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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 (DGKδ) 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 DGKδ expression had been knocked down and determined the effect of this on adipogenesis. We also performed lipidomic analysis to determine levels of the DGKδ product phosphatidic acid (PA), its substrate diacylglycerol (DAG) and triglyceride (TG).

Results—Inhibiting DGKδ expression prevents adipogenesis. DGKδ 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 DGKδ knockdown which significantly impaired the accumulation of DAG or PA species implicated in intracellular signaling. We also observed stimulation of the DAG activated kinase PKCδ in DGKδ knockdown cells, despite no increase in detectable species of DAG.

Conclusions—DGKδ 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; DGKδ

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. DGKδ has been implicated in insulin resistance in humans and whilst homozygous loss of DGKδ 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 DGKδ heterozygous mice. However, we observed that DGKδ is also clearly detectable in adipose tissue, another key metabolic tissue. We therefore sought to determine whether DGKδ 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 DGKδ (DGKδ shA: 5'-AAGACTTGTGGCAGCGTGTTA -3' DGKδ 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 DGKδ, DGKδ1, DGKδ2, SREBP1c, DGAT2, PPARγ2, aP2, lipin1β, adiponectin, adipsin, C/EBPβ and C/EBPδ mRNA expression as in (8). Primer/probe mix to assay C/EBPα 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 Choloform/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 × g, samples of supernatant containing 30 μg of protein were denatured and analysed by western blotting. Antibodies to Calnexin, PKCδ and phosphoT505-PKCδ 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 DGKδ have been reported with differing N termini (11). Using isoform specific primers we determined the expression of DGKδ1 (Fig. 1A) and DGKδ2 (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 DGKδ has been reported to affect insulin sensitivity and metabolism (6). To determine whether altered DGKδ 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 DGKδ1 mRNA expression in adipose tissue from ob/ob mice compared to their lean heterozygous littermates (Fig 1C). Consistent with the high expression levels of DGKδ in adipose tissue, we found that DGKδ expression was dramatically upregulated during adipogenesis in 3T3-L1 cells (Fig 1D). Together this suggested that DGKδ could have a role in the development or function of adipocytes. To examine this we generated 3T3-L1 preadipocytes in which DGKδ had been knocked down by stable expression of shRNAs targeting DGKδ. One of these, DGKδ-shA, gave a modest knockdown of DGKδ, whilst the second, DGKδ-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 DGKδ-shB expressing cells (Fig 2C).

Specific analysis of adipogenic gene expression revealed that DGKδ knockdown did not significantly affect the induction of the early transcriptional regulators of adipogenesis, C/EBPβ (Fig. 3A), or C/EBPδ (Fig. 3B), nor the early induction of the critical adipogenic transcription factor PPARγ up to day 3 of differentiation (Fig. 3C). However, the subsequent expression of PPARγ was significantly impaired in DGKδ knockdown cells, as was the induction of other adipocyte markers including the transcriptional factors C/EBPα (Fig. 3D), SREBP1c (Fig. 3E), the lipogenic enzymes DGAT2 (Fig. 3F) and Lipin1β (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 DGKδ 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 DGKδ

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 DGKδ 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/EBPα 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 DGKδ 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 adipogeneis in DGKδ 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 DGKδ knockdown cells (Fig. 4C).

To further probe the changes in these lipid species in DGKδ 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 DGKδ 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 DGKδ knockdown cells, suggesting few inherent differences in the synthesis of abundant lipid species between these cells 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 DGKδ 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 DGKδ 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 DGKδ 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 DGKδ knockdown (Fig. 4F). Indeed TG species containing longer medium chain fatty acids appeared to be decreased during adipogenesis.

A previous study implicating decreased DGKδ expression in the development of insulin resistance in skeletal muscle suggested that reduced DGKδ increased cellular DAG and led to the activation of PKCδ. We therefore assessed whether PKCδ activity could be altered in our DGKδ knockdown cells. This revealed that knockdown of DGKδ increased PKCδ activation (as assessed by Thr505 phosphorylation) in preadipocytes both immediately prior to differentiation and during the first 24hours of adipogenesis (Fig. 5A). Quantification of post-confluent preadipocytes exhibited more than 2-fold increased levels of PKCδ phosphorylation (Fig 5B). The increase in PKCδ activation due to reduced DGKδ 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 DGKδ 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 DGKδ 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 DGKδ knockdown preadipocytes following acute insulin treatment (not shown). We cannot rule out subtle alterations in the kinetics or quality of insulin signaling due to DGKδ knockdown. However, our data do not suggest that preadipocytes lacking DGKδ exhibit the insulin resistance observed in DGKδ deficient skeletal muscle cells making it very unlikely that this underlies the lack of adipogenesis mechanistically. These data also suggest both DGKδ inhibition and PKCδ activation may exert cell-type specific effects on insulin signaling.

DISCUSSION

Our study demonstrates that DGKδ 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 DGKδ in generating PA from DAG the clear implication is that the loss of DGKδ 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 DGKδ activity.

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

It is not clear whether PKCδ activation contributes to the failure of DGKδ knockdown cells to differentiate. However, the finding that PKCδ activity is increased in DGKδ knockdown cells is important as it strongly supports the notion that loss of DGKδ increases the abundance of a small pool of signaling DAG which is capable of activating PKCδ. As described above, we were not able to detect any individual species of DAG that were increased in DGKδ knockdown cells at day 0 (Fig.4E) when increased PKCδ activation is apparent (Fig. 5A). This indicates that the DAG species increased by DGKδ knockdown and responsible for activating PKCδ 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.

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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 DGKδ 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 DGKδ 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 DGKδ as a novel regulator of adipocyte differentiation.

Acknowledgments

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What is already known about this subject

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

What this study adds

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

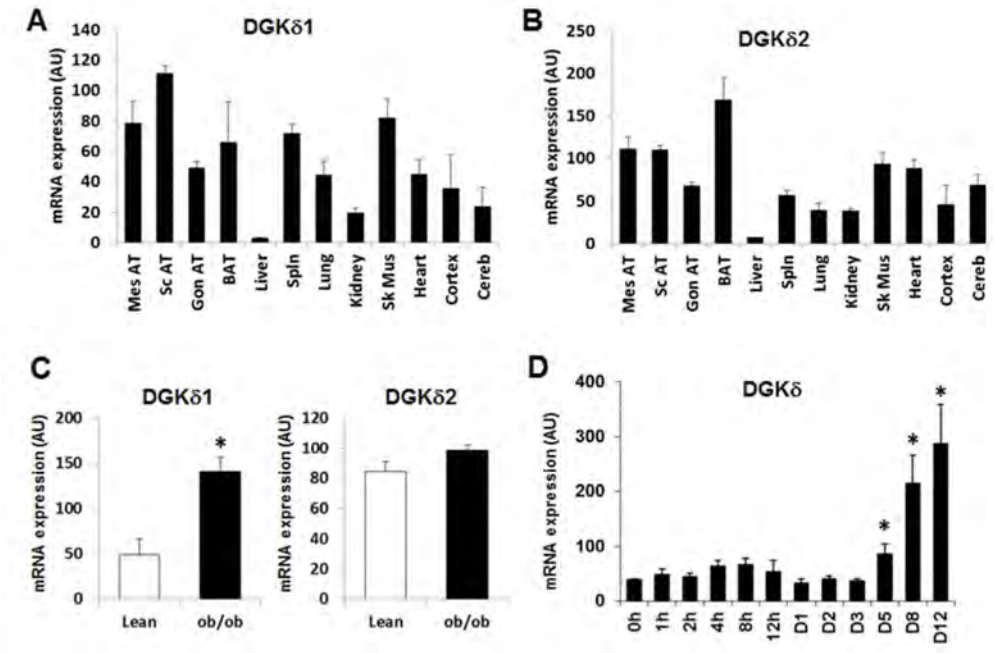

Figure 1.

DGKδ 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 DGKδ1 (**A**) or DGKδ2 (**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 ± SEM (n=5). (**C**) DGKδ1 and DGKδ2 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 ± 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 DGKδ was determined by real time PCR. Data are normalised to Cyclophilin A and are expressed as means ± 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.

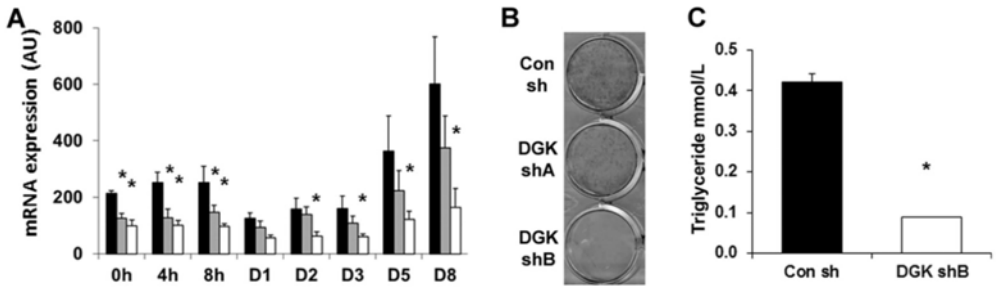

Figure 2.

DGKδ expression is required for adipocyte differentiation. 3T3-L1 preadipocytes were retrovirally transduced with control shRNA (black bars) or shRNA targeting DGKδ (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 DGKδ was determined by real time PCR. Data are normalised to Cyclophilin A and are expressed as means ± 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 DGKδ 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 DGKδ shB knockdown cells by enzymatic assay. Data are expressed as means ± SEM (n=4), * indicates significant difference in levels versus control cells, P<0.05 by students T-test.

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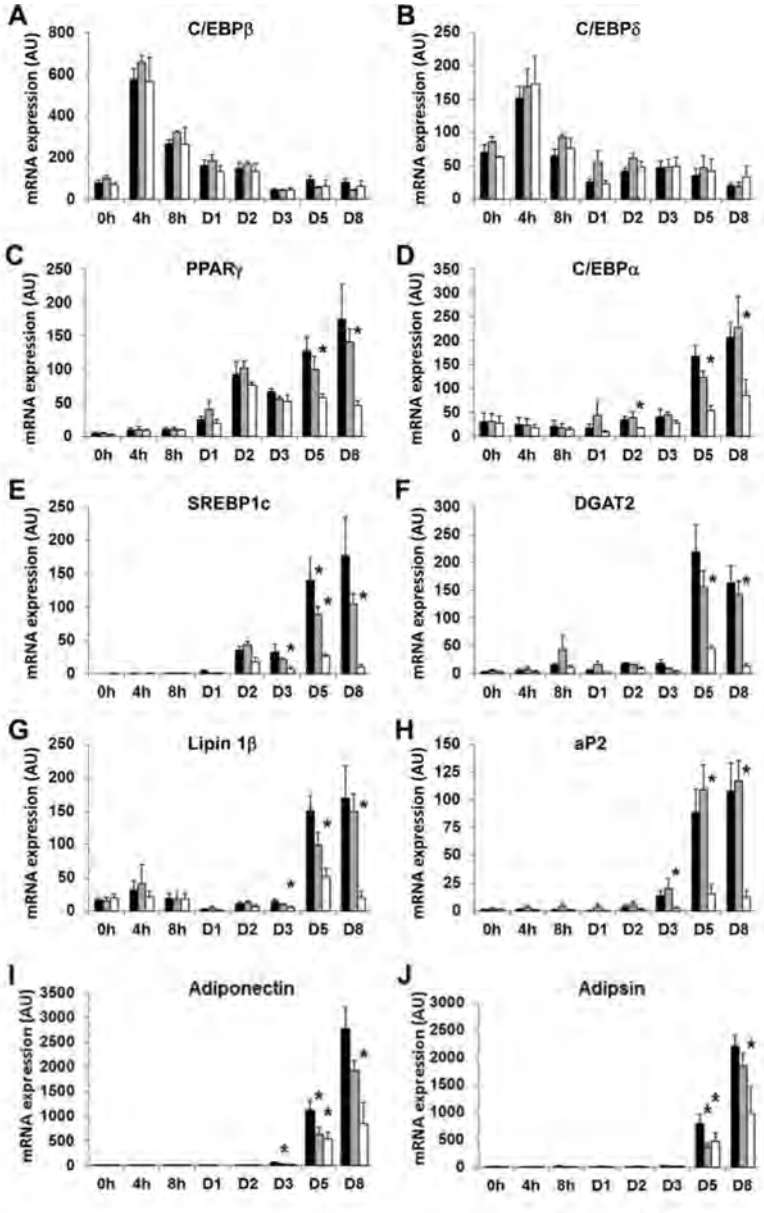

Figure 3.

Knockdown of DGKδ inhibits adipogenic gene expression. RNA was isolated from 3T3-L1 preadipocytes expressing control shRNA (black bars), DGKδ shRNA-A (grey bars) or DGKδ shRNA-B (white bars) that had been induced to differentiate for various times as indicated. Expression of mRNA encoding C/EBPβ (**A**), C/EBPδ (**B**), PPARγ (**C**), C/EBPα (**D**), SREBP1c (**E**), DGAT2 (**F**), Lipin 1β (**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 ± 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.

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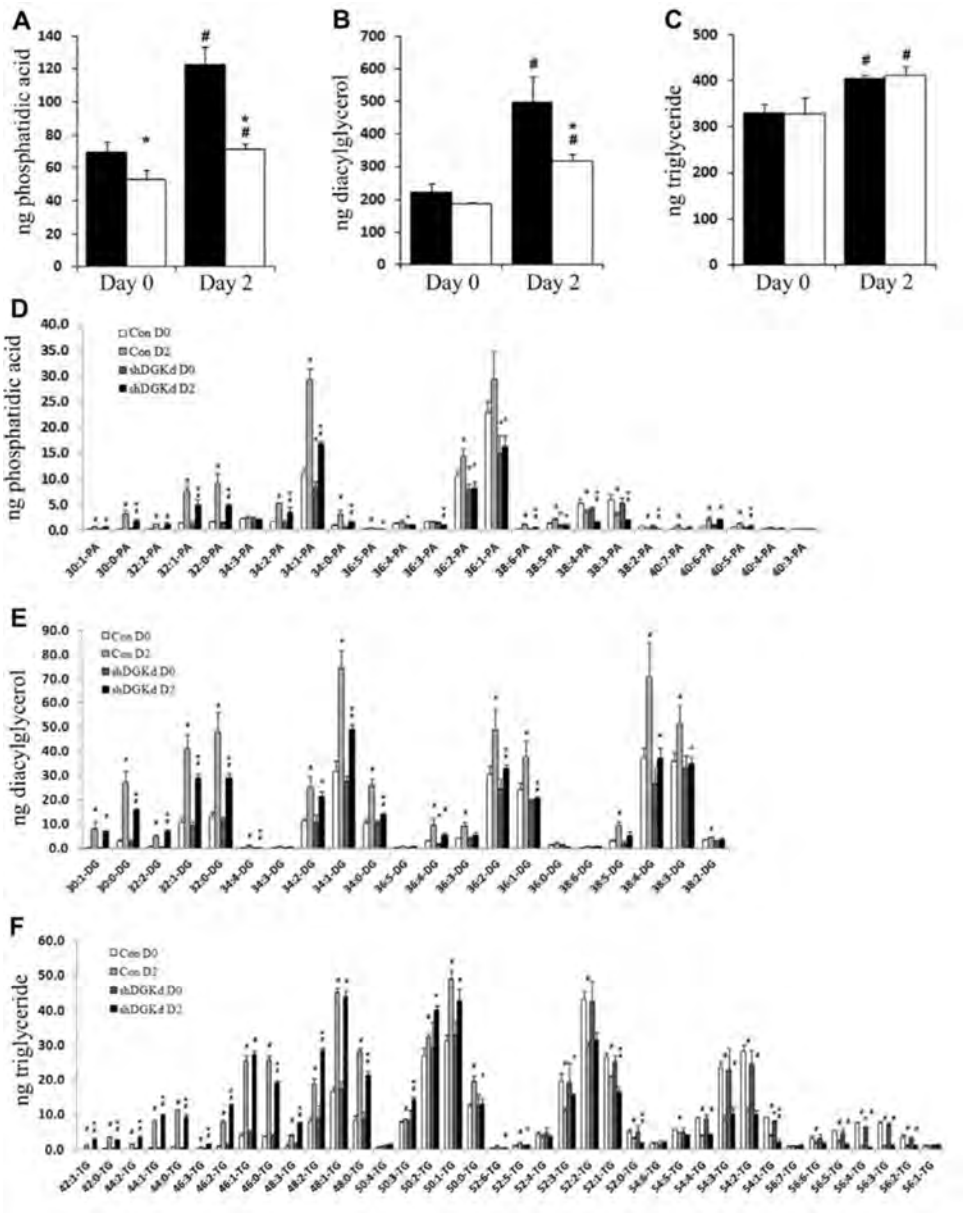

Figure 4.

Analysis of lipid species in differentiating control and DGKδ knockdown 3T3-L1 cells. Levels of total PA (**A**), DAG (**B**) and TG (**C**) were determined in extracts from control cells (black bars) or DGKδ shB knockdown cells (white bars) collected at day 0 or day 2 of differentiation as indicated. Data are shown in ng per 10μl sample as mean +/−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 DGKδ shB knockdown cells at day 0 (dark grey bars) or day 2 (black bars) of differentiation. Data are shown in ng per 10μl sample as mean +/−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, $p<0.05$.

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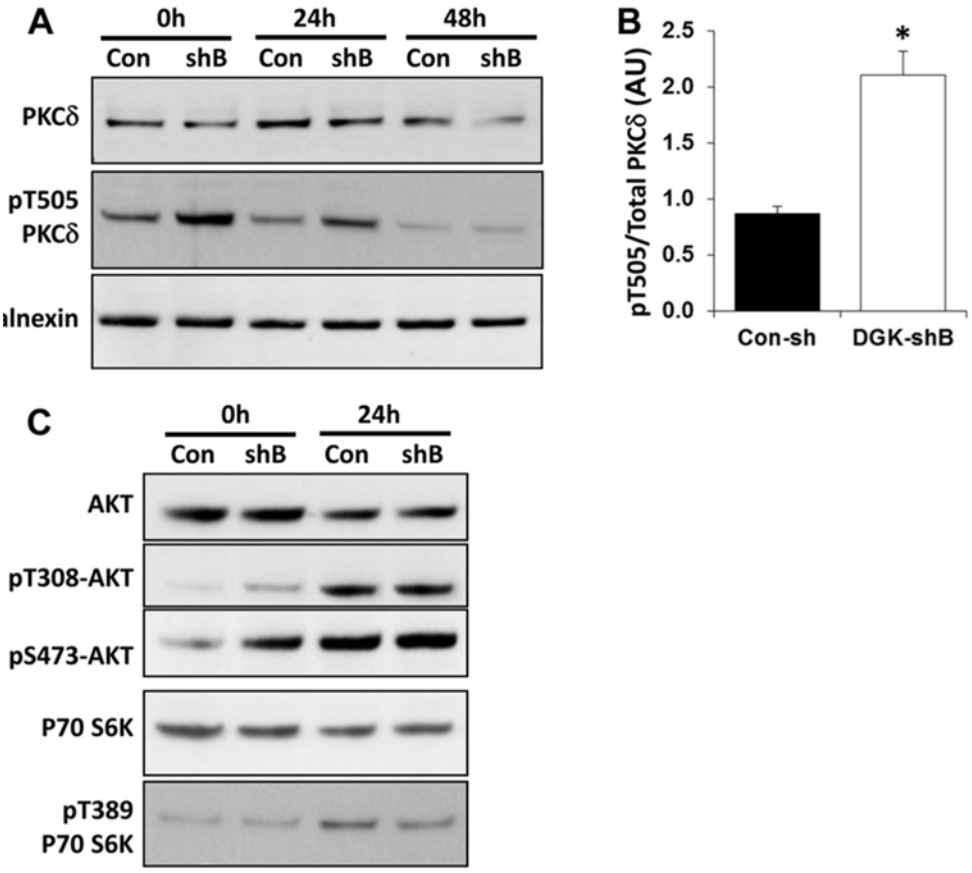

Figure 5.

Knockdown of DGKδ increases the activation of PKCδ. (**A**) Total cell lysates were isolated from 3T3-L1 preadipocytes expressing control shRNA (Con) or DGKδ shRNA-B (shB) that had been induced to differentiate for various times as indicated. 30 μg of cellular protein was separated by SDS-PAGE and western blotted for total PKCδ or phosphor-Thr505-PKCδ (pT505 PKCδ). Samples were also blotted for calnexin as a loading control. Epresentative blots are shown. (**B**) pT505 PKCδ signal vs total PKCδ signal was determined in blots of lysates isolated from 3T3-L1 preadipocytes expressing control shRNA (Con-sh) or DGKδ shRNA-B (DGK-shB) at day 0. Data shown are means +/−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 DGKδ 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.