Gastrointestinal, Hepatobiliary and Pancreatic Pathology

Prevention of Hepatic Fibrosis in a Murine Model of Metabolic Syndrome with Nonalcoholic Steatohepatitis

Laurie D. DeLeve,* Xiangdong Wang,*
Gary C. Kanel,[†] Roscoe D. Atkinson,[†]
and Robert S. McCuskey[‡]

From the Division of Gastrointestinal and Liver Diseases and the Research Center for Liver Diseases, and the Department of Pathology,[†] Keck School of Medicine, University of Southern California, Los Angeles, California; and the Department of Cell Biology and Anatomy,[‡] University of Arizona, Tucson, Arizona*

The endocannabinoid pathway plays an important role in the regulation of appetite and body weight, hepatic lipid metabolism, and fibrosis. Blockade of the endocannabinoid receptor CB1 with SR141716 promotes weight loss, reduces hepatocyte fatty acid synthesis, and is antifibrotic. D-4F, an apolipoprotein A-1 mimetic with antioxidant properties, is currently in clinical trials for the treatment of atherosclerosis. C57BL/6J mice were fed a high-fat diet for 7 months, followed by a 2.5-month treatment with either SR141716 or D-4F. SR141716 markedly improved body weight, liver weight, serum transaminases, insulin resistance, hyperglycemia, hypercholesterolemia, hyperleptinemia, and oxidative stress, accompanied by the significant prevention of fibrosis progression. D-4F improved hypercholesterolemia and hyperleptinemia without improvement in body weight, steatohepatitis, insulin resistance, or oxidative stress, and yet, there was significant prevention of fibrosis. D-4F prevented culture-induced activation of stellate cells *in vitro*. In summary, C57BL/6J mice given a high-fat diet developed features of metabolic syndrome with nonalcoholic steatohepatitis and fibrosis. Both SR141716 and D-4F prevented progression of fibrosis after onset of steatohepatitis, ie, a situation comparable to a common clinical scenario, with D-4F seeming to have a more general antifibrotic effect. Either compound therefore has the potential to be of clinical benefit. (Am J Pathol 2008, 173:993–1001; DOI: 10.2353/ajpath.2008.070720)

Intervention in the endocannabinoid pathway is of particular interest for nonalcoholic fatty liver disease because this pathway is involved in the regulation of appetite and body weight, hepatic lipid metabolism, and fibrosis. SR141716, an antagonist of the endocannabinoid receptor CB1, reduces food intake.¹ In the liver, a high-fat diet increases fatty acid synthesis and induces fatty liver in the liver and this effect is blocked by SR141716.¹ Hepatocytes express CB1 and activation of CB1 induces expression of SREBP-1, its target enzymes acetyl-coA carboxylase-1 and FAS, and increases fatty acid synthesis in the liver.¹ CB1 and CB2 expression are up-regulated in cirrhotic liver and expression on hepatic stellate cells (HSCs) is enhanced on activation of the stellate cell.^{2,3} Stimulation of the two endocannabinoid receptors has opposing effects on fibrosis: CB2 is antifibrotic³ and CB1 is profibrotic.² Blockade of CB1 with SR141716 before or during administration of experimental fibrogenic toxins prevents fibrosis.²

D-4F has not previously been studied in liver disease. D-4F is an 18 D-amino acid peptide that markedly reduces atherosclerosis in mouse models⁴ and that is currently being investigated in clinical trials for amelioration of atherosclerosis. D-4F has anti-oxidant properties: its lipid-associating structural motifs allow it to interact with phospholipids in cellular membranes and remove oxidized lipids⁵ and it decreases lipoprotein lipid hydroperoxides.⁶ Hepatic fibrogenesis is mediated by activation of HSCs. Oxidative stress from lipid peroxidation products plays a role in initiating stellate cell activation.^{7–12}

C57BL/6J mice develop obesity, insulin resistant diabetes mellitus, hypercholesterolemia, and hypertriglycer-

Supported by the National Institutes of Health (grant DK66423), the Morphology Core of the University of Southern California Research Center for Liver Diseases, and the Nonparenchymal Liver Cell Core of the Southern California Research Center for Alcoholic Liver and Pancreatic Diseases and Cirrhosis.

Accepted for publication June 18, 2008.

Address reprint requests to Laurie D. DeLeve, M.D., Ph.D., USC Keck School of Medicine, Div. of GI/Liver Diseases, 2011 Zonal Ave., HMR603, Los Angeles, CA 90033. E-mail: deleve@usc.edu.

idemia when fed a Western diet, a high-fat diet formulated to approximate the typical human diet in North America and Europe.¹³ We demonstrate that C57BL/6J mice given 9.5 months of a Western diet develop both steatohepatitis and significant fibrosis. Thus the high saturated fat diet model presented here represents a model of metabolic syndrome with advanced fatty liver disease. We have examined the effect of 2.5 months of treatment with either SR141716 or D-4F in this model.

Materials and Methods

Reagents

SR141716 was a kind gift from Sanofi-Aventis, Paris, France. SR141716 was dissolved in water with 0.1% Tween and given daily by gavage for 10 weeks. D-4F is an 18-mer of D-amino acids (Ac-D-W-F-K-A-F-Y-D-K-V-A-E-K-F-K-E-A-F-NH₂)⁴ that was commercially synthesized (A & A Laboratories, San Diego, CA) with 80% purity. D-4F was dissolved in the drinking water at a concentration of 60 μ g/ml of water for a calculated dose corrected for drug purity of 125 μ g of D-4F/mouse/day. The high-fat diet used was the RD Western diet (D12079B; Research Diets, New Brunswick, NJ), which contains 41% of calories from fat, 43% calories from carbohydrate, and 17% of calories from protein; this diet was designed to approximate the typical human diet of North America and Europe.

Animal Model

Studies were performed in male C57BL/6J mice (The Jackson Laboratory, Bar Harbor ME). Six groups were studied ($n = 3$ to 5; Figure 1) with four mice per group at the outset, except for group 2, which was started with $n = 5$; one mouse died in group 1. Groups 1, 2, and 3 received the Western diet for 9.5 months; during the last 2.5 months of the 9.5 months, group 2 received SR141716 10 mg/kg/day i.g., and group 3 received D-4F, 125 μ g/mouse/day, in the drinking water. Groups 4, 5, and 6 received the Western diet for 7 months followed by standard chow for 2.5 months; during the 2.5 months on standard chow, group 5 received SR141716 and group 6 received D-4F as described for groups 2 and 3, respectively. Additional control groups were studied: 6-month-old C57BL/6J mice as age-matched controls ($n = 5$) and C57BL/6J mice that received the Western diet for 6 months ($n = 6$). All protocols dealing with animals were reviewed and approved by the Animal Care and Use Committees at the University of Southern California and University of Arizona to ensure ethical and humane treatment of the animals. This study followed the guidelines outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Revised 1985) prepared by the National Academy of Sciences.

Sirius Red Staining

Sirius red staining was performed by the Morphology Core of the USC-UCLA Research Center for Alcoholic Liver and Pancreatic Diseases.

α -Smooth Muscle Actin (α -SMA)

Seven- μ m-thick sections of formalin-fixed, paraffin-embedded liver were used for α -SMA immunofluorescence staining. Slides were deparaffinized in xylene and serially rehydrated in graded ethanol (100 to 70%). Slides were treated with 0.1 mol/L Tris-HCl (pH 7.6)/0.05 Tween-20 for 5 minutes, incubated with 1 μ g/ml of proteinase K (Sigma, St. Louis, MO) for 15 minutes at room temperature, and then rinsed in water for 10 minutes. Slides were rinsed in 0.1 mol/L Tris-HCl with 2% goat serum for 5 minutes, incubated with monoclonal anti- α -SMA (1:5000, Sigma) for 30 minutes at room temperature, followed by a fluorescein isothiocyanate-conjugated goat anti-mouse IgG (1:75, Sigma) secondary antibody for 45 minutes at room temperature. After rinsing in 0.1 mol/L Tris-HCl/0.05% Tween-20 for 5 minutes followed by 0.1 mol/L Tris-HCl for 5 minutes, nuclear staining was performed with 4,6-diamidino-2-phenylindole diluted in methanol (1:1000, Sigma) for 5 minutes. Negative controls were performed by omitting the primary antibody.

Evaluation of Steatosis and/or Fibrosis

The slides were coded, without the pathologist knowing the specific treatment group that the slides represented. The

Figure 1. Protocol for the six treatment groups.

histology was graded according to a number of histological features. The degree of fat was graded as to the approximate percentage of hepatocytes containing fat: 0, absent; +/–, less than 5%; 1+, 5 to 25%; 2+, 26 to 50%; 3+, 51 to 75%; 4+, >75%. The fat was also assessed as to whether it was totally macrovesicular, predominantly macrovesicular, totally microvesicular, predominantly microvesicular, or mixed macro- and microvesicular. In addition, the zone was assessed as to whether the fat was perivenular, periportal, diffuse, or focal. Inflammation was graded as absent, scanty (1 to 3 foci per slide), present (scattered, with 4 to 10 foci per slide), present + (scattered, >10 foci per slide but not numerous), and present ++ (numerous). The type of inflammation was graded as totally mononuclear, predominantly mononuclear, totally neutrophilic, predominantly neutrophilic, and mixed mononuclear-neutrophilic. The hydropic change of hepatocytes was graded as absent, mild, and moderate/severe, with the location noted as perivenular, focal, or diffuse.

Whole Section-Scanning Morphometric Image Analysis

Tissue sections stained with Sirius red were scanned by a third-generation automated cellular imaging system (ACIS III), a highly automated microscope-based digital imaging system (Carl Zeiss MicroImaging AIS Inc., Aliso Viejo, CA). The color of the Sirius red staining in the digitized tissue sections was determined and applied to a pre-existing algorithm that provides the area of a target color in μm^2 . The outline of each digitized tissue section was manually traced using the computer's mouse. Areas of large portal tracts and central veins were also outlined manually. The total area (μm^2) of each tissue section and of the total area of large portal tracts and central veins were determined by the image analysis system. The area of fibrosis was calculated as the total area stained by Sirius red minus perivascular staining as a percentage of the size of the tissue section. This method has the advantage of scanning the entire section, rather than measuring fibrosis in randomly chosen fields.

Electron Microscopy

In a separate set of experimental animals ($n = 3$), routine methods were used to prepare liver specimens for transmission and scanning electron microscopy.^{14,15} Briefly, the livers were perfused through the portal vein with 0.1 mol/L Na-cacodylate buffer to aid in washing out blood. This was immediately followed with a fixative containing 1.5% glutaraldehyde in 0.1 mol/L cacodylate buffer, pH 7.4. For transmission electron microscopy, minced pieces of liver were further fixed for 2 hours, then washed in buffer, postfixated with 1% osmium tetroxide in 0.1 mol/L cacodylate buffer at 4°C, dehydrated through graded alcohols, and embedded in Spurr. Thin sections were cut on a Reichert Ultracut microtome, (Reichert-Jung Ultracut microtome, Vienna, Austria) examined and photographed using a Philips CM-12S electron microscope

(Philips Electronic Instruments, Eindhoven, The Netherlands). For scanning electron microscopy, pieces of perfused-fixed livers were dehydrated in a graded ethanol series, fractured, dried with hexamethyldisilazane, sputter-coated with 10-nm gold, and examined using a Philips XL30 scanning electron microscope.

In Vitro Studies

Murine HSCs were isolated by collagenase/pronase digestion and Stratan density gradient centrifugation.¹⁶ For α -SMA staining the HSCs were cultured on glass coverslips and fixed with 10% formaldehyde (EM Science, Gibbstown, NJ) for 20 minutes at room temperature. Cells were incubated with a mouse monoclonal anti- α -SMA (1:250 or 1:50, as indicated), followed by a rabbit anti-mouse IgG tetramethyl-rhodamine isothiocyanate conjugate (1:50; Santa Cruz Scientific, Santa Cruz, CA). Slides were examined using a Nikon (Melville, NY) PCM-2000 confocal microscope with a Nikon Eclipse TE300 microscope with a plan Apo 60 $\times$ /1.4 aperture oil immersion objective, 543-nm laser, and Simple PCI software from the C-Imaging series from Nikon/Compix Inc. (Cranberry Township, PA). Values were obtained by counting the number of α -SMA-positive cells in 15 randomly selected fields in triplicate. Oil red O staining was performed by the Histology Core of the USC Research Center for Liver Disease and slides were stained with oil red O for 10 minutes, rinsed briefly with 70% ethanol, counterstained with Harris' hematoxylin, and then treated with saturated sodium bicarbonate solution.

Biochemical Assays

Serum aspartate amino transferase (AST) and alanine aminotransferase (ALT) were measured with the Infinity AST and ALT liquid assay reagent (catalog number TR70121 and TR71121; Thermo Electron Corporation, Louisville, CO). Serum triglycerides and cholesterol were measured with the Infinity cholesterol assay reagent (catalog number TR22321 and TR13315, Thermo Electron Corporation). Serum glucose was measured with a glucose (HK) assay kit (catalog number GAHK-20, Sigma). Serum insulin was measured with the Mercodia Ultrasensitive mouse insulin enzyme-linked immunosorbent assay kit (catalog number 10-1150-01; ALPCO Diagnostics, Salem, NH). The homeostasis model assessment insulin resistance index (HOMA-IR) was calculated as [fasting glucose (mmol/L) $\times$ fasting insulin (microunits/ml)]/22.5.^{17,18} Serum leptin was measured with the TiterZyme EIA mouse leptin kit (catalog number 900-019; Assay Designs Inc., Ann Arbor, MI). Serum adiponectin was measured with the mouse adiponectin kit (catalog number RD 293023100; Biovendor LLC, Candler, NC). Thio-barbituric acid reactive substance (TBARS) was measured as previously described;^{19,20} in brief, liver was homogenized and diluted 1:1 in 10% ice cold trichloroacetic acid. Supernatant or a standard solution of 1,1,3,3-tetraethoxypropane (0.5 ml) was mixed with 0.5 ml of

Table 1. Biochemical Findings and Liver and Body Weights

	6-Month control	6-Month Western diet	7-Month Western diet followed by 2.5 months of		
			Western diet	Western diet + D-4F	Western diet + SR141716
Body weight (g)	28.1 ± 1.1	45.9 ± 1.2	40 ± 5.2	39.5 ± 0.8	29.1 ± 2.0
Liver weight (g)	1.34 ± 0.05	3.7 ± 0.2	2.74 ± 0.67	2.42 ± 0.04	1.54 ± 0.1
AST (U/L)	54.7 ± 10.0	250.7 ± 27.1	268 ± 29.6	211.9 ± 19.1	109.6 ± 10.4
ALT (U/L)	18.8 ± 4.0	300.0 ± 37.1	235.2 ± 23.5	277.3 ± 18.2	54.8 ± 12.4
Cholesterol (mg/dl)	90.0 ± 5.1	274.1 ± 22.1	288.0 ± 5.4	146.8 ± 2.1	112.2 ± 13.7
Triglycerides (mg/dl)	50.7 ± 7.7	32.4 ± 5.6	38.0 ± 4.4	40.0 ± 6.7	33.1 ± 2.2
Glucose (mg/dl)	65.0 ± 1.2	261.0 ± 24.2	270.2 ± 10.0	231.2 ± 18.0	136.3 ± 29.2
Insulin (ng/ml)	0.47 ± 0.06	2.21 ± 0.09	3.27 ± 3.0	3.7 ± 1.1	0.81 ± 0.15
HOMA-IR	2.2 ± 0.3	40.9 ± 4.2	62.8 ± 5.1	63.3 ± 23.5	8.8 ± 3.6
TBARS (nmol/g tissue)	18.7 ± 1.2	16.4 ± 0.4	27.1 ± 4.0	29.4 ± 2.4	17.1 ± 0.7
GSH (nmol/mg protein)	91.8 ± 3.8	71.2 ± 3.9	39.6 ± 4.8	50.1 ± 5.8	68.7 ± 2.0
Leptin (ng/ml)	1.89 ± 0.29	24.0 ± 2.2	32.62 ± 1.86	17.49 ± 1.41	2.36 ± 1.05
Adiponectin (μg/ml)	22.9 ± 0.3	31.1 ± 1.9	17.3 ± 0.8	15.3 ± 1.0	18.4 ± 1.8
	6-Month control	6-Month Western diet	7-Months Western diet followed by 2.5 months of		
			Chow	Chow + D-4F	Chow + SR141716
Body weight (g)	28.1 ± 1.1	45.9 ± 1.2	34.6 ± 1.2	35.9 ± 3.2	30.2 ± 1.1
Liver weight (g)	1.34 ± 0.05	3.7 ± 0.2	1.54 ± 0.09	1.67 ± 0.19	1.39 ± 0.1
AST (U/L)	54.7 ± 10.0	250.7 ± 27.1	107.2 ± 20.5	104.2 ± 22.4	69.1 ± 5.5
ALT (U/L)	18.8 ± 4.0	300.0 ± 37.1	44.0 ± 4.0	69.1 ± 21.5	59.1 ± 11.4
Cholesterol (mg/dl)	90.0 ± 5.1	274.1 ± 22.1	108.6 ± 4.7	120.8 ± 2.9	105.3 ± 4.7
Triglycerides (mg/dl)	50.7 ± 7.7	32.4 ± 5.6	36.2 ± 6.6	49.1 ± 1.4	47.0 ± 2.2
Glucose (mg/dl)	65.0 ± 1.2	261.0 ± 24.2	137.2 ± 18.6	159.2 ± 2.4	96.2 ± 3.0
Insulin (ng/ml)	0.47 ± 0.06	2.21 ± 0.09	1.78 ± 0.39	1.46 ± 0.2	0.89 ± 0.27
HOMA-IR	2.2 ± 0.3	40.9 ± 4.2	18.1 ± 5.1	16.5 ± 2.2	6.1 ± 2.0
TBARS (nmol/g tissue)	18.7 ± 1.2	16.4 ± 0.4	20.4 ± 0.3	20.0 ± 0.6	18.7 ± 1.09
GSH (nmol/mg protein)	91.8 ± 3.8	71.2 ± 3.9	85.1 ± 3.8	82.8 ± 5.2	73.8 ± 2.2
Leptin (ng/ml)	1.89 ± 0.29	24.0 ± 2.2	9.36 ± 0.76	8.70 ± 1.43	1.95 ± 0.72
Adiponectin (μg/ml)	22.9 ± 0.3	31.1 ± 1.9	19.3 ± 1.1	18.52 ± 1.5	16.5 ± 2.0

0.6% (w/v) 4,6-dihydroxy-2-thiopyrimidine. The solution was boiled for 30 minutes, cooled, and centrifuged. Absorbance was measured at 532 nm with a Shimadzu (Kyoto, Japan) UV-2101 PC spectrophotometer. To measure hepatic glutathione (GSH), liver was homogenized and diluted 1:1 in 10% ice-cold trichloroacetic acid. GSH in the supernatant was measured according to the recycling assay of Tietze.²¹

Statistical Analysis

Groups were compared by analysis of variance with post hoc analysis by least significant difference. $P < 0.05$ was considered statistically significant.

Results

Western Diet Model

Group 1 (Western diet for 9.5 months) had a 43% increase in body weight, hypercholesterolemia, hyperglycemia, hyperinsulinemia, marked insulin resistance (elevated HOMA-IR), and hyperleptinemia (Table 1). Liver weight was twice that of controls, AST and ALT were

elevated 5- to 12-fold, and there was evidence of oxidative stress in the liver with increased tissue TBARS and decreased GSH. Liver histology showed diffusely distributed marked steatosis, foci of mononuclear inflammatory cells, but no hydropic change in the hepatocytes (Table 2 and Figure 2). There was marked fibrosis with $4.6 \pm 1.9\%$ of surface area positive for Sirius red staining by whole section-scanning morphometric image analysis (Figures 2 and 3). Consistent with the fibrosis, there was increased α -SMA staining (Figure 4).

There were substantial differences in the model after 9.5 months on the Western diet versus 6 months: more pronounced hyperinsulinemia and insulin resistance (HOMA-IR), more evidence of oxidative stress in the liver with higher tissue TBARS and lower hepatic GSH, and higher leptin levels at 9.5 months (Table 1). The major histological difference (Table 2 and Figure 3) was that there was significantly less fibrosis after 6 than after 9.5 months of the Western diet. The surface area of fibrosis by whole section-scanning morphometric image analysis was $0.13 \pm 0.01\%$ for the 6-month control, $0.42 \pm 0.03\%$ after 6 months on the Western diet, and $4.6 \pm 1.9\%$ after 9.5 months on the Western diet ($P < 0.01$ for 6-month control versus 6 months of Western diet; $P < 0.01$ for 6 months versus 9.5 months

Table 2. Assessment of Steatosis and Inflammation

7 Months of Western diet followed by 2.5 months of	Fatty change		Inflammatory clusters [†]
	Degree of fat	Type*	
Western diet	4	Macrovesicular	4
	4	Mixed	3
	1	Macrovesicular	4
Western diet + D-4F	4	Macrovesicular	3
	4	Macrovesicular	3
	4	Macrovesicular	2
	4	Macrovesicular	3
Western diet + SR141716	4	Microvesicular	1
	3	Mixed	2
	±	Macrovesicular	2
	1	Mixed	2
	4	Microvesicular	2
Chow	2	Mixed	2
	1	Mixed	3
	1	Microvesicular	2
	1	Mixed	3
Chow + D-4F	2	Mixed	1
	0		2
	2	Mixed	2
	3	Mixed	2
	3	Microvesicular	2
Chow + SR141716	2	Microvesicular	2
	1	Microvesicular	2
	3	Microvesicular	1

*The type of fatty change listed indicates the predominant type present. Steatosis was diffusely distributed in mice that remained on the Western diet with or without D-4F, and perivenular in mice that switched to chow with or without D-4F; steatosis was diffuse or perivenular in mice on SR141716.

[†]Inflammatory clusters were composed of mononuclear cells.

Western diet). Electron microscopy of mice after 6 months on the Western diet demonstrated early capillarization with defenestration of sinusoidal endothelial cells (Figures 5 and 6) and formation of a basal lamina (Figure 6). Scanning electron microscopy demonstrated marked distortion of the sinusoids by fat-laden hepatocytes (Figure 6).

SR141716

In group 2 (Western diet with addition of SR141716 for the last 2.5 months), body weight decreased back to the normal range within 1 week (data not shown) and remained stable until mice were sacrificed. Compared to group 1 (9.5 months Western diet), group 2 demonstrated improvement in hyperglycemia, hyperinsulinemia and insulin resistance (HOMA-IR), and hypercholesterolemia (Table 1). Liver weight returned to near normal, AST and ALT improved, leptin levels dropped markedly, and evidence of oxidative stress (hepatic TBARS and GSH) improved. Histologically, inflammation was reduced. Hepatic steatosis was decreased in three of five mice and was more commonly predominantly microvesicular in pattern, but the marked decrease in liver weight in all mice treated with SR141716 suggests that histological assessment underestimates the decline in steatosis (Table 2). The area of fibrosis was reduced from $4.6 \pm 1.9\%$ in group 1 to $1.2 \pm 0.3\%$ in group 2 ($P < 0.005$; Figures 2 and 3) and α -SMA staining was decreased (Figure 4).

Figure 2. Liver histology. Representative sections of liver after 9.5 months. **A:** H&E stain; **B-G:** Sirius red stain. **A:** Liver after 9.5 months of Western diet (group 1) demonstrates steatohepatitis; **B:** liver after 9.5 months of Western diet (group 1) demonstrates fibrosis; **C:** 9.5 months of Western diet with D-4F during last 2.5 months (group 3); **D:** 9.5 months of Western diet with SR141716 during last 2.5 months (group 2); **E:** 7 months of Western diet followed by 2.5 months of standard chow (group 4); **F:** 7 months of Western diet followed by 2.5 months of standard chow plus D-4F (group 6); **G:** 7 months of Western diet followed by 2.5 months of standard chow plus SR141716 (group 5).

When compared to group 4 (switch to chow), group 5 (switch to chow plus SR141716) demonstrated greater decreases in body weight and liver weight, lower AST levels, greater improvement in hyperglycemia, hyperinsulinemia and insulin resistance (HOMA-IR), and in hyperleptinemia. Steatohepatitis was comparable, but the histological assessment underestimates the improvement in steatosis because the chow plus SR141716 group had normalization of liver weight and the group on chow alone did not. The switch to chow alone (group 4) did not significantly reduce progression of fibrosis unless SR141716 was added (group 5) (Figures 2 and 3).

Comparison of group 2 (Western diet, SR141716) versus group 4 (switch to chow) demonstrated that SR141716 more markedly reduced evidence of hyperinsulinemia and insulin resistance than the change in diet did (Table 1). Liver weight, AST and ALT, and histological evidence of inflammation were comparable in both groups. Even though group 2 had slightly

Figure 3. Morphometric image analysis. The figure demonstrates whole section-scanning morphometric image analysis of slides stained with Sirius red to determine extent of fibrosis. Control groups: 6-month control (open bar), 6 months of feeding with the Western diet (checked bars). Treatment groups received 7 months of Western diet followed by: 2.5 months of Western diet or chow as indicated (black bar), 2.5 months of D-4F and either Western diet or chow as indicated (hatched bar), or 2.5 months of SR141716 and either Western diet or chow as indicated (dotted bar). Single factor analysis of variance for all groups $P < 0.01$; * $P < 0.005$ by least significant difference compared to Western diet; § $P < 0.001$ compared to Western diet and $P = 0.05$ compared to chow for the last 2.5 months by least significant difference; †no significant difference versus 9.5 months of Western diet.

higher leptin levels, somewhat greater evidence of oxidative stress, and somewhat more steatosis, there was less progression of fibrosis in group 2: when contrasted with the Western diet (group 1), group 4 did not have a significant decrease in fibrosis and group 2 did (Figures 2 and 3).

D-4F

When mice received the Western diet for 9.5 months with addition of D-4F for the last 2.5 months (group 3), total cholesterol and leptin levels were decreased by half compared to mice on Western diet alone (group 1). There was no improvement in body weight, liver weight, serum transaminases, hyperglycemia, hyperinsulinemia, insulin resistance, or overall hepatic oxidative stress with D-4F

(Table 1). Histologically, there was no effect on steatosis and some evidence of a reduction in inflammation (Table 2). Nevertheless, D-4F reduced fibrosis from $4.6 \pm 1.9\%$ in group 1 to 1.25% in group 3 ($P < 0.005$; Figures 2 and 3).

Comparison of group 4 (switch to chow for the last 2.5 months) with group 6 (switch to chow plus D-4F) demonstrated less fibrosis in the group receiving D-4F: $0.9 \pm 0.2\%$ fibrosis in group 6 versus $2.8 \pm 0.7\%$ fibrosis in group 4 ($P = 0.05$) and perhaps a small decrease in inflammation (Table 2). No other effect was observed with chow plus D-4F compared to chow alone (Table 1).

Comparison of group 3 (Western diet, D-4F) with group 4 (switch to chow) demonstrated that group 4 had greater improvement in body and liver weight, steatohepatitis (AST, ALT, histological steatosis), hypercholesterolemia, hyperglycemia, insulin resistance, hyperleptinemia, and overall hepatic oxidative stress (TBARS, GSH). However, group 3 had a significant reduction in progression of fibrosis and group 4 did not (Figures 2 and 3).

To examine the mechanism of action of D-4F, murine HSCs were cultured on plastic for 7 days in the absence or presence of $10 \mu\text{g/ml}$ of D-4F. Without D-4F, $87 \pm 2\%$ of cells expressed α -SMA after 7 days as assessed by

Figure 4. α -SMA staining. **A:** Control; **B:** 9.5 months of Western diet; **C:** 7 months of Western diet followed by 2.5 months of chow; **D:** 9.5 months of Western diet with addition of SR141716 during the last 2.5 months; **E:** 9.5 months of Western diet with addition of D-4F during the last 2.5 months. α -SMA (green) stains activated HSCs. Erythrocytes (red) are visible in venules and sinusoids.

Figure 5. Transmission electron micrograph of the liver of a mouse that received 6 months of the Western diet, illustrating thickened sinusoidal endothelium (E), and a subendothelial basal lamina (arrow) and collagen deposition (CF) in the space of Disse. S, sinusoid lumen. Original magnification, $\times 6000$.

Figure 6. Scanning electron micrographs of livers from lean mice fed a normal chow diet (**A**, **C**) compared to obese mice fed the Western diet for 6 months (**B**, **D**). Compare the loss of fenestrae in **D** versus **C**. Note the sinusoids and their pattern distorted by the enlarged, fat-laden parenchymal cells in **B** versus **A**. The arrow in **D** points to the compression of the sinusoid caused by a ballooning hepatocyte containing a large fat droplet. Original magnifications: $\times 1000$ (**A**, **B**); $\times 8000$ (**C**, **D**).

confocal microscopy. In contrast, in the presence of D-4F 0% of HSCs expressed α -SMA after 7 days (see Figure 7). HSCs cultured in the presence of D-4F also maintained cytoplasmic fat droplets, in contrast to HSCs cultured without D-4F (Figure 7).

Discussion

C57BL/6J mice given 9.5 months of a high saturated fat diet formulated to approximate the typical human diet in North America and Europe¹³ developed nonalcoholic steatohepatitis with significant fibrosis and signs of the metabolic syndrome (obesity, insulin resistance, hyperglycemia, and hypercholesterolemia). This provides a model to examine interventions that prevent progression of fibrosis and to correlate the degree of fibrosis with features of steatohepatitis and the metabolic syndrome.

In mice remaining on the Western diet, SR141716 markedly improved body weight, liver weight, serum transaminases, insulin resistance, hyperglycemia, hypercholesterolemia, hyperleptinemia, and oxidative stress. This was accompanied with significant prevention of progression of fibrosis. In mice remaining on the Western diet and treated with D-4F there was improvement in hypercholesterolemia and hyperleptinemia, no improvement in body weight, steatohepatitis, insulin resistance, or oxidative stress, and yet there was significant prevention of fibrosis.

From a clinical perspective, the most important comparison may be the effect of switching to chow versus the use of either SR141716 or D-4F with continuation of the Western diet, ie, comparing the effect of switching to a healthier diet with the effect of medication and a continued high-fat diet. Treatment with SR141716 with continuation of the Western diet significantly prevented progression of fibrosis, whereas the switch to chow somewhat decreased fibrosis but not to a statistically

significant degree. SR141716 is known to prevent fibrosis by blocking CB1 in disparate models of experimental fibrosis.² The reduction in steatohepatitis and markers of oxidative stress was similar in mice receiving treatment with SR141716 with continuation of the Western diet and mice that were switched to chow. SR141716 more markedly reduced hyperinsulinemia and insulin resistance and nearly normalized leptin levels. The fall in leptin may have contributed to the antifibrotic effect.^{22–25} Adiponectin levels did not increase in mice treated with SR141716, in contrast to the marked increase in adiponectin seen in obese *fa/fa* rats treated with the drug²⁶; the difference in adiponectin response to SR141716 may reflect differences between the models used.

D-4F prevented progression of fibrosis to a comparable degree to SR141716 in mice continued on the Western diet. D-4F had no effect on steatohepatitis, and no effect on overall oxidative stress in liver tissue. The only extrahepatic effects of D-4F that were observed were reduction in leptin and cholesterol in the mice continued on the Western diet. However the combination of D-4F and a switch to chow reduced fibrosis more than chow alone and in this group D-4F had no additive effect on cholesterol and leptin compared to the switch to chow alone. The *in vitro* studies demonstrated that D-4F prevented HSC activation *in vitro*, which suggests that D-4F prevented fibrosis in the nonalcoholic fatty liver disease model through a more general antifibrotic effect.

Fat-laden hepatocytes are swollen, and further swelling occurs in nonalcoholic steatohepatitis because of hydropic change (ballooning) of hepatocytes. As can be appreciated in the scanning electron microscopy (Figure 6), such swelling causes sinusoidal distortion. *In vivo* microscopy of fatty liver demonstrates sinusoidal distortion with reduced intrasinusoidal volume and microvascular blood flow. Involvement of other cell types (sinusoi-

Figure 7. Effect of D-4F on primary murine HSCs. HSCs were cultured for 7 days in the presence (B, C, F) or absence (A, E) of D-4F. α -SMA staining (α -SMA antibody, 1:250 dilution) demonstrates that 87% of cells cultured in the absence of D-4F are positive for α -SMA (A), whereas none of the stellate cells cultured in the presence of D-4F express α -SMA fibers (B). C: Higher concentrations of α -SMA antibody (α -SMA antibody, 1:50 dilution) demonstrate the characteristic compact morphology of quiescent stellate cells after 7 days culture in the presence of D-4F. D: Oil red O staining of stellate cells after 1 day in culture demonstrates the characteristic lipid droplets. Stellate cells cultured for 7 days without D-4F demonstrate few lipid droplets (E), whereas stellate cells cultured in the presence of D-4F maintain significantly more lipid droplets (F).

dal endothelial cells, Kupffer cells, stellate cells) and recruitment of inflammatory cells and platelets lead to dysregulation of microvascular blood flow by adherent inflammatory cells and perisinusoidal matrix deposition (fibrosis). In animal models, the net effect of such changes is a marked reduction of sinusoidal space (~50% of control), and a decrease in the number of normally perfused sinusoids. It has been proposed that such microvascular damage could accentuate further liver injury and disease progression in nonalcoholic steatohepatitis.^{27,28}

In summary, both SR141716 and D-4F prevented progression of fibrosis after onset of steatohepatitis, ie, a situation comparable to a common clinical scenario, and either compound therefore has the potential to be of clinical benefit. SR141716 promoted weight loss, improved steatohepatitis, decreased insulin resistance, decreased leptin levels, and prevented fibrosis through antagonism of CB1. D-4F did not alter steatohepatitis, but

prevented stellate cell activation *in vitro* and may therefore be a more general antifibrotic agent.

References

- Osei-Hyiaman D, DePetrillo M, Pacher P, Liu J, Radaeva S, Batkai S, Harvey-White J, Mackie K, Offertaler L, Wang L, Kunos G: Endocannabinoid activation at hepatic CB1 receptors stimulates fatty acid synthesis and contributes to diet-induced obesity. *J Clin Invest* 2005, 115:1298–1305
- Teixeira-Clerc F, Julien B, Grenard P, Tran Van Nhieu J, Deveaux V, Li L, Serriere-Lanneau V, Ledent C, Mallat A, Lotersztajn S: CB1 cannabinoid receptor antagonism: a new strategy for the treatment of liver fibrosis. *Nat Med* 2006, 12:671–676
- Julien B, Grenard P, Teixeira-Clerc F, Van Nhieu JT, Li L, Karsak M, Zimmer A, Mallat A, Lotersztajn S: Antifibrogenic role of the cannabinoid receptor CB2 in the liver. *Gastroenterology* 2005, 128:742–755
- Navab M, Anantharamaiah GM, Hama S, Garber DW, Chaddha M, Hough G, Lallone R, Fogelman AM: Oral administration of an Apo A-I mimetic peptide synthesized from D-amino acids dramatically reduces atherosclerosis in mice independent of plasma cholesterol. *Circulation* 2002, 105:290–292
- Anantharamaiah GM, Mishra VK, Garber DW, Datta G, Handattu SP, Palgunachari MN, Chaddha M, Navab M, Reddy ST, Segrest JP, Fogelman AM, Anantharamaiah GM, Mishra VK, Garber DW, Datta G, Handattu SP, Palgunachari MN, Chaddha M, Navab M, Reddy ST, Segrest JP, Fogelman AM: Structural requirements for antioxidative and anti-inflammatory properties of apolipoprotein A-I mimetic peptides. *J Lipid Res* 2007, 48:1915–1923
- Navab M, Anantharamaiah GM, Reddy ST, Hama S, Hough G, Grijalva VR, Wagner AC, Frank JS, Datta G, Garber D, Fogelman AM: Oral D-4F causes formation of pre-beta high-density lipoprotein and improves high-density lipoprotein-mediated cholesterol efflux and reverse cholesterol transport from macrophages in apolipoprotein E-null mice. *Circulation* 2004, 109:3215–3220
- MacDonald GA, Bridle KR, Ward PJ, Walker NI, Houghlum K, George DK, Smith JL, Powell LW, Crawford DH, Ramm GA: Lipid peroxidation in hepatic steatosis in humans is associated with hepatic fibrosis and occurs predominately in acinar zone 3. *J Gastroenterol Hepatol* 2001, 16:599–606
- Nieto N, Friedman SL, Cederbaum AI: Cytochrome P450 2E1-derived reactive oxygen species mediate paracrine stimulation of collagen I protein synthesis by hepatic stellate cells. *J Biol Chem* 2002, 277:9853–9864
- Poli G: Pathogenesis of liver fibrosis: role of oxidative stress. *Mol Aspects Med* 2000, 21:49–98
- Schneiderhan W, Schmid-Kotsas A, Zhao J, Grunert A, Nussler A, Weidenbach H, Menke A, Schmid RM, Adler G, Bachem MG: Oxidized low-density lipoproteins bind to the scavenger receptor, CD36, of hepatic stellate cells and stimulate extracellular matrix synthesis. *Hepatology* 2001, 34:729–737
- Svegliati Baroni G, D'Ambrosio L, Ferretti G, Casini A, Di Sario A, Salzano R, Ridolfi F, Saccomanno S, Jezequel AM, Benedetti A: Fibrogenic effect of oxidative stress on rat hepatic stellate cells. *Hepatology* 1998, 27:720–726
- Tsukamoto H, Rippe R, Niemela O, Lin M: Roles of oxidative stress in activation of Kupffer and Ito cells in liver fibrogenesis. *J Gastroenterol Hepatol* 1995, 10(Suppl 1):S50–S53
- Merat S, Casanada F, Sutphin M, Palinski W, Reaven PD: Western-type diets induce insulin resistance and hyperinsulinemia in LDL receptor-deficient mice but do not increase aortic atherosclerosis compared with normoinsulinemic mice in which similar plasma cholesterol levels are achieved by a fructose-rich diet. *Arterioscler Thromb Vasc Biol* 1999, 19:1223–1230
- McCuskey RS: Microscopic Methods for Studying the Microvasculature of Internal Organs. Edited by Baker CH, Nastuk WF. New York, Academic Press, 1986, pp 247–264
- McCuskey RS, McCuskey PA: The fine structure and function of Kupffer cells. *J Electron Microscop Techniques* 1990, 14:237–246
- Tsukamoto H, Townner SJ, Ciofalo LM, French SW: Ethanol-induced liver fibrosis in rats fed high fat diet. *Hepatology* 1986, 6:814–822
- Xia Z, Sniderman AD, Cianflone K: Acylation-stimulating protein (ASP)

- deficiency induces obesity resistance and increased energy expenditure in ob/ob mice. *J Biol Chem* 2002, 277:45874–45879
18. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC: Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* 1985, 28:412–419
19. Wong SHY, Knight JA, Hopfer SM, Zaharia O, Leach Jr CN, Sunderman Jr FW: Lipoperoxides in plasma as measured by liquid-chromatographic separation of malondialdehyde-thiobarbituric acid adduct. *Clin Chem* 1987, 33:214–220
20. Wallin B, Rosengren B, Shertzer HG, Camejo G: Lipoprotein oxidation and measurement of thiobarbituric acid reacting substances formation in a single microtiter plate—its use for evaluation of antioxidants. *Anal Biochem* 1993, 208:10–15
21. Tietze F: Enzymic method for quantitative determination of nanogram amounts of total and oxidised glutathione: applications to mammalian blood and other tissues. *Anal Biochem* 1969, 27:502–522
22. Saxena NK, Saliba G, Floyd JJ, Anania FA: Leptin induces increased alpha2 (I) collagen gene expression in cultured rat hepatic stellate cells. *J Cell Biochem* 2003, 89:311–320
23. Honda H, Ikejima K, Hirose M, Yoshikawa M, Lang T, Enomoto N, Kitamura T, Takei Y, Sato N: Leptin is required for fibrogenic responses induced by thioacetamide in the murine liver. *Hepatology* 2002, 36:12–21
24. Lin S, Saxena NK, Ding X, Stein LL, Anania FA: Leptin increases tissue inhibitor of metalloproteinase 1 (TIMP-1) gene expression by a specificity protein 1/signal transducer and activator of transcription 3 mechanism. *Mol Endocrinol* 2006, 20:3376–3388
25. Ikejima K, Takei Y, Honda H, Hirose M, Yoshikawa M, Zhang Y-J, Lang T, Fukuda T, Yamashina S, Kitamura T, Sato N: Leptin receptor-mediated signaling regulates hepatic fibrogenesis and remodeling of extracellular matrix in the rat. *Gastroenterology* 2002, 122:1399–1410
26. Gary-Bobo M, Elachouri G, Gallas JF, Janiak P, Marini P, Ravinet-Trillou C, Chabbert M, Cruccioli N, Pfersdorff C, Roque C, Arnone M, Croci T, Soubrié P, Oury-Donat F, Maffrand JP, Scatton B, Lacheretz F, Le Fur G, Mohammed Bensaid JMH: Rimonabant reduces obesity-associated hepatic steatosis and features of metabolic syndrome in obese Zucker fa/fa rats. *Hepatology* 2007, 46:122–129
27. Farrell GC, Teoh NC, McCuskey RS: The hepatic microcirculation in fatty liver disease. *Anat Rec* 291:684–692
28. McCuskey RS, Ito Y, Robertson GR, McCuskey MK, Perry M, Farrell GC: Hepatic microvascular dysfunction during evolution of dietary steatohepatitis in mice. *Hepatology* 2004, 40:386–393