Running title: AGEs and insulin secretion

A**dvanced Glycation End Products (AGEs) are Cross-sectionally Associated with Insulin Secretion in Healthy Subjects.**

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Abstract

It has been postulated that chronic exposure to high levels of advanced glycation end products (AGEs), in particular from dietary sources, can impair insulin secretion. In the present study, we investigated the cross-sectional relationship between AGEs and acute insulin secretion during an intravenous glucose tolerance test (IVGTT) and following a 75g oral glucose tolerance test (OGTT) in healthy humans. We report the cross-sectional association between circulating AGE concentrations and insulin secretory function in healthy humans (17F:27M, aged 30±10 yrs) with a wide range of BMI (24.6 - 31.0 kg/m^2). Higher circulating concentrations of AGEs were related to increased first phase insulin secretion during IVGTT ($r= 0.43$; $p<0.05$) and lower 2-hour glucose concentrations during OGTT ($r=-0.31$; $p<0.05$). In addition, fasting ($r=-0.36$; $p<0.05$) and 2-hour glucose concentrations were negatively related to circulating levels of soluble

receptor for AGE (RAGE) isoforms ($r=-0.39$; $p<0.01$). In conclusion, in healthy humans, we show a cross-sectional association between advanced glycation end products and acute insulin secretion during glucose tolerance testing.

Abbreviations

AGE – Advanced glycation end product

HOMA-IR – Homeostatic model of insulin resistance

RAGE – Receptor for advanced glycation end products

IVGTT – Intravenous glucose tolerance test

OGTT – Oral glucose tolerance test

PCNA – Proliferating cell nuclear antigen

RSA –Rat serum albumin

BMI – Body mass index

Introduction

Insulin resistance and impaired insulin secretion the two major independent risk factors for type 2 diabetes (Weyer, Tataranni et al. 2001). Increased insulin secretion can develop as a primary defect or secondarily as a consequence of compensation for declining insulin sensitivity. In addition, abnormalities in insulin secretion play a central role in progression from impaired glucose tolerance (IGT) to type 2 diabetes (Pratley and Weyer 2002).

Diet represents an important environmental factor which can influence glycaemic control. Globalisation of the food chain has required changes in preparation techniques, which include the necessity to impart functional properties to food such as longer shelf life (Cordain, Eaton et al. 2005). Furthermore, increasing demands on family life and in some cases

socioeconomic pressures have increased the utilization of more rapid cooking methods and hence the intake of highly processed “fast food”. These changes have contributed to an increase in the consumption of foods containing increased amounts of advanced glycation end products (AGEs), which are important flavour and stabilization compounds in foods (Henle 2005). Studies have shown that there is uptake of a proportion of AGEs from the gut and despite efficient renal clearance, some AGEs are likely deposited in tissues (Vlassara, Striker et al. 1994, Miyata, Ueda et al. 1998, Hellwig, Geissler et al. 2009). Furthermore, there is preliminary evidence, primarily from animal models that increasing exposure to (Riboulet-Chavey, Pierron et al. 2006, Hagiwara, Gohda et al. 2009) or dietary intake of AGEs impairs insulin secretion and sensitivity (Hofmann, Dong et al. 2002, Sandu, Song et al. 2005, Coughlan and Forbes 2011, Forbes, Cowan et al. 2013). Excessive consumption of

saturated fat and glucose can also promote advanced glycation (Beisswenger, Delucia et al. 2005, Sandu, Song et al. 2005, Forbes, Cowan et al. 2013). In cross-sectional human studies, higher circulating concentrations of AGE modified proteins are associated with diabetes, renal or cardiovascular disease (Stam, Schalkwijk et al. 2006, Nin, Jorsal et al. 2011) and acute changes in insulin secretion following meal challenges (Vaaler, Hanssen et al. 1984, Rizkalla, Laromiguiere et al. 2007). Intervention studies specifically demonstrate that increased AGE intake is associated with insulin resistance (Birlouez-Aragon, Saavedra et al. 2010, Uribarri, Cai et al. 2011) and macrovascular dysfunction (Uribarri, Stirban et al. 2007, Stirban, Kotsi et al. 2012). Conversely we and others have shown that individuals who are obese have lower circulating AGEs concentrations (Sebekova, Somoza et al. 2009, Sourris, Lyons et al. 2013).

The receptor for advanced glycation end products (RAGE) is thought to bind to large ligands including AGE complexes (Tian, Avalos et al. 2007, Penfold, Coughlan et al. 2010) on cell surfaces and within the circulation (Zong, Madden et al. 2010) which has been associated with chronic disease (Schmidt, Yan et al. 1999, Yamamoto, Kato et al. 2001, Bierhaus, Haslbeck et al. 2004). We have recently shown that a chronic decline in the expression of RAGE is associated with a decline in insulin secretion and the development of experimental autoimmune diabetes and type 1 diabetes in children (Coughlan and Forbes 2011). In contrast however, some *in vitro* studies in isolated insulin producing cells lines have shown that acute exposure to AGEs can stimulate basal insulin secretion in the context of reduced glucose uptake (Kaneto, Fujii et al. 1996, Matsuoka, Kajimoto et al. 1997,

Uribarri, Cai et al. 2011). These disparities in the relationship between AGEs and insulin secretion may relate to the duration or degree of exposure to excess AGE concentrations. Polymorphism in the AGER gene which encodes for RAGE has also been demonstrated to associate with insulin resistance in humans (Sullivan, Futers et al. 2005). To further examine whether there is a relationship between circulating AGEs and insulin secretion, we performed a cross-sectional analysis of circulating AGE modified albumin and direct measures of insulin secretion in healthy humans.

Materials and Methods

Human Clinical Study

Volunteers were recruited (17 female, 27 male) between the ages of 18 and 50 years. Participants were non-diabetic, non-smokers at the time of the study and generally healthy according to data obtained from detailed physical examination, basic laboratory analyses (fasting plasma lipid levels, liver function tests, urea, creatinine and electrolytes). No participant had signs of acute or chronic infection, or took any medication or illicit drugs. Height and weight were measured and body mass index (BMI) calculated. The protocol was approved by the Alfred Hospital Ethics Committee and complied with the Declaration of Helsinki. All participants provided written informed consent prior to participation.

Metabolic testing in humans

At recruitment, an oral glucose tolerance test was performed after a 75g oral glucose load (OGTT) where glucose tolerance was determined by WHO 1999 criteria. For three days before further metabolic testing, participants were asked to abstain from strenuous exercise and caffeine intake. The first metabolic testing day in females took place in their follicular phase. Insulin sensitivity and secretion were assessed after a 12-h overnight fast on 2 separate days. A hyperinsulinemic-euglycemic glucose clamp was performed as previously described to assess insulin sensitivity (Sourris, Lyons et al. 2009) In brief, a primed continuous intravenous infusion of insulin (9mU/kg) was administered for 120 min at a constant rate of 40mU/m^2 body surface area per min. This infusion achieved plasma steady state insulin concentrations. Every 5 minutes during the clamp, plasma glucose was measured and the variable infusion of glucose adjusted in order to maintain blood glucose at a constant value of 5mmol/L. Finally, the

rate of total insulin stimulated glucose disposal (M; mg/kg/min) was calculated for the last 40 min of insulin infusion. In a subset of individuals (n=22; male 15: female 7), an intravenous glucose tolerance test (IVGTT) was then performed 7 days following the initial oral glucose tolerance test. The plasma increment of insulin at 3-5 minutes after the intravenous glucose bolus (25g) was calculated as area under the curve (AUC).

Biochemistry in Humans

Plasma glucose concentrations were measured by the glucose oxidase method (ELM 105, Radiometer Copenhagen, Denmark). Plasma insulin levels were measured by chemiluminescent microparticle immunoassay. Serum was collected for analysis of AGEs using an indirect ELISA as previously described (Coughlan and Forbes 2011). Soluble RAGE (sRAGE) and endogenous secretory RAGE (esRAGE)

were analysed in plasma samples using commercially available human sRAGE (R&D Systems, Minneapolis, MN, USA) and esRAGE ELISAs (B-Bridge International, USA) (Penfold, Coughlan et al. 2010).

Statistical Analyses

Statistical analyses were performed using SAS Jump Statistics Software (Cary, North Carolina, USA). Results are given as mean±SD (unless otherwise indicated). Correlations were performed using the Spearman correlation coefficient. Multiple regression analyses were used to assess the determinants of insulin secretion after adjusting for covariates. Statistical significance was assumed when $p<0.05$.

Results

AGEs and glucose metabolism in humans

All participants were healthy according to the physical examination i.e. they were normotensive, had normal full blood counts, liver and renal function tests (data not shown). The study participants were 30±10 years of age with an average BMI of 28±5 kg/m^2 (range: 24.6-31.4 kg/m^2) (Table 1) and a waist circumference of 93±13 cm. Male participants were more overweight but were more insulin sensitive than female participants (Table 1). Fasting plasma glucose concentrations were not different between males and females but were slightly elevated outside the normal clinical range (Table 1).

Two-hour plasma glucose concentrations during an OGTT were negatively related to increases in circulating AGE concentrations ($r=-0.31$; $p<0.05$) (Table 2). In the subgroup of

22 individuals where acute insulin secretion during IVGTT measurement was available, early insulin secretory function was positively related to AGE concentrations ($r=0.43$, $p<0.05$; Table 2). Circulating AGE concentrations were a significant determinant of early insulin secretory function independently of age, gender, BMI and waist circumference ($p=0.03$) and following additional adjustment for insulin sensitivity ($p=0.01$). Insulin sensitivity was not associated with serum AGE or circulating RAGE concentrations in this population (Table 2).

Circulating sRAGE and esRAGE concentrations were also inversely related to 2-hour plasma glucose concentrations during OGTT (Table 2). Fasting glucose was also negatively associated with circulating sRAGE concentrations (Table 2). There was a negative relationship between circulating sRAGE and fasting plasma insulin concentrations ($r=0.36$, $p=0.02$),

but no relationship with AGEs (r=0.23, p=0.1) and esRAGE (r=-0.22, p=0.2).

Discussion

Circulating AGEs associate with insulin secretion

We have demonstrated a novel positive association between circulating fasting concentrations of advanced glycation end products and acute insulin secretion during IVGTT in healthy humans. Furthermore, 2-hour plasma glucose concentrations during an OGTT were negatively related to increases in circulating AGE concentrations. Circulating concentrations of soluble and esRAGE were negatively associated with 2-hour glucose concentrations and esRAGE with acute insulin secretion during IVGTT. Interestingly, correction for adiposity did not affect these associations between higher circulating levels of AGEs and acute insulin secretion during IVGTT or 2 hour glucoses following OGTT.

Other *in vitro* studies (Uribarri, Cai et al. 2011, Puddu, Sanguineti et al. 2012) and our own chronic rat feeding of

AGEs for 24 weeks (Coughlan, Yap et al. 2011) have contrasted the present human study by suggesting that high concentrations of AGEs may impair glucose stimulated insulin secretion although these in vivo studies have been performed in lean male rats where there was no evidence of increased fat mass. Furthermore, in this previous in vivo study, we did not assess whether increases in AGEs firstly lead to enhanced compensatory insulin secretion at earlier time points following AGE exposure as might be seen in the compensatory phase of progressive injury to β-cells. Indeed, given that the individuals in the present study were healthy but many overweight, the association of AGEs with increases in insulin secretion may be indicative of hypersecretion of insulin in response to excess nutrient intake. Ultimately it is well understood that hyperinsulinemia due to compensation for increased obesity and insulin resistance, eventually progresses to a loss of first phase insulin secretion and type 2

diabetes. This may be why dietary restriction of AGE intake has shown greater insulin secretion in individuals with type 2 diabetes with prominent β-cell abnormalities in first phase insulin secretion during IVGTT (Uribarri, Cai et al. 2011).

Insulin resistance was not a determinant of AGE induced changes in insulin secretion

Advanced glycation end products have been previously reported to influence insulin sensitivity. Within this healthy population studied, insulin resistance as determined by gold-standard hyperinsulinaemic-euglycaemic clamp did not associate with circulating AGE concentrations. There are other previous studies which have shown a negative relationship between circulating AGE concentrations and insulin sensitivity measured by HOMA in obese humans, where AGE concentrations were found to be lower with obesity (Sebekova, Somoza et al. 2009, Chiavaroli, D'Adamo

et al. 2012). Furthermore, higher AGE concentrations were associated with higher acute phase insulin secretion in healthy but overweight humans in the present study, which could also be interpreted as improvements in β-cell function in the context of a glucose challenge. This discordance between circulating AGE concentrations, insulin sensitivity and glycaemic control warrants further investigation.

AGEs show an inverse relationship with circulating RAGE isoforms in overweight humans

Advanced glycation end products can exert their physiological actions via receptors such as RAGE. There have been a number of previous *in vitro* studies suggesting that interruption of RAGE signalling can increase insulin secretion and decrease excess formation of reactive oxygen species following treatment with AGEs in cell lines (Uribarri, Cai et al. 2011, Puddu, Sanguineti et al. 2012). In more chronic

progressive models however, it appears that eventually a decline in RAGE expression in pancreatic islets may be associated with impaired insulin secretion (Forbes, Soderlund et al. 2011). Each of these rodent models suggests, however, that more RAGE expression is associated with greater insulin expression and secretion, given that chronic models show a loss of insulin secretory capacity in the context of less RAGE expression. However, in our human population circulating isoforms of RAGE were increased in association with lower fasting and lower 2 hour plasma glucose levels during OGTT. In addition, acute insulin secretion on IVGTT was also negatively associated with esRAGE concentrations. Although surprising, these changes agree with the higher insulin secretion seen with increasing AGE concentrations observed in our overweight human population. The specific mechanisms by which this occurs however, remains to be fully delineated.

Conclusion

Taken together, these studies suggest that there is a relationship between circulating advanced glycation end products and insulin secretion in healthy humans, which could influence glycaemic control, but appeared to be independent of insulin resistance in this population. The association between AGEs and insulin secretion is likely to involve modulation of the AGE receptor, RAGE isoforms given their negative relationship with insulin secretion in the present study, although this remains to be fully ascertained in human interventional studies. Therefore changes in circulating AGE modified proteins and soluble RAGE as a result of numerous stressors such as consumption of processed diets, which may be high in AGE content, obesity and renal impairment may be risk factors for the development of early insulin hypersecretion and progressively, insulin secretory defects.

Indeed, changes in circulating and tissue AGE accumulation and/or their receptor RAGE, could therefore be risk factors for the development of type 2 diabetes *per se* which should be the subject of future studies in larger clinical cohorts.

Conflict of Interest Statement

There are no conflicts of interest, which exist for the data presented within this manuscript.

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TABLE 1: Anthropometric and metabolic parameters of study population

	All *N=44*	*Female* *N=17*	*Male* *N=27*
Age (years)	30 ± 10	30 ± 10	31 ± 10
Body Weight (kg)	84 ± 16	74 ± 12	90 ± 16*
Fasting plasma glucose (mmol/L)	4.8± 0.6	4.5±0.6	4.9± 0.5
2 hour plasma glucose OGTT (mmol/L)	5.1 ± 1.5	5.2 ± 1.5	5.1 ± 1.5
Insulin sensitivity - M (mg/kg/min)	9.0 ± 4.2	10.9 ± 4.6	7.8 ± 3.6*
Insulin secretion AUC 3-5 mins IVGTT(mU/L)	137± 68	103 ± 37	155 ± 75
Alkaline phosphatase (U/L)	68 ± 21	61 ± 15	72 ± 23*

Circulating AGEs (µmol/mol lysine)	860 ± 590 (227.0 - 1326)	829.8 ± 529.4 (239.8 - 1309)	882.8 ± 597.2 (221-1453)
esRAGE (ng/ml)	0.3 ± 0.1 (0.2-0.4)	0.31 ± 0.07 (0.26-0.34)	0.31 ± 0.11 (0.22-0.42)
Soluble RAGE (pg/ml)	944 ± 399 (672 - 1256)	894 ± 333 (624-1272)	978 ± 441 (675 - 1253)

Data are means ±SD, * p <0.05 male vs. female subjects Circulating AGEs, esRAGE and soluble RAGE are represented as means ±SD range is included in the brackets

Table 2: ***Pearson correlation coefficients.*** Relationships between circulating AGEs and

metabolic parameters in human participants. N=44 (apart from insulin secretion (n=22).

*P<0.05; **P<0.01

	Serum AGEs	**sRAGE**	**esRAGE**
Fasting glucose	-0.19	-0.36*	-0.20
2 hour glucose (OGTT)	-0.31*	-0.39**	-0.34*
Insulin sensitivity	0.21	-0.03	0.13
Insulin secretion (IVGTT AUC 3-5 mins)	0.43*	-0.28	-0.33*

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Table 1 ***Rodent physiological and metabolic parameters at week 4.***

Plasma glucose and glycated hemoglobin are included as measures of glycaemic control at the study end point (4 weeks).

	High AGE	**Low AGE**
Fasting Plasma Glucose (mmol/L)	7.0±0.8	7.1±1.0
Glycated Haemoglobin (%)	3.1±0.3	3.0±0.7
Body Weight (g)	458±22	439±31
Plasma AGEs (μmol/mol lysine)	253±74	250±80

Table 2: ***Pearson correlation coefficients.*** Relationships between circulating AGEs and metabolic parameters in human participants. N=44 (apart from insulin secretion (n=23).

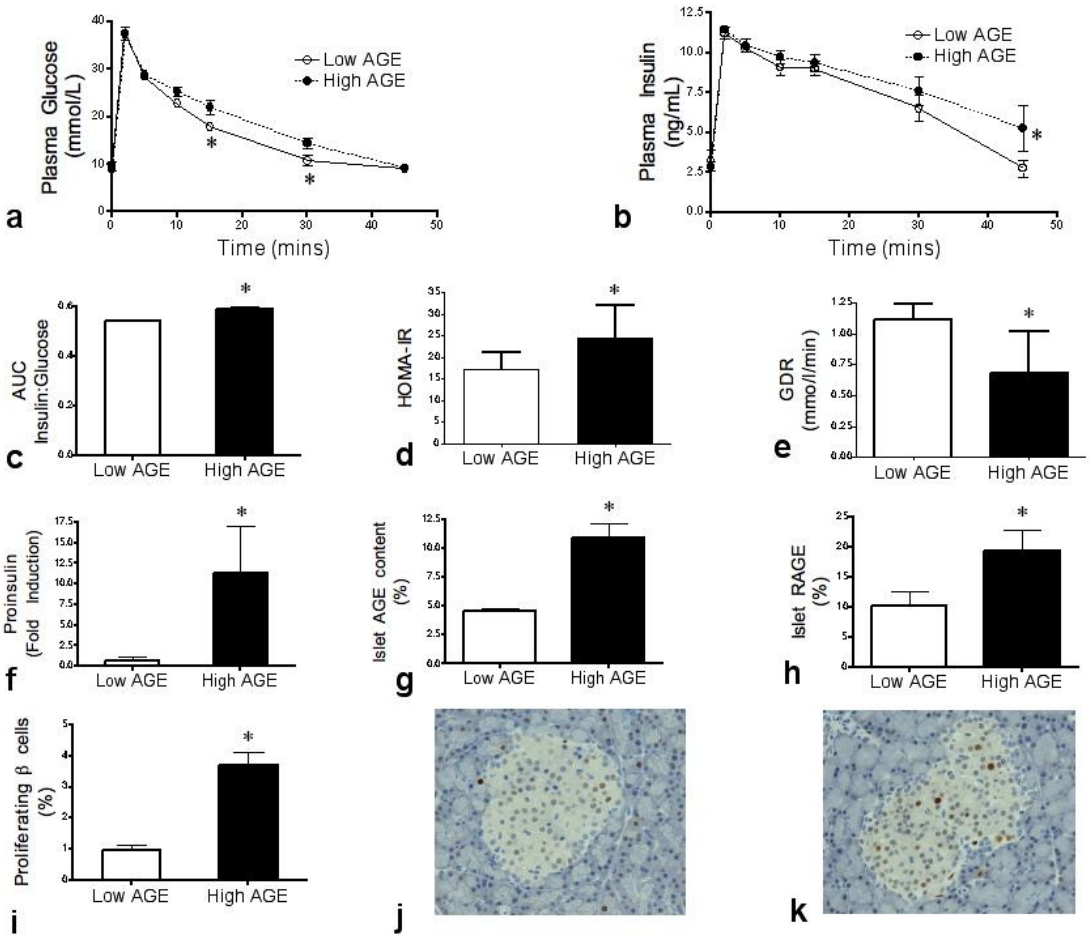
*P<0.05; **P<0.01

	Serum AGEs	**sRAGE**	**esRAGE**
Fasting glucose	-0.19	-0.36*	-0.20
2 hour glucose	-0.31*	-0.39**	-0.34*
Insulin sensitivity	0.21	-0.03	0.13
Insulin	0.43*	-0.28	-0.33*

secretion	

Figure 1: ***Insulin secretion and sensitivity following short term infusion of AGEs in rats.*** Groups of Sprague Dawley rats (n=10/group) were infused with 20mg/kg/day of AGE-RSA (High AGE) or RSA (low AGE) via subcutaneous minipump for 1 month. **a-c** Intravenous glucose tolerance testing parameters a) Plasma glucose concentrations; b) Plasma insulin concentrations; c) glucose disposal rate; d) HOMA-IR; e) AUC insulin:AUC glucose; f) Proinsulin gene expression; g) Islet AGE content; h) Islet RAGE content; **i-k** Islet proliferation using PCNA immunohistochemistry, g) Quantification; Representative Photomicrographs (x200) of h) Low AGE i) High AGE groups.

*P<0.05 vs Low AGE group.


a
Plasma Glucose (mmol/L)
Low AGE
High AGE
Time (mins)
b
Plasma Insulin (ng/mL)
Low AGE
High AGE
Time (mins)
c
AUC Insulin:Glucose
Low AGE
High AGE
d
HOMA-IR
Low AGE
High AGE
e
GDR (mmol/l/min)
Low AGE
High AGE
f
Proinsulin (Fold Induction)
Low AGE
High AGE
g
Islet AGE content (%)
Low AGE
High AGE
h
Islet RAGE (%)
Low AGE
High AGE
i
Proliferating β cells (%)
Low AGE
High AGE
j
k

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