Developmental changes in antioxidant enzymatic defences against oxidative stress in sheep placentomes

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Abstract

Early pregnancy is susceptible to oxidative stress, and thus characterisation of antioxidant systems and pro- and anti-apoptotic pathways would improve understanding of placental development and function. We aimed, therefore, to determine the activities of the antioxidant enzymes, copper/zinc-superoxide dismutase (SOD1), manganese-SOD (SOD2), catalase (CAT), glutathione (GSH) peroxidase (GPX) and GSH reductase (GSR); and to quantify the expression of BAX and MCL1 proteins in relation to the developmental changes in antioxidant defences in sheep placentomes sampled on days 35, 55 and 80 of pregnancy. Placentome progesterone content was analyzed to determine steroidogenic capacity. Malondialdehyde (MDA) and protein carbonyl were quantified in placentomes as biomarkers of lipid peroxidation and protein damage respectively. Placentome tissues demonstrated significantly increased content of

progesterone and MDA at day 80 of pregnancy and protein carbonyl as early as day 50 of pregnancy. Progesterone and MDA contents were not different between days 35 and 55 of pregnancy. While SOD1 and CAT activities did not alter significantly, SOD2 activity decreased from days 35 to 55. GPX activity increased from days 35 to 55 and increased further to day 80 of pregnancy. GSR activity increased from days 35 to 55 of pregnancy. BAX protein expression decreased, while MCL1 increased from days 35 to 55 and 80 of pregnancy. The increased GPX activity was associated with a decrease in the BAX/MCL1 protein expression ratio. Changes in the antioxidant enzymatic defences could be a part of placentome adaptation to reactive oxygen species-induced oxidative stress at specific early developmental stages of pregnancy.

Journal of Endocrinology (2010) **205**, 107–116

Introduction

Maintaining a physiological equilibrium between intracellular levels of antioxidants and the production of reactive oxygen species (ROS) is crucial for the survival of the developing organisms. Tightly controlled ROS generation appears to be one of the central elements in the mechanisms involved in cell function, growth, differentiation and death (Valko *et al.* 2007). An increase in ROS generation beyond the ability of antioxidative protection, i.e. oxidative stress, potentially leads to cellular damage. Irreversible modification of cellular components, such as lipids, proteins and nucleic acids, by ROS leads to cell damage and dysfunction (Halliwell & Gutteridge 1989). Mammalian cells have evolved a variety of antioxidant mechanisms to control ROS production and propagation (Fridovich 1999). Superoxide dismutases (SODs) are metalloenzymes that catalyze the dismutation of superoxide radical ($O_2^{\cdot-}$) into hydrogen peroxide (H_2O_2). Thus, they constitute the first enzymatic step that plays a vital role in the control of cellular $O_2^{\cdot-}$

production. Two distinct isoforms of SOD have been identified in mammals. Copper–zinc containing SOD (Cu, Zn-SOD or SOD1) is a dimeric protein, essentially located in the cytoplasm. Manganese containing SOD (Mn-SOD or SOD2) is a homotetrameric protein, located in the mitochondria. Glutathione (GSH) peroxidases (GPXs) are located within the mitochondrial matrix and the cytoplasm, and catalase (CAT) found primarily within peroxisomes, both belong to the secondary defence mechanism by catalyzing the conversion of H_2O_2 to H_2O . GSH reductase (GSR) catalyzes GSH disulphide to reduced GSH, with NADPH as the reducing agent. GPX reduces H_2O_2 or other hydro peroxides using GSH as donor substrate. In the presence of iron, H_2O_2 and $O_2^{\cdot-}$ can interact in a Haber–Weiss reaction (Kehrer 2000) to generate highly toxic hydroxyl radical ($OH^{\cdot}$). It follows that SOD1, SOD2, CAT, GPX and GSR represent co-ordinately operating defences against ROS propagation (Fig. 1). Studies of key antioxidant enzymatic activities are of utmost importance because they may indicate how tissue or organ might

Figure 1 Schematic representation of the pathways producing reactive oxygen species (ROS) and key cellular antioxidant enzymatic systems controlling ROS production. Superoxide radical (O₂^{•-}), hydrogen peroxide (H₂O₂), nitric oxide (NO[•]), peroxynitrite (ONOO⁻), hydroxyl radical (OH[•]), copper/zinc-superoxide dismutase (SOD1), manganese-SOD (SOD2), catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GSR), reduced glutathione (GSH), oxidized glutathione (GSSG), NADPH and oxidized NADP⁺.

respond to oxidative stress in oxidizing environment, such as during early placental development.

The effectiveness of antioxidant enzymes against ROS varies with the stage of human placental development (Sekiba & Yoshioka 1979, Takehara *et al.* 1990, Qanungo *et al.* 1999, Jauniaux *et al.* 2000, Qanungo & Mukherjee 2000). Oxidative stress during early human placental development (Myatt & Cui 2004) is associated with human pregnancy-related disorders, such as preeclampsia, embryonic resorption, spontaneous abortion and intra-uterine growth restriction (Agarwal & Allamaneni 2004). Furthermore, oxidative stress alters human placenta development and may be a general underlying mechanism that links altered placental function to foetal programming (Myatt 2006). Therefore, understanding of factors that control ROS production during early placental development is essential to improving reproductive efficiency of humans and domestic animals. Although antioxidant enzymatic defences may play a role in antioxidative processes during human placental development, no attention has been paid to the ROS scavenging systems in placenta of domestic animals, leaving a gap in our knowledge about the importance of antioxidant mechanisms to control ROS production and oxidative stress during early placental development.

Ruminants (sheep, goat and cattle) have a cotyledonary placenta. The appropriate development and function of discrete areas of functional placentation, the placentomes, are

central for optimal foetal growth and pregnancy outcomes. Rescue of the corpus luteum (CL) from cyclic luteal regression and maintenance of progesterone production are initially important events for the establishment of pregnancy in mammals. In both humans and sheep, the shift of progesterone production from the CL to the placenta occurs early in pregnancy, while the CL constitutes the principal source of progesterone throughout all or most of pregnancy in the mouse, rat, cat, rabbit, pig, goat, cow and horse (Ryan 1969). The early luteo-placental shift in progesterone production plays a crucial role in the maintenance of pregnancy beyond the lifespan of the CL in humans (Csapo *et al.* 1972) and sheep (Al-Gubory *et al.* 1999), allowing continued pre-natal development. Thus, compromised placental progesterone production is a potential risk factor for pregnancy outcomes in both species.

In early pregnancy, sheep placentomes undergo a series of morphological changes, which are characterized by cell proliferation and apoptosis (Riley *et al.* 2000). ROS-induced oxidative stress plays a role in apoptosis induction (Simon *et al.* 2000), at least in part, through changes in the expression of BCL-2-associated X protein, or BAX (Nakamura & Sakamoto 2001) which was the first identified pro-apoptotic member of the BCL-2 protein family. BCL-2-family proteins are key regulators of mitochondrial-related apoptosis pathways (Tsujimoto & Shimizu 2000). Activities of SOD1 and GPX are lower (Wang & Walsh 1996), and expression of BAX is higher in preeclamptic placenta than in normal full-term placenta (Cobellis *et al.* 2007). The antiapoptotic protein MCL1, a member of the BCL-2 protein family, preferentially inhibits BAX-induced mitochondrial cytochrome C release and apoptosis (Germain *et al.* 2008).

In order to improve understanding of the dynamics involved in early placental development and function in domestic animals, we aimed to investigate i) placentome steroidogenic capacity; ii) placentome content of malondialdehyde (MDA) and protein carbonyls as markers of lipid peroxidation and protein oxidation respectively; iii) the activities of key intracellular antioxidant enzymes (SOD1, SOD2, CAT, GPX and GSR) and iv) the expression of BAX and MCL1 proteins in relation to the developmental changes in antioxidant defences in sheep placentomes. In sheep, foetal chorionic villi interlock in predetermined regions of the maternal endometrium, known as caruncles, to form placentomes on and after the fourth week of pregnancy (Boshier 1969). Sheep placental cellular proliferation peaks between days 50 and 60 of pregnancy (Alexander 1964), and maximum placental weight is attained around day 80 of pregnancy (Ehrhardt & Bell 1995). Placentomes (cotyledonary and caruncular tissues) were therefore sampled from singleton-bearing ewes at three physiologically significant stages of gestation, i.e. days 35, 55 and 80, coinciding with the period of early placentation, rapid placental growth and with the peak in placental weight.

Materials and Methods

Animals and tissue collection

All procedures relating to care and use of animals were approved by the French Ministry of Agriculture according to the French regulation for animal experimentation (authorisation no. 78-34). Ewes of the Préalpes-du-Sud breed were treated for 14 days with intravaginal sponges containing 40 mg fluorogestone acetate (Intervet, Angers, France) to synchronize oestrus. Immediately after removal of the sponges, each ewe received an i.m. injection of 400 IU equine chorionic gonadotropin (Intervet). The ewes were mated at oestrus with fertile rams, twice at an interval of 12 h. The ewes were killed at a local abattoir in accordance with protocols approved by the local institutional animal use committee. After killing, the reproductive tracts were collected and immediately transported to the laboratory. Placentomes (cotyledonary and caruncular tissues) were sampled from singleton-bearing ewes at days 35 and 55, coinciding with the period of early placentation (Boshier 1969) and rapid placental development (Alexander 1964), and at day 80, coinciding with the peak in placental weight (Ehrhardt & Bell 1995). Ten representative placentomes were removed from the entire length of the two uterine horns of each pregnant ewe ($n=4$ ewes per gestational stage), snap frozen in liquid nitrogen and then stored at -80°C until processed for progesterone, MDA and protein carbonyl contents, the activities of SOD1, SOD2, CAT, GPX and GSR, and the expression of BAX and MCL1 proteins. The time that elapsed between the killing of the ewes and placentomes frozen in liquid nitrogen was <10 min.

Extraction of progesterone from placentomes

A simple extraction of progesterone from placentome tissues was performed as described previously for CL progesterone content (Marinelli *et al.* 2009), but instead of extracting progesterone with petroleum ether, it was extracted with ethanol. Briefly, tissues (wet weight ~ 600 mg) were homogenized in 3 ml of absolute ethanol (200 mg/ml, w/v), using an Ultra-Turrax homogenizer (Janke & Kunkel IKA-Labortechnik, Staufen, Germany). Homogenates were centrifuged (3500 g) for 30 min at 4°C , and the ethanol extracts were then decanted into glass tubes. The organic phase was dried under a stream of nitrogen in a water bath at 37°C . The dried extracts were dissolved in 0.5 ml phosphate buffer (0.1 M, pH 7.25) containing 0.1% (wt/v) gelatine, vortexed for 10 min at laboratory temperature and then the mixture assayed for progesterone. The recovery of tritiated progesterone in extracted placentome tissues was 82 ± 2.3 (means $\pm$ S.E.M.), and placentome progesterone contents were adjusted for recovery.

Progesterone RIA

Placentome ethanol extracts were analyzed for progesterone content in duplicate by a RIA as previously described

(Schanbacher 1979) and validated for sheep jugular venous plasma with slight modifications (Al-Gubory *et al.* 2006). Briefly, dextran-coated charcoal solution was used instead of polyethylene glycol for the separation of bound and free radioactivity. Tritiated progesterone (1,2,6,7- ^3H -progesterone, sp act 88 Ci/mmol) was obtained from Amersham, and a specific anti-progesterone antibody was obtained from the Institut Pasteur (Paris, France). Hormone preparations, progesterone tracer and other reagents were diluted in 0.1 M PBS (pH 7.25). Tritiated P4 (3000 c.p.m.) in 100 μl buffer, 100 μl progesterone antiserum (1/20 000 dilution), 20 μl progesterone standard, placentome extract samples, and 80 μl buffer were dispensed into the assay tubes. After an initial 2-h incubation at laboratory temperature (21°C) followed by 24-h incubation at 4°C , 2.2 ml cold charcoal-dextran solution containing 0.1% gelatine was added, and the tubes were centrifuged at 3000 g for 45 min. The supernatant was decanted into vials, and 3 ml scintillation fluid (Scintillator Plus; Perkin-Elmer Life & Analytical Sciences, Boston, MA, USA) was added to each vial. The radioactivity was counted in a Packard Tri-Carb Liquid Scintillation analyzer (model 2100 TR; Groningen, The Netherlands). Placentome content of progesterone was adjusted to picogram/milligram of tissue. To minimize assay variability, all placentome extract samples were analyzed in a single RIA. The limit of assay sensitivity was 0.1 ng/ml, and the intra-assay coefficient of variation was $<10\%$.

Antioxidant enzyme activity assays

Placentome tissues corresponding to each stage of pregnancy from each ewe were homogenized separately in cold phosphate buffer (50 mM, pH 7.4), and then the homogenates were centrifuged at 15 000 g for 30 min at 4°C . The resulting supernatant was used for determination of protein concentration (Lowry *et al.* 1951), MDA and carbonyl content and measurement of enzymatic activities. We used a standard SOD assay (Marklund & Marklund 1974), which has been validated for different sheep reproductive tissues (Al-Gubory *et al.* 2004, 2005, 2006, 2008, Garrel *et al.* 2007). Total SOD activity was measured using the pyrogallol assay based on the competition between pyrogallol oxidation by $\text{O}_2^{\cdot-}$ and superoxide dismutation by SOD. SOD2 is encoded in the nuclear chromatin, synthesized as a precursor in the cytoplasm and imported posttranslationally into the mitochondrial matrix in its mature form (Wispe *et al.* 1989). The freeze-thaw approach results in mitochondrial release of large amounts of SOD2 into the cytosol (Jin *et al.* 2005). This approach was used for measurement of SOD2 activity in cytosol-enriched placentome fraction. We determined the enzymatic activity of SOD2 by assaying for SOD activity in the presence of sodium cyanide, which selectively inhibits SOD1 but not SOD2 (Jin *et al.* 2005). SOD1 activity was calculated by subtracting SOD2 activity from total SOD activity. The rate of auto-oxidation is taken from the increase in the absorbance at 420 nm. One unit of SOD activity is

defined as the amount of the enzyme required to inhibit the rate of pyrogallol auto-oxidation by 50%. CAT activity was determined as described previously (Nzengue *et al.* 2008). Activity was assayed by determining the rate of decomposition of H_2O_2 by CAT in 10 mM potassium phosphate buffer (pH 7). The reaction rate was related to the amount of CAT present in the mixture. The rate of H_2O_2 decomposition by CAT was followed at 240 nm. One unit was defined as the decomposition of 1 mmol hydrogen peroxide per minute per milligram protein. GPX activity was measured using the GSR–NADPH method. Enzyme activity was determined by a coupled assay system in which oxidation of GSH was coupled to NADPH oxidation catalyzed by GSR. The rate of GSH oxidized by tertiary butyl hydroperoxide was evaluated by the decrease of NADPH in the presence of EDTA, excess reduced GSH and GSR. The rate of decrease in concentration of NADPH was recorded at 340 nm. GPX activity was expressed in terms of nanometer of NADPH oxidized per minute per milligram protein. GSR activity was assayed by the standard method of NADPH oxidation. In this assay, oxidized GSH is reduced to GSH by GSR, which oxidizes NADPH to NADP^+ . NADPH consumption was determined at 340 nm. Enzyme activity was expressed in terms of nanometer of NADPH oxidized per minute per milligram protein.

Malondialdehyde measurement

MDA, an end product of lipid peroxidation processes (Mukai & Goldstein 1976), is frequently used as a biomarker of oxidative stress. The most widely used method for the determination of MDA in biological materials is based on its reaction with thiobarbituric acid (TBA). Reversed-phase HPLC techniques in which the MDA–TBA adducts are separated from interfering substances (Londero & Lo Greco 1996) was used for determining MDA in placentome tissue homogenates. The breakdown product of 1,1,3,3-tetraethoxypropane (TEP) was used as standard. TEP undergoes hydrolysis to liberate stoichiometric amounts of MDA. Stock standard solution (480 μl of TEP in 100 ml ethanol) was prepared, and this primary solution was diluted to the concentrations of 0, 1, 2, 3, 4, 5 and 6 μM . Tissue extract aliquots or standards (100 μl) were mixed with 750 μl of 0.8% TBA. The tubes were placed in a water bath at 95 °C for 1 h, and then they were cooled. Samples were neutralized with methanol–NaOH mixture (pH 6). After centrifugation, 50 μl of protein-free supernatant were chromatographed in the HPLC system. The column used for the separation was Adsorbosphere C18 (5 μm particle diameter, 250 mm $\times$ 4.6 mm ID; Grace Davison Discovery Sciences Deerfield, IL, USA). The MDA–TBA adduct is eluted from the column with potassium dihydrogen phosphate buffer (10 mM, pH 6.0) – acetonitrile (17%). The quantification of MDA derivative was established by comparing the absorption to the standard curve of MDA equivalents generated by acid-catalyzed hydrolysis of TEP as micromoles per gram tissue protein.

Protein carbonyl measurement

Protein oxidation is an oxidative stress marker (Zusterzeel *et al.* 2001), and the oxidation of proteins is often analyzed by measuring the protein-bound carbonyl groups. Protein carbonyl content was determined in placentome tissue homogenates as described previously (Levine *et al.* 1994). Briefly, 1 ml 10% trichloroacetic acid (TCA) and 1 ml 10 mM 2,4-dinitrophenylhydrazine solution was added to aliquots of placentome homogenates, and the mixture was vortexed and incubated at 37 °C for 50 min. Next, 1 ml ice-cold TCA was added to the mixture. The pellet obtained after centrifugation at 3000 *g* for 10 min at 4 °C was washed three times with 1 ml ethanol/ethyl acetate (1:1, v:v). The last pellet was dissolved in 1 ml 6 M guanidine. After centrifugation at 12 000 *g* for 10 min, the absorbance of the supernatant was measured spectrophotometrically at 380 nm to quantify protein carbonyl, and its concentration was expressed as micromoles of carbonyl groups per gram of tissue protein.

1-D gel electrophoresis and western blot

Placentome tissues were processed for 1-D gel electrophoresis as described previously (Fowler *et al.* 2007a). Briefly, the frozen placentome tissues were blotted on filter paper and combined with lysis buffer (1 mg wet tissue weight: 5 μl lysis buffer) containing 0.01 M Tris–HCl, pH 7.4, 1 mM EDTA, 8 M urea, 0.05 M dithiothreitol, 10% (v/v) glycerol, 5% (v/v) NP40 and protease inhibitor cocktail (Roche Diagnostics). The tissues were placed in 2 ml tubes containing a stainless steel ball, and all were simultaneously lysed (Tissue Lyser, 2 min at maximum; Qiagen Ltd). Supernatants were transferred to Eppendorf tubes and centrifuged at 50 000 *g* for 20 min at 4 °C. Protein content of the final supernatant containing the soluble cellular proteins had been determined (RC-DC assay, Bio-Rad Laboratories Ltd), and the placentome extracts were stored at –80 °C. Individual lysates were electrophoresed (30 μg protein/lane) on 26-lane 1-DE 4–12% Bis–Tris gels (Invitrogen Ltd) under reducing conditions (MOPS buffer, Invitrogen) and transferred to immobilon-FL membrane (Millipore Ltd, Watford, UK) as described previously (Fowler *et al.* 2007b). See Blue plus 2 molecular weight markers (Invitrogen) were electrophoresed in three lanes of every gel. The membranes were blocked (overnight at 4 °C) with Odyssey Blocking Buffer (927–4000: LI-COR Biosciences Ltd, Cambridge, UK) and were incubated with primary antibodies (in blocking buffer) at 4 °C overnight: i) BAX (1:200: Santa-Cruz Biotechnology Inc., Santa Cruz, CA, USA, sc-493), ii) MCL1 (1:500: Santa-Cruz Biotechnology Inc., sc-95) combined with an anti- β -actin load control of differing species as appropriate (mouse 1:5000 AB6276; rabbit 1:10 000 AB8227, both Abcam Ltd, Cambridge, UK). The protein bands were visualized using an Odyssey infrared fluorescence imager (LI-COR), and the resulting electronic images were analyzed using Phoretix-1D

Figure 2 Progesterone contents in sheep placentomes ($n=10$ per ewe) collected from ewes at days 35 ($n=4$ ewes), 55 ($n=4$ ewes) and 80 ($n=4$ ewes) of pregnancy showing significant increases in progesterone with gestational stages. Values are means $\pm$ S.E.M. for the number of ewe used. ** $P<0.01$, *** $P<0.001$.

advanced software (Nonlinear Dynamics Ltd, Newcastle-upon-Tyne, UK) in order to determine the band volumes and molecular weights. This software calculates band volumes, based on constant lane width and automatic band selection, from the raw data of pixel area and intensity that are independent of operator-altered contrast or brightness. The specificity of the antibodies for the sheep proteins was tested by mixing the antibodies with blocking peptides, BAX (Santa Cruz Biotechnology; sc-493) at 5 μ g/ml antibody solution and MCL1 (Santa Cruz Biotechnology; sc-958) at 2 μ g/ml antibody solution, both incubated for 2 h, prior to use. The blocking peptides prevented immunodetection of the relevant bands compared with incubation with antibody alone, and therefore the bands quantified were BAX and MCL1. There were no significant differences in β -actin band volumes between the gestational stages indicating the suitability of this load control for the present study.

Statistical analysis

The enzymatic activities, progesterone contents, MDA and carbonyl contents were analyzed by one-way ANOVA and the Newman-Keuls multiple comparison test (PRISM Graph Pad version 2; Graph Pad Software, San Diego, CA, USA). Western blot analyses were performed using JMP (version 5.1; Thomas Learning, London, UK). Normality of data distribution was tested with the Shapiro-Wilk test, and nonnormally distributed data were log-transformed prior to analysis. The band volumes, normalized relative to β -actin expression separate for each lane, were compared using one-way ANOVA. The acceptable level of significance was set at $P<0.05$. Data are presented as the means $\pm$ S.E.M.

Results

Progesterone content in placentome tissue homogenates (Fig. 2) rose steadily from days 35 to 80 of pregnancy ($P<0.001$), but although the contents of progesterone were significantly different between days 55 and 80 ($P<0.01$) and between days 35 and 80 ($P<0.001$) of pregnancy, progesterone contents between days 35 and 55 of pregnancy were not significantly different.

Protein carbonyl content in placentome tissue homogenates significantly increased from days 35 to 55 ($P<0.05$) and then remained relatively stable at that level at day 80 of pregnancy (Fig. 3a). MDA content in placentome tissue homogenates (Fig. 3b) significantly increased from days 35 to 80 ($P<0.01$) and from days 55 to 80 ($P<0.001$) of pregnancy. MDA content was not significantly different between days 35 and 55 of pregnancy.

Figure 3 Protein carbonyl and malondialdehyde (MDA) contents in sheep placentomes ($n=10$ per ewe) collected from ewes at days 35 ($n=4$ ewes), 55 ($n=4$ ewes) and 80 ($n=4$ ewes) of pregnancy showing significant increases in (a) carbonyl and (b) MDA with gestational stages. Values are means $\pm$ S.E.M. for the number of ewe used. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

Enzymatic activities of SOD1, SOD2, CAT, GPX and GSR in sheep placentomes collected at days 35, 55 and 80 of pregnancy are shown in Fig. 4a–e. The activity of SOD1 (Fig. 4a) was not different between any stages of the pregnancy examined. The activity of SOD2 (Fig. 4b) significantly decreased from days 35 to 55 ($P<0.05$) and then remained relatively stable at that level at day 80 of pregnancy. The activity of CAT (Fig. 4c) was not different between any stages of the pregnancy examined. The activity of GPX (Fig. 4d) increased from days 35 to 55 ($P<0.05$) and increased further at day 80 of pregnancy ($P<0.001$, days 80 vs 35; $P<0.01$, days 80 vs 55). The activity of GSR (Fig. 4e) peaked at day 55, being significantly higher than that at days 35 and 80 of pregnancy ($P<0.05$).

Ovine BAX immunodetection was clear with a single band at 22 kDa, and ovine MCL1 immunodetection band was quantifiable at 40 kDa (Fig. 5a). The expression of BAX protein (Fig. 5b) significantly decreased from days 35 to 55 ($P<0.05$) and decreased further at day 80 of pregnancy ($P<0.01$, days 80 vs 35). The expression of MCL1 protein (Fig. 5c) significantly increased from days 35 to 55 ($P<0.05$) and from days 35 to 80 ($P<0.05$) of pregnancy. To assess the balance between pro- and anti-apoptotic signalling, the BAX/MCL1 ratios were calculated (Fig. 5d), revealing a marked switch from pro-apoptotic to anti-apoptotic signalling between days 35 and 55 and 80 of pregnancy ($P<0.05$).

Discussion

To the best of our knowledge, this is the first report of physiological changes in key antioxidant enzymatic pathways at specific early stages of placentome development in domestic animals. Interestingly, these changes coincide with the period of most rapid growth of the sheep placenta (Alexander 1964) and high steroidogenic activity (present study). Previous human studies have reported interesting but contradictory findings. Some of these studies showed an increase in SOD activity (Sekiba & Yoshioka 1979, Takehara *et al.* 1990, Qanungo *et al.* 1999), whereas one study showed no changes (Jauniaux *et al.* 2000). However, all these studies only measured total SOD activity which is not informative of the changes in activity of cytosolic SOD1 and/or mitochondrial SOD2. This is important because mitochondria are the major sites of endogenous $O_2^{\cdot-}$ production (Wallace 2005). $O_2^{\cdot-}$ is converted to H_2O_2 by mitochondrial matrix SOD2, and in the presence of reduced transition metals, it can be converted to $OH^{\cdot}$ (Kehrer 2000), suggesting that the mitochondria are the prime target for ROS-induced oxidative damage (Wallace 2005). In addition, H_2O_2 is more stable than $O_2^{\cdot-}$ and can diffuse out of the mitochondria into the cytoplasm and the nucleus where it can lead to cell damage (Wallace 2005). Elevated activity of SOD2 would, in theory, promote H_2O_2 production and propagation of highly reactive ROS in the mitochondria and cytoplasm.

Figure 4 Changes in the activities of (a) SOD1, (b) SOD2, (c) CAT, (d) GPX and (e) GSR in sheep placentomes ($n=10$ per ewe) collected from ewes at days 35 ($n=4$ ewes), 55 ($n=4$ ewes) and 80 ($n=4$ ewes) of pregnancy showing significant alterations in SOD2, GPX and GSR with gestational stages. Values are means $\pm$ s.e.m. for the number of ewe used. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

Figure 5 (a) Western blot (WB) analysis of MCL1 and BAX proteins expression in sheep placentomes ($n=10$ per ewe) collected from ewes at days 35 ($n=4$ ewes), 55 ($n=4$ ewes) and 80 ($n=4$ ewes) of pregnancy showing (b) decreases in BAX, (c) increases in MCL1 and (d) decreases in the pro-/anti-apoptotic index (BAX/MCL1 ratio). Band volumes are means $\pm$ S.E.M. normalized against β -actin load control values for the same lanes individually, for four ewes measured independently at each gestational stage. * $P<0.05$, ** $P<0.01$.

Furthermore, it may be assumed that elevated SOD2 activity may be directly harmful to the mitochondria through steady state O_2^- production. Our results show that SOD1 activity in sheep placentomes did not alter across early pregnancy, whereas that of SOD2 significantly decreased from days 35 to 55 of pregnancy. The finding that GPX activity increased and that of SOD2 decreased in murine fibroblasts transfected with a SOD1 expression vector (Kelner & Bagnell 1990) supports the hypothesis that a fine balance between antioxidant enzymes determines the cellular resistance to oxidative stress. The fact that $OH^\cdot$ can be formed by H_2O_2 metabolism via iron-catalyzed Fenton reaction and as by-products of lipid peroxidation, points to the importance of GPX within cells to

remove H_2O_2 before it reacts with metal catalyst to form extremely toxic ROS. Under physiologically relevant conditions of sheep placental development between days 35 and 55 of pregnancy (present study), placentome cells may respond to oxidative stress by increasing GPX and GSR activities and decreasing SOD2 activity.

GPX has selenocysteine within its active site, and therefore, it is selenium dependent for antioxidant activity. The retention of the placenta in selenium-deficient cattle suggests a role for selenium-GPX in pregnancy outcomes (Eger *et al.* 1985). The increased GPX and GSR activity in sheep placentomes during early pregnancy (present study), also reported during human placental development for GSR (Qanungo *et al.* 1999) and GPX (Jauniaux *et al.* 2000) activities, is probably an important adaptive mechanism protecting the early developing placentomes against ROS-mediated oxidative stress and cell damage. With respect to GPX activity measured in whole homogenate of human placental tissue, conflicting results have been reported, varying from unchanged (Takehara *et al.* 1990, Qanungo *et al.* 1999) or increased (Jauniaux *et al.* 2000) activity, as pregnancy advances. It may be that differences between these studies could be explained by experimental conditions, such as heterogeneity of whole placental tissues and variation in the timing of collection. GPX not only functions by removing H_2O_2 formed after the SOD-catalyzed dismutation reaction, but also detoxifies the lipid hydroperoxides (Hayes & McLellan 1999). The efficiency of GPX may be attributable to the fact that it is located in both the cytoplasm and the mitochondrial matrix, and that it can utilize both H_2O_2 and lipid peroxides as substrates. Increased GPX activity during early pregnancy (present study) would protect the developing placentomes from ROS-induced cell damage, suggesting that GPX may be the major enzyme in defending against ROS attack within the sheep feto-placental unit.

In the present study, we demonstrated that BAX protein expression decreased, whereas that of MCL1 increased from days 35 to 55 and 80 of pregnancy in sheep placentomes. The BAX/MCL1 ratio decreased as pregnancy progresses, revealing a marked switch from pro-apoptotic to anti-apoptotic signalling. It is interesting that the increased GPX activity we reported here in sheep placentomes during the first half of pregnancy was associated with a decrease in the BAX/MCL1 expression ratio. The induction of apoptosis in bovine luteal cells by downregulation of GPX (Nakamura *et al.* 2001) and the induction of apoptosis in cultured bovine luteal cells by simultaneous treatment with H_2O_2 and with a specific inhibitor of GPX (Nakamura & Sakamoto 2001) suggest a role for H_2O_2 in CL apoptosis, at least in part through upregulation of BAX mRNA expression (Nakamura & Sakamoto 2001). *In vitro* experiments also have shown that GPX overexpression in a human endothelial-like cell line downregulates BAX protein expression (Faucher *et al.* 2005). Considering these previous results, one can speculate that the increased GPX activity in sheep placentomes as pregnancy progresses (present study), and likely the maintained ability of

placentome cells to scavenge H_2O_2 , would contribute to the downregulation of pro-apoptotic BAX protein expression and ultimately the BAX/MCL1 ratio. We suggest that the enhanced activity of GPX in sheep placentomes early in pregnancy may act as protective mechanism to prevent local tissue damage by directing H_2O_2 to form water and, thereby, preventing the production and propagation of potentially harmful ROS during fetoplacental growth and development.

In the present study, MDA and carbonyl contents were measured in sheep placentomes during early developmental stages as being the end products of lipid peroxidation and protein damage respectively, and both as biomarkers of oxidative stress. Overall, the extent of placentome lipid peroxidation and protein oxidation increased markedly as gestation proceeds. MDA is a decomposition product of peroxidized polyunsaturated fatty acids (Mukai & Goldstein 1976). Elevated lipid peroxide levels have also been reported to be greater in third trimester than first trimester human placental tissue (Diamant *et al.* 1980) and are found to be produced by the human placenta during uncomplicated pregnancy (Walsh & Wang 1993).

The first and rate-limiting step in the synthesis of progesterone in all steroidogenic organs, including the placenta (Strauss *et al.* 1996), is the transfer of cholesterol from the outer mitochondrial membrane to the inner membrane where it is converted into pregnenolone by the enzyme cytochrome P-450 side-chain cleavage. It is well known that ROS are produced during enzyme reaction, particularly by the cytochrome P-450 family (Cross & Jones 1991) and by the respiratory system of mitochondria (Cadenas & Davies 2000), and thus, they are considered as a by-product of steroid synthesis. Increased placentome progesterone content on and after day 60 of pregnancy was accompanied by a corresponding increase in MDA content (present study). There is evidence that the inhibition of progesterone biosynthesis by a NADPH-dependent lipid peroxidation in human placental mitochondria is a consequence of cytochrome P-450 degradation due to lipid peroxidation (Klimek 1992). The increased MDA and carbonyl contents reported here in sheep placentomes support the hypothesis that pregnancy *per se* is a state of oxidative stress (Myatt & Cui 2004) arising from high placental metabolic and steroidogenic activities.

Knowledge of placentome contribution to progesterone production during the first 2 months of pregnancy in domestic animals, including sheep, is still limited, and the antioxidative mechanisms involved in the control of placentome synthesis of progesterone during early pregnancy have not been elucidated. Moore *et al.* (1972) demonstrated the presence of small amounts of progesterone in the uterine vein blood of ewes between days 40 and 60 of pregnancy. *In vitro* studies have showed that foetal cotyledons from sheep placentomes have the potential for *de novo* synthesis of progesterone from pregnenolone as early as day 45 of pregnancy (Koligian & Stormshak 1976). Placental production of progesterone has been shown to rise initially

between days 50 and 70 of pregnancy in sheep (Ricketts & Flint 1980). Taken together, these studies suggest that sheep placenta possess the capacity to synthesize and secrete progesterone prior to day 60 of gestation at which time the placenta begins producing progesterone in concentrations sufficient to maintain pregnancy. It was observed in the present *in vivo* study that ovine placentomes had already acquired appreciable steroidogenic capacity as early as day 35 of pregnancy. Increased placentome tissue progesterone contents on and after day 60 of pregnancy (present study), when the CL is no longer necessary to maintain pregnancy (Al-Gubory *et al.* 1999), indicates that the steroidogenic capacity of placentome cells increased as pregnancy advances. It was concluded that the interval between days 35 and 60 provides sufficient time to prepare the luteal-placental shift in progesterone production and secretion. ROS, particularly H_2O_2 , have been shown to inhibit steroidogenesis by blocking cholesterol transport into mitochondria of rat luteal cells (Behrman & Aten 1991). In the present study, the increased enzymatic activity of GPX in sheep placentomes as pregnancy progresses, and consequently the increased ability of placentome cells to scavenge H_2O_2 , would contribute to the maintenance of progesterone production.

In summary, we have demonstrated that the activities of the key antioxidant enzymes show major changes in sheep placentomes during early placental development. These data suggest that enhanced activities of GSH-related enzymes in placentomes during early pregnancy may act as protective mechanism against oxidative damage during early sheep fetoplacental development and growth. Adequate placental antioxidant status during early pregnancy could prevent and control those mechanisms and disorders induced by maternal environmental factors and associated oxidative stress that could lead to impairment of placental function, foetal growth and pregnancy outcomes.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This research did not receive any specific grant from any funding agency in the public, commercial or not-for-profit sector.

Acknowledgements

The authors would like to thank Krawiec Angele, Catherine Mangournet, Sandra Grange, Christine Tozzoli, Cécile Mounioz (CHU Grenoble), Philippe Bolifraud and Christian Poirier (INRA, Jouy-en-Josas) and Margaret Fraser (University of Aberdeen) for their excellent technical assistance. The authors also thank the staff of the sheep sheds of Brouëssy and Jouy-en-Josas for outstanding technical help and sheep management.

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Received in final form 5 January 2010

Accepted 22 January 2010

Made available online as an Accepted Preprint
22 January 2010