Treatment with SRT1720, a SIRT1 activator, ameliorates fatty liver with reduced expression of lipogenic enzymes in MSG mice

Yu Yamazaki,1 Isao Usui,1 Yukiko Kanatani,2 Yuji Matsuya,2 Koichi Tsumeyama,3 Shiho Fujisaka,1 Agussalim Bukhari,1 Hikari Suzuki,1 Satoko Senda,1 Shingo Imanishi,1 Kazuya Hirata,1 Manabu Ishiki,1 Ryuji Hayashi,1 Masaharu Urakaze,1 Hideo Nemoto,2 Masashi Kobayashi,4 and Kazuyuki Tobe1
1First Department of Internal Medicine, 2Graduate School of Medicine and Pharmaceutical Sciences, 3The Department of Diagnostic Pathology, 4University Hospital, University of Toyama, Toyama, Japan

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Monosodium glutamate-injected Institute of Cancer Research (ICR) mice (MSG mice) are a newly developed mouse model characterized by central obesity and insulin resistance, thereby establishing a vicious cycle (10). In fact, NAFLD is often related to insulin resistance and several metabolic disorders, including obesity, diabetes, dyslipidemia, and atherosclerosis (17, 18). Calorie restriction (CR) and weight reduction are useful treatments for NAFLD as well as for other metabolic disorders (1).

Silent information regulator-2 (Sir2) is a highly conserved protein found in organisms ranging from bacteria to humans. In mammals, seven homologs of Sir2, SIRT1–7, have been identified (4). The expression of Sir2 is induced by CR and is associated with many cellular functions (11). CR reportedly prolongs the life spans of many species, including yeast (12), Caenorhabditis elegans (28), and mice (12). Furthermore, it also decreases the development of age-associated disorders such as diabetes, cancer, and cardiovascular diseases (27). Interestingly, SIRT1 transgenic mice display some similarity to mice subjected to CR, i.e., their metabolic rate and locomotor activities are upregulated, and their longevity is prolonged. These results suggest that the activation of SIRT1 may play an important role in the beneficial effects of CR on longevity and metabolic diseases (5). The involvement of SIRT1 activation in the regulation of longevity or metabolism has also been confirmed in experiments using some SIRT1 activators, such as resveratrol. The administration of resveratrol to mice fed a high-fat diet improved glucose metabolism, reduced liver fat content, and prolonged the longevity of the mice (3). However, some recent studies have revealed that resveratrol is not necessarily specific to SIRT1 (5). Recently, SRT1720, a new chemical compound, has been identified as a specific activator of SIRT1. The activity of SRT1720 is 1,000-fold stronger than that of resveratrol. The administration of resveratrol to ob/ob mice or Zucker fa/fa rats improved whole glucose metabolism and reduced insulin resistance in the skeletal muscle and liver (19). Furthermore, a recent study has revealed that SRT1720 improves several metabolic disorders, including fatty liver, by enhancing lipid oxidation (7). However, the mechanisms responsible for this compound’s effects on the reduction of lipid accumulation in the liver have not been fully clarified.

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THE NUMBER OF PATIENTS WITH nonalcoholic fatty liver disease (NAFLD) is rapidly increasing worldwide; thus, strategies for preventing or treating this disease are urgently needed. As a possible model for the pathogenesis of NAFLD, the “two hit hypothesis” is widely accepted. The first hit (accumulation of lipid) makes the liver more sensitive to second hits, such as oxidative stress, endotoxins, or abnormal cytokine productions, which can cause inflammation and/or eventual fibrosis of the liver (6). Insulin resistance is also related to the development of NAFLD. Under an insulin-resistant condition, the supply of free fatty acids (FFA) from other tissues increases because of the impaired suppression of lipolysis, which may promote liver lipid accumulation. The accompanying increase in oxidative stress and proinflammatory cytokine production following liver lipid accumulation causes a further increase in insulin resistance, thereby establishing a vicious cycle (10). In fact, NAFLD is often related to insulin resistance and several metabolic disorders, including obesity, diabetes, dyslipidemia, and atherosclerosis (17, 18). Calorie restriction (CR) and weight reduction are useful treatments for NAFLD as well as for other metabolic disorders (1).

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process resembles the pathological and chemical findings of human NAFLD more closely than other models of obese mice (21). Thus MSG mice may be considered one of the best models available for studying the mechanisms of NAFLD.

In general, the hepatic fatty acid metabolism observed with NAFLD are characterized by 1) an increase in de novo lipogenesis in hepatocytes; 2) an increased influx of FFA in the liver from the other organs, such as fat tissue; and 3) the suppression or insufficiency of mitochondrial β-oxidation to the excessive FFA influx and a related increase in peroxisomal β-oxidation (14). In this study, we hypothesized that the activation of SIRT1 by SRT1720 may ameliorate fatty liver via the altered fatty acid metabolism. Thus we investigated the effects of SRT1720 treatment on the hepatic lipid accumulation and the lipid metabolisms in MSG mice. The direct effects of this drug on lipogenic genes were also examined in cultured HepG2 cells, a hepatoma cell line. Here, we provide evidence that the treatment with SRT1720 decreases the hepatic lipid content with reducing expressions of lipogenic genes.

MATERIALS AND METHODS

Preparation of SRT1720. We synthesized SRT1720, a SIRT1 activator, at the Laboratory of Medicinal Chemistry (Synthetic Organic Chemistry), Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, according to the previously published protocol (19) with a slight modification. The compound was first synthesized as a free base, and the structure and purity were then confirmed using nuclear magnetic resonance spectroscopy. The free base compound was transformed into an HCl salt upon treatment with ethereal/HCl in a chloroform solution. The deacetylation activity stimulated by this product was finally confirmed using CycLex SIRT1/Sirt2 Deacetylase Fluorometric Assay Kits according to the manufacturer’s protocol (BIOMOL International).

Animals. Four-week-old male MSG mice and control male ICR mice were purchased from the Institute for Animal Reproduction (Ibaragi, Japan). Twenty ICR mice and 12 MSG mice at 6 wk old were analyzed as pretreatment groups before starting SRT1720 treatment. SRT1720 was mixed with standard chow (200 mg·kg body wt⁻¹·day⁻¹) and orally administered to 6-wk-old MSG mice for 10 wk. Fifteen ICR mice, 15 MSG mice, and 15 MSG mice with SRT1720 treatment at 16 wk old were analyzed as posttreatment groups after a 10-wk treatment period. Mice were housed in a room with a 12:12-h light-dark cycle and were given free access to food and water. The current study, including the protocol, was reviewed and approved by the Animal Committee of the University of Toyama.

Quantitative RT-PCR. Total RNAs from mouse liver or HepG2 cells were extracted using an RNaseasy Mini kit (Qiagen, Tokyo, Japan). Quantitative RT-PCR was conducted according to the manufacturer’s protocol (Applied Biosystems, Foster City, CA). Briefly, we synthesized cDNA using random hexamers and TaqMan Reverse Transcription Reagents. Reverse-transcribed cDNA was mixed with PCR Master Mix and gene-specific Assays-on-Demand Gene Expression Products (Applied Biosystems, Foster City, CA); then amplified on a MX3000P (Stratagene). Results were normalized against the gene expression of 18S. For SIRT1 mRNA, the PCR products after 30 reaction cycles were run on an agarose gel and detected using ethidium bromide as a standard method.

Immunoprecipitation and immunoblotting. The livers were removed and homogenized for 1 min in lysis buffer containing 25 mM Tris·HCl (pH 7.4), 10 mM Na3VO4, 100 mM NaF, 50 mM Na2PO4, 10 mM EGTA, 10 mM EDTA, 5 mg leupeptin/ml, 5 mg aprotinin/ml, 2 mM phenylmethylsulfonyl fluoride, and 1% Nonidet P-40. Lysates were centrifuged to remove the insoluble materials. Immunoprecipitation and immunoblotting were performed as described previously (13), using anti-PGC-1α antibody (Santa Cruz Biotechnology), anti-acetylated lysine antibody (Cell Signaling), anti-SIRT1 antibody (Upstate), anti-β-actin antibody (Cell Signaling), anti-acetyl-CoA carboxylase (ACC) antibody (Cell Signaling), anti-FAS antibody (BD Bioscience), anti-sterol regulatory element-binding protein (SREBP)-1 antibody (Santa Cruz Biotechnology), anti-tumor necrosis factor (TNF)-α antibody (Cell Signaling), anti-monocyte chemotactic protein (MCP)-1 antibody (Cell Signaling), anti-glutathione peroxidase (Gpx) 1 antibody (Santa Cruz Biotechnology), anti-catalase antibody (abcam), and anti-superoxide dismutase (SOD) 1 antibody (Santa Cruz Biotechnology).

Histological analysis of liver. The livers were fixed overnight in buffered 20% formaldehyde, embedded in paraffin, and sectioned at a thickness of 4 μm. The sections were then stained with hematoxylin and eosin using a standard protocol. For lipid staining, frozen samples were sectioned at a thickness of 10 μm and stained with Sudan IV with hematoxylin used as a counterstain.

Biochemical assays. Plasma triglycerides (TG) and FFA levels were measured using enzymatic methods (Triglyceride E-test Wako, NEFA C-test; Wako, Osaka, Japan). Total cholesterol, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) levels were measured using the REFUROTORON system (Roche Diagnostics, Basel, Switzerland). Liver TG was extracted from liver homogenates using methanol and chloroform as previously described (8) and quantified using a commercially available kit to measure the concentration of TG (Triglyceride E-test).

Culture of HepG2 cells. HepG2 cells were purchased from American Type Culture Collection. They were cultured in Dulbecco’s modified Eagle’s medium (DMEM)-low glucose with 10% FCS, 100 U/ml of penicillin, and 100 mg/ml of streptomycin, as described previously (26). A cell steatosis model was established by culturing HepG2 cells in DMEM with a high concentration of glucose (25 mM) and insulin (100 nM) for 24 h, as described previously (9). Simultaneously, 0.1 or 1 mM SRT1720 was dissolved in dimethyl sulfoxide (DMSO), 5 μl of which was added to 5 ml medium to get the final concentrations, whereas the control group received 5 μl DMSO only.

Statistical analysis. All data are presented as means ± SD. Data were analyzed using an ANOVA or Student’s t-test. P values <0.05 were considered statistically significant.

RESULTS

Administration of SRT1720 did not affect body weight or food intake. SRT1720 was orally administered to male MSG mice at a dose of 200 mg/kg body wt for 10 wk (from the age of 6 to 16 wk). Deacetylation activity of SIRT1 in the liver was assessed by examining the acetylation of PGC-1α, one of the major substrates for SIRT1 (Fig. 1A) (23). Administration of SRT1720 reduced the acetylation level of PGC-1α almost to the control level (Fig. 1A), whereas it did not alter the expression of SIRT1 at either the mRNA (Fig. 1B) or protein levels (Fig. 1C) in the liver. The body weight (Fig. 1D) and the weight of the epididymal fat (data not shown) in 16-wk-old MSG mice was higher than those in control mice by ~20 and 60%, respectively. SRT1720 treatment did not significantly affect the body weight or food intake of the MSG mice (Fig. 1, D and E).

Administration of SRT1720 reduced fat accumulation in the liver and ameliorated liver dysfunction in MSG mice. The liver weights of the 16-wk-old MSG mice were higher than those of the lean ICR mice by 34% (Fig. 2A). Microscopic views with hematoxylin and eosin staining revealed a microvesicular clear cytoplasmic reaction of the hepatocytes predominantly in Zone 3 of the livers of MSG mice. Because staining with Sudan IV showed that these clear hepatocytes contained lipid droplets,
they were considered to represent the fatty degeneration of hepatocytes (Fig. 2C). The TG content in the livers of MSG mice was 3.7-fold higher than that in ICR mice (Fig. 2B). The administration of SRT1720 to MSG mice for 10 wk reduced fat accumulation by 21% (Fig. 2B and C), although it did not alter the liver weight significantly (Fig. 2A). The serum levels of aminotransferases were also elevated in MSG mice. The administration of SRT1720 lowered the AST level by 42%, whereas the ALT level was not altered (Fig. 2D). These results indicate that administration of SRT1720 ameliorated fat accumulation in the liver and liver dysfunction in MSG mice.

Expressions of genes involved in hepatic lipid synthesis were decreased by SRT1720 treatment. To determine how SRT1720 ameliorated the development of fatty liver in MSG mice, we measured the expression of mRNA or proteins involved in lipid synthesis, β-oxidation, and mitochondrial electron transport. As expected, the expressions of key enzymes for lipid synthesis in the liver, including SREBP-1c, ACC, and FAS, were increased in MSG mice, and were suppressed by SRT1720 treatment by 20–33%, at both mRNA and protein levels (Fig. 3A). The expressions of genes involved in fatty acid oxidation such as medium-chain acyl-CoA dehydrogenase and carnitine palmitoyltransferase-1b (Fig. 3B) and in mitochondrial metabolism such as PGC-1α, cytochrome C, ATP synthase subunit 5g1, and NADH ubiquinone oxidoreductase-α (Fig. 3C) were increased in MSG mice. The expressions of these genes were not enhanced but rather were suppressed or not altered by SRT1720 treatment, except for PGC-1α mRNA, which was significantly increased by SRT1720 treatment (Fig. 3B and C).

SRT1720 treatment decreased the expression of lipogenic genes in HepG2 cells. To clarify whether SRT1720 directly decreased the expression of lipogenic enzymes in the liver of MSG mice, the effects of SRT1720 treatment on lipogenic genes were also examined using cultured HepG2 cells, a human hepatoma cell line. As reported previously (26), HepG2 cells incubated with a high concentration of insulin (100 nM) for 24 h accumulated more lipid droplets (data not shown) and increased the expression of lipogenic genes, including SREBP-1c, ACC, and FAS (Fig. 4). Cotreatment with SRT1720 significantly decreased the expressions of these genes by 30–40% (Fig. 4), suggesting that SRT1720 treatment directly decreased de novo lipid synthesis in hepatocytes.

Administration of SRT1720 improved dyslipidemia in MSG mice. We next examined the lipid profiles of these mice, since elevated FFA level is often observed with fatty liver. MSG mice (16 wk old) showed dyslipidemia characterized by elevated serum levels of FFA, TG, and total cholesterol. Treatment with SRT1720 (10 wk) to MSG mice decreased the serum concentration of FFA, TG, and total cholesterol in MSG mice to almost the levels in control ICR mice (Fig. 5, A–C).

SRT1720 treatment decreased the expressions of genes related to inflammation and oxidative stress. Finally, the effects of SRT1720 treatment on inflammation and oxidative stress in the liver were examined. The expressions of proinflammatory cytokines, such as TNF-α and MCP-1, were increased in the livers of MSG mice and were significantly decreased by SRT1720 treatment (Fig. 6A). The expressions of F4/80, a pan-marker for macrophages, and of CD11c, a marker for...
classically activated M1 macrophages, were not altered in MSG mice compared with those in control ICR mice (Fig. 6A). Marker genes for oxidative stress signaling, such as Gpx1, catalase, and SOD1, were also increased in MSG mice and were significantly decreased by SRT1720 treatment (Fig. 6B). These results indicate that SRT1720 treatment may decrease inflammation and oxidative stress in the liver of MSG mice.

**DISCUSSION**

The activation of SIRT1, a well-known longevity-related gene, is involved in the improvement of several metabolic diseases (5). SRT1720, a newly synthesized SIRT1 activator, was recently reported to have beneficial effects on glucose metabolism and insulin sensitivity (7, 19). In the present study, we synthesized this drug and administered it to MSG mice, an obese and insulin-resistant mouse model. The activation of SIRT1 in the liver of MSG mice was confirmed by the observation that PGC-1α was deacetylated by SRT1720 treatment without affecting its expression levels (Fig. 1). We found that the administration of SRT1720 for 10 wk ameliorated the development of fatty liver in MSG mice (Fig. 2). As the mechanisms, the gene expressions involved in lipid synthesis, fatty acid oxidation, and mitochondrial metabolism as well as serum FFA level were increased in the livers of MSG mice and were decreased by the administration of SRT1720 (Figs. 3 and 5). These results suggest that the increase in TG accumulation in the liver of MSG mice may be explained by an increase in de novo lipid synthesis and/or FFA influx to the liver, but not by the insufficiency of mitochondrial metabolism or β-oxidation. On the contrary, the reduced accumulation of TG resulting from SRT1720 treatment may be due to a reduction in de novo lipid synthesis and/or FFA influx.

As reported previously (26), the treatment of HepG2 cells with a high concentration of insulin increased the number of...
lipid droplets (data not shown). Because the concentration of FFA in the culture media was similar between the control and insulin-treated cells, this cell culture model can be considered to mimic lipid accumulation through increased de novo lipid synthesis, not through an increase in FFA availability.

SRT1720 treatment reduced the number of lipid droplets and the expression of lipogenic enzymes such as SREBP-1c, ACC, and FAS (Fig. 4). These data suggest that the reduction in de novo lipid synthesis caused by SRT1720 treatment may be involved in the amelioration of fatty liver. A recent study reported that SIRT1 deacetylates LKB1, an AMP-activated protein kinase (AMPK) kinase, thus activating AMPK (15). This previous report supports our hypothesis that the expressions of lipogenic genes are suppressed by SRT1720 probably via the activation of AMPK.

We also examined the effects of SRT1720 on both inflammatory cytokines and oxidative stress. As shown in Fig. 6A, the expressions of inflammatory cytokines, such as TNF-α and MCP-1, were increased in MSG mice and were decreased by SRT1720 treatment. The expressions of F4/80, a pan-marker of macrophages, and of CD11c, a marker of classically activated macrophages, were not altered (Fig. 6B). Similarly, no remarkable increase in inflammatory cells was observed in a histological analysis of liver from MSG mice (Fig. 2C). These

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results suggest that the increased expression of inflammatory cytokines is not derived from newly recruited inflammatory cells, but possibly from hepatocytes. Because SIRT1 is reported to inhibit the transcriptional activity of nuclear factor (NF)-κB (24), the reduced expressions of such cytokines observed in SRT1720-treated mice might result from the inhibition of NF-κB activity. A recent study has reported that FFA infiltrating the liver directly inhibits mitochondrial function and stimulates reactive oxygen species (ROS) production (16). In this study, we observed that the expressions of several redox-balancing genes, such as Gpx1, catalase, and SOD1, were decreased by SRT1720 treatment (Fig. 6B). Because the serum FFA level was decreased in SRT1720-treated mice, a reduction in FFA influx in the liver may be involved in the lower ROS production and the resulting decrease in the expressions of such redox-related genes. Both inflammatory cytokines and oxidative stress are considered to be factors, which can progress NAFLD and cause more severe conditions of this disease (6). Reduced inflammation or oxidative stress by SRT1720 may lead to the prevention of these liver diseases. In the present study, SRT1720 treatment was started long term before the starting lipid accumulation, and the liver was ana-
lyzed just after developing fatty liver. Unfortunately, we have not performed the analysis using different treatment conditions, such as a longer treatment period or starting the treatment at the different stage of the disease. Thus it is thus far unclear whether SRT1720 may prevent the development of more severe liver disease or whether SRT1720 may reverse the already developed NAFLD. Further studies are necessary to show such possible effects of this drug. A recent study by Feige et al. (7) has revealed that SRT1720 improves several metabolic disorders, including fatty liver. They reported that the reduced serum FFA level, which might be caused by increased fat consumption in the skeletal muscle and brown adipocytes, was one of the mechanisms responsible for this compound’s effects on the reduction of lipid accumulation in the liver (7). Consistent with their result, we found that SRT1720 treatment lowered the serum FFA level in MSG mice, too (Fig. 5). On the other hand, the Feige group also reported that SRT1720 treatment enhanced the expressions of genes involved in lipid oxidation in the liver (7). However, in the present study, SRT1720 treatment failed to increase, but rather reduced, the expressions of these genes (Fig. 3B). If we assume that fatty acid oxidation in the liver is determined by both FFA-induced peroxisome proliferator-activated receptor-α activation (25) and SRT1720-induced LKB1 and AMPK activation, the reduction in FFA influx caused by SRT1720 may have a stronger effect on lipid oxidation than on the activation of LKB1 and AMPK in our model mice, whereas the opposite might be true in the different model mice used by Feige et al. Further studies are required to clarify this issue.

The present study in MSG mice has provided evidence that the lipid accumulation in liver can be reduced by the administration of SRT1720, a newly synthesized SIRT1 activator, without altering food intake or body weight. A decrease in de novo lipid synthesis and FFA influx in the liver were suggested to be responsible for the reduced lipid accumulation. Moreover, beneficial effects on lipid metabolism, glucose metabolism, and longevity can also be expected because of the improvement of inflammation and oxidative stress in the mice treated with this drug. Thus far, effective strategies are not available for the treatment of fatty liver other than the improvement of lifestyle-related factors, such as CR or body weight reduction. Importantly, the present study suggests that fatty liver might be ameliorated through the use of this synthesized compound.

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REFERENCES


