-Ac-LDL, Invitrogen; incubation at 37°C for 3 hours) and with lectin from Baindeiraea Simplicifolia BS-1 Isolectin B4 FITC conjugated (Sigma; incubation at 37°C for 1 hour). After staining, double positive cells were quantified by examining 15 random microscopic fields by two independent investigators (GG, RDR). Moreover, cells were investigated to the ability to express multiple endothelial cell markers (VE Cadherin, Flk-1, CD31, CD 34, eNOS) and to exclude expression of leukocyte or myeloid cells surface markers (CD45 and CD14) by automated flow cytometry. Finally, cells were tested for ability to form tubules in vitro when co-cultured with human umbilical vein endothelial cells (HUVECs). Thus, cells expressing all the characteristics reported in Table 1 were defined throughout this paper as EPCs.

βAR Binding Assay.

Membrane fractions obtained as described above were used for βAR radioligand binding assay using the nonselective βAR antagonist [125I]-Cyanopindolol (125I-CYP), as previously reported. Briefly, binding was allowed to occur for 1 h at 37°C as previously described. For competition isotherms, membrane (25 μg total protein) were incubated with 300 μM 125 I-CYP and increasing dilution of selective β1AR antagonist CGP. The percentage of β2ARs was calculated from the high affinity binding subpopulation using a dedicated software (Graph Pad Prism).

Western Immunoblot analysis.

Western Blots (WB) were performed to assess the cellular expression of β2AR, phosphorylated isophorn of AKT and RB on EPCs. Cell stimulation was performed by using β2AR agonist Fenoterol (FENO) 10^-8 mol/L, isoproterenol (ISO) 10^-8 mol/L for 3 or 6 hours, ISO 10^-8 mol/L for 3 or 6 hours after pre-treatment with selective β2AR antagonist ICI 118,551 (ICI) 10^-7 mol/L for 30 minutes. After stimulation, cells were collected with lysis buffer (LB-Tris 12.5 mmol, EDTA 5 mmol, EGTA 5 mmol) for β2AR and RIPA-SDS buffer for AKT and RB blot, whereas membrane fraction was obtained by lysis with MHLB buffer for β2AR blot. Cell lysates (50 μg) were therefore electrophoresed on SDS polyacrylamide gel and transferred on a nitrocellulose membrane according to standard procedures. Proteins of interest were visualized by specific antibodies (SantaCruz), anti-rabbit horseradish...
peroxidase-conjugated secondary antibodies (GE Healthcare) and standard chemiluminescence (Pierce Biotechnology, Rockford, Illinois) on autoradiographic films. After digitalization, densitometry quantification was performed with dedicated software (ImageQuant 5.0, Molecular Dynamics, Sunnyvale, California). Data are presented as arbitrary densitometry units (ADU) after normalization for total AKT or actin.

**Immunocytochemistry.**

Cells were grown in four chamber slides fibronectin coated and immunofluorescence performed as previously reported. Cells, cultured for 7 days as above indicated, were fixed with -20°C cold methanol and permeabilized with 0.01% Triton X-100 in PBS. Primary antibodies incubation with anti-Flk-1 (BD), anti-β2AR at a 1:100 dilution were performed at room temperature for one hour. Fluorescent-labeled secondary antibodies were incubated at room temperature for 1 hour. Images were taken by using an Eclipse E1000 Fluorescence Microscope (Nikon) and acquired by using Sigma Scan Pro software (Jandel). Images were optimized for contrast in Adobe PhotoShop, but no further manipulations were made.

**Real time-PCR analysis**

β2AR, Flk-1, vonWillebrand Factor and Smooth Muscle Actin (SMA) mRNA levels were determined by quantitative Real Time PCR (Step-One, Applied Biosystems, Milano, Italy). Total RNA was isolated and purified using Trizol reagent (Invitrogen) and reverse transcribed into cDNA using the Thermoscript RT-PCR System according to the manufacturer’s instructions. 18s RNA was used as endogenous control.

**Flow Cytometry.**

Fluorescence-activated cell sorter (FACS) analysis was used to detect the cell surface expression of the endothelial cell antigen Flk-1 (VEGF receptor-2) and other endothelial (CD34, CD31, eNOS, VE Cadherin, von Willebrand Factor) and leukocyte or myeloid (CD45, CD14) cell surface markers. Moreover, β2AR expression and co-expression of β2AR and EPCs marker Flk-1 was showed. Isotype specific conjugated anti IgG was used as a control.

**EPCs migration assay.**

EPCs migration assays were performed using a modified Boyden chamber assay as previously described. Briefly, VEGF (100 ng/ml) or saline in EBM-2 cell medium supplemented with 20% FBS was placed in the lower compartment of the modified Boyden chamber. Circulating MNCs, after 7 day culture on fibronectin coated wells, were harvested and 2.5 x 10^4 cells suspended in EBM-2 medium supplemented with 20% FBS were placed in the upper compartment of the chamber. After incubation at 37°C for 6 hours with ISO 10^-8 mol/L, cells migrating into the lower chamber were stained with DiI-Ac-LDL and FITC labeled lectin. Double positive cells were then counted manually in five random high power fields by two independent investigators (GG, RDR). To further confirm the defined EPCs phenotype, cells migrating in the lower chamber were tested by FACS for all the cells surface markers reported in Table 1.

**Matrigel assay.**

Matrigel assay was performed to detect vascular network formation in vitro on commercially available 96-well multi-dishes coated with growth factor-reduced Matrigel by Biocoat Angiogenesis system for Endothelial Cell Tube Formation (Becton Dickinson) on according to the manufacturer’s instructions. EPCs harvested from WT or β2-KO mice were plated fibronectin six-well multi-dishes and after 7 days culture were incubated with DiI-Ac-LDL for 3 hours to be detectable at fluorescence microscopy. Part of these cells were then stimulated with ISO10^-8 mol/L for 6 hours. Human umbilical vein endothelial cells (HUVECs) (4 x 10^4, Lonza) and the DiI-Ac-LDL-labeled EPCs (2 x 10^3) after ISO-stimulation
were cocultured and incubated at 37°C for 24 h in 200 μl of EGM-2 medium. Tubule formation was observed using an inverted phase-contrast fluorescence digital microscope (Nikon). Tubule formation was defined as a structure exhibiting a length 4 times its width. The average of the total number of complete tubes formed by cells was counted in 15 random fields by two independent investigators (GG, RDR) as previous described.

**VEGF quantification**

Cultured cells were starved overnight and then stimulated with ISO for 6 hours. Cultured medium was collected and VEGF production was revealed with an enzyme-linked immunosorbent assay using Mouse VEGF ELISA Kit (R&D) for the quantitative measurement of VEGF in cell culture supernatant, following the manufacturer instructions.

**Adenoviral constructs**

Adenovirus vectors encoding for the β2 AR or for an empty vector were a kind gift of Professor Walter J. Koch (Temple University, Philadelphia, USA).

**Animal models of ischemia.**

Mice or rats were subjected to unilateral hindlimb femoral artery removal as previously reported. Briefly, after anesthesia (tiletamine 50 mg/Kg and zolazepam 50 mg/Kg), the common right femoral artery was isolated and permanently closed with a non reabsorbable suture whereas the right femoral vein was clamped. Through an incision on the common right femoral artery distal to the suture, a plastic cannula was advanced into the artery and 1 x 10⁷ EPCs were injected. Afterwards, the common femoral artery was removed and the wound closed in layers. The mouse study included four study groups: controls (sham operated WT), KO (not treated KO), KO EPCs (KO treated with KO EPCs infusion) and WT EPCs (KO treated with WT EPCs infusion). Rats were divided in three study groups: controls (no EPCs infusion), EPCs (injection of empty virus treated-EPCs), and β2AR EPCs (β2 AR-infected EPCs). Controls were represented by sham operated rats.

**Blood-flow analysis.**

We used dyed bead perfusion to evaluate blood flow. We injected 6 x 10⁵ yellow dyed beads (Triton Technologies) diluted in 1 ml NaCl 0.9% through a polyethylene catheter introduced through right common carotid artery down to the abdominal aorta. After a lethal dose of pentobarbital, samples of the gastrocnemius muscle (520–880 mg) were collected and frozen with liquid nitrogen. Next, samples were homogenized and digested, the beads were collected and suspended in dimethylformamide. The release of dye was assessed by light absorption at a wavelength of 448 nm. Data are expressed as the ratio of dye extracted from ischemic to that extracted from non-ischemic muscle. Moreover, in rats we analyzed blood flow in vivo by digital angiography analysis at surgery time and up to three weeks. Animals were anesthetized and left common carotid artery was exposed as previously reported. Briefly, a 50 polyethylene catheter was advanced into the abdominal aorta right before the iliac bifurcation under fluoroscopic visualization (Advantix LCX, General Electrics). Maximal vasodilatation was obtained with nitro-glycerine (20 μg i.a.). An electronically regulated injector (ACIST Medical System Inc.) was used to deliver with constant pressure (900 psi) 0.2 ml of contrast medium (Iomeron 400, Bracco). The cine-frame number for TIMI frame count assessment was measured with a digital frame counter on the suitable cine-viewer monitor as previously described. All angiograms were filmed at 5 frame/sec and were analyzed by two blinded investigators. TIMI frame count was evaluated from the first frame in which the contrast medium was visualized in the iliac artery until the frame in which there was the full visualization of first paw artery bifurcation.

**Histological analysis.**

Capillary density within tibialis anterior muscle was used for histological study. Tissue specimens were
dissected and immediately fixed by immersion in phosphate buffered saline (0.01M, pH 7.2–7.4)/formalin for at least 12 h. Muscle samples (5 μm thick) were prepared and capillary endothelial cells were identified by immunostaining for CD31 (PECAM: Becton Dickinson) as previously reported. Ten random microscopic fields from three different sections in each tissue sample were examined. Capillary density was expressed as number of CD31 positive features per high power field (400 x) and the number of capillaries per muscle fiber as previously reported.

**Statistical analysis**

Results from at least 3 independent experiments are expressed as mean ± SD and categorical variables are expressed as absolute or percent value. Categorical variables were analyzed by chi square test, while continuous variable tested by 2-tailed t test. Comparisons between groups were performed by Student’s paired 2-tailed t test or ANOVA for experiments with more than 2 subgroups. Post hoc analysis and pair wise multiple comparisons were performed using the 2-sided t test with Bonferroni adjustment. Probability values <0.05 were considered statistically significant. All analyses were performed with SPSS 16.0 software (SPSS Inc).


Cells harvested from whole blood and cultured, as above described, for 7 days on fibronectin coated wells, were tested for the ability to express endothelial markers used to describe EPCs by FACS. As reported, these cells expressed on their surface Flk-1, CD31, CD34 that are typical marker of EPCs. As expected, CD45, a leukocyte marker, was not expressed. Isotype is reported for FL2-H and FL3-H as control.
Online Figure II. Scatchard plot of the βAR binding.

Scatchard plot analysis of the I-CYP ligand binding for total and β1AR in EPC cells.
Online Figure III. Effect of Adβ2AR delivery on EPCs.

To corroborate our findings, we investigated p-AKT, p-RB and Flk-1 expression on Adβ2AR infected EPCs after stimulation with ISO. Panel A: Western blot analysis showed that the ratio between active, phosphorilated form of AKT and total AKT significantly increased after 6 hours ISO stimulation and was further increased when stimulation was performed on Adβ2AR treated EPCs. Panel B: Western blot was performed to detect levels of phosphorilated form of Rb, as cellular proliferation marker, on EPCs. We found that p-Rb increase induced by stimulation with ISO was even greater after ISO stimulation of Adβ2AR-treated EPCs. Panel C: Then, we investigated Flk-1 expression on cells cultured as above described, performing a FACS analysis. We found that ISO stimulation of Adβ2AR-treated EPCs led to a more pronounced increase in the expression of Flk-1 on these cells. Taken together, these data show that the favorable effect of β2AR on EPCs proliferation and differentiation can even be ameliorated by increasing β2AR density through transgenic mechanism.