Adiponectin increases glucose transport, reduces inflammation, and controls vascular functions. Hence, we propose that treatment with a recombinant globular domain of adiponectin (rgAd110-244) has significant therapeutic potential to treat insulin resistance. Mice were fed for 3 months on a high-fat diet (HFD) to induce insulin resistance, diabetes, and moderate weight gain. The mice were first infused iv with different doses of rgAd110-244 (0.12, 0.4, and 1.2 μg/kg/min) for 5 h. Basal and insulin-sensitive glucose use rates were assessed by the use of a submaximal rate of insulin in the awake free-moving mouse. rgAd110-244 reduced, with dose dependence, epinephrine-induced hyperglycemia and HFD-induced insulin resistance by increasing whole-body glucose use (35% at the highest dose) and glycolysis rates. Similarly, the reduction of plasma free fatty acid concentrations by insulin was dramatically improved. Basal hepatic glucose production was unchanged by rgAd110-244 infusion. This acute rgAd110-244 treatment improved glucose homeostasis and was associated with an increased content of muscle phospho-Akt, glycogen synthase kinase-3β, and AMP-activated kinase. Second, HFD mice were chronically treated with sc rgAd110-244 injections (10, 30, and 100 μg/kg). Fasting glycemia and insulin-sensitive glucose use were improved by rgAd110-244 at the highest dose at completion of the treatment, with concomitant reduction in body weight gain. We here show for the first time that a recombinant adiponectin fragment (110-244 amino acids called rgAd110-244) is able to treat insulin-resistant diabetes. Our results strongly suggest further pharmacological investigation of rgAd110-244 with the objective of developing a new treatment of insulin-resistant diabetes. (Endocrinology 150: 4493–4501, 2009)
role of adiponectin in the control of whole-body insulin sensitivity, particularly by enhancing it in muscle and liver (6, 7) and increasing fatty acid oxidation in muscle (8). A similar conclusion was reached using mice with the adiponectin gene deleted (9). A molecular hypothesis has been proposed for the increased muscle insulin sensitivity by adiponectin, which involves the activation of the AMP-activated kinase (AMPK). Interestingly, the globular low-molecular-weight form of adiponectin was responsible for muscle activation, whereas the high-molecular-weight hexamer triggered both liver and muscle glucose metabolism (10). Importantly, in addition to glucose metabolism, lipid oxidation was regulated by adiponectin (11). Adiponectin phosphorylates acetyl-coenzyme A carboxylase (ACC), which inhibits its activity and prevents the accumulation of malonylCoA (11). Hence, the mitochondrial entry of acyl CoAs is no longer reduced, which allows for lipid oxidation to prevent lipotoxicity and excessive lipogenesis (13).

In conclusion, the above arguments are in favor of a strong potential role for adiponectin and adiponectin-like molecules as therapeutic agents for the control of insulin sensitivity and hyperglycemia. However, because the full length is able to form hexamer/trimers and other combined forms, it is difficult to decipher the role of each molecule on the control of glucose metabolism. This problem can, however, be overcome by the use of a recombinant trimeric-stable form. To address this issue, we used a pharmacological approach and treated high-fat diet (HFD)-fed diabetic mice with recombinant adiponectin globular fragment called gAd110-244 and assessed whole-body glucose and lipid metabolism. In addition, we elucidated some molecular signaling molecules involved in the control of glucose and lipid metabolism.

Materials and Methods

Generation of the tailor-made animal model: the HFD-fed mouse

All experimental procedures were performed according to the National Institutes of Health guidelines for the care and use of laboratory animals. Six-week-old male C57B6/6J mice (Charles River, L’Arbresle, France) were fasted a HFD for 12 wk, as previously described (14, 15). Five mice per cage were kept on an 0800–2000 h inverted dark to light cycle. At completion of this feeding period, body weight gain and ip glucose tolerance were determined to define a group of mice with similar excessive body weight gain and glucose tolerance status. The selection criteria were determined as follows. An ip injection of glucose (1 g/kg body weight) was given to 6-h-fasted mice. Blood glucose levels were monitored from the tip of the tail vein with a glucose meter (Roche Diagnostics, Meyland, France) from a 3.5-μl sample of tail blood at −30, 0, 30, 60, and 90 min after glucose injection. The area under the curve (AUC) of the glucose tolerance test was calculated for each HFD mouse. The mice characterized by an AUC ranging between the mean +2 SD and the mean +4 SD of the AUC from the normal chow-fed mice were included in the study. Similarly, mice showing body weight greater than the mean +1 SD and lower than the mean +3 SD of the normal chow-fed mice were included.

Production and purification of rgAd110-244

A cDNA encoding the C-terminal part of human adiponectin (Val110-Asn244 fragment) was introduced into the pET30a expression vector (Novagen, Madison, WI) to produce the recombinant globular domain of adiponectin (rgAd110-244). The resulting construct was used to transform Escherichia coli BL21 (DE3) (Novagen). Expression of rgAd110-244 was induced by the addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside into the culture medium and 3 h incubation after induction. Under these conditions, rgAd110-244 was expressed as insoluble aggregates. Inclusion bodies were isolated, solubilized, and the recovered rgAd110-244 purified by size exclusion chromatography under denaturing conditions. After analysis by SDS-PAGE under reducing conditions and staining by Coomassie blue, the fractions containing rgAd110-244 with purity higher than 95% were pooled. After renaturing, the trimeric rgAd110-244 was purified by gel filtration on a Superdex 200 column (Pharmacia, Uppsala, Sweden) and submitted to quality control analyses including purity determination, N-terminal sequencing, total amino acid composition, HPLC, and mass spectrometry.

Epinephrine-induced hyperglycemia in mice

All experimental procedures were performed according to the Cantonal Veterinary Office (Geneva, Switzerland) guidelines for the care and use of laboratory animals. Eight-week-old C57BL/6 mice (Elevage Janvier, Le Genest Saint Isle, France) were fasted for 4 h (n = 4–8). They received rgAd110-244 (1, 3, or 10 μg/kg, sc) 60 min, 30 min, and immediately before the challenge with epinephrine hydrochloride (Sigma-Aldrich, Buchs, Switzerland; 0.2 mg/kg, sc). This allowed obtaining a circulating pharmacological enrichment of the peptide in the mouse blood. Control animals received the vehicle (PBS containing 0.2% BSA). Thirty minutes after the challenge, the mice were anesthetized with isoflurane. Blood was sampled by retroorbital puncture using heparinized microcapillaries and kept on ice until glucose determination using a glucometer (model DEX 2, Intrset reference 5893; Bayer AG, Zurich, Switzerland).

Treatments

The selected diabetic obese mice underwent an acute or chronic treatment with rgAd110-244. In the acute treatment protocol, rgAd110-244 was infused within a 10-fold dose range of 0.12 and 0.4 and 1.2 μg/kg · min into the femoral vein. These concentrations were determined from previous sets of experiments that showed that a single sc injection of 1 mg/kg rgAd110-244 in mice 2 h before a lipopolysaccharide challenge significantly lowered the circulating concentration of TNF-α (data not shown). Therefore, based on this estimated efficacy and the importance of the delivery route in the present protocol, i.e., iv vs. sc, we assessed the effect of rgAd110-244 on glucose metabolism over a 10-fold range. These mice were compared with controls receiving an infusion of vehicle [0.2% BSA in PBS (pH 7.4)] or metformin (5 μg/kg · min), considered as a positive control.
For the chronic treatment protocol, the selected mice were distributed into five groups homogeneous for their body weight, body weight gain and glucose tolerance status. In the first three groups, three doses of rAd110-244 were assessed [10, 30, and 100 μg/kg], diluted in 0.2% BSA in PBS (pH 7.4)]. Animals were treated daily with a single sc injection at 0700 h, i.e. before the light was turned off. In the fourth group, the mice were treated with the buffer only and considered as the control group. In the fifth group of mice, metformin was used as the positive control (300 mg/kg · d).

During the first 3 wk of treatment, body weight gain, fasting glycemia, and glucose tolerance were determined. Ten days later, while the mice were still being treated, an indwelling iv catheter was placed in the left femoral vein of each animal. The treatments were continued for 5 more days to allow full recovery from the surgery.

Body weight follow-up

Mice were weighed twice weekly at 0800 h for 3 wk. On the last week of treatment, body weight was monitored the day of the surgery and the day of the infusions. Mice that did not recover from their presurgical body weight were discarded.

In vivo glucose use rate

To determine the rate of whole-body glucose use, an indwelling catheter was introduced into the femoral vein under anesthesia, sealed under the back skin, and glued onto the top of the skull, as previously described (16). The mice were allowed to recover for 4–6 d, 6 h before the assays. The whole-body glucose use rate was determined in basal and hyperinsulinemic-euglycemic conditions. In the basal state, 2-[3,3H]glucose (HPLC purified; NEN Life Science Products, Boston, MA) was continuously infused through the femoral vein at a rate of 10 μCi · kg⁻¹ · min⁻¹ for 3 h. Under hyperinsulinemic conditions, insulin was infused at a rate of 18 mU/kg · min⁻¹ for 3 h, and 2-[3,3H]glucose was infused at a rate of 30 μCi/kg · min⁻¹ to ensure a detectable plasma 2-[3,3H]glucose enrichment.

Through-out the infusion, blood glucose was assessed with a blood glucose meter from blood samples (3.5 μl) collected from the tip of the tail vein when needed. Euglycemia was maintained by periodically adjusting a variable infusion of 10% glucose.

Calculations

Calculations for glucose turnover measurements were made from parameters obtained during the last 60 min of the infusions in steady-state condition. Briefly, the 2-[3,3H]glucose-specific activity was calculated by dividing the 2-[3,3H]glucose enrichment by the plasma glucose concentration. The whole-body glucose turnover rate was calculated by dividing the rate of 2-[3,3H]glucose by the 2-[3,3H]glucose plasma-specific activity. For each mouse, the mean values were calculated and averaged with values from mice of the same group. Mice showing variations of the steady-state 2-[3,3H]glucose-specific activity greater than 15% during this time period were excluded from the study.

The whole-body glycolysis rate was determined by assessing the amount of tritiated water accumulated in the blood during the 2-[3,3H]glucose infusion. Whole-body glycogen synthesis was calculated as the difference between whole-body glucose turnover and whole-body glycolysis rate.

Blood parameters

Plasma glucose concentrations were determined using a glucose oxidase method (Trinder kit; Sigma Diagnostic, St. Louis, MO). Plasma insulin was determined by ELISA (Mercodia, Uppsala, Sweden). Plasma free fatty acid (FFA) concentrations were determined by an enzymatic colorimetric reaction using the enzymes acyl-CoA synthase and acyl-CoA dehydrogenase (NEFA C; Wako, Neuss, Germany).

Statistical analyses

The statistical significance of differences was analyzed by one-way ANOVA or two-way ANOVA (oral glucose tolerance test studies) followed by post hoc Bonferroni for multiple com-
Results

Protein purification and characterization

The expression of the recombinant *Escherichia coli* rgAd110-244 in insoluble inclusion bodies corresponded to 71.5% of the total inclusion body mass. After purification and refolding, about 5% of the total rgAd110-244 was obtained as soluble trimers. The final purity of rgAd110-244 measured by SDS-PAGE and analytical HPLC was 98%. Accurate protein concentration determination and amino acid composition of the rgAd110-244 was obtained by amino acid analysis. N-terminal analysis of the protein showed heterogeneity, with 65% of methoxylated rgAd110-244 and 35% of the molecules having the predicted N terminus starting at Val 110 (m/z = 15,800 and 15,682.6, respectively). The rgAd110-244 used in this study was tested for its endotoxin content, which was 3.3 EU/mg (QCL-BioWiththaker, Walkersville, MD).

Epinephrine-induced hyperglycemia in mice

Epinephrine (0.2 mg/kg, sc) increased blood glucose by 122% (*P < 0.001*). RgAd110-244, administered three times consecutively (60, 30, and 0 min) before the challenge of epinephrine at the doses of 1, 3, and 10 µg/kg (sc), decreased epinephrine-induced hyperglycemia in a dose-dependent manner, i.e. by 36, 42, and 78%, respectively (ED$_{50}$ = 4 µg/kg, sc, Fig. 1). The level of serum insulin was not affected by epinephrine or rgAd110-244 (data not shown).

Induction of diabetes in mice by high-fat feeding

The therapeutic potential of adiponectin has been assessed in HFD-fed mice as previously described (17). After 3 months of high-fat feeding, the cohort of mice was screened for the severity of diabetes by means of an ip glucose tolerance test. The AUC was calculated and body weight recorded. Then mild obese (29–36 g) and glucose-intolerant mice (diabetic index between 7,500 and 10,500) were selected and distributed to have a homogeneous population in each group.

Acute rgAd110-244 treatment controls fasting glycemia and whole-body glucose clearance rate

In this first protocol, continuous iv infusions of rgAd110-244 were performed at 0.4 and 0.12 and 1.2 µg/kg·min into the femoral vein and compared with sa-

line- and metformin-infused mice. The mean glycemia, as calculated between 60 and 120 min after the beginning of the infusions, was significantly reduced for the 1.2 and 0.4 µg/kg·min rgAd110-244 infusions when compared with the vehicle-infused mice (Fig. 2A). No effects were observed for the animals receiving the lowest dose of rgAd110-244 or for those treated with metformin (Fig. 2A). The endogenous glucose production rate was unchanged by the treatments (Fig. 2B). However, because the mean glycemia was different between groups, we calculated the glucose clearance rates and showed that only the highest doses of rgAd110-244 significantly increased the glucose clearance (Fig. 2C).

We next assessed whether an acute rgAd110-244 treatment could influence insulin sensitivity. Therefore, HFD mice were treated with an iv infusion of rgAd110-244 at 0.12, 0.4, or 1.2 µg/kg·min. The data show that rgAd110-244 increased insulin sensitivity in a dose-dependent manner and to a similar range to that observed in metformin-treated mice (Fig. 2D). This was due to an increased glycolytic rate (Fig. 2E), whereas the effect of metformin was due to an increased rate of glycogen synthesis (Fig. 2F).

Acute rgAd110-244 treatment increased insulin-regulated lipid metabolism

Adiponectin is a known regulator of lipid metabolism (10). We next assessed whether the recombinant protein could increase the effect of insulin on lipid clearance. In the same set of HFD mice, we assessed plasma FFA concentrations and calculated the lowering effect of insulin in the absence or presence of adiponectin. In the presence of adi-
Acute rgAd110-244 treatment increases phosphorylated signaling molecules

To delineate some molecular targets of rgAd110-244, we analyzed at completion of the clamp study some signaling molecules involved in the control of muscle energy metabolism. The phosphorylated to dephosphorylated ratios of AKT, NF-κB, GSK-3β, and AMPK were all increased when compared with saline infused mice (Fig. 4). Metformin similarly phosphorylated these proteins, but NF-κB phosphorylation remained specific to rgAd110-244. Conversely, P38 remained unchanged.

Chronic rgAd110-244 treatment controls body weight and fasting glycemia

To assess the therapeutic effect of rgAd110-244, three sets of HFD-fed mice were injected daily for 5 wk with three different doses of the recombinant hormone. In addition, two other sets were given oral metformin as a positive control or vehicle as a negative control. The data show that after the first 3 wk, fasting blood glucose concentrations were reduced in the rgAd110-244-treated mice at the dose of 100 µg/kg (Fig. 5A). The effect was similar to mice treated with metformin. Moreover, the chronic rgAd110-244 treatment significantly controlled whole-body glucose tolerance at the highest dose (Fig. 5, B and C). Body weight was slightly reduced by the highest dose of rgAd110-244 (Fig. 5D).

**Chronic rgAd110-244 treatment controls whole-body insulin sensitivity**

We next assessed whether the effect of rgAd110-244 on fasted glycemia was associated with increased insulin sensitivity. All groups of mice were clamped in hyperinsulinemia and euglycemia. The data show that insulin sensitivity was improved by rgAd110-244 with a dose as low as 10 µg/kg · d. Such improvement of insulin sensitivity was similarly obtained with metformin treatment (Fig. 6A). The glycolytic rate and glycogen synthesis were unchanged by the chronic rgAd110-244 treatments (Fig. 6B).

**Discussion**

The present data show that a chronic treatment with a recombinant rgAd110-244 molecule reduces epinephrine-induced hyperglycemia and improves insulin sensitivity and glucose tolerance of HFD-induced diabetic mice. A similar observation was obtained after a few hours of rgAd110-244 infusion. This mechanism could be due to an increased concentration of signaling molecules involved in energy metabolism.

Numerous studies pointed out the inverse relationship between insulin resistance and the plasma adiponectin concentration (18). Functional analyses including the generation of adiponectin transgenic or knockout mice revealed that adiponectin serves as an insulin-sensitizing adipokine (19). In mouse models of diabetes, such as the HFD-fed mouse, adiponectin production by the adipose tissue is reduced (20). Therefore, we synthesized a recombinant adiponectin-derived pro-
tein, rgAd110-244, and treated HFD-fed mice for 4 wk. Our data show that, unlike full-length adiponectin, our fragment can be easily produced and purified as a trimer from recombinant *E. coli* synthesizing the protein. Moreover, this process is also scalable to ensure a large production.

Using our protein and as expected, insulin resistance was totally reversed by the chronic treatment using a dose as low as 10 µg/kg·d. Both glycolysis and glycogen synthesis rates were also improved. The improved insulin action was, however, not sufficient to mainly impact whole-body glucose metabolism because glucose tolerance was not fully normalized despite a total correction of fasting glycaemia. This could be due to the reduced duration of the
treatment. However, when metformin was used as a positive control for the therapeutic efficacy of rgAd110-244, insulin sensitivity and glucose tolerance were improved, albeit with a larger dose of the drug. Therefore, we could also suggest that the action of our recombinant rgAd110-244 would be restricted to some tissues such as the muscles and the liver.

Adiponectin receptors are present on the plasma membrane of numerous cell types (19, 21). Adiponectin receptor (AdipoR)-1 and -2 serve as receptors for globular and full-length adiponectin and mediate increased AMPK, peroxisome proliferator-activated receptor-α ligand activities, and glucose uptake and fatty-acid oxidation in the muscles. In the liver it has been reported that an acute increase in circulating adiponectin triggers a transient decrease in basal glucose levels by inhibiting both the expression of hepatic gluconeogenic enzymes and the rate of endogenous glucose production in both wild-type mice and a type 2 diabetes mouse model (6, 7). The mechanisms involved the AMP-activated kinase (22). AdipoR1 is ubiquitously expressed, most abundantly in skeletal muscle, whereas AdipoR2 is most abundantly expressed in mouse liver (23). Therefore, because the chronic treatment controlled both fasting glycemia and insulin sensitivity, it is suggested that rgAd110-244 would trigger both muscle AdipoR2 receptors and liver AdipoR1. The blood glucose profiles during the glucose challenge were, however, only mildly controlled by the rgAd110-244 treatment, which suggests that the impaired insulin secretion induced by high-fat feeding was unchanged. Because adiponectin receptors have been described in pancreatic β-cells (24), it might also be suggested that adiponectin could increase insulin secretion, but our molecule would not show this because the glucose profiles obtained during the glucose challenge were not normalized. Importantly, the chronic treatment is most likely clearly associated with a general improvement of glucose metabolism. Therefore, the direct tissue targets of the recombinant rgAd110-244 cannot be determined after a chronic treatment.

To this aim we acutely infused rgAd110-244 in HFD mice and studied insulin action. The data show that a 5-h rgAd110-244 infusion dose-dependently increased whole-body insulin sensitivity. The glycolytic flux was mainly activated, whereas metformin triggered glycogen synthesis. In addition to glucose metabolism, rgAd110-244 dose-dependently increased the lowering effect of insulin on plasma FFA concentrations. This set of data are in agreement with previous reports (25). We show here that the lipid effect is acutely mediated by rgAd110-244 and could contribute to reduce lipotoxicity-induced insulin resistance.

To further analyze the molecular mechanisms related to rgAd110-244 action on insulin sensitivity, we analyzed the skeletal muscles at completion of the insulin and rgAd110-244 infusions. The data show that AKT, GSK-3β, and AMPK were all phosphorylated. Their respective contribution to the increased glucose metabolism cannot be determined but clearly AMPK would be a likely candidate as described elsewhere (26). Surprisingly, GSK-3β phosphorylation state was increased in both rgAd110-244- and metformin-treated mice. However, glycogen synthesis, a target of AMPK, was improved in the metformin-treated group only. This suggests that in addition to AMPK, other mechanisms that directly control glycogen synthesis are involved. Along the same line of surprising data were that NF-κB phosphorylation state was increased, suggesting a state of inflammation and hence insulin resistance. However, we recently described in other instances that the treatment of HFD-fed insulin-resistant mice by estrogens improved insulin sensitivity and also increased the inflammatory tone of adipose tissues and liver (27). Therefore, is some physiological circumstances, both insulin sensitivity and inflammation can coexist. It is noteworthy that when compared with our previous results using commercial adiponectin, (22), the

**FIG. 6.** Effect of chronic rgAd110-244 treatment on glucose fluxes under insulin action. HFD diabetic obese mice (n = 7–8) were infused with rgAd110-244 and metformin at the indicated doses for 5 h. A, The whole body glucose turnover and hepatic glucose production, B. Glycogen synthesis and glycogen synthesis rates (milligrams per kilogram per minute). Results are presented as mean ± st. *, Statistically different from vehicle-treated mice when P < 0.05. White bars, Vehicle (PBS 0.2% BSA), lightgray bars, rgAd110-244 (10 μg/kg · d); dark gray bars, rgAd110-244 (30 μg/kg · d); black bars, rgAd110-244 (100 μg/kg · d); hatched bars, metformin (300 mg/kg · d).
effect of rgAd110-244 on the liver seemed to be indirect in the current study. Basal glucose turnover remained unchanged but the clearance was increased. This was not due to a change in hepatic glucose production but rather to an increased use because the fasting glycemia was reduced after 2 h of infusion. The reason for this difference between our hormone preparation and the commercial molecules remains unknown.

In summary, rgAd110-244 is a recombinant adiponectin fragment that controls glucose and lipid metabolism by triggering muscle insulin sensitivity. This impacts on whole-body glucose metabolism normalizing fasting glycemia.

Acknowledgments

We thank Virginie Ogier and B. Bihain for their constructive discussions.

Address all correspondence and requests for reprints to: Dr. Thierry Sulpice, Physiogenex, Prologue Biotech, Rue Pierre et Marie Curie-BP 28 262, 31682 Labège Innpolop cedex, France. E-mail: t.sulpice@physiogenex.com.

This work was supported by Merck Serono S.A.-Geneva.

Disclosure Summary: T.S. and B.P.-M. are employed by Physiogenex S.A.S. C.M. was previously a postdoctoral fellow (Institut National de la Sante et de la Recherche Médicale). P.-A.V., P.G., and S.A.S. P.C. was previously a postdoctoral fellow (Institut National de la Sante et de la Recherche Médicale). R.B. is professor and research director (Institut National de la Sante et de la Recherche Médicale).

References


Downloaded from https://academic.oup.com/endo/article-abstract/150/10/4493/2455401 by guest on 18 February 2019


The Power of Programming: International Conference on Development Origins of Health and Disease
May 6–8, 2010, Munich, Germany
www.metabolic-programming.org

The conference is jointly organized by the “Early Nutrition Academy,” the “Early Nutrition Programming Project,” the “Developmental Origins of Health and Disease Society.” It will be an opportunity to share the latest results that have come out of the Early Nutrition Programming Project as well as other recent findings on the long-term effects of early nutrition. The conference brings together more than 250 clinicians and scientists from around the world.