Mitochondrial function and mitochondria-induced apoptosis in an overstimulated rat ovarian cycle

Ana Navarro,1 Rafael Torrejón,2 Manuel J. Bández, José M. López-Cepero,3 and Alberto Boveris4

1Department of Biochemistry and Molecular Biology and 2Department of Obstetrics and Gynecology, University Hospital of Puerto Real; 3Department of Cell Biology and Histology, School of Medicine, University of Cádiz, Cádiz, Spain; and 4School of Pharmacy and Biochemistry, University of Buenos Aires, Buenos Aires, Argentina

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Mitochondrial function and mitochondria-induced apoptosis in an overstimulated rat ovarian cycle. Am J Physiol Endocrinol Metab 289: E1101–E1109, 2005. First published July 12, 2005; doi:10.1152/ajpendo.00223.2005.—Female rats were treated with FSH (40 IU/kg) on the first and second diestrous days (D1 and D2) and with LH (40 IU/kg) on the proestrus (P) day to synchronize and maximize ovarian changes. Follicle area increased by 50% from D1 to P, and the estrus (E) phase showed multiple corpora lutea and massive apoptosis. Increased oxygen uptakes (42–102%) were determined in ovary slices and in isolated mitochondria in active state 3 along the proliferation phase (D1-D2-P) that returned to initial values in the E phase. Mitochondrial content and the electron transfer activities of complexes I and IV were also maximal in the P phase (20–79% higher than in D1). Production of NO by mitochondrial nitric oxide synthase (mtNOS), biochemically determined, and the mtNOS functional activity in regulating state 3 oxygen uptake were maximal in the P phase and 79–88% higher than at D1. The moderately increased rate of NO in the proliferative phase is associated with mitochondrial biogenesis, whereas the high rate of NO generation by mtNOS at phase P appears to trigger mitochondria-dependent apoptosis. The calculated fraction of ovary mitochondria in state 3 was at a minimal value at the P phase. Mitochondrial oxidative damage, with increased thiobarbituric acid-reactive substances and protein carbonyls, indicates progressive mitochondrial dysfunction between phases P and E. The roles of mitochondria as ATP provider, as a source of NO for mitochondrial proliferation and mitochondrial function in the P phase, and as a source of O2 and H2O2 appear well adapted to serve the proliferation-apoptosis sequence of the ovarian cycle.

ovarian follicle; oxygen uptake; mitochondrial nitric oxide synthase; respiratory chain; oxidative damage

OVARian CYCLE AND FOLLICULAR DEVELOPMENT are controlled by circulatory feedback between the ovarian hormones and the hypothalamic-pituitary axis (12) and the hormonal predominance of a dominant follicle determines its morphological changes and growth (13) by increasing diameter and the number of granulosa cells (28) whereas nondominant follicles become atretic.

During each cycle, the increased FSH concentration recruits growing antral follicles, and the concept of “cyclic recruitment” has been proposed to describe this rescue of follicles from degeneration (27). Follicles in the antral stage express receptors for FSH and become dependent on FSH stimulation for survival, proliferation, and expression of the LH receptor that after stimulation by the hypophysis reach ovulation and

formation of the corpus luteum. The number of corpora lutea formed depends, in a ratio of one to one, on the number of follicles that responded to the LH signal. In physiological conditions, FSH stimulates ovarian follicular growth, and LH controls their hormonal secretory capacity, with FSH and LH maximal levels at the end of the proestrus phase with maximal follicular growth simultaneous to ovulation. A decrease in the levels of LH pulse results in follicular cell death and in abortion of the generation of corpora lutea (10). The concept of physiological cell death in the ovary is a classical biological idea; the morphological description of apoptosis in rabbit Graafian follicles was already reported in 1885 (14).

Pituitary gonadotropins are the most important survival factors for ovarian follicle cells by promotion of the expression of local survival factors in ovarian follicles. Specifically, gonadotropins promote cell proliferation and suppress ovarian cell apoptosis by activation of cAMP-dependent pathways and by increasing the production of paracrine and autocrine factors such as estrogens, interleukin-1, nitric oxide, and insulin-like growth factor I (IGF-I). These factors promote cell survival and proliferation through activation of the nuclear estrogen receptor, the cGMP-dependent pathway, and protein tyrosine phosphorylation (23). Because the execution of the apoptosis program in ovarian follicles depends on the cooperative regulation of different paracrine and autocrine factors, it is likely that none of these factors is singularly obligatory in the control of follicle growth or demise. Instead, a balance of survival and apoptotic factors may decide whether a follicle will continue development or undergo apoptosis. How the various extracellular hormones and their intracellular signal transduction mediators are linked to the intracellular decision step of triggering apoptosis in ovarian follicles remains, however, largely undefined.

Laboratory rodents, e.g., mouse, rat, and hamster, have a reproductive strategy that allows them to ovulate, and thus potentially conceive, every 4–5 days. The high frequency of ovulation is possible because these mammals, although they ovulate spontaneously, do not develop a fully functional and secretory corpus luteum. Thus there is no inhibition of gonadotropin activity, which allows follicular development and ovulation to recur within a few days. The ovarian cycle in the rat is divided into estrus (E), or ovulation phase with a duration of ~12 h, usually in the evening; metestrus (M), or luteal phase, with a duration of 1 day; diestrus (D), or follicular phase with a duration of 1 (D1), or 2 (D2) days; and proestrus (P), or the

Address for reprint requests and other correspondence: A. Navarro, Departamento de Bioquímica y Biología Molecular, Facultad de Medicina, Plaza Fragela 9, 11003 Cádiz, Spain (e-mail: ana.navarro@uca.es).
end of follicular phase, with the highest levels of FSH and LH and a duration of ~12 h (36).

Although the histological changes and hormone dependence of the ovarian cycle in mammals are well known, including the biochemical signaling that follows the hormone receptor bindings, there is a paucity of information concerning the energetics and the mitochondrial function during the ovarian cycle. The objective of this study was to characterize ovary mitochondrial function in the phases of the ovarian cycle in the rat. Hormonal (FSH and LH) overstimulation was chosen as the experimental approach to synchronize and maximize the ovary treatments to increase the number of ovarian follicles in vivo fertilization (15, 16, 22), and the FSH and LH doses used in this study are similar to those used in human fertility treatments to increase the number of ovarian follicles in maturation.

MATERIALS AND METHODS

Animal protocol. Female Wistar rats, 3 mo old and of 250 ± 15 g in weight, were used (n = 18 per group). The animals were siblings grown at the Department of Experimental Animals of the University of Cádiz, housed in groups of 3 rats and kept at 22 ± 2°C with 12:12-h light-dark cycles and with full access to water and food. Animal experiments were carried out in accordance with the 86/609/CEE European Community regulations and the Guiding Principles for Research Involving Animals and Human Beings of the American Physiological Society.

Daily examination of vaginal smears, obtained by inserting a hyssop into the vagina and removing a sample of cells from the vaginal walls, under a low-power microscope allowed the selection of animals in M phase. Standard criteria were used to characterize the cycle phases using conventional Papanicolau staining (18). Rats were injected daily with recombinant human FSH (Follitropin beta, 40 IU/kg ip; Puregon, Organon Laboratory) on D1 and D2, and with recombinant human LH (Lutropin alfa, 40 IU/kg ip, Luveris; Serono, Biotech & Beyond) on the P day. Rats were daily killed (D1, D2, P, and E days; Fig. 1) by decapitation, ovaries were rapidly excised and weighed, and tissue samples were immediately processed for mitochondrial suspensions, containing ~20 mg protein/ml, were used immediately after isolation for oxygen uptake determination or frozen in liquid N2 and kept at −80°C. Mitochondrial samples, twice frozen and thawed, were homogenized each time by passage through a 15/10 tuberculin needle; the resulting mitochondrial membranes from disrupted mitochondria had a standard content of 0.16–0.21 nmol cytochrome aa3/mg protein and were used for determination of enzyme activities and oxidative stress markers. The protein content of the samples was determined using the Folin reagent and bovine serum albumin as standard.

Mitochondrial oxygen consumption. Oxygen uptake was determined with a Clark electrode in a 1.5-ml chamber at 30°C in an air-saturated reaction medium consisting of 0.23 M mannitol, 0.07 M sucrose, 20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 5 mM phosphate, 4 mM MgCl2, and 0.5–0.7 mg mitochondrial protein/ml, at pH 7.4 (7). Respiratory rates were determined with 10 mM succinate as substrate, and state 3 active respiration was established by addition of 0.5 mM ADP. Oxygen uptake is expressed in ng-at O per minute per milligram of protein.

Mitochondrial mass in the tissue. The content of mitochondria (mitochondrial mass) of the whole organ was calculated from the ratios of cytochrome oxidase activities in ovary homogenates and in isolated mitochondria (31, 37).

Mitochondrial nitric oxide synthase functional activity. The mitochondrial nitric oxide synthase (mNOS) functional activity was assayed by determination of the difference between the rates of state 3 mitochondrial oxygen consumption at maximal and minimal intramitochondrial NO levels (42). The first condition was achieved by supplementation of the mitochondrial suspensions with 0.2 mM l-arginine and 1 µM superoxide dismutase (SOD) and the second by addition of 1 mM nitro-l-arginine methyl ester (the NOS competitive inhibitor) and 20 µM oxyhemoglobin (HbO2).

Spectrophotometric determination of NO activity. Mitochondrial NO production was determined by the HbO2 oxidation assay as previously described (3). The reaction medium consisted of 0.1 mM NADPH, 0.2 mM arginine, 1 mM CaCl2, 4 mM Cu-Zn-SOD, 0.1 µM catalase, and 25 µM HbO2 heme in 50 mM phosphate buffer and 0.5–0.7 mg protein/ml, pH 7.4. A diode array-sensitive spectrophotometer (model 8453; Agilent, Palo Alto, CA) was used to follow the absorbance change at 577 nm with a reference wavelength at the isosbestic point of 591 nm (ε577ε591 = 11.2 mM−1·cm−1). Production of NO was calculated from the absorbance change that was inhibited by 2 mM N3-methyl-l-arginine, usually 92–96%, and expressed in nanomoles NO per minute per milligram of protein.

Mitochondrial superoxide dismutase activity (Mn-SOD). Dismutase activity was determined by the spectrophotometric adrenochrome assay (29) followed at 480 nm (ε = 4.0 mM−1·cm−1) in a reaction medium containing 1 mM epinephrine, 1 mM KCN, and 50
mM glycine-KOH (pH 10.0). One Misra-Fridovich unit of enzyme activity, 50% inhibition of the rate of spontaneous adrenochrome formation, is given by 1.8 nM Mn-SOD.

**Mitochondrial electron transfer activities.** The membrane-bound activities of complexes I-III, II-III, and IV were determined spectrophotometrically at 30°C with submitochondrial membranes suspended in 100 mM phosphate buffer (pH 7.4) added with the corresponding substrates (32, 33). For NADH-cytochrome c reductase (complexes I-III) and succinate-cytochrome c reductase (complexes II-III) activities, submitochondrial membranes were added with 0.2 mM NADH or with 20 mM succinate as substrates, 0.1 mM cytochrome c oxidized at 10 μM cytochrome c per milligram of protein, which gives rates of the order of mitochondrial electron transfer activities.

**Biochemical markers of oxidative stress.** The mitochondrial content of thiobarbituric acid-reactive substances (TBARS) and protein carbonyls was determined in submitochondrial membranes by the original assays of Fraga et al. (17) and of Oliver et al. (35), modified as previously described (31). Protein carbonyls are expressed in picomoles per milligram of mitochondrial protein.

**Statistics.** The numbers in the tables and figures are mean values ± SE. Differences between groups were analyzed by the Student-Newman-Keuls post hoc test after significant one-way ANOVA. A P value of <0.05 was considered biologically significant. Statistical analyses were carried out using a statistical package (SPSS 11.5 for Windows).

**RESULTS**

**Histological results.** Ovaries in the D1 phase showed several cohorts of medium-size (400–500 μm) follicles corresponding to recruited primordial follicles that had grown over the last 3–4 estrous cycles (Fig. 2). Some atretic follicles showed regressive changes with apoptotic bodies in the granulosa cells with basal membrane dissolution. Large nonfunctional corpora lutea from previous cycles were in different regression stages: from eosinophilic and highly vascularized endocrine cords in recent ones to highly pyknotic and apoptotic cell cords with lymphocyte infiltration and spongy degeneration in the oldest ones. The D2 phase showed enlarged follicles with a highly proliferative multilayered granulosa. About seven large cavitary follicles and 14 corpora lutea were usually observed in a midsagittal ovary section. The ovaries in the P phase had an increased number of large tertiary follicles, hyperplasia of follicular, luteal, and interstitial type of tissue gave relative area values of 1:6:7 in the D1 phase and of 2:6:7 in the P phase, showing that the follicular area was doubled at the end of the proliferative process. Follicle area was considered as the area of granulosa cells excluding follicular fluid area. The corpora lutea fraction represented nearly one-half of the ovary volume from D1 to P, and although they were not actively secreting progesterone, they exhibited a structural continuum of regression stages and apoptotic changes. Ovaries in the E phase showed multiple large and young corpora lutea that originated from recently ovulated follicles. Some nonovulated follicles showed cystic development or regressive changes in the granulosa cell layer. Corpora lutea were structurally heterogeneous, and a substantial number of them were involved in massive apoptosis. The apoptotic index was variable, indicating a sudden triggering of cell death only for cohorts of corpora lutea, presumably the oldest ones. A morphometric estimation indicates that the heterogeneous luteal compartment constitutes ~60% of the ovarian volume, whereas nonovulated follicles represented only ~4% of ovarian volume.

**Oxygen uptake of ovarian tissue.** Ovary oxygen consumption, determined in tissue cubes suspended in Krebs medium and followed with an oxygen electrode, progressively increased along with the FSH treatment and reached the highest respiratory rate at P, when the respiratory increase was 42% in relation to D1. In the E phase, ovary respiration decreased to levels similar to the ones shown in D1 (Fig. 3).

**Mitochondrial mass.** The content of ovary mitochondria, calculated from the ratio of cytochrome oxidase activity in ovary homogenates and in isolated mitochondria, was increased by 20% at the end of the proliferative phase in P (Table 1). Cytochrome oxidase activity was also maximal at P, when this enzymatic activity was increased by 38% in mitochondria and by 64% in the homogenate in relation to D1 and E (Table 1).

**Mitochondrial oxygen uptake.** The isolated ovary mitochondria were coupled with high rates of state 3 oxygen uptake and standard respiratory control ratios. Mitochondrial respiration in state 3, the maximal physiological rate of oxygen uptake and ATP production, also showed a progressive increase in oxygen uptake in the proliferative phases along with FSH treatment with a maximal rate of respiration in the P phase (Table 2 and Fig. 4). State 3 and state 4 mitochondrial respiration were increased 102 and 65%, respectively, in P compared with D1, whereas respiratory control, the ratio of both respiratory rates, was 23% higher in P compared with D1 (Table 1 and Fig. 4). In E, mitochondrial oxygen uptake and respiratory control decreased and approached the values of phase D1 (Table 2).

Mitochondrial enzymatic activities. The ovarian cycle produced marked changes in mitochondrial enzymatic activities
The activity of mtNOS activity was significantly increased (78%) in the P phase compared with the D1 phase (Table 5). A positive correlation was observed between mt-NOS enzymatic and functional activities (Fig. 5).

The activity of intramitochondrial Mn-SOD is frequently taken, when it is decreased, as an indication of mitochondrial oxidative stress. When measured in rat ovary mitochondria along the estrous cycle, Mn-SOD was observed to be de-
In 1-mm³ ovary cubes in air-saturated Krebs suspending medium at 30°C. Nos. near traces indicate O₂ uptake in ng-at O/min/g tissue. Table 4 gives a statistical presentation of the data.

Table 1. Mitochondrial content in rat ovary during the phases of the estrous cycle

<table>
<thead>
<tr>
<th>Ovarian phase</th>
<th>Homogenate, nmol·min⁻¹·g organ⁻¹</th>
<th>Mitochondria, nmol·min⁻¹·mg protein⁻¹</th>
<th>Mitochondrial Mass, mg protein/g organ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diestrus (D1)</td>
<td>2350 ± 140</td>
<td>260 ± 15</td>
<td>9.0 ± 0.4</td>
</tr>
<tr>
<td>Diestrus (D2)</td>
<td>2900 ± 160</td>
<td>330 ± 20</td>
<td>8.8 ± 0.4</td>
</tr>
<tr>
<td>Proestrus (P)</td>
<td>3850 ± 150*</td>
<td>360 ± 16*</td>
<td>10.7 ± 0.4*</td>
</tr>
<tr>
<td>Estrus (E)</td>
<td>2150 ± 120</td>
<td>245 ± 14</td>
<td>8.8 ± 0.5</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 9 rats per group. Content of mitochondria (mitochondrial mass) of the whole ovary was calculated from the ratios of cytochrome oxidase activity in organ homogenates and in isolated mitochondria (37). *P < 0.05, P vs. D1 or D1–D2.

Mitochondrial oxidative stress markers. The mitochondrial content of oxidative stress markers, protein carbonyls and malonaldehyde, this latter a lipid peroxidation by-product measured as TBARS, also showed maximal values in the P phase, values that were 42–45% higher than the ones corresponding to the other ovarian phases (Fig. 6).

DISCUSSION

In this study, overstimulation by FSH and LH was used as the experimental approach to synchronize and maximize ovarian changes. The rationale was to amplify the hormone-dependent tissue response that usually follows the proliferation-apoptosis pathway along the ovarian cycle.

The proliferative phases induced by FSH treatment promoted follicular growth and prevented follicular atresia, without granulosa or oocyte apoptosis detected in phases D1 and D2. Large and numerous follicles with hyperplasic granulosa and theca cell layers were the rule under FSH overstimulation. After LH treatment, a series of well-developed corpora lutea, albeit not functional, followed to the superovulation, and 1 day later, in phase E, these corpora lutea constituted a large fraction of the ovarian volume, much greater than the fraction usually observed in a physiological cycle. A large proportion of corpora lutea exhibited massive apoptosis, likely induced by the progesterone surge at proestrus evening (9). There is ample evidence that luteal regression involves apoptotic mechanisms in cycling rat ovaries (25, 40, 41). In summary, ovary morphological changes during the ovarian cycle describe a process in which the follicles undergo a clear cycle with two sequential phases: proliferation and apoptosis.

The general functional characteristics of ovary respiration and of ovary mitochondria, isolated from whole rat ovaries by using conventional methodology, are reported here. The lack of previous information is likely due to the limited amount of tissue of rodent ovaries. The clinical interest is evident; Seifer et al. (38) showed that women over the age of 38 yr have DNA damage (2). Mitochondrial DNA damage is considered a marker of oxidative stress and damage (2).

Ovaries showed a progressively increased whole organ oxygen uptake along the proliferation phase and the early luteinizing phase (D1, D2, and P) that returned to initial respiration values in E. This biphasic dependence of respiration along the ovarian cycle was characterized in this study in terms of...
mitochondrial function. Mitochondrial mass, expressed in milligrams of mitochondrial protein per gram of ovary, reaches maximal values at the P phase, and isolated mitochondria also showed maximal rates of specific respiration, referred to milligrams of protein, and in metabolic states 3 and 4 at the P phase. The pattern of respiration rates, with maximal values at P, agrees with the pattern of electron transfer activities of complexes I, II, and IV (NADH-ubiquinone reductase, succinate-ubiquinone reductase, and cytochrome oxidase). The activities of complexes I and IV were more selectively affected and could be taken as markers of mitochondrial biogenesis.

Interestingly, the calculated fraction of mitochondria in state 3 in the tissue was at a minimal value at P, suggesting a turning point for mitochondrial function and signaling to the cytosol at that specific time point of the ovarian cycle.

Concerning ovary slice respiration, the observed linear rates of oxygen uptake have to be interpreted as the result of a fast and random oscillation of mitochondria between states 3 and 4 in the cells, driven by local ATP demands, taking into account the marked difference in oxygen uptake of both mitochondrial states. It has been postulated that, under physiological conditions, a mitochondrial subpopulation is exposed to high ATP and another subpopulation is exposed to ADP levels that stimulate respiration. (39). It is then clear that the proliferative phase of the ovarian cycle shows a significantly increased mitochondrial respiration associated with an active synthesis of mitochondrial components and mitochondrial biogenesis, a process that is understood to serve to increased organ energy demands.

The activity of mtNOS was found 79% higher in phase P than in phase D1, and the increased biochemical activity was reflected in the increased mtNOS functional activity able to inhibit mitochondrial respiration. Although it is not clear how much mtNOS contributes to total cellular NO production in the

**Table 3. mtNOS functional activity in inhibition of mitochondrial respiration**

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Respiratory Rate, ng-at O/min·mg protein⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>State 3</td>
<td>D1  129 ± 3</td>
</tr>
<tr>
<td>+L-arginine (a)</td>
<td>D2  147 ± 4</td>
</tr>
<tr>
<td>+L-NAME (b)</td>
<td>P   260 ± 7*</td>
</tr>
<tr>
<td>mtNOS functional activity [b-a]</td>
<td>E   145 ± 4</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 9 rats per group. mtNOS, mitochondrial NO synthase; L-NAME, N⁵-nitro-L-arginine methyl ester. Respiratory rates were determined with mitochondria (0.4–0.6 mg/ml) supplemented with 10 mM succinate and 0.5 mM ADP. *P < 0.05, P vs. D1.

**Table 4. Fraction of mitochondria in states 3 and 4 when respiring in isolated ovary as a function of the phases of the ovarian cycle**

<table>
<thead>
<tr>
<th>Phase</th>
<th>Ovary O₂ Uptake, ng-at O/min·g organ⁻¹</th>
<th>Mitochondrial O₂ Uptake, ng-at O/min·mg protein⁻¹</th>
<th>Fraction of Mitochondria, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>State 3 + arginine</td>
<td>State 4</td>
<td>State 3</td>
</tr>
<tr>
<td>Diestrus (D1)</td>
<td>622±22</td>
<td>108±9</td>
<td>43±4</td>
</tr>
<tr>
<td>Diestrus (D2)</td>
<td>758±24</td>
<td>110±7</td>
<td>49±4</td>
</tr>
<tr>
<td>Proestrus (P)</td>
<td>882±22*</td>
<td>214±9*</td>
<td>71±6*</td>
</tr>
<tr>
<td>Estrus (E)</td>
<td>748±23</td>
<td>106±7</td>
<td>56±4</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 9 rats per group. Mitochondrial subpopulations in states 3 and 4 were calculated as indicated in the text and in Ref. 6. *P < 0.05; †P < 0.001, P vs. D1.
Table 5. Enzymatic and electron transfer activities in ovary mitochondria during the estrous cycle

<table>
<thead>
<tr>
<th>Activities</th>
<th>D1</th>
<th>D2</th>
<th>P</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzymatic activities</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mtNOS</td>
<td>1.4 ± 0.1</td>
<td>2.0 ± 0.1</td>
<td>2.5 ± 0.2*</td>
<td>1.8 ± 0.1</td>
</tr>
<tr>
<td>Mn-SOD</td>
<td>6.7 ± 0.5</td>
<td>6.1 ± 0.4</td>
<td>5.1 ± 0.4*</td>
<td>5.8 ± 0.4*</td>
</tr>
<tr>
<td>Electron transfer activities</td>
<td>Complexes I–III</td>
<td>359 ± 34</td>
<td>369 ± 36</td>
<td>462 ± 44*</td>
</tr>
<tr>
<td></td>
<td>Complexes II–III</td>
<td>111 ± 10</td>
<td>114 ± 11</td>
<td>132 ± 13</td>
</tr>
<tr>
<td></td>
<td>Complex IV</td>
<td>260 ± 25</td>
<td>335 ± 29</td>
<td>358 ± 30*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 9 rats per group. Electron transfer activities: complex I–III (NADH-cytochrome c reductase), complex II–III (succinate-cytochrome c reductase), and complex IV (cytochrome oxidase) are expressed in nmol cytochrome c reduced or oxidized·min⁻¹·mg protein⁻¹. Enzymatic activities: mtNOS is expressed as nmol NO·min⁻¹·mg mitochondrial protein⁻¹; mitochondrial superoxide dismutase (Mn-SOD) is expressed as pmol/mg mitochondrial protein. *P < 0.05, P vs. D1.

Ovary, the mtNOS contribution is not to be disregarded. For instance, it has been claimed that mtNOS provides 62% of total heart NO, with the remaining 38% due to endothelial NOS activity (21).

The observed increases of NO production, cytochrome oxidase activity, and mitochondrial mass in the ovary proliferative phases are consistent with the recently reported role of endogenous NO in mitochondrial biogenesis in mammals (34). Nisoli et al. (34) were able to elegantly describe how NO trigger mitochondrial biogenesis in a process mediated by cGMP and peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) that operates in adipocytes, brain, liver, heart, and HeLa cells. The process of mitochondrial biogenesis in the proliferative phase of the ovarian cycle seems driven by FSH, which activates the membrane receptors of ovarian cells and increases the levels of cytosolic messengers and the synthesis of mitochondrial components, specially mtNOS and complexes I and IV.

It has been recognized that NO increases the mitochondrial rates of O₂ and H₂O₂ production; then, a process that activates mtNOS triggers a feed-forward process of mitochondrial free-radical production. Mitochondria are an active source of NO (19, 20) and O₂⁻ (5), two free radicals that are able to sustain, likely mediated by peroxynitrite, a continuous free-radical chain reaction involving lipid peroxidation, and protein damage as a cytotoxic processes. The progressively increased rate of free-radical generation in phases D1 to P leads to cumulated mitochondrial oxidative damage, with increased TBARS and protein carbonyls, which are associated with mitochondrial dysfunction, a mitochondrial condition that triggers mitochondria-dependent apoptosis (26). Two types of tissues can be differentiated according to the rate of execution of the cell death program: tissues that show a fast apoptosis, such as ovary and thymus, and tissues that show a slow apoptosis, such as heart and brain (26). Bustamante et al. (11) described the kinetics of thapsigargin-dependent thymus apoptosis in terms of the t₀.₅, the time to reach the half-maximal response of each process. The sequence is: cytosolic Ca²⁺ (t₀.₅ = 2.5 min), mtNOS activity and cellular H₂O₂ steady-state level (t₀.₅ = 15 min), TBARS levels (t₀.₅ = 30 min), mitochondrial dysfunction (as decreased state 3 respiration and loss of membrane potential and cytochrome c; t₀.₅ = 101–133 min), caspase 3 activation (t₀.₅ = 210 min), and DNA laddering (t₀.₅ = 260 min). The same sequence seems to operate in the ovary, in which the increases in mtNOS activity and TBARS levels indicate the triggering of the NO-dependent oxidative mitochondrial damage that leads to mitochondria-dependent apoptosis. In this connection, Murray et al. (30) reported that addition of ascorbic acid to cultured mouse preantral follicles decreased the rate of apoptosis and increased the percentage of follicles that maintain basement membrane integrity. A protective role of the Bcl-2 family in the hormonal regulation of follicular atresia in rodents was also reported (24).

The fine regulation by H₂O₂ of the physiological cell cycle was advanced by Antunes and Cadenas (1), who observed in Jurkat T cells that H₂O₂ steady-state concentrations below 0.7 μM place cells in a proliferative state, whereas at 1.0–3.0 μM H₂O₂ cells develop apoptosis and that at levels higher than 3.0 μM H₂O₂ cells undergo necrosis. It is likely that NO exerts a similar fine regulation of mammalian cell cycle, as seems suggested by this study on ovary. Relatively low levels of NO drive the cell signaling for follicle proliferation, whereas rela-

![Fig. 5](http://ajpendo.physiology.org/)

**Fig. 5.** Correlation between mitochondrial NO synthase (mtNOS) biochemical activity, and mtNOS functional activity, expressed as the rate of mitochondrial state 3 oxygen uptake that is inhabitable by NO, the product of mtNOS activity (r² = 0.99). Experimental determinations are described in MATERIALS AND METHODS.

![Fig. 6](http://ajpendo.physiology.org/)

**Fig. 6.** Oxidation products, protein carbonyls and thiobarbituric acid-reactive substances (TBARS), in mitochondria from rat ovary during the ovarian cycle. The methods used to measure protein carbonyls and TBARS are described in MATERIALS AND METHODS (n = 36 rats). **P < 0.001.
tively high NO levels trigger mitochondria-dependent follicle apoptosis.

The general role of mitochondria as the ATP-provider powerhouse of the cell, as a source of NO to signal for mitochondrial proliferation and mitochondria-dependent apoptosis, and as the main intracellular source of O$_2^-$ and H$_2$O$_2$ (4, 6, 8), appears well adapted to serve the proliferation-apoptosis sequence of the ovarian cycle. Understanding of the mitochondrial role in the proliferation and death of the ovarian follicles will be useful to describe the physiology of the ovarian cycle, with applications in the development of contraceptives and methods for extending female reproductive life span for in vivo and in vitro fertilization.

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