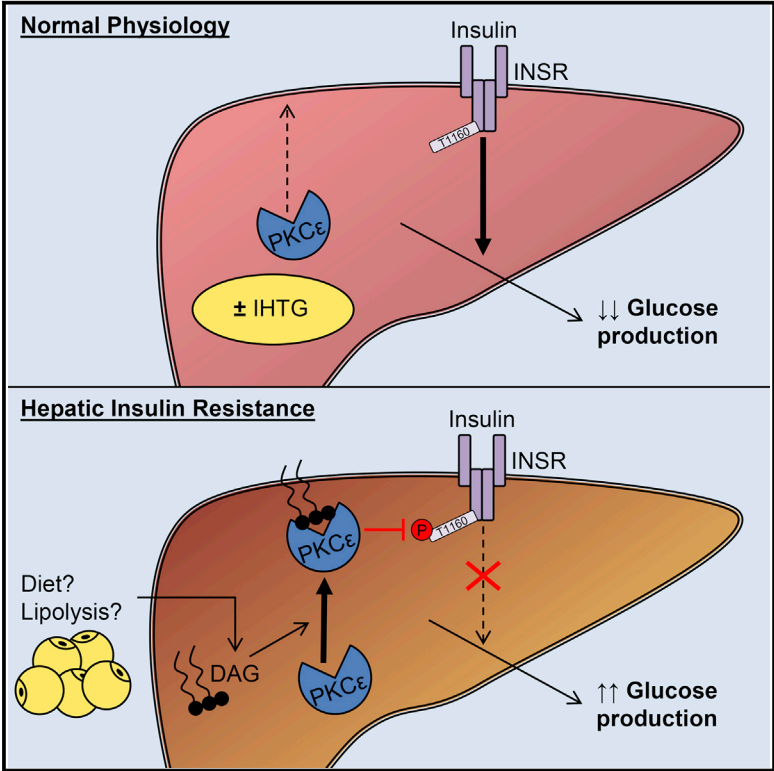


## Hepatic Diacylglycerol-Associated Protein Kinase Cε Translocation Links Hepatic Steatosis to Hepatic Insulin Resistance in Humans

### Graphical Abstract



### Authors

Kasper W. ter Horst, Pim W. Gilijamse, Ruth I. Versteeg, ..., Kitt F. Petersen, Gerald I. Shulman, Mireille J. Serlie

### Correspondence

m.j.serlie@amc.nl

### In Brief

ter Horst et al. show that simple hepatic steatosis in the context of NAFLD does not fully explain hepatic insulin resistance in obese humans. Impaired hepatic insulin action was characterized by accumulation of diacylglycerol subspecies in the cytosol of hepatocytes and translocation of PKCε from the cytosol to the membrane.

### Highlights

- The presence of hepatic steatosis is associated with insulin resistance
- Intrahepatic triglycerides are not sufficient for hepatic insulin resistance
- Diacylglycerol in hepatic cytosol predicts insulin inhibition of glucose production
- Diacylglycerol-associated insulin resistance is characterized by PKCε translocation

# Hepatic Diacylglycerol-Associated Protein Kinase C $\epsilon$ Translocation Links Hepatic Steatosis to Hepatic Insulin Resistance in Humans

Kasper W. ter Horst,<sup>1</sup> Pim W. Gijljamse,<sup>1</sup> Ruth I. Versteeg,<sup>1</sup> Mariette T. Ackermans,<sup>2</sup> Aart J. Nederveen,<sup>3</sup> Susanne E. la Fleur,<sup>1,2,11</sup> Johannes A. Romijn,<sup>4</sup> Max Nieuwdorp,<sup>5,6,7</sup> Dongyan Zhang,<sup>8</sup> Varman T. Samuel,<sup>9</sup> Daniel F. Vatner,<sup>9</sup> Kitt F. Petersen,<sup>9</sup> Gerald I. Shulman,<sup>8,9,10</sup> and Mireille J. Serlie<sup>1,12,\*</sup>

<sup>1</sup>Department of Endocrinology and Metabolism

<sup>2</sup>Department of Clinical Chemistry, Laboratory of Endocrinology

<sup>3</sup>Department of Radiology

<sup>4</sup>Department of Medicine

<sup>5</sup>Department of Vascular Medicine

Academic Medical Center, 1105AZ Amsterdam, the Netherlands

<sup>6</sup>Department of Internal Medicine

<sup>7</sup>Institute for Cardiovascular Research

VU University Medical Center, 1081HV Amsterdam, the Netherlands

<sup>8</sup>Howard Hughes Medical Institute, Yale University, New Haven, CT 06519, USA

<sup>9</sup>Department of Medicine

<sup>10</sup>Department of Cellular and Molecular Physiology

Yale University School of Medicine, New Haven, CT 06520, USA

<sup>11</sup>Metabolism and Reward Group, Netherlands Institute for Neuroscience, 1105BA Amsterdam, the Netherlands

<sup>12</sup>Lead Contact

\*Correspondence: [m.j.serlie@amc.nl](mailto:m.j.serlie@amc.nl)

<http://dx.doi.org/10.1016/j.celrep.2017.05.035>

## SUMMARY

Hepatic lipid accumulation has been implicated in the development of insulin resistance, but translational evidence in humans is limited. We investigated the relationship between liver fat and tissue-specific insulin sensitivity in 133 obese subjects. Although the presence of hepatic steatosis in obese subjects was associated with hepatic, adipose tissue, and peripheral insulin resistance, we found that intrahepatic triglycerides were not strictly sufficient or essential for hepatic insulin resistance. Thus, to examine the molecular mechanisms that link hepatic steatosis to hepatic insulin resistance, we comprehensively analyzed liver biopsies from a subset of 29 subjects. Here, hepatic cytosolic diacylglycerol content, but not hepatic ceramide content, was increased in subjects with hepatic insulin resistance. Moreover, cytosolic diacylglycerols were strongly associated with hepatic PKC $\epsilon$  activation, as reflected by PKC $\epsilon$  translocation to the plasma membrane. These results demonstrate the relevance of hepatic diacylglycerol-induced PKC $\epsilon$  activation in the pathogenesis of NAFLD-associated hepatic insulin resistance in humans.

## INTRODUCTION

Insulin resistance and non-alcoholic fatty liver disease (NAFLD) are common and consequential complications of obesity (Grundy, 2000). Ectopic triglyceride accumulation in the liver has clearly been linked to non-alcoholic steatohepatitis (NASH), liver cirrhosis, and hepatocellular carcinoma (Wong et al., 2014), but it is less clear whether there is a causal relationship between intrahepatic triglyceride (IHTG) accumulation and hepatic insulin resistance (Farese et al., 2012; Shulman, 2000).

Elevated IHTG content is associated with hepatic and/or muscle insulin resistance in most, but not in all, human studies (Bugianesi et al., 2005; Deivanayagam et al., 2008; Gastaldelli et al., 2007; Korenblat et al., 2008; Kotronen et al., 2008; Petersen et al., 2002, 2005; Sanyal et al., 2001; Seppälä-Lindroos et al., 2002). Moreover, in addition to triglycerides, it is now established that other (possibly more bioactive) lipid species may also accumulate in hepatocytes in the context of NAFLD. In several animal models, buildup of diacylglycerol (DAG) has been linked to hepatic insulin resistance through activation of protein kinase C $\epsilon$  (PKC $\epsilon$ ), which may directly interfere with hepatic insulin signaling (Jornayvaz et al., 2011; Samuel et al., 2004, 2007; Samuel and Shulman, 2016; Xia et al., 2015). In this regard, PKC $\epsilon$  has recently been shown to phosphorylate insulin receptor (INSR) threonine<sup>1160</sup>, which led to inhibition of INSR kinase activity (Petersen et al., 2016). Furthermore, mice with a threonine-to-alanine mutation at the homologous residue threonine<sup>1150</sup> (*Insr*<sup>T1150A</sup> mice) were protected from high-fat-diet-induced

**Table 1. Characteristics of Study Participants According to Extent of Hepatic Steatosis, n = 133**

|                                       | No Steatosis (n = 52) | Mild Steatosis (n = 41) | Severe Steatosis (n = 40)  | p      |
|---------------------------------------|-----------------------|-------------------------|----------------------------|--------|
| Female sex                            | 27 (51)               | 14 (34)                 | 18 (45)                    | 0.229  |
| Age (years)                           | 49 ± 2                | 51 ± 2                  | 49 ± 2                     | 0.634  |
| BMI (kg/m <sup>2</sup> )              | 39 ± 1                | 41 ± 1                  | 41 ± 1                     | 0.442  |
| Body fat (%)                          | 45 ± 1                | 45 ± 2                  | 46 ± 1                     | 0.695  |
| Fasting glucose (mmol/L)              | 5.2 ± 0.1             | 5.2 ± 0.1               | 5.4 ± 0.1                  | 0.301  |
| Triglycerides (mmol/L)                | 1.3 ± 0.1             | 1.6 ± 0.1               | 1.7 ± 0.2 <sup>a</sup>     | 0.039  |
| Cholesterol (mmol/L)                  | 4.9 ± 0.1             | 4.9 ± 0.2               | 5.0 ± 0.2                  | 0.671  |
| High-density lipoprotein (mmol/L)     | 1.2 ± 0.0             | 1.1 ± 0.1               | 1.1 ± 0.0                  | 0.287  |
| Low-density lipoprotein (mmol/L)      | 3.0 ± 0.1             | 3.1 ± 0.2               | 3.2 ± 0.1                  | 0.620  |
| Alanine aminotransferase (U/L)        | 26 ± 2                | 38 ± 3 <sup>b</sup>     | 40 ± 3 <sup>c</sup>        | <0.001 |
| Aspartate aminotransferase (U/L)      | 22 ± 1                | 30 ± 3                  | 32 ± 3 <sup>b</sup>        | 0.004  |
| Gamma-glutamyl transpeptidase (U/L)   | 34 ± 4                | 42 ± 7                  | 42 ± 4                     | 0.320  |
| C-reactive protein (mg/L)             | 4.9 ± 0.9             | 6.0 ± 1.0               | 7.6 ± 1.3                  | 0.197  |
| Resting energy expenditure (kcal/day) | 1,862 ± 48            | 2,000 ± 72              | 1,931 ± 54                 | 0.256  |
| IHTG (%)                              | 2.9 ± 0.2             | 10.4 ± 0.4 <sup>c</sup> | 23.3 ± 1.0, <sup>c,d</sup> | <0.001 |

Data are count (%) or mean ± SEM. No, mild, and severe steatosis were defined as intrahepatic triglycerides (IHTG) <5.56%, 5.56%–15%, or >15%, respectively.

<sup>a</sup>p < 0.05 versus no steatosis.

<sup>b</sup>p < 0.01 versus no steatosis.

<sup>c</sup>p < 0.001 versus no steatosis.

<sup>d</sup>p < 0.001 versus mild steatosis.

hepatic insulin resistance and displayed increased hepatic insulin signaling, suppression of hepatic glucose production, and hepatic glycogen synthesis compared with wild-type (WT) controls during hyperinsulinemic clamp studies. These results provide a strong mechanistic link for DAG-PKC $\epsilon$ -mediated hepatic insulin resistance in the context of NAFLD. In contrast, other studies have suggested that an increase in hepatic ceramide content is the major mediator of lipid-induced hepatic insulin resistance in the context of NAFLD (Chavez and Summers, 2012; Holland et al., 2011; Luukkonen et al., 2016), although the mechanism by which ceramides cause hepatic insulin resistance is less clear. In the few available translational studies, hepatic DAG content, but not hepatic ceramide content, correlated with indices of hepatic insulin resistance in obese humans (Kumashiro et al., 2011; Magkos et al., 2012).

There are also circumstances, in both animals and humans, in which hepatic insulin resistance is dissociated from hepatic steatosis (Amaro et al., 2010; Benhamed et al., 2012; Brown et al., 2010; Buettner et al., 2004; Monetti et al., 2007; Semple et al., 2009; Visser et al., 2011). Some, but not all, of these apparently conflicting observations may be the result of differences among studies in diagnosis and definition of fatty liver and/or insulin resistance (Farese et al., 2012). Moreover, a better understanding of the subcellular compartmentalization of hepatic DAG species and PKC $\epsilon$  may explain some of the apparent dissociations between hepatic steatosis and hepatic insulin resistance (Samuel and Shulman, 2016). For instance, short-term high-fat feeding promotes hepatic insulin resistance, which is associated with increases in cytosolic and membrane DAG content and activation of PKC $\epsilon$  as reflected by translocation of PKC $\epsilon$  from the cytosol to the plasma membrane (Samuel et al., 2004, 2007). In contrast,

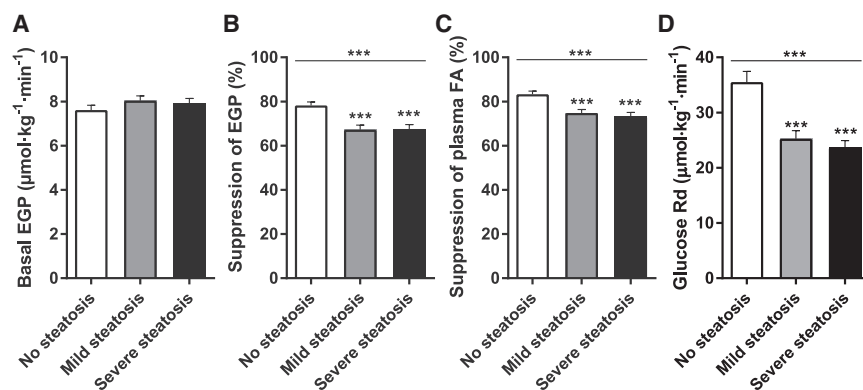
antisense oligonucleotide knockdown of hepatic CGI-58 leads to hepatic steatosis, which is associated with considerable accumulation of DAGs in lipid droplets (Cantley et al., 2013). Contrary to the high-fat-fed mice, this increase in lipid droplet DAGs is not associated with PKC $\epsilon$  activation or hepatic insulin resistance. It is currently unknown how these paradigms translate to humans.

Therefore, we here report a clinical study in obese non-diabetic humans aimed to (1) determine the relationship between liver fat content and insulin resistance using gold-standard metabolic flux measurements and (2) determine whether the subcellular distribution of individual hepatic lipid species and PKC $\epsilon$  in liver biopsies is relevant for hepatic insulin resistance in humans.

## RESULTS AND DISCUSSION

### The Presence of NAFLD, but Not the Extent of Hepatic Steatosis, Is Associated with Insulin Resistance in Obese Humans

To determine whether the accumulation of IHTG in the context of NAFLD relates to abnormalities in glucose metabolism and/or tissue-specific parameters of insulin action, we measured these metabolic fluxes using the two-step hyperinsulinemic-euglycemic clamp technique in a cohort of 133 obese adults. Fifty-two subjects had normal IHTG by proton magnetic resonance spectroscopy (<sup>1</sup>H-MRS) (no steatosis, IHTG <5.56% [Szczepaniak et al., 2005]). Of the 81 subjects with <sup>1</sup>H-MRS-defined hepatic steatosis, 41 subjects had mild steatosis (IHTG 5.56%–15%), and 40 subjects had severe steatosis (IHTG >15%). These groups did not differ in most baseline characteristics, but subjects with hepatic steatosis had higher plasma triglycerides and aminotransferases than those without steatosis (Table 1).



**Figure 1. The Presence of Hepatic Steatosis, but Not Its Extent, Is Associated with Insulin Resistance in Obese Humans**

(A) The basal rate of EGP was assessed after an overnight fast. (B) Hepatic insulin sensitivity is expressed as the insulin-mediated suppression of basal EGP. (C) Adipose tissue insulin sensitivity is expressed as the insulin-mediated suppression of circulating FA. (D) Peripheral insulin sensitivity is expressed as the insulin-stimulated Rd of glucose. Data are mean  $\pm$  SEM (n = 125–133). \*\*\*p  $\leq$  0.001.

Hepatic steatosis was not associated with differences in the basal rate of endogenous glucose production (EGP) (Figure 1A). However, subjects with mild or severe hepatic steatosis had higher fasting plasma insulin levels than subjects without hepatic steatosis (Table S1). Since fasting insulin  $\times$  basal EGP is an index of hepatic insulin resistance (Korenblat et al., 2008), this suggests that basal hepatic responsiveness to insulin may be impaired in obese subjects with steatosis (Figure S1A). Indeed, during the low-dose insulin step of the hyperinsulinemic-euglycemic clamp, insulin-mediated suppression of EGP was impaired in subjects with mild or severe hepatic steatosis, indicating hepatic insulin resistance (Figure 1B). In both steatosis groups, insulin suppression of plasma fatty acids (FA) and insulin stimulation of the glucose rate of disappearance (Rd) were also reduced, indicating adipose tissue and peripheral/muscle insulin resistance, respectively (Figures 1C, 1D, S1B, and S1C). Notably, there were no differences in any of these parameters between subjects with mild or severe hepatic steatosis (Figures 1B–1D). Finally, these results were not caused by differences in insulin clearance (Figures S1D and S1E) or levels of glucoregulatory hormones during the clamps (Table S1). Therefore, data from our large cohort of obese subjects show that the presence of liver steatosis is associated with impaired insulin sensitivity in the liver, adipose tissue, and muscle, but that the extent of hepatic steatosis does not predict the severity of insulin resistance.

#### IHTG Are Not Sufficient or Essential for the Development of Hepatic Insulin Resistance in Obese Humans

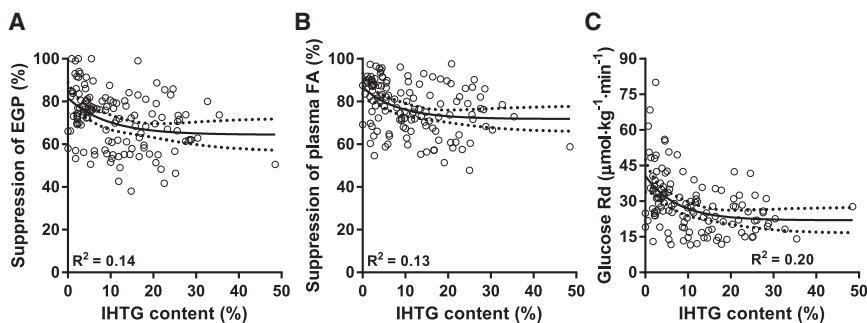
In agreement with the previous data, regression analysis confirmed that the relationship between IHTG and insulin suppression of EGP was best described by a nonlinear model: IHTG and hepatic insulin action were negatively correlated in the lower range of steatosis but dissociated in the higher range of steatosis (Figure 2A). More importantly, obese subjects with hepatic steatosis displayed nearly the same variation in hepatic insulin sensitivity as subjects without hepatic steatosis (suppression of EGP ranging from 38%–99% and 51%–100%, respectively). In fact, the distribution of both variables clearly shows that hepatic steatosis can occur without hepatic insulin resistance, and vice versa (Figure 2A). Therefore, IHTG are not strictly sufficient or necessary for hepatic insulin resistance (Farese

et al., 2012), at least in the conditions of our study. We observed similar relationships between IHTG and parameters of extrahepatic insulin sensitivity (Figures 2B and 2C). These observations are consistent with previous human studies that have demonstrated IHTG accumulation without insulin resistance (Amaro et al., 2010; Visser et al., 2011) or insulin resistance in the absence of IHTG (Semple et al., 2009). Therefore, the available data do not implicate that IHTGs are a cause for hepatic insulin resistance, and this indicates that (an)other factor(s) must be responsible for NAFLD-associated hepatic insulin resistance.

#### Hepatic Insulin Resistance Is Associated with DAG Accumulation in Cytosol

Non-invasive measurement methods such as <sup>1</sup>H-MRS cannot distinguish between inert lipids such as triglycerides (Yu et al., 2002) and bioactive lipids such as DAGs or ceramides. The subcellular distribution of these lipids may also be critical to their biological effects. Therefore, to determine whether these parameters are important for hepatic insulin resistance in obese humans, we obtained liver biopsies from a subset of subjects that underwent bariatric surgery <2 weeks after the clinical experiments (n = 29).

Liquid chromatography/tandem mass spectrometry (LC-MS/MS) analysis of snap-frozen liver biopsies revealed that neither hepatic ceramide (r = 0.32, p = 0.117) nor total DAG (r = 0.31, p = 0.109) content predicted hepatic insulin sensitivity in morbidly obese subjects (Figures S2A and S2B). However, DAG may be present in the cell membrane, cytosol, and lipid droplets (Cantley et al., 2013; Kumashiro et al., 2011). Using a subcellular fractionation method (Cantley et al., 2013), we separated liver biopsies into membrane and cytosolic fractions. Here, we observed that DAG accumulation in the cytosolic fraction (r = 0.43, p = 0.024), but not DAG accumulation in the membrane (r = 0.19, p = 0.342), predicted hepatic insulin resistance assessed by clamp (Figures S2C and S2D). Cytosolic DAG also strongly correlated with other parameters of hepatic insulin action including the Hepatic Insulin Sensitivity Index, HOMA-IR, and fasting plasma insulin levels (data not shown). Finally, we analyzed individual hepatic DAG species in relation to hepatic insulin sensitivity (Table S2). In the cytosolic fraction, hepatic DAGs composed of C18:1–C16:0, C16:0–C16:0, and C18:1–C18:1 were most abundant and also showed strong negative



**Figure 2. Relationships between IHTG Content and Tissue-Specific Measurements of Insulin Sensitivity in Obese Subjects Were Best Described by Nonlinear Models**

(A) Hepatic insulin sensitivity.  
(B) Adipose tissue insulin sensitivity.  
(C) Peripheral insulin sensitivity.  
Lines are best fit and 95% CI (n = 125–133).

correlations with suppression of EGP. In contrast, hepatic DAG composed of C20:4–C20:5 was highly abundant in the membrane fraction and positively correlated with hepatic insulin sensitivity. Individual hepatic ceramide species were not related to hepatic insulin action (Table S3).

#### Hepatic DAG-Associated Insulin Resistance and PKC $\epsilon$ Translocation

Hepatic DAGs are proposed to impair insulin signaling through activation of novel PKC (nPKC) (Farese et al., 2012; Nagle et al., 2009; Samuel and Shulman, 2016). In our prior study, we found that PKC $\epsilon$  was the major nPKC isoform expressed in human liver, and activation of PKC $\epsilon$  correlated with hepatic DAG content in the cytosol and HOMA-IR (Kumashiro et al., 2011). However, the association between PKC $\epsilon$  activation and insulin suppression of hepatic glucose output has not been reported in humans. Therefore, we measured PKC $\epsilon$  translocation to the cell membrane as an index of PKC $\epsilon$  activation (Kumashiro et al., 2011; Schmitz-Peiffer and Biden, 2008).

We compared liver biopsies from insulin-sensitive subjects (insulin suppression of EGP >80%, n = 10) with biopsies from insulin-resistant subjects (insulin suppression of EGP <65%, n = 8). The insulin-resistant subjects displayed both impaired fasting glucose concentrations and hyperinsulinemia (Table S4). In addition, insulin-resistant livers had a marked, 2.5-fold increase in cytosolic DAG content, but no difference in total DAG or ceramide content (Figures 3A–3D). In fact, all individual DAG species were increased in the hepatic cytosol of insulin-resistant subjects (Figure 3E). Notably, hepatic DAG-associated hepatic insulin resistance was characterized by a 2-fold increase in translocation of PKC $\epsilon$  from the cytosol to the membrane (Figures 3F and 3G). Taken together, this suggests that hepatic DAGs in the cytosol promote PKC $\epsilon$  translocation from the cytosol to the membrane where it can bind INSR and phosphorylate threonine<sup>1160</sup>, which, in turn, inhibits INSR kinase activity (Petersen et al., 2016).

#### Sources for Hepatic DAG Accumulation in Obese Humans

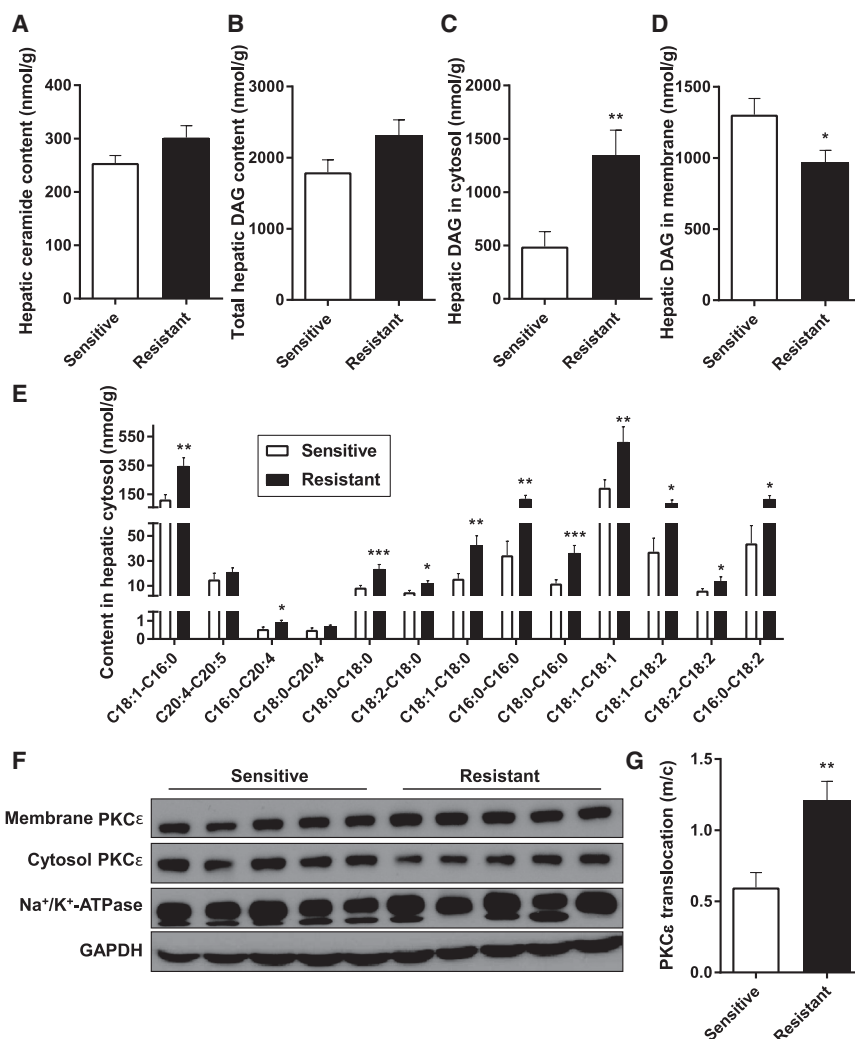
Hepatic DAG may accumulate from a variety of sources including circulating FA derived from adipose tissue lipolysis (Donnelly et al., 2005; Finck and Hall, 2015). To explore the potential role of adipose tissue lipolysis in promoting increased hepatic DAG content, we assessed rates of lipolysis measured by tracer dilution of [1,1,2,3,3-<sup>2</sup>H<sub>5</sub>]glycerol during hyperinsulinemic

and circulating FA levels during insulin infusion both strongly correlated with hepatic DAG accumulation, indicating that higher release of FA from lipolysis during hyperinsulinemic (postprandial) conditions is associated with increased cytosolic DAG content (Figures S3A and S3B). In line, hepatic lipid synthesis in rats is primarily driven by substrate (FA) delivery to the liver, which occurs in an insulin-independent manner (Vatner et al., 2015). Thus, these data are consistent with the possibility that most of the hepatic DAG derives from FA from increased lipolysis in insulin-resistant adipose tissue, underlining the importance of healthy insulin-sensitive adipose tissue in the prevention of ectopic lipid disposition and hepatic insulin resistance.

#### Perspective

In this study, we demonstrated that the presence of hepatic steatosis in obese subjects is associated with insulin resistance in the liver, adipose tissue, and peripheral tissues. In subjects with hepatic steatosis, however, the amount of IHTG per se did not additionally affect tissue-specific parameters of insulin action. Noteworthy, although all subjects were obese, there was a wide range in insulin sensitivity. The distribution of the data further showed that IHTG were neither sufficient nor necessary for the development of hepatic insulin resistance, indicating that another underlying mechanism must be responsible for impaired insulin suppression of EGP in the context of NAFLD. Recent animal studies showed that the major underlying mechanism for hepatic insulin resistance involves hepatic DAG-induced PKC $\epsilon$  activation (Kumashiro et al., 2011; Petersen et al., 2016; Samuel and Shulman, 2016), but efforts to translate this paradigm to humans have been limited. Therefore, we comprehensively analyzed liver samples from subjects with normal or impaired insulin suppression of EGP and found that hepatic DAG accumulation in the cytosol was increased in obese humans with hepatic insulin resistance. Moreover, this was strongly associated with hepatic PKC $\epsilon$  activation as reflected by its translocation to the cell membrane. In contrast, there was no relationship between hepatic ceramide content and hepatic insulin resistance in these subjects. Taken together, these results support a model for the pathogenesis of hepatic insulin resistance in NAFLD, in which DAGs in the cytosol promote translocation of PKC $\epsilon$  to the membrane, where it inhibits hepatocellular insulin signaling. Here, we substantiate the relevance of this pathway in human metabolic disease.

Some nuances with respect to our findings should be made. First, the above-mentioned mechanism for lipid-induced hepatic insulin resistance should be appreciated in light of



**Figure 3. Hepatic Insulin Resistance Was Strongly Associated with Elevated DAG Content in the Cytosolic Fraction and PKC $\epsilon$  Translocation**

(A) Hepatic ceramide content in obese subjects with normal hepatic insulin sensitivity or hepatic insulin resistance. (B) Total hepatic DAG content. (C) Hepatic DAG content in cytosolic fraction. (D) Hepatic DAG content in membrane fraction. (E) Individual DAG species in hepatic cytosol. Note the segmented y axis. (F) Representative bands show PKC $\epsilon$  translocation from cytosol to membrane. (G) Translocation of PKC $\epsilon$  from cytosol (c) to membrane (m) as an index of activation. Data are mean  $\pm$  SEM (n = 18–29). \*p < 0.05. \*\*p < 0.01. \*\*\*p = 0.001.

perform measurements in subcellular compartments but conclude that their data do not exclude the possibility that DAGs contribute to hepatic insulin resistance in humans, indicating that results are not strictly conflicting. In addition, the I148M gene variant in PNPLA3 was associated with hepatic steatosis without insulin resistance, whereas metabolic NAFLD was associated with a harmful hepatic lipidome (Luukkonen et al., 2016). We have also reported that hepatic steatosis in the context of familial hypobetalipoproteinemia is not associated with hepatic insulin resistance (Visser et al., 2011), suggesting that genetics may, in part, explain why some people with hepatic steatosis develop insulin resistance, whereas others do not. Unfortunately, we do not have genotype data in this study, but it will be of interest to determine whether the I148M and other variants play a role in the susceptibility to lipid mediated-insulin resistance. Third, there is ample (reverse causal) evidence suggesting that extrahepatic insulin resistance contributes to the development of hepatic steatosis. In individuals with muscle and adipose tissue insulin resistance, nutrients are diverted to the liver. Accompanied by a compensatory hyperinsulinemia, these conditions provide both substrate (glucose and FA) and stimulus (insulin signaling through sterol regulatory element-binding protein 1c) for hepatic triglyceride synthesis and de novo lipogenesis (Cohen et al., 2011; Perry et al., 2014; Petersen et al., 2007; Rabøl et al., 2011; Vatner et al., 2015). Finally, an important limitation in the interpretation of these data is the cross-sectional design of the study. However, we believe that obtaining serial liver biopsies in humans would be unethical in the absence of urgent medical indications, and numerous animal studies support a causal role for hepatic DAG accumulation and PKC $\epsilon$  activation in hepatic insulin resistance (Jornayvaz et al., 2011; Petersen et al., 2016; Samuel

interconnected defects in other organ systems. For instance, extrahepatic regulation of adipose tissue lipolysis and FA availability by insulin is another important regulator of hepatic gluconeogenesis through substrate-driven regulation of pyruvate carboxylase activity/flux by hepatic acetyl CoA, which is independent of canonical hepatic insulin signaling (Perry et al., 2015). Thus, PKC $\epsilon$  inhibition of hepatic insulin signaling may mainly affect insulin regulation of net hepatic glycogen synthesis. Second, previous studies have suggested that increased hepatic ceramide synthesis may be causal in lipid-induced hepatic insulin resistance (Chavez and Summers, 2012; Holland et al., 2011; Luukkonen et al., 2016), but ceramides were unrelated to hepatic insulin resistance in the present study. Others have also reported such dissociations (Kumashiro et al., 2011; Magkos et al., 2012; Perry et al., 2013). Some of these conflicting results may be due to differences in experimental techniques or limited sample size/power. In one study, “metabolic NAFLD” was associated with an increase in hepatic ceramides and elevated HOMA-IR, but also with an increase in four out of five DAG species (Luukkonen et al., 2016). The authors did not

et al., 2007; Samuel and Shulman, 2016). These data offer further support for the DAG-PKC $\epsilon$  hypothesis for lipid-induced hepatic insulin resistance in NAFLD and support the development of interventions that target hepatic DAG-induced PKC $\epsilon$  activation for the prevention and/or treatment of type 2 diabetes.

## EXPERIMENTAL PROCEDURES

### Subjects

Subjects participated in metabolic studies at the Academic Medical Center (Amsterdam, the Netherlands). Treatment-naïve subjects were included in this study if they were obese (BMI  $\geq 30$  kg/m<sup>2</sup>) with stable weight (<5% weight change) for >3 months prior to examinations, and if they had undergone a two-step hyperinsulinemic-euglycemic clamp study according to standard operating procedures and liver <sup>1</sup>H-MRS before any intervention. Subjects were excluded in case of a history of diabetes, use of alcohol (>2 units/day) or recreational drugs, use of antipsychotic or antidepressant medication, or any somatic disorder except for stable obesity-related conditions (i.e., NAFLD/NASH, dyslipidemia, hypertension, obstructive sleep apnea).

All subjects completed a medical evaluation including history, physical examination, and blood tests. Body composition was determined by bioelectrical impedance (Maltron BF-906). Resting energy expenditure was assessed by indirect calorimetry ( $V_{\max}$  Encore 29n; CareFusion).

All procedures were approved by the Academic Medical Center medical ethics committee, and all subjects provided written informed consent in accordance with the Declaration of Helsinki.

### Liver Fat Content

We assessed IHTG content by <sup>1</sup>H-MRS as described (van der Valk et al., 2014). This method has high diagnostic accuracy and high precision with low variability for assessment of hepatic steatosis in NAFLD (Dulai et al., 2016).

### Hyperinsulinemic-Euglycemic Clamps

Glucose kinetics and tissue-specific parameters of insulin sensitivity were assessed during a two-step hyperinsulinemic-euglycemic clamp study as described (ter Horst et al., 2015). Briefly, subjects were admitted to the metabolic unit after an overnight fast. A primed continuous infusion of the stable isotope-labeled glucose tracer [6,6-<sup>2</sup>H<sub>2</sub>]glucose (>99% enriched; Cambridge Isotopes) was started. In a subset of subjects (n = 29), we also infused the stable isotope-labeled glycerol tracer [1,1,2,3,3-<sup>2</sup>H<sub>5</sub>]glycerol (>99% enriched; Cambridge Isotopes). Basal rates of EGP and lipolysis were determined after 2 hr of tracer equilibration. Next, hepatic insulin sensitivity (expressed as suppression of basal EGP by insulin) and adipose tissue insulin sensitivity (expressed as suppression of circulating FA levels or suppression of glycerol Ra by insulin) were assessed after 2 hr of low-dose insulin infusion (Actrapid 20 mU · m<sup>-2</sup> · min<sup>-1</sup>; Novo Nordisk Farma). Finally, peripheral insulin sensitivity (expressed as insulin-stimulated Rd of glucose) was assessed after 2 hr of high-dose insulin infusion (60 mU · m<sup>-2</sup> · min<sup>-1</sup>). During hyperinsulinemia, plasma glucose was maintained at 5.0 mmol/L by frequent bedside monitoring and variable infusion of exogenous glucose (enriched with 1% [6,6-<sup>2</sup>H<sub>2</sub>] glucose).

### Liver Biopsies

A subset of subjects (n = 29) underwent bariatric surgery shortly (<2 weeks) after the clinical assessments. Subjects were instructed to maintain stable weight by consumption of a weight-maintenance diet in the preoperative period. During surgery, an experienced surgeon obtained a wedge biopsy from the right liver lobe. Biopsies were immediately snap-frozen in liquid nitrogen and stored at -80°C.

### Plasma Hormones and Metabolites

Glucose, insulin, glucagon, cortisol, and FA were measured as described (ter Horst et al., 2015). Enrichments of [6,6-<sup>2</sup>H<sub>2</sub>]glucose and [1,1,2,3,3-<sup>2</sup>H<sub>5</sub>]glycerol (tracer-to-tracee ratios) were measured by gas chromatography-MS (Ackermans et al., 2001).

### Hepatic Lipid Metabolites

Extraction, purification, and assessment of DAGs and ceramides from liver was performed by LC-MS/MS in a blinded fashion as described (Kumashiro et al., 2011). Subcellular fractionation into membrane and cytosolic compartments was performed as reported (Cantley et al., 2013).

### PKC $\epsilon$ Translocation Assay

Membrane translocation of PKC $\epsilon$  was assayed as described (Kumashiro et al., 2011) and expressed as the ratio of membrane protein band density over cytosol protein band density.

### Calculations

Fluxes (EGP, glucose Rd, and glycerol Ra) were calculated using modified versions of the Steele equations for the steady state (basal EGP and glycerol Ra) or non-steady state (during insulin infusion) and expressed as  $\mu\text{mol} \cdot (\text{kg body weight})^{-1} \cdot \text{min}^{-1}$  (Steele, 1959).

### Statistical Analyses

Groups were compared by two-tailed t test (two groups) or one-way ANOVA with Bonferroni correction (more than two groups). Correlations between continuous variables were evaluated by Pearson's correlation and regression analyses (linear and nonlinear). Findings were considered significant if  $p < 0.05$ . Statistical analyses were performed using IBM SPSS Statistics v.23 and GraphPad Prism v.6 (La Jolla).

## SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and four tables and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2017.05.035>.

## AUTHOR CONTRIBUTIONS

K.W.t.H. researched data, performed the analysis, contributed to discussions about the results, and wrote the manuscript. P.W.G. and R.I.V. researched data. M.T.A. and D.Z. were responsible for laboratory analyses. A.J.N. was responsible for <sup>1</sup>H-MRS experiments. S.E.I.F., J.A.R., M.N., D.F.V., V.T.S., K.F.P., G.I.S., and M.J.S. contributed to discussions about the results. All authors critically reviewed and approved the final manuscript.

## ACKNOWLEDGMENTS

The authors thank Erik Endert (Academic Medical Center) for expert advice on laboratory analyses and Mario Kahn (Yale University School of Medicine) for LC-MS/MS analyses of hepatic lipid species. This study was funded, in part, by grants from the EU (FP7-EU 305707), NWO (STW 12189), and the US Public Health Service (R01 AG-023686, R24 DK-085638, P30 DK-045735). M.N. is supported by grants VID1 2013 (016.146.327) and CVON 2012 young talent (IN-CONTROL).

Received: January 23, 2017

Revised: April 17, 2017

Accepted: May 10, 2017

Published: June 6, 2017

## REFERENCES

- Ackermans, M.T., Pereira Arias, A.M., Bisschop, P.H., Endert, E., Sauerwein, H.P., and Romijn, J.A. (2001). The quantification of gluconeogenesis in healthy men by (2)H<sub>2</sub>O and [2-(13)C]glycerol yields different results: Rates of gluconeogenesis in healthy men measured with (2)H<sub>2</sub>O are higher than those measured with [2-(13)C]glycerol. *J. Clin. Endocrinol. Metab.* 86, 2220–2226.
- Amaro, A., Fabbrini, E., Kars, M., Yue, P., Schechtman, K., Schonfeld, G., and Klein, S. (2010). Dissociation between intrahepatic triglyceride content and insulin resistance in familial hypobetalipoproteinemia. *Gastroenterology* 139, 149–153.

- Benhamed, F., Denechaud, P.D., Lemoine, M., Robichon, C., Moldes, M., Bertrand-Michel, J., Ratzju, V., Serfaty, L., Housset, C., Capeau, J., et al. (2012). The lipogenic transcription factor ChREBP dissociates hepatic steatosis from insulin resistance in mice and humans. *J. Clin. Invest.* **122**, 2176–2194.
- Brown, J.M., Betters, J.L., Lord, C., Ma, Y., Han, X., Yang, K., Alger, H.M., Melchior, J., Sawyer, J., Shah, R., et al. (2010). CGI-58 knockdown in mice causes hepatic steatosis but prevents diet-induced obesity and glucose intolerance. *J. Lipid Res.* **51**, 3306–3315.
- Buettner, R., Ottinger, I., Schölmerich, J., and Bollheimer, L.C. (2004). Preserved direct hepatic insulin action in rats with diet-induced hepatic steatosis. *Am. J. Physiol. Endocrinol. Metab.* **286**, E828–E833.
- Bugianesi, E., Gastaldelli, A., Vanni, E., Gambino, R., Cassader, M., Baldi, S., Ponti, V., Pagano, G., Ferrannini, E., and Rizzetto, M. (2005). Insulin resistance in non-diabetic patients with non-alcoholic fatty liver disease: Sites and mechanisms. *Diabetologia* **48**, 634–642.
- Cantley, J.L., Yoshimura, T., Camporez, J.P., Zhang, D., Jornayvaz, F.R., Kumashiro, N., Guebre-Egziabher, F., Jurczak, M.J., Kahn, M., Guigni, B.A., et al. (2013). CGI-58 knockdown sequesters diacylglycerols in lipid droplets/ER-preventing diacylglycerol-mediated hepatic insulin resistance. *Proc. Natl. Acad. Sci. USA* **110**, 1869–1874.
- Chavez, J.A., and Summers, S.A. (2012). A ceramide-centric view of insulin resistance. *Cell Metab.* **15**, 585–594.
- Cohen, J.C., Horton, J.D., and Hobbs, H.H. (2011). Human fatty liver disease: Old questions and new insights. *Science* **332**, 1519–1523.
- Deivanayagam, S., Mohammed, B.S., Vitola, B.E., Naguib, G.H., Keshen, T.H., Kirk, E.P., and Klein, S. (2008). Nonalcoholic fatty liver disease is associated with hepatic and skeletal muscle insulin resistance in overweight adolescents. *Am. J. Clin. Nutr.* **88**, 257–262.
- Donnelly, K.L., Smith, C.I., Schwarzenberg, S.J., Jessurun, J., Boldt, M.D., and Parks, E.J. (2005). Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease. *J. Clin. Invest.* **115**, 1343–1351.
- Dulai, P.S., Sirlin, C.B., and Looma, R. (2016). MRI and MRE for non-invasive quantitative assessment of hepatic steatosis and fibrosis in NAFLD and NASH: Clinical trials to clinical practice. *J. Hepatol.* **65**, 1006–1016.
- Farese, R.V., Jr., Zechner, R., Newgard, C.B., and Walther, T.C. (2012). The problem of establishing relationships between hepatic steatosis and hepatic insulin resistance. *Cell Metab.* **15**, 570–573.
- Finck, B.N., and Hall, A.M. (2015). Does diacylglycerol accumulation in fatty liver disease cause hepatic insulin resistance? *BioMed Res. Int.* **2015**, 104132.
- Gastaldelli, A., Cusi, K., Pettiti, M., Hardies, J., Miyazaki, Y., Berria, R., Buzzigoli, E., Sironi, A.M., Cersosimo, E., Ferrannini, E., and DeFronzo, R.A. (2007). Relationship between hepatic/visceral fat and hepatic insulin resistance in nondiabetic and type 2 diabetic subjects. *Gastroenterology* **133**, 496–506.
- Grundy, S.M. (2000). Metabolic complications of obesity. *Endocrine* **13**, 155–165.
- Holland, W.L., Miller, R.A., Wang, Z.V., Sun, K., Barth, B.M., Bui, H.H., Davis, K.E., Bikman, B.T., Halberg, N., Rutkowski, J.M., et al. (2011). Receptor-mediated activation of ceramidase activity initiates the pleiotropic actions of adiponectin. *Nat. Med.* **17**, 55–63.
- Jornayvaz, F.R., Birkenfeld, A.L., Jurczak, M.J., Kanda, S., Guigni, B.A., Jiang, D.C., Zhang, D., Lee, H.Y., Samuel, V.T., and Shulman, G.I. (2011). Hepatic insulin resistance in mice with hepatic overexpression of diacylglycerol acyltransferase 2. *Proc. Natl. Acad. Sci. USA* **108**, 5748–5752.
- Korenblat, K.M., Fabbrini, E., Mohammed, B.S., and Klein, S. (2008). Liver, muscle, and adipose tissue insulin action is directly related to intrahepatic triglyceride content in obese subjects. *Gastroenterology* **134**, 1369–1375.
- Kotronen, A., Juurinen, L., Tiikkainen, M., Vehkavaara, S., and Yki-Järvinen, H. (2008). Increased liver fat, impaired insulin clearance, and hepatic and adipose tissue insulin resistance in type 2 diabetes. *Gastroenterology* **135**, 122–130.
- Kumashiro, N., Erion, D.M., Zhang, D., Kahn, M., Beddow, S.A., Chu, X., Still, C.D., Gerhard, G.S., Han, X., Dziura, J., et al. (2011). Cellular mechanism of insulin resistance in nonalcoholic fatty liver disease. *Proc. Natl. Acad. Sci. USA* **108**, 16381–16385.
- Luukkonen, P.K., Zhou, Y., Sädevirta, S., Leivonen, M., Arola, J., Orešič, M., Hyötyläinen, T., and Yki-Järvinen, H. (2016). Hepatic ceramides dissociate steatosis and insulin resistance in patients with non-alcoholic fatty liver disease. *J. Hepatol.* **64**, 1167–1175.
- Magkos, F., Su, X., Bradley, D., Fabbrini, E., Conte, C., Eagon, J.C., Varela, J.E., Brunt, E.M., Patterson, B.W., and Klein, S. (2012). Intrahepatic diacylglycerol content is associated with hepatic insulin resistance in obese subjects. *Gastroenterology* **142**, 1444–6.e2.
- Monetti, M., Levin, M.C., Watt, M.J., Saján, M.P., Marmor, S., Hubbard, B.K., Stevens, R.D., Bain, J.R., Newgard, C.B., Farese, R.V., Sr., et al. (2007). Dissociation of hepatic steatosis and insulin resistance in mice overexpressing DGAT in the liver. *Cell Metab.* **6**, 69–78.
- Nagle, C.A., Klett, E.L., and Coleman, R.A. (2009). Hepatic triacylglycerol accumulation and insulin resistance. *J. Lipid Res.* **50** (Suppl), S74–S79.
- Perry, R.J., Kim, T., Zhang, X.M., Lee, H.Y., Pesta, D., Popov, V.B., Zhang, D., Rahimi, Y., Jurczak, M.J., Cline, G.W., et al. (2013). Reversal of hypertriglyceridemia, fatty liver disease, and insulin resistance by a liver-targeted mitochondrial uncoupler. *Cell Metab.* **18**, 740–748.
- Perry, R.J., Samuel, V.T., Petersen, K.F., and Shulman, G.I. (2014). The role of hepatic lipids in hepatic insulin resistance and type 2 diabetes. *Nature* **510**, 84–91.
- Perry, R.J., Camporez, J.P., Kursawe, R., Titchenell, P.M., Zhang, D., Perry, C.J., Jurczak, M.J., Abudukadier, A., Han, M.S., Zhang, X.M., et al. (2015). Hepatic acetyl CoA links adipose tissue inflammation to hepatic insulin resistance and type 2 diabetes. *Cell* **160**, 745–758.
- Petersen, K.F., Oral, E.A., Dufour, S., Befroy, D., Ariyan, C., Yu, C., Cline, G.W., DePaoli, A.M., Taylor, S.I., Gorden, P., and Shulman, G.I. (2002). Leptin reverses insulin resistance and hepatic steatosis in patients with severe lipodystrophy. *J. Clin. Invest.* **109**, 1345–1350.
- Petersen, K.F., Dufour, S., Befroy, D., Lehrke, M., Hendler, R.E., and Shulman, G.I. (2005). Reversal of nonalcoholic hepatic steatosis, hepatic insulin resistance, and hyperglycemia by moderate weight reduction in patients with type 2 diabetes. *Diabetes* **54**, 603–608.
- Petersen, K.F., Dufour, S., Savage, D.B., Bilz, S., Solomon, G., Yonemitsu, S., Cline, G.W., Befroy, D., Zemany, L., Kahn, B.B., et al. (2007). The role of skeletal muscle insulin resistance in the pathogenesis of the metabolic syndrome. *Proc. Natl. Acad. Sci. USA* **104**, 12587–12594.
- Petersen, M.C., Madiraju, A.K., Gassaway, B.M., Marcel, M., Nasiri, A.R., Butrico, G., Marcucci, M.J., Zhang, D., Abulizi, A., Zhang, X.M., et al. (2016). Insulin receptor Thr1160 phosphorylation mediates lipid-induced hepatic insulin resistance. *J. Clin. Invest.* **126**, 4361–4371.
- Rabøl, R., Petersen, K.F., Dufour, S., Flannery, C., and Shulman, G.I. (2011). Reversal of muscle insulin resistance with exercise reduces postprandial hepatic de novo lipogenesis in insulin resistant individuals. *Proc. Natl. Acad. Sci. USA* **108**, 13705–13709.
- Samuel, V.T., and Shulman, G.I. (2016). The pathogenesis of insulin resistance: Integrating signaling pathways and substrate flux. *J. Clin. Invest.* **126**, 12–22.
- Samuel, V.T., Liu, Z.X., Qu, X., Elder, B.D., Bilz, S., Befroy, D., Romanelli, A.J., and Shulman, G.I. (2004). Mechanism of hepatic insulin resistance in non-alcoholic fatty liver disease. *J. Biol. Chem.* **279**, 32345–32353.
- Samuel, V.T., Liu, Z.X., Wang, A., Beddow, S.A., Geisler, J.G., Kahn, M., Zhang, X.M., Monia, B.P., Bhanot, S., and Shulman, G.I. (2007). Inhibition of protein kinase Cepsilon prevents hepatic insulin resistance in nonalcoholic fatty liver disease. *J. Clin. Invest.* **117**, 739–745.
- Sanyal, A.J., Campbell-Sargent, C., Mirshahi, F., Rizzo, W.B., Contos, M.J., Sterling, R.K., Luketic, V.A., Shiffman, M.L., and Clore, J.N. (2001). Nonalcoholic steatohepatitis: Association of insulin resistance and mitochondrial abnormalities. *Gastroenterology* **120**, 1183–1192.
- Schmitz-Peiffer, C., and Biden, T.J. (2008). Protein kinase C function in muscle, liver, and beta-cells and its therapeutic implications for type 2 diabetes. *Diabetes* **57**, 1774–1783.



- Seemple, R.K., Sleight, A., Murgatroyd, P.R., Adams, C.A., Bluck, L., Jackson, S., Vottero, A., Kanabar, D., Charlton-Menys, V., Durrington, P., et al. (2009). Postreceptor insulin resistance contributes to human dyslipidemia and hepatic steatosis. *J. Clin. Invest.* *119*, 315–322.
- Seppälä-Lindroos, A., Vehkavaara, S., Häkkinen, A.M., Goto, T., Westerbacka, J., Sovijärvi, A., Halavaara, J., and Yki-Järvinen, H. (2002). Fat accumulation in the liver is associated with defects in insulin suppression of glucose production and serum free fatty acids independent of obesity in normal men. *J. Clin. Endocrinol. Metab.* *87*, 3023–3028.
- Shulman, G.I. (2000). Cellular mechanisms of insulin resistance. *J. Clin. Invest.* *106*, 171–176.
- Steele, R. (1959). Influences of glucose loading and of injected insulin on hepatic glucose output. *Ann. N Y Acad. Sci.* *82*, 420–430.
- Szczepaniak, L.S., Nurenberg, P., Leonard, D., Browning, J.D., Reingold, J.S., Grundy, S., Hobbs, H.H., and Dobbins, R.L. (2005). Magnetic resonance spectroscopy to measure hepatic triglyceride content: Prevalence of hepatic steatosis in the general population. *Am. J. Physiol. Endocrinol. Metab.* *288*, E462–E468.
- ter Horst, K.W., Gilijamse, P.W., Koopman, K.E., de Weijer, B.A., Brands, M., Kootte, R.S., Romijn, J.A., Ackermans, M.T., Nieuwdorp, M., Soeters, M.R., and Serlie, M.J. (2015). Insulin resistance in obesity can be reliably identified from fasting plasma insulin. *Int. J. Obes.* *39*, 1703–1709.
- van der Valk, F., Hassing, C., Visser, M., Thakkar, P., Mohanan, A., Pathak, K., Dutt, C., Chauthaiwale, V., Ackermans, M., Nederveen, A., et al. (2014). The effect of a diiodothyronine mimetic on insulin sensitivity in male cardio-metabolic patients: A double-blind randomized controlled trial. *PLoS ONE* *9*, e86890.
- Vatner, D.F., Majumdar, S.K., Kumashiro, N., Petersen, M.C., Rahimi, Y., Gattu, A.K., Bears, M., Camporez, J.P., Cline, G.W., Jurczak, M.J., et al. (2015). Insulin-independent regulation of hepatic triglyceride synthesis by fatty acids. *Proc. Natl. Acad. Sci. USA* *112*, 1143–1148.
- Visser, M.E., Lammers, N.M., Nederveen, A.J., van der Graaf, M., Heerschap, A., Ackermans, M.T., Sauerwein, H.P., Stroes, E.S., and Serlie, M.J. (2011). Hepatic steatosis does not cause insulin resistance in people with familial hypobetalipoproteinaemia. *Diabetologia* *54*, 2113–2121.
- Wong, R.J., Cheung, R., and Ahmed, A. (2014). Nonalcoholic steatohepatitis is the most rapidly growing indication for liver transplantation in patients with hepatocellular carcinoma in the U.S. *Hepatology* *59*, 2188–2195.
- Xia, J.Y., Holland, W.L., Kusminski, C.M., Sun, K., Sharma, A.X., Pearson, M.J., Sifuentes, A.J., McDonald, J.G., Gordillo, R., and Scherer, P.E. (2015). Targeted induction of ceramide degradation leads to improved systemic metabolism and reduced hepatic steatosis. *Cell Metab.* *22*, 266–278.
- Yu, C., Chen, Y., Cline, G.W., Zhang, D., Zong, H., Wang, Y., Bergeron, R., Kim, J.K., Cushman, S.W., Cooney, G.J., et al. (2002). Mechanism by which fatty acids inhibit insulin activation of insulin receptor substrate-1 (IRS-1)-associated phosphatidylinositol 3-kinase activity in muscle. *J. Biol. Chem.* *277*, 50230–50236.

**Supplemental Information**

**Hepatic Diacylglycerol-Associated Protein**

**Kinase C $\epsilon$  Translocation Links Hepatic**

**Steatosis to Hepatic Insulin Resistance in Humans**

**Kasper W. ter Horst, Pim W. Gilijamse, Ruth I. Versteeg, Mariette T. Ackermans, Aart J. Nederveen, Susanne E. la Fleur, Johannes A. Romijn, Max Nieuwdorp, Dongyan Zhang, Varman T. Samuel, Daniel F. Vatner, Kitt F. Petersen, Gerald I. Shulman, and Mireille J. Serlie**

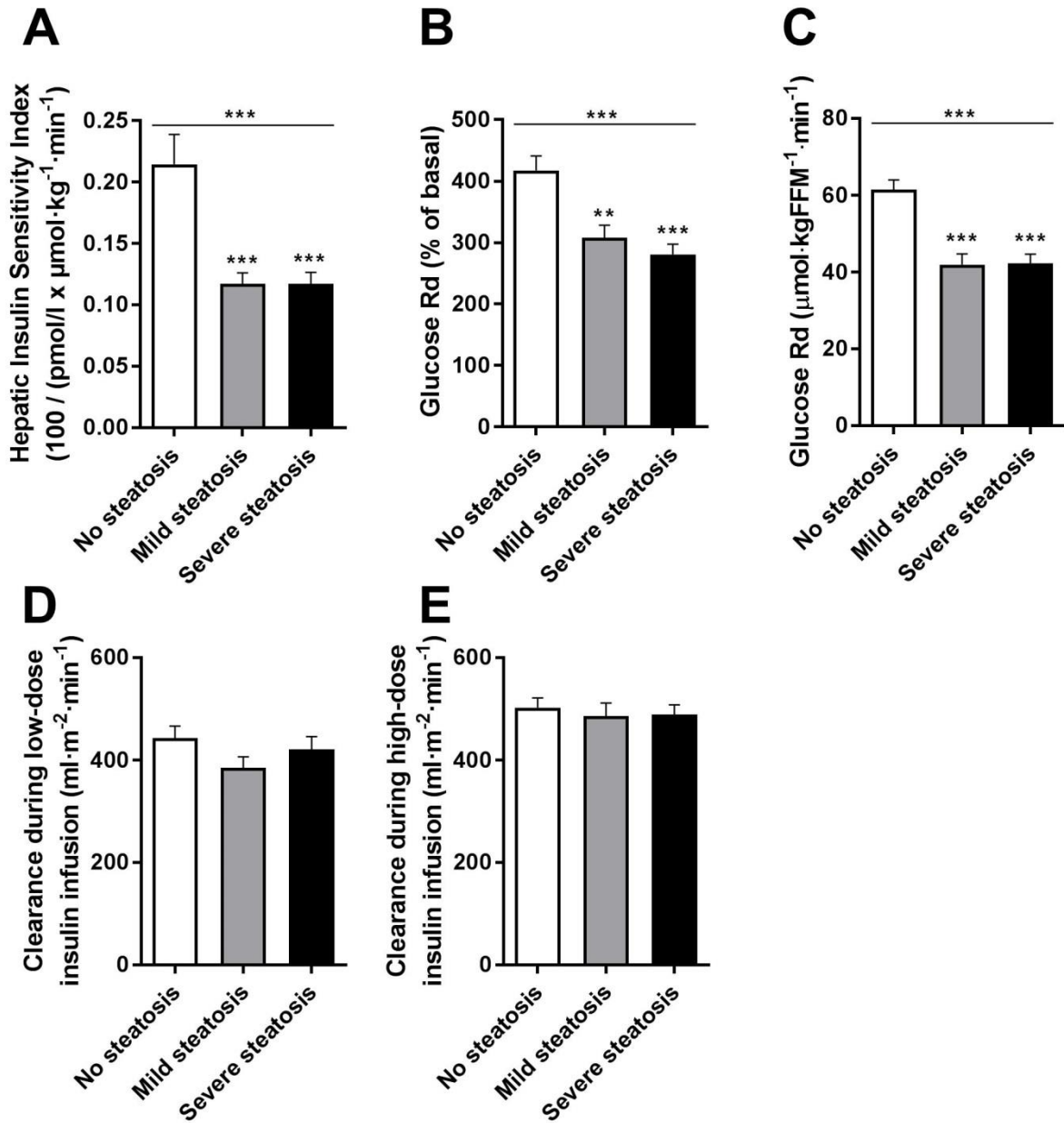
**Table S1. Hormones and Metabolites during Hyperinsulinemic-Euglycemic Clamp Studies. Related to Figures 1 and 2.**

|                                     | No steatosis<br>(n = 52) | Mild steatosis<br>(n = 41) | Severe steatosis<br>(n = 40) | p <sup>a</sup> |
|-------------------------------------|--------------------------|----------------------------|------------------------------|----------------|
| Basal (fasting)                     |                          |                            |                              |                |
| Insulin (pmol/l)                    | 84 ± 5                   | 131 ± 9**                  | 145 ± 16***                  | < 0.001        |
| Glucagon (ng/l)                     | 94 ± 5                   | 98 ± 6                     | 81 ± 5                       | 0.129          |
| Cortisol (nmol/l)                   | 228 ± 13                 | 214 ± 14                   | 213 ± 22                     | 0.754          |
| FA (mmol/l)                         | 0.66 ± 0.03              | 0.65 ± 0.03                | 0.63 ± 0.03                  | 0.702          |
| Low-dose insulin infusion (step 1)  |                          |                            |                              |                |
| Insulin (pmol/l)                    | 301 ± 13                 | 343 ± 18                   | 326 ± 24                     | 0.249          |
| Glucagon (ng/l)                     | 78 ± 4                   | 80 ± 5                     | 76 ± 4                       | 0.858          |
| Cortisol (nmol/l)                   | 271 ± 16                 | 252 ± 20                   | 259 ± 15                     | 0.562          |
| FA (mmol/l)                         | 0.11 ± 0.01              | 0.16 ± 0.01*               | 0.17 ± 0.01**                | 0.004          |
| High-dose insulin infusion (step 2) |                          |                            |                              |                |
| Insulin (pmol/l)                    | 765 ± 30                 | 811 ± 47                   | 777 ± 35                     | 0.664          |
| Glucagon (ng/l)                     | 67 ± 4                   | 70 ± 6                     | 65 ± 4                       | 0.778          |
| Cortisol (nmol/l)                   | 226 ± 16                 | 218 ± 20                   | 213 ± 15                     | 0.842          |
| FA (mmol/l)                         | 0.03 ± 0.00              | 0.04 ± 0.00                | 0.05 ± 0.00*                 | 0.009          |

Data are mean ± SEM. No, mild and severe hepatic steatosis were defined as IHTG < 5.56%, 5.56 – 15%, or > 15%, respectively. Fatty acids (FA), intrahepatic triglycerides (IHTG).

<sup>a</sup> Groups were compared by 1-way ANOVA with Bonferroni correction for multiple testing.

\* p < 0.05 vs no steatosis. \*\* p < 0.01 vs no steatosis. \*\*\* p < 0.001 vs no steatosis.



**Figure S1. Additional Metabolic Flux Data. Related to Figure 1.**

(A) The Hepatic Insulin Sensitivity Index was reduced in subjects with hepatic steatosis, but did not differ between subjects with mild or severe steatosis. The index was calculated as  $100 / (\text{fasting plasma insulin} \times \text{basal endogenous glucose production})$ .

(B) Expressing peripheral insulin sensitivity as percentage increase relative to basal glucose uptake yielded similar results.

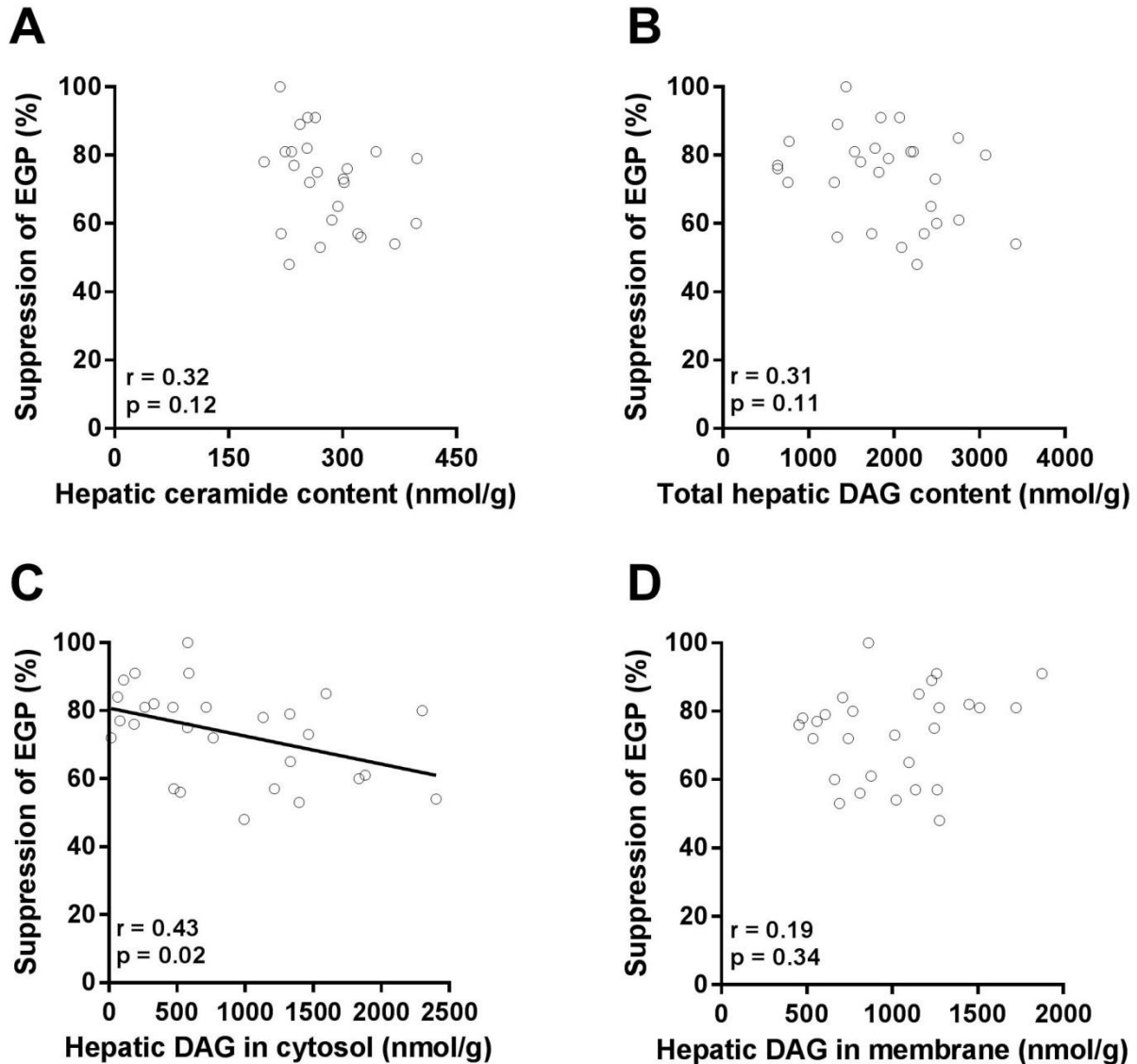
(C) Expressing peripheral insulin sensitivity relative to lean body mass yielded similar results.

(D) Insulin clearance during low-dose insulin infusion (step 1 of the clamp) did not differ between subjects with varying extent of steatosis. Insulin clearance was calculated as insulin infusion rate / plasma insulin concentration during the clamp.

(E) Insulin clearance during high-dose insulin infusion (step 2) did not differ between subjects with varying extent of steatosis.

Data are mean  $\pm$  SEM (n = 133). Rate of disappearance (Rd), fat-free mass (FFM).

\*\* p < 0.01 vs no steatosis. \*\*\* p < 0.001 vs no steatosis.



**Figure S2. Hepatic DAG Accumulation in the Cytosolic Fraction, but Not Total DAG Content, Membrane DAG Content, or Ceramide Content, Predicted Hepatic Insulin Sensitivity in Obese Subjects. Related to Figure 3.**

- (A) Hepatic ceramide content and insulin suppression of EGP.
- (B) Total hepatic DAG content and insulin suppression of EGP.
- (C) Hepatic DAG content in the cytosolic fraction and insulin suppression of EGP.
- (D) Hepatic DAG content in the membrane fraction and insulin suppression of EGP.

Lines were fitted by least squares method ( $n = 25-29$ ). Diacylglycerol (DAG), endogenous glucose production (EGP).

**Table S2. Correlations Between Individual Hepatic DAG Species and Insulin Sensitivity of EGP Suppression (n = 29). Related to Figure 3.**

| Species     | Cytosolic fraction |              |                                    | Membrane fraction |              |                                    |
|-------------|--------------------|--------------|------------------------------------|-------------------|--------------|------------------------------------|
|             | r <sup>a</sup>     | p            | Abundance<br>[mean ± SEM (nmol/g)] | r <sup>a</sup>    | p            | Abundance<br>[mean ± SEM (nmol/g)] |
| C18:1-C16:0 | <b>-0.44</b>       | <b>0.019</b> | <b>222.6 ± 33.9</b>                | 0.01              | 0.948        | 215.2 ± 14.6                       |
| C20:4-C20:5 | -0.12              | 0.551        | 17.1 ± 2.3                         | <b>0.41</b>       | <b>0.028</b> | <b>84.7 ± 6.9</b>                  |
| C16:0-C20:4 | -0.36              | 0.061        | 0.7 ± 0.1                          | 0.39              | 0.041        | 1.6 ± 0.1                          |
| C18:0-C20:4 | -0.27              | 0.172        | 0.6 ± 0.1                          | 0.41              | 0.032        | 2.0 ± 0.1                          |
| C18:0-C18:0 | -0.47              | 0.012        | 15.2 ± 2.1                         | -0.12             | 0.559        | 15.3 ± 0.9                         |
| C18:2-C18:0 | -0.33              | 0.083        | 7.9 ± 1.2                          | 0.34              | 0.078        | 12.9 ± 0.8                         |
| C18:1-C18:0 | -0.42              | 0.027        | 27.6 ± 4.2                         | 0.06              | 0.760        | 32.8 ± 2.4                         |
| C16:0-C16:0 | <b>-0.47</b>       | <b>0.013</b> | <b>73.5 ± 12.0</b>                 | -0.21             | 0.296        | 71.0 ± 4.5                         |
| C18:0-C16:0 | -0.45              | 0.015        | 23.5 ± 3.5                         | -0.10             | 0.630        | 20.6 ± 1.3                         |
| C18:1-C18:1 | <b>-0.41</b>       | <b>0.030</b> | <b>326.4 ± 51.2</b>                | 0.17              | 0.377        | 377.4 ± 34.2                       |
| C18:1-C18:2 | -0.33              | 0.091        | 59.6 ± 9.5                         | 0.34              | 0.076        | 65.8 ± 6.8                         |
| C18:2-C18:2 | -0.23              | 0.233        | 9.8 ± 1.6                          | 0.38              | 0.044        | 10.4 ± 1.1                         |
| C16:0-C18:2 | -0.33              | 0.085        | 79.0 ± 12.4                        | 0.29              | 0.130        | 100.9 ± 5.9                        |

Highly abundant species that were also related to hepatic insulin sensitivity are highlighted. Diacylglycerol (DAG), endogenous glucose production (EGP).

<sup>a</sup> Correlations were evaluated by Pearson's correlation coefficient.

**Table S3. Correlations Between Individual Hepatic Ceramide Species and Insulin Sensitivity of EGP Suppression (n = 25). Related to Figure 3.**

| Species | r <sup>a</sup> | p     | Abundance<br>[mean ± SEM (nmol/g)] |
|---------|----------------|-------|------------------------------------|
| C16:0   | -0.27          | 0.187 | 58.8 ± 2.1                         |
| C18:0   | -0.24          | 0.254 | 16.6 ± 0.6                         |
| C20:0   | -0.28          | 0.180 | 43.8 ± 6.6                         |
| C22:0   | -0.27          | 0.190 | 65.1 ± 2.4                         |
| C24:1   | -0.16          | 0.447 | 40.9 ± 1.2                         |
| C24:0   | -0.06          | 0.779 | 52.4 ± 2.1                         |

Endogenous glucose production (EGP).

<sup>a</sup> Correlations were evaluated by Pearson's correlation coefficient.

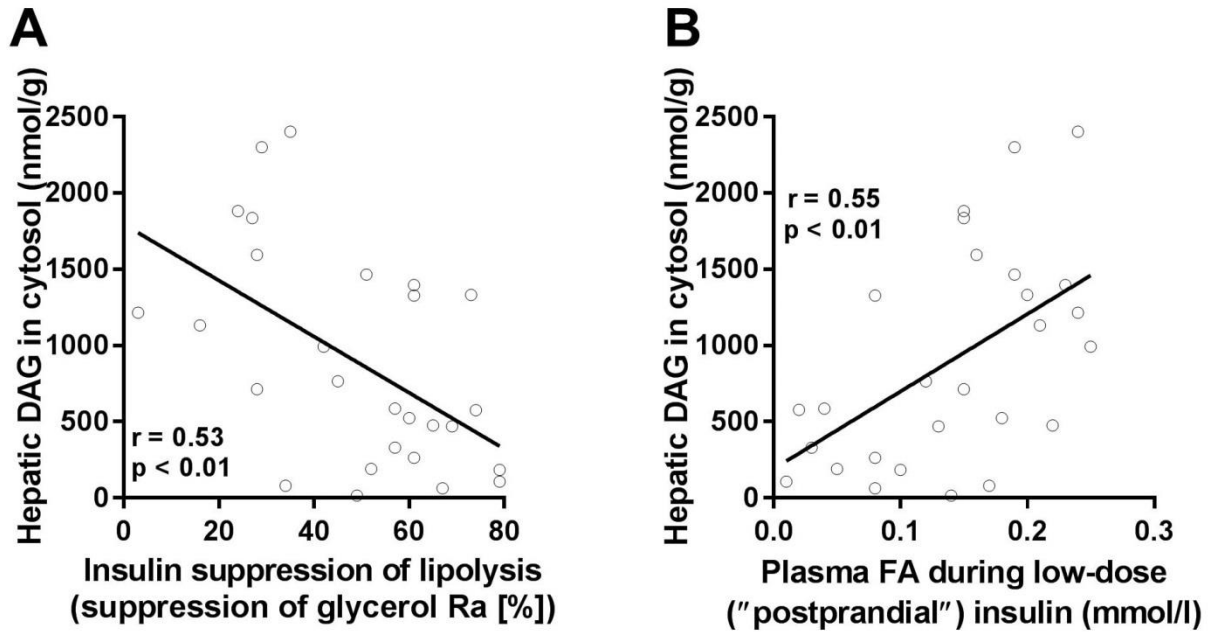


**Table S4. Characteristics of Subjects with Normal Hepatic Insulin Sensitivity or with Hepatic Insulin Resistance, Who Had Liver Biopsies Taken during Bariatric Surgery. Related to Figure 3.**

|   | Sensitive<br>(n = 10) | Resistant<br>(n = 8) | p <sup>a</sup> |
|---|-----------------------|----------------------|----------------|
| Female sex  | 6 (60)                | 3 (38)               | 0.343          |
| Age (years)   | 51 ± 2                | 45 ± 4               | 0.135          |
| Body mass index (kg/m <sup>2</sup> )                  | 41 ± 1                | 47 ± 3               | 0.055          |
| Body fat content (%)                                  | 45 ± 1                | 47 ± 3               | 0.603          |
| Fasting glucose (mmol/l)                              | 4.8 ± 0.1             | 5.5 ± 0.3            | 0.013          |
| Fasting insulin (pmol/l)                              | 115 ± 20              | 183 ± 20             | 0.029          |
| Basal EGP (μmol·kg <sup>-1</sup> ·min <sup>-1</sup> ) | 6.9 ± 0.3             | 7.2 ± 0.3            | 0.518          |
| Insulin suppression of EGP (%)                        | 87 ± 2                | 56 ± 1               | < 0.001        |
| IHTG content (%)                                      | 9.2 ± 3.0             | 14.2 ± 2.6           | 0.209          |

Data are count (%) or mean ± SEM. Hepatic insulin sensitivity and resistance were defined as insulin suppression of EGP > 80% and < 65%, respectively. Endogenous glucose production (EGP), intrahepatic triglyceride (IHTG).

<sup>a</sup> Groups were compared by 2-tailed independent samples t test.



**Figure S3. Release of FA from Lipolysis during Hyperinsulinemic Conditions Predicts Hepatic DAG Accumulation. Related to Figure 3.**

(A) The rate of lipolysis during hyperinsulinemic-euglycemic clamps was assessed by tracer-dilution of [1,1,2,3,3- $^2$ H<sub>5</sub>]glycerol.

(B) Plasma FA concentrations during hyperinsulinemia.

Data were fitted by least squares method ( $n = 28$ ). Diacylglycerol (DAG), fatty acids (FA).