Disruption of CXC Motif Chemokine Ligand-14 in Mice Ameliorates Obesity-induced Insulin Resistance*§

Noriko Nara‡1,2, Yuki Nakayama‡1, Shiki Okamoto‡3, Hiroshi Tamura‡4, Mari Kiyono‡1, Masatoshi Muraoka‡1, Kiyoko Tanaka‡6, Choji Taya‡1, Hiroshi Shitara‡1, Rie Ishii‡1, Hiromichi Yonekawa‡6, Yasuhiro Minokoshi‡6, and Takahiko Hara‡1,3

From the Stem Cell Project Group and the Laboratory of Mouse Model for Human Heritable Diseases, Tokyo Metropolitan Institute of Medical Science, Tokyo Metropolitan Organization for Medical Research, 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113-8613 and the Division of Endocrinology and Metabolism, National Institute for Physiological Sciences, 38 Nishigonaka Myodaiji, Okazaki, Aichi 444-8585, Japan

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In obese individuals, white adipose tissue (WAT) is infiltrated by large numbers of macrophages, resulting in enhanced inflammatory responses that contribute to insulin resistance. Here we show that expression of the CXC motif chemokine ligand-14 (CXCL14), which targets tissue macrophages, is elevated in WAT of obese mice fed a high fat diet (HFD) compared with lean mice fed a regular diet. We found that HFD-fed CXCL14-deficient mice have impaired WAT macrophage mobilization and improved insulin responsiveness. Insulin-stimulated phosphorylation of Akt kinase in skeletal muscle was severely attenuated in HFD-fed CXCL14+/− mice but not in HFD-fed CXCL14−/− mice. The insulin-sensitive phenotype of CXCL14+/− mice after HFD feeding was prominent in female mice but not in male mice. HFD-fed CXCL14+/− mice were protected from hyperglycemia, hyperinsulinemia, and hypoadiponectinemia and did not exhibit increased levels of circulating retinol-binding protein-4 and increased expression of interleukin-6 in WAT. Transgenic overexpression of CXCL14 in skeletal muscle restored obesity-induced insulin resistance in CXCL14−/− mice. CXCL14 attenuated insulin-stimulated glucose uptake in cultured myocytes and to a lesser extent in cultured adipocytes. These results demonstrate that CXCL14 is a critical chemotactant of WAT macrophages and a novel regulator of glucose metabolism that functions mainly in skeletal muscle.

The number of patients suffering from type 2 diabetes is increasing worldwide, due in large part to the increased incidence of obesity. To date, several key regulators of obesity-induced insulin resistance have been identified. In an obese mouse model, elevation of free fatty acids (FFAs), inflammatory cytokines, such as tumor necrosis factor-α and interleukin (IL)-6, endoplasmic reticulum stress, and reactive oxygen species inhibit insulin action, in part through the activation of c-Jun N-terminal kinase-1 and NF-κB-mediated signaling pathways, which are critical for obesity-induced insulin resistance (1–7). Obesity-associated down-regulation of circulating adiponectin blunts fatty acid oxidation and glucose uptake (8), whereas elevation of serum retinol-binding protein-4 (RBP4) levels in obese mice leads to a critical enhancement of gluconeogenesis in the liver (9).

Genetic analysis of WAT, which is the major producer of FFAs and adipokines in the body, from obese mice or patients revealed a positive correlation between macrophage accumulation and obesity-induced insulin resistance (10, 11). Moreover, myeloid-specific disruption of the IκB kinase β gene in mice (7) revealed that the inflammatory response of macrophages can trigger insulin resistance. Recent studies have shown that CC motif chemokine ligand-2 (CCL2) (also known as monocyte chemoattractant protein-1 or MCP-1) is up-regulated in WAT of HFD-induced obese mice (12, 13), and glucose metabolism is improved in HFD-fed obese mice lacking CCL2 (14) or the gene encoding the CCL2 receptor CCR2 (15). Furthermore, transgenic mice overexpressing CCL2 mRNA in adipose tissue exhibited enhanced macrophage infiltration into WAT and whole body insulin resistance (14, 16). CCL2 directly inhibits insulin-stimulated glucose uptake in cultured adipocytes and myocytes (16) and enhances glucose production in the liver of transgenic mice (14). These results indicate that the interaction of CCL2 and CCR2 contributes to WAT macrophage infiltration and impaired glucose metabolism in obese mice. However, in a different genetic background, blood glucose levels and the number of WAT macrophages in CCR2-deficient obese mice were similar to control mice (17). This suggests that there

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1 Both authors contributed equally to this work.
2 Recipient of a research fellowship from the Japanese Society for the Promotion of Science.
3 To whom correspondence should be addressed. Tel.: 81-3-4463-7595; Fax: 81-3-3823-1418; E-mail: thara@rinshoken.or.jp.

4 The abbreviations used are: FFA, free fatty acid; WAT, white adipose tissue; CXCL14, CXC motif chemokine ligand-14; HFD, high fat diet; IL-6, interleukin-6; RBP4, retinol-binding protein-4; CCL2, CC motif chemokine ligand-2; BAT, brown adipose tissue; FCS, fetal calf serum; FACS, fluorescence-activated cell sorting; SVCs, stromal and vascular cells; RD, regular diet; IITT, intraperitoneal insulin tolerance test; IGTT, intraperitoneal glucose tolerance test; DMEM, Dulbecco’s modified Eagle’s medium; RT, reverse transcriptase; 2-DG, 2-deoxyglucose; UCP, uncoupling protein; GLUT4, glucose transporter-4.
is an additional chemoattractant for WAT macrophages in obese animals. CXCL14 (originally designated as BRAK, BMAC, or Mip-2γ) is expressed in skeletal muscle, WAT, and brown adipose tissue (BAT), which suggests that it may play a role in myogenesis, adipogenesis, and metabolic regulation. CXCL14 is a chemoattractant for activated tissue macrophages and dendritic progenitor cells (18–24). Human monocytes acquire CXCL14 responsiveness and lose their chemotactic response to CCL2 after prostaglandin E2 treatment, suggesting that the role of CXCL14 may be distinct from other inflammatory monokines (25). However, the CXCL14 receptor has not yet been identified, and the physiological roles of CXCL14 have not been fully explored in animal models. In this study, we generated CXCL14-null mice and demonstrated that CXCL14 is involved in the obesity-associated infiltration of macrophages into WAT, alteration of serum adipokine levels, hepatic steatosis, and attenuation of insulin signaling in skeletal muscle, thereby contributing to whole body insulin resistance in HFD-induced obese mice.

EXPERIMENTAL PROCEDURES

Generation of CXCL14 Knock-out and Transgenic Mouse Lines—The CXCL14 targeting vector consisted of the genomic DNA sequence of mouse CXCL14 (GenBank accession numbers AC165347 and AC124395) containing a deletion of the translation initiation site and exon 2 and a Neo cassette insertion (supplemental Fig. S1A). TT2 embryonic stem cells derived from CBA × C57BL/6 F1 mice were electroporated with linearized targeting vector and cultured in the presence of Geneticin (G418) (350 μg/ml) for 10–12 days. Homologous recombination was assessed by PCR using P1 (5′-CATGCACTCAACTGATGTCAGACACAGAC-3′) and P2 (5′-CGAATGGGCTCACTGCTGTTGTCAG-3′) primers (product size 2,193 bp). Embryonic stem cells were microinjected into 8-cell-stage eggs of ICR mice to generate chimeras, which were bred to C57BL/6 mice to obtain F1 heterozygous mutant mice (C57BL/6-CBA mixed background). F1 and F2 mice were intercrossed to obtain CXCL14+/−, CXCL14+/+ and CXCL14−/− littersmates. For genotyping, PCR was carried out on tail fragment-derived DNA, using wild-type primers (5′-GACCGCTTCCGACGTC-3′ and 5′-GTCCGATCTAACCCTAGGTTG-3′) and mutant primers (5′-AGACCTGTACGGCGGCGAC-3′ and 5′-GTCCGATCTAACCCTAGGTTG-3′) (product size 251 bp). As CXCL14+/+ and CXCL14−/− mice were phenotypically indistinguishable, most experiments with mice were pre-approved by the ethical committee of the institute.

Southern Blot Analysis—High molecular weight DNA (10 μg) extracted from the spleen was digested with EcoRI. Southern blot hybridization was carried out according to standard procedures. The 390-bp [32P]dCTP-labeled probe was generated by PCR using the following primers: 5′-GCTTCAGATGAGATCCACAG-3′ and 5′-AGTAGACTGATGTCCTA-3′.

Northern Blot Analysis—Total RNA (20 μg) was isolated from the indicated tissues and organs using TRIzol (Invitrogen). Northern blot analysis was carried out using a PCR-amplified cDNA fragment as a probe. Sequences of the gene-specific primer sets used for PCR are listed in supplemental Table S1.

Cell Fractionation—Fragmented periovarian fat pads were digested in a solution of collagenase/dispase (1 mg/ml PBS; Roche Applied Science) for 45 min at 37 °C with gentle agitation. After centrifugation at 1200 rpm for 5 min, floating cells (adipocytes) were recovered, and cells in the pellet were incubated in a solution of biotinylated anti-Mac1 antibody (M1/70, 1:50 dilution; BD Biosciences) in PBS containing 5% fetal calf serum (FCS) on ice for 30 min, followed by incubation with fluorescein isothiocyanate-conjugated streptavidin (BD Biosciences) (10 μg/ml). Fluorescence intensity and number of viable cells were measured by fluorescence activated cell sorting (FACS), using a FACSaria system (BD Biosciences). Mac1-positive (Mac1+ cells) and Mac1-negative (Mac1−) cells were collected and designated as macrophages and stromal and vascular cells (SVCs), respectively.

Immunohistochemistry—For histological analysis, fat pads were fixed in 4% paraformaldehyde in PBS, and paraffin-embedded sections were prepared (5-μm sections). Sections were incubated with biotinylated F4/80 antibody (1:20 dilution; BM8, Caltag) at room temperature for 30 min, followed by incubation with horseradish peroxidase-conjugated streptavidin (1:50 dilution; Molecular Probes) and diaminobenzidine (Sigma), as described previously (27). For fatty liver analysis, fixed cryostat sections (7 μm thick) were stained with Oil Red O (Sigma; 1.5 mg/ml in 60% isopropyl alcohol) for 10 min at room temperature.

Chemotaxis Assay—Mac1+ mononuclear cells in the peripheral blood of 5-month-old C57BL/6 female mice were sorted by FACSaria and applied to the D-6 chamber (width, 260 μm; depth, 5 μm) of EZ-TAXIScan (Effector Cell Institute, Tokyo, Japan) (28) in RPMI 1640 medium (Sigma) containing 0.1% BSA in 20 mm HEPES, pH 8.0. Human CXCL14 (PeproTech, 100 nM) was added to a lower well of the chamber, and cell migration was recorded for 3 h at 37 °C.

Diet Study and Metabolic Measurements—Mice were fed a regular diet (RD) (12% fat, CE-2; CLEA, Tokyo) or an HFD (32% fat, HFD-32; CLEA). Initial studies were carried out using female mice fed an RD alone (11 months) or an HFD (6 months with RD and 3 months with HFD). We also used younger mice fed an HFD (2 months with RD and 3 months with HFD) and obtained similar results. For the intraperitoneal insulin toler-
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ance test (ITT) and intraperitoneal glucose tolerance test (IGTT), mice were fasted for 16 h and then injected intraperitoneally with human insulin (0.75 milliunits/g) or glucose (0.75 mg/g; Wako Pure Chemical Industries Ltd., Osaka, Japan), respectively. To measure insulin concentrations for the IGTT, glucose (2 mg/g) was administered. Blood glucose was measured using a Glustest Ace R glucometer (Sanwa Chemical, Nagoya, Japan). Levels of serum cholesterol, serum, and hepatic triglyceride and serum FFAs were analyzed using the corresponding enzyme-based measurement kits (Wako). To determine hepatic triglyceride concentrations, liver fragments (100 mg) were homogenized in 1 ml of 0.1 M KCl, and lipids were extracted from the homogenate with chloroform:methanol (2:1, v/v) (29). An aliquot of the organic phase was collected, dried, and resuspended in isopropl alcohol containing 1% Triton X-100. Serum concentrations of insulin, leptin, and adiponectin were determined using enzyme-linked immunosorbent assay kits from Shibayagi (Shibukawa, Gunma, Japan), Morinaga Chemical (Yokohama, Japan), and Otsuka Pharmaceutical Co., Ltd. (Tokyo, Japan), respectively.

Statistical Analysis—Statistical analysis was carried out using the unpaired Student’s t test and Statview J5.0 software (Abacus Concepts). A p value of <0.05 was considered to be significant.

Western Blot Analysis—For the in vivo analysis, mice were fasted for 16 h, injected with human insulin (10 milliunits/g) intraperitoneally, and then sacrificed 4 min after injection. Liver and skeletal muscle (tibialis anterior muscle) in obese mice were dissected and immediately frozen in liquid nitrogen. For in vitro analysis, confluent C2C12 myoblasts were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% horse serum for 4 days to induce myotube differentiation. After 16 h of serum starvation, cells were incubated with or without CXCL14 (100 ng) for 1 h followed by stimulation with insulin (10 ng/ml) at 37 °C for 10 min in the presence or absence of CXCL14. Total protein (20 μg) was subjected to Western blot analysis using a polyclonal antibody to phosphorylated Akt (Akt-pSer473) or nonphosphorylated Akt (Cell Signaling) according to the manufacturer’s instructions. CXCL14 in the brain and RBP4 in serum were detected using sheep anti-mouse CXCL14 antibody (R & D Systems) and rabbit anti-human RBP4 antibody (DAKO), respectively.

Reverse Transcriptase (RT)-PCR—First-strand cDNAs were prepared from total RNA (5 μg) using SuperScript II reverse transcriptase and a random primer (Invitrogen). One percent of the cDNA mixture was subjected to 30–40 cycles of PCR using the primer sets listed in supplemental Table S1, an annealing temperature of 57 °C, and ExTaq DNA polymerase (Takara, Otsu, Japan).

Measurement of 2-Deoxyglucose (2-DG) Uptake—Differentiated C2C12 myoblasts were generated as described for Western blot analysis. 3T3-L1 preadipocytes were cultured in DMEM containing 10% FCS at 37 °C. Two days after reaching confluence, cells were treated with 0.25 μM dexamethasone and 0.5 mM 3-isobutyl-1-methylxanthine for 48 h, followed by 10 μM insulin alone for an additional 48 h. The cells were then maintained for 6 days in DMEM containing 10% FCS. For the 2-DG uptake assay, C2C12-derived myocytes or 3T3-L1-derived adipocytes were serum-starved for 16 h, and then incubated with 0, 0.1, or 10 nm CXCL14 for 37 °C for 1 h. Cells were stimulated with insulin (10 ng/ml) for 30 min and incubated with 100 nm [1-3H]2-DG (0.8 μCi/ml, Amersham Biosciences) at 37 °C for 10 min in the presence or absence of CXCL14. Specific uptake of 2-DG was measured as described previously (30).

RESULTS

Obesity-associated Up-regulation of CXCL14 mRNA—In adult mice, CXCL14 was expressed in the brain, lung, kidney, ovary, skeletal muscle, uterus, and WAT (Fig. 1A). Expression of CXCL14 was strikingly elevated in WAT, BAT, and skeletal muscle (tibialis anterior muscle) in obese mice fed an HFD (Fig. 1B), and in ob/ob mice (Fig. 1C) compared with mice fed an RD. This was the first demonstration that CXCL14 is an obesity-induced gene. In WAT, the expression level of CXCL14 mRNA in SVCs and macrophages was equivalent in RD-fed and HFD-
General Properties of CXCL14-deficient Mice—To understand the physiological role of CXCL14, we generated heterozygous (CXCL14+/−) and homozygous (CXCL14−/−) CXCL14 mutant mice, according to standard gene targeting strategies (supplemental Fig. S1A). Southern, Northern, and Western blot analyses confirmed that region-specific recombination occurred and that CXCL14 transcripts and protein were absent in null mutant mice (supplemental Fig. S1B). The Mendelian frequency, CXCL14−/−/− adult mice were fertile and asymptomatic for severe diseases. However, CXCL14−/− and CXCL14+/− mice exhibited several differences in body weight and metabolic parameters. Female CXCL14−/− mice showed a more pronounced phenotype than male CXCL14−/− mice, particularly under HFD feeding conditions. Thus, the following experiments were conducted using female homozygous CXCL14−/− and heterozygous CXCL14+/− littermates.

Under RD feeding conditions, the mean body weight of CXCL14−/− mice (26.3 ± 1.3 g) was 24.6% less than that of CXCL14+/− mice (34.9 ± 2.3 g) (Fig. 2A). Periоварian WAT mass in RD-fed CXCL14−/− mice was significantly reduced, to 25.4% of CXCL14+/− mice (Fig. 2B). The livers of mice and kidney were comparable between the two strains of RD-fed mice (Fig. 2C). Under RD feeding conditions, CXCL14−/− mice ate 19.3% less than CXCL14+/− mice (Fig. 2D), which indicated that the lean phenotype and WAT hypertrophy of CXCL14−/− mice is because of decreased appetite. The total macrophage number in WAT was not significantly different between RD-fed CXCL14−/− and CXCL14+/− mice (Fig. 2B); thus, the decreased appetite of CXCL14−/− mice appeared to be independent of the macrophage chemoattractant activity of CXCL14 in adipose tissues.

Under HFD feeding conditions, whereas both CXCL14−/− and CXCL14+/− mice gained a significant amount of weight (Fig. 2, A and B), CXCL14−/− mice were 20.8% lighter than CXCL14+/− mice (Fig. 2A). In contrast to RD-feeding conditions, the periovarian WAT mass of CXCL14−/− mice increased under HFD-feeding conditions and was not significantly different from that of CXCL14+/− mice (3.4 ± 0.4 and 4.7 ± 0.6 g, respectively; p = 0.107) (Fig. 2B). To accurately determine the adiposity of HFD-fed CXCL14−/− mice, we analyzed a second group of HFD-fed female mice by CT scan. The estimated weights of total visceral fat and subcutaneous fat in the body were not statistically different (p = 0.0671 and 0.958, respectively) (Fig. 2E). Next, we compared fat cell size in HFD-fed CXCL14−/− mice and HFD-fed CXCL14+/− mice. As shown in supplemental Fig. S2, A–D, there was an HFD feeding-dependent hypertrophy of adipocytes in WAT of CXCL14−/− mice. These data collectively indicated that CXCL14−/− mice are not defective in adipogenesis and WAT development under HFD-feeding conditions. Although the WAT was enlarged in HFD-fed CXCL14−/− mice compared with HFD-fed CXCL14+/− mice, the dimensions (supplemental Fig. S3A) and mass (1.6 ± 0.1 and 3.8 ± 0.3 g, respectively) (Fig. 2D) of the livers were smaller in HFD-fed CXCL14−/− mice. The livers of HFD-fed CXCL14−/− mouse had less Oil Red O-positive lipid deposits compared with HFD-fed CXCL14+/− or CXCL14+/− mice (supplemental Fig. S3B). The amount of triglyceride in the livers of HFD-fed CXCL14−/− mice was 20.0% less than HFD-fed CXCL14+/− mice (supplemental Fig. S3C). These results indicated that CXCL14-deficient mice are partially protected from HFD-induced hepatic steatosis. In contrast to adipose tissue and skeletal muscle, HFD-induced up-regulation of CXCL14 mRNA was not observed in the livers of CXCL14+/− mice (supplemental Fig. S3D). Thus, the smaller size and decreased fat content in the livers of HFD-fed CXCL14−/− mice were likely an indirect
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effect of the CXCL14 deficiency. The kidney mass of HFD-fed CXCL14−/− mice was also smaller than HFD-fed CXCL14+/− mice (0.36 ± 0.02 and 0.45 ± 0.02 g, respectively) (Fig. 2D). The level of food intake of HFD-fed CXCL14−/− mice was not significantly different from that of HFD-fed CXCL14+/− mice (p = 0.097) (Fig. 2F). As mentioned above, although HFD-fed CXCL14−/− mice were able to convert excess energy into WAT, their body weight and organ masses were smaller than HFD-fed CXCL14+/− mice. This unusual phenotype implied that CXCL14−/− mice have central nervous system defects as well, because CXCL14 mRNA was also observed in the brain (Fig. 1A). The mean nasal-anal length of HFD-fed CXCL14−/− mice (9.22 ± 0.086 cm) was significantly shorter than that of HFD-fed CXCL14+/− mice (9.62 ± 0.073 cm; n = 5, p = 0.0077). Thus the lighter body weight of CXCL14 null mice may be because of a somatotropic defect.

Defective Macrophage Infiltration of WAT in HFD-fed CXCL14−/− Mice—Although there were a variety of phenotypic differences between CXCL14−/− and CXCL14+/− mice, both strains produced a comparable mass of WAT under HFD feeding conditions (Fig. 2, D and E). We next explored whether CXCL14 deficiency affected macrophage infiltration of WAT. Using FACS analysis, we observed that the total number of Mac1+ macrophages per periovarian WAT of RD-fed CXCL14+/− mice was not significantly different from that of CXCL14+/− mice (p = 0.254) (Fig. 3A). We observed a similar trend using immunohistochemical analysis of WAT sections with an anti-F4/80 antibody (Fig. 3B). In contrast, the number of Mac1+ macrophages in periovarian WAT of HFD-fed CXCL14+/− mice decreased to 28.0% of HFD-fed CXCL14+/− mice (Fig. 3A). Consistent with the FACS data, immunohistochemical analysis indicated that the frequency of F4/80+ macrophages surrounding mature adipocytes was decreased in WAT from HFD-fed CXCL14−/− mice compared with HFD-fed CXCL14+/− mice (Fig. 3B). Using an in vitro chemotaxis assay, we detected CXCL14-responsive cells in the Mac1+ population of peripheral blood mononuclear cells (Fig. 3C). These results indicated that CXCL14 is involved in the HFD-induced infiltration of macrophages into WAT. Note that the number of Mac1+ SVsCs in the WAT of HFD-fed CXCL14+/− mice was also reduced to 27.3% of that in HFD-fed CXCL14+/− mice (Fig. 3A). This result suggested that CXCL14 deficiency influences the migration of nonhematopoietic cells into WAT under HFD-feeding conditions. In the in vitro chemotaxis assay, we were unable to detect any migratory response to CXCL14 of Mac1− cells recovered from the WAT of HFD-fed CXCL14+/− mice (data not shown), indicating that Mac1− cells are mobilized by a secondary chemokine that is produced by the infiltrated macrophages, or that the CXCL14-responsiveness of Mac1− cells is lost soon after they enter WAT. The identification of the CXCL14 receptor would greatly facilitate the resolution of these two mechanisms.

Blood Glucose Level and Insulin Sensitivity of CXCL14-deficient Mice—Macrophage infiltration is thought to be one of the critical causes of obesity-induced insulin resistance. Therefore, we compared the blood glucose concentration and insulin responsiveness of CXCL14−/− and CXCL14+/− mice. Fasting blood glucose levels of RD-fed CXCL14−/− mice were slightly lower than those of RD-fed CXCL14+/− mice (79.6 ± 3.2 and 93.0 ± 6.4 mg/dl, respectively). Under HFD feeding conditions, the difference in fasting blood glucose concentration between CXCL14−/− and CXCL14+/− mice was more pronounced (127.0 ± 17.4 and 165.3 ± 15.0 mg/dl, respectively). An ITT revealed that HFD-fed CXCL14−/− mice were insulin-resistant, whereas HFD-fed CXCL14+/− mice exhibited a stronger insulin response to lower the blood glucose level (Fig. 4A). This trend was reproducible, although the extent of insulin responsiveness of HFD-fed CXCL14−/− mice in the ITT varied from experiment to experiment, most likely due to the mixed background of the mice. In comparison, CXCL14−/− mice under RD feeding conditions showed a marginally enhanced sensitivity to insulin (Fig. 4B), although this difference was most likely not statistically significant, based on an analysis of the areas under the curves in Fig. 4B (p = 0.0724). These results indicated that insulin sensitivity is associated with HFD-induced obesity in CXCL14−/− mice. Because the WAT mass of HFD-fed CXCL14−/− mice was similar to that of HFD-fed CXCL14+/− mice (Fig. 2, D and E), we were interested in whether the decreased infiltration of macrophages into the WAT and the lower fat content of the liver was associated with the amelioration of insulin resistance in HFD-fed CXCL14−/− mice. In an IGT, HFD-fed CXCL14−/− mice were glucose-intolerant (Fig. 4C).
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FIGURE 4. Protection from HFD-induced insulin resistance and hyperinsulinemia in CXCL14−/− mice. A and B, ITT of CXCL14−/− and CXCL14+/− female mice fed an HFD (A) or RD (B). C and D, IGTT of CXCL14−/− and CXCL14+/− female mice fed an HFD (C) or RD (D). E, concentrations of serum insulin in CXCL14−/− and CXCL14+/− female mice fed an RD (n = 5 for CXCL14−/−; n = 5 for CXCL14+/−). F, concentrations of serum insulin in HFD-fed CXCL14−/− and CXCL14+/− female mice during the IGTT (n = 3 for each). Values for the zero time point represent serum insulin concentrations of fasted mice. Data represent the means ± S.E. * p < 0.01; ** p < 0.05 compared with CXCL14+/− mice. A and B, number and mean body weights (g) ± S.E. for each group of mice are shown above the data legend.

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4C), and an analysis of the areas under the curves in Fig. 4C indicated that they were not statistically different (p = 0.688). In contrast, RD-fed CXCL14−/− mice exhibited better glucose disposal capacity (Fig. 4D) compared with CXCL14+/− mice, and this difference appeared to be statistically significant, based on analysis of the areas under the curves in Fig. 4D (p = 0.0126). These data indicated that CXCL14 deficiency sensitizes glucose adsorption capacity only in lean mice.

To explore whether there were gender-specific differences in the insulin sensitivity of HFD-fed CXCL14−/− mice, we performed ITTs and IGTTs using HFD-fed male mice. Although there was a slightly improved insulin response in the ITT in male mice when compared with CXCL14+/− mice (supplemental Fig. S4A), this difference was not statistically significant at any time point. The IGTT profile of HFD-fed CXCL14−/− male mice (supplemental Fig. S4B) was similar to that of HFD-fed CXCL14−/− female mice (Fig. 4C). These results demonstrated that the amelioration of HFD-induced insulin resistance in CXCL14−/− mice is largely dependent on gender.

Serum insulin concentrations of HFD-fed CXCL14−/− mice were significantly lower than those of HFD-fed CXCL14+/− mice (5.1 ± 1.5 and 13.9 ± 2.7 ng/ml, respectively) (Fig. 4E), which correlated with the improved insulin response of HFD-fed CXCL14−/− mice. This result indicated that CXCL14−/− mice are protected from HFD-induced hyperinsulinemia. However, the IGTT data clearly suggested that HFD-fed CXCL14−/− mice have a defect in glucose disposal. In fact, serum insulin levels of RD-fed CXCL14−/− mice were lower than RD-fed CXCL14+/− mice (0.52 ± 0.06 and 1.30 ± 0.23 ng/ml, respectively) (Fig. 4E). In addition, during the IGTT, serum insulin levels in HFD-fed CXCL14−/− mice were consistently lower than in HFD-fed CXCL14+/− mice (Fig. 4F). These results indicated that CXCL14 deficiency is associated not only with impaired insulin sensitivity but also impaired insulin production, which would result in CXCL14−/− mice being glucose-intolerant under HFD feeding conditions.

We next investigated the insulin response of the peripheral organs and tissues of HFD-fed CXCL14−/− mice. Consistent with the ITT results (Fig. 4A), insulin-stimulated phosphorylation of Ser115 of Akt in the liver (Fig. 5A), skeletal muscle (Fig. 5A), and periovarian WAT (Fig. 5B) of HFD-fed CXCL14−/− mice was much stronger than in the corresponding organs of HFD-fed CXCL14+/− mice. The improvement in insulin response in HFD-fed CXCL14−/− mice was most prominent in skeletal muscle. We observed a similar result in RD-fed control mice (Fig. 5A). These results demonstrated that insulin-mediated cellular responses in peripheral organs and tissues are preserved in HFD-fed CXCL14−/− mice.

Serum Lipids and Expression of Metabolic Regulator Genes in CXCL14−/− Mice—To examine the metabolic status of HFD-fed CXCL14−/− mice, we first examined the concentration of serum lipids in these animals. Serum cholesterol in HFD-fed CXCL14−/− mice was 22.4% lower than in CXCL14+/− mice (36.7 ± 2.0 and 47.3 ± 3.4 mg/dl, respec-
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the liver was decreased in HFD-fed CXCL14<sup>−/−</sup> mice (supplemental Fig. S3C), which indicated that the CXCL14-deficiency affects lipid turnover to some extent.

The expression levels of the mitochondrial genes uncoupling protein-1 (UCP-1) in BAT (supplemental Fig. S6A), and UCP-3 in WAT (supplemental Fig. S6B) and skeletal muscle (supplemental Fig. S6C) were similar in CXCL14<sup>+/−</sup> and CXCL14<sup>−/−</sup> mice under both RD and HFD feeding conditions. These data suggested that CXCL14 deficiency or HFD-induced up-regulation of CXCL14 does not influence the mRNA levels of these genes. In HFD-fed CXCL14<sup>−/−</sup> mice, the mRNA levels of glucose transporter-4 (GLUT4) in BAT and skeletal muscle was unaltered when compared with HFD-fed CXCL14<sup>+/−</sup> mice (supplemental Fig. S6, A and C). Of note, the expression of GLUT4 in WAT was enhanced in RD-fed CXCL14<sup>−/−</sup> mice compared with RD-fed CXCL14<sup>+/−</sup> mice (supplemental Fig. S6B). Down-regulation of GLUT4 mRNA occurred in both HFD-fed CXCL14<sup>−/−</sup> mice and HFD-fed CXCL14<sup>+/−</sup> mice (supplemental Fig. S6B). These results indicated that CXCL14 negatively regulates the expression of GLUT4 in WAT in RD feeding conditions but that CXCL14-mediated signaling is independent of obesity-induced down-regulation of GLUT4 in WAT, which is a common and crucial feature of insulin-resistant states (31).

Expression Levels of Adipokines and Cytokines in CXCL14-deficient Mice—We next examined the serum concentrations of leptin, adiponectin, and RBP4, which are key adipose tissue-derived regulators of obesity-associated type 2 diabetes. Serum leptin concentrations were lower in RD-fed CXCL14<sup>−/−</sup> mice compared with RD-fed CXCL14<sup>+/−</sup> mice (7.5 ± 2.3 and 18.7 ± 4.1 ng/ml, respectively) but were comparable in the two strains under HFD feeding conditions (Fig. 6A). These results correlated with the differences in WAT mass in mice fed an RD or an HFD (Fig. 2, C–E). In contrast, serum adiponectin levels were significantly higher in HFD-fed CXCL14<sup>−/−</sup> mice compared with HFD-fed CXCL14<sup>+/−</sup> mice (48.1 ± 2.5 and 22.7 ± 5.5 μg/ml, respectively) (Fig. 6B), indicating that CXCL14<sup>−/−</sup> mice were insensitive to HFD-induced hypoadiponectinemia. The ratio (w/w) of serum adiponectin per WAT mass in CXCL14<sup>−/−</sup> mice was 4.4-fold (RD feeding conditions) and 2.9-fold (HFD feeding conditions) higher than in CXCL14<sup>+/−</sup> mice. This result indicated that there is an inverse correlation between CXCL14 deficiency and serum adiponectin levels. The serum
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concentrations of RBP4 in CXCL14−/− mice were significantly lower than in CXCL14+/− mice under both RD and HFD feeding conditions (Fig. 6C). The levels of phosphoenolpyruvate kinase and glucose-6-phosphatase mRNAs in the livers of HFD-fed CXCL14+/− mice were much lower than in the livers of HFD-fed CXCL14−/− mice (supplemental Fig. S6D), which was consistent with previous reports showing that RBP4 enhances gluconeogenesis in the liver (9) and that adiponectin suppresses hepatic glucose production (8). Although precise measurements of glucose production and disposal rates in these mice would be required to establish a more definitive link, these results suggested that the decreased level of RBP4 and increased concentration of adiponectin in HFD-fed CXCL14−/− mice protect CXCL14−/− mice from obesity-induced gluconeogenesis in the liver and obesity-associated impairment of insulin signaling in WAT, liver and skeletal muscle.

IL-6 has been shown to be a crucial mediator of insulin resistance caused by the activation of NF-κB in hepatocytes (6). HFD feeding up-regulated the expression of IL-6 in WAT of CXCL14+/− mice but not CXCL14−/− mice (Fig. 6D). There were no significant changes in IL-1β and tumor necrosis factor-α mRNA levels in WAT of HFD-fed CXCL14+/− and HFD-fed CXCL14−/− mice (Fig. 6D). HFD-induced up-regulation of CCL2 occurred in both CXCL14+/− and CXCL14−/− mice (Fig. 6D), implying that obesity-induced up-regulation of CCL2 in WAT is independent of the action of CXCL14. There was limited mobilization of macrophages into the WAT of HFD-fed CXCL14−/− mice (see Fig. 3A). Thus, although CCL2 was up-regulated in WAT, CXCL14 likely plays a critical role in the chemotraction of WAT macrophages in HFD-induced obese mice.

Restoration of Insulin Resistance by Transgenic CXCL14 Expression and Attenuation of Insulin Signaling by CXCL14 in Cultured Myocytes—Based on our analysis of HFD-fed CXCL14+/− mice, CXCL14 appeared to be closely involved in obesity-induced insulin resistance. To determine whether reintroduction of CXCL14 reverted the insulin-sensitivity phenotype of HFD-fed CXCL14−/− mice, we generated a transgenic mouse line, referred to as mck-CXCL14-Tg, in which the murine CXCL14 cDNA was under the control of the mouse muscle creatine kinase promoter/enhancer (26). We then crossed mck-CXCL14-Tg mice with CXCL14−/− mice to generate the CXCL14−/−/Tg double mutant. The transgenic CXCL14 mRNA was expressed at a higher level than endogenous CXCL14 in skeletal muscle of mck-CXCL14-Tg mice (Fig. 7A). Under RD feeding conditions, the insulin response of double mutant CXCL14−/−/Tg mice in the IITT was indistinguish-
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able from that of CXCL14+/− or CXCL14−/− mice (Fig. 7B). However, under HFD feeding conditions, insulin sensitivity was significantly blunted in CXCL14−/− mice, similar to what was observed in HFD-fed CXCL14+/+ or HFD-fed CXCL14+/− mice (Fig. 7C). This result demonstrated that transgenic expression of CXCL14 in CXCL14−/− mice restores HFD-induced whole body insulin resistance. CXCL14 has insulin desensitizing activity in myocytes (see below); thus, additional obesity-associated factors most likely cooperate with CXCL14 in whole body insulin resistance. In the IITT, the mean body weight of HFD-fed CXCL14−/− mice was similar to HFD-fed CXCL14+/− mice, which indicated that CXCL14 can cause insulin resistance independent of the lighter body weight phenotype of HFD-fed CXCL14+/− mice. Because CXCL14 was specifically expressed in skeletal muscle of CXCL14+/− mice, production of CXCL14 in adipose tissues may not be absolutely required for HFD-induced insulin resistance in mice. The number of macrophages in WAT of HFD-fed CXCL14−/− mice was not fully restored to that of HFD-fed CXCL14+/+ mice, but it was slightly increased compared with HFD-fed CXCL14+/− mice (43.9 and 18.4%, respectively, of the number in HFD-fed CXCL14+/+ mice). Thus, we cannot rule out the possibility that WAT macrophages do not participate in CXCL14-mediated perturbation of the insulin response in obese mice.

Finally, we examined whether CXCL14 directly modulated insulin-mediated signal transduction in myocytes by analyzing Ser473 phosphorylation of Akt kinase and 2-DG uptake. In differentiated C2C12 cells that contained myotubes, insulin-stimulated phosphorylation of Akt was attenuated by treatment with CXCL14 (Fig. 7D). Based on densitometry analysis, phosphorylation of Akt in insulin plus CXCL14-treated cells was reduced to 59.2% of the level in cells stimulated with insulin alone. The insulin-stimulated uptake of 2-DG by C2C12-derived myocytes was significantly inhibited by CXCL14 in a dose-dependent fashion (Fig. 7E). To our knowledge, these results are the first demonstration that CXCL14 inhibits the insulin responsiveness of skeletal muscle cells in vitro. CXCL14 also inhibited glucose uptake in 3T3-L1-derived adipocytes (Fig. 7F), but the effect was very subtle when compared with the inhibitory effect of CXCL14 in cultured myocytes. We were unable to detect CXCL14 mRNA by RT-PCR in either C2C12-derived myocytes or 3T3-L1-derived adipocytes (data not shown); thus, we can exclude the possibility of desensitization by endogenous CXCL14. In light of our earlier observation of the clear insulin responsiveness in vivo of skeletal muscle in HFD-fed CXCL14−/− mice (Fig. 5A), these results suggested that skeletal muscle rather than adipose tissue is the major site of CXCL14 action.

DISCUSSION

In this study, using a genetic mouse model of obesity, we demonstrated that CXCL14 is a novel obesity-associated regulator of glucose metabolism in female mice. Although the level of CXCL14 protein in these mice has yet to be determined, our analysis of HFD-fed CXCL14+/+ mice indicated that obesity-induced up-regulation of CXCL14 in WAT promotes the infiltration of macrophages into WAT. We also showed that the enhanced transcription of CXCL14 in HFD-fed mice does not significantly affect adiposity but alters the expression of adipokines, including adiponectin, RBP4, and IL-6, and contributes to whole body insulin resistance. The increase of macrophages in WAT in CXCL14-null mice or a direct effect of CXCL14 likely plays an important role in this process. Among the peripheral organs and tissues that govern glucose metabolism, skeletal muscle was the most prominently effected in HFD-fed CXCL14+/− mice. Transgenic overexpression of CXCL14 in skeletal muscle restored insulin resistance in HFD-fed CXCL14+/− mice, which strongly suggests that skeletal muscle is a major target of CXCL14. We also presented evidence that CXCL14 indirectly participates in fatty liver formation, which may also have a large impact on glucose metabolism. Although CXCL14+/− mice were partially protected from weight gain under HFD feeding conditions, the total fat weight and the size of the adipocytes in WAT of CXCL14+/− mice were indistinguishable from HFD-fed CXCL14+/− mice. Previously, it was reported that CXCL14 has angiostatic activity, using the rat corneal micropocket assay (22). We did not observe any marked differences in the blood vessels in WAT, liver, and skeletal muscle in HFD-fed CXCL14+/− mice.

We demonstrated that CXCL14 mRNA is induced in multiple tissues in HFD-fed obese mice. Recently, it was shown that phosphorylation of c-Jun de-represses inflammation-associated gene expression in macrophages by removing the nuclear receptor-corepressor complex and that CXCL14 is one of the genes regulated by this complex (32). Therefore, increased FFAs, endoplasmic reticulum stress, and/or reactive oxygen species in WAT of obese mice might mediate c-Jun N-terminal kinase-1-induced transcription of CXCL14. Although additional experiments are need to understand the mechanism of CXCL14 regulation, transcriptional repression of CXCL14 or inhibition of CXCL14 activity via neutralizing antibodies or antagonists would provide valuable insight into the role of CXCL14 in whole body insulin resistance in HFD-induced obese mice.

Previous reports have demonstrated that CCL2 inhibits insulin-stimulated glucose uptake in adipocytes and skeletal muscle cells (12, 16). In this study, we showed that overexpression of CXCL14 in skeletal muscle confers insulin resistance in HFD-fed mice, and that CXCL14 directly attenuates insulin-stimulated glucose uptake in skeletal muscle cells in vitro. These results indicate that chemokines play a more crucial role in obesity-induced impairment of glucose metabolism than simply recruiting inflammatory cells into visceral WAT. Future studies with CXCL14-deficient mice will advance our understanding of how macrophage-mediated chronic inflammation in WAT contributes to insulin resistance, and how CXCL14 modulates glucose metabolism and the onset of type 2 diabetes.

Recently, Meuter et al. (33) reported that macrophages and dendritic cells function normally in CXCL14-deficient mice. Consistent with our results, they showed that CXCL14-null mice had lower body weights compared with wild-type littermate mice. Although it remains to be determined how CXCL14 deficiency leads to a lean phenotype, the results of this study illuminate a novel biological function of CXCL14 as a metabolic regulator.
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