Short Communication

Role of Phosphatidic Acid Phosphatase Domain Containing 2 in Squalestatin 1–Mediated Activation of the Constitutive Androstane Receptor in Primary Cultured Rat Hepatocytes

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ABSTRACT

Farnesyl pyrophosphate (FPP) is a branch-point intermediate in the mevalonate pathway that is normally converted mainly to squalene by squalene synthase in the first committed step of sterol biosynthesis. Treatment with the squalene synthase inhibitor squalestatin 1 (SQ1) causes accumulation of FPP, its dephosphorylated metabolite farnesol, and several oxidized farnesol-derived metabolites. In addition, SQ1 treatment of primary cultured rat hepatocytes increases CYP2B expression through a mechanism that requires FPP synthesis and activation of the constitutive androstane receptor (CAR). Because direct farnesol treatment also increases CYP2B expression, it seems likely that SQ1-mediated CAR activation requires FPP dephosphorylation to farnesol. The lipid phosphatase, phosphatidic acid phosphatase domain containing 2 (PPAPDC2), was recently reported to catalyze FPP dephosphorylation. We therefore determined the effect of overexpressing or knocking down PPAPDC2 on SQ1-mediated CAR activation in primary cultured rat hepatocytes. Cotransfection of rat hepatocytes with a plasmid expressing rat or human PPAPDC2 enhanced SQ1-mediated activation of a CAR-responsive reporter by 1.7- or 2.4-fold over the SQ1-mediated activation that was produced when hepatocytes were cotransfected with empty expression plasmid. Similarly, transduction of rat hepatocytes with a recombinant adenovirus expressing PPAPDC2 enhanced SQ1-mediated CYP2B1 mRNA induction by 1.4-fold over the induction that was seen in hepatocytes transfected with control adenovirus. Cotransfection with a short hairpin RNA targeting PPAPDC2 reduced SQ1-mediated CAR activation by approximately 80% relative to the activation that occurred in hepatocytes transfected with nontargeting short hairpin RNA. These results indicate that PPAPDC2 plays an important role in SQ1-mediated CAR activation, most likely by catalyzing the conversion of FPP to farnesol.

Introduction

The mevalonate pathway comprises the first portion of the cholesterol biosynthetic pathway, and its rate-limiting enzyme, 3-hydroxy-3-methylglutarylcoenzyme A reductase (HMGCR), is the target of the statin class of cholesterol-lowering drugs. The mevalonate pathway is a highly conserved process whereby acetyl-CoA is converted to a series of progressively elongated isoprenoids that include the branch-point metabolite, farnesyl pyrophosphate (FPP; also known as farnesyl diphosphate). Although FPP is normally converted mainly to squalene by squalene synthase in the first committed step of sterol biosynthesis, it is also metabolized to several nonsterol isoprenoids, including ubiquinone (coenzyme Q), dolichol, dolichyl phosphate, heme O, heme A, and isopentenyl transfer RNA (Goldstein and Brown, 1990). In addition, FPP and its metabolite geranylgeranyl pyrophosphate (GGPP) are substrates for protein prenylation reactions, which are required for cellular signaling by small GTPases (Zhang and Casey, 1996). FPP and GGPP are also precursors to the isoprenoids, farnesol and geranylgeraniol (Edwards and Ericsson, 1999). Under normal physiologic conditions, little FPP is dephosphorylated to farnesol; however, when squalene synthase is inhibited, FPP accumulates, leading to massive production of farnesol and its progressively oxidized metabolites (collectively known as farnesoids), farnesal, farnesoic acid, and a series of dicarboxylic acids that are excreted in urine (Bostedor et al., 1997; Vaidya et al., 1998).

We previously reported that treatment of primary cultured rat hepatocytes with the squalene synthase inhibitor, squalestatin 1 (SQ1), increases CYP2B1 expression by causing accumulation of an endogenous isoprenoid(s) that activates the constitutive androstane receptor (CAR; NR1I3) (Kocarek and Mercer-Haines, 2002; Jackson and Kocarek, 2008). Additional evidence implied that this effect required the conversion of FPP to farnesol, since direct treatment with farnesol caused CYP2B2 induction in cultured rat hepatocytes (Kocarek and Mercer-Haines, 2002) and rat liver (Horn et al., 2005). However, this possibility was not specifically addressed since the enzyme(s) responsible for converting FPP to farnesol was not known. Later, phosphatidic acid phosphatase domain containing 2 (PPAPDC2) was identified as a lipid phosphate phosphohydrolase that could convert presqualene diphosphate to presqualene monophosphate (Fukunaga...
et al., 2006). PPAPDC2 was subsequently shown to hydrolyze FPP and GGPP preferentially over several other phospholipid substrates in vitro and to deplete FPP levels when overexpressed in cells (Miriyala et al., 2010). These data suggested that PPAPDC2 could be an important component of the pathway for synthesis of isoprenoids. Therefore, the objective of this investigation was to determine the effect of PPAPDC2 expression on SQ1-mediated activation of CAR in primary cultured rat hepatocytes.

Materials and Methods

Materials. SQ1 was a gift from Bristol-Myers Squibb Co. (Stamford, CT) and pravastatin was from GlaxoSmithKline (Research Triangle Park, NC). Phenobarbital (PB) was purchased from Sigma-Aldrich (St. Louis, MO). Matrigel was purchased from Corning Life Sciences (Tewksbury, MA), PureCol from Advanced BioMatrix (San Diego, CA), and recombinant human insulin (Novolin R) from Novo Nordisk Pharmaceuticals, Inc. (Princeton, NJ). Williams’ E medium and Lipofectamine 2000 reagent were purchased from Invitrogen (Life Technologies, Grand Island, NY). SYBR Green PCR Master Mix was purchased from Applied Biosystems (Foster City, CA). Additional materials were obtained from the sources indicated below.

Primary Culture of Rat Hepatocytes. Adult male Sprague-Dawley rats (200–250 g) were purchased from Harlan Sprague-Dawley (Indianapolis, IN) and maintained in an Association for Assessment and Accreditation of Laboratory Animal Care–approved animal facility with free access to food and water for 1 week. Hepatocytes were isolated by two-step collagenase perfusion as described previously (Kocarek and Reddy, 1996). Freshly isolated hepatocytes were plated onto PureCol-coated plates at a density of 1.6 million hepatocytes per well for six-well plates (for RNA isolation) or 0.6 million hepatocytes per well for 12-well plates (for transfection experiments) and maintained at 37°C under a humidified atmosphere of 95% air/5% CO2 in Williams’ E medium supplemented with 0.25 U/ml insulin, 0.1 µM triamcinolone acetonide, 100 U/ml penicillin, and 100 µg/ml streptomycin. Culture medium was renewed every 24 hours and drug treatments were begun 48 hours after plating as described in the individual figure legends. Drugs were prepared as 1000× stock solutions in sterile water.

Overexpression and Knockdown of PPAPDC2 by Transient Transfection in Primary Cultured Rat Hepatocytes. A CAR-responsive firefly luciferase reporter plasmid containing approximately 2.4 kb of the CYP2B1 promoter–flanking region was previously described (Kocarek et al., 1998). An expression plasmid containing the rat PPAPDC2 cDNA and a cloning plasmid containing the human PPAPDC2 cDNA were purchased from GE Healthcare (Pittsburgh, PA). Human PPAPDC2 cDNA from the latter was transferred into an expression vector as described in the (Supplemental Methods). Four plasmids expressing 29-mer short hairpin RNAs (shRNAs) targeting rat PPAPDC2 and a nontargeting shRNA plasmid (TR30012) were purchased from Origene Technologies (Rockville, MD). The validation vector reporter assay that was used to evaluate the shRNAs for knockdown efficiency is described in the (Supplemental Methods).

For overexpression of rat or human PPAPDC2, hepatocytes were transiently transfected with Williams’ E medium containing 0.2 ml OptiMEM (Life Technologies) and a premixed complex of Lipofectamine 2000 (4 µl), the CYP2B1 reporter plasmid (1.2 µg), rat or human PPAPDC2 expression plasmid (50 ng), pRL-CMV luciferase reporter plasmid (1 ng), and pBlueScript II KS+ (350 ng; Stratagene Cloning Systems; Agilent Technologies, Santa Clara, CA) to adjust total DNA content to 1.6 µg. For knockdown, hepatocytes were transfected as described above, except that the complex contained 400 ng of nontargeting control or PPAPDC2-targeting shRNA instead of PPAPDC2 expression plasmid and pBlueScript II KS+. Five hours after transfection, medium was replaced with Williams’ E medium containing 0.8 mg/ml Matrigel. Drug treatments were begun the following day and repeated once after 24 hours. Hepatocytes were harvested 48 hours after initial treatment of measurement of firefly and Renilla luciferase activities using the Dual Luciferase Reporter Assay System and a GloMax luminometer (Promega Corporation, Madison, WI). Each transfection experiment included three wells per treatment group, and luciferase data were calculated as mean firefly/Renilla ratios and presented as described in the individual figure legends. Experiments were repeated in four independent rat hepatocyte preparations for overexpression and three for knockdown.

Overexpression of PPAPDC2 by Adenoviral Transduction in Primary Cultured Rat Hepatocytes. The generation of a recombinant adenovirus for expression of rat PPAPDC2 (Ad5.CMV-PPAPDC2) is described in the (Supplemental Methods). Twenty-four hours after plating, primary cultured rat hepatocytes were transduced with Ad5.CMV-PPAPDC2 or control adenovirus without insert (Ad5/375; O.D. 260 Inc., Boise, ID) at a multiplicity of infection of 5. After 5 hours of infection, the medium was replaced with Williams’ E medium containing 0.8 mg/ml Matrigel. Drug treatments were begun 24 hours after infection (two wells per treatment group) and were repeated once after 24 hours. Forty-eight hours after initial treatment, hepatocytes were harvested for RNA or protein extraction.

Quantitative Reverse-Transcription Polymerase Chain Reaction Analysis. Total RNA was extracted using the PureLink RNA isolation kit (Ambion, Carlsbad, CA) and processed for cDNA synthesis and quantitative reverse-transcription polymerase chain reaction as described in the (Supplemental Methods). CYP2B1 mRNA levels were normalized to TATA box-binding protein (assay identifier Rn.PT.51.2418050; Integrated DNA Technologies, Coralville, IA). Relative changes in mRNA levels were calculated using the comparative threshold cycle method (user bulletin 2; Applied Biosystems). All assays were performed in duplicate and were repeated in four independent experiments.

Western Blot Analysis of PPAPDC2. PPAPDC2 protein levels in primary cultured rat hepatocytes were measured by Western blot analysis 48 hours after adenoviral transduction using a rabbit polyclonal antibody (Origene Technologies). Additional details are presented in the (Supplemental Methods).

Statistical Analyses. Statistical analyses were performed using GraphPad Prism (version 6.00 for Mac OS X; GraphPad Software Inc., San Diego, CA) to perform two-way analysis of variance with the Newman–Keuls correction for multiple comparisons. P < 0.05 was considered significantly different. All results are presented as means ± S.E.M.

Results and Discussion

Based on the reported ability of PPAPDC2 to dephosphorylate FPP to farnesol (Miriyala et al., 2010), we hypothesized that overexpression of PPAPDC2 would enhance SQ1-mediated CAR activation in primary cultured rat hepatocytes. Therefore, rat hepatocyte cultures were cotransfected with an expression plasmid for PPAPDC2 (rat or human) or empty vector and a reporter plasmid containing the CAR-responsive region of CYP2B1 (Kocarek et al., 1998) and then treated with 0.1 µM SQ1 or 100 µM PB, the prototype CAR activator. SQ1 and PB treatment significantly increased CYP2B1 reporter activity by 7.1- and 22-fold, respectively, compared with the untreated control in hepatocytes transfected with empty vector (Fig. 1A). The SQ1-mediated induction of CYP2B1 reporter activity was significantly enhanced by overexpression of either rat or human PPAPDC2 compared with the SQ1-treated, empty vector–transfected group (1.7-fold and 2.4-fold enhancement, respectively). Therefore, rat and human PPAPDC2, which share 89% amino acid sequence similarity, were functionally comparable in their abilities to enhance SQ1-inducible CAR activation. PPAPDC2 overexpression did not affect reporter activity in either untreated or PB-treated hepatocytes, indicating that the enhancing effect of PPAPDC2 on CAR activity only occurred when squalene synthase was inhibited. Cotreatment of rat hepatocytes with the HMGCR inhibitor pravastatin (30 µM), which blocks production of mevalonate (and therefore FPP), abolished the effect of SQ1, without or with PPAPDC2 overexpression, on CAR activity (Fig. 1B), demonstrating the dependence of the PPAPDC2-enhanced CAR activation on the ongoing production of mevalonate and its isoprenoid metabolites.

To measure the effect of PPAPDC2 overexpression on an endogenous marker of CAR activation, we used a recombinant adenovirus to achieve high-efficiency transfer of rat PPAPDC2 (Ad5.CMV-PPAPDC2)
into the primary cultured rat hepatocytes. Transduction with PPAPDC2 at a multiplicity of infection of 5 resulted in an approximately 2.2-fold increase in PPAPDC2 immunoreactive protein content relative to that measured in empty adenoviral control vector–transduced cells after 48 hours (Fig. 1C). SQ1 treatment increased CYP2B1 mRNA content by approximately 27-fold in hepatocytes transduced with control vector and approximately 38-fold in PPAPDC2-transduced cells (Fig. 1C), for a significant 1.4-fold enhancement of SQ1-mediated CYP2B1 mRNA induction.

Overall, we interpret the PPAPDC2 overexpression data as follows. Under untreated conditions, most of the FPP is converted to squalene and relatively little is converted to farnesol, likely reflecting the relative affinities of the pathways. In addition, the farnesol that is produced is readily metabolized, so farnesol does not accumulate sufficiently to cause CAR activation. Increasing PPAPDC2 expression in untreated hepatocytes probably does not cause much diversion of FPP away from the squalene synthase pathway; therefore, the increase in farnesol production that occurs is still inadequate to cause effective CAR activation.

Fig. 1. Effect of PPAPDC2 overexpression on SQ1-mediated CAR activation in primary cultured rat hepatocytes. (A) Twenty-four hour–old primary cultures of rat hepatocytes were transiently transfected with a CAR-responsive reporter plasmid (CYP2B1-Luc) and either an empty expression plasmid (Empty) or an expression plasmid for rat (rPPAPDC2) or human PPAPDC2 (hPPAPDC2). Then, 24 hours after transfection, cultures were incubated in medium alone (UT) or containing 0.1 μM SQ1 or 100 μM PB. Each bar represents the mean ± S.E.M. of the mean normalized (firefly/Renilla) luciferase measurements from four independent experiments (n = 4). All values are normalized to the Empty/UT group. *P < 0.05; **P < 0.01 (significantly different from Empty/SQ1 group). (B) Rat hepatocytes were cotransfected with CYP2B1-Luc and either empty vector (Empty) or rPPAPDC2 and treated with medium alone (UT), 0.1 μM SQ1, 0.1 μM SQ1 and 30 μM pravastatin (Prava), or 100 μM PB. Forty-eight hours after drug treatment, transduced cultures were harvested for measurement of luciferase activity. Each bar represents the mean ± S.E.M. of mean normalized (firefly/Renilla) luciferase measurements from three independent experiments (n = 3). All values are normalized to the Empty/UT group. *P < 0.05 (significantly different from the Empty/SQ1 group). (C) Rat hepatocytes were transduced with empty adenoviral vector (Ad-375) or adenoviral vector expressing rPPAPDC2 (Ad5.CMV-PPAPDC2) and harvested after 48 hours for measurement of either rat PPAPDC2 or β-actin protein. The bar graph shows band intensity quantification of PPAPDC2 levels normalized to β-actin protein. (D) Rat hepatocytes were transduced with empty adenoviral vector (Ad-375) or adenoviral vector expressing rPPAPDC2 (Ad5.CMV-PPAPDC2), treated for 48 hours with medium alone (UT) or 0.1 μM SQ1, and harvested for measurement of CYP2B1 mRNA levels. Each bar represents the mean CYP2B1 levels ± S.E.M from four independent experiments normalized to the Ad-375/UT group (n = 4). *P < 0.05 (significantly different from Ad-375/SQ1 group).

Fig. 2. Effect of PPAPDC2 knockdown on SQ1-mediated CAR activation in primary cultured rat hepatocytes. (A) Validation of rPPAPDC2 knockdown. Twenty-four hours after plating, primary cultured rat hepatocytes were transiently transfected with a validation reporter vector for rat PPAPDC2 (rPPAPDC2-Luc) and either a nontargeting control shRNA (shNT) or a shRNA targeting rat PPAPDC2 (shrPPAPDC2). After 48 hours, hepatocytes were harvested for measurement of luciferase activity. Each bar represents the mean ± S.E.M. of mean normalized (firefly/Renilla) luciferase measurements from three independent experiments (n = 3). (B) Rat hepatocytes were transiently transfected with the CYP2B1-Luc reporter and either the shNT or shrPPAPDC2 plasmid. Then, 24 hours after transfection, cultures were incubated for 48 hours in medium alone (UT) or containing 0.1 μM SQ1 and harvested for measurement of luciferase activity. Each bar represents the mean ± S.E.M. of mean normalized (firefly/Renilla) luciferase measurements from three independent experiments (n = 3). All values are normalized to the shNT/UT group. *P < 0.05 (significantly different than the shNT/SQ1 group).
activation. When squalene synthase is inhibited, FPP accumulates, resulting in marked dephosphorylation to farnesol and substantial CAR activation. Under these conditions, PPAPDC2 overexpression is able to enhance the process enough to cause a further increase in CAR activation.

The above data show that supplemental the rat hepatocyte’s capacity to convert FPP to farnesol increased the ability of a drug that causes FPP accumulation to activate CAR, suggesting that FPP dephosphorylation to farnesol is a necessary step in the mechanism by which SQ1 treatment activates CAR. However, these data do not address whether PPAPDC2 is a major enzyme that normally performs this function in hepatocytes. We therefore evaluated the effect of knocking down PPAPDC2 on SQ1-mediated CAR activation by performing cotransfections with shRNA expression plasmids (nontargeting and PPAPDC2-targeting) and the CYP2B1 reporter plasmid. Transfection with the shRNA that produced the greatest knockdown of PPAPDC2 in a validation vector reporter assay (approximately 62% reduction; Fig. 2A) significantly attenuated SQ1-inducible CYP2B1 reporter activation (approximately 80% reduction; Fig. 2B), indicating that PPAPDC2 plays an essential role in SQ1-mediated CAR activation in primary cultured rat hepatocytes.

Our data demonstrate that PPAPDC2 plays an essential role in the mechanism by which SQ1 treatment causes CAR activation in primary cultured rat hepatocytes, most likely by catalyzing the conversion of FPP to farnesol. Either farnesol itself or a metabolite is then an endogenous CAR activator. Miriyala et al. (2010) established that PPAPDC2 catalyzes FPP dephosphorylation and that its overexpression can modulate FPP-dependent cellular processes. Overexpression of PPAPDC2 in yeast or mammalian cells decreased FPP levels and caused cytotoxicity. In human embryonic kidney 293 cells, overexpression of PPAPDC2, but not a catalytically defective mutant, caused cytostasis and accumulation of multineucleate cells. In mouse aortic vascular smooth muscle cells, PPAPDC2 overexpression affected processes associated with Rho family GTPase function, which is dependent on protein prenylation. Our data are the first to link PPAPDC2 to a signaling mechanism in normal hepatocytes.

Nonsterol isoprenoids play important roles in a variety of biologic processes, and the control of their cellular levels is therefore an important therapeutic approach for several pathologies (Buaesauc and Izzedine, 2007; Oldfield, 2010; Li et al., 2012). For example, the nitrogen-containing bisphosphonate class of drugs (e.g., alendronate, ibandronate) are first-line treatments for osteoporosis that produce their therapeutic effects by inhibiting FPP synthase and thus production of FPP and its metabolites, including GPP. Therefore, the bisphosphonates reduce geranylgeranylation of small GTPase proteins in the osteoclast, which leads to osteoclast apoptosis (Drake et al., 2015). The isoprenoids farnesol and geranylgeraniol themselves have been implicated in several biologic processes; for example, farnesol is a nonsterol regulator of HMGCR (Correll et al., 1994; Meigs and Simoni, 1997), and these alcohols can be converted back to FPP and GGPP through sequential monophosphorylation reactions (Thai et al., 1999). Therefore, the mechanisms controlling the interconversion of FPP and farnesol are likely important determinants of isoprenoid-regulated cellular processes. Our findings provide further insight into the role of PPAPDC2 as a modulator of hepatic physiology. Specifically, PPAPDC2 provides a metabolic link between the cholesterol biosynthetic pathway and the xenobiotic sensor CAR. Therefore, dietary, physiologic, pathophysiological, or pharmacological conditions that cause enhanced PPAPDC2-mediated conversion of FPP to farnesol could alter CAR-regulated processes, such as xenobiotic metabolism and transport.

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References


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Pant A and Kocarek TA. Role of phosphatidic acid phosphatase domain containing 2 (PPAPDC2) in squalestatin 1-mediated activation of the constitutive androstane receptor in primary cultured rat hepatocytes. Drug Metabolism andDisposition

Supplemental Methods

PPAPDC2 overexpression by transient transfection in primary cultured rat hepatocytes. A CAR-responsive firefly luciferase reporter plasmid containing ~2.4 Kb of the CYP2B1 5’-flanking region has been previously described (Kocarek et al., 1998). An expression plasmid (pExpress-1) containing the rat PPAPDC2 cDNA sequence (Mammalian Gene Collection Clone ID 7125531) and a cloning plasmid (pBluescript) containing the human PPAPDC2 cDNA (Mammalian Gene Collection Clone ID 5268486) were purchased from GE Healthcare (Pittsburgh, PA). An expression plasmid for human PPAPDC2 was prepared as follows. PCR was performed using the plasmid containing human PPAPDC2 cDNA as template, Pfu polymerase (Stratagene Cloning Systems; La Jolla, CA), and the following primer pairs: forward 5’-GCG AGCGGCCGCGCCACCATGCCAAGTCCCCGGAGGA-3’ and reverse 5’-GCGAGGATCCTCATCGTTGACTCCACAGT-3’ to amplify the coding region of human PPAPDC2 (nt 83 to 970 of NCBI Reference Sequence NM_203453.3). The PCR product was digested with NotI and BamHI and ligated into the corresponding sites of the pE1.1 vector (O.D. 260 Inc.; Boise, ID), into which the cytomegalovirus promoter and bovine growth hormone polyadenylation sequences of pcDNA3.1 had been pre-ligated at the BglII and Acc65I/KpnI sites, respectively. The sequence of this clone (and others
described below that were derived by PCR) was verified by the Applied Genomics Technology Center at Wayne State University.

Twenty-four hr after plating, cell culture medium was replaced and hepatocytes were transiently transfected with Williams’ Medium E containing 0.2ml of OptiMEM (Life Technologies; Grand Island, NY) and a premixed complex of Lipofectamine 2000 (4 μL), the CYP2B1 reporter plasmid (1.2 μg), rat or human PPAPDC2 expression plasmid (50 ng), pRL-CMV Renilla luciferase reporter plasmid (1 ng), and pBlueScript II KS+ (350 ng) (Agilent Technologies; Santa Clara, CA) to adjust total DNA content to 1.6 μg. Five hr following transfection, medium was replaced with Williams' Medium E containing 0.8 mg/ml Matrigel. Drug treatments were begun the following day and repeated once after 24 hr. Hepatocytes were harvested 48 hr after initial treatment for measurement of luciferase activities using the Dual Luciferase Reporter Assay System and a GloMax luminometer (Promega Corporation; Madison, WI) according to the manufacturer’s instructions. Luciferase data were calculated as firefly/Renilla ratios and presented as described in the figure legends. Experiments were repeated in four independent rat hepatocyte preparations.

**PPAPDC2 knockdown by transient transfection in primary cultured rat hepatocytes.** Four plasmids expressing 29-mer shRNAs targeting rat PPAPDC2 and a non-targeting shRNA plasmid (TR30012) were purchased from Origene Technologies (Rockville, MD). A validation vector (rPPAPDC2-Luc) was additionally prepared to evaluate knockdown of rat PPAPDC2. To prepare this vector, the rat PPAPDC
expression plasmid described above was used as a template to amplify the rat PPAPDC2 cDNA using the forward primer 5'-GCGAGCGGCCGCGCCACC CGGAGGACTATCGAGGGAC-3' and reverse primer 5'-GCGATAGCGGCCGCTGGATGGCTCTGGCTTAGG-3' and Pfu polymerase to amplify the coding region of rat PPAPDC2 (nt 64 to 1014 of NCBI Reference Sequence NM_001034854.1). The PCR product was digested with NotI and ligated into the corresponding site of the pCMV-LUC validation vector (TR30004; Origene Technologies), which is located downstream of the firefly luciferase coding region.

To evaluate the abilities of the different shRNA constructs to knock down PPAPDC2, primary cultured rat hepatocytes were incubated in Williams’ Medium E containing 0.2 ml of OptiMEM containing a premixed complex of 4 μl Lipofectamine 2000 with 50 ng PPAPDC2 validation vector, 200 ng of a PPAPDC2-targeting or non-targeting shRNA expression plasmid, 1 ng of pRL-CMV Renilla luciferase reporter plasmid, and 1350 ng of pBlueScript II KS+. Five hr following transfection, culture medium was replaced with Williams' Medium E containing 0.8 mg/ml Matrigel. The cells were then harvested 48 hr after transfection, and luciferase activities were measured as described above. The shRNA construct that produced the largest reduction in reporter activity compared to non-targeting control was TI713339, and this construct was used for further experiments.

To evaluate the effect of PPAPDC2 knockdown on SQ1-mediated CAR activation, primary cultured rat hepatocytes were transfected as described above, but using 1.2 μg of CYP2B1 reporter plasmid, 200 ng of shRNA construct (PPAPDC2-targeting or non-
targeting), and 200 ng pBluescript II KS⁺. After transfection and overnight incubation with Williams' Medium E containing Matrigel, drug treatments were performed as described in the individual figure legends (three wells per treatment group), repeated once after 24 hr. Forty-eight hr following initial treatment, cells were harvested for measurement of luciferase activities.

**PPAPDC2 overexpression by adenoviral transduction in primary cultured rat hepatocytes.** The generation of a recombinant adenovirus for expression of rat PPAPDC2 (Ad5.CMV-PPAPDC2) was performed as described below. The expression plasmid for rat PPAPDC2 described above was used as template to amplify rat PPAPDC2 cDNA using the following primers: forward primer 5’-GCGAGCGGCCGCGAAGCCTGTCTCCGGTCTG-3’ and reverse primer 5’-GCGGGATCCGGATGGCTCTGGCTTAGGT-3’ and *Pfu* polymerase to amplify the coding region of rat PPAPDC2 (nt 2 to 1012 of NCBI Reference Sequence NM_001034854.1). The PCR product was ligated into the *Not*I and *Bam*HI sites of the pE1.1 shuttle vector, containing the cytomegalovirus promoter and bovine growth hormone polyadenylation sequences, as described above. This shuttle plasmid was then provided to O.D. 260 Inc., who prepared the recombinant adenovirus expressing rat PPAPDC2 (Ad5.CMV-PPAPDC2). The titer of the recombinant adenovirus as well as of the control adenovirus (Ad-375; O.D. 260 Inc.) was determined using the QuickTiter Adenovirus Titer ELISA Kit (Cell Biolabs, Inc.; San Diego, CA). Twenty-four hr following plating, primary cultured rat hepatocytes were transduced with Ad5.CMV-PPAPDC2 or control adenovirus without insert (Ad-375) at a multiplicity of infection of
5. After 5 hr of infection, medium was replaced with Williams' Medium E containing 0.8 mg/ml Matrigel. Drug treatments were begun 24 hr after infection (two wells per treatment group), and treatments were repeated once after 24 hr. Forty-eight hr following the initial treatments, cells were harvested for RNA or protein extraction as described below.

**Quantitative reverse-transcription polymerase chain reaction analysis.** Primary cultured rat hepatocytes were harvested and total RNA was extracted and column purified using the Purelink RNA isolation kit (Ambion; Carlsbad, CA). cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Life Technologies), following the manufacturer’s instructions. Primers used to detect CYP2B1 (Forward – 5’-CAACCCTTGATGACCGCAGTA-3’ and Reverse – 5’-TTCAGTGTCTTGGAGAAGCAG -3’) and primers for the TATA box-binding protein (TBP; Assay ID Rn.PT.51.24118050) were purchased from Integrated DNA Technologies (IDT; Coralville, IA). CYP2B1 mRNA levels were measured using Power SYBR Green Master Mix in the StepOne Plus Real Time PCR System (Applied Biosystems; Foster City, CA). The concentration of each primer was 150 nM, and the real-time cycling conditions were: Initial activation step at 95 °C (15 min) and 40 cycles of melting (95 °C, 15 s) and annealing/extension (60 °C, 1 min). Relative changes in mRNA levels were quantified using the comparative CT (ΔΔCT) method (User bulletin no.2, Applied Biosystems). All assays were performed in duplicate in four independent experiments.
Western blot analysis. After 48 hr of treatment, cultured hepatocytes were incubated with gentle agitation on ice for 60 min with ice-cold phosphate-buffered saline (PBS) containing 5 mM EDTA to dissolve the Matrigel. The cells were then scraped into tubes and centrifuged briefly at 1000 rpm for 5 min at 4°C. Supernatants were aspirated and cells washed twice with ice-cold PBS. Following the final wash, cells were lysed in ice-cold RIPA buffer [50 mM Tris, 150 mM NaCl, 0.2% sodium dodecyl sulfate (SDS), 0.25% sodium deoxycholate, 1% Triton X-100, 1 mM EDTA] containing Halt Protease Inhibitor Cocktail (ThermoFisher Scientific; Rockford, IL) by passing the suspension through a 24 gauge needle several times. After extensive vortexing, the lysates were centrifuged at 14,000 x g for 15 min at 4 °C and the protein concentrations of the supernatants were determined using the bicinchoninic acid assay (Sigma-Aldrich; St. Louis, MO) with bovine serum albumin as the standard. Lysate samples (20 µg protein) were diluted in Laemmli Sample Buffer, denatured at 100°C for 5 min, resolved on SDS-polyacrylamide gels (10% acrylamide), and electrophoretically transferred onto polyvinylidene difluoride membranes (BioRad; Hercules, CA). Following transfer, membranes were incubated in blocking buffer [5% nonfat dry milk diluted in Tris-buffered saline (10 mM Tris, 150 mM NaCl, pH 7.4) containing 0.05% Tween-20 (TBST)] for 1 hr at room temperature and then incubated overnight at 4°C with gentle rocking in blocking buffer containing rabbit polyclonal PPAPDC2 antibody (TA306886; Origene Technologies) diluted 1:15,000. The following day, blots were washed 3 times with TBST and then incubated 1 hr at room temperature in blocking buffer containing horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (Santa Cruz Biotechnology; Dallas, TX) diluted 1:20,000. Following washing, immunoreactive bands
were visualized using enhanced chemiluminescence according to the manufacturer’s instructions (GE Healthcare) and a FluorChem E detection system (ProteinSimple, San Jose, CA). Blots were subsequently incubated in stripping buffer (0.1 mM glycine, 1% SDS, 0.05% Tween-20, pH 2.2), blocked, and re-developed with a mouse monoclonal β-actin antibody (Sigma-Aldrich) diluted 1:250 in blocking buffer followed by anti-mouse IgG secondary antibody diluted 1:20,000 (Santa Cruz Biotechnology). Band densities were quantified using ImageJ software (Rasband, 2012).

Reference