Pitavastatin Ameliorates Severe Hepatic Steatosis in Aromatase-Deficient (Ar–/–) Mice

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ABSTRACT: Tamoxifen is a potent antagonist of estrogen, and hepatic steatosis is a frequent complication in adjuvant tamoxifen for breast cancer. Impaired hepatic FA β-oxidation in peroxisomes, microsomes, and mitochondria results in progression of massive hepatic steatosis in estrogen deficiency. This impairment, although latent, is potentially serious: About 3% of the general population in the United States is now suffering from nonalcoholic steatohepatitis associated with obesity and hyperlipidemia. Therefore, in the present study we tried to restore impaired hepatic FA β-oxidation by administering a novel statin, pitavastatin, to aromatase-deficient (Ar–/–) mice defective in intrinsic estrogen synthesis. Northern blot analysis of Ar–/– mice liver revealed a significant restoration of mRNA expression of essential enzymes involved in FA β-oxidation such as very long fatty acyl-CoA synthetase in peroxisome, peroxisomal fatty acyl-CoA oxidase, and medium-chain acyl-CoA dehydrogenase. Severe hepatic steatosis observed in Ar–/– mice substantially regressed. Consistent findings were obtained in the *in vitro* assays of FA β-oxidation activity. These findings demonstrate that pitavastatin is capable of restoring impaired FA β-oxidation *in vivo via* the peroxisome proliferator-activated receptor-α-mediated signaling pathway and is potent enough to ameliorate severe hepatic steatosis in mice deficient in intrinsic estrogen.

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Uptake, utilization, and secretion rates of FA are tightly controlled to meet energy demands and to maintain the cellular lipid content in hepatocytes. The major lipid catabolic pathways in the liver are peroxisomal and mitochondrial FA β-oxidation, which are regulated at the level of gene expression during development and in response to diverse physiological stimuli (1–3). The deterioration of gene expression of FA-metabolizing enzymes in hepatocytes may result in massive hepatic steatosis.

FA metabolism is impaired in the aromatase-deficient (Ar–/–) mouse. It has a reduced gene expression of FA-metabolizing enzymes in hepatocytes and spontaneously develops massive hepatic steatosis, a condition observed in one-third of breast cancer patients treated with the estrogen antagonist tamoxifen (4,5). Thus, it is an elegant model of estrogen deficiency mimicking that of breast cancer patients treated with tamoxifen.

Adjuvant tamoxifen became a standard treatment for women with early breast cancer in the 1990s, and although some adverse effects have been reported (6), this treatment undoubtedly outweighs the risks of the adverse effects. A major problem, however, is that tamoxifen induces nonalcoholic steatohepatitis (NASH) and liver cirrhosis (7–10). Rapid progression of hepatic steatosis was also noted in 36% of nonobese, nondiabetic breast cancer patients treated with tamoxifen (11), where a body mass index (BMI: kg/m^2) greater than 23 was reported as a significant risk factor for progressive hepatic steatosis (5).

Today, hepatic steatosis is no longer regarded as a benign lesion, since chronic accumulation of hepatic TG sometimes leads to NASH, liver fibrosis, and cirrhosis (12). In two-thirds of the general population in the United States, the BMI exceeds 23 and about 3% of the general population is now suffering from NASH associated with obesity (13). Twenty percent of NASH patients are expected to develop liver cirrhosis within 10 yr (14), yet breast cancer patients are advised to take tamoxifen for 5 yr (15). It is difficult to ignore such a risk for 5 yr when a breast cancer patient is obese, particularly since tamoxifen is known to impair FA β-oxidation in at least in one-third of nonobese nondiabetic Japanese women (4,11,16). In breast cancer patients treated with tamoxifen, physicians need medicines that can either decrease fat deposition in the liver or prevent the development of hepatic steatosis. Fibrates, one type of peroxisome proliferators, are the first-line medicines because they were shown to ameliorate massive hepatic steatosis in tamoxifen-treated patients by activating FA β-oxidation in the liver through peroxisome proliferator-activated receptor-α (PPAR-α)-mediated signaling (4,5,16).

Recently, however, hypercholesterolemia has become a frequent concern both in the general population and in breast cancer patients. Fibrates sometimes fail to reduce plasma cholesterol levels sufficiently in these patients. Coadministration of a statin with a fibrate is one option, but it is not recommended since such coadministration is believed to increase the incidence rate of rhabdomyolysis, a rare but serious com-

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Abbreviations: AOX, peroxisomal acyl-CoA oxidase; Ar+/+ mouse, wild-type mouse; apo, apolipoprotein; Ar–/– mouse, aromatase-deficient mouse; BMI, body mass index; CYP2E1, microsomal cytochrome P450 2E1; CYP4A1, microsomal cytochrome P450 4A1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MCAD, medium-chain acyl-CoA dehydrogenase; NASH, nonalcoholic steatohepatitis; PPAR-α, peroxisome proliferator-activated receptor-α; VLACS, very long fatty acyl-CoA synthetase.

plication (17). For this reason, a statin with intrinsic agonistic effects on PPAR-α such as pitavastatin, would be preferable for those patients (18–20).

In the present study, we investigated the hypolipidemic effects of pitavastatin and its fat-eliminating capacity on spontaneously developed fatty liver in Ar–/– mice.

MATERIALS AND METHODS

Mice. The aromatase gene (*cyp 19* gene) was disrupted by homologous recombination (21). In brief, an 87-bp fragment located within exon 9 of the *cyp 19* gene in E14-1 cells (embryonic stem cells) was replaced with a neomycin resistance gene derived from *pMC1*-neo. Selected embryonic stem cells were micro-injected into the C57BL/6J blastocytes to generate chimeric mice. Chimeric male mice were then mated with C57BL/6J female mice to generate mice heterozygous for the mutation. Heterozygous mice were mated to obtain aromatase null (Ar–/–) mice because of the infertility of homozygous males and females. Ar–/– male mice aged 16 wk and their wild-type male siblings (Ar+/+, C57BL/6J) were used in the present study. A conventional maintenance diet (CE-2) containing 25.4 w/w% of protein and 4.4 w/w% of fat (Clea Japan, Suita, Japan) was used in this study, which approximates the composition of the human diet. The Ar+/+ group was fed a CE-2 diet for 8 wk. Ar–/– mice were divided into two groups and also fed for 8 wk: The Ar–/– group was fed a CE-2 diet, and the Ar–/– + pitavastatin group was fed a CE-2 diet supplemented with 0.014% pitavastatin, a potent novel statin with an intrinsic agonistic effect on PPAR-α (kindly provided by Kowa, Nagoya, Japan). Genotypes of mice were determined by a PCR using genomic DNA isolated from tail tips. Animal care and experiments were carried out in accordance with institutional animal care regulations.

Light microscopic observations. Liver tissues were routinely fixed in 10% phosphate-buffered formalin (pH 7.4), embedded in paraffin, and sectioned for hematoxylin and eosin staining. The degree of hepatic steatosis in whole specimens was classified into four grades according to the distribution pattern of the fat vacuoles as follows: 0 = no or few fat droplets in the lobules, I = a few fat droplets in the lobules, II = fat droplets restricted to zone 3, III = fat droplets in zones 3 and 2, IV = numerous fat droplets in zones 3 and 2.

Analysis of mRNA expression for enzymes involved in FA β-oxidation. mRNA analysis was performed by Northern blotting. Total liver RNA was obtained from fresh liver using the acid guanidinium thiocyanate/phenol/chloroform extraction method. RNA was separated on 1% agarose gel and transferred to a nylon membrane. The membranes were incubated with ^{32}P-labeled cDNA probes and analyzed on a Fuji system analyzer (Fuji Photo Film, Tokyo, Japan). The cDNA used for Northern blotting included catalase, very long fatty acyl-CoA synthetase (VLACS), peroxisomal acyl-CoA oxidase (AOX), medium-chain acyl-CoA dehydrogenase (MCAD), cytochrome P450 2E1 (CYP2E1), cytochrome P450 4A1 (CYP4A1), apolipoprotein A4, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin (4). Changes in mRNA levels were estimated by densitometric scanning of autoradiograms and analyzed by NIH Image 1.52 to show a relative ratio to the findings in Ar+/+. The number of animals used for mRNA analysis was five in each group.

FA β-oxidation activity. FA β-oxidation activity was measured as described previously (22). In brief, fresh liver was homogenized in 4 vol of 0.25 M sucrose containing 1 mM EDTA in a Potter–Elvehjem homogenizer using a tight-fitting Teflon pestle. Homogenate (1–10 mg) was incubated with the assay medium in 0.2 mL of 150 mM KCl, 10 mM HEPES (pH 7.2), 0.1 mM EDTA, 1 mM potassium phosphate buffer (pH 7.2), 5 mM malonate, 10 mM $MgCl_2$, 1 mM carnitine, 0.15% BSA, 5 mM ATP, and 50 μM of each FA (10^5 cpm for radioactive substrates; 55 mCi/mmol; American Radiolabeled Chemicals, St. Louis, MO): [1-^{14}C]tetracosanoic acid (24:0), [1-^{14}C]palmitic acid (16:0), or [1-^{14}C]lauric acid (12:0). The reaction was run for 30 min at 25°C and stopped by the addition of 0.2 mL of 0.6 N perchloric acid. The mixture was centrifuged at 2,000 × *g* for 10 min, and the unreacted FA in the supernatant was removed using 2 mL of *n*-hexane with three extractions. Radioactive degradation products in the water phase were counted. FA β-oxidation activity was expressed as nmol/min/liver. In some experiments using [1-^{14}C]palmitic acid, KCN, a potent inhibitor of the mitochondrial respiratory chain, was added to the assay medium to inhibit potent mitochondrial activity.

FA analysis. The liver was homogenized and TG were extracted using chloroform/methanol (2:1, vol/vol). TG were then further extracted with water/chloroform (1:1, vol/vol) and quantified.

Statistical analysis. Data were analyzed using Student's *t*-test or Wilcoxon's signed rank test.

RESULTS

Light microscopy observation. Hepatic steatosis was limited to zone 3 (centrilobular zone in the lobules) in Ar–/– mice at 10 wk of age. However, the mice progressively developed massive hepatic steatosis, although there was a slight variation in the severity (Fig. 1A). Steatosis of liver cells in zone 1 (periportal) was absent or slight. Liver cells in zones 3 and 2 (centrilobular and intermediate zones in the lobules) showed marked microvesicular steatosis in Ar–/– mice. Some large vacuoles were also seen. This zonal difference of steatosis within the liver lobules was clear. Grade IV hepatic steatosis regressed to grades II or III in Ar–/– mice when treated with pitavastatin ($P < 0.03$; Table 1, Fig. 1B). Their wild-type siblings (Ar+/+) did not develop hepatic steatosis (Fig. 1C).

mRNA analysis. To clarify whether pitavastatin could restore deteriorated hepatic lipid metabolism, mRNA expression of hepatic peroxisomal (catalase, VLACS, AOX), mitochondrial (MCAD), and microsomal (CYP2E1 and CYP4A1) enzymes was analyzed by Northern blot analyses in the Ar+/+, Ar–/–, and Ar–/– + pitavastatin groups (Fig. 2).

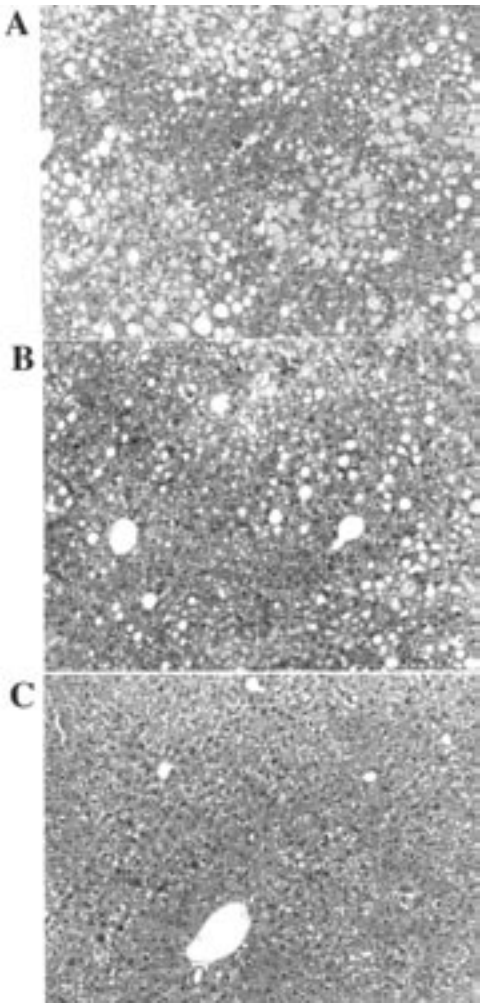

FIG. 1. Steatosis of the liver in aromatase (Ar)–/– mice and its attenuation by pitavastatin. Ar–/– mice spontaneously developed massive steatosis of the liver. Light micrographs show livers of Ar–/– mice at 24 wk of age. (A) Numerous fat droplets in zones 3 and 2 (centrilobular and intermediate zones in the lobules) of Ar–/– mice liver at 24 wk of age; (B) a few fat droplets in the lobules of Ar–/– mice liver at 24 wk of age treated with pitavastatin; (C) no fat droplets in the lobules of Ar+/+ mice liver at 24 wk of age.

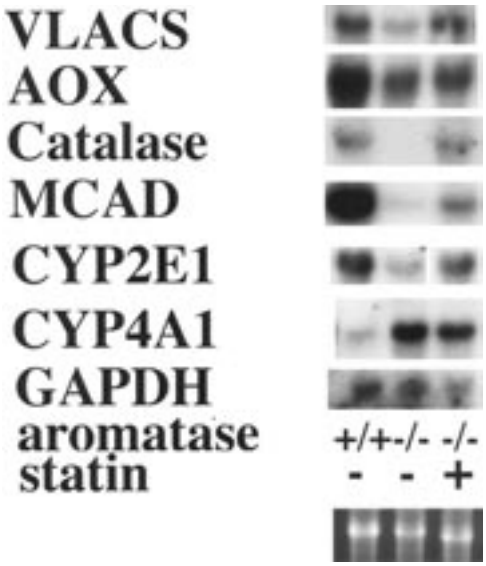

FIG. 2. Northern blot analysis of Ar–/– liver. mRNA expression of hepatic peroxisomal, mitochondrial, and microsomal enzymes was analyzed. The expression of most enzymes for FA β-oxidation was diminished in Ar–/– mice and pitavastatin was partially recovered. In contrast, microsomal cytochrome P450 4A1 (CYP4A1) mRNA expression was enhanced in Ar–/– mice and pitavastatin attenuated the enhanced expression. VLACS, very long chain fatty acyl-CoA synthetase; AOX, peroxisomal acyl-CoA oxidase; MCAD, medium-chain acyl-CoA dehydrogenase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CYP2E1, cytochrome P450 2E1; for other abbreviation see Figure 1.

mRNA expression of mitochondrial MCAD and of three peroxisome-associated enzymes (VLACS, AOX, and catalase) was significantly lower in Ar–/– mice than in Ar+/+ mice (0.23 ± 0.12, 0.33 ± 0.13, 0.28 ± 0.11, and 0.18 ± 0.09 fold-change, respectively, compared to Ar+/+), whereas microsomal CYP4A1 mRNA expression was significantly higher in the Ar–/– mice (7.64 ± 1.07 fold-change compared to Ar+/+; $P = 0.043$ in the Wilcoxon signed rank test). Administration of pitavastatin significantly restored mRNA expression of MCAD and of VLACS, AOX, and catalase (3.15 ± 0.44, 2.39 ± 0.23, 1.41 ± 0.15, and 3.59 ± 0.42 fold-change, respectively, compared to Ar–/–; $P = 0.043$ in the Wilcoxon signed rank test), whereas microsomal CYP4A1 mRNA expression (0.38 ± 0.18) was attenuated by pitavastatin treatment ($P = 0.043$ in the Wilcoxon signed rank test), although CYP4A1 is well known to be induced by PPAR-α stimulation (23).

FA β-oxidation activity. The basal levels of FA β-oxidation activity in Ar–/– mice were lower compared with those in C57BL/6J using [1-^{14}C]24:0 (1.82 ± 0.21 vs. 6.55 ± 0.39 nmol/min/g protein, $P < 0.001$), [1-^{14}C]16:0 (35.5 ± 5.8 vs. 123.7 ± 5.5 nmol/min/g protein, $P < 0.001$), or [1-^{14}C]12:0 (7.7 ± 0.5 vs. 25.6 ± 2.1 nmol/min/g protein, $P < 0.01$) as substrates (Fig. 3). FA β-oxidation activities were significantly enhanced by administration of pitavastatin (2.77 ± 0.23, 48.6 ± 7.0, and 11.6 ± 2.6 nmol/min/g protein; $P < 0.01$, 0.05, and 0.05, respectively), whereas they were significantly lower

TABLE 1
Grade of Hepatic Steatosis in three Groups of Mice

Grade	Description[a]	Ar+/+ group (n = 10)	Ar–/– group (n = 10)	Ar–/–+ statin group (n = 10)
0	No or few fat droplets in the lobules	8	0	0
I	A few fat droplets in the lobules	1	0	0
II	Fat droplets restricted to zone 3	1	0	3
III	Fat droplets in zones 3 and 2	0	3	5
IV	Numberous fat droplets in zones 3 and 2	0	7	2

[a]Zone 3, centrilobular area; zone 2, intermediate area.

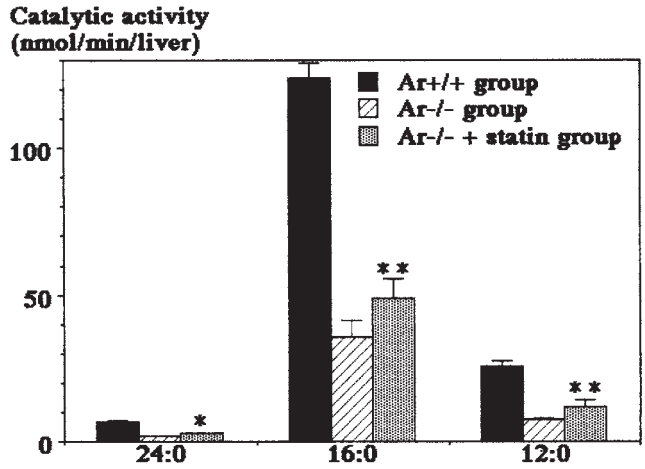

FIG. 3. Effects of pitavastatin on lipid β-oxidation activity in the liver. Peroxisomal β-oxidation capacity was assessed using tetracosanoic acid (24:0) as substrate and mitochondrial β-oxidation capacity using palmitic acid (16:0) and lauric acid (12:0). Bars represent the mean ± SD from at least five samples in each group, and asterisks denote a statistically significant difference ($P < 0.001$) compared with the values obtained with the wild-type group (Ar+/+) and the Ar–/– mice group treated with pitavastatin (Ar–/– + statin).

than those in wild-type mice (Ar+/+) ($P < 0.001$, $P < 0.001$, and $P < 0.001$, respectively). The specificity of the β-oxidation assay was confirmed by KCN, which inhibited the assay at a rate of 91–94%.

Plasma and hepatic TG content. Levels of plasma TG (128.1 ± 36.2 mg/dL) and total cholesterol (107.5 ± 19.6 mg/dL) of Ar–/– mice were higher than those of Ar+/+ mice (80.6 ± 20.3 mg/dL and 71.7 ± 22.1 mg/dL, respectively, $P < 0.05$). Pitavastatin administration resulted in a significant reduction in levels of plasma TG (44.0 ± 8.2 mg/dL, $P < 0.01$) and total cholesterol (47.5 ± 8.7 mg/dL, $P < 0.001$) of Ar–/– mice. The hepatic content of TG of Ar–/– mice was clearly higher than that of Ar+/+ mice (126.1 ± 7.9 vs. 29.4 ± 3.3 mg/g liver, $P < 0.001$). In Ar–/– mice the hepatic content of TG was significantly diminished by administration of pitavastatin (126.1 ± 7.9 vs. 101.3 ± 17.2 mg/g liver, $P < 0.001$) (Fig. 4).

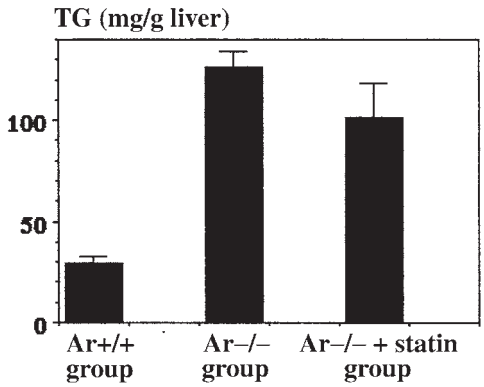

FIG. 4. TG content in the liver. Hepatic TG content in Ar–/– mice was clearly higher than that in Ar–/– mice treated with pitavastatin (Ar–/– + statin) or their wild-type siblings (Ar+/+).

DISCUSSION

The clinical benefits of cholesterol reduction by HMG-CoA reductase inhibitors (statins) have been established in large-scale primary and secondary prevention studies for coronary heart disease (24,25). However, successful reduction of LDL-cholesterol has revealed the contribution of other atherogenic lipoproteins, such as TG-rich lipoproteins, in the development of coronary events. Therefore, statins possessing TG-lowering properties have been newly developed.

Pitavastatin is a potent novel synthetic inhibitor of HMG-CoA reductase, that has shown lowering effects on plasma total cholesterol and TG (19,20). Two possible mechanisms appear to cooperate in lowering plasma TG levels during pitavastatin treatment (18). One mechanism is an inhibition of the assembly and secretion of VLDL, a common mechanism observed in statins. The other is an enhanced cycling of hepatic LDL receptors. These pathways contribute to the elimination of plasma TG but result in hepatic accumulation of TG unless they are oxidized efficiently.

FA are physiological ligands for PPAR-α, and PPAR-α has been implicated in the control of cellular lipid utilization (4). TG are accumulated in Ar–/– mouse liver, but our molecular and enzymological analyses revealed that mRNA expression and activities of enzymes involved in peroxisomal and mitochondrial FA β-oxidation are markedly reduced in Ar–/– mouse liver. Therefore, this is the most suitable model for investigating whether pitavastatin is able to activate the peroxisomal and mitochondrial FA β-oxidation and CYP450 pathways sufficiently. Otherwise, an inhibition of the assembly and secretion of VLDL induced by pitavastatin may easily exaggerate hepatic steatosis further in the liver of Ar–/– mice.

VLACS and AOX are involved in the first two steps of peroxisomal FA β-oxidation, by which very long chain FA are exclusively metabolized, and MCAD is a rate-limiting enzyme catalyzing the mitochondrial oxidation of medium-chain fatty acyl thioesters produced by peroxisomal β-oxidation of long-chain FA (26,27). Northern blot analysis and enzyme activities revealed that pitavastatin efficiently activated PPAR-α-dependent FA metabolizing pathways in Ar–/– mice. Indeed, not only plasma levels of total cholesterol and TG but also fat deposition in hepatocytes were reduced by pitavastatin treatment. These observations suggest that pitavastatin has an agonistic effect on PPAR-α and that pitavastatin can ameliorate severe hepatic steatosis in Ar–/– mice through the PPAR-α-mediated activation of the peroxisomal and mitochondrial FA β-oxidation pathways (26–28), including most known PPAR-α target genes encoding enzymes involved in hepatocellular FA β-oxidation. This is the first evidence *in vivo* that pitavastatin activates the peroxisomal and mitochondrial FA β-oxidation pathways.

Our knowledge is still limited about the regulatory mechanisms involved in maintaining cellular lipid content under normal physiological conditions or in the context of disease states. As such, present laboratory findings are important, as they support the idea of administering pitavastatin to tamox-

ifen-treated breast cancer patients with fibrate-resistant hypercholesterolemia to lower both plasma total cholesterol and TG. It is worth noting as well that physicians need to minimize the effects of obesity and diabetes mellitus that deteriorate FA homeostasis in the liver when adjuvant tamoxifen is being administered to breast cancer patients.

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