Spironolactone Improves Glucose and Lipid Metabolism by Ameliorating Hepatic Steatosis and Inflammation and Suppressing Enhanced Gluconeogenesis Induced by High-Fat and High-Fructose Diet

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Recent evidence suggests that treatment with mineralocorticoid receptor antagonist suppressed local inflammation in vascular tissues or cardiomyocytes; therefore, we examined the effect of spironolactone on glucose and lipid metabolism in a mouse model with diet-induced diabetes and nonalcoholic fatty liver disease. C57BL/6 mice were fed either the control diet, 60% fat diet with 30% fructose water (HFFD), or HFFD with spironolactone for 8 wk. HFFD mice demonstrated apparent phenotypes of metabolic syndrome, including insulin resistance, hypertension, dyslipidemia, and fatty liver. Although treatment with spironolactone did not affect the increased calorie intake and body weight by HFFD, the increments of epididymal fat weight, blood pressure, serum triglyceride, free fatty acids, leptin, and total cholesterol levels were significantly suppressed. Elevation of blood glucose during glucose and insulin tolerance tests in HFFD mice was significantly lowered by spironolactone. Notably, increased glucose levels during pyruvate tolerance test in HFFD mice were almost completely ameliorated to control levels by the treatment. Staining with hematoxylin-eosin (HE) and Oil-red-O demonstrated marked accumulation of triglycerides in the centrilobular part of the hepatic lobule in HFFD mice, and these accumulations were effectively improved by spironolactone. Concomitantly HFFD feeding markedly up-regulated hepatic mRNA expression of proinflammatory cytokines (TNFα, IL-6, and monocyte chemoattractant protein-1), gluconeogenic gene phosphoenolpyruvate carboxykinase, transcription factor carbohydrate response element binding protein, and its downstream lipogenic enzymes, all of which were significantly suppressed by spironolactone. These results indicate that inhibition of mineralocorticoid receptor might be a beneficial therapeutic approach for diet-induced phenotypes of metabolic syndrome and fatty liver. **(*Endocrinology* 151: 2040–2049, 2010)**

Insulin plays a crucial role in the regulation of whole-body glucose and lipid metabolism (1). The major pathogenesis of metabolic syndrome is the development of insulin resistance caused by the accumulation of visceral fat, which promotes the elevation of blood pressure, dyslipidemia, and dysregulation of glucose metabolism (2). Among the target tissues of insulin, the liver is the principal regulator of glucose and lipid metabolism by controlling hepatic glucose production, glycogen storage, and lipogenesis (2). In metabolic syndrome with insulin resistance,

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Abbreviations: ACC, Acetyl-CoA carboxylase; AUC, area under the curve; ChREBP, carbohydrate response element binding protein; FAS, fatty acid synthase; FFA, free fatty acid; HE, hematoxylin-eosin; HFFD, high-fat diet with high-fructose water; HOMA, homeostasis model assessment; IRS, insulin receptor substrate; MCP, monocyte chemoattractant protein; MR, mineralocorticoid receptor; NAFLD, nonalcoholic fatty liver disease; PGC, peroxisome proliferator-activated receptor-γ coactivator; RAAS, renin-angiotensin-aldosterone system; ROS, reactive oxygen species; siRNA, small interfering RNA; SP, spironolactone; SREBP, sterol response element binding protein.

the liver is profoundly affected by excess dietary nutrients from the intestines and inflammatory adipocytokines from enlarged visceral adipose tissues (3, 4). Thus, fatty liver is considered as a representative characteristic of metabolic syndrome (3–5). Nonalcoholic fatty liver disease (NAFLD) includes a broad spectrum of manifestations of fatty liver, ranging from steatosis alone, steatosis with inflammation, steatosis with hepatocyte injury, or steatosis with sinusoidal fibrosis in relation to the progress of the pathological state. Epidemiological studies screened by ^{1}H-nuclear magnetic resonance spectroscopy have shown that about 30% of the adult population in North America have NAFLD (6).

The renin-angiotensin-aldosterone system (RAAS) was originally recognized as a coordinated hormone cascade maintaining salt-water balance and blood pressure (7). In addition to the crucial role of angiotensin 2 in the pathogenesis of cardiovascular and renal organ damage, recent studies suggest that RAAS is implicated in the development of insulin resistance in metabolic syndrome (7). Elevation of angiotensin 2 has been shown to induce insulin resistance via reactive oxygen species (ROS) production in various tissues, including vascular smooth muscle and skeletal muscle in metabolic syndrome (7, 8). Furthermore, either angiotensin 2 type 1 receptor antagonist or genetic knockout of angiotensin 2 type 1 receptor is known to effectively attenuate lipid accumulation in the liver (9). Importantly, increasing evidences demonstrated that aldosterone *per se* also promotes inflammation and ROS production in vessels (10, 11), kidney (12, 13), and heart (14), independently of angiotensin 2. However, the *in vivo* impact of mineralocorticoid receptor (MR) inhibition on impaired glucose and lipid metabolism in metabolic syndrome is largely unknown. Thus, we hypothesized that administration of MR antagonist might ameliorate hepatic insulin resistance, hepatic steatosis, and systemic glucose and lipid metabolism by suppressing hepatic inflammation and ROS production induced by excess ingestion of the dietary nutrient.

Because increased fat intake and soft drink consumption are closely associated with a higher prevalence of metabolic syndrome and NAFLD (5, 15, 16), we prepared mice fed a 60% high-fat diet with 30% fructose water (HFFD) for 8 wk. These mice showed apparent phenotypes of metabolic syndrome, including elevation of blood pressure, dyslipidemia, impaired glucose tolerance, and NAFLD. In the present study, we examined the effect of spironolactone (SP) administration on glucose metabolism using the glucose tolerance test, insulin tolerance test, and pyruvate tolerance test in HFFD mice. In addition, the impact on hepatic steatosis was investigated by histological analysis. Furthermore, the expression levels of lipogenic enzymes and inflammatory cytokines in the liver were compared between HFFD and HFFD with SP mice.

Materials and Methods

Materials

Human regular insulin (Humulin R) was provided by Eli Lilly (Indianapolis, IN). Controlled-release tablets of spironolactone were purchased from Innovative Research of America (Sarasota, FL). A polyclonal anti-insulin receptor substrate (IRS)-1 antibody, a polyclonal anti-IRS2 antibody, and a polyclonal anti-Akt2 antibody were from Upstate Biotechnology (Lake Placid, NY). A polyclonal anti-Ser^{473} phospho-specific Akt antibody was obtained from Cell Signaling Technology Inc. (Beverly, MA). All other reagents were of analytical grade and purchased from Sigma-Aldrich Corp. (St. Louis, MO) or Wako Pure Chemical Industries (Osaka, Japan).

Animal procedures

Male C57BL6/J mice were maintained under standard light (12 h light, 12 h dark cycle), temperature (24 ± 1 C), and humidity (55 ± 10%) conditions and provided with food and water *ad libitum*. For the control group, mice were fed a normal chow diet composed of 4.8% fat (CE-12; Clea Japan Inc., Tokyo, Japan) and water. For the HFFD group, mice were fed chow with 60% fat (D12492; Research Diets Inc., New Brunswick, NJ) and 30% fructose water for 8 wk after 8 wk of normal diet feeding. For spironolactone treatment, a controlled-release tablet of spironolactone (400 μg/d; 16 mg/kg daily) was sc implanted in the posterior cervical portion, as described previously (17, 18), and then mice were fed HFFD. The sham operation was similarly conducted in both control and HFFD mice. Blood pressure and heart rate were measured at the mouse tail by noninvasive sphygmomanometer (MK-2000ST; Muromachi Kikai Co. Ltd., Tokyo, Japan). All experimental procedures used in this study were approved by the Committee of Animal Experiments at University of Toyama. In some of the studies, different numbers of mice were analyzed because part of the group was used for histological analysis.

Measurements of serum parameters

Mice were deprived of food overnight, and blood samples were collected from the orbital sinus under anesthesia. After centrifugation at 15,000 × *g* for 1 min, the supernatants of the blood samples were separated and subjected to measurements of serum insulin and leptin levels using the ELISA kit (Morinaga, Kanagawa, Japan). Serum glucose levels were measured with a FreeStyle Kissei (Kissei Pharmaceutical Co. Ltd., Tokyo, Japan). Serum levels of alanine aminotransferase, cholesterol, triglyceride, and free fatty acid (FFA) were determined by colorimetric kit (Wako, Tokyo, Japan). To estimate insulin resistance in mice, the homeostasis model assessment (HOMA) index was calculated by the formula: fasting plasma insulin (microinternational units per milliliter) × fasting plasma glucose (millimoles per liter)/22.5.

Glucose, insulin, and pyruvate tolerance tests

For the glucose tolerance test and pyruvate tolerance test, mice fasted for 16 h were injected ip with glucose (2 g/kg body

weight) and pyruvate (2 g/kg body weight), respectively. For the insulin tolerance test, human regular insulin (0.75 U/kg body weight) was ip injected into random-fed mice. Blood samples were collected from the tail vein 0, 15, 30, 60, and 120 min after injection.

Histological analysis

Mice were anesthetized with pentobarbital, and the liver was perfused with ice-cold PBS and embedded in paraffin, and sections of 4 μm thickness were cut. Each section was routinely stained with hematoxylin and eosin. Frozen sections of formalin-fixed liver were stained with Oil-red O using standard techniques.

RT-PCR

Total RNA was extracted from the liver of mice by TRIsure (Nippon Genetics, Tokyo, Japan). cDNA synthesis was performed by SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) using 1 μg total RNA as previously described (19, 20). Quantitative real-time PCR was performed with SYBR Premix Ex Taq (Takara Bio, Shiga, Japan), using the Mx3000p real-time PCR system according to the manufacturer's instructions (Stratagene, La Jolla, CA). The primer pairs used are listed in Table 1. The primers for MR were prepared by a QuantiTect Primer assay system (QIAGEN, Tokyo, Japan). The PCR conditions used were as follows: initial denaturation [95 C for 10 sec, followed by 40 cycles of 95 C for 10 sec (denaturation), 62 C for 20 sec (annealing), and 72 C for 15 sec (extension)]. The relative expression levels of objective mRNAs were calculated as a ratio to those of 18S rRNA.

Cell culture and small interfering RNA (siRNA)-mediated knockdown of MR

Primary hepatocytes were isolated from the liver of male Sprague Dawley rats (8 wk old) with the use of collagenase. The isolated hepatocytes were seeded at a density of 2.6×10^6 cells per 60-mm dish (coated with rat collagen I) in medium A [medium 199 containing Earle's salt, penicillin (100 U/ml) and streptomycin (100 μg/ml)] supplemented with 10% fetal calf serum, 100 nM dexamethasone, 1 nM insulin, and 100 nM triiodothyronine as described previously (21). HepG2 cells were grown and passed in DMEM (Life Technologies, Inc., Carlsbad, CA) supplemented with 10% fetal calf serum. For MR knockdown, siRNA for MR and scrambled negative control (Invitrogen stealth RNAi system; Life Technologies) were incorporated into HepG2 cells by using Lipofectamine RNAiMax transfection reagent (Life Technologies) as described in the manufacture's protocol.

Western blot analysis

Western blotting was performed as described previously (19, 21, 22). In brief, primary hepatocytes or HepG2 cells were serum deprived and treated with various concentrations of aldosterone for 16 h. Then the cells were stimulated with 17 nM insulin for 5 min. Spironolactone was added 1 h before the treatment with aldosterone. The cell lysates were separated by SDS-PAGE, transferred onto membranes, and immunoblotted. Densitometric analysis was conducted directly from the blotted membrane by using LAS-4000 lumino-image analyzer system (Fujifilm, Tokyo, Japan).

Statistical analysis

Data are expressed as the means ± SE. P values were determined by one-way ANOVA with Bonferroni's correction, and $P < 0.05$ was considered significant.

Results

Characteristics of control, HFFD, and HFFD + SP mice

Male C57BL6/J mice grown with standard diet were separated into three groups at 8 wk old: mice fed standard diet and water (control), high-fat diet and 30% fructose

TABLE 1. Primers for real-time PCR

Genes	Forward primer	Reverse primer
PGC-1α	GCCCGGTACAGTGAGTGTTC	CTGGGCCGTTTAGTCTTCCT
PGC-1β	GCTCTGACGCTCTGAAGGAC	CACCGAAGTGAGGTGCTTATG
ChREBP	GATGGTGCGAACAGCTCTTCT	CTGGGCTGTGTCATGGTGAA
SREBP1c	GATGTGCGAACTGGACACAG	CATAGGGGGCGTCAAACAG
FAS	ATCCTGGAACGAGAACACGATCT	AGAGACGTGTCACTCCTGGACTT
ACC	GGGCACAGACCGTGGTAGTT	CAGGATCAGCTGGGATACTGAGT
SCD1	TGGGTTGGCTGCTTGTG	GCGTGGGCAGGATGAAG
PPARγ	TCGCTGATGCACTGCCTATG	TGTCAAAGGAATGCGAGTGGTC
PPARα	CGGGAAAGACCAGCAACAAC	TGGCAGCAGTGGAAGAATCG
CPT1a	CAAAGATCAATCGGACCCTAGAC	CGCCACTCACGATGTTCTTC
TNFα	AAGCCTGTAGCCCACGTCGTA	GGCACCACTAGTTGGTTGTCTTTG
IL-6	ATGGATGCTACCAAACTGGAT	TGAAGGACTCTGGCTTTGTCT
MCP1	CCACTCACCTGCTGCTACTCAT	TGGTGATCCTCTTGTAGCTCTCC
IRS1	CTCTACACCCGAGACGAACAC	TGGGCCTTTGCCCGATTATG
IRS2	GGAGAACCCAGACCCTAAGCTACT	GATGCCTTTGAGGCCTTCAC
PEPCK	CAGGATCGAAAGCAAGACAGT	AAGTCCTCTTCCGACATCCAG
G6Pase	GAAAAAGCCAACGTATGGATTCC	CAGCAAGGTAGATCCGGGA
Cyp11β2	TGGCTGAAGATGATACAGATCCT	CACTGTGCCTGAAAATGGGC
18S rRNA	AGTTCCAFCACATTTTGCGAG	TCATCCTCCGTGAGTTCTCCA

TABLE 2. Characteristics of mice after 8-wk experimental period

	Control	HFFD	HFFD + SP
Weight gain from 8 to 16 wk (g)	4.0 ± 0.5 (10)	9.2 ± 0.7 (20)[a]	8.7 ± 0.5 (20)[a]
Body weight (g)	28.4 ± 0.5 (10)	34.0 ± 0.9 (20)[a]	32.0 ± 0.9 (20)[a]
Food intake (g/d)	2.4 ± 0.3 (8)	1.5 ± 0.2 (11)[a]	1.4 ± 0.2 (9)[a]
Water intake (ml/d)	4.1 ± 0.2 (8)	3.3 ± 0.2 (11)[a]	3.3 ± 0.2 (9)[a]
Total calorie intake (kcal/d)	8.2 ± 1.0 (8)	11.7 ± 1.2 (11)[a]	11.2 ± 1.1 (9)[a]
Liver weight (g)	1.26 ± 0.09 (10)	1.37 ± 0.09 (10)	1.24 ± 0.10 (10)
Epididymal fat weight (g)	0.25 ± 0.02 (6)	1.49 ± 0.08 (6)[a]	1.08 ± 0.06 (6)[a,b]

Values are the means ± SE. Numbers of mice are indicated in *parentheses*.

[a] $P < 0.01$ compared with control.

[b] $P < 0.05$.

water (HFFD), and high-fat diet and 30% fructose water receiving spironolactone (400 μg/d) for 8 wk (HFFD + SP). The characteristics of mice at the age of 16 wk are shown in Tables 2 and 3. Body weight, body weight gain during the 8-wk experimental period, and daily caloric intake were significantly higher in HFFD and HFFD + SP mice than in the control group. Liver weight was not significantly changed among these three groups. In contrast, epididymal fat weight was significantly increased in HFFD mice and was significantly reduced by treatment with spironolactone. Systolic blood pressure was significantly increased at 8 wk in HFFD mice, and spironolactone effectively suppressed systolic blood pressure at 4 and 8 wk. Diastolic blood pressure and heart rate were unchanged among the three groups. Thus, HFFD augmented body weight and epididymal fat weight gain with elevation of systolic blood pressure, and spironolactone effectively suppressed epididymal fat gain and lowered systolic blood pressure induced by HFFD.

Serum analysis of control, HFFD, and HFFD + SP mice

Serum biochemical analysis under fasted conditions was conducted after 8 wk of the experimental period (Fig. 1). In accordance with body weight gain, serum fasting glucose, insulin levels, and estimated HOMA index were significantly elevated in HFFD mice, and these were ameliorated in HFFD + SP mice. The estimated HOMA index of control, HFFD, and HFFD + SP mice was 2.08 ± 0.31, 7.67 ± 0.91, and 4.91 ± 1.21, respectively. Concomitantly, triglyceride, total cholesterol, FFA, and serum leptin levels were significantly elevated in HFFD mice, and spironolactone effectively ameliorated these metabolic abnormalities. The serum level of alanine aminotransferase was not apparently changed among the three groups (data not shown).

HFFD-induced glucose intolerance and insulin resistance are effectively ameliorated by spironolactone

Because HFFD mice demonstrated apparent phenotypes of metabolic syndrome, we next examined glucose metabolism and insulin sensitivity by glucose and insulin tolerance tests. HFFD mice showed a significantly higher glucose concentration at any time after ip glucose injection (Fig. 2A). The glucose area under the curve (AUC) in glucose tolerance test increased by 74.2% in HFFD mice compared with control mice. In the insulin tolerance test, blood glucose concentration at 15, 60, and 120 min was significantly higher in HFFD mice (Fig. 2B). The glucose

TABLE 3. Blood pressure (BP, mm Hg) and heart rate (HR, beats/min) of mice at 0, 4, and 8 wk of the experimental period

	Control	HFFD	HFFD + SP
BP at 0 wk	97.2 ± 2.1/67.4 ± 6.4	95.4 ± 2.1/50.0 ± 4.5	100.4 ± 2.3/54.6 ± 4.4
BP at 4 wk	99.6 ± 2.2/57.2 ± 2.4	98.5 ± 2.6/46.3 ± 3.2	81.7 ± 4.3/46.1 ± 2.1[b]
BP at 8 wk	93.1 ± 3.3/54.5 ± 3.7	105.9 ± 2.4/46.7 ± 3.4[a]	91.3 ± 2.4/41.3 ± 3.5[b]
HR at 0 wk	575.3 ± 27.7	598.4 ± 25.1	545.4 ± 29.0
HR at 4 wk	665.8 ± 21.5	710.0 ± 11.7	698.3 ± 12.3
HR at 8 wk	673.0 ± 18.7	632.2 ± 33.2	643.2 ± 24.4

Values are the means ± SE. Control, n = 10; HFFD, n = 10; HFFD + SP, n = 10. Mice were fed for 8 wk on normal chow (control), HFFD, or HFFD + SP.

[a] $P < 0.01$ compared with control.

[b] $P < 0.01$ compared with HFFD.

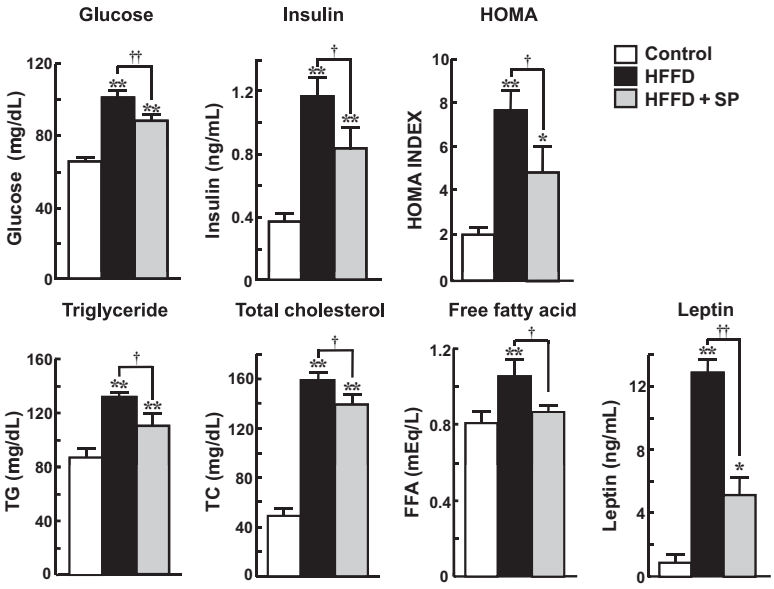

FIG. 1. Serum measurements of fasting glucose, insulin, HOMA, triglycerides, total cholesterol, FFA, and leptin levels in the mice after an 8-wk experimental period. Mice were fed normal chow (control), HFFD, or HFFD + SP for 8 wk. After overnight fasting, blood samples were analyzed in mice. *, $P < 0.05$, **, $P < 0.01$ compared with control; †, $P < 0.05$, ††, $P < 0.01$ compared with HFFD. Ten animals were used in each condition.

AUC in insulin tolerance test increased by 23.4% in HFFD mice compared with control mice. In contrast, the elevation of blood glucose in the glucose tolerance test and insulin tolerance test was effectively improved by coadministration of spironolactone with HFFD. The glucose AUC in the glucose tolerance test and insulin tolerance test decreased by 17.9 and 33.7% in HFFD + SP mice compared with HFFD mice, respectively. Elevation of fasting blood glucose and insulin levels was also observed at 4 wk of HFFD feeding. Impaired glucose tolerance and insulin resistance caused by the HFFD feeding were effectively ameliorated by treatment with spironolactone. Additional experiments were performed to examine the effect of spironolactone in control mice fed normal chow diet. Treatment with spironolactone did not affect glucose tolerance and insulin sensitivity in the mice (Supplemental Fig. 1 published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org). These results indicate that HFFD feeding is an optimal model of diet-induced glucose intolerance and insulin resistance, and administration of spironolactone successfully ameliorates the impaired glucose homeostasis.

To further investigate the degree of hepatic insulin resistance in HFFD mice, we used the pyruvate tolerance test to investigate abnormal hepatic glucose production (Fig. 2C). HFFD mice showed a significantly higher concentration of glucose at any time points. The glucose AUC in pyruvate tolerance test increased by 47% in HFFD mice compared with control mice. Importantly, the elevation of the glucose level was significantly lowered in HFFD + SP mice and was almost identical with the level in control mice (glucose AUC in pyruvate tolerance test in HFFD + SP mice was 102% of control mice), indicating that HFFD-induced hepatic insulin resistance was effectively ameliorated by spironolactone administration.

Spironolactone prevents the development of HFFD-induced hepatic steatosis

Because the elevation of hepatic glucose production during pyruvate tolerance test was effectively ameliorated in HFFD + SP mice, we next performed histological analysis of the liver in these mice (Fig. 3). In HE staining, marked vacuolar degeneration was seen in HFFD mice, indicating fat accumulation in the liver. These vacuolar changes tended to be located in the centrilobular part of the hepatic lobule. This vacuolar degeneration was apparently reduced in HFFD + SP mice. To further investigate the existence of fatty liver in HFFD mice, we prepared frozen sections of liver and stained with Oil-red O (Fig. 3B).

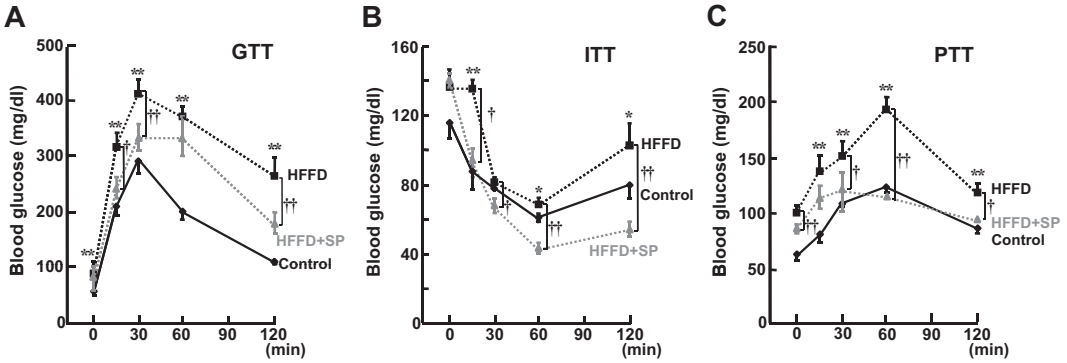

FIG. 2. Glucose, insulin, and pyruvate tolerance tests in mice after 8 wk exposure to HFFD and HFFD + SP. Mice were fed normal chow (control), HFFD, or HFFD + SP for 8 wk. Glucose tolerance test (A, GTT), insulin tolerance test (B, ITT), and pyruvate tolerance test (C, PTT) were studied. Values are the means ± SE (n = 5–7). *, $P < 0.05$, **, $P < 0.01$ compared with control; †, $P < 0.05$, ††, $P < 0.01$ compared with HFFD.

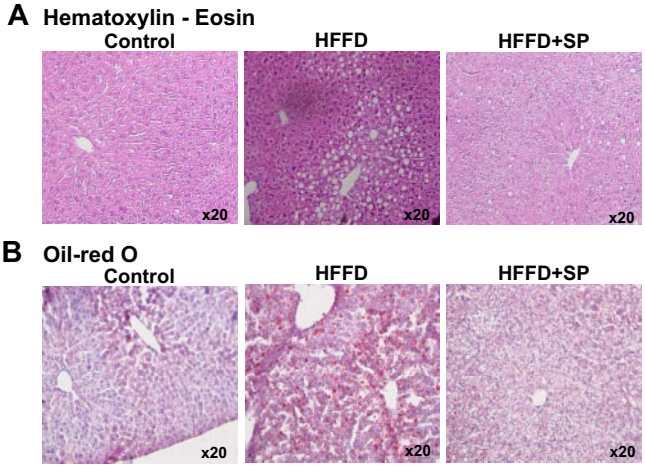

FIG. 3. Histological examination of liver in control, HFFD, and HFFD + SP mice. Mice were fed normal chow (control), HFFD, or HFFD + SP for 8 wk. Liver of each mouse was perfused with PBS, embedded, and cut as described in *Materials and Methods* (n = 8). Each section was stained with HE (A) or Oil-red O (B). Representative photomicrographs of liver sections in control, HFFD, and HFFD + SP mice are shown.

Lipid droplets were obvious in HFFD mice, and the number of lipid droplets was clearly decreased in HFFD + SP mice.

Spironolactone effectively suppresses HFFD-induced up-regulation of lipogenic and inflammatory genes in the liver

Because spironolactone effectively improved serum dyslipidemia, glucose intolerance, insulin resistance, as well as histological changes in fat accumulation in the liver, we further examined the expression of genes that control glucose and lipid metabolism in the liver by real-time PCR. Figure 4A shows the relative mRNA expression of lipogenic transcriptional factors and the downstream lipogenic enzymes. Hepatic mRNA expressions of both peroxisome proliferator-activated receptor-γ coactivator (PGC)1α and PGC1β were significantly elevated by 70.6 and 28.8% in HFFD mice, which were apparently decreased to 21.2 and 47.4%, respectively, in HFFD + SP mice compared with HFFD mice (Fig. 4A). Interestingly, treatment with spironolactone also reduced the mRNA expressions of PGC1α and PGC1β to levels significantly below controls in mice fed normal chow diet (Supplemental Fig. 2).

Among crucial transcriptional factors for lipogenesis, mRNA expression of carbohydrate response element binding protein (ChREBP) was increased by 139.4% in HFFD mice and significantly decreased to 48.0% of HFFD values in HFFD + SP mice. Along this line, mRNA expressions of downstream lipogenic enzymes of acetyl-CoA carboxylase (ACC) and stearoyl-CoA desaturase were significantly up-regulated by 152.1 and 340.8% in HFFD mice. Expression of fatty acid synthase (FAS) also tended to increase in HFFD mice. In contrast, the mRNA expression levels of these lipogenic enzymes were significantly suppressed by spironolactone administration to 80.0, 29.5, and 35.5%, respectively, compared with HFFD mice. Interestingly, the expression of sterol response element binding protein 1c (SREPB1c), another master regulator of *de novo* lipogenesis, was not apparently affected by HFFD or HFFD + SP. Although the expression of peroxisome proliferator-activated receptor-α was significantly reduced in HFFD + SP mice, the expression of carnitine palmitoyltransferase-1a was not apparently altered in any condition (Fig. 4B).

Concerning proinflammatory cytokines, hepatic mRNA expression of TNFα was significantly elevated by 78.8% in HFFD mice (Fig. 4C). There was also a trend toward an increased expression of IL-6 in HFFD mice. In addition, remarkable up-regulation by 459.3% of monocyte chemoattractant protein (MCP)-1 was observed in HFFD mice. Notably, these up-regulations of proinflammatory cytokines were markedly suppressed by coadministration of spironolactone. Thus, spironolactone decreased the expres-

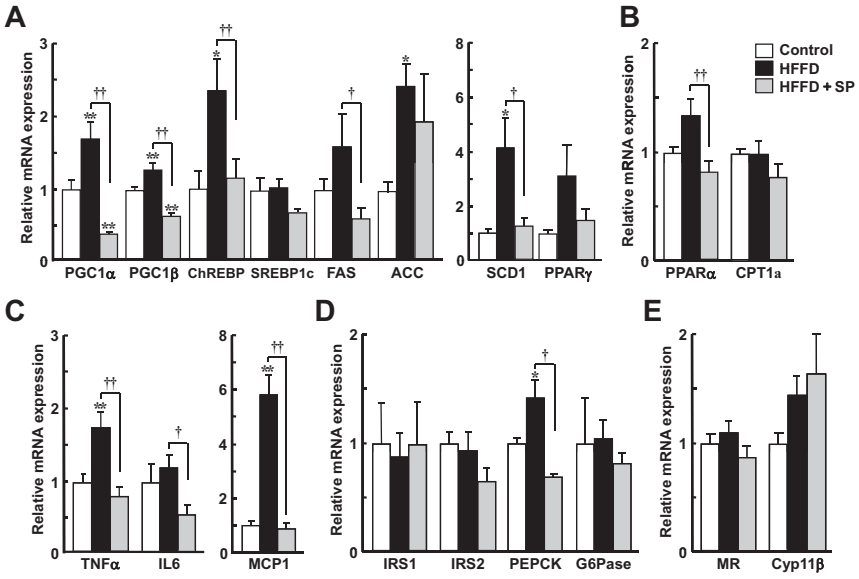

FIG. 4. Effect of HFFD and HFFD + SP on the gene expression in the liver. Mice were fed normal chow (control), HFFD, or HFFD + SP for 8 wk. Relative expression levels of transcription factors related to lipogenesis and its target enzymes (A), genes related to β-oxidation (B), proinflammation genes (C), genes controlling glucose metabolism (D), and aldosterone-related genes (E) in the liver were analyzed by SYBR-green real-time PCR as described in *Materials and Methods*. Values are the means ± SE. Number of animals: PGC1α, PGC1β, SREBP1c, FAS, ACC, and MCP1, n = 6; MR and Cyp11β2, control, n = 7; HFFD, n = 11; HFFD + SP, n = 6; other genes, n = 12. *, $P < 0.05$, **, $P < 0.01$ compared with control; †, $P < 0.05$, ††, $P < 0.01$ compared with HFFD.

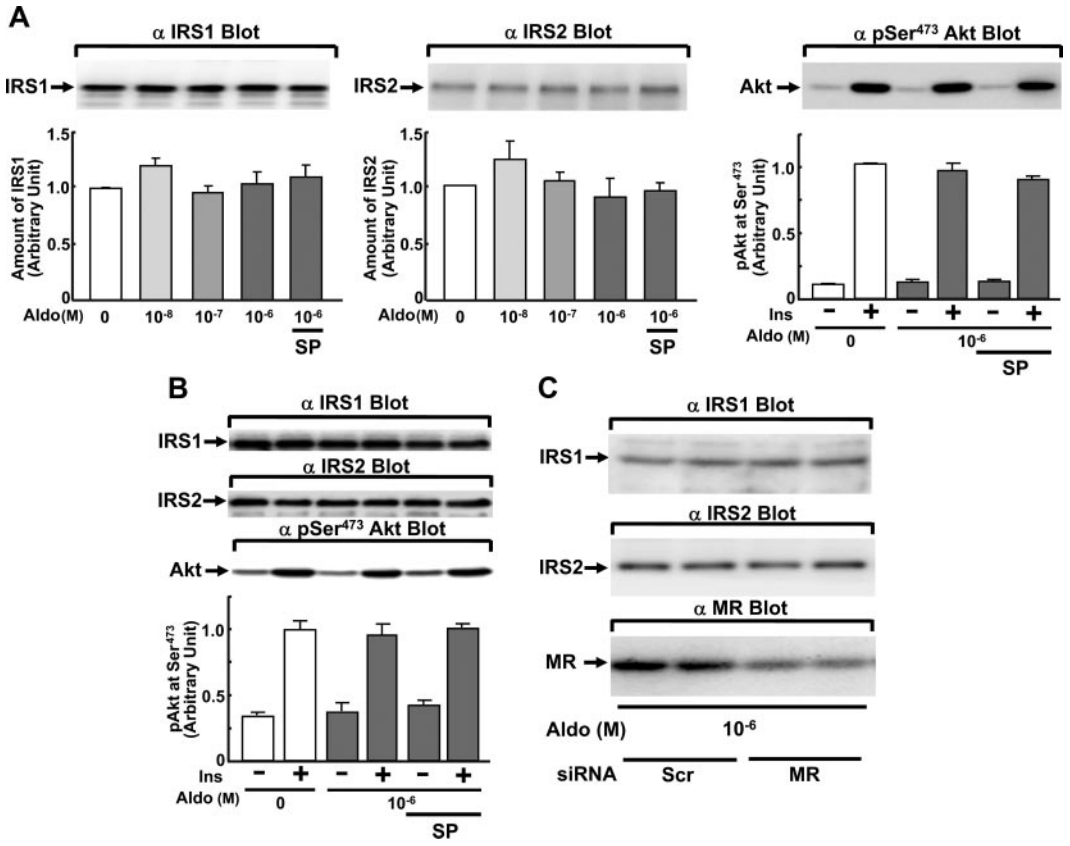

FIG. 5. Effect of inhibition and knockdown of MR on the metabolic signaling of insulin in rat primary hepatocyte and HepG2 cells. Rat primary hepatocytes (A) and HepG2 cells (B and C) were serum starved and treated with aldosterone at 10^{-8} to 10^{-6} M for 16 h. Spironolactone was administered 1 h before aldosterone treatment. siRNA for MR or scrambled control siRNA was transfected to HepG2 cells 24 h before serum starvation by using Lipofectamine RNAiMax transfection reagent (Life Technologies). Cells were treated without or with 17 nM insulin for 5 min, and the total cell lysates were subjected to Western blotting. Transfection of the scrambled siRNA did not affect the expression of MR compared with control (data not shown). Results are the means ± SE of four separate experiments.

sions of TNFα, IL-6, and MCP1 to 44.4, 44.3, and 15.4%, respectively, compared with HFFD mice. Although the expression levels of TNFα and IL-6 appeared to be lower in HFFD + SP mice compared with control mice, these changes were not statistically significant.

Insulin is the principal regulator of glucose and lipid metabolism by allosterically controlling the expressions of various metabolic enzymes involved in gluconeogenesis. Because HFFD mice showed insulin resistance and elevation of blood glucose during the pyruvate tolerance test, we examined mRNA expressions of genes involved in insulin signaling and gluconeogenic enzymes (Fig. 4D). Neither IRS1 nor IRS2 expression was affected by HFFD feeding, at least in the fasting condition. In contrast, the expression of phosphoenolpyruvate carboxykinase was increased by 40.7% in HFFD mice and effectively suppressed to 49.1% in HFFD + SP mice compared with HFFD mice. The expression of glucose-6-phosphatase was not altered in either HFFD or HFFD + SP mice. On the other hand, the mRNA expression of MR was not changed and that of Cyp11β2 (aldosterone synthase) tended to increase in HFFD and HFFD + SP mice (Fig. 4E).

Inhibition and knockdown of MR do not affect metabolic signaling of insulin in primary hepatocytes and HepG2 cells

Because spironolactone effectively ameliorated systemic glucose and lipid metabolism induced by HFFD feeding, we examined the direct effect of aldosterone, a ligand of MR, on the metabolic signaling of insulin in the hepatocytes. In contrast to the *in vivo* effect, treatment with aldosterone at 10^{-8} to 10^{-6} M for 16 h did not affect the expression of IRS1 and IRS2 in primary hepatocytes (Fig. 5A). In addition, insulin-induced phosphorylation of Akt was not affected by treatment with aldosterone or aldosterone plus spironolactone. Similarly, aldosterone did not affect the expression of IRS1 and IRS2 and insulin-induced phosphorylation of Akt in HepG2 cells (Fig. 5B). Moreover, pretreatment with spironolactone (Fig. 5B) or knockdown of MR (Fig. 5C) in combination with aldosterone treatment did not affect the expression of IRS proteins in HepG2 cells.

Discussion

NAFLD is the most representative characteristic of the liver in metabolic syndrome (3–6). Excess calorie intake with increased lipid ingestion is the central mechanism of the increasing prevalence of metabolic syndrome with NAFLD (4–6). Increasing consumption of soft drinks with fructose has also been shown to be strongly associated with a high prevalence of NAFLD (15, 16). Because fructose is a lipogenic component, its consumption promotes the development of an atherogenic lipid profile, elevation of postprandial hypertriglycemia, and elevation of apolipoprotein B concentration, compared with glucose consumption (3). To reflect modern dietary conditions, we prepared C57BL/6 mice fed a 60% high-fat diet with 30% high-fructose water for 8 wk. HFFD mice showed elevation of systolic blood pressure, dyslipidemia, and impaired glucose tolerance with hepatic insulin resistance. Histological examination revealed the marked accumulation of lipid droplets in the liver, indicating that mice fed HFFD exhibited typical phenotypes of diet-induced metabolic syndrome with NAFLD. Furthermore, we found that treatment with spironolactone effectively ameliorated impaired glucose and lipid metabolism, reduced epididymal fat weight, and improved histological changes of fatty liver in HFFD mice. Thus, an MR antagonist might be a beneficial novel option for the treatment of patients with diet-induced metabolic syndrome and/or NAFLD, in addition to its wide usage as an antihypertensive agent and diuretic.

Apparent elevation of systolic blood pressure was observed in mice fed HFFD for 8 wk (Table 3), similarly to the observation under hyperinsulinemic or hyperleptinemic conditions in mice (23). We speculate that elevation of the peripheral vascular tonus may be due to obesity-induced activation of the sympathetic nervous system. Alternatively, the facilitation of RAAS in response to body weight gain might be the determining factor because body weight reduction has been shown to reduce RAAS in plasma and adipose tissue, which in turn decreases systolic blood pressures in women (24). Because serum aldosterone levels were not significantly altered between control and HFFD mice (data not shown), systemic elevation of the aldosterone level does not appear to be involved in the phenotype of metabolic syndrome caused by HFFD.

HFFD mice demonstrated apparent systemic insulin resistance, as shown in glucose and insulin tolerance tests (Fig. 2, A and B). In addition, the pyruvate tolerance test revealed that hepatic glucose production was excessive in HFFD mice compared with mice fed the normal chow diet (Fig. 2C). Therefore, insulin sensitivity appears to be impaired in HFFD mice, mainly due to hepatic insulin resistance. Interestingly, elevation of glucose production in the pyruvate tolerance test was almost completely normalized by spironolactone. Consistently, the enhanced expression of phosphoenolpyruvate carboxykinase, a hepatic gene for gluconeogenesis, in HFFD mice was effectively suppressed in HFFD + SP mice (Fig. 4D). Histological analysis showed the marked accumulation of lipid droplets in the centrilobular part of the hepatic lobule in HFFD mice (Fig. 3), which is a histological feature of steatosis in NAFLD (26, 27). Importantly, these abnormalities in HFFD mice were ameliorated to control levels in HFFD + SP mice (Figs. 1 and 2). Taken together, spironolactone appears to have a great impact on the hepatic system to prevent systemic metabolic dysregulation, at least caused by HFFD feeding.

The abnormal profiles of hepatic expression of lipogenic transcription factors and enzymes under HFFD consumption imply that high-energy feeding effectively augmented *de novo* lipogenesis in the liver (Fig. 4). Carbohydrates and insulin coordinately regulate *de novo* lipogenesis from glucose (28, 29). Excessive glucose influx promotes the formation of xylulose-5-phosphate by the hexose monophosphate pathway, which activates protein phosphatase-2A to dephosphorylate and activate ChREBP (28, 29). ChREBP controls about half of hepatic lipogenesis by regulating glycolytic and lipogenic gene transcription, including ACC, FAS, and stearoyl-CoA desaturase-1 (28, 29). On the other hand, insulin and oxysterols are generally known to activate the SREBP family of transcriptional factors that regulate other hepatic lipogenesis genes (30, 31). In addition, high-fat feeding induces the PGC1 family of coactivators, including PGC1α and particularly PGC1β, which coordinately enhance SREBP1c-mediated up-regulation of lipogenic enzymes (30, 31). Indeed, we observed that HFFD administration significantly up-regulated mRNA expressions of PGC1α, PGC1β, and ChREBP in the liver. Nevertheless, we did not find any changes in the mRNA expression of SREBP1c by HFFD feeding (Fig. 4C).

This unexpected result may be attributed to the animal species used because high-fat or high-fructose feeding has been consistently reported to stimulate hepatic SREBP1c expression and the development of fatty liver in rats (31, 32), whereas several conflicting data exist in mice (15, 27, 33–36). Moreover, the association between polymorphism in the promoter region of the SREBP1c gene and the lower susceptibility of SREBP1c expression in response to fructose consumption was demonstrated in some strains of mice, including C57BL/6 mice (36). Interestingly, the expression levels of mRNA for both PGC1α and PGC1β were significantly lower in HFFD + SP mice compared with control mice. In addition, spironolactone suppressed the expressions of PGC1s in mice fed normal chow diet (Supplemental Fig. 2). These results imply that spironolactone directly suppresses expressions of PGC1α and PGC1β. Alternatively, continu-

ous inhibition of MR with spironolactone may have an impact on the suppression because PGC1α and PGC1β are coactivators of MR (37). Further studies are required to clarify the underlying mechanism of the suppression.

Our results do not rule out the possibility that the ameliorating effect of spironolactone is mediated via insulin target tissues other than the liver. Administration of spironolactone to rats expressing the mouse renin gene improved insulin-stimulated glucose uptake in the skeletal muscle by suppressing the production of ROS (18). Treatment with an MR antagonist ameliorated the elevation of circulating levels of triglyceride and glucose by inhibiting proinflammatory cytokine production and by increasing adiponectin production from white adipose tissue in db/db mice (38). The present study showed that increased expressions of TNFα, IL-6, and MCP1 in the liver of HFFD mice are significantly suppressed in HFFD + SP mice (Fig. 4C). Because HFFD + SP mice demonstrated reduced epididymal fat weight and circulating FFA and leptin levels, it is possible that spironolactone suppresses hepatic steatosis and the expression of proinflammatory cytokines by preventing hepatic influx of adipocytokines or FFA from enlarged adipose tissues. Along this line, it has been reported that the source of accumulated triglyceride in the liver is 59% from serum FFA, 26.1% from *de novo* lipogenesis, and 14.9% from diet in patients with NAFLD (39).

Previous *in vitro* studies have shown that aldosterone induces insulin resistance in adipocytes and hepatocytes (22, 40). Although aldosterone attenuates the metabolic signaling of insulin via ROS-mediated degradation of IRS proteins in 3T3-L1 adipocytes (22) and enhanced mRNA expression of gluconeogenic enzymes in primary cultured hepatocytes (40), these *in vitro* effects are reported to be mediated mainly via glucocorticoid receptors but not via MR (22, 40). Indeed, neither treatment with aldosterone, spironolactone, nor knockdown of MR affected the expressions of IRS1, IRS2, and insulin-induced Akt phosphorylation in the primary hepatocytes and HepG2 cells (Fig. 5). In contrast, the present results indicate that MR antagonist can effectively ameliorate insulin resistance *in vivo*, consistent with the previous observations in db/db and ob/ob mice (38, 41).

The liver is composed of various heterogeneous cells, including hepatocytes, Kupffer cells, stellate cells, and sinusoidal endothelial cells. These hepatic cells interact with each other via the actions of various hormonal factors (3, 4, 42), and proinflammatory cytokines are mainly secreted from hepatic Kupffer cells or stellate cells in metabolic syndrome. Therefore, we consider that MR antagonist could break down proinflammatory cytokine networks in specific types of hepatic cells and effectively ameliorate systemic insulin resistance. On the other hand, in enlarged adipose tissue, migration of macrophage is known to play a crucial role in the development of insulin resistance (25). In fact, treatment of MR antagonist significantly prevented macrophage infiltration and its crown-like structure formation, resulting in the suppression of proinflammatory cytokine production in adipose tissue of ob/ob and db/db mice (25). It is of note that the mRNA expression of MR was not changed in the liver of HFFD mice, whereas MR expression has been shown to be increased in adipose tissue of ob/ob and db/db mice (41). Therefore, the cell-type and organ specific modification of MR expression may occur depending on dietary nutrient condition. Further studies are required to clarify the mechanism by which spironolactone ameliorates glucose and lipid metabolism in metabolic syndrome.

In conclusion, spironolactone improved HFFD-induced glucose intolerance, hyperlipidemia, and hepatic steatosis, presumably by ameliorating insulin resistance and inflammation in the liver. Although a large-scale human trial is needed to clarify the antidiabetic effect of MR antagonist, these results indicate that inhibition of MR might be a beneficial therapeutic approach to improve diet-induced metabolic syndrome, including systemic insulin resistance and NAFLD.

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References

1. **Saltiel AR, Kahn CR** 2001 Insulin signalling and the regulation of glucose and lipid metabolism. Nature 414:799–806
2. **Cornier MA, Dabelea D, Hernandez TL, Lindstrom RC, Steig AJ, Stob NR, Van Pelt RE, Wang H, Eckel RH** 2008 The metabolic syndrome. Endocrinology 29:777–822
3. **Tilg H, Hotamisligil GS** 2006 Nonalcoholic fatty liver disease: cytokine-adipokine interplay and regulation of insulin resistance. Gastroenterology 131:934–945
4. **Shoelson SE, Herrero L, Naaz A** 2007 Obesity, inflammation, and insulin resistance. Gastroenterology 132:2169–2180
5. **Stanhope KL, Havel PJ** 2008 Endocrine and metabolic effects of consuming beverages sweetened with fructose, glucose, sucrose, or high-fructose corn syrup. Am J Clin Nutr 88:1733S–1737S
6. **Greenfield V, Cheung O, Sanyal AJ** 2008 Recent advances in nonalcoholic fatty liver disease. Curr Opin Gastroenterol 24:320–327

7. **Giacchetti G, Sechi LA, Rilli S, Carey RM** 2005 The renin-angiotensin-aldosterone system, glucose metabolism and diabetes. Trends Endocrinol Metab 16:120–126
8. **Wei Y, Sowers JR, Nistala R, Gong H, Uptergrove GM, Clark SE, Morris EM, Szary N, Manrique C, Stump CS** 2006 Angiotensin II-induced NADPH oxidase activation impairs insulin signaling in skeletal muscle cells. J Biol Chem 281:35137–35146
9. **Nabeshima Y, Tazuma S, Kanno K, Hyogo H, Chayama K** 2009 Deletion of angiotensin II type I receptor reduces hepatic steatosis. J Hepatol 50:1226–1235
10. **Rajagopalan S, Duquaine D, King S, Pitt B, Patel P** 2002 Mineralocorticoid receptor antagonism in experimental atherosclerosis. Circulation 105:2212–2216
11. **Leopold JA, Dam A, Maron BA, Scribner AW, Liao R, Handy DE, Stanton RC, Pitt B, Loscalzo J** 2007 Aldosterone impairs vascular reactivity by decreasing glucose-6-phosphate dehydrogenase activity. Nat Med 13:189–197
12. **Miyata K, Rahman M, Shokoji T, Nagai Y, Zhang GX, Sun GP, Kimura S, Yukimura T, Kiyomoto H, Kohno M, Abe Y, Nishiyama A** 2005 Aldosterone stimulates reactive oxygen species production through activation of NADPH oxidase in rat mesangial cells. J Am Soc Nephrol 16:2906–2912
13. **Fujisawa G, Okada K, Muto S, Fujita N, Itabashi N, Kusano E, Ishibashi S** 2004 Spironolactone prevents early renal injury in streptozotocin-induced diabetic rats. Kidney Int 66:1493–1502
14. **Sun Y, Zhang J, Lu L, Chen SS, Quinn MT, Weber KT** 2002 Aldosterone-induced inflammation in the rat heart: role of oxidative stress. Am J Pathol 161:1773–1781
15. **Dhingra R, Sullivan L, Jacques PF, Wang TJ, Fox CS, Meigs JB, D'Agostino RB, Gaziano JM, Vasan RS** 2007 Soft drink consumption and risk of developing cardiometabolic risk factors and the metabolic syndrome in middle-aged adults in the community. Circulation 116:480–488
16. **Ouyang X, Cirillo P, Sautin Y, McCall S, Bruchette JL, Diehl AM, Johnson RJ, Abdelmalek MF** 2008 Fructose consumption as a risk factor for non-alcoholic fatty liver disease. J Hepatol 48:993–999
17. **Cassis LA, Helton MJ, Howatt DA, King VL, Daugherty A** 2005 Aldosterone does not mediate angiotensin II-induced atherosclerosis and abdominal aortic aneurysms. Br J Pharmacol 144:443–448
18. **Lastra G, Whaley-Connell A, Manrique C, Habibi J, Gutweiler AA, Appesh L, Hayden MR, Wei Y, Ferrario C, Sowers JR** 2008 Low-dose spironolactone reduces reactive oxygen species generation and improves insulin-stimulated glucose transport in skeletal muscle in the TG(mRen2)27 rat. Am J Physiol Endocrinol Metab 295:E110–E116
19. **Wada T, Naito M, Kenmochi H, Tsuneki H, Sasaoka T** 2007 Chronic nicotine exposure enhances insulin-induced mitogenic signaling via up-regulation of α7 nicotinic receptors in isolated rat aortic smooth muscle cells. Endocrinology 148:790–799
20. **Kagawa S, Soeda Y, Ishihara H, Oya T, Sasahara M, Yaguchi S, Oshita R, Wada T, Tsuneki H, Sasaoka T** 2008 Impact of transgenic overexpression of SH2-containing inositol 5′-phosphatase 2 on glucose metabolism and insulin signaling in mice. Endocrinology 149:642–650
21. **Wada T, Azegami M, Sugiyama M, Tsuneki H, Sasaoka T** 2008 Characteristics of signalling properties mediated by long-acting insulin analogue glargine and detemir in target cells of insulin. Diabetes Res Clin Pract 81:269–277
22. **Wada T, Ohshima S, Fujisawa E, Koya D, Tsuneki H, Sasaoka T** 2009 Aldosterone inhibits insulin-induced glucose uptake by degradation of insulin receptor substrate (IRS) 1 and IRS2 via a reactive oxygen species-mediated pathway in 3T3-L1 adipocytes. Endocrinology 150:1662–1669
23. **Grassi G** 2006 Sympathetic overdrive and cardiovascular risk in the metabolic syndrome. Hypertens Res 29:839–847
24. **Engeli S, Böhnke J, Gorzelniak K, Janke J, Schling P, Bader M, Luft FC, Sharma AM** 2005 Weight loss and the renin-angiotensin-aldosterone system. Hypertension 45:356–362
25. **Wellen KE, Hotamisligil GS** 2003 Obesity-induced inflammatory changes in adipose tissue. J Clin Invest 112:1785–1788
26. **Sanyal AJ; American Gastroenterological Association** 2002 AGA technical review on nonalcoholic fatty liver disease. Gastroenterology 123:1705–1725
27. **Inoue M, Ohtake T, Motomura W, Takahashi N, Hosoki Y, Miyoshi S, Suzuki Y, Saito H, Kohgo Y, Okumura T** 2005 Increased expression of PPARγ in high fat diet-induced liver steatosis in mice. Biochem Biophys Res Commun 336:215–222
28. **Uyeda K, Repa JJ** 2006 Carbohydrate response element binding protein, ChREBP, a transcription factor coupling hepatic glucose utilization and lipid synthesis. Cell Metab 4:107–110
29. **Iizuka K, Horikawa Y** 2008 ChREBP: a glucose-activated transcription factor involved in the development of metabolic syndrome. Endocr J 55:617–624
30. **Repa JJ, Liang G, Ou J, Bashmakov Y, Lobaccaro JM, Shimomura I, Shan B, Brown MS, Goldstein JL, Mangelsdorf DJ** 2000 Regulation of mouse sterol regulatory element-binding protein-1c gene (SREBP-1c) by oxysterol receptors, LXRα and LXRβ. Genes Dev 14:2819–2830
31. **Lin J, Yang R, Tarr PT, Wu PH, Handschin C, Li S, Yang W, Pei L, Uldry M, Tontonoz P, Newgard CB, Spiegelman BM** 2005 Hyperlipidemic effects of dietary saturated fats mediated through PGC-1β coactivation of SREBP. Cell 120:261–273
32. **Nagai Y, Nishio Y, Nakamura T, Maegawa H, Kikkawa R, Kashiwagi A** 2002 Amelioration of high fructose-induced metabolic derangements by activation of PPARα. Am J Physiol Endocrinol Metab 282:E1180–E1190
33. **Murase T, Mizuno T, Omachi T, Onizawa K, Komine Y, Kondo H, Hase T, Tokimitsu I** 2001 Dietary diacylglycerol suppresses high fat and high sucrose diet-induced body fat accumulation in C57BL/6J mice. J Lipid Res 42:372–378
34. **Toye AA, Dumas ME, Blancher C, Rothwell AR, Fearnside JF, Wilder SP, Bihoreau MT, Cloarec O, Azzouzi I, Young S, Barton RH, Holmes E, McCarthy MI, Tatoud R, Nicholson JK, Scott J, Gauguier D** 2007 Subtle metabolic and liver gene transcriptional changes underlie diet-induced fatty liver susceptibility in insulin-resistant mice. Diabetologia 50:1867–1879
35. **Kim S, Sohn I, Ahn JI, Lee KH, Lee YS, Lee YS** 2004 Hepatic gene expression profiles in a long-term high-fat diet-induced obesity mouse model. Gene 340:99–109
36. **Nagata R, Nishio Y, Sekine O, Nagai Y, Maeno Y, Ugi S, Maegawa H, Kashiwagi A** 2004 Single nucleotide polymorphism (−468 Gly to A) at the promoter region of SREBP-1c associates with genetic defect of fructose-induced hepatic lipogenesis. J Biol Chem 279:29031–29042
37. **Knutti D, Kaul A, Kralli A** 2000 A tissue-specific coactivator of steroid receptors, identified in a functional genetic screen. Mol Cell Biol 20:2411–2422
38. **Guo C, Ricchiuti V, Lian BQ, Yao TM, Coutinho P, Romero JR, Li J, Williams GH, Adler GK** 2008 Mineralocorticoid receptor blockade reverses obesity-related changes in expression of adiponectin, peroxisome proliferator-activated receptor-γ, and proinflammatory adipokines. Circulation 117:2253–2261
39. **Donnelly KL, Smith CI, Schwarzenberg SJ, Jessurun J, Boldt MD, Parks EJ** 2005 Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease. J Clin Invest 115:1343–1351
40. **Yamashita R, Kikuchi T, Mori Y, Aoki K, Kaburagi Y, Yasuda K, Sekihara H** 2004 Aldosterone stimulates gene expression of hepatic gluconeogenic enzymes through the glucocorticoid receptor in a manner independent of the protein kinase B cascade. Endocr J 51:243–251
41. **Hirata A, Maeda N, Hiuge A, Hibuse T, Fujita K, Okada T, Kihara S, Funahashi T, Shimomura I** 2009 Blockade of mineralocorticoid receptor reverses adipocyte dysfunction and insulin resistance in obese mice. Cardiovasc Res 84:164–172
42. **Leclercq IA, Da Silva Morais A, Schroyen B, Van Hul N, Geerts A** 2007 Insulin resistance in hepatocytes and sinusoidal liver cells: mechanisms and consequences. J Hepatol 47:142–156