ABSTRACT

Gwen Yung-Hsin Hwarng:

Finding links between obesity and diabetes: Using diacylglycerol kinase to regulate insulin signaling

(Under direction of Rosalind A. Coleman)

In the U.S., two-thirds of adults are overweight or obese, with 35.7% of them - nearly 78 million people – obese. What used to be a disease that only afflicted adults is now also increasingly being diagnosed in children, contributing to ever-growing obesity rates. In particular, obesity and diabetes are closely linked, with obese people at higher risk for developing type 2 diabetes. All this only further intensifies the urgent need to address obesity and type 2 diabetes. In particular, we need to understand how the two conditions are linked and what metabolic processes are involved, in order to develop treatment and prevention strategies that will ultimately reduce the burden of type 2 diabetes on healthcare.

My project looks at how the triacylglycerol synthesis and insulin signaling pathways are linked. In particular, we are studying how lipid intermediates cause insulin resistance in hepatocytes. We are interested in whether phosphatidic acid (PA) and diacylglycerol (DAG) affect Akt phosphorylation, and the mechanism by which PA and DAG might inhibit insulin signaling. Diacylglycerol kinase (DGK) is the enzyme that catalyzes conversion of DAG to PA, and was used as a tool to manipulate the cellular content of PA and DAG. The content and activity of DGK was adjusted by DGK overexpression in mouse hepatocytes, thus altering the balance of PA and DAG. The physiological consequences were then used to determine how insulin signaling is affected. Our hypothesis is that DGK regulates insulin signaling by changing intracellular PA and DAG levels. The isoform used was the DGKθ isoform. Our results showed that PA is associated with impaired insulin action in mouse hepatocytes, but DAG is not.

ACKNOWLEDGEMENTS

I would like to thank Dr. Rosalind A. Coleman and Dr. Eric L. Klett for sharing your knowledge and skills with me and pushing me beyond what I thought I was capable of. Our conversations, discussions, and debates always challenge and inspire me. I would especially like to recognize the efforts of my mentor, Dr. Chongben Zhang, for guiding me every step of the way through this project and teaching me everything I know about lab research. You have been instrumental in shaping the past two years of my Carolina experience and for that I am so grateful. I would also like to thank everyone in the Coleman lab for supporting me in this project. I have grown tremendously from our time working together in the lab, and seeing your smiling faces at my honors thesis public defense made me realize how much I appreciate you all.

I would also like to thank my community here in Chapel Hill, as well as family and friends back home in Singapore, for your love and warmth. It is from all of you that I have learned what it means to be genuine.

Most importantly, I would like to give all glory, honor, and praise to my Father in heaven.

"Every good and perfect gift is from above, coming down from the Father of the heavenly lights, who does not change like shifting shadows." James 1:17

This project was supported by the Tom and Elizabeth Long Excellence Fund for Honors, administered by Honors Carolina.

TABLE OF CONTENTS

LIST OF TABLES……vi

LIST OF FIGURES……vii

LIST OF ABBREVIATIONS……..……viii

CHAPTER 1: INTRODUCTION……..1

1.1 Obesity and diabetes……...1

1.2 The insulin signaling pathway……1

1.3 The GPAT pathway……3

1.4 Lipid metabolism and insulin resistance……5

CHAPTER 2: STUDY AIMS AND HYPOTHESIS……6

CHAPTER 3: MATERIALS AND METHODS……...8

3.1 Materials……....8

3.2 Methods……8

3.2.1 DGK activity assay……...8

3.2.2 Western blot analysis……....9

3.3 Other methods……....9

3.3.1 Mouse liver perfusion, hepatocyte isolation, and culture…….....9

3.3.2 cDNA cloning, lentiviral packaging, and hepatocyte infection……...…...10

3.3.3 Lipid extraction and PA and DAG assays……..10

3.4 Statistical analysis……10

CHAPTER 4: RESULTS……11

4.1 DGKθ overexpression impaired Akt phosphorylation in mouse hepatocytes…….....11

4.2 DGKθ overexpression increased cellular content of PA,

and decreased cellular content of DAG……12

CHAPTER 5: DISCUSSION……..14

5.1 Molecular mechanisms of insulin resistance……14

5.2 Lipid metabolism and insulin resistance……...15

5.3 DGK……..16

5.4 DGK-derived PA is associated with impaired insulin action, but DAG is not……....17

5.5 Limitations……18

5.6 Future direction……18

REFERENCES……20

LIST OF TABLES

Table 1. Cellular content of PA species in mouse primary hepatocytes with GFP or DGKθ overexpression (pmol/mg protein)……..……13

Table 2. Cellular content of DAG species in mouse primary hepatocytes with GFP or DGKθ overexpression (pmol/mg protein)..……..……...13

LIST OF FIGURES

Figure 1. Key steps in the insulin signaling pathway……2

Figure 2. Enzymes and lipid intermediates of the GPAT pathway……4

Figure 3. Lipid intermediates and the insulin signaling pathway……..7

Figure 4. DGKθ overexpression impaired Akt phosphorylation in mouse hepatocytes……..……11

Figure 5. DGKθ overexpression increased cellular content of PA,
and decreased cellular content of DAG……...12

Figure 6. Structures of ten mammalian DGK isoforms classified into five types……...16

Figure 7. Structural diagram of DGKθ……17

Figure 8. DGKθ mRNA expression in rat……17

LIST OF ABBREVIATIONS

ACSL	acyl-CoA synthetase
AGPAT	1-acyglycerol-3-phosphate acyltransferase
ATP	adenosine triphosphate
CRD	cysteine-rich domain
DAG	diacylglycerol
DGAT	diacylglycerol acyltransferase
DGK	diacylglycerol kinase
ER	endoplasmic reticulum
G3P	glycerol-3-phosphate
GLUT	glucose transporter
GPAT	glycerol-3-phosphate acyltransferase
IR	insulin receptor
IRS	insulin receptor substrate
LPA	lysophosphatidic acid
mTOR	mammalian target of rapamycin
mTORC	mammalian target of rapamycin complex
PA	phosphatidic acid
PAPase/lipin	phosphatidic acid phosphohydrolase
PC	phosphatidylcholine
PDK1	3-phosphoinositidedependent protein kinase-1
PH	pleckstrin homology
PI3K	phosphatidylinositol 3-kinase
PIP2	phosphatidylinositol-4,5-bisphosphate
PIP3	phosphatidylinositol-(3,4,5)-triphosphate

PKC	protein kinase C
PLD	phospholipase D
RA	Ras-associating domain
S473	serine473
T2D	type 2 diabetes
T308	threonine308
TAG	triacylglycerol

CHAPTER 1: INTRODUCTION

1.1 Obesity and diabetes

In the U.S., two-thirds of adults are overweight or obese, with 35.7% of them - nearly 78 million people – obese[1]. What used to be a disease that only afflicted adults is now increasingly being diagnosed in children, contributing to ever-growing obesity rates[2]. Furthermore, obesity is related to some of the leading causes of preventable death such as type 2 diabetes (T2D), heart disease, and cancer[1].

In particular, obesity and diabetes are closely linked, with obese people at higher risk for developing T2D. In 2012, the total estimated cost of diabetes in the US was $245 billion[3] and it is estimated that by 2050, 1 in 3 US adults will have diabetes[4]. T2D accounts for 90-95% of all diabetes cases.

The need to address obesity and T2D is critical. It is important to understand how the two conditions are linked and what metabolic processes are involved, in order to develop treatment and prevention strategies that will ultimately reduce the burden of T2D on healthcare costs and health outcomes.

1.2 The insulin signaling pathway

T2D is characterized by high blood glucose levels due to insulin resistance, which occurs when the insulin signaling pathway is impaired[5]. Insulin is a hormone that is secreted by the pancreas and is responsible for maintaining normal glucose levels in the blood by increasing glucose uptake by glucose transporter (GLUT) 4, decreasing glucose production by the liver, and increasing glycogen synthesis. Resistance to insulin action occurs due to a defect in the insulin signaling pathway, and thus it is crucial to understand how the pathway normally works. Some key steps of the pathway are shown in Figure 1.

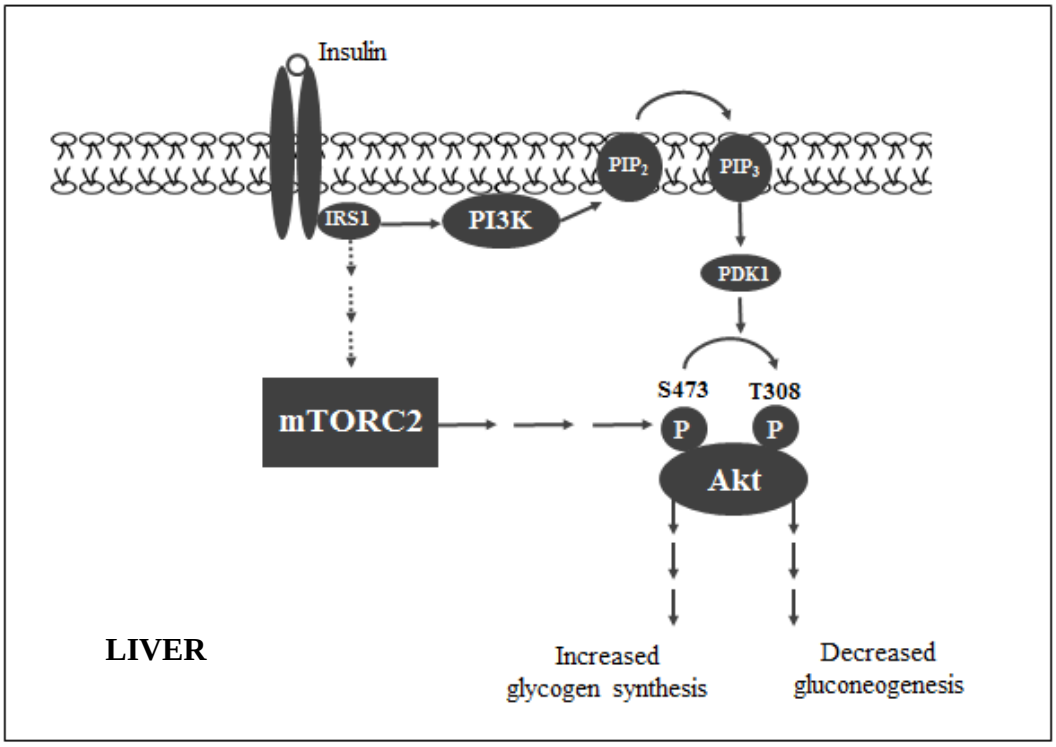


Figure 1. Key steps in the insulin signaling pathway. Insulin binds to the insulin receptor (IR), and autophosphorylation of specific tyrosine residues results in activation of insulin receptor substrate (IRS) 1. IRS1 activation is responsible for the subsequent generation of downstream signals to phosphatidylinositol 3-kinase (PI3K), phosphatidylinositol 4,5-bisphosphate (PIP_2), and phosphatidylinositol-(3,4,5)-triphosphate (PIP_3). PIP_3 recruits 3-phosphoinositidedependent protein kinase-1 (PDK1) and Akt to the plasma membrane. Subsequent Akt phosphorylation results in a sequence of downstream signaling that increases the rate of glycogen synthesis and decreases the rate of gluconeogenesis in the liver. IRS1 also triggers an enzyme phosphorylation cascade which may activate the mammalian target of rapamycin complex 2 (mTORC2).

When insulin binds to the extracellular insulin binding site on the insulin receptor (IR), the tyrosine kinases on the receptor are activated, leading to autophosphorylation of specific tyrosine residues[6]. This results in the binding of insulin receptor substrate (IRS) 1 to the IR and activation of IRS1. The activation of IRS1 is responsible for the subsequent generation of downstream signals. IRS1 binds to phosphatidylinositol 3-kinase (PI3K), which activates various signaling lipids[7]. One of these lipids is phosphatidylinositol 4,5-bisphosphate (PIP_2), a phospholipid component of the cell membrane which is phosphorylated by PI3K to form phosphatidylinositol-(3,4,5)-triphosphate (PIP_3). PIP_3 recruits 3-

phosphoinositidedependent protein kinase-1 (PDK1) and Akt to the plasma membrane[6]. Akt, a serine/threonine kinase, is then activated by phosphorylation at two amino acid sites, Serine473 (S473) and Threonine308 (T308). Akt phosphorylation results in a sequence of downstream signaling that eventually increases the rate of glycogen synthesis and decreases the rate of gluconeogenesis in the liver. Glycogen synthase converts glucose into glycogen for storage, whereas gluconeogenesis is the process by which glucose is produced from non-carbohydrate sources like amino acids. Fatty acid oxidation provides the adenosine triphosphate (ATP) required to power gluconeogenesis. These two processes are crucial for maintaining normal plasma glucose levels. IRS1 also triggers an enzyme phosphorylation cascade which may eventually activate the mammalian target of rapamycin complex 2 (mTORC2). mTORC2 is a complex of proteins that work together to phosphorylate Akt at S473[8]. When both S473 and T308 are phosphorylated, Akt is fully activated, leading to the Akt-dependent phosphorylation of many substrates. In adipose and muscle, this results in GLUT4 translocation and glucose uptake. Many of insulin's other effects are regulated by Akt.

1.3 The GPAT pathway

Another pathway that has been implicated in the development of insulin resistance is the glycerol-3-phosphate acyltransferase (GPAT) pathway[9]. This is the pathway by which triacylglycerol (TAG) is synthesized from fatty acids and glycerol-3-phosphates (G3P). The reactions of the main lipid intermediates and enzymes of this pathway are shown in Figure 2.

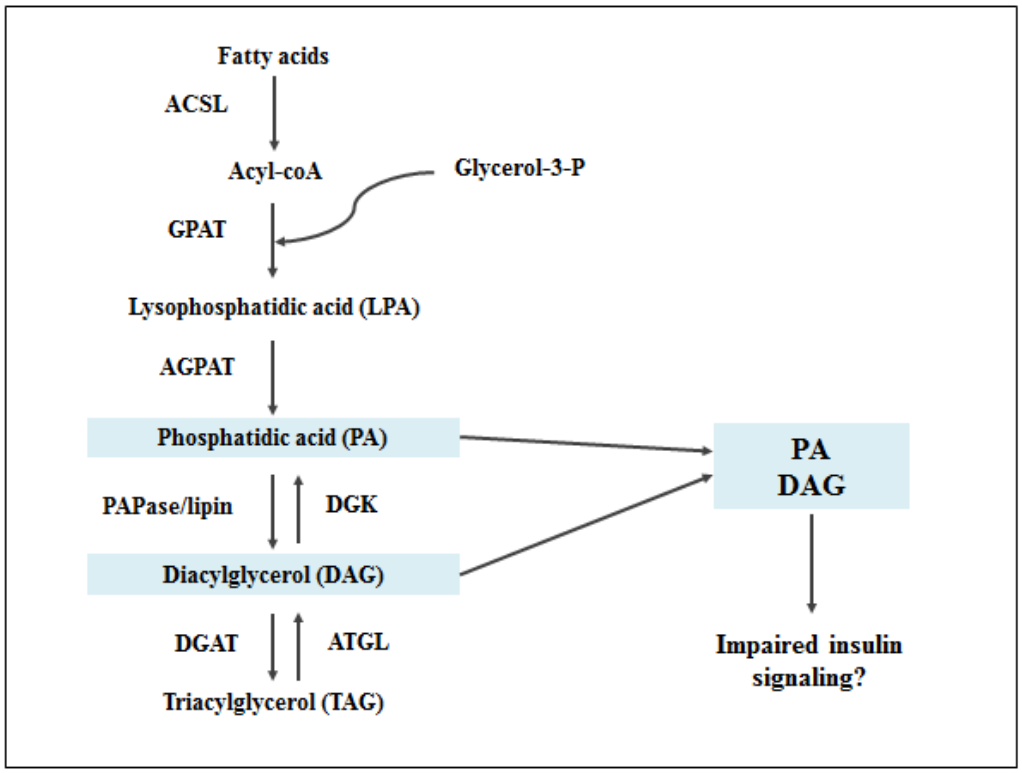


Figure 2. Enzymes and lipid intermediates of the GPAT pathway. Fatty acids enter the GPAT pathway, eventually being converted to triacylglycerol (TAG) through several reactions catalyzed by various enzymes. Lipid intermediates in this pathway include lysophosphatidic acid (LPA), phosphatidic acid (PA), and diacylglycerol (DAG). This project focused on whether either of two lipid intermediates, PA or DAG, impairs insulin signaling.

Initially, a fatty acid is converted to acyl-CoA by acyl-CoA synthetase (ACSL). GPAT catalyzes the reaction of acyl-CoA and G3P to form lysophosphatidic acid (LPA). 1-acyglycerol-3-phosphate acyltransferase (AGPAT) catalyzes the reaction of LPA and another acyl-CoA to form phosphatidic acid (PA). Diacylglycerol (DAG) is formed from PA through the removal of a phosphate group by phosphatidic acid phosphohydrolase (PAPase/lipin). Finally, diacylglycerol acyltransferase (DGAT) catalyzes the reaction of DAG and a third fatty acid to form TAG. LPA, PA, and DAG are lipid intermediates which can also act as signaling molecules in other pathways. This project focused on whether either of two lipid intermediates, PA or DAG, impairs insulin signaling. Another enzyme,

diacylglycerol kinase (DGK), plays the antagonistic role of converting DAG back to PA with the addition of a phosphate group.

1.4 Lipid metabolism and insulin resistance

Lipid intermediates modulate insulin signaling and have been implicated in insulin resistance. Lipid-induced insulin resistance in human muscle has been associated with changes in DAG and certain protein kinase C (PKC) isoforms[10]. DAG activates certain PKC isoforms, which could lead to serine or threonine phosphorylation of the IR or IRS1, thus disrupting insulin signaling. PA is another important signaling molecule which has been shown to inhibit insulin signaling to Akt by disrupting the mTOR-rictor association[8]. Besides being produced by GPAT/AGPAT-catalyzed reactions, PA is also derived from the phosphorylation of DAG by DGK. DGK can thus alter the cellular content of DAG and PA reciprocally[11]. The focus of this project was to determine how lipid intermediates in the GPAT pathway, specifically PA and DAG, are associated with signaling molecules in the insulin signaling pathway in mouse hepatocytes, using DGK as a tool to manipulate the cellular content of PA and DAG.

CHAPTER 2: STUDY AIMS AND HYPOTHESIS

Our project looks at how the TAG synthesis and insulin signaling pathways are linked in mouse hepatocytes. In particular, we are studying how lipid intermediates may cause insulin resistance. In determining how lipid intermediates in the GPAT pathway are associated with signaling molecules in the insulin signaling pathway, we raised two main questions. These two questions are summarized in Figure 3.

1. Do the relative amounts of PA and DAG decrease Akt phosphorylation?

   Since Akt phosphorylation is a key step in the insulin signaling pathway, it is important to determine whether the relative amounts of PA and DAG decrease Akt phosphorylation.

2. What is the mechanism by which PA or DAG decreases Akt phosphorylation?

   Our previous studies suggest that PA and Akt phosphorylation could be linked by mTORC2[8]. mTORC2 phosphorylates Akt at S473, thereby activating downstream signaling.

DGK is the enzyme that catalyzes the conversion of DAG to PA, and was used as a tool to manipulate the cellular content of PA and DAG. We hypothesize that DGK can regulate insulin signaling by altering the cellular content of PA and DAG.

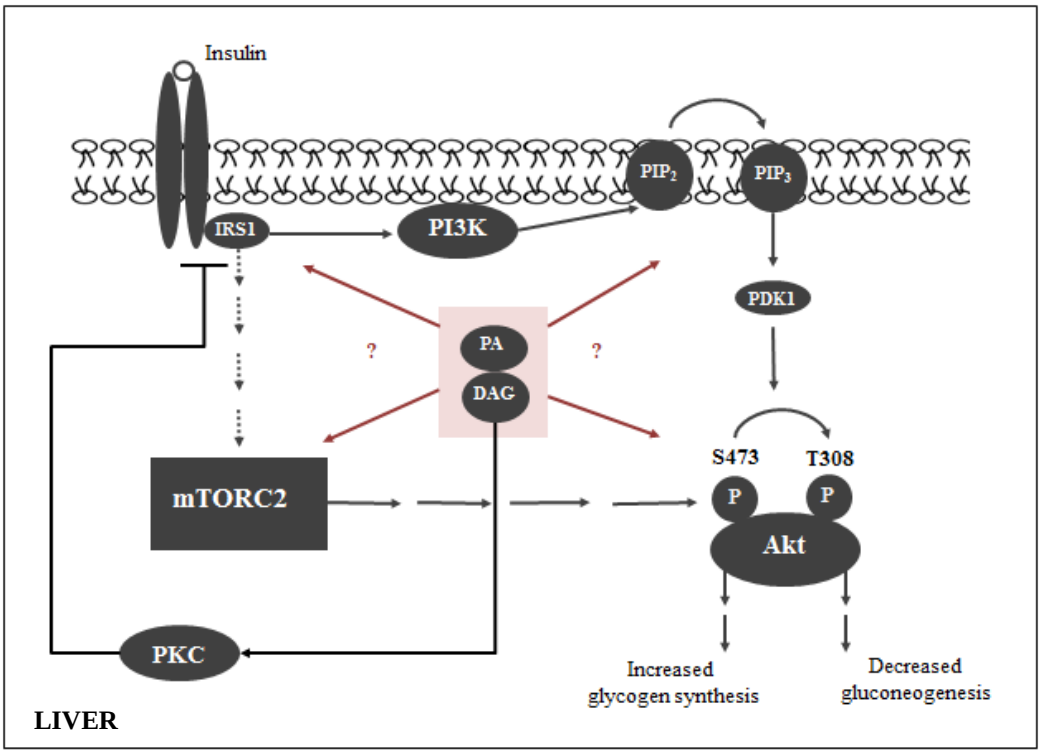


Figure 3. Lipid intermediates and the insulin signaling pathway. Our project looks at how the TAG synthesis and insulin signaling pathways are linked. In particular, we are studying how lipid intermediates may cause insulin resistance.

CHAPTER 3: MATERIALS AND METHODS

This chapter will go into detail about the methods that I participated in, followed by a summary of methods that were crucial for data collection and that I observed but did not participate in.

3.1 Materials

Primary antibodies were from Cell Signaling Technology (MA). Secondary antibodies and SuperSignal West Pico Chemiluminescent Substrate were from Thermo Scientific. Anti-DGKθ antibodies were from Santa Cruz Biotechnology (CA). Anti-α-tubulin antibodies, insulin (human recombinant), phosphatase inhibitor cocktails 1 and 2, ATP, and CHAPS were from Sigma (MO). Inactive Akt1 was from SignalChem (British Columbia, Canada). Protease inhibitor tablets were from Roche (CT). Cell culture media and reagents were from Invitrogen (CA).

3.2 Methods

3.2.1 DGK activity assay

The DGK activity assay used DAG/phosphatidylserine (PS)/ octyl glucoside (OG) mixed micelles[12]. A cold assay buffer (40 mM Hepes, pH 7.5, 120 mM NaCl, 1 mM EDTA, 10 mM pyrophosphate, 10 mM glycerophosphate, 50 mM NaF, 0.5 mM Na_3VO_4, EDTA-free protease inhibitors, 1% phosphatase inhibitor mixture 2 and 3, 0.3% CHAPS) was used to lyse the hepatocytes on ice. Cell lysates were then centrifuged at 13,000×g for 10 min. Supernatants were transferred to new tubes, and the reaction was initiated by adding 10 μl of the cell supernatant (containing 10-30 μg protein) to 90 μl of the reaction system. The reaction system contained DAG/PS/OG mixed micelles (8% mol DAG, 16% mol PS, 55 mM OG) and the reaction mixture (100 μM DTPA, 50 mM imidazole-HCl, 50 mM NaCl, 1.5

mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, and 1 mM γ-^{32}P[ATP] mix containing 2 μCi γ-^{32}P[ATP]). The reaction was terminated by adding 1 ml 1% perchloric acid and 1 ml chloroform. This was followed by vortexing and washing twice with 2 ml of 1% perchloric acid. An aliquot of the lower organic phase was removed for scintillation counting.

3.2.2 Western blot analysis

Hepatocytes were harvested in lysis buffer (20 mM Tris-HCl, pH 7.5, 0.1 mM Na_3VO_4, 25 mM NaF, 25 mM glycerophosphate, 2 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 0.3% Triton X-100). The lysates were mixed 1:1 with 2x Laemmli sample buffer and boiled before loading onto SDS-PAGE. Gels were run at 180V for 1 h in running buffer (25 mM Tris base, 190 mM glycine, 0.1% SDS, pH 8.3) and then transferred to nitrocellulose membrane at 75V for 1 h in transfer buffer (25 mM Tris base, 190 mM glycine, 20% methanol, pH 8.3). The membrane was blocked with 5% milk for 30 min and incubated with primary antibodies overnight at 4°C. After primary antibody incubation, the membrane was washed three times for 5 min each with washing buffer (20mM NaCl, 20mM Tris-Cl, 0.05% Tween-20, pH 7.6). Incubation with secondary antibodies was done for 30 min at room temperature. After secondary antibody incubation, the membrane was washed five times for 5 min each with washing buffer. The membrane was incubated with SuperSignal West Pico Chemiluminescent Substrate for 5 min at room temperature. Horseradish peroxidase-conjugated secondary antibodies were detected by exposing the gel to X-ray film. The film was converted to digital image by an Epson scanner (Perfection 2400), and the images were cropped using Photoshop CS2.

3.3 Other methods

3.3.1 Mouse liver perfusion, hepatocyte isolation, and culture

Hepatocytes were isolated from livers of 8-15 week old wild type C57/B6J mice and cultured overnight in William's medium E (10% FBS, 1% penicillin/streptomycin and 4 mM glutamine). Hepatocytes were infected for 24 h with constructs of the different enzymes in FBS-free D/MEM (4.5 g/L

glucose, 1% penicillin/streptomycin and 4 mM glutamine), followed by specific treatments according to the experiment design.

3.3.2 cDNA cloning, lentiviral packaging, and hepatocyte infection

Lentiviral constructs for GFP and mouse DGKθ were gifts from Dr. Daniel M. Raben (The Johns Hopkins University, Baltimore, MD). The constructs were transfected into HEK293T cells with plasmids pCMV delta-R8.2 and PCMV-VSV-G (Addgene, Cambridge, MA). The crude lentiviruses (about 1-2×10^8 TU/ml) produced in D/MEM (4.5 g/L glucose, 30% FBS, 0.1% penicillin/streptomycin and 4 mM glutamine) were concentrated by 2 h ultracentrifugation at 25,000×g at 4℃; the lentivirus pellet was reconstituted in PBS to obtain concentrated lentivirus with a titer of $1\text{-}2\times10^{10}$ TU/ml. MOIs of 10 were used for mouse hepatocyte lentiviral infection.

3.3.3 Lipid extraction and PA and DAG assays

Total cell lipid was extracted and analyzed by LC/MS. The amount of PA and DAG species in the biological samples was calculated from the peak areas obtained using Analyst 1.5 (Applied Biosystems). Raw peak areas were corrected and transformed into amounts of analyte using standard curves. Glycerolipids were quantified with 0.1 nmol 17:0 LPA and 15:0 DAG as internal standards for PA and DAG respectively. Amounts of PA and DAG were quantified and normalized to the protein concentrations of the cellular lysates.

3.4 Statistical analysis

Values are expressed as means ± SE. Student's t test was used to compare between groups (GFP and DGKθ) of the same treatment (basal, or insulin-stimulated) or same lipid species (PA, or DAG). GFP was set as the control. Data represent at least 3 independent experiments. $P < 0.05$ was considered significant.

CHAPTER 4: RESULTS

4.1 DGKθ overexpression impaired Akt phosphorylation in mouse hepatocytes

DGK is a key enzyme which phosphorylates DAG to produce PA, thus it occupies an important position in the modulation of DAG and PA concentrations. To investigate whether the product of DGK, PA, plays a role in regulating insulin sensitivity, we overexpressed DGKθ in mouse primary hepatocytes. Insulin signaling was examined by measuring the effects of DGK overexpression on Akt phosphorylation. Western blotting showed that DGKθ was overexpressed (Figure 4A). A DGK activity assay further confirmed a corresponding increase in DGK activity by 4-fold (Figure 4B). Western blotting also showed that following DGKθ overexpression, there was no Akt phosphorylation at S473 and T308, representing blocked insulin signaling (Figure 4C). These results indicate that insulin signaling is disrupted when DGK is overexpressed in hepatocytes.

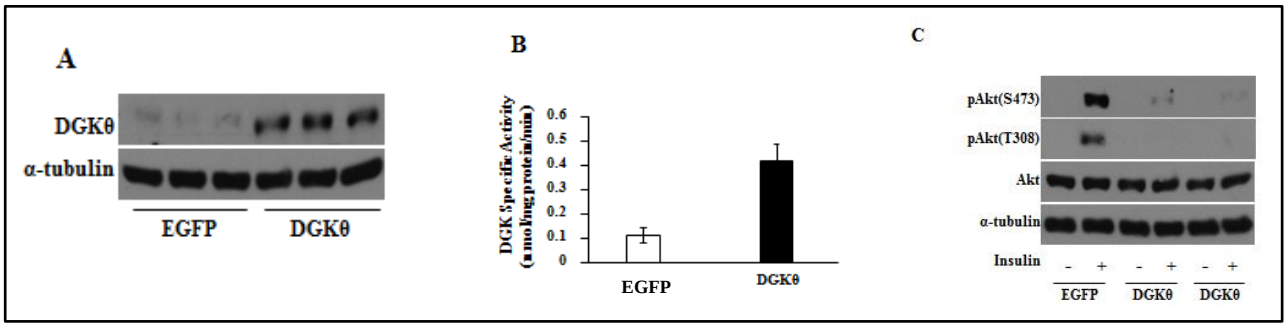


Figure 4. DGKθ overexpression impaired Akt phosphorylation in mouse hepatocytes. Mouse primary hepatocytes were infected with EGFP or DGKθ lentiviruses for 24 h. Cells were (A) lysed and subjected to Western blotting; or (B) lysed for cell lysate preparations and subjected to DGK activity assay; or (C) treated with or without insulin (100 nM) for 10 min, followed by cell lysate preparation for Western blotting. *, $P < 0.05$ compared to EGFP.

4.2 DGKθ overexpression increased cellular content of PA, and decreased cellular content of DAG

Since DGK regulates both PA and DAG, we looked at the cellular content of PA and DAG to find out how concentrations of lipid metabolites produced by DGK overexpression were associated with impaired insulin signaling in mouse hepatocytes. DGKθ overexpression increased total PA, 16:0-PA, and other PA species by 2.3 fold each, and increased 16:0-16:0-PA by 9.4 fold (Figures 5A and 5B, Table 1). DGKθ overexpression decreased total DAG, 16:0-DAG, and other DAG species by 71.1%, 90.6%, and 27.2% respectively, and decreased 16:0-16:0-DAG by 95.4% (Figures 5C and 5D, Table 2). These results suggest that the cellular content of PA, but not DAG, is associated with impaired insulin signaling in mouse hepatocytes. In particular, increased 16:0-16:0-PA is strongly associated with impaired insulin signaling.

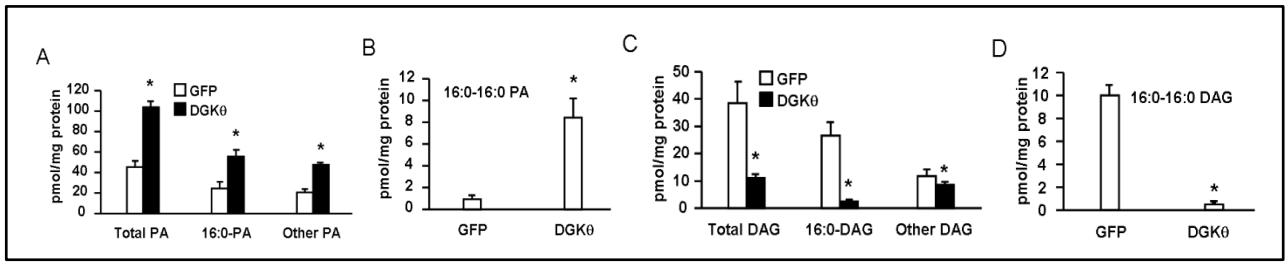


Figure 5. DGKθ overexpression increased cellular content of PA, and decreased cellular content of DAG. Lipids from mouse primary hepatocytes with GFP or DGKθ overexpression were assayed. (A) Total PA, 16:0-PA and other PA. (B) 16:0-16:0 PA. (C) Total DAG, 16:0-DAG and other DAG. (D) 16:0-16:0 DAG. *, $P < 0.05$ compared to GFP.

Table 1. Cellular content of PA species in mouse primary hepatocytes with GFP or DGKθ overexpression (pmol/mg protein)

PA species	GFP	DGKθ
16:0-16:0	0.90 ± 0.33	8.45 ± 2.15*
16:0-18:0	1.15 ± 0.41	2.49 ± 0.33*
16:0-18:1	15.31 ± 6.10	22.70 ± 4.06
16:0-18:2	7.22 ± 0.47	22.18 ± 5.16*
18:0-18:1	5.79 ± 0.98	7.17 ± 0.86
18:0-18:2	1.53 ± 0.18	0.24 ± 0.11
18:1-18:1	12.08 ± 3.71	36.82 ± 1.54*
18:1-18:2	1.35 ± 0.23	3.53 ± 0.76*

*, $P < 0.05$ compared to GFP.

Table 2. Cellular content of DAG species in mouse primary hepatocytes with GFP or DGKθ overexpression (pmol/mg protein).

DAG species	GFP	DGKθ
16:0-16:0	10.00 ± 0.93	0.46 ± 0.08*
16:0-18:0	-	-
16:0-18:1	12.20 ± 0.45	1.23 ± 0.20*
16:0-18:2	4.43 ± 0.58	0.81 ± 0.09*
18:0-18:1	-	-
18:0-18:2	-	-
18:1-18:1	8.52 ± 0.69	6.90 ± 1.02*
18:1-18:2	3.30 ± 0.72	1.70 ± 0.54*
18:2-18:2	-	-

*, $P < 0.05$ compared to GFP.

CHAPTER 5: DISCUSSION

5.1 Molecular mechanisms of insulin resistance

Many molecular mechanisms have been implicated in the development of insulin resistance, including inflammation, endoplasmic reticulum (ER) stress, mitochondrial dysfunction, and dysfunctional lipid metabolism.

One of the mechanisms that have been proposed to explain insulin resistance is inflammation, which arises because of the phosphorylation of serine residues of IRS1 upon exposure of cells to elevated levels of free fatty acids[13]. Inflammation has been understood to reduce the ability of IRS1 to associate with the IR and inhibit downstream signaling of the IR. The subsequent enzyme cascade is interrupted and propagation of the insulin signal is blocked.

Another mechanism that provides an understanding of insulin resistance is ER stress[14]. The ER is crucial in the synthesis and processing of proteins, but under certain stress conditions such as obesity and diabetes, the ER fails to fold and export proteins. ER stress leads to insulin resistance by activating abnormal phosphorylation of IRS1, which inhibits insulin receptor signaling[15]. ER stress also modifies the expression of proteins like Akt, which is involved in insulin signaling. ER stress ultimately disrupts insulin signaling, causing insulin resistance.

Mitochondrial dysfunction is also a mechanism associated with insulin resistance and is caused by an imbalance between energy intake and expenditure[16]. Oxidative stress can happen as a result of excessive intake of nutrients, causing mitochondrial dysfunction by damaging proteins, DNA, and lipids in membrane components. This increases intracellular fatty acid metabolites that disrupt insulin signaling.

5.2 Lipid metabolism and insulin resistance

The causes of insulin resistance remain controversial, but lipid intermediates have been implicated as playing important roles in modulating insulin signaling and contributing to insulin resistance. Previous studies have concluded that instead of the final product TAG causing insulin resistance, the defect could be caused by intermediates in the lipogenic pathway[8]. GPAT1 overexpression induces insulin resistance, revealing an association between the insulin signaling and lipogenic pathway[17]. In particular, GPAT1 overexpression leads to decreased pAkt. Thus, an important next step is to determine whether any of the lipid intermediates inhibits insulin signaling and the mechanism by which they function.

DAG has been reported as the link between lipid metabolism and insulin resistance because it can activate certain PKC isoforms, which could lead to serine or threonine phosphorylation of the IR or IRS1 and disrupt insulin signaling[10]. However, the relationship between DAG and insulin resistance remains unclear. In human skeletal muscle, lipid infusion reduces insulin sensitivity but does not alter intramyocellular DAG content[18]. Also, while diabetic patients have higher intramyocellular TAG content, they have similar DAG content as non-diabetic subjects[19]. These studies show that whether DAG contributes to insulin resistance remains unclear.

Another lipid intermediate is PA, which inhibits insulin signaling through a variety of ways. PA derived from GPAT or AGPAT inhibits insulin signaling to Akt by disrupting mTOR-rictor[8]. PA from another source, phospholipase D (PLD)-catalyzed phosphatidylcholine (PC) hydrolysis, also inhibits insulin signaling[20]. It activates mTORC1 and enhances IRS1 phosphorylation at serine sites. A third source of PA is DAG phosphorylation by DGK[11]. To investigate whether PA derived from DAG phosphorylation is involved in insulin resistance, we overexpressed DGKθ in mouse primary hepatocytes, and examined its effects on insulin signaling and the cellular PA and DAG content.

5.3 DGK

DGK is a family of enzymes that catalyzes the conversion of DAG to PA, using phosphate from ATP[11]. Ten mammalian isoforms of DGK have been discovered so far, and are further classified into five types based on their structures (Figure 6)[21]. Their distinct structures and subcellular localizations suggest that each isoform performs its own specific functions[22].

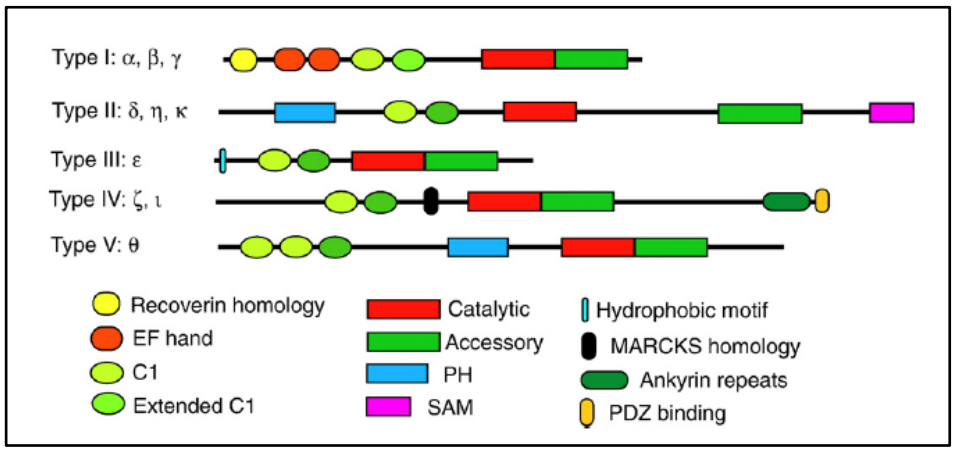


Figure 6. Structures of ten mammalian DGK isoforms classified into five types. DGKθ is the only type V isoform. (Topham and Epand, 2009)

The isoform we used in our studies was DGKθ. DGKθ is the only type V isoform and its structure begins with a proline-rich region (Figure 7)[23]. It has cysteine-rich domains (CRDs) and could possibly have a Ras-associating domain (RA), in addition to a pleckstrin homology (PH) domain, catalytic region, and two binding sites for SF-1 and RhoA[23]. DGKθ is a cytosolic protein whose activity is regulated by translocation to the nucleus[24] or plasma membrane[25]. Of the three DGK isoforms that are expressed in the liver – δ, η, and θ – DGKθ has the highest expression[26]. In addition to the liver, DGKθ is also expressed in the brain, intestine, and duodenum (Figure 8)[26, 27]. As the major isoform in the liver, DGKθ may play important roles in regulating hepatic insulin sensitivity and contribute to hepatic insulin resistance.

DGK occupies a pivotal position in cell signaling because it catalyzes the conversion of DAG to PA, thus decreasing intracellular DAG levels while increasing intracellular PA levels. In the current

study, we used DGK as a tool to manipulate DAG and PA content, in order to study whether the relative amounts of DAG and PA inhibit insulin signaling.

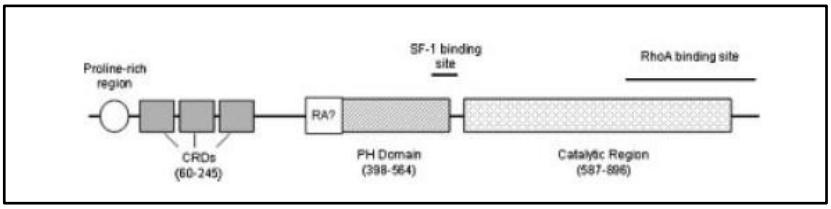


Figure 7. Structural diagram of DGKθ. The structure of DGKθ begins with a proline-rich region and cysteine-rich domains (CRDs). It could possibly have a Ras-associating domain (RA), in addition to a pleckstrin homology (PH) domain and catalytic region, as well as two binding sites for SF-1 and RhoA. (Tu-Sekine and Raben, 2009)

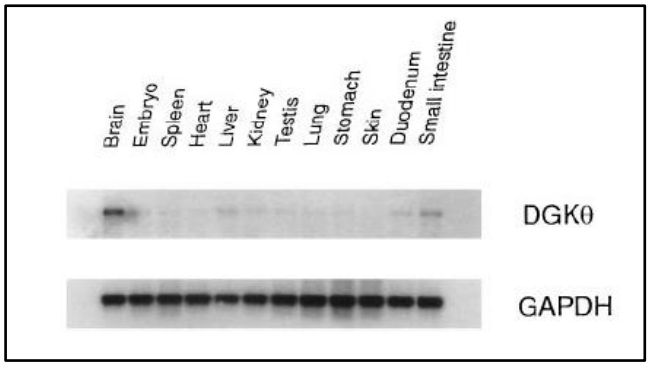


Figure 8. DGKθ mRNA expression in rat. DGKθ is most highly expressed in the brain, small intestine, and liver. (Houssa et al., 1997)

5.4 DGK-derived PA is associated with impaired insulin action, but DAG is not

The results of this study showed that PA is associated with impaired insulin action in mouse primary hepatocytes, but DAG is not. Our previous studies showed that overexpressing GPAT1 or AGPAT2 in mouse hepatocytes inhibited insulin signaling, accompanied by increased cellular content of both PA and DAG[8]. This suggested possible roles for both PA and DAG in disrupting insulin signaling. In the current study, overexpressing DGKθ inhibited insulin signaling in mouse hepatocytes, with an increased cellular content of total PA and 16:0-16:0 PA. DGKθ overexpression also decreased, rather than increased, total content of DAG. In summary, our results suggest that there is an association

between insulin signaling inhibition and the cellular PA content, but not the cellular DAG content, in mouse hepatocytes.

5.5 Limitations

A major limitation of this study is that only one DGK isoform was used, out of ten possible DGK isoforms. These isoforms all have different structures, subcellular localizations, and tissue distributions. Thus, while our conclusions may hold true for DGKθ, other DGK isoforms may have different effects on PA and DAG concentrations, and affect insulin signaling differently.

Another limitation is the lack of direct evidence to support the association between impaired insulin signaling and the cellular content of 16:0-16:0 PA. Several questions arise of whether palmitic acid supplementation to the culture medium will impair insulin signaling in the cultured hepatocytes, or whether palmitic acid supplementation to the diet will cause insulin resistance in mice.

In addition, hepatocytes were grown in William's Medium E (2.0 g/L glucose), which has a higher glucose concentration than normal physiologic fasting blood glucose levels of 0.7-1.0 g/L. However, William's Medium E has been recommended and widely used in hepatocyte culture for such experiments.

5.6 Future direction

Globalization has increased access to higher caloric diets and reduced physical activity. In combination with population growth, increased survival, and an aging population, this just means that the trend of T2D is likely to continue increasing worldwide. T2D is closely associated with insulin resistance, and thus many molecular mechanisms relating to insulin resistance have been implicated in the development of T2D. A major contributor to insulin resistance appears to arise from defects in the normal functioning of the insulin signaling pathway caused by lipid intermediates in the *de novo* lipogenic pathway.

In this work, we have explored further how the elevated content of lipid intermediates is associated with insulin resistance. By overexpressing DGKθ, we showed that insulin signaling was impaired, accompanied by an increase in cellular content of total PA and a concomitant decrease in cellular content of total DAG. These data suggest that PA, but not DAG, is associated with impaired insulin action in isolated mouse hepatocytes. However, the mechanism by which PA impairs insulin signaling is still not well understood. A possible future direction would be to elucidate the mechanism by which PA impairs insulin signaling. We have previously reported that GPAT1-derived PA disrupts mTOR-rictor assembly, thereby inhibiting insulin signaling. It will be important to answer the question of how PA, and in particular 16:0-16:0 PA, interacts with mTOR or with rictor to disrupt mTOR-rictor association, thus deepening our understanding of the lipid species that contribute to hepatic insulin resistance and revealing the mechanism by which these lipid intermediates cause hepatic insulin resistance.

REFERENCES

1. Adult Obesity Facts. August 2013. Available from: http://www.cdc.gov/obesity/data/adult.html (Accessed March 2014).
2. Childhood Overweight and Obesity. August 2013. Available from: http://www.cdc.gov/obesity/childhood/index.html (Accessed March 2014).
3. Economic costs of diabetes in the U.S. in 2012. Diabetes Care. 2013;36(4):1033-46.
4. Diabetes, August 2011. Available from: http://www.cdc.gov/chronicdisease/resources/publications/aag/ddt.htm (Accessed March 2014).
5. Pessin JE, Saltiel AR. Signaling pathways in insulin action: molecular targets of insulin resistance.
6. White MF. Insulin signaling in health and disease. Science. 2003;302(5651):1710-1.
7. Song G, Ouyang G, Bao S. The activation of Akt/PKB signaling pathway and cell survival. J Cellular Mol Med. 2005;9(1):59-71.
8. Zhang C, Wendel AA, Keogh MR, Harris TE, Chen J, Coleman RA. Glycerolipid signals alter mTOR complex 2 (mTORC2) to diminish insulin signaling. Proceedings of the National Academy of Sciences. 109(5):1667-1672.
9. Holland WL, Knotts TA, Chavez JA, Wang L, Hoehn KL, Summers SA. Lipid Mediators of Insulin Resistance. Nutrition Reviews. 2007;65:S39-S46.
10. Itani SI, Ruderman NB, Schmieder F, Boden G. Lipid-Induced Insulin Resistance in Human Muscle Is Associated With Changes in Diacylglycerol, Protein Kinase C, and I B- . Diabetes. 2002;51(7):2005-2011.
11. Mérida I, Avila-flores A, Merino E. Diacylglycerol kinases: at the hub of cell signalling. Biochem J. 2008;409(1):1-18.
12. Epand RM, Topham MK. Measurement of mammalian diacylglycerol kinase activity in vitro and in cells. Meth Enzymol. 2007;434:293-304.

13. Wellen KE, Hotamisligil GS. Inflammation, stress, and diabetes. J Clin Invest. 2005;115(5):1111-9.
14. Ozcan U, Cao Q, Yilmaz E, et al. Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes. Science. 2004;306(5695):457-61.
15. Nakatani Y, Kaneto H, Kawamori D, et al. Involvement of endoplasmic reticulum stress in insulin resistance and diabetes. J Biol Chem. 2005;280(1):847-51.
16. Kim JA, Wei Y, Sowers JR. Role of mitochondrial dysfunction in insulin resistance. Circ Res. 2008;102(4):401-14.
17. Nagle CA, An J, Shiota M, et al. Hepatic overexpression of glycerol-sn-3-phosphate acyltransferase 1 in rats causes insulin resistance. J Biol Chem. 2007;282(20):14807-15.
18. Høeg LD, Sjøberg KA, Jeppesen J, et al. Lipid-induced insulin resistance affects women less than men and is not accompanied by inflammation or impaired proximal insulin signaling. Diabetes. 2011;60(1):64-73.
19. Anastasiou CA, Kavouras SA, Lentzas Y, Gova A, Sidossis LS, Melidonis A. Diabetes mellitus is associated with increased intramyocellular triglyceride, but not diglyceride, content in obese humans. Metab Clin Exp. 2009;58(11):1636-42.
20. Fang Y, Vilella-bach M, Bachmann R, Flanigan A, Chen J. Phosphatidic acid-mediated mitogenic activation of mTOR signaling. Science. 2001;294(5548):1942-5.
21. Topham MK, Epand RM. Mammalian diacylglycerol kinases: molecular interactions and biological functions of selected isoforms. Biochim Biophys Acta. 2009;1790(6):416-24.
22. Sakane F, Imai S, Kai M, Yasuda S, Kanoh H. Diacylglycerol kinases: why so many of them?. Biochim Biophys Acta. 2007;1771(7):793-806.
23. Tu-sekine B, Raben DM. Regulation of DGK-theta. J Cell Physiol. 2009;220(3):548-52.
24. Tabellini G, Bortul R, Santi S, et al. Diacylglycerol kinase-theta is localized in the speckle domains of the nucleus. Exp Cell Res. 2003;287(1):143-54.

25. Walker AJ, Draeger A, Houssa B, Van blitterswijk WJ, Ohanian V, Ohanian J. Diacylglycerol kinase theta is translocated and phosphoinositide 3-kinase-dependently activated by noradrenaline but not angiotensin II in intact small arteries. Biochem J. 2001;353(Pt 1):129-137.
26. Houssa, B., Schaap, D., van der Wal, J., Goto, K., Kondo, H., Yamakawa, A., Shibata, M., Takenawa, T., and van Blitterswijk, W.J. Cloning of a novel human diacylglycerol kinase (DGKθ) containing three cysteine-rich domains, a proline-rich region, and a pleckstrin homology domain with an overlapping Ras-associating domain. J Biol Chem. 1997:272:10422-10428.
27. van Blitterswijk WJ, Houssa B. Properties and functions of diacylglycerol kinases. Cellular Signalling. 2000;12(9-10):595-605.