GASTROINTESTINAL, HEPATOBILIARY, AND PANCREATIC PATHOLOGY

Beneficial Effects of the Peroxisome Proliferator-Activated Receptor α/γ Agonist Aloglitazar on Progressive Hepatic and Splanchnic Abnormalities in Cirrhotic Rats with Portal Hypertension

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Recent studies have reported that peroxisome proliferator-activated receptor α (PPAR α) agonist decreases intrahepatic resistance, whereas PPAR γ agonist reduces portosystemic shunts (PSSs) and splanchnic angiogenesis in cirrhotic rats. The present study investigated the effects of a 21-day treatment with the dual PPAR α/γ agonist aloglitazar (Ale) on progressive abnormalities in bile-duct–ligated and thioacetamide-induced cirrhotic rats with portal hypertension (PH). *In vivo* and *in vitro* effects were evaluated. Chronic Ale treatment significantly up-regulated PPAR α /PPAR γ receptors and down-regulated tumor necrosis factor- α (TNF- α) and NF- κ B expression in the liver, splanchnic tissues, collateral vessels, and intestines of cirrhotic rats with PH. Notably, Ale improved PH by the suppression of systemic/tissue inflammation, hepatic fibrosis, hepatic Rho-kinase–mediated endothelin-1 hyperresponsiveness, intrahepatic/mesenteric angiogenesis, vascular endothelial growth factor expression, PSS, intestinal mucosal injury, and hyperpermeability in cirrhotic rats. Acute Ale treatment inhibited TNF- α –enhanced endothelin-1–induced contraction of primary hepatic stellate cells, vascular endothelial growth factor–induced migration/angiogenesis of liver sinusoidal endothelial cells, and TNF- α –induced disruption of Caco-2 cell monolayer-epithelial barrier. The present study suggested that Ale can potentially treat relevant abnormalities through the inhibition of inflammatory, vasoconstrictive, angiogenic, and mucosal-disrupted pathogenic markers in cirrhosis. Overall, chronic Ale treatment ameliorated PH syndrome by the suppression of hepatic fibrogenesis, neoangiogenesis, vasoconstrictor hyperresponsiveness, splanchnic vasodilatation, and PSS; and decreased intestinal mucosal injury and hyperpermeability in cirrhotic rats. (*Am J Pathol* 2018, 188: 1608–1624; <https://doi.org/10.1016/j.ajpath.2018.03.018>)

Increased portal inflow resistance and splanchnic hyperdynamic circulation are the primary factors in the pathophysiology of portal hypertension (PH).¹ In cirrhotic livers, progressive fibrogenesis, intrahepatic neoangiogenesis, and enhanced vasoconstrictive response to endothelin-1 (ET-1) by the activated Rho-kinase–myosin light chain kinase pathway results in increased portal inflow resistance.^{2–6} Meanwhile, splanchnic vasodilatation, neoangiogenesis, and portosystemic collateralization contribute to splanchnic hyperdynamic circulation.^{1,7} Intestinal hyperpermeability

and associated systemic inflammation are important pathogenic factors in the development of hemodynamic disarrangement in cirrhosis.^{8,9} Nonselective β -blockers, which

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lack splanchnic and intestinal effects, have been the mainstay of pharmacologic therapy for PH, but with possible adverse effects, including dizziness, fatigue, cardiac insufficiency, bronchospasm, and decreased survival in patients with ascites.¹⁰ Therefore, there is an urgent need to find drugs that can simultaneously treat the multifaceted abnormalities of cirrhotic PH.

Both peroxisome proliferator-activated receptor α (PPAR α) and PPAR γ are expressed on vascular endothelial cells and regulate inflammation and neoangiogenesis.^{11–13} Specifically, PPAR α is predominantly expressed in the liver, whereas PPAR γ is primarily expressed in hepatic, immune, endothelial, and smooth muscle cells.^{11–13} PPAR α and PPAR γ agonists, such as fenofibrate and pioglitazone, are widely used to treat diabetes mellitus and hyperlipidemia.¹⁴ Interestingly, both PPAR α and PPAR γ agonists have been reported to have anti-inflammatory effects.^{12,15}

Studies in patients with primary biliary cirrhosis and nonalcoholic fatty liver disease have suggested that PPAR α and PPAR γ agonists can improve liver function.^{16–19} In carbon tetrachloride–induced cirrhotic rats, a 7-day regimen of fenofibrate significantly reduced portal pressure (PP) by decreasing hepatic fibrosis and intrahepatic resistance.²⁰ Nonetheless, in that study, splanchnic and collateral circulations were not evaluated in cirrhotic rats receiving fenofibrate treatment.²⁰ In bile duct–ligated cirrhotic and portal vein–ligated noncirrhotic rats, a 7-day regimen of pioglitazone significantly decreased portosystemic shunting (PSS) via inhibition of splanchnic inflammation and neoangiogenesis.²¹ Both in cirrhotic and noncirrhotic rats with PH in that study, PPAR γ agonist treatment did not reduce hepatic fibrosis, superior mesenteric arterial (SMA) blood flow (BF), and PP.²¹ However, hepatic microcirculation was not evaluated in that study.¹⁹ Moreover, benefits of fenofibrate and pioglitazone treatment, focusing on either hepatic or splanchnic circulation, can be explained by specific distribution of PPAR α and PPAR γ receptors in these circulations.^{20,21} Taken together,^{4,7,13,20,21} combined treatment with PPAR α and PPAR γ agonists may exert synergistic effects for simultaneously treating systemic, splanchnic, and hepatic hemodynamic dysarrangements in cirrhosis.

Clinically, glucose intolerance and diabetic mellitus are frequently reported in patients with cirrhosis.²² Nonetheless, the levels of lipids and lipoproteins in patients with cirrhosis diminish progressively with the increasing severity of the disease.²³ With safety concerns regarding the adverse effects, including fluid retention, weight gain, congestive heart failure, and elevation in markers of cardiovascular diseases (eg, homocysteine), renal disease (eg, creatinine), and liver dysfunction (eg, alanine aminotransferase and γ -glutamyl transpeptidase), long-term use of PPAR α and PPAR γ agonists is not suggested.^{24–26} Therefore, it is necessary to find new PPAR agonists that can be used for the long-term in chronic cirrhotic PH without severe adverse effects.

Recently, newly developed dual PPAR α/γ agonists, such as aleglitazar, muraglitazar, and tesaglitazar, with anti-inflammatory effects, have been reported.^{27–31} Until now, the therapeutic potentials of dual PPAR α/γ agonists have not been explored in cirrhotic rats with PH. Moreover, unlike muraglitazar, with higher affinity toward PPAR γ , and tesaglitazar, with higher affinity toward PPAR α , aleglitazar is a novel, potent, and neutral ligand for both PPAR α and PPAR γ .^{28,30,31}

In this study, we aimed to evaluate the possible systemic, splanchnic, and hepatic effects of chronic aleglitazar, a dual PPAR α/γ agonist, treatment in cirrhotic PH rats.

Materials and Methods

Approval for this study was obtained from the Institutional Ethics Review Committee of the University of Yang-Ming (Taipei, Taiwan). All procedures were performed according to the guidelines of the Institutional Animal Care and Use Committee at the University of Yang-Ming and the National Research Council's *Guide for the Care and Use of Laboratory Animals*.³²

Tumor necrosis factor- α (TNF- α), IL-1 β , transforming growth factor- β 1 (TGF- β 1), and vascular endothelial growth factor (VEGF) enzyme-linked immunosorbent assay kits were purchased from R&D Systems Inc. (Minneapolis, MN). Antibodies were purchased from Abcam (Cambridge, MA), for α -smooth muscle actin (α -SMA), IL-1 β , occludin, claudin-1, zonula occludens protein 1 (ZO-1), and p-VEGF receptor 2 (p-VEGFR2; Santa Cruz Biotechnology Dallas TX), for CD31, TNF- α , NF- κ Bp65, RhoA, Rho kinase, and p-moesin-thr-558; Cayman Chemical (Ann Harbor, MI), for PPAR α and PPAR γ ; and Invitrogen (Carlsbad, CA), for phosphorylated myosin light chain kinase. All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Animals

To develop cirrhotic animals with PH, thioacetamide (TAA; 200 mg/kg, three times per week) was administered for 12 weeks, and common bile duct ligation (BDL) was performed in male Sprague-Dawley rats (weighing 200 to 250 g). The dose of aleglitazar that significantly suppressed systemic and tissue inflammation was administered according to previous studies.^{30,31} For evaluation of therapeutic effects on progressive abnormalities of cirrhotic PH, aleglitazar [Ale (0.3 mg/kg per day), a dual PPAR α/γ agonist] was administered along with TAA to the rats for 21 days ($n = 9$) since 12 weeks after TAA administration. Meanwhile, similar dose and duration of aleglitazar was administered since 3 weeks after BDL in biliary cirrhotic rats with PH ($n = 9$). In parallel, sham-operated rats and TAA- or BDL-induced cirrhotic rats with PH receiving similar duration of vehicle (sterile saline solution) served as controls (sham-V, TAA-V, and BDL-V; $n = 6$). Body weight and energy consumption were recorded weekly throughout the experiments.

Table 1 Primer of Rat Gene Used for Quantitative Real-Time PCR Analysis

Gene	Primer sequence	
	Forward primer	Reverse primer
<i>Tnfa</i>	5'-GCTCACAATGTCTGTGCTTAGAG-3'	5'-GCAGTAGCCACAGCTCCAG-3'
<i>Tnfr2</i>	5'-GAATACTATCACCAGACAGCTCAGATGTGC-3'	5'-TATCCGTGGATGAAGTCGTGTTGGAGAACG-3'
<i>Nfkbp65</i>	5'-GGCCTCATCCACATGAACCT-3'	5'-CACTGTCACCTGGAAGCAGA-3'
<i>Il1b</i>	5'-ACAGATGAAGTGCTCCTTCCA-3'	5'-GTCGGAGATTTCGTAGCTGGAT-3'
<i>Col1a1</i>	5'-ACTGGTACATCAGCCCAAACCC-3'	5'-GGAATCCATCGGTTCATGCTCT-3'
<i>Ctgf</i>	5'-CAAGCTGCCCCGGGAAAT-3'	5'-CGGTCCTTGGGCTCATCA-3'
<i>Rhoa</i>	5'-GGCAGGATATGGCAAACAGG-3'	5'-TCCGTCTTTGGTCTTTGCTGA-3'
<i>Rock1</i>	5'-CCCGATCATCCCTAGAAC-3'	5'-TTGGAGCAAGCTGTCGACTG-3'
<i>Vegf</i>	5'-CTACCTCCACCATGCCAAGT-3'	5'-GCAGTAGCTGCGCTGATAGA-3'
<i>Angpt1</i>	5'-GGGAGGTTGGACTGTAAT-3'	5'-CTTTATCCCATTCAGTTT-3'
<i>Vegfr2</i>	5'-AAGCAAATGCTCAGCAGGAT-3'	5'-TAGGCAGGGAGAGTCCAGAA-3'
<i>Ppara</i>	5'-TGCGGACTACCAGTACTTAG-3'	5'-CGACACTCGATGTTTCAGTGC-3'
<i>Pparg2</i>	5'-CTCTGGGAGATTCTCCTGTTGA-3'	5'-GGTGGGCCAGAATGGCATCT-3'
<i>Ocln</i>	5'-TCCTATAAATCCACGCCGGTTC-3'	5'-CTCAAAGTTACCACCGCTGCTG-3'
<i>Cldn1</i>	5'-GCACATACCTTCATGTGGCTCAG-3'	5'-TGGAACAGAGCACAAACATGTCA-3'
<i>Zo1</i>	5'-CGGGACTGTTGGTATTGGCTAGA-3'	5'-GGCCAGGGCCATAGTAAAGTTTG-3'
<i>18s</i>	5'-GTAACCCGTTGAACCCCAT-3'	5'-CCATCCAATCGGTAGTAGCG-3'

Grouping

In the first series of experiments on BDL- and TAA-cirrhotic rats, systemic and splanchnic hemodynamics were measured. Then, *in situ* liver perfusion study was performed; at the same time, mesenteric vascular bed, splanchnic tissue, and collateral vessels were collected for various measurements.

In the second series of experiments, regional BF, PSS, portal venous inflow, and collateral BF were measured and calculated by the color microsphere method. In the third series of experiments, all rats were included for evaluation of intestinal permeability by fluorescein isothiocyanate–dextran–based and Evans Blue–permeated methods. Then, liver samples, aorta, splanchnic tissue, collateral vessels, and terminal ileum were collected to measure various pathogenic factors.

Hemodynamics and Measurement of Various Vasoactive Substances

Under ketamine (100 mg/kg) anesthesia, femoral veins and arteries/portal veins were cannulated with PE-50 tubing to monitor mean arterial pressure, heart rate, and PP as well as to withdraw blood samples. Cardiac output (CO) was measured using the transit-time ultrasound-dilution method. Stroke volume (mL/beat) was calculated as follows: CO (mL/minute)/heart rate (beats/minute). The cardiac index (CI) was then calculated using the following formula: CI = CO/body weight.

Systemic vascular resistance was calculated according to the following formula: systemic vascular resistance = mean arterial pressure (mm Hg) $\times$ 80/CI.

The ileocolic vein was cannulated with PE-10 tubing for measuring PP. PP was monitored using a polygraph (Gould Inc., Valley View, OH) via strain-gauge transducers (Viggo-Spectramed, Oxford, CA). Meanwhile, a flow probe

was placed around the SMA and connected to a flowmeter (Transonic Systems, Ithaca, NY) for continuous monitoring of SMA BF. The degree of mucosal damage in the terminal ileum lumen was assessed by a semiquantitative grading system.³³ Immunohistochemical staining of PPAR α , TNF- α , and NF- κ Bp65 indexes in ileal tissues was calculated as the product of the staining intensity score (0, 1, 2, and 3) and the proportion of positive cells (0, 1, 2, and 3). Serum TNF- α , VEGF, and IL-1 β levels were determined with commercially available enzyme-linked immunosorbent assay kits (R&D Systems).

In Situ Liver Perfusion Experiment

In situ liver perfusion was performed in a recirculating system, as previously described.^{3,20} Portal perfusion pressure (PPP)–response curves, which represented intrahepatic resistance (IHR) to ET-1 (10^{-10} , 10^{-9} , 3×10^{-9} , 10^{-8} , and 3×10^{-8} mol/L), were evaluated in perfused rat livers to explore the effects of chronic aleglitazar treatment on hepatic vasoconstrictor ET-1 hyperresponsiveness. In cirrhotic rat liver, acute effect of aleglitazar on PPP–response curves to ET-1 was assessed after pretreatment with vehicle or aleglitazar (100 nmol/L) 30 minutes before perfusion. IHR was calculated according to the formula: IHR (mm Hg $\cdot$ mL $\cdot$ minute $\cdot$ g) = PPP $\cdot$ Q $\cdot$ liver weight (wet), where PPP is the portal perfusion pressure (mm Hg) corrected for the resistance of the portal vein cannula and Q is the constant flow rate (20 mL/minute). Preliminary studies were performed with 10, 100, 300, and 500 nmol/L of aleglitazar to determine the lowest effective concentration to attenuate hepatic ET-1 hyperresponsiveness, which was 100 nmol/L. In addition, the role of Rho kinase was examined on the effects of aleglitazar on hepatic ET-1 hyperresponsiveness by a Rho-kinase inhibitor (Y-27632; 10

Table 2 Effects of Ale (Dual PPAR α/γ Agonist) Treatment on the Metabolic Parameters and Cytokines

Metabolic parameter/cytokine	Sham-V	BDL-V	BDL-Ale	TAA-V	TAA-Ale
Body weight, g	456 $\pm$ 10	380 $\pm$ 7	398 $\pm$ 13	402 $\pm$ 11	409 $\pm$ 8
Liver weight, g	21.5 $\pm$ 1.4	19.3 $\pm$ 0.8	20.6 $\pm$ 1.7	20.4 $\pm$ 3.1	21.6 $\pm$ 2
Serum cholesterol, mg/dL	84.2 $\pm$ 3.5	89 $\pm$ 4	79 $\pm$ 13	80 $\pm$ 11	78 $\pm$ 14
Serum triglycerides, mg/dL	47.1 $\pm$ 5.8	43 $\pm$ 10	39 $\pm$ 16	41 $\pm$ 8	40 $\pm$ 9
Serum alanine aminotransferase, IU/L	16.7 $\pm$ 1.5	110 $\pm$ 6 ^{††}	102 $\pm$ 11	96 $\pm$ 13 [†]	89 $\pm$ 7
Serum aspartate aminotransferase, IU/L	18.9 $\pm$ 2.3	250 $\pm$ 11 ^{††}	244 $\pm$ 33	180 $\pm$ 5 [†]	178 $\pm$ 9
Serum albumin, g/L	3.6 $\pm$ 2.3	2.7 $\pm$ 1.5 [†]	3.1 $\pm$ 0.9*	2.9 $\pm$ 1.2 [†]	3.3 $\pm$ 0.2*
Serum tumor necrosis factor- α level, pg/mL	30.2 $\pm$ 6.4	81.3 $\pm$ 6.2 [†]	44 $\pm$ 7.6*	85 $\pm$ 12 [†]	40 $\pm$ 5.1*
Serum IL-1 β level, pg/mL	132 $\pm$ 11	180 $\pm$ 23 [†]	166 $\pm$ 9	158 $\pm$ 7 [†]	142 $\pm$ 14
Serum vascular endothelial growth factor level, pg/mL	6.8 $\pm$ 3.1	75 $\pm$ 11 ^{††}	68 $\pm$ 17	64 $\pm$ 9 [†]	59 $\pm$ 8
Hepatic hydroxyproline content, μ g/g tissue	514 $\pm$ 72	1215 $\pm$ 246 ^{††}	789 $\pm$ 115*	1302 $\pm$ 308 ^{††}	695 $\pm$ 88*
Hepatic TGF- β 1 content, ng/mg tissue	35.2 $\pm$ 3.9	81.3 $\pm$ 7.2 ^{††}	52.4 $\pm$ 8.6*	85.4 $\pm$ 3.2 ^{††}	80.9 $\pm$ 5.5*

Data are expressed as means $\pm$ SD.

* P < 0.05 versus BDL-V/TAA-V rats.

[†] P < 0.05, ^{††} P < 0.01 versus sham-V rats.

Ale, aleglitazar; BDL, bile duct ligation; PPAR, peroxisome proliferator-activated receptor; TAA, thioacetamide; TGF- β 1, transforming growth factor- β 1 (a fibrogenesis-driving cytokine); V, vehicle.

μ mol/L) by Y-27632 preincubation 30 minutes before aleglitazar (100 nmol/L) administration. Similarly, the role of PPAR γ on aleglitazar-related effects on ET-1 hyper-responsiveness was evaluated by preincubation of PPAR γ antagonist (GW9662; 10 μ mol/L) before aleglitazar administration in the isolated liver perfusion system.

Hepatic Angiogenic, Fibrogenic, and Rho-Kinase Pathway Markers

In particular, protein and mRNA expression levels of hepatic PPAR α , PPAR γ , TNF- α , NF- κ Bp65, IL-1 β , collagen I, connective tissue growth factor (CTGF), VEGF, RhoA,

Rho kinase, p-moesin-thr-558, and phosphorylated myosin light chain kinase were measured using appropriate antibodies and primers (Table 1). Hepatic fibrosis was determined with Sirius Red and α -SMA staining. In addition, hepatic hydroxyproline content and TGF- β 1 levels were measured. For hepatic hydroxyproline content measurement, three small fragments of each liver were pooled, homogenized in distilled water, lyophilized, and hydrolyzed overnight in 6 N HCl at 110°C (10 mg of dry liver power/mL HCl 6 N). The hydrolysates were then treated with activated charcoal, filtered, evaporated, and resuspended in distilled water. Aliquots of the hydrolysates were used to measure hydroxyproline content spectrophotometrically by

Table 3 Effects of Ale (Dual PPAR α/γ Agonist) Treatment on the Splanchnic and Hepatic Hemodynamic Parameters

Hemodynamic parameter	Sham-V	BDL-V	BDL-Ale	TAA-V	TAA-Ale
Portal pressure, mm Hg	5.6 $\pm$ 0.5	16.7 $\pm$ 1.8 ^{††}	12.9 $\pm$ 1.1*	15.4 $\pm$ 2.0 ^{††}	11.2 $\pm$ 1.5*
Superior mesenteric arterial blood flow, mL/minute	2.8 $\pm$ 0.9	7.9 $\pm$ 0.1 [†]	5.1 $\pm$ 0.3*	6.8 $\pm$ 0.4 [†]	4.9 $\pm$ 0.2*
Superior mesenteric arterial resistance, mm Hg/mL per minute/100 g	34.8 $\pm$ 3.5	8.6 $\pm$ 0.9 ^{††}	16.3 $\pm$ 3.5**	9.5 $\pm$ 1.1 ^{††}	16.1 $\pm$ 3.9**
Portosystemic shunting, %	-	88 $\pm$ 6	73 $\pm$ 8*	81 $\pm$ 7	69 $\pm$ 10*
Portal venous inflow, mL/minute per 100 g	4.0 $\pm$ 0.3	5.8 $\pm$ 0.9 [†]	4.3 $\pm$ 0.5*	5.7 $\pm$ 0.4 [†]	4.5 $\pm$ 0.6*
Collateral blood flow, mL/minute $\times$ 100 g	-	3.7 $\pm$ 0.2	1.9 $\pm$ 0.5*	3.8 $\pm$ 0.7	2.3 $\pm$ 0.4*
Splanchnic arterial resistance, mL/minute per 100 g liver weight	59 $\pm$ 13	21 $\pm$ 6 ^{††}	35 $\pm$ 2*	19 $\pm$ 11 ^{††}	32 $\pm$ 4*
Hepatic arterial blood flow, mL/minute $\times$ 100 g	1.8 $\pm$ 0.4	5.3 $\pm$ 1.3 ^{††}	4.9 $\pm$ 0.8	4.9 $\pm$ 1.6 ^{††}	4.6 $\pm$ 1.1
Hepatic arterial resistance, mm Hg*minute*100 g/mL liver weight	1.3 $\pm$ 0.2	5.4 $\pm$ 0.7 ^{††}	4.8 $\pm$ 1.6	5.2 $\pm$ 0.8 ^{††}	5.1 $\pm$ 1.3
Baseline portal perfusion pressure, mm Hg	8.2 $\pm$ 1.9	12.1 $\pm$ 2.4 [†]	10.8 $\pm$ 1.3*	12.2 $\pm$ 1.8 [†]	10.9 $\pm$ 0.9*
Baseline intrahepatic resistance, mm Hg/mL per minute/g liver weight	13.2 $\pm$ 1.1	31.3 $\pm$ 2.8 [†]	26.2 $\pm$ 0.6*	29.9 $\pm$ 1.5 [†]	25.4 $\pm$ 0.9*

Data are expressed as means $\pm$ SD.

Collateral blood flow = (portosystemic shunting $\times$ portal venous inflow)/100.

* P < 0.05, ** P < 0.01 versus BDL-V/TAA-V rats.

[†] P < 0.05, ^{††} P < 0.01 versus sham-V rats.

Ale, aleglitazar; BDL, bile duct ligation; PPAR, peroxisome proliferator-activated receptor; TAA, thioacetamide; V, vehicle.

Figure 1 Effects of aleglitazar (Ale), a dual PPAR α/γ agonist, on hepatic marker expression in rats by immunohistochemical staining. Images (A) and bar graphs (B) of hepatic marker expressions. Sham-V, bile duct ligation (BDL)-V, and thioacetamide (TAA)-V indicate rats receiving chronic vehicle treatment; BDL-Ale/TAA-Ale indicate rats receiving chronic aleglitazar treatment. * $P < 0.05$ versus BDL-V/TAA-V rat livers. Original magnification, $\times 20$ (A). FOV, field of view; α -SMA, α -smooth muscle actin.

reacting with Ehrlich's reagent. Hepatic hydroxyproline content was expressed as micrograms/milligram of tissue. Hepatic level of TGF- β in liver homogenate was determined using enzyme-linked immunosorbent assay kits. The severity of intrahepatic angiogenesis was evaluated by counting the number of CD31-positive vessels by immunohistochemical or immunofluorescence staining.³³

Effects of Aleglitazar on HSC Contraction and LSEC Tube Formation and Migration

Hepatic stellate cells (HSCs) and liver sinusoidal endothelial cells (LSECs) were isolated from noncirrhotic Sprague-Dawley rats.^{2,20} As per previous setting,² for gel contraction assay, first-passage primary HSCs were used on day 14 after isolation, because these cells are comparable to cells of fibrotic liver. In total, 3×10^3 cells/well were plated onto collagen gels, which is adequate to produce significant collagen gel contraction. Ale (100 nmol/L), Ale + Y-27632 (Rho-kinase inhibitor; 10 μ mol/L), Ale + GW9662 (PPAR γ antagonist; 10 μ mol/L), or Ale + GW1929 (PPAR α antagonist; 10 μ mol/L) was preincubated for 24 hours before

the addition of ET-1 (20 nmol/L) or ET-1 + TNF- α (0.05 ng/mL); the buffer group served as the controls. Contractile effects were observed within 1 hour at this concentration. Preliminary studies revealed that Ale (100 nmol/L) showed the strongest effect in terms of ET-1-induced TNF- α -enhanced contraction. In the following 3 hours, the gels were imaged every 1 hour using a three charged-couple device video camera (Sony Corp., Tokyo, Japan). The surface areas of the collagen gels were measured using IPP image analysis software version 6.0 (Media Cybernetics, Silver Spring, MD), and the images were processed using Adobe Photoshop 3.0 (Adobe Systems, San Jose, CA).

As per previous setting,³³ LSEC migration was assessed using a chemotaxis chamber with inserts equipped with an 8-mm-pore membrane of 0.3 cm² placed in 24-well culture dishes forming the upper and lower compartments of the assay (Transwell; Corning Costar, Cambridge, MA). The lower compartment contained only the medium (buffer) (Dulbecco's modified Eagle's medium/0.2% bovine serum albumin) or the medium with drugs precoated with type I collagen (50 mg/L). The upper compartment of the chemotaxis filter equipment was seeded with LSECs

Table 4 Effects of Ale (Dual PPAR α/γ Agonist) Treatment on the Systemic Hemodynamic Parameters

Hemodynamic parameter	Sham-V	BDL-V	BDL-Ale	TAA-V	TAA-Ale
Mean arterial pressure, mm Hg	103 $\pm$ 8	84 $\pm$ 5	96 $\pm$ 3	81 $\pm$ 11	90 $\pm$ 4
Heart rate, beats/minute	279 $\pm$ 43	298 $\pm$ 61	305 $\pm$ 39	311 $\pm$ 74	298 $\pm$ 67
Cardiac output, mL/minute	112 $\pm$ 7.9	231.5 $\pm$ 4.1 ^{††}	168.7 $\pm$ 13.5 ^{**}	229.8 $\pm$ 3.2 ^{††}	170.4 $\pm$ 9.2 ^{**}
Cardiac index, mL*minute/100 g	24.6 $\pm$ 5.7	61.1 $\pm$ 7.3 ^{††}	42.2 $\pm$ 8.1 [*]	57.2 $\pm$ 4.6 ^{††}	41.7 $\pm$ 3.9 [*]
Stroke volume, mL/beats	0.42 $\pm$ 0.08	0.78 $\pm$ 0.06 [†]	0.55 $\pm$ 0.04	0.74 $\pm$ 0.03 [†]	0.57 $\pm$ 0.02
Systemic vascular resistance, mm Hg/mL per minute/100 g	4.2 $\pm$ 0.2	1.4 $\pm$ 0.6 ^{††}	2.5 $\pm$ 0.8	1.39 $\pm$ 0.3 ^{††}	2.2 $\pm$ 0.9

Data are expressed as means $\pm$ SD.

* P < 0.05, ** P < 0.01 versus BDL-V/TAA-V rats.

[†] P < 0.05, ^{††} P < 0.01 versus sham-V rats.

Ale, aleglitazar; BDL, bile duct ligation; PPAR, peroxisome proliferator-activated receptor; TAA, thioacetamide; V, vehicle.

(3 to 3.5 $\times 10^5$ cells/mL), which were serum starved for 24 hours, in 150 μ L of a serum-free medium. For drugs, the concentration of TNF- α , aleglitazar, Y-27632, GW9662, and GW1929 was similar to that used in the collagen gel contraction assay. LSECs were pretreated with buffer, VEGF (50 ng/mL), VEGF + TNF- α , Ale + VEGF + TNF- α , Y-27632 + Ale + TNF- α + VEGF, GW9662 + Ale + TNF- α + VEGF, and GW1929 + Ale + TNF- α + VEGF. The entire chamber was incubated at 37°C for another 6 hours to allow possible migration of cells. At the end of experiment, LSECs remaining on the upper surface of filters were removed with cotton tips. The membranes were fixed in 100% methanol, stained with May-Grunwald-Giemsa, mounted with glycerol on glass slides, and examined under microscope. LSECs adhering to the underside of membranes were counted in 10 random high-power fields ($\times 400$). Then, the migration index (MI) was calculated, as follows: MI (fold change) = number of LSECs transmigrating in the presence of different combined drugs/number of LSECs transmigrating in the buffer group.

An *in vitro* Matrigel tube formation angiogenesis assay kit (Kurabo, Tokyo, Japan) was used to assess the formation of capillary-like structure of LSECs, as in our previous setting.³³ Similar to migration assays, LSECs were pretreated with buffer, VEGF, VEGF + TNF- α , Ale + VEGF + TNF- α , Y-27632 + Ale + TNF- α + VEGF, GW9662 + Ale + TNF- α + VEGF, and GW1929 + Ale + TNF- α + VEGF. After a 30-hour incubation, LSECs were fixed, and the images were captured using an Olympus Inverted Research Microscope (Olympus, Tokyo, Japan) coupled with an Olympus C-5050 Zoom digital camera. Images were prepared in Adobe Photoshop 7.0 (Adobe) and exported to IPP image analysis software package for identification of endothelial cell tubule-like networks. The total tubule length (≥ 30 μ m) was derived for each of the four randomly chosen fields, and the total area of the culture surfaces covered by LSECs was determined in the same fields. Then, the angiogenic index was calculated, as follows: angiogenic index (fold change) = number of LSECs transmigrating in the presence of different combined drugs/number of LSECs transmigrating in the buffer group.

For both migration and angiogenic assays, the mean count was determined from four independent experiments and expressed as means $\pm$ SD.

In addition, the expression of RhoA, Rho-kinase, angiopoietin-1, VEGFR2, and β -actin mRNA in lysates of primary HSCs and LSECs with various pretreatments (n = 6 in each group) was determined.

Evaluation of the Mesenteric Vascular Density with Immunofluorescence Study

Mesenteric angiogenesis (mesenteric window vascular length and area) was measured with fluorescein isothiocyanate-labeled CD31 immunofluorescence staining.³³

PSS and Regional BF Analysis

PSS ratio was determined by injection of 30,000 of 15- μ m yellow microspheres (Dye Track; Triton Technology, San Diego, CA) into the spleen. Then, the livers and lungs were dissected. A reference sample was obtained for 1 minute at a rate of 0.65 mL/minute using a continuous withdrawal pump (Hugo Sachs Electronic, March, Germany). The number of microspheres was determined according to the manufacturer's instructions. In brief, 3000 blue microspheres (Dye Track) were added as the internal control. Tissue was digested to retrieve the pellets containing microspheres.³³ Then, acidified Cellosolve acetate (Spectrum Chemicals, Gardens, CA) was added, and absorption was measured at 448 nm (yellow) and 670 nm (blue) with a spectrophotometer (Shimadzu, Columbia, MD). Spillover between wavelengths was corrected with the matrix inversion technique. Next, 300,000 yellow microspheres were suspended in 0.3 mL saline containing 0.05% Tween and injected into the left ventricle 10 seconds after the withdrawal pump was started. On completion of the hemodynamic measurements, the animals were sacrificed, and the lungs, liver, kidney, stomach, intestine, pancreas, and spleen were resected. The tissues were weighed, minced with scissors, digested by adding 4 mol/L KOH at 14 mL/g of tissue and 2% Tween, and boiled for 1 hour. The blood reference sample was digested by adding 3.8 mL of 5.3 mol/L KOH and 0.5 mL of Tween and subsequent boiling for 1 hour. The digested tissues and blood

Figure 2 Effects of aleglitazar (Ale) treatments on cirrhotic livers. **A** and **B**: Evaluation of the roles of Rho kinase on endothelin-1 (ET-1)—induced portal perfusion pressure (PPP)—response curves with vehicle (V), Ale (10 μ M/L), or pioglitazone (Pio; PPAR γ agonist, 10 μ M/L) preincubation in thioacetamide (TAA)—V perfused rat livers. **C**: Effects of preincubation of PPAR α agonist fenofibrate (Feno; 20 μ M/L) on the ET-1—induced PPP—response curve of cirrhotic-V rat livers. **D**: Hepatic mRNA expression (fold/18S) in rats with either chronic vehicle or aleglitazar treatment. * P < 0.05 versus bile duct ligation (BDL)—V/TAA-V rat livers or vehicle group; † P < 0.05 versus Ale group.

samples were vortex mixed and filtered using Whatman Nuclepore filters (Whatman International, Maidstone, UK). The color of the filtered microspheres was dissolved in 0.2 mL N,N-dimethylphthalein. Thereafter, the number of microspheres per organ and organ perfusion BF were calculated using Microsoft Excel 2007 software (Microsoft, Redmond, WA) obtained from Triton Technologies.

Portal venous flow, the total BF within the portal system, was calculated as the sum of the BF to the stomach, spleen, intestines, pancreas, and mesentery. Collateral blood flow (mL/minute $\times$ 100 g) was estimated as portal venous inflow $\times$ PSS/100. Splanchnic vascular resistance was calculated as the ratio of splanchnic perfusion pressure and splanchnic BF, without including hepatic arterial flow. Hepatic arterial blood flow was estimated as PP/the sum of gastrointestinal and splenic perfusion minus shunt flow. Hepatic arterial resistance was calculated as the ratio of PP/hepatic arterial blood flow.

Intestinal Mucosal Injury and Permeability Study

Histologic assessment was performed in blinded manner by two pathologists in two hematoxylin and eosin—stained terminal ileal section slides from each ileal tissue block of three animals in each group. The intestinal mucosal injury was

classified using a semiquantitative grading system, as previously described,³³ where a numerical score was assigned on the basis of the type of mucosal and submucosal damage, as follows: 0, normal mucosal villi; 1, development of a subepithelial space, usually at the tip of the villus, with capillary congestion; 2, extension of the subepithelial space with increased lifting of the epithelial layer; 3, massive epithelial lifting down the sides of villi; 4, denuded villi with lamina propria and dilated capillaries exposed/increased cellularity of the lamina propria; and 5, digestion and disintegration of the lamina propria, hemorrhage, and ulceration. Intestinal PPAR α , TNF- α , and NF- κ Bp65 expressions were assessed by immunohistochemical staining. Intestinal permeability was measured by fluorescein isothiocyanate—dextran— and Evans Blue—based intestinal permeability methods, as previously described.³⁴

Effects of Aleglitazar on TNF- α —Induced Caco-2 Cell Monolayer Barrier Dysfunction

Caco-2 cells were purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan). Then, effects of various treatments on barrier integrity of differentiated Caco-2 monolayers were evaluated by application of vehicle, TNF- α (50 ng/mL), TNF- α + Ale

Figure 3 Effects of aleglitazar (Ale) on perfused rat liver to endothelin-1 (ET-1)-induced vasoconstrictive response. ET-1-induced portal perfusion pressure [PPP; absolute percentage change (Δ) from baseline]-response curves (**A** and **B**) and area under the curves (AUCs; **C**) in chronic aleglitazar-treated cirrhotic rats. Evaluation of the roles of Rho kinase on ET-1-induced PPP-response curves (**D** and **E**) and AUC (**F**) with vehicle (V), Ale (10 μ mol/L), or pioglitazone (Pio; PPAR γ agonist, 10 μ mol/L) preincubation in bile duct ligation (BDL)-V perfused rat livers. Y27632 indicates Rho-kinase inhibitor (10 μ mol/L). * P < 0.05 versus BDL-V/thioacetamide (TAA)-V rat livers or vehicle group; † P < 0.05 versus Ale group.

(100 nmol/L), GW9662 + Ale + TNF- α , and GW1929 + Ale + TNF- α to its apical and basolateral compartments for 48 hours. The doses applied were based on previous literature.³⁵ Preliminary tests revealed that 50 nmol/L TNF- α , from 15, 30, 50, and 100 nmol/L, was the lowest effective concentration to induce disruption of the epithelial barrier function. Then, barrier integrity of Caco-2 monolayer was determined by measuring apical-to-basolateral flux of a fluorescent marker [fluorescein sulfonic acid (FSA; 200 μ g/mL; 478 Da)], as described previously.³⁶ The excitation and emission spectra were calculated using the following formula: Clearance (nL/hour/cm²) = Fab/([FSA]_a × S).

where Fab is the apical-to-basolateral flux of FSA (light units/hour), [FSA]_a is the concentration at baseline (light units/nL), and S is the surface area (0.3 cm²). In addition, expression of *PPARA*, *PPARG*, *ZOI*, *CLDN1*, *OCLN*, and *ACTB* mRNA in cell lysate of Caco-2 monolayer with various preincubation conditions (n = 6 in each group) was measured.

Results

All cirrhotic (including BDL-V and TAA-V) rats had microscopic cirrhosis and PH as well as high serum alanine aminotransferase (ALT)/aspartate aminotransferase (AST) and low serum albumin levels (Tables 2 and 3 and Figure 1). There were no significant differences in body weight, liver weight, serum cholesterol, and triglycerides levels between cirrhotic rats and sham-V rats. In comparison with cirrhotic-V rats, chronic

aleglitazar treatment significantly increased serum albumin but did not modify AST, ALT, body weight, liver weight, serum cholesterol, and triglyceride levels in cirrhotic-Ale rats.

Aleglitazar Treatment Improves Systemic, Portal, and Splanchnic Hemodynamics in Cirrhotic Rats

In addition to typical hemodynamics (higher CO, CI, and stroke volume, and lower systemic vascular resistance), cirrhotic-V rats had higher serum TNF- α , IL-1 β , and VEGF levels than in sham-V rats (Tables 2 and 4). Although other parameters were unchanged, cirrhotic rats treated with a 21-day regimen of aleglitazar had significantly lower CO, CI, and serum TNF- α than cirrhotic-V rats.

Typical portal and splanchnic hyperdynamic circulations, including markedly high PP, SMA BF, SMA resistance, portal venous inflow, PSS, hepatic arterial blood flow, and HAV as well as low splanchnic arterial resistance were observed in cirrhotic-V rats compared with those in sham-V rats (Tables 3 and 4). Although no changes were observed in hepatic arterial blood flow and HAR, other aforementioned portal and splanchnic hemodynamics were significantly normalized by chronic aleglitazar treatment in cirrhotic-Ale rats.

Antihepatic Fibrosis Effects of Aleglitazar in Cirrhotic Rats

In addition to reduced hepatic fibrosis (Figure 1), chronic aleglitazar treatment significantly decreased levels of

Figure 4 Effects of aleglitazar (Ale) treatment on hepatic protein expression and primary hepatic stellate cell (HSC) contractility by collagen gel assay. **A** and **B**: Representative images (**A**) and bar graphs (**B**) of hepatic protein expression in rats with either chronic vehicle or aleglitazar treatments. **C** and **D**: Representative images (**C**) and bar graphs (**D**) of percentage change of surface area compared with buffer group, which indicated the effects of different administration on endothelin-1 (ET-1)-induced TNF- α -enhanced HSC contraction. **E**: mRNA expression in lysate of HSCs (%/buffer group). * $P < 0.05$ versus bile duct ligation-vehicle (BDL-V)/thioacetamide (TAA)-V group or ET-1 group; $^{\dagger}P < 0.05$ versus ET-1 + TNF- α group; $^{\ddagger}P < 0.05$ versus ET-1 + TNF- α + Ale group. p-MLCK, phosphorylated myosin light chain kinase.

hepatic fibrogenic and angiogenic markers TNF- α , NF- κ Bp65, IL-1 β , VEGF, VEGFR2, collagen I, and CTGF in cirrhotic-Ale rats compared with those in cirrhotic-V rats (Table 2 and Figures 1 and 2D). Notably, the simultaneous decrease in hepatic fibrosis and PP indicated that aleglitazar-related reduction in PP in cirrhotic-Ale rats was partly contributed by its antihepatic fibrotic effects.

Aleglitazar Improves ET-1 Hyper-Responsiveness in Cirrhotic Rat Livers through PPAR γ -Dependent Inhibition of RhoA/Rho-Kinase Signals

In comparison with sham-V rat perfused livers, baseline PPP and calculated IHR were significantly higher in cirrhotic-V rat liver, whereas chronic aleglitazar treatment significantly reduces PPP and IHR (Table 3). In parallel with the up-regulation of PPAR α and PPAR γ protein expression, the ET-1 hyperresponsiveness was significantly attenuated, represented as less prominent PPP-response curves and low area under the curve, by chronic aleglitazar treatment in cirrhotic rat livers (Figure 3, A–C, and Figure 4, A and B).

In comparison with cirrhotic-Ale rat livers, PPAR α/γ activation was associated with the down-regulation of

RhoA/Rho kinase and its downstream signals (p-moesin-thr-588 and phosphorylated myosin light chain kinase) expression (Figure 1 and Figure 4, A and B). In comparison with buffer group, the PPP-response curve revealed that acute aleglitazar administration attenuated ET-1 hyperresponsiveness in perfused rat cirrhotic-V (BDL-V and TAA-V) livers (Figure 2A and Figure 3, D and F). Furthermore, Y27632, a RhoA kinase inhibitor, preincubation before aleglitazar administration abolished the aleglitazar-related attenuation of ET-1 hyperresponsiveness in cirrhotic-V rat livers (Figure 2A and Figure 3, D and F).

Taken together, these results indicated that the attenuation of ET-1 hyperresponsiveness by aleglitazar is mainly contributed by the suppression of RhoA/Rho kinase/p-moesin/phosphorylated myosin light chain kinase cascades in cirrhotic rat liver. Of treatment with 5, 10, and 20 μ mol/L of pioglitazone, the preliminary tests revealed that the lowest effective pioglitazone concentration to inhibit ET-1-induced constrictive-response curves in cirrhotic rat perfused liver was 10 μ mol/L (Figure 2B and Figure 3, E and F).

By contrast, acute fenofibrate (15, 25, and 30 μ mol/L) pretreatment did not significantly modify hepatic ET-1 hyperresponsiveness in cirrhotic-V rat livers (Figure 2C). In

Figure 5 *In vivo* and *in vitro* effects of aleglitazar (Ale) treatment on the severity of hepatic angiogenesis. **A** and **B**: Images (**A**) and bar graph (**B**) for the CD31 fluorescein isothiocyanate staining in rat livers. **C**: mRNA expression in lysate of liver sinusoidal endothelial cells (LSECs; %/buffer group). **D** and **E**: Representative images (**D**) and bar graphs (**E**) of the effects of different pretreatments on vascular endothelial growth factor (VEGF)—induced TNF- α —enhanced LSEC migration [vertical black lines in images: boundary between compartment that initially filled with LSECs and compartment that was initially without any cells (central part)]. **F** and **G**: Representative images (**F**) and bar graphs (**G**) of the effects of different pretreatments on VEGF-induced TNF- α —enhanced LSEC tube formation. ** $P < 0.01$ versus VEGF-preincubated groups; † $P < 0.05$ versus VEGF + TNF- α —preincubated group; ‡ $P < 0.05$ versus VEGF + TNF- α + Ale—preincubated group. Scale bars = 20 μ m (**D** and **F**). Original magnification, $\times 10$ (**A**); $\times 40$ (**D** and **F**). BDL, bile duct ligation; TAA, thioacetamide; V, vehicle.

acute liver perfusion study, the degree of pioglitazone-induced attenuation of ET-1 hyperresponsiveness was similar to that of aleglitazar (with similar degree of change in area under the curve compared with the vehicle pretreatment group). In cirrhotic-V rat livers, Y27632 preincubation abolished the pioglitazone-related attenuation of ET-1 hyperresponsiveness, indicating that the effects were PPAR γ mediated rather than PPAR α mediated (Figure 2A and Figure 3, E and F).

Aleglitazar Inhibits ET-1—Stimulated Primary HSC Contractility through PPAR γ Activation

In comparison with buffer group, ET-1 or ET-1 + TNF- α incubation induced a significant decrease in the HSC-contained collagen gel surface area, representing HSC contraction, with the decrease being higher in the ET-1 + TNF- α group than that in ET-1 group (Figure 4, C and D). Comparison of Ale + ET-1 + TNF- α and ET-1 + TNF- α groups showed that acute aleglitazar coincubation inhibited ET-1 + TNF- α —induced HSC contraction. Furthermore, Rho-kinase inhibitor (Y27632) or PPAR γ antagonist (GW9662) preincubation reversed the aleglitazar-related decrease in the degree of ET-1 + TNF- α —induced HSC

contraction. By contrast, PPAR α antagonist (GW1929) preincubation did not modify aleglitazar-related suppression in ET-1 + TNF- α —induced HSC contraction (Figure 4, C and D). In HSC lysate, ET-1 + TNF- α preincubation resulted in an up-regulation in RhoA and Rho-kinase mRNA expression, which was suppressed by aleglitazar coincubation (Figure 4E). Notably, the dose of aleglitazar (100 nmol/L) used in this experiment significantly up-regulated PPAR α and PPAR γ mRNA, whereas doses of GW1929 and GW9662 adequately inhibited aleglitazar—up-regulated PPAR α /PPAR γ expression. Meanwhile, the dose of Y27632 used in this experiment adequately inhibited ET-1 + TNF- α —enhanced RhoA and Rho-kinase mRNA expression.

Aleglitazar Decreases Intrahepatic Angiogenesis and LSEC Migration/Angiogenic Index through PPAR γ Activation

With respect to intrahepatic angiogenesis in cirrhotic rat, the hepatic CD31-stained area was significantly lower in the aleglitazar-treated group than that in the vehicle-treated group (Figure 5, A–C). In the LSEC lysate, VEGF + TNF- α

Figure 6 Effects of aleglitazar (Ale) treatment on the severity of mesenteric angiogenic and inflammatory markers. **A–C:** Images (**A**) and bar graph (**B** and **C**) of CD31 fluorescein isothiocyanate staining in rat mesentery. **D** and **E:** Representative images (**D**) and bar graphs (%/ β -actin; **E**) of splanchnic tissue protein expression in rats receiving either chronic vehicle (V) or aleglitazar treatments. **F:** mRNA expression in splanchnic tissue (fold/18S) of all rats. **G** and **H:** Representative images (**G**) and bar graphs (%/ β -actin; **H**) of collateral vessel protein expression in rats receiving either chronic vehicle or aleglitazar treatments. **I:** mRNA expression in collateral vessels (fold/18S) in all rats. * $P < 0.05$ versus bile duct ligation (BDL)–V/thioacetamide (TAA)–V groups. Original magnification, $\times 20$ (**A**). p-VEGFR2, phosphorylated vascular endothelial growth factor type II receptor.

preincubation resulted in up-regulation of angiopoietin-1 and *VEGFR2* mRNA expression, which was reduced by aleglitazar coinubation (Figure 5C). Similar to the HSC culture system, the doses of aleglitazar, GW9662, and GW1929 used effectively inhibited PPAR α and PPAR γ mRNA expression.

In comparison with buffer group, VEGF incubation induced a significant increase in the LSEC migration and tube formation, which was further enhanced by VEGF + TNF- α incubation (Figure 5, D–G). Ale preincubation reduced the VEGF + TNF- α –induced LSEC migration and tube formation. PPAR γ antagonist (GW9662), rather than PPAR α antagonist (GW1929), preadministration abolished the aleglitazar-related decrease in the degree of VEGF + TNF- α –induced LSEC migration and tube formation (Figure 5, D–G).

Aleglitazar Inhibits Mesenteric Angiogenesis and Hyperemia in Cirrhotic Rats

In line with the improvement of splanchnic hyperemia (decrease in SMA BF and increase in SMA resistance and splanchnic arterial resistance), mesenteric angiogenesis was

significantly inhibited in cirrhotic-Ale rats (Table 3 and Figure 6, A–C). In the splanchnic and collateral vessel tissues of cirrhotic-Ale rats, significant inhibition of angiogenic (angiopoietin-1, *VEGFR2*, and p-VEGFR2) and inflammatory (TNF- α and NF- κ Bp65) marker expression was parallel to aleglitazar-induced up-regulation of PPAR α , PPAR γ , and PPAR γ 2 expression (Figure 6, D–I).

PPAR α -Specific Effects of Aleglitazar Inhibit Intestinal Mucosal Injury and Correct Intestinal Hyperpermeability

The improvement in intestinal mucosal injury was associated with the up-regulation of intestinal PPAR α and down-regulation of intestinal TNF- α , NF- κ Bp65, and TNF receptor II expression in cirrhotic-Ale rats compared with those in cirrhotic-V rats (Figure 7, A, B, and D–F, and Figure 8). Meanwhile, intestinal hyperpermeability was normalized by aleglitazar-related up-regulation of intestinal PPAR α expression and normalization of the expression of tight-junction proteins (occludin, claudin-1, and ZO-1) in cirrhotic-Ale rats (Figure 7, C–F, and Figure 8). Intestinal

Figure 7 Effects of chronic aleglitazar (Ale) treatment on intestinal mucosal injury and hyperpermeability. **A** and **B**: Images (**A**) and bar graph (**B**) for hematoxylin and eosin staining of rat small intestinal mucosal injury Suzuki score. **C**: Bar graphs of fluorescein isothiocyanate–dextran (FD-4) and Evans Blue (EB)–based intestinal permeability assay. **D** and **E**: Representative images (**D**) and bar graphs (%/β-actin; **E**) of terminal ileum tissue protein expression in rats receiving either chronic vehicle (V) or aleglitazar treatments. **F**: mRNA expression in terminal ileum tissue (fold/18S) in all rats. **P* < 0.05 versus bile duct ligation (BDL)–V/thioacetamide (TAA)–V groups. Original magnification, ×200 (**A**). ZO-1, zonula occludens protein 1.

PPARγ2 expression was similar between cirrhotic-V and cirrhotic-Ale rats (Figure 7F).

Aleglitazar Prevents the TNF-α–Induced Disruption of Caco-2 Monolayer–Epithelial Barrier through PPARα Activation

TNF-α disrupted the barrier function of Caco-2 cells in a dose-dependent manner, as demonstrated by the incremental increased clearance of FSA and down-regulation of occludin, claudin-1, and ZO-1 mRNA expression (Figure 9, A and C). Aleglitazar pretreatment significantly prevented TNF-α–induced disruption of Caco-2 cell–monolayer barrier function, as demonstrated by the decrease in the amount of FSA clearance and normalized occludin, claudin-1, and ZO-1 mRNA expression (Figure 9, B and D). Furthermore, GW1929 (PPARα antagonist), rather than GW9662 (PPARγ antagonist), pretreatment abolished the aforementioned aleglitazar-related protective effects in Caco-2 cell monolayer barrier disruption.

Discussion

PH accounts for the majority of morbidity and mortality rates observed in patients with cirrhosis. PH in patients with

cirrhosis is primarily initiated through increases in the levels of circulating soluble TNF receptor and lipopolysaccharide-stimulated monocyte-produced TNF-α,³⁷ and this elevation correlates with the severity of liver disease. In PH rats, higher plasma TNF levels after treatment with endotoxin or recombinant TNF are observed than those in sham-operated rats.³⁸ Increased circulating TNF-α level has been proved to be a major pathogenic factor that contributes to the development of multifaceted abnormalities in hyperdynamic and portal-hypertensive syndrome.^{39,40} Anti-TNF-α treatment in rats blunts the development of hyperdynamic circulation and decreases PP in PH rats.^{39,40} Therefore, in cirrhotic PH rats of this study, the multisystem beneficial effects of chronic aleglitazar treatment were associated with the simultaneous suppression of systemic, hepatic, splanchnic, and intestinal TNF-α and NF-κB signals.

PPARγ is a negative regulator of macrophage activation, and synthetic PPARγ ligands are the potential agents to treat human diseases that involve activated macrophages as pathogenic factors.⁴¹ Both PPARα and PPARγ agonists can inhibit lipopolysaccharide-induced TNF-α expression in rat primary cardiomyocytes in a dose-dependent manner.⁴² An *in vitro* study revealed that aleglitazar can attenuate TNF-α–mediated inflammation in human adipocytes.⁴³ In addition to extensive human and animal studies on metabolic profiles and cardiovascular risk,^{44,45} the potential for

Figure 8 Effects of chronic aleglitazar (Ale) treatment on various intestinal pathogenic markers. Images (A) and bar graphs (B) of immunohistochemical staining of small intestine marker expression in all rats. The staining index was determined by calculating the ratio of the positive-stained area/area of the whole microscopic field. * $P < 0.05$ versus bile duct ligation—vehicle (BDL-V)/thioacetamide (TAA)—V groups. Original magnification, $\times 200$ (A).

application of aleglitazar has been evaluated in hyperglycemia-induced cardiomyocyte dysfunction, mice and human diabetes-related organ dysfunction, and a cellular model of inflamed human adipose tissue.^{46–48} Recent studies have reported hepatic or splanchnic benefits for application of either PPAR α or PPAR γ agonist in cirrhotic PH animals.^{20,21} There is, therefore, an urgent medical need to evaluate the potential of dual PPAR α/γ agonist, with lower potential adverse effects, for treating multifaceted abnormalities of cirrhotic PH syndrome.

In vivo and *in vitro* studies revealed that up-regulation of hepatic, splanchnic, and intestinal PPAR α and PPAR γ levels by chronic aleglitazar treatment was accompanied with significant suppression of systemic and local inflammatory markers in cirrhotic rats with PH. Specifically, it was found that the beneficial effects of aleglitazar in hepatic microenvironment act through PPAR γ activation. However, the beneficial effects of aleglitazar in the splanchnic system act through both PPAR α and PPAR γ activation. Finally, the beneficial effects of aleglitazar in the intestine act primarily through PPAR α activation. Overall, all above-mentioned therapeutic effects of aleglitazar are because of its anti-inflammatory and anti-TNF- α action in multifaceted abnormalities of cirrhotic rats with PH.

In human dermal microvascular endothelial cells, TNF- α can induce ET-1 gene expression in a dose-dependent manner.⁴⁹ Endotoxin, which can stimulate TNF- α release,

pretreatment enhances portal and sinusoidal contractile response to ET-1 in isolated rat liver perfusion system.⁵⁰ PPAR activators directly inhibit thrombin-induced ET-1 production in human vascular endothelial cells.¹¹

In this study, the cirrhotic rats were characterized by increased systemic and hepatic TNF- α levels. In parallel to the hepatic ET-1 hyperresponsiveness in rat perfused livers, TNF- α coinubation enhanced ET-1-induced primary rat HSC contraction in this study. Furthermore, inhibition of ET-1 + TNF- α -induced HSC contraction by acute and chronic aleglitazar treatment was associated with the suppression of hepatic ET-1 hyperresponsiveness in perfused cirrhotic rat livers.

Similar to previous studies,^{12,15,30} anti-inflammatory cytokine effects were observed in the mesentery and small intestine of cirrhotic rats receiving chronic aleglitazar treatment. Liver transaminases are released into the bloodstream when liver cells are damaged.⁵¹ Serum ALT and AST are the best indicators of hepatic damage, including necrosis and dysfunction.⁵² Notably, the serum AST/ALT ratio of 1 in the cirrhotic rats was the typical cirrhotic pattern of the liver function test.⁵³ Until now, the effects of chronic PPAR α/γ agonist treatment on serum AST/ALT have not been explored.^{27–31} In advanced cirrhosis complicated with PH, hepatic dysfunction, rather than necrosis, is the dominant type of hepatic damage. Serum albumin is an important marker of hepatic dysfunction.⁵⁴ This might probably be the reason that

Figure 9 Effects of aleglitazar (Ale) on TNF- α -induced Caco-2 cell monolayer—epithelial barrier dysfunction. **A:** Dose-dependent effects of TNF- α on Caco-2 cell-barrier dysfunction. **B:** Effects of acute aleglitazar pretreatment on TNF- α -induced Caco-2 cell barrier dysfunction. **C:** Dose-dependent effects of TNF- α on tight junction protein expression (/buffer group) in cell lysate of Caco-2 cell monolayer. **D:** Effects of acute aleglitazar pretreatment on TNF- α -suppressed tight junction protein expression (/buffer group) in cell lysates of the Caco-2 cell monolayer. * $P < 0.05$ versus lower-dose TNF- α group; † $P < 0.05$ versus TNF- α group; ‡ $P < 0.05$ versus TNF- α + Ale group. FSA, fluorescein sulfonic acid.

chronic aleglitazar treatment normalized serum albumin levels rather than serum AST/ALT levels in the cirrhotic rats.

In cirrhotic rats, chronic PPAR α agonist treatment—related improvement in hepatic endothelial dysfunction is accompanied with a reduction in hepatic fibrosis.²⁰ Without evaluating the antihepatic fibrogenic effects, a recent study reported the beneficial effects of PPAR γ agonist on splanchnic circulation in cirrhotic rats.²¹ TGF- β 1 is a fibrogenesis-driving cytokine. CTGF is also a highly profibrogenic molecule that is overexpressed in fibrotic liver and is transcriptionally activated by TGF- β . CTGF and TGF- β can act in synergy for extracellular matrix synthesis. Collagen I is the main component of extracellular matrix that is accumulated in cirrhotic livers. In nonalcoholic steatohepatitis mice, chronic PPAR γ agonist treatment has been reported to ameliorate hepatic fibrosis by inhibiting HSC activation and suppressing TGF- β 1/CTGF expression.⁵⁵ TNF- α can activate HSCs and accelerate hepatic fibrosis.⁵⁶ α -SMA is a well-known marker for activated HSCs. TNF- α has a direct stimulatory effect on collagen synthesis.⁵⁷ Accordingly, in cirrhotic rats of this study, the anti-TNF- α and PPAR α / γ activation effects of aleglitazar were associated with a significant reduction in hepatic fibrosis, demonstrated by decreased hepatic Sirius red and α -SMA stained—positive area, reduced hepatic hydroxyproline content/TGF β 1 level, and down-regulation of collagen I/CTGF expression.

During cirrhosis progression, tissue hypoxia in liver leads to an increased expression of proangiogenic cytokine, such as TGF- β , and results in intrahepatic angiogenesis.⁵⁸ In

return, abnormal intrahepatic angiogenesis stimulates hepatic fibrogenesis and eventually liver failure, cirrhosis, and PH. Thus, in patients with cirrhosis, significant positive correlations were observed between levels of hepatic VEGFR2 and α -SMA, between levels of hepatic VEGFR2 and Sirius red—stained positive area, and between levels of hepatic VEGFR2 and child-Pugh class.⁵⁹ In the cirrhotic rats receiving aleglitazar treatment, improvement in tissue hypoxia with simultaneous amelioration of hepatic endothelial dysfunction and intrahepatic angiogenesis (decreased hepatic VEGF and VEGFR2 expression and CD31-stained positive area) also contributed to reduced hepatic fibrogenesis.

In chronic inflammatory disease, such as PH, increased circulating TNF- α is associated with multifaceted hemodynamic dysarrangements.^{33,39,40,60,61} *In vivo* microscopy-based evaluation revealed a direct acute vasodilatory effect of TNF- α on rat cremaster muscle microcirculation.⁶² In an *in situ* perfusion system, bacterial translocation—related TNF- α overproduction was associated with the impairment of mesenteric vasculature contractility in ascitic cirrhotic rats.⁶³ Therefore, in this study, it is reasonable to conclude that the anti-TNF- α effects of aleglitazar are associated with the normalization of SMA and splanchnic arterial resistance in cirrhotic rats.

Angiogenesis is a pathologic hallmark of PH.^{4,7} Recent data suggest that intrahepatic and splanchnic neoangiogenesis are involved in sinusoidal resistance, hepatic fibrosis, and PH in cirrhosis.^{4,7} By inhibition of splanchnic

tissue damage and inflammation, anti-angiogenesis treatment improves PH and decreases liver damage, intrahepatic fibrosis, and hepatic/splanchnic inflammation in cirrhotic rats. In this study, the aleglitazar-related *in vivo* attenuation of intrahepatic/mesenteric angiogenesis and PSS were validated by *in vitro* anti-angiogenic effects of aleglitazar in liver sinusoidal cell endothelial cells.

It has been reported that tight junction–related proteins (occludin, claudin-1, and ZO-1) are primarily responsible for maintaining intestinal barrier function.⁶⁴ TNF- α can result in the disruption of intestinal tight junctions, epithelial cell death, and intestinal inflammation.⁶⁵ Binding of TNF- α to the TNF receptor, which activates NF- κ B in intestinal epithelial cells, contributes to the increase in intestinal epithelial tight junction permeability.⁶⁶ In this study, the chronic aleglitazar-related amelioration of intestinal hyperpermeability in cirrhotic rats was associated with the restoration of intestinal tight junction protein expression and suppression of TNF- α –TNF receptor II–NF- κ B cascades. In particular, a direct anti-inflammatory effect of aleglitazar was observed at the cellular level in the Caco-2 culture system. Interestingly, the protective effects and mechanisms of aleglitazar on TNF- α –induced mucosal barrier dysfunction have been validated by an *in vitro* study in the Caco-2 cell monolayer.

In conclusion, chronic treatment with the dual PPAR α/γ agonist, aleglitazar, decreased portal inflow resistance, improved systemic/splanchnic hyperdynamic circulation, and ameliorated intestinal hyperpermeability in cirrhotic rats with PH. These effects occur via the inhibition of systemic/local inflammation and TNF- α overproduction. These findings as a whole imply that treatment with a dual PPAR α/γ agonist may help simultaneously control multifaceted abnormalities of PH syndromes in cirrhosis.

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Supplemental Data

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