Blockade of Cannabinoid Receptor 1 Improves Insulin Resistance, Lipid Metabolism, and Diabetic Nephropathy in db/db Mice

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The endocannabinoid system is important in the pathogenesis of obesity-related metabolic disorders. However, the effect of inhibiting the endocannabinoid system in type 2 diabetic nephropathy is unclear. Therefore, we examined the effect of the cannabinoid (CB)1 receptor antagonist, SR141716, on insulin resistance and diabetic nephropathy in db/db mice. Six-week-old db/db mice were treated with the CB1-specific antagonist SR141716 (10 mg/kg/d) for 3 months. Treatment with SR141716 significantly improved insulin resistance and lipid abnormalities. Concomitantly, CB1 antagonism improved cardiac functional and morphological abnormality, hepatic steatosis, and phenotypic changes of adipocytes into small differentiated forms, associated with increased adiponectin expression and decreased lipid hydroperoxide levels. CB1 receptor was overexpressed in diabetic kidneys, especially in podocytes. Treatment with the SR141716 markedly decreased urinary albumin excretion and mesangial expansion and suppressed profibrotic and proinflammatory cytokine synthesis. Furthermore, SR141716 improved renal lipid metabolism and decreased urinary 8-isoprostane levels, renal lipid hydroperoxide content, and renal lipid content. In cultured podocytes, high-glucose stimulation increased CB1 receptor expression, and SR141716 treatment abolished high-glucose-induced up-regulation of collagen and plasminogen activator inhibitor-1 synthesis. Additionally, knockdown of CB1 receptor expression by stealth small interfering RNA abolished high-glucose-induced sterol-regulatory element-binding protein-1 expression in podocytes. These findings suggest that CB1 blockade improves insulin resistance and protect against renal injury through both metabolic and antifibrotic effects in type 2 diabetic nephropathy. (Endocrinology 153: 1387–1396, 2012)

Type 2 diabetes mellitus is characterized by progressive loss of pancreatic β-cell function and insulin resistance that usually occurs with advancing age, inactivity, and weight gain (1). The disease accounts for substantial morbidity and mortality from adverse effects on cardiovascular risk and disease-specific complications (2). The increasing prevalence of obesity is accompanying an increase of type 2 diabetes mellitus, and the diabetic nephropathy is the most common cause of end-stage renal disease (2).

The endocannabinoid system (ECS) contains endogenous lipid mediators of adiposity and insulin resistance.
that interact with the G protein-coupled cannabinoid (CB) receptors (CB1 and CB2 receptors) that recognize plant-derived CB and regulate a broad range of physiological functions (3). CB1 receptors are expressed at very high levels in the brain but are also present at much lower yet functionally relevant concentrations in various peripheral tissues, such as liver, skeletal muscle, pancreas, and fat, where their activation contributes to obesity-related metabolic and hormonal abnormalities (4–7). Several animal and human studies reported that the ECS is activated in the presence of obesity or diabetes mellitus and contributes to the development of metabolic syndrome (8–10). In animal models of obesity and metabolic syndrome, as well as in humans, genetic deficiency or blockade of CB1 has demonstrated beneficial effects in terms of metabolic alterations (11–13).

A recent study showed that the CB1 receptors are expressed at a low level within the glomeruli and that CB1 receptor blockade ameliorates proteinuria in an animal model of obesity-induced nephropathy (14). In addition, the CB1 receptor is overexpressed by glomerular podocytes, and blockade of the CB1 receptor in experimental type 1 diabetes mellitus ameliorates albuminuria, possibly via prevention of podocyte loss (15). These observations suggest the possibility that the CB1 receptor in glomeruli may be involved in the development of diabetic nephropathy.

Considering that type 2 diabetes mellitus has characteristics of metabolic alterations, such as insulin resistance and dyslipidemia, blockade of ECS by CB1 receptor antagonists may provide organ-protective effects, including against diabetic nephropathy. However, the pathophysiology of ECS inhibition in type 2 diabetic nephropathy has not been elucidated. Therefore, we investigated the effect of the specific CB1 receptor antagonist SR141716 on insulin resistance and diabetic nephropathy under the hypothesis that inhibition of CB1 receptor can provide renal protection through direct effects on the kidney and amelioration of systemic insulin resistance in db/db mice.

### Materials and Methods

#### Animal experiments

Six-week-old male diabetic db/db mice (C57BLKS/J-lepr<sup>db</sup>/lepr<sup>db</sup>) and male nondiabetic db/m mice (C57BLKS/J-lepr<sup>db</sup>/+) were purchased from The Jackson Laboratory (Sacramento, CA). Diabetic db/db mice were divided into two groups of diabetic controls (n = 8) and drug-treated diabetic mice (n = 12). The specific CB1 receptor antagonist SR141716 was kindly supplied by the National Institute of Mental Health Chemical Synthesis and Drug Supply Program (Research Triangle Institute International, Research Triangle Park, NC). Mice were given either normal water or water mixed with 10 mg/kg SR141716 for 12 wk from 8 wk of age. All mice were provided with a standard diet and water and were maintained in a temperature (23 ± 2°C) and humidity (55 ± 5%)-controlled room with a 12-h light, 12-h dark cycle. Daily amounts of water intake were checked at regular intervals to confirm drug dose. During experiments, food intake, water intake, urine volume, body weight, fasting plasma glucose concentration, and glycated hemoglobin (HbA1c) levels were measured each month. Plasma glucose levels were measured using a glucose oxidase-based method, plasma sodium and potassium levels were measured by flame photometry, and creatinine levels were determined using a HPLC method. Plasma insulin levels and plasma adiponectin levels were measured using an ELISA kit (Linco Research, St. Charles, MO). The homeostasis model assessment index (HOMA-IR) was calculated by the equation fasting glucose (mmol/liter) × fasting insulin (mU/liter)/22.5. Plasma triglyceride and cholesterol analyses were performed using a GPO-Trinder kit (Sigma-Aldrich, St. Louis, MO). Plasma lipoprotein profiles were measured using a fast protein HPLC system. Insulin tolerance tests (ITT) and glucose tolerance tests (GTT) determined the insulin resistance and glucose intolerance of each group at the end of the study. ITT was conducted by ip injection of 0.75 U/kg regular insulin in fasted mice with blood glucose measurements at 0, 30, 60, 90, and 120 min. GTT was performed by oral gavage of 3 g dextrose/kg after a 5-h fast, and blood samples were collected through the tail vein. Lipids from the hepatic, adipose, and renal cortical tissues were extracted as described by Bligh and Dyer (16). Total cholesterol and triglyceride contents were measured using a commercial kit (Wako Chemicals, Richmond, VA). Plasma and urinary levels of 8-isoprostane were measured using an ELISA kit (Cayman Chemical, Ann Arbor, MI). The extent of peroxidative reaction in the hepatic, adipose tissue, and kidneys was determined by directly measuring lipid hydroperoxides (LPO) using an LPO assay kit (Cayman Chemical) as described previously (17). To determine urinary albumin excretion, individual mice were caged once each month in a metabolic cage, and urine was collected for 24 h. Urinary albumin concentrations were determined duplicate by competitive ELISA (Shibayagi, Shibukawa, Japan) after correction by urinary creatinine concentrations.

To determine drug cardiovascular effects, systolic blood pressure and echocardiograms were performed at the end of the study. Systolic blood pressure was measured using unanesthetized mice with tail-cuff plethysmography (LE 5001-Pressure Meter; Letica SA, Barcelona, Spain) after acclimatization to tail-cuff for 1 wk. Transthoracic echocardiogram was performed as described previously (17). After 3 months of treatment, mice were killed under anesthesia by ip injection of sodium pentobarbital (50 mg/kg), and heart, epididymal fat, liver, and kidney tissues were weighed and subsequently snap frozen in liquid nitrogen. All experiments were conducted in accordance with National Institutes of Health guidelines and with approval of the Korea University Institutional Animal Care and Use Committee.

#### Analysis of gene expression by real-time quantitative PCR

Total RNA was extracted from the renal cortical tissues, adipose tissue, and experimental cells with TRIzol reagent and fur-
ther purified using an RNase Mini kit (QIAGEN, Valencia, CA). Primer sequences are in Supplemental Table 1, published on The Endocrine Society’s Journals Online web site at http://endo.endojournals.org. Quantitative gene expression was performed on a LightCycler 1.5 system (Roche Diagnostics Corp., Indianapolis, IN) using SYBR Green technology. In brief, 10 μl of SYBR Green master mix were added to 1 μl of RNA (corresponding to 50 ng of total RNA) and 900 nmol/liter of forward and reverse primers in a reaction volume of 20 μl. Real-time RT-PCR was performed for 10 min at 50°C and 5 min at 95°C. A total of 22–30 cycles of 10 sec at 95°C and 30 sec at 60°C was used. On completion of cycling, samples were heated to 95°C to verify that a single PCR product was obtained. The ratio of each gene to β-actin level (relative gene expression number) was calculated by subtracting the threshold cycle number of the target gene from that of β-actin and raising two to the power of this difference.

Histopathological evaluation and immunohistochemistry

Cardiac, aortic, hepatic, and adipose tissues were fixed for 48 h with 10% paraformaldehyde at 4°C, dehydrated, embedded in paraffin, cut into 4-μm thick slices, and stained with periodic acid-Schiff, Masson’s trichrome, and hematoxylin and eosin. Glomerular mesangial expansion was scored semiquantitatively, and the percentage of mesangial matrix occupying each glomerulus was rated as 0–4 as follows: 0, 0%; 1, less than 25%; 2, 25–50%; 3, 50–75%; and 4, more than 75%. For immunohistochemical staining, renal tissues were sliced into 4-μm sections. Slides were transferred to a 10 mmol/liter citrate buffer solution at pH 6.0 and heated at 80°C for 30 min for TGFβ1 and CB1 receptor staining. Alternatively, sections were transferred to a Biogenex Retrievit (pH 8.0) (InnoGenex, San Ramon, CA) and microwaved for 10–20 min for antigen retrieval before plasminogen activator inhibitor-1 (PAI-1) staining. For type IV collagen staining, slides were treated with trypsin (one tablet per 1 ml of H2O) for 20 min for antigen retrieval. To block endogenous peroxidase activity, 3.0% H2O2 in methanol was applied for 20 min, followed by incubating at room temperature for 60 min with 3% BSA/3% normal goat serum (type IV collagen and CB1 receptor), 15 min with 10% powerblock (PAI-1), or 30 min with 20% normal sheep serum (TGFβ1). Slides were incubated overnight at 4°C with a rabbit polyclonal anti-TGFβ1 antibody (1:100; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), rabbit monoclonal anti-CB1 receptor antibody (1:100; Abcam, Cambridge, MA), rabbit polyclonal antitype IV collagen antibody (1:150; BioDesign International, Sarco, ME), or rabbit polyclonal anti-PAI-1 antibody (1:60; American Diagnostica, Stamford, CT). Slides were incubated with secondary antibodies for 30 min, and slides were incubated at room temperature with 0.05% 3,3-diaminobenzidine containing 0.01% H2O2 and counterstained with Mayer’s hematoxylin. The quantitation of immunohistochemical staining was calculated as described previously (18). Frozen brain cross-sections from C57BL/6j were used as positive control for CB1 receptor immunostaining. A pathologist carried out the histological examinations in a blinded manner.

Protein extraction and Western blot analysis

For Western blotting, 40 μg of protein were electrophoresed on 10% SDS-PAGE minigel. Proteins were transferred onto a polyvinylidene difluoride membrane, the membrane was hybridized in blocking buffer overnight at 4°C with rabbit polyclonal anti-CB1 receptor antibody (1:500; Abcam), rabbit polyclonal anti-PAI-1 antibody (1:500; Santa Cruz Biotechnology, Inc.), sterol-regulatory element-binding protein (SREBP)-1c antibody (1:500; Santa Cruz Biotechnology, Inc.), and type IV collagen antibody (1:500; Santa Cruz Biotechnology, Inc.). Mouse monoclonal anti-β-actin antibody (1:5000; Sigma-Aldrich) was hybridized in blocking buffer at 4°C, and the membrane was incubated with horseradish peroxidase-conjugated secondary antibody diluted 1:1000 for 60 min at room temperature. The detection of specific signals used the enhanced chemiluminescence method (Amersham, Buckinghamshire, UK).

Podocyte culture and stealth RNA interference for CB1 receptor

Because immunostaining for CB1 receptor was preferentially detected in glomerular podocytes in db/db mice, we used podocytes to define the molecular mechanism of CB1 receptor. Cultivation of podocytes was done as described previously (19). Podocytes were cultivated on 100-mm dishes and serum restricted for 24 h. Afterwards, cells were treated with 30 mM D-glucose from 30 min to 96 h to evaluate whether high-glucose stimuli increased CB1 receptor expression. To evaluate the effects of CB1 receptor antagonist, 100 nm SR141716 was added to the cells 1 h before treatment with high glucose for 48 h. Three independent experiments were performed to determine the protein expression levels. Because the CB1 receptor antagonist showed a beneficial effect in renal lipid metabolism and SR141716 treatment most profoundly suppressed SREBP-1 expression in renal cortical tissues, we next used small interfering RNA (siRNA) for the CB1 receptor to investigate whether CB1 receptor regulate renal lipid metabolism. Mouse CB1 receptor mRNA was specifically knocked down using commercially available siRNA oligonucleotides.

The sequences of stealth siRNA were designed using BLOCK-iT RNAi Designer (Invitrogen Life Technologies, Gaithersburg, MD) to target base pairs 1107–1131 of the mouse CB1 receptor (sense strand, 5-AAC AGC UGG AAG ACA UCA AAG CUG U-3; anti-sense strand, 5-ACA GCU UUG UUG ACU UCC ACG UGU U-3). Before transfection, podocytes were maintained in RPMI1640 with 10% fetal calf serum, then transfected with 100 pmol siRNA using Lipofectamine RNAiMax (Invitrogen Life Technologies) per the manufacturer’s instructions. Transfection for 24 h induced a 70% reduction in CB1 receptor mRNA expression, podocytes were transfected for 24 h with siRNA and cultivated in serum-free and antibiotic-free RPMI 1640. Cells were made quiescent for 16 h and treated with 30 mM high-glucose medium for 48 h. Three independent experiments were performed to quantitate the CB1 receptor and SREBP-1c protein expression. Stealth RNA interference negative control duplexes were used as controls.

Statistical analysis

A nonparametric analysis was used because of the relatively few samples. Results were expressed as mean ± SEM. Multiple comparisons were done using Wilcoxon rank sum tests and Bon-
Results

Biochemical and physical parameters of experimental animals

Tables 1 and 2 show biochemical and physical results for each experimental group. No significant differences in body weight, fasting plasma glucose levels, or plasma electrolyte levels were observed between the groups. Organ mass showed a decreased tendency in the SR141716 group, but this did not reach significance; however, heart weight was significantly decreased in the SR141716-treatment group. Food intake was significantly decreased after 3 months with SR141716, and water intake was transiently increased by SR141716. Although no difference was seen in systolic blood pressure, left ventricular mass index (LVMI) was significantly decreased by SR141716 treatment (Supplemental Table 2). HbA1c levels did not show a significant difference at 3 months. Interestingly, plasma creatinine concentration was significantly decreased in the CB1 receptor antagonist group, urinary albumin excretion was significantly decreased after antagonist treatment even after 1 month of treatment, and the antialbuminuric effects of CB1 receptor antagonist treatment persisted throughout the entire study period (Fig. 1).

Effect of CB1 receptor antagonist on metabolic parameters in experimental animals

As shown in Fig. 2, the improvement in insulin resistance and glucose intolerance by SR141716 treatment was confirmed by an ip ITT and oral GTT. Consistent with the improved insulin resistance, SR141716 treatment significantly decreased plasma total cholesterol, low-density lipoprotein-cholesterol and triglyceride levels. In addition, plasma insulin levels and HOMA-IR were significantly higher in control mice than SR141716-treated mice. In

TABLE 1. Physical and metabolic parameters during experimental periods in experimental animals

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>SR141716</th>
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<tr>
<td>Body weight (g)</td>
<td>45.62 ± 1.42</td>
<td>44.60 ± 0.84</td>
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<tr>
<td>UV (m/d)</td>
<td>3.6 ± 0.9</td>
<td>4.1 ± 1.0</td>
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<tr>
<td>Food Intake (g/d)</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td>Water Intake (m/d)</td>
<td>N.A.</td>
<td>N.A.</td>
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<tr>
<td>FPG (mg/dl)</td>
<td>629 ± 42</td>
<td>617 ± 43</td>
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<tr>
<td>Hba1c (%)</td>
<td>11.9 ± 0.5</td>
<td>10.4 ± 0.4*</td>
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<tr>
<td>Baseline</td>
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<td>Two months</td>
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<td>Three months</td>
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Values are expressed as means ± SEM. BW, Body weight; Na, sodium; K, potassium.

* P < 0.05 vs. control.

** P < 0.01 vs. control.

*** P < 0.001 vs. control.

was seen in systolic blood pressure, left ventricular mass index (LVMI) was significantly decreased by SR141716 treatment (Supplemental Table 2). HbA1c levels did not show a significant difference at 3 months. Interestingly, plasma creatinine concentration was significantly decreased in the CB1 receptor antagonist group, urinary albumin excretion was significantly decreased after antagonist treatment even after 1 month of treatment, and the antialbuminuric effects of CB1 receptor antagonist treatment persisted throughout the entire study period (Fig. 1).
addition, plasma levels of adiponectin were significantly increased in the SR141716 group (Table 2).

Expression of CB1 receptor in glomerular cells, adipocytes, and diabetic kidneys

We first performed RT-PCR for the CB1 receptor mRNA in glomerular cells and adipocytes to confirm the presence of CB1 receptor. In addition, we performed immunostaining for the CB1 receptor in 8-wk-old db/m and db/db mice to elucidate whether the CB1 receptor is overexpressed in diabetic kidneys. As shown in Supplemental Fig. 1, most glomerular cells and adipocytes showed the presence of the CB1 receptor. Figure 3 shows that most CB1 receptor was preferentially expressed in glomerular podocytes and that CB1 receptor expression was 1.8-fold greater in diabetic db/db glomeruli than in control non-diabetic mice. Furthermore, CB1 receptor expression increased in cultured podocytes after 48 h of high-glucose stimulus.

Effects of the CB1 receptor antagonist on histological changes of organs of experimental animals

As shown in Supplemental Fig. 2, SR141716 treatment induced phenotypic changes in adipocytes, causing differentiation into small adipocytes, decreased hepatic steatosis, and improved medial hyperplasia in the aorta. In line with the improvement in LVMI, fibrotic change in cardiac tissues was markedly decreased after SR141716 treatment. Supplemental Fig. 3 shows representative renal pathology and immunohistochemical staining for TGFβ1, type IV collagen, and PAI-1. Consistent with marked attenuation of albuminuria, the immunostaining score for profibrotic molecules were markedly improved in the SR141716 treatment group (Supplemental Fig. 4). Although glomerular immunostaining score for CB1 receptor was significantly higher in diabetic db/db glomeruli than in nondiabetic db/m mice (Fig. 3), its expression did not show difference with or without CB1 receptor antagonist treatment in diabetic db/db mice (Supplemental Fig. 4).

Effects of the CB1 receptor antagonist on inflammatory, fibrotic molecules, and renal lipid metabolism in kidney and adipose tissue

We next examined the effect of CB1 receptor inhibition on inflammatory and fibrotic molecule synthesis and renal lipid metabolism in the kidney. SR141716 treatment significantly suppressed the expression of profibrotic or pro-inflammatory genes in kidney and adipose tissue.

![Graphs showing effects of SR141716 on histological changes and lipid metabolism](https://example.com/graphs.png)

**FIG. 2.** Effects of SR141716 on insulin resistance, glucose intolerance, and metabolic parameters in experimental animals. ITT (A), GTT (B), plasma lipid concentrations (C), and HOMA-IR and plasma insulin concentrations (D). ITT and GTT data are expressed as percentage of basal glucose levels caused by high basal glucose levels. T-Chol, Total cholesterol; TG, triglyceride; LDL, low-density lipoprotein; HDL, high-density lipoprotein. Comparisons were performed between the control db/db group and the SR141716 treatment group at the same time points. Data are means ± SEM. A and B, Black circle, db/db; white circle, db/db+SR141716. C and D, Black bars, db/db; white bars, db/db+SR141716. *, P < 0.05; **, P < 0.01; ***, P < 0.001, db/db vs. db/db+SR141716.
inflammatory molecules in the kidney (Supplemental Fig. 5A). Of interest, genes known to be involved in lipotoxicity, such as SREBP-1, showed a significant decrease in the SR141716 treatment group. However, in adipose tissue, the expression of TNFα, visfatin, and PAI-1 were significantly inhibited by SR141716 treatment. In addition, the expression of peroxisome proliferator-activated receptor-γ significantly increased with SR141716 treatment (Supplemental Fig. 5B).

Effects of the CB1 receptor antagonist on lipid peroxidation and oxidative stress in experimental animals

Because SR141716 treatment improved systemic metabolic abnormalities, we examined whether these improvements were derived from lipotoxicity correction. As shown in Fig. 4, cholesterol and triglyceride levels in renal, adipose, and liver tissues were significantly decreased by SR141716 treatment; however, cholesterol in the adipose tissues increased significantly. Because oxidative stress in a diabetic milieu causes peroxidation of lipids and leads to cellular dysfunction, we also evaluated changes in LPO in tissues and 8-isoprostane in plasma and urine. Interestingly, LPO levels in kidney, liver, and adipose tissues were significantly suppressed by SR141716 treatment (Fig. 5A). Furthermore, plasma and urinary 8-isoprostane levels were markedly decreased by SR141716 treatment, consistent with the LPO results (Fig. 5, B and C).

Effects of CB1 receptor inhibition by CB1 receptor antagonist or CB1 receptor knockdown in cultured podocytes

Because the CB1 receptor was preferentially activated in podocytes in the diabetic kidney, we performed in vitro experiments using podocytes. As shown in Fig. 6, A and D, CB1 receptor expression significantly increased after high-glucose stimulation in podocytes but not in mesangial cells. In addition, SR141716 treatment decreased high-glucose-induced collagen and PAI-1 protein synthesis (Fig. 6, B and E). Because SR141716 treatment most profoundly suppressed SREBP-1 expression in the diabetic kidney, we performed siRNA experiments to determine if CB1 receptor knockdown was associated with decreased SREBP-1 synthesis. As shown in Fig. 6, C and F, silencing of the CB1 receptor induced reduction in CB1 receptor expression, and a marked decrease in high-glucose-induced up-regulation of the active cleaved form of SREBP-1 protein (p68) synthesis.

Discussion

Accumulation of excess lipids in nonadipose tissues induces cellular dysfunction, and this phenomenon, known as lipotoxicity, may play an important role in tissue injury pathogenesis (20, 21). Lipid deposition in the kidney is not a rare phenomenon, and previous reports suggested an important role for renal lipid metabolism in diabetic nephropathy pathogenesis (22, 23). In this study, we observed that CB1 receptor antagonist significantly improved plasma lipid abnormalities. This finding agrees with previous reports that CB1 receptor antagonist improved dyslipidemia in mice with genetic or diet-induced obesity (24–26). We also observed that altered morphological changes in the heart, liver, and aorta in diabetes were improved by treatment with a CB1 receptor antagonist. These results were in agreement with previous reports showing that a CB1 receptor antagonist improves obesity-related metabolic alterations in human and experimental animals (14, 27–29).

Many studies suggest the central effect of CB1 receptor antagonist, including loss of appetite and weight loss. In the present study, it may be possible that CB1 receptor antagonist affect food intake via the central receptor as
shown in decrease food intake after 3 months of treatment, leading to improvement in metabolic alterations. However, we did not observe weight loss and decrease in fat mass. These results suggest weight-independent improvements in metabolic alterations, which imply that these effects were caused by inhibition of the CB1 receptor in peripheral tissues.

In terms of renal function, SR141716 markedly decreased urinary albumin excretion and structural changes and oxidative stresses. Because SR141716 improved systemic insulin resistance and dyslipidemia, renoprotective effects of CB1 blockade are responsible, at least in part, for the direct effects of SR141716 as well as improvement of systemic metabolic alterations.

In the present study, we observed that cholesterol content in adipose tissue was significantly increased after CB1 receptor antagonist treatment. However, previous studies show that adipocyte cholesterol content is strongly cor-
related with lipid droplet content in adipocytes (30). In addition, cholesterol level in plasma membranes is important for the function of caveolin, which serves as the intracellular signal for ongoing lipid droplet biogenesis during adipocyte differentiation (31). Furthermore, depletion of cholesterol from plasma membranes disrupts caveolae and attenuate insulin receptor signaling and downstream metabolic effector systems, including glucose transport in rat adipocytes (32). Taken together, these results suggest that increased cholesterol content in adipose tissue may be a reflection of lipid droplet biogenesis during adipocyte maturation.

Increasing evidence indicates that insulin resistance is associated with inflammatory changes in adipose tissue (33, 34). Of interest, CB1 receptor inhibition induces a more insulin-sensitive phenotype in small differentiated adipocytes (35). In line with this phenotypic change, TNFα and PAI-1 gene expression significantly decreased and peroxisome proliferator-activated receptor-γ gene expression significantly increased after CB1 receptor inhibition. Additionally, the plasma level of adiponectin was significantly increased after CB1 receptor inhibition. Taken together, these results suggest that the CB1 receptor antagonist decreased inflammation and oxidative stress in adipose tissue, leading to improved insulin resistance.

The most important finding in this study is that a CB1 receptor antagonist mitigated the urinary excretion of albumin and mesangial expansion in renal tissues, accompanied by suppression of the synthesis of profibrotic and proinflammatory molecules. Furthermore, we observed that the CB1 receptor was overexpressed in podocytes in the glomeruli of db/db mice compared with control db/m mice. This result is in agreement with a previous report in streptozotocin-induced type 1 diabetic mice (15). We also found that CB1 receptor expression was markedly upregulated in response to high-glucose stimulation in podocytes but not in mesangial cells. In addition, we observed that CB1 receptor antagonist abolished this high-glucose-mediated fibrotic protein production. These results imply that the CB1 receptor is present and overexpressed in diabetic podocytes and suggest a direct effect of the CB1 receptor antagonist in the kidney.

**FIG. 6.** Synthesis of CB1 receptor in response to high-glucose stimulation, effect of SR141716 on collagen and PAI-1 synthesis, and effect of stealth siRNA for CB1 receptor on CB1 receptor and SREBP-1c expression in cultured podocytes. A, Representative Western blotting for CB1 receptor under high-glucose conditions in MC and podocytes. B, Representative Western blotting for type IV collagen and PAI-1 in response to high glucose and SR141716 in podocytes. C, Representative Western blotting for CB1 receptor and SREBP-1c in cultured podocytes with or without transfection with siRNA for CB1 receptor under high-glucose stimulation. D, Densitometric analysis of CB1 receptor proteins in MC and podocytes. E, Densitometric analysis of type IV collagen and PAI-1 proteins in podocytes. F, Densitometric analysis of CB1 receptor and SREBP-1c proteins in podocytes; 100 nm SR141716 was added to the cells 1 h before treatment with high glucose. Densitometric data are shown as means ± SEM of three independent experiments with triplicate dishes. NG, Normal glucose (5 mM D-glucose); HG, high glucose (30 mM of D-glucose); MC, mesangial cell. *, P < 0.05; **, P < 0.01; ***, P < 0.001 NG vs. HG or NG+siRNA; ##, P < 0.01; ###, P < 0.001 HG vs. HG+SR141716 or HG+siRNA.
Of interest, SR141716 suppressed the expression of mRNA levels of genes involved in cholesterol synthesis, such as SREBP-1. In addition, we observed that SREBP-1 expression was up-regulated under high-glucose conditions in podocytes. Furthermore, silencing the CB1 receptor suppressed SREBP-1 expression in podocytes. SREBP-1 is a transcription factor regulating the synthesis of fatty acid and triglyceride (36). Although its function in lipid accumulation in the diabetic kidney is still not completely clear, the potential involvement of SREBP-1 in diabetic kidney diseases has been described in transgenic mice overexpressing SREBP-1c, and in both type 1 and 2 diabetic mice (23, 37, 38). Taken together, these results suggest that inhibition of CB1 receptors partially improves renal function by improving renal lipid metabolic abnormalities.

In the present study, the CB1 receptor antagonist induced phenotypic changes of adipocytes into small differentiated forms. Although it is generally considered that small adipocyte is an insulin sensitive phenotype, recent studies show that insulin resistance in obesity does not depend primarily on increased numbers of large adipocytes (39, 40). Rather, it is the consequence of the failure of a remodeling of subset of very small adipocytes into fully differentiated adipocytes (39, 40). So the beneficial effects of CB1 receptor antagonist in this study may be derived from improvement of systemic lipid parameters as well as improvement of inflammation and oxidative stress in adipose tissue.

In conclusion, the CB1 receptor antagonist SR141716 improved insulin resistance and protected against renal injury via several mechanisms, including improvement in renal lipid metabolism, an antifibrotic effect and an antioxidative effect in type 2 diabetic nephropathy. These findings suggest that antagonism of CB1 receptors may be a potential therapeutic approach in the treatment of type 2 diabetes mellitus and diabetic nephropathy.

Acknowledgments

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References