nature medicine

LETTERS

PGC-1 promotes insulin resistance in liver through PPAR-α-dependent induction of TRB-3

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Insulin resistance is a major hallmark in the development of type 2 diabetes, which is characterized by an impaired ability of insulin to inhibit glucose output from the liver and to promote glucose uptake in muscle[1,2]. The nuclear hormone receptor coactivator PGC-1 (peroxisome proliferator-activated (PPAR)-γ coactivator-1) has been implicated in the onset of type 2 diabetes. Hepatic PGC-1 expression is elevated in mouse models of this disease, where it promotes constitutive activation of gluconeogenesis and fatty acid oxidation through its association with the nuclear hormone receptors HNF-4 and PPAR-α, respectively[3–5]. Here we show that PGC-1-deficient mice, generated by adenoviral delivery of PGC-1 RNA interference (RNAi) to the liver, experience fasting hypoglycemia. Hepatic insulin sensitivity was enhanced in PGC-1-deficient mice, reflecting in part the reduced expression of the mammalian *tribbles* homolog TRB-3, a fasting-inducible inhibitor of the serine-threonine kinase Akt/PKB (ref. 6). We show here that, in the liver, TRB-3 is a target for PPAR-α. Knockdown of hepatic TRB-3 expression improved glucose tolerance, whereas hepatic overexpression of TRB-3 reversed the insulin-sensitive phenotype of PGC-1-deficient mice. These results indicate a link between nuclear hormone receptor and insulin signaling pathways, and suggest a potential role for TRB-3 inhibitors in the treatment of type 2 diabetes.

Fasting triggers a series of hormonal and nutritional cues that promote energy balance by stimulating glucose output and lipid breakdown in the liver[7]. For example, pancreatic glucagon induces expression of the gluconeogenic program, through protein kinase A–mediated phosphorylation of the nuclear factor cAMP response element binding protein (CREB)[8,9]. Influx of free fatty acids from adipose tissue stimulates β-oxidation through the nuclear receptor PPAR-α[10]. The expression of both gluconeogenic and β-oxidation genes is further potentiated by the nuclear hormone receptor coactivator PGC-1, a target of CREB whose levels are increased during fasting and in diabetes[3,4].

To determine whether PGC-1 is necessary for glucose and fatty acid homeostasis, we prepared an RNAi adenovirus targeting PGC-1. After tail-vein injection into mice, the PGC-1 RNAi adenovirus disrupted hepatic PGC-1 expression relative to control virus expressing green fluorescent protein (GFP; **Fig. 1a**). Livers of PGC-1 knockdown mice had threefold higher triglyceride levels compared with GFP-injected mice (**Fig. 1b**). Fasting glucose levels were also reduced in PGC-1-deficient mice, along with mRNAs encoding the gluconeogenic enzymes phosphoenolpyruvate carboxykinase (PEPCK) and glucose 6-phosphatase (**Figs. 1c–e**). Similar effects of PGC-1 knockdown were observed in cultures of rat hepatocytes treated with forskolin plus dexamethasone to mimic the fasted state (**Supplementary Fig. 1** online). In parallel with these changes, levels in the liver of mRNAs encoding fatty acid oxidation enzymes (carnitine palmitoyl transferase, medium-chain acyl-CoA dehydrogenase (MCAD) and acyl-CoA oxidase) were decreased by 10- to 15-fold (**Fig. 1f**). Despite its importance in promoting mitochondrial function in skeletal muscle[11], PGC-1 knockdown had marginal effects in the liver on mRNAs encoding the mitochondrial proteins cytochrome c and cyclooxygenase-4 (**Supplementary Fig. 2** online). Indeed, hepatic expression of the genes encoding cytochrome c and cyclooxygenase-4 did not vary appreciably in fasting compared with re-fed animals (**Supplementary Fig. 3** online), and there was no difference in body weight observed between control and PGC-1-deficient mice (**Supplementary Fig. 4** online). These results indicate that the PGC-1-dependent activation of gluconeogenesis and fatty acid oxidation in liver is crucial for glucose and lipid homeostasis during fasting.

PGC-1 expression is induced in mouse models of diabetes, where it seems to contribute to insulin resistance[3,4]. Indeed, injection of *db*/*db* diabetic mice with PGC-1 RNAi adenovirus reduced fasting blood glucose levels to normal (**Fig. 1d**). To investigate whether PGC-1 interferes with insulin signaling in liver, we conducted glucose tolerance tests on wild-type and diabetic mice transduced with adenoviruses expressing GFP (control) or PGC-1 RNAi. After glucose injection, GFP-injected mice showed a higher excursion of blood glucose relative to PGC-1-deficient animals; these levels remained ele-

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Published online 25 April 2004; doi:10.1038/nm1044

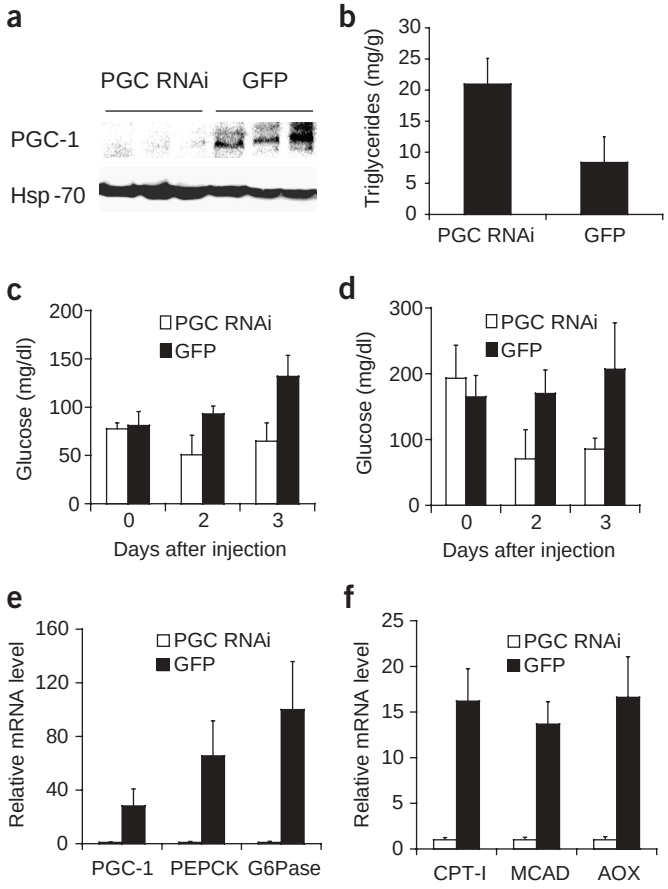

Figure 1 PGC-1 deficiency promotes fasting hypoglycemia and altered hepatic lipid metabolism. (**a**) Western blot analysis of PGC-1 and Hsp-70 proteins from livers of 16-h fasted mice injected with adenovirus expressing PGC-1 RNAi or control GFP ($n = 3$). (**b**) Triglycerides in livers of mice injected with GFP or PGC-1 RNAi adenovirus ($n = 4$). (**c**,**d**) Fasting blood glucose in wild-type (**c**; $n = 7$) and diabetic (**d**; $n = 8$) male mice injected with either GFP or PGC-1 RNAi adenovirus. (**e**) Q-PCR analysis of gluconeogenic gene expression using liver RNA from 16-h fasted mice injected with control or PGC-1 RNAi adenovirus ($n = 3$). (**f**) Q-PCR analysis of hepatic β-oxidation genes using RNA samples described in **e** ($n = 3$). Data in **b**–**f** represent mean ± s.e.m. G6Pase, glucose 6-phosphatase; CPT-I, carnitine palmitoyl transferase I; AOX, acyl-CoA oxidase.

vated in control mice compared with PGC-1 RNAi mice for up to 2 h (**Fig. 2a**). PGC-1 RNAi adenovirus had stronger effects on insulin sensitivity in diabetic mice, suggesting that PGC-1 induces the expression of genes that inhibit hepatic insulin signaling (**Fig. 2b**). Indeed, in insulin tolerance tests, PGC-1-deficient mice experienced a greater reduction in blood glucose after 60 min than did GFP-infected control mice (**Supplementary Fig. 5** online).

To further evaluate the effects of PGC-1 deficiency on tissue insulin sensitivity, we conducted euglycemic–hyperinsulinemic glucose clamp experiments on adult male Wistar rats transduced with PGC-1 RNAi or GFP adenoviruses. After a 6-h fast, basal hepatic glucose output (HGO), measured by infusion of [^{3}H]glucose, was 25% lower in PGC-1 RNAi rats than in control GFP-infected rats ($P < 0.01$; **Fig. 2c**). HGO was suppressed by insulin infusion in both groups, but insulin had greater effects on PGC-1-deficient rats, reducing HGO by 75 ± 3.2% in those animals compared with 58 ± 4.3% ($P < 0.01$) in control rats (**Fig. 2d**). Seventy to eighty percent of the insulin-stimulated glucose disposal rate *in vivo* represents skeletal muscle glucose uptake; this rate did not differ between the two groups of rats (**Fig. 2e**). These results suggest that hepatic PGC-1 knockdown reduces basal HGO and increases hepatic insulin sensitivity without affecting insulin action.

In the fed state, insulin inhibits hepatic glucose output through a cascade of phospholipid-dependent kinases that activate Akt/PKB by phosphorylating it at Thr308 and Ser473 (refs. 12,13). Consistent with its ability to reduce HGO, the PGC-1 RNAi adenovirus increased basal and insulin-stimulated Ser473 phosphorylation of Akt in livers of diabetic mice (**Fig. 2f**). Ser9 phosphorylation of glycogen synthase kinase-3β (GSK-3β), a substrate of Akt, was also induced in PGC-1-deficient mice, indicating that PGC-1 knockdown potentiates hepatic Akt activity.

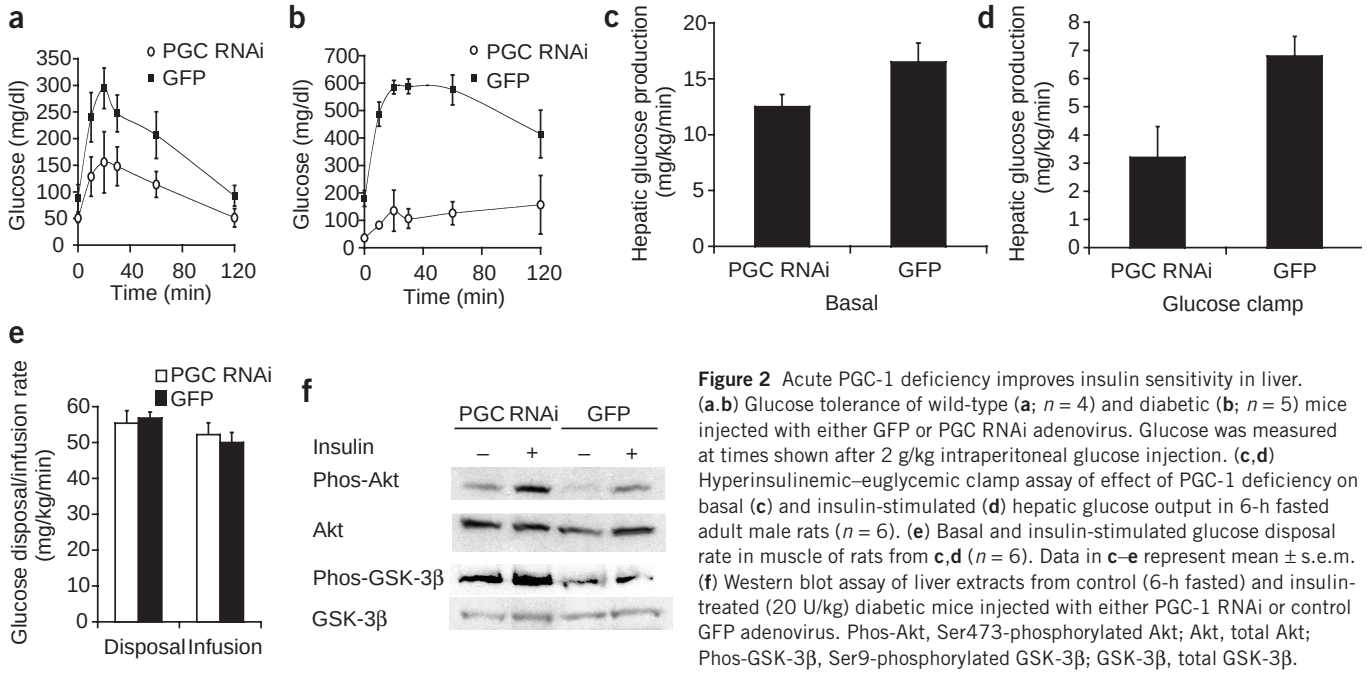

Figure 2 Acute PGC-1 deficiency improves insulin sensitivity in liver. (**a**,**b**) Glucose tolerance of wild-type (**a**; $n = 4$) and diabetic (**b**; $n = 5$) mice injected with either GFP or PGC RNAi adenovirus. Glucose was measured at times shown after 2 g/kg intraperitoneal glucose injection. (**c**,**d**) Hyperinsulinemic–euglycemic clamp assay of effect of PGC-1 deficiency on basal (**c**) and insulin-stimulated (**d**) hepatic glucose output in 6-h fasted adult male rats ($n = 6$). (**e**) Basal and insulin-stimulated glucose disposal rate in muscle of rats from **c**,**d** ($n = 6$). Data in **c**–**e** represent mean ± s.e.m. (**f**) Western blot assay of liver extracts from control (6-h fasted) and insulin-treated (20 U/kg) diabetic mice injected with either PGC-1 RNAi or control GFP adenovirus. Phos-Akt, Ser473-phosphorylated Akt; Akt, total Akt; Phos-GSK-3β, Ser9-phosphorylated GSK-3β; GSK-3β, total GSK-3β.

TRB-3 is induced in liver during fasting and in diabetic mice, where it inhibits insulin signaling by binding to and preventing activation of Akt/PKB[6]. TRB-3 protein was markedly reduced in PGC-1-deficient mice compared with control diabetic mice, as assayed by western blotting (**Fig. 3a**). Conversely, overexpression of PGC-1 in primary rat hepatocytes increased TRB-3 mRNA (**Fig. 3b**), suggesting that PGC-1 modulates hepatic insulin sensitivity by stimulating TRB-3 expression.

TRB-3 levels in liver are elevated in the fatty liver dystrophic mouse[14], a model of lipodystrophy. This prompted us to examine whether regulators of hepatic fatty acid metabolism, such as PPAR-α, promote TRB-3 expression in a PGC-1-dependent manner. Treatment with the PPAR-α agonist GW7,647 (ref. 15) increased TRB-3 mRNA twofold in primary hepatocytes; this effect was reversed by cotreatment with insulin (**Fig. 3b**). Both TRB-3 mRNA and PGC-1 mRNA were elevated in LIRKO mice, which have a knockout of the insulin receptor in liver[16], indicating that insulin negatively regulates TRB-3 expression during feeding (**Fig. 3c**).

To examine the importance of PPAR-α in modulating hepatic TRB-3 expression, we added a PPAR-α-specific ligand, WY-14,643, to the diets of control and PPAR-α-deficient mice. WY-14,643 induced a 2.5-fold increase in TRB-3 mRNA in wild-type mice, as well as a fourfold increase in the fatty acid oxidation enzyme MCAD (**Fig. 3d**). The PPAR-α ligand had no effect on TRB-3 or MCAD in PPAR-α-deficient animals, suggesting that TRB-3 is a target for PPAR-α regulation in liver (**Fig. 3d**). To further test the importance of PPAR-α for PGC-1-dependent induction of TRB-3, we infected PPAR-α-deficient mice with the PGC-1 RNAi adenovirus. There was no decrease in hepatic TRB-3 protein in PPAR-α-deficient mice after RNAi-mediated knockdown of PGC-1 (**Fig. 3e**), indicating that PPAR-α is indeed required to induce TRB-3.

The human TRB-3 promoter contains a number of consensus PPAR-α regulatory elements. We evaluated the importance of these sites for TRB-3 gene regulation. Overexpression of PPAR-α and its heterodimeric partner RXR[17] induced a twofold increase in TRB-3 luciferase reporter activity, as compared to empty vector, in HepG2 hepatoma cells, and treatment with WY-14,643 ligand further potentiated reporter activity by twofold (**Fig. 3f**). Promoter deletion studies indicate that sequences between –750 and –350 on the TRB-3 promoter mediate PPAR-α-dependent induction by its ligand (**Fig. 3f**).

The induction of TRB-3 by PPAR-α and PGC-1 during fasting prompted us to examine the role of TRB-3 in mediating the effects of PGC-1 on glucose metabolism. After injection with TRB-3-encoding adenovirus, expression of TRB-3 in the liver of PGC-1-deficient mice was comparable to that in diabetic mice (**Fig. 4a**). By contrast with the insulin-sensitizing effects of PGC-1 RNAi adenovirus alone, mice injected with both TRB-3 and PGC-1 RNAi adenoviruses had glucose excursion profiles comparable to those of control GFP-injected mice (**Fig. 4b**). The effect of TRB-3 alone on maximal glucose excursion in glucose tolerance assays (1.4-fold increase)[6] was intermediate relative to mice injected with TRB-3 and PGC-1 RNAi viruses (1.8-fold increase; **Fig. 4b**). PGC-1 protein was equally reduced in mice injected with PGC-1 RNAi virus alone and mice injected with TRB-3 plus PGC-1 RNAi, which argues against a nonspecific effect of TRB-3 adenovirus on PGC-1 RNAi expression (**Supplementary Fig. 6** online).

To determine whether changes in TRB-3 expression modulate insulin sensitivity in liver, we prepared a TRB-3 RNAi adenovirus. Mice transduced with TRB-3 RNAi virus experienced a reduction in TRB-3 protein relative to control mice, according to western blot assays of liver extracts (**Fig. 4c**). Consistent with these effects, mice subjected to TRB-3 RNAi showed improved glucose tolerance compared with control mice (**Fig. 4d**). These results argue that induction of TRB-3 by PGC-1 inhibits insulin signaling during fasting, and may contribute to insulin resistance in diabetic mice (**Fig. 4e**).

The hypoglycemic fatty liver phenotype in PGC-1 knockdown mice is reminiscent of the phenotype of PPAR-α-deficient mice, which have hepatic steatosis and hypoglycemia[18,19]. Disruption of fatty acid oxidation in this setting seems to induce steatosis by diverting the pool of intracellular free fatty acids toward triglyceride storage. PPAR-α-deficient mice also show increased insulin sensitivity in

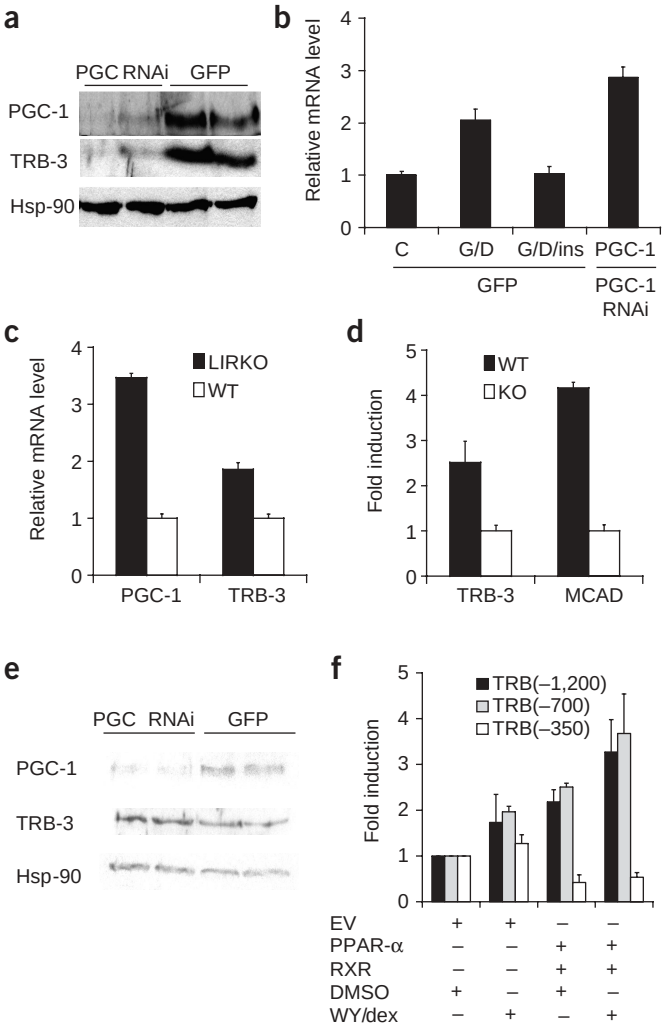

Figure 3 PGC-1 promotes expression of TRB-3 through PPAR-α. (**a**) Western blot analysis of PGC-1 and TRB-3 from fasted livers of diabetic mice injected with either GFP, PGC-1 or Hsp-90 RNAi adenovirus. (**b**) Q-PCR analysis of TRB-3 mRNA in primary hepatocytes infected with either GFP or PGC-1 adenovirus. C, control; G, GW7,647; D, dexamethasone; Ins, insulin. (**c**) PGC-1 and TRB-3 mRNA in either Lox*P* control animals (WT) or liver-specific insulin receptor knockout (LIRKO) mice fed *ad libitum*. (**d**) Hepatic TRB-3 and MCAD mRNA in wild-type (WT) or PPAR-α-deficient (KO) mice fed *ad libitum* for 4 d, with the PPAR-α-agonist WY-14,643; fold induction by ligand (as compared to mice not receiving WY-14,643) is shown. (**e**) Western blot analysis of PGC-1 and TRB-3 in livers of 6-h fasted PPAR-α-deficient mice injected with either GFP, PGC-1 or Hsp-90 RNAi adenovirus. (**f**) Co-transfection assay of HepG2 cells using TRB-3 luciferase reporters extending from –170 to either –1,200, –700 or –350 and fused to the herpesvirus thymidine kinase promoter. EV, empty vector; Wy/dex, WY-14,643 plus dexamethasone. Data in **b**–**d**,**f** represent mean ± s.e.m.

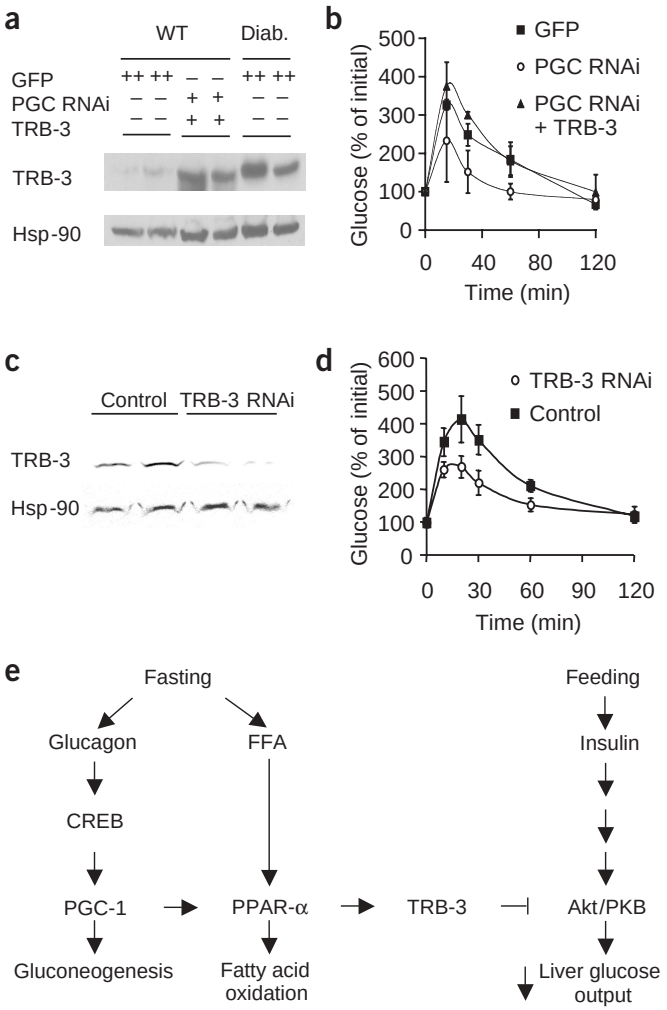

Figure 4 PGC-1 promotes insulin resistance by inducing TRB-3 expression in liver. (**a**) Western blot assay of hepatic TRB-3 and Hsp-90 in wild-type (WT) and diabetic (Diab.) mice injected with GFP, or PGC RNAi plus TRB-3, adenoviruses. GFP-expressing adenovirus was included to equalize the amount of adenovirus injected. ++ and +, twice the amount of GFP adenovirus was injected compared to PCG RNAi or TRB-3 adenovirus. (**b**) Glucose tolerance of fasted wild-type mice injected with GFP, PGC-1 RNAi, or PGC RNAi plus TRB-3 adenovirus. Glucose levels were monitored at times shown. (**c**) Western blot assay of hepatic TRB-3 and Hsp-90 in wild-type mice injected with TRB-3 RNAi or nonspecific control adenovirus. (**d**) Glucose tolerance of wild-type mice injected with TRB-3 RNAi or nonspecific control adenovirus. Glucose levels were measured as above. Data in **b**,**d** represent mean ± s.e.m. (**e**) Fasting stimulates expression of gluconeogenesis and fatty acid (FFA) oxidation through CREB-dependent induction of PGC-1. PPAR-α/PGC-1 pathway interferes with insulin signaling by stimulating expression of TRB-3.

the context of LDL receptor[20] and ApoE knockouts[21], indicating that the effects of PGC-1 on insulin signaling are likely to reflect the action of PGC-1 on the PPAR-α pathway.

Free fatty acids are thought to inhibit glucose use[22] in part by disrupting components of the insulin signaling cascade in liver[23] and skeletal muscle[24]. Notably, hepatic TRB-3 is elevated in fatty liver dystrophic mice, which have high levels of circulating lipids and are intolerant to glucose during suckling[14,25]. It will be of interest to examine whether inappropriate activation of the PPAR-α pathway in this and other lipodystrophies promotes insulin resistance through TRB-3.

METHODS

Plasmids. TRB-3 promoter sequences were PCR-amplified from human genomic DNA and inserted into the TK-Luc vector to generate the hTRB-3(–1,200/–170)TK-Luc construct. Expression vectors for PPAR-α and RXR were described previously[17]. PEPCK promoter sequences were PCR-amplified from rat genomic DNA and inserted into the pGL3 basic vector to generate the PEPCK(–549/+49)Luc reporter. The pU6-PGC-1 RNAi construct was generated using the mouse PGC-1 coding sequence 5′-GGTG-GATTGAAGTGGTGTAGA-3′ under the control of the human U6 promoter.

Culture of primary hepatocytes. Primary hepatocytes were prepared from 200- to 300-g Sprague-Dawley rats by collagenase perfusion[26] and plated with medium 199 (Invitrogen). After 3–6 h of attachment, cells were infected with either the GFP or PGC-1 adenoviruses for 16 h. Cells were then maintained in serum-free medium without dexamethasone for 8 h and subjected to various treatments, including 100 nM dexamethasone, 1 nM GW7647 or 100 nM insulin for 16 h.

Transfection assays. HepG2 cells were maintained with Ham's F12 medium (Invitrogen). For transfection, we used FuGENE 6 reagent (Roche Applied Science) with 50 ng luciferase construct, 25 ng β-galactosidase plasmid and 25 ng each of PPAR-α and RXR expression vectors. Cells were treated with either DMSO or WY-14,643 (50 nM; Sigma) and dexamethasone (50 nM) for 16 h before collection. Luciferase activity was normalized to β-galactosidase activity. For transfection of primary hepatocytes, we used 1.2 μg luciferase construct, 0.4 μg RNAi construct and 0.4 μg β-galactosidase plasmid with F1 reagent (Targeting Systems). Forty-four hours after transfection, cells were stimulated with either 10 μM forskolin plus 100 nM dexamethasone, or with DMSO vehicle for 4 h.

Recombinant adenoviruses. Adenoviruses expressing GFP only, nonspecific control, PGC-1 or TRB-3 were described previously[4,6]. Adenoviruses for PGC-1 RNAi and TRB-3 RNAi were generated as described[6]. All viruses were CsCl purified.

Animal experiments. Male, 7-week-old C57BL/6 mice or *db*/*db* diabetic mice were purchased from Harlan Laboratories or The Jackson Laboratory. Recombinant adenovirus (0.5×10^9 PFU) was delivered by tail-vein injection to mice anesthetized with isoflurane. To measure fasting blood glucose, we fasted the mice for 16 h (but allowed them free access to water). Liver sections were fixed in 10% formaldehyde and analyzed by fluorescence microscopy to evaluate infection efficiency. PPAR-α-deficient mice (provided by F.J. Gonzalez, National Institutes of Health) were backcrossed for several generations to C57BL/6 mice. For fibrate feeding, mice were given a 0.5% carboxymethylcellulose solution, with or without WY-14,643 (50 mg/kg/d; ChemSyn), by gavage for 4 d. RNA was isolated using TRIzol solution (Invitrogen). Liver RNA was pooled for quantitative PCR (Q-PCR) analysis.

Glucose tolerance test. Mice fasted for 16 h were injected intraperitoneally with 2 g glucose per kg body weight. Blood glucose was measured from tail-vein blood collected at the designated times.

Euglycemic-hyperinsulinemic clamp studies. Nine-week old Wistar rats were implanted with two catheters in the jugular vein and carotid artery. Two days after adenovirus injection, we began euglycemic-hyperinsulinemic clamp studies with a priming injection (2.5 μCi in 0.5ml) and constant infusion (0.04 μCi/min) of D-[3-^{3}H]glucose (New England Nuclear) into rats fasted for 6 h, as previously described[27,28]. After 60 min of tracer equilibration and basal sampling at time 0, glucose (50% dextrose, variable infusion; Abbott) and tracer plus insulin (25 mU/kg/min; Novolin R, Novo Nordisk) were infused into the jugular vein. Blood samples were drawn at 10-min intervals and analyzed for glucose content (YSI 2300 Glucose Analyzer, Yellow Springs Instruments). Blood samples were also taken at –60 min, 0 min and 120 min for determination of glucose specific activity.

Measurement of metabolites. Blood glucose was monitored from tail-vein blood using an automatic glucose monitor (One Touch, Lifescan). Liver triglycerides were measured by colorimetric assay kits (Sigma).

Q-PCR. Total RNA from either primary hepatocytes or liver was extracted using RNeasy mini-kit (Qiagen). cDNA generated by Superscript II enzyme (Invitrogen) was analyzed by Q-PCR using a SYBR green PCR kit and an ABIPRISM 7700 Sequence detector (Perkin Elmer). All data was normalized to ribosomal L32 expression.

Western blot analysis. Western blot assays were done as described[6]. Antibody to TRB-3 was described previously[6]. Antibody to PGC-1 was generated using mouse PGC-1 (amino acids 1–390) as an epitope. For insulin signaling experiments, mice fasted for 6 h were injected intraperitoneally with 20 U insulin per kg body weight. Antibodies to Akt, Ser473-phosphorylated Akt, GSK-3β and Ser9-phosphorylated GSK-3β were from Cell Signaling. Antibodies to heat shock protein-90 (Hsp-90) or Hsp-70 (Santa Cruz Biotechnology) were used as loading controls.

Note: Supplementary information is available on the Nature Medicine website.

ACKNOWLEDGMENTS

We thank K. Suter and L. Vera for performing injections, and C.R. Kahn (Joslin Diabetes Center) for LIRKO mice. This work was supported by National Institutes of Health grant GM RO1 37828 (M.M.), the American Diabetes Association, the Hillblom Foundation and Deutsche Forschungsgemeinschaft grant He3260/1-1 (S.H.).

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

Received 27 January; accepted 6 April 2004
Published online at http://www.nature.com/naturemedicine/

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