Mechanism of Hepatic Insulin Resistance in Non-alcoholic Fatty Liver Disease*

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In recent years, there has been an increasing appreciation for the significance of non-alcoholic fatty liver disease (NAFLD). Although the true prevalence is unknown, estimates of the prevalence of NAFLD in the general population range from 5 to 20% and up to 75% of patients with obesity and diabetes mellitus (1–3). While it is accepted that hepatic fat accumulation is linked to insulin resistance, the exact mechanism is unclear (4). Some investigators have postulated that with insulin resistance, the combination of elevated plasma concentrations of glucose and fatty acids promote hepatic fatty acid synthesis and impair β-oxidation leading to hepatic steatosis (4, 5). In contrast, others have proposed that hepatic fat accumulation and hepatic insulin resistance can occur without the development of peripheral insulin resistance (6, 7). However, the mechanism by which hepatic fat accumulation might lead to hepatic insulin resistance has not been resolved.

Determining the steps between hepatic fat accumulation and hepatic insulin resistance requires models in which hepatic fat accumulation occurs without peripheral fat accumulation. In a study examining the time course of hepatic and peripheral insulin resistance, Kraegen et al. (7) reported that rats fed a high fat diet for 3 days developed hepatic insulin resistance prior to the development of peripheral insulin resistance (7). We reasoned that feeding rats for a short duration would therefore provide an excellent model of NAFLD in which we could study the effect of hepatic fat accumulation on hepatic insulin responsiveness without the confounding effects of peripheral insulin resistance.

In the current study, rats were subjected to a 3 day high fat diet to simulate NAFLD. Glucose metabolism and insulin response were then determined with a hyperinsulinemic-euglycemic clamp. A low dose of the mitochondrial uncoupler, 2,4-dinitrophenol, was used to increase energy expenditure and prevent hepatic fat accumulation. In this way, it was possible to determine if the hepatic insulin resistance specifically depended on hepatic fat accumulation. In addition, the model was used to determine the impact of hepatic fat accumulation on the insulin signaling pathway, glycogen synthase (GS) activation, and possible mediators of fat-induced hepatic insulin resistance.

EXPERIMENTAL PROCEDURES

Animals and Diets—Normal, adult male Sprague-Dawley rats (300–350 g) were obtained from Charles River Labs (Wilmington, MA). The rats were placed on a 12-h daytime cycle and provided ad libitum access to food and water, except when specified by experimental protocol. They were housed individually and had their food consumption and weights measured daily. Rats received either regular rodent chow (60% CHO/10% fat/30% protein) or a high fat diet (26% CHO/59% fat/15% protein). Safflower oil was the major constituent of the high fat diet (Dyets Inc., Bethlehem, PA). Animals were fasted for 12 h prior to any study. The Yale Animal Care and Use Committee approved all protocols.

Hyperinsulinemic-Euglycemic Clamps—Five days prior to the clamp, indwelling catheters were implanted into the right jugular vein extending to the right atrium, and the right carotid artery extending to the aortic arch. The catheters were externalized through a subcutaneous channel at the back of the neck, sealed with a polyvinylpyrrolidone/heparin solution, and closed. Animals were allowed 2 days to recover from surgery before starting on the diet. After 3 days of either a control or high fat diet, the animals were fasted for 12 h prior to the clamp. A...
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primed (25 mg/kg/continuous 0.25 mg/kg/min) infusion of [U-13C]glucose (>98%, Cambridge Isotope Laboratories, Andover, MA) was started at 0 min. From 90 to 120 min of the basal period, plasma samples are obtained every 10 min to determine the plasma enrichment of glucose[1-13C]. After the basal period, the animals received a primed (150 milliunits/kg/continuous 4 milliunits/kg/min) infusion of insulin and a variable infusion of unlabeled 20% glucose to maintain euglycemia (<100 mg/dl). Plasma samples were taken every 10 min to determine the steady state enrichment of glucose[1-13C] from 90 to 120 min of the hyperinsulinemic-euglycemic clamp. At the end of the clamp, the tissues were harvested in situ with aluminum tongs precooled in liquid nitrogen and stored at −80 °C.

Plasma samples were deproteinized with 5 volumes of 100% methanol, and precipitated with 1:1 acetic acid/dry ice solution. The precipitate was dissolved in 0.1 M NaOH. The resulting pentacetate derivative of glucose was then analyzed by GCMS analysis using a Hewlett-Packard 5890 gas chromatograph interfaced to a Hewlett-Packard 5971A mass selective detector operating the chemical ionization mode. Glucose[1-13C] enrichment was determined from the ratio of m/z 337:331.

Glucose incorporation into glycogen under hyperinsulinemic-euglycemic conditions was done by omitting the basal infusion to avoid contaminating the glycogen pool with [U-13C]glucose and by using 20% glucose that was 20% enriched with [U-13C]glucose. This higher level of plasma enrichment insured satisfactory detection of [U-13C]glucose incorporation. The glycogen was subsequently isolated and completely digested with amylglucosidase. The resulting glucose concentration was measured by the glucose oxidase method (Glucose Analyzer II, Beckman Instruments, Fullerton, CA). Glucose[1-13C] enrichment was then analyzed by GCMS as described above.

2-Deoxyglucose Uptake in Vitro—Measurement of 2-deoxyglucose uptake in isolated soleus strips was done as described previously (9). After an overnight fast, rats were anesthetized and had soleus muscles dissected out. The soleus muscles were split to yield −30 mg strips, which were held under resting tension between metal clips. The muscle strips were allowed 40 min of recovery in Krebs-Henseleit bicarbonate buffer (KHB) supplemented with 2 mM pyruvate, 0.1% bovine serum albumin (BSA), and 1 µCi/ml [3H]2-deoxyglucose. After further washing of the buffer, the muscles were incubated with horseradish peroxidase-conjugated IgG fraction of goat anti-rabbit IgG (Bio-Rad) diluted 1:5000 in PBS-T for 2 h. 2-Pc-Jun-GST signal was then normalized to the amount of JNK antibody to determine the efficiency of the immunoprecipitation. The 2-Pc-Jun-GST signal was then normalized to the amount of JNK present in the immunoprecipitates.

JNK1 Immunoprecipitation and Activity—For JNK1 immunoprecipitation, 100 mg of liver tissues were lysed with Triton X-100 lysis buffer (50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 2 mM Na3VO4, 20 mM Na2HPO4, 100 mM NaF, 1% Triton X-100, 2 mM phenylmethylsulfonyl fluoride, 1 µg/ml pepstatin, and leupeptin, pH 7.4). The 8 mg of cell lysate was preincubated with protein A/G-Sepharose for 1 h at 4 °C with gentle rocking. Either polyclonal anti-IRS-1 or polyclonal anti-JNK1 antibodies were added and incubated at 4 °C overnight with gentle rocking. Immune complexes were collected by incubation with protein A/G, washed three times with 1 ml of ice-cold lysis buffer, resuspended in Laemmli sample buffer, and separated using 8% SDS-PAGE. For association of JNK1 and IRS2, 20 µg of rabbit polyclonal IRS2 was linked to gel matrix using the Seize Primary kit (Pierce). This was then incubated with 2 µg of preincubated cell lysate at 4 °C overnight. After washing three times with TBS, the proteins were eluted in three fractions with elution buffer. The first fraction contained the majority of the immunocomplexes and was used for radioimmunoprecipitation analysis. Proteins were electrophoretically transferred to Immobilon-P membranes (Millipore, Billerica, MA) and immunoblotted with the appropriate antibody followed by detection using ECL chemiluminescence (Amersham Biosciences). JNK1 activity assay was measured using the SAPK/JNK assay kit (Upstate, Charlottesville, VA). After autoradiography was performed to detect 32P-labeled c-Jun-GST, the membrane was blotted with anti-JNK antibody to determine the efficiency of the immunoprecipitation. The 32P-c-Jun-GST signal was then normalized to the amount of JNK present in the immunoprecipitates.

mRNA Analyses—Liver and muscle were harvested in situ using tongs precooled in liquid nitrogen. The mRNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA). Transcripts were analyzed by Northern blot using 32P-labeled cDNA probes for pyruvate carboxylase, phosphoenolpyruvate carboxykinase, fructose-1,6-bisphosphatase, and glucose-6-phosphatase and normalized to β-actin. Images obtained on a phosphorimager screen were analyzed using the Storm system with ImageQuant software (Amersham Biosciences).

Calculations—Rates of whole body glucose uptake and basal glucose turnover were determined as the ratio of the [U-13C]glucose infusion rate (mg per kg per minute) to the atom percent enrichment of glucose[1-13C] (%) during steady state of the basal and clamped periods. Endogenous glucose production (EGP) during the clamp procedure was determined by subtracting the glucose infusion rate from whole body glucose uptake.

Statistics—All values are presented as the mean ± S.E. A two-way Student’s t test was performed to determine difference between the control and treated group. Significance was accepted at p < 0.05. For multiple comparisons between groups, ANOVA was performed followed by Bonferroni’s t test.

RESULTS

Baseline Characteristics—The average caloric intake and weight gain were similar in both control and high fat-fed animals (Table 1). Plasma glucose concentrations were not different between the two groups. While there was a trend for increased peripheral insulin, this did not reach statistical significance (16 ± 4 versus 31 ± 6 microunits/ml, p = 0.08, n = 10 per group). There were no differences in portal insulin (46 ± 16

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TABLE I
Characteristics of rats fed 3 days of a control or high fat diet

|                      | Control  | Fat-fed  | DNP-treated |
|----------------------|----------|----------|-------------|
| Weight (g)           | 332 ± 3  | 329 ± 4  | 321 ± 6     |
| Weight gain (g)      | 23 ± 1   | 22 ± 2   | 15 ± 2°b    |
| Caloric consumption  | 354 ± 11 | 345 ± 9.6| 321 ± 10    |
| Glucose (mg/dl)      | 120 ± 3  | 114 ± 2  | 112 ± 3     |

°p = 0.008 versus Control.
°p = 0.019 versus Fat-fed by Bonferroni’s t test.

versus 63 ± 16 microunits/ml) or portal glucagon concentration (81 ± 8 versus 79 ± 13 pg/ml). Plasma leptin concentration in the FF group increased in a similar fashion to what has previously been reported (1.2 ± 0.14 versus 2.2 ± 0.2 mg/ml, p = 0.002) (17). Adiponectin concentrations were not significantly different between the two groups (2658 ± 283 versus 3302 ± 279 ng/ml, p = 0.12).

Plasma fatty acid concentration was measured at several points throughout the day (Table II). Immediately after food withdrawal, both peripheral and portal FA concentration were elevated in the fat-fed group. Thereafter, a 4 h, 8 h, and after an overnight fast, plasma fatty acid concentration in the peripheral blood was identical between the two groups. Surprisingly, the portal FA concentration after an overnight fast was nearly 50% lower in the fat-fed group compared with the control group. In addition, mesenteric weight, an indicator of visceral fat stores, was unchanged (2.80 ± 0.32 versus 2.96 ± 0.20, p = 0.49).

As shown in Fig. 1a, hepatic triglyceride content was increased in the fat-fed rats after 3 days of high fat feeding (4.9 ± 0.8 versus 16.2 ± 2.5 mg/g liver, p = 0.004). In contrast, there was no change in muscle triglyceride content (1.5 ± 0.2 versus 1.8 ± 0.2 mg/g muscle). Total fatty acyl-CoA concentrations were measured by LC/MS/MS (Fig. 1b). Fat feeding results in a 3-fold elevation in hepatic total fatty acyl-CoA (58.7 ± 4.5 versus 163.2 ± 23.2 nmol/g, p = 0.004) in the liver without a significant change in the muscle (12.4 ± 0.9 versus 20.8 ± 4.4, p = 0.11). Analysis of the species of fatty acyl-CoA revealed the major species in the tissues reflected the major dietary species (18:2 fatty acid or linoleic acid).

DNP Therapy for Fat-fed Animals—2,4-Dinitrophenol, has been used to promote fat oxidation by increasing energy expenditure through mitochondrial uncoupling (18). We used this agent as a pharmacological tool to prevent hepatic fat accumulation in fat-fed rats and examined whether or not this would prevent the development of hepatic insulin resistance. Separate groups of rats were subjected to either 3 days of fat feeding with 0.3 mg/g 2,4-dinitrophenol (16 mg/kg/day). During previous studies have shown that at doses below 20 mg/kg/day, no adverse affects have been observed (19). Compared with the control and fat-fed group, total weight gain over the 3 days was reduced by 30% in the DNP-treated group. There was no difference in intrahepatic ATP content, as assessed by 31P magnetic resonance spectroscopy. Analysis of liver fatty acyl-CoA content showed that DNP treatment in fat-fed rats prevented an increase in hepatic fat content (Fig. 2a). Plasma FA concentrations from both peripheral and portal samples were not different from the fat-fed animals (0.76 ± 0.08 and 0.78 ± 0.08 mmol/g for peripheral and portal samples, respectively).

Assessment of Peripheral Insulin Action—In order to assess the effects of 2,4-DNP on peripheral glucose metabolism 2-deoxyglucose uptake was performed in isolated soleus muscle strips and in vivo during hyperinsulinemic-euglycemic clamp conditions. There was no difference in basal 2-deoxyglucose uptake between any groups. Insulin increased the uptake of 2-deoxyglucose over the basal state similarly in soleus strips for all groups (fold increase over basal: 1.47 ± 0.44 versus 1.62 ± 0.30 versus 2.01 ± 0.56, for control, fat-fed, and DNP, respectively. ANOVA p = 0.56). There was no significant difference in 2-deoxyglucose uptake in the gastrocnemius muscle between control and fat-fed rats during the hyperinsulinemic-euglycemic clamp (71.7 ± 11 versus 48.0 ± 11 nmol/g/min, p = 0.17). DNP treatment did increase 2-deoxyglucose uptake as compared with the fat-fed rats but not the control rats (106.3 ± 11.9 nmol/g/min, p = 0.005 versus fat fed, p = 0.06 versus cont). Neither fasting nor DNP treatment altered epididymal adipose tissue 2-deoxyglucose uptake during hyperinsulinemic-euglycemic clamp conditions (10.3 ± 1.8 versus 17.0 ± 1.7 versus 12.0 ± 2.5 nmol/g/min for control, fat-fed, and DNP, respectively, ANOVA p = 0.10). In addition, during the hyperinsulinemic-euglycemic clamp, FA levels were suppressed to an
equal degree in all three groups (0.35 ± 0.12 versus 0.36 ± 0.02 versus 0.29 ± 0.04 mm for control, fat-fed, and DNP-treated respectively).

Hyperinsulinemic-Euglycemic Clamp—During the hyperinsulinemic-euglycemic clamp, insulin-stimulated peripheral glucose metabolism was similar between the control and fat-fed groups (23.2 ± 1.3 versus 25.4 ± 1.3 mg/kg/min, p = 0.27, Fig. 2b). Basal endogenous glucose production was similar in the control and fat-fed group (4.7 ± 0.4 versus 5.4 ± 0.4 mg/kg/min, p = 0.31). In contrast, insulin suppression of endogenous glucose production was impaired in the fat-fed group compared with the control group (74 ± 18% versus 8 ± 3%, p < 0.001) (Fig. 2c). DNP treatment did not affect either insulin-stimulated whole body glucose metabolism or basal endogenous glucose production (Fig. 2b and c). However, the ability of insulin to suppress endogenous glucose production was improved in the DNP group compared with the fat-fed group (74 ± 18% versus 8 ± 3%, p < 0.001) (Fig. 2c). DNP treatment improved insulin suppression of endogenous glucose production in the fat-fed group (8 ± 3% versus 39 ± 12%, p = 0.016, Fig. 2c). Furthermore, a positive linear correlation between liver triglyceride content and clamped EGP was observed (r² = 0.52, Fig. 2d).

Effect of Fat Feeding on Insulin Signaling Pathway—To determine the mechanism of fat-induced hepatic insulin resistance, the insulin signaling cascade was dissected into its key components. Activation of IR, IRS-1, and IRS-2 was determined by measuring the degree of tyrosine phosphorylation. The activity of the downstream kinases, IRS-2-associated PI 3-kinase, AKT2, and GSK3 were measured directly by immunoprecipitating the appropriate kinase and quantifying the phosphorylation on the target substrate. The results are reported as insulin-stimulated values compared with unstimulated values. As shown in Fig. 3c the increase in IR tyrosine phosphorylation was equal in both groups. However, the increase in both IRS-1 and IRS-2 tyrosine phosphorylation was diminished in the fat-fed group (Fig. 3, b and c). This block in IRS tyrosine phosphorylation was reflected in diminished activation of IRS-associated PI 3-kinase. Both IRS-1- and IRS-2-associated PI 3-kinase activity increased with insulin in the control rats but was unchanged in the fat-fed rats (Fig. 3d and e). This defect in insulin-stimulated IRS-1 and IRS-2 PI 3-kinase activity was prevented by DNP treatment in the fat-fed animals. This proximal block in the signaling cascade was propagated in the downstream signaling kinases. AKT2 activity increased 3.2 ± 0.15-fold with insulin stimulation in the control rats versus 1.0 ± 0.06-fold in the fat-fed rats (Fig. 4a). Again, this block in insulin-stimulated AKT2 activation was prevented with DNP treatment (fold increase 5.4 ± 1.1). Glycogen synthase kinase 3 (GSK-3) tonically phosphorylates and inactivates glycogen synthase. When it is phosphorylated by AKT2 it is inactivated. Thus the net effect would be to allow dephosphorylation and activation of glycogen synthase. GSK-3 activity was decreased to a greater extent in the control animals than in the fat-fed animals (Fig. 4b). DNP treatment in fat-fed
animals maintained the ability of insulin to deactivate GSK-3.

Effect of Fat Feeding on Glycogen Synthase and Glycogen Synthesis—The activity of GS in liver homogenates was measured in the basal and insulin-stimulated state. As shown in Fig. 5a, the ability of insulin to increase GS activity was diminished in the fat-fed animals. Insulin increased total GS activity 4.7 ± 0.5-fold in the control animals, but only by 2.4 ± 0.2-fold in the fat-fed animals (p = 0.002). Glycogen synthesis was also assessed in vivo during a hyperinsulinemic-euglycemic clamp by comparing the enrichment of [U-13C]glucose in plasma versus glycogen. As shown in Fig. 5b, the percent of glycogen synthesized via the direct pathway was 28% in the control compared with 11% in the fat-fed group (p = 0.04) consistent with increased gluconeogenesis in the fat-fed animals.

Effect of Fat Feeding on Protein Kinase C Activity—Activation of the novel PKC (PKC-θ, -δ, and -βIII) has been implicated in the pathogenesis of peripheral insulin resistance in rodents and humans (10, 20). We assessed the activities of the major hepatic isoforms of PKC (α, β, δ, ε, and θ) to determine if a
similar activation occurred in the liver. Measuring the relative abundance of the particular PKC isoform in the membrane and cytosol fractions reflected PKC activation. An increase in the membrane to cytosol ratio was taken as an indication of cytosol fractions reflected PKC activation. An increase in the abundance of the particular PKC isoform in the membrane and similar activation occurred in the liver. Measuring the relative abundance of the particular PKC isoform in the membrane and cytosol fractions reflected PKC activation.

Effect of Fat Feeding on JNK1 Activity—Recently, the c-Jun N-terminal kinase 1 (JNK1) has been found to play a role in the pathogenesis of fat-induced insulin resistance (22). The activity of JNK1 in immunoprecipitates was assessed against a synthetic c-Jun substrate. As shown in Fig. 7b, fat feeding resulted in a ~300% increase in JNK1 activity. This activation was prevented by DNP treatment. JNK1 is a serine/threonine kinase, which has putative phosphorylation sites on IRS-1 and IRS-2. In a co-immunoprecipitation experiment, JNK1 was found to bind to IRS-1 and IRS2 in both control and fat-fed rats (Fig. 7, b and d). Ser307 has previously been identified as a site for JNK serine phosphorylation. No difference in Ser307 phosphorylation was found between control and fat-fed rats (Fig. 7c).

DISCUSSION

Although an association between NAFLD and hepatic insulin resistance is clear, a causal relationship between hepatic fat accumulation and hepatic insulin resistance has not been established. In this report, we provide evidence to support the causal relationship between hepatic fat accumulation and hepatic insulin resistance. First, we show a specific relationship between hepatic fat accumulation and hepatic insulin resistance. Second, we demonstrate that preventing hepatic fat accumulation abrogates the development of hepatic insulin resistance. Third, we demonstrate a "dose" relationship between fat accumulation and insulin resistance. Finally, we provide evidence to suggest a cellular mechanism whereby hepatic fat accumulation can lead to hepatic insulin resistance.

The value of the current model is the specific increase in hepatic fat content without significant alteration of peripheral fat content. Three days of high fat feeding nearly triples hepatic TG and fatty acyl-CoA content. The similarity in fat composition between the liver and diet, both having an abundance of 18:2 fatty acid, suggest that the source of the liver fat is the diet. This magnitude of change was absent in the muscle.
Three days of high fat feeding did not alter fasting plasma glucose concentration or the basal rate of EGP. Insulin-stimulated whole body glucose utilization was similar in both groups, demonstrating that peripheral insulin sensitivity was unaltered. In contrast, short term high fat feeding resulted in marked hepatic insulin resistance: in the control group EGP was suppressed by 74% but only by 8% in the fat-fed group. Thus this model allowed us to examine the impact of hepatic fat accumulation on hepatic insulin action without the confounding influence of peripheral fat accumulation.

The association between fatty acids and hepatic insulin action has been previously studied using intravenous infusion of lipid + heparin to acutely raise fatty acids (23–25). These studies conclude that the apparent defects in hepatic glucose metabolism are a consequence of insulin resistance in the visceral adipose beds. In the present study, fat feeding elevated plasma fatty acids only in the immediate post-prandial state. At 4 h, 8 h, and after an overnight fast, the FA concentration was similar in the control and fat-fed group. Surprisingly, after an overnight fast, portal FA concentration was actually lower in the fat-fed animals than the control animals. Adipose insulin sensitivity was unchanged by fat feeding as demonstrated by epididymal 2-deoxyglucose uptake and suppression of plasma FA during the hyperinsulinemic-euglycemic clamp. Finally, mesenteric weight was unchanged by fat feeding, suggesting that visceral fat stores were similar. Thus, in this model, hepatic insulin resistance develops without any evident adipose tissue insulin resistance.

If hepatic fat accumulation is truly required for the development of hepatic insulin resistance, then preventing fat accumulation in fat-fed rats should prevent hepatic insulin resistance. This was accomplished by using a nontoxic dose of the mitochondrial uncoupler 2,4-dinitrophenol. DNP carries protons across the inner mitochondrial membrane and dissipates the potential of the proton gradient as heat. We hypothesized that low doses of DNP would increase energy expenditure and prevent hepatic fat accumulation in rats subjected to the same high fat diet. The concentration of DNP added to the high fat diet (0.3 mg/g), resulted in an average dose of 16.3 mg/kg/day. Based on a previous study of DNP toxicity, doses below 20 mg/kg resulted in no detectable adverse affects (19). Although there was no significant decrease in food intake, there was a
JNK1 immunoprecipitates are analyzed by immunoblotting for IRS1. Liver whole cell lysate is used as a positive control for IRS1. Primary kit, Pierce) were analyzed by immunoblotting for JNK1. IRS2 immunoprecipitates (using the Seize 

Evaluation of JNK activity. a, activity of JNK1 in immunoprecipitates against c-Jun target sequence. Values represent the mean ± S.E. of four animals per group. †, p < 0.005 versus control and #, p < 0.005 versus fat-fed by Bonferroni’s t test. b, association of IRS1 and JNK. Liver whole cell lysate is used as a positive control for IRS1. JNK1 immunoprecipitates are analyzed by immunoblotting for IRS1. c, liver IRS1 Ser307 phosphorylation. Whole cell lysates in basal and insulin-stimulated state used as a control for Ser307 phosphorylation. d, association of IRS2 and JNK1. Liver whole cell lysate is used as a positive control for JNK1. IRS2 immunoprecipitates (using the Seize Primary kit, Pierce) were analyzed by immunoblotting for JNK1.

30% reduction in weight gain suggesting some degree of metabolic uncoupling. However, the degree of uncoupling had no effect on intrahepatic ATP concentration. DNP also had no adverse affects on peripheral insulin action.

DNP treatment of fat-fed rats prevented the accumulation of fat and fatty acid metabolites within the liver. DNP treatment in fat-fed rats improved the hepatic insulin responsiveness, as gauged by the ability to suppress EGP during the hyperinsulinemic-euglycemic clamp. Furthermore, there was a direct linear relationship between hepatic fat content and hepatic insulin responsiveness, as reflected by EGP during the hyperinsulinemic-euglycemic clamp. These results demonstrate that preventing hepatic fat accumulation in rats on a high fat diet ameliorates hepatic insulin resistance.

We also examined the insulin signaling cascade to determine the mechanism whereby hepatic fat accumulation impairs hepatic insulin action. Although there was no effect of fat feeding on insulin receptor tyrosine phosphorylation, insulin-stimulated IRS-1 and IRS-2 tyrosine phosphorylation was blunted in the fat-fed animals. Furthermore, insulin activation of AKT2 and inactivation of GSK3 was impaired in the fat-fed animals. DNP treatment in fat-fed animals prevented the development of this proximal block in the insulin signaling cascade and preserved insulin-stimulated AKT2 activation and GSK3 inactivation. Thus hepatic fat accumulation appears to be specifically linked to the development of this impaired insulin signaling in fat-fed animals.

Ultimately, the defect in the signaling pathway affected insulin activation of glycogen synthase GS activity. As compared with a 4.7-fold insulin-stimulated increase in GS activity in the control group, GS activity increased by only 2.4-fold in the fat-fed group. Glycogen synthesis was also assessed in vivo using [U-13C]glucose, to determine the relative contributions of the direct and indirect (or gluconeogenic) pathways of glycogen synthesis. The percent of glycogen synthesized via the indirect pathway was 72% in the control group versus 89% in the fat-fed group demonstrating that hepatic steatosis is associated with increased gluconeogenesis. The mechanism for this increase is unclear. Transcription of the key gluconeogenic enzymes (phosphoenolpyruvate carboxykinase, pyruvate carboxylase, fructose-1,6-bisphosphatase, and glucose-6-phosphatase) was not altered by fat feeding (data not shown). The increase could be due to either allosteric activation or covalent modification of one of these enzymes or increased flux through this pathway driven by the availability of intracellular substrates (i.e. glycerol, Ref. 26).

Taken together, these data suggest that hepatic fat accumulation alone is insufficient to increase EGP but that it does cause hepatic insulin resistance. This can be attributed in part to decreased insulin-stimulated tyrosine phosphorylation of IRS-1 and IRS-2, which in turn blocks the ability of insulin to activate glycogen synthase and diminishes the ability of the liver to store glucose as glycogen. In addition, hepatic steatosis was associated with increased gluconeogenesis. This may maintain EGP under hyperinsulinemic conditions, given that higher insulin concentrations are required to suppress gluconeogenesis compared with glycogenolysis (27).

Previous reports have implicated activation of the novel PKCs in the pathogenesis of skeletal muscle insulin resistance in rodents (PKC-θ) (10, 11) and humans (PKC-δ and βII) (20). As shown in Fig. 6a, of all the isoforms assayed PKC-ε, a novel PKC, appeared to be the only one activated. In addition the finding that DNP treatment prevents activation of PKC-ε suggests that that its activation is specifically linked to hepatic steatosis. Lam et al. (21) have identified PKC-δ as a possible mediator for fat-induced hepatic insulin resistance (21). As opposed to a dietary challenge, they compared saline infused rats to Intralipid + heparin infused. Intralipid + heparin acutely raised plasma FA levels and cause both peripheral and hepatic insulin resistance, as compared with saline infused controls. Thus, the increase in circulating FA level and the development of peripheral insulin resistance distinguish this model from the dietary perturbation employed in this study and make direct comparison difficult. In the current study, there was neither increased membrane translocation of PKC-δ nor increased activity of PKC-δ immunoprecipitated from whole cell lysates. Based on the data presented, we conclude that hepatic PKC-ε is activated by accumulation of an intracellular fatty acid metabolite and may play role in the pathogenesis of hepatic insulin resistance. This may be analogous to the role other novel PKCs play in the pathogenesis of peripheral insulin resistance.

One possible target of PKC-ε is the c-Jun N-terminal kinase 1 (JNK1), a member of the mitogen-activated protein kinases. Studies in B lymphocytes and mouse epidermal cells suggest that JNK1 is activated by PKC in response to phorbol esters and ultraviolet light, respectively (28, 29). JNK1 was shown to play a key role in the pathogenesis of fat-induced insulin resistance, possibly caused by serine phosphorylation of IRS-1 (22, 30). JNK1 activity in liver extracts was also increased in

**Fig. 7.** Evaluation of JNK Activity. a, activity of JNK1 in immunoprecipitates against c-Jun target sequence. Values represent the mean ± S.E. of four animals per group. †, p < 0.005 versus control and #, p < 0.005 versus fat-fed by Bonferroni’s t test. b, association of IRS1 and JNK. Liver whole cell lysate is used as a positive control for IRS1. JNK1 immunoprecipitates are analyzed by immunoblotting for IRS1. c, liver IRS1 Ser307 phosphorylation. Whole cell lysates in basal and insulin-stimulated state used as a control for Ser307 phosphorylation. d, association of IRS2 and JNK1. Liver whole cell lysate is used as a positive control for JNK1. IRS2 immunoprecipitates (using the Seize Primary kit, Pierce) were analyzed by immunoblotting for JNK1.
the fat-fed animals. JNK1 was found to bind both IRS1 and IRS2 in both control and fat-fed rats. Despite this association and the increased JNK1 activity with fat feeding, we were unable to detect an increase in IRS1 Ser307 phosphorylation. Although other reports have shown increased hepatic IRS1 Ser307 phosphorylation (22, 31), the models used are different than the one employed here in that they are models of chronic insulin resistance affecting both the periphery and the liver. JNK-mediated serine phosphorylation of IRS1 may be a later event in the pathogenesis of insulin resistance that is not detectable in the early stages described here. Thus, while hepatic fat accumulation appears to be associated with increased JNK1 activity, the target proteins for this kinase remain unknown. Finally, both PKC-ε and JNK1 activation were prevented by DNP treatment, again suggesting that they have a specific role between hepatic fat accumulation and hepatic insulin resistance. Further studies will need to be conducted to determine the exact role of each of serine/threonine kinases in the pathogenesis of fat induced hepatic insulin resistance.

In conclusion, the data presented in this manuscript support a causal role for intracellular hepatic fat accumulation in the pathogenesis of hepatic insulin resistance. Three days of high fat feeding specifically causes hepatic fat accumulation and hepatic insulin resistance in the absence of significant peripheral fat accumulation or peripheral insulin resistance. These changes were also not associated with any increases in visceral fat mass or portal vein fatty acid concentrations. Fat-induced hepatic insulin resistance may result from activation of PKC-ε and/or JNK1, which may then lead to impaired IRS-1 and IRS-2 tyrosine phosphorylation. This block in the insulin signaling pathway then limits the ability of insulin to activate glycogen synthase. In addition, fat accumulation increases the contribution of gluconeogenesis to total EGP. Increased mitochondrial uncoupling with low dose DNP therapy in fat-fed animals prevented hepatic fat accumulation and activation of PKC-ε and JNK1. This in turn preserved the insulin signaling cascade and attenuated the development of hepatic insulin resistance. These studies support the causal link between hepatic fat accumulation and hepatic insulin resistance.

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