

Plasma Adiponectin Concentration Is Associated With Skeletal Muscle Insulin Receptor Tyrosine Phosphorylation, and Low Plasma Concentration Precedes a Decrease in Whole-Body Insulin Sensitivity in Humans

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Adiponectin, the most abundant adipose-specific protein, has been found to be negatively associated with degree of adiposity and positively associated with insulin sensitivity in Pima Indians and other populations. Moreover, adiponectin administration to rodents has been shown to increase insulin-induced tyrosine phosphorylation of the insulin receptor (IR) and also increase whole-body insulin sensitivity. To further characterize the relationship between plasma adiponectin concentration and insulin sensitivity in humans, we examined 1) the cross-sectional association between plasma adiponectin concentration and skeletal muscle IR tyrosine phosphorylation and 2) the prospective effect of plasma adiponectin concentration at baseline on change in insulin sensitivity. Fasting plasma adiponectin concentration, body composition (hydrodensitometry or dual energy X-ray absorptiometry), insulin sensitivity (insulin-stimulated glucose disposal, hyperinsulinemic clamp), and glucose tolerance (75-g oral glucose tolerance test) were measured in 55 Pima Indians (47 men and 8 women, aged 31 ± 8 years, body fat $29 \pm 8\%$ [mean \pm SD]; 50 with normal glucose tolerance, 3 with impaired glucose tolerance, and 2 with diabetes). Group 1 (19 subjects) underwent skeletal muscle biopsies for the measurement of basal and insulin-stimulated tyrosine phosphorylation of the IR (stimulated by 100 nmol/l insulin). The fold increase after insulin stimulation was calculated as the ratio between maximal and basal phosphorylation.

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ELISA, enzyme-linked immunosorbent assay; EMBS, estimated metabolic body size (fat - free mass + 17.7 kg); IR, insulin receptor; IRS-1, IR substrate-1; OGTT, oral glucose tolerance test.

Group 2 (38 subjects) had follow-up measurements of insulin-stimulated glucose disposal. Cross-sectionally, plasma adiponectin concentration was positively associated with insulin-stimulated glucose disposal ($r = 0.58$, $P < 0.0001$) and negatively associated with percent body fat ($r = -0.62$, $P < 0.0001$) in the whole group. In group 1 plasma adiponectin was negatively associated with the basal ($r = -0.65$, $P = 0.003$) and positively associated with the fold increase in IR tyrosine phosphorylation ($r = 0.69$, $P = 0.001$) before and after the adjustment for percent body fat ($r = -0.58$, $P = 0.01$ and $r = 0.54$, $P = 0.02$, respectively). Longitudinally, after adjustment for age, sex, and percent body fat, low plasma adiponectin concentration at baseline was associated with a decrease in insulin sensitivity ($P = 0.04$). In conclusion, our cross-sectional data suggest a role of physiological concentration of fasting plasma adiponectin in the regulation of skeletal muscle IR tyrosine phosphorylation. Prospectively, low plasma adiponectin concentration at baseline precedes a decrease in insulin sensitivity. Our data indicate that adiponectin plays an important role in regulation of insulin sensitivity in humans. *Diabetes* 50:1884–1888, 2002

Adipose tissue serves not only as an energy storage organ, but also secretes hormones and metabolites that are thought to regulate insulin sensitivity and energy metabolism (1,2). Adiponectin, the most abundant adipose-specific protein, is exclusively expressed in and secreted from adipose tissue (3–5). Plasma adiponectin concentration is decreased in individuals with obesity (4–6) and type 2 diabetes (7) and is more closely related to whole-body insulin sensitivity than to adiposity (8). In rhesus monkeys, plasma adiponectin concentration has been shown to decrease in parallel with increases in insulin sensitivity during the progression to type 2 diabetes (9). Whereas these findings provide circumstantial evidence that adiponectin may modulate insulin sensitivity, more recent studies demonstrate that administration of adiponectin to rodents increases insulin-induced tyrosine phosphorylation of the insulin receptor (IR) in skeletal muscle, resulting in improved glucose

TABLE 1
Anthropometrical and metabolic characteristics for subjects included in the analyses

	All	Group 1	Group 2		
	<i>n</i> = 55 (47 men and 8 women)	<i>n</i> = 19 (17 men and 2 women)	<i>n</i> = 38 (32 men and 6 women)		<i>P</i> *
			Basal	Follow-up	
Age (years)	31 ± 8	32 ± 8	28 ± 7	31 ± 7	
Height (cm)	171 ± 7	172 ± 8	170 ± 7	170 ± 7	<0.65
Weight (kg)	95 ± 24	105 ± 23	92 ± 23	98 ± 25	<0.001
Body fat (%)	29 ± 8	32 ± 8	29 ± 8	30 ± 6	<0.05
Fasting glucose (mmol/l)	4.7 ± 0.6	4.8 ± 0.7	4.6 ± 0.4	4.8 ± 0.4	<0.01
2-h glucose (mmol/l)	5.8 ± 1.9	6.4 ± 2.5	5.4 ± 1.3	6.5 ± 1.6	<0.001
Fasting insulin (pmol/l)	238 ± 122	275 ± 146	217 ± 104	244 ± 97	0.06
2-h insulin (pmol/l)	1,014 ± 1,158	1,428 ± 1,620	771 ± 751	1,218 ± 1,152	<0.05
Insulin-stimulated glucose disposal (mg · kg EMBS ⁻¹ · min ⁻¹)	2.97 ± 1.6	2.79 ± 1.48	3.10 ± 1.63	2.7 ± 0.99	0.05
Adiponectin (μg/ml)	6.87 ± 2.74	5.47 ± 1.83	7.58 ± 2.80	—	—

Data are means ± SE. Group 1: subjects who underwent skeletal muscle biopsies; group 2: subjects with baseline and follow-up measurements of insulin-stimulated glucose disposal. *Paired *t* test between basal and follow-up.

tolerance in these animals (10). The role of adiponectin in modulating insulin signaling in humans remains unknown.

In vivo insulin-mediated glucose disposal occurs primarily in skeletal muscle (11). In Pima Indians, obesity and insulin resistance are associated with impairments of skeletal muscle IR tyrosine phosphorylation in response to insulin (12). To further characterize the relationship between adiponectin and glucose/insulin metabolism in humans, we examined 1) the cross-sectional association between plasma adiponectin concentration and skeletal muscle IR tyrosine phosphorylation and 2) the prospective association between plasma adiponectin concentration at baseline and change in whole-body insulin sensitivity.

RESEARCH DESIGN AND METHODS

Subjects. A total of 55 Pima Indians (Table 1) who were participants in ongoing studies of the pathogenesis of obesity and type 2 diabetes were included in this analysis. Subjects for the present analysis were selected from participants of a longitudinal study of the risk factors of obesity and type 2 diabetes and had been admitted to the metabolic ward for measurements including muscle insulin-stimulated IR phosphorylation (12) and plasma adiponectin (8). All subjects were between 18 and 50 years of age and were nonsmokers at the time of the study. Except for two subjects with type 2 diabetes, all subjects were healthy according to a physical examination and routine laboratory tests. Subjects were then invited back at approximately annual intervals for repeated oral glucose tolerance tests (OGTTs) and, in a subgroup of subjects, for repeated assessment of insulin sensitivity. The protocol was approved by the Tribal Council of the Gila River Indian Community and by the Institutional Review Board of the National Institute of Diabetes and Digestive and Kidney Diseases, and all subjects provided written informed consent before participation.

Cross-sectional analyses were carried out in all subjects (*n* = 55) who were characterized for plasma adiponectin concentration, glucose tolerance, percent body fat, insulin sensitivity; in addition, a subgroup (group 1, *n* = 19) underwent muscle biopsies for measurement of skeletal muscle IR tyrosine phosphorylation. Subjects included in this analysis represented a wide range of glucose tolerance (14 normal glucose tolerant, 3 impaired glucose tolerant, and 2 type 2 diabetic subjects, according to the 1997 American Diabetes Association diagnostic criteria (13)).

Prospective analyses were performed in a subgroup of subjects (group 2, *n* = 38) who were normal glucose tolerant at baseline, were nondiabetic at follow-up, and had baseline measurements of plasma adiponectin concentration, percent body fat, 2-h glucose, insulin-stimulated glucose disposal, and follow-up measurements of insulin-stimulated glucose disposal (group 2).

Methods. Subjects were admitted for 8–10 days to the National Institutes of Health Clinical Research Unit in Phoenix, AZ, where they were fed a weight-maintaining diet (50% of calories as carbohydrate, 30% as fat, and 20% as protein) and abstained from strenuous exercise. After at least 3 days on the

diet, subjects underwent a series of tests for the assessment of body composition, glucose tolerance, insulin sensitivity, and (in selected cases) skeletal muscle biopsies.

Body composition was estimated by underwater weighing with determination of residual lung volume by helium dilution (14) or by total body dual-energy X-ray absorptiometry (DPX-L; Lunar, Madison, WI) (15,16). Percent body fat, fat mass, and fat-free mass were calculated as previously described (17), and a conversion equation (16) was used to make measurements comparable between the two methods.

After a 12-h overnight fast, subjects underwent a 75-g OGTT. Baseline blood samples were drawn for the determination of fasting plasma glucose, insulin, and adiponectin concentrations. Plasma glucose concentration was determined by the glucose oxidase method (Beckman Instruments, Fullerton, CA) in the fasting state and 2 h after glucose ingestion for the assessment of glucose tolerance, according to the 1997 American Diabetes Association diagnostic criteria (13). Plasma insulin concentration was determined by an automated immunoassay (Access; Beckman Instruments). Blood samples for the measurement of fasting plasma adiponectin concentration were drawn with prechilled syringes, transferred into prechilled EDTA tubes, and immediately placed on ice. All tubes were cold centrifuged (+4°C) within several minutes of collection and stored at -70°C until assayed at the Department of Internal Medicine and Molecular Sciences, Osaka University, Osaka, Japan. Fasting plasma adiponectin concentration was determined using a validated sandwich enzyme-linked immunosorbent assay (ELISA) employing an adiponectin-specific antibody (intra-assay and interassay coefficients of variation 3.3 and 7.4%, respectively).

Hyperinsulinemic-euglycemic glucose clamp. Insulin action was assessed at physiological insulin concentrations during a hyperinsulinemic-euglycemic glucose clamp as previously described (18,19). In brief, after an overnight fast, a primed continuous intravenous insulin infusion was administered for 100 min at a constant rate of 40 mU · m⁻² body surface area · min⁻¹ leading to steady-state plasma insulin concentrations. Plasma glucose concentration was maintained at ~5.5 mmol/l with a variable infusion of a 20% glucose solution. The rate of total insulin-stimulated glucose disposal was calculated for the last 40 min of insulin infusion. The insulin-stimulated glucose disposal values were additionally adjusted for endogenous glucose production (measured by a primed [30-μCi] continuous [0.3 μCi/min] 3-³H-glucose infusion), steady-state plasma glucose, and insulin concentration as previously described (18) and were normalized to estimated metabolic body size (EMBS) (EMBS = fat-free mass + 17.7 kg).

IR tyrosine kinase activity. Percutaneous needle biopsies of the vastus lateralis muscle were performed under local anesthesia (1% lidocaine) after a 12-h overnight fast and 2 days after the hyperinsulinemic-euglycemic glucose clamp. Muscle samples were blotted dry, quick frozen in liquid nitrogen, and stored at -70°C until analysis. To prepare soluble extracts of muscle tissue for IR tyrosine phosphorylation measurements, ~100–150 mg frozen tissue was pulverized under liquid nitrogen, and the resultant powder was homogenized in 0.75–1.5 ml buffer [20 mmol/l tris(hydroxymethyl) aminomethane (Tris), 5 mmol/l MgCl₂, 1 mmol/l phenylmethylsulfonyl fluoride (PMSF), 2 μmol/l leupeptin, and 2 μmol/l pepstatin, pH 8.7] at 4°C using a Polytron homogenizer

(Kinematica, Lucerne, Switzerland) for 10 s at a setting of 9. Triton X-100 was added to a final concentration of 1%, and the homogenate was solubilized for 60 min at 4°C. The material was then centrifuged at 100,000g for 60 min at 4°C and the supernatant collected and stored at -70°C. Muscle IR tyrosine phosphorylation was determined using an ELISA (12,20). Ninety-six-well microtiter plates were coated with 0.2 µg anti-IR antibody MA-20, 100 µl solubilized muscle extract (600 pg IR/ml) was added to each well, and receptors were allowed to bind overnight at 4°C. The following morning, receptors were preincubated for 15 min at 22°C in 80 µl assay buffer without or with insulin (final concentration 100 nmol/l). The phosphorylation reaction was started by adding 20 µl of a 50-µmol/l ATP solution and allowed to continue for 60 min at 22°C. The amount of tyrosine phosphorylated IR was quantified with biotinylated anti-phosphotyrosine antibody, to which peroxidase-conjugated streptavidin was added. The absorption at 450 nm of each well was measured in a microtiter plate reader (DuPont NEN, Boston, MA). Basal and maximal IR tyrosine phosphorylation were measured at 0 and 100 nmol/l insulin, respectively. The fold increase after insulin stimulation was calculated as the ratio between maximal and basal phosphorylation. As previously reported, this measure is highly correlated to the ability of the IRs to act as a tyrosine kinase and to phosphorylate exogenous substrates, such as the artificial substrate poly-(Glu,Tyr), after adding ATP (12).

Statistical analyses. Statistical analyses were performed using the software of the SAS Institute (Cary, NC). Results are given as means ± SD. Fasting plasma adiponectin and insulin-stimulated glucose disposal were logarithmically transformed to approximate a normal distribution. Differences between anthropometrical and metabolic characteristics at baseline and follow-up were assessed by Student's *t* test.

In cross-sectional analyses, relationships between plasma adiponectin concentration, percent body fat, insulin-stimulated glucose disposal, and IR tyrosine phosphorylation were examined by calculation of Pearson's correlation coefficients. Partial correlation was used to examine the relationships between plasma adiponectin and phosphorylation status, independent of percent body fat.

In prospective analyses, the predictive effect of plasma adiponectin concentration at baseline on change (follow-up adjusted for baseline) in insulin-stimulated glucose disposal was evaluated using multiple linear regression models. Models were adjusted for sex, follow-up age, change in percent body fat, and time of follow-up.

RESULTS

The anthropometrical and metabolic characteristics for the subjects included in the cross-sectional and longitudinal analyses are summarized in Table 1.

Cross-sectional analysis. Plasma adiponectin concentration was positively associated with insulin-stimulated glucose disposal ($r = 0.58$, $P < 0.0001$) and negatively associated with percent body fat ($r = -0.62$, $P < 0.0001$) in 55 subjects. In group 1 ($n = 19$), plasma adiponectin concentration was negatively associated with basal ($r = -0.65$, $P = 0.003$) and positively associated with the fold increase in skeletal muscle IR tyrosine phosphorylation ($r = 0.69$, $P = 0.001$) (Fig. 1) before and after adjustment for percent body fat in a partial correlation ($r = -0.58$, $P = 0.01$ and $r = 0.54$, $P = 0.02$, respectively). Plasma adiponectin concentration was also negatively associated with basal skeletal muscle IR tyrosine phosphorylation after adjustment for percent body fat and fasting plasma insulin concentration ($r = -0.55$, $P = 0.02$).

Prospective analysis. Low plasma adiponectin concentration at baseline was associated with a decrease in insulin-stimulated glucose disposal after adjustment for sex, age at follow-up, time of follow-up, and change in percent body fat ($P = 0.04$) (Table 2 and Fig. 2).

DISCUSSION

In the present study, we found that a low fasting plasma adiponectin concentration was cross-sectionally associated with a high basal and low insulin-stimulated skeletal muscle IR tyrosine phosphorylation and prospectively associated with a decrease in insulin sensitivity. Our

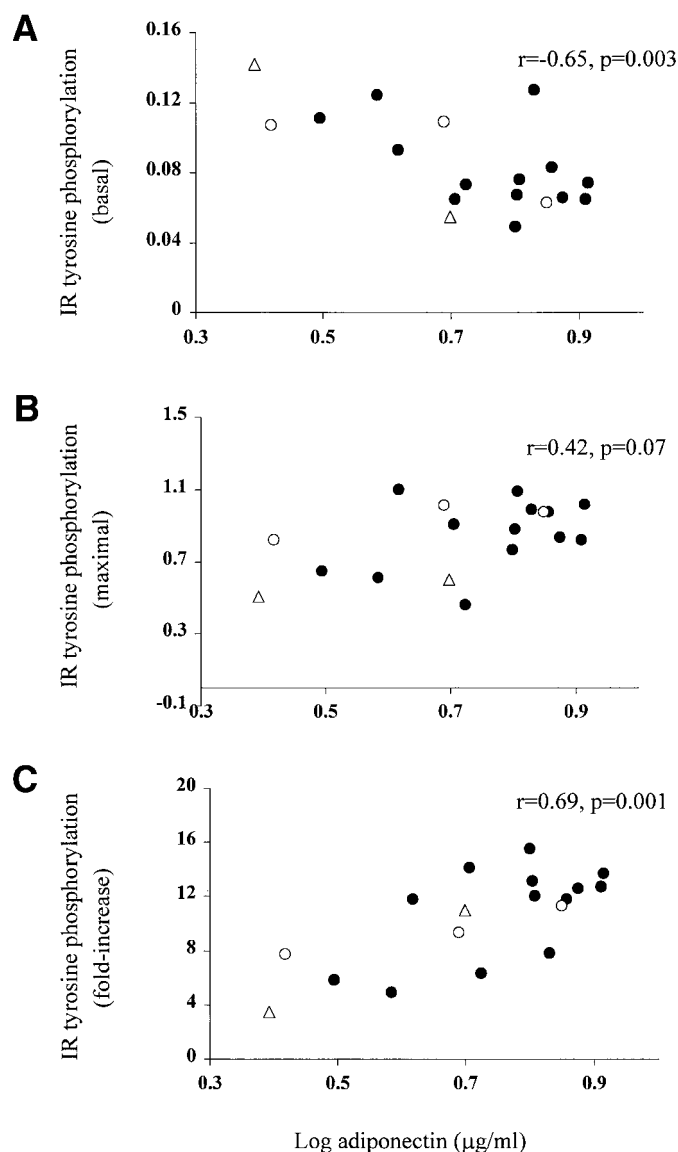


FIG. 1. Relationship between fasting plasma adiponectin concentration and basal (A), maximal insulin stimulation (B), and fold increase (C) in IR tyrosine phosphorylation. ●, subjects with normal glucose tolerance; ○, subjects with impaired glucose tolerance; and △, subjects with type 2 diabetes.

results are consistent with the notion that adiponectin plays a role in insulin sensitivity in humans.

We have previously established that adiponectin is associated with whole-body insulin sensitivity in humans (8). We now show that low plasma adiponectin concentration is associated with a high basal and decreased insulin-stimulated tyrosine phosphorylation of the IR in skeletal muscle, which is consistent with experimental observations in rodents (10). A high basal level of phosphorylation of the IR at tyrosine residues has been shown to be associated with hyperinsulinemia in the sand rat model of type 2 diabetes (21). In humans, little is known about the relationships between basal IR tyrosine phosphorylation and insulin sensitivity. Nevertheless, a high basal phosphorylation of IR substrate-1 (IRS-1), which is downstream of tyrosine phosphorylation of the IR (22), has been associated with insulin resistance and type 2 diabetes (23,24). Increased basal phosphorylation of IRS-1 would

TABLE 2
General linear regression model with insulin-stimulated glucose disposal at follow-up as dependent variable

	Estimate	SE	P
Independent variable			
Intercept	0.36	0.159	0.03
Age at follow-up (years)	-0.002	0.002	0.28
Sex	0.092	0.041	0.034
Body fat at follow-up (%)	-0.014	0.004	0.002
Body fat at baseline (%)	0.004	0.003	0.30
Insulin-stimulated glucose disposal at baseline ($\text{mg} \cdot \text{kg} \text{EMBS}^{-1} \cdot \text{min}^{-1}$)	0.244	0.120	0.023
Time of follow-up (years)	<-0.001	<0.001	0.83
Adiponectin at baseline ($\mu\text{g}/\text{ml}$)	0.252	0.120	0.044

thus reflect high basal IR tyrosine kinase activity and be compatible with our data. In addition, high IR tyrosine phosphorylation in the basal state has been associated with fasting hyperinsulinemia in an animal model (21).

Previously, we have shown that fasting hyperinsulinemia is associated with low plasma adiponectin concentration (8). Here we report a negative correlation between basal IR tyrosine phosphorylation and plasma adiponectin concentration. We hypothesize that adiponectin decreases basal phosphorylation by promoting insulin signaling downstream of the IR. This might result in increased insulin sensitivity and decreased fasting plasma insulin concentration. Therefore, when adiponectin concentration is low, fasting hyperinsulinemia, which is associated with decreased insulin sensitivity, may increase basal phosphorylation of the IR. Moreover, we found a relationship between adiponectin and basal phosphorylation independent of fasting plasma insulin, suggesting that factors other than insulin, such as tumor necrosis factor- α (25), may be determinants of basal tyrosine phosphorylation.

Skeletal muscle insulin-stimulated IR tyrosine phosphorylation is an important step in the insulin-signaling cascade and has been shown to decrease with increasing insulin resistance (12). In addition, we have previously shown that the degree of insulin-stimulated phosphorylation of immunocaptured IR correlated strongly with its kinase activity (12). Impaired skeletal muscle tyrosine kinase activity has been shown in obesity and type 2 diabetes (26–28) and has been suggested to be an early or

primary event in the development of insulin resistance. In our study, subjects with a high basal skeletal muscle tyrosine phosphorylation of the IR also have the lowest fold increase in tyrosine phosphorylation upon insulin stimulation. This may suggest that the relationship between the fold increase in phosphorylation and plasma adiponectin concentration may be a function of basal phosphorylation.

We also investigated whether a low plasma adiponectin concentration at baseline has an effect on future changes in whole-body insulin sensitivity. We found that a low fasting plasma adiponectin concentration at baseline is associated with a decrease in insulin sensitivity, independently of changes in percent body fat. Thus, the presented results extend our previous observation of a close association between hypo adiponectinemia and insulin resistance (8) by indicating that a low plasma adiponectin concentration precedes the decline in insulin sensitivity. Possible mechanisms for the insulin-sensitizing effects of adiponectin other than its direct effect on skeletal muscle IR tyrosine phosphorylation may include increased lipid oxidation in muscle (29) and enhancement of hepatic insulin action (30), possibly also by interaction with insulin signaling.

In summary, our cross-sectional data suggest a role of physiological concentration of fasting plasma adiponectin in the regulation of skeletal muscle IR tyrosine kinase activity in humans. Prospectively, low plasma adiponectin concentration at baseline precedes a decrease in insulin sensitivity. Our data indicate that adiponectin plays an important role in regulation of insulin sensitivity in humans.

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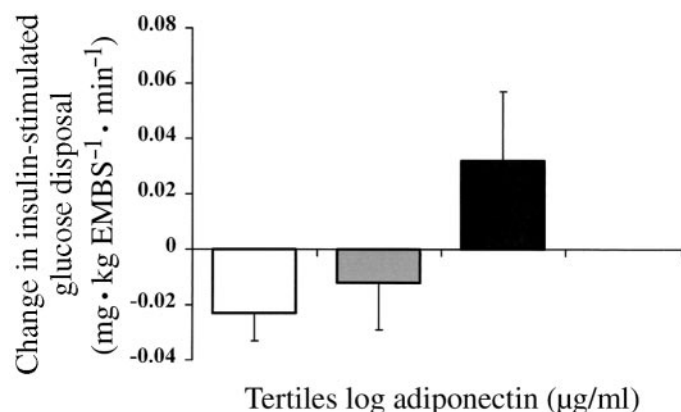


FIG. 2. Relationship between fasting plasma adiponectin concentration at baseline, represented as tertiles and change in insulin sensitivity (insulin-stimulated glucose disposal, follow-up adjusted for baseline) adjusted for sex, change in percent body fat, and age at follow-up. Data are means \pm SE).

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