Obesity, ectopic lipids, and insulin resistance

*Tissue-specific defects in nutrient handling*

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Citation for published version (APA):
CHAPTER 10

Hepatic diacylglycerol-associated protein kinase Cε translocation links hepatic steatosis to hepatic insulin resistance in humans


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 PKCε activation as reflected by PKCε translocation to the plasma membrane. These results demonstrate the relevance of hepatic diacylglycerol-induced PKCε 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 [312]. Ectopic triglyceride accumulation in the liver has clearly been linked to non-alcoholic steatohepatitis (NASH), liver cirrhosis, and hepatocellular carcinoma [405], but it is less clear whether there is a causal relationship between intrahepatic triglyceride (IHTG) accumulation and hepatic insulin resistance [55,406].

Elevated IHTG content is associated with hepatic and/or muscle insulin resistance in most, but not in all, human studies [69-71,407-412]. 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 Cε (PKCε), which may directly interfere with hepatic insulin signaling [21,60,72,73,413]. In this regard, PKCε has recently been shown to phosphorylate insulin receptor (INSR) threonine1160, which led to inhibition of INSR kinase activity [414]. Furthermore, mice with a threonine-to-alanine mutation at the homologous residue threonine1150 (InsrT1150A mice) were protected from high fat diet-induced hepatic insulin resistance and displayed increased hepatic insulin signaling, suppression of hepatic glucose production, and hepatic glycogen synthesis compared with WT controls during hyperinsulinemic clamp studies. These results provide a strong mechanistic link for DAG-PKCε-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 [415-417], 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 [418,419].

There are also circumstances, in both animals and humans, in which hepatic insulin resistance is dissociated from hepatic steatosis [64,420-425]. 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 [406]. Moreover, a better understanding of the subcellular compartmentalization of hepatic DAG species and PKCε may explain some of the apparent dissociations between hepatic steatosis and hepatic insulin resistance [21]. 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 PKCε as reflected by translocation of PKCε from the cytosol to the plasma membrane [60,73]. In contrast, antisense oligonucleotide knockdown of hepatic CGI-58 leads to hepatic steatosis, which is associated with considerable accumulation of DAGs in lipid droplets [74]. Contrary to the high fat-fed mice, this increase in lipid droplet DAGs is not associated with PKCε 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 (i) determine the relationship between liver fat content and insulin resistance using gold-standard metabolic flux measurements and (ii) determine whether the subcellular distribution of individual hepatic lipid species and PKCε 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 2-step hyperinsulinemic-euglycemic clamp technique in a cohort of 133 obese adults. Fifty-two subjects had normal IHTG by proton magnetic resonance spectroscopy (1H-MRS) (no steatosis, IHTG <5.56% [322]). Of the 81 subjects with 1H-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 10.1).

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<tr>
<th>Table 10.1. Characteristics of study participants according to extent of hepatic steatosis (n=133).</th>
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<tr>
<td>(n=52)</td>
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<tr>
<td>Female sex</td>
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<td>Age (years)</td>
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<tr>
<td>BMI (kg/m2)</td>
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<tr>
<td>Body fat (%)</td>
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<tr>
<td>Fasting glucose (mmol/l)</td>
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<tr>
<td>Triglycerides (mmol/l)</td>
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<td>AST (U/l)</td>
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<tr>
<td>γ-Glutamyl transpeptidase (U/l)</td>
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<tr>
<td>CRP (mg/l)</td>
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<tr>
<td>REE (kcal/day)</td>
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<td>IHTG (%)</td>
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Data are count (%) or mean ± SEM. ¹ No, mild, and severe steatosis were defined as IHTG <5.56%, 5.56–15%, or >15%, respectively. * p<0.05 vs no steatosis. ** p<0.01 vs no steatosis. *** p<0.001 vs no steatosis. † p<0.001 vs mild steatosis.

Hepatic steatosis was not associated with differences in the basal rate of endogenous glucose production (EGP) (Figure 10.1A). However, subjects with mild or severe
hepatic steatosis had higher fasting plasma insulin levels than subjects without hepatic steatosis (Supplemental Table S1). Since fasting insulin x basal EGP is an index of hepatic insulin resistance [70], this suggests that basal hepatic responsiveness to insulin may be impaired in obese subjects with steatosis (Supplemental 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 10.1B). In both steatosis groups, insulin suppression of plasma fatty free acids (FFA) and insulin stimulation of the glucose rate of disappearance (Rd) were also reduced, indicating adipose tissue and peripheral/muscle insulin resistance, respectively (Figures 10.1C-D and Supplemental Figures S1B-C). Notably, there were no differences in any of these parameters between subjects with mild or severe hepatic steatosis (Figures 10.1B-D). Finally, these results were not caused by differences in insulin clearance (Supplemental Figures S1D-E) or levels of glucoregulatory hormones during the clamps (Supplemental 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.

![Figure 10.1](image)

**Figure 10.1.** 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 FFA. (D) Peripheral insulin sensitivity is expressed as the insulin-stimulated Rd of glucose. Data are mean ± SEM (n=125-133). *** p≤0.001.

**Hepatic triglycerides 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 10.2A). 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 10.2A). Therefore, IHTG are not strictly sufficient or necessary for hepatic insulin resistance [406], at least in the conditions of our study. We observed similar relationships between IHTG and parameters of extrahepatic insulin sensitivity (Figures 10.2B-C). These observations are consistent with previous human studies that have demonstrated IHTG accumulation without insulin resistance [64,420] or insulin resistance in the absence of IHTG [425]. 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.

Figure 10.2 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).

Hepatic insulin resistance is associated with DAG accumulation in cytosol
Non-invasive measurement methods such as 1H-MRS cannot distinguish between inert lipids such as triglycerides [65] 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 week 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 (Supplemental Figures S2A-B). However, DAG may be present in the cell membrane, cytosol, and lipid droplets [74,418]. Using a subcellular fractionation method [74], 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 (Supplemental Figures S2C-D). 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 (not shown).
Finally, we analyzed individual hepatic DAG species in relation to hepatic insulin sensitivity (Supplemental 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 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 (Supplemental Table S3).

**Hepatic DAG-associated insulin resistance and PKCe translocation**

Hepatic DAGs are proposed to impair insulin signaling through activation of novel PKC [21, 406, 426]. In our prior study, we found that PKCe was the major novel PKC isoform expressed in human liver, and activation of PKCe correlated with hepatic DAG content in the cytosol and HOMA-IR [418]. However, the association between PKCe activation and insulin suppression of hepatic glucose output has not been reported in humans. Therefore, we measured PKCe translocation to the cell membrane as an index of PKCe activation [418, 427].

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 (Supplemental 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 10.3A-D). In fact, all individual DAG species were increased in the hepatic cytosol of insulin-resistant subjects (Figure 10.3E). Notably, hepatic DAG-associated hepatic insulin resistance was characterized by a 2-fold increase in translocation of PKCe from the cytosol to the membrane (Figures 10.3F-G). Taken together, this suggests that hepatic DAGs in the cytosol promote PKCe translocation from the cytosol to the membrane where it can bind INSR and phosphorylate threonine1160, which in turn inhibits INSR kinase activity [414].

**Sources for hepatic DAG accumulation in obese humans**

Hepatic DAG may accumulate from a variety of sources including circulating FFA derived from adipose tissue lipolysis [53, 428]. 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, 3H]glycerol during hyperinsulinemic conditions. Using this approach, we found that insulin-mediated suppression of the glycerol rate of appearance (Ra) and circulating FFA levels during insulin infusion both strongly correlated with hepatic DAG accumulation, indicating that higher release of FFA from lipolysis during hyperinsulinemic (postprandial) conditions is associated with increased cytosolic DAG content (Supplemental Figures S3A-B). In line, hepatic lipid synthesis in rats is primarily driven by substrate (FFA) delivery to the liver, which occurs in an insulin-independent manner [159]. Thus, these data are consistent with the possibility that most of the hepatic DAG derives from FFA 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.
Figure 10.3. Hepatic insulin resistance was strongly associated with elevated DAG content in the cytosolic fraction and PKCε 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 PKCε translocation from cytosol to membrane. (G) Translocation of PKCε from cytosol (c) to membrane (m) as an index of activation. Data are mean ± SEM (n=18-29). * p<0.05. ** p<0.01. *** p=0.001.
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 PKCε activation [21,414,418], 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 PKCε 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 PKCε 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. Firstly, the above-mentioned mechanism for lipid-induced hepatic insulin resistance should be appreciated in light of interconnected defects in other organ systems. For instance, extrahepatic regulation of adipose tissue lipolysis and FFA 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 [75]. Thus, PKCε inhibition of hepatic insulin signaling may mainly affect insulin regulation of net hepatic glycogen synthesis.

Secondly, previous studies have suggested that increased hepatic ceramide synthesis may be causal in lipid-induced hepatic insulin resistance [415-417], but ceramides were unrelated to hepatic insulin resistance in the present study. Others have also reported such dissociations [418,419,429]. 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 4 out of 5 DAG species [417]. The authors did not 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 [417]. We have also reported that hepatic steatosis in the context of familial hypobetalipoproteinemia is not
associated with hepatic insulin resistance [64], 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 if the I148M and other variants play a role in the susceptibility to lipid mediated-insulin resistance.

Thirdly, 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 FFA) and stimulus (insulin signaling through sterol regulatory element-binding protein 1c) for hepatic triglyceride synthesis and de novo lipogenesis [59,159,430-432].

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 PKCε activation in hepatic insulin resistance [21,72,73,414]. These data offer further support for the DAG-PK- Cε hypothesis for lipid-induced hepatic insulin resistance in NAFLD and support the development of interventions that target hepatic DAG-induced PKCε 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, Netherlands). Treatment-naive subjects were included in this study if they were obese (body mass index (BMI) ≥30 kg/m²) with stable weight (<5% weight change) for >3 months prior to examinations, and if they had undergone a 2-step hyperinsulinemic-euglycemic clamp study according to standard operating procedures and liver 1H-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 (that is, NAFLD/NASH, dyslipidemia, hypertension, or 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; Rayleigh, UK). Resting energy expenditure was assessed by indirect calorimetry (Vmax Encore 29n; CareFusion, San Diego, CA, USA). 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 1H-MRS as described [135]. This method has high diagnostic accuracy and high precision with low variability for assessment of hepatic steatosis in NAFLD [323].
**Hyperinsulinemic-euglycemic clamps**

Glucose kinetics and tissue-specific parameters of insulin sensitivity were assessed during a 2-step hyperinsulinemic-euglycemic clamp study as described [184]. Briefly, subjects were admitted to the metabolic unit after an overnight fast. A primed continuous infusion of the stable isotope-labeled glucose tracer \([6,6-^2\text{H}_2]\text{glucose} (>99\% \text{ enriched}; \text{Cambridge Isotopes, Andover, MA, USA})\) was started. In a subset of subjects (n=29), we also infused the stable isotope-labeled glycerol tracer \([1,1,2,3,3-^2\text{H}_5]\text{glycerol} (>99\% \text{ enriched}; \text{Cambridge Isotopes, Andover, MA, USA})\). Basal rates of EGP and lipolysis were determined after 2 h 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 FFA levels or suppression of glycerol Ra by insulin) were assessed after 2 h of low-dose insulin infusion (Actrapid 20 mU·m⁻²·min⁻¹; Novo Nordisk Farma, Alphen aan de Rijn, Netherlands). Finally, peripheral insulin sensitivity (expressed as insulin-stimulated Rd of glucose) was assessed after 2 h of high-dose insulin infusion (60 mU·m⁻²·min⁻¹). During hyperinsulinemia, plasma glucose was maintained at 5.0 mmol/l by frequent bedside monitoring and variable infusion of exogenous glucose (enriched with 1% \([6,6-^2\text{H}_2]\text{glucose}\)).

**Liver biopsies**

A subset of subjects (n=29) underwent bariatric surgery shortly (<2 wk) 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 FFA were measured as described [184]. Enrichments of \([6,6-^2\text{H}_2]\text{glucose}\) and \([1,1,2,3,3-^2\text{H}_5]\text{glycerol}\) (tracer-to-tracee ratios) were measured by gas chromatography-mass spectrometry [136].

**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 [418]. Subcellular fractionation into membrane and cytosolic compartments was performed as reported [74].

**PKCε translocation assay**

Membrane translocation of PKCε was assayed as described [418] 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 µmol·(kg body weight)⁻¹·min⁻¹ [41].
Statistical analyses
Groups were compared by 2-tailed t test (2 groups) or 1-way ANOVA with Bonferroni correction (>2 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 v23 (Armonk, NY, USA) and GraphPad Prism v6 (La Jolla, CA, USA).

Supplemental information to this chapter is available online (http://www.cell.com/cell-reports).