The conceptus induces a switch in protein expression and activities of superoxide dismutase 1 and 2 in the sheep endometrium during early pregnancy

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Abstract

There has been a growing interest in the importance of superoxide dismutases (SODs) in the regulation of endometrial function. However, little is known about endometrial SOD1 and SOD2 protein expression and activity associated with early conceptus development. We aimed to investigate changes in protein levels and activities of SOD1 and SOD2 in the sheep caruncular (CAR) and intercaruncular (ICAR) endometrium during the transition from pre-implantation (day 14) to implantation (day 16) and post-implantation (day 18) periods of pregnancy. Lipid peroxidation was assessed by measuring CAR and ICAR malondialdehyde (MDA) content. SOD1 activity increased from day 14 to day 18 ($P<0.05$) in CAR and from day 14 to day 18 ($P<0.05$) and from day 16 to day 18 ($P<0.01$) in ICAR. SOD1 protein level increased from day 16 to day 18 ($P<0.05$) in CAR and from days 14 to days 16 and 18 ($P<0.05$) in ICAR. SOD2 activity increased from day 16 to day 18 ($P<0.05$) in CAR and from days 14 and 16 to day 18 ($P<0.001$) in ICAR. SOD2 protein level increased from day 14 to day 18 ($P<0.05$) in CAR and from days 14 and 16 to day 18 ($P<0.05$) in ICAR. The content of MDA in CAR or ICAR did not alter significantly between stages of pregnancy. In conclusion, the early post-implanting conceptus co-ordinately up-regulates SOD1 and SOD2 protein expression and bioactivity within CAR and ICAR. By the maintenance of adequate endometrium SOD1 and SOD2 activity, the conceptus limits lipid peroxidation during the peri-implantation period of pregnancy.

Introduction

The superoxide dismutase (SOD) family is a ubiquitously distributed group of metalloenzymes that catalyze the dismutation of superoxide radicals ($^{\bullet}O_2^-$) into hydrogen peroxide (H_2O_2). By scavenging $^{\bullet}O_2^-$, which is a precursor molecule for all other reactive oxygen species (ROS), SOD is the first line of defense against cellular oxidative damage and its subsequent effects on tissues of biological systems. Copper-zinc containing SOD (Cu, Zn-SOD or SOD1) is a dimeric protein, essentially located in the cytoplasm, whereas manganese-containing SOD (Mn-SOD or SOD2) is a homotetrameric protein, located in the mitochondria (McCord et al., 1971). The selenium glutathione peroxidases (seGPXs or GPXs), located within the mitochondrial matrix and the cytoplasm, are responsible for the conversion of H_2O_2 to water (Hayes and McLellan, 1993). Studies have indicated that SODs may have important roles in rodent (Laloraya et al., 1991; Jain et al., 2000), human (Sugino et al., 1996; Sugino et al., 2002a, 2002b; Lucic and Milicevic, 2011) and sheep (Al-Gubory and Garrel, 2012; Al-Gubory et al., 2014) endometrial function early in pregnancy.

The enzyme activity is primarily determined by protein expression level. The corresponding changes in protein levels and enzyme activities of SOD1 and SOD2 in mammalian endometrium associated with early conceptus (embryo and associated extraembryonic placental membranes) development have not been fully described. The sheep is a useful animal model to explore the endometrial antioxidant machinery and its regulation during the oestrous cycle (Al-Gubory et al. 2008) and early pregnancy (Al-Gubory and Garrel, 2012). The ovine uterine endometrium consists of large numbers of well-delimited aglandular caruncules (CAR) and glandular intercaruncular (ICAR) areas. The CAR and ICAR endometrium are histoarchitecturally different and play different roles in the establishment of pregnancy (Cooke et al., 2013). The glands of ICAR areas produce a complex mixture of growth factors, cytokines, adhesion proteins and enzymes to support early conceptus development whereas the CAR areas allow conceptus attachment of and early placentation (Filant and Spencer, 2014). Our hypothesis is that the conceptus-derived factors influence the uterine environment favourably for conceptus attachment via the regulation of SOD1 and SOD2 protein expression in sheep endometrium. To test our hypothesis, CAR and ICAR tissues from pregnant ewes were used to characterize peri-

implantation specific alterations of protein expression and activities of SOD1 and SOD2, GPX activity and the content of malondialdehyde (MDA), a biomarker of lipid peroxidation and oxidative stress.

Materials and Methods

Animals and management

The French Ministry of Agriculture approved all procedures relating to care and use of animals according to the French regulation for animal experimentation (authorization no° 78-34). Ewes of the Préalpes-du-Sud breed (18 months of age) were used in this study. All the ewes were treated for 14 days with intravaginal sponges containing 40 mg fluorogestone acetate (Intervet, Angers, France) to synchronize oestrous. Immediately after removal of the sponge, each ewe received an intramuscular injection of 400 IU of equine chorionic gonadotropin (eCG, Intervet). Ewes were mated twice with fertile rams of the same breed, at an interval of 12 h during the synchronized oestrus.

Endometrial tissue collection

The ewes were slaughtered at a local abattoir in accordance with protocols approved by the local institutional animal use committee at the Institut National de la Recherche Agronomique (INRA, Jouy-en-Josas, France). Ewes (n=4 ewes per group) were randomly allocated for slaughter at the pre-implantation period (day14), initial conceptus implantation (day 16) and the early conceptus post-implantation period (day 18). Immediately after slaughter of the ewes, the reproductive tracts were collected, placed on crushed ice and transported to the laboratory. All subsequent manipulation of the tissue was performed at 4 °C. The uterine horns were opened and the CAR and ICAR endometrial tissues were separately dissected from the entire two uterine horns of each ewe, snap-frozen in liquid nitrogen and then stored at -80°C until processed for activities of the $^{\bullet}O_2^-$ scavenging antioxidant enzymes, SOD1 and SOD2, the H_2O_2 scavenging antioxidant enzyme, GPX, and the content of MDA.

Malondialdehyde measurement

The content of MDA in CAR and ICAR endometrial tissues was determined by reversed-phase high performance liquid chromatography (HPLC) in which the MDA-thiobarbituric acid (TBA) adducts are

separated from interfering substances (Londero and Lo Greco, 1996). The breakdown product of 1,1,3,3-tetraethoxypropane (TEP) was used as a 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 (95°C, 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 x 4.6 mm ID). 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 µmoles per g tissue protein.

Antioxidant enzyme activity assays

The CAR and ICAR endometrial tissues 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, 4 °C. The resulting supernatant was used for determination of protein concentration (Lowry et al., 1951). Enzyme activities of SOD1 and SOD2 were determined as described previously (Al-Gubory and Garrel, 2012). Total SOD activity was measured using the pyrogallol assay based on the competition between pyrogallol oxidation by $^{\bullet}O_2^-$, and O_2^- dismutation by SOD. Enzymatic activity of SOD2 was determined 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. GPX activity was measured using the glutathione reductase (GR)-NADPH method. Activity was determined by a coupled assay system (Nzengue et al., 2008) in which oxidation of glutathione (GSH) was coupled to NADPH oxidation catalysed by GR. The rate of GSH oxidized by tertiary butyl hydroperoxide was evaluated by the decrease of NADPH in the presence of ethylenediaminetetraacetic

acid (EDTA), excess GSH and GR. The rate of decrease in concentration of NADPH was recorded at 340 nm.

Western blot

CAR and ICAR endometrial tissue lysates were loaded (30 µg protein/lane) onto 26-lane 1DE gels (NUPAGE Novex Midi gels, 4-12 %, Invitrogen) under reducing conditions and then electroblotted onto immobilon-FL membrane (Millipore Ltd, Watford, UK) as described previously (Fowler et al., 2008). After blotting, membranes were incubated in blocking buffer, 1:1 Odyssey blocking buffer (LI-COR Biosciences UK Ltd, Cambridge, UK) and PBS, at 4°C overnight. Primary antibodies were diluted in Odyssey blocking buffer 1:1 with 0.2 µm filtered PBST as follows: rabbit anti-Cu/Zn superoxide dismutase (SOD1, Abnova, Taipei City, Taiwan, PAB14492), 2 µg/ml; mouse anti-Mn superoxide dismutase (SOD2: AbCam Ltd, Cambridge, UK, ab16956), 1-10000; rabbit anti-Alpha Tubulin (AbCam Ltd, Cambridge, UK, ab4074), 1µg/ml. The membranes were incubated with primary antibodies at 4°C overnight and then incubated with secondary antibodies for 60 min at room temperature. Secondary antibodies including anti-mouse IgG IRDYe™800 (all secondary antibodies were provided from LI-COR, Cambridge, UK, 610-732-124), 1-10,000 and anti-mouse IRDYe™700DX (610-730-124) 1-5,000 were diluted in Odyssey blocking buffer 1:1 with 0.2 µm filtered PBST + 0.01% SDS. Following washing the membranes, the digital images were captured using Odyssey LI-COR Infrared Imager (LI-COR, Cambridge, UK). The band volumes and molecular weights (kDa) were then obtained following a background subtraction using Phoretix-1D Advanced software (Nonlinear Dynamics).

Statistical analysis

Statistical significance was determined by one-way ANOVA. After ANOVA, the Newman-Keuls multiple comparison test (PRISM Graph Pad version 2; Graph Pad Software, San Diego, CA) was applied to analyse differences between groups. The acceptable level of significance was set at $P<0.05$. Data are presented as the mean ± SEM.

156 **Results**

157 MDA content was not significantly different between stages of pregnancy examined in either CAR or
158 ICAR endometrium (Figure 1). At day 14 of pregnancy, the CAR endometrium demonstrated greater
159 ($P<0.05$) MDA content than the ICAR endometrium. In contrast enzymatic activities of total SOD but
160 not GPX showed stage-specific changes in the CAR and ICAR endometrium (Figure 2). In the CAR
161 endometrium, total SOD activity increased ($P<0.05$) from day 16 to day 18 of pregnancy. In the ICAR
162 endometrium, total SOD activity increased from day 14 to day 18 ($P<0.01$) and from day 16 to day 18
163 ($P<0.001$) of pregnancy. In the CAR and ICAR endometrium tissues, total activity GPX was not
164 different between the three stages of pregnancy examined.
165
166 Enzymatic activity and protein expression of SOD1 in the CAR and ICAR endometrium collected
167 during early pregnancy are shown in figure 3. In the CAR endometrium, SOD1 activity increased
168 ($P<0.05$) from day 14 to day 18 of pregnancy. In the ICAR endometrium, SOD1 activity increased
169 from day 14 to day 18 ($P<0.05$) and from day 16 to day 18 ($P<0.01$) of pregnancy. SOD1 protein in
170 the CAR and ICAR endometrium was detected at the expected molecular weight of 16 kDa on the
171 immunoblotted membranes. In the CAR endometrium, SOD1 protein expression increased ($P<0.05$)
172 from day 16 to day 18 of pregnancy. In the ICAR endometrium, SOD1 protein expression increased
173 ($P<0.05$) from day 14 to day 16 and from day 14 to day 18 of pregnancy.
174
175 Enzymatic activity and protein expression of SOD2 in the CAR and ICAR endometrium showed
176 similar, but not identical, patterns to SOD1 between days 14-18 of pregnancy (Figure 4). In the CAR
177 endometrium, activity of SOD2 increased ($P<0.05$) from day 16 to day 18 of pregnancy. In the ICAR
178 endometrium, activity of SOD2 increased ($P<0.001$) from day 14 to day 18 and from day 16 to day 18
179 of pregnancy. SOD2 Protein in the CAR and ICAR endometrium was detected at the expected
180 molecular weight of 24 kDa on the immunoblotted membranes. In the CAR endometrium, expression
181 of SOD2 protein increased ($P<0.05$) from day 14 to day 18 of pregnancy. The expression of SOD2
182 protein tended to be increased in CAR endometrium from day 16 to day 18 of pregnancy, although not

attaining statistical significance. In the ICAR endometrium, expression of SOD2 protein increased ($P<0.05$) from day 14 to day 18 and from day 16 to day 18 of pregnancy.

Discussion

In the present study, we observed differences in the activity and protein level of both SOD isoenzymes between implantation and post-implantation periods of pregnancy. Specifically, a sharp rise in the activities of SOD1 and SOD2 in CAR and ICAR endometrium was observed at the early conceptus post-implantation period in association with an increase in protein expression of SOD1 and SOD2.

Under physiological conditions, the mitochondria are the major sites of $^{\bullet}O_2^-$ radicals, a class of ROS that act in the control of cell function (Dröge, 2002) or threaten cell survival by their transformation to more highly reactive ROS (Ježek and Hlavatá, 2005). Members of the SOD family have major roles in the first line of antioxidant defence by catalysing the dismutation of $^{\bullet}O_2^-$ radicals. There is substantial evidence that SOD1 and SOD2 play crucial roles in the process of implantation and successful establishment of pregnancy. Defects in conceptus implantation or premature death of the foetuses have been observed in mutant mice lacking SOD1 (Ho et al., 1998). Neonatal lethality (Li et al., 1995) or postnatal development restriction (Lebovitz et al., 1996) has been reported in mutant mice lacking SOD2. The control of $^{\bullet}O_2^-$ radical production by both SOD1 and SOD2 is the first enzymatic defence pathway protecting human endometrial tissue from oxidative stress (Sugino, 2007). Our study suggests that increased activities of SOD1 and SOD2 in sheep CAR and ICAR endometrium during the transition from conceptus implantation to post-implantation periods represent a protective mechanism against oxidative damage during early pregnancy.

There is evidence to suggest that conceptus-derived factors regulate protein expression and enzyme activities of SOD1 and SOD2 during the peri-implantation period. In the human endometrium, SOD2 activity decreases in the late secretory phase of the menstrual cycle, but increases early in pregnancy (Sugino et al., 1996). SOD2 protein was found to be abundant in sheep CAR endometrium during early pregnancy, whereas it declined at the end of the oestrous cycle (Al-Gubory et al., 2014). Taken

together with other studies, our present data suggest that conceptus play a role in the up-regulation of SOD1 and SOD2 protein expression and activities in the endometrium, thus contributing to the establishment of pregnancy. The existence of conceptus-derived proteins that modulate the endometrial free-radical SOD scavenging system early in pregnancy means that the endometrium dynamically responds to the developing conceptus to control the production of $^{\bullet}O_2^-$ radicals before their transformation to highly reactive ROS. Well-designed studies are necessary to further characterise conceptus-derived molecules and to understand mechanisms whereby they exert paracrine action within the endometrium.

There may be an alternative mechanism for the increase of SOD2 protein expression and activities in the endometrium at the time of implantation. The endometrium around implantation is a cytokine-rich environment because a variety of immune cells including macrophages increase at the implantation site and produce cytokines (Tamura et al., 2011). Cytokines induce $^{\bullet}O_2^-$ radical production in the mitochondria. However, SOD2 is immediately induced by cytokines and scavenges $^{\bullet}O_2^-$ radicals in the mitochondria, indicating the protective roles of SOD2 against cytokine-mediated oxidative stress (Karube-Harada et al., 2001; Sugino et al., 2002c). In fact, blockage of SOD2 induction causes cell death due to oxidative stress in human endometrial cells (Sugino et al., 2002c). Therefore, SOD2 works protectively against oxidative stress in the endometrium for successful pregnancy at the time of implantation (Matsuoka et al., 2010).

The toxicity of $^{\bullet}O_2^-$ is based on generation of downstream products of $^{\bullet}O_2^-$, mainly hydroxyl radical ($^{\bullet}OH$) and peroxynitrite ($ONOO^-$). The $^{\bullet}OH$ radicals can be formed in the presence of $^{\bullet}O_2^-$ and H_2O_2 via the Haber-Weiss reaction (Kehrer, 2000). The $^{\bullet}O_2^-$ radicals that escape dismutation by SODs may react with nitric oxide ($NO^{\bullet}$) in a reaction controlled by the rate of diffusion of both radicals to form $ONOO^-$ (Radi et al., 1991). Both the $^{\bullet}OH$ and $ONOO^-$ are highly reactive and toxic ROS (Halliwell and Gutteridge, 2007), which can mediate peroxidation of polyunsaturated fatty acids abundantly present in cell membranes. Therefore, the control of $^{\bullet}O_2^-$ production by SODs is an important component of the first line of defence against membrane lipid peroxidation. The increased activity of

239 cytosolic SOD1 and mitochondrial SOD2 in CAR and ICAR endometrial tissues from day 16 to day
240 18 of pregnancy are likely sufficient to maintain levels of the lipid peroxidation end-product, MDA,
241 relatively stable explaining why their levels were unchanged during the peri-implantation periods
242 (present study). Endometrial cells may use the "SOD switch" reported here during the transition from
243 conceptus implantation to post-implantation periods to control $^{\bullet}O_2^-$ radical production within both the
244 mitochondria and cytoplasm, while avoiding peroxidative damage to mitochondrial and cytoplasmic
245 membranes. Post-implantation metabolism constitutes a critical stage of early pregnancy due to high
246 susceptibility of the developing conceptuses to oxidative damage. Endometrium antioxidant enzyme
247 pathways may play important roles in protecting the implantation conceptus against the deleterious
248 effects of ROS produced during the switch from the pre-implantation anaerobic metabolism to post-
249 implantation aerobic metabolism. There is evidence that defective uterine environment in association
250 with inappropriate expression of ROS-scavenging antioxidant enzymes contributes to early pregnancy
251 failure in domestic ruminates (Ramos et al. 2015). Under physiologically relevant conditions of the
252 sheep endometrium and conceptus development between implantation (day 16) and post-implantation
253 (day 18) periods of pregnancy, CAR and ICAR may respond to oxidative stress by increasing SOD1
254 and SOD2 activities. By the maintenance of adequate •O2− scavenging antioxidant activity, lipid
255 peroxidation can be held in check in the endometrium during the peri-implantation period.
256
257 In conclusion, our data shows for the first time that the early post-implanting conceptus co-ordinately
258 up-regulates SOD1 and SOD2 protein expression and bioactivity within CAR and ICAR
259 endometrium. By maintaining adequate SOD1 and SOD2 antioxidant activity, the conceptus limits
260 lipid peroxidation within the endometrium during the peri-implantation period of pregnancy. The
261 increased protein expression and enzyme activities of SOD1 and SOD2 in sheep CAR and ICAR
262 endometrium during the transition from conceptus pre-implantation to post-implantation period are
263 probably important mechanisms to ensure the establishment of pregnancy. Well-designed studies on
264 regulation of SOD1 and SOD2 proteins might provide important clues to endometrial receptivity and
265 implantation physiology.
266

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Conflict of interest

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

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Author contributions

KHA conceived and designed the study, prepared the animal model, performed tissue collection, acquisition and statistical analysis of data, wrote the manuscript and acted as corresponding author. CG provided reagents and materials and took responsibility for the integrity and the accuracy of the biochemical analysis. PAF contributed reagents and materials and helped in data interpretation and manuscript preparation. NS made critical revisions of the manuscript for important scientific content.

Figure 1. Malondialdehyde (MDA) content in the caruncular (CAR) and intercaruncular (ICAR) endometrial tissues collected at days 14 (P14), 16 (P16) and 18 (P18) of pregnancy. Values are means ± SEM for four ewes per group. The acceptable level of significance was set at $P<0.05$. * = $P<0.05$, ** = $P<0.01$, *** = $P<0.001$.

Figure 2. Enzymatic activities of total SOD or GPX in caruncular (CAR) and intercaruncular (ICAR) endometrial tissues collected at days 14 (P14), 16 (P16) and 18 (P18) of pregnancy. Values are means ± SEM for four ewes per group. The acceptable level of significance was set at $P<0.05$. * = $P<0.05$, ** = $P<0.01$, *** = $P<0.001$.

Figure 3. Enzyme activity (A, B) and protein expression (C,D) of copper-zinc superoxide dismutase (SOD1) and manganese superoxide dismutase (SOD2) in sheep caruncular endometrial tissues collected at days 14 (P14), 16 (P16) and 18 (P18) of pregnancy. In all Western blot there were no changes in alpha tubulin band volumes between groups (E), indicating its validity as a load control. Normalized band volumes are shown as means ± SEM for four ewes per group. The acceptable level of significance was set at $P<0.05$. * = $P<0.05$.

Figure 4. Enzyme activity (A, B) and protein expression (C,D) of copper-zinc superoxide dismutase (SOD1) and manganese superoxide dismutase (SOD2) in sheep intercaruncular collected at days 14 (P14), 16 (P16) and 18 (P18) of pregnancy. In all Western blot, there were no changes in alpha tubulin band volumes between groups (E), indicating its validity as a load control. Normalized band volumes are shown as means ± SEM for four ewes per group. The acceptable level of significance was set at $P<0.05$. * = $P<0.05$, ** = $P<0.01$, *** = $P<0.001$.

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Figure 1

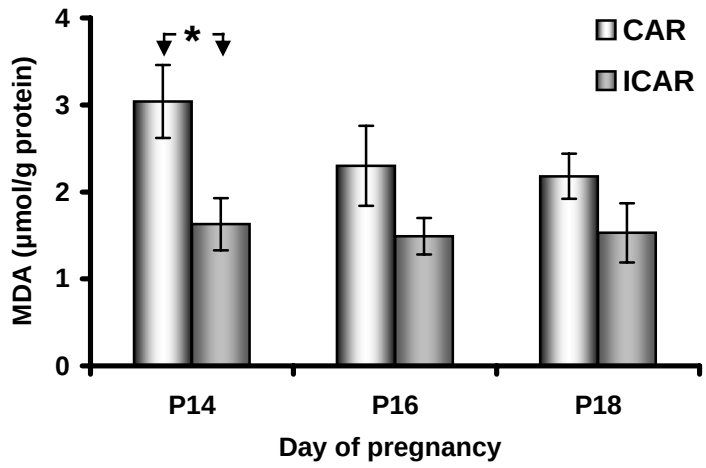

4
3
2
1
0
MDA (µmol/g protein)
*
CAR
ICAR
P14
P16
P18
Day of pregnancy

Figure 2

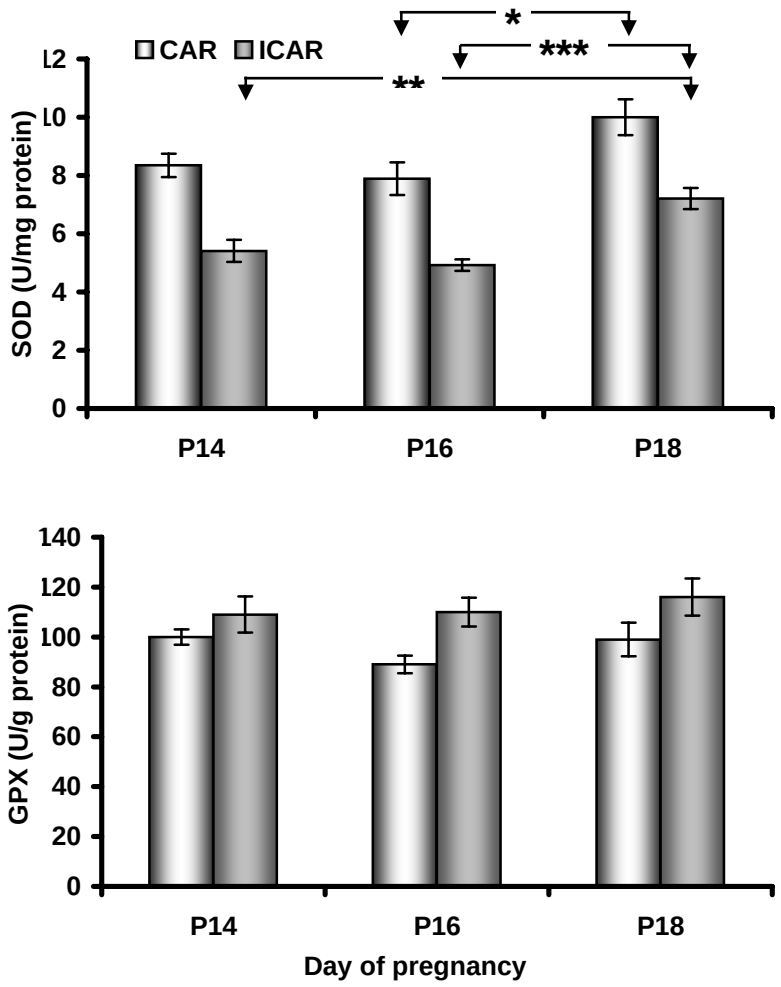

CAR
ICAR
*
***
**
SOD (U/mg protein)
12
10
8
6
4
2
0
P14
P16
P18
GPX (U/g protein)
140
120
100
80
60
40
20
0
P14
P16
P18
Day of pregnancy

Figure 3

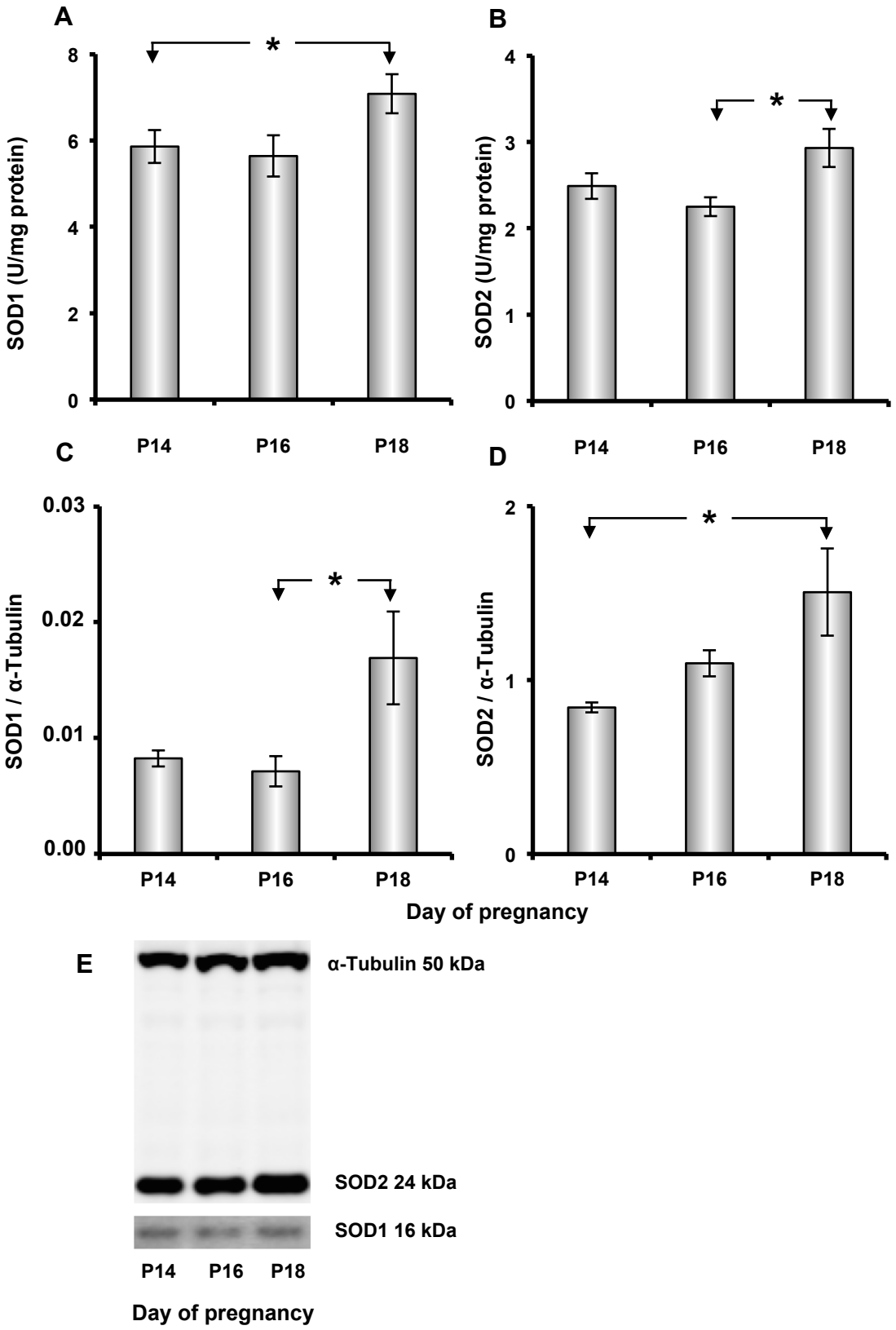
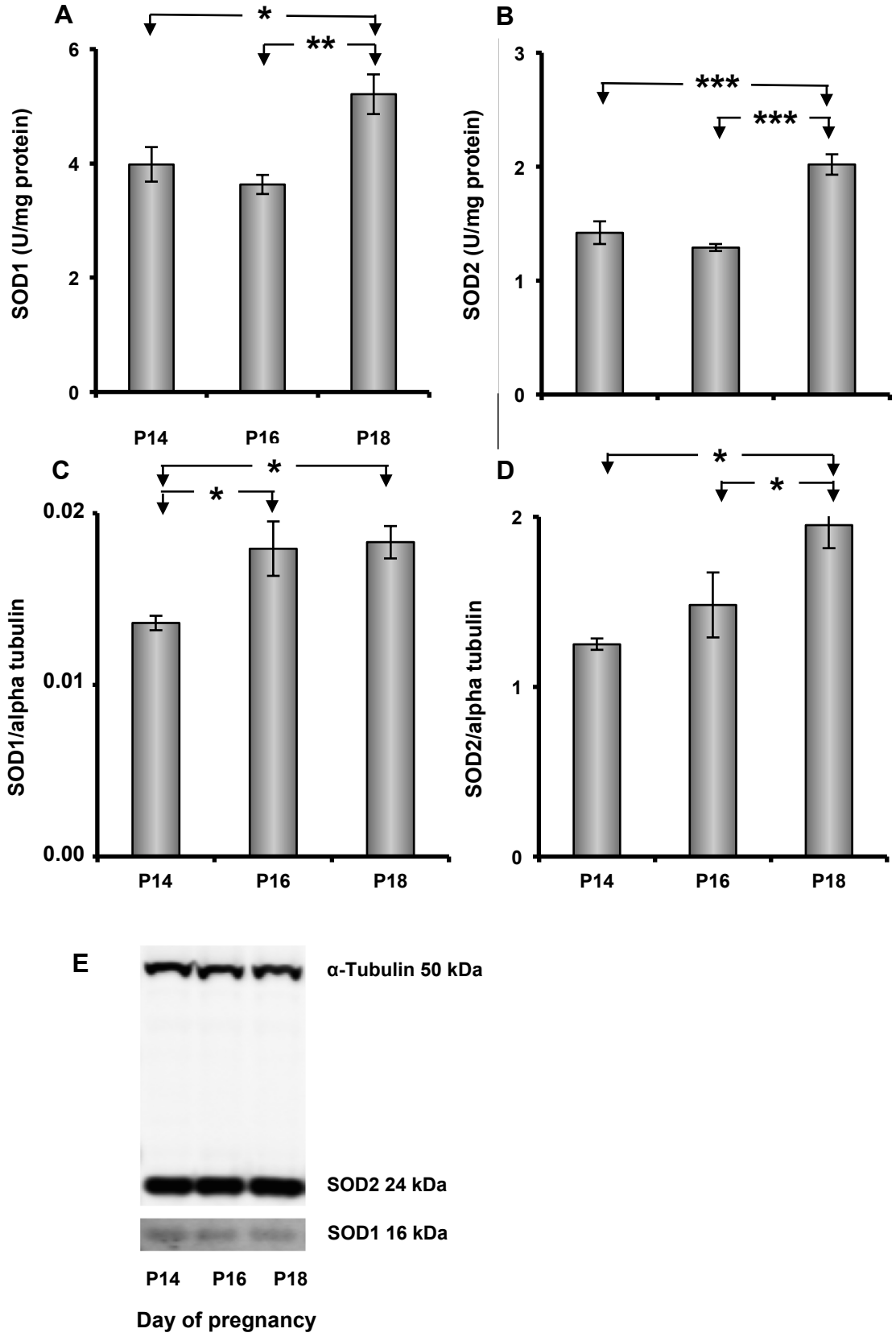

A
B
C
D
E
SOD1 (U/mg protein)
SOD2 (U/mg protein)
SOD1 / α-Tubulin
SOD2 / α-Tubulin
P14
P16
P18
Day of pregnancy
α-Tubulin 50 kDa
SOD2 24 kDa
SOD1 16 kDa
*

Figure 4


A
B
C
D
E
SOD1 (U/mg protein)
SOD2 (U/mg protein)
SOD1/alpha tubulin
SOD2/alpha tubulin
P14
P16
P18
α-Tubulin 50 kDa
SOD2 24 kDa
SOD1 16 kDa
Day of pregnancy