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Role of Hypolipidemic Drug Clofibrate in Altering Iron Regulatory Proteins IRP1 and IRP2 Activities and Hepatic Iron Metabolism in Rats Fed a Low-Iron Diet

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In addition to reducing the expression of transferrin and ceruloplasmin genes, hypolipidemic peroxisome proliferators may alter iron homeostasis in the liver. Therefore, this study investigates the effects of clofibrate on proteins related to liver iron metabolism in a rat model using a 2 × 2 experimental design: two dose levels of clofibrate in diet (0 and 0.5%) and two dietary iron levels (35 ppm as normal level and 15 ppm as low-iron diet). Twenty-four Wistar rats were assigned to the four diets and fed for 6 weeks. Subsequent measurements of iron parameters in the blood and the liver indicated that, in addition to mild anemia and the reduction in serum iron and total iron-binding capacity, clofibrate treatment altered IRP1 and IRP2 activities differentially and increased mitochondrial aconitase both at activity and protein levels. At both normal and low-iron intakes, clofibrate caused a 50% reduction in serum iron and TIBC with a corresponding reduction in transferrin mRNA. The RNA-binding activities of IRP1 were selectively activated by clofibrate treatment even though liver iron concentration was not depleted. The RNA-binding activity of IRP2 was selectively activated by the low iron intake and correlated with an increase of transferrin receptor mRNA, while clofibrate treatment offset the effects of the low iron intake. © 2002 Elsevier Science (USA)

Key Words: **clofibrate; iron regulatory proteins; mitochondrial aconitase; transferrin receptor; rats.**

Fibrates such as clofibrate, fenofibrate, and gemfibrozil are an established class of drugs for treating hypertriglyceridemia and combined hyperlipidemia. Fibrates are peroxisome proliferators (PPs), which cause hepatomegaly, proliferation of peroxisomes, and induction of many enzymes involved in β-oxidation and ω-oxidation of fatty acids in rodents (Schoonjans *et al.,* 1996). Although incapable of inducing peroxisome proliferation in humans, fibrates alter the expression of enzymes regulating serum cholesterol and lipid homeostasis (Auwerx, 1992; Schoonjans *et al.,* 1996).

PPs also caused transcriptional repression in the liver. Genes down-regulated by PPs include those related to lipid metabolism, such as apoE, apoAI, apo AIV, and apo CIII (Schoonjans *et al.,* 1996), and those not obviously involved in lipid homeostasis, such as transferrin (Tf) (Hertz *et al.,* 1996), ceruloplasmin (Cp), and P-type ATPase 7B (ATP7B) (Eagon *et al.,* 1999). Transferrin is highly expressed in the adult mammalian liver and is secreted by hepatocytes into the serum, where it functions as an iron-transport protein (Crichton and Charloteaux-Wauters, 1987). A lack of transferrin causes iron accumulation in the liver (Hayashi *et al.,* 1993). Meanwhile, ceruloplasmin is a copper-containing serum protein exhibiting ferroxidase activity, which accelerates the incorporation of iron into apotransferrin (Harris *et al.,* 1999). Aceruloplasminemic subjects (Harris, 1998) and Cp-null mice (Harris *et al.,* 1999) have marked iron accumulation in hepatocytes and reticuloendothelial cells.

At the cellular level, iron storage and uptake are coordinately regulated posttranscriptionally by interactions between cytoplasmic factors, iron regulatory proteins 1 and 2 (IRP1 and IRP2), and the *cis*-regulating iron-responsive element (IRE) located in the 3′-untranslated region (UTR) of transferrin receptor (TfR) mRNA as well as in the 5′-UTRs of mRNAs for ferritin, mitochondrial aconitase (m-ACO), and erythroid-specific 5-aminolevulinic acid synthase (Hentz and Kühn, 1996). When cellular iron becomes limiting, IRP1 is recruited into the high-affinity binding state, and IRP2 is synthesized in active form. The binding of IRPs to the IRE in the 5′-UTR represses the translation of ferritin and m-ACO, while an association of IRPs with IREs in the 3′-UTR stabilizes the TfR mRNA transcript. On the other hand, the expansion of the labile iron pool causes IRP1 inactivation and IRP2 degradation to result in an efficient translation of ferritin and m-ACO mRNA and rapid degradation of TfR mRNA.

Powanda *et al.* (1978) were probably the first to describe the effects of clofibrate on iron and copper metabolism; serum Tf concentration and Cp ferroxidase activity were both reduced after 1 week of treatment, while the concentration of hepatic iron increased, but no copper accumulation occurred. Meanwhile, Hertz *et al.* (1996) demonstrated that other fibrate com-

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pounds caused a 50% reduction of serum iron and total iron-binding capacity (TIBC), corresponding to a reduced Tf mRNA level in rat as well as in a hepatoma cell line. PPs also caused a reduction of liver Cp and ATP7B mRNA and serum Cp activity (Anderson *et al.,* 1999; Eagon *et al.,* 1999). In contrast to the outcome of 1 week of treatment (Powanda *et al.*, 1978), clofibrate caused a 3.3-fold increase of liver copper, but did not affect liver iron after a 60-day exposure (Eagon *et al.*, 1999).

Adverse outcomes associated with fibrate treatment include hepatotoxicity and anemia. Abnormal liver function tests including elevation of serum glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) have occurred during fibrate administration (Sirtori *et al.,* 1992). Increases to more than three times the upper limit of normal values have been recorded in controlled multiple-dose trials of fenofibrate (Schrefer, 2001). As for anemia, mild to moderate reduction of hemoglobin and hematocrit has been documented. In The Coronary Drug Project (The Coronary Drug Project Research Group, 1975), the clofibrate treatment group recorded a significant lowering of hematocrit (Hct) values (from 46 to 44.8%) compared to the placebo group (from 46.2 to 45.9%) and an elevated prevalence of anemia indicated by abnormal hematocrit values (Hct $<$ 38%, 7.9 vs 4.1%; Hct $<$ 36%, 3.4 vs 1.7%) after 5 years of follow-up. Therefore, we hypothesized that the onset of PP-induced anemia may depend on dietary iron level and that PP may alter hepatic iron homeostasis. Consequently, we investigated the effect of clofibrate on iron status, hepatic IRP activities, and the expression of hepatic ferritin, TfR, and m-ACO in rats fed a semisynthetic low-iron diet.

MATERIALS AND METHODS

Chemicals. The following chemicals were used: Clofibrate (ethyl 2-(4-chlorophenoxy)-2-methylpropionate) (Fluka Chemie, Germany), Serum iron/UIBC kit and Serum Copper kit (RANDOX, Antrim, UK), horseradish peroxidase-conjugated goat anti-rabbit IgG (Vectastain, Vector Laboratories), PVDF transfer membrane (PolyScreen, NEN Life Science, Boston, MA), α-[^{32}P]CTP (3000 Ci/mmol or 111 TBq/mmol, NEN–DuPont, Boston, MA), positively charged nylon membrane for hybridization, GeneScreen Plus (NEN Life Science), X-OMAT AR film and Bio-Max film (Kodak), reagents for *in vitro* transcription, DNA labeling kit, CTP4A Western blotting kit, ECL Western blotting detection system, and Micro Spin G-50 column (Amersham Pharmarcia Biotech Inc.). Other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) and Merck (Taiwan).

Animals and treatment. Twenty-four weanling, male Wistar rats (Laboratory Animal Center, College of Medicine, National Taiwan University), weighing 148 $\pm$ 10 g, were housed individually in stainless-steel cages with wire-mesh floors in a temperature-, humidity-, and light-controlled room. Animal care and handling conformed to the *NSC Guide for the Care and Use of Laboratory Animals* (National Science Council, 1993). The basal diet was AIN-76 formula (AIN, 1977) modified to contain 15% soybean oil (Table 1). Rats (six each group) were assigned to the four diets with treatment of clofibrate (0 and 0.5%) and dietary iron levels (35 and 15 ppm) in 2 $\times$ 2 experimental design. The 35 ppm Fe is the normal dietary level for rats, while the 15 ppm Fe is a suboptimal level that will deplete liver Fe store and may cause mild anemia (Chen *et al.,* 1997). Food and deionized water were freely available. After 42 days of dietary treatment, rats were killed by carbon dioxide asphyxiation, and blood and liver were collected for analysis. Serum was separated by low-speed centrifugation and was frozen at -70°C until analyzed.

TABLE 1
Compositions of Semisynthetic Normal and Low-Iron Diets[a] Containing Clofibrate

	Composition (%)			
	No clofibrate		0.5% Clofibrate	
Ingredients of diets	35 ppm Fe	15 ppm Fe	35 ppm Fe	15 ppm Fe
Casein	20	20	20	20
Corn starch	55	55	55	55
Soybean oil	15	15	15	15
Vitamin mixture AIN-76[a]	1	1	1	1
Mineral mixture[a] (iron omitted)	3.5	3.5	3.5	3.5
Cellulose	5	5	5	5
Choline	0.2	0.2	0.2	0.2
Methionine	0.3	0.3	0.3	0.3
Clofibrate[b]	—	—	0.5	0.5
Total amount	100	100	100	100
Iron content[c] (ppm)	35	15	35	15

[a] Based on AIN-76 formula (AIN, 1977), in which the 35 ppm Fe is normal dietary level for rats, while the 15 ppm Fe is a suboptimal level that will deplete liver Fe store and may cause mild anemia.
[b] Ethyl 2-(4-chlorophenoxy)-2-methylpropionate (Fluka Chemie).
[c] Iron source: ferric citrate ($FeC_6H_5O_7$; Fe–16–20%)

Blood analyses. Hemoglobin concentration was determined by the cyanmethemoglobin method using Drabkin's solution (Oser, 1965). Serum iron and TIBC were determined using the serum iron/UIBC kit. Serum copper was determined using the Serum Copper kit. Serum ceruloplasmin was assessed as ferroxidase activity by measuring the oxidation of *o*-diansidine dihydrochloride, and one unit of activity was defined as oxidation of 1 μmol substrate per minute (Schosinsky *et al.,* 1974).

Liver Fe and Cu. Fractions of liver were digested with nitric acid and hydrogen peroxide in a microwave digestion oven (Milestone, Microwave Laboratory System, Italy). Iron and copper were measured by atomic absorption spectrometry (Hitachi 180-30). The steady concentration of desferrioxamine (DFO)-chelatable iron was measured by electron paramagnetic resonance spectroscopy (EPR) as described by Cairo *et al.* (1995) and Yegorov *et al.* (1993). Briefly, 0.05 ml of 10 mM desferrioxamine mesylate was added to 0.5 ml of liver homogenate (50% w/v in 20 mM Tris–HCI and 135 mM KCl, pH 7.4), incubated at room temperature for 20 min, and then placed in a quartz tube, frozen in liquid nitrogen, and stored at -70°C before analysis. EPR spectra were recorded on a Brucker 200 spectrometer (NTHU Instrument Center, National Tsing Hua University, Hsin-chu, Taiwan) at liquid nitrogen temperature under the following conditions: Klystron frequency, 9.12 GHz; power, 20 mW; modulation amplitude, 2.0 mT. The intensity of the EPR signal at $g = 4.3$ was measured to estimate DFO-chelatable Fe concentration. A calibration plot was obtained by adding to a series of control homogenate incremental volumes of a freshly prepared $FeSO_4$ solution of predetermined concentration, and the homogenate was treated as described above.

Preparation of cellular fractions. Liver cytosol, mitochondria, and microsome were obtained by differential centrifugation modified from Chen *et al.* (1997). Liver homogenate (25% w/v) was prepared by homogenizing a fraction of liver in ice-cold HDSC buffer (50 mM Hepes, pH 7.4, 1 mM dithiothreitol, 0.25 M sucrose, 2 mM trisodium citrate, 0.5 mg/L leupeptin and 0.2 mM

TABLE 2
Oligonucleotides for Reverse Transcriptase–PCR Cloning

Protein	Sequence of 5′ sense (1) and 5′ antisense (2) strands	Probe size (bp)	Sequence no.	GenBank access no.	Reference
Transferrin	(1) 5′ TggCT CaggA ACACT TTggc (2) 5′ ggAgC ACAgC CTTgA CTgAA	658	893–1550	D38380	Hoshino *et al.* (1996)
Transferrin receptor	(1) 5′ ggTAA ACTgg TCCAT gcTAA T (2) 5′ CATTG TTCAC AgTgA gcTTC	451	275–725	M58040	Roberts and Griswold (1990)
β-Actin	(1) 5′ gTggg CCgCT CTAgg CACCA (2) 5′ CTCTT TgATg TCACg CACgA TTTC	540	103–642	55574	Nudel *et al.* (1983)

phenylmethylsulfonyl fluoride) using a Potter–Elvehiem homogenizer. The mitochondria pellet was obtained from the postnuclear supernatant by centrifugation at 15,000*g* for 30 min, washed three times to reduce cytosolic contamination, resuspended in HDSC, and stored at −70°C. The postmitochondrial supernatant was further centrifuged at 105,000*g* for 1 h (Ultra-centrifuge, 50.4 Ti Rotor, Beckman). The supernatant was designated as the cytosol fraction and the pellet was designated as the microsomes. The microsome pellet was suspended in 0.05 M phosphate buffer containing 1 mM EDTA at pH 7.7, stored at −70°C, and used for CYP4A analysis.

Electrophoretic mobility shift assay of IRPs activity. IRPs–IRE binding activity was determined by gel retardation analysis using a ^{32}P-labeled IRE-containing RNA probe of the first 73 nucleotides from rat L-ferritin 5′-UTR in the absence or presence of 2-mercaptoethanol (2-ME) (Chen *et al.*, 1997; Schalinske and Eisenstein, 1996). Rat L-ferritin cDNA (kindly provided by Dr. R. S. Eisenstein, University of Wisconsin, Madison, WI) was linearized by *Sma*I digestion and used as template. ^{32}P-labeled IRE probe was produced by *in vitro* transcription using α-[^{32}P]CTP, Riboprobe Germini II Core System (Promega) and T7 RNA polymerase (Boeringer Mannheim, Germany) and then purified by a 10% acrylamide/8 M urea gel. The specific radioactivity of the probe was estimated to be about 4700−4900 cpm/fmol.

For the binding assay, 7.5 μg of cytosol protein (measured with the Lowry method; Lowry *et al.*, 1951) was incubated with a saturating level of ^{32}P-labeled IRE probe (final concentration at 1 nmol/L) in the presence of reaction buffer (10 mM Hepes, pH 7.6, 3 mM $MgCl_2$, 40 mM KCl, 1 mM DTT, and 5% glycerol) in a final volume of 20 μl for 10 min at room temperature and then 2 μl heparin (5 mg/mL) was added and incubated for another 5 min (Barton *et al.*, 1990). When measuring total IRP1 in the cytosol, 1.5 μg of cytosol protein was used in the binding reaction and incubation was carried out in the presence of 2-ME.

The IRP-bound probe was separated from the free probe on a 4% nondenaturing polyacrylamide gel (60:1 acrylamide/bisacrylamide) in 0.5× Tris borate–EDTA buffer and electrophoresed (Hofer SE-600, with a cooling system) at 300 V for 1.5 h at 5°C. The gel was then dried on filter paper (Gel drier Model 283, Bio-Rad), and an autoradiogram on X-OMAT AR film was prepared. The bands corresponding to IRE-bound IRP1 and IRP2 were cut out and counted in a liquid scintillation counter (Beckman, LS 5000 CE).

Western blot analysis of cytochrome P450 4A, ferritin, and mitochondrial aconitase. For the analysis of CYP 4A, the microsomal fraction containing 5 μg of protein was resolved on a 12% SDS–polyacrylamide gel, transferred to PVDF membranes, detected by a commercial CYP4A Western blotting kit, and visualized with an ECL Western blotting detection system. For the analysis of ferritin, liver cytosol was heated at 75°C for 10 min and then was centrifuged at 10,000*g* for 20 min, and protein in the supernatant was resolved on a 15% SDS–polyacrylamide gel, transferred to PVDF membranes, detected with rabbit anti-rat ferritin polyclonal antibodies (Shaw and Chen, 1993) and horseradish peroxidase-conjugated goat anti-rabbit IgG, and the immunoreactive, 19–21-kDa protein was visualized with an enchanced chemiluminescence of ECL detection regents (Amersham Pharmarcia Biotech Inc.). For the analysis of m-ACO, the mitochondrial fraction containing 10 μg of protein was resolved on a 10% SDS–polyacrylamide gel, transferred to PVDF membranes, detected with rabbit anti-human m-ACO polyclonal antibody (kindly provided by Dr. H.-H. Zhuang, Memorial University of Chang Gung) and alkaline phosphatase-conjugated goat anti-rabbit IgG (Tropix), and visualized with an enhanced chemiluminescence substrate CDP-star (Tropix). The results of the Western blotting analyses were recorded on Bio-Max films and quantified by densitometry (Microcomputer image device-M4 V3.0).

RNA preparation and Northern blot analysis of transferrin and transferrin receptor. Total RNA was extracted from liver using TRIZOL reagent (GIBCO-BRL, Grand Island, NY). All the cDNAs used as probes were obtained by cloning RT–PCR products of total RNA prepared from the liver of control rats, and their identities were confirmed by sequencing. The synthetic oligonucleotides used to amplify respective cDNA probe are listed in Table 2. The cDNA probes was prepared using a DNA labeling kit and purified by a Micro Spin G-50 column. Ten and 40 μg of total liver RNA was used for Tf and TfR analysis, respectively. RNA was separated on a formaldehyde–agarose gel (1%) and transferred to a nylon membrane. Following UV/cross-linking (Hoefer), membrane was hybridized to ^{32}P-labeled probes ($>5 \times 10^8$ cpm/μg DNA) using β-actin as an internal standard. All the results were recorded on X-OMAT AR films and quantified by densitometry.

Aconitase enzyme assay. Activity of m-ACO was determined by the coupled reaction of aconitase (EC 4.2.1.3) and isocitrate dehydrogenase (EC 1.1.142) (Rose and O'Connell, 1967). One unit of enzyme activity was defined as the reduction of 1 μmol $NADP^+$ per minute.

Statistical analysis. The main effects of dietary iron level and clofibrate were evaluated by two-way ANOVA. The differences among the four groups were analyzed by Duncan's Multiple Range test. The statistical significance level was set at $p < 0.05$. All statistics were carried out on the SAS System (Version 6.12, SAS Institute, Cary, NC).

RESULTS

Growth Repression, Hepatomegaly, and Liver Enzymes

Clofibrate treatment reduced body weight and weight gain in the normal iron group by 21 and 30%, respectively, and in the low-iron group by 14 and 21%, respectively (Table 3). Growth suppression by clofibrate treatment was greater at normal iron intakes than at low iron intakes. Since food intakes were reduced by less than 10%, the suppression occurred mainly due to reduced feed efficiency, indicating inefficient energy utilization.

Clofibrate-treated rats displayed marked hepatomegaly (Table 4). Liver weights increased 10 and 30% and relative liver weights increased 40 and 50% at normal and low iron intakes, respectively. The hepatomegaly in terms of final liver weight

TABLE 3
Growth and Feed Efficiency of Rats as Affected by Low Iron Intake and Clofibrate Treatment

Treatment and Fe group[a]	No clofibrate[b]		0.5% Clofibrate[b]		*p* value of two-way ANOVA	
	35 ppm Fe	15 ppm Fe	35 ppm Fe	15 ppm Fe	Fe	Clofibrate
Final body weight (g/rat)	417 ± 41^{1}	378 ± 24^{2}	330 ± 15^{2}	325 ± 22^{2}	0.43	0.0001
Body weight gain (g/day/rat)	6.7 ± 1^{1}	5.8 ± 0.7^{2}	4.7 ± 0.3^{3}	4.5 ± 0.5^{3}	0.043	0.0001
Food intake (g/rat/day)	19.1 ± 2.3^{1}	$17.3 \pm 1.3^{1,2}$	17.4 ± 1.4^{2}	16.1 ± 1.5^{2}	0.26	0.047
Feed efficiency[c,d]	0.35 ± 0.01^{1}	0.33 ± 0.02^{1}	0.27 ± 0.01^{2}	0.27 ± 0.01^{2}	0.17	0.0001

[a] The 35 ppm Fe is normal dietary level for rats, while the 15 ppm Fe is a suboptimal level that will deplete liver Fe store and may cause mild anemia.
[b] Each value represents group mean ± SD of six rats. Different superscript numbers in each row indicate significant difference by Duncan's Multiple Range test at $p < 0.05$.
[c] Feed efficiency = body weight gain (g/day/rat) ÷ feed intake (g/day/rat).
[d] Log-transformed data were analyzed.

was comparable between the two clofibrate-treated groups, regardless of iron levels. While maintained within normal ranges, serum GOT and GPT were significantly elevated by clofibrate treatment, and this phenomenon was aggravated by low iron intake. As expected, clofibrate treatment induced significant expression of CYP 4A in microsomes (Fig. 1), which was not compromised by the low iron intake.

Indices of Iron Status

Both clofibrate treatment and low iron intake caused reduction in hemoglobin concentration (significant main effects at $p < 0.05$, Table 5). Low iron intake or clofibrate alone reduced hemoglobin by 8%, while the decrease in hemoglobin in the clofibrate groups was not significantly influenced by dietary iron levels. In contrast, clofibrate, but not iron intake, significantly affected serum iron and TIBC, both of which were reduced by 50% regardless of dietary iron levels. Meanwhile, transferrin saturation remained normal for all four groups and was not influenced by clofibrate or iron intakes (Table 5).

Hepatic ferritin (Fig. 2) and iron (Table 6) levels changed with dietary iron intakes and were significantly lower at low iron intakes. Clofibrate treatment caused total liver iron to increase by 25 and 86% in the normal and low-iron groups, respectively (Table 6). Meanwhile, clofibrate caused an 114 and 35% increase of DFO-chelatable iron at normal and low iron intakes, respectively; while low iron intake caused a 54 and 27% decrease with and without clofibrate treatment, respectively. At normal iron intake, the clofibrate-treated rats exhibited the highest concentration of DFO-chelatable iron, which was 2.2 times that of the untreated counterpart. Furthermore, the clofibrate treatment caused a twofold increase in m-ACO activity at both normal and low iron intakes (Table 6). The expression of m-ACO protein increased with clofibrate treatment and was significantly higher at low iron intakes (Fig. 3).

Transferrin and Transferrin Receptor

Clofibrate treatment significantly reduced hepatic Tf mRNA level by approximately 50% at both normal and low iron

TABLE 4
Liver Weights and Liver Function Indicators in Rats as Affected by Low Iron Intake and Clofibrate Treatment

Treatment and Fe group[a]	No clofibrate[b]		0.5% Clofibrate[b]		*p* value of two-way ANOVA	
	35 ppm Fe	15 ppm Fe	35 ppm Fe	15 ppm Fe	Fe	Clofibrate
Liver weight (g)[c]	23.8 ± 2.4^{1}	20.4 ± 1.4^{2}	26.2 ± 0.9^{3}	26.9 ± 1.0^{3}	0.06	0.0001
Relative liver weight (% body weight)	5.7 ± 0.2^{1}	5.4 ± 0.2^{1}	7.9 ± 0.3^{2}	8.3 ± 0.5^{2}	0.24	0.0001
GOT[d] (U/L)	58 ± 8^{1}	$64 \pm 5^{1,2}$	$61 \pm 6^{1,2}$	73 ± 6^{2}	0.04	0.0238
GPT[d] (U/L)	25 ± 4^{1}	27 ± 5^{1}	28 ± 4^{1}	37 ± 9^{2}	0.04	0.0065

[a] The 35 ppm Fe is normal dietary level for rats, while the 15 ppm Fe is a suboptimal level that will deplete liver Fe store and may cause mild anemia.
[b] Each value represents sample mean ± SD of six rats. Different superscript numbers in each row indicate significant difference by Duncan's Multiple Range test at $p < 0.05$.
[c] Iron and clofibrate interaction was statistically significant by two-way ANOVA at $p < 0.05$.
[d] Log-transformed data were analyzed.

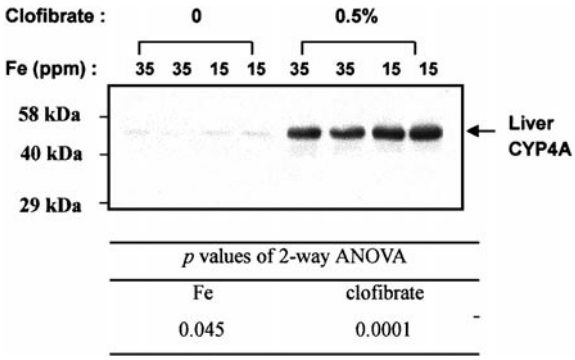


FIG. 1. A representative gel electrophoresis and immunoblotting of microsomal CYP4A in the rat liver as affected by a low-iron diet and clofibrate treatment. The 35 ppm Fe is normal dietary level for rats, while the 15 ppm Fe is a suboptimal level which will deplete liver Fe store and may cause mild anemia. CYP4A abundance was determined by Western blot analysis using 5 μ g microsomal protein. Each lane represents one rat and results of six rats were quantified by densitometry and statistically analyzed with two-way ANOVA (significant level set at $p < 0.05$).

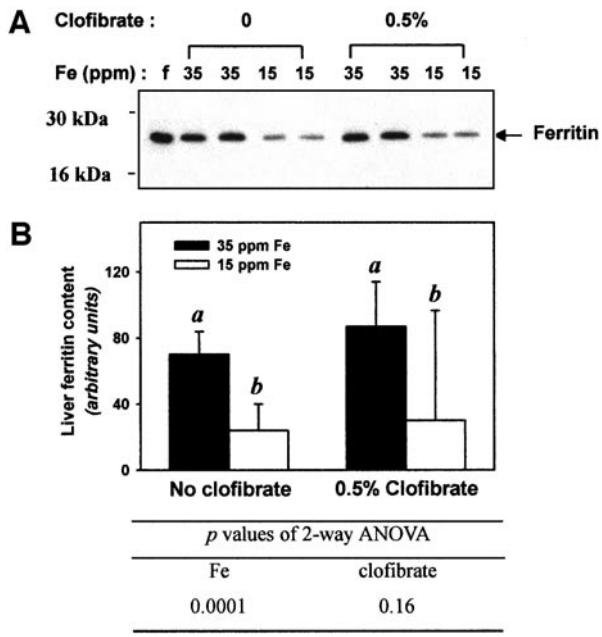


FIG. 2. Ferritin in the rat liver as affected by a low-iron diet and clofibrate treatment. The 35 ppm Fe is normal dietary level for rats, while the 15 ppm Fe is a suboptimal level which will deplete liver Fe store and may cause mild anemia. Ferritin was determined by Western blot analysis using 70 μ g cytosolic protein (A) and results were quantified by densitometry (B). In A, lane f represents rat ferritin purchased from Sigma Co. In B, the values are mean $\pm$ SD of six rats and bars not sharing the same superscript letters are significantly different from one another by Duncan's Multiple Range test ($p < 0.05$).

intakes (Fig. 4A). Both dietary iron intake and clofibrate significantly affected hepatic TfR mRNA level; which was reduced by clofibrate, while increased by low iron intake. Without clofibrate, low iron intake caused TfR mRNA to increase by twofold, but this effect disappeared with clofibrate treatment (Fig. 4B).

Hepatic IRP1 and IRP2 Activities

The main effects of iron intake or clofibrate treatment on IRP1 activity were not significant (Fig. 5A). Nevertheless, the clofibrate-treated group had significantly higher IRP1 activity than the untreated counterpart at normal iron intake (Fig. 5B). Without clofibrate treatment, IRP2 activity was significantly enhanced by low iron intake, but clofibrate treatment offset this effect (Fig. 5C).

Serum and Hepatic Copper

Clofibrate caused serum copper to decrease by 30 and 18% at normal and low iron intakes, respectively. Serum ceruloplasmin ferroxidase activity decreased with serum copper levels (Table 7). In contrast, clofibrate caused hepatic copper to increase by more than twofold (Table 7). The low iron intake did not affect serum ceruloplasmin and copper levels, but it increased hepatic copper accumulation by approximately 69% with or without clofibrate treatment (Table 7).

DISCUSSION

Previous studies (Anderson *et al.*, 1999; Eagon *et al.*, 1999; Hertz *et al.*, 1996; Powanda *et al.*, 1978) have demonstrated

TABLE 5
Hematological Indices of Iron Status of Rats as Affected by Low Iron Intake and Clofibrate Treatment

	No clofibrate[b]		0.5% Clofibrate[b]		*p* value of two-way ANOVA	
Treatment and Fe group[a]	35 ppm Fe	15 ppm Fe	35 ppm Fe	15 ppm Fe	Fe	Clofibrate
Hemoglobin (mmol/L)	2.4 ± 0.1^1	2.2 ± 0.1^2	2.2 ± 0.1^2	2.1 ± 0.1^2	0.0295	0.0062
Serum Fe (μ mol/L)	42 ± 3^1	42 ± 6^1	21 ± 4^2	22 ± 2^2	0.76	0.0001
TIBC (μ mol/L)	98 ± 7^1	103 ± 6^1	52 ± 8^2	55 ± 8^2	0.60	0.0001
Transferrin saturation (%)	43 ± 4	41 ± 5	40 ± 9	42 ± 9	0.95	0.79

[a] The 35 ppm Fe is normal dietary level for rats, while the 15 ppm Fe is a suboptimal level that will deplete liver Fe store and may cause mild anemia.
[b] Each value is sample mean $\pm$ SD of six rats. Different superscript numbers in each row indicate significant difference by Duncan's Multiple Range test at $p < 0.05$.

TABLE 6
Hepatic Iron Content and Mitochondrial Aconitase Activity of Rats as Affected by Low Iron Intake and Clofibrate Treatment

Treatment and Fe group[a]	No clofibrate[b]		0.5% Clofibrate[b]		*p* value of two-way ANOVA	
	35 ppm Fe	15 ppm Fe	35 ppm Fe	15 ppm Fe	Fe	Clofibrate
Hepatic Fe						
(μmol/g liver)	0.84 ± 0.19^{1}	0.36 ± 0.11^{2}	0.94 ± 0.20^{1}	0.47 ± 0.11^{2}	0.0001	0.068
(μmol/ whole liver)	20 ± 4^{2}	7 ± 2^{3}	25 ± 5^{1}	13 ± 3^{4}	0.0001	0.0019
DFO-chelatable Fe[c] (nmol/g liver)	11.3 ± 3.8^{2}	8.3 ± 2.1^{2}	24.2 ± 8.5^{1}	11.2 ± 2.7^{2}	0.0008	0.0008
Mitochondrial aconitase activity[d]						
(mU/mg mitochondrial protein)	19.1 ± 1.6^{2}	17.0 ± 1.5^{2}	41.8 ± 4.8^{1}	42.1 ± 3.8^{1}	0.45	0.0001

[a] The 35 ppm Fe is normal dietary level for rats, while the 15 ppm Fe is a suboptimal level that will deplete liver Fe store and may cause mild anemia.
[b] Each value is sample mean $\pm$ SD of six rats. Different superscript numbers in each row indicate significant difference by Duncan's Multiple Range test at $p < 0.05$.
[c] Iron and clofibrate interaction was statistically significant by two-way ANOVA at $p < 0.05$.
[d] One enzyme unit = 1 μmol $NADP^{+}$ consumed per min in a coupling reaction of aconitase and isocitrate dehydrogenase.

that PPs suppress the expression of transferrin, ceruloplasmin, and ATP7B and reduce the concentrations of serum iron and copper. Complementing previous results, this study reveals that clofibrate alters internal iron exchange and liver iron homeostasis. The physiological implication of the adaptation can be partially explained.

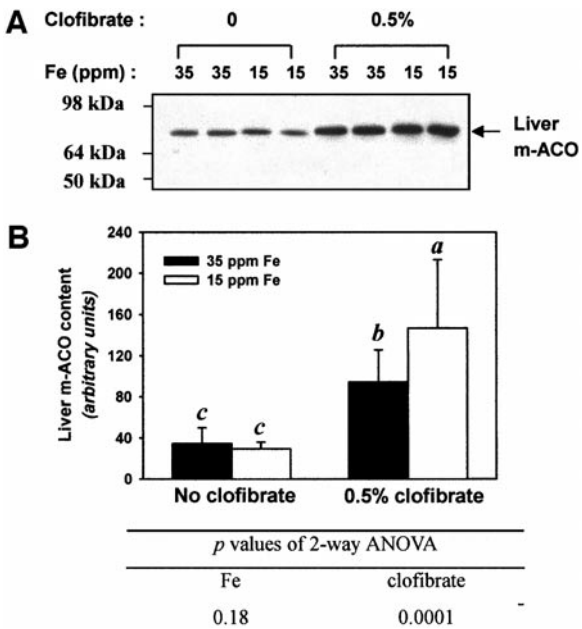
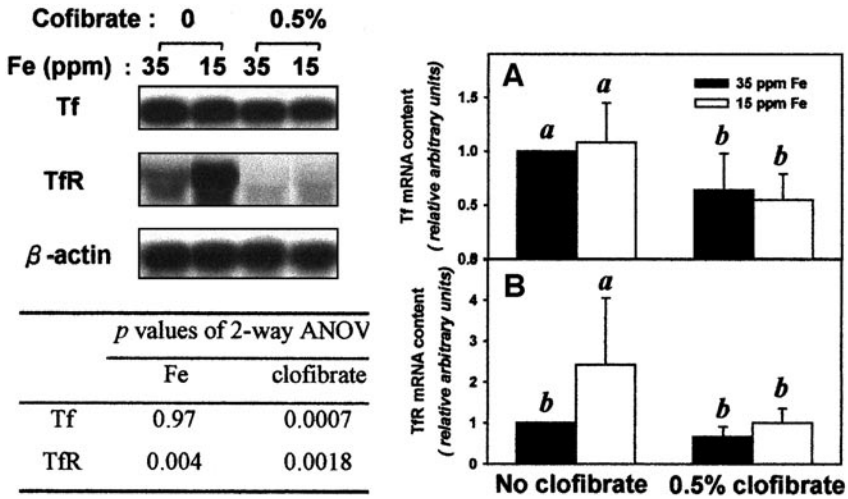


FIG. 3. Mitochondrial aconitase (m-ACO) protein level in the rat liver as affected by a low-iron diet and clofibrate treatment. The 35 ppm Fe is normal dietary level for rats, while the 15 ppm Fe is a suboptimal level which will deplete liver Fe store and may cause mild anemia. Abundance was determined by Western blot analysis using 10 μ g mitochondrial protein (A) and results were quantified by densitometry (B). In A, each lane represents one rat. In B, the values are mean $\pm$ SD of six rats and bars not sharing the same superscript letters are significantly different from one another by Duncan's Multiple Range test ($p < 0.05$).

Clofibrate and Internal Iron Exchange

Since plasma Tf level is dominated by liver Tf expression, the reduction in TIBC owing to clofibrate treatment can be fully accounted for by the reduction in Tf mRNA. Recycling of scavenged hemoglobin iron to erythropoietic marrow via plasma transferrin represents the single largest iron flux in the body (Fairbanks, 1994). Although liver iron was not depleted, and the labile iron pool represented by DFO-chelatable Fe was expanded, clofibrate treatment resulted in anemia herein, indicating that the 50% reduction of serum Tf actually limits iron efflux from the storage sites as well as the availability of iron for erythropoiesis. The blood outcome observed in rats is applicable to PP-treated patients, who frequently displayed a 2 to 4% reduction in hemoglobin concentrations (Schrefer, 2001). Ceruloplasmin is also essential for hepatic iron efflux (Harris *et al.*, 1999); however, Cp at 70% of the normal levels was adequate for iron mobilization in the clofibrate-treated rats (Cohen *et al.*, 1983; Roeser *et al.*, 1970). Therefore, the reduction in transferrin is fully responsible for the clofibrate-induced anemia.

Two questions relevant to PP-induced anemia are raised: whether this mild anemia worsens at suboptimal iron intake and whether iron supplement alleviates it. Herein, the result of a two-way ANOVA ($p = 0.0295$ for Fe) indicates a significant main effect of iron intake on hemoglobin concentration (Table 5). Nevertheless, the hemoglobin level of the clofibrate-treated groups was not reduced by low iron intake, most likely due to up-regulation of the number of transferrin receptors and increased iron uptake hence in the iron-depleted erythroblasts, thus partially compensating for the reduced iron supply from the liver. However, the fraction of the recycling hemoglobin iron is reduced with decreased TIBC and will not match the rate of hemoglobin degradation, thus providing an explanation for the elevated anemia rate in the clinical follow-up (The Coronary Drug Project, 1975).



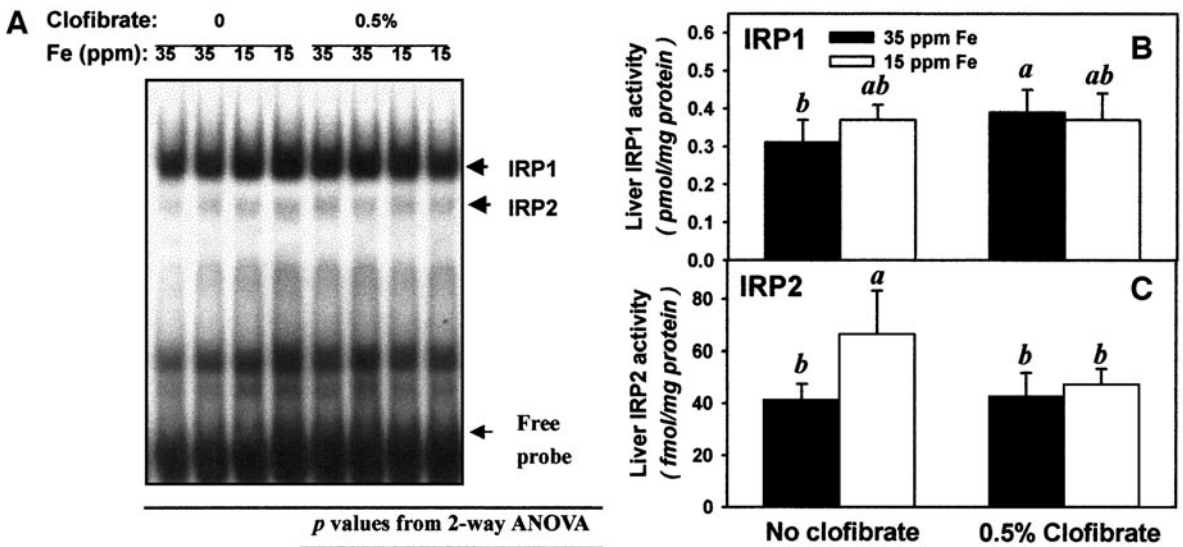
	p values of 2-way ANOV	
	Fe	clofibrate
Tf	0.97	0.0007
TfR	0.004	0.0018

FIG. 4. Abundance of (A) transferrin, Tf, and (B) transferrin receptor, TfR mRNAs in the rat liver as affected by a low-iron diet and clofibrate treatment. The 35 ppm Fe is normal dietary level for rats, while the 15 ppm Fe is a suboptimal level which will deplete liver Fe store and may cause mild anemia. Abundance was determined by Northern blot analysis (total RNA 10 μ g for Tf and 40 μ g for TfR) and results were quantified by densitometry normalized to β-actin mRNA. The values are mean $\pm$ SD of six rats and bars in each panel not sharing the same superscript letters are significantly different from one another by Duncan's Multiple Range test ($p < 0.05$).

Iron supplement does not eliminate the limit of the reduced TIBC, therefore, it does not correct PP-induced anemia.

Clofibrate and Liver Iron Accumulation

Previous studies are inconsistent regarding PP-induced iron accumulation in the liver; short-term drug exposure increased liver iron content (Powanda *et al.*, 1978), while prolonged exposure did not (Eagon *et al.*, 1999). The 42-day exposure herein actually caused iron loading, as indicated by the percentage increase in total iron (25–86%) exceeding that of the proportion of hepatomegaly (10–30%). On the other hand, tumors induced by chronic PP administration are strongly



	p values from 2-way ANOVA	
	Fe	clofibrate
IRP1 activity	0.41	0.093
IRP2 activity	0.0012	0.068

FIG. 5. Cytosolic IRP1 and IRP2 activities in the rat liver as affected by a low-iron diet and clofibrate treatment. IRPs activities were determined by EMSA and bound IRP1 and IRP2 as well as free probe are indicated (A). The 35 ppm Fe is normal dietary level for rats, while the 15 ppm Fe is a sub-optimal level which will deplete liver Fe store and may cause mild anemia. Activities were quantified by scintillation counting (B and C). The values are mean $\pm$ SD of six rats and bars not sharing the same superscript letters are significantly different from one another by Duncan's Multiple Range test ($p < 0.05$).

TABLE 7
Serum and Hepatic Copper Indices of Rats as Affected by Low Iron Intake and Clofibrate Treatment

	No clofibrate[b]		0.5% Clofibrate[b]		*p* value of two-way ANOVA	
Treatment and Fe group[a]	35 ppm Fe	15 ppm Fe	35 ppm Fe	15 ppm Fe	Fe	Clofibrate
Serum Cu (μ mol/L)[c]	17 ± 1^{1}	15 ± 1^{2}	12 ± 1^{3}	12 ± 1^{3}	0.11	0.0001
Ceruloplasmin (U/L)[d]	121 ± 16^{1}	111 ± 16^{1}	91 ± 5^{2}	85 ± 8^{2}	0.12	0.0001
Hepatic Cu (nmol/g liver)[e]	52 ± 15^{2}	88 ± 36^{2}	$125 \pm 88^{1,2}$	210 ± 139^{1}	0.0255	0.0039
Cu:Fe molar ratio[e]	0.06 ± 0.02^{2}	0.27 ± 0.15^{1}	0.12 ± 0.08^{2}	0.51 ± 0.41^{1}	0.0001	0.0489

[a] The 35 ppm Fe is normal dietary level for rats, while the 15 ppm Fe is a suboptimal level that will deplete liver Fe store and may cause mild anemia.
[b] Each value is sample mean $\pm$ SD of six rats. Different superscript numbers in each row indicate significant difference by Duncan's Multiple Range test at $p < 0.05$.
[c] Iron and clofibrate interaction was statistically significant by two-way ANOVA at $p < 0.05$.
[d] One unit = 1 μmol *o*-diansidine dihydrochloride oxidized per minute by the ferroxidase reaction of ceruloplasmin.
[e] Data were log transformed for statistical analysis.

resistant to iron accumulation even given very high levels of dietary iron (Rao *et al.*, 1986). Taking together, a time-dependent effect of PP on hepatic iron homeostasis is indicated in that liver iron increases transiently in the initial stage of PP-induced liver hyperplasia preceding the development of hepatocellular carcinomas (HCC). The excess iron may work for functional and synthetic purposes, because numerous PP-induced enzymes (cytochrome P450s, peroxisomal oxidases, and mitochondrial enzymes) are Fe-containing proteins. Clofibrate treatment did not reduce liver ferritin levels, suggesting that intracellular iron is conserved by the reduction of transferrin-mediated iron efflux. Therefore, the altering of iron homeostasis by PPs is a physiological adaptation to conserve iron in liver cells for metabolic functions.

Differential Regulation of IRPs

As an essential nutrient, cellular iron is closely regulated through the action of TfR and ferritin, the proteins for iron uptake and storage, respectively. Ferritin and TfR are regulated posttranscriptionally by the IRP/IRE system (Cairo and Pietrangelo, 2000). It is well established that RNA binding activities of both IRP1 and IRP2 in the liver are influenced by dietary iron intakes (Chen *et al.*, 1997, 1998) and generally change with similar kinetics when activated by iron deficiency or deactivated by iron excess (Guo *et al.*, 1994; Henderson and Kühn, 1995). Herein, we demonstrated that IRP1 and IRP2 reacted differentially, with IRP2 activity increasing, while IRP1 activity remained unchanged, in response to the low-iron diet (15 ppm Fe), which provided 42% of the recommended dietary iron level for rats and caused mild iron-deficiency symptoms as indicated by an 8% reduction in hemoglobin concentration and a 14% reduction in body weight gain. This result supports the observation by Chen *et al.* (1997) that IRP2 is more sensitive than IRP1 to *in vivo* changes in iron level.

Additionally, we provide the first *in vivo* observation that the change in TfR mRNA was connected with the change in IRP2 activity, both increasing at the low iron intake and being simultaneously suppressed by clofibrate treatment. A similar correlation has been documented in NO-treated RAW 264.7 cells previously (Kim and Ponka, 1999). Such a connection does not exist between IRP1 activity and TfR mRNA, since TfR mRNA did not change as IRP1 activity increased in the clofibrate-treated, normal iron group herein. Thus, corroborating the previous findings that IRP2 is more effective than IRP1 in modulating ferritin synthesis *in vivo* (Chen *et al.*, 1997), the present study proves a similar IRP2 effect on the stability of TfR mRNA.

Herein, clofibrate treatment selectively activated IRP1 despite the expanded liver iron and DFO-chelatable Fe concentrations. This activation cannot be attributed to cellular iron depletion and is best explained by some H_2O_2-triggered pathways. Recent investigations have proven that H_2O_2 treatment causes IRP1, but not IRP2, to bind IREs rapidly in cell models (Pantopoulos and Hentz, 1995; Pantopoulos *et al.*, 1996) and in perfused liver (Mueller *et al.*, 2001). Furthermore, liver IRP1 is activated by H_2O_2 leaking from the mitochondrial respiratory chain (Pantopoulos *et al.*, 1997). In PP-treated livers, H_2O_2 increases because of elevated activity of peroxisomal fatty acyl-CoA oxidase and microsomal cytochrome P450 enyzems, while the activity of H_2O_2-degrading enzymes, peroxisomal catalase and cytosolic glutathione peroxidase, is relatively insufficient (Yeldandi *et al.*, 2000). The leakage of H_2O_2 from peroxisomes and undegraded H_2O_2 in cytosol may serve as a signal to activate IRP1. Additionally, the H_2O_2 activation of IRP1 was followed by the suppression of ferritin synthesis and an increase in TfR numbers (Pantopoulos and Hentze, 1995; Caltagirone *et al.*, 2001), implying an expansion of the intracellular free-iron pool, which is now demonstrated in the clofibrate-treated, normal iron group herein, indicating that

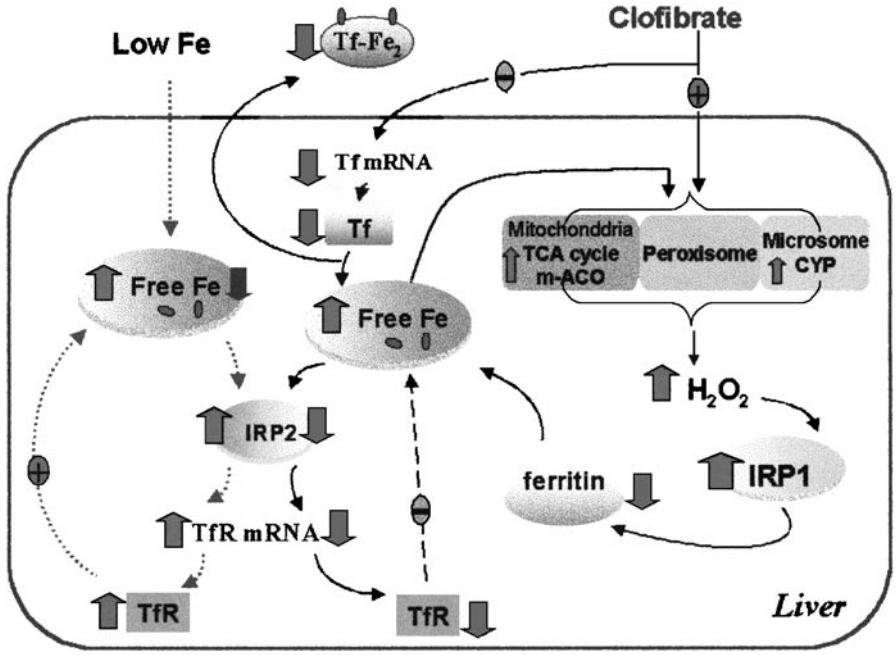


FIG. 6. Iron homeostasis in the liver affected by a low-iron diet and clofibrate treatment. When iron intake is low, IRP2 is activated by the depleted free-iron pool in the cell, which will increase the stability of transferrin receptor (TfR) mRNA and the number of transferrin receptors. When clofibrate is administered, iron efflux from the liver decreases due to suppressed expression of transferrin (Tf) gene and decreased iron transport in the serum. Clofibrate elevates peroxisomal oxidase, microsomal CYP and mitochondrial aconitase (m-ACO) activities, which may increase intracellular H_2O_2 level. IRP1 activated by H_2O_2 suppresses ferritin synthesis, thus expanding the intracellular free iron pool, which allows more iron to be available for the biosynthesis of iron-containing enzymes in response to clofibrate treatment on the one hand, and inactivates IRP2 to reduce TfR mRNA and receptor numbers on the other hand. Thus, a new steady state of iron homeostasis will be established.

peroxisome-derived oxidative stress can activate IRP1 via H_2O_2 production and alter iron homeostasis.

Metabolic Adaptations of m-ACO

The mRNA of m-ACO, the TCA enzyme converting citrate to isocitrate, contains an IRE in the 5′-UTR and is subjected to translational regulation by IRP/IRE binding (Schalinske *et al.*, 1998). Dietary iron deficiency reduces both the level and the activity of m-ACO protein in rat liver in a time- and dose-dependent manner (Chen *et al.*, 1997, 1998). However, neither the activity nor the protein level of m-ACO decreased when IRP2 was activated by the low-iron diet herein, which is the first *in vivo* demonstration of differential regulation between m-ACO and TfR mRNA by IRP2, probably due to differing affinities between IRP2 and IRE isoforms from different mRNAs (Theil and Eisenstein, 2000). It is noted that the two clofibrate-treated groups displayed similar m-ACO activities, while differing in protein levels. An explanation for this discrepancy is that the assembly of holo-*m*-ACO was limited at low iron intake with an excess of nonfunctional apoprotein in the cell.

Regardless of iron status, clofibrate treatment caused m-ACO activity to increase by 133%, while clofibrate also enhanced the activity of three rate-limiting enzymes in the TCA cycle, citrate synthase, nicotinamide-linked isocitrate dehydrogenase, and α-ketoglutarate dehydrogenase, by 24, 54, and 153%, respectively (Prager *et al.*, 1993; Schon *et al.*, 1994). In the clofibrate-treated liver, the production of acetyl-CoA is elevated and leads to increased citrate formation via citrate synthase (Ball *et al.*, 1979). Accumulating citrate acts as an allosteric activator of acetyl-CoA carboxylase and favors fatty acid synthesis. The enhanced activities of m-ACO and rate-limiting enzymes makes it possible to accommodate higher acetyl-CoA influx and citrate flux, owing to PP-induced β-oxidation of fatty acids.

Clinical Implication of Liver Copper Accumulation

Clofibrate-induced copper accumulation in the liver was not significant during a 5-day exposure to PP (Powanda *et al.*, 1978), while it increased markedly with prolonged exposure (Eagon *et al.*, 1999). The copper loading was specific as verified by the twofold increase in Cu to Fe mole ratio of the treated groups (Table 7). It is well established that excess copper acts as a prooxidant to promote the formation of free radicals and the harmful oxidation of lipids and proteins. In the hereditary Wilson's disease, excess copper causes hepatocellular necrosis of the liver, as well as portal and periportal inflammation, and ultimately fibrosis (Pfeil and Lynn, 1999). Since elevated serum GOT and GPT have been observed occasionally in patients taking PP drugs (Sirtori *et al.*, 1992),

copper-induced oxidative stress may be a major contributor to PP-induced liver toxicity. Few clinical trials have monitored copper status, mainly because of a lack of feasible blood markers. Since Cp and liver copper change inversely during PP treatment, serum Cp may be used to check the risk of hepatic copper loading.

In conclusion, the present study demonstrates that changes in hepatic iron homeostasis by clofibrate involve a differential regulation of IRP1 and IRP2 with changes in m-ACO both at protein and enzyme activity levels, and a model is proposed to depict these effects (Fig. 6). Clofibrate suppresses Tf expression and reduces hepatic iron efflux to maintain an expanded free-iron pool in the liver cell via differential regulation of IRP1 and IRP2 activities, in order to conserve iron for the synthesis of a variety of Fe-containing enzymes, including m-ACO, which are up-regulated by hypolipidemic peroxisomal proliferators for metabolic functions. We also observe that TIBC, Cp, and liver copper levels rapidly returned to normal while clofibrate was discontinued (data not shown); periodic discontinuation of fribrate drugs needs to be considered in order to alleviate PP-induced anemia and liver copper toxicity.

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