

## CHEMERIN IS A NOVEL ADIPOCYTE-DERIVED FACTOR INDUCING INSULIN RESISTANCE IN PRIMARY HUMAN SKELETAL MUSCLE CELLS

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**Running title:** Chemerin and muscle insulin resistance

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Submitted 24 February 2009 and accepted 10 August 2009.

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*Objective:* Chemerin is an adipokine that affects adipogenesis and glucose homeostasis in adipocytes and increases with BMI in humans. This study aimed at investigating the regulation of chemerin release and its effects on glucose metabolism in skeletal muscle cells.

*Research Design and Methods:* Human skeletal muscle cells were treated with chemerin to study insulin signaling, glucose uptake and activation of stress kinases. The release of chemerin was analyzed from in vitro differentiated human adipocytes and adipose tissue explants from 27 lean and 26 obese patients.

*Results:* Human adipocytes express chemerin and CMKLR1 differentiation-dependently and secrete chemerin (15 ng/ml from  $10^6$  cells). This process is slightly but significantly increased by TNF $\alpha$  and markedly inhibited by over 80 % by PPAR $\gamma$  activation. Adipose tissue explants from obese patients are characterized by significantly higher chemerin secretion as compared to lean controls (21 ng and 8 ng from  $10^7$  cells, respectively). Chemerin release is correlated with BMI, waist-hip-ratio and adipocyte volume. Furthermore, higher chemerin release is associated with insulin resistance at the level of lipogenesis and insulin-induced antilipolysis in adipocytes. Chemerin induces insulin resistance in human skeletal muscle cells at the level of IRS1, Akt and GSK3 phosphorylation and glucose uptake. Furthermore, chemerin activates p38 MAPK, NF- $\kappa$ B and ERK1/2. Inhibition of ERK prevents chemerin-induced insulin resistance pointing to participation of this pathway in chemerin action.

*Conclusions:* Adipocyte-derived secretion of chemerin may be involved in the negative crosstalk between adipose tissue and skeletal muscle contributing to the negative relationship between obesity and insulin sensitivity.

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) and increased adipose tissue mass, especially in the visceral compartment, is now described as 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 proinflammatory adipokines such as tumor necrosis factor (TNF) $\alpha$ , interleukin (IL)-6, angiotensinogen and resistin (7; 8). It is likely that some of these secreted molecules may be factors underlying the association of increased body fat to insulin resistance in peripheral organs such as skeletal muscle. We previously demonstrated that skeletal muscle cells treated with conditioned medium from adipocytes or the adipokine monocyte-chemotactic protein (MCP)-1 are characterized by an impairment of insulin signaling and glucose uptake (9; 10) and could thereby define the mechanism of a negative crosstalk between adipose tissue and skeletal muscle.

Recently, the rapidly growing adipokine-family was expanded by chemerin, a secreted chemoattractant protein. Initially discovered in body fluids associated with inflammatory processes (11), chemerin and its receptor, chemokine-like receptor 1 (CMKLR1) (or ChemR23) are also highly

expressed in adipose tissue (12; 13). In adipocytes, chemerin and CMKLR1 are necessary for adipogenesis (13). *In vivo* data revealed that chemerin is elevated in adipose tissue of diabetic *P. obesus* compared to controls (12). However, no difference in chemerin levels between diabetic and control patients could be observed despite a correlation of chemerin levels with BMI, blood triglycerides and blood pressure (12). As skeletal muscle is the major postprandial glucose uptaking organ, the present study was meant to describe effects of the novel adipokine chemerin on skeletal muscle insulin sensitivity in the context of the negative crosstalk between adipose tissue and skeletal muscle.

## RESEARCH DESIGN 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 GE Healthcare (München, Germany) and by Sigma (München, Germany). Polyclonal antibodies anti-phospho GSK3 $\alpha/\beta$  (Ser21/9), anti-phospho-Akt (Ser473), anti-phospho-NF- $\kappa$ B (p65) (Ser536), anti-phospho-ERK1/2 (Thr202/Tyr204) and anti-phospho p38 MAPK (Thr180/Tyr182) were supplied by Cell Signaling Technology (Frankfurt, Germany) and anti-tubulin from Calbiochem (Merck Biosciences, Schwalbach, Germany). Chemerin, CMKLR1, actin and adiponectin antibodies were purchased from Abcam (Cambridge, UK) and an antibody against MHC from Upstate (San Diego, CA). HRP-conjugated goat-anti-rabbit and -anti-mouse IgG antibodies were from Promega (Mannheim, Germany). Collagenase NB4 standard grade was obtained from Serva (Heidelberg, Germany) and culture media from Gibco (Berlin, Germany). Recombinant human chemerin (E-coli derived, molecular mass 16 kDa) was supplied by R&D Systems

(Wiesbaden-Nordenstadt, Germany). TNF $\alpha$  was purchased from Sigma and adiponectin from Biovendor (Heidelberg, Germany). Primary human skeletal muscle cells and supplement pack for growth medium were delivered by PromoCell (Heidelberg, Germany). 2-Deoxy-D-[1-14C] glucose was purchased from GE Healthcare. The ELISA kit for phospho-IRS-1(Ser307) (coated antibody against mouse IRS-1 and detection antibody against rabbit phospho-IRS-1 (Ser307)) was purchased from Cell Signaling Technology. 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. Primary human skeletal muscle cells of three different healthy Caucasian donors were supplied as proliferating myoblasts ( $5 \times 10^5$  cells) and cultured as described in our earlier study (14). For an individual experiment, myoblasts were seeded in six-well culture dishes at a density of  $10^5$  cells per well and were cultured in  $\alpha$ -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  $\alpha$ -modified Eagles medium for 4 days before using them in experiments. The expression of the myogenin markers, myogenin, myoD and MHC reached a plateau at 4 days until at least 6 days of culture. Experiments were repeated with the same donor but always with a different cryo-conserved aliquot of cells. The cells were always used in the 4<sup>th</sup> passage.

**Adipocyte isolation and culture.** Adipose tissue samples were obtained from the mammary fat of normal or moderate overweight women 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 (14). Isolated cell pellets were resuspended in Dulbecco's modified Eagles/Hams F12 medium supplemented with 10 % FCS and kept in culture for 16 h. After washing, culture was continued in an adipocyte differentiation medium (DMEM/F12, 33  $\mu$ M biotin, 17  $\mu$ M d-pantothenic acid, 66 nM insulin, 1 nM triiodo-L-thyronin, 100 nM cortisol, 10  $\mu$ g/ml apo-transferrin, 50  $\mu$ g/ $\mu$ l gentamycin, 15 mM HEPES, 14 mM NaHCO<sub>3</sub>, pH 7.4) supplemented with 5 $\mu$ M troglitazone for 3 initial days. After 15 days, 70-90 % 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 experiments or generation of adipocyte-conditioned medium, as recently described by us (15). Briefly, after *in vitro* differentiation, adipocytes were washed with skeletal muscle cell differentiation medium and conditioned medium (CM) was then generated by culturing adipocytes for 48 h in the same medium followed by collection of the medium.

We used subcutaneous adipose tissue from both abdominal and mammary areas (see also Clinical studies). There was no evidence of apparent differences in chemerin effects using preadipocytes from the two sources.

**Clinical studies of chemerin release from adipose tissue.** Twenty-six obese (body mass index, BMI,  $\geq 30$  kg/m<sup>2</sup>) otherwise healthy and twenty-seven lean (BMI < 25 kg/m<sup>2</sup>) healthy women were investigated in

the morning after an overnight fast. They were investigated about 1 week prior to expected start of the menstruation period. Their age (mean  $\pm$  SD years) was  $36\pm 7$  and  $35\pm 8$ , respectively. A venous blood sample was obtained for the analysis of glucose and insulin to be used as estimation of insulin sensitivity *in vivo*, HOMA index as described (16). Thereafter an abdominal subcutaneous adipose tissue biopsy was obtained by needle aspiration as described (17). One part of the tissue was used for measurements of chemerin release as described (18). In brief, about 300 mg of tissue was incubated at  $37^{\circ}\text{C}$  for 2 hours in an albumin buffer (pH 7.4) and chemerin in the medium was determined and related to the number of fat cells incubated or for the adipose tissue wet weight. Methodological experiments revealed that chemerin release was linear with time for at least 3 h suggesting no important cell damage. Another part of the tissue was subjected to collagenase treatment and mean adipocyte volume and weight were determined as described (19). Isolated fat cells were incubated for 2 hours at  $37^{\circ}\text{C}$  in an albumin concentration buffer without or with increasing concentrations of insulin. Lipolysis or lipogenesis (incorporation of  $^{14}\text{C}$  glucose into lipids) was determined as described (19). From the insulin concentration response curves the following parameters were determined as described (19): (i) half maximum effective concentration which was transformed to a negative logarithm value (pD2), (ii) lipolysis or lipogenesis at maximum effective insulin concentration expressed either per number of fat cells or per lipid weight of the incubated cells and (iii) basal lipolysis or lipogenesis. The study was explained in detail to each woman and a written informed consent was obtained. The investigation was approved by the ethics committee at Karolinska Institutet.

***Measurement of chemerin in CM and supernatant from adipose tissue explants.***

Chemerin ELISA kits were purchased from Biovendor (Heidelberg, Germany) and used according to the manufacturer. The intra- and inter-assay coefficients of variation for the chemerin ELISA were 6.1 % and 7.6 %, respectively.

***Immunoblotting.*** Muscle cells and adipocytes were treated as indicated and lysed in a buffer containing 50 mM HEPES (pH 7.4), 1 % (v/v) Triton-X, PhosStop and Complete protease inhibitor cocktail from Roche. After incubation for 2 h at  $4^{\circ}\text{C}$  the suspension was centrifuged at  $10,000 \times g$  for 15 min. Thereafter 5  $\mu\text{g}$  of lysates were separated by SDS-PAGE using 10 % or 15 % horizontal gels and transferred to polyvinylidene fluoride (PVDF) filters in a semidry blotting apparatus (20). For detection filters were blocked with TBS containing 0.1 % Tween-20 and 5 % non-fat dry milk and subsequently incubated overnight with a dilution of appropriate antibodies in TBS containing 0.1 % Tween-20 and 5 % non-fat dry milk or 5 % BSA. After washing, filters were incubated with secondary HRP-coupled antibody and processed for enhanced chemiluminescence (ECL) detection using Uptilight (Interchim, France) or Immobilon Western Detection Reagents (Millipore, Schwalbach, Germany). Signals were visualized and evaluated on a LUMI Imager work station (Boehringer Mannheim, Mannheim, Germany).

***Glucose uptake in skeletal muscle cells.*** Four days after start of differentiation, skeletal muscle cells were used for glucose uptake experiments. Uptake of 2-desoxyglucose was measured for 2 hours after an acute 30 min insulin stimulus ( $10^{-7}$  M insulin) as described before (21).

***Presentation of data and statistics.*** Data are expressed as mean  $\pm$  SEM. Unpaired two-tailed Student's t test or one-way ANOVA (post-hoc test: Newman-Keuls multiple comparison test) were used to determine statistical significance. All

statistical analyses were done using Prism (GraphPad, La Jolla, CA, USA) considering a P value of less than 0.05 as statistically significant. Corresponding significance levels are indicated in the figures.

## RESULTS

***Chemerin and CMKLR1 expression in primary human adipocytes and skeletal muscle cells.*** In adipocytes, we can detect chemerin protein throughout differentiation (Fig. 1A). Chemerin levels increase with differentiation but decrease at the end of differentiation to levels similar to preadipocytes at day 0. In comparison to chemerin, adiponectin expression is only detectable after 3 days of differentiation and remains high until day 13 of differentiation. CMKLR1 levels also increase during differentiation and decrease again with full differentiation after 13 days (Fig. 1A) very similar to chemerin but reaching the maximal expression already at day 1 of differentiation.

Skeletal muscle cells do not express chemerin but CMKLR1 (Fig. 1B). The expression of CMKLR1 is highest in undifferentiated myoblasts and decreases during differentiation. After 6 to 8 days of differentiation when myotube formation is completed and fused skeletal muscle cells are characterized by a high expression of the myogenic marker myosin heavy chain (MHC) the levels of CMKLR1 in the myotubes are 60 % lower compared to myoblasts.

***Regulation of chemerin expression and secretion from adipocytes.*** Chemerin expression in human adipocytes is upregulated by TNF $\alpha$  but not influenced by neither adiponectin nor troglitazone treatment (Fig. 2A). In the same cells, adiponectin is upregulated by troglitazone treatment in accordance with the literature (22; 23). CMKLR1 expression is not regulated by any of the treatments.

Human *in vitro* differentiated adipocytes secrete measurable amounts of

chemerin over 48 hours (Fig. 2B). The secretion of chemerin increases constantly over 48 hours to 15 ng/ml from  $10^6$  cells. The secretion of chemerin is slightly but significantly upregulated by TNF $\alpha$  in accordance with increased chemerin expression in this situation (Fig. 2A and C). Adiponectin does not influence chemerin secretion from adipocytes while troglitazone-treatment leads to a marked decrease by over 80 % in secretion of this adipokine. It can be concluded that at least in this setting, peroxisome proliferator-activated receptor (PPAR) $\gamma$  activation leads to decreased chemerin release with unchanged intracellular chemerin levels (Fig. 2A and C).

We also tested if macrophages isolated from human adipose tissue with a method described by Curat et al. (24) secrete chemerin. Chemerin could not be detected in culture media from these macrophages as well as in media from skeletal muscle cells (data not shown).

***Chemerin secretion from adipose tissue explants increases with obesity.*** Measurement of chemerin release over 2 hours from adipose tissue biopsies obtained from lean and obese female subjects revealed a significantly higher release of chemerin in obesity (Fig. 3). Chemerin secretion is significantly higher when expressed as ng release per g adipose tissue explant and also when expressed in relation to fat cell number.

Chemerin release correlates with BMI, waist-hip-ratio and fat cell volume (Fig. 4) while no correlation could be found for clinical parameters such as insulin, HOMA, blood lipids and blood pressure (data not shown). However, a significant relationship could be found between secretion of chemerin and insulin sensitivity of adipocyte lipogenesis and insulin-stimulated antilipolysis. At the same time, chemerin does not correlate with basal nor maximal insulin-inhibited lipolysis or lipogenesis (data not shown).

***Chemerin impairs insulin signaling and glucose uptake in skeletal muscle cells.***

After overnight incubation with chemerin, skeletal muscle cells showed a marked decrease in insulin-stimulated Akt phosphorylation at Ser 473, glycogen synthase kinase (GSK)3 $\alpha$  phosphorylation at Ser 21 and GSK3 $\beta$  phosphorylation at Ser 9 (Fig. 5A and B). This effect was dose-dependent as treatment with 250 ng/ml of chemerin showed a trend towards induction of insulin resistance while 1  $\mu$ g/ml of chemerin provoked a significant decrease in insulin-stimulated phosphorylation of Akt and GSK3 $\alpha/\beta$ . Expression of Akt and GSK3 $\alpha/\beta$  remained unchanged by treatment with chemerin (data not shown). Upstream of Akt, chemerin increases basal serine phosphorylation of IRS-1 and further increases insulin-stimulated phosphorylation of this substrate (Fig. 5C). This IRS-1 serine site is targeted by several kinases and known to negatively modulate insulin action.

To test if the observed effect on insulin signaling also translates into changes of glucose homeostasis in the skeletal muscle cell, we measured glucose uptake. Treatment with chemerin significantly decreases insulin-stimulated glucose uptake with a slight but not significant parallel impairment of basal glucose uptake (Fig. 5D). Overall, the insulin-stimulated increase of glucose uptake over the respective basal level was significantly lower in skeletal muscle cells treated with chemerin ( $910 \pm 110$  versus  $620 \pm 80$  cpm/ $3.5 \times 10^5$  cells, respectively,  $n=3$ ).

Chemerin levels are lower in CM than the concentrations used to stimulate skeletal muscle cells. Therefore, we also used a combination of CM containing various adipokines besides chemerin and added chemerin in high concentrations to analyze if higher chemerin levels can add to the effect of CM to induce insulin resistance as described in earlier work (10; 15). CM and chemerin induce insulin resistance to a similar degree

while addition of chemerin to CM further decreases insulin-stimulated Akt phosphorylation (Fig. 6). This additive effect of CM containing low chemerin concentrations and added chemerin in a high concentration might indicate the stimulation of different pathways by adipokines present in CM and chemerin.

***Chemerin activates the NF- $\kappa$ B pathway and MAP kinases in skeletal muscle cells.***

Different pathways were analyzed in order to identify mechanisms that might mediate the effect of chemerin. Chemerin rapidly activates p38 mitogen-activated protein (MAP) kinase and extracellular signal-regulated kinase (ERK) 1/2 in a dose-dependent manner (Fig. 7A and B). The activation of these kinases reaches its maximum after 30 min, and the phosphorylation of both kinases slowly decreases. The nuclear factor (NF)- $\kappa$ B pathway is activated in a more prolonged fashion reaching a maximal activation of p65 by chemerin after 60 min. After 24 hours of chemerin treatment, there was no significant increase in p38 MAP kinase phosphorylation but still significant activation of ERK and p65 (Fig. 7C). The expression of p38 MAP kinase, ERK 1/2 and p65 remained unchanged by treatment with chemerin (data not shown).

***ERK-inhibition partially restores insulin sensitivity in chemerin-treated skeletal muscle cells.***

To analyze the role of ERK in the impairment of insulin signaling by chemerin, we pre-incubated skeletal muscle cells with the specific ERK inhibitor PD 98059. In short term experiments (Fig. 8A), preincubation with the ERK inhibitor completely blocks ERK activation by TNF $\alpha$  and chemerin. After overnight incubation, the ERK inhibitor has no effect on insulin signaling itself but can prevent the impairment of insulin signaling by chemerin partially (Fig. 8B). Similarly, inhibition of ERK can prevent a chemerin-induced decrease in glucose uptake (Fig. 8C). We

conclude that ERK activation is necessary for the specific effect of chemerin on insulin signaling in skeletal muscle cells but that other pathways could also be involved in this process as ERK inhibition is not able to completely reverse chemerin-induced reduction in insulin-stimulated Akt phosphorylation.

## DISCUSSION

Adipokines such as TNF $\alpha$  and IL-6 are significantly increased in obesity and are good predictors for the development of type 2 diabetes (25; 26). TNF $\alpha$ , IL-6 and MCP-1 have been identified to contribute *in vitro* to insulin resistance (27; 28) and might be regulators of insulin sensitivity. Increased concentrations of the chemokines MCP-1, IL-8 and IP-10 have been described to be associated with incident type 2 diabetes (29; 30). Their role as important immune mediators in physiological and pathological processes might also translate into increased macrophage infiltration into adipose tissue as it has been observed in obesity (31-33). Therefore, chemoattractant proteins represent an ideal link between obesity-associated changes in adipose tissue and an increased risk for metabolic diseases such as type 2 diabetes.

Chemerin is a novel chemokine and adipokine with a described role in host survival defense including complement fibronolysis and coagulation. While first described as a chemokine occurring in fluids during inflammatory processes such as cancer and rheumatoid arthritis (11), chemerin is also expressed in adipose tissue. We could demonstrate that *in vitro* differentiated adipocytes release measurable amounts of chemerin and express CMKLR1. Several studies describe similarly that chemerin mRNA expression increases with adipogenesis in 3T3 L1 adipocytes but two of the studies comprise completely divergent data on the regulation of the CMKLR1 (12;

13; 34). In human adipocytes chemerin and CMKLR1 mRNA expression increase with differentiation (13) the effect being more pronounced for CMKLR1. We could also find an increased expression of chemerin and CMKLR1 at the protein level over differentiation that was however not lasting until the end of differentiation. The use of freshly isolated subcutaneous adipocytes and a distinct differentiation protocol might explain this difference. In adipose tissue, chemerin can also be found in the stroma-vascular fraction pointing to a contribution of various adipose tissue cell types to chemerin production. Unable to detect chemerin release from macrophages isolated from adipose tissue, we can demonstrate that this cell type being so critical for adipose tissue inflammation does not contribute to chemerin release. However, it has been described that macrophages express CMKLR1 and are chemerin responsive (35). Chemerin is expressed similarly in human preadipocytes and adipocytes making it possible that adipocytes and preadipocytes are the main cells secreting this adipokine within adipose tissue.

The current knowledge of chemerin is more complicated including more targets than chemerin and CMKLR1. Chemerin is synthesized as prochemerin with low affinity to CMKLR1 (11). Prochemerin is rapidly converted into chemerin by proteolytic cleavage of a carboxy-terminal peptide involving serine proteases of the coagulation and inflammation cascades (11). C-terminal peptides derived from chemerin by cysteine protease cleavage bind to CMKLR1 with much higher affinity than chemerin itself and exerts potent anti-inflammatory effects on activated macrophages (36; 37). This divergent effect of chemerin and chemerin-derived peptides can be explained by binding to other receptors recently identified such as the G-protein coupled receptors GPR1 and CCRL2 (37; 38). In detail, chemerin binds

with its C-terminus to CMKLR1 directly activating cells but chemerin can also bind CCRL2 on its N-terminal domain and present the C-terminus to CMKLR1 on neighboring cells. Differently, chemerin-derived peptides can only bind to CMKLR1 and inhibit an inflammatory response, a process that is similarly known for other chemokines such as MCP-1 or RANTES (39; 40). In this study, we observed a pro-inflammatory action of chemerin on skeletal muscle cells. The role of the novel chemerin receptors and chemerin-derived peptides in the context of obesity and type 2 diabetes is not known. Further work is also necessary to assess the effects of chemerin-derived peptides in this respect.

Chemerin expression is not increased in adipose tissue of genetically obese mice (13) or even lower in db/db mice (41) but higher in obese, insulin-resistant P. obesus (12). The initial study on chemerin in humans revealed a correlation of chemerin blood levels with BMI independent of glucose tolerance (12). Very recent publications could also demonstrate an association of chemerin with markers of the metabolic syndrome and inflammation (42-44). We could show that the release of chemerin is clearly increased from adipose tissue explants of obese patients as compared to lean controls. Furthermore, chemerin release from adipose tissue correlates with waist-hip-ratio and fat cell volume while no correlation could be found with blood pressure and HOMA. Interestingly, chemerin secretion is negatively correlated with insulin sensitivity of the adipocytes as higher chemerin release is associated with lower insulin sensitivity of lipogenesis and lower insulin-stimulated antilipolysis. In conclusion, we could show that chemerin is released by human adipose tissue and in obesity its amounts are increased. Chemerin correlates with insulin sensitivity of the fat cell potentially leading to local insulin resistance in obesity. Thus, chemerin might, clinically, serve as a marker

for body composition. Its possible role as marker for metabolic control and homeostasis needs to be explored further.

We demonstrate in this study that chemerin secretion by adipocytes is regulated *in vitro* and that the release of chemerin from adipose tissue explants correlates with various features of the metabolic syndrome. It would be interesting to also study the *in vivo* effect of chemerin. This is unfortunately not possible because chemerin is not registered for therapeutic use in humans. Chemerin is secreted at concentrations of about 15 ng/ml from  $10^6$  cells which is relatively low compared to the concentrations used on the skeletal muscle cells of 250 ng/ml to 1  $\mu$ g/ml. The latter reflect serum concentrations of this chemokine in lean ( $249 \pm 71$  ng/ml measured in 142 patients (12)) and obese patients (measured chemerin levels in 4 morbidly obese women of  $674 \pm 37$  ng/ml, BMI of 48-55, unpublished data). Contribution of liver, lung and other chemerin producing organs to chemerin blood levels have to be taken into account.

In accordance with chemerin secretion being elevated from adipose tissue of obese patients and published data on chemerin being related to obesity (12), the expression and secretion of this chemokine by adipocytes is upregulated by TNF $\alpha$ . Interestingly, the PPAR $\gamma$  agonist troglitazone strongly suppresses the release of chemerin while having no effect on its expression. This observation might add to the wide area of action of these compounds (45). A very recent publication revealed that chemerin secretion is increased by insulin in adipose tissue explants while metformin is able to reduce chemerin secretion (46). Metformin also reduces chemerin blood levels without decreasing BMI in women with polycystic ovary syndrome.

A role for chemerin was not only described in chemoattraction and macrophage infiltration into adipose tissue but also in

adipogenesis and adipocyte metabolism (13; 34; 41). Chemerin is necessary for normal adipogenesis but, as we could show here, also induces insulin resistance in peripheral tissues such as skeletal muscle. We observe a clear inhibitory effect of chemerin on skeletal muscle cell glucose uptake but in 3T3 adipocytes chemerin has the opposite effect (41), namely increasing insulin-stimulated glucose uptake. Thus, chemerin may exert different action in endocrine and paracrine/autocrine ways. Other chemokines such as MCP-1 are also known to induce insulin resistance in skeletal muscle cells also involving activation of ERK1/2 similarly to chemerin (9). However, there are also differences in their mode of action as MCP-1 does not activate the NF- $\kappa$ B pathway while chemerin does.

In summary, our data show that skeletal muscle is a target tissue for chemerin that might be involved in the negative crosstalk between skeletal muscle and adipose tissue. The possible role of chemerin 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. Other adipokines already tested for their ability to disturb insulin-stimulated glucose homeostasis in skeletal muscle are also involved in inflammation and it must be

emphasized that there is most likely not a single adipokine that is fully responsible for obesity-associated metabolic complications including insulin resistance in skeletal muscle. It is apparent that the role of adipokines in obesity and its associated metabolic complications is complex involving numerous proteins which may act independently or in consonance. A rather complicated interplay between a huge number of adipokines and their overlapping physiological effects adds to other more environmental or genetic factors to decide for the development of type 2 diabetes.

#### **ACKNOWLEDGMENTS**

This work was supported by the Ministerium für Wissenschaft und Forschung des Landes Nordrhein-Westfalen, the Bundesministerium für Gesundheit, European Union COST Action BM0602, Swedish Research Council, Swedish Heart and Lung Foundation, Swedish Diabetes Association and the Commission of the European Communities (Collaborative Project ADAPT, contract number HEALTH-F2-2008-201100). We wish to thank Prof. Liebau and her team, Dept. of Plastic Surgery, Florence-Nightingale-Hospital Düsseldorf, for support in obtaining adipose tissue samples. The secretarial assistance of Birgit Hurow and the technical help of Kerstin Wahlen is gratefully acknowledged.

## REFERENCES

1. Bosello O, Zamboni M: Visceral obesity and metabolic syndrome. *Obes Rev* 1:47-56, 2000
2. Grundy SM, Brewer HB, Jr., Cleeman JI, Smith SC, Jr., Lenfant C: Definition of metabolic syndrome: Report of the National Heart, Lung, and Blood Institute/American Heart Association conference on scientific issues related to definition. *Circulation* 109:433-438, 2004
3. Kahn BB, Flier JS: Obesity and insulin resistance. *J Clin Invest* 106:473-481, 2000
4. Bloomgarden ZT: Obesity and diabetes. *Diabetes Care* 23:1584-1590, 2000
5. Felber JP, Golay A: Pathways from obesity to diabetes. *Int J Obes Relat Metab Disord* 26 Suppl 2:S39-45, 2002
6. Finegood DT: Obesity, inflammation and type II diabetes. *Int J Obes Relat Metab Disord* 27 Suppl 3:S4-5, 2003
7. Arner P: Regional differences in protein production by human adipose tissue. *Biochem Soc Trans* 29:72-75, 2001
8. Trayhurn P, Beattie JH: Physiological role of adipose tissue: white adipose tissue as an endocrine and secretory organ. *Proc Nutr Soc* 60:329-339, 2001
9. Sell H, Dietze-Schroeder D, Kaiser U, Eckel J: Monocyte chemotactic protein-1 is a potential player in the negative cross-talk between adipose tissue and skeletal muscle. *Endocrinology* 147:2458-2467, 2006
10. Sell H, Eckardt K, Taube A, Tews D, Gurgui M, Van Echten-Deckert G, Eckel J: Skeletal muscle insulin resistance induced by adipocyte-conditioned medium: underlying mechanisms and reversibility. *Am J Physiol Endocrinol Metab* 294:E1070-1077, 2008
11. Wittamer V, Franssen JD, Vulcano M, Mirjolet JF, Le Poul E, Migeotte I, Brezillon S, Tyldesley R, Blanpain C, Detheux M, Mantovani A, Sozzani S, Vassart G, Parmentier M, Communi D: Specific recruitment of antigen-presenting cells by chemerin, a novel processed ligand from human inflammatory fluids. *J Exp Med* 198:977-985, 2003
12. Bozaoglu K, Bolton K, McMillan J, Zimmet P, Jowett J, Collier G, Walder K, Segal D: Chemerin is a novel adipokine associated with obesity and metabolic syndrome. *Endocrinology* 148:4687-4694, 2007
13. Goralski KB, McCarthy TC, Hanniman EA, Zabel BA, Butcher EC, Parlee SD, Muruganandan S, Sinal CJ: Chemerin, a novel adipokine that regulates adipogenesis and adipocyte metabolism. *J Biol Chem* 282:28175-28188, 2007
14. Dietze D, Koenen M, Rohrig K, Horikoshi H, Hauner H, Eckel J: Impairment of insulin signaling in human skeletal muscle cells by co-culture with human adipocytes. *Diabetes* 51:2369-2376, 2002
15. Dietze-Schroeder D, Sell H, Uhlig M, Koenen M, Eckel J: Autocrine action of adiponectin on human fat cells prevents the release of insulin resistance-inducing factors. *Diabetes* 54:2003-2011, 2005
16. Bonora E, Targher G, Alberiche M, Bonadonna RC, Saggiani F, Zenere MB, Monauni T, Muggeo M: Homeostasis model assessment closely mirrors the glucose clamp technique in the assessment of insulin sensitivity: studies in subjects with various degrees of glucose tolerance and insulin sensitivity. *Diabetes Care* 23:57-63, 2000
17. Kolaczynski JW, Morales LM, Moore JH, Jr., Considine RV, Pietrzakowski Z, Noto PF, Colberg J, Caro JF: A new technique for biopsy of human abdominal fat under local anaesthesia with Lidocaine. *Int J Obes Relat Metab Disord* 18:161-166, 1994

18. Lonnqvist F, Nordfors L, Jansson M, Thorne A, Schalling M, Arner P: Leptin secretion from adipose tissue in women. Relationship to plasma levels and gene expression. *J Clin Invest* 99:2398-2404, 1997
19. Lofgren P, Hoffstedt J, Naslund E, Wiren M, Arner P: Prospective and controlled studies of the actions of insulin and catecholamine in fat cells of obese women following weight reduction. *Diabetologia* 48:2334-2342, 2005
20. Wichelhaus A, Russ M, Petersen S, Eckel J: G protein expression and adenylate cyclase regulation in ventricular cardiomyocytes from STZ-diabetic rats. *Am J Physiol* 267:H548-555, 1994
21. Uhlig M, Passlack W, Eckel J: Functional role of Rab11 in GLUT4 trafficking in cardiomyocytes. *Mol Cell Endocrinol* 235:1-9, 2005
22. Maeda N, Takahashi M, Funahashi T, Kihara S, Nishizawa H, Kishida K, Nagaretani H, Matsuda M, Komuro R, Ouchi N, Kuriyama H, Hotta K, Nakamura T, Shimomura I, Matsuzawa Y: PPARgamma ligands increase expression and plasma concentrations of adiponectin, an adipose-derived protein. *Diabetes* 50:2094-2099, 2001
23. Phillips SA, Ciaraldi TP, Kong AP, Bandukwala R, Aroda V, Carter L, Baxi S, Mudaliar SR, Henry RR: Modulation of circulating and adipose tissue adiponectin levels by antidiabetic therapy. *Diabetes* 52:667-674, 2003
24. Curat CA, Miranville A, Sengenès C, Diehl M, Tonus C, Busse R, Bouloumie A: From blood monocytes to adipose tissue-resident macrophages: induction of diapedesis by human mature adipocytes. *Diabetes* 53:1285-1292, 2004
25. Fain JN, Bahouth SW, Madan AK: TNFalpha release by the nonfat cells of human adipose tissue. *Int J Obes Relat Metab Disord* 28:616-622, 2004
26. Festa A, D'Agostino R, Jr., Tracy RP, Haffner SM: Elevated levels of acute-phase proteins and plasminogen activator inhibitor-1 predict the development of type 2 diabetes: the insulin resistance atherosclerosis study. *Diabetes* 51:1131-1137, 2002
27. Sell H, Dietze-Schroeder D, Eckel J: The adipocyte-myocyte axis in insulin resistance. *Trends Endocrinol Metab* 17:416-422, 2006
28. Sell H, Eckel J: Monocyte chemotactic protein-1 and its role in insulin resistance. *Curr Opin Lipidol* 18:258-262, 2007
29. Herder C, Haastert B, Muller-Scholze S, Koenig W, Thorand B, Holle R, Wichmann HE, Scherbaum WA, Martin S, Kolb H: Association of systemic chemokine concentrations with impaired glucose tolerance and type 2 diabetes: results from the Cooperative Health Research in the Region of Augsburg Survey S4 (KORA S4). *Diabetes* 54 Suppl 2:S11-17, 2005
30. Herder C, Baumert J, Thorand B, Koenig W, de Jager W, Meisinger C, Illig T, Martin S, Kolb H: Chemokines as risk factors for type 2 diabetes: results from the MONICA/KORA Augsburg study, 1984-2002. *Diabetologia* 49:921-929, 2006
31. Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW, Jr.: Obesity is associated with macrophage accumulation in adipose tissue. *J Clin Invest* 112:1796-1808, 2003
32. Weisberg SP, Hunter D, Huber R, Lemieux J, Slaymaker S, Vaddi K, Charo I, Leibel RL, Ferrante AW, Jr.: CCR2 modulates inflammatory and metabolic effects of high-fat feeding. *J Clin Invest* 116:115-124, 2006
33. Kanda H, Tateya S, Tamori Y, Kotani K, Hiasa K, Kitazawa R, Kitazawa S, Miyachi H, Maeda S, Egashira K, Kasuga M: MCP-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity. *J Clin Invest* 116:1494-1505, 2006

34. Roh SG, Song SH, Choi KC, Katoh K, Wittamer V, Parmentier M, Sasaki S: Chemerin--a new adipokine that modulates adipogenesis via its own receptor. *Biochem Biophys Res Commun* 362:1013-1018, 2007
35. Zabel BA, Ohyama T, Zuniga L, Kim JY, Johnston B, Allen SJ, Guido DG, Handel TM, Butcher EC: Chemokine-like receptor 1 expression by macrophages in vivo: regulation by TGF-beta and TLR ligands. *Exp Hematol* 34:1106-1114, 2006
36. Wittamer V, Gregoire F, Robberecht P, Vassart G, Communi D, Parmentier M: The C-terminal nonapeptide of mature chemerin activates the chemerin receptor with low nanomolar potency. *J Biol Chem* 279:9956-9962, 2004
37. Cash JL, Hart R, Russ A, Dixon JP, Colledge WH, Doran J, Hendrick AG, Carlton MB, Greaves DR: Synthetic chemerin-derived peptides suppress inflammation through ChemR23. *J Exp Med* 205:767-775, 2008
38. Zabel BA, Nakae S, Zuniga L, Kim JY, Ohyama T, Alt C, Pan J, Suto H, Soler D, Allen SJ, Handel TM, Song CH, Galli SJ, Butcher EC: Mast cell-expressed orphan receptor CCRL2 binds chemerin and is required for optimal induction of IgE-mediated passive cutaneous anaphylaxis. *J Exp Med* 205:2207-2220, 2008
39. Zhang Y, Rollins BJ: A dominant negative inhibitor indicates that monocyte chemoattractant protein 1 functions as a dimer. *Mol Cell Biol* 15:4851-4855, 1995
40. Proudfoot AE, Power CA, Hoogewerf AJ, Montjovent MO, Borlat F, Offord RE, Wells TN: Extension of recombinant human RANTES by the retention of the initiating methionine produces a potent antagonist. *J Biol Chem* 271:2599-2603, 1996
41. Takahashi M, Takahashi Y, Takahashi K, Zolotaryov FN, Hong KS, Kitazawa R, Iida K, Okimura Y, Kaji H, Kitazawa S, Kasuga M, Chihara K: Chemerin enhances insulin signaling and potentiates insulin-stimulated glucose uptake in 3T3-L1 adipocytes. *FEBS Lett* 582:573-578, 2008
42. Bozaoglu K, Segal D, Shields KA, Cummings N, Curran JE, Comuzzie AG, Mahaney MC, Rainwater DL, Vandeberg JL, Maccluer JW, Collier G, Blangero J, Walder K, Jowett J: Chemerin is associated with metabolic syndrome phenotypes in a Mexican American Population. *J Clin Endocrinol Metab*, in press
43. Lehrke M, Becker A, Greif M, Stark R, Laubender R, von Ziegler F, Leberherz C, Tittus J, Reiser M, Becker C, Goke B, Leber A, Parhofer K, Broedl U: Chemerin is associated with markers of inflammation and components of the metabolic syndrome but does not predict coronary atherosclerosis. *Eur J Endocrinol*, in press
44. Weigert J, Neumeier M, Wanninger J, Filarsky M, Bauer S, Wiest R, Farkas S, Scherer MN, Schaffler A, Aslanidis C, Scholmerich J, Buechler C: Systemic chemerin is related to inflammation rather than obesity in type 2 diabetes. *Clin Endocrinol (Oxf)*, in press
45. Arner P: The adipocyte in insulin resistance: key molecules and the impact of the thiazolidinediones. *Trends Endocrinol Metab* 14:137-145, 2003
46. Tan BK, Chen J, Farhatullah S, Adya R, Kaur J, Heutling D, Lewandowski KC, O'Hare JP, Lehnert H, Randeve HS: Insulin and Metformin Regulate Circulating and Adipose Tissue Chemerin. *Diabetes*, 10.2337/db08-1528

### **Figure legends**

**Fig. 1.** Expression of chemerin and CMKLR1 in human skeletal muscle cells and adipocytes. **A:** Human adipocytes from different donors were differentiated for the indicated time and total cell lysates were resolved by SDS-Page. Western blots for chemerin, adiponectin and CMKLR1 as well as normalization for actin are shown. Data are mean values  $\pm$  SEM of 3-4 independent experiments. All data were normalized to the level of actin expression and are expressed relative to the expression level at day 0. \*significantly different from day 0. **B:** Skeletal muscle cells from different donors were differentiated for the indicated time and total cell lysates were resolved by SDS-Page. Western blots for CMKLR1 and MHC as well as normalization for tubulin are shown. Data are mean values  $\pm$  SEM of 3 independent experiments. All data were normalized to the level of tubulin expression and are expressed relative to the expression level at day 0. The right panel shows that skeletal muscle cells from 2 different donors (SkM1 and SkM2) have no expression of chemerin as compared to adipocytes harvested at day 0, 1 and 3 of differentiation. \*significantly different from day 0.

**Fig. 2.** Regulation of chemerin and CMKLR1 expression and chemerin secretion in human adipocytes. **A:** Human adipocytes from different donors were differentiated and incubated with either 2.5 nM TNF $\alpha$ , 5 nM adiponectin or 5  $\mu$ M troglitazone overnight. Total cell lysates were resolved by SDS-Page. Western blots for chemerin, adiponectin and CMKLR1 as well as normalization for actin are shown. Data are mean values  $\pm$  SEM of 3-4 independent experiments. All data were normalized to the level of actin expression and are expressed relative to the unstimulated control. \*significantly different control. **B:** Human adipocytes from different donors were differentiated and conditioned medium was collected after different periods of incubation. The release of chemerin was analyzed using a chemerin ELISA. Data are mean values  $\pm$  SEM of 3 independent experiments. **C:** Human adipocytes from different donors were treated with 2.5 nM TNF $\alpha$ , 5 nM adiponectin or 5  $\mu$ M troglitazone overnight and the conditioned medium was collected for chemerin measurement. Data are mean values  $\pm$  SEM of 3 independent experiments. All data are expressed relative to the unstimulated control. \*significantly different from control.

**Fig. 3.** Secretion of chemerin from adipose tissue explants derived from lean and obese females. Adipose tissue explants were treated as detailed in the Methods section and the release of chemerin was measured by ELISA. Mean  $\pm$  SEM of tissue explants from 53 individuals. \*significantly different from lean controls.

**Fig. 4.** Correlation of chemerin release from adipose tissue explants with different parameters. Adipose tissue explants were treated as detailed in the Methods section and the release of chemerin was measured by ELISA. Chemerin release was correlated with BMI, Waist-hip-ratio and adipocyte volume. Lipogenesis and insulin-stimulated antilipolysis was measured as described in the Methods section. Values for antilipolysis are not normally distributed but the correlation remains significant using a nonparametric test (Spearman Rank Test).

**Fig. 5.** Effect of chemerin on insulin signaling and glucose uptake in human skeletal muscle cells. **A:** Myocytes from different donors were cultured with increasing concentrations of chemerin (250 ng/ml and 1  $\mu$ g/ml) for 24h. After acute stimulation with insulin, total cell lysates were resolved by SDS-PAGE and immunoblotted with phosphospecific Akt antibody and tubulin

antibody. Data are mean values  $\pm$  SEM of 5 independent experiments. All data were normalized to the level of tubulin expression and are expressed relative to the insulin-stimulated control value. **B:** Myocytes from 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  $\pm$  SEM of 4 independent experiments. All data were normalized to the level of tubulin expression and are expressed relative to the insulin-stimulated control value. **C and D:** Skeletal muscle cells were cultured for 24 h in absence or presence of chemerin (1  $\mu$ g/ml). IRS1 phosphorylation and glucose uptake was assessed after acute stimulation with insulin, as outlined in the Methods section. Mean  $\pm$  SEM of 3 independent experiments. \* significantly different from insulin-stimulated control, § significantly different from respective insulin-stimulated control.

**Fig. 6.** Additive effect of chemerin and CM on insulin signaling in skeletal muscle cells. Skeletal muscle cells from different donors were incubated with chemerin, CM or a combination of both overnight. After insulin stimulation, total cell lysates were resolved by SDS-Page and immunoblotted with a phosphospecific antibody for Akt and tubulin for loading control. Representative blots are shown. Mean  $\pm$  SEM of 3-4 independent experiments. \*significantly different from respective basal, § significantly different from respective insulin-stimulated control.

**Fig. 7.** Chemerin signaling in skeletal muscle cells. **A:** Skeletal muscle cells from different donors were cultured with chemerin for 30 min and as a control with 2.5 nM TNF $\alpha$  for 10 min. Total cell lysates were resolved by SDS-Page and immunoblotted with phosphospecific antibodies for p38 MAP kinase, the p65 subunit of NF- $\kappa$ B (p65) and ERK1/2 and tubulin for loading control. Representative blots are shown. **B:** Skeletal muscle cells from different donors were cultured with chemerin for 10 to 120 min. Total cell lysates were resolved by SDS-Page and immunoblotted with phosphospecific antibodies for p38 MAPK, the p65 subunit of NF- $\kappa$ B (p65) and ERK1/2 and tubulin for loading control. Mean  $\pm$  SEM of 4-5 independent experiments. \*significantly different from unstimulated control. **C:** Skeletal muscle cells from different donors were cultured with different concentrations of chemerin for 24 hours. Total cell lysates were resolved by SDS-Page and immunoblotted with phosphospecific antibodies for p38 MAP kinase, the p65 subunit of NF- $\kappa$ B (p65) and ERK1/2 and tubulin for loading control. Mean  $\pm$  SEM of 4-5 independent experiments. \*significantly different from unstimulated control.

**Fig. 8.** Prevention of chemerin-induced insulin resistance by ERK-inhibition. **A:** Skeletal muscle cells from different donors were precultured with or without 50  $\mu$ M of the specific ERK inhibitor PD 98059 for 15 min before starting the treatment with chemerin or TNF $\alpha$ . The cells were then treated with chemerin for 30 min and as a control with 2.5 nM TNF $\alpha$  for 10 min. Total cell lysates were resolved by SDS-Page and immunoblotted with a phosphospecific antibody for ERK1/2 and tubulin for loading control. Representative blots are shown. **B and C:** After pretreatment for 15 min with PD 98059 (50  $\mu$ M), skeletal muscle cells from different donors were treated with chemerin overnight. After insulin stimulation, total cell lysates were resolved by SDS-Page and immunoblotted with a phosphospecific antibody for Akt and tubulin for loading control. Representative blots are shown. Mean  $\pm$  SEM of 4 independent experiments. Glucose uptake was measured as outlined in the Methods section. Mean  $\pm$  SEM of 3 independent experiments. \*significantly different from respective insulin-stimulated control.

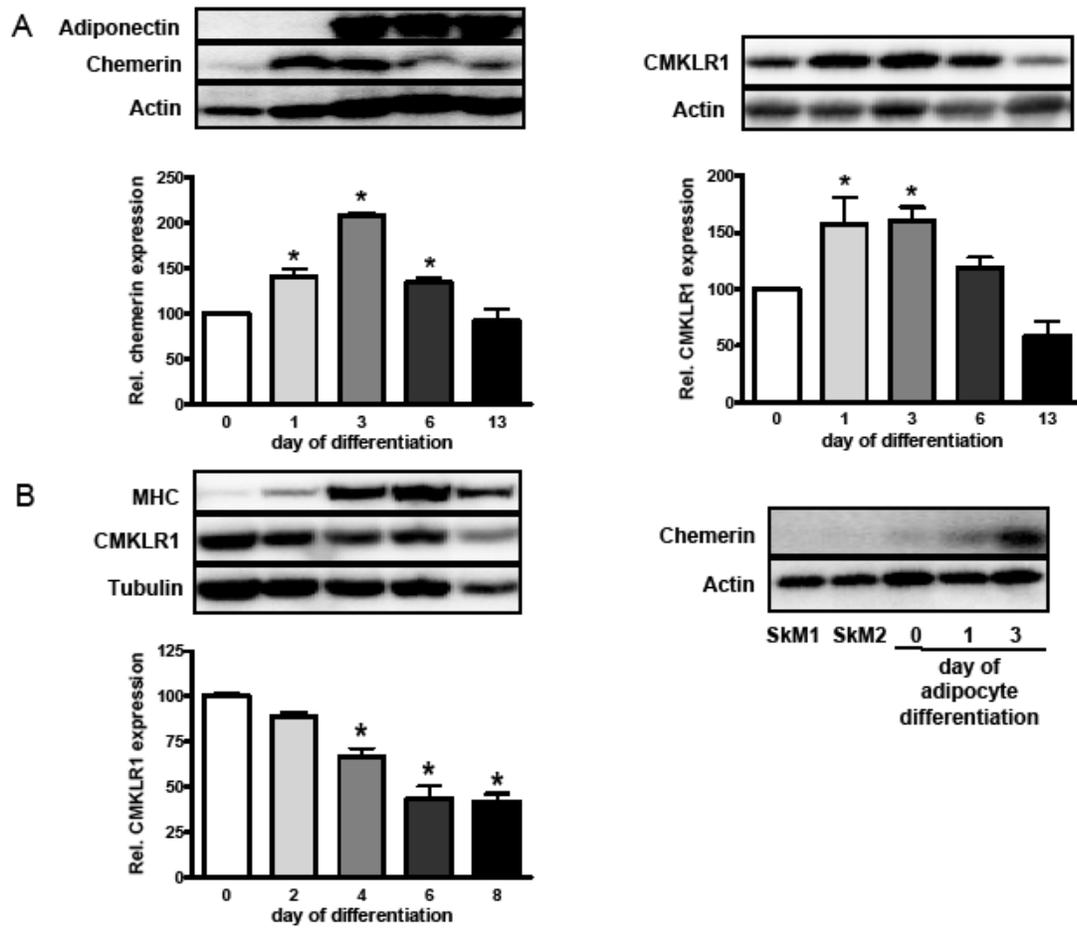


Figure 1

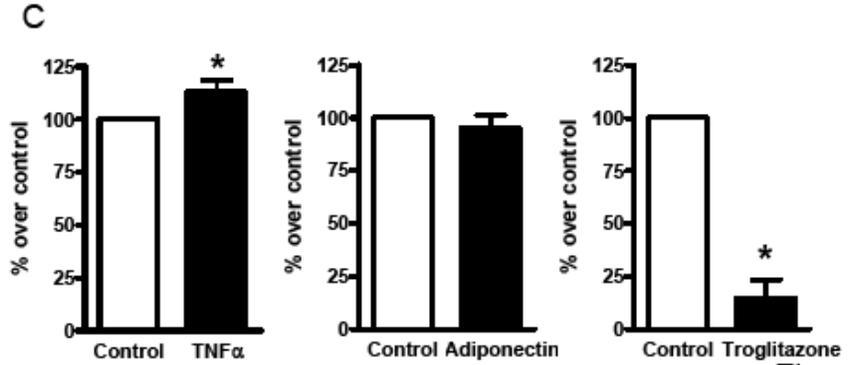
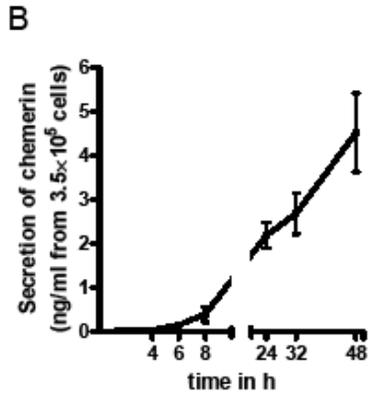
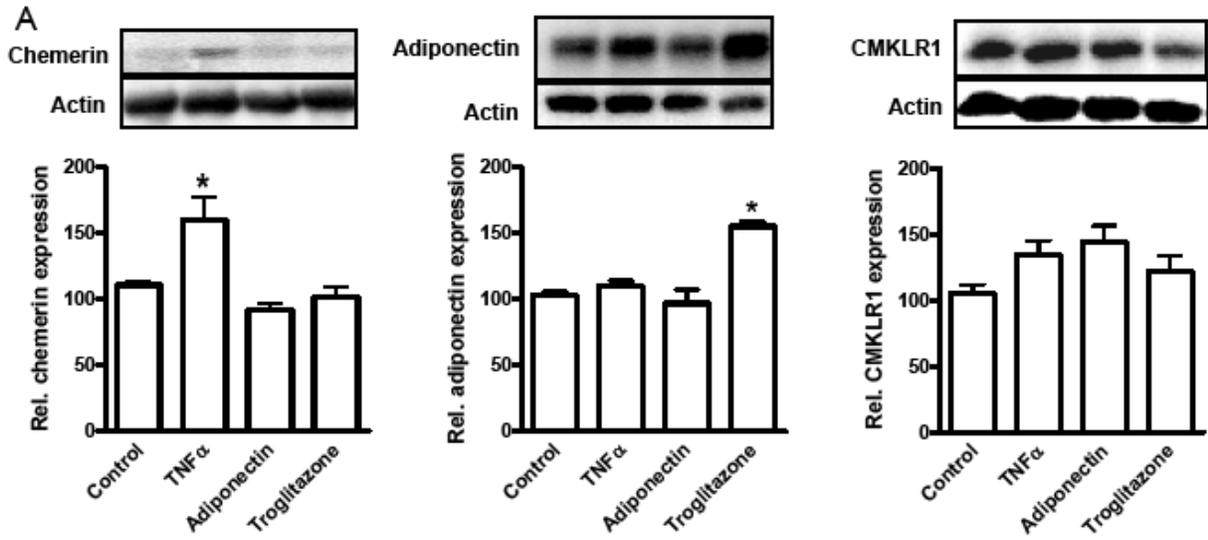


Figure 2

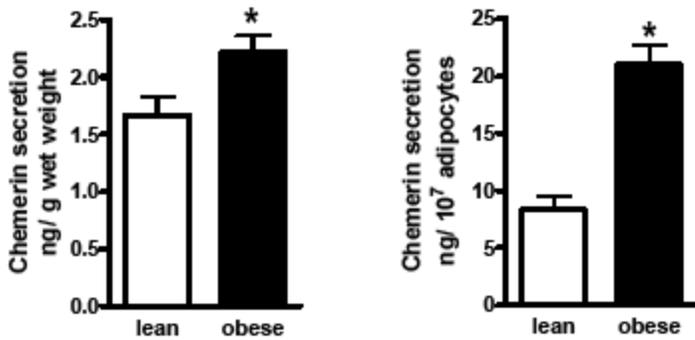


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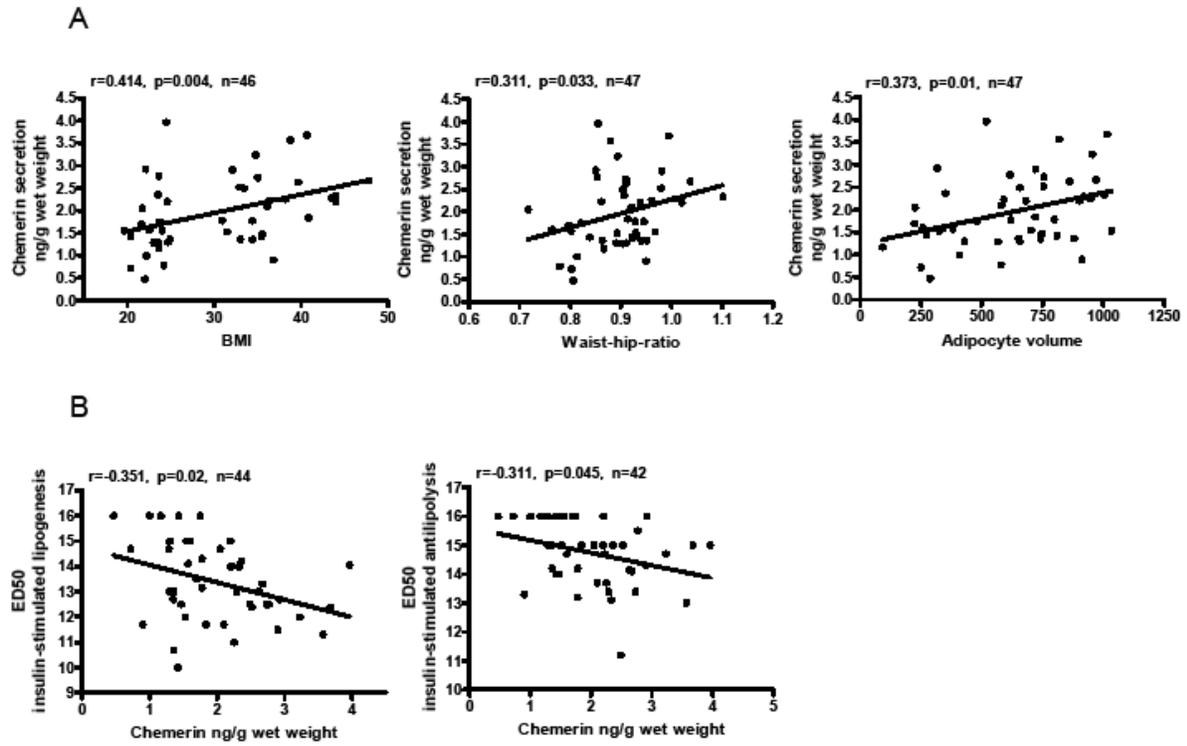


Figure 4

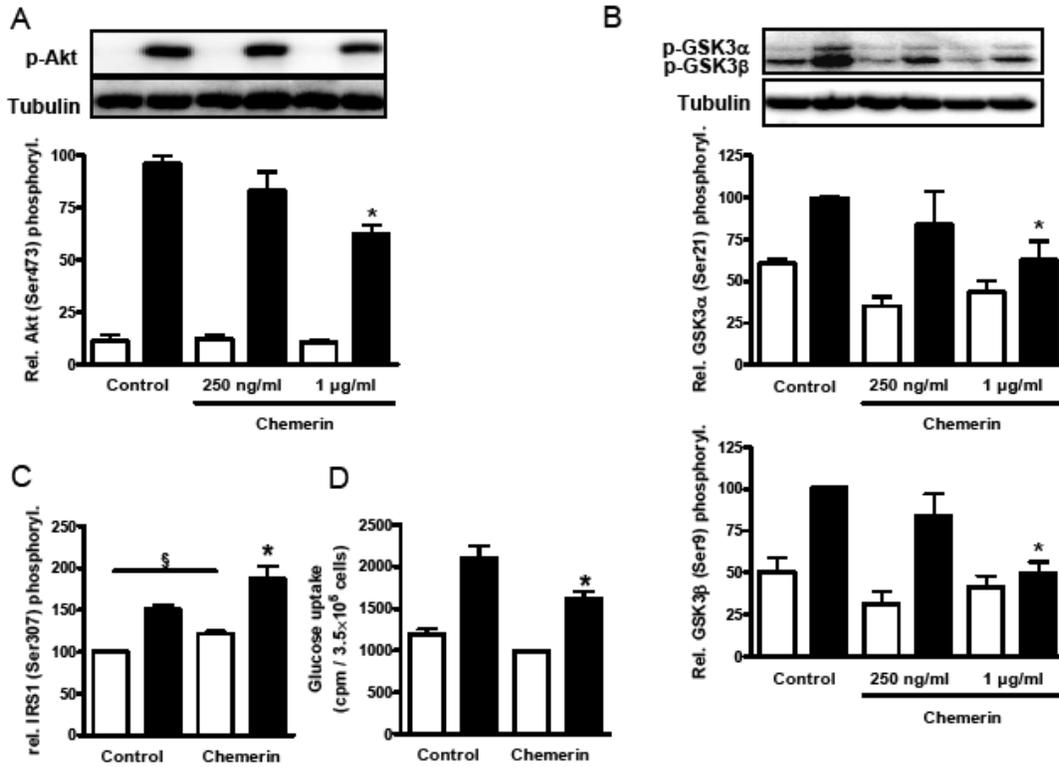


Figure 5

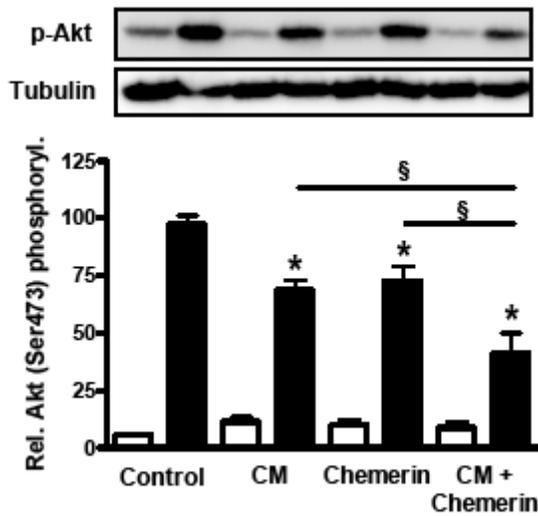
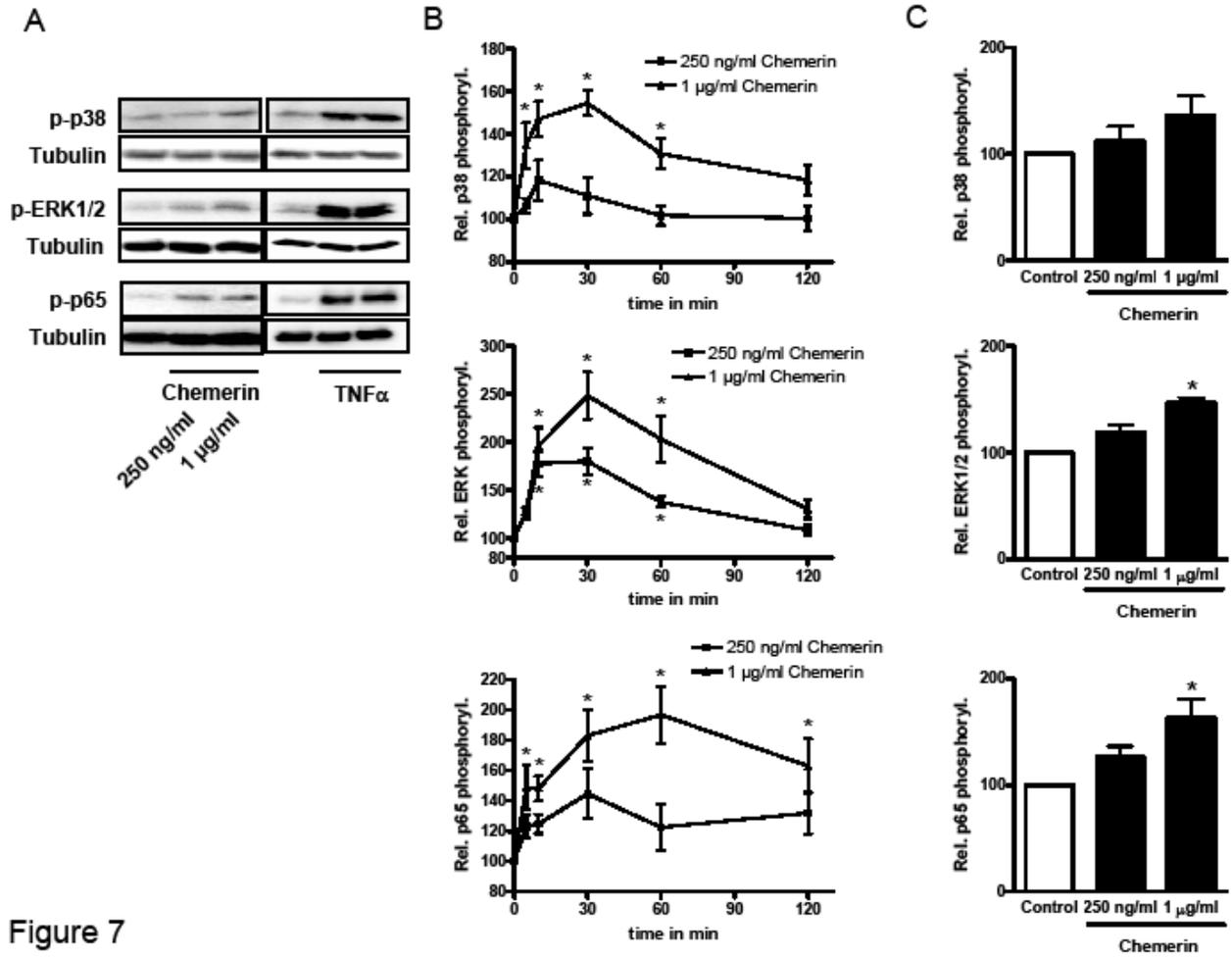


Figure 6



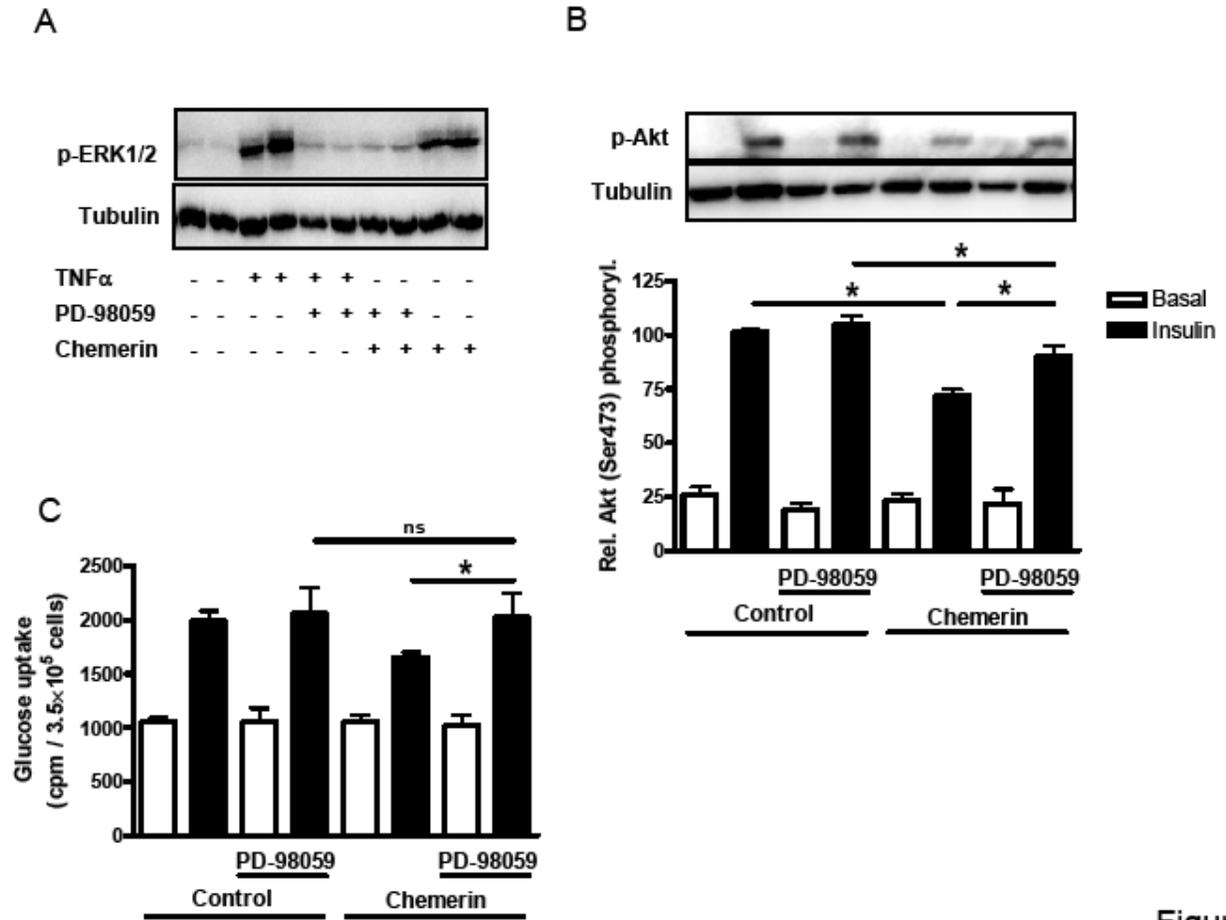


Figure 8