Regular Article

Protective role of adiponectin on endothelial dysfunction induced by AGEs: A clinical and experimental approach

Serena Del Turco *, Teresa Navarra, Amalia Gastaldelli, Giuseppina Basta

CNR, Institute of Clinical Physiology, San Cataldo Research Area, Via Moruzzi, 1,56124 Pisa, Italy

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ABSTRACT

Objective: Obesity is characterized by low levels of adiponectin, an adipocytes derived hormone, and by an inflammatory component. Endothelial dysfunction is often found in overweight/obesity, diabetes, and atherosclerosis. Advanced glycation end products (AGEs) induce endothelial dysfunction and are linked to diabetes and increased atherogenicity and inflammation. The aim of the study was to investigate the possible link between adiponectin and N(epsilon)-(carboxymethyl) lysine (CML), the predominant adduct of circulating AGEs in overweight patients, and, in an in vitro model, to test the hypothesis that adiponectin acts as modulator of endothelial dysfunction, induced by AGEs.
Results: In 108 overweight patients, plasma levels of CML correlated inversely with adiponectin levels. Pre-incubation of human vein endothelial cells (HUVECs) with physiological concentrations of adiponectin, followed by stimulation with AGEs, reduced vascular adhesion molecule-1 (VCAM-1) and E-selectin expression, as assessed by surface enzyme immunoassay.
Conclusions: Taken together, these findings demonstrate an inverse correlation between CML and adiponectin levels in overweight patients and a protective role of adiponectin on endothelial dysfunction induced by AGEs, suggesting its key role in the treatment of the vascular complications of obesity/metabolic syndrome.

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Introduction

Adiponectin is an adipokine specifically secreted by adipose cells that plays an important role in the modulation of insulin sensitivity, inflammation, and endothelial function. Low plasma adiponectin concentrations are associated with higher prevalence of overweight/ obesity (Arita et al., 1999; Shimabukuro et al., 2003), insulin resistance (Fernandez-Real et al., 2004), type 2 diabetes (Li et al., 2009), hypertension (Adamczak et al., 2003), and atherosclerosis. Adiponectin modulates endothelial cell dysfunction induced by proinflammatory stimuli, countering the adverse cellular effects of both increased oxidative stress and cytokine stimulations (Ouchi et al., 1999).

Endothelial dysfunction has been proposed to be an early and pivotal event of atherosclerotic disease, providing an important common denominator between several diseases, such as hypertension, overweight/obesity (Meyers and Gokce, 2007; Pierce et al., 2008), and diabetes (Basta et al., 2004; Yonekura et al., 2005).

Advanced glycation end products (AGEs) are a heterogeneous group of compounds formed by nonenzymatic glycation of proteins and lipids, whose increased levels contribute to the development of vascular complications in diabetes, inducing endothelial dysfunction (Basta et al., 2002; Yonekura et al., 2005). Several mechanisms by which AGEs lead to endothelial dysfunction have been proposed, such as the accumulation of AGEs in the extracellular matrix, the binding of circulating AGEs to the endothelial surface receptor for AGEs (RAGE) with subsequent generation of reactive oxygen species (ROS), the cytokine release, and the expression of cell adhesion molecules (Basta et al., 2004).

Here, we have investigated the possible link between adiponectin and N(epsilon)-(carboxymethyl) lysine (CML), the predominant adduct of circulating AGEs, in a group of overweight patients, and tested the hypothesis that adiponectin may act as endogenous modulator of endothelial cell dysfunction, induced by AGEs and evaluated by the expression of adhesion molecules.

Methods

Subjects and blood collection

We analyzed plasma samples collected from 108 subjects without evidence of either cardiac, hepatic, or renal diseases that previously participated to another study (Basta et al., 2006). Subjects with a body mass index (BMI) >25 kg/m² were stratified according to glucose

tolerance evaluated by oral glucose tolerance test (OGTT; 75 g) being either normal (NGT; $n=39$) or impaired glucose tolerant (IFG and/or IGT; $n=27$) or with previous diagnosis of type 2 diabetes ($n=42$). Subject characteristics are shown in Table 1. Subjects are non-smoking, had normal serum creatinine (i.e., <1.2 mg/dl) and did not take any medication known to affect glucose tolerance. Hypertension was defined as blood pressure greater than 140/90 mm Hg. The study protocol was approved by the Institutional Review Board of the University of Pisa, and all subjects gave written informed consent to the study.

Blood samples were drawn in tubes without additives, containing heparin or citrate (for routine biochemistry). For CML and adiponectin determination, blood samples were collected in tubes containing Na_2EDTA and aprotinin, centrifuged at 4 °C and immediately divided in aliquots. Plasma and serum samples were stored at −80 °C until analysis.

Determination of plasma adiponectin levels

Plasma levels of total adiponectin were measured by enzyme immunoassay (LINCO Research Inc., Missouri, USA), according to manufacturer's instructions.

Determination of plasma CML levels

Plasma CML levels were measured in triplicate by an in-house competitive ELISA using the mouse F(ab′)2 fragment anti-AGE monoclonal antibody (clone 6D12) (ICN Biochemical Division, Aurora, OH), as described previously (Kaloudi et al., 2007).

Cell culture and experimental design

All experiments were performed in human umbilical vein endothelial cells (HUVECs), isolated, and grown as described (Massaro et al., 2002). Before the indicated treatments, the cells were made quiescent by replacing the growth medium with UltraCulture Serum-free Medium (BioWhitaker, Walkersville, Inc., USA) for 24 h. HUVECs were pretreated with recombinant globular adiponectin (gAcrp30) expressed in *Escherichia coli* (Peprotech, Inc., Rocky Hill, NJ) for 10 h and then stimulated with AGEs (containing about 80% CML-adducts), prepared as described (Basta et al., 2002), for 18 h and 10 h to induce VCAM-1 and E-selectin, respectively, as validated in our previous study (Basta et al., 2002). Cytotoxicity of treatment with adiponectin was tested as described in our previous
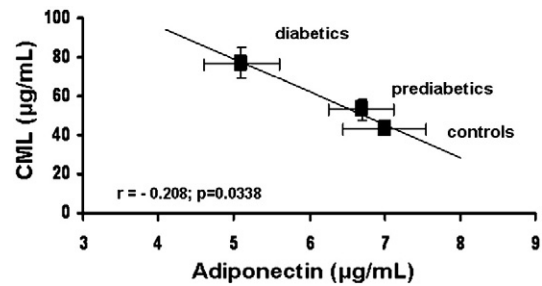

Fig. 1. Correlation between adiponectin and CML in control, prediabetic, and diabetic subjects. Data are expressed as mean ± SEM. In the insert is showed the partial coefficient of correlation (r) and the P-value of the entire group of study subjects.

study (Del Turco et al., 2007). All experiments were performed in triplicate.

Detection of cell surface adhesion molecules

Assay of cell surface molecules was performed by cell surface enzyme immunoassay as described by us previously (Basta et al., 2002; Lazzerini et al., 2009).

Statistical analysis

Data normally distributed are given as mean ± SD unless specified. HOMA has a skewed distribution and is expressed as median and interquartile range. Comparisons of continuous variables were compared by Student's t-test. A Pearson's correlation test was used to determine the relationship among variables. A multivariate analysis was used to evaluate the correlation between adiponectin and CML levels after adjustment for age, BMI, HOMA, and mean blood pressure (MBP). Results are given as partial correlation coefficients. Multiple differences were evaluated by analysis of variance (ANOVA) followed by Bonferroni post hoc test. Data were analyzed by StatView 5.0.1 software (SAS, Cary, NC, USA).
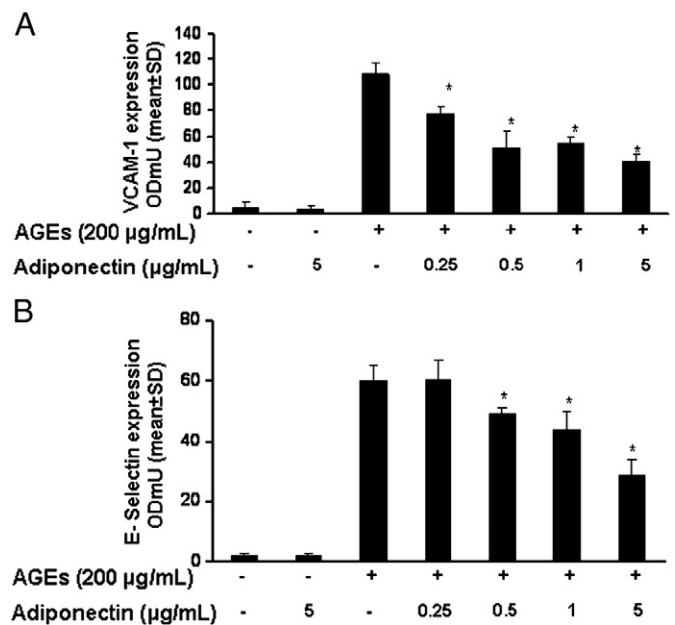
Table 1
Patient characteristics.

	Controls (N = 108)	Prediabetics (N=27)	Diabetics (N=42)
Gender (female/male)	14/25	4/23	13/29
Age (y)	41 ± 9	47 ± 9*	59 ± 5[§,†]
BMI (kg·m^{-2})	27 ± 3	28 ± 4	29 ± 3
HT (yes/no)	28/11	19/8	20/22
MBP (mm Hg)	93 ± 15	97 ± 14	96 ± 9
CML (µg/mL)	43 ± 21	53 ± 29	77 ± 52[**,§]
Adiponectin (µg/mL)	7 ± 3	6.7 ± 2	5 ± 3[*,**]
HOMA (mM • pM)	19 [4–41]	23 [3–65]	31 ± [8–105][§]
Fasting plasma glucose (mg/dl)	91 ± 6	107 ± 11[§]	149 ± 35[§,†]
Cholesterol (mg/dl)	186 ± 35	197 ± 33	201 ± 34
HDL (mg/dl)	46 ± 13	43 ± 9	50 ± 14[**]
Triglycerides (mg/dl)	94 ± 65	110 ± 65	126 ± 48[*]

HT indicates hypertension; BMI, body mass index; CRP, C-reactive protein; HOMA, homeostasis model assessment; MBP, mean blood pressure.
$P<0.05$ vs. control subjects, ** $P<0.05$ vs. prediabetic subjects, [§] $P<0.001$ vs. control subjects, [†] $P<0.001$ vs. prediabetic subjects.

Fig. 2. Effects of adiponectin on VCAM-1 and E-selectin expression. HUVECs were pretreated for 10 h with indicated amounts of adiponectin then treated with AGEs (200 µg/mL) for 18 h (A) and 10 h (B). Data are expressed as mean ± SD and are representative of three independent experiments. *$P<0.001$ vs. treatment with AGEs alone.

Results

Clinical results: correlation between adiponectin and CML plasma levels

The demographic characteristics of the subjects are shown in Table 1. Of 108 subjects, 39 were normal, 42 were diabetic, and 27 were prediabetic, as resulted by oral glucose tolerance test (OGTT).

Our results showed that adiponectin levels were inversely associated with insulin resistance [homeostasis model assessment (HOMA)] ($r = -0.286$; $p = 0.045$), high density lipoproteins (HDL) ($r = -0.307$; $p = 0.0022$), glucose ($r = -0.235$; $p = 0.0206$), triglycerides ($r = -0.228$; $p = 0.0252$) and, more interestingly, correlated inversely with CML levels in all subjects ($r = -0.208$; $p = 0.0338$), after adjustment for age, BMI, HOMA, and mean blood pressure (MBP). This correlation is well represented in Fig. 1, where the patients were divided into three groups: prediabetic, diabetic, and normal glucose tolerant subjects.

In vitro results: adiponectin inhibits the AGE-induced endothelial expression of adhesion molecules

AGEs (200 μg/mL) enhanced VCAM-1 and E-selectin expression as previously demonstrated by us (Basta et al., 2002). The pretreatment with physiological concentrations of adiponectin (0.25–0.5–1–5 μg/mL) for 10 h inhibited the endothelial surface expression of VCAM-1 (Fig. 2A) and E-selectin (Fig. 2B) in concentration-dependent manner. Adiponectin alone, also when the highest concentration was used, had no significant effect on adhesion molecule expression.

Discussion

In the present study, we provide evidence of an inverse relationship between adiponectin and CML levels in insulin resistant overweight subjects and we identify adiponectin as a modulator of endothelial dysfunction induced by AGEs.

Being overweight is the major risk factor for developing type 2 diabetes and cardiovascular disease. Many evidences suggest that insulin resistance and obesity are closely correlated to endothelial dysfunction and development of cardiovascular disease (Meyers et al., 2007; Pierce et al., 2008), but the underlying mechanisms remain in part unknown. In particular, the endothelial dysfunction in overweight/obese adults is related to increased abdominal visceral fat that contributes directly or indirectly to impaired endothelial function by releasing proinflammatory cytokines and adipokines, such as leptin, inhibiting adiponectin release and increasing oxidative stress (Williams et al., 2002). A relationship between hypoadiponectinemia and endothelial dysfunction has been previously shown in overweight patients (Shimabukuro et al., 2003), demonstrating that loss of adiponectin effects enhanced endothelial injury in obese people. Subjects with more elevated levels of adiponectin present a significant reduction of the risk of myocardial infarction (Pischon et al., 2004). In particular, decreased plasma adiponectin levels were related to the development of coronary artery disease (CAD), suggesting that its measurement may be helpful in assessment of CAD risk (Ouchi et al., 1999). However, it has been observed that the heart volume in obese mice overexpressing adiponectin, but metabolically healthy, was significantly larger than that of obese mice, suggesting that excess adiponectin leads to cardiomyopathy. Whether this effect is a direct effect of increased adiponectin levels or the result of the overall increase in weight is not known to date (Kim et al., 2007). The link between adiponectin and endothelial dysfunction in different pathological conditions may be also explained by its anti-atherogenic and anti-inflammatory properties (Ouchi et al., 1999; Ouchi et al., 2000; Plant et al., 2008). Data in vitro have demonstrated that adiponectin modulates the vascular response to lipid and inflammatory stimuli, such as TNF-α, inhibiting the activation of endothelial nuclear

transcription factor (NF-κB signaling), involved in the adhesion molecule expression (Ouchi et al., 2000), and the production of reactive oxygen species in hyperglycemic conditions (Ouedraogo et al., 2006).

Since our results show that, in overweight patients, there is an inverse correlation between adiponectin and CML levels, it is possible to speculate that the down-regulation of adiponectin, related to the presence of insulin resistance and obesity, may be a critical factor in the development of endothelial dysfunction induced by AGEs. This suggestion is also supported by our in vitro findings showing a protective effect of physiologically concentration of adiponectin on endothelial dysfunction induced by AGEs.

Endothelial dysfunction represents a key early step in atherogenesis, typically associated with decreased vasodilation and increased expression of vascular adhesion molecules (Libby, 2002), and AGEs, one of risk factors of endothelial dysfunction, are linked to increased atherogenicity and inflammation. It is known that AGEs induce the expression of endothelial adhesion molecule (Basta et al., 2002). Thus, interaction of AGEs with endothelial cells, as well as with other cells accumulating within the atherosclerotic plaque, provides a mechanism to augment vascular dysfunction.

Obesity has an inflammatory component, but interestingly, there are no studies on the relationship between AGEs levels and metabolic features of overweight/obesity.

It has been demonstrated that, in healthy subjects, plasma levels of soluble form of RAGE (sRAGE), a receptor which acts as a decoy for AGEs, were negatively correlated with BMI, and plasma sRAGE was significantly lower in overweight subjects free of diabetes compared to lean subjects supporting a possible protective role for this receptor before any evidence of diabetic or vascular complications (Norata et al., 2009).

A recent study has demonstrated a possible link between AGEs and obesity showing that low caloric diet reduces AGEs levels in obese Japanese men plausibly by a reduction in glycation/lipoxidation due to the caloric restriction or the decreased intake of food containing glycotoxins. Likewise, it has been demonstrated that a meal with high AGEs content decreases postprandial adiponectin and leptin levels (Stirban et al., 2008). In vitro data have shown that AGEs impair the leptin secretion (Unno et al., 2004). However, the details of the mechanisms involved remain unknown.

Our findings suggest that adiponectin exerts a protective effect against the endothelial dysfunction induced by AGEs, at least in part, by decreasing adhesion molecule expression, and provides direct evidence of the protective role of adiponectin in the pathogenesis of the vascular complications of obesity/metabolic syndrome. However, further investigation is needed to elucidate the intracellular signals by which physiological concentrations of adiponectin suppresses AGE-induced adhesion molecule expression, so to provide insight into therapies.

Acknowledgments

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