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## An Increase in the Circulating Concentration of Monocyte Chemoattractant Protein-1 Elicits Systemic Insulin Resistance Irrespective of Adipose Tissue Inflammation in Mice

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Chronic inflammation in adipose tissue is thought to be important for the development of insulin resistance in obesity. Furthermore, the level of monocyte chemoattractant protein-1 (MCP-1) is increased not only in adipose tissue but also in the circulation in association with obesity. However, it has remained unclear to what extent the increased circulating level of MCP-1 contributes to insulin resistance. We have now examined the relevance of circulating MCP-1 to the development of insulin resistance in mice. The plasma concentration of MCP-1 was increased chronically or acutely in mice to the level observed in obese animals by chronic subcutaneous infusion of recombinant MCP-1 with an osmotic pump or by acute intravenous infusion of MCP-1 with an infusion pump, respectively. Whole-body metabolic parameters as well as inflammatory changes in adipose tissue were examined. A chronic increase in the circulating level of MCP-1 induced insulin resistance, macrophage infiltration into adipose tissue, and an increase in hepatic triacylglycerol content. An acute increase in the circulating MCP-1 concentration also induced insulin resistance but not macrophage infiltration into adipose tissue. In addition, inhibition of signaling by MCP-1 and its receptor CCR2 by administration of a novel CCR2 antagonist ameliorated insulin resistance in mice fed a high-fat diet without affecting macrophage infiltration into adipose tissue. These data indicate that an increase in the concentration of MCP-1 in the circulation is sufficient to induce systemic insulin resistance irrespective of adipose tissue inflammation. (*Endocrinology* 151: 971–979, 2010)

The number of individuals with type 2 diabetes is increasing as a result of the aging population, urbanization, and physical inactivity. In particular, the marked increase in the worldwide prevalence of obesity is a substantial contributing factor to the increased incidence of diabetes (1). Over the past decade, obesity research has revealed that adipose tissue is indispensable for appropriate glucose and lipid metabolism as well as energy homeostasis (2). In addition to its role in energy storage, adipose tissue is an active endocrine organ and secretes a variety of bioactive molecules, known as adipokines, that

include leptin, adiponectin, resistin, TNF- $\alpha$ , IL-6, and monocyte chemoattractant protein-1 (MCP-1). Some adipokines are proinflammatory whereas others are antiinflammatory. Changes in adipokine expression induced by obesity are associated with systemic insulin resistance and reflect chronic inflammation in adipose tissue (3, 4). These findings suggest that obesity, insulin resistance, and type 2 diabetes are all associated with chronic inflammation characterized by abnormal cytokine production, up-regulation of acute-phase reactants, and activation of a network of inflammatory signaling pathways (5).

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Abbreviations: CCR2, Chemokine (C-C motif) receptor 2; G6Pase, glucose-6-phosphatase; HGP, hepatic glucose production; MCP-1, monocyte chemoattractant protein-1; NEFA, nonesterified fatty acid; SREBP1c, sterol regulatory element-binding protein 1c; TAG, triacylglycerol.

The inflammatory response associated with obesity appears to be triggered by and to be largely restricted to adipose tissue, although other metabolically important organs, in particular the liver, may also be affected during the course of the disease (6). In both humans and mice, the number of bone marrow-derived macrophages that have infiltrated into adipose tissue correlates with obesity and adipocyte size (7). Adipose tissue in obese subjects is thus characterized by macrophage infiltration (7, 8). These macrophages possess the characteristics of M1 or classically activated macrophages (9) and are a substantial source of TNF- $\alpha$ , IL-6, and MCP-1 in white adipose tissue of obese mice and humans (7, 8). These observations suggest that macrophage infiltration into adipose tissue is an early contributing event to the development of systemic insulin resistance. The increased expression of MCP-1 in white adipose tissue associated with obesity is thought to play an important role in recruiting monocytes to adipose tissue. Indeed, transgenic mice that overexpress MCP-1 specifically in adipocytes develop adipose tissue inflammation and insulin resistance without obesity (10, 11). In addition, mice deficient in MCP-1 or its receptor chemokine (C-C motif) receptor 2 (CCR2) manifest a reduced susceptibility to the development of adipose tissue inflammation and insulin resistance when fed a high-fat diet (10, 12). *In vitro* studies have also shown that recombinant MCP-1 induces insulin resistance in 3T3-L1 adipocytes and C2C12 myotubes (11, 13). However, other studies have found that MCP-1-deficient mice exhibit an increased susceptibility to the development of inflammation in white adipose tissue when fed a high-fat diet (14, 15).

The abundance of MCP-1 mRNA in adipose tissue has been shown to be increased in obese individuals (16) and to correlate with body mass index (17). The serum concentration of MCP-1 was also found to be higher in patients with type 2 diabetes than in normal subjects (18). These observations suggest that MCP-1 in adipose tissue or in circulating blood may contribute to the development of insulin resistance in humans. However, the mechanism by which MCP-1 might contribute to insulin resistance has remained unknown. Furthermore, it has not been clear whether macrophage infiltration into adipose tissue is required for the induction of insulin resistance by MCP-1, or whether an increase in the circulating concentration of MCP-1 is sufficient for such induction. To address these questions, we have now examined the effects of an increase in the plasma concentration of MCP-1 resulting from short-term (acute) or long-term (chronic) administration of recombinant MCP-1 in mice. In addition, we examined whether inhibition of MCP-1-CCR2 signaling by administration of a novel CCR2 antagonist in mice might ame-

liorate insulin resistance irrespective of adipose tissue inflammation.

## Materials and Methods

### Experimental animals

C57BL/6N mice were obtained from CLEA Japan (Tokyo, Japan), and a high-fat diet (56% of calories from fat) was from Oriental Yeast (Tokyo, Japan). All mice were maintained under a 12-h light, 12-h dark cycle and had access to food and water *ad libitum*. The experimental protocols were performed with male mice in accordance with the guidelines of the animal ethics committee of Kobe University Graduate School of Medicine.

### Acute or chronic administration of recombinant MCP-1

For chronic administration of MCP-1, the recombinant mouse protein (R&D Systems, Minneapolis, MN) was dissolved in PBS containing 0.1% BSA and administered at the rate of 10 ng/h for 14 d via a micro-osmotic pump (Alzet Model 1002; Durect, Cupertino, CA) implanted sc in the back of 11-wk-old mice. Saline was injected at the same rate via the same apparatus in control mice. For acute administration, MCP-1 was injected directly with the use of an infusion pump into a catheter inserted into the internal jugular vein of 13-wk-old mice for hyperinsulinemic-euglycemic clamp analysis. MCP-1 administration was initiated at a rate of 15 ng/h beginning 30 min before the onset of the clamp analysis.

### Administration of a CCR2 antagonist

A novel CCR2 antagonist, 2-[[2-amino-5-(trifluoromethoxy)phenyl]formamido]-N-[(3R)-1-[[4-(3-amino-5-methyl-1,2-oxazol-4-yl)thiophen-2-yl]methyl]pyrrolidin-3-yl]acetamide (TEI-K03134), was obtained from Teijin Pharma (Tokyo, Japan) and dissolved in 0.5% lactic acid. Either TEI-K03134 (150 mg/kg of body mass) or vehicle (0.5% lactic acid) was administered by oral gavage twice a day for 21 d to 13-wk-old mice that had been fed a high-fat diet for 8 wk; the mice continued to receive the high-fat diet during treatment.

### Measurement of metabolic parameters

The plasma concentrations of insulin and MCP-1 were measured with an insulin assay kit (Morinaga Institute of Biological Science, Yokohama, Japan) and a Quantikine M mouse MCP-1 ELISA kit (R&D Systems), respectively. The nonesterified fatty acid (NEFA) concentration in serum was measured with the use of a NEFA-C test (Wako, Osaka, Japan). Serum adiponectin level was measured with ELISA kits from Otsuka Pharmaceutical (Tokyo, Japan). Lipids were extracted from liver homogenates with isopropanol, and the triacylglycerol (TAG) content was determined with the use of a Triglyceride E test (Wako). Food intake was measured by weighing the food given at the beginning and that remaining at the end of each day for 1 wk.

### Hyperinsulinemic-euglycemic clamp analysis

Five to seven days before the clamp, mice were anesthetized with sodium pentobarbital (80–100 mg/kg, ip) and a catheter was inserted into the right internal jugular vein for infusion. The

analysis was performed under nonstressful conditions with conscious mice that had been deprived of food for 4 h. The [ $3\text{-}^3\text{H}$ ]glucose was infused for 2 h at a rate of  $0.05\ \mu\text{Ci}/\text{min}$  before initiation of the clamp, and a blood sample was collected at the end of this period to estimate basal glucose turnover. After a bolus injection of [ $3\text{-}^3\text{H}$ ]glucose ( $10\ \mu\text{Ci}$ ; NEN Life Science Products, Boston, MA) and the onset of subsequent continuous infusion of [ $3\text{-}^3\text{H}$ ]glucose ( $0.1\ \mu\text{Ci}/\text{min}$ ), a hyperinsulinemic-euglycemic clamp was applied for 120 min with continuous infusion of insulin at a rate of 2.5 and 4.0 mU/kg·min in the experiment of MCP-1 administration and in that of CCR2 antagonist administration, respectively. Plasma glucose concentration was monitored every 10 min, and 30% glucose was infused at a variable rate to maintain plasma glucose concentration at about 110 mg/dl. Blood samples were collected 80, 90, 100, 110, and 120 min after the onset of the clamp for determination of the plasma concentrations of [ $3\text{-}^3\text{H}$ ]glucose and  $^3\text{H}_2\text{O}$ . The rates of glucose disposal and hepatic glucose production (HGP) were calculated as described (19).

### Isolation of total RNA and quantitative RT-PCR analysis

Total RNA was extracted from adipose tissue or liver with the use of TRIzol reagent (Invitrogen, Carlsbad, CA) and was subjected to reverse transcription. The resulting cDNA was subjected to real-time PCR analysis with specific primers and SYBR Green PCR Master reagents (PerkinElmer Life Sciences, Waltham, MA) and with the use of a Sequence Detector (model 7900; PE Applied Biosystems, Foster City, CA). The relative abundance of target mRNAs was calculated with 36B4 mRNA as the invariant control.

### Immunohistochemical analysis

Adipose tissue was fixed for 24–48 h with 10% paraformaldehyde at 4 C, dehydrated, embedded in paraffin, and sectioned at a thickness of  $5\ \mu\text{m}$ . The sections were mounted on glass slides, depleted of paraffin with xylene, exposed to microwave radiation (500 W for 5 min) in 0.01 mol/liter sodium citrate buffer (pH 6.0), and treated both with a Biotin Blocking System (Dako, Glostrup, Denmark) to block endogenous biotin and avidin binding sites and with PBS containing 20% FBS to block Fc receptors. They were then incubated overnight at 4 C with a mouse monoclonal antibody to Mac3 (M3/84; BD Biosciences, San Jose, CA) at a dilution of 1:100, after which immune complexes were detected with biotinylated secondary antibodies (BD Biosciences), horseradish peroxidase-conjugated streptavidin (Dako), and the peroxidase substrate diaminobenzidine (Dako). The sections were stained with hematoxylin (Muto Pure Chemicals, Tokyo, Japan), after which mounting solution (Matsunami Glass, Kishiwada, Japan) and cover slips were added and the slides were observed with a light microscope.

### Flow cytometry

The stromal vascular fraction of mouse epididymal adipose tissue was prepared as described (10), and red blood cells present in this fraction were lysed with the use of Pharm Lyse (BD Biosciences). The remaining cells were suspended in PBS containing 2 mmol/liter EDTA, exposed to FcBLOCK (BD Biosciences) for 20 min, and then labeled with phycoerythrin-conjugated antibodies to mouse CD11b (M1/70; BD Biosciences) and fluorescein isothiocyanate-conjugated antibodies to mouse CD45 (BD

Biosciences). Dead cells were stained with 7-amino-actinomycin D (BD Biosciences), and live cells positive for both CD11b and CD45 (macrophages) were quantitated by flow cytometry with a FACSCalibur analyzer (BD Biosciences) (20).

### Measurement of the effect of the CCR2 antagonist on ligand binding

The inhibitory effect of TEI-K03134 on ligand binding was determined with the use of CHO-K1 cells expressing human CCR2. The cells were incubated for 1 h at 25 C with 0.1 nmol/liter  $^{125}\text{I}$ -labeled recombinant MCP-1 in a solution containing 25 mmol/liter HEPES-NaOH (pH 7.4), 1 mmol/liter  $\text{CaCl}_2$ , 0.5% BSA, and 5 mmol/liter  $\text{MgCl}_2$ . Nonspecific binding was determined in the presence of 0.03  $\mu\text{mol}/\text{liter}$  MCP-1. The  $\text{IC}_{50}$  value was determined by nonlinear, least squares regression analysis with the use of MathIQ software (ID Business Solutions, Guildford, UK). The inhibition constant ( $K_i$ ) for TEI-K03134 was calculated from the observed  $\text{IC}_{50}$  with the use of the equation of Cheng and Prusoff (21).

### Statistical analysis

Data are presented as means  $\pm$  SEM and were analyzed by Student's *t* test. A *P* of  $<0.05$  was considered statistically significant.

## Results

### Chronic administration of MCP-1 in sc tissue induces insulin resistance

We previously generated transgenic mice in which MCP-1 is overexpressed almost exclusively in adipocytes under the control of the promoter-enhancer of the  $\alpha\text{P2}$  gene (10). The plasma level of MCP-1 in these transgenic mice is similar to that in obese mice (10). In addition, the transgenic animals manifest systemic insulin resistance together with macrophage infiltration into adipose tissue. To clarify whether increased expression of MCP-1 in adipose tissue or an increase in the plasma concentration of MCP-1 is more important for inducing insulin resistance, we administered recombinant MCP-1 in C57BL/6N mice via an sc implanted osmotic pump. MCP-1 infusion for 14 d resulted in an approximately 2-fold increase in the plasma MCP-1 concentration (to 100.7 pg/ml) compared with that of control mice (Table 1). This level of plasma MCP-1 is similar to that observed in obese mice (10). MCP-1 infusion for 14 d had no effect on body mass, food intake, fasting plasma glucose concentration, or serum adiponectin level (Table 1). However, it induced a significant increase in the serum NEFA concentration and showed a tendency to increase the plasma insulin level in the fed condition (Table 1).

We next investigated the effect of chronic MCP-1 administration on insulin sensitivity with the use of hyperinsulinemic-euglycemic clamp analysis. We confirmed

**TABLE 1.** Effects of long-term administration of recombinant MCP-1 on metabolic parameters in mice

Parameter	Control	Recombinant MCP-1	P
Plasma MCP-1 (pg/ml)	44.6 ± 3.5	100.7 ± 6.4	<b>&lt;0.01</b>
Body mass (g)	27.0 ± 0.19	26.5 ± 0.3	0.30
Food intake (g/d)	4.4 ± 0.14	4.2 ± 0.13	0.34
Fasting plasma glucose (mg/dl)	71.9 ± 2.8	67.3 ± 3.8	0.35
Fed plasma insulin (pg/ml)	1080 ± 73.6	1767.9 ± 278.7	0.06
Serum NEFA (mEq/liter)	1.10 ± 0.07	1.32 ± 0.06	<b>0.03</b>
Serum adiponectin (μg/ml)	19.4 ± 0.62	20.3 ± 1.54	0.15

Each parameter was measured after the administration of recombinant MCP-1 for 14 d. Data are means ± SEM from eight mice per group. P values of <0.05 are shown in *bold*.

that plasma glucose was actually clamped at euglycemic levels and was almost same between control and MCP-1-treated mice during the latter half of hyperinsulinemic-euglycemic clamp period (control mice, 108.8 ± 3.8 mg/dl; MCP-1-treated mice, 108.4 ± 1.7 mg/dl; P, not significant) (supplemental Fig. S1, published as supplemental data on The Endocrine Society's Journals Online web site at <http://endo.endojournals.org>). In this condition, the glucose infusion rate was reduced by 65% in mice treated with MCP-1 compared with that in control mice (Fig. 1A and supplemental Fig. S2). This result implies that MCP-1 infusion induced systemic insulin resistance on the assumption that plasma insulin levels during the clamp period were same between two groups, although we did not measure them actually. HGP during the clamp period was also markedly increased in mice treated with MCP-1, although basal HGP was not altered (Fig. 1A). Insulin's ability to suppress HGP during clamp was significantly impaired in mice treated with MCP-1 (Fig. 1B). These results were thus indicative of the presence of hepatic insulin resistance in mice subjected to chronic administration of MCP-1.

#### Chronic administration of MCP-1 in sc tissue induces macrophage infiltration into adipose tissue

Transgenic mice in which MCP-1 is overexpressed in adipose tissue manifest macrophage infiltration into this tissue (10, 11). We therefore next investigated the inflammatory status of adipose tissue in mice subjected to chronic subcutaneous infusion of MCP-1. Immunohistochemical analysis with a monoclonal antibody to Mac3 revealed macrophage infiltration in epididymal adipose tissue of mice infused with MCP-1 but not in that of control mice (Fig. 1C). Quantitative analysis by flow cytometry with antibodies to CD11b and to CD45 confirmed the greater extent of macrophage infiltration into adipose tissue of the MCP-1-treated animals (Fig. 1C). We also examined the expression of genes whose products are associated with inflammation. Quantitative RT-PCR analysis revealed that the amounts of the mRNAs for F4/80, CD68,

and CD11c in epididymal adipose tissue were all increased in mice subjected to chronic administration of MCP-1 compared with those in control mice (Fig. 1D). These results suggested that an increase in the plasma MCP-1 concentration similar to that observed in obese mice was sufficient to induce macrophage infiltration into adipose tissue, even if the source of the MCP-1 was not adipose tissue.

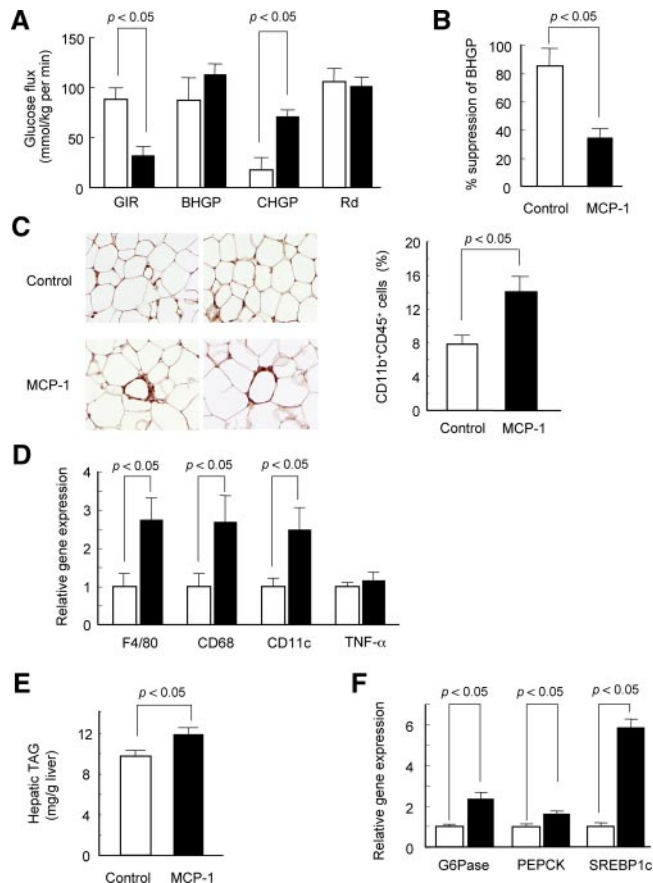
#### Chronic administration of MCP-1 in sc tissue induces hepatic steatosis

Transgenic mice overexpressing MCP-1 in adipose tissue develop hepatic steatosis (10). We found that sc MCP-1 infusion also increased the hepatic TAG content (Fig. 1E). Expression of the gluconeogenic gene for glucose-6-phosphatase (G6Pase) and the lipogenic gene for sterol regulatory element-binding protein 1c (SREBP1c) in the liver is also increased in adipocyte-specific MCP-1 transgenic mice (10). Similarly, we found that the hepatic expression of G6Pase and phosphoenolpyruvate carboxykinase genes as well as that of the SREBP1c gene were increased in mice subjected to chronic MCP-1 administration (Fig. 1F), likely contributing to the increased HGP during the hyperinsulinemic-euglycemic clamp and the increased hepatic TAG content in these animals. Mice subjected to chronic sc infusion of MCP-1 thus show a phenotype that is similar to that of adipocyte-specific MCP-1 transgenic mice (10, 11) and characterized by insulin resistance, adipose tissue inflammation, and hepatic steatosis.

#### Acute administration of MCP-1 in circulating blood induces insulin resistance without macrophage infiltration into adipose tissue

Chronic MCP-1 administration in sc tissue induced not only insulin resistance but also concomitant macrophage infiltration into adipose tissue. These results therefore did not clarify whether MCP-1 elicited systemic insulin resistance irrespective of adipose tissue inflammation. We thus next subjected mice to short-term administration of MCP-1 to determine the acute effect of MCP-1 on insulin





**FIG. 1.** Effects of chronic infusion of recombinant MCP-1 in mice on insulin sensitivity, inflammatory status of adipose tissue, and liver metabolism. A, Mice were subjected to infusion of recombinant MCP-1 via a sc implanted osmotic pump for 14 d. Both MCP-1-treated ( $n = 5$ ) and control ( $n = 4$ ) mice were then subjected to hyperinsulinemic-euglycemic clamp analysis. Glucose infusion rate (GIR), the rate of insulin-stimulated glucose disposal (Rd), and HGP during the basal or clamp periods (BHGP and CHGP, respectively) were determined. B, Percentage of suppression of basal HGP during the clamp period. C, Epididymal adipose tissue of MCP-1-treated or control mice was subjected to immunohistochemical analysis with antibodies to Mac3 (*left panel*); magnification,  $\times 200$ . The proportion of CD11b<sup>+</sup>CD45<sup>+</sup> cells (macrophages) in the stromal vascular fraction of epididymal adipose tissue was also quantitated by flow cytometry (*right panel*).  $n = 5$ . D, Quantitative RT-PCR analysis of inflammation-related gene expression in epididymal adipose tissue of MCP-1-treated ( $n = 9$ ) or control ( $n = 8$ ) mice. Data are expressed relative to the corresponding value for control mice. E, Hepatic TAG content of MCP-1-treated or control mice. Data are expressed as milligrams of TAG per gram of liver wet weight.  $n = 5$ . F, Quantitative RT-PCR analysis of hepatic gene expression related to gluconeogenesis (G6Pase, phosphoenolpyruvate carboxykinase) or lipid synthesis (SREBP1c) in MCP-1-treated ( $n = 9$ ) or control ( $n = 8$ ) mice. Data are expressed relative to the corresponding value for control mice. All quantitative data are means  $\pm$  SEM, with *white and black bars* indicating control and MCP-1-treated mice, respectively.

sensitivity. We administered recombinant MCP-1 directly into the internal jugular vein with the use of an infusion pump. At 30 min after the onset of MCP-1 infusion, the mice were subjected to hyperinsulinemic-euglycemic clamp analysis with further continuous infusion of MCP-1. Metabolic parameters were determined during

the 2-h basal period and the subsequent 2-h clamp period. At the end of the hyperinsulinemic-euglycemic clamp experiment (4.5 h after the start of MCP-1 infusion), the plasma MCP-1 concentration was approximately 110 pg/ml (Table 2), which was about twice that in control mice and similar to the value observed in obese mice (10). The serum NEFA concentration was not affected by acute MCP-1 administration (Table 2). However, the glucose infusion rate was reduced by 41% in MCP-1-treated mice compared with that in control mice (Fig. 2A). HGP during the clamp period was significantly increased in MCP-1-treated mice (Fig. 2A). Suppression of basal HGP in response to insulin was also smaller in MCP-1-treated mice (Fig. 2B). In addition, this effect was accompanied by a significant decrease in the rate of glucose disappearance (Fig. 2A). These results were thus indicative of insulin resistance in both skeletal muscle and liver of mice subjected to acute administration of MCP-1.

Immunohistochemical and flow cytometric analyses revealed no difference in macrophage infiltration into adipose tissue between control mice and MCP-1-treated animals after the glucose clamp experiment (Fig. 2C), suggesting that an increase in the circulating MCP-1 concentration to the level apparent in obese mice is sufficient to induce insulin resistance irrespective of macrophage infiltration into adipose tissue.

### A CCR2 antagonist ameliorates insulin resistance in mice with high-fat diet-induced obesity

CCR2 is a major receptor for MCP-1 and regulates monocyte and macrophage chemotaxis and local inflammatory responses (22). Mice deficient in CCR2 exhibit a reduced number of macrophages and a lower level of inflammation in adipose tissue, a reduced level of hepatic steatosis, and improved systemic glucose homeostasis and insulin sensitivity when fed a high-fat diet compared with control mice (12). We therefore next examined the effects of TEI-K03134, a novel pharmacological antagonist of CCR2 (Fig. 3A). We found that the  $IC_{50}$  and inhibition constant ( $K_i$ ) values of this compound for inhibition of MCP-1 binding to cells expressing human CCR2 were  $8.73 \pm 1.27$  and  $3.37 \pm 0.49$  nmol/liter, respectively.

Mice fed a high-fat diet for 8 wk were previously shown to manifest increased expression of the genes for MCP-1, CD68, and TNF- $\alpha$  in adipose tissue as well as an increased plasma concentration of MCP-1 (23). Oral administration of TEI-K03134 for 3 wk in mice fed a high-fat diet for 8 wk induced a significant decrease in the fasting plasma glucose level without affecting either body mass, food intake, plasma insulin level in the fed condition, serum NEFA concentration, or serum adiponectin level (Table 3). Administration of TEI-K03134 for the same period did

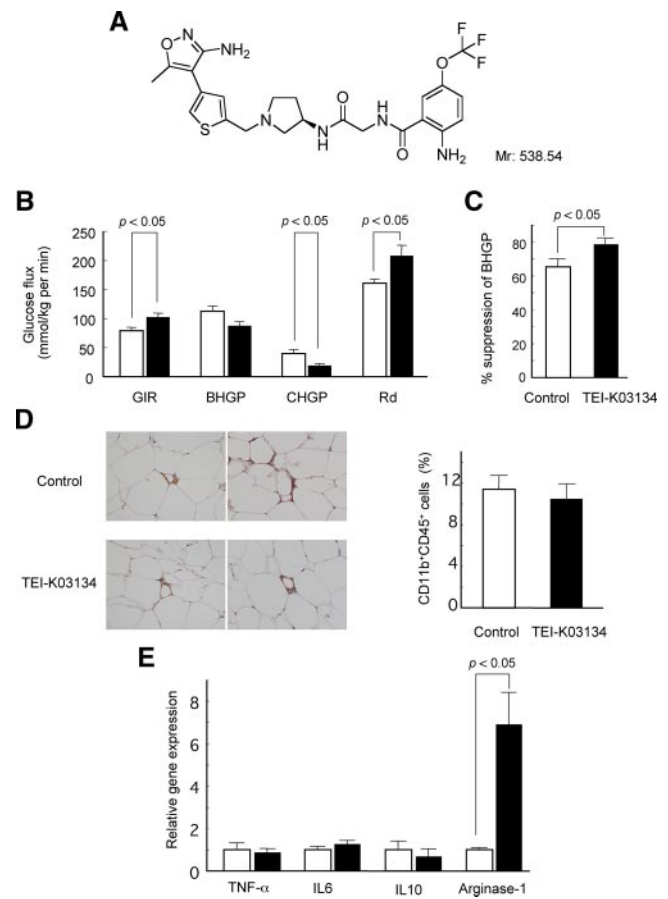
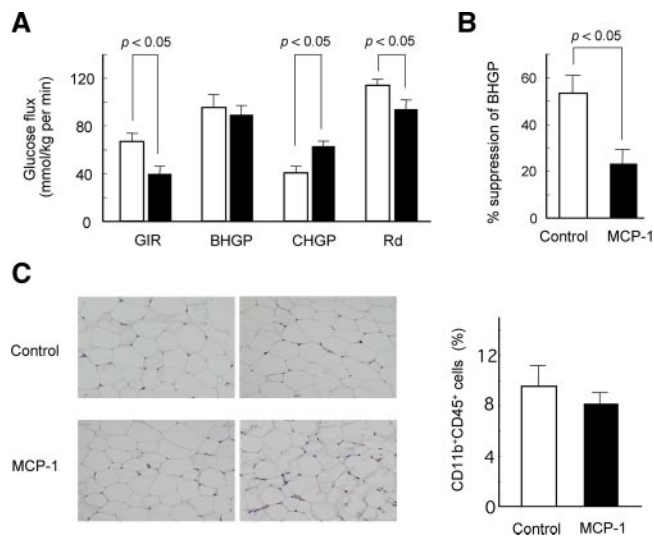
**TABLE 2.** Effects of short-term administration of recombinant MCP-1 on plasma MCP-1 and serum NEFA concentrations in mice

Parameter	Control	Recombinant MCP-1	<i>P</i>
Plasma MCP-1 (pg/ml)	55.0 ± 12.0	110.3 ± 15.6	<b>0.01</b>
Serum NEFA (mEq/liter)	0.83 ± 0.04	0.75 ± 0.05	0.25

Each parameter was measured at the end of the hyperinsulinemic-euglycemic clamp analysis in control ( $n = 13$ ) and MCP-1-treated ( $n = 12$ ) mice. Data are means ± SEM. *P* values of < 0.05 are shown in *bold*.

not affect any of these parameters in mice fed a standard diet (supplemental Table S1). Hyperinsulinemic-euglycemic clamp analysis in mice fed a high-fat diet revealed that both the glucose infusion rate and glucose disposal were significantly increased, whereas HGP during the clamp period was significantly decreased, by administration of TEI-K03134 (Fig. 3B). In fact, suppression of basal HGP by insulin was significantly larger in TEI-K03134-treated mice (Fig. 3C). This suggests that inhibition of MCP-1-CCR2 signaling ameliorated diet-induced insulin resistance. In addition, we found that administration of TEI-K03134 for 3 wk did not affect macrophage infiltration into adipose tissue of mice fed a high-fat diet (Fig. 3D), suggesting that inhibition of the MCP-1-CCR2 pathway improves insulin sensitivity irrespective of such macrophage infiltration. This latter finding is consistent with our

observation that an acute increase in the circulating level of MCP-1 induced insulin resistance in a manner independent of macrophage infiltration. Furthermore, we inves-



**TABLE 3.** Effects of the CCR2 antagonist TEI-K03134 on metabolic parameters in mice fed a high-fat diet

Parameter	Control	TEI-K03134	P
Body mass (g)	32.2 ± 1.15	31.6 ± 0.65	0.63
Food intake (g/d)	2.1 ± 0.07	2.1 ± 0.1	0.37
Fasting plasma glucose (mg/dl)	132 ± 4.8	104 ± 5.0	<b>0.03</b>
Fed plasma insulin (pg/ml)	1565.3 ± 166.9	1635.6 ± 121.4	0.75
Serum NEFA (mEq/liter)	1.44 ± 0.18	1.56 ± 0.09	0.46
Serum adiponectin (μg/ml)	20.1 ± 0.98	19.8 ± 1.26	0.85

Each parameter was measured after the administration of TEI-K03134 for 21 d in mice fed a high-fat diet for 8 wk. Data are means ± SEM ( $n = 5$ ). *P* values of < 0.05 are shown in *bold*.

tigated the macrophage characters in adipose tissue by analyzing the expressions of M1 (TNF- $\alpha$  and IL-6) and M2 (IL-10 and arginase-1) macrophage markers (9). We found that expressions of TNF- $\alpha$ , IL-6, and IL-10 were not changed by administration of TEI-K03134, although arginase-1 expression was increased significantly (Fig. 3E). This may suggest the possibility that inhibition of the MCP-1-CCR2 pathway partly modulated the macrophage characters in adipose tissue of mice fed a high-fat diet.

## Discussion

The circulating concentration of MCP-1 is increased concomitant with the up-regulation of MCP-1 expression in adipose tissue associated with obesity (7, 8, 10, 13). The up-regulation of MCP-1 in adipose tissue is thought to result from the generation of reactive oxygen species and endoplasmic reticulum stress induced in adipocytes by the excess intake of nutrients in obesity and is thought to reflect an inflammatory response in this tissue (24–26). The increased production of MCP-1 by adipocytes results in the recruitment of macrophages to adipose tissue and consequent enhancement of inflammation as a result of the release of proinflammatory cytokines and down-regulation of adiponectin. These effects are thought to underlie the development of insulin resistance in liver and skeletal muscle. However, it has not been clear whether inflammation in adipose tissue is indispensable for the development of insulin resistance. We have now shown that a chronic increase in the circulating level of MCP-1 in mice similar to that observed in obese animals resulted in the development of systemic insulin resistance together with inflammatory changes in adipose tissue. In contrast, a similar acute increase in the plasma concentration of MCP-1 led to systemic insulin resistance without macrophage infiltration into adipose tissue. Furthermore, inhibition of MCP-1-CCR2 signaling improved insulin resistance in mice fed a high-fat diet without affecting the extent of macrophage accumulation in adipose tissue. Our findings thus indicate that an increase in the concentration of MCP-1 in the circulation is sufficient to induce systemic

insulin resistance irrespective of inflammatory changes in adipose tissue.

MCP-1 is secreted by a variety of cell types, including mononuclear cells, endothelial cells, fibroblasts, and adipocytes. However, given that MCP-1 expression is increased predominantly in adipose tissue in obese mice (10, 13), the source of the increased amount of MCP-1 in the circulating blood in obese animals is likely adipose tissue. Our present data now suggest that a chronic increase in the circulating level of MCP-1 induces insulin resistance and adipose tissue inflammation, even if the primary source of the MCP-1 is not adipose tissue. MCP-1 was previously shown to induce insulin resistance directly in 3T3-L1 adipocytes (13), C2C12 myotubules, and isolated skeletal muscle (11) *in vitro*, suggesting that elevated levels of MCP-1 in the circulation directly induce insulin resistance in insulin target tissues. Indeed, the circulating MCP-1 level was found to correlate with the homeostasis model assessment of insulin resistance but not with body mass index in Japanese individuals with type 2 diabetes (27). It was also associated with indices of insulin resistance regardless of body adiposity in Italian subjects (28). These observations suggest that elevated levels of MCP-1 in the circulation induce insulin resistance irrespective of obesity. The precise mechanism by which an increase in the circulating concentration of MCP-1 induces macrophage infiltration into adipose tissue remains unclear. However, MCP-1 activates ERK, c-Jun NH<sub>2</sub>-terminal kinase, and p38 MAPK in endothelial cells (29). Given that ERK and c-Jun NH<sub>2</sub>-terminal kinase contribute to the inflammatory response by activating the transcription factor activator protein-1, an increase in the circulating level of MCP-1 may up-regulate the expression of adhesion molecules in vascular endothelial cells of adipose tissue (30), leading to macrophage infiltration into this tissue. Such infiltration of macrophages into adipose tissue specifically may reflect the different expression patterns of adhesion molecules apparent among vascular endothelial cells from different tissues (31).

With the use of a novel CCR2 antagonist, TEI-K03134, we found that inhibition of MCP-1-CCR2 signaling ameliorated insulin resistance in mice fed a high-fat diet. Al-



though inhibition of CCR2 signaling by a CCR2 antagonist was previously shown to improve insulin resistance in mice with diet-induced or genetic obesity (12, 32), we further found that inhibition of macrophage infiltration into adipose tissue was not required for amelioration of insulin resistance by a CCR2 antagonist. Although we are not able to exclude the possibility that administration of TEI-K03134 for a longer period might have reduced the extent of macrophage infiltration into adipose tissue of mice fed a high-fat diet, we found that inhibition of CCR2 signaling was able to ameliorate insulin resistance without such an effect. A CCR2 antagonist was also recently shown to ameliorate insulin resistance and hepatic steatosis in a mouse model of lipoatrophic diabetes (A-ZIP/F-1 transgenic mice), in which the level of MCP-1 in liver and serum is highly increased in spite of adipose tissue loss (33). These findings together with our present results suggest that an increase in the circulating level of MCP-1 can induce systemic insulin resistance independently of adipose tissue inflammation.

In summary, we have found that an increase in the circulating concentration of MCP-1 is sufficient to provoke insulin resistance directly in insulin target tissues in the absence of macrophage infiltration into adipose tissue. In addition, persistent elevation of circulating MCP-1 induces macrophage accumulation and inflammatory changes in adipose tissue, leading to further exacerbation of insulin resistance. Our results indicate that plasma MCP-1 is not merely a marker of systemic inflammation or insulin resistance but an important inducer of insulin resistance and adipose tissue inflammation. They also suggest that CCR2 antagonists might prove beneficial for the treatment of type 2 diabetes and metabolic syndrome.

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## References

1. Wild S, Roglic G, Green A, Sicree R, King H 2004 Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. *Diabetes Care* 27:1047–1053
2. Rosen ED, Spiegelman BM 2006 Adipocytes as regulators of energy balance and glucose homeostasis. *Nature* 444:847–853
3. Schenk S, Saberi M, Olefsky JM 2008 Insulin sensitivity: modulation by nutrients and inflammation. *J Clin Invest* 118:2992–3002
4. Kasuga M 2006 Insulin resistance and pancreatic  $\beta$  cell failure. *J Clin Invest* 116:1756–1760
5. Wellen KE, Hotamisligil GS 2005 Inflammation, stress, and diabetes. *J Clin Invest* 115:1111–1119
6. Shoelson SE, Lee J, Goldfine AB 2006 Inflammation and insulin resistance. *J Clin Invest* 116:1793–1801
7. Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante Jr AW 2003 Obesity is associated with macrophage accumulation in adipose tissue. *J Clin Invest* 112:1796–1808
8. Xu H, Barnes GT, Yang Q, Tan G, Yang D, Chou CJ, Sole J, Nichols A, Ross JS, Tartaglia LA, Chen H 2003 Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *J Clin Invest* 112:1821–1830
9. Lumeng CN, Bodzin JL, Saltiel AR 2007 Obesity induces a phenotypic switch in adipose tissue macrophage polarization. *J Clin Invest* 117:175–184
10. Kanda H, Tateya S, Tamori Y, Kotani K, Hiasa K, Kitazawa R, Kitazawa S, Miyachi H, Maeda S, Egashira K, Kasuga M 2006 MCP-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity. *J Clin Invest* 116:1494–1505
11. Kamei N, Tobe K, Suzuki R, Ohsugi M, Watanabe T, Kubota N, Ohtsuka-Kawatari N, Kumagai K, Sakamoto K, Kobayashi M, Yamauchi T, Ueki K, Oishi Y, Nishimura S, Manabe I, Hashimoto H, Ohnishi Y, Ogata H, Tokuyama K, Tsunoda M, Ide T, Murakami K, Nagai R, Kadowaki T 2006 Overexpression of monocyte chemoattractant protein-1 in adipose tissues causes macrophage recruitment and insulin resistance. *J Biol Chem* 281:26602–26614
12. Weisberg SP, Hunter D, Huber R, Lemieux J, Slaymaker S, Vaddi K, Charo I, Leibel RL, Ferrante Jr AW 2006 CCR2 modulates inflammatory and metabolic effects of high-fat feeding. *J Clin Invest* 116:115–124
13. Sartipy P, Loskutoff DJ 2003 Monocyte chemoattractant protein 1 in obesity and insulin resistance. *Proc Natl Acad Sci USA* 100:7265–7270
14. Inouye KE, Shi H, Howard JK, Daly CH, Lord GM, Rollins BJ, Flier JS 2007 Absence of CC chemokine ligand 2 does not limit obesity-associated infiltration of macrophages into adipose tissue. *Diabetes* 56:2242–2250
15. Kirk EA, Sagawa ZK, McDonald TO, O'Brien KD, Heinecke JW 2008 Macrophage chemoattractant protein-1 deficiency fails to restrain macrophage infiltration into adipose tissue. *Diabetes* 57:1254–1261
16. Bruun JM, Lihn AS, Pedersen SB, Richelsen B 2005 Monocyte chemoattractant protein-1 release is higher in visceral than subcutaneous human adipose tissue (AT): implication of macrophages resident in the AT. *J Clin Endocrinol Metab* 90:2282–2289
17. Christiansen T, Richelsen B, Bruun JM 2005 Monocyte chemoattractant protein-1 is produced in isolated adipocytes, associated with adiposity and reduced after weight loss in morbid obese subjects. *Int J Obes* 29:146–150
18. Mine S, Okada Y, Tanikawa T, Kawahara C, Tabata T, Tanaka Y 2006 Increased expression levels of monocyte CCR2 and monocyte chemoattractant protein-1 in patients with diabetes mellitus. *Biochem Biophys Res Commun* 344:780–785
19. Kim JK, Zisman A, Fillmore JJ, Peroni OD, Kotani K, Perret P, Zong H, Dong J, Kahn CR, Kahn BB, Shulman GI 2001 Glucose toxicity

- and the development of diabetes in mice with muscle-specific inactivation of GLUT4. *J Clin Invest* 108:153–160
20. Curat CA, Miranville A, Sengenès C, Diehl M, Tonus C, Busse R, Bouloumié A 2004 From blood monocytes to adipose tissue-resident macrophages: induction of diapedesis by human mature adipocytes. *Diabetes* 53:1285–1292
  21. Cheng Y, Prusoff WH 1973 Relationship between the inhibition constant (K<sub>1</sub>) and the concentration of inhibitor which causes 50 per cent inhibition (I<sub>50</sub>) of an enzymatic reaction. *Biochem Pharmacol* 22:3099–3108
  22. Charo IF, Myers SJ, Herman A, Franci C, Connolly AJ, Coughlin SR 1994 Molecular cloning and functional expression of two monocyte chemoattractant protein 1 receptors reveals alternative splicing of the carboxyl-terminal tails. *Proc Natl Acad Sci USA* 91:2752–2756
  23. Chen A, Mumick S, Zhang C, Lamb J, Dai H, Weingarth D, Mudgett J, Chen H, MacNeil DJ, Reitman ML, Qian S 2005 Diet induction of monocyte chemoattractant protein-1 and its impact on obesity. *Obes Res* 13:1311–1320
  24. Furukawa S, Fujita T, Shimabukuro M, Iwaki M, Yamada Y, Nakajima Y, Nakayama O, Makishima M, Matsuda M, Shimomura I 2004 Increased oxidative stress in obesity and its impact on metabolic syndrome. *J Clin Invest* 114:1752–1761
  25. Lin Y, Berg AH, Iyengar P, Lam TK, Giacca A, Combs TP, Rajala MW, Du X, Rollman B, Li W, Hawkins M, Barzilai N, Rhodes CJ, Fantus IG, Brownlee M, Scherer PE 2005 The hyperglycemia-induced inflammatory response in adipocytes: the role of reactive oxygen species. *J Biol Chem* 280:4617–4626
  26. Ozcan U, Cao Q, Yilmaz E, Lee AH, Iwakoshi NN, Ozdelen E, Tuncman G, Görgün C, Glimcher LH, Hotamisligil GS 2004 Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes. *Science* 306:457–461
  27. Kouyama K, Miyake K, Zenibayashi M, Hirota Y, Teranishi T, Tamori Y, Kanda H, Sakaguchi K, Ohara T, Kasuga M 2008 Association of serum MCP-1 concentration and MCP-1 polymorphism with insulin resistance in Japanese individuals with obese type 2 diabetes. *Kobe J Med Sci* 53:345–354
  28. Piemonti L, Calori G, Mercalli A, Lattuada G, Monti P, Garancini MP, Costantino F, Ruotolo G, Luzi L, Perseghin G 2003 Fasting plasma leptin, tumor necrosis factor- $\alpha$  receptor 2, and monocyte chemoattracting protein 1 concentration in a population of glucose-tolerant and glucose-intolerant women: impact on cardiovascular mortality. *Diabetes Care* 26:2883–2889
  29. Werle M, Schmal U, Hanna K, Kreuzer J 2002 MCP-1 induces activation of MAP-kinases ERK, JNK and p38 MAPK in human endothelial cells. *Cardiovasc Res* 56:284–292
  30. Ahmad M, Theofanidis P, Medford RM 1998 Role of activating protein-1 in the regulation of the vascular cell adhesion molecule-1 gene expression by tumor necrosis factor- $\alpha$ . *J Biol Chem* 273:4616–4621
  31. Imbert E, Poot AA, Figdor CG, Feijen J 2001 Expression of leukocyte adhesion molecules by endothelial cells seeded on various polymer surfaces. *J Biomed Mater Res* 56:376–381
  32. Tamura Y, Sugimoto M, Murayama T, Ueda Y, Kanamori H, Ono K, Ariyasu H, Akamizu T, Kita T, Yokode M, Arai H 2008 Inhibition of CCR2 ameliorates insulin resistance and hepatic steatosis in db/db mice. *Arterioscler Thromb Vasc Biol* 28:2195–2201
  33. Yang SJ, IglayReger HB, Kadouh HC, Bodary PF 2009 Inhibition of the chemokine (C-C motif) ligand 2/chemokine (C-C motif) receptor 2 pathway attenuates hyperglycaemia and inflammation in a mouse model of hepatic steatosis and lipodystrophy. *Diabetologia* 52:972–981