Overexpression of Monocyte Chemoattractant Protein-1 in Adipose Tissues Causes Macrophage Recruitment and Insulin Resistance*

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Adipose tissue expression and circulating concentrations of monocyte chemoattractant protein-1 (MCP-1) correlate positively with adiposity. To ascertain the roles of MCP-1 overexpression in adipose tissue, we generated transgenic mice by utilizing the adipocyte P2 (aP2) promoter (aP2-MCP-1 mice). These mice had higher plasma MCP-1 concentrations and increased macrophage accumulation in adipose tissues, as confirmed by immunochemical, flow cytometric, and gene expression analyses. Tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) (14), monocyte chemoattractant protein-1 (MCP-1) (15, 16), and plasminogen activator inhibitor-1 (17). These inflammatory molecules may have local effects on white adipose tissue and plasma non-esterified fatty acid levels were increased in transgenic mice. aP2-MCP-1 mice showed insulin resistance, suggesting that inflammatory changes in adipose tissues may be involved in the development of insulin resistance. Insulin resistance in aP2-MCP-1 mice was confirmed by hyperinsulinemic euglycemic clamp studies showing that transgenic mice had lower rates of glucose disappearance and higher endogenous glucose production than wild-type mice. Consistent with this, insulin-induced phosphorylations of Akt were significantly decreased in both skeletal muscles and livers of aP2-MCP-1 mice. MCP-1 pretreatment of isolated skeletal muscle blunted insulin-stimulated glucose uptake, which was partially restored by treatment with the MEK inhibitor U0126, suggesting that circulating MCP-1 may contribute to insulin resistance in aP2-MCP-1 mice. We concluded that both paracrine and endocrine effects of MCP-1 may contribute to the development of insulin resistance in aP2-MCP-1 mice.

Obesity correlates closely with insulin resistance (1, 2). We have demonstrated that the size of adipocytes is inversely related to insulin sensitivity (3–5); namely, larger adipocytes are associated with insulin resistance, smaller adipocytes, with insulin sensitivity. Energy excess results in adipocyte hypertrophy, which in turn exerts deleterious effects on insulin sensitivity. Larger adipocytes are less insulin-sensitive as shown by impaired insulin stimulated glucose uptake. Moreover, diet-induced hypertrophy of adipocytes leads to changes in the profile of adipokines toward a deterioration of insulin sensitivity, particularly with decreased adiponectin levels (6, 7).

Recent studies have shown that obesity is associated not only with larger adipocytes but also with increasing numbers of infiltrating macrophages in adipose tissues (8–10). These adipose tissue macrophages are currently considered to be a major cause of obesity-associated chronic low grade inflammation (2, 11) via secretion of a wide variety of inflammatory molecules (12), including tumor necrosis factor-α (TNF-α)2, (13), interleukin-6 (IL-6) (14), monocyte chemoattractant protein-1 (MCP-1) (15, 16), and plasminogen activator inhibitor-1 (17). These inflammatory molecules may have local effects on white adipose tissue (WAT) physiology as well as potential systemic effects on other organs, which culminate in insulin resistance (12). The molecular signals that trigger the macrophage accumulation in obese WAT are, however, not yet known. How macrophage accumulation in adipose tissues causes systemic insulin resistance is currently unknown.

2 The abbreviations used are: TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; MCP-1, monocyte chemoattractant protein-1; WAT, white adipose tissue; CCR2, C-C motif chemokine receptor 2; TG, transgenic; aP2, adipocyte P2; WT, wild type; ITT, insulin tolerance test; NEFA, non-esterified fatty acid; IR, insulin receptor; IRS, IR substrate; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated extracellular signal protein kinase; BAT, brown adipose tissue; MMP12, matrix metallopeptidase 12; PPARγ, peroxisome proliferator-activated receptor γ; SVC, stromal-vascular cell; 2-DG, 2-deoxyglucose; Rd, rate of glucose disappearance; EGP, endogenous glucose production; ANOVA, analysis of variance; eWAT, epididymal WAT; HF, high fat; NC, normal chow.

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Among inflammatory molecules up-regulated in adipose tissues of obese animals and humans, MCP-1 has been viewed as one of the likely candidate adipokines initiating macrophage infiltration of the adipose tissue and inducing systemic insulin resistance. MCP-1 is a member of the CC chemokine family and promotes migration of inflammatory cells by chemotaxis and integrin activation (18), and it has been reported to recruit monocytes from the blood into atherosclerotic lesions, thereby promoting foam cell formation (19–21). MCP-1 expression in adipose tissue and plasma MCP-1 levels have been found to correlate positively with the degree of obesity (9, 10, 16, 22). In addition, increased expression of this chemokine in adipose tissue precedes the expression of other macrophage markers during the development of obesity (10). A recent report on mice lacking C-C motif chemokine receptor-2 (CCR2), a receptor for MCP-1, and other several chemokines suggested the MCP-1/CCR2 pathway to influence the development of obesity and insulin resistance via adipose macrophage accumulation and inflammation (23). Thus, we hypothesized that MCP-1 may serve as a signal that triggers inflammation by attracting macrophages into adipose tissues as well as an adipokine that causes insulin resistance by directly affecting insulin signaling in other organs.

In this study, we assessed the effect of adipose overexpression of MCP-1 on the development of insulin resistance by generating transgenic (TG) mice under the adipocyte P2 (aP2) promoter. The TG mice showed increased macrophage accumulation in adipose tissues with higher plasma MCP-1 concentrations than littermate wild-type (WT) mice. The TG mice were insulin-resistant as shown by insulin tolerance test (ITT), hyperinsulinemic euglycemic clamp studies, and insulin signal studies. Because the TG mice displayed increased gene expression of TNF-α and IL-6 as well as higher plasma concentrations of non-esterified fatty acids (NEFAs), adipocyte dysfunction caused by macrophage accumulation in adipose tissue may contribute to the development of systemic insulin resistance. In addition, we demonstrated that MCP-1 directly attenuated insulin signaling in myotube cells and insulin-stimulated glucose uptake in isolated skeletal muscle, suggesting that higher circulating MCP-1 may have a direct negative impact on insulin-stimulated glucose uptake in aP2-MCP-1 mice. Thus, we conclude that both macrophage accumulation leading to adipocyte dysfunction (local effects on adipose tissues) and direct effects of circulating MCP-1 on insulin target organs (endocrine effects) contribute to the development of insulin resistance in aP2-MCP-1 mice.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Recombinant mouse CCL2/JE/MCP-1 protein was purchased from R&D Systems Inc. (Minneapolis, MN). U0126 was purchased from Calbiochem. 2-Deoxy-D-[1-14C]glucose and L-[1-3H]glucose were purchased from Amer sham Radiolabeled Chemicals Inc. (St. Louis, MO). Mouse monoclonal anti-phosphotyrosine antibody 4G10 (PY), rabbit polyclonal antibodies to insulin receptor substrate (IRS)-1, IRS-2, and the phosphatidylinositol 3-kinase p85 regulatory subunit were purchased from Upstate Biotechnology Inc. Rabbit polyclonal antibody to insulin receptor β (IRβ) was purchased from Santa Cruz Biotechnology. Rabbit polyclonal antibodies against p44/42 MAPK, phosphor-p44/42 MAPK, Akt, and phospho-Akt (Ser-473) were purchased from Cell Signaling Technology.

**Cell Culture, Differentiation, and in Vitro Assay**—C2C12 mouse skeletal myoblast cell lines were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in humidified 5% CO2, 95% air at 37 °C and cultured to confluence. To induce differentiation, cells were switched to media containing Dulbecco’s modified Eagle’s medium, 2.5% horse serum, 100 units/ml penicillin, and 100 μg/ml streptomycin for the indicated time periods. For the Western blotting analyses, cells were serum-deprived for 10 h in media and treated with 10 nm MCP-1 for 5 min to detect the extracellular signal-regulated kinase (ERK) activation. C2C12 cells were treated with 1–10 nm MCP-1 for 30 min before 10 nm insulin stimulation to test activation of insulin signaling. In some experiments cells were pretreated with U0126, an inhibitor of mitogen-activated extracellular signal protein kinase (MEK), for 30 min before MCP-1 addition.

**Generation of TG Mice Expressing MCP-1 in Adipose Tissues**—A murine MCP-1 coding sequence cDNA for insertion was prepared by cloning reverse transcriptase-PCR products from mouse macrophage mRNA into a 2.1-TOP cloning vector (Invitrogen). For overexpression in adipose tissues, transgene expression was targeted to adipose tissue using the mouse aP2 promoter (24) kindly provided by Dr. Bruce Spiegelman (Dana Farber Institute, Boston, MA). The transgene consisted of 5.4 kilobases of the aP2 gene promoter linked to rabbit β-globin, the 447 bp MCP-1 cDNA, and a polyadenylation sequence (Fig. 2A). The construct was inserted into a pUC19 vector (Nippon Gene Co., Ltd.) and cloned. The purified Ascl-Ascl fragment was microinjected into the pronuclei of fertilized DBF2 eggs. The recipient eggs were [C57BL/6 × DBA2] F2 hybrids. TG founder or F2 mice were identified by Southern blot analysis of tail DNAs using the cDNA probe to the BamHI/BamHI site in MCP-1 and PCR. The primers used for genotyping PCR were as follows: 5’ primer, 5’-CATCTGTCCCTTCTCTTATGGTTC3’, and 3’ primer, 5’-CTAGTTCACGTGCACGTGGTC3’. From the 13 lines of TG mice obtained, we selected three lines showing graded expression of MCP-1 and designated them low (L), middle (M), and high (H). The founder and TG descendants were bred onto a C57BL/6 background for two generations. The F2 TG mice and their littermates were used in experiments. TG mice served as heterozygotes.

**Animal Care**—ob/ob mice with a C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME). C57BL/6 mice were purchased from CLEA Japan (Tokyo, Japan). Mice were housed under a 12-h light-dark cycle and given ad libitum access to normal chow MF consisting of 25% (w/w) protein, 53% carbohydrates, 6% fat, and 8% water (Oriental Yeast Co., Ltd., Osaka, Japan) or a high fat diet 32 consisting of 25.5% (w/w) protein, 2.9% fiber, 4.0% ash, 29.4% carbohydrates, 32% fat, and 6.2% water (CLEA Japan Inc., Tokyo, Japan). All experiments in this study were performed on male mice. The animal care and procedures for the experiments were approved by the Animal Care Committee of the University of Tokyo.
RNA Preparation and Northern Blot Analysis—Mice were sacrificed after a 6-h fast and the epididymal fat pad (for epididymal WAT), subcutaneous fat (for subcutaneous WAT), brown adipose tissue (BAT), liver, spleen, kidney, heart, and muscle were excised. Total RNA was prepared from tissues using TRIzol Reagent (Invitrogen) according to the manufacturer’s instructions. Northern blot analysis was performed with 15 μg of total RNA according to the standard protocol. Total RNA was loaded onto a 1.3% agarose gel then transferred to anylon membrane (Hybond N+; Amersham Biosciences). MCP-1 coding sequence cDNA was used as the probe template. The corresponding bands were quantified by exposure of BAS 2000 to the filters and measurement with BAStation software (Fuji Film, Tokyo, Japan).

Quantitative Reverse Transcriptase-PCR—Total RNA was extracted from various tissues or C2C12 cells with TRIzol reagent according to the manufacturer’s instructions. After treatment with RQ1 RNase-free DNase (Promega, Madison, WI) to remove genomic DNA, cDNA was synthesized with MultiScribe reverse transcriptase (Applied Biosystems, Foster City, CA), and TaqMan quantitative PCR (50 °C for 2 min, 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min) was then performed with the ABI Prism 7900 PCR instrument (Applied Biosystems) to amplify samples for MCP-1, F4/80, CD68, matrix metallopeptidase 12 (MMP12), glucose-6-phosphatase, TNF-α, IL-6, resistin, adiponectin, leptin, peroxisome proliferator-activated receptor γ (PPARγ), CCR2, and cyclophilin cDNA. The primers used for cyclophilin were as described previously (25), and those for the other reactions were purchased from Applied Biosystems. The relative abundance of transcripts was normalized to constitutive expression of cyclophilin mRNA.

Isolation of Adipocytes and Stromal-vascular Cells (26)—Mice were anesthetized, and epididymal white fat pads were removed. The fat pads were rinsed in saline and cut into small pieces, then digested with collagenase (Sigma-Aldrich) with Krebs-Henseleit-HEPES buffer, pH 7.4, supplemented with 20 mg/ml of bovine serum albumin and 2 mmol/liter glucose at 37 °C in a shaking water bath for 45 min. Then digested tissues were filtered through mesh and fractionated by brief centrifugation (1000 rpm). Floating cells were adipocytes, and the pellet was nonadipocytes (stromal-vascular cells (SVCs)). Both cell types were rinsed three times with Krebs-Henseleit-HEPES buffer and used in RNA extraction or flow cytometry analysis.

Flow Cytometry Analysis (9, 23)—In the SVCs red blood cells were lysed and removed by a 15-min incubation in Pharm Lyse (BD Biosciences) at 4 °C. The SVCs were rinsed twice and resuspended in Pharmingen stain buffer (BD Biosciences). The cell number was calculated by hemocytometry, and the cells were incubated with FcBlock (BD Biosciences) for 10 min at 4 °C before the incubation with either anti-mouse CD11b antibodies conju-
gated with Alexa Fluor488 (Caltag Laboratories) or anti-mouse F4/80 antibodies conjugated with phycoerythrin (Caltag Laboratories) or each of the matching isotopes as controls for 30 min at 4 °C. After incubation with the antibodies, the cells were rinsed twice and resuspended in Pharamingen stain buffer. After labeling with TO-PRO-3 (Invitrogen), the cells were analyzed by FACSCalibur (BD Biosciences). Data acquisition and analysis were performed using CellQuest Pro software (BD Biosciences). Dead cells were gated out by a combination of forward scatter side scatter (FSC/SSC) and TO-PRO-3 dot plots. The numbers of macrophages in epididymal white adipose tissues were calculated by multiplying the number of SVCs by the percentage of CD11d and F4/80 double positive cells.

Immunoprecipitation and Western Blot Analysis—Tissues and cells were homogenized and lysed with ice-cold buffer A (25 mM Tris-HCl, pH 7.4, 10 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM EGTA, and 1 mM phenylmethylsulfonyl fluoride). After centrifugation, immunoprecipitation of liver and muscle proteins was performed as described previously (27) with some modifications. Samples were separated on polyacrylamide gels and transferred to Hybond-P PVDF transfer membrane (Amersham Biosciences). After incubating the membrane with antibodies, bands were detected by ECL detection reagents (Amersham Biosciences).

Histological and Immunohistochemical Analysis of WAT—An epididymal fat pad was removed from each animal, fixed in 10% formaldehyde/phosphate-buffered saline, and maintained at 4 °C for 2 days. Fixed specimens were dehydrated and embedded in paraffin. The fat pad was then cut into 5-μm sections at 50-μm intervals and then mounted on silanized slides. After deparaffinization, the sections were stained with rat monoclonal F4/80 antibody (Serotec Ltd.) at a 1:1000 concentration followed by counter-
staining with hematoxylin. The adipocyte area was manually traced and analyzed with Win ROOF software (Mitani Co., Ltd., Chiba, Japan). The area was measured in four high-power fields (275,000 μm²/field) from different sections, and the histogram was drawn by analyzing 6 mice per group according to methods described previously (4) with modifications. The adipocyte area was measured in 400 or more cells per mouse on normal chow or in 180 or more cells per mouse on the high fat diet. The total number of nuclei and the number of F4/80 positive nuclei were counted in four different high-power fields from each of four different sections. The nuclei of more than 2000 cells per mouse on normal chow or more than 1000 cells per mouse on the high fat diet were counted. The ratio of F4/80 positive nuclei was calculated as the number of nuclei of F4/80-expressing cells divided by the total number of nuclei in sections of a sample.

**Measurement of 2-Deoxyglucose (2-DG) Uptake**—This assay was performed as described previously (28) with some modifications. The soleus muscles of 9-week-old C57BL/6 mice were removed from the hindlimbs, ligated around each tendon using silk surgical thread, and attached across a plastic holder. The muscles were incubated for 10 min at 37 °C in Krebs-Ringer phosphate buffer, pH 7.4, containing 5 mM HEPES, 3% bovine serum albumin, and 2 mM sodium pyruvate (buffer A). For MCP-1 pretreatment, the muscles were incubated in buffer A containing 0, 0.1, 1, or 10 nM MCP-1 for 30 min at 37 °C before insulin treatment. The muscles were incubated with or without 10 nM insulin in buffer A containing 0, 0.1, 1, or 10 nM MCP-1 for 30 min at 37 °C before insulin treatment. The muscles were incubated with or without 10 nM insulin in buffer A containing 0, 0.1, 1, or 10 nM MCP-1 for 30 min at 37 °C before insulin treatment. The muscles were incubated with or without 10 nM insulin in buffer A containing 0, 0.1, 1, or 10 nM MCP-1 for 30 min at 37 °C before insulin treatment. The muscles were incubated with or without 10 nM insulin in buffer A containing 0, 0.1, 1, or 10 nM MCP-1 for 30 min at 37 °C before insulin treatment.
added to all of the buffers. The buffers were continuously gassed with 95% O₂, 5% CO₂ in a shaking incubator. To terminate the reaction, the muscles were washed 3 times with chilled buffer A and then dissolved in 5N NaOH. The samples were neutralized with 5N HCl and dissolved in ACSII (Amersham Biosciences). 14C and 3H specific activities were counted by a liquid scintillation counter (Packard Instrument Co.). The specific uptake of 2-DG was calculated by subtracting the nonspecific uptake of L-glucose from total uptake 2-DG uptake.

**Plasma MCP-1, Adiponectin, Leptin, and NEFA Measures**—Mice were fasted for 6 h before plasma was obtained. Plasma MCP-1, adiponectin, and leptin levels were determined with a mouse JE/MCP-1 enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems), mouse adiponectin ELISA kit (Otsuka Pharmaceutical Co., Ltd, Tokyo, Japan), and mouse leptin ELISA kit (R&D Systems), respectively. Plasma NEFAs (Wako Pure Chemical Industries Ltd., Osaka, Japan) were assayed by enzymatic methods.

**Measurement of Tissue Triglyceride Contents**—Liver and muscle tissues were homogenized, and their triglyceride contents were determined as described previously (6).

**Hyperinsulinemic-Euglycemic Clamp Study**—Clamp studies were carried out as described previously (29). In brief, 4–5 days before the study, an infusion catheter was inserted into the right jugular vein under general anesthesia with sodium pentobarbital. Studies were performed on mice under conscious and unstressed conditions after a 6-h fast. A primed-continuous infusion of insulin (Humulin R, Lilly) was given (3.0 milliunits/kg/min for normal chow (NC) fed mice and 10.0 milliunits/kg/min for high fat (HF) diet-fed mice), and the blood glucose concentration, monitored every 5 min, was maintained at ~120 mg/dl by administration of glucose (5 g of glucose/10 ml enriched to ~20% with [6,6-²H₂]glucose (Sigma)) for 120 min. Blood was sampled via tail-tip bleeds at 90, 105, and 120 min for determination of the rate of glucose disappearance (Rd). Rd was calculated according to non-steady-state equations (29), and endogenous glucose production (EGP) was calculated as the difference between Rd and exogenous glucose infusion rates (29).

**Statistical Analysis**—Results were expressed as the means ± S.E. Differences between groups were examined for statistical significance using Student’s t test, analysis of variance (ANOVA) with
Fisher's protected least significant difference test, or ANOVA with the Games-Howell test.

RESULTS

Increased MCP-1 Expression in WAT of Obesity Model Mice—

The macrophage content of adipose tissue has been reported to be increased by obesity and to correlate positively with insulin resistance (9, 10, 15, 22). To identify potential regulators of macrophage accumulation, we studied the expression profiles of epididymal adipose tissue from WT animals and PPARγ+/− mice (4) on the high fat diet using oligonucleotide microarrays (Murine Genome Array U74 Version 2, Affymetrix Inc.). The expression of MCP-1, a powerful chemokine involved in macrophage chemotaxis and recruitment, was up-regulated by 2.7-fold in WT mice as compared with PPARγ+/− mice, which showed protection from obesity and insulin resistance even on the high fat diet (data not shown). MCP-1 expression was increased by 4.5-fold in ob/ob mice together with enhanced expressions of macrophage markers including F4/80, CD68, and MMP12 (Fig. 1A). As reported previously, we also observed increased plasma concentrations of MCP-1 in ob/ob mice (Fig. 1B). C57BL/6 mice on a high fat diet showed increased expressions of MCP-1 and CD68 (Fig. 1C) in epididymal WAT as compared with control mice on a normal chow diet. These enhanced gene expressions were observed as early as 8 weeks of high fat feeding and became even more pronounced thereafter. Increased plasma levels of MCP-1 were observed after 18 weeks of the high fat diet (Fig. 1D). Thus, the accumulation of macrophages in WAT preceded the elevation of plasma MCP-1 with high fat feeding. As shown in Fig. 1E, high fat diet feeding led to increased MCP-1 mRNA only in WAT and BAT.

Generation of TG Mice Overexpressing MCP-1 in Adipose Tissues—

Having demonstrated insulin resistance to be associated with enhanced MCP-1 expression in adipose tissue, we hypothesized that increased expression of this chemokine in these tissues may lead to systemic insulin resistance. Thus, we generated TG mice overexpressing MCP-1 in adipose tissue, utilizing the aP2 promoter (Fig. 2A). We obtained three lines of TG mice with graded expressions of MCP-1, designated low (L), middle (M), and high (H), with Line L having the fewest copies of the transgene (Fig. 2B). All of these lines expressed MCP-1 abundantly in WAT as well as BAT (Fig. 2C), although minimal MCP-1 expressions were observed in the spleen, heart, and muscle. Expression analyses

![Graphs of glucose levels and insulin levels during ITT and GTT tests for different lines of TG mice.](https://example.com/graphs.png)
revealed MCP-1 expression to be markedly increased in WAT as well as BAT in TG mice of Line M (Fig. 2D). In this analysis, enhanced expression of MCP-1 mRNA was also noted in the liver, spleen, and muscle. This was presumably due to aP2 activity in macrophages as well as adipocytes residing in these organs, as previously reported (30). When determined after adipose tissues had been fractioned into adipocytes and SVCs in Line M mice, MCP-1 expressions were observed mainly in adipocytes, although a small but significant increase in the expression of this gene was also seen in SVCs in the Line M mice (Fig. 2E).

Plasma MCP-1 concentrations were determined. All three lines had elevated MCP-1 concentrations as compared with littermate controls (Line L, 78.9 ± 6.8 pg/ml on normal chow and 66.6 ± 2.0 pg/ml on high fat; Line M, 161.6 ± 6.8 pg/ml on normal chow and 213.7 ± 14.2 pg/ml on high fat; Line H, 189.1 ± 17.2 pg/ml on normal chow and 238.8 ± 8.5 pg/ml on high fat) (Fig. 2F). Of note, the MCP-1 elevations in aP2-MCP-1 TG mice rose to levels between those of C57BL/6 mice and ob/ob mice (Fig. 1B).

aP2-MCP-1 TG Mice Show Normal Adiposity and Increased Infiltration of Macrophages into WAT—To assess the roles of MCP-1 overexpression and macrophage recruitment in adipose tissue in the development of insulin resistance, we fed either normal chow or a high fat diet to the three lines of male aP2-MCP-1 TG mice as well as their littermate controls. aP2-MCP-1 TG mice gained weight to the same extent as their WT littermates did on either normal chow or the high fat diet (Fig. 3A). We further examined the weight of adipose tissue, the size of adipocytes, and the number of accumulated macrophages in the epididymal WAT in Line M of the aP2-MCP-1 TG mice on either normal chow or after 8 weeks of high fat diet feeding. Although neither epididymal WAT weight nor adipocyte size differed between aP2-MCP-1 TG mice and WT littermates (Fig. 3, B and D), immunohistochemistry showed the number and the distribution of macrophages to be altered in aP2-MCP-1 mice. As shown in Fig. 3C, more macrophages were scattered throughout adipose tissue in aP2-MCP-1 TG mice than in WT control mice. When fed a high fat diet for 8 weeks, aP2-MCP-1 TG mice exhibited more areas of aggregated F4/80-positive nuclei surrounding adipocytes, which formed a typical crown. Morphometric analysis showed that aP2-MCP-1 TG mice had more F4/80 positive nuclei in adipose tissues (Fig. 3E), further confirming that these mice had greater accumulation of macrophages in adipose tissues. Furthermore, flow cytometric analyses of stromal vascular fraction in Line M mice showed that the percentage of CD11b and F4/80 double-positive cells to be increased in TG mice (41.0 ± 1.0%) as compared with WT mice (34.7 ± 1.3%; p < 0.01 versus TG mice) on a normal chow diet. We also observed an increase in the total number of macrophages in the stromal vascular fraction of epididymal WAT in TG mice compared with that in WT mice (Fig. 3F). Expression analyses of F4/80, CD68, and MMP12 in WAT revealed the expressions of these macrophage markers to be increased in TG mice as compared with WT mice, and the differences became significant on a high fat diet (Fig. 3G). Although they were not statistically different, the expressions of macrophage markers in TG mice fed on a normal chow diet tended to be higher than those in WT mice.

FIGURE 6. Hyperinsulinemic euglycemic clamp studies demonstrate insulin resistance in skeletal muscle and liver of aP2-MCP-1 TG mice. A–B, hyperinsulinemic euglycemic clamp study showed glucose infusion rates (GIR), EGP, and Rd in aP2-MCP-1 TG (filled bars) and WT (open bars) mice of Line M on NC (A) or a HF diet (B) (n = 6 per group). Values are the means ± S.E. *p < 0.05, by Student’s t test. C, expressions of glucose-6-phosphatase (G6Pase) in the livers of WT (open bars) and TG (filled bars) mice (n = 6 per group) of Line M on the HF diet after the clamp studies. Values are the means ± S.E. p < 0.05 (*) by Student’s t test.

TG Animals Have High TNF-α and IL-6 Expressions in WAT and High Plasma NEFAs—Gene expression analysis in the Line M revealed the expressions of TNF-α and IL-6 to be increased in WAT of TG mice (Fig. 4A). On the contrary, however, the expressions of adipokines including resistin, adiponectin, and leptin were not altered. We also observed the plasma NEFA concentration to be increased in TG mice, whereas plasma adiponectin and leptin levels were not changed (Fig. 4B).

aP2-MCP-1 TG Mice Were Insulin-resistant and Glucose-intolerant—To assess insulin sensitivity in the aP2-MCP-1 TG animals, we performed ITT. In all three lines of the aP2-MCP-1 mice, whether on a normal or a high fat diet, the glucose lowering effect of insulin was impaired (Fig. 5A). Oral glucose tolerance test demonstrated mice of TG Line L, which had low MCP-1 expression, to not have altered glucose metabolism on either the normal diet or the high fat diet (Fig. 5B). Although both the M and the H Line had normal glucose metabolism after glucose loading on a normal diet, these mice showed impaired glucose tolerance when fed a high fat diet. As shown in Fig. 4C, the plasma insulin concentrations of the aP2-MCP-1 TG mice tended to be higher than those of control littermates.

Hyperinsulinemic Euglycemic Clamp Study Revealed Insulin Resistance in Skeletal Muscle and Liver of aP2-MCP-1 TG Mice—To further examine the mechanisms of the insulin resistance in TG mice, we performed a hyperinsulinemic euglycemic clamp study on Line M mice (Fig. 6, A and B). The TG mice required less glucose to be infused to maintain euglycemia on both the normal chow and the high fat diet. EGP appeared to be higher and Rd tended to be lower in the TG mice than in their littermate counterparts while on normal chow. The difference in EGP and Rd increased, becoming statistically significant when WT and TG mice were fed a high fat diet. These
observations indicate insulin resistance in muscle and liver. Hepatic glucose-6-phosphatase expression levels were higher in TG than in WT mice on a high fat diet, which is consistent with the increased hepatic glucose production in aP2-MCP-1 mice (Fig. 6C). No significant differences in triglyceride contents were observed in either muscle or hepatic tissues between WT and TG mice of Line M whether fed normal chow or a high fat diet (data not shown).

MCP-1 Overexpression in WAT Decreased Tyrosine Phosphorylation of IR and IRS Proteins in Both Skeletal Muscle and Liver—To further examine the mechanisms of insulin resistance in aP2-MCP-1 mice, we studied the insulin signaling pathways in skeletal muscle and the liver in WT and TG mice of Line M on normal chow or high fat diets (Figs. 7 and 8). In muscle, Akt phosphorylation was decreased in TG mice as compared with WT mice on both normal chow and a high fat diet (Fig. 7A). In addition, tyrosine phosphorylation of the IR was decreased in TG mice (Fig. 7B). Likewise, insulin-stimulated tyrosine phosphorylation of IRS-1 (Fig. 7C) and insulin-stimulated association of p85 with IRS-1 (Fig. 7D) were also decreased in TG mice. TG mice showed no significant difference in insulin-stimulated tyrosine phosphorylation of IRS-2 (Fig. 7E) or the amount of IRS-2-associated p85 (Fig. 7F) in muscle.

In the liver we observed reductions in phosphorylated Akt and tyrosine phosphorylation of IR with insulin stimulation (Fig. 8, A and B), similar to the reductions seen in muscle. A significant decrease in insulin-stimulated IRS-1 tyrosine phosphorylation was seen in the TG mice fed a normal diet (Fig. 8C), whereas the amount of IRS-1-associated p85 in TG mice was not significantly decreased (Fig. 8D). There was also a significant decrease in insulin-stimulated tyrosine phosphorylation of IRS-2 (Fig. 8E) and the amount of IRS-2-associated p85 (Fig. 8F) in the liver.

MCP-1 Directly Impaired Insulin Signaling in Myotube Cells and Isolated Skeletal Muscle—aP2-MCP-1 mice displayed not only macrophage accumulation in adipose tissue but also higher plasma MCP-1 concentrations. We presumed that circulating MCP-1 might directly affect insulin signaling in skeletal muscle. Because CCR2, the receptor for MCP-1, is expressed in skeletal muscle (Fig. 9A) and in C2C12-differentiated myotube cells (Fig. 9B), we assessed whether MCP-1 has a direct effect on insulin signaling via ERK phosphorylation utilizing the C2C12 cell line and isolated skeletal muscle. Incubation with 10 nM MCP-1 for 5 min in C2C12 myotubes stimulated ERK phosphorylation, which was reversed by a 30-min
preincubation with 100 nM U0126, a specific MEK inhibitor (Fig. 9C). We found that pretreatment of the cells with 1–10 nM MCP-1 for 30 min significantly attenuated insulin-stimulated Akt phosphorylation (Fig. 9D), which was restored, albeit only partially, by preincubation with 100 nM U0126. In addition, preincubation with MCP-1 blunted insulin-stimulated 2-deoxyglucose uptake in isolated skeletal muscle, but this uptake was also partially restored by preincubation with U0126 (Fig. 9E).

Circulating MCP-1 secreted by adipocytes of aP2-MCP-1 mice may directly attenuate insulin signaling in skeletal muscle in an endocrine fashion (Fig. 10B). We have shown that MCP-1 treatment of C2C12 myotube cells blunted insulin signals, including Akt phosphorylation (Fig. 9D). We further showed that MCP-1 attenuated insulin-stimulated glucose uptake in isolated skeletal muscle (Fig. 9E). These negative effects of MCP-1 on insulin action were partially restored by the addition of the MEK inhibitor

**FIGURE 8.** Insulin signals in the livers of aP2-MCP-1 TG mice of Line M were also attenuated as compared with those of WT mice. A, equal amounts of liver protein in total lysates were immunoblotted with anti-phospho-Akt (pAkt) and anti-Akt antibodies (n = 4 per group). Phosphorylated-Akt and the Akt signal were quantified with a scanning imager. The relative ratio of Akt phosphorylation was calculated after normalization with the total Akt signal and then normalized in WT mice without insulin (Ins). B, lysates were immunoprecipitated with anti-insulin receptor β subunit antibodies (αIRβ), and the immunoprecipitates (IP) were subjected to Western blotting with αPY and αIRβ. C–D, lysates were immunoprecipitated with αIRS-1 and immunoblotted with αPY (C), αPY (D), and αIRS-1 (C–D). E–F, lysates were immunoprecipitated with αIRS-2 and immunoblotted with αPY (E), αPY (E), and αIRS-2 (E–F). Values are the means ± S.E. p < 0.05 (*) and p < 0.01 (**), compared with WT mice under the same conditions by ANOVA.

**DISCUSSION**

In this study we demonstrated that overexpression of MCP-1 in adipose tissue caused systemic insulin resistance, which was shown by ITT (Fig. 5A) and confirmed by a hyperinsulinemic euglycemic clamp study and the assessment of insulin signaling (Figs. 6–8). The aP2-MCP-1 mice had an increased number of adipose tissue macrophages with higher plasma MCP-1 levels. We assume that both the paracrine and the endocrine effects of MCP-1 contributed to the development of insulin resistance in aP2-MCP-1 mice, although it is difficult to quantify the degree to which each of these effects contributes to the development of insulin resistance.

Changes in proinflammatory adipokines may contribute to the development of insulin resistance in this murine model (Fig 10A). Although there were no changes in adiponectin or leptin levels in our aP2-MCP-1 TG mice, we observed elevations of TNF-α and IL-6 mRNA in WAT. We assume the increased expressions of TNF-α and IL-6 gene to be attributable to the increased macrophage accumulation. In our preliminary experiments large amounts of TNF-α and IL-6 were secreted by cells in the stromal-vascular fraction whereas significant amounts were also from the adipocytes themselves (31, 32) (data not shown). TNF-α and IL-6 from accumulated macrophages may work locally on adipocytes (33), causing changes in metabolic and endocrine functions as they do systemically on skeletal muscle and the liver. MCP-1 secreted by adipocytes may locally affect insulin signaling in adipocytes (22), thereby contributing to the increased release of NEFAs. Elevated NEFAs may also participate in the exacerbation of insulin resistance in aP2-MCP-1 mice (34–36).
U0126, suggesting that ERK activation is part of the mechanism by which MCP-1 blunts insulin signaling. These data indicated that circulating MCP-1 may contribute to the development of insulin resistance in aP2-MCP-1 mice.

Results of a hyperinsulinemnic euglycemic study indicated that aP2-MCP-1 mice have lower Rd and higher EGP (Fig. 6). Insulin resistance in skeletal muscle and liver was confirmed by an insulin signaling study (Figs. 7 and 8). In fact, insulin-stimulated Akt phosphorylation was blunted in both skeletal muscle and liver. Analyses of insulin signaling revealed slight but significant attenuation of tyrosine phosphorylation of the insulin receptor in the liver as well as skeletal muscle. A difference was also seen in tyrosine phosphorylation of IRS proteins. We and others have demonstrated that IRS-1 plays a major role in insulin signaling in skeletal muscle, whereas IRS-2 predominantly functions in the liver (27, 29, 37–41). In muscle, reduction of tyrosine phosphorylation and less IRS-1-associated p85 were apparent, whereas IRS-2 tyrosine phosphorylation and IRS-2-associated p85 were reduced in the liver. Decreased tyrosine phosphorylation of IRS-1 or IRS-2 in skeletal muscle or the liver, respectively, was responsible for the insulin signaling defects shown by reduced Akt phosphorylation in these two tissues.

The roles of MCP-1 in adipose tissue inflammation and systemic insulin resistance were also supported by the metabolic phenotypes of mice lacking CCR2, a receptor for MCP-1 (CCL2), MCP-2 (CCL8), and MCP-3 (CCL7) (23). The CCR2/H11002 mice showed improved systemic glucose homeostasis and insulin sensitivity accompanied by reduced macrophage contents and an altered inflammatory profile in adipose tissue while on a high fat diet. These mice showed reduced food intake with attenuated obesity. They exhibited increased adiponectin and leptin gene expressions. However, the adiposity of our aP2-MCP-1 mice was comparable with that in WT mice, and there were no significant changes in adiponectin or leptin gene expressions. Our data suggested that CCR2 ligands other than MCP-1 may be involved in CCR2 signaling so far as metabolic phenotypes are concerned.

While this manuscript was being revised, glucose metabolism in aP2-MCP-1 transgenic mice was described by Kanda et
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FIGURE 10. Overexpression of MCP-1 in adipose tissues causes insulin resistance in both a paracrine and an endocrine manner. A and B, we propose that both increased accumulation of macrophages in adipose tissue (A) and higher plasma levels of MCP-1 (B) contributed to the development of insulin resistance in aP2-MCP-1 TG mice. A, MCP-1 produced by adipocytes induced recruitment of macrophages to adipose tissue, thereby causing inflammation. In fact, increased gene expression of inflammatory secretory molecules such as TNF-α and IL-6 was observed in adipose tissue of aP2-MCP-1 mice. In addition, plasma NEFA levels were increased in TG mice. Increased NEFA levels as well as TNF-α and IL-6 gene expressions may contribute to the development of insulin resistance. B, circulating plasma MCP-1 may also contribute to the development of systemic insulin resistance in aP2-MCP-1 mice. We demonstrated that MCP-1 blunted insulin signaling and insulin-stimulated glucose uptake in myotube cells and isolated skeletal muscle, respectively. Alternation of these two pathways may cause insulin resistance. GIR, glucose infusion rates.

It is noteworthy that the role of MCP-1 expression in attracting macrophages in WAT and BAT seems to be different. We observed an increased expression of MCP-1 gene in BAT compared with that in WAT under both normal chow and a high fat diet (Fig. 2, C and D). The expression of macrophage markers in BAT, however, was not increased as much as that in WAT. These data suggested that MCP-1 expression is not sufficient for macrophage accumulation, and an additional factor(s), which is differentially expressed in between WAT and BAT, may be required for macrophage accumulation.

The findings derived from our aP2-MCP-1 mice corroborate the link between inflammation in adipose tissues and systemic insulin resistance. First, our TG mice showed greater macrophage accumulation in adipose tissue, and the aggregated macrophages were more readily apparent when the transgenic mice were fed a high fat diet. Second, although adipose cell size did not differ in our aP2-MCP-1 mice, the functions of adipocytes were altered, as evidenced by increased plasma concentrations of NEFAs. Finally, our TG mice were insulin-resistant as shown by insulin tolerance tests as well as hyperinsulinemic euglycemic clamp study.

In conclusion, transgenic overexpression of MCP-1 in adipose tissue resulted in systemic insulin resistance. We propose that both paracrine and endocrine effects of MCP-1 contribute to the development of insulin resistance in aP2-MCP-1 mice (Fig 10).

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