Adenovirus-Mediated Adiponectin Expression Augments Skeletal Muscle Insulin Sensitivity in Male Wistar Rats

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In this study, we investigated the chronic in vivo effect of adiponectin on insulin sensitivity and glucose metabolism by overexpressing the adiponectin protein in male Wistar rats using intravenous administration of an adenovirus (Adv-Adipo). Virally infected liver secreted adiponectin as high and low molecular weight complexes. After 7 days of physiological or supraphysiological hyperadiponectinemia, the animals displayed enhanced insulin sensitivity during the glucose tolerance and insulin tolerance tests. Glucose clamp studies performed at submaximal and maximal insulin infusion rates (4 and 25 mU · kg^{-1} · min^{-1}, respectively) also demonstrated increased insulin sensitivity in Adv-Adipo animals, with the insulin-stimulated glucose disposal rate being increased by 20–67%. In contrast, insulin's effect on the suppression of hepatic glucose output and plasma free fatty acid levels was not enhanced in Adv-Adipo rats compared with controls, suggesting that high levels of adiponectin expression in the liver may lead to a local desensitization. Consistent with the clamp data, the activation of AMP-activated protein kinase was significantly enhanced in skeletal muscle (by 50%) but not in liver. One interesting finding was that in male Wistar rats, both AdipoR1 and AdipoR2 expression levels were higher in skeletal muscle than in liver, as it is the case in humans. These results indicate that chronic adiponectin treatment enhances insulin sensitivity and could serve as a therapy for human insulin resistance.
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Insulin resistance characterizes Syndrome X and type 2 diabetes and is defined as a failure of insulin target tissues such as adipose, liver, and skeletal muscle to respond normally to insulin (1). Skeletal muscle accounts for the major component of in vivo insulin-mediated glucose disposal; in insulin-resistant states, insulin stimulation fails to adequately enhance glucose uptake into this tissue (2–4). In the liver, insulin-induced suppression of hepatic glucose output (HGO) is attenuated in most insulin-resistant states. In insulin-resistant adipose tissue, insulin inhibition of lipolysis is impaired. Beyond their role in lipid storage and release, adipocytes can influence whole-body insulin sensitivity through the secretion of adipokines that mediate auto/paracrine and endocrine effects.

It has been proposed that the adipocyte-derived hormone adiponectin, also known as Acrp30, AdipoQ, and GBP28 (5–8), plays an important role in the regulation of energy homeostasis and glucose metabolism. Decreased serum levels of adiponectin are a common feature of obesity in humans and rodents (9–12) and also correlate with lowered indexes of insulin sensitivity (13), leading to the hypothesis that decreased serum adiponectin levels are contributory and not simply diagnostic of in vivo insulin resistance. The striking increase in adiponectin levels in response to treatment with the antidiabetic, insulin-sensitizing agents thiazolidinediones (TZDs) further underscores the potential insulin-sensitizing effects of adiponectin. Pharmacological studies have shown that recombinant, full-length adiponectin suppresses endogenous glucose production by enhancing hepatic insulin sensitivity in mice and primary hepatocytes (14,15). Furthermore, the globular domain of adiponectin can decrease elevated fatty acid levels by increasing fatty acid oxidation in muscle (16,17). Long-term expression of adiponectin using adeno-associated virus vectors has been shown to improve insulin sensitivity through modulation of hepatic gluconeogenesis and lipogenesis in female SD rats with diet-induced obesity (18). Recently, another transgenic model of hyperadiponectinemia was generated by expressing a mutant form of adiponectin (ΔGly-adiponectin) that blocks the degradation of endogenous protein in the secretory pathway of adipocytes (19). This resulted in a net increase in circulating adiponectin levels in the physiological range, which improved glucose tolerance by reducing hepatic glucose production.

Circulating adiponectin exists predominantly as low

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Adv, adenovirus; AMPK, AMP-activated protein kinase; ELISA, enzyme-linked immunosorbent assay; FFA, free fatty acid; GDR, glucose disposal rate; GINF, glucose infusion rate; GTT, glucose tolerance test; HF, high fat; HGO, hepatic glucose output; HMW, high molecular weight; IR, insulin receptor; IRS, insulin receptor substrate; ISGDR, insulin-stimulated GDR; ITT, insulin tolerance test; LMW, low molecular weight; RIA, radioimmunoassay; TZD, thiazolidinedione.

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molecular weight (LMW) hexamers and high molecular weight (HMW) complexes that are believed to possess differing biological activities (20,21). In addition, it has been demonstrated that the ratio between these oligomeric forms (HMW to LMW), not absolute amounts of circulating adiponectin, is critical in determining TZD-mediated improvements in insulin sensitivity (22). Yamauchi et al. (23) cloned two distinct receptors for adiponectin in mouse and human, AdipoR1 and AdipoR2. AdipoR1 is a high-affinity receptor for globular adiponectin and a low-affinity receptor for the full-length ligand; AdipoR2 is an intermediate-affinity receptor for both forms of adiponectin. In mice, AdipoR1 is abundantly expressed in skeletal muscle, whereas AdipoR2 is predominantly found in the liver. In humans, AdipoR1 and AdipoR2 expression levels are higher in skeletal muscle than in liver (online supplement of ref. 23), suggesting that the tissue distribution of adiponectin receptors varies among animal species. Recently, another receptor for the hexameric and HMW species of adiponectin has been identified, T-cadherin, a glycosylphosphatidylinositol-anchored protein of unknown cellular functions expressed in endothelial and smooth muscle cells (24). To elicit its cellular actions, adiponectin likely binds to these cell surface receptors, triggering signaling cascades that culminate in improved insulin action and/or sensitivity. Studies have shown that adiponectin can augment fatty acid oxidation and AMP-activated protein kinase (AMPK) as well as coenzyme A carboxylase activity (25). However, the mechanisms of action of this adipokine in vivo, especially in liver and skeletal muscle, remain to be fully elucidated.

Because the complete physiological role of adiponectin and its chronic actions in vivo are still unclear, we investigated the role of adiponectin in insulin and glucose metabolism using an adenovirus to chronically overexpress adiponectin in male Wistar rats. Our results show that elevated levels of circulating adiponectin enhance glucose disposal in skeletal muscle, likely because of an increase in AMPK activity.

RESEARCH DESIGN AND METHODS

Subcloning of the mouse adiponectin cDNA by RT-PCR. The mouse full-length adiponectin cDNA (765 bp) was amplified from mouse adipose tissue using the 5′-CAGGATGCTACTGTTGCAAGC-3′ sense and 5′-TGGGTAGTTGCAGTCAGTTGG-3′ antisense primers, subcloned into the pCR2.1-TOPO (Invitrogen, Carlsbad, CA) and sequenced, confirming that the clones corresponded to the mouse adiponectin (GenBank accession number AF304466).

Construction of recombinant adenoviruses. The adiponectin adenovirus (Adv-Adipo) and lacZ adenovirus (Adv-LacZ) viruses were generated and purified using a previously described protocol (26).

Animal studies. Wistar rats (age 8 weeks; Harlan, Indianapolis, IN) were housed individually under controlled light/dark (12/12 h) and temperature conditions and had free access to water and a standard rat diet. They were injected intravenously from the tail with 0.4×10^9 (low dosage) or 1×10^9 (high dosage) plaque-forming units of adiponectin or lacZ viruses per rat. All procedures were performed in accordance with the Guide for Care and Use of Laboratory Animals of the National Institutes of Health and were approved by the Animal Subjects Committee of the University of California, San Diego.

The rats were implanted with three catheters (Micro-Renathane MRE-033, 0.033 cm in OD and 0.014 cm in ID; Braintree Scientific, Braintree, MA) on day 3 after the adenovirus injection, as previously described (26). The animals were given 4 days to fully recover from the surgery. On postinjection day 7, insulin sensitivity was assessed using a glucose tolerance test (GTT), insulin tolerance test (ITT), or euglycemic-hyperinsulinemic clamp. The rats were fasted for 6 h before the start of any experiment.

For the GTT, the rats were injected with 2 g/kg body wt of dextrose (50% dextrose; Abbott, Chicago, IL) in the jugular vein. Blood samples were collected at $t = 0$, 15, 30, 60, 90, and 120 min from the carotid artery. For the ITT, the rats were injected with 0.75 units/kg body wt of human insulin (Novolin R; Novo Nordisk, Copenhagen, Denmark). Blood samples were collected after 0, 15, 30, and 60 min.

The euglycemic-hyperinsulinemic clamp experiments began with a priming injection (2.5 μCi/0.5 ml) and constant infusion (0.04 μCi/min) of D-[3-^{3}H]glucose (Du Pont-NEN, Boston, MA). After 60 min of tracer equilibration and basal sampling at $t = -10$ and 0, glucose (50% dextrose, variable infusion; Abbott) and tracer (0.12 μCi/min) plus insulin (4 or 25 mU · kg^{-1} · min^{-1}; Novolin R; Novo Nordisk) were infused into the jugular vein, as previously described (26).

Small blood samples (60 μl) were drawn at 10-min intervals and immediately analyzed for glucose (YSI 2300 Glucose Analyzer; Yellow Springs Instruments, Yellow Springs, OH) to maintain the integrity of the glucose clamp throughout the duration of the experiment. Blood samples were taken at $t = -60$ (start of experiment), −10, 0 (basal), 110, and 120 (end of experiment) min to determine glucose-specific activity and insulin, free fatty acid (FFA), and adiponectin content. To ensure accuracy, basal and terminal sampling was performed twice at 10-min intervals. We confirmed that steady-state conditions were achieved at the end of the clamp before obtaining the terminal blood specimen by measuring blood glucose every 10 min and ensuring that steady state for glucose infusion and plasma glucose levels was maintained for a minimum of 20 min before final sampling. We defined a steady-state blood glucose concentration as one where the glucose concentration and infusion rate fluctuated by ≤3 mg/dl and ≤5%, respectively, over 10 min. All blood samples were immediately centrifuged, and plasma was stored at −80°C for subsequent analysis. After terminal blood sampling at 120 min, animals were promptly killed with pentobarbital (180 mg/kg). Tissues were taken and immediately frozen in liquid nitrogen and stored at −80°C for subsequent metabolic analysis.

High-fat feeding study. Male Wistar rats (age 6 weeks) were fed a high-fat (HF) diet (DYeT#101582; Dyets, Bethlehem, PA) for 4 weeks (for a complete description of the diet, see 27). After 3 weeks on the diet, animals were injected with the indicated adenovirus; then 1 week later, the euglycemic-hyperinsulinemic clamp experiments were performed as detailed above.

Analytic procedures. Plasma glucose was assayed by the glucose oxidase method (Yellow Springs). Basal plasma insulin was quantified using a rat insulin enzyme-linked immunosorbent assay (ELISA) kit (Crystal Chem, Chicago, IL), and insulin values during the clamp studies were measured with a human-specific insulin radioimmunoassay (RIA) kit (Linco Research, St. Charles, MO). Plasma glucose-specific activity was measured in duplicate after zinc sulfate and barium hydroxide deproteinization. Plasma FFA, leptin, resistin, and adiponectin levels were determined using the nonesterified fatty acid C-test (Wako Pure Chemical, Richmond, VA); rat leptin enzyme immunoassay kit (Assay Designs, Ann Arbor, MI); mouse resistin RIA kit (Linco Research), which cross-reacts with rat resistin; and mouse/rat adiponectin ELISA kit (B-Bridge, San Jose, CA), respectively. Serum alanine aminotransferase and aspartate aminotransferase levels were enzymatically measured with a commercial kit (Sigma, St. Louis, MO).

Red quadriceps muscle and liver tissue used for metabolic analysis were taken from the animals immediately after they were killed, rinsed several times in cold saline to remove blood, frozen in liquid nitrogen, and stored at −80°C. Care was taken to avoid taking sections of the liver lobes containing large blood vessels. Tissues were homogenized in buffer containing phosphatase and protease inhibitors. After a 10-min incubation, the lysates were clarified by centrifugation (10,000*g* at 4°C), quantified for total protein, and analyzed by immunoblotting using AMPK-α and phospho-AMPK-α (Thr172) antibodies, as previously described (26).

Analysis of the oligomeric distribution of adiponectin in Adv-Adipo and Adv-LacZ rats. Fractionation and quantification of HMW and LMW adiponectin forms in plasma and liver extracts were performed as previously described (20). Briefly, plasma or liver extract from Adv-LacZ and Adv-Adipo animals was diluted 1:10 in 10 mmol/l HEPES (pH 8.0) and 125 mmol/l NaCl, layered onto 5–20% sucrose step gradients, and spun. Then 150-μl fractions were sequentially retrieved from the top of the gradient and analyzed by Western blotting using an antibody against the murine NH_2-terminal hypervariable region of adiponectin (EDDVTTTEELAPALV) (15). This antibody is specific for mouse adiponectin and does not cross-react with rat adiponectin. Blots were decorated with an ^{125}I-derivatized, secondary goat anti-rabbit antibody (Amersham, Piscataway, NJ) and analyzed with a PhosphorImager (Amersham). Fractions 4–6 (hexamers) and 9–11 (HMW adiponectin) from velocity sedimentation were quantitated with ImageQuant software.

Analysis of AdipoR1 and AdipoR2 mRNA distribution by Northern blot analysis. Northern blot analysis of red quadriceps muscle and liver tissue was performed with 20 μg of total RNA, as previously described (28). RNA-containing membranes were probed with mouse AdipoR1 and AdipoR2 cDNA

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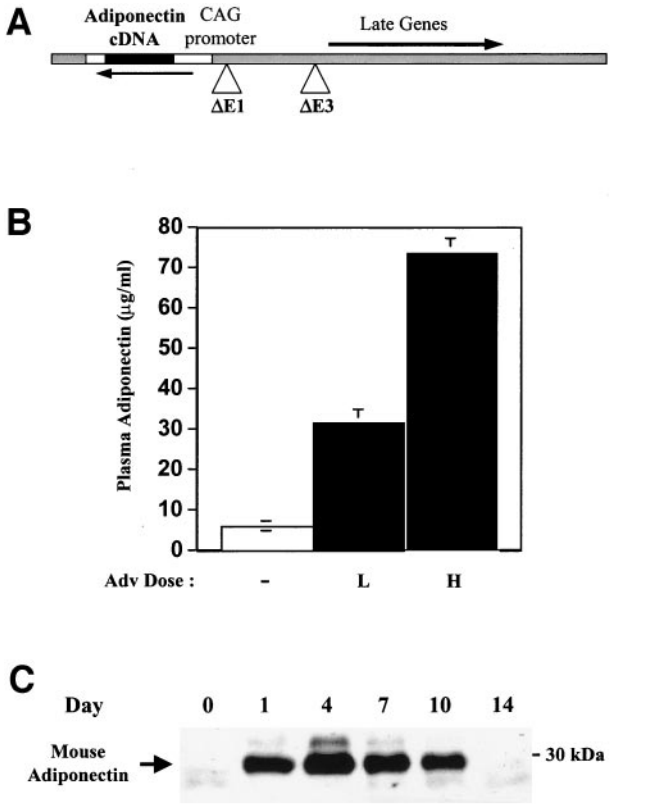

FIG. 1. Adenoviral transduction of mouse adiponectin. *A*: Structure of Adv encoding mouse adiponectin. *B*: Plasma adiponectin levels on postinjection day 7 by ELISA. L, low dosage; H, high dosage; □, Adv-LacZ (n = 17); ■, Adv-Adipo (low dosage, n = 2; high dosage, n = 18). Data are means ± SE. *C*: Representative time course of plasma mouse adiponectin in Adv-Adipo rats (high dosage) as analyzed by SDS-PAGE and immunoblotting.

probes generated by RT-PCR using mouse skeletal muscle and liver total RNA and the following primers: 5′-CCAGCCAGATGTCTTCCCACAAAG-3′ and 5′-CTCAGAGAAGGGAGTCGTCGGTAC-3′ (AdipoR1) and 5′-GAAGGGTTTATGGG CATGTCCCCG-3′ and 5′-CCTGGAGAGTATCACAGTGCATCC-3′ (AdipoR2) (GenBank accession numbers BC014875 and XM_132831, respectively).

Calculations. HGO and the glucose disposal rate (GDR) were calculated for the basal period and steady-state portion of the glucose clamp using the Steele equation for steady-state conditions (29). The insulin-stimulated GDR (ISGDR) reflects the ability of insulin to increase the GDR above the basal value and was calculated by subtracting each animal's basal HGO value from the final GDR achieved at the end of the clamp period. Data calculation and statistical analysis were performed using the StatView program (Abacus Concepts, Berkley, CA). Data are means ± SE. Statistical significance was tested with repeated measures using ANOVA. Statistical significance was defined as $P < 0.05$.

RESULTS

Plasma adiponectin levels in Adv-Adipo rats. To study the chronic effects of adiponectin in vivo, we overexpressed the adiponectin protein by injecting male Wistar rats with Adv-Adipo or control Adv-LacZ. To accomplish this, a replication-defective Adv expressing the mouse adiponectin cDNA from a CAG promoter was constructed (Fig. 1*A*). Previous studies have shown that intravenously administered adenoviruses are targeted to the liver, with >90% of hepatocytes expressing virally encoded proteins (26,30–32). We therefore performed X-gal staining of liver sections from Adv-LacZ rats; microscopy analysis demonstrated that the β-galactosidase enzyme was expressed in >90% of hepatocytes (data not shown).

To confirm adiponectin protein expression in vivo, adiponectin levels were measured from plasma samples obtained on postinjection day 7 using a mouse/rat adiponectin ELISA kit. A low and high dosage of injected Adv increased the expression of adiponectin by ~3- and 10-fold, respectively (Fig. 1*B*). Adv-Adipo–infected liver cells thus secreted high levels of adiponectin into the circulation, reaching physiological and supraphysiological concentrations.

To determine the duration of adiponectin protein expression, plasma samples were collected at multiple time points after Adv injection. Figure 1*C* shows the time course of adiponectin content in the plasma of Adv-Adipo rats. Plasma levels increased fairly rapidly on day 1, were sustained until day 7, and declined gradually from day 7 to day 14. To elicit the chronically physiological function of adiponectin, we therefore conducted all subsequent in vivo experiments on postinjection day 7.

Overall animal characteristics. Table 1 illustrates some of the general characteristics of the Adv-Adipo and control Adv-LacZ animals in the basal state on postinjection day 7. Liver function was normal in both groups, with aspartate aminotransferase and alanine aminotransferase values being within the normal range, indicating an absence of hepatic toxicity induced by the Adv injection. Body weights were not different between the control and experimental groups (292.2 ± 4.8 vs. 289.4 ± 4.4 g). In the fasting state, glucose, insulin, FFA, resistin, and leptin levels were the same between the two groups.

GTT and ITT. On postinjection day 7, GTTs were performed on 6-h–fasted rats injected with glucose (2 g/kg body wt) into the jugular vein. As seen in Fig. 2*A*, the fasting and GTT glucose levels showed no significant difference between the Adv-LacZ and Adv-Adipo (high viral dosage) groups. Fasting insulin levels also showed no significant difference between the two groups (Fig. 2*B*). However, after the glucose load, the insulin levels were significantly lower in the Adv-Adipo animals (50–60%) after 15 and 30 min ($P < 0.05$), suggesting enhanced insulin action in hyperadiponectinemic animals.

ITTs (0.75 units/kg body wt) performed on 6-h–fasted rats indicated increased overall insulin sensitivity. Thus, the glucose-lowering effect of insulin was significantly increased in Adv-Adipo (high viral dosage) rats compared with Adv-LacZ controls at 30, 45, and 60 min ($P < 0.05$) (Fig. 2*C*).

Euglycemic-hyperinsulinemic clamp studies. To directly examine the metabolic impact of adiponectin overexpression on insulin sensitivity, we next subjected both groups of rats to euglycemic-hyperinsulinemic clamps at maximal and submaximal (25 mU and 4 mU · kg^{-1} · min^{-1}) insulin infusion rates. Steady-state glucose and insulin levels during the clamp studies were similar in the two groups (Table 1). During these studies, we measured insulin stimulation of whole-body GDR, suppression of HGO, and inhibition of FFA release into the circulation. The glucose infusion rate (GINF) required to maintain euglycemia was increased by 15 and 25% at maximal and submaximal insulin stimulation in Adv-Adipo rats, respectively, showing enhanced overall insulin sensitivity (Fig. 3*A*). To assess the insulin-stimulated component of glucose disposal, the ISGDR was calculated. As seen in Fig.

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TABLE 1
Plasma measurements in the basal state and during euglycemic-hyperinsulinemic clamps

	Normal diet		HF diet	
	Adv-LacZ	Adv-Adipo	Adv-LacZ	Adv-Adipo
Body weight (g)	292.2 ± 4.8	289.4 ± 4.4	384.1 ± 12.8	402.1 ± 11.8
Basal (*n*)	17	18	9	9
Glucose (mg/dl)	129.7 ± 3.5	124.6 ± 4.9	133.9 ± 4.7	129.6 ± 4.0
Insulin (ng/ml)	0.73 ± 0.05	0.75 ± 0.04	1.07 ± 0.06*	0.84 ± 0.06
FFA (mmol/l)	1.09 ± 0.14	1.17 ± 0.09	1.19 ± 0.16	1.25 ± 0.12
Resistin (ng/ml)	3.5 ± 0.3	3.0 ± 0.4	ND	ND
Leptin (ng/ml)	7.7 ± 3.1	8.1 ± 1.8	ND	ND
Liver function				
Aspartate aminotransferase (IU)	35.4 ± 1.5	32.4 ± 0.7	56.4 ± 2.5	54.8 ± 3.8
Alanine aminotransferase (IU)	38.2 ± 1.3	35.2 ± 0.8	44.4 ± 2.4	44.0 ± 2.5
Clamp				
Maximal insulin (*n*)	10	10	9	9
Glucose (mg/dl)	151.1 ± 0.6	151.0 ± 0.9	148.8 ± 1.2	151.1 ± 1.3
Insulin (ng/ml)	25.22 ± 1.10	24.77 ± 1.09	24.32 ± 3.27	24.45 ± 0.90
FFA (mmol/l)	0.08 ± 0.02	0.07 ± 0.02	0.08 ± 0.01	0.08 ± 0.01
Submaximal insulin (*n*)	7	8	—	—
Glucose (mg/dl)	150.3 ± 1.6	151.4 ± 1.9	ND	ND
Insulin (ng/ml)	3.52 ± 0.16	3.49 ± 0.16	ND	ND
FFA (mmol/l)	0.38 ± 0.06	0.32 ± 0.05	ND	ND

Data are means ± SE. *$P < 0.05$. ND, not determined.

3*A*, the ISGDR (ISGDR = total GDR − basal HGO value) was increased by 20%, from 43.8 ± 2.5 to 52.7 ± 1.0 mg · kg^{-1} · min^{-1} ($P < 0.01$), at maximal insulin stimulation and by 67%, from 9.9 ± 1.6 to 14.3 ± 0.7 mg · kg^{-1} · min^{-1} ($P < 0.05$), at submaximal insulin stimulation in Adv-LacZ and Adv-Adipo rats, respectively. Similarly, the GINF and ISGDR increased by 18 and 24%, respectively, at maximal insulin stimulation in low-dosage Adv-Adipo−injected rats ($P < 0.01$) (Fig. 3*A*), confirming that adiponectin also enhances glucose metabolism at near physiological concentrations in male rats. Because skeletal muscle accounts for the great majority of ISGDR, these results indicate that adiponectin enhances insulin sensitivity in skeletal muscle.

Basal HGO values were not different between Adv-LacZ and Adv-Adipo animals (15.2 ± 1.4 and 14.7 ± 0.8 mg · kg^{-1} · min^{-1}) (Fig. 3*B*). During the clamp studies, insulin inhibition of HGO was unexpectedly not enhanced in the Adv-Adipo group compared with the control group at either maximal (3.8 ± 0.5 vs. 4.0 ± 0.4 mg · kg^{-1} · min^{-1}) or submaximal (7.4 ± 0.4 mg vs. 7.9 ± 0.5 mg · kg^{-1} · min^{-1}) insulin infusion rates.

Plasma FFA levels in the basal state and during the euglycemic-hyperinsulinemic clamp. To assess whether adiponectin overexpression affected adipose tissue, plasma FFA levels were measured (Fig. 3*C*). In the basal state and during the euglycemic-hyperinsulinemic clamp, plasma FFA levels were not significantly different between the two groups.

HF-feeding studies. We also studied Adv-LacZ and Adv-Adipo animals on an HF diet fed for 4 weeks to assess the potential protective effects of overexpressed adiponectin on the development of insulin resistance. In the basal state in Adv-LacZ animals, HF feeding led to a 33% increase in body weight, 30% increase in fasting insulin levels, no

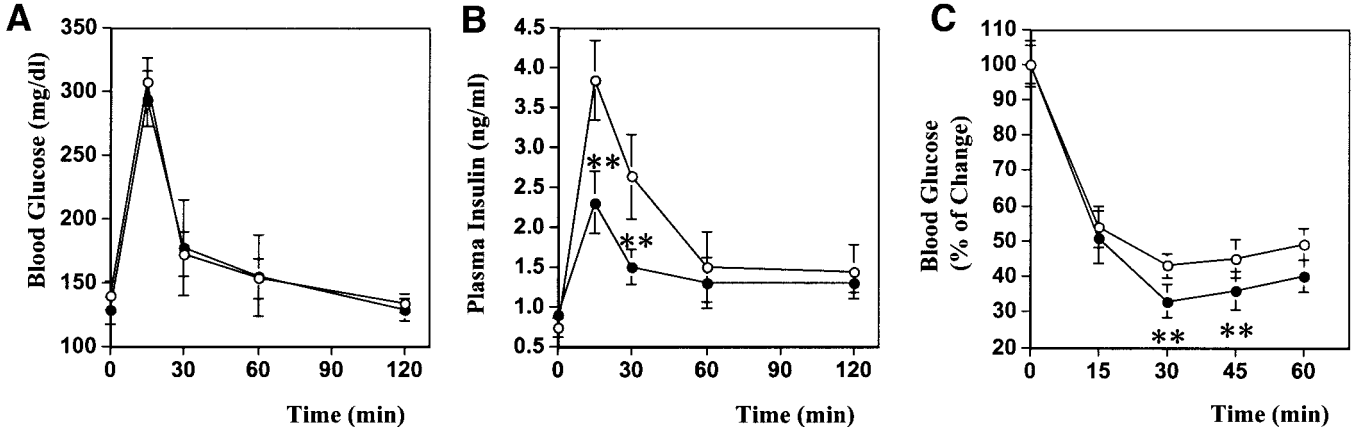

FIG. 2. GTT and ITT in male Wistar rats on postinjection day 7. Glucose (*A*) and insulin (*B*) curves from the GTT in Adv-LacZ (□; $n = 8$) and Adv-Adipo (■; $n = 8$) rats injected with 2 g/kg body wt of glucose after a 6-h fast. *C*: Glucose curves from the ITT in Adv-LacZ (□; $n = 8$) and Adv-Adipo (■; $n = 8$) rats injected with 0.75 units/kg body wt of human insulin after a 6-h fast. Plasma glucose levels were normalized to those at $t = 0$ min in each group (100%). Data are means ± SE. **$P < 0.05$.

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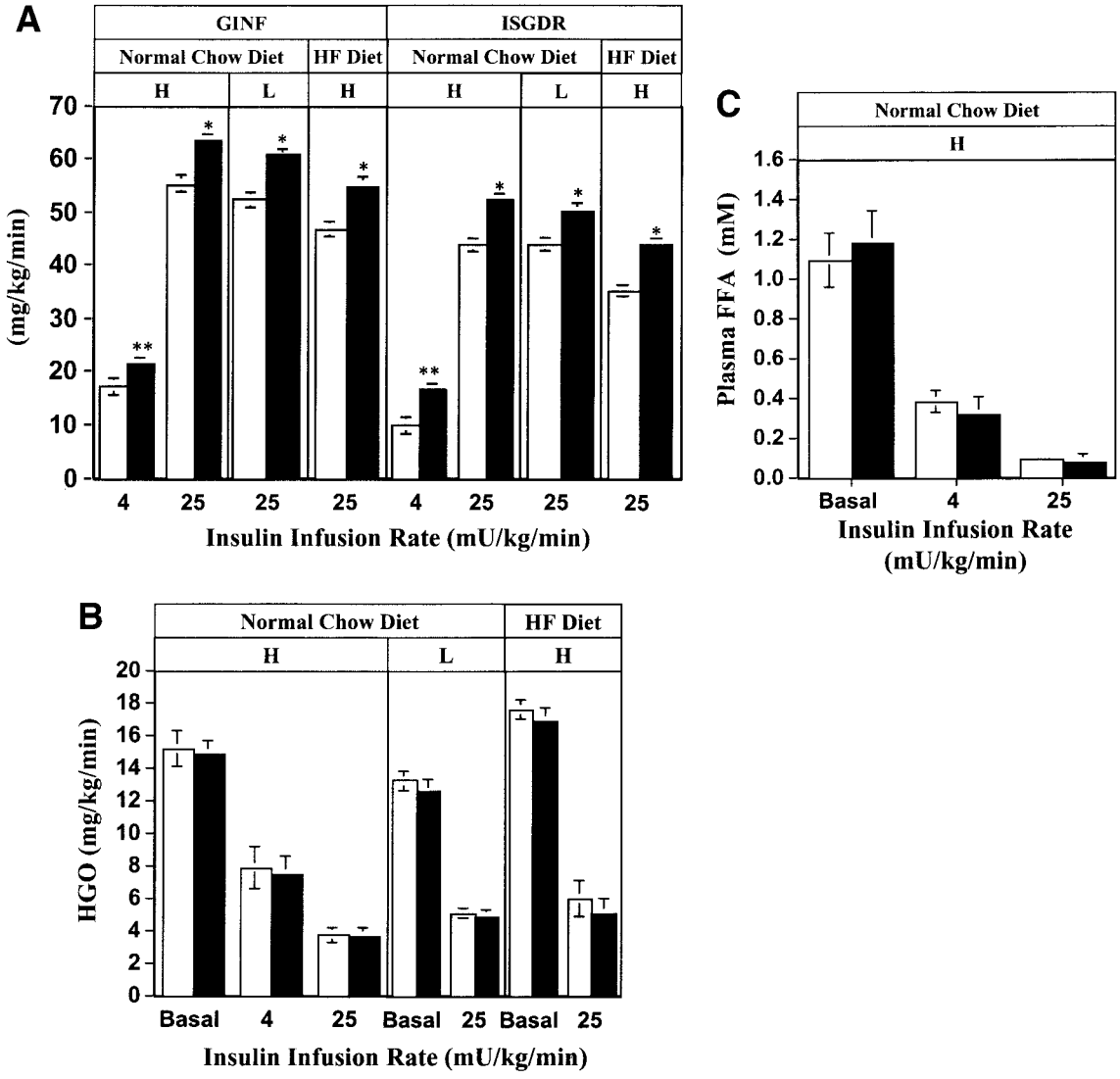

FIG. 3. GINF and ISGDR, HGO, and plasma FFA concentrations during euglycemic-hyperinsulinemic clamps on postinjection day 7 in rats given a high or low viral dosage and fed a normal or HF diet. *A*: GINF and ISGDR at 4 or 25 mU · kg^{-1} · min^{-1} insulin infusion rates. □, Adv-LacZ rats (n = 7, 10, 6, and 9, respectively); ■, Adv-Adipo rats (n = 8, 10, 6, and 9, respectively). *B*: Basal and hyperinsulinemic clamp HGO at 4 or 25 mU · kg^{-1} · min^{-1} insulin infusion rates. □, Adv-LacZ rats (n = 17, 7, 10, 6, 6, 9, and 9, respectively); ■, Adv-Adipo rats (n = 18, 8, 10, 6, 6, 9, and 9, respectively). *C*: Plasma FFA levels in the basal state and during the hyperinsulinemic clamp at 4 or 25 mU · kg^{-1} · min^{-1} insulin infusion rates. □, Adv-LacZ rats (n = 17, 7, and 10, respectively); ■, Adv-Adipo rats (n = 18, 8, and 10, respectively). Data are means ± SE. H, high viral dosage; L, low viral dosage. *P < 0.01, **P < 0.05.

change in fasting plasma glucose or FFA levels (Table 1), and a 14% increase in HGO (17.6 ± 0.6 mg · kg^{-1} · min^{-1}; $P < 0.05$) (Fig. 3*B*) compared with Adv-LacZ animals on a normal diet. When euglycemic-hyperinsulinemic clamp studies were performed in HF-fed Adv-LacZ animals, the GINF required to maintain euglycemia and the ISGDR were decreased by 18 and 24% (35.3 ± 0.7 mg · kg^{-1} · min^{-1}; $P < 0.01$) (Fig. 3*A*), respectively, compared with the Adv-LacZ animals fed a normal diet (Fig. 3*A*). In addition, insulin's ability to suppress HGO was diminished from 75 to 66% ($P < 0.05$), consistent with the HF diet–induced insulin-resistant state. In the Adv-Adipo animals, the HF diet also led to a 33% increase in body weight, with no change in fasting glucose or circulating FFA levels (Table 1). Compared with the Adv-LacZ rats, the effect of the HF diet in elevating basal insulin levels was attenuated (Table 1). As seen in the clamp studies (Fig. 3*A*), GINF and ISGDR were higher by 18 ($P < 0.01$) and 22% (43.2 ± 2.0 mg · kg^{-1} · min^{-1}; $P < 0.01$), respectively, in the Adv-Adipo compared with Adv-LacZ rats, whereas insulin suppression of HGO was the same between the two groups. The GINF and ISGDR values in the HF-fed Adv-Adipo animals were comparable to the values in normal diet–fed control animals (Fig. 3*A*), demonstrating that hyperadiponectinemia prevented the HF diet–induced insulin-resistant state.

Insulin signaling studies. To assess the potential cellular mechanisms of the chronic adiponectin-induced increase in insulin sensitivity, we obtained skeletal muscle and liver tissue samples at the end of the maximal euglycemic-hyperinsulinemic clamp from rats on the normal diet. These samples, which represent the fully insulinized state at the termination of the glucose clamp study, were homogenized and the protein lysates were subjected to immunoblotting.

We first determined whether adiponectin affects activa-

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tion of upstream insulin-signaling components by measuring insulin receptor (IR) and insulin receptor substrate-1 (IRS-1) protein levels and phosphorylation status in skeletal muscle and liver samples. Chronic hyperadiponectinemia did not alter IR or IRS-1 protein levels or tyrosine phosphorylation in response to insulin in either tissue (data not shown). We also measured Akt phosphorylation (Ser473) in skeletal muscle and liver samples obtained from rats in the basal state and at the end of the maximal euglycemic-hyperinsulinemic experiments. Insulin led to a marked stimulation of Akt phosphorylation in skeletal muscle and liver in both Adv-LacZ and Adv-Adipo animals (data not shown). These results suggest that chronic hyperadiponectinemia augments skeletal muscle insulin sensitivity via a pathway that is independent of early IR-mediated signaling events.

Adiponectin increases AMPK activation in response to insulin in skeletal muscle. Because adiponectin can increase AMPK activity (25), we assessed whether adiponectin overexpression alters AMPK activity by measuring phospho-AMPK (Thr172) and total AMPK protein in skeletal muscle and liver samples obtained at the end of the clamp studies from normal diet–fed rats. As illustrated in Fig. 4, AMPK phosphorylation was increased by 58% ($P < 0.05$) in Adv-Adipo skeletal muscle compared with in control animals, whereas total AMPK protein content remained unchanged. In contrast, AMPK phosphorylation and protein content were unchanged in liver. These data are consistent with the results from euglycemic-hyperinsulinemic clamp experiments demonstrating that skeletal muscle is a target tissue of adiponectin.

Oligomeric distribution of plasma adiponectin in Adv-Adipo and Adv-LacZ rats. To further understand the effects of overexpressed adiponectin, we measured the oligomeric distribution of circulating adiponectin in the Adv-Adipo animals. Adiponectin forms LMW and HMW complexes consisting of 6 or 12–18 subunits, as assessed by gel filtration or velocity gradient studies; these species are thought to possess differing and possibly tissue-specific biological activities (20,21). To determine the nature of the oligomers formed by adenovirus-encoded adiponectin in normal diet–fed Adv-Adipo rats, serum and intrahepatic HMW and LMW adiponectin content was quantified in velocity sedimentation experiments using sucrose step gradients, ultracentrifugation, and immunoblotting (see RESEARCH DESIGN AND METHODS). As illustrated in Fig. 5*A*, adiponectin protein produced from Adv-Adipo in hepatocytes circulates in the form of HMW as well as LMW oligomers, as in the case of adipocyte-derived adiponectin (19). No mouse adiponectin was detected in Adv-LacZ control rats. Quantification of HMW/total adiponectin revealed that ~27% of adiponectin of hepatic origin circulated as HMW complexes in low-dosage Adv-Adipo animals (Fig. 5*B*), a level that is comparable with the proportion of adipocyte-derived HMW adiponectin in rodents and humans (19,20). This distribution was dosage dependent, as ~41% of adiponectin from the liver circulated as HMW forms in high-dosage Adv-Adipo animals. Measurement of the intrahepatic adiponectin oligomer content indicated that ~29% of intracellular adiponectin was in the HMW form (Fig. 5*B*).

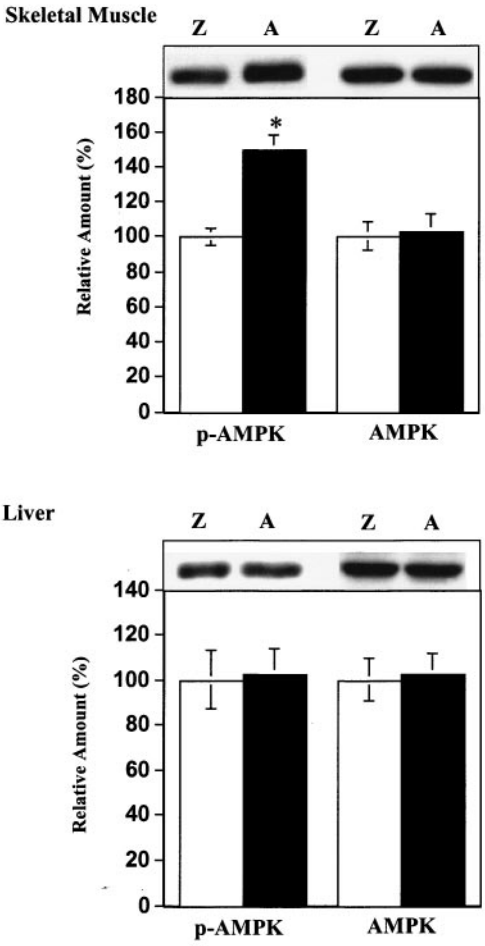

FIG. 4. Increased AMPK phosphorylation in skeletal muscle but not in liver in Adv-Adipo rats. Insulin-stimulated skeletal muscle and liver samples were collected from normal diet–fed Adv-LacZ (□ and Z) and Adv-Adipo (■ and A) rats at the end of the hyperinsulinemic clamp and processed for immunoblotting analysis using anti–phospho-specific AMPK (Thr172) and anti-AMPK antibodies. The bar graphs show data quantification by National Institutes of Health Image for total and phosphorylated AMPK protein levels in both tissues. Data are means ± SE of four animals for each group and are expressed relative to control Adv-LacZ values (defined as 100%).

AdipoR1 and AdipoR2 mRNAs are more abundant in rat skeletal muscle than in liver. Because the distribution of AdipoR1 and AdipoR2 expression varies between target tissues and among animal species, we hypothesized that adiponectin receptor distribution and/or upregulation by adiponectin overexpression could play a role in enhancing insulin sensitivity in rat skeletal muscle. We performed Northern blot analysis on total RNA from male rat red quadriceps skeletal muscle and liver on postinjection day 7. In Adv-LacZ and Adv-Adipo animals, AdipoR1 and AdipoR2 mRNA expression was ~1.9- and ~3.5-fold higher in skeletal muscle than in liver, respectively (Fig. 6). Chronic hyperadiponectinemia did not alter expression of AdipoR1 or AdipoR2 in either tissue. Interestingly, the tissue distribution of the two receptors was different from that in mouse, where AdipoR1 is predominant in muscle and AdipoR2 is most abundant in liver, but similar to that in humans, underscoring the usefulness of this animal model in investigating adiponectin biology.

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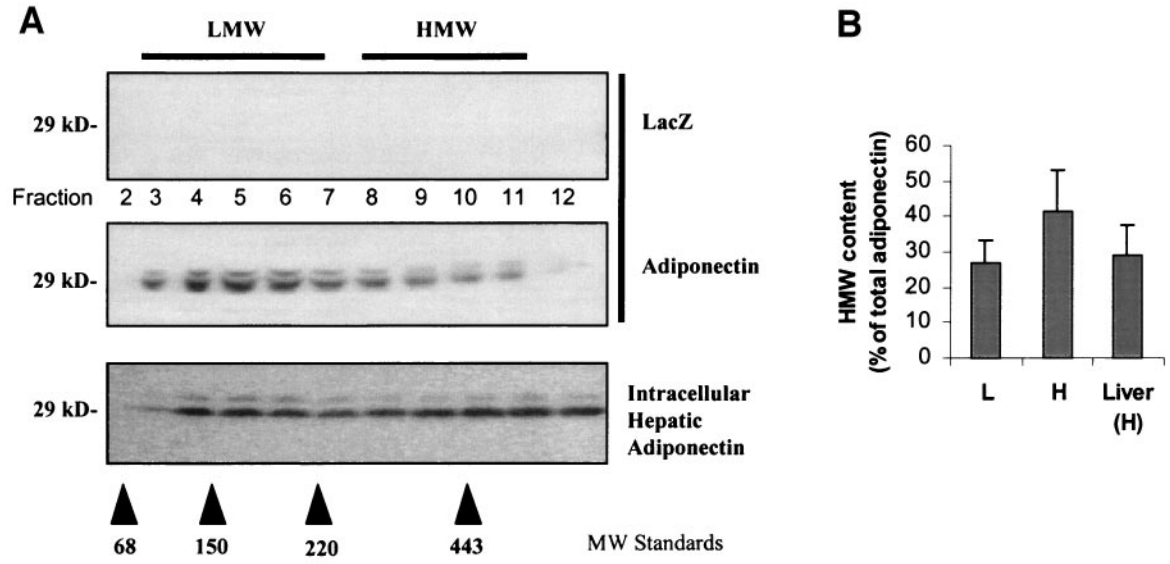

FIG. 5. Oligomeric distribution of plasma adiponectin in Adv-LacZ and Adv-Adipo rats. Plasma samples were collected on postinjection day 7 from normal diet–fed Adv-LacZ and Adv-Adipo animals, and adiponectin oligomers were analyzed by velocity sedimentation, standard SDS-PAGE, and Western blotting. *A*: Representative distribution of adiponectin oligomers from control Adv-LacZ and Adv-Adipo plasma and Adv-Adipo liver. Hepatocyte-derived adiponectin was synthesized and secreted into the circulation as LMW and HMW forms in Adv-Adipo animals. *B*: Quantification of HMW adiponectin content in plasma and liver of Adv-Adipo rats. L, low-dosage Adv-Adipo rats (plasma); H, high-dosage Adv-Adipo rats (plasma); Liver (H), liver from high-dosage Adv-Adipo rats.

DISCUSSION

Adiponectin is a recently described adipocyte-secreted polypeptide that can exert systemic effects on insulin sensitivity, lipid metabolism, and inflammatory processes (10,12,17,33,34). Circulating levels of adiponectin are decreased in a variety of insulin-resistant states in humans (34) and animals (12), including obesity and type 2 diabetes (10). To deliver ectopic expression of adiponectin in vivo and provide a physiologically relevant ligand capable of exerting systemic metabolic effects, we developed an animal model of adiponectin overexpression using adenovirus-mediated gene transfer. The mouse adiponectin cDNA was cloned into an adenovirus vector and the Adv-Adipo was given to rats, where it is targeted to the liver, with >90% of hepatocytes being infected. In turn, the liver cells secreted adiponectin into the circulation, creating a chronic adiponectinemic state. Low and high dosages of adenovirus were used to allow us to study the effects of adiponectin on glucose metabolism at physiological and supraphysiological levels.

It is of interest that ~27–41% of the circulating adiponectin in the Adv-Adipo animals was in the HMW form. Because the adenovirus construct is targeted to the liver and the antibody used in our studies recognizes mouse but not rat adiponectin, this means that the circulating adiponectin measured in these experiments was exclusively the Adv-Adipo derived from hepatocytes. The presence of a normal percentage of HMW indicates that hepatocytes express the intracellular machinery necessary for producing the HMW species, just like adipose tissue, and that the mechanisms that lead to HMW secretion must also exist and operate in liver cells. We noted that ~29% of intrahepatic adiponectin was in the HMW form, in contrast to the oligomeric distribution of endogenous adiponectin in adipose tissue, where >90% of adiponectin exists in the HMW form (19). This finding suggests that although liver cells and adipocytes possess the capability to generate both HMW and LMW components, a late-stage secretory event involving oligomer interconversion may be a regulated and/or tissue-specific process.

It is well known that an HF diet leads to insulin resistance (27), a phenomenon that was also demonstrated in the current study. Previous in vitro studies have shown that adiponectin will increase fatty acid oxidation in skeletal muscle (17,25). This fits with our results showing an increase in AMPK phosphorylation and insulin sensitivity in skeletal muscle from the Adv-Adipo animals. The adiponectin-induced increase in fatty acid oxidation would also predict that hyperadiponectinemia can alleviate the effects of an HF diet to cause insulin resistance. Again, this is fully consistent with our findings showing that when Adv-Adipo animals were placed on an HF diet, they were completely protected from the effects of this diet in causing decreased insulin sensitivity. Stimulation of AMPK activity may not be the only means by which hyperadiponectinemia enhances insulin sensitivity, however. Adiponectin has been shown to exert anti-inflammatory effects (34,35), and it is possible that downregulation of circulating tumor necrosis factor-α and interleukin-6 levels and/or inhibition of proinflammatory signaling processes might also be involved in the insulin-sensitizing effects (36).

Yamauchi et al. (23) have published the cloning of two separate receptors for adiponectin, AdipoR1 and AdipoR2, and have indicated that adiponectin exerts biological effects on lipid and glucose metabolism through these receptors. In addition, Yamauchi and colleagues (37) have recently shown that these receptors are subject to regulation by fasting, feeding, hyperglycemia, and hyperinsulinemia in mice. In our own studies, we used Northern blot analysis to measure AdipoR1 and AdipoR2 receptors in muscle and liver tissue from control and Adv-Adipo rats, and our results have shown that hyperadiponectinemia has no effect on regulating R1 and R2 mRNA levels. In an

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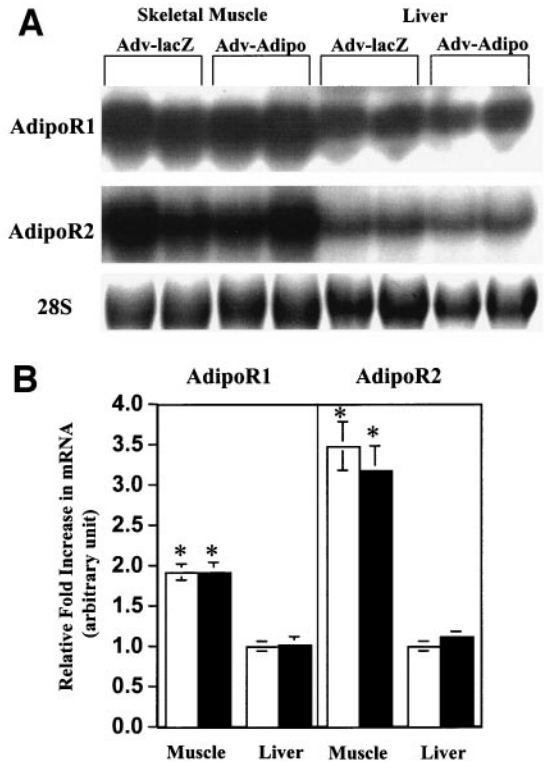

FIG. 6. AdipoR1 and AdipoR2 mRNAs are more abundant in skeletal muscle of Adv-LacZ and Adv-Adipo rats. Total RNA was obtained from red quadriceps muscle and liver tissue of normal diet–fed Adv-LacZ (□) and Adv-Adipo (■) rats on postinjection day 7. *A*: Comparative expression by Northern blot analysis. *B*: Data quantification by National Institutes of Health Image for AdipoR1 and AdipoR2 mRNA expression levels, which were normalized to 28S levels. Data are means ± SE of six animals for each group, and are expressed relative to the value in liver of control Adv-LacZ rats, which was set at 100%. *$P < 0.01$.

important finding, we observed that the distribution and expression patterns of the two receptors are markedly different between rats and mice. Thus, in mice, R1 is predominantly expressed in skeletal muscle, whereas R2 is the dominant component in liver. In our studies (Fig. 6), we found that both R1 and R2 are more highly expressed in skeletal muscle than in liver and that this pattern of expression is comparable with what has been reported in humans (online supplement of ref 23). These findings raise the possibility that results in the rat model may be more appropriately extrapolated to human physiology. In this study, we did not examine the expression levels of T-cadherin, a newly identified candidate receptor for hexameric and HMW adiponectin (24). T-cadherin has been previously reported to be expressed in muscle and liver tissue (38), in muscle cells (24), and at low levels in hepatocytes (24).

A central finding in our studies is that chronically elevated levels of adiponectin enhance overall insulin sensitivity, as manifested by an increase in insulin-stimulated glucose disposal into skeletal muscle. This observation is consistent with several previous reports that have demonstrated positive effects of adiponectin on glucose metabolism and insulin sensitivity (15,19,25). Thus, Kubota et al. (39) and Okamoto et al. (36) have reported that adiponectin knockout mice display insulin resistance. In contrast, Ma et al. (40) were unable to find glucose intolerance or insulin resistance, even on an HF diet, in adiponectin knockout mice. In complementary studies, transgenic overexpression of adiponectin leads to increased insulin sensitivity (14,18,19). In addition, various studies using acute administration of the adiponectin globular domain (16,41) or the full-length protein (14,15, 17) have shown a variety of glucose- and insulin-lowering and insulin-sensitizing effects.

The in vitro effects of the globular form of adiponectin on enhancing insulin action in muscle have been well described (25,36). However, in vivo studies have demonstrated that hyperadiponectinemia induces improvements in hepatic insulin sensitivity (14,15,18,19). In the current studies, we found that chronic hyperadiponectinemia leads to increased muscle insulin action, as demonstrated by the enhanced insulin-stimulated GDR, whereas no effects on insulin's ability to suppress hepatic glucose production were observed. This contrasts with the two published transgenic models of hyperadiponectinemia (18,19), which have demonstrated enhanced hepatic insulin sensitivity (18,19) associated with higher AMPK activity in the liver (19). Although we do not know the precise mechanisms underlying these differences in experimental results, several possibilities exist, some of which likely arise from differing experimental designs. *1*) One of the above transgenic studies was conducted in mice; as noted above, it has been reported that AdipoR1 is more abundant in skeletal muscle and AdipoR2 is more abundant in liver in this species. In contrast, as seen in Fig. 6, we found that both AdipoR1 and AdipoR2 are more highly expressed in skeletal muscle than in liver in rats. This pattern of receptor expression mimics the expression pattern in humans, but differs from that in mice (23). Thus increased expression of AdipoR2 in skeletal muscle may mediate a greater response of adiponectin in this tissue in rats. The other transgenic study used female SD rats on a HF diet. To date, the adiponectin receptor expression patterns in liver and skeletal muscle of female rats on a normal and HF diet have not been investigated. Thus it is possible that, just as nutritional status and hyperglycemia can regulate adiponectin receptor (37), sex, strain, and diet (in addition to age) can alter adiponectin receptor expression and/or function in target tissues to create differences in responses to ligands. *2*) The rats used in our studies had normal hepatic insulin sensitivity, and it is well recognized that other insulin-sensitizing agents, such as TZDs, have little, if any, effect on enhancing hepatic insulin action beyond the normal state (14). *3*) It has been suggested that the LMW component of adiponectin can antagonize the actions of the HMW fraction (22); given that our hyperadiponectinemic rats had increased circulating LMW as well as HMW fractions, perhaps this led to antagonism of adiponectin's biological effects, at least in the liver. *4*) Previous reports have shown clear-cut effects of adiponectin on skeletal muscle in vitro (36), and some of these effects have been attributed to actions of the globular head domain fragment. Although we were been unable to detect globular head adiponectin in our Adv-Adipo animals, we cannot exclude the possibility that small amounts were present that contributed to the effects on muscle insulin sensitivity. *5*) An alternate plausible explanation is that chronic adenovirus-mediated, liver-derived expression re-

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sults in postreceptor desensitization of the hepatic adiponectin signaling system by high local concentrations of the ligand.

In summary, we have demonstrated that physiologically and supraphysiologically elevated levels of adiponectin enhance insulin sensitivity in skeletal muscle via an AMPK-dependent pathway without affecting hepatic and adipose insulin sensitivity in rats. We attribute this tissue-specific effect to the high circulating HMW adiponectin levels and the preferential distribution of AdipoR1 and AdipoR2 receptors in skeletal muscle in Wistar rats. This receptor expression pattern in rats is similar to that found in humans, but differs from that found in mice, suggesting that this rat model may be more relevant to physiological events in humans.

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