Superoxide dismutase and glutathione peroxidase activities in bovine placenta : spectrophotometric and electrophoretic analysis

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SUMMARY

Reactive oxygen species are unavoidable products of metabolism and in excess they can be dangerous for tissues and cells. Superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px), among others, protect tissues and cells against reactive oxygen species. The aim of the study was the description of SOD and GSH-Px from bovine placenta by use of spectrophotometric and electrophoretic analysis according to time and mode of delivery.

The placental samples were obtained immediately after spontaneous delivery at term as well as preterm and term caesarian section. Animals were divided into 3 groups : A-preterm caesarian section, B-term caesarian section, C-term spontaneous delivery. The activity of examined enzymes was measured spectrophotometrically in maternal and fetal part of placenta and expressed in nkat/g prot for GSH-Px and in Units/g prot for SOD. Results of zymografic electrophoresis were expressed in optical density of bands.

SOD activity was significantly the highest in preterm group (maternal and fetal placenta : 7.27 ± 0.69 U/g, 8.85 ± 0.95 U/g respectively). In term groups the enzyme activity significantly decreased (B - maternal and fetal placenta : 3.93 ± 0.22 U/g, 2.79 ± 0.18 U/g respectively and C - maternal and fetal placenta : 4.48 ± 0.59 U/g and 2.91 ± 0.44 U/g respectively). The molecular weights of enzymes determined by electrophoresis were 35 kDa and 85 kDa for SOD and GSH-Px respectively. GSH-Px activity increased towards parturition and was significantly higher in fetal than in maternal part of placenta in all groups examined (A 114.32 ± 9.15 vs 54.18 ± 6.81 ; B 181.44 ± 12.65 vs 117.39 ± 7.28 ; C 135.11 ± 14.65 vs 87.15 ± 10.25nkat/g).

In conclusion, the activity of SOD and GSH-Px in bovine placenta depended on time and mode of delivery.

KEY-WORDS : bovine placenta - superoxide dismutase - glutathione peroxidase - spectrophotometry - electrophoresis.

RÉSUMÉ

La superoxyde dismutase et la glutathion peroxydase dans le placenta de vache : analyse spectrophotométrique et électrophorétique. Par M. KANKOFER.

Les formes réactives de l'oxygène constituent des produits inévitables du métabolisme, mais en excès, elles sont délétères pour les tissus et les cellules. La superoxyde dismutase et la glutathion peroxydase, entre autres, protègent les tissus et les cellules contre les formes réactives de l'oxygène. L'objectif de ce travail est de mettre en évidence et de mesurer les activités de la superoxyde dismutase et de la glutathion peroxydase du placenta de vache, par analyses spectrophotométriques et électrophorétiques en fonction du temps et du type de part.

Les échantillons de placenta ont été prélevés directement après un vêlage spontané à terme (groupe C) ou réalisé par césarienne (groupe B), et lors d'une césarienne réalisée avant le terme (groupe A). L'activité des enzymes étudiés a été mesurée par spectrophotométrie dans la partie maternelle et foetale du placenta et exprimée en nkat/g de protéine dans le cas de la glutathion peroxydase et en Units/g protéine dans le cas de la superoxyde dismutase. Après électrophorèse les densités optiques des bandes correspondant à une activité enzymatique ont été mesurées.

L'activité de la superoxyde dismutase a été significativement la plus élevée dans les placentas obtenus avant terme (groupe A : partie maternelle 7.27 ± 0.69 U/g et partie foetale 8.85 ± 0.95 U/g). Dans les groupes à terme l'activité de l'enzyme baissait significativement (groupe B : partie maternelle 3.93 ± 0.22 U/g et partie foetale 2.79 ± 0.18 U/g ; groupe C : partie maternelle 4.48 ± 0.59 U/g et partie foetale 2.91 ± 0.44 U/g). L'activité de la glutathion peroxydase augmentait avec la durée de la gestation et était significativement plus élevée dans la partie foetale du placenta que dans la partie maternelle de celui-ci dans tous les groupes considérés (groupe A 114.32 ± 9.15 vs 54.18 ± 6.81 ; groupe B 181.44 ± 12.65 vs 117.39 ± 7.28 ; groupe C 135.11 ± 14.65 vs 87.15 ± 10.25 nkat/g). Les poids moléculaires déterminés par zymographie sont de 35 kDa pour l'activité de la SOD et de 85 kDa pour celle de la GSH-Px. Les densités optiques déterminées à partir des fractions correspondantes ont confirmé les résultats spectrophotométriques. En conclusion, les activités de la superoxyde dismutase et de la glutathion peroxydase dans le placenta des vaches dépend de la proximité du terme et du mode de parturition.

MOTS-CLÉS : placenta - superoxyde dismutase - glutathion peroxydase - vache - spectrophotométrie - électrophorèse.

Introduction

Superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px), belong to the members of enzymatic antioxidative defence mechanisms against reactive oxygen species (ROS), and protect macromolecules, cells and cell membranes from peroxidative damage [10].

SOD catalyses the dismutation of superoxide anion radical into oxygen and hydrogen peroxide, that, in turn, can be removed by GSH-Px and catalase (CAT) [4]. SOD and GSH-Px activities were described in liver, lungs, platelets and erythrocytes [1, 6]. GSH-Px [12] and SOD [13] activities, measured by spectrophotometry, were reported in bovine placental tissues. However, no electrophoretic analysis of these enzymatic proteins from bovine placenta were performed. Zymography, which is based on enzyme specific staining of polyacrylamide gels after electrophoresis, allows for the determination of molecular weight of enzymatic proteins and also supports the spectrophotometric determinations.

The imbalance between production and neutralisation of reactive oxygen species, which may occur when antioxidative system is not efficient enough, leads to peroxidative damage of macromolecules and in consequence the disturbances of metabolic pathways, injury of tissues and clinical symptoms of illnesses.

The changes in steroid hormone [11] and eicosanoid [7] concentrations, which occur shortly before parturition, might be the source of ROS. But these metabolic pathways are also susceptible to peroxidative damage [8, 25] and should be protected from ROS.

The aim of the study was spectrophotometric determination of SOD and GSH-Px activities and partial description of these enzymatic proteins using electrophoretic analysis with respect to time and mode of delivery in bovine placental tissues.

Material and methods

Placentomes were collected from healthy, pregnant cows of White-Black breed immediately after spontaneous delivery via the vagina or during caesarian section before expected term (265-272 days of pregnancy) and at term (282-288 days of pregnancy). The samples were divided into 3 groups according to time and mode of delivery as follows :

A - preterm caesarian section (n = 16)

B - term caesarian section (n = 16)

C - term spontaneous delivery (n = 16)

Placental samples were collected on ice, washed with 0.9 % NaCl, separated manually into maternal and fetal parts, frozen in liquid nitrogen and stored at -70°C until used.

SPECTROPHOTOMETRIC DETERMINATION OF SOD [24]

Samples were homogenized in phosphate buffer (0.1 mol/l, pH 7.4) using an Ultra Turrax T 25 (Ikawerk, Janke and Kunkel Inc., Staufen, Germany) for 5 min at the speed 10 000 rev/min and centrifuged for 20 min at 3000 x g. One ml of homogenate was centrifuged again for 10 min at 15 000 x g. Chloroform:ethanol (3 : 5) (0.8 ml) was added to the supernatant and centrifuged for 10 min at 2 000 x g. The whole procedure was performed at 4°C. Enzyme activity was determined using a spectrophotometric method based on the inhibition of the spontaneous degradation of adrenaline to adrenochrom at pH 10.2 by SOD. The reaction mixture contained 1.8 ml carbonate buffer (0.05 mol/l, pH 10.2), 100 µl of homogenate and 100 µl of adrenaline (18 mg/10 ml 0.1 mol/l HCl, Sigma). The increase in absorbance at 340 nm (Ultrospec 2000, Pharmacia, Sweden) from zero during 10 min was compared with control in which the homogenate was replaced by 0.9 % NaCl. The activity of SOD expressed as the percentage of inhibition of spontaneous degradation of adrenaline and was recalculated into SOD units per protein content. Under conditions stated above 50 % of inhibition was taken as 1 U SOD. Intra-assay 8.8 % (n = 10) and inter-assay 9.0 % (n = 10) coefficients of variation were established.

SPECTROPHOTOMETRIC DETERMINATION OF GSH-PX [20]

Samples were homogenized in phosphate buffer as mentioned above and centrifuged for 20 min at 3000 x g. The whole procedure was performed at 4°C. The reaction mixture contained 100 µl of NADPH solution (8.4 mmol/l, Sigma), 10 µl glutathione reductase (GSSG-R, 100 U/mg protein/ml, Sigma), 10 µl sodium azide (1.125 mol/l, Sigma), 100 µl glutathione (0.02 mol/l, GSH, Sigma) filled with phosphate buffer (0.05 mol/l, pH 7.0) up to the volume of 2.8 ml and 100 µl of supernatant. The enzymatic reaction was initiated by addition of 100 µl of 0.022 mol/l H_2O_2. The rate of GSSG formation was measured by the following decrease in absorbance of the mixture at 340 nm, as NADPH was converted to $NADP^+$, between 2^{nd} and 4^{th} minute after initiation of the reaction, using a cuvette with a 1 cm light path. The activity was expressed as nanokatals (nkat) per protein content. Intra-assay 8.1 % (n = 10) and inter-assay 8.7 % (n = 10) coefficients of variation were established.

ELECTROPHORETIC ANALYSIS

Homogenates of maternal and fetal part of placenta were subjected to polyacrylamide gel electrophoresis [5] using 10 % gels and non-reducing conditions (Mighty Small SE 250, Hoefer, Pharmacia, Sweden). The running buffer was 0.025 mol/l Tris-0.192 mol/l glycine, pH 8.3. The amount of protein loaded at each line was the same (20 µg) and a constant voltage of 100 V was applied. The samples were mixed with 2x sample buffer without reducing agent (4 % SDS, 20 % glycerol, 0.125 mol/l Tris/HCl, 0.01 % bromophenol blue, pH 6.8). After electrophoresis, the gels were washed in 2.5 % Triton X-100 for 15 min. Then washed again for 15 min in distilled water. For the determination of SOD activity [19], the gels were incubated in solution containing 2 mmol dianisidine, 0.1 mmol riboflavin, 10 mmol potassium phosphate buffer at pH 7.2 for 1 h at room temperature. Then, after rinse with water, the gels were illuminated for 10 min. Brown bands against pale yellow background were conside-

red as SOD. For the determination of GSH-Px [19] the gels were stained in solution containing 2 mmol dianisidine, 10 mmol potassium phosphate buffer at pH 7.2 for 1 h followed by a 15 min incubation in 0.1 mmol H_2O_2. Brown bands against pale yellow background, but localised at different positions, were considered as GSH-Px. During one experiment, two gels were electrophoresed parallely. The first contained homogenates and molecular weight standards : myosin (200 kDa), β galactosidase (116 kDa), phosphorylase (97 kDa), serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa) and was stained with Coomasie Brillant Blue. The second gel containing one homogenate sample inactivated by heating was stained for enzyme activity. The inhibition studies with cyanide were performed using 1.2 mM/l KCN. The gels were scanned (Calibrating Imagining Densitometer GS-710, BioRad) and analysed using computer programme Quantity One (BioRad). Optical density and molecular weight of bands were measured.

PROTEIN CONTENT DETERMINATION

The protein content of supernatants was determined using Lowry's method [16] and bovine serum albumin as standard.

STATISTICAL EVALUATION

Paired observations of enzyme activity and protein content were averaged and compared using ANOVA and multiple range t-Tukey test.

Results

The results of spectrophotometric determinations and semiquantitative electrophoretic analysis are presented in table I.

SOD activity was the highest in preterm caesarian section group. In this group, the activity was significantly ($p < 0.05$) lower in maternal than in fetal part of placenta. In groups B and C the activity decreased in comparison with group A and was significantly ($p < 0.05$) lower in fetal than in maternal part. The lowest values were observed in group B (term caesarian section) but they did not differ significantly between respective tissues in group C (spontaneous delivery).

GSH-Px activity was the highest in group B. The activity was significantly ($p < 0.05$) higher in fetal than in maternal part in all groups examined. The activity of enzyme increased towards parturition - the differences, observed between groups before term (A) and at term (B and C) within the same tissue were statistically significant.

The localisation of enzymatic bands was compared with protein molecular weight standards. The fraction of SOD activity refered to molecular weight of about 35 kDa and GSH-Px activity of about 85 kDa. Because of the light colours of gels and the lack of a good contrast, only GSH-Px activity is presented as the example (Figure 1). The gels incubated with presence of KCN showed no activity of SOD. The lines where inactivated homogenate was loaded also did not show any visible bands of enzyme activity.

Groups	SOD		GSH-Px	
	U/g prot (mean±SD)	OD	nkat/g prot (mean±SD)	OD
A-Maternal	7.27 (0.69) [a]	0.22	54.18 (6.81) [a]	0.04
A- Fetal	8.85 (0.95) [b]	0.24	114.32 (9.15) [b]	0.09
B-Maternal	3.93 (0.22) [c]	0.10	117.39 (7.28) [b]	0.10
B- Fetal	2.79 (0.18) [d]	0.09	181.44 (12.65) [c]	0.19
C-Maternal	4.48 (0.59) [c]	0.12	87.15 (10.25) [d]	0.07
C – Fetal	2.91 (0.44) [d]	0.06	135.11 (14.65) [e]	0.15

Legend : A - Preterm caesarian section group (n = 16), B - term caesarian section group (n = 16), C - term spontaneous delivery group (n = 16) ; values with different superscripts are significantly different from each other at $p < 0.05$; OD - optical density of bands : for GSH-Px values measured in gel presented in figure 1, for SOD values measured in gel electrophoresed paralelly with mentioned above.

TABLE I. — The activities of SOD and GSH-Px in bovine placental tissues (spectrophotometric and electrophoretic results).

Discussion

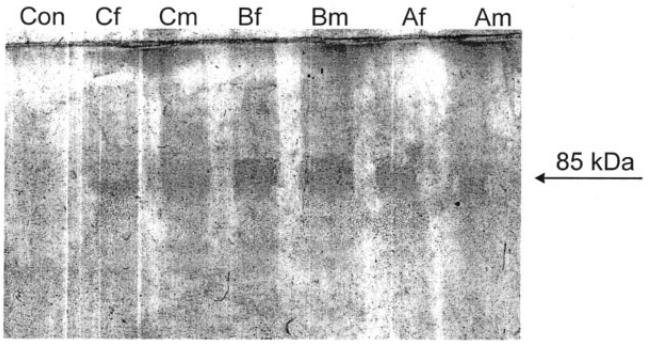
The goal of antioxidative defence systems is to remove ROS in order to maintain the balance between their production and neutralisation [18]. The mechanism of action of these systems, however, differs from each other and is based on enzymatic reactions as well as non-enzymatic systems.

SOD is a very stable enzyme which exists in mammalian organisms in three forms. Cytoplasmic form (SOD-1) is a homodimer with a molecular weight of about 32 kDa. It contains Cu and Zn and is susceptible to cyanide as well as hydrogen peroxide inhibition [21]. Mitochondrial form (SOD-2) is a tetramer with a molecular weight of about 80 kDa and contains Mn. Extracellular form (EC-SOD) is also a tetramer containing Cu, Zn as well as sugar residues [17]. The mechanism of SOD action is based on redox reactions of Cu ions and superoxide anion radicals leading to production of oxygen and hydrogen peroxide [2].

Hydrogen peroxide is catabolised by GSH-Px and CAT [4]. GSH-Px is a tetramer with a molecular weight of about 84 kDa and contains Se atoms which are indispensable for its catalytic activity. The mechanism of GSH-Px action is based on redox abilities of thiol groups of glutathione and the reduction of not only hydrogen peroxide, but also organic peroxides such as : lipid peroxides and probably protein peroxides [26]. GSH-Px can reduce only free peroxides. That is the reason why its activity depends on the activity of phospholipase A_2 which liberates fatty acids residues from phospholipids.

The importance of GSH-Px is also connected with its participation in arachidonic acid cascade and a possible indirect influence on prostaglandin levels [9].

Electrophoretic patterns in non-reducing conditions performed in this study, confirmed the presence of bands related to SOD and GSH-Px activities in placental tissues. Molecular weight of enzymes, determined in this study, corresponded to previous studies. Moreover, SOD found in bovine placenta tissues would be the cytoplasmic Cu/Zn dependent form



Lanes : Am - sample of maternal part of group A
Af - sample of fetal part of group A
Bm - sample of maternal part of group B
Bf - sample of fetal part of group B
Cm - sample of maternal part of group C
Cf - sample of fetal part of group C
Con - sample inactivated by heating

FIGURE 1. — Electrophoretic pattern of GSH-Px activity in bovine placental tissues.

(SOD-1). The positive staining was based on SOD's acceleration of photooxidation of dianisidine, sensitised by riboflavin. GSH-Px could also be stained by this procedure due to the photochemical production of H_2O_2 [19]. As a result, the sites where enzymatic proteins were localised were darker than the background. The intensity of bands, which reflected enzyme activity, supported spectrophotometric results. The comparison of spectrophotometric results of both enzymes examined showed generally a negative relationship. SOD activity decreased towards parturition while GSH-Px activity increased at the same time. This non-uniform pattern may be due to different mechanisms of action of SOD and GSH-Px where GSH-Px removes the product of SOD catalytic action.

GSH-Px activity increased towards parturition. Previous studies using analogous samples have evidenced that glutathione and other thiol compound concentrations were enhanced in the vinicity of parturition [14, 15].

The experiments of BRZEZIŃSKA-ŚLEBODZIŃSKA *et al.* [3] on red blood cell GSH-Px activity and glutathione concentration showed an increase of these 2 parameters during last two weeks before parturition.

During the periparturient period, changes in hormone concentrations, essential for luteolysis and parturition, can be susceptible to ROS imbalance. Intensive steroid metabolism leading to the increase of estrogen production and the decrease of progesterone quantity and prostaglandin metabolism might be sources of ROS. On the other hand, these metabolic ways could be disturbed by an uncontrolled ROS increase. Particularly, ROS could inhibit the activity of enzymes involved in steroid [23] and arachidonic acid cascade [22]. Efficient antioxidative systems are able to protect against such disturbances and can control ROS accumulation.

In conclusion, the presence of SOD and GSH-Px activity in bovine placenta was confirmed by two different analytical methods. The activity of examined enzymes depended, although in a non-uniform pattern, on time and mode of delivery. It may suggest that the periparturient period is protected from peroxidative damage using different enzymatic mechanisms of action.

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