MCP-1 is a potential player in the negative crosstalk between adipose tissue and skeletal muscle

Henrike Sell, Daniela Dietze-Schroeder, Ulrike Kaiser and Jürgen Eckel

Institute of Clinical Biochemistry and Pathobiochemistry, German Diabetes Center, Düsseldorf, Germany

Running title: MCP-1 and muscle insulin resistance

Key words: MCP-1; Insulin resistance; skeletal muscle; adipose tissue; cellular crosstalk

Revised Version (January 2006)

Address for correspondence:

Prof. Dr. Jürgen Eckel
German Diabetes Center
Auf'm Hennekamp 65
D-40225 Düsseldorf
Germany
Tel: +49 211 3382561
Fax: +49 211 3382697
E-mail: eckel@uni-duesseldorf.de
Internet: www-public.rz.uni-duesseldorf.de/~eckel

H.S., D.D-S., U.K., J.E. have nothing to declare.
**Abbreviations**

CCR, chemokine CC motif receptor; CM, conditioned medium; CXCR, chemokine CXC motif receptor; ECL, enhanced chemiluminescence; FCS, fetal calf serum; TBS, Tris-buffered saline; MCP-1, monocyte chemotactic protein-1; MIP-1, macrophage inflammatory protein-1
Abstract

Adipose tissue is a major secretory and endocrine active organ producing a variety of bioactive proteins which may regulate energy metabolism and insulin sensitivity. In several studies, we could already show that adipocyte-secretory products induce skeletal muscle insulin resistance. However, the precise nature of these factors has remained elusive. Human adipocytes were found to secrete various cytokines including IL-6, IL-8, MIP-1α/β and MCP-1. Among these candidates, MCP-1 alone impaired insulin signaling in skeletal muscle cells at doses similar to its physiological plasma concentrations (200 pg/ml) whereas IL-6, IL-8 and MIP-1β were effective at very high concentrations only. In addition, MCP-1 significantly reduced insulin-stimulated glucose uptake in the myocytes. Expression analysis of chemokine receptors in skeletal muscle cells revealed the presence of CXCR1/2 and CCR1/2/4/5/10. The action of MCP-1 on insulin signaling in skeletal muscle cells occurs via ERK1/2 activation but does not involve activation of the NF-κB pathway. In conclusion, our data show that adipocytes secrete various adipokines that may be involved in the negative crosstalk between adipose tissue and skeletal muscle. Human skeletal muscle cells are highly sensitive towards MCP-1 which impairs insulin signaling and glucose uptake at concentrations even below that found in the circulation. Other cytokines that are released by adipocytes, however, impair insulin action only at supraphysiological concentrations. Therefore, MCP-1 may represent a molecular link in the negative crosstalk between adipose tissue and skeletal muscle assigning a completely novel important role to MCP-1 besides inflammation.
Introduction

Obesity is one of the most serious health hazards, especially in the Western World. Frequently, obesity is accompanied by metabolic disturbances such as insulin resistance, hyperglycemia, dyslipidemia, hypertension and other components of the metabolic syndrome (1, 2). Insulin resistance is a hallmark of obesity emerging early in the metabolic syndrome and is highly associated with increased visceral adipose tissue mass. The concept of adipose tissue as a major secretory and endocrine active organ producing a variety of bioactive proteins which may regulate energy metabolism and insulin sensitivity is now widely accepted (3). Increased adipose tissue mass, especially in the visceral compartment, represents one of the major risk factors for the development of type 2 diabetes (4-6). Adipocytes from obese subjects are characterized by altered metabolic and endocrine function leading to an increased secretion of adipocytokines and proinflammatory molecules including TNFα, IL-6, angiotensinogen and resistin (7, 8). It is likely that some of these secreted molecules may be factors underlying the key association of excess body fat to insulin resistance in peripheral organs such as skeletal muscle. We recently demonstrated that skeletal muscle cells co-cultured with human adipocytes exhibit an impairment of insulin signaling and GLUT4 translocation (9, 10) and defined thereby the mechanism of a negative crosstalk between adipose tissue and skeletal muscle.

MCP-1 is a chemokine and member of the small inducible cytokine family and plays a crucial role in the recruitment of monocytes and T lymphocytes into tissues (11). It is expressed by adipocytes (12) and a number of other cell types including smooth muscle and endothelial cells when exposed to inflammatory stimuli (13). MCP-1 is overexpressed in obese rodents (14, 15) and reaches significantly higher plasma levels in diabetic patients (16). Its overexpression especially in epicardial
adipose tissue is thought to increase the inflammatory burden of arteries (17). In 3T3-L1 adipocytes, MCP-1 expression is increased by TNFα, insulin, growth hormone and IL-6 (18). 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 (14). The role of MCP-1 in skeletal muscle insulin action still needs to be established.

We recently reported that the autocrine action of adiponectin ameliorates the insulin resistance-inducing capacity of adipocyte-conditioned medium concomitant with reduced secretion levels of various cytokines (10). These cytokines include IL-6, IL-8, MCP-1, MIP-1α and -1β. In the present investigation we have assessed the effect of these cytokines on insulin signaling and downstream insulin action in primary human skeletal muscle cells. The data show that MCP-1 is a prominent inducer of insulin resistance in human skeletal muscle, which assigns a completely novel important role to MCP-1 besides its role in inflammation and infiltration of monocytes to adipose tissue.
Material and Methods

**Material.** Bovine serum albumin (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 GSK3α/β (Ser21/9), anti-phospho-Akt (Ser473), anti-phospho-NF-κB (P65), anti-phospho-ERK1/2 (Ser536) and anti-Akt were supplied by Cell Signaling Technology (Frankfurt, Germany), anti-actin from Santa Cruz Biotechnology (Heidelberg, Germany) and anti-tubulin from Callbiochem (Merck Biosciences, Schwalbach, Germany). Anti-GSK3α/β was from Stressgene (Victoria, Canada). Antibodies for CCR4 and CCR10 came from Imgenex (San Diego, CA, USA) and the one for CCR2 from Alexis (San Diego, CA, USA). HRP-conjugated goat-anti-rabbit and –anti-mouse IgG antibodies were from Promega (Mannheim, Germany). Cytokine protein arrays (RayBio® Custom Array) were purchased from RayBiotech (Norcross, GA, USA). Collagenase CLS type 1 was obtained from Worthington (Freehold, N.J., USA) and culture media were obtained from Gibco (Berlin, Germany). The cytokines IL-6, IL-8, MCP-1 and MIP-1β were purchased from Hölzel Diagnostics (Cologne, Germany) and TNFα from Sigma. Primary human skeletal muscle cells and supplement pack for growth medium were obtained from PromoCell (Heidelberg, Germany). 2-Deoxy-D-[1-14C] glucose was purchased from Amersham Biosciences Europe (Freiburg, Germany). All other chemicals were of the highest analytical grade commercially available and were purchased from Sigma.

**Adipocyte isolation and culture.** Adipose tissue samples were obtained from the mammary fat of normal or moderate overweight women (BMI 24.9 ± 3.5, aged between 21 and 52) undergoing surgical mammary reduction. The procedure to
obtain adipose tissue was approved by the ethical 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 about 10 mg in
weight. Preadipocytes were isolated by collagenase digestion as previously
described by us (19). Isolated cell pellets were resuspended in Dulbecco’s modified
Eagles/Hams F12 medium supplemented with 10% FCS, seeded on membrane
inserts (3.5 x 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
(DMEM/F12, 33 µM biotin, 17 µM d-pantothenic acid, 66 nM insulin, 1 nM triiodo-L-
thyronin, 100 nM cortisol, 10 µg/ml apo-transferrin, 50 µg/µl gentamycin, 15 mM
HEPES, 14 mM 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 generation of
adipocyte-conditioned medium, as recently described by us (20). Briefly, after in vitro
differentiation, adipocytes were incubated for 24 h in skeletal muscle cell
differentiation medium containing 1 pM insulin. Conditioned medium was then
generated by culturing adipocytes for 48 h in the same medium followed by collection
of the medium.

The purity of adipocytes in the culture was analyzed by morphological means (Fig.
1). Cells that do not differentiate most likely are preadipocytes that may contribute to
the results. Isolated macrophages do not adhere to the culture dishes and are
washed away. Adhesion of stromal cells is prevented by the elimination of
erthrocytes by the appropriate lysis buffer during preadipocyte isolation (21).
Furthermore, the use of a two-step filtration process before seeding of cells
substantially eliminates endothelial cells (22).
**Measurement of cytokine protein levels in adipocyte-conditioned medium**

Adipocyte-conditioned medium from was hybridized with the array membranes according to the protocol supplied by the manufacturer. Briefly, membranes were blocked by the furnished blocking solution and then incubated with 1.2 ml of conditioned medium over night 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 RT. After washing, HRP-conjugated streptavidin was added to the membranes for 1 h at RT. The signal was developed with detection buffers and directly detected using a LUMI Imager system (Roche Diagnostics, Mannheim, Germany). The signal intensity was normalized to internal positive signals on the membrane.

**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 four healthy Caucasian donors (male, 5 and 9 y (M5, M9); female, 10 and 48 y (F10, F48)) were supplied as proliferating myoblasts (5 x 10^5 cells) and cultured as described in our earlier study (9). For an individual experiment, myoblasts were seeded in six-well culture dishes (9.6 cm²/well) at a density of 10^5 cells per well and were cultured in α-modified Eagles/Hams 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 Eagles medium for 4 days.
Primer and RT-PCR

Total RNA was extracted from differentiated human skeletal muscle cells using the RNeasy kit from Qiagen (Hilden, Germany). cDNA was generated with an Omniscript RT kit from Qiagen and PCR performed with PuRe Taq Read-To-Go PCR beads from Amersham Biosciences Europe using primers shown in Table 1 (12).

Immunoblotting. Muscle cells were treated as indicated and lysed in a buffer containing 50 mM Tris/HCl (pH 7.4), 1% (vol/vol) NP-40, 0.25% (vol/vol) sodium-deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM Na3VO4 and Complete protease inhibitor cocktail from Roche Diagnostics. After incubation for 2 h at 4°C the suspension was centrifuged at 10,000 x g for 10 min. Thereafter 8 µg of lysates were separated by SDS-PAGE using 10% horizontal gels and transferred to polyvinylidene fluoride (PVDF) filters in a semidry blotting apparatus (23). For detection filters were blocked with TBS containing 0.1% Tween-20 and 5% non-fat dry milk and subsequently incubated over night with a 1:1000 dilution of appropriate antibodies. After extensive washing, filters were incubated with secondary HRP-coupled antibody and processed for enhanced chemiluminescene (ECL) detection using SuperSignal Substrate (Pierce, Rockford, Ill., USA). Signals were visualized and evaluated on a LUMI Imager work station using image analysis software (Boehringer Mannheim, Mannheim, Germany).

Assay of glucose uptake

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 (22) and were used for analysis after an additional 48 h
incubation. Uptake of 2-desoxy-glucose was measured for 30 min after an acute 30 min insulin stimulus (10^{-7} M insulin) as described before (24).

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

Adipocyte-conditioned medium impairs insulin signaling and contains various cytokines.

Conditioned medium (CM) of differentiated human adipocytes impairs insulin signaling at the level of Akt and GSK3α phosphorylation while GSK3β phosphorylation is modestly but significantly decreased (Fig. 2A and 2B). Furthermore, CM impairs insulin action on GLUT4 translocation in primary human skeletal muscle cells, as described in several studies from our laboratory (9,10,20). We concluded that adipocyte-derived factors impair insulin signaling and downstream insulin action in skeletal muscle cells. Several adipocyte-derived factors could be described to be secreted from adipocytes and could be found in CM of differentiated human adipocytes such as IL-6, IL-8, MCP-1, MIP-1α and MIP-1β (Fig. 2C). Very recently, we demonstrated the autocrine control of the release of these adipocyte secretory products by adiponectin (10).

Human skeletal muscle cells express different CXCR and CCR.

In order to test if differentiated human skeletal muscle cells express chemokine receptors, we analyzed chemokine CXC motif receptor (CXCR) and chemokine CC motif receptor (CCR) expression using RT-PCR. Our results show that human skeletal myotubes express both CXCR1 and CXCR2, important receptors for IL-8 (Fig. 3A). Furthermore, skeletal muscle cells express CCR1, CCR2, CCR4, CCR5 and CCR10 mRNA. Using Western blot analysis, the protein expression of CCR2, CCR4 and CCR10 could also be demonstrated (Fig. 3B). These receptors mediate the action of MCP-1, MIP-1α and MIP-1β, CCR2 representing the main receptor for MCP-1. Comparing the expression of these receptors between skeletal muscle cells and adipocytes, we observed that the abundance of CCR2 is significantly higher in...
skeletal muscle cells but that CCR4 and CCR10 are expressed at similar levels Fig. 3B). It is noteworthy that CCR4 protein expression is similar in skeletal muscle cells and adipocytes while CCR4 mRNA is visibly more abundant in adipocytes. This might be due to different processing of CCR4 mRNA in these two cell types or different half-live of the receptor.

*Human skeletal muscle cells are highly sensitive towards MCP-1.*

Fig. 4 shows that concentrations as low as 0.1-times the physiological plasma level (20 pg/ml) significantly reduce insulin-stimulated Akt phosphorylation in skeletal muscle cells. At a physiological dose (200 pg/ml), MCP-1 is able to decrease insulin signaling to the same extent as adipocyte-conditioned medium, however, not significantly increasing its effect upon application of higher doses. Downstream from Akt, insulin-stimulated GSK3α and β phosphorylation is also significantly impaired by MCP-1 (Fig. 4C). However, MCP-1 does not affect the expression of Akt and GSK3α/β (Fig. 4D)

To test the effect of MCP-1 further downstream of Akt, we also measured insulin-stimulated glucose uptake. Physiological concentrations of MCP-1 as well as 10-times over physiological concentration significantly impaired insulin-stimulated glucose uptake reducing it to about 65% of the control situation (Fig. 5). This impairment of glucose uptake is in good agreement with data obtained in adipocytes where 5-times over physiological concentration (1 ng/ml) of MCP-1 reduced insulin-stimulated glucose uptake by 25% after long term treatment (14).

In skeletal muscle cells, MCP-1 signals via ERK1/2 (Fig. 6A). ERK has already been described as a key mediator of MCP-1 signaling in human endothelial cells and macrophages (25, 26). In human smooth muscle cells, MCP-1 induced migration is also mediated via ERK activation (27). Interestingly, MCP-1 does not activate the NF-
κB pathway (Fig. 6A). These data contrast with our findings obtained with adipocyte-conditioned medium that was shown to contain MCP-1 (9) clearly indicating that adipocyte-derived factors other than MCP-1 must be responsible for NF-κB activation by adipocyte-conditioned medium. Significant activation of ERK1/2 with MCP-1 could be obtained with doses as low as 0.1x physiological serum concentration (20 pg/ml) in short term (10’) and long term (overnight) exposure (Fig. 6B). This again shows that skeletal muscle cells are highly sensitive towards MCP-1. ERK expression remains unaffected by MCP-1 (Fig. 6C).

Inhibition of ERK by the specific ERK inhibitor PD 98059 prevents the impairment of insulin signaling by MCP-1.

To analyze the role of ERK in the impairment of insulin signaling by MCP-1, we preincubated the skeletal muscle cells with the specific ERK inhibitor PD 98059 and analyzed the effect of MCP-1 in skeletal muscle cells with inhibited ERK1/2. In short term experiments (Fig. 7A), we could clearly show that a preincubation with the ERK inhibitor completely blocks ERK activation by TNFα and MCP-1. In the long term, the ERK inhibitor alone has no effect on insulin signaling but can prevent the impairment of insulin signaling by MCP-1 (Fig. 7B). We conclude that ERK activation is necessary for the specific effect of MCP-1 on insulin signaling in skeletal muscle cells. A similar involvement of ERK in the impairment of insulin signaling in smooth muscle cells by angiotensin II was recently published (28) making it possible that ERK activation could play a role in signaling pathways of different cytokines which are thought to induce insulin resistance.

IL-6, MIP-1β and IL-8 decrease insulin-stimulated Akt phosphorylation only at very high, supraphysiological doses.
When testing other cytokines present in adipocyte-conditioned medium, we were not able to find candidates to induce insulin resistance in skeletal muscle cells with a potency similar to MCP-1. IL-6 produced a slight reduction of insulin-stimulated Akt phosphorylation when used at concentrations as high as 50,000-times over circulating plasma levels (250 ng/ml) (Fig. 8A). When IL-6 is employed at lower concentrations, it is even leading to a slight but not significant increase in Akt phosphorylation. Similar observations were made in short term experiments (29) where IL-6 is acting as a metabolic stimulator similar to insulin rather than an insulin resistance inducing agent in skeletal muscle. MIP-1β produced a dose-dependent decrease in insulin signaling, but also at concentrations much higher than its circulating level of 250 pg/ml. Interestingly, MIP-1β is decreasing insulin-stimulated Akt phosphorylation by about 60% when used at 125 ng/ml, a concentration 500-times over circulating levels (Fig. 8B). Possibly, MIP-1β could play a role in restricted areas where MIP-1β concentrations could reach such high concentrations. As for IL-8, this chemokine has also to be used at supraphysiological concentrations to significantly impair insulin signaling in skeletal muscle cells making it unlikely to be a major candidate in the negative crosstalk between adipose tissue and skeletal muscle (Fig. 8C).
Discussion

Adipocyte-derived factors such as TNFα and IL-6 are significantly increased in obesity and are good predictors for the development of type 2 diabetes (30, 31). Obesity thereby contributes to a pro-inflammatory milieu and it is now recognized that adipose tissue functions as an endocrine organ secreting a variety of pro-inflammatory factors. Adipocytokines are in fact emerging regulators of insulin sensitivity. Prominent members of the adipocytokine family such as TNFα, PAI-1, IL-6 and resistin have been identified to contribute in vitro to insulin resistance. TNFα, for example, is upregulated in obesity in animal models (32, 33) but its role in humans is controversial (34). In rodents, TNFα increases serine phosphorylation of IRS1 and attenuates insulin signaling including PI3 kinase (35) and downregulates the expression of IRS and GLUT4 (36). However, in humans, adipocytokines which could clearly induce insulin resistance are not known.

Adiponectin is the only adipocytokine known to be downregulated in obesity. Very recently, we reported on adiponectin acting as an autocrine regulator of adipokine secretion of the human fat cell (10). By decreasing cytokine release by the adipocyte, adiponectin prevents the impairment of insulin signaling in a co-culture model of human adipocytes and skeletal muscle cells. Some of the adiponectin-regulated cytokines such as IL-6, IL-8 and MCP-1 are already known to be related to obesity and diabetes. Others such as MIP-1α and MIP-1β are related to inflammation and tissue remodeling. IL-6 and IL-8 are well-known to be induced in the obese state in humans and rodents (14, 15, 37, 38). Elevated plasma concentrations of these adipokines in obese and insulin resistant patients may contribute to the insulin-resistant state observed in obesity. IL-6 is expressed both by adipose tissue and skeletal muscle (39) but its role in skeletal muscle remains controversial (29, 40). It is
shown in this study that only extremely high concentrations of IL-6 and IL-8 produced a slight impairment of insulin signaling in human skeletal muscle cells, making it unlikely that IL-6 or IL-8 alone are sufficient to induce muscle insulin resistance.

MCP-1 has already been shown to be clearly associated to the obese state in humans and rodents (14, 15, 39). MCP-1 is secreted by various cells including human adipocytes but also cells of the vasculature when stimulated with pro-inflammatory factors (12, 13). MCP-1 is a well characterized chemokine when it comes to its role in the recruitment of monocytes and memory T lymphocytes into tissue (11). However, many chemokines have been shown to possess physiological activities going far beyond the recruitment of immune cells. This is also the case for MCP-1 for which insulin-resistance inducing capacities have been postulated in adipocytes (14). Furthermore, MCP-1 was shown to have angiogenic effects in endothelial cells and may therefore play a role during adipose tissue expansion and remodeling in obesity (41). The effect of MCP-1 in accelerating wound healing involving vessel formation also points out this angiogenic action of MCP-1 (42). The induction of insulin resistance in skeletal muscle cells as shown in this study adds a new aspect to the role of MCP-1. Taken together with former studies, it can undoubtedly be said that MCP-1 can alter the function of tissues involved in the insulin resistant state. Skeletal muscle (43) and adipose tissue both produce MCP-1 and may in the inflamed and obese state increase the release of MCP-1 inducing then insulin resistance in both tissues. This adds a complete new feature to the negative crosstalk between adipose tissue and skeletal muscle pointing towards a close relationship between obesity, inflammation and diabetes, as already postulated in many publications (5, 6, 8, 44, 45).

MCP-1 has in vivo relevance related to diabetes and obesity, as already shown in different studies. Its expression is increased in obese mice, especially in
white adipose tissue (14). As for skeletal muscle, MCP-1 is increased in the injured state and can be induced by interferon-γ (46). In this respect, it needs to be assessed which of both MCP-1 producing tissues is contributing more to MCP-1 plasma levels that are increased in diabetic patients (16). It is likely that skeletal muscle also contributes to increased MCP-1 plasma levels since it has been shown to be induced in muscle of patients with inflammatory myopathies (43).

Lower MCP-1 levels due to a MCP-1 G-2518 gene variant (47) were shown to protect from the development of diabetes. As for the treatment of diabetes, it has been demonstrated that rosiglitazone (48) and exercise (49) both reduce plasma levels of MCP-1 significantly making it possible that MCP-1 reduction is an important point in improving insulin action in diabetic patients. In hypertensive and hypercholesterolemic patients, MCP-1 could be significantly reduced by treatment with a combination of simvastatin and losartan improving endothelial function (50). In our study, we could show that very low concentrations of MCP-1 are effective in inducing skeletal muscle insulin resistance. This would suggest that already small overweight, which is potentially associated with a slight increase in MCP-1 may contribute to insulin resistance in skeletal muscle and possibly underlies early steps in the development of the metabolic syndrome. This question needs to be addressed in future clinical studies in order to confirm the role of MCP-1 in the development of insulin resistance.

MCP-1 is an interesting candidate that may play a role in the negative crosstalk between adipose tissue and skeletal muscle but it is certainly not the molecule solely causing the induction of insulin resistance in skeletal muscle cells by adipocyte-conditioned medium. Since MCP-1 is highly concentrated in adipocyte-conditioned medium (31.2 ± 6.7 ng/ml) (10) and is downregulated by adiponectin to half this level that is still higher than the concentrations used in this study to induce
insulin resistance in skeletal muscle, we hypothesize that adipocyte-conditioned medium must contain unknown adipokines that positively influence insulin action and are able to prevent induction of insulin resistance by MCP-1. These adipokines need to be identified in the future.

In summary, our data show that several adipokines might be involved in the negative crosstalk between skeletal muscle and adipose tissue. MCP-1 is a candidate of special interest since it is highly effective in inducing insulin resistance in skeletal muscle cells. We therefore suggest that this cytokine which is regulated by adiponectin and which is clearly associated with the obese state and diabetes, may represent a molecular link between obesity and skeletal muscle insulin resistance. The possible role of MCP-1 as a connecting link between obesity and diabetes needs however to be established by further studies, since cell types other than adipocytes secrete this cytokine and may contribute to its effect on skeletal muscle cells. The other adipocytokines tested in this study 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 to fully understand their meaning for human physiology.
Acknowledgements

This work was supported by the Ministerium für Wissenschaft und Forschung des Landes Nordrhein-Westfalen, the Bundesministerium für Gesundheit, European Union COST Action B17, and the Deutsche Forschungsgemeinschaft (EC 64/11-1). We wish to thank Prof. R. Olbrisch and his team, Dept. of Plastic Surgery, Florence-Nightingale-Hospital Düsseldorf, for support in obtaining adipose tissue samples. The secretarial assistance of Birgit Hurow is gratefully acknowledged.
References


29. Weigert C, Hennige AM, Brodbeck K, Haring HU, Schleicher ED 2005 Interleukin-6 (IL-6) acts as insulin sensitizer on glycogen synthesis in human skeletal muscle cells by phosphorylation of Ser-473 of Akt. Am J Physiol Endocrinol Metab


38. Rotter V, Nagaev I, Smith U 2003 Interleukin-6 (IL-6) induces insulin resistance in 3T3-L1 adipocytes and is, like IL-8 and tumor necrosis factor-alpha, overexpressed in human fat cells from insulin-resistant subjects. J Biol Chem 278:45777-84


40. Carey AL, Febbraio MA 2004 Interleukin-6 and insulin sensitivity: friend or foe? Diabetologia 47:1135-42


Legends to Figures

Fig. 1. Morphological analysis of primary adipocyte cultures. A: Light microscopy of preadipocytes one day after seeding. B: Micrograph of fully differentiated human adipocytes demonstrating that the majority of preadipocytes differentiates into adipocytes. A minor contribution of undifferentiated preadipocytes and small amounts of other possible contaminating cells cannot be ruled out completely.

Fig. 2. Effect of adipocyte-conditioned medium (CM) on insulin-signaling in skeletal muscle cells and secretion pattern of differentiated human adipocytes.

A and B: Akt and GSK3 phosphorylation in the myocytes were analyzed after acute stimulation with insulin (100 nM, 10 min). Total cell lysates were resolved by SDS-PAGE and immunoblotted with phosphospecific Akt/GSK3 and Akt/GSK3 antibodies. One exemplary western blot is shown to illustrate the impairment of insulin signaling by adipocyte conditioned-medium. Data are mean values ± SEM of 4 independent experiments. All data were normalized to the level of Actin expression and are expressed relative to the insulin-stimulated control value. *significantly different from insulin-stimulated control. C: Cytokine array membranes (each detecting 20 different cytokines) were incubated with adipocyte-conditioned medium. One representative array is presented. 1: adiponectin; 2: intracellular adhesion molecule 1; 3: IL-6; 4: IL-8; 5: MCP-1; 6: macrophage-derived chemokine; 7: MIP-1α; 8: MIP-1β; 9: osteoprotegerin; 10: TIMP-1; and 11: TIMP-2. Pos, positive control for normalization.

Fig. 3. Expression of chemokine receptors in human skeletal muscle cells. A:
Total RNA was extracted from differentiated human skeletal muscle cells and adipocytes. RT-PCR for CXCR1, CXCR2, CCR1, CCR2, CCR4, CCR5, CCR10 and
18S was performed as outlined in Methods with at least 3 repeated experiments. Representative agarose gels of PCR products are shown for two skeletal muscle cell donors (M9 and F48) as well as for a fat cell control (FC). **B:** Skeletal muscle cells from two different donors (M9 and F48) and two fat cell donors (FC1 and FC2) were differentiated and total cell lysates resolved by SDS page. Western blots for CCR2, CCR4 and CCR10 as well as normalization for actin are shown. Data are mean values ± SEM of 4 independent experiments. All data were normalized to the level of Actin expression and are expressed relative to the expression level of M9. *significantly different from skeletal muscle cells (SkMcs).

**Fig. 4. Effect of MCP-1 on insulin signaling in human skeletal muscle cells. A:** Myocytes from four different donors were cultured with increasing concentrations of MCP-1 (1x physiological level: 200 pg/ml MCP-1) for 18h. After acute stimulation with insulin, total cell lysates were resolved by SDS-PAGE and immunoblotted with phosphospecific Akt antibody and Actin antibody. Data are mean values ± SEM of 5-6 independent experiments. All data were normalized to the level of Actin expression and are expressed relative to the insulin-stimulated control value. *significantly different from insulin-stimulated control. B:** Skeletal muscle cells from four different donors were cultured with increasing concentrations of MCP-1 (0.1 x physiological level: 20 pg/ml MCP-1). Data from 5-6 independent experiments are presented as a dose-response-curve of insulin-stimulated Akt phosphorylation. *significantly different from untreated control. C:** Myocytes from two different donors were cultured as outlined in A. After acute stimulation with insulin, total cell lysates were resolved by SDS-PAGE and immunoblotted with phosphospecific GSK3 antibody and GSK3 antibody. Data are mean values ± SEM of 4 independent experiments. All data were normalized to the level of GSK3 expression and are expressed relative to the insulin-
stimulated control value. *significantly different from insulin-stimulated control. D: Akt and GSK3 expression after overnight stimulation with MCP-1 relative to tubulin as a stable marker was analyzed in 10 independent experiments.

**Fig. 5. Effect of MCP-1 on glucose uptake in skeletal muscle cells.** Skeletal muscle cells were first adenotransfected with GLUT4myc and then cultured for 18 h in absence or presence of a physiological level of MCP-1 (200 pg/ml) or a 10-times over physiological concentration. Glucose uptake was assessed after acute stimulation with insulin, as outlined in the Methods section. Mean ± SEM of 4 independent experiments. *significantly different from insulin-stimulated control.

**Fig. 6. MCP-1 signaling in skeletal muscle cells.** A: Skeletal muscle cells from three different donors were cultured with 10-times over physiological concentration of MCP-1 (200 pg/ml) for the indicated time periods. As a control for ERK and NF-κB activation, 2.5 nM TNFα was used. Total cell lysates were resolved by SDS-Page and immunoblotted with phosphospecific antibodies for the P65 subunit of NF-κB (p-P65) and ERK1/2 and tubulin for loading control. Representative blots are shown. Mean ± SEM of 4 independent experiments. *significantly different from control. B: Skeletal muscle cells from three different donors were cultured with increasing concentrations of MCP-1 (0.01x 2 pg/ml) for 10 min and overnight. As a control for ERK activation, a 10 min stimulus with 2.5 nM TNFα was used. Total cell lysates were resolved by SDS-Page and immunoblotted with phosphospecific antibodies for ERK1/2 and tubulin for loading control. Representative blots are shown. Mean ± SEM of 3 independent experiments. *significantly different from control. C: ERK expression after overnight stimulation with MCP-1 relative to tubulin as a stable marker was analyzed in 4 independent experiments.
Fig. 7. ERK inhibition prevents impairment of insulin signaling by MCP-1. A: Skeletal muscle cells from two different donors were precultured with or without 50 µM of the specific ERK inhibitor PD 98059 for 15 min before starting the treatment with MCP-1 or TNFα. Cells were then treated either with a 10-times over physiological concentration of MCP-1 (2 ng/ml) for 30 min or 2.5 nM TNFα for 10 min. Total cell lysates were resolved by SDS-Page and immunoblotted with phosphospecific antibodies for the ERK1/2 and tubulin for loading control. Representative blots are shown. Mean ± SEM of 4 independent experiments. *significantly different from control. B: After pretreatment for 15 min with PD 98059 (50 µM) skeletal muscle cells from two different donors were cultured with a 10-times over physiological concentration of MCP-1 (2 ng/ml) overnight. Total cell lysates were resolved by SDS-Page and immunoblotted with phosphospecific antibodies for Akt and tubulin for loading control. Representative blots are shown. Mean ± SEM of 4 independent experiments. *significantly different from insulin-stimulated control.

Fig. 8. Effect of IL-6, IL-8 and MIP-1β on insulin signaling in human skeletal muscle cells. Skeletal muscle cells from three different donors were cultured with increasing concentrations of A IL-6 (500x over physiological level: 2.5 ng/ml IL-6), B MIP-1β (50x over physiological level: 12.5 ng/ml MIP-1β) and C IL-8 (10³x over physiological level: 5 ng/ml IL-8). After acute stimulation with insulin, total cell lysates were resolved by SDS-PAGE and immunoblotted with phosphospecific Akt antibody and actin antibody. Data are mean values ± SEM of 3-4 independent experiments. All data were normalized to the level of Actin expression and are expressed relative to the insulin-stimulated control value. *significantly different from corresponding control.
<table>
<thead>
<tr>
<th>Target</th>
<th>Forward primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCR1</td>
<td>CCATTGCTGAAACTGAAGAGG</td>
<td>TTGTTTGGATGGTAAGCCTGG</td>
</tr>
<tr>
<td>CXCR2</td>
<td>CGAAGGACCGTCTACTCATC</td>
<td>AGTGTGCCCTGAAGAAGAGC</td>
</tr>
<tr>
<td>CCR1</td>
<td>ACGAAAGCCTACGAGAGTG</td>
<td>GGTGAACAGGAAGTCTTTGG</td>
</tr>
<tr>
<td>CCR2</td>
<td>GATTACGGTGCTCCTGTC</td>
<td>GCCACAGACATAAACAGAATC</td>
</tr>
<tr>
<td>CCR4</td>
<td>AGCACTTGTTATACTGAGCG</td>
<td>CCACCACGGCAAGATCATC</td>
</tr>
<tr>
<td>CCR5</td>
<td>GACAAACTCTCCCTTCACTC</td>
<td>ACAAGTCTCTCGCTGGTTTC</td>
</tr>
<tr>
<td>CCR10</td>
<td>AGAGCCTGCTCCTTGTAC</td>
<td>AGCCTCAACAAAGACACAAAC</td>
</tr>
<tr>
<td>18S</td>
<td>CGATGCTCTTAGCTGAGTGT</td>
<td>GGTCAGAAGATTTCCACCTCT</td>
</tr>
</tbody>
</table>
Figure 1

(A) preadipocytes

(B) in vitro differentiated adipocytes

20x 40x
Figure 2
Figure 3
Figure 4
Figure 5

2-Deoxyglucose uptake (% of basal control)

- Basal
- Insulin

Control 1x MCP-1 10x MCP-1
Figure 6
Figure 7
Akt serine phosphorylation (arbitrary units)

Control 50x IL-6 5-10^3x IL-6 5-10^4x IL-6

*Control 500x MIP1 500x MIP1

A

Akt serine phosphorylation (arbitrary units)

Control 50x MIP1β 500x MIP1β

*Basal Insulin

B

Akt serine phosphorylation (arbitrary units)

Control 10^2x IL-8 10^4x IL-8

*Control

C

Figure 8