CLINICAL STUDY

Decreased concentration of adiponectin together with a selective reduction of its high molecular weight oligomers is involved in metabolic complications of myotonic dystrophy type 1

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Abstract

Objective: The hormone adiponectin exerts beneficial pleiotropic effects on biological and metabolic processes. Although a well-recognized insulin sensitizer, its characteristic has yet to be clearly defined. Myotonic dystrophy type 1 (DM1) is a rare genetic disorder that features muscle wasting and metabolic comorbidity, and patients have an increased risk of developing type 2 diabetes. We analyzed circulating levels of adiponectin and its oligomers to determine whether their expression correlates with metabolic alterations in DM1 patients.

Design and methods: We measured the anthropometric and biochemical features and three insulin resistance (IR) indices (homeostasis model assessment, quantitative insulin sensitivity check index, and McAuley) of 21 DM1 patients and of 82 age-, sex-, and weight-matched controls. In the blood samples of patients and controls, adiponectin levels were measured by ELISA, and its oligomers were characterized by using western blotting and gel filtration. The adiponectin gene was molecularly analyzed in patients.

Results: DM1 patients had significantly higher body mass index, waist circumference, triglycerides (TGs), glucose, tumor necrosis factor α , and IR; conversely, they had significantly lower concentrations of total serum adiponectin with a selective, pronounced decrease of its high molecular weight (HMW) oligomers. There was a strong negative correlation between adiponectin and TGs in DM1 patients.

Conclusions: Our results endorse the hypothesis that decreased expression of adiponectin together with a selective reduction of its HMW oligomers contributes to the worsening of IR and its metabolic complications in DM1 patients. These findings suggest that adiponectin and HMW oligomers may serve as biomarkers and are promising therapeutic agents for IR and its consequences in DM1.

European Journal of Endocrinology 165 969–975

Introduction

Adiponectin (ACRP30 or ADIPOQ) is a secretory protein exclusively synthesized by adipose tissue. Owing to its anti-inflammatory, antiatherogenic, and antidiabetic properties, ACRP30 exerts beneficial and protective effects on energy metabolism and cell differentiation and proliferation (1). Low concentrations of serum ACRP30 are related to two metabolic disorders, namely obesity and insulin resistance (IR) (1, 2). Moreover, low ACRP30 levels have been implicated in cancer pathogenesis (3). Single-nucleotide polymorphisms (SNPs) and adiponectin gene (*ACDC* or *ADIPOQ*) haplotypes have been associated with obesity, type 2 diabetes mellitus (T2DM), metabolic syndrome, and coronary artery disease (CAD) (2, 4, 5).

The effects of ACRP30 are mediated by ADIPOR1 and ADIPOR2 receptors, which are ubiquitous, although the former is mainly expressed in skeletal muscle and the latter reaches its highest levels in the liver (4). ACRP30 is a protein secreted in serum at high levels that vary depending on sex, body fat distribution, and metabolic status (1, 2, 4). A striking feature of ACRP30 is the ability to assemble in various oligomeric complexes, namely trimers (low molecular weight (LMW)), hexamers (medium MW (MMW)), and oligomers larger than hexamers (high MW (HMW)) (1, 4, 6). The state of ACRP30 oligomerization is critical for its signal transduction pathway and for the regulation of its biological activities (1, 4, 6). A large body of evidence indicates that HMW oligomers are the main bioactive

forms that mediate ACRP30's insulin-sensitizing effects and several metabolic abnormalities. The rare human adiponectin mutations G84R and G90S result in a decrease in HMW oligomer levels that are closely associated with T2DM (4, 6).

Myotonic dystrophy type 1 (DM1) is a multisystem autosomal dominant disorder characterized by progressive muscle wasting and weakness (7). The primary defect in DM1 is the expansion of a CTG triplet repeat in the 3'-UTR of the myotonic dystrophy protein kinase (*DMPK*) gene. The complex phenotype of DM1 is usually characterized by metabolic dysfunctions such as IR and hyperinsulinemia, hypertriglyceridemia, increased fat mass, and a fourfold higher risk of developing T2DM (8–11). The pathogenetic mechanism leading to these metabolic dysfunctions in DM1 remains unclear but it is closely linked to disorders of muscle cells containing reduced levels of insulin receptor and muscle-specific chloride-channel mRNAs, and elevated levels of tumor necrosis factor α (TNF α) (12–14).

To date, only two studies have evaluated ACRP30 expression in DM1 patients (15, 16), although DM1 is considered a good model for the study of metabolic perturbations in IR with adequate islet compensation. To determine whether the metabolic perturbations in DM1 are associated with deranged adiponectin expression, we measured circulating levels of ACRP30 and characterized its oligomerization pattern. We also evaluated whether molecular alterations of the *ACDC* gene were involved in the metabolic complications of the DM1 phenotype. Finally, we examined the correlations between total ACRP30 expression and several biochemical parameters in 21 DM1 patients and 82 age-, sex-, and weight-matched healthy controls.

Subjects and methods

Subjects

Twenty-one patients with the adult form of DM1 were recruited from outpatients attending the neurological clinic of the Second University of Naples (South Italy). Diagnoses were made based on clinical findings and genetic analyses according to a standard protocol (17). The severity of muscular involvement was assessed using the muscular impairment rating scale (17) and ranged from two (minimal signs) to five (severe weakness). Nine patients had normal electrocardiograms. The remaining 12 patients were affected by first-degree atrioventricular block and/or bundle branch block. Conventional echocardiography revealed normal left ventricular dimensions, wall thickness, and global systolic function (left ventricular ejection fraction 50–77%, mean $\pm$ s.d., 64.1 ± 8.7). Left ventricular ejection fraction is a parameter for the diagnosis, prognosis, and treatment of various types of heart disease (18). No patient presented signs and/or symptoms of CAD. Eighty-two age-, sex-, and weight-matched healthy volunteers served as controls.

The anthropometric and biochemical features of the study participants are shown in Table 1. Height, weight, and waist circumference at umbilicus were measured while the subject was standing. Body mass index (BMI) was calculated as the body weight (kg) divided by height (m) squared. Systolic and diastolic blood pressures were measured after the subject had been seated for at least 10 min. Creatinine clearance was estimated with the Cockcroft–Gault formula, which is widely used in cross-sectional studies, being easy to use, reproducible, and inexpensive (19).

Table 1 Anthropometric and biochemical characteristics of study participants. Data are expressed as mean (s.d.) and median.

Parameters	Controls <i>n</i> =82	Patients <i>n</i> =21	<i>P</i> values
Age (years)	39.2 (15.8); 33.5	44.5 (16.9); 46.5	NS
Sex (male/female)	43/39	8/13	NS
Weight (kg)	68.2 (13.4); 68.5	69.9 (10.7); 66.5	NS
BMI (kg/m ²)	23.2 (2.9); 23.4	25.7 (3.6); 25.2	0.001
Waist circumference (cm)	84.9 (11.6); 89	97.4 (8.6); 95	<0.001
Total cholesterol (mg/dl)	194.5 (38.9); 190.0	210.8 (51.3); 198.5	NS
Triglycerides (mg/dl)	95.2 (53.9); 82.5	244.1 (186.0); 146	<0.001
Glucose (mg/dl)	81.6 (11.7); 80.0	108.5 (42.2); 100.0	<0.001
TNF α (pg/ml)	4.0 (2.1); 3.6	6.8 (2.4); 6.8	<0.001
Insulin t0 (mU/l)	6.6 (1.8); 6.0	13.8 (9.1); 10.8	<0.001
HOMA	1.3 (0.2); 1.3	3.6 (2.2); 3.0	<0.001
QUICKI	0.4 (0.0); 0.4	0.33 (0.0); 0.3	<0.001
McAuley	8.7 (1.4); 9.2	5.6 (1.8); 5.5	<0.001
Fibrinogen (mg/dl)	274.7 (44.6); 268.7	336.7 (73.7); 312.0	<0.01
Estimated creatinine clearance (ml/min)	103.1 (71.7); 94.0	97.4 (29.1); 95.5	NS
Left ventricular ejection fraction (%)	66.1 (2.6); 65.9	64.1 (8.7); 65.0	NS
Total ACRP30 (μ g/ml)	29.0 (7.9); 27.7	22.0 (9.5); 19.8	<0.001

The statistical significance was established at $P < 0.05$. NS, not significant; BMI, body mass index; HOMA, homeostasis model assessment; QUICKI, quantitative sensitivity check index.

Informed consent was obtained from each patient and each volunteer. The study was approved by the Local Ethics Committee and was conducted in accordance with ethical principles stated in the most recent version of the Declaration of Helsinki.

Laboratory measurements

For laboratory studies, 2 ml blood samples were collected after a 12 h overnight fast and immediately centrifuged at room temperature. Serum levels of total cholesterol, triglycerides (TGs), glucose, and fibrinogen were determined with standard enzymatic methods (Hitachi Modular, Roche). Serum TNF α and insulin were measured by immunoassays (Immunolight 2000, Medical System, Genova, Italy and Human TNF α Quantikine, R&D Systems, Minneapolis, MN, USA respectively).

A standard oral glucose tolerance test (OGTT) was performed as described elsewhere (13). After a 12 h overnight fast, peripheral venous blood samples were collected before and 30, 60, 120, and 180 min after ingestion of 75 g glucose. IR was estimated according to the homeostasis model assessment (HOMA), calculated as (fasting glucose $\times$ fasting insulin)/22.5 (20). The quantitative insulin sensitivity check index (QUICKI) and the McAuley index were calculated as described previously (20). In detail, QUICKI is calculated as $1/(\log \text{ insulin} + \log \text{ glucose in mg/dl})$; McAuley calculated as $\exp [2.63 - 0.28 \ln (\text{insulin in mU/l}) - 0.31 \ln (\text{TG in mmol/l})]$. Patients were considered insulin resistant when HOMA was ≥ 2.6 , QUICKI ≤ 0.33 , and McAuley ≤ 5.8 .

Measurement of serum ACRP30 by ELISA

ACRP30 concentrations were measured with an ELISA using a polyclonal antibody produced in-house vs a human ACRP30 amino acid fragment (H₂N-ETTTQ-GPGVLLPLPKG-COOH) as described previously (21). ACRP30 was measured three times in triplicate.

Western blotting analysis

Serum samples were purified using Aurum Affi-GEL Blue columns (Bio-Rad). Proteins were quantified with Bradford's method (Bio-Rad), and 10 μ g purified serum was treated in Laemmli buffer with and without 10 mmol/l dithiothreitol (DTT) and loaded on a 10% SDS-PAGE. The western blotting analysis was performed as described previously (21). All experiments were performed in triplicate.

Gel filtration analysis

The distribution of ACRP30 oligomers in serum was analyzed on a Superdex 200 10/300 GL column

connected to a fast protein liquid chromatography system (Amersham Biosciences Biotech) as previously reported (21). Two hundred microliters of serum samples were fractionated at 0.5 ml/min using a PBS containing 5 mM DTT as elution buffer. Fractions (250 μ l) were collected and the concentration of ACRP30 oligomers was measured by ELISA as reported above; the oligomeric composition was assessed by western blotting. The column was calibrated using apo-ferritin (440 kDa), amylase (200 kDa), and glyceraldehyde-3-P dehydrogenase (160 and 46 kDa) as molecular weight standards (Sigma–Aldrich).

Molecular analysis of the ACDC gene

Blood samples (2 ml) were collected by venipuncture in EDTA and genomic DNA was extracted using a standard salting out/ethanol precipitation. Exon regions, the exon–intron boundaries, and the promoter region of ACDC gene were amplified with an in-house primer set using the PCR protocol, as described previously (5). PCR products were electrophoresed on a 1% agarose gel and both strands were sequenced with an automated procedure using the 3100 Genetic analyzer (Applied Biosystems, Foster City, CA, USA). PCR fragments were sequenced with the same primers used for PCR amplification.

Statistical analysis

Data were analyzed using the SPSS (v 10.0) Software package (SPSS, Inc., Chicago, IL, USA). Variables were examined for outliers and extreme values by the box and normal quantile–quantile plots, for normal distribution by the Kolmogorov–Smirnov and Shapiro–Wilk tests, and for homogeneity of variance by the Levene test. When normal distribution could not be accepted, variable transformations (square, square root, logarithmic, reciprocal of square root, or reciprocal transformations) were reviewed. Since normality was not reached for most variables, we used the following non-parametric tests: the Mann–Whitney *U* test for group differences and the Spearman rank coefficient (r_s) for correlation analysis. The χ^2 test was used to compare sex ratio. To overcome confounder effects of variables on adiponectin levels, we used a general linear model for analysis of covariance (ANCOVA) and stepwise multiple regression analysis (*F* to enter > 3.84). Statistical significance was established at $P < 0.05$.

Results

The anthropometric and biochemical features of DM1 patients and healthy controls are listed in Table 1. DM1 patients and the age-, sex-, and weight-matched controls had comparable systolic/diastolic pressure (data not shown), total cholesterol, estimated creatinine

clearance, and left ventricular ejection fraction, whereas DM1 patients had significantly higher BMI, waist circumference, and TG, glucose, TNF α , insulin, and fibrinogen levels ($P < 0.001$). HOMA, QUICKI, and McAuley indicated that DM1 patients had significantly higher IR than controls ($P < 0.001$). OGTT, performed in 13 DM1 patients, revealed serum levels of glucose and insulin indicative of glucose intolerance and hyperinsulinemia (data not shown). In addition, DM1 patients had significantly lower concentrations of total ACRP30 ($P < 0.001$). The difference in total ACRP30, TG, and TNF α levels between controls and patients persisted after adjustment for age, sex, and BMI (data not shown).

ANCOVA was performed to remove confounder effects of variables that influence ACRP30 levels. The analysis, adjusted for age, sex, BMI, waist circumference, total cholesterol, TG, glucose, insulin, HOMA, McAuley, estimated creatinine clearance, and left ventricular ejection fraction, confirmed the significant difference in ACRP30 concentrations between the two groups ($F = 4.2$; df 1.66; $P = 0.045$). Total ACRP30 levels correlated negatively with age ($r_s = -0.46$, $P < 0.001$), BMI ($r_s = -0.37$, $P = 0.001$), and waist circumference ($r_s = -0.40$, $P < 0.001$) in the control group. Total ACRP30 levels correlated negatively with TG levels ($r_s = -0.59$, $P = 0.006$) in the DM1 group. Multiple regression analysis with age, sex, BMI, waist circumference, total cholesterol, TG, glucose, insulin, HOMA, McAuley, estimated creatinine clearance, and left ventricular ejection fraction as independent variables showed that total ACRP30 levels remained negatively correlated with age ($r^2 = 0.18$, $F = 8.3$, $P = 0.007$) in the control group and with TG ($r^2 = 0.22$, $F = 5.14$, $P = 0.036$) in the DM1 group.

To verify the decrease in ACRP30 serum levels reported above, we examined the serum of control subjects and DM1 patients by western blotting in non-reducing and reducing conditions. As shown in Fig. 1A, a single band of molecular weight > 250 kDa corresponding to ACRP30 appeared under non-reducing conditions. Densitometric analysis (Fig. 1B) showed that the total amount of ACRP30 was 1.7-fold lower in DM1 patients than in controls (21721 ± 11120 pixels vs 36123 ± 8674 pixels respectively; $P < 0.01$). These results are similar to the results of ELISA and confirm that the total amount of ACRP30 was significantly lower in the serum of DM1 patients than in control subjects. Western blotting, performed under reducing conditions, revealed three major molecular mass species corresponding to LMW, MMW, and HMW ACRP30 oligomers (~ 60 , ~ 150 , and > 250 kDa respectively). Figure 1C shows the distribution of ACRP30 oligomers in the serum of representative controls (lanes 1 and 2) and DM1 patients (lanes 3–8). The pattern of ACRP30 oligomers differed between most DM1 patients and controls, the level of HMW and MMW oligomers being very low in patients. Western blotting analysis revealed

Figure 1 Western blotting analysis of serum ACRP30 in controls and DM1 patients in non-reducing (A and B) and reducing (C and D) conditions. (A) Representative blot image for total serum ACRP30 in controls (lanes 1 and 2) and in DM1 patients (lanes 3–8). (B) Graphic representation of pixel analysis performed with ImageJ Software showing that total serum ACRP30 expression is significantly lower in DM1 patients than in controls (* $P < 0.01$). Values are reported as pixel mean $\pm$ SD of three experiments expressed as percentage vs control. (C) Representative blot showing the ACRP30 oligomers (HMW, MMW, and LMW) in the serum of controls (lanes 1 and 2) and DM1 patients (lanes 3–8). (D) Graphic representation of pixel analysis performed using ImageJ Software (available from <http://rsbweb.nih.gov/ij/>) showing that HMW and MMW expression is significantly lower in DM1 patients than in controls (* $P < 0.01$). Values are reported as mean $\pm$ SD of three experiments.

that the difference in total ACRP30 levels between the two groups in non-reducing conditions is due to a lower level of HMW and MMW oligomers in DM1 patients vs controls. In particular, HMW oligomers were more than fourfold higher in controls than in DM1 patients, and MMW oligomers were 2.5-fold higher in controls than in DM1 patients. LMW oligomers did not differ between the two groups (Fig. 1D). The total amount of ACRP30 (i.e. the sum of the HMW, MMW, and LMW oligomers) was 1.6-fold lower in DM1 than in controls. Taken together, our results indicate that the lower amount of ACRP30 in most DM1 patients was primarily due to a decrease in HMW oligomers and secondarily to a decrease in MMW, whereas LMW ACRP30 oligomers remained unchanged.

To study the association of ACRP30 with DM1 in more detail, we evaluated the distribution of its oligomers in the serum of DM1 patients and controls by FPLC-gel filtration chromatography. Figure 2A shows the oligomerization state of the fractions of two representative DM1 patients and a representative control obtained by ELISA. The western blotting analysis of these fractions confirmed that ACRP30 HMW oligomers were more abundant in controls than in DM1 patients (Fig. 2B).

The molecular analysis of SNPs in the *ACDC* gene of DM1 patients revealed six variants previously reported in Caucasian populations (5). All variants were detected in the heterozygous state and four of them were located in the promoter region (c. -11426 A>G, c. -11391 G>A, c. -11377 C>G, and c. -11156 insCA), one in exon 2 (c.45 T>G p.G15G), and one in intron 2 (c.214+62G>T; data not shown).

Discussion

To our knowledge, this is the first study to characterize the distribution of circulating ACRP30 oligomers in patients affected by DM1. We demonstrate that ACRP30 expression is altered in these patients. In particular, total serum ACRP30 was significantly lower in DM1 patients than in controls due to a pronounced, selective decrease in its HMW oligomers. BMI, waist circumference, TG, insulin, TNF α , and fibrinogen were significantly higher in our DM1 patients than in age-, sex-, and body weight-matched controls. Their glucose levels were within normal range, although the results of OGTT were indicative of IR and glucose intolerance. Moreover, in patients we found a strong negative correlation between ACRP30 and TG levels, but no

significant association with the other parameters was analyzed. In our control group, there was a significant negative correlation between ACRP30 and age, waist circumference, and BMI, but only age remained significantly correlated after multiple regression analysis.

The clinical features of DM1 patients are often complicated by metabolic abnormalities and hormonal deregulation including IR (8–11, 22). ACRP30 and HMW oligomers are potent insulin sensitizers. In fact, the increase in serum ACRP30 by genetic and/or pharmacological approaches can alleviate metabolic dysfunctions (23, 24).

Here, we show that ACRP30 and its HMW oligomers are involved in the metabolic phenotype of DM1 patients. Our results are consistent with the report of Matsumura *et al.* (15) who showed that ACRP30 concentrations were lower in patients with glucose concentrations comparable to those of our patients (i.e. between 90 and 110 mg/dl). They are also in agreement with Abe *et al.* (16) who reported that serum ACRP30 concentrations improved in two DM1 patients with T2DM after treatment with pioglitazone. However, these two earlier studies did not measure the expression of HMW oligomers, which is the oligomer that accounts for most of the biological effects of ACRP30 (4, 6). In this study, we found a pronounced and preferential reduction of HMW oligomers in the serum of DM1 patients. Hypoadiponectinemia and the inability to form HMW oligomers can be due to SNPs and/or mutations of the *ACDC* gene (2, 4). In particular, two rare mutations, G84R and G90S, are closely associated with IR and T2DM (4, 6, 25). In our molecular analysis of the *ACDC* gene of DM1 patients (data not shown), we found only previously described SNPs (2, 5) and we are unable to determine whether they contribute to the genetic background of the patients' metabolic complication due to our small sample size. However, a number of studies have shown that CAD, weight gain, and IR are characterized by low concentrations of HMW and that these oligomers are more closely correlated with HOMA alterations and metabolic syndrome than total ACRP30 (25–27). It has recently been reported that a decrease in HMW oligomers is an independent risk factor for progression to T2DM in Japanese Americans and a predictor of CAD (25, 27, 28). Thus, the decrease in total ACRP30 we observed, associated with the preferential reduction of HMW oligomers, may help to explain the decreased insulin sensitivity in patients affected by DM1.

It is not known why ACRP30 decreases in DM1 patients, but it is well established that elevated levels of insulin and TG are associated with low total ACRP30 levels (25, 29). Our DM1 patients had a large waist circumference and elevated TG levels (which correlated negatively with ACRP30 levels) as reported for subjects with primary hypertriglyceridemia (30, 31).

Figure 2 Analysis of oligomerization state of ACRP30 by FPLC-gel filtration. An aliquot of each collected fraction was subjected to ELISA and western blotting analysis. (A) Graphic representation of the ELISA of one control and two DM1 patients and (B) the relative western blotting images of FPLC fractions showing that the expression of HMW and of MMW, albeit to a lesser extent, is lower in DM1 patients, whereas there was no between-group difference in the expression of LMW oligomers.

Our DM1 patients had high levels of TNF α ; a potent inflammatory cytokine affects the function of not only muscle but also adipose tissues and thus induces IR and obesity (13, 14). In particular, TNF α inhibits the expression of ACRP30 and vice versa (32–34). Therefore, in DM1 patients, one could envisage a vicious cycle of IR, altered muscle and adipose tissue functions, and hypoadiponectinemia in which each of these factors strengthen the others. It is conceivable that this vicious cycle could be interrupted by exogenously increasing ACRP30 levels, which would result in a reduction of the metabolic complications of DM1.

A limitation of our study is the sample size, because DM1 is a rare disease. Nevertheless, ACRP30 levels in our patients were significantly lower than in controls even after adjusting for several confounding variables.

In conclusion, our results demonstrate that DM1 patients are characterized by low levels of total serum ACRP30 and by a pronounced and selective decrease in HMW oligomers. This preferential decrease in HMW oligomers is of interest because HMW oligomers are better correlated with insulin sensitivity than total ACRP30 – a finding suggesting that their reduction could contribute to IR in DM1, as reported for other diseases with metabolic alterations similar to those found in DM1 (25–27). However, although it remains to be established whether ACRP30 is directly involved in IR in DM1 patients, measurement of its serum levels might represent an additional criterion for the staging of metabolic abnormalities in DM1. Further studies are needed to better understand the role of ACRP30 in DM1, but our findings indicate that ACRP30, and in particular HMW oligomers, may be used as biomarkers of metabolic perturbations in DM1 patients and represent promising therapeutic agents against IR and its complications.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This study was supported by the Regione Campania (DGRC n. 1901 del 22/12/2009), the MIUR (grant number: PS35–126/IND), the IRCCS-SDN Foundation, and the Ministero della Salute.

Acknowledgements

We thank Rita Ciaramella for her invaluable collaboration and Jean Ann Gilder (Scientific Communication srl, Naples, Italy) for revising and editing the text.

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Received 16 June 2011

Revised version received 17 September 2011

Accepted 28 September 2011