Arginase inhibition enhances angiogenesis in endothelial cells exposed to hypoxia

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Abstract

Hypoxia-induced arginase elevation plays an essential role in several vascular diseases but influence of arginase on hypoxia-mediated angiogenesis is completely unknown. In this study, *in-vitro* network formation in bovine aortic endothelial cells (BAEC) was examined after exposure to hypoxia for 24 hours with or without arginase inhibition. Arginase activity, protein levels of the two arginase isoforms, eNOS, and VEGF as well as production of NO and ROS were examined to determine the involvement of arginase in hypoxia-mediated angiogenesis. Hypoxia elevated arginase activity and arginase 2 expression but reduced active p-eNOS^{Ser1177} and NO levels in BAEC. Additionally, both VEGF protein levels and endothelial elongation and network formation were reduced with continued hypoxia, whereas ROS levels increased and NO levels decreased. Arginase inhibition limited ROS, restored NO formation and VEGF expression and prevented the reduction of angiogenesis. These results suggest a fundamental role of arginase activity in regulating angiogenic function.

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Keywords

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1. Introduction

Arginase, an ureohydrolase enzyme, has been shown to be involved in the several vascular diseases such as hypertension, atherosclerosis, and diabetic coronary vascular dysfunction (Yang & Ming, 2006; Sasaki *et al.*, 2007; Romero *et al.*, 2008). The detrimental effects of elevated arginase activity in these conditions are mostly attributed to its capability to inhibit nitric oxide (NO) synthesis by competing with endothelial NO synthase (eNOS) for their common substrate, L-arginine (Morris, 2002, 2009). When the L-arginine supply needed for eNOS activity is insufficient, NOS can become uncoupled, producing less NO and forming superoxide (O_2^-). Further, remaining NO can react with O_2^- to form the toxic oxidant peroxynitrite ($ONOO^-$) (Forstermann & Munzel, 2006). NO plays a pivotal role in vasodilation and angiogenesis (Dulak *et al.*, 2000; Jones *et al.*, 2004; Morris *et al.*, 2008; Berchner-Pfannschmidt *et al.*, 2010). Reduced NO bioavailability within the vessel wall leads to vasoconstriction, vascular remodeling and an aberrant neovasculature (Madigan & Zuckerbraun, 2013).

Many factors that trigger endothelial dysfunction also up-regulate arginase expression and activity. Hypoxia is one of these factors. Hypoxia has been reported to increase arginase-2 expression and activity in human umbilical vein and pulmonary microvascular endothelial cells (Krotova *et al.*, 2010; Prieto *et al.*, 2011). Also, oxidative stress due to generation of reactive oxygen species (ROS), including superoxide and peroxynitrite, contributes to elevated arginase expression/activity and eNOS uncoupling (Thengchaisri *et al.*, 2006; Sankaralingam *et al.*, 2010; Chandra *et al.*, 2012). Moreover, hypoxia is a major mediator of angiogenesis. Hypoxia-mediated angiogenesis due to elevated levels of hypoxia-induced factor-1α (HIF-1α) and vascular endothelial growth factor (VEGF) has been well demonstrated (Fong, 2009; Takenaga, 2011; Ahluwalia & Tarnawski, 2012). NO also is importantly involved in regulation of hypoxia-mediated angiogenesis through a NO/HIF-1α/ VEGF axis (Kimura *et al.*, 2000; Kimura *et al.*, 2001; Kimura & Esumi, 2003). Hence, arginase may alter this process via its reduction of NO. However, no studies on a role of arginase in hypoxia-mediated angiogenesis have been reported. The present study examined the hypothesis that enhanced arginase activity during hypoxia limits the angiogenic process. *In-vitro* network formation in endothelial cells was examined after exposure to hypoxia (2% O_2) for 12 and 24 hours with or without arginase inhibition. Protein levels of the two arginase isoforms, eNOS and VEGF in addition of NO and ROS production were examined to determine the role of arginase in hypoxia-mediated angiogenesis.

2. Materials and Methods

2.1 Cell culture and hypoxia

Bovine aortic endothelial cells (BAECs, Cell Application Inc, CA, USA) were used from passages 4 to 7. Cells were cultured in normoxia (21% O_2) incubator to 70% confluence and

were maintained for 2 hrs in low-arginine M-199 medium (Invitrogen, Carlsbad, CA, USA) containing 0.2% FBS and 50 μM L-arginine, before treatment. The cells were pretreated with the arginase inhibitor 2(S)-amino-6-boronohexanoic acid (ABH, 100 μm, a gift of Dr. Dan Berkowitz) or vehicle (1xPBS) for 1 hr. Thereafter, cells were maintained with a gas mixture (5% CO_2-balanced N_2) to obtain 2% O_2 in a hypoxia chamber for 12 or 24 hrs. Normoxia control group cells were incubated in 21% O_2 conditions. This concentration of ABH, 100 μM inhibits arginase activity by over 90% in endothelial cells for over 24 hrs (Steppan *et al.*, 2013).

2.2 Arginase activity

BAEC were washed in PBS and incubated in lysis buffer (50 mM Tris-HCl) containing protease inhibitors. Cell lysates were centrifuged at 12,000 g for 10 min. The enzyme was activated by heating the lysate at 56°C for 10 min in 25 mM Tris buffer (pH 7.4) containing 5 mM $MnCl_2$. L-arginine hydrolysis was then conducted by incubating 50 μl of the activated lysate with 50 μl of 0.5 M L-arginine (pH 9.7) at 37°C for 60 min. The reaction was stopped in acid medium (H_2SO_4:H_3PO_4:H_2O=1:3:7 v/v). The concentration of urea was determined after adding 25 μl of 9% α-isonitrosopropiophenone. Protein concentration in the lysates was determined by a BCA assay (Thermo Scientific, IL, USA). Results were normalized to arginase activity in normoxia control group and expressed as arbitrary unit.

2.3 Western-blot

BAEC were homogenized in a RIPA Lysis Buffer (EMD Millipore, MA, USA) supplied with phosphatase inhibitor cocktail (Sigma-Aldrich, MO, USA) and protease inhibitor cocktail (Sigma-Aldrich, MO, USA). 20μg protein samples were subjected to 10% SDS-PAGE. Proteins were transferred onto a nitrocellulose membrane and the membrane was blocked with 5% BSA and incubated with primary polyclonal chicken anti-arginase I (1:10,000, gift of Dr. S.M. Morris, Jr.), rabbit anti-arginase II (1:250, SantaCruz Biotech, Texas, USA), rabbit anti-eNOS(1:4000, Santacruz Biotech, Texas, USA), monoclonal mouse anti-eNOS phosphorylated at serine-1177 or threonine-495 (1:1000, BD Bioscience, CA, USA), human anti-VEGF antibody (LifeSpan BioScience, WA, USA), mouse anti-β-actin (1:5000, Sigma-Aldrich, MO, USA) overnight at 4°C. After incubation with secondary antibodies, proteins were detected using an enhanced chemiluminescence and quantified by densitometry. Results were normalized to β-actin.

2.4 Measurement of NO levels and production

Intracellular NO levels at 24 hr were monitored using the NO-specific fluorescent dye 4,5 -diaminofluorescein diacetate (DAF-2 DA, Calbiochem, USA). Thereafter, cells were stained with oxygenated DAF-2 DA solution (10 mM) for 30min in the darkness at 37C, in a shaking water bath. This experimental procedure in saturated oxygen ensures that NO, rather than O_2, is the limiting factor in the reaction and the fluorescence is directly proportional to NO. Negative controls for DAF-2 DA were incubated in NOS inhibitor L-NAME (0.1mM) throughout the experimental period. Fluorescence was monitored using fluorescence microscope (excitation, 488 nm; emission, 610 nm) at 20x magnification. Nine pictures were captured in each well (n=3) and the fluorescent intensity was quantified using Image J

software. Results were presented as mean fluorescent intensity per field and expressed as percent of control.

Cumulative production of NO by BAEC was also assessed. After 24 hr exposure to normoxia, hypoxia or hypoxia+ABH, the medium was collected. NO levels in 30μl of media was measured using NO-specific chemiluminescence in a Sievers Nitric Oxide Analyzer as previously described (Shatanawi *et al.*, 2011).

2.5 *In-vitro* EC network formation assay

Cells were collected for in-vitro tube formation assay. ABH (100 μM) or PBS (as control) was added to the wells of 96-well plate before 30 μl Matrigel (reduced growth factor basement membrane matrix, BD Bioscience, USA) mixed with 60 μl medium containing 1.5 $\times 10^4$ cells were seeded. After 12 hr and 24 hr hypoxia exposure, the network-like structures were visualized and captured using microscope (Axiovert 25, Carl Zesis) at 10x magnification. Five pictures were taken for each well and five wells for each group. Network length in each field was analyzed and quantified using NIH ImageJ. Results were presented as network length in each field and expressed as percent of control.

2.6 Reactive Oxygen Species (ROS)

Dichlorofluorescein (DCF) is the oxidation product of the reagent 2′,7′-dichlorofluorescein diacetate (H_2DCFDA; Molecular Probes, CellRox Oxidative Stress Deep Red Reagents, Life technologies, NY, USA). It is a marker of cellular oxidation by hydrogen peroxide, peroxynitrite, and hydroxy radicals. Cells were incubated with low-arginine M199 medium containing 5 μM H_2DCFDA at 37°C for 30mins. Fluorescence was monitored using fluorescence microscope (excitation, 640 nm; emission, 665 nm) at 20x magnification. Nine pictures were captured in each well (n=3) and the fluorescent intensity was quantified using NIH ImageJ software. Results were presented as mean fluorescent intensity in each field and expressed as percent of control.

2.7 Statistical Analysis

All the values are presented as mean ± S.E.M. Student's t-test or one-way analysis of variance (ANOVA) with Bonferroni post-test was used to evaluate differences between the groups as appropriate. P<0.05 was considered significant. All statistical analysis was calculated by using GraphPad Prism (V5.01).

3. Results

3.1 *In-vitro* tube-like formation and VEGF165 expression

We first determined the effects of hypoxia exposure on angiogenic function as shown by the alignment of ECs in tube-like networks and VEGF165 protein levels as shown by Western blot. At 12 hr of exposure to hypoxia, ECs showed significantly increased VEGF165 levels. The increase in VEGF165 was accompanied by EC elongation and alignment, but network formation was not evident. Both VEGF165 levels and EC elongation/alignment in hypoxia were significantly increased by ABH treatment (Figure 1). In normoxic conditions, EC exhibited only small spiky protrusions. After 24 hrs of hypoxia exposure, well-formed EC

networks were observed in the normoxia control cultures. However, the network length per field in hypoxia was less than observed in normoxia control, being 63% of that level. ABH treatment partially prevented this hypoxia-induced reduction in tube-like formation (88% of control level) (Figure 2a-d). VEGF165 protein levels in the hypoxia group were reduced to 53% of control and ABH prevented this decline (Figure 2e-f). We therefore used the 24 hrs hypoxia treatment for further analyses of the role of arginase activity in this process.

3.2 Arginase expression and activity

Hypoxia increased protein levels of arginase 2 in BAEC but did not change in arginase 1 expression compared with normoxia control. In hypoxia group, arginase 2 protein expression was increased by 87% over normoxia control. Pretreatment with ABH significantly inhibited the hypoxia-induced increase in arginase expression. Hypoxia-induced elevation of arginase activity also was partially inhibited by ABH, but activity still was higher than that of normoxia control (Figure 3).

3.3 eNOS expression

Total eNOS protein was unaltered after 24 hr hypoxia exposure, and was not affected by ABH treatment. However, hypoxia reduced protein levels of eNOS phosphorylated at serine-1177 (p-eNOS^{Ser1177}) to 73% of the normoxia control. In contrast, phosphorylation at threonine-495(p-eNOS^{Thr495}) was increased by 91% over normoxia control values. ABH pretreatment prevented the changes in p-eNOS^{Ser1177} and p-eNOS^{Thr495} caused by hypoxia (Figure 4). These data indicate that eNOS activity, determined by p-eNOS levels, is affected by arginase.

3.4 NO levels and production

Mean fluorescent intensity of DAF-2 staining for intracellular NO showed that hypoxia markedly decreased intracellular NO levels to 13% of the normoxia control. Extracellular NO accumulation in culture medium, detected by the NO analyzer during hypoxia exposure, declined to 35% of the normoxia control. ABH pretreatment partially prevented the reduction in NO levels and production in response to hypoxia, with values only falling to 44% and 53% of respective controls (Figure 5).

3.5 ROS generation

Twenty-four hours of hypoxia exposure significantly increased reactive oxygen species generation in EC cytoplasm to 96% over normoxia control. But ABH pretreatment reduced the hypoxia-induced ROS generation to only 66% above control (Figure 6).

4. Discussion

Hypoxia is a major trigger of elevated arginase expression and/or activity in several pathological conditions such as pulmonary arterial hypertension (PAH), cardiovascular dysfunction, and atherosclerosis (Xu *et al.*, 2004; Yang & Ming, 2006; Romero *et al.*, 2008). Furthermore, hypoxia-mediated angiogenesis is a feature of these diseases. For example, hypoxia-mediated neovascularization of pulmonary artery vasa vasorum has been found in PAH and intraplaque hypoxia-induced angiogenesis also has been reported to be present in

atherosclerosis (Kuwahara *et al.*, 2002; Gao *et al.*, 2012). However, the role of arginase in hypoxia-mediated angiogenesis has not been determined.

In present study, bovine endothelial cells (BAEC) were grown in normoxic conditions to 70% confluence and then subjected to 2% oxygen, a concentration commonly used for hypoxia studies. After 12 hr of hypoxia exposure, levels of VEGF were significantly increased as compared to the normoxia control cells and the ECs began to elongate and align in primitive networks. Both VEGF and network alignment were significantly increased by ABH treatment. After 24 hr hypoxia exposure, well-formed EC networks were observed. However, compared with normoxia control, there was a significantly lower network length along with a corresponding decrease in VEGF protein levels. Arginase inhibition with ABH partially prevented the suppressive effects of hypoxia.

Hypoxia is a well-known inducer of angiogenesis (Fong, 2009; Takenaga, 2011; Ahluwalia & Tarnawski, 2012). However, sustained hypoxia conditions, such as ischemia, are detrimental for the vasculature through high levels of ROS formation (Webster, 2012). ROS have been noted to be significantly increased in ischemic hindlimbs of wild-type mice, but not in mice deficient in Nox2- NADPH oxidase (NOX2). The NOX2 mice exhibited resistance against ROS production, preserved eNOS expression and improved VEGF-dependent angiogenesis (Haddad *et al.*, 2009). In our study, a significantly higher level of ROS and decreased VEGF levels occurred in hypoxia group. This could explain the impaired network formation after 24 hr of hypoxia exposure.

Importantly, inhibition of arginase with ABH partially prevented the hypoxia-induced impairment of network formation and the hypoxia-related decline in VEGF, which possibly was achieved through its actions to maintain NO production and subsequent VEGF expression (Kimura & Esumi, 2003; Fraisl, 2013). Under the low oxygen tension, NO donors at low concentrations have a synergetic effect on the VEGF gene activation by promoting HIF-1α (Kimura & Esumi, 2003; Abe *et al.*, 2013). Site-directed mutational analysis of the VEGF gene promoter has revealed that a HIF-1 binding site (HBS) and its downstream HIF-1α ancillary sequence (HAS) within the hypoxia response elements (HRE) are required as *cis*-elements for the transcriptional activation of VEGF by NO (Kimura *et al.*, 2001). Therefore, NO-induced VEGF expression can be partially mediated by activation and subsequent binding of HIF-1α (Kimura *et al.*, 2000). Moreover, increased endogenous NO due to overexpressed eNOS or elevated eNOS activity has been shown to enhance angiogenesis in a human colon tumor cell line and vascular smooth muscle cells (VSMC) (Jenkins *et al.*, 1995; Dulak *et al.*, 2000). Additionally, the protective role of ABH pretreatment in network formation can be partially attributed to its action to reduce ROS levels in hypoxia. Inhibition of arginase has been shown to reduce the ROS production from uncoupled eNOS in previous studies (Reid *et al.*, 2007; Kim *et al.*, 2009; Jung *et al.*, 2010).

More recent studies also report the protective role of arginase inhibition in oxidative stress. Systemic treatment of rat with an arginase inhibitor N-(ω)-hydroxy-nor-L-arginine (nor-NOHA) was shown to significantly prevent both hypoxia-increased tissue level of ROS and lipid peroxides in hypoxic hearts. Nor-NOHA treatment also significantly ameliorated the peroxynitrite formation in hypoxic hearts (Singh *et al.*, 2014). Further, nitroglycerin (NTG)-

induced increases of ROS levels in aorta isolated from wild type mice were not observed in aorta pretreated with the arginase inhibitor (s)-(2-boronethyl)-L-cysteine HCl (BEC), nor in aorta isolated from Arg2$^{-/-}$ mice. BEC pretreatment also significantly reduced the ROS levels in NTG-treated HUVEC (Khong *et al.*, 2012).

In parallel with elevating ROS levels, hypoxia significantly elevated arginase activity and arginase 2 protein level, but had no influence on arginase 1 levels. Hypoxia-induced arginase expression appears to involve arginase activity, possibly due to increased levels of ROS which drive arginase expression (Thengchaisri *et al.*, 2006; Sankaralingam *et al.*, 2010; Chandra *et al.*, 2012). Hypoxia-induced arginase 2 expression and activity are also reported in human umbilical vein endothelial cells (HUVEC) (Prieto *et al.*, 2011) and human pulmonary microvascular endothelium (HMVEC) (Krotova *et al.*, 2010). Hypoxia-inducible factor 2α (HIF-2α) likely mediates this induction since the silencing of HIF-2α but not HIF-1α prevented activation of arginase 2 by hypoxia (Krotova *et al.*, 2010). In contrast, hypoxia-induced enhancement of arginase 1 expression and arginase activity was found in rat cardiac and aortic tissue, which was linked to the hypoxia-mediated c-Jun recruitment to AP-1 binding site on ARG1 promoter (Singh *et al.*, 2014). The discrepancy in arginase isoforms induced by hypoxia can be explained by reports that arginase 1 is the predominant isoforms in the rat endothelium while arginase 2 is the major isoforms in human aortic and human umbilical vein endothelial cells (Ming *et al.*, 2004; Ryoo *et al.*, 2006; Gao *et al.*, 2007).

Previous studies have shown that ROS (Zhang *et al.*, 2009), including H_2O_2 (Thengchaisri *et al.*, 2006) and $ONOO^-$ (Sankaralingam *et al.*, 2010) can up-regulate arginase expression. Our recent studies further confirm that this up-regulation of arginase by ROS occurs through the Rho/ROCK pathway and that pretreatment with inhibitors of Rho kinase or protein kinase C (PKC) can prevent this increase (Chandra *et al.*, 2012). Given that arginase inhibition can reduce ROS levels in hypoxic tissue (Takemoto *et al.*, 2002; Gonon *et al.*, 2012), we project that reduced arginase 2 expression in the hypoxia+ABH group is due to decreased ROS production via arginase inhibition.

Hypoxia-induced arginase elevation appears to contribute to the marked reduction in NO production via competition for L-arginine and eNOS uncoupling. Reduced active eNOS (p-eNOS^{Ser1177}) levels and increased levels of inactive eNOS (p-eNOS^{Thr495}) indicate reduced eNOS activity during hypoxia. Although ABH prevented these changes in p-eNOS levels caused by hypoxia, NO levels and production in hypoxia+ABH groups were still significantly below that of normoxic control. This implies that eNOS function is substantially impaired by hypoxia. However, ABH partially preserved NO synthesis seemingly by attenuating hypoxia-induced arginase expression and activity. Results from *in-vitro* studies of hypoxia on activation of eNOS has been controversial (Hoffmann *et al.*, 2001; Takemoto *et al.*, 2002; Prieto *et al.*, 2011; Krause *et al.*, 2012). The reasons for the variability of eNOS expression in these studies may reflect differences in cell species and location, oxygen concentration and exposure duration.

Our data shows that pretreatment with the arginase inhibitor ABH is protective in angiogenesis after sustained hypoxia exposure. To our knowledge, ours is the first study to

show a detrimental role of arginase in limiting angiogenesis *in vitro*. Further work is needed to understand the influence of arginase on angiogenic function *in vivo*. However, our group has shown that deletion of arginase 2 and inhibition of arginase promotes vascular repair in a model of ischemic retinopathy by a mechanism involving normalization of NOS function and reduced superoxide formation (Suwanpradid et al., Manuscript In Review). A protective role of arginase inhibition also has been demonstrated in rodent hearts subjected to ischemic-reperfusion myocardium or to hypobaric hypoxia by restoring eNOS coupling and NO production (Gonon *et al.*, 2012; Singh *et al.*, 2014). In line with these previous studies, our present results suggest that hypoxia elevates arginase activity and arginase 2 expression but reduces active p-eNOS^{Ser1177} and NO levels in BAEC. Furthermore, the impaired network formation and reduced VEGF protein levels with continued hypoxia appear to have occurred because of rising level of ROS and falling levels of NO. However, arginase inhibition prevented this reduction of angiogenesis. These effects appear to be related to a maintained NO availability and sustained VEGF protein levels.

5. Conclusion

In summary, we project that the protective role of arginase inhibition is derived from increased NO production and availability and the subsequent sustained VEGF levels. Additional studies on the role of arginase in hypoxia-mediated angiogenesis and VEGF expression are needed to understand the mechanism or pathways more clearly.

Acknowledgments

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Highlights

- We investigated the role of arginase in hypoxia-mediated angiogenesis.

- Endothelial cell (EC) arginase expression and activity are elevated by hypoxia.

- Inhibition of arginase enhances EC network formation and NO levels.

- Arginase inhibition also limits ROS formation and elevates VEGF levels.
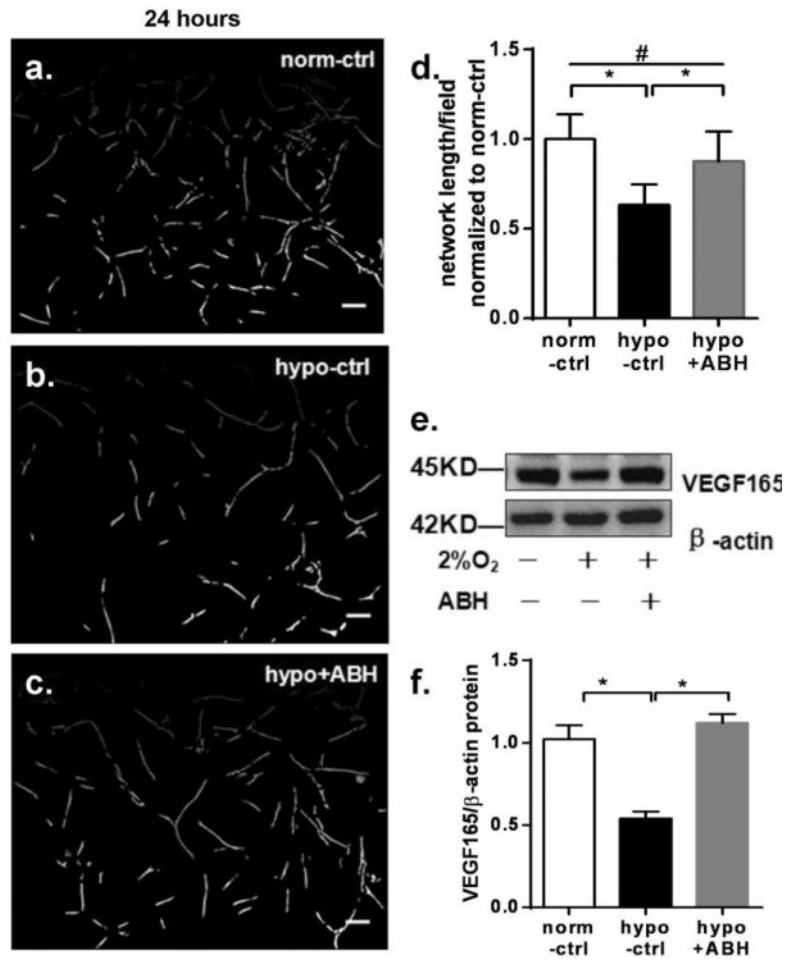
- Arginase plays a fundamental role in angiogenesis
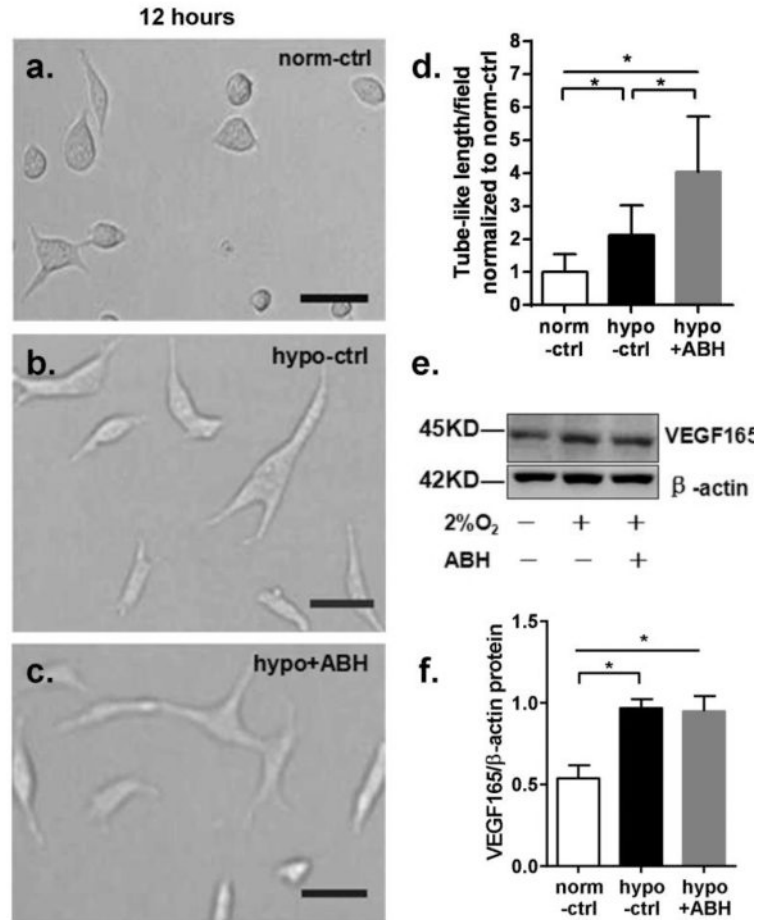
Figure 1.
Effect of 12 hr exposure on *in-vitro* angiogenesis of BAEC cultured in Matrigel and VEGF protein levels in BAEC. Magnification is 40X. Scale bar length is 20 μm. (a) Cells in normoxia control group. (b) Cells in hypoxia group. (c) Cells in hypoxia+ABH group. (d) Mean length of tube-like structures among groups. Values are normalized to normoxia control. (e)Western blot for VEGF165 normalized to β-actin in BAEC exposed to either normoxia(-) or hypoxia (2% O2, +) and with (+) or without ABH (-). (f) VEGF 165/β-actin levels. N=4-6 wells. *P < 0.01.
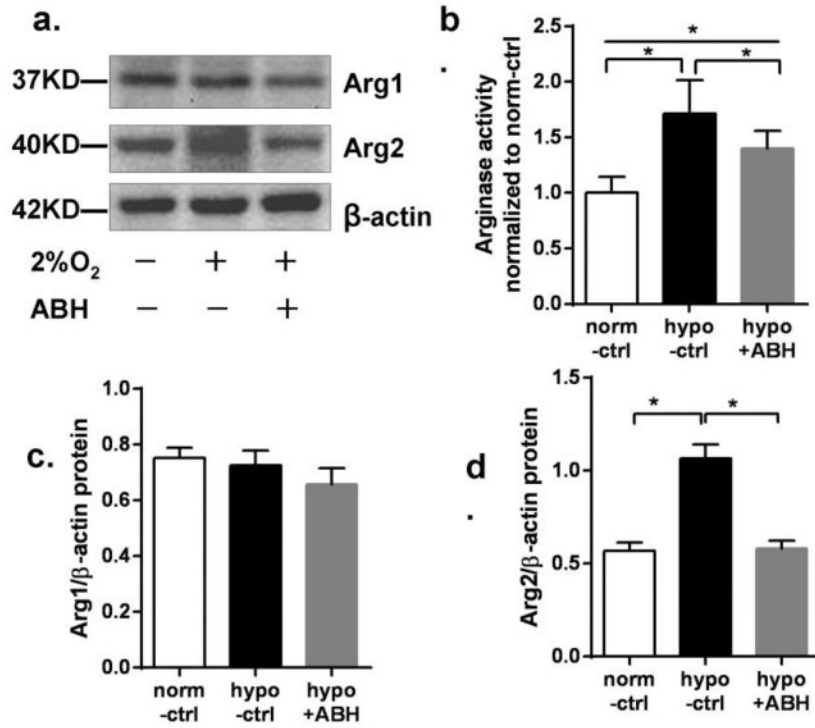
Figure 2.
Effect of 24 hr exposure on *in-vitro* angiogenesis of BAEC cultured in Matrigel and VEGF protein levels in BAEC. Images a-c are normoxia control, hypoxia control and hypoxia +ABH group, respectively (10X). Scale bar length is 50 μm. (d) Mean length of tube-like structures among groups. Values are normalized to normoxia control. (e) Western blot for VEGF165 and β-actin in BAEC exposed to either normoxia (-) or hypoxia (2% O2, +) and with (+) or without ABH (-). (f). VEGF165 /β-actin levels. N=4-6 wells. *P < 0.01,#P < 0.05.

Figure 3.
Arginase expression and arginase activity in BAEC exposed to hypoxia for 24 hr. (a) Western blot for arginase 1 (Arg1) and arginase 2 (Arg2) normalized to β-actin in BAEC exposed to either normoxia (-) or hypoxia (2% O_2, +) and with (+) or without ABH (-). (b) Arginase activity in BAEC, normalized to normoxia control. (c) Argl/β-actin levels and (d) Arg2/β-actin levels. N=4-6. *P < 0.01.
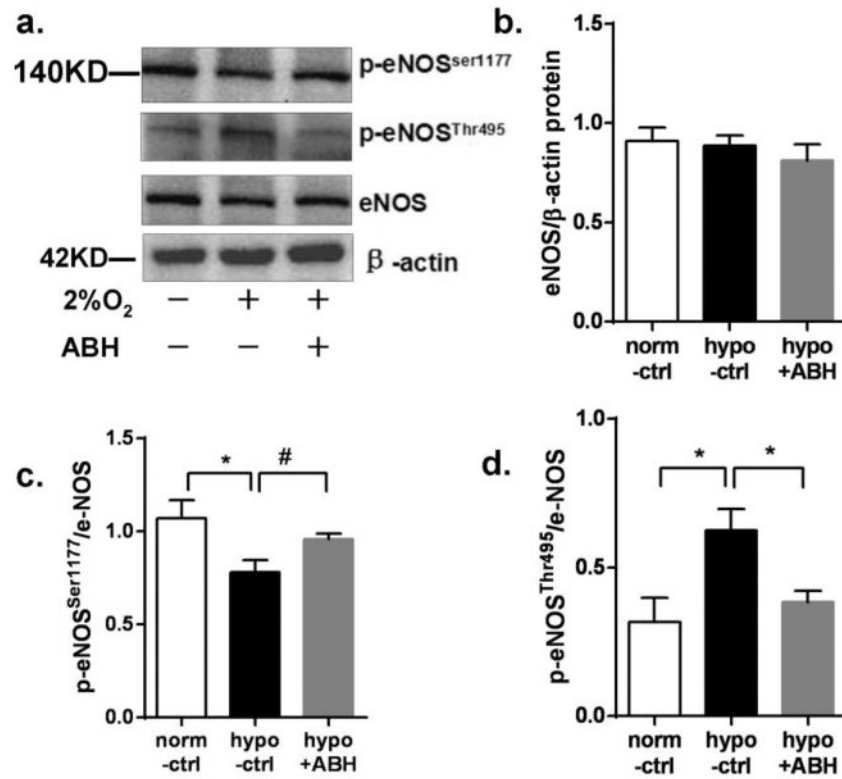
Figure 4.
eNOS expression in BAEC exposed to hypoxia or 24 hr. (a) Western blot or p-eNOS[Ser1177], p-eNOS[Thr495] and β-actin in BAEC exposed to either normoxia (-) or hypoxia (2% O_2, +) and with (+) or without ABH (-). Panels b-d are e-NOS, p-eNOS[Ser1177], p-eNOS[Thr495]/β-actin levels, respectively N=4-6. *P < 0.01, #P < 0.05.
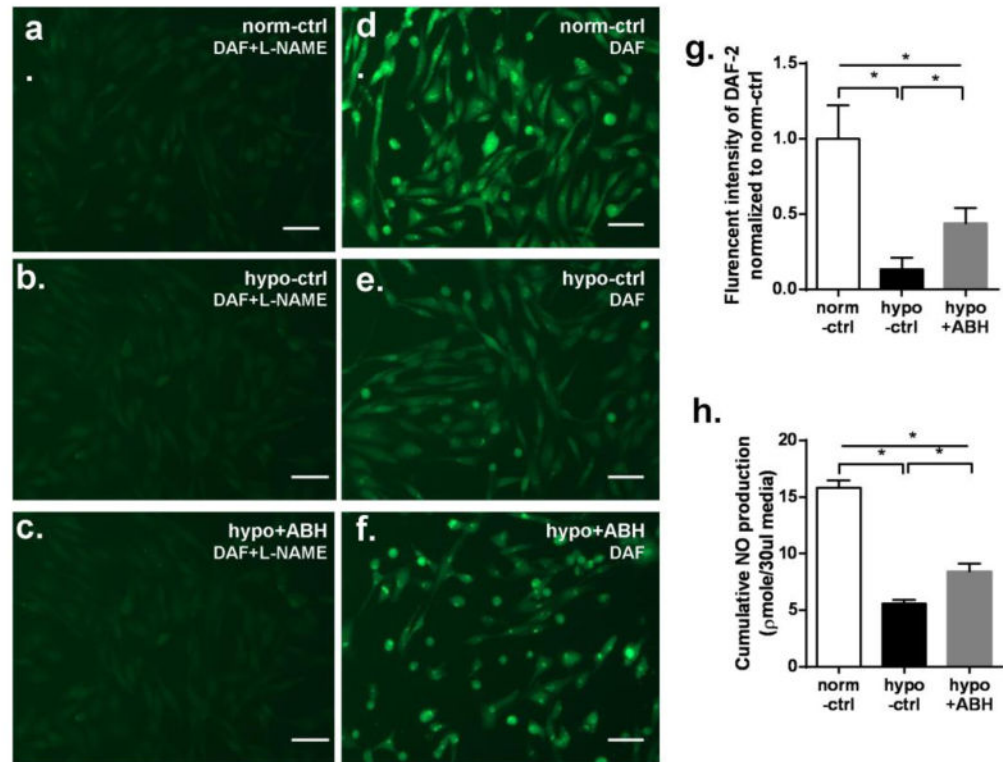
Figure 5.
NO production in BAEC exposed to hypoxia for 24 hr. (a-f) NO-specific fluorescence of DAF-2 in BAEC exposed to hypoxia for 24 hr (20x) with L-NAME (a-c) and without L-NAME (d-f). Scale bar length is 50μm. (g) Mean DAF-2 fluorescent intensity levels in EC without L-NAME at 24 hr among groups. Values are normalized to normoxia control. (h) Cumulative NO production measured by chemiluminescence over 24 hr among groups. N=5 wells. *P < 0.01.
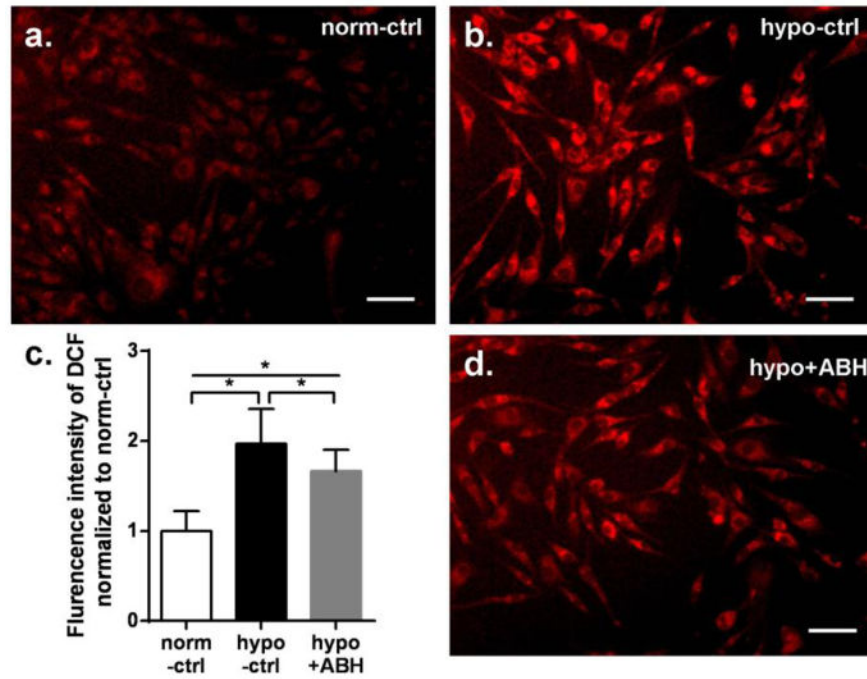
Figure 6.
ROS-specific fluorescence of DCF in BAEC exposed to hypoxia for 24 hr. Images a, b, d
are for normoxia control, hypoxia control and hypoxia+ABH group, respectively (20x).
Scale bar length is 50μm. (c) Mean DCF fluorescent intensity among groups. Values are
normalized to normoxia control. N=4-6 wells. *P < 0.01.