Peptides 63 (2015) 150–155

Contents lists available at ScienceDirect

Peptides

journal homepage: www.elsevier.com/locate/peptides

Evaluation of salivary adiponectin profile in obese patients

E. Nigro[a], P. Piombino[b], O. Scudiero[a,c], M.L. Monaco[a], P. Schettino[d], A. Chambery[e,f], A. Daniele[a,e,*]

[a] CEINGE-Biotecnologie Avanzate Scarl, Via Salvatore 486, 80145 Napoli, Italy
[b] Dipartimento di Agraria, Università degli Studi di Napoli "Federico II", Portici, Italy
[c] Dipartimento di Medicina Molecolare e Biotecnologie Mediche, Università di Napoli Federico II, Napoli, Italy
[d] Divisione di Oncologia Chirurgica, "F. Magrassi-A. Lanzara" Dipartimento di Medicina e Chirurgia Clinica e Sperimentale, Seconda Università degli Studi di Napoli, 80131 Napoli, Italy
[e] Dipartimento di Scienze e Tecnologie Ambientali Biologiche Farmaceutiche, Seconda Università degli Studi di Napoli, Via Vivaldi 42, 81100 Caserta, Italy
[f] IRCCS Multimedica, Milan, Italy

ARTICLE INFO

Article history:
Received 20 October 2014
Received in revised form
24 November 2014
Accepted 24 November 2014
Available online 4 December 2014

Keywords:
Adiponectin
HMW oligomers
Super HMW
Obesity
Saliva
Biomarkers

ABSTRACT

Obesity is a chronic inflammatory disease significantly risen worldwide, especially among children. Adipokines, secreted from adipose tissue, are hormones involved in various cellular processes such as energy metabolism and inflammation. Among the others, adiponectin is gaining increasing interest for its insulin-sentitizing, anti-atherogenic and anti-inflammatory properties. This adipokine undergoes different post-translational modifications, after which it circulates as oligomers of high, medium and low molecular weight (HMW, MMW, LMW); HMW are the most biologically active oligomers. Serum adiponectin levels as well as the amount of its oligomers are inversely correlated to BMI and closely associated with obesity and related diseases. In this study, we analyzed total adiponectin expression and its oligomeric profile in saliva samples from 27 obese compared to 27 age- and sex-matched controls. Moreover, we compared adiponectin oligomerization between serum and saliva samples. The analysis of the different adiponectin oligomers reveals a slightly higher expression of total, HMW and LMW salivary adiponectin in obese patients compared to controls. Finally, FPLC analysis evidenced that HMW oligomers in saliva have a higher molecular weight than in serum confirming the presence of more complex oligomers in saliva, previously identified as super HMW (S-HMW). Saliva is considered a potential source of novel biomarkers for the diagnosis of metabolic disorders. The assessment of total adiponectin and its oligomeric profiles in saliva samples may represent a promising biological marker for the analysis of metabolic diseases.

Introduction

Obesity, one of the most common chronic inflammatory diseases, has reached epidemic proportions globally, with an increasing incidence among children [21]. Obesity represents a risk factor for the development of type 2 diabetes and cardiovascular diseases, pathologies significantly associated to high mortality [2,4,27,11]. The increase of fat mass is associated with altered expression of various adipokines, hormones involved in lipid and glucose metabolism, inflammation, and atherosclerosis [20]. Among the others, adiponectin is an adipokine gaining increasing interest for its insulin-sensitizing, anti-inflammatory and anti-atherogenic effects [13,14]. Adiponectin levels are inversely correlated to BMI and insulin sensitivity, and are closely associated with obesity, metabolic syndromes, coronary heart disease, and lung diseases [7,8]. Adiponectin is produced as a monomer of 28 kDa that, through several post-translational modifications, self-assemblies as oligomers of different molecular weight: high molecular weight (HMW), medium molecular weight (MMW), and low molecular weight (LMW) [6,13,18]. There is a great interest about the oligomeric state of adiponectin since it affects its biological activity [24]. In particular, *in vitro* studies revealed HMW as the most biologically active oligomers while *in vitro* studies demonstrated that HMW are more strongly associated with metabolic disorders representing a very useful diagnostic marker [12]. The pathologic correlation between HMW levels and metabolic diseases sustains research in this field. In fact, HMW oligomers have been shown to possess insulin-sensitizing and anti-atherogenic properties [12]. Adiponectin acts through

* Corresponding author at: CEINGE-Biotecnologie Avanzate, Via Gaetano Salvatore, 486, 80145 Napoli, Italy. Tel.: +39 081 3737856; fax: +39 081 3737808.
E-mail address: aurora.daniele@unina2.it (A. Daniele).

http://dx.doi.org/10.1016/j.peptides.2014.11.007
0196-9781/

two receptors (AdipoRs) widely expressed in several organs, tissues and cell lines [13,14,30,31]. A third receptor, T-cadherin, has a crucial role representing a storage depot for adiponectin [31]. Therefore, adiponectin and HMW oligomers are considered valuable biomarkers in obesity and metabolic disorders [21].

As a diagnostic fluid, saliva offers several advantages over blood; saliva collection is non-invasive and therefore stress-free for patients, especially for children [5,10]. Actually, researchers have attempted to find early molecular biomarkers of obesity and abnormal proteins have been identified in patient bodily fluids but very few studies have evaluated salivary adiponectin levels as a possible new diagnostic marker [1,3,16,17,19,26–30]. Recently, the presence of adiponectin and of very high molecular weight oligomers (S-HMW) have been identified [13]. In the present study, we analyzed total adiponectin expression and its oligomeric profile in saliva samples of obese patients compared to control subjects. Moreover, we compared adiponectin oligomerization profile between saliva and serum samples from obese patients with respect to controls, by using western blotting and FPLC analysis.

Materials and methods

Subjects recruitment, serum and saliva samples

Two-seven severely obese subjects and 27 control subjects were recruited from San Giovanni Bosco Hospital (ASL NA1, Naples, Italy), among bariatric surgery candidates; all subjects were aged between 20 and 68 years; obese patients with type 2 diabetes mellitus were not included in the study. Underweight (body mass index, BMI < 18.5) and overweight/pre-obese ($25 \geq$ BMI ≤ 29.9) candidates were excluded. BMI, kg/m^2 categories are defined according to the World Health Organization guidelines (http://www.who.int/bmi). Forty-eight healthy age-matched volunteers were recruited as lean controls. Participants were instructed not to consume food and beverages for at least 2.5 h prior to 10:30 a.m. Water drinking was allowed before the saliva collection started. Then, subjects were asked to wash teeth (new toothbrush and toothpaste were provided and were the same for all donors) and finally to rinse mouth several times with tap water to avoid any contamination from food/beverage residues and/or toothpaste flavorings. After 1.5 h from teeth brushing (12:00 a.m.), the collection of whole resting saliva was started. Saliva samples were shortly centrifuged at 13,200 rpm. Participants were instructed as previously described [23]. The anthropometrical features of the patients and controls are shown in Table 1. Before each assay, saliva samples were centrifuged at 13,200 rpm. Ten sera samples of severely obese subjects and ten sera samples of control subjects were recruited as previous described [8]. The biochemical and phenotypical characteristics of sera from obese patients and controls are from De Rosa et al. [8]. Both samples were processed in the following procedure: sterile serum and saliva samples were recruited and maintained on ice, immediately under sterile hood aliquoted in small volumes and kept frozen (−80 °C) until analysis. The study, approved by the local Ethics Committee, was conducted in accordance with ethical principles stated in the most recent version of the Declaration of Helsinki. The informed consent was obtained from each subject prior to inclusion in the study.

Table 1
Anthropometric features of study participants. *n*: number of donors.

Antropometric features	Obese patients (27)	Controls (27)
Sex	Male ($n = 27$)	Male ($n = 27$)
Age (years)	21–29 ($n = 9$)	20–27 ($n = 10$)
	30–36 ($n = 4$)	30–39 ($n = 7$)
	42–47 ($n = 7$)	40–48 ($n = 6$)
	52–58 ($n = 5$)	51–52 ($n = 3$)
	64–68 ($n = 2$)	60 ($n = 1$)
Normal weight ($18.5 \leq$ BMI ≤ 24.9)		($n = 27$)
Obese class I ($30 \leq$ BMI ≤ 34.9)	($n = 7$)	
Obese class II ($35 \leq$ BMI ≤ 39.9)	($n = 4$)	
Obese class III (BMI ≥ 40)	($n = 16$)	
Smokers	($n = 10$)	($n = 9$)

From Ref. [23].

Measurement of adiponectin concentration by ELISA test

Total saliva concentration of adiponectin was measured by enzyme-linked immunosorbent assay (ELISA) utilizing a polyclonal antibody, in house produced, *versus* a human adiponectin sequence region (H_2N-ETTTQGPGVLLPLPKG-COOH) as previously reported [7]. Calibration curve was performed and quantified by human recombinant adiponectin from Phoenix Pharmaceuticals, CA used as a standard. Each sample was diluted 1:10 and assayed three times in triplicate.

Western blotting analysis

Total protein content was quantified in saliva and sera samples by Bradford's method (Bio-Rad, Hercules, CA, USA) and 5 μg of total proteins were treated with 1× Laemmli buffer, heated at 95 °C for 10 min and loaded under non-reducing conditions on 10% SDS-PAGE gel and western blotting analyses were performed as previously described [8]. The blots were developed by ECL (Amersham Biosciences, NJ, USA) with the use of Kodak BioMax Light film, digitalized with a scanner (1200 dpi) and analyzed by densitometry with the ImageJ software (http://rsbweb.nih.gov.ij/). Each sample was tested three times in duplicate.

Gel filtration analysis

The oligomeric distribution of adiponectin in saliva and serum samples was analyzed by gel filtration chromatography on a Superdex 200 10/300 GL column connected to a fast protein liquid chromatography system (Amersham Pharmacia Biotech, Uppsala, Sweden). In detail, for saliva samples, about 270 μg of total proteins contained in about 300 μL were fractionated at 0.5 mL/min using a 100 mmol/L PBS, pH 7.4 elution buffer; for sera samples, about 1875 μg of total proteins contained in 300 μL were fractionated at 0.5 mL/min using a 100 mmol/L PBS, pH 7.4 elution buffer. The column was calibrated using ferritin (440 kDa), aldolase (158 kDa) and ovalbumin (44 kDa) (GE Healthcare). Moreover, Blue Dextran 2000 was used to determine the void volume of the column (GE Healthcare). This analysis was performed on 10 controls and 10 obese patients. Fractions (250 μL) were collected and the occurrence of adiponectin oligomers was tested using western blotting analysis.

Statistical analysis

Data are expressed as means ± SE. Two groups were compared by the two-tailed unpaired Student's *t*-test and Mann–Whitney *U*-test. Multiple comparisons were evaluated by one-way analysis of variance (ANOVA). The statistical significance was established at $p < 0.05$. All statistical analyses were performed using the Stat View software 5.0.1.0.