Endothelium

Endothelial Cells Are Able to Synthesize and Release Catecholamines Both In Vitro and In Vivo

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See Editorial Commentary, pp 12–14

Abstract—Recently it has been demonstrated that catecholamines are produced and used by macrophages and mediate immune response. The aim of this study is to verify whether endothelial cells (ECs), which are of myeloid origin, can produce catecholamines. We demonstrated that genes coding for tyrosine hydroxylase, Dopa decarboxylase, dopamine β hydroxylase (DβH), and phenylethanolamine-N-methyl transferase, enzymes involved in the synthesis of catecholamines, are all expressed in basal conditions in bovine aorta ECs, and their expression is enhanced in response to hypoxia. Moreover, hypoxia enhances catecholamine release. To evaluate the signal transduction pathway that regulates catecholamine synthesis in ECs, we overexpressed in bovine aorta ECs either protein kinase A (PKA) or the transcription factor cAMP response element binding, because PKA/cAMP response element binding activation induces tyrosine hydroxylase transcription and activity in response to stress. Both cAMP response element binding and PKA overexpression enhance DβH and phenylethanolamine-N-methyl transferase gene expression and catecholamine release, whereas H89, inhibitor of PKA, exerts the opposite effect, evidencing the role of PKA/cAMP response element binding transduction pathway in the regulation of catecholamine release in bovine aorta ECs. We then evaluated by immunohistochemistry the expression of tyrosine hydroxylase, Dopa decarboxylase, DβH, and phenylethanolamine-N-methyl transferase in femoral arteries from hindlimbs of C57Bl/6 mice 3 days after removal of the common femoral artery to induce chronic ischemia. Ischemia evokes tyrosine hydroxylase, Dopa decarboxylase, DβH, and phenylethanolamine-N-methyl transferase expression in the endothelium. Finally, the pharmacological inhibition of catecholamine release by fusaric acid, an inhibitor of DβH, reduces the ability of ECs to form network-like structures on Matrigel matrix. In conclusion, our study demonstrates for the first time that ECs are able to synthesize and release catecholamines in response to ischemia. (*Hypertension*. 2012;60:129-136.) ● **Online Data Supplement**

Key Words: catecholamines ■ ischemia ■ endothelium ■ angiogenesis

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Endothelial cells (ECs) cover the interior surface of blood vessels throughout the entire circulatory system, and they are involved in many aspects of vascular biology.[1–4] Indeed, angiogenesis is a phenomenon intimately associated with EC migration and proliferation during embryonic development.[5] Similarly, ECs play major roles in immune and inflammatory reactions by regulating lymphocyte and leukocyte migration into tissues by means of direct interaction with ECs.[6] Moreover, ECs have an important role in the regulation of the vascular tone by releasing vasoactive agents controlling smooth muscle cell proliferation and contractility.

Indeed, ECs are known to release both vasodilators (NO) and vasoconstrictors (thromboxane, platelet-derived growth factor, and endothelin 1) in response to local and circulating stimuli.[7–14] To this purpose, the endothelium is regulated in a fine way by a series of receptors that are expressed on its surface, including adrenergic receptors (ARs), such as α_1 and β_2AR.[15,16] Circulating catecholamines are thought to be the natural agonist of these receptors, giving the fact that ECs are not reached by sympathetic innervation.[1]

The endothelium derives from the embrional mesenchimal sheet, so it does the population of myeloid lineage-restricted bone marrow progenitors.[17] The common features between the 2 lineages are suggested by the observation that endothelial progenitors are released in the bloodstream from bone

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marrow myelocytic progenitors.[18] This finding is consistent with results from several laboratories that have reported circulating cells with myeloid features contributing to human angiogenesis.[19–22]

Circulating catecholamines, dopamine, norepinephrine (NE), and epinephrine (EPI), regulate vascular tone via αAR activation, leading to vasoconstriction in most systemic arteries and veins. They are synthesized from the amino acid precursor l-tyrosine. Tyrosine hydroxylase (TH) is the first rate-limiting enzyme in catecholamine synthesis that catalyzes the conversion of tyrosine to l-dihydroxyphenylalanine. This latter is converted to dopamine by Dopa decarboxylase (DDC). On turn, dopamine is converted to NE by dopamine β hydroxylase (DβH), and NE is converted to EPI by phenylethanolamine-*N*-methyl transferase (PNMT).[23–26] Once released, catecholamines are quickly inactivated by 2 enzymes that are responsible for their catabolism, catechol-oxymethyltransferase and monoamine oxidase.[27,28] Monoamine oxidase catalyzes the oxidative deamination of amines, and catechol-oxymethyltransferase methylates the hydroxyl group meta to the side chain of catecholamines.[29] Until a few years ago, catecholamines were considered mainly as conventional neurotransmitters and neuroendocrine mediators, synthesized in the adrenal medulla. However, it has been demonstrated recently that they are also produced and used by cells of the immune system.[30–36] Indeed, catecholamines are actively produced by macrophages and have the capacity to act in an autocrine way on ARs to regulate macrophage production of interleukin 1β, which has a key role in the inflammatory response.[37]

Giving the common myeloid origin of ECs and macrophages and the ability of cells of the immune system to produce catecholamines, the aim of this study was to verify whether ECs were also able to autonomously synthesize and release catecholamines for signaling purposes.

Materials and Methods

All of the experiments were performed as described previously.[38] Extended details of Methods are described in the online-only Data Supplement.

Results

ECs Produce Catecholamines

To assess the ability of ECs to synthesize catecholamines, we first evaluated the expression of genes coding for the enzymes involved in the synthesis of catecholamines by real-time RT-PCR both in basal conditions and in response to hypoxia. We performed a time course experiment evaluating enzyme gene expression at 1, 3, 6, 16, and 24 hours of hypoxia (Figure 1A). Hypoxia-induced TH, DDC, DβH, and PNMT gene expressions respect to basal conditions in a time-dependent manner. At 16 hours of hypoxia, we found the maximum expression of these enzymes, and then cells start to die. Based on such data, we chose to incubate cells in hypoxic conditions for 16 hours, because it is an intermediate time between enzyme maximum expression and cell death. We confirmed this result by Western blot analysis. Figure 1B shows that hypoxia increased protein levels of TH, DDC, DβH, and PNMT in whole cell lysates. Total lysate from
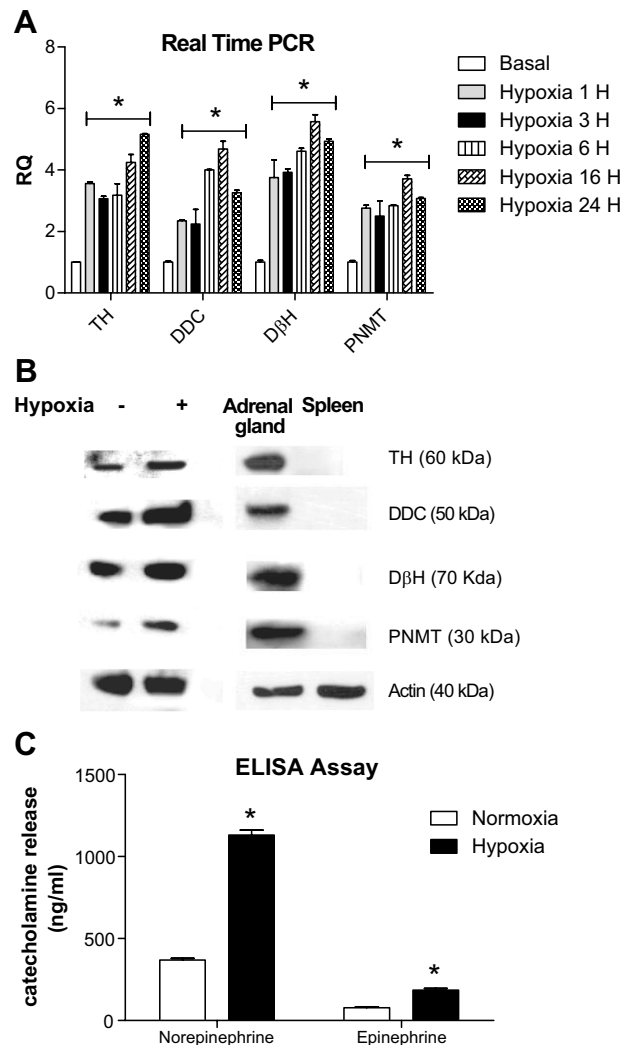

Figure 1. Endothelial cells synthesize catecholamines in response to hypoxia. **A**, RNA from bovine aorta endothelial cells (BAECs) was converted to cDNA and analyzed by real-time PCR to evaluate the expression of tyrosine hydroxylase (TH), Dopa decarboxylase (DDC), dopamine β hydroxylase (DβH), and phenylethanolamine-*N*-methyl transferase (PNMT) in response to hypoxia (1, ▥; 3, ■; 6, vertical lines; 16, ▨; and 24 hours, dotted). All of the enzymes are expressed in basal conditions and hypoxia enhanced their expression in a time-dependent manner; *P<0.05 vs normoxia. Results are the mean of 5 independent experiments. **B**, TH, DDC, DβH, and PNMT were evaluated in whole cell lysates by Western blot. Hypoxia increases TH, DDC, DβH, and PNMT levels. Lysates from adrenal gland and spleen were used, respectively, as positive and negative controls. Actin was used as loading control. Images are representative of 3 independent experiments. **C**, Catecholamines release was evaluated by ELISA assay in the culture medium of BAECs in basal conditions and after 16 hours of hypoxia. Hypoxia induces both norepinephrine (NE) and epinephrine (EPI) release. *P<0.05 vs normoxia. Results are the mean of 5 independent experiments. □, normoxia; ■, hypoxia.

mouse adrenal glands and spleens were used, respectively, as positive and negative controls. We also verified enzyme expression in bovine aorta ECs by immunohistochemistry. TH, DDC, DβH, and PNMT were all expressed in basal condition, and their expression was enhanced by hypoxic stimulus (Figure S1, available in the online-only Data Supplement). Immunohistochemistry shows that DDC, DβH, and
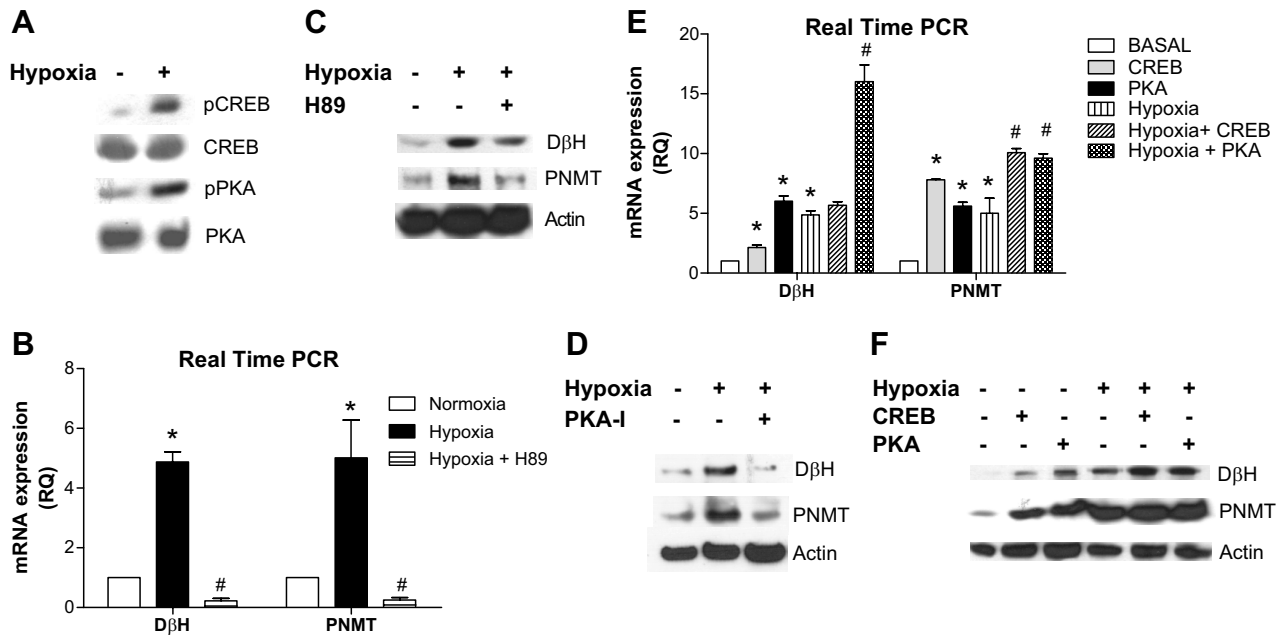
Figure 2. Endothelial cell (EC) regulates catecholamine release by hypoxia-induced protein kinase A (PKA)/cAMP response element binding (CREB) signaling activation. **A**, We evaluated hypoxia-induced CREB and PKA activation by Western blot using an antibody against the phosphorylated form of CREB and PKA. Hypoxia induces both CREB and PKA activation. Images are representative of 3 independent experiments. **B** and **C**, To evaluate the effect of PKA/CREB signaling on catecholamines release in bovine aorta ECs (BAECs), we analyzed by real-time PCR and Western blot the expression of dopamine β hydroxylase (DβH) and phenylethanolamine-*N*-methyl transferase (PNMT) in response to a selective inhibitor of PKA, H89. H89 inhibits both DβH and PNMT gene expression (**B**) and protein levels (**C**) in response to hypoxia. Actin was used as loading control for western blot analysis; *$P<0.05$ vs normoxia, #$P<0.05$ vs hypoxia. Results are the mean of 5 independent experiments. **B**, □, normoxia; ■, hypoxia; horizontal stripe, hypoxia+H89. **D**, To confirm the involvement of PKA in this phenomenon, we inhibited PKA activity by means of a peptidic inhibitor that is more selective and specific respect to H89, peptidic inhibitor of PKA (PKA-I). PKA-I inhibits both DβH and PNMT protein levels in response to hypoxia. Actin was used as loading control. **E** and **F**, To confirm these data, we overexpressed in cells CREB and PKA by transient transfection and analyzed DβH and PNMT levels. Both CREB and PKA overexpression increase DβH and PNMT gene expression (**E**) and protein levels (**F**) both in basal condition and in response to hypoxia. Actin was used as loading control for Western blot analysis; *$P<0.05$ vs normoxia, #$P<0.05$ vs hypoxia. Images are representative of 3 independent experiments. **E**, □, basal; ▦, CREB; ■, PKA; vertical lines, hypoxia; ▨, hypoxia+CREB; dots, hypoxia+PKA.

PNMT presented a cytosolic and perinuclear localization, whereas TH was mainly localized in the nucleus. Finally, to confirm the ability of bovine aorta ECs (BAECs) to produce catecholamines, we analyzed NE and EPI release in the culture medium by ELISA assay (Figure 1C). Hypoxia enhanced both NE and EPI release with respect to normoxia.

Hypoxia Regulates Catecholamine Synthesis in EC by Activation of Protein Kinase A/cAMP Response Element Binding Signaling

To assess the signal transduction pathway that regulates catecholamine synthesis in ECs, we evaluated protein kinase A (PKA) signaling, because it is known that PKA/cAMP response element binding (CREB) activation induces TH transcription and activity in response to stress.[39,40] We first analyzed by Western blot the effect of hypoxia on the activation of CREB and PKA. Figure 2A shows that hypoxia induces the expression of the phosphorylated and activated forms of both CREB and PKA, confirming that, also in BAECs, hypoxia regulates PKA/CREB signaling. Based on such data, we evaluated whether hypoxia-induced PKA/CREB activation regulates catecholamine synthesis in BAECs. In these experiments, we focused on DβH and PNMT expression, being the key enzymes for NE and EPI

synthesis. To assess whether this signaling was involved in the regulation of catecholamine synthesis, we pharmacologically inhibited PKA/CREB signaling using a selective inhibitor of PKA, H89. This latter inhibited hypoxia induced DβH and PNMT gene expression (DβH, $-73.7\pm0.03\%$ and PNMT, $-75.2\pm0.04\%$ versus hypoxia; Figure 2B) and prevented hypoxia-evoked increase of DβH and PNMT protein levels (Figure 2C). To further confirm these data, we used a peptidic inhibitor of PKA (PKA-I), which is more specific and selective respect to H89. PKA-I reduced both DβH and PNMT protein levels in response to hypoxia (Figure 2D). These data demonstrate that, in BAECs, hypoxia induces PKA/CREB signaling, and the activation of this pathway regulates catecholamine synthesis. To confirm these results, we overexpressed in BAECs both PKA and CREB by means of transient transfection. Both CREB and PKA overexpression enhanced DβH (CREB, $+2.1\pm0.09$; PKA, $+6\pm2.01$-fold of basal) and PNMT (CREB, $+9.8\pm0.3$; PKA, $+5.6\pm1.7$-fold of basal) gene expression in basal conditions (Figure 2E). PKA overexpression further enhanced the response to hypoxia (DβH $+16\pm0.6$ and PNMT $+9.6\pm0.2$ versus hypoxia; Figure 2E). Accordingly, Western blot analysis showed that CREB and PKA overexpression increased DβH and PNMT protein levels, and PKA overexpression
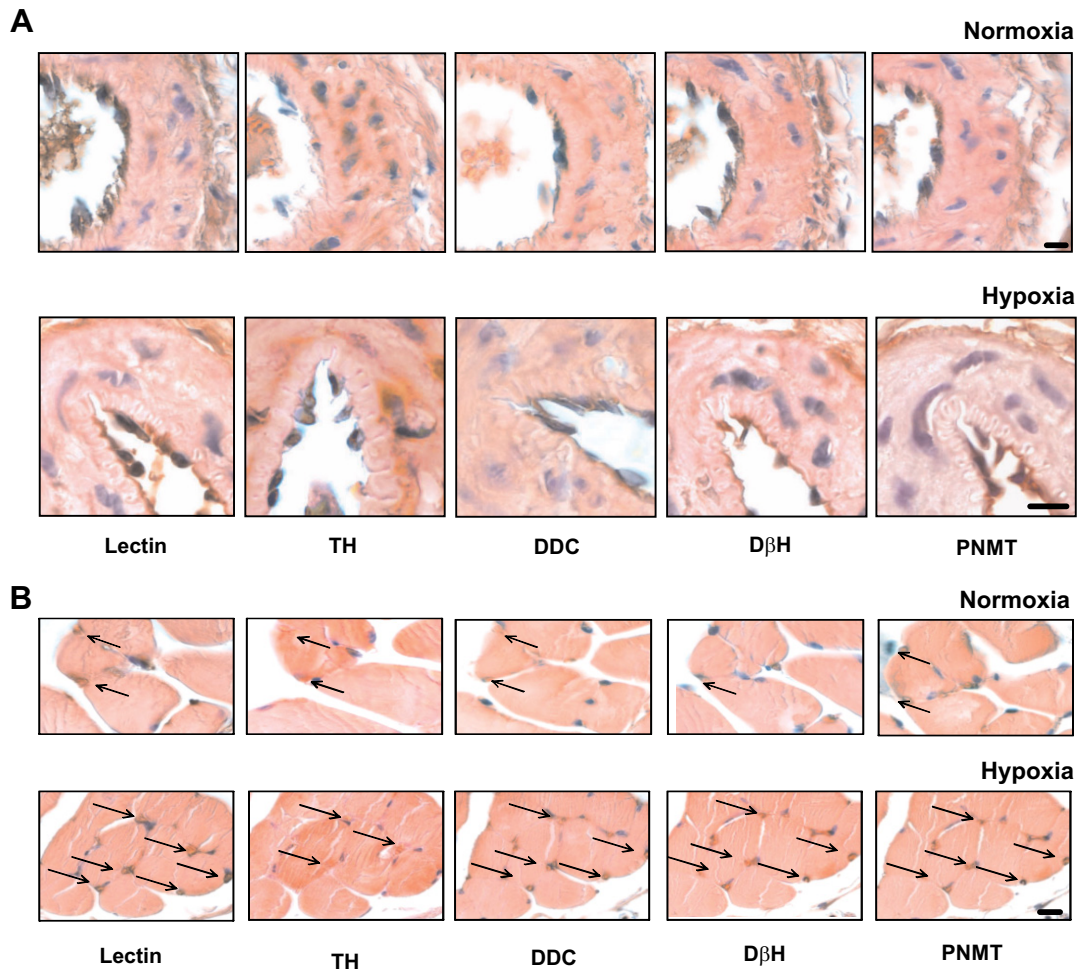
Figure 3. Endothelial cell (EC) produce catecholamines in vivo. Immunohistochemical analysis of tyrosine hydroxylase (TH), Dopa decarboxylase (DDC), dopamine β hydroxylase (DβH), and phenylethanolamine-*N*-methyl transferase (PNMT) expression was performed in superficial femoral arteries from hindlimbs of C57/black mice 3 days after removal of the common femoral artery. Ischemia induces TH, DDC, DβH, and PNMT expression in the endothelium of femoral arteries (**A**) and in capillaries (**B**), evidenced in the femoral muscle by lectin staining. The expression of TH in endothelium is just evident at a higher magnification. TH is also expressed in the muscle tissue, and its expression is enhanced in response to ischemia (**B**). Scale bar is 20 μm. Images are representative of 3 independent experiments.

further increased the response to hypoxia (Figure 2F). Moreover, PKA/CREB signaling also regulated catecholamine release in the culture medium (Figure S2). Indeed, H89 inhibited hypoxia-induced NE (Figure S2A) and EPI (Figure S2B) release. CREB overexpression caused NE (Figure S2C) and EPI (Figure S2D) release. In these conditions, hypoxia was no longer able to induce further accumulation (Figure S2C and S2D). Also, PKA overexpression increased catecholamine release (Figure S2C and S2D), and in these cells hypoxia was still able to induce a modest increase of catecholamine release. These data indicate that hypoxia-induced activation of the PKA/CREB signaling transduction pathway regulates catecholamine synthesis and release in BAECs.

Endothelium Produces Catecholamines In Vivo

To confirm in vitro data, we evaluated in vivo by immunohistochemistry the expression of TH, DDC, DβH, and PNMT in superficial femoral arteries from hindlimbs of C57Bl/6 mice 3 days after surgery. Ischemia induced DDC, DβH, and

PNMT expression in the endothelium of femoral arteries (Figure 3A) and in capillaries, highlighted in the femoral muscle by lectin staining (Figure 3B). On the contrary, TH was maximally expressed in the cytoplasm of muscle cells, although a higher magnification also revealed an endothelial localization. Negative control with secondary antibody only was shown in Figure S3.

Endothelium-Dependent Catecholamine Release Has a Key Role in Angiogenesis In Vitro

To evaluate the physiopathological role of endothelium-dependent catecholamine release in vitro, we evaluated the ability of BAECs to form network-like structures on Matrigel matrix in the presence or absence of a selective inhibitor of DβH, fusaric acid (FA). Figure S4 shows that hypoxia induced vascular network formation and FA inhibited the response to hypoxia (Figure S4A). To confirm that the effect of FA on angiogenesis is ascribed to its ability to inhibit catecholamine release, we evaluated NE and EPI release in the culture medium by ELISA assay (Figure S4B and S4C).

FA inhibited hypoxia-dependent catecholamine release (NE, $-32\pm1.7\%$; EPI, $-37\pm2.1\%$ versus hypoxia). These data suggest that ECs autonomously produce catecholamines that have a key role in the regulation of EC proangiogenic responses. To avoid the possibility that FA-dependent inhibition of angiogenesis in vitro was attributed to a toxic effect of the drug, we performed a proliferation assay using different dosages of FA. FA turned out to be not toxic for cells at doses from 10 nmol/L to ≤500 μmol/L (data not shown).

β_2AR Is Involved in Catecholamine-Induced Angiogenesis in BAECs

We have demonstrated previously that β_2AR has a key role in angiogenesis both in vitro and in vivo.[16,41] In particular, we demonstrated in vivo that β_2AR regulates angiogenesis in vivo in response to ischemia, because angiogenesis is impaired in β_2AR knockout mice and is ameliorated by reinstatement of β_2AR. Here we tested this effect also on hypoxia-induced angiogenesis in BAECs. To this aim we performed a real-time PCR analysis to evaluate the effect of β_2AR inhibition by the selective inhibitor ICI 118.551 on vascular endothelial growth factor gene expression. Our data show that β_2AR blockade is able to reduce vascular endothelial growth factor expression induced by hypoxia (Figure 4A), suggesting a key role for this receptor in catecholamine-induced angiogenesis in response to hypoxia. Furthermore, we evaluated whether catecholamine-induced β_2AR-dependent angiogenesis could, in turn, be regulated by β_2AR itself. To this aim, we performed a real-time PCR experiment to evaluate DβH and PNMT gene expression in cells with selective blockade of β_2AR by ICI 118.551. The blockade of β_2AR inhibited both DβH (Figure 4B) and PNMT (Figure 4C) gene expression in response to hypoxia. These data suggest that catecholamines from ECs could promote their own synthesis in a paracrine positive feedback manner by means of β_2AR activation.

Discussion

We report here, for the first time, that ECs are capable of synthesizing catecholamines because they have the complete intracellular machinery for the generation and release of NE and EPI. In vitro, ECs expressed all of the enzymes involved in the synthesis of catecholamines in basal conditions, and this expression is enhanced in response to hypoxia. We also identified the molecular mechanism that regulates this phenomenon. Indeed, we demonstrated that hypoxia-induced catecholamine release in the culture medium is regulated by the activation of PKA/CREB signaling, which is already known to regulate most important endothelial functions, such as endothelial NO synthase gene transcriptional activation.[42] These results are paralleled by data from an in vivo model of chronic ischemia, where we demonstrate that TH, DDC, DβH, and PNMT are expressed in the endothelium of the femoral artery. TH expression in endothelium, despite the other enzymes, is difficult to detect at low magnification and needs a higher enlargement. This is because of the nuclear localization of TH in BAECs, as demonstrated by immuno-histochemical analysis (Figure S1), which is difficult to detect in vivo in capillaries. Moreover, the nuclear staining is masked by hematoxylin counterstain. This nuclear localization of TH is suggested also by other reports, in human epidermal melanocytes[43] and in perikarya of rat ventral tegmental area.[44] Our in vivo results also show that TH is localized in the cytoplasm of muscle cells, and it was never described before. However, further experiments are needed to clarify this aspect.

ECs play a critical role in the control of vascular function because they participate in all aspects of vascular homeostasis but also in physiological or pathological processes like vascular wall remodeling, inflammation, or thrombosis. In particular, they have a key role in angiogenesis, in coagulation and fibrinolysis, and in the regulation of vascular tone, as well as in inflammatory reactions and in tumor neoangiogenesis.[45–50] ECs are capable of synthesizing and releasing a variety of substances that may exert autocrine, paracrine, or endocrine effects. Indeed, they contribute to the regulation of blood pressure and blood flow by releasing vasodilators such as NO and prostacyclin, as well as vasoconstrictors, including endothelin and platelet-activating factor, in response to stimuli.[14,51,52] Given the evidence that ischemia enhances plasma levels of NE,[53] it has been shown recently that catecholamines contribute to arteriogenesis and angiogenesis in pathological conditions such as hindlimb ischemia[54] and that they contribute to angiogenesis in the wound-healing process.[55] Based on our and previous data, we hypothesize that the ability of ECs to synthesize and release catecholamines is an autoregulatory physiological mechanism in response to hypoxia to induce neovascularization. Indeed, the inhibition of catecholamine release by FA reduced the proangiogenic phenotype of ECs in vitro. This finding puts the ARs on ECs in a different perspective. Indeed, the lack of terminal innervations on ECs has sustained the concept that circulating catecholamines are the natural stimulants to endothelial ARs. Our data allow for the promoting of a new scenario in which ECs produce catecholamines that act in a paracrine way to sustain proangiogenic phenotypes in conditions such as ischemia (Figure 4D). Furthermore, these data are well in agreement with previous reports showing the ability of ECs to produce catecholamine catabolism enzymes, such as monoamine oxidase and catechol-oxymethyltransferase.[56]

We have identified previously the β_2AR as the adrenergic receptor involved in the regulation of angiogenesis in ECs.[16,41] Here we demonstrated that β_2AR is activated by catecholamines released by ECs to induce angiogenesis, and it, in turn, regulates catecholamines release. A future direction of research will address the signaling linking hypoxia and PKA/CREB activation given the potential role of this pathway in the activation of the endothelium. In conclusion, our study demonstrates for the first time the ability of ECs to synthesize and release catecholamines in response to hypoxia (in vitro) and ischemia (in vivo) and the involvement of this phenomenon in the regulation of angiogenesis.

Perspectives

Our data indicate ECs as a source of catecholamine synthesis and release. This is a further advance in the understanding of endothelial function and physiology that could be useful to
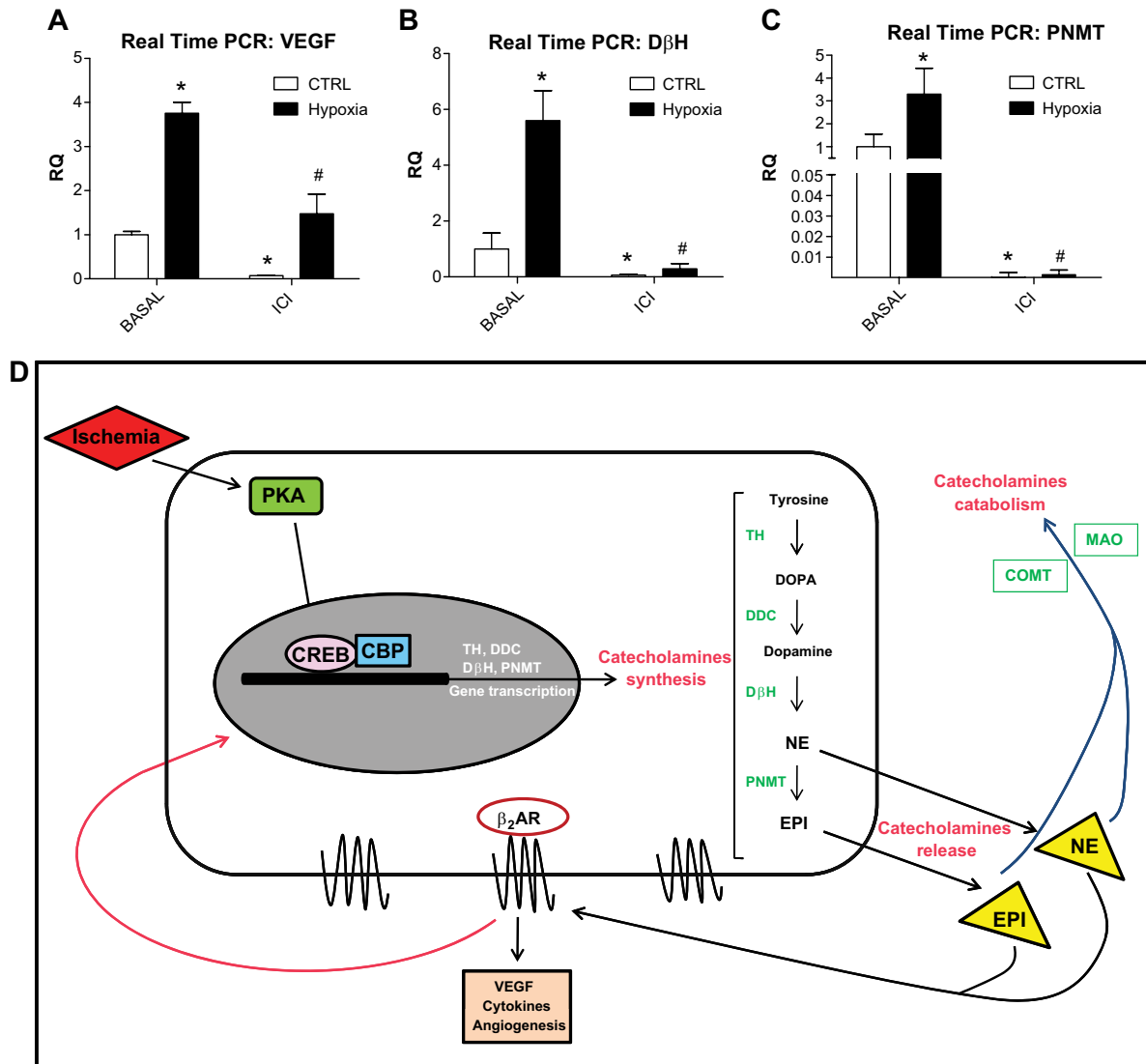
Figure 4. β_2-adrenergic receptor (AR) is involved in catecholamine-induced angiogenesis in bovine aorta endothelial cells (BAECs). BAECs were treated with ICI 118.551, 100 nmol/L, for 30 minutes and incubated in the hypoxic chamber for 16 hours. **A** through **C**, RNA from BAECs was converted to cDNA and analyzed by real-time PCR to evaluate the expression of vascular endothelial growth factor (VEGF; **A**), dopamine β hydroxylase (DβH; **B**), and phenylethanolamine-*N*-methyl transferase (PNMT; **C**) in response to hypoxia. All of the enzymes are expressed in basal conditions, and hypoxia enhanced their expression. ICI inhibits basal gene expression and reduces the response to hypoxia; *$P<0.05$ vs normoxia, #$P<0.05$ vs hypoxia. Results are the mean of 5 independent experiments. □, control (CTRL); ■, hypoxia. **D**, Schematic summary of our results: in endothelial cells, ischemia activates induced protein kinase A (PKA)/cAMP response element binding (CREB) signaling inducing the transcription of genes coding for tyrosine hydroxylase (TH), Dopa decarboxylase (DDC), DβH, and PNMT. All of these enzymes participate in catecholamine synthesis. Norepinephrine (NE) and epinephrine (EPI) are then released and act in a paracrine way on β2 adrenergic receptors to promote VEGF production and angiogenesis. The enzymes catechol-oxymethyltransferase (COMT) and monoamine oxidase (MAO) regulate catecholamine catabolism.

determine targeted therapies for many diseases, including cancer, cardiovascular disease, and inflammatory conditions.

Disclosures

None.

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Novelty and Significance

What Is New?

- ECs respond to stress-releasing catecholamines. These catecholamines stimulate receptors on EC accelerating regenerating processes, such as angiogenesis.

What Is Relevant?

- Endothelial dysfunction seen in hypertension might alter the ability of ECs to release catecholamines in response to stress. This impairment alters the ability of ECs to adapt to conditions such as ischemia or inflammation.

Summary

We demonstrate that ECs present the enzymes to produce and release NE and EPI, in response to ischemia. These substances, in turn, further sustain the activation of endothelial function, such as angiogenesis.

Endothelial Cells Are Able to Synthesize and Release Catecholamines Both In Vitro and In Vivo

Daniela Sorriento, Gaetano Santulli, Carmine Del Giudice, Antonio Anastasio, Bruno Trimarco and Guido Iaccarino

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ONLINE SUPPLEMENT SECTION

Endothelial cells are able to synthesize and release catecholamines both *in vitro* and *in vivo*

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Supplemental Methods

Cell Culture

Bovine aorta EC (BAEC), purchased from Lonza, were cultured in Dulbecco's MEM (DMEM) supplemented with 10% foetal bovine serum (FBS) at 37°C in 95% air and 5% CO2.

Plasmids

p-CREB and p-PKA were purchased from Stratagene; p-PKA-I was a kind gift of Prof. Antonio Feliciello (Federico II University of Naples). Transient transfection of these plasmids was performed using Lipofectamine 2000 (Invitrogen) in 70% confluent BAEC, accordingly to manufacturer instructions.

Hypoxia

24 hours after transient transfection, culture medium was replaced with hypoxia-medium (116 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO4, 26.2 mM NaHCO3, 1 mM NaH2PO4, 1.8 mM CaCl2, 0.01 mM glycine and 0.001 (% w/v) phenol red), previously saturated for 10 min at 1 atm with 95% N2 and 5% CO2 mixture. In some plates H89 (10^{-6}M, Sigma Aldrich), a selective inhibitor of PKA, was added to the culture medium for 30 minutes and then plates were incubated at 37°C in an anaerobic chamber (hypoxia chamber) filled with the same gas mixture for 16 hours.

Western Blot

BAEC were lysed in RIPA/SDS buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0, 25% deoxycholate, 9,4 mg/50 ml sodium orthovanadate, 20% SDS]. Protein concentration was determined by using BCA assay kit (Pierce). Total extracts were electrophoresed by SDS/PAGE and transferred to nitrocellulose. Endogenous pCREB, pPKA, TH (Cell signaling), Actin, DDC, DβH and PNMT (Santacruz) were visualized by specific antibodies, anti-rabbit HRP-conjugated secondary antibody (Santa Cruz) and standard chemiluminescence (Pierce).

Immunocytochemistry and Immunohistochemistry

Transfected cells were grown in chamber slides (Nunc, LabTek). Cells were then fixed with -20°C cold methanol and permeabilized with 0.01% Triton X-100 in PBS. Primary antibodies incubation with anti-TH, anti-DDC, anti-DbH and anti-PNMT (Santacruz) at a 1:50 dilution were performed at room temperature for two hours. Specific secondary antibodies (Santacruz) at a 1:100 dilution were incubated at room temperature for 1 hour. The peroxidase was revealed in presence of 0,03% hydrogen peroxide and of an electron donor, 2,5% diaminobenzidine, which becomes visible as a brown precipitate. Cells were then counterstained with hematoxylin, then dehydradated, cleared with histolemon and coverslipped. For immunohistochemistry, femoral muscles and arteries were fixed in formaline, embedded in paraffin and sectioned at 5 μm with a rotary microtome. Sections were dewaxed, rehydrated and immunostaining was performed by the peroxidase anti-peroxidase (PAP) method as described above. 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 RT-PCR

Total RNA was isolated using Trizol reagent (Invitrogen) and cDNA was synthetized by means of Thermo-Script RT-PCR System (Invitrogen), following the manufacturer instruction. After reverse transcription reaction, real-time quantitative polymerase chain reaction (PCR) was performed with the SYBR Green Real Time PCR master mix kit (Applied Biosystems). The reaction was visualized by SYBR Green Analysis (Applied Biosystem) on StepOne instrument (Applied Biosystem).

Primers for gene expression analysis were as follows:

TH: FOR 5'AGCCTGGCCTTCCGCGTGTTCCAG3'
                REV 5'CTACGCCTCCCGCATCCAGCGCCC3'

DDC: FOR, 5'CCAGAGACATTTGAGGCCAT3'

	REV 5'TCCAGCCAGAAACGCCTCT3',
DBH:	FOR 5'GAACATCAGCTATGCGCAGGA3'
	REV 5' AAAAGGCCTCTTGAAGAGCAG3'
PNMT:	FOR 5'TACCTCCGCAACAACTACGC3'
	REV 5' CTGTATACGCTCCAGTCGAA3'
18S:	FOR 5'GTAACCCGTTGAACCCCATT3'
	REV 5'CCATCCAATCGGTAGTAGCG3'
VEGF:	FOR 5'CAGGCTGTCGTAACGATGAA3'
	REV 5'TTTCTTGCGCTTTCGTTTTT3'

All values obtained were normalized to the values obtained with the 18S primers (endogenous control) and relative to the reference sample (basal control) using the formula $2^{-\Delta\Delta Ct}$. Obtained results are expressed as relative quantification (RQ).

ELISA assay

The release of catecholamines in the culture medium was analyzed by ELISA assay (Pantec), accordingly to the manufacturer instructions. Cell were transfected and incubated in the hypoxia chamber for 16 hours as described above. In some experiments Fusaric Acid (Sigma, 10μM) was added to the culture medium before hypoxic stimulus to inhibit catecholamines release. Culture medium was collected and used for the ELISA assay.

Angiogenesis *in vitro*

Angiogenesis *in vitro* was performed on Matrigel matrix as previously described [1].

In Vivo Study.

Experiments were carried out, in accordance to Federico II University guidelines and to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals, on 12-week-old C57Bl/6 mice, which had access to food and water *ad libitum*. The model of unilateral hindlimb (HL) ischemia was prepared as described previously [1]. Briefly, anesthesia was performed with an intramuscular injection of a mixture of tiletamine (50 mg/kg) and zolazepam (50 mg/kg); the right common femoral artery was exposed, isolated and permanently closed after the emergence from the inguinal ligament with a non-reabsorbable suture (5–0 silk, Ethicon) whereas the femoral vein was clamped. Afterwards, the common femoral artery was removed and the wound closed in layers. After 3 days, femoral muscles and arteries were extracted from both legs of five animals/group, fixed in formalin and included in paraffin. Paraffin-embedded sections were stained for hematoxylin and eosin. Five micrometer-thick sections were processed for the triple-layered immunohistochemical PAP (peroxidase anti-peroxidase) method as described above. For negative controls, the primary anti-serum was omitted. Lectin staining (1:100, Sigma Aldrich) was used to identify capillaries.

Statistical anlaysis

All values are presented as mean±SEM. Two-way ANOVA was performed to compare the different parameters between the different groups. A P value < 0.05 was considered to be significant. Statistics were computed with GraphPad Prism version 5.01 (GraphPad Software).

Supplemental References

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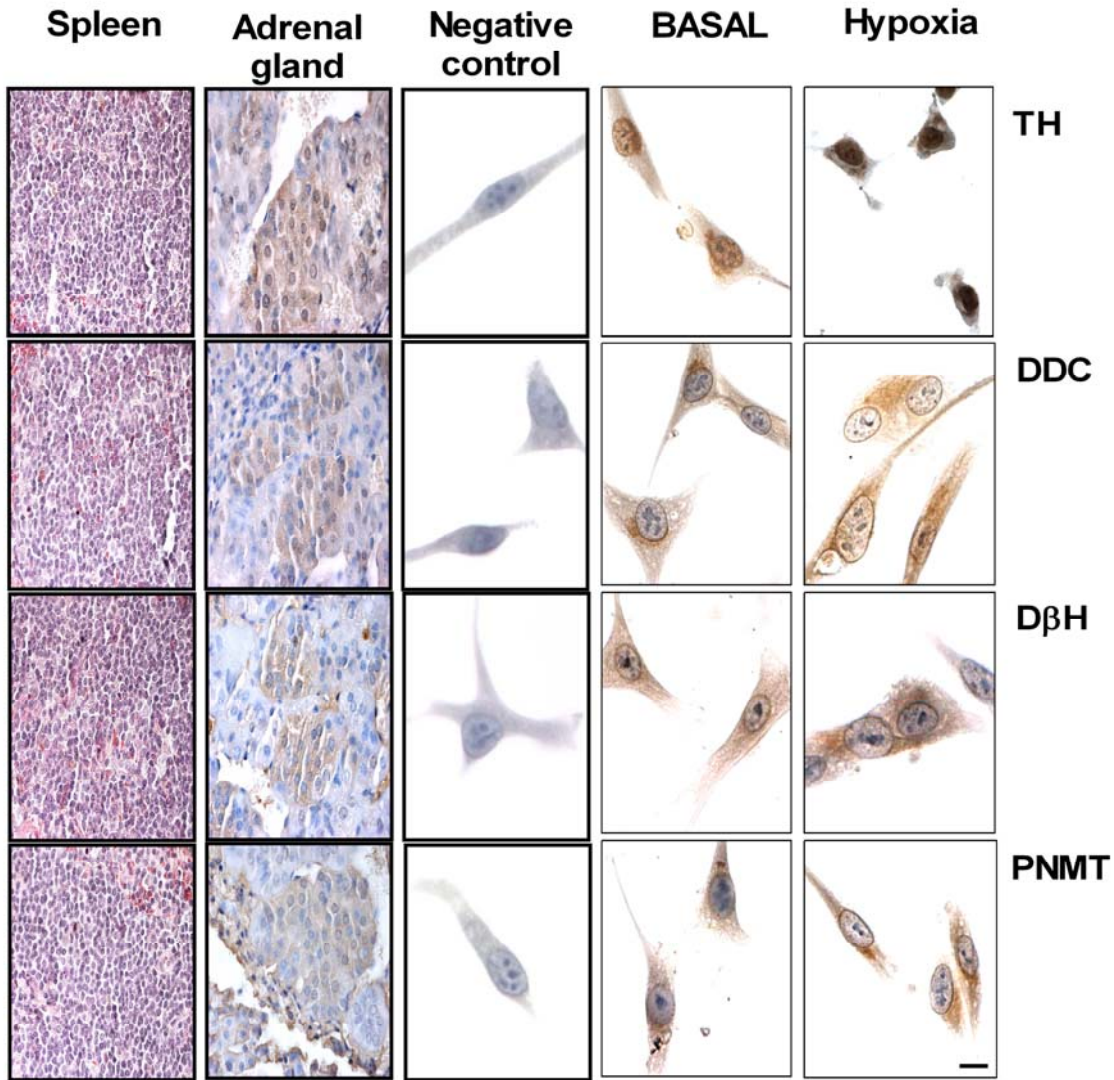
Supplemental Figures

FIGURE S1

Figure S1. Immunohistochemistry analysis of the expression of TH, DDC, DβH and PNMT in BAEC.
Paraffin embedded sections from adrenal gland and spleen and methanol fixed EC were analyzed by immunohistochemistry using the PAP peroxidase anti-peroxidase system. DDC, DβH and PNMT are localized in the cytosol of EC and their expression is enhanced after 16 hours of hypoxia. TH present a nuclear localization. Scale bar is 20 μm. Adrenal gland was used as positive control and spleen as negative control. A negative control in EC was performed incubating cells with secondary antibody only to avoid false positive. Images are representative of 3 independent experiments.
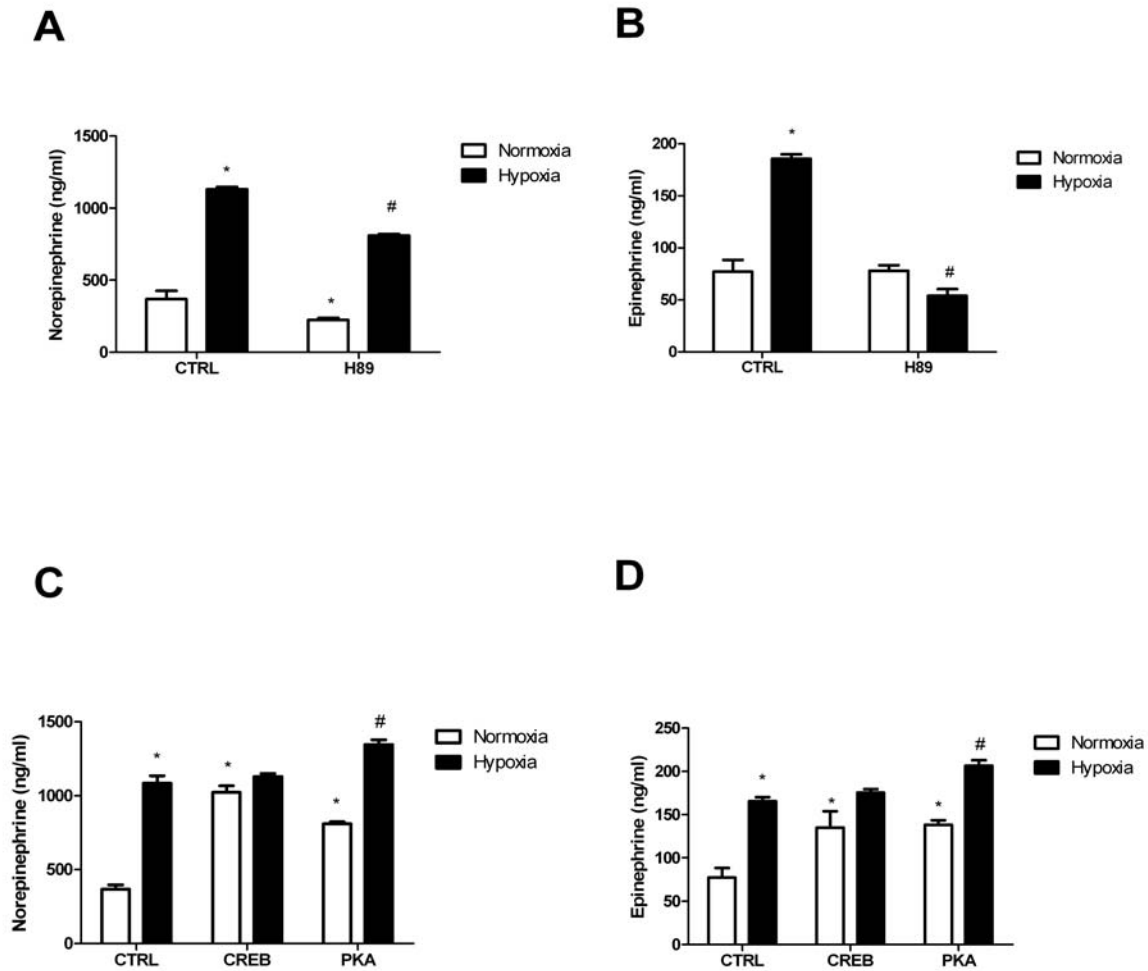
FIGURE S2

Figure S2: PKA/CREB regulates catecholamines release in the culture medium
BAEC were transfected with p-CREB and p-PKA or treated with H89 and then incubated in the
hypoxic chamber for 16 hours. Culture mediums were collected and used for ELISA assay. **A-D)**
H89 inhibits catecholamines release induced by hypoxia (A-B). CREB and PKA overexpresion
increase NE (C) and EPI (D) release respect to basal levels. PKA overexpression further increases
the response to hypoxia; * $p<0.05$ vs normoxia, # $p<0.05$ vs hypoxia. Results are the mean of 5
independent experiments.

FIGURE S3

A

Normoxia

Hypoxia

TH   DDC   DβH   PNMT

B

Normoxia
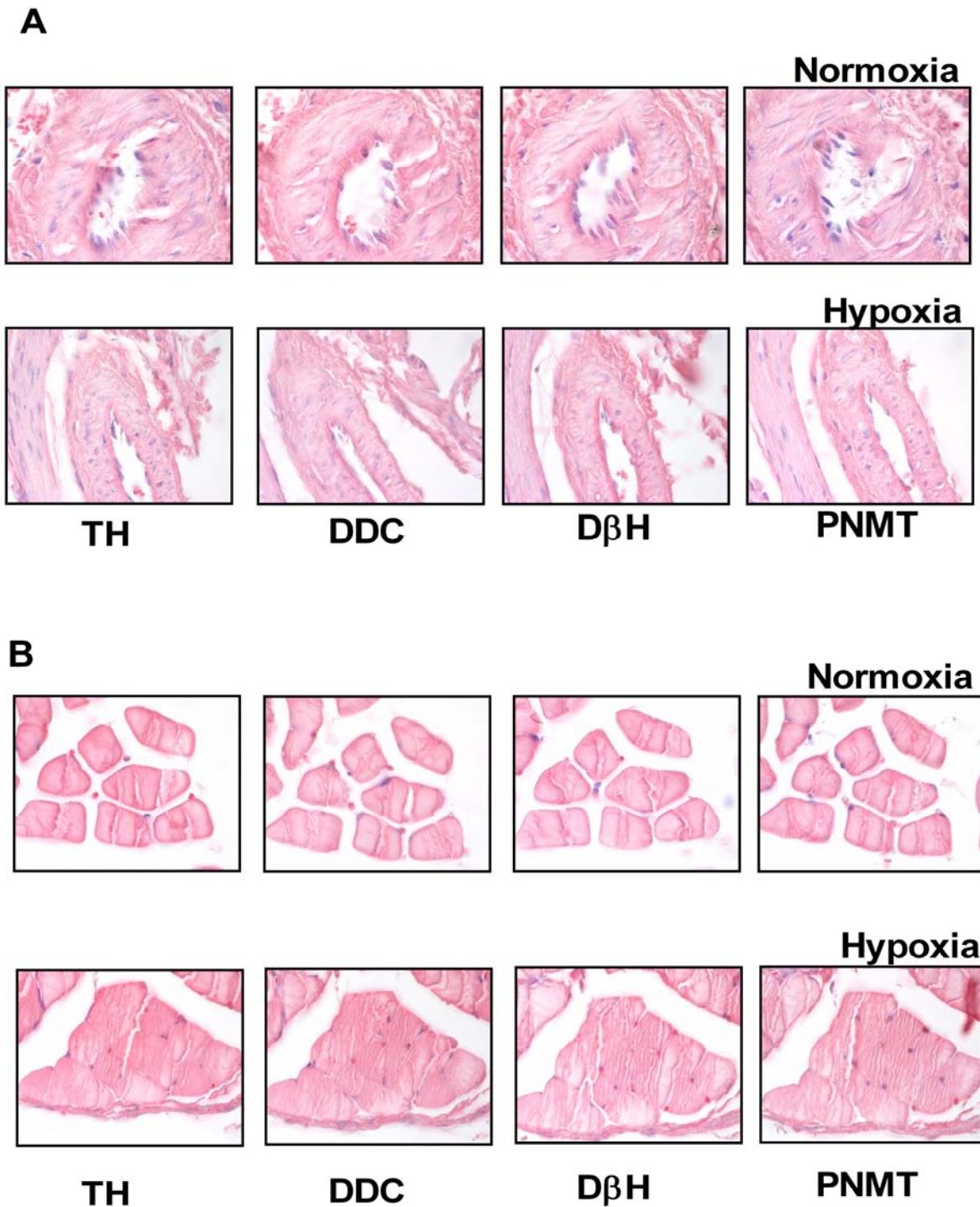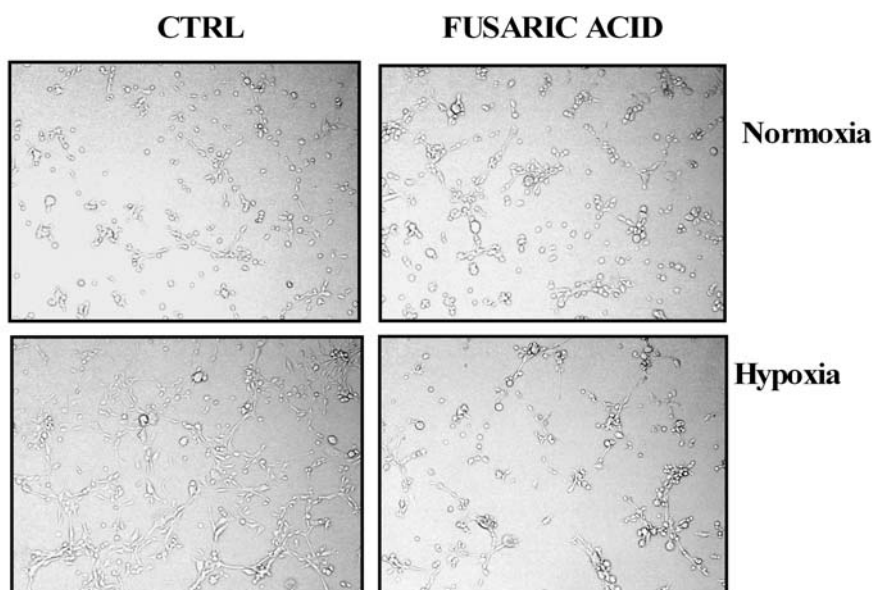
Hypoxia

TH   DDC   DβH   PNMT

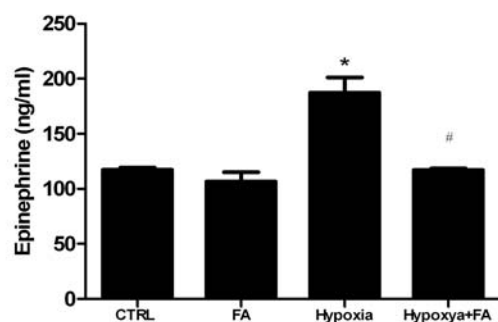**Figure S3: EC produce catecholamines in vivo: negative control**
To confirm the specificity of immunohistochemical data about TH, DDC, DβH and PNMT expression in BAEC (Fig. 3) we performed a negative control incubating tissues with secondary antibody only [femoral arteries (**A**) and in capillaries (**B**)].
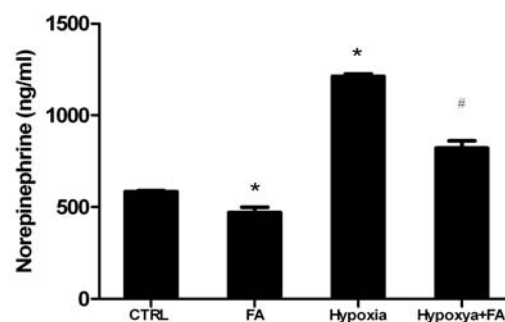
FIGURE S4

A

B **C**

Figure S4: EC dependent catecholamines release has a key role in angiogenesis *in vitro*.
BAEC were treated with fusaric acid (FA, 10 μM) for 30 min and incubated in the hypoxic chamber for 16 hours. **A**) Cells were plated on matrigel matrix and the formation of network-like structures was analyzed by microscopy. FA inhibits hypoxia induced BAEC organization in tubular structures. Images are representative of 3 independent experiments. **B-C**) Cultured mediums were collected and used for ELISA assay. FA inhibits hypoxia-induced norepinephrine (NE, B) and epinephrine (EPI, C) release. * $p<0.05$ vs normoxia, # $p<0.05$ vs hypoxia. Results are the mean of 5 independent experiments.