Original research

Advanced glycation end-products and insulin signaling in granulosa cells

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Abstract

Advanced glycation end-products (AGEs) may interfere with insulin intracellular signaling and glucose transport in human granulosa cells, potentially affecting ovarian function, follicular growth, linked with diminished fertility. The potential interaction of AGEs with insulin signaling pathways and glucose transport was investigated in human granulosa KGN cells. KGN cells were cultured with variable concentrations of human glycated albumin (HGA, 50–200 μg/mL) or insulin (100 ng/mL). Combined treatments of KGN cells with insulin (100 ng/mL) and HGA (200 μg/mL) were also performed. p-AKT levels and glucose transporter type 4 (Glut-4) translocation analysis were performed by Western blot. Phosphatidylinositol-3-kinase (PI3K)-specific signaling was checked by using the PI3K-inhibitor, LY294002. p-AKT levels were significantly increased following insulin treatment compared to basal levels or HGA exposure. This insulin-mediated AKT-phosphorylation was PI3K-specific and it was inhibited after combined treatment of insulin and HGA. Furthermore, Glut-4 translocation from the cytoplasm to the membrane compartments of KGN cells was remarkably reduced after the combined treatment of insulin and HGA. The present findings support that AGEs interfere with insulin signaling in granulosa cells and prevent Glut-4 membrane translocation suggesting that intra ovarian AGEs accumulation, from endogenous or exogenous sources, may contribute to the pathophysiology of states characterized with anovulation and insulin resistance such as polycystic ovary syndrome.

Keywords: Glucose transporter type 4, granulosa. polycystic ovary syndrome, insulin signaling, insulin resistance

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Introduction

Advanced glycation end-products (AGEs) present a group of heterogeneous compounds of high reactivity with increased cross-linking potential and detrimental effects over normal tissue physiology.[1,2] They are formed from non-enzymatic reaction of reducing sugars with the amino groups of proteins, nucleic acids, and lipids, causing tissue injury both directly (through trapping and cross-linking) and indirectly by binding to specific receptors for AGEs (RAGE) on the surface of various cell types.[1–9] Elevated serum AGEs levels have been observed in patients with hyperglycemia, insulin resistance and/or oxidative stress, such as diabetes, renal insufficiency, polycystic ovary syndrome (PCOS), rheumatoid arthritis, atherosclerosis, and aging.[3,10–15] Naturally occurring glycated albumin is a sensitive indicator of glycemic control by diabetic patients[16] whereas other circulating AGE forms such as glyoxal, methylglyoxal, and *N*-(carboxymethyl)lysine (CML) are also found elevated in diabetic and PCOS patients.[3,17]

Further investigation on the potential impact of increased circulating AGEs in PCOS pathology, a common metabolic and reproductive abnormality of young women, demonstrated the localization of AGE-modified proteins and their receptor RAGE in human ovarian tissue. Specifically, a differential qualitative distribution of AGE, RAGE, and their signaling mediator, NF-κB was observed in the granulosa cell layer of PCOS ovaries as compared to controls, indicating their localized effects.[3] Additional studies performed in an experimental animal model of normal female rats assigned to high- or low-in-AGEs content diet for three months revealed increased AGEs deposition and RAGE expression in ovarian tissue of high- compared to low-AGE-fed animals.[12] This observation was followed by

reduced activity of ovarian glyoxalase I (GLO-I), the major detoxifying enzyme of AGEs, in the granulosa cells of the high-AGE-fed animals, associated with hormonal abnormalities affecting progesterone, estrogens as well as androgens.[4]

Based on these studies it was hypothesized that AGEs may influence proper functioning of granulosa cells, possibly affecting ovarian physiology. This was supported by a retrospective study of assisted reproductive technology patients, in which accumulation of AGEs in serum and follicular fluid correlated highly with poor follicular and embryonic development and a lower likelihood of ongoing pregnancy.[18] Additionally, an investigation of ovulatory and anovulatory PCOS women revealed that AGEs levels, along with the number of follicles and the presence of anovulation were positively correlated with the levels of anti-Mullerian hormone (AMH). AMH being produced by granulosa cells is considered as an indicator of disturbed ovulatory process.[19]

Furthermore, AGEs interfere with insulin signaling pathways of several target tissues and are implicated in insulin resistance mechanisms. Insulin resistance in classic and non-classic insulin target tissues has been shown to be a major contributor in PCOS pathophysiology.[12] More specifically in granulosa cells recent data suggest that insulin action may also be affected.[20–23] The objective of the present study was to scrutinize how AGEs could affect insulin signaling in human granulosa cells, a question that so far was not answered. In the normal ovary, the activity of AKT, regulated downstream of phosphatidylinositol-3-kinase (PI3K), controls follicle and oocyte development by adjusting the amount of primordial follicles and the transition from quiescent to growing phase.[24] Previous studies have shown that deterioration of the PI3K (phosphatidylinositol-4,5-bisphosphate 3-kinase)/PTEN (phosphatase and tensin homolog)/AKT (protein kinase B, PKB or AKT) pathway is able to impair follicular development, intrafollicular oocyte development, and ovulation and therefore is critical denominator of ovarian function.[25] Similarly, decreased presence of glucose transporter type 4 (Glut-4) on cell surface impairs glucose uptake and metabolism, thereby aggravating follicle growth.[20] Taking into account that older studies provided evidence that PCOS-derived granulosa cells are characterized by impaired insulin action,[26,27] the missing link between metabolic dysregulation and PCOS was the mechanism and the molecules by which the latter is mediated. Therefore in the present study we focused on how AGEs could affect the first stage of this procedure and not granulosa cell function that has been already addressed by some studies. Thus, in these cells, we investigated the possibility of AGEs to interfere with AKT phosphorylation via activation of PI3K, affecting the translocation of the insulin-sensitive Glut-4 to the membrane surface.

Materials and methods

Cell cultures

A steroidogenic human ovarian granulosa cell line, designated KGN was obtained from RIKEN Bioresource center (Tsukuba, Japan) and established as previously described.[28] Steroid analysis and further characterization of KGN cells confirmed their applicability as a useful model to study steroidogenesis, cell growth, and apoptosis of human granulosa cells.[28] KGN cells were maintained in Dulbecco's modified Eagle's medium/F-12 (Cambrex, Walkerville, MD) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Biochrom, Berlin, Germany), 100 U/mL penicillin/streptomycin (Cambrex) at 37°C in a humidified atmosphere of 5% CO_2.

Since naturally occurring glycated albumin presents a sensitive glycemic indicator[16] commercially available human glycated albumin (HGA) was used as a representative AGE form throughout the study. KGN cells were treated with variable concentrations of HGA (50–200 μg/mL, Sigma-Aldrich St. Louis, MO) or human insulin (100 ng/mL, Lilly, Indianapolis, IN). Similar treatments have been also performed with human standard albumin (HSA) as control of the effect of albumin. Combined treatments of KGN cells with insulin (100 ng/mL) and HGA (200 μg/mL) were also performed with or without prior treatment with the PI3K inhibitor, LY294002 (Sigma-Aldrich).

Western blot analysis

KGN cells were seeded in six-well plated and grown up to 70% confluency in DMEM/F-12 containing 10% FBS. Twenty four hours prior to various treatments, the growth medium was changed to 0.5% FBS. For single or combination experiments, the cells were either treated with HGA for 24 h or serum starved for 3 h prior to insulin exposure for 1 h. For the experiments with the PI3K inhibitor LY294002, cells were incubated for 2 h with 25 μmol/L LY294002 during the serum-starved period. The cells extracts were obtained by lysis of the cells in RIPA buffer (50 mmol/L Tris-HCl; 150 mmol/L NaCl, Sigma, St. Louis, MO) containing 0.55% Nonidet P-40, protease inhibitors (1 mmol/L phenylmethylsulfonyl fluoride (PMSF) (Sigma), 10 μg/mL aprotinin, and 10 μg/mL leupeptin (Sigma); and phosphatase inhibitors (1 mmol/L sodium orthovanadate, 1 mmol/L NaF (Sigma). After 30 min incubation on ice, the lysates were cleared by centrifugation (14,000 rpm, 15 min, and 4°C). Protein concentrations were determined by Bio-Rad protein assay (BIO-RAD Laboratories, Hercules, CA) and Western blot analysis was performed as previously described with some modifications.[29–31] Briefly, equal amount of cell lysates (20 μg) were heated at 95°C for 5 min, electrophoresed on 12% SDS-PAGE under denaturing conditions, and transferred onto nitrocellulose membrane (BIO-RAD). The blots were blocked with TBS-T (20 mmol/L Tris–HCl, pH 7.6, 137 mmol/L NaCl, and 0.1% Tween 20) containing 5% non-fat dried milk at room temperature for 1 h. The membranes were probed overnight with primary antibody against phospho-AKT (1:1000 dilution) or total AKT (1:1000 dilution) in TBS/T containing 5% bovine serum albumin (BSA) (Cell Signaling, Beverly, MA). The blots were washed and followed by incubation with a secondary goat antibody raised against rabbit IgG conjugated to

horseradish peroxidase (HRP, 1:2000 dilution; sc-2004 Santa Cruz Biotechnology, CA). The bands were visualized by exposing the blots to x-ray film after incubation with freshly made ECL substrate for 3 min (SuperSignal, Pierce Biotechnology, Rockford, IL). Western blot experiments were repeated three independent times. Band densitometric analysis was performed using Image J software and normalized to the corresponding actin levels.

Protein fractionation

KGN cells were cultured in T75 culture flasks, starved overnight, and then treated with insulin (100 ng/mL), HGA (0.2 mg/mL), and insulin combined with HGA for 1 h. After treatment the media was removed and the different protein fractions were collected using ProteoExtract Native Membrane Protein Extraction Kit (Calbiochem, La Jolla, CA) according to manufactures' instructions. To verify that the separation of the protein lysates into cytosol and membrane-enriched fraction, Western blot with NA^+K^+-ATPase antibody (Cell Signaling) was performed.

Glut-4 translocation

For the analysis of Glut-4 translocation experiments, immunoblotting of the cytosolic and membrane fractions was performed by using a Glut-4 specific antibody (Santa Cruz Biotechnology). Following treatments, equal amounts of membrane proteins and cytosolic proteins were analyzed by SDS-PAGE and electrotransferred onto nitrocellulose membrane. The blots were blocked with TBS-T (20 mmol/L Tris–HCl, pH 7.6, 137 mmol/L NaCl, and 0.1% Tween 20) containing 5% non-fat dried milk at room temperature for 1 h. The membranes were probed overnight with primary antibodies against Glut-4 containing 5% non-fat milk. Immunoreactive bands were visualized using HRP-conjugated goat anti-rabbit IgG for polyclonal antibodies (1:2000 dilution; sc-2004 Santa Cruz Biotechnology) with the enhanced chemiluminescence (ECL) detection technique (SuperSignal, Pierce Biotechnology). The bands were visualized by exposing the blots to x-ray film. Glut-4 translocation experiments were repeated three independent times.

Statistical analysis

Data represent means ± SD of triplicate observations from at least three independent experiments. Activation of AKT was determined by taking the mean densitometry values of the ratio of phosphorylated:total AKT and normalizing to the control. Statistical analysis was performed by using ANOVA for comparisons of more than two groups. A Levene's test was always used to assess the equality of variances among groups and depending on its result, a Bonferroni or Games-Howell post hoc test was used for pairwise comparisons. The level of statistical significances was set at $P < 0.05$.

Results

Effects of HGA and insulin on AKT activation in human granulosa KGN cells

The interaction of HGA with key components of insulin signaling was investigated relative to AKT phosphorylation via activation of PI3K. Variable concentrations of HGA (50–200 μg/mL) were tested on AKT activation compared to insulin (100 ng/mL) and untreated control (Figure 1). The concentrations of HGA that we used were chosen according to previous literature.[32,33] KGN cells treated with insulin for 60 min showed significantly increased phosphorylation of AKT compared to control or HGA exposure ($P < 0.05$, Figure 1).

We then explored the combined effects of insulin and HGA on phosphorylation of AKT and PI3K specificity (Figure 2). Insulin-induced phosphorylation of AKT was significantly reduced in the presence of HGA ($P < 0.05$), although HGA alone did not affect AKT phosphorylation (Figure 1). In order to determine whether non-glycated albumin (human standard insulin, HSA)—and not only its glycated form—had any effect in AKT phosphorylation in the presence or absence of insulin, we performed a series of control experiments in which KGN cells were treated with HGA, HSA, insulin, and combinations of them (Supplementary Figure 1). Indeed, the standard insulin did not deteriorate insulin-mediated AKT activation in granulosa cells as the HGA did.

Moreover, preincubation of KGN cells with the specific PI3K inhibitor, LY294002, attenuated AKT phosphorylation to below basal levels across all treatment groups (Figure 2). Current literature[34] has shown that cell survival of KGN cells is affected mainly after a 48 h treatment of cells with LY294002. Similarly to the experiments of Rice *et al.*,[20] in our experiments, the 2 h incubation of LY294002 that we performed did not affect cell survival as indicated also by the total AKT levels that were not altered after treatments compared to control (Figure 2).

Effects of HGA and insulin on Glut-4 translocation to the membrane

Insulin-induced activation of PI3K pathway has been previously shown to be involved in the translocation of the major glucose transporter, Glut-4 from the cytoplasm to the plasma membrane.[35,36] We carried on investigating the potential effect of HGA in this pathway.

Immunoblotting with NA^+K^+-ATPase antibody verified that the protein fractionation protocol was effective in separating the protein lysates into cytosol and membrane-enriched fractions, with NA^+K^+-ATPase being detected predominantly in the membrane rather than in the cytosolic fraction (Figure 3a). Western blotting of Glut-4 revealed several glycosylated bands ranging from 44 to 62 kDa as previously reported.[37] For densitometric analysis we correlated changes of all bands detected including the main glycosylated band which resolves at around 59 kDa (Figures 3b, 4). There were more glycosylated Glut-4 variants in the cytosolic fraction compared to the membrane fraction of the control group (Figure 4).

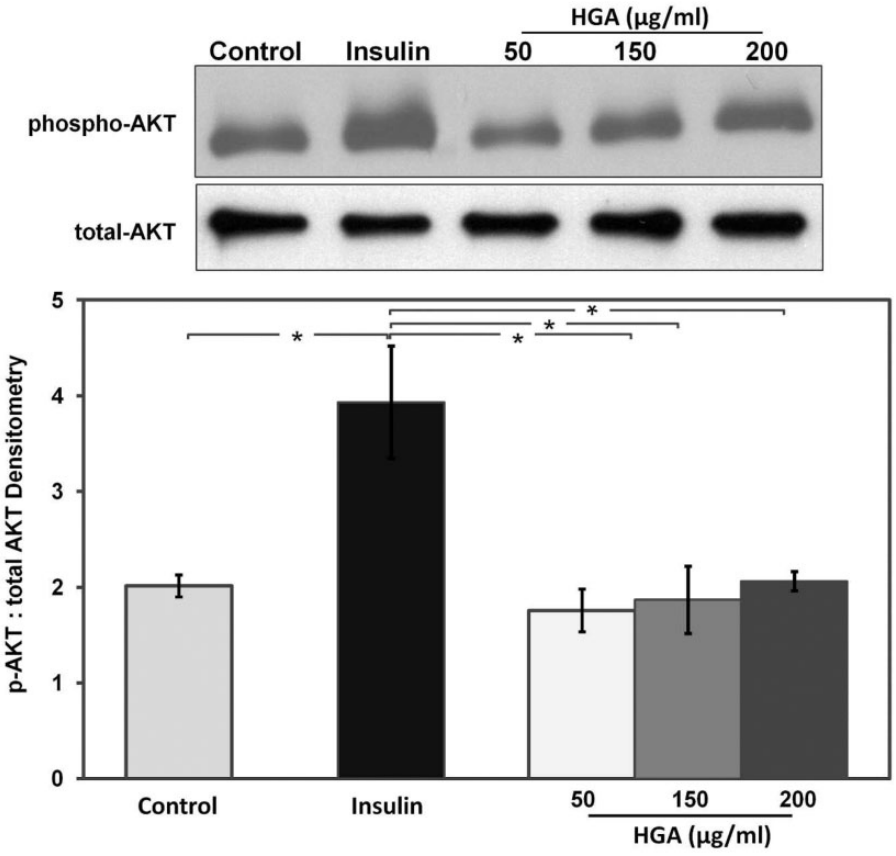

Figure 1 (a) Western blot analysis of insulin and variable HGA treatment (50–200 μg/mL) on phosphorylated (phospho) and total AKT protein expression in KGN cells after 1 h incubation. Untreated control was used for estimation of basal levels. (b) Graph of the densitometric analysis of the effect of insulin and HGA on activation of AKT (phospho-AKT): total AKT in KGN cells. *$P < 0.05$. Data are representative of three independent experiments

Insulin treatment revealed more glycosylated Glut-4 variants in both compartments and particularly membrane fraction compared with basal membrane fraction ($P < 0.001$). HGA alone increased Glut-4 in the cytosolic fraction compared with basal ($P < 0.001$) and reduced further movement to the membrane compared with basal ($P < 0.001$). The combinational treatment of insulin and HGA reduced Glut-4 translocation to the membrane even further ($P < 0.001$ compared to basal and insulin alone) and remarkably increased the cytosolic fraction ($P < 0.001$ compared to basal and insulin alone, Figure 4).

Discussion

We have shown for the first time a direct interaction of AGEs (HGA) with key components of the insulin-signaling pathway in human ovarian granulosa cells, highlighting the potential role of AGEs in the homeostasis of the ovarian and follicular microenvironment. This is especially relevant to insulin-resistance states such as PCOS, where elevated circulating AGE levels have been observed in normoglycemic, insulin resistant women with PCOS.[11,19] Our scientific interest is focused on the role of AGEs in relation to PCOS pathology as a distinct finding associated with hyperandrogenemia and insulin resistance.[11,19] With their prolonged half-life and ability to act as signaling molecules, AGEs may gradually accumulate in the ovary. *In-vivo* studies have shown that rodents fed with high AGE diet exhibit increased circulating AGE levels that are correlated with increased deposition of these agents in ovarian granulose cell layer.[3] Clinical evidence for this view supports the hypothesis that AGEs are good candidates as predictive markers and therapeutic targets in new strategies for improving reproductive counseling in specific populations, i.e. women with failed assisted reproductive techniques.[18] Similarly, our recent study by Tantalaki *et al*. showed that modifications of dietary AGEs intake are influencing the metabolic, hormonal, and oxidative stress profile of women with PCOS, highlighting the hypothesis that AGEs could be implicated in pathogenetic or therapeutic aspects of this group of patients.[38]

Therein we provided evidence that AGEs are capable of inhibiting insulin-mediated AKT activation in granulosa cells. Interestingly, exogenous administration of combined insulin and AGEs treatment markedly reduced the observed insulin-mediated AKT activation in granulosa KGN cells. We have shown that HGA could specifically impair insulin signaling as the inhibition of PI3K cascade was not observed when granulosa cells were co-treated with human unglycated albumin and insulin. Additionally we observed that HGA did alter AKT phosphorylation only when this pathway was stimulated by insulin and not when KGN cells were treated with HGA

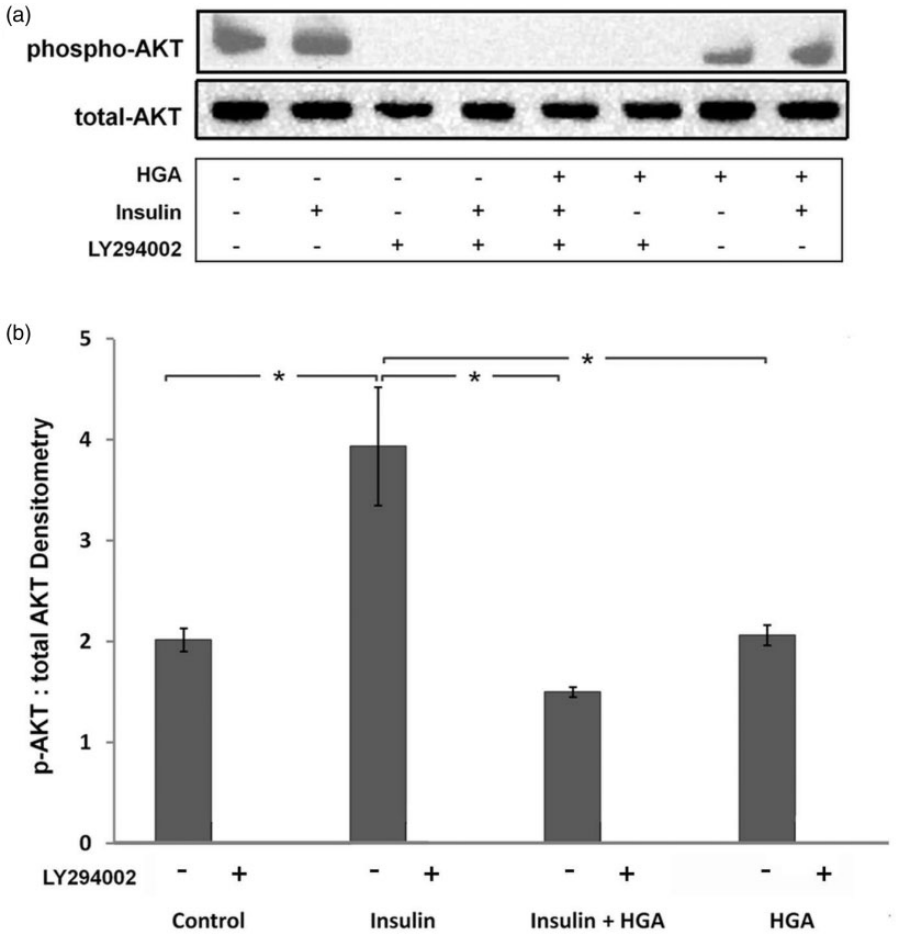

Figure 2 (a) Western blot of phosphorylated (phospho) and total AKT protein expression after 1 h treatment of KGN cells with insulin (100 ng/mL), HGA (200 μg/mL), or their combination. Cells were also exposed to PI3K inhibitor LY294002 (LY) prior treatments. (b) Graph of the densitometric analysis of the effect of insulin, HGA, and their combination on activation of AKT (phospho-AKT): total AKT in KGN cells. $^{*}P < 0.05$, $^{**}P < 0.01$. Data are representative of three independent experiments

alone. These findings were consistent with other studies in L6 skeletal muscle cells[33,39] and in INS-1E rat beta cells where either AGEs or methylglyoxal affected insulin-induced phosphorylation.[32,39,40] In these studies it was demonstrated that AGEs altered insulin receptor substrate (IRS) function and hence stimulation of the downstream PI3K signaling cascade. It seems that the beneficial effects of insulin metabolic signals can be impeded by AGEs. Under our experimental conditions insulin-induced AKT phosphorylation was PI3K-mediated as it was demonstrated by its reduction when a specific PI3K inhibitor was used. These findings fortify the suggestion that AGEs ''hamper'' on insulin are upstream and through PI3K.

Glucose uptake in insulin-sensitive tissues such as fat and muscle is mediated through glucose transporters with major one being the Glut-4. This is present in a highly complex intracellular tubule–vesicular network that is connected to endosomal Golgi system. Upon stimulation of insulin receptor, a rapid shift of Glut-4 takes place from the *trans* Golgi network into endosomes, leading to increased rate of Glut-4 exocytosis from the vesicles and reduction in the degree of endocytosis. The resulting outcome is movement of Glut-4 to the plasma membrane where it increases the formation of aqueous pores, facilitating glucose cell entry.[41] As expected the treatment of human granulosa KGN cells with insulin increased Glut-4 translocation, however, as Berenguer et al. and Chiu et al. for substrates other than AGEs, have reported, Glut-4 translocation could also be insulin-independent. Glut-4 translocation could be also insulin-independent as Berenguer *et al.* and Chiu *et al*. for substrates other than AGEs have reported.[42,43] Besides, the combined administration of insulin and AGEs reduced Glut-4 translocation even further. Our findings suggest that HGA participates in glucose metabolism of human granulosa cells. Since AGEs block PI3K-dependent AKT activation in insulin-stimulated granulosa KGN cells, it is possible that their inhibitory effect on Glut-4 translocation is mediated through down-regulation of the PI3K pathway. These data were consistent with another study in L6 skeletal muscles cells where HGA was able to prevent Glut-4 translocation induced by insulin.[33]

Taken all together, these data suggest that increased AGEs presence interferes with insulin-stimulated glucose uptake in the ovary implying a potential contributing factor to the insulin resistance observed in granulosa cells

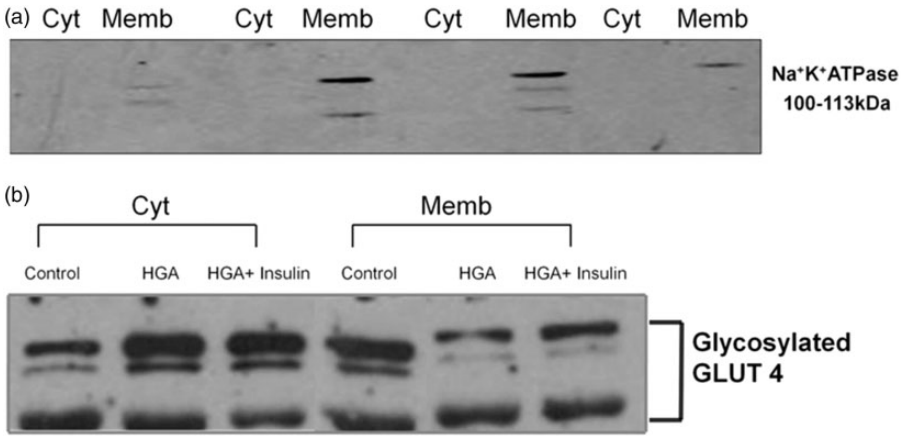

Figure 3 (a) NA^+K^+-ATPase protein expression from Western immunoblotting in KGN cells cultured with HGA for 24 h, then serum-starved for 3 h, and exposed acutely to insulin for 30 min. Cells lysates were separated into cytosolic (Cyt) and membrane (Memb) enriched fractions using a sucrose lysis buffer and ultracentrifugation, before Western blot with an antibody to NA^+K^+-ATPase (a plasma membrane bound protein). NA^+K^+-ATPase is detected predominantly in the membrane fraction, thereby verifying the fractionation protocol. (b) Western immunoblotting illustrating glycosylated bands (44–62 kDa) of Glut-4 in cytosolic (Cyt) and membrane (Memb) fractions in KGN cells untreated or single-treated with HGA or combined with insulin. Data are representative of three independent experiments

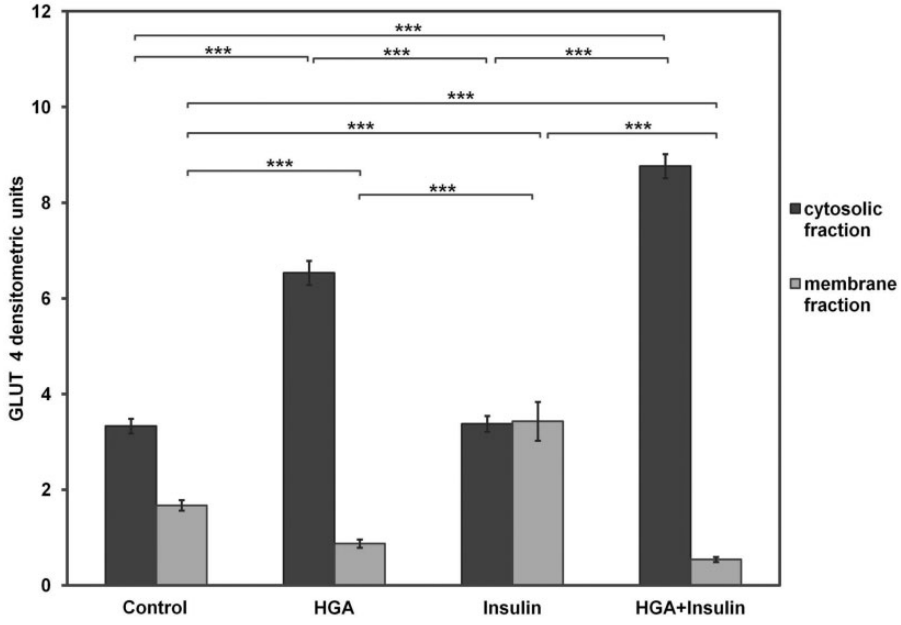

Figure 4 Graph of the densitometry analysis of the effect of HGA and insulin exposure (30 min) on the expression of glycosylated Glut-4 in KGN cells. ***$P < 0.001$

of anovulatory PCOS women. However, it still remains to be more meticulously investigated whether dysregulation in the metabolism of granulosa cells indeed affects follicular growth and oocyte quality. The latter may also be affected by other pathways such as those of RAGE receptor, SR-A or CD36 that are being activated by AGEs and could be active in the ovarian cells.[9] Indeed in a study by Riboulet-Chavey *et al*., aminoguanidine, an inhibitor of AGEs/RAGE axis was able to prevent all the biological responses produced by methylglyoxal, a precursor of AGEs, thus connecting the presence of AGEs with deteriorated insulin action.[39]

In conclusion, we have shown a direct inhibitory effect of AGEs on insulin signaling in human granulosa cells, resulting in prevention of membrane translocation of Glut-4 and implying a compromised metabolic fuel intake by granulosa cells, in states with insulin resistance and ovarian dysfunction, like PCOS. However these results should be considered cautiously since the dysregulation of one molecular pathway, despite its importance, cannot explain the pathology of the disease. However, the systemic metabolic disturbance and insulin resistance that are present in PCOS patients are probably mirroring what is taking place at a molecular level in the human granulosa cells and other cells, contributing thus to the reproductive abnormalities that accompany the disease. Therefore it is important to investigate how molecular disturbances could affect the

initiation or progression of PCOS when looking the disease from a Systems Biology point of view and further *in-vivo* studies are needed on this direction. Additionally, future molecular studies have to more intensively explore how disturbances in the metabolic machinery of granulosa cells could affect their reproduction-related features as well as how AGEs account for the function of other cells in the ovarian environment such as the thecal cells that produce androgens. The data from the present study support our hypothesis that intra ovarian AGEs accumulation, from endogenous or exogenous sources, via interference with insulin signaling may contribute to the pathophysiology of states characterized with anovulation and insulin resistance such as PCOS. Simultaneously, these observations may prove to be of clinical importance, suggesting that an AGE-restricted diet could constitute a novel treatment strategy against PCOS in the future.

Author contributions: Authors contributed significantly to the study and have agreed to the manuscript's content: EDK designed the study and involved in interpretation of data writing. AC and EP involved in experimental work. DK, AC, and EP wrote the paper. MK supervised and guided all the experimental steps of the study.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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