論文の内容の要旨

論文題名　An analysis of oxidative stress and SIRT3 in ovarian granulosa cells

（酸化ストレスとSIRT3の卵巣顆粒膜細胞における機能解析）

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Introduction

Ovarian lifespan is the main determinant of female reproductive function. The normal folliculogenesis and luteinization processes of ovary are prerequisite to avoid female infertility. The production of ROS and antioxidants in a body is normally balanced by converting ROS to water. Oxidative stress (OS), however, occurs when increased ROS levels disrupt cellular redox circuits, inducing various damages. OS like a double-edged sword precipitates a range of normal physiological processes and pathologies in female reproductive systems. Ovulation, as an example, is impaired by the depletion of overabundant post-LH surge inflammatory precursors, which generates ROS. However, the excess oxidants can contribute to a number of gynecological diseases, such as endometriosis, polycystic ovary syndrome, and infertility and pregnancy complications such as spontaneous abortions, recurrent pregnancy loss, and preeclampsia.

Sirtuins are a family of proteins that can extend the lifespan in several lower model organisms. SIRT3, which predominantly resides in mitochondria, has recently emerged as a key player in regulating metabolic and respiratory pathway.SIRT3-deficient mice exhibit phenotypes of cardiac hypertrophy and age-related hearing loss due to the high levels of ROS. The mechanisms of SIRT3 regulate ROS homeostasis includes activation of antioxidant enzymes and other important molecules, such as SOD1, Foxo3a. Additionally, SIRT3 has been shown to protect in vitro fertilized murine preimplantation embryos against oxidative stress and protect cells from stress-mediated cell death by Ku70 deacetylation and NF-Kb activation in cardiomyocytes. In the present experiment, hence, we further focus on the expression and function of one sirtuin, SIRT3 in the human ovary.

MATERIALS AND METHODS

Human ovarian tissue for immunohistochemistry

The human ovarian tissues were obtained by hysterectomy from 12 female patients. Immunohistochemical analysis was performed by standard procedures. Sections were then incubated with the anti-SIRT3 rabbit polyclonal antibody (ab86671; Abcam Ltd) overnight at 4°C. Negative controls were incubated with PBS instead of the antibody.

COV434 cell line and Western blotting

The COV434 cell line were seeded onto 10 cm dishes cultured in growth medium in a humidified atmosphere of 5% CO2 and 95% air at 37°C. Upon reaching confluence, the cells were incubated with 1ml of serum-free medium with varying concentrations (250, 500 μM) of H_2O_2. After 12 hours incubation, the cells were subjected to western blot. The membranes were blotted overnight at 4°C with primary antibodies, including anti-SIRT3 (1:1000, C73E3; Cell Signaling Technology, Inc), anti-catalase (1:2000, ab16731; Abcam Ltd), and anti-superoxide dismutase 1 (SOD1) (1:1000, ab13499; Abcam Ltd). The secondary antibodies are anti-rabbit IgG (1:3000, 7074S) and anti-mouse IgG (1:3000, 7076S; Cell Signaling). The expression of target proteins was internally normalized to the optical density of β-actin (sc-47778; Santa Cruz Biotechnology, Inc) by Image J software.

Purification, culture and management of primary human GCs

The patients were recruited from the IVF-ET center at the University of Tokyo Hospital. Parameters of patients are shown in table 1. The follicular fluid was centrifuged and the supernatant was removed. After layered onto Ficoll-Paque PLUS solution (GE Healthcare) to remove the red blood cells, the cells in the interface were collected into PBS, then centrifuged and resuspended in culture medium and seeded onto 12-well plates at a density of 2×10^5 cells/mL in DMEM/F12 (Invitrogen). To evaluate the effect of ROS on SIRT3 expression, various concentrations of H_2O_2 (100, 200, and 400 μM) were added to the GCs in serum-free growth medium for 24 hours. To determine whether HCG affects SIRT3 expression, the GCs were incubated with various concentrations of HCG (0.01, 0.1, and 1 U/ml) in serum-free medium for 24 hours. Then both samples were subjected to quantitative real-time PCR.

Transfection with SIRT3 siRNA and Cell viability counting

The seeded GCs were transfected with 100 nM of siRNA (Invitrogen, oligo no. HSS177402) for 48 hours in Opti-MEM (Life Technologies), using Lipofectamine RNAiMAX (Life Technologies). After transfection, the GCs were covered with serum-free medium, which was subjected to a progesterone concentration assay, and living cell count.

RNA extraction and quantitative real-time PCR

Total RNA was extracted from GCs, using an RNeasy mini kit (QIAGEN) and RT was performed using ReverTra Dash (TOYOBO). Then real-time PCR was performed using the LightCycler (Roche Diagnostic GmbH). The primer sets used are described in table 2.

Detection of intracellular ROS

The GCs were incubated with 10 μM fluoroprobe carboxymethyl H_2 dichlorofluor-escein diacetate (CM-H_2DCFDA) in PBS for 20 minutes at 37°C, and counterstained with 10 nM of SYTO 61 red fluorescent nucleic acid stain (Life Technologies) in PBS for 5 minutes, and then immediately observed under a laser scanning confocal microscope (D-Eclipse C1; Nikon), with an excitation wavelength of 480 nm and an emission wavelength of 505 to 530 nm.

Measurement of progesterone secretion

The above-mentioned serum-free medium collected 72 hours after transfection of SIRT3 siRNA was thawed, and the progesterone concentration was determined using a Progesterone EIA (enzyme immunoassay) Kit (Cayman Chemical Co).

Statistical analysis

Data were analyzed by SPSS statistical software (SPSS Inc). The differences between the 2 groups were analyzed using the Mann-Whitney *U* test. Multiple comparisons between more than 2 groups were analyzed by one-way ANOVA and post hoc tests. Values of $P < .05$ were considered significant.

RESULTS

Expression of SIRT3 in human ovaries

The results of immunohistochemistry shows SIRT3 protein were predominantly in the human GCs at various stages of follicles (Fig. 6A-E). The corpus albican (Fig. 6G) and negative control (Fig. 6H) which was incubated with PBS, did not exhibit any SIRT3 expression staining.

H_2O_2 upregulated the protein expression of antioxidants and SIRT3 in COV434 cell line and human GCs

The two antioxidants, SOD1 and catalase were remarkably increased under the condition of high levels of H_2O_2 by Western blot. Simultaneously, the expression of SIRT3 protein also increased. Quantitative PCR further displayed similar results, which is that the mRNA expression of catalase and SOD1 and SIRT3 increased markedly after exposure to 400 μM H_2O_2.

Knockdown of endogenous SIRT3 induced generation of ROS in GCs

CM-H_2DCFDA fluorescence images show that, compared with the control group (Fig. 9B), the depletion of endogenous SIRT3 by siRNA incubation caused tremendous ROS generation in the GCs (Fig. 9E). The fluorescence intensity of GCs depleted of endogenous SIRT3 was approximately 4-fold higher than that of the control GCs by Image J. SIRT3 deletion by siRNA did not cause statistically significant mortality in the live cell number between the knock down group and control group (Fig. 10).

Stimulation of luteinization by HCG down-regulated anti-oxidant signaling

As expected, HCG stimulated the luteinization function, which was verified by the induction of steroidogenic acute regulatory protein (StAR) mRNA, a representative luteinization-associated factor in GCs. In addition, HCG down regulated the mRNA expression of SIRT3, catalase, and SOD1 (Fig. 11).

Role of SIRT3 in the folliculogenesis and luteinization

SIRT3 mRNA levels were effectively suppressed after 48 hours of incubation of 100 nM SIRT3 siRNA (Fig. 12). The mRNA expression of folliculogenesis-associated molecules including aromatase (P450arom) and 17β-hydroxysteroid dehydrogenase 1 (17β-HSD1) was significantly decreased, so did the three luteinization-associated genes, StAR, 3β-hydroxysteroid dehydrogenase 1 (3β-HSD1) and the cholesterol side-chain cleavage enzyme (P450scc) in Fig. 13. Besides, the progesterone immunoassay

displayed significantly lower levels of progesterone concentration after SIRT3 gene was knocked down as compared to SIRT3 gene normal group (Fig. 14).

Discussion

A large number of previous studies documented the H_2O_2-induced SIRT3 expression elevation in diverse models such as in mice suffered with CR or fasting and in 4-cell mouse embryos. Besides, the increased activities of endogenous antioxidant enzymes, SOD2 with isocitrate dehydrogenase 2, were displayed when SIRT3 was overexpressed by lentivirus transfection in primary rat cortical neurons. Further, the present study revealed the simulatneouly increased SIRT3 expression when the expression of SOD1 and catalase was upregulated in the human GCs (Fig.7 and 8), implying that the concomitant SIRT3 rise possibly serves as a feedback mechanism to manipulate intracellular ROS levels similar to catalase and SOD1.

HCG has recently been found to function as a non-enzyme antioxidant in human cytrophoblasts stimulated with H_2O_2 in vitro, promoting the survival of human cytotrophoblastic cells. Furthermore, another research shows the protective effects exerted by HCG against peroxidation by H_2O_2 on vascular endothelial function. Considering above complex role of HCG, the inverse correlation between HCG stimulation and decreased antioxidants and SIRT3 may probably be due to competition among antioxidants.

Our data first showed after SIRT3 gene was ablated by siRNA transfection, the folliculogenesis and luteinization related molecules and the progesterone secretion significantly decreased compared to the vehicle (Fig. 12-14). This would be resulted from a reduction of cAMP, protein kinase C inhibition and the activities of intracellular calcium, or non-AMP dependent steroidogenesis, which was found at the presence of excess H_2O_2 in cells.

Conclusionly, our study suggested that SIRT3 could function as an antioxidant, like SOD1 and catalse, to counteract the excess cellular ROS in the human GCs, and at the end markedly upregulated the folliculogenesis- and luteinization-related molecules and further significantly increased the progesterone secretion. The protective effect of SIRT3 may be an important clinical implication.