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粒線體功能對胚胎發育影響之研究(2/3)

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Paper 1: Calcium stimulate mitochondrial biogenesis in human granulosa cells

Abstract

Ovarian granulosa cells are known to play a key role in regulating ovarian physiology. Age increases apoptosis in the follicular granulosa cells and subsequently decreased ovarian fecundity. The aging ovary also contains fewer follicles that contain fewer granulosa cells. The viability of the follicular granulosa cells may be essential for development of the oocyte. Ionized calcium plays an important role in a variety of biological processes, including gene expression, cell cycle regulation, and cell death. In order to study the ability of mitohcondrial biogenesis in human granulosa cells, the mitochondrial marker proteins including nuclear encoded NADH subunit and mitochondrial encoded COX I were determined by treatment with calcium ionophore A23187. We showed that their expressions in human granulosa cells are increased by changes in cytosolic Ca^{2+} using the ionophore A23187. Treatment of granulosa cells with 0.5 μM of A23187 120 hours increased NADH subunit and COX I subunit up to 2.6-fold and 2.4-fold, respectively. Raising Ca^{2+} by exposing granulosa cells to 1 μM of A23187 for 48 hours significant increases in mitochondrial transcription factor (mtTFA) gene expression up to 2.9-fold. Our results indicate that the adaptive response of granulosa cells to an increase Ca^{2+} may up-regulate mitochondrial proteins and indicate the mtTFA implicated in such mitochondrial biogenesis pathway. We also first provide evidence of the ability mitochondrial biogenesis maintain in human granulosa cells.

Introduction

Ovarian granulose cells play a major role in regulating ovarian physiology, including ovulation and luteal regression (1). Granulosa cells secrete a wide variety of growth factors that may attenuate gonadotrophin action in the ovary in a paracrine-autocrine processes (2-3). Most of these factors do not directly affect the oocyte but exerts their action via granulosa cells. The presence of granulose cells appears to be beneficial for oocyte maturation and early development (4). Nevertheless, granulosa cells might have also a negative effect upon the oocyte. It has been demonstrated that the increased apoptotic potential in oocytes of aged mice is due to the presence of granulosa cells (5). The decline in reproductive ability in women with age is associated with loss of follicles and decrease in oocyte quality. Aging associated apoptosis increasing in the follicular granulosa cells and consequently decreases ovarian fecundity were also determined (6-7).

In eukaryotic cells, mitochondria are specialized organelles that catalyze the formation of ATP. Two distinct genomes exists in all eukaryotic cells. One is located in the nucleus and is transmitted in a Mendelian fashion, whereas the other is located in mitochondria and is transmitted by maternal inheritance. The mitochondria in an oocyte must have produced and stored all the energy required for the resumption of meiosis II, fertilization, and development of the embryo (8-9). Deficiency in mitochondrial ATP production may be associated with the impairment of oocyte fertilization (10-11). The normal function of mitochondria in the follicular granulosa cells may participate with growth factor production and subsequent paracrine effects for development of the oocyte. Ionized calcium plays an important role in a variety of biological processes, including gene expression, cell cycle regulation, and cell death. In this study the ability of mitohcondrial biogenesis in

human granulosa cells were determined by calcium incubated with human granulosa cells.

Material and methods

Collection of human granulosa cells

We collected human granulosa cells from patients undergoing in vitro fertilization by gonadotrophin-stimulated cycles. This study was approved by the institutional review board of Taipei Medical University Hospital. Follicular aspirates from each patient were pooled, and were centrifuged for 10 minutes at 800 X g and room temperature.

Cell culture and drug treatment

A23187 was purchased from Sigma. It was prepared as stock solution in Me_2SO at concentrations of 0.25, 0.5 and 1 μM, respectively. The granulosa cells were cultured with HTF medium contained 2% human plasma at 37°C incubator. The dose-dependent effects of A23187 on mitochondrial respiratory chain subunits were evaluated at final concentrations ranging 0.25 to 1 μM. Total RNA extracted from harvested cells was used as templates and cDNA was prepared using the RNA extraction kit and reverse transcription polymerase chain reaction (RT-PCR) kit from Ambion (Austin, TX). The sequences of the oligonucleotide primers used for detection mtTFA mRNA were listed as follows: Forwar: Reverse: .

Result

To study the effects of ionic calcium on mitochondrial biogenesis, the mitochondrial marker proteins were determined. Western blot analysis were used to determine the effect of A23187 treatment on nuclear-encoded NADH subunit and mitochondrial-encoded COX I levels. Dosage of A23187 were used including 0.25, 0.5 and 1 μM showed a progressive increase in NADH and COX I levels by up to 2.5-fold and 2.3-fold ($p<0.05$) (Fig. 1). The effect of A23187 on both nuclear- and mitochondrial-encoded subunit were also time-dependent (Fig. 2). By 120 h of exposure to the ionophore, the levels of NADH and COX I were increased by 2.6- and 2.4-fold, respectively ($p<0.05$). To determine the possible roles participate in mitochondrial biogenesis, the mitochondrial transcription factor A (mtTFA) were examined. The expression levels of mtTFA were determined by treatment granulosa cells with A23187 ranging from 0.25 to 1 μM coupled with 24 or 48 h incubation time. By exposure 24 and 48 h in 0.5 and 1 μM A23187, mtTFA mRNA levels showed 2.5- (0.5μM+24h), 2.9- (0.5μM+48h), 3.1- (1μM+24h) and 2.9-folds (1μM+48h), respectively. Taken together, these data show a calcium-dependent increase in both nuclear-encoded NADH and mitochondrial-encoded COX I expression levels and indicate the mtTFA implicated in mitochondrial biogenesis.

Discussion

Mitochondrial biogenesis requires the symphonious expression of the mtDNA and nuclear genes that both encode mitochondrial proteins and their control factors. One of these control factors was mitochondrial transcription factor A (mtTFA), which activates mitochondrial DNA transcription and replication (12). The cells respond to environment changes include energy demand should be reflected in the physiological state of mitochondria. Our data indicate increased expression levels of mtTFA mRNA in the human granulosa cells following Ca^{2+} stimulation. The data presented in this study also showed mitochondrial biogenesis respond to mtTFA gene expression. This correlates well with Ca^{2+} mediate increased mitochondrial biogenesis in muscle (13), mitogen-stimulated effects in human lymphocytes (14) and murine splenocytes (15).

Calcium (Ca^{2+}) acts as a second messenger in a variety of biological processes (16,17). In this study, the effect of Ca^{2+} on both nuclear- and mitochondrial-encoded subunit were dose- and time-dependent. The expression levels of mtTFA were also changed dependent on Ca^{2+}. Previous studies have provided evidence that cytosolic Ca^{2+} concentration is involved in regulating mitochondrial biogenesis in skeletal muscle (13,18). The adaption of mitochondria involves induction of mtTFA (13). These experimental evidences support Ca^{2+} may influence mtTFA expression and subsequently response to mitochondrial biogenesis. They also indicate Ca^{2+} is one of the signals that mediate mitochondrial biogenesis in the differentiated granulosa cells.

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Paper 2: Multiple rearrangements of mitochondrial DNA in human luteinized granulosa cells

To determine the rearrangement of mitochondrial DNA in luteinized granulosa cells for evaluation of the fertilization capacity of oocytes and development of embryos. Multiple deletions of mtDNA were found in luteinized granulosa cells from IVF patients. The 4977-bp deletion was the most frequent deletion in human granulosa cells. There was no significantly difference between mtDNA deletions of granulosa cells with the fertilization capacity of oocytes and development of embryos. In order to determine the relationship of proportions of mtDNA rearrangements with aging process, the granulosa cells were grouped into three different cohorts according to maternal age: younger than 32 years old, between 32 and 37 years old, and older than 37 years. There was no statistical correlation between patient age and the frequency of occurrence of the multiple mtDNA deletions. However, the increase in granulosa cell apoptosis was associated with an increase in mtDNA deletions. Accumulation of mtDNA deletions may contribute to mitochondrial dysfunction and impaired ATP production. We conclude that the accumulation of rearranged mtDNA in granulosa cells may not interfere with fertilization of human oocytes and further embryonic development, however, associated with apoptosis processes.

Material and method

Polymerase Chain Reaction

Oocytes and embryos were stored in 20 μL of 1X PCR buffer containing 0.05 mg/mL of proteinase K, 20 mM of dithiothreitol (DTT), and 1.7 μM of SDS. After digestion for 1 hour at 56°C and 10 minutes of heat-inactivation of proteinase K at 95°C, the total DNA in the solution was then used as template for the PCR assay. The sequences of the oligonucleotide primers used in this study are listed as follows: H1 (np 8285–8304, CTCTAGAGCCCACTGTAAAG), H2 (np 8781–8800, CGGACTCCTGCCTCACTCAT), H3 (np 9207–9226, ATGACCCACCAATCACATGC), L1 (np 13650–13631, GGGGAAGCGAGGTTGACCTG), and L2 (np 14145–14126, TGTGATTAGGAGTAGGGTTA), L3 (np 15896–15877, TACAAGGACAGGCCCATTTG) and L4 (np 16410–16391, GAGGATGGTGGTCAAGGGAC).

Semiquantitative RT-PCR

Total RNA extracted from human granulosa cells was used as templates and cDNA was prepared using the RNA extraction and reverse transcription polymerase chain reaction (RT-PCR) kit from Ambion (Austin, TX). The RT-PCR amplifications were performed with 3 μL of cDNA in a total volume of 50 μL of amplification buffer, 40 pmol of specific primers, and 2.5 units of Taq DNA polymerase (Life Technologies, Grand Island, NY). The sequences of the oligonucleotide primers used in this study are listed as follows: ND2 (Forward, np5101-5120, TAACTACTACCGCATTCCTA; Reverse, np5400-5381, CGTTGTTAGATATGGGGAGT), GAPDH (Forward, CCTTCATTGACCTCAAC; Reverse, AGTTGTCATGGATGACC). For semiquantitative amplification, each

cycle was carried out 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 to optimize the linear range of amplification. The PCR reactions were optimized with respect to annealing temperature and numbers of PCR cycles. Each PCR product was run through a 2% agarose gel and was visualized with ethidium bromide staining.

Result

To determine whether the existence of mtDNA deletions in luteinized granulosa cells affect the fertilization capacity of oocytes and development of embryos. The DNA were extracted from granulosa cells to determine the mtDNA rearrange by PCR using multiple pairs of primers. Multiple deletions of mtDNA were found in luterized granulosa cells from IVF patients. The 4977-bp deletion was the most frequent deletion in human granulosa cells. There was no significantly difference between mtDNA deletions of granulosa cells with the fertilization capacity of oocytes and development of embryos. In order to determine the relationship of proportions of mtDNA rearrangements with aging process, the granulosa cells were grouped into three different cohorts according to maternal age: younger than 32 years old, between 32 and 37 years old, and older than 37 years. Frequencies of occurrence of mtDNA deletions in human granulosa cells of different age cohorts were listed in Table 1. Percentages of 4977-bp rearranged mtDNA were 43.8%, 39.1% and 47.4% in three age-related cohorts, respectively. There was no statistical correlation between patient age and the frequency of occurrence of the 4977-bp mtDNA deletion as well as multiple mtDNA deletions. Whether coexistence of rearranged mtDNA in oocytes and its surrounding granulosa cells were also determined. The data showed mtDNA deletions distributed randomly between oocytes and granulosa cells, higher proportions of rearranged mtDNA in oocytes (Fig. 1, lanes 2, 3, 6 and 7) and higher frequencies in granulosa were determined (Fig.1, lanes 1 and 5). Independent existence of rearranged mtDNA in oocytes and granulosa cells were shown in Fig. 1. Expression levels of mitochondrial RNA in granulosa cells of different age group were determined by using semiquantitative reverse transcription of polymerase chain reaction (Fig. 2). The transcripts of mitochondria NADH dehydrogenase subunit 2 (ND2) were determined and normalization to GAPDH gene, with no statistically

significant differences among different age cohort.

Discussion

In this study, aging-independent existence of rearranged mtDNA in granulosa cells indicates that aging process was not the major role on accumulation of mtDNA mutations in granulosa cells. However, rearranged mtDNA may contribute to apoptosis of granulosa cells. Independent existence of rearranged mtDNA in oocytes and granulosa cells support that the accumulation of rearranged mtDNA in granulosa cells may not interfere with fertilization of human oocytes and further embryonic development.