Liver-specific Knockdown of JNK1 Up-regulates Proliferator-activated Receptor γ Coactivator 1β and Increases Plasma Triglyceride despite Reduced Glucose and Insulin Levels in Diet-induced Obese Mice*

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The c-Jun N-terminal kinases (JNKs) have been implicated in the development of insulin resistance, diabetes, and obesity. Genetic disruption of JNK1, but not JNK2, improves insulin sensitivity in diet-induced obese (DIO) mice. We applied RNA interference to investigate the specific role of hepatic JNK1 in contributing to insulin resistance in DIO mice. Adenovirus-mediated delivery of JNK1 short-hairpin RNA (Ad-shJNK1) resulted in almost complete knockdown of hepatic JNK1 protein without affecting JNK1 protein in other tissues. Liver-specific knockdown of JNK1 resulted in significant reductions in circulating insulin and glucose levels, by 57 and 16%, respectively. At the molecular level, JNK1 knockdown mice had sustained and significant increase of hepatic Akt phosphorylation. Furthermore, knockdown of JNK1 enhanced insulin signaling in vitro. Unexpectedly, plasma triglyceride levels were robustly elevated upon hepatic JNK1 knockdown. Concomitantly, expression of proliferator-activated receptor γ coactivator 1β, glucokinase, and microsomal triacylglycerol transfer protein was increased. Further gene expression analysis demonstrated that knockdown of JNK1 up-regulates the hepatic expression of clusters of genes in glycolysis and several genes in triglyceride synthesis pathways. Our results demonstrate that liver-specific knockdown of JNK1 lowers circulating glucose and insulin levels but increases triglyceride levels in DIO mice.

Obesity has become an epidemic worldwide and is strongly associated with the development of insulin resistance and type 2 diabetes (1). Clinical evidence has further demonstrated a correlation between obesity and type 2 diabetes (2, 3). Genes with a high fat diet, mice with genetic disruption of JNK1, but not JNK2, exhibit significantly improved insulin sensitivity and enhanced insulin signaling (4). Knock-out of JNK1 in leptin-deficient (ob/ob) mice results in significant reduction of circulating glucose and insulin levels (4). However, the tissue-specific contribution of JNK1 to insulin resistance has not been investigated.

Short double-stranded RNA, 19–29 nucleotides in length, can specifically silence target genes without inducing a toxic interferon response (10, 11). Systemic delivery of recombinant adenovirus has been shown to specifically target liver without affecting other tissues, such as brain, muscle, adipose or potentially own stock and/or hold stock options in Abbott Laboratories. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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5 The abbreviations used are: JNK, c-Jun N-terminal kinase; Akt, a serine/threonine kinase/protein kinase B; CyTC, cytochrome c; DIO, diet-induced obese; GFP, green fluorescent protein; IRS-1, insulin receptor substrate 1; MTP, microsomal triacylglycerol transfer protein; PPAR, peroxisome proliferator-activated receptor; PGC-1α, PPARγ coactivator 1α; PGC-1β, PPARγ coactivator 1β; SCD1, stearoyl-CoA desaturase 1; shGFP, short-hairpin RNA against GFP; shJNK1, short-hairpin RNA against JNK1; shRNA, short-hairpin RNA; siRNA, short-interfering RNA; SREBP1, sterol regulatory element binding factor 1; VLDL, very-low-density lipoprotein; Ad, adenovirus; LDL, low density lipoprotein; P3K, phosphatidylinositol 3-kinase; siRNA, small interference RNA; RNAi, RNA interference; ACSL4, acyl-CoA synthetase 4; DGAT1, diacylglycerol acyltransferase 1; Akt-p, Akt phosphorylation.

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pose, and heart (12). To understand the liver-specific contribution of JNK1 to insulin resistance, we applied adenovirus-mediated delivery of short hairpin RNA (shRNA) for JNK1 in an animal model of mild obesity and insulin resistance, the DIO mouse. An advantage of this approach over transgenic methods is the ability to determine the effects of acute inhibition of an enzyme or pathway in an adult animal; a more relevant validation tool when considering potential effects of pharmacologic intervention. Our results indicate that liver-specific knockdown of JNK1 improved whole body insulin sensitivity and enhanced hepatic insulin signaling in DIO mice. Surprisingly, significant knockdown of hepatic JNK1 also resulted in a robust increase in circulating triglyceride levels, suggesting a role of JNK1 in lipid metabolism. Knockdown of JNK1 significantly increased the expression of PPARγ coactivator-1β (PGC-1β). PGC-1β has been implicated as a key regulator affecting hepatic lipogenesis and lipoprotein secretion (13, 14). An analysis of gene expression changes provides an explanation for increased circulating triglycerides and lowered blood glucose and insulin levels found in JNK1-knockdown DIO mice.

**EXPERIMENTAL PROCEDURES**

_Generation of Synthetic siRNAs, Vectors Expressing shRNAs, and Recombinant Adenoviruses—_An RNAi sequence specific for mouse JNK1 was selected to specifically match only mouse JNK1 when analyzed using a genome-wide Blast search ([www.ncbi.nlm.nih.gov/blast/Blast.cgi](http://www.ncbi.nlm.nih.gov/blast/Blast.cgi)). The sense sequence for the JNK1 RNAi sequence contains 21 nucleotides: 5’-GCA-GAAGCAACGGTGAACA-3’. To create an shRNA, an 8-nucleotide loop (TCAAGACT) was placed to separate the sense and antisense sequences for RNAi, and the entire sequence was cloned into a pENTR™ vector driven by the U6 promoter (Invitrogen). In _vitro_ testing6 showed it to be efficacious, and the shRNA was transferred to an adenoviral pAd/BLOC-KIT™ DEST vector by Gateway LR recombination (Invitrogen). Recombinant adenovirus containing JNK1 shRNA (Ad-shJNK1) was generated by transfection of the vector into HEK293 cells as described previously (12). The control adenovirus containing shRNA targeting GFP (Ad-shGFP) has been described previously (15). Human JNK1 siRNA and control siRNA (catalog no. 003514) were purchased from Dharmaco Inc. A recombinant adenovirus overexpressing cDNA for β-galactosidase was obtained from Millennium Pharmaceuticals.

_Cell Culture and Transfection—_Primary hepatocytes from CD1 mice were prepared as previously described (16). The cells were plated into 6-well collagen-coated plates and allowed to reach 50% confluency. The hepatocytes were treated with adenoviruses containing shRNAs at a concentration of 5 × 10⁹ viral particles per milliliter of culture medium for 18 h. Cell incubation was continued in fresh medium for a further 24 h before collection and analysis. Human JNK1 siRNAs were transfected into HepG2 cells using TransIT-TKO reagent (Mirus Inc.). Forty-eight hours after transfection, cell lysates were collected and analyzed further.

_Animal Studies—_Male C57BL/6J mice (Jackson Laboratories) were housed on a 12-h light-dark cycle and allowed free access to water and a high fat diet (Research Diet D12492I, 60% kcal from fat) for 16–18 weeks. Adenoviruses were purified and diluted in saline solution, and 2 × 10¹¹ viral particles were delivered into the animals by a single tail-vein injection on day 0 as described previously (17). Animals were fed _ad libitum_ and were sacrificed at day 5 after adenovirus injection. Blood and tissue samples were collected for further analysis. Tissue samples were snap-frozen in liquid nitrogen. For fasting studies, animals on day 5 were fasted overnight for 16 h and were then sacrificed at day 6. An insulin tolerance test was performed in DIO mice at day 5 after adenovirus injection, after a 4-h fast. Insulin was delivered by intraperitoneal injection of 0.25 unit/kg insulin (Humulin-R, Lilly). Blood glucose levels were monitored every 30 min post injection, up to 120 min. Blood glucose levels were measured using a Precision PCx glucose meter (Abbott Laboratories). The Abbott Laboratories Animal Care and Use Committee approved all procedures.

_Sample Analysis—_Liver and fat tissues were pulverized and homogenized in lysis buffer as described before (17). Western blot was performed and protein-antibody complexes were visualized using ECL plus (PerkinElmer Life Science) as suggested by the manufacturer. Immunoprecipitation assays were performed with anti-phosphotyrosine antibodies followed by Western analysis detecting insulin receptor and IRS-1. Co-immunoprecipitation analysis was performed with anti-IRS-1 antibodies followed by Western analysis detecting PI3K subunit p85. JNK antibody (sc571), which detects both JNK1 and JNK2, glucokinase, and SREBP1 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against IRS-1, IRS-1-p-Ser-307, insulin receptor, PI3K p85, phosphotyrosine, Akt, and Akt-p-Ser-473 were purchased from Cell Signaling Technology (Beverly, MA). An electromembraniluminescent enzyme-linked immunosorbent assay kit was used to detect c-Jun phosphorylation (catalog no. K111CGD, Meso-Scale-Discovery).

Real-time PCR analysis was performed on cDNA prepared from total RNA isolated from mouse tissues using TRIzol™ and Qiagen RNeasy kits. The real-time PCR reaction used SYBR Green supermix (catalog no. 170-8862, Bio-Rad), and data were analyzed as previously described (17). The mRNA levels were normalized with 18 S RNA and data were represented relative to the control Ad-shGFP-treated group. Primer sequences for different genes are shown in Table I. The hepatic RNA samples from _ad libitum_ fed mice were analyzed by using an Affymetrix microarray. Five µg of total RNA from each sample was used to prepare a biotin-labeled cRNA target using standard Affymetrix protocols (Enzo Biochem). 10 µg of prepared cRNA for each sample was hybridized with an Affymetrix Mouse Genome 430A 2.0 array. After hybridization and chip scanning, quality control data were evaluated to ensure all chip data were of good quality. Image analysis and data normalization were performed using Affymetrix and Rosetta Resolver 6.0 software (Rosetta Inpharmatics) using the Resolver Affymetrix error model. Within Resolver, expression levels for each sample
were compared against the average of the vehicle-treated control group. Data from individual probe sets were grouped by Entrez Gene ID (18) for visualization by gene expression heat map as described in each figure. Genes that had statistically significant differences \((p < 0.05)\) of >1.5-fold versus the control Ad-shGFP-treated group were investigated using GenMapp 2.1 (19) and MappFinder (20) software to identify the most over-represented, significant categories of gene ontology and biological pathways.

Blood glucose levels were monitored in tail-snip samples as described in the previous section. Plasma insulin was measured using kits from ALPCO. Plasma free fatty acids were measured using kits from ThermoElectron Corp. Plasma triglyceride and cholesterol profiling was performed using Infinity kits from ThermoElectron Corp. Liver histological analysis was performed using \( \beta \)-galactosidase antibodies (Cortex Biochem). Plasma triglyceride and cholesterol profiling was performed with a SMART fast protein liquid chromatography system (Pfizer) using a Superose 6 PC 3.2/30 column (Amersham Biosciences). Pooled plasma samples (50 \( \mu l \)) were loaded onto the column. The elution flow rate was 40 \( \mu l/min \) in a running buffer consisting of 0.15 \( M \) NaCl and 0.05 \( M \) sodium phosphate, pH 7.0. Fractions of 40 \( \mu l \) were collected, and triglyceride and cholesterol contents were determined using enzymatic kit assays (Infinity, ThermoElectron Corp.).

**Statistical Analysis**—Data are expressed as the mean ± S.E. Statistical significance was determined by using the unpaired Student’s \( t \) test. Statistical significance was assumed at \( p < 0.05 \).

**RESULTS**

**Adenovirus-mediated Delivery of JNK1 shRNA Induced Robust Reduction of Hepatic JNK1 Protein in DIO Mice**—Recombinant adenovirus expressing an effective shRNA against JNK1 (Ad-shJNK1) was designed, and we confirmed its robust knockdown of JNK1 protein in primary mouse hepatocytes.\(^6\) To evaluate the transduction efficiency of adenovirus-mediated delivery in DIO mice, recombinant adenovirus expressing the \( \beta \)-galactosidase gene was delivered by a single tail-vein injection. Histological evaluation demonstrated that delivery of \( 2 \times 10^{11} \) viral particles expressing \( \beta \)-galactosidase resulted in almost 100% transduction of liver cells 5 days after injection (Fig. 1A). We used the same viral dose for delivery of Ad-shJNK1 or a control recombinant adenovirus expressing shRNA against GFP protein (Ad-shGFP) into DIO mice. Knockdown was expressed relative to the Ad-shGFP control. A robust 95% reduction of hepatic JNK1 protein was observed (Fig. 1B). Knockdown of JNK1 by Ad-shJNK1 was specific to JNK1 and did not affect JNK2 protein levels in liver (Fig. 1B).

**Liver-specific Knockdown of JNK1 Reduced Circulating Glucose and Insulin Levels in DIO Mice**—To determine the effects of decreased hepatic JNK1 protein on whole body metabolic parameters, we evaluated plasma from DIO mice on day 5 after a single injection of Ad-shJNK1. In ad libitum fed mice, Ad-shJNK1 treatment resulted in a significant 16% reduction in blood glucose levels compared with control Ad-shGFP treatment (144 ± 14 versus 172 ± 28 mg/dl, respectively) (Fig. 2A). Plasma insulin levels were also significantly lowered, by 57%, in Ad-shJNK1-treated mice compared with control Ad-shGFP-treated mice (0.31 ± 0.07 versus 0.72 ± 0.31 ng/ml, respectively) (Fig. 2B). No significant changes in blood glucose and plasma insulin levels were observed in the control Ad-shGFP-treated mice compared with vehicle-treated mice. Consistent with results in fed mice, liver-specific knockdown of JNK1 also led to reduced blood glucose and insulin levels in 16-h-fasted DIO mice (data not shown). There was no obvious liver toxicity associated with Ad-shRNA delivery, as determined by histological analysis, as well as measurement of plasma alanine aminotransferase and aspartate aminotransferase (data not shown). On day 5 after Ad-shRNA delivery, an insulin tolerance test was performed in 4-h-fasted DIO mice. A single intraperitoneal injection of 0.25 unit/kg insulin was given, and blood glucose levels were monitored every 30 min for 2 h. Ad-shJNK1-
initial point during the insulin tolerance test. We did not observe any significant changes in body weight or epididymal fat mass with treatment. Taken together, our data demonstrate that liver-specific knockdown of JNK1 significantly decreases glucose and insulin levels and tends to improve insulin sensitivity in DIO mice.

**RNAi-mediated JNK1 Knockdown Enhanced Hepatic Insulin Signaling in Vivo and in Vitro—**Increased phosphorylation of the serine/threonine kinase Akt, also called protein kinase B, has been considered a marker of enhanced insulin sensitivity. In our studies, liver-specific knockdown of JNK1 protein by Ad-shJNK1 in *ad libitum* fed mice resulted in a significant 3-fold increase in hepatic Akt phosphorylation at serine 473 (Akt-p-Ser-473) (Fig. 3A). After a 16-h fast, hepatic Akt-p-Ser-473 was also significantly increased, >10-fold compared with levels observed in control Ad-shGFP-treated mice (Fig. 3B). No change of total Akt proteins was observed among different groups of treated mice (Fig. 3, A and B). Additionally, Akt-p-Ser-473 was significantly increased after an insulin tolerance test in DIO mice treated with Ad-shJNK1 (data not shown). Knockdown of JNK1 did not change Akt-p-Ser-473 in epididymal fat tissue compared with control groups in *ad libitum* fed mice (data not shown). Furthermore, knockdown of JNK1 increased phosphorylation of glycogen synthase kinase 3, a downstream target of AKT, supportive of enhanced hepatic insulin signaling in DIO mice (data not shown).

To examine the effects of JNK1 knockdown on insulin signaling *in vitro*, primary mouse hepatocytes were treated with Ad-shJNK1 or control Ad-shGFP for 2 days followed by a 20-h chronic incubation with 10 μM insulin. Consistent with our *in vivo* results, Western blot analysis demonstrated >3-fold increased Akt-p-Ser-473 without changes to total Akt protein, concomitant with robust knockdown of JNK1 protein by Ad-shJNK1 (Fig. 3C). Compared with control Ad-shGFP-treated cells, knockdown of JNK1 resulted in enhanced response of Akt-p-Ser-473 upon acute treatment with insulin (10 nm insulin for 10 min, data not shown). We further applied RNAi to knock down JNK1 in human hepatoma-derived HepG2 cells. HepG2 cells were transfected with 100 nM of an effective human JNK1 siRNA or control non-targeting siRNA for 48 h, followed by incubation with 10 μM insulin for 20 h to induce an insulin resistance condition. Compared with control non-targeting siRNA-transfected cells, Western blot analysis demonstrated that human JNK1 siRNA robustly reduced JNK1 protein. Also consistent with our *in vivo* Ad-shJNK1 results, knockdown of JNK1 in HepG2 cells by human JNK1 siRNA resulted in significantly increased Akt-p-Ser-473 in response to chronic insulin treatment (Fig. 3D), without affecting total Akt analyzed by densitometric analysis (graphed) of 6–8 animals per group (p < 0.0001). *D* electrochemiluminescent enzyme-linked immunosorbent assay analysis for hepatic c-Jun phosphorylation relative to Ad-shGFP controls (6–8 animals in each group; p < 0.01). * and # represent significant differences between Ad-shJNK1 and control Ad-shGFP or vehicle-treated mice, respectively.

treated mice had significantly reduced blood glucose levels at several time points compared with control Ad-shGFP-treated mice (Fig. 2C). The insulin tolerance test results were also plotted as the percentage of initial glucose level at each time point (Fig. 2D). Compared with control Ad-shGFP-treated mice, Ad-shJNK1-treated mice had reduced blood glucose levels at each time point. These differences failed to reach statistical significance because one out of the seven animals in the Ad-shJNK1-treated group had an unexplained robust increase (the remaining six mice had a robust significant reduction) in blood glucose levels compared with the

**FIGURE 1.** Adenovirus-mediated delivery of JNK1 shRNA induced robust reduction of hepatic JNK1 protein in DIO mice. **A,** histological staining for β-galactosidase in liver after treatment with vehicle or adenovirus expressing β-galactosidase. **B,** Western analysis of JNK1 and JNK2 in liver, C, JNK1 in fat tissue after adenovirus administration. Densitometric analysis (graphed) of 6–8 animals per group (p < 0.0001). **D,** electrochemiluminescent enzyme-linked immunosorbent assay analysis for hepatic c-Jun phosphorylation relative to Ad-shGFP controls (6–8 animals in each group; p < 0.01). * and # represent significant differences between Ad-shJNK1 and control Ad-shGFP or vehicle-treated mice, respectively.
that knockdown of JNK1 enhances insulin signaling in vivo and in vitro.

Liver-specific Knockdown of JNK1 Robustly Increased Circulating Triglyceride Levels Associated with Increased Hepatic PGC-1β, Glucokinase, and MTP Expression—Plasma triglyceride levels were examined following recombinant adenoviral treatment in ad libitum fed and 16-h-fasted DIO mice. To our surprise, in ad libitum fed mice, liver-specific knockdown of JNK1 by Ad-shJNK1 resulted in a robust 2.8-fold increase in circulating triglyceride levels compared with control Ad-shGFP treatment (250 ± 84 versus 88 ± 38 mg/dl, respectively) (Fig. 4A). In 16-h-fasted mice, Ad-shJNK1 treatment resulted in a 3.7-fold increase of plasma triglyceride levels compared with control Ad-shGFP treatment (280 ± 46 versus 76 ± 26 mg/dl, respectively) (Fig. 4B). In the fasted mice, Ad-shJNK1 treatment resulted in no significant change in hepatic triglyceride levels, compared with control Ad-shGFP treatment (118 ± 35 versus 123 ± 31 mg/g, respectively). However, in ad libitum fed mice, Ad-shJNK1 treatment resulted in a significant increase in hepatic triglyceride levels compared with control Ad-shGFP treatment (95 ± 8 versus 51 ± 5 mg/g, respectively). Plasma free fatty acid levels were significantly elevated by 40% in JNK1 knockdown ad libitum fed mice compared with Ad-shGFP-treated mice (0.85 ± 0.2 versus 0.60 ± 0.18 mM). Plasma total cholesterol levels tended to be lower in Ad-shJNK1-treated ad libitum fed mice compared with Ad-shGFP-treated mice (117 ± 16 versus 141 ± 29 mg/dl, respectively), but this difference did not reach statistical significance (Fig. 4C). Plasma lipoprotein analysis by fast-protein liquid chromatography showed a robust 5-fold increase of triglyceride levels in the VLDL fraction of Ad-shJNK1-treated ad libitum fed mice compared with Ad-shGFP-treated mice (Fig. 4, D and E). Additionally, the Ad-shJNK1-treated mice had significantly increased cholesterol levels in the VLDL fraction and reduced cholesterol levels in LDL and HDL fractions compared with Ad-shGFP-treated mice (Fig. 4D). Similar lipoprotein changes were observed in 16-h fasted mice as a result of liver-specific JNK1 knockdown (data not shown).

To investigate potential mechanisms for increased plasma triglyceride, we analyzed liver expression of several key genes involved in lipogenesis and lipoprotein secretion at day 5 after recombinant adenoviral treatment in the ad libitum fed mice. Compared with control Ad-shGFP treatment, real-time PCR analysis demonstrated that liver-specific knockdown of JNK1 resulted in a robust and significant 3.8-fold induction of PGC-1β expression (Fig. 5A). PGC-1β expression, in contrast, did not show significant changes as a result of JNK1 knockdown. In addition, expression of cytochrome c, a well known PGC-1 target gene, was significantly elevated by 2-fold in the same mice, supportive of increased PGC-1β activity in these animals (Fig. 5A). The mRNA level of the transcription factor SREBP1, a master regulator for hepatic lipogenesis, was modestly but not significantly reduced in the JNK1-knockdown mice (Fig. 5A). Western blot analysis demonstrated a significant

![FIGURE 2. Adenovirus-mediated delivery of JNK1 shRNA significantly lowered the blood glucose and plasma insulin levels in DIO mice. A, blood glucose levels (18–20 animals per group; p < 0.0001). B, plasma insulin levels (6–8 animals per group; p < 0.05) in ad libitum fed DIO mice at day 5 after adenovirus administration. C, glucose levels and percent changes of initial glucose levels (D) after an insulin tolerance test in DIO mice at day 5 after adenovirus administration (7 animals per group; p < 0.05). * and # represent significant differences between Ad-shJNK1 and control Ad-shGFP or vehicle-treated mice, respectively.](https://example.com)
JNK1 Up-regulates PGC-1β and Increases Plasma Triglyceride

A

Vehicle
Ad-shGFP
Ad-shJNK1

Anti-phospho-Akt

Total Akt

B

Vehicle
Ad-shGFP
Ad-shJNK1

Anti-phospho-Akt

Total Akt

C

Viral dose (VP/ml)
Ad-shJNK1
Ad-shGFP

5 x 10^3
10 x 10^3

Anti-JNK

Total Akt

D

siRNA
100 nM JNK1 siRNA
Control

Anti-JNK

Total Akt

E

IB:IR

IP: p-tyrosine

Insulin

Control siRNA

JNK1 siRNA

15
60
15
60

F

IB:PI3K

IP: IRS-1

Insulin

Control siRNA

JNK1 siRNA

15
60
15
60

FIGURE 3. RNAi-mediated JNK1 knockdown enhanced insulin signaling in vivo and in vitro. A and B, Western blots of hepatic Akt-p-Ser-473 and total Akt in ad libitum fed and 16-h-fasted DIO mice after adenoviral administration. Densitometric analysis (graphed) of 6–8 animals per group (p < 0.01). C, Western analysis of JNK1, Akt-p-Ser-473, and total Akt in primary mouse hepatocytes treated with Ad-shGFP or Ad-shJNK1. Data are representative of three independent experiments. D, Western analysis of JNK1 and Akt-p-Ser-473 in HepG2 cells treated with control siRNA, or siRNA against human JNK1 in response to chronic 10 μM insulin treatment. E, tyrosine phosphorylation of insulin receptor (IR) and IRS-1 was measured by immunoprecipitating tyrosine-phosphorylated proteins followed by Western analysis of IR and IRS-1 in HepG2 cells treated with 10 nM insulin for 15 and 60 min. F, association of IRS-1 with PI3K subunit, p85, was measured by co-immunoprecipitating IRS-1 and p85 proteins with IRS-1 antibody followed by Western analysis of p85 in HepG2 cells treated with 10 nM insulin. * and # represent significant differences between Ad-shJNK1 and control Ad-shGFP or vehicle-treated mice, respectively.

46% reduction of SREBP1 protein in JNK1-knockdown mice (Fig. 5C). Despite reduced expression of SREBP1, real-time PCR analysis showed unchanged expression of several SREBP1-target genes, including FAS, SC1, ACC1, DGAT2, HMGCR, and LDLR in JNK1-knockdown mice (data not shown). In contrast, JNK1 knockdown resulted in a significant 70% increase over the control in the expression of glucokinase, another target gene of SREBP1 (22) (Fig. 5B). Glucokinase protein levels, measured after Western blotting and using densitometric analysis, were increased to about the same extent (Fig. 5C). Hepatic microsomal triacylglycerol transfer protein (MTP), which is the key enzyme for VLDL synthesis and secretion, was significantly increased by 70% in JNK1 knockdown mice (Fig. 5B). Consistent with this finding, expression of MTP was significantly increased by 1.8-fold in the microarray analysis (data not shown). Hepatic overexpression of either glucokinase or MTP has been shown by others to result in increased circulating triglyceride levels in rodents (23, 24). Overall, our data suggest that increased hepatic expression of PGC-1β, glucokinase, and MTP upon JNK1 knockdown could contribute to the elevated plasma triglyceride levels.

Liver-specific Knockdown of JNK1 Increased the Expression of Clusters of Genes in Glycolysis and Several Genes in Triglyceride Synthesis Pathways—To further investigate the mechanism of reduced circulating glucose and insulin, and increased plasma triglyceride levels in JNK1 knockdown mice, expression of metabolic pathway genes in ad libitum fed mice was analyzed by Affymetrix GeneChip array as described under “Experimental Procedures.” Genes that had statistically significant differences (p < 0.05) of >1.5-fold versus the control Ad-shGFP-treated group were investigated using the Gladstone Institute’s GenMapp and MapperFinder software to identify the biological pathways and categories of related genes with coordinated gene regulation. Consistent with the real-time PCR data, PGC-1β, but not PGC-1α, was increased by 2.2-fold in the JNK1 knockdown group. The majority of genes in mitochondrial biogenesis, oxidative phosphorylation, fatty acid oxidation, and tri-chloroacetic acid cycle were significantly up-regulated upon JNK1 knockdown. Glycolysis and gluconeogenesis pathway analysis demonstrated that knockdown of
increased hepatic triglyceride synthesis but not fatty acid synthesis in JNK1 knockdown mice.

**DISCUSSION**

With adenovirus-mediated expression of an effective JNK1 shRNA, we achieved near complete knockdown of liver JNK1 without affecting JNK1 in other tissues. Several important findings have emerged from this study. First, liver-specific knockdown of JNK1 significantly lowers circulating glucose and insulin levels in DIO mice. Knockdown of JNK1 enhanced hepatic insulin signaling in vivo and in vitro. Gene expression analysis showed that the glycolysis but not the gluconeogenesis pathway was up-regulated upon JNK1 knockdown, which could contribute to reduced glucose and insulin levels. Second, hepatic JNK1 knockdown significantly increased circulating triglyceride levels and was associated with increased PGC-1β expression. Furthermore, knockdown of JNK1 up-regulated genes in lipoprotein secretion and triglyceride synthesis.

It is interesting to observe that JNK1 appears to play an important role in lipid metabolism. Increased plasma triglyceride levels were not reported in JNK1 knock-out mice fed a high fat diet (4). It is possible that knock-out of JNK1 in other tissues could obviate or negate elevation of circulating triglyceride levels due to liver-specific JNK1 effects. Another possible explanation is that we knocked down hepatic JNK1 in insulin-resistant obese mice, whereas mice with genetic disruption of JNK1 did not develop diet-induced obesity and insulin resistance and were, therefore, metabolically different. Several other studies designed to investigate inhibition of JNK activity, through use of a JNK inhibitory peptide, overexpression of a dominant-negative JNK, and use of JNK small molecule inhibitors, also demonstrated improved insulin sensitivity without increased plasma triglyceride levels in obese diabetic mice (3, 25, 26).
However, these studies did not specifically target liver JNK1, and the specificity and potency of the inhibitors in vivo was not described. Although we selected JNK1 RNAi sequence to match only JNK1, it is possible that increased plasma triglycerides result from off-target effects of RNAi, a technical limitation of RNAi technology. However, in our study, this is unlikely to be the case. We applied different RNAi sequences in vitro to target either human or mouse JNK1 and observed increased Akt-p with JNK1 knockdown in both systems. The sustained increase in hepatic Akt activity resulting from JNK1 knockdown could contribute to lowered blood glucose and insulin levels and increased circulating triglycerides: it has previously been reported that adenovirus-mediated hepatic overexpression of constitutively activated Akt significantly lowered blood glucose and insulin levels and robustly increased plasma triglycerides (28). Although insulin activates lipogenesis and SREBP1, the role of Akt in activation of SREBP1 is controversial (29, 30). Recent studies showed that atypical protein kinase C plays a major role in mediating insulin effects on SREBP1 expression through the IRS-2/PI3K pathway in liver (31). In our study, hepatic knockdown of JNK1 resulted in mildly reduced SREBP1 levels despite sustained increases in Akt phosphorylation.

What could be the mechanism for increased circulating triglyceride levels in DIO mice as a result of liver-specific knockdown of JNK1? Our study showed that knockdown of JNK1 increased the expression of genes involved in lipoprotein secretion, glycolysis, and triglyceride synthesis pathways, which could contribute to the increased plasma triglyceride levels in DIO mice (Fig. 7). First, expression of MTP gene was up-regulated upon JNK1 knockdown, indicating increased lipoprotein secretion. Hepatic overexpression of MTP has been shown by others to result in increased circulating triglyceride levels in rodents (24). Second, microarray analysis showed that the majority of genes in the glycolysis pathway were up-regulated, including that for glucokinase (23). It could also contribute to the increased circulating triglyceride levels we observed, because activation of glycolysis by hepatic overexpression of glucokinase gene increases circulating triglyceride levels in rats (23). However, mildly overexpressed glucokinase or pharmacological glucokinase activators appear not to increase circulating triglyceride levels in rodents (23, 32, 33). In our study, activation of glycolysis in JNK1 knockdown mice could increase lipogenesis through the provision of increased substrates to lipogenesis in the context of unchanged expression of fatty acid synthesis genes. Third, several genes in triglyceride synthesis were up-regulated in JNK1 knockdown mice, including DGAT1, glycerol kinase, and ACSL4, whereas the rest of the genes involved in fatty acid and triglyceride syn-

![Diagram of glycolysis and gluconeogenesis pathways](https://example.com/glycolysis-diagram.png)
thesis pathways were unchanged. This indicates that the triglyceride synthesis pathway was activated upon JNK1 knockdown. Plasma free fatty acid levels were significantly increased in JNK1 knockdown mice, possibly due to increased lipolysis from fat tissue because of lowered insulin levels. The activation of hepatic triglyceride synthesis and increased plasma free fatty acid levels could contribute to increased plasma triglyceride levels in JNK1 knockdown mice.

How does knockdown of JNK1 up-regulate the expression of genes in glycolysis, lipoprotein secretion, and triglyceride synthesis? PGC-1β has recently been identified not only as a key activator of hepatic lipogenesis through co-activation of SREBP1 but also as a key activator of lipoprotein secretion through co-activation of LXRα and Foxa2 and a concomitant increase in MTP expression (13, 14, 27). The expression of hepatic PGC-1β is strongly induced by dietary fats (14). Hepatic overexpression of PGC-1β significantly increases circulating triglyceride levels in rodents (14). In our study, liver-specific knockdown of JNK1 in DIO mice significantly increased PGC-1β expression. As a consequence, this could lead to up-regulation of hepatic MTP expression. Increased PGC-1β could also lead to up-regulation of genes in glycolysis and triglyceride synthesis, such as glucokinase and DGAT1, because they are target genes for SREBP1. Despite the somewhat reduced expression level of SREBP1 we observed with JNK1 knockdown, increased glucokinase and DGAT1 expression and maintained expression of other SREBP1 target genes could be explained by greater SREBP1 activity achieved through activation of SREBP1 as a result of increased PGC-1β expression. We cannot exclude, however, the possibility that other mechanisms may contribute to activate these pathways and increase plasma triglyceride levels. From our results, we hypothesize that the increased expression of PGC-1β provides the link between JNK1 knockdown and the increased plasma triglyceride levels (Fig. 7).

In summary, there is support from the existing literature that increased activity of Akt, or increased expression of PGC1β, glucokinase, or MTP, can result in increased plasma triglycerides in rodents. Our observation of increased expression of PGC-1β, MTP, and glucokinase and sustained elevation of Akt activity in liver-specific JNK1 knockdown mice sheds light on potential mechanisms to explain how knockdown of JNK1 affects triglyceride metabolism and secretion in vivo. Increased circulating triglycerides as a consequence of lowered hepatic JNK1 activity could have deleterious consequences in humans, such as increased risk of cardiovascular disease for diabetic and obese patients already at high risk and increased islet β-cell death. Further studies are required to evaluate the undesired increase in circulating triglyceride levels and the potential ramifications for drug development targeting JNK1 activity.

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REFERENCES
1. Wellen, K. E., and Hotamisligil, G. S. (2005) J. Clin. Invest. 115, 1111–1119
2. Manning, A. M., and Davis, R. J. (2003) Nat. Rev. Drug Discov. 2, 554–565
3. Bennett, B. L., Satoh, Y., and Lewis, A. J. (2003) Curr. Opin. Pharmacol. 3, 420–425
4. Hirosumi, J., Tuncman, G., Chang, L., Gorgun, C. Z., Uysal, K. T., Maeda, K., Karin, M., and Hotamisligil, G. S. (2002) Nature 420, 333–336
5. Davis, R. J. (2000) Cell 103, 239–252
6. Carlson, C. J., Koterski, S., Sciotti, R. J., Poccard, G. B., and Rondinone, C. M. (2003) Diabetes 52, 634–641
7. Aguirre, V., Uchida, T., Yenush, L., Davis, R., and White, M. F. (2000) J. Biol. Chem. 275, 9047–9054
8. Gao, Z., Zhang, X., Zuberi, A., Hwang, D., Quon, M. J., Lefevre, M., and Ye, J. (2004) Mol. Endocrinol. 18, 2024–2034
9. Ozcan, U., Cao, Q., Yilmaz, E., Lee, A. H., Iwakoshi, N. N., Ozdelen, E., Tuncman, G., Gorgun, C., Glimcher, L. H., and Hotamisligil, G. S. (2004) Science 306, 457–461
10. Novina, C. D., and Sharp, P. A. (2004) Nature 430, 161–164
11. Dorsett, Y., and Tuschl, T. (2004) Nat. Rev. Drug Discov. 3, 318–329
12. Becker, T. C., Noel, R. J., Coats, W. S., Gomez-Foix, A. M., Alam, T., Gerard, R. D., and Newgard, C. B. (1994) Methods Cell Biol. 43, 161–189
13. Lin, J., Yang, R., Tarr, P. T., Wu, P. H., Handschin, C., Li, S., Yang, W., Pei, L., Uldry, M., Fontenoz, P., Newgard, C. B., and Spiegelman, B. M. (2005) Cell 120, 261–273
14. Wolfrum, C., and Stoffel, M. (2006) Cell Metab. 3, 99–110
15. Huang, A., Chen, Y., Wang, X., Zhao, S., Su, N., and White, D. W. (2004) FEBS Lett. 558, 69–73
16. Yang, R., Cao, L., Gasa, R., Brady, M. J., Sherry, A. D., and Newgard, C. B. (2002) J. Biol. Chem. 277, 1514–1523
17. Yang, R., and Newgard, C. B. (2003) J. Biol. Chem. 278, 23418–23425
18. Maglott, D., Ostell, J., Pruitt, K. D., and Tatusova, T. (2005) Nucleic Acids Res. 33, D39–D45
19. Dahlquist, K. D., Salomonis, N., Vranizan, K., Lawlor, S. C., and Conklin, B. R. (2002) Nat. Genet. 31, 19–20
20. Doniger, S. W., Salomonis, N., Dahlquist, K. D., Vranizan, K., Lawlor, S. C., and Conklin, B. R. (2003) Genome Biology 4:R7
21. Wilcox, D. M., Yang, R., Morgan, S. J., Nguyen, P. T., Voorbach, M. J., Jung,
JNK1 Up-regulates PGC-1β and Increases Plasma Triglyceride

P. M., Haasch, D. L., Lin, E., Bush, E. N., Op Menorth, T. J., Jacobson, P. B., Collins, C. A., Rondinone, C. M., Surowy, T., and Landschulz, K. T. (2007) J. RNAi Gene Silencing 3, 225–236

22. Foretz, M., Guichard, C., Ferre, P., and Foufelle, F. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 12737–12742

23. O’Doherty, R. M., Lehman, D. L., Telemaque-Potts, S., and Newgard, C. B. (1999) Diabetes 48, 2022–2027

24. Tietge, U. J., Bakillah, A., Maugerais, C., Tsukamoto, K., Hussain, M., and Rader, D. J. (1999) J. Lipid Res. 40, 2134–2139

25. Kaneto, H., Nakatani, Y., Miyatuka, T., Kawamori, D., Matsuoka, T. A., Matsuhisa, M., Kajimoto, Y., Ichijou, H., Yamasaki, Y., and Hori, M. (2004) Nat. Med. 10, 1128–1132

26. Nakatani, Y., Kaneto, H., Kawamori, D., Hatazaki, M., Miyatsuka, T., Matsuoka, T. A., Kajimoto, Y., Matsuhisa, M., Yamasaki, Y., and Hori, M. (2004) J. Biol. Chem. 279, 45803–45809

27. Spann, N. J., Kang, S., Li, A. C., Chen, A. Z., Newberry, E. P., Davidson, N. O., Hui, S. T., and Davis, R. A. (2006) J. Biol. Chem. 281, 33066–33077

28. Ono, H., Shimano, H., Katagiri, H., Yahagi, N., Sakoda, H., Onishi, Y., Anai, M., Ogihara, T., Fujishiro, M., Viana, A. Y., Fukushima, Y., Abe, M., Shoji, N., Kikuchi, M., Yamada, N., Oka, Y., and Asano, T. (2003) Diabetes 52, 2905–2913

29. Fleischmann, M., and Iynedjian, P. B. (2000) Biochem. J. 349, 13–17

30. Matsumoto, M., Ogawa, W., Akimoto, K., Inoue, H., Miyake, K., Furukawa, K., Hayashi, Y., Iguchi, H., Matsuki, Y., Hiramatsu, R., Shimano, H., Yamada, N., Ohno, S., Kasuga, M., and Noda, T. (2003) J. Clin. Invest. 112, 935–944

31. Farese, R. V., Sajjan, M. P., and Standart, M. L. (2005) Exp. Biol. Med. 230, 593–605

32. Desai, U. J., Slosberg, E. D., Boettcher, B. R., Caplan, S. L., Fanelli, B., Stephan, Z., Gunther, V. J., Kaleko, M., and Connelly, S. (2001) Diabetes 50, 2287–2295

33. Grimsby, J., Sarabu, R., Corbett, W. L., Haynes, N. E., Bizzarro, F. T., Coffey, J. W., Guertin, K. R., Hilliard, D. W., Kester, R. F., Mahaney, P. E., Marcus, L., Qi, L., Spence, C. L., Tengi, J., Magnuson, M. A., Chu, C. A., Dvorozniak, M. T., Matschinsky, F. M., and Grippo, J. F. (2003) Science 301, 370–373