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Knockdown of Diacylglycerol Kinase Delta Inhibits Adipocyte Differentiation and Alters Lipid Synthesis

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Abstract

Objective—Decreased expression of diacylglycerol kinase delta (DGK δ) has been linked to insulin resistance in humans and mice and is abundantly expressed in adipose tissue. We therefore examined its role in adipogenesis.

Design and Methods—We generated 3T3-L1 preadipocytes in which DGK δ expression had been knocked down and determined the effect of this on adipogenesis. We also performed lipidomic analysis to determine levels of the DGK δ product phosphatidic acid (PA), its substrate diacylglycerol (DAG) and triglyceride (TG).

Results—Inhibiting DGK δ expression prevents adipogenesis. DGK δ knockdown in differentiating adipocytes blunted the increase in total levels of PA and DAG but did not affect the early rise in TG levels. DAG or PA species acting as TG precursors were only modestly reduced by DGK δ knockdown which significantly impaired the accumulation of DAG or PA species implicated in intracellular signaling. We also observed stimulation of the DAG activated kinase PKC δ in DGK δ knockdown cells, despite no increase in detectable species of DAG.

Conclusions—DGK δ is a novel regulator of adipogenesis and phosphorylates a quantitatively small pool of signaling DAG important for differentiation and indirectly affects overall levels of signaling DAG and PA species distinct from those acting as precursors for TG synthesis.

Keywords

Diacylglycerol; adipogenesis; lipids; phosphatidic acid; DGK

INTRODUCTION

The appropriately regulated development of metabolically active adipocytes is essential for human health. This is illustrated by the occurrence of metabolic disease with pathogenically decreased or increased adiposity in lipodystrophy or obesity respectively (1). Hence a better understanding of processes regulating adipocyte development and function may provide novel targets for improving metabolic disease.

Adipogenesis requires the induction of a complex and highly regulated program of gene expression which is co-ordinated with significant alterations in intracellular lipids (1, 2).

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These include signaling lipids as well as triglyceride (TG), the major component of the lipid droplet which ultimately defines the mature adipocyte and occupies the majority of the cell. The cross-talk between the lipid biosynthetic pathways and the transcriptional regulation of adipogenesis is poorly understood. However, this communication is clearly critical, as illustrated by the inhibition of adipogenic gene expression in cells lacking the key lipogenic enzyme 1-acylglycerol-3-phosphate O-acyltransferase 2 (AGPAT2) and severe lipodystrophy in individuals in which the *AGPAT2* gene is disrupted (3).

The diacylglycerol kinase (DGK) family of enzymes phosphorylates diacylglycerol (DAG) to generate phosphatidic acid (PA) and in mammals comprises of 10 members with diverse signaling and physiological roles (4, 5). DGKs may regulate intracellular signaling both by decreasing levels of DAG or increasing levels of PA as both lipids have been shown to have key signaling functions. DGK has been implicated in insulin resistance in humans and whilst homozygous loss of DGK in mice causes perinatal lethality, heterozygous disruption also leads to insulin resistance (6, 7). This has been shown to involve reduced insulin sensitivity in the skeletal muscle of DGK heterozygous mice. However, we observed that DGK is also clearly detectable in adipose tissue, another key metabolic tissue. We therefore sought to determine whether DGK loss might specifically affect the development or function of adipocytes.

METHODS AND PROCEDURES

Cell Culture

3T3-L1 preadipocytes were maintained and differentiated as described in (8). Populations of 3T3-L1 preadipocytes stably expressing shRNA sequences targeting DGK (DGK shA: 5'-AAGACTTGTGGCAGCGTGTTA -3' DGK shB: 5'-AACTTCGGACCAGCATTCTGT-3') or a control shRNA targeting luciferase were generated using the pSiren retroQ kit (BD Biosciences) according to the manufacturer's instructions. Retrovirus production, 3T3-L1 infection and selection were essentially as described in (8). Oil red O staining of lipids was performed as previously described (9).

RNA Isolation, cDNA Synthesis and Real Time PCR

Total RNA was extracted from mouse tissue as in (9) or from cell cultures using an RNeasy kit (Qiagen). Primer Express, version 1.0 software (Perkin Elmer Applied Biosystems) was used to design the probes and primers for real time quantitative PCR to determine total DGK, DGK 1, DGK 2, SREBP1c, DGAT2, PPAR 2, aP2, lipin1, adiponectin, adipisin, C/EBP and C/EBP mRNA expression as in (8). Primer/probe mix to assay C/EBP was obtained from Applied Biosystems. RNA was reverse transcribed using Moloney murine leukemia virus-reverse transcriptase and random hexamer primers (Promega). The resulting cDNA was used in real time-PCR assays with ABI Taqman or Sybr green master mix (Applied Biosystems) according to the manufacturer's instructions. mRNA expression was normalised to 18S rRNA (tissue samples) or cyclophilin A mRNA (cell culture samples).

Lipidomic Studies

Cells were grown to 2 days post-confluence and then collected in PBS at day 0 or day 2 of differentiation. Cell pellets were spiked with internal standards and extracted with modified Folch method using two rounds of chloroform:methanol:NaCl extraction. Lower phases were dried and resuspended in 50µl of Chloroform/Methanol (1:1). 10µl of each sample was separated on normal phase silica gel column and subjected to mass spectrometry (MS) analysis using a Shimadzu IT-TOF LC/MS/MS system hyphenated with a five-channel online degasser, four-pump, column oven and autosampler with cooler Prominence HPLC.

Accurate mass (5-10ppm accuracy) and tandem MS were used to identify and quantify molecular species, confirmed by reference to appropriate lipid standard.

Western Blotting

Protein samples were extracted by scraping in lysis buffer containing 1% NP40, followed by sonication as described previously (10). After centrifugation for 10 min at $13,000 \times g$, samples of supernatant containing 30 μg of protein were denatured and analysed by western blotting. Antibodies to Calnexin, PKC and phosphoT505-PKC were from Abcam, total and phosphor-specific antibodies to AKT and p70S6 kinase were from Cell Signalling. Quantitative analysis of western blots was performed using Image J.

Statistics

Statistical analyses were performed using t-tests or ANOVA for multiple comparisons.

RESULTS

Two isoforms of DGK have been reported with differing N termini (11). Using isoform specific primers we determined the expression of DGK 1 (Fig. 1A) and DGK 2 (Fig. 1B) in various mouse tissues. This revealed that both isoforms were widely expressed with significant expression in various depots of white adipose tissue, at least as great as that in skeletal muscle where DGK has been reported to affect insulin sensitivity and metabolism (6). To determine whether altered DGK expression might be altered in obesity we also assayed DGK expression in subcutaneous adipose tissue samples from leptin deficient ob/ob murine model of hyperphagia. This revealed increased DGK 1 mRNA expression in adipose tissue from ob/ob mice compared to their lean heterozygous littermates (Fig 1C). Consistent with the high expression levels of DGK in adipose tissue, we found that DGK expression was dramatically upregulated during adipogenesis in 3T3-L1 cells (Fig 1D). Together this suggested that DGK could have a role in the development or function of adipocytes. To examine this we generated 3T3-L1 preadipocytes in which DGK had been knocked down by stable expression of shRNAs targeting DGK. One of these, DGK-shA, gave a modest knockdown of DGK, whilst the second, DGK-shB, gave a more robust knockdown (Fig 2A). When these cells were induced to differentiate for 8 days DGK-shB expressing cells were unable to form adipocytes as determined by oil-red O staining whilst DGK-shA expressing cells showed an intermediate phenotype (Fig 2B). This was confirmed by quantitative assay of TG in control and DGK-shB expressing cells (Fig 2C).

Specific analysis of adipogenic gene expression revealed that DGK knockdown did not significantly affect the induction of the early transcriptional regulators of adipogenesis, C/EBP (Fig. 3A), or C/EBP (Fig. 3B), nor the early induction of the critical adipogenic transcription factor PPAR up to day 3 of differentiation (Fig. 3C). However, the subsequent expression of PPAR was significantly impaired in DGK knockdown cells, as was the induction of other adipocyte markers including the transcriptional factors C/EBP (Fig. 3D), SREBP1c (Fig. 3E), the lipogenic enzymes DGAT2 (Fig. 3F) and Lipin1 (Fig. 3G), the fatty acid binding protein aP2 (Fig. 3H) the adipokine adiponectin (Fig. 3I) and adipsin (Fig. 3J). Together these data demonstrate that the induction of DGK expression plays an essential role in adipocyte differentiation.

The differentiation of adipocytes involves the co-ordinated induction of a complex program of gene expression and the accumulation of lipid droplets comprised mainly of TG. TG synthesis involves the stepwise addition of acyl groups to glycerol-3-phosphate to produce monoacylglycerol-3-phosphate (MG) then PA which is dephosphorylated to produce DAG by lipin enzymes before the addition of a final acyl chain to generate TG. As DGK

generates PA from DAG, opposing the actions of lipin enzymes, its requirement for adipogenesis appears paradoxical when viewed in the context of TG synthesis. We therefore determined the effect of DGK knockdown on the levels of PA, DAG and TG species in these cells to gain further insight. Our gene expression analyses revealed that the induction of the critical adipogenic transcriptional regulator C/EBP β was significantly reduced as early as at day 2 of adipogenesis (Fig. 3D). Hence we performed our lipid analyses in cells immediately prior to and 2 days following the induction of differentiation so that alterations observed were more likely to be causative, rather than the downstream consequences, of altered adipogenesis.

Analysis of total cellular levels of PA revealed that the increase observed in control cells during the first two days of differentiation was severely blunted in DGK knockdown cells (Fig. 4A). Whilst this might appear consistent with the loss of diacylglycerol kinase activity we also observed similar failure to increase DAG levels during adipogenesis in DGK knockdown cells (Fig. 4B). Although both PA and DAG are proposed to act as precursors of TG synthesis, the modest but significant increase in total TG levels observed in control cells during the first 2 days of differentiation was entirely preserved in DGK knockdown cells (Fig. 4C).

To further probe the changes in these lipid species in DGK knockdown cells we more specifically examined the profile of different lipid species. This revealed significant heterogeneity in both the direction and extent of changes in individual species between time points in control cells and between control and DGK knockdown cells during differentiation. During differentiation of control cells some species of PA such as 32:0 PA were significantly increased whilst others such as 38:3 PA were decreased (Fig. 4D). Differentiation increased the levels of a greater number of DAG species (Fig. 4E). There were remarkably few differences in the levels of any species tested between undifferentiated control and DGK knockdown cells, suggesting few inherent differences in the synthesis of abundant lipid species between these cell lines (Fig. 4D& E). However, it is notable that PA species (34:1, 36:1, 36:2) and DAG species (38:4, 38:3) that have been reported to have signaling functions are elevated during differentiation (Fig. 4D&E) (12-14). These differentiation-associated increases were reduced or ablated in the DGK knockdown cells pointing either to the signals being important for differentiation or that differentiation increases the generation of these lipid signals. In contrast, other DAG and PA species particularly those containing shorter or medium chain fatty acids were less abundant and any increases during adipogenesis appeared only modestly affected by inhibition of DGK expression. The differentiation-associated increase in the polyunsaturated DAG species (38:4, 38:3) suggests an associated elevation in phospholipase C activity since these DAG species are generally generated through PtdIns(4,5)P $_2$ hydrolysis, although whether this is itself regulated by DGK is unclear (12, 13). A similar analysis of TG species in these cells showed that most species that were increased during early adipogenesis contained predominantly shorter medium chain fatty acids and were unaffected by DGK knockdown (Fig. 4F). Indeed TG species containing longer medium chain fatty acids appeared to be decreased during adipogenesis.

A previous study implicating decreased DGK expression in the development of insulin resistance in skeletal muscle suggested that reduced DGK increased cellular DAG and led to the activation of PKC δ . We therefore assessed whether PKC δ activity could be altered in our DGK knockdown cells. This revealed that knockdown of DGK increased PKC δ activation (as assessed by Thr505 phosphorylation) in preadipocytes both immediately prior to differentiation and during the first 24 hours of adipogenesis (Fig. 5A). Quantification of post-confluent preadipocytes exhibited more than 2-fold increased levels of PKC δ phosphorylation (Fig 5B). The increase in PKC δ activation due to reduced DGK in skeletal

muscle is reported to lead to impaired insulin signaling (6). As insulin is a key component of the adipogenic media used here we investigated whether the failure of adipogenesis in DGK knockdown cells could result from impaired insulin action. However, we observed similarly robust activation of the key insulin sensitive signaling kinases AKT and p70S6 kinase in both control and DGK knockdown cells during the first 24 hours of adipogenesis (Fig. 5C). In addition we observed no differences in the stimulation of these enzymes in post-confluent control or DGK knockdown preadipocytes following acute insulin treatment (not shown). We cannot rule out subtle alterations in the kinetics or quality of insulin signaling due to DGK knockdown. However, our data do not suggest that preadipocytes lacking DGK exhibit the insulin resistance observed in DGK deficient skeletal muscle cells making it very unlikely that this underlies the lack of adipogenesis mechanistically. These data also suggest both DGK inhibition and PKC activation may exert cell-type specific effects on insulin signaling.

DISCUSSION

Our study demonstrates that DGK plays an important role in adipocyte differentiation and that its loss prevents the appropriate induction of both the full transcriptional program regulating this process and co-incident changes in lipid species. Given the role of DGK in generating PA from DAG the clear implication is that the loss of DGK either prevents the removal of DAG species that inhibit adipogenesis or the production of PA species that, directly or indirectly, promote adipocyte differentiation. In an effort to identify these lipids we performed comprehensive lipidomic analysis both immediately prior to adipogenic induction and at a key early stage of this process, when our gene expression data suggests that a defect in this process first becomes apparent. However, we were unable to identify any individual species of PA which was decreased whose corresponding DAG precursor was increased. Rather the alterations observed in all lipid species measured are consistent with the overall failure of adipogenesis. Hence, whilst we believe these data provide a valuable catalogue of the changes in specific PA, DAG and TG species during early adipogenesis they did not highlight a specific pool directly affected by DGK activity.

Interestingly, the early induction of PPAR expression was preserved in DGK knockdown cells, whilst its subsequent expression, and that of several PPAR target genes, was inhibited. This would be consistent with a failure to appropriately activate PPAR once expressed. Several studies have proposed the existence of a lipid species acting as an endogenous PPAR ligand (15). Whilst highly speculative at this stage, this offers the intriguing possibility that by altering lipid synthesis pathways DGK loss may affect the generation of this PPAR ligand.

It is not clear whether PKC activation contributes to the failure of DGK knockdown cells to differentiate. However, the finding that PKC activity is increased in DGK knockdown cells is important as it strongly supports the notion that loss of DGK increases the abundance of a small pool of signaling DAG which is capable of activating PKC. As described above, we were not able to detect any individual species of DAG that were increased in DGK knockdown cells at day 0 (Fig.4E) when increased PKC activation is apparent (Fig. 5A). This indicates that the DAG species increased by DGK knockdown and responsible for activating PKC is/are either below the level of detection or masked by the presence of more abundant DAG species of the same mass in a different subcellular location. Overall we propose that this quantitatively small pool of DAG has a critical proadipogenic signaling role and that loss of this may lead to inhibition of adipogenesis resulting in failure to appropriately regulate production of high abundance PA and DAG species indirectly. The data also imply that the elevation in these signaling lipid species may control the expression of adipocyte differentiation-related genes.

Overall our detailed analysis of lipid species reveals a complexity of changes that are masked in measures of total PA, DAG or TG levels. In addition our study highlights the difficulty of identifying and examining the roles of individual signaling lipids, even using highly sensitive state of the art techniques. However, we believe that the data from our novel and detailed lipidomic analyses provide valuable insights to inform future studies dissecting the involvement of specific signaling lipids in adipogenesis. Our data suggest that DGK does not directly or indirectly alter greatly the levels of PA and DAG species that act as precursors for the synthesis of TG at this early stage of adipogenesis. This may explain the apparent paradox that DGK expression rises during adipogenesis and is abundantly expressed in adipocytes, when one might predict its activity would oppose that of enzymes driving TG accumulation. Whilst further work is required to determine the precise underlying mechanism, our study reveals DGK as a novel regulator of adipocyte differentiation.

Acknowledgments

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What is already known about this subject

- Inhibition of DGK can contribute to insulin resistance and metabolic disease.
- DGK converts diacylglycerol to phosphatidic acid.
- Diacylglycerol and phosphatidic acid can act both as important signaling lipids and as precursors for the synthesis of triglyceride.

What this study adds

- DGK is required for adipocyte differentiation.
- DGK knockdown alters intracellular lipids and the activation of the diacylglycerol-sensitive protein kinase C .
- Different pools of intracellular diacylglycerol and phosphatidic acid species act as precursors of triacylglycerol or signaling lipids.

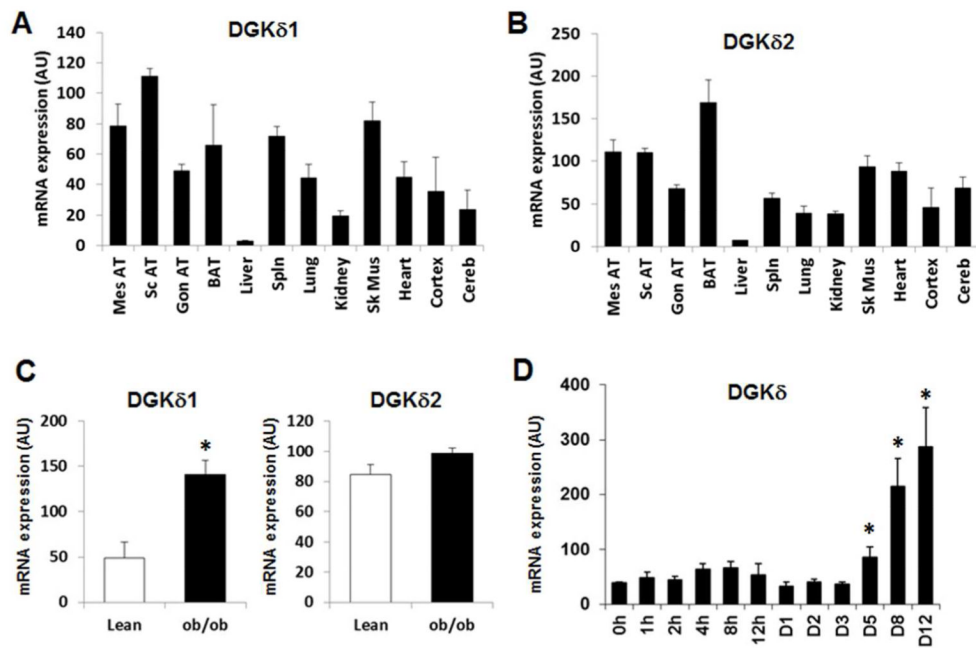


Figure 1.

DGK is abundantly expressed in adipose tissue and adipocytes. mRNA was extracted from murine mesenteric (Mes AT), subcutaneous (Sc AT), gonadal (Gon AT) adipose tissue, brown adipose tissue (BAT), liver, spleen (Spln), lung, kidney, skeletal muscle (SkMus), heart, cortex or cerebellum (Cereb) as indicated. Expression of mRNA encoding DGK 1 (A) or DGK 2 (B) was determined in each tissue by real-time PCR using isoform specific primers. Data are normalised to 18S ribosomal RNA and are expressed as means \pm SEM (n=5). (C) DGK 1 and DGK 2 expression was similarly determined in subcutaneous adipose tissue samples from 12 week old ob/ob mice and their heterozygous littermates. Data are normalised to 18S ribosomal RNA and are expressed as means \pm SEM (n=5). * indicates significant difference from expression in heterozygous mice, $P < 0.05$. (D) 3T3-L1 preadipocytes were induced to differentiate for various times and total RNA isolated using RNEasy kit (Qiagen, UK). Expression of mRNA encoding DGK was determined by real time PCR. Data are normalised to Cyclophilin A and are expressed as means \pm SEM (n=3). * indicates significant difference in expression from control shRNA at the same timepoint, $P < 0.05$ by ANOVA with post hoc Tukey's test.

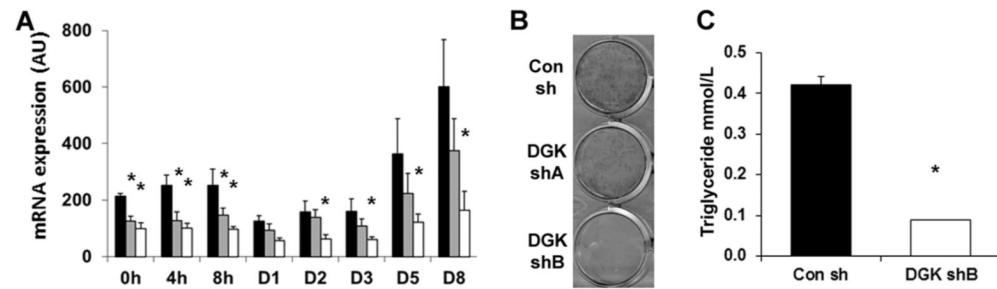


Figure 2.

DGK expression is required for adipocyte differentiation. 3T3-L1 preadipocytes were retrovirally transduced with control shRNA (black bars) or shRNA targeting DGK (shRNA-A grey bars, shRNA-B white bars). **(A)** Cells were induced to differentiate for various times, total RNA isolated and expression of mRNA encoding DGK was determined by real time PCR. Data are normalised to Cyclophilin A and are expressed as means \pm SEM (n=4). * indicates significant difference in expression from control shRNA at the same timepoint, $P < 0.05$ by ANOVA with post hoc Tukey's test. **(B)** control or DGK knockdown cells were differentiated for 8 days and stained with oil-red O to visualise lipid accumulation. **(C)** Total cellular triglyceride levels determined in control or DGK shB knockdown cells by enzymatic assay. Data are expressed as means \pm SEM (n=4), * indicates significant difference in levels versus control cells, $P < 0.05$ by students T-test.

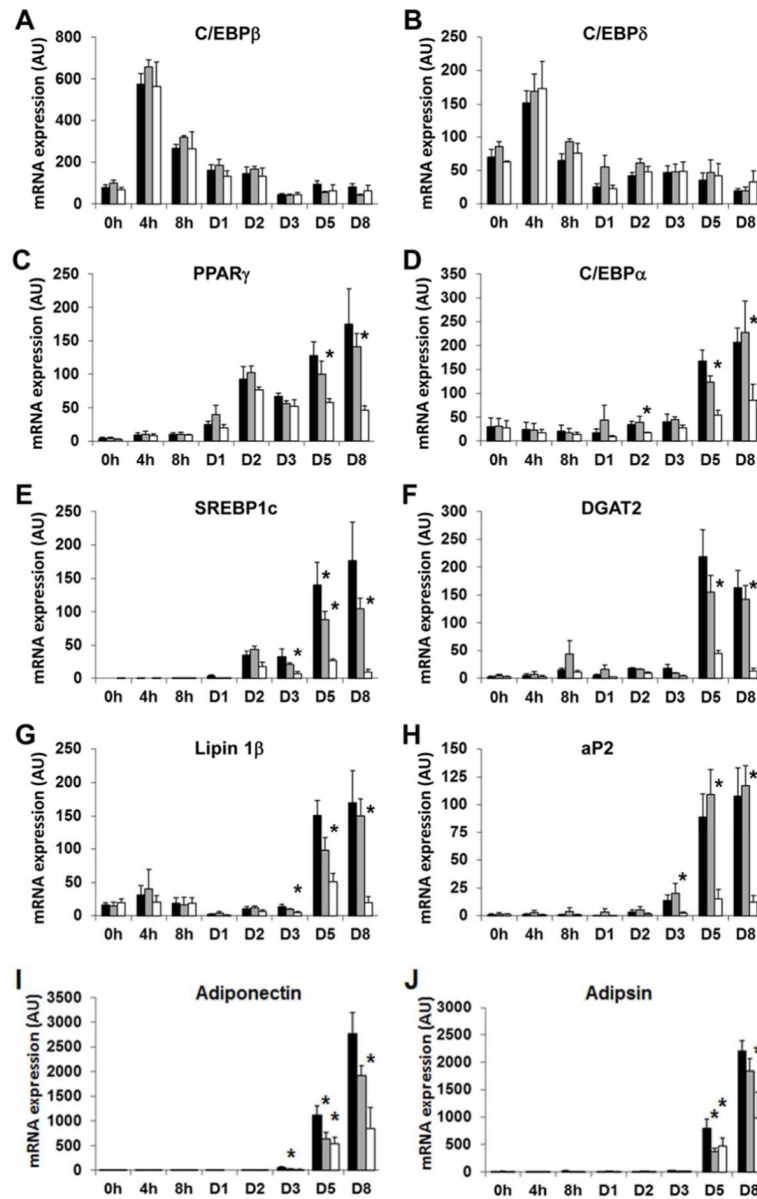


Figure 3. Knockdown of *DGK* inhibits adipogenic gene expression. RNA was isolated from 3T3-L1 preadipocytes expressing control shRNA (black bars), *DGK* shRNA-A (grey bars) or *DGK* shRNA-B (white bars) that had been induced to differentiate for various times as indicated. Expression of mRNA encoding C/EBP β (A), C/EBP δ (B), PPAR γ (C), C/EBP α (D), SREBP1c (E), DGAT2 (F), Lipin 1 β (G), aP2 (H), adiponectin (I) and adipsin (J) was determined by real-time PCR. Data are normalised to Cyclophilin A and are expressed as means \pm SEM (n=3). * indicates significant difference in expression from control shRNA at the same timepoint, P<0.05 by ANOVA with post hoc Tukey's test.

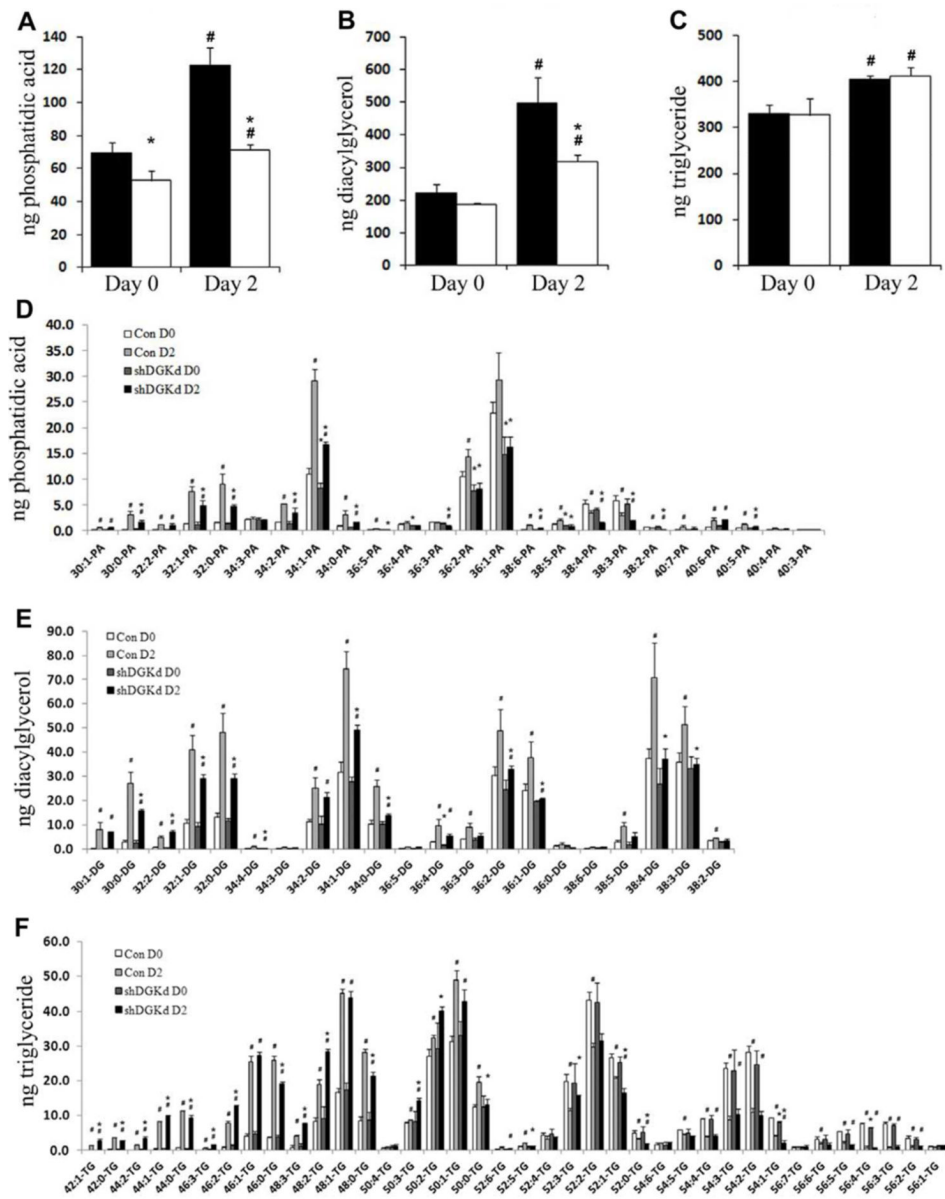


Figure 4.

Analysis of lipid species in differentiating control and DGK knockdown 3T3-L1 cells. Levels of total PA (A), DAG (B) and TG (C) were determined in extracts from control cells (black bars) or DGK shB knockdown cells (white bars) collected at day 0 or day 2 of differentiation as indicated. Data are shown in ng per 10 μ l sample as mean \pm S.D. n=3. Individual species of PA (D), DAG (E) and TG (F) were determined in control cells at day 0 (white bars) or day 2 (light grey bars), and DGK shB knockdown cells at day 0 (dark grey bars) or day 2 (black bars) of differentiation. Data are shown in ng per 10 μ l sample as mean \pm SD n=3. * indicates significant difference from levels in control cells at the same time point, # indicates significant difference from day 0 levels in the same cells by students T-test, p<0.05.

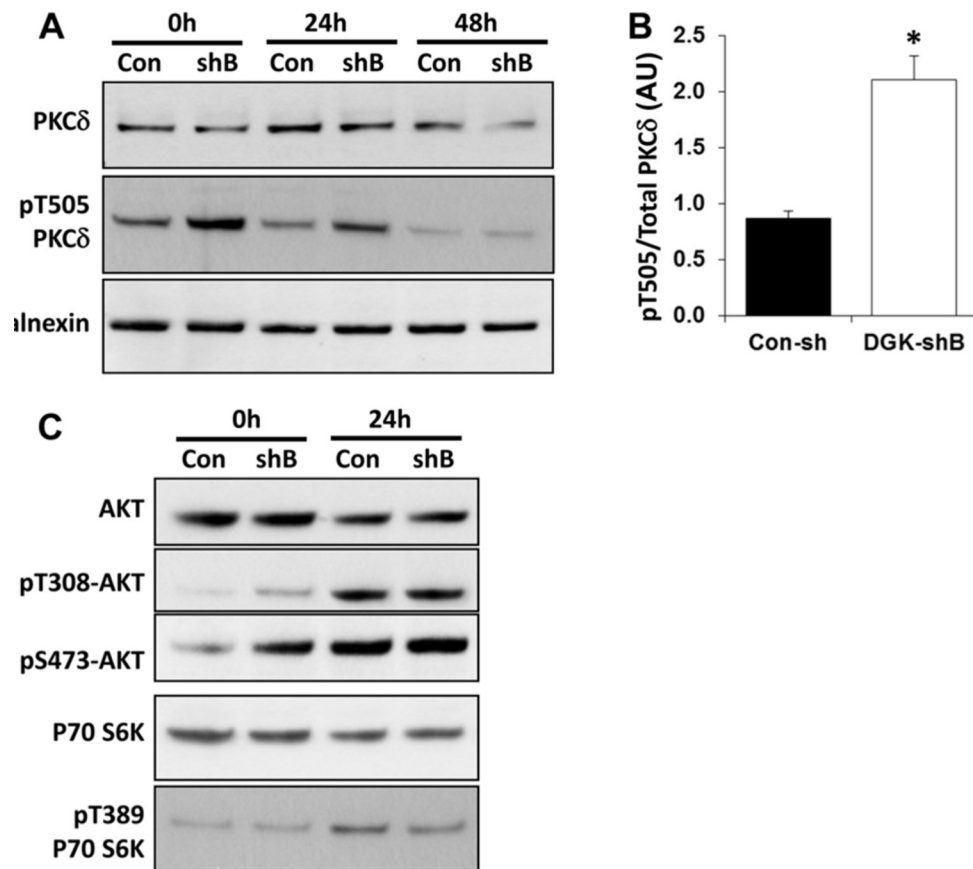


Figure 5.

Knockdown of DGK increases the activation of PKC. (A) Total cell lysates were isolated from 3T3-L1 preadipocytes expressing control shRNA (Con) or DGK shRNA-B (shB) that had been induced to differentiate for various times as indicated. 30 μ g of cellular protein was separated by SDS-PAGE and western blotted for total PKC or phospho-Thr505-PKC (pT505 PKC). Samples were also blotted for calnexin as a loading control. Representative blots are shown. (B) pT505 PKC signal vs total PKC signal was determined in blots of lysates isolated from 3T3-L1 preadipocytes expressing control shRNA (Con-sh) or DGK shRNA-B (DGK-shB) at day 0. Data shown are means \pm SEM n=4. * indicates significant difference from levels in control cells. (C) Total cell lysates were isolated from 3T3-L1 preadipocytes expressing control shRNA (Con) or DGK shRNA-B (shB) induced to differentiate for the times as indicated were western blotted for total AKT, phospho-Thr308-AKT (pT308 AKT), phospho-Ser473-AKT (pS473 AKT), total p70 S6 kinase (P70 S6K) or phospho-Thr389-p70 S6 kinase (pT389 P70 S6K). Blots are representative of 3 independent experiments.