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 (Hiddenhausen, 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 anti-mouse 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 × 10⁵ 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 10⁵ cells/well and were cultured in α-modified Eagle’s/Ham’s F-12 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 and used for experiments.

Adipocyte isolation and culture. Adipose tissue samples were obtained from the mammary fat of normal or moderately overweight patients having a different secretion pattern compared with lean donors, with the release of proinflammatory factors and adipokines being increased (28). In fact, these adipocyte-derived molecules could be key contributors to the development of muscle resistance and other diseases such as endothelial dysfunction and atherosclerosis (36). In vitro, we were able to show that adipocyte-conditioned medium (CM) containing various adipokines induces insulin resistance in skeletal muscle cells (7, 9). The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Women (body mass index 24.5 ± 0.9, aged between 23 and 41 yr) undergoing surgical mammmary reduction. The procedure to obtain adipose tissue was approved by the ethics committee of Heinrich-Heine-University, Duesseldorf, 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/F transduced with SMAD3 and SMAD4 under conditions for 4 h with adducts of small or large lipid droplets. These cells were then used for generation of CM, as previously described by us (8). Briefly, after in vitro differentiation, adipocytes were incubated for 48 h in skeletal muscle cell differentiation medium. This CM was then harvested, centrifuged to remove any cell debris, and immediately frozen in aliquots for future use. CM from 350,000 adipocytes was used to stimulate one cavity in a six-well plate of skeletal muscle cells. In control experiments, skeletal muscle cell differentiation medium was incubated for 48 h without adipocytes and tested upon its effect on skeletal muscle. No difference in insulin signaling could be found using this medium compared with fresh skeletal muscle cell differentiation medium (data not shown).

**Immunoblotting.** Muscle cells were treated as indicated and lysed in a buffer containing 50 mM HEPES (pH 7.4), 1% (vol/vol) Triton X, 1 mM Na3VO4, 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 incubated with secondary HRP-coupled antibody and developed with a chemiluminescence detection system. The ability of cell lysates to cleave the specific caspase-3 substrate was quantified by fluorometry using an excitation wavelength of 390 nm and an emission wavelength of 460 nm with a microplate reader.

**Quantitative evaluation of ceramide.** Lipids from skeletal muscle cells were extracted in chloroform-methanol-water (2:1:0, 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 (38). 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 observed at 55°C for 10 min. Lipids were identified by their Rf 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**. CM-induced insulin resistance of insulin signaling in skeletal muscle cells is reversible process. CM of differentiated human adipocytes impairs insulin signaling at the level of Akt in human skeletal muscle cells (Fig. 1A). Insulin-stimulated GSK-3α/β phosphorylation is only slightly decreased by CM treatment, whereas basal phosphorylation is significantly increased, leading to an insignificant insulin effect (Fig. 1B). Withdrawal of CM for 24 or 48 h reestablishes normal insulin signaling in skeletal muscle cells, with Akt and GSK-3α phosphorylation being similar to control and GSK-3β phosphorylation being even higher than in the control situation.

**Insulin resistance is accompanied by reduced expression of 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 $\approx 500 \text{ pg} \cdot \text{ml}^{-1} \cdot 24 \text{ h}^{-1}$ IL-6, skeletal muscle cells exhibit lower secretion of this cytokine with $23 \pm 1 \text{ pg} \cdot \text{ml}^{-1} \cdot 24 \text{ h}^{-1}$ ($n = 5$). 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 ($94 \pm 12 \text{ pg} \cdot \text{ml}^{-1} \cdot 24 \text{ h}^{-1}$; $n = 5$) compared with adipocytes ($\approx 500 \text{ pg} \cdot \text{ml}^{-1} \cdot 24 \text{ h}^{-1}$).
pg·ml⁻¹·24 h⁻¹). 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].

Insulin-resistant skeletal muscle cells contain higher ceramide levels. Ceramide constitutes a well-known player in insulin resistance. Fatty acids and ceramide can induce insulin resistance in skeletal muscle cells (26, 37). Analysis of lipid extracts by thin-layer chromatography revealed a nearly threefold increase of ceramide content in insulin-resistant skeletal muscle cells compared with controls (Fig. 8).

DISCUSSION

Adipose tissue expansion and increased release of adipokines have been shown to play a crucial role in the induction of

Fig. 5. Effect of CM treatment on skeletal muscle cell secretion. Differentiated skeletal muscle cells from 2–3 donors were treated with CM for 24 h. After being washed twice with PBS, cells were given fresh differentiation medium for 24 h, followed by medium collection. Differentiation medium was then added again for 24 h and collected for the 48-h time point. IL-6 (A), IL-8 (B), and monocyte chemotactic protein-1 (MCP-1; C) secretion from the myotubes were analyzed by ELISA. Data are means ± SE (n = 3–4). *Significantly different from control.

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 stud-
ies 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 dis-
turbed 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
culpit 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 myo-
genic 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 re-
duced in skeletal muscle lysates from diabetic patients com-
pared with controls (20). We also observed a reduction in SDH
activity in CM-treated skeletal muscle cells, indicating a pos-
sible 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 produc-
tion 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
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 palmit-
tate-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 ap-
proach 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 intra-
cellular increase in skeletal muscle cell NO might contribute
to insulin resistance (41). Furthermore, diabetic patients are char-
acterized by higher blood levels of nitrates and nitrites as well

![Fig. 7. Effect of CM treatment on skeletal muscle succinate dehydrogenase (SDH) activity.](image)

![Fig. 8. Effect of adipocyte CM on ceramide content in skeletal muscle cells.](image)
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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