Skeletal muscle insulin resistance induced by adipocyte-conditioned medium: underlying mechanisms and reversibility

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The development of insulin resistance is a reversible process. Reduction of adipose tissue mass by weight loss is a validated approach to reverse insulin resistance (11, 25). In parallel with improved insulin sensitivity, weight reduction also normalizes adipokine blood level, which has been demonstrated for IL-6 (5), high-molecular-weight adiponectin (2), monocyte chemotactic protein-1 (MCP-1) (4), and TNFα (19). It could be shown that insulin resistance disappears in cultured skeletal muscle biopsies from obese patients (3, 22), demonstrating that insulin resistance might be a reversible feature that can be acquired with obesity. However, other studies in muscle biopsies from obese and diabetic patients demonstrated that insulin resistance is retained in culture (3, 13, 39). This study was aimed at analyzing reversibility of adipocyte-induced insulin resistance in skeletal muscle cells and underlying mechanisms.

MATERIALS AND METHODS

Materials. BSA (fraction V, fatty acid free) was obtained from Roth (Karlsruhe, Germany). Reagents for SDS-PAGE were supplied by Amersham Pharmacia Biotech (Braunschweig, Germany) and by Sigma (München, Germany). Polyclonal antibodies anti-phospho-GSK-3α/β (Ser21/9), anti-phospho-Akt (Ser473), and anti-glucose transporter 4 (GLUT4) were supplied by Cell Signaling Technology (Frankfurt, Germany) and anti-tubulin by Calbiochem (Darmstadt, Germany). Antibodies for myogenin came from Acris (Hiddenhagen, Germany), for MyoD from Imgenex (San Diego, CA), and the one for myosin heavy chain (MHC) from Upstate (San Diego, CA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit and goat antiamouse IgG antibodies were purchased 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). All other chemicals were of the highest analytical grade commercially available and were purchased from Sigma.

Culture of human skeletal muscle cells. Primary human skeletal muscle cells of four healthy Caucasian donors (2 males, 9 and 47 yr of age; 2 females, 10 and 48 yr of age) were supplied as proliferating myoblasts (5 × 10^5 cells) and cultured as described previously (9). For an individual experiment, myoblasts were seeded in six-well culture dishes (9.6 cm²/well) at a density of 10^5 cells. The development of insulin resistance is a reversible process. Reduction of adipose tissue mass by weight loss is a validated approach to reverse insulin resistance (11, 25). In parallel with improved insulin sensitivity, weight reduction also normalizes adipokine blood level, which has been demonstrated for IL-6 (5), high-molecular-weight adiponectin (2), monocyte chemotactic protein-1 (MCP-1) (4), and TNFα (19). It could be shown that insulin resistance disappears in cultured skeletal muscle biopsies from obese patients (3, 22), demonstrating that insulin resistance might be a reversible feature that can be acquired with obesity. However, other studies in muscle biopsies from obese and diabetic patients demonstrated that insulin resistance is retained in culture (3, 13, 39). This study was aimed at analyzing reversibility of adipocyte-induced insulin resistance in skeletal muscle cells and underlying mechanisms.

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Adipocyte isolation and culture. Adipose tissue samples were obtained from the mammary fat of normal or moderately overweight
women (body mass index 24.5 ± 0.9, aged between 23 and 41 yr) undergoing surgical mammary reduction. The procedure to obtain adipose tissue was approved by the ethics committee of Heinrich-Heine-University, Dusseldorf, Germany. All subjects were healthy, 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 described previously [12]. Isolated cell pellets were resuspended in Dulbecco’s modified Eagle’s/Ham’s F-12 medium supplemented with 10% FBS, seeded on membrane inserts (3.5 × 105/4.3 cm2) or on a six-well dish culture, and kept in culture for 16 h. After washing, culture was continued in an adipocyte differentiation medium (DMEM-F-12, 33 mM glutamine, 10 mM sodium pyruvate, and Complete Protease Inhibitor Cocktail from Roche Diagnostics. After incubation for 2 h at 4°C, the suspension was centrifuged at 13,000 g for 15 min. Thereafter, 5 μg of lysates was separated by SDS-PAGE using 10% horizontal gels and transferred to polyvinylidene fluoride filters in a semidry blotting apparatus. For detection, filters were blocked with Tris-buffered saline containing 5% (vol/vol) nonfat dry milk and subsequently incubated overnight with the appropriate antibodies. After extensive washing, filters were incubated with secondary HRP-coupled antibody and processed for enhanced chemiluminescence detection using Uplight (Interchim, France). Signals were visualized and evaluated on a LUMI Imager workstation using image analysis software (Boehringer Mannheim, Mannheim, Germany).

Measurement of reactive oxygen species and nitric oxide production in skeletal muscle cells. Differentiated skeletal muscle cells were treated with CM overnight to induce insulin resistance. Then, cells were washed in PBS without Ca/Mg and used for the assay. For measurement of reactive oxygen species (ROS), cells were incubated in 10 mM 2′,7′-dichlorodihydrofluorescein diacetate (Molecular Probes, Karlsruhe, Germany) solved in phenol red-free DMEM for 30 min. As a positive control, cells were treated with 0.3% H2O2 for 30 min in parallel to 2′,7′-dichlorodihydrofluorescein diacetate incubation. For measurement of nitric oxide (NO), skeletal muscle cells were incubated with 10 mM 4-amino-5-methylamino-2′,7′-diaminofluorescein diacetate (Molecular Probes) solved in phenol red-free DMEM for 30 min. As a positive control for NO production, cells were also treated with 500 μM SNAP (Calbiochem, Darmstadt, Germany) for 30 min in parallel with 4-amino-5-methylamino-2′,7′-diaminofluorescein diacetate. Afterward, cells were lysed in the above-mentioned lysis buffer and fluorescence measured using an excitation wavelength of 595 nm on a Fluostar-P (SLT, Salzburg, Austria).

Measurement of SDH activity in skeletal muscle cells. Differentiated skeletal muscle cells were incubated with CM for the indicated time and lysed in homogenization buffer containing 250 mM glucose, 10 mM Tris-HCl, 0.5 mM EGTA, and 0.5 mM DTT. Succinate dehydrogenase (SDH) activity was measured according to Pennington’s method [23]. Briefly, ~200 μg of cell lysate was incubated with 10 mM sodium succinate in 50 mM NaH2PO4 buffer for 20 min at 37°C. Five millimolar p-iodonitrotetrazolium violet solved in 50 mM NaH2PO4 buffer was added to a final concentration of 0.5 mM for an additional 10 min at 37°C. The reaction was stopped by an ethylacetate-ethanol-trichloroacetic acid solution (5:5:1, vol/vol/wt). Immediately after 2-min centrifugation at 13,000 g, the supernatant was measured at 490 nm on a spectrophotometer (Beckman, Krefeld, Germany).

Measurement of apoptosis. Apoptosis was monitored by assessment of caspase-3 activity, and nuclear fragmentation in skeletal muscle cells was treated with CM. The DEVD-cleaving activity of the caspase-3 class of cystein proteases was determined in cell lysates using Ac-DEVD-AMC (BD Biosciences, Heidelberg, Germany) as fluorescent substrate according to the manufacturer’s protocol. The ability of cell lysates to cleave the specific caspase-3 substrate was quantified by spectrophorometry using an excitation wavelength of 390 nm and an emission wavelength of 460 nm with a microplate reader. For detection of nuclear fragmentation, the cells were double-stained with Hoechst 33342 and propidium iodide. Skeletal muscle cells were washed twice with PBS and stained with 10 μg/ml Hoechst 33342 and 1 μg/ml propidium iodide at 37°C for 15 min. Fluorescence was observed under a Leica DM IRB fluorescence microscope. At least 400 cells were counted for each experiment. Cells with condensed or fragmented nuclei were defined to be apoptotic, and cells with normally shaped nuclei were supposed to be viable.

Quantitative evaluation of ceramide. Lipids from skeletal muscle cells were extracted in chloroform-methanol-water (2:1:0.01, vol/vol/vol) for 24 h at 48°C. Lipid extracts were applied to thin-layer Silica Gel 60 plates (Merck, Darmstadt, Germany) as described earlier [28]. Ceramides were resolved twice using chloroform-methanol-acetic acid (190:9:1, vol/vol/vol) as developing systems. Following development, plates were air-dried, sprayed with 8% (wt/vol) H2PO4 and charred at 180°C for 10 min. Lipids were identified by their RI value, using authentic lipid samples as references. Individual lipid bands obtained by thin-layer chromatography were evaluated by densitometry (Shimadzu, Kyoto, Japan). Assuming constant cholesterol amounts in all samples, densitometric data obtained for ceramide were normalized to cholesterol.

Presentation of data and statistics. Statistical analysis was performed by ANOVA. All statistical analyses were done using Statview (SAS, Cary, NC), considering a P value of <0.05 as statistically significant. Corresponding significance levels are indicated in the figures.

RESULTS

CM-induced insulin resistance of insulin signaling in skeletal muscle cells is a reversible process. CM of differentiated human adipocytes impairs insulin signaling at the level of Akt and myogenic transcription factors in skeletal muscle cells and an irreversible downregulation of myogenin. During differentiation, skeletal muscle cells display an increased expression of...
myogenin, MHC, and MyoD, all of which are markers of myogenesis (Fig. 2, A–C). Analysis of myogenic transcription factors revealed that CM-treated skeletal muscle cells have significantly reduced expression of myogenin, MHC, and MyoD (Fig. 3, A–C). Skeletal muscle cells display an increasing GLUT4 level (Fig. 4A, top). However, CM treatment did not affect GLUT4 expression in differentiated myotubes (Fig. 4A, bottom), and the cells exhibited an unaltered morphology compared with control cells (Fig. 4B). Withdrawal of CM for 24 or 48 h reverses the downregulation of MHC and MyoD, whereas
the expression of myogenin remains decreased over the whole period compared with control (Fig. 3). Thus, despite reestablished insulin signaling, skeletal muscle cells do not normalize myogenin expression after CM treatment and withdrawal.

**CM-treated skeletal muscle cells are characterized by a partially irreversible secretory dysfunction.** Skeletal muscle cells secrete various myokines, including IL-6, IL-8, and MCP-1. Compared with adipocytes that secrete ~500 pg·ml⁻¹·24 h⁻¹ IL-6, skeletal muscle cells exhibit lower secretion of this cytokine with 23 ± 1 pg·ml⁻¹·24 h⁻¹ (n = 5). Treatment with CM leads to a significantly lower IL-6 secretion during the first 24 h of regeneration of myotubes (Fig. 5A). Forty-eight hours after CM withdrawal, however, IL-6 secretion is comparable with control cells.

IL-8 secretion is also lower in skeletal muscle cells (94 ± 12 pg·ml⁻¹·24 h⁻¹; n = 5) compared with adipocytes (~500 pg·ml⁻¹·24 h⁻¹).

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Fig. 3. Effect of CM treatment and CM withdrawal on myogenic markers. Differentiated skeletal muscle cells from 2–3 donors were treated with CM for 24 h and lysed directly or after regeneration for 24 or 48 h. Lysates were used for Western blot as described in Fig. 1 and detected with myogenin (A), MHC (B), and MyoD (C) antibodies. Data are tubulin-normalized means ± SE (n = 6–12). *Significantly different from control.

Fig. 4. Glucose transporter 4 (GLUT4) expression and morphology of insulin-resistant skeletal myotubes. A: myoblasts and differentiated skeletal muscle cells from 3 donors were analyzed for GLUT4 expression during differentiation (top), and differentiated skeletal muscle cells from 2–3 donors were treated with CM for 24 h (bottom) and lysed. Lysates were used for Western blot as described in Fig. 2 and detected with a GLUT4 antibody. Data are tubulin-normalized means ± SE (n = 3 during differentiation and n = 6–12 for CM treatment). *Significantly different from day 0 of differentiation. B: myotubes were treated with CM for 24 h, and a representative micrograph showing unaltered myotube morphology in insulin-resistant skeletal muscle cells is presented. Magnification ×4.
CM-treated skeletal muscle cells display significantly impaired IL-8 secretion over the whole regeneration period of 48 h compared with control. This suggests that IL-8 secretion might be irreversibly disturbed in insulin-resistant myocytes (Fig. 5B).

MCP-1 is a cytokine robustly released from human adipocytes (~3 ng·ml⁻¹·24 h⁻¹) but also secreted at low levels from myotubes (37 ± 11 pg·ml⁻¹·24 h⁻¹; n = 5). Induction of insulin resistance in skeletal muscle cells significantly stimulates MCP-1 secretion after 24 h of regeneration with an additional increase after 48 h (Fig. 5C).

Insulin-resistant skeletal muscle cells exhibit increased oxidative stress and decreased mitochondrial capacity but no apoptosis. ROS and NO are both potential players in the induction of insulin resistance. As presented in Fig. 6, a significant increase in both ROS and NO production was observed in skeletal muscle cells treated with CM. SDH activity was measured in whole cell lysates of skeletal muscle cells to assess oxidative capacity. CM treatment slightly but significantly reduced SDH activity in whole cell lysates after 24 h (Fig. 7). Longer incubation with CM over 96 h further reduced the level of SDH activity. The parallel induction of insulin resistance and oxidative stress cannot, however, be assigned to apoptosis in skeletal muscle cells. Measurement of caspase-3 activity revealed no increase in CM-treated cells compared with controls [1.08 ± 0.13 vs. 1.06 ± 0.17 arbitrary units, significantly different from control (Fig. 6)].

Fig. 6. Effect of CM treatment on skeletal muscle reactive oxygen species (ROS) and nitric oxide (NO) production. Differentiated skeletal muscle cells from 2 donors were treated with CM for 24 h and subsequently analyzed for their capacity to produce ROS and NO as described in Materials. As a positive (pos) control, cells were treated for 30 min prior to the beginning of the experiment with H₂O₂ and SNAP. Data are means ± SE (n = 3–4). *Significantly different from control.
insulin resistance (14). We could demonstrate in several studies that adipocyte-derived factors can induce insulin resistance in skeletal muscle cells in vitro (7, 9, 32). The data presented here now demonstrate that CM-treated skeletal muscle cells are characterized not only by impaired insulin signaling but also by various other defects. Insulin-resistant skeletal muscle cells downregulate the expression of myogenin and display oxidative stress, lower mitochondrial capacity, and higher ceramide content. Furthermore, insulin-resistant myotubes have disturbed secretion of the myokines IL-6, IL-8, and MCP-1.

In vitro-differentiated skeletal muscle cells are characterized by a high abundance of myogenic transcription factors such as myogenin and MyoD. We demonstrate here for the first time that adipocyte-derived factors lead to a marked downregulation of myogenin in skeletal muscle cells. It is known from the literature that TNFα suppresses the differentiation process in C2C12 myoblasts (34), but nothing is known about its effect on differentiated cells. However, CM contains very low doses of TNFα (<0.02 pmol/l (7)], making it probable that another adipokine with a higher concentration in CM might be the culprit for downregulation of myogenin. The loss of myogenin in insulin-resistant skeletal muscle cells is, however, associated with a conservation of skeletal muscle phenotype, as myotubes display normal morphology and GLUT4 expression. However, it cannot be completely ruled out that the downregulation of multiple markers, including MyoD, MHC, and SDS, points to a dedifferentiation of skeletal muscle cells, and it is impossible so far to speculate on the meaning of this finding for the situation in skeletal muscle in vivo.

IL-6, IL-8, and MCP-1 are known secretory products from skeletal muscle with different roles in myogenesis, exercise, inflammation, and insulin sensitivity. Increased IL-6 levels are associated with insulin resistance in vivo (16), but short-term treatment of skeletal muscle cells with IL-6 can increase insulin sensitivity (40). The reported increase of IL-6 during exercise (21) makes it likely that IL-6 has completely different acute and chronic effects. As for myogenesis, IL-6 is a pro-myogenic factor (1) explaining the parallel decrease of myogenic markers and IL-6 secretion in the myotubes. Both IL-8 and MCP-1 are proinflammatory chemokines that are increased in serum of obese and diabetic patients (17, 29, 30). MCP-1 is a potent inducer of insulin resistance in skeletal muscle cells (32) and plays a role in myopathies (6). TNFα and IFNγ have been described to induce MCP-1 transcription in myoblasts (6). Although IL-8 secretion is almost completely inhibited in CM-treated skeletal muscle cells, MCP-1 release increases, pointing to an inflammatory effect of CM.

SDH activity is known to be slightly but significantly reduced in skeletal muscle lysates from diabetic patients compared with controls (20). We also observed a reduction in SDH activity in CM-treated skeletal muscle cells, indicating a possible role of decreased oxidative capacity in the initiation of skeletal muscle cell insulin resistance. Notably, in diabetic patients, reduced oxidative capacity in parallel with increased glycolytic activity is due to a significant alteration of skeletal muscle fiber composition.

Oxidative stress is a result of increased ROS or NO production and can lead to oxidation and damage of DNA, protein, and lipids (18). Increasing ROS production as observed in our model could cause damage to mitochondria and so-called mitoptosis and explain the loss of mitochondria observed in states with increased oxidative stress such as insulin resistance and diabetes. Thus, increased ROS or NO levels could also explain decreased SDH activity in insulin-resistant skeletal muscle cells. Other work in L6 muscle cells shows that palmitylate-induced insulin resistance is also characterized by higher levels of ROS and NO (27). However, it should be noted that fatty acids are barely detectable in CM when an HPLC approach is used (data not shown). Therefore, we conclude that adipocyte-derived factors produce an increase in ROS and NO similar to that produced by fatty acids.

NO and inducible NO synthase are known to be increased in the diabetic state and are linked to chronic inflammation (15). However, it is not known how NO induces or exacerbates insulin resistance. In C2C12 skeletal muscle cells, the NO donor SNAP inhibits Akt activity, making it possible that an intracellular increase in skeletal muscle cell NO might contribute to insulin resistance (41). Furthermore, diabetic patients are characterized by higher blood levels of nitrates and nitrites as well...
as higher expression of inducible NO synthase in skeletal muscle (35). In our primary myotubes we also observed an increase in NO production after treatment with CM, which might, together with ROS, contribute to the development of insulin resistance. It should be noted in this context that CM-treated skeletal muscle cells are not apoptotic, as shown by unaltered percentage of cells with nuclear fragmentation and similar caspase-3 activity, compared with controls, so NO and ROS elevation cannot be attributed to apoptosis.

The sphingolipid ceramide is described to be a possible link between obesity and diabetes. Fatty acids and resulting higher levels of ceramide can induce insulin resistance in skeletal muscle cells (26, 37). In this study, insulin-resistant skeletal muscle cells are also characterized by increased ceramide levels, which may contribute to adipokine-induced insulin resistance and illustrate disturbed lipid metabolism.

In this study, we were able to show that adipocyte-induced insulin resistance is a reversible process in skeletal muscle cells, at least at the level of insulin signaling. However, some alterations are not fully reversible and may illustrate longer-lasting damage to the myotubes by one-time treatment with CM. Skeletal muscle cells display long-lasting myogenin downregulation and secretory defects of IL-8 and MCP-1. Differentiation of skeletal muscle involves a group of transcription factors, including myogenin and MyoD, that activate muscle-specific gene expression and each have a distinct function during myogenesis (24). In our model, we observe a loss of myogenin expression with preservation of muscle phenotype. At this point, we cannot evaluate the physiological impact of the loss of myogenin. Our data clearly show that the loss of myogenin is unrelated to early steps in insulin signaling, myotube morphology, and GLUT4 expression. Certainly, our model of in vitro-differentiated skeletal muscle cells has limitations as to how our findings on downregulation of myogenic markers underlies obesity-related insulin resistance in vivo. Future work should be aimed to relate our findings to the in vivo situation in diabetic and obese patients in this respect. In summary, we could demonstrate that adipocyte-derived insulin resistance in skeletal muscle cells impacts on various aspects of skeletal muscle cell physiology. The analysis of mechanisms involved in skeletal muscle insulin resistance and its reversibility might lead to a better understanding of this process and a possible discovery of muscular targets for the treatment of type 2 diabetes.

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