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REVIEW ARTICLE *Reproductive biology*

Advanced glycation end products and their relevance in female reproduction

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STUDY QUESTION: Do advanced glycation end products (AGEs) and their receptors play a role in female reproduction?

SUMMARY ANSWER: AGEs might contribute to the etiology of polycystic ovary syndrome (PCOS) and infertility.

WHAT IS KNOWN ALREADY: The endogenous AGEs are produced in the body by chemical reactions. Exogenous sources of AGEs are diet and smoking. AGEs have been proposed to be among the main intermediaries involved in several diseases, such as metabolic syndrome, type 2 diabetes mellitus, cardiovascular disease, ovarian aging, inflammation, neurodegenerative disorders and PCOS.

STUDY DESIGN, SIZE, DURATION: A systematic review was performed for all available basic science and clinical peer-reviewed articles published in PubMed from 1987 to date. Abstracts of annual meetings of the Endocrine Society and American Society for Reproductive Medicine were also reviewed.

PARTICIPANTS/MATERIALS, SETTING, METHODS: A total of 275 publications and scientific abstracts were identified from the initial search. Sixty-two papers and four published scientific abstracts were selected for full review. The main outcomes were the regulatory effects of AGEs on: (i) granulosa cells, adipocyte physiology, obesity and insulin resistance in women with PCOS and in polycystic ovary animal models and (ii) infertility and measures of ovarian reserve.

MAIN RESULTS AND THE ROLE OF CHANCE: There is an intricate relationship between the AGE-RAGE (receptor for AGEs) system and some aspects of PCOS, such as granulosa cell dysfunction, adipocyte pathophysiology, obesity and insulin resistance. Additionally, irregular ovarian AGE signaling might in part explain the abnormal ovarian histology observed in women with PCOS. The ovarian dysfunction due to AGEs in women without PCOS suggests a role for the AGE-RAGE system in the ovarian follicular environment, and might relate to assisted reproduction technology outcome and measures of ovarian reserve.

LIMITATIONS, REASONS FOR CAUTION: The body of literature currently available limits these findings. The results obtained from granulosa cell lines and animal models may not fully extrapolate to humans.

WIDER IMPLICATIONS OF THE FINDINGS: This review underscores a critical need to unveil the exact mechanistic actions of AGEs in reproductive physiology and more specifically the hypothalamic–pituitary–ovarian axis. AGE inhibitors might present an emerging therapeutic approach with significant applications in the context of PCOS and infertility.

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Key words: advanced glycation end products / polycystic ovary syndrome / infertility / obesity / insulin resistance

Introduction

Advanced glycation end products (AGEs) are the end products of a chemical procedure called the Maillard reaction in which the carbonyl group of carbohydrates reacts non-enzymatically with primary amino groups of proteins (Brownlee *et al.*, 1988; Unoki and Yamagishi, 2008). This glycation reaction involves the formation of chemically reversible early glycosylation products with proteins, so-called Schiff bases and Amadori adducts, for example HbA1C (Brownlee, 1991). It was not until around 1980 that researchers began recognizing the significance of the complex, late-stage Maillard processes as mediators of the complications of diabetes and aging (Monnier *et al.*, 1981). Subsequently, proteins bearing Amadori product have come to be referred to as glycated proteins (distinguishing them from enzymatically glycosylated proteins),

while the process of Amadori product formation is termed glycation. The later-stage, complex pigments and cross links formed from glycated protein during the *in vivo* Maillard reaction have come to be known as AGEs (Bucala and Cerami, 1992).

AGEs have been proposed to be among the main intermediaries of several diseases such as type 2 diabetes mellitus (T2DM), obesity, metabolic syndrome (MetS), cardiovascular disease (CVD), aging, inflammation and neurodegenerative disorders (Yamagishi *et al.*, 2005; Diamanti-Kandarakis *et al.*, 2007d, Tatone and Amicarelli, 2013). Along with the appreciation of these problems, there has been recognition that AGEs have a wider range of actions, including in reproduction. This review will focus on the regulatory effects of AGEs on granulosa cells, adipocyte physiology, obesity and insulin resistance in women with polycystic ovary syndrome (PCOS) and in polycystic ovary (PCO) animal models. It will also tackle the relationship between the AGE system and infertility as well as ovarian reserve in women of reproductive age. Finally, it will address potential therapeutic relevance pertaining to PCOS and infertility via targeting the AGE pathway.

Methods

Search strategy and data extraction

A systematic review was performed for all available basic science (*in vitro* and *in vivo*) and clinical peer-reviewed articles (prospective, retrospective and review articles) published in English from 1987 to date in PubMed. Additionally, references from all relevant articles were checked and hand searches of the abstracts of annual meetings of the Endocrine Society and American Society for Reproductive Medicine were performed. In addition to using 'advanced glycation end-products' as a keyword search in PubMed, the following keywords were used: 'receptor', 'inhibitor', 'obesity', 'insulin resistance, 'hyperandrogenism', 'PCOS', 'granulosa cell', 'infertility', 'assisted reproductive technology', '*in vitro* fertilization', 'ovarian reserve', and 'anti-Mullerian hormone'. Data were extracted from the text, tables and graphs in the manuscripts.

Results

A total of 275 manuscripts and abstracts were identified during the initial search. After reviewing titles and abstracts, 62 papers and 4 published scientific abstracts were selected for full review.

Biochemistry and effect of AGEs

AGEs

Among the most important post-translational modifications is the non-enzymatic modification of proteins, lipids and nucleic acids by glucose, converting them to AGEs (Inagi, 2011; Piperi *et al.*, 2012) (Fig. 1). Advanced glycation results in irreversible cross linking of proteins, loss of protein structure and function, followed by apoptosis. Once formed, AGEs may damage cellular structures via a number of mechanisms, including the formation of cross links between key molecules in the basement membrane of the extracellular matrix (ECM) and interaction with receptors on the cell surfaces thus altering cellular function (Inagi, 2011; Piperi *et al.*, 2012). AGEs constitute a heterogeneous group of compounds of >20 members, such as the 1,2-dicarbonyl precursor compounds glyoxal, methylglyoxal (MG), *N*-carboxymethyl-lysine (CML) and pentosidine (Fig. 1). Pentosidine and CML have been well characterized and used as markers of AGE accumulation in various tissues (Diamanti-Kandarakis *et al.*, 2007c).

AGEs are usually formed slowly under physiological conditions. Hyperglycemia, insulin resistance, obesity, aging, oxidative stress and hypoxia accelerate the generation of AGE precursors (Yamagishi *et al.*, 2005). The accumulation of AGEs is associated with variable pathologies such as T2DM, MetS, CVD, aging (including ovarian aging), inflammation, neurodegenerative disorders, obesity and even PCOS (Yamagishi *et al.*, 2005; Diamanti-Kandarakis *et al.*, 2007d; Tatone and Amicarelli, 2013). The action of AGEs is classified as receptor independent (such as cross links with ECM) or receptor dependent (Piperi *et al.*, 2012). AGEs circulate and act on cell surface receptors, such as the receptor for AGE (RAGE) which is a member of the immunoglobulin superfamily of receptors (Kalea *et al.*, 2009). The AGE-RAGE interaction leads to activation of secondary messenger activating nuclear factor-kappa B leading to the development of a proinflammatory state, cellular toxicity and damage (Fig. 2) (Kalea *et al.*, 2009).

RAGE

RAGE has transmembrane, cytosolic and extracellular domains (Basta, 2008). RAGE is expressed in many tissues including ovaries and is most abundant in the heart, lung, skeletal muscle, vessel wall and ovaries (Basta, 2008). It is also present in monocytes, macrophages and lymphocytes (Basta, 2008). RAGE is down-regulated in most organs during normal life. With aging, RAGE expression increases again, possibly due to the accumulation of RAGE ligands, which in turn up-regulate receptor expression (Yan *et al.*, 2007; Basta, 2008; Kalea *et al.*, 2009). In the cases of diabetes, inflammation, atherosclerosis and PCOS, there is a marked induction of RAGE due to the action of its ligands and to several mediators from activated inflammatory cells (Dunaif *et al.*, 1989; Diamanti-Kandarakis *et al.*, 2005; Yan *et al.*, 2007; Kalea *et al.*, 2009). In turn, the binding of ligand to RAGE induces further up-regulation of the receptor causing a positive feedback loop (Kalea *et al.*, 2009).

sRAGE

In addition to the normal receptor, forms of RAGE lacking both the cytosolic and the transmembrane domains have been described (Fig. 1) (Diamanti-Kandarakis *et al.*, 2005; Diamanti-Kandarakis, 2006). These forms of AGE receptors are, therefore, secreted extracellularly, can be detected in circulating blood, and are called soluble RAGEs (sRAGE) (Diamanti-Kandarakis *et al.*, 2005; Diamanti-Kandarakis, 2006). This is of importance because sRAGEs can bind their ligands (AGEs) in the circulation, thus preventing the adverse intracellular events of the AGE-RAGE axis. In contrast to RAGE, sRAGE is often considered the 'good' receptor since their levels have been shown to be down-regulated in hyperglycemia and inversely associated with the severity of some vascular complications (Basta *et al.*, 2006).

Generic factors inducing AGEs production

Serum and tissue (including ovary) AGE levels seem to depend on endogenous and exogenous sources (Goldberg *et al.*, 2004). The endogenous AGEs are produced in the body by chemical reactions. Exogenous sources of AGEs are diet and smoking. Human and animal studies have shown that serum and tissue AGEs can be influenced by diet (Goldberg *et al.*, 2004; Diamanti-Kandarakis *et al.*, 2007c). Foods high in protein and fat, such as meat, cheese and egg yolk, are rich in AGEs (Goldberg *et al.*,

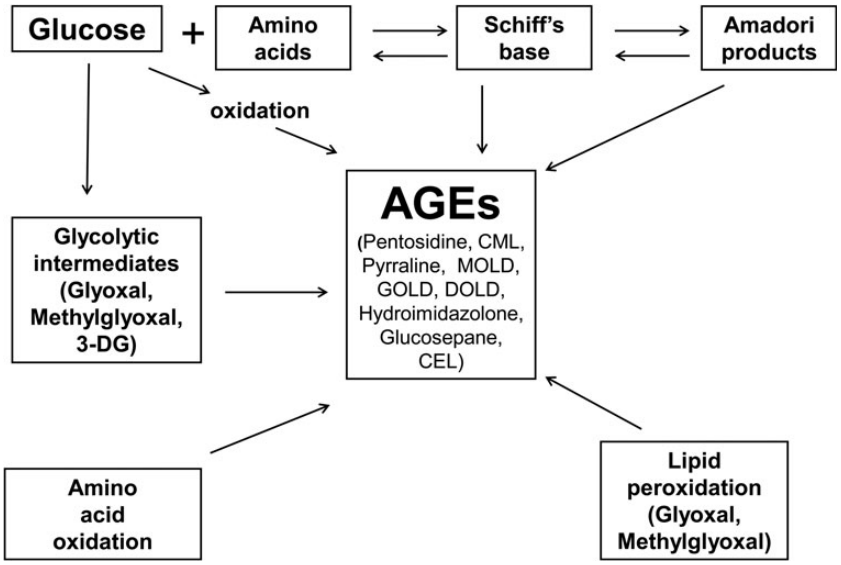

Figure 1 A scheme of the complex Maillard reaction and formation of advanced glycation end-products (AGEs). 3-DG, 3-deoxyglucosone, CML, carboxymethyllysine, MOLD, MG-lysine dimer, GOLD, glyoxal lysine dimer, DOLD, 3-deoxyglucosone lysine dimer, CEL, carboxyethyllysine.

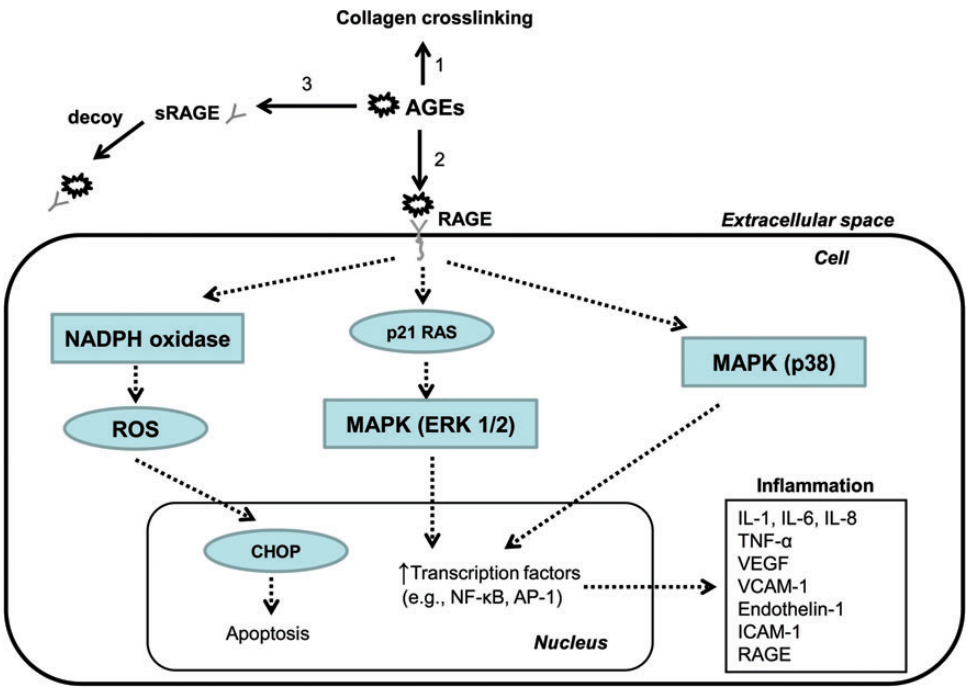

Figure 2 A simplified diagram of the pathogenic effects of AGEs. AGEs could damage cellular structures via formation of cross links between key molecules in the basement membrane of the ECM, e.g. collagen (1). The interaction with cellular AGE receptors (RAGE) induces inflammatory reactions and oxidative stress leading to increased transcription of inflammatory genes and apoptosis (2). The circulating receptor for AGEs (sRAGE) acts as decoy by binding the circulating AGEs, thus conferring a potential protective role (3). NF-kB, nuclear factor kappa B; AP-1, activator protein-1; ROS, reactive oxygen species; CHOP, CCAAT-enhancer-binding protein homologous protein; MAPK, mitogen-activated protein kinase; NADPH, nicotinamide adenine dinucleotide phosphate. IL, interleukin; TNF-α, tumor necrosis factor α, VEGF, vascular endothelial growth factor; VCAM-1, vascular cell adhesion protein 1; ICAM-1, intercellular adhesion molecule 1; RAGE, receptor for advanced glycation end-products.

2004). Additionally, contemporary methods of cooking (precooked fast-food meals heated in high temperatures) dramatically increase serum AGE concentration (Goldberg *et al.*, 2004). A study in female rats has shown that a high-AGE diet for 6 months caused an increase in serum AGE levels as well as higher RAGE deposition in the ovarian tissue compared with animals fed on a low-AGE diet and controls (Diamanti-Kandarakis *et al.*, 2007c). Additionally, it was found that fasting glucose, insulin and testosterone levels, as well as the weight of the ovaries, were increased in animals fed on a high-AGE diet compared with the controls and animals on a low-AGE diet (Diamanti-Kandarakis *et al.*, 2007c). These findings of AGEs deposition in the ovarian tissue in conjunction with an altered metabolic profile and elevated testosterone levels provide evidence for a dual effect of dietary AGEs on aspects of reproductive and metabolic function.

Studies in animals and humans with diabetes indicated that dietary AGE restriction results in significant reduction of circulating AGE levels leading to improved insulin sensitivity and preventing progression of atherosclerosis (Hofmann *et al.*, 2002; Vlassara and Palace, 2002; Uribarri *et al.*, 2003). A diet low in AGEs and administration of an AGE blocker, aminoguanide, effectively reduced serum AGE concentration in diabetes (He *et al.*, 1999; Uribarri *et al.*, 2003; Diamanti-Kandarakis *et al.*, 2007a). Additionally, studies in humans have revealed that the administration of a lipase inhibitor (orlistat), a medication used clinically for weight loss, resulted in a decrease of AGE absorption and serum levels in women with PCOS following the ingestion of an AGE-rich meal (Diamanti-Kandarakis *et al.*, 2007b).

Smoking is another exogenous source of AGEs (Cerami *et al.*, 1997). Reactive glycation products are present in aqueous extracts of tobacco and in tobacco smoke in a form that can rapidly react with proteins to form AGEs both *in vitro* and *in vivo* (Cerami *et al.*, 1997). This reaction can be inhibited by aminoguanidine, a known inhibitor of AGE formation. Additionally, serum AGE levels in cigarette smokers were shown to be significantly higher than those in non-smokers.

The relationship between AGEs and aspects of PCOS

AGEs and inflammation

Circulating AGEs (such as CML and MG derivatives) correlate with indicators of inflammation [such as C-reactive protein (CRP)] and oxidative stress across all ages (Uribarri *et al.*, 2007). Additionally, the accumulation of AGEs in tissues induces cellular oxidative stress and promotes inflammation (Vlassara *et al.*, 2002). The endogenous AGE production, along with AGEs from exogenous sources, promote a systemic glycoxidant burden, oxidant stress and cell activation, thus enhancing vulnerability of target tissues to injury (Vlassara *et al.*, 2002; Negrean *et al.*, 2007). Dietary AGE restriction in humans has been associated with a significant reduction of inflammation markers, such as plasma CRP, tumor necrosis factor-α and vascular cell adhesion molecule-1 (Uribarri *et al.*, 2011; Luevano-Contreras *et al.*, 2013). Using recently developed RAGE-depleted mice, Liliensiek *et al*. (2004) showed that the absence of RAGE protects mice from lethal multibacterial peritonitis and sepsis caused by cecal ligation and puncture. The decoy receptor sRAGE has been shown to successfully reduce inflammatory responses in many models (Park *et al.*, 1998; Bucciarelli *et al.*, 2002). Moreover, sRAGE is now shown to be expressed in a wide variety of human tissues including vascular endothelium, pneumocytes, pancreatic beta cells, monocyte/macrophages and various epithelial cells, many of which play pivotal roles in local inflammation (Cheng *et al.*, 2005). All these findings strongly implicate that AGE-RAGE signaling plays an important role in modulating local inflammation.

AGEs and insulin resistance

PCOS is a complex, heterogeneous endocrine disorder characterized by chronic anovulation, hyperandrogenism and PCO that affects 5–10% of women of reproductive age worldwide (Azziz *et al.*, 2004). In addition to its reproductive consequences, PCOS is associated with an increased prevalence of cardiometabolic aberrations including obesity, MetS, insulin resistance, T2DM, all leading to the development of CVD (Azziz *et al.*, 2004; Wild *et al.*, 2010).

The etiology of PCOS remains largely unknown. Although historically characterized as a disorder of androgen excess, recent studies have identified insulin resistance as a significant contributor and possible mediator of the underlying pathophysiology (Diamanti-Kandarakis, 2006). Insulin resistance is defined as a physiologic state at which a greater amount of insulin than normal is required to elicit an appropriate response. Approximately 50–70% of women with PCOS have some degree of insulin resistance (Diamanti-Kandarakis, 2006). In addition to contributing to the hyperandrogenism associated with PCOS, insulin resistance is also linked to the development of impaired glucose tolerance and T2DM (Legro *et al.*, 1999). Glucose concentration remains within a normal range as long as the secretion of insulin from pancreatic β-cells overcomes the insulin resistance. Impaired glucose tolerance or T2DM develops when the β-cell compensatory response becomes insufficient (Mantzoros and Flier, 1995). Although obesity appears to augment the degree of insulin resistance, the prevalence of impaired glucose tolerance (10.3%) and T2DM (1.5%) is also increased in non-obese women with PCOS (Dunaif *et al.*, 1989).

Multiple actions of insulin may contribute to hyperandrogenism (Burghen *et al.*, 1980). Although several studies have shown a correlation between fasting insulin levels and androgen concentration, it remains unclear if hyperandrogenism is a result of hyperinsulinemia or vice versa (Burghen *et al.*, 1980). Both insulin-like growth factor-1 (IGF-1) and insulin are potent stimulators of ovarian androgen production, an action likely mediated via the insulin receptor (Burghen *et al.*, 1980; Barbieri *et al.*, 1988). Additionally, it is possible that the increased levels of circulating insulin potentiate the effect of LH on ovarian theca cells. Another mechanism of the possible hyperandrogenism seen in PCOS is the insulin-mediated inhibition of sex hormone-binding globulin resulting in an increase in unbound androgen available for delivery to target tissues (Lindstedt *et al.*, 1991).

Since oxidative stress generation and inflammation are closely associated with insulin resistance, it is conceivable that the AGE-RAGE system could play a role in the pathogenesis of insulin resistance observed in PCOS (Unoki and Yamagishi, 2008). The AGE-RAGE system has been directly linked to insulin resistance independent of circulating glucose levels, weight and over-nutrition/obesity. A report by Cai *et al.* (2012) identified AGEs as a non-traditional risk factor for insulin resistance in non-obese mice independent of over-nutrition. In that study, exposure to an isocaloric diet in which the typical thermally promoted AGEs were largely substituted by synthetic MG derivatives of a single protein (MG^{+}) caused a phenotypic shift consisting of weight gain, adiposity, insulin resistance and ultimately diabetes. The insulin resistance occurred in the animals even before alterations in blood glucose levels. This newly

identified link between exogenous AGEs and metabolic dysfunction is supported by the evident activation of inflammation in both macrophages and adipocytes, and the markedly impaired insulin-signaling pathways in insulin-sensitive tissues of MG^{+} mice independent of glucose levels. Another study showed that obese RAGE knockout mice (RAGE −/−) have a higher homestasis model assessment (HOMA) index when compared with obese RAGE +/+ mice (Leuner *et al.*, 2012). A recent study in overweight women reported that consumption of a diet low in AGEs improves insulin sensitivity (Mark *et al.*, 2013). These mechanistic findings thus provide an important framework for elucidating the role of AGE-RAGE system in insulin resistance.

AGEs and adiposity

Approximately 30–75% of women with PCOS are obese (Ehrmann, 2005). While obesity may have a central role in the development of PCOS in susceptible individuals, it certainly appears to exacerbate the clinical and metabolic features of the disorder. Obese women with PCOS are more likely to experience more severe sequelae, such as hyperandrogenism and MetS, than those with a normal BMI (Kirchengast and Huber, 2001; Norman *et al.*, 2004). Additionally, modest weight loss has been shown to regulate menses, improve reproductive performance and hirsutism, decrease androgen and insulin serum levels and improve insulin sensitivity indices in individuals with PCOS (Norman *et al.*, 2004). Additionally, the distribution and morphology of the adipose tissue appears to be a significant contributor to the pathophysiology underlying PCOS. A majority of women with PCOS have an abdominal distribution of adiposity (central obesity), independent of BMI: an effect likely associated with elevated circulating androgens (Kirchengast and Huber, 2001).

A relationship between adiposity and reproduction has been reported (Merhi, 2007, 2009; Merhi and Pal, 2007; Merhi *et al.*, 2008, 2009). AGEs have been directly linked to adipocyte physiology (Jia *et al.*, 2012). Studies using cell lines derived from adipose tissue (3T30L1) have shown that AGE (specifically MG) stimulate adipogenesis (Jia *et al.*, 2012). In an animal model, RAGE knockout mice (RAGE −/−) showed an accelerated weight gain compared with RAGE +/+ mice (Leuner *et al.*, 2012). As mentioned above, sRAGE isoforms may act as a binding protein, removing circulating AGEs and protecting tissues from AGE-elicited damage. Indeed, in diabetic and non-diabetic subjects, plasma sRAGE inversely correlates with components of MetS, including obesity and insulin resistance (Koyama *et al.*, 2005).

Obese women have significantly lower sRAGE and adiponectin concentrations (Vazzana *et al.*, 2012). This observation confirms previous findings that sRAGE correlates with adiponectin, both in diabetic and non-diabetic subjects (Choi *et al.*, 2009). Interestingly, diet-induced weight loss has been shown to increase serum sRAGE levels by 150% (Vazzana *et al.*, 2012). These data taken together reveal that the AGE-RAGE system plays a role in the development of obesity.

Serum and ovarian AGE-RAGE system in PCOS

Insulin resistant women with PCOS without hyperglycemia have elevated serum AGE levels and increased RAGE expression in their circulating monocytes (Diamanti-Kandarakis *et al.*, 2005). Additionally, serum AGE levels are positively correlated with testosterone level, free androgen index, insulin, HOMA and waist-to-hip ratio in women with PCOS without hyperglycemia (Burghen *et al.*, 1980). Another study has shown that increased serum AGE level is a distinct finding in non-insulin resistant lean women with PCOS suggesting that serum AGEs are elevated in PCOS independently of the presence of insulin resistance (Diamanti-Kandarakis *et al.*, 2008).

Recent studies have demonstrated that RAGE- and AGE-modified proteins are expressed in human ovarian tissue (Fujii and Nakayama, 2010; Tatone and Amicarelli, 2013). In ovarian tissue samples, women with PCOS have increased AGEs and RAGE expression in theca and granulosa cell layers compared with normal women (Diamanti-Kandarakis *et al.*, 2007d). Additionally, a differential qualitative distribution of AGE and RAGE subunit was observed in women with PCOS compared with healthy controls, where a more pronounced staining density of both AGE and RAGE was observed in the granulosa cell layer of PCOS ovaries (Diamanti-Kandarakis *et al.*, 2007d).

The ovaries of women with PCOS have alterations in enzymes responsible for collagen synthesis (Henmi *et al.*, 2001). Lysyl oxidase enzyme is one of the key enzymes in the ovary responsible for collagen and elastin cross linking in the organization of ECM during follicular development (Harlow *et al.*, 2003). A study has shown that the deposition of excess collagen in PCO tissue may, in part, be due to AGE-mediated stimulation of lysyl oxidase activity (Papachroni *et al.*, 2010). These results indicate that AGE signaling could regulate ovarian follicular ECM organization in PCOS. Other data demonstrated that AGEs could reduce the activity of some 'good' detoxifying enzymes (such as glyoxalase-I) in the ovary of PCOS rats (Kandaraki *et al.*, 2012).

Ovarian dysfunction linked to AGEs

Diamanti-Kandarakis and her group have performed highly valuable and informative work on the role of the AGE-RAGE system in PCOS (Diamanti-Kandarakis *et al.*, 2013; Piperi *et al.*, 2013). They have recently demonstrated that the AGE-RAGE system might be responsible for the ovulation failure that characterizes PCOS. For instance, in a human granulosa cell line model (KGN), the authors demonstrated that AGEs *in vitro* interfere with LH action leading to a sustained abnormal activation of the ERK1/2 pathway—a crucial pathway for directing normal follicular development and initiating the ovulation process (Diamanti-Kandarakis *et al.*, 2013). The authors concluded that this inappropriate sustained activation of the ERK1/2 pathway in KGN granulosa cells by AGEs may be partly responsible for the impaired follicular development and hence ovulatory dysfunction associated with PCOS. Piperi *et al.* (2013) postulated that AGEs within the ovary alter glucose metabolism and folliculogenesis. They grew KGN cells in culture with insulin alone or with human glycated albumin (HGA; rich with AGEs) alone, or with a combination of insulin and HGA. They then assessed the activation of Akt followed by the analysis of GLUT-4 (a glucose transporter) translocation from the cytoplasm to the membrane compartments of KGN cells. The authors demonstrated that HGA alone inhibited the insulin-mediated Akt phosphorylation and significantly reduced GLUT-4 in the granulosa cell membrane (Piperi *et al.*, 2013) (Fig. 3). The combined treatment of insulin and HGA significantly reduced GLUT-4 translocation to the membrane. They concluded that AGEs might be responsible for reduced glucose uptake by granulosa cells, potentially altering follicular growth. Hence, the AGE-RAGE system might contribute to the ovarian dysfunction observed in insulin-resistance states, such as PCOS (Fig. 4). Because AGEs are elevated in the serum of women with PCOS, Christakou *et al.* (2013) investigated whether oral contraceptives (OCPs) or metformin affect serum AGEs levels in women with PCOS. They randomized women with PCOS ($n = 109$) to receive either OCP or metformin

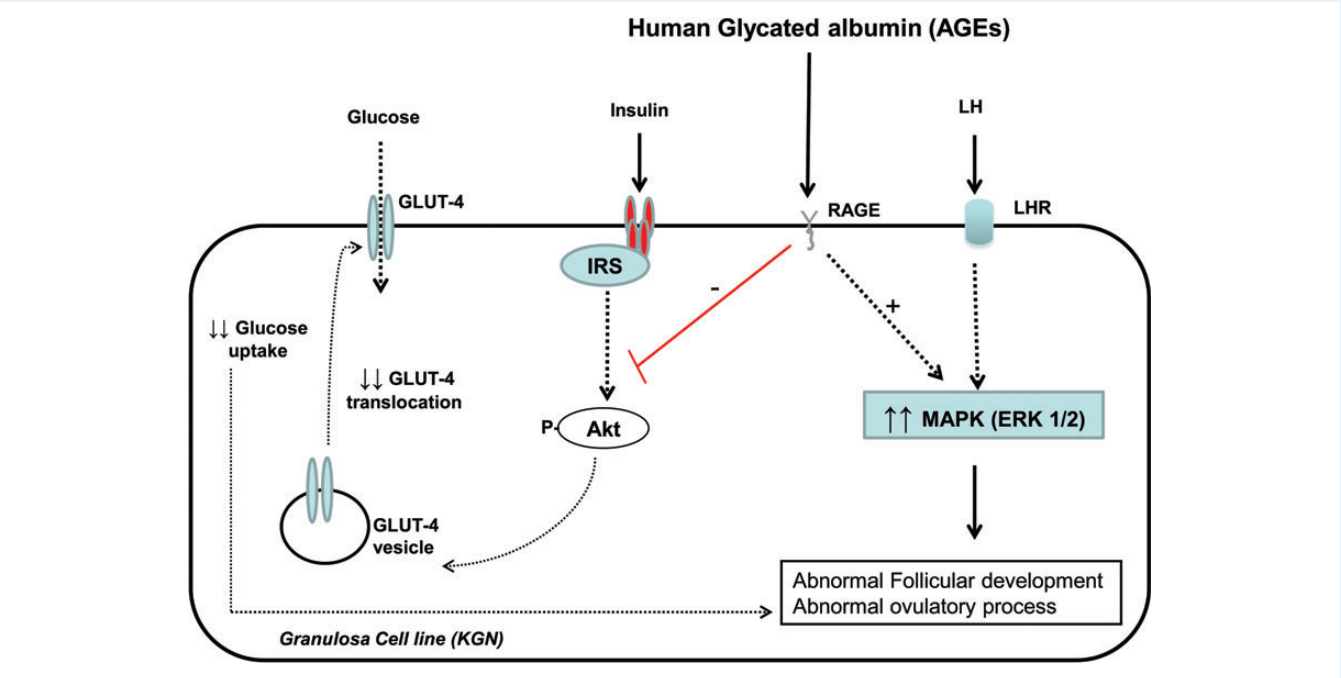

Figure 3 Insulin increases GLUT-4 translocation and glucose uptake in granulosa cell through Akt pathway. HGA-containing AGEs inhibits insulin-induced Akt phosphorylation and reduces GLUT-4 on granulosa cell membrane, thus reducing intracellular glucose uptake. HGA also interferes with LH action by causing a sustained activation of the ERK1/2 pathway, a crucial pathway for follicular development. AGE, advanced glycation end product; RAGE, AGE receptor; LHR, LH receptor; IRS, insulin receptor substrate; PIP3, phosphatidylinositol-3,4,5-trisphosphate; GLUT-4, glucose transporter type 4; MAPK, mitogen-activated protein kinase.

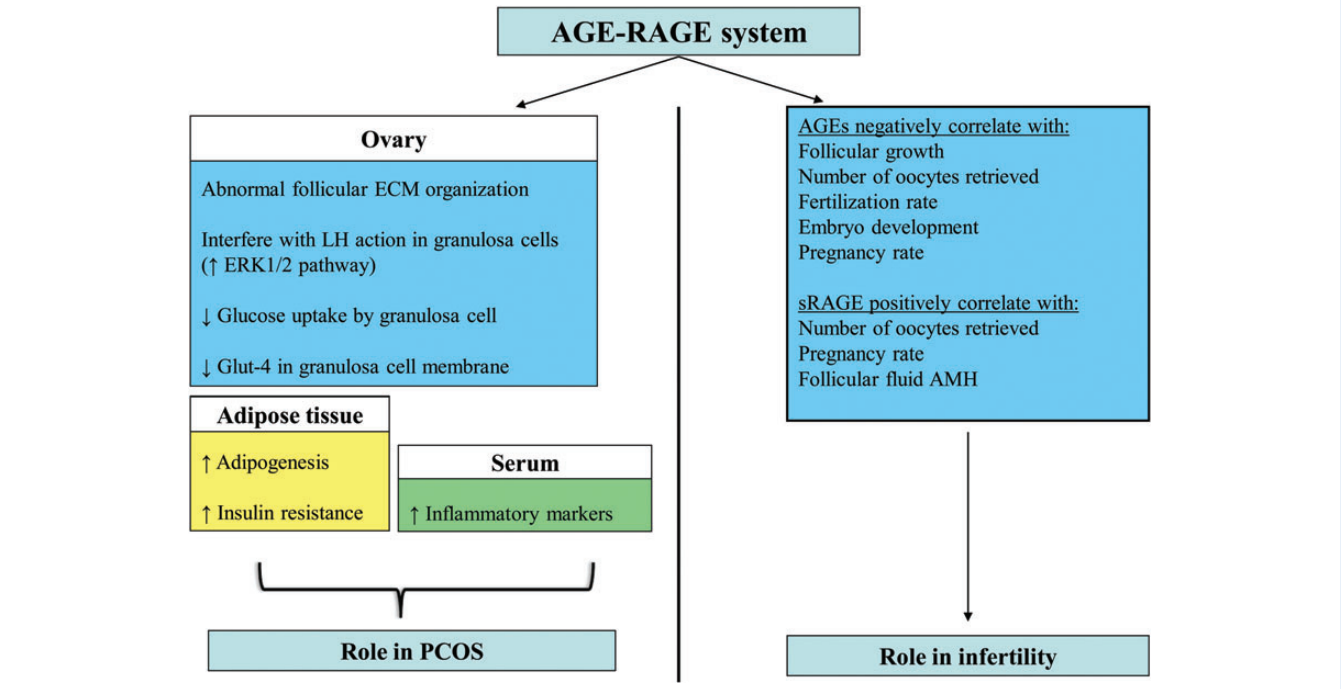

Figure 4 Relationship of the AGE-RAGE system with PCOS and infertility. In PCOS, there is an increase activity of this system at the serum, adipose tissue and ovary levels. In the ovary, AGEs cause: (1) abnormal ovarian ECM organization, (2) abnormal stimulation of the ERK1/2 pathway in granulosa cells altering LH action and (3) low glucose uptake by granulosa cells via lowering glucose transporters (GLUT-4) on granulosa cell membrane. In infertility, AGEs are negatively, while sRAGE are positively, correlated with ART outcome and measures of ovarian reserve, as reflected by AMH level. AGE, advanced glycation end product; RAGE, AGE receptor; GLUT-4, glucose transporter type4; PCOS, polycystic ovary syndrome; AMH, anti-Mullerian hormone.

(850 mg twice daily) for 6 months and determined serum AGE levels at baseline, and after 3 and 6 months of treatment. Their results indicated that serum AGE levels were significantly reduced in all groups at 6 months of treatment compared with baseline, but the percentage reduction was significantly greater in the metformin group compared with the OCP group. Although they showed that metformin may be superior to OCP in reducing serum AGE levels, this does not necessarily mean that metformin is better than OCP in alleviating the cardiovascular risk associated with PCOS. Whether metformin lowers cardiovascular risks in PCOS women via the AGE-RAGE system remains to be determined.

We (Ambroggio *et al*., 2013) and others (Malickova *et al.*, 2010; Fujii and Nakayama, 2010; Jinno *et al.*, 2011) have documented a relationship between the AGE-RAGE system and infertility. Jinno *et al*. (2011) measured the levels of toxic AGE (TAGE), pentosidine and CML in blood and follicular fluid of 157 patients undergoing IVF. They analyzed the association between these levels and assisted reproduction technology (ART) outcomes and pre-ART clinical factors. Their results revealed that the accumulation of TAGE, pentosidine and CML in the follicular fluid and TAGE in serum negatively correlated ($P < 0.05$) with follicular growth, fertilization and embryonic development. Lower concentrations of pentosidine in the follicular fluid and TAGE in the serum were the most significant predictors for achievement of ongoing pregnancy, acting independently of conventional determinants, such as age and Day 3 FSH level. Additionally, elevation of serum TAGE >7.24 U/ml appeared to indicate ovarian dysfunction causing diminished fertility, even at a young age (<40 years old) or with normal Day 3 FSH (below 10 IU/l). These data indicate that there is a clinical evidence for an important role of AGE accumulation in ovarian dysfunction and poorer outcome in women with elevated AGEs and undergoing IVF.

Another study (Malickova *et al.*, 2010) evaluated sRAGE levels in serum and follicular fluid of 33 women undergoing IVF. The control group of serum samples was collected from 35 healthy females. Their results indicated that follicular fluid sRAGE levels were several fold higher than serum levels ($P < 0.001$) suggesting an outflow of sRAGE to the follicular compartment in human ovaries. Additionally, it was found that serum levels of sRAGE in women after controlled ovarian hyperstimulation (COH) were significantly lower than in controls ($P = 0.045$). The authors also found a significant negative correlation between serum sRAGE levels and the number of stimulated follicles ($r = -0.71, P = 0.01$) and retrieved oocytes ($r = -0.54$, $P = 0.048$). Women in that study who conceived following IVF showed significantly higher sRAGE levels in the follicular fluid compared with women who did not conceive ($P = 0.031$). A similar study by Fujii and Nakayama (2010) evaluated follicular fluid and plasma sRAGE levels in 28 participants who underwent IVF and found a positive correlation between follicular fluid and plasma sRAGE levels and a borderline positive correlation between follicular fluid sRAGE and the number of collected oocytes ($r = 0.25$; $P = 0.05$). These data show that the decoy sRAGE, via binding circulating and follicular fluid AGEs, might be able to serve as a useful biological marker of the follicular environment.

We recently examined the relationship between the AGE-RAGE system and measures of ovarian reserve, specifically anti-Mullerian hormone (AMH) synthesis and release (Ambroggio *et al*., 2013). We therefore studied 34 women of reproductive age who underwent COH followed by oocyte retrieval for IVF. The objective of this study was to investigate the hypothesis that follicular fluid sRAGE concentration in women undergoing IVF is an indicator of ovarian reserve, as manifested by follicular fluid AMH and the number of oocytes retrieved. We collected cumulus granulosa cells then AMH and its receptor (AMHR-II) mRNA expression were quantified using RT–PCR, as previously described (Jayaprakasan *et al.*, 2010). Follicular fluid sRAGE and AMH protein levels were also measured by enzyme-linked immunosorbent assay. Our results showed that there was a trend for a negative correlation between sRAGE and age ($r = -0.34$, $P = 0.09$). The higher the follicular fluid sRAGE, the fewer IUs of gonadotrophin needed per cycle, independent of age, BMI and Day 3 FSH ($r = -0.4, P = 0.04$). Follicular fluid sRAGE positively correlated with number of oocytes retrieved ($r = 0.57$, $P = 0.02$). After adjusting for age, BMI, Day 3 FSH and IUs of gonadotrophin required, sRAGE predicted the number of oocytes retrieved ($R^2 = 0.27$, $P = 0.045$). Additionally, there was a positive correlation between follicular fluid sRAGE and follicular fluid AMH protein levels ($r = 0.5$, $P = 0.008$) while, in contrast, RT–PCR results showed no correlation between follicular fluid sRAGE and AMH or AMHR-II mRNA levels, suggesting that sRAGE has an effect on AMH release rather than AMH synthesis in granulosa cells. In brief, our data supported a positive relationship between sRAGE and ovarian response to COH, as manifested by oocyte quantity, and also suggested that sRAGE may relate to AMH protein levels, and hence may be a marker of better reproductive environment as well as reproductive potential The function of sRAGE in the follicular fluid is still unclear but it may reflect the activity of the AGE-RAGE system in ovarian follicles.

Altogether, the literature to date indicates that AGEs have a negative impact on reproductive outcome in women undergoing ART (Fig. 4). Elevated AGE levels in women without PCOS appear to be related to diminished ovarian reserve or functionally abnormal folliculogenesis. Whether plasma AGE measurement may facilitate early detection of diminished female fertility, when Day 3 FSH is still normal, is yet to be determined. The pathological significance of these harmful inflammatory AGE molecules in follicular health clearly requires further investigation. Targeting AGEs might offer potential therapeutic options for the treatment of diminished ovarian response.

Of note, because of its inflammatory character, a role of the AGE-RAGE axis on the endometrium, such as an effect on embryo implantation and menstrual irregularity, cannot be excluded. Studies on endometrial endometriotic tissue have shown that there is an elevated expression of RAGE when compared with healthy endometrial tissue (Sharma *et al*., 2010). This suggests that the AGE-RAGE system could, in part, be responsible for the inflammatory processes occurring in endometriosis. The role of AGEs at the endometrial level in normal and pathological conditions needs to be explored as data on this topic are not yet available.

Targeting the AGE-RAGE system

The mechanism of AGE formation is only partially understood, making it difficult to identify the precise chemical products responsible for *in vivo* damage and thus impeding the development of specific inhibitors. Direct intervention on the AGE-RAGE system might lead to new and more targeted therapeutic approaches. AGE inhibitors can be either synthetic or natural products (Peng *et al.*, 2011). This section will discuss some of the most commonly used inhibitors:

Inhibitors of AGE formation

Aminoguanidine is a well-established AGE inhibitor that has the ability to sequester the toxic 1,2-dicarbonyl compounds and render them into

non-toxic triazines (Desai and Wu, 2007; Carvalho *et al.*, 2011). Aminoguanidine prevents AGE formation by interacting with derivatives of early glycation products that are not bound to proteins (Desai and Wu, 2007; Carvalho *et al.*, 2011). In animal models with diabetes, aminoguanidine treatment increased arterial elasticity, decreased vascular AGE accumulation as well as the severity of atherosclerotic plaques and reduced accumulation of fibronectin and laminin in the extracellular membrane of streptozotocin-induced diabetic rats with diabetic nephropathy (Hu *et al.*, 2011). In PCO-like phenotype animals (D-galactose-induced animal model) with elevated serum AGEs, elevated serum AMH (possible serum indicator for PCOS) (Dewailly *et al.*, 2010) and elevated testosterone levels, aminoguanidine administration was able to reverse these hormonal imbalances with subsequent normalization in serum AMH and testosterone levels (Park and Choi, 2012).

Inhibitors of AGE absorption

A novel approach to reduce the deleterious effects of AGEs is to block their absorption with oral adsorbents (Ueda *et al.*, 2006; Yamagishi *et al.*, 2007), since many diets contain AGEs (as mentioned above). AST-120, an oral adsorbent, is able to bind AGEs, such as CML, and decrease their serum levels in diabetic and non-diabetic subjects with chronic renal failure (Ueda *et al.*, 2006; Yamagishi *et al.*, 2007). To date, there are no data pertaining to AGE absorption inhibitors and PCOS/infertility.

Anti-diabetic agent

Metformin, a medication already in use to treat diabetes, might have a positive impact on the AGE-RAGE pathway, beyond glycemic control (Diamanti-Kandarakis *et al.*, 2007a). Metformin prevented AGE-induced cell death and RAGE protein expression in osteoblastic cells in culture (Schurman *et al.*, 2008), and protected Schwann cells from apoptosis induced by MG (Ota *et al.*, 2007). In addition, the drug was able to reduce RAGE and lectin-like oxidized receptor 1 expression in endothelial cells exposed to high glucose levels or AGEs (Ouslimani *et al.*, 2007). The dimethylguanidine metformin is a glucose-lowering agent with antioxidant properties and a widely used insulin sensitizer in the management of PCOS (Eisenhardt *et al.*, 2006). Metformin acts as a moderate AGE inhibitor through its reaction with MG to form dihydroimidazolone, a less potent derivative (Diamanti-Kandarakis *et al.*, 2007a). In women with PCOS, treatment with metformin reduced AGE levels which, in the normotolerant group, occurred without significant changes in body weight and metabolic parameters (Diamanti-Kandarakis *et al.*, 2007a). These beneficial actions of the drug are likely to be due, at least in part, to the reduction of oxidative stress associated with metformin (Marchetti *et al.*, 2004).

Future research

Future research focusing on understanding the clinical effect of AGEs on germ cells, aged oocytes, mitochondrial dysfunction and the follicular microenvironment during different stages of development is needed. By focusing on the AGE-RAGE system as a potential therapeutic target for women with diminished ovarian reserve, an interaction between basic scientists and clinical researchers is vital in order to develop prevention and treatment modalities for age-related subfertility. Human granulosa cells collected from women who undergo IVF provide a valuable model for studying AGEs.

Conclusion

The AGE-RAGE system has been implicated in the pathogenesis of multiple metabolic diseases and more recently PCOS and infertility. This system has been targeted in PCO animal models with promising results at the level of the hormonal imbalances and granulosa cell dysfunction observed in this disease (Park and Choi, 2012). Clearly the application of AGEs (which are elevated in serum of women with PCOS) *in vitro* has a direct effect on granulosa cells by making these cells behave in a manner similar to those in women with PCOS. Hence, targeting the AGE-RAGE system could be an area of potential therapy with its possible reversal of the ovarian dysfunction observed in PCOS. Additionally, there is an increased awareness that the accumulation of AGE products at the level of the ovarian follicle might trigger early ovarian aging (Tatone and Amicarelli, 2013), which might be significant in infertile women with diminished ovarian reserve. This is supported by our recent findings of a positive correlation between follicular fluid sRAGE levels and AMH protein concentration. The potential accumulation of AGEs in the ovary may account for compromised efficiency of vascularization and for activation of oxidative stress response through interaction with cellular RAGE (Tatone *et al.*, 2008). Similar to sRAGE, follicular fluid AMH reflects ovarian health and constitutes a useful biological marker of the follicular environment (Hirobe *et al.*, 1992). Within follicles, AMH is expressed exclusively by granulosa cells with mitotic activity (Hirobe *et al.*, 1992, 1994) presumably because it interacts with mitogenic growth factors during follicle development (i.e. epidermal growth factor, transforming growth factor-b and IGF-I) (May *et al.*, 1988; Kim *et al.*, 1992). Therefore, a positive relationship between intrafollicular AMH and sRAGE concentrations further suggests that AGEs play a role in the inhibition of cellular proliferation or a role in enhancing granulosa cell apoptosis. It is unclear whether the AMH system has a role in the mechanistic effect of sRAGE on the number of oocytes retrieved. It is well known that AMH release inhibits, in a paracrine fashion, the depletion of the oocyte pool by slowing down growth followed by atresia of follicles containing the oocytes (Durlinger *et al.*, 2002). Thus, clearly a favorable interrelationship exists between sRAGE and AMH in the follicular environment. Altogether, the AGE-RAGE system could represent a potential therapeutic target in women with a diagnosis of diminished ovarian reserve undergoing ART. Finally, this review underscores a critical need to unveil the exact mechanistic actions of AGEs in reproductive physiology and, more specifically, the hypothalamic–pituitary–ovarian axis.

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