Short-Term Therapy with Peroxisome Proliferation-Activator Receptor-α Agonist Wy-14,643 Protects Murine Fatty Liver Against Ischemia–Reperfusion Injury

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Steatosis increases operative morbidity/mortality from ischemia–reperfusion injury (IRI); few pharmacological approaches have been protective. Using novel genetic/dietary models of nonalcoholic steatohepatitis (NASH) and simple steatosis (SS) in *Alms1* mutant (*foz/foz*) mice, we characterized severity of IRI in NASH versus SS and lean liver and tested our hypothesis that the lipid-lowering effects of the peroxisome proliferation-activator receptor (PPAR)-α agonist Wy-14,643 would be hepatoprotective. Mice were subjected to 60-minute partial hepatic IRI. Microvascular changes were assessed at 15-minute reperfusion by *in vivo* microscopy, injury at 24 hours by serum alanine aminotransferase (ALT), and hepatic necrosis area. Injury and inflammation mediators were determined by way of immunoblotting for intercellular cellular adhesion molecule, vascular cellular adhesion molecule, p38, c-jun N-terminal kinase, IκB-α, interleukin (IL)-1a, IL-12, tumor necrosis factor-α (TNF-α) and IL-6, cell cycle by cyclin D1 and proliferating cell nuclear antigen immunohistochemistry. In *foz/foz* mice fed a high-fat diet (HFD) to cause NASH or chow (SS), IRI was exacerbated compared with HFD-fed or chow-fed wild-type littermates by ALT release; corresponding necrotic areas were 60 ± 22% NASH, 29 ± 9% SS versus 7 ± 1% lean. Microvasculature of NASH or SS livers was narrowed by enormous lipid-filled hepatocytes, significantly reducing numbers of perfused sinusoids, all exacerbated by IRI. Wy-14,643 reduced steatosis in NASH and SS livers, whereas PPAR-α stimulation conferred substantial hepatoprotection against IRI by ALT release, with reductions in vascular cellular adhesion molecule-1, IL-1a, TNF-α, IL-12, activated nuclear factor-κB (NF-κB), p38, IL-6 production and cell cycle entry. *Conclusion:* NASH and SS livers are both more susceptible to IRI. Mechanisms include possible distortion of the microvasculature by swollen fat-laden hepatocytes, and enhanced production of several cytokines. The beneficial effects of Wy-14,643 may be exerted by dampening adhesion molecule and cytokine responses, and activating NF-κB, IL-6 production, and p38 kinase to effect cell cycle entry. (HEPATOLOGY 2010;51:996-1006.)

Nonalcoholic fatty liver disease, the most common cause of steatosis, is associated with visceral obesity and insulin resistance. With more severe risk factors (obesity, type 2 diabetes, metabolic syndrome), steatosis may be complicated by hepatocellular injury and liver inflammation in that part of the nonalcoholic fatty liver disease spectrum referred to as nonalcoholic steatohepatitis (NASH). The fatty liver is sensitive to ischemia–reperfusion injury (IRI), and this limits the use of fatty donor livers for transplantation.[1,2] Between 15% and 50% of livers from cadaveric brain-dead adult and child donors for liver transplantation exhibit significant fatty liver disease, defined by more than 30% of hepatocytes showing steatosis.[1-4] Several clinical and experimental studies have demonstrated that primary graft nonfunction resulting in liver failure is more frequent when such grafts are used for transplantation.[1-5] Further, the more extensive the steatosis, the poorer the graft sur-

Abbreviations: ALT, alanine aminotransferase; HFD, high-fat diet; ICAM-1, intercellular cellular adhesion molecule-1; IL, interleukin; IRI, ischemia–reperfusion injury; NASH, nonalcoholic steatohepatitis; NF-κB, nuclear factor-κB; PCNA, proliferating cell nuclear antigen; PPAR-α, peroxisome proliferation-activator receptor-α; SD, standard deviation; SS, simple liver steatosis; TNF-α, tumor necrosis factor-α; VCAM-1, vascular cell adhesion molecule-1; WT, wild-type.

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vival, with up to 80% primary graft nonfunction in severely steatotic livers.[6] In most liver transplantation centers, donor livers with fatty change classified as moderate (30%-60% of cells involved) or severe (>60%) are discarded.[1-7] Although the reasons for the increased susceptibility of fatty liver to injury during hepatic surgery and liver transplantation have been the subject of several investigations,[8] little progress has been made in identifying hepatoprotective strategies against IRI in such livers.

We recently reported a new obese mouse strain (Fat Aussie) with insulin resistance and type 2 diabetes that undergoes the transition from simple steatosis (SS) to fibrosing steatohepatitis (NASH) when fed a high-fat diet (HFD).[9] The Fat Aussie mouse inherits a spontaneous mutation (*foz*), an 11-base pair truncating deletion in exon 8 of *Alms1*, the gene responsible for Alström syndrome in humans. Alström syndrome is a rare monozygotic form of hyperphagic obesity complicated by early onset insulin resistance, type 2 diabetes and severe liver disease, including cirrhosis; the Alms1 mutant (*foz/foz*) mouse recapitulates these and other features.[9] We hypothesized that hepatic IRI would be more severe in SS and NASH murine liver, and tested whether the peroxisome proliferation-activator receptor (PPAR)-α agonist, Wy-14,643 was hepatoprotective against this form of injury on the basis of its lipid-depleting and/or anti-inflammatory effects.

In the present study, we first showed that livers from *foz/foz* mice with NASH or SS are more susceptible to IRI than lean livers, and that the mechanisms of such injury in fatty liver differ from those in lean liver. We then tested whether there was a beneficial effect of depleting steatotic livers of lipid using a potent PPAR-α agonist, Wy-14,643, to enhance fatty acid oxidation.[10] Finally, we identified additional anti-inflammatory and proproliferative effects of PPAR-α stimulation in relation to hepatoprotection against IRI in SS and NASH. These findings offer a novel strategy to prevent IRI in human fatty liver before surgery or organ donation.

Materials and Methods

Murine Model of Partial Hepatic IRI and Administration of Wy-14,643. All animal protocols and studies complied with the highest International Criteria of Animal Experiments and were approved by the Australian National University's Animal Ethics Committee. The *foz/foz* mice and their lean, wild-type (WT) littermates were bred and housed in the Australian National University Medical School Animal House at The Canberra Hospital under standard environmental and housing conditions. After weaning, male *foz/foz* mice and their WT littermates were fed a commercial pellet diet (5.4% fat by weight) or HFD (23% fat by weight, SF03-020, Specialty Feeds, Glen Forrest, Western Australia) for 8 weeks and allowed water *ad libitum*. Wy-14,643 (0.1% wt/wt in diet) was administered for 10 days prior to surgery. The diet was well tolerated by the animals with no side effects, as described.[10] The mean body weights of mice after 8 weeks of diet were as follows: WT NC 30 ± 1.2 g; WT HFD 35 ± 1.1 g; foz NC 52 ± 1.7 g; foz HFD 63 ± 1.8 g. The design of the IRI experiments was modified slightly from that previously used by us,[11,12] because preliminary experiments using 90-minute ischemia caused mortality in obese mice; the design was refined to 60-minute ischemia. In other respects, anesthesia, the method of occlusion of the left lateral and median lobes vascular pedicle, and reperfusion were performed as described.[11,12] After 2-24 hours reperfusion, animals were killed by way of exsanguination, blood was collected by way of cardiac puncture for preparation of plasma, and livers were harvested for tissue studies (minimum of 4, up to n = 5 per time point per treatment group). One group of control mice was subjected to anesthesia/sham laparotomy. Liver samples were collected from the postischemic lobes and immediately frozen in liquid nitrogen, or fixed in phosphate-buffered formalin prior to embedment in paraffin.

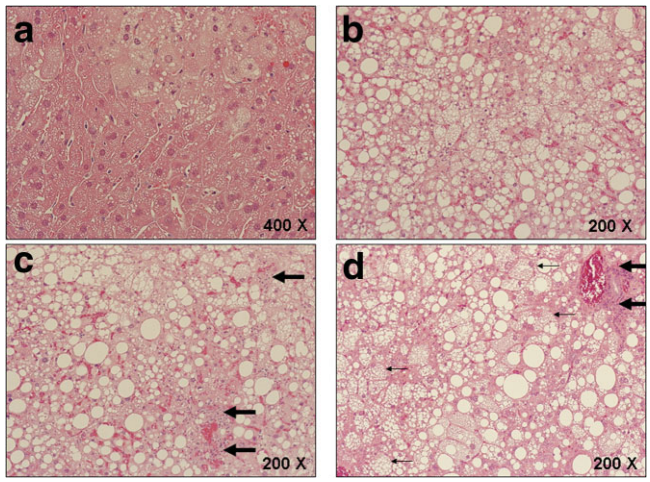

Fig. 1. Histology from sham-operated mice with lean livers, simple steatosis, and NASH. After weaning, (A) WT mice fed an HFD (WT HFD) and (B) *foz/foz* mice fed normal chow (foz NC) for 8 weeks developed simple steatosis, whereas (C,D) *foz/foz* mice fed an HFD for a similar period developed NASH. Virtually all hepatocytes contain (A) microvesicular steatosis with minimal lobular inflammation and (B) mixed microvesicular/macrovesicular steatosis. (C,D) Grade 4 steatosis (mixed microvesicular and macrovesicular steatosis) and foci of inflammation (thick arrows) are noted with ballooned hepatocytes (thin arrows). Hematoxylin-eosin staining of representative liver sections shown at magnifications indicated. Sections are representative of five experiments.

Assessing the Severity of Liver Injury. Severity of liver injury was determined from serum levels of alanine aminotransferase (ALT) and liver histology (Fig. 1). Se-

Table 1. Primer Pairs and Probe Sequences Used for Real-Time Polymerase Chain Reaction

Target	Sequences	Genbank Accession No.
ICAM-1	Forward: 5′-TCGGGAAGGGAGCCAAGTAACT-3′ Reverse: 5′-GATCCTCCGAGCTGGCATT-3′	BC016198
VCAM-1	Forward: 5′-CTACAAAGTCTACATCTCTCCCAGGAA-3′ Reverse: 5′-CCTCGCTGGAACAGGTCATT-3′	BC029823

rum ALT was assayed by automated procedures in the Department of Pathology, The Canberra Hospital. Consecutive liver sections (4 μm) were cut from paraffin-embedded livers and stained with hematoxylin-eosin to evaluate the extent of hepatic necrosis. Areas of hepatic necrosis were measured in five low-power fields (magnification $\times$4) using Image Pro 5.1 software (Media Cybernetics, Silver Spring, MD).

Proliferating Cell Nuclear Antigen Immunohistochemistry. Proliferating cell nuclear antigen (PCNA) immunohistochemical analysis was performed using the Santa Cruz immunostaining kit (Santa Cruz Biotechnology, Santa Cruz, CA).

In Vivo *Microscopy.* Hepatic microvascular alterations were examined using established high-resolution *in vivo* microscopic methods under ketamine/xylazine anesthesia, as reported.[11] A compound binocular inverted stage microscope adapted for *in vivo* microscopy was equipped to provide transillumination, epi-illumination, as well as video microscopy using a digital camera, video monitor, and recorder (Olympus IX71, Mount Waverley, Victoria, Australia). The liver was exteriorized through a left subcostal incision and positioned over a window of optical-grade mica in a specially designed tray mounted on a microscopic stage. The liver was covered by a piece of Saran wrap (Dow Chemical Co., Midland, MI), which held it in position and limited movement. Homeostasis was ensured by a constant irrigation of the liver with normal saline maintained at body temperature. Microvascular events were observed and recorded in naïve or Wy-14,643–treated mice subjected to 60-minute ischemia and 15-minute reperfusion (n = 4 per experimental group). The relative adequacy of blood perfusion through the sinusoids was evaluated by counting the number of sinusoids exhibiting blood flow in five periportal and five centrilobular regions in each animal. Transmitted bright-field and epi-illuminated fluorescence images were obtained simultaneously to provide images of the sinusoidal wall and blood flow simultaneously. The number of perfused sinusoids was expressed as a percentage of total number of sinusoids, regardless of blood flow per region.

Measurement of Hepatic Messenger RNA Levels by Real-Time Polymerase Chain Reaction. RNA was extracted using TRIzol (Invitrogen Life Technologies, Carlsbad, CA) and complementary DNA was synthesised using Superscript III (Invitrogen). Semiquantitative real-time polymerase chain reaction analysis using Platinum SYBYR Green qPCR SuperMix UDG (Invitrogen) was performed using a Corbett Rotor-Gene 3000 (Corbett Research, Sydney, Australia). Values were normalized to 18S or glyceraldehyde 3-phosphate dehydrogenase. Primer and gene details are listed in Table 1.

Western Blot Analysis of Signaling and Inflammatory Intermediates. Liver homogenates in protein lysis buffer were resolved by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis under reducing conditions.[10-12] The conditions for detecting cyclin D1, IκB-α, p38, phosphorylated p38, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) (Santa Cruz Biotechnology, Santa Cruz, CA) protein by immunoblotting have been reported.[10-12] Nuclear extracts and cytoplasmic fractions were prepared from whole liver and subjected to immunoblotting for nuclear factor-κB (NF-κB) p65 (Santa Cruz Biotechnology).

Measurement of Cytokines and Chemokines in Serum. An antibody-coupled bead-based serum assay system was used to quantify release of several cytokines and chemokines (Bio-Plex Mouse 23plex assay) at 2-hour reperfusion following 60-minute ischemia, with high sensitivity and a dynamic range of detection up to 32,000 pg/mL: interleukin (IL)-1α, IL-6, IL-12(p70), and tumor necrosis factor-α (TNF-α).

Statistical Analyses. Analysis of variance with *post hoc* analysis and Bonferroni correction were used for the comparison of data from different groups. Results are presented as the mean $\pm$ standard deviation (SD) and were considered significant at $P < 0.05$.

Results

NASH Predisposes to More Severe Hepatic IRI than SS, but Fat Depletion by PPAR-α Agonist, Wy-14,643 Protects Against IRI in Both Types of Fatty Liver Disease. Chow-fed *foz/foz* mice developed SS, whereas WT mice had lean livers unless fed an HFD, which also caused SS. In contrast, HFD-fed *foz/foz* mice developed severe steatohepatitis (NASH), which is associated with

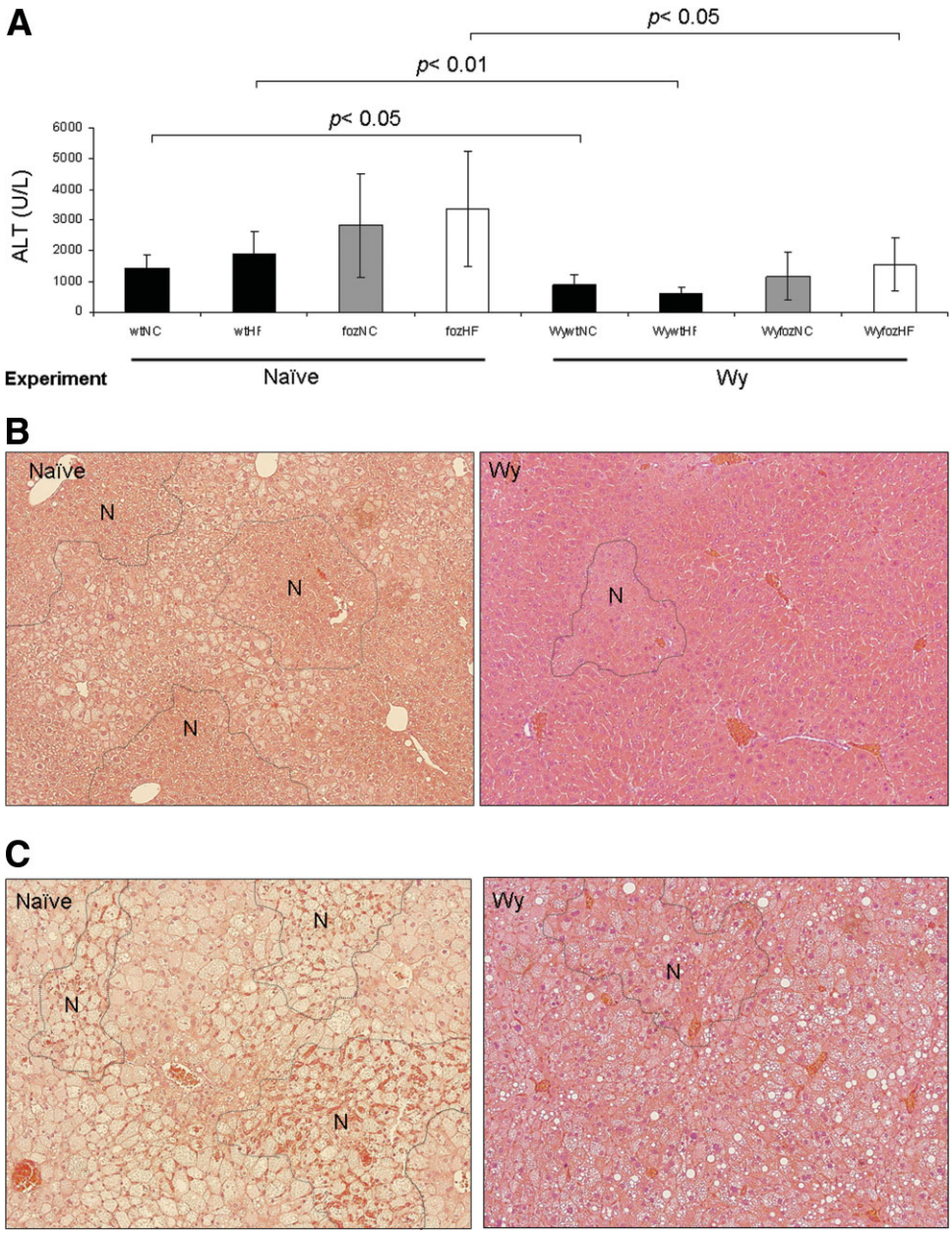

Fig. 2. ALT levels and hepatic necrotic area after IRI in NASH, steatotic and lean liver, and effects of pretreatment with Wy-14,643. (A) Hepatic IRI in naïve and Wy-14,643–pretreated mice subjected to 60 minutes of liver ischemia. Serum ALT levels were determined after 24 hours of reperfusion. Data are expressed as the mean ± SD (n = 5 mice per group). (B) Liver histology from naïve and Wy-14,643–pretreated WT mice fed an HFD and subjected to 60 minutes of hepatic ischemia and 24-hour reperfusion. Hematoxylin-eosin staining of representative liver sections (magnification ×200). Note marked steatosis in liver sections from naïve mice. N, necrotic areas. Sections are representative of five experiments. (C) Liver histology from a naïve *foz/foz* mouse fed an HFD and a Wy-14,643–pretreated animal subjected to 60 minutes of hepatic ischemia and 24-hour reperfusion. Hematoxylin-eosin staining of representative NASH liver sections (magnification ×200). N, necrotic areas. Sections are representative of livers of five experiments.

elevated serum ALT levels even in sham-operated animals (Fig. 2A,B). When subjected to 90 minutes of ischemia followed by 24 hours of reperfusion, there was substantial liver damage in this NASH model with ≈15-fold elevated serum ALT levels (15,000 ± 3500 U/L). Because there was mortality in 33% (2/6 mice), the ischemic interval was reduced to 60 minutes for subsequent studies. After 10 days of Wy-14,643 treatment in HFD or chow-fed *foz/foz* mice, histological analysis revealed a reduction in microsteatotic and macrosteatotic change, as well as fewer inflammatory foci compared with untreated animals (Fig. 2C,D). Wy-14,643 pretreatment in both the NASH and SS models proved to be protective against IRI, with a significant and substantial diminution in ALT levels (Fig. 2B) and areas of hepatocellular necrosis (Table 2, Fig. 2C,D).

Perturbed Microvascular Perfusion in Fatty Liver Is Exacerbated by Ischemia–Reperfusion but Is Not Restored by Pretreatment with PPAR-α Agonist. Visualization of the hepatic microcirculation by way of *in vivo* microscopy allows direct observation of the dynamics of sinusoidal blood flow, the impairment of which can be appreciated from the number of sinusoids in which flow is arrested. At all time points in WT or *foz/foz* animals fed an HFD, *in vivo* microscopy revealed enormous lipid-filled hepatocytes impinging on and narrowing the lumen of sinusoids (Supporting Video). As a result, the vessels appeared tortuous and irregular in caliber. Flow rates were

Table 2. Liver Necrotic Area in Mice Subjected to 60-Minute Ischemia and 24-Hour Reperfusion

Experimental Group	Liver Pathology	Naïve	Wy-14,643 Pretreated
WT NC	Lean	19 ± 2	7.1 ± 1.3*
WT HFD	Simple steatosis	29 ± 9.3	18 ± 5.7*
Foz NC	Simple steatosis	38 ± 15	19 ± 3.2*
Foz HFD	Steatohepatitis	60 ± 22	19 ± 8.6*

Values are expressed as the mean ± SD (n = 4 mice per group). The area of necrosis is expressed as a percentage of total area of liver section at low magnification (×4).

Abbreviations: Foz NC, *foz/foz* mice fed normal chow; Foz HFD, *foz/foz* mice fed a high-fat diet; WT HFD, wild-type mice fed a high-fat diet; WT NC, wild-type mice fed normal chow.

*$P < 0.01$ compared with naïve mice in the corresponding experimental group.

slow in contrast to sham-operated lean liver from chow-fed WT mice. Quantitative assessment of sinusoidal perfusion showed that a significant proportion of sinusoids had minimal or no flow compared with lean liver (Supporting Video). The sluggish perfusion in steatotic and NASH livers appeared to be further aggravated in animals subjected to 60-minute ischemia and 15-minute reperfusion; in some regions, sinusoidal flow was completely arrested (Fig. 3A). However, despite histological and biochemical reductions in hepatic steatosis (Fig. 2C,D) and a significant enhancement of sinusoidal blood flow in lean livers after IRI (Fig. 3B), Wy-14,643 failed to restore (or may have further impaired) sinusoidal flow in livers with SS or NASH (Fig. 3B).

Effects of Fatty Liver Pathology and Wy-14,643 Treatment on Inflammatory Mediators and Cytokines During Hepatic Ischemia–Reperfusion. ICAM-1 mediates the adhesion of leukocytes to activated endothelium through integrins, induces firm arrest of inflammatory cells at the vascular surface, and promotes leukocyte extravasation.[13] VCAM-1 is transcriptionally induced on sinusoidal endothelial cells but can also be expressed by macrophages and other cell types. Its interaction with integrins induces signals in endothelial cells that trigger changes in their morphology, thereby allowing leukocyte transmigration.[14] Following 60-minute ischemia and 24-hour reperfusion, there was at least a three-fold increase in hepatic messenger RNA levels of VCAM-1 (Fig. 4A) and a two-fold up-regulation of ICAM-1 (Fig. 4B) in naïve compared with sham-operated mouse livers. Wy-14,643 abrogated VCAM-1 messenger RNA expression (Fig. 4A) in all mice irrespective of genotype or diet. The real-time polymerase chain reaction findings were corroborated by way of western immunoblotting of liver lysates for VCAM-1, which revealed a striking reduction in its protein expression in Wy-14,643–treated lean and fatty livers subjected to IRI (Fig. 4C). The effect of Wy-14,643 on ICAM-1 transcript and protein levels was significant in lean livers; however, despite similar trends, the apparent suppression in steatotic and NASH livers was not significant (Fig. 4B,C). Wy-14,643 pretreatment also suppressed production of the proinflammatory cytokines IL-1a and IL-12 in livers with SS and NASH compared with naïve livers, whereas significant reduction in TNF release was only observed in NASH livers from HFD-fed *foz/foz* mice (Fig. 4D). Taken together, these findings show varying patterns of cytokine production after hepatic IRI according to underlying liver pathology, with IL-12 increases in both SS and NASH but significant increases in IL-12 and a trend of increasing TNF only in NASH livers. Correspondingly, PPAR-α agonist treatment appears to have different inhibitory effects on pathways involved with inflammatory recruitment and proinflammatory cytokine production in lean and fatty liver, with suppression of all cytokines studied being most consistent in NASH livers.

Wy-14,643 Treatment Activates NF-κB (by IκB-α Degradation, p65 Nuclear Expression), Triggers IL-6 Release and Induces Cell Cycle Entry. NF-κB governs transcriptional regulation of genes involved in cell cycle control, cell death, and cytoprotection against injury. In resting hepatocytes, NF-κB is sequestered in the cytosol bound to inhibitory proteins, among which IκB-α is most abundant. The transactivation of NF-κB requires phosphorylation, ubiquitination, and degradation of IκB-α for the liberation of NF-κB protein and transport to the nucleus. As described,[12] levels of IκB-α in whole liver homogenates decreased at 2-hour reperfusion (data not shown), but more impressive degradation was evident at 24-hour reperfusion in Wy-14,643–treated mice (Fig. 5A). NF-κB translocation was demonstrated by heightened p65 expression in nuclear extracts from the same samples compared with their respective cytosolic fractions (Fig. 5B). These findings are consistent with the well-described effects of PPAR-α agonists on NF-κB. Exposure to a single dose of Wy-14,643 has been shown to cause rapid activation of NF-κB in Kupffer cells and hepatocytes, an effect thought to be dependent on nicotinamide adenine dinucleotide phosphate oxidase.[15]

Of note, long-term feeding experiments in mice with Wy-14,643 can cause hepatomegaly by peroxisomal proliferation of hepatocytes, and induce hepatocellular proliferation.[15,16] In the present study, 10 days of pretreatment of Wy-14,643 did not significantly increase hepatocellular proliferation compared with untreated animals. However, following IRI, Wy-14,643–treated mice showed cell cycle entry evident by way of PCNA immunostaining (Fig. 5B) as well as cyclin D1 protein expression by way of immunoblotting (Fig. 5C). Interestingly, Wy-14,643 treatment also significantly enhanced the production of the pro-proliferative cytokine IL-6 in serum from all mice subjected to IRI,

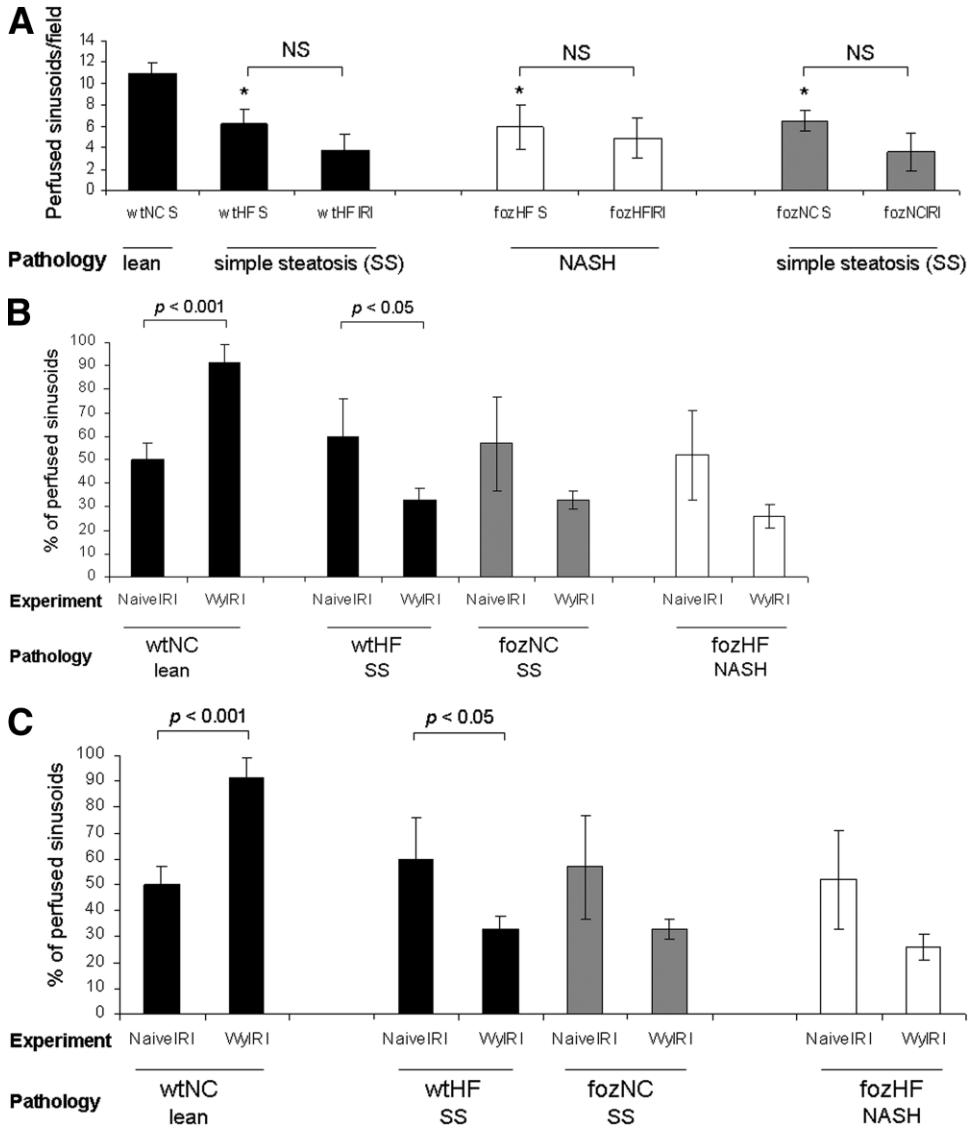

Fig. 3. Microvascular perfusion in fatty livers is exacerbated by IRI but not restored by Wy-14,643. The Supporting online Video shows *in vivo* microscopic images of the hepatic microcirculation in an obese *foz/foz* mouse fed an HFD. Note the sluggish flow in the distorted sinusoidal network, with a serpiginous appearance of vessels of varied caliber in *foz/foz* liver (original magnification ×1000). (A) Number of perfused sinusoids in centrilobular and periportal areas of livers from animals subjected to 60 minutes of ischemia and 15 minutes of reperfusion compared with sham-operated controls (n = 4 mice per group; five centrilobular and five periportal fields assessed per mouse). Mice with lean (WT NC), steatotic liver (WT HFD, foz NC), and NASH (foz HFD) are denoted. (B) Percentage of perfused sinusoids in centrilobular and periportal areas of livers from naïve and Wy-14,643–pretreated animals subjected to 60 minutes of ischemia and 15 minutes of reperfusion (n = 4 mice per group; five centrilobular and five periportal fields assessed/mouse).

regardless of genotype or diet (Fig. 5D). Because IL-6 is likely the pivotal cytokine that mediates hepatoprotection and cell cycle entry from ischemic preconditioning,[12] we conclude that Wy-14,643 pretreatment in the setting of ischemia reperfusion in fatty (or lean) livers can accelerate and enhance cell cycle entry of hepatocytes, thereby contributing to the observed hepatoprotection conferred by this compound.

Wy-14,643 Activates Stress-Activated Protein Kinases, p38, and c-jun N-Terminal Kinase-1. Induction of cyclin D1, one of the key steps in the pathway of resting cells to enter the cell cycle is under the regulatory control of the stress- and mitogen-activated protein kinases, p38 kinase, and c-jun N-terminal kinase.[17] In earlier work using lean livers, we reported that ischemic preconditioning against hepatic IRI activated p38 but suppressed c-jun N-terminal kinase phosphorylation, a pathway that we and others have characterized as pro-injurious during IRI.[12,17] In the present study, we determined p38 kinase activation by measuring relative amounts of native and phosphorylated p38 protein in liver homogenates at 24-hour reperfusion in naïve and

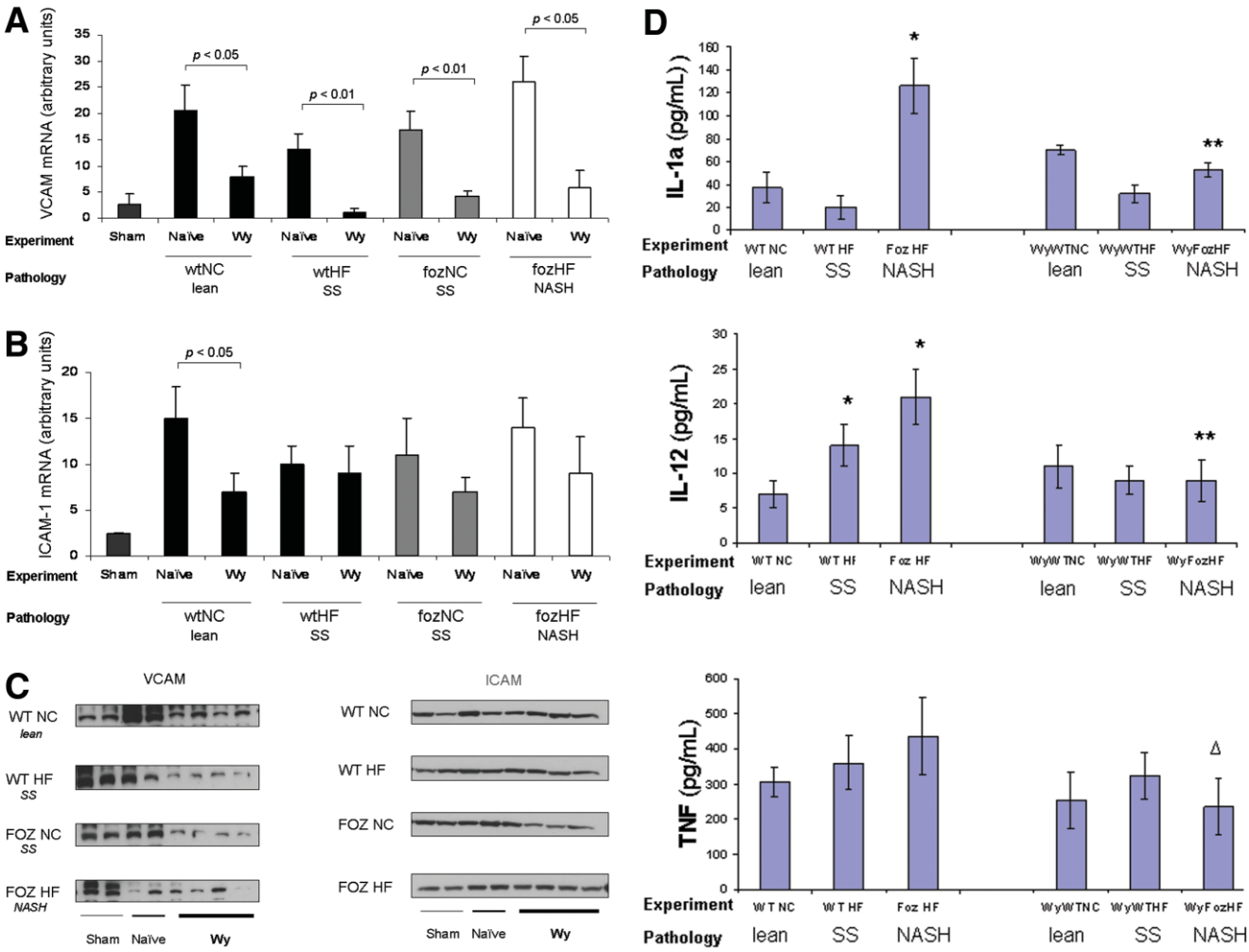

Fig. 4. Effects of Wy-14,643 on hepatic VCAM-1 and ICAM-1 expression during IRI. (A) VCAM-1 and (B) ICAM-1 messenger RNA levels were determined by way of real-time polymerase chain reaction, normalized to glyceraldehyde 3-phosphate dehydrogenase and expressed in arbitrary units. Abbreviations are as for Fig. 3. Results are expressed as the mean ± SD (n = 4 mice per group). Significant differences are denoted by *P* values between groups. (C) VCAM-1 and ICAM-1 immunoblots are from sham-operated, naïve, and Wy-14,643-treated mice subjected to 60 minutes of ischemia, 24 hours of reperfusion. Results are representative of four experiments at least in triplicate. Each lane contains 20 μg protein of liver homogenate from a single mouse at the indicated times of reperfusion. β-Actin was used as the loading control (not shown to minimize panels). (D) Release of serum IL-1a, IL-12, and TNF by antibody-coupled bead-based assay. Results are expressed as the mean ± SD (n = 4 mice per group). *$P < 0.05$ compared with WT NC (lean) or WT HFD (SS) mice. **,$^{\Delta}P < 0.05$ compared with naïve HFD-fed *foz/foz* mice with NASH.

Wy-14,643–treated mice fed normal chow or an HFD. Wy-14,643 treated mice showed increased levels of p38-phosphoprotein at 24-hour reperfusion compared with naïve and sham-operated mice, irrespective of diet and corresponding liver pathology (Fig. 6). Whereas activation of c-jun N-terminal kinase-1 by phosphorylation was apparent in treated livers, levels were not significantly different between naïve and Wy-14,643 pretreated mice (data not shown).

Discussion

Experiments on IRI and organ preservation for transplantation in several dietary and genetic rodent models have provided solid evidence that steatotic liver predisposes to IRI and graft failure of affected livers.[8] The present study used a model with the pathophysiological hallmarks of NASH (obesity, dyslipidemia, diabetes, hypoadiponectinemia), together with the appropriate spectrum of liver pathology. When fed commercial rodent chow, *foz/foz* mouse livers show extensive steatosis, even at 3 months. When fed an HFD, weight gain and the metabolic changes are accentuated and livers show severe NASH with fibrosis.[9] We also produced steatosis in WT mice fed an HFD for 8 weeks, thereby allowing us to compare livers showing NASH and two models of simple steatosis, as well as lean liver.[18-20] The highly reproducible phenotype of liver disease and metabolic syndrome with type 2 diabetes in these obese mice, together with the

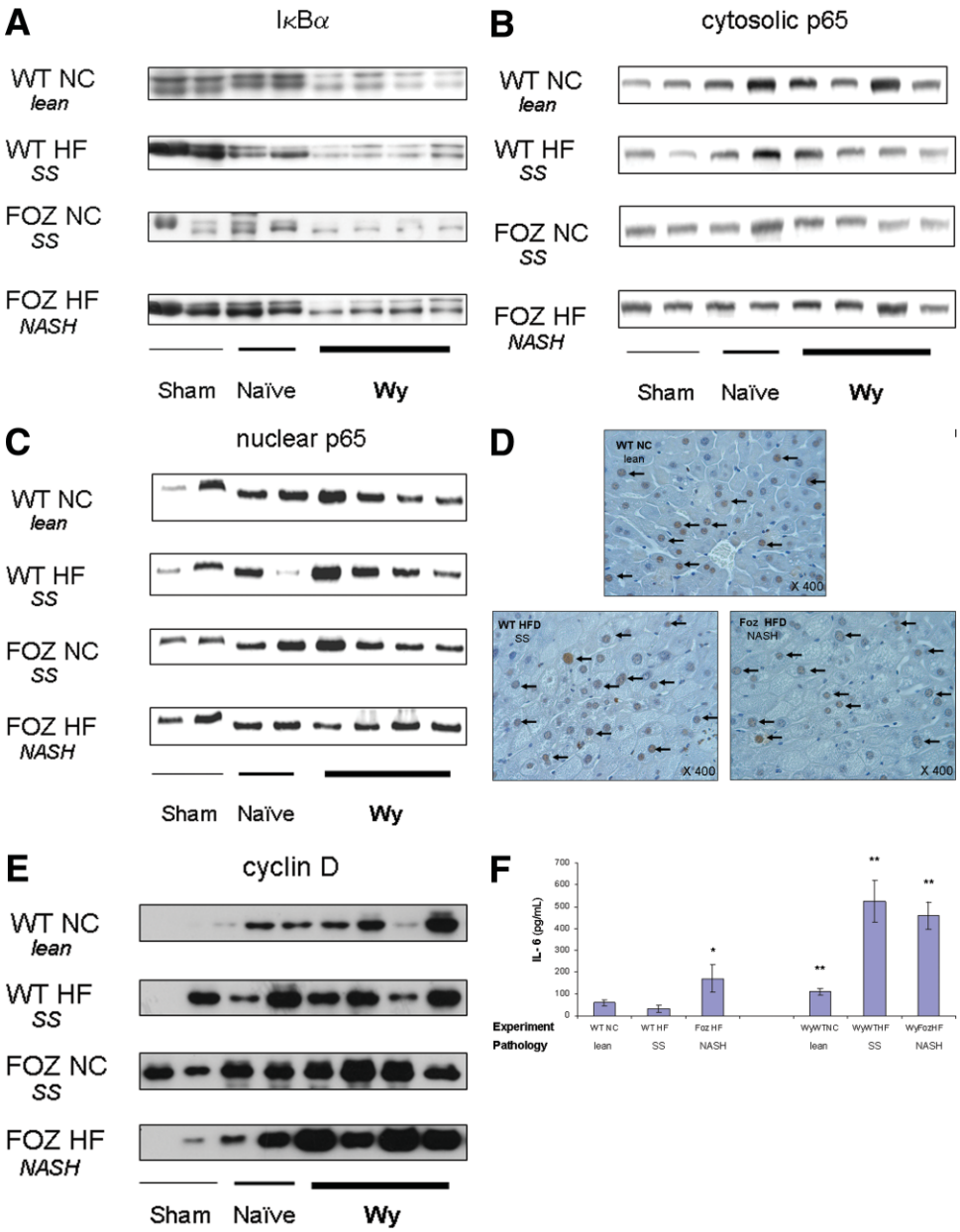

Fig. 5. Effects of Wy-14,643 on IκBα, nuclear p65, cyclin D1, and PCNA expression in naïve and treated liver subjected to IRI. (A) IκBα expression by way of western blotting. Each lane contains a sample (20 μg/lane) from a single mouse sacrificed at 24 hours of reperfusion after 60 minutes of ischemia. Blots are representative of four experiments performed at least in duplicate. (B,C) p65 nuclear and cytoplasmic expression by way of western blotting indicative of NF-κB translocation. Each lane contains nuclear extract or cytoplasmic fraction (20 μg/lane) from the corresponding liver samples in (A). Blots are representative of four experiments performed at least in duplicate. (D) PCNA immunostaining of liver sections after 60 minutes of ischemia and 24 hours of reperfusion. Multiple PCNA-positive staining nuclei (arrows) are evident in Wy-14,643–pretreated lean, steatotic, and NASH livers. Hematoxylin-eosin with PCNA staining (original magnification ×400). (E) Hepatic cyclin D1 expression by immunoblotting in naïve and Wy-14,643–treated mouse liver lysates. Each lane contains a sample (20 μg/lane) from a single mouse sacrificed at 24 hours of reperfusion after 60 minutes of ischemia. Blots are representative of four experiments performed at least in duplicate. (F) Serum IL-6 concentration by antibody-coupled bead-based assay. Results are expressed as the mean ± SD (n = 4 mice per group). *$P < 0.05$ compared with WT NC (lean). **$P < 0.05$ compared with naïve animals of corresponding genotype and diet.

facility to compare steatohepatitis with SS, makes this a suitable model to examine microvascular changes in fatty liver disease and in which to test putative protective agents, the main purpose of the present study.[20-24]

The substantial changes in blood flow noted in livers with both NASH and SS in the present study are consistent with other studies in fatty liver disease.[22,23] Using Doppler flowmetry, Seifalian et al. demonstrated reduced sinusoidal perfusion in fatty human liver donors compared with healthy livers.[25] Analogous studies in rabbits with diet-induced steatosis confirmed that this reduction in perfusion correlated with the severity of fat accumula-

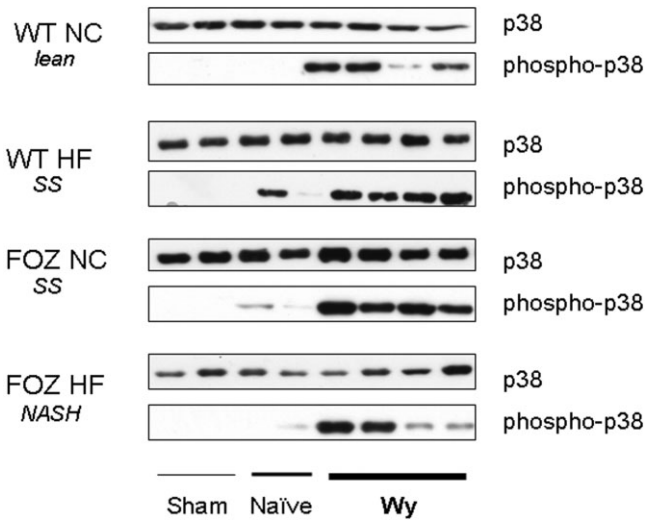

Fig. 6. Activation of p38 evaluated by way of western blotting in naïve and Wy-14,643–pretreated mouse liver homogenates. Mice were subjected to 60 minutes of ischemia and 24 hours of reperfusion. Representative blots of native p38 and phosphorylated p38 protein expression are shown. Each lane contains 20 μg protein from a different mouse. Blots are representative of four experiments performed at least in duplicate.

tion in hepatocytes.[26] Here, we have demonstrated that the reductions in sinusoidal perfusion appear to arise initially from the effects of enlarged hepatic parenchymal cells, swollen with accumulated lipid, which widen the parenchymal cell plates and narrow and distort the lumens of sinusoids. Other investigators have shown that as a result of the structural alterations around them, the sinusoids become inefficient conduits of blood with resulting impairment of tissue perfusion, evidenced by the significant reductions in the numbers of perfused sinusoids per microscopic field.[22,27-29]

Not surprisingly, the impaired hepatic microcirculation in fatty liver disease has been implicated in the decreased tolerance of the fatty liver to IRI,[28,30] as well as drugs and toxins.[31] Patients with fatty liver experience significantly higher mortality rates (15%) after hepatic surgery compared with lean individuals (2%). To date, there are no available therapies against IRI in the fatty liver that are practical and affordable. In this study, we show that the short-term treatment of mice with steatotic livers and NASH with Wy-14,643, a potent PPAR-α agonist, depletes lipid from hepatocytes and protects fatty livers from IRI. However, contrary to our initial hypothesis, the protective effect of Wy-14,643–mediated lipid reduction in NASH and SS livers could not be attributed to improved microcirculatory blood flow, which was increased after Wy-14,643 treatment only in lean livers. Instead, we found that the profile of cytokine release after IRI differed both quantitatively and qualitatively between NASH versus SS versus lean livers, and that Wy-14,643 restored the exaggerated profile noted in NASH livers to that found in lean livers.

From liver histology, hepatocellular necrosis appeared to be the major mode of cell death following IRI in the models of NASH and SS in the present study. Furthermore, necrotic cell death was significantly abrogated by Wy-14,643 pretreatment. This hepatoprotective effect seems likely to be multifactorial, because, in addition to reduction of hepatic lipid content, PPAR-α–stimulation caused activation of NF-κB and p38 kinase, release of IL-6 and induction of a hepatoproliferative response through these signaling pathways. Further evidence that the mechanism for Wy-14,643–mediated protection against IRI in fatty livers involves dampening of the inflammatory response came from studies of adhesion molecule expression. Thus, hepatic expression of VCAM and ICAM-1, molecules that facilitate transendothelial migration of leukocytes, increased in fatty livers compared with lean livers 24 hours following IRI. Wy-14,643 downregulated VCAM and ICAM-1 messenger RNA and VCAM protein; the discordance is consistent with other reports.[10]

IL-1, IL-12, and TNF have all been implicated as proinflammatory cytokines with direct roles in the induction and development of hepatic IRI. Specific blockade of IL-12 or TNF protects against hepatic IRI in mice, whereas TNF-null mice manifest minimal liver injury following ischemia–reperfusion.[32-34] Fatty liver (particularly NASH) was associated with greater increases in these three proinflammatory cytokines (particularly IL-12), whereas Wy-14,643 pretreatment significantly diminished their production after hepatic IRI compared with naïve livers with the same pathology; the most significant effects were attenuation of IL-12 and TNF release in NASH livers from HFD-fed *foz/foz* mice.

We have also shown that IL-6 is released during ischemic preconditioning, and exogenously administered IL-6 in comparable "physiological" doses confers hepatoprotection and a pro-proliferative stimulus against IRI in lean mouse livers.[35,36] Using pharmacological doses of IL-6, others have confirmed the hepatoprotective effects of IL-6 in both lean and fatty livers (SS) in genetic rodent models of nonalcoholic fatty liver disease.[36,37] In our study, Wy-14,643 significantly enhanced production of IL-6 in mice subjected to IRI, regardless of genotype or diet, but the effect in mice with NASH or SS was approximately five-fold greater than in lean mice. There was corresponding enhancement of cyclin D1 in mice with fatty liver, supporting the proposal that IL-6 plays an important role in the hepatoprotective and pro-prolifera-

tive effects of PPAR-α stimulation against hepatic IRI in NASH and SS.

Although rodents express ≈10-fold higher levels of PPAR-α than humans,[38] PPAR-α agonists are clinically and functionally relevant as fibrate therapy against hyperlipidemia and to reduce hypertriglyceridemia and complications of peripheral vascular disease in patients with type 2 diabetes, the latter unrelated to lipid-lowering effects.[39] PPAR-α agonists also partially inhibit activation of human T lymphocytes and reduce their cytokine production, all desired effects against hepatic IRI.[40] Despite their potentially beneficial roles, PPAR-α agonists should be used judiciously. Long-term exposure to peroxisome proliferators in rodents can lead to increase in peroxisomes, hepatocellular proliferation, oxidative DNA damage, suppression of apoptosis, and, in chronic feeding experiments, hepatocellular adenoma and carcinoma (though the latter effects have not been reported in humans).[15,40-43] Short-term administration in humans (1-10 days) would be unlikely to produce permanent genotoxic effects. In the present study, the 10-day treatment regimen with oral Wy-14,643 did not cause significant hepatomegaly, but in mice subjected to IRI, this PPAR-α agonist induced cell cycle entry evident by PCNA and cyclin D1. The only pharmacological approach to prevent IRI in fatty livers shown experimentally to be effective in preliminary studies is IL-6. For reasons related to high cost and potential proinflammatory and injurious effects of high-dose IL-6, this form of hepatoprotective therapy has never been trialed clinically. The present results point to a mechanism for raising endogenous levels of IL-6 by a simple, inexpensive pharmacological therapy (PPAR-α agonist) that is unlikely to confer deleterious effects of this cytokine, and further studies are warranted.

In conclusion, NASH and simple steatosis contribute to mortality after liver surgery and acute graft failure during liver transplantation because of the increased susceptibility of fatty livers to oxidative forms of liver injury. Microcirculatory and cellular changes in the fatty liver sensitize the organ to the effects of IRI, toxins, and drugs, leading to an exacerbated response to liver damage. In Asia, 90% of all liver transplantations performed are from living related donors, whereas up to 10% of liver transplantations in North America are from live donors.[44-46] Of those livers, between 15% and 50% exhibit significant fatty liver disease.[44] Use of a PPAR-α agonist may be a simple, inexpensive, and safe preoperative intervention that opens up the potential for rendering steatotic livers—especially those from living donors—safe for transplantation.

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