Circulating Concentrations of the Adipocyte Protein Adiponectin Are Decreased in Parallel With Reduced Insulin Sensitivity During the Progression to Type 2 Diabetes in Rhesus Monkeys

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Adiponectin is an adipose-specific plasma protein whose plasma concentrations are decreased in obese subjects and type 2 diabetic patients. This protein possesses putative antiatherogenic and anti-inflammatory properties. In the current study, we have analyzed the relationship between adiponectin and insulin resistance in rhesus monkeys (*Macaca mulatta*), which spontaneously develop obesity and which subsequently frequently progress to overt type 2 diabetes. The plasma levels of adiponectin were decreased in obese and diabetic monkeys as in humans. Prospective longitudinal studies revealed that the plasma levels of adiponectin declined at an early phase of obesity and remained decreased after the development of type 2 diabetes. Hyperinsulinemic-euglycemic clamp studies revealed that the obese monkeys with lower plasma adiponectin showed significantly lower insulin-stimulated peripheral glucose uptake (*M* rate). The plasma levels of adiponectin were significantly correlated to *M* rate ($r = 0.66$, $P < 0.001$). Longitudinally, the plasma adiponectin decreased in parallel to the progression of insulin resistance. No clear association was found between the plasma levels of adiponectin and its mRNA levels in adipose tissue. These results suggest that reduction in circulating adiponectin may be related to the development of insulin resistance. *Diabetes* 50:1126–1133, 2001

Insulin resistance is one of the important risk factors associated with atherosclerosis and diabetes. Insulin resistance often accompanies visceral fat accumulation (1). Recent studies have provided evidence that adipose tissue may play a crucial role in the development of insulin resistance, type 2 diabetes, and their complications through the secretion of a variety of biologically active molecules (adipocytokines) (1). Hotamisligil et al. (2) reported that tumor necrosis factor-α (TNF-α) overproduced in adipose tissue of obesity contributes to the development of insulin resistance. Leptin is an adipose-specific hormone contributing to the regulation of energy expenditure and food intake (3). Leptin also affects insulin sensitivity and may participate in the development of hypertension (4–8). Plasminogen activator inhibitor-1 (PAI-1) increases in obesity and diabetes and may play a part in thrombosis and the development of vascular disease (9–11). These adipocytokines may cause the atherosclerotic vascular disease in type 2 diabetes directly or through the development of insulin resistance.

Adiponectin is a novel adipose-specific collagen-like molecule that belongs to the collectin family (12–14). Adiponectin bound to collagens I, III, and V (major components of the vascular intima) in a solid-phase binding assay and accumulated in the vascular wall when the endothelial barrier was damaged (15). The concentration of adiponectin in plasma ranged from 5 to 10 μg/ml in healthy humans (14). Obese patients, type 2 diabetic patients, and patients with coronary artery disease showed significantly lower levels of plasma adiponectin (14,16,17). We found that administration of adiponectin decreased the attachment of monocytic cell line THP-1 cells to human aortic endothelial cells (16,18), which is an early event in atherosclerotic vascular damage. Adiponectin decreases the expression of multiple adhesion molecules, including in endothelial cells via the modulation of NFκB signaling (16,18). Adiponectin also dramatically suppressed the secretion of TNF-α from human monocyte-macrophages (19). These clinical and experimental observations suggest that adiponectin plays some protective role against the atherosclerotic vascular change and that the decreased plasma adiponectin in type 2 diabetic patients may contribute to the development of atherosclerotic complications. The mechanism of decreased plasma adiponectin in type 2 diabetes, however, has not yet been clarified.

Many rhesus monkeys (*Macaca mulatta*) spontaneously develop obesity and subsequently develop type 2 diabetes, so they are excellent nonhuman primate models of human type 2 diabetes (20–23). Sequential metabolic changes during the development of obesity and diabetes can be

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AIR, acute insulin response; ELISA, enzyme-linked immunosorbent assay; FFM, fat-free mass; HSP83, heat shock protein 83; K_G, glucose disappearance rate; *M* rate, insulin-stimulated glucose uptake rate; PAI-1, plasminogen activator inhibitor-1; PCR, polymerase chain reaction; RT, reverse transcriptase; TNF-α, tumor necrosis factor-α.

followed in this model. A decrease in insulin sensitivity and a progressive increase of fasting plasma insulin (followed by a decline in β-cell function) precedes the manifestation of diabetes in rhesus monkeys (20–22). In the current study, we investigated the plasma concentrations and adipose mRNA expression of adiponectin during the development of obesity, insulin resistance, and type 2 diabetes in rhesus monkeys.

RESEARCH DESIGN AND METHODS

Male rhesus monkeys (*Macaca mulatta*) were individually housed and maintained in accordance with the National Academy of Sciences guidelines for the care and use of laboratory animals. Either the nutritionally complete liquid diet Ensure (Ross Laboratories, Columbus, OH) or Monkey Chow (Purina Mills, St. Louis, MO) and fresh water were provided to monkeys ad libitum (8 h/day). For measurement of adiponectin and leptin, the monkeys to be studied were sorted into the following three groups: lean (body fat <22%), obese (body fat >22%), or type 2 diabetic. Diabetes was diagnosed according to American Diabetes Association criteria (24) (fasting plasma glucose >7 mmol/l).

Procedures. Plasma samples were obtained under light anesthesia (ketamine hydrochloride 10 mg/kg body wt) after a 16-h fast. Plasma samples were frozen and kept at −80°C for later assays. Subcutaneous adipose tissues were obtained under ketamine hydrochloride, as described above, or immediately after anesthetization by intravenous sodium pentobarbital. Tissue samples were immediately frozen in liquid nitrogen and stored at −165°C.

After a 16-h fast, the monkeys were placed under light anesthesia (ketamine hydrochloride 10 mg/kg body wt), and an intravenous glucose tolerance test (0.5 ml/kg of 50% dextrose) was carried out with sampling at 1, 3, 5, 7, 10, 15, and 20 min. The glucose disappearance rate (K_G) was calculated using the 5- and 20-min time points (20). Acute insulin response (AIR) was determined to be the mean change in plasma insulin per minute from basal levels for the period 0–10 min during the intravenous glucose tolerance test (22). Hyperinsulinemic-euglycemic (2,400 pmol · m^{-2} body surface area · min^{-1} insulin infusion) clamps were carried out (21). The plasma glucose was maintained at ~4.7 mmol/l to estimate insulin-stimulated glucose uptake rate (*M* rate). *M* rate was corrected for fat-free mass (FFM), an estimate of metabolically active mass, as described previously (21). Percentage body fat was determined by the tritiated water dilution method (25).

Enzyme-linked immunosorbent assay of plasma adiponectin and leptin. We measured plasma adiponectin levels in monkeys using the enzyme-linked immunosorbent assay (ELISA) system developed for the measurement of human plasma adiponectin concentrations, as described previously (14). Human recombinant adiponectin was used as a standard. The affinity of the monoclonal anti-adiponectin antibody (used as the first antibody of ELISA) to the monkey adiponectin was as strong as that to human adiponectin; however, the affinity of the polyclonal antibody (which was used for the second antibody) was weaker. Thus, the values were indicated as an arbitrary unit. Plasma samples from three monkeys with high, medium, and low levels of plasma adiponectin, respectively, were diluted 4:1, 2:1, and 4:3 into phosphate-buffered saline and measured by ELISA. The observed values were highly correlated to the expected values ($r = 1.00$), verifying that this ELISA system can detect monkey adiponectin quantitatively. The concentrations of leptin were determined by ELISA, as described previously (26,27).

Sequencing of the coding region of monkey adiponectin cDNA. The following oligonucleotides were synthesized using the human adiponectin sequence (12): 5′-TGATTCCATACCAGAGGGGCTCA-3′ (−25 to −3) and 5′-GGAGGAGGCTCTGAGTTAGTGGT-3′ (738–760). The full coding sequence of monkey adiponectin was amplified by reverse transcriptase (RT)-polymerase chain reaction (PCR), as described before (26). The PCR fragment was cloned into pCR 2.1 (Invitrogen, Carlsbad, CA) and DNA from several clones was sequenced using an ABI Prism dRhodamine Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA polymerase FS (Perkin-Elmer, Norwalk, CT).

Northern blot analysis. Total cellular RNA was prepared from monkey subcutaneous adipose tissue using TRIzol reagent (Life Technologies, Bethesda, MD). A total of 10 μg of total RNA was electrophoresed and transferred to a nylon membrane (Hybond-N; Amersham International, Buckinghamshire, U.K.). The membranes were hybridized in QuikHyb hybridization solution (Stratagene, La Jolla, CA). The *Eco*RI fragment of monkey adiponectin cDNA was used as a probe. Human heat shock protein 83 (HSP83) was used as an internal control as described previously (26,28). The probes were labeled with [α-^{32}P]2′-deoxycytidine-5′-triphosphate (3,000 Ci/mmol) (Amersham), using a random-primer DNA labeling system (Amersham). The relative mRNA levels of adiponectin and HSP83 were quantitated using a BAS5000 phosphor imaging system (Fuji Photo Film, Tokyo).

Statistical analysis. Data are expressed as means ± SE. Significant group differences were determined by one-way analysis of variance, and significant *F* values were further tested by the Fisher multiple comparison method. Linear relationships between key variables were tested using Pearson's correlation coefficient. Multiple linear regression analysis was performed to evaluate the independent relationship of the studied variables.

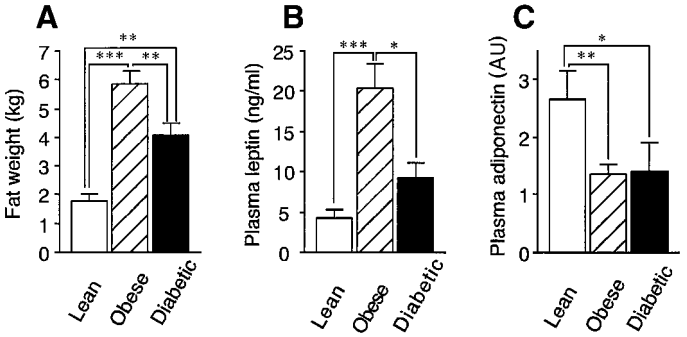

FIG. 1. Fat weight (*A*) and the plasma levels of leptin (*B*) and adiponectin (*C*) in lean, hyperinsulinemic obese, and type 2 diabetic rhesus monkeys. The plasma levels of adiponectin and leptin from lean ($n = 14$), obese ($n = 23$), and type 2 diabetic ($n = 10$) monkeys were measured by ELISA. Values represent means ± SE. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

RESULTS

Plasma adiponectin concentration in obese and type 2 diabetic monkeys. First, we evaluated the plasma concentrations of adiponectin and leptin in the lean monkeys ($n = 14$, age 10 ± 2 years, 10.5 ± 0.7 kg body wt, 16 ± 1% body fat); the obese monkeys ($n = 23$, age 16 ± 1 years, 16.7 ± 0.7 kg body wt, 34 ± 1% body fat); and the diabetic monkeys ($n = 10$, age 23 ± 1 years, 13.2 ± 0.6 kg body wt, 31 ± 2% body fat). The lean group showed normal fasting glucose (3.3 ± 0.1 mmol/l) and insulin (351 ± 31 pmol/l) levels. The obese group showed normal fasting glucose levels (4.0 ± 0.2 mmol/l), but they had significantly higher plasma insulin levels (1,009 ± 278 pmol/l, $P < 0.05$) compared with normal monkeys. In the diabetic group, fasting plasma glucose levels were elevated (11.2 ± 0.9 mmol/l) and fasting plasma insulin levels had declined from previously elevated levels to the normal range (251 ± 56 pmol/l), as described previously (20–23). The obese monkeys showed significantly higher plasma leptin concentrations than did lean monkeys (20.4 ± 3.0 vs. 4.3 ± 1.0 ng/ml, $P < 0.001$) as reported previously (Fig. 1) (26,27). In contrast, the plasma adiponectin concentrations in obese monkeys were significantly lower than in lean monkeys (1.4 ± 0.2 vs. 2.7 ± 0.5, $P < 0.01$) (Fig. 1). When the monkeys developed diabetes, the fat weight decreased. Accordingly, plasma leptin levels in the diabetic monkeys returned to near lean levels (Fig. 1). However, the plasma adiponectin levels in diabetic monkeys remained lower than those in lean monkeys (Fig. 1). These results were similar to our previous data in humans (17).

Longitudinal changes of plasma adiponectin during the development of obesity and diabetes. Longitudinal changes of plasma adiponectin were investigated in the individual monkeys during the development of obesity and type 2 diabetes. The time course during the development of type 2 diabetes has been divided into eight phases (20). At phase 1, monkeys are young (age <10 years), lean

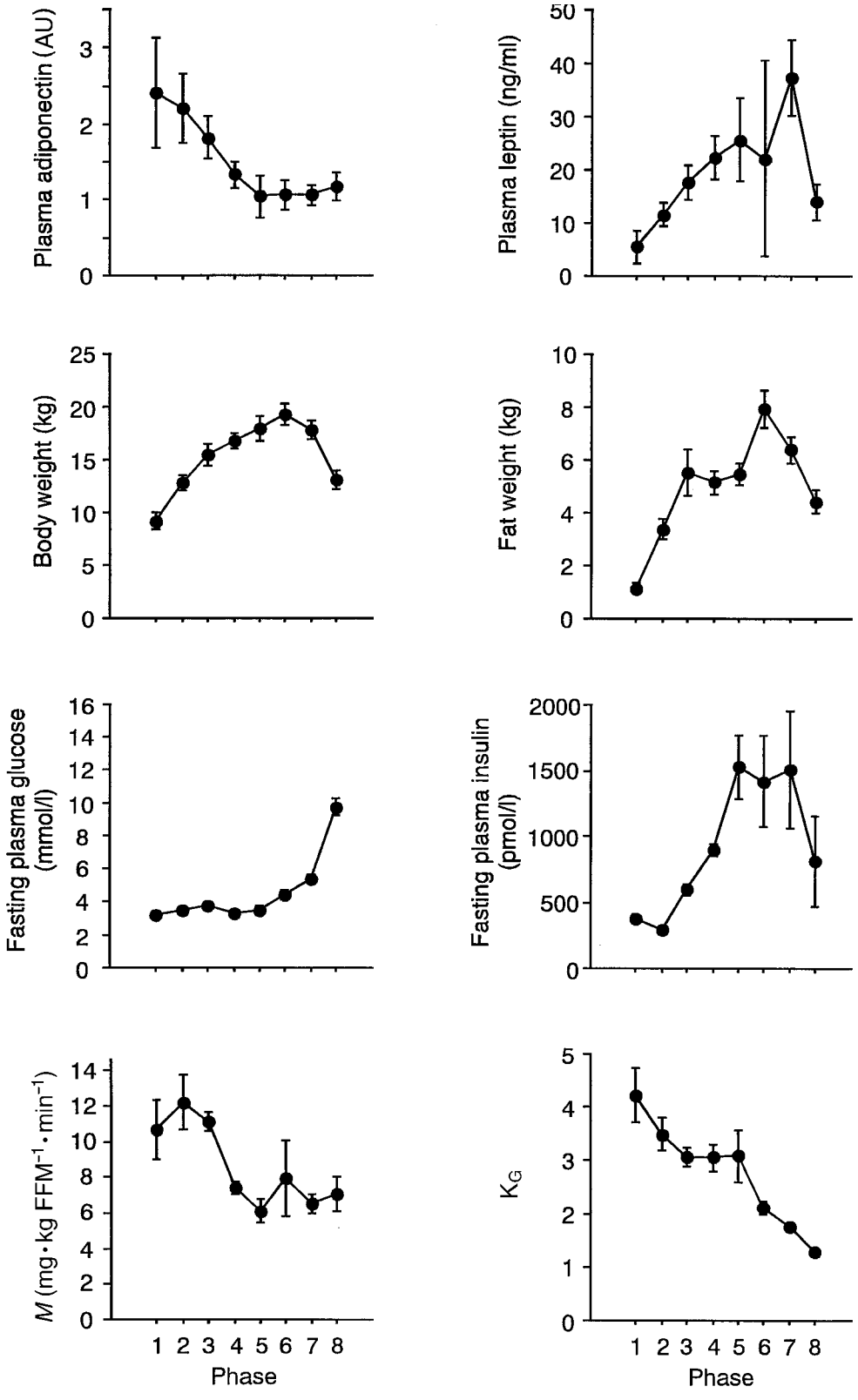

FIG. 2. Longitudinal changes in plasma adiponectin, plasma leptin, body weight, fat weight, plasma glucose, plasma insulin, *M* rate, and K_G. The plasma levels of adiponectin and leptin were analyzed by ELISA. *M* rate was determined by a hyperinsulinemic-euglycemic clamp, as described in RESEARCH DESIGN AND METHODS. Data are shown as means ± SE. $n = 7$ in phase 1, $n = 14$ in phase 2, $n = 14$ in phase 3, $n = 11$ in phase 4, $n = 7$ in phase 5, $n = 6$ in phase 6, $n = 16$ in phase 7, and $n = 12$ in phase 8.

(body fat <22%), and have normal fasting plasma insulin and glucose levels (Fig. 2). At phase 2, the monkeys are lean and normal, although they are middle-aged (>10 years). High plasma levels of adiponectin were observed at these phases (Fig. 2). During phases 3 through 5, monkeys became obese, and progressive increases in fasting plasma insulin and leptin were observed. Plasma levels of adiponectin were greatly decreased in this phase. Obesity and

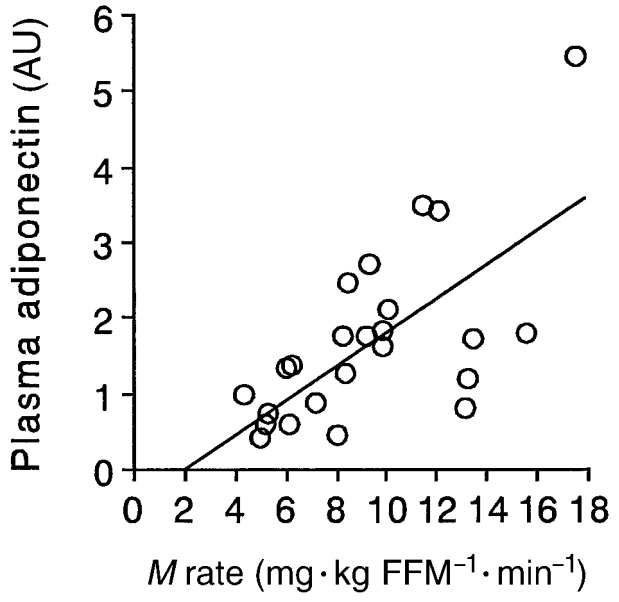

FIG. 3. Correlation between *M* rate and plasma adiponectin in 24 independent nondiabetic monkeys. *M* rate was determined by the hyperinsulinemic-euglycemic clamp method, as described in RESEARCH DESIGN AND METHODS. The plasma levels of adiponectin were analyzed by ELISA. $r = 0.66$; $P < 0.001$.

hyperinsulinemia were prominent at phases 5 through 7. The deterioration of K_G values (the index of glucose tolerance) became evident. The plasma levels of leptin further increased during these phases, reflecting the progression of obesity. The plasma levels of adiponectin reached quite low levels (Fig. 2). At phase 8, fasting plasma insulin levels dropped and the fasting plasma glucose increased. The plasma leptin levels were decreased in association with the decrease in body and fat weight. In contrast, the plasma adiponectin levels remained at low levels (Fig. 2).

Relationship between plasma adiponectin concentration and insulin sensitivity. Obese monkeys are often insulin resistant and hyperinsulinemic. The degree of insulin resistance varies in the individual obese monkey (21,23). In rhesus monkeys, the plasma levels of adiponectin decreased in phases 3–5. Insulin sensitivity decreased in phases 3–5 preceding the development of diabetes (21). The change in the plasma levels of adiponectin was similar to the change in insulin sensitivity (Fig. 2). Thus, the reduction of plasma adiponectin concentration might be related to the development of insulin resistance. We evaluated *M* rates using the euglycemic-hyperinsulinemic technique in 24 nondiabetic monkeys. Plasma adiponectin concentrations were closely correlated with *M* rates ($r = 0.66$, $P < 0.001$, Fig. 3) as well as with body weight ($r =$

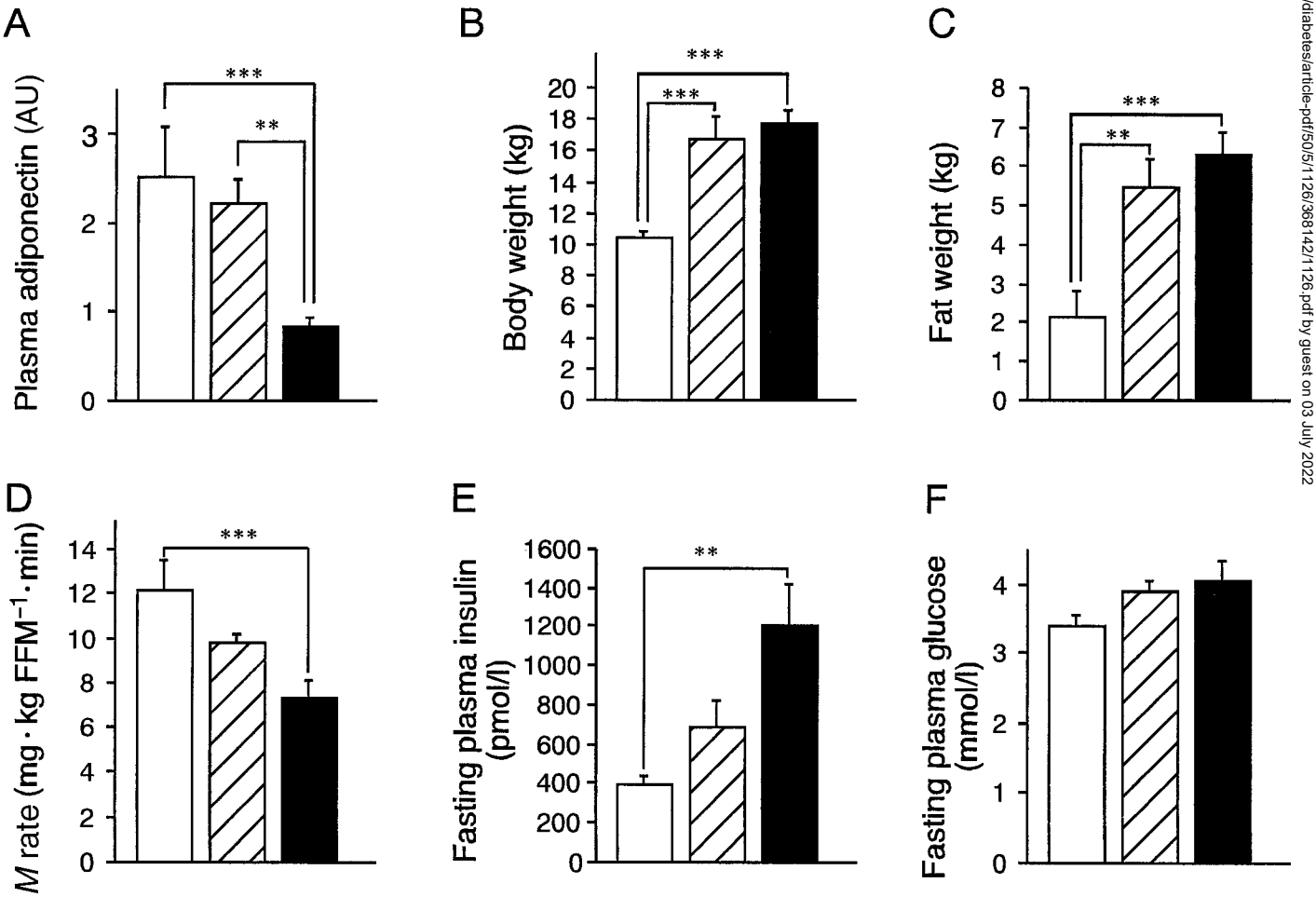

FIG. 4. Plasma adiponectin (*A*), body weight (*B*), fat weight (*C*), *M* rate (*D*), fasting plasma insulin (*E*), and fasting plasma glucose (*F*) in lean (□), obese with hyperadiponectinemia (▨), and obese with hypoadiponectinemia (■) monkeys. The plasma levels of adiponectin were analyzed by ELISA. ** $P < 0.01$; *** $P < 0.001$.

-0.46, $P < 0.05$), body fat weight ($r = -0.42$, $P < 0.05$), and plasma insulin ($r = -0.41$, $P < 0.05$). Plasma adiponectin levels were not correlated with age, K_G, or AIR ($r = -0.12$, 0.15, and -0.21, respectively). Multiple regression analysis showed that decreased M rate ($P < 0.05$) and increased fat weight ($P < 0.05$) were significantly and independently associated with decreased levels of adiponectin. Fasting plasma insulin was not significantly associated with the plasma levels of adiponectin, because fasting plasma insulin levels progressed through an inverted ∪–shaped curve. We divided nondiabetic monkeys into the following three groups: lean (body fat <22%, $n = 7$), obese with high plasma levels of adiponectin (body fat >22%, adiponectin >1.4, $n = 9$), and obese with low plasma levels of adiponectin (body fat >22%, adiponectin <1.4, $n = 8$). The body weight, body fat, and plasma glucose levels were not different in the obese monkeys with higher and lower levels of adiponectin (Fig. 4). In the obese monkeys with lower levels of plasma adiponectin, the M rate was significantly lower than it was in those with higher levels of plasma adiponectin (t test, $P < 0.05$) (Fig. 4). Thus, hypoadiponectinemia could be related to insulin resistance.

Longitudinally, the M rate began to decrease at the earliest phase of obesity then reached and sustained low levels in subsequent phases. The plasma levels of adiponectin decreased as the monkeys developed insulin resistance (Fig. 2). The close relationship was indicated in this prospective longitudinal study.

Plasma adiponectin concentrations and mRNA levels in adipose tissue. Plasma adiponectin concentrations decreased when the monkeys became obese. To clarify whether the plasma adiponectin levels are determined by the mRNA expression levels in the adipose tissue, we measured the adiponectin mRNA levels in subcutaneous adipose tissue. We first cloned the monkey adiponectin cDNA. Sequence analysis revealed that the predicted monkey adiponectin protein (excluding the signal peptide) was identical in length (224 amino acids) and showed a 96% identity with human adiponectin (12,13) (Fig. 5*A*). Northern blot analysis showed that monkey adiponectin mRNA was expressed exclusively in adipose tissue, as observed in humans in earlier studies (Fig. 5*B*) (12,13).

Leptin mRNA levels were significantly correlated to fat weight ($r = 0.62$, $P < 0.01$) as previously reported (26). On the other hand, adiponectin mRNA levels were not correlated to fat weight ($r = -0.11$) (Fig. 6) or M rate ($r = 0.36$). No clear correlation between the plasma and mRNA levels of adiponectin was observed ($r = -0.02$). Plasma leptin levels were significantly correlated with leptin mRNA levels in adipose tissue ($r = 0.60$, $P < 0.01$) (Fig. 6). Adjustment of each mRNA level for HSP83 produced similar results, as shown in Fig. 6.

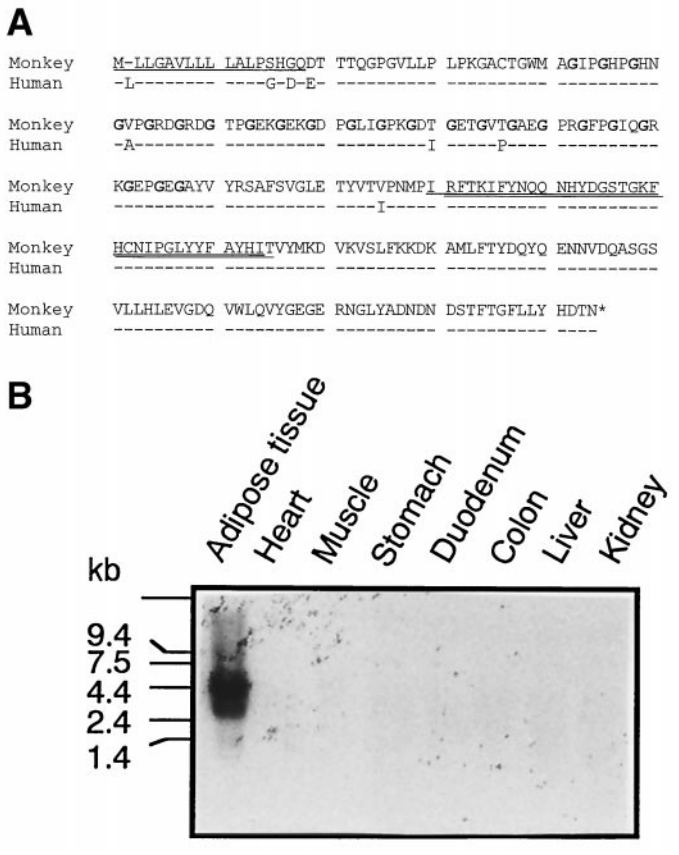

FIG. 5. Amino acid sequence and expression of rhesus monkey adiponectin. *A*: Comparison of the amino acid sequences from monkey and human adiponectin. Amino acids that are identical to the monkey sequence are represented by dashed lines. The predicted signal sequence is underlined. The glycine in a region encoding Gly-X-Y triplets (Glys-X-Y repeats) is indicated in bold type. The noncollagenous region, which is homologous to VIII collagen, X collagen, and the C chain of C1q, is double-underlined. *B*: Tissue distribution of monkey adiponectin mRNA. Total RNA (10 μg) isolated from various tissues was applied to each lane. Northern blot analysis was carried out as described in RESEARCH DESIGN AND METHODS.

DISCUSSION

Adiponectin is an adipose-specific plasma protein that inhibits the expression of adhesion molecules in endothelial cells and the secretion of TNF-α from monocyte-macrophages (16,18,19). Therefore, it possesses possible antiatherogenic and anti-inflammatory properties. The plasma levels of adiponectin were decreased in obese subjects and in patients with type 2 diabetes (14,17). The mechanism of reduced adiponectin in these metabolic disorders has not been clear. Rhesus monkeys provide excellent models of human obesity and type 2 diabetes (20–23), and the natural histories of obesity and of diabetes have been clearly demonstrated in this model. Plasma adiponectin concentrations were decreased in rhesus monkeys with obesity and in those with type 2 diabetes. The current data were compatible with our previous data observed in humans (14,17). One of the important observations in this study was the demonstration of longitudinal changes in plasma adiponectin levels during the development of obesity and type 2 diabetes. The plasma levels of adiponectin began to decrease in the earliest stage of obesity, when the insulin resistance and hyperinsulinemia were progressing. This pattern was very similar to the change in insulin sensitivity as measured by the M rates, suggesting the close relationship between the M rate and the plasma adiponectin levels. Indeed, the plasma levels of adiponectin were strongly correlated to the M rates. Moreover, when the obese monkeys were divided into hyperadiponectinemic and hypoadiponectinemic groups, the hypoadiponectinemic monkeys were more insulin-resistant than the hyperadiponectinemic ones. These re-

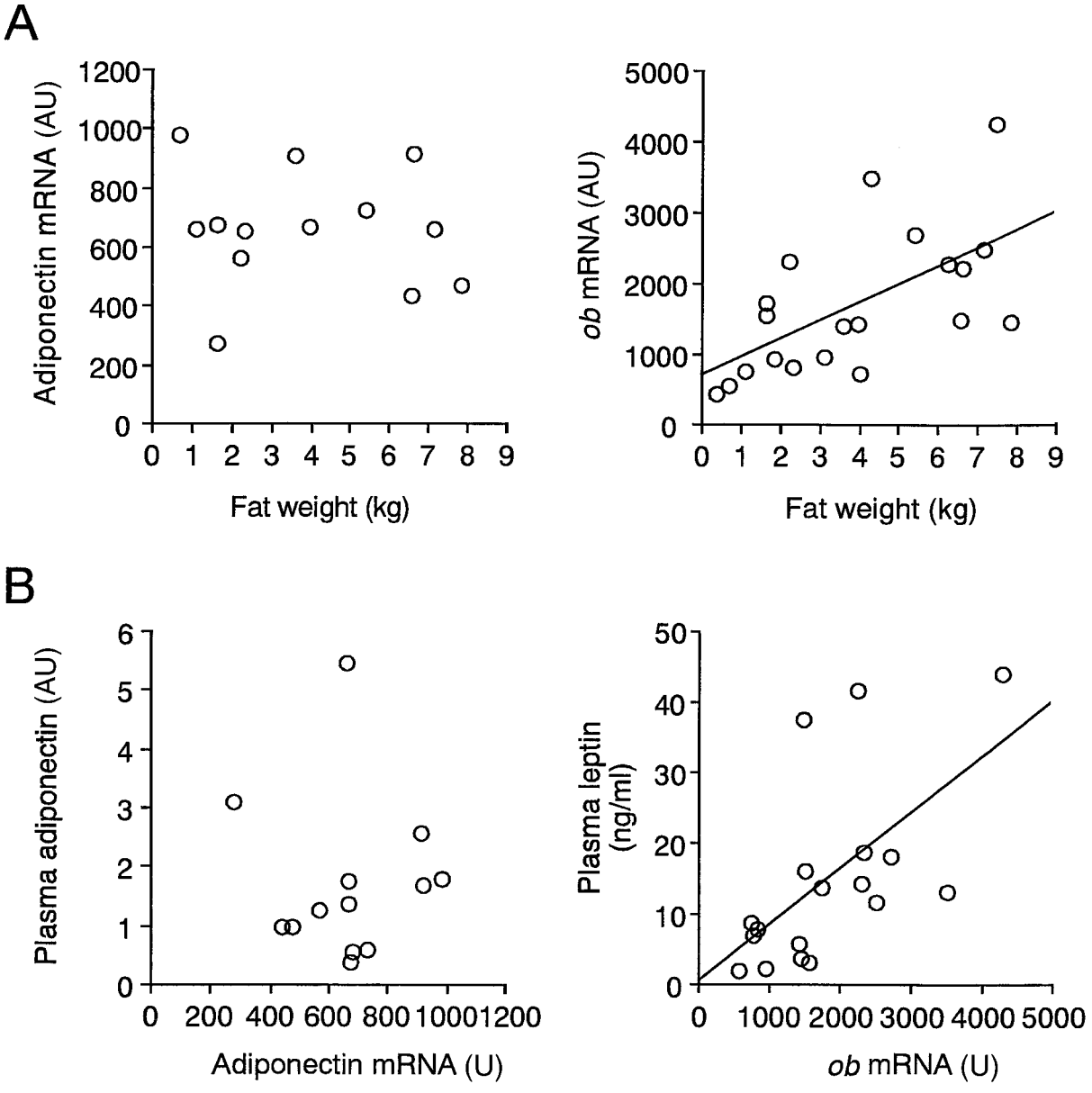

FIG. 6. ***A*: The correlation between mRNA levels of adiponectin and fat weight ($r = -0.11$, NS) and between mRNA levels of *ob* (leptin) and fat weight ($r = 0.62$, $P = 0.01$). A total of 5 μg of total RNA was electrophoresed. Hybridization was performed as described in RESEARCH DESIGN AND METHODS. *B*: Correlation between the plasma and mRNA levels of adiponectin ($r = -0.02$, NS) and between the plasma leptin and *ob* mRNA levels of ($r = 0.60$, $P = 0.01$). The mRNA levels of adiponectin and *ob* were determined by Northern blot hybridization. The plasma levels of adiponectin and leptin were analyzed by ELISA.**

sults suggested that hypoadiponectinemia associates with insulin resistance.

Insulin binding to adipocytes was reduced in obese and diabetic monkeys (29). Basal and insulin-stimulated glucose utilization dropped markedly as hyperinsulinemia progressed into diabetes (29). A defect in the covalent activation of glycogen synthase by in vivo insulin has been identified in insulin resistance and type 2 diabetes in subcutaneous adipose tissue (30). Thus, adipose tissue as well as skeletal muscle plays an important role in insulin resistance in rhesus monkeys (30,31). Insulin regulates secretion of various molecules from adipocytes (32,33). Scherer et al. (34) demonstrated that the secretion of the mouse adiponectin homologue Acrp30 was stimulated by insulin in 3T3-11 cells. Secretion of adiponectin from adipocytes may be disturbed in the insulin-resistant state. One of the possible mechanisms of insulin resistance in obesity is overproduction of TNF-α from adipose tissue. TNF-α suppresses the release of leptin and PAI-1 from adipocytes (35–40). Thus, the increased TNF-α may also reduce the secretion of adiponectin from adipose tissue in insulin-resistant obesity.

Recent studies have determined that the cellularity of adipose tissue is heterogeneous and that the size of adipocytes is also importantly related to the insulin resistance. Okuno et al. (41) reported that the adipocytes in Zucker obese rats with insulin resistance were larger than those in lean littermates without insulin resistance. Treatment with the insulin-sensitizing thiazolidinedione troglitazone increased the number of small adipocytes and improved insulin sensitivity in Zucker obese rats without changing the total adiposity. Therefore, small adipocytes

may be more insulin-sensitive than larger ones. Insulin-sensitive small adipocytes may secrete more adiponectin, and obese monkeys with more of the small adipocytes (i.e., hyperplastic obesity) may have higher plasma adiponectin levels than obese monkeys with hypertrophic obesity.

We have reported that adiponectin suppressed the TNF-α signaling in endothelial cells (16). Although we have not examined this specifically, adiponectin may attenuate the TNF-α signaling in adipocytes and protect them from the development of insulin resistance. TNF-α increases in obesity (2) and may suppress the secretion of adiponectin from adipocytes. The decreased adiponectin in obesity may further deteriorate insulin resistance caused by TNF-α. In insulin resistance, secretion of adiponectin would decrease further. Such a vicious cycle may be involved in the insulin-resistant state.

It is not clear whether the plasma level of adiponectin is determined at mRNA levels in humans. In this study, we assayed the mRNA levels of adiponectin using monkey adipose tissue. The mRNA levels of adiponectin did not correlate with either adiposity or insulin sensitivity. Plasma adiponectin levels did not correlate with mRNA levels in adipose tissue in the current study. Therefore, the plasma adiponectin concentration in rhesus monkeys may be regulated at a posttranscriptional level, including the translation and/or secretion level.

In summary, the antiatherogenic plasma protein adiponectin decreased before the onset of diabetes, in parallel with the decrease of insulin sensitivity. Hypoadiponectinemia is closely related to insulin resistance. Our present data suggests that the decreased plasma levels of adiponectin may be related to the development of insulin resistance and possibly of atherosclerosis.

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