Calcium-dependent up-regulation of mitochondrial electron transfer chain gene expressions in human luteinized granulosa cells

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Objective: To evaluate the transcription and translation ability of mitochondria in terminally differentiated granulosa cells, these cells were incubated with ionic calcium.
Design: Prospective laboratory research.
Setting: In vitro fertilization laboratory in a university hospital.
Patient(s): Granulosa cells were harvested from 50 female patients undergoing IVF.
Intervention(s): Analysis of mitochondrial gene expression by semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) and of mitochondrial-encoded proteins by Western blot.
Main Outcome Measure(s): Comparison of the RNA expression levels of genes including cytochrome *c* oxidase subunit I (COX I), adenosine triphosphate synthase 6 (ATPase 6), flavoprotein, and succinate-ubiquinone oxidoreductase, and protein levels of COX I and flavoprotein in different calcium ion treatment groups.
Result(s): There were dose-dependent increases in RNA expressions of the four genes analyzed from granulosa cells cultured in a serial concentration of calcium ions. This effect was abolished when cells were preincubated with the extracellular calcium-chelating agent, Ethylene glycol-bis (2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA). The effect of ionic calcium on both the nuclear- and mitochondrial-encoded subunits also was determined. Expression levels of mitochondrial transcription factor A in RNA significantly increased in granulosa cells that were exposed for 24 and 48 hours to 0.5 and 1 μM A23187.
Conclusion(s): The present study is the first report to present calcium-dependent increases in the transcription and translation levels of both nuclear-encoded and mitochondrial-encoded mitochondrial respiratory enzyme subunits and also indicates that mitochondrial transcription factor A is involved in mitochondrial biogenesis. (Fertil Steril® 2005;84(Suppl 2):1104–8. ©2005 by American Society for Reproductive Medicine.)

Key Words: Granulosa cells, mitochondria, RT-PCR

In eukaryotic cells, mitochondria are special organelles that are responsible for the synthesis of adenosine triphosphate (ATP). Two distinct genomes exist in all eukaryotic cells. One is located in the nucleus and is transmitted in a mendelian fashion, whereas the other is located within the mitochondria and is transmitted through maternal lineage. Human mitochondrial (mt) DNA is a circular, histone-free molecule composed of 16.6 kb of DNA, present in one or more copies in every mitochondrion. It encodes 13 protein subunits of a total of about 80 constituting the oxidative phosphorylation system, with the remainder being encoded by nuclear genes that are imported into the mitochondrion.

Human mtDNA also encodes 2 ribosomal RNAs and 22 transfer RNAs. The oxidative phosphorylation capacity of mitochondria is determined by the interplay between nuclear and mitochondrial genes. Whereas mtDNA encodes 13 polypeptides that are the components of the respiratory system, nuclear DNA encodes the majority of respiratory chain proteins and all of the proteins and enzymes that regulate the replication and transcription of mtDNA (1).

In mammals, an essential component of the mitochondrial transcription initiation complex is mitochondrial transcription factor A (mtTFA), a nuclear-encoded 25-kDa protein that bends and unwinds mtDNA upon binding and that belongs to the high-mobility-group (HMG)-box family of proteins (2, 3). Mitochondrial transcripton factor A regulates both mitochondrial transcription and replication by permitting transcription of the RNA primer to commence from the unwinding L-strand DNA (4). The gene of mtTFA has been isolated in humans and is structured with 7 exons and 6 introns (5).

Granulosa cells play a major role in regulating ovarian physiology, including ovulation and luteal regression (6). Granulosa cells secrete a wide variety of growth factors that

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may attenuate gonadotropin's action in the ovary in paracrine–autocrine processes (7, 8). Most of these factors do not directly affect oocytes but exert their actions via granulosa cells. The presence of granulosa cells appears to be beneficial for oocyte maturation and early development (9). Nevertheless, granulosa cells also might have a negative effect upon oocytes. It has been demonstrated that the increased apoptotic potential in oocytes of aged mice is because of the presence of cumulus cells (10).

The decline in reproductive ability in women as they age is associated with a loss of follicles and a decrease in oocyte quality. Aging-associated apoptosis increases in follicular granulosa cells and consequently decreases ovarian fecundity (11, 12). In the present study, to determine the mitochondrial biogenesis ability of human granulosa cells, the expressions of the RNA of five genes including cytochrome *c* oxidase subunit I (COX I), ATP synthase 6 (ATPase 6), flavoprotein, succinate-ubiquinone oxidoreductase (SDHA), and mtTFA were determined. Nuclear-encoded flavoprotein and mitochondrial-encoded COX I protein expression levels also were detected.

MATERIALS AND METHODS

Granulosa Cells in Culture

The institutional review board of Taipei Medical University Hospital approved the study, which we intended to perform on granulosa cells discarded during an IVF program. Human granulosa cells were collected from patients who were recruited into an IVF-embryo transfer (IVF-ET) program. Ovarian stimulation was performed by desensitization using a gonadotropin-releasing hormone (GnRH) agonist followed by treatment with gonadotropins (follicle-stimulating hormone (FSH) and human menopausal gonadotropin (hMG)). Ovulation was induced by using human chorionic gonadotropin (hCG).

Oocytes then were retrieved by transvaginal ultrasonography-guided aspiration 34–36 hours after hCG administration. Granulosa cells were harvested from follicular aspirates by centrifugation through Ficoll-Paque, washed, and suspended in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO) containing 50 μg/mL of uridine supplemented with 10% fetal bovine serum, 100 IU/mL of penicillin, and 100 μg/mL of streptomycin. Human granulosa cells were grown at 37°C in a humidified atmosphere with 5% CO_2 for 3 days before the experimental treatment. A23187 was purchased from Sigma (St. Louis, MO). One millimole of A23187 dissolved in dimethyl sulfoxide was prepared freshly before study and diluted into Dulbecco's modified Eagle's medium just before use.

Extraction of RNA and Semiquantitative Reverse-Transcription-Polymerase Chain Reaction Analysis

Total RNA was extracted from cells with Trizol reagent according to the manufacturer's instructions. Total RNA (1–5 μg) was reverse-transcribed into cDNA using oligo(dT)18 as the primer and into MMLV reverse transcriptase (Ambion, Austin, TX), and 1 μL of the cDNA template was used separately to amplify different mRNAs. Amplifications by reverse-transcription polymerase chain reaction (RT-PCR) were performed with 1 mL of cDNA in a total volume of 25 μL of amplification buffer, 10 pmol of specific primers, and 2.5 U of Taq DNA polymerase (Life Technologies, Grand Island, NY).

Sequences of the oligonucleotide primers used in this study are listed as follows: mtTFA (forward, CCGGAGGGTCGCACGCGGGT; reverse, CAGATGAAAACCACCTCAAT), COX I (forward, GTCCTATCAATAGGAGCTGT; reverse, TTCGAAGCGAAGGCTTCTC), flavo-protein (forward, GCTCAGTATCCAGTAGTGGA; reverse, CCCTTCACGGTGTCGTAGAA), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; forward, ACCACAGTCCATGCCATCA; reverse, TCCACCACCCTGTTGCTGTA). The predicted sizes of the mtTFA, COX I, flavoprotein, and GAPDH RT-PCR products were 235, 299, 230, and 453 bp, respectively. For semiquantitative amplification, each cycle was performed at 92°C for 30 seconds, 58°C for 30 seconds, and 72°C for 60 seconds. The reactions were analyzed after 15, 20, 25, 30, 35, and 40 cycles, respectively, to optimize the linear range of amplification. The PCR reactions were optimized with respect to the annealing temperature and number of PCR cycles (13).

Each PCR product was run through a 2% agarose gel and was visualized with ethidium bromide staining. Cycle-dependent amplification of the housekeeping GAPDH mRNA was almost identical in different clones, which allowed semiquantitative comparison of mtDNA PCR products obtained with each sample by densitometric analysis. The relative expression levels of the PCR products were determined with an imaging densitometer, and results are expressed as a ratio of analyzed genes divided by GAPDH.

Western Blot Analysis

Total protein was extracted from harvested cells by using lysis buffer (25 mM Tris-phosphate, pH 7.8; 2 mM dithiothreitol; 10% glycerol; and 1% Triton X-100) with a protease inhibitor cocktail (Roche, Mannheim, Germany). The cell lysate was cleared of cell debris by low-speed centrifugation at 10,000 × *g* for 5 minutes. The protein concentration was measured by using a BCA protein assay kit (Pierce, Rockford, IL). Proteins were resolved on polyacrylamide gels, and then proteins were transferred onto a polyvinyldene diflouride membrane (PVDF; Amersham Pharmacia, Piscataway, NJ). Membranes were blocked for 1 hour at 4°C with 10% skim milk in TBST buffer (1 M Tris-HCl, 100 mM NaCl, and 1% Tween-20).

Blots were probed with the following primary antibodies: a monoclonal antibody against cytochrome *c* oxidase subunit I, a monoclonal antibody against flavoprotein (Molecular

Probes, Eugene, OR), or a polyclonal antibody against GAPDH. Blots then were incubated with the appropriate horseradish peroxidase–conjugated anti-IgG antibody. Antibody-bound protein was detected by using enhanced chemiluminescence and exposure to film.

Statistical Analysis

All experiments were repeated at least three times. An analysis of variance (ANOVA) was used to test statistically significant differences ($P<.05$) between experimental groups.

RESULTS

To test whether mitochondria in terminal-differentiated granulosa cells preserve their transcription and translation ability, human granulosa cells were incubated with ionic calcium. Expressions in RNA of four genes, COX I, ATPase 6, flavoprotein, and SDHA, were analyzed by semiquantitative RT-PCR. Three different A23187 dosages of 0.25, 0.5, and 1 μM were used to treat human granulosa cells for 48 hours. There were dose-dependent increases in the RNA expressions of the 4 genes analyzed (Fig. 1). Both mitochondrial-encoded mitochondrial protein transcripts, COX I and ATPase6, and nuclear-encoded mitochondrial protein transcripts, flavoprotein and SDHA, were significantly elevated compared with the control. This effect was abolished when granulosa cells were preincubated with the extracellular calcium (Ca^{2+})-chelating agent, Ethylene glycol-bis (2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA) (Fig. 2).

Western blot analysis was used to determine the effect of ionic calcium on nuclear-encoded flavoprotein and mitochondrial-encoded COX I protein expression levels. The effect of ionic calcium on both the nuclear- and mitochondrial-encoded subunits was also time-dependent (Fig. 3). However, calcium induction of protein expression in granulosa cell also was abolished by EGTA. To determine the possible factors participating in mitochondrial respiratory enzyme subunit transcription and translation, mtTFA was examined. Expression levels of mtTFA were determined by treatment of granulosa cells with A23187 (at 0.5–1 μM) for 24 or 48 hours of incubation time.

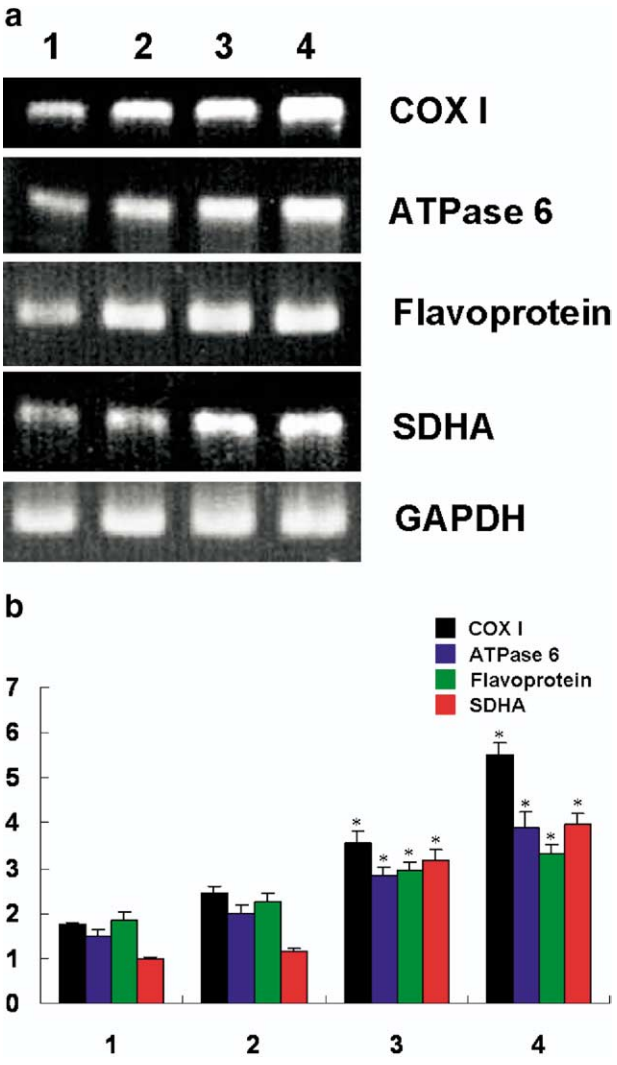
The RNA expression levels of mtTFA significantly increased in granulosa cells exposed for 24 and 48 hours to 0.5–1 μM A23187 (Fig. 4). Taken together, these data show calcium-dependent increases in transcription and translation levels of both nuclear-encoded and mitochondrial-encoded mitochondrial respiratory enzyme subunits and indicate that mtTFA is involved in mitochondrial biogenesis.

DISCUSSION

In this study, cohorts of every surrounding granulosa cell from five IVF oocytes were lysed to harvest total RNA and determine the semiquantative expression level of RNA by RT-PCR. To rule out limitations of RT-PCR, various replication cycles (15, 20, 25, 30, 35, and 40 cycles) were decided upon to identify the optimal cycles with RT-PCR products in a linear range as in a previous study (13). The mtDNA transcripts are polycistronic (14, 15), which means that each gene is separated after precise endonucleolytic excision of the tRNAs from the nascent transcripts. In the present study, we determined that two mtDNA-transcribed genes, COX I

FIGURE 1

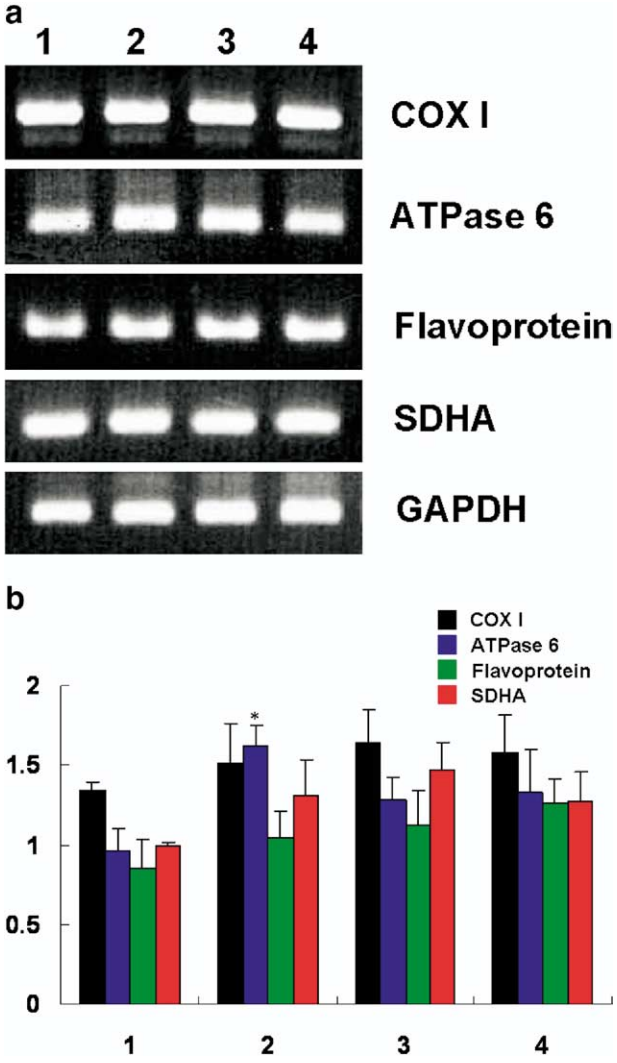
Semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of COX I, ATPase 6, flavoprotein, and succinate-ubiquinone oxireductase (SDHA) gene expressions in human granulosa cells treated with three different A23187 dosages for 48 hours. The RNA was extracted and reverse-transcribed into cDNA, and then PCR amplification was respectively performed with primers for the four genes. *Lanes 1* to *4* are 0, 0.25, 0.5, and 1 μM of A23187, respectively.



Au. Calcium-dependent mitochondrial gene transcription and translation. Fertil Steril 2005.

FIGURE 2

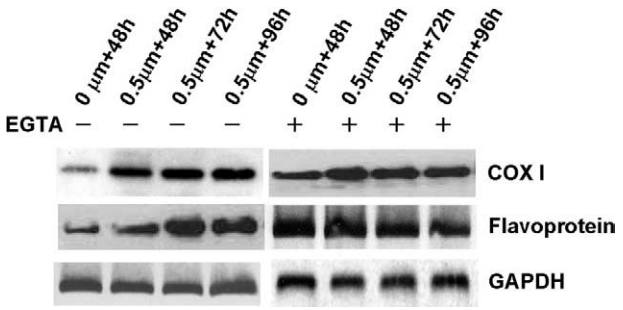
Semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of COX I, ATPase 6, flavoprotein, and succinate-ubiquinone oxireductase (SDHA) gene expressions in human granulosa cells pretreated with the Ca^{2+}-chelating agent, EGTA, then treated with three different A23187 dosages for 48 hours. *Lanes 1* to *4* are 0, 0.25, 0.5, and 1 μM of A23187, respectively.



Au. Calcium-dependent mitochondrial gene transcription and translation. Fertil Steril 2005.

FIGURE 3

Western blot analysis of nuclear- and mtDNA-encoded proteins in human granulosa cells. COX I = mtDNA-encoded cytochrome *c* oxidase subunit I; flavoprotein = nuclear-encoded flavoprotein; EGTA+ = cells pretreated with the Ca^{2+}-chelating agent, EGTA, then respectively treated with 0.5 μM of A23187 for 48, 72, and 96 hours.



Au. Calcium-dependent mitochondrial gene transcription and translation. Fertil Steril 2005.

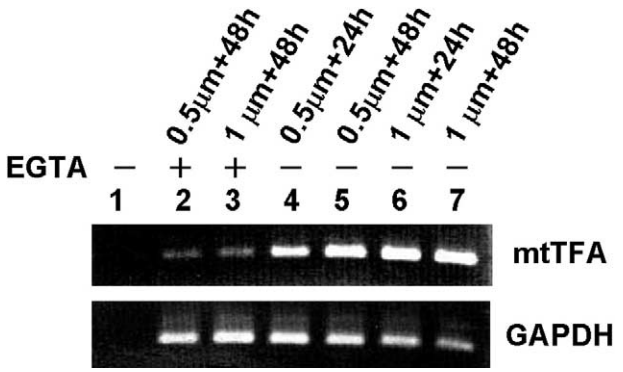
and ATPase6, may be representative of mtDNA gene expression.

Although different mtDNA transcripts encode different protein assemblies to one of five mitochondrial complexes, in our previous report, the expression levels of the eight genes, including ND2, COI, COII, ATPase 6, COIII, ND3, ND6, and Cyt b, were similar in unfertilized oocytes, arrested embryos, and tripronucleate zygotes. The polycistronic mtDNA transcripts are consistent, with different mtRNA expression levels showing the same pattern in the same cell (13, 16, 17).

Calcium acts as a second messenger in a variety of biological processes, including fertilization, embryo develop-

FIGURE 4

Semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of mtTFA gene expression in human granulosa cells treated with EGTA and/or A23187. *Lane 1,* RNA was extracted and without reverse-transcribed into cDNA, and then PCR amplification was performed with primers for the mtTFA gene. EGTA+ = cells pretreated with the Ca^{2+}-chelating agent, EGTA, then treated with A23187.



Au. Calcium-dependent mitochondrial gene transcription and translation. Fertil Steril 2005.

ment, and cell proliferation and death (18, 19). The mechanisms underlying the induction of calcium signals by pituitary FSH or the release of ATP by autonomic nerves in granulosa cells are controversial (20, 21). It is uncertain whether the mobilized cytosolic calcium is caused by mobilization from intracellular sources or to Ca^{2+} influx. Previous studies have provided evidence that cytosolic Ca^{2+} concentration is involved in regulating mitochondrial biogenesis in skeletal muscle (22, 23). In the present study, the induction of RNA and the protein expression levels of extracellular Ca^{2+} on both nuclear- and mitochondrial-encoded respiratory chain subunits were dose and time dependent. Calcium-induced mitochondrial biogenesis was abolished in the presence of EGTA. These novel data demonstrate that extracellular Ca^{2+} can stimulate mitochondrial biogenesis in human granulosa cells.

Biogenesis of the mitochondrial oxidative phosphorylation enzyme complex requires the symphonious expression of mtDNA and nuclear genes which both encode mitochondrial proteins and their control factors. One of these control factors is mtTFA, which plays a major role in regulating mtDNA transcription and replication (4, 24, 25). Our data indicate increased expression levels of mtTFA mRNA in human granulosa cells after Ca^{2+} stimulation (Fig. 4). The data presented in this study also showed that both mitochondrial biogenesis and mtTFA gene expression were induced by Ca^{2+}.

Cell responses to environmental changes including energy demands should be reflected in the physiological state of the mitochondria. This correlates well with Ca^{2+}-mediated increases in mitochondrial biogenesis in muscle (23) and mitogen-stimulated effects in human lymphocytes (26) and murine splenocytes (27). However, more data are needed to clarify the correlation between mtTFA and mitochondrial biogenesis in this study. This experimental evidence indicated that Ca^{2+} is one of the signals that mediate mitochondrial biogenesis in differentiated luteinized granulosa cells.

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