ADIPONECTIN IS ONE OF THE MOST abundant plasma proteins, and its circulating level shows an inverse correlation with body mass index. Many studies have established a strong independent correlation between adiponectin levels, in particular the high molecular weight (HMW) form, and various components of the metabolic syndrome (2, 17–19, 24, 25, 29). Indeed, adiponectin has now been shown to mediate potent anti-diabetic effects due to its insulin-mimetic and insulin-sensitizing actions as well as many other beneficial physiological effects, including anti-inflammatory, anti-atherosclerotic, and cardioprotective actions (8, 11, 13, 35).

The adiponectin gene product is a 30-kDa protein, and extensive posttranslational modifications play a vital role in oligomerization of these monomers to produce the circulating trimeric (low molecular weight; LMW), hexameric (medium molecular weight; MMW), and HMW (28) forms. An intracellular signaling role for the globular C-terminal domain of adiponectin has also been proposed (12). Use of thiazolidinediones has become a common and successful, albeit somewhat controversial, approach for the treatment of diabetes (1). These drugs, such as rosiglitazone, mediate their beneficial anti-diabetic effects at least in part via stimulating adiponectin synthesis and secretion from adipocytes (22), thus increasing circulating adiponectin levels and enhancing its function.

Until recently, it was thought that adipocytes were the only site of adiponectin synthesis and secretion. However, several studies have now uncovered the potential for adiponectin production by cardiomyocytes and skeletal muscle cells (4–6, 10, 33). We have used RT-PCR, Western blot, and immunohistochemistry to demonstrate that adiponectin is expressed in mouse skeletal muscles and differentiated L6 myotubes (15). This is in keeping with the growing awareness of the ability of skeletal muscle to produce and secrete biologically active hormones, collectively referred to as myokines (21). More work is clearly needed to further understand the control of adiponectin production in nonadipocyte cell types such as skeletal muscle, and to determine the functional significance of such production. In the current study, we examine the regulation of adiponectin expression in L6 rat skeletal muscle cell lines by hyperglycemia and the thiazolidinedione rosiglitazone as well as the functional significance of local adiponectin overexpression on insulin sensitivity. We also used genetic and dietary animal models of obesity, insulin resistance, and diabetes to examine the regulation of skeletal muscle adiponectin expression level and profile in impaired metabolic states.

MATERIALS AND METHODS

Materials. 2-Deoxy-D-[3H]glucose were purchased from Amer sham (Quebec, Canada). Insulin (Humulin) was purchased from Eli Lilly (Toronto, Canada). The cell culture medium [α-minimum essential medium (α-MEM); 5 mM glucose], FBS, and antibiotic/antimycotic solution were purchased from Wisent (St.-Bruno, QC). TRIzol reagent was from Invitrogen Life Technologies (Burlington, ON). Total protein kinase B (Akt) antibody, polyclonal phosphospecific antibodies to Akt (Thr308), and horseradish peroxidase (HRP)-
conjugated anti-rabbit IgG and Platinum SYBR Green qPCR SuperMix UDG Kit were from Cell Signaling Technology (Beverly, MA). Rosiglitazone was from SynFine (Richmond Hill, ON). Polyvinylidene difluoride (PVDF) membrane was from Bio-Rad (Burlington, ON) and chemiluminescence reagent plus from PerkinElmer (Boston, MA). All other reagents used were of the highest purity available.

**Cell culture.** Rat L6 skeletal myoblasts were grown in α-MEM containing 10% (vol/vol) FBS (growth medium) under 5% CO2 at 37°C. For experimental procedures, cells were cultured in α-MEM (5 mM glucose) containing 2% (vol/vol) FBS plus 1% antibiotic/antimycotic until differentiated into myotubes. Cells were then treated with or without final concentrations of 25 mM glucose or 1, 10, and 100 nM insulin or 10 μM rosiglitazone (3) as indicated in the legends for Figs. 1–6.

**Experimental animals.** Male Wistar rats (Charles River, St. Constant, QC) weighing 250–300 g upon arrival were randomly allocated to treatment groups. Animals were placed in temperature- and humidity-controlled rooms (21 ± 2°C, 35–40%), with a daily 12:12-h light-dark cycle (lights on at 0700). Animal facilities met the guidelines of the Canadian Council on Animal Care, and the protocols were approved by the Animal Care Committee of Laval University. Animals were acclimated to their environment for a minimum of 5 days and consumed a nonpurified chow rodent diet ad libitum. After the acclimation period, rats were fed an obesity-promoting diet, high-fat high-sucrose (HFHS). HFHS diet provided 19.7% of energy as carbohydrate (sucrose), 65.4% as fat (lard and corn oil), and 15.0% as protein (casein and t-cystine). HFHS animals received rosiglitazone (5 mg/kg body wt) or vehicle twice daily for the last 28-day period as previously described (30). Rosiglitazone was prepared in acidified double-distilled water (with HCl at pH 2.5) for adequate dissolution and administered orally at 5 mg/kg. At the end of the treatment period, skeletal muscles (gastrocnemius) were collected, frozen in liquid nitrogen, and kept in −80°C until analysis.

Male lean wild-type and obese db/db mice (Charles River, Montreal, Canada) were fed on chow diet and housed in plastic cages in animal quarters maintained at 22°C with a 12:12-h dark-light schedule. At 14 wk of age, lean wild-type and obese db/db mice were fasted for 5 h before death. Skeletal muscles [extensor digitorum longus (EDL) and soleus] were collected, frozen in liquid nitrogen, and kept in −80°C until analysis.

**Analysis of adiponectin expression by real-time quantitative PCR.** cDNAs were synthesized by reverse transcription with 1 μg total RNA. Primer sequences and their respective PCR fragment length were as follows: β-actin (133 bp): forward 5'-CTTGCTCCATCTCTAGGCCTT-3', reverse 5'-CTCTCAGCTTGTTGGAAC-3'; adiponectin (101 bp): forward 5'-GCAAGAGATGGCACTCCTGGA-3', reverse 5'-GCCCTACGCTCTTGGAAC-3'(Invitrogen Life Technologies). Adiponectin RNA levels in L6 myotubes and EDL, soleus, and gastrocnemius skeletal muscles were determined by using platinum the SYBR Green qPCR SuperMix UDG Kit. L6 myotubes were incubated with glucose (25 mM) or rosiglitazone (10 μM) for the times indicated, whereas EDL, soleus, and gastrocnemius skeletal muscles were isolated from animals, and then RNA was extracted and reverse transcribed to cDNA. Cycling conditions consisted of an initial denaturation step of 95°C for 3 min followed by 40 cycles of

![Fig. 1. Effect of rosiglitazone on adiponectin expression, glucose uptake, and protein kinase B (Akt) phosphorylation in L6 skeletal muscle cells. L6 skeletal muscle cells were grown in 2% FBS α-minimum essential medium (α-MEM) until differentiated into myotubes, followed by 24 h treatment with 10 μM rosiglitazone (Rosi). At the end of the treatment, cells were prepared for quantitative PCR and Western blot analysis of mRNA and intracellular protein expression; media were collected for ELISA kit analysis of secreted adiponectin; 2-deoxyglucose uptake and phosphorylation of Akt were measured with or without additional 20 min insulin treatment. A: total RNA was extracted, and quantitative PCR was performed to measure the relative adiponectin mRNA levels between dimethyl sulfoxide (DMSO; control) and Rosi treatment. B: relative expression of multimer [high molecular weight (HMW)], hexamer [medium molecular weight (MMW)], and trimer [low molecular weight (LMW)] adiponectin in cell lysates (intracellular) between DMSO (control) and Rosi treatment. C: total secreted level of adiponectin between DMSO (control) and Rosi treatment. D: 2-Deoxyglucose uptake level was measured under basal condition and in response to 1 or 10 mM insulin (Ins) stimulation with DMSO (control) and Rosi treatment. E: representative immunoblot and quantitative analysis of phosphorylated Akt (Thr308). Also shown is the immunoblot for total Akt protein level. A–C: values represent means ± SE of n = 6 experiments; *P < 0.05 vs. DMSO (*) and vs. DMSO + insulin (#).
95°C for 30 s/65°C (adiponectin) or 60°C (β-actin) for 30 s/72°C for 30 s.

Overexpression of adiponectin in L6 cells. Rat L6 skeletal muscle myoblasts were induced to differentiate into myotubes after being transfected with mammalian expression vector pcDNA3-Ad-F (27) to overexpress full-length adiponectin by using the calcium phosphate-DNA precipitates method. Briefly, a procedure based on slow mixing of HEPES-buffered saline containing sodium phosphate with a CaCl₂ solution containing plasmid DNA (pcDNA3 empty vector and pcDNA3-Ad-F) allows a DNA-phosphate precipitate that adheres to the cell surface and is then taken up by the cell. Cells were incubated with transfection media for 16 h and then recovered and induced into the myotube in growth media (2% FBS, vol/vol) until further analysis.

Preparation of muscle homogenates for Western blotting. Skeletal muscle strips snap-frozen in liquid nitrogen were first ground into powder then mixed with 100 μl lysis buffer (30 mM HEPES, pH 7.4, 2.5 mM EGTA, 3 mM EDTA, 70 mM KCl, 20 mM β-glycerophosphate, 20 mM NaF, 1 mM Na₃VO₃, 200 μM phenylmethylsulfonyl fluoride, 1 μM peptatin A, 10 μM E-64, 1 μM leupeptin, and 0.1% Nonidet P-40) per 10 mg tissue and incubated on a rotating rocker for 1 h at 4°C. Samples were centrifuged at 10,000 rpm for 10 min in 4°C, and only supernatants were collected for Western blot analysis.

Measurement of glucose uptake. Cells were transfected to overexpress adiponectin or incubated with glucose (25 mM) for the first 24 h followed by rosiglitazone treatment for another 20 h, and subsequently serum-starved in the continued presence of all treatments for the final 4 h in 24-well plates before glucose uptake assay. After treatments of 1 or 10 nM of insulin for the last 20 min, glucose uptake was determined as previously described (7). Briefly, cells were incubated in transport solution (140 mM NaCl, 20 mM HEPES-Na, 2.5 mM MgSO₄, 1 mM CaCl₂, 5 mM KCl, and 0.5 μCi/ml 2-deoxy-[3H]glucose, pH 7.4) for 5 min. Nonspecific uptake was measured in the presence of cytochalasin B (10 μM). Cells were then lysed with 1 M KOH, and aliquots were transferred to scintillation vials for 3H radioactivity counting. Calculation was made as picomoles per microgram per minute.

Western blot analysis. Western blotting was conducted essentially as described previously (7) where, after treatments described previously, L6 cells were lysed in 1× Laemmli sample buffer containing 10% (vol/vol) β-mercaptoethanol, passed through a syringe several times, and heated (65°C, 5 min). Cell lysates were then centrifuged for 5 min in a benchtop microfuge (13,000 rpm), and ~30 μg protein were resolved by 10% SDS-PAGE and immunoblotted onto PVDF membrane. Membranes were blocked with 3% BSA dissolved in 1× wash buffer solution of 50 mM Tris base, 150 mM NaCl, 1% Triton X-100, and 1% Nonidet P-40 for 1 h. Membranes were incubated overnight with primary antibodies at the following dilutions: phospho-Akt (Thr308) (1:1,000) and total Akt (1:1,000). Membranes were then washed four times in 1× wash buffer for 15 min each at room temperature and incubated with appropriate HRP-coupled secondary antibody (1:10,000) for 1 h. Membranes were washed five times in 1× wash buffer for 10 min each, and proteins were visualized using enhanced chemiluminescence. Nondenaturing, nonreducing conditions were applied to allow analysis of different forms of adiponectin (HMW >250 kDa, MMW = 180 kDa, and LMW = 90 kDa) with in-house polyclonal anti-adiponectin antibody in the concentration of 1 μg/ml and anti-rabbit HRP-coupled secondary antibody (1:10,000).

Content of each specific form of adiponectin (HMW, MMW, and LMW) and total or phosphorylated Akt were then determined via densitometric scanning.

Hyperinsulinemic euglycemic clamp. Whole body insulin-mediated glucose disposal was assessed in unrestrained conscious animals by the hyperinsulinemic-euglycemic clamp technique, as previously described (23). Briefly, catheters were inserted in the left jugular vein and in the right carotid artery. Rats were allowed to recover from surgery for 4–5 days before the clamp procedure was performed. At that time, the food intake and body weights of all rats had returned to 80 and 90% of their presurgery values, respectively. Rats were fasted overnight (12–14 h) before receiving an infusion of saline (basal) or insulin at 4 μJ·kg⁻¹·min⁻¹ for 120 min by means of syringe pump (Razel, Stamford, CT). Venous catheters were used for the multiple infusions, and blood samples were obtained from the carotid artery.

Adiponectin and insulin determinations. Analysis of adiponectin production by L6 cells was examined by determining the content in conditioned media using an in-house adiponectin assay as previously described (32). The antimouse adiponectin antibody used in this study was affinity-purified using recombinant mouse adiponectin. Affinity
ligand recognized all forms of adiponectin and did not cross-react with human adiponectin and a panel of other adipokines/cytokines (31). Intra- and interassay coefficients of variance were 5.3–7.6 and 4.1–6.4%, respectively. Lower limits of detection for this assay are 0.5 ng/ml of adiponectin protein. The total adiponectin is the sum of all forms of adiponectin intracellularly from whole skeletal muscle lysate. Serum insulin level was measured using RIA methods (RI-13K; Linco Research, St. Charles, MO), and total adiponectin levels were determined using EIA (44-ADPR-0434; ALPCO Diagnostics, Salem, NH).

**RESULTS**

We first expanded upon our previous demonstration of adiponectin mRNA and protein synthesis in L6 cells (15) by demonstrating that treating L6 cells with rosiglitazone increased adiponectin mRNA expression levels by 

**Fig. 3.** Effect of hyperglycemia and rosiglitazone on adiponectin expression and glucose uptake in L6 skeletal muscle cells. L6 skeletal muscle cells were grown in 2% FBS α-MEM until differentiated into myotubes; 24 h pretreatment with 25 mM glucose followed by treatment with or without 10 μM rosiglitazone (Rosi) for another 24 h. At the end of the treatment, cells were prepared for quantitative PCR and 2-deoxyglucose uptake. A: total RNA was extracted and quantitative PCR was performed to measure the relative adiponectin mRNA levels between control (Con), 25 mM glucose (HG), and HG + Rosi treatment. B: 2-deoxyglucose uptake level was measured under basal conditions and in response to 1 or 10 nM insulin stimulation with control, 25 mM glucose, Rosi, and HG + Rosi treatment. A: values represent means ± SE of n = 4; P < 0.05 vs. Con (*) and vs. HG (#). B: values represent means ± SE of n = 4; P < 0.05 vs. Con (*), vs. Con + insulin (#) and vs. HG + insulin ($)
rosiglitazone (Fig. 1C). The potential functional significance of increased muscle adiponectin production in response to rosiglitazone was examined via analysis of glucose uptake where we demonstrated using submaximal insulin concentrations [1 and 10 nM (Fig. 1D)], but not 100 nM (data not shown), that rosiglitazone could increase insulin sensitivity without significantly altering basal glucose uptake (Fig. 1D). The enhanced insulin action in the presence of rosiglitazone was also apparent upon analysis of Akt (Thr308) phosphorylation (Fig. 1E).

To further investigate the autocrine role of adiponectin production in skeletal muscle cells and as a mechanism explaining the effect of rosiglitazone in L6 cells, we transfected cells with a vector encoding full-length adiponectin. Figure 2A shows that overexpression of adiponectin led to higher levels of HMW, MMW, and LMW adiponectin, especially in the form of HMW, both intracellularly in L6 myotubes as well as secreted from these cells. Overexpression of adiponectin elicited a small but significant increase in basal glucose uptake and also resulted in significant enhancement of glucose uptake stimulated by 1 or 10 nM insulin, but no significant increase was observed at 100 nM (Fig. 2B). Although no increase in Akt phosphorylation was detected upon adiponectin overexpression, an enhanced level of insulin-stimulated Akt (Thr308) phosphorylation was observed (Fig. 2C).

Whether adiponectin expression levels in skeletal muscle cells could be regulated by physiologically relevant parameters was first tested in vitro by exposing L6 cells to hyperglycemic conditions. A significant decrease in adiponectin expression in L6 myotubes was observed after growth in 25 mM glucose for 24 h, and this could be corrected by rosiglitazone treatment (Fig. 3A). Furthermore, rosiglitazone can also reverse the insulin resistance caused by hyperglycemia, perhaps at least in part by restoring the local adiponectin expression profile. Cells grown in 25 mM glucose show a small decreased basal glucose uptake level and significantly inhibited insulin-induced glucose uptake, and this can be fully restored after additional rosiglitazone treatment (Fig. 3B).

We next used the HFHS-fed rat, a well-established animal model of obesity and insulin resistance (16), to examine changes in gastrocnemius muscle adiponectin expression. HFHS-fed rats exhibited increased adiposity and were insulin-resistant as determined by the hyperinsulinemic-euglycemic clamp technique (Fig. 4). As expected, despite a higher body weight gain (Fig. 4A), rosiglitazone treatment was found to improve whole body insulin sensitivity in HFHS-fed obese rats as indicated by normalization of the glucose infusion rate during the clamp and of fasting insulinemia (Fig. 4, B and C). Rosiglitazone was found to significantly increase the circulating amount of adiponectin although there was no reduction in high-fat-fed animals compared with chow-fed animals (Fig. 4D). Importantly, we observed a significant decrease in adiponectin mRNA expression in gastrocnemius of these obese insulin-resistant rats, and this was fully corrected by rosiglitazone treatment (Fig. 5A). There was no decrease in total adiponectin protein expression upon HFHS feeding, and rosiglitazone elicited a small but significant increase in total

![Fig. 5](http://ajpendo.physiology.org/)
adiponectin protein expression (Fig. 5B). Importantly, despite no decrease in total adiponectin content, upon analysis of the profile of adiponectin multimers expressed within gastrocnemius under these conditions, we observed an altered profile consisting of a selective decrease in the HMW-to-total adiponectin ratio and an apparent increase in the LMW-to-total adiponectin ratio after HFHS feeding (Fig. 5, C and E). The altered ratio of each specific form to total adiponectin was normalized when animals were treated with rosiglitazone (Fig. 5C).

Adiponectin expression was then examined in EDL (predominantly type 2, fast-twitch, glycolytic fibers) and soleus (predominantly type 1, slow-twitch, oxidative fibers) muscle from control or obese diabetic db/db mice. In EDL, there was a slight decrease in total intracellular adiponectin expression that reflected decreased HMW-to-total and higher LMW-to-total ratios (Fig. 6, A and B). There was a significant reduction in total adiponectin protein in soleus, and analysis of changes in the profile of distinct forms showed a significantly decreased HMW-to-total adiponectin ratio and increases in MMW-to-total adiponectin ratios (Fig. 6, C and D). Note that we did not detect a change in adiponectin receptor expression in the muscle samples used here (data not shown).

**DISCUSSION**

The work presented here analyzes adiponectin production by skeletal muscle, its functional significance, and alterations in obese and/or diabetic animal models. Although until recently it was thought that adipocytes were the only site of adiponectin synthesis and secretion, there is good rationale for this work, with several studies having now uncovered the potential for adiponectin production by skeletal muscle cells and also cardiomyocytes (4, 5, 10, 33). We previously used RT-PCR, Western blot, and immunohistochemistry to demonstrate that adiponectin is expressed in mouse skeletal muscles and differentiated L6 myotubes, but not skeletal muscle from adiponectin knockout mice (15). Notably, comparison of wild-type and adiponectin knockout mice suggested that the expression of adiponectin in skeletal muscle influenced muscle phenotype and function. Hence, we performed the current study to further understand the control of adiponectin production in skeletal muscle and, most importantly, to determine the functional significance of production in skeletal muscle via analyzing regulation of metabolism.

Rosioglitazone is a potent inducer of adiponectin synthesis and secretion from adipocytes (20, 34), and here we examined whether rosioglitazone changes adiponectin mRNA and protein expression in L6 cells. Our data demonstrated that rosioglitazone increased adiponectin mRNA expression levels as well as all oligomeric forms of adiponectin, indicating that skeletal muscle cells have the capacity to posttranslationally modify adiponectin and allow formation of biologically active forms (26). Acute treatment of L6 with another thiazolidinedione, troglitazone, has previously been shown to act directly on skeletal muscle cells to cause a small but significant increase in glucose uptake (14). Here we demonstrate prolonged exposure to rosioglitazone can enhance insulin-induced but not basal glucose uptake. Because we proposed that adiponectin production by skeletal muscle may have important autocrine effects, we then directly examined the potential functional significance of increased muscle adiponectin production and considered both direct and insulin-sensing effects of adiponectin. The effect of rosioglitazone on glucose uptake was additive with that of insulin, and we further observed that, at submaximal insulin concentrations, rosioglitazone could increase insulin sensitivity when we measured glucose uptake and Akt (Thr308) phosphorylation. These initial experiments were suggestive of a poten-
tional role for adiponectin, and we then directly investigated this possibility via transfecting L6 cells with a vector expressing the full-length adiponectin gene. This led to enhanced expression of adiponectin and secretion of HMW, MMW, and LMW adiponectin in the medium by these cells. Functionally, overexpression of adiponectin led to a small but significant increase in basal glucose uptake and resulted in enhancement of glucose uptake stimulated by 10 or 10 nM insulin. Although no increase in Akt phosphorylation was detected upon adiponectin overexpression alone, an enhanced level of insulin-stimulated (10 nM) Akt (Thr^308) phosphorylation was observed. Our study focused on insulin-stimulated mechanisms leading to glucose uptake rather than AMP-activated protein kinase-dependent effects, and we also noted no change in basal glucose uptake upon rosiglitazone treatment. Overall, the overexpression of adiponectin or rosiglitazone-induced increase in adiponectin correlated with altered metabolic effects, primarily mediated by enhancement of insulin sensitivity. This in vitro data clearly suggest a potentially important role for local effects of adiponectin, and we next translated our study to more physiologically relevant analyses using animal models of obesity and/or diabetes.

Many studies have shown that adiponectin levels, and in particular the proportion of the HMW form, are reduced in obese and/or diabetic individuals (2, 17–19, 24, 25, 29). Here, we tested whether adiponectin levels were reduced in skeletal muscle from obese/diabetic animal models, and if so whether it could be corrected by a thiazolidinedione such as rosiglitazone. We used the db/db mouse as a model of obesity and diabetes and observed decreases in adiponectin expression in both soleus and EDL muscles of these animals. The HFHS-fed rat was also studied here as an animal model of obesity and insulin resistance (16). HFHS-fed rats exhibited increased adiposity and insulin-resistant as determined by the hyperinsulinemic-euglycemic clamp technique. Interestingly, we demonstrated that, in HFHS rats, there was a significant decrease in adiponectin mRNA as well as HMW-to-total protein expression in gastrocnemius muscle but with no changes in circulating adiponectin level, suggesting that the changes in local adiponectin profiles may be at least partly responsible for skeletal muscle insulin resistance in these animal models. This analysis of mRNA and distinct forms is of significance and in support of our previous work (15), since it further demonstrates that skeletal muscle adiponectin content does not simply reflect circulating levels. Importantly, the decreased muscle adiponectin expression in HFHS animals was fully corrected by rosiglitazone treatment, which also correlated with improvements in whole body insulin sensitivity. We observed no change in expression of adiponectin receptors (data not shown) in skeletal muscles from HFHS rosiglitazone animals, which confirmed that metabolic disorders in the above animal models are due to the change of local adiponectin expression profile. Interestingly, a recent study indicated that adiponectin level, determined by immunostaining, appeared to decrease in the fast pad of HFHS mice, perhaps indicating distinct local regulation of adiponectin in skeletal muscle and adipose tissue (9).

In summary, this work indicates that skeletal muscle cells can synthesize and secrete adiponectin, which then exerts local metabolic effects, at least in part via enhancing insulin sensitivity. We also demonstrate that muscle adiponectin expression levels change in disease states and that rosiglitazone treatment can both restore deficits in adiponectin expression and improve skeletal muscle insulin sensitivity and whole body glucose homeostasis. Ultimately, it could be envisaged that, if adiponectin produced by skeletal muscle has important and beneficial autocrine/paracrine effects, then therapeutic approaches aimed at enhancing adiponectin expression may be of significant value.

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