Role of nitric oxide in placental vascular development and function

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1. Introduction

Placental vascular development represents a crucial process for adequate fetal development. In fact, pregnancy pathologies, such as, gestational diabetes mellitus (GDM), intrauterine growth restriction (IUGR) and pre-eclampsia (PE) are related with vascular dysfunction in the placental bed [1,2]. The etiology of this dysfunction remains to be fully understood [3,4] but even though these conditions have detrimental effects on the fetus in late gestation they seem to be influenced by early embryo development, and are exacerbated by environmental cues through gestation such as oxidative stress, local oxygen tension and metabolic disorders [5–7].

The placenta originates from the outer cell layer of the morula, called trophoblast, which proliferates after implantation, invades the decidua and differentiates into cytotrophoblast and syncytiotrophoblast, forming the primary villi. These villi are invaded by mesenchymal cells derived from the embryo (secondary villi) which through vasculogenesis generate vascular capillaries, forming the tertiary villi. These villi continuously undergo transformation and maturation during gestation and this includes (1) further differentiation of cytotrophoblast to syncytiotrophoblast, (2) branching angiogenesis and growth of the vascular tree, and (3) longitudinal growth of capillaries and maturation of vascular vessels [8,9]. Thus, placental vasculogenesis, angiogenesis and vascular function are interrelated processes that influence fetal growth throughout gestation.

Nitric oxide (NO) is one of the most pleiotropic signaling molecules at systemic and cellular levels, participating in vascular tone regulation, cellular respiration, proliferation, apoptosis and gene expression. Indeed NO actively participates in trophoblast invasion, placental development and represents the main vasodilator in this tissue. Despite the large number of studies addressing the role of NO in the placenta, its participation in placental vascular development and the effect of altered levels of NO on placental function remains to be clarified. This review draws a time-line of the participation of NO throughout placental vascular development, from the differentiation of vascular precursors to the consolidation of vascular function are considered. The influence of NO on cell types involved in the origin of the placental vasculature and the expression and function of the nitric oxide synthases (NOS) throughout pregnancy are described. The developmental processes involved in the placental vascular bed are considered, such as the participation of NO in placental vasculogenesis and angiogenesis through VEGF and Angiopoietin signaling molecules. The role of NO in vascular function once the placental vascular tree has developed, in normal pregnancy as well as in pregnancy-related diseases, is then discussed.

2. Expression of nitric oxide synthases in the placenta

During gestation NOS isoform expression is dynamically regulated in the placenta (Fig. 1), which show heterogeneous properties
and distribution (Table 1). In fact, at least eNOS and iNOS have been found in diverse species since early stages of placental development [15–19]. The iNOS isoform is expressed mainly at the fetomaternal interface in the first stages of pregnancy [20]. Apparently, there is an increase in iNOS activity throughout pregnancy which peaks at mid gestation [21–24]. On the other hand, in human first trimester placenta, eNOS is expressed in syncytiotrophoblast, early endo- 


derm [1]. Additionally, there is growing evidence for a crucial role of the yolk sac in embryo and placental vascular development [40]. Indeed, using Ncx-1 knockout mice which fail to initiate cardiac contraction Lux et al. [41] showed that all the hematopoietic progenitor cells emerge from the yolk sac. Furthermore, a study in mice yolk sac demonstrated that vasculogenesis at this level is initiated by NO [42]. These authors described the spatio-temporal expression pattern of iNOS and eNOS, which was related to vasculogenesis in the yolk sac. In the first stage, at 7 days of embryonic development (E7.0), iNOS-derived NO synthesized by endodermal cells induces the differentiation of adjacent extra-embryonic mesodermal cells to form a primary capillary plexus. After that, eNOS expression increases in the yolk sac mesodermal cells [36,42] accompanied by a decrease in iNOS expression in the endoderm [42]. Experimental inhibition of NOS activity at E6.5 completely arrests the development of the primary capillary plexus [42]. Altogether, these data suggest that NO could be crucial for placental vasculogenesis.

4. Nitric oxide and placental angiogenesis

Growth and consolidation of the placental vascular tree occurs by angiogenesis. In this process single vessels are formed by endothelial precursor cells (EPC) which differentiate into endothelial cells, and/or proliferate from endothelial cells. These vessels can grow in two ways, (1) non-branching angiogenesis, which implies an increase in the length of the villous vessels, and (2) branching angiogenesis, in which multiple short capillary loops are formed [8], increasing the vascular surface area. After these processes have taken place, the vessels mature and their structures stabilize. Additional maturation and specialization in the vascular system is influenced by environmental cues, such as blood flow, oxygen tension, oxidative stress which could be signaled by epigenetic mechanisms [43]. All these factors have been implicated in the development and function of the human placenta [5–7].

Participation of NO in angiogenesis is more clearly established than it is in vasculogenesis. Angiogenesis has been studied in vitro using endothelial cells from the human umbilical vein (HUVEC). Animals with experimentally-induced eNOS deficiency show defective vascular development throughout the vascular tree, which is mainly associated with decreased vessel maturation and disorganization [35,44]. Despite the initial evidence that showed an inhibitory role for NO in angiogenesis [45], further studies have demonstrated that exogenous NO [46,47] or over-expression of eNOS [48,49] induce proliferation of endothelial cells and angiogenesis. In contrast, inhibition of NOS [50,51] or deletion of the eNOS gene [52] are accompanied by deficient angiogenesis in the embryo and placenta. Additionally, NO induces proliferation in fetal endothelial cells coming from different vascular beds [53–55] and regulates the caliber of central vessels [56].

The main molecular mediators of angiogenesis, VEGF and angiopeptin [57] depend mainly on NO synthesis to induce new vessel formation. In endothelial cells VEGF induces eNOS-dependent NO synthesis through the activation of VEGFR-1 [58] and VEGFR-2 [59]. It has been shown that VEGF-induced angiogenesis requires NO synthesis [32] derived from eNOS activity.
Moreover, migration of endothelial cells and EPC is regulated by NO. VEGF-induced migration requires the activation of eNOS through PKCδ [33], Akt [63] and HSP90 [64]. Nitric oxide facilitates cell migration in angiogenesis, increasing the expression of adhesion molecules (i.e. integrin αvβ3) [65,66] and extracellular matrix metalloproteases (i.e. MT1-MMP, MMP-9 and MMP-13) allowing the invasion of endothelial cells and new vessel formation [67-69]. Angiopoietin activates eNOS via PI3K/Akt [70] and ERK1/2 [71] pathways, leading to proliferation and angiogenesis. Additionally, Ang-1 increases the expression of eNOS [72], whilst eNOS over-expression enhances the angiogenic response to Ang-1 [49]. Moreover, the Ang-1/eNOS pathway contributes to vessel maturation and stabilization [49,72]. In this context, capillary formation and maintenance is influenced by the expression of VE-cadherin, which is regulated by VEGF and Ang-1 [73,74]. It has been shown that NO induces the expression and function of VE-cadherin [75-77] modifying the stability and permeability of the endothelium.

Additionally, NO donors induce proliferation in ovine placental endothelial cells via activation of MEK1/2 [54]. On the other hand, eNOS [36] and Akt [78] knockout mice show reduced placental development with decreased vascularization. Moreover, NO increases the levels of VEGF [46] and Ang-1 [79,80] in endothelium. All these data show that NO is not only an effector of angiogenic pathways, but an inducer and positive feedback signal for Ang-1- and VEGF-induced angiogenesis.

5. Placental angiogenesis in diseases of pregnancy

Placental dysfunction is present in some diseases of pregnancy (i.e. GDM, IUGR and PE) [12,81], and accompanied by abnormal expression of angiogenic mediators [82-86] and altered NO synthesis [2]. Interestingly Akt null mice fetuses present IUGR [37], detectable as early as E8.5 [38], a time at which the placental vascular development depends only on angiogenesis. In various models of IUGR the expression of eNOS and angiogenic factors is higher during the first half of pregnancy compared with normal fetuses [19,87-89]. However, by the end of gestation these levels fall below those seen in normal placenta [19,29,87,88] suggesting a possible failure of the compensatory mechanisms which normally sustain placental development and the consequently, fetal growth.

In contrast to IUGR, placenta from GDM pregnancies are larger than normal [90] showing decreased formation of terminal villi and increased numbers of intermediate villi in relation to gestational age [81]. Studies in rats have suggested that the increased growth and vascularization in GDM are associated with higher levels of MMP-2 and MMP-9, the activities of which are positively regulated by NO [91]. These data suggest that NO not only regulates endothelial cell proliferation and migration in the placenta, but participates in the stabilization of the new vessels.

However, there is no well established level of NO required for adequate placental angiogenesis. High NO levels can prevent angiogenesis [45,52,93], and its effect on cell survival and proliferation depends on its concentration [94]. This could explain in part why pregnancy diseases, such as IUGR and PE, show higher levels of placental NO [56-58] and nitrosative stress [99] without adequate placental vascularization.

6. Nitric oxide in placental vascular maturation

The adequate morphology and function of the vascular tree at different levels (i.e. arteries/veins, and conduit/resistance/exchange vessels) are modulated by environmental cues, such as; blood flow, oxygen levels, oxidative stress and epigenetic factors [43]. In addition, these signals have a functional relationship with the eNOS-derived NO. Shear stress induces the differentiation of human placenta-derived pluripotent cells to endothelial cells [100], and the specialization of endothelial progenitor cells to "arterial" endothelial cells [101]. In this context, the ability of new endothelial cells to generate vessels depends on the release of NO from pre-existing endothelial cells [102]. Additionally, shear stress is the main stimulus for NO release in placental vessels [103] and for angiogenesis via eNOS activation [104,105]. Indeed in the focal adhesion kinase (FAK – a critical shear stress sensor) knockout mouse there is impaired placental vascular development [106]. Thus, it can be proposed that the increase in placental blood flow during gestation [23,107] drives placental vascular maturation, in part, through NO synthesis.

Hypoxia is an important factor that regulates placental development and angiogenesis. In fact, a fine tuning of oxygen levels throughout pregnancy are required, being hypoxia associated with beneficial and detrimental effects on development [6]. It stimulates trophoblast invasion, differentiation, survival [6], angiogenesis [108,109] and vasculogenesis [108]. The angiogenic effect of hypoxia can occur via VEGF and eNOS, which are activated by hypoxia-inducible factor-α (HIF-α), a key transcription factor operating in hypoxia [108,109]. However, chronic hypoxia is associated with reduced fetal and placental development [110], and in the mouse embryo high level of VEGF and eNOS, HDAC inhibition and hypoxia increases the formation and maintenance of trophoblast invasion, differentiation, survival [6], and angiogenesis [137]. Furthermore, hypoxia-induced angiogenesis is also associated with endothelial cell apoptosis via p53 [112] and nuclear factor κB (NF-κB) [113]. There is some uncertainty about the effect of hypoxia on eNOS expression and activity. Some reports have shown an induction of eNOS expression in hypoxia [114-116], whilst others suggest a down-regulation [117-119]. These differences could be explained, in part, by the different vascular beds, endothelial cell types (i.e. arteries [114,116] and veins [117-119]). Additionally, NO has a dual effect on HIF levels: short exposures to hypoxia (2 h) activates eNOS and increases HIF-1α [120,121], however, after 4 h of hypoxia NO increases the levels of prolyl hydroxylases (PHD), which under normal oxygen levels induce HIF-1α degradation [121,122]. Alternatively, NO-dependent activation of PHD can occur through a change in oxygen consumption [123] via inhibition of mitochondrial respiration [124]. Prolonged exposure to hypoxia is associated with decreased eNOS activity, endothelial cell apoptosis and deficient placental vascular development, indicating that placental angiogenesis is very sensitive to oxygen levels. This suggests that NO participates in responses to acute hypoxia via inducing HIF and contributes to a negative feedback process in chronic hypoxia.

During placental vascular maturation and development epigenetic mechanisms play a key role. The expression of genes implicated in trophoblast invasion are influenced by histone post-translational modifications (HPTM) [125], whilst placental growth and function are regulated by DNA methylation (imprinting) and HPTM [126-128]. Moreover, IUGR placenta show epigenetic modifications [129-131] which correlate with abnormal placental development. On the other hand, the first stages of vascular development are controlled mainly by genetic processes; however, endothelial specialization and vessel maturation are controlled by epigenetic mechanisms [43]. There is growing evidence that endothelial precursor cell differentiation, endothelial cell specialization, and shear stress- and hypoxia-induced responses are modulated by epigenetic mechanisms, such as, DNA methylation, HPTM and non-coding small RNA-mediated mechanisms [132,133]. Shear stress- and VEGF-induced differentiation of endothelial precursor cells activate histone deacetylase (HDAC) 1 and 3, increasing the expression of VEGFR-2 and eNOS [134]. HDAC inhibition reduces the VEGF-induced angiogenesis through down-regulation of VEGFR-1 and -2 expression [135], whilst histone methyl transferase activity is required for migration and vessel sprouting [136]. Additionally, hypoxia-induced angiogenesis requires HDAC1 activity, which
 decreases the expression of von Hippel-Lindau, increasing HIF-1α levels [137]. Interestingly, reduced angiogenesis induced by HDAC inhibition is not reversed by VEGF supplementation, but is restored by NO restitution with S-nitroso-N-acetylpenicillamine (SNAP) [138]. The promoter region of the eNOS gene shows a cell-specific pattern of epigenetic modifications [133]. There are subtle differences in the epigenetic markers between micro and macrovascular endothelial cells [139] arguing for a role for the differential expression of eNOS in different vascular beds. Altogether these data show that in endothelial cells eNOS expression is highly regulated by epigenetic mechanisms with important effects on angiogenesis. Additionally, there is growing evidence that NO exerts control on epigenetic mechanisms [reviewed in Illi et al. [140]].

7. Nitric oxide and placental vascular function

The placenta lacks adrenergic and cholinergic innervation [141], thus its vascular tone is regulated mainly by local factors derived from the endothelium and blood cells. Studies in the later 1980's and early 1990's show that the endothelium from chorionic and umbilical vessels releases NO [142,143]. Further studies demonstrated that in umbilical vessels the production of NO and its vasodilator effect is higher than that of prostacyclin (PGI2) [144,145]. Like other vascular beds, placental vessels express the molecular mediators of the classical NO-dependent pathway which includes the soluble guanylate cyclase (sGC) [146], cGMP-dependent protein kinase and cGMP-specific phosphodiesterases [147,148]. Nitric oxide exerts its effects via activation of the sGC [149] and modulation of potassium channel activity (BKCa) [150,151] (Fig. 2). The NO synthesis at this level can be induced by histamine [142], adenosine [143,152], ATP [143], calcitonin gene-related peptide (CGRP) [153,154], and shear stress [103,155]. The later is an important stimulus which activates eNOS through its phosphorylation at serine 1177 via ERK1/2 and Akt in placental endothelium [156] increasing its long term expression [157].

Oxygen level is the most important factor controlling placental vascular reactivity; hypoxia in vivo and ex vivo increases placental vascular tone. In isolated placental vessels low oxygen levels reduce the maximal response to vasodilator agents [158] and increase that to vasoconstrictors [158–160]. Studies in sheep have shown that NO synthesis plays a key role maintaining placental blood flow during acute episodes of hypoxemia [161]. In perfused human cotyledons NOS inhibition and hypoxia independently increase placental perfusion pressure to a similar degree, effects prevented by NO donors [162], suggesting that the effect of hypoxia is mediated in part via low NOS activity. The effect of hypoxia in placental vasculature depends on the vessels studied (Fig. 3). In isolated chorionic arteries hypoxia increases the maximal response to KCl and the thromboxaneA2 mimetic U46619 [159,160]. However, the maximal response to the NO-donor SNAP is unaffected by acute changes in the PO2 [160] in these vessels. In contrast, in chorionic and umbilical veins an acute reduction on PO2 has a positive effect on the vasodilator response to NO [160] and NOS activity [163], whilst there is no change in the response to vasoconstrictors [160]. The effect of PO2 on NO-induced responses in umbilical arteries has not been resolved; Lovren & Triggle [150] showed that the maximal vasodilator response and sensitivity to NO were reduced at low PO2 (<55 mmHg, slightly higher than normal fetal PO2, which is 25–30 mmHg) compared with higher oxygen levels (>300 mmHg); however Leung et al. [164] observed no changes in NO-induced vasodilation with changes in pO2. The study of the molecular mechanisms activated by hypoxia in placental endothelium has been carried out only in HUVEC. It has been demonstrated that HUVEC exposed to 24 h of hypoxia show a decrease in L-arginine transport and eNOS activity [117,118].

8. Placental vascular reactivity and nitric oxide synthesis in diseases of pregnancy

As previously discussed, in normal conditions NO is the main vasodilator in the placenta. For this reason several studies have characterized alteration in placental NO synthesis in an attempt to explain the vascular dysfunction observed in pregnancy diseases, such as IUGR, PE and GDM. Alterations in placental vascular reactivity in these conditions are associated with changes in vascular tree structure and vasoactive response pathways. In IUGR and
IUGR/PE placentae the villous tree has longer capillaries with fewer branches [165] and lower caliber umbilical vessels [166,167] compared with normal placenta. However, there are no alterations in the maximal responses to vasoconstrictor agents in IUGR chorionic vessels [168,169]. In contrast, the response to NO-dependent vasodilator agents is decreased in IUGR and PE. IUGR chorionic arteries show a reduced vasodilator response to VEGF and PIGF [168], whilst in PE the response to CGRP is practically absent [170]. It is noteworthy that in these vessels the response to exogenous NO is not altered [171], however the amplitude and frequency of NO-dependent spontaneous tone oscillations are reduced in IUGR chorionic arteries and can be invoked in normal vessels by inhibiting NO synthesis [172]. As other metabolic pathways which use L-arginine, such arginases, could compete with eNOS in the placent al endothelium, they may play a role under these circumstances. It has been shown that arginase-2 expression is increased in chorionic villi endothelium in PE [173]. Furthermore, we have recently demonstrated in HUVEC that arginase-2 levels and activity are up-regulated by hypoxia [174]; however the role of arginase activity in the control of placent al vascular reactivity remains to be determined. These data suggest that vascular dysfunction in diseases of pregnancy is mainly due to changes in vessel structure and activation of NO-synthesis pathways by vasodilator agents rather than by altered responses to NO and vasoconstrictors.

The expression of NOS and NO-metabolites levels in placent al endothelium in pregnancy pathologies cannot be easily correlated with the vascular dysfunction observed. Rutherford and colleagues [95] reported increased levels of NOS in chorionic villi of IUGR and PE placentae and these were decreased in umbilical vessels compared with normal placenta. Similarly, increased levels of NO-metabolites in umbilical cord blood [96] and placental tissue [98] of IUGR pregnancies have been reported. The source of this NO is still unclear. Initial studies carried out by Myatt and colleagues [26] found increased levels of eNOS in the endothelium of IUGR and PE placentae. However, other authors have found decreased levels of eNOS in these conditions [30,173] that can be correlated with the vascular dysfunction determined in vivo [30]. Additionally, in HUVEC from PE [175] and IUGR [118] pregnancies, decreased eNOS expression and activity can be observed. Apparently, HUVEC chronically exposed to hypoxia, as occurs in IUGR pregnancies, do not regulate the L-arginine/NO pathway in response to hypoxia and present a persistent hypoxia-like phenotype [117,118]. An explanation for the increased levels of NO-metabolites together with increased placental vascular resistance in IUGR and PE could be due to higher iNOS expression. Giannubilo and colleagues [30] found that eNOS protein levels negatively correlate with the pulsatility Index (PI) determined by Doppler ultrasound, whilst INOS levels in IUGR placent al endothelium show a positive correlation with PI. Additionally, normal HUVEC exposed to PE-deri ved umbilical plasma show higher iNOS expression [176]. Under these conditions increased levels of nitrosative stress have been described [99], HUVEC exposed to oxidative stress down-regulate eNOS and up-regulate iNOS expression [177], effects which are increased by shear stress and lead to nitrosative stress and cell apoptosis [178]. Increased endothelial iNOS expression has been implicated in vascular dysfunction [179,180] and in animal models of diabetes [181,182], atherosclerosis [179] and aging [183]. Thus NO and NO-metabolite levels are not necessarily associated with improved placental endothelial function and further studies are required to determine the source of NO (i.e. eNOS or iNOS) in diseases of pregnancy characterized by placental vascular dysfunction.

Acknowledgements

FONDECYT 1080534 & 1110977 (PC); CONICYT-ACT-73 (PC); CONICYT-24100107 (BJK); British Heart Foundation (MAH).

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B.J. Krause et al. / Placenta 32 (2011) 797–805


