Changes in the glucose-6-phosphate dehydrogenase activity in granulosa cells during follicular atresia in ewes

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Abstract

Apoptosis of granulosa cells during follicular atresia is preceded by oxidative stress, partly due to a drop in the antioxidant glutathione (GSH). Under oxidative stress, GSH regeneration is dependent on the adequate supply of NADPH by glucose-6-phosphate dehydrogenase (G6PD). In this study, we analyzed the changes of G6PD, GSH, and oxidative stress of granulosa cells and follicular liquid and its association with apoptosis during atresia of small (4–6 mm) and large (> 6 mm) sheep antral follicles. G6PD activity was found to be higher in granulosa cells of healthy small rather than large follicles, with similar GSH concentration in both cases. During atresia, increased apoptosis and protein oxidation, as well as a drop in GSH levels, were observed in follicles of both sizes. Furthermore, the activity of G6PD decreased in atretic small follicles, but not in large ones. GSH decreased and protein oxidation increased in follicular fluid. This was dependent on the degree of atresia, whereas the changes in G6PD activity were based on the type of follicle.

Introduction

The development of morphologically and steroidogenically mature follicles depends on granulosa cell survival (Hsueh et al. 1994, 1996). Although hormonal stimuli, intracellular signals, and metabolic processes are determinants of the death of these cells, the inducing factor involved in follicular atresia by way of apoptosis is still unknown (Tilly et al. 1992). Apparently, the intracellular redox state is a critical factor affecting the apoptosis cascade by altering the equilibrium between cell survival signals and the progression to death (Jolly et al. 1997).

Reactive oxygen species (ROS) play a key role in intracellular signaling, and recent evidence has suggested their participation in apoptosis and follicular atresia (Jolly et al. 1997). Electron transport associated with steroidogenesis, in addition to mitochondrial metabolism, constitutes important pathways for the production of free radicals in steroidogenically active cells, such as granulosa cells of antral follicles (Rapoport et al. 1995, Hsueh et al. 1996, Tilly 1996), in spite of the fact that these radicals are efficiently neutralized by antioxidant molecules (Aten et al. 1992). Under certain stimuli, this equilibrium can be disrupted, leading to a state of oxidative stress. Hence, it has been proposed that inadequate protection against ROS constitutes a trigger for follicular atresia. Therefore, the survival of follicles subjected to gonadotropin stimulation is related to an increase in the expression of antioxidant enzymes (Tilly & Tilly 1995), while treatment with antioxidants blocks granulosa cell apoptosis in cultured follicles (Tilly 1996). Among the most noteworthy deleterious effects of ROS on follicular cells is the inhibition of induction of steroidogenesis by gonadotrophic hormone with the loss of follicular function (Margolin et al. 1990).
Changes in the homeostasis of the reduced form of glutathione (GSH) and in the intracellular redox equilibrium constitute some of the first events in apoptosis (Lu 1999). GSH contributes to antioxidant metabolism through its action, either as a proton donor or as a cofactor for nucleophilic conjugations. It has been demonstrated that it detoxifies a wide range of oxygen and nitrogen reactive species. The antiapoptotic effect of FSH is mediated in part by increased synthesis of GSH, resulting in a decrease in ROS production and survival of granulosa cells in cultured preovulatory follicles (Tsai-Turton & Luderer 2006). Alterations in the reduced/oxidized GSH (GSH/GSSG) ratio precede the induction of the apoptotic signal in the mitochondrion and fragmentation of chromatin, coinciding with an increase in caspase-3 expression. Restoration of the GSH/GSSG equilibrium with the administration of niacinamide and/or GSH inhibits apoptosis (Lu 1999, Pias 2002). GSH participates in diverse processes related to oocyte maturation (Nasr-Esfahani & Johnson 1992), follicular growth and development (Agarwal et al. 2003), and its concentration is dependent on the day of the estrous cycle (Kim et al. 2007).

The antioxidant activity of GSH depends on its reduced form, which is regenerated from GSSG through the action of glutathione reductase. In this context, glucose-6-phosphate dehydrogenase (G6PD) has been proposed as a cell enzyme aiding in survival against oxidative stress by generating NADPH required for GSH regeneration (Leopold & Loscalzo 2000, Filosa et al. 2003, Jiang et al. 2003, Díaz-Flores et al. 2006). G6PD activity increases in growing cells and prevents cell death during oxidative stress (Tian et al. 1998, 1999); hence, its inhibition brings about a drop in NADPH levels, a decrease in cell growth (Endo et al. 1993), and an exacerbation of damage caused by free radicals (Filosa et al. 2003). Likewise, it has been demonstrated that an increase in its expression provides better protection against cell death caused by H₂O₂, through the increase in GSH (Salvemini et al. 1999, Filosa et al. 2003). The aforementioned information led us to suppose that a decrease in G6PD activity must be associated with the start and progression of apoptosis in follicular atresia. Thus, the present study is aimed at determining the temporal relationship between G6PD activity, GSH concentration, protein oxidation levels, and the degree of apoptosis in granulosa cells during the atresia of antral follicles of two different sizes.

Results

Apoptosis frequency and oxidative stress in atretic follicles

The results show that the degree of atresia is directly proportional to the percentage of apoptotic cells and inversely proportional to viable cells. Figure 1A depicts the results of the cytometric analysis. Cells that initiated apoptosis are shown in the right lower quadrant (Annexin-V-FITC+/PI−), while those in the upper right quadrant correspond to late apoptosis (Annexin-V-FITC+/PI+). In both types of follicle, a progressive increase in the apoptosis index of granulosa cells can be observed with respect to the degree of atresia (Fig. 1B). The highest percentage of apoptotic cells was found in atresia 3 versus atresia 1 and 2; *P<0.001 when comparing atresia 3 versus atresia 1 and 2; **P<0.01 when comparing atresia 1 versus atresia 2. (C) Carbonyl groups exposed. Determination of dinitrophenylhydrazones on proteins oxidized. *P<0.001 when comparing atresia 3 versus atresia 1 and 2; **P<0.05 when comparing atresia 1 versus atresia 2.
Figure 1C shows that protein oxidation was significantly higher in large atretic follicles 2 and 3 versus large healthy follicles (P<0.001). In small follicles, a 3.3-fold increase was observed in atresia 3, and 2.4-times in atresia 2 (P<0.001) with respect to atresia 1, although the difference between atresia 1 and 2 follicles was not significant. Atretic large follicles depicted a higher oxidative damage than smaller follicles (5.7±1.8 vs 3.7±0.5 μmol/mg protein respectively).

**G6PD activity and GSH concentration in granulosa cells of atretic follicles**

G6PD activity in healthy small follicles (atresia 1) was statistically higher (P<0.001), markedly dropping (1.8 times) in atresia 2, and even more in atresia 3 (2.8 times). This decrease paralleled the GSH drop associated with the progression of atresia (23.9±4.2, 9.5±2.7, and 6.8±1.3 μmol/mg protein, atresia 1, 2, and 3 respectively). However, no significant differences were observed in G6PD activity in the large follicles at different degrees of atresia, although this enzyme did have a tendency to decrease in granulosa cells dependent on the degree of atresia, being more evident in atresia 3 (1.3 times; Fig. 2A). On the other hand, GSH concentrations in these follicles decreased in a much larger proportion: 1.9- and 2.8-fold in atresia 2 and 3 respectively as compared with atresia 1 (P<0.001; Fig. 2B).

**Steroid hormones**

Figure 3 depicts that a high and statistically significant increase in DHEA concentrations occurs only in the fluid of atresia 3 follicles. A 2.3-fold increase was observed in atresia 3 with respect to atresia 2, and 2.9-times versus atresia 1 in large follicles (P<0.01). A similar pattern was observed in small follicles.

**G6PD activity, GSH concentration, and carbonyl groups in the liquid of atretic follicles**

Specific activity of G6PD and GSH concentrations in the atretic follicular fluid were lower than those found in granulosa cells (already of 10- and 5-times respectively) in all kinds of follicles. Figure 4B depicts G6PD activity in the fluid of large follicles, which is 1.1- and 1.6-times lower in atresia 2 and 3 respectively, than in atresia 1, but without significant differences. The follicular liquid of healthy small follicles (atresia 1) possesses higher G6PD activity than that of large follicles, which dropped significantly (P<0.001) only in atretic small follicles. In parallel, GSH concentrations also dropped sharply (1.4-fold in atresia 2 and 2.2-fold in atresia 3; Fig. 4C) in both large and small follicles. Figure 4A shows a significant increase in the degree of protein oxidation (0.6±0.1, 1.9±0.4, 3.4±0.8 μmol/mg protein in atresia 1, 2, and 3 respectively) in large follicles, with no differences found between follicular sizes.

**Discussion**

In this study, we used sheep antral follicles of two different sizes – small (4–6 mm) and large follicles (>6 mm) – to assess changes in G6PD, GSH, and oxidative stress associated to the increase in apoptosis of granulosa cells during follicular atresia. The percentage of apoptotic granulosa cells and the degree of oxidative stress increased during progression toward atresia, in addition to a decrease in GSH concentration. G6PD-specific

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**Figure 2** G6PD activity and GSH concentration in granulosa cells from differently sized atretic follicles. (A) G6PD activity. (B) GSH concentration. *P<0.001 when comparing atresia 1 versus atresia 2 and 3. **P<0.05 when comparing atresia 2 versus atresia 3. The graph presents the mean±s.d.

**Figure 3** DHEA concentration in the liquid of differently sized atretic follicles. *Means significant differences in atresia 3 follicles versus atresia 1 and 2 (P<0.01). Bars represent mean±s.d.
activity is dependent on follicular size with higher activity occurring in small healthy follicles than that in the non-atretic large ones. In the small follicles, activity dropped concomitantly with progression toward follicular atresia, whereas, in the large follicles, no significant changes were seen during atresia. These results indicate that the antioxidant mechanisms of the small follicles are more efficient since their potential to produce NADPH is greater than that in large follicles. It also suggests that a decrease in G6PD activity and GSH depletion are involved in the induction of increased oxidative stress leading to apoptosis during small follicle atresia.

We observed that oxidative stress prevails in follicular atresia, as manifested by the higher oxidation of proteins due to the free radical effects, in addition to an increase in the apoptosis index (Fig. 1B and C). These results demonstrate that the oxidizing environment in the atretic follicles contributes to the damage of macromolecules and is associated to the apoptotic cascade in granulosa cells. Therefore, activation of the antioxidant system becomes relevant for cell survival and prevention of apoptosis. In this context, GSH plays a pivotal role as a protector against ROS (Agarwal et al. 2003), particularly in the ovary where its depletion increases antral follicular atresia in rats (Tsai-Turton & Luderer 2006), and in the apoptosis of granulosa cells induced by toxicants (Tsai-Turton et al. 2007a, 2007b). In this study, GSH concentrations in granulosa cells and in the follicular liquid were inversely related to the severity of the atresia (Figs 2B and 4C).

The difference in G6PD-specific activity in the small and large follicles, observed in this study, constitutes a relevant finding. Small follicle granulosa cells revealed a higher activity of this enzyme (almost twice as high) than large follicles, and only in the small follicles did G6PD activity decrease concomitantly with GSH depletion, dependent on the degree of atresia. These results point to the relevance of this enzyme in cellular proliferation, steroidogenesis, and the antioxidant protection of small follicles (Fig. 2A), and suggests that in these follicles, the increase in oxidative stress and the decrease in GSH concentrations associated with atresia are in part a consequence of reduced NADPH availability due to the drop in G6PD activity.

G6PD as a supplier of NADPH is indispensable for replenishing GSH in oxidative stress conditions of diverse cells (Leopold & Loscalzo 2000, Filosa et al. 2003, Jiang et al. 2003). The over-expression of G6PD protects against H2O2-induced cellular death through GSH production (Salvemini et al. 1999) and, by contrast, the deficiency of G6PD increases susceptibility to oxidative stress and apoptosis (Pandolfil et al. 1995, Filosa et al. 2003, Jiang et al. 2003). This indicates that cells with high G6PD activity respond efficiently to NADPH requirements for maintaining cellular redox states, antioxidant systems, and cell survival.

The lower activity of G6PD in large follicles could be due to the lower rate of cell proliferation of these follicles in relation to small follicles (Jablonka-Shariff et al. 1994, 1996). Also, this could indicate a lower capacity of large follicles to respond to oxidative stress and explains the higher degree of protein oxidation observed during atresia of this type of follicles (Jablonka-Shariff et al. 1994). Our results are consistent with the regulation of GSH concentration by de novo synthesis, as well as by NADPH-dependent recycling under oxidative stress conditions, since G6PD activity in large atretic follicles did not show concomitant changes associated to the decrease in GSH concentration.
NADPH can be supplied by malate dehydrogenase and isocitrate dehydrogenase, which in rat ovary depict an activity equal or higher to that shown by other tissues (Flint & Denton 1970). More studies are required for establishing the relevance of these enzymes in the response to oxidative stress in granulosa cells.

On the other hand, it is known that the antiapoptotic effect of FSH in cultured follicles (Tilly & Tilly 1995) is partially due to its stimulatory effect on the de novo synthesis of GSH (Tsai-Turton & Luderer 2006). Glutamate cysteine ligase (GCL), which catalyzes γ-glutamylcysteine synthesis, is needed for the de novo synthesis of GSH and expressed in the granulosa cells of healthy follicles. Its expression is stimulated by gonadotropin treatment (Tsai-Turton & Luderer 2005), whereas atretic follicles are devoid of GCL (Luderer et al. 2003). Hence, the increase in GCL levels can increase GSH levels in healthy preovulatory follicles.

The decrease in G6PD activity during atresia in small follicles can be explained either by a decrease in the enzyme’s concentration, oxidative modifications (Oliver et al. 1987), or by the increase in one of its inhibitors, DHEA (Tian et al. 1998). In this study, we observed an increase in the concentration of oxidized proteins of granulosa cells and follicular liquid. It is thought that G6PD could be part of these oxidized proteins (Figs 1C and 4A). Treatment of G6PD with an in vitro oxidizing system or with secondary products of lipid oxidation, such as hydroxynonenal, induces oxidative changes in this enzyme that increases its heat instability and leads to the loss of its biological activity (Tsai-Turton & Luderer 2006). Estrogen concentration in non-atretic follicles depends on different degrees of proliferation and sensitivity to oxidative stress. Furthermore, this indicates that GSH concentration in non-atretic follicles depends on additional factors in addition to G6PD activity, such as de novo synthesis of GSH or activity of other NADPH-generating enzymes. The lower G6PD activity in the large follicles was associated to a greater susceptibility to oxidative stress, which produced in them a higher degree of protein oxidation during follicular atresia. The induction of apoptosis of granulosa cells during atresia in small follicles is associated to GSH depletion probably due to a low NADPH supply caused by a decrease in G6PD activity.

### Materials and Methods

#### Reagents

Annexin-V-Fluos was obtained from Roche Applied Science and dichlorofluorescein-diacetate from Molecular Probes (Leiden, The Netherlands), Coat-A-count kits for estradiol (E_2) and progesterone (P_4) quantification were purchased from Diagnostic Products Corporation (Los Angeles, CA, USA), and DHEA RIA DSL 9000 active was acquired from Diagnostic Systems Laboratories (Webster, TX, USA). GSH, glucose-6-phosphate, NADP, and other analytical grade reagents were purchased from Sigma Chemical Co.

#### Procurement and classification of follicles

Ovaries from non-gestating sheep were obtained from a local slaughterhouse and transported to the laboratory (transport time never exceeded 60 min) in 0.01 M PBS (pH 7.4) at 4 °C. Once the antral follicles were obtained, they were separated by size into two groups (small <6 mm) and large (>6 mm) follicles, were washed twice in cold PBS, and classified under a stereomicroscope (magnification 120×), dependent on the degree of atresia into one of three groups: atresia 1) healthy follicles abundantly vascularized, of brilliant appearance, and without the apparent detachment of the granulosa layer; atresia 2) follicles with a slight decrease in vascularization and with small detachments of the granulosa layer and atresia 3) follicles with a marked decrease in vascularization and abundant detachments of the granulosa layer and of grayish appearance (Rosales-Torres et al. 2000). To corroborate the morphological criteria for the classification of follicular atresia, E_2 and P_4 concentrations were determined in the cell-free follicular liquid.
Sample processing

After classification, the follicular liquid was extracted using an insulin syringe (25×16 mm needle), and immediately centrifuged at 600 g for 10 min at 4 °C to separate the granulosa cells. Follicles were opened to recover the granulosa cells adhered to the walls (Ballesteros et al. 1992). After washing the granulosa cells with PBS, viability was determined with Trypan blue (follicles that showed percentage viability of granulosa cells higher than 90% were included), and the apoptosis index was measured using flow cytometry. Remnant cells were homogenized (glass-Teflon) in 250 μl of PBS (pH 7.4) and centrifuged at 20 000 g for 10 min at 4 °C in order to separate the cell debris from the supernatant. Protein and GSH concentration, G6PD activity, and carbonyl groups levels in proteins were determined on the same day. The cell-free follicular liquid (obtained by centrifugation at 12 000 g for 20 min) was subjected to a similar process and a fraction was stored at −70 °C for E2, P4 and DHEA quantification.

Apoptosis index

Cells were washed twice in cold PBS by centrifugation at 250 g for 5 min, and were resuspended with 0.1 ml of the staining solution: 10 μl of Annexin-V-Fluos (10 μg/ml; conjugated with FITC) and 10 μl of propidium iodide (PI, 50 μg/ml) in 1 ml HEPES buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 5 mM CaCl2) and incubated for 15 min in the absence of light. Afterward, 0.4 ml of the HEPES buffer was added, proceeding then with the flow cytometry analysis (BD FACSAria; Becton Dickinson System & Co., San Jose´, C A, USA). We analyzed 30 000 cells per follicle. Fluorescence emission was measured at 515 nm for FITC and at 610 nm for PI, with excitation at 488 nm. Data were analyzed using the FACSDIVA program for flow cytometry. Simultaneous staining with Annexin-V-FITC and PI highlighted discriminate cells with more severe plasmatic membrane alterations having undergone late apoptosis or necrosis. The sum of Annexin-V-FITC+/PI− and Annexin-V-FITC+/PI+ yields the total apoptosis index.

GSH concentration

Fractions of follicular liquid and granulosa cells were supplemented with an equal volume of 5% (v/v) metaphosphoric acid, and centrifuged at 3000 g for 10 min at 4 °C. GSH concentration was measured in the supernatant using a GSH assay Kit (Calbiochem, San Diego, CA, USA).

Enzyme activity assay

G6PD activity was measured by quantifying NADPH production in the follicular liquid and granulosa cell homogenate. After centrifugation for 30 min at 15 000 g, the supernatants were collected and G6PD activity was analyzed spectrophotometrically (Rudack et al. 1971). Proteins were quantified using the Lowry method (Lowry et al. 1951) with BSA as standard. Enzyme activity was expressed as mU per mg protein per minute under assay conditions. 1 mU equals 1 nmol of NADPH produced per minute.

Protein carbonyl groups

Proteins were precipitated using 20% (w/v) trichloroacetic acid (TCA), centrifuged at 500 g for 10 min at 4 °C, washed thrice with 5% (w/v) TCA, and thrice with chloroform. The precipitate was resuspended in 1 ml of 2% (w/v) 2,4-dinitrophenylhydrazine in 200 mM HCl and incubated at 37 °C for 60 min with stirring at an interval of 5 min. Afterward, proteins were precipitated with 20% (w/v) TCA, centrifuged, and washed with 5% (w/v) TCA and finally washed with ethanol/ethyl acetate (1/1 v/v). The pellets were dissolved in 6 M guanidine-HCl, incubated for 10 min at 37 °C. Absorbance was measured at 370 nm to detect dinitrophenylhydrazone formation. Molar extinction coefficient for DPNH (E=22 000/M−1 per cm) was used for calculating carbonyl concentration (Halliwell & Gutteridge 1992).

Steroid hormone determination

Concentrations of E2, P4, and DHEA were measured by radioimmunoanalysis, using the Coat-A-count E2, Coat-A-count P4, and DHEA RIA DSL 9000 active kits, following the manufacturer’s instructions. The intra-assay CVs were 5.07, 2.89, and 3.05%, while the inter-assay CVs were 4.9, 5.9, and 10.8% for E2, P4, and DHEA respectively.

The E2/P4 ratio in healthy (atresia 1) follicles was > 1 and < 1 in atretic follicles (data not shown). These results agree with previously reported data (Burke et al. 2005) and support the morphological classification of follicles.

Statistical analysis

Two or three follicles of each size were assessed on every fourth day. All the determinations were done in duplicate. Data were analyzed by two-way ANOVA using a general linear model with the SAS software (SAS User’s Guide, 197; SAS Institute Inc., Cary, NC, USA). Statistically significant differences among groups were determined applying Tukey’s test. Results are expressed as mean±S.D. P<0.05 was considered statistically significant.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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