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Diacylglycerol kinase θ couples farnesoid X receptor-dependent bile acid signalling to Akt activation and glucose homoeostasis in hepatocytes

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Abstract

DGKs (diacylglycerol kinases) catalyse the conversion of diacylglycerol into PA (phosphatidic acid), a positive modulator of mTOR (mammalian target of rapamycin). We have found that chenodeoxycholic acid and the synthetic FXR (farnesoid X receptor) ligand GW4064 induce the mRNA and protein expression of DGKθ in the HepG2 cell line and in primary human hepatocytes. Reporter gene studies using 1.5 kB of the DGKθ promoter fused to the luciferase gene revealed that bile acids increase DGKθ transcriptional activity. Mutation of putative FXR-binding sites attenuated the ability of GW4046 to increase DGKθ luciferase activity. Consistent with this finding, ChIP (chromatin immunoprecipitation) assays demonstrated that bile acid signalling increased the recruitment of FXR to the DGKθ promoter. Furthermore, GW4064 evoked a time-dependent increase in the cellular concentration of PA. We also found that GW4064 and PA promote the phosphorylation of mTOR, Akt and FoxO1 (forkhead box O1), and that silencing DGKθ expression significantly abrogated the ability of GW4046 to promote the phosphorylation of these PA-regulated targets. DGKθ was also required for bile-acid-dependent decreased glucose production. Taken together, our results establish DGKθ as a key mediator of bile-acid-stimulated modulation of mTORC2 (mTOR complex 2), the Akt pathway and glucose homoeostasis.

Keywords

bile acid; diacylglycerol kinase θ (DGKθ); farnesoid X receptor (FXR); hepatocyte; phosphatidic acid

INTRODUCTION

Bile acids are amphipathic molecules that regulate the elimination of cholesterol. In humans there are two major bile acid biosynthetic pathways, neutral and acidic, that are initiated by cholesterol 7α-hydroxylase (CYP7A1) and mitochondrial sterol 27-hydroxylase (CYP27A1) respectively. The neutral pathway produces primary bile acids [CA (cholic acid) and CDCA (chenodeoxycholic acid)] and secondary bile acids {DCA (deoxycholic acid) and lithocholic acid [1]}, whereas CDCA is the only product in the acidic pathway. Under normal physiological conditions, the neutral pathway is the primary bile acid biosynthetic pathway [2]. Besides their well-established roles in cholesterol metabolism, bile acids are emerging

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AUTHOR CONTRIBUTION

Kai Cai and Marion Sewer participated in research design. Kai Cai conducted the experiments. Kai Cai and Marion Sewer performed data analysis and wrote the paper.

as key signalling mediators that evoke changes in various physiological processes by serving as ligands for TGR5, a G-protein-coupled receptor [3] and the nuclear receptor FXR (farnesoid X receptor; NR1H4) [4]. As the endogenous ligand for FXR, CDCA is a more potent ligand for FXR than DCA and LCA (lithocholic acid) [5]. However, FXR is also activated by synthetic agonists, such as GW4064 [6] and 6α-ECDCA (6α-ethyl-chenodeoxycholic acid) [7].

FXR is expressed in liver, intestine, pancreas, kidney and adrenal glands [6,8,9] and regulates the expression of target genes by binding to their promoters in a ligand-dependent manner. A pivotal function of FXR is to induce the expression of SHP (small heterodimer partner), a nuclear receptor that suppresses CYP7A1 [10]. FXR also regulates cholesterol production and lipid metabolism [11–14], a function that was demonstrated in FXR-null mice, where hepatic cholesterol, triacylglycerol and high-density lipoprotein expression are increased [14,15]. Furthermore, FXR plays an important role in the regulation of carbohydrate metabolism [17]. FXR suppresses PEPCK (phosphoenolpyruvate carboxykinase) and G6Pase (glucose 6-phosphatase), thereby regulating gluconeogenesis and glucose homoeostasis [18].

DGKs (diacylglycerol kinases) phosphorylate DAG (diacylglycerol) to produce PA (phosphatidic acid) [19–21]. There are ten mammalian isoforms of DGK, which are organized into five categories on the basis of the presence of specific domains identified in their primary sequence. DGKθ is the sole member of group V and harbours three cysteine-rich domains [22,23], compared with two for other DGK isoforms. DGKθ has been implicated in various physiological processes [24–28]. Translocation of DGKθ from the cytosol to the plasma membrane facilitates interaction with the EGFR (epidermal growth factor receptor) and inhibits the ability of protein kinase C to terminate EGFR signalling [29]. The synergistic interaction of DGKθ with acidic phospholipids and polybasic proteins such as Tau and histone H1 promote phospholipid-dependent activation of the enzyme [30]. Finally, adenosine A2a receptor-initiated repression of DGKθ by the small GTPase RhoA confers tolerance to ischaemia/reperfusion injury in hepatocytes [31].

The functional importance of PA as a second messenger that mediates various intracellular signalling pathway by regulation of protein kinases and phosphatases is well established [32]. PA has been shown to positively [33,34] and negatively [35] regulate mTOR (mammalian target of rapamycin) signalling. Moreover, a role for DGK activity in the regulation of mTOR-dependent pathways has been reported [36,37]; however, the precise mechanism or specific DGK isoform that regulates PA-dependent mTOR signalling was unclear. In the present study, we show that DGKθ gene expression (encoded by *DGKQ*) and activity are in response to bile acid stimulation. The DGKθ-produced PA increases the phosphorylation of mTOR, Akt and FoxO1 (forkhead box O1), thus suppressing gluconeogenic gene expression. In summary, we show that DGKθ plays an important role in hepatic bile acid-regulated glucose production.

EXPERIMENTAL

Materials

GW4064 and CDCA were obtained from Sigma–Aldrich), and PA (egg PA; catalogue number 840101) was from Avanti Polar Lipids.

Cell culture

HepG2 cells were obtained from the A.T.C.C. (Manassas, VA, U.S.A.) and cultured in Eagle's minimum essential medium (Mediatech) supplemented with 10 % FBS (Mediatech) and antibiotics (1× penicillin-streptomycin/amphotericin B; Lonza). DGKθ-knockdown cells

were generated as described previously [38]. The following lots of primary human hepatocyte cultures were purchased from XenoTech: lot 1147 (35-year-old male), lot 1152 (69-year-old female), lot 1155 (43-year-old female) and lot 1157 (59-year-old female). Primary human hepatocytes were plated in William's E medium (Invitrogen) containing FBS, ITS (insulin-transfemin-selenium) (Mediatech), dexamethasone (0.1 μM) and antibiotics (1× penicillin-streptomycin/amphotericin B; Lonza).

Transient transfection and reporter gene analysis

HepG2 cells were subcultured on to 24-well plates and transfected with 20 ng of pGL3-DGKθ [38], 0.5 ng of pRL-CMV (Promega) and 25 ng of pcDNA3.1-FXR (provided by Dr David D. Moore, Baylor College of Medicine, Houston, TX, U.S.A.) using Genejuice® (Novagen). At 24 h after transfection, the cells were treated with 1 nM GW4064 for 24 h and the transcriptional activity of the DGKθ reporter gene was measured using a dual luciferase assay kit (Promega). Firefly (pGL3-DGKθ) luciferase activity were normalized to *Renilla* luciferase activity (pRL-CMV) and expressed as fold change over the mean of the untreated control group.

RNA isolation and real-time RT-PCR

Cells were subcultured on to 12-well plates and 24 h later were treated with 100 μM CDCA, 1 nM GW4064 or 10 μM PA for 24 h. Total RNA was extracted using Iso-RNA Lysis Regent (5 Prime) and amplified using a One-Step SYBR Green RT-PCR Kit (Thermo Fisher Scientific) and the following primers: DGKθ forward, 5′-CGTTCTCCGTACTGCTGTC-3′ and reverse, 5′-G-TCTGCCGTGTCGTTCTC-3′; DGKα forward, 5′-GCGAGGA-GGCTGGTGAGTC-3′ and reverse, 5′-TGGAAGATGGGAGG-CAGGATG-3′; DGKδ forward, 5′-GCACAGAACCTACAGA-ACC-3′ and reverse, 5′-GCGACCACCTCCAGAATC-3′, DGKζ forward, 5′-AGCAAGAAGAAGAAGAGG-3′ and reverse, 5′-GGATTGAGATACCAGAGG-3′; and β-actin forward, 5′-ACG-GCTCCGGCATGTGCAAG-3′ and reverse, 5′-TGACGATGCC-GTGCTGCATG-3′. DGKθ expression was normalized to β-actin content and calculated using the cycle threshold ($\Delta\Delta C_T$) method.

Western blotting

Cells were subcultured on to six-well plates and treated with 100 μM CDCA or 1 nM GW4064 for 48 h and harvested into RIPA buffer [50 mM Tris/HCl, pH 7.4, 1 % Nonidet P40, 0.25 % sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 150 nM aprotinin, 1 mM leupeptin, 1 mM E-64 and 500 mM 4-(2-aminoethyl)benzenesulfonylfluoride]. Cells were then lysed by sonication (one 5 s burst) followed by incubation on ice for 30 min. Lysates were centrifuged at 13 800 ***g*** for 10 min at 4 °C and the supernatant was collected for analysis by SDS/PAGE. Protein concentrations were determined using the BCA Protein Assay (Pierce). Aliquots of each sample (25 μg of protein) were run on SDS/PAGE (8 % gels) and transferred on to PVDF membranes (Millipore). Blots were probed with primary antibodies overnight. Anti-DGKθ antibody (1:1000 dilution, HPA026797) was obtained from Sigma–Aldrich. Anti-Akt antibody (1:1000 dilution, sc-8312), anti-phospho-Akt (1:1000 dilution, sc-7985) and anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (1:5000 dilution, sc-25778) antibodies were obtained from Santa Cruz Biotechnology. Anti-(total mTOR) (1:1500 dilution, 2983P), anti-phospho-mTOR(Ser^{2448}) (1:1000 dilution, 2971S), anti-(total FoxO1) (1:1500 dilution, 2880S) and anti-phospho-FoxO1(Thr^{24}) (1:1000 dilution, 9464P) antibodies were obtained from Cell Signaling Technology. Anti-rabbit secondary antibody was obtained from GE Healthcare (ECF Western Blotting Reagent). Blots were scanned on a VersaDoc 4000 (Bio-Rad Laboratories) and densitometric analysis was carried out using Quantity One software (Bio-Rad Laboratories).

ChIP (chromatin immunoprecipitation)

ChIP assays were performed as described previously [39]. Briefly, HepG2 cells were subcultured on to 15-cm-diameter dishes and treated with 1 nM GW4064 for 60 min. Cells were treated with 1 % formaldehyde for 10 min at room temperature and then incubated with 0.125 M glycine for 5 min. After washing twice with PBS, cells were harvested into RIPA buffer. The purified chromatin solutions were immunoprecipitated using 5 μg of anti-(acetyl-histone H3) (17-615, Millipore), anti-FXR (Santa Cruz Biotechnology) or anti-IgG (Millipore) antibodies and Protein A/G PLUS (Santa Cruz Biotechnology). Real-time PCR was carried out to amplify region − 1000 to − 700 bp of the DGKθ promoter [38] using the following primer sets: forward 5′-CAGAGTCCACAGCCCCCAGCCCCTTTCAGG-3′ and reverse 5′-CTGCCTCGTGCGCGCCACGGGTCTTGTTCA-3′. Output (immunoprecipitated promoter region) was normalized to input DNA. PCR products were run on 2 % agarose gels.

PA assay

HepG2 cells were grown on six-well plates and then treated with 1 nM GW4064 for 24 h to 72 h and total or nuclear (Nuclei Pure Kit; Sigma) lipids were harvested. PA content was quantified using a Total PA kit (Cayman Chemical) and a SpectraMax M5 MultiMode Microplate Reader (Molecular Devices) at an excitation wavelength of 530–540 nM and an emission wavelength of 585–595 nm. Data were quantified using SoftMax Pro Software (Molecular Devices).

DAG assay

The DAG assay was performed as described previously [38]. HepG2 cells or human hepatocytes were cultured on to six-well plates and treated with 1 nM GW4064 for 72 h. The amount of DAG in each sample was determined using a Human DAG ELISA kit (MyBioSource).

Glucose measurement assay

Wild-type and DGKθ knockdown HepG2 cells were seeded on to a 96-well plate with the normal growth medium at 37 °C in 5 % CO_2. The next day, the medium was removed and changed to 100 μl of glucose-free medium and treated with GW4064 or PA for 48 h. A volume of 50 μl of medium was collected from each well and glucose output in HepG2 cells was determined by using the Amplex Red glucose kit (Invitrogen) [40]. A standard calibration curve of glucose (0.9–18 ng/ml) was prepared using the stock solution of glucose (72 μg/ml) in a volume of 50 μl. Standards and samples were both incubated with Amplex Red working solution in the dark for 30 min. The fluorescence was measured at a SpectraMax M5 Multi-Mode Microplate Reader (Molecular Devices).

Statistical analysis

One-way ANOVA and Tukey-Kramer multiple comparisons were performed using GraphPad Prism version 5.00 (GraphPad Software). The significant difference value was set as $P < 0.05$.

RESULTS

Bile acids induce DGKθ gene expression

As discussed above, bile acid signalling has been linked to PA metabolism [41]. Therefore we treated HepG2 cells with 100 μM CDCA or 1 nM GW4064 and assessed the effect on DGKθ expression. As shown in Figure 1(A), CDCA and GW4064 increased the mRNA expression of DGKθ 2.5- and 4-fold respectively. Consistent with an induction in the

expression of mRNA, the protein levels of DGKθ were also increased in response to bile acid signalling, with GW4064 resulting in a 1.7-fold increase in the protein expression of DGKθ (Figure 1B). Significantly, the stimulatory effect of bile acids was also observed in primary human hepatocytes, where GW4064 evoked a 3.9-and 2.1-fold increase in DGKθ mRNA (Figure 1C) and protein expression (Figure 1D) respectively. In contrast, other DGK isoforms that have been shown to be expressed in hepatocytes [31] were not induced by GW4064 treatment.

FXR stimulates DGKθ expression

To examine further the mechanism by which bile acids induce DGKθ expression, HepG2 cells were transfected with a reporter construct that contained 1.5 kB of the DGKθ promoter fused to the luciferase gene [38]. As shown in Figure 2(A), GW4064 increased the transcriptional activity of the reporter gene by 3.9-fold. Overexpression of FXR increased luciferase activity by 3.7-fold, and GW4064 further increased FXR-dependent reporter gene activity by 9.2-fold (Figure 2A). *In silico* analysis of the promoter revealed the presence of two putative FXR response elements at − 910/− 904 (M1) and − 817/− 809 (M2) upstream of the translation start site. Although mutation of the M1 site had no significant effect on the ability of GW4064 or FXR to increase DGKθ reporter gene activity, mutation of M2 abrogated GW4064-or FXR-dependent luciferase activity (Figure 2B). However, mutation of the M1 and M2 sites decreased the ability of GW4064 to activate FXR-dependent reporter gene activity by 42 % and 60 % respectively. Reporter gene studies were consistent with ChIP assays where we found that GW4064 increased acetylation of histone H3 at a region of the DGKθ promoter that encompasses the FXR-binding site, which was concomitant with a 5-fold increase in the recruitment of FXR (Figure 3).

Bile acids induce PA concentration in HepG2 cells

Next we assessed the effect of FXR activation on the cellular concentration of PA and found that stimulation of HepG2 cells with 1 nM GW4064 increased the production of PA in a time-dependent manner, with a 3.7-fold increase observed after 72 h of stimulation (Figure 4A). The treatment with the DGK inhibitor R59949 reduced basal PA production and attenuated GW4064-stimulated PA biosynthesis, suggesting a prominent role for DGK activity in conferring increased PA production in response to bile acid synthesis. Since R59949 inhibits all DGK isoforms, we generated a cell line where the expression of DGKθ was stably silenced using shRNAs targeted against the lipid kinase (Figure 4B) and determined the relative contribution of DGKθ to GW4064-stimulated PA production. As shown in Figure 4(C), suppressing DGKθ expression resulted in a 55 % reduction in PA production, suggesting that DGKθ plays a predominant role in the response to GW4064 and that other isoforms contribute to the response. However, analysis of the mRNA expression of all other DGKθ isoforms in expressed in HepG2 cells and in human primary hepatocytes revealed that DGKθ was the sole isoform that exhibited an induction in mRNA expression in response to GW4064 (Figure 1C). Thus it is possible that bile acid signalling may regulate other DGK isoforms via non-transcriptional mechanisms. Concomitant with an increase in cellular PA, DAG content was reduced by 40 %with the GW4064 treatment, and the decrease was less in DGKθ-knockdown cells (Figure 4D). Consistent with the effect of GW4064 in HepG2 cells, the FXR agonist increased PA levels by 2.1-fold (Figure 4E) while decreasing DAG levels by 34 % (Figure 4F) in human hepatocytes.

Effect of silencing of DGKθ on GW4064-stimulated target gene activity and expression

PA is a well-established second messenger that regulates multiple proteins, including phosphatidylinositol 4-phosphate 5-kinase [42], Raf kinase [43], PKCε (protein kinase Cε) and PKCζ [44,45], sphingosine kinase [46] and the tyrosine phosphatase SHP-1 [47]. In

addition, PA activates mTOR-dependent downstream signalling [33] and DGKζ-produced PA mediates mTOR signalling in HEK (human embryonic kidney)-293 cells [36]. On the basis of these previously published data and our findings from the present study demonstrating that bile acids induce DGKθ expression, we sought to investigate the functional significance of DGKθ-produced PA as it relates to mTOR signalling. As shown in Figure 5(A), both GW4064 and PA increase the cellular content of Ser^{2448}-phosphorylated mTOR [49] in wild-type cells; however, GW4064 is unable to stimulate mTOR phosphorylation in DGKθ-knockdown cells. Consistent with these data, GW4064 activates phosphorylation of the mTOR target Akt at Thr^{473} [50,51], which plays a central role in inhibition of FoxO1 and leads to constitutive suppression of glucose production [52] in wild-type HepG2 cells, but not in cells where DGKθ expression is silenced. In contrast, we were unable to detect an increase in the phosphorylation of the mTORC1 (mTOR complex 1) downstream target p70 S6 kinase in HepG2 cells or primary human hepatocytes (results not shown), suggesting selective activation of the mTORC2 signalling pathway.

Bile acids have been reported to down-regulate gluconeogenic genes, including PEPCK and G6Pase [53]. Thus we assessed the role of DGKθ-catalysed PA production in regulating gluconeogenic gene expression. As shown in Figure 5(B), both GW4064 and PA potently suppressed the mRNA expression of PEPCK and G6Pase in wild-type HepG2 cells. GW4064 suppressed G6Pase expression by 62 % in wild-type cells and by 29 % in DGKθ-knockdown cells. Unexpectedly, silencing DGKθ reduced the mRNA expression of G6Pase by 46 %. Although GW4064 reduced PEPCK mRNA expression by 68 % in wild-type HepG2 cells, silencing DGKθ resulted in only a 43 % decrease in PEPCK mRNA expression, suggesting a role for DGKθ-dependent PA production in regulating hepatic gluconeogenic gene expression. These expression data were supported by glucose measurements, which indicated that GW4064 and PA suppressed glucose production in wild-type cells by 35 % and 65 % respectively (Figure 5C). In contrast, GW406 had no significant effect on glucose concentrations in the DGKθ-knockdown cell line, whereas PA reduced glucose production by 47 %. Importantly, GW4064 and PA increased the phosphorylation of mTOR, Akt and FoxO1 (Figure 5D) and suppressed the mRNA expression of G6Pase and PEPCK (Figure 5E) in primary human hepatocytes.

DISCUSSION

Bile acids are important signalling molecules that regulate hepatic cholesterol, triacylglycerol and glucose homoeostasis [2,14,54]. Previous studies have demonstrated that bile acids and insulin act co-operatively to regulate glucose storage in primary rodent hepatocytes [54,55]. In particular, a clinical study has shown that the activation of FXR is beneficial for the treatment of hypertriacylglycerolaemia [54]. DGKs play a pivotal role in balancing the cellular concentrations of two lipid second messengers, PA and DAG [21,33,56–58]. Since the first DGK was discovered [59], ten DGKs isoforms have been identified. Aberrant DGK function has been associated with several pathophysiological states, an expected consequence of the regulation of these enzymes in multiple tissues. Hepatic DAG accumulation can activate the predominant PKC isoform (PKCε) in liver, which has been strongly associated with hepatic insulin resistance [60,61], a major risk factor of Type 2 diabetes [62]. Mounting evidence indicates that bile acids regulate glucose homoeostasis. Recent research has demonstrated PEPCK and G6Pase are repressed by taurocholate in primary rat hepatocytes [53]. Moreover, the Akt pathway has been shown to have a central role in bile-acid-regulated glucose production [53,55].

DGKθ is modulated by PKCε [64], protein–protein interaction with RhoA [65] and phospholipid activation [66]. Moreover, recent studies have shown that the interaction of DGKθ with polybasic proteins such as histone H1 and Tau positively regulate the enzyme

[30]. Our previous studies established a role for DGKθ in adrenocortical glucocorticoid production, where the kinase binds to the nuclear receptor SF-1 (steroidogenic factor-1) [67]. We have also recently shown that the expression of DGKθ in human adrenocortical cells is transcriptionally regulated by the cAMP signalling pathway, which induces the expression of DGKθ via a mechanism that requires SREBP1 (sterol-regulatory-element-binding protein 1) and SF-1 [38]. However, less is known about the factors that regulate the expression of DGKθ in liver or about the functional significance of the lipid kinase in hepatic cellular processes. Work by Baldanzi et al. [31] showed that DAG accumulation during adenosine-dependent hepatocyte preconditioning is regulated by DGKθ. In the present study we show that CDCA and GW4064 positively regulate the mRNA and protein expression of DGKθ (Figure 1) and identify a novel regulatory pathway whereby the lipid kinase contributes to the regulation of glucose homoeostasis (Figure 6). Significantly, we did not observe a similar effect on other DGK isoforms in primary human hepatocytes or HepG2 cells. It is worth noting that published studies have demonstrated that DGKθ is most abundant DGK isoform expressed in human hepatocytes and was identified as the major isoform that mediates the response to signalling pathways that result in DAG accumulation [31].

Our luciferase reporter assays revealed that FXR positively regulated the transcriptional activity of the DGKθ reporter gene (Figure 2), a finding that was supported by ChIP studies, which found that GW4064 stimulated the recruitment of FXR to the endogenous DGKθ promoter (Figure 3). FXR regulates multiple genes involved in bile acid metabolism, including SHP [53]. Moreover, the links between bile acid signalling and lipid metabolism are well established [11,70,71], and bile acid receptors are emerging as attractive targets for the treatment of varied pathophysiological disease states, including cardiovascular disease and dyslipidaemia [72,73].

We found that the cellular PA concentration increases in response to GW4064 treatment and that DGKθ plays a predominant role in bile acid-stimulated PA production in HepG2 cells (Figure 4). As discussed earlier, PA increases the phosphorylation of Akt at Ser^{473} [34]. PLD1 (phospholipase D1) and PLD2 have been shown to be required for PA-dependent mTOR activation in skeletal muscle [75]. Moreover, the increase in PLD activity induced by amino acids and glucose confers elevated mTOR activation in multiple cancer cell lines, including MDA-MB-231 breast cancer cells and T24 bladder cancer cells [76]. The present study identifies that DGKθ is another source for PA production and mTORC2 activation in hepatocytes. A role for DGK activity in mTOR activation has been previously demonstrated in HEK-293 cells where overexpression of DGKθ identified a role for the isoform in mTOR signalling [36]. As described above, hepatic mTOR plays a critical role in regulation of the Akt–FoxO1 axis [77,78]. The physiological function of hepatic Akt is essential for maintaining glucose homoeostasis through increasing the phosphorylation of FoxO1, which abolished the transcriptional regulation effect of FoxO1 on gluconeogenesis genes [52]. The effect of silencing DGKθ on mTORC2 signalling and gluconeogenic gene expression (Figure 5) provides support for the role that this lipid kinase plays in regulating hepatic glucose homoeostasis. However, the inability of suppressing DGKθ to completely abrogate the repressive effects of GW4064 on gluconeogenic gene expression suggest that other DGK isoforms contribute to the PA-dependent hepatic glucose metabolism. Additionally, the effect of silencing DGKθ on the basal expression of G6Pase (Figure 5B) suggests that there are multiple mechanisms by which DGKθ may regulate glucose metabolism. In conclusion, we identify a novel role for DGKθ in hepatic bile acid signalling and glucose homoeostasis.

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Abbreviations used

CDCA	chenodeoxycholic acid
ChIP	chromatin immunoprecipitation
DAG	diacylglycerol
DCA	deoxycholic acid
DGK	diacylglycerol kinase
EGFR	epidermal growth factor receptor
FoxO1	forkhead box O1
FXR	farnesoid X receptor
G6Pase	glucose 6-phosphatase
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HEK	human embryonic kidney
mTOR	mammalian target of rapamycin
mTORC	mTOR complex
PA	phosphatidic acid
PEPCK	phosphoenolpyruvate carboxykinase
PKC	protein kinase C
PLD	phospholipase D
SHP	small heterodimer partner
SF-1	steroidogenic factor-1

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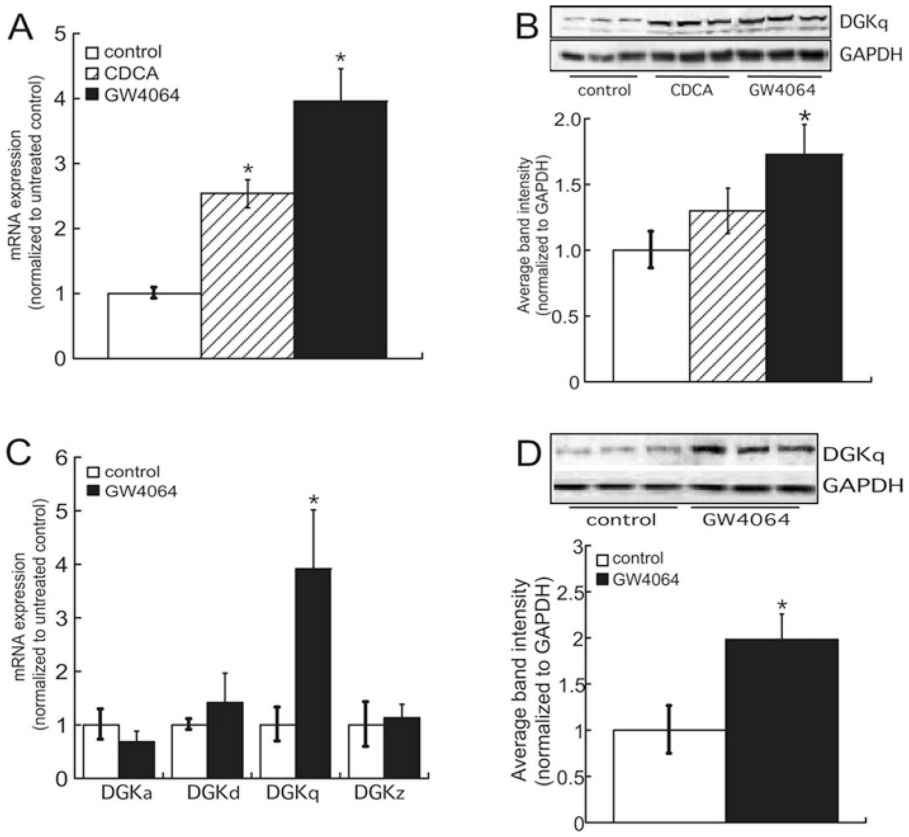

Figure 1. Bile acids increase DGKθ mRNA and protein expression

(**A**) HepG2 cells were cultured on to 12-well plates and treated for 24 h with 100 μM CDCA or 1 nM GW4064. Total RNA was isolated for analysis of DGKθ and β-actin mRNA expression by qRT-PCR (quantitative reverse transcription–PCR). Data are shown as fold change in DGKθ mRNA expression and normalized to the mRNA expression of β-actin. The data are the means ± S.E.M. for three separate experiments, each performed in triplicate. *P < 0.05, statistically different from untreated control group. (**B**) Representative blots from analysis of lysates isolated from HepG2 cells that were treated for 48 h with 100 μM CDCA or 1 nM GW4064. The graph depicts densitometric analysis of DGKθ (DGKq) protein expression normalized to GAPDH. The data are the means ± S.E.M. for three separate experiments, each carried out in triplicate. *P < 0.05, a statistically significant difference compared with untreated controls. (**C**) RNA was isolated from human hepatocytes that were treated for 24 h with 1 nM GW4064 and subjected to qRT-PCR. Data are shown as fold change in DGKα (DGKa), DGKσ (DGKd), DGKζ (DGKz) and DGKθ mRNA expression, normalized to the mRNA expression of β-actin, and represent the means ± S.E.M. of four donors with cells plated in triplicate. (**D**) Representative immunoblot of DGKθ protein expression in human hepatocytes that were treated for 72 h with 1 nM GW4064. The data are the means ± S.E.M. of four donors, with each experiment carried out in triplicate. *P < 0.05, a statistically significant difference compared with untreated controls.

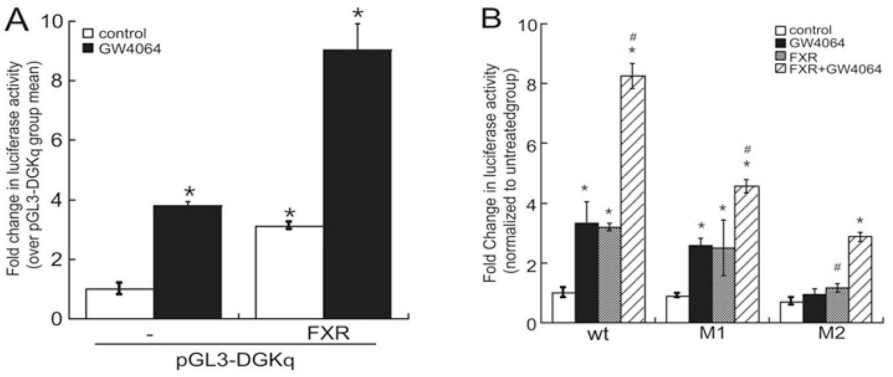

Figure 2. FXR increases DGKθ reporter gene activity

(**A**) HepG2 cells were transiently transfected with pGL3-DGKθ (pGL3-DGKq), pRL-CMV and pCMX-hFXR, and then treated with 1 nM GW4064 for 16 h. Luciferase activity in lysates isolated from control and GW4064-treated cells was measured by luminometry. The results were normalized to the luciferase activity of *Renilla* gene and expressed as the fold change in pGL3-DGKθ reporter gene activity over the untreated control group mean and represent the means ± S.E.M. for three separate experiments, each performed in triplicate. *$P < 0.05$, a statistically significant difference from the untreated control group. (**B**) HepG2 cells were transiently transfected with wild-type (wt) or mutant (M1 or M2) pGL3-DGKθ, pRL-CMV and pCMX-hFXR expression plasmids. * and # indicate a statistically significant difference ($P < 0.05$) from untreated control group and untreated FXR group respectively.

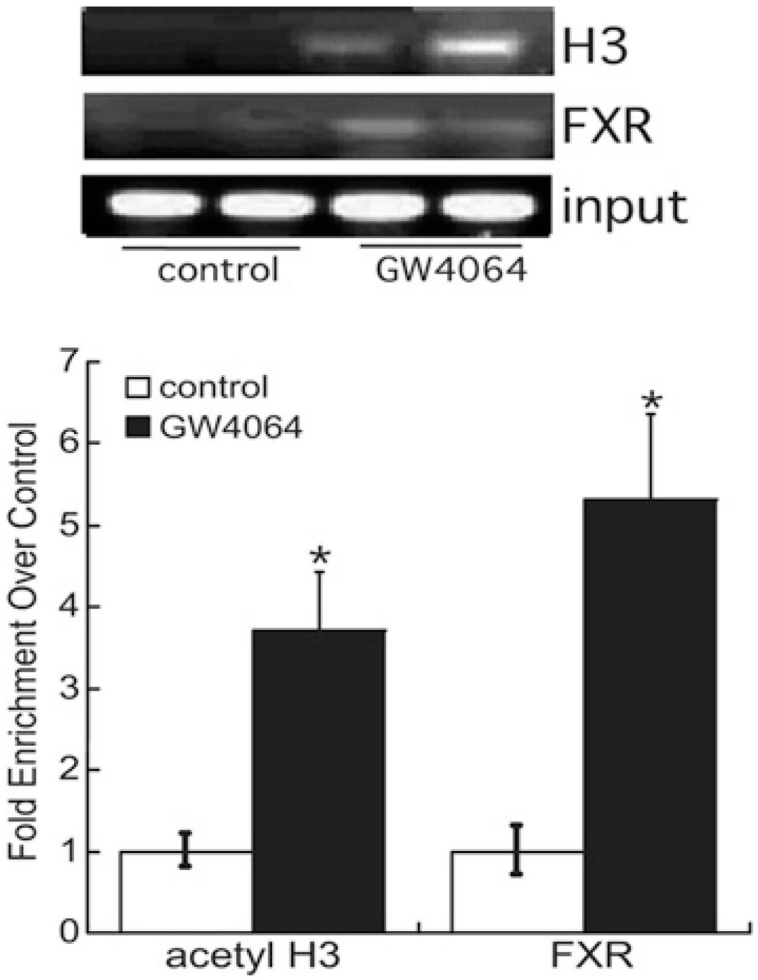

Figure 3. GW4064 stimulates FXR recruitment to the DGKθ promoter

HepG2 cells were incubated with 1 nM GW4064 for 60 min, and then cross-linked with 1 % formaldehyde. The sheared chromatin was harvested and immunoprecipitated with antibodies against FXR or acetyl-histone H3 and recruitment to the DGKθ promoter (−1000/− 700) assessed by qPCR. DNA purified was quantified by real-time PCR and normalized to the $\Delta\Delta C_T$ values of input DNA. The results are expressed as fold change over untreated control. *$P < 0.05$, a statistically significant difference from untreated control group.

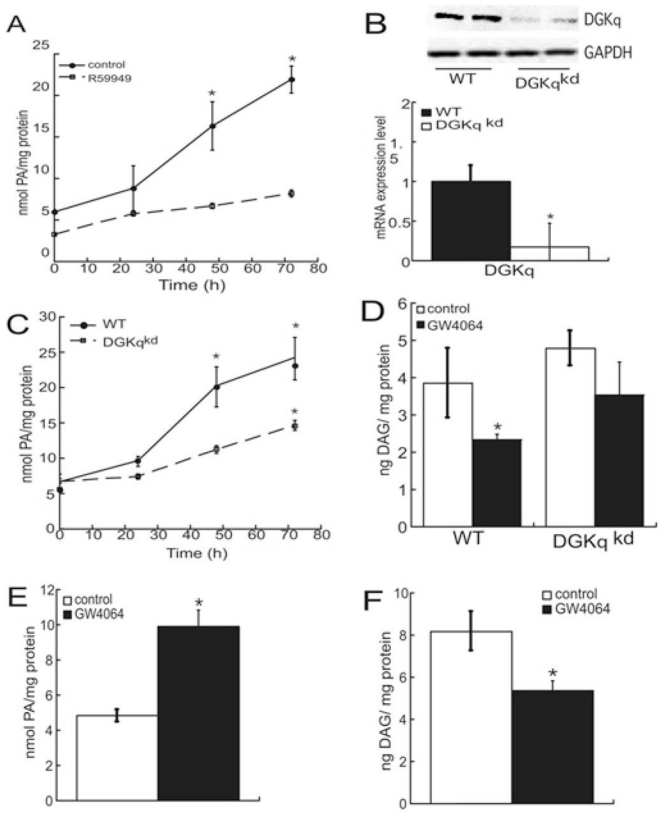

Figure 4. Bile acids induce PA production

(**A**) HepG2 cells were treated with 1 nM GW4064 and R59949 from 24 h to 72 h and PA levels were quantified by ELISA. (**B**) Lysates from wild-type (wt) and DGKθ-knockdown (DGKq^{kd}) cells were harvested and analysed using SDS/PAGE and Western blotting using anti-DGKθ (top) and anti-GAPDH (bottom) antibodies. DGKθ mRNA expression was quantified in wild-type and DGKθ-knockdown HepG2 cells by qRT-PCR (quantitative reverse transcription–PCR). Data are shown as fold change in DGKθ mRNA expression and normalized to β-actin mRNA expression. (**C**) Wild-type and DGKθ-knockdown cells were treated with 1 nM GW4064 and the cellular amount of PA was measured and normalized to the protein concentration. (**D**) HepG2 cells and DGKθ-knockdown HepG2 cells were treated with 1 nM GW4064 for 72 h. Cellular DAG levels were measured by ELISA kit. The graph shows the means ± S.E.M. for three separate experiments, each performed in triplicate. *P < 0.05 indicate a statistically significant difference from the untreated wild-type control group. Human hepatocytes from donor 4 were cultured on to six-well plates and incubated with 1 nM GW4064 for 72 h. Cellular PA (**E**) and DAG (**F**) concentration were quantified by PA assay and DAG assay respectively. *P < 0.05, a statistically significant difference from the untreated control group.

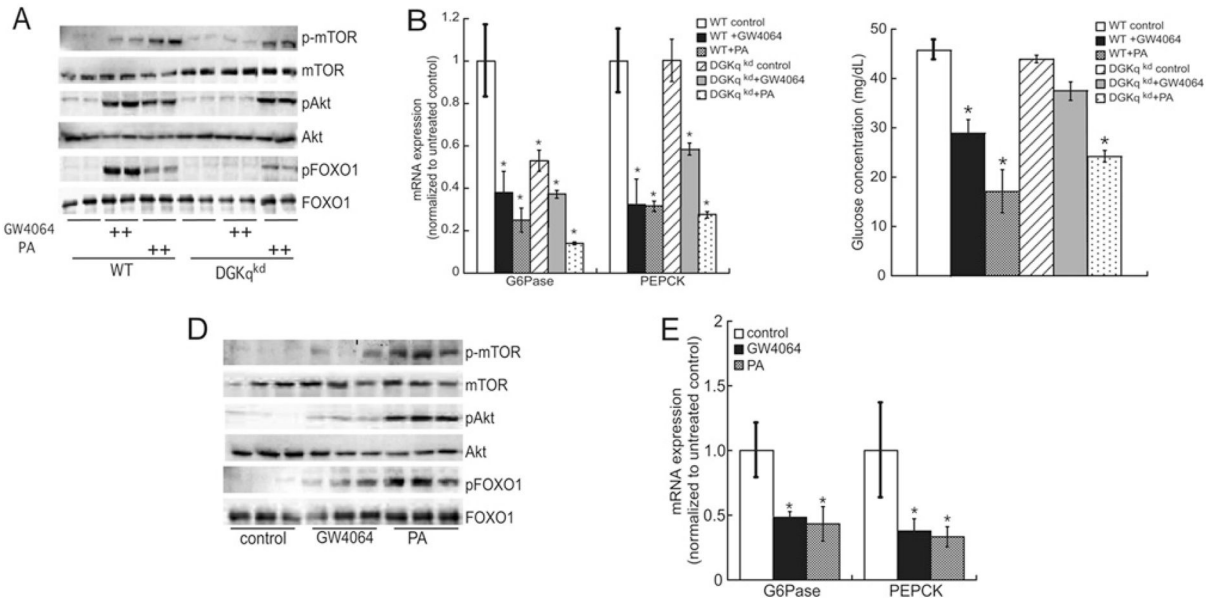

Figure 5. DGKθ is required for bile-acid-stimulated regulation of the mTOR pathway

(**A**) Wild-type (WT) and DGKθ-knockdown (DGKq^{kd}) HepG2 cells were treated with 1 nM GW4064 or 10 μM DLPA for 48 h respectively. The levels of phosphorylated (p) and total mTOR, Akt and FoxO1 were evaluated by Western blotting using the antibodies described in the Experimental section. (**B**) RNA was isolated from wild-type and DGKθ-knockdown HepG2 cells that were treated with 1 nM GW4064 or 10 μM DLPA for 24 h. The mRNA expression of PEPCK and G6Pase was normalized to β-actin. Data are graphed as fold change over wild-type control and represent the means ± S.E.M. for three independent experiments, each performed in triplicate. *$P < 0.05$, a statistically significant difference from untreated group. (**C**) HepG2 cells (wild-type and DGKθ-knockdown) were cultured in glucose-free medium and endogenous glucose production was measured after treatment with 1 nM GW4064 or 10 μM PA for 48 h. Data are graphed as the mean of duplicate measurements and represent three independent experiments, each performed in at least triplicate. *$P < 0.05$, a statistically significant difference from the wild-type untreated control group. (**D**) Primary human hepatocytes were incubated with 1 nM GW4064 or 10 μM PA for 48 h and proteins were isolated using SDS/PAGE and Western blot analysis. Blots were incubated with antibodies against phosphorylated (p) and total mTOR, Akt and FoxO1. (**E**) Primary human hepatocytes were treated with 1 nM GW4064 or 10 μM PA for 24 h and RNA was isolated for real-time RT (reverse transcription)–PCR. G6Pase and PEPCK mRNA expression was normalized to β-actin and data are shown as means ± S.E.M. for four donors, with each independent experiment carried out in triplicate. *$P < 0.05$, a statistically significant difference compared with untreated controls.

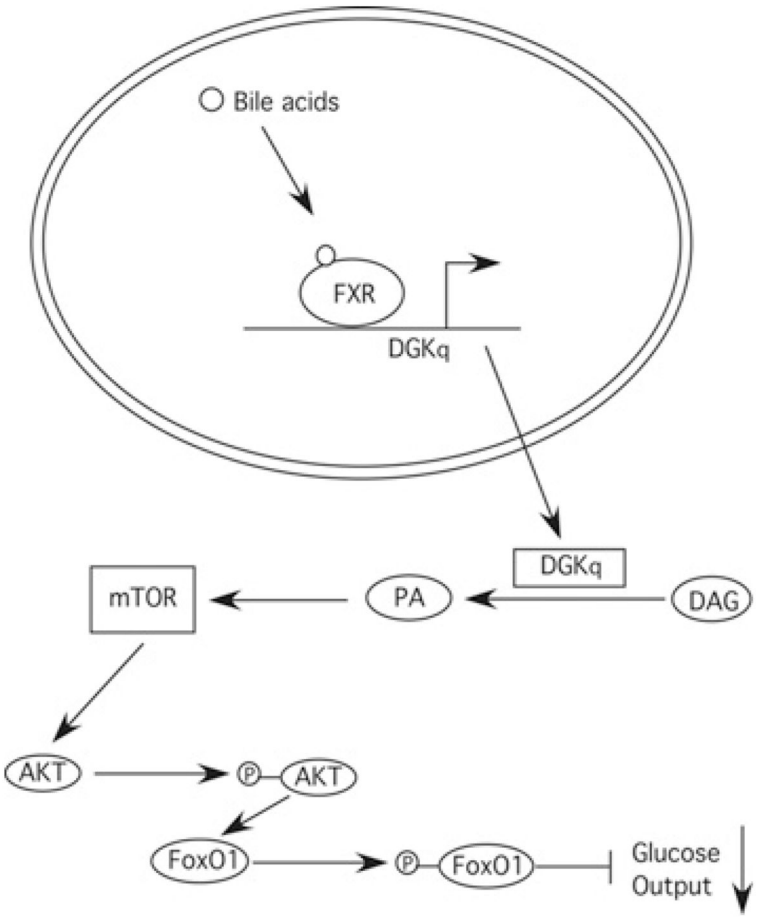

Figure 6. Model for the role of DGKθ in bile-acid-mediated regulation of glucose production

Bile acids induce DGKθ (DGKq) gene expression resulting in increased cellular PA. PA activates mTOR signalling leading to increased phosphorylation of Akt and FoxO1, and ultimately decreased glucose production owing to a suppression of gluconeogenic gene expression.