Hyperglycemia- and hyperinsulinemia-induced alteration of adiponectin receptor expression and adiponectin effects in L6 myoblasts

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Abstract

Adiponectin has been shown to regulate glucose and fatty acid uptake and metabolism in skeletal muscle. Here we investigated the role of the recently cloned adiponectin receptor (AdipoR) isoforms in mediating effects of both globular (gAd) and full-length (fAd) adiponectin, and their regulation by hyperglycemia (25 mM, 20 h) and hyperinsulinemia (100 nM, 20 h). We used L6 rat skeletal muscle cells, which were found to express both AdipoR1 and AdipoR2 mRNA in a ratio of over 6:1 respectively. Hyperglycemia and hyperinsulinemia both decreased AdipoR1 receptor expression by approximately 50%, while the latter induced an increase of approximately threefold in AdipoR2 expression. The ability of gAd to increase GLUT4 myc translocation, glucose uptake, fatty acid uptake and oxidation, as well as AMP-activated protein kinase (AMPK) and acetyl-CoA carboxylase (ACC) phosphorylation, was decreased by both hyperglycemia and hyperinsulinemia. Interestingly, hyperinsulinemia induced the ability of fAd to elicit fatty acid uptake and enhanced fatty acid oxidation in response to fAd. In summary, our results suggest that both hyperglycemia and hyperinsulinemia cause gAd resistance in rat skeletal muscle cells. However, hyperinsulinemia induces a switch toward increased fAd sensitivity in these cells.

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Introduction

The association between obesity and diabetes has prompted great interest in the metabolic effects of adipokines (Kershaw & Flier 2004). In this study, we focus on adiponectin (Acrp30), a 247-amino-acid peptide with sequence similarity to complement factor C1q and structural homology to tumor necrosis factor (TNF)- α (Scherer *et al.* 1995). Adiponectin mRNA expression and circulating levels are reduced in obese (Statnick *et al.* 2000, Halleux *et al.* 2001, Yang *et al.* 2001, Fisher *et al.* 2002, Milan *et al.* 2002, Altomonte *et al.* 2003) and lipotrophic (Yamauchi *et al.* 2001, Vigouroux *et al.* 2003) humans or rodent models. Adiponectin has been shown to have antidiabetic, anti-inflammatory and antiatherosclerotic effects (Diez & Iglesias 2003, Haluzik *et al.* 2004). Indeed, adiponectin can both improve insulin sensitivity and mediate direct effects on glucose and fatty acid metabolism in skeletal muscle, adipocytes and liver (for reviews, see Berg *et al.* 2002, Tsao *et al.* 2002, Goldfine & Kahn 2003, Haluzik *et al.* 2004).

Adiponectin contains 22 collagen repeats in the N-terminal domain and a C1q-like C-terminal globular domain (Scherer *et al.* 1995). Regulation of physiologic responses to adiponectin is complicated and occurs at

many levels, including formation of oligomers, enzymatic cleavage and availability of its receptor isoforms. Full-length adiponectin (fAd), which is thought to mediate primarily effects in liver, may be cleaved (Waki *et al.* 2005) to liberate a fragment containing the globular domain (gAd), which can also directly mediate metabolic effects, particularly in skeletal muscle (Fruebis *et al.* 2001, Yamauchi *et al.* 2003, Ceddia *et al.* 2005). Adiponectin monomers can form trimeric or higher order complexes that may each have different potency for, and end-point responses in, different tissues (Tsao *et al.* 2002a, 2003, Waki *et al.* 2003). Although some debate exists, circulating fAd has been found in monomeric, trimeric, hexameric and higher order forms, whereas gAd exists as both a monomer and trimer (Fruebis *et al.* 2001, Yamauchi *et al.* 2002, 2003, Pajvani *et al.* 2003, 2004, Shklyayev *et al.* 2003, Shoji *et al.* 2004, Wang *et al.* 2004). While the high-, medium- and low-molecular-weight complexes of fAd are the primary circulating forms of adiponectin, recent evidence for localized cleavage to produce gAd makes it imperative to study the effect of gAd, particularly in muscle. Two adiponectin receptor (AdipoR) isoforms were recently discovered (Yamauchi *et al.* 2003); AdipoR1 has a high affinity for gAd and low affinity for fAd, whereas AdipoR2 exhibits intermediate

binding affinity for both gAd and fAd. A potential pathophysiologic role for alterations in AdipoR is supported by a positive correlation between receptor expression and insulin resistance (Civitarese *et al.* 2004) and plasma insulin levels (Inukai *et al.* 2004, Tsuchida *et al.* 2004), lower AdipoR1 and AdipoR2 expression in patients with a family history of diabetes (Civitarese *et al.* 2004), and increased skeletal muscle AdipoR1 expression in streptozotocin (STZ)-diabetic mice (Inukai *et al.* 2004, Tsuchida *et al.* 2004). Here we characterized the effect of chronic insulin and glucose treatment on AdipoR expression and investigated whether the observed changes correlated with alterations in the functional effects of gAd and fAd on glucose and fatty acid uptake and metabolism in rat skeletal muscle cells.

Materials and methods

Materials

[1-¹⁴C]Oleic acid and 2-deoxy-D-[³H]glucose were purchased from Amersham (Quebec, Canada). BODIPY-conjugated palmitate was from Molecular Probes (Eugene, OR, USA). Insulin (Humulin) was purchased from Eli Lilly (Toronto, Canada). Recombinant human globular adiponectin (gAd) was purchased from Peprotech (Ottawa, Canada). Antifade solution was from Dako (Kyoto, Japan). TRIzol reagent and Platinum SYBR Green qPCR SuperMix UDG Kit were from Invitrogen Life Technologies (Burlington, Canada). Anti-myc monoclonal antibody, AMP-activated protein kinase (AMPK)- α 1 and AMPK- α 2 were from Santa Cruz (Santa Cruz, CA, USA). All other antibodies are from Cell Signalling (Beverly, MA, USA). PVDF membrane and HRP-linked secondary antibodies were from BioRad (Burlington, Canada). *o*-Phenylenediamine dihydrochloride (OPD) and all other chemicals and primers were from Sigma. All cell culture components were from Wisent (Quebec, Canada).

Cell culture

Rat L6 skeletal myoblasts were grown in α -minimum essential medium (α MEM) containing 10% (v/v) fetal bovine serum (FBS) (growth medium) under 5% CO₂ at 37 °C. We used wild-type L6 cells or cells stably transfected to overexpress stably GLUT4 harboring a myc epitope on the first exofacial loop of the transporter (L6 GLUT4 myc cells, a kind gift from Dr Amira Klip, Hospital for Sick Children, Toronto). This facilitated accurate quantitative analysis of GLUT4 translocation in intact cells. For experimental procedures, cells were cultured in α MEM (5 mM glucose) containing 2% (v/v) FBS plus 1% antibiotic/antimycotic, which was supplemented with or without final concentrations of 25 mM glucose or 100 nM insulin, as indicated in figure legends.

Analysis of adiponectin receptors expression by real-time quantitative PCR

cDNAs were synthesized by reverse transcription with 1 μ g total RNA. Primers for real-time quantitative PCR were based on sequences of adiponectin receptors in L6 myoblasts (see Results). Primer sequences and their respective PCR fragment length were as follows:

● AdipoR1 (158 bp): forward 5'-GCTGGCCTTTA TGCTGCTCG-3', reverse 5'-TCTAGGCCGTAACG GAATTC-3'

● AdipoR2 (415 bp): forward 5'-CCCTCTGCAAG AGAAAGTGG-3', reverse 5'-TAGCCAGCCTATCT GCCCTA-3'

● β -actin (133 bp): forward 5'-CTGTGCCCATCTA TGAGGGT-3', reverse 5'-CTCTCAGCTTGGTGGT GAA-3'.

AdipoR1 and AdipoR2 RNA levels in L6 myoblasts were determined by using platinum SYBR Green qPCR SuperMix UDG Kit. L6 myoblasts were incubated with insulin (100 nM) or glucose (25 mM) for the times indicated, 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 95 °C for 30 s/60 °C (AdipoR1 and AdipoR2) or 65 °C (β -actin) for 30 s/72 °C for 30 s.

Production of globular and full-length adiponectin

For this work, we chose to express and purify recombinant gAd by subcloning murine gAd cDNA (a kind gift from Dr Philipp Scherer, Albert Einstein College of Medicine, New York City) into the pTrisEx expression vector (Novagen). Expression of the N-terminal His₆-tagged fusion protein was then induced in *E. coli* with IPTG. Recombinant gAd was isolated from the bacterial cell lysate with a nickel column according to the manufacturer's instruction (Sigma). Mass spectrometry analysis confirmed the production and isolation of gAd (data not shown). We have also used commercially available human gAd (Peprotech) to validate our results and, despite batch to batch variability, we found similar results with both. It has been suggested that bacterially produced fAd is inactive (Berg *et al.* 2001, Yamauchi *et al.* 2001, 2002) and that fAd must be appropriately post-translationally modified before it can inhibit gluconeogenesis, increase fatty acid oxidation, reduce steatosis, increase CPT-1 activity and decrease acetyl-CoA carboxylase (ACC) activity in liver (Wang *et al.* 2002, Xu *et al.* 2003). Therefore, here we used full-length post-translationally modified Ad produced in a mammalian expression system, as previously described (Wang *et al.* 2002, Xu *et al.* 2003).

Measurement of glucose uptake

Cells were incubated with insulin (100 nM) or glucose (25 mM) for 20 h, and subsequently serum-starved in the

continued presence of insulin or glucose for the final 4 h in 24-well plates prior to further treatment. Cells were washed twice with PBS and treated with gAd (1 µg/ml, 2 h) or fAd (5 µg/ml, 2 h). After treatments, glucose uptake was determined as previously described (Ceddia *et al.* 2005). 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-D-[³H]glucose (pH 7.4)) for 5 min at room temperature. 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.

Determination of GLUT4 translocation

The overexpression of GLUT4 with an exofacial myc epitope allows accurate quantification of cell-surface GLUT4 content in intact cells. L6-GLUT4 myc cells were pretreated as described above for glucose uptake, and then cell surface GLUT4 content was determined by an antibody-coupled colorimetric assay, as previously described (Ceddia *et al.* 2005). Cells were first incubated with primary antibody solution (anti-myc monoclonal antibody) for 1 h at 4 °C, and fixed with 3% paraformaldehyde for 3 min. The fixative was then neutralized by 10 mM glycine for 10 min on ice. Cells were then treated with blocking buffer (3% BSA, 5% goat serum in PBS) for 30 min and incubated with secondary antibody solution (HRP-conjugated antimouse antibody) for 1 h at 4 °C. Finally, 1 ml OPD reagent was added in each well for 30 min, the reaction was stopped by adding 3 M HCl, and the absorbance of supernatant was measured at 492 nm.

Measurement of fatty acid oxidation

Fatty acid oxidation was measured by the production of ¹⁴CO₂ from [1-¹⁴C]oleate, as previously described (Ceddia *et al.* 2005). Briefly, cells in 60 × 15 mm Petri dishes were incubated with insulin (100 nM) or glucose (25 mM) for 20 h and subsequently serum-starved for 4 h. After this incubation period, cells were treated with gAd (1 µg/ml, 2 h) or fAd (5 µg/ml, 2 h) prepared in serum-free medium containing 0.3 µCi/ml [1-¹⁴C]oleic acid. [1-¹⁴C]Oleate and cold oleate (0.5 mM final concentration) were complexed with defatted BSA at a 2.5:1 ratio. Each Petri dish was sealed with parafilm with a piece of Whatman paper taped facing the inside of the petri dish. The Whatman paper was wet with 100 µl phenylethylamine/methanol (1:1) to trap the CO₂ produced during the incubation period. After the 2-h incubation, 200 µl H₂SO₄ (4 mol/l) was added to the cells and incubated for 1 h at 37 °C. Finally, the sealing from each Petri dish was removed, and the pieces of

Whatman paper were carefully transferred to scintillation vials for radioactivity counting.

Measurement of fatty acid uptake

To determine fatty acid uptake, we used fluorescent palmitate as previously described (Hirsch *et al.* 1998, Stahl *et al.* 2002) with slight modifications. Cells were grown on cover slips and incubated with insulin (100 nM) or glucose (25 mM) for 20 h, and subsequently serum-starved for the final 4 h. Culture medium was removed, and cells washed twice with PBS containing 0.1% fatty acid-free albumin. After this incubation period, cells were treated with gAd (1 µg/ml) or fAd (5 µg/ml) for the times indicated in figure legends. BODIPY-conjugated palmitate (1 µM) was prepared in serum-free αMEM and added to cells. After incubation at 37 °C for 2 min, cells were washed three times and cover slips were fixed on glass slides with antifade solution. Nonspecific uptake was determined by competition with 5 mM palmitate. For confocal microscopy analysis, BODIPY-conjugated fatty acids were excited at 488 nm with an Olympus 300 multiline argon laser. Fluorescent images were obtained with Fluoview software, and fluorescence intensity was quantitated by ImageJ software.

Western blot analysis

Cells were cultured in six-well plates and incubated with insulin (100 nM) or glucose (25 mM) for 20 h where indicated, serum-starved for 4 h, and then stimulated with gAd (1 µg/ml, 5 min) or fAd (5 µg/ml, 5 min). Subsequently, as we previously described (Sweeney *et al.* 2001, Tajmir *et al.* 2003), cells were lysed in 1 SDS sample buffer (62.5 mM Tris-HCl (pH 6.8), 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.01% w/v bromophenol blue, and protease and phosphatase inhibitors (1 µM Na₃VO₄, 1 µM leupeptin, 1 µM pepstatin, 1 µM okadaic acid and 1 µM PMSF)), passed through a syringe several times and heated (65 °C) for 5 min. Cell lysates were then centrifuged for 5 min in a benchtop microfuge (16 294 g), and approximately 30 µg protein was resolved by 10% SDS-PAGE and then 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% NP-40 for 1 h. Membranes were incubated overnight with primary antibodies at the following dilutions: phospho-AMPK (1:500), AMPK-α1 (1:500), AMPK-α2 (1:500), phospho-ACC (1:1000), ACC (1:1000) and β-actin (1:1000). Membranes were then washed four times in 1 × wash buffer for 15 min each at room temperature and then incubated with HRP-coupled secondary antibody (1:10 000) for 1 h. Membranes were washed five times in 1 × wash buffer

Table 1 Expression of AdipoR1 and AdipoR2 isoforms in L6 myoblasts and rat soleus and EDL muscle. We used quantitative real-time PCR, with β -actin as reference, to show that the L6 myoblasts used in this study expressed over six-fold more AdipoR1 than AdipoR2 mRNA. Analysis of glycolytic (EDL) and oxidative (soleus) rat skeletal muscle demonstrated that L6 cells and EDL expressed a similar AdipoR isoform profile (over 6 : 1). Almost no AdipoR2 was detectable in soleus, giving rise to a ratio of approximately 4000-fold more AdipoR1. Results represent mean $\pm$ S.E.M. of $n=3$, and in each case the level of AdipoR1 expression was assigned an arbitrary value of 1, and AdipoR2 content was expressed relative to this value

	Relative expression ratio	
	AdipoR1	AdipoR2
L6 myoblasts	1	0.14 $\pm$ 0.02
Rat EDL	1	0.16 $\pm$ 0.03
Rat soleus	1	(2.5 $\times$ 10 ⁻⁴)

for 10 min each, and proteins were visualized by enhanced chemiluminescence and quantified by the Scion Image program (Scion Corporation, Frederick, MD, USA).

Results

Analysis of AdipoR1 and AdipoR2 sequence and expression in L6 cells

When we initiated this work, the human and mouse, but not rat, AdipoR sequences had been published (Yamauchi *et al.* 2003), and so we began by cloning and sequencing rat AdipoR1 and AdipoR2 from L6 cells. This was done by extracting total RNA and then cDNA synthesis and PCR amplification with primers designed to recognize mouse AdipoR. The resulting PCR fragment was ligated to pDrive cloning vector, and this was followed by colony screening, plasmid detection and sequencing. We found that rat AdipoR1 comprises a 1128 bp cDNA sequence which shares 94% and 90% identity to mouse and human AdipoR1 respectively. These cDNAs encoded proteins showing 98% and 93% identity to mouse and human AdipoR1 respectively. The 1161 bp AdipoR2 sequence shares 97% and 90% identity to mouse and human AdipoR2 respectively, and 99% and 96% identity at the amino-acid level. The sequences have been deposited in Genbank with accession nos DQ148391 for AdipoR1 and DQ148392 for AdipoR2. We also used quantitative real-time PCR, with β -actin as reference, to show that the L6 myoblasts used in this study express both AdipoR1 and AdipoR2 in a ratio of over 6:1 (Table 1). We used rat extensor digitorum longus (EDL) and soleus muscle for comparison and found a very similar AdipoR expression

profile in EDL and L6 myoblasts. In soleus, a very low, and barely detectable, AdipoR2 copy number was observed (Table 1).

Alteration in AdipoR1 and AdipoR2 expression induced by hyperglycemia and hyperinsulinemia

We used quantitative real-time PCR to investigate the effect of glucose (25 mM, 20 h) and insulin (100 nM, 20 h) on AdipoR1 and AdipoR2 mRNA expression, using β -actin as reference. Both hyperglycemia and hyperinsulinemia caused a significant reduction in AdipoR1 expression to approximately 50% of the original level (Fig. 1A). Hyperinsulinemia increased AdipoR2 expression by almost threefold whereas hyperglycemia had no effect on the expression level of AdipoR2 (Fig. 1B).

Hyperglycemia- and hyperinsulinemia-induced changes in glucose uptake and GLUT4myc translocation in response to gAd and fAd

We have demonstrated previously that proper hydroxylation and glycosylation of lysine residues in fAd play a critical role in determining activity of this protein (Wang *et al.* 2002, Xu *et al.* 2003). Examination of metabolic responses in liver demonstrated that fAd produced in mammalian expression systems, with appropriate hydroxylation and glycosylation, is significantly more biologically active than fAd produced in bacteria (Wang *et al.* 2002, Xu *et al.* 2003). Therefore, for this study, we used fAd produced in COS cells, as previously described by us (Wang *et al.* 2002, Xu *et al.* 2003). We and others have demonstrated previously that gAd expressed and purified from bacteria exhibits full activity (Ceddia *et al.* 2005). We tested whether the ability of gAd or fAd to regulate GLUT4 myc translocation and glucose uptake in L6 cells (Ceddia *et al.* 2005) may be altered by hyperglycemia or hyperinsulinemia. We found that gAd, but not fAd, increases glucose uptake (Fig. 2A). When cells were grown in a high concentration of insulin, the ability of gAd to increase glucose uptake was lost, but fAd now elicited an increase in glucose uptake of $\sim$ 1.4-fold (Fig. 2B). The ability of gAd to elicit glucose uptake was reduced by hyperglycemia (Fig. 2C). Absolute values for glucose uptake were 16.55 $\pm$ 2.73 pmol/min per mg protein (control) and 25.33 $\pm$ 3.49 and 19.22 $\pm$ 0.95 for high insulin and high glucose conditions respectively. An increase in the amount of GLUT4 myc detected at the cell surface of intact cells was observed in response to gAd, but not fAd (Fig. 3A). A smaller increase in GLUT4 myc translocation in response to gAd was observed under both hyperinsulinemic and hyperglycemic conditions, and there was still no effect of fAd (Fig. 3B and C).

Figure 1 Regulation of AdipoR1 and AdipoR2 expression by hyperinsulinemia and hyperglycemia. The effect of growing cells in the presence of insulin (HI: 100 nM, 20 h) or glucose (HG: 25 mM, 20 h) on AdipoR mRNA expression with respect to untreated cells (Con) is shown in panels A and B. Values are expressed as mean±S.E.M. of $n \geq 3$. * $P < 0.05$ with respect to control.

Regulation of fatty acid uptake by gAd and fAd in the presence of hyperglycemia or hyperinsulinemia

We first examined the time-dependent ability of gAd to induce fluorescent fatty acid uptake in L6 cells, and, as shown in Fig. 4A, we found a maximal increase after 2 h. This time of treatment was chosen to investigate the

effect of gAd (Fig. 4B) and fAd (Fig. 4C) on fatty acid uptake under control, hyperinsulinemic and hyperglycemic conditions. gAd increased fatty acid uptake by 1.6-fold under normal conditions, an effect which was abolished by hyperinsulinemia and reduced by hyperglycemia (Fig. 4B). A small but significant increase in fatty acid uptake was observed in response to fAd only

Figure 2 Effect of gAd and fAd on glucose uptake and regulation by hyperinsulinemia and hyperglycemia. Uptake of 2-deoxyglucose was measured in response to gAd (1 µg/ml, 2 h) or fAd (5 µg/ml, 2 h) in cells grown under normal growth conditions (A) or in the presence of (B) insulin (100 nM, 20 h) or (C) glucose (25 mM, 20 h). Values are expressed as mean±S.E.M. of $n \geq 3$ where each condition was assayed in triplicate. * $P < 0.05$ with respect to control.

Figure 3 Effect of gAd and fAd on GLUT4 translocation and regulation by hyperinsulinemia and hyperglycemia. The amount of GLUT4 detected at the cell surface was measured in L6-GLUT4 myc cells in response to gAd (1 μ g/ml, 2 h) or fAd (5 μ g/ml, 2 h) in cells grown under normal growth conditions (A) or in the presence of (B) insulin (100 nM, 20 h) or (C) glucose (25 mM, 20 h). Values are expressed as mean $\pm$ S.E.M. of $n \geq 3$ experiments where each condition was assayed in triplicate. * $P < 0.05$ with respect to control.

under hyperinsulinemic conditions (Fig. 4C). As can be seen, both hyperinsulinemia and hyperglycemia elevate the basal level of fatty acid uptake.

Regulation of fatty acid oxidation by gAd and fAd in the presence of hyperglycemia or hyperinsulinemia

Both gAd and fAd were found to increase fatty acid oxidation significantly (Fig. 5A), and this was prevented by hyperglycemia (Fig. 5C). Hyperinsulinemia reduced basal fatty acid oxidation levels, and abolished the ability of gAd and enhanced the ability of fAd to increase oxidation (Fig. 5B).

Regulation of AMPK and ACC phosphorylation by gAd and fAd in the presence of hyperglycemia or hyperinsulinemia

Figure 6A demonstrates that gAd significantly increases AMPK phosphorylation in L6 cells (average 1.51-fold from $n \geq 3$) under normal growth conditions, but not in the presence of hyperinsulinemia (0.95-fold) or hyperglycemia (1.14-fold). ACC phosphorylation is also stimulated by gAd (1.49-fold) (Fig. 6C), and this effect is attenuated by both hyperinsulinemia (1.17-fold) and hyperglycemia (1.13-fold). We also examined the effect of hyperinsulinemia and hyperglycemia on expression of AMPK isoforms and found that hyperinsulinemia causes an increase in AMPK- $\alpha 2$ expression (Fig. 6B). ACC

expression was reduced by hyperinsulinemia and unaltered by hyperglycemia (Fig. 6D). There was a small but not significant stimulation of AMPK phosphorylation in response to fAd under normal and hyperglycemic conditions and no apparent change in the presence of hyperinsulinemia (Fig. 7A). ACC phosphorylation was significantly increased by fAd, 1.65-fold under normal conditions and 1.23-fold under hyperinsulinemia (Fig. 7B). This effect of fAd was not apparent (1.1-fold) under hyperglycemia (Fig. 7B).

Discussion

There is currently great interest in the role played by adiponectin in glucose homeostasis; however, correlative studies represent the bulk of literature available to date. In this study, we have established a system to examine regulation of glucose and lipid metabolism by both gAd and fAd in skeletal muscle under conditions commonly observed in obese and diabetic individuals, namely, prolonged hyperglycemia or hyperinsulinemia. The recent identification of adiponectin receptors (Yamauchi *et al.* 2003) was an important step in understanding regulation of adiponectin function. However, regulation of adiponectin function is complex, as different oligomeric forms of fAd and gAd mediate distinct effects (Tsao *et al.* 2002a, 2003, Waki *et al.* 2003). Although some debate exists, circulating fAd has been found in monomeric, trimeric, hexameric and higher-order

Figure 4 Effect of gAd and fAd on fatty acid uptake and regulation by hyperinsulinemia and hyperglycemia. The time course of gAd-stimulated fatty acid uptake was first established (A). Subsequently, uptake of BODIPY-palmitate was measured in response to (B) gAd (1 µg/ml, 2 h) or (C) fAd (5 µg/ml, 2 h) in cells grown under normal growth conditions (control (Con)) or in the presence of insulin (100 nM, 20 h) or glucose (25 mM, 20 h). Representative images for each experiment are shown, and quantitative values, obtained by analyzing at least 20 individual cells from each independent experiment, are expressed as mean ± S.E.M. * $P < 0.05$ compared with control.

forms, whereas gAd exists as both a monomer and a trimer (Fruebis *et al.* 2001, Yamauchi *et al.* 2002, 2003, Pajvani *et al.* 2003, 2004, Shklyav *et al.* 2003, Shoji *et al.* 2004, Wang *et al.* 2004). A fragment of adiponectin containing the globular domain can mediate metabolic effects, particularly in skeletal muscle (Fruebis *et al.* 2001, Yamauchi *et al.* 2003, Ceddia *et al.* 2005). Thus, cleavage of full-length adiponectin to liberate the globular domain by enzymes such as the recently characterized leukocyte elastase (Waki *et al.* 2005) may represent an important regulatory step in the function of adiponectin (Pajvani *et al.* 2003).

Using quantitative PCR, we show that L6 rat skeletal myoblasts express over six times more AdipoR1 than AdipoR2 mRNA. This is in keeping with their observed greater sensitivity to gAd (Ceddia *et al.* 2005), since it has been demonstrated that AdipoR1 contains both a high-

and intermediate-affinity binding site for gAd, whereas AdipoR2 contains intermediate- and low-affinity binding sites for fAd (Yamauchi *et al.* 2003). Indeed, use of siRNA to remove AdipoR1 in C2C12 cells almost totally eliminated gAd binding but had little effect on fAd binding (Yamauchi *et al.* 2003). The converse was observed upon siRNA-induced reduction in AdipoR2 expression (Yamauchi *et al.* 2003). Previous work in mice also found that AdipoR1 expression was highest in muscle (Civitarese *et al.* 2004), while another study demonstrated that human muscle contained 1.8-fold more AdipoR1 than AdipoR2 mRNA (Staiger *et al.* 2004). Here we show that EDL expresses a very similar ratio of AdipoR1 to AdipoR2 to that found in L6 cells, whereas soleus muscle appears to express little AdipoR2. Using epitope-tagged AdipoR, we and others have found that commercially available AdipoR antibodies

Figure 5 Effect of gAd and fAd on fatty acid oxidation and regulation by hyperinsulinemia and hyperglycemia. The production of $^{14}\text{CO}_2$ from $[1\text{-}^{14}\text{C}]$ oleate was examined in response to gAd (1 $\mu\text{g}/\text{ml}$, 2 h) or fAd (5 $\mu\text{g}/\text{ml}$, 2 h) in cells grown under normal growth conditions (A) or in the presence of (B) insulin (100 nM, 20 h) or (C) glucose (25 mM, 20 h). Values are expressed as mean $\pm$ S.E.M. of $n \geq 3$. * $P < 0.05$ with respect to control.

are unreliable; therefore, we are in the process of making AdipoR antibodies. As we demonstrate here, regulation of AdipoR expression may be an important mechanism determining the function of adiponectin.

Increased AdipoR1 expression in skeletal muscle of STZ-diabetic mice was corrected by addition of insulin (Inukai *et al.* 2004, Tsuchida *et al.* 2004), and in C2C12 cells, insulin (24 h) reduced AdipoR1 expression (Inukai *et al.* 2004, Tsuchida *et al.* 2004). However, the reason why another study found no significant change in C2C12 myotube AdipoR1 mRNA expression in response to 28-h treatment with insulin is unclear (Staiger *et al.* 2004). Insulin did not alter AdipoR1 or AdipoR2 in adipocytes (Fasshauer *et al.* 2004), and expression of AdipoR1 and AdipoR2 in liver was not significantly changed in hypo- or hyperinsulinemic mouse models (Inukai *et al.* 2004). Here we showed that insulin decreased AdipoR1 expression in rat skeletal muscle cells and, importantly, increased AdipoR2 expression. This raises the interesting possibility that hyperinsulinemia may make skeletal muscle cells resistant to gAd while inducing sensitivity to fAd. AdipoR1 expression was decreased in hyperglycemic, obese db/db mice (Inukai *et al.* 2004). AdipoR1 and AdipoR2 were decreased in skeletal muscle of hyperglycemic ob/ob mice (Tsuchida *et al.* 2004) and increased in muscle and liver of fasted mice (Tsuchida *et al.* 2004). One study, which should be interpreted cautiously, has failed to detect any change in AdipoR mRNA expression in muscle of type 2 diabetic

patients (Debard *et al.* 2004). We show here that glucose decreased AdipoR1 expression, without altering AdipoR2, suggesting that hyperglycemia may decrease the function of gAd.

We have demonstrated previously that gAd (1 $\mu\text{g}/\text{ml}$, 2 h) increased glucose uptake in L6 cells by causing increased GLUT4 content at the cell surface (Ceddia *et al.* 2005). Concentrations of gAd of 0.5–2.5 $\mu\text{g}/\text{ml}$ (30 min) were found to increase glucose uptake significantly in C2C12 cells (Yamauchi *et al.* 2002) or rat EDL muscle (Tomas *et al.* 2002). It should be noted that in our own studies and the published literature, metabolic responses mediated by adiponectin are typically of a small but significant magnitude, and *in vivo* studies have validated the physiologic importance of the metabolic effects of adiponectin. Here we found a decrease in gAd-stimulated glucose uptake and cell-surface GLUT4 under hyperinsulinemic and hyperglycemic conditions. This is akin to previous studies to reduce AdipoR1 expression artificially with siRNA, whereupon decreased gAd-stimulated, but not fAd-stimulated, glucose uptake and fatty acid oxidation were observed in C2C12 cells (Wang *et al.* 2004). We attribute the fact that fAd increased glucose uptake without altering GLUT4 translocation to glucose transport occurring through the elevated GLUT1 content found in cells grown in high insulin. We and others have shown previously that gAd increases fatty acid oxidation in skeletal muscle or cultured cells (Fruebis *et al.* 2001, Yamauchi *et al.* 2002, 2003, Ceddia *et al.* 2005). We now

Figure 6 Effect of hyperinsulinemia and hyperglycemia on AMPK and ACC expression and their phosphorylation by gAd. The effects of gAd (1 μ g/ml, 5 min) on AMPK and ACC phosphorylation are shown in panels A and C respectively, using cells grown under normal conditions or in the presence of insulin (100 nM, 20 h) or glucose (25 mM, 20 h). The effect of insulin (HI: 100 nM, 20 h) or glucose (HG: 25 mM, 20 h) on expression of AMPK- α 1 and AMPK- α 2 isoforms is shown in panel B, and its effect on total ACC expression is shown in panel D, with β -actin used as a control in each case as shown. Representative immunoblots are shown for each quantitative analysis performed in experiments repeated at least three times. * $P < 0.05$ compared with control.

also have demonstrated that stimulation of both fatty acid uptake and oxidation by gAd is lost when cells are grown in hyperinsulinemic or hyperglycemic conditions. It should also be borne in mind that both cause an elevated level of basal fatty acid uptake that may contribute to the lower magnitude of response elicited by gAd under these conditions. Thus, our results suggest that alterations in the expression level of AdipoR1 may represent an important potential node in determining the response of skeletal muscle cells to gAd.

The effects of fAd are thought to be mediated by AdipoR2 and under normal conditions occur primarily

in liver. However, fAd (2.5 μ g/ml, 30–60 min) has been shown to stimulate glucose uptake and fatty acid oxidation in C2C12 cells (Yamauchi *et al.* 2002). Here we show that fAd does not alter cell-surface GLUT4 myc content, glucose or fatty acid uptake in L6 cells. Most interestingly, we show that the increased AdipoR2 expression induced by hyperinsulinemia correlates with induction of the ability of fAd to stimulate glucose and fatty acid uptake and to enhance further fatty acid oxidation. The physiologic significance of this novel observation must now be further investigated. Surprisingly, fAd increased glucose uptake, but not GLUT4

Figure 7 Effect of fAd on AMPK and ACC phosphorylation and regulation by hyperinsulinemia and hyperglycemia. The effects of fAd (5 µg/ml, 5 min) on AMPK and ACC phosphorylation are shown in panels A and B respectively, using cells grown under normal conditions or in the presence of insulin (100 nM, 20 h) or glucose (25 mM, 20 h). Representative immunoblots are shown for each and quantitative analysis performed on experiments repeated at least three times. * $P < 0.05$ compared with control.

myc translocation, under hyperinsulinemic conditions, and it may be that this occurs via the increased GLUT1 content found under these conditions (data not shown). Hyperglycemia-induced alterations in expression of a protein that plays a critical role in fatty acid oxidation may represent a nonspecific reason for the decrease in ACC phosphorylation and fatty acid oxidation mediated by fAd under these conditions.

Regulation of AMPK phosphorylation plays an important role in mediating many effects of adiponectin, including glucose uptake and fatty acid metabolism. Treatment of C2C12 cells, rat extensor digitorum longus or soleus muscle with gAd and high concentrations of fAd resulted in increased AMPK activity (Tomas *et al.* 2002, Yamauchi *et al.* 2002). Furthermore, increased glucose uptake in response to both gAd and fAd in C2C12 cells was blocked by a dominant negative version

of AMPK (Yamauchi *et al.* 2002). Here we confirm that gAd increased AMPK phosphorylation in L6 cells and show that this effect was prevented by both hyperinsulinemia and hyperglycemia. AMPK also phosphorylates and inhibits activity of ACC, and treatment of C2C12 cells, rat extensor digitorum longus or soleus with gAd or fAd was shown to increase ACC α and ACC β phosphorylation and decrease malonyl Co-A levels by 30% (Tomas *et al.* 2002, Yamauchi *et al.* 2002). The increased ACC phosphorylation and fatty acid oxidation in response to both gAd and fAd in C2C12 cells was blocked by a dominant negative version of AMPK (Yamauchi *et al.* 2002). Importantly, our results show that gAd increased ACC phosphorylation, and this effect was attenuated when AdipoR1 expression was reduced by hyperinsulinemia or hyperglycemia. ACC phosphorylation was significantly increased by fAd under normal and hyperinsulinemic conditions, but not in the presence of hyperglycemia, a result which is in keeping with the lack of oleate oxidation observed in response to fAd under the latter conditions. We observe that under some conditions AMPK and ACC phosphorylation in response to adiponectin do not correlate. This is not entirely surprising and has been documented previously in one of the first studies to examine the effect of adiponectin on skeletal muscle glucose uptake and metabolism (Tomas *et al.* 2002). The authors attributed this observation to temporal reasons or regulation of an alternative ACC kinase by adiponectin. Indeed, although AMPK activation is often regarded as being a prerequisite for ACC phosphorylation, several publications have demonstrated that several kinases can regulate ACC phosphorylation, albeit on additional residues including protein kinase A (Winder *et al.* 1997).

In this study, we have characterized the effect of insulin and glucose on AdipoR expression in L6 rat skeletal muscle cells. AdipoR1 expression is decreased by both hyperinsulinemia and hyperglycemia, while AdipoR2 expression is induced by hyperinsulinemia. Importantly, we have correlated these changes with alterations in the functional effects of gAd and fAd on glucose and fatty acid uptake and metabolism. In summary, our results suggest that both hyperglycemia and hyperinsulinemia cause gAd resistance in rat skeletal muscle cells. However, hyperinsulinemia induces a switch toward induction of, or increase in, fAd sensitivity in these cells. Given the recent interest in the differential effects of various adiponectin forms – globular or various full-length oligomeric forms – we believe that our results may be of major pathophysiologic significance.

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