Preserved placental oxygenation and development during severe systemic hypoxia

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Submitted 6 April 2005; accepted in final form 26 September 2005

Schäffer, Leonhard, Johannes Vogel, Christian Breymann, Max Gassmann, and Hugo H. Marti. Preserved placental oxygenation and development during severe systemic hypoxia. Am J Physiol Regul Integr Comp Physiol 290: R844–R851, 2006. First published September 29, 2005; doi:10.1152/ajpregu.00237.2005.—Local tissue oxygenation profoundly influences placental development. To elucidate the impact of hypoxia on cellular and molecular adaptation in vivo, pregnant mice at embryonic days 7.5–11.5 were exposed to reduced environmental oxygen (6–7% O2) for various periods of time. Hypoxia-inducible factor (HIF)-1α mRNA was highly expressed in the placenta, whereas HIF-2α was predominantly found in the decidua, indicating that HIF-1 is a relevant oxygen-dependent factor involved in placental development. During severe hypoxia, HIF-1α protein was strongly induced in the periphery but, however, not in the labyrinth layer of the placenta. Accordingly, no indication for tissue hypoxia in this central area was detected with 2-(2-nitro-1-imidazolyl)-N-(2,2,3,3,3-pentafluoropropyl)acetamide staining and VEGF expression as hypoxic markers. The absence of significant tissue hypoxia was reflected by preserved placental architecture and trophoblast differentiation. In the search for mechanisms preventing local hypoxia, we found upregulation of endothelial nitric oxide synthase (NOS) expression in the labyrinth layer. Inhibition of NOS activity by Nω-nitro-l-arginine methyl ester application resulted in ubiquitous placental tissue hypoxia. Our results show that placental oxygenation is preserved even during severe systemic hypoxia and imply that NOS-mediated mechanisms are involved to protect the placenta from maternal hypoxia.

Disturbance of physiological oxygen concentrations may lead to abnormal placental development. In preeclampsia, a severe pregnancy disorder, important cellular developmental steps are compromised and have been attributed to disturbed oxygen levels (5, 16–18, 39). Furthermore, various forms of intrauterine growth retardation have been associated with distinct villous vascular alterations that potentially are related to inadequate changes in placental oxygen partial pressure (25). An untimely switch in oxygen tension or a failure in proper cellular oxygen sensing may therefore be responsible for deregulated placental structure and function corresponding to the situation observed in these pregnancy disorders.

Adaptation to altering oxygen levels is mainly mediated by the hypoxia-inducible transcription factors hypoxia-inducible factor (HIF)-1α and HIF-2. HIFs are heterodimers composed of an α- and a β-subunit, the latter being identical to the aryl hydrocarbon receptor nuclear translocator (ARNT) (47). On hypoxic stabilization, HIF-1 transactivates oxygen-regulated genes such as inducible nitric oxide synthase (iNOS) (32) and VEGF (15). VEGF promotes new vessel growth, leading to enhanced blood flow and thereby increasing the amount of oxygen delivered to the hypoxic tissue (30). Similarly, NO generated by NOS serves as a key signaling molecule in the regulation of vascular tone by mediating endothelium-dependent vascular relaxation and thus can increase local blood flow (37). Because of its ubiquitous expression, HIF-1α represents the master regulator of oxygen-dependent gene transcription (21, 48). The human placenta shows a time-dependent expression of HIF-1α, with high levels in early pregnancy followed by a drop at later stages when oxygen levels are believed to increase (8). In keeping with this, preeclamptic placentas have been observed to alter HIF-1α expression (8, 38). Mice deficient for the common subunit HIF-1β/ARNT, which have lost responsiveness to hypoxia, display dramatically reduced numbers of spongiotrophoblast cells, whereas the amount of trophoblast giant cells is increased. Furthermore, vascularization of the placental labyrinth is impaired in these mice (1). These observations support the crucial role of adequate placental oxygenation and suggest that oxygen-dependent transcription factors and their target genes are important for the regulation of trophoblast cell fate at the fetomaternal border and angiogenesis during normal placentation and disease.

Considering the impact of varying oxygen levels during this highly sensitive period establishing placental circulation, we aimed to analyze mechanisms of adaptive cellular and molecular responses during events of reduced oxygen levels. To this end, mice were exposed to severe environmental hypoxia...
during early pregnancy, when critical steps for proper placental cellular and vascular development occur. Analysis of placental architecture and distribution of trophoblast subpopulations at the fetomaternal border, as well as distribution of placental oxygen levels modulating expression of oxygen-regulated transcription factors and their target genes during placentaion revealed highly adaptive maintenance of placental oxygenation and development.

MATERIALS AND METHODS

Animals. All experiments were approved by the Kantonalen Veterinäramt Zürich. Eight- to ten-week-old NMRI outbred mice were mated, and noon after detection of the vaginal plug was designated as E0.5. Pregnant mice at E7.5–E10.5 were subjected to systemic normobaric hypoxia by substituting oxygen with nitrogen, using a Digamix 2M 302/a-f pump (H. Woesthoff, Bochum, Germany) at a constant gas flow rate of 37 l/min in a Perpes chamber. Mice had access to food and water ad libitum. Animals were allowed to adapt to the hypoxic environment by gradually decreasing the inspired O2 fraction (FiO2) from 0.21 to 0.07 or 0.06 within 1 h (44) and were then kept for either 4 h (FiO2 = 0.06) or 24 h (FiO2 = 0.07) in hypoxia and killed immediately thereafter. To map hypoxic regions in the placenta in vivo, 250 μl of 10 mM 2-(2-nitro-1H-imidazol-1-yl)-N-(2,2,3,3,3-pentafluoropropy)acetamide (EF-5) (26, 27) was administered intravenously before exposure to 4 h of hypoxia or normoxia. To inhibit endogenous NOS activity, N-nitro-l-arginine methyl ester (l-NAME; Sigma, St. Louis, MO; 40 mg/kg), according to Ruschitzka et al. (42), was administered intraperitoneally before exposure to 4 h of hypoxia or normoxia. After hypoxic exposure, animals were immediately killed by decapitation. In a separate set of experiments, after 24-h hypoxic exposure, animals were allowed to reoxygenate at room air for an additional 24 h before tissue harvesting to exclude the possibility that delayed effects of hypoxic exposure on cellular changes were missed. Dissected placentas were embedded in Tissue Tek (Sakura, Zoeterwoude, The Netherlands), snap frozen in liquid nitrogen-cooled 2-methylbutane, and stored at −80°C until further processing.

In situ hybridization. In situ hybridization was performed essentially as described previously (4). cDNAs encoding for mouse 4311 and placental lactogen-1 (PL.Lac1) were kindly provided by Celeste Simon (Abramson Family Cancer Research Institute, University of Pennsylvania School of Medicine, Philadelphia, PA). For generation of hybridization probes, 638-bp EcoRI (4311) and 619-bp BamHI-NorI (PL.Lac1) fragments were inserted into appropriately opened pBluescript KSII vectors (Stratagene). The VEGF probe has been described previously (4). Single-stranded sense or antisense cRNA probes were generated by in vitro transcription of the resulting plasmids, BS2.11 (4311) and BS3.8 (PL.Lac1), using 100 μCi of 35S-labeled UTP and T3 or T7 RNA polymerases as described by the manufacturer (Stratagene). Ten-micrometer sections of frozen placentas were cut with a cryostat and melted on silane-coated glass slides. Sections were incubated in 2× SSC at 70°C, digested with Pronase (40 μg/ml), fixed in 4% paraformaldehyde, and acetylated with acetic anhydride diluted 1:400 in 0.1 M triethanolamine. Hybridization was performed in buffer containing 50% formamide, 10% dextran sulfate, 10 mM Tris-HCl (pH 7.5), 10 mM sodium phosphate (pH 6.8), 2× SSC, 5 mM EDTA, 150 μg/ml yeast tRNA, 0.1 mM UTP, 1 mM adenosine 5’-O-(2-thiodiphosphate), 1 mM adenosine 5’-O-(3-thio-triphosphate), 10 mM dithiothreitol, 10 mM 2-mercaptoethanol, and 2.5–3 × 106 cpm/ml 35S-labeled RNA probe overnight at 48°C. Slides were washed in 2× SSC-50% formamide at 37°C for 3 h, digested with RNase (20 μg/ml) for 15 min, washed again in 2× SSC-50% formamide overnight, and dehydrated in graded ethanol. Slides were coated with Kodak NTB-2 emulsion (Eastman Kodak) diluted 1:1 in water and exposed for 6–21 days. Slides were developed and counterstained with 0.02%toluidine blue, air dried, and mounted.

Immunohistochemistry. Frozen placentas were cut into 10-μm perpendicular serial sections at or close to the level of umbilical cord insertion to provide maximal comparability. For platelet endothelial cell adhesion molecule (PECAM)-1 (1:100, MEC 13.3 rat anti-mouse monoclonal antibody), Flik-1 (1:87, rat anti-mouse monoclonal antibody; Ref. 29), endothelial NOS (eNOS; 1:100, rabbit anti-human polyclonal antibody; Santa Cruz), and iNOS (1:100, rabbit anti-mouse polyclonal antibody; Santa Cruz) staining, dried sections were fixed and permeabilized in 100% acetone at −20°C for 20 min, air dried, rehydrated in PBS, and blocked for 30 min in 100% goat or rabbit serum. After incubation with primary antibody (60 min at room temperature (RT)) and biotinylated secondary goat anti-rabbit or rabbit anti-rat IgG (H+L) antibody (Vector Laboratories) for 30 min, immunolocalization was performed with the Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA). For HIF-1α immunohistochemistry, dried sections were fixed in 4% paraformaldehyde in PBS for 10 min. All primary and secondary antibodies were diluted in Tris-buffered saline (pH 7.4) containing 0.1% Tween 20 and 10% FCS. Sections were incubated with our chicken polyclonal anti-HIF-1α affinity-purified IgY antibody (7) (1:50) for 20 h at 4°C. A peroxidase-conjugated rabbit anti-chicken IgY antibody (1:100; Pierce, Rockford, IL) was added for 45 min at RT, followed by a peroxidase-conjugated goat anti-rabbit IgG antibody (1:100; Dako, Carpenteria, CA) for a further 45 min. All reactions were visualized with an AEC Substrate Kit (Vector Laboratories). Control sections were treated identically except for replacement of the primary antibody by Tris-buffered saline containing 0.1% Tween 20 and 10% FCS. Sections were counterstained with hematoxylin and mounted in Dako Paramount aqueous mounting medium.

Detection of the in vivo hypoxia marker EF-5 has been described in detail elsewhere (26). EF-5 is a 2-nitroimidazole compound that is reduced under hypoxic conditions facilitating the formation of covalent linkages with cellular protein thiols, thereby demarcating regions of hypoxia (26, 27). Briefly, 10-μm sections were placed into fresh paraformaldehyde for 60 min at 0°C. After being washed in ice-cold PBS, sections were blocked with blocking solution (PBS containing 0.3% Tween 20 (PBSst), 1.5% albumin, 20% (vol/vol) fresh skim milk, and 5% mouse serum) overnight at 0°C. After being washed in PBS, sections were incubated with ELK3–51 antibody (1:30; Ref. 26), diluted in PBSst containing 1.5% albumin, for 6 h at 4°C. Sections were rinsed in cold PBS, PBSst, and PBS for a total of 3 h, mounted in Dako Paramount aqueous mounting medium, and stored in darkness.

Digital quantification of expression levels of eNOS, iNOS, and PECAM was performed with an image analyzing system (MCID, Imaging Research, St. Catherines, ON, Canada). For quantification of the staining intensity of eNOS, iNOS, and PECAM, relative optical densities (ROD) were determined in digital images of the placental sections. In addition, the ratio between target area (Tgt) and scan area was determined. This measure provides information about the proportion of the stained area to total larynch area. Shrinkage could result in an increased ROD because the proteins could move more closely together. Therefore, a real increase in expression level requires an increased ROD at an unchanged and/or increased ratio between Tgt and scan area or an unchanged ROD at an increased ratio between Tgt and scan area.

Statistics. Values for ROD and the ratio of Tgt and scan area were analyzed with a two-tailed t-test for unpaired samples with Bonferroni correction. The level of statistical significance was set at P < 0.05.

RESULTS

Severe hypoxia does not alter placental morphology and trophoblast development. Mice were exposed to severe hypoxia during critical steps of placental development, and the
distribution of trophoblast subpopulations was analyzed by in situ hybridization with specific markers for spongiotrophoblasts (4311) and trophoblast giant cells (Pl.Lac1). Expression levels of 4311 and Pl.Lac1 were similar in E8.5 (n = 3), E9.5 (n = 3), and E10.5 (n = 3) placentas after 24-h exposure to 7% O₂ compared with normoxic controls, indicating comparable ratios and normal differentiation of spongiotrophoblasts and trophoblast giant cells, respectively (Fig. 1). Furthermore, regional distribution of 4311 and Pl.Lac1 gene expression was unchanged, suggesting no gross alteration in the placental architecture of hypoxic mice. Reoxygenation of pregnant mice after hypoxic exposure for an additional 24 h before placental harvesting did not lead to structural differences either (data not shown).

This lack of morphological changes in the placenta from hypoxic mothers led us to analyze expression of molecular markers for tissue hypoxia. VEGF is induced by hypoxia in a HIF-1-dependent manner (15) and controls the vascular network in the placental labyrinth (3). Thus in situ hybridization studies were performed in placentas of hypoxic mice exposed to 7% O₂ for 24 h at E9.5 (n = 3) and E10.5 (n = 3). In the normoxic control placenta, VEGF was mainly expressed in the

![Fig. 1. Systemic hypoxia does not alter differentiation of trophoblasts. Transmission and dark-field images show the distribution of trophoblast subpopulations at the fetomaternal border in placentas of normoxic (20% O₂) and hypoxic (7% O₂ for 24 h) pregnant mice at embryonic day (E)8.5 (n = 3), E9.5 (n = 3), and E10.5 (n = 3). Localization and ratio of spongiotrophoblasts (expressing 4311) and trophoblast giant cells (expressing placental lactogen-1, Pl.Lac1) were analyzed by 35S in situ hybridization in serial sections. AS, antisense; S, sense control. Magnification: ×50.](http://ajpregu.physiology.org/)
labyrinth layer as described previously (3). However, no increase in VEGF expression was observed in the placental labyrinth layer of hypoxic mice, whereas VEGF was clearly upregulated in the decidua (Fig. 2). Inspection of vascular structures visualized by immunohistochemistry using the endothelial marker PECAM-1 as well as Flk-1, a marker for newly formed blood vessels, showed no major differences between normoxic and hypoxic mice (Fig. 2). To further quantify placental vascularity, the space covered by PECAM-1-positive vascular structures was morphometrically measured, again revealing no difference between normoxic and hypoxic mice (see Fig. 6).

**HIFs and placental oxygen levels are regionally distributed.** Because hypoxic signals are mediated through HIFs, and considering that hypoxic VEGF expression is controlled by HIF-1 (15) and HIF-2 (13), the presence and distribution of the oxygen-dependent α-subunits HIF-1α and HIF-2α were directly analyzed by in situ hybridization. In placentas from normoxic animals (n = 4), a strong signal for HIF-1α mRNA was detected throughout the placenta, gradually increasing toward the fetomaternal border, whereas expression was low in the maternal decidua (Fig. 3). In contrast, HIF-2α mRNA was strongly expressed in the maternal decidua and only low levels were detected in the placenta (Fig. 3). During hypoxic exposure (n = 4), no major change in HIF-1α mRNA expression was detected, as expected considering that hypoxic regulation of this factor occurs mainly through protein stabilization (21, 48), whereas HIF-2α mRNA expression appeared to be induced in the decidua but clearly not in the placenta (Fig. 3).

For analysis of HIF-1α protein levels, animals were exposed to prolonged (24 h) and short-term (4 h) hypoxia because it has been shown that prolonged hypoxia may lead to downregulation of HIF-1α protein (44). HIF-1α protein was barely detectable in placentas of normoxic mice and mainly confined to singular trophoblast giant cells at peripheral areas of the placenta (n = 6; Fig. 4A). In contrast, a strong HIF-1α signal was observed in the trophoblast giant and adjacent spongiotrophoblast cell layer after 4 h of hypoxia (6% O2, n = 7; Fig. 4A). HIF-1α was highly expressed in the peripheral areas of the placenta, whereas signal intensity decreased toward the center. Interestingly, no HIF-1α signal was detected in the labyrinth layer of the placenta, although HIF-1α mRNA was present there (Fig. 3), suggestive of normal tissue oxygenation. After prolonged exposure to 24 h of hypoxia (n = 3) HIF-1α was strongly expressed in the same areas as observed after 4 h of hypoxic exposure (data not shown). Of note, in placentas of early development (E8.5), when oxygen levels are believed to be low, no immunohistochemical signal for HIF-1α expression was observed in the ectoplacental cone (n = 3; Fig. 4B). As observed above, on hypoxic exposure HIF-1α was induced in the trophoblast border zone but not in the central area.

To exclude the possibility that the absence of HIF-1α protein in the placental labyrinth was due to limited immunohistochemistry sensitivity, we aimed to directly map hypoxic re-

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**Fig. 2.** No alteration in VEGF expression and angiogenesis in the placenta during hypoxia. Vascular pattern and newly formed blood vessels were assessed by immunohistochemistry using platelet endothelial cell adhesion molecule (PECAM)-1 and Flk-1 antibodies at E10.5 (n = 3). Both markers revealed no major alteration after hypoxic exposure (7% O2 for 24 h). VEGF expression was analyzed by 35S in situ hybridization at E10.5 (n = 3). Note induction of VEGF in the maternal decidua but not in the placenta during hypoxia. D, decidua; P, placenta. Magnification: ×50 (PECAM-1, VEGF), ×100 (Flk-1).

**Fig. 3.** Placental hypoxia-inducible factor (HIF)-1α and -2α mRNA expression pattern is complementary. Local distribution of HIF-1α and -2α mRNA levels in placentas from normoxic (n = 4) and hypoxic (n = 4; 7% O2 for 24 h) mice were analyzed by 35S in situ hybridization at E10.5. Note that HIF-1α is mainly expressed in the placenta, whereas HIF-2α is mainly expressed in the decidua. Magnification: ×50.
regions in the placenta in vivo, using the hypoxia marker EF-5. As shown in Fig. 4C, during hypoxia a strong EF-5 signal was obtained in outer placental areas of the trophoblast giant cell layer and the spongiotrophoblast cell layer, with decreasing intensity toward the placental center (n = 6). However, no increase in EF-5 signal intensity was detectable in the inner labyrinth layer compared with normoxic controls. The hypoxic regions identified by EF-5 coincided well with HIF-1α protein expression (Fig. 4A). The maternal decidua displayed a slight increase in hypoxic signal activity.

*Increased eNOS expression is involved in placental protection from hypoxia.* We assumed that elevated placental blood flow by NO-mediated vasodilation is a mechanism to maintain proper oxygen homeostasis in the placenta’s central region. To test this hypothesis, pregnant mice at E10.5 and E11.5 were exposed to acute (4 h, n = 3) and prolonged (24 h, n = 5) hypoxia and placentas were analyzed for eNOS and iNOS expression by immunohistochemistry. In normoxic controls, iNOS was expressed in all layers of the placenta, whereas eNOS expression was confined to the placental labyrinth layer only (Fig. 5). Acute hypoxia did not lead to alterations in NOS expression (data not shown). However, placentas of the prolonged-hypoxia group displayed clearly enhanced eNOS expression, whereas no evidence for increased iNOS expression was detected (Fig. 5). Morphometric analysis revealed that eNOS expression increased significantly by 23% compared with normoxic controls, whereas iNOS expression remained unchanged (Fig. 6). For direct comparability of normoxic and hypoxic tissue, care was taken to analyze matched target areas for each digital scan (Fig. 6).

To confirm NOS as a protective factor for placental tissue oxygenation, NOS activity was blocked by L-NAME. On intravenous L-NAME administration, a strong increase in hypoxia signal activity as analyzed by EF-5 and HIF-1α staining was found in the placental labyrinth layer (n = 7; Fig. 4, A and C).

**DISCUSSION**

Our main findings are that 1) despite exposure of pregnant mice to severe environmental hypoxia, placental architecture, vascular structure, and trophoblast composition were well preserved and 2) severe systemic hypoxemia did not significantly alter placental oxygen levels at the fetomaternal exchange area during placentation, whereas the placental periphery became clearly hypoxic. Our results suggest that the placenta is protected from hypoxia in the area of fetomaternal exchange by NOS upregulation.

Previous experiments showed that hypoxia (3% O₂) can lead to altered differentiation of trophoblast cells (1). In those experiments, however, cells were maintained under hypoxia in vitro for 4 days. The absence of major changes in placental architecture in our in vivo setting might reflect the different experimental conditions. Precursor cells reflecting the situation in vivo may not face such a dramatic hypoxic exposure that leads to the alterations observed in vitro. Indeed, major areas of the placenta did not become hypoxic when the inspiratory oxygen content was lowered to 6% O₂ for 4 h. Even after 24-h exposure to 7% O₂, we found no signs of hypoxia in the placental labyrinth layer, as judged from the absent upregulation of the hypoxia marker VEGF. Accordingly, we did not observe any changes in vascularity, as judged from the unal-
tered PECAM-1 and Flk-1 staining. Hypoxic exposure time might have been too short to result in visible changes. Indeed, during prolonged hypoxia, e.g., in high-altitude residents, a greater vascularity accompanied by lowered HIF-1 activity was observed during hypoxic adaptation of the placenta (46). On the other hand, in a comparable short-term hypoxia model (6-h exposure to 6% O2), we found clearly increased VEGF expression in all organs analyzed (31). Furthermore, an increased number of PECAM-1-positive cells could be found in hypoxic brain areas within 12–24 h after onset of cerebral ischemia (29). In analogy, the placental labyrinth layer appears to be specifically protected from shorter episodes of hypoxia.

The precise roles of the hypoxia-inducible transcription factors HIF-1 and HIF-2 in the murine placenta remain to be established. Mice lacking HIF-1β/ARNT display a dramatically reduced labyrinth and spongiotrophoblast layer and increased numbers of giant cells (1). Those authors concluded that oxygen levels regulate cell fate determination in vivo and that HIF-1 is essential for mammalian placentation. Furthermore, data from human placenta suggest HIF-1α as a major factor involved in trophoblast differentiation (8). In contrast, no early placental aberrations in HIF-1α-deficient mice have been observed (22). It has been suggested that HIF-2 may compensate for the absence of HIF-1 (1). Our data, showing disperse expression of HIF-1α and HIF-2α, with HIF-1α being mainly expressed in the placenta but HIF-2α mainly localized to the maternal decidua, imply complementary rather than redundant functions for HIF-1 and HIF-2 in the placenta. Indeed, differential roles for HIFs due to distinct localization in the murine uterus were also suggested during the peri-implantation period (12). The complex and as yet not fully understood regulation and interaction of hypoxia-inducible factors and other growth factors makes it difficult to draw a definite conclusion concerning the role of HIF-1α and the involved compensatory mechanisms.

Interestingly, during early placentation when placental blood flow is not yet established, we were unable to detect HIF-1α in the ectoplacental cone (Fig. 4B). Placental cellular oxygen sensitivity may be dependent on gestational age. Nevertheless, HIF-1α expression was induced in our system during hypoxic exposure, indicating that oxygen sensitivity is preserved at least in the outer trophoblast layer, albeit not in centrally lying intermediate-type cells. The lack of HIF-1α signal in these cells may indicate a different threshold in response to changes

![Fig. 5. Endothelial (e)NOS is induced during systemic hypoxia in the placental labyrinth: immunohistochemical analysis for eNOS (n = 5) and inducible (i)NOS (n = 3) protein expression in placentas of normoxic and hypoxic mice at E10.5. Note that signal intensity for eNOS but not iNOS protein is markedly increased in the placental labyrinth after 24 h of hypoxia (7% O2). Magnification: ×50, details marked by boxes ×630.](image)

Fig. 6. eNOS but not iNOS is significantly induced during systemic hypoxia in the placental labyrinth. Relative optical density of eNOS expression was significantly higher in the labyrinth layer during hypoxia (Hx; 7% O2, 24 h) (solid bars) compared with normoxia (Nx) (open bars), whereas iNOS expression remained unchanged. PECAM-1 expression as a surrogate marker for vascular density was not altered. The increase of relative optical density was not due to shrinkage of the tissue because the ratio between target area (proportion of stained area) and scan area (total labyrinth area) remained unchanged for all proteins in normoxic (light gray bars) compared with hypoxic (dark gray bars) animals, *P < 0.001, n = 5 (eNOS), 2 (iNOS), or 3 (PECAM-1).
in oxygen tissue partial pressure at this developmental stage. Taken together, placental local expression of HIF-1 and -2 is diverse and HIF-1 rather than HIF-2 may be involved in oxygen-dependent developmental processes within the placenta. Furthermore, oxygen sensitivity may be different in various cellular subpopulations.

We have shown preservation of normal tissue oxygenation in the placental labyrinth even during severe systemic hypoxia and provide evidence for hypoxia-induced activation of NOS being responsible for tissue protection. NOS catalyzes the production of NO that represents a potent vasodilator (9, 19). In mammals, three NOS isoforms have been identified: neuronal NOS, iNOS, and eNOS. Whereas iNOS expression is induced by cytokines, hypoxia, and other agents (20, 32), eNOS is constitutively expressed but is known to be activated by physical and metabolic stimuli such as shear stress and hypoxia as well (6, 9, 35). Indeed, it was recently shown that the eNOS promoter contains a hypoxia response element and thus might be a HIF target gene (11). Expression of iNOS and eNOS has been observed in human and murine placenta (10, 41, 45). Thus hypoxic upregulation of placental NOS expression may result in NO-mediated vasodilation, thereby increasing placental blood flow and reconstituting oxygen supply to the placenta. Indeed, in pregnant sheep chronic hypoxia selectively augments eNOS expression and activates endothelium-dependent relaxation in uterine arteries (49). Our results expand these findings by showing that eNOS was upregulated also inside the placenta in the labyrinth layer of mice during prolonged hypoxia. Apparently, eNOS is upregulated in human placentas from high-altitude pregnancies as well (Tissot van Patot M, unpublished observation). Consistent with our results, an increase in uterine blood flow velocity, probably as a result of downstream vasodilation, has been observed in pregnant women residing at high altitude (50). Applying the NOS inhibitor l-NAME, we demonstrated the crucial role of NOS upregulation as a mechanism for preserved tissue oxygenation. Because l-NAME inhibits all NOS isoforms, we cannot exclude the possibility that inhibition of iNOS also contributed to the observed effects, although no hypoxic iNOS upregulation was found. Furthermore, systemic cardiovascular effects of l-NAME might have influenced placental perfusion, as l-NAME increases arterial pressure and reduces heart rate and cardiac output (24, 34). Finally, hypoxic changes in metabolic parameters such as glucose levels or acid-base status might have influenced placental oxygenation as well. We observed reduced food intake and activity of hypoxic mice, including a decrease in ventilatory oxygen consumption and carbon dioxide production (43). Furthermore, tissue glycogen levels have been shown to decrease in hypoxic rats (36), and inhibition of brown adipose tissue sympathetic nerve activity during hypoxia has been suggested to directly contribute to the hypoxia-evoked reduction in body temperature and oxygen consumption serving as adaptive responses to decreased oxygen availability (28).

Although an important role for NOS in preserving placental perfusion during hypoxia has been postulated (49), to our knowledge this is the first report showing selective inhibition of NOS activity resulting in a severe reduction of placental oxygenation. In conclusion, our results show that murine placental development is relatively resistant to prolonged systemic hypoxia and indicate NO synthesis as a potent protective mechanism to maintain placental tissue oxygenation and function during systemic hypoxia.

ACKNOWLEDGMENTS

The authors thank R. Landwehr and B. Saam for technical assistance, Celeste Simon for the gift of plasmids, A. Scheid for valuable discussion, E. Beinder and R. H. Wenger for critically reading the manuscript, and R. Zimmermann, R. Huch, and C. Bauer for support.

GRANTS

This work was supported by the Stiftung für wissenschaftliche Forschung an der Universität Zürich (to L. Schafer) and the Swiss National Science Foundation (to M. Gassmann).

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