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

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**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 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) [4].

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 homeostasis [18].

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 transcripational 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 homeostasis.

Key words: bile acid, diacylglycerol kinase θ (DGKθ), farnesoid X receptor (FXR), hepatocyte, phosphatidic acid.
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/amphoterocin 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 was 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′-CGTTCTCCGACTGCTGTC-3′ and reverse, 5′-GTCTGGCTGTGCTTCTC-3′; DGKα forward, 5′-GGGAGGGAGGACTGATGC-3′; DGKζ forward, 5′-GGAGTGGATACCGAGG-3′ and reverse, 5′-AGATGGTTGAG-3′; and DGKβ forward, 5′-ACGGCAGTCGCCAGCC-3′ and reverse, 5′-TGACAGTGGTGCTGCTGATG-3′. DGKθ expression was normalized to β-actin content and calculated using the cycle threshold (ΔΔCt) 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/Cl, pH 7.4, 1% Nonidet P40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 150 mM 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(Ser404) (1:1000 dilution, 2971S), anti−total FoxO1 (1:1500 dilution, 28805) and anti-phospho-FoxO1(Thr38) (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′-CAGAGTCCACAGCCCCAGCCCCCTTTTCAGG-3′ and reverse 5′-CTGCTCTGTTGCGGGCACCAGGGTTCTGTGTC-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 Multi-Mode 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

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Bile acids induce DGKθ mRNA and protein expression

(A) HepG2 cells were cultured on 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), DGKd (DGKd), DGKc (DGKc), 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.

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 activity.
Figure 2 FXR increases DGKθ reporter gene activity

(A) HepG2 cells were transiently transfected with pGL3-DGKθ (pGL3-DGKp), 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.

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 DGK 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 Ce)
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 homeostasis [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 (PKCζ) 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 homeostasis. 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 Ser473 [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 DGKα 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 homeostasis 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 gluconegenic gene expression (Figure 5) provides support for the role that this lipid kinase plays in regulating hepatic glucose homeostasis. However, the inability of suppressing DGKθ to completely abrogate the repressive effects of GW4064 on gluconegenic 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 homeostasis.

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.

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