Glucocorticoids and cyclic AMP selectively increase hepatic lipin-1 expression, and insulin acts antagonistically

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Abstract Glucocorticoids (GCs) increase hepatic phosphatidate phosphatase (PAP1) activity. This is important in enhancing the liver's capacity for storing fatty acids as triacylglycerols (TAGs) that can be used subsequently for β-oxidation or VLDL secretion. PAP1 catalyzes the conversion of phosphatidate to diacylglycerol, a key substrate for TAG and phospholipid biosynthesis. PAP1 enzymes in liver include lipin-1A and -1B (alternatively spliced isoforms) and two distinct gene products, lipin-2 and lipin-3. We determined the mechanisms by which the composite PAP1 activity is regulated using rat and mouse hepatocytes. Levels of lipin-1A and -1B mRNA were increased by dexamethasone (dex; a synthetic GC), and this resulted in increased lipin-1 synthesis, protein levels, and PAP1 activity. The stimulatory effect of dex on lipin-1 expression was enhanced by glucagon or cAMP and antagonized by insulin. Lipin-2 and lipin-3 mRNA were not increased by dex/cAMP, indicating that increased PAP1 activity is attributable specifically to enhanced lipin-1 expression. This work provides the first evidence for the differential regulation of lipin activities. Selective lipin-1 expression explains the GC and cAMP effects on increased hepatic PAP1 activity, which occurs in hepatic steatosis during starvation, diabetes, stress, and ethanol consumption.—Manmontri, B., M. Sariahmetoglu, J. Donkor, M. B. Khalil, M. Sundaram, Z. Yao, K. Reue, R. Lehner, and D. N. Brindley. **Glucocorticoids and cyclic AMP selectively increase hepatic lipin-1 expression, and insulin acts antagonistically. *J. Lipid Res.* 2008.** 49: **1056–1067.**

Supplementary key words β-oxidation • diabetes • ethanol ingestion • fasting • glucagon • hypertriglyceridemia • steatosis • triacylglycerol synthesis

Mammalian phosphatidate phosphatase (PAP1) activity is Mg^{2+}-dependent and is inhibited by *N*-ethylmaleimide (1). These characteristics distinguish mammalian PAP1 from PAP2 activities that also convert phosphatidate (PA) to diacylglycerol (DAG). PAP2 is now commonly known as a family of lipid phosphate phosphatases (LPPs) that dephosphorylate a variety of lipid phosphate esters. The LPPs are mainly involved in regulating signal transduction (2). By contrast, PAP1 appears to be specific for PA as a substrate (3, 4) and is a required enzyme in the biosynthesis of triacylglycerol (TAG), phosphatidylcholine, and phosphatidylethanolamine (5).

Our previous work showed that injecting rats with cortisol or corticotropin produced marked increases in PAP1 activity in the liver (6, 7). Subsequent work with rat hepatocytes demonstrated that the glucocorticoid (GC) effect in increasing PAP1 activity was synergized by glucagon and inhibited by insulin (8, 9). We showed that these GC-induced increases in PAP1 activity provide the extra capacity for the liver to sequester excess FAs as TAG when these FAs are not immediately required for β-oxidation (5). The interaction of GC with insulin explains the diurnal rhythm of PAP1 activity in rat livers (10). The GC effect is also consistent with increases in hepatic PAP1 seen after sham operations or in liver remnants after partial hepatectomy (11), in starvation (6), diabetes (12), insulin resistance (13), and hypoxia (14), and in toxic conditions (5). Increases in hepatic PAP1 also occur in response to dietary modification in rodents, for instance, when glucose or starch is replaced by fructose, sorbitol, glycerol, or ethanol (15), and these effects are exaggerated by high-fat feeding (16). These changes in PAP1 are

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Abbreviations: CPTcAMP, 8-(4-chlorophenylthio) cyclic AMP; DAG, diacylglycerol; dex, dexamethasone; ER, endoplasmic reticulum; GC, glucocorticoid; LPP, lipid phosphate phosphatase; PA, phosphatidate; PAP1, phosphatidate phosphatase; PGC-1α, peroxisome proliferator-activated receptor-coactivator-1α; TAG, triacylglycerol.

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also associated with increased GC concentrations relative to insulin. PAP1 activity is also increased in the livers of baboons (17) and human alcoholics (18). The involvement of GC in ethanol-induced increases in PAP1 activity is confirmed because this is attenuated in adrenalectomized rats (19).

The physiological expression of PAP1 activity involves a FA-induced translocation of the reservoir of cytosolic PAP1 to become functional on membranes of the endoplasmic reticulum (ER), where PA is synthesized (20, 21). The activity of the membrane-bound PAP1 correlates closely with the conversion of PA to DAG and the synthesis of TAG and phosphatidylcholine in intact rat hepatocytes (21).

Further work in this area was severely hampered because of the inability of any group to purify or identify the structure of PAP1. This situation changed with a publication by Han, Wu, and Carman (4), who identified the yeast PAP1 (PAH1; previously known as SMP2) as an ortholog of mammalian lipin. They also showed that recombinant mammalian lipin-1 had PAP1 activity. Mammals express a family of lipins consisting of lipin-1A and its splice variant lipin-1B, plus lipin-2 and lipin-3 (22). In mature adipocytes, lipin-1A is preferentially located in the nucleus, whereas most of the lipin-1B is found in the cytosol (23). Our recent studies demonstrated that all of these lipins possess Mg^{2+}-dependent PAP1 activity and that they are expressed in a tissue-specific manner (3). For example, lipin-1 provides the majority, if not all, of the PAP1 activity in white and brown adipose tissue, skeletal muscle, and heart, whereas liver expresses lipin-1, -2, and -3 (3, 24). This observation explains why the *fld* mouse, which has a null mutation in the *Lpin1* gene, exhibits lipodystrophy, because lipin-1 is required for the development of mature adipocytes by regulating the expression of peroxisome proliferator-activated receptor γ (PPARγ) and TAG synthesis (25). The *fld* mouse also develops a fatty liver and hypertriglyceridemia in the preweaning period, which indicates an ability of the liver to synthesize and secrete TAG (26). This capacity in the *fld* mouse is explained by the expression of PAP1 activity through lipin-2 and -3 (3, 24). In fact, livers from *fld* mice show normal PAP1 activity and increased lipin-3 mRNA levels, presumably as an adaptive response to the lack of lipin-1 (3). In addition to controlling TAG synthesis, lipin-1 increases the capacity of the liver for β-oxidation in fasting by facilitating transcriptional regulation by peroxisome proliferator-activated receptor-coactivator-1α (PGC-1α) and PPARα (27).

The discovery that the liver expresses lipin-1A, -1B, -2, and -3 (3) provokes the question of which lipins respond to hormonal regulation to explain the observed physiological changes in the composite PAP1 activity. Answering this question and describing the mechanisms that control the expression of the different lipins are essential to establishing their functions in hepatic metabolism and for understanding the hormonal regulation of their expression. To investigate this, we compared the responses of primary cultures of rat and mouse hepatocytes over a time course after treatment with hormones and 8-(4-chlorophenylthio) cyclic AMP (CPTcAMP). The results show that the members of the lipin family were differentially regulated by dexamethasone (dex), glucagon, and insulin. Dex with glucagon or CPTcAMP markedly increased total PAP1 activity, and this effect was accounted for by the increased synthesis of lipin-1. Insulin attenuated the dex- + CPTcAMP-induced increases in lipin-1 synthesis. These results provide the first evidence for the differential regulation of the activity of different lipins in the liver. They help to explain how the composite changes in PAP1 (lipin) activity may coordinate increased TAG synthesis, β-oxidation, and VLDL secretion in conditions of starvation, metabolic stress, insulin resistance, and diabetes.

METHODS

Materials

Dex, CPTcAMP, insulin, and glucagon was purchased from Sigma-Aldrich (St. Louis, MO). Rabbit polyclonal antibodies against lipin-1 were prepared in accordance with University of Ottawa Heart Institute regulations relating to Animal Care Procedures using the peptide SKTDSPSRKKDKRSRHLGADG essentially as described previously (28). The antibody was used at a dilution of 1:500. Mouse monoclonal antibody for the V5 tag and GAPDH were purchased from Invitrogen and Sigma and used at dilutions of 1:1,000 and 1:5,000, respectively. Secondary antibodies were IRDye 800 goat anti-rabbit IgG (Rockland Immunochemicals, Gilbertsville, PA) and goat anti-mouse IgG conjugated to Alexa Fluor 680 (Molecular Probes, Eugene, OR).

Preparation and culture of hepatocytes

Hepatocytes were prepared from male Sprague-Dawley rats (200–540 g) or C57BL/6J mice (22.5–32 g) as described previously (29). They were plated onto collagen-coated dishes in DMEM containing 15% serum in an atmosphere of 95% air and 5% CO_2 for 45–90 min to allow attachment. The medium was changed to remove nonviable cells, and the hepatocytes were incubated for a further 4 h to allow them to spread. The medium was then changed, and the hepatocytes were incubated for different times in serum-free medium containing 0.1% BSA with the addition of hormones or agonists as indicated. All incubations contained 0.5% DMSO, which was used as a vehicle for dex.

Gene expression analysis in fasting/refeeding conditions

Livers were harvested from 16 week old female C57BL/6J mice after fasting for 16 h (fasted samples) or fasting for 16 h followed by refeeding for 4 h (refed samples). Total RNA was isolated with TRIzol (Invitrogen, Carlsbad, CA) and cDNA synthesized from 2 μg of RNA using the Omniscript reverse transcriptase kit (Qiagen, Valencia, CA). Real-time RT-PCR was performed with the iCycler (Bio-Rad, Hercules, CA) using SYBR Green PCR reagents (Qiagen) as described previously (25). Gene expression was normalized to levels of β_2-microglobin and 18S rRNA. Primers used for this fasting study are listed in **Table 1**.

RNA quantitation for hepatocyte samples by real-time RT-PCR

RNA was collected using the RNAqueous Kit (Ambion, Inc., Austin, TX) according to the manufacturer's directions. Reverse

TABLE 1. Oligonucleotide primers used for real-time RT-PCR in fasting experiments with mice

Protein	Forward Primer	Reverse Primer	Reference
Mouse 18s rRNA	accgcagctaggaataatgga	gcctcagttccgaaaacca	
Mouse β_2-microglobin	gtctttcagcaaggactggtc	caaatgcggcatcttcaaacc	48
Mouse lipin-1A	ggtcccccagccccagtcctt	gcagcctgtggcaattca	23
Mouse lipin-1B	cagcctggtagattgccaga	gcagcctgtggcaattca	23
Mouse lipin-2	agttgaccccatcaccgtag	cccaaagcatcagacttggt	3
Mouse lipin-3	tggaattgggatgacaaggt	cactgcaagtacccccttggt	3
Mouse PGC-1α	ctcacagagacactggacagt	tgtagctgagctgagtgttgg	
Mouse PPARα	aatgcaattcgctttggaag	ggccttgaccttgttcatgt	48

PGC-1α, peroxisome proliferator-activated receptor-coactivator-1α; PPARα, peroxisome proliferator-activated receptor α. Where not referenced, primers were designed by Primer3 or Primer Express version 2.0 software using default parameters.

transcription was performed using SuperScript II, random primers, and RNaseOUT according to instructions from the supplier (Invitrogen). PCR was performed on an iCycler (Bio-Rad) using SYBR Green PCR reagents (Applied Biosystems, Foster City, CA). Primer sequences for PCR are listed in **Table 2**. Gene expression was normalized to the housekeeping genes cyclophilin A and GAPDH. In initial experiments, the relative changes in mRNA expression for the lipins were essentially the same when expressed relative to both reference mRNAs; therefore, we routinely expressed results relative to cyclophilin A mRNA.

Measurement of PAP1 activity

Hepatocytes were lysed in 0.25 M sucrose containing 2 mM dithiothreitol, 0.15% Tween 20, and a protease inhibitor cocktail (Sigma). We developed the following assay specifically to give accurate measurements of relative PAP1 activity in tissue and cell homogenates, which are able to metabolize PA by several different routes (3, 30). We chose to measure the formation of DAG from PA labeled with [^{3}H]palmitate (3, 30) in preference to the release of water-soluble ^{32}P from [^{32}P]PA. In liver or hepatocyte homogenates, glycerol-^{32}P can be produced by phospholipase A action, and this product is further converted to inorganic ^{32}P (31). Thus, this latter assay with crude enzyme preparations has to be used with care to ensure that the measured ^{32}P is only produced by PAP activity (32). We also mixed the PA in the molar ratio of 3:2 with nonradioactive PC, because this form of the substrate maximizes PAP1 activity relative to that of PAP2 (1). In our assays, ~90% of the PAP activity is from PAP1 (3). Had we used Triton X-100 to solubilize the PA, this would extract lipids, hydrophobic proteins, and amphiphilic proteins from the homogenates, and the advantage of this "defined substrate" would immediately be lost. Moreover, the use of PA dissolved in micelles of Triton X-100 favors PAP2 activity relative to that of PAP1 (1). It is important in assays for PAP1 to eliminate the contribution from PAP2, and this is best done in these mammalian systems by inhibiting PAP1 activity with *N*-ethylmaleimide rather than by trying to eliminate the effects of endogenous Mg^{2+} (30, 33). This value was then subtracted from the total activity to give PAP1 activity. Thus, maximizing the PAP1 activity relative to PAP2 makes this correction smaller and leads to greater precision in estimating PAP1 activity in tissue and cell homogenates. We also discovered that adding Tween-20 to the homogenates stabilizes and increases PAP1 activity (34). In addition, Tween-20 appears to minimize the breakdown of the DAG product by lipases in the assay, making the use of tetrahydrolipstatin to inhibit this activity unnecessary (3).

Briefly, samples were assayed in 100 mM Tris/maleate buffer, pH 6.5, 5 mM $MgCl_2$, 2 mM dithiothreitol, 2 mg/ml FA-poor BSA, 0.6 mM PA labeled with [^{3}H]palmitate (~1×10^5 dpm/assay), and 0.4 mM phosphatidylcholine. The [^{3}H]DAG product was purified using alumina and then quantitated by scintillation counting (33). Parallel incubations were performed in the presence of excess (8 mM) *N*-ethylmaleimide to inhibit PAP1 and to compensate for any PAP2 (LPP) activity in this assay (30). The amount of cell homogenate was adjusted so that the formation of DAG consumed <15% of the PA added. Reaction rates were measured at three different protein concentrations to ensure the proportionality of the assay

TABLE 2. Oligonucleotide primers used for real-time RT-PCR in hepatocyte experiments

Protein	Forward Primer	Reverse Primer	Reference
Cyclophilin A	caccgtgttcttcgacatcac	ccagtgctcagagctcgaaag	49
Mouse GAPDH	tgtgtccgtcgtggatctga	cctgcttcaccaccttcttga	
Mouse lipin-1A	gcctgctcgtgaatcctct	cgatgcatcccgacagcgt	23
Mouse lipin-1B	cagcctggtagattgccaga	gcagcctgtggcaattca	23
Rat lipin-1	tcactacccagtaccagggc	tgagtccaatcctttcccag	
Rat lipin-1B	agcagcctggtagattgtca	taaggggctggagtctttcat	
Rat and mouse lipin-2	tagatgcagaccctgttccc	ctggtgctggcttcttttgt	
Rat and mouse lipin-3	aaagactggacacaccaggg	tgctggatatcactcaggca	
Mouse PGC-1α	ggcacgcagccctattca	cgacacggagagttaaaggaaga	
Mouse PPARα	actacggagttcacgcatgtg	ttgtcgtacaccagcttcagc	50
Rat PGC-1α	cacaacgcggacagaactga	ccgcagatttacggtgcatt	
Rat PPARα	tggagtccacgcatgtgaag	cgccagctttagccgaatag	

Where not referenced, primers were designed by Primer3 or Primer Express version 2.0 software using default parameters.

for each sample, so that the relative rates of PAP1 activity can be calculated.

Expression of recombinant lipins

Lipin-1A, -1B, -2, and -3 that were tagged with a V5 epitope were expressed in HEK 293 cells as described previously (3), and these were used to test the specificity of the lipin-1 antibody.

Western blot analysis

Protein concentrations in cell lysates were determined using the Bradford protein assay (Bio-Rad). Identical amounts of protein (100 μg) were mixed with a commercial loading buffer (Invitrogen), and proteins were separated by SDS-PAGE (35) using 8% gels. The proteins were transferred onto nitrocellulose membranes (Bio-Rad), which were blocked with Odyssey® blocking buffer (Li-Cor Biosciences, Lincoln, NE). Membranes were then incubated with rabbit antibodies for lipin-1 or mouse monoclonal antibody for GAPDH or V5. The membranes were then washed and incubated with IRDye 800 goat anti-rabbit IgG and goat anti-mouse IgG conjugated to Alexa Fluor 680, respectively. Simultaneous images obtained at 700 and 800 nm were quantified using the Odyssey® Imager System (Li-Cor).

Statistical analysis

The significance of differences among treatments was analyzed using a Newman-Keuls post hoc test for a one-way ANOVA or a Bonferroni test with a two-way ANOVA.

RESULTS

To investigate how the expression of different lipins is regulated in the liver, we used primary cultures of mouse hepatocytes to relate this work to various mouse genetic models that exist or will be created. We also used rat hepatocytes, because our earlier work showing marked hormone-induced changes in PAP1 activity was performed with the rat. We incubated the hepatocytes for various times with an optimum (100 nM) concentration (9) of dex, a synthetic GC, because the natural corticosterone is efficiently degraded by hepatocytes (9). We also determined interactions of dex with glucagon, CPTcAMP, or insulin.

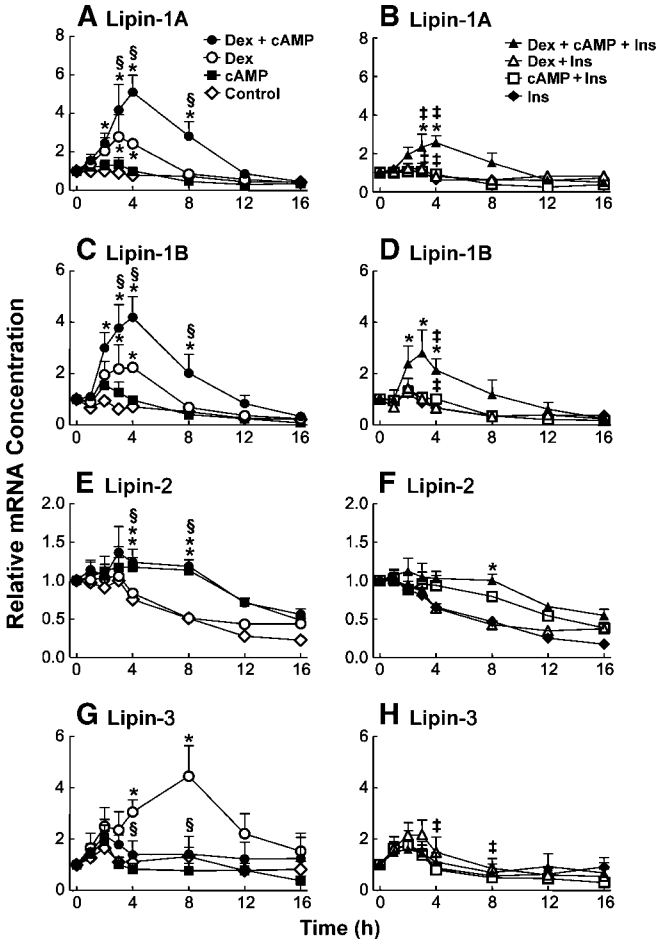

Fig. 1. Interaction of dexamethasone (dex), cAMP, and insulin in controlling mRNA expression for lipin in mouse hepatocytes. Mouse hepatocytes were incubated for the times shown with 100 nM dex (Dex), 100 μM 8-(4-chlorophenylthio) cyclic AMP (CPTcAMP; cAMP), and 100 nM insulin (Ins) alone or in combination as indicated. Relative mRNA concentrations for the different lipins were measured (A–H) by real-time RT-PCR and expressed relative to that for cyclophilin A. Results are expressed as means ± SEM for 3–15 independent experiments. The significance of the differences ($P < 0.05$) is indicated as follows: * different from the untreated control value; § dex alone different from dex + CPTcAMP treatment; ‡ incubation with insulin decreases the effect of dex alone or dex + CPTcAMP.

Hormonal regulation of the expression of mRNA for lipin-1A and -1B

In mouse hepatocytes, the relative mRNA levels for lipin-1A and -1B were increased by dex and reached a peak after ~4 h (**Fig. 1A, C**). This dex effect was amplified by the presence of CPTcAMP (Fig. 1A, C) or by glucagon in the case of lipin-1B (**Fig. 2A**). CPTcAMP or glucagon alone had no significant effect in increasing the mRNA for lipin-1A or -1B compared with the untreated (control) incubations (Figs. 1A, C, 2A). Insulin alone had no significant effect on the relative mRNA concentrations for lipin-1A or -1B compared with the nontreated control. However, it attenuated the effects of dex alone and dex with CPTcAMP or glucagon (Figs. 1B, D, 2A).

For work with rat hepatocytes, we chose to use primers for total lipin-1 and lipin-1B, because at the beginning of this work we were uncertain about the nucleotide sequences for these lipins. The levels of mRNA for lipin-1 and -1B in the nontreated controls declined as the incubation proceeded (**Fig. 3A, C**). Dex increased the levels of these mRNAs relative to the value at time 0 of incubation and even more so compared with the nontreated control at the equivalent time. Maximum increases were obtained after 4–8 h of incubation. CPTcAMP or glucagon alone had no significant effect on the levels of mRNA for lipin-1 and -1B (Figs. 2B, 3A, B). Although CPTcAMP or glucagon appeared to have a slight effect in augmenting the dex-induced increase in the levels of mRNA for total lipin-1 and -1B, this only reached statistical significance for lipin-1 and -1B at the 8 and 4 h, respectively. Insulin alone had no sig-

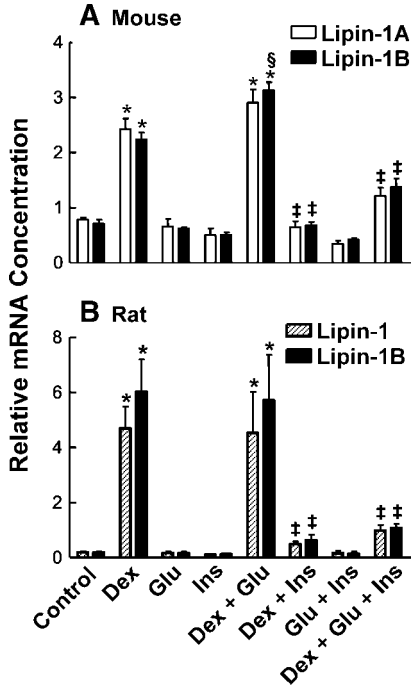

Fig. 2. Interaction of dex, glucagon, and insulin in controlling mRNA expression for lipin-1 in mouse and rat hepatocytes. Mouse (A) and rat (B) hepatocytes were incubated for 4 and 8 h, respectively, with 100 nM dex (Dex), 10 nM glucagon (Glu), and 100 nM insulin (Ins) alone or in combination as indicated. The relative mRNA concentrations for the different lipins were measured by real-time RT-PCR and expressed relative to that for cyclophilin A. Results are means ± SEM for 3–15 independent experiments for the mouse and for 3 to 8 experiments for the rat. The significance of the differences ($P < 0.05$) is indicated as follows: * different from the untreated control value; § dex alone is significantly different from dex + CPTcAMP treatment; ‡ incubation with insulin decreases the effect of dex alone or dex + glucagon.

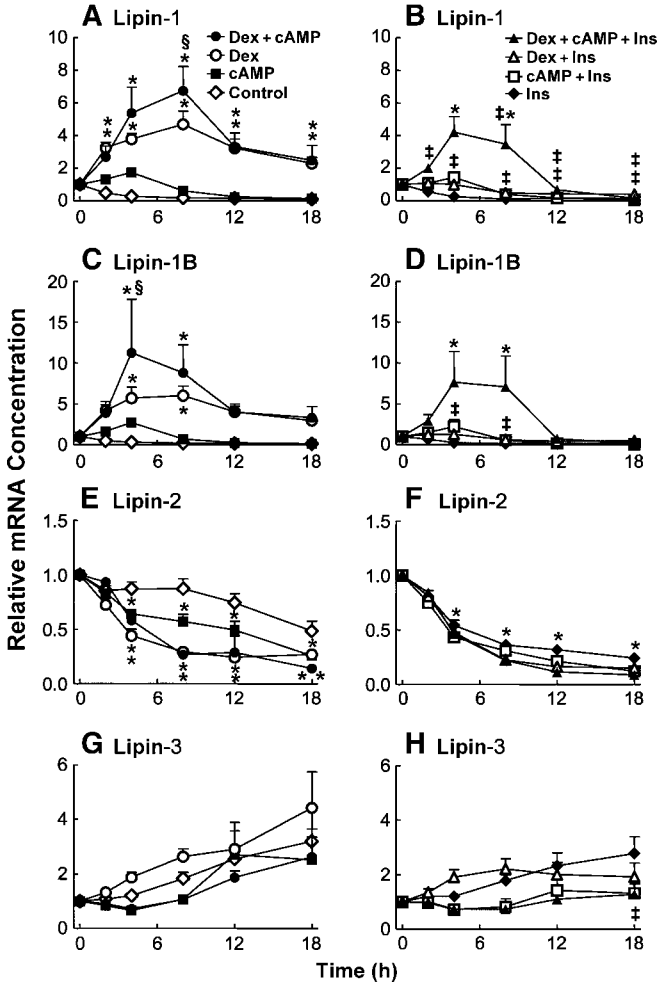

Fig. 3. Interaction of dex, cAMP, and insulin in controlling mRNA expression for lipin in rat hepatocytes. Rat hepatocytes were incubated for various times with 100 nM dex (Dex), 100 μM CPTcAMP (cAMP), and 100 nM insulin (Ins) alone or in combination as indicated. The relative mRNA concentrations for the different lipins were measured (A–H) by real-time RT-PCR and expressed relative to that for cyclophilin A. Results are expressed as means ± SEM for three to eight independent experiments. The significance of the differences ($P < 0.05$) is indicated as follows: * different from the untreated control value; § dex alone different from dex + CPTcAMP treatment; ‡ incubation with insulin decreases the effect of dex alone or dex + CPTcAMP.

nificant effect on mRNA levels for lipin-1 or -1B, but it decreased the effects of dex alone, or dex with CPTcAMP, or glucagon (Figs. 2B, 3B, D).

Actinomycin D blocked the dex effect on the levels of mRNA for lipin-1A or -1B in mouse hepatocytes (**Fig. 4**) and for lipin-1 and -1B in rat hepatocytes (results not shown). This demonstrates that the increase in mRNA depends on increased transcription. Conversely, the dex-induced expression of mRNA for lipin-1 and -1B in rat hepatocytes was not decreased by the presence of cycloheximide, an inhibitor of protein synthesis (results not shown). With mouse hepatocytes, cycloheximide decreased the relative dex-induced increase in mRNA for lipin-1A and -1B (Fig. 4D, E).

Hormonal regulation of expression of mRNA for lipin-2

For mouse hepatocytes, no treatment that we used increased lipin-2 mRNA levels (Fig. 1E, F). In fact, the expression of mRNA for lipin-2 decreased during the incubation period in the nontreated controls and in dex- or insulin-treated hepatocytes. The presence of CPTcAMP alone, or in combination with dex, or insulin maintained the mRNA at the starting level for ~8 h. For rat hepatocytes, lipin-2 mRNA levels decreased over the period of incubation under all conditions (Fig. 3E, F).

Hormonal regulation of expression of mRNA for lipin-3

Treatment of mouse hepatocytes with dex increased the relative lipin-3 mRNA concentrations by ~4-fold after 8 h (Fig. 1G). CPTcAMP or insulin alone had no significant effect relative to the nontreated control, but both decreased the dex-induced increase in lipin-3 mRNA (Fig. 1G, H). Treatment with actinomycin D or cycloheximide also blocked the dex-induced increase in mRNA (Fig. 4C, F). These inhibitors also attenuated the expression of lipin-3 mRNA in the nontreated control hepatocytes after 8 h (Fig. 4C, F) or 4 h (results not

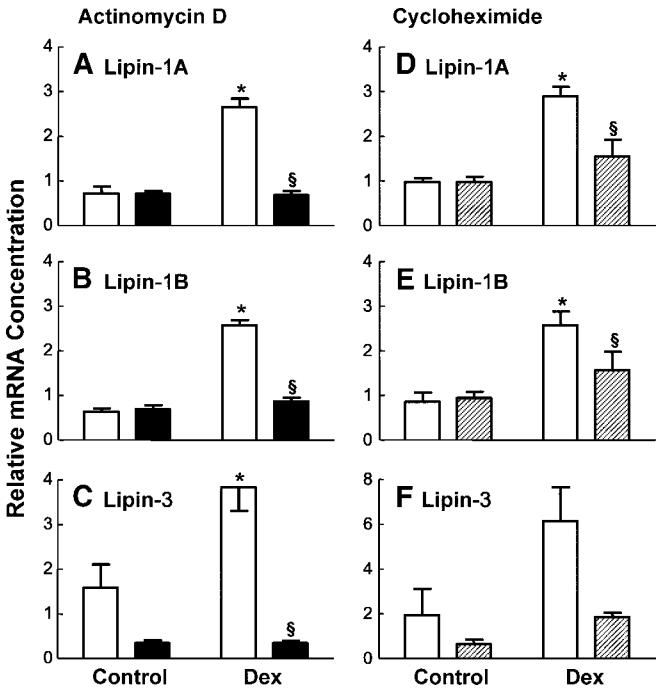

Fig. 4. Effects of actinomycin D and cycloheximide on the dex-induced increase in mRNA for lipin-1A, -1B, and -3. Mouse hepatocytes were treated with or without dex (Dex) in the presence or absence of 10 μg/ml actinomycin D (cells were also preincubated for 30 min with actinomycin D) (A–C) or 5 μg/ml cycloheximide (D–F). To measure mRNA production for lipin-1A and -1B or lipin-3, the hepatocytes were incubated for 4 or 8 h, respectively, based upon the time required to achieve optimum stimulation of mRNA production as show in Fig. 1. White columns show values for incubations in the absence of inhibitor, whereas black and hatched columns indicate the presence of actinomycin D or cycloheximide, respectively. Results are means ± SEM for three independent experiments, except for F, where means ± ranges are shown for two experiments. The significance of the differences ($P < 0.05$), as evaluated with a Student's *t*-test, is indicated as follows: * dex treatment different from the untreated control value; § the actinomycin D or cycloheximide result is different from the equivalent incubation without these inhibitors.

shown). One explanation for these results is that the transcription of lipin-3 mRNA in mouse hepatocytes is dependent on the rapid synthesis of an unidentified protein.

In rat hepatocytes, none of the hormonal treatments changed lipin-3 mRNA levels relative to the nontreated control (Fig. 3G, H). There appeared to be a gradual increase in lipin-3 mRNA levels during the 18 h of incubation even in the nontreated control (Fig. 3G, H). This effect was blocked by actinomycin D or cycloheximide (results not shown).

Relationship of the expression of mRNA for peroxisome proliferator-activated receptor-coactivator-1α and PPARα to that of lipin-1

Full induction of hepatic lipin-1 expression under conditions such as fasting requires the presence of peroxisome proliferator-activated receptor-coactivator-1α (PGC-1α), and lipin-1 also interacts physically with both PGC-1α and PPARα (27). To understand whether the effects of dex, CPTcAMP, and insulin on the expression of mRNA for lipin-1A or -1B depend upon prior changes in the transcription for PGC-1α and PPARα, we determined the time course and hormonal requirements for expression.

In mouse hepatocytes, the maximum effect of CPTcAMP in increasing mRNA expression for PGC-1α was achieved by 4 h. Dex alone had no significant effect on the expression of mRNA for PGC-1α, but it synergized the action of CPTcAMP (**Fig. 5A**). Insulin did not significantly affect these actions of CPTcAMP and dex + CPTcAMP (Fig. 5B).

Dex produced maximum increases in mRNA expression for PPARα by 4 h (Fig. 5C). CPTcAMP alone did not change the mRNA expression, but there was an indication that it might have delayed the maximum expression of PPARα. Insulin did not modify the effect of dex or dex + CPTcAMP significantly (Fig. 5D).

In rat hepatocytes, CPTcAMP increased mRNA expression for PGC-1α, especially in the presence of dex. The maximum increase occurred after ~8 h (**Fig. 6A, B**). Insulin had no significant effect in inhibiting these actions of CPTcAMP and dex + CPTcAMP.

Dex alone increased the mRNA for PPARα after 8 h (Fig. 6C, D), but in contrast to mouse hepatocytes, CPTcAMP partly attenuated this effect. There was also a pronounced effect of insulin in attenuating the dex-induced increase in mRNA for PPARα, which was not seen in mouse hepatocytes.

These results from mouse and rat hepatocytes show that the induction of mRNA expression for PGC-1α and PPARα by CPTcAMP and dex, respectively, occurs at the same time rather than preceding that for lipin-1A and -1B. Dex alone does not increase the expression of PGC-1α mRNA; therefore, the dex-induced production of lipin-1 mRNA cannot rely on an indirect effect through PGC-1α production. Also, insulin does not block the increase in mRNA for PGC-1α in mouse or rat hepatocytes. Therefore, the insulin effect in decreasing the production of mRNA for lipin-1A and -1B cannot be mediated by decreasing the transcription regulation of *Lpin1* by regulating PGC-1α expression.

Dex and CPTcAMP increase PAP1 activity in mouse and rat hepatocytes, and insulin partially blocks these effects

These experiments were designed to relate the changes in the lipin mRNA concentrations to those in PAP1 activity. Dex increased total PAP1 activity in mouse hepatocytes by ~1.8-fold after 8 h of incubation compared with the activity at the beginning of the incubation (**Fig. 7A**). The dex-induced increase in PAP1 activity was enhanced to >2-fold when CPTcAMP was added together with dex (Fig. 7A). These increases in PAP1 activity remained until at least 16 h. When added alone, CPTcAMP had no significant effect on PAP1 activity. Insulin alone also had no significant effect on PAP1 activity, but it partially reversed the increases produced by dex or dex + CPTcAMP (Fig. 7B).

In rat hepatocytes, dex increased PAP1 activity by ~2.5-fold after incubation for 12 or 18 h compared with

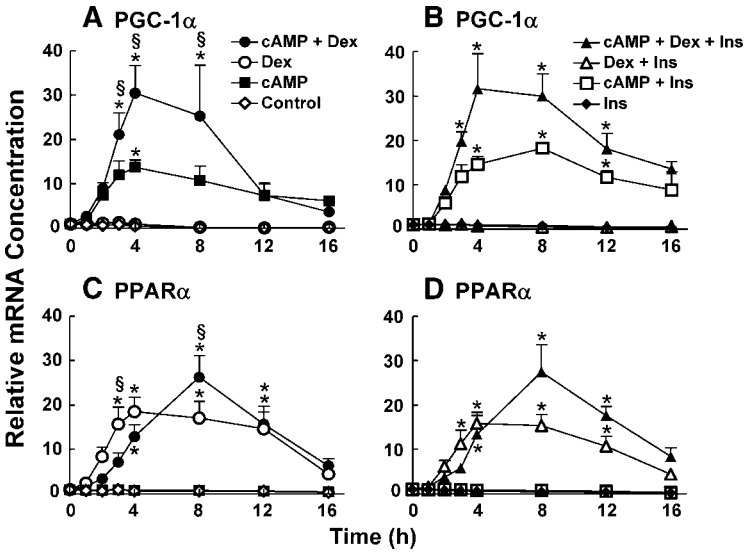

Fig. 5. Interaction of dex, cAMP, and insulin in controlling mRNA expression for peroxisome proliferator-activated receptor α (PPARα) and peroxisome proliferator-activated receptor-coactivator-1α (PGC-1α) in mouse hepatocytes. Mouse hepatocytes were incubated for the times shown with 100 nM dex (Dex), 100 μM CPTcAMP (cAMP), and 100 nM insulin (Ins) alone or in combination as indicated. Relative mRNA concentrations for PGC-1α (A, B) and PPARα (C, D) were measured by real-time RT-PCR and expressed relative to that for cyclophilin A. Results are expressed as means ± SEM for three independent experiments. The significance of the differences ($P < 0.05$) is indicated as follows: * different from the untreated control value; § dex alone different from dex + CPTcAMP treatment.

the activity at the beginning of the incubation (Fig. 7C). CPTcAMP did not increase PAP1 activity, but it prevented the decline of up to 70% in activity that occurred after 18 h in the untreated controls or in insulin-treated cells (Fig. 7C, D). These results are compatible with the known stability of PAP1 activity in rat hepatocytes. PAP1 activity in untreated or insulin-treated cells has a half-life of 5–7 h, and this is increased to 12 h by glucagon (through cAMP) (36). Adding CPTcAMP together with dex in the present experiments produced a synergistic effect, and increases of >4-fold in PAP1 activity were obtained after 12 and 18 h (Fig. 7C). Insulin attenuated the actions of dex alone or dex + CPTcAMP (Fig. 7D).

Dex and CPTcAMP increase lipin-1 synthesis and protein levels, and insulin attenuates these effects

The purpose of these experiments was to investigate whether the dex-induced increases in lipin-1 mRNA expression result in increased expression of lipin-1 protein. To do this, we first validated the quality of the antibody that we used. **Figure 8A** shows the results of a Western blot for recombinant lipin-1A, -1B, -2, and -3 containing a V5 tag that were individually expressed in HEK 293 cells (3). The blots were probed simultaneously with mouse monoclonal anti-V5 antibody and rabbit polyclonal anti-lipin-1 antibody. The results show coincident detection of lipin-1A and -1B by the anti-lipin-1 antibody and V5 antibodies. There was no cross-reactivity of the anti-lipin-1 antibody with lipin-2 and -3. The second test was to compare the Western blots from adipose tissue of wild-type mice and *fld* mice, which are deficient in lipin-1 (3). The rabbit antibody detected lipin-1 in the sample from a wild-type mouse, but there was no response with the same amount of protein from the *fld* mouse (Fig. 8B). These results provide evidence that the lipin-1 antibody can specifically detect lipin-1A and -1B.

Figure 8B also shows the Western blots for lipin-1 from mouse and rat hepatocytes that were incubated for 8 and 12 h, respectively, with the various combinations of hormones and CPTcAMP based upon the

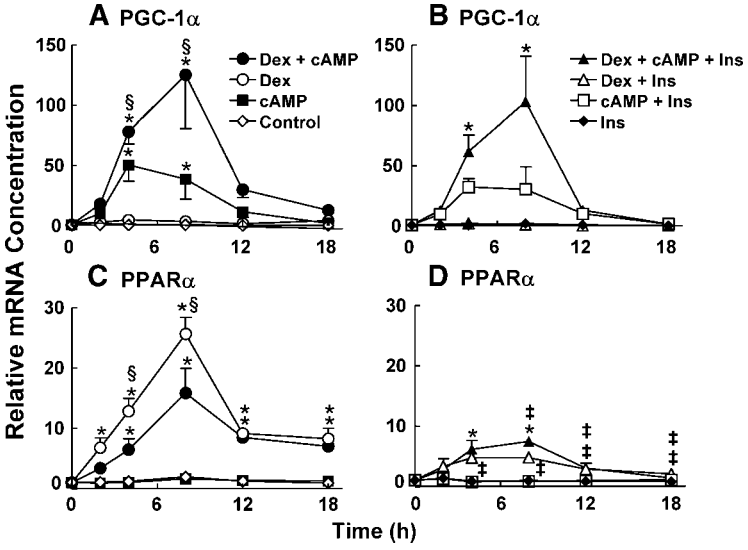

Fig. 6. Interaction of dex, cAMP, and insulin in controlling mRNA expression for PPARα and PGC-1α in rat hepatocytes. Rat hepatocytes were incubated for the times shown with 100 nM dex (Dex), 100 μM CPTcAMP (cAMP), and 100 nM insulin (Ins) alone or in combination as indicated. Relative mRNA concentrations for PGC-1α (A, B) and PPARα (C, D) were measured by real-time RT-PCR and expressed relative to that for cyclophilin A. Results are expressed as means ± SEM for three independent experiments. The significance of the differences ($P < 0.05$) is indicated as follows: * different from the untreated control value; § dex alone different from dex + CPTcAMP treatment; ‡ incubation with insulin decreases the effect of dex alone or dex + CPTcAMP.

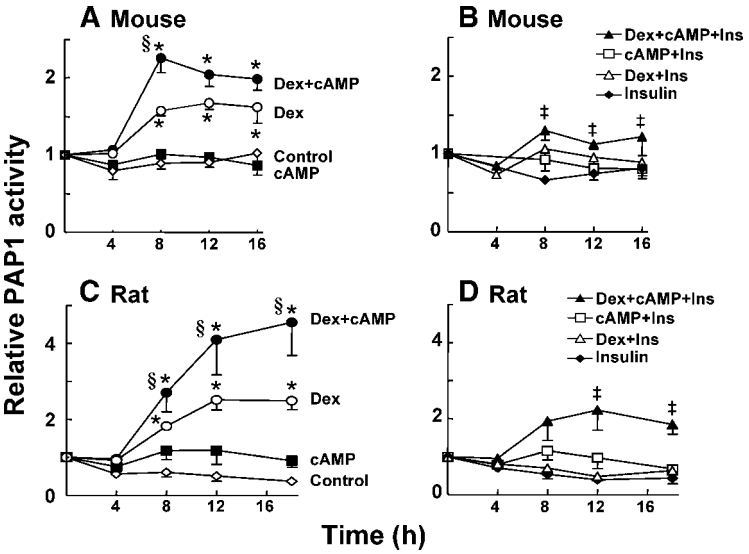

Fig. 7. Interaction of dex, glucagon, and insulin in controlling phosphatidate phosphatase (PAP1) activity in mouse and rat hepatocytes. Mouse (A, B) and rat (C, D) hepatocytes were incubated for various times with 100 nM dex (Dex), 100 μM CPTcAMP (cAMP), and 100 nM insulin (Ins) alone or in combination as indicated. The average PAP1 specific activities at the beginning of the incubation were 32 ± 15 (n = 7) and 36 ± 12 (n = 5) nmol diacylglycerol produced per min per mg protein for mouse and rat hepatocytes, respectively. The results are expressed relative to the initial untreated value, which was normalized to 1. Results are means ± SEM for three to nine independent experiments. The significance of the differences ($P < 0.05$) is indicated as follows: * different from the untreated control value; § dex alone is significantly different from dex + CPTcAMP treatment; ‡ incubation with insulin decreases the effect of dex alone or dex + CPTcAMP.

changes in PAP1 activity from Fig. 7. The same amount of protein from these samples was loaded onto each lane, and GAPDH was used as an additional loading control (Fig. 8B). The results for lipin-1 were expressed relative to GAPDH, and the average results are illustrated in Fig. 8C. Lipin-1 was detected as multiple bands, which probably represent different phosphorylation states (24). Incubation of mouse hepatocytes with dex increased the relative lipin-1 protein levels by ~2.5-fold, and this was further increased to ~4.5-fold when CPTcAMP was

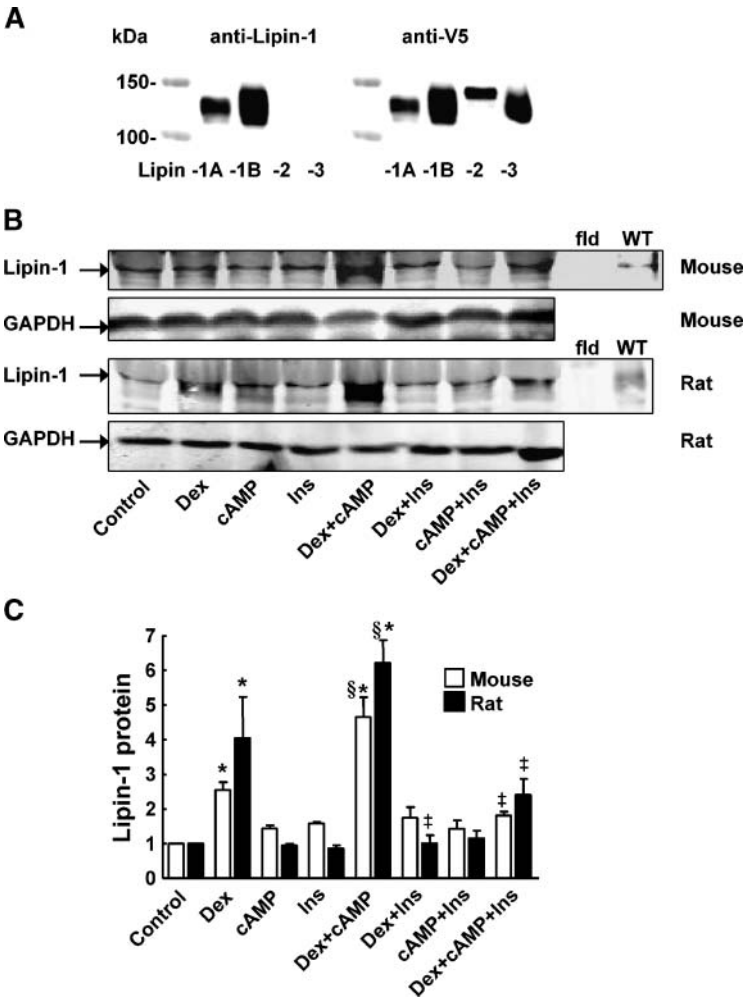

Fig. 8. Interaction of dex, glucagon, and insulin in controlling lipin-1 expression in mouse and rat hepatocytes. A: Western blots for different V5-tagged lipins that were detected simultaneously with a rabbit polyclonal anti-lipin-1 antibody and a mouse monoclonal anti-V5 antibody. B: Representative Western blots for mouse and rat hepatocytes that were incubated for 8 and 12 h, respectively, with 100 nM dex (Dex), 100 μM CPTcAMP (cAMP), and 100 nM insulin (Ins) alone or in combination as indicated. A marker for lipin-1 is shown on the right side, where adipose tissue extracts from wild-type (WT) and *fld* mice were used. The lower Western blots are for GAPDH, which was used as a loading control. C: Means ± SEM for the relative expression of lipin-1 after normalization against GAPDH for each treatment. Results are for three to four independent experiments. The significance of the differences ($P < 0.05$) is indicated as follows: * different from the untreated control value; § dex alone is significantly different from dex + CPTcAMP treatment; ‡ incubation with insulin decreases the effect of dex alone or dex + CPTcAMP.

present (Fig. 8C). CPTcAMP had no significant effect on lipin-1 protein levels when added alone. Insulin alone had no effect on lipin-1 protein levels, but it attenuated the effects of dex and dex + CPTcAMP. The results are expressed relative to the nontreated control at the 8 h time point. This value was ~90% of that at the beginning of the incubation.

Incubation of rat hepatocytes with dex increased the relative lipin-1 protein levels by ~4-fold, and this increased to ~6-fold when CPTcAMP was also present (Fig. 8B, C). Incubation with CPTcAMP alone had no significant effect on lipin-1 protein levels. Insulin alone also had no significant effect, but it blocked the effects of dex and attenuated the actions of dex + CPTcAMP. Lipin-1 protein levels decreased by ~50% in the nontreated controls after the 12 h incubation (results not shown). Therefore, the relative increases in the expression of lipin-1 with dex and dex + CPTcAMP that are shown in Fig. 8B would be approximately halved if expressed relative to the value at the beginning of the incubation. By comparison, the results for the mouse would only be affected by ~10%.

The dex-induced increases in lipin-1 protein levels in rat and mouse hepatocytes were blocked by actinomycin D or cycloheximide, and this was reflected in the lack of increase in PAP1 activity (**Fig. 9A, B**). These combined results demonstrate that the dex-induced increase in lipin-1 and PAP1 activity depends upon increases in both transcription and translation.

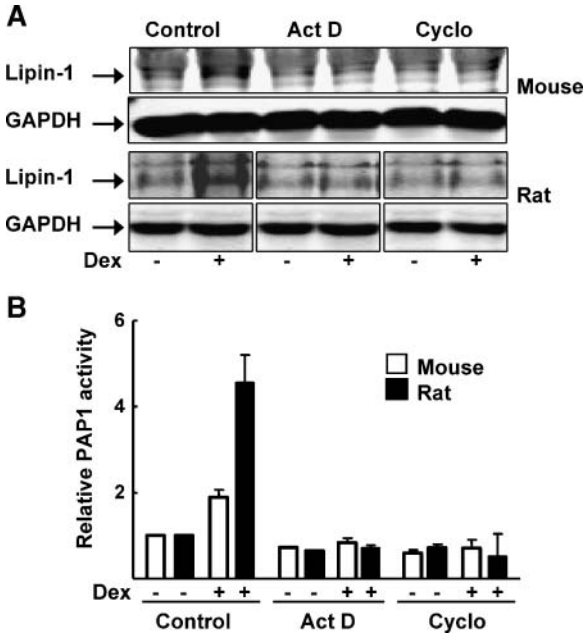

Fig. 9. Actinomycin D and cycloheximide block the dex-induced expression of lipin-1 and PAP1 activity. Mouse and rat hepatocytes were preincubated with or without 10 μg/ml actinomycin D (Act D) or 5 μg/ml cycloheximide (Cyclo) for 30 min. The inhibitors were maintained in the subsequent incubations in the presence or absence of 100 nM dex, which for mouse and rat hepatocytes were 8 and 12 h, respectively. These times were based upon the results in Fig. 4. A: Representative Western blots. B: PAP1 activity relative to the equivalent incubation in the absence of inhibitors or dex. Results are means ± SEM for three independent experiments with mouse hepatocytes and means ± ranges for two experiments with rat hepatocytes.

Effects of fasting mice on the expression of mRNA for different lipins in the liver

The next series of experiments were performed to relate our observations with hepatocytes to the effects of fasting mice on the expression of mRNA for different lipins, PPARα, and PGC-1α in the liver. mRNA concentrations for lipin-1A, -1B, and -2 were increased, on average, by 4.3-, 3.2-, and 2.4-fold respectively, but there was no significant change in mRNA for lipin-3 (**Fig. 10**). Fasting also increased the mRNA for PGC-1α by 2.4-fold, as expected (37), although the apparent increase for PPARα did not reach statistical significance.

DISCUSSION

The recent identification of lipin proteins as PAP1 enzymes made it possible to investigate the molecular basis for the modulation of PAP1 activity that occurs in various physiological and pathological conditions. The liver expresses lipin-1A, -1B, -2, and -3; therefore, it was essential to elucidate how each lipin responds to GC, glucagon, and insulin to understand the functions of individual lipins and the reason for changes in the composite PAP1 activity. Our present experiments with rat and mouse hepatocytes demonstrate a level of hormonal control that is preserved between these rodent species. The lipin-1A and -1B isoforms, alternative splice variants of the *Lpin1* gene, are induced at the transcriptional level by the action of dex. This dex action, especially in mouse hepatocytes, was synergized by glucagon, whereas insulin attenuated this effect. Insulin can block the effects of glucagon by stimulating phosphodiesterase activity and diminishing the increase in cAMP. However, we also used CPTcAMP, which is not readily degraded by phosphodi-

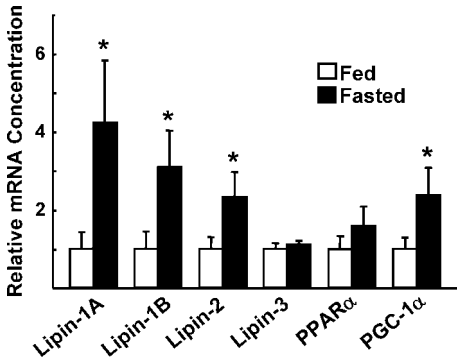

Fig. 10. Effects of fasting on mRNA expression for the lipins, PPARα, and PGC-1α in mouse liver. mRNA concentrations were measured by real-time PCR in livers from C57BL/6J mice that were fasted for 16 h (fasted samples) or fasted for 16 h and refed for 4 h (fed samples). Results are means ± SD for three mice in each group, and significant differences compared with the fed values are indicated (* $P < 0.05$).

esterase (38), and observed the same insulin attenuation of *Lpin1* transcription. This result indicates that insulin exerts a more direct effect on lipin-1 expression.

There is no evidence that the hormonal combinations we used regulate the relative expression of the lipin-1 splice isoforms in hepatocytes. The dex-induced increases in PAP1 activity depended upon transcription of the *Lpin1* gene and increased lipin-1 mRNA, which was followed by increases in lipin-1 synthesis and protein expression. This conclusion is supported by the effects of actinomycin D and cycloheximide, which blocked the increase in these parameters.

Our results demonstrate that the expression of lipin-2 and -3 is regulated in a distinct manner from that of lipin-1. Incubation of mouse and rat hepatocytes with dex resulted in a time-dependent decrease in lipin-2 mRNA. Dex did produce small increases in lipin-3 mRNA in mouse hepatocytes, but this effect was blocked by CPT-cAMP. In rat hepatocytes, there was no significant effect of dex or dex + CPTcAMP on lipin-3 mRNA expression relative to nontreated controls. Therefore, increased transcription of the *Lpin2* and *Lpin3* genes did not contribute to the dex + CPTcAMP-induced increase in PAP1 activity in isolated rat or mouse hepatocytes, which depended upon increased transcription and translation. At present, we are unable to perform satisfactory Western blot analysis for lipin-2 and -3 because we lack convincing evidence that the antibodies we possess selectively identify lipin-2 or -3 in hepatocyte extracts.

Our conclusions that the dex- and dex + cAMP-induced increases in PAP1 activity are accounted for by increased lipin-1 expression are strongly supported by studies in vivo. Livers of fasted *fld* mice, which do not express lipin-1, do not show increases in PAP1 activity compared with ~2-fold increases in control mice (24). Lipin-1 transcription and lipin-1 protein levels in the livers of wild-type mice are increased after fasting or dex injection (27). In our experiments with fasted mice, we also observed increases in mRNA for lipin-1A, -1B, and PGC-1α but not for lipin-3. Surprisingly, the relative mRNA concentration for lipin-2 was doubled in the livers of fasted mice. This is unlikely to have occurred by GC action, based upon our hepatocyte work. It could have resulted from a cAMP effect, because this second messenger appeared to increase the relative concentration of lipin-2 mRNA in mouse hepatocytes relative to the nontreated, or dex-treated, cells. However, the physiological significance of the increased lipin-2 mRNA in fasted mice is uncertain, because of the lack of increase in hepatic PAP1 activity in *fld* mice after fasting (24). The combined results from experiments in vivo strongly support our conclusion that increased lipin-1 expression accounts for the increased PAP1 activity that occurs when there is increased GC action compared with insulin.

However, there could be other factors that contribute to the observed increase in PAP1 activity. First, we established that glucagon, through cAMP, increases the half-life of GC-induced PAP1 activity (which we now ascribe to lipin-1) from ~7 to 12 h (36). This could contribute to the increased expression of the lipin-1 protein that is produced by dex + CPTcAMP. Second, CPTcAMP could affect PAP1 activity through its phosphorylation, and we provided indirect evidence for this before the lipins were discovered (8, 39). Direct evidence for the phosphorylation of yeast PAP1 (PAH1) has now been obtained (40). In adipose tissue, the level of lipin-1 phosphorylation is controlled by the balance of signaling from insulin versus cAMP (24, 28). These changes in lipin-1 phosphorylation were not reflected in changes in PAP1 activity as measured in vitro (24), although treatment of liver cytosol with phosphatases did decrease total PAP1 activity in earlier work (39). The major effect of lipin-1 phosphorylation appears to be to regulate its subcellular distribution (24) and its physiological expression (5).

A third explanation for the regulation of lipin-1 and PAP1 activity is through interaction with other proteins, including PGC-1α and PPARα (27). Not only does lipin-1 amplify signaling by PGC-1α and PPARα, but PGC-1α is required as a coamplifier of lipin-1 expression in the liver. Thus, induction of lipin-1 expression in fasted or in dex-treated mice is partially attenuated when PGC-1α is totally deficient, indicating a partial dependence on PGC-1α. Hepatic lipin-1 expression is also increased in type 1 and type 2 diabetes, conditions in which PGC-1α expression is increased (27). Our results showing that cycloheximide partially inhibits the dex-induced production of lipin-1 mRNA in mouse hepatocytes indicate that the induced expression of cotranscriptional regulators, including PGC-1α, may be required for full lipin-1 expression. In rat hepatocytes, cycloheximide produced no significant decrease in lipin-1 mRNA expression. This could indicate that the endogenous levels of cotranscriptional regulators for lipin-1 mRNA production in rat hepatocytes are sufficient to sustain high levels of transcriptional induction. Significantly, CPTcAMP had only a marginal effect in increasing the dex-induced production of lipin-1 mRNA in rat hepatocytes, whereas the effect was more marked in mouse hepatocytes.

As expected from previous work (37, 41), mRNA for PGC-1α was increased by CTPcAMP in mouse and rat hepatocytes (Figs. 5, 6). Dex alone had no significant effect on the relative mRNA for PGC-1α, but it strongly synergized the CPTcAMP action. By contrast, dex was effective in increasing mRNA expression for PPARα, as expected (42), and there was little if any effect of CPTcAMP. These combined observations are compatible with the increased expression of PGC-1α and PPARα in starvation and diabetes (37, 41). Although insulin decreases the signaling effects of PGC-1α in vivo (37, 41), there was no significant effect of insulin in directly decreasing the expression of PGC-1α mRNA in mouse and rat hepatocytes. Our results for PGC-1α agree with previous work with mouse hepatocytes (41). Although lipin-1 expression depends partly on the presence of PGC-1α (27), the effects of dex + CPTcAMP in increasing the mRNA for PGC-1α and PPARα do not precede the maximum expression of mRNA for lipin-1A or -1B. Also, the inhibition of lipin-1 mRNA expression by insulin probably results from a direct action

on transcription of the *Lpin1* gene, because insulin did not block the stimulated increase in mRNA for PGC-1α. In the case of PPARα, we did see an attenuation of the dex induction in mRNA concentrations in rat hepatocytes, but this insulin effect was not observed with the mouse hepatocytes.

In liver, the interaction of lipin-1 with PGC-1α and PPARα promotes the transcriptional regulation of enzymes involved in β-oxidation, and this is also a response to starvation and diabetes (27). We proposed that the GC-induced increase in PAP1 activity could be an adaptive response to protect the liver against an increased FA load and lipotoxicity in starvation and diabetes (5, 8). However, the accumulation of TAG in the liver could also be lipotoxic (43). We now show that lipin-1 specifically is responsible for the GC-induced increase in PAP1 activity, which we previously showed to result in increased hepatic glycerolipid synthesis (7). As FA concentrations increase and exceed the capacity for β-oxidation, PAP1 translocates to ER membranes to facilitate the storage of the excess FA as TAG in fat droplets, resulting in steatosis (5). The FA-induced translocation of lipin-1 occurs in adipocytes (24). TAG synthesis in the liver should not be viewed simply as antagonistic to β-oxidation. It is a companion pathway, because these TAG stores are turned over and the FA is used for oxidation (5). Increased lipin-1 expression, in addition to facilitating TAG synthesis, also promotes the transcription of key enzymes in FA oxidation (27).

Alternatively, the stored TAGs are hydrolyzed, and together with exogenous FAs they are reesterified for VLDL production. The secretions of TAG and apolipoprotein B are also increased by GC, and insulin antagonizes this action (44–46). Hepatic PAP1 activity is also positively correlated with circulating TAG (5, 13, 47), and the changes in the level of lipin-1 are probably partly responsible for the regulation of VLDL secretion.

We also established that the hepatic responses of PAP1 to GC, glucagon, and insulin are coregulated with those of enzymes involved in controlling gluconeogenesis (5, 9). Transcription of key gluconeogenic enzymes is also regulated through PGC-1α and PPARα. The coregulation of lipin-1 expression and the physical interactions of lipin-1 with PGC-1α and PPARα (27) could help to modulate and integrate gluconeogenesis with the increased capacity for hepatic TAG synthesis and β-oxidation in starvation and diabetes. In this respect, it may be significant that the livers of *fld* mice show a 40% decrease in hepatic glucose production in fasting (48).

In conclusion, GC increases the transcription of the *Lpin1* gene. cAMP synergizes this GC effect in specifically increasing the expression of lipin-1 mRNA in rat and mouse hepatocytes, and there was no significant increase in mRNA for lipin-2 and -3. The effect of dex + CPTcAMP in increasing lipin-1 mRNA production was attenuated by insulin. This control of the transcription of the *Lpin1* gene explains the increased synthesis and expression of lipin-1A and -1B. This in turn accounts for the increased PAP1 activity that is observed after treating hepatocytes with dex + CPTcAMP or in vivo in starvation and diabetes. The FA-stimulated association of lipin-1 with the nucleus probably enables it to regulate transcription and increase FA oxidation in starvation and diabetes. Higher expression of lipin-1 also increases the capacity for hepatic glycerolipid synthesis and secretion in VLDL. This capacity is expressed as FA accumulates and lipin-1 translocates to the ER, where TAGs are produced. This lipin-1-induced increase in TAG synthesis is reflected in vivo in the steatosis that is observed in starvation, diabetes, ischemia, toxic conditions, and ethanol intoxication.

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REFERENCES

1. Jamal, Z., A. Martin, A. Gomez-Munoz, and D. N. Brindley. 1991. Plasma membrane fractions from rat liver contain a phosphatidate phosphohydrolase distinct from that in the endoplasmic reticulum and cytosol. *J. Biol. Chem.* **266:** 2988–2996.
2. Brindley, D. N. 2004. Lipid phosphate phosphatases and related proteins: signaling functions in development, cell division, and cancer. *J. Cell. Biochem.* **92:** 900–912.
3. Donkor, J., M. Sariahmetoglu, J. Dewald, D. N. Brindley, and K. Reue. 2007. Three mammalian lipins act as phosphatidate phosphatases with distinct tissue expression patterns. *J. Biol. Chem.* **282:** 3450–3457.
4. Han, G. S., W. I. Wu, and G. M. Carman. 2006. The *Saccharomyces cerevisiae* lipin homolog is a Mg^{2+}-dependent phosphatidate phosphatase enzyme. *J. Biol. Chem.* **281:** 9210–9218.
5. Brindley, D. N. 1988. Phosphatidate Phosphohydrolase: Its Role in Glycerolipid Synthesis. CRC Press, Boca Raton, FL. 21–77.
6. Sturton, R. G., S. C. Butterwith, S. L. Burditt, and D. N. Brindley. 1981. Effects of starvation, corticotropin injection and ethanol feeding on the activity and amount of phosphatidate phosphohydrolase in rat liver. *FEBS Lett.* **126:** 297–300.
7. Glenny, H. P., and D. N. Brindley. 1978. The effects of cortisol, corticotropin and thyroxine on the synthesis of glycerolipids and on the phosphatidate phosphohydrolase activity in rat liver. *Biochem. J.* **176:** 777–784.
8. Pittner, R. A., R. Fears, and D. N. Brindley. 1985. Interactions of insulin, glucagon and dexamethasone in controlling the activity of glycerol phosphate acyltransferase and the activity and subcellular distribution of phosphatidate phosphohydrolase in cultured rat hepatocytes. *Biochem. J.* **230:** 525–534.
9. Pittner, R. A., R. Fears, and D. N. Brindley. 1985. Effects of cyclic AMP, glucocorticoids and insulin on the activities of phosphatidate phosphohydrolase, tyrosine aminotransferase and glycerol kinase in isolated rat hepatocytes in relation to the control of triacylglycerol synthesis and gluconeogenesis. *Biochem. J.* **225:** 455–462.
10. Knox, A. M., R. G. Sturton, J. Cooling, and D. N. Brindley. 1979. Control of hepatic triacylglycerol synthesis. Diurnal variations in hepatic phosphatidate phosphohydrolase activity and in the concentrations of circulating insulin and corticosterone in rats. *Biochem. J.* **180:** 441–443.
11. Mangiapane, E. H., K. A. Lloyd-Davies, and D. N. Brindley. 1973. A study of some enzymes of glycerolipid biosynthesis in rat liver after subtotal hepatectomy. *Biochem. J.* **134:** 103–112.

12. Whiting, P. H., M. Bowley, R. G. Sturton, P. H. Pritchard, D. N. Brindley, and J. N. Hawthorne. 1977. The effect of chronic diabetes, induced by streptozotocin, on the activities of some enzymes of glycerolipid synthesis in rat liver. *Biochem. J.* **168:** 147–153.
13. Jamal, Z., A. Martin, A. Gomez-Munoz, P. Hales, E. Chang, J. C. Russell, and D. N. Brindley. 1992. Phosphatidate phosphohydrolases in liver, heart and adipose tissue of the JCR:LA corpulent rat and the lean genotypes: implications for glycerolipid synthesis and signal transduction. *Int. J. Obes. Relat. Metab. Disord.* **16:** 789–799.
14. Kinnula, V. L., and I. Hassinen. 1978. Metabolic adaptation to hypoxia. Redox state of the cellular free NAD pools, phosphorylation state of the adenylate system and the (Na^+-K^+)-stimulated ATP-ase in rat liver. *Acta Physiol. Scand.* **104:** 109–116.
15. Sturton, R. G., P. H. Pritchard, L. Y. Han, and D. N. Brindley. 1978. The involvement of phosphatidate phosphohydrolase and phospholipase A activities in the control of hepatic glycerolipid synthesis. Effects of acute feeding with glucose, fructose, sorbitol, glycerol and ethanol. *Biochem. J.* **174:** 667–670.
16. Brindley, D. N., J. Cooling, H. P. Glenny, S. L. Burditt, and I. S. McKechnie. 1981. Effects of chronic modification of dietary fat and carbohydrate on the insulin, corticosterone and metabolic responses of rats fed acutely with glucose, fructose or ethanol. *Biochem. J.* **200:** 275–283.
17. Savolainen, M. J., E. Baraona, P. Pikkarainen, and C. S. Lieber. 1984. Hepatic triacylglycerol synthesizing activity during progression of alcoholic liver injury in the baboon. *J. Lipid Res.* **25:** 813–820.
18. Simpson, K. J., S. Venkatesan, A. Martin, D. N. Brindley, and T. J. Peters. 1995. Activity and subcellular distribution of phosphatidate phosphohydrolase (EC 3.1.3.4) in alcoholic liver disease. *Alcohol Alcohol.* **30:** 31–36.
19. Brindley, D. N., J. Cooling, S. L. Burditt, P. H. Pritchard, S. Pawson, and R. G. Sturton. 1979. The involvement of glucocorticoids in regulating the activity of phosphatidate phosphohydrolase and the synthesis of triacylglycerols in the liver. Effects of feeding rats with glucose, sorbitol, fructose, glycerol and ethanol. *Biochem. J.* **180:** 195–199.
20. Cascales, C., E. H. Mangiapane, and D. N. Brindley. 1984. Oleic acid promotes the activation and translocation of phosphatidate phosphohydrolase from the cytosol to particulate fractions of isolated rat hepatocytes. *Biochem. J.* **219:** 911–916.
21. Martin, A., R. Hopewell, P. Martin-Sanz, J. E. Morgan, and D. N. Brindley. 1986. Relationship between the displacement of phosphatidate phosphohydrolase from the membrane-associated compartment by chlorpromazine and the inhibition of the synthesis of triacylglycerol and phosphatidylcholine in rat hepatocytes. *Biochim. Biophys. Acta.* **876:** 581–591.
22. Peterfy, M., J. Phan, P. Xu, and K. Reue. 2001. Lipodystrophy in the fld mouse results from mutation of a new gene encoding a nuclear protein, lipin. *Nat. Genet.* **27:** 121–124.
23. Peterfy, M., J. Phan, and K. Reue. 2005. Alternatively spliced lipin isoforms exhibit distinct expression pattern, subcellular localization, and role in adipogenesis. *J. Biol. Chem.* **280:** 32883–32889.
24. Harris, T. E., T. A. Huffman, A. Chi, J. Shabanowitz, D. F. Hunt, A. Kumar, and J. C. Lawrence, Jr. 2007. Insulin controls subcellular localization and multisite phosphorylation of the phosphatidic acid phosphatase, lipin 1. *J. Biol. Chem.* **282:** 277–286.
25. Phan, J., M. Peterfy, and K. Reue. 2004. Lipin expression preceding peroxisome proliferator-activated receptor-gamma is critical for adipogenesis in vivo and in vitro. *J. Biol. Chem.* **279:** 29558–29564.
26. Langner, C. A., E. H. Birkenmeier, O. Ben-Zeev, M. C. Schotz, H. O. Sweet, M. T. Davisson, and J. I. Gordon. 1989. The fatty liver dystrophy (fld) mutation. A new mutant mouse with a developmental abnormality in triglyceride metabolism and associated tissue-specific defects in lipoprotein lipase and hepatic lipase activities. *J. Biol. Chem.* **264:** 7994–8003.
27. Finck, B. N., M. C. Gropler, Z. Chen, T. C. Leone, M. A. Croce, T. E. Harris, J. C. Lawrence, Jr., and D. P. Kelly. 2006. Lipin 1 is an inducible amplifier of the hepatic PGC-1alpha/PPARalpha regulatory pathway. *Cell Metab.* **4:** 199–210.
28. Huffman, T. A., I. Mothe-Satney, and J. C. Lawrence, Jr. 2002. Insulin-stimulated phosphorylation of lipin mediated by the mammalian target of rapamycin. *Proc. Natl. Acad. Sci. USA.* **99:** 1047–1052.
29. Sahoo, D., T. C. Trischuk, T. Chan, V. A. Drover, S. Ho, G. Chimini, L. B. Agellon, R. Agnihotri, G. A. Francis, and R. Lehner. 2004. ABCA1-dependent lipid efflux to apolipoprotein A-I mediates HDL particle formation and decreases VLDL secretion from murine hepatocytes. *J. Lipid Res.* **45:** 1122–1131.
30. Martin, A., A. Gomez-Munoz, Z. Jamal, and D. N. Brindley. 1991. Characterization and assay of phosphatidate phosphatase. *Methods Enzymol.* **197:** 553–563.
31. Sturton, R. G., and D. N. Brindley. 1978. Problems encountered in measuring the activity of phosphatidate phosphohydrolase. *Biochem. J.* **171:** 263–266.
32. Jasinska, R., Q. X. Zhang, C. Pilquil, I. Singh, J. Xu, J. Dewald, D. A. Dillon, L. G. Berthiaume, G. M. Carman, D. W. Waggoner, et al. 1999. Lipid phosphate phosphohydrolase-1 degrades exogenous glycerolipid and sphingolipid phosphate esters. *Biochem. J.* **340:** 677–686.
33. Martin, A., P. Hales, and D. N. Brindley. 1987. A rapid assay for measuring the activity and the Mg^{2+} and Ca^{2+} requirements of phosphatidate phosphohydrolase in cytosolic and microsomal fractions of rat liver. *Biochem. J.* **245:** 347–355.
34. Butterwith, S. C., R. Hopewell, and D. N. Brindley. 1984. Partial purification and characterization of the soluble phosphatidate phosphohydrolase of rat liver. *Biochem. J.* **220:** 825–833.
35. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* **227:** 680–685.
36. Pittner, R. A., R. Fears, and D. N. Brindley. 1986. Effects of insulin, glucagon, dexamethasone, cyclic GMP and spermine on the stability of phosphatidate phosphohydrolase activity in cultured rat hepatocytes. *Biochem. J.* **240:** 253–257.
37. Puigserver, P., and B. M. Spiegelman. 2003. Peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC-1 alpha): transcriptional coactivator and metabolic regulator. *Endocr. Rev.* **24:** 78–90.
38. Miller, J. P., A. H. Beck, L. N. Simon, and R. B. Meyer, Jr. 1975. Induction of hepatic tyrosine aminotransferase in vivo by derivatives of cyclic adenosine 3′:5′-monophosphate. *J. Biol. Chem.* **250:** 426–431.
39. Butterwith, S. C., A. Martin, and D. N. Brindley. 1984. Can phosphorylation of phosphatidate phosphohydrolase by a cyclic AMP-dependent mechanism regulate its activity and subcellular distribution and control hepatic glycerolipid synthesis? *Biochem. J.* **222:** 487–493.
40. O'Hara, L., G. S. Han, S. Peak-Chew, N. Grimsey, G. M. Carman, and S. Siniossoglou. 2006. Control of phospholipid synthesis by phosphorylation of the yeast lipin Pah1p/Smp2p Mg^{2+}-dependent phosphatidate phosphatase. *J. Biol. Chem.* **281:** 34537–34548.
41. Puigserver, P., J. Rhee, J. Donovan, C. J. Walkey, J. C. Yoon, F. Oriente, Y. Kitamura, J. Altomonte, H. Dong, D. Accili, et al. 2003. Insulin-regulated hepatic gluconeogenesis through FOXO1-PGC-1alpha interaction. *Nature.* **423:** 550–555.
42. Lemberger, T., B. Staels, R. Saladin, B. Desvergne, J. Auwerx, and W. Wahli. 1994. Regulation of the peroxisome proliferator-activated receptor alpha gene by glucocorticoids. *J. Biol. Chem.* **269:** 24527–24530.
43. Unger, R. H. 2002. Lipotoxic diseases. *Annu. Rev. Med.* **53:** 319–336.
44. Wang, C. N., R. S. McLeod, Z. Yao, and D. N. Brindley. 1995. Effects of dexamethasone on the synthesis, degradation, and secretion of apolipoprotein B in cultured rat hepatocytes. *Arterioscler. Thromb. Vasc. Biol.* **15:** 1481–1491.
45. Mangiapane, E. H., and D. N. Brindley. 1986. Effects of dexamethasone and insulin on the synthesis of triacylglycerols and phosphatidylcholine and the secretion of very-low-density lipoproteins and lysophosphatidylcholine by monolayer cultures of rat hepatocytes. *Biochem. J.* **233:** 151–160.
46. Martin-Sanz, P., J. E. Vance, and D. N. Brindley. 1990. Stimulation of apolipoprotein secretion in very-low-density and high-density lipoproteins from cultured rat hepatocytes by dexamethasone. *Biochem. J.* **271:** 575–583.
47. Sessions, V. A., A. Martin, A. Gomez-Munoz, D. N. Brindley, and A. M. Salter. 1993. Cholesterol feeding induces hypertriglyceridaemia in hamsters and increases the activity of the Mg(2+)-dependent phosphatidate phosphohydrolase in the liver. *Biochim. Biophys. Acta.* **1166:** 238–243.
48. Xu, J., W. N. Lee, J. Phan, M. F. Saad, K. Reue, and I. J. Kurland. 2006. Lipin deficiency impairs diurnal metabolic fuel switching. *Diabetes.* **55:** 3429–3438.
49. Morris, K. E., L. M. Schang, and D. N. Brindley. 2006. Lipid phosphate phosphatase-2 activity regulates S-phase entry of the cell cycle in Rat2 fibroblasts. *J. Biol. Chem.* **281:** 9297–9306.
50. Yang, J., N. Sambandam, X. Han, R. W. Gross, M. Courtois, A. Kovacs, M. Febbraio, B. N. Finck, and D. P. Kelly. 2007. CD36 deficiency rescues lipotoxic cardiomyopathy. *Circ. Res.* **100:** 1208–1217.