Diacylglycerol - PKC Epsilon - Insulin Receptor as a Key Regulatory Axis of Hepatic and White Adipose Tissue Insulin Signaling

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Abstract

Diacylglycerol - PKC Epsilon - Insulin Receptor as a Key Regulatory Axis of Hepatic and White Adipose Tissue Insulin Signaling

Kun Lyu

2021

Type 2 diabetes (T2D) is a chronic condition that currently affects 463 million people worldwide and is expected to affect 700 million people by the year of 2045. People with T2D suffer from many downstream conditions, such as cardiovascular diseases and stroke, vision loss, renal function impairment and amputations due to limb extremity damages, and are also much more susceptible to severe COVID-19 conditions. This devastating disease not only inflicts severe personal suffering on the individuals affected but also inflicts a huge economic burden on the society. For example, the annual cost of diagnosed diabetes in United States is estimated to be $327B in 2017. Therefore, understanding the mechanisms underlying the pathogenesis of T2D is of great importance, and effective therapeutic inventions can then be developed to remediate the situation.

Insulin resistance is the primary characteristic of T2D, where insulin fails to activate downstream signaling pathways, causing excessive glucose build-up in the circulation and tissues starved from critical nutrients. In the human body, liver serves as the center of numerous metabolic functions and is pivotal in regulating glucose homeostasis. Hepatic insulin resistance (HIR) significantly contributes to hyperglycemia via increased hepatic glucose output and decreased hepatic glucose storage. Understanding the mechanism of HIR is thus critical. Nonalcoholic fatty liver disease (NAFLD) is strongly associated with HIR, however, the key lipid species and molecular mechanisms linking these conditions are widely debated. We developed
a subcellular fractionation method to quantify diacylglycerol (DAG) stereoisomers and ceramides in the endoplasmic reticulum (ER), mitochondria, plasma membrane (PM), lipid droplets and cytosol. Acute knockdown (KD) of diacylglycerol acyltransferase-2 (DGAT2) in liver induced HIR in rats. This was due to PM sn-1,2-DAG accumulation, which promoted PKCε activation, and insulin receptor kinase (IRK)-T1160 phosphorylation resulting in decreased IRK-Y1162 phosphorylation. Liver PM sn-1,2-DAG content and IRK-T1160 phosphorylation were also higher in humans with HIR. In rats, liver-specific PKCε KD ameliorated high-fat diet (HFD)-induced HIR by lowering IRK-T1160 phosphorylation, while liver-specific overexpression of constitutively active PKCε-induced HIR by promoting IRK-T1160 phosphorylation.

White adipose tissue (WAT) is also of great regulatory importance in glucose metabolism. Insulin-mediated suppression of WAT lipolysis is an important anabolic function that is dysregulated in the state of overnutrition. However, the mechanism of short-term HFD-induced WAT insulin resistance is poorly understood. Based on our studies in the liver and preliminary studies in the WAT, we hypothesize that a short-term HFD causes WAT insulin resistance through a similar mechanism. Increases in PM sn-1,2-DAG content, which promotes PKCε activation, impairs insulin signaling by phosphorylating IRK-T1160. To test this hypothesis, we assessed WAT insulin action in 7-day HFD-fed versus regular chow diet-fed rats during a hyperinsulinemic-euglycemic clamp. HFD feeding caused WAT insulin resistance, reflected by reductions in both insulin-mediated WAT glucose uptake and suppression of WAT lipolysis. These changes were specifically associated with increased PM sn-1,2-DAG content, increased PKCε activation and impaired insulin-stimulated IRK-Y1162 phosphorylation. In order to examine the role of IRK-T1160 phosphorylation in mediating lipid-induced WAT insulin resistance, we examined these same parameters in short-term HFD-fed IRK\textsuperscript{T1150A} mice (IRK-T1150 is the mouse homolog of human IRK-T1160). Similar to the rat study, short-term HFD feeding induced WAT insulin resistance in WT control mice but failed to induce WAT insulin resistance in IRK\textsuperscript{T1150A} mice.
Taken together these data demonstrate that the PM \( \text{sn-1,2-DAG - PKC}_\varepsilon - \text{IRK-T1160} \) phosphorylation pathway plays an important role in mediating lipid-induced hepatic and WAT insulin resistance and represents a potential therapeutic target to improve insulin sensitivity in the liver and WAT.
Diacylglycerol - PKC Epsilon - Insulin Receptor as a Key Regulatory Axis of Hepatic and White Adipose Tissue Insulin Signaling

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Presented to the Faculty of the Graduate School

Of

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In Candidacy for the Degree of

Doctor of Philosophy

By

Kun Lyu

Dissertation Director: Gerald I. Shulman

Dissertation Committee Chair: David Zenisek

June 2021
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Chapter 1

Hepatic Insulin Signaling and Resistance
The postprandial rise in plasma insulin concentration activates hepatic insulin signaling pathways (Figure 1), which promote the storage of ingested carbohydrates into hepatic glycogen and suppress endogenous glucose production (EGP) to maintain normoglycemia within a relatively narrow range. Decreased hepatic insulin action disrupts these processes and contributes to fasting and postprandial hyperglycemia in type 2 diabetes (T2D) [1]. While the cellular and molecular mechanisms responsible for hepatic insulin resistance (HIR) are unresolved, ectopic lipid accumulation in the liver, leading to nonalcoholic fatty liver disease (NAFLD), is strongly associated with HIR and often precedes the development of T2D [2]. However, the key lipid species and molecular mechanisms by which ectopic lipids mediate HIR are widely debated, with pathways mediated by DAGs and ceramides positioned at the center of this controversy [3-5].

Diacylglycerols (DAGs), the penultimate intermediate in triglyceride synthesis, have been shown to be highly associated with hepatic steatosis and HIR in both rodents and humans [6-12]. In liver, DAGs have been proposed to induce HIR by activating PKCε [8, 13-15]. When activated, PKCε phosphorylates T1160, a key threonine residue on insulin receptor kinase (IRK), which is situated between two critical IRK activation sites, Y1158 and Y1162, to destabilize IRK’s activation loop [16]. However, there are still gaps in this mechanistic model and experimental support establishing the direct causal relationship between DAG accumulation and the development of HIR is lacking. Further, the role of hepatic PKCε and IRK-T1160 phosphorylation in regulating hepatic glucose metabolism has recently been challenged [17].

Among three DAG stereoisomers (sn-1,2-DAG, sn-2,3-DAG and sn-1,3-DAG), sn-1,2-DAG was shown to be the only stereoisomer that activates novel PKCs [18-20] and therefore is potentially the key DAG stereoisomer that is responsible for impeding insulin signaling. In addition, the subcellular distribution of DAGs is likely important in this regard. For example, in mouse models of hepatic CGI-58 KD and Hdac3 depletion, accumulation of lipid droplet DAGs are not associated with HIR [21, 22]. Therefore, it is essential to determine the associations between
subcellular DAG stereoisomer content, liver-specific PKCε activity, IRK-T1160 phosphorylation and hepatic insulin action in vivo.
Figure 1. Hepatic Insulin Signaling

The binding of insulin triggers insulin receptor’s activation through its dimerization and autophosphorylation on critical tyrosine residues. Activated insulin receptor recruits and activates a series of downstream effects to promote hepatic glycogen synthesis and suppress gluconeogenesis.
Chapter 2

A Membrane-Bound Diacylglycerol Species Induces PKCε-Mediated Hepatic Insulin Resistance
Introduction

To address the questions on the associations between subcellular diacylglycerol (DAG) stereoisomer content, liver-specific PKCɛ activity, insulin receptor kinase (IRK)-T1160 phosphorylation and hepatic insulin action in vivo, we developed a liquid-chromatography tandem mass spectrometry (LC-MS/MS) method to quantify DAG stereoisomers, as well as ceramides in five hepatocellular compartments: endoplasmic reticulum (ER), mitochondria, plasma membrane (PM), lipid droplet and cytosol. We then applied these methods in combination with in vivo assessment of hepatic insulin signaling (pIRK-T1160, pIRK-Y1162, pAkt-S473, pGSK3β-S9 and pFOXO1-S256 and endogenous glucose production (EGP), utilizing the hyperinsulinemic-glucose clamp technique, in awake rats with acute liver-specific diacylglycerol acyltransferase-2 (DGAT2) knockdown (KD) to acutely raise hepatic DAG content. Next, in order to determine if the results translate to humans, we quantified liver DAG stereoisomers and ceramides in the five subcellular compartments as well as pIRK-T1160 in humans with and without nonalcoholic fatty liver disease (NAFLD) and hepatic insulin resistance (HIR). Finally, in order to determine the role of PKCɛ in regulating hepatic insulin signaling, we assessed hepatic glycogen synthesis, EGP and hepatic insulin signaling in liver-specific PKCɛ KD rats and rats with liver-specific overexpression (OE) of constitutively activated PKCɛ. Using this comprehensive approach, we show that an accumulation of PM sn-1,2-DAG content is sufficient to cause HIR at the level of IRK, which in turn could be attributed to activation of PKCɛ and phosphorylation of IRK-T1160 in vivo. We further establish that liver PKCɛ is both necessary and sufficient in the development of lipid-induced HIR.
Results

Acute Hepatic DGAT2 KD Induces HIR

DGAT2 is the critical ER-located enzyme that catalyzes the esterification of DAGs with acyl-CoAs to produce triglyceride. We previously reported that chronic hepatic DGAT2 KD paradoxically reverses high-fat diet (HFD)-induced DAG accumulation and HIR, at least partially due to suppression of SREBP-1c-mediated lipogenesis [23]. However, we hypothesized that an acute DGAT2 KD might provide a narrow window in which DAGs transiently accumulate, allowing us to further delineate their association with PKCε activation and HIR. To test this hypothesis, we dosed regular chow-fed male Sprague Dawley rats with an antisense oligonucleotide (ASO) targeting DGAT2. Two days after the dosing, liver DGAT2 protein content was ~50% lower in acute hepatic DGAT2 KD rats compared to the control group and no KD effect was observed in white adipose tissue (WAT) (Figure 2A). During a hyperinsulinemic-euglycemic clamp (Figures 3A-3D), the acute hepatic DGAT2 KD impaired insulin’s ability to suppress EGP (Figure 2B). Acute hepatic DGAT2 KD impaired hepatic insulin signaling at the level of IRK. Insulin-stimulated IRK-Y1162 phosphorylation was >50% lower and was accompanied by impaired downstream insulin-stimulated Akt-S473, GSK3β-S9 and FOXO1-S256 phosphorylation in acute hepatic DGAT2 KD rats compared to the control group (Figure 2C). This defect in hepatic insulin signaling was associated with ~2-fold higher PKCε activation (Figure 2D). PKCε has been previously shown to phosphorylate IRK at T1160 to impair hepatic insulin signaling [16]. We used a phospho-specific monoclonal antibody to assay the phosphorylation of this residue in liver and observed ~75% higher pIRK-T1160 in acute hepatic DGAT2 KD rats compared to the control group (Figure 2E).
**Figure 2. Acute Hepatic DGAT2 KD Induces HIR**

(A) Hepatic and WAT DGAT2 protein content measured by western blot (top) and with its quantification (bottom).

(B) EGP and its suppression by insulin during a hyperinsulinemic-euglycemic clamp in Ctrl versus acute hepatic DGAT2 KD rats.

(C) Levels of insulin-stimulated liver pIRK-Y1162, pAkt-S473, pGSK3β-S9 and pFOXO1-S256 as measured by western blot (top) and with its quantification (bottom).

(D) Liver PKCε translocation from cytosol to membrane as measured by western blot (top) and with its quantification (bottom).

(E) Levels of liver pIRK-T1160 in Ctrl versus acute hepatic DGAT2 KD rats as measured by western blot (top) and with its quantification (bottom).

In all panels, data are the mean±S.E.M. In (A), (C) and (E), n = 6 per group. In (B), n = 8 per group. In (D), n = 5 or 6 per group. *p < 0.05, **p < 0.01 and ***p < 0.001.
**Figure 3. Hepatic DGAT2 KD Hyperinsulinemc-Euglycemic Clamp**

(A) Fasted body weight, fasting and clamp insulin levels, plasma glucose and glucose infusion rate during hyperinsulinemic-euglycemic clamp in Ctrl versus DGAT2 KD rats.

(B) Plasma NEFA and its suppression by insulin during clamp.

(C) Insulin-stimulated whole-body glucose turnover during clamp.

(D) Liver triglyceride content in Ctrl versus DGAT2 KD rats.

In all panels, data are the mean ± SEM of n = 8 per group.
Liver PM sn-1,2-DAG Content Tracks with HIR in Rats and Humans

Next to examine the associations of DAG stereoisomer content in different subcellular compartments with HIR, we separated liver tissues into ER, mitochondria, PM, lipid droplet and cytosol using differential centrifugation (Figures 4A and 5A) and measured their DAG stereoisomer content utilizing a novel LC-MS/MS method (Figure 4B). Acute hepatic DGAT2 KD caused the sn-1,2-DAG content to be ~50% higher in the ER and ~80% higher in the PM (Figure 4C). In contrast, there were no discernible differences observed for sn-2,3- and sn-1,3-DAGs (Figures 4D and 4E). We also observed slightly higher hepatic cytosolic C16:0, C22:0 and C24:0 ceramide content following acute DGAT2 KD, potentially due to substrate (acyl-CoA) accumulation (Figure 5B). In order to determine if these results would translate into humans, we also examined liver tissues from a cohort of human individuals who were either hepatic insulin-sensitive or hepatic insulin-resistant as previously characterized using hyperinsulinemic clamp methodology (Table 1) [6]. Consistent with the results in rodents, liver PM sn-1,2-DAG content was ~5-fold higher in individuals with HIR and NAFLD compared to individuals without HIR (Figure 4F), without discernible differences in ceramide content (Figure 5C). Furthermore, the higher PM sn-1,2-DAG content was associated with an ~3-fold higher level of pIRK-T1160 in individuals with HIR compared to individuals without HIR (Figure 5D).
Figure 4. Liver PM sn-1,2-DAG Content Tracks with HIR in Rats and Humans

(A) Separation of five subcellular compartments in liver measured by western blot.

(B) Representative chromatogram (n = 13) of DAG stereoisomer separation on LC-MS/MS.

(C) Liver sn-1,2-DAG, (D) liver sn-2,3-DAG, and (E) liver sn-1,3 DAG content in five subcellular compartments in Ctrl versus DGAT2 KD rats.

(F) Liver sn-1,2-DAG, (G) liver sn-2,3-DAG, and (H) liver sn-1,3-DAG content in five subcellular compartments in human individuals who were insulin-sensitive (black) or insulin-resistant (red).

In all panels, data are the mean±S.E.M. In (C), (D) and (E), n = 8 per group. In (F), (G) and (H), n = 4 per group. *p < 0.05, **p < 0.01 and ***p < 0.001.
Figure 5. Subcellular Content of Lipids in DGAT2 KD Rats and Human Subjects

(A) Liver DAG stereoisomer content measured with and without the fractionation method to determine the lipid content recovery of the fractionation method.

(B) and (C) Liver ceramide species content in five subcellular compartments.

(D) Human liver IRK-T1160 phosphorylation as measured by western blot (top) and with its quantification (bottom).

In all panels, data are the mean ± SEM. In (A), n = 3 per group. In (B), n = 8 per group. In (C), n = 4 per group. In (D), n = 5 per group. *p < 0.05 and **p < 0.01.
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**Table 1. Baseline Characteristics of Human Subjects**
Liver-Specific PKCε KD Ameliorates HFD- and Acute DGAT2 KD-Induced HIR

A recent study in liver-specific PKCε knockout mice challenged the notion that hepatic PKCε activation is required for the development of lipid-induced HIR [17], seemingly refuting a prior study using 2'-O-methoxyethyl (2'-MOE) ASO against PKCε in which the KD of both hepatic and WAT PKCε were associated with improvements in hepatic and WAT insulin action in awake rats [13]. To further examine the role of PKCε in regulating insulin action in the liver, we used a next-generation 2'-MOE ASO additionally modified with N-acetyl galactosamine (GalNAc). GalNAc modification facilitates ASO’s delivery into hepatocytes [24], permitting liver-specific PKCε KD in adult rats over the course of 3-week ASO dosing. MOE-GalNAc-modified PKCε ASO lowered liver PKCε protein content by ~70% without altering WAT PKCε protein content (Figure 6A).

Insulin action was then assessed in these rats after a 4-day HFD feeding which is a common model to induce acute hepatic steatosis and HIR. During a hyperinsulinemic-hyperglycemic clamp (Figures 7A-7F), liver-specific PKCε KD improved insulin’s ability to suppress EGP (Figure 6B).

We chose to use hyperinsulinemic-hyperglycemic clamp since both hyperinsulinemia and hyperglycemia are required for maximumly promoting hepatic glycogen synthesis [25], and therefore, it provides an optimal condition to detect potential differences in the rates of glycogen synthesis during the hyperinsulinemic clamp. Hepatic glycogen synthesis (specifically the direct pathway: glucose → glucose-6-phosphate → UDP glucose → glycogen) is the most robust in vivo flux indicator of direct insulin action on hepatic glucose metabolism, while the suppression of EGP is also influenced indirectly (i.e. independent of insulin signaling in liver) by insulin’s regulation of WAT lipolysis [2, 26]. Liver-specific PKCε KD led to an ~2-fold higher insulin-stimulated hepatic glycogen synthesis rate compared to the control group, resulting in higher post-clamp hepatic glycogen content (Figure 6C).

We then assayed key components of the insulin signaling pathway and found that in rats treated with the liver-specific PKCε ASO, the improved hepatic insulin sensitivity was associated
with ~3-fold higher insulin-stimulated pIRK-Y1162 (Figure 6D). This in turn was accompanied by significant improvements in downstream insulin-stimulated Akt-S473, GSK3β-S9 and FOXO1-S256 phosphorylation (Figures 6D and 6E). We also observed that there was markedly lower pIRK-T1160 levels with liver-specific PKCε KD compared to the control group (Figure 6F).

Furthermore, with the same 4-day HFD feeding, rats with liver-specific PKCε KD had lower plasma glucose and insulin concentrations during an oral glucose tolerance test (oGTT) despite similar body weights (Figure 6G), demonstrating that hepatic PKCε activation contributes to HFD-induced hyperglycemia and more prominently hyperinsulinemia during a physiological feeding behavior by directly mediating HIR. Additionally, in regular chow-fed rats, liver-specific PKCε KD abrogated acute DGAT2 KD’s induction of HIR during a hyperinsulinemic-hyperglycemic clamp (Figures 6H and 7G), indicating that hepatic PKCε is the necessary mediator of PM sn-1,2-DAG accumulation-induced HIR in the acute DGAT2 KD model. Liver-specific PKCε KD in regular chow-fed rats, however, did not further enhance hepatic insulin sensitivity (Figure 7H).

Thus, liver-specific KD of PKCε with an MOE-GalNAc-modified liver-targeted ASO for PKCε specifically ameliorated lipid-induced HIR in HFD-fed rats.
Figure 6. Liver-Specific PKCe KD Ameliorates HFD- and Acute DGAT2 KD-Induced HIR

(A) Hepatic and WAT PKCe protein content measured by western blot (top) and with its quantification (bottom).

(B) EGP and its suppression by insulin during a hyperinsulinemic-hyperglycemic clamp in Ctrl versus hepatic PKCe KD rats.

(C) Hepatic glycogen synthesis rate during a hyperinsulinemic-hyperglycemic clamp and post-clamp hepatic glycogen content in Ctrl versus hepatic PKCe KD rats.

(D) Levels of insulin-stimulated liver pIRK-Y1162, pAkt-S473 and (E) pGSK3β-S9 and pFOXO1-S256 as measured by western blot (top) and with its quantification (bottom).

(F) Levels of liver pIRK-T1160 as measured by western blot (top) and with its quantification (bottom) in Ctrl versus hepatic PKCe KD rats.

(G) Fasted body weight, plasma glucose and insulin levels during an oGTT in Ctrl versus hepatic PKCe KD rats.

(H) EGP, EGP’s suppression by insulin and hepatic glycogen synthesis rate during a hyperinsulinemic-hyperglycemic clamp and post-clamp hepatic glycogen content in Ctrl versus hepatic DGAT2 and PKCe KD rats.

In all panels, data are the mean±S.E.M. In (A), (D), (E) and (F), n = 6 per group. In (B), (C) and (H), n = 7 per group. In (G), n = 9 per group. *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001.
Figure 7. Liver-Specific PKCε KD Hyperinsulinemic-Hyperglycemic Clamp

(A) Fasted body weight, fasting and clamp insulin levels, plasma glucose and glucose infusion rate during clamp in HFD-fed Ctrl versus hepatic PKCε KD rats.

(B) Plasma NEFA and its suppression by insulin during clamp in HFD-fed Ctrl versus hepatic PKCε KD rats.

(C) Insulin-stimulated whole-body glucose turnover during clamp in HFD-fed Ctrl versus hepatic PKCε KD rats.

(D) Liver triglyceride content in HFD-fed Ctrl versus hepatic PKCε KD rats.

(E) Liver DAG stereoisomer content in five subcellular compartments in HFD-fed Ctrl versus hepatic PKCε KD rats.

(F) Liver ceramide species content in five subcellular compartments in HFD-fed Ctrl versus hepatic PKCε KD rats.

(G) Fasted body weight, fasting and clamp insulin levels, plasma glucose and glucose infusion rate during clamp in Ctrl versus hepatic DGAT2 + PKCε KD rats.

(H) EGP, EGP’s suppression by insulin and hepatic glycogen synthesis rate during a hyperinsulinemic-hyperglycemic clamp and post-clamp hepatic glycogen content, fasted body weight, fasting and clamp insulin levels, plasma glucose and glucose infusion rate during clamp in regular chow diet-fed Ctrl versus hepatic PKCε KD rats.

In all panels, data are the mean ± SEM. In (E) and (F), n = 6 per group. In all the other panels, n = 7 per group. *p < 0.05 and **p < 0.01.
Liver-Specific OE of Constitutively Active PKCε Induces HIR

Taken together, these data so far demonstrate that PKCε is necessary for the development of sn-1,2-DAG-mediated HIR. To determine whether PKCε activation per se is sufficient to cause HIR, we used an AAV vector to overexpress a constitutively active isoform of PKCε (PKCε-A159E) [27] driven by the liver-specific promoter, thyroxine binding globulin (TBG), in regular chow-fed rats. The A159E mutation prevents the binding of the pseudo-substrate domain to the catalytic domain, rendering the kinase constitutively active [27]. The AAV injection doubled the amount of total PKCε content in the liver with ~6-fold higher translocation without altering WAT PKCε content (Figure 8A). The higher hepatic content of constitutively active PKCε impaired insulin-stimulated hepatic glycogen synthesis by ~40% during a hyperinsulinemic-hyperglycemic clamp, resulting in lower post-clamp hepatic glycogen content (Figures 8B and 9A-9G). HIR was observed at the level of IRK, reflected by >50% lower insulin-stimulated pIRK-Y1162 levels, accompanied by impaired downstream insulin-stimulated Akt-S473, GSK3β-S9 and FOXO1-S256 phosphorylation in hepatic PKCε OE rats compared to the control group (Figures 8C and 8D). Furthermore, we also observed that there was ~2-fold higher pIRK-T1160 levels with liver-specific OE of constitutively active PKCε (Figure 8E).

Despite marked reductions in hepatic insulin signaling and hepatic glycogen synthesis, hepatic OE of PKCε-A159E did not affect EGP during the clamp (Figure 9E). This result is consistent with prior studies demonstrating a relatively minor role for direct hepatic insulin signaling in regulating hepatic glucose production in mice and rats without hepatic steatosis and the critical role of insulin to suppress WAT lipolysis to regulate hepatic glucose production by the indirect pathway [2, 11, 28, 29]. Consistent with this model, plasma non-esterified fatty acid (NEFA) concentrations were equally suppressed by >90% in both groups resulting in near complete suppression of EGP in both groups during the clamp due to the indirect pathway regulation (Figure 9B).
Taken together, these data support the sufficiency of hepatic PKCε in mediating HIR. Additionally, regular chow-fed rats with liver-specific OE of constitutively active PKCε had higher plasma glucose and insulin concentrations during an oGTT despite similar body weights (Figure 8F), demonstrating that hepatic PKCε activation per se is sufficient to drive hyperglycemia and more prominently hyperinsulinemia during a physiological feeding behavior by directly mediating HIR.
Figure 8. Liver-Specific OE of Constitutively Active PKCε Induces HIR

(A) Hepatic PKCε protein content, hepatic PKCε translocation from cytosol to membrane and WAT PKCε content measured by western blot (top) and its quantification (bottom).

(B) Hepatic glycogen synthesis rate during a hyperinsulinemic-hyperglycemic clamp and post-clamp liver glycogen content in Ctrl versus hepatic PKCε OE rats.

(C) and (D) Levels of insulin-stimulated liver pIRK-Y1162, pAkt-S473, pGSK3β-S9 and pFOXO1-S256 as measured by western blot (top) and with its quantification (bottom).

(E) Levels of liver pIRK-T1160 as measured by western blot (top) and with its quantification (bottom).

(F) Fasted body weight, plasma glucose and insulin levels during an oGTT in Ctrl versus hepatic PKCε OE rats.

In all panels, data are the mean±S.E.M. In (A), n = 5 or 7 per group. In (B), n = 8 per group. In (C), (D) and (E), n = 6 per group. In (F), n = 9 per group. *p < 0.05, **p < 0.01 and ***p < 0.001.
Figure 9. Liver-Specific OE of Constitutively Active PKCε Hyperinsulinemic-Hyperglycemic Clamp

(A) Fasted body weight, fasting and clamp insulin levels, plasma glucose and glucose infusion rate during clamp in Ctrl versus hepatic PKCε OE rats.

(B) Plasma NEFA and its suppression by insulin during clamp.

(C) Insulin-stimulated whole-body glucose turnover during clamp.

(D) Liver triglyceride in Ctrl versus hepatic PKCε OE rats.

(E) EGP and its suppression by insulin during clamp.

(F) Liver DAG stereoisomer content in five subcellular compartments in Ctrl versus hepatic OE rats.

(G) Liver ceramide species content in five subcellular compartments in Ctrl versus hepatic OE rats.

In all panels, data are the mean ± SEM. In (F) and (G), n = 6 per group. In all the other panels, n = 8 per group. *p < 0.05.
**Discussion**

NAFLD and HIR are both highly prevalent conditions that are strongly associated with each other, but the underlying molecular links between them remain widely debated. Multiple studies have reported the dissociation between hepatic triglyceride accumulation and HIR [12, 21, 22, 30-33], suggesting that triglycerides are likely not the mediating factor in causing HIR. Instead, among all the lipid species associated with NAFLD, DAGs and ceramides are the leading candidates thought to be involved [3-5, 28]. Since the mechanisms currently proposed for ceramides to induce hepatic insulin resistance target the distal insulin signaling steps (e.g. at the level of Akt) [3], they cannot explain the occurrence of proximal defects in hepatic insulin signaling at the level of the insulin receptor [13, 34].

We established the importance of PM sn-1,2-DAGs in the development of HIR using a model of acute DGAT2 KD. Since DGAT2 has been shown to be an ER-resident protein [35, 36], the accumulation of sn-1,2-DAGs in the ER following the acute hepatic DGAT2 KD is likely a direct result of transient substrate backup, while the extra DAGs are rapidly shuttled to other membrane compartments such as the PM, where they promote translocation of PKCε to the PM to induce insulin resistance.

Consistent with the rodent studies, we found that liver PM sn-1,2-DAG content and pIRK-T1160 levels also tracked with HIR in humans. The concomitant accumulation of PM sn-2,3-DAGs and sn-1,3-DAGs in the livers of individuals with HIR may simply reflect the condition of chronic excessive fatty acid supply, lipogenesis and/or lipolysis in the livers with NAFLD and likely does not contribute to the defects in hepatic insulin signaling. We also observed an accumulation of lipid droplet DAGs in liver samples from humans with HIR, consistent with our prior report where we saw a similar accumulation in lipid droplet DAGs in liver samples of humans with HIR using a relatively crude three-compartment subcellular fractionation method [8]. However, this likely reflects the concurrent expansion of the lipid droplet pool in fatty liver in subjects with obesity.
under chronic conditions of increased substrate flux to the liver. Consistent with the hypothesis, we have previously demonstrated that this pool of lipid droplet DAGs does not contribute directly to HIR as demonstrated in a mouse model where CGI-58 ASO treated mice, with a marked accumulation of hepatic lipid droplet DAG content, were protected from lipid-induced HIR and manifested reduced hepatic PKCε translocation [21]. In addition, in the study of Ter Horst et al. [6], higher cytosolic DAGs were detected in human livers with HIR. However, the high cytosolic DAG content reported likely reflects contamination from lipid droplet and membrane fractions, while our new fractionation methodology utilized in the present study has been our best attempt to resolve content of DAGs and ceramides in different subcellular compartments.

Our finding on the importance of PM sn-1,2-DAGs in mediating PKCε translocation, IRK-T1160 phosphorylation and HIR is consistent with studies demonstrating that only sn-1,2-DAGs activate novel PKCs [18-20], as well as that the PM is the primary site of action for PKCε to induce HIR [13, 16]. Since lipid droplet lipolysis has been shown to produce mostly sn-2,3- and sn-1,3-DAGs, which do not activate novel PKCs [37], our data could also provide an explanation for a few experimental models where CGI-58 and HSL (enzymes in lipolysis pathway) inhibition caused accumulation of DAGs (likely sn-2,3- and sn-1,3-DAGs) in the absence of PKCε activation and HIR [32, 38, 39].

In contrast to the consistent association between PM sn-1,2-DAGs and HIR, we observed no consistent association of any ceramide species in any subcellular compartment with HIR. This is consistent with prior studies that have dissociated hepatic ceramide content from HIR [7, 9, 40].

Multiple studies have suggested the involvement of PKCε in mediating lipid-induced HIR [8, 13-15], though many of the interventions used in these studies affected PKCε globally or in multiple tissues. In this regard, a recent study challenged the role of hepatic PKCε in regulating hepatic glucose metabolism [17]. Consistent with our prior study [13], Brandon et al. found that WAT PKCε has a role in the regulation of glucose metabolism. However, in contrast to the
conclusions of Brandon et al., we find that hepatic PKCε also has an important role in lipid-mediated HIR and hypothesize that the discrepancies between our study and their study may be due to multiple technical issues. Firstly, as hepatic glucose production receives regulatory input from multiple sources in addition to the liver (e.g. WAT and skeletal muscle), and that insulin’s acute effects to suppress hepatic glucose production reflects insulin’s ability to directly stimulate hepatic glycogen synthesis and indirectly inhibit hepatic gluconeogenesis through inhibition of WAT lipolysis [2, 11, 26, 28, 29], it is essential to assess insulin’s direct activation of hepatic insulin signaling parameters (e.g. IRK-Y1162, Akt-S473, GSK3β-S9 and FOXO1-S256 phosphorylation), as well as hepatic glycogen synthesis, to reliably assess the relatively subtle direct effects of insulin on hepatic glucose metabolism and HIR. Unfortunately, none of these parameters were assessed in the liver-specific PKCε knockout mice in the previous study [17].

Secondly, there may have been technical differences between the studies that preclude direct comparison. For example, the plasma glucose concentrations in some of the experiments of the previous study [17] were reported to be 12-13 mM for the basal state and increased to 30-35 mM during the GTTs. Despite this severe hyperglycemia, plasma insulin response curves during the GTTs were relatively flat without a physiological response to the extremely high plasma glucose concentrations, suggesting that these mice may have been stressed during the procedures, which in turn will mask any effects that PKCε may have on hepatic insulin action. Thirdly, the Cre-lox genetic knockout mouse model used in the previous study [17] may naturally produce different results compared to the short-term gene KD and OE rat models utilized in our studies due to potential developmental effects or strong compensatory effects in long-term genetic manipulation conditions. Finally, while it is possible that species differences might also explain the different observations, we do not think this is likely given that we and others have established a strong relationship between hepatic DAG content, PKCε translocation and HIR in mice [10, 41, 42] and most recently demonstrated that IRK-T1160A mice are protected from lipid-induced HIR [16].
In order to address these limitations, we performed a series of studies in short-term genetically modified awake rats. There are multiple advantages and strengths of the awake rat model compared to the awake mouse model including the fact that rates of EGP and insulin-stimulated whole-body glucose turnover in the awake rats are much closer to rates of EGP and turnover in humans than mice [2, 26], and compared to a life-long genetic knockout model, short-term genetic modulation eliminates potential confounding developmental compensatory effects on hepatic insulin action. Using this approach, our results support both the necessity and sufficiency of hepatic PKCε activation in mediating HIR (Figure 10).

Taken together with the fact that IRK-T1160 is highly conserved throughout evolution down to Drosophila melanogaster, we can hypothesize that the PM sn-1,2-DAG - PKCε - IRK-T1160 axis may be evolutionarily preserved to limit insulin’s effects on promoting hepatic glycogen synthesis and reducing EGP during re-feeding following fasting and starvation with the intention to preserve blood glucose in favor of supporting central nervous system function [2]. Furthermore, given the presence of PKCε in other insulin responsive tissues, such as skeletal muscle and WAT, it is likely that PM sn-1,2-DAG activation of PKCε may also be playing a similar role in causing lipid-induced insulin resistance in these tissues. Finally, given that PKCε has multiple targets besides IRK-T1160, it is likely that PKCε activation may also impact additional downstream targets to alter insulin action in these tissues [43].

Limitations of Study
The number of human liver samples from subjects with and without NAFLD and HIR were relatively small, and we were limited by the amount of tissue available from each biopsy. We therefore had to prioritize our analyses to yield the most novel information. We therefore prioritized these samples to assess DAG stereoisomers and ceramides in the five compartments in subjects with adequate liver tissues and subjected the rest of the tissue samples to an IRK-T1160
phosphorylation assay. Unfortunately, we did not have enough remaining tissues to assess PKC\(_{\varepsilon}\) translocation in these liver biopsies and future studies will be required to further address this relationship. However, we have previously established that increased PKC\(_{\varepsilon}\) translocation correlates with HIR in humans with NAFLD in the same cohort of human subjects [6].

Additionally, our study mostly investigated the importance of PM sn-1,2-DAGs and hepatic PKC\(_{\varepsilon}\) in relatively acute models of HIR. However, our data from human subjects suggest that the proposed model may also play an important role in long-term conditions of lipid-induced HIR associated with NAFLD.
Figure 10. The Working Model of Lipid-Induced Hepatic Insulin Resistance
# Methods

## Key Resources Table

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Software and Algorithms

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Experimental Model and Subject Details

Rat Models

All animal studies were approved by the Yale University Institutional Animal Care and Use Committee and were performed in accordance with all regulatory standards. 150-250 g male Sprague-Dawley rats at ~6-8 weeks of age were purchased from Charles River Laboratories (Wilmington, MA) and were housed in the Yale Animal Resources Center at 23°C under 12-hour light (7:00am-7:00pm)/12-hour dark cycles (7:00pm-7:00am). The rats were fed ad libitum with free access to water and were in good health condition. Upon arrival from the vendor, the rats remained on their regular living condition without any experimental perturbations for at least 3-4 days for acclimation and stress-minimization. The rats were group housed (2-3 per cage) until they underwent surgery under general isoflurane-induced anesthesia for insertion of polyethylene catheters in the common carotid artery (PE50 tubing, Instech Solomon, Plymouth Meeting, PA), the jugular vein (PE90 tubing, Instech) and the stomach (PE90 tubing, Instech) or injection of AAV solution into the portal vein, after which they were singly housed until time of killing. The rats were either fed with a regular chow diet (Harlan Teklad #2018, Indianapolis, IN) or a safflower oil-based
HFD with 60 kcal% from fat (Dyets #112245, Bethlehem, PA) for the amount of time as indicated in the main text. The rats were randomly allocated into experimental groups with matched body weight.

**Human Subjects**

Human subjects were recruited from the outpatient clinics of two obesity centers in the Amsterdam metropolitan area. Inclusion and exclusion criteria have been described [6]. Liver fat content was measured using proton magnetic resonance spectroscopy as described [6]. Insulin sensitivity was measured during a two-step hyperinsulinemic-euglycemic clamp using a stable isotope-labeled glucose tracer as described [6]. Liver biopsies were collected from the right liver lobe by an experienced surgeon during bariatric surgeries scheduled <2 weeks after clinical assessments. All human studies were approved by the Amsterdam University Medical Center medical ethics committee. The study was registered in the Netherlands Trial Register (NTR4666). All subjects provided written informed consent prior to inclusion. Please refer to Table S1 for the baseline characteristics of the human subjects included in this study.

**Method Details**

**Animal Studies**

At the end of all animal studies, rats were killed by intravenous pentobarbital sodium injection (150 mg/kg) with tissues and plasma collected for further analysis. Tissues were rapidly harvested and snap-frozen with clamps precooled in liquid nitrogen and then stored at -80°C for further analyses. For clamps, all tracers, insulin and glucose were infused via a catheter placed in the carotid artery at least one week before the study, and all blood collections were performed via a catheter placed in the jugular vein together with the arterial catheter.

For the DGAT2 ASO dosing, control and DGAT2 ASO (dissolved in PBS) were dosed once at the dose of 100 mg/kg via intraperitoneal (IP) injection two days before the clamp/tissue
collection studies. For the PKC\(_e\) ASO dosing, control and PKC\(_e\) ASO (dissolved in PBS) were dosed twice per week at the dose of 0.75 mg/kg via IP injection for three weeks prior to the clamp/oGTT/tissue collection studies. For the PKC\(_e\) A159E AAV dosing, control (AAV8-TBG-eGFP, dissolved in PBS with 5% glycerol) and PKC\(_e\) A159E AAV (AAV8-TBG-rPKCe A159E, dissolved in PBS with 5% glycerol) were dosed once at the dose of 2e13 GC/kg via portal vein injection four weeks prior to the clamp/oGTT/tissue collection studies.

Hyperinsulinemic-Glucose Clamps

For the hyperinsulinemic-euglycemic clamp, after 14-16 hours of overnight fasting, \([1,2,3,4,5,6,6-^2\text{H}_7]\text{glucose}\) was infused at the rate of 0.1 mg/[kg-min] for 2 hours during basal infusion (-120 min to 0 min) to measure basal glucose turnover rates. Blood samples were collected at -20 min, -10 min and 0 min to measure plasma glucose, insulin, NEFA concentrations and tracer atom percent excess (APE). Immediately after the basal infusion, insulin was infused at the rate of 4 mU/[kg-min] to achieve physiological hyperinsulinemia during clamp (0 min to 120 min). At the same time, 20% dextrose was infused at variable rates to maintain plasma glucose level at \(\sim 100-110 \text{ mg/dL}\). Infusion of \([1,2,3,4,5,6,6-^2\text{H}_7]\text{glucose}\) continued at the rate of 0.1 mg/[kg-min] during the clamp to measure insulin-stimulated glucose turnover rates. Blood samples were collected at 100 min, 110 min and 120 min to measure plasma glucose, insulin, NEFA concentrations and tracer APE. At the end of the clamp, rats were euthanized with tissues collected as described above.

For the hyperinsulinemic-hyperglycemic clamp, after 14-16 hours of overnight fasting, \([6,6-^2\text{H}_2]\text{glucose}\) was infused at the rate of 0.1 mg/[kg-min] for 2 hours during basal infusion (-120 min to 0 min) to measure basal glucose turnover rates. Blood samples were collected at -20 min, -10 min and 0 min to measure plasma glucose, insulin, NEFA concentrations and tracer APE. Immediately after the basal infusion, insulin was infused at the rate of 4 mU/[kg-min] to achieve physiological hyperinsulinemia during clamp (0 min to 90 min). A 1:1 mixture of somatostatin-14
and somatostatin-28 (w/w) was also infused at the rate of 4 μg/[kg-min] to suppress endogenous insulin secretion. At the same time, 20% dextrose labeled with 50% [13C₆]glucose was infused at variable rates to maintain plasma glucose level at ~150-160 mg/dL, measure rates of insulin-stimulated hepatic glycogen synthesis and glucose turnover. Blood samples were collected at 10 min, 20 min, 30 min, 40 min, 50 min, 60 min, 75 min and 90 min to measure plasma glucose, insulin, NEFA concentrations and tracer APE. At the end of the clamp, rats were euthanized with tissues collected as described above.

**Oral Glucose Tolerance Tests**

After 14-16 hours of overnight fasting, a single dose of 1 g/kg dextrose was injected via the gastric catheter placed at least one week before the study, and blood samples were collected at 0 min, 15 min, 30 min, 45 min, 60 min, 90 min and 120 min via the jugular vein catheter placed together with the gastric catheter to measure plasma glucose and insulin levels. At the end of the oral glucose tolerance tests, rats were euthanized with tissues collected as described above.

**Flux Measurements**

All turnover rates were calculated based on the following equation:

\[
\text{Turnover} = \left( \frac{\text{Tracer APE}}{\text{Plasma APE}} - 1 \right) \times \text{Infusion rate}
\]

APE designates the atom percent enrichment measured by gas chromatography-mass spectrometry (GC-MS). Briefly, glucose samples were deproteinized with 1:1 ZnSO₄:Ba(OH)₂, derivatized with 1:1 acetic anhydride:pyridine (v/v) at 65°C with methanol added afterwards to quench the reaction. Derivatized samples were then analyzed on GC-MS with CI mode to measure enrichment.
For the calculation of glycogen synthesis rates, liver glycogen m+6 APE ($X_m^{glycogen}$) and plasma glucose m+6 APE ($X_m^{glucose}$) were determined by GC-MS. Liver UDP-glucose m+6 APE ($X_m^{UDP-glucose}$) was determined by liquid chromatography-MS/MS (LC-MS/MS). Rates of liver glycogen synthesis direct pathway $V_{direct}$ were calculated based on the following equation:

$$V_{direct} = \frac{X_m^{glycogen} \cdot Glycogen_{total}}{\int_0^{90} X_m^{glucose} \, dt}$$

The fractional direct pathway contribution $D$ was calculated as:

$$D = \frac{X_m^{UDP-glucose}}{X_m^{plasma \, glucose, \, t = 90}}$$

Total glycogen synthesis rates $V_{total}$ were calculated as:

$$V_{total} = \frac{V_{direct}}{D}$$

To measure liver total glycogen, liver samples were first homogenized in 0.6 N perchloric acid (PCA). Part of the homogenate was then neutralized with 1M KHCO$_3$ and digested with amylloglucosidase (2 mg/mL) dissolved in acetate buffer (0.4 M, pH 4.8) for 2 hours at 37-40°C. Glucose levels were determined in samples before (free glycose) and after the amylloglucosidase digestion (total glucose) to calculate liver total glycogen content.

To measure liver glycogen m+6 APE, liver samples were ethanol-precipitated from PCA homogenates, dialyzed extensively against deionized water to remove free glucose before digested with amylloglucosidase. Digested glucose samples were then derivatized as described above prior to GC-MS analysis.

**Biochemical Analyses**

Plasma glucose was measured enzymatically using the YSI Glucose Analyzer (Yellow Springs, OH). Plasma insulin was measured by the Yale Diabetes Research Center Radioimmunoassay Core using radioimmunoassay. Plasma NEFA was measured using the enzymatic colorimetric
method by Wako reagents (Wako Diagnostics, Mountain View, CA). Liver triglyceride was first extracted with 2:1 chloroform:methanol (v/v). Samples were then mixed with sulfuric acid (1 M) and centrifugated to achieve phase separation. The organic phase was used to measure triglyceride content with the Sekisui triglyceride-SL reagent spectrophotometrically. Liver glycogen content measurement was described above.

**Tissue Analyses**

DAGs and ceramides were extracted from tissues with 2:1 chloroform:methanol (v/v) with 0.01% butylated hydroxytoluene. Known amounts of internal standards (1,2-dinonadecanoin for DAGs, N-heptadecanoyl-D-erythro-sphingosine for ceramides) were added during the extraction process. Afterwards, samples were dried down and re-dissolved in 95:5:0.5 hexane/methylene chloride/ethyl ether (v/v/v) before analyzed on LC/MS-MS as described below.

For western blots, tissues were homogenized in ice-cold homogenization buffer (20 mM Tris-HCl, pH 7.4, 5 mM EDTA, 0.25 mM EGTA, 10 mM Na₃P₂O₇, 1% Nonidet P-40, and protease and phosphatase inhibitor mixtures; Roche Diagnostics) and centrifuged at 13,000 g at 4 °C for 10 min to collect supernatant. Protein concentration was then determined by the Bradford method (Thermo Scientific). Protein samples were then diluted to 2 μg/μL with the addition of SDS buffer and β-mercaptoethanol and boiled for 5 min at 95°C. Afterwards, 20 μg of protein was loaded and resolved by SDS/PAGE using 4–12% gradient gels (Life Technologies) and transferred to polyvinylidene difluoride membranes (DuPont) using semi-dry transfer. Membranes were then blocked for 60 min at room temperature in 5% bovine serum albumin (BSA) and incubated overnight with primary antibody. After washing, membranes were incubated with horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology) for 1 hour. Detection was performed with enhanced chemiluminescence.

For assaying the IRK-T1160 phosphorylation, after protein concentration quantitation, protein samples were first immunoprecipitated by Dynabeads M-270 Epoxy (Invitrogen)
conjugated with D2 anti-IR alpha-subunit antibody. Primary antibody solution was diluted 1:100 – 1:200 for pIRK-T1160 detection.

**PKCe Translocation Assay**

Tissue samples were homogenized in ice-cold buffer A (20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.25 mM EGTA, 250 mM sucrose, and freshly added protease and phosphatase inhibitors, Roche Diagnostics). Debris was removed by centrifugation (5 min, 280 g, 4°C). Lysate was centrifuged (60 min, 100,000 g, 4°C), and an aliquot of the supernatant was saved as the cytosolic fraction. The pellet was washed once in ice-cold buffer B (250 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.25 mM EGTA, 2% Triton X-100, and freshly added protease and phosphatase inhibitors). The pellet was resuspended in buffer B by sonication, incubated at 4°C for 45 minutes to solubilize membrane proteins, and centrifuged (60 min, 100,000 g, 4°C). An aliquot of the supernatant was saved as the membrane fraction. The resulting protein samples were subjected to western blot analysis as described above. The ratio between membrane PKCe (normalized to Na-K ATPase intensity) to cytosolic PKCe (normalized to GAPDH intensity) was calculated as an index of PKCe translocation.

**Five-Compartment Ultracentrifugation Fractionation**

The protocol was adapted and modified based on previous studies [44, 45]. Fresh tissues (60-150 mg) were first homogenized in cold (4°C) TES buffer (250 mM sucrose, 10 mM Tris - pH 7.4, 0.5 mM EDTA) with a Dounce homogenizer. The homogenate was then centrifuged (at 12,000 rpm with SS-34 rotor or 17,000 g, 15 min, 4°C) to separate pellet A and supernatant A. The top lipid layer was collected as lipid droplet fraction. Pellet A was washed by being resuspended in TES buffer and centrifuged (at 12,000 rpm with SS-34 rotor or 17,000 g, 20 min, 4°C). Pellet A was then collected and resuspended in TES buffer and gently layered on top of 1.12 M sucrose
buffer cushion in ultracentrifuge tubes. The pellet A samples were then centrifuged (at 35,000 rpm with TLS-55 rotor or 105,000 g, 20 min, 4°C) to separate pellet B, interface B and supernatant B. The interface B was collected, resuspended in TES buffer and centrifuged (at 37,000 rpm with TLA-100.2 rotor or 60,000 g, 9 min, 4°C) to obtain pellet C. Pellet C was then washed by being resuspended in TES buffer and centrifuged (at 37,000 rpm with TLA-100.2 rotor or 60,000 g, 9 min, 4°C) and was designated as plasma membrane fraction. The pellet B was washed by being resuspended in TES buffer and centrifuged (at 12,000 rpm with SS-34 rotor or 17,000 g, 15 min, 4°C) and was collected as mitochondria fraction. The supernatant A was centrifuged (at 65,000 rpm with Ti-70.1 rotor or 390,000 g, 75 min, 4°C) to separate pellet D and supernatant D. Pellet D was washed by being resuspended in TES buffer and centrifuged (at 65,000 rpm with Ti-70.1 rotor or 390,000 g, 60 min, 4°C) and was collected as the endoplasmic reticulum fraction. Supernatant D was collected as the cytosol fraction.

**Diacylglycerol Stereoisomer Separation and Quantitation**

Chiral analysis of DAGs were performed by LC-MS/MS using electrospray ionization on an AB Sciex Qtrap 6500 interfaced to Shimadzu UFLC with 2 LC-20AD pumps (and degassers), SIL-20AC xR autosampler. Chromatographic separation was performed using Luna 5u Silica (100A, 250x2.0mm) and LUX 5u Cellulose-1 (250x4.6mm) columns connected in series with an isocratic solvent of hexane:isopropanol (300:7) with a flow rate of 0.6 mL/min. TAGs elute between ~4 to 8 minutes, and DAGs elute between ~11 and 17 minutes. For DAGs with the same FA composition, the order of elution was sn-1,3-DAG → sn-2,3-DAG → sn-1,2-DAG. For any specific stereoisomer (i.e., sn-1,2-DAG, sn-2,3-DAG, or sn-1,3-DAG) the order of elution generally increases with the degree of unsaturation. For example, C18:1 C18:1 → C18:1 C18:2 → C18:2 C18:2. Standards were used to establish retention times, matrix effects, and response relative to the internal standard (1,2-Dinonadecanoin). No matrix effects were detected in the five subcellular compartments. However, the position of the FAs on the DAGs have not been established. For
example, for the $sn$-1,2-DAG assigned as C18:0 C16:0, C16:0 could be on either the $sn$-1 or the $sn$-2 position. In the process of optimization, it was found that the use of cartridges (e.g., DiOH) or silica TLC plates to pre-separate the DAGs from TAGs results leads to intramolecular trans-esterification (e.g. the pre-separation of $sn$-1,2-DAGs will produce a mixture of $sn$-1,2-, $sn$-2,3-, and $sn$-1,3-DAGs). Thus, no additional separation steps following centrifugation to obtain cellular fraction were done prior to LC-MS/MS analysis.

**Generation of Rabbit-Mouse Chimeric Anti-plRK-T1160 Monoclonal Antibody**

All experimental procedures involving animals were performed under a license granted by the national Danish authority, The Animal Experiment Inspectorate. Rabbits were immunized four times with a 1:1 mixture of CYEpTDY and YEpTDYC conjugated to keyhole limpet hemocyanin via the terminal cysteines. All immunizations were performed subcutaneously using 50 µg of antigen in Ribi adjuvant. Blood was drawn ten days after the last immunization and used for sera tests. Splenic B cells were harvested from rabbits with best titers and single-cell sorted on a Sony SH800 cell sorter using FITC-labelled anti-rabbit IgG (Jackson ImmunoResearch) and Biotin-GGSGGSDIYEpTDYYRKG labelled with Streptavidin-PE (Jackson ImmunoResearch). Sytox red (Invitrogen) was included as a dead live stain. Sorted cells were expanded in vitro for 10 days on a layer of irradiated EL4-B5 cells, and the culture supernatant was harvested and used for ELISA screening. ELISA-positive clones were defined as binding the peptide used for immunization, but not binding the non-phosphorylated version of the same peptide. Hits were cloned as described in Lightwood et al. 2006 [46]. In short, the heavy/light chain variable regions were cloned using RT-PCR with gene specific primers and the amplicons inserted into an expression vector containing mouse IgG1/kappa constant regions. The construct was expressed in HEK cells, and the specificity of the chimeric rabbit/mouse antibody was validated by ELISA.

**Quantification and Statistical Analysis**
Most of our data follow normal distribution, and comparisons were performed using the unpaired 2-tailed Student’s t-test, with significance defined as a p value < 0.05. For data that do not follow normal distribution, non-parametric test was used to calculate the p value. GraphPad Prism 8.0 (San Diego, CA) was used for all statistical analysis. In most cases, n = 6-8 per group, unless otherwise indicated in the figure legends. Data are presented as the mean ± SEM.

All the tracer APE analyses (measured by GC-MS), DAG and ceramide content analyses (measured by LC-MS/MS) and insulin level assays were performed in a blinded fashion. For the animal studies, sample sizes were preselected using power calculation. Exclusion was applied only when the technical procedure failed (e.g. catheter failure causing inability to complete the clamp). Approximately 1 or 2 out of 10 samples in the animal studies were exclude based on this criterion.
Chapter 3

White Adipose Tissue Insulin Signaling and Resistance
White adipose tissue (WAT) insulin action promotes GLUT4-mediated WAT glucose uptake and suppresses lipolysis (Figure 11). Obesity-related metabolic diseases such as type 2 diabetes (T2D) and metabolic-associated fatty liver disease (MAFLD) are often accompanied by WAT dysfunction [2, 47]. One aspect of WAT dysfunction is WAT insulin resistance, which is partially characterized by insulin’s reduced ability to suppress lipolysis, resulting in higher rates of fatty acid delivery to liver and skeletal muscle [2, 48]. Ectopic lipid accumulation in insulin-responsive tissues such as liver and skeletal muscle leads to insulin resistance via the accumulation of diacylglycerol (DAG) in the plasma membrane (PM) and subsequent translocation and activation of novel PKCs (nPKCs) [1, 2, 13]. In liver, accumulation of sn-1,2-DAGs in the PM activates PKCε which then phosphorylates insulin receptor kinase (IRK) at T1160 to impair IRK’s activation and subsequent activation of downstream signaling events [2, 13, 16, 49]. This model can explain the development of lipid-induced liver and skeletal muscle insulin resistance in obese rodents and humans [1, 2, 13, 16, 49-52] as well as the mechanism by which weight loss [40], adiponectin [53], and liver-targeted mitochondrial uncouplers reverse insulin resistance in high-fat diet (HFD)-fed obese [9, 54] and lipodystrophic [10] insulin-resistant rodents.

Despite these insights into insulin resistance in other tissues, the pathogenesis of WAT insulin resistance remains unclear. Multiple factors have already been implicated, including low-grade inflammation, altered adipokine secretion and hypoxia [29, 55, 56]. However, further studies have suggested that WAT insulin resistance can develop early following overfeeding as a primary event. For example, overfed human subjects can develop peripheral insulin resistance prior to WAT immune cell infiltration [57, 58].
The binding of insulin triggers insulin receptor’s activation through its dimerization and autophosphorylation on critical tyrosine residues. Activated insulin receptor recruits and activates a series of downstream effects to promote GLUT4-mediated white adipose tissue glucose uptake and suppress lipolysis.

Figure 11. White Adipose Tissue Insulin Signaling
Chapter 4

Short-Term Overnutrition Induces White Adipose Tissue Insulin Resistance through \( sn-1,2- \) Diacylglycerol - PK\( \varepsilon \) - Insulin Receptor Kinase T1160 Phosphorylation
**Introduction**

Similar to its critical role in lipid-induced hepatic insulin resistance, PKC\(\varepsilon\) appears to be also important in the pathogenesis of white adipose tissue (WAT) insulin resistance. Silencing of both hepatic and WAT PKC\(\varepsilon\) with an antisense oligonucleotide improved WAT insulin action, reflected by increased insulin-stimulated WAT glucose uptake in high-fat diet (HFD)-fed rats [13]. Consistent with these results, Brandon and colleagues recently demonstrated improvement of glucose tolerance in HFD-fed WAT-specific PKC\(\varepsilon\) knockout mice [17]. However, they did not document any alterations in the WAT insulin signaling pathway or in insulin's suppression of WAT lipolysis in these mice [17]. Furthermore, there was no conclusion on whether the diacylglycerol (DAG) - PKC\(\varepsilon\) - insulin receptor kinase (IRK)-T1160 pathway was involved in WAT insulin action regulation or whether another distinct molecular pathway is regulated by PKC\(\varepsilon\) activation to impact glucose tolerance. In this study, we examine the hypothesis that the DAG - PKC\(\varepsilon\) - IRK-T1160 pathway serves as an early contributor to short-term HFD-induced WAT insulin resistance which hinders the ability of insulin to suppress WAT lipolysis and promote WAT glucose uptake.

In order to test this hypothesis, we first explored this pathway in male Sprague Dawley rats fed with HFD versus regular chow diet (RC) for 7 days, a time frame in which HFD feeding may induce WAT insulin resistance without causing WAT inflammation in rats [29]. We measured the content of DAG stereoisomers in five subcellular compartments – plasma membrane (PM), endoplasmic reticulum (ER), mitochondria (Mito), cytosol (C) and lipid droplet (LD), assayed the translocation of different PKCs and the activation of certain key steps in insulin signaling pathway. We further quantified the impact of WAT insulin resistance with a hyperinsulinemic-euglycemic clamp (HEC) study, using a combination of stable and radiolabeled isotope tracers to measure the alterations in whole-body and WAT fatty acid flux and glucose metabolism. Furthermore, to determine whether phosphorylation of IRK-T1160 is necessary for lipid-induced WAT insulin
resistance, we performed the same HEC with stable and radiolabeled isotope tracers in WT versus IRK\textsuperscript{T1150A} mice on HFD to assess their WAT insulin sensitivity using these same methods.

**Results**

**Seven-Day HFD Causes WAT Insulin Resistance**

We performed a HEC combined with $[^2\text{H}_7]$glucose, $[^2\text{H}_5]$glycerol and $[^{13}\text{C}_{16}]$palmitate infusions in male Sprague-Dawley rats fed with either RC or a 7-day HFD (Figures 12A and 12B). There was no significant difference in body weight, fasting plasma glucose and insulin concentrations between the RC and HFD group (Table 2). We quantified fatty acid and glycerol turnover in the basal (fasted) state and 30 min after a primed (25 mU/kg-min x 5 min), continuous (2.5 mU/kg-min) insulin infusion. This short HEC clamp was sufficient to establish a steady state in fatty acid and glycerol turnover (Figures 12C and 12D) and allowed us to study changes in insulin action and signaling mediating the suppression of WAT lipolysis. There were no significant differences in fasting non-esterified fatty acid (NEFA) concentrations or rates of whole-body lipolysis between the groups (Figures 12E, 13A, 13C and 13E). However, after 7-day HFD, the ability of insulin to suppress WAT lipolysis was impaired, and we observed less suppression of NEFA concentration (76±3% versus 54±6%, $p < 0.001$, Figures 13A and 13B). Consistent with these results, we found that insulin's suppression of whole-body glycerol turnover (46±5% versus 23±4%, $p < 0.01$, Figures 13C and 13D) and fatty acid turnover (37±2% versus 23±3%, $p < 0.001$, Figures 12E, 12F, 13E and 13F) during the HEC clamp were also impaired with 7-day HFD feeding. Further, we performed a 140-min HEC to quantify insulin-stimulated glucose uptake in WAT. Rats fed with a 7-day HFD exhibited a ~50% reduction in insulin-stimulated WAT glucose uptake (Figures 12G, 12H and 13G).

In summary, 7 days of HFD feeding caused WAT insulin resistance reflected by reductions in insulin-mediated suppression of WAT lipolysis and glucose uptake.
Table 2. Characteristics of the Rats in Hyperinsulinemic-Euglycemic Clamp

Data are presented as mean ± SEM of n = 8-10 per group, with comparisons by the 2-tailed unpaired Student’s t-test.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>RC</th>
<th>7-day HFD</th>
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<tbody>
<tr>
<td>Body weight (g)</td>
<td>309.0 ± 4.6</td>
<td>315.9 ± 4.5</td>
</tr>
<tr>
<td>Basal plasma glucose (mg/dL)</td>
<td>118.9 ± 4.80</td>
<td>113.0 ± 2.5</td>
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<tr>
<td>Basal plasma insulin (µU/mL)</td>
<td>7.3 ± 1.5</td>
<td>9.7 ± 1.3</td>
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<tr>
<td>Clamp plasma glucose (mg/dL)</td>
<td>112.6 ± 4.1</td>
<td>123.3 ± 5.0</td>
</tr>
<tr>
<td>Clamp plasma insulin (µU/mL)</td>
<td>75.7 ± 4.9</td>
<td>78.5 ± 7.4</td>
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</table>
Figure 12. Hyperinsulinemic-Euglycemic Clamp in RC- versus 7-Day HFD-Fed Rats
(A) and (B) Time course of plasma glucose and glucose infusion rates during a 30-min hyperinsulinemic-euglycemic clamp.
(C) and (D) Whole-body palmitate turnover and its suppression by insulin during the clamp.
(E) and (F) Time course of glycerol m+5 APE and palmitate m+16 APE during the clamp.
(G) and (H) Time course of plasma glucose and glucose infusion rates during a 140-min HEC.
In all panels, data are the mean ± SEM of n = 6-10 per group, with comparisons by 2-tailed unpaired Student’s t-test. *p < 0.05, **p < 0.01, ***p < 0.001.
Figure 13. Seven-Day HFD Causes WAT Insulin Resistance as Reflected by Reductions in WAT Glucose Uptake and Insulin’s Suppression of WAT Lipolysis

(A) Plasma NEFA under basal (overnight fasting) and hyperinsulinemic-euglycemic clamp conditions.

(B) Insulin’s suppression of plasma NEFA during the clamp.

(C) and (D) Whole-body glycerol turnover and its suppression by insulin during the hyperinsulinemic-euglycemic clamp.

(E) and (F) Whole-body fatty acid turnover and its suppression by insulin during the hyperinsulinemic-euglycemic clamp.

(G) Insulin-stimulated WAT glucose uptake.

In all panels, data are the mean ± SEM of n = 7-10 per group, with comparisons by 2-tailed unpaired Student's t-test. *p < 0.05, **p < 0.01, ***P < 0.001.
Seven-Day HFD Impairs Insulin-Stimulated Signaling Cascade Activation in WAT

We next explored potential mechanisms by which HFD impairs insulin’s suppression of WAT lipolysis by examining the components of the insulin signaling cascade that regulate the key lipolytic enzymes in epididymal WAT. Insulin-stimulated phosphorylation of both IRK and Akt were decreased in HFD-fed rats compared to the RC group (Figures 14A and 14B), suggesting that the defect in insulin action could be attributed to impaired IRK activation. The canonical pathway by which insulin suppresses WAT lipolysis is mainly through activation of phosphodiesterase 3B (PDE3B), which then degrades cyclic adenosine monophosphate (cAMP) to halt PKA-mediated phosphorylation of lipolytic enzymes, leading to decreased activity of HSL and reduced LD protection from perilipin. Thus, we measured insulin-stimulated PDE3B phosphorylation, cAMP level and PKA activity in WAT. Rats subjected to 7-day HFD exhibited decreased insulin-stimulated phosphorylation of PDE3B, and this was associated with higher cAMP levels and PKA activity in WAT (Figures 14C-14E). These changes were associated with increased phosphorylation of key lipolytic proteins - hormone-sensitive lipase (HSL) at S660, perilipin at S522, and adipose triglyceride lipase (ATGL) at S406 (Figures 14F-14H). Basal levels of these key WAT insulin signaling proteins were unchanged (Figure 15E).

In summary, 7 days of HFD-feeding impaired insulin signaling in WAT at the level of IRK, limiting the ability of insulin to suppress activity of the downstream proteins mediating WAT lipolysis.
Figure 14. Seven-Day HFD Feeding Impairs Insulin-Stimulated Signaling Cascade Activation in WAT and is Associated with Increases in Plasma Membrane sn-1,2-DAG Content and PKCε Translocation

(A), (B) and (C) Insulin-stimulated phosphorylation of IRK, Akt and PDE3B in WAT.

(D) and (E) WAT cAMP level and PKA activity during the hyperinsulinemic-euglycemic clamp.

(F), (G) and (H) Insulin-stimulated phosphorylation of HSL, perilipin and ATGL.

(I) WAT PKCε translocation as measured by its membrane / cytosol ratio.

(J) Separation of five subcellular compartments in WAT – plasma membrane (PM), mitochondria (Mito), endoplasmic reticulum (ER), cytosol (C) and lipid droplet (LD).

(K) WAT sn-1,2-DAG content in five subcellular compartments.

In (A)-(H) panels, samples (after overnight fasting) were under hyperinsulinemic-euglycemic clamp conditions. Data are the mean ± SEM of n = 5-10 per group. In (I)-(K) panels, samples were under 6-hr fasting basal condition, data are the mean ± SEM of n = 4-5 per group. In all panels, groups are compared by 2-tailed unpaired Student’s t-test. *p < 0.05, **p < 0.01, ***p < 0.001.
Figure 15. DAG Compartmental Profile in WAT of RC- versus 7-Day HFD-Fed Rats

(A) WAT total DAG proportion in five subcellular compartments.

(B) WAT total DAG content in five subcellular compartments.

(C) WAT sn-2,3-DAG content in five subcellular compartments.

(D) WAT sn-1,3-DAG content in five subcellular compartments.

(E) WAT protein content in basal condition.

In all panels, samples were under basal condition (6-hr fasting), data are the mean ± SEM of n = 5 per group, with comparisons by 2-tailed unpaired Student’s t-test.
### Table 3. WAT sn-1,2-DAG Individual Species Content in RC- versus 7-Day HFD-Fed Rats

Data are presented as mean ± SEM of n = 4-5 per group, with comparisons by the 2-tailed unpaired Student's t-test.
Seven-Day HFD Increases WAT PM sn-1,2-DAG Content and PKCε Translocation

Both HFD-induced WAT insulin resistance and hepatic insulin resistance have an insulin signaling defect at the level of IRK activation. In liver, impaired IRK activation is attributed to activation of PKCε, and we hypothesized that a short-term HFD may also lead to activation of PKCε in WAT. As there is little data on the role of PKCs in regulating WAT physiology, we first assayed the activation of certain major PKC isoforms. Specifically, we measured translocation (from cytosol to membrane, an index of activation) of both conventional and novel PKC isoforms in WAT in RC versus 7-day HFD-fed rats. The translocation of PKCε, as reflected by its membrane / cytosol ratio, increased by ~two-fold in HFD-fed rats compared to the RC-fed group (Figure 14I). In contrast, the membrane translocation of other PKC isoforms including α, β, θ and δ were unaltered by the 7-day HFD feeding (Figures 16A-16D). PKCs are activated by DAGs, specifically, sn-1,2-DAGs [18-20]. DAGs are present in multiple subcellular compartments, such as the ER, Mito, PM, LD and cytosol. Thus, we developed an assay to separate five subcellular compartments in WAT (Figure 14J). Next, we measured the concentration of DAG stereoisomers in each fraction. As expected, approximately 90% of total DAG were located in the lipid droplet fraction (Figure 15A). There were no differences in total DAG and sn-2,3-, sn-1,3-DAG concentrations between the groups (Figure 15B-15D). However, we observed an ~two-fold increase of sn-1,2-DAG content in the PM compartment (Figure 14K, Table 3). This was specifically associated with WAT PKCε membrane translocation (Figure 14I) but not translocation of other isoforms (Figures 16A-16D). These changes occurred without evidence of WAT inflammation. Expression of genes associated with WAT inflammation and hypoxia were unchanged (Figure 16E).

Taken together, these data suggest that activation of the PKCε pathway, by a short-term HFD, is triggered by accumulation of PM-associated sn-1,2-DAGs and that the sn-1,2-DAG - PKCε pathway could be the primary driver of impaired WAT insulin signaling in short-term overnutrition.
Figure 16. Translocation of PKCs and Expression of Genes Related to Inflammation and Hypoxia in WAT of RC- versus 7-Day HFD-Fed Rats

(A) WAT PKCα translocation as measured by its membrane / cytosol ratio.

(B) WAT PKCβ translocation as measured by its membrane / cytosol ratio.

(C) WAT PKCδ translocation as measured by its membrane / cytosol ratio.

(D) WAT PKCε translocation as measured by its membrane / cytosol ratio.

(E) Expression of WAT genes associated with inflammation and hypoxia.

In all panels, rats were under basal condition (6-hr fasting), data are the mean ± SEM of n = 4-6 per group, with comparisons by 2-tailed unpaired Student’s t-test.
**IRK^{T1150A} Mice Are Protected from A 7-Day HFD-Induced WAT Insulin Resistance**

We had previously identified IRK-T1160 (human homolog of mouse IRK-T1150) as a specific residue that is phosphorylated by PKCε in the liver [16]. Phosphorylation of this residue decreases the tyrosine kinase activity of IRK and its activation of downstream signaling events. Mutation of this residue from a threonine to an alanine (i.e. IRK^{T1150A}) shields IRK from this pathogenic phosphorylation and preserves hepatic insulin signaling and hepatic insulin sensitivity in HFD-fed mice [16]. In our previous study we did not observe any alterations in WAT insulin action in HFD-fed IRK^{T1150A} mice [16]. However, those assessments of WAT metabolism were performed during the final stages of a 140-min hyperinsulinemic-euglycemic clamp with an insulin infusion rate of 2.5 mU/(kg-min). Suppression of WAT lipolysis occurs rapidly after the onset of hyperinsulinemia [29], and the degree of WAT insulin resistance after just several days of HFD feeding is subtle and can be surmounted with high plasma insulin concentrations. Thus, any differences in WAT lipolysis may have been obscured in our previous studies involving the IRK^{T1150A} mice.

In order to address this possibility, we performed a much shorter 30-min hyperinsulinemic-euglycemic clamp study with a lower-dose insulin infusion rate [2.0 mU/(kg-min)] to evaluate WAT insulin action in IRK^{T1150A} mice subjected to 7-day HFD (Figures 17A-17E). As observed previously, there were no significant differences in body composition, overnight fasting plasma glucose, insulin and NEFA concentrations as well as whole-body rates of WAT lipolysis between the WT and IRK^{T1150A} group (Table 4, Figures 17A-17F, 18A, 18C and 18E). Nevertheless, IRK^{T1150A} mice retained the ability of insulin to suppress WAT lipolysis as reflected by lower plasma NEFA concentration, whole-body glycerol turnover and fatty acid turnover (Figures 17G and 18A-18F) during the hyperinsulinemic-euglycemic clamp. In addition, WAT insulin signaling was preserved in IRK^{T1150A} mice, reflected by higher insulin-stimulated IRK-Y1162 phosphorylation and Akt-S473 phosphorylation compared to HFD-fed WT mice (Figures 19A and 19B). We also examined the impact of 7-day HFD feeding on the downstream proteins that regulate WAT lipolysis. In contrast to the WT mice subjected to 7-day HFD feeding, IRK^{T1150A} mice displayed
increased PDE3B activity, which subsequently resulted in lower cAMP levels and thereby lower PKA activity (Figures 19C-19E). Consequently, phosphorylation of HSL, perilipin and ATGL is lower in IRK\textsuperscript{T1150A} mice (Figures 19F-19H), consistent with the preservation of insulin-mediated suppression of WAT lipolysis. These data demonstrate that phosphorylation of IRK-T1160 is required for the development of WAT insulin resistance after a 7-day HFD. Taken together with our prior studies [9, 10, 13, 16, 40, 49-54], these findings suggest that lipid-induced liver, muscle and WAT insulin resistance develop as a consequence of a common pathway involving increases in plasma membrane sn-1,2-DAG content leading to PKCe activation and the consequent impairment of IRK activation due to increased IRK-T1160 phosphorylation.

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<td>Body weight (g)</td>
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<td>23.6 ± 0.4</td>
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<tr>
<td>Fat mass (g)</td>
<td>2.7 ± 0.4</td>
<td>2.4 ± 0.2</td>
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<tr>
<td>Basal plasma glucose (mg/dL)</td>
<td>128.5 ± 11.9</td>
<td>126.8 ± 4.9</td>
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<tr>
<td>Basal plasma insulin (µU/mL)</td>
<td>8.5 ± 0.6</td>
<td>8.3 ± 0.9</td>
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<tr>
<td>Clamp plasma glucose (mg/dL)</td>
<td>98 ± 7.4</td>
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<tr>
<td>Clamp plasma insulin (µU/mL)</td>
<td>36.1 ± 4.7</td>
<td>38.9 ± 4.2</td>
</tr>
</tbody>
</table>

Table 4. Characteristics of WT versus IRK\textsuperscript{T1150A} Mice in Hyperinsulinemic-Euglycemic Clamp

Data are presented as mean ± SEM of n = 6 per group, with comparisons by the 2-tailed unpaired Student’s t-test.
Figure A: Plasma glucose (mg/dL) over time (min) for WT and IRK^{T1150A}.

Figure B: Glucose infusion rate [mg (kg.min)^{-1}] over time (min) for WT and IRK^{T1150A}.

Figure C: Body weight (g) comparison between WT and IRK^{T1150A}.

Figure D: Fat mass (g) comparison between WT and IRK^{T1150A}.

Figure E: Plasma insulin (mU/mL) at basal and clamp conditions for WT and IRK^{T1150A}.

Figure F: Palmitate turnover [nmol (kg.min)^{-1}] at basal and clamp conditions for WT and IRK^{T1150A}.

Figure G: Suppression of palmitate turnover (%/basal) for WT and IRK^{T1150A}.
**Figure 17. Hyperinsulinemic-Euglycemic Clamp in HFD-Fed WT versus IRK^{T1158A} Mice**

(A) and (B) Time course of plasma glucose and glucose infusion rates.

(C) and (D) Body weight and fat mass.

(E) Plasma insulin concentrations under basal and clamp conditions.

(F) and (G) Whole-body palmitate turnover and its suppression by insulin during the hyperinsulinemic-euglycemic clamp.

In all panels, data are the mean ± SEM of n = 6 per group, with comparisons by 2-tailed unpaired Student’s t-test. *p < 0.05.
Figure 18. IRK$^{T1150A}$ Mice Retain Insulin’s Ability to Suppress WAT Lipolysis After 7-Day HFD

(A) Plasma NEFA under basal (overnight fasting) and hyperinsulinemic-euglycemic clamp conditions.
(B) Insulin’s suppression of plasma NEFA during the hyperinsulinemic-euglycemic clamp.
(C) and (D) Whole-body glycerol turnover and its suppression by insulin during the hyperinsulinemic-euglycemic clamp.
(E) and (F) Whole-body fatty acid turnover and its suppression by insulin during the hyperinsulinemic-euglycemic clamp.

In all panels, data are the mean ± SEM of n = 5-6 per group, with comparisons by 2-tailed unpaired Student’s t-test. *p < 0.05, **p < 0.01, ***p < 0.001.
Figure 19. IRK^{T1150A} Mice Are Protected from HFD-Induced WAT Insulin Resistance

(A)-(C) Insulin-stimulated phosphorylation of IRK, Akt and PDE3B in WAT.

(D) and (E) WAT cAMP level and PKA activity during the clamp.

(F)-(H) Insulin-stimulated phosphorylation of HSL, perilipin and ATGL.

In all panels, mice (after overnight fasting) were under hyperinsulinemic-euglycemic clamp condition, data are the mean ± SEM of n = 6 per group, with comparisons by 2-tailed unpaired Student’s t-test. *p < 0.05, **p < 0.01, ***p < 0.001.
Discussion

Appropriate energy storage and release in healthy WAT is critical for survival under calorie scarce conditions as well as for proper nutrient distribution during feeding. Under conditions of overnutrition this process is dysregulated, especially in individuals with inherited predisposition to restrained adipocyte capacity [59]. Insulin regulates energy storage in WAT, in part by suppressing lipolysis. Dysregulated WAT lipolysis (along with a decreased capacity for adipocyte expansion) could promote ectopic lipid accumulation and, consequently, insulin resistance in liver and skeletal muscle [1, 2, 60-62]. Increased WAT lipolysis will also increase hepatic gluconeogenesis by providing increased delivery of glycerol and NEFA to the liver, which in turn will both promote increased gluconeogenesis [2, 29]. As such, insulin’s regulation of WAT lipolysis serves as a key component in the network of inter-organ communication and regulation of hepatic glucose production [2, 29]. Defects in insulin’s suppression of WAT lipolysis may occur early during the transition from insulin-sensitive to insulin-resistant, with defects detectable as early as 7-10 days of HFD feeding in rodents [21]. This early defect precedes other well-characterized changes in the adipocytes (i.e. inflammation, hypoxia, necrosis, etc.) which will also promote dysregulated WAT metabolism and increased lipolysis. The data presented here now provide a mechanistic underpinning for this phenomenon. Specifically, the PM sn-1,2-DAG - PKCε - IRK-T1160 pathway that is responsible for lipid-induce hepatic insulin resistance, which occurs in nonalcoholic fatty liver disease (NAFLD), may also account for lipid-induced WAT insulin resistance in the early stage of overnutrition.

Firstly, impaired WAT insulin signaling occurs rapidly during HFD feeding. We detected WAT insulin resistance after only 7 days of HFD feeding with impaired insulin-mediated WAT glucose uptake and suppression of WAT lipolysis, which was accompanied by impaired activation of key insulin signaling steps initiating at the level of IRK tyrosine auto-phosphorylation. Importantly, this subtle development of WAT insulin resistance manifests earlier than other
alterations such as inflammation and hypoxia in epididymal WAT, which may predispose WAT to more severe metabolic disturbances with prolonged HFD feeding.

WAT insulin resistance can be attributed to a proximal defect in insulin signaling at the level of IRK which ultimately impacts the regulation of key lipolytic enzymes. The defect in IRK activation leads to impaired Akt and PDE3B phosphorylation. As a consequence, the decrease in PDE3B activity permits higher levels of cAMP which then promotes PKA activity. PKA directly phosphorylates and activates HSL [63, 64]. PKA also phosphorylates perilipin, which promotes the release of CGI-58 (a key co-effector of ATGL) and the recruitment of HSL to LDs [65]. Some have proposed that insulin may regulate WAT lipolysis in an Akt-independent pathway. Choi et al. demonstrated a noncanonical Akt-independent, phosphoinositide-3 kinase-dependent pathway regulating WAT lipolysis by selectively altering PKA targets perilipin and HSL [66]. This pathway would also be impacted by the proximal defect in IRK activation and thus is consistent with our proposed model of WAT insulin resistance.

Short-term HFD feeding increases the content of sn-1,2-DAGs in the PM of adipocytes. Though an adipocyte is mainly comprised of a massive lipid droplet, important signaling lipids are also present in other subcellular compartments. Here we assessed the content of DAG stereoisomers in five WAT subcellular compartments. As expected, LD was the largest reservoir of DAGs accounting for ~90% of total DAGs. However, in epididymal WAT, there was no difference in LD sn-1,2-DAG content following short-term HFD feeding. In contrast, the PM DAGs account for only ~1% of total DAGs in WAT, but HFD feeding caused an approximately two-fold increase in PM sn-1,2-DAG content.

sn-1,2-DAGs are the primary DAG product of the re-esterification pathway, while previous studies demonstrated that sn-2,3- and sn-1,3-DAGs are primarily generated through the lipolytic pathway [37]. Therefore, a short-term HFD condition will likely promote more accumulation of sn-1,2-DAGs due to the increased flux of fatty acids into the re-esterification pathway. As for the compartment specificity, it's likely that in short-term HFD (e.g. 7-day), the other membrane
compartments – ER and mitochondria have the ability to maintain a relatively steady pool of lipids, due to their relatively larger baseline lipid content and/or higher lipid handling capacity (transport or oxidation), while PM has a relatively small baseline lipid content and therefore is more prone to relatively large fold changes in sn-1,2-DAGs. In LDs, DAGs mostly originate from lipolysis, and since basal rates of lipolysis do not change under this condition, DAG content in LDs is not expected to change very much. However, in long-term (e.g. chronic HFD) conditions, DAG content will likely increase in LDs and potentially other subcellular compartments.

The two-fold increase in PM sn-1,2-DAGs was associated with an approximately two-fold increase in PKCε translocation which has previously been implicated to play a role in WAT insulin action. PKCε antisense oligonucleotide treatment improved insulin-stimulated WAT glucose uptake in 3-day HFD-fed rats [13]. Consistent with these results, Brandon et al. found that WAT-specific PKCε knockout mice displayed improved glucose tolerance on HFD, indicating that WAT PKCε activation may be an essential step in the development of WAT insulin resistance [17]. However, there are some notable differences between the study of Brandon et al. and the present work. Brandon et al. reported that WAT-specific deletion of PKCε improved glucose tolerance in chronically HFD-fed mice, but they did not detect alterations in insulin’s regulation of WAT lipolysis or insulin-stimulated glucose uptake in WAT explant after 1-week HFD. They also did not detect differences in plasma fatty acid concentrations during a glucose tolerance test. In contrast, our relatively low-dose hyperinsulinemic-euglycemic clamp studies, combined with stable isotopic measurements of lipolytic rates, provided us with a more sensitive approach to detect differences in insulin’s regulation of WAT lipolysis in vivo.

We have previously identified IRK-T1160 as a specific target phosphorylated by PKCε, leading to inhibition of IRK activity in the liver [16]. Phosphorylation of the T1160 residue in IRK activation loop is predicted to destabilize its active configuration, thereby inhibiting IRK activity. As such, IRK^{T1150A} mice are protected from HFD-induced hepatic insulin resistance [16]. WAT is
exquisitely sensitive to the antilipolytic effects of insulin [67] and thus we were most likely unable to detect differences in WAT insulin action due to the saturating dose of insulin administered in our prior hyperinsulinemic-euglycemic clamp studies in the IRK\textsuperscript{T1150A} mice. In order to address this issue, we used a lower insulin infusion rate [2.0 mU/(kg-min) versus 2.5 mU/(kg-min)] for a shorter time (30 min versus 140 min) in the current study to better assess WAT insulin action in WT versus IRK\textsuperscript{T1150A} mice. Under this new experimental condition, we demonstrated that IRK\textsuperscript{T1150A} mice were protected from HFD-induced WAT insulin resistance. WAT insulin signaling was preserved at the level of IRK activity down through Akt phosphorylation, thereby preserving insulin’s ability to suppress phosphorylation of perilipin, HSL and ATGL through regulating PDE3B activity and cAMP content. In summary, we demonstrated that PKC\textepsilon-mediated IRK-T1160 phosphorylation mediates lipid-induced WAT insulin resistance by inhibiting IRK activity. Taken together, these data demonstrate that PM sn-1,2-DAG - PKC\textepsilon - IRK-T1160 phosphorylation is a critical pathway in the pathogenesis of short-term HFD-induced WAT insulin resistance (Figure 20). Alterations in this pathway may occur during the early stage of WAT lipid over-influx, resulting in impaired insulin’s regulation of WAT lipolysis and glucose uptake. Though the defect in WAT is subtle, it may be a necessary predisposition to more severe WAT dysfunction as well as for ectopic lipid deposition and insulin resistance in liver and skeletal muscle in more prolonged states of overnutrition.

Furthermore, given that PKC\textepsilon has many additional targets in addition to IRK-T1160, it is likely that PM sn-1,2-DAG induced PKC\textepsilon activation will impact many additional targets that affect WAT lipid and glucose metabolism independent of changes in insulin receptor kinase activity [43]. Thus, longer-term studies are needed to investigate if eliminating WAT insulin resistance could ameliorate other metabolic disturbances and whether these results translate to humans under conditions of short-term overnutrition and obesity. Moreover, these data identify the PM sn-1,2-
DAG - PKC\textsubscript{e} - IRK-T1160 phosphorylation pathway as a new potential therapeutic target to treat metabolic dysfunctions that are associated with WAT insulin resistance.
Figure 20. The Working Model of Lipid-Induced White Adipose Tissue Insulin Resistance
Methods

Animals

All animal studies were approved by the Yale University Institutional Animal Care and Use Committee and were performed in accordance with all regulatory standards. Male Sprague-Dawley rats weighing approximately 250g were obtained from Charles River Laboratories (Wilmington, MA) and were maintained on a 12-hour light/12-hour dark cycle. After 1 week of acclimation, rats underwent surgery for placement of polyethylene catheters in the common carotid artery (PE50 tubing, Instech Solomon, Plymouth Meeting, PA) and the jugular vein (PE90 tubing, Instech), and then fed either a regular chow diet (Harlan Teklad #2018, Madison, WI: 18% calories from fat, 58% from carbohydrate, 24% from protein) or a high-fat diet (Dyets #112245, Bethlehem, PA: 59% calories from fat, 26% from carbohydrate, 15% from protein), ad lib for 7 days.

Mice were generated and housed in the Yale Animal Resources Center under a 12-hour light/12-hour dark cycle and received ad libitum access to food and water. IRK^{T1150A} mice were generated as previously reported [16]. Male mice were studied at 14-18 weeks of age. Catheters were placed in the jugular vein 7-9 days before the hyperinsulinemic-euglycemic clamp. Mice were fed either a regular chow diet (Harlan Teklad #2018: 18% fat, 58% carbohydrate, and 24% protein) or a high-fat diet (Research Diets D12492: 60% fat, 20% carbohydrate, and 20% protein).

Hyperinsulinemic-Euglycemic Clamps

After either 7 days of regular chow or high-fat diet feeding, rats were fasted overnight. The rats underwent a basal intra-arterial prime infusion of \([1,2,3,4,5,6,6\text{-}^{2}\text{H}_2]\text{glucose} [1.5 \text{mg/(kg-min)}], [^{13}\text{C}_{16}]\text{palmitate} [0.5 \text{mg/(kg-min)}] \text{and} [1,1,2,3,3\text{-}^{2}\text{H}_5]\text{glycerol} [1.5 \text{mg/(kg-min)}] \text{for} 5 \text{min}, \text{followed by} \text{a continuous infusion at rate of} 0.15 \text{mg/(kg-min)}, 0.05 \text{mg/(kg-min)} \text{and} 0.15 \text{mg/(kg-min)} \text{respectively for} 115 \text{min}. \text{Blood samples were taken from the jugular vein catheter at} 100, 110, \text{and} 120 \text{min of the basal infusion for measurement of lipid turnover. A short (30 min)
A hyperinsulinemic-euglycemic clamp was then conducted starting with a primed/continuous infusion of human insulin [prime 25 mU/(kg-min) for 5 min, continuous 2.5 mU/(kg-min)] and a variable infusion of 20% dextrose to maintain euglycemia (~110 mg/dL). Blood samples were drawn from the venous catheter at 10, 20, 30 min of the clamp, with 20 and 30 min time points to measure clamp lipid turnover. Plasma insulin levels at the 120 min of basal infusion and 30 min of the clamp were measured by radioimmunoassay in the Yale Diabetes Research Center. GC/MS was used to measure plasma glycerol and palmitate atom percent excess (APE), as we have described [29], as well as calculating rates of lipolysis by correcting for the percentage of individual fatty acid content among the content of total fatty acids. Glycerol and palmitate turnover was calculated as \(Turnover = \left(\frac{Tracer\ APE}{Plasma\ APE} - 1\right) \times Infusion\ rate\). Another set of 140-min clamp study was performed as previously described [13]. A 200 µCi bolus of 2-deoxy-[1-14C]-glucose (PerkinElmer) was injected at 120-min to monitor tissue-specific insulin-stimulated glucose uptake. For the assay of WAT glucose uptake, WAT samples were homogenized, and the supernatants were transferred to an ion-exchange column to separate 14C-2-deoxyglucose-6-phosphate from 2-deoxy-[1-14C]-glucose as previously described [68]. At the end of the clamp, rats were euthanized with pentobarbital sodium (150 mg/kg), and tissues were immediately harvested and frozen with tongs pre-cooled in liquid N2. Tissues and plasma were stored at -80 °C for subsequent analyses.

For the mouse clamp, study cohorts consisted of homozygous male IRK\(^{T1150A}\) mice and littermate male WT controls which were generated by our group [16]. After 7 days of high-fat feeding, mice were fasted overnight. Awake mice under gentle tail restraint were first infused with \([^{13}C_{16}]\)palmitate [0.3 mg/(kg-min)] and \([^2H_5]\)glycerol [0.075 mg/(kg-min)] for 120 minutes to measure lipid turnover. A short (30 min) hyperinsulinemic-euglycemic clamp was then performed with a primed, continuous infusion of human insulin [prime 4.8 mU/(kg-min) for 3 min, continuous 2.0 mU/(kg-min)] and a variable infusion of 20% dextrose to maintain euglycemia (~120 mg/dL).
At the end of the clamp, mice were euthanized with pentobarbital sodium (150 mg/kg), and tissues were immediately harvested and snap-frozen in liquid N₂. Tissues and plasma were stored at -80 °C for subsequent analyses.

**Biochemical Analysis**

Plasma glucose concentrations were measured using the YSI Glucose Analyzer (Yellow Springs, OH). Plasma insulin was measured by radioimmunoassay. Plasma NEFA concentration was measured spectrophotometrically using a Wako reagent (Wako Diagnostics). cAMP was measured by cAMP ELISA kit (Enzo Life Science) in accordance with the protocol. PKA activity was measured by PKA colorimetric activity kit (Invitrogen).

**Immunoblotting and IP**

WAT lysates were prepared in RIPA buffer with protease inhibitors (cOmplete MINI, Roche) and phosphatase inhibitors (PhosSTOP, Roche). Protein was measured by the BCA assay (Pierce) and equal amount of protein extraction was mixed with sample buffer containing 5% β-mercaptoethanol. After running the samples in 4% to 12% Tris-glycine gels (Novex), proteins were electrotransferred to Immobilon P PVDF membranes (Millipore) by semi-dry transfer. Membranes were blocked in 5% BSA for 1 hour at room temperature and then probed overnight at 4°C with primary antibodies. The antibodies were obtained from Cell Signaling Technology (IRK: Cat#3025, pIRK-Y1162: Cat#3918, Akt: Cat#2920, pAkt-S473: Cat#4060, perilipin: Cat#9349, HSL: Cat#4107, pHSL-S660: Cat#4126, ATGL: Cat#2138, GAPDH: Cat#5174); abcam (pATGL-S406: Cat#ab135093, Na-K ATPase: Cat#ab7671, Calnexin: Cat#ab22595, VDAC: Cat#ab14734); ThermoFisher (PDE3B: Cat#14-1973-82); FabGennix International Incorporated (pPDE3B: Cat#PPD3B-140AP); VALA Sciences (pPerilipin-S522: Cat#4856) and BD Biosciences (PKCε: Cat#610086). Membranes were then washed in TBST and incubated for 1 hour at room temperature with secondary antibodies (Cell Signaling). After washing in TBST for
three times, antibody binding was detected by enhanced chemiluminescence (Pierce). Films were
developed within the linear dynamic range of signal intensity and then were scanned for digital
analysis. Densitometry was performed using ImageJ software.

IP was performed using 1-2 mg protein from lysates prepared as described above, the lysate
was incubated with antibody PDE3B (ThermoFisher) overnight for 16 hours and then mixed with
protein A/G agarose beads (Santa Cruz Biotechnology Inc.) for 4 hours. Immune complexes were
washed extensively in lysis buffer and eluted in Laemmli buffer for immunoblot analysis.

**PKCε Translocation Assay**

PKCε membrane / cytosol ratio in freeze clamped WAT was assessed by western blot as
previously reported [15] with slight modifications and assumed to represent PKCε activity in WAT.
Briefly, 400-500 mg WAT from rats fasted for 6 hours was homogenized in ice-cold buffer A
containing 20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.25 mM EGTA, 250 mM sucrose with protease
inhibitors (Complete MINI, Roche). Lysate was centrifuged (60 min, 100,000 g, 4°C) to separate
the membrane and cytosol from lipid droplet. The supernatant was saved as the cytosolic fraction.
The pellet was washed once in ice-cold buffer A to remove all the lipid droplet and cytosol and
then was resuspended in buffer B containing 250 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.25 mM
EGTA, 2% Triton X-100 with protease inhibitors by sonication, incubated at 4°C for 45 minutes to
solubilize membrane proteins, and centrifuged (60 min, 100,000 g, 4°C). The supernatant was
saved as the membrane fraction. Equal amounts of protein were subjected to measure
PKCε membrane / cytosol ratio by immunoblotting.

**DAG Subcellular Fractionation Assay**

Subcellular fractionation was performed as described previously [44] with modifications. 300-350
mg epididymal WAT were first homogenized in cold (4°C) TES buffer (250 mM sucrose, 10 mM
Tris - pH 7.4, 0.5 mM EDTA) with a Dounce-type tissue grinder (Kontes no. 21). All subsequent centrifugation steps were performed at 4°C. The homogenate was centrifuged at 12,000 rpm (or 17,000 g) in an SS-34 rotor for 15 min to separate pellet A and supernatant A (including the top lipid layer). Pellet A was washed by being resuspended in buffer A and centrifuged at 12,000 rpm (or 17,000 g) in an SS-34 rotor for 20 min. Pellet A was then resuspended in buffer A and layered on top of 1.12 M sucrose solution in a 2 mL ultracentrifuge tube and was centrifuged in a TLS-55 rotor at 35,000 rpm (or 105,000 g) for 20 min to separate interface B and pellet B. Interface B was collected and resuspended with buffer A and then centrifuged in a TLA-100.2 rotor at 37,000 rpm (or 60,000 g) for 9 min to obtain pellet C. Pellet C was washed by being resuspended in buffer A and centrifuged in a TLA-100.2 rotor at 37,000 rpm (or 60,000 g) for 9 min and was saved as plasma membrane fraction. Pellet B was washed by being resuspended in buffer A and centrifuged at 12,000 rpm (or 17,000 g) in an SS-34 rotor for 15 min and was collected as mitochondrial fraction. Supernatant A was centrifuged at 65,000 rpm (or 390,000 g) in Ti-70.1 rotor for 75 min to separate pellet D, supernatant D (collected as cytosol fraction) and the top lipid layer (collected as lipid droplet fraction). Pellet D was washed by being resuspended in buffer A and centrifuged at 65,000 rpm (or 390,000 g) in Ti-70.1 rotor for 60 min and was collected as ER fraction. DAGs were extracted from the five compartmentations and measured by LC-MS/MS as described previously [21, 49].

**Statistical Analysis**

Comparisons were performed using the 2-tailed Student's t-test, unpaired with significance defined as a p value < 0.05. GraphPad Prism 8.0 (San Diego, CA) was used for all statistical analysis. Data are presented as the mean ± SEM.