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Synthesis of Ascorbate and Urate in the Ovary of Water Buffalo

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Blood flow interruption is associated with oxygen depletion and loss of factors for function and survival in downstream tissues or cells. Hypoxia and absence of gonadotropins trigger apoptosis and atresia in the ovary. We studied the antioxidant response of follicular cells to plasma deprivation in ovaries dissected from water buffalo. Aliquots of follicular fluid were aspirated from each antral follicle, before and during incubation of the ovaries at 39 °C. Urate, ascorbate, retinol and α-tocopherol in the fluid were, titrated by High Performance Liquid Chromatography (HPLC) with spectrophotometric or spectrofluorimetric detection. The total antioxidant capacity of follicular fluid was determined as absorbance decrease, following addition of a source of radical chromophores. The more the incubation progressed, the higher levels of urate, ascorbate and total antioxidant capacity were found. Conversely, changes in concentration of the liposoluble antioxidants were not observed. Ascorbate synthesizing activity in the follicle was demonstrated by detecting the enzyme L-gulono-γ-lactone oxidase in microsomes prepared from granulosa cells. These cells were also analyzed for the expression of the enzyme CPP32. The enzyme level, measured as DEVD-*p*-nitroanilide cleaving activity, was found related with the immunoreactivity to anti-CPP32 antibodies. Negative correlation between the enzyme activity (which is known to be induced by peroxynitrite) and the follicular level of urate (which scavenges peroxynitrite) was also observed. The amount of nitrotyrosine, a product of peroxynitrite attack on proteins, was measured in follicular fluids by Enzyme Linked ImmunoSorbent Assay (ELISA). This amount was found positively correlated with the CPP32 activity, and negatively correlated with the urate level in follicular fluid. Alterations in concentrations of ascorbate or urate may be associated with oxidative stress during follicular atresia.

Keywords: Follicular fluid, granulosa cells, L-gulono-γ-lactone oxidase, nitrotyrosine

INTRODUCTION

The developmental potential of the human oocyte was suggested to be related to the oxygen level in follicular fluid.[1] Most of the follicles do not develop to the ovulatory status, but undergo a program of biochemical and morphological changes leading to programmed cell death

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(apoptosis) and massive destruction of their structure (atresia).[2–4] This process is regulated by a cascade activation of cytosolic proteases, called caspases.[5] Activation of CPP32 (also known as Caspase-3) is a key step in execution of apoptosis.[6–8] It is promoted by different events including oxidative stress by peroxynitrite,[9–11] a reactive nitrogen species which can be formed by combination of superoxide anion and nitric oxide radicals,[12] yet scavenged by urate or ascorbate.[13] Peroxynitrite, or its protonated form, is a powerful one- and two-electron oxidant which is known to modify lipids, proteins and DNA, and can be detected by increased nitrotyrosine levels in the injured tissue.[12,14]

Apoptosis is normally suppressed in the ovary by gonadotropins and other survival factors,[2–4] but it is induced by hypoxia.[15–17] Oxygen deprivation was suggested to stimulate apoptosis by causing ATP depletion, ionic imbalances and a paradoxical increase of the level of reactive oxygen species.[7,15,18,19] In particular it was suggested that, during hypoxia, oxidative stress occurs by mechanisms involving superoxide[20] and peroxynitrite.[7,14,21] These results support the hypothesis that apoptosis in follicular cells might be suppressed by ascorbate or other inhibitors of oxidative stress.[22–25] In ovaries from water buffalo, the follicular levels of ascorbate and urate were previously found higher than those in plasma, thus suggesting that efficient antioxidant mechanisms might occur in the follicle to limit or prevent damages of the oocyte structure.[26] Depletion of cellular oxygen, such as in the case of hypoxia or hypoxic ischemia, and loss of blood-mediated supply of survival factors for follicular cells can be obtained just by dissecting and removing the ovary from the animal, yet incubating at body temperature. In these conditions, the follicular level of urate is expected to increase, as a result of ATP degradation,[27] while that of ascorbate would rise only if this antioxidant is synthesized in the follicle. To address this hypothesis, we measured urate and ascorbate levels in water buffalo follicular fluid, immediately and later after the ovary dissection, and analyzed the granulosa cells for their possible equipment with the ascorbate synthesizing enzyme, namely L-gulono-γ-lactone oxidase (E.C. 1.1.3.8, GLO). Moreover, in this study we report data on CPP32 activation in granulosa cells, and peroxynitrite stress in follicular fluids with different concentrations of urate. Urate and ascorbate synthesis in the follicle are discussed in terms of cell response to oxidative stress, as counteracting loss of function and atresia.

MATERIALS AND METHODS

Materials

The ovaries of water buffalo (*Bubalus bubalis*) were obtained, at random stages of the reproductive cycle, at a local slaughterhouse. Chemicals of the highest purity, bovine serum albumin (BSA), donkey anti-sheep horseradish peroxidase-conjugated (DAS-HRP) IgG, goat anti-rabbit horseradish peroxidase-conjugated (GAR-HRP) IgG and chromatography standards were purchased from Sigma-Aldrich (Milan, Italy). Rabbit anti-CPP32 of Zymed (San Francisco, CA, USA), nitrated BSA (nitrotyrosine-BSA) of Cayman Chemical (Ann Arbor, MI, USA) and sheep anti-nitrotyrosine IgG of Oxis (Portland, OR, USA) were used. The Nucleosil 100-NH_2 column (5 µm particle size, 250 × 4.6 mm I.D.) was obtained from Macherey-Nagel (Düren, Germany) and the Nova-Pak C18 column (4 µm particle size, 150 × 3.9 mm I.D.) from Waters (Milford, MA, USA). The resin Sephadex G25 was purchased from Pharmacia (Milan, Italy). Polystyrene 96-wells plates were from Corning-Costar (Concorezzo, Italy).

Preparation of Follicular Fluids and Granulosa Cells

The ovaries were carefully dissected within 40–60 minutes from the animal death, and either

immediately used or incubated in Tris buffered saline solution (TBS: 130 mM NaCl, 20 mM Tris-HCl, pH 7.3) at 39 °C. In the latter case, the ovaries were immersed in degassed buffer, 5 cm from the air. Follicular aspirates were collected from follicles (diameter = 6–8 mm) protruding at the surface of the ovary by a syringe equipped with a 22-gauge needle. The follicular aspirates were then centrifuged (1,000 g for 10 minutes at 4 °C) to quickly separate fluid from particulate fraction. After filtration through 0.1 μm nylon membrane, fluids were centrifuged again (10,000 g for 10 minutes at 4 °C) to remove molecular aggregates. The resulting supernatants, if free from hemoglobin contamination, were frozen in liquid nitrogen, and analyzed within two hours from the animal death. Syringe needles approached the follicle antrum from the stroma side. Repeated penetrations were done at 0, 30 and 60 minutes to collect the aspirates: 30–50 μl of fluid was prepared from each aspiration.

Granulosa cells were collected by a round edged spatula from cut open follicles of ice-cold ovaries. In particular, after fluid removal, a large incision was made on the follicle surface and, after washing the exposed cavity with 10 ml of ice-cold TBS, the granulosa layer was soaked by blotting paper before scraping on the cells.

Titration of Antioxidants in Follicular Fluids

Follicular fluids were analyzed for their content of antioxidants. The total antioxidant capacity (TAC) was measured, in samples reacted with the radical 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate), as decrease of the absorbance at 734 nm, and expressed as μM concentration of Trolox equivalents according to a published procedure.[28]

Samples used for titration of ascorbate and urate were prepared by mixing 100 μl of ice-cold ethanol with 15 μl of fluid. The mixture was centrifuged (12,000 g for 1 minute at 4 °C), and the protein pellet was discarded. A Nucleosil 100-NH_2 column was injected with 20 μl of supernatant. Chromatography was carried out at a flow rate of 0.7 ml per minute with 50 mM NaH_2PO_4:CH_3CN (35:65, v:v) as mobile phase. Ascorbic and uric acids were separately eluted and identified on the basis of their retention times or co-chromatography of purified standards and by their absorbance spectrum, as displayed by a diode array detector. Absorbance at 275 nm (A_{275}) and calibration curves, obtained by injecting known amounts of purified standards, were used for quantitative analysis. In particular, standard solutions for ascorbic and uric acids were freshly prepared in 0.2 mM metaphosphoric acid containing 5 mM EDTA, at concentration ranging from 5 to 160 μM and from 15 to 500 μM, respectively. Calibration curves $A_{275} = a + bx$, as obtained by plotting six duplicates, displayed the trend of straight lines ($r^2 \geq .9997$).

Samples for titration of retinol and α-tocopherol were prepared by mixing 15 μl of fluid with 200 μl of ice-cold isopropanol-methanol (1:1, v:v). After centrifugation (12,000 g for 1 minute at 4 °C), 20 μl of supernatant were analyzed by HPLC. The reverse phase system represented by the Nova-Pack C18 column and the mobile phase, consisting of a mixture of isopropanol:methanol:water (46.25:46.25:7.5, v:v:v) were chosen. The chromatography was carried out at 0.8 ml per minute, and a programmable fluorescence spectrometer was used. In particular, retinol was detected by setting $\lambda_{EX} = 325$ nm and $\lambda_{EM} = 465$ nm from 0 to 5 minutes, while α- and γ-tocopherol were detected by $\lambda_{EX} = 295$ nm and $\lambda_{EM} = 335$ nm from 5 to 8 minutes. Stock solutions of retinol or α-tocopherol were prepared in ethanol; the solution titer was assessed by spectrometry.[29] Stock solutions were diluted with ethanol to obtain working standard solutions: 25–800 nM and 0.1–3.6 μM for retinol and α-tocopherol respectively. The calibration curve, obtained by plotting six duplicates, displayed the trend of straight lines for retinol ($r^2 \geq .9995$) and α-tocopherol ($r^2 \geq .9997$).

GLO Assay

Microsomes from liver of rat or water buffalo and granulosa cells of water buffalo were prepared in 50 mM K-phosphate, 5 mM $MgCl_2$, pH 7.4 at 37 °C, according to Chatterjee.[30] The protein amount was determined according to Markwell.[31] Protein values ranging from 1.8 to 2.4 mg/ml, in suspensions from 240 to 300 µl, were found in different preparations of liver microsomes. Microsomal suspensions from granulosa cells were never larger than 80 µl and never displayed protein concentration higher than 0.6 mg/ml. One volume of 5 mM L-gulono-γ-lactone was added to liver or granulosa microsomal suspension to trigger the ascorbate synthesis. The mixture was incubated at 37 °C under stirring. Aliquots of the reaction mixture (15 µl) were taken up both at the beginning and during incubation. The amount of *in vitro* synthesized ascorbate was measured by HPLC, as described above, and expressed as nmoles of product per mg of proteins.

A lower amount of microsomes, compared to those used in the method described above, were used for enzyme qualitative assay. This procedure is based on the electrophoretic analysis of partially purified GLO, performed essentially according to Nishikimi.[32] In details, the microsomes were suspended in buffer A (1.5% Tween-20, 20 mM Tris-HCl pH 8.0, 1 mM EDTA), and centrifuged for 90 minutes at 100,000 g. Then 4 M ammonium sulfate was added to the supernatant up to a final concentration of 161 mg/ml. After 30 minutes at room temperature, the mixture was centrifuged for 10 minutes at 10,000 g. The supernatant was taken and further ammonium sulfate was added (148 mg/ml). The salted out material was recovered, after 30 minutes at room temperature, by centrifugation for 10 minutes at 10,000 g. The resulting pellet was dissolved in 900 µl of buffer B (0.1% Triton X-100, 20 mM Tris-HCl pH 8, 10 mM KCl, 1 mM EDTA). This solution was loaded on a Sephadex G-25 column (1.5 × 13 cm), which was equilibrated and eluted with buffer C (0.1% Triton X-100, 20 mM Tris-HCl pH 8) at 10 ml per hour flow rate. Fractions of 500 µl were collected, and the proteins content was determined. Fractions with the highest microsome content were analyzed for the presence of GLO by electrophoresis on polyacrylamide gel, as previously described.[32]

Assay of the CPP32 Activity and Titration of CPP32 Antigens

Granulosa cells were analyzed for CPP32 activity. HPB-ALL cells induced for apoptosis with Fas ligand were used as positive control. Negative control consisted of HPB-ALL cells without induction. As previously reported,[33] 2×10^6 cells were lysed, and resulting soluble fractions were then incubated with the enzyme substrate DEVD-*p*NA (*p*-nitroanilide-derivatized aspartyl-glutamyl-valyl-aspartate). Protein concentration in the soluble fractions was determined, and adjusted to 6–8 mg per ml. Samples and controls were incubated, in duplicates, in 96-well microtiter plates. The chromophore *p*NA, when freed of DEVD by the enzyme dependent substrate cleavage, was titrated by spectrophotometry at 405 nm. The enzyme activity was calculated as units (nmoles of freed *p*NA per hour) per mg of protein.

CPP32 immunorelated antigens were titrated, by ELISA, in 2–3 µl aliquots from the same cell soluble fraction which was used to analyze the enzyme activity. The protein concentration of each aliquot was adjusted to 0.04 µg/µl. Then, dilutions of 1:10, 1:50 and 1:100 in ELISA coating buffer (7 mM Na_2CO_3, 17 mM $NaHCO_3$, 1.5 mM NaN_3, pH 9.6) were done, and 50 µl from each diluted solution were loaded, in duplicates, into the wells of the microtiter plate. Protein coating of plastic was carried out overnight at 4 °C. Unattached material was removed by two washes with buffer T-TBS (TBS containing 0.05% Tween-20) and two washes with 0.5 M NaCl, 20 mM Tris-HCl, pH 7.3. Remaining plastic reactive

sites were blocked by incubation (1 hour, 37 °C) with TBS supplemented with 0.5% BSA. After BSA treatment, the wells were washed, as described above, and loaded with 50 µl of the rabbit anti-CPP32 antibody (previously diluted 1:2000 with T-TBS supplemented with 0.25% BSA). After one hour of incubation at 37 °C, the wells were washed, loaded with 50 µl of GAR-HRP antibody (diluted 1:5000 as the primary antibody). After further incubation for 1 hour at 37 °C, the samples were washed again. Finally, 100 µl of a color development solution (20 mg of o-phenylendiamine in 50 ml of 0.1 M sodium phosphate at pH 5, supplemented with 120 µl of 3% H_2O_2) were added to each well. After 60 minutes of incubation at 37 °C, for color developing, the reaction was stopped by addition of 50 µl of 2.5 M H_2SO_4. The absorbance at 492 nm (A_{492}) of the solution was measured by a microplate reader. Samples processed with the omission of primary antibody treatment were used to determine the background.

Titration of Nitrotyrosine in Follicular Fluid Proteins

Nitrated BSA was purchased at a concentration of 0.4 mg/ml. The extent of tyrosine nitration was determined by measuring the absorbance at 430 nm, using $\epsilon = 4{,}500\,M^{-1}\,cm^{-1}$ at pH 9.6, according to a published procedure:[34] 9.3 residues of nitrotyrosine per BSA molecule were detected. The concentration of nitrotyrosine in follicular fluid proteins was determined by ELISA. Samples were prepared, in duplicate, by diluting the fluid (1:500, 1:2000, 1:4000, 1:6000, 1:8000 and 1:20,000) with the ELISA coating buffer. Standard curves were constructed with serial dilutions of nitrated BSA. Coating, treatments with antibodies (sheep anti-nitrotyrosine IgG and DAS-HRP IgG), washing and color development were carried on as described for the analysis of CPP32 immunorelated antigens. The non specific binding of the antibodies (background) was assessed by competitive inhibition with 10 mM nitrotyrosine.[34] Nitrotyrosines were expressed as µmoles of nitro-BSA equivalents.

Statistical Analysis

The samples for the titration of proteins, CPP32-immunorelated antigens or nitrotyrosines, and the determination of the activities of CPP32 or GLO were processed at least in triplicate. Averages ± standard deviations (SD) were calculated from the experimental data. Titration of retinol, α-tocopherol, ascorbate or urate was carried out on duplicates. The program "Graph Pad Prism 3" (Graph Pad Software, San Diego, CA, USA) was used to obtain trend curves, perform regression analysis and calculate significance.

RESULTS

Titration of Ascorbate and Urate in Follicular Fluids from Incubated Ovaries

Water buffalo ovaries ($N = 12$), each containing a single large antral follicle, were incubated at 39 °C (the body temperature of water buffalo) immediately after dissection. Follicular fluids were prepared from follicle aspirates collected before ($t = 0$ minutes) and during incubation ($t = 30$–60 minutes). Only fluids free of hemoglobin were analyzed. The TAC and the concentrations of retinol, α-tocopherol, ascorbate and urate were determined in each of the selected fluids. These levels fluctuated over wide ranges (Table I). The values of retinol and α-tocopherol, obtained from single incubated follicles, did not essentially differ from those before incubation. Conversely, the fluids displayed significantly higher values of TAC, ascorbate and urate as the incubation progressed (Figure 1). These data suggest that part of the increasing TAC level, during incubation, might be accounted for by the increase of the water-soluble antioxidants analyzed. The levels of ascorbate and urate were found significantly correlated ($p < .001$).

TABLE I Concentration (μM) of follicular fluid antioxidants in ovaries before and after incubation for 30 or 60 minutes

Antioxidant	Concentration (average ± SD) μM		
	0 min	30 min	60 min
Retinol	1.03 ± 0.24	1.08 ± 0.26	0.99 ± 0.21
α-Tocopherol	1.76 ± 0.56	1.68 ± 0.46	1.90 ± 0.53
Ascorbate	32.00 ± 9.12	58.65 ± 55.19	117.91 ± 64.80
Urate	117.81 ± 49.19	169.66 ± 76.37	243.76 ± 78.40
TAC	199.33 ± 82.89	240.00 ± 127.94	326.11 ± 102.90

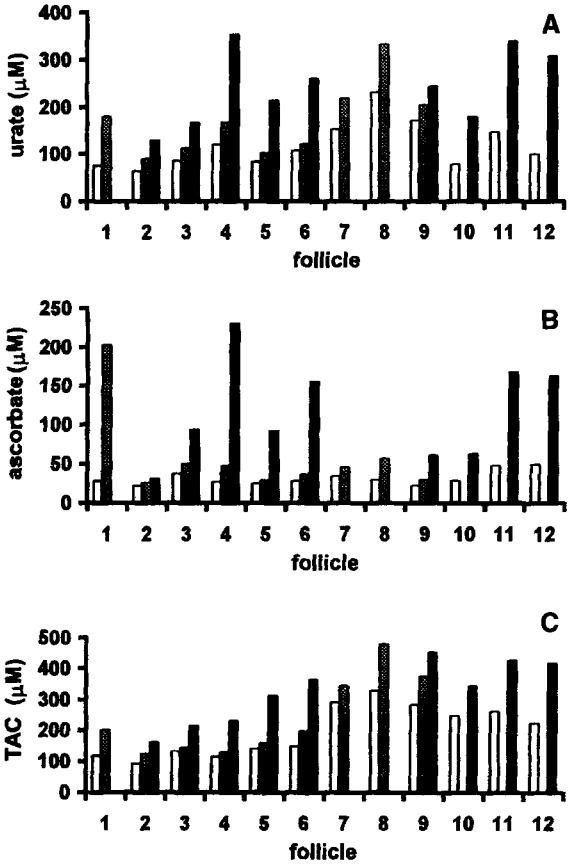

FIGURE 1 Increase of urate, ascorbate or TAC titer in follicular fluid during ovary incubation. The concentration of urate and ascorbate (μM) (panel A and B respectively) or TAC (μmoles of Trolox equivalents/liter) (panel C) was measured in fluids prepared from follicles of ovaries incubated at 39 °C. Each follicle was penetrated at 0, 30 and 60 minutes of incubation for the fluid aspiration (open, dotted and filled bars respectively). Aspirates with hemoglobin contamination were discarded. Each bar represents one determination.

Detection of GLO in Microsomes from Liver and Granulosa Cells of Water Buffalo

The cell endoplasmic reticulum is the endogenous source of ascorbate in most species of animals.[30,35] The enzyme producing this vitamin, namely GLO, was previously suggested to be expressed only in liver or kidney microsomes.[35] To investigate whether water buffalo synthesizes ascorbate and such a synthesis occurs in the follicle, we incubated microsomes from liver and granulosa cells with the GLO substrate and analyzed the resulting reaction mixtures for the amount of product. The analysis was carried out by HPLC with detector of UV absorbance. Microsomes of rat liver, which synthesize ascorbate,[35] were incubated as positive control. Ascorbate was produced by liver of both species, and its amount was found increasing upon incubation (Figure 2). Ascorbate was not detected in samples containing microsomes of granulosa cells. This result, while indicating that GLO is present in liver of water buffalo, suggests that granulosa cells might not express the enzyme or, more likely, were a too poor source

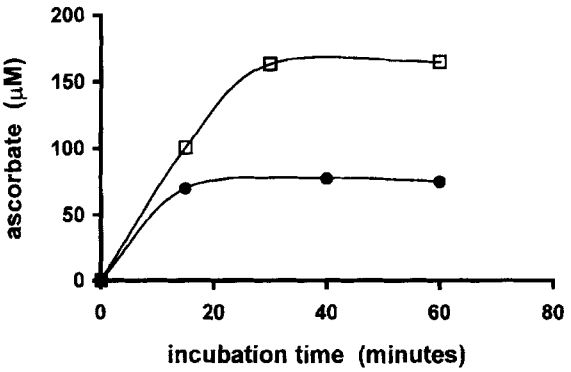

FIGURE 2 Synthesis of ascorbate in liver microsomes of water buffalo or rat. Liver microsomes of water buffalo (filled circles) or rat (open squares) were incubated at 39 °C, for 30 or 60 minutes, with L-gulono-γ-lactone as GLO substrate. The level of ascorbate, when measured just after the substrate addition (time = 0 minutes), was taken as background. The values obtained during the incubation were expressed, after background subtraction, as nmoles per mg of protein. Typical results from one experiment are shown: comparable data were obtained from two more experiments (with separate preparations of microsomes).

of microsomes (and therefore GLO) for effective detection of ascorbate in the system used. Thus we tried to carry out a different procedure to assay the enzyme, requiring amounts of microsomes smaller than those used in the above described experiment. The activity of GLO was detected by incubating a polyacrylamide gel, following electrophoresis of microsomal proteins, in a solution containing both the substrate and a reagent for hydrogen peroxide. This oxidant is actually formed with equimolar amounts of ascorbate.[30] The enzyme activity was detected as protein band. The electrophoretic mobility of the buffalo bands was lower than that of the rat band (Figure 3), suggesting the enzyme structure displays different charge/mass ratios in the two species. These results indicate that GLO is expressed in water buffaloes and demonstrate that ascorbate can be synthesized in the follicle.

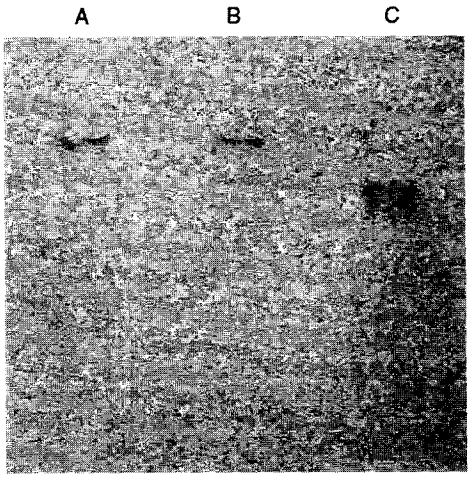

FIGURE 3 Detection of GLO activity in microsomes from granulosa cells of water buffalo. Microsomal proteins (40 μg) from water buffalo liver (lane A) or granulosa cells (lane B) were analyzed by electrophoresis on 7% polyacrylamide slab gel at pH 8.9. The proteins were fractionated at 200 V for 1 hour, in the presence of 1% Triton-X100. GLO was stained by a color developing solution which contained L-gulono-γ-lactone, phenazine methosulfate and nitroblue tetrazolium at pH 7.5. Details of the procedure are reported elsewhere.[34] Microsomal proteins from rat liver (lane C) were used as positive control of the enzyme activity.

Correlation of Urate and Nitrotyrosine Levels in Follicular Fluid

Urate is claimed to be a good scavenger of peroxynitrite.[13,14] This oxidant, which is formed into the cell, can diffuse through the plasma membrane and attack tyrosines in the extracellular fluid. High levels of nitrotyrosine residues, in follicular fluid proteins, are expected when low amounts of urate are produced and excreted by granulosa cells in the fluid. To support this hypothesis, the amount of nitrotyrosine in follicular fluids ($N = 20$) was determined, using specific antibodies in an ELISA procedure, and plotted vs the level of urate. Ovaries, stressed by blood flow deprivation and incubated for 1 hour at 39 °C, were used. The two parameters were found negatively correlated ($p < .001$) as shown in Figure 4. These results suggest that urate scavenges nitrating species in follicular fluid.

Correlations of CPP32 Expression in Granulosa Cells with Different Fluid Levels of Nitrotyrosine and Urate

The nitrosative stress was suggested to be involved in the activation of CPP32.[9–11] The

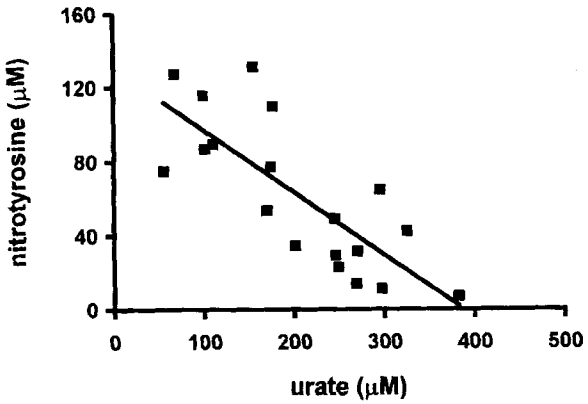

FIGURE 4 Nitrotyrosine and urate levels in follicular fluids of incubated ovaries. Follicular fluids ($N = 20$) were prepared from ovaries incubated at 39 °C for 1 hour. The levels of urate and nitrotyrosine were expressed as μmolar concentration. Each sample was analyzed in triplicate and the mean is represented: deviations over 5% from this value were not found. The results are from two separate experiments.

granulosa cells from different follicles ($N = 12$) were analyzed for the presence of CPP32 active forms or antigens. Cell homogenates were used to carry on the assay of the enzyme activity or the immunoreactivity to anti-CPP32 antibodies. The enzyme activity was found positively correlated with the amount of CPP32 immunorelated antigens ($p < .01$). This result indicates that CPP32 is expressed in granulosa cells of blood-deprived follicles.

The follicular fluid levels of nitrotyrosine or urate, which could not be measured in the cell homogenates, were assumed to reflect the cytosolic levels of the two molecules, as both can diffuse from their cell production site to the fluid compartment. The nitrotyrosine level was found positively correlated with the CPP32 activity ($p < .002$) (Figure 5, panel A). On the other hand, the urate level was found negatively correlated with both the CPP32 activity ($p < .005$) and the immunoreactivity to anti-CPP32 antibodies ($p < .01$) (Figure 5, panel B). These data suggest that, in granulosa cells, the activation of CCP32 is stimulated by nitrating species, and prevented by urate.

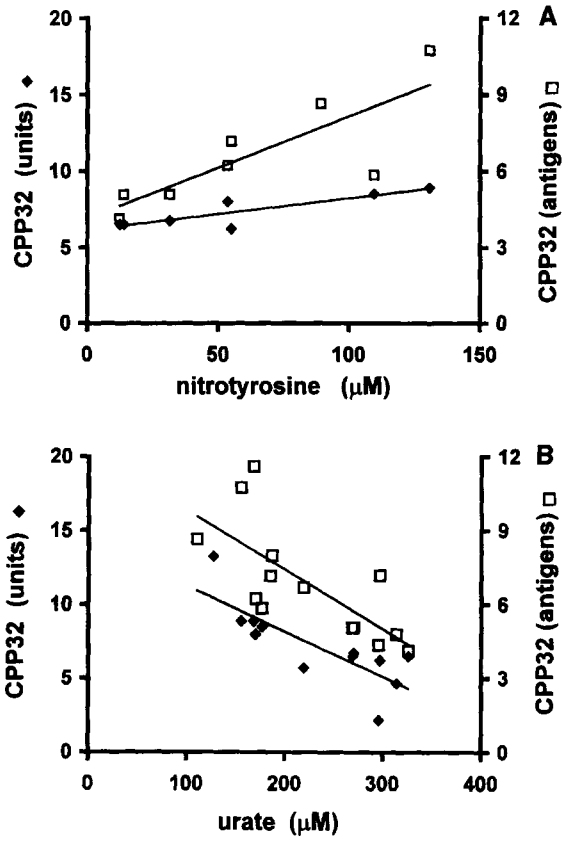

FIGURE 5 Correlations of immunoreactivity to anti-CPP32 antibodies, and CPP32 activity from granulosa cells with the nitrotyrosine or urate level in homologous follicular fluid. Follicular fluids, and homogenates of homologous granulosa cells were prepared from ovaries incubated at 39 °C for 1 hour. The μmolar concentration of nitrotyrosine (panel A) or urate (panel B) was determined in each fluid. The homogenate soluble fractions were assayed for CPP32 activity (using DEVD-*p*NA as substrate), and concentration of CPP32-immunorelated antigens (using GAR-HRP antibodies and ortho-phenylendiamine to detect the primary immunocomplexes). The enzyme activity is expressed as units (nmoles of *p*-nitroaniline produced per hour per mg of protein). The immunoreactivity is expressed as absorbance at 492 nm (OD per hour per mg of protein). Each point represents the mean from three determinations. Deviations over 5% from the mean were not found.

DISCUSSION

The supply of ascorbate to the ovary was previously suggested to be a limiting factor in the ability of the preovulatory follicle to grow in response to gonadotropin stimulation.[36] The finding of the FSH induction of ascorbic acid accumulation in granulosa cells of developing follicles supported the hypothesis that this antioxidant might be required for preventing apoptosis and atresia.[22,23]

In this paper, we report that the ascorbate level in the follicular fluid of water buffalo, after the ovary dissection that removes the cell exogenous source (i.e. plasma), can increase up to seven times in half an hour. This increase could be explained by considering the granulosa cells as releasing into the antrum the ascorbate, which they previously took up from the circulation. However, it is conceivable that, in addition to the transport from plasma, the cells themselves are another source of ascorbate. Ascorbate can be produced by the enzyme GLO in liver and kidney of most animals, except human and very few species.[30,35] In this paper, we demonstrate that the enzyme is present in water buffalo, and might be active not only in liver but also

in granulosa cells. This finding suggests that a local ascorbate synthesis might be required to counteract the oxidative stress in the large antral follicle. The fact that the origin site is just the action site for ascorbate represents an evident advantage as regard the speed and, possibly, the control of the antioxidative response in the follicle. Furthermore, a local antioxidant response offers more expediency than a systemic one because it might be more intense or, for the same intensity in the follicular compartment, less energy consuming. In this context also the local biosynthesis of other antioxidants can be discussed. We observed that, in ovaries dissected and incubated without perfusion, the ascorbate follicular accumulation is accompanied by urate production. One of the causes of tissue oxidative stress is the oxygen deprivation as resulting from ischemia.[14] The absence of blood flow in the ovary results in increasing hypoxia as tissue residual oxygen is consumed. Thus, our experimental conditions simulate interruption of ovarian blood flow, a circumstance analogous to that caused by ischemia. Our data are in agreement with the finding of increased cell concentration for both urate and ascorbate, after oxygen consumption or ischemia.[14–15,27,37] Both ascorbate and urate are scavengers of peroxynitrite.[13] This oxidant may stimulate the release of cytochrome c from the mitochondrion,[38–39] which triggers the Caspase cascade activation.[40] Peroxynitrite can directly activate CPP32 and react with other molecules, as mentioned above, or cross the plasma membrane[14] thus acting outside the origin site. Therefore the level of nitrotyrosine, a peroxynitrite footprint,[41,42] in the extracellular fluid might reflect the activation of CPP32 into the cell. Our results support this hypothesis. In fact, we found the nitrotyrosine level in follicular fluid positively correlated with both CPP32 activity and CPP32-like antigens from extracts of granulosa cells. CPP32 activation was also found in trophic factor-deprived cells.[43] Ovarian atresia, mostly affecting granulosa cells,[4] is thought to be caused by apoptosis[44] which is in turn stimulated by blood deprivation[45] while prevented by FSH.[2,46,47] In the context of the cell death induction by deprivation of oxygen, nutrients, and growth or survival factors, we propose to consider the synthesis of urate and ascorbate as (part of) the cell response to peroxynitrite formation. The death program is known to be canceled when the number or intensity of pro-apoptotic stimuli is low,[48] and inhibitors of CPP32 activation, such as bcl-2,[2] are expressed. Urate and ascorbate might be synthesized to counteract oxidative stress and resist to apoptosis stimuli. The protective role of these scavengers might be of vital importance for follicular cells during transient interruptions of blood flow in the ovary, or just when low concentration of dissolved oxygen is circulating.

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