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Inhibition of the NLRP3 inflammasome prevents ovarian aging

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Inflammation is a hallmark of aging and is negatively affecting female fertility. In this study, we evaluate the role of the NLRP3 inflammasome in ovarian aging and female fertility. Age-dependent increased expression of NLRP3 in the ovary was observed in WT mice during reproductive aging. High expression of NLRP3, caspase-1, and IL-1β was also observed in granulosa cells from patients with ovarian insufficiency. Ablation of NLRP3 improved the survival and pregnancy rates and increased anti-Müllerian hormone levels and autophagy rates in ovaries. Deficiency of NLRP3 also reduced serum FSH and estradiol levels. Consistent with these results, pharmacological inhibition of NLRP3 using a direct NLRP3 inhibitor, MCC950, improved fertility in female mice to levels comparable to those of Nlrp3−/− mice. These results suggest that the NLRP3 inflammasome is implicated in the age-dependent loss of female fertility and position this inflammasome as a potential new therapeutic target for the treatment of infertility.

INTRODUCTION

Aging is a natural process in all animals involving a progressive impairment of physiological and metabolic homeostasis characterized by many changes in body composition, insulin resistance, mitochondrial and autophagy dysfunction, inflammation, and hormonal dysregulation (1). From a clinical point of view, aging is associated with many signs of illness such as cardiovascular, neurodegenerative, and metabolic disorders, which are known as age-dependent diseases. These pathologies can be managed by different strategies including pharmacological intervention, lifestyle modifications, and prevention of harmful environmental exposures. However, the ovarian aging process is currently a pharmacologically uncontrollable process that impairs female fertility; the infertility correlates with a rapid decline after age 35 and is attributable to the impairment of the quantity and/or the quality of oocytes (2). Female infertility is exacerbated by socioeconomic changes in developed countries, which enable women to progressively delay the age at which they have their first child. As a consequence, there is increased demand for the treatment of infertility (3). One of the key events that contribute to ovarian aging includes follicular atresia as it is associated with the breakdown of the ovarian follicles in both physiological and premature ovarian aging (2). Follicular atresia, as an associated event to cellular aging, show many pathophysiological alterations associated with physiological aging such as mitochondrial dysfunction, oxidative stress, and inflammation, and as aging itself, it is sensitive to stress (1–3).

Although inflammation may negatively affect female fertility (4), the underlying molecular mechanisms are poorly understood. Preliminary studies in animal models have shown that genetic deletion of tumor necrosis factor (TNF) receptor improves female fertility and deletion of interleukin-1α (IL-1α) mice has prolonged the life span of the ovaries (5, 6).

The NLR family pyrin domain containing 3 (NLRP3) inflammasome is one of the most well-studied inflammasomes in humans and mice (1). It is a multiprotein complex comprising NLRP3 itself as an intracellular sensor, the adapter protein ASC [apoptosis-associated speck-like protein containing a CARD (caspase recruitment domain)], and procaspase-1. Two steps are required for the activation of this protein platform. The first step, also known as priming signals, causes the transcription of inflammasome constituents, a response that is mediated by nuclear factor κB and other pathways such as p38 mitogen-activated protein kinase (7). Signal two, or triggering, is induced by pathogen-associated molecular patterns, and stress-associated signals or host-derived damage-associated molecular patterns lead to NLRP3 oligomerization complex assembly (8), and caspase-1 activation activated caspase-1 process pro-IL-1β and pro-IL-18 to IL-1β and IL-18, respectively. This inflammasome is also involved in the processing of the pore-forming protein gasdermin D whose excessive pore formation can cause an inflammatory form of cell death known as pyroptosis (8). The NLRP3 inflammasome is activated by a range of danger and stress signals (8), some of which increase during aging (1). Thus, NLRP3 inflammasome can contribute to a vicious cycle of low-grade inflammation that occurs during aging.
Genetic deletion of Nlrp3 in mice has been shown to improve life span and health by attenuating multiple age-related degenerative changes such as cardiac aging, insulin sensitivity with glycermic control, and bone loss (9, 10). However, the role of the NLRP3 inflammasome in ovarian aging and female age-related fertility decline has not been studied. Therefore, we sought to determine whether genetic deletion of NLRP3 could have an effect on ovarian aging and potentially prevent the decline of female fertility.

RESULTS
The NLRP3 inflammasome is activated during ovarian aging
To evaluate the role of the NLRP3 inflammasome in ovarian aging, we examined ovaries from female mice of different ages. Wild-type (WT) C57BL/6J female mice exhibited a substantial increase in body weight over a 12-month period (Fig. 1A) and a significant decrease in anti-Müllerian hormone (AMH) after the sixth month of life (Fig. 1B). NLRP3 protein expression was increased from the fourth month onwards along the levels of active caspase-1 (p20) and an age-dependent increase of active IL-1β (p17; Fig. 1, C and D). NLRP3 expression was observed in the cytoplasm of granulosa cells (GCs) and follicles of different status including atretic follicles, in the cytoplasm of oocytes, and in the glandular epithelium of the uterus (Fig. 1E, negative control and magnification in figs. S1 to S3).

Notably, NLRP3 expression in these tissues was higher in aged animals. NLRP3 expression was inversely correlated with serum AMH levels (Fig. 1F). Since autophagic dysfunction has also been linked to aging, we analyzed the expression of the components in this pathway. We observed an increase in the expression of microtubule-associated protein 1 light chain 3 (LC3-II) and other proteins involved in clearance pathways such as p62/SQSTM1 beginning at 6 months (Fig. 1, C and D).

To translate our murine studies to humans, we evaluated NLRP3 inflammasome activation in GCs from human patients with accelerated ovarian aging disease. Diminished ovarian reserve (DOR) is a critical fertility defect characterized by an anticipated impairment of the follicular reserve for which pathophysiological mechanisms are not understood. Different genetic and molecular alterations have been proposed, such as mutations in genes associated with DNA repair or meiosis, bioenergetics alterations, and mitochondrial dysfunction (2). The clinical characteristics of patients with DOR and controls are listed in Table S1, and low ovarian reserve was confirmed by transvaginal ultrasound. Patients with DOR showed altered hormonal status compared to the control group (Table S1). Follicle-stimulating hormone (FSH) levels in these patients were above 10 mU/ml, and estradiol (E2; a sexual hormone involved in the regulation of the estrous and menstrual female reproductive cycles) levels were above 60 pg/ml. We found significantly increased NLRP3 mRNA and protein expression in GCs from patients with DOR compared to healthy controls. We also observed elevated protein expression of active caspase-1 (p20) and IL-1β (p17) (Fig. 1, G and H). However, plasma levels of TNF-α, but not IL-1β and IL-18, were increased in patients with DOR compared to healthy controls (Table S1).

NLRP3 expression correlates with reproductive aging
To evaluate the impact of NLRP3 expression on female fertility during aging, we monitored the life span and reproductive capacity of Nlrp3−/− and WT littersmate Kaplan–Meier survival curve showed an increase in mean life span of 37% and maximum life span of 24% in Nlrp3−/− (Fig. 2A), whereas body weights did not differ between both groups (Fig. 2B). Twelve-month-old female Nlrp3−/− mice exhibited a significant decrease in glucose serum levels at the Oral glucose tolerance tests (OGTT) peak (15 min) compared to old WT mice (Fig. 2A), indicating a higher glucose tolerance consistent with a trend toward lower values of the area under the curve of the glucose tolerance test (Fig. 2B). Furthermore, 12-month-old female Nlrp3−/− mice showed no significant difference in serum levels of leptin compared to WT but an increased level of adiponectin (Fig. 2C, D). Twenty-eight-month-old WT animals displayed increased age-related alopecia compared to Nlrp3 knockout (KO) mice (Fig. 2C).

AMH serum levels were similar in 2-month-old WT and Nlrp3−/− mice but were afterward significantly higher in mutant mice compared to WT mice (Fig. 2D). Consistent with serum levels, ovarian AMH protein levels were higher in aged (12 months old) Nlrp3−/− mice compared to WT mice (Fig. 2D). In addition, the levels of the sex hormones E2 and FSH increased between 4 and 12 months in WT mice but not in Nlrp3−/− mice (Fig. 2, E and F). The WT group showed a markedly enlarged uterine size and weight (hypertrophic uterus) compared to Nlrp3−/− (Fig. 2G). We also analyzed the number of ovarian follicles by histological staining in ovaries from 4- and 12-month-old Nlrp3−/− mice, and they showed more dynamic activity at 12 months and a greater percentage of follicles at 4 and 12 months compared with WT mice. Nlrp3−/− ovaries retained a larger pool of follicles, which appeared more active in folliculogenesis and contained many corpora lutea, observations suggesting successful ovulation (Fig. 2, H and I).

A significant reduction of the pregnancy rate was observed in WT mice during aging but was markedly preserved in aged (12-month-old) Nlrp3−/− mice compared with WT mice (Fig. 2A). These observations were consistent with AMH and ovari data. Mean litter size was also significantly larger in 12-month-old Nlrp3−/− mice compared with littersmate mice as in young mice (4 months; Fig. 2B). The loss of the ASC, which has a pivotal role in the assembly of several inflammasomes (8), did not affect the age-dependent loss of female fertility. Asc−/− mice showed similar levels of AMH, FSH, and E2 during aging and reduced AMH protein expression (Fig. S5, A to C). The pregnancy rate and mean litter size in Asc−/− mice were not statistically different compared with WT mice (Fig. S5, D and E). These observations suggest that NLRP3 attenuates the process of ovarian aging independent of ASC expression.

To reinforce the role of NLRP3 in female fertility, we studied the effects of a gain-of-function NLRP3 mutant associated with neonatal onset multisystem inflammatory disease (NOMID) in mice (11), which showed data consistent with infertility (Fig. S6, A to D).

Age-related autophagic and apoptotic changes in the ovaries are prevented by Nlrp3 ablation
Blockade of autophagic flux and accumulation of undegraded substrates in the form of autophagosomes are linked to aging (12). Furthermore, apoptosis and autophagy are involved in the process of oocyte loss (13, 14). Nlrp3−/− female mice showed increased levels of ATG12, beclin 1, and LC3-II protein and reduced expression of p62/SQSTM1 compared with an accumulation of LC3-I with p62-aged WT mice associated with an impairment of autophagy (Fig. 3C). Apoptosis has been associated with ovary aging and inflammation (6). The expression of the proapoptotic protein BCL2-associated X protein (BAX) was also substantially lower in 12-month-old Nlrp3−/− ovaries compared with WT ovaries despite no significant changes in the antiapoptotic

Fig. 1. NLRP3 signaling is associated with ovarian aging. (A) Body weight progression of female C57BL/6J mice evaluated per month. (B) Mean serum AMH levels to evaluate the progression of ovarian reserve during aging. N = 8 per group. (C and D) Western blot analysis with representative blots including NLRP3, caspase-1, IL-1β, LC3-I, and p62 levels in the ovary of WT mice at different ages. Densitometric analysis is shown as mean ± SD, n = 10 mice. *P < 0.05, **P < 0.005, and ***P < 0.001; 2 months old versus other ages. N = 6 to 8 per group. (E) Immunofluorescence (F) visualization of NLRP3 (green) and nuclei (blue) in ovary and uterus tissues from young and old WT mice. Oocyte (asterisk), GCs (arrows), corpus luteum (CL), and follicular atresia (FA), DAPI, 4',6-diamidino-2-phenylindole. (F) Correlation of NLRP3 expression versus serum AMH levels during aging. The correlation was established by calculating correlation coefficients. (G) Human NLRP3 transcript expression levels were determined in GCs by real-time quantitative reverse transcription polymerase chain reaction (PCR); n = 20 for control and n = 20 for DOR groups. (H) Western blot analysis with representative blots including NLRP3, caspase-1, and IL-1β levels in GCs from four representative patients with DOR compared to four representative age-matched controls. Densitometric analysis is shown as mean ± SD. *P < 0.05, **P < 0.005, and ***P < 0.001; control versus patients.