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Role of nitric oxide on mitochondrial biogenesis during the ovarian cycle

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1. ABSTRACT

The mitochondrial changes in the ovary during the ovarian cycle are adapted to a cyclically increased-decreased energy demand. In the proliferative phase, the increased energy needs are sustained by the recruitment of mitochondria in active state 3, by an increased tissue O_2 consumption and ATP production and by an increase in the number of mitochondria. In the phase of decreased energy needs, mitochondria-dependent apoptosis reduces tissue and mitochondrial O_2 uptake. The ovary morphological changes during the cycle describe a process in which the follicles undergo a clear cycle with two sequential phases: proliferation and apoptosis. The follicular growth stimulated by FSH characterizes a tissue that shows a quick cell proliferation. During the ovarian cycle, tissue and mitochondrial O_2 uptakes, mitochondrial mass, mitochondrial NO production and cytochrome oxidase activity exhibit a biphasic pattern, with marked increases in the ovary proliferative phases. Relatively low levels of NO seem to drive the cell signaling for follicle proliferation, whereas relatively high NO levels trigger mitochondria-dependent follicle apoptosis.

2. INTRODUCTION

Under physiological conditions, adaptation of cell metabolism to energy demands is basically made by adjustment of mitochondrial function. Mitochondria are the main intracellular cellular site of energy production and mitochondrial oxidative phosphorylation is coupled to the electron transfer in the respiratory chain. A physiological situation that demands an increase in energy supply can be sustained for by the switching of mitochondria from resting state 4 to active state 3, with the corresponding increase in oxygen consumption and ATP production, or by an increase in the number of mitochondria.

Usually, mitochondrial biogenesis is a concept that explain the formation of new mitochondria from pre-existing organelles by growth and division (1). Mitochondria divide themselves during cell mitosis and each daughter cell keeps the physiological number of mitochondria (2). However, in physiological situations that demand a highly increased energy production, such as in cellular differentiation (3) or in prolonged adaptation to a low temperature environment (4), a marked increase in

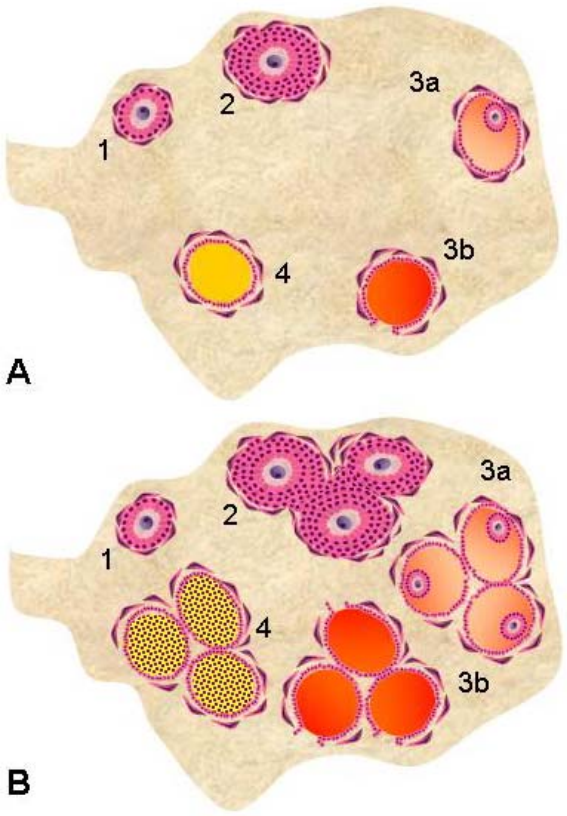

Figure 1. Scheme of the ovarian cycle. **A**. Normal ovarian cycle in rats: *(1)* Ovary at phase D1: primary follicle; *(2)* Ovary at phase D2: growing of follicle; *(3)* Ovary at phase P: *(3a)* preovulatory follicle, and *(3b)* ovulatory follicle; *(4)* Ovary at phase E: corpora lutea. B. Over-stimulated ovarian cycle in rats: *(1)* Ovary at phase D1: primary follicle; *(2)* Ovary at phase D2: increased number of recruited and highly proliferative follicles; *(3)* Ovary at phase P: (3a) large and multiple preovulatory follicles, and *(3b)* multiple ovulatory follicle; *(4)* Ovary at phase E: corpora lutea with massive apoptosis.

mitochondrial mass without cell division has been reported. Thus, mitochondrial biogenesis affords a physiological mechanism of cell adaptation to increase energy demand.

3. THE OVARIAN CYCLE

The ovarian cycle is the term used to describe the series of events associated with a developing oocyte, or egg cell, within the ovaries. Ovarian cycle and follicular development are controlled by circulatory feedback between the ovarian hormones and the hypothalamic-pituitary axis (5). Gonadotropin releasing hormone (GnRH) is secreted by the hypothalamus and stimulates the release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary gland.

3.1. Phases of the mammalian ovarian cycle

FSH initiates follicular growth and the secretion of estrogens by the growth follicles. During each cycle the increased FSH concentration recruit growing antral follicles and the concept of "cyclic recruitment" has been proposed to describe this rescue of follicles from degeneration (6). FSH, in turn, causes a burst of activity in a certain population, about 20% of ovarian follicles. In these follicles, proliferation and growth of granulosa and teca cells occur with the secretion of a fluid rich in estrogens and with the formation of an antrum or pocket within the follicles. The hormonal predominance of a dominant follicle determines its morphological changes and growth (7) by increasing diameter and the number of granulose cells (8), whereas non dominant follicles become atretic.

In physiological conditions FSH stimulates ovarian follicular growth and LH controls their hormonal secretory capacity: Maximal levels of FSH and LH are observed at the end of the proestrus phase with maximal follicular growth and simultaneous ovulation. These hormones induce rapid follicular swelling, and a portion of the follicular wall thins as the volume expands. Fluid begins to leak through this weakened wall area; soon after it ruptures, it extrudes the ovum.

Specifically, gonadotropins promote cell proliferation and suppress ovarian cell apoptosis by activation of the cAMP-dependent pathway 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 (9).

A decrease in the levels of circulating LH results in follicular cell death and in abortion of the generation of corpora lutea (10). Very large quantities of estrogen and progesterone are secreted by the follicle after ovulation, particularly when it has become a fully formed corpus luteum. Consequently, both the hypothalamus and the pituitary gland are greatly inhibited, and the circulating levels of both FSH and LH are very low. Follicular growth is therefore suppressed. Except for the dominant follicle, the ovary is functionally quiescent. The involution of the corpus luteum removes the feedback inhibition of the hypothalamic-hypophyseal axis, restoring the normal function feed back function. The release of both FSH, which stimulates growth of the follicles, and LH, which controls their secretory capacity increase as the hypophyseal part of the ovarian cycle, being the increase in FSH relatively more marked than the increase in LH, and a new cycle is initiated (11).

3.2. The ovarian cycle in laboratory rodents

Laboratory rodents, *e.g.*, mice, rats and hamsters, 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, while they ovulate spontaneously, they 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 occur within a few days. Figure 1.A show a scheme of the ovarian cycle in the

rat, which is divided in: estrus (E), or ovulation phase with a duration of about 12 hours, usually in the evening; metestrus (M), or luteal phase with a duration of 1 day; diestrus (D) or follicular phase with a duration of one (D_1), or two (D_2) days; and proestrus (P), or the end of follicular phase, with the highest levels of FSH and LH and a duration of about 12 hours (12).

3.3. The growth of the ovarian follicle demands a high supply of energy

The follicular growth stimulated by FSH constitutes a physiological situation characterized by a fast cell proliferation, an endergonic process that absolutely requires a sustained high rate of ATP production.

Although the histological changes and the hormone dependence of the ovarian cycle in mammals are well known, including the biochemical features of the signalling that follows to hormone-receptor bindings, there is a paucity of information concerning the energetics and the mitochondrial function during the ovarian cycle. It is presumable that a mitochondrial adaptation would be necessary during the ovarian cycle to match the highly increased energy demands.

3.4. The over-stimulated rat ovarian cycle

Hormonal, FSH and LH, over-stimulation has been chosen as the experimental approach to synchronize and maximize the ovary changes to study the mitochondrial adaptation during the ovarian cycle. High doses of FSH and LH are currently used for *in vivo* fertilization (13-15), and the used FSH and LH doses are similar to the ones used in human fertility treatments to increase the number of ovarian follicles in maturation. Rats were injected daily with recombinant human follicle stimulating hormone (FSH; Follitropin beta, Puregon®, Organon Laboratory) 40 IU/kg, ip, in the first and second day of diestrus (D1 and D2), and with recombinant human luteotropic hormone (LH; Lutropin alfa, Luveris®; Serono, Biotech & Beyond) 40 IU/kg, ip, in the proestrus (P) day (16).

A scheme of the changes in the ovarian cycle in the over-stimulated rats is shown in Figure 1-B.The proliferative phase induced by FSH treatment promoted follicular growth and prevented follicular atresia, without granulosa or oocyte apoptosis detected in phases D1 and D2. After LH treatment, a series of well developed corpora lutea, although not functional, followed to the super-ovulation, and one day later, 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 high progesterone levels at proestrus evening (17). There is ample evidence that luteal regression involves apoptotic mechanisms in cycling rat ovaries (18-20). 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.

4. REGULATION OF MITOCHONDRIAL BIOGENESIS

4.1. Genetic control

The biogenesis and function of mitochondria rely upon the regulated expression of nuclear genes. Recent evidence points to both transcriptional activators and co-activators as important mediators of mitochondrial maintenance and proliferation. Several sequence-specific activators including NRF-1, NRF-2, Sp1, YY1, CREB and MEF-2/E-box factors, among others, have been implicated in the expression of proteins constitutive of the mitochondrial respiratory chain (21-24). Notably, recognition sites for NRF-1, NRF-2 and Sp1 are common to most nuclear genes encoding respiratory subunits, mitochondrial transcription and replication factors, as well as certain heme biosynthetic enzymes and components of the protein import machinery. Moreover, genetic evidence supports a role for NRF-1 in the maintenance of mtDNA during embryonic development. Despite these advances, the means by which multiple transcription factors are integrated into a program of mitochondrial biogenesis remains an open question. New insight into this problem came with the discovery of the transcriptional co-activators, PGC-1, alpha and beta. PGC-1alpha can up-regulate nuclear genes that are required for mitochondrial biogenesis in part through a direct interaction with NRF-1 (24). PGC-1beta coactivates estrogen receptor-related receptors that increase the expression of Acyl-Coa dehydrogenase (25). PGC-1beta expression is positively associated with lipid oxidation and negatively related to glucose oxidation, whereas PGC-1alpha expression is positively related to glucose uptake and oxidation and negatively related to lipid oxidation (26).

4.2. Epigenetic control

Epigenetic factors have been involved in mitochondrial biogenesis: the inhibition of oxidative phosphorylation in mitochondrial myopathies (27); the uncoupling of mitochondria by thyroid hormone (28-30); the modification of the mitochondrial membrane composition (31); and pharmacological products as Diazepam (32), induce mitochondrial proliferation.

Recently, nitric oxide (NO) has been implicated in mitochondrial biogenesis by the stimulation of guanylate cyclase, generation of cGMP and activation of PGC1-alpha (33,34). Chronic treatment with NO donors produced a complete inhibition of brown fat cell proliferation with increased mitochondrial mass (35). The new mitochondria were able to carry out a coupled respiration with ATP production (34).

5. NITRIC OXIDE AND MITOCHONDRIA

Nitric oxide (NO) is synthesized by an enzymatic reaction catalyzed by a nitric oxide synthase (NOS) that has arginine, NADPH and O_2 as substrates and that produces citrulline, H_2O and NO as reaction products. Three different genomic NOS have been identified; they are named neuronal NOS (nNOS or NOS-1); inducible or macrophague NOS (iNOS or NOS-2) and endothelial NOS

(eNOS or NOS-3) (36). Recently, Ghafourifar and Richter (37) and Giulivi *et al.* (38) described the mitochondrial production of NO by a specialized isoform of NOS, the mitochondrial nitric oxide synthase (mtNOS) (39) that carries out a classical NOS reaction, requiring NADPH, arginine, O_2 and Ca^{2+}/calmodulin for enzyme activity (40,41).

Nitric oxide acts on mitochondrial respiration at two main levels. In the arterioles NO produces vasodilation and increases blood flow and O_2 delivery to the tissues. Moreover, NO directly favours O_2 dissotiation from hemoglobin (42). In a whole, vascular NO increases the O_2 supply to cells and mitochondria. In the cell NO inhibits cytochrome oxidase activity and mitochondrial respiration.

5.1. NO as regulator of mitochondrial respiration

Nitric oxide has three target sites at the mitochondrial respiratory chain in which NO, directly or indirectly after peroxynitrite formation, inhibits electron transfer. The three sites are: NADH-dehydrogenase (complex I); ubiquinol-cytochrome c reductase (complex III); and cytochrome oxidase (complex IV).

Cytochrome oxidase activity is inhibited by NO in a competitive form with oxygen (43-47). NO is the first molecule that fulfills the requirement for a physiological modulator of cytochrome oxidase activity with an O_2-competitive mode of binding and inhibition. Nitric oxide is intramitochondrially produced by mtNOS at a fair rate near the target site, and it has been calculated that endogenous mtNOS activity inhibits mitochondrial respiration in the tissues by 18-25 % (48).

Peroxynitrite, the product of NO and superoxide radical ($O_2^{\cdot-}$), inhibits in a close to irreversible manner both complex I (46,49) and III (50,51).

Superoxide radical was established as the stoichiometric precursor of mitochondrial H_2O_2 (52-54). The mitochondrial production of H_2O_2 was characterized as a by-product of electron transfer by the auto-oxidation of components of the respiratory chain in a process called "electron leak" (55,56). The majority of mitochondrial $O_2^{\cdot-}$, 70-80 %, is vectorially released to the mitochondrial matrix, but recently it was found that a 20-30 % is released to the mitochondrial intermembrane space (57,58). Both free radicals, $O_2^{\cdot-}$ and NO, are metabolized into the mitochondrial matrix through the formation of relatively stable and non-radical species, H_2O_2 and $ONOO^-$ (59). However, these stable species are also potentially harmful; they yield, after homolytic splitting, $HO^{\cdot}$ (60), a strong oxidant which abstracts hydrogen atoms and initiates the free radical reactions of the lipoperoxidation process. This free radical process, with initiation, propagation, inhibition and termination reactions, generates $ROO^{\cdot}$ and $^1O_2$.

5.2. NO and mitochondrial signalling

Nitric oxide was initially recognized as an intercellular messenger (36) and later as an intracellular regulator (43,44,51,61). At present, $O_2^{\cdot-}$, H_2O_2 and NO are considered part of an integrated system of mitochondrial signaling for cellular regulation (62).

There is evidence that H_2O_2 and NO modulate mitogen-activated protein kinases (MAPK), the widespread integral components of intracellular phosphorylation and dephosphorylation signaling cascades involved in cell survival, proliferation, differentiation, and death (63). The interactions are complex and seem to involve GSH and $ONOO^-$ (64,65). Both, H_2O_2 and NO diffusing from mitochondria to the cytosol appear to constitute a pleiotropic signal for a series of cellular processes, among them JNK signaling (66,67). Nitric oxide diffusing out of mitochondria may also inactivate JNK1 via S-nitrosylation (66). The concept of narrow concentration ranges of the messenger molecules for different or opposite biological actions, as observed for H_2O_2 levels in the proliferation/apoptosis transition (68) is now considered for effector systems with two regulators, H_2O_2 and NO, with the result of four different responses for the combination of two signals with two, low or high, levels of each signal.

6. MITOCHONDRIAL FUNCTION DURING THE OVARIAN CYCLE

Over-stimulation by FSH and LH was used as the experimental approach to synchronize and maximize ovarian changes and study the mitochondrial function in rats (16). In Figure 1 we schematized the morphological changes in the over-stimulated rat ovarian cycle.

6.1. Tissue and mitochondrial oxygen uptake during the ovarian cycle and changes in mitochondrial mass

In the ovary of stimulated rats we determined both tissue O_2 uptake and mitochondrial O_2 consumption, and the changes in mitochondrial mass during the cycle. Tissue O_2 uptake during the ovarian cycle, determined in ovary cubes suspended in Krebs medium and followed with a Clark-type O_2 electrode, progressively increased along with the FSH treatment and reached the highest respiratory rate at the proestrus phase (P), when the respiratory increase was 42 % in relation to D1. In the estrus phase, ovary respiration decreased to levels similar to the ones shown in D1 (16) (Figure 2).

Mitochondrial respiration in state 3, the maximal physiological rate of oxygen uptake and ATP production, also showed a progressive increase in O_2 uptake in the proliferative phases along with FSH treatment with a maximal rate of respiration in the P phase (16) (Figure 2). It was assumed, as a starting concept, that mitochondrial O_2 uptake wholly accounts for ovary respiration and that mitochondria oscillate between state 3 and state 4. 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 signalling to the cytosol at that specific time point of the ovarian cycle.

Mitochondrial mass, expressed in mg mitochondrial protein/g ovary, calculated from the ratio of cytochrome oxidase activity in ovary homogenates and in

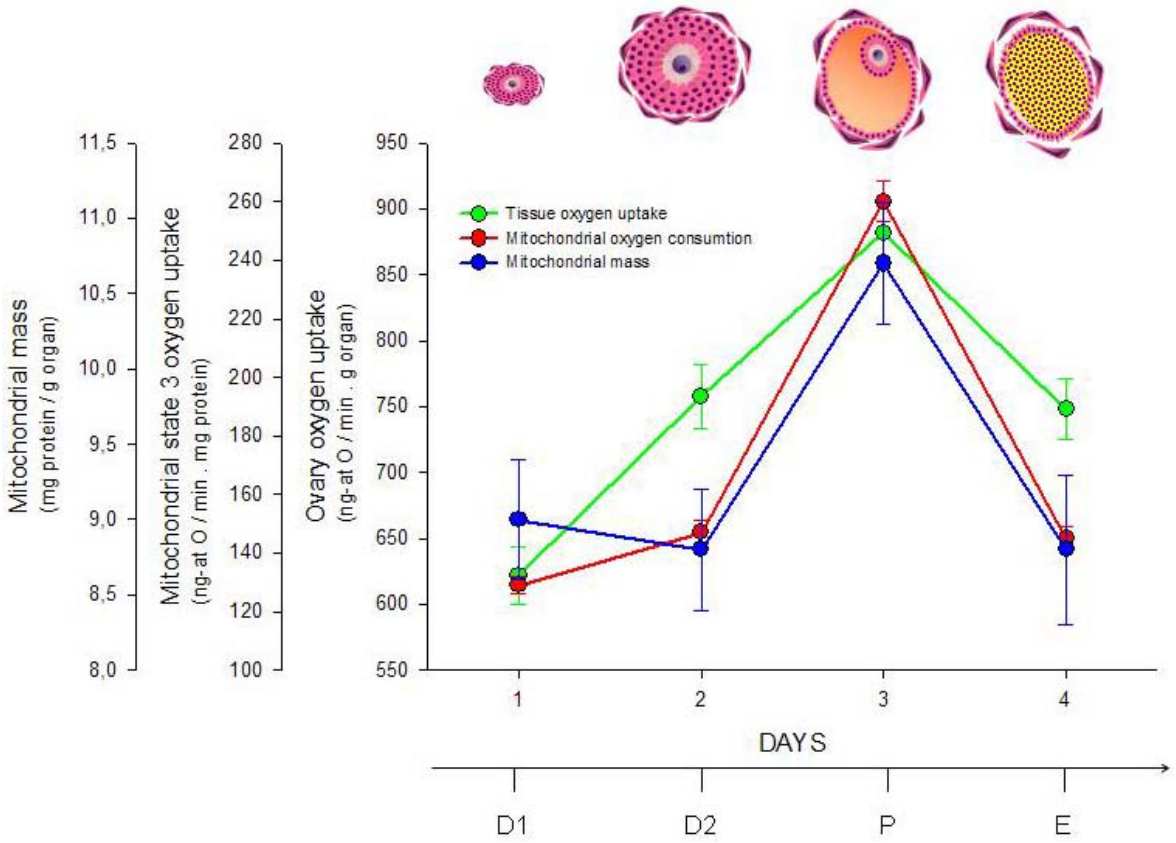

Figure 2. Tissue and mitochondrial O_2 uptake during the rat over-stimulated ovarian cycle and changes in mitochondrial mass. Tissue and mitochondrial O_2 consumption were determined polarographically in ovary pieces of $1mm^3$ and in isolated mitochondria with a Clark-type electrode (Hansatech Instrumens Ltd.) as described in Navarro *et al.* (16) and in Boveris *et al.* (80). Oxygen uptake is expressed in ng-at O / min and referred to g of tissue or mg of mitochondrial protein. Respiratory rates were determined with 10 mM succinate as substrate and state 3 active respiration was established by addition of 0.5 mM ADP. 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 (16,81,82).

isolated mitochondria, was increased by 20 % at the end of the proliferative phase in P (16) (Figure 2).

Concerning ovary slices respiration, the observed linear rates of O_2 uptake have to be interpreted as the result of a fast and at random oscillation of mitochondria between states 3 and 4 in the cells, driven by local ATP demands, taking into account the marked difference in O_2 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. (69). 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 the increased organ energy demands.

This biphasic dependence of respiration along the ovarian cycle was characterized in this study in terms of mitochondrial function.

6.2. The mitochondrial electron transfer activities during the ovarian cycle

The membrane-bound mitochondrial electron transfer activities also reached a maximal value in ovary mitochondrial membranes at the P phase (Table 5). NADH-cytochrome c reductase activity (Complex I-III) was increased by 29 %; succinate-cytochrome c reductase activity (Complex II-III) was increased by 19 %; and cytochrome oxidase (Complex IV) was increased by 38 %, in comparison with the D1 phase (16). 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 up-regulated and can be taken as markers of mitochondrial biogenesis (Figure 3).

6.3. Mitochondrial oxidative damage during the ovarian cycle

The mitochondrial content of oxidative stress markers, protein carbonyls and malonaldehyde, this latter a

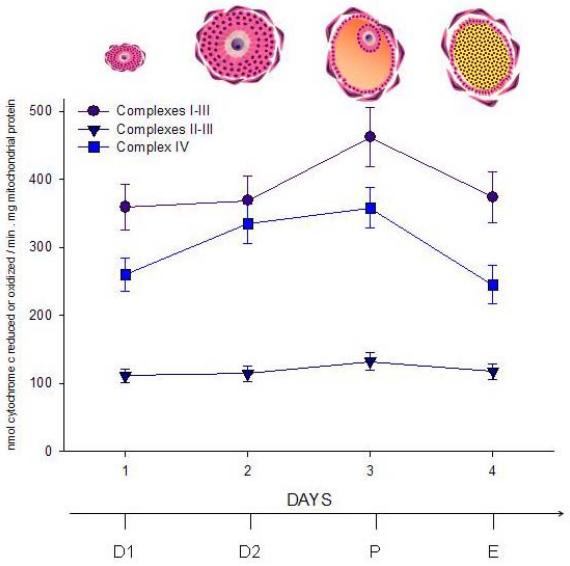

Figure 3. The mitochondrial electron transfer activities during the rat over-stimulated ovarian cycle. The membrane-bound activities of Complexes I-III, II-III, and IV were determined spectrophotometrically at 30 °C with submitochondrial membranes suspended in 100 mM phosphate buffer (pH 7.4) added with the corresponding substrates (16,83,84).

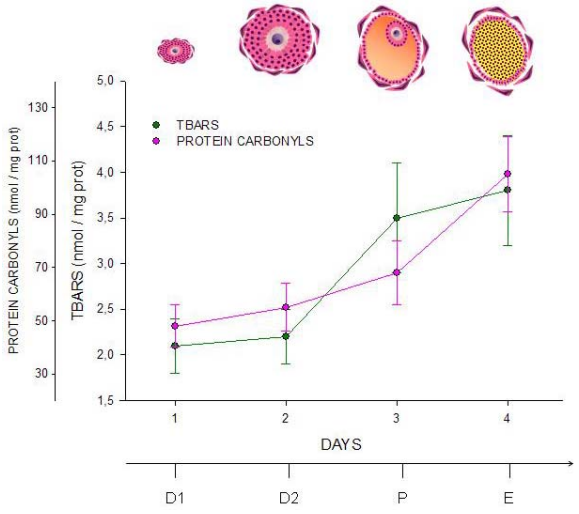

Figure 4. Mitochondrial oxidative damage during the rat over-stimulated ovarian cycle. The mitochondrial content of thiobarbituric acid-reactive substances (TBARS) and protein carbonyls were determined in submitochondrial membranes by the original assays of Fraga *et al.* (85) and of Oliver *et al.* (86), modified as described (82).

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 (16) (Figure 4). 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 is associated with mitochondrial dysfunction, a condition that is capable of triggering mitochondria-dependent apoptosis (70).

6.4. Mitochondrial nitric oxide production during the ovarian cycle

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 (16) (Figure 5). Although it is not clear how much mtNOS contributes to total cellular NO production in the 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 eNOS activity (71).

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 (33). The process of mitochondrial biogenesis in the proliferative phase of the ovarian cycle seems driven by FSH that activate the membrane receptors of ovarian cells and that 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 O_2^- and H_2O_2 production; then, a process that activates mtNOS triggers a feed-forward process of mitochondrial free radical production. Mitochondria are an active source of NO (72,73) and O_2^- (74), two free-radicals which 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 (70).

Two types of tissues can be differentiated according to the rate of execution of the cell death program: tissues that show a fast apoptosis, as ovary and thymus, and tissues that show a slow apoptosis, as the heart and the brain (70). Bustamante *et al.* (75) described the kinetics of thapsigargin-dependent thymus apoptosis in terms of the $t_{0.5}$, the time to reach the half-maximal response of each process. The sequence is: cytosolic Ca^{2+} ($t_{0.5}$ = 2.5 min), mtNOS activity and cellular H_2O_2 steady-state level ($t_{0.5}$ = 15 min), TBARS levels ($t_{0.5}$ = 30 min), mitochondrial dysfunction (as decreased state 3 respiration and loss of membrane potential and cytochrome c; $t_{0.5}$ = 101-133 min), caspase 3 activation ($t_{0.5}$ = 210 min), and DNA laddering ($t_{0.5}$ = 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.* (76)

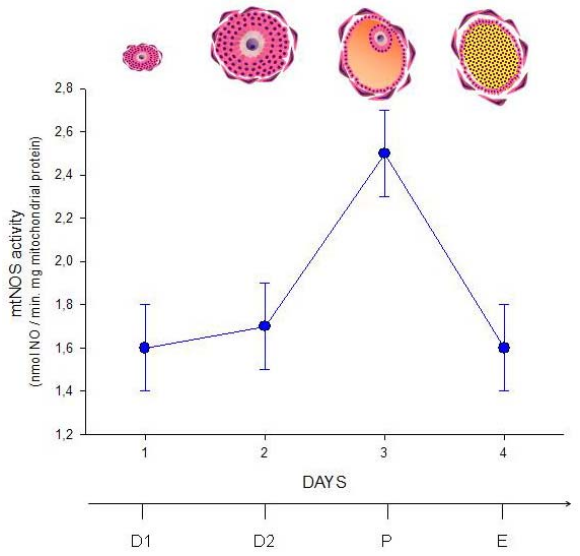

Figure 5. Mitochondrial nitric oxide production during the rat over-stimulated ovarian cycle. Mitochondrial NO production was determined by the oxyhemoglobin (HbO_2) oxidation assay as described (87). Production of NO was calculated from the absorbance change that was inhibited by 2 mM N^G-methyl-L-arginine, usually 92-96 %, and expressed in nmol NO/min.mg protein.

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 (9).

The fine regulation by H_2O_2 of the physiological cell cycle was advanced by Antunes and Cadenas (77) that observed in Jurkat T-cells that H_2O_2 steady state concentrations below 0.7 μM place cells in a proliferative state, whereas at 1.0-3.0 microM H_2O_2 cells develop apoptosis, and that at levels higher than 3.0 microM H_2O_2 cells undergo necrosis. It is likely that NO exerts a similar fine regulation of ovarian cell cycle. Relatively low levels of NO drive the cell signaling for follicle proliferation, whereas relatively high NO levels trigger mitochondria-dependent follicle apoptosis.

7. PERSPECTIVE

Mitochondrial NO production, with changes in the NO generation during the ovarian cycle according to the energy demands of the cells, could afford a mechanism to signal the proliferation-apoptosis sequence in the ovary. Thus, the role of mitochondria as source of NO and of O_2^- and H_2O_2 (78,79) appears well adapted to serve in the cellular and mitochondrial mass changes during 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 human gynecology.

8. ACKNOWLEDGMENTS

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