Histochem Cell Biol (2004) 122:369–382
DOI 10.1007/s00418-004-0677-x

REVIEW

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Oxidative stress in the placenta

Accepted: 7 June 2004 / Published online: 10 July 2004

Abstract Pregnancy is a state of oxidative stress arising from increased placental mitochondrial activity and production of reactive oxygen species (ROS), mainly superoxide anion. The placenta also produces other ROS including nitric oxide, carbon monoxide, and peroxynitrite which have pronounced effects on placental function including trophoblast proliferation and differentiation and vascular reactivity. Excessive production of ROS may occur at certain windows in placental development and in pathologic pregnancies, such as those complicated by preeclampsia and/or IUGR, overpowering antioxidant defenses with deleterious outcome. In the first trimester, establishment of blood flow into the intervillous space is associated with a burst of oxidative stress. The inability to mount an effective antioxidant defense against this results in early pregnancy loss. In late gestation increased oxidative stress is seen in pregnancies complicated by diabetes, IUGR, and preeclampsia in association with increased trophoblast apoptosis and deportation and altered placental vascular reactivity. Evidence for this oxidative stress includes increased lipid peroxides and isoprostanes and decreased expression and activity of antioxidants. The interaction of nitric oxide and superoxide produces peroxynitrite, a powerful prooxidant with diverse deleterious effects including nitration of tyrosine residues on proteins thus altering function. Nitrative stress, subsequent to oxidative stress is seen in the placenta in preeclampsia and diabetes in association with altered placental function.

Keywords Placenta · Oxidative stress · Nitric oxide · Superoxide · Peroxynitrite

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Introduction

Oxidative stress is described as an imbalance in the production of reactive oxygen species (ROS) and the ability of antioxidant defenses to scavenge them. It can arise from increased production of ROS and/or a decrease in antioxidant capacity. These ROS are free radicals and they induce cellular damage by acting on proteins and lipids. Pregnancy per se is a state of oxidative stress (Wisdom et al. 1991) arising from the increased metabolic activity in placental mitochondria and reduced scavenging power of antioxidants. Oxidative stress has been clearly shown in placental tissue. However, in certain pathologic pregnancies, i.e., those complicated by maternal diabetes, preeclampsia, or early pregnancy loss, a heightened level of oxidative stress is encountered. This increased oxidative stress can then affect placental function. This review will describe the determinants of oxidative stress in the placenta, review the evidence for increased oxidative stress in pathologic pregnancies, and discuss the functional consequences.

Generation of ROS in the placenta

The most common ROS is the superoxide anion ($O_2^{\cdot -}$) formed as the first step in the one-electron reduction of molecular oxygen. Superoxide is generated in living cells by NADPH oxidase, xanthine oxidase, flavin enzymes, and enzymes in the mitochondrial electron transport chain (METC). The reactivity due to the unpaired electron in superoxide then leads to the production of other ROS including the hydroxyl radical and hydrogen peroxide. Reactive oxygen species are recognized to be signal transduction agents in addition to their well-defined roles in combating pathogens. Superoxide can also be generated under certain conditions by enzymatic pathways that produce other ROS including nitric oxide and carbon monoxide which have vasoactive effects in the placenta but are also realized to have roles in signal transduction.

The pathways leading to the production of these ROS are described in more detail below.

Enzymatic pathways

Mitochondrial electron transport chain

The mitochondria are responsible for consumption of the majority of oxygen and ATP generation. It has been estimated that about 2–3% of all electrons in the METC leak out of the mitochondria, estimated to produce 160–320 mmol superoxide per day by a 60 kg woman (Chance et al. 1979). Although METC complex III was considered as the major ROS generation site (Chen et al. 2003), Kudin et al. (2004) found that inhibition of complex I resulted in a greater V_{max} for H_2O_2 generation (0.68 nmol/min per mg with rotenone, a complex I inhibitor, vs 0.14 nmol/min per mg with antimycin A, a complex III inhibitor) in isolated rat and human brain mitochondria. Inhibition of METC complex I with rotenone caused apoptosis in HL60, a leukemia cell, and in HT1080, a fibrosarcoma cell, via ROS generation showing a functional effect of superoxide generation (Li et al. 2003). Similarly, hypoxia may cause halting of complete mitochondrial oxygen reduction thus generating superoxide. Hypoxia may alter METC complex I in lung epithelial cells (Li et al. 2002), and in cultured pulmonary artery myocytes, hypoxia-induced ROS generation was attenuated by an inhibitor of METC complex I. This may explain the apparent paradox of hypoxia-induced oxidative stress. In addition, ROS seem to act as second messengers for oxygen sensing in the mitochondria since antioxidants abolished hypoxic pulmonary vasoconstriction without affecting contraction to U46619, and the hypoxic response was absent in cells that were depleted of METC (Waypa et al. 2001). The participation of METC in early pregnancy is not clear. However, mitochondrial mass in the placenta increases both with gestational age, suggesting increased contribution of METC to ROS generation, and in placental pathologies such as preeclampsia whereby a hampered oxygen sensing was suggested (Wang and Walsh 1998).

Xanthine oxidoreductase

Xanthine oxidoreductase exists in two functionally distinct forms. Under normal conditions, the larger part of the enzyme occurs as an NAD(+)-dependent dehydrogenase form (XDH) which converts hypoxanthine to xanthine with reduction of NAD to NADH. The dehydrogenase can be transformed under various pathophysiological conditions, i.e., hypoxia or cytokine stimulation, to an oxygen-dependent oxidase form (XO). Xanthine oxidase then catalyses the oxidation of xanthine to uric acid, with accompanying superoxide production. Hypoxia has been shown to upregulate expression of XO (Hassoun et al. 1994). However, studies in bovine pulmonary vascular endothelial cells showed that XO was quickly inactivated by in vitro and in vivo reoxygenation (Terada et al. 1988; Partridge et al. 1992), suggesting XO may serve as an initial source but not a prolonged source of ROS generation. This may have implications during early pregnancy (see later).

Xanthine oxidase was first described in villous trophoblast, stroma and endothelial cells by Many et al. (1996) with a modest increase in expression seen throughout gestation. Increased expression of XO mRNA in villous endothelial cells and extravillous trophoblast accompanied by decreased superoxide dismutase (SOD) expression in the same cells, was subsequently described in preeclamptic women (Many et al. 1996, 2000). However, XO activity was reportedly absent in fresh human placenta (Wajner and Harkness 1989). Therefore, the involvement of XO in generation of placental oxidative stress is not confirmed. Furthermore, placental transport of uric acid and hypoxanthine, the substrate of XDH, are very limited (van Kreel and van Dijk 1977) suggesting XO may only be a minor generator of superoxide in the placenta.

NADPH oxidase

The enzyme NADPH oxidase is capable of generating large amounts of ROS in neutrophils, macrophages, and monocytes (Babior et al. 2002). This phagocytic isoform is a multimeric enzyme consisting of five unique peptides, p22phox, p47phox, p67phox, Rac, and gp91phox. Under appropriate stimulation these subunits assemble at the cell membrane. A flavocytochrome consisting of two membrane-bound peptides of 22 and 91 kDa (p22phox and gp91phox or Nox2, respectively) makes up the redox pathway, and contains binding sites for NADPH, flavin, and heme. The placental macrophage, the Hofbauer cell, has been shown to express subunits of this phagocytic NADPH oxidase (Myatt et al. unpublished data; Fig. 1).

An oxidase activity was detected and was confined to the microvillous membrane of syncytiotrophoblast with non-specific cytochemical methods (Matsubara and Sato 2001). More recently, Manes (2001) isolated a protein consisting of a 58- and a 33-kDa subunit from term placenta, presumably from syncytiotrophoblast. In vitro, this placental NADPH oxidase is constitutively active and differs from that of phagocytes in several biochemical properties suggesting another isoform exists in placenta. Recently, several homologues of human gp91phox subunit have been identified in somatic cell types by molecular cloning (Table 1). The products are characterized by five to six transmembrane domains with a highly homologous C-terminus NADPH/FAD binding region. Although the physiological function of the non-phagocytic Nox genes remains largely unknown, some of these novel Nox isoforms have been shown to generate ROS in reoxygenation models (Caraceni et al. 1995; Zulueta et al. 1995; Souren et al. 1997; Al-Mehdi et al. 1998; Spranger et al. 1998; De Deken et al. 2000). Vascular endothelial growth factor (VEGF) mRNA, VEGF receptors, and

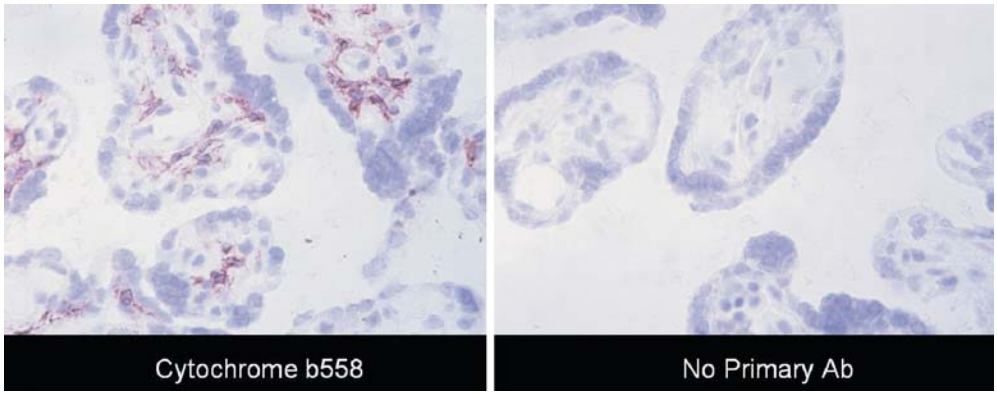

Fig. 1 Expression of Nox2 in human villous tissue. Frozen term villous tissue was immunostained with anti-cytochrome b558 antisera (*left panel*) or with the absence of primary antibody (*right panel*) using the Vectastain ABC kit. Magnification ×400

Table 1 Features of Nox genes

	Nox1	Nox2	Nox3	Nox4	Nox5	Duox1	Duox2
Chromosome	X	X	6	11	15	15	15
Peptide size	564	569	569	578	566	1,551	1,548
ROS generation	Yes	Yes	Unknown	Yes	Yes	Unknown	Yes
Regulation	EGF	Ca^{2+}, Akt	Unknown	Ang II	Ca^{2+}	Ca^{2+}, cAMP	Ca^{2+}, cAMP
Function	Proliferation	Host defense	Unknown	O_2 sensing	Proliferation	Unknown	Proliferation

metalloproteinase activity were markedly increased in Nox1-expressing cells (Arbiser et al. 2002). It has also been found that Nox5 induces apoptosis in prostate epithelial cells (Brar et al. 2003). These novel NADPH oxidase enzymes are possibly involved in the pathophysiology of atherosclerosis and hypertension and can be stimulated by ligands, such as angiotensin II, PDGF, cytokines, and thrombin, changes in hemodynamic forces, and cellular metabolism (Griendling et al. 2000) to produce superoxide.

Our laboratory has recently identified two Nox isoforms, Nox1 and Nox5, in cytotrophoblasts isolated from term human placenta (Cui et al. unpublished observations). Employing anti-Nox1 and anti-Nox5 peptide antisera, we observed specific staining in syncytiotrophoblast and villous vascular endothelial cells by immunohistochemistry (Fig. 2). We postulate that these novel Nox enzymes may play an important role in both ROS generation and oxygen sensing.

Nitric oxide synthase

Nitric oxide is an active vasodilator in the fetal placental vasculature (Myatt et al. 1991) where it maintains basal vascular tone and attenuates the action of vasoconstrictors. However, it may fulfill other roles such as anti-adhesive and anti-aggregatory roles in syncytiotrophoblast, immunomodulatory roles in Hofbauer cells, together with being a signal transduction agent. We first described that the type III or endothelial nitric oxide synthase (eNOS) isoform was immunolocalized to villous vascular endothelium and syncytiotrophoblast (Eis et al. 1995). In vitro expression of eNOS increased in syncytiotrophoblast as they differentiated from cytotrophoblast (Eis et al. 1995). In contrast type II or inducible NOS is expressed in Hofbauer cells (Myatt et al. 1997b). It is controversial whether eNOS is expressed in extravillous trophoblast cells where it may play a role in regulation of trophoblast invasion (Eis et al. 1995; Martin and Conrad 2000; Lyall 2003). We have found that expression of eNOS was increased in vascular endothelium with preeclampsia (Myatt et al. 1997a) perhaps as a compensatory response to vasoconstriction-induced increased shear stress across endothelial cells seen in these placentas. In conjunction with superoxide generation from NADPH oxidase by the same cells, this may lead to increased formation of the powerful prooxidant peroxynitrite ($ONOO^-$) which nitrates tyrosine residues on proteins thus covalently modifying protein function (Beckman and Koppenol 1996).

Heme oxygenase

Carbon monoxide is a vasodilator in the placenta (Lyall et al. 2000) and is synthesized by the enzyme heme oxygenase (HO). There is little of the HO-1 isoform in the placenta (Lyall et al. 2000; McLean et al. 2000; Yoshiki et al. 2000; Barber et al. 2001) whereas HO-2 is found in vascular endothelium and villous and extravillous trophoblast. A reduction in HO-2 expression was found in endothelial cells in preeclampsia (Barber et al. 2001).

Formation of peroxynitrite

Nitric oxide is inactivated by superoxide anion (Moncada et al. 1991) which, therefore, limits its bioactivity. Conversely, the activity of nitric oxide is prolonged by the presence of SOD which removes superoxide. The action

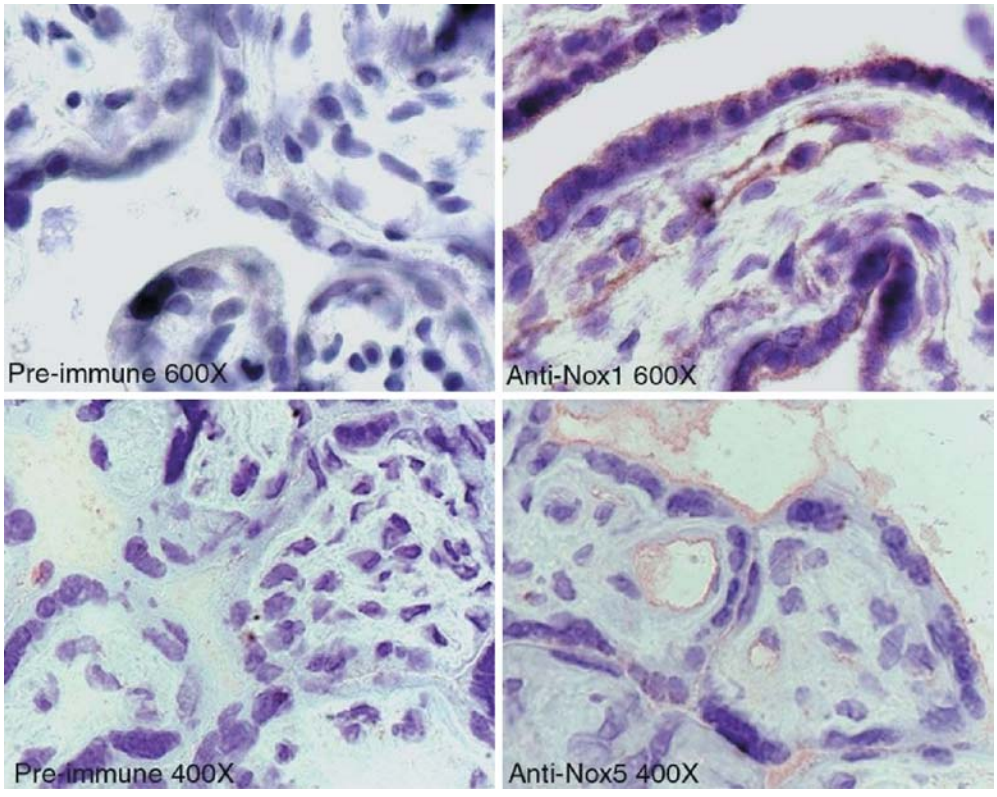

Fig. 2 Expression of Nox1 and Nox5 in human villous tissue. Frozen villous tissue sections were immunostained using polyclonal antibodies specific for Nox1 or Nox5 (*right panels*). Preimmune sera was used as immunologic control (*left panels*)

of superoxide per se is limited by its low lipid solubility, limited membrane transport, and by its removal by SOD. However, when tissues are induced to simultaneously produce both nitric oxide and superoxide in a concentrated and localized manner by inflammatory stimuli, sepsis, and ischemia/reperfusion, nitric oxide and superoxide react to produce peroxynitrite, a potent long-lived oxidant at a diffusion-limited rate constant. Peroxynitrite anion ($ONOO^-$) is a powerful oxidant of a variety of biomolecules (Beckman et al. 1990) and is cytotoxic as it inhibits mitochondrial electron transport resulting in inhibition of cellular respiration (Radi et al. 1994), oxidizes sulfhydryl groups in proteins, initiates lipid peroxidation without the requirement for transition metals (Radi et al. 1991), and nitrates aromatic amino acids such as tyrosine (Ischiropoulos et al. 1992), thus affecting many signal transduction pathways. Peroxynitrite can also increase DNA breaks which can lead to initiation of a futile DNA repair cycle by activation of poly (ADP-ribose) polymerase, resulting in depletion of cellular NAD^+ and ATP stores (Zhang et al. 1994). In the isolated perfused rat heart, peroxynitrite impairs relaxation (Villa et al. 1994) and it causes vascular dysfunction in rats by selective impairment of adrenoreceptors when given systemically (Benkusky et al. 1999). Peroxynitrite production can be indirectly localized by the presence of nitrotyrosine residues (Beckman et al. 1994). Nitrotyrosine residues have been demonstrated in human atherosclerotic plaques (Beckman et al. 1994) and in lung sections of patients and animals with acute lung injury (Haddad et al. 1994) indicating areas of cellular damage including vascular damage. Ischemia-reperfusion causes increased formation of nitrotyrosine in cardiac tissue and inhibition of NOS enzyme reduces both the level of protein nitration and reperfusion injury (Wang and Zweier 1996; Liu et al. 1997; Zweier et al. 2001; Baker et al. 2002) which supports the role both of nitric oxide and protein tyrosine nitration.

When immunostaining placental tissues from pregnancies complicated by preeclampsia we found increased nitrotyrosine residues in the placenta, especially in the vascular endothelium when compared to normotensive pregnancies (Myatt et al. 1996). Later extension of these studies to pregestational diabetes showed abundant nitrotyrosine immunostaining in the vascular endothelium and villous stroma (Fig. 3) when compared to control patients (Lyall et al. 1998). Thus, we have evidence that the oxidative stress of pregnancies complicated by preeclampsia or pregestational diabetes is associated with generation and action of peroxynitrite as assessed by nitrotyrosine immunostaining.

Antioxidants in the placenta

All the major antioxidant systems including Mn and Cu/ZnSOD, catalase, glutathione, glutathione peroxidase, glutathione *S*-transferase, thiol/disulfide oxidoreductase, and vitamins C and E are present in the placenta. We find that the two SOD isoforms show cell-specific expression patterns in the placenta (Fig. 4). The mitochondrial MnSOD isoform is faintly expressed in syncytiotropho-

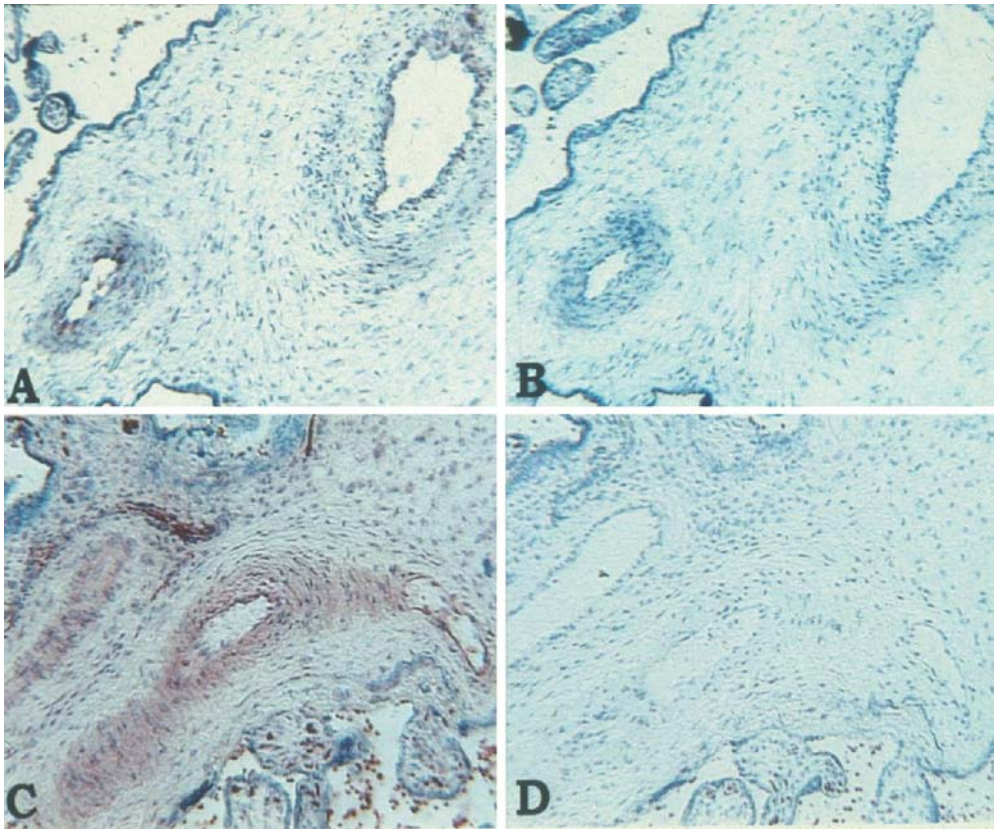

Fig. 3A–D Expression of nitrotyrosine residues in villous tissue of normal and diabetic pregnancies. Frozen sections from normal term (**A**, **B**) or diabetic (**C**, **D**) pregnancies were immunostained with a monoclonal anti-nitrotyrosine antibody using the Vectastain ABC kit. Control incubations (**B**, **D**) contained no primary antibody. Magnification ×125

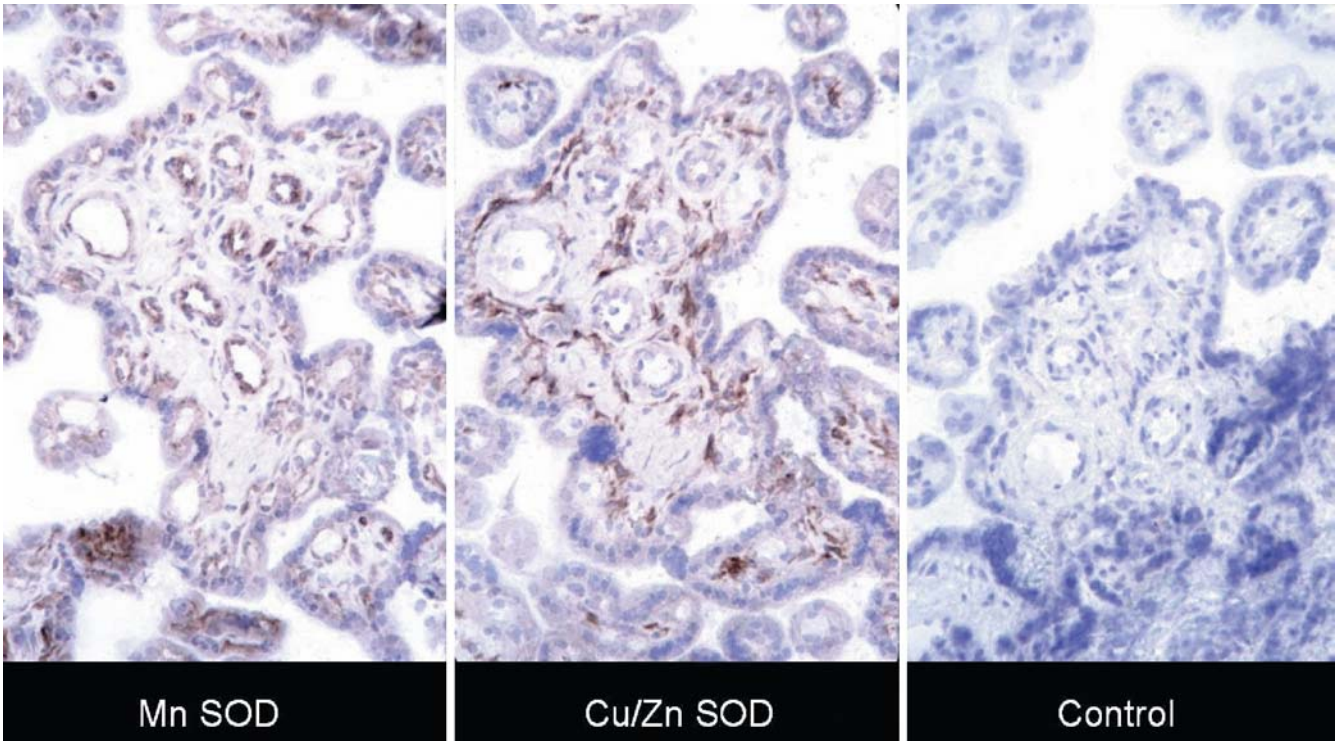

Fig. 4 Expression of manganese (*MnSOD*) and copper/zinc (*Cu/ZnSOD*) superoxide dismutase isoforms in villous tissue. Serial sections of frozen term villous tissue were immunostained using polyclonal MnSOD or monoclonal Cu/ZnSOD antibodies with the Vectastain ABC kit. Control sections omitted the primary antibody. Magnification ×250

blast but very intensely in villous vascular endothelium whereas the cytosolic Cu/ZnSOD isoform is intensely expressed in villous stroma, probably in the Hofbauer cell but only faintly in trophoblast (Myatt et al. 1997c). There is evidence for regional differences in the expression of antioxidant enzymes in the placenta perhaps in relation to the degree of oxygenation (Hempstock et al. 2003a) with the central region being well-oxygenated compared to the periphery, owing to the direction of maternal blood flow. However, central villi are morphologically and enzymatically immature compared to peripheral villi. Activity of catalase and glutathione peroxidase was higher in central than peripheral villi, but no difference was detected for total SOD. Also the mRNA concentration was higher in the center for catalase and for glutathione peroxidase but no differences were found for Cu/ZnSOD or MnSOD. Thus, it appears that the activities of catalase and glutathione peroxidase may reflect gradients established by the pattern of maternal intralobular blood flow, and that oxygen tension may be a regulatory factor in vitro (Hempstock et al. 2003a).

Evidence for oxidative stress in normal and pathologic pregnancies

Oxidative stress in early pregnancy

The placenta receives oxygenation from the maternal circulation and is positioned in an oxygen gradient between the mother and fetus. The placenta and fetus exist in a hypoxic environment during early pregnancy as the intrauterine oxygen tension is extremely low (pO_2<20 mmHg, ~5% O_2) at 8 weeks, (Jauniaux et al. 2000) prior to establishment of blood flow into the intervillous space. The placenta and fetus depend at this time on histiotrophic nutrition. However, the O_2 tension rises steeply at the end of first trimester when the invasion of trophoblast allows the occluded uterine spiral arteries to open and the pO_2 in the intervillous space reaches 50 mmHg (Jauniaux et al. 2000). The hypoxia/reoxygenation at this time imposes an ischemia-reperfusion insult. Therefore, the placenta is subjected to hypoxia and then hypoxia/reoxygenation in the first trimester of gestation.

In early pregnancy this increase in O_2 tension is associated with increase in mRNA of the antioxidant enzymes catalase, glutathione peroxidase, and SOD within placental tissues. The reperfusion of placenta between 8 and 9 weeks may be responsible for expression of heat shock protein (HSP) 70 and formation of nitrotyrosine residues (markers for oxidative stress) in syncytiotrophoblast (Jauniaux et al. 2000). The magnitude of oxidative stress may depend on both the severity of the insult and on the effectiveness of the placental antioxidant defenses. The cytotrophoblast begins to synthesize SOD by 11 weeks, while syncytiotrophoblast SOD increases by 16 weeks. This may explain why first trimester cytotrophoblast survives better in low pO_2 in culture and displays a better defense to pO_2 increase in second trimester (Watson et al. 1998). Similarly, syncytiotrophoblast and mitochondrial morphology improved, and mitochondrial activity was retained for 6 h and more if 8- to 10-week-old tissue was placed into a low oxygen environment immediately after removal from the uterus.

Burton's group also established an in vitro model using term placental tissues that recapitulated the ischemia/reperfusion seen in early pregnancy and also purported to occur in preeclampsia. Rapid generation of ROS was detected by fluorescent dye in different placental cell types with an intensity of villous endothelium >syncytiotrophoblast >stromal cells. Concomitantly, increased concentrations of HSP72, nitrotyrosine, and 4-HNE (markers of oxidative stress) were seen in placental endothelial and smooth muscle cells and syncytiotrophoblast (Hung et al. 2001). Preloading placental tissues with ROS scavengers desferrioxamine and α-phenyl-*N*-tert-butylnitrone reduced the level of oxidative stress seen. These data show the ability of placental tissues to generate ROS and the necessity for adequate antioxidants to scavenge them.

Oxidative stress and miscarriage

Using Doppler ultrasonography Jauniaux et al. (2003) showed that onset of intervillous blood flow increased with gestational age, being detected in 9 of 25 cases at 8–9 weeks but in 18 of 20 cases at 12–13 weeks. However, in abnormal pregnancies, which end in fetal demise, flow was detected in nearly all cases at 8–9 weeks and regional differences in flow to the placenta were observed between the normal and abnormal pregnancies. Early flow was restricted to the peripheral regions of most normal placentas but was most common in central regions or throughout the placenta in missed miscarriages. In comparison, immunoreactivity for HSP70 and nitrotyrosine residues (markers of oxidative stress) was greater in samples from peripheral than from central regions of normal placentas and from missed miscarriages compared to controls. The data suggest that oxidative damage to the trophoblast, induced by premature and widespread onset of the maternal placental circulation, is a key factor in early pregnancy loss (Jauniaux et al. 2003).

This group also determined if the disorganized and early onset of the maternal blood flow to the placenta seen in miscarriage was associated with excessive levels of oxidative damage and stress in placental tissues (Hempstock et al. 2003b). Indeed morphological and immunohistochemical markers of cellular stress and damage, including HSP70, nitrotyrosine residues, and lipid peroxidation, were increased in tissues obtained from missed miscarriages compared with controls particularly those with gestation shorter than 77 days and with evidence of recent fetal demise. Increased apoptosis and decreased numbers of mitotic cells were seen indicating that oxidative stress overwhelms cellular antioxidant defense systems. These data reinforce the concept that placental oxidative stress with resultant damage to the syncytio-

trophoblast, secondary to early onset of the maternal circulation, may be a mechanism contributing to early fetal loss (Hempstock et al. 2003b).

Oxidative stress in preeclampsia

Preeclampsia is the clinical syndrome of edema, hypertension, and proteinuria in the pregnant woman and occurs primarily in nulliparous women in their third trimester. Approximately 7% of pregnancies in the United States are affected by preeclampsia which is the leading cause of fetal growth restriction, indicated premature delivery, and maternal death. The underlying maternal pathophysiology involves generalized arteriolar constriction and intravascular depletion that can produce symptoms related to ischemia, necrosis, and hemorrhage of organs resulting in poor perfusion of the maternal and fetal circulations of the placenta leading to abnormal fetal growth and development. Shallow trophoblast invasion is the major pathologic finding in the preeclamptic placenta, particularly those that are early-onset. Since maximal placental trophoblast invasion occurs at the end of the first trimester when pO_2 increases sharply, a developmental failure of oxygen handling by trophoblast has been suggested in preeclampsia. In addition, hypoxia-reoxygenation has been shown to cause apoptosis in many cells, and an increased deportation of trophoblast fragments secondary to increased apoptosis in the syncytiotrophoblast is observed in preeclampsia (Allaire et al. 2000; Redman and Sargent 2000; Leung et al. 2001; Ishihara et al. 2002).

Exposure of the mother to the shed placental trophoblast may be responsible for onset of systemic inflammation in the mother. Maternal leukocytes from preeclamptic individuals were hyperactive in regard to constitutive and cytokine-induced ROS generation as compared to normal subjects (Gervasi et al. 2001; Lee et al. 2003). A significant increase of neutrophil-endothelial adhesion and adhesion molecules expression is seen in neutrophils exposed to conditioned medium derived from preeclamptic placental villous culture compared to normal placental culture (Wang et al. 2001). Sera obtained from patients with severe preeclampsia stimulated isolated healthy neutrophils significantly more than sera from normal subjects (Zusterzeel et al. 2001b). In addition, neutrophils are stimulated by shed syncytiotrophoblast microvillous membranes to generate ROS in women with preeclampsia (Aly et al. 2004). Tumor necrosis factor α (TNF_α), a cytokine produced mainly by macrophages, was abnormally increased in preeclamptic placentas (Conrad et al. 1998) perhaps in response to hypoxia. TNF_α might contribute to the increased oxidative stress by induction of oxygen free radicals (Wang and Walsh 1996b).

An agonistic autoimmune antibody to angiotensin II type 1 receptor has been reported to develop in preeclampsia patients which triggers receptor-mediated NADPH oxidase activation in the placenta (Dechend et al. 2003). Therefore, NADPH oxidase might be responsible for generating and/or sensing the oxidative stress and might be abnormally regulated in preeclamptic pregnancies. However, employing cerium as a capturing agent for enzymatic histochemistry, Matsubara and Sato (2001) did not find differences in distribution pattern and NADPH oxidase enzyme intensities among normal, preeclamptic, and IUGR placentas. Currently our laboratory is using specific antibodies to NADPH oxidase isoforms to map differences in expression.

In preeclamptic patients there is increased placental oxidative potential. Increased superoxide generation was observed employing a direct electron paramagnetic spin trap resonance technique (Sikkema et al. 2001). Wang and Walsh (1998) have reported that the amount of mitochondrial protein is 47% greater and the activity of the mitochondrial enzyme, citrate synthase, 56% greater in the preeclamptic placenta as compared to normal placentas, indicating an increase in the mitochondria number. These investigators also observed much higher lipid peroxide generation from preeclamptic mitochondria. However, Matsubara et al. (1997) found that mitochondrial cytochrome c oxidase activity was significantly decreased in trophoblast from preeclampsia. This may have resulted from prolonged hypoxia which has been shown to inhibit cytochrome c activity in isolated rat hepatocytes mitochondria (Chandel et al. 1995). However, these observations suggest that the increased amount of mitochondria may be a compensatory response, and that trophoblast cell mitochondrial dysfunction could be associated with preeclampsia.

Lipid peroxide formation, a marker of oxidative stress, is increased during pregnancy and preeclampsia (Casasco et al. 1997; Morris et al. 1998; Wang and Walsh 1998; Mutlu-Turkoglu et al. 1999). These lipid peroxides are produced mainly in the placenta due to membrane disruption by ROS. Microvillous membrane lipid peroxide concentration can be quantitated as malondialdehyde (MDA) in syncytiotrophoblast plasma membranes. A twofold increase of MDA along with increased arachidonic acid content was observed in preeclamptic women as compared to healthy subjects (Cester et al. 1994). Higher concentrations of MDA were found in the culture media of placental explants from preeclampsia patients, a finding recapitulated by culture of normal tissue in media containing XO/xanthine (Walsh et al. 2000). Concentrations of the isoprostane 8-iso-$PGF_{2\alpha}$, a specific marker for oxidative stress, are also seen in the preeclamptic placenta (Walsh et al. 2000).

Placental and decidual protein carbonyl levels (biomarkers for ROS-mediated protein damage) were higher in preeclampsia with HELLP than in normal pregnancy, whereas FRAP levels (a marker for antioxidant capacity) were lower (Zusterzeel et al. 2001a). The level of protein thiol/disulfide oxidoreductases (thioredoxin, glutaredoxin, and protein disulfide isomerase) were increased two- to threefold in preeclampsia placenta, indicating an adaptive protection against oxidative stress in trophoblast (Shibata et al. 2001). As an indirect indication of excessive ROS

generation, nitrotyrosine content is increased in placental villous vessel endothelium in preeclampsia (Myatt et al. 1996; Adams et al. 2000).

Often there is upregulation of antioxidant defenses in response to oxidative stress but persistent overwhelming oxidative stress leads to consumption and depression of antioxidants. Thus increased glutathione levels (Knapen et al. 1999) and increased glutathione peroxidase (Knapen et al. 1999) and catalase (Wang and Walsh 1996a) activities have been described in the placenta in preeclampsia. In contrast decreased mRNA expression and activity of Cu/ZnSOD (Wang and Walsh 2001), SOD, glutathione, and glutathione peroxidase (Wang and Walsh 1996a) have been reported together with reduced glutathione *S*-transferase (Zusterzeel et al. 1999) but no change in vitamin E (Poranen et al. 1998). The magnitude of oxidative stress and antioxidant changes correlate well with diastolic blood pressure (Madazli et al. 2002). However, no differences in localization or intensity of SOD isoforms were found between normal or preeclampsia tissues (Myatt et al. 1997c). Overall the severity of preeclampsia correlates with loss of antioxidant power.

Together these data suggest a loss of the balance in preeclampsia between ROS production and antioxidant scavenging capacity which is maintained under normal physiological conditions in the placenta. However, while we have appreciable data on oxidative stress and expression of antioxidant enzymes there is as yet no clear picture identifying the enzymes that are responsible for ROS generation and the site of these enzymes.

Oxidative stress in diabetic pregnancy

Diabetes during pregnancy encompasses a range of disease entities including gestational diabetes and overt diabetes mellitus. True gestational diabetes mellitus is an impairment in carbohydrate metabolism that first manifests during pregnancy. Pregestational diabetes mellitus complicates 0.25% of pregnancies, and, despite improvements in perinatal care over the last several decades, there still exists significant morbidity and mortality particularly in cases of poorly controlled glucose homeostasis (Garner et al. 1990). Like preeclampsia, diabetes is also characterized as a state of endothelial dysfunction and reactive nitrogen and oxygen species contribute to the progression of diabetes (Honing et al. 1998; Rosen et al. 2001). Women who are diabetic have a four times greater rate of development of preeclampsia suggesting their preexisting endothelial dysfunction may predispose them to preeclampsia. In diabetes, ROS including superoxide are thought to be produced as a result of prolonged periods of exposure to hyperglycemia, which is known to cause nonenzymatic glycation of plasma proteins (Tames et al. 1992). This superoxide, in the absence of appropriate levels of scavengers, may lead to an imbalance between prooxidants and antioxidants and produce a state of oxidative stress.

We determined the presence and level of expression of nitrotyrosine residues in placental villous tissue of diabetic pregnancies as an index of vascular damage linked to oxidative stress (Lyall et al. 1998). Villous tissue was collected from ten White Class C and D pregestational diabetic patients and ten normotensive controls matched for gestational age. Serial sections of tissue were immunostained for nitrotyrosine, eNOS, and MnSOD. All tissues demonstrated immunostaining for eNOS in both syncytiotrophoblast and stem villous vascular endothelium with no apparent differences between groups. Significantly more intense nitrotyrosine staining was apparent in vascular endothelium and villous stroma of diabetic placentas (Fig. 3). The endothelium of large villous vessels of diabetic tissues also showed more intense immunostaining for MnSOD (Fig. 5). In these diabetic pregnancies, we were unable to show increased eNOS, unlike previous findings in preeclamptic pregnancies. The presence of nitrotyrosine may indicate vascular damage in the diabetic placenta due to peroxynitrite action formed from increased synthesis/interaction of nitric oxide and superoxide. The apparently paradoxical increase in MnSOD expression may be an adaptive response to increased superoxide generation.

Functional consequences of oxidative stress in placental physiology

Trophoblast proliferation, invasion and migration, fusion and apoptosis, are processes required for normal embryo plantation and placental angiogenesis which eventually determine fetus growth (Fig. 6). However, whether and how these cellular events are linked and regulated remains largely unknown. Changes in pO_2 trigger several differentiation processes, including erythropoiesis and vasculogenesis/angiogenesis. Such processes are often medicated by oxygen-regulated expression of hormones or growth factors. Hypoxia-induced factor is a transcription factor that plays a central role in induction of VEGF, TGF, and erythropoietin.

Trophoblast fusion/differentiation

In vitro, isolated cytotrophoblasts aggregate and fuse to form a syncytium, a process associated with disappearance of several cellular markers (for example, desmoplakin) while hCG secretion increases. Exposure to 9% O_2 impairs cell fusion and differentiation since desmoplakin and E-cadherin persist and a 70% decrease of hCG secretion was observed, suggesting cytotrophoblast aggregation without fusion (Alsat et al. 1996). Impaired fusion and differentiation were also observed following overexpression of Cu/ZnSOD, a gene product of chromosome 21, in cytotrophoblasts (Frendo et al. 2001). Interestingly, higher levels of Cu/ZnSOD mRNA, protein, as well as enzymatic activity were observed in trisomy 21-affected placentas, where there is a defect in

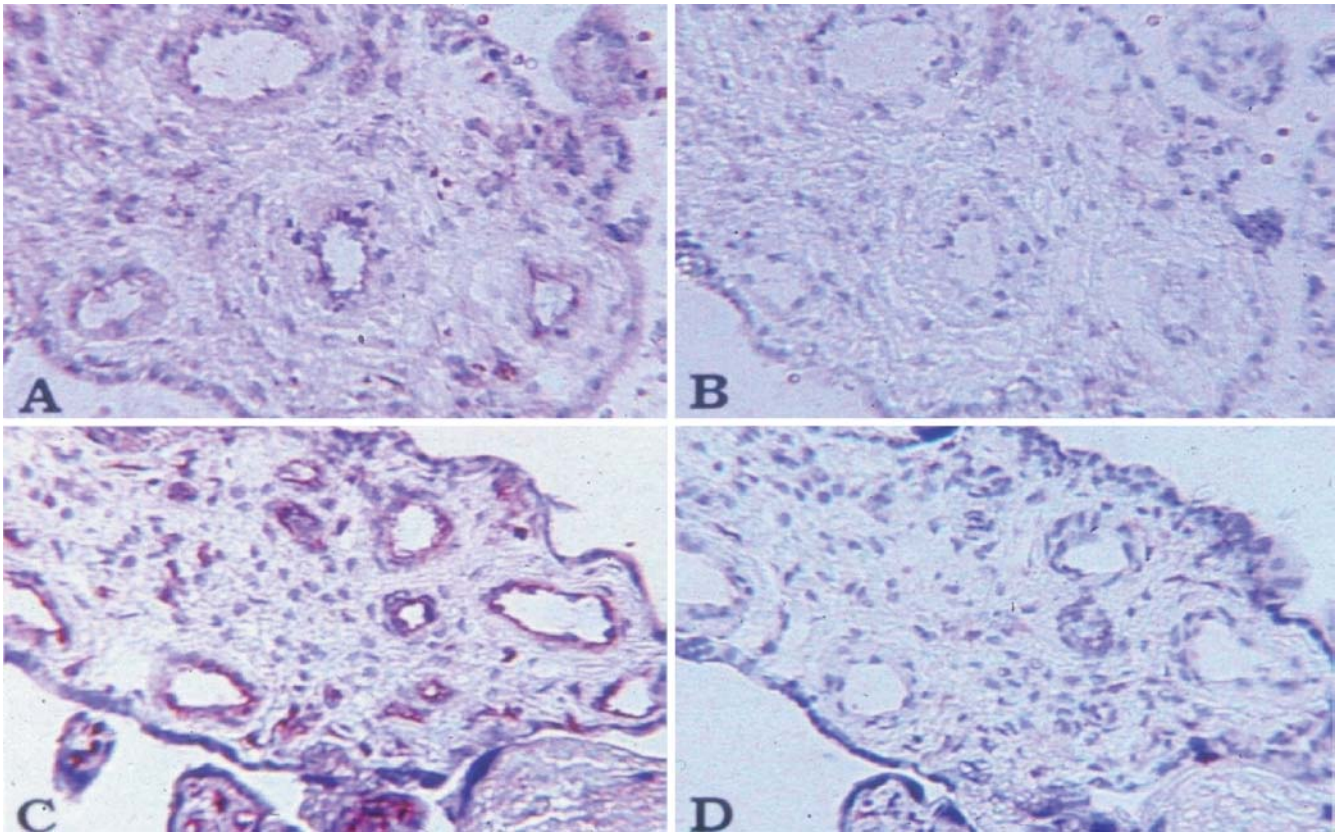

Fig. 5A–D Expression of MnSOD in villous tissue of normal and diabetic pregnancies. Frozen sections from normal term (**A**, **B**) or diabetic (**C**, **D**) pregnancies were immunostained with a polyclonal anti-MnSOD antibody using the Vectastain ABC kit. Control incubations (**B**, **D**) contained no primary antibody. Magnification ×125

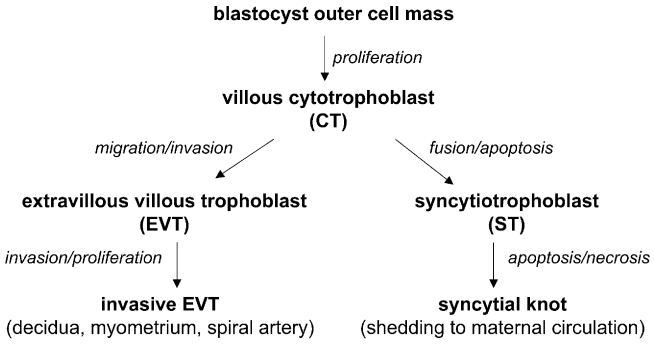

Fig. 6 Pathways of trophoblast proliferation and differentiation

syncytiotrophoblast formation and a decrease in the production of pregnancy-specific hormones. Choriocarcinoma cells have been widely employed as a convenient approach to mimic trophoblast cellular events. BeWo, JEG-3, and JAR cells may be induced to fuse and differentiate in vitro (Wice et al. 1990; Vaughan and Walsh 2002; Kudo et al. 2003). Forskolin-induced BeWo cell fusion (determined by a quantitative flow cytometry assay) was reversibly suppressed in 2% oxygen compared to 20% oxygen. This was associated with suppressed secretion of hCG (Kudo et al. 2003). In addition, H_2O_2 dose-dependently decreases hCG secretion in JEG-3 (McAleer and Tuan 2001).

Trophoblast proliferation

Hypoxia, however, has been shown to promote cytotrophoblast and extravillous trophoblast proliferation (Caniggia et al. 2000). Huppertz et al. (2003) observed that the proliferation rate of cytotrophoblast was decreased when exposed to higher pO_2. Recently, Fisher's group has employed proteomics to analyze protein expression of first trimester cytotrophoblast and observed a twofold decrease of MnSOD and a threefold decrease of 1-Cys peroxiredoxin, both antioxidant enzymes, when cells were exposed to extreme hypoxia (2% O_2; Hoang et al. 2001). Since hypoxia changes the differentiation property of the first trimester cytotrophoblast to proliferation (Genbacev et al. 1996, 1997), these results reinforce the existence of oxidative stress in early placenta which potentiates cell proliferation.

Oxidative stress and trophoblast apoptosis

Although morphological measurements of placental constituents and the villous surface area in placentas from uncomplicated pregnancies from 10 weeks of gestation to term reveal no evidence for placental senescence up to term, the rate of growth of the villous surface area decreases gradually from approximately 34 weeks gestation onward (Boyd 1984). The number of nuclei in cytotrophoblast and syncytiotrophoblast continuously increased while the percentage of cytotrophoblast maintains at 12–13% throughout villous arborizations (Sen et al. 1979). An increase of syncytial knot formation is observed during gestation. Thus apoptosis occurs in normal placental tissues, mainly in villous trophoblast (Smith et al. 1997b; Mayhew et al. 1999). Apoptosis is, however, enhanced in pregnancies complicated by preeclampsia (Leung et al. 2001) and also IUGR (Smith et al. 1997a) where the inadequate perfusion of the intervillous space is thought to give rise to oxidative stress.

Reactive oxygen species mediate cytokine-induced trophoblast apoptosis (Smith et al. 1999) and pO_2 differentially regulates apoptosis events. Both basal and TNF_α-induced apoptosis were highest when term cytotrophoblast was exposed to 0% O_2 (pO_2<10 mmHg; Kilani et al. 2003). Cytotrophoblast was resistant to apoptosis when cultured at intermediate oxygen levels, i.e., 2–5% O_2. Severe hypoxia (2% O_2), however, increased syncytiotrophoblast necrotic shedding (Huppertz et al. 2003). Similarly, apoptosis of chorion leave trophoblast is induced by oxidative stress (Ohyama et al. 2001).

Effect of ROS on placental angiogenesis

Placental angiogenesis is regulated by maternal and placental growth hormones. Placental growth factor (PIGF) belongs to the VEGF family of growth factors and significant expression of PIGF is found in the placenta (Maglione et al. 1991, 1993; Hauser and Weich 1993), with the primary site of synthesis being trophoblast (Khaliq et al. 1996; Shore et al. 1997; Vuorela et al. 1997). Recently hypoxia was shown to differentially regulate PIGF and VEGF expression; PIGF is downregulated by hypoxia while VEGF mRNA is upregulated (Khaliq et al. 1996). Elevated VEGF mRNA was detected in trophoblast-derived cells which were cultured under hypoxic conditions known to cause ROS generation (Taylor et al. 1997). This might be due to ROS increasing VEGF mRNA stability (Kuroki et al. 1996). Exogenous VEGF induces ^{3}H-thymidine incorporation an effect mediated by $p42/p44^{MAPK}$ via induction of eNOS in BeWo cells (Cha et al. 2001).

The mitogen-activated protein kinases (MAPK) are activated by oxidative stress in various cell types (Torres 2003). MAPK cascades seem to be involved in placental development since mutation in the mouse gene resulted in deficient phenotypes (Giroux et al. 1999; Adams et al. 2000; Mudgett et al. 2000; Yang et al. 2000). Targeted disruption of the $p38\alpha^{SAPK}$ gene results in homozygous embryonic lethality due to severe defects in placental development which appear to be secondary to insufficient oxygen and nutrient transfer across the placenta (Adams et al. 2000). In particular, $p38\alpha^{SAPK}$ mutant placentas display a lack of vascularization of the labyrinth layer as well as increased rates of apoptosis, consistent with a defect in placental angiogenesis and lack of expression of VEGF and angiopoietin. When the placental defect was rescued, $p38\alpha^{SAPK}(-/-)$ embryos developed to term and were normal in appearance (Mudgett et al. 2000).

Effect of oxidative stress on placental vascular reactivity

Oxidative stress may increase production of superoxide and nitric oxide, leading to formation of prooxidant peroxynitrite to cause vascular dysfunction. We find nitrotyrosine residues in the placenta in preeclampsia and diabetes but the functional significance remains to be fully elucidated. We sought to directly determine (Kossenjans et al. 2000) whether peroxynitrite caused a functional deficit in the placental vascular bed in placentas of pregnancies complicated by preeclampsia or diabetes by comparing the responses of diabetic, preeclamptic, and normal placentas to increasing concentrations of the vasoconstrictors U46619 and angiotensin II and the vasodilators glyceryl trinitrate and prostacyclin. In addition, we determined the response to these agents in normal placentas before and after treatment with authentic peroxynitrite for 30 min. Interestingly, we found that the responses to both vasoconstrictors and vasodilators were significantly attenuated in diabetic and preeclamptic placentas compared to controls. Similarly, the responses to U46619, glyceryl trinitrate, and prostacyclin, but not angiotensin II, were significantly attenuated in a normal placenta following peroxynitrite treatment. Immunostaining for nitrotyrosine residues confirmed that nitrotyrosine residues were present in the placenta from preeclamptic and diabetic pregnancies, but also that peroxynitrite treatment of the placental vasculature led to the formation of nitrotyrosine residues (Kossenjans et al. 2000). Taken together, these data suggest but do not prove a cause-and-effect relationship whereby peroxynitrite formation in the placental vasculature is capable of attenuating vascular responses. A significant attenuation of the vasoconstrictor response to U46619 in the fetal-placental circulation of women with preeclampsia was also reported (Read et al. 1999), although no effect was seen on vasodilator responses to prostacyclin. Similarly, Wilkes et al. (1994) found responses to U46619 to be attenuated in the fetal-placental vasculature of diabetic pregnancies accompanied by reduction of the affinity of thromboxane receptors.

Protein tyrosine nitration has been detected under apparently normal physiologic conditions (Greenacre and Ischiropoulos 2001) in numerous tissues including endothelial cells, fibroblasts, and vascular smooth muscle cells (Davidge et al. 1998; Frustaci et al. 2000; Kajstura et al. 2001). Several nitrated proteins have been identified in-

cluding myofibrillar creatine kinase (Mihm et al. 2001a), PGI_2 synthase (Zou et al. 1999), and heart succinyl-Co-A:3-oxoacid CoA transferase (Turko et al. 2001), and structural proteins such as myosin heavy chain, α-actinin, and desmin (Mihm et al. 2001b). Low levels of protein nitration may be a physiologic regulatory mechanism in redox regulation for signaling pathways by changing tyrosine into a negatively charge hydrophilic nitrotyrosine moiety and changing the function of a protein. A gain of function as well as no effect on function were reported for tyrosine nitrated proteins (Gole et al. 2000; Balafanova et al. 2002). More commonly, however, inhibition of function is found (Ischiropoulos 1998; Greenacre and Ischiropoulos 2001). Tyrosine nitration may function as a feedback inhibitory mechanism as $ONOO^-$ inhibits inducible NOS activity (Robinson et al. 2001) and inhibits XO activity and O_2^- activity (Lee et al. 2000). Conversely as $ONOO^-$ inhibits SOD activity it may exacerbate oxidative stress (MacMillan-Crow et al. 1998). The concept of protein nitration functioning as a posttranslational modification akin to phosphorylation is attractive and has been studied. Proteomics (2D gel electrophoresis and mass spectrometry) has been used to identify nitrated proteins (Aulak et al. 2001). A putative denitrase activity has been demonstrated (Gow et al. 1996; Kuo et al. 1999, 2002), however, neither the enzyme responsible nor the reaction product have been identified to date.

Our subsequent studies will identify the protein targets that are covalently modified by peroxynitrite.

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