Expression of superoxide dismutase and xanthine oxidase in myometrium, fetal membranes and placenta during normal human pregnancy and parturition

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Superoxide, an agent which attenuates the half-life of nitric oxide, is metabolized and synthesized by superoxide dismutase (SOD) and xanthine oxidase, respectively. Over the last few years much work has focused on the role of nitric oxide in human parturition. The aim of this study was to determine whether the onset of human parturition is associated with a change in the expression of copper/zinc superoxide dismutase (Cu/Zn SOD), manganese superoxide dismutase (Mn SOD) or xanthine oxidase within the uterus. Samples of myometrium, placenta, decidua and fetal membranes were obtained from women before and after the onset of labour at term. Immunocytochemistry was used to localize Cu/Zn SOD, Mn SOD and xanthine oxidase and measure SOD enzyme activity. Cu/Zn and Mn SOD-like immunoreactivity was detected in syncytiotrophoblast cells, villous stromal cells and endothelial cells of blood vessels in the placenta. In the myometrium Cu/Zn and Mn SOD were localized to myocytes and endothelial cells and to some vascular smooth muscle cells. In the fetal membranes we observed staining for Cu/Zn SOD and Mn SOD in the amnion, chorion, extravillous trophoblast and decidua. There was no difference in SOD enzyme activity or staining intensity for SOD between different cell types before and during labour. Xanthine oxidase immunoreactivity was identified in each of the tissues examined and again there was no difference in immunostaining in tissues obtained from women delivered before or after the onset of labour. These results show that the pregnant uterus is capable of both synthesizing and degrading superoxide and suggest that superoxide dismutase and xanthine oxidase may play a role in the maintenance of uterine quiescence during pregnancy, but not in the initiation of parturition.
Key words: nitric oxide/parturition/superoxide dismutase/ uterus/xanthine oxidase

Introduction

Nitric oxide synthases (NOS) are a group of isoenzymes responsible for the generation of nitric oxide (NO) from L-arginine. NO is a mediator of diverse biological activities including vasodilatation, neurotransmission and inhibition of platelet adhesion and aggregation (Moncada *et al.*, 1991). NO

may also play a role in the maintenance of myometrial quiescence during pregnancy, and a decrease in NO may contribute to the onset of parturition at term. The administration of NO donors has been shown to abolish rat myometrial contractions both *in vitro* and *in vivo* (Natuzzi *et al.*, 1993) and a similar inhibitory effect of NO donors on human myometrial contractions has also been shown (Lees *et al.*, 1994; Buhimschi *et al.*, 1995; Norman *et al.*, 1997). A role for NO in the control of rat and rabbit parturition is supported by a variety of studies. NO production within the uterus is high during pregnancy, but decreases at term thus initiating uterine activity at the onset of parturition (Sladek *et al.*, 1993; Natuzzi *et al.*, 1993; Yallampalli *et al.*, 1993). In contrast, in human parturition there is no evidence that NOS activity is decreased following the onset of labour either in the myometrium itself or in placenta or fetal membranes (Ramsay *et al.*, 1996; Di Iulio *et al.*, 1996; Thomson *et al.*, 1997). However, a change in NO activity may control parturition by mechanisms other than NO production. The biological actions of NO are abrogated via direct reaction with superoxide anion (Huie and Padmaja, 1993; McCall *et al.*, 1989). Moreover superoxide dismutase (SOD), which converts superoxide to hydrogen peroxide and oxygen (Klug-Roth *et al.*, 1973), prolongs the biological half-life of NO by removing superoxide. SOD exists as three isoenzymes, which are widely distributed. An intracellular copper/zinc-containing (Cu/Zn SOD) enzyme is found predominantly in the cytoplasm and nucleus (Crapo *et al.*, 1992), a manganese-containing SOD (Mn SOD) is found predominantly in mitochondria (Weisiger and Fridovich, 1973) and a third isoenzyme, extracellular SOD, is found in the extracellular matrix (Marklund, 1984). The importance of SOD in the augmentation of NO activity is illustrated by experiments showing that diethyldithiocarbamate (DETCA), an inhibitor of Cu/Zn SOD, diminishes NO-mediated relaxation of vascular smooth muscle (Rubanyi, 1988; Mugge *et al.*, 1991; Omar *et al.*, 1991).

Whereas SOD breaks down superoxide anion, xanthine oxidase synthesizes superoxide. Xanthine oxidase appears to be one of the major superoxide-producing enzymes in vessel walls (Jarasch *et al.*, 1986). NO-induced relaxation can be potentiated in blood vessels by xanthine oxidase inhibitors (Miyamoyo *et al.*, 1996), suggesting that xanthine oxidase is an important regulator of NO activity.

We postulated that expression and activity of SOD and xanthine oxidase might change with the onset of labour in such a way that the biological activity of NO would be attenuated. The purpose of this study was to test this hypothesis by determining the activity and localization of Cu/Zn, Mn

SOD and xanthine oxidase in human myometrium, placenta and fetal membranes before and after the onset of labour.

Materials and methods

Tissue collection

Twenty-two women undergoing Caesarean section at term (>37 weeks gestation) were recruited to the study. Eleven had an elective Caesarean section prior to the onset of labour. The other eleven were delivered by emergency Caesarean section during active labour (cervical dilatation >4 cm). Women were excluded from the study if they had a multiple pregnancy, evidence of active infection or if their labour had been induced. A myometrial strip was obtained from the upper margin of the lower segment uterine incision from each woman, in addition to biopsies of full-thickness placenta and the fetal membranes with attached decidua capsularis. Each of the biopsies was divided; half was fixed in 10% neutral buffered formalin (BDH, UK) and embedded in paraffin. The other half was snap-frozen in liquid nitrogen and stored at –70°C. Informed consent was obtained from each woman and the study was approved by the Local Research Ethics Committee (Glasgow Royal Infirmary University NHS Trust, Research Ethics Committee).

Immunocytochemistry

Sections (5 μm thick) of paraffin-embedded tissues were cut and mounted on silane-coated slides, heated to 60°C for 30 min, cleared in xylene and rehydrated in a graded alcohol series. Negative control slides were incubated without primary antibody. Two independent observers, blind to patient data, scored the intensity of staining in five different microscope fields. Intensity and consistency of staining was scored on a scale of 0–6 (0 = staining absent, 1 = weak, variable staining, 2 = weak, consistent staining, 3 = moderate, variable staining, 4 = moderate, consistent staining, 5 = strong, variable staining, 6 = strong, consistent staining). Data were analysed using contingency tables and χ^2-test.

Immunocytochemistry for Cu/Zn SOD

Immunocytochemistry was carried out using a monoclonal antibody against human Cu/Zn SOD (Sigma, Poole, UK) diluted 1/200, and an anti-mouse IgG peroxidase kit (Vectastain Elite ABC kit, Vector, Peterborough) as the detection system. Immunoreactive Cu/Zn SOD was localized using 1 mg/ml diaminobenzidine tetrahydrochloride (DAB, Sigma) and 0.02% H_2O_2 in 50 mM Tris–HCl, pH 7.6. Following immunocytochemistry sections were washed in distilled water, counterstained with Harris haematoxylin and mounted in DPX (BDH, UK).

Immunocytochemistry for Mn SOD

Immunocytochemistry was performed using a polyclonal antibody raised against human Mn SOD (The Binding Site, Birmingham, UK) diluted 1/250. Sections were pre-treated with a 0.01% (w/v) solution of protease type XXIV (Sigma) in 0.01% (w/v) calcium chloride for 10 min at 37°C then washed in water and phosphate-buffered saline (PBS), pH 7.5. Antibody binding was detected using an anti-sheep IgG peroxidase kit (Vectastain Elite ABC kit) and DAB as substrate.
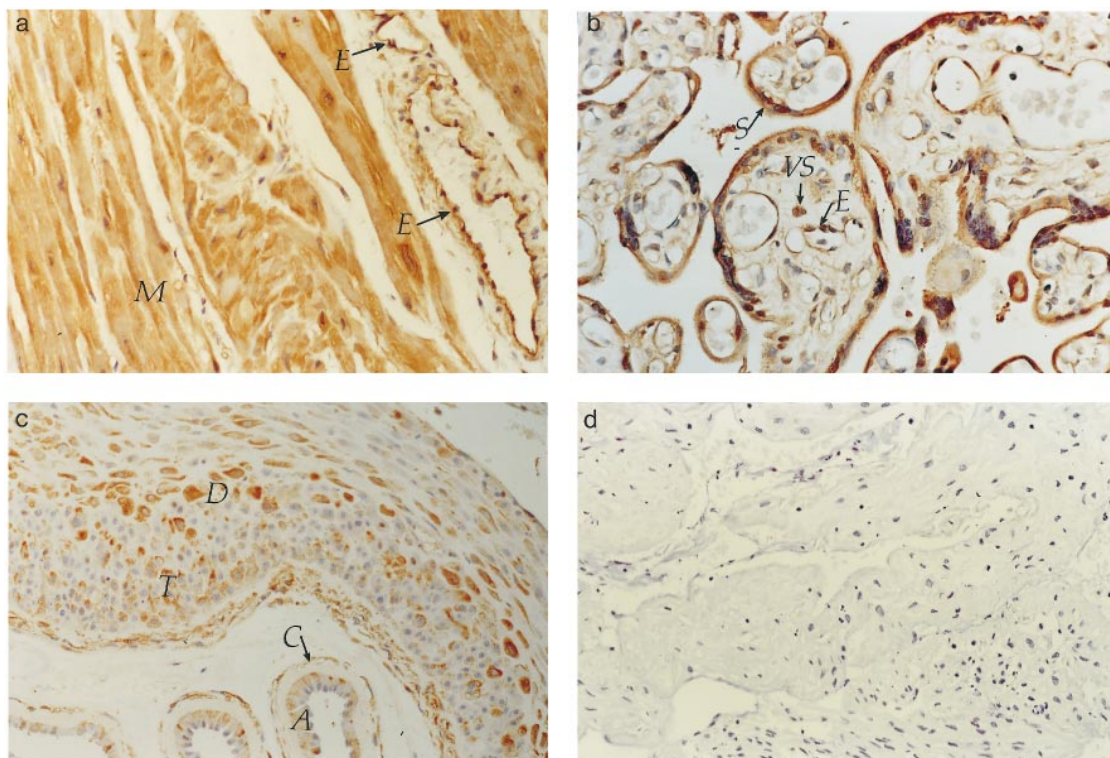
Figure 1. Localization of Cu/Zn superoxide dismutase (Cu/Zn SOD) protein in myometrium, placenta and fetal membranes with attached decidua. (**a**) Cu/Zn SOD localized to myocytes and endothelium of blood vessels in myometrium (arrows). Original magnification ×300. (**b**) In the placenta intense Cu/Zn SOD immunoreactivity was observed in the syncytiotrophoblast whereas endothelial cells of blood vessels and villous stromal cells exhibited moderate staining. Original magnification ×470. (**c**) Faint staining for Cu/Zn SOD staining in amniotic epithelium and moderate staining in chorion and extravillous trophoblast was observed in fetal membranes. Attached decidua showed intense Cu/Zn SOD staining. Original magnification ×230. (**d**) A negative control slide of myometrium without primary antibody exhibited no reactivity. M = myocytes, E = endothelium, VS = villous stromal cell, S = syncytiotrophoblast, A = amnion, C = chorion, T = extravillous trophoblast, D = decidua.
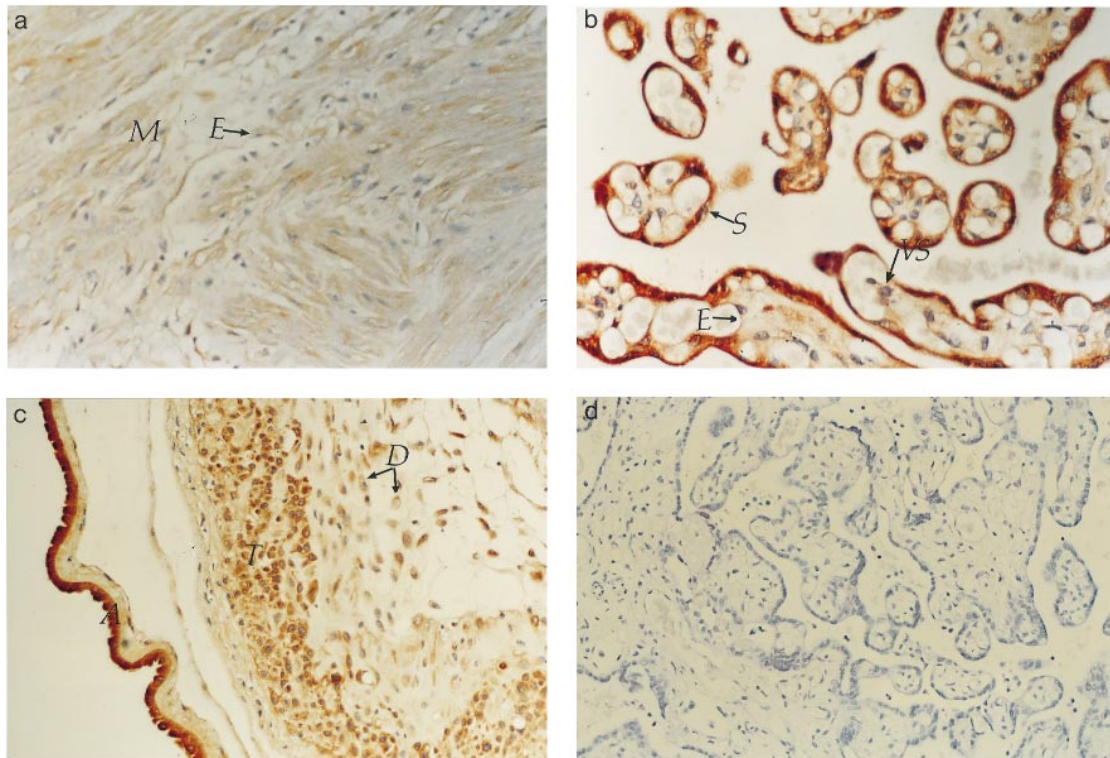
Figure 2. Localization of Mn superoxide dismutase (Mn SOD) protein in myometrium, placenta and fetal membranes with attached decidua. (**a**) Faint Mn SOD staining was observed in myocytes and endothelium of blood vessels in myometrium. Original magnification ×370. (**b**) Mn SOD was localized to syncytiotrophoblast, endothelium and stromal cells of placental villi. Original magnification ×470. (**c**) Localization of Mn SOD to amniotic epithelium, extravillous trophoblast and decidua. Original magnification ×180. (**d**) A negative control slide of placenta without primary antibody exhibited no reactivity. See Figure 1 for abbreviations.
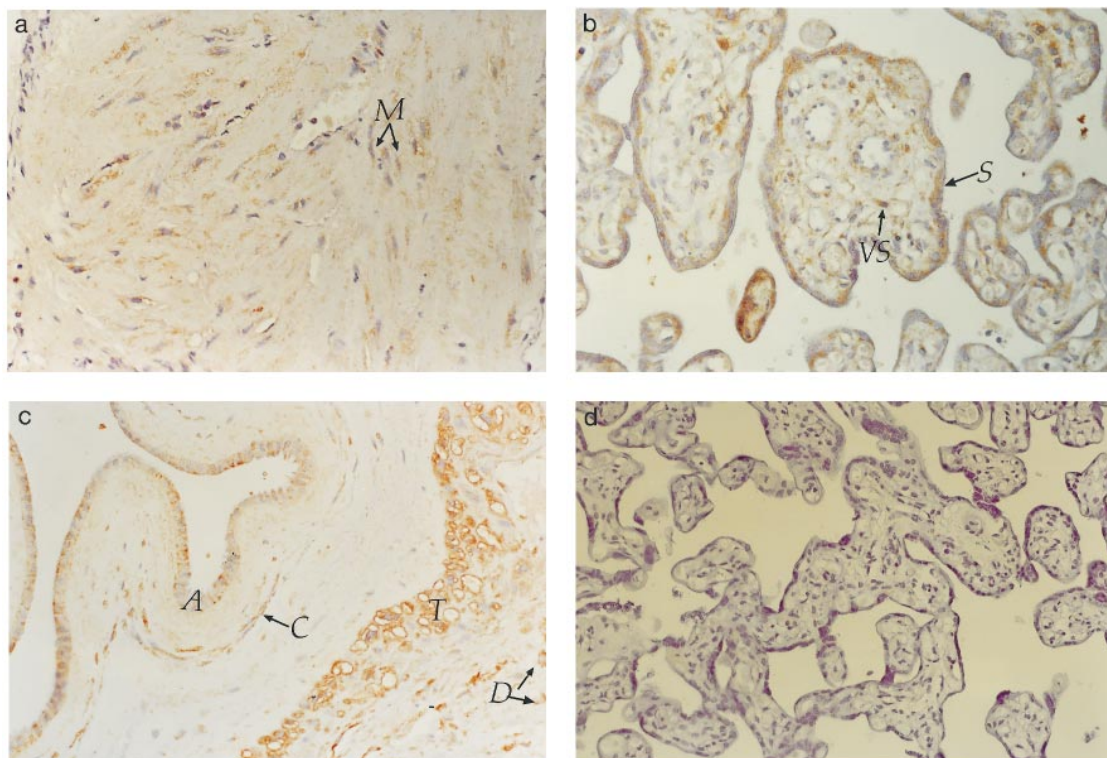
Figure 3. Immunostaining of myometrium, placenta and fetal membranes with attached decidua using a xanthine oxidase antibody. Original magnification ×300. (**a**) Faint xanthine oxidase staining in myocytes in myometrium. (**b**) Moderate staining in syncytiotrophoblast and villous stromal cells of the placenta. Original magnification ×370. (**c**) Xanthine oxidase staining was observed in amnion, chorion, extravillous trophoblast and decidua. Original magnification ×300. (**d**) A negative control slide of placenta without primary antibody exhibited no reactivity. See Figure 1 for abbreviations.

Immunocytochemistry for xanthine oxidase

Immunocytochemistry was carried out on paraffin-embedded sections using a polyclonal antibody raised against purified xanthine oxidase from bovine buttermilk (Lorne Laboratories, Reading, UK). This antibody has been shown to cross-react with human xanthine oxidase. The antibody was diluted 1/1000 and antibody binding was detected on protease-treated sections using an anti-rabbit IgG peroxidase kit (Vectastain Elite ABC kit).

Isolation of mitochondria for SOD assay

Frozen tissue was homogenized in homogenization medium (HM) containing 0.3 M sucrose, 5 mM [morpholino]propanesulphonic acid (MOPS) and 1 mM EDTA and the resultant homogenate spun at 2500 g for 5 min. An aliquot of the supernatant was collected for assay of total SOD activity and the remainder was spun at 10 000 g for 10 min to separate mitochondrial and cytoplasmic cell fractions. The supernatant was collected (cytoplasmic fraction) for SOD assay and the pellet was resuspended in HM medium. Homogenization and low and high speed centrifugation was repeated and the pellet obtained was again resuspended in HM (mitochondrial fraction). The purity of this mitochondrial fraction was determined using transmission electron microscopy.

Determination of SOD activity

Frozen tissue was homogenized in HM and spun at 2500 g for 5 min to remove cell debris. SOD activity was determined using an assay kit from Calbiochem (Nottingham, UK). This kit makes use of a proprietary reagent that undergoes alkaline auto-oxidation which is accelerated by SOD (Nebot et al., 1993). Interference from haemoglobin was eliminated by precipitation prior to assay using ice-cold ethanol/chloroform 62.5/37.5 (v/v) followed by centrifugation at 3000 g for 5 min at 4°C. SOD activity was determined from the Vs/Vc ratio of the auto-oxidation rates measured in the presence (Vs) and absence (Vc) of homogenized tissue. The data obtained was expressed as SOD activity units (U-525) per mg protein. One SOD activity unit is the activity that doubles the auto-oxidation background (Vs/Vc = 2). Protein concentration was measured by the method of Lowry (1951). The detection limit of the SOD assay is 0.2 U/ml. The intra-assay coefficient of variation was determined by calculating Vs/Vc on 12 replicate samples of homogenate of placental tissue. The mean of the obtained data was 1.69, the SD was 0.09 and the CV was 5.2%. The inter-assay mean was 1.74, the SD was 0.09 and CV was 5.2%.

Results

Immunocytochemistry for Cu/Zn SOD in myometrium, placenta and fetal membranes

Cu/Zn SOD was identified in each of the tissues examined (Figure 1a–c). There was no difference in localization or intensity of staining of Cu/Zn SOD in myometrium, placenta or membranes obtained from women delivered before or after the onset of labour. In the myometrium Cu/Zn SOD was localized to myocytes and endothelial cells of myometrial blood vessels (Figure 1a). In the placenta intense immunostaining was observed in the syncytiotrophoblast whilst endothelial cells of villous vessels and villous stromal cells exhibited moderate staining (Figure 1b). In the fetal membranes there was either a lack of staining or only very weak staining in the amnion, moderate staining in the chorion and extra-villous trophoblast and intense staining in decidua (Figure 1c). Specificity of

antibody binding was confirmed by the presence of immuno-staining for Cu/Zn SOD in epithelial cells in human endometrium, hepatocytes in human liver and epithelial cells in human kidney.

Immunocytochemistry for Mn SOD in myometrium, placenta and fetal membranes

The cellular distribution of Mn SOD cells in myometrium and placenta was similar to that of Cu/Zn SOD although the intensity of staining varied (Figure 2a–c). Mn SOD staining was localized to myocytes and endothelial cells in the myometrium (Figure 2a), although the staining was less intense than that observed with the Cu/Zn SOD antibody. In the placenta, intense staining of the syncytiotrohoblast and moderate staining of villous vessel endothelial cells and villous stromal cells was observed (Figure 2b). In the fetal membranes, in contrast to results with Cu/Zn SOD, intense staining of amnion and decidua, moderate staining of extravillous trophoblast and faint staining of chorion was observed (Figure 2c). There was no difference in the localization of staining or in the intensity of staining between tissue obtained before or after the onset of labour. Specificity of antibody binding was confirmed by the presence of immunostaining for Mn SOD in epithelial cells in human endometrium, hepatocytes in human liver and epithelial cells in human kidney and epithelial cells in human lung.

SOD activity in myometrium, placenta and fetal membranes obtained prior to or during term labour

SOD activity in each of the tissues studied is shown in Table I. There was no significant difference in total or mitochondrial SOD activity between tissue obtained before and after labour. Electron microscopy confirmed that the isolation procedure for mitochondria resulted in a cell fraction consisting mainly of mitochondria.

Immunocytochemistry for xanthine oxidase in myometrium, placenta and fetal membranes

Xanthine oxidase immunoreactivity was identified in each of the tissues examined. There was no difference in localization or intensity of xanthine oxidase immunostaining in myometrium, placenta or membranes obtained from women delivered before or after the onset of labour (Figure 3a–c). In the myometrium, myocytes showed a variation of staining intensity for xanthine oxidase ranging from absent to moderate (Figure 3a). In myometrial vessel endothelial cells there was either a lack of staining or very weak staining. In the placenta weak to moderate immunostaining was observed in the syncytiotrophoblast and villous stromal cells whilst endothelial cells of villous vessels exhibited only weak staining (Figure 3b). In the fetal membranes there was a variation in staining intensity ranging from weak to strong in amnion, moderate to strong staining in the chorion and extravillous trophoblast and intense staining in decidua (Figure 3c).

Discussion

The results detailed in this study show that Cu/Zn, Mn SOD and xanthine oxidase are present in human myometrium,

Table I. Total and mitochondrial superoxide dismutase (SOD) activity (mean ± SD, units/mg protein)

Tissue	Total SOD activity ($n = 9$)		Mitochondrial SOD activity ($n = 8$)	
	Not in labour	In labour	Not in labour	In labour
Myometrium	7.7 ± 4.1	11.8 ± 7.0	ND	ND
Placenta	30.6 ± 7.2	28.6 ± 14.5	ND	ND
Membranes	10.1 ± 8.9	5.0 ± 3.0	5.7 ± 5.8	3.7 ± 4.4

Not significant comparing tissues before and after labour using the Mann–Whitney U-test. ND = not determined.

placenta and fetal membranes and decidua but that there is no difference in expression of these proteins as determined by immunocytochemistry between tissue obtained before or after labour. In addition we have shown that these tissues possess superoxide dismutase activity and that there is no difference in total SOD activity between tissue obtained before or after the onset of labour.

The enzyme superoxide dismutase is now implicated in an increasing number of clinical disorders. Mutations in the Cu/Zn SOD gene are responsible for familial amyotrophic lateral sclerosis (ALS), an autosomal dominant degenerative disorder affecting motor neurons in the cortex, brain stem and spinal cord (Rosen et al., 1993). Affected individuals have a reduction of Cu/Zn SOD in their erythrocytes which may lead to oxyradical cell injury. In Down's syndrome, levels of Cu/Zn SOD are elevated by up to 50% (Garber et al., 1979), probably due to the presence of the extra copy of chromosome 21, on which the Cu/Zn gene is located (Tan et al., 1973). Evidence that CuZn SOD may be involved in the pathophysiology of Down's syndrome is suggested by experiments showing that overexpression of the Cu/Zn SOD gene may impair neurotransmitter transport and alter neuromuscular junctions (Elroy-Stein et al., 1988; Avraham et al., 1991).

We postulated that the interaction between SOD and NO might be important in the regulation of the onset of human parturition. Superoxide, which is a product of both oxidative metabolism and the inflammatory process, interferes with the biological activity of NO by converting it to peroxynitrite (Beckman and Croe, 1993). In contrast, superoxide dismutase converts superoxide to hydrogen peroxide and oxygen, and by removing superoxide anion prolongs the biological half-life of nitric oxide. The precise mechanism by which the myometrium remains quiescent during pregnancy, and is then converted to an actively contracting organ at the onset of parturition, is unknown. Animal studies have suggested a role for the smooth muscle relaxant NO. Uterine production of NO is high during rat and rabbit pregnancy, and declines at the onset of parturition, initiating myometrial contractility (Sladek et al., 1993; Natuzzi et al., 1993; Yallampalli et al., 1993). Although in human pregnancy NO is produced within the uterus, a decline in NO production at the onset of parturition has not been demonstrated (Thomson et al., 1997). Both Cu/Zn SOD and Mn SOD may be important in promoting myometrial relaxation. It is believed that NO produced within the placenta, decidua, fetal membranes or myometrium may induce guanylate cyclase activity

and thereby maintain myometrial quiescence during pregnancy. The biological activity of placental, decidual or myometrial NO will be potentiated by cytoplasmic Cu/Zn SOD in these tissues. In mitochondria, NO inhibits oxidative phosphorylation by binding to cytochrome oxidase and inhibiting electron transport (Bates et al., 1995). This will in turn decrease ATP production, attenuating a large range of cellular processes including muscle contraction (Brown, 1992). If the biological activity of mitochondrial NO is increased, for example by Mn SOD, the effect of NO on inhibition of smooth muscle contraction should be enhanced. Changes in myometrial Mn SOD may therefore have profound effects on myometrial contractility.

Previous studies have also reported SOD and xanthine oxidase within the human reproductive tract. Cu/Zn SOD has been identified within the endometrium, and increases immediately prior to implantation (Narimoto et al., 1991; Sugino et al., 1996). Furthermore, decidual cells of early pregnancy show strong immunostaining for Cu/Zn and Mn SOD (Sugino et al., 1996). These results agree with our own demonstration of intense immunostaining for Cu/Zn SOD and Mn SOD in decidual cells at term, and suggest that these enzymes may be expressed in the decidua throughout gestation. In vitro in the placenta, MnSOD but not Cu/Zn SOD is induced in cultured human trophoblast during differentiation (Church et al., 1992). These results appear to be at variance with our own showing both Cu/Zn and MnSOD expression in the placenta, but these differences may be explained by the fact that they were obtained in vitro. Xanthine oxidase activity and mRNA have previously been detected in placenta (Many et al., 1996). Immunostaining for xanthine oxidase has previously been detected in villous trophoblast and non-villous trophoblast of the fetal membranes, but in contrast to our data was not identified within the villous stroma (Many et al., 1996).

If SOD does not play a role in the initiation of parturition, what is its function in the reproductive tissues within which it has been identified? After fertilization, embryonic and endometrial expression of both Cu/Zn SOD and Mn SOD has been observed (Harvey et al., 1995; Sugino et al., 1996). It has been suggested that such expression is important for successful implantation, by protecting the blastocyst from damage by superoxide radicals. The function of decidual SOD expression is not known, but expression of Mn SOD may protect the decidua from TNF-mediated cell damage. Decidual cells and predecidual cells produce tumour necrosis factor (TNF) (Tabibzadeh, 1991) and TNF production increases during labour (Opsjon et al., 1993). TNF may damage cells via superoxide anion production (Zimmerman et al., 1989), but TNF-resistant cells produce Mn SOD which protects them from the cytotoxicity of TNF (Wong and Goedell, 1988). Mn SOD is itself induced by TNF (Wong and Goeddel, 1988), and it may be that in decidua Mn SOD is expressed in order to protect decidual cells from high local concentrations of TNF.

In the placenta, SOD may be important in preventing lipid peroxidation. Lipid peroxidation involves oxidative or enzymatic conversion of unsaturated fatty acids to primary products known as lipid hydroperoxides and a variety of secondary metabolites (Carpenter, 1981; Taylor and Morris,

1983). Peroxidation of membrane-associated fatty acids and cholesterol alters membrane fluidity and may eventually induce widespread membrane damage (Kagan, 1988). Pre-eclampsia is associated with elevated levels of lipid peroxidation products and it has been suggested that lipid peroxidation may play a role in aetiology of the disease (Hubel *et al.*, 1989). If lipid peroxidation is not prevented during pregnancy, diseases such as pre-eclampsia may ensue. Moreover, placental and systemic SOD activity and mRNA is lower in pre-eclampsia compared with normal pregnancy (Wang and Walsh, 1996; Poranen *et al.*, 1996), suggesting that SOD plays a role in the prevention of pre-eclampsia.

We have failed to show a decline in SOD activity following the onset of labour. Notwithstanding, the SOD present within the uterus may play a role in maintaining myometrial quiescence prior to the onset of parturition. There is some evidence that the uterus is more sensitive to the tocolytic effects of NO *in vivo* during pregnancy compared with isolated myometrial strips *in vitro* (Lees *et al.*, 1994; Rowlands *et al.*, 1996; Norman *et al.*, 1997). Studies *in vitro* have shown that the inhibitory effect of NO donors on myometrial contractility can be potentiated by the inclusion of placental tissue within the organ bath (Segal and Datta, 1996). Taken together, these results suggest that the placenta contains an agent which enhances the effect of NO during pregnancy. This agent could be SOD.

In conclusion we have detected systems for synthesis and destruction of superoxide in the myometrium and the surrounding placenta, fetal membranes and decidua and have discussed ways in which alterations in the balance between NO and superoxide production and destruction may affect myometrial contractility. We have failed to demonstrate a change in superoxide production or destruction following the onset of labour and it seems unlikely that these processes are implicated in the initiation of labour. The widespread distribution of Cu/Zn and Mn SOD in reproductive tissues suggests that these enzymes may be important in other reproductive processes such as implantation, the prevention of pre-eclampsia and the maintenance of myometrial quiesence during pregnancy.

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