

Quantification and regulation of the subcellular distribution of bile acid coenzyme A:amino acid *N*-acyltransferase activity in rat liver

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Abstract Bile acid coenzyme A:amino acid *N*-acyltransferase (BAT) is responsible for the amidation of bile acids with the amino acids glycine and taurine. To quantify total BAT activity in liver subcellular organelles, livers from young adult male and female Sprague-Dawley rats were fractionated into multiple subcellular compartments. In male and female rats, 65–75% of total liver BAT activity was found in the cytosol, 15–17% was found in the peroxisomes, and 5–10% was found in the heavy mitochondrial fraction. After clofibrate treatment, male rats displayed an increase in peroxisomal BAT specific activity and a decrease in cytosolic BAT specific activity, whereas females showed an opposite response. However, there was no overall change in BAT specific activity in whole liver homogenate. Treatment with rosiglitazone or cholestyramine had no effect on BAT activity in any subcellular compartment. These experiments indicate that the majority of BAT activity in the rat liver resides in the cytosol. Approximately 15% of BAT activity is present in the peroxisomal matrix. **■** These data support the novel finding that clofibrate treatment does not directly regulate BAT activity but does alter the subcellular localization of BAT.—Styles, N. A., J. L. Falany, S. Barnes, and C. N. Falany. **Quantification and regulation of the subcellular distribution of bile acid coenzyme A:amino acid *N*-acyltransferase activity in rat liver.** *J. Lipid Res.* 2007. 48: 1305–1315.

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Bile acids are critical for the digestion of dietary cholesterol and are the end products of cholesterol metabolism. In liver, bile acid synthesis accounts for 90% of all cholesterol that is metabolized (1, 2). Because of their hydrophobic and cytotoxic properties, bile acids in humans are amidated with glycine or taurine to form bile salts before their excretion into bile. Bile salts form mixed micelles in the intestinal lumen to increase the solubilization of dietary lipids (including cholesterol), which are

then transported to the liver. The conjugation of bile acids with glycine or taurine significantly decreases their P_{K_a} compared with that of unconjugated bile acids, ensuring a sustained aqueous solubility for bile salts over the wide range of pH conditions found within the intestinal tract (3). Under normal circumstances, >99% of bile acids in bile are amidated, indicating that the liver has a substantial ability to form bile salts (4). Bile salts excreted in the bile can be deconjugated by bacteria in the gut, and the primary bile acids can be enzymatically modified by the bacteria to form secondary bile acids. Both primary and secondary bile acids are then efficiently reabsorbed through the terminal ileum and transported back to the liver via the portal vein. Each cycle of this enterohepatic recirculation system recovers ~95% of bile acids secreted into the small intestines and delivers conjugated bile acids, deconjugated bile acids, and both tertiary and newly formed secondary bile acids back to hepatocytes to be conjugated and excreted again. Failure to efficiently form bile amides can lead to cholestasis as well as a loss of fat and fat-soluble vitamin absorption (1, 5).

Bile acid biosynthesis from cholesterol is a complex multistep process involving at least 17 enzymes across several subcellular compartments (1). The conjugation of bile acids in the liver with glycine or taurine is the terminal part of this process before secretion of the bile acids into bile. Bile acid amidation involves the successive action of the enzymes bile acid coenzyme A ligase (BAL) and bile acid coenzyme A:amino acid *N*-acyltransferase (BAT). BAL has been found localized to the endoplasmic reticulum (6) as well as the plasma membrane (7) of hepatocytes, whereas BAT protein and activity have been reported in liver cytosol, mitochondria, peroxisomes, and microsomes (6, 8–12). BAL catalyzes the formation of a bile acid acylthioester between CoA and a 27 or 24 carbon bile acid (11, 13). The C_{27} bile acid-CoA thioesters are produced in peroxisomes during the metabolism of cholesterol (14), where they undergo β -oxidation of the cholestanic side chain before BAT cleaves the acyl-thioester bond and at-

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taches a glycine or taurine to the resulting C₂₄ bile acid. Newly synthesized C₂₄ bile acids are formed in peroxisomes. However, because an individual bile acid molecule may undergo enterohepatic circulation several times, any deconjugated bile acids returning to the liver must be reconstituted.

Investigators have proposed that the deconjugated C₂₄ bile acids returning to the liver from the gut are reactivated by BAL in the microsomes (15) and by BAT in the cytosol (10). BAT activity in peroxisomes would have a role in conjugating newly synthesized bile acids, whereas BAT present in other subcellular compartments would conjugate recycled bile acids (10). After subfractionation of rat liver, Kase and Bjorkhem (10) found the highest specific BAT activity in the peroxisomal fraction and a lower specific activity in the microsomal fraction, leading them to predict a bimodal distribution of BAT to the peroxisomes and microsomes, with no cytosolic component. However, when human liver samples were subfractionated, BAT was found to have a bimodal distribution to the peroxisomes and cytosol (16). Experiments using green fluorescent protein and human BAT in human fibroblasts suggested a predominantly cytosolic localization of BAT (17). The relative levels of the subcellular pools of BAT are not known, as quantification of total cytosolic and peroxisomal BAT activities has not been accurately determined. Understanding the multiplicity of the subcellular localization and regulation of BAT expression in the liver will provide further insight into the cellular compartmentalization of bile acid synthesis and metabolism.

Solaas et al. (8) reported differential regulation of cytosolic and peroxisomal BAT in male mice treated with WY-14,643, a peroxisome proliferator-activated receptor α (PPAR α) agonist. They found a small decrease in cytosolic BAT activity and a large decrease in peroxisomal BAT activity, leading to the theory that there might be two distinct BATs in mice, one cytosolic and one peroxisomal, that are regulated differently. Concurrently, He, Barnes, and Falany (6) demonstrated that PPAR α activation by clofibrate in female rats increases cytosolic activity and protein levels of BAT. The discrepancies in BAT regulation could be attributable to physiological species variation, as mice have a gallbladder but rats do not. It is also possible that there is not only species variation but sexual differences in the regulation of BAT through PPAR α . Because peroxisome-proliferating agents can regulate cytosolic BAT expression and activity in rats, it is likely that there is only one form of rat BAT that can be moved between the cytosol and peroxisomes. To date, only a single BAT cDNA or gene has been identified in rats (6). Here, we describe the regulation and localization of the multiple pools of BAT activity in rat liver and the effects that clofibrate induction and gender differences play in regulating these pools.

MATERIALS AND METHODS

Materials

Clofibrate was purchased from ICN Biomedicals, Inc. (Aurora, OH). OptiPrep and rabbit anti-human PMP70 polyclonal

antibody were obtained from Sigma (St. Louis, MO). Mouse anti-ATP synthase- β was obtained from BD Biosciences (San Jose, CA). [α -³²P]dCTP (3,000 Ci/mmol) and [³H]taurine (31 Ci/mmol) were from Amersham Pharmacia Biotech (Piscataway, NJ). The STAT-60 RNA isolation reagent was purchased from Tel-Test (Friendswood, TX). Quik-Hyb was from Stratagene (La Jolla, CA).

Animals and treatment

Young adult female (178–192 g) and male (265–300 g) Sprague-Dawley rats were purchased from Charles River (Wilmington, MA). The rats were allowed to acclimate in the University of Alabama at Birmingham animal care facility for 1 week and were maintained on a 12 h light/dark cycle at a temperature of 18–22°C. The rats were allowed free access to food (Purina Certified Rodent Diet) and water for the duration of the study. After acclimatization, animals were randomly sorted into experimental groups of eight rats. For the peroxisomal induction studies, clofibrate in corn oil was administered daily by gavage at a dose of 250 mg/kg for 7 days. Control animals received an equivalent dose of corn oil. For the bile acid sequestration experiments, 5% cholestyramine resin mixed with diet was given for 9 days, whereas control animals received the equivalent diet with no cholestyramine. Food intake and body weight were measured daily. Bile was collected by cannulation of the bile duct of anesthetized animals using PE10 polyethylene tubing.

Preparation of rat liver subcellular fractions

For the isolation of subcellular fractions of liver, rats were weighed and then anesthetized with metaphane before removal of livers. The livers were blotted and weighed, then transferred to ice-cold homogenization medium B (0.25 M sucrose, 1 mM EDTA, 0.1% ethanol, and 10 mM HEPES-NaOH, pH 7.4), minced, and homogenized with a motorized homogenizer (PowerGen 700; Fisher Scientific) with a sawtooth generator. Liver homogenates were centrifuged at 500 *g* for 10 min to yield a heavy mitochondrial pellet and a light mitochondrial supernatant fraction. The heavy mitochondrial pellets were then resuspended in homogenization buffer B. The supernatant fractions from the initial spins were centrifuged at 17,000 *g* for 15 min to separate a crude peroxisomal pellet from a supernatant fraction composed of cytosol and microsomes. The supernatants were centrifuged at 100,000 *g* for 50 min to resolve microsomes from cytosol.

To purify peroxisomes, the crude peroxisomal pellets were resuspended in homogenization buffer B and mixed with an equal volume of a 50:50 mixture of OptiPrep (50% iodixanol) and homogenization buffer C (0.25 M sucrose, 6 mM EDTA, 0.6% ethanol, and 60 mM MOPS-NaOH, pH 7.4). The suspensions were centrifuged at 150,000 *g* for 4 h in a fixed-angle rotor (Beckman 50.2 Ti). Fractions were collected from the self-generated gradients by upward displacement. For analysis of subcellular BAT and BAL distribution, BAT activity and immunoblot analysis and BAL immunoblot analysis were performed with each gradient fraction as well as with the cytosolic and microsomal fractions as described below.

Immunoblot analysis

For immunoblot analysis, rat liver total homogenate protein or rat liver subcellular fractions were resolved by SDS-PAGE and electrotransferred to nitrocellulose membranes (7). Membranes were blocked with 5% nonfat milk followed by incubation with a 1:5,000 dilution of rabbit anti-mouse BAT antibody described previously or a 1:2,500 dilution of rabbit anti-rat BAL antibody (6). To identify the peroxisome-containing fractions, a polyclonal rabbit antibody to PMP70 was used at a 1:2,000 dilution.

Membranes were then incubated with a 1:20,000 dilution of goat anti-rabbit IgG conjugated with horseradish peroxidase (Southern Biotech, Birmingham, AL). Immunoconjugates were visualized using the Supersignal West Pico System (Pierce).

BAT assay

Rat liver BAT activity was determined using the radioassay described by Johnson, Barnes, and Diasio (18), in which [^3H]taurine or [^3H]glycine is conjugated to unlabeled cholyl-CoA to form ^3H -labeled bile acid conjugates. Cholyl-CoA was synthesized from cholic acid and CoA by the method of Shah and Staple (19) with modifications described previously (20).

The standard assay mixture contained 100 mM potassium phosphate (pH 8.4), 0.45 mM cholyl-CoA, and 0.025 μCi of the corresponding ^3H -labeled amino acid in a final concentration of 0.25 mM and a total volume of 50 μl . Reactions were initiated by the addition of cholyl-CoA, incubated at 37°C for 15 min, and terminated by the addition of 0.5 ml of 100 mM sodium phosphate (pH 2.0) containing 1% SDS. Radioactive conjugates were then extracted from unreacted labeled amino acid with water-saturated *n*-butanol and quantified by scintillation spectroscopy.

Northern blot analysis

Total RNA was purified from rat livers using RNA-STAT60. A full-length rat BAT cDNA probe (7) was labeled with [α - ^{32}P]dCTP by random priming (Ready-To-Go DNA Labeled Beads; Amersham Biosciences). For Northern blot analysis, 20 μg of total RNA was separated on an agarose-formaldehyde denaturing gel, transferred to a nylon membrane, and linked to the membrane by exposure to ultraviolet light. The membrane was prehybridized in Quik-Hyb buffer for 30 min at 68°C and then hybridized for 1 h at 68°C in the presence of the ^{32}P -labeled rat BAT cDNA probe in accordance with the Quik-Hyb protocol. The membrane was washed twice for 15 min with 2 \times SSC and 0.1% SDS at room temperature followed by two more 15 min washes with 0.1 \times SSC and 0.1% SDS at 60°C before exposure by autoradiography at -70°C with an intensifying screen.

Liquid chromatography/electrospray ionization/multiple reaction ion monitoring mass spectrometry

For mass spectrometric analysis of bile acids and bile salts in rat bile, 1 μl of bile samples taken from control and cholestyramine-treated rats was diluted to 1 ml in water for extraction over a 6 ml SepPak C18 column (Waters). The column was equilibrated by washing three times with 6 ml of methanol, then three times with 6 ml of water. The samples were eluted from the column with 100% methanol, and the eluants were evaporated to dryness under a stream of air and resolubilized with 80% methanol. The extracted bile samples were analyzed by reverse-phase HPLC (Shimadzu) using a 2.0 \times 100 mm Phenomenex Luna 3 μ Phenyl-Hexyl column and a dual solvent system. Solvent A was 10 mM ammonium acetate in 10% aqueous acetonitrile, and solvent B was 10 mM ammonium acetate in 100% acetonitrile. The samples were run on a linear gradient from 100% solvent A to 100% solvent B over a 10 min time course. The eluate was split 1:1, with 0.1 ml/min passed into the electrospray ionization interface of a triple quadrupole mass spectrometer (MDS Sciex API 4000). For negative ion spectra, the electrospray needle voltage and the orifice potential were set at -4,900 and -60 to -70 V, respectively. Mass spectra were collected over an *m/z* range of 300–600. In the multiple reaction ion monitoring mode, parent ion/daughter ion pairs were observed to specifically measure individual bile acids and bile salts when possible. The isomeric dihydroxy bile acids deoxycholate and chenodeoxycholate (and their glycine and taurine conjugates) are indistinguishable

because they have identical masses. Hippuric acid was used as an internal standard. Mass transitions were 448/74 for chenodeoxycholylglycine and deoxycholylglycine, 498/124 for chenodeoxycholyltaurine and deoxycholyltaurine, 407/289 for cholic acid, 391/345 for chenodeoxycholic acid and deoxycholic acid, 514/95 for cholyltaurine, and 464/402 for cholylglycine. For bile acid analysis, data were combined as dihydroxy and trihydroxy bile acids, because several bile acids have identical masses and are not resolved and identified by LC-MS.

RESULTS

Subcellular fractionation of rat liver

To determine the subcellular localization of BAT and BAL in rat liver, total homogenate, cytosol, microsomes, crude peroxisomes, and heavy mitochondrial fractions were isolated from rat liver via differential centrifugation. To obtain a purified peroxisomal fraction, the crude peroxisomal fraction was further resolved via iodixanol gradient centrifugation (6). Fractions were defined by visual separation of gradient interfaces and collected by upward displacement, as described in Materials and Methods. Immunoblot analysis indicated the presence of peroxisomes in fractions 1–6 by the presence of PMP70, a peroxisomal membrane marker protein (21). Fractions 5–8 contained some mitochondrial protein, as indicated by the presence of low levels of ATP synthase- β (Fig. 1A). According to immunoblot analysis, BAT colocalized with PMP70, and BAT activity analysis indicated that the fractions with the highest immunoreactive protein also had the highest BAT specific activity. A purified peroxisome fraction was obtained by pooling fractions 1–4 of the iodixanol gradient.

Some peroxisomes are fragile and rupture during homogenization of tissue, releasing their contents into the cytosol. This may provide an explanation for the dual localization of BAT to the peroxisomes and cytosol (22). To assess whether the protocol used resulted in minimal lysis of peroxisomes, livers from young adult Sprague-Dawley rats were deliberately frozen overnight at -70°C before undergoing fractionation. Freezing the livers compromised the integrity of some of the peroxisomes, resulting in a change in the distribution of BAT activity in the iodixanol gradient compared with the distribution of BAT activity in the iodixanol gradient obtained during isolation of fresh rat liver (Fig. 1B). Lysing the peroxisomes by freezing the liver before fractionation also resulted in the contamination of the cytosol, microsomes, and heavy mitochondrial fractions with PMP70 (Fig. 1B).

Sexual differences in subcellular BAT protein and activity

The total homogenate, cytosol, microsomes, purified peroxisomes, and heavy mitochondria obtained from rat liver were immunoblotted for BAT and other organelle selective proteins (Fig. 2A). The highest concentration of immunoreactive BAT protein was found in peroxisomes, but BAT was also detected in cytosol and the heavy mitochondrial fractions (Fig. 2A). Immunoreactive BAL was not found in the peroxisomal or cytosolic fractions but

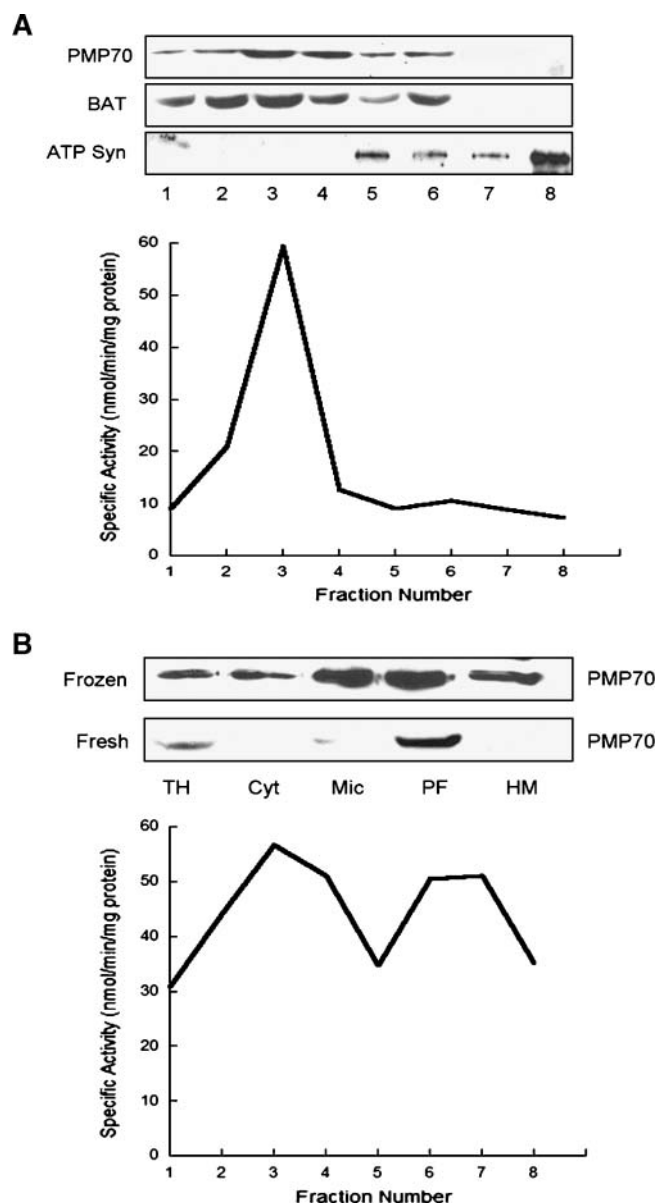


Fig. 1. Subcellular localization of bile acid coenzyme A:amino acid *N*-acyltransferase (BAT). Immunoblot analysis of male rat liver subcellular fractions. A: Iodixanol gradients resulting from centrifugation of fresh liver fractions isolated from untreated male Sprague-Dawley rats were analyzed for PMP70, ATP synthase- β (ATP Syn), and BAT content via immunoblot analysis. The lower graph represents the specific BAT activity of the corresponding gradient fractions. B: Livers removed from untreated male Sprague-Dawley rats were frozen overnight at -70°C before undergoing the subcellular fractionation procedure described in Materials and Methods. The resulting fractions (Frozen), along with fractions obtained from unfrozen liver (Fresh), were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with an anti-PMP70 antibody. Lane 1, total homogenate (TH); lane 2, cytosol (Cyt); lane 3, microsomes (Mic); lane 4, peroxisomes (PF); lane 5, heavy mitochondria (HM) ($100\ \mu\text{g}$ of each fraction). The lower graph represents the specific BAT activity of the iodixanol gradient fractions obtained from male rat livers frozen overnight.

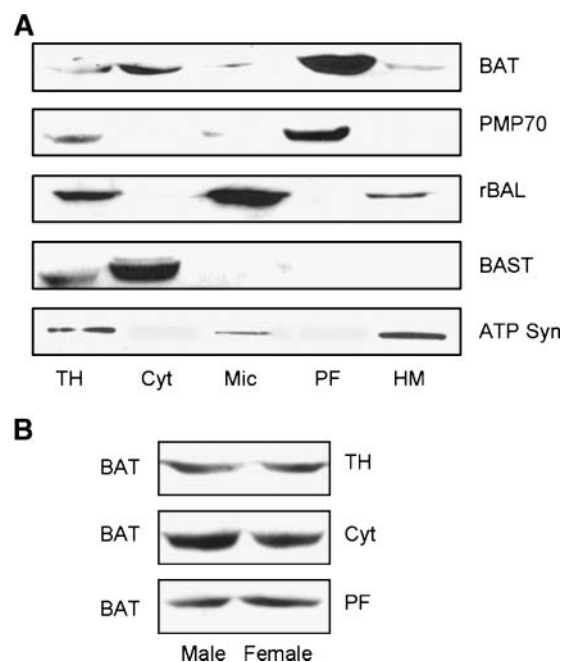


Fig. 2. Subcellular localization of BAT protein. A: Liver isolated from males was subfractionated as described in Materials and Methods and resolved by SDS-PAGE, transferred to nitrocellulose, and probed with antibodies to BAT, rat bile acid coenzyme A ligase (rBAL), PMP70, ATP-synthase β (ATP Syn), and bile acid sulfotransferase (BAST). Lane 1, total homogenate (TH); lane 2, cytosol (Cyt); lane 3, microsomes (Mic); lane 4, peroxisomes (PF); lane 5, heavy mitochondria (HM) ($100\ \mu\text{g}$ of each fraction). B: Immunoblot analysis using a rabbit anti-BAT antibody of $100\ \mu\text{g}$ of untreated male and female rat liver subcellular fractions of total homogenate (TH), cytosol (Cyt), and peroxisomes (PF) after resolution by SDS-PAGE and transfer to nitrocellulose.

only in the microsomal fraction. PMP70, a peroxisomal membrane protein used as a peroxisomal marker, was present almost totally in the peroxisomal fraction. ATP synthase- β subunit was used as a mitochondrial marker but also showed some staining in the microsomal fraction, indicating mitochondrial contamination. Rat bile acid sulfotransferase, used to denote the presence of cytosol, was found only in the cytosolic fraction (23) (Fig. 2A).

When BAT immunoreactive protein levels were compared between untreated male and female rats, there was no significant difference between the levels of BAT in total liver homogenate and peroxisomes; however, males had higher amounts of BAT in the cytosol than did females (Fig. 2B). To confirm that BAT immunoreactivity was a good indication of BAT activity, cholytaurine formation was measured in different rat liver subcellular fractions using [^3H]taurine as a substrate. The highest specific activity for cholytaurine formation was found in the peroxisomal fraction in both males and females and was ~ 7 -fold higher than the specific activity found in the cytosol (Fig. 3A). Male rats had significantly higher BAT specific activity in the cytosolic fraction than did female rats, but no difference in peroxisomal BAT specific activity or total homogenate specific activity was detected. When total BAT activity in 7 g of liver was calculated, cytosol

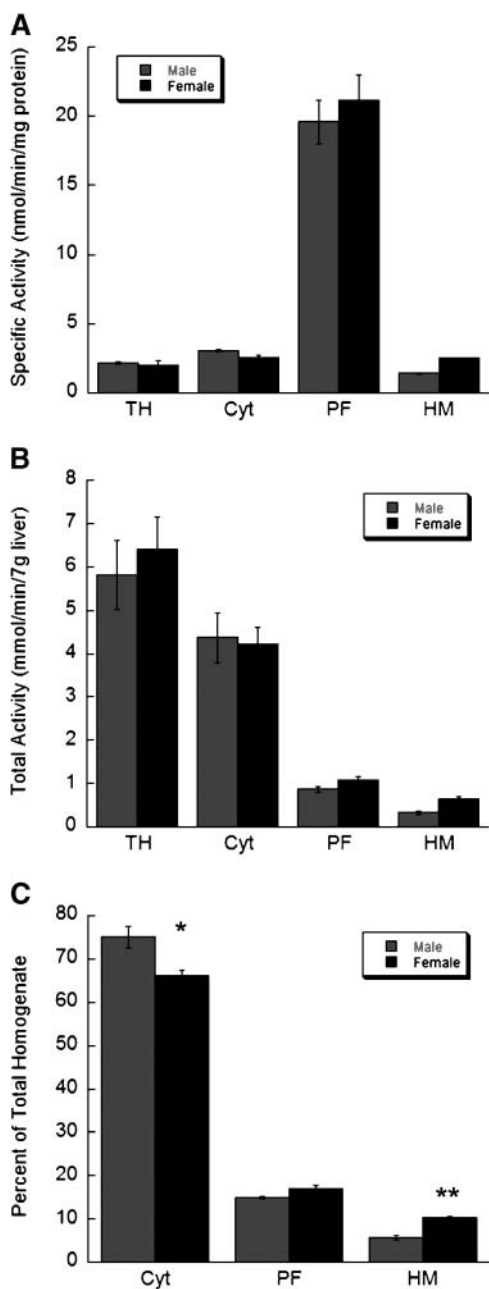


Fig. 3. Subcellular localization of BAT activity. A: BAT specific activity (nmol/min/mg protein) in male (gray bars) and female (black bars) rat liver subcellular fractions as measured by tau-rocholate production using 0.25 mM taurine as a substrate. B: Total BAT activity (mmol/min/7 g liver) in subcellular fractions in 7 g of liver. C: Subcellular fraction activity as a percentage of total homogenate. Total activity in each fraction was divided by the total homogenate activity in equivalent volumes of liver. TH, total homogenate; Cyt, cytosol; PF, peroxisomes; HM, heavy mitochondria. Asterisks indicate significant differences between male and female untreated rats (* $P < 0.05$, ** $P < 0.01$; $n = 8$ for each group). Error bars represent SEM.

contributed the majority of the activity compared with peroxisomes, heavy mitochondria, and microsomes (Fig. 3B). There was no difference in total BAT activity in total homogenate, cytosol, or peroxisomal fractions per 7 g of liver between male and female rats (Fig. 3B). When the

data were expressed as the percentage contribution by each fraction toward total homogenate activity, in male rats, 75% of the BAT activity was present in the cytosol, 15% was contributed by the peroxisomes, and 5% was attributed to the heavy mitochondrial fraction (Fig. 3C). In female rats, 66% of BAT activity was contributed by the cytosolic fraction, 17% was found in the peroxisomal fraction, and 10% was found in the heavy mitochondrial fraction. The contribution of microsomal BAT activity to the total homogenate activity was negligible, because centrifugation and resuspension of microsomes in buffer removed 97% of the initial microsomal BAT activity (data not shown). Therefore, BAT activity found in microsomes was regarded as cytosolic contamination and included as cytosolic activity in the context of total activity calculations.

BAT message

Total RNA isolated from the livers of untreated and clofibrate-treated male and female rats showed a single band when hybridized with a full-length rat BAT cDNA probe, consistent with previous reports (6) that only one form of BAT is present in these tissues (Fig. 4). Clofibrate treatment appeared to slightly decrease BAT message levels in male rat liver and to increase BAT message in

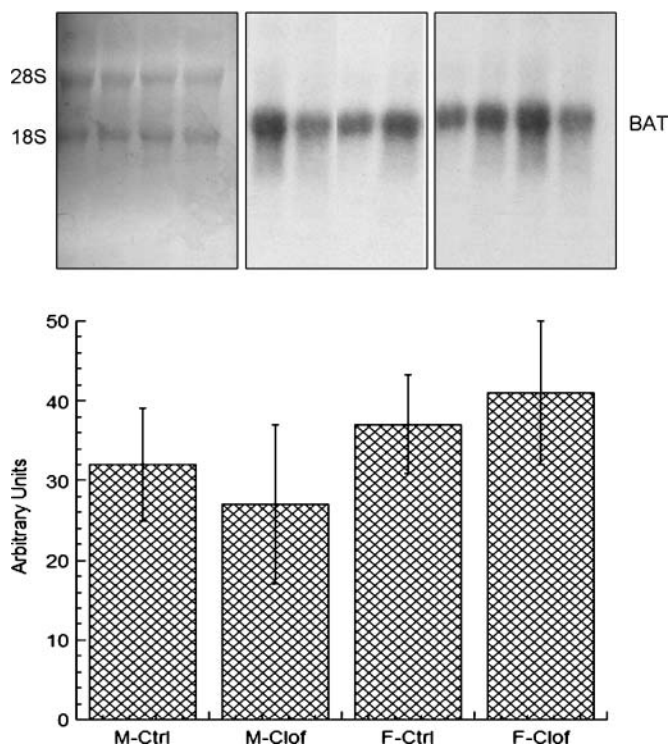


Fig. 4. Regulation of BAT message by clofibrate. Densitometric analysis of Northern blots depicting rat BAT mRNA expression in livers of untreated male rats (M-Ctrl), clofibrate-treated male rats (M-Clof), untreated female rats (F-Ctrl), and clofibrate-treated female rats (F-Clof) performed three separate times. Two separate gels (middle and right) are shown above the graph. Lane 1, M-Ctrl; lane 2, M-Clof; lane 3, F-Ctrl; lane 4, F-Clof. Total RNA (20 μ g) was hybridized with a full-length rat BAT cDNA probe as described in Materials and Methods. Standards for loading control were 18S and 28S RNA, shown in the left gel. Error bars represent SEM.

TABLE 1. Effect of clofibrate treatment on the liver and body weight of male and female Sprague-Dawley rats

Group	n	Body Weight	Liver Weight	Liver Percentage of Body Weight
			<i>g</i>	
Male control	8	274.2 ± 11.4	11.4 ± 0.7	4.2 ± 0.2
Male clofibrate	8	277.8 ± 9.7	15.5 ± 0.7 ^a	5.6 ± 0.3 ^a
Female control	8	209.1 ± 4.2	7.9 ± 0.3	3.8 ± 0.1
Female clofibrate	8	213.0 ± 4.7	11.1 ± 0.6 ^a	5.2 ± 0.2 ^a

^aSignificant difference between clofibrate-treated and control rats of the same gender ($P < 0.01$).

female rats; however, densitometric analysis of BAT message in the livers of four animals per group indicated that neither of these results was significant (Fig. 4).

Clofibrate treatment changes the distribution of BAT activity and protein in a sex-dependent manner

Solaas et al. (8) have reported a decrease in peroxisomal BAT protein after treating male mice with a PPAR α agonist, and previous work from our laboratory (6) reports an increase in cytosolic BAT protein after treatment of female rats with the peroxisome-proliferating agent clofibrate. Therefore, male and female rats were treated with clofibrate to discern any changes in the subcellular localization of BAT associated with the sex of the rats.

Clofibrate treatment for 7 days had no effect on the body weight for rats of either sex but did significantly increase liver weight. This effect resulted in a significant increase in liver as a percentage of body weight in both male and female rats compared with their respective gender controls (Table 1). Changes in BAT activity in liver subcellular fractions after clofibrate treatment are noted in Table 2. The total peroxisomal protein increased by 75% in 10 g of liver from male rats and by 50% in 7 g of liver from female rats after clofibrate treatment (Table 2). Total protein increased by 13% in 10 g of liver from male rats and by almost 10% in 7 g of liver from female rats after clofibrate treatment (Table 2).

Immunoblot analysis of BAT levels in the cytosolic fractions of control and clofibrate-treated liver showed an increase in cytosolic BAT immunoreactive protein in female rats and a decrease in cytosolic BAT in males (Fig. 5A). Peroxisomal fractions showed an opposite response, with an increase in peroxisomal BAT protein in males and a decrease in females (Fig. 5A). Both sexes showed an increase in PMP70, indicating a proliferation of peroxisomes, but no significant change in total BAT protein levels (Fig. 5B). The large increase in PMP70 in males does not necessarily correlate directly with a complementary increase in the number of functional peroxisomes (24) but does indicate a response to clofibrate.

TABLE 2. Changes in BAT activity in liver subcellular fractions after clofibrate treatment

Variable	Protein	Total Protein	Protein Percentage of TH	Specific BAT Activity	Total BAT Activity	BAT Activity Percentage of TH
	<i>mg/ml</i>	<i>mg</i>		<i>pmol/min/mg protein</i>	<i>nmol/min/10 g (males) or 7 g (females) liver</i>	
Male control						
TH	96.9 ± 6.7	3,874 ± 241	100.00	2,146 ± 92	8,314 ± 1,129	100.0
Cytosol	45.9 ± 2.6	2,025 ± 49	52.3 ± 0.8	3,078 ± 49	6,235 ± 0.827	75 ± 2.3
Peroxisomes	8.0 ± 0.9	64 ± 7.0	1.6 ± 0.2	19,603 ± 2,162	1,247 ± 0.191	15 ± 0.2
Heavy mitochondria	43.5 ± 4.0	331 ± 38	8.5 ± 3.1	1,416 ± 21	0.468 ± 0.058	5.6 ± 0.5
Microsomes	30.8 ± 1.1	658 ± 18	17.0 ± 3.1	—	—	—
Fractions 5–8	Varied	487 ± 55	12.6 ± 2.5	—	—	—
Male clofibrate						
TH	111.2 ± 4.1	4,394 ± 162	100.00	2,173 ± 114	9,549 ± 1,782	100.0
Cytosol	45.0 ± 0.4	2,079 ± 8	47.3 ± 0.1	2,710 ± 100	5,634 ± 0.126	59 ± 2.5
Peroxisomes	13.8 ± 2.4	111 ± 19	2.5 ± 0.2	26,788 ± 1,558	2.96 ± 0.755	31 ± 2.2
Heavy mitochondria	44.1 ± 1.2	350 ± 16	8.0 ± 2.3	1,599 ± 20	0.560 ± 0.134	5.9 ± 0.9
Microsomes	38.5 ± 1.7	790 ± 27.2	17.9 ± 0.9	—	—	—
Fractions 5–8	—	886 ± 47	20.0 ± 1.6	—	—	—
Female control						
TH	114.5 ± 4.7	3,149 ± 14	100.00	2,031 ± 287	6,395 ± 0.152	100.0
Cytosol	47.7 ± 2.6	1,646 ± 33	52.3 ± 1.0	2,563 ± 93	4,220 ± 0.384	66 ± 1.5
Peroxisomes	6.4 ± 0.6	51 ± 5	1.63 ± 0.3	21,133 ± 3,651	1,087 ± 0.080	17 ± 0.7
Heavy mitochondria	32.5 ± 7.7	252 ± 87	8.0 ± 2.4	2,584 ± 79	0.652 ± 0.052	10.2 ± 0.4
Microsomes	20.2 ± 2.7	449 ± 43.2	14.3 ± 4.4	—	—	—
Fractions 5–8	—	391 ± 61	12.42 ± 1.9	—	—	—
Female clofibrate						
TH	125.3 ± 5.3	3,459 ± 186	100.00	1,843 ± 126	6,374 ± 0.998	100.0
Cytosol	46.8 ± 2.8	1,707 ± 43.4	49.3 ± 0.9	2,763 ± 118	4,717 ± 0.651	74 ± 1.2
Peroxisomes	9.9 ± 1.3	77 ± 10.4	2.2 ± 0.4	92,53 ± 3,040	0.714 ± 0.112	11.2 ± 1.0
Heavy mitochondria	35.0 ± 11.4	280 ± 42.1	8.1 ± 2.7	2,205 ± 200	0.618 ± 0.177	9.7 ± 1.0
Microsomes	25.7 ± 0.3	551 ± 9.6	15.9 ± 0.6	—	—	—
Fractions 5–8	—	425 ± 109	12.3 ± 4.0	—	—	—

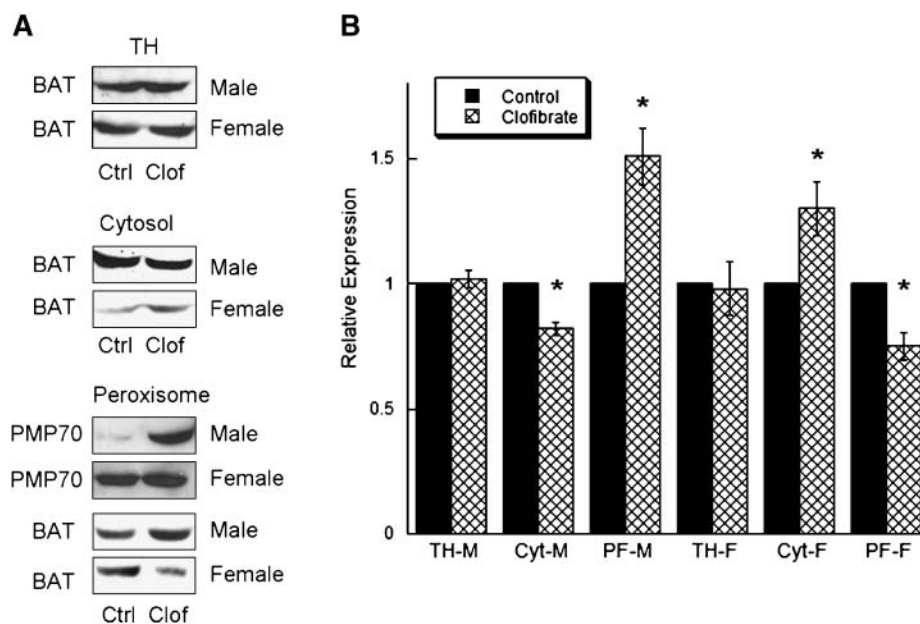


Fig. 5. Regulation of BAT in liver subcellular fractions by clofibrate. A: Immunoblot analysis of 100 μ g of male and female rat liver subcellular fractions after (Clf) or without (Ctrl) clofibrate treatment using anti-BAT and anti-PMP70 antibodies. TH, total homogenate. B: Quantification by densitometric analysis of immunoblotted BAT protein in rat liver subcellular fractions of total homogenate (TH), cytosol (Cyt), and peroxisomes (PF) with (hatched bars) or without (black bars) clofibrate treatment. F, female; M, male. Values are expressed as a fraction of average control values (no treatment). Asterisks indicate significant differences between clofibrate-treated and control rats of the same sex ($P < 0.05$; $n = 6$ for each group). Error bars represent SEM.

To determine whether the change in BAT protein levels coincided with a change in BAT activity, the specific activities of the different rat liver fractions in males and females were calculated before and after clofibrate treatment (Table 2). BAT specific activity from total liver homogenates of both sexes did not change significantly after clofibrate treatment (Fig. 6A). Male BAT specific activity decreased by 11% in the cytosolic fraction compared with male controls after clofibrate treatment and increased by 37% in the peroxisomal fraction (Fig. 6B, C). In contrast, female BAT specific activity increased by 10% in the cytosolic fraction and decreased by 57% in the peroxisomes after clofibrate treatment (Fig. 6B, C). These data show the same trends as the immunoblot results (Fig. 5). When the BAT activity data are expressed as a percentage of total homogenate activity, male rats showed a decrease of 15% of total activity contributed by the cytosol and a 16% increase in the total activity contributed by the peroxisomes. In female rats, the cytosolic contribution to the total activity was increased by 8%, whereas there was a 6% decrease in peroxisomal contribution (Fig. 6D). BAT activity present in the heavy mitochondrial fraction was unchanged in both sexes. When total BAT activities were calculated to account for the total liver weights of each animal, increases in total homogenate, cytosol, and peroxisomal BAT activity were found in all clofibrate-treated animals compared with their respective gender controls, as a result of the increased liver weights (data not shown).

Bile acid sequestration does not affect BAT localization or activity

The advantages of a shift in the localization of BAT between the cytosol and peroxisomes are not entirely clear. If peroxisomal BAT activity has a major role in de novo bile acid biosynthesis, then circumstances requiring newly synthesized bile acids could theoretically result in an increase in peroxisomal BAT activity. To test this hypothesis, adult male Sprague-Dawley rats were treated for 9 days with a 5% cholestyramine diet before removal and fractionation of their livers. There were no differences between control and treated rats in the amount of food consumed during the experiment or in body and liver weights (data not shown). Mass spectrometric analysis of bile collected from the animals showed an increase in the taurine-conjugated primary trihydroxy bile salts and a decrease in the taurine and glycine dihydroxy bile acid salts after cholestyramine treatment (Table 3). However, analysis of BAT activity using taurine as a substrate showed no change between control and treated rats in total liver homogenate, cytosol, or peroxisomal BAT activity (Fig. 7).

DISCUSSION

Reports of the subcellular localization of BAT in hepatocytes have been inconsistent in the recent literature. Different groups of investigators have proposed that BAT resides predominantly in the cytosol (17), predominantly in peroxisomes (10), or in both simultaneously

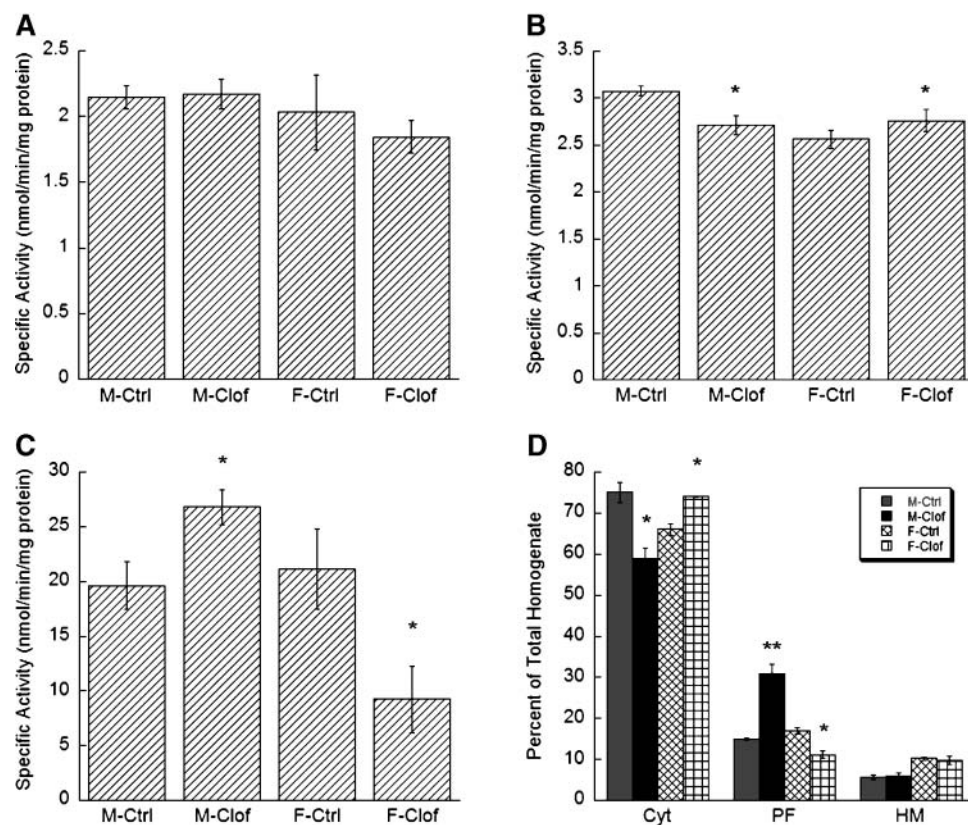


Fig. 6. Regulation of BAT activity in male and female rat liver subcellular fractions by clofibrate. BAT activity in subcellular fractions of livers of untreated male rats (M-Ctrl), clofibrate-treated male rats (M-Clof), untreated female rats (F-Ctrl), and clofibrate-treated female rats (F-Clof) was measured via a BAT radioassay using 0.25 mM taurine as a substrate. A: Specific BAT activity measured in 20 μ g of rat total liver homogenate from four experimental groups. B: Specific BAT activity measured in 20 μ g of rat liver cytosol from four experimental groups. C: Specific BAT activity in 5 μ g of rat liver peroxisomes from four experimental groups. D: Percentage of total BAT activity in 7 g of liver from each subcellular fraction from four experimental groups. Cyt, cytosol; PF, peroxisomes; HM, heavy mitochondria. Total BAT activity in each fraction was divided by the total homogenate activity in equivalent volumes of liver. Asterisks indicate significant differences between clofibrate-treated and control rats of the same sex (* $P < 0.05$, ** $P < 0.01$; $n = 8$ for each group). Units in A–C are nmol/min/mg protein. Error bars represent SEM.

(6, 16). Other investigators have hypothesized that there might be two distinct forms of BAT, one cytosolic and one peroxisomal (8). In this study, the contributions of the different subcellular compartments in rat liver to total BAT activity were evaluated, and our results demonstrate that the majority of BAT activity in male and female rats (75% and 64% of the total liver homogenate activity, respectively) resides in the cytosol. A smaller, yet signifi-

cant, portion of BAT activity (~15–17% in both sexes) is localized to peroxisomes. Because of the low levels of peroxisomal protein in the liver, this results in the highest BAT specific activity in peroxisomes. The remaining BAT activity in both genders can be found in the heavy mitochondrial fraction, although it is possible that this activity is simply attributable to contamination by unlysed cells. The large amount of total BAT activity found in the

TABLE 3. Concentration of bile acids in rat bile before and after cholestyramine treatment

Rat	Treatment	Trihydroxy	Dihydroxy	TriHy-Gly	TriHy-Tau	DiHy-Gly	DiHy-Tau
A	Control	12.9	4.58	13	163	5.4	21.5
B	Control	14.9	5.17	9.14	208	2.56	23.1
C	Control	33.3	5.64	10.7	262	4.86	20
D	Cholestyramine	<1	2.19	5.51	323	<1	12
E	Cholestyramine	14	4.53	3.08	274	<1	8.93

Bile collected from each rat was analyzed by liquid chromatography/electrospray ionization/multiple reaction ion monitoring mass spectrometry as indicated in Materials and Methods. All measurements are in μ M; <1 indicates a value of <1 μ M. Trihydroxy, trihydroxylated bile acids; Dihydroxy, dihydroxylated bile acids; TriHy-Gly, trihydroxylated bile acids conjugated with glycine; TriHy-Tau, trihydroxylated bile acids conjugated with taurine; DiHy-Gly, dihydroxylated bile acids conjugated with glycine; DiHy-Tau, dihydroxylated bile acids conjugated with taurine.

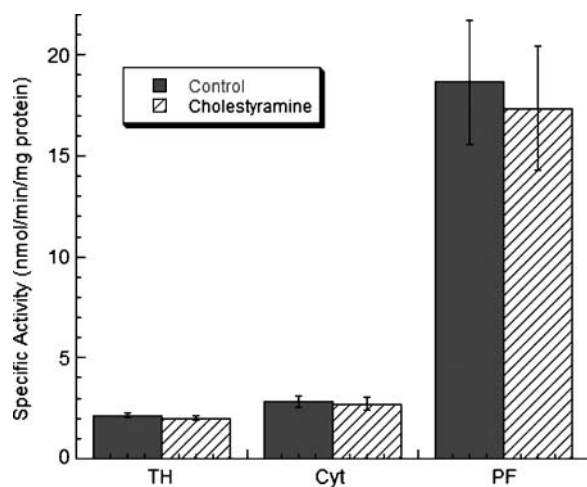


Fig. 7. Effect of cholestyramine on subcellular liver BAT activity. Specific BAT activity in total liver homogenate (TH) or liver subcellular cytosol (Cyt) and peroxisome (PF) fractions of untreated (closed bars) and cholestyramine-treated (hatched bars) rats measured via BAT radioassay. The fractions were assayed separately using 0.25 mM taurine as a substrate. For each group, $n = 4$. Error bars represent SEM.

cytosol, as well as the absence of PMP70 in that compartment, indicates that BAT found in the cytosol is probably not the result of peroxisomal disruption, as long as the liver is not frozen before fractionation.

Because of the liver's ability to efficiently recycle bile acids, the de novo synthesis of bile acids would be expected to be relatively small compared with the amount of bile acids in the enterohepatic circulation. If peroxisomal BAT activity is responsible for conjugating newly synthesized bile acids, and BAT activity outside of the peroxisome is responsible for conjugating recycled bile acids, as suggested by Kase and Bjorkhem (10), then one would expect to find a much greater proportion of BAT activity in the cytosol than in peroxisomes. Zhang, Barnes, and Diasio (25) reported that only 18% of radiolabeled chenodeoxycholyglycine is found intact after one pass of enterohepatic circulation, suggesting that glycine conjugates of bile acids are deconjugated extensively in the intestine. The deamidated bile acids would require conjugation by BAT activity in the cytosol after reabsorption by the hepatocytes. Unlike mice and humans, rats lack a gallbladder, leading to a constant enterohepatic circulation of bile acids, because they cannot be stored. It is possible that this physiologic difference could account for the different subcellular distribution of BAT in rats compared with mice and humans. Species differences in BAT isoforms are well noted, including sequence homology and substrate specificity; mice are able to form only taurine conjugates, whereas rats and humans make both taurine and glycine conjugates (6, 26, 27).

Sex differences in bile acid levels were noted by Kurtz et al. (28), who reported that 64% of nonsulfated bile acids in the liver are found in the cytosolic fraction of male Wistar rats and that female rat liver cytosol contains 68% of the nonsulfated bile acids. In rat liver homogenates,


female Wistar rats have higher concentrations of the primary bile acids cholic acid and chenodeoxycholic acid, whereas males tend to have higher concentrations of the secondary bile acids such as deoxycholic acid and lithocholic acid (26). Sex differences in PPAR α expression have also been reported and indicate that male rats have higher levels of PPAR α message and protein in liver and show a greater response to ligands such as clofibrate (29). PPAR α is also differentially regulated by sex hormones such as testosterone (30) and 17 β -estradiol (29). Bile acids in rats were found to be unaffected by ethinylestradiol treatment (31), but 17 β -estradiol treatment can lead to cholestasis by inhibiting proteins involved in bile salt transport (32). Cholestasis can also develop when bile acids are not conjugated efficiently, but the effects of testosterone and estrogen treatment on BAT activity remain unknown.

Clofibrate treatment of male and female rats resulted in sexual differences in hepatic BAT protein and activity (Table 2). In male rats treated with clofibrate, the total BAT activity contributed by the cytosol was decreased by 17%, whereas a 15–16% increase in the total activity contributed by peroxisomes was found. In female rats, the cytosolic contribution to the total activity was increased by ~8%, whereas a 5% decrease in peroxisomal contribution was observed. The calculated shifts in activity are such that the total decrease in activity from the peroxisomes can be reasonably accounted for by the total increase in the cytosol in females, and a similar result occurs in males. These findings, along with data that specific and total BAT activities for equivalent liver weights of total homogenates are unchanged after clofibrate treatment, suggest that PPAR α activation regulates the localization of BAT rather than total expression levels. If the increased liver weights of clofibrate-treated male and female rats are considered, total liver BAT activity increases significantly, suggesting an increased ability to conjugate bile acids regardless of localization.

Clofibrate treatment for 7 days did not significantly affect BAT message expression in either male or female rats. This lack of effect on BAT message levels is consistent with several recent microarray studies in rats, rat primary hepatocytes, and mouse primary hepatocytes (33–35) but contradicts a report from our own laboratory published previously (6). Although changes in gene expression are an important consideration, the concentration of BAT protein in the liver was unchanged after clofibrate treatment, regardless of whether the expression of BAT message was altered or not. Proteomic analysis of male rat liver tissue treated in identically to the rats in this study failed to find any change in BAT protein, supporting these results (36).

The physiologic advantages of a shift in the localization of BAT between the cytosol and peroxisomes are not entirely clear. Treatment with cholestyramine to stimulate the de novo synthesis of bile acids in rat liver had no effect on rat BAT activity in whole liver homogenate or its subcellular compartments. Cholyltaurine levels in bile were increased after cholestyramine treatment, which indi-

cates that there is sufficient BAT activity to efficiently conjugate the increase in newly synthesized bile acids. Therefore, it may be beneficial to examine the localization of BAT protein and activity in circumstances in which BAT message has been found to be increased. According to recent results (37), farnesoid X receptor activation in male rats increases BAT and BAL messages but inhibits proteins responsible for initiating bile acid biosynthesis.

This report has identified the subcellular distribution of BAT activity in rat liver and compared the differences in BAT activity in these compartments between males and females. These experiments indicate that the majority of BAT activity in the rat liver resides in the cytosol; however, smaller but significant amounts of BAT are also found in peroxisomes. These results support the novel finding that PPAR α activation via clofibrate treatment regulates the level of BAT activity in the cytosolic and peroxisomal compartments differently in male and female rats. The localization of the majority of BAT in the cytosol is consistent with the requirement for efficient reamidation of bile acids in the enterohepatic circulation, whereas BAT activity in the peroxisomal matrix is responsible for the amidation of newly synthesized bile acids. The movement of BAT between these cellular compartments also supports the concept of a single form of BAT in hepatocytes. 

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