Stimulation of cardiac cardiolipin biosynthesis by PPARα activation

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Abstract **The role of peroxisome proliferator-activated receptor α (PPARα)-stimulated phospholipase A_2 (PLA_2) in cardiac mitochondrial cardiolipin (CL) biosynthesis was examined in both in vivo and in vitro models. Treatment of rat heart H9c2 cells with clofibrate increased the expression and activity of 14 kDa PLA_2 but did not affect the pool size of CL. Clofibrate treatment stimulated de novo CL biosynthesis via an increase in phosphatidylglycerolphosphate (PGP) synthase activity, accounting for the unaltered CL content. Cardiac PLA_2, PGP synthase, and CDP-1,2-diacyl-*sn*-glycerol synthase (CDS-2) activities and CDS-2 mRNA levels were elevated in mice fed clofibrate for 14 days compared with controls. In PPARα-null mice, clofibrate feeding did not alter cardiac PLA_2, PGP synthase activities, or CDS-2 activity and mRNA level, confirming that these enzymes are regulated by PPARα activation. In contrast to mouse heart, clofibrate treatment did not affect the activity or mRNA levels of CDS-2 in H9c2 cells, indicating that CDS-2 is regulated differently in rat heart H9c2 cells in vitro and in mouse heart in vivo. These results clearly indicate that cardiac CL de novo biosynthesis is stimulated by PPARα activation in responsive rodent models and that CDS-2 is an example of an enzyme that exhibits alternative regulation in vivo and in cultured cell lines. This study is the first to demonstrate that CL de novo biosynthesis is regulated by PPARα activation.**—Jiang, Y. J., B. Lu, F. Y. Xu, J. Gartshore, W. A. Taylor, A. J. Halayko, F. J. Gonzalez, J. Takasaki, P. C. Choy, and G. M. Hatch. **Stimulation of cardiac cardiolipin biosynthesis by PPARα activation.** ***J. Lipid Res.*** **2004.** 45: **244–252.**

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Phospholipids are important structural and functional components of the biological membrane (1). Structurally, as major components of the biological membrane, they define the compartmentalization of organelles and the protective barrier, the cell membrane, that surrounds cells. An important class of phospholipids are the polyglycerophospholipids. The major polyglycerophospholipid found in most mammalian tissues is bis-(1,2-diacyl-*sn*-glycero-3-phospho)-1′,3′-*sn*-glycerol or cardiolipin (CL) (2). CL was first isolated from beef heart in 1942 by Pangborn (3). In rat liver, CL was initially shown to be associated exclusively with the inner mitochondrial membrane (4). However, using various fractionation techniques, CL was identified also in the mitochondrial outer membrane of rat liver mitochondria (5). In rat heart, CL is a major phospholipid component that constitutes ∼15% of the entire phospholipid phosphorus mass of the organ (6, 7). CL is involved in modulation of the activity of a number of mitochondrial membrane enzymes involved in the generation of ATP (8). It is well documented that alteration in the content and the molecular species composition of CL alters mitochondrial enzyme activity and oxygen consumption in mammalian mitochondria (9, 10). Recently, CL has been implicated as an important mitochondrial factor that may be involved in the regulation of apoptosis (11, 12). Thus, maintenance of the appropriate content of CL in cardiac mitochondria is essential for proper cardiac function.

The de novo biosynthesis of CL in the rat heart occurs via the CDP-1,2-diacyl-*sn*-glycerol (CDP-DG) pathway (2). Phosphatidic acid (PA) is converted to CDP-DG by CDP-DG synthase (CDS; EC 2.2.7.41) (13). In the rat heart and in embryonic rat heart-derived H9c2 myoblast cells, CDS may be a rate-limiting enzyme for CL biosynthesis (7, 14).

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Abbreviations: CDP-DG, CDP-1,2-diacyl-*sn*-glycerol; CDS, CDP-DG synthase; CL, cardiolipin; PA, phosphatidic acid; PG, phosphatidylglycerol; PGP, phosphatidylglycerolphosphate; PLA_2, phospholipase A_2; PPARα, peroxisome proliferator-activated receptor α.

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The CDP-DG formed condenses with glycerol-3-phosphate to form phosphatidylglycerol (PG) catalyzed by PG phosphate (PGP) synthase (EC 2.7.8.5) and PGP phosphatase (EC 3.1.3.27) (13). In the final step, PG is converted to CL by condensation with CDP-DG catalyzed by CL synthase (15). CL may be hydrolyzed by mitochondrial phospholipase A_2 (PLA_2) (2). Previously, we demonstrated that mitochondrial PLA_2 in rat embryonic H9c2 myoblastic cells could be activated by short-chain ceramides or tumor necrosis factor-α (TNFα) (16). This activation of mitochondrial PLA_2 led to an increase in CL biosynthesis that was mediated by stimulation of mitochondrial PGP synthase activity.

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors that belong to the nuclear hormone receptor superfamily (17–19). Three forms have been identified: PPARα, PPARβ, and PPARγ. It is well documented that the rat heart contains PPARα. Ethyl-*p*-chlorophenoxyisobutyrate (clofibrate) is a synthetic lipid-lowering agent used in clinical practice. The effect of clofibrate on lipid metabolism in rodent heart is mediated by PPARα after heterodimering with the retinoid X receptor and interacting with PPAR response elements in the promotor regions of target genes. A recent study indicated that the mRNA of uncoupling protein-2 was elevated in neonatal cardiomyocytes and embryonic rat heart-derived H9c2 myoblast cells, probably through PPARα activation (20). However, the presence of PPARα receptors in H9c2 cells was not examined in that study. Because PPARα activation stimulates PLA_2 activity (21, 22), the role of this receptor in CL biosynthesis was examined in embryonic rat heart H9c2 myoblastic cells and mouse heart. The results show that activation of PPARα mediates increased CL biosynthesis and metabolism via an increase in mitochondrial PLA_2 and PGP synthase activities. However, activation of CDS-2, a rate-limiting enzyme of CL biosynthesis in rat heart embryonic H9c2 cells, is not necessary for the PPARα agonist-mediated stimulation of de novo CL biosynthesis.

MATERIALS AND METHODS

Materials

[^{14}C]Glycerol-3-phosphate, [5-^{3}H]CTP, [1,3-^{3}H]glycerol, and [1-^{14}C]oleate were obtained from either Dupont (Mississauga, Ontario, Canada) or Amersham (Oakville, Ontario, Canada). [^{14}C]phosphatidylglycerol was synthesized from [^{14}C]glycerol-3-phosphate (14). DMEM and FBS were products of Canadian Life Technologies (GIBCO; Burlington, Ontario, Canada). Lipid standards were obtained from Serdary Research Laboratories (Englewood Cliffs, NJ). Thin-layer plates (silica gel G; 0.25 mm thickness) were obtained from Fisher Scientific (Winnipeg, Canada). Ecolite scintillant was obtained from ICN Biochemicals (Montreal, Canada). Rat heart H9c2 myoblastic cells were obtained from the American Type Culture Collection. Rabbit anti-rat PPARα polyclonal antibody and anti-rat actin antibody were purchased from Affinity BioReagents, Inc. (Golden, CO). Monoclonal anti-cPLA_2 antibody was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). For confocal immunocytochemistry, secondary FITC-conjugated goat anti-rabbit antibodies were obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Rabbit anti-rat PLA_2-IIA polyclonal antibody (protein A purified) was a generous gift from Dr. J. Takasaki (Molecular Medicine Laboratories, Institute for Drug Discovery Research, Yamanouchi Pharmaceutical Co., Ltd.). The RPN 2108 ECL Western blotting analysis system was used for PPARα and PLA_2 expression studies and was obtained from Amersham Pharmacia Biotech UK, Ltd. (Buckinghamshire, UK). Kodak X-OMAT film was obtained from Eastman Kodak Co. (Rochester, NY). All other biochemicals were certified American Chemical Society grade or better and obtained from either Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific.

Animals

Male C57BL/6N × Sv/129 mice (25–35 g) were obtained from Central Animal Care Services (University of Manitoba, Winnipeg, Manitoba, Canada). PPARα-null (−/−) mice were a generous gift from Dr. Frank Gonzalez (National Institutes of Health). Treatment of mice conformed to the guidelines of the Canadian Council on Animal Care. The mice were maintained on rat chow and water ad libitum. All animals were kept in identical housing units on a cycle of 12 h of light and 12 h of darkness. In treatment groups, mice were fed 0.5% clofibrate in a chow diet for 14 days (23).

Culture, radiolabeling, and harvesting of H9c2 cells

Rat heart H9c2 myoblastic cells were grown and incubated in DMEM containing 10% FBS until 90% confluence was achieved. Cells were then incubated for 48 h with DMEM containing 10% FBS in the absence or presence of 200 μM clofibrate or 5 μM WY14,643. The medium was changed once after 24 h. In radiolabeling experiments, cells were then incubated with DMEM containing 10% FBS for up to 3 h with 0.1 μM [1,3-^{3}H]glycerol (10 mCi/ml medium). The medium was removed and the cells were washed twice with ice-cold saline and then harvested from the dish with 2 ml of methanol-water (1:1, v/v) for lipid extraction. CL was separated from other phospholipids by two-dimensional TLC, and radioactivity incorporated into CL and other phospholipids was determined as described (14). In some experiments, H9c2 cells were preincubated with 0.1 μM [1-^{14}C]oleate (1.0 mCi/ml medium) for 24 h and then incubated in the absence or presence of 200 μM clofibrate for up to 8 h, and the radioactivity incorporated into CL was determined.

Determination of in vitro enzyme activities

H9c2 cells were incubated for 48 h with DMEM containing 10% FBS in the absence or presence of 200 μM clofibrate. The medium was changed once after 24 h. Subsequently, the cells were washed twice with ice-cold saline and harvested with 2 ml of homogenization buffer (10 mM Tris-HCL, pH 7.4, and 0.25 M sucrose). The cells were homogenized with 15 strokes of a Dounce A homogenizer. The homogenate (designated the cell lysate) was centrifuged at 1,000 *g* for 5 min, and the resulting supernatant was centrifuged at 10,000 *g* for 15 min. The pellet was resuspended in 0.5 ml of homogenization buffer and designated the mitochondrial fraction. CDS, PGP synthase, and CL synthase activities were determined as described (14). In some experiments, 200 μM clofibrate was added directly to the enzyme incubation mixture of mitochondrial fractions prepared from untreated H9c2 cells, and CDS, PGP synthase, and CL synthase activities were determined. PLA_2 activity was determined as described (24). PA phosphohydrolase activity also was determined as described (25).

Electrophoresis and Western blot analysis

H9c2 cells were incubated for 48 h with DMEM containing 10% FBS in the absence or presence of 50 μM clofibrate. The medium was changed once after 24 h. The cells were harvested and homogenized, and mitochondrial fractions were prepared as described

above. A 50 μg aliquot of the homogenate or mitochondrial fraction was subjected to SDS-7.5% PAGE with molecular weight standards using a Bio-Rad (Hercules, CA) Mini-Protean® II Dual Slab Cell electrophoresis unit. Proteins were transferred from the gel onto polyvinylidene difluoride (PVDF) membranes by incubation for 30 min at 15 V using a Bio-Rad Trans-Blot SD Semi-Dry Transfer Cell. Expression of PPARα was examined by incubating the PVDF membrane with the polyclonal anti-rat PPARα antibody (1:1,000 dilution) dissolved in Tris-buffered saline (TBS) containing 0.1% Tween 20 and 2% skim milk overnight at 4°C. Subsequently, the membrane was washed and incubated with peroxidase-labeled anti-rabbit secondary antibody (1:5,000) for 5–30 min at room temperature. Protein bands in the membrane were visualized by enhanced chemiluminescence. For detection of 14 kDa PLA_2, after transfer to PVDF membranes, the membranes containing protein samples were incubated with monoclonal anti-PLA_2 antibody (1:1,000). The membrane was then washed and incubated with the peroxidase-labeled anti-rabbit secondary antibodies (1:5,000) for 5–30 min at room temperature. The relative intensities of the bands were analyzed by scanning the film and subsequently determined using Scion Image software (22).

Immunofluorescence confocal microscopy analysis

Sterilized glass coverslips were transferred to individual wells of 12-well culture plates. H9c2 cells were seeded on the plates (40,000 cells/well) for 24 h. Cells were then incubated for 48 h with DMEM containing 10% FBS in the absence or presence of 50 μM clofibrate (40,000 cells/well). The medium was changed once after 24 h. The medium was then removed, and the cells were fixed with 3% paraformaldehyde for 15 min and then permeabilized using 3% paraformaldehyde and 0.3% Triton X-100 for 5 min at room temperature. The cells were next rinsed twice with buffer (10 mM MES, 150 mM NaCl, 5 mM EGTA, 5 mM $MgCl_2$, and 5 mM glucose, pH 6.1), stored in sealed culture chambers with 1 ml of TBS buffer (20 mM Tris-HCl, 150 mM NaCl, 2.0 mM EGTA, and 2.0 mM $MgCl_2$, pH 7.2), and stored. Cells could be stored for up to 2 weeks before analysis. The cells were then incubated with TBS containing 1% BSA and 2% sheep serum for 1 h. Coverslips were removed and inverted onto 20 μl of the primary anti-$cPLA_2$ antibody and incubated overnight at 4°C in a humidified chamber. The coverslip was then placed cell side up in 12-well culture dishes that were then immediately filled with 3 ml of TBS buffer containing 0.1% Tween 20. After 10 min, the buffer was removed and washed twice with TBS buffer containing 0.1% Tween 20. Coverslips were removed, inverted onto 20 μl of the secondary antibody (diluted 1:100), and incubated in the dark for 2–3 h at room temperature in a humidified chamber. The coverslips were then washed three times as described above with TBS buffer containing 0.1% Tween 20. DNA of the cells was stained with propidium iodide (1 μg/ml) in TBS buffer containing RNase (40 μg/ml). After staining, coverslips were washed three times with double- distilled water and then twice with TBS buffer containing 0.1% Tween 20. Coverslips were then washed three times with double-distilled water and mounted cell side down onto glass slides prespotted with ProLong AntiFade mounting medium (Molecular Probes, Inc., Eugene, OR). Confocal image stacks, consisting of three to five sections obtained at 0.5 mm vertical intervals, were obtained using an Olympus (Tokyo, Japan) LX70 inverted microscope equipped with FluoView Laser Scanning hardware, including argon and krypton lasers to generate 488 and 568 nm lines for the detection of FITC and propidium iodide fluorescence, respectively.

RT-PCR analysis

The cDNA for CDS-2 was amplified with a pair of specific primers synthesized by Invitrogen™ Life Technologies (Carlsbad, CA). The rat CDS-2 primers used for the RT-PCR experiments were previously described (26): CDS-2 (U), 5′-GAATCAGAGTCGGAAGCA; and CDS-2 (L), 5′-ACCAGGGCAAGTCGTAG. H9c2 cells were incubated for up to 72 h with 200 μM clofibrate, and total RNA was isolated using the Trizol reagent according to the manufacturer's instructions. The RNA pellet was suspended in autoclaved, double-distilled water and quantitated by absorbance at 260 nm using the 260:280 nm ratio as an index of purity. The integrity of the RNA was confirmed by denaturing agarose gel electrophoresis of the isolated RNA sample. The first-strand cDNA from 1 μg of total RNA was synthesized using 150 U of Moloney murine leukemia virus RT, 25 pmol of random hexamer primer, 20 U of RNase inhibitor, 1 mM dithiothreitol, and 10 pmol each of the four deoxynucleotides, in a total volume of 15 μl. The reaction mixture was incubated at 37°C for 1 h and terminated by boiling the sample at 95°C for 5 min. An aliquot of the resulting cDNA preparation was used directly for each amplification reaction. PCR was performed in 20 μl reaction mixtures containing 8 pmol of primer, 8 pmol of each deoxynucleotide trisphosphate, and 0.4 U of *Taq* DNA polymerase. The mixture was overlaid with 30 μl of mineral oil to prevent evaporation and was incubated in a Perkin-Elmer (Foster City, CA) DNA Thermal Cycler under the following conditions. For the PCR of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) CDS-2: 25–30 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 55–60°C, and extension for 2 min at 72°C. The amplified RT-PCR product was analyzed by 1.2–1.8% agarose gel electrophoresis in 1× TAE buffer (40 mM Tris acetate and 2 mM sodium EDTA) and visualized by staining with 0.5 μg/μl ethidium bromide. The GAPDH band was used as an internal control. An increase in the level of a specific mRNA is caused by an increase in its rate of synthesis, a decrease in its rate of degradation, or a combination of these two processes. Hence, mRNA stability assays were conducted using actinomycin D as an inhibitor of RNA synthesis. H9c2 cells were incubated with 200 μM clofibrate for 48 h, and 5 μg/ml actinomycin D was added after clofibrate treatment. The mRNA levels for CDS-2 and GAPDH were determined by RT-PCR at 4 h intervals after the actinomycin treatment. No apparent changes in mRNA degradation were observed within a 24 h period, indicating that clofibrate did not cause any change in the degradation of CDS-2 mRNA.

Based on the primer pair used for rat CDS-2, a homologous primer pair for mouse, 5′-GAATCAGAGTCCGAAGCA and 5′-CTACGACCTGCCCTGGT (GenBank identifier gi31542373), was designed for RT-PCR of CDS-2 in mouse heart (GenBank identifier gi31542373). Primers for β-actin were 5′-GTGGGGCGCCCCAGGCACCA-3′ and 5′-CTCCTTAATGTCACGCACGATTTG-3′ The conditions for amplification were 25 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 55°C, and extension for 30 s at 72°C. The amplified RT-PCR product was analyzed as described above.

Other determinations

Protein was determined by the method of Lowry et al. (27). Student's *t*-test was used to determine statistical significance. The level of significance was defined as $P < 0.05$.

RESULTS

Clofibrate stimulates the nuclear association of PPARα in H9c2 cells

Acute activation of mitochondrial PLA_2 by TNFα or short-chain ceramide resulted in the stimulation of CL biosynthesis in rat heart H9c2 cardiac myoblast cells (16).

Previous studies indicated that PPARα activation resulted in an increase in PLA_2 activity in human premonocytic cells and in rat mesangial cells (21, 22). Because the heart contains PPARα, we examined whether activation of PLA_2 through a TNFα- and ceramide-independent mechanism stimulated CL biosynthesis. Initially, we examined the nuclear expression of PPARα in H9c2 cells with polyclonal anti-rat PPARα antibody. H9c2 cells were grown to 90% confluence and harvested, and nuclear fractions were prepared. PPARα receptor was expressed in the nuclear fraction of H9c2 cells (**Fig. 1**).

PPARα activation in rat Morris cells by ciprofibrate resulted in increased nuclear association of PPARα (28). We examined whether the addition to H9c2 cells of clofibrate, a known pharmacological activator of PPARα, resulted in increased nuclear association of PPARα. H9c2 cells were incubated for up to 4 h with 200 μM clofibrate and then harvested, and nuclear fractions were prepared. We previously demonstrated that this concentration of clofibrate was shown to result in PPARα activation in SW872 preadipocytes (22). Incubation of H9c2 cells with clofibrate resulted in a rapid increase in nuclear PPARα compared with untreated control cells (Fig. 1). It is known that clofibrate may be toxic to tissue culture cells in high concentrations. We observed that 48 h of incubation of H9c2 cells with 200 μM clofibrate did not affect the ability of these cells to exclude Trypan blue (98% exclusion). Thus, H9c2 cells contain PPARα, and clofibrate addition to H9c2 cells resulted in the activation of PPARα.

Activation of PPARα in H9c2 cells stimulates mitochondrial PLA_2 activity

To determine whether the activation of PPARα in H9c2 cells increased PLA_2 activity, cells were incubated in the absence or presence of 200 μM clofibrate for 48 h and lysed, and PLA_2 activity was determined. As seen in **Table 1**, treatment of H9c2 cells with clofibrate resulted in a 32% increase ($P < 0.05$) in membrane PLA_2 activity compared with control cells. We examined the mechanism for the increase in PLA_2 activity. We previously demonstrated that the expression of cytosolic PLA_2 activity and protein expression were upregulated by PPARα in human premonocytic cells (22). H9c2 cells were incubated for 48 h with 200 μM clofibrate, and the presence of cytosolic PLA_2 was examined by immunofluorescence confocal microscopy. Clofibrate incubation of H9c2 cells resulted in an increase in cytosolic PLA_2 that was chiefly marked by the appearance of numerous dense foci of cytosolic staining near the periphery of each cell (**Fig. 2**). Thus, PPARα activation by clofibrate resulted in an increase in the expression of cytosolic PLA_2. However, because cytosolic PLA_2 does not have access to CL in mitochondria (29), we examined 14 kDa PLA_2 activity and protein expression in mitochondria. H9c2 cells were incubated for 48 h with 200 μM clofibrate and lysed, mitochondrial fractions were prepared, PLA_2 activity was determined, and the presence of 14 kDa PLA_2 was examined by Western blot analysis. In-

TABLE 1. PLA_2 activities in H9c2 cells treated with clofibrate

Sample	Enzyme Activity	
	Control	Clofibrate
	pmol/min/mg protein	
Cell lysates	56 ± 7	74 ± 5[a]
Mitochondrial fraction	150 ± 14	201 ± 21[a]

H9c2 cells were incubated in the absence or presence of 200 μM clofibrate for 48 h. The cells were lysed, and enzyme activities were determined in the cell lysates and in mitochondrial fractions. Values represent means ± SD of three dishes. PLA_2, phospholipase A_2.
[a] $P < 0.05$.

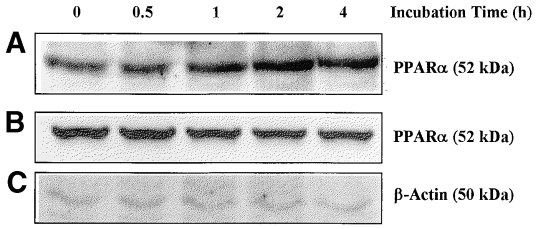

Fig. 1. Western blot analysis of peroxisome proliferator-activated receptor α (PPARα) expression in rat heart H9c2 myoblastic cells. H9c2 cells were preincubated in the absence or presence of 200 μM clofibrate for up to 4 h, and nuclear fractions were prepared. Expression of PPARα was examined using a polyclonal anti-rat PPARα antibody as described in Materials and Methods (primary anti-PPARα antibody, 1:500 dilution; secondary antibody, 1:2,000 dilution). A: Plus clofibrate. B: Minus clofibrate. C: Actin control (plus clofibrate). Representative blots are shown.

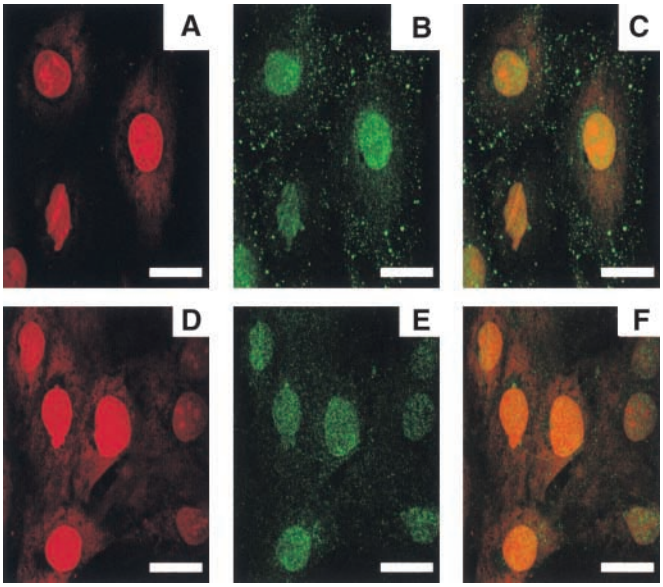

Fig. 2. Phospholipase A_2 ($cPLA_2$) expression in rat heart H9c2 myoblastic cells treated with clofibrate. Cells were permeabilized and pretreated with polyclonal anti-rat $cPLA_2$ antibody followed by secondary antibody and visualized by confocal microscopy as described in Materials and Methods. A–C: Clofibrate-treated cells. D–F: Untreated cells. A and D show nuclear stains; B and E show secondary antibody stains; and C and F show superimpositions of nuclear and secondary antibody stains. Representative micrographs are shown. Bars = 40 μm.

cubation of H9c2 cells with clofibrate resulted in a 34% increase ($P < 0.05$) in mitochondrial PLA_2 activity (Table 1) and a corresponding ~40% increase in 14 kDa PLA_2 protein associated with cell lysate membranes and mitochondrial membranes compared with controls (**Fig. 3A**, B). The 14 kDa PLA_2 protein was not observed in cytosolic fractions, indicating that there was no loss of the enzyme to the cytosol during subcellular fractionation (Fig. 3B). Thus, PPARα activation by clofibrate resulted in increases in mitochondrial 14 kDa PLA_2 enzyme activity and protein expression in H9c2 cells.

Activation of PPARα in H9c2 cells stimulates CL biosynthesis

Because mitochondrial PLA_2 activity was elevated in H9c2 cells treated with clofibrate, we examined if the pool size of CL was reduced in these cells. The CL pool size was 7.5 ± 0.2 nmol/mg cellular protein in H9c2 cells (average of three separate dishes) and was unaltered by clofibrate treatment (7.6 ± 0.2 nmol/mg protein). We examined the metabolism of CL in cells incubated with clofibrate using a pulse-chase experimental approach. H9c2 cells were incubated for 24 h with [1-^{14}C]oleate, the medium was removed, the cells were incubated in the absence or presence of 200 μM clofibrate for up to 8 h, and the radioactivity incorporated into CL was determined. Radioactivity incorporated into CL after 24 h of incubation with [1-^{14}C]oleate was 4.73 ± 0.21 × 10^4 dpm/mg protein (**Fig. 4**). Radioactivity associated with CL was reduced during the chase in control cells but was reduced at a greater rate in cells treated with clofibrate. Thus, the presence of clofibrate resulted in an increase in CL turnover in H9c2 cells. Because the pool size of CL was unaltered yet mitochondrial PLA_2 and CL metabolism were increased by clofibrate treatment, it was possible that CL biosynthesis was

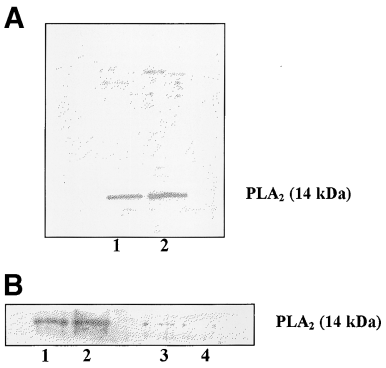

Fig. 3. Expression of 14 kDa PLA_2 in rat heart H9c2 myoblastic cells treated with clofibrate. H9c2 cells were preincubated in the absence or presence of 200 μM clofibrate for 48 h. The cells were then lysed, and mitochondrial fractions were prepared. PLA_2 protein was determined in lysates (A) and mitochondrial fractions (B) using Western blot analysis, and the relative intensities of the bands were analyzed using Scion Image software. A: Lane 1, control cells; lane 2, clofibrate-treated cells. B: Lane 1, mitochondria from control cells; lane 2, mitochondria from clofibrate-treated cells; lane 3, cytosol from control cells; lane 4, cytosol from clofibrate-treated cells. Representative blots are shown.

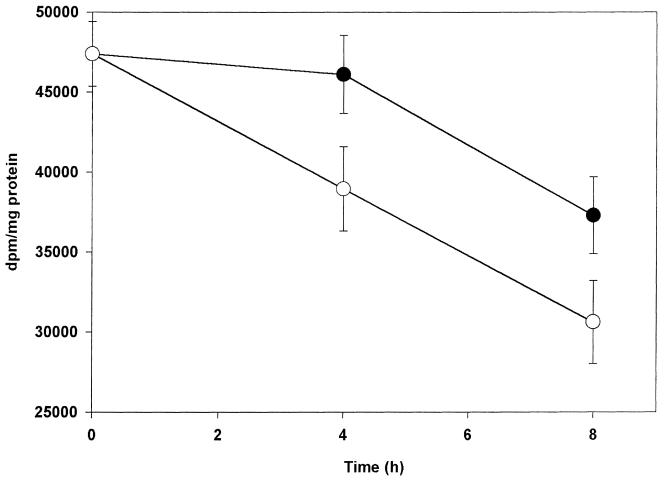

Fig. 4. Cardiolipin (CL) turnover in H9c2 cells treated with clofibrate. H9c2 cells were incubated for 24 h with [1-^{14}C]oleate and then incubated in the absence of presence of 200 μM clofibrate for up to 8 h, and the radioactivity in CL was determined as described in Materials and Methods. Closed circles, control cells; open circles, clofibrate-treated cells. Values represent means ± SD of three dishes.

increased in clofibrate-treated cells to maintain the CL pool size.

H9c2 cells were incubated with 200 μM clofibrate or 5 μM WY14,643 (a specific PPARα agonist) for 48 h followed by incubation with 0.1 μM [1,3-^{3}H]glycerol for 1 h, and radioactivity incorporated into phospholipids was determined. We previously demonstrated that this concentration of WY14,643 resulted in PPARα activation in SW872 preadipocytes (22). [1,3-^{3}H]Glycerol incorporation into CL was increased by 2-fold ($P < 0.05$) in clofibrate-treated cells and by 1.9-fold ($P < 0.05$) in WY14,643-treated cells compared with controls (**Table 2**). In addition, [1,3-^{3}H]glycerol incorporation into PG, the immediate phospholipid precursor of CL, was increased by 34% ($P < 0.05$) in clofibrate-treated cells and by 30% ($P < 0.05$) in WY14,643-treated cells compared with controls. Total uptake of radioactivity into H9c2 cells was 3.11 × 10^6 dpm/mg and was unaltered by clofibrate or WY14,643 treatment. Thus, the increase in glycerol incorporation into PG and CL in clofibrate- or WY14,643-treated cells was not attributable

TABLE 2. Incorporation of [1,3-^{3}H]glycerol into CL and PG in H9c2 cells treated with clofibrate or WY14,643

	Incorporation of [1,3-^{3}H]Glycerol		
Phospholipid	Control	Clofibrate	WY14,643
	dpm × 10^2/mg		
CL	8.4 ± 1.7	16.5 ± 3.8[a]	15.9 ± 2.7[a]
PG	40.9 ± 4.8	54.8 ± 4.7[a]	53.2 ± 3.8[a]

H9c2 cells were incubated in the absence or presence of 200 μM clofibrate or 5 μM WY14,643 for 48 h followed by incubation with 0.1 μM [1,3-^{3}H]glycerol (10 μCi/dish) for 1 h. Cardiolipin (CL) and phosphatidylglycerol (PG) were isolated by TLC, and the radioactivity incorporated into these was determined. Values represent means ± SD of three dishes.

[a] $P < 0.05$.

to an increase in the uptake of labeled glycerol into H9c2 cells.

The mechanism for the increase in the incorporation of [1,3-^{3}H]glycerol into CL was determined by assay of the enzymes of the CDP-DG pathway in mitochondrial fractions prepared from control and clofibrate-treated cells. H9c2 cells were incubated for 48 h with 200 μM clofibrate, the cells were isolated and homogenized, and mitochondrial fractions were prepared. CDS, PGP synthase, and CL synthase activities were determined in these mitochondrial fractions. Treatment of cells with clofibrate resulted in a 37% increase ($P < 0.05$) in the activity of PGP synthase (**Table 3**). In contrast, CDS and CL synthase activities were unaltered compared with those of controls. As previously shown (25), 20% of total PA phosphohydrolase activity was localized to membranes of H9c2 cells, and treatment with clofibrate did not alter the amount of activity associated with membranes (data not shown). Finally, the addition of clofibrate to the incubation mixture of mitochondrial fractions prepared from untreated H9c2 cells was examined. CDS, PGP synthase, and CL synthase activities were 2.5 ± 0.4, 498 ± 47, and 3.0 ± 0.5 pmol/min/mg protein, respectively, and were unaltered by the presence of 200 μM clofibrate in the incubation mixture.

Activation of PPARα in mice stimulates CL biosynthesis and metabolism

To confirm the PPARα agonist-mediated stimulation of cardiac CL biosynthesis, cardiac CL metabolism was examined in another PPARα-responsive rodent model. Mice were fed 0.5% clofibrate for 14 days, the hearts were removed, and the activities of the mitochondrial CL biosynthetic enzymes and PLA_2 activity were determined. As seen in **Table 4**, clofibrate treatment of mice resulted in a 36% ($P < 0.05$) and a 75% ($P < 0.05$) increase in cardiac PLA_2 and PGP synthase activities, respectively, compared with those of untreated mice. CL synthase activity was unaltered by clofibrate treatment. In contrast to H9c2 cells, CDS activity was increased by 3-fold ($P < 0.05$) in clofibrate-treated mice compared with untreated mice. These data appeared to indicate that CDS activity is regulated differently by clofibrate in these two different PPARα-responsive rodent models. To further confirm this and to confirm that PLA_2 and PGP synthase activities are indeed regulated by PPARα activation, the activities of these enzymes were examined in PPARα-null mice treated with clofibrate. PLA_2, CDS, PGP synthase, and CL synthase activities were unaltered in clofibrate-treated PPARα-null mice compared with controls. Thus, cardiac mitochondrial PLA_2, PGP synthase, and CDS activities are regulated by PPARα activation in mouse heart.

CDS-1 mRNA is not expressed in mammalian heart (30). Thus, we examined the expression of CDS-2 in H9c2 cells treated with clofibrate. The validity of RT-PCR quantitation for both rat and mouse CDS-2 is provided in **Fig. 5**. As seen in **Fig. 6**, the expression of CDS-2 mRNA in

TABLE 3. Mitochondrial CDS, PGP synthase, and CL synthase activities in H9c2 cells treated with clofibrate

Sample	Control	+ Clofibrate
	pmol/min/mg protein	
CDS	2.8 ± 0.2	2.8 ± 0.3
PGP synthase	510 ± 30	678 ± 62[a]
CL synthase	3.4 ± 0.4	3.1 ± 0.5

H9c2 cells were incubated in the absence or presence of 200 μM clofibrate for 48 h. Mitochondrial fractions were then prepared, and mitochondrial CDP-1,2-diacyl-*sn*-glycerol synthase (CDS), phosphatidylglycerolphosphate (PGP) synthase, and CL synthase activities were determined. Values represent means ± SD of four dishes.

[a] $P < 0.05$.

TABLE 4. Mitochondrial CDS, PGP synthase, CL synthase, and PLA_2 activities in wild-type and PPARα-null mice treated with clofibrate

Sample	Wild-type		PPAR Null	
	Control	+ Clofibrate	Control	+ Clofibrate
	pmol/min/mg protein			
CDS	0.19 ± 0.04	0.57 ± 0.06[a]	0.33 ± 0.02	0.32 ± 0.06
PGP synthase	57 ± 4	100 ± 8[a]	88 ± 5	95 ± 7
CL synthase	3.5 ± 0.5	3.9 ± 0.2	3.4 ± 0.5	3.0 ± 0.4
PLA_2	2.8 ± 0.2	3.8 ± 0.4[a]	3.2 ± 0.4	3.2 ± 0.5

Mice were fed 0.5% clofibrate for 14 days, cardiac mitochondrial fractions were prepared, and mitochondrial CDS, PGP synthase, CL synthase, and PLA_2 activities were determined. Values represent means ± SD of three animals.

[a] $P < 0.05$.

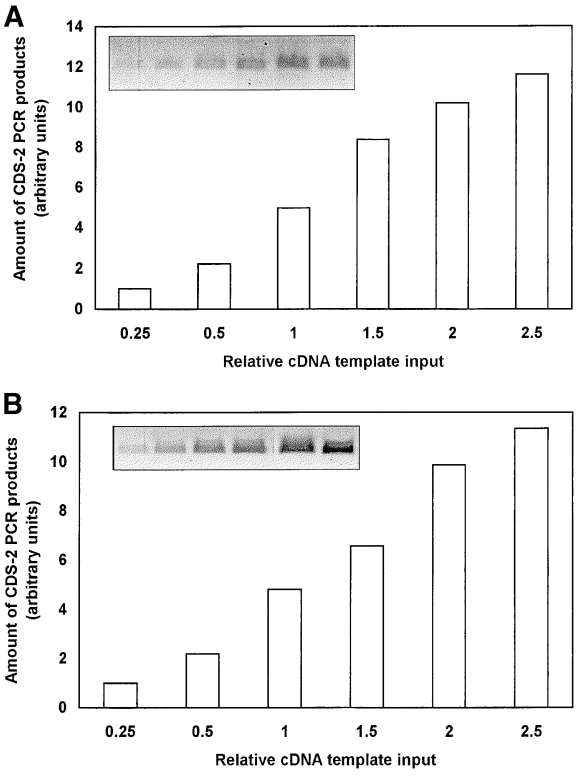

Fig. 5. Validity of mRNA quantitation for rat H9c2 cells and mouse heart CDP-1,2-diacyl-*sn*-glycerol synthase (CDS-2). Under the RT-PCR conditions used, the levels of the PCR products were dependent on the amount of templates used in the reaction. At least a 10-fold difference of cDNA levels of CDS-2 was detected. A: Rat heart H9c2 cells. B: Mouse heart.

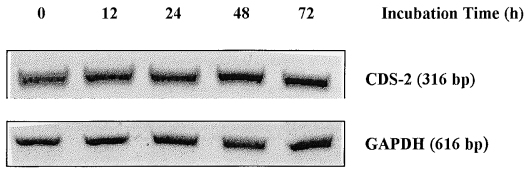

Fig. 6. Effect of clofibrate on CDS-2 mRNA levels in H9c2 cells. H9c2 cells were incubated for up to 72 h with 200 μM clofibrate, and mRNA levels were determined by RT-PCR as described in Materials and Methods. Representative blots are shown. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

H9c2 cells was unaltered by incubation with clofibrate for up to 72 h. Thus, CDS-2 is not upregulated during PPARα activation in H9c2 cells. We then examined CDS-2 mRNA expression in hearts of wild-type and PPARα-null mice. As seen in **Fig. 7**, the expression of cardiac CDS-2 mRNA was increased by 59% in wild-type but not in PPARα-null mice treated with clofibrate.

We conclude that CL de novo biosynthesis is regulated by PPARα activation in both mouse heart and rat heart embryonic H9c2 cells. However, because CDS activity and mRNA expression were unresponsive to PPARα agonist activation in rat heart H9c2 cells but cardiac CDS activity and mRNA expression were increased in mice treated with clofibrate, we hypothesize that this enzyme may be differentially regulated in different PPARα-responsive rodent models.

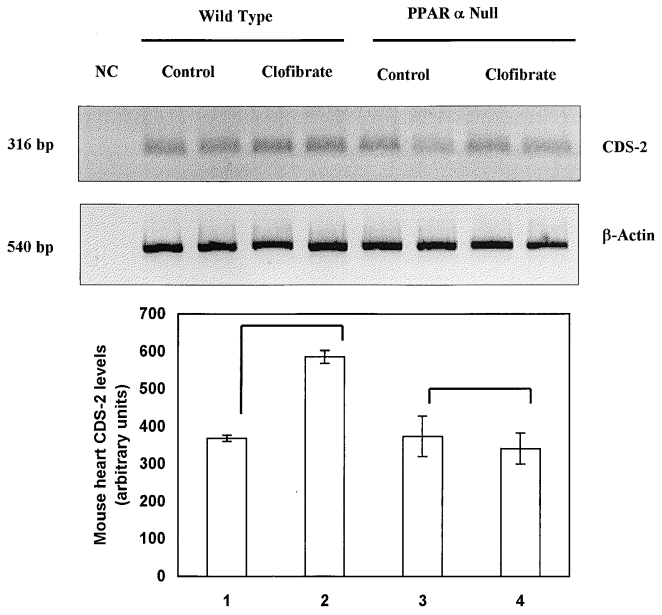

Fig. 7. Effect of clofibrate on CDS-2 mRNA levels in wild-type and PPARα-null mice. Wild-type and four PPARα-null mice were fed either a control or a clofibrate diet for 14 days (two animals from each group). The hearts were removed, and mRNA levels were determined by RT-PCR as described in Materials and Methods. The blots represent the two animals from each group. The relative intensities of the bands were analyzed using Scion Image software. The average relative intensities for the two animals from each group were plotted for wild-type mice (lane 1) versus clofibrate-fed wild-type mice (lane 2) and for PPARα-null mice (lane 3) versus PPARα-null mice fed clofibrate (lane 4). NC, negative control. Values represent means ± SD of two experiments.

DISCUSSION

The objective of this study was to examine whether CL biosynthesis was regulated by PPARα. The results indicate the following: *1*) rat heart myoblastic H9c2 cells contain PPARα; *2*) clofibrate treatment of H9c2 cells stimulates the nuclear association of PPARα; *3*) clofibrate stimulation of PPARα in H9c2 cells results in an increase in mitochondrial PLA_2 and CL de novo biosynthesis; *4*) the mechanism for the increase in CL biosynthesis in H9c2 cells is an increase in PGP synthase activities; and *5*) CDS-2 is a PPARα agonist-responsive gene in mouse heart but not in rat heart H9c2 cells.

The fibrates, including clofibrate, fenofibrate, and gemfibrozil, are a class of drugs that effectively lower plasma levels of VLDLs and triacylglycerides in humans, primates, and rodent species (17–19, 31–33). These drugs mediate their effect by binding to PPARα and enhancing the expression of specific target genes. Western blot analysis revealed the presence of PPARα receptors in rat heart myoblastic H9c2 cells.

Incubation of human premonocytic SW872 preadipocytes and rat mesangial cells with clofibrate results in the activation of $cPLA_2$ and $sPLA_2$ enzyme activities, respectively (21, 22). Treatment of H9c2 cells with clofibrate resulted in an increase of both $cPLA_2$ and the expression and activity of 14 kDa PLA_2-IIA in mitochondrial fractions. However, the pool size of CL remained unaltered. We previously demonstrated that activation of mitochondrial PLA_2 by TNFα or *N*-acetylsphingosine produced a compensatory increase in de novo CL biosynthesis via the activation of PGP synthase activity to maintain the intracellular CL pool (16). Thus, 14 kDa PLA_2-IIA is identified as a potential PPARα target gene in H9c2 cells.

Incubation of H9c2 cells with clofibrate resulted in an increase in [1,3-^{3}H]glycerol incorporation into CL and its immediate phospholipid precursor, PG. Some lipid-lowering or hypolipidemic drugs are known to inhibit PA phosphohydrolase activity, causing a redirection of lipid synthesis away from DG production toward CDP-DG-utilizing reactions (e.g., PG and phosphatidylinositol). Thus, incubation of cells with these compounds could erroneously affect [1,3-^{3}H]glycerol incorporation into various phospholipids. Clofibrate had little effect on the synthesis of glycerides, phospholipids, phosphatidate phosphohydrolase, or DG acyltransferase activities in rat liver slices (34). In agreement with these observations, PA phosphohydrolase activity in rat myoblastic H9c2 cells was unaltered by clofibrate preincubation. Thus, the observed increase in [1,3-^{3}H]glycerol incorporation into PG and CL was attributable to a direct effect on the enzymes of the CL biosynthetic pathway.

Incubation of rat heart H9c2 cells with clofibrate resulted in an increase in PGP synthase activity that would explain the increase in CL biosynthesis from [1,3-^{3}H]glyc-

erol in these cells. CDS may be a rate-limiting enzyme of CL biosynthesis in the rat heart and in rat embryonic heart H9c2 cells (7, 14). There are two known isoforms of the enzyme, CDS-1 and CDS-2, and CDS-1 is not expressed in mammalian heart (30). Surprisingly, treatment of H9c2 cells with clofibrate did not affect CDS enzyme activity. In addition, clofibrate administration directly to H9c2 cell mitochondrial fractions did not affect CDS activity (data not shown). Finally, clofibrate treatment did not affect CDS-2 mRNA levels in H9c2 cells. These data clearly indicate that CDS-2 is not a PPARα target gene in rat heart H9c2 cells. In contrast to rat H9c2 cells, CDS activity was increased by 3-fold in cardiac mitochondrial fractions prepared from mice treated with clofibrate but was unaltered when PPARα-null mice were treated with clofibrate. In addition, CDS mRNA level was increased by 59% in hearts from mice treated with clofibrate but was unaltered when PPARα-null mice were treated with clofibrate. These data clearly indicate that CDS is a PPARα agonist-responsive gene in mouse heart but not in rat heart H9c2 cells. Finally, clofibrate treatment stimulated cardiac mitochondrial PLA_2 and PGP synthase activities in mouse. These enzyme activities were unaltered in PPARα-null mice treated with clofibrate, indicating that they are responsive to PPARα agonist stimulation. Taken together, using two different rodent models, the data clearly indicate that de novo CL biosynthesis is regulated by PPARα. It is still not certain whether these effects are directly or indirectly regulated by PPARα.

What is the implication of increased mitochondrial CL biosynthesis during clofibrate treatment of rat H9c2 cells and mouse heart? Mice and rats are particularly responsive to the biological effects of fibrates (31, 32). In contrast, there is currently no direct evidence to indicate that guinea pigs and humans are responsive to the proliferative effects of these drugs. Mitochondria appear to be one of the principal intracellular targets for nutritional and pharmacological control of lipid metabolism mediated by the fibrates (35). Fenofibrate feeding increased rat liver mitochondrial β-oxidation, and this was associated with a decrease in triacylglyceride synthesis and secretion. In the rat heart, increased CL biosynthesis and remodeling were associated with increased mitochondrial β-oxidation (36–38). Thus, the clofibrate-induced increase in CL biosynthesis and metabolism in H9c2 cells and mouse heart may be linked to increased mitochondrial metabolism required for cellular proliferation in these rodents. Alternatively, the increase in CL biosynthesis may be a compensatory mechanism by the cell to maintain the CL pool when CL is degraded by phospholipases. We previously demonstrated that the addition of the proapoptotic factor TNFα to H9c2 cells resulted in the activation of mitochondrial PLA_2 activity and increased CL degradation, which in turn resulted in the activation of PGP synthase and increased CL biosynthesis from [1,3-^{3}H]glycerol (16). Given the importance of CL loss (11, 12) and the accumulation of its immediate metabolic product, monolysocardiolipin (39), in mitochondrion-mediated apoptosis, it is possible that rapid CL synthesis is required in response to proapoptotic stimuli-mediated CL degradation to restore cellular homeostasis and prevent a cell's entrance into apoptosis. Hence, increased CL biosynthesis may be a protective mechanism against apoptosis.

In summary, rat heart myoblastic H9c2 cells contain PPARα, and its activation results in an increase in de novo CL biosynthesis. In addition, PGP synthase and PLA_2 were identified as possible PPARα agonist-responsive enzymes involved in the regulation of CL biosynthesis in the mouse heart. Furthermore, CDS-2 was identified as a PPARα agonist-responsive gene and appears to be differentially regulated by PPARα activation between rat cardiac H9c2 cells and mouse heart models.

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REFERENCES

1. White, D. A. 1973. The phospholipid composition of mammalian tissues. *In* Form and Function of Phospholipids. G. B. Ansell, J. N. Hawthorne, and R. M. C. Dawson, editors. Elsevier Science Publishers, Amsterdam. 441–483.
2. Hostetler, K. Y. 1982. Polyglycerophospholipids. *In* Phospholipids. J. N. Hawthorne and G. B. Ansell, editors. Elsevier Science Publishers, Amsterdam. 215–261.
3. Pangborn, M. C. 1942. Isolation and purification of a serologically active phospholipid from beef heart. *J. Biol. Chem.* **143:** 247–256.
4. Stoffel, W., and H-G. Schiefer. 1968. Biosynthesis and composition of phosphatides in outer and inner mitochondrial membranes. *Hoppe-Seyler's Z. Physiol. Chem.* **349:** 1017–1026.
5. Hovius, R., H. Lambrechts, K. Nicolay, and B. de Kruijff. 1990. Improved methods to isolate and subfractionate rat liver mitochondria. Lipid composition of the inner and outer membrane. *Biochim. Biophys. Acta.* **1021:** 217–226.
6. Poorthuis, B. J. H. M., P. J. Yazaki, and K. Y. Hostetler. 1976. An improved two-dimensional thin layer chromatography system for the separation of phosphatidylglycerol and its derivatives. *J. Lipid Res.* **17:** 433–437.
7. Hatch, G. M. 1994. Cardiolipin biosynthesis in the isolated heart. *Biochem. J.* **297:** 201–208.
8. Hoch, F. L. 1992. Cardiolipins and biomembrane function. *Biochim. Biophys. Acta.* **1113:** 71–133.
9. Yamaoka-Koseki, S., R. Urade, and M. Kito. 1991. Cardiolipins from rats fed different dietary lipids affect bovine heart cytochrome c oxidase activity. *J. Nutr.* **121:** 956–958.
10. Ohtsuka, T., M. Nishijima, K. Suzuki, and Y. Akamatsu. 1993. Mitochondrial dysfunction of a cultured Chinese hamster ovary cell mutant defective in cardiolipin. *J. Biol. Chem.* **268:** 22914–22919.
11. Esposti, M. D. 2002. Lipids, cardiolipin and apoptosis: a greasy license to kill. *Cell Death Differ.* **9:** 234–236.
12. McMillann, and W. Dowhan. 2002. Cardiolipin and apoptosis. *Biochim. Biophys. Acta.* **1585:** 97–107.
13. Kiyasu, J. Y., R. A. Pieringer, H. Paulus, and E. P. Kennedy. 1963. The biosynthesis of phosphatidylglycerol. *J. Biol. Chem.* **238:** 2293–2298.
14. 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.
15. Hostetler, K. Y., H. Van den Bosch, and L. L. Van Deneen. 1971. Biosynthesis of cardiolipin in rat liver mitochondria. *Biochim. Biophys. Acta.* **239:** 113–119.
16. Xu, F., S. L. Kelly, and G. M. Hatch. 1999. N-Acetylsphingosine stimulates phosphatidylglycerolphosphate synthase activity in H9c2 cardiac cells. *Biochem. J.* **337:** 483–490.
17. Braissant, O., F. Foufelle, C. Scotto, M. Dauca, and W. Wahil. 1996. Differential expression of peroxisome proliferator-activated receptors (PPAR's): tissue distribution of PPAR-alpha, -beta, and -gamma in the adult rat. *Endocrinology.* **137:** 354–366.

18. Bishop-Bailey, D. 2000. Peroxisome proliferator-activated receptors in the cardiovascular system. *Br. J. Pharmacol.* **129:** 823–834.
19. Staels, B., and J. Auwerx. 1998. Regulation of apoA-1 gene expression by fibrates. *Atherosclerosis.* **137 (Suppl.):** 19–23.
20. Van der Lee, K. A., P. H. Willemsen, G. J. Van der Vusse, and M. Van Bilsen. 2000. Effects of fatty acids on uncoupling protein-2 expression in the rat heart. *FASEB J.* **14:** 495–502.
21. Scholz-Pedretti, K., A. Gans, K. F. Beck, J. Pfeilschifter, and M. Kaszkin. 2002. Potentiation of TNF-alpha-stimulated group IIA phospholipase A_2 expression by peroxisome proliferator-activated receptor alpha activators in rat mesangial cells. *J. Am. Soc. Nephrol.* **13:** 611–620.
22. Jiang, Y. J., G. M. Hatch, D. Mymin, T. Dembinski, E. A. Kroeger, and P. C. Choy. 2001. Modulation of cytosolic phospholipase A_2 by PPAR activators in human preadipocytes. *J. Lipid Res.* **42:** 716–724.
23. Pan, D. A., M. K. Mater, A. P. Thelen, M. Peters, F. J. Gonzalez, and D. J. Jump. 2000. Evidence against the peroxisome proliferator-activated receptor alpha (PPARalpha) as the mediator for polyunsaturated fatty acid suppression of hepatic L-pyruvate kinase gene transcription. *J. Lipid Res.* **41:** 742–751.
24. Hatch, G. M., S. G. Cao, and A. Angel. 1995. Decrease in cardiac phosphatidylglycerol in streptozotocin-induced diabetic rats: evidence for distinct pools of phosphatidylglycerol in the heart. *Biochem. J.* **306:** 759–764.
25. Xu, F. Y., W. A. Taylor, J. A. Hurd, and G. M. Hatch. 2003. Etomoxir mediates differential metabolic channeling of fatty acid and glycerol precursors into cardiolipin in H9c2 cells. *J. Lipid Res.* **44:** 415–423.
26. Volta, M., A. Bulfone, C. Gattuso, E. Rossi, M. Mariani, G. G. Consalez, O. Zuffardi, A. Ballabio, S. Banfi, and B. Franco. 1999. Identification and characterization of CDS2, a mammalian homolog of the Drosophila CDP-synthase gene. *Genomics.* **55:** 68–77.
27. 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.
28. Rodriguez, C., V. Noe, A. Caberero, C. J. Ciudad, and J. C. Laguna. 2000. Differences in the formation of PPARalpha-RXR/acoPPRE complexes between responsive and non-responsive species upon fibrate activation. *Mol. Pharmacol.* **58:** 185–193.
29. Liu, J., T. Takano, J. Papillon, A. Khadir, and A. V. Cybulsky. 2001. Cytosolic phospholipase A2 associates with plasma membrane, endoplasmic reticulum and nuclear membrane in glomerular epithelial cells. *Biochem. J.* **353:** 79–90.
30. Lykidis, A., P. D. Jackson, C. O. Rock, and S. Jackowski. 1997. The role of CDP-diacylglycerol synthase and phosphatidylinositol synthase activity levels in the regulation of cellular phosphatidylinositol content. *J. Biol. Chem.* **272:** 33402–33407.
31. Bentley, P., I. Calder, C. Elcombe, P. Grasso, D. Stringer, and H-J. Wiegand. 1993. Hepatic peroxisome proliferation in rodents and its significance to humans. *Food Chem. Toxicol.* **31:** 857–907.
32. Cattley, R. C., J. Deluca, C. Elcombe, P. Fenner-Crisp, B. G. Lake, D. S. Marsman, T. A. Pastoor, J. A. Popp, D. E. Robinson, B. Schwetz, J. Tugwood, and W. Wahli. 1998. Do peroxisome proliferating compounds pose a hepatocarcinogenic hazard to humans? *Regul. Toxicol. Pharmacol.* **27:** 47–60.
33. Winegar, D. A., J-L. Su, and S. A. Kliewer. 2000. Role of peroxisome proliferator-activated receptors in atherosclerosis. *Curr. Opin. Cardiovasc. Pulmon. Renal Invest. Drugs.* **2:** 233–243.
34. Brindley, D. N., and M. Bowley. 1975. Drugs affecting the synthesis of glycerides and phospholipids in rat liver. The effects of clofibrate, halofenate, fenfluramine, amphetamine, cinchocaine, chlorpromazine, dimethylimipramine, mepyramine and some of their derivatives. *Biochem. J.* **148:** 461–469.
35. Froyland, L., L. Madsen, H. Vaagenes, G. K. Totland, J. Auwerx, H. Kryvi, B. Staels, and R. K. Berge. 1997. Mitochondrion is the principal target for nutritional and pharmacological control of triglyceride metabolism. *J. Lipid Res.* **38:** 1851–1858.
36. Paradies, G., and F. M. Ruggiero. 1988. The effect of hypothyroidism on the transport of pyruvate in rat heart mitochondria. *Biochim. Biophys. Acta.* **935:** 79–86.
37. Cao, S. G., P. Cheng, A. Angel, and G. M. Hatch. 1995. Thyroxine stimulates phosphatidylglycerolphosphate synthase activity in rat heart mitochondria. *Biochim. Biophys. Acta.* **1256:** 241–244.
38. Mutter, T., V. W. Dolinsky, B. J. Ma, W. A. Taylor, and G. M. Hatch. 2000. Thyroxine regulation of monolysocardiolipin acyltransferase activity in rat heart. *Biochem. J.* **346:** 403–406.
39. Esposti, M. D., I. M. Cristea, S. J. Gaskell, Y. Nakao, and C. Dive. 2003. Proapoptotic Bid binds to monolysocardiolipin, a new molecular connection between mitochondrial membranes and cell death. *Cell Death Differ.* **10:** 1300–1309.