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Insulin and adiponectin inhibit the TNFα-induced ADMA accumulation in human endothelial cells The role of DDAH

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Abstract

Objective: Insulin and adiponectin exert important effects on the vasculature. We wanted to explore whether the eNOS inhibitor asymmetric dimethylarginine (ADMA) contribute to their effects.
Methods: Human umbilical vein endothelial cells (HUVECs) and human coronary artery endothelial cells (HCAECs) were incubated with growth medium in the presence or absence of tumor necrosis factor-alpha (TNFα), D-glucose, insulin or adiponectin. Further, cells exposed to TNFα for 24 h were co-stimulated with insulin or adiponectin for additional 24 h. Concentrations of ADMA in conditioned media and activity of dimethylarginine dimethylaminohydrolase (DDAH) in cell lysates were determined.
Results: The dose-dependent TNFα-induced ADMA accumulation was significantly inhibited when co-stimulated with insulin or adiponectin in both cell lines ($p < 0.01$ for all), accompanied by significant increases in DDAH activity in all conditions. Insulin alone resulted in a significant, but inversely dose-dependent accumulation of ADMA as compared to control cells in both cell lines, accompanied by increased DDAH activity. Adiponectin alone tended, dose-dependently to decrease ADMA, but without an increase of DDAH activity.
Conclusion: The results indicate that ADMA accumulation in human cultured endothelial cells is influenced by both insulin and adiponectin, and both mediators counteract the TNFα-induced accumulation of ADMA through the DDAH pathway.

Keywords: Adiponectin; ADMA; DDAH; Insulin; Human cultured endothelial cells

1. Introduction

There is growing evidence that endothelial dysfunction, reflected by an impaired nitric oxide (NO)-mediated vasodilatation [1], is an early step in the development of atherosclerosis [2]. The endogenous NO synthase (NOS) inhibitor asymmetric dimethylarginine (ADMA) is associated with reduced NO production and impaired endothelium-dependent vasodilatation [3]. Plasma ADMA concentrations have been found significantly increased in a variety of disease entities, including patients with chronic renal diseases and hypercholesterolemia [3–7]. Previous studies have demonstrated that ADMA concentrations are strongly associated with components related to the metabolic syndrome [7,8], in which the clustering of factors such as obesity, dyslipidaemia, hypertension and insulin resistance contributes to a substantial increase in the risk of cardiovascular diseases [9,10].

Hyperinsulinemia is an important component of the metabolic syndrome, and there is evidence that insulin *per se* has important direct effects on the vasculature, exerting both vasodilating and vasoconstricting effects [11,12]. How-

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ever, the exact mechanisms by which insulin exerts its effect on the vasculature are still not fully understood, and whether hyperinsulinemia contributes to endothelial dysfunction in insulin resistance remains debatable.

Adiponectin is secreted by adipocytes, and mimics several metabolic effects of insulin. Plasma concentrations of adiponectin are reduced in obese humans, and decreased concentrations are associated with insulin resistance and hyperinsulinemia [13]. In addition, adiponectin seems to have antiatherogenic properties, and decreased concentrations have been demonstrated in patients with coronary artery disease [14]. However, the direct relationship between adiponectin and endothelial dysfunction has not yet been fully explored.

Whereas glucose has been found to be stimulatory to ADMA accumulation in human endothelial cells [15], no such data exist with regard to insulin. The present study was therefore designed to investigate whether the formation of ADMA in cultured human endothelial cells is directly influenced by insulin or adiponectin in the ranges of human physiological concentrations. As plasma concentrations of insulin has been strongly correlated to ADMA concentrations in chronic insulin resistance states [8,16], but was shown to reduce ADMA concentrations during acute hyperinsulinemia [17], we hypothesised that insulin might exert either stimulating or suppressing effects on ADMA accumulation in cell cultures. Based on the strong association previously shown between ADMA and obesity, another hypothesis was that adiponectin would decrease the formation of ADMA. Our final hypothesis was that any effects of insulin and adiponectin on ADMA concentrations might be regulated by the activity of the degrading enzyme dimethylarginine dimethylaminohydrolase (DDAH).

2. Methods

2.1. Cell subculture preparation

Human umbilical vein endothelial cells (HUVECs), obtained from a caucasian female donor, and human coronary artery endothelial cells (HCAECs), obtained from a black male donor (Cambrex, Kerviers, Belgium, for both) were cultured according to the manufacturer's instructions and aliquoted in batches for use in the respective experiments. Briefly, the endothelial cells were seeded at a density of 5000 cells/cm^2 and grown in endothelial basal medium (Cambrex) supplemented with human recombinant epidermal growth factor, hydrocortisone, human fibroblast growth factor basic with heparin, human vascular endothelial growth factor, human recombinant insulin-like growth factor, ascorbic acid, gentamicin, amphotericin and fetal bovine serum (10%) until 80–90% confluence (3–4 days) in a humidified atmosphere (5% CO_2, 37 °C). After detachement with trypsin/EDTA (Cambrex), the endothelial cells were counted in a Bürker cell chamber and the viability was determined by means of trypan blue staining, final concentration 0.2% (Sigma Chemicals Co., St. Louis, MO, USA) and was regularly >90%.

2.2. Study protocol

For the experiments, the HUVECs and the HCAECs were obtained on sixth and seventh passage, respectively. The cells were passaged into 12-well culture dishes at a density of 4×10^4 cells/mL, and incubated for 24 h until 70–80% confluence. The cells were washed twice with phosphate buffered saline water (PAA Laboratories GmbH, Austria) and replaced with fresh growth medium (1 mL) in the absence (control) or presence of tumor necrosis factor-alpha (TNFα, 1.0 ng/mL) (Sigma) (the latter serving as positive control [18]), D-glucose (5.0, 15.0 or 30.0 mM) (Merck, Darmstadt, Germany), insulin (0.1, 1.0 or 10.0 nM) (Actrapid®, Novo Nordisk, Bagsværd, Denmark) or adiponectin (10.0 or 20.0 μg/mL) (R&D Systems Europe, Abingdon, UK) for 24 or 48 h. To further evaluate the influence of insulin and adiponectin, cells were exposed to TNFα (1.0 ng/mL) for 24 h and thereafter to insulin (0.1, 1.0 or 10.0 nM) or adiponectin (10.0 or 20.0 μg/mL) for additional 24 h. These procedures were repeated on three separate occasions in duplicates. At the end of the 24 or 48 h incubation period, the conditioned media and the cell lysates in 1 mL H_2O were saved for further measurements.

2.3. Total protein determination

The total cellular protein concentration was measured using BCA protein assay kit (Pierce, Bonn, Germany).

2.4. ADMA determination

Concentration of ADMA in the conditioned medium from all experiments in both cell lines was measured by high performance liquid chromatography and precolumn derivatization with *o*-phthaldialdehyde, as previously described [7].

2.5. DDAH activity assay

The DDAH activity in HUVECs and HCAECs was estimated by directly measuring the amount of ADMA metabolized by the enzyme [15]. On ice, cell lysates were divided into two aliqotes, and ADMA added to a final concentration of 500 μM. To inactivate DDAH, 5-sulphosalicylic acid (5-SSA, 30%) (Sigma) was immediately added to one part, thus providing baseline (0%) DDAH activity. The second lysate was incubated at 37 °C for 2 h before addition of 5-SSA (30%). The ADMA concentration in each aliqote was measured as described above. The difference in ADMA concentration between the two lysates reflects the DDAH activity. DDAH activity in the control cells was defined as 100% and the DDAH activity under the different conditions