Inhibition of carnitine palmitoyltransferase in the rat small intestine reduces export of triacylglycerol into the lymph

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Abstract **Following digestion of dietary triacylglycerol (TAG), intestinal epithelial cells absorb fatty acids and monoacylglycerols that are resynthesized into TAG by enzymes located on the endoplasmic reticulum (ER). A study in rat liver (Abo-Hashema, K. A., M. H. Cake, G. W. Power, and D. J. Clarke. 1999. Evidence for TAG synthesis in the lumen of microsomes via a lipolysis-esterification pathway involving carnitine acyltransferases. *J. Biol. Chem.* 274: 35577–35582) showed that there is a carnitine-dependent ER lumenal synthesis of TAG. We wanted to test the hypothesis that a similar pathway was present in rat intestine by utilizing etomoxir, a specific inhibitor of carnitine palmitoyltransferase (CPT). Intraduodenal infusion of etomoxir inhibited CPT activity in the ER by 69%. Etomoxir did not affect either the uptake of intraduodenally infused [^{3}H]glyceryltrioleate by the intestinal mucosa or the production of mucosal [^{3}H]TAG, excluding the possibility that etomoxir interfered with TAG absorption or synthesis. Etomoxir did not inhibit protein synthesis, glucose, cholesterol or palmitate absorption or metabolism, or ATP concentrations. Etomoxir substantially (74%) diminished lymph TAG output from intralumenally infused glyceryltrioleate. In conclusion, these data strongly support the hypothesis that an ER CPT system exists and is necessary for processing dietary TAG into chylomicrons. The significant reduction in lymphatic output of chylomicron TAG on etomoxir treatment suggests that the major source of chylomicron TAG is a diacylglyceroltransferase on the lumenal surface of the ER.**—Washington, L., G. A. Cook, and C. M. Mansbach, II. **Inhibition of carnitine palmitoyltransferase in the rat small intestine reduces export of triacylglycerol into the lymph.** ***J. Lipid Res.*** **2003.** 44: **1395–1403.**

Supplementary key words lipid absorption • diacylglycerol acyltransferase • lymph etomoxir

The intestinal epithelial absorptive cell has little control over the rate of entry of the absorbed products of lipid digestion, fatty acids (FAs) and monoacylglycerols (MAGs). These must be disposed of rapidly, otherwise the cells risk destruction of their cellular membranes. The enterocytes are able to store some of the FA (and MAG) bound to liver FA binding protein (L-FABP), which is expressed in the intestine (1). Most of the remainder is rapidly resynthesized into triacylglycerol (TAG) (2). The synthetic steps take place on the membrane of the endoplasmic reticulum (ER), which is the site of the neutral lipid acyl transferases. Exactly on which face of the ER membrane the last enzyme in this pathway, diacylglycerol acyltransferase (DGAT) [which acylates diacylglycerol (DAG) to TAG], exists is controversial.

A pathway for the synthesis of TAG within the lumen of the ER of liver cells, presumably leading to the synthesis of VLDL particles, has been identified that utilizes cytosolic TAG and acyl-CoA to produce TAG within the ER lumen. The ER lumenal TAG was found to consist of two acyl groups from the cytosolic TAG and one acyl group from cytosolic FA-CoA (3). This pathway of intralumenal ER synthesis of TAG was shown to be carnitine dependent and was inhibited by glybenclamide, a potent inhibitor of carnitine acyltransferase (3). These investigators predicted the existence of a lipase outside the ER and a second DGAT within the hepatic ER lumen. A lipase is known to exist in intestinal cytosol (4), and two DGATs have recently been identified in the intestine by Farese's laboratory (5, 6).

Studies in liver by Coleman and Bell (7, 8) showed that DGAT was on the cytosolic face of the ER, as suggested by protease digestion experiments with and without ER membrane permeation. These studies were later challenged by Saggerson and by Zammit, who found that only on liver ER membrane disruption could the full activity of DGAT be expressed (9–11), suggesting that DGAT was in part a latent enzyme; that is, in order for full activity to be

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observed, the ER membrane must be permeabilized. These data indicate that a second DGAT activity is needed for the maximal rate of chylomicron formation and export. This led to the hypothesis that there is a DGAT II that is present on the lumenal side of the ER membrane.

Molecular support for there being two DGATs has come from Farese's laboratory (5, 6), whose studies have shown that there are at least two DGATs that are expressed in the intestine in mice. These studies were not able to define their topology with respect to the ER membrane, however. More recent work from Farese's laboratory (12) has shown that DGAT1 knockout mice were able to synthesize absorbed lipid to TAG in the intestine; however, their ability to mount a chylomicronemia was greatly impaired following a high-lipid diet.

The well-characterized carnitine palmitoyltransferase (CPT) enzyme system of mammalian mitochondria is essential for translocation of FAs into mitochondria for β-oxidation. It consists of two enzymes, CPT-I and CPT-II (13). Following activation of FAs to produce acyl-CoA thioesters, CPT-I, located in the mitochondrial outer membrane, catalyzes the transfer of acyl groups from acyl-CoA to carnitine to produce acylcarnitine. Cytoplasmic acylcarnitine is exchanged for matrix carnitine by a specific mitochondrial antiport protein called carnitine-acylcarnitine translocase. CPT-II, located inside the mitochondrial matrix, then regenerates acyl-CoA for β-oxidation by catalyzing a reaction that is the reverse of the CPT-I reaction. The net effect of the CPT enzyme system, linked to the carnitine-acylcarnitine translocase, is net movement of acyl-groups across the inner mitochondrial membrane. This mitochondrial CPT system exists in all mammalian cells, including small intestine (14). Although two isoforms of CPT-I, known as CPT-Iα and CPT-Iβ, have been cloned (15–17), only CPT-Iα is expressed in the liver and small intestine (14). Mitochondrial CPT-I is inhibited by malonyl-CoA, which is a precursor for FA synthesis (13), and the sensitivity of CPT-Iα to malonyl-CoA inhibition is increased by insulin and decreased by fasting (18–20).

There has been increasing evidence that a similar if not identical CPT system also exists in the ER of some cells for the purpose of transporting acyl groups into the lumen for production of TAG that would be subsequently packaged into lipoproteins for export from the cell. Two different transport schemes involving lumenal DGAT and a carnitine acyltransferase system have been proposed that would allow cytosolic acyl-CoA to be translocated across the ER membrane to be incorporated into lumenal TAG [ref. (10), see Scheme 1; ref (9), see Fig. 3].

The presence of CPT activity in the ER was first reported in 1976 (21), and in 1990, it was reported that both rough and smooth ER contained a CPT activity that was malonyl-CoA inhibitable (22). Finally, in 1995, two proteins with CPT activity were solubilized from liver ER, only one of which was inhibitable by malonyl-CoA (23). These observations indicate that a CPT system exists in ER that is very similar to that in mitochondria. Malonyl-CoA-sensitive CPT activities of hepatic peroxisomes and ER have been shown to alter their sensitivity to malonyl-CoA inhibition during the fasting-feeding cycle (9, 24). These observations suggest a strong physiological connection among these CPT activities. In addition to malonyl-CoA, microsomal and mitochondrial CPTs have been shown to be inhibited by the hypoglycemic sulfonylureas and by etomoxir (24–26).

An additional interest in the question of the presence of two DGATs is our prior findings of the ER as the site at which TAG is split between TAG that is to enter the prechylomicron TAG pool, and TAG that is to enter the TAG storage pool and not be transported from the intestine in chylomicrons (27, 28). These data showed that TAG that is likely to enter the storage pool, as identified by its large complement of endogenous acyl groups and by its lack of transport to the Golgi, remains on the cytosolic face of the ER, and potentially oils out to form cytosolic lipid droplets. By contrast, TAG that is to enter the prechylomicron pool, as identified by its diet-derived acyl groups and its transport to the Golgi, is found in the ER lumen. The transverse location of DGATs in the ER membrane may play a role in the distribution of TAG to either of the two TAG pools.

The goal of the present study was to test the hypothesis that DGAT1, functionally important for chylomicron synthesis, is sited at least in part on the lumenal side of the ER membrane. We were able to test this thesis by using a specific CPT-1α inhibitor, etomoxir (29). If the hypothesis was correct, then the output of TAG into the lymph should be reduced in the presence of the inhibitor. Alternatively, if etomoxir did not curtail TAG delivery into the lymph, it would suggest that both DGATs are on the cytosolic face of the ER. Because of the potential confusion generated by the competing numbering systems for DGAT on the cytosolic face of the ER membrane and DGAT situated on the ER lumenal side of the membrane, in this paper we will use the terms DGAT-C for cytosolic and DGAT-l for lumenal DGAT to make clear the topology of the enzyme in question.

MATERIALS AND METHODS

Materials

Sodium (+)-etomoxir was obtained from Dr. H. P. O. Wolf, Allensbach, Germany. The etomoxir solution was made fresh for each experiment. L-[methyl-^{3}H]carnitine was purchased from Amersham (Piscataway, NJ). All other chemicals were obtained from Sigma (St. Louis, MO).

Animals

Physiological studies. Sprague Dawley rats weighing 200–300 g (Harlan, Indianapolis, IN) were brought to the laboratory in a nonfasting state. The mesenteric lymph duct was cannulated and the rats infused overnight with a 5% glucose, 0.15 M NaCl, and 5 mM KCl solution at 3.0 ml/h through a duodenal cannula (27). The next day, an infusion was started of either 1 mM etomoxir in the glucose-saline solution or the glucose-saline solution alone at 4.5 ml/h. After 2 h of infusion, a 30 mM glyceryl [[^{3}H]trioleate ([^{3}H]TO)] infusion was begun in both groups of rats for the next 6 h (30) [(specific activity 22,669 dpm/μmol), TO (99% TO, Sigma), [^{3}H]TO (New England Nuclear, Boston, MA), 10

mM taurocholate, 0.15 M NaCl, and 10 mM Tris-HCl, pH 7.0, as a sonicated emulsion]. In the rat preloaded with etomoxir, the [^{3}H]TO infusion was supplemented with 1 mM etomoxir. Lymph was collected on ice each hour during the [^{3}H]TO infusion. Chylomicrons were isolated from the lymph (30). TAG was extracted using a differential organic solvent method (31).

In studies to determine the absorption of [^{3}H]TO, lymph duct-intact rats were treated as above, except that after 6 h of [^{3}H]TO infusion, the entire intestine was removed and the lumen flushed with 5 ml saline. The total ^{3}H dpm remaining in the lumen was calculated by extracting 0.1 ml of the total lumenal fluid (32), using 0.1 ml HCl as a wash to be certain that all the FAs were extracted into the organic phase (2). The proximal half intestine from these rats was used to determine the percentage of ^{3}H dpm incorporated into [^{3}H]TAG. The mucosa was scraped from the intestine, homogenized in buffer, and 1 ml of the homogenate extracted (32). FA, MAG, and DAG were separated from TAG by thin-layer chromatography (TLC) (2), and the FA, MAG, DAG, and the TAG bands' radioactivities determined using a Packard TriCarb spectrophotometer (Packard Instrument Co., Downer's Grove, IL, model 1500).

Studies to show that etomoxir did not affect protein, phospholipid, or TAG synthesis were performed in lymph duct-intact rats infused as above through a duodenal cannula. The next day the rats received either etomoxir (1 mM) or saline for 2 h at 4.5 ml/h. The infusate was then changed to include glyceryl([^{14}C]oleoyl)TO [30 mM (23,000 dpm/ml, as a sonicated emulsion prepared as above)] and [^{3}H]leucine (330,000 dpm/ml) for 6 h at 4.5 ml/h. At the end of the infusion period, the rat was given an overdose of pentobarbital, and the mucosa from the proximal half of the intestine was harvested. The mucosa was homogenized and aliquots obtained for Folch extraction and TCA precipitation. Neutral lipids were separated by TLC (2), as were phospholipids (33). Incorporation of [^{3}H]leucine into TCA-precipitable protein was determined by adding 0.5 ml of 5% TCA to 0.5 ml of mucosal homogenate. The TCA precipitate was obtained, and 0.5 ml of NCS tissue solubilizer (Amersham, Arlington Heights, IL) was added. Radioactivity was determined after the addition of scintillant to 100 μl of solubilized precipitate. The percentage of total ^{3}H dpm in the mucosa that was precipitated by TCA was determined by comparing the ^{3}H dpm in the TCA precipitate to that of the whole homogenate.

Studies evaluating the effect of etomoxir on glucose, palmitate, and cholesterol absorption and metabolism were performed in rats infused with etomoxir or saline as above. For cholesterol absorption, 16.8×10^6 [^{3}H]cholesterol dpm (New England Nuclear) was infused with 2.6 mM cholesterol and the TO emulsion (135 μmol TO/h) as above for 6 h. For glucose absorption, 2.9×10^6 [^{3}H]glucose dpm (New England Nuclear) was added to a 1% glucose infusion for the last hour of a 6 h glucose infusion along with a 135 μmol/h TO emulsion. Palmitate absorption and metabolism was studied by injecting 1 ml of air at 5 h 55 min of a TO emulsion (135 μmol/h) infusion, rapidly infusing 1 ml of the emulsion supplemented with [^{14}C]palmitate, and then continuing the [^{14}C]palmitate in the TO emulsion for an additional 5 min (total dpm infused, 6×10^5 ^{14}C dpm, New England Nuclear). For each probe, etomoxir or saline was infused for 2 h prior to the start of the 6 h lipid infusion. At the end of the infusion, the rat was given an overdose of pentobarbital ip, and the intestines and cecum were removed. The intestinal lumen and cecum was flushed with 5 ml of 8 mM taurocholate in 0.15 M NaCl, placed in an iced glass/Teflon homogenizer, and homogenized. 1 ml was extracted (32). Dpms were determined both in the aqueous/methanolic and organic phases. Neutral lipids and cholesterol were separated by TLC and the respective dpm determined.

Analytical methods

TAG was quantitated chemically after separation of the neutral lipids by TLC (33). Cholesterol was separated from cholesterol esters similarly (33).

Assay of CPT activity

The procedure of Bremer (18) as modified previously (34) was used to measure CPT activity in microsomes. Final concentrations in a total volume of 1 ml at 37°C were 80 mM sucrose, 70 mM KCl, 70 mM imidazole (pH 7.0), 1 mM EGTA, 1 μg of antimycin A, and 2 mg of BSA. A 5 min preincubation period was initiated by the addition of myristoyl-CoA. The reaction was started with L-carnitine [0.4 mCi/mmol L-[methyl-^{3}H]carnitine (New England Nuclear)] and stopped after 10 min by adding 4 ml of 1.0 M perchloric acid. The assay was linear with respect to time for 30 min.

Inhibition of microsomal CPT by etomoxiryl-CoA, the inhibitory form of etomoxir, was examined in vitro to examine the effects of etomoxiryl-CoA on microsomal CPT activity in liver and small intestine. For these experiments, tissue preparations were preincubated with and without etomoxir with 3 mM ATP, 50 μM Co-A, 1 mM reduced glutathione, and 5 mM $MgCl_2$ to produce etomoxiryl-CoA. The production of etomoxiryl-CoA was essential for inhibition of hepatic microsomal CPT (**Table 1**) and for ER CPT of the small intestine (**Fig. 1**). Initial experiments were carried out using microsomes sedimenting at 100,000 *g* during a 60 min centrifugation of the supernatant fraction remaining after centrifugation of liver homogenate at 20,000 *g* for 30 min. Catalase and citrate synthase were used as marker enzymes to ensure that peroxisomes and mitochondria, respectively, had been removed from the microsomal preparations. These preparations were >90% free of peroxisomal and mitochondrial CPT. Final assessment of inhibition of microsomal CPT by etomoxiryl-CoA was carried out using purified intestinal ER (35).

Irreversible inhibition of intestinal ER CPT following infusion of the small intestine with a 1 mM solution of etomoxir was assessed by purification of the intestinal ER membranes followed by assay of CPT activity.

The ATP content of the intestine was measured in rats intraduodenally perfused with etomoxir (1 mM) or saline for 2 h, followed by a TO emulsion (135 μmol/h) containing either etomoxir 1 mM or saline for 6 h. The intestines were rapidly re-

TABLE 1. The ability of dietary probes to be absorbed by the intestine of etomoxir treated rats[e]

	Absorption Probe					
	Palmitate		Glucose		Cholesterol	
Etomoxir[a]	−	+	−	+	−	+
% absorption	73	80	93	93	95	98
% in cecum	0	0	0	0	0	0
% in prox. mucosa	15	14	0	0	9	3
% in dist. mucosa	2	2	0	0	8	4
% in aqueous[b]	0	0	0	0	NA[d]	NA[d]
% est. to TAG[c]	60	78	NA[d]	NA[d]	NA[d]	NA[d]
% est. to ester[c]	NA[d]	NA[d]	NA[d]	NA[d]	21	13

TAG, triacylglycerol.

[a] 1 mM etomoxir was (+) or was not (−) included in the 135 μmol/h trioleate infusate.

[b] The percentage of dpm in the aqueous/methanolic phase of a Folch extract.

[c] The percentage of total dpm esterified to either TAG or cholesterol ester in the mucosa.

[d] Not applicable.

[e] The data are the mean of two experiments. For dpm infused and other infusion conditions, see Materials and Methods.

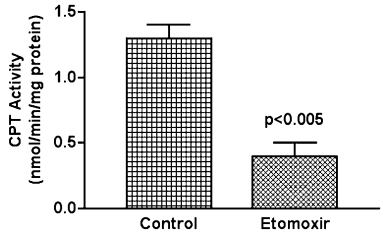

Fig. 1. Inhibition by etomoxir of carnitine palmitoyltransferase (CPT) activity in rat intestinal endoplasmic reticulum (ER). CPT activity was measured (Materials and Methods) in the purified ER fraction of rat small intestines after the intraduodenal infusion of etomoxir (1 mM) or saline for 2 h. Values are means ± SEM for nine rats in each group ($P < 0.005$).

TABLE 2. Effect of etomoxir on [^{14}C]oleate incorporation into triacylglycerol and phospholipids

	TAG[a,b]	PL[a,c]	PC[a,d]	PS[a,d]	PE[a,d]
Control	97 ± 5%	3 ± 0.2%	70 ± 2%	15 ± 1%	15 ± 1%
Etomoxir	94 ± 4%	6 ± 1%	68 ± 2%	16 ± 1%	16 ± 1%

PC, phosphatidylcholine; PE, phosphatidylethanolamine; PL, phospholipid; PS, phosphatidylserine; TAG, triacylglycerol.

[a] The data are the mean ± SEM (n = 4).

[b] The total [^{14}C]TAG dpm were 171,225 ± 11,158 in controls and 191,965 ± 17,505 in etomoxir-infused rats.

[c] The total [^{14}C]PL dpm were 5,850 ± 521 in controls and 7,440 ± 856 in etomoxir-infused rats.

[d] The percentages given for each specific PL are the number of ^{14}C dpm in each specific PL as compared with the ^{14}C dpm in the total PL × 100.

moved and drained of lumenal fluid. The intestine was cut in half and rapidly freeze-clamped between the aluminum discs of Wollenberger clamps (36) that had been precooled in liquid nitrogen. After grinding the intestinal tissue to a fine powder under liquid nitrogen, the tissue powder was extracted with perchloric acid and the extract was neutralized with potassium carbonate (36). The neutralized tissue extract was analyzed for ATP content by the enzymatic method of Lamprecht and Trautschold as modified by Lund, Cornell, and Krebs (37).

Statistical methods

Statistical differences between groups were tested by Student's *t*-test (InStat, GraphPad, San Diego, CA) using the nonpaired Student's *t*-test.

RESULTS

We postulated that by inhibiting microsomal CPT activity, we could block entry of FA-CoA into the ER lumen and thus disrupt TAG synthesis on the lumenal side of the ER membrane. To test this hypothesis, we first used glyburide, a known inhibitor of CPT (26), to test the effect of CPT inhibition on lymph TAG output. Although glyburide did reduce lymph TAG output (data not shown), we noted that lymph flow fell by ~50% during the treatment period. These data suggested that glyburide inhibited more than CPT, such as inhibition of energy metabolism (38). This forced us to turn to etomoxir, a more specific CPT inhibitor.

To test if etomoxir also had nonspecific effects on mucosal lipid and protein metabolism, we first infused etomoxir intraduodenally into rats for a total of 8 h with an intraduodenal infusion of [^{14}C]TO (135 μmol/h) and [^{3}H]leucine during the last 6 h. Because of the long perfusion period, the majority of the mucosal ^{3}H dpm was TCA precipitable, 70.5 ± 5% in saline-infused controls and 68 ± 6% in rats infused with etomoxir ($P > 0.05$). As shown in **Table 2**, there were no differences in the percentage of ^{14}C dpm distributed into mucosal TAG, phospholipids, or specific phospholipids between the group of rats infused with saline versus that infused with etomoxir.

The effect of etomoxir on palmitate, glucose, and cholesterol absorption and metabolism is shown in Table 1. Although palmitate was only infused for 5 min, ~75% of the lipid was absorbed. None was found in the cecum. The majority of the [^{14}C]palmitate had already been transported out of the mucosa, but that which remained was predominantly esterified. No water-soluble metabolic products were found in either the treated or control intestines, consistent with our prior observations (28). Glucose absorption was nearly complete in both groups (Table 1), and no glucose dpm was found in the mucosa at the conclusion of the experiment, suggesting rapid transport from the mucosa. Etomoxir also did not affect cholesterol absorption (Table 1). The vast majority of cholesterol in both groups was found neither in the mucosa or bowel lumen, suggesting that it had been absorbed into the body of the rat. Etomoxir modestly impaired the esterification of the small amount of cholesterol remaining in the mucosa (Table 1). ATP levels were found to be comparable in both groups, suggesting that etomoxir did not impair high-energy phosphate generation (proximal intestine, control vs. etomoxir: 1.71 ± 0.03 vs. 1.76 ± 0.05 μmol/g weight; distal intestine, control vs. etomoxir: 1.84 ± 0.03 vs. 1.82 ± 0.05 μmol/g weight). We conclude that etomoxir did not have generalized effects on nutrient absorption or cellular metabolism in the intestine.

To determine if etomoxiryl-CoA could inhibit microsomal CPT, ER was isolated from rat liver and small intestine. Using liver, inhibition of ER CPT activity was found to depend on formation of the CoA ester of etomoxir (**Table 3**). The observation of CoA dependency, combined

TABLE 3. Inhibition of carnitine palmitoyltransferase activity in liver microsomes by etomoxir

Additions to Assay	% Inhibition
40 μM Etomoxir	9 ± 3
40 μM Etomoxir + CoA	14 ± 5
40 μM Etomoxir + ATP + Mg + CoA	98 ± 1
20 μM Etomoxir + ATP + Mg + CoA	97 ± 2
10 μM Etomoxir + ATP + Mg + CoA	96 ± 2
1 μM Etomoxir + ATP + Mg + CoA	91 ± 3

Isolated liver microsomes were used to test etomoxir as an inhibitor of microsomal carnitine palmitoyltransferase (CPT) activity. All assays were started by addition of [^{3}H]carnitine after a 10 min preincubation of microsomes. Liver CPT activity was 2.2 ± 0.2 nmol/min/mg protein. Results are presented as mean ± SEM (n = 4 or 5).

with the observation of similar levels of strong inhibition using a 1 μM to 40 μM range of etomoxir concentrations, supports the conclusion that etomoxir must first be converted to etomoxiryl-CoA for it to become the potent inhibitor of CPT observed in our studies. Inhibition was, as expected, irreversible from previous reports of inhibition of CPT in mitochondria (29). Sedimentation and resuspension of ER in inhibitor-free medium following incubation with etomoxir, ATP, CoASH, and Mg2+ did not restore CPT activity (data not shown). Infusion of the small intestine for 2 h with etomoxir resulted in 69 ± 2% inhibition of CPT activity in the purified ER fraction of the small intestine when compared with saline-infused controls (Fig. 1).

After we established that etomoxiryl-CoA inhibited CPT activity in the intestinal mucosa, we wished first to determine if the infusion of etomoxir influenced either the rate of absorption of [^{3}H]TO or the ability of the intestine to process the absorbed hydrolytic products of the intraduodenally infused [^{3}H]TO into mucosal [^{3}H]TAG. After 6 h of [^{3}H]TO infusion, 98 ± 2% of the radiolabel was absorbed in the group receiving etomoxir, as compared with 98 ± 0.8% in the control group. These data indicate almost complete absorption of the infused [^{3}H]TO in both groups of rats. The absorbed radiolabel was almost entirely converted into [^{3}H]TAG in the mucosa of both groups of rats, 97 ± 0.5% in the etomoxir group and 94 ± 2% in the control group. In summary, these data indicate that etomoxir had no effect, either on the lumenal absorptive rate of the infused [^{3}H]TO or on the ability of the intestine to convert the absorbed lipid to TAG, confirming the lack of toxicity of etomoxir as shown in Tables 1 and 2.

The effect of etomoxir on the mass of TAG remaining in the mucosa after 6 h of lipid infusion is shown in **Fig. 2** (left pair of bars). Etomoxir treatment did not alter the amount of lipid in the mucosa. However, there was a significant (43%) increase in the [^{3}H]TAG dpm in the mucosa in the etomoxir-infused rats versus controls (Fig. 2, right pair of bars). This, combined with the nonsignificant reduction in mucosal TAG mass in the treatment group, resulted in a 74% increase in the [^{3}H]TAG-specific activity in the etomoxir-treated group as compared with the control group (**Fig. 3**). In comparing the specific activity of the [^{3}H]TO in the infusate to the TAG-specific activity in the mucosa in the two groups of rats, we found that the etomoxir-treated group had 87% of the specific activity of the infusate, indicating that most of the mucosal TAG derived from the infused [^{3}H]TO. By contrast, the specific activity of the TAG in the control group was only 50% that of the infusate, indicating a significant admixture of endogenous acyl groups. These data support our previous findings (39, 40).

Since etomoxir was expected to have an effect on the oxidation of FA, we determined the ^{3}H dpm in the aqueous/methanolic phase of Folch extracts of the mucosa. Similar to the short-term infusion conditions reported in Table 1, only small amounts of ^{3}H dpm were found, 97 ± 1 dpm in the etomoxir infused group and 122 ± 1 dpm in the control group as compared with 1.7 × 10^6 dpm and 1.2 × 10^6 dpm in the chloroform phase of the extract, respectively. These data suggest either that the products of FA metabolism are rapidly removed from the enterocyte or FA is poorly metabolized in enterocytes (41, 42).

We next wanted to determine the effect of etomoxir on the ability of the rat to export the absorbed TAG into the lymph. The mass of TAG appearing in lymph in response to an intraduodenal TO infusion in rats infused concomitantly with etomoxir or saline is shown in **Fig. 4**. In the control group, the amount of chylomicron TAG rapidly increased over the infusion period and reached, as expected, a plateau at 5 h of infusion (30). By contrast, the etomoxir-infused rats had a significantly reduced output of chylomicron TAG at each hour of lipid infusion. At steady state (Hours 5 and 6), the etomoxir-infused rats had a 74% reduction in TAG output into the lymph.

The amount of [^{3}H]TAG dpm appearing in the lymph increased in response to the [^{3}H]TO infusion (**Fig. 5**). In the control (saline) group, the amount of [^{3}H]TAG in the lymph progressively increased until reaching a plateau between 5 and 6 h of infusion, which was 54% of the input rate of [^{3}H]TO. The time required to reach a steady-state

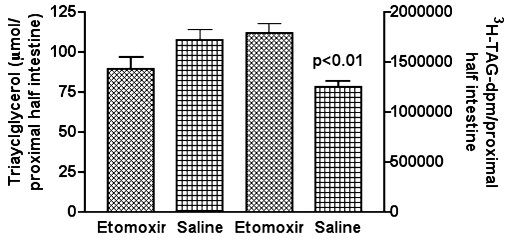

Fig. 2. Effect of etomoxir (hashed marks) or saline (grid marks) on the mass of triacylglycerol (TAG) and the number of ^{3}H dpm remaining in the mucosa after a 6 h infusion of 3 × 10^6 dpm/h [^{3}H]glyceryltrioleate, 135 μmol/h. Etomoxir (1 mM in glucose saline) or glucose saline alone was infused intraduodenally for 2 h prior to the onset of the 6 h lipid infusion and continued during the lipid infusion. At 6 h of lipid infusion, the proximal half of the intestine was harvested and the mass (left ordinate) and [^{3}H]TAG dpm (right ordinate) measured as in Materials and Methods. The left pair of bars reflects the mass and the right pair the ^{3}H dpm. The data are the mean ± SEM (n = 4). The *P* value indicates a significant difference between the amount of radiolabeled TAG in the etomoxir group versus the control.

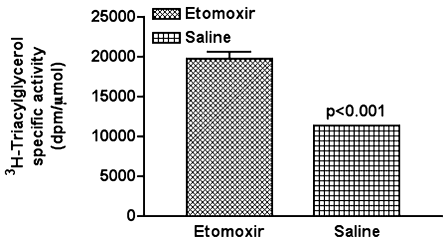

Fig. 3. The specific activity of mucosal triacylglcyerol (TAG) in response to etomoxir. The data from Fig. 2 were used to calculate the specific activity (^{3}H dpm/μmol TAG) of the TAG in the mucosa of the proximal half of the intestine. The *P* value is indicated above the saline-infused control bar. The data are the mean ± SEM (n = 4).

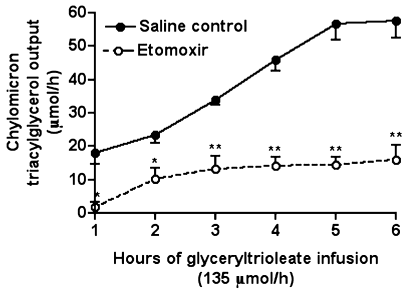

Fig. 4. The effect of etomoxir on the chylomicron output of TAG in μmol/h. Lymph fistula rats prepared as in Materials and Methods were infused with etomoxir (open circles) or saline (closed circles) and trioleate (TO) as in Fig. 2. Lymph was collected hourly, the chylomicron fraction isolated, and the TAG mass measured as in Materials and Methods. The data are the mean ± SEM (n = 4). $*P < 0.5$. $**P < 0.01$.

output of TAG into the lymph and the percentage of infusate TAG appearing in the lymph at steady state is consistent with our prior work (30, 43). By contrast, the etomoxir group had a blunted response to the [^{3}H]TO infusion. At each hour except the first hour of lipid infusion, the rats treated with etomoxir exported significantly less [^{3}H]TAG dpm into the lymph. At the steady state, 5–6 h of infusion, the etomoxir group had a 70% reduction in the output of [^{3}H]TAG into the lymph as compared with the saline infused group.

DISCUSSION

In the present study, we took advantage of the inability of FA-CoAs to cross the microsomal membrane without utilizing a CPT-dependent pathway to test the hypothesis that DGAT is, at least in part, sited on the lumenal side of the ER membrane. If DGAT is in part a latent enzyme, then inhibition of CPT should reduce TAG synthesis in

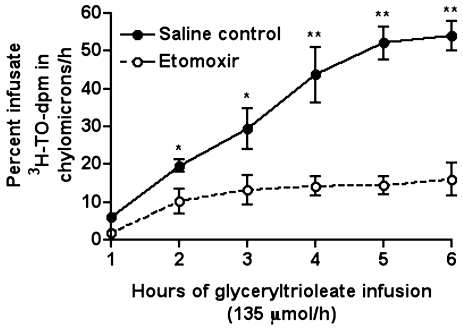

Fig. 5. The effect of etomoxir on the lymph output of [^{3}H]TAG. Etomoxir (open circles) or saline (closed circles) plus [^{3}H]TO (135 μmol/h, 22,669 dpm/μmol) was infused intraduodenally into lymph duct-cannulated rats for 6 h, as described in Materials and Methods. Lymph was collected on ice in hourly samples, and the total [^{3}H]TAG dpm was measured. The data are expressed as the percentage of infused [^{3}H]TO dpm appearing in the lymph each hour in chylomicrons. $*P < 0.05$. $**P < 0.01$. The data are the mean ± SEM (n = 4).

the ER lumen and, as a consequence, potentially reduce TAG output into the lymph as chylomicron-TAG because it is the ER-lumenal TAG that appears in the lymph as chylomicron-TAG (2, 27).

The data presented in the current report show a dramatic (~74%) reduction in the output of TAG into the lymph on treatment with etomoxir, supporting our hypothesis. The inhibitor did not appear to damage the intestinal absorptive cells, interfere with protein or phospholipid synthesis, or interfere with cholesterol, glucose, amino acid absorption, FA absorption or metabolism, or ATP generation. The lack of evidence of glucose metabolism supports previous work by Windmueller and Spaeth in which only 3% of lumenal glucose was metabolized (44). Similarly, FA metabolism to ketone bodies is limited in the intestine (41, 45). Dietary TAG and FA absorption was nearly complete in long-term (6 h) and short-term (5 min) infusions in treated and control rats, respectively. Further, the ability of the intestine to esterify the absorbed lipid to TAG was unimpaired by etomoxir, and almost all the absorbed lipid was found to be in TAG in both groups. These data also indicate that etomoxir does not inhibit DGAT activity in intestine. However, in a recent paper using a rat heart-derived cell line, Xu et al. (46) showed that etomoxiryl-CoA inhibited DGAT and that incorporation of [^{14}C]palmitate into TAG was reduced by etomoxir. In Xu et al.'s, study, the assay that was used to measure DGAT activity would measure DGAT-C. Here we show no inhibition of incorporation of either [^{14}C]palmitate in short-term or [^{3}H]oleate (from [^{3}H]TO) in long-term infusion conditions into mucosal TAG, excluding the possibility of etomoxir's inhibition of DGAT-C in the intestine. While we cannot rule out the possibility that etomoxir inhibited DGAT-l in our study, it seems unlikely that DGAT-l would be inhibited and DGAT-C would not. Furthermore, since it has been shown that DGAT1, which we believe to be DGAT-l, accounts for 85% of total intestinal DGAT activity (12), its inhibition by etomoxir should have been reflected in a reduced TAG synthetic rate in the mucosa. It was not. In summary, the reduction in lymph TAG output seen in the treated rats is likely due to the effect of the drug on CPT activity and not a nonspecific inhibitory effect.

If all TAG synthesis takes place on the cytosolic side of the ER, as originally proposed by Coleman and Bell (8), then TAG output into the lymph as chylomicron-TAG should have been unaffected by etomoxir. Alternatively, if ER DGAT activity is at least in part on the lumenal side of the ER membrane as proposed by Owen et al. (10), then the inhibition of CPT activity by etomoxir should block entry of FA-CoA into the lumen of the ER and thus reduce the synthetic rate of TAG by DGAT-l in proportion to the percentage of the total DGAT activity that is expressed on the ER lumenal membrane. In turn, this should reduce the output of TAG into the lymph. We found that etomoxir greatly reduced TAG delivery into the lymph, supporting the hypothesis that a functional DGAT exists on the lumenal side of the ER membrane. As judged by the amount of reduction in steady-state TAG output in re-

sponse to etomoxir, the majority of DGAT that is functional in chylomicron formation is on the ER lumenal hemi-leaflet.

The existence of DGAT on the lumen side of the ER membrane, as proposed by Owen et al. (10) and confirmed by Abo-Hashema et al. (3), provides a mechanism whereby neutral lipids can be rapidly moved across the ER membrane. If DGAT were only on the cytosolic side of the membrane, then the TAG synthesized as the end product of the enzyme must traverse the ER membrane to gain entry into the ER lumen. TAG has limited solubility in membranes, 3% (47), so that the rate of its translocation could become limiting, as suggested by our prior studies in which graded increases of TO were delivered intraduodenally to rats (27). By contrast, if TAG was synthesized, at least in part, on the lumenal side of the ER membrane, then its substrates, DAG and FA-CoA, could theoretically cross the ER membrane more easily than TAG. Carnitine acylation/deacylation to translocate the FA-CoA is a very efficient mechanism to move FA across at least mitochondrial membranes (13). The other DGAT substrate, DAG, has also been shown to translocate across membranes rapidly (48, 49). While the evidence is not clear that the CPT enzyme system is responsible for the translocation of FA-CoAs across the ER membrane in the intestine (49), the data from our etomoxir studies suggest that it is. This speculation is supported by Abo-Hashema et al. (3), who found that ER lumenal synthesis of TAG was carnitine dependent.

Most studies locate the major FA transporters, FAT/CD36 and plasma membrane bound-FA binding protein, to the plasma membrane, making active transport of FA to the ER lumen doubtful. Intracellular transmembrane FA transport always requires a carnitine-dependent system (50).

These speculations, based on biochemical and physiological studies, have been greatly supported by new molecular evidence regarding the cloning of DGAT-l (5) and DGAT-C (6), both of which are expressed in mouse intestine. Initial studies on the functionality of these two DGATs have shown that mice, whose DGAT-l gene is disrupted, absorb dietary lipid adequately and synthesize TAG, but have a reduced ability to transport the absorbed lipid in chylomicrons (12). In addition, these mice demonstrated lipid vacuoles in the intestine when placed on a high-fat diet. These data, combined with prior studies, suggest the information presented in **Fig. 6**. We propose that DGAT-l is sited on the lumenal face of the ER membrane because its absence results in greatly impaired chylomicron formation (12). This location is supported by the reduction in chylomicron output by the intestine due to etomoxir, ~74%, which is close to the total DGAT activity expressed by the intestine due to DGAT-l, ~85% (12). Etomoxir would not affect chylomicron output if both DGATs were located on the cytosolic face of the ER membrane. We propose that DGAT-C is sited on the cytosolic face of the ER membrane because TAG synthesis continues in the intestine despite the absence of DGAT-l. However, we postulate that the TAG produced by DGAT-C is

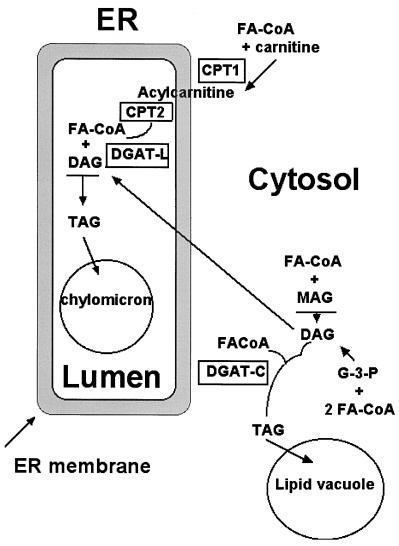

Fig. 6. The flow of lipid in enterocytes. Fatty acid (FA)-CoA plus carnitine are converted to FA-carnitine by CPT-1 on the cytosolic face of the ER membrane, and FA-CoA is reformed on the lumenal side of the membrane by CPT2. FA-CoA plus diacylglycerol (DAG) yields TAG as mediated by the enzyme lumenal DAG acyltransferase (DGAT-l). DAG, formed from FA-CoA and either monoacylglycerol (MAG) or glycerol-3-phosphate (G-3-P), is shown by the long arrow to translocate across the ER membrane to be available as a substrate for cytosolic DGAT (DGAT-C). The TAG, once synthesized, enters the forming chylomicron. On the cytosolic face of the ER membrane, FA-CoA and MAG form DAG, or DAG can be formed by acylating G-3-P. DAG is used by DGAT-C on the cytosolic face of the ER membrane to form TAG. Part of the TAG formed does not traverse the ER membrane, but enters a forming lipid vacuole. Some of the TAG synthesized in this manner does enter the ER lumen and participates in chylomicron formation (not shown). This figure is partially based on a diagram presented by Owen et al. (10).

poorly transported across the ER membrane, resulting in impaired chylomicron formation and its accumulation in the cytosol as lipid vacuoles. That TAG can accumulate on the cytosolic face of the ER membrane and not be transported as chylomicrons is suggested by our prior studies in which graded increases of TO were infused intraduodenally in rats (27). However, since our data do not directly address the location of the DGAT isoforms, the conformation of the location of the DGAT isoforms shown in Fig. 6 awaits further study.

The increase in the specific activity of mucosal [^{3}H]TAG in the etomoxir-treated rats versus the controls indicates a preferential acylation to TAG using dietary [^{3}H]oleate or [^{3}H]MAG in the treated group. A potential explanation for this finding is that by blocking dietary [^{3}H]FA-CoA translocation across the ER membrane, etomoxir treatment results in [^{3}H]FA accumulating on the cytosolic surface of the ER. This [^{3}H]FA effectively competes for endogenous FA as eventual substrate for the cytosolic face-located DGAT-C, producing TAG enriched with [^{3}H]FA acyl groups as compared with controls.

Regardless of the mechanism by which TAG gets to the ER lumen, its lumenal disposition is important to its even-

tual exit from the ER to the Golgi, and finally its exocytosis in a mature chylomicron. We have previously shown that TAG within the ER lumen is protected from lipolytic attack and that the TAG that is so protected is likely to be associated with chylomicrons (27). Further, electron micrographic studies have shown that TAG in the lumen of the ER is a precursor of chylomicron TAG (51). By contrast, TAG that remains on the cytosolic face of the ER is not likely to become a chylomicron-TAG precursor (27).

In summary, we propose that DGAT-l is functionally active in the intestine and that the majority of TAG in the lymph is synthesized by this ER lumenally-disposed enzyme.

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