Inhibition of CCR2 Ameliorates Insulin Resistance and Hepatic Steatosis in db/db Mice

Yukinori Tamura, Masayuki Sugimoto, Toshinori Murayama, Yukihiko Ueda, Hiroshi Kanamori, Koh Ono, Hiroyuki Ariyasu, Takashi Akamizu, Toru Kita, Masayuki Yokode, Hidenori Arai

Objective—Recently, adipose tissue inflammation induced by macrophage infiltration through MCP-1/C-C chemokine receptor-2 (CCR2) pathway is considered to play a role in the development of visceral obesity and insulin resistance. In the present study, to further examine the role of CCR2 in the development of obesity and type 2 diabetes, we studied the effect of pharmacological inhibition of CCR2 from the early stage of obesity in db/db mice.

Methods and Results—Db/+m (lean control) and db/db mice were fed with a standard diet with or without 0.005% propagermanium, as a CCR2 inhibitor for 12 weeks from 6 weeks of age. Propagermanium treatment decreased body weight gain, visceral fat accumulation, and the size of adipocytes only in db/db mice. Further, propagermanium suppressed macrophage accumulation and inflammation in adipose tissue. Propagermanium treatment also ameliorated glucose tolerance and insulin sensitivity, and decreased hepatic triglyceride contents in db/db mice.

Conclusions—Propagermanium improved obesity and related metabolic disorders, such as insulin resistance and hepatic steatosis by suppressing inflammation in adipose tissue. Our data indicate that inhibition of CCR2 could improve obesity and type 2 diabetes by interfering adipose tissue inflammation, and that propagermanium may be a beneficial drug for the treatment of the metabolic syndrome. (Arterioscler Thromb Vase Biol. 2008;28:2195-2201.)

Key Words: cytokines ■ diabetes mellitus ■ insulin resistance ■ macrophages ■ receptors

The metabolic syndrome, characterized by a clustering of visceral obesity, impaired glucose tolerance, hypertension, and dyslipidemia, is a major cause of type 2 diabetes mellitus and cardiovascular disease.1 Visceral obesity and insulin resistance are thought to represent common underlying factors of the syndrome.2 Therefore, it is very important to clarify the mechanism of the development of obesity and insulin resistance and to establish the therapeutic method based on its mechanism.

Many reports have shown that obesity is associated with a state of chronic, low-grade inflammation, suggesting that inflammation may be a potential mechanism whereby obesity leads to insulin resistance.3 Indeed, obesity and insulin resistance are strongly associated with systemic markers of inflammation, and clinically, inflammation has been recognized as a major predictor of atherosclerotic disease.3,4 The adipose tissue is an important endocrine organ that secretes many biologically active molecules, such as leptin, adiponectin, tumor necrosis factor (TNF-α), and monocyte chemoattractant protein 1 (MCP-1), which are collectively termed adipocytokines.5–8 Dysregulated production of proinflammatory and antiinflammatory adipocytokines seen in visceral fat obesity is associated with the metabolic syndrome,5,9 suggesting that inflammatory changes in the adipose tissue may contribute to the development of many aspects of the metabolic syndrome and result in type 2 diabetes and atherosclerosis.

Recent studies have demonstrated that obese adipose tissue is characterized by increased infiltration of macrophages, suggesting that they are important sources of inflammation in adipose tissue.10,11 C-C chemokine receptor-2 (CCR2), known as a receptor for MCP-1, play a role in monocyte/macrophage recruitment and macrophage-dependent inflammatory response and the development of atherosclerosis.12 Mouse models have demonstrated that adipose tissue macrophages (ATMs) accumulation via the MCP-1/CCR2 pathway both necessary and sufficient for the development of insulin resistance with obesity. Mice with targeted deletions in the genes for MCP-1 or CCR2 have decreased ATM content, decreased inflammation in adipose tissue, and protection from high-fat diet–induced insulin resistance.13,14 In contrast, mice overexpressing MCP-1 have increased numbers of ATMs along with increased insulin resistance.13,15 Although these data suggest that the recruitment of ATMs through MCP-1/CCR2 is required for the development of type 2 diabetes with obesity, the effect of postnatal inhibition of...
CCR2 on obesity and type 2 diabetes during the development of these pathological states is not fully understood.

An organic germanium compound, propagermanium, has been clinically used for the treatment of hepatitis B virus-induced chronic hepatitis in Japan. Previous studies demonstrated that this drug potently inhibits the infiltration of macrophages in vivo and in vitro. One of the mechanisms of this drug is mediated through the inhibition of CCR2 function.

In the present study, we examined the preventive effect of administration with propagermanium from the early stage of obesity on the development of obesity-associated metabolic disorders and type 2 diabetes in db/db mice.

Methods

Materials

Propagermanium (3-oxygenmethylpropinic acid polymer) was kindly provided by Sanwa Kagaku Kenkyusho Co (Nagoya, Japan).

Animal Preparation and Experimental Design

Male db/db mice and db/+ m mice were obtained from Charles River (Yokohama, Japan) at 5 weeks of age. Two kinds of mice were divided further into 2 groups (control and drug treated, n = 10 in each group). The mice were fed with normal chow without additional supplementation (nontreated group) or with chow supplementation with 0.005% propagermanium (propagermanium-treated group) for 12 weeks from 6 weeks of age. This concentration of propagermanium in the feeding chow results in a dose of 5 mg/kg body weight.

Body Fat Composition Analysis

For computed tomography (CT) analysis of body fat composition, mice were anesthetized with intraperitoneal injection of pentobarbital sodium (Nembutal; Dainippon Sumitomo Pharma Co Ltd) and then scanned using a LaTheta (LCT-100 mol/L) experimental animal CT system (Aloka). Contiguous 5-mm slice images between head and tail were used for quantitative assessment using LaTheta software (version 1.00). Visceral fat, subcutaneous fat, and muscle were distinguished and evaluated quantitatively.

Quantitative Real-Time Polymerase Chain Reaction

Total RNA was extracted from frozen adipose tissue (200 mg) and liver tissue (50 mg) using RNeasy mini kit (Qiagen). The cDNA was synthesized from total RNA using Super Script III (Invitrogen). Real-time PCR was performed on an ABI PRISM 7700 using the SYBR GREEN PCR Master Mix (Applied Biosystems). Primer sets were synthesized from total RNA using Super Script III (Invitrogen). Real-time PCR was performed on an ABI PRISM 7700 using the SYBR GREEN PCR Master Mix (Applied Biosystems). Primer sets were synthesized from total RNA using Super Script III (Invitrogen).

Analysis of Metabolic Parameters

Plasma insulin concentration was measured with an insulin assay kit (Morinaga Institute of Biological Science). Cholesterol and triglyceride were measured with Cholesterol E and Triglyceride E test (Wako pure Chemical Industries Ltd), respectively. Plasma adiponectin level was measured with an adiponectin ELISA kit (Otsuka Pharmaceutical Co Ltd). For glucose tolerance tests, mice were deprived of food for 16 to 24 hours and then injected i.p. with glucose (1.5 g/kg body mass). For insulin tolerance tests, mice were injected i.p. with human regular insulin (0.5 U/kg for db/+ m and 2 U/kg for db/db mice; Eli Lilly and Co). Blood samples were collected before and after injection, and plasma glucose concentration was measured with a Glustest Ace (Sanwa Kagaku Kenkyusho Co).

Measurement of Hepatic Triglyceride Content

Hepatic triglyceride contents were measured as previously described. Tissue triglyceride contents were expressed mg/mg protein.

Histological Analysis

Macrophage in epididymal fat pads were visualized by F4/80 immunostaining and quantified as described previously. The size of adipocyte area was measured with the use of Image Pro plus software version 3.0.1 (Media Cybernetics Inc).

Statistical Analysis

All data were expressed as means ± SD. The statistical significance of differences was assessed by the unpaired t test and 1-way ANOVA. Differences with P < 0.05 were regarded as significant. All statistical analyses were performed using StatView version 5.0 software (SAS Institute).

Results

Inhibition of CCR2 Suppressed Body Weight Gain With No Effect on Food Intake in Obese Mice

Propagermanium treatment had no effect on body weight in db/+ m mice. In db/db mice, however, propagermanium treatment caused a decrease in body weight compared with control group. We found no difference in food intake with or without propagermanium treatment in both db/+ m and db/db mice (data not shown), indicating that propagermanium suppressed weight gain only in db/db mice without causing appetite loss.

Effect of Inhibition of CCR2 on Lipid and Glucose Metabolism in db/db Mice

There was no difference in the fasting plasma triglyceride (TG) or total cholesterol (T-Cho) between nontreated and propagermanium-treated db/+ m mice. However, propagermanium treatment reduced fasting plasma TG, whereas propagermanium had no effect on plasma T-Cho in db/db mice (Table). No difference was observed in fasting blood glucose, HbA1c, or plasma insulin concentrations with or without propagermanium treatment in both db/+ m and db/db mice (data not shown), indicating that propagermanium suppressed weight gain only in db/db mice without causing appetite loss.
tolerance by propagermanium treatment. In addition, propagermanium treatment increased expression of glucose transporter-2 and 4 (GLUT2, 4) mRNA in adipose tissue, liver, and muscle (supplemental Figure IIIA through IIE), suggesting that propagermanium improved insulin action in all insulin sensitive organs in db/db mice. Furthermore, we observed that propagermanium decreased plasma insulin concentration (Table) and improved insulin sensitivity and glucose tolerance (Figure 1A and 1B, right) in weight-matched db/db mice at 9 weeks of age, suggesting that propagermanium improves insulin resistance before the suppression of weight gain in db/db mice.

**Effect of Inhibition of CCR2 on Adiposity in Obese Mice**

Propagermanium treatment reduced visceral fat mass only in db/db mice (supplemental Figure IA and IB). Epididymal fat mass was also decreased in propagermanium-treated db/db mice compared with nontreated db/db mice (supplemental Figure IC).

We next measured adipocyte size by histological analysis. Although propagermanium treatment had no effect on average adipocyte size in db/+m mice (1702 ± 84 versus 1660 ± 100 μm²; Figure 2A), the average adipocyte size of propagermanium-treated db/db mice was 40% lower than those of nontreated db/db mice (4332 ± 960 versus 7588 ± 1360 μm²; P < 0.001).

Adipose tissue expression of adiponectin mRNA was markedly decreased in db/db mice by 50% compared with nontreated db/+m mice at 18 weeks of age (Figure 2C). However, propagermanium treatment increased adipose tissue adiponectin expression by 1.4-fold in db/db mice at 18 weeks of age. Similar to the gene expression, propagermanium-treated db/db mice showed 1.3-fold higher plasma adiponectin levels than nontreated db/db mice (17.1 ± 0.9 versus 13.0 ± 0.9 μg/mL; P < 0.01; Figure 2C). In weight-matched db/db mice at 9 weeks of age, propagermanium increased expression of adiponectin mRNA (supplemental Figure IIB), but not plasma adiponectin level (supplemental Figure VA).

**Effect of Inhibition of CCR2 on ATM Accumulation and Adipose Tissue Inflammation in Obese Mice**

The percentage of F4/80-positive cells in adipose tissue was increased in db/db mice compared with db/+m mice (38.1 ± 10.3 versus 4.3 ± 1.0%; P < 0.001; Figure 3A). However, propagermanium treatment reduced macrophage infiltration in adipose tissue of db/db mice (24.6 ± 8.1 versus 38.1 ± 10.3%; P < 0.001; Figure 3A). Consistently, F4/80 (Emr1) and CD68 mRNA levels were significantly decreased in propagermanium-treated db/db mice compared with nontreated db/db mice, whereas propagermanium had no effect on the expression of F4/80 and CD68 mRNA in db/+m mice,

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**Table. Characteristics of db/tm and db/db Mice Treated With or Without Propagermanium**

<table>
<thead>
<tr>
<th></th>
<th>Con (18 Weeks of Age)</th>
<th>Pro (18 Weeks of Age)</th>
<th>Con (9 Weeks of Age)</th>
<th>Pro (9 Weeks of Age)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>32.1 ± 1.0</td>
<td>33.3 ± 1.4</td>
<td>54.1 ± 3.5</td>
<td>47.0 ± 4.3**</td>
</tr>
<tr>
<td>Glucose, mg/dl</td>
<td>75.6 ± 9.9</td>
<td>81.7 ± 8.6</td>
<td>540.4 ± 103.3</td>
<td>436.8 ± 83.3**</td>
</tr>
<tr>
<td>HbA1c, %</td>
<td>2.6 ± 0.1</td>
<td>2.5 ± 0.1</td>
<td>9.4 ± 0.7</td>
<td>8.0 ± 1.4**</td>
</tr>
<tr>
<td>Plasma insulin, ng/ml</td>
<td>1.1 ± 0.6</td>
<td>1.3 ± 0.8</td>
<td>3.4 ± 0.7</td>
<td>1.9 ± 0.7**</td>
</tr>
<tr>
<td>Plasma TG, mg/dl</td>
<td>87.2 ± 9.9</td>
<td>88.0 ± 23.1</td>
<td>164.4 ± 49.9</td>
<td>117.6 ± 31.6**</td>
</tr>
<tr>
<td>Plasma T-Chol, mg/dl</td>
<td>90.6 ± 11.4</td>
<td>81.6 ± 9.9</td>
<td>203.3 ± 16.3</td>
<td>208.5 ± 22.9</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SD. **P < 0.01 vs control group in each mice.
indicating that propagermanium reduced ATM accumulation only in obese mice (Figure 3B). In accordance with the results of macrophage infiltration, MCP-1 and TNF-α mRNA expression in adipose tissue was markedly reduced in propagermanium-treated db/db mice by 60% and 65%, respectively, compared with nontreated db/db mice (Figure 3C). We also observed that propagermanium treatment reduced ATM accumulation and suppressed adipose inflammation in adiposity-matched db/db mice at 9 weeks of age (supplemental Figure IIA and IIB), suggesting that propagermanium suppresses adipose tissue inflammation by reduction of ATM content in obese mice.

Effect of Inhibition of CCR2 on Hepatic Steatosis in Obese Mice
Liver tissue appearance in nontreated db/db mice was more whitish in color than that in db/+m mice. However, the liver in propagermanium-treated db/db mice looked more dark-red than that in nontreated db/db mice (Figure 4A). The liver weight was increased by 2-fold in nontreated db/db mice compared with nontreated db/+m mice (3.4±0.3 versus 1.3±0.4 g, P<0.001). However, the liver weight of propagermanium-treated db/db mice was reduced by 30% compared with that of nontreated db/db mice (2.3±0.1 versus 3.4±0.3 g, P<0.01). Similarly, nontreated db/db mice had more than 3-fold higher hepatic TG contents than nontreated db/+m mice (0.66±0.23 versus 0.18±0.08 mg/mg protein; P<0.001; Figure 4A). Propagermanium-treated db/db mice showed 45% lower hepatic TG contents than nontreated db/db mice (0.34±0.08 versus 0.66±0.23 mg/mg protein, P<0.01). Consistently, the expression of sterol regulatory element binding protein-1c (SREBP-1c), a transcription factor that regulates the expression of genes important in lipid synthesis, was decreased by 30% in propagermanium-treated db/db mice, suggesting that reduced hepatic TG content by propagermanium treatment is caused by a decrease in hepatic lipogenesis (Figure 4B).

Expression of TNF-α mRNA in the liver was increased in db/db mice compared with db/+m mice, and its increase was compared with nontreated db/+m mice (3.4±0.3 versus 1.3±0.4 g, P<0.001). However, the liver weight of propagermanium-treated db/db mice was reduced by 30% compared with that of nontreated db/db mice (2.3±0.1 versus 3.4±0.3 g, P<0.01). Similarly, nontreated db/db mice had more than 3-fold higher hepatic TG contents than nontreated db/+m mice (0.66±0.23 versus 0.18±0.08 mg/mg protein; P<0.001; Figure 4A). Propagermanium-treated db/db mice showed 45% lower hepatic TG contents than nontreated db/db mice (0.34±0.08 versus 0.66±0.23 mg/mg protein, P<0.01). Consistently, the expression of sterol regulatory element binding protein-1c (SREBP-1c), a transcription factor that regulates the expression of genes important in lipid synthesis, was decreased by 30% in propagermanium-treated db/db mice, suggesting that reduced hepatic TG content by propagermanium treatment is caused by a decrease in hepatic lipogenesis (Figure 4B).

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inhibited by propagermanium treatment (Figure 4C) suggesting that propagermanium also suppresses the liver tissue inflammation. In addition, we also detected amelioration of hepatic steatosis and hepatic inflammation in adiposity-matched db/db mice at 9 weeks of age (supplemental Figure IIC and IID).

**Discussion**

In this study, we have clearly shown that treatment with propagermanium improved obesity, insulin resistance, and hepatic steatosis by inhibiting macrophage infiltration into adipose tissue through the suppression of CCR2 function during the development of obesity in db/db mice.

Propagermanium has been used as a therapeutic agent against hepatitis B virus–induced chronic hepatitis in Japan. Previous reports showed that propagermanium reduces monocyte/macrophage infiltration into the liver and inhibits liver damage in 2 different experimental mouse liver injury models. In addition, another report showed that propagermanium attenuates atherogenesis via the inhibition of macrophage infiltration into the lesion area in apoE-deficient mice. Furthermore, Yokochi et al demonstrated that this drug suppressed monocyte migration in vitro and macrophage infiltration in vivo. Thus the inhibition of CCR2 function is shown as a potential mechanism of this drug. It does not affect the function of other chemokines, such as interleukin (IL)-8 or RANTES, except MCP-1 and MCP-3, which are the ligands for CCR2. These results suggest that the inhibitory effect of propagermanium on monocyte migration would be CCR2 dependent. The molecular mechanism of the action of propagermanium has been shown that it targets glycosylphosphatidylinositol-anchored proteins that are closely associated with CCR2 and interferes with MCP-1–induced monocyte chemotaxis.

In this study we demonstrated that inhibition of CCR2 function by propagermanium treatment reduced macrophage infiltration in adipose tissue. We also found that propagermanium treatment suppressed adipose tissue inflammation, as well as decreased TNF-α and MCP-1 expression. Recent data suggest that the expression of proinflammatory cytokines by adipose tissue is in part attributable to the expression of these cytokines by nonadipocytes, including macrophages. Further, Suganami et al have recently developed an in vitro coculture system composed of adipocytes and macrophages and demonstrated that a paracrine loop involving free fatty acids (FFAs) and TNF-α derived from adipocytes and macrophages, respectively, establishes a vicious cycle that augments the inflammatory changes in both adipocytes and macrophages: i.e., marked upregulation of proinflammatory cytokines such as MCP-1 and TNF-α and downregulation of antiinflammatory cytokine adiponectin. These reports and our results suggest that macrophage infiltration via CCR2 play a pivotal role in the induced inflammatory changes and dysregulated adipokine expression in adipose tissue.

Recent investigation suggests that adipose tissue inflammatory changes contribute to the development of insulin resistance. It has been shown that adipose tissue–derived proinflammatory cytokines such as TNF-α and IL-6 can actually cause insulin resistance in experimental models. In this study, propagermanium treatment only for 3 weeks reduced plasma IL-6 level in db/db mice (supplemental Figure VB), suggesting that propagermanium suppressed systemic inflammation by inhibiting ATM accumulation in db/db mice. Further, TNF-α dose-dependently reduces the expression of adiponectin in adipocytes by suppressing its promoter activity. Adiponectin works as an insulin-sensitizing agent and hence a decrease in plasma adiponectin is related to insulin resistance in obesity. In this study, we observed that adiponectin expression in adipose tissue and plasma adiponectin level decreased in db/db mice compared with db/+m mice, as well as impaired insulin sensitivity and glucose tolerance. However, we could improve insulin resistance by propagermanium treatment possibly by suppressing adipose tissue inflammation and increasing plasma adiponectin level. This result strongly suggests that dysregulated adipokine expression and secretion induced by macrophage infiltration through CCR2 causes insulin resistance in obesity.

In the present study, we successfully ameliorated hepatic steatosis by propagermanium treatment in db/db mice. Several reports suggest that insulin resistance contributes to the development of hepatic steatosis by impairing the ability of insulin to suppress lipolysis, leading to increased delivery of FFAs to the liver. In this study, propagermanium decreased hepatic expression of CD36, which takes up FFA into liver, and hepatic FFA in db/db mice at 18 weeks of age, but not at 9 weeks age (supplemental Figure IV). These findings suggest that propagermanium decreases FFA influx into liver in db/db mice. Increased de novo lipogenesis may also contribute to hepatic fat accumulation. This increased de novo lipogenesis may be a result of insulin resistance and the resulting hyperinsulinemia in subjects with hepatic steatosis, as insulin stimulates lipogenic enzymes via SREBP-1c even in an insulin-resistant state. Thus, improved hyperinsulinemia and decreased SREBP-1c by propagermanium treatment could also be involved in amelioration of hepatic steatosis.
We also observed that propagermanium treatment markedly suppressed liver inflammation, as shown by decreased MCP-1 and TNF-α expression. Cai et al reported that inflammatory gene expression increases in liver along with the increase in adiposity, without increasing a number of Kupffer cells, suggesting that hepatocyte lipid accumulation by increasing FFA delivery from adipose tissue might induce a subacute inflammatory response in the liver. Further, Arkan et al showed that suppression of liver-specific inflammatory response by hepatocyte-specific deletion of IKKβ protects from high fat diet-induced systemic insulin resistance and hepatic steatosis in mice. These reports suggest that liver tissue inflammation is responsible for impairment of systemic insulin resistance and hepatic steatosis. Therefore, suppression of liver inflammation through the reduction of FFAs delivery from adipose tissue and lipogenesis in liver by propagermanium treatment might also cause amelioration of insulin resistance and hepatic steatosis in db/db mice. However, we also found that propagermanium decreased expression of CD68 mRNA in liver of db/db mice (supplemental Figure VI). Therefore, propagermanium might affect Kupffer cell number or activity in liver of db/db mice.

We found that inhibition of CCR2 by propagermanium treatment suppressed body weight gain mainly by reducing visceral fat mass and liver weight without affecting food intake in db/db mice. Although Weisberg et al report that obese CCR2-deficient mice induced by HFD had lower body weight compared with obese wild-type mice, they discussed that this phenomenon may be caused by modulated feeding behavior by deletion of CCR2 in central nerve system. In the present study, however, oral inhibition of CCR2 did not affect food intake, suggesting that propagermanium might not affect CCR2 function in central nerve system. In addition, we found that propagermanium treatment only for 3 weeks increased the enzymes associated lipid oxidation including carnitine palmitoyltransferase (CPT) and acyl-coenzyme A (CoA) oxidase (ACOX) in muscle and liver of db/db mice as well as reduction of plasma IL-6 level (supplemental Figures III and VB). These data suggest suppressed systemic inflammation by propagermanium treatment causes increased energy expenditure in db/db mice. Furthermore, we detected an increase in plasma adiponectin by propagermanium treatment in db/db mice from 15 weeks of age (supplemental Figure VA). MCP-1–deficient mice, which develop improved insulin resistance, obesity and diabetes. However, further study using a pure CCR2 antagonist would be necessary for the confirmation of these findings.

In summary, we have shown that blockade of CCR2 function ameliorated both insulin resistance and hepatic steatosis along with decreased macrophage infiltration into adipose tissue and liver of obese mice. Our results thus suggest that CCR2 plays an important role in the pathogenesis of the metabolic syndrome and propagermanium may be a promising drug for treatment of the metabolic syndrome.

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Disclosures
None.

References


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Supplementary Methods

Materials
Antibodies directed to total AMPK, phosphorylated (Thr172) AMPKα and β-actin were purchased from Cell Signaling. Antibodies directed to CD36 was purchased from R&D systems Inc.

Quantitative Real-time PCR
Total RNA was extracted from frozen adipose tissue (200 mg), liver and muscle tissue (50 mg) using RNeasy mini kit (Qiagen, Valencia, CA). The cDNA was synthesized from total RNA using Super Script III (Invitrogen). Real-time polymerase chain reaction was performed on an ABI PRISM 7700 using the SYBR GREEN polymerase chain reaction Master Mix (Applied Biosystems, Warrington, UK). Primer sets were following; CD36 forward; TGTACCTGGGAGTTGGCGAG, CD36 reverse; CTGCTGTCTTTGCCACGTC, Acyl CoA oxidase forward; ATGAATCCCGATCTGCGCAAGGAGC, Acyl CoA oxidase reverse; AAAGGCATGTAACCCCGTAGCCTCC, CPT1a forward; GTCCCAGCTGTCAAGGATAC, CPT1a reverse; GGAAGTATTGAAGAGTGGC, CPT1b forward; CAAGTTCAGAGACGAACGC, CPT1b reverse; TCAAGAGCTGTTCTCCGAAACTG, HSL forward; CTGCTGACCATCAACCGAC, HSL reverse; CGATGGAGAGAGTCTGCA, LPL forward; TTCTCCTCCTACTCCTCCTC, LPL reverse; TGTCCCTAGCTGTGCTCTCA, GLUT4 forward; CATGGCTGTCGCTGTCTTCA, GLUT4 reverse; AAACCATGCGGCAAATGA, GLUT2 forward; GGCTAATTTCAGGACTGGT, GLUT2 reverse; TTTCTTCCCGCTGACTTCTT, β-actin forward; TACCACGGCATTTGTGATTC, β-actin reverse; TTTGATGTCACGCGATTT, The mRNA levels were normalized relative to the amount of β-actin and expressed in arbitrary units.

Immunoblot analysis
Cell lysates were prepared by the addition of lysis buffer (cell signaling). After heated at 95 °C for 5 min, equal protein concentrations of the cell lysates were subjected to SDS-polyacrylamide (12%) gel electrophoresis and transferred onto nitrocellulose membranes (PROTRAN, Schleicher & Schuell) by electroblotting. After preincubation with blocking buffer (PBS containing 0.1% Tween 20 and 5% nonfat dry milk) for 1 h at room temperature, blotted membranes were incubated with each primary antibody for
1 h at room temperature or overnight at 4 °C, followed by washing twice with blocking buffer. Membranes were then incubated with a horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (Amersham) for 30 min at room temperature, followed by washing three times in PBS containing 0.1% Tween 20, and visualized by ECL western blotting detection reagents (Amersham)

**Measurement of adipocytokine concentration**
Plasma IL-6 was measured by ELISA kit (eBioscience). Plasma resistin was measured by ELISA kit (R&D systems)

**Measurement of hepatic free fatty acid contents**
For determination of hepatic free fatty acid contents in db/+m and db/db mice, liver tissue (50 mg) was homogenized for 2 min at 20 Hz in 500 ml PBS with a Mixer Mill MM 300 (Retsch GmbH & Co, Haan, Germany). The tissue-suspending solution (equal to 0.1 mg protein) was adjusted 1.25 ml with PBS and was added with 5 ml chloroform/methanol (2:1, vol/vol), and was extracted for 16 hours at 4 °C. The extract was centrifuged at 2000 g for 20 min. The organic layer was collected and dried and the residue was dissolved in isopropanol and assayed for free fatty acid content with a NEFA C test (Wako). Tissue free fatty acid contents were expressed µmol/mg protein.
Supplementary Figure 4

A

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B

C

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Bar graphs showing:

- B: CD36 mRNA levels in db/+m and db/db mice at 18 weeks for db/+m and db/db mice at 9 weeks.
- C: Liver FFA levels in db/+m and db/db mice.
Supplementary Figure 6

A

B

CD68 mRNA (Arbitrary Unit)

0 0.2 0.4 0.6 0.8 1.0 1.2 1.4 1.6

db/+m (18w) db/db (18w)

CD68 mRNA (Arbitrary Unit)

0 0.2 0.4 0.6 0.8 1.0 1.2 1.4 1.6

db/db (9w)
Supplementary Figure Legend

Supplementary Figure 1
A: Representative CT images of propagermanium-treated (Pro) or non-treated (Con) db/+m and db/db mice at the L3 level. B, C: CT-estimated amount of visceral fat weight (B), and epididymal fat weight (C) in propagermanium-treated (closed columns) or non-treated (open columns) db/+m and db/db mice. Values are expressed as means ± S.D. *, p<0.05; **, p<0.01; vs. non-treated db/db mice (n=8 per group).

Supplementary Figure 2
A, B; Effect of propagermanium on the fraction of ATMs (F4/80-stained cell/ total cell) in eWAT (A), adipokine expression in eWAT (B) of db/db mice at 9 weeks of age. C; Effect of propagermanium on hepatic TG content in db/db mice at 9 weeks of age. D; Effect of propagermanium on TNF-alpha and SREBP-1c expression in liver of db/db mice at 9 weeks of age. Values are expressed as means ± S.D. *, p<0.05 and **, p<0.01 compared with non-treated db/db mice (n=9 per group).

Supplementary Figure 3
A, B; Effect of Pro treatment on expression of CPT1a (carnithine palmitoyltransferase 1a), ACOX (acyl CoA oxidase) and GLUT2 mRNA in liver from db/m and db/db mice at 18 weeks (A), 9 weeks (B) of age. C, D; Effect of Pro treatment on expression of CPT1b, ACOX and GLUT4 mRNA in muscle from db/+m and db/db mice at 18 weeks (C), 9 weeks (D) of age. E; Effect of Pro treatment on expression of GLUT4 mRNA in adipose tissue from db/db mice at 9 weeks of age. F; Effect of Pro treatment on expression of Phospho-AMPK (p-AMPK) and total AMPK (t-AMPK) protein in liver and muscle from db/db mice at 18 weeks of age. Values are expressed as mean ± SD. *; p<0.05 vs db/db control (n=10 in each group).

Supplementary Figure 4
A, B; Effect of Pro treatment on expression of CD36 protein (A) and mRNA (B) in liver from db/m or db/db mice at 9, 18 weeks of age. C; Effect of Pro treatment on free fatty acid content in liver from db/m or db/db mice at 18 weeks of age. Values are expressed as mean ± SD. *; p<0.05, **; p<0.01 vs db/db control (n=10 in each group).

Supplementary Figure 5
A; Time-dependent effect of Pro treatment on plasma adiponectin level in db/+m and
db/db mice. B; Effect of Pro treatment on plasma IL-6 level in db/+m and db/db mice at 9, 18 weeks of age. Values are expressed as mean ± SD. *; p<0.05, **; p<0.01 vs db/db control (n=10 in each group).

Supplementary Figure 6
A, B; Effect of Pro treatment on expression of CD68 mRNA in liver of db/+m and db/db mice at 18 weeks (A) or 9 weeks (B) of age. Values are expressed as mean ± SD. *; p<0.05, **; p<0.01 vs db/db control (n=10 in each group).
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