Etomoxir mediates differential metabolic channeling of fatty acid and glycerol precursors into cardiolipin in H9c2 cells

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Abstract **We examined the effect of etomoxir treatment on de novo cardiolipin (CL) biosynthesis in H9c2 cardiac myoblast cells. Etomoxir treatment did not affect the activities of the CL biosynthetic and remodeling enzymes but caused a reduction in [1-^{14}C]palmitic acid or [1-^{14}C]oleic acid incorporation into CL. The mechanism was a decrease in fatty acid flux through the de novo pathway of CL biosynthesis via a redirection of lipid synthesis toward 1,2-diacyl-*sn*-glycerol utilizing reactions mediated by a 35% increase ($P < 0.05$) in membrane phosphatidate phosphohydrolase activity. In contrast, etomoxir treatment increased [1,3-^{3}H]glycerol incorporation into CL. The mechanism was a 33% increase ($P < 0.05$) in glycerol kinase activity, which produced an increased glycerol flux through the de novo pathway of CL biosynthesis. Etomoxir treatment inhibited 1,2-diacyl-*sn*-glycerol acyltransferase activity by 81% ($P < 0.05$), thereby channeling both glycerol and fatty acid away from 1,2,3-triacyl-*sn*-glycerol utilization toward phosphatidylcholine and phosphatidylethanolamine biosynthesis. In contrast, etomoxir inhibited *myo*-[^{3}H]inositol incorporation into phosphatidylinositol and the mechanism was an inhibition in inositol uptake. Etomoxir did not affect [^{3}H]serine uptake but resulted in an increased formation of phosphatidylethanolamine derived from phosphatidylserine. The results indicate that etomoxir treatment has diverse effects on de novo glycerolipid biosynthesis from various metabolic precursors. In addition, etomoxir mediates a distinct and differential metabolic channeling of glycerol and fatty acid precursors into CL.**—Xu, F. Y., W. A Taylor, J. A. Hurd, and G. M. Hatch. **Etomoxir mediates differential metabolic channeling of fatty acid and glycerol precursors into cardiolipin in H9c2 cells.** ***J. Lipid Res.*** **2003.** 44: **415–423.**

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Cardiolipin (CL) is a major membrane phospholipid in most mammalian cells and is localized primarily to the inner mitochondrial membrane where it comprises approximately 20% of the mitochondrial phospholipid mass (1–3). In addition, CL has been identified in the outer mitochondrial membrane (4, 5). CL was shown to be required for the reconstituted activity of a number of key mammalian mitochondrial enzymes involved in cellular energy metabolism (6–14). More recently, peroxidation of CL in mitochondria of rat basophile leukemia cells resulted in dissociation of cytochrome c from mitochondrial inner membranes, an initial step in cytochrome c-mediated apoptosis (15). In mammalian tissues, CL contains four fatty acid side chains occupied primarily by unsaturated fatty acids of 16–18 carbons in length (16). The fatty acyl species appeared to be the important structural requirement for the high protein binding affinity of CL (17). Dietary modification of the fatty acyl species composition of CL was shown to alter the oxygen consumption in cardiac mitochondria (18, 19). The specific activity of cytochrome c oxidase reconstituted with CL varied markedly and significantly with different molecular species of the phospholipid. Thus, the appropriate fatty acyl composition of CL is important for optimum mitochondrial respiratory activity.

CL de novo biosynthesis begins with the conversion of phosphatidic acid (PtdOH) to cytidine-5′-diphosphate-1,2-diacyl-*sn*-glycerol (CDP-DG) catalyzed by PtdOH:CTP cytidylyltransferase (EC 2.7.7.41) (20). CDP-DG is then condensed with *sn*-glycerol-3-phosphate to form phosphatidylglycerol (PtdGro) phosphate catalyzed by PtdGro phosphate synthase (EC 2.7.8.5). The PtdGro phosphate is immediately converted to PtdGro by a highly active PtdGro phosphate phosphatase (EC 3.1.3.27). Finally, PtdGro is con-

Abbreviations: AT, monolysocardiolipin acyltransferase; CDP-DG, cytidine-5′-diphosphate 1,2-diacyl-*sn*-glycerol; CL, cardiolipin; DG, 1,2-diacyl-*sn*-glycerol; DGAT, 1,2-diacyl-*sn*-glycerol acyltransferase; GPAT, *sn*-glycerol-3-phosphate acyltransferase; MLCL, monolysocardiolipin; PAP, phosphatidate phosphohydrolase; PLA_2, phospholipase A_2; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdGro, phosphatidylglycerol; PtdIns, phosphatidylinositol; PtdOH, phosphatidic acid; PtdSer, phosphatidylserine; SM, sphingomyelin; TG, 1,2,3-triacyl-*sn*-glycerol.

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densed with another molecule of CDP-DG to form CL catalyzed by CL synthase (21). The de novo CL biosynthetic enzymes lack fatty acyl species specificity (22, 23). Thus, like other phospholipids, newly synthesized CL must be rapidly remodeled by a deacylation-reacylation pathway to obtain fatty acyl groups (24). The enzyme responsible for CL deacylation in the mitochondria is phospholipase A_2 (25–27). We have characterized the activity of monolysocardiolipin acyltransferase (MLCL AT) specific for the acylation of MLCL to CL in the heart and mammalian tissues (28).

Recent studies support the hypothesis of functionally independent acyl-CoA pools within mammalian cells that may be channeled toward specific fates rather than being freely available for all possible enzymatic reactions (29–32). Etomoxir is a member of the oxirane carboxylic acid carnitine palmitoyl transferase I inhibitors and has been suggested as a therapeutic agent for the treatment of heart failure (33–35). Acute etomoxir treatment irreversibly inhibits the activity of carnitine palmitoyltransferase I. As a result, fatty acid import into the mitochondria and β-oxidation is reduced, whereas cytosolic fatty acid accumulates and glucose oxidation is elevated. Prolonged incubation (24 h) with etomoxir produces diverse effects on the expression of several metabolic enzymes (36). It had been postulated that acute etomoxir treatment might stimulate de novo phospholipid biosynthesis in the mammalian heart (37). However, this had never been demonstrated. In this study, we examined if acute etomoxir treatment of H9c2 cardiac myoblast cells indeed stimulated de novo CL and phospholipid biosynthesis, and if etomoxir mediated distinct metabolic channeling of fatty acid and glycerol into CL. Our results indicate that etomoxir treatment of H9c2 cardiac myoblast cells produces a diverse plethora of effects on overall de novo glycerolipid biosynthesis from various metabolic precursors. In addition, etomoxir produces a distinct and differential metabolic channeling of glycerol and fatty acid precursors into CL in H9c2 cells.

MATERIALS AND METHODS

Rat heart H9c2 myoblastic cells were obtained from American Type Culture Collection. [^{14}C]glycerol-3-phosphate, [5-^{3}H]CTP, [1,3-^{3}H]glycerol, [1-^{14}C]palmitic acid, [1-^{14}C]oleic acid, [1-^{14}C]oleoyl-CoA, [^{3}H]serine, *myo*-[^{3}H]inositol, [^{3}H]ethanolamine, and [*methyl*-^{3}H]choline were obtained from either Dupont, Mississauga, Ontario, or Amersham, Oakville, Ontario, Canada. [^{14}C]PtdGro was synthesized from [^{14}C]glycerol-3-phosphate as previously described (38). Sodium etomoxir, 2-[6-(4-chlorophenoxy)hexyl]oxirane-2-carboxylate was obtained from Research Biochemicals Incorporated, Natick, MD. Etomoxiryl-CoA was synthesized as described (39). DMEM and fetal bovine serum were products of Canadian Life Technologies (GIBCO), Burlington, Onatrio, Canada. Lipid standards were obtained from Serdary Research Laboratories, Englewood Cliffs, NJ. MLCL was obtained from Avanti Polar Lipids, Alabaster, AL. Thin layer plates (silica gel G, 0.25 mm thickness) were obtained from Fisher Scientific, Winnipeg, Canada. Ecolite scintillant was obtained from ICN Biochemical, Montreal, Quebec, Canada. All other biochemicals were certified ACS grade or better and obtained from either Sigma Chemical Co., MO or Fisher Scientific.

Rat heart H9c2 myoblastic cells were incubated in DMEM containing 10% fetal bovine serum until near confluence. In some experiments, cells were preincubated for 2 h with DMEM (serum-free) in the absence or presence of 1–80 μM etomoxir and then incubated for 2 h with 0.1 mM [1-^{14}C]oleic acid (10 μCi/dish, bound to BSA in a 1:1 molar ratio). In other experiments, cells were preincubated for 2 h plus or minus 40 μM etomoxir and then incubated for 2 h with 0.1 μM or 0.1 mM [1,3-^{3}H]glycerol (10 μCi/dish), 0.1 mM [1-^{14}C]oleic acid (2 μCi/dish, bound to BSA in a 1:1 molar ratio), 0.1 mM [1-^{14}C]palmitic acid (2 μCi/dish, bound to BSA in a 1:1 molar ratio), 28 μM [^{3}H]ethanolamine (2 μCi/dish), 28 μM [*methyl*-^{3}H]choline (2 μCi/dish), 0.4 mM [^{3}H]serine (20 μCi/dish), or 40 μM *myo*-[^{3}H]inositol (10 μCi/dish). The medium was removed and the cells washed twice with ice-cold saline and then harvested from the dish with 2 ml methanol-water (1:1, v/v) for lipid extraction. An aliquot of the homogenate was taken for the determination of total uptake of radioactivity into cells. Phospholipids were then isolated and radioactivity in these determined as previously described (38).

For enzyme studies, H9c2 cells were washed twice with ice-cold saline and harvested with 2 ml homogenization buffer (10 mM Tris-HCL, pH 7.4, 0.25 M sucrose). The cells were homogenized with 15 strokes of a Dounce A homogenizer. The homogenate was centrifuged at 1,000 *g* for 5 min. The resulting supernatant was centrifuged at 10,000 *g* for 15 min. The resulting pellet was resuspended in 1 ml of homogenizing buffer and designated the mitochondrial fraction. The resulting supernatant was centrifuged at 100,000 *g* for 60 min to obtain the cytosolic fraction. The resulting pellet from this centrifugation was resuspended in 1 ml of homogenizing buffer and designated the microsomal fraction. Glycerol kinase in cytosol was determined by measuring the conversion of [1,3-^{3}H]glycerol to [1,3-^{3}H]glycerol-3-phosphate as described (40). Mitochondrial PtdOH:CTP cytidylyltransferase activity was determined by measuring the conversion of [5-^{3}H]CTP and PtdOH to CDP-[^{3}H]DG as described (38). Mitochondrial PtdGro phosphate synthase and PtdGro phosphate phosphatase combined activities were determined by measuring the conversion of [^{14}C]glycerol-3-phosphate and CDP-DG to [^{14}C]PtdGro as described (38). Mitochondrial CL synthase activity was determined by measuring the conversion of [^{14}C]PtdGro and CDP-DG to [^{14}C]CL as described (38). Mitochondrial phospholipase A_2 (PLA_2) was determined by measuring the conversion of [^{14}C]PtdGro to lyso[^{14}C]PtdGro as described (41). Mitochondrial MLCL AT activity was determined by measuring the conversion of [1-^{14}C]oleoyl-CoA and MLCL to [^{14}C]CL as described (28). Mitochondrial and microsomal *sn*-glycerol-3-phosphate acyltransferase (GPAT) activities were determined as described (42). Microsomal 1,2-diacyl-*sn*-glycerol acyltransferase (DGAT) activity was determined as described (43). Total cell and membrane phosphatidate phosphohydrolase (PAP) activity was determined as described (44). In some experiments microsomal DGAT and cytosolic glycerol kinase activities were determined in the absence or presence of 30 μM etomoxiryl-CoA. The fatty acid composition of CL in H9c2 cells was determined as described (45). Phospholipid phosphorus was determined as described (46). Protein was determined as described (47), and Student's *t*-test was used for statistical analysis. The level of significance was defined as $P < 0.05$.

RESULTS

Etomoxir reduces CL biosynthesis from fatty acids

Fatty acid import into mitochondria and its subsequent β-oxidation is mediated by carnitine palmitoyltransferase

I and II (48). Etomoxir, 2-[6-(4-chlorophenoxy)hexyl] oxirane-2-carboxylate, is a compound known to inhibit β-oxidation in mitochondria by irreversibly inhibiting carnitine palmitoyltransferase I activity (33). It was postulated that etomoxir stimulated phospholipid biosynthesis in mammalian heart (37). However, the effect of etomoxir on the biosynthesis of CL, a phospholipid exclusively found in mitochondria, had not been investigated. We chose H9c2 cardiac myoblast cells since it is a cell line derived from embryonic rat heart and these cells readily take up radiolabeled glycerol and fatty acids and incorporate them into CL (38). H9c2 cells were preincubated with 1–80 μM etomoxir for 2 h and incubated with 0.1 mM [1-^{14}C]oleic acid bound to albumin (1:1 molar ratio) for 2 h, and radioactivity incorporated into CL determined. These concentrations of etomoxir have been used in various cell culture models to inhibit carnitine palmitoyltransferase I activity (49, 50). Oleic acid (C18:1) was chosen for these experiments since this is the major unsaturated fatty acid found in CL in H9c2 cells (**Table 1**). The 2 h treatment was chosen to avoid the complicated effects of altered gene expression seen during prolonged incubation (24 h) with this compound (36). In H9c2 cells, CL was comprised mainly of myristic (C14:0), palmitic (C16:0), and oleic (C18:1) acids. Incubation of H9c2 cells with etomoxir resulted in a concentration-dependent reduction in [1-^{14}C]oleic acid incorporation into CL (**Fig. 1**). For subsequent studies, we chose to use 40 μM etomoxir since the reduction in [1-^{14}C]oleic acid incorporation into CL was maximum at this concentration. The presence of 40 μM etomoxir in the medium of H9c2 cells did not alter the percent fatty acid composition of CL (Table 1). We next examined incorporation of [1-^{14}C]oleic acid into lipid metabolites of the CDP-DG pathway. Incubation of cells with 40 μM etomoxir caused a 41% ($P < 0.05$) and 31% ($P < 0.05$) reduction in [1-^{14}C]oleic acid incorporated into CL and PtdGro, respectively, compared with controls (**Table 2**). In contrast, radioactivity incorporated into PtdOH was unaltered. Radioactivity incorporated into CDP-DG was low, twice background (data not shown). Total radioactivity associated with the cells was unaltered by etomoxir treatment. Thus, etomoxir treatment reduced [1-^{14}C]oleic acid incorporation into PtdGro and CL.

The mechanism for the reduction in [1-^{14}C]oleic acid incorporation into PtdGro and CL was examined. The phospholipid phosphorus concentration of these cells was

TABLE 1. The fatty acid composition of cardiolipin in H9c2 cells treated with etomoxir

% Fatty Acid	Control	+ Etomoxir
Myristate (C14:0)	3.6	4.2
Palmitate (C16:0)	49.1	47.2
Oleate (C18:1)	37.3	38.2
All others	10.0	10.4

H9c2 cells were incubated for 2 h in the absence or presence of 40 μM etomoxir. Cardiolipin (CL) was isolated and the percent fatty acid composition of CL determined. Results represent the mean of two experiments.

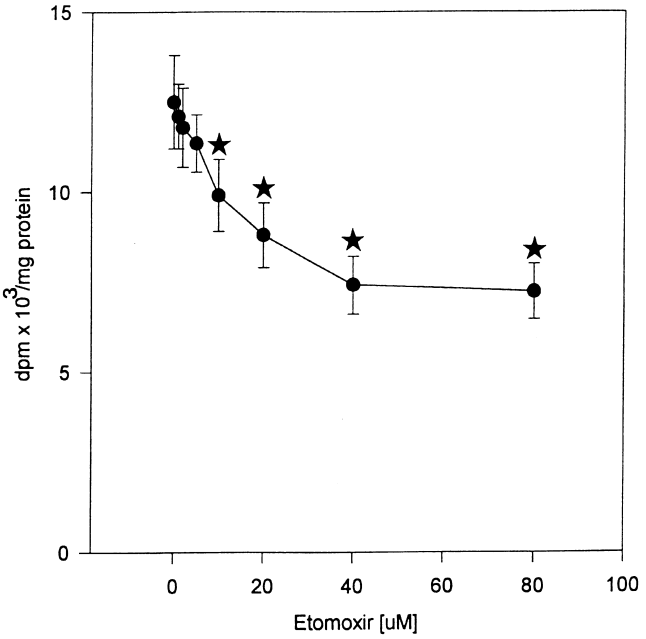

Fig. 1. Incorporation of [1-^{14}C]oleic acid into cardiolipin (CL) in H9c2 cells treated with various concentrations of etomoxir. H9c2 cells were preincubated for 2 h in the absence or presence of 1–80 μM etomoxir and then incubated for 2 h with 0.1 mM [1-^{14}C]oleic acid (10 μCi/dish) bound to 0.1 mM albumin, and the radioactivity incorporated into CL determined. Results represent the mean ± SD of three separate experiments. *$P < 0.05$.

124 ± 14 nmol/mg protein and was unaltered by pretreatment with etomoxir for 2 h. The percent phospholipid phosphorus of CL in these cells was 4.2% and was unaltered by pretreatment with etomoxir for 2 h. Since [1-^{14}C]oleic acid incorporated into PtdOH was unaltered, we examined if the reduction in [1-^{14}C]oleic acid incorporated into PtdGro and CL was due to a reduction in the activities of the enzymes of the CDP-DG pathway of CL biosynthesis. Cells were incubated for 2 h in the absence or presence of 40 μM etomoxir. The cells were homogenized, mitochondrial fractions prepared, and the activities of the biosynthetic enzymes determined. As seen in **Table 3**, etomoxir did not affect mitochondrial PtdOH:CTP cytidylyltransferase, PtdGro phosphate synthase/phosphatase, or CL synthase activities. Thus, the reduction in [1-

TABLE 2. Incorporation of [1-^{14}C]oleic acid into PtdOH, PtdGro, and CL in H9c2 cells treated with etomoxir

	Control	+ Etomoxir
	dpm × 10^3/mg protein	
CL	1.7 ± 0.1	1.0 ± 0.1[a]
PtdGro	3.2 ± 0.3	2.2 ± 0.2[a]
PtdOH	0.2 ± 0.1	0.2 ± 0.1
Total cellular incorporation	1370.1 ± 120.2	1420.7 ± 100.2

H9c2 cells were preincubated for 2 h in the absence or presence of 40 μM etomoxir, incubated with 0.1 mM [1-^{14}C]oleic acid (2 μCi/dish), and the radioactivity incorporated into cells, phosphatidic acid (PtdOH), phosphatidylglycerol (PtdGro), and CL determined. Results represent the mean ± SD of six experiments.

[a] $P < 0.05$.

TABLE 3. PtdOH:CTP cytidylyltransferase, PtdGro phosphate synthase/phosphatase, CL synthase, phospholipase A_2 (PLA_2), monolysocardiolipin (MLCL) acyltransferase (AT), *sn*-glycerol-3-phosphate acyltransferase (GPAT), 1,2-diacyl-*sn*-glycerol acyltransferase (DGAT), phosphatidate phosphohydrolase (PAP), and glycerol kinase activities in H9c2 cells treated with etomoxir

	Control	+ Etomoxir
	pmol/min.mg protein	
PtdOH:CTP cytidylyltransferase	2.5 ± 0.4	2.4 ± 0.2
PtdGro phosphate synthase/phosphatase	52.3 ± 7.2	46.4 ± 5.2
CL synthase	4.1 ± 1.0	4.2 ± 1.2
PLA_2	154.4 ± 17.2	161.4 ± 20.3
MLCL AT	268.4 ± 16.3	260.2 ± 22.4
GPAT		
mitochondrial	27.3 ± 4.3	26.5 ± 2.4
microsomal	31.8 ± 2.6	35.9 ± 3.6
DGAT	106 ± 15	20 ± 5[a]
Glycerol kinase	0.9 ± 0.1	1.2 ± 0.2[a]
	Percent of Total Activity on Membranes	
PAP	20 ± 2	27 ± 3[a]

H9c2 cells were incubated for 2 h in the absence of presence of 40 μM etomoxir. Mitochondrial, microsomal, and cytosolic fractions were prepared and PtdOH:CTP cytidylyltransferase, PtdGro phosphate synthase/phosphatase, CL synthase, MLCL AT, PLA_2, GPAT, DGAT, PAP, and glycerol kinase activities determined. The results represent the mean ± SD of five separate experiments.

[a] $P < 0.05$.

TABLE 4. Incorporation of [1-^{14}C]palmitic acid into glycerophospholipids and neutral lipids in H9c2 cells treated with etomoxir

	Control	+ Etomoxir
	dpm × 10^3/mg protein	
CL	1.3 ± 0.2	0.7 ± 0.1[a]
PtdGro	10.5 ± 1.0	8.0 ± 0.8[a]
PtdIns	3.8 ± 0.2	2.4 ± 0.3[a]
PtdOH	1.2 ± 0.4	1.4 ± 0.2
PtdEtn	34.7 ± 6.4	52.8 ± 3.8[a]
PtdCho	306.7 ± 20.4	371.1 ± 26.2[a]
PtdSer	0.6 ± 0.2	2.3 ± 0.2[a]
DG	122 ± 16	235 ± 25[a]
TG	450 ± 32	202 ± 25[a]
Total incorporation into cells	1631 ± 100	1743 ± 111

PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdIns, phosphatidylinositol; PtdSer, phosphatidylserine. H9c2 cells were incubated for 2 h in the absence or presence of 40 μM etomoxir and then incubated with 0.1 mM [1-^{14}C]palmitic acid (2 μCi/dish), and the radioactivity incorporated into glycerophospholipids, 1,2-diacyl-*sn*-glycerol (DG), and 1,2,3-triacyl-*sn*-glycerol (TG) determined. Results represent the mean ± SD of six experiments.

[a] $P < 0.05$.

^{14}C]oleic acid incorporation into PtdGro and CL was not due to alterations in the activities of the enzymes of de novo CL biosynthesis.

Unsaturated fatty acids enter into phospholipids mainly by deacylation-reacylation pathways (51). Since [1-^{14}C]oleic acid incorporation into PtdGro and CL were reduced to a similar extent and PtdGro is the immediate precursor to CL, it was possible that the reduction in radioactivity incorporated into CL was due to a either a reduction in the specific radioactivity of its immediate precursor, PtdGro, or a reduction in CL remodeling. To test this hypothesis, cells were preincubated with 40 μM etomoxir for 2 h then incubated with [1-^{14}C]palmitic acid for 2 h and radioactivity incorporated into lipids determined. In rat heart, palmitic acid enters CL mainly by de novo biosynthesis (28). As seen in **Table 4**, incubation of cells with 40 μM etomoxir caused a 46% ($P < 0.05$), 24% ($P < 0.05$), and 37% ($P < 0.05$) reduction in [1-^{14}C]palmitic acid incorporated into CL, PtdGro, and phosphatidylinositol (PtdIns), respectively, compared with controls. In contrast, radioactivity incorporated into phosphatidylethanolamine (PtdEtn), phosphatidylcholine (PtdCho), and phosphatidylserine (PtdSer) were elevated 52% ($P < 0.05$), 21% ($P < 0.05$), and 3.8-fold ($P < 0.05$), respectively, in etomoxir-treated cells compared with controls. Radioactivity incorporated into CDP-DG was low, twice background (data not shown). [1-^{14}C]palmitic acid incorporated into DG was increased 93% ($P < 0.05$), and incorporation into 1,2,3-triacyl-*sn*-glycerol (TG) decreased 55% ($P < 0.05$) in etomoxir-treated cells compared with control. Total radioactivity associated with the cells was unaltered by etomoxir treatment. We next examined if etomoxir affected mitochondrial PLA_2 and MLCL AT activities, the CL remodeling enzymes. H9c2 cells were incubated with 40 μM etomoxir for 2 h. The cells were homogenized, mitochondrial fractions prepared, and these enzyme activities determined. Etomoxir treatment did not affect MLCL AT and PLA_2 activities (Table 3). Thus, the reduction in [1-^{14}C]fatty acid incorporation into CL was due to the reduction in radioactivity of its immediate precursor, PtdGro, and not due to alterations in CL remodeling. In addition, etomoxir treatment stimulated [1-^{14}C]palmitic acid incorporation into PtdCho, PtdEtn, PtdSer, and DG and reduced [1-^{14}C]palmitic acid incorporation into TG (Table 4).

PtdCho and PtdEtn de novo biosynthesis utilizes DG, and PtdSer de novo biosynthesis utilizes PtdEtn and PtdCho as PtdOH precursors. Since [1-^{14}C]palmitic acid incorporated into DG was elevated, and [1-^{14}C]palmitic acid incorporated into CL, PtdGro, and PtdIns reduced, it was possible that etomoxir treatment stimulated microsomal GPAT activity or resulted in a re-direction of phospholipid biosynthesis away from CDP-DG-utilizing reactions toward DG-utilizing reactions. Cells were incubated plus or minus 40 μM etomoxir for 2 h, then microsomal and mitochondrial GPAT activities determined. Microsomal and mitochondrial GPAT activities were unaltered in cells treated with etomoxir (Table 3). PAP lies at a branch point in glycerolipid biosynthesis and controls the direction of PtdOH utilization (51). Cells were preincubated plus or minus 40 μM etomoxir for 2 h, then total and membrane PAP activity determined. Total cellular PAP activity was 0.65 ± 0.10 mU/total units lactate dehydrogenase and was unaltered by etomoxir treatment (average of five experiments). In contrast, membrane PAP was increased 35% ($P < 0.05$) in etomoxir-treated cells compared with control. Membrane PAP activity was 0.13 ± 0.01 mU/total units lactate dehydrogenase and was unaltered by 40 μM etomoxiryl-CoA treatment (average of three experiments). Since treatment of membranes directly with etomoxiryl-CoA did not

affect in vitro PAP activity, it is concluded that the increase in membrane PAP activity seen in cells treated with etomoxir was caused by the elevation in cytosolic fatty acid produced by the etomoxir-mediated inhibition of mitochondrial β-oxidation. Thus, the reduction in fatty acid incorporation into CL, PtdGro, and PtdIns in etomoxir-treated cells was due to a re-direction of glycerolipid biosynthesis away from CDP-DG-utilizing reactions toward DG-utilizing reactions.

Surprisingly, etomoxir reduced [1-^{14}C]palmitic acid incorporation into TG. We examined if etomoxir altered DGAT activity. H9c2 cells were incubated with 40 μM etomoxir for 2 h. The cells were then homogenized and microsomal fractions prepared and DGAT activities determined. DGAT activity was reduced 81% ($P < 0.05$) in etomoxir-treated cells compared with controls (Table 3). The mechanism of the etomoxir-mediated inhibition of DGAT activity was determined. Microsomal membranes from H9c2 cells were prepared and DGAT activity determined in the absence or presence of 40 μM etomoxiryl-CoA. DGAT activity was 94 ± 15 pmole/min·mg protein in control membranes and was reduced 67% ($P < 0.05$) to 31 ± 2 pmole/min·mg protein in membranes incubated with etomoxiryl-CoA. Since [1-^{14}C]palmitic acid incorporation into DG was elevated and DGAT activity reduced, we conclude that the reduction in [1-^{14}C]palmitic acid incorporation into TG was due to a decrease in flux of fatty acids toward TG biosynthesis mediated by an inhibition in DGAT activity.

Etomoxir treatment produces diverse effects on de novo phospholipid biosynthesis from head group precursors in H9c2 cells

Since etomoxir inhibited CL formation from exogenous fatty acid in H9c2 cells, we anticipated that etomoxir would also reduce CL biosynthesis from the glycerol head group precursor. To examine if etomoxir treatment altered de novo CL biosynthesis from glycerol, H9c2 cardiac myoblast cells were preincubated for 2 h in the absence or presence of 40 μM etomoxir and then incubated with 0.1 μM [1,3-^{3}H]glycerol for 2 h, and radioactivity incorporated into CL and intermediates of the CDP-DG pathway of CL biosynthesis was determined. Surprisingly, incubation of cells with etomoxir resulted in 67% ($P < 0.05$), 48% ($P < 0.05$), and 50% ($P < 0.05$) increase in [1,3-^{3}H]glycerol incorporation into CL, PtdGro, and PtdOH, respectively, compared with controls (**Table 5**). Radioactivity incorporated into CDP-DG was twice background (data not shown). Total uptake of [1,3-^{3}H]glycerol in H9c2 cells was unaltered by the presence of etomoxir. Since the specific activity of the radioactive glycerol may have been diluted by the intracellular glycerol concentration, the experiment was repeated with 0.1 mM [1,3-^{3}H]glycerol in the medium. The use of 0.1 mM glycerol is representative of plasma glycerol concentrations (53). Similar to the results with 0.1 μM glycerol, CL, PtdGro, and PtdOH biosynthesis were elevated and total uptake of [1,3-^{3}H]glycerol was unaltered by etomoxir treatment in these experiments (Table 5). Thus, etomoxir treatment of H9c2 cells apparently enhanced de novo CL biosynthesis from [1,3-^{3}H]glycerol.

TABLE 5. Incorporation of [1,3-^{3}H]glycerol into glycerolipids in H9c2 cells treated with etomoxir

	Control	+ Etomoxir
	dpm × 10^{3}/mg protein	
With 0.1 μM Glycerol		
CL	0.9 ± 0.2	1.5 ± 0.2[a]
PtdGro	5.2 ± 0.7	7.7 ± 0.5[a]
PtdOH	0.4 ± 0.1	0.6 ± 0.1[a]
Total uptake of [1,3-^{3}H]Glycerol	1916 ± 187	1969 ± 167
With 0.1 mM Glycerol		
CL	0.8 ± 0.1	1.2 ± 0.1[a]
PtdGro	3.2 ± 0.3	4.2 ± 0.3[a]
PtdOH	0.3 ± 0.1	0.5 ± 0.1[a]
PtdIns	0.8 ± 0.1	1.2 ± 0.1[a]
PtdSer	0.6 ± 0.1	0.9 ± 0.1[a]
PtdCho	319 ± 42	809 ± 77[a]
PtdEtn	353 ± 64	962 ± 81[a]
DG	7.6 ± 0.8	5.1 ± 0.4[a]
TG	74.2 ± 5.4	15.2 ± 2.1[a]
Total uptake of [1,3-^{3}H]Glycerol	2456 ± 168	2440 ± 139

H9c2 cells were preincubated for 2 h in the absence or presence of 40 μM etomoxir and then incubated with 0.1 μM or 0.1 mM [1,3-^{3}H]-glycerol and the radioactivity incorporated into glycerolipids determined. Results represent the mean ± SD of six experiments.

[a] $P < 0.05$.

We examined the mechanism for the etomoxir-mediated increase in glycerol incorporation into CL. Since glycerol kinase is the rate-limiting step in muscle cell glycerol metabolism (54), we examined if glycerol kinase activity was elevated in these cells. Cells were incubated for 2 h in the absence or presence of 40 μM etomoxir, and cytosol prepared and glycerol kinase activity determined. Glycerol kinase activity was elevated 33% ($P < 0.05$) in etomoxir-treated cells compared with control (Table 3). The mechanism for the etomoxir-mediated elevation in glycerol kinase activity was determined. Cytosolic fractions were prepared from H9c2 cells and glycerol kinase activity was determined in the absence or presence of 40 μM etomoxiryl-CoA. The presence of etomoxiryl-CoA in the incubation mixture stimulated glycerol kinase in vitro activity 67% ($P < 0.05$) from 0.9 ± 0.2 to 1.6 ± 0.1 nmol/min · mg protein. Since the activities of the de novo CL biosynthetic enzymes, GPAT activities, and glycerol uptake were unaltered, the elevation in radioactivity incorporated into CL in etomoxir treated cells was due to an increase in glycerol flux through the CDP-DG pathway mediated by an increase in glycerol kinase activity.

Since [1,3-^{3}H]glycerol incorporation into PtdOH was increased by etomoxir treatment, we examined if incorporation into other glycerolipids was altered. As seen in Table 5, radioactivity incorporated into PtdIns, PtdSer, PtdCho, and PtdEtn was elevated 50% ($P < 0.05$), 50% ($P < 0.05$), 2.5-fold ($P < 0.05$), and 2.7-fold ($P < 0.05$), respectively, in etomoxir-treated cells compared with controls, indicating an elevation in biosynthesis of these lipids from [1,3-^{3}H]glycerol, which would be expected if glycerol kinase activity was elevated. In contrast, incorporation of [1,3-^{3}H]glycerol into DG and TG was reduced 33% ($P < 0.05$) and 80% ($P < 0.05$), respectively, compared with

controls. Since DGAT activity was reduced in etomoxir-treated cells (Table 3), we interpret this data to indicate that [1,3-^{3}H]glycerol was preferentially channeled toward glycerophospholipid biosynthesis at the expense of TG biosynthesis.

We next examined the effect of etomoxir on the biosynthesis of other phospholipids from their de novo pathways. H9c2 cells preincubated with 40 μM etomoxir for 2 h were then incubated with 28 μM [*methyl*-^{3}H]choline or 28 μM [^{3}H]ethanolamine for 2 h. [*Methyl*-^{3}H]choline incorporation into PtdCho was increased 31% ($P < 0.05$) and [^{3}H]ethanolamine incorporation into PtdEtn was increased 32% ($P < 0.05$) in cells incubated with etomoxir compared with controls (**Table 6**). In addition, [*methyl*-^{3}H]choline incorporation into sphingomyelin (SM) was increased 28% ($P < 0.05$) in cells incubated with etomoxir compared with controls. Total uptake of [*methyl*-^{3}H]choline or [^{3}H]ethanolamine into H9c2 cells was unaltered by etomoxir treatment. Thus, the presence of etomoxir resulted in a stimulation of de novo PtdCho and PtdEtn biosynthesis by their respective CDP-choline and CDP-ethanolamine pathways, consistent with the observed increase in biosynthesis from [1-^{14}C]palmitic acid and [1,3-^{3}H]glycerol.

H9c2 cells preincubated with 40 μM etomoxir for 2 h were then incubated with *myo*-[^{3}H]inositol or [^{3}H]serine, and the radioactivity incorporated into PtdIns and PtdSer examined. Treatment of cells with etomoxir resulted in a 61% ($P < 0.05$) decrease in radioactivity incorporated into PtdIns compared with controls (**Table 7**). Total uptake of inositol was reduced 26% ($P < 0.05$) in etomoxir-treated cells compared with controls, which would explain why incorporation of *myo*-[^{3}H]inositol into PtdIns was reduced. Total uptake of [^{3}H]serine into cells was unaltered by etomoxir. Treatment of cells with etomoxir resulted in a 22% ($P < 0.05$) decrease in radioactivity incorporated into PtdSer compared with controls. In contrast, etomoxir treatment resulted in a 47% ($P < 0.05$) increase in radioactivity incorporated into PtdEtn compared with controls. The reduction in radioactivity incorporated into PtdSer was quantitatively accounted for by the elevation in PtdEtn, suggesting that etomoxir stimulated PtdEtn synthesis from PtdSer. Thus, etomoxir inhibited de novo PtdIns biosynthesis and stimulated PtdEtn biosynthesis from PtdSer. Taken together, the above results clearly indicate that etomoxir treatment has diverse effects on de novo phospholipid biosynthesis from various metabolic precursors. In addition, etomoxir mediates a distinct and differential metabolic channeling of glycerol and fatty acid precursors into CL.

TABLE 6. Incorporation of [*methyl*-^{3}H]choline into PtdCho and SM and [^{3}H]ethanolamine incorporation into PtdEtn in etomoxir-treated cells

	Control	+ Etomoxir
	dpm × 10³/mg protein	
PtdCho	259.1 ± 29.2	339.3 ± 27.4[a]
SM	1.8 ± 0.1	2.3 ± 0.2[a]
Total uptake of [*methyl*-^{3}H]choline	1388.6 ± 127.4	1428.0 ± 227.7
PtdEtn	4.1 ± 0.4	5.4 ± 0.4
Total uptake of [^{3}H]ethanolamine	139.9 ± 16	150.1 ± 22

H9c2 cells were preincubated for 2 h in the absence or presence of 40 μM etomoxir and then incubated for 2 h in the presence of [*methyl*-^{3}H]choline or [^{3}H]ethanolamine, and the radioactivity incorporated into PtdCho, sphingomyelin (SM), and PtdEtn determined. The results represent the mean ± SD of four experiments.

[a] $P < 0.05$.

TABLE 7. Incorporation of *myo*-[^{3}H]inositol into PtdIns and [^{3}H]serine incorporation into PtdSer and PtdEtn in etomoxir-treated cells

	Control	+ Etomoxir
	dpm × 10³/mg protein	
myo-[^{3}H]Inositol incubated cells		
PtdIns	1.8 ± 0.3	0.7 ± 0.2[a]
Total uptake of radioactivity	901.1 ± 143.7	667.4 ± 73.4[a]
[^{3}H]Serine incubated cells		
PtdSer	27.2 ± 1.6	21.2 ± 1.8[a]
PtdEtn	8.3 ± 0.9	12.2 ± 0.7[a]
Total uptake of radioactivity	3665.5 ± 86.9	3612.2 ± 172.7

H9c2 cells were preincubated for 2 h in the absence or presence of 40 μM etomoxir, and then incubated for 2 h in the presence of *myo*-[^{3}H]inositol or [^{3}H]serine, and the radioactivity incorporated into PtdIns, PtdSer, and PtdEtn determined. The results represent the mean ± SD of four experiments.

[a] $P < 0.05$.

DISCUSSION

The objective of this study was to examine if etomoxir treatment stimulated CL biosynthesis in H9c2 cells and if etomoxir mediated metabolic channeling of fatty acid and glycerol precursors into CL. Our results indicate that etomoxir treatment of H9c2 cardiac myoblast cells produces a diverse plethora of effects on overall de novo glycerolipid biosynthesis from various metabolic precursors. In addition, etomoxir produces a distinct and differential metabolic channeling of glycerol and fatty acid precursors into CL in H9c2 cardiac myoblast cells.

The final steps of de novo CL biosynthesis have been shown to occur on the inner side of the inner mitochondrial membrane (55). Etomoxir is taken up by cells and rapidly converted to etomoxiryl-CoA, a potent inhibitor of mitochondrial fatty acid import and β-oxidation. The result is an accumulation of extramitochondrial fatty acid and an elevation in glucose oxidation. Treatment of H9c2 cells with etomoxir reduced the incorporation of [1-^{14}C]fatty acids into CL and PtdGro in H9c2 cardiac myoblast cells but did not affect total incorporation of radioactivity into these cells. In addition, the activities of the enzymes of the CDP-DG pathway of CL biosynthesis were unaltered by etomoxir treatment. These results indicated that the reduction in fatty acid incorporation into CL was not due to a reduction in [1-^{14}C]fatty acid uptake or a decrease in the de novo CL biosynthetic enzymes. In contrast, incorporation of [1-^{14}C]fatty acid into PtdCho and PtdEtn were elevated in etomoxir-treated cells. Finally, radioactive fatty acid incorporation into PtdIns was reduced by etomoxir treatment. These results initially indicated

that etomoxir treatment mediated a redirection of phospholipid biosynthesis from fatty acid precursors away from CDP-DG-utilizing pathways and toward DG-utilizing pathways. Recent studies have indicated that there may be selectivity of fatty acyl-CoA pools for various glycerolipid biosynthetic pathways (29–32). In the current study, the redirection in fatty acyl-CoA selectivity for lipid synthesis was mediated by an increase in membrane PAP activity in etomoxir-treated cells. However, in vitro PAP activity was unaltered in membranes incubated directly with etomoxiryl-CoA. Incubation of cells with etomoxir will produce an elevation in extramitochondrial fatty acid, and increase in extramitochondrial fatty acid should activate membrane PAP activity in vivo (52). Thus, the mechanism for the etomoxir-mediated reduction in fatty acid incorporation into PtdGro and CL appeared to be a decrease in fatty acid flux through the CDP-DG pathway.

Etomoxir treatment of H9c2 cells resulted in an elevation in the [1-^{14}C]palmitic acid-labeled DG pool available for PtdCho and PtdEtn biosynthesis. The etomoxir-mediated stimulation in PtdCho and PtdEtn biosynthesis from [1-^{14}C]palmitic acid was confirmed in the [*methyl*-^{3}H]choline and [^{3}H]ethanolamine labeling experiments. In these experiments, PtdCho and PtdEtn de novo biosynthesis by their respective CDP-choline and CDP-ethanolamine pathways were elevated by etomoxir. The large increase in [1-^{14}C]palmitic acid incorporated into PtdSer observed in the presence of etomoxir was likely due to an increase in the specific radioactivity of its immediate precursors, PtdEtn and PtdCho. Elevated phospholipid synthesis by etomoxir-induced inhibition of mitochondrial β-oxidation had been postulated (37), but until the current study had not been directly demonstrated. A previous study showed that incubation of murine hematopoietic cell lines with etomoxir stimulated sphingolipid synthesis (56). We observed elevated [*methyl*-^{3}H]choline incorporation into SM in H9c2 cells incubated with etomoxir consistent with an etomoxir-mediated elevation in sphingolipid synthesis. Surprisingly, etomoxir treatment reduced [1-^{14}C]palmitic acid incorporation into TG. DGAT activity was markedly reduced in these cells and etomoxiryl-CoA inhibited DGAT in vitro activity. These data suggested that the etomoxir-mediated reduction in fatty acid incorporation into TG was due to a preferential channeling of DG for glycerophospholipid synthesis over TG synthesis and support the hypothesis of selectivity of acute regulation of fatty acyl-CoA pools for various glycerolipid biosynthetic pathways (57).

The de novo enzymes for CL biosynthesis show little acyl group specificity (22, 23), and MLCL AT activity may be rate-limiting for the molecular remodeling of CL (58). Thus, it was possible that the reduction in [1-^{14}C]oleic acid incorporation into CL was due to a reduced availability of intramitochondrial fatty acid for CL remodeling or to alteration in the activity of the CL remodeling enzymes. Palmitic acid enters into rat heart CL mainly by the de novo biosynthetic pathway (28). Since a similar reduction in radioactivity incorporation into CL was seen with both [1-^{14}C]palmitic acid and [1-^{14}C]oleic acid, and the activities of the CL remodeling enzymes in mitochondrial fractions were unaltered in etomoxir-treated cells, it can be concluded that the reduction in [1-^{14}C]fatty acid incorporation into CL was not due to a reduced availability of intra-mitochondrial fatty acid for CL remodeling. In support of this was the observation that the reduction in [1-^{14}C]fatty acid incorporation into PtdGro, the immediate precursor of CL, was similar to that of CL in etomoxir-treated cells. In addition, mitochondrial GPAT activity was unaltered by etomoxir treatment. A previous study on the topography of PtdOH synthesis in rat liver mitochondria had indicated that lysoPtdOH and PtdOH are formed from *sn*-glycerol-3-phosphate and palmitoyl-CoA on the outer surface of the mitochondrial outer membrane and that this is followed by movement of PtdOH to the inner membrane for PtdGro and CL biosynthesis (59).

We expected to see a reduction in [1,3-^{3}H]glycerol head group incorporation into CL in cells treated with etomoxir since [1-^{14}C]fatty acid incorporation into CL was reduced and the activities of the enzymes of the CDP-DG pathway of CL biosynthesis were unaltered. Surprisingly, the opposite was observed. Treatment of cells with etomoxir resulted in an increase in [1,3-^{3}H]glycerol incorporation into CL and other glycerophospholipids. Since total [1,3-^{3}H]glycerol incorporation into cells was unaltered, these data indicated that etomoxir treatment mediated metabolic channeling of glycerol into CL and other glycerophospholipids. A recent study had implicated glycerol kinase as a rate-limiting step in muscle cell glycerol metabolism (54). We observed an increase in glycerol kinase activity in etomoxir-treated cells and etomoxiryl-CoA stimulated glycerol kinase in vitro activity. The increase in glycerol kinase activity in etomoxir-treated cells would explain the elevation in [1,3-^{3}H]glycerol incorporation into CL and other glycerophospholipids since GPAT activities were unaltered. [1,3-^{3}H]glycerol incorporation into TG was reduced by etomxoir treatment, indicating channeling of glycerol away from TG biosynthesis. The large reduction in DGAT activity observed in etomoxir-treated cells was likely responsible for the reduction in TG synthesis from glycerol. Surprisingly, [1,3-^{3}H]glycerol incorporation into DG was reduced in exotomoxir-treated cells. These data indicate the existence of separate pools of DG available for glycerolipid synthesis in H9c2 cells: a pool that may be rapidly utilized for glycerophospholipid synthesis and a pool utilized for general glycerolipid synthesis.

Surprisingly, etomoxir did not affect all de novo phospholipid biosynthesis from head group precursors in an identical manner. PtdIns de novo biosynthesis from *myo*-inositol was markedly inhibited in H9c2 cells in the presence of etomoxir. The mechanism was an etomoxir-mediated reduction in uptake of *myo*-inositol. Uptake of serine was unaltered in H9c2 cells treated with etomoxir, and PtdEtn biosynthesis from serine was elevated. At first glance it appeared that PtdSer biosynthesis from serine was reduced in etomoxir-treated cells. However, PtdEtn is synthesized from PtdSer by PtdSer decarboxylase in the mitochondria (60). Since the combined amount of radio-

activity incorporated into both PtdSer and PtdEtn was equivalent in both control and etomoxir-treated cells, it can be concluded that PtdSer biosynthesis was unaltered by etomoxir treatment and that PtdSer decarboxylation to PtdEtn was increased by etomoxir treatment.

The etomoxir-mediated alterations in de novo phospholipid biosynthesis are acute effects since etomoxir-induced alteration in gene expression requires prolonged periods (24 h) of incubation (36, 49, 50). In support of this was the observation that CL fatty acid composition and content were unaltered by incubation of H9c2 cells with etomoxir. A previous study had indicated that short-term incubation with etomoxir did not alter the fatty acid pattern of PtdCho in type I and type II rat muscle (61). In summary, our results indicate that etomoxir treatment has diverse effects on overall de novo glycerolipid biosynthesis from various metabolic precursors. In addition, etomoxir mediates a distinct and differential metabolic channeling of glycerol and fatty acid precursors into CL.

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REFERENCES

1. Hostetler, K. Y. 1982. Polyglycerophospholipids: phosphatidylglycerol, diphosphatidylglycerol and bis(monoacylglycerol)phosphate. *In* Phospholipids. J. N. Hawthorne and G. B. Ansell, editors. Elsevier Press, Amsterdam. 215–261.
2. Daum, G. 1985. Lipids of mitochondria. *Biochim. Biophys. Acta.* **882:** 1–42.
3. Poorthuis, B. J., P. J. Yazaki, and K. Y. Hostetler. 1976. An improved two-dimensional thin layer chromatrography system for the separation of phosphatidylglycerol and its derivatives. *J. Lipid Res.* **17:** 433–437.
4. Hovius, R., H. Lambrechts, K. Nicolay, and B. de Kruijff. 1990. Improved methods to isolate and subfractionate rat liver mitochondria. Lipid composition of the outer mitochondrial membrane. *Biochim. Biophys. Acta.* **1021:** 217–226.
5. Hovius, R., J. Thijssen, P. van der Linden, K. Nicolay, and B. de Kruijff. 1993. Phospholipid asymmetry of the outer membrane of rat liver mitochondria. Evidence for the presence of cardiolipin on the outside of the outer membrane. *FEBS Lett.* **330:** 71–76.
6. Vik, S. B., G. Georgevich, and R. A. Capaldi. 1981. Diphosphatidylglycerol is required for optimal activity of beef heart cytochrome c oxidase. *Proc. Natl. Acad. Sci. USA.* **78:** 1456–1460.
7. Fiol, C. J., and L. L. Bieber. 1984. Sigmoidal kinetics of purified beef heart mitochondrial carnitine palmitoyltransferase. *J. Biol. Chem.* **259:** 13084–13088.
8. Muller, M., R. Moser, D. Cheneval, and E. Carafoli. 1985. Cardiolipin is the membrane receptor for creatine phosphokinase. *J. Biol. Chem.* **260:** 3839–3843.
9. Hutson, S. M., S. Roten, and R. S. Kaplan. 1990. Solubilization and reconstitution of the branched-chain-alpha-keto acid transporter from rat heart mitochondria. *Proc. Natl. Acad. Sci. USA.* **87:** 1028–1031.
10. Kaplan, R. S., J. A. Mayor, N. Johnston, and D. L. Oliveira. 1990. Purification and characterization of the reconstitutively active tricarboxylate transporter from rat liver mitochondria. *J. Biol. Chem.* **265:** 13379–13385.
11. Belezani, Z. S., and V. Janesik. 1989. Role of cardiolipin in the function of the L-glycerol-3-phosphate dehydrogenase. *Biochem. Biophys. Res. Commun.* **159:** 132–139.
12. Kadenbach, B., P. Mende, H. V. Kolbe, I. Stipani, and F. Palmieri. 1982. The mitochondrial phosphate carrier has an essential requirement for cardiolipin. *FEBS Lett.* **139:** 109–112.
13. Hoffman, B., A. Stockl, M. Schlame, K. Beyer, and M. Klingenberg. 1994. The reconstituted ADP/ATP carrier activity has an absolute requirement for cardiolipin as shown in cysteine mutants. *J. Biol. Chem.* **269:** 1940–1944.
14. Eble, K. S., W. B. Coleman, R. R. Hantgan, and C. C. Cunningham. 1990. Tightly associated cardiolipin in bovine heart mitochondrial ATP synthase as analysed by ^{31}P nuclear magnetic resonance spectroscopy. *J. Biol. Chem.* **265:** 19434–19440.
15. Nomura, K., H. Imai, T. Koumura, T. Kobayashi, and Y. Nakagawa. 2000. Mitochondrial hydroperoxide glutathione peroxidase inhibits the release of cytochrome c from mitochondria by suppressing the peroxidation of cardiolipin in hypoglycemia-induced apoptosis. *Biochem. J.* **351:** 183–193.
16. Schlame, M., S. Brody, and K. Y. Hostetler. 1993. Mitochondrial cardiolipin in diverse eukaryotes: comparison of biosynthetic reactions and molecular acyl species. *Eur. J. Biochem.* **212:** 727–735.
17. Schlame, M., L. Hovath, and L. Vigh. 1990. Relationship between lipid saturation and lipid-protein interaction in liver mitochondria modified by catalytic hydrogenation with reference to cardiolipin molecular species. *Biochem. J.* **265:** 79–85.
18. Yamaoka, S., R. Urade, and M. Kido. 1990. Cardiolipin molecular species in rat heart mitochondria are sensitive to essential fatty acid-deficient dietary lipids. *J. Nutr.* **120:** 415–421.
19. Yamaoka-Koseki, S., R. Urade, and M. Kito. 1991. Cardiolipins from rats fed different diets affect bovine heart cytochrome c oxidase activity. *J. Nutr.* **121:** 956–958.
20. Kiyasu, J. Y., R. A. Pieriniger, H. Paulus, and E. P. Kennedy. 1963. The biosynthesis of phosphatidylglycerol. *J. Biol. Chem.* **238:** 2293–2298.
21. Hostetler, K. Y., H. Van den Bosch, and L. L. M. Van Deenen. 1971. Biosynthesis of cardiolipin in rat liver. *Biochim. Biphys. Acta.* **239:** 113–119.
22. Rustow, B., M. Schlame, H. Rabe, G. Reichman, and D. Kunze. 1989. Species pattern of phosphatidic acid, diacylglycerol, CDP-diacylglycerol and phosphatidylglycerol synthesized *de novo* in rat liver mitochondria. *Biochim. Biophys. Acta.* **1002:** 261–263.
23. Hostetler, K. Y., J. M. Galesloot, P. Boer, and H. van den Bosch. 1975. Further studies on the formation of cardiolipin and phosphatidylglycerol in rat liver mitochondria: effect of divalent cations and the fatty acid composition of CDP-diglyceride. *Biochim. Biophys. Acta.* **380:** 382–389.
24. Schlame, M., and B. Rustow. 1990. Lysocardiolipin formation and regulation in isolated rat liver mitochondria. *Biochem. J.* **272:** 589–595.
25. Nachbaur, J., and P. M. Vignais. 1968. Localization of phospholipase A_2 in outer membrane of mitochondria. *Biochem. Biophys. Res. Commun.* **33:** 315–320.
26. Severina, E. P., and I. Evtodienko. 1981. Phospholipase A_2 localization in mitochondria. *Biokhimiia.* **46:** 1199–1201.
27. Levrat, C., and P. Louisot. 1992. Dual localization of the mitochondrial phospholipase A_2: outer membrane contact sites and inner membrane. *Biochem. Biophys. Res. Commun.* **183:** 719–724.
28. Ma, B. J., W. A. Taylor, V. Dolinsky, and G. M. Hatch. 1999. Acylation of monolysocardiolipin in rat heart. *J. Lipid Res.* **40:** 1837–1845.
29. Muoio, D. M., T. M. Lewin, P. Weidmar, and R. A. Coleman. 2000. Acyl-CoA's are functionally channelled in liver: Potential role of acyl-CoA synthetase. *Am. J. Physiol.* **279:** E1366–E1373.
30. Fulgencio, J. P., C. Kohl, J. Girard, and J. P. Pegorier. 1996. Troglitazone inhibits fatty acid oxidation and esterification, and gluconeogenesis in isolated hepatocytes from starved rats. *Diabetes.* **45:** 1556–1562.
31. Coleman, R. A., T. M. Lewin, and D. M. Muoio. 2000. Physiological and nutritional regulation of enzymes of triacylglycerol synthesis. *Annu. Rev. Nutr.* **20:** 77–103.
32. Hatch, G. M., A. J. Smith, F. Y. Xu, A. M. Hall, and D. A. Bernlohr. 2002. FATP1 channels exogenous FA into 1,2,3-triacyl-sn-glycerol and down-regulates sphingomyelin and cholesterol metabolism in growing 293 cells. *J. Lipid Res.* **43:**. In press.
33. Bartlett, K., D. M. Turnball, and H. S. A. Sherratt. 1984. Inhibition of hepatic and skeletal muscle carnitine palmitoyltransferase I by 2[5(4-chlorophenyl)pentyl]-oxirane-2-carboxyl-CoA. *Biochem. Soc. Trans.* **12:** 688–689.
34. Bristow, M. 2000. Etomoxir: a new approach to treatment of heart failure. *Lancet.* **356:** 1621–1622.
35. Schmidt-Schweda, S., and C. Holubarsch. 2000. First clinical trial with etomoxir in patients with chronic congestive heart failure. *Clin. Sci.* **99:** 27–35.

36. Hegardt, F. G., D. Serra, and G. Asins. 1995. Influence of etomoxir on the expression of several genes in liver, testes and heart. *Gen. Pharmacol.* **26:** 897–904.
37. Dhalla, N. S., V. Elimban, and H. Rupp. 1992. Paradoxical role of lipid metabolism in heart function and dysfunction. *Mol. Cell. Biochem.* **116:** 3–9.
38. Hatch, G. M., and G. McClarty. 1996. Regulation of cardiolipin biosynthesis in H9c2 cardiac myoblast cells by cytidine-5′-triphosphate. *J. Biol. Chem.* **271:** 25810–25816.
39. Morillas, M., J. Clotet, B. Rubi, D. Serra, J. Arino, F. G. Hegardt, and G. Asins. 2000. Inhibition by extomoxir of rat liver carnitine octanoyltransferase is produced through the coordinate interaction with two histidine residues. *Biochem. J.* **351:** 495–502.
40. Thorner, J. W. 1975. Glycerol kinase. *Methods Enzymol.* **42:** 148–156.
41. Hatch, G. M., S. G. Cao, and A. Angel. 1995. Decrease in cardiac phosphatidylglycerol in streptozotocin-induced diabetic rats does not affect cardiolipin biosynthesis: evidence for distinct pools of phosphatidylglycerol in the heart. *Biochem. J.* **306:** 759–764.
42. Haldar, D., and A. Vancura. 1992. Glycerophosphate acyltransferase from liver. *Methods Enzymol.* **209:** 64–72.
43. Coleman, R. A. 1992. Diacylglycerol acyltyransferase and monoacylglycerol acyltransferase from liver and intestine. *Methods Enzymol.* **209:** 98–102.
44. Gomez-Munoz, A., G. M. Hatch, A. Martin, Z. Jamal, D. E. Vance, and D. N. Brindley. 1992. Effects of okadaic acid on the activities of two distinct phosphatidate phosphohydrolases in rat hepatocytes. *FEBS Lett.* **301:** 101–106.
45. Tardi, P. J., J. J. Mukherjee, and P. C. Choy. 1992. The quantitation of long chain acyl-CoA in mammalian tissue. *Lipids.* **27:** 65–67.
46. Rouser, G., S. Fleischer, and A. Yamamoto. 1970. Two dimensional thin layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. *Lipids.* **5:** 494–496.
47. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
48. Kerner, J., and C. Hoppel. 2000. Fatty acid import into mitochondria. *Biochim. Biophys. Acta.* **1486:** 1–17.
49. Spurway, T. D., H. S. A. Sherratt, C. I. Pogson, and L. Agius. 1997. The flux control coefficient of carnitine palmitoyltransferase I on palmitate beta-oxidation in rat hepatocyte cultures. *Biochem. J.* **323:** 119–122.
50. Cabrero, A., M. Alegret, R. Sanchez, T. Adzet, J. C. Laguna, and M. Vazquez. 1999. Etomoxir, sodium-2-[6-(4-chlorophenoxy)hexyl]-oxirane-2-carboxylate upregulates uncoupling protein-3 mRNA levels in primary culture of rat preadipocytes. *Biochem. Biophys. Res. Commun.* **263:** 87–93.
51. Akesson, B. 1970. Initial esterification and conversion of intrapotally injected [1–14C]linoleic acid in rat liver. *Biochim. Biophys. Acta.* **218:** 57–70.
52. Brindley, D. N., and D. Waggoner. 1998. Mammalian lipid phosphatidate phosphohydrolases. *J. Biol. Chem.* **273:** 24281–24284.
53. Robinson, J., and E. A. Newsholme. 1969. The effects of dietary conditions and glycerol concentration on glycerol uptake in rat liver and kidney-cortex slices. *Biochem. J.* **122:** 449–453.
54. Montell, E., C. Lerin, C. B. Newgard, and A. M. Gomez-Foix. 2002. Effects of modulation of glycerol kinase expression on lipid and carbohydrate metabolism in human muscle cells. *J. Biol. Chem.* **277:** 2682–2686.
55. Schlame, M., and D. Halder. 1993. Cardiolipin is synthesized on the matrix side of the inner membrane in rat liver mitochondria. *J. Biol. Chem.* **268:** 74–79.
56. Paumen, M. B., Y. Ishida, M. Muramatsu, M. Yamamoto, and T. Honjo. 1997. Inhibition of carnitine palmitoyltransferase I augments sphingolipid synthesis and palmitate-induced apoptosis. *J. Biol. Chem.* **272:** 3324–3329.
57. Lewin, T. M., J. H. Kim, D. A. Granger, J. E. Vance, and R. A. Coleman. 2001. Acyl-CoA synthetase isoforms 1, 4 and 5 are present in different subcellular membranes in rat liver and can be inhibited independently. *J. Biol. Chem.* **276:** 24674–24679.
58. Mutter, T., D. W. Dolinsky, B. J. Ma, W. A. Taylor, and G. M. Hatch. 2000. Thyroxine regulation of monolysocardiolipin activity in rat heart. *Biochem. J.* **346:** 403–406.
59. Chakraborty, T. R., A. Vancura, V. S. Balija, and D. Haldar. 1999. Phosphatidic acid synthesis in mitochondria: Topography of formation and transmembrane migration. *J. Biol. Chem.* **274:** 29786–29790.
60. Butler, M. M., and W. Thompson. 1975. Transfer of phosphatidylserine from liposomes or microsomes to mitochondria. Stimulation by a cell supernatant factor. *Biochim. Biophys. Acta.* **388:** 52–57.
61. Blackard, W. G., J. Li, J. N. Clore, and W. B. Rizzo. 1997. Phospholipid fatty acid composition in type I and type II rat muscle. *Lipids.* **32:** 193–198.