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Regulation of Lipid Flux between Liver and Adipose Tissue during Transient Hepatic Steatosis in Carnitine-depleted Rats*

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Rats with carnitine deficiency due to trimethylhydrazinium propionate (mildronate) administered at 80 mg/100 g body weight per day for 10 days developed liver steatosis only upon fasting. This study aimed to determine whether the transient steatosis resulted from triglyceride accumulation due to the amount of fatty acids preserved through impaired fatty acid oxidation and/or from up-regulation of lipid exchange between liver and adipose tissue. In liver, mildronate decreased the carnitine content by ~13-fold and, in fasted rats, lowered the palmitate oxidation rate by 50% in the perfused organ, increased 9-fold the triglyceride content, and doubled the hepatic very low density lipoprotein secretion rate. Concomitantly, triglyceridemia was 13-fold greater than in controls. Hepatic carnitine palmitoyltransferase I activity and palmitate oxidation capacities measured *in vitro* were increased after treatment. Gene expression of hepatic proteins involved in fatty acid oxidation, triglyceride formation, and lipid uptake were all increased and were associated with increased hepatic free fatty acid content in treated rats. In periepididymal adipose tissue, mildronate markedly increased lipoprotein lipase and hormone-sensitive lipase activities in fed and fasted rats, respectively. On refeeding, carnitine-depleted rats exhibited a rapid decrease in blood triglycerides and free fatty acids, then after ~2 h, a marked drop of liver triglycerides and a progressive decrease in liver free fatty acids. Data show that up-regulation of liver activities, peripheral lipolysis, and lipoprotein lipase activity were likely essential factors for excess fat deposit and release alternately occurring in liver and adipose tissue of carnitine-depleted rats during the fed/fasted transition.

Fat is mainly deposited in adipose tissue, but is also observed in liver biopsies of humans suffering from disorders originating, for instance, from alcohol abuse, diabetes (1), or intoxications (2). Fat storage usually results from the imbalance of the partitioning of lipids between their utilization as energy sources and their preservation or synthesis as triglycerides (TG)[2] initially provided by excess feeding or increased lipogenesis (3). Liver steatosis may also exist when the lipoprotein secretion mechanisms are impaired (4, 5). Genetic models of animal obesity (6, 7) and overweight humans (8, 9) have provided information on regulations occurring in adipose tissue and liver, in which fat deposit may be the consequence of actions mediated by insulin, such as inhibition of fatty acid (FA) oxidation (10) or increased lipogenesis (11, 12), and even of hypothalamic injuries (13). Experimental studies have been undertaken to amplify the inhibition of the FA oxidation pathway with drugs that reduce the carnitine-dependent transfer of FA into mitochondria, for example, etomoxir for the carnitine palmitoyltransferase (CPT) I step (14), L-aminocarnitine for the CPT II step (15), or 3,2,2,2-trimethylhydrazinium propionate (mildronate) for liver carnitine biosynthesis (16, 17). Under normal conditions, liver mitochondrial FA oxidation is inhibited via the insulin secretion in the fed state, whereas TG formation and VLDL secretion are increased (18) without liver fat accumulation. In the present study, we have tried to reproduce the early stage of induction of liver steatosis to know whether liver fat accumulation is associated with severe impairments or appropriate reactions of lipid metabolic pathways, and if a correction for liver steatosis is still possible. In this goal, the inhibition of FA oxidation occurring in the fed state was extended to the fasted state through the mildronate-mediated carnitine depletion to obtain, in a minimum of time, the accumulation of fat in the liver (16, 17). The experimental procedure also aimed to amplify the relationship between liver and adipose tissue in terms of up- or down-regulation occurring during the fed/fasted state transition.

EXPERIMENTAL PROCEDURES

Animals and Treatments

Official French regulations (number 87848) for the care and use of laboratory animals were followed (number 03056) throughout. Male Wistar rats weighing about 170 g were

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[2] The abbreviations used are: TG, triglyceride; VLDL, very low density lipoprotein; CPT I, carnitine palmitoyltransferase I; DGAT, diacylglycerol acyltransferase; FA, fatty acid; FAT/CD36, fatty acid translocase; GPAT, glycerol phosphate acyltransferase; HSL, hormone-sensitive lipase; KB, ketone bodies; LPL, lipoprotein lipase; PAT, periepididymal adipose tissue; PPAR, peroxisome proliferator-activated receptor; FFA, free fatty acid.

obtained from the Centre d'élevage Dépré (Saint-Doulchard, France). They were housed 1 week before the beginning of the study in a controlled environment and were fed a standard non-purified diet (AO4, UAR Epinay-sur-Orge, France) containing 3.5% lipids, with free access to tap water. Animals individually housed in stainless steel cages received 80 mg of mildronate (Grindex, Riga, Latvia) per 100 g body weight per day for 10 days. They were daily fed 3 g of the powdered AO4 diet wetted with 2 ml of water containing the drug at ~10:00 a.m., then 20 g of the same diet wetted with 15 ml of water at ~2:00 p.m. Mildronate-treated and control rats were used in the fed state (experiment 1) or fasted for 18 h (experiment 2). For the refeeding procedure, 18-h fasted rats were given 10 g of powdered diet wetted with 6 ml of water and were used for blood sampling and organ weighing at regular time interval over the 4 first hours (with 4 rats per time point). Anesthesia was performed by intraperitoneal route with sodium pentobarbital kept at 37 °C.

Liver Secretion and Palmitate Perfusion

Rates of hepatic VLDL-apoB secretion were measured according to the procedure using Triton WR-1339 (19). Liver perfusion was performed via the portal vein toward the liver in a recirculating and oxygenating system using the apparatus and techniques described in Ref. 20 with details modified as follows. The perfusion medium consisted of washed bovine erythrocytes suspended to a hematocrit value of 20% in Krebs bicarbonate buffer, pH 7.4, containing 4% bovine serum albumin. [1-^{14}C]Palmitic acid (15 MBq/mmol) from PerkinElmer Life Sciences, as a sodium salt, was initially bound to bovine serum albumin of the buffer in a 2:1 molar ratio. For the perfusion of fed rat livers, the medium contained 0.25 mIU insulin/ml. Over the 40-min perfusion period, the effluent gas was passed through two successive CO_2 traps, each containing 150 ml of Carbo-Sorb E (PerkinElmer), that were changed every 10 min. Samples of the final perfusion fluid were placed into vials fitted with conical plastic tubes containing 1.5 ml of Hyamine (PerkinElmer) and were acidified with 3 ml of 10% (w/v) perchloric acid for 2 h. Radioactivity of CO_2 trapped into Carbo-Sorb E from the gas flow and into Hyamine after acidification of the perfusion medium was determined after mixing in Permafluor E+ and Ultima Gold XR (PerkinElmer), respectively. The radioactivity of acid-soluble products represented by short molecules, mainly ketone bodies (KB), derived from β-oxidation reactions, was measured after filtration of the acidified medium as previously described (21). After the end of the perfusion with labeled palmitic acid, livers were rinsed with a medium devoid of labeled FA for 3 min. They were then extracted for total lipids according to Folch *et al.* (22) after addition of 1,2,3-triheptadecanoylglycerol and pentadecanoic acid, as internal standards for TG and free FA (FFA) determinations, respectively. Lipid classes were separated by thin-layer chromatography on silica gel and measured after gas liquid chromatography of constitutive FA methyl esters (23).

Hepatic Enzyme Activities

Activities were studied using whole liver homogenates and/or mitochondrial, microsomal, or cytosolic fractions isolated as previously described (21).

Mitochondrial Activities—Respiration with palmitoylcarnitine, octanoate, glutamate/malate, and succinate as substrates was measured polarographically at 30 °C with a Clark-type oxygen electrode (Yellow Springs Instruments, Yellow Springs, OH) as in Ref. 24. Oxidation of [1-^{14}C]palmitate was performed using whole homogenates and isolated mitochondria (21, 25). Long-chain acyl-CoA synthetase and CPT I activities were measured with [1-N^6-etheno]CoA (26) and L-[*methyl*-^{14}C]carnitine (Amersham Biosciences), respectively. Sensitivity of CPT I to malonyl-CoA inhibition was estimated as in Ref. 27. Monoamine oxidase (28), cytochrome *c* oxidase, hydroxybutyryl-CoA dehydrogenase (29), citrate synthase, and glycerol-3-phosphate acyltransferase (mtGPAT) (30) were measured in liver homogenates and/or isolated mitochondria (31, 32) as indicated. Total mitochondrial protein (milligrams per g of liver) was calculated by dividing the activity of monoamine oxidase, cytochrome *c* oxidase, or citrate synthase expressed per g of liver (using homogenates; as in Table 2) by the activity of corresponding enzymes expressed per mg of protein of isolated mitochondrial fractions.

Microsomal Activities—Activities of aryl-ester hydrolase (31) and glycerol-3-phosphate acyltransferase (30) were measured in liver homogenates and isolated fractions, respectively.

Peroxisomal Activities—[1-^{14}C]Palmitate oxidation (25) and CN^--insensitive palmitoyl-CoA-dependent NAD^+ reduction (33) that is described as the peroxisomal fatty acid-oxidizing system, were directly measured on whole liver homogenates.

Cytosolic Activities—Phosphofructokinase (29) and γ-butyrobetaine hydroxylase (34) activities were measured in the clear phase (cytosol) obtained after the sedimentation of microsomes (21).

Adipose Tissue Enzyme Activities

Hormone-sensitive lipase (HSL) activities were measured with adipocytes freshly isolated from periepididymal adipose tissues (PAT) according to the Rodbell procedure (35). Each assay used 50,000 adipocytes in 1 ml of Krebs-glucose-Hepes medium containing 4% bovine serum albumin, isoproterenol at concentrations ranging from 10^{-8} to 10^{-5} M and, for adipocytes from fed rats, 1 mIU of insulin. After 45 min of incubation at 37 °C, the amount of glycerol released in the medium was measured using the TG-GPO-Trinder kit from Sigma. Lipoprotein lipase (LPL) activity was determined on PAT extracts as described by Iverius and Ostlund-Lindquist (36) using 0.1–2 mM triolein emulsified with tri(9,10-[^{3}H]oleoyl)glycerol (8 KBq/assay) (PerkinElmer) as a substrate. LPL activities were calculated from the radioactivity of [^{3}H]oleate released through the activity of all lipases measured in 0.1 M NaCl medium corrected by non-LPL activities measured in 1 M NaCl medium (37, 38).

Liver, Blood, and Adipose Tissue Analyses

Liver carnitine content was estimated after alkaline hydrolysis of esterified forms, according to the radiochemical proce-

TABLE 1

Body, blood, liver, and adipose tissue parameters in fed and fasted mildronate-treated rats

Results are means ± S.E. ($n = 6$). Blood was left to clot for 30 min at room temperature and serum was obtained by centrifugation at 6500 × *g* for 10 min. Liver parameters are expressed per g wet tissue.

	Fed state (experiment 1)		Fasted state (experiment 2)	
	Control	Treated	Control	Treated
Body weight (g)	310 ± 8[a]	298 ± 7[a]	278 ± 4[b]	267 ± 8[b]
Serum				
Total carnitine (μmol liter^{-1})	65.5 ± 15[a]	6.2 ± 0.9[b]	57.9 ± 6.5[a]	4.0 ± 0.3[b]
Triglycerides (g liter^{-1})	0.95 ± 0.09[a]	1.15 ± 0.13[a]	0.35 ± 0.07[b]	4.6 ± 1.5[c]
Phospholipids (g liter^{-1})	1.58 ± 0.15	1.42 ± 0.12	1.32 ± 0.12	1.39 ± 0.18
Cholesterol (g liter^{-1})	0.84 ± 0.12	0.64 ± 0.08	0.81 ± 0.09	0.66 ± 0.10
Free fatty acids (mg liter^{-1})	0.05 ± 0.01[a]	0.04 ± 0.01[a]	0.13 ± 0.02[b]	0.22 ± 0.03[c]
Glucose (g liter^{-1})	0.95 ± 0.06[a]	1.05 ± 0.08[a]	0.80 ± 0.07[b]	0.55 ± 0.05[c]
Ketone bodies (mg liter^{-1})	5 ± 2[a]	7 ± 4[a]	87.5 ± 6.2[b]	93.0 ± 5.3[b]
Epinephrine (pg liter^{-1})	200 ± 28	222 ± 30	207 ± 12	183 ± 24
Liver				
Total mass (% of body wt)	4.02 ± 0.15[a]	4.46 ± 0.20[a]	3.05 ± 0.06[b]	3.85 ± 0.05[a]
Total carnitine (nmol g^{-1})	420 ± 45[a]	32 ± 5[b]	534 ± 38[a]	40 ± 4[b]
Triglycerides (mg g^{-1})	4.0 ± 0.4[a]	15.0 ± 1.3[b]	14.2 ± 2.1[b]	130 ± 15[c]
Phospholipids (mg g^{-1})	19.9 ± 0.8[a]	15.5 ± 0.6[b]	25.6 ± 1.0[c]	16.5 ± 1.0[b]
Glycogen (mg g^{-1})	38 ± 6[a]	41 ± 4[a]	7.9 ± 0.9[b]	1.8 ± 0.2[c]
Free fatty acids (mg g^{-1})	0.67 ± 0.02[a]	0.62 ± 0.04[a]	0.81 ± 0.05[b]	1.38 ± 0.11[c]
Total acyl-CoA (nmol g^{-1})	30.6 ± 3.8[a]	27.3 ± 2.4[a]	67.5 ± 3.0[b]	64.8 ± 2.6[b]
Malonyl-CoA (nmol g^{-1})	61.2 ± 10.1[a]	50.5 ± 8.2[a]	34.3 ± 8.3[b]	23.0 ± 6.2[b]
Periepididymal adipose tissue				
Total mass (% of body wt)	1.31 ± 0.03[a]	1.34 ± 0.06[a]	1.30 ± 0.04[a]	1.15 ± 0.04[b]
Triglycerides (mg 100 g body wt^{-1})	1164 ± 25[a]	1137 ± 58[a]	1095 ± 73[a]	914 ± 42[b]

[a,b,c] Values in the same row with different superscript letters are statistically different at $p < 0.05$.

dure of McGarry and Foster (39) using [^{14}C]acetyl-CoA (PerkinElmer). Liver malonyl-CoA and acyl-CoA contents were measured by high pressure liquid chromatography (21) and fluorometric methods (40), respectively. Glycogen contents were determined as described in Ref. 41. For the refeeding experiment, blood samples were collected from the iliolumbar vein (draining blood from dorsal muscles and from a large part of adipose tissue masses), from the upper part of the abdominal vena cava (venous liver blood), from the portal vein, and from the abdominal aorta. Concentrations of glucose, TG, and FFA in serum were measured with glucose-Trinder and TG-GPO-Trinder kits from Sigma, and NEFA-kit from Wako (Neuss, Germany), respectively. Catecholamines were determined by the procedure described in Ref. 42.

Gene Expression

Total mRNA was extracted from tissues by the Tri-Reagent method adapted from the procedure of Chomczynski and Sacchi (43). Tri-Reagent was provided by Euromedex (Souffelweyersheim, France). One-step cDNA synthesis and conventional PCR were performed as described in Ref. 44. Primer pairs were designed using "Primers!" software and synthesized by MWG-Biotech AG (Ebersberg, Germany). The sequence of the forward and reverse primers used were: 5′-GGATCTACAATTCCCCTCTGC-3′ and 5′-GCAAAATAGGTCTGCCGACA-3′ for *CptIα*, 5′-AGGTATGGCCACTTTGGGA-3′ and 5′-AGCTTCAGGGTTTGTCGGA-3′ for *CptIβ*, 5′-GGTAGGAACATTCGTGCAGA-3′ and 5′-AGACAAAGTGGGATAGTCATGG-3′ for hydroxyacyl-CoA dehydrogenase (*HAD*), 5′-GGTGGTATGGTGTCGTACTTGA-3′ and 5′-GAATCTTGGGGAGTTTATCTGC-3′ for acyl-CoA-oxidase (*Aco*), 5′-CTCATGTTTTGTTTGGGACTTC- 3′ and 5′-ATCCTCGGTGCCTTGTGT-3′ for *mtGpat*, 5′-TGCTGCTACATGTGGTTAACCT-3′ and 5′-GCTGGGTGAAAAAGAGCATC-3′ for *Dgat1*, 5′-AGCGGCCTGTGAGAGTTTAT-3′ and 5′-CGGGTGATGATGTCTGATGT-3′ for *Ctp-pct*, 5′-CTCCCATTCTTCCAAAGCCT-3′ and 5′-CCTTGGCACCAGCTACTTGT-3′ for *apoB*, 5′-GAGGAGAACTCTGTTCCGAGAG-3′ and 5′-GATCTAGTGTGATGCCATTTGG-3′ for low density lipoprotein receptor (*LDLR*), 5′-ACTTTGTAGGGCATCTGAGAGC-3′ and 5′-GAATCGCTGTAACAACGTGG-3′ for *Lpl*, 5′-TTACTGGAGCCGTTATTGGTG-3′ and 5′-CTGTCTTTGGGGTCCTGAGTTA-3′ for *Fat*/*Cd36*, 5′-CAGGTCACCAAGTAATCACCA-3′ and 5′-AGGAGTTATGCACCGTGGTT-3′ for *Fabp-pm*, 5′-CAGTACTGCCGTTTCCACAA-3′ and 5′-CATCCCGTCTTTGTTCATCA-3′ for *Pparα*, 5′-CATGCTTGTGAAGGATGCAAG-3′ and 5′-TTCTGAAACCGACAGTACTGACAT-3′ for *Pparγ2*, 5′-CGTTGAATACCTGGAAGGAAGA-3′ and 5′-TCTTCTCTCTCACACCTCGGA-3′ for *Hsl*, 5′-AATCGTGCGTGACATCAAAG-3′ and 5′-GAAAAGAGCCTCAGGGCAT-3′ for *β-actin*. For each gene studied, *β-actin* was used for normalization.

Statistics

Results are expressed as mean ± S.E. When appropriate, data were subjected to one-way analysis of variance followed by Student's *t* test analysis. When variances and numbers of mice were unequal, means were tested by a Kruskal-Wallis nonparametric test. Differences were considered statistically significant at $p < 0.05$, except for semi-quantitative PCR-related results that were considered significant at $p < 0.001$.

RESULTS

Blood and Liver Parameters—Table 1 shows that in both fed and fasted mildronate-treated rats, total carnitine content of blood and liver was dramatically lower than in untreated rats. The other parameters measured in fed animals were not altered by the treatment, except for a slight increase in liver TG, relative

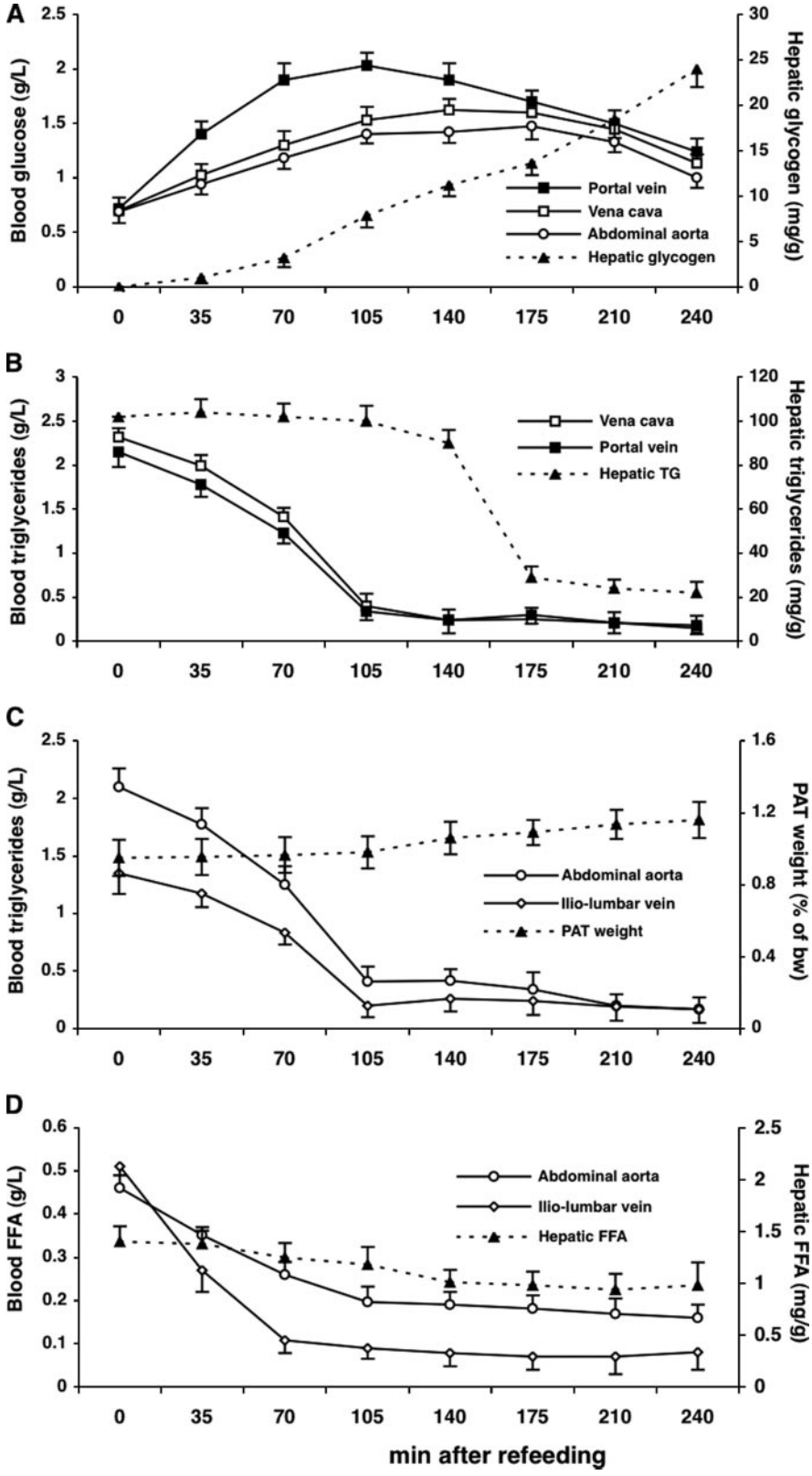

FIGURE 1. **Effect of refeeding on blood and tissue parameters in mildronate-treated rats used after a 18-h fast.** *A*, blood glucose and liver glycogen. *B*, blood and liver TG. *C*, blood TG and weight of periepididymal adipose tissue. *D*, blood and hepatic FFA. Values are means ± S.E. ($n = 4$).

colored and accompanied in cells with microvesicular fat accumulation (data not shown). Concomitantly TG content of blood and liver was 13- and 9-fold increased, respectively. There was significantly more FFA in blood and liver, without any change in hepatic acyl-CoA content. In fasted rats, the treatment resulted in a lowering of blood glucose and liver glycogen. When mildronate-treated rats used after a 18-h fast were refed over ~4 h, the earliest changes were the increase in blood glucose that was more elevated in portal vein than in the upper part of the vena cava, and the reappearance of liver glycogen whose content was linear with the refeeding times used (Fig. 1*A*). Concomitantly, there was a nearly immediate drop of blood TG (Fig. 1, *B* and *C*) and FFA (Fig. 1*D*) that was significantly more marked in venous blood of the ilio-lumbar region draining large masses of adipose tissue than in arterial blood. However, the high TG content of the steatotic liver upon fasting was maintained for ~2 h after refeeding, then rapidly regressed with a progressive reversal toward normal values (Fig. 1*B*). There was a slow increase in PAT weight (Fig. 1*C*), with values significantly different at $p < 0.05$ only between 0 and 240 min of refeeding. During the acute decrease in liver TG, it could be constantly noted in all rats, taken individually, slightly greater TG concentrations in venous blood drained from liver than in the portal vein (data not shown). FFA contents of liver, which were significantly decreased in fed rats (Table 1), tended to decrease over the 4-h refeeding period (Fig. 1*D*).

Liver FA Perfusion—After perfusion of livers isolated from fed rats, palmitate oxidation rate, as estimated through the production of labeled CO_2 and acid-soluble products was comparable in control and treated rats, but was much lower than when rats were used upon fasting (Fig. 2, *A versus B*). However, when livers were isolated from fasted rats, palmitate oxidation in the treated group was ~50% of that in the control one (Fig. 2*B*). Esterification of palmitate

to controls. By contrast in rats sacrificed in the fasted state, although the relative liver weight was a little greater after treatment, the liver surface and transverse section appeared cream-

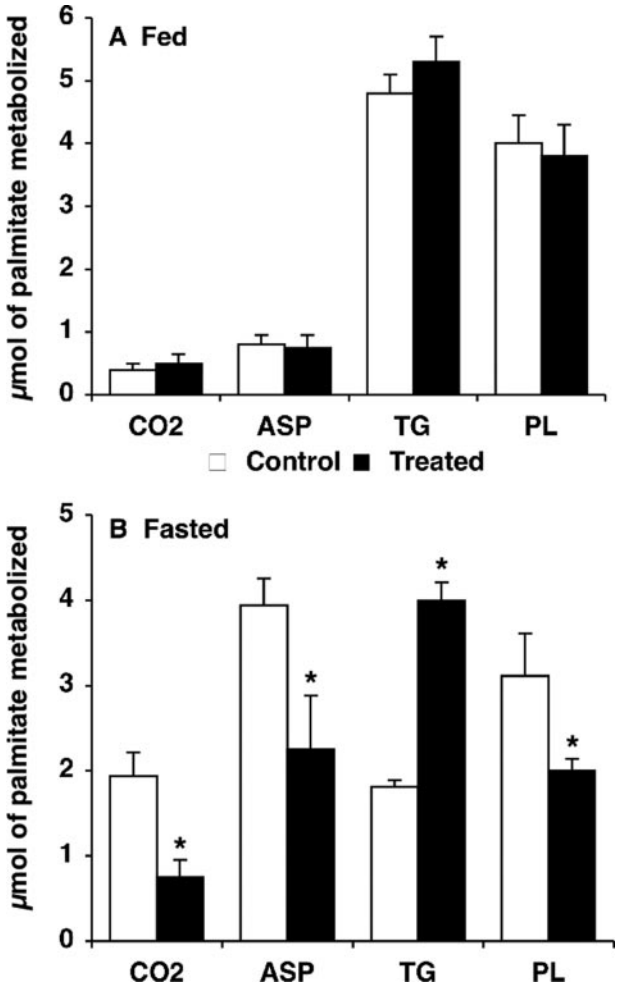

FIGURE 2. **Palmitate oxidation and esterification in the perfused liver of control and mildronate-treated rats used in the fed (*A*) or fasted (*B*) state.** Values are means ($n = 4$) and are expressed as μmol of palmitate oxidized to CO_2 and acid-soluble products (*ASP*) or esterified to TG and phospholipids (*PL*) over 90 min of perfusion in rats untreated (*control*) or daily receiving 0.8 g of mildronate per kg of body weight for 10 days. The data are given per 100 g body weight. *T-bars* indicate the S.E. An *asterisk* indicates a statistically significant difference between treated and control rats at $p < 0.05$.

into TG or phospholipids was comparable in fed rats of either group (Fig. 2*A*). By contrast, in fasted rats, mildronate doubled the incorporation of labeled palmitate into TG and halved that into phospholipids (Fig. 2*B*).

Liver FA Oxidation-related Enzymes—Table 2 indicates that, in rats used in either the fed or fasted state, mildronate treatment did not alter activities related to glycolysis (as phosphofructokinase in cytosol), mitochondria (as monoamine oxidase for outer membranes, cytochrome oxidase for inner membranes and citrate synthase for matrix), or microsomes (as aryl-ester hydrolase). The comparable activities of the above mitochondrial marker enzymes of livers in either nutritional state showed that treatment did not alter the protein content of mitochondria expressed per g or per whole liver (Table 2). However, under the same experimental conditions, mildronate strongly reduced the activity of cytosolic γ-butyrobetaine hydroxylase, the rate-limiting step of carnitine synthesis, whereas it markedly increased the activity of part (peroxisomal fatty acid-oxidizing system) or total peroxisomal carnitine-independent FA oxidation. Furthermore, among the mitochondrial enzymes or enzymatic sequences assayed in the presence of exogenous carnitine for FA oxidation (Table 3), only those acting on long or medium chain FA exhibited significantly greater activities after mildronate treatment, irrespective of the fed or fasted state. This was the case for [1-^{14}C]palmitate oxidation (measured in the presence of carnitine), for CPT I activity, and for respiration on palmitoylcarnitine or octanoate, but not for shorter substrates of the respiratory chain (glutamate, malate, and succinate) nor for hydroxybutyryl-CoA used for the measurement of hydroxyacyl-CoA dehydrogenase activity. Liver malonyl-CoA contents that were nearly 2-fold greater in fed than in the fasted state were not altered by mildronate treatment in each of these nutritional states (Table 1). In parallel, the sensitivity of CPT I to malonyl-CoA inhibition was, as expected in normal rats, much greater in the fed state than on fasting, but was unaffected by mildronate treatment under each nutritional condition (Table 3). It should be noted that the *in vitro* mito-

TABLE 2

Effects of mildronate treatment on specific enzyme activities of hepatocyte compartments

Results are means ± S.E. ($n = 6$). PFK, phosphofructokinase; γ-BBH, γ-butyrobetaine hydroxylase; MAO, monoamine oxidase; Cytox, cytochrome *c* oxidase; GPAT, glycerol-3-phosphate acyltransferase with mtGPAT for mitochondria and GPAT for microsomes; PFAOS, peroxisomal fatty acid oxidizing system. Values were given per g wet liver or mg of protein of cell fractions.

	Fed state (experiment 1)		Fasted state (experiment 2)	
	Control	Treated	Control	Treated
Cytosol				
PFK (IU g tissue^{-1})	7.66 ± 1.25	8.70 ± 1.07	9.29 ± 1.62	9.64 ± 0.92
γ-BBH (mIU g tissue^{-1})	20.5 ± 2.1^a	2.4 ± 0.5^b	23.0 ± 1.8^a	3.4 ± 0.8^b
Mitochondria				
MAO (mIU g tissue^{-1})	772 ± 35	895 ± 43	826 ± 53	947 ± 43
Cytox (IU g tissue^{-1})	109 ± 15	118 ± 12	119 ± 10	117 ± 13
Citrate synthase (IU g tissue^{-1})	11.4 ± 1.5	9.8 ± 1.4	13.3 ± 1.4	11.4 ± 1.1
mtGPAT (mIU mg of mito.protein^{-1})	1.32 ± 0.17	1.25 ± 0.12	1.55 ± 0.10	1.46 ± 0.15
Mitochondrial protein				
(mg g tissue^{-1})	86 ± 10	84 ± 8	103 ± 11	99 ± 7
Microsomes				
Aryl-ester hydrolase (IU g tissue^{-1})	137 ± 13	145 ± 17	161 ± 19	175 ± 36
GPAT (mIU mg micro.protein^{-1})	1.42 ± 0.12^a	2.43 ± 0.20^b	1.65 ± 0.12^a	2.65 ± 0.14^b
Peroxisomes				
PFAOS (mIU g tissue^{-1})	0.95 ± 0.07^a	8.1 ± 0.8^b	1.18 ± 0.03^c	10.5 ± 1.4^d
Carnitine-independent palmitate oxidation (mIU g tissue^{-1})	140 ± 10^a	172 ± 12^b	161 ± 7^b	204 ± 11^c

^{a,b,c,d} Values in a same row with different superscript letters are significant different at $p < 0.05$.

TABLE 3

Effects of mildronate treatment on sequential and individual enzyme activities related to fatty acid oxidation in liver mitochondria

Results are means ± S.E. ($n = 6$). Measurements were made on liver homogenates (per g of liver) or mitochondrial fractions (per mg of mitochondrial protein). The measurement of activities was performed for oxidation of [1-^{14}C]palmitate from labeled CO_2 and acid-soluble products, for acyl-CoA synthetase with palmitate from the fluorescent and acid-precipitable palmitoyletheno-CoA, for carnitine palmitoyltransferase I from butanol-extractable [^{3}H]acylcarnitine, with IC_{50} as the concentration of malonyl-CoA halving CPT I activity, for respiration on palmitoylcarnitine, octanoate, glutamate/malate, or succinate from oxygen consumption with mitochondria respiring in the presence of ADP (state 3), and for hydroxyacyl-CoA dehydrogenase from NAD^+ reduction using hydroxybutyryl-CoA as a substrate.

	Fed state (experiment 1)		Fasted state (experiment 2)	
	Control	Treated	Control	Treated
Oxidation of [1-^{14}C]palmitate (with 1 mM carnitine)				
Nanomole/min g liver^{-1}	61 ± 7^a	102 ± 12^b	74 ± 6^a	133 ± 10^b
Nanomole/min mg mitochondrial protein^{-1}	6.2 ± 0.5^a	10.8 ± 0.7^b	6.1 ± 0.4^a	11.4 ± 0.8^b
Acyl-CoA synthetase activity (at 50 μM palmitate)				
Nanomole/min mg mitochondrial protein^{-1}	18 ± 2	22 ± 4	17 ± 2	20 ± 3
Carnitine palmitoyltransferase I (at 40 μM palmitoyl-CoA)				
Nanomole/min mg mitochondrial protein^{-1}	2.92 ± 0.20^a	5.4 ± 0.3^b	2.80 ± 0.15^a	5.0 ± 0.2^b
IC_{50} malonyl-CoA (μM)	2.82 ± 0.07^a	2.75 ± 0.04^b	28.1 ± 2.1^b	30.0 ± 2.9^b
Respiration on palmitoylcarnitine (at 16 μM) in state 3				
Nanoatom O/min mg mitochondrial protein^{-1}	33.2 ± 2.5^a	59.5 ± 6.2^b	31.2 ± 1.8^a	63.1 ± 7.8^b
Respiration on octanoate (at 200 μM) in state 3				
Nanoatom O/min mg mitochondrial protein^{-1}	22.0 ± 1.3^a	39.7 ± 2.9^b	19.2 ± 1.4^a	40.3 ± 3.1^b
Hydroxyacyl-CoA dehydrogenase				
Micromole/min g liver^{-1}	10.2 ± 2.0	11.2 ± 2.3	12.6 ± 3.6	10.9 ± 3.1
Micromole/min mg mitochondrial protein^{-1}	6.3 ± 0.95	6.18 ± 1.05	5.59 ± 1.22	6.97 ± 1.15
Respiration on glutamate (5 mM)/malate (5 mM) in state 3				
Nanoatom O/min mg mitochondrial protein^{-1}	225 ± 10	228 ± 8	236 ± 9	233 ± 9
Respiration on succinate (5 mM) in state 3				
Nanoatom O/min mg mitochondrial protein^{-1}	287 ± 13	298 ± 15	289 ± 15	291 ± 18

^{a,b} Values in the same row with different superscript letters are statistically different at $p < 0.05$.

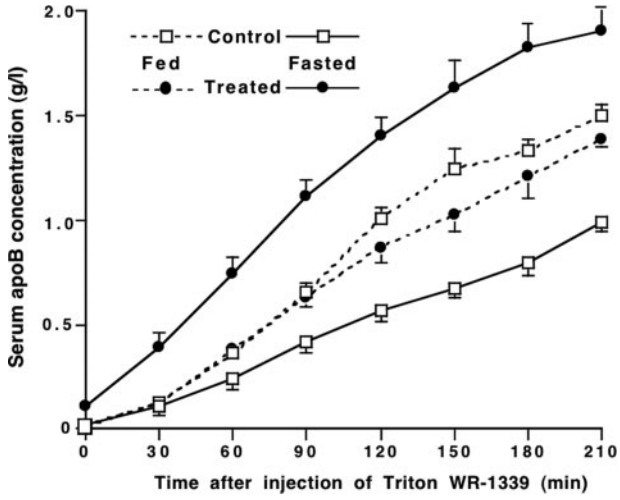

FIGURE 3. **Effect of mildronate treatment on liver VLDL-apoB secretion rate in rats used in the fed or fasted state.** Serum apoB concentration was determined after intravenous injection of Triton WR-1339. Data are means ($n = 5$) and *T-bars* indicate the S.E. Only the values between treated and control rats used in the fasted state are significantly different at $p < 0.01$.

chondrial palmitate oxidation using liver of normal fasted rats (measured as in Table 3) was unaltered when the incubation medium contained a wide range of mildronate or γ-butyrobetaine concentrations (data not shown).

Liver FA Esterification and Lipoprotein Secretion—The marked increase in serum TG concentrations only in mildronate-treated rats used in the fasted state (Table 1) was associated with greater hepatic microsomal GPAT activities (Table 2) and a 2-fold increase in VLDL-apoB secretion (Fig. 3). In fed rats, lipoprotein secretion was comparable in treated and control rats with values intermediate between those obtained in the fasted state (Fig. 3).

Expression of Step Involved in Oxidation, Esterification, and Transport of FA in Hepatocytes—In the liver of rats used in the fed state, mildronate did not alter markedly mRNA levels of α- and β-isoforms of CPT I (Fig. 4*A*). When treated rats were used upon fasting, mRNA levels of enzymes involved in long chain FA oxidation in mitochondria (CPT I isoforms and hydroxyacyl-CoA dehydrogenase) and peroxisomes (acyl-CoA oxidase) were all greater than in controls (Fig. 4*B*). After treatment, mRNA levels of mitochondrial GPAT were unaltered in fed and fasted rats by comparison with their respective controls. By contrast, mRNA contents of microsomal DGAT1, a key enzyme of TG synthesis, were doubled by mildronate upon fasting (Fig. 4*B*). However, mildronate did not significantly alter mRNA levels of microsomal CTP:phosphocholine cytidylyltransferase, catalyzing a key step of phospholipid synthesis, in both fed and fasted rats. mRNA levels of proteins involved in the transfer of lipids into hepatocytes were increased markedly for low density lipoprotein receptor and more slightly for FAT/CD36 and FABPpm after mildronate treatment only in fasted rats (Fig. 4*B*). Furthermore, mildronate changed neither mRNA expression of PPARα nor that of PPARγ2.

Adipocytes, Lipid Flux-related Activities, and mRNA Expression Levels—PAT weight and TG content were significantly lower in treated rats than in controls, but only when rats were used in the fasted state (Table 1). LPL activities were very low in PAT extracts from treated and control rats upon fasting, but were in the fed state about 60% greater in mildronate-treated rats than in controls (Fig. 5). Isoproterenol-stimulated lipase (HSL) activities determined in adipocytes isolated from fed rats were very low and comparable in treated and control groups, but when adipocytes were obtained from fasted rats, HSL activities were ~2-fold greater after mildronate treatment (Fig. 6). Mildronate or γ-butyrobetaine added to incubation media at

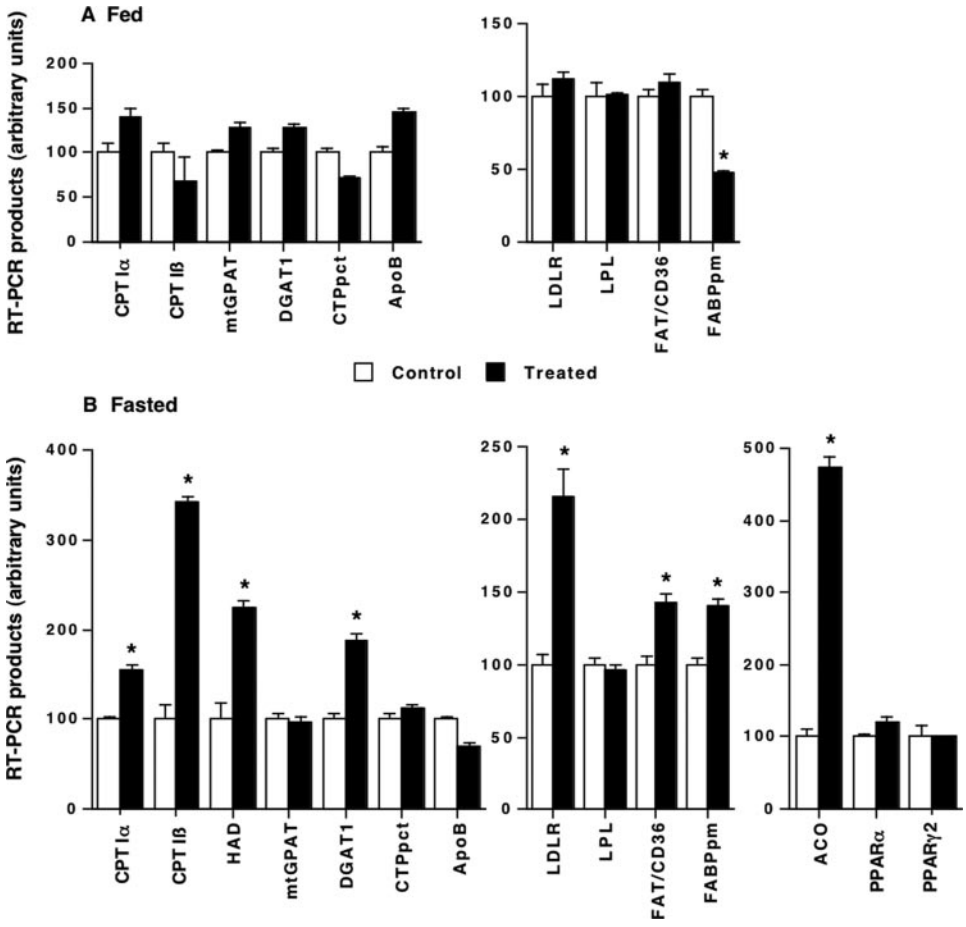

FIGURE 4. **Effect of mildronate treatment on the mRNA levels of liver enzymes from rats used in the fed (*A*) or fasted (*B*) state, regarding FA oxidation with α and β isoforms of CPT I, long chain hydroxyacyl-CoA dehydrogenase (*HAD*) and acyl-CoA oxidase (*ACO*), esterification with mtGPAT, DGAT1 and CTP: phosphocholine cytidylyltransferase (*CTPpct*), VLDL secretion (*apoB*), lipid transfer into hepatocytes (low density lipoprotein receptor or *LDLR*, *LPL*, *FAT/CD36*, and *FABPpm*), and nuclear receptors (*PPARα* and γ2).** Results are means (*T-bars* indicate the S.E.; $n = 4$) and were obtained through the reverse transcriptase-PCR method using total mRNA. Values are standardized by calibration with β-actin and are expressed as percentages of those in controls. An *asterisk* indicates a statistically significant difference between treated and control rats at $p < 0.001$.

concentrations ranging from 0.1 to 10 mM did not affect HSL activities of adipocytes isolated from fed or fasted control rats (data not shown). In PAT from fed rats, mRNA levels of LPL and FAT/CD36 were significantly lower in treated rats than in controls (Fig. 7*A*), but when treated rats were used upon fasting, mRNAs encoding FAT/CD36, DGAT1, and HSL were all markedly greater than in controls (Fig. 7*B*).

DISCUSSION

Perfusion of liver isolated from fasted normal rats gives rise to a maximum production of KB (20), but when rats are fed, the synthesis of KB is markedly reduced and associated with an increase in TG formation (20). These latter effects were reproduced using fasted rats whose livers were perfused with an inhibitor of mitochondrial acylcarnitine transport (20, 45). Nevertheless, in the whole animal, information on the origin(s) of hepatic TG was limited. Consequently, under conditions of low FA oxidation rates, we were interested to know (*a*) whether the rapid shift of available FA toward esterification within the perfused liver as above (20) could be associated with regulation of both the FA oxidation and esterification pathways, and (*b*) if the level of hepatic esterification was even more amplified *in vivo* when the liver was provided with more circulating lipids. To address this aim, we artificially decreased the level of mitochondrial FA oxidation in rats receiving 80 mg of mildronate/100 g body weight day^{-1} over 10 days. Our data show that the marked fat deposition observed in the liver of mildronate-treated rats after a 18-h fast nearly disappeared after ~4 h of refeeding. These results strongly contrasted with the much lower TG accumulation obtained using 4-fold less mildronate over at least a double time period (46). The puzzling transient steatosis apparent only during fasting indicated that the liver esterification capacities were increased and strongly suggested that a large amount of fat accumulating within liver cells originated from other tissues. This prompted us to also investigate some related biochemical events occurring in adipose tissue. The starting point for induction of steatosis might be ascribed to the marked decrease in hepatic γ-butyrobetaine hydroxylase activity that is specifically inhibited by mildronate (16, 17, 47, 48). This enzyme is almost exclusively located in hepatocytes and is required for carnitine synthesis (49). The decrease in hepatic carnitine after mildronate treatment was markedly greater than in previous studies performed under milder conditions (17, 47). A still more severe reduction of carnitine content was observed in other organs, such as heart (44) and skeletal muscle (data not shown), in which mildronate was also shown to inhibit the transport of carnitine into cells (50). Because CPT I catalyzes the key step of the mitochondrial FA oxidation pathway, and necessarily requires carnitine for its activity, a decrease in FA oxidation rates through mildronate treatment was expected in mitochondria of all organs. Yet, the expected decreased FA oxidation in muscle of mildronate-treated rats used in the fasted or fed state was never associated with any lipid infiltration (44). This suggests that the increased FA esterification due to artificial inhibition of FA oxidation in the perfused liver of normal rats used upon fasting (20) was quantitatively of too low extent to trigger steatosis, but was sufficient to initiate, in the whole body of mildronate-treated rats, regulations amplifying the lipid flux to liver and finally producing liver steatosis. The effectiveness of the events induced by mildronate was investigated in

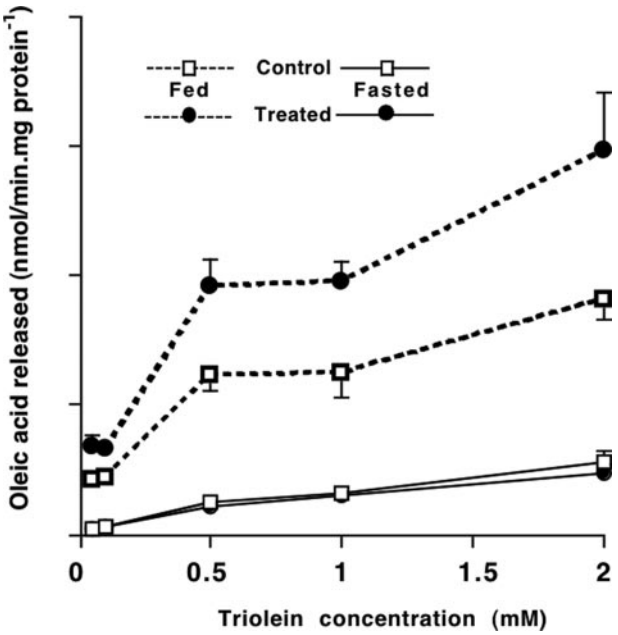

FIGURE 5. **Effect of mildronate treatment on LPL activity in the periepididymal adipose tissue of fed or fasted rats.** Values are means ± S.E. ($n = 5$) and *T-bars* indicate the S.E. All values between treated and control rats used in the fed state are statistically different at $p < 0.01$.

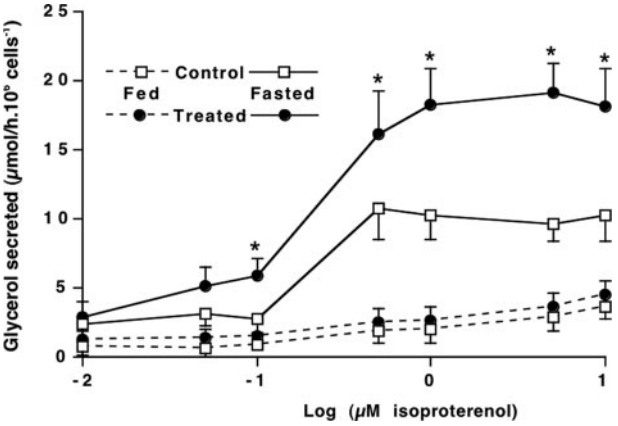

FIGURE 6. **Effect of mildronate treatment on the HSL activity measured in adipocytes of periepididymal adipose tissue of fed and fasted rats.** Values are means ± S.E. ($n = 5$) and *T-bars* indicate the S.E. Lipolytic activities were determined from the amount of glycerol released from endogenous triglycerides in the presence of increasing concentrations of isoproterenol. Only values between treated and control rats used in the fasted state are statistically different (with *asterisk*) at $p < 0.05$.

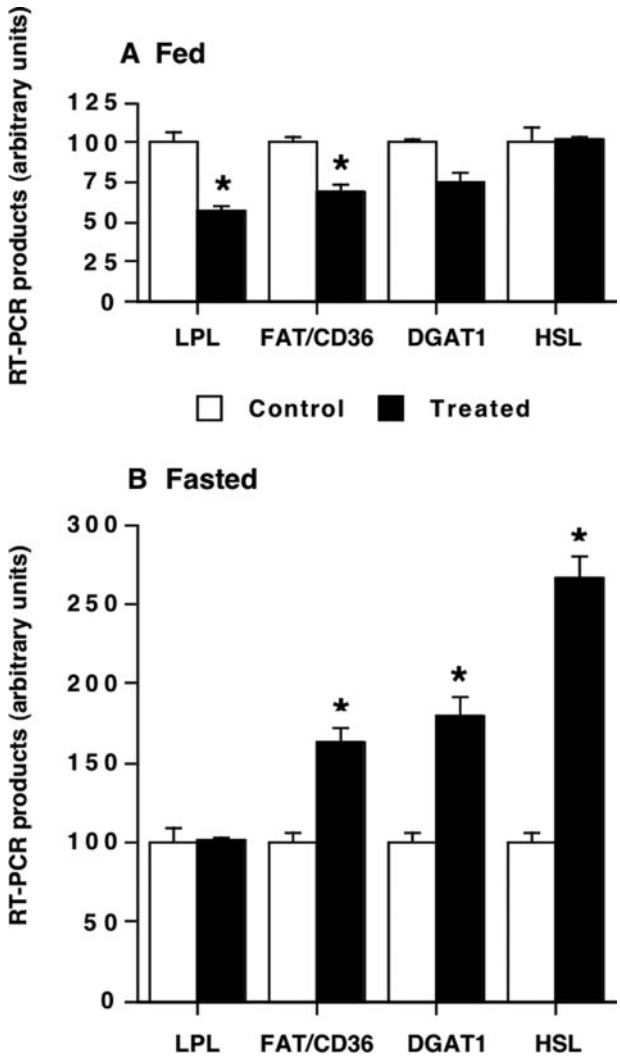

FIGURE 7. **Effect of mildronate treatment on the levels of LPL, FAT/CD36, DGAT1, and HSL mRNAs extracted from the periepididymal adipose tissue of fed (*A*) or fasted (*B*) rats.** Results are means (*T-bars* indicate the S.E.; $n = 4$) and were obtained through the reverse transcriptase-PCR procedure using total mRNA. Values were standardized by calibration with β-actin and expressed as percentages of those in controls. An *asterisk* indicates a statistically significant difference between treated and control rats at $p < 0.001$.

both liver and adipose tissue because these organs are strongly interdependent through their respective lipid secretions.

Regulation of Liver FA Oxidation-related Activities—The amount of palmitate oxidized during the liver perfusion was strongly reduced in control and mildronate-treated rats when they were fed. This was due to the expected physiological inhibition of FA oxidation through malonyl-CoA when insulin secretion was increased via blood glucose from dietary origin (20). Indeed, liver glucose uptake and glycogen synthesis are also characteristic effects of insulin. When rats were used upon fasting, palmitate oxidation of treated rat liver was reduced only by ~50% despite the severe drop of hepatic carnitine content. After treatment, energy requirements of fasted rats appeared to be met less by FA than by carbohydrates (see the low levels of blood glucose and liver glycogen). KB that are nearly exclusively synthesized within liver cells from β-oxidation products were recovered in comparable concentrations in blood of both control and treated rats (Table 1). This surprising observation is consistent with results of another study using the same drug over longer times (46). Indeed, it has been shown that acetyl-CoA released in low amounts from poorly active β-oxidation reactions more easily enters the KB synthesis pathway than the tricarboxylic acid cycle that is inversely more used under conditions of stimulated β-oxidation (51). This suggests that blood KB are unreliable markers of liver β-oxidation, at least in whole animals. Thus in liver perfusion and cell assay, the actual β-oxidation rates of ^{14}C-labeled FA are much more accurately given by the sum of radioactivity of CO_2 and acid-soluble products (including both KB and tricarboxylic acid cycle-related small molecules). The reasons for the non-negligible rates of FA oxidation in the perfused liver of mildronate-treated rats after a

18-h fast were further investigated from the following data. First, liver mitochondrial protein content was unchanged after treatment. Second, the oxidation of labeled palmitate and the respiration of palmitate or octanoate carried out in the presence of whole liver homogenates or isolated mitochondria were ~2-fold greater after treatment in rats used in the fed or fasted state. The increased oxidation capacities were apparent only *in vitro* because exogenous carnitine was added. Furthermore, these facts were limited to long and medium chain FA, which precisely indicated that the improvement was related to enzymes using carnitine for the transport of FA into mitochondria. These increased activities were associated with increased peroxisomal oxidation of palmitate, at least in the very active first steps of the peroxisomal FA oxidation pathway (Table 2). On the contrary, mildronate did not alter the activity of reactions acting on small molecules such as citrate, malate, glutamate, succinate, or hydroxybutyryl-CoA. The increase in FA-related activities was greater than that in rats given lower daily amounts of mildronate (17, 47), even over a longer time (46), and seemed to disagree with our results of liver perfusion with palmitate (Fig. 2). In mildronate-treated rats, these qualitatively different results originated from carnitine that was artificially used *in vitro* to trigger maximum enzyme activities, but whose actual content in liver cells was too low in treated rats, despite the increased enzyme capacities, for normal rates of FA oxidation. Yet the increased FA oxidation, in terms of capacities shown *in vitro*, demonstrated that the activities and/or amounts of involved enzymes were increased. Indeed, mRNA levels of the two isoforms of CPT I, of long chain hydroxyacyl-CoA dehydrogenase for mitochondria, and of acyl-CoA oxidase for peroxisomes, as measured on fasted rats, were all increased. However, such inductions did not seem to depend on the increase in PPARα or PPARγ mRNA contents. The amount of acyl-CoA, considered as a ligand for PPAR (52), was similar in the liver of both treated and untreated rats, and still lower in fed rats, which should limit the direct participation of these molecules in the above inductions. By contrast, the greater amount of FFA in liver cells of mildronate-treated rats used in the fasted state could represent the starting point of the CPT I induction, which has been shown to be PPARα-independent (53). Conflicting results related to amounts of acyl-CoA and mitochondrial protein per g of liver, and to activities of the respiratory chain using small molecules as substrates, and of CPT I, were reported in rats treated with less mildronate over longer times (46). This could correspond to the effects of belated up- and down-regulation consecutively to primary regulation induced by the initial carnitine depletion (*i.e.* depressed FA oxidation). Furthermore, the liver steatosis we describe here did not resemble at all that triggered through *trans*-10, *cis*-12 conjugated linoleic acid administration, at least partly because it was specifically associated with a strong PPARγ induction (54). Our experimental procedure with a relatively high dose of mildronate administered over only 10 days was sufficient to induce only some activities related to long and medium chain FA β-oxidation.

Regulation of Liver FA Esterification-related Activities—Mildronate treatment triggered a dramatic increase in liver and blood TG in rats used in the fasted state, whereas these parameters were reversed upon refeeding. After a 18-h fast, several possibilities (changes in rates of FA oxidation and esterification, *de novo* FA synthesis, lipoprotein secretion, and lipid uptake) could account for the liver TG enrichment. 1) In the perfused liver isolated from untreated rats, FA that are usually β-oxidized under normal conditions were partially preserved with the immediate inhibition of FA oxidation through carnitine depletion and were simply diverted to TG synthesis (20), the short time of the experiment excluding any change in the amounts of esterification enzymes. 2) In the perfused liver of mildronate-treated rats used upon fasting, the greater incorporation of palmitate into an intracellular larger bulk of TG, relative to controls, suggested that the increased TG production was also the consequence of more abundant esterification enzymes. Indeed, microsomal GPAT activity (Table 2), as a first step toward TG synthesis (55), and mRNA levels of DGAT1 (Fig. 4*B*), acting at the end of TG synthesis pathway, were both increased after treatment. 3) Stimulation of the *de novo* FA synthesis pathway might increase the abundance of liver TG after treatment, but this adaptation is very unlikely in fasted animals (*i.e.* in the absence of insulin secretion). Indeed, malonyl-CoA as the precursor of FA was found in very low and comparable amounts within liver cells of both control and treated rats. Under lipogenic conditions, CPT I was also demonstrated to be much more sensitive to malonyl-CoA inhibition (56), which was the case for the control and treated groups used in the fed state, but not at all when used in the fasted state (see IC_{50} values in Table 3). In addition, it has been shown that mildronate did not alter insulin secretion as young rats bred from mildronate-treated mothers exhibit both severe liver steatosis and normal levels of blood and pancreatic insulin (57). 4) If the mechanisms relative to lipoprotein formation and secretion had been impaired, the retention of TG would have easily accounted for the liver steatosis upon fasting. But the hepatic lipoprotein secretion rates in fasted treated rats were always greater than those in the other groups, which indicated that the hepatic secretion function was even stimulated during the steatotic state, as it has already been observed under other similar conditions (4, 5, 58, 59). Nearly comparable apoB mRNA contents in treated rats *versus* controls upon fasting could appear surprising. Indeed several authors also reported increased levels of circulating apoB associated with unchanged or lower contents of apoB mRNA (60, 61) and suggested that hepatic apoB secretion is regulated by post-transcriptional mechanisms. 5) Fat accumulating within liver cells could arise from greater uptake of circulating lipoproteins such as LDL, because the mRNA level of LDL receptors was 100% greater in the liver of treated rats on fasting. The slight rise in mRNA levels of FAT/CD36 and FABPpm would constitute complementary inductions allowing the increased transport of FA released from lipoproteins into hepatocytes (62).

At the beginning of the refeeding period, the major liver-related events were (*a*) the glycemia relatively elevated in portal vein and recovered lowered in the effluent blood of liver, which represents, as already noted, a specific effect of insulin on liver carbohydrate metabolism, (*b*) the maintenance of the elevated liver TG content for ~2 h after refeeding, which could be attributed to decreased hepatic lipoprotein secretion rates as meas-

ured after treatment in fed rats and to persisting lipoprotein uptake by hepatocytes as suggested by the much greater amounts of mRNA of low density lipoprotein receptor found in the previous fast period. However, the rapid decrease in blood TG, despite the maintenance of steatosis during ~2 h after refeeding, and the absence of increase in blood TG during the steatosis regression after ~2 h were surprising and addressed the questions of the primary origin of excess hepatic TG in treated rats upon fasting and of the fate of hepatic TG during the liver steatosis regression on refeeding.

Adipose Tissue Function-related Activities and Regulations—The liver fat content growing only during the fast period in mildronate-treated rats suggested that the liver was provided more abundantly with FA from extrahepatic organs, in particular from adipose tissue, that alone is capable of releasing relatively large amounts of FFA. During the postprandial period, the role of PAT, as of any fat storage tissue, is to remove excess fat from blood and, during the fasting period, to release FFA into blood. LPL activity of PAT, which was already clearly greater in fed control rats than in the fasted ones, was about twice as high in fed mildronate-treated rats as in fed controls (Fig. 4). Consequently, because of the elevated blood TG concentration after treatment in fasted rats and the increased insulin secretion on refeeding, a greater flux of FA from lipoproteins would enter fat cells and produce TG, causing the replenishment of PAT. This was obvious not long after the refeeding time by the rapid drop of blood TG and the negative difference of both TG and FFA concentrations between venous and arterial sides of adipose tissues. These facts emphasized the efficiency of adipose tissue to clear up blood from fat under insulin status. Yet, the increased LPL activity observed in PAT of fed treated rats was associated with decreased LPL mRNA levels. This inconsistent result was, however, explained in studies showing that increased LPL activity and secretion were attributable to positive effects of insulin on mRNA stability and changes at the post-translational level, but not to an increase in *Lpl* gene expression (63–65). Conversely, when rats were used in the fasted state, adipocytes isolated from the treated group exhibited a HSL activity about twice that in the control group and much greater than in fed treated rats (Fig. 6). However, *in vivo*, although blood epinephrine was found in about comparable concentrations irrespective of treatment and nutritional state (Table 1), a more elevated HSL activity could be expected through adrenergic action in treated rats upon fasting, *i.e.* in the absence of insulin. Furthermore, with the greater HSL activity acting on adipose masses previously replenished in treated rats during the postprandial period, the amount of FFA released during the fasting period should be maximum and about equivalent to FA under free and esterified forms cleared up from blood, as seen above, on refeeding. The isoproterenol-stimulated HSL activity met the markedly increased mRNA level of the enzyme, which strongly suggested that the amount of HSL enzyme in adipocytes was likely increased during the treatment. As a consequence, released FFA would be actively removed, as well as lipoproteins, from blood by liver cells whose capacity to esterify FA into TG was increased in treated rats with the consecutive development of liver steatosis. Surprisingly, mRNA levels of both FAT/CD36 and DGAT1 in adipose tissue were increased in treated rats upon fasting, but tended to decrease when they were fed (Fig. 7). One can hypothesize that FFA as possible modulators of *Fat/Cd36* and *Dgat1* expression (66, 67) were all the more effective as they were released more abundantly through the HSL activity usually stimulated on fasting. Inversely on refeeding, DGAT1, whose activity had likely been up-regulated in the previous fasting period, would be immediately useful, because FFA released from lipoproteins through LPL activity would be more rapidly sequestered into TG of adipocytes.

Under the experimental conditions described, mildronate did not alter the normal metabolic functions of liver and adipose tissue, and was likely responsible, at least in part via increased intracellular FFA concentrations, for appropriate regulations amplifying the alternate flux of lipids that normally occurs moderately between both organs. The study emphasized the converse capability of liver and adipose tissue to rapidly exchange lipids. The data also indicate that the early stages of liver steatosis do not trigger dramatic biochemical defects, allowing the possible reversibility of the liver fat accumulation.

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