Placenta 33 (2012) 360–366

Contents lists available at SciVerse ScienceDirect

Placenta

journal homepage: www.elsevier.com/locate/placenta

Role of arginase-2 and eNOS in the differential vascular reactivity and hypoxia-induced endothelial response in umbilical arteries and veins

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ARTICLE INFO

Article history:
Accepted 4 February 2012

Keywords:
Umbilical
Artery
Vein
Arginase
eNOS
Endothelium
Vasodilation
Hypoxia

ABSTRACT

The main vasodilator in the placenta is nitric oxide (NO), which is synthesized by endothelial NO synthase (eNOS). Arginase-2 competes with eNOS for L-arginine, and its activity has been related with vascular dysfunction. Recently, we showed that hypoxia induces arginase-2, and decreases eNOS activity in human umbilical vein endothelial cells (HUVEC). However there is evidence that vascular responses to hypoxia are not similar throughout the placental vascular tree. We studied whether arginase-2 plays a role controlling vascular tone in human umbilical vessels, and the changes in the expression of arginase-2 and eNOS proteins by hypoxia in endothelial cells from umbilical arteries (HUAEC) and veins (HUVEC). In isolated umbilical vessels the presence of eNOS and arginase-2 was determined in the endothelium, and the NO-dependent vasoactive responses in the presence and absence of S-(2-boronoethyl)-L-cysteine (BEC, arginase inhibitor) were studied. Additionally, HUAEC and HUVEC were exposed (0–24 h) to hypoxia (2% O_2) or normoxia (5% O_2), and protein levels of eNOS (total and phosphorylated at serine-1177) and arginase-2 were determined. In umbilical arteries and veins arginase-2 and eNOS were detected mainly at the endothelium. BEC induced a higher concentration-dependent relaxation in umbilical arteries than veins, and these responses were NOS-dependent. In HUAEC exposed to hypoxia there were no changes in eNOS and arginase-2 levels, however there was a significant increase of p-eNOS. In contrast, HUVEC showed an increase in arginase-2 and a reduction of p-eNOS in response to hypoxia. These results show that arginases have a vascular role in placental vessels counteracting the NOS-dependent relaxation, which is differentially regulated in placental artery and vein endothelial cells.

1. Introduction

Placental blood flow and its control during gestation are crucial for fetal development. The main vasodilator in the placenta is nitric oxide (NO) [1,2], a free radical synthesized mainly by the endothelial NO synthase (eNOS). In the placenta eNOS activity is mainly increased in response to shear stress[3], via a mechanism involving its phosphorylation at serine-1177 (p-eNOS^{1177}) by several stimuli [4]. Among several mechanisms controlling eNOS activity, this phosphorylation is considered an eNOS activity biomarker [5]. Additionally, endothelial-dependent NO release can be triggered at lower degree by agonists such as the calcitonin gene-related peptide (CGRP) [6–8].

Hypoxia is an important factor that regulates at short- and long-term NO synthesis and its vasoactive effects in placental vessels [4]. Placental vasculature responds to acute hypoxia with an increase in vascular resistance in an NOS-independent manner [9–12]. Apparently this response is restricted to small stem villi arteries, whilst larger arteries respond with an NOS-dependent vasodilation [11]. Additionally, in this hypoxic condition, the vasodilator response of placental arteries to NO-donors is unaltered, whilst in veins this response is increased [13]. On the other hand, chronic fetal hypoxia is associated with a decrease in eNOS expression and activity, and altered NO synthesis [4]. Umbilical vein endothelium from patients with sickle cell disease has lower levels of eNOS protein [14]. In cultured human umbilical vein endothelial cells (HUVEC) it has been shown that 24 h of hypoxia reduces L-arginine transport and decreases the level of activated eNOS [15]; these hypoxic characteristics are present in HUVEC from intrauterine growth restriction (IUGR) in normoxia and they are not further

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doi:10.1016/j.placenta.2012.02.006

affected by hypoxia [15]. Conversely, in sheep fetuses exposed for 15 days to hypoxia no changes in the expression of eNOS in the placental villous endothelium have been described [16]. The effect of chronic hypoxia on eNOS expression in human placental arteries is not clear. Previous studies have shown normal [17], increased [18] or decreased [19,20] eNOS levels in placental endothelium from IUGR and preeclampsia (PE). Based on these data we propose that in the umbilical vessels eNOS levels and activity are differentially regulated by hypoxia between arteries and veins.

Endothelial NOS activity is highly dependent on the availability of L-arginine [21,22]. Thus enzymes related with the metabolism of this amino acid, such as arginases have risen as determinant factors that could regulate endothelial NO synthesis leading to the modulation of vascular function [22]. Although there are two arginase isoforms, arginase-2 is the main isoform expressed in human endothelial cells in several vascular beds including the placenta [19,23] and umbilical vein endothelial cells (HUVEC) [24,25]. Normally, arginase activity is involved in placental and fetal growth [26,27] and the immune hypo-responsiveness of the mother [28]. In HUVEC, arginase-2 controls NOS activity restricting L-arginine availability [24]. Arginase-2 levels are increased in the placental endothelium of PE placentae [19]. Recently we have demonstrated that hypoxia increases arginase-2 expression and activity in HUVEC. The reduced eNOS activity induced by hypoxia is partially reverted by arginase inhibition [25]. However, there is no information addressing whether arginase-2 plays a physiological role as modulator of NO synthesis in placental endothelium.

Here we determined the presence of arginase-2 in the endothelium of umbilical arteries and veins, and its participation in the regulation NOS-dependent vasodilation. Additionally, we studied whether these placental endothelial cells from arteries and veins have a similar response to hypoxia, in terms of L-arginine/NO pathway. For this purpose, the protein levels of eNOS, p-eNOS^{1177}, and arginase-2 were determined in cultured endothelial cells from umbilical arteries (HUAEC) and veins (HUVEC) exposed to hypoxia.

2. Materials and methods

2.1. Study participants

Pregnant women attending routine antenatal care at the Maternity of the Hospital Clínico Pontificia Universidad Católica, Santiago, Chile, were invited to participate in the study. The women included in this study were nonsmoking, normotensive, and did not have preeclampsia, pregestational or gestational diabetes mellitus and none were on regular medication. Written consent was obtained from those who agreed to participate. The investigation conformed to the principles outlined in the Declaration of Helsinki, and the protocol was approved by the ethics committee of the Faculty of Medicine of the Pontificia Universidad Católica de Chile.

2.2. Wire myography

Umbilical arteries and veins (~3 cm from the insertion point in the placenta) from normal pregnancies were dissected and the Warton's jelly and connective tissue was removed. Vessel segments of 2 mm were mounted in a wire myograph (model 610A; Danish Myo Technology A/S, Aarhus, Denmark), maintained at 37 °C in Krebs buffer (in mmol/l: 118.5 NaCl, 25 $NaHCO_3$, 4.7 KCl, 2.5 $CaCl_2$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 5.5 D-glucose) with constant bubbling (5% CO_2 in air). Isometric force was recorded using a PowerLab data acquisition hardware (ADInstruments, Castle Hill, Australia) and LabChart (version 6; ADInstruments) software. After 30 min of equilibration, vessel internal circumferences were determined by measuring the maximal active force in response to KCl (65 mmol/l) as described [29]. To evaluate the participation of arginase activity in the vessels tone, ring vessels were preconstricted with 31.25 mmol/l KCl and the isometric force in response to cumulative concentrations of the arginase inhibitor S-(2-boronoethyl)-L-cysteine (BEC, 10^{-9}–10^{-5} mol/l) was measured. In order to determine the effect of the arginase activity on the NOS-dependent vasodilation, the response to calcitonin gene-related peptide (CGRP, 10^{-10}–10^{-7} mol/l) was determined in the absence or presence of BEC (10 μmol/l) with or without the NOS inhibitor N^G-nitro-L-arginine (L-NA, 100 μmol/l). Responses were expressed as a percentage of relaxation relative to maximal effects of KCl 31.2 mmol/l (%Kmax) and adjusted to concentration response curves.

2.3. Immunohistochemistry

Umbilical cords were washed with cold phosphate buffer saline solution (PBS, in mmol/l: 136 NaCl, 2.7 KCl, 7.8 Na_2HPO_4, 1.5 KH_2PO_4, pH 7.4), dissected in segments of 5 mm, treated overnight with paraformaldehyde (4% in PBS) and included in paraffin. Deparaffinized and rehydrated histological sections of 4 μm were subjected to heat-induced antigen retrieval using 100 mmol/l citrate buffer (pH 6.0) in a steam cooker for 15 min at 95 °C. Samples were treated with 3% H_2O_2 in PBS for 30 min to quench endogenous peroxidase activity. After rinsing in PBS for 5 min, all slides were incubated for 1 h with protein block solution (Cas-Block, Zymed Laboratories, South San Francisco, CA, USA). Sections were incubated for 18 h at 4 °C with primary rabbit anti-eNOS (4 μg/ml), rabbit anti-arginase-2 (4 μg/ml) and mouse anti-vWF (1 μg/ml) antibodies. Immunostaining was performed using HRP-conjugated secondary antibody and binding was determined with NovaRED kit (Vector, Burlingame, CA, USA), treating for 4 min. Slides were counterstained with Harris hematoxylin and permanently mounted. Specificity of the staining was determined by incubation of sections in the absence of the primary antibody. Sections were examined in an IX81-Olympus microscope, and the images were captured using a digital camera (Olympus DP-71) and DP-BSW software (Olympus) [30].

2.4. Cell isolation and culture

Umbilical cords were obtained immediately after delivery and transported from the maternity ward to our laboratory, where both human umbilical artery (HUAEC) and vein endothelial cells (HUVEC) were isolated from the same cord as described [15]. Briefly, umbilical arteries and veins were rinsed with warm (37 °C) PBS and endothelial cells were isolated by collagenase (0.2 mg/ml) digestion and cultured (37 °C, 5% CO_2) up to passage 2 in medium 199 (M199) containing 5 mmol/l D-glucose, 10% new born calf serum, 10% fetal calf serum, 3.2 mmol/l L-glutamine and 100 U/mL penicillin–streptomycin. The medium was fully replaced every 2 days until confluence.

2.5. Hypoxia

Cells were exposed (0, 6 and 24 h, 37 °C) to a gas mixture (5% CO_2-balanced N_2) to obtain 5% O_2 [normoxia, oxygen partial pressure (PO_2) ~33.9 mmHg] or 2% O_2 (PO_2 ~13.5 mmHg) in a hypoxia chamber connected to a PROOX 110 device (BioSpherix, NY, USA) provided with an oxygen sensor [15]. Control experiments were performed in cells cultured for 24 h in normoxia.

2.6. Arginase activity

Total urea production from L-arginine (60 min, 37 °C, 50 mmol/l L-arginine) was measured in whole protein extract from HUAEC and HUVEC as described [25]. Briefly, cells were washed (×2) with cold PBS (4 °C, pH 7.4) and incubated (5 min, on ice) with lysis buffer [1 μmol/l pepstatine A, 1 μmol/l leupeptine, 200 μmol/l phenylmethylsulfonyl fluoride (PMSF), 50 mmol/l Tris-HCl (pH 7.5), 0.2% Triton X-100]. Cell lysate was sonicated (20 pulses, 150 Watts, ×3) and total protein content was determined by Bradford method (BioRad, CA, USA). Aliquots (100 μg) of tissue lysate were preincubated (10 min, 55 °C) with 10 mmol/l $MnCl_2$ in 25 mmol/l Tris-HCl buffer (pH 7.5) and then mixed with 50 mmol/l L-arginine (60 min, 37 °C, pH 7.4). Reaction was stopped by addition (400 μL) of an acid mix (H_2SO_4:H_3PO_4:H_2O = 1:3:7 v/v). The reaction mix was then incubated (45 min, 100 °C) with 9% α-isonitrosopropiophenone (25 μL) for colorimetric determination of urea. Aliquots of 200 μL were then transferred to a 96-well plate and absorbance at 540 nm was measured in a microplate spectrophotometer (Thermo Labsystems, Waltham, MA, USA).

2.7. Western blotting

Proteins from freshly isolated endothelium and cultured HUAEC and HUVEC (70 μg) were separated in polyacrylamide gel (8–10%) electrophoresis, transferred to 0.45 μm nitrocellulose membranes (BioRad), blocked with 5% skimmed milk in Tris buffered saline (TBS) with 0.1% Tween, and probed with primary polyclonal rabbit anti-eNOS (0.4 μg/ml) (Santa Cruz Biotechnology, CA, USA), monoclonal mouse anti-eNOS phosphorylated at serine-1177 (p-eNOS^{1177}) (0.25 μg/ml) (BD Transduction Laboratories, CA, USA), polyclonal rabbit anti-arginase-2 (1 μg/ml) (Santa Cruz Biotechnology) or monoclonal mouse anti-ß-actin (0.4 μg/ml) (Sigma) antibodies. Membranes were washed in TBS/0.1% Tween, and incubated (1 h, 22 °C) in TBS/0.1% Tween containing horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse secondary antibodies. Proteins were detected by enhanced chemiluminescence and quantified by densitometry using Image J (NIH, USA) as described [15].

2.8. Statistical analysis

Values are mean ± S.E.M., where *n* indicates the number of different placentae from which the umbilical cord, vessels and endothelial cells were obtained. Comparisons between two and more groups were performed by means of Student's

unpaired *t*-test and analysis of variance (ANOVA), respectively. If the ANOVA demonstrated a significant interaction between variables, post hoc analyses were performed by Dunns when comparing selected groups and multiple-comparison Bonferroni correction test. Data from isolated vessel reactivity were adjusted to dose–response curves from which maximal response was obtained. Comparison of curves and maximal responses under different conditions were analyzed by ANOVA. All the analyses were carried out with the statistical software Graphpad Prism 5.03 (GraphPad Software Inc., San Diego, CA, USA). $P < 0.05$ was considered the cut-off for statistical significance.

3. Results

3.1. Expression of arginase-2 and eNOS in umbilical vessels endothelium

Umbilical arteries were distinguished from the umbilical vein by their higher post-natal vasoconstriction and the thickness of the vascular wall. In serial sections of umbilical vessels a strong labeling of vWF (Fig. 1A and D) was observed at the inner surface of the vessels. Arginase-2 protein (Fig. 1C and F) was detected either in arteries and vein and it was preferentially located in the endothelial layer. Similarly, in these vessels the presence of eNOS protein (Fig. 1B and E) was mainly limited to the endothelium.

3.2. Myography of umbilical vessels

The vasoactive responses of isolated umbilical arteries and veins were determined using a wire myograph bath. In pre-constricted umbilical arteries and veins, the arginase inhibitor BEC induced a concentration-dependent relaxation (Fig. 2A and B), which was higher in arteries (19.7 ± 2.4 %Kmax) than veins (7.1 ± 0.8 %Kmax) (Fig. 2C). These responses were significantly reduced when vessels were preincubated with the NOS inhibitor L-NA (100 μM) in both arteries (2.4 ± 0.4 %Kmax) and veins (1.2 ± 0.5 %Kmax) (Fig. 2A–C).

To determine the effect of arginase activity on NOS-dependent vasodilation, we evaluated the response to CGRP, which exerts its effects in fetoplacental vessels partially through eNOS activation [7,8]. CGRP induced a concentration-dependent relaxation in umbilical vessels that was similar in arteries (18.4 ± 2.9 %Kmax) (Fig. 3A) and veins (14.3 ± 2.1 %Kmax) (Fig. 3B). CGRP-induced relaxations in arteries and veins were almost completely inhibited (4.3 ± 1.7 and 0.4 ± 1.0 %Kmax respectively) by pre-incubation with L-NA (Fig. 3A and B). Subsequently, the maximal response to a single concentration of CGRP (100 nM) was determined in umbilical vessels preincubated with BEC (10 μM). Arginase inhibition induced a ~2.5 fold increase in the relaxation to CGRP in arteries (46.7 ± 11.9 %Kmax) and veins (42.0 ± 1.6 %Kmax), and this effect was prevented by co-incubation of BEC with L-NA (Fig. 3C and D).

3.3. Protein levels of eNOS and p-eNOS^{1177} in umbilical artery and vein endothelial cells exposed to hypoxia

Protein levels of eNOS and p-eNOS^{1177} (an index of the *in vivo* eNOS activity [5]) were determined in total cell extracts from HUAEC and HUVEC cultured in hypoxia (0, 6 and 24 h). In normoxia, relative levels of eNOS were lower in HUAEC (0.61 ± 0.12) compared with HUVEC (0.95 ± 0.13) (Fig. 4A), with comparable levels of p-eNOS^{1177} in both cell types. Hypoxia had no effect on the total protein levels of eNOS neither in HUAEC nor HUVEC (Fig. 4B and C). In contrast, endothelial cells from arteries and veins showed a differential regulation of p-eNOS by hypoxia. In HUAEC exposed to hypoxia, p-eNOS^{1177} was strongly increased after 6 h of hypoxia (~2 fold), that was maintained at 24 h (Fig. 4B and D). As previously reported [15], in HUVEC hypoxia induced a decrease in p-eNOS^{1177} levels at 6 hours that persist even at 24 h (Fig. 4B and D).

3.4. Protein levels of arginase-2 and arginase activity in umbilical artery and vein endothelial cells exposed to hypoxia

In normoxia, arginase-2 protein expression in total cell extracts was not different between HUAEC and HUVEC (Fig. 5A). However, hypoxia (6 and 24 h) increased arginase-2 protein levels in HUVEC (~2.3 fold), as we have recently described [25], without changes in HUAEC (Fig. 5B and C). Similarly, in hypoxia there was an important increase in arginase activity in HUVEC (~2.4 fold) which was unaltered in HUAEC (Fig. 5D).

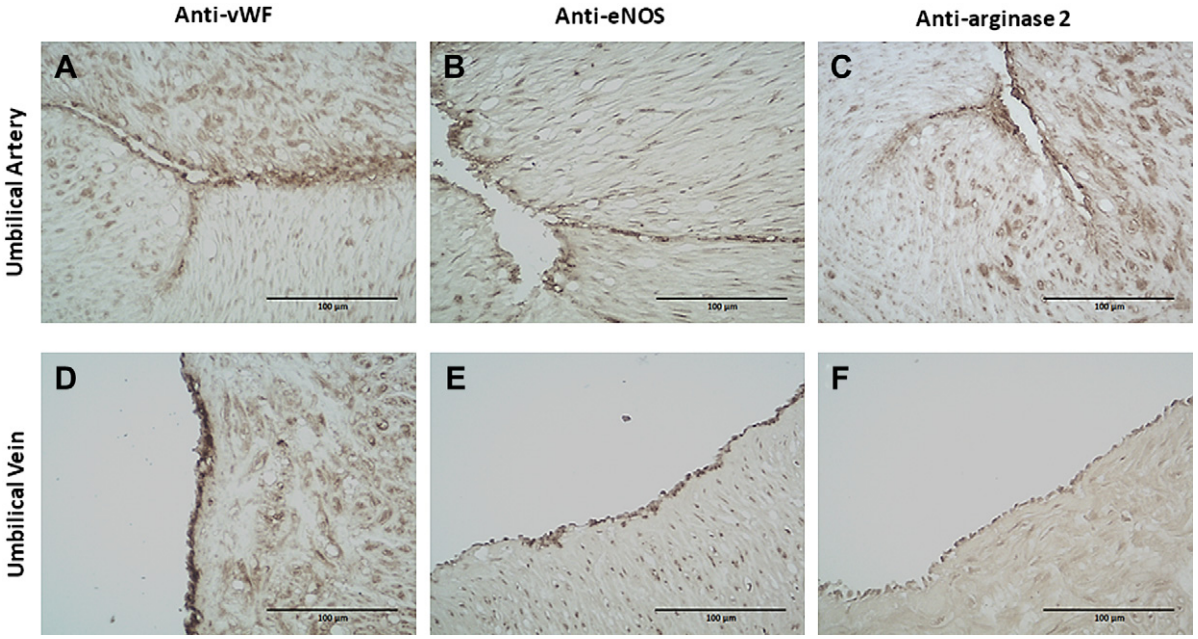

Fig. 1. Expression of eNOS and arginase-2 in umbilical vessels. The presence of von Willebrand Factor (vWF) (A, D), eNOS (B,E) and arginase-2 (C, F) in umbilical arteries (A, B and C) and veins (D, E and F) was detected in paraffin-embedded umbilical cords ($n = 4$) by peroxidase-coupled immunohistochemistry.

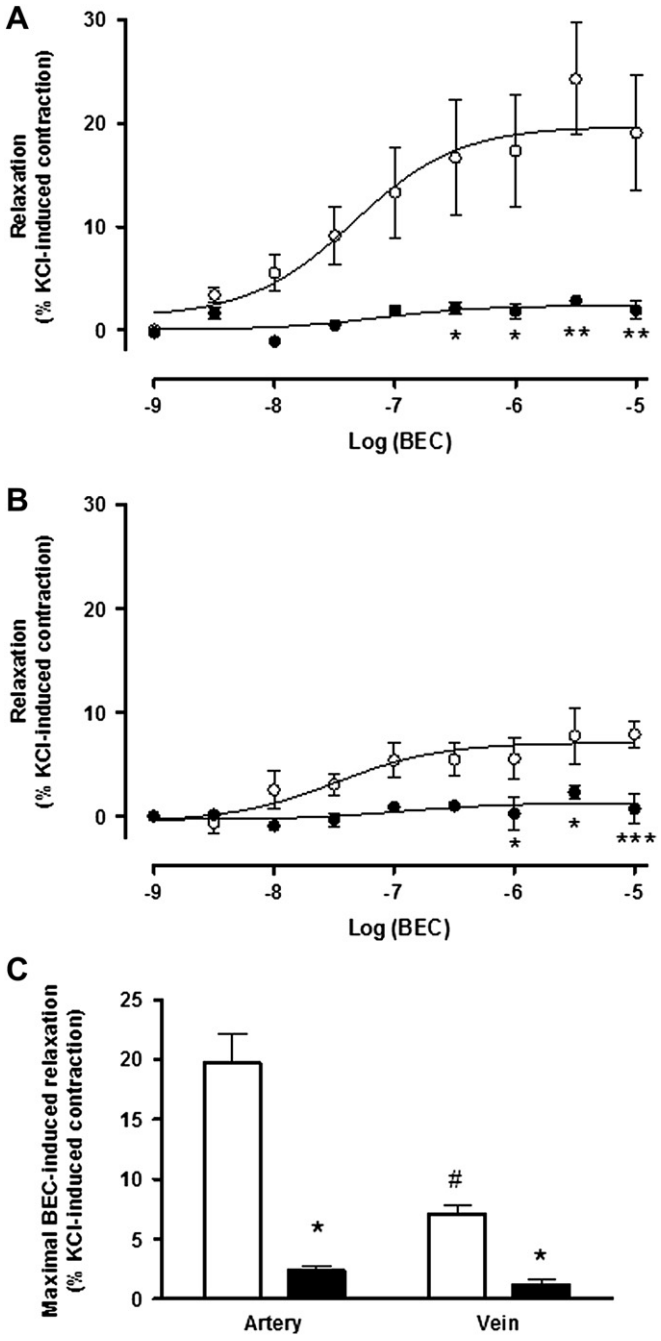

Fig. 2. Effect of arginase inhibition on isolated umbilical vessels tone. Concentration–response curves to the arginase inhibitor S-(2-boronoethyl)-L-cysteine-HCl (BEC, 10^{-9}–10^{-5} mol/l) in umbilical arteries (A, $n = 6$) and veins (B, $n = 6$) in the absence (open circles) and presence (solid circles) of the NOS inhibitor N^G-nitro-L-arginine (L-NA, 100 μmol/l). (C) Maximal relaxation induced by BEC in umbilical arteries and veins derived from (A) and (B). Values are mean ± S.E.M. $^*p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ versus corresponding values in absence of L-NA, $\#p < 0.05$ versus artery in the absence of L-NA.

4. Discussion

This study demonstrates that arginase-2 is present in the endothelium of umbilical vessels and its activity has a role as a negative regulator of the NOS-dependent vasodilation. In basal conditions this effect is different in arteries and veins, whilst it is similar when the eNOS- pathway is activated. On the other hand, the regulation of eNOS and arginase-2 proteins by hypoxia is different in umbilical endothelial cells from arteries and veins. Arterial endothelial cells express lower levels of eNOS compared with HUVEC, and these levels are not altered by hypoxia. Furthermore, HUAEC exposed to hypoxia showed unchanged levels of arginase-2 and increased levels of eNOS phosphorylation suggesting that arterial endothelial cells would maintain the NOS activity upon hypoxia. In contrast in vein endothelial cells, as it has been previously described [15,25], hypoxia decreased eNOS activation and increased arginase-2 expression and activity.

4.1. Vascular role of arginase in umbilical vessels

In the placenta, due to the lack of adrenergic and cholinergic innervations [31], local factors derived from the endothelium play a key role in the regulation of the vascular tone. The release of NO from umbilical and chorionic endothelium has been described since the late 1980s [32,33] at the same time when its vascular function was discovered. In placenta, the vasodilator effects of NO are higher than prostacyclin [1,2], and acts through cGMP-dependent [34] and -independent pathways [35,36]. Thus, the regulation of eNOS expression and activity, and NO bioavailability have been studied as altered mechanisms which could explain the placental vascular dysfunction present in some gestational pathologies [4,21]. Activity of eNOS is strongly influenced by the availability of its substrate L-arginine [21,22]. Arginases compete with eNOS by their common substrate L-arginine [24] and they have been implicated in the vascular dysfunction present in atherosclerosis [37], hypertension [38] and aging [22]. Arginase-2 is the predominant isoform expressed in the human endothelium as well as fetoplacental vasculature [19,23–25]. This study showed for the first time the presence of arginase-2 in the endothelium of umbilical arteries and veins. Furthermore, we demonstrated that arginase activity has a vasoconstrictor effect on the basal tone of umbilical vessels counteracting NOS activity.

Noteworthy, the effect of arginase activity on the basal vessel tone was totally dependent on NOS activity and it was different in arteries and veins; arginase inhibition induces a higher relaxation in umbilical arteries than veins. The latter could be explained by differences in the eNOS/arginase ratio [22] present in the vessel type studied; thus in the umbilical artery there could be a lower eNOS/arginase ratio compared with veins having the arginase inhibition a higher vasodilator effect in the former. Despite that protein expression *in vitro* could differ from its *in vivo* expression, this idea is supported by the protein levels of eNOS and arginase-2 present in freshly isolated (data not shown) as well as cultured HUAEC and HUVEC. Furthermore, other studies in freshly isolated endothelium show that HUAEC and HUVEC present similar eNOS protein levels, but the latter has ~6 fold higher NOS activity [39]. This data suggests the presence of alternative mechanisms controlling basal eNOS activity in these cells, which could include the competition with different arginase levels. Another explanation for the differential effects of arginase inhibition on vessel reactivity could be a different responsiveness to NO in umbilical arteries and veins; in this case the arginase inhibition would induce similar amounts of NO by the NOS activation. However this option can be neglected due to the fact that different studies have shown similar responsiveness to exogenous NO in umbilical arteries and veins [1,35,40].

The strongest physiological stimuli for the release of NO in placental vasculature is shear stress, which induces eNOS phosphorylation at serine-1177 (long-term activation) via ERK1/2 and Akt [3], increasing its expression in the long term [41]. Additionally, eNOS activity can be trigger by the action of CGRP [6] which induces NO-dependent vasodilation in isolated umbilical and chorionic arteries and veins [7]. Furthermore, *in vivo* CGRP participates in the

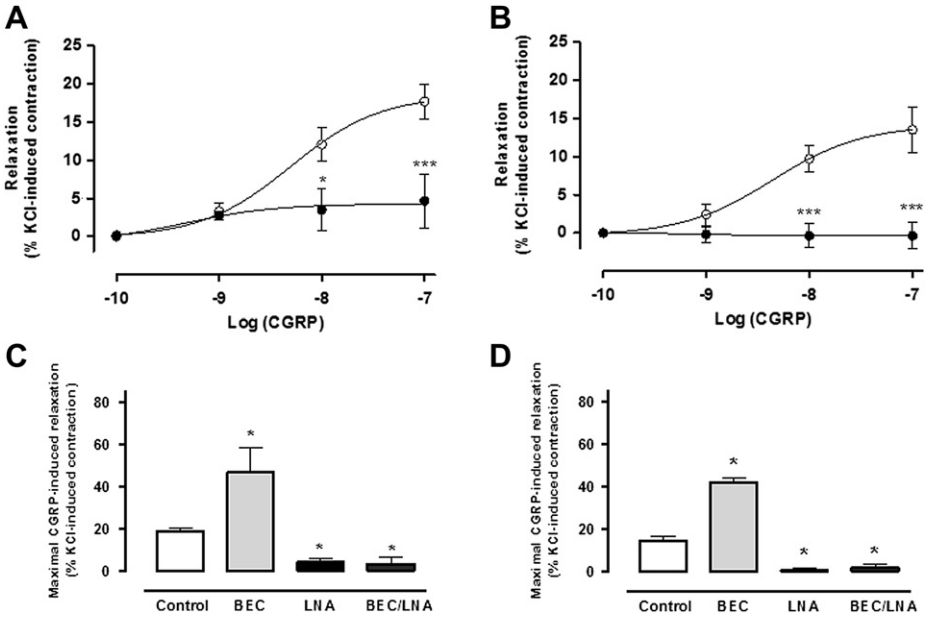

Fig. 3. Effect of arginase inhibition on NOS-dependent vasodilation in umbilical vessels. Concentration–response curves to calcitonin gene-related peptide (CGRP, 10^{-10}–10^{-7}) in umbilical arteries (A, $n = 6$) and veins (B, $n = 6$) in the absence (open circles) or presence (solid circles) of the N^G-nitro-L-arginine (L-NA, 100 μmol/l). Maximal CGRP-induced relaxation in arteries (C) and veins (D) in the presence or absence of S-(2-boronoethyl)-L-cysteine-HCl (BEC, 10^{-5} mol/l), L-NA (100 μmol/l) and BEC with L-NA. Values are mean ± S.E.M. $^{***}p < 0.01$ versus corresponding values in the absence of L-NA, $^{*}p < 0.05$ versus control.

control of the placental vascular tone in normoxia [8] and hypoxia [42], and this *in vivo* action is completely dependent on NO synthesis [8]. In this study were determined the effects of arginase inhibition on NO-dependent CGRP-induced relaxation. In umbilical vessels, the vasodilator response to CGRP was similar in arteries and veins and it was almost completely dependent on NOS activity. Additionally, arginase inhibition induces a 2.5-fold increase in the maximal response to CGRP, thus arginase has a role not only on the basal tone but also when eNOS is activated by extracellular stimuli.

4.2. Differential response to hypoxia in artery and vein endothelium

Here we demonstrated that 24 h of hypoxia differentially regulate the expression of eNOS and arginase-2 in human umbilical artery and vein endothelial cells. Hypoxia is an important regulator of placental vascular tone. Acute hypoxia increases placental vascular resistance [9,11] and this effect occurs mainly by vasoconstriction of the small chorionic arteries [11] without participation of the NO pathway [10,12]. However, long-term exposure to

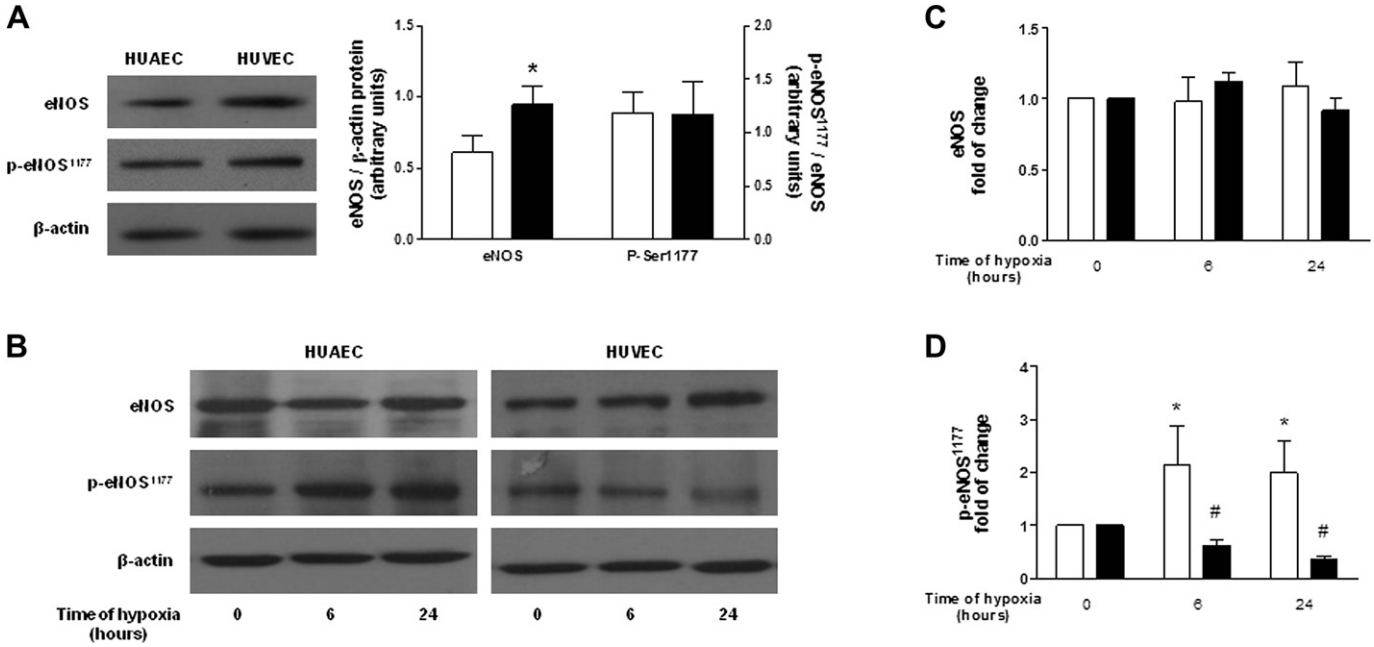

Fig. 4. eNOS and p-eNOS^{1177} protein expression in HUAEC and HUVEC exposed to hypoxia. (A) Western blot of total eNOS, p-eNOS^{1177} and ß-actin (internal reference) proteins in whole cell extracts of HUAEC (open bars, $n = 8$) and HUVEC (solid bars, $n = 8$) in normoxia (5% oxygen). (B) Representative blots of eNOS, p-eNOS^{1177} and ß-actin proteins in HUAEC and HUVEC exposed for 0, 6 and 24 h to hypoxia (2% oxygen). Time 0 indicates cells in 5% oxygen. (C) Total eNOS/ß-actin protein ratio densitometries for HUAEC (open bars) and HUVEC (solid bars) exposed to hypoxia, normalized to control. (D) p-eNOS^{1177}/total eNOS protein ratio densitometries as in C. Values are mean ± S.E.M. $^{*}p < 0.05$ versus HUAEC in normoxia, $\#p < 0.05$ versus HUVEC in normoxia.

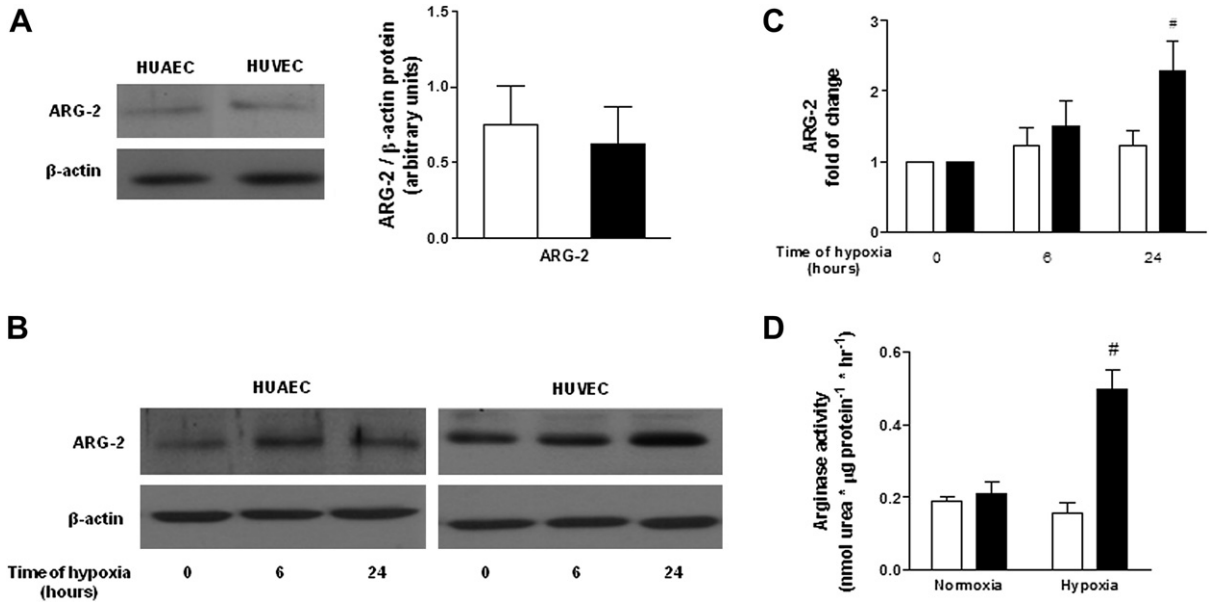

Fig. 5. Arginase-2 protein expression and arginase activity in HUAEC and HUVEC exposed to hypoxia. (A) Western blot for total arginase-2 and ß-actin (internal reference) in whole cell extracts of HUAEC (open bars, $n = 8$) and HUVEC (solid bars, $n = 8$) exposed to normoxia (5% oxygen). (B) Representative blots for proteins arginase-2 and ß-actin in HUAEC and HUVEC exposed for 0, 6 and 24 h to hypoxia (2% oxygen). Time 0 indicates cells in 5% oxygen. (C) Total arginase-2/ß-actin protein ratio densitometries for HUAEC (open bars) and HUVEC (solid bars) exposed to hypoxia, normalized to control. (D) Arginase activity was measured as urea formation from L-arginine (37 °C) in cell lysates of HUAEC (open bars) and HUVEC (solid bars) exposed to hypoxia for 24 h. Values are mean ± S.E.M. #$p < 0.05$ versus HUVEC in normoxia.

hypoxia, as well as pregnancy pathologies related with chronic hypoxia (IUGR and PE), are accompanied with changes in placental vascular reactivity, which include alterations in the L-arginine/NO pathways [4,21]. In PE, arginase-2 protein levels are increased whilst eNOS expression is decreased in placental endothelium [19].

Recently, we have shown in HUVEC that arginase-2 expression and activity are induced by hypoxia [25]. Furthermore, in hypoxia these cells reduce eNOS activity [15,25] and this decrease is partially reverted by arginase inhibition [25]. Our results showed that umbilical artery endothelial cells respond to hypoxia with an initial increase in eNOS activation, as is suggested by the p-eNOS^{1177} levels at 6 hours of hypoxia, without changes in the protein levels of total eNOS and arginase-2. In contrast, hypoxia in umbilical vein endothelial cells reduced eNOS activation and this was accompanied with increased protein levels for arginase-2. This data suggests that umbilical artery endothelium responds to hypoxia maintaining its capacity to synthesize NO whilst venous endothelium restrains the activation of this pathway. Despite that under physiological conditions higher levels of PO_2 in the umbilical vein (30–45 mmHg) [43,44] compared to umbilical arteries (15–35 mmHg) [43,45] have been reported, for the purposes of this study both endothelial cell types were exposed to similar oxygen levels for normoxia and hypoxia. These oxygen levels (5% and 2%) were selected in order to determine the response to a comparable hypoxic challenge, being the later in the range of those reported in umbilical vein and arteries of IUGR fetuses [44,46]. The consequences of a longer exposure (>24 h) to hypoxia on the L-arginine- and NO-related pathways have not been studied in isolated placental endothelial cells. However, it is possible to speculate that the initial differential response observed in HUAEC and HUVEC in this study, is maintained after chronic hypoxia, as in IUGR, where eNOS is upregulated in the umbilical artery [47] and dowregulated in the umbilical vein [15].

Noteworthy, the different responses determined in this study could be important in acute episodes of hypoxia, when placental microvessels are constricted [11] and the umbilical artery blood flow increases [10]. The higher microvascular placental tone will increase the transmural pressure and shear stress in larger placental arteries requiring a vasodilator response in the later. In contrast, at the venous side a higher tone would contribute to prevent an over-flow through placental shunts. These ideas agree with the model proposed by Talbert and Sebire [48] to explain the auto-regulation of placental blood flow, and the results from Hampl et al. [11] that suggests a relaxation in chorionic arteries in response to hypoxia. If the differential response to hypoxia in HUAEC and HUVEC, is reflecting differences in arteries and veins at the microvascular level needs to be addressed. There is no consensus regarding a clear distinction between micro and macrovascular endothelium, as it has been established for arterial and venous endothelium [49,50]. In fact, initial studies in placental endothelial cells showed difference in the response to and release of vascular factors between micro (venous and arterial) and macrovascular (HUVEC) cells [51]. However, this same group later demonstrated important differences between placental microvascular arterial and venous endothelial cells at the trascriptomic and functional levels [52] as well as in response to changes in oxygen levels [53].

In summary, here we provide *ex vivo* evidence for a vascular role of arginase activity in the placenta, controlling NOS activity. Noteworthy, the regulation of L-arginine/NO pathway by hypoxia in endothelial cells from normal placentae shows a heterogeneous response between arteries and veins, suggesting a complex control of the inward and outward placental blood flow, in order to improve the nutrient exchange during episodes of hypoxia.

Conflicts of interest

None.

Sources of funding

This study was supported by Fondo Nacional de Desarrollo Científico y Tecnológico (FONDECYT 1080534, 1110977) and Programa de Investigación Interdisciplinario (PIA), Comisión Nacional de Investigación en Ciencia y Tecnología (CONICYT) (Anillos ACT-

73), Chile. Fellowship Apoyo a la Realización de la Tesis Doctoral, CONICYT (AT-24090200), Chile.

Acknowledgments

B. J. Krause, E. Muñoz-Urrutia and C.P. Prieto hold CONICYT-PhD (Chile) fellowships. The authors thank the excellent technical support of Paulina Arias with immunohistochemistry of umbilical vessels.

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