Impairment of Insulin Signaling in Human Skeletal Muscle Cells by Co-Culture With Human Adipocytes

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Adipocyte factors play a major role in the induction of insulin resistance in skeletal muscle. To analyze this cross-talk, we established a system of co-culture of human fat and skeletal muscle cells. Cells of three muscle donors were kept in co-culture with cells of various fat cell donors, and insulin signaling was subsequently analyzed in myocytes. Insulin-induced tyrosine phosphorylation of insulin receptor substrate (IRS)-1 was completely blocked, with unaltered expression of IRS-1. Troglitazone increased insulin action on IRS-1 phosphorylation, in both the absence and presence of co-culture. Insulin-regulated activation of Akt kinase in the myocytes was significantly reduced after co-culture, with troglitazone restoring insulin action. Addition of tumor necrosis factor (TNF)-α (2.5 nmol/l) to myocytes for 48 h reduced IRS-1 expression and inhibited IRS-1 and Akt phosphorylation comparable to the effect of co-culture. Lower doses of TNF-α were ineffective. After co-culture, TNF-α in the culture medium was below the detection limit of 0.3 pmol/l. A very low level of resistin was detected in the supernatant of myocytes, but not of adipocytes. In conclusion, the release of fat cell factors induces insulin resistance in human skeletal muscle cells; however, TNF-α and resistin appear not to be involved in this process. *Diabetes* 51:2369–2376, 2002

Obesity is almost invariably associated with insulin resistance and represents one of the major risk factors for the development of type 2 diabetes. It is now believed that a negative cross-talk between excess body fat and skeletal muscle disturbs insulin signaling in muscle and finally generates muscle insulin resistance, representing a key feature of type 2 diabetes that is observed early in the prediabetic state (1,2). Recent studies have shown that adipose tissue can be regarded as a major secretory and endocrine organ, and a variety of factors released by adipose cells potentially mediate skeletal muscle insulin resistance (3,4). These factors include free fatty acids, tumor necrosis factor (TNF)-α, angiotensinogen, interleukin-6, and the recently discovered hormone resistin. However, the precise role of these factors for human insulin resistance has remained elusive.

Substantial evidence supports a key role for TNF-α in the insulin resistance of obesity, at least in rodents (5). Thus, overexpression of TNF-α from adipose tissue was commonly observed in different rodent models of obesity (6,7), and direct exposure of isolated cells to TNF-α inhibits insulin signaling and induces a state of insulin resistance (8–10). In contrast to rodents, however, TNF-α is expressed at much lower levels in human adipose tissue (11), and antibody-mediated neutralization of TNF-α completely failed to improve insulin sensitivity in obese humans (12). Recent studies in murine models suggest that a novel adipocyte peptide hormone, resistin, may represent the link between obesity and insulin resistance (13). Again, recent data by Savage et al. (14) show that this hypothesis may not readily translate to humans, since human resistin was found to be predominantly expressed in mononuclear cells and was low or even absent in adipocytes. In light of these considerations, additional work using primary human cells is needed to characterize the cross-talk between human fat and muscle cells. Furthermore, a recent study by Goodpaster et al. (15) using cross-sectional computed tomography imaging has shown that adipose tissue dispersed within muscle strongly correlated to insulin resistance. Thus, a paracrine interaction of fat and muscle cells may represent a key step in the development of muscle insulin resistance.

In an attempt to study these processes under more physiological conditions, we have established a novel system of co-culture of differentiated human adipocytes and human skeletal muscle cells. Using this model, we addressed the following: *1*) whether co-culture disturbs insulin signaling in skeletal muscle cells and whether this can be prevented by thiazolidinediones, *2*) whether the effect of co-culture can be mimicked by TNF-α, and *3*) whether TNF-α and resistin are released under co-culture conditions. Our data show that adipocytes induce a profound paracrine perturbation of insulin signaling in skeletal muscle cells. However, neither TNF-α nor resistin is involved in this process.

RESEARCH DESIGN AND METHODS

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

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ECL, enhanced chemiluminescence; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; IRS, insulin receptor substrate; PI, phosphatidylinositol; PPAR-γ, peroxisome proliferator–activated receptor-γ; TBS, Tris-buffered saline; TNF, tumor necrosis factor.

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Germany), protein A trisacryl beads were from Pierce (Rockford, IL), and human recombinant TNF-α was from Sigma. Polyclonal anti–IRS-1 antiserum was a gift from Dr. J.A. Maassen (Leiden, the Netherlands). Polyclonal antibodies anti–phospho-Akt (Ser473) and anti-Akt were supplied by Cell Signaling Technology (Frankfurt, Germany). The anti-phosphotyrosine antibody (RC-20) coupled to horseradish peroxidase (HRP) was from Transduction Laboratories (Lexington, KY). HRP-conjugated goat anti-rabbit IgG antibody was from Promega (Mannheim, Germany). Collagenase CLS type 1 was obtained from Worthington (Freehold, NJ), and culture media were obtained from Gibco (Berlin, Germany). Primary human skeletal muscle cells and supplement pack for growth medium were obtained from PromoCell (Heidelberg, Germany). Sterile plasticware for tissue culture was purchased from Falcon (Becton Dickinson, Heidelberg, Germany). Membrane inserts for six-well culture dishes with a pore size of 0.4 μm (pore density 1.6×10^6 per 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 are 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 three healthy Caucasian donors (male, 9 and 28 years of age [M9, M28] and female, 10 years old [F10]) were supplied as proliferating myoblasts (5×10^5 cells). For all donors, chronic diseases, including diabetes and a family history of diabetes, were excluded. These cells were grown to confluence in 25-cm^2 flasks, trypsinized, and subsequently seeded in 175-cm^2 flasks at a density of 1×10^6 cells. After two passages, ~5–7.5 × 10^7 cells were harvested and stored until further use as frozen aliquots containing 2×10^6 myoblasts. For an individual experiment, myoblasts were then seeded in six-well culture dishes (9.6 cm^2/well) at a density of 10^5 cells per well and cultured in α-modified Eagle's/Ham's F-12 medium containing Skeletal Muscle Cell Growth Medium Supplement Pack (PromoCell) to near confluence. The cells were then differentiated and fused by culture in α-modified Eagle's medium supplemented with 2% FCS for 7 days. The myocytes were cultured in differentiation medium without FCS for 24 h before being used for any experiment.

Adipocyte isolation and culture. Adipose tissue samples were obtained from the mammary fat of 12 normal-weight or moderately overweight women (BMI 22–29.4 kg/m^2, between 29 and 50 years of age) undergoing surgical mammary reduction. All subjects were healthy 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. Preadipocytes were isolated by collagenase digestion as previously described (16). 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) in a six-well culture dish, and kept in culture for 16 h. After washing, culture was continued in an adipocyte differentiation medium, as detailed previously (16). After a period of 15 days, 60–80% of seeded preadipocytes developed to differentiated adipose cells, as defined by cytoplasma completely filled with small or large lipid droplets. This population of cells, subsequently designated as differentiated adipocytes, was used for the co-culture experiments. Preliminary data in our laboratory have shown a similar hormonal control of adipose differentiation in preadipose cells from mammary adipose tissue and the subcutaneous abdominal depot. Furthermore, the release of leptin and plasminogen activator inhibitor 1 (PAI-1) was found to be comparable from the two depots.

Co-culture. 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 (8 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 separated by the membrane of the insert. Distance from the bottom of the culture dish to the membrane was 0.9 mm. Co-culture was conducted for 48 h. Integrity of the two cell types was routinely checked by light microscopy at the end of the co-culture period.

Immunoprecipitation. 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 the supernatant (0.3 mg protein) was used for immunoprecipitation. Anti–IRS-1 antiserum (5 μl) was preadsorbed to 20 μl protein A trisacryl beads, which were then added to the solubilized cell supernatant and incubated overnight at 4°C with gentle rotation. The immunopellet was collected by centrifugation and washed three times with ice-cold Tris-buffered saline (TBS). The pellet was incubated for 10 min at 95°C with 20 μl electrophoresis buffer and centrifuged, and the complete supernatant was used for Western blot analysis.

Immunoblotting. Immunoprecipitates or total cell lysates were separated by SDS-PAGE using gradient (8–18%) horizontal gels and transferred to polyvinylidene difluoride filters in a semidry blotting apparatus (17). For phosphotyrosine detection, filters were blocked for 60 min in TBS containing 0.05% Tween-20 and 1% BSA. Thereafter, filters were incubated overnight with a 1:10,000 dilution of the anti-phosphotyrosine antibody (RC-20) coupled to HRP and subsequently processed for enhanced chemiluminescence (ECL) detection using SuperSignal Substrate (Pierce). For detection of IRS-1, *p*-Akt or Akt filters were blocked with TBS containing 0.05% Tween-20 and 5% nonfat dry milk and incubated overnight with a 1:1,000 dilution of appropriate antibody. After extensive washing, filters were incubated with goat anti-rabbit HRP-coupled antibody and processed for ECL detection. Signals were visualized and evaluated on a LUMI Imager workstation using image analysis software (Boehringer Mannheim).

ELISA for TNF-α and resistin. TNF-α and resistin secreted by adipocytes and human skeletal muscle cells into the culture medium were quantified using ELISA kits from either Research Diagnostics or Phoenix Pharmaceuticals (Belmont, CA). Differentiated adipocytes and skeletal muscle cells were incubated for 48 h either individually or under co-culture conditions using the culture medium outlined above. After that time, the cell culture supernatants were collected and concentrated using Vivaspin Columns (Sartorius, Göttingen, Germany). The assay was then performed as recommended by the manufacturer using duplicate samples for all determinations. Sensitivity of the assay procedure was ensured by addition of known amounts of TNF-α or resistin.

Presentation of data and statistics. All data analysis was performed using Prism (GraphPad, San Diego, CA) or t-ease (ISI, Philadelphia) statistical software. Significance of reported differences was evaluated using the null hypothesis and *t* statistics for paired data. A P value <0.05 was considered statistically significant.

RESULTS

Insulin signaling in human skeletal muscle cells after co-culture with adipocytes. Human skeletal muscle cells in primary culture have been shown to represent a valuable tool for studies of insulin action in this tissue (18–20). We have now co-cultured these cells with differentiated human adipocytes to investigate the cross-talk between the tissues. Initial experiments indicated that the two cell types can be kept in the same medium and can be co-cultured for at least 48 h without any gross morphological alterations. Using this protocol, cells of three skeletal muscle donors were co-cultured with adipocytes of three to five different fat cell donors; after removal of the fat cells, the myocytes were acutely stimulated with insulin. As presented in Fig. 1*A*, a strong increase in insulin-stimulated tyrosine phosphorylation of IRS-1 was observed under control conditions.

After co-culture, this insulin response was largely reduced in skeletal muscle cells from donors M9 and F10. In cells from donor M28, a reproducible increase in the basal tyrosine phosphorylation of IRS-1 occurred after co-culture, with no additional effect of insulin (Fig. 1*A*). Quantification of the data for the three individuals indicated that insulin was unable to produce a significant stimulation of tyrosine phosphorylation of IRS-1 in skeletal muscle cells subjected to co-culture with adipocytes (Fig. 1*B*). It should be noted that the level of IRS-1 expression in the myocytes was not affected by the co-culture (see Fig. 3*B*).

We next assessed the effect of the thiazolidinedione compound troglitazone on the induction of insulin resistance in the co-culture system. When added for 48 h in the absence of adipocytes, troglitazone sensitized the skeletal muscle cells toward insulin, with a twofold increase in the

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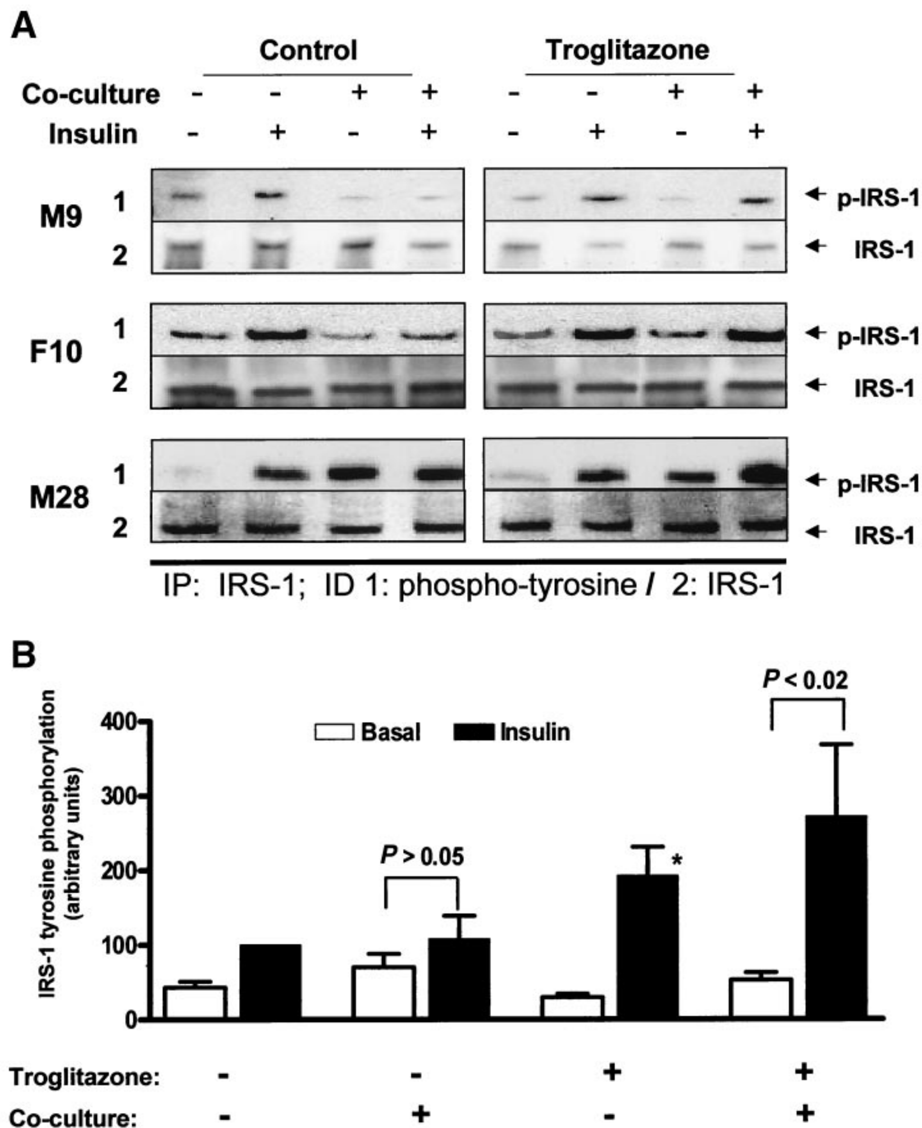

FIG. 1. Muscle cells were incubated for 48 h in co-culture with adipocytes, as detailed in RESEARCH DESIGN AND METHODS. Fat cells were then removed, and the myocytes were stimulated with 100 nmol/l insulin (10 min) followed by cell lysis and immunoprecipitation of IRS-1. *A*: IRS-1 immunoprecipitates were resolved by SDS-PAGE and immunoblotted with anti-phosphotyrosine and anti–IRS-1 antisera. Signals were visualized using ECL detection. Representative blots for the three muscle cell donors are shown. *B*: Western blots were quantified on a LUMI Imager workstation using image analysis software. Results were corrected for the actual amount of IRS-1 present in the immunoprecipitates and are expressed relative to the insulin-stimulated control, which was set as 100. Data are means ± SE of 10–12 experiments from three different muscle cell donors (M9, $n = 3$; F10, $n = 4$; M28, $n = 3$–5). *Significantly different from insulin-stimulated control, $P < 0.05$.

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effect of the hormone on the tyrosine phosphorylation of IRS-1 (Fig. 1*A* and *B*), without affecting IRS-1 expression. Addition of troglitazone to the co-culture *1*) completely prevented the perturbation of insulin signaling at the level of IRS-1 (Fig. 1*A*) and *2*) sensitized the skeletal muscle cells toward insulin to the same degree as in the absence of adipocytes (Fig. 1*B*).

As presented in Fig. 2, the co-culture conditions were also found to induce prominent insulin resistance in the skeletal muscle cells at the level of the serine/threonine kinase Akt, a central component of downstream insulin signaling to glucose transport and glycogen synthesis (21).

Under control conditions, insulin produced a three- to fourfold increase in the Ser473 phosphorylation of Akt in the myocytes, with a significant ($P < 0.001$) reduction of this response (by 60–70%) after co-culture with adipocytes (Fig. 2*A* and *B*). It is worth noting that in cells from donor F10, we observed a reproducible increase in the basal serine phosphorylation of Akt, with no additional effect of insulin (Fig. 2*A*). As found for IRS-1, the expression of Akt was not affected by the co-culture conditions. Troglitazone strongly sensitized the skeletal muscle cells toward insulin-mediated activation of Akt, with a parallel 1.5- to 2-fold increase in the protein expression of this enzyme (Fig. 2). The sensitizing effect of troglitazone was not observed under co-culture conditions; however, the thiazolidinedione compound restored a completely normal insulin response of Akt serine phosphorylation in the skeletal muscle cells (Fig. 2).

Effect of TNF-α on insulin signaling in human skeletal muscle cells. The role of TNF-α in the development of insulin resistance under co-culture conditions was assessed by culturing human skeletal muscle cells for 48 h in the presence of a high dose of the cytokine. Direct addition

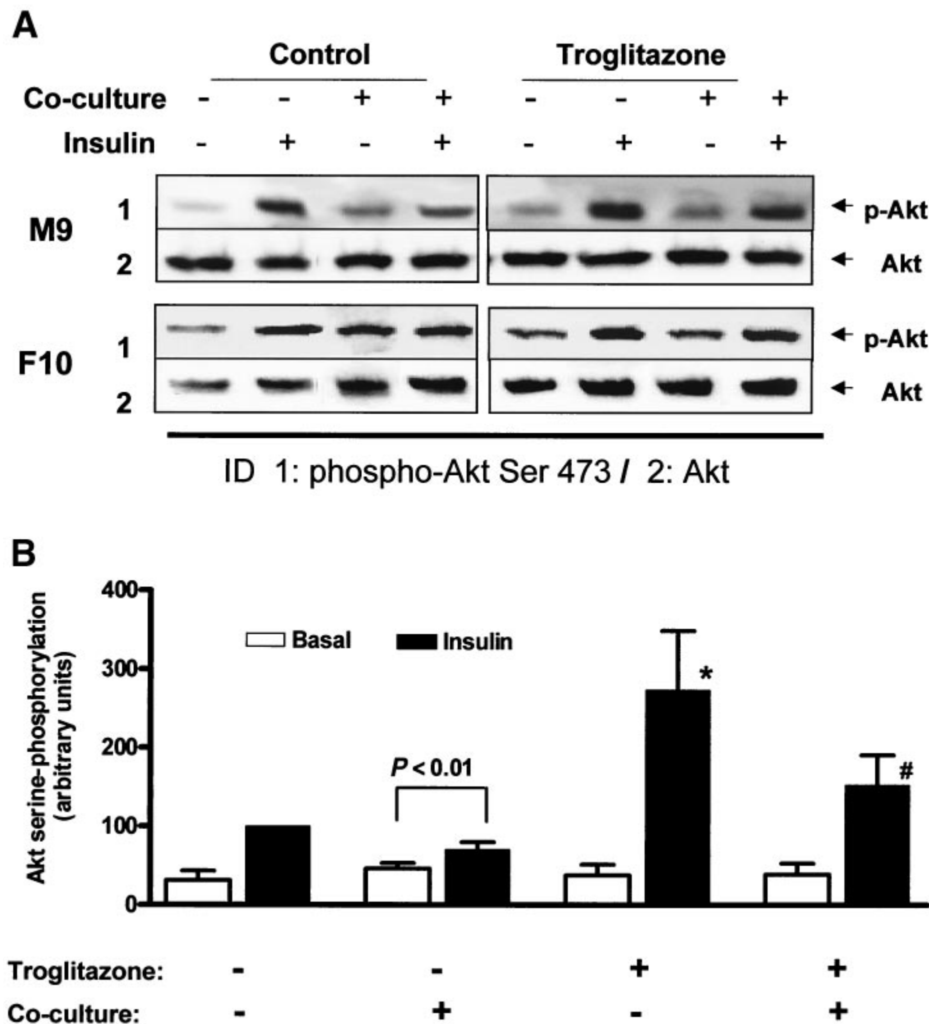

FIG. 2. ***A*****: Human skeletal muscle cells were subjected to co-culture and acutely stimulated with insulin (100 nmol/l, 10 min), as described in Fig. 1. Total cell lysates (19–20 mg/lane) were resolved by SDS-PAGE and immunoblotted with phosphospecific Akt antibody and Akt antibody. Each donor is represented by typical blot.** ***B*****: Quantification of immunoblots was performed 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 mean values ± SE of seven independent experiments from two different muscle cell donors (M9, *n* = 3; F10, *n* = 3–4). *Significantly different from all other insulin-stimulated values, *P* < 0.05; #not significantly different from control, *P* > 0.05.**

of TNF-α to the skeletal muscle cells was found to reduce the protein expression of IRS-1 by ~40% (Fig. 3*A*), with troglitazone preventing this reduction. In contrast, unaltered IRS-1 expression was observed in myocytes subjected to co-culture (Fig. 3*B*).

Akt protein expression remained unaffected by TNF-α. As presented in Fig. 4, insulin signaling to both IRS-1 and Akt was completely blocked in skeletal muscle cells cultured in the presence of 2.5 nmol/l TNF-α. Troglitazone ameliorated this insulin resistance, in agreement with our findings in skeletal muscle cells subjected to co-culture with adipocytes. It should be noted that all data on protein phosphorylation were corrected for the actual amount of IRS-1 and Akt present in the immunoprecipitates and lysates, respectively.

The data obtained so far indicated that TNF-α was able to mimic the effect of co-culture on insulin signaling in human skeletal muscle cells. It was therefore of interest to study this effect of TNF-α using lower, physiologically more relevant doses. The dose-response relationship for IRS-1 and Akt expression and phosphorylation is presented in Fig. 5*A* and *B*.

As can be seen from the data, insulin signaling to IRS-1 remained unaffected at 0.15 and 1 nmol/l TNF-α, with a dose-dependent reduction of IRS-1 expression. Insulin-induced signaling to Akt was also reduced in a dose-dependent fashion by TNF-α; however, even at 1 nmol/l of the cytokine, this reduction was not more than ~30% (Fig. 5*B*). Only at 2.5 nmol/l TNF-α was the perturbation of insulin signaling in the skeletal muscle cells comparable to the results obtained in the co-culture of myocytes and adipocytes.

Secretion of TNF-α and resistin by skeletal muscle cells and adipocytes. The dose-response data described above make it unlikely that TNF-α represents a major mediator of insulin resistance in the co-culture system. To further address this issue, attempts were made to quantify the TNF-α concentration in the cell culture supernatant of skeletal muscle cells, adipocytes, and the cells in co-culture using a highly sensitive TNF-α ELISA assay (de-

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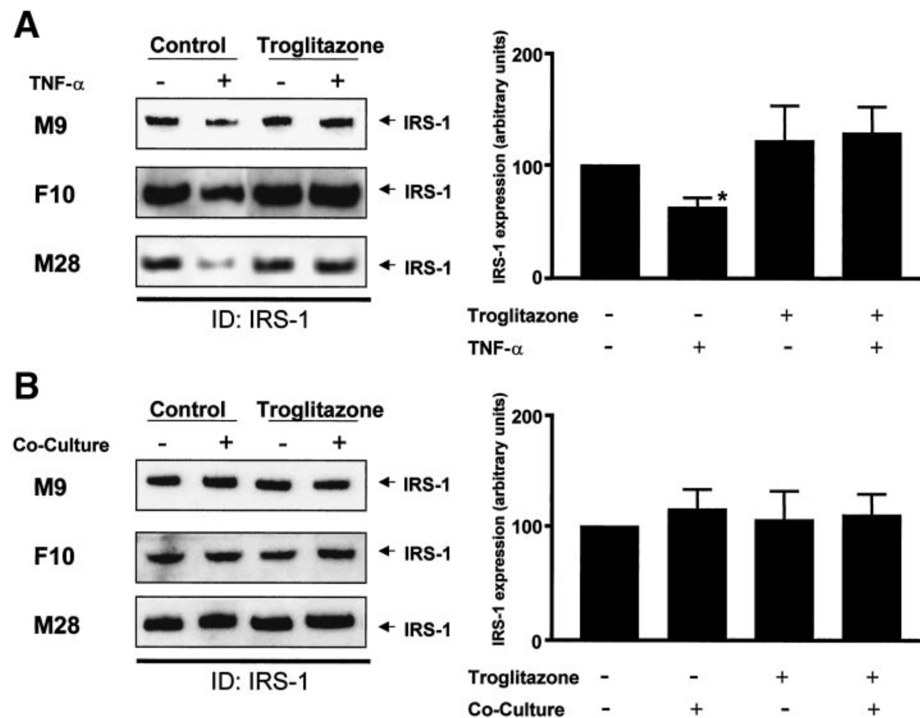

FIG. 3. ***A*: Muscle cells were incubated for 48 h in the absence or presence of 2.5 nmol/l TNF-α without or with 5 mmol/l troglitazone. After 24-h incubation, the medium was completely removed and replaced by fresh differentiation medium supplemented with TNF-α and troglitazone, as indicated. Cells were lysed, and total cell lysates were resolved by SDS-PAGE and immunoblotted with anti–IRS-1 antiserum. Results are expressed relative to the control value (defined as 100). Data are means ± SE of 13 independent experiments from three different muscle cell donors (M9, $n = 3–5$; F10, $n = 3–5$; M28, $n = 3$). Representative blots are shown. *Significantly different from control, $P < 0.005$. *B*: Muscle cells were subjected to co-culture as described in Fig. 1. Total cell lysates were resolved by SDS-PAGE and immunoblotted with anti–IRS-1 antiserum. Quantification was performed as in *A*. Data are means ± SE of 12 independent experiments from three different muscle cell donors.**

tection limit, 0.2 pmol/l). Cell culture supernatants were collected and concentrated 10-fold. Essentially no TNF-α was detected under these conditions after 48 h culture. When 0.3 pmol/l of TNF-α was added to the culture medium, this amount of the cytokine was reproducibly detected, thus validating the sensitivity of the assay.

The same analysis was subsequently performed for the recently detected hormone resistin. This peptide has been implicated as an adipocytokine leading to insulin resistance (13). We used a resistin ELISA with a detection limit of 5 pg/ml. As shown in Fig. 6, skeletal muscle cells were found to secrete a measurable amount of resistin (10–20 pg/ml) into the culture medium, whereas the hormone remained below the detection level in the supernatant of the differentiated adipocytes. Under co-culture conditions and in the presence of troglitazone, resistin was also detected in the culture medium, with a trend for even lower values compared with skeletal muscle cells alone (Fig. 6).

DISCUSSION

Skeletal muscle accounts for ~80% of total glucose disposal under insulin-stimulated conditions (22), and defects of insulin action in this tissue are central to the pathogenesis of type 2 diabetes. Based on animal studies, it has been postulated that the overproduction of TNF-α and resistin by adipose tissue may link obesity to type 2 diabetes (5,13). However, it remains questionable if this concept may be translated to humans (12,14). Furthermore, adipose tissue is also dispersed within muscle (15). This intramuscular fat increases with obesity (23), which may lead to a paracrine interaction between adipose and muscle cells potentially involving much lower concentrations of adipocyte factors. We report here for the first time on the co-culture of human adipocytes and human skeletal muscle cells, a generally accepted approach for studies on the paracrine interaction between two cell types (24,25). Our results support the notion of a significant cross-talk between fat and muscle cells but exclude the involvement of TNF-α and resistin in this process.

A key finding of the present investigation is the observation of a profound disturbance of insulin signaling in muscle cells subjected to co-culture. This involves the absence of insulin-stimulated phosphorylation of IRS-1 and the reduced ability of the hormone to stimulate Akt-kinase, without affecting the expression of these proteins. Recent studies on insulin signaling in human skeletal muscle of obese type 2 diabetic patients have shown that insulin was completely unable to stimulate the phosphatidylinositol (PI) 3-kinase pathway in muscle biopsies of these subjects, as measured by the absence of both insulin-regulated phosphorylation of IRS-1 and activation of PI 3-kinase (26,27). These data are in excellent agreement with the present results obtained in the co-culture system and define the IRS-1/PI 3-kinase pathway as a key step in the development of insulin resistance. An apparent difference between adolescent and adult muscle donors was observed, concerning the potential mechanism underlying the absence of insulin-stimulated phosphorylation of IRS-1 (see Fig. 1). However, several lines of evidence support the view that this difference more likely reflects an inter-individual variation in the sensitivity of the myotubes

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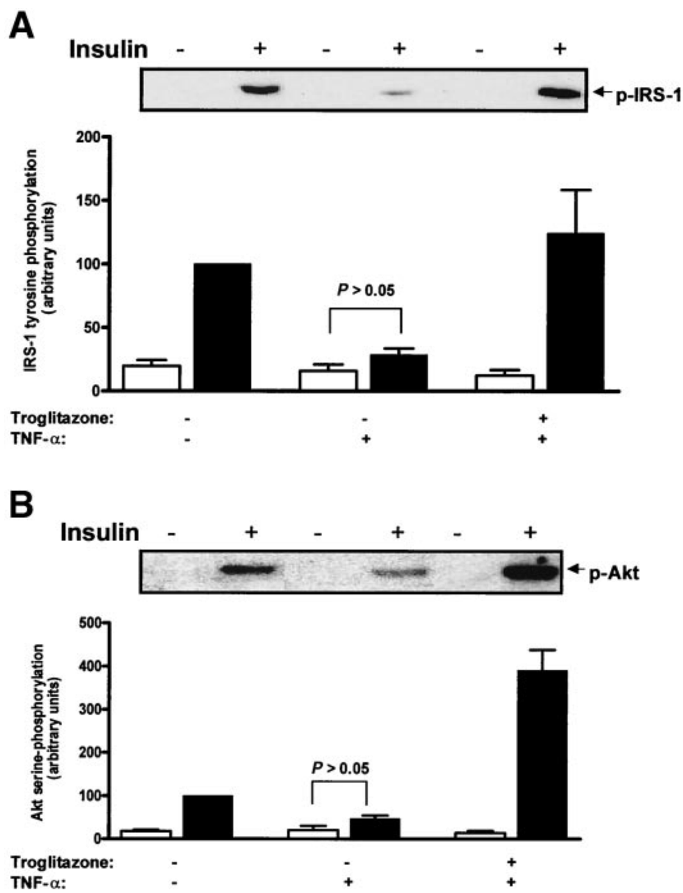

FIG. 4. ***A*****: Cells were cultured for 48 h with TNF-α and troglitazone, as detailed in Fig. 3. After stimulation with insulin (100 nmol/l for 10 min), IRS-1 was immunoprecipitated, and the tyrosine phosphorylation of IRS-1 was determined and quantified as described in Fig. 1. The inset shows a representative Western blot. Data are means ± SE of 15 independent experiments using skeletal muscle cells of the three different donors. Data are expressed relative to the insulin-stimulated control.** ***B*****: Cells were cultured as outlined for *A*, and total cell lysates were used for the analysis of insulin-induced serine phosphorylation of Akt, as detailed in Fig. 2. The inset shows a representative Western blot. Data represent means ± SE of 10 independent experiments using skeletal muscle cells of the three different donors. All data were normalized to the level of Akt expression and are expressed relative to the insulin-stimulated control.**

toward the modulatory crosstalk with adipocytes. First, in contrast to rodent models, the proliferative capacity and the program of myogenic differentiation were found to be identical in 9-year-old and 60-year-old humans (28,29). Second, we have determined the differentiation-dependent gene expression profile (including insulin receptor, IRS-1, and IRS-2) of myoblasts from the three donors and did not detect any difference (J.E. and T. Gromke, unpublished data). Third, a consistent response toward TNF-α was observed for the three individuals (Figs. 3 and 4). At the present stage, the precise mechanism leading to the abrogation of insulin-stimulated tyrosine phosphorylation of IRS-1 after co-culture remains unknown.

Using in vitro incubations of skeletal muscle strips from type 2 diabetic patients, Krook et al. (30) reported a marked reduction in insulin-stimulated Akt kinase at high concentrations of the hormone. In subsequent studies using in vivo insulin stimulation, however, the phosphorylation and activation of the three Akt isoforms was found to be normal in skeletal muscle from obese type 2 diabetic subjects (31), and the role of Akt for human insulin resistance has been questioned. In contrast, our data suggest that the negative cross-talk between fat and muscle may largely abrogate insulin-regulated activation of Akt. Some limitations of our in vitro system may explain these discrepancies. Thus, *1*) desensitization of Akt may be transient and reversible, *2*) fiber type–specific insulin signaling (32) must be considered, and *3*) only a subset of skeletal muscle tissue is subject to the paracrine interaction in vivo, making potential alterations undetectable in a large muscle sample. Interestingly, in a recent study, Storgaard et al. (33) reported a tendency for reduced activation of Akt in skeletal muscle of first-degree relatives of type 2 diabetic patients after in vivo insulin stimulation. Additional work will be needed to clarify the role of Akt in skeletal muscle insulin resistance.

The thiazolidinedione compound troglitazone completely prevented the insulin resistance in skeletal muscle cells when present during the co-culture with adipocytes. This observation excludes nonspecific alterations of the myocytes and shows that our co-culture approach should be useful for defining novel pharmacological interventions of the cross-talk between fat and muscle. It is generally assumed that troglitazone acts primarily on adipocytes by binding to the nuclear peroxisome proliferator–activated receptor-γ (PPAR-γ) and that the improvement of insulin responsiveness in skeletal muscle of type 2 diabetic patients is indirect (34). This may result from a decrease in elevated free fatty acid levels and a change in the body distribution pattern of adipose tissue (35). As reported by Park et al. (36), PPAR-γ is also present in primary cultured human skeletal muscle cells, with increased expression upon culture with troglitazone. We have confirmed this increased expression of PPAR-γ in our cultures of human skeletal muscle cells (data not shown) and show here a prominent sensitization of insulin signaling in skeletal muscle cells exposed to troglitazone. Our data do not exclude effects of troglitazone on the release of adipocyte factors; nevertheless, it is evident that the direct effect on human skeletal muscle cells is sufficient to prevent the insulin resistance induced by the cross-talk with adipocytes.

When added at high concentrations, TNF-α was able to mimic the disturbance of insulin signaling found after co-culture; however, concentrations $<$1,000 pmol/l were marginally effective. This result differs markedly from our earlier study on human adipocytes, where we reported that TNF-α at 10 pmol/l was able to attenuate insulin signaling (10). Thus, auto-/paracrine effects of TNF-α on adipocytes may be much more relevant for the induction of insulin resistance in adipose tissue than the effects of the cytokine on skeletal muscle. This notion is strongly supported by a recent study on human skeletal muscle cells showing no effect of 300 pmol/l TNF-α on insulin-mediated activation of Akt kinase (19). Importantly, we were unable to detect TNF-α in the co-culture medium despite using a highly sensitive assay with a detection limit of 0.2 pmol/l. Taking into account that the culture medium was concentrated 10-fold, the TNF-α level during the co-culture can be estimated to be $<$0.02 pmol/l. These considerations strongly suggest that TNF-α is not responsible for the insulin resistance observed in skeletal muscle

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FIG. 5. *A*: Myocytes were cultured with TNF-α for 48 h using the indicated concentrations of the cytokine. Cell lysates were then analyzed by immunoblotting using antisera for IRS-1 and Akt. Results are expressed relative to the untreated control value and represent means ± SE of 10 independent experiments using cells of donor M9 and F10. Significantly different from control, *$P < 0.05$; #$P < 0.005$. *B*: Cells were cultured as in *A*, and the insulin-induced tyrosine phosphorylation of IRS-1 and the serine phosphorylation of Akt were determined by Western blotting and quantified as described in Figs. 1 and 2. Data are means ± SE of 10 independent experiments. *Significantly different from control, $P < 0.05$.

cells after the co-culture procedure. Consistent with this suggestion, no correlation between insulin sensitivity and subcutaneous adipose tissue TNF-α expression could be detected in obese type 2 diabetic patients (37). Furthermore, TNF-α is expressed at much lower levels in human adipose tissue compared with rodents (11). However, it cannot be ruled out that TNF-α induces muscle insulin resistance in a synergistic fashion together with other yet unidentified adipocyte factors.

The peptide resistin represents such a recently detected novel adipocyte factor, and based on rodent studies, it has been postulated that resistin acts as a hormonal link between obesity and insulin resistance (13). We show here that resistin is undetectable in the supernatant of differentiated mammary human adipocytes and therefore conclude that resistin does not mediate the negative cross-talk between fat and muscle in our co-culture system. Our findings agree with two recent reports showing low/absent expression of resistin in human adipocytes, with a high interindividual variability and no correlation to BMI (14,38). Instead, resistin was found to be predominantly expressed in mononuclear cells (14). Interestingly, resistin appears to be secreted by untreated skeletal muscle cells, with a tendency to a reduction in the co-culture. This clearly rules out resistin as a mediator of insulin resistance in human skeletal muscle. In addition to cytokines, free fatty acids may also be involved in the negative cross-talk between fat and muscle (39). However, studies by Kausch et al. (40) suggest that palmitate, 2-bromo-palmitate, and linoleate do not modify insulin signaling in primary cultured human skeletal muscle cells. Initial experiments in our laboratory confirm these findings and make it unlikely that the results obtained in the co-culture can be explained by the release of free fatty acids.

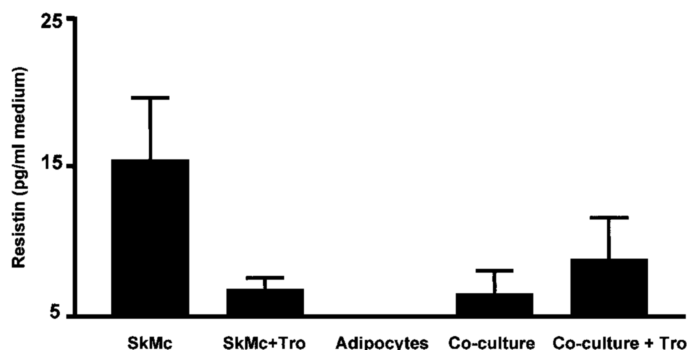

FIG. 6. Culture media from skeletal muscle cells (SkMc), adipocytes, troglitazone (Tro)-treated skeletal muscle cells, and co-culture were collected and concentrated 10-fold, and resistin was determined using a resistin ELISA kit. Data are means ± SE ($n = 8$) using skeletal muscle cells of donor M9 and F10. There are no statistically significant differences. The *y*-axis starts at detection limit.

In summary, we describe here the co-culture of human skeletal muscle cells and human adipocytes as a novel model system for studies on the paracrine interaction between fat and muscle. Our data show that adipocytes

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induce a rapid disturbance of insulin signaling in skeletal muscle cells that is not mediated by TNF-α and resistin. It is suggested that a complex array of adipocyte factors is responsible for generating muscle insulin resistance.

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