Sterol Regulatory Element-Binding Protein 2 (SREBP2) Activation after Excess Triglyceride Storage Induces Chemerin in Hypertrophic Adipocytes

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Chemerin is an adipokine whose systemic concentration and adipose tissue expression is increased in obesity. Chemerin is highly abundant in adipocytes, yet the molecular mechanisms mediating its further induction in obesity have not been clarified. Adipocyte hypertrophy contributes to dysregulated adipokine synthesis, and we hypothesized that excess loading with free fatty acids (FFA) stimulates chemerin synthesis. Chemerin was expressed in mature adipocytes, and differentiation of 3T3-L1 cells in the presence of FFA further increased its level. TNF and IL-6 were induced by FFA, but concentrations were too low to up-regulate chemerin. Sterol regulatory element-binding protein 2 (SREBP2) was activated in these cells, indicative for cholesterol shortage. Suppression of cholesterol synthesis by lovastatin led to activation of SREBP2 and increased chemerin, and supplementation with mevalonate reversed this effect. Knockdown of SREBP2 reduced basal and FFA-induced chemerin. EMSA confirmed binding of 3T3-L1 adipocyte nuclear proteins to a SREBP site in the chemerin promotor. SREBP2 was activated and chemerin was induced in adipose tissue of mice fed a high-fat diet, and higher systemic levels seem to be derived from adipocytes. Lipopolysaccharide-mediated elevation of chemerin was similarly effective as induction by FFA, indicating that both mechanisms are equally important. Chemokine-like receptor 1 was not altered by the incubations mentioned above, and higher expression in fat of mice fed a high-fat diet may reflect increased number of adipose tissue-resident macrophages in obesity. In conclusion, the current data show that adipocyte hypertrophy and chronic inflammation are equally important in inducing chemerin synthesis. (***Endocrinology*** **152: 26–35, 2011)**

Dysfunctional adipose tissue in obesity is characterized by adipocyte hypertrophy, macrophage infiltration, and reduced insulin sensitivity. Impaired adipocyte physiology contributes to elevated systemic free fatty acids (FFA), chronic inflammation, and peripheral insulin resistance (1–4).

So far, the mechanisms for adipocyte dysfunction in obesity have not been clarified. Chronic inflammation alters adipocyte function and impairs insulin sensitivity (4). Disturbed cholesterol balance is a characteristic feature of enlarged adipocytes and also plays a prominent role in the dysregulation of adipocyte metabolism (2, 5, 6). In obesity, the transcription factor sterol regulatory element-binding protein 2 (SREBP2), which activates genes in cholesterol biosynthesis like 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase is activated, whereas SREBP1c, mainly regulating FFA synthesis, is not affected or even reduced (7–9). Endocrine function of hypertrophic adipocytes is disturbed and may further affect cell function by paracrine/autocrine mechanisms. One consequence

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in U.S.A.

doi: 10.1210/en.2010-1157 Received October 5, 2010. Accepted October 14, 2010.
First Published Online November 17, 2010

Abbreviations: CMKLR1, Chemokine-like receptor 1; FABP4, fatty acid-binding protein 4; FFA, free fatty acids; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HFD, high-fat diet; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; HSL, hormone-sensitive lipase; LPS, lipopolysaccharide; OA, oleic acid; PA, palmitic acid; siRNA, small interfering RNA; SREBP2, sterol regulatory element-binding protein 2.

of altered adipocyte function is the increase of the circulating levels of most adipokines, which control metabolism of other tissues like liver and muscle (4, 10).

Chemerin is a recently identified adipokine whose circulating levels positively correlate with markers of the metabolic syndrome including body mass index and systemic triglycerides. Chemerin, furthermore, positively correlates with circulating levels of inflammatory cytokines (11–14). Adipose tissue explants of obese donors secrete higher levels of chemerin compared with explants of lean controls, and chemerin in the supernatants negatively correlates with insulin sensitivity of these cells (15). Chronic low-grade inflammation in obesity is characterized by elevated systemic and adipose tissue levels of proinflammatoy cytokines. Adipocyte chemerin synthesis is induced by proinflammatory cytokines like IL-1β and TNF, and TNF also increases circulating chemerin in mice (16–18). Therefore, elevated systemic chemerin in human obesity and mouse models of obesity have been suggested to originate from inflammatory cytokines, further stimulating its production in adipocytes (14, 18).

Chemerin is an agonist of the G protein-coupled receptor chemokine-like receptor 1 (CMKLR1), which is expressed by adipocytes allowing autocrine and/or paracrine effects of chemerin (11, 19, 20). Recombinant chemerin inhibits insulin-induced glucose uptake in adipocytes *in vitro* (17), whereas application of recombinant chemerin during a glucose tolerance test has no effect on adipose tissue glucose incorporation in mice (21). Here, impaired glucose uptake is observed only in the liver of mice with mutated leptin receptor (db/db mice) but not in wild-type animals (21). Interestingly, insulin elevates chemerin in human adipose tissue explants *in vitro*, and systemic chemerin increases after prolonged hyperinsulinemia in healthy individuals (22), indicating induction of chemerin as a potential mechanism to impair insulin activity.

CMKLR1 was originally known for its expression in immune cells, and later on it was detected in skeletal muscle cells. Chemerin stimulates chemotaxis of macrophages and dendritic cells, whereas it induces insulin resistance in the latter (15, 23). Whether CMKLR1 mediates chemerin-induced insulin resistance in these cells, however, has not been proven so far. Importantly, neither macrophages nor skeletal muscle cells produce chemerin (15).

Adipocyte hypertrophy develops under conditions of chronic energy surplus and causes an increased release of most adipokines and inflammatory cytokines (4, 24, 25). The current study intended to further characterize the molecular mechanisms contributing to elevated adipocyte chemerin synthesis in obesity.

Materials and Methods

Materials

ELISA for murine chemerin, murine TNF, and murine IL-6 and antibodies to detect chemerin by immunoblot were from R&D Systems (Wiesbaden-Nordenstadt, Germany). The antibody to analyze CMKLR1 was from Abcam (Cambridge, UK). Antibodies against hormone-sensitive lipase (HSL), fatty acid-binding protein 4 (FABP4), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and cyclophilin A were from New England Biolabs GmbH (Frankfurt, Germany). SREBP2 antibody was ordered from Cayman Chemicals (IBL International GmbH, Hamburg, Germany). Lipopolysaccharide (LPS) (*Escherichia coli* serotype 055:B5), lovastatin, and mevalonate were ordered from Sigma (Deisenhofen, Germany). The preparation of nuclear extracts was performed using the nuclear extract kit (Active Motif, Rixensart, Belgium) according to manufacturer's instructions.

ELISA

ELISA was performed as recommended by the distributor. Supernatant was used undiluted for murine TNF and IL-6 and diluted 1- to 5-fold for chemerin determinations.

Adipocyte cell culture

3T3-L1 preadipocytes were purchased from the American Type Culture Collection (Manassas, VA) and cultured at 37 C and 5% CO_2 in DMEM (Biochrom, Berlin, Germany) supplemented with 10% newborn calf serum (Sigma Bioscience, Deisenhofen, Germany) and 1% penicillin/streptomycin (PAN, Aidenbach, Germany). For adipogenesis, 3T3-L1 preadipocytes were grown to confluence and differentiated into adipocytes by DMEM/F12/glutamate medium (Lonza, Basel, Switzerland) supplemented with 0.5 mM 3-isobutylmethylxanthine (Serva, Heidelberg, Germany), 10^{-7} M corticosterone, 10^{-7} M insulin, 200 mM ascorbate, 2 μg/ml transferrin, 5% newborn calf serum, 1 mM biotin, 17 mM panthothenate (all procured from Sigma Bioscience), 1% penicillin/streptomycin, and 300 mg/liter Pedersen-fetuin (MP Biomedicals, Illkirch, France) for 7 d. Differentiation medium was replaced at d 3 and 6 of adipogenesis. Thereafter, the cells were exposed to DMEM/F12/glutamate medium with 10^{-9} M insulin for 24 h. This was followed by incubation with DMEM/F12/glutamate medium until the cells reached the fully differentiated phenotype, which was controlled by light microscopy for the existence of a more rounded cell shape and the typical appearance of extensive lipid droplet accumulation.

For isolation of primary mouse cells, adipose tissue was dissected and incubated for 30 min at 37 C in Krebs-Ringer bicarbonate buffer with collagenase (AL Labortechnik, Friedmühle, Austria). The suspension was filtrated through a 100-μM filter and centrifuged at 400 $\times$ *g*. Floating adipocytes were removed, and stromal vascular cells and adipocytes were washed with Krebs-Ringer bicarbonate buffer containing 4% BSA. Purified cells were cultivated for 24 h before harvesting.

Knockdown of SREBP2

SREBP2 small interfering RNAs (siRNAs) and Silencer Negative Control 1 siRNA were from Applied Biosystems (Darmstadt, Germany). SREBP2 siRNAs (CUGGUACGCUGGUUACUCAtt, CAGCCUUUGAUAUACCAGAtt, and GCAGUACAGCGGUCAUUCAtt) were pooled, and preadipocytes were transfected us-

ing the siRNA Xtreme transfection reagent from Roche (Mannheim, Germany) and subsequently differentiated to adipocytes as described above.

Fatty acid treatment

Palmitic acid (PA) and oleic acid (OA) were purchased from Sigma. The 200 mM PA and OA stock solutions were prepared in ethanol by heating at 70 C, and 100 μl of 200 mM PA and OA stock solution was added to 900 μl of a 10% fatty acid-free BSA solution (Roche) to obtain a 20 mM stock solution and incubated at 55 C. The BSA-bound FFA stock solutions were added to the medium during differentiation or for 24 h after the adipocytes were fully differentiated.

Measurement of intracellular triglyceride concentration

Intracellular triglyceride concentrations were measured using the GPO-PAP micro-test (purchased from Roche). Triglyceride concentration was divided by intracellular total protein concentration, and these ratios are given as arbitrary units.

Electrophoretic mobility shift assay

The Lightshift chemiluminescent kit (Thermo Fisher Scientific, Bonn, Germany) was used according to the instructions of the manufacturer. Nuclear extracts (4 μg) were incubated with 50 ng/μl poly deoxyinosine-deoxycytidine in a volume of 20 μl binding buffer [100 mmol Tris, 500 mmol KCl, 10 mmol dithiothreitol (pH 7.5)] containing 2.5% glycerol, 5 mmol $MgCl_2$, and 0.05% Nonidet P-40. Chemerin mouse promoter sequence was obtained using Genomatix Software (Genomatix Software GmbH, München, Germany), and one SREBP binding site was identified by TFSEARCH (Supplemental Fig. 1, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org). The oligonucleotides (5′-biotin-CTT TCT AGT GGA AGA TCA CCT GGT CAA GCG GGG ATC T-3′) containing the SREBP binding site (which is *underlined* in the sequence) were annealed with their complementary strands and added to the reaction mixture. The corresponding mutated oligonucleotides (5′-biotin-CTT TCT AGT GGA AGA **TTG TAC** GGT CAA GCG GGG ATC T-3′; mutated nucleotides are given in *bold*) were used as control. For competition experiments, nuclear extracts were preincubated for 10 min with a 200-fold molar excess of nonmutated, nonlabeled oligonucleotides. The reaction mixture was incubated for 20 min at room temperature. DNA-protein complexes were separated by 4% polyacrylamide gel in 0.5% Tris-borate-EDTA buffer at 100 V using 0.5% Tris-borate-EDTA buffer as electrophoresis buffer, followed by transfer to a positively charged nylon membrane. The membrane was dried and cross-linked for 15 min with face down on a transilluminator by exposing it to 321 nm UV light. Detection of binding activity was performed with streptavidin-horseradish peroxidase conjugate antibody following the manufacturer's protocol.

Monitoring of gene expression by real-time RT-PCR

The mRNA expression of chemerin, SREBP2, SREBP1c, HMG-CoA reductase, and GAPDH was investigated by quan-

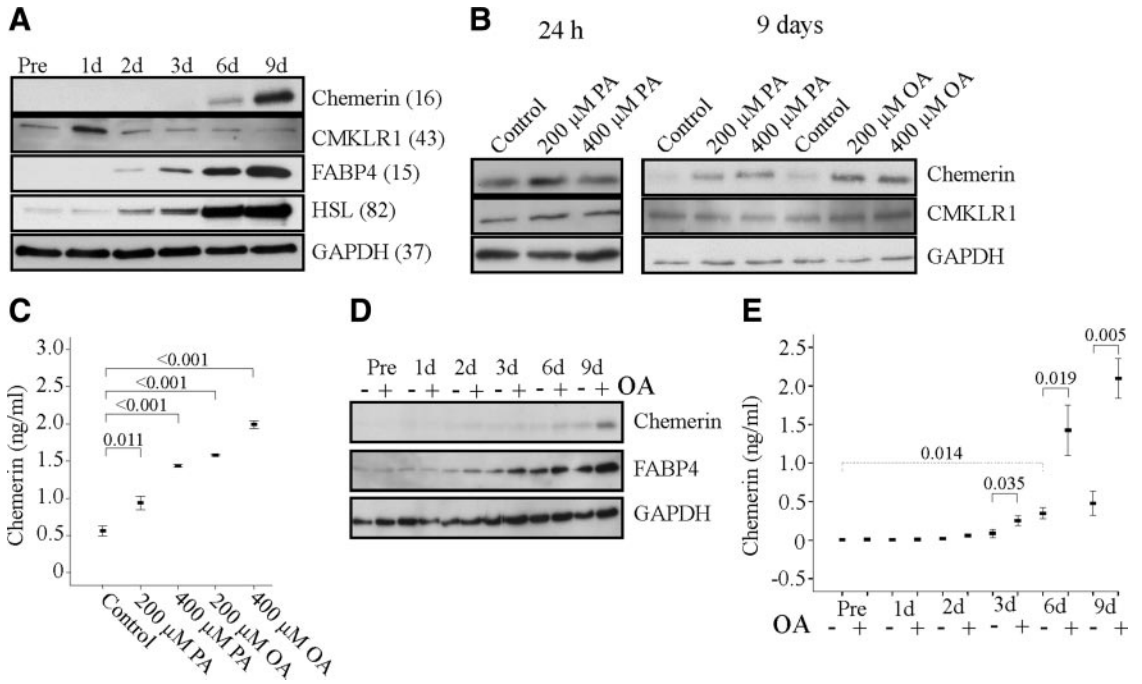

FIG. 1. FFA are potent chemerin inductors. A, Chemerin, CMKLR1, FABP4, HSL, and GAPDH were determined in preadipocytes (pre) and in 1-, 2-, 3-, 6-, and 9-d differentiated adipocytes of the murine adipocyte cell line 3T3-L1. One representative immunoblot of three independent experiments is shown. The *numbers in brackets* indicate the molecular mass of the respective proteins in kilodaltons. B, Chemerin, CMKLR1, and GAPDH in 3T3-L1 cells incubated with PA for 24 h or differentiated in the presence of PA or OA for 9 d. One representative immunoblot of three independent experiments is shown. C, Soluble chemerin in the supernatant of 3T3-L1 differentiated in the presence of PA and OA. Data of four independent experiments are given. D, Chemerin and FABP4 in preadipocytes (Pre) incubated with (+) or without (−) oleic acid for 24 h and in 3T3-L1 cells differentiated with (+) or without (−) OA for the indicated times. One representative immunoblot of two independent experiments is shown. E, Chemerin in the supernatant of the cells described in D. Data of four independent experiments are shown. The *dotted line* indicates that 3 d after inducing differentiation, a significant up-regulation of chemerin was observed.

titative real-time PCR using SYBR Green. Total cellular RNA was isolated with TRIzol reagent from GIBCO (Carlsbad, CA), and 1 μg RNA was reverse transcribed using the Promega reverse transcription system (Promega, Madison, WI) in a volume of 40 μl; 2 μl of the cDNA was used for amplification in glass capillaries (LightCycler) using exon-spanning PCR primers specific for mouse chemerin, SREBP2, SREBP1c, HMG-CoA reductase, and GAPDH (Supplemental Table 1). These oligonucleotides were synthesized by Metabion (Planegg-Martinsried, Germany). Amplification in the LightCycler capillaries was for 40 cycles with initial incubation of 10 min at 95 C for activation of *Taq* polymerase. Cycling parameters were 15 sec at 95 C, 10 sec at 62 C, and 10 sec at 72 C. Real-time RT-PCR was performed as recently described, and the specificity of the PCRs was confirmed by sequencing of the amplified DNA fragments (Geneart, Regensburg, Germany) (26, 27). For quantification of the results, RNA of 3T3-L1 adipocytes was reverse transcribed, and cDNA was serially diluted and used to create a standard curve for each of the genes analyzed The second-derivative maximum method was used for quantification with the LightCycler software (http://www.roche-applied-science.com/sis/rtpcr/lightcycler/lightcycler_docs/technical_notes/lc_11_updated.pdf). Values were normalized to GAPDH mRNA expression.

Animals

Female C57/Bl6 mice were purchased from Charles River Laboratories (Sulzfeld, Germany) at 8 wk of age and housed in a 22 C controlled room under a 12-h light, 12-h dark cycle with free access to food and water. After acclimatization, mice were divided into four groups (four to five mice per group) and fed either a standard diet [3% (wt/wt) fat] or the so-called Paigen diet [1.25% (wt/wt) cholesterol, 0.5% (wt/wt) cholic acid, and 17% (wt/wt) fat] for 8 or 24 wk (28). This diet has been developed by the group of B. Paigen as an atherogenic diet (29). Mice (four to five mice per group) were also fed a Paigen diet with increasing fat content (17, 25, and 35%) or a Paigen diet with 35% fat not supplemented with cholic acid for 8 wk. All animal procedures were approved by the local committee on animal research and complied with the German Law on Animal Protection as well as the Universities Federation for Animal Welfare Handbook on the Care and Management of Laboratory Animals (1999).

SDS-PAGE and immunoblotting

Adipocytes were solubilized in radioimmunoprecipitation assay lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% vol/vol Nonidet P-40, 0.5% vol/vol deoxycholic acid, and 0.1% (vol/vol) sodium dodecyl sulfate]. Proteins (10–20 μg) were separated by SDS-PAGE and were transferred to polyvinylidene difluoride membranes (Bio-Rad, Munich, Germany). Incubations with antibodies (dilution 1:1000) were performed in 1.5% BSA in PBS and 0.1% Tween overnight. Detection of the immune complexes was carried out with the enhanced chemiluminescence Western blot detection system (Amersham Pharmacia, Deisenhofen, Germany).

Statistical analysis

Data are presented as mean ± SD (SPSS version 12.0). Statistical differences were analyzed by paired Student's *t* test or two-tailed Mann-Whitney *U* test, and a value of $P < 0.05$ was regarded as statistically significant.

Results

Chemerin and CMKLR1 expression in 3T3-L1 mouse adipocytes during differentiation

The murine 3T3-L1 cell line was used as an *in vitro* model for adipogenesis (30, 31), and HSL and FABP4 were analyzed as markers for differentiation (Fig. 1A). Chemerin was not detected in preadipocytes and differentiated cells until 6 d after initiation of differentiation but was highly abundant in fully differentiated (9 d) adipocytes (Fig. 1A). CMKLR1 was already detected in preadipocytes, was induced during early differentiation (1 d), and subsequently decreased to levels similar to nondifferentiated cells (Fig. 1A).

High levels of systemic free fatty acids and increased storage of triglycerides in adipocytes are associated with obesity. Short-term incubation (24 h) of fully differentiated cells with FFA did not induce cellular chemerin levels (Fig. 1B). To evaluate the effects of a chronic supply with FFA, 3T3-L1 cells were differentiated in medium where PA or OA (32) (200 or 400 μM) had been added. Chemerin, CMKLR1, and GAPDH were analyzed by immunoblot in the 9-d differentiated 3T3-L1 cells. Differentiation in the presence of 200 or 400 μM FFA induced chemerin independent of the type of lipid used, whereas CMKLR1 was not affected (Fig. 1B). Chemerin was also measured in the supernatants, and PA and OA significantly elevated soluble chemerin. The induction was more

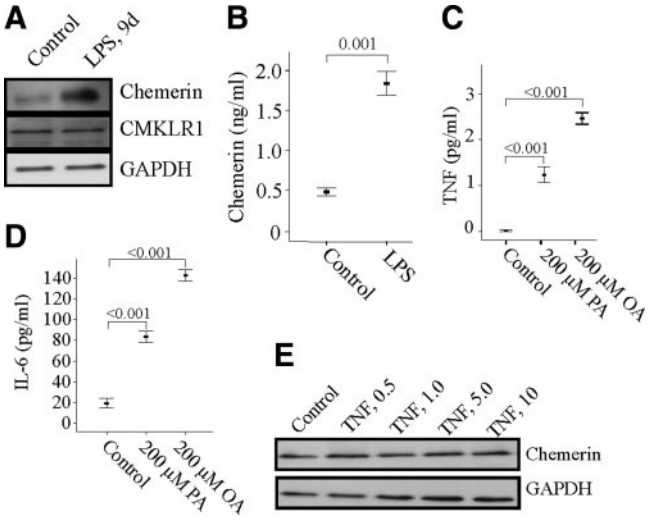

FIG. 2. LPS induces chemerin, whereas TNF and IL-6 have no effect. A, Chemerin in 3T3-L1 cells differentiated in the presence of LPS. One representative immunoblot of three independent experiments is shown. B, Soluble chemerin in the supernatant of 3T3-L1 cells described in A. C and D, 3T3-L1 cells were differentiated in the presence of 200 μM PA or OA, and TNF (C) and IL-6 (D) were measured in the supernatants. Data of five independent experiments are shown. E, 3T3-L1 cells were differentiated in the presence of TNF (0.5, 1.0, 5.0, and 10.0 pg/ml), and chemerin and GAPDH were analyzed by immunoblot. One representative immunoblot of three independent experiments is shown.

pronounced in cells differentiated in the presence of OA compared with PA for both concentrations used (Fig. 1C). Triglyceride content was similarly enhanced by PA and OA incubation (data not shown), excluding different efficiencies of fat accumulation as a potential explanation for increased chemerin release by the latter.

To find out whether FFA induce chemerin during differentiation, chemerin was determined in 3T3-L1 cells differentiated in the absence or presence of 400 μM OA for 1, 2, 3, 6, and 9 d. Chemerin was induced by OA treatment in the cell lysate of 6- and 9-d differentiated cells (Fig. 1D). FABP4, which is induced by FFA (33), was analyzed as control and was higher in cells differentiated in the presence of OA from 2 d on compared with the respective controls (Fig. 1D). Soluble chemerin in the supernatants was even increased after 3 d of differentiation in the presence of OA, but the effect was much more prominent in the 6- and 9-d differentiated cells (Fig. 1E).

Differentiation in the presence of LPS (10 ng/ml) significantly induced cellular and soluble chemerin but not CMKLR1 (Fig. 2, A and B).

TNF and IL-6 levels in 3T3-L1 cells differentiated in the presence of FFA

Differentiation in the presence of FFA significantly increased TNF and IL-6 in the supernatants. OA was more effective in inducing both cytokines (Fig. 2, C and D). To find out whether these increased concentrations of TNF, IL-6, or both contribute to elevated chemerin in 3T3-L1 cells differentiated in the presence of high amounts of FFA, differentiation was performed in the presence of these cytokines. 3T3-L1 cells were differentiated in the presence of TNF or IL-6 using similar concentrations as measured in the supernatants of these cells, namely 0.5, 1.0, 5.0, and 10 pg/ml TNF and 50, 100, and 200 pg/ml IL-6. Three independent experiments were performed, but chemerin was not induced. A representative immunoblot of TNF-incubated adipocytes is shown (Fig. 2E). Even when cells were differentiated in the presence of both cytokines (10 pg/ml TNF and 200 pg/ml IL-6), chemerin was not changed (data not shown).

Activation of SREBP2 is associated with elevated adipocyte chemerin

Excess storage of triglycerides in hypertrophic adipocytes leads to a deficit of cellular cholesterol and activation of SREBP2 (34). To test whether cholesterol depletion contributes to chemerin induction, 3T3-L1 cells were differentiated for 7 d, and then lovastatin (5 μM and 10 μM) was added until d 9, the period with a high triglyceride deposition. Cholesterol content was significantly reduced (data not shown), and as expected, the active form of SREBP2 was increased (Fig. 3A). Furthermore, induction of chemerin was observed by both concentrations of lo-

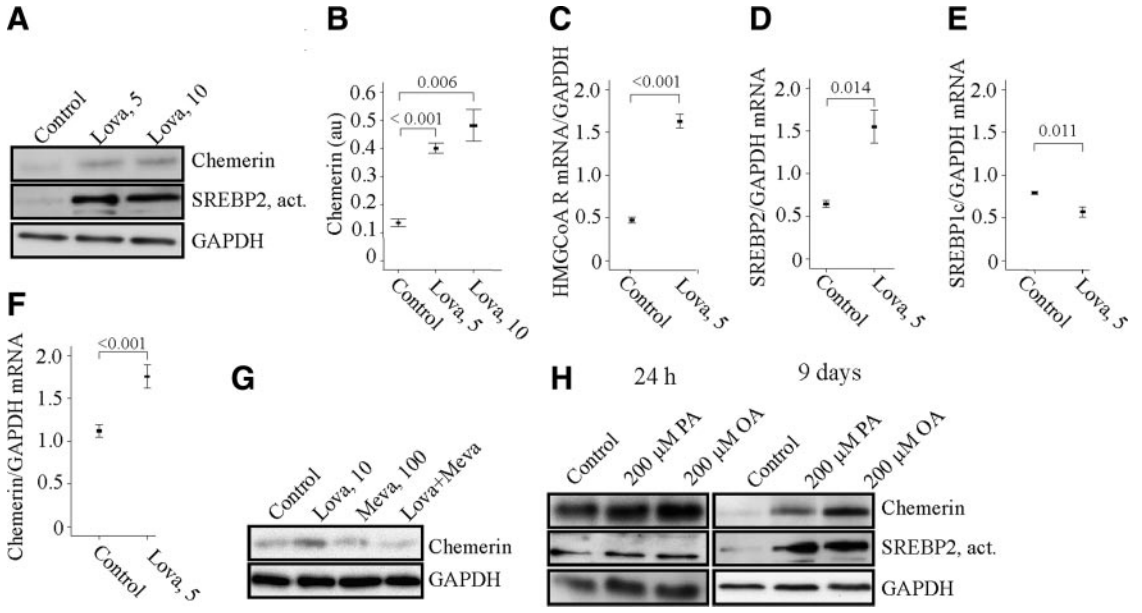

FIG. 3. Cholesterol shortage induces chemerin. A, Chemerin, active (act.) SREBP2, and GAPDH in 3T3-L1 cells incubated with 5 or 10 μM lovastatin added during the last 3 d of differentiation. B, Quantification of chemerin immunoblots of three independent experiments where a representative result is shown in A. C, HMG-CoA reductase mRNA in lovastatin-incubated cells. Data of three independent experiments are shown. D, SREBP2 mRNA in lovastatin-incubated cells. Data of three independent experiments are shown. E, SREBP1c mRNA in lovastatin-incubated cells. Data of three independent experiments are shown. F, Chemerin mRNA expression in 3T3-L1 cells incubated with 5 μM lovastatin. Data of five experiments have been calculated. G, Chemerin in 3T3-L1 cells where lovastatin, mevalonate (Meva), or both have been added during the last 3 d of differentiation. One representative immunoblot of three independent experiments is shown. H, Chemerin, active SREBP2, and GAPDH in 3T3-L1 cells incubated with PA or OA for 24 h or differentiated in the presence of PA and OA. One representative immunoblot of three independent experiments is shown.

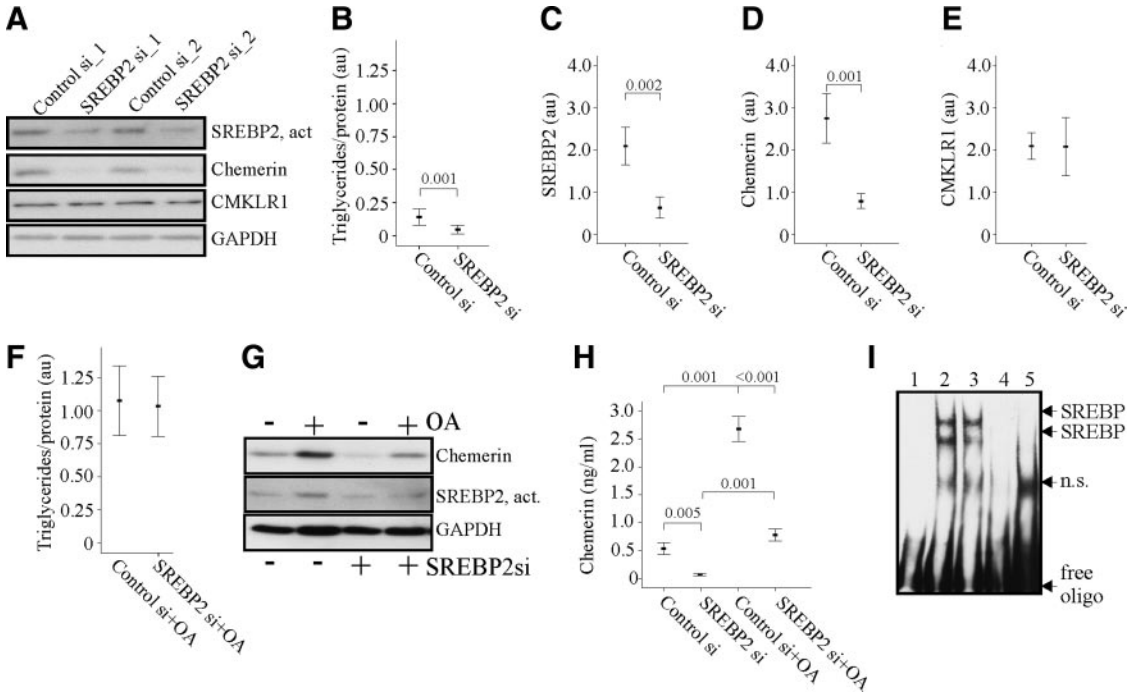

FIG. 4. Knockdown of SREBP2 impairs chemerin synthesis. A, Active (act.) SREBP2, chemerin, CMKLR1, and GAPDH in 3T3-L1 cells differentiated from preadipocytes treated with control or SREBP2 siRNA. Two representative immunoblots of four independent experiments are shown. B, Triglyceride content normalized to total cellular protein in SREBP2 siRNA-treated cells and the respective controls. Data of four independent experiments are shown. C, Quantification of the SREBP2 immunoblots (four independent experiments) partly shown in A. D, Quantification of the chemerin immunoblots (four independent experiments) partly shown in A. E, Quantification of the CMKLR1 immunoblots (four independent experiments) partly shown in A. F, SREBP2 siRNA-treated preadipocytes and the respective controls were differentiated in the presence of OA, and triglyceride content normalized to total cellular protein is shown. Data of four independent experiments were calculated. G, Chemerin and active SREBP2 in 3T3-L1 cells differentiated from preadipocytes treated with control or SREBP2 siRNA in the presence (+) or absence (−) of OA. One representative immunoblot of four independent experiments is shown. H, Chemerin was measured in the supernatants of the cells described in G. I, The binding of nuclear extracts (NE1 and NE2) from 3T3-L1 adipocytes to the SREBP binding site and to a mutated SREBP binding site were studied by EMSA. Competition experiments were performed using unlabeled probe. Lane 1, Free probe; lane 2, NE1; lane 3, NE2; lane 4, NE1 preincubated with 500-fold molar excess of unlabeled probe; lane 5, NE1 plus mutated probe (n.s., not specific). One representative EMSA of two independent experiments is shown. au, Arbitrary units.

vastatin used (Fig. 3, A and B). The mRNA expression of HMG-CoA reductase, a SREBP2-regulated gene (35), was increased in cells incubated with 5 μM lovastatin (Fig. 3C). SREBP2 mRNA was also induced, whereas SREBP1c was reduced (Fig. 3, D and E). Most importantly, chemerin mRNA expression was strongly increased after lovastatin treatment (Fig. 3F).

Lovastatin inhibits HMG-CoA reductase activity and mevalonate synthesis, and supplementation of growth medium with mevalonate can restore cholesterol synthesis. Simultaneous incubation with mevalonate (100 μM) reversed induction of chemerin by lovastatin, confirming that cholesterol deficit is linked to chemerin up-regulation (Fig. 3G). SREBP2 was also activated in 3T3-L1 cells differentiated in the presence of 200 μM PA or OA but not during 24 h incubation (Fig. 3H).

Knockdown of SREBP2 lowers basal and FFA-induced chemerin synthesis

To find out whether SREBP2 regulates chemerin synthesis, SREBP2 was knocked down by siRNA in preadipocytes, and chemerin and SREBP2 protein was determined in the differentiated cells (Fig. 4A). Cellular cholesterol was 48.8 ± 11.2% compared with control siRNA transfected cells (set to 100%, data not shown), and triglyceride storage was also markedly lower (Fig. 4B). SREBP2 protein was reduced to about 25%, cellular chemerin to about 15%, and soluble chemerin to about 7% in SREBP2 siRNA-treated adipocytes compared with the respective control treated cells, and abundance of CMKLR1 was not affected (Fig. 4, A–D and H). Reduced chemerin synthesis may be related to suppression of SREBP2 but may also be a consequence of impaired triglyceride storage of these cells (Fig. 4B). When differentiation of 3T3-L1 cells was performed in the presence of 400 μM OA, cells transfected with control or SREBP2 siRNA accumulated similar levels of triglyceride (Fig. 4F), but up-regulation of SREBP2 and chemerin was still impaired in SREBP2 siRNA-treated cells (Fig. 4, G and H).

The chemerin promoter has a SREBP binding site (Supplemental Fig. 1), and binding of nuclear proteins to this

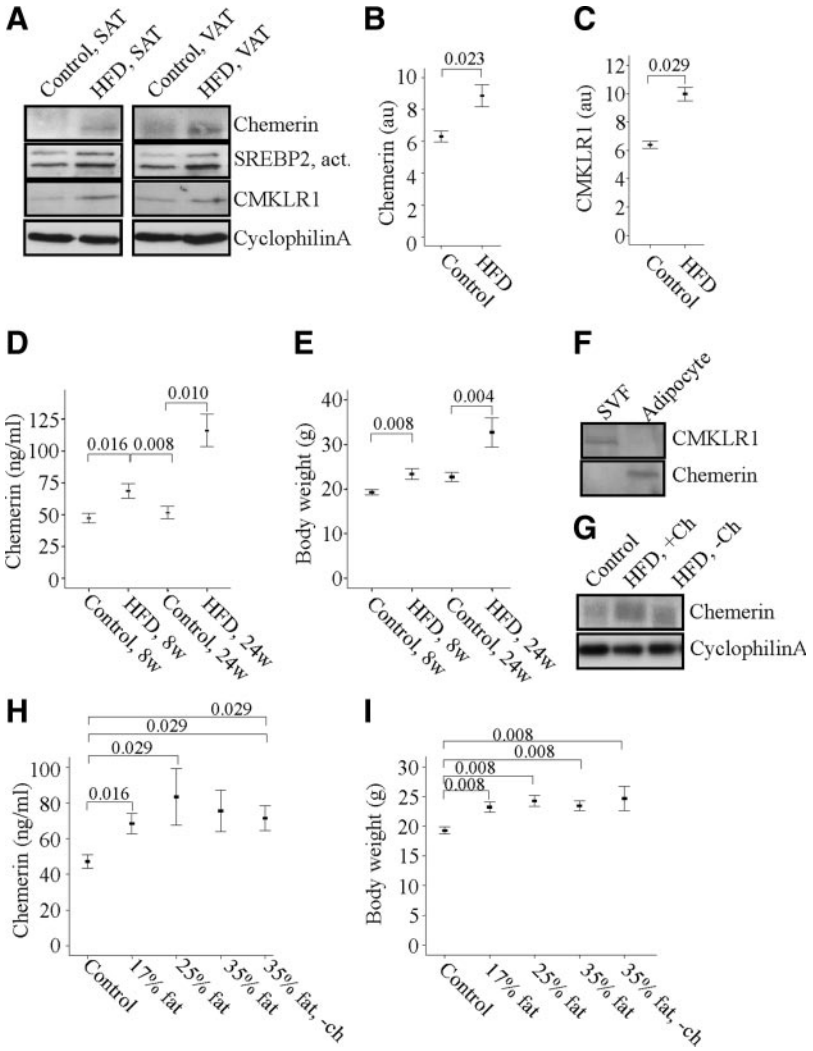

FIG. 5. Adipose tissue and systemic chemerin in mice on a HFD. A, Chemerin, active (act.) SREBP2, CMKLR1, and cyclophilin A in sc adipose tissue (SAT) and visceral adipose tissue (VAT) of mice fed a standard diet (control) or a HFD. B, Quantification of chemerin in VAT of four to five animals per group. C, Quantification of CMKLR1 in VAT of four to five animals per group. D, Chemerin in serum of mice (five animals per group) fed a standard diet (control) or a HFD for 8 or 24 wk. E, Body weight of these animals. F, CMKLR1 and chemerin in stromal vascular cells (SVF) and adipocytes isolated from visceral fat of mice. One of two independent experiments is shown. G, Chemerin in sc fat of mice fed a standard diet (control), a HFD supplemented with cholic acid (HFD, +Ch), or the identical diet not supplemented with cholic acid (HFD, −Ch). One representative immunoblot of three independent experiments is shown. H, Chemerin in serum of mice fed a standard diet (control) or a HFD with increasing fat content for 8 wk. Chemerin was also measured in the serum of mice fed a diet with 35% fat not supplemented with cholic acid (35%, −ch, four animals). I, Body weight of these mice. au, Arbitrary units.

site was detected by EMSA when nuclear extracts of differentiated 3T3-L1 were used. Binding was blocked by preincubation with 500-fold molar excess of unlabeled probe and was not observed when an oligonucleotide with a mutated SREBP site was used (Fig. 4I).

Adipose tissue chemerin and SREBP2 activation in mice on a high-fat diet (HFD)

Induction of chemerin by FFA suggests that HFD-mediated obesity may be associated with elevated adipose tissue chemerin. In sc as well as visceral adipose tissue of mice fed a HFD for 24 wk, chemerin was similarly elevated compared with fat of lean animals (Fig. 5, A and B, and data not shown). CMKLR1 was also upregulated in both fat depots of HFD animals (Fig. 5, A and C). CMKLR1 is more abundant in the stromal vascular fraction compared with adipocytes (Fig. 5F), suggesting that increased numbers of adipose tissue-resident macrophages in obesity (36) account for higher CMKLR1.

Similar to related studies, circulating chemerin and body weight were significantly higher in HFD animals (Fig. 5, D and E). Systemic chemerin and body weight were even increased in the serum of animals kept on the Paigen diet for just 8 wk (Fig. 5, D and E). Interestingly, mice kept on HFD for 8 wk and mice kept on a standard chow for 24 wk had similar body weight, whereas chemerin was significantly higher in the HFD-fed animals (Fig. 5, D and E).

The diet used was supplemented with cholic acid, and to exclude that this component is the main reason for chemerin induction, adipose tissue of mice kept on the identical diet without cholic acid was also analyzed, and chemerin was similarly upregulated (Fig. 5G). Serum chemerin was similarly induced in mice kept on otherwise identical diets when cholic acid was omitted (Fig. 5H).

It was also tested whether increasing concentrations of fat in the diet are associated with higher chemerin. Chemerin was already increased in the serum of mice kept on the diet with 17% fat for 8 wk. Higher fat content (25 and 35%) in the diet did not further increase chemerin (Fig. 5H). However, body weight of all the mice kept on the different diets was significantly higher compared with control animals but was comparable within the HFD groups, suggesting that increasing fat content in the chow may not necessarily cause additional weight gain (Fig. 5I).

To test whether adipose tissue-produced chemerin may be relevant to determine systemic levels, chemerin protein abundance was analyzed in different murine tissues. Chemerin was highly expressed in brown adipose tissue and sc and visceral fat depots and was not detected in liver, pancreas, spleen, brain, and skeletal muscle (Fig. 6). This

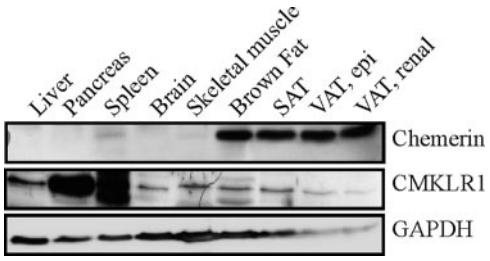

FIG. 6. Chemerin and CMKLR1 in different rodent tissues. Chemerin and CMKLR1 in different rodent tissues (epididymal, epi). One representative immunoblot of two independent experiments is shown.

demonstrates that chemerin is exclusively/mainly synthesized in fat tissues. CMKLR1 was highest in the spleen, second highest in the pancreas, and much less abundant in liver and skeletal muscle. Low expression was detected in brain and adipose tissues.

Discussion

Chemerin is a newly identified adipokine whose systemic levels are elevated in obesity and positively correlate with markers of the metabolic syndrome such as body mass index, triglycerides, high-sensitivity C-reactive protein, or leptin (11, 12, 14). Chemerin and CMKLR1 are expressed in adipocytes (19), and the current experiments confirm that cellular chemerin is strongly induced in differentiated 3T3-L1 adipocytes (19, 20). CMKLR1 is transiently increased in 1-d differentiated cells and is otherwise similar in preadipocytes and mature adipocytes, pointing to an important function of CMKLR1 in early differentiation. This hypothesis is supported by recent experiments where knockdown of chemerin or its receptor in preadipocytes impairs subsequent differentiation but has no overt effect on adipocyte phenotype when knocked down 4 d after initiating differentiation of 3T3-L1 cells (19).

Chemerin seems to be preferentially synthesized by adipocytes, indicating that higher production in adipose tissue of obese donors is due to higher adipocyte synthesis (15). The elevated number of adipose tissue-resident macrophages and activated preadipocytes are suggested to significantly contribute to local and systemic chronic inflammation in obesity (37). IL-1β and TNF increase adipocyte chemerin, and elevation of serum TNF leads to higher systemic chemerin in mice, suggesting that inflammatory cytokines are involved in the up-regulation of chemerin in obesity (15, 17, 18). In accordance with this hypothesis, LPS, a well described proinflammatory stimulus inducing the release of proinflammatory cytokines in adipocytes (38), strongly enhances chemerin synthesis in 3T3-L1 cells as demonstrated herein.

Chronic caloric surplus causes excess storage of triglycerides in adipocytes (2). As an *in vitro* model, incubation with FFA has been established to increase adipocyte hypertrophy (39, 40). Short-term incubation of differentiated adipocytes elevates fat storage but has no effect on chemerin synthesis. Therefore, to apply a more physiological model, 3T3-L1 fibroblasts are differentiated in the presence of FFA, and mature adipocytes are characterized by elevated triglyceride storage, higher chemerin synthesis, and activated SREBP2. This transcription factor is activated by proteolysis when membrane cholesterol content is reduced (35) and is activated in hypertrophic adipocytes (7, 9). To further confirm that reduced cholesterol content increases chemerin synthesis, cellular cholesterol has been depleted by lovastatin. In these adipocytes, SREBP2 is activated and elevated chemerin mRNA and protein levels indicate that higher chemerin protein is at least partly explained by transcriptional mechanisms. SREBP2 mRNA is also strongly increased, whereas SREBP1c expression is reduced. SREBP1c is also down-regulated in adipose tissue of obese subjects, suggesting that SREBP1c most likely does not contribute to higher chemerin synthesis (7, 8). Restoration of cholesterol homeostasis by mevalonate abrogates lovastatin-induced chemerin synthesis, thereby confirming that low cholesterol is directly linked to higher chemerin.

Human and mouse chemerin promoter have a SREBP binding site, and nuclear proteins of differentiated adipocytes specifically bind to this site. These data suggest that SREBP2 induces up-regulation of chemerin in hypertrophied adipocytes. However, triglycerides, cholesterol, and chemerin are strongly reduced in adipocytes differentiated from 3T3-L1 preadipocytes treated with SREBP2 siRNA, and therefore, these experiments are not suitable to identify SREBP2 as the central regulator of chemerin expression. However, SREBP2 siRNA-treated cells accumulate similar levels of triglycerides as control treated cells when differentiated in the presence of FFA where induction of chemerin is still markedly impaired. Therefore, it is likely that SREBP2 is the central transcription factor mediating FFA-induced chemerin synthesis.

Systemic concentrations of chemerin are increased in mouse models of obesity and human adiposity (11, 14, 18) and are also elevated in the diet-induced obesity mouse model used in the current study. Whereas longer feeding of the HFD further induces body weight and chemerin, higher fat content in the diet does not increase weight gain or chemerin. Higher fat in the diets has been shown to accelerate onset of diabetes without enhancing obesity in New Zealand obese mice (41). This indicates that insulin sensitivity of the mice fed a more fat-rich diet might be further reduced, although this has not been determined in the current study. Studies in humans cannot show a further

increase of chemerin in insulin resistance (11, 14), and therefore, similar systemic chemerin levels in the mice are in agreement with these findings.

The HFD used in the current experiments contains cholesterol contributing to inflammation (42) and may more adequately mimic human nutrition. Cholic acid supplementation of this diet mainly exerts profibrotic effects in the liver (42) but does not further increase adipose tissue or systemic chemerin in line with human data where systemic chemerin is linked to hepatic inflammation and not to liver fibrosis (43).

Although circulating chemerin is higher in obesity, there are conflicting reports regarding expression of adipose tissue chemerin, which was found either elevated or unchanged in obesity (11, 18, 21). In the mouse model used herein, chemerin and its receptor are induced in sc and visceral fat of animals kept on a HFD when compared with mice fed a standard chow. SREBP2 is 2- to 3-fold more activated in both fat depots of mice fed a hypercaloric chow, suggesting that cholesterol deficit, subsequent activation of SREBP2, and induction of chemerin may represent a scenario explaining higher chemerin levels. CMKLR1 is also induced in fat of obese mice and most likely is explained by the elevated number of tissue-resident macrophages in obesity (36). CMKLR1 protein is not changed upon differentiation of 3T3-L1 cells in the presence of FFA, and LPS, IL-6, or TNF (15) do not affect its protein levels in adipocytes, arguing against induction of CMKLR1 in hypertrophic cells. Stromal vascular cells express higher levels of CMKLR1, further suggesting that an altered cellular composition of adipose tissue in obesity may cause CMKLR1 induction.

Analysis of different rodent tissues reveals that chemerin is highly expressed in adipose tissues, strongly supporting the hypothesis that higher synthesis in fat may considerably contribute to elevated systemic levels in obesity. Chemerin is similarly abundant in white and brown fat, and this is in accordance with recent data where chemerin is detected in the supernatant of brown adipocytes (17). CMKLR1 is highly abundant in the spleen, in agreement with its expression in immune cells (44). Interestingly, pancreas has high levels of CMKLR1, and recently impaired insulin release in chemerin-treated obese mice has been reported, suggesting that chemerin may regulate insulin processing or secretion. CMKLR1 is also found in adipose tissue and is slightly more abundant in the liver as has been already indicated by mRNA expression analysis (21, 45).

In conclusion, the current study demonstrates that adipocyte hypertrophy is associated with cholesterol deprivation and subsequent activation of SREBP2, which strongly up-regulates chemerin.

Acknowledgments

The technical assistance of Yvonne Hader and Kerstin Neumeier is greatly appreciated.

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This work was supported by grants from the Regensburger Forschungsförderung in der Medizin (ReForM C).

Disclosure Summary: The authors have nothing to disclose.

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