Ischemic Neoangiogenesis Enhanced by β_2-Adrenergic Receptor Overexpression

A Novel Role for the Endothelial Adrenergic System

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Abstract—β_2-Adrenergic receptors (β_2ARs) are widely expressed, although their physiological relevance in many tissues is not yet fully understood. In vascular endothelial cells, they regulate NO release and vessel tone. Here we provide novel evidence that β_2ARs can regulate neoangiogenesis in response to chronic ischemia. We used in vivo adenoviral-mediated gene transfer of the human β_2AR to the endothelium of the rat femoral artery and increased β_2AR signaling resulting in ameliorated angiographic blood flow and hindlimb perfusion after chronic ischemia. Histological analysis confirmed that β_2AR overexpression also produced benefits on capillary density. The same maneuver partially rescued impaired angiogenesis in hypertensive SHR rats, whereas gene delivery of the G-protein–coupling defective mutant Ile164 β_2AR failed to provide ameliorations. Stimulation of endogenous and overexpressed β_2AR on endothelial cells in vitro was found to regulate cell number by inducing proliferation and [^{3}H]-thymidine incorporation through means of extracellular receptor-activated kinase and vascular endothelial growth factor. The β_2AR also has novel effects on endothelial cell number through stimulation of proapoptosis and antiapoptosis pathways involving p38 mitogen-activated protein kinase and PI3-kinase/Akt activation. Therefore, β_2ARs play a critical role in endothelial cell proliferation and function including revascularization, suggesting a novel and physiologically relevant role in neoangiogenesis in response to ischemia. (***Circ Res*. 2005;97:0-0.)**

Key Words: angiogenesis ■ rats ■ polymorphism ■ hypertension ■ in vivo digital angiography

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 often, the same receptor can activate multiple endothelial functions.

Adult angiogenesis only occurs in particular conditions such as wound healing, tumorogenesis, hypoxia, and chronic ischemia.[2] It is a phenomenon intimately associated with endothelial cell (EC) proliferation, which appears to be under control of the extracellular receptor-activated kinase (ERK)/mitogen-activated protein kinase (MAPK)–mediated signaling cascades.[2] The most important system regulating angiogenesis is the cytokine vascular endothelial growth factor (VEGF), although a number of other cytokines and hormones acting through various tyrosine kinase and G-protein–coupled receptors are also implicated in this process.

The adrenergic system is the major regulator of cardiac and vascular function, and evidence is mounting for the relevance of this system in the control of endothelial vasodilation through means of α_{2-} and β-adrenergic receptors (βARs). In particular, β_2ARs, the most abundant βARs in the vasculature,[3,4] modulate the release of NO, causing endothelium-dependent vasodilation.[5] β_2ARs are G-protein–coupled receptors activated by adrenergic catecholamines and promote a series of intracellular signal transduction pathways, leading to multiple cell-specific responses.[6,7] Recently, it was proposed that β_2ARs modulate cell proliferation, at least in fibroblasts, by activating ERK/MAPK through pathways dependent on β-arrestins.[8] The physiological implications of such an in vitro observation have not yet been fully investigated in vivo.

Altogether, the above considerations provide the background for the current investigation into the role of β_2ARs in

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	Total Binding Mean±ES (pmol/L)	β_2 Binding Mean±SE (pmol/L)
Nonischemic contralateral HL	59.3±6.1	32±4.7
Ischemic HL	29.7±0.9*	14.2±1.1*
Ischemic+ADβ2AR-wt HL	55.5±1.4#	n.d.
Ischemic+ADβ2AR-164 HL	55.2±1.4#	n.d.

Total βAR and β_2AR density were analyzed rat tibial muscle from the ischemic and nonischemic hindlimb (HL); *$P<0.05$ vs nonischemic HL; #$P<0.03$ vs ischemic HL. n.d. indicates nondetectable.

the control of angiogenesis. Accordingly, we evaluated whether β_2ARs on ECs could enhance ischemia-induced angiogenesis in vivo. Also, in ECs, we evaluated the in vitro effects of β_2AR overexpression on cell signaling and biology.

Materials and Methods

Cell Culture, [^{3}H]-Thymidine Incorporation, βAR Radioligand Binding, Adenoviral Constructs, Cell Proliferation, Angiogenesis In Vitro Assay, Western Blot, VEGF Production, Animals, Surgical Procedures, Digital Angiography, and Hindlimb Muscle Histology

Experimental procedures were performed as described previously.[5,9–13] Extended details are provided in the online data supplement, available at http://circres.ahajournals.org.

Results

Effects of In Vivo β_2AR Gene Transfer to the Endothelium During Chronic Ischemia in Wistar Kyoto Rats

We studied neoangiogenesis in a well-characterized rat model of chronic hindlimb ischemia in the rat.[14] Our control group consisted of pooled data collected from sham-treated and AdEmpty virus–treated rats because no differences were found between these two treatments (supplemental Table I). In this model, 15 days after resection of the femoral artery, we observed a reduction in βAR density within the ischemic hindlimb, which was attributable to a selective downregulation of β_2ARs, the major βAR subtype found in this tissue (Table). The numbers included in the Table reflect the sum of βARs expressed on all cell types present in the hindlimb area, including, but not solely, ECs. Also, 15 days after femoral artery resection, there was impairment in limb perfusion assessed by digital angiography (Figure 1A; supplemental video 1). These hemodynamic changes were paralleled by the rarefaction in capillary (<5 μm) density in the anterior tibial muscle compared with the contralateral normal limb (Figure 1C and 1D). In a subset of rats, we injected the Adβ_2AR-wt into the femoral artery at the time of surgery. Consistent with previous results,[15] this resulted in β_2AR overexpression in muscle, arterial capillaries, and venous (Figure 1E), which was double that of endogenous βAR density found in the rat hindlimb (Table). After 15 days of ischemia, rats receiving Adβ_2AR-wt had significantly improved hindlimb perfusion with increased blood flow compared with untreated rats (Figure 1A and 1B; supplemental video 2). Similarly, histological analysis of the anterior tibial muscle revealed restored capillary density because of Adβ_2AR-wt treatment (Figure 1C and 1D). These positive benefits in vivo appear to be attributable to enhanced β_2AR signaling because delivery of Adβ_2AR-Ile164 (Figure 1A through 1D) resulted in no beneficial effects on hindlimb hemodynamic or histology. The Ile164β_2AR mutation is a naturally occurring human polymorphism that causes a severe impairment in the G-protein coupling.[16–19]

Chronic ischemia leads to increased systemic VEGF levels, a potent proangiogenesis cytokine, and levels that typically return to baseline values once the ischemia is eliminated.[20,21] We examined VEGF levels in our model in the blood and in the nonischemic contralateral hindlimb. In both samples, VEGF-165 levels were significantly lower in ischemic rats treated with Adβ_2AR-wt compared with control and Adβ_2AR-Ile164 rats, suggesting that β_2AR overexpression significantly lessens ischemia (Figure 1F and 1G). Overall, these results suggest that β_2ARs can regulate key endothelial cellular functions in vivo in response to chronic ischemia.

Impaired Angiogenesis Ameliorated by In Vivo β_2AR Gene Transfer to the Endothelium During Chronic Ischemia in Spontaneously Hypertensive Rats

To further explore the role of endothelial β_2AR in pathophysiological conditions, we repeated the in vivo experiment using the angiogenesis-impaired hypertensive SHR rat.[22] Previously in this model, we have shown that reduced vascular and endothelial βAR signaling and vasodilation is corrected by β_2AR gene transfer.[5] Fifteen days after resection of the femoral artery in SHR, there was a dramatic impairment of hindlimb perfusion, evidenced by the elevated occurrence of blistering, necrosis, or self-inflicted amputation of the ischemic paw (occurrence of necrosis, amputation, and blistering: SHR 35%; Wistar Kyoto [WKY] 0%; $P<0.05$; χ^2 test). This loss of perfusion was confirmed by digital angiography (Figure 1A), dyed microsphere dilution (Figure 1B), and by histological analysis (Figure 1D). In SHR treated with the ADβ2AR-wt, we observed a partial correction of this phenotype indicated by the reduced occurrence of blistering (10%; $P<0.05$; χ^2 test) and the ameliorated hemodynamic and histological parameters (Figure 1A, 1B, and 1D).

Finally, the analysis by Western blot of VEGF performed on the contralateral nonischemic hindlimb showed that systemic levels of this cytokine were reduced in the Adβ_2AR-wt SHR compared with the nontreated group, suggesting that local ischemia was resolved (Figure 1G).

Effects of β_2AR Signaling on EC Proliferation

Our in vivo results do not let us discern whether the protective effects of β_2AR overexpression in the rat hindlimb arterial endothelium after ischemic damage are attributable to an effect on cell proliferation or cell survival. These mechanistic questions were addressed in vitro using ECs in culture. The overall angiogenic properties of β_2AR were recapitulated under conditions in which stimulated human ECs organize in network forming tubules. The βAR agonist isoproterenol (ISO) increased the number of connections among endothelial tubules in vitro, and this response was magnified by

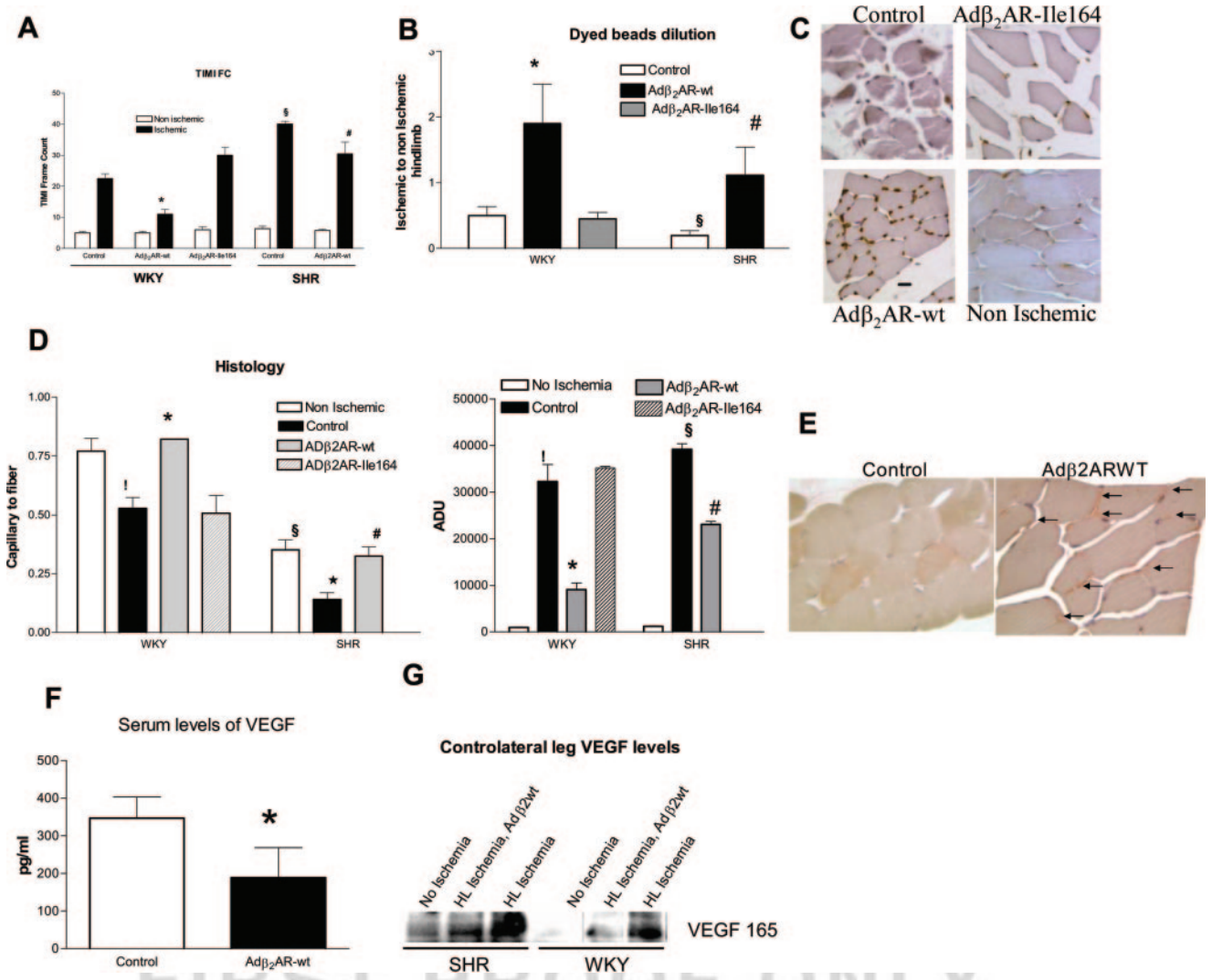

Figure 1. Increased neoangiogenetic responses by β_2AR-wt gene transfer during chronic ischemia in vivo. A, TIMI frames count (FC). After 15 days of chronic ischemia, digital angiographies evidenced a reduced number of TIMI FCs in ischemic hindlimbs treated with Adβ_2AR-wt with respect to control rats (*$P<0.05$). Adβ_2AR-Ile164 did not modify the number of TIMI FCs respect to controls. SHR have impaired angiogenesis because TIMI FC was significantly higher compared with control WKY (§$P<0.05$). Also in SHR, Adβ_2AR-wt significantly improved ischemia (#$P<0.05$ vs controls). B, Dyed beads dilution assay, where Adβ_2AR-wt but not Adβ_2AR-Ile164 attenuated blood perfusion in ischemic hindlimb respect to controls. Shown is the ischemic to nonischemic ratio of dyed beads content per mg of hindlimb muscle tissue (*$P<0.05$). In SHR, there is reduced blood flow under control conditions compared with WKY rats (§$P<0.05$) and Adβ_2AR-wt treatment restores blood flow in the ischemic hindlimb (#$P<0.05$ vs control). C and D, Lectin BS-I staining of capillaries in the rat hindlimb. Magnification $\times 40$; bar$=10$ μm. Chronic ischemia in WKY produced a rarefaction on the capillary density of anterior tibial muscle evaluated as number of capillary corrected for number of muscle fibers (!$P<0,05$ vs nonischemic). Adβ_2AR-wt corrected capillary density respect to control (*$P<0.05$), whereas Adβ_2AR-Ile164 did not produce any significant effect. In SHR, capillary density is lower in nonischemic tissue compared with WKY (§$P<0.05$), and ischemia further reduces this score (⋆$P<0.05$ vs nonischemic). Adβ_2AR-wt enhances the capillary density in the ischemic district also in this rat strain (#$P<0.05$ vs control). E, Immunohistochemistry of overexpressed β_2AR using a specific antibody (see supplemental data). β_2ARs were visualized as a red reaction product. In ischemic muscle, the intra-arterial infusion of Adβ_2AR-wt produces an increase in β_2AR expression, which is most localized in the perivascular spaces (arrows). F, Systemic levels of VEGF on serum. Systemic VEGF-165 was used as marker of ischemia and assessed on serum by ELISA. Fifteen days after femoral artery resection, serum VEGF-165 levels were significantly lower in the Adβ_2AR-wt–treated rats (*$P<0.05$) vs controls. G, Systemic levels of VEGF on nonischemic contralateral muscle. We evaluated VEGF levels on contralateral hindlimbs by WB (inset) as an indicator of systemic VEGF. Using muscle of rats that were not subjected to femoral artery resection as non ischemic reference (no Ischemia), we found that ischemia (control) caused an increase in systemic levels of VEFG in WKY (!$P<0.02$ vs nonischemic) and in SHR (§$P<0.05$ vs nonischemic). In WKY rats, Adβ_2AR-wt but not Adβ_2AR-Ile164 delivery leads to a limitation of the ischemic insult and consequently to a reduction of systemic levels of VEGF after 15 days (*$P<0.05$ vs control). In SHR, Adβ_2AR-wt also reduced VEGF levels, indicating an ameliorated ischemic hindlimb perfusion (#$P<0.05$ vs control; ADU indicates arbitrary densitometry units).

Adβ_2AR-wt but not Adβ2AR-Ile164 (Figure 2A). Importantly, ISO-mediated tubule formation was similar to that induced by VEGF, used as positive control.

Because angiogenesis is intimately associated to EC proliferation, we explored whether stimulation of β_2ARs in ECs can increase cell number. In subconfluent bovine aorta ECs (BAECs), the addition of ISO caused a biphasic response with an initial increase, followed by a drop in cell number after 36 hours (Figure 2B). Loss of cell viability is probably related to multiple effects of ISO, which are dependent on

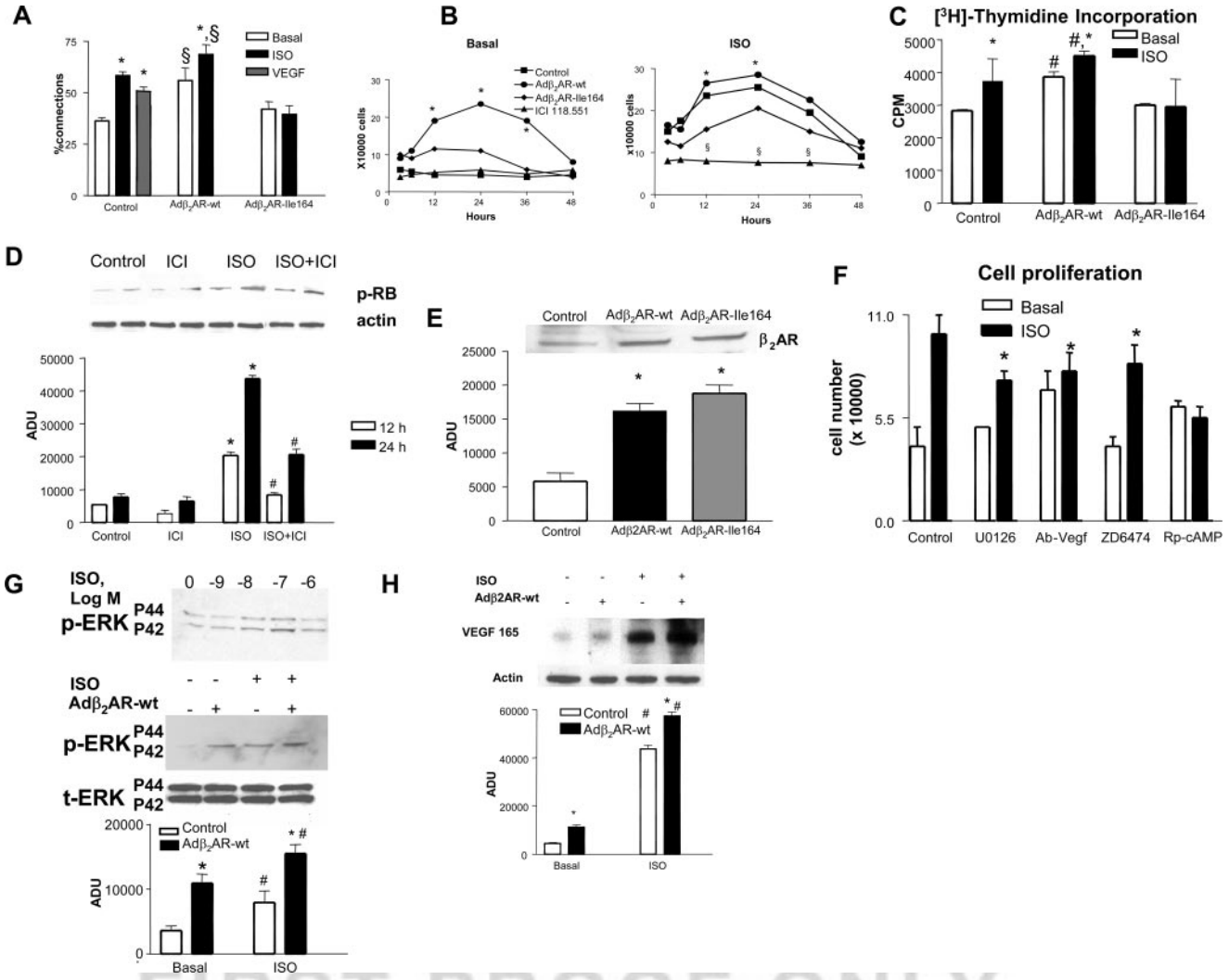

Figure 2. In vitro effects of β_2AR stimulation on EC biology. A, Angiogenic properties of β_2ARs. Human ECs were exposed to the Adβ_2AR-wt or the Adβ_2AR-Ile164 and cocultured with other human cells in a specially designed medium in a 24-well plate. Every three days, ISO (10^{-8} mol/L) was added to the cultures. On the eleventh day, cells were visualized by staining for anti-human CD31 (platelet-endothelial cell adhesion molecule-1). In this setting, cells tend to form a tubule-like shape and organize in a network. Images were digitalized and scored with an image analysis software to count the number of cellular connections and the total tubule length. In control cells, ISO stimulation produces an increased number of connections, and this response was enhanced by Adβ_2AR-wt but not Adβ_2AR-Ile164. VEGF-165 (100 nmol/L) was used as positive control (*$P<0.05$ vs basal; §$P<0.05$ vs the respective condition in control; n=2 in duplicate). B, Proliferative response in subconfluent BAECs. Adβ_2AR-wt but not Adβ_2AR-Ile164 produced a biphasic response, which was initially proliferative with a maximal effect at 24 hours, and then cell availability reduced at 36 and 48 hours (*$P<0.05$ vs control). ISO (10^{-8} mol/L) caused an increase of cells number, followed by a drop in cell number observed at 36 and 48 hours. The β_2AR antagonist ICI (10^{-6} mol/L) prevented the changes in cell number induced by ISO (§$P<0.02$ vs control), whereas Adβ_2AR-wt but not Adβ_2AR-Ile164 enhanced ISO response (*$P<0.05$ vs control; n=3 to 5 in triplicate). C, DNA synthesis by [^{3}H]-thymidine incorporation. The activation of βAR by ISO (10^{-8} mol/L) caused increased DNA synthesis. The same effect was obtained in Adβ_2AR-wt but not Adβ2AR-Ile164. The maximal response was observed in ISO-stimulated Adβ2AR-wt cells (*$P<0.05$ vs basal; #$P<0.05$ vs control; n=3 to 5 in duplicate). D, Progression in cell cycle assessed by Rb phosphorylation. This protein regulates cell cycle progression through the restriction point within the G1 phase. By Western blot at 12 and 24 hours of stimulation (inset), ISO (10^{-8} mol/L), caused Rb phosphorylation, antagonized by the β_2AR antagonist ICI (10^{-6} mol/L; ADU indicates arbitrary densitometry units; *$P<0.05$ vs control; #$P<0.05$ vs ISO; n=3 in duplicate) E, β_2AR overexpression in BAECs. To test the level of overexpression induced by Adβ_2AR-wt and Adβ_2AR-Ile164 in BAECs, we performed a Western blot of β_2AR on membrane extracts (inset). Densitometric analysis (bar graph) showed a similar 3-fold increase of the β_2AR density induced by both adenoviruses (*$P<0.05$ vs control; n=3 in triplicate). F, Role of ERK/MAPK activation and VEGF production on ISO (10^{-8} mol/L) induced cell proliferation. The MAPK kinase inhibitor UO126 (10^{-6} mol/L), the antibody to VEGF (0.2 μg/mL) as well as the VEGF receptor inhibitor ZD6474 (10^{-6} mol/L), attenuated to a similar extent the proliferation induced by ISO (10^{-8} mol/L). Also, the cAMP antagonist Rp-cAMP (10^{-5} mol/L) similarly prevented ISO proliferation (*$P<0.05$ vs control; ANOVA; n=3 to 5 experiments, repeated in triplicate). G, ERK/MAPK activation. Western blot of activated (phosphorylated; p-ERK) ERK1/2 after ISO treatment. Equal amount of proteins were confirmed via blotting for total ERK/MAPK. Representative blots are presented in the inset. Densitometric analysis (bar graph) show that βAR stimulation caused ERK/MAPK activation with a maximal effect at 10^{-7} mol/L ISO. Adβ_2AR-wt treatment induced ERK activation the ISO (10^{-7} mol/L) response was enhanced (*$P<0.05$ vs control; #$P<0.05$ vs basal; n=3 in duplicate). H, VEGF production in BAECs measured by Western blot (inset) of VEGF-165. Shown are VEGF-165 levels after 6 hours of serum starvation. Equal amount of proteins were verified by blotting for actin. Adβ_2AR-wt and ISO (10^{-8} mol/L) increased VEGF-165 production with a maximal effect in presence of both conditions (*$P<0.05$ vs control; #$P<0.05$ vs basal; n=3 in duplicate).

time and dosage, because it can be observed after 24 hours, when using higher doses of ISO ($>10^{-8}$ mol/L; supplemental Figure I). The response to βAR stimulation is largely dependent on endogenous β_2AR because cell proliferation can be prevented by the selective β_2AR inhibitor ICI 118 551 (ICI; Figure 2B). Consistent with an effect on cell proliferation, βAR stimulation by ISO resulted in an increase in [^{3}H]-thymidine incorporation (Figure 2C), an index of DNA synthesis, and phosphorylation of retinoblastoma protein RB, which removes the inhibition of E2F in the nucleus causing the cell cycle progression from G1 to S[23]. This response in BAEC was attributable to β_2AR signaling because it was attenuated by the β_2AR antagonist ICI (Figure 2D).

For in vitro analysis of the effects of added wild-type or mutant (Ile164) β_2AR density on EC function, adenoviral-mediated β_2AR overexpression was obtained (Figure 2E), causing an increase in total βAR density (from 29.7±0.9 to 55.5±1.4 fmol/mg and 55.2±1.4 fmol/mg protein in Adβ_2AR-wt and Adβ_2AR-Ile164, respectively). Overexpression of the wild-type but not the Ile164 mutant β_2AR resulted in increased cell proliferation and DNA synthesis as measured by [^{3}H]-thymidine incorporation, and ISO further enhanced these mitogenic responses (Figure 2B and 2C). The need of an intact β_2AR signaling through the cAMP second messenger to achieve cell proliferation was verified by the antagonist RP-cAMP because the presence of this inhibitor attenuated the proliferative response after 24 hours of ISO treatment (Figure 2F).

Angiogenesis is largely dependent on ERK/MAPK activation[24–26] because it can promote EC proliferation and expression of VEGF, which, in turn, sustains the proangiogenic phenotype.[27] Using BAECs, we found that ISO leads to significant ERK activation (Figure 2G) and VEGF production (Figure 2H). Moreover, overexpression of the wild-type β_2AR enhanced ERK/MAPK activation (Figure 2G). The β_2AR-Ile164 failed to induce the potentiation of ERK/MAPK observed with the β_2AR-wt (supplemental Figure II). To further assess the role of β_2AR-mediated ERK/MAPK activation in BAEC proliferation, we used an inhibitor of ERK/MAPK activation, U0126 (10^{-6} mol/L), and observed reduced BAEC proliferation in response to ISO (Figure 2F). Because β_2ARs can stimulate VEGF production, we evaluated whether this cytokine is responsible for the proliferative effects of ECs after ISO. VEGF signaling was inhibited by using either an antibody to VEGF (200 ng/mL) or an inhibitor of the VEGF receptor. In both cases, BAEC proliferation in response to ISO was reduced (Figure 2F). These results suggest that β_2AR-mediated EC proliferation is dependent, at least in part, on VEGF production and release.

Effects of β_2AR Signaling on EC Apoptosis

Our cell proliferation data demonstrate that chronic exposure to ISO causes a loss in cell viability after 36 hours (Figure 2B). This is probably initiated by serum deprivation but is clearly further sustained by chronic βAR activation. EC number may also be the result of cellular apoptosis under the control of βARs. Indeed, a previous report has shown that the β_2AR in neonatal cardiac myocytes produces proapoptotic and antiapoptotic effects, and this signaling involves the p38 MAPK and PI3-kinase/Akt pathways.[28] We have recently shown that in endothelium, β_2ARs activate Akt.[29] Other reports have documented the ability of βARs to stimulate p38 MAPK,[28,30] which is considered an important mechanism of receptor-mediated apoptosis.[31,32] Using BAECs, we first determined whether β_2AR stimulation could result in Akt or p38 MAPK activation, and indeed, this was found with endogenous βARs as well as overexpressed β_2ARs (Figure 3A and 3B). Next, we examined apoptosis using caspase-3 cleavage as a marker. Serum starvation and ISO reproducibly activated caspase-3 in BAECs, and both responses were significantly greater in ECs overexpressing the wild-type β_2AR (Figure 3C). The involvement of p38 MAPK is suggested by the negative regulation of the p38/MAPK inhibitor SB203580 on β_2AR-mediated caspase-3 cleavage (Figure 3D). Overall, these apoptosis data correlate with the observed reduction in cell number at 36 and 48 hours observed in cell proliferation experiments (Figure 2B).

Importantly, we also examined whether β_2ARs have any effect on prosurvival pathways mediated by PI3-kinase/Akt. We repeated the above apoptosis assays in the presence of inhibitors of Akt activation and found that caspase-3 cleavage was significantly enhanced after ISO in Adβ_2AR-wt and control cells (Figure 3D; supplemental Figure III). Overall, our data show that β_2ARs exert a dualistic effect on cell survival, with activation and inhibition of apoptosis through means of p38 MAPK and PI3-kinase/Akt, respectively.

Discussion

We verified that the β_2AR is an endogenous mediator of angiogenesis in vivo, using a rat model of neovascularization and adrenergic activation attributable to hindlimb ischemia.[14] The observed downregulation β_2AR density suggests a role for this receptor in the compensatory response to chronic ischemia and adrenergic activation. Adenoviral-mediated overexpression of wild-type β_2AR in vivo improves neoangiogenesis activity documented by digital imaging and perfusion techniques. Moreover, histological analysis showed that β_2AR signaling preserves and enhances the number of capillaries in the ischemic area. The implications of this phenomenon were further tested in SHR, which experience impaired βAR signaling and angiogenesis. In this model, the removal of the femoral artery causes severe perfusion deficit with necrosis and self-inflicted amputations of the ischemic limb. These alterations were all attenuated after intrafemoral artery delivery of the Adβ_2AR-wt. Mechanistic studies show that β_2ARs control EC proliferation as well as stimulating proapoptotic and antiapoptotic pathways. These novel effects of β_2ARs in ECs are in addition to their recently discovered role on NO release and regulation of vascular tone.[5,29] βAR stimulation activates proliferative and cell death and survival pathways, but the β_2AR-mediated EC proliferative mechanism appears to be relevant for therapeutic angiogenesis.

It is noteworthy that β_2ARs can induce cell proliferation and promote cell survival in other tissues; in particular, Kim et al recently described that β_2ARs expressed on cardiac fibroblasts can induce cell proliferation through ERK/MAPK-dependent mechanisms.[8] Our current data demonstrate that this signaling paradigm also exists in ECs because

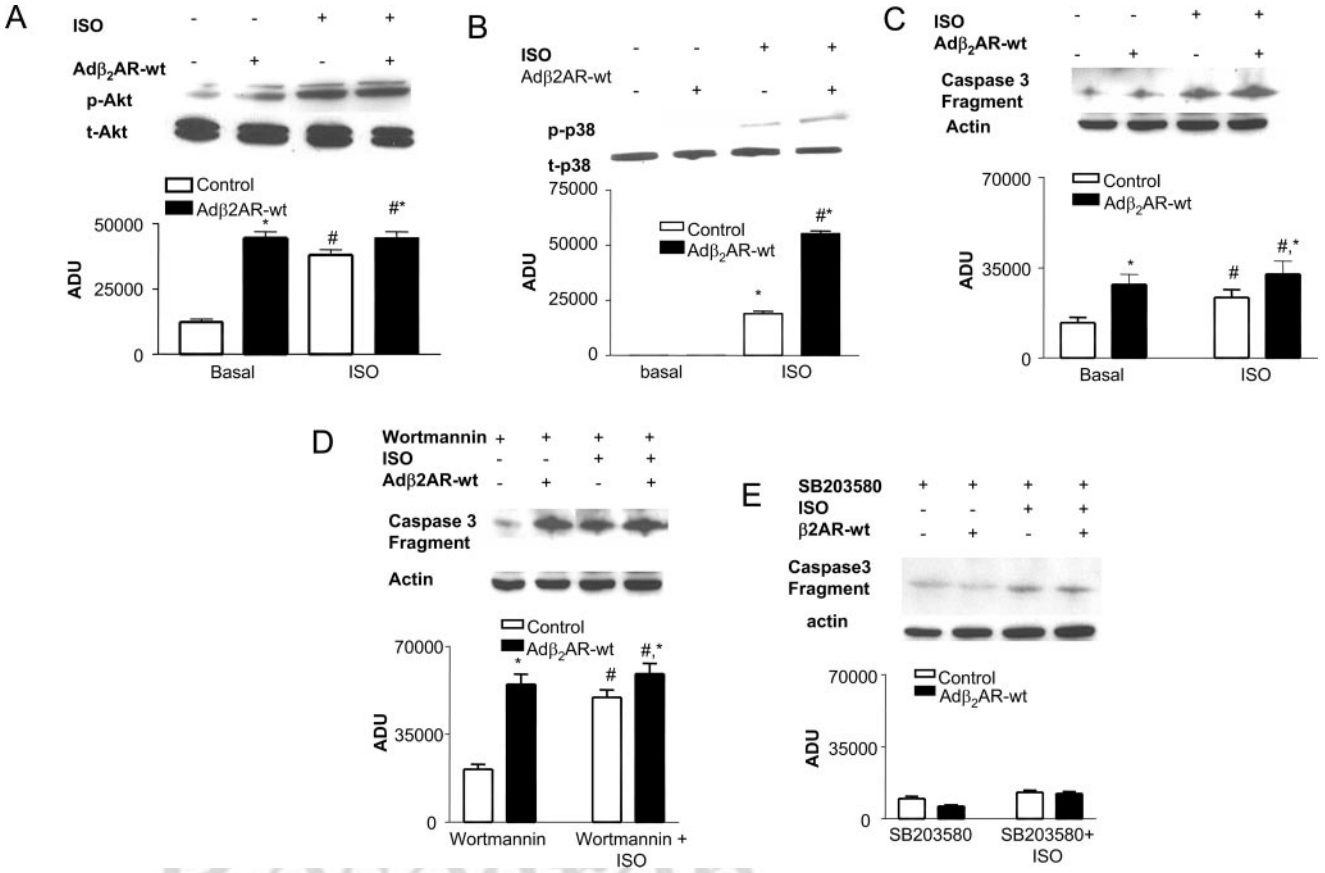

Figure 3. β_2AR regulation of apoptosis in BAEC. A, Akt activation assessed by Western blotting (p-Akt). Blots were corrected by total Akt (insets). ISO (10^{-7} mol/L) induced Akt activation with a maximal effect in Adβ_2AR-wt cells (ADU indicates arbitrary densitometry units; *$P<0.05$ vs control; #$P<0.05$ vs basal; n=3 in duplicate). B, p38/MAPK activation determined by Western blot of phosphorylated (activated) p38/MAPK using cell lysates of BAECs and corrected for blotting of total p38 MAPK. ISO (10^{-7} mol/L) induced p38 activation with a maximal effect in Adβ2AR-wt cells (*$P<0.05$ vs control; #$P<0.05$ vs basal; n=3 in duplicate). C, Caspase-3 cleavage assessed as a marker of cell apoptosis. Densitometric analysis of Western blots for cleaved caspase-3 was corrected by actin blotting. ISO (10^{-8} mol/L) and Adβ_2AR-wt induced caspase-3 cleavage. The presence of both conditions produced a maximal effect (*$P<0.05$ vs control; #$P<0.05$ vs basal; n=3 in triplicate). D, Effects of Akt inhibition on caspase-3 cleavage. Wortmannin (10^{-5} mol/L), an inhibitor of Akt/PKB activation, enhanced caspase-3 cleavage in response to serum starvation and ISO. The maximal activation of caspase-3 was observed in Adβ_2AR-wt either with or without ISO (*$P<0.05$ vs control; #$P<0.05$ vs base; n=3 in triplicate). A similar result was observed using another inhibitor of Akt activation: LY 294002 (supplemental Figure III). E, Effects of p38 MAPK inhibition on caspase 3 cleavage. SB 203580 (10^{-5} mol/L) attenuated caspase-3 cleavage in a similar manner in control and Adβ_2AR-wt cells, with and without ISO (10^{-8} mol/L; n=3 in triplicate).

β_2AR stimulation increased EC number, and this cellular proliferative effect is blocked by ERK/MAPK inhibition. Although we did not investigate the whole signal transduction involved in the proangiogenic phenotype induced by β_2AR in ECs, our data are suggestive that cAMP production is important in this response.

β_2ARs exert a positive effect on EC ERK/MAPK activation by at least two mechanisms. First, stimulation of endothelial βARs and wild-type β_2AR overexpression directly activate ERK/MAPK. Second, β_2AR stimulation can induce the release of VEGF, which can also activate ERK/MAPK.[26] ERK/MAPK inhibition totally prevents EC proliferation, demonstrating that this kinase is critical for βAR-mediated cell mitogenesis and proliferation. The ability of βARs to induce VEGF production and release was reported previously for β_1ARs and β_3ARs in adipose tissue.[33] Here, we show that this effect can also be demonstrated for the β_2ARs found on ECs. Our in vitro results may appear in contrast with the in vivo observation of lower systemic VEGF in the Adβ_2AR-wt treated rats. On the contrary, both results testify the facilitation of angiogenesis mediated by an early boost of VEGF production that chronically causes a faster resolution of ischemia with the consequent return of systemic VEGF toward lower levels.

Concerning cell survival, Morisco et al have also shown in neonatal cardiomyocytes that β_2AR signaling can prevent apoptosis through Akt activation.[34] Zhu et al have confirmed this observation in adult cardiomyocytes.[28] In ECs, too, β_2AR activation leads to Akt-mediated apoptosis protection. Furthermore, in ECs, β_2ARs can also stimulate apoptosis through a p38/MAPK mechanism. The net effect of proapoptotic and antiapoptotic signaling results in an eventual loss of cell number when βARs are chronically activated, whereas in the short term, there is an increase in cell number.

The effects of β_2AR signaling on EC biology are clearly dependent on the overall integrity of the total signal transduction pathway. Indeed, overexpression of the naturally occurring Ile164 β_2AR mutant failed to induce the enhance-

ment of neoangiogenesis observed in vivo in the ischemic hindlimb. This mutant receptor is largely nonfunctional and poorly activates downstream G-protein–mediated signaling events. In transgenic mice with cardiac overexpression (≈45-fold over total βAR density) of this receptor, the changes in cardiac contractility induced by βAR stimulation were not different from those of nontransgenic littermate.[17] Furthermore, in patients harboring this polymorphism in heterozygosity, β_2AR-dependent responses to exercise (cardiac index, stroke volume, systemic vascular resistance) were all reduced by ≥50%.[19] In vitro, the EC_{50} of the mutant receptor to epinephrine is 5× higher than that of the wild type.[16] Our results add to these data, showing that its overexpression did not alter the angiogenic response of ischemic hindlimb, nor in vitro EC proliferation, thymidine incorporation, and apoptosis. These results work also as a further control, ruling out the hypothesis of a "mass effect" because of overexpression-induced transactivation of non-β_2AR via heterodimerization.[35] Our results imply that patients carrying this polymorphism may have an impairment of angiogenic response, in particular, in those conditions that are associated to sympathetic activation, such as chronic ischemia or cardiac dysfunction. Indeed, Liggett et al showed reduced survival in heart failure patients harboring the Ile164 mutant of the β_2AR gene,[36] thus suggesting a more profound inadequacy of the adaptive responses of these patients to the challenged hemodynamic condition. Our data provide the ground to interpret these findings as the result of the impaired angiogenesis associated to this polymorphism, leading to a reduced myocardial perfusion in a situation in which angiogenesis largely determines cardiac function and growth.[37]

The sympathetic nervous system regulates in vivo blood vessel growth, although this process was believed to be mediated by αARs and limited to vascular smooth muscle cell proliferation.[38] Recently, another sympathetic neurotransmitter, the neuropeptide Y, has been demonstrated to be a potent angiogenic factor in vivo through means of the endothelial Y2 receptors.[39] It is therefore emerging that the sympathetic nervous system is an important determinant of neoangiogenesis, acting through means of catecholamines and neuropeptides. As further support to this notion, mice lacking the ability to produce catecholamines are not able to fully develop during fetal life.[40]

In conclusion, we provide evidence that β_2ARs are involved in the control of EC biology with implications in neoangiogenesis in response to ischemia. This is a novel finding carrying critical relevance in the current and future treatment of chronic ischemia. Therapeutic angiogenesis is the ultimate rescue of ischemic tissue, pursued using soluble growth factors such as VEGF, with the limitation of the potential facilitation of tumor genesis or growth. Our data suggest that therapeutic angiogenesis in ischemic tissue might be more efficiently and selectively achieved when associated to activation of β_2AR signaling by means of available classical drugs or novel molecular tools such as adenoviral-mediated β_2AR gene transfer.

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References

1. Schmidt HH, Walter U. NO at work. *Cell*. 1994;78:919–925.
2. Bussolino F, Mantovani A, Persico G. Molecular mechanisms of blood vessel formation. *Trends Biochem Sci*. 1997;22:251–256.
3. Osswald W, Guimaraes S. Adrenergic mechanisms in blood vessels: morphological and pharmacological aspects. *Rev Physiol Biochem Pharmacol*. 1983;96:53–122.
4. Guimaraes S, Moura D. Vascular adrenoceptors: an update. *Pharmacol Rev*. 2001;53:319–356.
5. Iaccarino G, Cipolletta E, Fiorillo A, Annecchiarico M, Ciccarelli M, Cimini V, Koch WJ, Trimarco B. Beta(2)-adrenergic receptor gene delivery to the endothelium corrects impaired adrenergic vasorelaxation in hypertension. *Circulation*. 2002;106:349–355.
6. Lefkowitz RJ, Caron MG. The adrenergic receptors. *Adv Second Messenger Phosphoprotein Res*. 1990;24:1–8.
7. Hall RA, Lefkowitz RJ. Regulation of G-protein–coupled receptor signaling by scaffold proteins. *Circ Res*. 2002;91:672–680.
8. Kim J, Eckhart AD, Eguchi S, Koch WJ. Beta-adrenergic receptor-mediated DNA synthesis in cardiac fibroblasts is dependent on transactivation of the epidermal growth factor receptor and subsequent activation of extracellular signal-regulated kinases. *J Biol Chem*. 2002;277:32116–32123.
9. Iaccarino G, Smithwick LA, Lefkowitz RJ, Koch WJ. Targeting Gbeta gamma signaling in arterial vascular smooth muscle proliferation: a novel strategy to limit restenosis. *Proc Natl Acad Sci U S A*. 1999;96:3945–3950.
10. Xiao RP, Tomhave ED, Wang DJ, Ji X, Boluyt MO, Cheng H, Lakatta EG, Koch WJ. Age-associated reductions in cardiac beta1- and beta2-adrenergic responses without changes in inhibitory G-proteins or receptor kinases. *J Clin Invest*. 1998;101:1273–1282.
11. Iaccarino G, Barbato E, Cipolleta E, Esposito A, Fiorillo A, Koch WJ, Trimarco B. Cardiac betaARK1 upregulation induced by chronic salt deprivation in rats. *Hypertension*. 2001;38:255–260.
12. Chesley A, Lundberg MS, Asai T, Xiao RP, Ohtani S, Lakatta EG, Crow MT. The beta(2)-adrenergic receptor delivers an antiapoptotic signal to cardiac myocytes through G(i)-dependent coupling to phosphatidylinositol 3′-kinase. *Circ Res*. 2000;87:1172–1179.
13. Cui QL, Zheng WH, Quirion R, Almazan G. Inhibition of Src-like kinases reveals Akt-dependent and -independent pathways in insulin-like growth factor I-mediated oligodendrocyte progenitor survival. *J Biol Chem*. 2005;280:8918–8928.
14. Lee EW, Michalkiewicz M, Kitlinska J, Kalezic I, Switalska H, Yoo P, Sangkharat A, Ji H, Li L, Michalkiewicz T, Ljubisavljevic M, Johansson H, Grant DS, Zukowska Z. Neuropeptide Y induces ischemic angiogenesis and restores function of ischemic skeletal muscles. *J Clin Invest*. 2003;111:1853–1862.
15. Suzuki M, Iso-o N, Takeshita S, Tsukamoto K, Mori I, Sato T, Ohno M, Nagai R, Ishizaka N. Facilitated angiogenesis induced by heme oxygenase-1 gene transfer in a rat model of hindlimb ischemia. *Biochem Biophys Res Commun*. 2003;302:138–143.
16. Green SA, Cole G, Jacinto M, Innis M, Liggett SB. A polymorphism of the human beta 2-adrenergic receptor within the fourth transmembrane domain alters ligand binding and functional properties of the receptor. *J Biol Chem*. 1993;268:23116–23121.
17. Turki J, Lorenz JN, Green SA, Donnelly ET, Jacinto M, Liggett SB. Myocardial signaling defects and impaired cardiac function of a human beta 2-adrenergic receptor polymorphism expressed in transgenic mice. *Proc Natl Acad Sci U S A*. 1996;93:10483–10488.
18. Green SA, Rathz DA, Schuster AJ, Liggett SB. The Ile164 beta(2)-adrenoceptor polymorphism alters salmeterol exosite binding and conventional agonist coupling to G(s). *Eur J Pharmacol*. 2001;421:141–147.
19. Wagoner LE, Craft LL, Singh B, Suresh DP, Zengel PW, McGuire N, Abraham WT, Chenier TC, Dorn GW II, Liggett SB. Polymorphisms of the beta(2)-adrenergic receptor determine exercise capacity in patients with heart failure. *Circ Res*. 2000;86:834–840.
20. Seko Y, Imai Y, Suzuki S, Kamijukkoku S, Hayasaki K, Sakomura Y, Tobe K, Kadowaki T, Maekawa H, Takahashi N, Yazaki Y. Serum levels of vascular endothelial growth factor in patients with acute myocardial infarction undergoing reperfusion therapy. *Clin Sci (Lond)*. 1997;92:453–454.

21. McLaren M, Newton DJ, Khan F, Belch JJ. Vascular endothelial growth factor in patients with critical limb ischemia before and after amputation. *Int Angiol*. 2002;21:165–168.
22. Emanueli C, Salis MB, Stacca T, Gaspa L, Chao J, Chao L, Piana A, Madeddu P. Rescue of impaired angiogenesis in spontaneously hypertensive rats by intramuscular human tissue kallikrein gene transfer. *Hypertension*. 2001;38:136–141.
23. Deshpande A, Sicinski P, Hinds PW. Cyclins and cdks in development and cancer: a perspective. *Oncogene*. 2005;24:2909–2915.
24. Sosnowski RG, Feldman S, Feramisco JR. Interference with endogenous ras function inhibits cellular responses to wounding. *J Cell Biol*. 1993; 121:113–119.
25. Meadows KN, Bryant P, Vincent PA, Pumiglia KM. Activated Ras induces a proangiogenic phenotype in primary endothelial cells. *Oncogene*. 2004;23:192–200.
26. Parenti A, Morbidelli L, Cui XL, Douglas JG, Hood JD, Granger HJ, Ledda F, Ziche M. Nitric oxide is an upstream signal of vascular endothelial growth factor-induced extracellular signal-regulated kinase1/2 activation in postcapillary endothelium. *J Biol Chem*. 1998;273: 4220–4226.
27. Zachary I. VEGF signaling: integration and multitasking in endothelial cell biology. *Biochem Soc Trans*. 2003;31:1171–1177.
28. Zhu WZ, Zheng M, Koch WJ, Lefkowitz RJ, Kobilka BK, Xiao RP. Dual modulation of cell survival and cell death by beta(2)-adrenergic signaling in adult mouse cardiac myocytes. *Proc Natl Acad Sci U S A*. 2001;98: 1607–1612.
29. Iaccarino G, Ciccarelli M, Sorriento D, Cipolletta E, Cerullo V, Iovino GL, Paudice A, Elia A, Santulli G, Campanile A, Arcucci O, Pastore L, Salvatore F, Condorelli G, Trimarco B. AKT participates in endothelial dysfunction in hypertension. *Circulation*. 2004;109:2587–2593.
30. Magne S, Couchie D, Pecker F, Pavoine C. Beta(2)-adrenergic receptor agonists increase intracellular free Ca(2+) concentration cycling in ventricular cardiomyocytes through p38 and p42/44 MAPK-mediated cytosolic phospholipase A(2) activation. *J Biol Chem*. 2001;276: 39539–39548.
31. Sarkar D, Su ZZ, Lebedeva IV, Sauane M, Gopalkrishnan RV, Valerie K, Dent P, Fisher PB. MDA-7 (IL-24) Mediates selective apoptosis in human melanoma cells by inducing the coordinated overexpression of the GADD family of genes by means of p38 MAPK. *Proc Natl Acad Sci U S A*. 2002;99:10054–10059.
32. Porras A, Zuluaga S, Black E, Valladares A, Alvarez AM, Ambrosino C, Benito M, Nebreda AR. P38 alpha mitogen-activated protein kinase sensitizes cells to apoptosis induced by different stimuli. *Mol Biol Cell*. 2004;15:922–933.
33. Fredriksson JM, Lindquist JM, Bronnikov GE, Nedergaard J. Norepinephrine induces vascular endothelial growth factor gene expression in brown adipocytes through a beta-adrenoreceptor/cAMP/protein kinase A pathway involving Src but independently of Erk1/2. *J Biol Chem*. 2000; 275:13802–13811.
34. Morisco C, Zebrowski D, Condorelli G, Tsichlis P, Vatner SF, Sadoshima J. The Akt-glycogen synthase kinase 3beta pathway regulates transcription of atrial natriuretic factor induced by beta-adrenergic receptor stimulation in cardiac myocytes. *J Biol Chem*. 2000;275:14466–14475.
35. Bulenger S, Marullo S, Bouvier M. Emerging role of homo- and heterodimerization in G-protein-coupled receptor biosynthesis and maturation. *Trends Pharmacol Sci*. 2005;26:131–137.
36. Liggett SB, Wagoner LE, Craft LL, Hornung RW, Hoit BD, McIntosh TC, Walsh RA. The Ile164 beta2-adrenergic receptor polymorphism adversely affects the outcome of congestive heart failure. *J Clin Invest*. 1998;102:1534–1539.
37. Shiojima I, Sato K, Izumiya Y, Schiekofer S, Ito M, Liao R, Colucci WS, Walsh K. Disruption of coordinated cardiac hypertrophy and angiogenesis contributes to the transition to heart failure. *J Clin Invest*. 2005; 115:2108–2118.
38. Bevan RD. Trophic effects of peripheral adrenergic nerves on vascular structure. *Hypertension*. 1984;6:III19–III26.
39. Zukowska Z, Grant DS, Lee EW. Neuropeptide Y: a novel mechanism for ischemic angiogenesis. *Trends Cardiovasc Med*. 2003;13:86–92.
40. Thomas SA, Matsumoto AM, Palmiter RD. Noradrenaline is essential for mouse fetal development. *Nature*. 1995;374:643–646.