Advanced glycation end products and their receptor contribute to ovarian ageing

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STUDY QUESTION: Do advanced glycation end products (AGE) and the receptor for advanced glycation end products (RAGE) affect the cells of the human ovarian follicle?

SUMMARY ANSWER: AGE accumulate on the surface of ovarian granulosa–lutein (GL) cells and monocytes by binding to RAGE and other receptors with possible functional effects on these cells.

WHAT IS KNOWN ALREADY: AGE and RAGE are expressed in granulosa and theca cells, as well as in luteinized cells derived from the ovary.

STUDY DESIGN, SIZE, DURATION: In this prospective cohort study, human follicle fluid-derived cells were isolated from aspirates of ovarian follicles of women who underwent assisted reproduction treatment.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Immunofluorescence microscopy and multi-colour flow cytometry were used to determine the presence of AGE and RAGE on the surface of follicular fluid-derived cells and to characterize downstream effects of RAGE activation.

MAIN RESULTS AND THE ROLE OF CHANCE: GL cells and ovarian monocytes were found to contain AGE and RAGE and to bind AGE–bovine serum albumin (BSA) in correlation with the patients’ chronological age. AGE–BSA and BSA failed to induce significantly the cleavage of caspase-3, phosphorylation of nuclear factor-kB or the binding of annexin V (the latter was marginally increased). AGE-fibronectin was found to induce detachment of cultured GL cells in vitro.

LIMITATIONS, REASONS FOR CAUTION: The impact of AGE and RAGE in the ovary, shown here in cells in culture, remains to be affirmed in clinical settings.

WIDER IMPLICATIONS OF THE FINDINGS: The ligands of RAGE and their effects in the ovary remain uncertain but this study implies that AGEs in the form of structural long-lived extracellular matrix proteins, rather than soluble AGEs, may play a role in the decline of ovarian function during ageing.

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Key words: female infertility / assisted reproduction / cell culture / cell signalling / advanced glycation end products

Introduction

Human fetal ovaries are endowed with ~6 million oocytes surrounded by somatic granulosa cells. The pool of primordial follicles is gradually depleted by atresia so that ~1 million follicles remain at birth, ~300 000 at menarche and during reproductive life an average of 1000 follicles are lost each month (Byskov, 1986; Faddy and Gosden, 1996; te Velde and Pearson, 2002). Ovarian ageing is characterized by an accelerating loss of follicles beyond 37 years of age, when the pool is <25 000, and menopause commences when the remaining follicles are fewer than
1000 at the mean age of 51 years (Faddy et al., 1992; Faddy and Gosden, 1996; te Velde and Pearson, 2002). Age-related decline of the ovarian follicle pool is closely related to the decline of ovarian function and a reduction in female reproductive potential (te Velde and Pearson, 2002). Indeed, during assisted reproduction treatment (ART), the age of the woman is probably the most important factor influencing clinical outcome, as the pregnancy rate decreases from 43.6% at age 25 years to 21.0% at 41 years and older ($P < 0.001$) (Stensen et al., 2010). The mechanisms that induce a declining follicle pool and diminishing oocyte quality are likely to involve generic processes of senescence, such as accumulation of unrepairred DNA damage, telomere loss, deterioration of mitochondrial function and accumulation of altered proteins (Kirkwood, 2008).

Advanced glycation end products (AGEs) are a heterogeneous, complex group of compounds that are formed through the browning process called Maillard reaction, when a reducing sugar reacts non-enzymatically with amino acids in proteins, lipids or DNA (Thorpe and Baynes, 1996; Luevano-Contreras and Chapman-Novakofski, 2010). AGEs are suspected to be involved in the pathogenesis and progression of several diseases including diabetes mellitus, cardiovascular disease and Alzheimer’s disease, as well as ageing in general (Brownlee et al., 1984; Kirstein et al., 1990; Vitek et al., 1990). AGEs cause tissue injury directly through protein cross-linking or indirectly by binding to multi-ligand transmembrane receptors, known as RAGE (receptor for advanced glycation end products). RAGEs belong to the immunoglobulin superfamily and are expressed by several cell types, for example endothelial and smooth muscle cells (Schmidt et al., 2000; Neeper et al., 1992). Activation of RAGE has multiple downstream intracellular effects, such as activation of NAD(P)H oxidase, mitogen-activated protein kinases (MAPKs) and the transcription factor NF-$\kappa$B (nuclear factor kappa B) (Lander et al., 1997; Brownlee, 2001; Xu and Kyriakis, 2003). These effects result in increased intracellular oxidative stress and up-regulated expression of genes for cytokines, growth factors and cell-adhesion molecules, such as tumour necrosis factor $\alpha$ (TNF$\alpha$), as well as various pro-inflammatory cytokines [interleukin (IL)-$\alpha$, IL-6] and an increased expression of RAGE itself (Li and Schmidt, 1997; Mohamed et al., 1999; Neumann et al., 1999; Basta et al., 2004; Goldin et al., 2006). This results in a positive feedback pro-inflammatory cycle and increased reactive oxidative stress (Mohamed et al., 1999; Neumann et al., 1999; Schmidt et al., 2001).

During reproductive ageing, it has been suggested that intraovarian accumulation of AGE compounds compromises the vascular supply of the ovary and induces an oxidative stress-response through interaction with RAGE (Tatone et al., 2008). It is also an intriguing possibility that accumulation of ageing-related molecular damage in ovarian somatic cells stimulates innate immune responses and contributes to a decline in ovarian function. In the present study we aimed to determine whether follicular fluid-derived cells (FFDCs) express AGE and RAGE and the possible intracellular downstream effects of RAGE activation.

**Materials and Methods**

**Reagents**

Antibodies and reagents were purchased from the following suppliers: rabbit anti-AGE, rabbit anti-RAGE (Abcam, Cambridge, UK); anti-cleaved caspase-3, rabbit anti-phospho-NF-$\kappa$B p65 (Ser536)-Alexa Fluor 488 conjugate (BioNordika, Lysaker, Norway); anti-CD14-PerCP, anti-CD45-PE (eBioScience, San Diego, CA, USA); anti-CD45-fluorescein isothiocyanate (FITC), anti-CD31-FITC, anti-CD16-PE (BD, Franklin Lakes, NJ, USA); anti-CD3-FITC (Diaotec, Oslo, Norway); anti-CD8-BPE (DakoCytomation, Glostrup, Denmark); normal rabbit IgG and goat anti-rabbit IgG (RPE; Southern Biotech, Birmingham, AL, USA); AGE–bovine serum albumin (BSA) and control BSA (antibodies-online, Atlanta, GA); Ficol Pague 1 PLUS (GE Healthcare, Oslo, Norway); phosphate-buffered saline (PBS), HBSS, DMEM, fetal calf serum (FCS); Invitrogen, Carlsbad, CA, USA); staurosporine, phorbol 12-myristate 13-acetate (PMA), paraformaldehyde, glycine, goat serum, Tween 20, medicinal gelatin, RPMI, albumin, bis-benzimide, mannan, d-mannose, d-galactose, percoll, DNase, hyase, collagenase, fibronectin (Sigma-Aldrich, Oslo, Norway).

**In-house solutions and reagents**

DYNAL buffer consisted of 2% FCS, 20 mM EDTA in PBS (pH 7.2), ACK buffer contained 150 mM NH$_4$Cl, 10 mM NaHCO$_3$, 0.1 M EDTA (pH 7.2).

Conjugation of proteins with FITC proceeded as follows: AGE–BSA (2 mg/ml), BSA (2 mg/ml) and recombinant human hGC (rhCG, 1000 IU/ml; Ovitrelle; Merck Serono, Darmstadt, Germany) were suspended in 0.1 M sodium carbonate buffer solution (pH 9.0), mixed with 1 mg/ml FITC in DMSO, and incubated at 4°C overnight. The unbound FITC was separated from the conjugate by filtration through an NAP-5 fine-sized gel matrix column. The molar ratio of fluorescein to protein was estimated by measuring the absorbance at 495 and 280 nm with a spectrophotometer (NanoDrop 2000; Thermo Scientific, Germany).

Conjugation with Alexa Fluor 488 was performed with Alexa Fluor 488 protein labelling kit (Invitrogen).

For the glycation of albumin, fibronectin, FCS and the preparation of AGE-modified follicle fluid, 50 mg/m BSA, 0.33 mg/ml fibronectin, 10% FCS in PBS or fluid from aspirates of blood-free pre-ovulatory follicles were incubated with 0.5 M glucose in PBS at 50°C for 7 days in a rotating hybridization chamber (Bhatwadekar and Ghole, 2005). Appropriate controls were prepared by either (a) omitting glucose and incubating the solution at 50°C for 7 days or (b) adding the glucose but incubating the solution at 4°C for 7 days. Slide-a-lyzer dialysis cassettes (Thermo Scientific) were used to remove the excess glucose after incubation.

**Cell culture**

**Isolation of follicle fluid-derived cells**

Human FFDC (the heterogeneous mixture of cells present in follicular fluid) were isolated from aspirates of follicles from women who underwent ART. Prior to follicle aspiration, women received controlled ovarian stimulation according to the luteal-phase GnRH agonist (Suprecur; Sanofi-Aventis, Norway) down-regulation protocol with daily injections of either recombinant FSH (Puregon; MSD, The Netherlands or Gonal F; Merck Serono) or purified urinary gonadotrophins (Menopur; Ferring, Switzerland). Final follicular maturation was induced with 6500 IU rhCG (Ovitrelle). Collection of follicular fluid and peripheral blood was approved by the Regional Committee for Medical and Health Research Ethics, Health Region South (No. S-05058), and written informed consent was obtained from the participants for collection of the follicle fluid.

Follicle fluid was collected during follicle aspiration and all fluid from a single patient was pooled. Although this design obscures the heterogeneity of ovarian follicles (i.e. follicles of varying sizes and stages of maturation may have contributed to the experiments), our data show that there was no systematic bias in terms of oocyte maturity in the sample of women who had contributed samples to the cell culture experiments. The Supplementary data, Table S1 compares the clinical data of our sample to the whole cohort of women who had received treatment during the same 3 year period while these experiments were conducted and shows that women...
selected for this study were not outliers, presented with normal ovarian re-
sponse to stimulation and had comparable oocyte maturity and treatment
outcome. The FFDCs were isolated by either of two methods with compar-
able results. The first method involved haemolysis with ACK buffer, enzym-
atic dispersion with DNase, hyase and collagenase followed by mechanical
dispersion with a needle. Density gradient centrifugation with Percoll was the
final step in the procedure and cells at the interphase were collected and
suspended in HBSS, PBS with 10% FCS or RPMI with 1% BSA (Fedorchak
et al., 2010). The alternative method involved an initial centrifugation on a
Ficoll cushion, enzymatic dispersion of cells at the interphase with DNase,
hyase and collagenase, and mechanical dispersion with a needle. The cells
were suspended in either HBSS or PBS with 10% FCS for flow cytometric
analysis or cultured in RPMI with 1% BSA.

Isolation of peripheral blood mononuclear cells
Blood samples were drawn from the women donating follicle fluid on the day of
follicle aspiration. To isolate peripheral blood mononuclear cells (PBMCs),
heparinized blood was subjected to Ficoll gradient centrifugation and the cells
in the interphase were collected. The cells were then washed twice and
re-suspended in HBSS or PBS with 10% FCS on ice, as appropriate.

Isolation of human umbilical vein endothelial cells
Human umbilical vein endothelial cells (HUVECs) were isolated from the
umbilical vein as described (Polec et al., 2011) and cultured in MCD131
with 7.5% FCS, trypsinized and re-suspended in medium on ice until
further analysis.

Immunofluorescence microscopy
GL cells and PBMC were cultured in RPMI with 10% FCS on flamed-sterilized
glass coverslips coated with 0.2% gelatin. Monolayers were fixed with 4% para-
formaldehyde in PBS, permeabilized with 0.1% Triton X-100 or 0.2% Tween
20, washed in 0.1 M glycine in PBS and incubated in blocking buffer 1 (BB1; 1% goat
serum, 0.2% Tween 20 and 0.001% sodium azide in PBS). The cells were
labelled with primary antibodies (rabbit anti-AGE, 1:50 dilution or anti-
RAGE, 1:50 dilution) in BB1 for 1 h at 37°C, washed three times in 0.2%
Tween 20 in PBS, followed by staining with Alexa 488-conjugated goat anti-
rabbit IgG (1:500 dilution) as secondary antibody for 30 min at 37°C. Normal
rabbit IgG (1:50 dilution) was used as a negative control. Nuclei were counter-
stained with bis-benzimide (1:1000 dilution in PBS) or DAPI, and the cov-
erslips were mounted with Vectashield (Vector Labs, Burlingame, CA, USA).
Images were taken with a Carl Zeiss epifluorescent microscope equipped
with a Spot camera, and using constant exposure settings in an experiment.

Flow cytometry
FFDCs, PBMCs and HUVECs were either fixed as above or were fixed and
permeabilized with Cytofix/Cytoperm (BD Biosciences, CA, USA) to
detect cell surface specific or intracellular immunofluorescence, respect-
ively. The cells were incubated at room temperature for 30 min in blocking
buffer 2 (BB2; 1% goat serum in PBS with or without 0.2% Tween 20), and
exposed to primary antibodies (rabbit anti-AGE, 1:50 dilution or rabbit anti-
RAGE, 1:50 dilution) at 37°C for 30 min, washed twice in BB2, and incubated
with secondary antibody for 30 min at 37°C followed by analysis with an
FACScan instrument (BD) using the Cellquest 4.0 software (BD) or FlowJo
for data analysis. Normal rabbit IgG (1:1000 dilution) was used as the negative control.
Anti-CD14-PerCP (1:5 dilution), anti-CD45-PE (1:5 dilution), anti-CD45-FITC (1:5 dilution),
anti-CD3-PE (1:5 dilution), anti-CD16-PE (1:5 dilution), anti-CD8-PE (1:5 dilution) and anti-CD31-FITC (1:5 dilution)
were used to characterize leucocyte subsets. Propidium iodide (PI, 10 ng/ml) was added immediately before analysis to identify and exclude
necrotic cells.

Binding of conjugated AGE–BSA and BSA by cells
For visualization of uptake of AGE–BSA and BSA by GL cells, isolated GL cells were
incubated on flamed-sterilized coverslips in DMEM with 10% FCS over-
night. The cells were then washed twice with pre-warmed DMEM and
exposed to 1:50 dilution of Alexa Fluor 488-conjugated AGE–BSA or Alexa Fluor 488-conjugated BSA overnight. The samples were then
washed twice with PBS, fixed with 4% parafomaldehyde, washed with
glycine in PBS and counterstained with DAPI. Images were taken with a
Carl Zeiss epifluorescent microscope and a Spot camera.

For quantification of uptake of AGE–BSA and BSA, isolated FFDCs were
pre-incubated in RPMI with 1% BSA for 15 min. Afterwards, the cells were incubated with (a) FITC-conjugated AGE–BSA (1: 50 dilution); (b) FITC-
conjugated BSA (1:50 dilution); (c) Alexa Fluor 488-conjugated AGE–BSA
(1:50 dilution) or (d) Alexa Fluor 488-conjugated BSA (1:50 dilution) on
ice for 1.5 h and washed twice with DYNAL buffer. Anti-CD14-PerCP
(1:5 dilution) and anti-CD45-PE (1:5 dilution) were then added and incu-
bated for 30 min on ice. The cells were washed twice with DYNAL buffer
and analysed using FACS. To examine whether AGES bind unspecifically
to certain structures on the cell surface, the GL cells were blocked with 0.8%
BSA in DYNAL buffer followed by incubation with (a) 1.6 mg/ml mannan; (b)
16 mg/ml D-mannose or (c) 16 mg/ml D-galactose for 30 min on ice. The
cells were subsequently incubated with FITC-conjugated AGE–BSA (1:50 di-
lution), FITC-conjugated BSA (1:50 dilution), dextran-FITC (1:50 dilution) or
Lucifer Yellow (LY; 1:50 dilution) for 1.5 h on ice, followed by flow cytometry.

Binding of rhCG by GL cells
FFDCs were pre-incubated in blocking buffer 3 (BB3) with either 0.5% AGE–
BSA or 0.5% BSA for 30 min, followed by incubation with FITC-conjugated
rhCG (1:25 dilution) for 1.5 h at 37°C. Finally, cells were washed with
DYNAL, labelled with anti-CD45-PE (1:5 dilution) and anti-CD14-PerCP
(1:5 dilution), and analysed with FACS.

Apoptosis and cell signaling
Annexin V
The Annexin V FITC Apoptosis Detection kit (BD Pharmigen) was used to
detect Annexin V positive cells. FFDCs were cultured in gelatin-coated
six-well dishes in DMEM with 10% FCS and 50 μg/ml gentamicin overnight.
The cells were then washed with PBS and incubated in DMEM with 0.1% BSA
supplemented with (a) 10 μg/ml AGE–BSA; (b) 10 μg/ml BSA; (c) 20 μg/ml
AGE–BSA and (d) 20 μg/ml BSA overnight. The cells were then washed
twice with pre-warmed PBS, and 5 mM EDTA in PBS was added to detach
cells. Cells were incubated with FITC-conjugated Annexin V (1:20 dilution)
in 1 × binding buffer for 15 min at room temperature, protected from
light. To induce apoptosis, 1 μg/ml staurosporine was added to positive controls.
PI (10 ng/ml) was added prior to analysis by FACS to gate necrotic
cells.

NF-κB activation
For visualization of NF-κB activation, PBMCs were suspended in HBSS and
incubated in 200 μg/ml AGE–BSA or 200 μg/ml BSA for 1 h at 37°C.
The cells were then fixed with 4% paraformaldehyde and cytosplined onto
slides. The slides were then washed in Photoflo (Kodak), dried and
incubated with Alexa Fluor 488-conjugated phospho-NF-κB p65 (Ser536)
antibody (1:50 dilution) at 4°C overnight. Images were taken with a Carl
Zeiss epifluorescent microscope. Phorbol-12-myristate-13-acetate (PMA,
50 ng/ml) was used to induce activation of NF-κB in positive controls.

For quantification of NF-κB activation, PBMCs were incubated with
200 μg/ml AGE–BSA or 200 μg/ml BSA for 1 h at 37°C, fixed and permea-
bilized with Cytofix/Cytoperm for 20 min on ice and washed in 0.05% Tween
glycine in PBS and counterstained with DAPI. Images were taken with a
Carl Zeiss epifluorescent microscope and a Spot camera.
20, 100 mM NaF and 1 mM sodium vanadate in PBS. The cells were then stained with Alexa Fluor 488-conjugated anti-phospho-NF-κB (25 μg/ml; 1:50 dilution in 0.05% Tween 20/SF/SV and 1% BSA in PBS) at 4°C overnight, protected from light and washed twice with PBS/Tween 20/NaF/vanadate. PI (10 mg/ml) was added before flow cytometric analysis to gate necrotic cells and 100 ng/ml PMA was used to induce NF-κB activation in positive controls.

GL cells cultured on gelatin-coated 6-well dishes in DMEM with 10% FCS and 50 μg/ml gentamicin overnight were incubated for an additional day in either (a) 200 μg/ml AGE–BSA, (b) 200 μg/ml BSA, (c) follicle fluid (FF) incubated without glucose at 50°C for 7 days, (d) FF exposed to glucose at 50°C for 7 days or (e) FF exposed to glucose at 4°C for 7 days. PBS/5 mM EDTA was used to detach cells from the dish, and the cells were fixed with 4% paraformaldehyde, permeabilized with 90% ice-cold methanol and washed twice with PBS/Tween 20/NaF/vanadate. The cells were labelled with Alexa488-conjugated phospho-NF-κB p65 (25 μg/ml; 1:50 dilution in Tween 20/NaF/vanadate and 1% BSA in PBS), and incubated for 2.5 h at 37°C, followed by flow cytometry. PMA (50 ng/ml) was used to induce activation of NF-κB in positive controls.

Cleaved caspase-3

GL cells were cultured in gelatin-coated 6-well dishes in DMEM with 10% FCS overnight. The cells were then washed in DMEM, and cultured overnight in either (a) 0.1 mg/ml AGE–BSA, (b) 0.1 mg/ml BSA, (c) FF incubated without glucose at 50°C for 7 days, (d) FF exposed to glucose at 50°C for 7 days or (e) FF exposed to glucose at 4°C for 7 days. PBS/5 mM EDTA was used to harvest the cells from the dish. The cells were then fixed with 4% paraformaldehyde, permeabilized with 90% ice-cold methanol and washed twice with Tween 20/SF/SV in PBS. The cells were then labelled with Alexa 488-conjugated anti-cleaved caspase-3 (1:50 dilution in Tween 20/NaF/vanadate and 1% BSA in PBS) for 2.5 h at 37°C, followed by FACS. Positive controls were incubated with 1 μg/ml staurosporine to induce caspase-3 activation.

Cell detachment

GL cells were incubated overnight in DMEM on dishes coated with (a) AGE-modified fibronectin, (b) fibronectin incubated without glucose at 50°C for 7 days, (c) fibronectin incubated with glucose at 4°C for 7 days, (d) AGE-modified FCS, (e) FCS incubated without glucose at 50°C for 7 days or (f) FCS incubated with glucose at 4°C for 7 days. Detached cells were collected and stained with acridine orange/ethidium bromide (AO/EB) and counted using an epifluorescent microscope.

Statistics

Pearson correlation, Student’s t-test and analysis of variance were calculated where appropriate. Statistical Package for Social Sciences version 18 (SPSS, USA) was used for data analysis, and P < 0.05 was considered statistically significant.

Results

Expression of AGE and RAGE on the surface of FFDC, PBMC and HUVEC

Both GL cells (CD14+) and monocyes (CD14+) in the follicle fluid were found to express AGE on the cell surface (Fig. 1A) and anti-AGE decorated larger patches or domains of the surface of non-permeabilized cells (Fig. 1B).

Both GL cells (CD14+) and monocyes (CD14+) were found to express RAGE, although the density of RAGE-expressing cells was low (Fig. 1A and 1B). Specific RAGE-related fluorescence intensity in GL cells correlated with the patients’ chronological age (n = 6; r = 0.93, P = 0.004; Fig. 1E).

Peripheral monocytes were found to express AGE and RAGE (Fig. 1C), whereas HUVEC lacked the expression of AGE and RAGE (Fig. 1D).

Both CD14+/CD16bright and CD14+/CD16dim populations of monocytes were found to express AGE (Supplementary data, Fig. S1A and Fig. R1). Increased expression of RAGE by CD14+/CD16bright monocytes was observed, but CD14+/CD8- and CD14+/CD3+ lymphocytes from the follicle fluid were not found to express AGE or RAGE.

Binding of AGE–BSA and BSA by FFDC

GL cells, monocytes and lymphocytes gated by CD14- and CD45-positive cells (Fig. 2A and B) were found to bind AGE–BSA (Fig. 2C; n = 10); GL cells: P = 0.001; monocytes: P = 0.023; lymphocytes: P = 0.003: all P values for AGE–BSA versus BSA). The binding of AGE–BSA on the surface of GL cells was found to correlate with the patients’ chronological age (Fig. 2D; n = 10; r = 0.688; P = 0.027). The experiments were repeated with AGE–albumin conjugated with FITC yielding comparable results (n = 13; data not shown).

Mannan, D-mannose or D-galactose failed to affect AGE–BSA binding by monocytes, lymphocytes or GL cells (Fig. 2E), suggesting that AGE does not bind to cell surface-associated molecules unspecifically.

The cell-associated fluorescence intensity of hCG was not affected by the presence of AGE–BSA (n = 16; P = 0.97; Fig. 2F).

Downstream effects of AGE binding in GL cells in vitro

In GL cells, AGE–BSA and BSA failed to induce cleavage of caspase-3 (n = 8; Fig. 3B), phosphorylation of NF-κB (n = 2; data not shown) or increase binding of annexin in vitro (n = 10; Fig. 3A). These experiments were repeated with separate batches of glycated proteins that were prepared by prolonged exposure of albumin or human follicular fluid to high glucose at 50°C for 7 days. Glycated albumin and follicular fluid activated neither caspase-3 nor NF-κB in cultured GL cells in vitro (n = 2; data not shown).

AGE-fibronectin was found to induce cell death in vitro (n = 3; Fig. 3C), whereas AGE–FCS (n = 1) had no effect on cell detachment and survival.

Discussion

We found that AGE-modified proteins are present on the surface of freshly isolated human GL cells and intrafollicular monocytes, suggesting that ovarian cells may be exposed to AGE-related damage in vivo. We also observed that granulosa cells and ovarian monocytes bind AGE-modified albumin in vitro and that specific RAGE receptors are present on the surface of these cells. These data imply that AGEs, which are involved in the structural and functional modification of cellular proteins, the formation of cross links between molecules in the basement membrane and the extracellular matrix (ECM), and activation of the RAGE receptor that leads to production of reactive oxygen species (Brownlee, 2001), may also be involved in the decline of ovarian function.

Our results are in agreement with the reports on the expression of AGE and RAGE in granulosa and theca cells, as well as in luteinized...
Figure 1 Detection of advanced glycation end products (AGE) and the receptor (RAGE) on the surface of follicle fluid-derived cells (FFDC), cultured granulosa lutein (GL) cells, peripheral blood mononuclear cells (PBMC) and endothelial cells from human umbilical vein (HUVEC). (A) FFDCs were labelled with anti-AGE or anti-RAGE and anti-CD14 (n = 6). The cells were gated on the forward scatter (FSC, cell size), and CD14+ cells were defined as monocytes and CD14− cells as GL cells. Unstained cells were used as negative control. (B) Cultured GL cells, derived from follicle fluid, were labelled with anti-AGE or anti-RAGE followed by PE-conjugated secondary antibody (RPE; n = 3). Normal rabbit IgG was used as negative control. (C) PBMCs were labelled with anti-AGE or anti-RAGE and anti-CD14 (n = 6). The monocytes (CD14+1) were gated on the FSC. (D) HUVEC cells were labelled with anti-AGE or anti-RAGE and anti-CD31 (n = 2). (E) Correlation between patient’s age and proportion of RAGE-expressing CD14− cells GL cells and CD14+ monocytes (n = 6; GL cells: r = 0.93; P = 0.004, monocytes: r = 0.54; P = 0.14).
Figure 2 Binding of AGE-albumin (AGE–BSA) by FFDCs in vitro. (A) FFDCs were incubated with Alexa 488-conjugated AGE–BSA and BSA, and immunostained with anti-CD14-PerCP and anti-CD45-PE (n = 10). On FACS, monocytes and lymphocytes were gated on FSC. CD14+CD45+ are defined as monocytes, CD14−CD45+ as lymphocytes and CD14−CD45− as GL cells. (B) Binding of Alexa 488-conjugated AGE–BSA and Alexa 488-conjugated BSA by GL cells gated as described. (C) Proportion of AGE–BSA and BSA (mean ± SE; n = 10; GL cells: P = 0.001, monocytes: P = 0.023, lymphocytes: P = 0.003). (D) Proportion of AGE–BSA-binding GL cells in correlation with the patient’s age (n = 10; r = 0.688; P = 0.03). (E) FFDCs were pre-incubated with mannan, D-mannose or D-galactose, followed by exposure to FITC-conjugated AGE–BSA, FITC-conjugated dextran or Lucifer Yellow. Mean fluorescence intensity of gated cells relative to pre-incubation in BSA is given on a grey scale. (F) FFDCs were pre-incubated with AGE–BSA or BSA, followed by exposure to FITC-conjugated recombinant hCG (rhCG; n = 16). CD14+CD45+ granulosa cells were identified by appropriate markers. RhCG-specific mean fluorescence intensity of 16 experiments are shown in bar chart (P = 0.97 for difference between pre-incubation with BSA versus AGE–BSA). FFDC, follicle fluid-derived cells; GL, granulosa lutein.
Figure 3 Downstream effects of AGE binding in GL cells in vitro. (A) GL cells were incubated with BSA or AGE–BSA. Annexin V-specific mean fluorescence intensity (SD) was quantified with FACS ($n = 9; P = 0.80$). Staurosporine is an activator of apoptosis and positive control. (B) GL cells were incubated with AGE–BSA or BSA, fixed, permeabilized and expression of cleaved caspase 3 was determined with FACS ($n = 8$). (C) GL cells were cultured on a plate pre-coated with AGE–fibronectin. Detached dead cells were counted after staining with acridine orange and ethidium bromide, and the mean number of counted detached cells (SD) is presented here. As controls, culture plates were coated with fibronectin that were treated in the absence of glucose at $50^\circ C$, as well as fibronectin that was treated in the presence of glucose at $4^\circ C$ ($n = 3; P = 0.38$). GL, granulosa lutein.
cells (Diamanti-Kandarakis et al., 2007b). Notably, RAGE has been found to be expressed at low levels in normal tissues and vessels, but increased expression was observed in endothelial cells and macrophages in chorionic villi from first trimester human placenta (Schmidt et al., 2001; Konishi et al., 2004). Cellular expression of RAGE is controlled by a positive feedback loop where accumulation of AGEs and TNF-α promote RAGE expression through transcription factors NF-κB and increase transcription of TNF-α (Neepere et al., 1992; Li and Schmidt, 1997; Schmidt et al., 2001; Basta et al., 2004). Consistent with ligand-induced up-regulation, we found that the expression of RAGE by GL cells was increased in older women.

The interaction of AGEs with the cell surface receptor RAGE is likely to perpetuate the ageing process through induction of inflammatory reactions. Indeed, we found an increased expression of RAGE by intrafollicular monocytes that also expressed the CD14<sup>+</sup>CD16<sup>+</sup>bright markers, consistent with a pro-inflammatory role of RAGE (Ziegler-Heitbrock, 2007). Follicular fluid-derived cells (FFDCs) is a heterogeneous population consisting of granulosa cells and various leukocytes (Fedorcsak et al., 2007). Leukocytes may contaminate the pre-ovulatory follicle as a result of bleeding during follicle aspiration, but the surgical procedure alone cannot entirely account for the presence of immune cells (Smith et al., 2005). Indeed, ovulation itself involves an inflammatory process (Espey, 1980), including tissue remodelling during follicle rupture (Bukulmez and Arici, 2000; Fedorcsak et al., 2010) and release of chemokines, cytokines and angiogenic factors (Polec et al., 2011). An erroneously increased inflammatory reaction may have clinical implications, since increased concentrations of proinflammatory cytokines (IL-1β, IL-12, IL-8 and TNF-α) in the follicular fluid is associated with a reduced ovarian response during hormone stimulation for IVF (Opoien et al., 2013).

Although the factors that activate immune cells in the context of normal or deranged ovarian function are yet to be identified, we suggest that AGE may contribute to immune processes in the ovary. AGEs had been shown to increase transcription of TNF-α by activation of RAGE (Schmidt et al., 2001), and the inflammatory cascade so initiated can attract polymorphonuclear leukocytes to sites of inflammation and prime processes leading to further generation of AGEs. The continued presence of AGEs may lead to sustained expression of proinflammatory mediators following a cycle of attraction and activation of inflammatory effector cells.

Supporting the notion that ovarian follicles are continuously exposed to the effects of AGEs, we found that GL cells, monocytes and lymphocytes bind AGE–albumin in vitro, indicating that there are unoccupied binding sites for AGE on the surface of ovarian cells. AGE–BSA was highly bound by GL cells and the rate of binding correlated with the patient’s chronological age, supporting findings of age-related expression of RAGE. However, exposure of cultured cells to AGE–BSA failed to activate NF-κB signalling or apoptosis in this study, suggesting that AGE–BSA is not a physiological ligand regulating granulosa cell survival, which may have several explanations. Soluble AGE, like AGE–BSA, has been shown to activate monocytes, whereas AGE-modified structural proteins of the basement membranes or AGE absorbed in the ECM attract and then reduce the migration of monocytes through a mechanism termed apoptosis (Schmidt et al., 1999). Formation of AGEs in the ECM occurs on proteins with a slow turnover rate, and AGEs may induce permanent abnormalities in ECM function (Goldin et al., 2006). In fact, the effect of AGE on the vascular endothelium is more related to modification of structural proteins, including the ECM, rather than soluble AGE-modified proteins that are deposited in the plaques. Cellular effects of AGE may be related to internalization of the RAGE-bound ligand and differ according to cells’ endocytic capacity, or AGEs may bind multiple cell surface proteins and their cellular effect may depend on the context of cells’ receptor repertoire (Tamura et al., 2003). Collectively, it is plausible that soluble AGE–BSA lacks a pro-apoptotic effect on granulosa cells and may not be a major factor that brings about physiological effects in the ovary. Probably the long-lived ECM proteins, with more prolonged exposure to AGEs, are the ligands of RAGE or other AGE sensors on the granulosa cells. Physiologically relevant ovarian AGE-modified proteins remain to be identified.

Given the high abundance and uncertain nature of AGE binding sites in granulosa cells, we explored whether cell surface-associated lectins participate in AGE binding and whether AGEs interfere with ligand binding by gonadotrophin receptors. We found that AGEs do not interfere with binding of hCG to its receptors on GL cells, suggesting that AGEs do not interrupt the LH signal before ovulation. However, AGE may influence hCG action by inhibiting secretion of hCG by trophoblasts, and thus AGEs may contribute to dysfunction of the corpus luteum and the developing placenta (Konishi et al., 2004).

The sources of ovarian AGEs are currently unknown. AGEs may be produced endogenously in the ovary, but exogenous factors, such as tobacco smoking and diet, may also contribute to the AGE deposition in the ovary. In rats, ingested AGEs led to increased serum AGE levels and higher expression of AGE and RAGE in the ovary (Diamanti-Kandarakis et al., 2007a). The AGE content of the diet depends on nutrient composition and food processing (Krajcovicová-Kudličkova et al., 2002), and the Western diet is believed to be an important source of AGEs. Tobacco smoking has been proposed as a source of compounds that can readily increase in vivo concentrations of AGEs (Cerami et al., 1997), and may contribute to increased AGE levels also in the ovary.

A soluble isoform of RAGE (sRAGE) is believed to function as a decoy that sequesters oxidative ligands, interrupts intracellular signaling (Hofmann et al., 1999; Fujisawa et al., 2013; Selvin et al., 2013) and may thus alter AGE–RAGE interactions associated with reproductive dysfunction during ageing. However, the concentration of sRAGE in the follicle fluid does not correlate with women’s biological age (Fuji and Nakayama, 2010), although high intrafollicular concentrations of sRAGE in the leading follicle may predict poor embryo quality in ovarian stimulation cycles (Bonetti et al., 2013).

Although the present data suggest that the ovarian follicles are exposed to AGE-induced damage in vivo, the nature of this damage may be difficult to assess in our model. Indeed, the ECM of non-ative follicles that are aspirated during IVF may typically undergo considerable turnover and remodelling during maturation (Rodgers et al., 2003). Furthermore, these healthy follicles may contain granulosa cells that are not representative of the ageing ovary or states of decline of ovarian function, such as diminished ovarian reserve and poor response to stimulation.

In conclusion, we report that the ovarian granulosa cells and monoocytes are exposed to AGEs in vivo, express RAGE and bind AGEs on the cell surface. RAGE expression on GL cells appears to correlate with the chronological age of the patient, implying an up-regulation of the receptor by positive feedback systems. The physiological ligands of RAGE and their effects in the ovary remain uncertain, and there may be an indication that structural long-lived ECM proteins, rather than soluble AGE, play a role in the decline of ovarian function.
Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

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Authors’ roles

M.H.S: designing the study, performing the experiments, analysis and interpretation of data, drafting the article and final approval. P.F.: designing the study, performing some of the experiments, analysis and interpretation of data, drafting the article, revising it critically for important intellectual content and final approval of the version to be published. T.T. substantial contributions to design, revising the article critically for important intellectual content and final approval of the version to be published. R.S.: substantial contributions to design, revising the article critically for important intellectual content and final approval of the version to be published.

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Conflict of interest

The authors have no conflicts of interests.

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Advanced glycation end products and ovarian ageing


