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# SINGLE ORAL CHALLENGE BY ADVANCED GLYCATION END PRODUCTS ACUTELY IMPAIRS ENDOTHELIAL FUNCTION IN DIABETIC AND NONDIABETIC SUBJECTS

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**Short running title:** Dietary AGEs and endothelial dysfunction

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## ABSTRACT

**Objective:** The current study was designed to test the acute effects of dietary advanced glycation end products (AGEs) on endothelial function of diabetic and non-diabetic subjects.

**Research Design and Methods:** Flow-mediated dilatation (FMD) of the brachial artery and serum levels of AGEs, plasminogen activator inhibitor (PAI-1), vascular cell adhesion molecule (VCAM-1) and glucose were assessed before and after a single oral AGE challenge (~$1.8x10^6$ AGE U) in 44 diabetic and 10 non-diabetic subjects.

**Results:** The diabetic patients had higher baseline levels of serum AGEs (p=0.020), PAI-1 (p=NS) and VCAM-1 (p=0.033) and lower baseline values of FMD compared with non-diabetic subjects (p=0.032). Ninety minutes after a single oral AGE challenge, serum AGEs and PAI-1 levels increased and FMD decreased significantly in both healthy (AGEs: 7.2$\pm$0.5 to 9.3$\pm$1 U/ml, p=0.014; PAI-1: 5.4$\pm$0.4 to 6.8$\pm$0.4 ng/ml, p=0.007; and FMD: 9.9$\pm$0.7 to 7.4$\pm$0.9 %, p=0.019) and diabetic subjects (AGEs: 10.5$\pm$0.7 to 14.2$\pm$1 U/ml, p=0.020; PAI-1: 6.5$\pm$1 to 10$\pm$2 ng/ml, p=0.030 and FMD: 5.4$\pm$0.4 to 4.0$\pm$0.3 %, p=0.032). Serum glucose and VCAM-1 levels remained unchanged.

**Conclusions:** Significant increases in serum AGEs can occur together with altered clinical measures of endothelial dysfunction in diabetic and non-diabetic subjects following a single modest AGE-rich meal. Thus, repeated or chronic exposure to high AGE diets could over time lead to, and/or accelerate vascular disease.

## INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality among patients with diabetes mellitus (1,2). Elevated levels of oxidants such as advanced glycation end products (AGEs) are known promoters of high oxidative stress (OS) playing a significant role in the pathogenesis of diabetic CVD (3-5). While AGEs are better known as products of hyperglycemia, they are also abundant in standard Westernized diet (6-9). AGEs are especially elevated in dietary mixtures of proteins, lipids and sugars processed under elevated temperatures, as in broiling, roasting or grilling (6,7). Specific immunoassays provide practical and reliable means for the detection of representative types of AGEs that are present in human serum, urine, tissues, as well as in dietary products (8,10,11). Among the more frequently employed assays are immunoreactive probes for $N^{\varepsilon}$-carboxymethyl-lysine (CML) or methyl-glyoxal (MG) derivatives, serving as examples of dietary oxidants, shown to share the pro-inflammatory and pro-oxidant properties of the endogenous AGEs, based on in vitro studies (8) and animal models of CVD (12-14). The reduction of certain dietary AGEs has been associated with the prevention of CVD in mice (12-14), while in diabetic patients, their restriction has produced significant decreases in serum AGE levels, in parallel with a reduction in circulating vascular cell adhesion molecule (VCAM-1), tumor necrosis factor (TNF$\alpha$) and C-reactive protein (CRP), despite sustained hyperglycemia (15), strengthening the link between chronic exposure to exogenous AGEs, elevated OS and diabetic vascular disease.

Recently, we showed that the administration of a single full meal rich in AGEs to human subjects led to acute impairment of arterial vasodilatation in response to ischemia (FMD), a result interpreted as an early indication of endothelial dysfunction (16). A similar vasodilatory defect has been described after an oral load of glucose or fatty acids (17-19). Since the AGE-rich meal used in the above mentioned study (16) also contained carbohydrates and fatty acids it was difficult to distinguish a purely AGE-related effect on FMD. The current study was designed to further assess the hypothesis that dietary AGEs can acutely alter endothelial function in human subjects with or without pre-existing diabetes mellitus. An AGE-rich beverage free of carbohydrates or lipids or other known vasoactive substances was administered to diabetic as well as healthy subjects and its acute effects on arterial endothelial function were assessed, based on FMD in response to ischemia and circulating levels of VCAM-1 and plasminogen activator inhibitor (PAI-1). The findings were consistent with abnormal endothelial response to an oral AGE test in normal persons, and a worsening of pre-existing vascular dysfunction in diabetic subjects.

## PATIENTS AND METHODS

A group of 44 stable diabetic subjects and 10 healthy subjects underwent non-invasive measurement of brachial artery flow-mediated

dilatation (FMD) with simultaneous measurement of circulating levels of AGEs, PAI-1, VCAM-1 and glucose, before and after the ingestion of 300 ml of an AGE-rich test beverage. We chose to repeat measurements at 90 minutes based on preliminary pilot data. The diabetic patients had an average duration of diabetes of 15 $\pm$ 1.5 years (Table 1). There were 23 type 1 and 21 type 2 diabetic patients. Most of the patients had no clinical evidence of diabetic complications. Ten patients had diabetic retinopathy, three had peripheral artery disease and one had coronary artery disease. None of the patients had evidence of nephropathy, as determined by a lack of microalbuminuria or proteinuria and normal serum creatinine. Any patient with symptoms suggestive of diabetic gastroparesis was excluded. Medications, except for basal insulin in type 1 diabetic subjects, were not administered for 12 hours prior to the study to avoid potential interferences with the measured parameters. Medications included insulin (patient n=36), aspirin (n=10), ACE-inhibitors (n=6), statins (n=4), beta-blockers (n=3) and fibrates (n=1). Healthy subjects had normal renal function and had no clinical evidence of diabetes or CVD (Table 1).

Levels of AGEs in serum and in the test beverage were measured by ELISA using a monoclonal antibody against CML-KLH (4G9 mab, Alteon, Inc, Northvale, NJ) (8,10,11). Plasma samples were tested for PAI-1 and serum samples for VCAM-1 using commercially available ELISA kits (15, 20).

The oral AGE challenge beverage (300 ml) contained $1.8 \times 10^6$ AGE units, but neither carbohydrates nor lipids. This beverage was prepared from glucose and caffeine-free Coca Cola$^R$ light, which was concentrated 10 times by rotary evaporation at room temperature. Twelve subjects who had a drop of FMD greater than 20% with the test beverage accepted to be retested on a subsequent day, after replacing the AGE beverage with 300 ml of drinking water to exclude the potential influences of gastrointestinal distention and incretin release on FMD.

**Flow-mediated arterial vasodilation (FMD)**

FMD was assessed by measuring the response of brachial artery diameter to reactive hyperemia, as previously described (21). Endothelium-dependent dilatation was defined as the percent change in arterial diameter following reactive hyperemia, relative to the baseline diameter. Repeated measurements of recorded images showed a coefficient of variation of 0.22 ± 0.16%. The day-to-day variability of FMD investigated in 13 subjects was -0.05 ±1.6%, range: -3.1 to +2.44%.

Subjects presented to the testing unit in the morning after an overnight fast and measurements were performed immediately before, and 90 and 150 minutes after the ingestion of the AGE-rich beverage.

**Statistical analysis**

All data are given as mean $\pm$ SEM, unless otherwise stated. Differences of means between groups were analyzed by the paired or unpaired Student t test. All reported p values are based on two-sided tests. Statistical significant difference was

defined as a p value < 0.05. All data analysis was performed using the SPSS statistical program (SPSS 14.0 for Windows, Chicago, Illinois).

## Results

Diabetic subjects had higher fasting serum AGE (sAGE) levels, based on CML, (10.5 ± 0.7 U/ml versus 7.1 ± 0.5 U/ml, p=0.020), higher VCAM-1 (1407 ± 115 ng/ml versus 846 ± 158 ng/ml, p=0.033), PAI-1 (6.5 ± 0.8 ng/ml versus 5.4 ± 0.4 ng/ml, p=NS) and lower FMD (5.4 ± 0.4 % versus 9.9 ± 0.7 %, p=0.032) compared to healthy controls (Table 1). The administration of the oral AGE challenge to diabetic patients was followed at 90 min by a significant increase in serum AGE above the baseline (p=0.001; Figure 1A) and PAI-1 levels (p=0.028; Figure 2A). At 90 min there was also a significant decrease in maximal arterial dilatation following ischemia below the baseline (FMD) (p=0.000; Figure 3A). There were no significant changes in serum levels of glucose during this period (157 ± 16 mg/dl versus 153 ± 15 mg/dl, p=NS). Also, there were no significant changes in plasma levels of VCAM-1 (1407 ± 115 ng/ml versus 1252 ± 104 ng/ml, p=NS).

Similar significant changes in sAGE, PAI-1 and FMD were observed in the non-diabetic subjects 90 min after exposure to the same oral AGE challenge (Figure 1B, 2B, 3B). By 150 minutes, FMD values were at baseline (diabetics: 5.5 ± 0.5%; healthy: 9.6 ± 0.7%) although serum AGE levels (diabetics: 17 ± 2 U/ml; healthy: 13 ± 2 U/ml) and PAI-1 (diabetics: 11.7 ± 1 ng/ml; healthy: 7.3 ± 0.5 ng/ml) were still elevated.

There were no significant differences in the percent change of sAGE, PAI-1 or FMD between diabetic patients and healthy subjects. There were no significant associations between baseline levels of sAGE and either PAI-1 or FMD in diabetic patients or in healthy subjects.

In 12 diabetic subjects, who underwent repeated FMD testing with water, no significant arterial changes were noted between the baseline and the 90-minute points (0 min: 5.2 ± 0.7 % and 90 min: 5.6 ± 0.7 %) although they manifested a significant change in FMD when tested with the oral AGE challenge (0 min: 5.2 ± 0.6 % and 90 min: 3.08 ± 0.38; p=0.000).

## DISCUSSION

The evidence presented indicates that a single oral load of an AGE-rich beverage free of glucose or lipids to diabetic, as well as to healthy subjects is associated with a significant rise of serum AGE levels in parallel with an acute impairment of endothelial function, as reflected both by a decrease in arterial vasodilatation in response to ischemia and by an increase in circulating PAI-1 levels.

The significant decrease in brachial artery vasodilatation after ingestion of an AGE-rich beverage was similar to the impaired vasodilatation observed after an oral load of glucose or fatty acids (17-19). The AGE-rich beverage used in the present study did not contain glucose, lipids or other substances known to impede vascular dilatory capacity or to affect endothelial cell properties. These effects were therefore attributable to the AGE load ingested, which in addition to the prototype CML tested here, could also include other

AGE intermediates, known to mediate endothelial injury, i.e. glyoxal or methyl-glyoxal derivatives (22). The contribution of additional non-AGE chemical substances is also possible, although none has been known thus far. We have recently reported that the ingestion of a single solid AGE-rich meal is followed by a significant impairment of FMD, which can persist for six hours (16). The shorter duration of the vasodilatory impairment observed in the current study could reflect the absence of glucose and lipids, the involvement of different AGE compounds and their effects on vascular cells, or variable rates of absorption and elimination, relative to those ingested with a mixed meal (16). Alternatively, this could be due to the induction of a counter-regulatory hemodynamic response to the oral load, i.e. release of prostaglandins, a less likely explanation.

AGEs, whether endogenous or exogenously derived, have significant and direct effects on endothelial cells, including increased expression and release of VCAM-1 (23, 24) and decreased endothelial nitric oxide synthase (eNOS) (25, 26). Particularly noteworthy were the findings from the healthy subjects, the vascular endothelium of which seemed on the whole equally vulnerable to an acute AGE load as that of the diabetic patients. Indeed, the percent change of FMD values as well as PAI-1 and AGE levels were not significantly different in healthy subjects compared to diabetic. In the present study we did not investigate endothelium-independent vasodilation in response to nitroglycerin because the marked and often persistent vasodilation produced by this agent would have interfered with the second measurement of FMD, 90 min after the baseline test. However, this was addressed in our previous report in which endothelium-independent vasodilatation was not altered significantly after a single high AGE meal (16). This could be due to the time-limited impact on tissue AGE levels by a single load. Rather, acutely elevated circulating AGEs could alter endothelium-dependent response by decreasing NO production by endothelial cells (25, 26) or by inactivating NO (27), causing a decrease in NO bioavailability.

Based on the estimated daily AGE consumption by human subjects of 16 x $10^6$ AGE Eq/ day) (6), the amount of AGEs administered during the oral AGE test was similar to that of a regular meal**.** While a larger challenge might have provoked greater vascular dysfunction, the study was designed so as to provide a more physiologic window through which specific events and their significance could be observed. Because the consumption of similar amounts of AGEs is quite common in the general population, similar endothelial insults may occur repeatedly and often. Since endothelial dysfunction represents an early step in the development of atherosclerosis (21), repeated endothelial disturbances of the endothelial integrity may over time lead to CVD in healthy persons, or exacerbate pre-existing vascular disease in those with diabetes.

The diabetic patients in this study had high CML levels and established endothelial dysfunction, independently of the presence of

diabetic complications and despite an apparently good glycemic control. Thus impaired baseline FMD in diabetic subjects, even when under fair glycemic control may in part be due to the higher steady-state serum AGE levels, relative to that of healthy subjects, possibly involving both, endothelium-dependent and –non-dependent mechanisms. Higher AGE deposits in the subendothelial matrix of the diabetic patients could quench NO released by the endothelium and could account for the abnormal baseline values in this group (27). It has indeed been suggested that AGEs are more potent inhibitors of endothelial NO activity than high glucose (26).

The current data are consistent with previous findings demonstrating a rapid increase of serum AGE levels following a single oral AGE challenge in both diabetic and healthy subjects (28). Also, a direct association has been recently found in healthy human subjects between chronically ingested CML and circulating CML, other AGEs, i.e. MG-derivatives, and CRP (29). Moreover, circulating CML levels were associated with markers of oxidative stress, i.e. 8-isoprostane and insulin resistance (29). Of note, diet derived AGEs were found to be the best predictors of high serum AGEs (29). These studies indicate that, regardless of hyperglycemia, high serum AGEs, whether native or dietary in origin, could signal and/or contribute to alterations in oxidative stress and vascular cell inflammatory state, which could in turn subvert vascular wall function (29).

AGE-rich beverages, e.g. cocoa with or without sugar, which contains about 0.6 AGE Eq in a serving of 250 ml, (6) are often ingested with regular meals and together or independently can contribute to vascular injury. By comparison, low dietary AGE intake can reduce levels of serum AGEs and inflammatory markers in diabetic (15) or renal failure patients (20, 30). These findings strongly support the view that exogenous AGEs are not only important contributors to the body's AGE pool, but also a significant risk to the vascular integrity of both diabetic and healthy persons.

It has been determined that the content of AGEs in foods relates strongly to the amount of exposure to heat (6, 7) and that their toxic consequences may depend on a chronic pattern of consumption (29). The current report demonstrates that even a single, modest in size oral AGE challenge can provoke a significant vascular insult in both healthy persons and in diabetic subjects. It is therefore reasonable to conclude that, during recurrent ingestion of high AGE foods, multiple insults to the vasculature can result in persistent endothelial dysfunction and, over time, in overt vascular disease. Further studies are needed to dissect the mechanistic links between oral AGEs and diabetic or non-diabetic vascular disease and to establish methods for avoiding high dietary AGE intake as an effective non-pharmacological intervention for the diabetic, as well as the diabetes-prone population of today.

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**Table 1. Characteristics of the study population**

| Parameter | Diabetic subjects | Healthy subjects |
|---|---|---|
| **n** | 44 | 10 |
| **Male:Female ratio** | 36:8 | 5:5 |
| **Age (years)** | 50.5 ± 2 | 43 ± 4* |
| **BMI** | 25.7 ± 0.5 | 25 ± 2 |
| **HgbA1c** | 8.6 ± 0.3 | N/A |
| **Fasting blood glucose (mg/dl)** | 157 ± 16 | 100 ± 7* |
| **Serum creatinine (mg/dl)** | 0.8 ± 0.02 | 0.9 ± 0.02 |
| **Serum AGE (U/ml)** | 10.5 ± 0.7 | 7.1 ± 0.5* |
| **PAI-1 (ng/ml)** | 6.5 ± 0.8 | 5.4 ± 0.4 |
| **VCAM-1 (ng/ml)** | 1407 ± 115 | 846 ± 158* |
| **FMD (%)** | 5.4 ± 0.4 | 9.9 ± 0.7* |

* Statistical significant difference between diabetic and non-diabetic subjects ($p<0.05$)

## Figure legends

**Figure 1. Effect of an oral AGE-challenge test on serum AGEs (sAGE) in diabetic patients (A) and in healthy controls (B)**
Values were obtained at baseline and at 90 min after the ingestion of an AGE-challenge (300 ml) by 44 diabetic and 10 healthy subjects. Mean differences between baseline and 90 min are statistically significant ($p<0.05$).

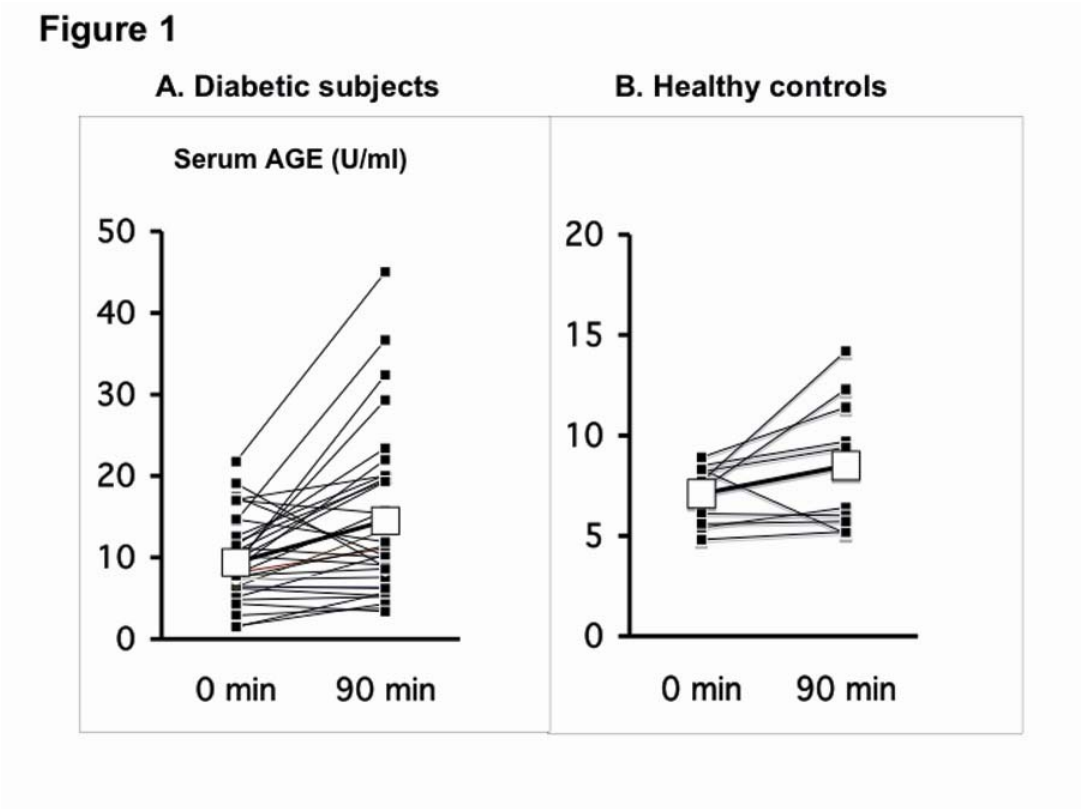

**Figure 2. Effect of an oral AGE-challenge test on circulating PAI-1 levels in diabetic patients (A) and in healthy controls (B)**

Values were obtained at baseline and at 90 min after the ingestion of an AGE-challenge (300 ml) by 44 diabetic and 10 healthy subjects. Mean differences between baseline and 90 min are statistically significant ($p<0.05$).

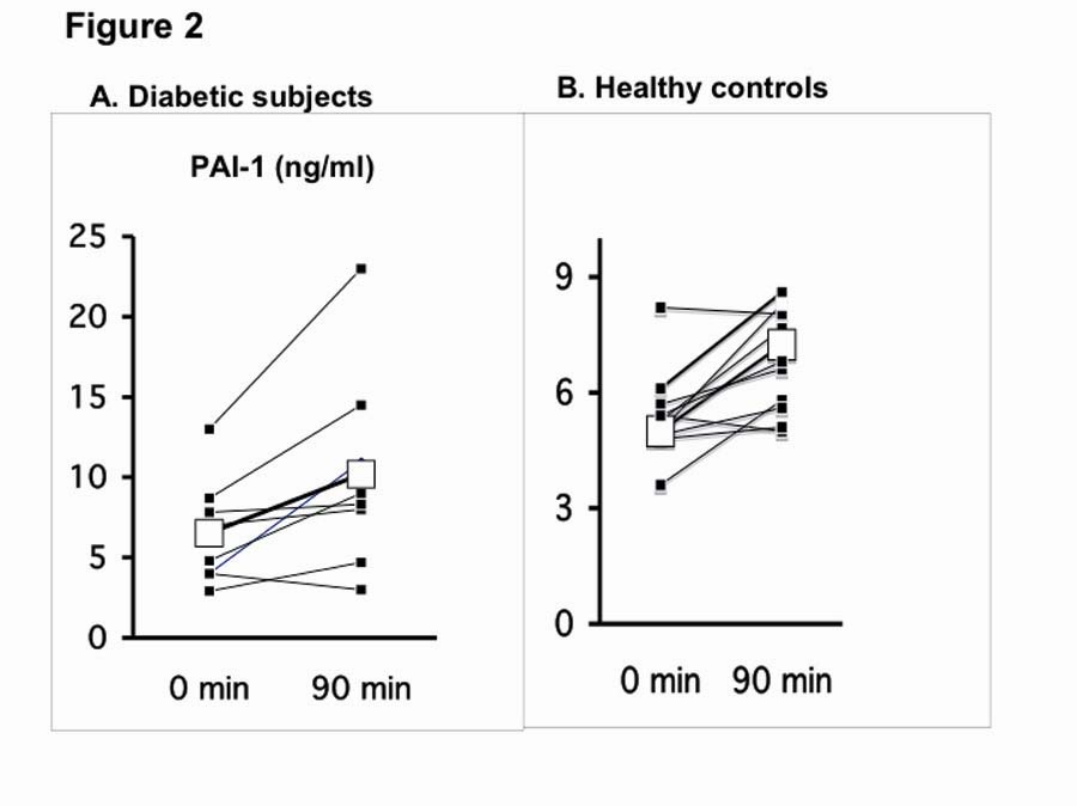

**Figure 3. Effect of an oral AGE-challenge test on flow-mediated vasodilation (FMD) in diabetic patients (A) and in healthy controls (B)**
Values were obtained at baseline and at 90 min after the ingestion of an AGE-challenge (300 ml) by 44 diabetic and 10 healthy subjects. Mean differences between baseline and 90 min are statistically significant ($p<0.05$).