ChemR23 knockout mice display mild obesity but no deficit in adipocyte differentiation

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Abstract

Chemerin was initially described as a chemoattractant factor for leukocyte populations. More recently, the protein has also been reported to be an adipokine, regulating adipocyte differentiation *in vitro* via its receptor ChemR23, and to be correlated with BMI and other parameters of the metabolic syndrome in humans. The aim of this study was to investigate the role of the chemerin/ChemR23 axis in the regulation of metabolism *in vivo*, using a mouse knockout (KO) model for *ChemR23* (*Cmklr1*) in a C57BL/6 genetic background. Body weight and adipose tissue mass did not differ significantly in young animals, but were significantly higher in *ChemR23* KO mice aged above 12 months. Glucose tolerance was unaffected. No significant modifications in the levels of blood lipids were observed and no increase in the levels of inflammatory markers was observed in the adipose tissue of KO mice. A high-fat diet did not exacerbate the obese phenotype in *ChemR23* KO mice. No obvious defect in adipocyte differentiation was detected, while a marker of lipogenic activity (GPD1 expression) was found to be elevated. In conclusion, the chemerin/ChemR23 system does not appear to play a major role in adipocyte differentiation *in vivo*, but it may be involved in adipose tissue homeostasis.

Key Words

- G-protein-coupled receptor
- adipokines
- inflammation

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Introduction

Obesity and associated disorders, such as type 2 diabetes, metabolic syndrome, and cardiovascular diseases, constitute a rapidly growing health problem in Western countries. According to the World Health Organization, worldwide obesity has doubled since 1980 (Pi-Sunyer 2002, Karlsson & Beck 2010). In this context, a better understanding of adipose tissue biology could contribute to the control of energy metabolism and obesity, at least in the most extreme situations. White adipose tissue (WAT) contributes to endocrine signaling by the secretion of a set of factors, collectively named adipokines,

expressed and secreted by adipocytes and delivered locally and into the systemic circulation. Adipokines have various functions in the control of metabolism, insulin responses, and inflammation (Galic *et al.* 2010). Some adipokines also influence adipose tissue development and inflammatory cell recruitment. Leptin and adiponectin are the most studied members of the adipokine family (Maffei *et al.* 1995, Hu *et al.* 1996), but several new members have been discovered recently (MacDougald & Burant 2007, Wozniak *et al.* 2009, Ouchi *et al.* 2011), and since 2007, chemerin has been included in this family of extracellular

mediators (Bozaoglu *et al.* 2007, Goralski *et al.* 2007, Ernst & Sinal 2010).

Chemerin is a chemoattractant protein synthesized and secreted as an inactive precursor, prochemerin, which is activated by various serine proteases, including cathepsin G, neutrophil elastase, and proteases of the coagulation cascade (Wittamer *et al.* 2003). Chemerin acts via its G-protein-coupled receptor ChemR23, expressed in dendritic cells and macrophages, and displays anti-inflammatory properties (Luangsay *et al.* 2009, Bondue *et al.* 2011). Strong expression of chemerin and ChemR23 in human and murine adipose tissue (Bozaoglu *et al.* 2007, Goralski *et al.* 2007, Mussig *et al.* 2009) indicates that chemerin might also act as an adipokine. In humans, blood chemerin levels have been described to be correlated with BMI, circulating triglyceride levels, blood pressure, and various other parameters of the metabolic syndrome (Goralski *et al.* 2007, Bozaoglu *et al.* 2009, Mussig *et al.* 2009, Sell *et al.* 2010). In mice, the expression of chemerin in adipocytes is upregulated under a high-fat diet (HFD; Roh *et al.* 2007, Ernst *et al.* 2010) and is increased in obese/diabetic *db/db* mice (Ernst *et al.* 2010, Parlee *et al.* 2010) and in diabetic *Psammomys obesus* (Goralski *et al.* 2007). The administration of recombinant chemerin has also been reported to reduce glucose uptake, increasing glucose intolerance in *db/db* mice and diet-induced obesity models (Ernst *et al.* 2010). Recently, Ernst *et al.* (2012) have reported that regardless of diet, *ChemR23* (*Cmklr1*) knock-out (KO) mice display lower food intake, body mass, and body fat percentage, with decreased hepatic and adipose tissue inflammation and relative glucose intolerance. Many *in vitro* studies have reported the effects of the chemerin/ChemR23 system on adipocytes. Chemerin has been proposed to control the differentiation of 3T3-L1 preadipocytes via ChemR23 (Bozaoglu *et al.* 2007, Goralski *et al.* 2007, Roh *et al.* 2007, Muruganandan *et al.* 2010), to promote lipolysis (Roh *et al.* 2007), and to potentiate insulin-stimulated glucose uptake in adipocytes (Takahashi *et al.* 2008). Taken together, these previous studies indicate a role for the chemerin/ChemR23 system in adipose tissue function and metabolism. However, many aspects remain controversial, as results are often conflicting according to studies, even when using very similar models.

The aim of this study was to investigate further *in vivo* whether chemerin and ChemR23 play a role in the regulation of body weight and energy metabolism in mice, by acting on adipocyte differentiation and function, insulin sensitivity, and inflammatory status.

Materials and methods

Mice

As has been described previously (Luangsay *et al.* 2009), *ChemR23* KO mice were obtained from Deltagen (San Mateo, CA, USA) and backcrossed for 13 generations into a C57BL/6 background. Heterozygous mice (*ChemR23*^{+/-}) were then interbred to generate *ChemR23* KO mice (*ChemR23*^{-/-}) and their WT controls (*ChemR23*^{+/+}). The mice were kept in a specific pathogen-free (SPF) environment under a 12 h light:12 h darkness cycle with unlimited access to food and water. The mice were fed standard laboratory diet with 3.5% fat (Carfil, Oud-Turnhout, Belgium). Food intake was recorded in 4-month-old mice as the difference between preweighed food and food remaining 48 h later. Blood and tissue samples were collected under anesthesia at different ages from three different groups of mice to evaluate the effects of invalidation during life: young (12 weeks), middle-aged (8 months), and old (14 months). All the procedures involving animals were reviewed and approved by the local ethics committee (Commission d'Ethique du Bien-Etre Animal, Université Libre de Bruxelles).

Adipose tissue analysis

Inguinal fat pads were collected and weighed, and the right pads were processed for histology or stored at -80 °C for RNA extraction. Histological samples were fixed in 4% paraformaldehyde (PFA), dehydrated, and embedded in paraffin. Sections (7 µm) were stained with hematoxylin and eosin and assessed by light microscopy. The section area of adipocytes was measured using the ImageJ Software (National Institute of Health, Bethesda, MD, USA). Total DNA was extracted from 25 mg of frozen samples of adipose tissue using the DNeasy Kit (Qiagen, Hilden, Germany) and eluted using 200 µl of buffer; the result of the assays was analyzed as an estimate of the total number of cells (adipocytes, immature cells, immune cells, etc.) for the same weight of fat. Total RNA was extracted from frozen tissue using the RNeasy Kit (Qiagen). First-strand cDNA was synthesized using Superscript III reverse transcriptase and random hexamers as primers following the manufacturer's instructions (Invitrogen). Samples of cDNA were diluted 1:25 in nuclease-free water, and real-time quantitative PCR was carried out in a 20 µl volume, containing 8 µl diluted cDNA template, 1.25 µM forward and reverse primers, and PCR SYBR Green I Mix (Qiagen). PCR conditions were 95 °C for 15 min, followed by 40 cycles of 94 °C for 15 s, 58 °C for

20 s, and 72 °C for 20 s in a 7500 Fast Real Time PCR system (Applied Biosystems). After amplification, melting curve analysis was carried out, and relative expression values were calculated using the $\Delta\Delta C_t$ method and the *Canx* and *Ywhaz* genes as controls. The sequences of primers were as follows: *Tnf α* (*Tnf*) forward, 5'-CAGACCCTCACACTCAGATCA-3'; *Tnf α* reverse, 5'-CACTTGGTGGTTTGTCTACGA-3'; *Il1 β* (*Il1b*) forward, 5'-CAACCAACAAGTGATATTCTCCATG-3'; *Il1 β* reverse, 5'-GATCCACACTCTCCAGCTGCA-3'; *Il6* forward, 5'-GAGGATACCACTCCCAACAGACC-3'; *Il6* reverse, 5'-AAGTGCATCATCGTTGTTTCATACA-3'; *Pparg* (*Pparg*) forward, 5'-TCCGAAGAACCATCCGATT-3'; *Pparg* reverse, 5'-TTTGTGGATCCGGCAGTTAAG-3'; *Dlk1* forward, 5'-CGTGATCAATGGTTCTCCCT-3'; *Dlk1* reverse, 5'-AGGGGTACAGCTGTTGGTTG-3'; *lipin 1* forward, 5'-CCATTCACAGCGAGTCTTCA-3'; *lipin 1* reverse, 5'-TGGAAGGGGAATCTGTCTTG-3'; *Gpd1* forward, 5'-ATGCTCGCACAGAATCCACAC-3'; *Gpd1* reverse, 5'-AACCGGCAGCCCTTGACTTG-3'; *Lpl* forward, 5'-GGGCTCTGCCTGAGTTGTAG-3'; *Lpl* reverse, 5'-CCATCCTCAGTCCAAGAA-3'; *chemerin* (*Rarres2*) forward, 5'-CCAACTGCCCAAGAAGGA-3'; *chemerin* (*Rarres2*) reverse, 5'-AATGCAGGCCAGGCATTTC-3'; *ChemR23* forward, 5'-GTTCAACATCTTTTGGCCGAT-3'; *ChemR23* reverse, 5'-TCCCGAACAACCAGTGGTA-3'; *Gpr1* forward, 5'-GTGGTCATTGCCTTCTTGGT-3'; *Gpr1* reverse, 5'-GGCTCTTGTTTCAGCACTC-3'; *Canx* forward, 5'-TTGCTGACTCCTTTGACAGAGG-3'; *Canx* reverse, 5'-CCACTTCCATCATATTTGGCA-3'; *Ywhaz* forward, 5'-TGCAACGATGTACTGTCTCTTTTG-3'; and *Ywhaz* reverse, 5'-CGGTAGTAGTCACCCTTCATTTTCA-3'.

In vitro studies

Freshly collected adipose tissue samples were digested using collagenase I (Sigma–Aldrich) at 37 °C for 40–60 min, with intermittent pipetting. The suspension was added to 30 ml DMEM/F12 and passed through a 100 μ m filter. The filtrate was centrifuged, and the pellet was resuspended in DMEM/F12. Red blood cells were lysed using RBC lysis buffer, and after lavage, the final pellet was resuspended in 1 ml of 3% DMEM/calf serum and 300 000 cells were seeded per each well in a 12-well plate and incubated at 37 °C in a 5% CO₂ incubator. After 1 week, adipocyte differentiation was induced by the addition of 5 μ g/ml insulin, 50 mM indomethacin, 1 μ g/ml dexamethasone, 100 mM 3-isobutyl-1-methylxanthine (IBMX) in 3% DMEM/calf serum for 1 week. The cells were fixed using 4% PFA, and lipid droplets were stained using Oil Red O to quantify differentiation. Microscopic images

were taken using an inverted light microscope, and the number of differentiated cells was determined using the ImageJ Software and compared with the total number of cells in each well.

Cytokine assays

The mice that were fasted for 16 h were anesthetized using thiopental (10 mg/kg), and blood was collected from the portal vein. Serum was immediately separated and frozen at –20 °C until use. Triglyceride and cholesterol levels were determined on an automated clinical chemistry analyzer in the Erasme Hospital. Insulin (Mercodia, Uppsala, Sweden), and leptin, adiponectin, and chemerin (R&D Systems, Abington, UK) levels were determined by ELISA, according to the manufacturer's instructions. Serum samples collected from the HFD-fed mice were assayed for TNF α and IL6 levels using a cytometric bead array-based immunoassay (CBA Flex Sets, BD Biosciences Europe, Erembodegem, Belgium), a dual-laser flow cytometer FACSCalibur (BD Biosciences Europe), and the FCAP Array Software (BD Biosciences Europe), following the manufacturer's instructions.

Indirect calorimetry

The mice were analyzed for whole energy expenditure, oxygen consumption, and carbon dioxide production (VO₂ and VCO₂, where V is volume), respiratory exchange rate (RER = VCO₂/VO₂), and spontaneous activities using calorimetric cages with bedding, food, and water (Labmaster, TSE Systems GmbH, Bad Homburg, Germany). The mice were allowed to acclimatize in individual cages for 48 h before the experiment. Data were recorded every 40 min during the entire experiment. The ratio of gas concentrations was determined using an indirect open-circuit calorimeter (Arch *et al.* 2006), which can monitor O₂ and CO₂ concentrations. The flow was calibrated with O₂ and CO₂ mixtures of known concentrations before the start of the experiment. Total energy expenditure was calculated according to the Weir equation (Weir 1949) normalized to lean body mass. The mice had free access to food and water *ad libitum*. Cages were equipped with infrared light beams, allowing the measurement of the locomotor activity of mice throughout the experiment.

High-fat diet

For 20 weeks (starting at the age of 5 weeks), the mice were fed a HFD in which 46% of the calories were derived from

lipids (Purified diet 235HF, Safe, Augy, France). Control mice were fed a standard pellet diet in which 5% of the calories were provided as fat (Diet 210, Safe). The mice were weighed weekly until the 19th week, and blood was collected under anesthesia after 20 weeks. After coagulation, the serum was separated and frozen at -20°C until use. Inguinal fat pads were frozen in liquid nitrogen and stored at -80°C for subsequent quantitative RT-PCR (qRT-PCR) analysis.

Insulin and glucose tolerance tests

Insulin and glucose tolerance tests were carried out on 8-week-old mice fasted for respectively 4 and 16 h. After an i.p. injection of recombinant human insulin (Actrapid, Novo Nordisk, Copenhagen, Denmark; 0.5 mU/g body weight) or glucose (2 mg/g body weight), blood was collected from the tail vein and glucose levels were measured using a glucometer (One Touch Ultra, Lifescan, Beerse, Belgium) before and 30, 60, and 120 min after glucose injection or 30, 45, and 60 min after insulin injection.

Statistical analyses

Data are expressed as means $\pm$ S.E.M. Unpaired two-tailed Student's *t*-test, two-way ANOVA, or Mann-Whitney *U* tests were used to determine statistical significance depending on the normality of the data. All the statistical analyses were carried out using Statview (SAS Institute, Cary, NC, USA, v 5.0.1) considering a *P* value of <0.05 as significant.

Results

Progressive and moderate obesity develops in ChemR23 KO mice

To determine the role of the chemerin/ChemR23 system in metabolism in mice, we first measured the body weight of ChemR23 KO mice and their littermate controls fed a regular (low-fat) chow at three different endpoints in three different groups of male mice: young (12 weeks), middle age (8 months), and old (14 months), to determine any difference depending on age. As shown in Fig. 1A, no significant difference was observed in 12-week-old mice, but the weight of male KO mice increased faster than that of control mice, and by 14 months, their weight was, on average, 10% higher than that of control mice (31.9 ± 0.8 vs 28.8 ± 0.7 g, respectively, $P=0.01$). Interestingly, this moderately obese phenotype was absent in female mice

(Supplementary Figure 1, see section on supplementary data given at the end of this article). Food intake was recorded in 4-month-old male mice, but no significant difference could be observed (Fig. 1B). Inguinal fat pads were collected from 12-week-, 8-week-, and 14-month-old male mice, as a marker of WAT mass. The weight of the fat pads was higher in ChemR23 KO mice at 8 months and was 60% higher at 14 months (Fig. 1C, $P=0.0079$). Histological examination of fat tissue (Fig. 1D) revealed adipocytes of increased diameter in 14-month-old KO mice (Fig. 1E, $P=0.02$), while the estimation of the number of cells in WAT by the quantification of DNA content revealed no apparent change in this parameter (Fig. 1F).

Blood lipid, glucose, and insulin levels were measured in 12-week-old (before the onset of mild obesity in ChemR23 KO mice) and 14-month-old, regular chow-fed male mice that were overweight. Cholesterol levels were found to change with age, but were not affected by the genotype (Table 1). Similarly, triglyceride values, glucose levels, and insulin levels were not significantly different between the ChemR23 KO and WT mice.

Because activity-related energy expenditure is a main contributor to energy balance, we examined locomotor activity and energy expenditure by indirect calorimetry in 4-month-old male mice (before the onset of the mild obesity phenotype in ChemR23 KO mice). No difference was detected in ambulatory activity in the dark and light phases (Supplementary Figure 2A, see section on supplementary data given at the end of this article), carbon dioxide production (Supplementary Figure 2B), oxygen consumption (Supplementary Figure 2C), or respiratory quotient (Supplementary Figure 2D), indicating that the lack of ChemR23 does not modify the type of substrate used for energy consumption.

The circulating levels of chemerin and two other major adipokines (leptin and adiponectin) were measured in 14-month-old WT and KO male mice. As shown in Fig. 2, serum adiponectin levels were similar in both the genotypes (Fig. 2A), and serum leptin levels were increased in ChemR23 KO mice without reaching significance (Fig. 2B). Interestingly, the total chemerin levels were decreased by 34% in ChemR23 KO mice (Fig. 2C, 316 ± 25 vs 210 ± 23 ng/ml for control mice, $P=0.029$), despite the higher body weight of these mice.

Influence of HFD

To determine whether ChemR23 KO mice would display more overt obesity on being fed hypercaloric diets, mice (5 weeks old) were fed a HFD (46% lipid-derived calories)

Figure 1

Body weight and adipose tissue mass of *ChemR23* KO mice. (A) Male mice were weighed at 12 weeks ($n=6$ per group), 8 months ($n=4$ per group), and 14 months (WT, $n=8$; KO, $n=15$). (B) Daily food intake was recorded in 4-month-old WT and KO male mice ($n=6$ per group). (C) Inguinal adipose tissue samples were collected from 12-week-old ($n=6$ per group), 8-week-old ($n=4$ per group), and 14-month-old (WT, $n=8$; KO, $n=15$) male mice and weighed. (D) Histology of inguinal adipose tissue samples collected from 14-month-old WT and *ChemR23* KO mice was studied after

hematoxylin and eosin staining of paraffin sections (original magnification, $20\times$). (E) Mean section area of adipocytes was determined in the sections collected from WT and *ChemR23* KO mice (WT, $n=4$; KO, $n=6$). (F) DNA content (ng) of the adipose tissue of WT and *ChemR23* KO mice was determined ($n=8$ per group). Black columns, WT mice; white columns, *ChemR23* KO mice. The unpaired two-tailed Student's *t*-test was used, $*P<0.05$.

for 20 weeks. As shown in Fig. 3A, the HFD-fed WT and *ChemR23* KO mice exhibited a higher increase in body weight compared with their respective controls fed a standard diet after 19 weeks. However, weight increment was not higher in *ChemR23* KO mice. After 19 weeks, there

was no difference in the body weight of the standard diet-fed WT mice and HFD-fed mice. An increase in the levels of cholesterol (Fig. 3B), mainly associated with LDLs was observed, with no difference being observed with regard to genotype.

Table 1 Metabolic parameters of 12-week-old and 14-month-old WT and *ChemR23* KO mice. $n=4-8$ per group

	WT mice (12 weeks)	<i>ChemR23</i> KO mice (12 weeks)	WT mice (14 months)	<i>ChemR23</i> KO mice (14 months)
Cholesterol (mg/dl)	96.0 $\pm$ 2.6	102 $\pm$ 6.7	64.7 $\pm$ 5.2	70.2 $\pm$ 6.2
Triglycerides (mg/dl)	25.7 $\pm$ 13.7	38.0 $\pm$ 11.7	34.9 $\pm$ 3.9	26.4 $\pm$ 7.5
Glucose (mg/dl)	120 $\pm$ 5	93 $\pm$ 2	93 $\pm$ 6	91 $\pm$ 3
Insulin (μ g/l)	0.53 $\pm$ 0.03	0.66 $\pm$ 0.06	0.65 $\pm$ 0.07	0.51 $\pm$ 0.02

Figure 2

Blood adipokine levels. Adiponectin (A), leptin (B), and total chemerin (C) levels were measured by ELISA of the serum of 14-month-old WT (black columns, $n=8$) and KO (white column, $n=15$) mice. The nonparametric Mann–Whitney U test was used, $*P<0.05$.

Although blood chemerin levels were not affected by HFD (Fig. 3C), chemerin transcript levels in adipose tissue were significantly increased in the HFD-fed mice independent of genotype (Fig. 3D), in agreement with the findings reported by Roh *et al.* (2007), and this diet led to an increase in *ChemR23* transcript levels in the adipose tissue of WT mice (Fig. 3E); data for *ChemR23* KO mice are not reported because no transcripts were detected. The inflammatory status of the HFD-fed mice was evaluated after 20 weeks, but neither the circulating levels of $\text{TNF}\alpha$ and IL6 (Fig. 3F) nor the relative expression of $\text{TNF}\alpha$ and IL1 (Fig. 3G) in adipose tissue was altered. Therefore, it appears that under the conditions of our experiment, *ChemR23* deficiency and HFD led to an increased body weight gain, but these effects did not appear to be additive.

Figure 3

Effects of high-fat diet (HFD) on the body weight, blood lipids, chemerin/ChemR23 expression, and proinflammatory cytokines in adipose tissue. Body weight (A) was measured once per week in CTL WT (black columns, $n=4$), HF WT (black columns, $n=9$), CTL *ChemR23* KO (white columns, $n=5$), and HF *ChemR23* KO (white columns, $n=10$) mice after 4 and 20 weeks of diet. Blood lipid parameters were analyzed after 20 weeks in (B) CTL WT (black columns, $n=4$), HF WT (dashed black columns, $n=9$), CTL *ChemR23* KO (white columns, $n=5$), and HF *ChemR23*

KO (dashed white columns, $n=10$) mice. Two-way ANOVA followed by Fisher's *post hoc* test was used, $*P<0.05$. Chemerin (C) and $\text{TNF}\alpha$ and IL6 (F) levels were measured by ELISA of the serum of WT (black columns) and *ChemR23* KO (white columns) mice after 20 weeks of being fed the control (CTL) diet or HFD. Relative transcript levels of chemerin (D), *ChemR23* (E), and $\text{TNF}\alpha$ and IL1 (G) were estimated by qRT-PCR of the adipose tissue of the same groups (WT, $n=5$ per group; KO, $n=7$ per group). The nonparametric Mann–Whitney U test was used, $*P<0.05$.

Role of the chemerin/ChemR23 system in glucose metabolism

To investigate the role played by the chemerin/ChemR23 system in glucose homeostasis, we carried out tolerance tests for glucose and insulin in 8-week-old WT and *ChemR23* KO mice. At this age, there is no significant difference in body weight. After i.p. injection of glucose, a strong increase in glucose levels was observed at 30 min, followed by a progressive return to baseline by 120 min (Fig. 4A and B). No difference in the hyperglycemia curves or in the insulin levels measured 30 and 60 min after glucose injection was observed between WT and *ChemR23* KO mice (Fig. 4C). Following insulin injection, glucose levels dropped moderately in WT and *ChemR23* KO mice (Fig. 4D and E). All these data call into question the anti-insulin properties of the chemerin/ChemR23 system *in vivo*.

Adipocyte differentiation and activity markers in *ChemR23* KO mice

The qRT-PCR analysis of WAT markers (Fig. 5A) indicated an increased expression of glycerol phosphate dehydrogenase (GPD1), an enzyme involved in lipogenesis, in

14-month-old *ChemR23* KO mice, but no changes in the expression of peroxisome proliferator-activated receptor γ (PPAR γ) or lipoprotein lipase, implicated in lipolysis, were detected. No differences in the expression of the preadipocyte marker preadipocyte factor 1 (PREP1, *Dlk1*) and lipin 1 were observed, suggesting that ChemR23 is not essential for adipocyte maturation *in vivo*. *ChemR23* KO mice exhibited the same level of expression of GPR1 in adipose tissue. The relative expression of proinflammatory cytokines IL1 β , IL6, and TNF α , and chemokine MCP1 (CCL2) was similar for both the genotypes (Fig. 5B). Moreover, *in vitro* culture of preadipocytes obtained from WT and *ChemR23* KO mice resulted in the same level of differentiation after the addition of insulin, dexamethasone, indomethacin, and IBMX (Fig. 5C and D).

Discussion

In this study, we investigated the role of ChemR23, a G-protein-coupled receptor expressed in human and murine adipose tissue, in glucose and lipid metabolism *in vivo*. ChemR23 is the receptor of chemerin, described as a new adipokine. Under the standard environmental conditions and diet used in the present study, male (but not female) *ChemR23* KO mice developed a moderate

Figure 4

Results of glucose and insulin tolerance tests. A glucose tolerance test was carried out in 8-week-old mice after a 16-h fast by i.p. injection of glucose (2 mg/g body weight). Glucose levels were measured at 30, 60, and 120 min (A, $n=8$ per group) and area under the curve was calculated (B). Blood insulin levels were measured by ELISA at 30 and 60 min (C, $n=5$ per group). An insulin tolerance test was carried out after a 16-h fast by i.p. injection of human insulin (0.5 mU/g body weight). Glucose levels were measured at 20, 30, 45, 60, and 120 min (D, $n=12$ per group) and area under the curve was calculated (E). Squares and black columns, WT mice; circles and white columns, *ChemR23* KO mice. Nonparametric Mann-Whitney U test was used.

Figure 5

Differentiation and inflammation markers in the adipose tissue of *ChemR23* KO and WT mice. The relative expression of the markers of (A) adipocyte differentiation (PREF1, lipin1, and PPAR γ), lipogenesis (GPDH), and lipolysis (LPL) and (B) inflammation (IL1 β , IL6, TNF α , MCP1, ChemR23, GPR1, and chemerin) in the adipose tissue of 14-month-old WT (black columns, $n=7$) and *ChemR23* KO (white columns, $n=9$) mice was measured by qRT-PCR. The unpaired two-tailed Student's t -test was used, $*P<0.05$. (C) Differentiation of the preadipocytes of WT and *ChemR23* KO mice after staining of lipid droplets using Oil Red O (original magnification, 20 $\times$). (D) Representative quantification (%) of the differentiated cells in WT and *ChemR23* KO mice. Black columns, WT mice; white columns, *ChemR23* KO mice.

mature-onset obesity phenotype when compared with their WT littermates. This obesity is associated with a significant increase in body fat content. We did not observe any change in food intake or a decrease in energy expenditure that might explain the progressive increase in body weight. However, given the slow development of this obesity, the difference in energy balance might remain below the level of detection, particularly for food intake, which is difficult to assess precisely. This phenotype is not in agreement with the recently published data reporting lower body mass, body fat percentage, and food intake in *ChemR23* KO mice (Ernst *et al.* 2012). Differences in regimen might explain these differences. Indeed, the standard low-fat diets used in both the studies differ (3.5% kcal from fat in the present study vs 10% in the study of Ernst *et al.* (2012)). In the present study, we also observed an influence of sex, as obesity did not reach significance in female mice, and therefore data were collected only for male mice. Ernst *et al.* (2012) did not report the sex of animals used or differences according to sex.

Histological analyses of adipose tissue indicated hypertrophy of adipocytes in *ChemR23* KO mice. There was also overexpression of the lipogenic enzyme GPD1, a major actor in lipid biogenesis acting at the level of

glycerol synthesis, suggesting a role for ChemR23 in the negative control of lipogenesis. Previous *in vitro* studies have shown that recombinant chemerin could enhance lipolysis in adipocytes (Roh *et al.* 2007), while knockdown of the gene encoding chemerin has been reported to inhibit lipolysis (Goralski *et al.* 2007). These *in vitro* data are consistent with the *in vivo* results of the present study and further support the hypothesis that chemerin has an important role in the negative control of the lipolytic function of adipocytes.

Recently, a chemerin-dependent exacerbation of glucose intolerance has been observed in mouse models of obesity and diabetes (Ernst *et al.* 2010), while, conversely, glucose intolerance has also been reported in *ChemR23* KO mice (Ernst *et al.* 2012). In the present study, the KO of *ChemR23* led to increased body weight but not to basal hyperglycemia or deficient insulin secretion, even in the context of glucose or insulin tolerance tests. In 14-month-old mice, no difference in basal insulin levels was observed, despite the difference in fat mass between WT and *ChemR23* KO mice. Ernst *et al.* (2012) reported a reduction in glucose-induced insulin secretion *in vivo*, but no effect of *ChemR23* knockdown on insulin release in murine MIN6 pancreatic β -cells, suggesting that the involvement of the chemerin/ChemR23 system might be indirect.

Mice fed a HFD have been characterized as a model of obesity and associated type 2 diabetes (Winzell & Ahren 2004). Such a regimen indeed leads to increased body weight gain and progressive alteration of glucose tolerance. On being fed a HFD, no difference in body weight and cholesterol levels were observed between *ChemR23* KO and control mice, indicating that the chemerin/ChemR23 system has more effect under normocaloric conditions. HFD promoted the upregulation of chemerin expression in WAT, in accordance with the findings reported by Bauer *et al.* (2011), and ChemR23 expression was also upregulated. The upregulation of chemerin expression in adipose tissue was not associated with higher chemerin levels in blood, suggesting that WAT might not represent the main source of circulating chemerin, even in mice with increased adipose tissue mass. Despite the increase in fat mass, we did not observe an increase in local or circulating levels of cytokines or their transcripts.

Previous *in vitro* studies have established a correlation between adipocyte differentiation stage and chemerin expression levels (Bozaoglu *et al.* 2007, Goralski *et al.* 2007, Roh *et al.* 2007, Takahashi *et al.* 2008) and indicated a major role for the chemerin/ChemR23 axis in the control of the differentiation of mesenchymal stem cells into mature adipocytes (Muruganandan *et al.* 2010, 2011). The differentiation of preadipocytes into adipocytes requires the suppression of the signaling of the preadipocyte marker PREF1 (Sul *et al.* 2000, Sul 2009). In the present study, the adipose tissue of *ChemR23* KO mice appeared to be mature and functional. Similar expression levels of PREF1, lipin 1, and the adipocyte differentiation marker PPAR γ were observed in both the genotypes. Fat tissue mass was observed to increase in (male) KO mice, with no apparent change in the number of cells, but with an increase in lipid accumulation in each cell. Such observations do not appear to support the conclusions of previous *in vitro* studies and the major role of chemerin and ChemR23 in adipocyte differentiation. Moreover, the results of our *in vitro* studies indicate a similar differentiation of preadipocytes from the adipose tissue of WT and *ChemR23* KO mice (Fig. 5C and D). To draw conclusions regarding the real role of the chemerin/ChemR23 system in adipocyte differentiation, more investigations by independent groups should be carried out in the future using primary cultures of mesenchymal stem cells or preadipocytes obtained from WT and *ChemR23* KO mice.

Studies in humans have demonstrated a strong correlation between circulating chemerin levels and

markers of inflammation and the metabolic syndrome (Lehrke *et al.* 2009, Spiroglou *et al.* 2010, Weigert *et al.* 2010, Dong *et al.* 2011, Gao *et al.* 2011, Ernst *et al.* 2012). Yet, the circulating levels of TNF α and expression of proinflammatory cytokines in adipose tissue were not increased in *ChemR23*-deficient mice, even after they had been fed a HFD for 20 weeks. In their recent study, Ernst *et al.* (2012) have suggested that ChemR23 could be involved in the recruitment of leukocytes to the liver and WAT, as HFD-fed *ChemR23* KO mice displayed lower levels of TNF α in the liver and lower levels of TNF α and IL6 in WAT when compared with WT control mice. No such finding was observed in our study. Differences between the two studies include the diets fed to the mice. We chose to use a moderately HFD (46% lipid-derived calories), because we foresaw that *ChemR23* KO mice might display an increased sensitivity to such a diet, while Ernst *et al.* used a 60% lipid-derived diet. Also, as has been stated above, it is unclear whether male or female mice (or both) were used in the latter study, and we observed sex-specific differences in terms of body weight gain. Finally, Ernst *et al.* did not describe the nature of the control group (littermates or independent animals of the C57BL/6 genetic background), and this may significantly affect the results. Throughout our study, we used as controls age-matched WT mice derived from the same breeding colony as the KO mice. Finally, the sanitary status of the animals might contribute to differences, given the role of chemerin in inflammation. The mice used in the present study were maintained in a SPF environment, while the sanitary status of the animals was not specified by Ernst *et al.*

In summary, through this study, we provide evidence that the chemerin/ChemR23 system is not essential for adipocyte differentiation *in vivo*, but that it plays a role in the control of body weight and energy metabolism. However, differences in the results of this study and other reports in the literature, as well as between published reports, suggest that this role is complex, associated with other signaling pathways and that the net effect is highly dependent on the precise experimental setting. If we extrapolate our data to correlations previously made for human populations, the overproduction of chemerin in obese patients might constitute an adaptive pathway aimed at correcting metabolic imbalance.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JOE-13-0106>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References

- Arch JR, Hislop D, Wang SJ & Speakman JR 2006 Some mathematical and technical issues in the measurement and interpretation of open-circuit indirect calorimetry in small animals. *International Journal of Obesity* **30** 1322–1331. (doi:10.1038/sj.ijo.0803280)
- Bauer S, Wanninger J, Schmidhofer S, Weigert J, Neumeier M, Dorn C, Hellerbrand C, Zimara N, Schaffler A, Aslanidis C *et al.* 2011 Sterol regulatory element-binding protein 2 (SREBP2) activation after excess triglyceride storage induces chemerin in hypertrophic adipocytes. *Endocrinology* **152** 26–35. (doi:10.1210/en.2010-1157)
- Bondue B, Wittamer V & Parmentier M 2011 Chemerin and its receptors in leukocyte trafficking, inflammation and metabolism. *Cytokine & Growth Factor Reviews* **22** 331–338. (doi:10.1016/j.cytogfr.2011.11.004)
- Bozaoglu K, Bolton K, McMillan J, Zimmet P, Jowett J, Collier G, Walder K & Segal D 2007 Chemerin is a novel adipokine associated with obesity and metabolic syndrome. *Endocrinology* **148** 4687–4694. (doi:10.1210/en.2007-0175)
- Bozaoglu K, Segal D, Shields KA, Cummings N, Curran JE, Comuzzie AG, Mahaney MC, Rainwater DL, VandeBerg JL, MacCluer JW *et al.* 2009 Chemerin is associated with metabolic syndrome phenotypes in a Mexican-American population. *Journal of Clinical Endocrinology and Metabolism* **94** 3085–3088. (doi:10.1210/jc.2008-1833)
- Dong B, Ji W & Zhang Y 2011 Elevated serum chemerin levels are associated with the presence of coronary artery disease in patients with metabolic syndrome. *Internal Medicine* **50** 1093–1097. (doi:10.2169/internalmedicine.50.5025)
- Ernst MC & Sinal CJ 2010 Chemerin: at the crossroads of inflammation and obesity. *Trends in Endocrinology and Metabolism* **21** 660–667. (doi:10.1016/j.tem.2010.08.001)
- Ernst MC, Issa M, Goralski KB & Sinal CJ 2010 Chemerin exacerbates glucose intolerance in mouse models of obesity and diabetes. *Endocrinology* **151** 1998–2007. (doi:10.1210/en.2009-1098)
- Ernst MC, Haidl ID, Zuniga LA, Dranse HJ, Rourke JL, Zabel BA, Butcher EC & Sinal CJ 2012 Disruption of the chemokine-like receptor-1 (CMKRL1) is associated with reduced adiposity and glucose intolerance. *Endocrinology* **153** 672–682. (doi:10.1210/en.2011-1490)
- Galic S, Oakhill JS & Steinberg GR 2010 Adipose tissue as an endocrine organ. *Molecular and Cellular Endocrinology* **316** 129–139. (doi:10.1016/j.mce.2009.08.018)
- Gao X, Mi S, Zhang F, Gong F, Lai Y, Gao F, Zhang X, Wang L & Tao H 2011 Association of chemerin mRNA expression in human epicardial adipose tissue with coronary atherosclerosis. *Cardiovascular Diabetology* **10** 87. (doi:10.1186/1475-2840-10-87)
- Goralski KB, McCarthy TC, Hanniman EA, Zabel BA, Butcher EC, Parlee SD, Muruganandan S & Sinal CJ 2007 Chemerin, a novel adipokine that regulates adipogenesis and adipocyte metabolism. *Journal of Biological Chemistry* **282** 28175–28188. (doi:10.1074/jbc.M700793200)
- Hu E, Liang P & Spiegelman BM 1996 AdipoQ is a novel adipose-specific gene dysregulated in obesity. *Journal of Biological Chemistry* **271** 10697–10703. (doi:10.1074/jbc.271.18.10697)
- Karlsson EA & Beck MA 2010 The burden of obesity on infectious disease. *Experimental Biology and Medicine* **235** 1412–1424. (doi:10.1258/ebm.2010.010227)
- Lehrke M, Becker A, Greif M, Stark R, Laubender RP, von Ziegler F, Lebherz C, Tittus J, Reiser M, Becker C *et al.* 2009 Chemerin is associated with markers of inflammation and components of the metabolic syndrome but does not predict coronary atherosclerosis. *European Journal of Endocrinology* **161** 339–344. (doi:10.1530/EJE-09-0380)
- Luangsay S, Wittamer V, Bondue B, De Henau O, Rouger L, Brait M, Franssen JD, de Nadai P, Huaux F & Parmentier M 2009 Mouse ChemR23 is expressed in dendritic cell subsets and macrophages, and mediates an anti-inflammatory activity of chemerin in a lung disease model. *Journal of Immunology* **183** 6489–6499. (doi:10.4049/jimmunol.0901037)
- MacDougald OA & Burant CF 2007 The rapidly expanding family of adipokines. *Cell Metabolism* **6** 159–161. (doi:10.1016/j.cmet.2007.08.010)
- Maffei M, Fei H, Lee GH, Dani C, Leroy P, Zhang Y, Proenca R, Negrel R, Ailhaud G & Friedman JM 1995 Increased expression in adipocytes of *ob* RNA in mice with lesions of the hypothalamus and with mutations at the *db* locus. *PNAS* **92** 6957–6960. (doi:10.1073/pnas.92.15.6957)
- Muruganandan S, Parlee SD, Rourke JL, Ernst MC, Goralski KB & Sinal CJ 2010 Chemerin, a novel peroxisome proliferator-activated receptor γ (PPAR γ) target gene that promotes mesenchymal stem cell adipogenesis. *Journal of Biological Chemistry* **286** 23982–23995. (doi:10.1074/jbc.M111.220491)
- Muruganandan S, Roman AA & Sinal CJ 2011 Role of chemerin/CMKLR1 signaling in adipogenesis and osteoblastogenesis of bone marrow stem cells. *Journal of Bone and Mineral Research* **25** 222. (doi:10.1359/jbmr.091106)
- Mussig K, Staiger H, Machicao F, Thamer C, Machann J, Schick F, Claussen CD, Stefan N, Fritsche A & Haring HU 2009 *RARRES2* encoding the novel adipokine chemerin, is a genetic determinant of disproportionate regional body fat distribution: a comparative magnetic resonance imaging study. *Metabolism* **58** 519–524. (doi:10.1016/j.metabol.2008.11.011)
- Ouchi N, Parker JL, Lugus JJ & Walsh K 2011 Adipokines in inflammation and metabolic disease. *Nature Reviews. Immunology* **11** 85–97. (doi:10.1038/nri2921)
- Parlee SD, Ernst MC, Muruganandan S, Sinal CJ & Goralski KB 2010 Serum chemerin levels vary with time of day and are modified by obesity and tumor necrosis factor- α . *Endocrinology* **151** 2590–2602. (doi:10.1210/en.2009-0794)
- Pi-Sunyer FX 2002 The obesity epidemic: pathophysiology and consequences of obesity. *Obesity Research* **10**(Suppl 2) 97S–104S. (doi:10.1038/oby.2002.202)
- Roh SG, Song SH, Choi KC, Katoh K, Wittamer V, Parmentier M & Sasaki S 2007 Chemerin – a new adipokine that modulates adipogenesis via its own receptor. *Biochemical and Biophysical Research Communications* **362** 1013–1018. (doi:10.1016/j.bbrc.2007.08.104)
- Sell H, Divoux A, Poitou C, Basdevant A, Bouillot JL, Bedossa P, Tordjman J, Eckel J & Clement K 2010 Chemerin correlates with markers for fatty liver in morbidly obese patients and strongly decreases after weight loss induced by bariatric surgery. *Journal of Clinical Endocrinology and Metabolism* **95** 2892–2896. (doi:10.1210/jc.2009-2374)
- Spiroglou SG, Kostopoulos CG, Varakis JN & Papadaki HH 2010 Adipokines in periaortic and epicardial adipose tissue: differential expression and relation to atherosclerosis. *Journal of Atherosclerosis and Thrombosis* **17** 115–130. (doi:10.5551/jat.1735)
- Sul HS 2009 Minireview: Pref-1: role in adipogenesis and mesenchymal cell fate. *Molecular Endocrinology* **23** 1717–1725. (doi:10.1210/me.2009-0160)

- Sul HS, Smas C, Mei B & Zhou L 2000 Function of pref-1 as an inhibitor of adipocyte differentiation. *International Journal of Obesity and Related Metabolic Disorders* **24**(Suppl 4) S15–S19. (doi:10.1038/sj.ijo.0801494)
- Takahashi M, Takahashi Y, Takahashi K, Zolotaryov FN, Hong KS, Kitazawa R, Iida K, Okimura Y, Kaji H, Kitazawa S *et al.* 2008 Chemerin enhances insulin signaling and potentiates insulin-stimulated glucose uptake in 3T3-L1 adipocytes. *FEBS Letters* **582** 573–578. (doi:10.1016/j.febslet.2008.01.023)
- Weigert J, Neumeier M, Wanninger J, Filarsky M, Bauer S, Wiest R, Farkas S, Scherer MN, Schaffler A, Aslanidis C *et al.* 2010 Systemic chemerin is related to inflammation rather than obesity in type 2 diabetes. *Clinical Endocrinology* **72** 342. (doi:10.1111/j.1365-2265.2009.03664.x)
- Weir JB 1949 New methods for calculating metabolic rate with special reference to protein metabolism. *Journal of Physiology* **109** 1–9.
- Winzell MS & Ahren B 2004 The high-fat diet-fed mouse: a model for studying mechanisms and treatment of impaired glucose tolerance and type 2 diabetes. *Diabetes* **53**(Suppl 3) S215–S219. (doi:10.2337/diabetes.53.suppl_3.S215)
- Wittamer V, Franssen JD, Vulcano M, Mirjolet JF, Le Poul E, Migeotte I, Brezillon S, Tyldesley R, Blanpain C, Detheux M *et al.* 2003 Specific recruitment of antigen-presenting cells by chemerin, a novel processed ligand from human inflammatory fluids. *Journal of Experimental Medicine* **198** 977–985. (doi:10.1084/jem.20030382)
- Wozniak SE, Gee LL, Wachtel MS & Frezza EE 2009 Adipose tissue: the new endocrine organ? A review article *Digestive Diseases and Sciences* **54** 1847–1856. (doi:10.1007/s10620-008-0585-3)

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