Delivery of adiponectin gene to skeletal muscle using ultrasound targeted microbubbles improves insulin sensitivity and whole body glucose homeostasis

Vivian Vu,†* Ying Liu,†* Sanjana Sen,† Aimin Xu,‡ and Gary Sweeney†

†Department of Biology, York University, Toronto, Canada; and ‡Department of Medicine and Department of Pharmacology and Pharmacy, University of Hong Kong, Pokfulam, Hong Kong

Submitted 2 October 2012; accepted in final form 4 November 2012

Vu V, Liu Y, Sen S, Xu A, Sweeney G. Delivery of adiponectin gene to skeletal muscle using ultrasound targeted microbubbles improves insulin sensitivity and whole body glucose homeostasis. Am J Physiol Endocrinol Metab 304: E168–E175, 2013. First published November 6, 2012; doi:10.1152/ajpendo.00493.2012.—Numerous studies have shown that adiponectin confers antidiabetic effects via both insulin-like and insulin-sensitizing actions. The majority of adiponectin in circulation is derived from adipocytes; however, other tissues such as skeletal muscle can produce adiponectin. This study was designed to investigate the functional significance of adiponectin produced by skeletal muscle. We encapsulated the adiponectin gene in lipid-coated microspheres filled with octafluoropropane gas that were injected into the systemic circulation and destroyed within the microvasculature of skeletal muscle using ultrasound. We first demonstrated safe and successful targeting of luciferase and green fluorescent protein reporter genes to skeletal muscle using this approach and then confirmed efficient overexpression of adiponectin mRNA and oligomeric protein forms. Glucose tolerance test indicated that overexpression of adiponectin in skeletal muscle was able to improve glucose intolerance induced by feeding mice a high-fat diet (HFD), and this correlated with improved skeletal muscle insulin signaling. We then performed hyperinsulinemic-euglycemic clamp studies and demonstrated that adiponectin overexpression attenuated the decreases in glucose infusion rate, glucose disposal, and increase in glucose appearance induced by HFD. Ultrasound-targeted microbubble destruction (UTMD) delivery of adiponectin to skeletal muscle also enhanced serum adiponectin levels and improved hepatic insulin sensitivity. In conclusion, our data show that UTMD efficiently delivers adiponectin to skeletal muscle and that this improves insulin sensitivity and glucose homeostasis.

MATERIALS AND METHODS

Materials. High-fat diet (HFD) was purchased from Research Diets. The HFD provided 20% kcal from protein, 20% kcal from carbohydrate, and 60% kcal from fat compared with the control regular chow diet (LabDiets, Brentwood, MO), which provided 20%...
kcal from protein, 70% kcal from carbohydrate, and 10% kcal from fat. DEFINITY perfutren lipid microsphere microbubbles were from Lantheus Medical Imaging (Saint-Laurent, QC, Canada). QIAGEN Plasmid Giga Kit was from QIAGEN (Toronto, ON, Canada). [3H]glucose was from PerkinElmer (Woodbridge, ON), and human insulin (Humulin R) was from Eli Lilly (Toronto, ON). Polyclonal antibodies to phosphospecific protein kinase B (Akt) [threonine-308 (T308) and serine-473 (S473)], Akt2, the adaptor protein containing pleckstrin homology domain, phosphotyrosine binding domain, and leucine zipper motif (APPL) 1 and GAPDH as well as horseradish peroxidase-conjugated anti-rabbit-IgG were from Cell Signaling Technology (Beverly, MA). Polyclonal antibodies to adiponectin and APP2 were produced in-house. Antibodies to adiponectin receptors 1 and 2 (AdipoR1 and AdipoR2) were a kind gift from Dr. Tony Clementz, AstraZeneca (Molndal, Sweden). Chemiluminescence reagent plus was obtained from PerkinElmer (Boston, MA), and the Luciferase Assay System was from Promega (Madison, WI). Bradford Reagent and polyvinylidene difluoride membranes were from Bio-Rad Laboratories (Burlington, ON, Canada). All other reagents used were of the highest purity available.

**Experimental animals.** C57BL/6 male mice were purchased from Charles River Laboratories (Montreal, QC, Canada), housed in a temperature-controlled environment under a 12:12-h light-dark cycle, and were fed ad libitum. Animal facilities met the guidelines of the Canadian Council on Animal Care, and all protocols used were approved by the Animal Care Committee, York University. For diet-induced obesity studies, animals received HFD commencing at 6 wk of age, with corresponding age-matched controls receiving regular chow for 6 or 12 wk.

**Plasmid DNA preparation and purification.** pGL3-control luciferase vector was purchased from Promega, and myogenin promoter-driven luciferase (31) was a kind gift from Dr. John C. McDermott (York University, Toronto). Full-length adiponectin (fAd) and enhanced green fluorescent protein-tagged APP21 (eGFP-APP21) plasmid were produced in-house. An empty pCDNA3 vector was used as an empty vector (EV) control. All vectors were first transformed into Escherichia coli followed by overnight incubation in 37°C with agitation and antibiotics. All plasmids were then purified using the QIAGEN Plasmid Giga Kit according to the manufacturer’s protocol.

**Ultrasound-targeted microbubble delivery.** One milligram of plasmid DNA mixed with 100 µl DEFINITY microbubbles was infused at a constant rate of 50 µl/min via catheters inserted in the left/right jugular vein of mice. A 1-MHz, 2.0-cm² ultrasound signal (Vevo SoniGene; Visual Sonics, Toronto, ON) at 50% duty cycle (pulsed signal) was applied to each hind leg to induce microbubble destruction and transfection. In initial experiments the right hind leg was used as the control untransfected leg, and the left leg was used for luciferase or green fluorescent protein expression. All mice were monitored after the experiment for normal behavior.

**Luciferase assay.** To quantify expression of the luciferase transgene, total protein was extracted from hind leg skeletal muscle following UTMD transfection with lysis buffer (20 mm Tris-HCl, pH 7.4, 0.1% Triton X-100). Luciferase assay was performed according to the manufacturer’s protocol, and luciferase activity was quantified using a luminometer (Berthold Lumat, 9501) and standardized by total protein determined by the Bradford protein assay. Relative luciferase units (RLU) for each sample were determined from triple readings and expressed as RLU per microgram protein (fold change above readings obtained using lysates from control leg muscle).

**Immunofluorescent and bright-field microscopy.** Ten-micrometer cryostat sections were obtained from mouse hind leg skeletal muscle, and green fluorescence was examined using fluorescence microscopy on the Olympus BX51 confocal microscope (Olympus, Seattle, WA) with a ×10 objective. Corresponding bright-field images were also acquired.

**Hyperinsulinemic-euglycemic clamp.** Whole body insulin sensitivity was determined by hyperinsulinemic-euglycemic clamp in unrestrained, conscious mice as previously described (6) 1 wk after UTMD transfection of hind leg skeletal muscle with EV or fAd plasmids. Briefly, right jugular vein and left carotid artery catheters were embedded in animals 4 days before the hyperinsulinemic-euglycemic clamp procedure. Mice were fasted for 5–6 h before commencement of the clamp procedure, weighed, and stabilized with a constant infusion of [3H]glucose tracer for 90 min. Basal levels of blood glucose were set as the average blood glucose level + 0.5 mmol/l measured at −90, −30, −20, −10, and 0 min during the stabilization period. At 0 min, a continuous intravenous infusion of purified human insulin was started and maintained for 120 min at a constant speed of 4.0 nU/kg·min⁻¹. Thirty percent dextrose solution was infused at a variable rate to maintain blood glucose at the previous set basal glucose range while carotid artery blood samples were taken at 10-min intervals to monitor plasma glucose concentrations. Erythrocytes from corresponding chow-fed or HFD blood donor animals were suspended in saline and reinfused in the animals at a continuous rate to minimize stress and maintain erythrocyte volume fraction. Blood samples collected during the clamp procedure and radioactive activities in serum samples were analyzed, and calculations were made based on the radioactivity readings to represent whole body glucose turnover rate, glucose disappearance rate (Rd), glucose appearance rate (Ra), and glycotoxic rate.

**Glucose tolerance test and analysis of muscle and liver insulin signaling.** To perform the glucose tolerance test animals were starved 5–6 h before receiving a bolus intraperitoneal injection of glucose (2 g/kg body wt). Tail vein blood samples were collected after 15, 30, 60, and 90 min, and the blood glucose level (mmol/l) was determined with a glucometer (Conture; Bayer). A bolus insulin dose of 4 U/kg body wt was injected via the tail vein of anesthetized mice, and skeletal muscle from both hind legs and liver tissue were excised 15 min following insulin injection and snap-frozen in liquid nitrogen for later analysis.

**Tissue and serum analysis by Western blotting.** Skeletal muscle and liver homogenates were obtained from powdered tissue samples lysed with buffer containing phosphatase and protease inhibitors (30 mM HEPES, pH 7.4, 2.5 mM ethylene glycol tetraacetic acid, 3 mM ethylenediaminetetraacetic acid, 70 mM KCl, 20 mM β-glycerophosphate, 20 mM NaF, 1 mM Na3VO4, 200 µM phenylmethylsulfonyl fluoride, 1 µM pepstatin A, 10 µM E-64, 1 µM leupeptin, and 0.1% Nonidet P-40). All samples were standardized by total protein and analyzed by SDS-PAGE. Serum and tissue adiponectin were determined by immunoblotting for multimeric and monomer total adiponectin under nonreducing/nondenaturing conditions and denaturing/reducing conditions, respectively, as described previously (25).

**Statistical analysis.** Data were analyzed by Graphpad Prism 5, and statistical analysis was performed using the Student’s t-test and one-way ANOVA followed by Tukey’s Multiple-Comparison Test. Data are expressed as means ± SE where P < 0.05 was considered statistically significant.

**RESULTS**

To establish UTMD as an effective method for in vivo plasmid-based gene transfer in skeletal muscle of C57BL/6 mice, we first used reporter gene constructs to evaluate the efficiency of transfection. First, a pGL3-control luciferase vector was infused, and ultrasound signal was applied to the left hind leg while the right hind leg served as a negative nontransfected control. A strong signal for luciferase activity was detected in the ultrasound-targeted leg (Fig. 1A). We next used a construct
where luciferase was driven by a myogenin promoter and observed a slightly higher increase in luciferase activity in the UTMD-transfected leg (Fig. 1B). Finally, to confirm tissue-specific protein expression as a consequence of UTMD, an eGFP-APPL1 construct was used. APPL1 was not used for functional significance in this study but rather to allow for fluorescent detection of this transgene in muscle. An increase in green fluorescence was detected in UTMD-transfected skeletal muscle sections (Fig. 1C) but not in other tissues (liver, kidney, etc.) isolated from the same animal (data not shown). When these experimental conditions were used to transfected muscle with a plasmid encoding fAd, a significant increase in skeletal muscle total, HMW, and hexameric adiponectin expression was detected (Fig. 1, D–G).

To examine if adiponectin overexpression in muscle had any functional consequences, we first conducted a glucose tolerance test in mice receiving EV or adiponectin by UTMD. We fed mice HFD, and, as expected, we found decreased glucose tolerance compared with the chow-fed age-matched control group (Fig. 2, A and B). A striking improvement in glucose tolerance was observed in HFD-fed mice with overexpression of adiponectin in skeletal muscle (Fig. 2, A and B). To study this observation in more detail, mice were subjected to a hyperinsulinemic-euglycemic clamp study that was conducted 7 days after delivery of adiponectin transgene by UTMD. As expected, a significant difference was seen in the glucose infusion rate (GIR) between chow-fed and HFD animals receiving EV UTMD transfection (Fig. 3A). Skeletal muscle overexpression of fAd significantly improved GIR, inferring improved insulin sensitivity in these mice. Whole body glucose turnover rate was calculated as the ratio of postinsulin infusion to basal levels (post:basal) for glucose Ra and glucose Rd. A significant difference in post:basal Ra (Fig. 3B) was detected between the HFD EV group compared with the chow-fed EV group, indicating decreased response to insulin-suppressed glucose production in the liver of HFD EV animals. These mice also showed reduced post:basal Rg levels compared with chow-fed EV animals (Fig. 3B) and thus a decreased response to insulin-stimulated glucose disposal in peripheral tissues. A significant correction of HFD-induced defects in Rg and Rd was achieved upon skeletal muscle fAd overexpression (Fig. 3B), demonstrating enhanced insulin responsiveness in both liver and peripheral tissues. Glycolytic rate was determined from the accumulation of \(^{3}H_{2}O\) in plasma samples and is an indicator of utilization of glucose as an energy substrate. The basal and postinsulin glycolytic rates (Fig. 3C) were similar across all groups.
We next directly examined the influence of UTMD-mediated adiponectin overexpression on insulin sensitivity in skeletal muscle. Following a bolus intravenous injection of 4 U/kg body wt insulin, phosphorylation of Akt was examined. As expected, a decrease in insulin-stimulated Akt signaling was observed in animals fed HFD, and our data demonstrated a significant improvement in Akt signaling (T308) in skeletal muscle of HFD-fed mice after adiponectin expression (Fig. 4, A and B). An apparent improvement in insulin-stimulated phosphorylation of S473 was also observed after adiponectin was expressed in skeletal muscle of HFD-fed mice (Fig. 4, C and D). We observed that HFD induced an increase in total expression of Akt2 isoform in skeletal muscle that was reduced to similar levels to the chow-fed control group by adiponectin overexpression (Fig. 4E). Neither HFD nor adiponectin transgene expression had an effect on the expression of AdipoR1, AdipoR2, APPL1, or APPL2 (Fig. 4F).

Data from hyperinsulinemic-euglycemic clamp studies also indicated that hepatic insulin sensitivity was improved in HFD-fed mice after adiponectin transgene expression (Fig. 3B). Therefore, we examined insulin-stimulated Akt phosphorylation in liver of the mice described above. A significant reduction in insulin-stimulated Akt phosphorylation at T308 occurred after HFD, and this was partially improved after adiponectin expression (Fig. 5, A and B). The changes observed upon study of S473 phosphorylation were less pronounced although again there was an apparent reduction in HFD-fed mice (Fig. 5, C and D). This was not altered by muscle adiponectin expression (Fig. 5, C and D). In liver we did not detect any change in Akt isoform expression among groups (Fig. 5, A and C), and neither HFD nor adiponectin transgene expression had an effect on hepatic expression of AdipoR1, AdipoR2, APPL1, or APPL2 (Fig. 5E). Because our data suggested cross talk between muscle and liver, we next examined serum adiponectin levels and found that overexpression of adiponectin transgene in skeletal muscle correlated with a small but significant increase in total serum levels of adiponectin and in particular a selective increase in the content of the oligomeric HMW form (Fig. 5, G-K). This increased adiponectin circulating level also led to a reduction of HFD-induced triglyceride accumulation in liver (Fig. 5F).

**DISCUSSION**

This study was designed to examine the functional effects of adiponectin synthesized and secreted by skeletal muscle. The ability of adiponectin to improve insulin sensitivity and whole body glucose homeostasis in animal models of obesity and diabetes has been well documented (12, 42). Adiponectin is best known as an adipocyte-derived hormone, although there is...
evidence that adiponectin can be produced by a variety of
tissues, including skeletal muscle (5, 14, 15, 19, 23, 25).
However, the functional and physiological significance of
adiponectin from nonadipose sources is not well established.
We used UTMD to specifically overexpress adiponectin in
skeletal muscle and examine the metabolic consequences in
mice fed a HFD to induce obesity and insulin resistance.

Use of UTMD for tissue-specific gene delivery has proven
effective in various tissues, including heart (3, 11, 37), pancreas
(8–10), kidney (7, 17) and skeletal muscle (27, 36, 40, 43).
Gene delivery via UTMD in adult mice is in fact preferable
to models where the gene is eliminated or overexpressed
constitutively, which are often complicated by occurrence of
compensative mechanisms. Systemic delivery of plasmid DNA
is possible, since its encapsulation in microbubbles serves to
protect from degradation by DNases. Application of ultrasound
at the tissue of choice primarily causes microbubble destruc-
tion, thus increasing local concentration of transgene yet also
allowing for efficient transfection via cavitation and tissue pore
formation (21, 30). In this study we first optimized our exper-
imental protocol to establish conditions that conferred effective
overexpression of luminescent and fluorescent reporter gene
expression. Subsequently, this UTMD protocol was effective
in enhancing muscle expression of total as well as HMW and
hexameric forms of adiponectin. This clearly indicates that
skeletal muscle has the molecular machinery to posttransla-
tionally modify adiponectin and allow oligomerization to bi-
ologically active forms (24, 25, 41).

We then tested the functional metabolic significance of
overexpressing adiponectin in skeletal muscle. Importantly, we
first observed that overexpression of adiponectin caused a
significant improvement in the plasma glucose excursion ob-
served upon glucose challenge. To investigate this improved
whole body glucose tolerance in more detail, we subjected
mice to hyperinsulinemic-euglycemic clamp study (6). As
expected, mice fed HFD showed a decreased GIR compared
with normal chow-fed controls, indicating development of
insulin resistance. In keeping with data from preliminary glu-
cose tolerance tests, overexpressing adiponectin only in skel-
etal muscle of these mice caused a significant recovery of
HFD-decreased GIR. Hyperinsulinemic-euglycemic clamp
analysis (6) allows determination of changes in insulin-stimu-
lated glucose uptake by peripheral tissues, including skeletal
muscle (post:basal Ra) and insulin-inhibited glucose produc-
tion by liver (post:basal Rl). As expected HFD induced defects
in regulation of both glucose disposal and appearance com-
pared with normal chow-fed mice. We observed a significant
improvement in insulin-stimulated glucose disposal in HFD-
fed mice in which UTMD was used to overexpress adiponectin
in muscle. It has been estimated that 80% of serum glucose is
cleared via being uptaken into skeletal muscle, indicating an
important autocrine effect of adiponectin is likely in our study.
Fig. 5. Serum adiponectin, insulin, and adiponectin signaling, lipid analysis in liver. Mice were fed either commercial chow or 60% HF diet at the age of 6 wk. After 12 wk HF diet, we used UTMD to deliver either pGL3 (EV) or fAd plasmid to hind leg skeletal muscles. Serum samples were collected pre- and post-UTMD procedure. Seven days post-UTMD, animals were starved 5â€“6 h, and insulin was administered as a bolus injection (4 U/kg body wt) via tail vein. Fifteen minutes after injection, liver tissue was collected, and homogenates were prepared and then analyzed by Western blotting. Representative Western blot of p-Akt [T308 (A), S473 (C), total Akt2 (A and C)] and quantitative analysis [T308 (B), S473 (D)]. E: representative Western blot for AdipoR1, AdipoR2, APPL1, and APPL2. F: liver triglyceride content (nmol/μg protein). G-K: representative Western blot and quantitative analysis of serum adiponectin level. Data represent means ± SE. *P < 0.05, chow-fed UTMD-EV vs. 60% HFD UTMD-EV, #P < 0.05, 60% HFD UTMD-EV vs. 60% HFD UTMD-fAd, and αP < 0.05 vs. pre-UTMD; n = 4–5.
We also observed improved insulin-stimulated Akt phosphorylation in muscle of mice after adiponectin expression, which is in keeping with previous work that has shown improved basal glucose uptake and insulin-stimulated Akt phosphorylation and glucose uptake in L6 skeletal muscle cells overexpressing adiponectin (25). The improved insulin action was independent of altered expression of AdipoR1, AdipoR2, APPL1, or APPL2, four major proteins involved in adiponectin's signaling pathway in skeletal muscle, since no changes were observed between groups of animals studied here (13, 29, 39, 42). A decreased level of adiponectin has been detected in skeletal muscle of obese and diabetic animals, was enhanced when the animals were treated with rosiglitazone, and correlated with improved whole body glucose homeostasis (25). Other studies have also indicated a potentially important physiological effect of adiponectin produced by skeletal muscle. For example, elevated expression in response to proinflammatory cytokines or lipopolysaccharide has been suggested to represent a local anti-inflammatory protection mechanism (14, 19). Therefore, it is clear that skeletal muscle produces adiponectin, that this is altered in disease states, and that it may have more than one functional consequence.

Data from hyperinsulinemic-euglycemic clamp study also indicated that adiponectin overexpression in skeletal muscle improved HFD-induced defects in insulin-inhibited glucose production by liver, and analysis of Akt phosphorylation indicated improved hepatic insulin sensitivity. This suggests that cross talk between muscle and liver occurred, and, to investigate the potential role of adiponectin as the factor that mediated this cross talk, we first examined changes in serum adiponectin content. Previous estimates suggested that adiponectin derived from skeletal muscle represents only 1/174 of total circulating levels (15). Nevertheless, although a relatively small contributor to the total circulating pool, changes in muscle adiponectin levels can be of potential physiological significance. Indeed, animals with muscle-specific PPAR-γ deletion displayed reduced circulating adiponectin (16). Our analysis of serum adiponectin revealed a small but significant increase in mice in which adiponectin, as opposed to EV, was overexpressed using UTMD. Interestingly, this small change was accounted for by increased amounts of HMW adiponectin, the form that is thought to be most biologically active in liver and to mediate antidiabetic effects (32). Changes in hepatic insulin sensitivity occurred independently of changes in expression of adiponectin receptors or their adaptor protein isoforms (13, 29, 39, 42). Whether the small increase in HMW adiponectin is enough to contribute to improved whole body glucose homeostasis, this alludes to the possibility that elevating muscle adiponectin production therapeutically can be sufficient to capitalize on the antidiabetic effects of adiponectin in obese and diabetic patients.

GRANTS
Funding for this study was provided by the Canadian Institutes of Health Research via an operating grant to G. Sweeney. V. Vu was supported by a Doctoral Student Research Award from the Canadian Diabetes Association and a Graduate Scholarship from the Government of Ontario.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS
Author contributions: V.V., Y.L., A.X., and G.S. conception and design of research; V.V., Y.L., and S.S. performed experiments; V.V., Y.L., and G.S. analyzed data; V.V. and Y.L. prepared figures; V.V. and Y.L. drafted manuscript; V.V., Y.L., A.X., and G.S. approved final version of manuscript; V.V., Y.L. and G.S. edited and revised manuscript.

REFERENCES
17. Hou CC, Wang W, Huang XR, Fu P, Chen TH, Sheikh-Hamad D, Lan HY. Ultrasound-microbubble-mediated gene transfer of inducible Smad7...


