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Research paper

Are sirtuins markers of ovarian aging?

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ABSTRACT

Sirtuins, a family of nicotinamide adenine dinucleotide (NAD)-dependent deacetylases that play diverse roles in regulating metabolism, cell proliferation, and genome stability, have been implicated in mammalian aging and age-related diseases, including cancers, metabolic disorders, and neurodegenerative diseases. Ovarian aging is thought to be characterized by a gradual decrease in both the number of follicles and the quality of oocytes. Ovarian reserve is indicated by the number of primordial follicles. In this study, ovarian reserve was assessed in mice of different ages and mice subjected to caloric restriction (CR) and chemotherapy (2 commonly used models for ovarian aging research) by counting primordial follicles and determining the expression levels of SIRT1, SIRT3, and SIRT6 to explore the relationship between ovarian function and sirtuin expression. A gradual decline in the number of follicles (especially primordial follicles) was observed in aging mice and mice subjected to chemotherapy. Histological analysis showed that CR mice displayed a significantly greater number of primordial follicles and less atretic follicles. Western blot analysis indicated that expression levels of SIRT1, SIRT3, and SIRT6 were significantly decreased in the ovaries of aged mice and mice treated with chemotherapy, but increased in CR mice. SIRT1, SIRT3, and SIRT6 all showed a significantly positive correlation with the numbers of primordial follicles ($r^2 = 0.6399$, $P < 0.0001$; $r^2 = 0.5445$, $P < 0.001$; and $r^2 = 0.4956$, $P < 0.0001$, respectively). These results indicate that SIRT1, SIRT3 and SIRT6 are closely related to ovarian reserve, and suggest that these sirtuins may be markers of ovarian aging.

1. Introduction

The sirtuin (SIRT) family of nicotinamide adenine dinucleotide (NAD)-dependent deacetylases plays diverse roles in regulating metabolism, cell proliferation, and genome stability, and has been implicated in mammalian aging and age-related diseases, including cancers, metabolic disorders, and neurodegenerative diseases (Finley and Haigis, 2012; Firestein et al., 2008; Jiang et al., 2011; Kaplon et al., 2013; Sebastian et al., 2012). The first link between sirtuins and longevity was made 16 years ago in yeasts. Many studies in diverse experimental models have been performed to elucidate the relationship between sirtuins, lifespan, and age-associated dysfunction. To date, 7 homologs (SIRT1-7) of the yeast *SIR2* gene have been identified in mammals. SIRT1 and SIRT6, which are mainly localized in cell nuclei, regulate the transcription of genes and DNA repair, while SIRT3 regulates mitochondrial bioenergetics. The anti-aging effects of mammalian homologs of the SIRT family (SIRT1, SIRT3, and SIRT6) are the most extensively studied members in relation to the effects of caloric restriction (CR) on longevity. A large body of evidence now indicates that these sirtuins suppress age-related dysfunction and increase longevity by enhancing the expression of SIRT1 or SIRT6 (Camins et al., 2010; Giblin et al., 2014; Park et al., 2013; Satoh et al., 2011). Overall, the available data point to important roles for sirtuins in promoting mammalian health, and perhaps in modulating the aging process. However, the anti-aging mechanism of sirtuins remains elusive.

The ovarian aging process is parallel to that of somatic aging in that the underlying mechanisms have a remarkable similarity to the mechanisms of general aging. The ovarian aging process is thought to be characterized by a gradual decrease in both the quantity and quality of the oocytes in the ovarian cortex (te Velde and Pearson, 2002). Inevitably,

Abbreviations: AFC, antral follicle count; AMH, anti-Müllerian hormone; ANOVA, analysis of variance; CR, caloric restriction; CTX, cyclophosphamide; FOXOs, Forkhead box class O transcription factors; FSH, follicle-stimulating hormone; GDH, glutamate dehydrogenase; H&E, hematoxylin and eosin; HSF1, heat shock transcription factor 1; NAD, nicotinamide adenine dinucleotide; NF-κB, nuclear factor-kappaB; mTOR, mammalian target of rapamycin; NC, normal control; PBS, phosphate-buffered saline; PGC-1, peroxisome proliferator-activated receptor-gamma coactivator-1; PMSF, phenylmethanesulfonyl fluoride; POF, premature ovarian failure; RIPA, radio-immunoprecipitation assay; ROS, reactive oxygen species; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SIRT, sirtuin; SOD2, superoxide dismutase 2; UCP1, uncoupling protein 1.

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menopause is the final step in the process of ovarian aging. The primordial follicle pool serves as the source of developing follicles in mammals, and reflects the lifespan of ovaries in mice, undergoing an inevitable decline during their lifetimes (Broekmans et al., 2007; Hansen et al., 2008; McGee and Hsueh, 2000). A decreased ovarian antral follicle count and primordial follicle pool suggest a lower ovarian reserve (Broekmans et al., 2009; Hansen, 2013; Li et al., 2012).

Several researchers have found that sirtuins play some role in ovarian function. Zhang et al. (2013) found that rapamycin preserves the follicle pool reserve and prolongs the ovarian lifespan of female rats via modulating mammalian target of rapamycin (mTOR) activation and sirtuin expression. SIRT1 activator (SRT1720) was found to improve follicle reserve and prolong the ovarian lifespan of diet-induced obese female mice by activating SIRT1 and suppressing mTOR signaling (Zhou et al., 2014). Fu et al. (2014) found that SIRT3 positively regulates the expression of folliculogenesis and luteinization-related genes, and progesterone secretion by manipulating oxidative stress in human luteinized granulosa cells. While it is known that sirtuins are closely related to mammalian longevity and aging, do they regulate ovarian aging?

This study aimed to explore the relationship between ovarian reserve and the expression of SIRT1, SIRT3, and SIRT6 in the ovaries of mice with physiological ovarian aging and mice studied in 2 experimental models — a cyclophosphamide (CTX)-induced premature ovarian failure (POF) model, and a CR-induced ovarian reserve protection model.

2. Materials and methods

2.1. Mice and treatments

C57BL/6 mice were used in these studies and were purchased from the Beijing HFK Bio-Technology Company (Beijing, PR China). All of the experimental procedures involving the animals were approved by the Ethics Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology. The mice received humane care according to the Guide for the Care and Use of Laboratory Animals of the Chinese Academy of Sciences. All mice were adult females aged either 6 weeks, 2 months, 8 months, or 1.5 years, and weighed 16–19 g. They were fed in a specific pathogen-free (SPF) house at controlled temperature (25 °C) and light (12 h light, 12 h dark) conditions.

For the POF model, 6 adult female mice aged 6 weeks were given a single intraperitoneal injection of 500 μL phosphate-buffered saline (PBS) or an equal volume containing CTX 120 mg/kg (Sigma-Aldrich, CAS No. 6055–19–2). Ovaries were collected 7 days after CTX treatment. For the CR model, 6-week old adult female mice were randomly divided into 2 groups: a normal control (NC) group (fed ad libitum) and the 25% caloric restriction (CR) group (fed with 75% of the food of the NC group). Ovaries were harvested after 8 weeks of treatment. The mice aged 2 months, 8 months, and 1.5 years were at sexual maturity, reproductive aging, and ovarian aging stages, respectively (Gosden et al., 1983; Hansen et al., 2008; Li et al., 2014). All mice were sacrificed by cervical dislocation. Their ovaries were harvested, and the right-side ovaries were snap frozen and stored at −80 °C for protein extraction, while left-side ovaries were fixed in 4% paraformaldehyde at 4 °C for histological sectioning.

2.2. Follicle counting

Paraffin-embedded ovaries were longitudinally and serially sectioned (4 μm) and every fifth section was mounted on a glass slide. Routine hematoxylin and eosin (H&E) staining was performed for histological examination. The slides were analyzed under a microscope by 2 people who were blind to the origin of the sections. Only follicles containing an oocyte were counted to avoid any duplication. Follicles were classified as either: *primordial follicles* (oocyte surrounded by a single layer of squamous granulosa cells); *primary follicles* (intact enlarged oocyte with a visible nucleus and one layer of cuboidal granulosa cells); *secondary follicles* (2 or 3 layers of cuboidal granulosa cells without an antral space); or *antral follicles* (emerging antral spaces and atretic follicles, with apoptotic bodies of granulosa cells and fragmentation of the oocyte nucleus) (Myers et al., 2004).

2.3. Western blot analysis

Ovaries were homogenized in a radio-immunoprecipitation assay (RIPA) with phenylmethanesulfonyl fluoride (PMSF) and a Teflon-glass homogenizer on ice. After centrifugation (12,000 rpm for 15 min at 4 °C), the supernatants were collected for protein analysis. Protein concentrations were determined by the bicinchoninic acid (BCA) Protein Assay. For each sample, 40 μg of protein samples were separated by SDS-PAGE and transferred onto nitrocellulose membranes (BioTrace™ NT, USA). Tris-buffered saline with 0.1% Tween 20 (TBST) buffer containing 5% non-fat dry milk was used to block non-specific binding at room temperature for 1 h. The membranes were then incubated with a primary antibody against SIRT1 (Abcam, UK, ab110304, 1:8000 dilution), SIRT3 (Abcam, UK, ab86671, 1:500 dilution), SIRT6 (Abcam, UK, ab135566, 1:100 dilution), or β-actin (Sigma, USA, 1:1000 dilution) overnight at 4 °C, followed by incubation with a horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibody (Sigma, USA, A0545, A6154, 1:3000 dilution) for 30 min at room temperature and washed three times with TBST. Bands were visualized by the ECL Plus Western blotting Detection System (Amersham, USA). Band intensities were analyzed using Quantity One software (Bio-Rad Laboratories Pty Ltd., USA). β-Actin protein levels were used as a control to verify equal protein loading.

2.4. Statistical analysis

All results were expressed as means ± SEM and analyzed by the SPSS® 17.0 software. A paired Student's t test or one-way analysis of variance (ANOVA) was used to compare data between groups, with significance defined as $P < 0.05$. The relationship between ovarian expression levels of sirtuin proteins (SIRT1, SIRT3, and SIRT6) and the primordial follicle numbers of the mice was further analyzed using linear regression. Correlation coefficients (r) were tested for each SIRT.

3. Results

3.1. Increased expression levels of SIRT1, SIRT3, and SIRT6 proteins in the ovaries of mice whose ovarian reserve was protected by CR

CR without malnutrition has been shown to delay aging and increase longevity in diverse experimental models. Some researchers have also reported that CR increases ovarian reserve. The mean number of primordial follicles in the CR group was significantly greater than in the normal control (NC) group (112.5 ± 12.49 vs 85.0 ± 6.55, respectively; $P < 0.05$) [Fig. 1C], and the percentage of the total number of follicles that were primordial follicles was greater in the CR group (33.2% ± 0.9% vs 26.2% ± 0.4%, respectively) [Fig. 4A]. However, the mean numbers of primary follicles (104.6 ± 9.7 vs 102.5 ± 11.4), secondary follicles (69.8 ± 9.6 vs 73.0 ± 8.7), and antral follicles (19.5 ± 6.3 vs 12.5 ± 0.2) were not significantly different between the 2 groups. For atretic follicles, the mean number and percentage in the CR group were almost half those of the NC group (26.7 ± 1.1 vs 48.6 ± 5.1, respectively; and 7.9% ± 0.4% vs 15% ± 0.7%, respectively). These results indicate that the CR increases ovarian reserve and reduces follicular atresia.

Ovarian expression levels of SIRT1, SIRT3, and SIRT6 proteins were significantly higher in CR mice in comparison with NC mice (1.41-fold, 1.47-fold, and 1.40-fold higher, respectively) [Fig. 1E].