Expression of human chemerin induces insulin resistance in the skeletal muscle but does not affect weight, lipid levels and atherosclerosis in LDL receptor knockout mice on high fat diet

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Running head: Metabolic effect of chemerin in vivo

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Objective. Chemerin is a recently discovered hepatoadipokine that regulates adipocyte differentiation as well as chemotaxis and activation of dendritic cells and macrophages. Chemerin was reported to modulate insulin sensitivity in adipocytes and skeletal muscle cells in vitro and to exacerbate glucose intolerance in several mouse models in vivo. In humans, chemerin was shown to be associated with multiple components of the metabolic syndrome including body mass index, triglycerides, HDL-cholesterol and hypertension. This study was aimed to examine the effect of chemerin on weight, glucose and lipid metabolism as well as atherosclerosis in vivo.

Research Design and Methods. We used recombinant adeno-associated virus to express human chemerin in LDL receptor knockout mice on high fat diet.

Results. Expression of chemerin did not significantly alter weight, lipid levels, and extent of atherosclerosis. Chemerin, however, significantly increased glucose levels during the intraperitoneal glucose tolerance test without affecting endogenous insulin levels and the insulin tolerance test. Chemerin reduced insulin-stimulated Akt1 phosphorylation and activation of 5’AMP activated protein kinase (AMPK) in the skeletal muscle, but had no effect on Akt phosphorylation and insulin-stimulated AMPK activation in the liver and gonadal adipose tissue.

Conclusions. Chemerin induces insulin resistance in the skeletal muscle in vivo. Chemerin is involved in the cross talk between liver, adipose tissue and skeletal muscle.

Chemerin was initially described in 2003 as a novel chemoattractant protein (1) that upon enzymatic proteolysis modulates chemotaxis and activation of dendritic cells and macrophages through distinct G protein-coupled receptors such as CMKLR1, GPR1 and CCRL2 (2-4). More recently, chemerin was reported as a hepatoadipokine that regulates adipocyte differentiation in an autocrine/paracrine manner via CMKLR1 and modulates the expression of adipocyte genes involved in glucose and lipid homeostasis (5,6). Conflicting data exist regarding the effect of chemerin on insulin signaling in adipocytes in vitro. Kralisch et al. (7) demonstrated that chemerin downregulated insulin-stimulated glucose uptake in 3T3-L1 adipocytes, while Takahasi et al. (8) reported the opposite. In skeletal muscle cells, chemerin was shown to impair insulin signaling and glucose uptake, a process partially mediated by activation of the extracellular signal-regulated kinases (ERK) 1/2 (9). In humans, chemerin correlated with systemic markers of inflammation such as hsCRP, IL-6 and TNF-α, and was associated with components of the metabolic syndrome including body mass index, triglycerides, HDL-cholesterol and hypertension in different populations (5,10-13). In morbidly obese patients undergoing bariatric surgery, weight loss was associated with improvement of various metabolic parameters and a sustained reduction of chemerin levels (14,15). Taken together, available data suggest that chemerin may play a role in glucose and lipid metabolism as well as inflammation. Surprisingly little data, however, are currently available about the potential effects of chemerin on the regulation of these parameters in vivo.
Recently, Ernst et al. (16) reported that acute administration of recombinant human chemerin exacerbated glucose intolerance, lowered serum insulin levels, and decreased tissue glucose uptake in ob/ob, db/db, and diet-induced obese (DIO) but not in normoglycemic wildtype mice. As acute injection of chemerin may not reflect the effect of chronically elevated chemerin levels seen in obesity we designed the present study to investigate the impact of long-term chemerin overexpression on body weight, glucose and lipid metabolism as well as atherosclerosis in LDL receptor knockout (LDLRKO) mice on a high fat diet. As human chemerin fully binds to and activates murine CMKLR1 (17), we expressed human chemerin using a recombinant adeno-associated virus (AAV)-based vector in this well established mouse model of diet-induced insulin resistance, dyslipidemia and atherosclerosis.

MATERIALS AND METHODS

Generation of recombinant adeno-associated viral constructs. All constructs were expressed under control of the thyroxine binding globulin promoter (18,19). Transgenes were full length human chemerin (preprochemerin, aa 1-163) and β-galactosidase (LacZ) as control. To produce AAV vectors encapsidated in an AAV8 capsid (AAV2.8), a pseudotyping strategy was performed. Vectors (AAV.chemerin, AAV.lacZ) were purified using a standard cesium sedimentation method and titers were determined via TaqMan analysis using probes and primers targeting the BGH poly(A+) region of the vectors (18,19).

Animal studies. Male LDLRKO (B6.12957-LDLr<sup>tm1Her</sup>/J) mice were obtained from Charles River Laboratories (Germany, Sulzfeld, Germany). Mice (5 weeks of age) were kept on a high fat diet (0.21% cholesterol, 21% butterfat; ssniff, Soest, Germany) for 16 weeks. Blood was drawn after 12h of overnight fasting before and several timepoints after virus injection. The study was approved by the District Government of Upper Bavaria.

Intraperitoneal glucose tolerance test (ipGTT) and insulin tolerance test (ITT) were performed 12 and 14 weeks after AAV injection, respectively (detailed description given in the supplement available online at http://diabetes.diabetesjournals.org).

At the end of the study (16 weeks after injection) mice (n=12/group; one death/group during the study) were injected intraperitoneally with insulin (n=6/group) (0.02 U/g body weight) or saline (n=6) to determine protein levels and phosphorylation of Akt1 and 5’AMP-activated protein kinase (AMPK)-α. Mice were sacrificed 15 min after injection and organs were harvested. Liver, skeletal muscle, and gonadal adipose tissue were freeze-clamped in liquid nitrogen <i>in situ</i> and stored at -80°C for further analysis. Aortas of LDLRKO mice were harvested for <i>en face</i> quantification of atherosclerotic lesions.

Blood sample assays. Human and murine serum chemerin levels were determined using commercially available ELISAs (R&D Systems, Wiesbaden, Germany). Plasma insulin levels were measured with an ultrasensitive insulin immunoassay (Alpc0, Salem, MA, USA). Serum total cholesterol, triglyceride and HDL-cholesterol levels were measured enzymatically on an Alcyon 300 Analyzer (Abbott GmbH & Co, Wiesbaden, Germany) using commercial reagents (Diasys, Holzheim, Germany).

Analysis of mRNA expression. Total RNA was extracted as previously described (19). Real-Time PCR was performed using the Realplex<sup>4</sup> Mastercycler (Eppendorf, Hamburg, Germany). Expression levels of genes of interest were determined using the
"Assay on demand" TaqMan-Primer-Probes (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's instructions (see supplemental data for primer/probe information).

**Immunoblotting. Chemerin expression.** One μl of murine and human serum samples were subjected to 10% BisTris gel, transferred to a PVDF membrane (Immobilon-P, Zefa-Laborservice GmbH, Germany), and human chemerin was detected with goat anti-human chemerin antibody (concentration 1:1000, R&D Systems, Wiesbaden, Germany) and peroxidase-conjugated AffiniPureRabbit anti-goat IgG (H+L) (Jackson ImmunoResearch, concentration 1:10,000, Baltimore, USA) as the secondary antibody.

**Insulin signaling and activation of AMPKα.** Liver, skeletal muscle, and adipose tissue samples were homogenized in RIPA-buffer (50mM Tris-HCl, pH 7.4; 1% NP-40; 0.25% Na-deoxycholate; 150mM NaCl; 1mM EDTA; 1mM PMSF; 1µg/ml of each aprotinin, leupeptin, pepstatin; 1mM Na3VO4; 1mM NaF) and lysates were subjected to 10% BisTris gel and transferred to PVDF membrane (Immobilon-P, Zefa-Laborservice GmbH, Germany). Anti Akt1 (C73H10) (concentration 1:1000), Phospho-Akt (Thr308) (C31E5) (concentration 1:1000), AMPKα (23A3) (concentration 1:1000), Phospho-AMPKα (Thr172) (D79.5E) (concentration 1:1000) and anti-rabbit IgG HRP-linked antibodies (concentration 1:10,000) were obtained from Cell Signaling (Danvers, MA, USA). Anti-Actin antibody was purchased from Sigma (Sigma, St. Louis, USA).

**Histological analysis.** For the quantification of the aortic plaques, the mouse aorta was carefully harvested and stained with Sudan IV (Sigma, Germany) as previously described (18,19). Extent of lesion area was determined using Nikon Nis Elements D3.0 (Nikon, Düsseldorf, Germany). Data are reported as the percentage of the aortic surface covered by lesions (total surface area of the atherosclerotic lesions divided by the total surface area of the aorta).

**Statistical Analysis.** Data are expressed as the mean ± SEM. Analysis of the data was performed using the unpaired Student’s t-test. Statistical significance for all comparisons was assigned at p<0.05.

**RESULTS**

To examine the effect of chemerin expression on body weight, glucose and lipid metabolism as well as atherosclerosis, LDLRKO mice on high fat diet were injected with 1x10^{13} particles of AAV.chemerin or control (AAV.lacZ). Injection of AAV.chemerin resulted in specific and sustained chemerin expression over the course of the study with serum levels comparable to those seen in healthy human volunteers (Figure 1A,B). Expression of human chemerin did not alter murine chemerin serum levels (Figure 1C).

Mice injected with AAV.chemerin or AAV.lacZ had a similar change in body weight over the 16 week study period (Figure 1, supplemental data). Compared with control mice, chemerin-expressing mice showed significantly increased glucose levels during both the ipGTT without affecting endogenous insulin levels and the ITT (Figure 2) indicating that overexpression of human chemerin induces insulin resistance in LDLRKO mice. To determine whether expression of chemerin resulted in a tissue-specific pattern of insulin resistance, insulin signaling was examined in liver, adipose tissue and skeletal muscle. Expression of chemerin substantially reduced insulin-stimulated Akt1 phosphorylation and AMPKα activation in the skeletal muscle (Figure 3). In contrast, mice injected with AAV.chemerin showed no effect on basal and insulin-stimulated hepatic Akt1 phosphorylation, AMPKα activation and the mRNA expression of the gluconeogenic enzymes G6P and
PEPCK (Figure 2, supplemental data). In gonadal adipose tissue, expression of chemerin slightly decreased phospho-AMPKα levels in the absence of insulin but did not alter insulin-stimulated AMPKα activation and Akt1 signaling (Figure 3, supplemental data). In conclusion, expression of chemerin induced glucose intolerance via induction of insulin resistance in the skeletal muscle.

Mice expressing chemerin did not show significantly altered total cholesterol, triglyceride, HDL-cholesterol, and non HDL-cholesterol levels compared to controls (Figure 4, supplemental data). Likewise, expression of chemerin did not change the mean atherosclerotic lesion area as determined by en face analysis of the entire aorta (Figure 4).

**DISCUSSION**

Our study was aimed to determine the effect of human chemerin on weight, glucose and lipid metabolism, as well as atherosclerosis in LDLRKO mice on high fat diet. We demonstrate that expression of chemerin with serum levels comparable to those seen in healthy human volunteers induced insulin resistance in the skeletal muscle with increased glucose levels during both the ipGTT and the ITT. Expression of chemerin, however, did not significantly alter weight, lipid levels, and extent of atherosclerotic lesion area.

Ernst et al. (16) reported that acute administration of recombinant human chemerin exacerbated glucose intolerance and lowered serum insulin levels in ob/ob and db/db mice but had no effect on glucose tolerance in wildtype mice on chow diet. Chemerin injection of DIO mice also resulted in impaired glucose tolerance but (except for the 2h timepoint) did not reduce insulin levels during the GTT. Chemerin significantly decreased liver but not skeletal muscle glucose uptake in db/db mice. Consistent with Ernst et al. (16), long-term expression of human chemerin in our diet-induced mouse model of insulin resistance and atherosclerosis resulted in glucose intolerance without altering insulin levels. We did not assess the effect of chemerin on glucose uptake in different tissues. However, given the results of the present study, we would have expected decreased glucose uptake in the skeletal muscle consistent with data from Sell et al. demonstrating that chemerin impaired insulin signaling and glucose uptake in skeletal muscle cells *in vitro* (9). Unfortunately, Ernst et al. (16) did not report the effect of acute chemerin administration on glucose uptake in DIO mice. Further studies including clamp techniques are necessary to better characterize the effect of chemerin on glucose metabolism *in vivo*, and to address the mechanism by which chemerin reduced insulin-stimulated Akt1 phosphorylation and AMPKα activation in the skeletal muscle. *In vitro*, Sell et al. (9) identified activation of p38 MAP kinase, ERK-1/2, and p56 as potential mechanisms that might mediate the effect of chemerin on insulin resistance in skeletal muscle cells.

In contrast to epidemiological data (5,12,13), chemerin had no significant effect on triglyceride and HDL-cholesterol levels in our study. Studies in mice with a more human-like lipoprotein metabolism (e.g. APOE*3-Leiden.CETP transgenic mice) may be required to reproduce the lipid phenotype seen in humans.

Lack of chemerin on atherosclerotic lesion area does not contradict previous studies demonstrating that chemerin modulates activation of immune cells in inflamed tissues of patients with skin disorders (20,21) or mouse models of peritonitis and lung disease (3,17). One could speculate that chemerin may affect early atherosclerotic plaque development and/or plaque morphology rather than extent of atherosclerotic lesion area. In patients with stable chest pain, however, chemerin was not
associated with coronary atherosclerotic plaque morphology as determined by CT-angiography (12).

Chemerin is synthesized as a secreted precursor, prochemerin, which is poorly active on the chemerin receptor CMKLR1 (1). Prochemerin can be rapidly converted into inactive, pro-inflammatory, or anti-inflammatory chemerin variants by proteolytic removal of carboxy-terminal peptides mediated by distinct proteases of the coagulation and inflammation cascades (3,22-24). Unfortunately, the antibodies used in our study for Western blot analysis, human and murine chemerin ELISAs detect prochemerin, chemerin and other proteolytically processed forms but do not allow to distinguish between these peptides. Since distinct chemerin forms differ substantially in their bioactivity, the significance for total chemerin assessment is limited complicating the interpretation of our results. It is reasonable to speculate that since both human and murine chemerin activate the murine chemerin receptor CMKLR1 (17), overall increased chemerin levels resulted in glucose intolerance and insulin resistance in the skeletal muscle of LDLRKO mice on high fat diet. However, it is also conceivable that expression of chemerin altered its enzymatic proteolysis thus modulating both murine and human chemerin bioactivity.

In summary, in the present study we determined the effect of human chemerin on weight, glucose and lipid metabolism, and atherosclerosis in vivo. We demonstrate that expression of chemerin induced insulin resistance in the skeletal muscle of LDLRKO mice on high fat diet. Expression of chemerin, however, did not significantly alter weight, lipid levels, and extent of atherosclerotic lesion area.

Author Contributions. M.B. wrote manuscript, researched data and revised manuscript. K.R. researched data and reviewed/edited manuscript. C.L. researched data, contributed to discussion and reviewed/edited manuscript. J.Z. researched data and reviewed/edited manuscript. B.G. contributed to discussion, reviewed/edited manuscript. K.G.P contributed to discussion, reviewed/edited manuscript. M.L. designed study, contributed to discussion, reviewed/edited manuscript. U.C.B designed study, wrote manuscript and revised manuscript.

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REFERENCES


FIGURE LEGENDS

Figure 1. Western blot analysis of chemerin expression in serum samples of LDLRKO mice 16 weeks after injection with 1x10^13 particles of AAV.lacZ or AAV.chemerin, and a healthy human volunteer (A). Expression of chemerin in LDLRKO mice 16 weeks after injection with 1x10^13 particles of AAV.lacZ or AAV.chemerin (n=12/group), and in three healthy human volunteers as assessed by ELISA (B). Murine chemerin levels in LDLRKO mice 16 weeks after injection with 1x10^13 particles of AAV.lacZ or AAV.chemerin (n=12/group) as assessed by ELISA (C).

Figure 2. Effect of chemerin on glucose (A) and insulin (B) levels during the ipGTT in LDLRKO mice 12 weeks after injection with AAV.lacZ (black triangle) or AAV.chemerin (white square) (n=10/group). Effect of chemerin on glucose levels during the ITT (C) in LDLRKO mice 14 weeks after injection with AAV.lacZ (black triangle) or AAV.chemerin (white square) (n=10/group). *p<0.05, **p=0.01.

Figure 3. Effect of chemerin on basal and insulin-stimulated Akt1 phosphorylation (A, B) and AMPKα activation (C, D) in the skeletal muscle of LDLRKO mice injected with AAV.lacZ or AAV.chemerin (data are representative for n=6/group).

Figure 4. Effect of chemerin on mean atherosclerotic lesion area as determined by en face analysis of the entire aorta in LDLRKO mice injected with AAV.lacZ (n=11) or AAV.chemerin (n=12)
Figure 1

A

LacZ  Chemerin  human serum

α-hChemerin

B

Concentration (ng/ml)

LacZ  Chemerin  human serum

C

Concentration (ng/ml)

LacZ  Chemerin  human serum
Figure 3

A

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B

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Figure 4

A

Lac Z | Chemerin

B

Plaque area (% aortic area)

LacZ (n=11) | Chemerin (n=12)