Autocrine Action of Adiponectin on Human Fat Cells Prevents the Release of Insulin Resistance–Inducing Factors

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The adipocyte hormone adiponectin is negatively correlated with obesity and insulin resistance and may exert an important antidiabetes function. In this study, primary human skeletal muscle cells were cocultured with human fat cells or incubated with adipocyte-conditioned medium in the presence or absence of the globular domain of adiponectin (gAcrp30) to analyze its capacity to restore normal insulin signaling in the muscle cells. Human skeletal muscle cells cocultured with adipocytes or treated with adipocyte-conditioned medium showed an impaired Akt and glycogen synthase kinase 3 serine phosphorylation in response to insulin. Furthermore, insulin-stimulated GLUT4 translocation was reduced by adipocyte-conditioned medium. Impaired insulin signaling was normalized upon addition of gAcrp30 to the coculture. Further, adipocyte-conditioned medium generated in the presence of gAcrp30 was unable to perturb insulin-stimulated Akt phosphorylation. Concomitant addition of gAcrp30 and adipocyte-conditioned medium to the myocytes failed to restore normal insulin action. Protein array analysis of adipocyte-conditioned medium indicated that the secretion of at least eight different cytokines was diminished in response to gAcrp30. We therefore suggest that adiponectin operates as a key regulator of adipocyte secretory function. This autocrine action may prevent the induction of skeletal muscle insulin resistance and may partly explain the antidiabetes action of this hormone. *Diabetes* 54: 2003–2011, 2005

It is now well accepted that adipose tissue represents a major secretory and endocrine active organ producing a variety of factors that may regulate energy metabolism and insulin sensitivity (1). Increased adipose tissue mass, especially in the visceral compartment, is associated with insulin resistance, hyperglycemia, dyslipidemia, hypertension, and other components of the metabolic syndrome (2,3) and represents one of the major risk factors for the development of type 2 diabetes (4–6). Adipocytes from obese subjects exhibit an altered endocrine function and secretory profile leading to an increased release of adipocytokines and proinflammatory molecules including tumor necrosis factor (TNF)α, interleukin (IL)-6, angiotensinogen, and resistin (7,8). Some of these factors play a key role in the induction of skeletal muscle insulin resistance in rodents (9); however, their precise role in humans remains controversial (10).

Adiponectin or adipocyte complement–related protein of 30 kDa (Acrp30) is the only known adipocytokine in which plasma levels are decreased in obesity and type 2 diabetes (11–13). Low adiponectin plasma levels are good indicators of insulin resistance and the development of diabetes (14,15). In studies with obese and diabetic rodents, it was further shown that intravenous application of adiponectin leads to normalized insulin sensitivity (16,17). Many studies focused on the physiological importance of adiponectin and support the notion of an antidiabetes action of this hormone. However, the cellular and molecular basis of this effect remains poorly understood, and little is known about the role of adiponectin in the cross talk between adipose tissue and skeletal muscle. Known effects of adiponectin in vitro include an antiapoptotic action on pancreatic β-cells (18) and an anti-inflammatory and vasoprotective function in vascular endothelial cells (12,19). Furthermore, adiponectin secretion by 3T3 adipocytes is decreased by proinflammatory adipocytokines like IL-6 (20).

In the present study, we took advantage of our recently described coculture model of human adipocytes and skeletal muscle cells (10) to elucidate the role of adiponectin in the cross talk between adipose tissue and skeletal muscle. We report here that adiponectin prevents the induction of muscle insulin resistance by reducing the release of fat cell secretory produts. These factors include IL-6, IL-8, growth-regulated oncogene (GRO)-α, monocyte chemotactic protein (MCP)-1, macrophage inflammatory protein (MIP)-1α and -1β, and tissue inhibitor of metalloproteinase (TIMP)-1 and TIMP-2. We suggest that adiponectin acts as a key regulator of adipocyte secretory function and that this autocrine action may contribute to the antidiabetes effect of this hormone.

RESEARCH DESIGN AND METHODS

BSA (fraction V, fatty acid free) was obtained from Boehringer (Mannheim, Germany). Reagents for SDS-PAGE were supplied by Amersham Pharmacia

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Acrp30, adipocyte complement–related protein of 30 kDa; gAcrp30, globular domain of Acrp30; GSK, glycogen synthase kinase; GRO, growth-regulated oncogene; HGF, hepatocyte growth factor; HRP, horseradish peroxidase; IGFBP, IGF-binding protein; IL, interleukin; MCP, monocyte chemotactic protein; MIP, macrophage inflammatory protein; sTNFR, soluble TNF receptor; TIMP, tissue inhibitor of metalloproteinase; TNF, tumor necrosis factor.

Biotech (Braunschweig, Germany) and Sigma (München, Germany). The recombinant COOH-terminal globular domain of adiponectin and full-length adiponectin were products from Tebu (Offenbach, Germany). Polyclonal antibodies anti–phospho-glycogen synthase kinase (GSK) 3α/β (Ser21/9), anti–phospho-Akt (Ser473), and anti-Akt were supplied by Cell Signaling Technology (Frankfurt, Germany). Anti-GSK3α/β was from Stressgene (Victoria, Canada). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody was from Promega (Mannheim, Germany). Cytokine protein arrays (RayBio Antibody Array C Series 1000) were purchased from RayBiotech (Norcross, GA). Collagenase CLS type 1 was obtained from Worthington (Freehold, NJ), and culture media were obtained from Gibco (Berlin, Germany). The cytokines IL-6, IL-8, MCP-1, MIP-1α, and MIP-1β were purchased from Hölzel Diagnostics (Cologne, Germany). Primary human skeletal muscle cells and supplement pack for growth medium were obtained from PromoCell (Heidelberg, Germany). Membrane inserts for six-well culture dishes with a pore size of 0.4 μm (pore density $1.6 \times 10^6/cm^2$) and insert companion plates were supplied by FALCON. All other chemicals were of the highest analytical grade commercially available and were purchased from Sigma.

Culture of human skeletal muscle cells. Satellite cells were isolated from M. rectus abdominis by enzymatic digestion with trypsin followed by a purification step with fibroblast-specific magnetic beads to prevent contamination with fibroblasts. After two passages, the myoblasts were characterized by the manufacturer (PromoCell) using immunohistochemical detection of sarcomeric myosin in differentiated cultures at 100% confluence (8 days). Primary human skeletal muscle cells of four healthy Caucasian donors (male, 9 and 5 years [M9, M5]; female, 10 and 48 years [F10, F48]) were supplied as proliferating myoblasts (5×10^5 cells) and cultured as described in our earlier study (10). For an individual experiment, myoblasts were seeded in six-well culture dishes (9.6 cm^2/well) at a density of 10^5 cells per well and were cultured in α-modified Eagle's/Ham's F12 medium containing Skeletal Muscle Cell Growth Medium Supplement Pack up to near confluence. The cells were then differentiated and fused by culture in α-modified Eagle's medium for 4 days.

Adipocyte isolation and culture. Adipose tissue samples were obtained from the mammary fat of 13 normal or moderately overweight women (BMI 19.2–31 kg/m2, aged between 21 and 52 years) undergoing surgical mammary reduction. All subjects were healthy, were free of medication, and had no evidence of diabetes according to routine laboratory tests. Adipose tissue samples were dissected from other tissues and minced in pieces of ~10 mg in weight. Preadipocytes were isolated by collagenase digestion as previously described (21). Isolated cell pellets were resuspended in Dulbecco's modified Eagle's/Ham's F12 medium supplemented with 10% FCS, seeded on membrane inserts (3.5×10^5/4.3 cm^2) or in a six-well culture dish, and kept in culture for 16 h. After washing, culture was continued in an adipocyte differentiation medium (Dulbecco's modified Eagle's medium/F12, 33 μmol/l biotin, 17 μmol/l D-pantothenic acid, 66 nmol/l insulin, 1 nmol/l triiodo-L-thyronin, 100 nmol/l cortisol, 10 μg/ml apo-transferrin, 50 μg/μl gentamicin, 15 mmol/l HEPES, and 14 mmol/l $NaHCO_3$, pH 7.4). After 15 days, 60–80% of seeded preadipocytes developed to differentiated adipose cells, as defined by cytoplasm completely filled with small or large lipid droplets. These cells were then used for the coculture experiments and for generation of adipocyte-conditioned medium, as recently described (22).

Coculture. Coculture of human fat and muscle cells was conducted according to our recently published protocol (10). Briefly, after in vitro differentiation of preadipocytes on membrane inserts, the adipocytes were washed once with PBS and then incubated for 24 h in skeletal muscle cell differentiation medium containing 1 pmol/l insulin. Thereafter, adipocytes were washed twice with PBS and individual membrane inserts were subsequently transferred to the culture plates containing differentiated (4 days) myocytes in α-modified Eagle's medium containing 1 pmol/l insulin. This resulted in an assembly of the two cell types sharing the culture medium but being separated by the membrane of the insert at a distance of 0.9 mm from the bottom. Coculture was conducted for 48 h. Integrity of both cell types was routinely checked by light microscopy at the end of the coculture period.

Conditioned medium was generated by culturing adipocytes for 48 h using exactly the same protocol as described for the coculture followed by collection of the medium. This material was lyophilized, reconstituted, and finally added to myotubes. As a control, we used lyophilized adipocyte medium.

Assay of GLUT4 translocation. Recombinant, replication-defective adenoviral vectors were generated with the AdenoVator system from QBiogene (Heidelberg, Germany). Three days after start of differentiation, skeletal muscle cells were infected with recombinant adenoviruses encoding GLUT4myc and used for analysis after an additional 48-h incubation. After stimulation with 10^{-7} mol/l insulin for 30 min, GLUT4 translocation was measured based on the protocol described by Kanai et al. (23).

Primer and RT-PCR. Total RNA was extracted from differentiated human skeletal muscle cells and adipocytes by using Trizol (Roche Diagnostics) following the manufacturer's protocol. The reverse transcription reaction and the following PCR were performed with the One Step RT-PCR kit (Qiagen, Hilden, Germany), as previously described (24).

Measurement of cytokine protein levels in adipocyte-conditioned medium. Adipocyte-conditioned media generated in absence or presence of 5 nmol/l globular domain of adiponectin (gAcrp30) from different fat donors ($n = 5$) were hybridized with the array membranes according to the protocol supplied by the manufacturer. Briefly, membranes were blocked by furnished blocking solution and then incubated with 1.2 ml of conditioned medium overnight at 4°C. Membranes were then washed according to the manufacturer's protocol and incubated with the mix of biotin-conjugated antibodies for 2 h at room temperature. After washing, HRP-conjugated streptavidin was added to the membranes for 1 h at room temperature. The signal was developed with detection buffers and directly detected using a LUMI Imager system (Roche Diagnostics). The signal intensity was normalized to internal positive signals on the membrane. For each fat donor, conditioned medium generated in presence of 5 nmol/l gAcrp30 and control medium without addition of adiponectin was analyzed at the same time and cytokine signals compared. Cytokine concentrations in conditioned medium were determined with the same cytokine array. A mix of the five measured cytokines was analyzed twice using different concentrations to assure linearity of the assay. Absolute cytokine concentrations were calculated based on the calibration curve. The level of adiponectin in adipocyte-conditioned media was determined using an enzyme-linked immunosorbent assay kit from B-Bridge International (Sunnyvale, CA). The assay was performed as recommended by the manufacturer using duplicate samples for all determinations.

Immunoblotting. Muscle cells were treated as indicated and lysed in a buffer containing 50 mmol/l Tris/HCl (pH 7.4), 1% (vol/vol) NP-40, 0.25% (vol/vol) sodium-deoxycholate, 150 mmol/l NaCl, 1 mmol/l EGTA, 1 mmol/l Na_3VO_4, and protease inhibitor cocktail. After incubation for 2 h at 4°C the suspension was centrifuged at 10,000*g* for 10 min. Thereafter, total cell lysates were separated by SDS-PAGE using gradient (8–18%) horizontal gels and transferred to polyvinylidene fluoride filters in a semidry blotting apparatus (25). For detection of p-Akt, Akt, p-GSK3α/β, and GSK3α/β, filters were blocked with Tris-buffered saline containing 0.1% Tween-20 and 5% nonfat dry milk and subsequently incubated overnight with a 1:1,000 dilution of appropriate antibodies. After extensive washing, filters were incubated with goat anti-rabbit or goat anti-chicken (in case of GSK3) HRP-coupled antibody and processed for enhanced chemiluminescene detection using SuperSignal Substrate (Pierce, Rockford, IL). Signals were visualized and evaluated on a LUMI Imager workstation using image analysis software (Boehringer Mannheim).

Statistical analysis was carried out by using either Student's t test or ANOVA for comparison of more than two variables. All statistical analyses were done using Prism software (Graphpad, San Diego, CA). A P value <0.05 was considered statistically significant. Corresponding significance levels are indicated in the figures.

RESULTS

Adiponectin ameliorates impairment of insulin signaling in skeletal muscle cells cocultured with human adipocytes. As we were previously able to demonstrate, coculture of human adipocytes and human skeletal muscle cells leads to a rapid disturbance of insulin signaling in the muscle cell, as seen by a prominent reduction in the efficiency of insulin to promote insulin receptor substrate–1/Akt phosphorylation (10). As presented in Fig. 1*A*, this impairment of insulin signaling could also be detected further downstream at the level of GSK3. Coculture conditions increased basal phosphorylation of GSK3 and concomitantly reduced the effect of insulin on this process. Thus, the fold stimulation was reduced from 2.5 ± 0.2 to 1.6 ± 0.3 and from 3.7 ± 0.9 to 1.9 ± 0.4 ($n = 12$) for GSK3α and GSK3β serine phosphorylation, respectively. Most importantly, adipocyte-conditioned medium, which mimicks the coculture condition (22), profoundly reduced insulin-stimulated GLUT4myc translocation in human skeletal muscle cells (Fig. 1*B*). This assay detects the movement of GLUT4 to the cell surface, a major effect of insulin in this tissue. We therefore conclude that adipo-

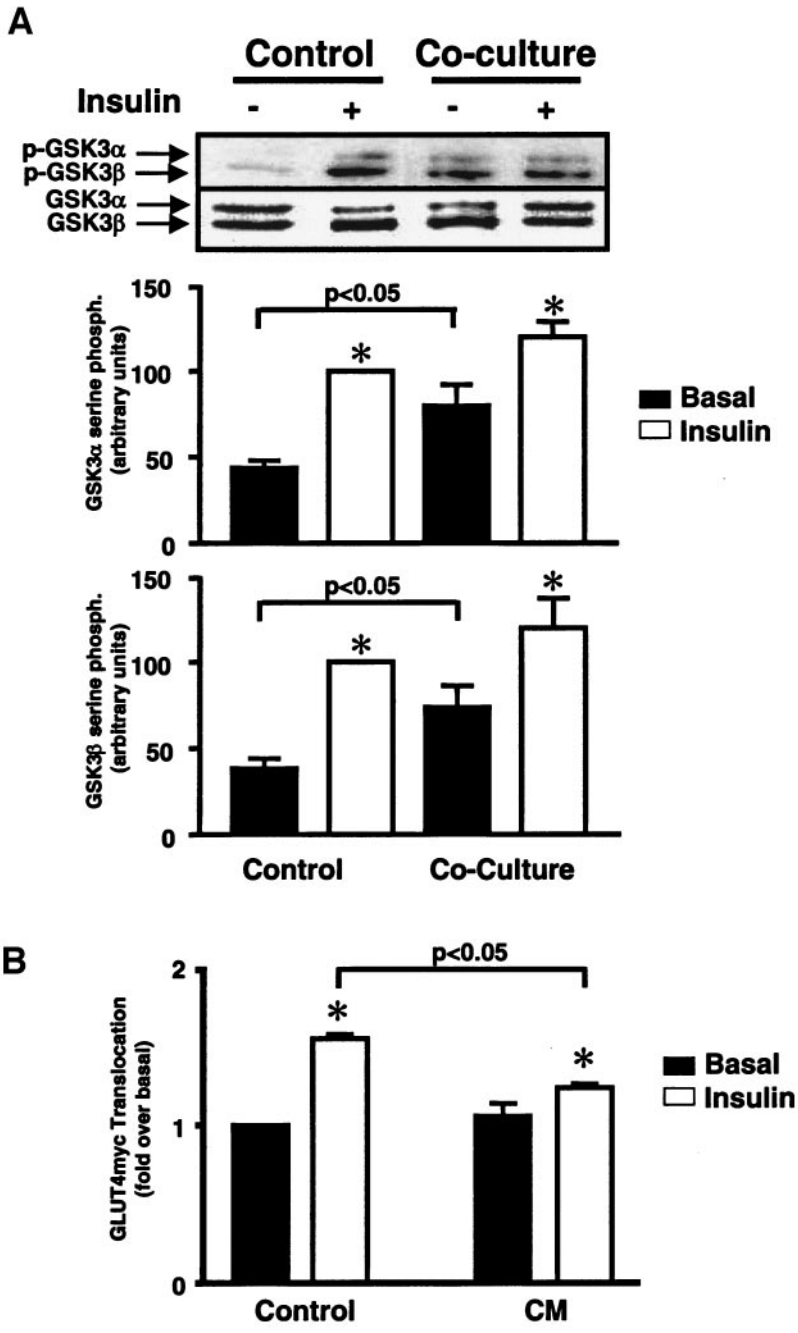

FIG. 1. Effect of coculture on insulin signaling and effect of adipocyte-conditioned medium on insulin-stimulated GLUT4 translocation in human skeletal muscle cells. *A*: Skeletal muscle cells from three different donors (M5: male, 5 years old; M9: male, 9 years old; F10: female, 10 years old) were subjected to coculture with differentiated human adipocytes, and GSK3 phosporylation in the myocytes was analyzed after acute stimulation with insulin (100 nmol/l, 10 min). Total cell lysates were resolved by SDS-PAGE and immunoblotted with phosphospecific GSK3 and GSK3 antibodies. Western blots were quantified on a LUMI Imager workstation using image analysis software. Data are means ± SE of 12 independent experiments and are normalized to GSK3 expression levels. *Significantly different from corresponding basal level. *B*: Skeletal muscle cells were cultured for 18 h in absence or presence of conditioned medium from two different adipocyte donors. Translocation of GLUT4myc was assessed after acute stimulation with insulin, as outlined in RESEARCH DESIGN AND METHODS. Data are means ± SE of four independent experiments. *Significantly different from corresponding basal level.

cyte-derived factors impair both insulin signaling and downstream insulin action in the myocytes.

To investigate whether adiponectin has the capacity to prevent the induction of impaired myocyte insulin signaling under coculture conditions, adipocytes and skeletal muscle cells were cocultured for 48 h in the absence or presence of 10 nmol/l gAcrp30. After coculture, skeletal muscle cells were acutely stimulated with insulin, and downstream signaling was assessed at the level of the serine/threonine kinase Akt, a key mediator of insulin action on glucose transport and glycogen synthesis (26). As reported earlier, coculture leads to a significant reduction of insulin-induced serine phosphorylation of Akt without affecting basal phosphorylation or expression level of the kinase (Fig. 2*A*). As can be seen from the data, the presence of adiponectin during the coculture period clearly prevents the impairment of insulin signaling in the muscle cells. Under these conditions, Akt serine phosphorylation after acute insulin stimulation was not significantly different from the control situation (Fig. 2*A*).

To check if the two cell types express the receptors for adiponectin, we analyzed the cells using RT-PCR. Our results show that in vitro–differentiated human skeletal muscle cells and adipocytes express both adiponectin receptor 1 and 2 (Fig. 2*B*). As shown in Fig. 2*C*, adiponectin has no direct effect on insulin-stimulated Akt phosphorylation in skeletal muscle cells. Thus, adiponectin-mediated prevention of impaired insulin signaling in the myocytes (Fig. 2*A*) cannot be explained by augmented insulin responsiveness of Akt phosphorylation in these cells.

Autocrine action of adiponectin on human adipocytes prevents the release of insulin resistance–inducing

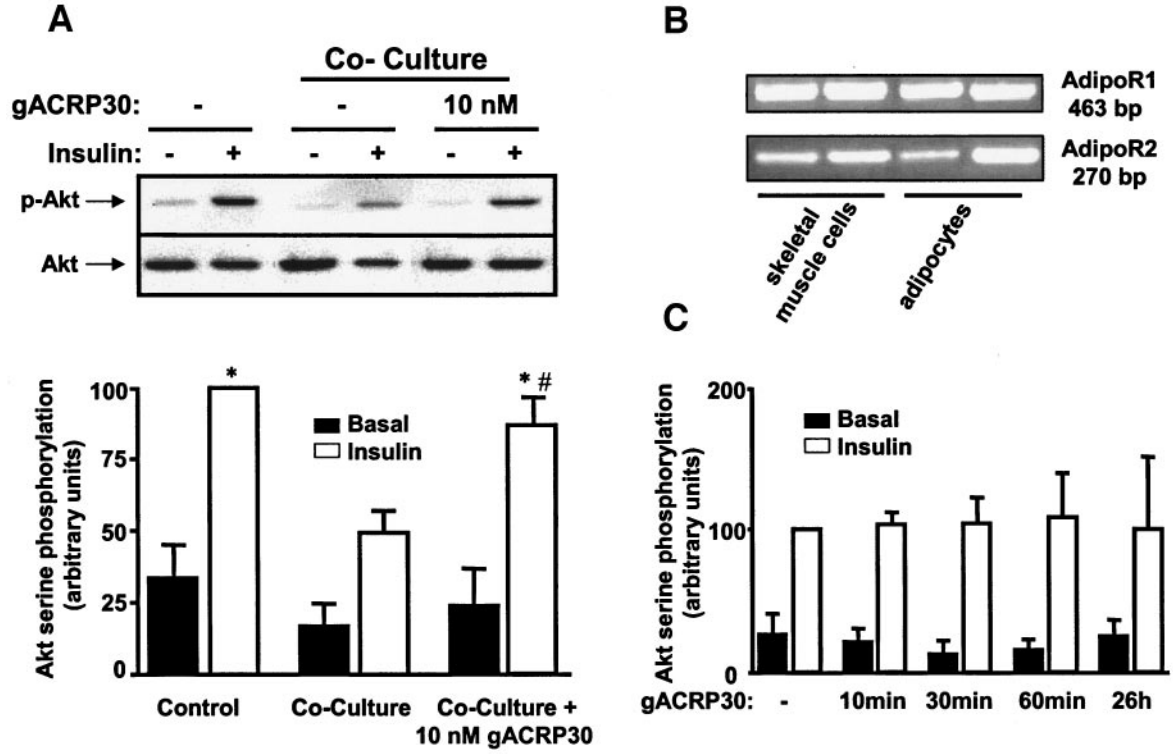

FIG. 2. Effect of adiponectin on insulin signaling in human skeletal muscle cells subjected to coculture with human adipocytes. *A*: Human skeletal muscle cells were subjected to coculture in the absence or presence of 10 nmol/l gAcrp30 for 48 h and were subsequently stimulated with insulin (100 nmol/l, 10 min). *Upper panel:* Total cell lysates (8 μg/lane) were resolved by SDS-PAGE and immunoblotted with phosphospecific Akt antibody (p-Akt) and Akt antibody. *Lower panel:* Western blots were quantified as described in Fig. 1. All data were normalized to Akt protein expression and are expressed relative to the insulin-stimulated control value. Data are means ± SE of four independent experiments from three different muscle cell donors. *Significantly different from insulin-stimulated coculture ($P < 0.001$); #not significantly different from control ($P > 0.05$). *B*: Total RNA was extracted from differentiated human skeletal muscle cells and adipocytes and RT-PCR for adiponectin receptor-1 and -2 (AdipoR1 and AdipoR2) was performed as outlined in RESEARCH DESIGN AND METHODS. *C*: Skeletal muscle cells were incubated without or with 10 nmol/l gAcrp30 for the indicated times followed by acute stimulation with insulin. Akt phosphorylation was determined as outlined above. Data are means ± SE of four independent experiments from four different muscle cell donors (M5, M9, F10, F48: $n = 1$ each).

factors. We next addressed the question of whether skeletal muscle cells incubated with adipocyte-conditioned medium and adiponectin are protected from impaired insulin signaling. Adipocyte-conditioned medium impaired insulin signaling in skeletal muscle cells at the level of Akt serine phosphorylation by 50–60% without affecting the expression level of this enzyme (Fig. 3). Concomitant incubation of muscle cells with adiponectin and adipocyte-conditioned medium did not prevent impairment of insulin signaling, making it likely that the results observed in the coculture reflect adiponectin action on the fat cells.

To provide evidence for an autocrine action of adiponectin, fat cells from the same fat cell donors were cultured for 48 h in the absence or presence of 5 nmol/l gAcrp30 and the conditioned medium was processed for experiments with myotubes. As presented in Fig. 4, impairment of insulin-stimulated Akt phosphorylation by adipocyte-conditioned medium was nearly abolished when generated in the presence of adiponectin. This experiment was repeated with the same result using full-length adiponectin. Thus, when using cells from muscle donors M9 and F48, adipocyte-conditioned medium reduced ($P = 0.006$) insulin-stimulated Akt phosphorylation to 59.5 ± 12.5% of control, whereas 92 ± 1% of insulin-stimulated control was observed with medium generated in the presence of full-length adiponectin. Furthermore, adipocyte-conditioned medium induced a significant impairment of insulin signaling downstream of Akt (69.9 ± 3.5 and 86.4 ± 4.1% [$n = 4$] of insulin-stimulated control for GSK3α and -β phosphorylation, respectively). This inhibitory action was completely abolished when testing adipocyte-conditioned medium generated in the presence of gAcrp30 (99.2 ± 8.9 and 100.9 ± 2.9% of insulin-stimulated control for phospho-GSK3α and -β, respectively). These observations suggest that adiponectin can counteract induction of skeletal muscle insulin resistance by autocrine action on adipocytes. This notion is supported by the determination of endogenous adiponectin in adipocyte-conditioned media. As presented in Table 1, a very low concentration of adiponectin was associated with inhibitory activity on insulin signaling in skeletal muscle cells. This inhibitory activity was not found in conditioned media with 10- to 20-fold higher endogenous adiponectin levels (Table 1). It is worth noting that the addition of gAcrp30 for 48 h did not alter the adiponectin release by the adipocytes. Furthermore, we observed lower MCP-1, MIP-1α, and MIP-1β concentrations (reduced to 40, 30, and 10%, respectively) in conditioned media with higher endogenous adiponectin that were not used for further experiments. It should be noted that low adiponectin is unrelated to any clinical background of the donors, since adipose tissue was always obtained from healthy subjects.

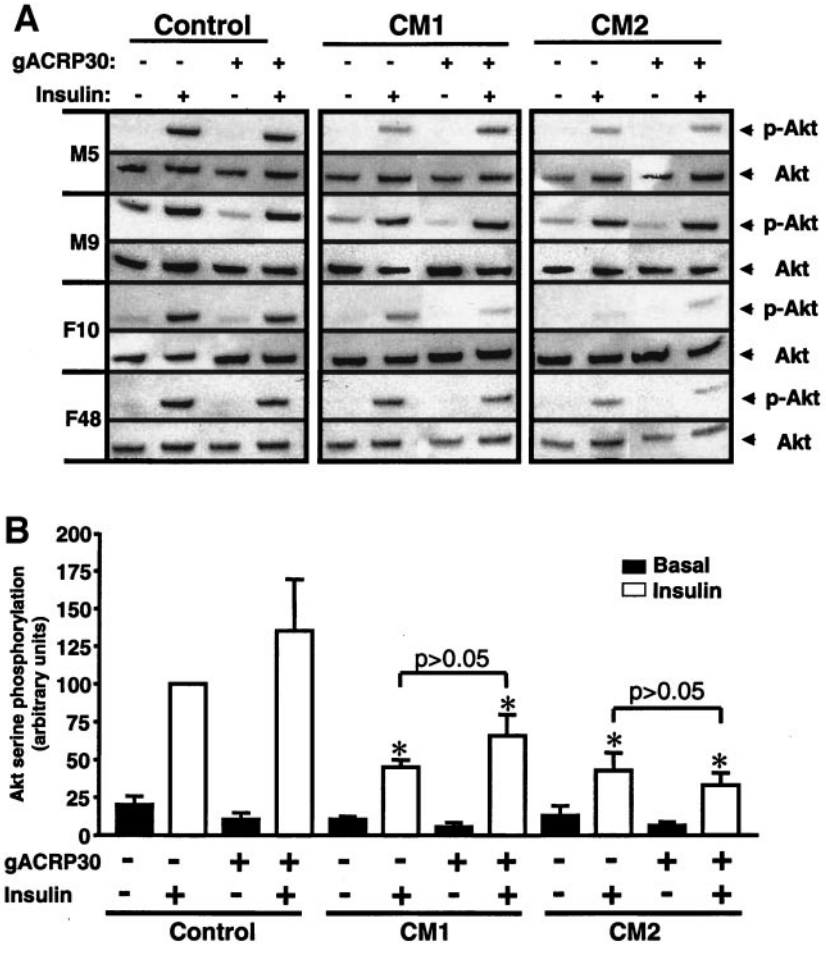

FIG. 3. Effect of adipocyte-conditioned medium on insulin signaling in myocytes in absence or presence of adiponectin. *A*: Myocytes from four different donors (M5, M9, F10, and F48) were cultured in the presence of adipocyte-conditioned medium from two different donors (CM1 and CM2) for 18 h with or without 10 nmol/l adiponectin. After acute stimulation with insulin, total cell lysates were resolved by SDS-PAGE and immunoblotted with phosphospecific Akt antibody and Akt antibody. *B*: Blots were quantified as described in Fig. 1. Data are means ± SE of six independent experiments. All data were normalized to the level of Akt expression and are expressed relative to the insulin-stimulated control value. *Significantly different from control ($P < 0.01$).

Adiponectin downregulates cytokine secretion from human adipocytes. To identify adipocytokines that are secreted by differentiated human adipocytes and regulated by adiponectin, we analyzed adipocyte-conditioned medium by a cytokine protein array detecting 120 different cytokines. In conditioned medium from five different fat donors, the following cytokines were identified: adiponectin, GRO-α, hepatocyte growth factor (HGF), IGF-binding protein (IGFBP)-3, IL-6, IL-8, MCP-1, macrophage-derived chemokine, MIP-1α, MIP-1β, osteoprotegerin, soluble TNF receptor 2 (sTNFR 2), TIMP-1, and TIMP-2. In conditioned media from some individuals we also detected angiogenin, fibroblast growth factor-9, intercellular adhesion molecule-1, neutrophil activating peptide-2, TNFα, TNFβ, and plasminogen activator receptor. To evaluate the effect of adiponectin treatment on secretion of adipocytokines, we analyzed adipocyte-conditioned medium generated in the presence of 5 nmol/l gAcrp30 in comparison to the respective control adipocyte-conditioned medium (Fig. 5*A*). Macrophage-derived chemokine, IGFBP-3, and HGF secretion was not affected by adiponectin treatment. However, adiponectin reduced IL-6, IL-8, and GRO-α secretion by 50–60% (Fig. 5*B*). Several members of the small inducible cytokine family such as MCP-1, MIP-1α, and MIP-1β were also significantly reduced. From the two TNF-related proteins, only osteoprotegerin was significantly reduced. TIMP-1 and TIMP-2, two cytokines that are thought to play a role in adipocyte differentiation, were also significantly reduced in medium generated in the presence of adiponectin (Fig. 5*B*). A comparable reduction in adipokine secretion was also observed in response to 5 nmol/l full-length adiponectin (percent of untreated control: IL-6 52 ± 3, IL-8 52 ± 14, MCP-1 55 ± 8, MIP-1α 61 ± 15, MIP-1β 66 ± 12, osteoprotegerin 53 ± 14, TIMP-1 39 ± 7, TIMP-2 44 ± 13; $n = 3$). The concentrations of several adiponectin-regulated cytokines in the supernatant of cultured human adipocytes are presented in Table 2. As reported earlier, a very high amount of IL-6 is released by these cells (27), whereas IL-8 and MCP-1 are present at 50- and 200-fold concentrations above the physiological level, in excellent agreement with recent observations (28,29).

DISCUSSION

Adipocyte-derived factors such as TNFα and IL-6 are significantly increased in obesity and are good predictors of the development of type 2 diabetes (30,31). Obesity thereby contributes to a proinflammatory milieu, and it is now recognized that adipose tissue functions as an endocrine organ secreting a variety of proinflammatory factors. Adiponectin is the only adipocytokine known to be downregulated in obesity; however, little is known about the role of adiponectin in the cross talk between adipose tissue and skeletal muscle itself. We report here for the first time on adiponectin acting as an autocrine regulator of adipokine secretion of the human fat cell. By decreasing cytokine release by the adipocyte, adiponectin prevents the impairment of insulin signaling in a coculture model of

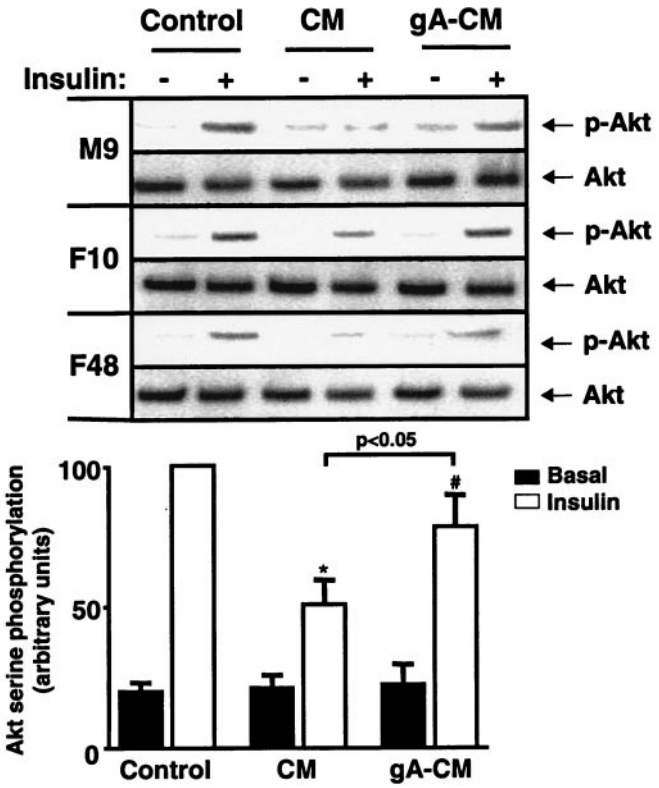

FIG. 4. Analysis of adipocyte-conditioned medium generated in the presence of adiponectin. Skeletal muscle cells from three different donors were cultured under control conditions or were incubated with adipocyte-conditioned medium generated in the presence or absence of 5 nmol/l gAcrp30. After acute stimulation with insulin, Akt serine phosphorylation and Akt expression was assessed. Data are means ± SE of four independent experiments. All data were normalized to the level of Akt expression and are expressed relative to the insulin-stimulated control value. *Significantly different from insulin stimulated control ($P < 0.001$); #not significantly different from insulin stimulated control ($P < 0.05$).

human adipocytes and skeletal muscle cells. This is in accordance with the view that the cross talk between inflammatory and metabolic signaling pathways may elicit insulin resistance and extends this concept to human skeletal muscle. Adiponectin secretion is very low by in vitro–differentiated human adipocytes in accordance with studies using freshly isolated mature adipocytes in long-term culture (32). Furthermore, we found here that the low level of adiponectin correlated with the release of insulin resistance–inducing factors by adipocytes. Therefore, the coculture represents an ideal model of adiponectin deficiency to study the role of adiponectin in the cross talk between fat and muscle. It is worth noting that most of our experiments were conducted using the globular head domain of adiponectin. Adiponectin prevails in serum as a trimer, hexamer, or high–molecular weight form, with controversial results being reported on the biological activity of these isoforms (33). The globular head domain of adiponectin is generated by leukocyte elastase (34) and became detectable in human plasma at low abundance when using immunoprecipitation with a globular head–specific antiserum (35). Furthermore, the head domain binds to adiponectin receptors 1 and 2, and transgenic expression protects *ob/ob* mice from diabetes (36). However, the physiological relevance of gAcrp30 remains controversial, since other studies failed to detect it in human serum samples using nonreducing and non–heat-denaturing SDS-PAGE (37) or velocity sedimentation (38). Nevertheless, the high efficiency of gAcrp30 in animal studies highlights the potential role of this molecule for future drug development.

TABLE 1
Adiponectin concentrations in adipocyte-conditioned media

Conditioned medium	Adiponectin concentration (ng/ml)	Inhibition of Akt phosphorylation (%)
CM 1	1.13	55 ± 5 (6)
CM 2	0.43	57 ± 12 (6)
CM 3	0.83	29 ± 6 (3)
CM 4	0.74	43 ± 7 (5)
CM 5	0.74	30 ± 10 (5)
CM 6	0.80	38 ± 4 (2)
CM 7	9.06	ND
CM 8	17.40	ND

Data are means ± SE (no. of experiments). Conditioned medium (CM) of eight different donors was generated as outlined in RESEARCH DESIGN AND METHODS. Duplicate samples were analyzed using an adiponectin enzyme-linked immunosorbent assay kit. Inhibition of insulin-stimulated Akt phosphorylation was determined as outlined in RESEARCH DESIGN AND METHODS. ND, inhibition not detectable.

Earlier studies focused on adiponectin action in skeletal muscle and adipose tissue. Adiponectin was found to activate AMP kinase in skeletal muscle (39) depending on the muscle type (40). In myocytes, adiponectin may also activate the nuclear factor-κB pathway (41). Furthermore, in this tissue, adiponectin treatment leads to enhanced fat oxidation and glucose transport. Adiponectin expression in adipocytes is regulated by various compounds such as TNFα and growth hormone, which also influence insulin sensitivity (42,43), and adiponectin treatment leads to increased glucose uptake (44). In the current investigation, we focused on the cross talk between these two tissues, which are both critical players regarding insulin resistance and diabetes. We propose a model of autocrine/paracrine adiponectin action on adipose tissue as a pivotal determinant of muscle insulin sensitivity. In this respect, it was shown here that adiponectin had no effect on insulin signaling at the level of Akt in the muscle cells, neither alone nor in combination with adipocyte-conditioned medium, although these cells express both types of adiponectin receptors. Thus, in our model the beneficial effect of adiponectin on muscle insulin signaling is clearly adipocyte dependent since *1*) it was observed upon addition to the coculture and *2*) the insulin resistance–inducing effect of conditioned medium was ameliorated when generated in the presence of adiponectin. A paracrine effect of adiponectin on fat cell formation in bone marrow (45) and fat cell differentiation (46) was already proposed. Furthermore, adiponectin is considered an autocrine regulator of energy metabolism (47). An autocrine regulation of cytokine secretion in adipocytes adds a new mechanism to the pleiotropic action potential of adiponectin.

In this study, we identified several cytokines exhibiting adiponectin-regulated secretion from differentiated human adipocytes. Some of these adiponectin-regulated cytokines such as IL-6, IL-8, and MCP-1 are known to be related to obesity and diabetes. Others such as GRO-α, TIMP-1

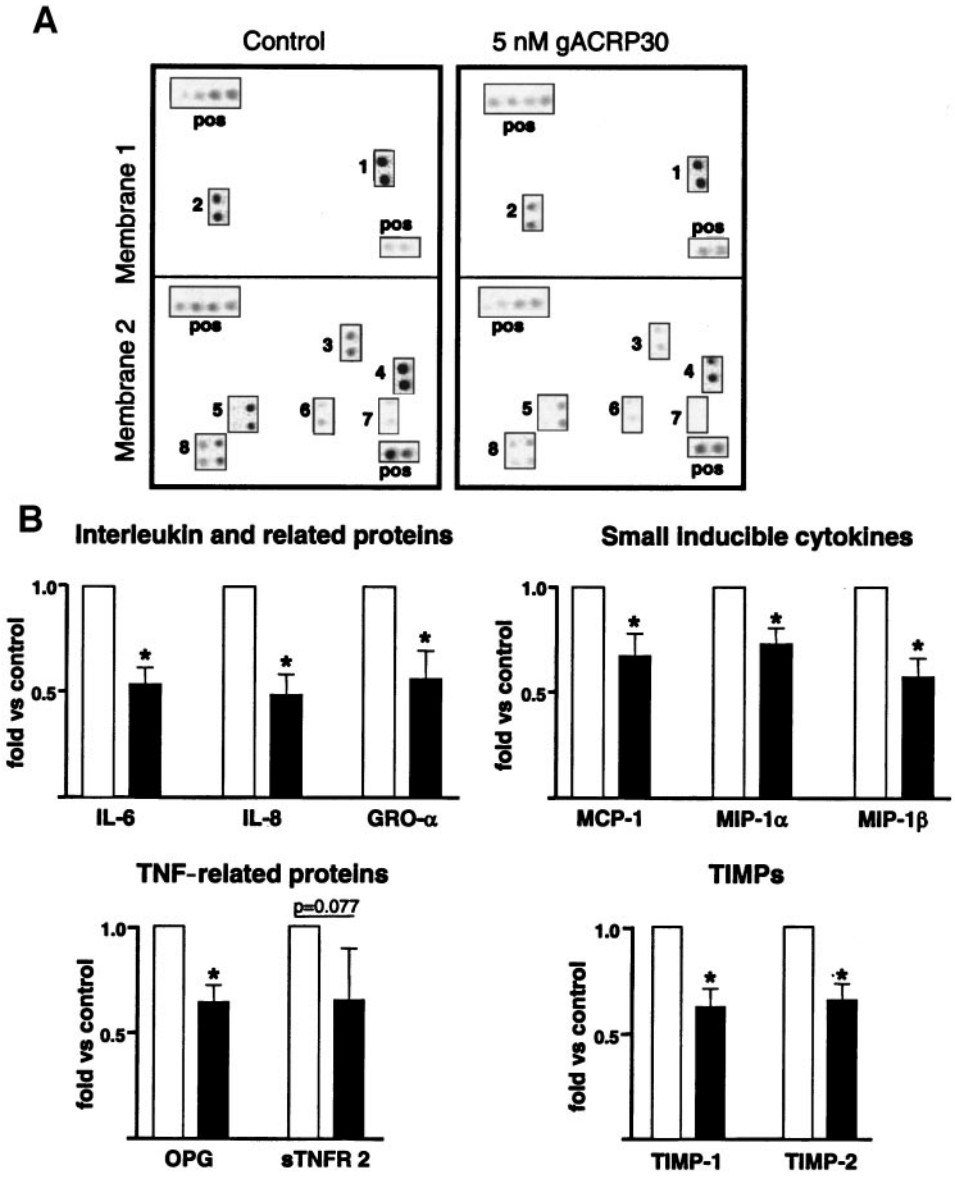

FIG. 5. Regulation of adipocyte cytokine secretion by adiponectin. *A*: Cytokine array membranes (membrane 1 and 2, each detecting 60 different cytokines) were incubated with adipocyte-conditioned medium that was generated in absence or presence of 5 nmol/l adiponectin. After washing, the membranes were incubated with a mix of biotin-conjugated antibodies and processed for detection on the LUMI Imager system, as described in RESEARCH DESIGN AND METHODS. Two representative arrays with adipocyte-conditioned media from the same donor culture are presented: *1)* IL-6, *2*) MCP-1, *3)* GRO-α, *4)* IL-8, *5)* MIP-1α and −1β, *6)* osteoprotegerin, *7)* sTNFR2, and *8)* TIMP-1 and -2. Pos, positive control for normalization. *B*: Protein arrays were quantified using image analysis software. Data are means ± SE of five independent adipocyte cultures from five different donors, each run in the absence or presence of gAcrp30. *$P < 0.05$ compared with control medium. OPG, osteoprotegerin.

and -2, and MIP-1 are related to inflammation and tissue remodeling, but their relation to adipose tissue, obesity, and diabetes is less clear. IL-6, IL-8, and MCP-1 are well known to be induced in the obese state in humans and rodents (48–51). IL-6 is expressed both by adipose tissue and skeletal muscle (52). Elevated plasma concentrations of these adipokines in obese and insulin-resistant patients may contribute to the insulin-resistant state observed in obesity. Interestingly, adiponectin concentrations are inversely correlated to IL-6 plasma concentrations, insulin sensitivity, and obesity in full agreement with our findings (48). However, the role of IL-6 in skeletal muscle remains controversial with publications both supporting and not supporting involvement of IL-6 in impaired insulin action (53). Our recent study showed that only extremely high concentrations of IL-6 produced a slight impairment of insulin signaling in human skeletal muscle cells, making it unlikely that IL-6 alone is sufficient to induce muscle insulin resistance (27). IL-8 is secreted from adipose tissue, and its plasma levels are elevated in obesity (49,54). Since IL-8 expression is increased by proinflammatory cytokines such as TNFα and IL-1, it may be involved in obesity-related complications. The potent reduction of IL-8 secretion by adiponectin shown here supports the notion (49) that this cytokine may play a role in the induction of insulin resistance, most likely in concert with other adipokines.

We show here for the first time that GRO-α is secreted

TABLE 2
Cytokine concentrations in adipocyte-conditioned medium

Cytokine	Concentration (ng/ml)
IL-6	22.0 ± 6.0
IL-8	0.26 ± 0.08
MCP-1	31.2 ± 6.7
MIP-1α	0.20 ± 0.06
MIP-1β	1.09 ± 0.32

Data are means ± SE of five adipocyte cultures. Cytokine concentrations in adipocyte-conditioned medium without addition of gAcrp30 were analyzed by protein cytokine array as detailed in RESEARCH DESIGN AND METHODS. A mix of the five cytokines was measured at different concentrations and used for calibration.

from human adipocytes. This cytokine is structurally related to IL-8 and considered to attract neutrophils to the site of inflammation and may play a role in inflammation, angiongenesis and tumorigenesis (55). Its role in adipose tissue and the regulation of insulin sensitivity needs to be defined. MCP-1 is clearly associated with the obese state (50,51). Its overexpression, especially in epicardial adipose tissue, is thought to increase the inflammatory burden of arteries (56). In adipocytes, MCP-1 expression is increased by TNFα, insulin, growth hormone, and IL-6 (57). Treatment of 3T3-L1 adipocytes with MCP-1 was found to impair glucose uptake, indicating that this cytokine may contribute to the pathogenesis of insulin resistance (50), although the effect of MCP-1 on skeletal muscle insulin action needs to be established.

TIMP-1 and -2, in combination with the matrix metalloproteinases, exert key functions in extracellular matrix remodeling. As for TIMP-1, it was shown to be strongly induced in obesity (58). Matrix metalloproteinases and TIMP activity may be essential for adipogenesis since changes in cell-matrix interaction must accompany adipocyte hypertrophy as well as recruitment and differentiation of adipocyte precursors. This is supported by the observation that TIMP-1 knockout mice are less sensitive to the induction of obesity (59). By decreasing secretion of TIMPs, adiponectin may decrease adipocyte hypertrophy and fat accumulation. Thus, adiponectin could directly contribute to adipose tissue remodeling by increasing the number of smaller adipocytes which are known to better retain free fatty acids and contribute to increased insulin sensitivity. MIP-1α is expressed by human adipocytes, but its secretion decreases upon differentiation (28), a feature shared with IL-8. Regulation of MIP-1α by adiponectin may also contribute to decreased adipocyte hypertrophy. Thus, in addition to regulating the release of inflammatory cytokines that may interfere with insulin signaling in the muscle cell, adiponectin may also exert its antidiabetes action by regulating fat cell differentiation and growth.

In summary, our data show that adiponectin acts as a key regulator of cytokine secretion in adipose tissue. We therefore suggest that cytokines regulated by adiponectin may represent a molecular link between obesity and skeletal muscle insulin resistance. These adipocytokines are involved in inflammation, tissue remodeling, and angiogenesis, but their role in obesity and the development of skeletal muscle insulin resistance needs to be further analyzed.

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