HEPATOCYTE CYP2E1 OVEREXPRESSION AND STEATOHEPATITIS
LEAD TO IMPAIRED HEPATIC INSULIN SIGNALING

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Running Title: CYP2E1 overexpression impairs hepatic insulin signaling

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Summary

Insulin resistance and increased cytochrome P450 2E1 (CYP2E1) expression are both associated with and mechanistically implicated in the development of nonalcoholic fatty liver disease (NAFLD). Although currently viewed as distinct factors, insulin resistance and CYP2E1 expression may be interrelated through the ability of CYP2E1-induced oxidant stress to impair hepatic insulin signaling. To test this possibility, the effects of in vitro and in vivo CYP2E1 overexpression on hepatocyte insulin signaling were examined. CYP2E1 overexpression in a hepatocyte cell line decreased tyrosine phosphorylation of insulin receptor substrate (IRS)-1 and IRS-2 in response to insulin. CYP2E1 overexpression was also associated with increased inhibitory serine 307 and 636/639 IRS-1 phosphorylation. In parallel, the effects of insulin on Akt activation, glycogen synthase kinase 3 and FoxO1a phosphorylation, and glucose secretion were all significantly decreased in CYP2E1 overexpressing cells. This inhibition of insulin signaling by CYP2E1 overexpression was partially c-Jun N-terminal kinase (JNK) dependent. In the methionine and choline deficient diet mouse model of steatohepatitis with CYP2E1 overexpression, insulin-induced IRS-1, IRS-2 and Akt phosphorylation were similarly decreased. These findings indicate that increased hepatocyte CYP2E1 expression and the presence of steatohepatitis result in the down regulation of insulin signaling, potentially contributing to the insulin resistance associated with NAFLD.
Introduction

Nonalcoholic fatty liver disease (NAFLD) is a chronic disease characterized by hepatic steatosis and varying degrees of liver injury, inflammation and fibrosis. The prominent association of insulin resistance or type 2 diabetes mellitus with NAFLD has suggested that these conditions may contribute to the pathogenesis of NAFLD by promoting the initial development of steatosis, and/or the subsequent progression to the state of liver injury and inflammation termed nonalcoholic steatohepatitis (NASH) (1-3). NAFLD may therefore represent a hepatic manifestation of the metabolic syndrome (3,4). However, the function of insulin resistance in the development and progression of NAFLD remains to be established experimentally. An additional, and as yet unexamined, mechanism underlying the co-association of these two conditions could be that NAFLD-related changes in hepatic physiology act to promote insulin resistance.

The liver is an important regulator of carbohydrate homeostasis that stores or releases glucose according to metabolic demands. This process is under the control of insulin through signal transduction pathways initiated by insulin binding to the hepatocyte insulin receptor. Following this ligand-receptor interaction, a class of molecules known as insulin receptor substrates (IRS) are recruited and activated by tyrosine phosphorylation (5,6). The critical role of IRS in insulin responsiveness has been demonstrated by studies in which deletion of IRS-1 or IRS-2 resulted in hepatic insulin resistance and symptoms mimicking diabetes including increased gluconeogenesis and glycogenolysis (7-9). Tyrosine phosphorylated IRS recruits additional signaling molecules containing Src-homology-2 (SH2) domains, including phosphatidylinositol 3-kinase, Grb2, and Shp2. These signaling molecules activate downstream effectors that mediate the metabolic and growth stimulatory effects of insulin (10). In contrast,
increased phosphorylation of IRS serine residues results in decreased tyrosine phosphorylation, and the termination of insulin signaling (11,12). Tyrosine phosphatases (13) and serine kinases including c-Jun N-terminal kinase (JNK) (14), protein kinase C (PKC) (15), the mammalian target of rapamycin (12,16), and IκB kinase β (17) have been mechanistically implicated in this process. The principle downstream effector of insulin signaling is the protein kinase Akt as demonstrated by the fact that animals lacking the Akt2 isoform develop insulin resistance secondary to the loss of insulin’s effects on the liver (18). Upon activation, Akt phosphorylates and inactivates the inhibitory kinase of glycogen synthase, glycogen synthase kinase 3 (GSK3), and members of the forkhead box transcription factor family. These effects mediate an inhibition of gluconeogenesis and glycogenolysis (19).

One proposed mechanism of the decreased insulin signaling that underlies insulin resistance is oxidative stress. Oxidants activate stress-activated signaling pathways that have been implicated in the development of insulin resistance including JNK and PKC (20,21). Interestingly, oxidative stress is also an important component of NAFLD. Increased expression of the prooxidant cytochrome P450 2E1 (CYP2E1) isoform and elevated levels of lipid peroxidation occur in both the human disease and animal models of NASH (22-24). CYP2E1 has enhanced NADPH oxidase activity resulting in increased production of the reactive oxygen species superoxide and hydrogen peroxide by redox cycling of endogenous and exogenous substrates (25). These facts led us to hypothesize that chronic oxidative stress generated through CYP2E1 induction may promote the development of hepatic insulin resistance in NAFLD. To test this hypothesis we examined the effects of in vitro hepatocyte CYP2E1 overexpression and the in vivo development of steatohepatitis associated with CYP2E1 overexpression on insulin signaling.
Experimental Procedures

Cells and culture conditions

Studies were performed in the rat hepatocyte line RALA255-10G cultured as previously described (26). This hepatocyte cell line is conditionally immortalized with a mutant SV40 virus expressing a temperature-sensitive T antigen (27). Cells were cultured in Dulbecco’s modified Eagle medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 4% fetal bovine serum (Gemini, Woodland, CA), and antibiotics (Invitrogen) at the permissive temperature of 33°C in 60 mm dishes (Falcon Becton Dickinson, Lincoln Park, NJ). For experiments, cells were cultured in DMEM, 2% fetal bovine serum, antibiotics and 1 μM dexamethasone at the restrictive temperature of 37°C for 3 days and then placed in serum-free medium containing dexamethasone for 18 h. Under these conditions, T antigen expression is suppressed, the cells are nontransformed, and they display a differentiated hepatocyte phenotype (27).

From wild-type (WT) RALA255-10G cells, clonal cell lines with increased CYP2E1 expression were established by stable transfection with a pCI-Neo expression vector containing the human CYP2E1 cDNA, as previously described (28). Clones with increased CYP2E1 expression (S-CYP cells) were identified by both Western blotting for CYP2E1 protein levels, and chlorzoxazone assay for enzyme activity (28,29). A polyclonal cell line transfected with the pCI-Neo vector lacking any insert (VEC cells) was also established (29).

RALA hepatocytes were treated with 100 nM insulin (Sigma, St. Louis, MO) for the times indicated, and 20 ng/ml recombinant rat tumor necrosis factor-α (TNF) (R&D Systems, Minneapolis, MN) for 30 min.
**Protein isolation and subcellular fractionation**

For the isolation of total protein cells were harvested in phosphate buffered saline, centrifuged and resuspended in cell lysis buffer containing 20 mM Tris, pH 7.5, 1% Triton X-100, 1 mM ethylenediamine tetraacetic acid (EDTA), 1 mM ethylene glycol-bis(2-aminoethylether)-tetraacetic acid, and protease and phosphatase inhibitors previously described (29).

Subcellular fractionation of nuclear and cytosolic proteins was performed by hypotonic-hypertonic cell lysis. Cells were cultured in 100 mm tissue culture dishes as described above and lysed in hypotonic buffer containing 10 mM HEPES, pH 7.4, 10 mM sodium chloride, 0.1 mM EDTA, 0.4% nonident P-40 (NP-40), 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml pepstatin A, 1 μg/ml leupeptin, 5 mM sodium pyrophosphate, 2 mM β-glycerophosphate, and 2 mM sodium orthovanadate. Lysates were centrifuged at 800 g at 4°C to remove the cellular debris and subsequently centrifuged at 100,000 g at 4°C for 60 min. The resultant supernatant was the cytosolic fraction. The nuclei containing pellets were lysed for 30 min in hypertonic lysis buffer containing 20 mM HEPES, pH 7.4, 400 mM sodium chloride, 1 mM EDTA, 1% NP-40, and protease and phosphatase inhibitors. Protein concentrations were determined by the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA), according to the manufacturer’s instructions.

**Immunoprecipitations and Western blotting**

For immunoprecipitations, 500 μg of total cellular protein were incubated overnight with 0.5 μg of anti-IRS-1 or IRS-2 antibody and Protein A agarose beads (Sigma). Immunoprecipitates were washed three times in cold lysis buffer and agarose-bound protein
recovered in 2x sodium dodecyl sulfate (SDS) loading buffer containing 50 mM Tris, pH 6.8, 2% SDS, 0.1% bromophenol blue and 10% glycerol by boiling for 5 min.

Western blotting was performed by denaturing 50 μg of protein at 100°C for 5 min in Laemmli sample buffer containing 62.5 mM Tris, pH 6.8, 2% SDS, 25% glycerol, 0.01% bromophenol blue and 5% β-mercaptoethanol. Samples were applied to 8% SDS gels and resolved at 100 V over 3 h. Proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH) in transfer buffer containing 25 mM Tris, pH 8.3, 192 mM glycine, 0.01% SDS, and 15% methanol using a Bio-Rad Trans-blot SD semidry transfer cell to which 150 mA were applied for 90 min. Membranes were blocked in 5% non-fat dry milk, 20 mM Tris, pH 7.5, 500 mM sodium chloride, and 0.5% Tween 20 (TBS-T) for 1 h. Rabbit anti-IRS-1, mouse anti-phospho tyrosine, rabbit anti-phospho serine 307 IRS-1, rabbit anti-phospho serine 636/639 IRS-1, rabbit anti-phospho Akt, rabbit anti-Akt, rabbit anti-phospho GSK3, rabbit anti-GSK3, rabbit anti-phospho FKHR/RL1 (Cell Signaling, Beverly, MA), rabbit anti-IRS-2, rabbit anti-FKHR (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-Nopp140 (kindly provided by U.T. Meier) (30), and rabbit anti-protein disulfide isomerase (PDI) (kindly provided by R.J. Stockert) (31) antibodies were used as primary antibodies at 1:1,000 to 1:4,000 dilutions in 5% bovine serum albumin for 18 h at 4°C. Membranes were exposed to goat anti-rabbit and anti-mouse secondary antibodies conjugated with horseradish peroxidase (KPL, Gaithersburg, MD) at a dilution of 1:10,000 in 5% non-fat milk TBS-T for 1 h at room temperature. Signals were detected with a chemiluminescence detection system (Western Lightning Chemiluminescence Plus, PerkinElmer, Boston, MA) and exposure to x-ray film.
**Akt activity assay**

Akt activity was measured in cell lysates using an Akt assay kit (Cell Signaling) according to the manufacturer’s instructions. Akt was precipitated from 250 μg of cellular protein with an anti-Akt monoclonal antibody covalently bound to agarose beads. After washing, kinase reactions were performed in the presence of 200 μM ATP and a GSK3 fusion protein as substrate. Samples were resolved on 8% SDS polyacrylamide gels and immunoblotting performed as described previously. Phosphorylated GSK3 fusion protein was detected with a phospho-GSK3 antibody directed against serine residues 9 and 21. As a control for the loading of equivalent amounts of protein among samples, levels of total GSK3 were examined with a phosphorylation-independent GSK3 antibody.

**RNA isolation and Northern blotting**

RNA was extracted by cesium chloride gradient centrifugation as previously described (32). Cells were grown in 100 mm tissue culture dishes as described above, harvested in phosphate buffered saline, centrifuged and homogenized in 4 M guanidine thiocyanate. RNA was obtained by centrifugation in 5.7 M cesium chloride followed by repeated precipitations in ethanol. Steady-state RNA levels were determined by Northern blot analysis, as previously described (26). Briefly, 25 μg of total RNA were denaturated in 500 mM glyoxal, 45% dimethylsulfoxide, and 10 mM phosphate buffer, resolved on a 1% agarose gel, transferred to GeneScreen membrane (PerkinElmer) and baked for 2 h at 80°C. Membranes were prehybridized overnight, and then hybridized with [32P]dCTP (PerkinElmer) labeled cDNA probes for rat phosphoenolpyruvate carboxykinase (PEPCK) (33) or α-tubulin (34) for 17 h at 42°C. Membranes were washed and exposed to x-ray film as previously described (32).
**Measurement of glucose secretion**

Cells were cultured in 35 mm dishes as described above. The medium was changed to glucose-free medium (Sigma) supplemented with 20 mM sodium lactate and 2 mM sodium pyruvate. Some cells were then treated for 3 h with 100 nM insulin after which glucose concentrations in the medium were determined using a commercial kit (Sigma). Results were normalized to total protein and expressed as the percentage decrease of glucose secretion in insulin treated cells as compared to untreated controls.

**Adenovirus infection**

Replication-deficient adenoviruses expressing the *Escherichia coli* β-galactosidase gene (Ad5LacZ) (35), dominant negative JNK1 (AdJNK1dn), or dominant negative JNK2 (AdJNK2dn, kindly provided by J.D. Molkentin) (36) were employed. Adenoviruses were amplified in 293 cells, purified by banding twice on cesium chloride gradients and titered by plaque assay. Cells were infected at a multiplicity of infection of 20 for 3 h at 37°C 24 h prior to experiments, as previously described (26).

**JNK activity assay**

JNK activity was measured in cell lysates using a SAPK/JNK assay kit (Cell Signaling). An N-terminal c-Jun fusion protein bound to glutathione sepharose beads was used to immobilize JNK from cell lysates containing 125 μg of total protein. After washing, the kinase reaction was performed in the presence of 100 μM ATP using the c-Jun fusion protein as substrate. Samples were resolved on 12% SDS polyacrylamide gels, and the amount of phosphorylated c-Jun detected with an antibody specific for c-Jun phosphorylated at serine
residue 63. The equivalence of sample loading among reaction mixtures was assessed by immunoblotting with a rabbit phosphorylation-independent c-Jun antibody (Santa Cruz Biotechnology).

Animal model

All studies were approved by the Animal Care and Use Committee of the Albert Einstein College of Medicine, and followed the National Institutes of Health guidelines on the care and use of animals. C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) were maintained in 12 h light/dark cycles with unlimited access to food and water. The mice were fed a methionine and choline deficient diet (MCD diet) or a corresponding control diet supplemented with 300 mg of DL-methionine and 200 mg of choline per 100 g (ICN Biomedicals Inc., Costa Mesa, CA). To accustom the animals to the high sucrose diet, all animals were initially placed on the control diet for 2 weeks. Animals were then randomly assigned to 4 additional weeks of either control diet or MCD diet. After 4 weeks, some of the animals were fasted overnight, anesthetized with ether and given a portal vein injection of normal saline or 50 μmoles insulin/kg body weight in normal saline. Liver tissue was excised 3 min following injection and frozen in liquid nitrogen. Portions of liver were sonicated in homogenization buffer containing 1 mM sodium bicarbonate, 0.5 mM calcium chloride and protease and phosphatase inhibitors as described above, centrifuged at 100,000 g at 4°C for 2 h, and the resulting supernatant used for Western blotting.

Statistical analysis

All numerical results are expressed as mean ± SE and represent data from a minimum of three independent experiments. Calculations were made with Sigma Plot 2000 (SPSS Science,
Chicago, IL). The intensities of signals on immunoblots were quantitated using a FluorChem densitometer (Alpha Innotech, San Leonardo, CA) and expressed numerically relative to the appropriate untreated control cell line. Statistical significance was determined by the Student's t test.
Results

*CYP2E1 overexpression in RALA hepatocytes decreases IRS activation in response to insulin*

IRS-1 and IRS-2 are adaptor proteins that mediate the activation of downstream effectors of insulin signaling. Upon insulin stimulation, these molecules are recruited to the insulin receptor, activated through tyrosine phosphorylation, and then down regulated by inhibitory serine phosphorylation (5,6). To determine whether CYP2E1 overexpression decreased hepatocyte insulin signaling, we examined baseline and insulin-stimulated levels of IRS-1 and IRS-2 phosphorylation in cells with differential levels of CYP2E1 expression. Studies were performed in polyclonal cells transfected with an empty expression vector (VEC cells), and a RALA hepatocyte clone that overexpressed CYP2E1 following stable transfection with a CYP2E1 expression vector (S-CYP15 cells). Increased levels of CYP2E1 protein and catalytic activity have been previously demonstrated in S-CYP15 cells (28,29). By Western blotting, constitutive IRS-1 protein expression was increased approximately 4-fold in S-CYP15 cells as compared to VEC cells (Fig. 1A). The levels of IRS-1 were unaffected in either cell line by insulin for 30 min (Fig. 1A), although IRS-1 levels did decrease with longer insulin treatment (data not shown). IRS-1 tyrosine phosphorylation was not detectable in untreated cells, but occurred within 5 min of insulin treatment in both cell types (Fig. 1A). The amount of insulin-induced IRS-1 tyrosine phosphorylation relative to the level of total IRS-1 was significantly greater in VEC cells than in S-CYP15 cells (Fig. 1B). Phosphorylation of the inhibitory serine 307 IRS-1 residue was absent in untreated VEC cells but present constitutively at high levels in S-CYP15 cells (Fig. 1A). After insulin treatment, levels of serine 307 phosphorylation increased in both cell types but remained 2-fold greater in S-CYP15 cells as compared to VEC cells after 30 min of insulin treatment (Fig. 1A). Findings were similar for a second inhibitory IRS-1 serine
residue, serine 636/639. Levels of IRS-1 phosphorylation at serine 636/639 were elevated in untreated S-CYP15 cells, and further increased relative to VEC cells with insulin treatment (Fig. 1A). S-CYP15 cells therefore have decreased levels of IRS-1 tyrosine phosphorylation in response to insulin in association with constitutive and insulin-induced increases in inhibitory IRS-1 serine phosphorylation.

Insulin signaling in the liver is transduced almost equally by IRS-1 and IRS-2 (7,9). To determine whether CYP2E1 overexpression also affected IRS-2 activation, levels of total and tyrosine phosphorylated IRS-2 were examined in the two cell lines. Constitutive levels of IRS-2 were equivalent in the two cell types and decreased with insulin treatment (Fig. 1A). A 6-fold increase in IRS-2 tyrosine phosphorylation resulted from insulin treatment of VEC cells (Fig. 1A). In contrast, levels of insulin-induced IRS-2 tyrosine phosphorylation in S-CYP15 cells were only 25% of those in VEC cells (Fig. 1A). Thus, CYP2E1 overexpression is associated with decreased insulin-induced tyrosine phosphorylation of both IRS-1 and IRS-2.

Alterations in IRS phosphorylation correlate with CYP2E1 expression in additional RALA hepatocyte clones

To insure that the differences in total and phosphorylated IRS-1 and IRS-2 in S-CYP15 cells were secondary to increased CYP2E1 expression and not nonspecific clonal variation, insulin-induced IRS phosphorylation was examined in two additional S-CYP clones. The clone S-CYP29 has increased CYP2E1 protein and catalytic activity similar to S-CYP15 cells (29). In contrast, S-CYP43 cells fail to overexpress CYP2E1 despite transfection with the CYP2E1 expression plasmid, as previously determined by Western blotting and enzyme activity assays (29). The S-CYP29 clone had a 2-fold increase in IRS-1 protein levels similar to the increase in
S-CYP15 cells, whereas IRS-1 levels in S-CYP43 cells were equivalent to those in VEC cells (Fig. 2). Also similar to S-CYP15 cells, relative tyrosine phosphorylation in S-CYP29 cells was reduced 50% following insulin treatment for 10 min as compared to VEC and S-CYP43 cells (Fig. 2). Inhibitory phosphorylation at serine residues 307 and 636/639 was significantly increased in S-CYP29 cells untreated and following insulin treatment (Fig. 2). In contrast, S-CYP43 cells had low levels of serine 307 and serine 636/639 phosphorylation even after insulin treatment, identical to findings in VEC cells (Fig. 2). Tyrosine phosphorylation of IRS-2 following insulin treatment was also reduced in clone S-CYP29, similar to S-CYP15 cells, whereas levels in S-CYP43 cells were equivalent to those in VEC cells (Fig. 2). Therefore findings of IRS-1 and IRS-2 tyrosine hypophosphorylation and IRS-1 serine hyperphosphorylation correlated with levels of CYP2E1 expression among four lines of RALA hepatocytes.

**S-CYP15 cells have impaired activation of downstream effectors of insulin signaling**

Although CYP2E1-overexpressing cells had significantly decreased IRS-1 and IRS-2 tyrosine phosphorylation in response to insulin, lower levels of tyrosine phosphorylation were present leaving the overall impact of CYP2E1 overexpression on insulin signaling unclear. To assess the functional significance of this decrease in IRS tyrosine phosphorylation, the effects of CYP2E1 overexpression on the activation of downstream effectors of insulin signaling were determined. The serine/threonine protein kinase Akt is an important mediator of insulin's effects on hepatocytes and other cell types (37,38). Activated Akt phosphorylates many cellular substrates that in turn regulate glucose transport and glycogen synthesis (39). Akt phosphorylation in response to insulin was therefore examined as a measure of the effects of
decreased IRS tyrosine phosphorylation on insulin signaling in S-CYP15 cells. A low level of phosphorylated Akt was detected in untreated VEC cells but not S-CYP15 cells (Fig. 3A). Increased phosphorylation occurred within 15 min of insulin treatment in both cell types, however Akt phosphorylation was decreased by approximately 60% in S-CYP15 cells as compared to VEC cells at the time points examined (Fig. 3A). Levels of total Akt were equivalent in both cell types and unaffected by insulin treatment (Fig. 3A).

A prominent substrate of Akt is GSK3. GSK3 phosphorylation in response to insulin inhibits kinase activity, resulting in decreased phosphorylation and suppression of the glyconeogenic enzyme glycogen synthase. Akt activity was assessed by an in vitro kinase activity assay using GSK3 as substrate. Akt activity increased 19-fold in VEC cells after 30 min of insulin treatment (Fig. 3B). Akt activity also increased in insulin treated S-CYP15 cells, but to a level that was only 37% of that in VEC cells (Fig. 3B). Equivalent levels of total GSK3 among samples demonstrated the loading of equal amounts of reaction product (Fig. 3B). Levels of GSK3 phosphorylation in response to insulin were also examined by immunoblotting. In VEC cells, insulin induced a 6-fold increase in the level of phosphorylated GSK3, while levels in S-CYP15 cells were only approximately 33% of those in VEC cells (Fig. 3C). Levels of total GSK3 were equivalent in the two cell types and unaffected by insulin treatment (Fig. 3C). Therefore the reduction in IRS tyrosine phosphorylation in S-CYP15 cells markedly decreased Akt activation and GSK3 phosphorylation indicating that CYP2E1 overexpression significantly impaired insulin signaling.
**CYP2E1 overexpression causes impaired phosphorylation of FoxO1a and increased levels of PEPCK**

The effects of insulin on gene expression are ultimately mediated in part by a member of the winged helix/forkhead transcription factor family FoxO1a (FKHR) (40,41). FoxO transcription factors are phosphorylated in response to insulin resulting in their export from the nucleus and the abrogation of their transcriptional activity (42). To determine the effect of the CYP2E1-induced reduction in insulin signaling on FoxO1a activation, levels of phosphorylated FoxO1a were examined by immunoblotting. Low levels of phospho-FoxO1a were present in untreated VEC cells and increased significantly with insulin treatment (Fig. 4A). Phospho-FoxO1a was not detectable in S-CYP15 cells untreated, or even after insulin treatment (Fig. 4A). Levels of total FoxO1a were unaffected by insulin treatment in either cell type (Fig. 4A). Equivalent levels of the constitutively expressed PDI indicated equal protein loading among samples (Fig. 4A).

To further document the impairment of FoxO1a phosphorylation in S-CYP15 cells, the effects of insulin stimulation on the nuclear and cytosolic levels of this protein were investigated. The amount of nuclear FoxO1a was similar in S-CYP15 and VEC cells (Fig. 4B). In the absence of insulin, cytosolic FoxO1a was detectable only in VEC cells with the phospho-FoxO1a antibody (Fig. 4B). Insulin treatment led to an increase in cytosolic total and phospho-FoxO1a in VEC cells, but FoxO1a was still undetectable in the cytosol of S-CYP15 cells (Fig. 4B). To confirm the purity and equivalence of protein amounts among subcellular fractions, the nuclear and cytosolic extracts were immunoblotted with antibodies against the nuclear protein Nopp140 (30), and the cytosolic protein PDI (31). Nopp140 was detectable only in the nuclear fractions...
whereas PDI expression was restricted to the cytosolic isolates (Fig. 4B). Levels of both proteins were equivalent among samples from the two cell types.

One of the genes transcriptionally regulated by the FoxO transcription factors is the gluconeogenic enzyme PEPCK (43,44). To determine whether constitutive differences in FoxO1a activity between cell types translated into changes in PEPCK expression, steady-state mRNA levels of PEPCK were analyzed by Northern blotting. S-CYP cells had a 2-fold increase in PEPCK mRNA as compared to VEC cells that paralleled the finding of increased nuclear FoxO1a in these cells (Fig. 4C). Levels of α-tubulin mRNA were equivalent between the two cell types (Fig. 4C).

**Insulin fails to inhibit glucose secretion in S-CYP cells**

As an additional assessment of the functional effects of CYP2E1 expression on hepatocyte insulin responsiveness, the ability of insulin to inhibit RALA hepatocyte glucose production was examined. The amount of glucose secreted into the medium was assayed in WT, VEC, and S-CYP15 cells after 3 h of insulin treatment. Glucose production by WT and VEC cells was reduced by approximately 25% within this time period in response to insulin (Fig. 5). The inhibition of glucose secretion by insulin in S-CYP15 cells was significantly reduced by 70% in comparison to WT and VEC cells (Fig. 5). Thus, decreased insulin signaling secondary to CYP2E1 overexpression resulted in a failure of insulin to suppress RALA hepatocyte glucose secretion.
Decreased insulin signaling in S-CYP cells is partially reversed by inhibition of JNK signaling

Previous studies have demonstrated that CYP2E1 overexpression causes an activation of JNK mitogen-activated protein kinase (MAPK) (45). JNK is known to phosphorylate inhibitory IRS serine residues in other cell types and in nonhepatic and hepatic tissue (14,20). The possibility that JNK overactivation mediated the CYP2E1-induced reduction in IRS phosphorylation and insulin signaling was investigated by infecting S-CYP15 cells with adenoviruses expressing a dominant negative JNK1 (AdJNK1dn) or dominant negative JNK2 (AdJNK2dn) (36). As a control for the nonspecific effects of viral infection, cells were also infected with an adenovirus expressing β-galactosidase (Ad5LacZ). To determine the effectiveness of the JNK dominant negatives, JNK activity was examined in infected cells untreated and after treatment with TNF, a known activator of JNK in RALA hepatocytes (46). Infection with AdJNK1dn and AdJNK2dn completely inhibited JNK activity in untreated cells and decreased TNF-induced activity 54% and 83%, respectively (Fig. 6A). Next we examined the effect of JNK inhibition on S-CYP15 cell IRS phosphorylation in response to insulin. Insulin-induced IRS-1 tyrosine phosphorylation was unaffected by infection with either dominant negative JNK adenovirus (Fig. 6B). However, IRS-2 tyrosine phosphorylation was significantly increased by JNK inhibition. When compared to S-CYP15 cells infected with Ad5LacZ, AdJNK1dn infected cells had a 1.8-fold increase in IRS-2 tyrosine phosphorylation after 30 min of insulin treatment (Fig. 6B). An even a greater 2.9-fold increase in tyrosine phosphorylated IRS-2 occurred in insulin-treated cells infected with AdJNK2dn (Fig. 6B). Chemical inhibitors of the MAPK extracellular signal-regulated kinase 1/2, PKC or the mammalian target of rapamycin did not affect levels of IRS tyrosine or serine phosphorylation in
S-CYP15 cells (data not shown). Thus, decreased insulin signaling in CYP2E1 overexpressing cells is in part JNK dependent, but additional unidentified mechanisms are involved.

**Hepatic insulin signaling is impaired in a murine model of steatohepatitis with CYP2E1 overexpression**

Animals fed a diet deficient in methionine and choline (MCD diet) develop hepatic steatosis and necroinflammation in parallel with increased CYP2E1 expression and lipid peroxidation (23). To determine whether steatohepatitis in association with CYP2E1 overexpression may affect hepatocyte insulin signaling, constitutive and insulin-stimulated IRS-1 and IRS-2 phosphorylation were examined in this murine model. C57BL/6 mice were fed an MCD diet or control diet containing methionine and choline for 4 weeks. Consistent with prior reports (23,47), MCD diet fed mice developed elevated alanine aminotransferase levels, increased hepatic triglyceride content and histologic evidence of steatohepatitis (data not shown). Insulin responsiveness in these animals was assessed by levels of hepatic IRS-1 and IRS-2 phosphorylation after an intraportal insulin injection. Levels of total IRS-1 and IRS-2 were similar in control and MCD diet fed animals (Fig. 7A). An insulin bolus induced a marked increase in IRS-1 and IRS-2 tyrosine phosphorylation in the livers of control diet fed animals (Fig. 7A). In contrast, in MCD diet fed animals with steatohepatitis minimal tyrosine phosphorylation of IRS-1 and IRS-2 occurred in response to insulin (Fig. 7A). In parallel to their decreased level of IRS tyrosine phosphorylation, mice on the MCD diet had higher levels of IRS-1 serine 307 and 636/639 phosphorylation constitutively and after insulin injection (Fig. 7A). To evaluate the effect of decreased IRS phosphorylation in MCD diet fed animals on downstream effectors of insulin signaling, levels of Akt phosphorylation were assessed by immunoblotting.
In parallel to the impaired IRS tyrosine phosphorylation in animals on MCD diet, levels of Akt phosphorylation although variable, were significantly reduced by greater than 50% in MCD diet fed mice as compared to control diet fed mice (Fig. 7B). Levels of total Akt protein were equivalent among animals on the two diets (Fig. 7B). Thus, hepatic insulin signaling is impaired in an in vivo model of steatohepatitis associated with CYP2E1 overexpression as manifested by decreased IRS tyrosine phosphorylation and Akt activation.
Discussion

The high prevalence of insulin resistance in humans with NAFLD has led to the concept that hepatic insulin resistance plays an important role in the development of this disease (48). NAFLD may result in part from an increased supply of fatty acids caused by peripheral insulin resistance, and therefore constitute a hepatic manifestation of the metabolic syndrome (3). However, the liver is not only an end organ for the effects of insulin resistance, but also an active regulator of glucose homeostasis. Studies have documented that inappropriate glucose production by the liver is an important component of the diabetic state (49,50). Therefore it is possible that the strong association between insulin resistance and hepatic steatohepatitis may result in part from NAFLD-induced changes in liver physiology that promote insulin resistance through impaired hepatic insulin signaling.

A common denominator in the pathogenesis of insulin resistance and NASH is increased oxidative stress (23,51). Hepatic induction of the prooxidant enzyme CYP2E1 occurs in both NAFLD and type 2 diabetes (22,52). We therefore selected a nontransformed hepatocyte cell line with CYP2E1 overexpression as a physiologically relevant model to examine the effects of NAFLD-associated chronic oxidative stress on hepatocyte insulin resistance. The following effects of CYP2E1 overexpression on IRS signaling were demonstrated: (1) levels of IRS-1 were increased; (2) relative IRS-1 tyrosine phosphorylation in response to insulin was decreased; (3) inhibitory IRS-1 serine phosphorylation was increased constitutively and after insulin treatment; and (4) IRS-2 tyrosine phosphorylation in response to insulin was decreased. CYP2E1 overexpression therefore reduced stimulatory tyrosine phosphorylation of both IRS-1 and IRS-2 while increasing inhibitory IRS-1 serine phosphorylation. The reduction in insulin-induced tyrosine phosphorylation was presumably secondary to a primary increase in serine
phosphorylation as decreased tyrosine phosphorylation of IRS-1 has been attributed to increased levels of serine phosphorylation (11). Supportive of this possibility was that serine phosphorylation of IRS-1 was constitutively increased in RALA hepatocytes prior to insulin stimulation. Insulin induced an even greater increase in serine phosphorylation in CYP2E1 overexpressing RALA hepatocytes, suggesting that these cells were additionally primed for a more rapid phosphorylation of inhibitory serine residues. In contrast to studies of insulin resistance in nonhepatic cell types (12,14,51), IRS-1 levels were up-regulated rather than decreased in S-CYP cells. This finding may represent a cellular compensation for the reduction in insulin signaling.

In hepatocytes in vivo, both IRS-1 and IRS-2 are required for normal insulin signaling (7). The only partial inhibition of IRS-1 and IRS-2 tyrosine phosphorylation in S-CYP cells left the overall effect of CYP2E1 overexpression on cellular insulin signaling uncertain. However, the effects on IRS significantly impaired hepatocellular insulin signaling as evidenced by: (1) decreased phosphorylation and activation of the downstream effector kinase Akt; (2) reduced insulin-induced phosphorylation of GSK3; and (3) impaired inactivation of the transcription factor FoxO1a. Hepatocytes express both Akt1 and Akt2. Deletion of Akt2 in mice results in hyperglycemia and hepatic insulin resistance (18,53), while liver specific overexpression of Akt results in marked hypoglycemia (37). In times of nutrient excess, Akt acts to both suppress gluconeogenesis and activate glyconeogenesis. Glyconeogenesis increases following phosphorylation and inactivation of GSK3. Accordingly GSK3 phosphorylation occurred in VEC cells following insulin treatment, while levels of phospho-GSK3 in S-CYP cells were minimally affected by insulin.
Suppression of gluconeogenesis depends on Akt-induced inactivation of the forkhead transcription factor FoxO1a that results from its phosphorylation and nuclear export (54). This transcription factor regulates the expression of the gluconeogenic enzyme PEPCK as liver specific inhibition of FoxO1a has been demonstrated to decrease gluconeogenic activity in parallel with reduced transcription of PEPCK (40,55). Our findings demonstrate that CYP2E1 overexpression decreased FoxO1a phosphorylation and nuclear export following insulin stimulation. Taken together these findings are consistent with impaired activation of downstream effectors of the insulin signaling pathway in CYP2E1 overexpressing cells that parallels the upstream defect in IRS activation.

The finding of impaired inhibition of glucose production further supports the significance of the CYP2E1-induced changes in insulin signaling. Excessive hepatic glucose production contributes to fasting hyperglycemia in type 2 diabetes, and the mechanism implicated in this effect is increased transcription of the gluconeogenic enzyme PEPCK (55). Accordingly, we found an increased steady-state mRNA level of PEPCK in S-CYP15 cells. The significance of this finding is that CYP2E1-induced changes in insulin signaling led to a biological effect associated with type 2 diabetes mellitus, the increased expression of a gluconeogenic enzyme.

Previously we reported that CYP2E1 overexpression induces activation of the MAPK signaling pathway JNK (45). JNK activation in cultured cells (14), and in animal models (20,56,57), has been implicated in the inhibition of insulin signaling. In our cell culture system adenoviral expression of a dominant negative JNK1 or JNK2 reversed the decrease in insulin-induced tyrosine phosphorylation of IRS-2 in S-CYP15 cells. The present data therefore provide additional evidence for the importance of JNK activation in the promotion of hepatic insulin resistance. However, activation of IRS-1 was unaffected by JNK inhibition suggesting that
significant differences exist in the mechanisms of inhibitory phosphorylation of IRS-1 and IRS-2 in RALA hepatocytes. The lack of effect on IRS-1 was surprising in light of the finding of increased levels of IRS-1 serine 307 phosphorylation in S-CYP cells and reports in nonhepatic cell types of JNK mediating phosphorylation at this site (58). Prominent among other signaling pathways previously implicated in promoting insulin resistance and potentially affected by CYP2E1-induced oxidative stress are PKC and suppressor of cytokine signaling-3 (SOCS-3) (59,60). Western blot analysis revealed no effect of CYP2E1 overexpression on levels of PKC isoforms and suppressor of cytokine signaling-3 (data not shown). Expression of p53 is induced by oxidative stress and has recently been demonstrated to have a critical role in the development of steatohepatitis (61). However, p53 up-regulation was also not observed in CYP2E1 overexpressing RALA hepatocytes (data not shown). Despite additional studies with inhibitors of other potential negative regulatory pathways of insulin signaling, the present investigations could not determine the mechanism of CYP2E1-induced inhibition of IRS-1 signaling.

Our in vitro findings are supported by evidence of impaired insulin signaling in a rodent model of steatohepatitis associated with CYP2E1 overexpression. The MCD diet leads to a progressive steatohepatitis accompanied by CYP2E1 induction and increased lipid peroxidation (23). This mouse model therefore mimics important features of the human disease. Similar to findings in hepatocytes overexpressing CYP2E1, these animals had decreased hepatic insulin responsiveness as evidenced by reduced tyrosine phosphorylation of IRS-1 and IRS-2 and increased serine phosphorylation of IRS-1. The changes in IRS phosphorylation were sufficient to significantly down regulate effectors of insulin signaling as Akt phosphorylation was also reduced in MCD diet fed animals. MCD diet induced steatohepatitis does not affect peripheral insulin resistance (47), clearly demonstrating that the liver disease by itself was sufficient to
induce hepatic insulin resistance. The MCD diet model is more complex than the cell culture system studied, and the in vivo hepatic insulin resistance may be a function of more than just CYP2E1 overexpression. Other factors such as the increase in intracellular triglycerides may promote hepatic insulin resistance in this model. Recently Samuel et al. demonstrated that a brief period of a high fat diet induced steatosis associated with hepatic insulin resistance (62). Thus, hepatic insulin resistance in the MCD diet may result in part just from the lipid accumulation in this model.

The present studies suggest that the interrelationship between insulin resistance and NAFLD may be more complex than the simple concept that insulin resistance serves to promote the development NAFLD. Once liver disease is established, NAFLD-induced impairment of insulin signaling may then further promote the diabetic state. In support of this concept are findings of reduced suppression of hepatic glucose production by insulin in NAFLD patients as compared to controls (3,24). The present studies suggest that one mechanism of this effect may be through the effects of CYP2E1 overexpression. Aggressive treatment of NAFLD in diabetics may therefore be critical not only for the prevention of the chronic complications of liver disease, but also for the successful treatment of their hyperglycemic and hyperinsulinemic state.
Acknowledgments

We thank U. Thomas Meier for the Nopp140 antibody, Richard J. Stockert for the PDI antibody, Daryl K. Granner for the rat PEPCK cDNA, and Jeffrey D. Molkentin for the JNK adenoviruses.
Footnotes

1The abbreviations used are: CYP2E1, cytochrome P450 2E1; GSK3, glycogen synthase kinase 3; IRS, insulin receptor substrate; JNK, c-Jun N-terminal kinase; MCD diet, methionine choline deficient diet; MAPK, mitogen-activated protein kinase; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; PDI, protein disulfide isomerase; PEPCK, phosphoenolpyruvate carboxykinase; PKC, protein kinase C; TNF, tumor necrosis factor-α.
References


Figure Legends

Fig. 1. **IRS-1 and IRS-2 phosphorylation are altered in CYP2E1 overexpressing RALA hepatocytes.** (A) Levels of total and phosphorylated IRS-1 and IRS-2 were determined in VEC and S-CYP15 (S-CYP) cells untreated, and treated with insulin for the indicated number of minutes. Immunoblotting (IB) and immunoprecipitations (IP) were performed with antibodies directed against total IRS-1 (IRS-1), phospho-tyrosine peptides (P-Tyr), IRS-1 phosphorylated at serine 307 (P-S 307), IRS-1 phosphorylated at serine 636/639 (P-S 636/9), or total IRS-2 (IRS-2). The immunoblots are representative of three independent experiments. Numerical results under the respective Western blots indicate the relative signal intensity obtained by densitometric scanning of these three independent experiments. The numbers represent the signal intensity relative to untreated VEC cells. The constitutive and insulin-stimulated increases in IRS-1, IRS-1 phosphorylated at serine 307, and IRS-1 phosphorylated at serine 636/639 in S-CYP cells as compared to VEC cells were statistically significant (\( p < 0.02 \)). The decrease in IRS-2 tyrosine phosphorylation in insulin-treated S-CYP cells as compared to VEC cells was also statistically significant (\( p < 0.01 \)). (B) The relative amount of IRS-1 tyrosine phosphorylation was determined in cells untreated (\( \square \)) or treated with insulin for the indicated number of minutes by densitometric analysis of three independent experiments. The results are expressed as the ratio of tyrosine phosphorylated IRS-1 to total IRS-1 (* \( p < 0.05 \)).

Fig. 2. **Changes in IRS phosphorylation correlate with CYP2E1 overexpression in additional cell clones.** VEC cells, the CYP2E1 overexpressing clones S-CYP15 (S15) and S-CYP29 (S29), and S-CYP43 (S43) cells that lack CYP2E1 activity, were untreated or treated with insulin for 10 min. Cell lysates were immunoblotted (IB) or immunoprecipitated (IP) with
an anti-IRS-1 (IRS-1) or anti-IRS-2 (IRS-2) antibody. Immunoprecipitates were immunoblotted with antibodies directed against tyrosine phosphorylated proteins (P-Tyr), phospho-serine 307 of IRS-1 (P-S 307), or phospho-serine 636/639 of IRS-1 (P-S 636/9). Results are representative of three independent experiments.

Fig. 3. **Insulin induced Akt and GSK3 activation is decreased in CYP2E1 overexpressing hepatocytes.** (A and C) Western blots of whole cell lysates from VEC and S-CYP15 (S-CYP) cells untreated or treated with insulin for the indicated number of minutes. Immunoblots were performed with antibodies directed against phosphorylated (P-Akt) or phosphorylation-independent (Akt) Akt, and phosphorylated (P-GSK3) or phosphorylation-independent (GSK3) GSK3. (B) Akt kinase activity using GSK as a substrate was determined in VEC and S-CYP15 cells untreated or treated with insulin for the indicated number of minutes. Amounts of phosphorylated GSK3 and total GSK3 as a control for protein loading were determined by immunoblotting. Numerical results represent the relative signal intensity obtained by densitometric analysis of three independent experiments.

Fig. 4. **S-CYP15 cells have decreased FoxO1a phosphorylation and increased steady-state PEPCK gene expression.** (A) VEC and S-CYP15 cells were untreated or treated with insulin for the indicated number of minutes. Total protein was isolated and immunoblotted with antibodies against phosphorylated FoxO1a (P-FoxO1a), total FoxO1a (FoxO1a), or protein disulfide isomerase (PDI) as a control for protein loading. (B) Nuclear and cytosolic proteins were isolated from VEC and S-CYP15 cells untreated or treated with insulin for 10 min and immunoblotted with the same antibodies along with an antibody against Nopp140. (C) Steady-
state levels of PEPCK mRNA were determined by Northern blotting of total RNA from VEC and S-CYP15 cells. RNA was hybridized with cDNA probes for PEPCK or α-tubulin as indicated. Findings are representative of three independent experiments.

Fig. 5. Insulin-induced inhibition of glucose secretion is impaired in CYP2E1 overexpressing cells. Wild-type (WT), VEC and S-CYP15 (S-CYP) cells were treated with insulin for 3 h and then their culture medium assayed for glucose concentration. Results are expressed as the percentage inhibition of glucose secretion of data derived from three independent experiments performed in duplicate (*p<0.01 compared to WT or VEC cells).

Fig. 6. JNK inhibition up-regulates tyrosine phosphorylation of IRS-2 in CYP2E1 overexpressing cells. (A) JNK activity was determined in S-CYP15 cells infected with adenoviruses expressing β-galactosidase (Ad5LacZ), a dominant negative JNK1 (AdJNK1dn), or a dominant negative JNK2 (AdJNK2dn) for 24 h prior to treatment with TNF for 30 min. An in vitro kinase assay employing c-Jun as a substrate was performed as described in Experimental Procedures. Amounts of phosphorylated c-Jun (P-c-Jun) and total c-Jun (c-Jun) as a control were determined by immunoblotting. (B) Immunoprecipitations (IP) and immunoblotting (IB) were performed with antibodies directed against total IRS-1 (IRS-1), total IRS-2 (IRS-2) and phospho-tyrosine peptides (P-Tyr). Numerical results below each lane represent the relative signal intensities normalized to insulin-treated Ad5LacZ-infected cells as obtained from densitometric scanning of three independent experiments.
Fig. 7. **IRS tyrosine phosphorylation and Akt activation in response to insulin are decreased in MCD diet fed animals.** (A) C57BL/6 mice fed a control or MCD diet for 4 weeks received an intraportal injection of saline as a control or insulin. Portions of liver were harvested 3 min after injection, protein isolated from portions of liver, and samples immunoblotted with anti-IRS-1 (IRS-1) or anti-IRS-2 (IRS-2) antibodies. Protein lysates were also immunoprecipitated (IP) with anti-IRS-1 or anti-IRS-2 antibodies and immunoblotted with anti-phospho-tyrosine (P-Tyr), anti-phospho-serine 307 IRS-1 (P-S 307), or anti-phospho-serine 636/639 IRS-1 (P-S 636/9) antibodies. (B) Western blots of the same liver isolates with antibodies directed against phospho- (P-Akt) or total (Akt) Akt. Results are representative of 4 animals per data point.
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Figure 7A
Figure 7B
Hepatocyte CYP2E1 overexpression and steatohepatitis lead to impaired hepatic insulin signaling
Jorn M. Schattenberg, Yongjun Wang, Rajat Singh, Raina M. Rigoli and Mark J. Czaja

J. Biol. Chem. published online January 4, 2005

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