Androgens Increase Accumulation of Advanced Glycation End Products in Granulosa Cells by Activating ER Stress in PCOS

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Polycystic ovary syndrome (PCOS) is associated with hyperandrogenism, and we previously found that androgens activate endoplasmic reticulum (ER) stress in granulosa cells from patients with PCOS. In addition, recent studies demonstrated the accumulation of advanced glycation end products (AGEs) in granulosa cells from PCOS patients, which contribute to the pathology. Therefore, we hypothesized that androgens upregulate the receptor for AGEs (RAGE) expression in granulosa cells by activating ER stress, thereby increasing the accumulation of AGEs in these cells and contributing to the pathology. In the present study, we show that testosterone increases RAGE expression and AGE accumulation in cultured human granulosa-lutein cells (GLCs), and this is reduced by pretreatment with tauroursodeoxycholic acid (TUDCA), an ER stress inhibitor in clinical use. Knockdown of the transcription factor C/EBP homologous protein (CHOP), an unfolded protein response factor activated by ER stress, inhibits testosterone-induced RAGE expression and AGE accumulation. The expression of RAGE and the accumulation of AGEs are upregulated in granulosa cells from PCOS patients and dehydroepiandrosterone-induced PCOS mice. Administration of the RAGE inhibitor FPS-ZM1 or TUDCA to PCOS mice reduces RAGE expression and AGE accumulation in granulosa cells, improves their estrous cycle, and reduces the number of atretic antral follicles. In summary, our findings indicate that hyperandrogenism in PCOS increases the expression of RAGE and accumulation of AGEs in the ovary by activating ER stress, and that targeting the AGE-RAGE system, either by using a RAGE inhibitor or a clinically available ER stress inhibitor, may represent a novel approach to PCOS therapy. **(*Endocrinology* 161: 1–13, 2020)**

Key Words: androgen, advanced glycation end product, granulosa cell, polycystic ovary syndrome, receptor for advanced glycation end products, unfolded protein response

Polycystic ovary syndrome (PCOS) is the most common endocrine and metabolic disorder in women of reproductive age, and hyperandrogenism is a key factor in its pathophysiology (1-4). Previously, we demonstrated that androgen activates endoplasmic reticulum (ER) stress in granulosa cells from PCOS patients and mice, and that this contributes to the pathology of PCOS, including ovarian fibrosis and the growth arrest of antral follicles (5, 6). ER stress, which involves the accumulation of unfolded or misfolded proteins in the ER, is incited by various physiological and pathological conditions that increase the demand for protein folding or attenuate the protein-folding capacity of the ER (7, 8). It results in the activation of several signal transduction

ISSN Online 1945-7170
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Received 4 November 2019. Accepted 4 February 2020.
First Published Online 5 February 2020.
Corrected and Typeset 24 February 2020.

Abbreviations: AGEs, advanced glycation end products; AR, androgen receptor; cDNA, complementary DNA; CHOP, C/EBP homologous protein; DHEA, dehydroepiandrosterone; DMSO, dimethyl sulfoxide; ER, endoplasmic reticulum; ESR, estrogen receptor; GLCs, granulosa-lutein cells; mRNA, messenger RNA; PCOS, polycystic ovary syndrome; qPCR, quantitative polymerase chain reaction; RAGE, receptor for advanced glycation end products; siRNA, small interfering RNA; TUDCA, tauroursodeoxycholic acid; UPR, unfolded protein response.

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cascades, collectively termed *the unfolded protein response* (UPR), which affects a wide variety of cellular functions (5, 6, 9-12).

It has been recently recognized that advanced glycation end products (AGEs) accumulate in the granulosa cells of PCOS patients (13) and contribute to the pathology by affecting a number of cellular processes, such as steroidogenesis, glucose metabolism, and the production of proinflammatory cytokines (10, 14, 15). AGEs are produced by the Maillard reaction, in which the carbonyl groups of carbohydrates react nonenzymatically with the primary amino groups of proteins, which bind to the receptor for AGEs (RAGE) and activate downstream signaling (16-18). AGEs accumulate in several tissues under various pathological conditions and during normal aging (16, 19); however, the mechanisms that regulate the expression of RAGE and the accumulation of AGEs are largely unknown.

On the basis of these findings, we hypothesized that androgens upregulate RAGE expression in granulosa cells by activating ER stress, and this increases the local accumulation of AGEs and contributes to PCOS pathology. To test this hypothesis, we determined the effects of testosterone on RAGE expression and AGE accumulation in cultured human granulosa-lutein cells (GLCs), and the role of ER stress, in particular that of the UPR transcription factor C/EBP homologous protein (CHOP). We then measured the expression of RAGE and the accumulation of AGEs in the granulosa cells of PCOS patients and in those of mice with dehydroepiandrosterone (DHEA)-induced PCOS. We also determined the in vivo effects of a RAGE inhibitor (FPS-ZM1) and a clinically available ER stress inhibitor (tauroursodeoxycholic acid; TUDCA) on the accumulation of AGEs in granulosa cells, in the estrous cycle, and in atretic antral follicle formation in a mouse model of PCOS. FPS-ZM1 is a recently developed RAGE-specific inhibitor, for which therapeutic effects were demonstrated in several pathologies associated with the accumulation of AGEs, including Alzheimer disease, in animal models (20-22). TUDCA has been used to treat liver diseases and to dissolve gallstones (23), and recent studies revealed that TUDCA functions as a chemical chaperone that attenuates protein misfolding and ameliorates ER stress (24).

Materials and Methods

Human samples

Women undergoing in vitro fertilization were recruited from the University of Tokyo Hospital, Matsumoto Ladies' Clinic, Hamada Clinic, and Akihabara Clinic. To compare the messenger RNA (mRNA) and protein expression of RAGE between patients with and without PCOS, we measured this in GLCs obtained from 11 PCOS patients and 10 control patients. Ovaries harvested from 3 PCOS patients and 3 control patients were also evaluated immunohistochemically. PCOS was diagnosed accordingly to the Rotterdam criteria (25), and the inclusion criteria for the control group were as follows: normal ovulatory cycle, no endocrine abnormalities, and normal ovarian morphology, confirmed ultrasonographically or histologically. Written informed consent was obtained from all the participants, and the experimental procedures were approved by the University of Tokyo Institutional Review Board with approval number 3594-(6). The study was conducted in accordance with the principles of the Declaration of Helsinki.

Mouse model of polycystic ovary syndrome

A well-established model of PCOS, created by the administration of DHEA to mice, was used in this study (26-28). Forty 3-week-old female Balb/C mice were obtained from Japan SLC (Hamamatsu) and were allocated to 4 groups (control, PCOS, PCOS plus FPS-ZM1, and POCS plus TUDCA). Mice in the control group were orally administered normal saline, subcutaneously injected with sesame oil, and intraperitoneally administered dimethyl sulfoxide (DMSO). Mice in the PCOS group were orally administered normal saline, subcutaneously injected with DHEA (6 mg/100 g of body weight; Sigma-Aldrich), and intraperitoneally injected with DMSO. Mice in the PCOS plus FPS-ZM1 group were orally administered normal saline, subcutaneously injected with DHEA, and intraperitoneally injected with FPS-ZM1 (0.1 mg/100 g of body weight; Cayman Chemical). Mice in the PCOS plus TUDCA group were orally administered TUDCA (50 mg/100 g of body weight; Tokyo Chemical Industry), subcutaneously injected with DHEA, and intraperitoneally injected with DMSO. The treatments were administered for 20 days, and the mice were humanely killed on day 21, when their ovaries were harvested. The doses of each substance used were determined in previous studies (6, 12, 22, 29). All the procedures described here were conducted in accordance with accepted standards of humane animal care, and specifically according to the guidelines and regulations of the University of Tokyo Committee on the Use and Care of Animals.

Isolation and culture of human granulosa-lutein cells

The retrieval of GLCs from follicular fluid was carried out as previously described (5, 10). Briefly, granulosa cell aggregates were dispersed using 0.2% hyaluronidase at 37°C for 30 minutes. Then, the suspension was slowly layered over Ficoll-Paque (GE Healthcare, Buckinghamshire, UK) and centrifuged at 700 × *g* for 30 minutes. The trilayer created by this density gradient separation technique contained the GLCs at the interface, and they were collected; resuspended in DMEM/F12 (Invitrogen) containing 10% fetal bovine serum (BioWest) and antibiotics (penicillin 100 U/mL, streptomycin 0.2 mg/mL, and amphotericin B 250 ng/mL; Sigma-Aldrich). GLCs were adjusted to a density of 2×10^5 cells/mL and cultured in

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6-well or 48-well plates. All GLCs were precultured for 3 to 5 days before treatment at 37°C in a humidified atmosphere containing 5% CO_2. After the preculture, GLCs were characterized as corresponding to the nonluteinized granulosa cells at early antral stage (30).

Treatment of human granulosa-lutein cells

To determine the effects of testosterone on the expression of RAGE, human GLCs were treated with 0.5, 1, 5, 10, or 50 μg/mL testosterone (Tokyo Chemical Industry) for 3 to 24 hours. To determine the most appropriate working concentration of testosterone, we initially determined the effects of various concentrations of testosterone on the induction of RAGE expression at the beginning of this study, with reference to concentrations previously used (5, 31-36). As shown in Fig. 1, testosterone increased *RAGE* mRNA expression at 5 μg/mL and increased it further, in a dose-dependent manner, up to 50 μg/mL. Therefore, we employed 50 μg/mL testosterone as a model of hyperandrogenism in cultured human GLCs in subsequent experiments. On the basis of the results of a time-course experiment, cells were incubated with testosterone for 24 hours in subsequent experiments.

Next, we determined the effect of ER stress inhibition on testosterone-induced RAGE expression. GLCs were treated with 1 mg/ml TUDCA for 24 hours, and then with testosterone for a further 24 hours. Pretreatment with TUDCA was performed as described previously (5, 6, 12). To knock down CHOP, small interfering RNA (siRNA) was obtained from Dharmacon (GE Healthcare) in the form of SMARTpools: ON-TARGETplus Human *DDIT3* siRNA (L004819-00-0005) and negative control ON-TARGETplus Non-targeting Pool (D-001810-10-20). GLCs were transfected with 50 nM siRNA for 24 hours in Opti-MEM Reduced Serum Medium (Thermo Fisher) using Lipofectamine RNAiMAX (Thermo Fisher). After transfection, this medium was removed and the GLCs were treated with testosterone for 24 hours. To evaluate the involvement of the androgen receptor (AR) in the mechanism of testosterone-induced RAGE expression, GLCs were treated with testosterone after preincubation with flutamide (Sigma-Aldrich), an AR antagonist, at 5 μM for 48 hours, or with siRNA targeting AR (SMARTpool: ON-TARGETplus human siRNA AR, L-003400-00-0020; Dharmacon, GE Healthcare). To examine the effects of testosterone mediated through conversion to estradiol, GLCs were treated with testosterone after preincubation with letrozole (Letrozole, L6545, Sigma-Aldrich), an aromatase inhibitor, at 1 μM for 48 hours, or with siRNA targeting estrogen receptor 2 (ESR2), a dominant ESR in granulosa cells (SMARTpool:

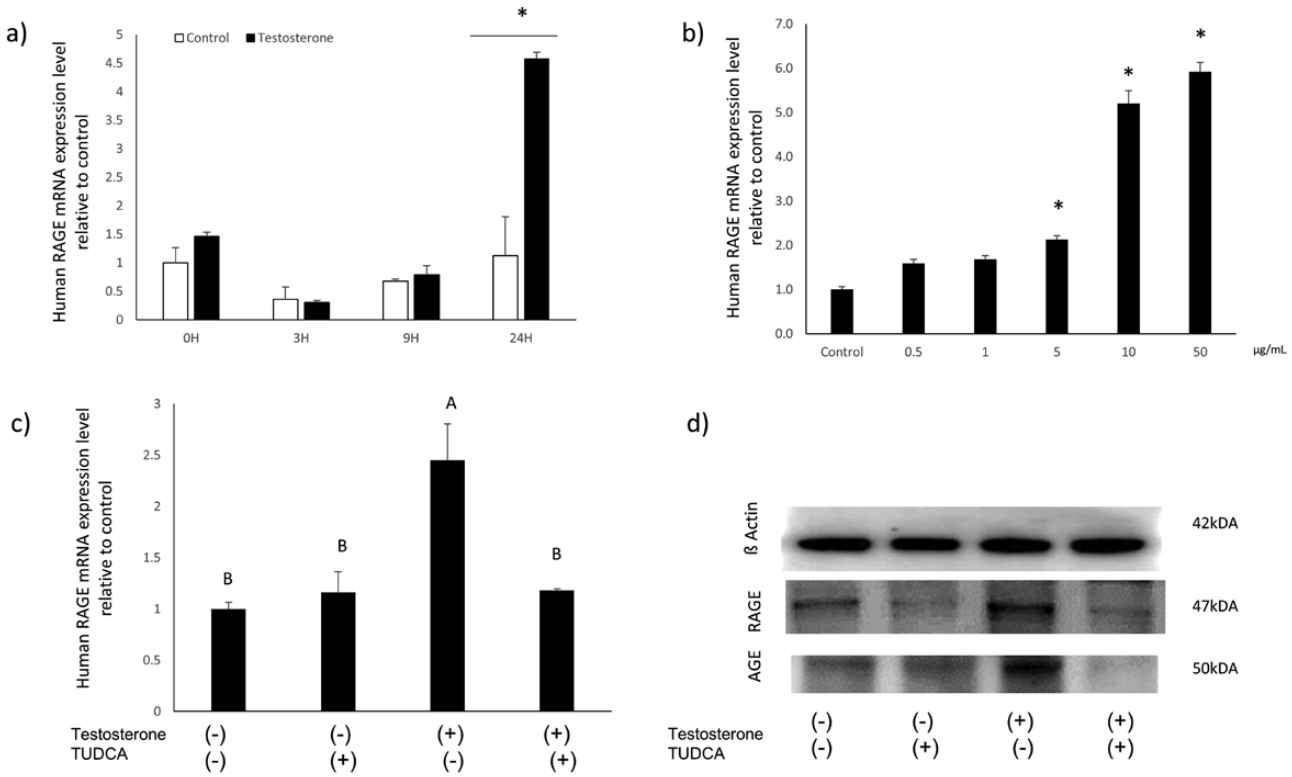

Figure 1. Testosterone increases receptor for advanced glycation end products (RAGE) expression in cultured human granulosa-lutein cells (GLCs), and this effect is reduced by inhibition of endoplasmic reticulum (ER) stress. A, GLCs treated with 50 μg/mL testosterone for 0, 3, 9, and 24 hours. B, GLCs treated with 0.5, 1, 5, 10, or 50 μg/mL testosterone for 24 hours. C and D, GLCs preincubated with the ER stress inhibitor tauroursodeoxycholic acid (TUDCA) (1 mg/mL) for 24 hours and then incubated with 50 μg/ml testosterone for 24 hours. A to C, *RAGE* messenger RNA expression in GLCs measured using real-time polymerase chain reaction and normalized to *GAPDH* expression. The values are the means ± SEM of triplicate or quadruplicate experiments, expressed relative to the mean control value. D, Western blotting was performed using anti-RAGE or anti-AGE antibodies, with β-actin as the loading control. Results are representative of 3 different samples and at least 3 independent experiments per sample. Letters denote significant differences among groups. **P* less than .05.

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ON-TARGETplus human siRNA ESR2, L-003402-00-0020; Dharmacon, GE Healthcare). The siRNA pool used as a negative control and the method of transfection were as described for the knockdown of CHOP. The doses of flutamide (5, 37-39) and letrozole (40, 41) were determined according to the previous papers.

RNA extraction, reverse transcription, and quantitative real-time polymerase chain reaction

To obtain RNA from GLCs and to synthesize the corresponding cDNA, a SuperPrep Cell Lysis & RT Kit for quantitative polymerase chain reaction (qPCR) (Toyoba) was used. *RAGE* mRNA expression was measured using real-time qPCR and the obtained complementary DNA (cDNA). The 20 µL reaction volumes contained 10 µL KOD SYBR qPCR Mix (TOYOBO), 0.5 µL forward and reverse primers, and 2 µL cDNA. The analysis was performed using the Light Cycler System (Roche Diagnostic GmBH), and the target mRNA expression levels that were obtained were normalized to the corresponding expression levels of *GAPDH* mRNA, which served as a reference gene, and relative expression values are shown in each figure. The primer sequences were as follows: *RAGE* (sense, 5'-GGAAAGGAGACCAAGTCCAA-3'; antisense, 5'-CATCCAAGTGCCAGCTAAGA-3') and *GAPDH* (sense, 5'-TGGACCTGACCTGCCGTCTA-3'; antisense, 5'-CTGCTTCACCACCTTCTTGA-3'). The cycling conditions were 98°C, 60°C, and 68°C for 10, 10, and 30 seconds, respectively, for 40 cycles. For all the in vitro experiments, samples were analyzed in triplicate or quadruplicate.

Immunoblotting

PhosphoSafe Extraction Reagent (Novogen) was used to extract soluble proteins from the GLCs. The suspensions were sonicated for 10 minutes and then centrifuged to remove the insoluble material. The supernatants were collected, and the concentration of protein in each sample was measured using the BioRad protein assay reagent. A total of 30 µg/70 µg of each protein lysate was separated using 10% or 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels and transferred to membranes using a Trans-Blot Turbo Transfer System (BioRad). The membranes were then incubated in 5% skim milk in tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 hour at room temperature. Membranes blotted with 30 µg protein per lane were probed with an anti-RAGE antibody (RRID: AB_777613, 1:1000; Abcam [42]), and those blotted with 70 µg protein per lane were probed with an anti-AGE antibody (RRID: AB_447638, 1:1000; Abcam [43]) overnight at 4°C. Horseradish peroxidase-conjugated secondary antibody (anti-rabbit RRID: AB_2099233; Cell Signaling Technology, Danvers, MA [44]) was then applied at a 1:800 dilution. Chemiluminescence was detected using ECL Select Detection Reagent (GE Healthcare) and exposure times for RAGE and AGE of 0.5 and 1 second, respectively. Images were acquired on an ImageQuant LAS 4000 Mini luminescent image analyzer (GE Healthcare). Anti-β-actin antibody (RRID: AB_476697, 1:2000; Sigma-Aldrich [45]) was used to demonstrate the expression of β-actin, which was used as the loading control, and all the experiments were repeated 3 times. Quantification of intensity of bands was conducted using ImageJ software (National Institutes of Health) (46).

Immunohistochemistry

Human and mouse ovarian sections were immunostained using RAGE (RRID: AB_777613, 1:800; Abcam [42]) or AGE (RRID: AB_447638, 1:800; Abcam [43]) antibodies and the EnVision+ Dual Link System/HRP (DAB; Dako). Isotype-specific immunoglobulin G was used as the negative control, and antigen retrieval was performed using Target Retrieval Solution (Dako). These procedures were repeated 3 times, independently, for each sample. The images were analyzed using ImageJ software (National Institutes of Health) (46). The number of positively stained granulosa cells were counted in 5 randomly selected follicles in ovaries from 3 different patients or 5 different mice.

Examination of vaginal smears

Estrous cycle for each mouse was analyzed by obtaining vaginal smear daily between 9 AM and 11 AM from day 12 of treatment until the day of humane killing. The detached vaginal cells were collected via vaginal saline lavage and transfer onto a clean glass. Air-dried smears were treated with Diff-Quick Solution (Sysmex). Mice estrous cycle was examined under light microscopy and the stage was determined as previously described (47). Briefly, smears with predominantly nucleated epithelial cells were classified as proestrus. An estrus vaginal smear consisted mainly of cornified epithelial cells. Metestrus smear had an equal proportion of leukocytes, cornified, and nucleated epithelial cells. Finally, in the diestrus stage, the smear consisted primarily of leukocytes.

Quantification of atretic antral follicles

Ovaries from mice were fixed and embedded in paraffin, serially sectioned at 5 µm thickness, and then stained with hematoxylin and eosin to assess the distribution and classification of follicles. Atretic follicles were identified by disorganization, shrinkage, or loss of the granulosa cell wall; hypertrophy of theca cells; and disintegration of cumulus cells (48, 49), independent of the presence of an oocyte (50). The stained sections were examined by light microscopy using an Olympus BX50 Fluorescence Microscope. The number of atretic antral follicles were counted on every sixth section across the entire ovary, as previously described (51). To ensure each atretic antral follicle was counted only once, adjacent sections were also analyzed.

Statistical analysis

Data were analyzed using JMP Pro 11 software (SAS Institute). For paired comparisons, the Student t test was used, whereas for multiple comparisons, the Tukey-Kramer honest significant difference test was used. *P* less than .05 was

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considered to represent statistical significance, and the data are displayed as means ± SEM.

Results

Testosterone increases RAGE expression in cultured human GLCs, and this is reduced by the inhibition of ER stress

To determine the effect of testosterone on *RAGE* mRNA expression, we treated human GLCs with 50 µg/mL testosterone for 0, 3, 9, and 24 hours. The expression of *RAGE* mRNA was significantly higher in cells exposed to 24 hours of testosterone than in control cells (Fig. 1A). As shown in Fig. 1B, the induction of *RAGE* mRNA expression by testosterone was dose dependent, as illustrated by significant differences in expression after treatment with 5, 10, or 50 µg/mL for 24 hours. To determine whether ER stress participates in testosterone-induced RAGE expression, GLCs were pretreated with an ER stress inhibitor, TUDCA, and then treated with testosterone. As shown in Fig. 1C and 1D, pretreatment with TUDCA abrogated the testosterone-induced RAGE mRNA and protein expression. We also examined the accumulation of AGEs in GLCs treated with testosterone. As shown in Fig. 1D, treatment with testosterone increased AGE immunoreactivity in GLCs, and this was reduced by pretreatment with TUDCA, similar to the pattern for RAGE expression.

Testosterone-induced RAGE expression is mediated by the UPR transcription factor CHOP in cultured human GLCs

Next, to determine the molecular mechanisms involved in the upregulation of RAGE expression by testosterone, we knocked down CHOP, a transcription factor that is activated during ER stress, by RNA interference. Following testosterone treatment, CHOP-deficient cells demonstrated significantly lower expression of *RAGE* mRNA than control siRNA-treated cells (Fig. 2A). This finding was confirmed by Western blotting, which showed a concomitant reduction in AGE immunoreactivity following CHOP silencing (Fig. 2B).

Both AR and ESR mediate testosterone-induced RAGE expression in cultured human GLCs

Pretreatment with flutamide, an AR antagonist, significantly attenuated the induction of *RAGE* mRNA expression by testosterone (Fig. 3A). To further explore the role of AR signaling in this process, *RAGE* mRNA expression was measured in AR-deficient GLCs treated with testosterone. As shown in Fig. 3B, the knockdown significantly attenuated the testosterone-induced mRNA expression of *RAGE*. To examine the effects of testosterone mediated through conversion to estradiol, GLCs were treated with testosterone after preincubation with letrozole. As shown in Fig. 3C, pretreatment with letrozole significantly attenuated the induction of *RAGE* mRNA expression by testosterone. In addition, the knockdown of ESR2, a dominant ESR in human granulosa cells, significantly attenuated the testosterone-induced mRNA expression of RAGE (Fig. 3D).

The expression of RAGE and the accumulation of AGEs are higher in the granulosa cells of PCOS patients than in those of control participants

As shown in Fig. 4, GLCs harvested from PCOS patients exhibited higher expression of *RAGE* mRNA and protein than those from control participants. To confirm the upregulation of RAGE in the granulosa cells of PCOS patients, immunohistochemical analysis was performed. As shown in Fig. 5A to 5E, RAGE immunoreactivity was also higher in granulosa cells from PCOS patients than in those from control participants. Immunohistochemical analysis using an anti-AGE antibody revealed a concomitant increase in the accumulation of AGEs in the granulosa cells of PCOS patients (Fig. 5F to 5J).

RAGE or ER stress inhibition reduces RAGE expression and AGE accumulation in granulosa cells, and improves the estrous cycle and ovarian morphology of mice with PCOS

Consistent with the results obtained in human ovaries, the immunoreactivities of RAGE and AGE were higher in the granulosa cells of PCOS mice (Fig. 6 and Fig. 7). As shown in Figs. 6 and 7, treatment of PCOS mice with a RAGE inhibitor, FPS-ZM1, reduced RAGE expression and AGE accumulation in granulosa cells, indicating that systemic administration of FPS-ZM1 effectively inhibits the local AGE-RAGE system in the ovary. Examination of vaginal smears revealed an improvement in the estrous cycles of FPS-ZM1–treated PCOS mice, with a significant reduction in the number of acyclic animals, although their cycles were not completely normalized (Fig. 8C and 8E). The number of atretic follicles was also lower in the PCOS + FPS-ZM1 group than in the untreated PCOS mice (Fig. 8F).

On the basis of the results of the in vitro experiments, we wondered whether ER stress inhibition with TUDCA would have similar effects to those of FPS-ZM1 treatment. Administration of TUDCA reduced RAGE expression (Fig. 6) and AGE accumulation (Fig. 7) in the granulosa cells of PCOS mice. Furthermore, all PCOS mice treated with TUDCA exhibited estrous cycles,

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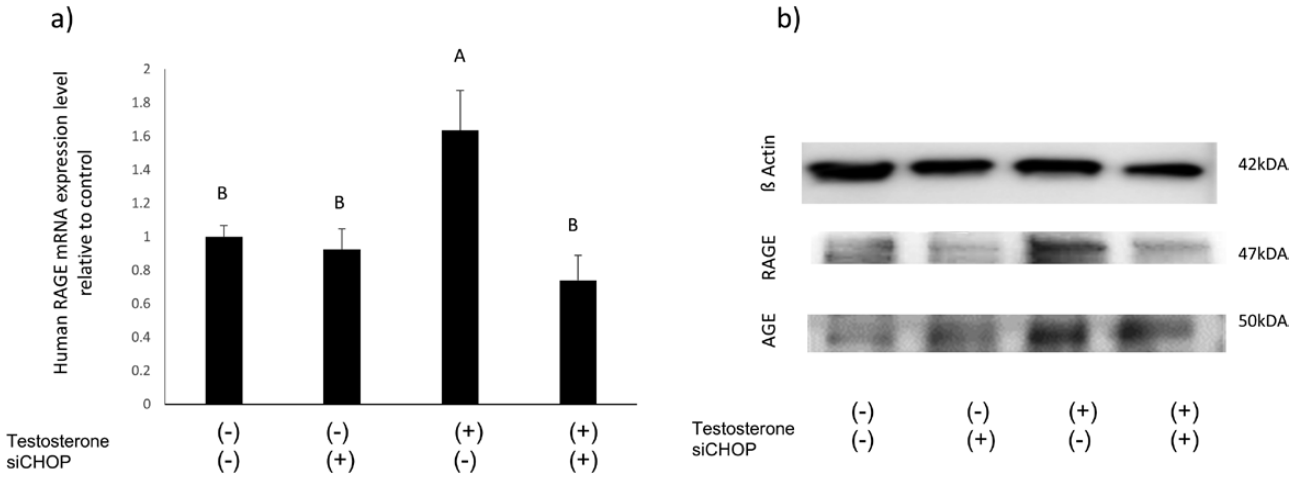

Figure 2. C/EBP homologous protein (CHOP) mediates testosterone-induced receptor for advanced glycation end products (RAGE) expression. Granulosa-lutein cells (GLCs) were transfected with CHOP small interfering RNA (siRNA) (50 nM) or negative control siRNA (50 nM) for 24 hours and then treated with 50 μg/mL testosterone for 24 hours. A, *RAGE* messenger RNA expression in GLCs was measured using real-time polymerase chain reaction and was normalized to *GAPDH* expression. The values are the means ± SEM of triplicate or quadruplicate experiments, expressed relative to the mean control value. B, Western blotting was performed using anti-RAGE or anti-AGE antibodies, with β-actin as the loading control. The results are representative of at least 3 independent experiments using 3 different samples. Letters denote significant differences among groups.

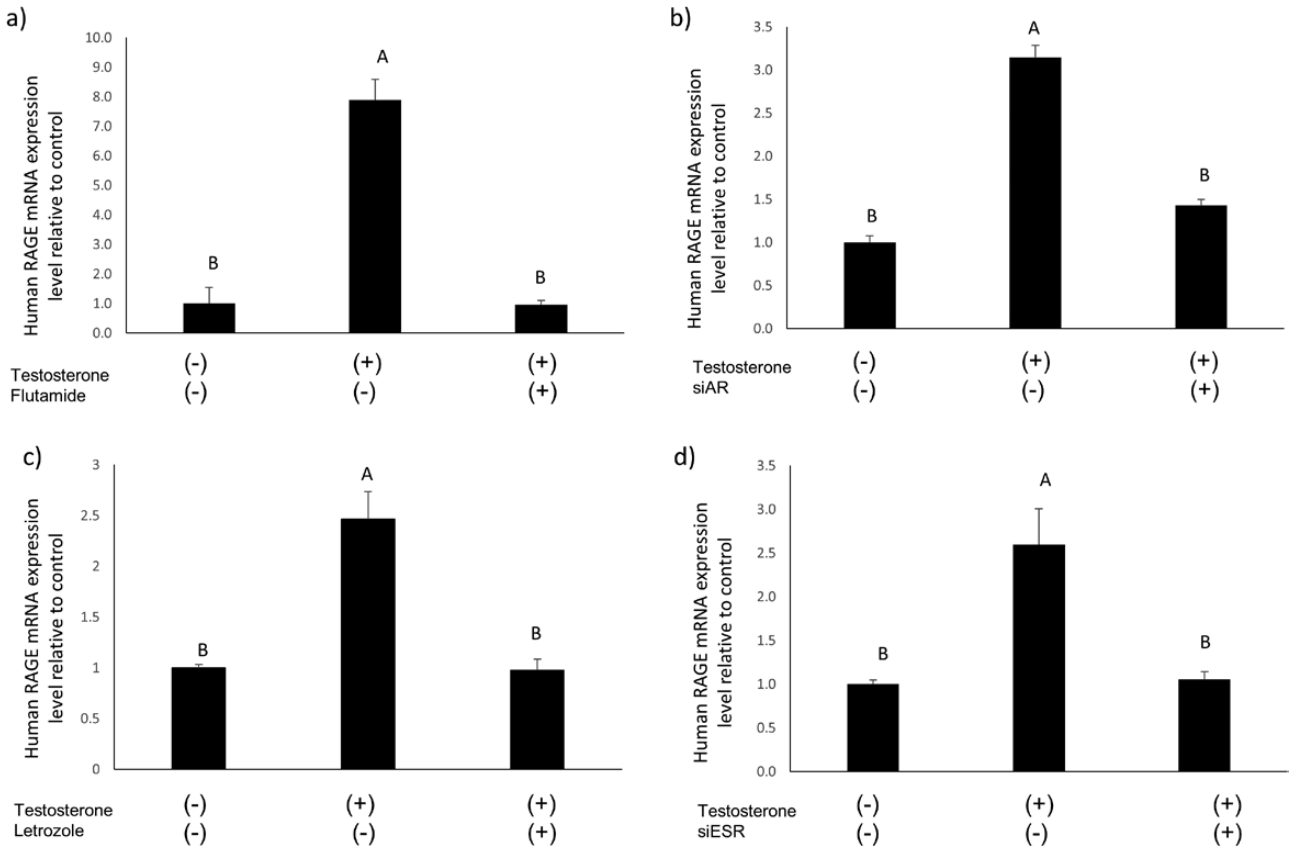

Figure 3. Both androgen receptor (AR) and estrogen receptor (ESR) mediate testosterone-induced *RAGE* messenger RNA (mRNA) expression in cultured human granulosa-lutein cells (GLCs). A, GLCs were preincubated with 5 μM flutamide for 48 hours and then incubated with 50 μg/mL testosterone for 24 hours. B, GLCs were transfected with AR small interfering RNA (siRNA) (50 nM) or negative control (50 nM), and then treated with 50 μg/mL testosterone for 24 hours. C, GLCs were preincubated with 1 μM letrozole for 48 hours and then incubated with 50 μg/mL testosterone for 24 hours. D, GLCs were transfected with ESR2 siRNA (50 nM) or negative control (50 nM), and then treated with 50 μg/mL testosterone for 24 hours. *RAGE* mRNA expression was measured using real-time polymerase chain reaction and was normalized to *GAPDH* expression. The values are the means ± SEM of triplicate or quadruplicate experiments, expressed relative to the mean control value. The letters denote significant differences among groups.

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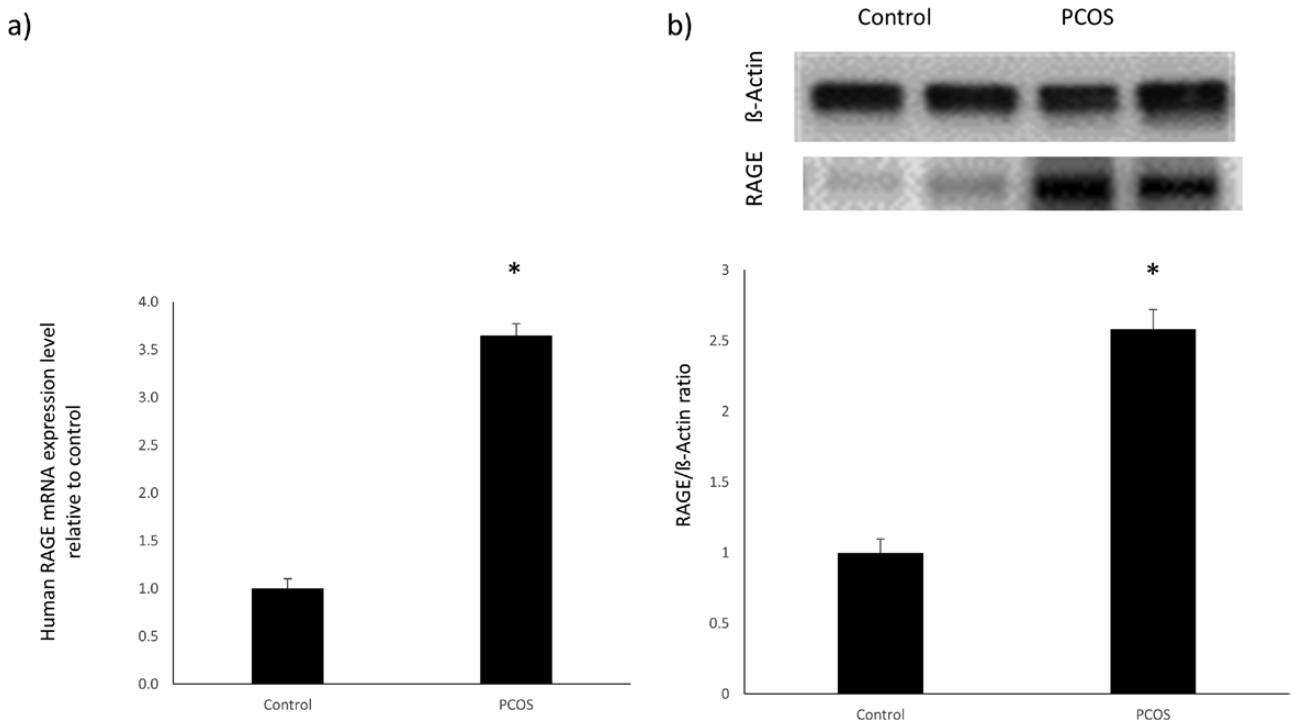

Figure 4. Expression of receptor for advanced glycation end products (RAGE) in granulosa-lutein cells (GLCs) from control and polycystic ovary syndrome (PCOS) patients. A, *RAGE* messenger RNA expression in GLCs from control participants (n = 10) and from patients with PCOS (n = 11) was measured using real-time polymerase chain reaction and was normalized to GAPDH expression. The values are means ± SEM, expressed relative to the mean control value. B, Western blotting was performed using anti-RAGE antibody, with β-actin as the loading control, using the same set of samples as used in A. The results of quantitative analysis shown are means ± SEM, expressed relative to the mean control value. Two representative blots from different patients in each group are also shown. **P* less than .05 compared with controls.

although they were not completely normal (Fig. 8D to 8E). The number of atretic follicles was also lower in the TUDCA-treated group (Fig. 8F).

Discussion

The present study shows that testosterone increases the expression of RAGE and the accumulation of AGEs in human GLCs, and that this is mediated by the UPR transcription factor CHOP. The expression of RAGE and the accumulation of AGEs were higher in the granulosa cells of PCOS patients and in a mouse model of PCOS than those of control participants and in control mice. The administration of a RAGE inhibitor (FPS-ZM1) or a clinically available ER stress inhibitor (TUDCA) to PCOS mice reduced the expression of RAGE and the accumulation of AGEs in granulosa cells, and this was accompanied by an improvement in estrous cycling and a reduction in the number of atretic antral follicles.

In cultured human GLCs, RAGE expression was increased by testosterone. Very few studies have examined the underlying mechanisms that regulate expression of RAGE to date. AGEs themselves induce RAGE expression in various types of human cells including GLCs (52-55); thus it seems that the AGE-RAGE system represents a vicious cycle, regardless of the affected cell type. In human vascular endothelial cells, tumor necrosis factor-α and 17β-estradiol also induce RAGE expression (53). The present study reveals that in granulosa cells, testosterone contributes to the induction of RAGE, in addition to AGEs themselves. Pretreatment with the AR antagonist flutamide or knockdown of AR using siRNA, as well as pretreatment with the aromatase inhibitor letrozole or knockdown of ESR, inhibits the stimulatory effect of testosterone on RAGE expression. These results imply that AR and ESR signaling are both involved in testosterone-induced RAGE expression, although the respective role of AR and ESR signaling, as well as which signaling plays more critical roles in this process, remains to be elucidated. Intriguingly, several lines of evidence indicate a strong association between AGEs and hyperandrogenism (56): Serum AGEs and testosterone are positively correlated in PCOS patients (57), and the consumption of a high-AGE diet results in increases in RAGE expression and the deposition of AGEs in the ovary, which is associated with higher serum testosterone concentrations (58, 60). Therefore, it is plausible that the AGE-RAGE system in granulosa cells is coordinately upregulated by AGEs and testosterone in related conditions, including PCOS.

ER stress is activated by testosterone and mediates the increase in RAGE expression and the accumulation of

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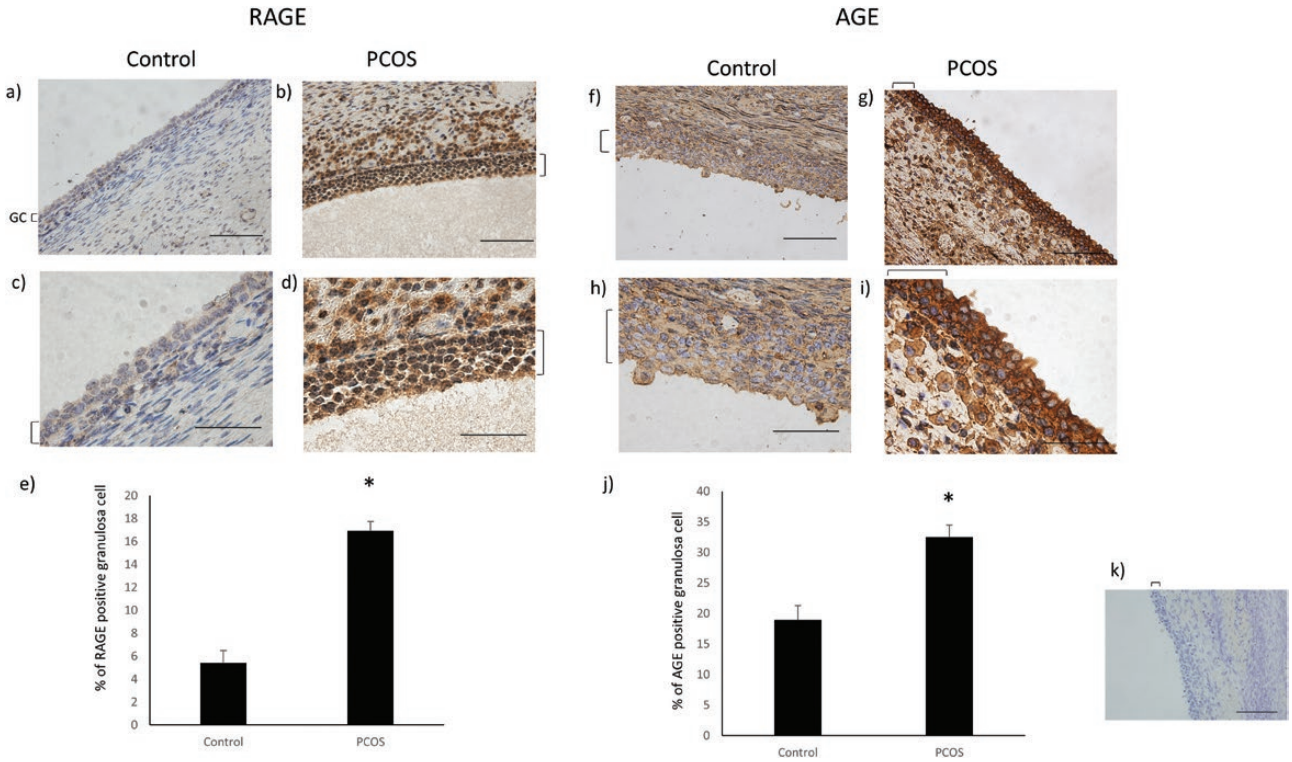

Figure 5. Expression of receptor for advanced glycation end products (RAGE) and accumulation of AGEs in granulosa cells from control participants and patients diagnosed with polycystic ovary syndrome (PCOS). Immunohistochemical analysis was performed on the ovaries of 3 patients with PCOS and 3 without. A to D, Cross-sections of ovaries were stained with anti-RAGE antibody and counterstained with hematoxylin; E, quantitative analysis. F to I, Cross-sections of ovaries were stained with anti-AGE antibody and counterstained with hematoxylin; J, quantitative analysis. A control section showing the background level of staining, to which isotype immunoglobulin G and hematoxylin were applied, is shown in K. For representative ovarian cross-sections, the lower panels C and D show highly magnified views of A and B, and the lower panels H and I show highly magnified views of F and G. Scale bars, (a, b, f, g, k), 100 μm; (c, d, h, i), 50 μm. *P less than .05 vs control. GC, granulosa cells layers.

AGEs in human GLCs. Of interest, there is substantial evidence that the AGE-RAGE system activates ER stress in various cells, including granulosa cells, as previously reported (10, 61, 62); however, this is the first report to show that ER stress is involved in the activation of the AGE-RAGE system. The results of the interference assay suggest that CHOP, a UPR transcription factor induced by ER stress, mediates the increase in RAGE expression and AGE accumulation induced by testosterone. We focused on CHOP, among various UPR factors, because RAGE expression is transcriptionally regulated in human vascular endothelial cells (53, 54), and the promoter region of RAGE harbors multiple CHOP binding sites. It is plausible that other UPR transcription factors may also be involved in this process. We previously demonstrated that ER stress is activated by testosterone, and that the activation of ER stress is required for testosterone-induced apoptosis in these cells (5). When these findings are taken together with those of the present study, it appears that testosterone modulates various cellular functions in granulosa cells via UPR pathways.

The expression of RAGE and the accumulation of AGEs were increased in the granulosa cells of PCOS patients and in those of mice with DHEA-induced PCOS. These findings are consistent with those of a previous study (13). Immunohistochemical examination of ovarian sections from PCOS women demonstrated stronger localization of both AGEs and RAGE in the granulosa cell layer than in that of healthy controls (13). In addition, several studies reported higher serum AGE concentrations (56) and lower concentrations of soluble RAGE, a decoy receptor for AGE that serves as an inhibitor of the AGE-RAGE system, in follicular fluid (63, 64). AGEs are formed exogenously, consumed in the diet or inhaled when smoking, or endogenously; the latter is accelerated by aging, hyperglycemia, and obesity (56). Evidence for the potential involvement of AGEs in PCOS is further strengthened by the finding that rodents fed a high-AGE diet exhibit PCOS-like phenotypes (52, 65). AGEs seem to have both systemic and local effects on the pathogenesis of PCOS; they alter the function of various cell types, including vascular endothelial cells, adipocytes, and granulosa cells (56). In granulosa cells, AGEs increase the production of estradiol and proinflammatory cytokines (10, 15), and they reduce glucose uptake (14).

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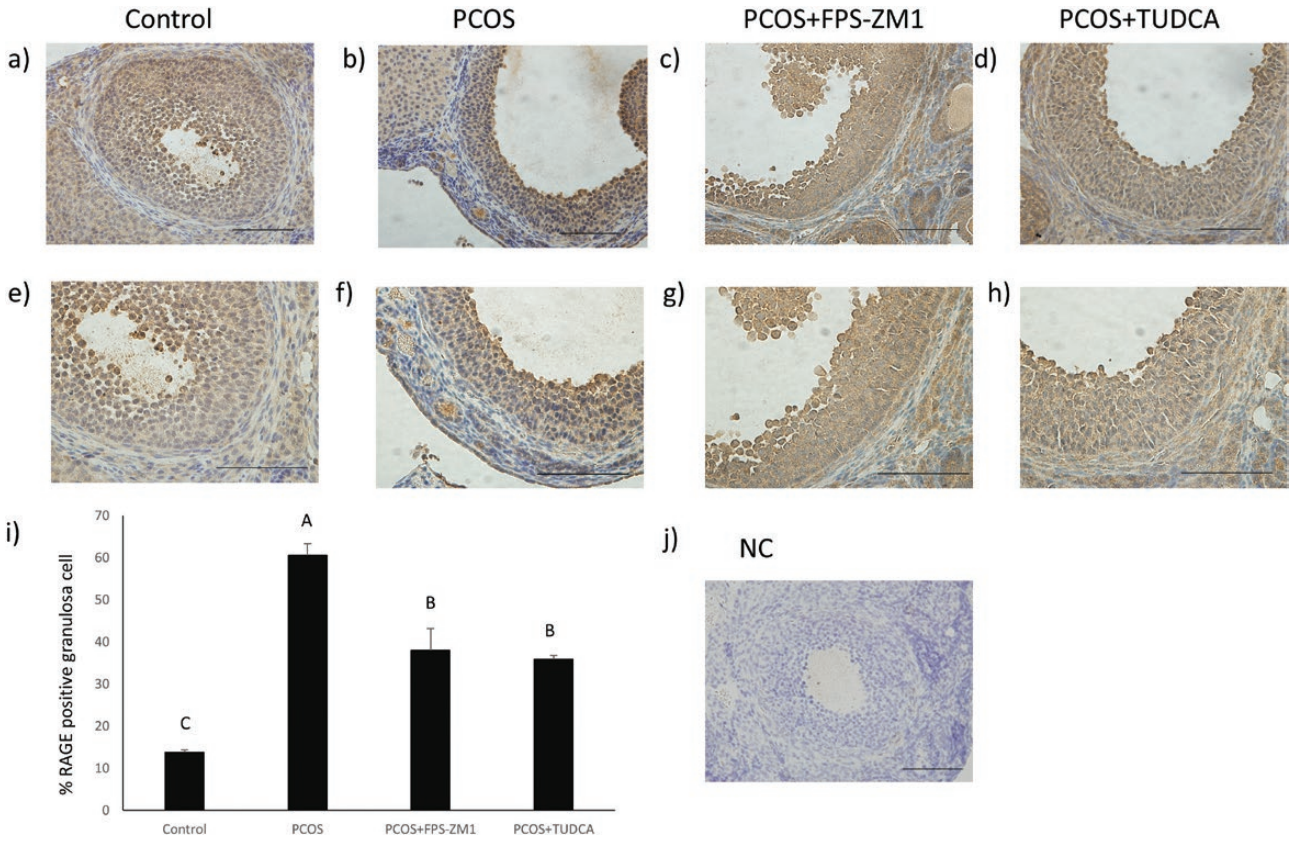

Figure 6. Effect of a receptor for advanced glycation end products (RAGE) inhibitor and an endoplasmic reticulum (ER) stress inhibitor on RAGE expression in the granulosa cells of polycystic ovary syndrome (PCOS) mice. Mice were subjected to 1 of 4 treatments: oral administration of saline, subcutaneous (SC) injection of sesame oil, and intraperitoneal (IP) administration of dimethyl sulfoxide (DMSO) (control, n = 10), oral administration of saline, SC dehydroepiandrosterone (DHEA) 6 mg/100 g of body weight, and IP DMSO (PCOS, n = 10), oral administration of saline, SC DHEA, and IP administration of the RAGE inhibitor FPS-ZM1 0.1 mg/100g of body weight (PCOS + FPS-ZM1, n = 10), and oral administration of the ER stress inhibitor tauroursodeoxycholic acid (TUDCA) 50 mg/100 g of body weight, SC DHEA, and IP DMSO (PCOS + TUDCA, n = 10). SC, IP, and oral administration were performed daily for 20 days. Ovaries were collected on day 21, and immunohistochemical analysis was performed. A to H, Cross-sections through the ovaries were immunostained with anti-RAGE antibodies and counterstained with hematoxylin; I, quantitative analysis. A control section showing the background level of staining, to which isotype immunoglobulin G and hematoxylin were applied, is shown in J. For representative ovarian cross-sections, the lower panels E to H show highly magnified views of A to D. All scale bars represent 100 μm. Letters denote significant differences among treatment groups. NC, negative control.

In the present study, administration of a RAGE inhibitor (FPS-ZM1) to PCOS mice reduced the expression of RAGE and the accumulation of AGEs in granulosa cells. FPS-ZM1 is a RAGE-specific inhibitor that binds to the V-domain of RAGE (20). Because ligand-RAGE interactions activate nuclear factor-κB, which increases the transcription of RAGE (52), administration of FPS-ZM1 not only inhibits the binding of AGEs to RAGE but also reduces RAGE expression. The present results suggest that systemic administration of FPS-ZM1 effectively attenuates local AGE-RAGE activation in the ovary, and that the inhibition of RAGE may represent a novel therapeutic approach in PCOS.

We also evaluated the effects of the inhibition of ER stress in PCOS mice. Treatment with the ER stress inhibitor TUDCA had similar effects to those of FPS-ZM1: It reduced the expression of RAGE and the accumulation of AGEs in granulosa cells, it improved the estrous cycle, and it reduced the number of atretic antral follicles in PCOS mice. We previously showed that systemic administration of an ER stress inhibitor significantly reduces ER stress in granulosa cells (5, 6). When taken together with the results of the in vitro experiments, it appears that a reduction in ER stress in granulosa cells achieved by the systemic administration of TUDCA contributes to a reduction in RAGE expression and AGE accumulation in these cells. An advantage of TUDCA is its safety in humans, which has been established through clinical use in the treatment of liver disease over many years (23), whereas there are no substances that directly inhibit the AGE-RAGE system that can be used clinically at present, such as an inhibitor of the formation of AGEs or an inhibitor for the interaction between AGEs and RAGE (66, 67).

The reductions in RAGE expression and AGE accumulation in granulosa cells shown in the present study

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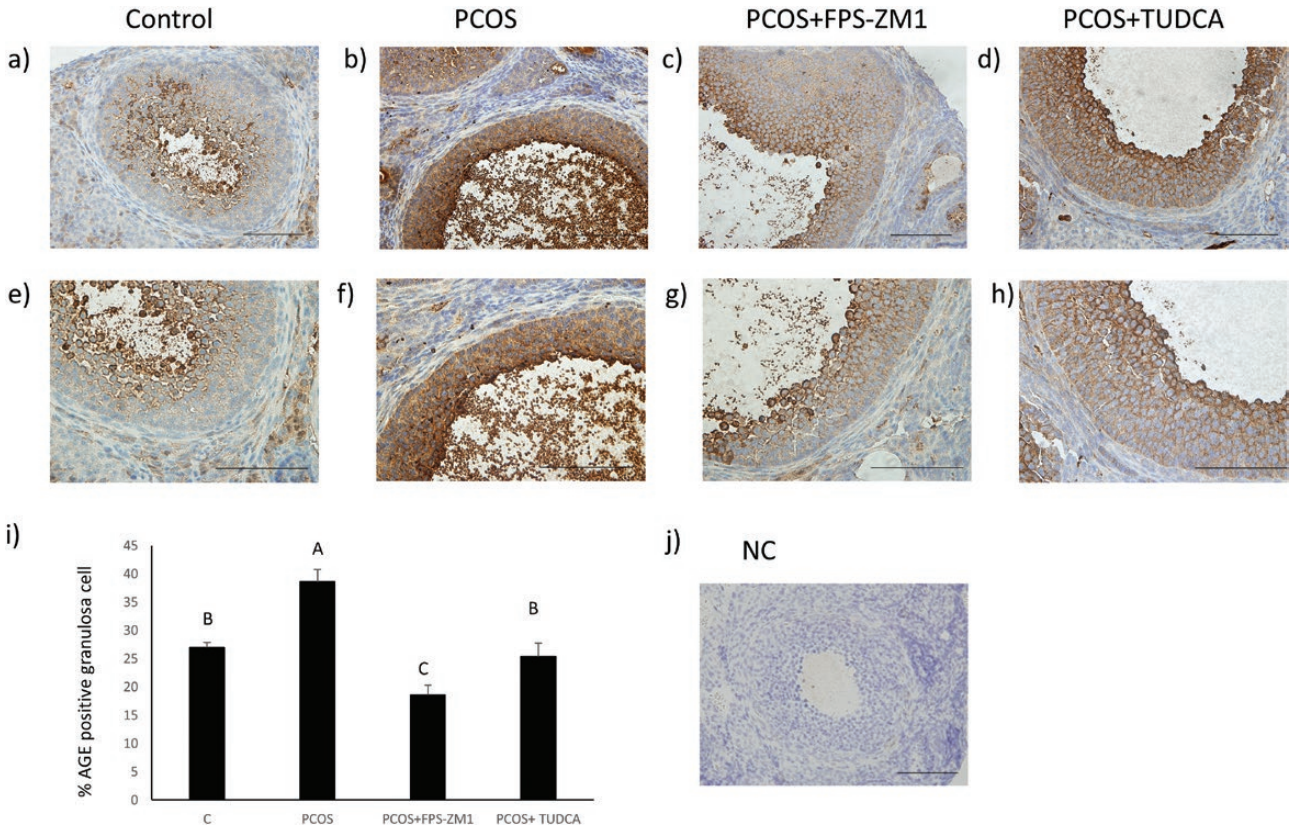

Figure 7. Effect of a receptor for advanced glycation end products (RAGE) inhibitor and an endoplasmic reticulum (ER) stress inhibitor on AGE accumulation in the granulosa cells of polycystic ovary syndrome (PCOS) mice. Mice were subjected to 1 of 4 treatments: oral administration of saline, subcutaneous (SC) injection of sesame oil, and intraperitoneal (IP) administration of dimethyl sulfoxide (DMSO) (control, n = 10), oral administration of saline, SC dehydroepiandrosterone (DHEA) 6 mg/100 g of body weight, and IP DMSO (PCOS, n = 10), oral administration of saline, SC DHEA, and IP administration of the RAGE inhibitor FPS-ZM1 0.1 mg/100g of body weight (PCOS + FPS-ZM1, n = 10), and oral administration of the ER stress inhibitor tauroursodeoxycholic acid (TUDCA) 50 mg/100 g of body weight, SC DHEA, and IP DMSO (PCOS + TUDCA, n = 10). SC, IP, and oral administration were performed daily for 20 days. Ovaries were collected on day 21, and immunohistochemical analysis was performed. A to H, Cross-sections through the ovaries were immunostained with anti-AGE antibodies and counterstained with hematoxylin; I, quantitative analysis. A control section showing the background level of staining, to which isotype immunoglobulin G and hematoxylin were applied, is shown in J. For representative ovarian cross-sections, the lower panels E to H show highly magnified views of A to D. All scale bars represent 100 μm. Letters denote significant differences among treatment groups. NC, negative control.

are not the only mechanisms by which TUDCA may have therapeutic effects on PCOS. Our previous studies showed that ER stress is activated in granulosa cells of PCOS patients and that the administration of ER stress inhibitors reduces ovarian fibrosis, by reducing the secretion of profibrotic factors from granulosa cells, and reduces the apoptosis of granulosa cells in antral follicles (5, 6). Therefore, it is possible that the inhibition of ER stress in granulosa cells has beneficial effects in PCOS, by inhibiting both the AGE-RAGE system and the UPR independent of AGE-RAGE system, which is activated in the follicular environment of PCOS ovaries. However, the potential systemic effects of ER stress inhibition remain to be elucidated, including the effects of altering RAGE and UPR signaling in tissues outside the ovary, as do the effects of ER stress on the formation of AGEs. The present study shows that TUDCA improves the estrous cycle and attenuates atretic antral follicle formation. However, these findings are not consistent with those of our previous study, which failed to show these improvements, although ovarian fibrosis was significantly lower after TUDCA treatment (6). We speculate that this discrepancy may be due to differences in the settings of the studies. In the present study, we used double the number of mice in each group compared with the previous study, and the number of sections taken from each ovary was also increased to facilitate detection of the atretic antral follicles present. In addition, multiple observers were involved in the reading of vaginal smears to ensure consistency of interpretation (68). Thus, the more careful and thorough examination of the phenotype in the present study should have yielded a more accurate interpretation.

A potential limitation of the present study would be that it is not determined whether the concentrations of testosterone usually used in human GLC culture studies are appropriate as a model of in vivo hyperandrogenism, because the environment of the in vitro GLC culture system is different from the in vivo follicular environment

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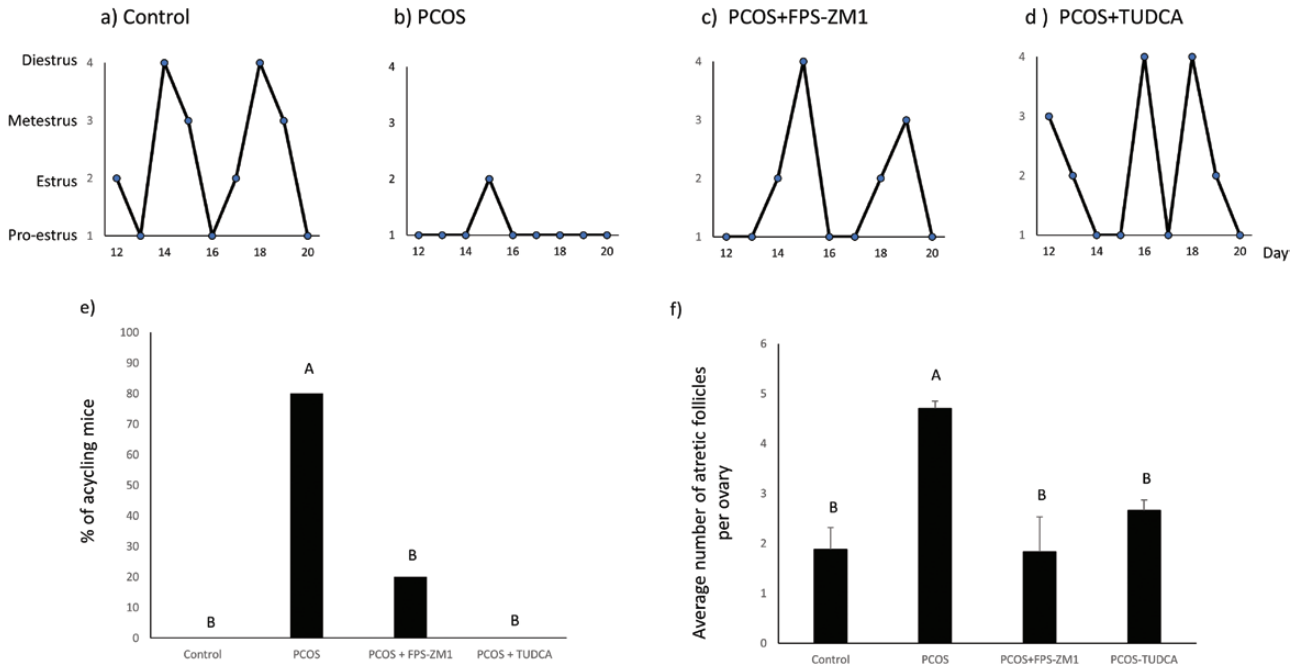

Figure 8. Effect of a receptor for advanced glycation end products (RAGE) inhibitor and an endoplasmic reticulum (ER) stress inhibitor on the estrous cycles and ovarian morphology of polycystic ovary syndrome (PCOS) mice. Treatment with a RAGE inhibitor (FPS-ZM1) or an ER stress inhibitor (tauroursodeoxycholic acid [TUDCA]) improves the estrous cycle of PCOS mice. Mice were subjected to 1 of 4 treatments: oral administration of saline, subcutaneous (SC) injection of sesame oil, and intraperitoneal (IP) administration of dimethyl sulfoxide (DMSO) (control, n = 10), oral administration of saline, SC dehydroepiandrosterone (DHEA) 6 mg/100 g of body weight, and IP DMSO (PCOS, n = 10), oral administration of saline, SC DHEA, and IP administration of the RAGE inhibitor FPS-ZM1 0.1 mg/100 g of body weight (PCOS + FPS-ZM1, n = 10), and oral administration of the ER stress inhibitor TUDCA 50 mg/100 g of body weight, SC DHEA, and IP DMSO (PCOS + TUDCA, n = 10). A to E, Examination of vaginal smears was performed daily between days 12 and 20 of treatment. A to D, Representative estrous cycles for each group. E, The percentage of mice that were acyclic (constant diestrus) and F, the mean number of atretic follicles per section. Letters denote significant differences among treatment groups.

where various intraovarian factors interact each other. Furthermore, the concentrations of testosterone in follicular fluid of antral follicles of PCOS women, not of preovulatory follicles, are still unknown.

In summary, we showed that testosterone increases the expression of RAGE and the accumulation of AGEs in granulosa cells via the activation of ER stress. Treatment of PCOS mice with a RAGE inhibitor or an ER stress inhibitor reduces RAGE expression and AGE accumulation in granulosa cells, improves the estrous cycle, and reduces the number of atretic antral follicles. Our findings suggest that the hyperandrogenism of PCOS increases the accumulation of AGEs in the ovary by activating ER stress, and that targeting the AGE-RAGE system, either by using a RAGE inhibitor or a clinically available ER stress inhibitor, may provide a novel therapeutic approach for PCOS.

Acknowledgments

The authors thank Dr Kazunori Matsumoto from Matsumoto Ladies' Clinic and Dr Xiaohui Tang from Akihabara ART Clinic for providing the human samples.

Financial Support: This work was supported by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (Grants 19k09749 to M.H., 19k24021 to N.T., 19k24045 to Y.U., and 19h00319 to N.O.), a grant from the Takeda Science Foundation (to M.H.), a grant from the Yakult Bio-Science Foundation (to M.H.), a grant from the College Women's Association of Japan (to J.M.K.A.), and a grant from the Japan Agency for Medical Research and Development (18gk0110014h003 to Y.O.).

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Disclosure Summary: The authors have nothing to disclose.

References

1. Dumesic DA, Oberfield SE, Stener-Victorin E, Marshall JC, Laven JS, Legro RS. Scientific statement on the diagnostic criteria, epidemiology, pathophysiology, and molecular genetics of polycystic ovary syndrome. *Endocr Rev.* 2015;**36**(5):487–525.

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2. Escobar-Morreale HF. Polycystic ovary syndrome: definition, aetiology, diagnosis and treatment. *Nat Rev Endocrinol*. 2018;**14**(5):270–284.
3. McCartney CR, Marshall JC. Clinical practice. Polycystic ovary syndrome. *N Engl J Med*. 2016;**375**(1):54–64.
4. Rosenfield RL, Ehrmann DA. The pathogenesis of polycystic ovary syndrome (PCOS): the hypothesis of PCOS as functional ovarian hyperandrogenism revisited. *Endocr Rev*. 2016;**37**(5):467–520.
5. Azhary JMK, Harada M, Takahashi N, et al. Endoplasmic reticulum stress activated by androgen enhances apoptosis of granulosa cells via induction of death receptor 5 in PCOS. *Endocrinology*. 2019;**160**(1):119–132.
6. Takahashi N, Harada M, Hirota Y, et al. Activation of endoplasmic reticulum stress in granulosa cells from patients with polycystic ovary syndrome contributes to ovarian fibrosis. *Sci Rep*. 2017;**7**(1):10824.
7. Ozcan L, Tabas I. Role of endoplasmic reticulum stress in metabolic disease and other disorders. *Annu Rev Med*. 2012;**63**:317–328.
8. Walter P, Ron D. The unfolded protein response: from stress pathway to homeostatic regulation. *Science*. 2011;**334**(6059):1081–1086.
9. Ron D, Walter P. Signal integration in the endoplasmic reticulum unfolded protein response. *Nat Rev Mol Cell Biol*. 2007;**8**(7):519–529.
10. Takahashi N, Harada M, Azhary JMK, et al. Accumulation of advanced glycation end products in follicles is associated with poor oocyte developmental competence. *Mol Hum Reprod*. 2019;**25**(11):684–694.
11. Takahashi N, Harada M, Hirota Y, et al. A potential role for endoplasmic reticulum stress in progesterone deficiency in obese women. *Endocrinology*. 2017;**158**(1):84–97.
12. Takahashi N, Harada M, Hirota Y, et al. A potential role of endoplasmic reticulum stress in development of ovarian hyperstimulation syndrome. *Mol Cell Endocrinol*. 2016;**428**:161–169.
13. Diamanti-Kandarakis E, Piperi C, Patsouris E, et al. Immunohistochemical localization of advanced glycation end-products (AGEs) and their receptor (RAGE) in polycystic and normal ovaries. *Histochem Cell Biol*. 2007;**127**(6):581–589.
14. Diamanti-Kandarakis E, Chatzigeorgiou A, Papageorgiou E, Koundouras D, Koutsilieris M. Advanced glycation end-products and insulin signaling in granulosa cells. *Exp Biol Med (Maywood)*. 2016;**241**(13):1438–1445.
15. Merhi Z, Buyuk E, Cipolla MJ. Advanced glycation end products alter steroidogenic gene expression by granulosa cells: an effect partially reversible by vitamin D. *Mol Hum Reprod*. 2018;**24**(6):318–326.
16. Unoki H, Yamagishi S. Advanced glycation end products and insulin resistance. *Curr Pharm Des*. 2008;**14**(10):987–989.
17. Santilli F, Vazzana N, Bucciarelli LG, Davì G. Soluble forms of RAGE in human diseases: clinical and therapeutical implications. *Curr Med Chem*. 2009;**16**(8):940–952.
18. Piperi C, Adamopoulos C, Dalagiorgou G, Diamanti-Kandarakis E, Papavassiliou AG. Crosstalk between advanced glycation and endoplasmic reticulum stress: emerging therapeutic targeting for metabolic diseases. *J Clin Endocrinol Metab*. 2012;**97**(7):2231–2242.
19. Vlassara H, Palace MR. Diabetes and advanced glycation endproducts. *J Intern Med*. 2002;**251**(2):87–101.
20. Deane R, Singh I, Sagare AP, et al. A multimodal RAGE-specific inhibitor reduces amyloid β-mediated brain disorder in a mouse model of Alzheimer disease. *J Clin Invest*. 2012;**122**(4):1377–1392.
21. Gasparotto J, Ribeiro CT, Bortolin RC, et al. Targeted inhibition of RAGE in substantia nigra of rats blocks 6-OHDA-induced dopaminergic denervation. *Sci Rep*. 2017;**7**(1):8795.
22. Sanajou D, Ghorbani Haghjo A, Argani H, et al. Reduction of renal tubular injury with a RAGE inhibitor FPS-ZM1, valsartan and their combination in streptozotocin-induced diabetes in the rat. *Eur J Pharmacol*. 2019;**842**:40–48.
23. Boatright JH, Nickerson JM, Moring AG, Pardue MT. Bile acids in treatment of ocular disease. *J Ocul Biol Dis Infor*. 2009;**2**(3):149–159.
24. Hetz C, Chevet E, Harding HP. Targeting the unfolded protein response in disease. *Nat Rev Drug Discov*. 2013;**12**(9):703–719.
25. Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group. Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome. *Fertil Steril*. 2004;**81**(1):19–25.
26. Elia E, Sander V, Luchetti CG, et al. The mechanisms involved in the action of metformin in regulating ovarian function in hyperandrogenized mice. *Mol Hum Reprod*. 2006;**12**(8):475–481.
27. Lai H, Jia X, Yu Q, et al. High-fat diet induces significant metabolic disorders in a mouse model of polycystic ovary syndrome. *Biol Reprod*. 2014;**91**(5):127.
28. Solano ME, Elia E, Luchetti CG, et al. Metformin prevents embryonic resorption induced by hyperandrogenisation with dehydroepiandrosterone in mice. *Reprod Fertil Dev*. 2006;**18**(5):533–544.
29. Sanajou D, Ghorbani Haghjo A, Argani H, et al. FPS-ZM1 and valsartan combination protects better against glomerular filtration barrier damage in streptozotocin-induced diabetic rats. *J Physiol Biochem*. 2018;**74**(3):467–478.
30. Ophir L, Yung Y, Maman E, et al. Establishment and validation of a model for non-luteinized human mural granulosa cell culture. *Mol Cell Endocrinol*. 2014;**384**(1-2):165–174.
31. Zhao KK, Cui YG, Jiang YQ, et al. Effect of HSP10 on apoptosis induced by testosterone in cultured mouse ovarian granulosa cells. *Eur J Obstet Gynecol Reprod Biol*. 2013;**171**(2):301–306.
32. Crisosto N, Sir-Petermann T, Greiner M, et al. Testosterone-induced downregulation of anti-Müllerian hormone expression in granulosa cells from small bovine follicles. *Endocrine*. 2009;**36**(2):339–345.
33. Garcia-Velasco JA, Rodríguez S, Agudo D, Pacheco A, Schneider J, Pellicer A. FSH receptor in vitro modulation by testosterone and hCG in human luteinized granulosa cells. *Eur J Obstet Gynecol Reprod Biol*. 2012;**165**(2):259–264.
34. Michael AE, Glenn C, Wood PJ, Webb RJ, Pellatt L, Mason HD. Ovarian 11β-hydroxysteroid dehydrogenase (11βHSD) activity is suppressed in women with anovulatory polycystic ovary syndrome (PCOS): apparent role for ovarian androgens. *J Clin Endocrinol Metab*. 2013;**98**(8):3375–3383.
35. Yang M, Du J, Lu D, et al. Increased expression of kindlin 2 in luteinized granulosa cells correlates with androgen receptor level in patients with polycystic ovary syndrome having hyperandrogenemia. *Reprod Sci*. 2014;**21**(6):696–703.
36. He H, Li T, Yin D, et al. HOXA10 expression is decreased by testosterone in luteinized granulosa cells in vitro. *Mol Med Rep*. 2012;**6**(1):51–56.
37. Zhang Y, Xu Y, Kuai Y, Wang S, Xue Q, Shang J. Effect of testosterone on the Connexin37 of sexual mature mouse cumulus oocyte complex. *J Ovarian Res*. 2016;**9**(1):82.
38. Yang F, Ruan YC, Yang YJ, et al. Follicular hyperandrogenism downregulates aromatase in luteinized granulosa cells in polycystic ovary syndrome women. *Reproduction*. 2015;**150**(4):289–296.
39. Liu T, Zhao H, Wang J, et al. The role of fructose-1,6-bisphosphatase 1 in abnormal development of ovarian follicles caused by high testosterone concentration. *Mol Med Rep*. 2017;**16**(5):6489–6498.
40. Lu X, Wu Y, Gao XH, Wang YW, Wang L, Sun XX. Effect of letrozole on estradiol production and P450 aromatase messenger RNA expression of cultured luteinized granulosa cells from women with and without endometriosis. *Fertil Steril*. 2012;**98**(1):131–135.
41. Li K, Kang H, Wang Y, Hai T, Rong G, Sun H. Letrozole-induced functional changes in carcinoma-associated fibroblasts and their influence on breast cancer cell biology. *Med Oncol*. 2016;**33**(7):64.
42. https://scicrunch.org/resolver/RRID: AB_777613.

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43. https://scicrunch.org/resolver/RRID:AB_447638.
44. https://scicrunch.org/resolver/RRID: AB_2099233.
45. https://scicrunch.org/resolver/RRID: AB_476697.
46. Schneider CA, Rasband WS, Eliceiri KW. NIH image to ImageJ: 25 years of image analysis. *Nat Methods*. 2012;**9**(7):671–675.
47. Byers SL, Wiles MV, Dunn SL, Taft RA. Mouse estrous cycle identification tool and images. *PloS One*. 2012;**7**(4):e35538.
48. Osman P. Rate and course of atresia during follicular development in the adult cyclic rat. *J Reprod Fertil*. 1985;**73**(1):261–270.
49. Kauffman AS, Thackray VG, Ryan GE, et al. A novel letrozole model recapitulates both the reproductive and metabolic phenotypes of polycystic ovary syndrome in female mice. *Biol Reprod*. 2015;**93**(3):69.
50. Meng L, Jan SZ, Hamer G, et al. Preantral follicular atresia occurs mainly through autophagy, while antral follicles degenerate mostly through apoptosis. *Biol Reprod*. 2018;**99**(4):853–863.
51. Winship AL, Bakai M, Sarma U, Liew SH, Hutt KJ. Dacarbazine depletes the ovarian reserve in mice and depletion is enhanced with age. *Sci Rep*. 2018;**8**(1):6516.
52. Lin PH, Chang CC, Wu KH, et al. Dietary glycotoxins, advanced glycation end products, inhibit cell proliferation and progesterone secretion in ovarian granulosa cells and mimic PCOS-like symptoms. *Biomolecules*. 2019;**9**(8):327.
53. Tanaka N, Yonekura H, Yamagishi S, Fujimori H, Yamamoto Y, Yamamoto H. The receptor for advanced glycation end products is induced by the glycation products themselves and tumor necrosis factor-alpha through nuclear factor-kappa B, and by 17beta-estradiol through Sp-1 in human vascular endothelial cells. *J Biol Chem*. 2000;**275**(33):25781–25790.
54. Mukherjee TK, Reynolds PR, Hoidal JR. Differential effect of estrogen receptor alpha and beta agonists on the receptor for advanced glycation end product expression in human microvascular endothelial cells. *Biochim Biophys Acta*. 2005;**1745**(3):300–309.
55. Chen XJ, Wu WJ, Zhou Q, et al. Advanced glycation end-products induce oxidative stress through the Sirt1/Nrf2 axis by interacting with the receptor of AGEs under diabetic conditions. [Published online ahead of print October 15, 2018.] *J Cell Biochem*. 2019;**120**:2159–2170.
56. Merhi Z, Kandaraki EA, Diamanti-Kandarakis E. Implications and future perspectives of AGEs in PCOS pathophysiology. *Trends Endocrinol Metab*. 2019;**30**(3):150–162.
57. Diamanti-Kandarakis E, Katsikis I, Piperi C, et al. Increased serum advanced glycation end-products is a distinct finding in lean women with polycystic ovary syndrome (PCOS). *Clin Endocrinol (Oxf)*. 2008;**69**(4):634–641.
58. Diamanti-Kandarakis E, Piperi C, Korkolopoulou P, et al. Accumulation of dietary glycotoxins in the reproductive system of normal female rats. *J Mol Med (Berl)*. 2007;**85**(12):1413–1420.
59. Chatzigeorgiou A, Kandaraki E, Piperi C, et al. Dietary glycotoxins affect scavenger receptor expression and the hormonal profile of female rats. *J Endocrinol*. 2013;**218**(3):331–337.
60. Tantalaki E, Piperi C, Livadas S, et al. Impact of dietary modification of advanced glycation end products (AGEs) on the hormonal and metabolic profile of women with polycystic ovary syndrome (PCOS). *Hormones (Athens)*. 2014;**13**(1):65–73.
61. Antoniotti GS, Coughlan M, Salamonsen LA, Evans J. Obesity associated advanced glycation end products within the human uterine cavity adversely impact endometrial function and embryo implantation competence. *Hum Reprod*. 2018;**33**(4):654–665.
62. Chen Y, Liu CP, Xu KF, et al. Effect of taurine-conjugated ursodeoxycholic acid on endoplasmic reticulum stress and apoptosis induced by advanced glycation end products in cultured mouse podocytes. *Am J Nephrol*. 2008;**28**(6):1014–1022.
63. Wang B, Li J, Yang Q, Zhang F, Hao M, Guo Y. Decreased levels of sRAGE in follicular fluid from patients with PCOS. *Reproduction*. 2017;**153**(3):285–292.
64. Garg D, Grazi R, Lambert-Messerlian GM, Merhi Z. Correlation between follicular fluid levels of sRAGE and vitamin D in women with PCOS. *J Assist Reprod Genet*. 2017;**34**(11):1507–1513.
65. Park JH, Choi TS. Polycystic ovary syndrome (PCOS)-like phenotypes in the d-galactose-induced aging mouse model. *Biochem Biophys Res Commun*. 2012;**427**(4):701–704.
66. Prasad K. AGE-RAGE stress: a changing landscape in pathology and treatment of Alzheimer's disease. *Mol Cell Biochem*. 2019;**459**(1-2):95–112.
67. Sanajou D, Ghorbani Haghjo A, Argani H, Aslani S. AGE-RAGE axis blockade in diabetic nephropathy: current status and future directions. *Eur J Pharmacol*. 2018;**833**:158–164.
68. Cora MC, Kooistra L, Travlos G. Vaginal cytology of the laboratory rat and mouse: review and criteria for the staging of the estrous cycle using stained vaginal smears. *Toxicol Pathol*. 2015;**43**(6):776–793.

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