NF-κB–inducing kinase (NIK) promotes hyperglycemia and glucose intolerance in obesity by augmenting glucagon action

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The canonical inhibitor of nuclear factor κB kinase subunit β (IKK-β)–nuclear factor of κ light polypeptide gene enhancer in B cells 1 (NF-κB1) pathway has been well documented to promote insulin resistance; however, the noncanonical NF-κB–inducing kinase (NIK)–NF-κB2 pathway is not well understood in obesity. Additionally, the contribution of counter-regulatory hormones, particularly glucagon, to hyperglycemia in obesity is unclear. Here we show that NIK promotes glucagon responses in obesity. Hepatic NIK was abnormally activated in mice with dietary or genetic obesity. Systemic deletion of Map3k14, encoding NIK, resulted in reduced glucagon responses and hepatic glucose production (HGP). Obesity is associated with high glucagon responses, and liver-specific inhibition of NIK led to lower glucagon responses and HGP and protected against hyperglycemia and glucose intolerance in obese mice. Conversely, hepatocyte-specific overexpression of NIK resulted in higher glucagon responses and HGP. In isolated mouse livers and primary hepatocytes, NIK also promoted glucagon action and glucose production, at least in part by increasing cAMP response element-binding (CREB) stability. Therefore, overactivation of liver NIK in obesity promotes hyperglycemia and glucose intolerance by increasing the hyperglycemic response to glucagon and other factors that activate CREB.

Obesity is associated with chronic inflammation that is believed to contribute to the pathogenesis of type 2 diabetes¹–². Proinflammatory cytokines activate the canonical IKK-β–NF-κB1 and the jun N-terminal kinase (JNK) pathways, which is believed to impair glucose metabolism by increasing insulin resistance³–⁵. Blood glucose concentrations are determined by a balance between insulin and counter-regulatory hormones (for example, glucagon, catecholamines, glucocorticoids or growth hormone)⁶. In the fasting state, glucagon is secreted by pancreatic α cells and increases HGP by stimulating hepatic glycogenolysis and gluconeogenesis⁶. Glucagon stimulates the phosphorylation and activation of CREB through the cAMP–protein kinase A pathway, and CREB in turn activates the transcription of key gluconeogenic enzymes, including phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase)⁷. In rodents with type 1 diabetes, glucagon action is high and contributes to hyperglycemia and glucose intolerance⁸–¹⁰. Blood glucagon concentrations are elevated in rodents with insulin deficiency, and leptin treatments normalize hyperglucagonemia and hyperglycemia⁸,⁹. Deletion of glucagon receptors prevents streptozotocin (STZ)-induced hyperglycemia and glucose intolerance¹⁰. In a type 2 diabetes model, leptin treatments also attenuate hyperglucagonemia, leading to improvement in hyperglycemia and glucose intolerance¹¹. Insulin suppresses glucagon secretion from α cells¹²; therefore, α cell insulin resistance may lead to hyperglucagonemia, which contributes to hyperglycemia in type 2 diabetes.

A subset of cytokines also stimulates the noncanonical NIK–NF-κB2 pathway; however, the metabolic function of this pathway has not been examined. It is also unclear whether inflammation alters glucagon responses. NIK, also called MAP3K14 (NM_003954), is an essential upstream serine/threonine kinase of the noncanonical NIK–NF-κB2 pathway¹³. NIK protein concentrations are extremely low in quiescent cells as a result of rapid degradation, and cytokines and oxidative stress increase NIK protein stability, leading to NIK activation¹⁴. NIK has been reported to regulate B and T cell development in mice¹⁵,¹⁶. In this study we show that liver NIK is aberrantly activated in obesity and increases HGP by promoting glucagon action in mice, thus contributing to hyperglycemia and glucose intolerance. NIK promotes glucagon action, at least in part, by increasing CREB stability.

RESULTS

NIK is abnormally activated in the livers of obese mice

We measured NIK activity in two commonly used mouse models of obesity: dietary obesity and genetic obesity with leptin deficiency (ob/ob). We immunopurified NIK protein from the livers of mice from these two models and subjected it to in vitro kinase assays using the glutathione-S transferase (GST)–IKK-α (amino acids 108–368) fusion protein as a substrate. GST–IKK-α contains an NIK phosphorylation site (IKK-α Ser176) and lacks catalytic activity¹⁷. NIK activity in the

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liver was tenfold higher in ob/ob mice (at 13 weeks of age) than in wild-type (WT) mice (Fig. 1a). Liver NIK activity was also 14-fold higher in mice fed a high-fat diet (HFD) than in mice fed a normal chow diet (Fig. 1b). We were able to detect NIK protein in the ob/ob mice (Fig. 1a) but not in the WT mice because of its rapid degradation.\(^\text{18,19}\). The concentrations of the active form (p52) of NF-κB2 in the liver were higher in ob/ob mice than in WT mice and in HFD-fed mice than in chow-fed mice (Fig. 1c). In contrast, NIK activity in the skeletal muscles was similar between normal chow- and HFD-fed WT mice (Supplementary Fig. 1a).

Obesity is associated with chronic inflammation, oxidative stress and steatosis in the liver.\(^\text{1,2}\). To determine whether these factors contribute to NIK activation, we examined the ability of tumor necrosis factor α (TNF-α) (which mimics inflammation), hydrogen peroxide (which mimics oxidative stress) and palmitic acid (which mimics steatosis) to stimulate NIK activity. We introduced recombinant NIK into mouse primary hepatocytes by NIK adenoviral infection, and we then treated the cells with each of the compounds. Treatment with TNF-α, hydrogen peroxide or palmitic acid increased both NIK auto-phosphorylation and the ability of NIK to phosphorylate GST–IKK-α (Fig. 1d). TNF-α, hydrogen peroxide and palmitic acid also stimulated endogenous NIK in hepatocytes (Supplementary Fig. 1b).

**Inhibition of liver NIK improves glucose metabolism**

To examine the metabolic function of NIK in vivo, we characterized previously generated NIK knockout mice.\(^\text{15}\). The active form of NF-κB2 (p52) was absent in WT but not knockout hepatocytes (Supplementary Fig. 2a). TNF-α similarly stimulated the degradation of inhibitor of κ light polypeptide gene enhancer in B cells (IkB) as well as the phosphorylation of IκB, p38 and JNK in both WT and knockout hepatocytes (Supplementary Fig. 2b). These results verify the previous findings that NIK is required for both the canonical, NF-κB pathways and the noncanonical, NF-κB pathways. Notably, knockout mice had hypoglycemia (Fig. 2a) and greater glucose tolerance compared to WT littermates (Fig. 2b). HGP, estimated by pyruvate tolerance tests (PTT), was lower in knockout than in WT mice (Fig. 2c).
Plasma insulin concentrations (Supplementary Fig. 2c) and insulin tolerance (Supplementary Fig. 2d) were relatively normal in the knockout mice.

To examine the role of liver NIK, we selectively inhibited NIK in the liver by adenovirally overexpressing kinase-inactive NIK(KA). NIK(KA) contains substitutions of Lys429 and Lys430 with alanines and acts as a dominant-negative mutant of NIK20,21. We detected the expression of Flag-tagged NIK(KA) and inhibition of NIK activity in the livers of mice infected with the NIK(KA) adenovirus but not when a β-galactosidase adenovirus control was used (Fig. 2e). We fed mice an HFD for 12 weeks and infected them with NIK(KA) or β-galactosidase adenoviruses. HFD promoted hyperglycemia; however, liver-specific inhibition of NIK significantly ameliorated the HFD-induced hyperglycemia (Fig. 2f), hyperinsulinemia (Supplementary Fig. 3a), glucose intolerance (Supplementary Fig. 3g) and insulin resistance (Supplementary Fig. 3h). HGP, estimated by PTT, was also lower in mice infected with NIK(KA) adenovirus than those infected with β-galactosidase adenovirus (Fig. 2g). The body weights and liver sizes were similar between the NIK(KA) and β-galactosidase groups (Supplementary Fig. 3b,c).

In db/db mice, which lack functional leptin receptors, blood glucose concentration was inversely correlated with NIK(KA) expression after infection with NIK(KA) adenovirus. We detected NIK(KA) in the livers of these mice 13 d after infection, but it was undetectable after 53 days (Fig. 2h). The blood glucose concentration in these mice was lowest at 10–20 d after NIK(KA) adenoviral infection and then increased progressively to reach concentrations at 40 d after infection that were similar to those in db/db mice that we infected with the β-galactosidase adenovirus (Fig. 2h). Glucose intolerance (Fig. 2i), insulin resistance (Fig. 2i) and hyperinsulinemia (Supplementary Fig. 3d) were also significantly lower in the NIK(KA) group than in the β-galactosidase group. Body weight and liver TNF-α expression were similar between the NIK(KA) and β-galactosidase groups (Supplementary Fig. 3e,f).

In ob/ob mice, liver-specific overexpression of NIK(KA) also reduced hyperglycemia (Supplementary Fig. 3g), glucose intolerance (Supplementary Fig. 3h), insulin resistance (Supplementary Fig. 3i) and HGP (Supplementary Fig. 3j). To further study NIK in hepatocytes, we generated STOP-NIK(KA) adenoviruses (Fig. 3a). In these adenoviruses, NIK(KA) expression was blocked by a STOP cassette and was able to be reactivated by Cre-mediated excision of the STOP cassette. To verify hepatocyte-specific expression of NIK(KA), we infected WT and albumin-Cre+/- mice with STOP-NIK(KA) or GFP adenoviruses. We detected NIK(KA) after infection with STOP-NIK(KA) adenovirus in the livers of albumin-Cre+/- but not WT mice (Fig. 3b). We fed the albumin-Cre+/- mice an HFD and infected them with STOP-NIK(KA) or GFP adenoviruses. Blood glucose concentrations (Fig. 3c), glucose intolerance (Fig. 3d) and HGP (Fig. 3e) were significantly lower in the STOP-NIK(KA) than the GFP groups. In contrast, STOP-NIK(KA) adenoviral infection neither lowered blood glucose concentrations nor improved glucose intolerance in WT mice (data not shown). Taken together, these data indicate that liver-specific inhibition of NIK decreases hyperglycemia and glucose intolerance in both dietary and genetic obesity independently of changes in body weight.

To determine whether activation of liver NIK is sufficient to alter glucose metabolism, we generated mice with hepatocyte-specific overexpression of Flag-tagged NIK (HepNIK mice) using previously generated STOP-NIK (HepCON mice) mice22. In HepCON mice with the genotype STOP-NIK+/+; Cre-/-, a STOP-Map3k14 (also known as STOP-Nik) transgene was knocked in the Rosa26 locus (Fig. 3f), and the STOP cassette blocked the expression of Flag-tagged NIK (Fig. 3g). To create the HepNIK mice (with the genotype STOP-Nik+/-; Cre+/-), we crossed HepCON mice with albumin-Cre mice. In HepNIK mice, Cre-mediated excision of the STOP sequences was restricted to the hepatocytes, resulting in modest expression of Flag-tagged NIK in the liver but not in the pancreas, muscle, brain, white fat, brown fat, spleen, kidney or heart (Fig. 3g). Glucose tolerance and pyruvate tolerance were impaired in HepNIK males fed a normal chow diet compared to HepCON mice on the same diet (Fig. 3h,i). HepNIK females fed an HFD also developed hyperglycemia and glucose intolerance (Supplementary Fig. 4a,b).

NIK promotes HGP by enhancing glucagon action

To directly measure HGP, we isolated livers from NIK knockout and WT mice and subjected them to ex vivo gluconeogenesis assays. In WT mice, the amount of hepatic gluconeogenesis was 1.4-fold higher in the fasting state than in the fed state (Fig. 4a). There was 92% less fasting-stimulated...
NIK promotes the stimulation of glucose production by glucagon. (a) Gluconeogenesis in isolated WT and knockout livers (n = 5). (b) Glucose production in primary WT and knockout hepatocytes treated with dibutyryl-CAMP (CAMP) and dexamethasone (dex) (n = 8). (c) Relative mRNA abundance of hepatic PEPCK and G6Pase in WT (n = 9) and knockout (n = 9) males (9 weeks old) and hepatic G6Pase activity in WT (n = 7) and knockout (n = 6) males (19 weeks old). (d,e) Relative mRNA abundance of hepatic PEPCK and G6Pase in db/db males infected with β-galactosidase (n = 5) or NIK(KA) adenoviruses (n = 6) and in HepCON (n = 10) and HepNIK males (n = 7) (e). (f) Results from WT and knockout mice (9 weeks old) fasted overnight and stimulated with insulin. Shown are liver extracts immunoblotted with antibody to Akt phosphorylated at Thr308 (pThr308) and G6Pase activity. Deletion of NIK or NIK(KA) did not impair insulin-stimulated glucose production. Concomitant with these findings, the production of insulin-stimulated hepatic G6Pase was largely blocked by NIK deletion (Fig. 4c). Conversely, glucagon-stimulated glucose production (Supplementary Fig. 5). In contrast, overexpression of kinase-inactive, dominant-negative I KK-β (KA) did not alter basal or glucagon-stimulated glucose production (Supplementary Fig. 5). Therefore, NIK enhances glucagon stimulation of HGP independently of IKK-β.

NIK mediates increased glucagon action in obesity

To examine glucagon responses in obesity, we performed glucagon tolerance tests (GTTs) in WT mice fed an HFD for 6 weeks. Blood glucose concentrations were significantly higher at each time point after glucagon injection in mice fed an HFD than in mice fed a normal chow diet (Fig. 5a). The glucagon-stimulated increase in blood glucose concentration was larger in HFD-fed than in chow-fed mice (Fig. 5b). Liver-specific inhibition of NIK significantly decreased the glucagon responses in mice infected with the NIK(KA) adenovirus compared to those infected with the β-galactosidase adenovirus (Fig. 5c).

As we found earlier that inflammation, oxidative stress or steatosis can induce NIK activity and that NIK promotes glucagon action, we next explored if these three conditions also enhance glucagon action and if this was dependent on NIK expression. Thus, we pretreated primary hepatocytes with TNF-α, hydrogen peroxide and palmitic acid before glucagon stimulation. TNF-α, hydrogen peroxide and palmitic acid all significantly increased the expression of both the basal and glucagon-stimulated glucose production in WT hepatocytes (Fig. 5d). Deletion of Map3k14 greatly attenuated the ability of the three compounds to enhance glucagon responses in knockout compared to WT hepatocytes (Fig. 5d,e). Treatment with TNF-α, hydrogen peroxide and palmitic acid significantly increased glucagon-stimulated expression of PEPCK and G6Pase in WT hepatocytes (Fig. 5f). Deletion of Map3k14 largely blocked the effects of these compounds in knockout hepatocytes (Fig. 5g).
NIK phosphorylates CREB and increases CREB stability

CREB mediates the stimulation of gluconeogenesis by glucagon. CREB protein concentrations in both the liver and in purified hepatocytes were lower in NIK knockout mice than in WT littermates (Fig. 6a). CREB phosphorylation at Ser133 was also lower in knockout than in WT mice (Fig. 6a). Liver-specific restoration of CREB reversed glucagon resistance in the knockout mice (Supplementary Fig. 6). Conversely, liver CREB concentrations were higher in HepNIK than in HepCON mice (Fig. 6b). Liver CREB concentrations were also higher in mice with either dietary (HFD compared to chow diet) or genetic (ob/ob compared to WT) obesity (Fig. 6c); in agreement with these findings, liver NIK activity was abnormally high in HFD-fed and ob/ob mice (Fig. 1). Notably, hepatic CREB mRNA levels were similar between knockout and WT mice (Supplementary Fig. 7a,b) and between HepCON and HepNIK mice (Supplementary Fig. 7c).

We hypothesized that NIK increases CREB protein stability. To test this idea, we co-introduced into primary hepatocytes by adenoviral infection Flag-tagged CREB along with either β-galactosidase, NIK or NIK(KA). CREB mRNA levels were similar between cells infected with the β-galactosidase, NIK and NIK(KA) adenoviruses (Supplementary Fig. 7d); however, CREB protein concentrations were higher in NIK-expressing and lower in NIK(KA)-expressing cells.
compared to in β-galactosidase–expressing cells (Supplementary Fig. 7e). To determine whether proteasomes are involved in CREB degradation, we infected knockout and WT hepatocytes with CREB adenoviruses and treated them with MG132, a proteasome inhibitor. CREB concentrations were lower in knockout than in WT cells, and treatment with MG132 markedly increased the amount of recombinant CREB in knockout cells (Fig. 6d). MG132 also increased the amount of endogenous CREB in knockout hepatocytes (Fig. 6e). In agreement, CREB ubiquitination was higher in knockout than in WT hepatocytes (Supplementary Fig. 7f).

To determine whether NIK phosphorylates CREB, we performed in vitro kinase assays using immunopurified CREB, NIK and NIK(KA). CREB was robustly phosphorylated by NIK but not NIK(KA) (Fig. 6f). We identified Ser114 and Ser142 as phosphorylation sites in NIK-overexpressing hepatocytes using liquid chromatography–tandem mass spectrometry proteomics approaches. However, NIK did not phosphorylate a CREB mutant lacking the Ser111, Ser114 and Ser142 sites (Supplementary Fig. 8a). We replaced Ser98, Ser108, Ser111, Ser114, Ser117, Ser121, Ser129, Ser142, Ser143, Ser156, Ser271, Ser340 and Thr100 (13 known phosphorylation sites) with alanines in CREB (termed here 13S-A). NIK was unable to phosphorylate 13S-A (Fig. 6g).

To determine whether these 13 sites are involved in the regulation of CREB stability by NIK, we introduced CREB or 13S-A into HepG2 cells expressing low amounts of recombinant NIK. 13S-A migrated slightly faster than CREB in SDS-PAGE gels; NIK increased the abundance of CREB but not 13S-A proteins (Fig. 6h). However, NIK at high concentrations increased the amount of both CREB and 13S-A proteins (Supplementary Fig. 8b,c). NIK at low concentrations increased the abundance of 2S-A (Ser117 and Ser121 to alanines, Ser114 and Ser117 to alanines or Ser114 and Ser117 to alanines or Ser114 and Ser117 to alanines) (Supplementary Fig. 8d) but not 3S-A (Ser114, Ser117 and Ser121 to alanines) or 4S-A (Ser111, Ser114, Ser117 and Ser121 to alanines) proteins (Fig. 6i). In contrast, NIK at high concentrations increased the abundance of 3S-A proteins (Supplementary Fig. 8e). Therefore, NIK is able to increase CREB stability by both phosphorylation-dependent and phosphorylation-independent mechanisms.

**DISCUSSION**

We here identify NIK overactivation in the liver as a contributing factor to hyperglycemia and glucose intolerance in obesity. NIK was aberrantly activated in the livers of mice with either dietary or genetic obesity. Systemic deletion of Map3k14 led to decreased blood glucose concentrations and improved glucose tolerance. In both dietary and genetic obesity, liver-specific inhibition of NIK markedly improved hyperglycemia and glucose intolerance. In agreement, hepatocyte-specific overexpression of NIK was sufficient to promote glucose intolerance.

We have discovered a new function of NIK in liver glucose metabolism. NIK promotes HGP by enhancing the hyperglycemic response to glucagon, a key counter-regulatory hormone. In mice, deletion of Map3k14 resulted in a reduction in HGP, glucagon resistance and compensatory hyperglycagomina. Similarly, liver-specific inhibition of NIK also decreased HGP and impaired glucagon responses. Conversely, hepatocyte-specific overexpression of NIK resulted in higher HGP and glucagon responses. In isolated livers and primary hepatocytes, deletion of Map3k14 decreased both basal and glucagon-stimulated HGP. At molecular levels, NIK phosphorylated CREB and increased CREB protein stability by both phosphorylation-dependent and phosphorylation-independent mechanisms. Deletion of Map3k14 decreased, whereas NIK overexpression increased, the amounts of hepatic CREB protein but not the levels of CREB mRNA. CREB mediates glucagon-stimulated hepatic glucoseogenesis23–25. Consistently, systemic deletion of Map3k14 or liver-specific inhibition of NIK decreased, whereas hepatocyte-specific overexpression increased, the expression of glucosegenic PEPCK and G6Pase, two CREB targets. Therefore, NIK is a newly identified positive regulator of hepatic glucoseogenesis, enhancing the action of glucagon and/or other factors that activate the protein kinase A (PKA)–CREB pathway. Notably, the transcriptional activity of 13S-A was slightly higher than that of WT CREB (data not shown), raising the possibility that phosphorylation of CREB by NIK may inhibit CREB activity. The NIK-induced increase in CREB stability may overcome the inhibition of CREB activity by NIK, promoting glucagon responses. However, our data do not exclude the possibility that NIK may regulate glucose metabolism by other mechanisms, in addition to increasing CREB stability and glucagon responses.

We show that obesity is associated with elevated glucagon responses. Liver-specific inhibition of NIK decreased glucagon responses, suggesting that overactivation of liver NIK is the major contributor to abnormal glucagon responses in obesity. Notably, TNF-α, hydrogen peroxide and palmitic acid activated NIK in hepatocytes, suggesting that inflammation, oxidative stress and/or steatosis contributes to NIK overactivation in obesity. Under normal conditions, NIK is recruited to the baculoviral IAP repeat containing 1 (cIAP1) and cIAP2 E3 ligases by a TNF receptor–associated factor 3 (TRAF3)–TRAF2 complex, leading to the ubiquitination and proteasome-mediated degradation of NIK28–30. Cytokine stimulation promotes the degradation of TRAF3, resulting in NIK stabilization and activation39,30. In future studies, it will be crucial to examine how inflammation, oxidative stress and steatosis promote the activation of hepatic NIK in obesity. We also found that TNF-α, hydrogen peroxide and palmitic acid enhanced glucagon-stimulated glucose production and the expression of glucosegenic genes in an NIK-dependent manner in primary hepatocytes. These results suggest that hepatic inflammation, oxidative stress and/or steatosis activate NIK, which in turn promotes glucagon responses and HGP, thereby contributing to hyperglycemia and glucose intolerance in obesity.

In conclusion, we show that obesity is associated with the activation of the noncanonical NIK–NF-κB2 pathway in addition to the canonical IKK-β–NF-κB1 pathway in the liver. The NIK pathway increases HGP by enhancing glucagon and/or other counter-regulatory hormone responses, whereas the IKK-β pathway promotes insulin resistance. Both elevated counter-regulatory hormone responses and insulin resistance contribute to hyperglycemia and glucose intolerance in obesity. Thus, NIK may be a new therapeutic target for the treatment of type 2 diabetes.

**METHODS**

Methods and any associated references are available in the online version of the paper.

*Note: Supplementary information is available in the online version of the paper.*
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AUTHOR CONTRIBUTIONS
L.R. and L.S. designed the experiments and prepared the manuscript. L.S., Y.Z., Z.C., D.R., K.W. C., L.J. and H.S. performed experiments. Y.S. generated the STOP-NIK mice.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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ONLINE METHODS

Mice. ob/ob, db/db, albumin-Cre and WT male mice (in a C57BL/6 background) for the HFD experiments were from the Jackson Laboratory. NIK knockout and WT mice (in a 129/Sv and C57BL/6 mixed background) were from R. Schreiber (Washington University School of Medicine, St. Louis, Missouri). STOP-NIK mice (in a C57BL/6 background) were from K. Rajewsky (Harvard Medical School, Boston, Massachusetts). STOP-NIK mice were crossed with albumin-Cre mice to generate HepCON and HepNIK mice (in a C57BL/6 background). Mice were housed on a 12-h light and 12-h dark cycle in the Unit for Laboratory Animal Medicine at the University of Michigan and fed either a normal chow diet (9% fat; Lab Diet) or an HFD (45% fat; Research Diets). Liver and hepatocyte glucose production assays.

Glucose, pyruvate, insulin and glucagon tolerance tests. Glucose (2 g kg\(^{-1}\) body weight) and insulin (1 unit kg\(^{-1}\) body weight) tolerance tests have been described previously\(^{35–37}\). Briefly, mouse livers were isolated and perfused with Kreb-Ringer bicarbonate (KRB) buffer sequentially with Kreb-Ringer bicarbonate (KRB) buffer (286 mM NaCl, 10 mM Na\(_2\)HPO\(_4\), 4.5 mM KCl, 1.8 mM CaCl\(_2\), 1.18 mM MgCl\(_2\), 1.17 mM KH\(_2\)PO\(_4\), 25 mM glucose, 4.5 mM HEPES, pH 7.4) for 10 min, glycogenolysis buffer (KRB buffer supplemented with 0.5% BSA and gluconeogenic substrates (10 mM lactate and 5 mM pyruvate) in the presence or absence of 50 nM glucagon (or 10 nM N6,2'-O-dibutylryl-cAMP and 100 nM dexamethasone). Hepatocytes were incubated for 4 h at 37 °C, and the culture medium was collected and used to measure glucose concentrations. Glucose production was normalized to the total hepatocyte protein concentrations. In a few experiments, the HFD groups were pretreated with a combination of TNF-\(\alpha\) (10 ng ml\(^{-1}\)), hydrogen peroxide (100 nM) and palmitic acid (100 μM) for 4 h before the glucose production assays.

In vitro kinase assays. NIK was immunopurified using antibody to NIK (endogonic NIK) or Flag (recombinant NIK) and incubated with \(\gamma\)\(^{32P}\)-ATP (2 μCi) and GST–IKK-\(\alpha\) (amino acids 108–368) fusion protein (0.5 μg) in a kinase buffer (20 μM HEPES, pH 7.5–7.6, 33 μM ATP 10 μM MgCl\(_2\), 50 mM NaCl, 1 mM dithiothreitol (DTT), 10 μg ml\(^{-1}\) aprotinin, 10 μg ml\(^{-1}\) leupeptin and 1 mM Na\(_2\)VO\(_4\)) at 30 °C for 30 min. The reactions were stopped by adding SDS-PAGE loading buffer and boiled for 5 min. Proteins were resolved by SDS-PAGE, transferred onto a nitrocellulose membrane and visualized by autoradiography. The membranes were subsequently immunoblotted with antibodies against GST, NIK or Flag. Primary hepatocytes were treated with TNF-\(\alpha\) (10 ng ml\(^{-1}\)), hydrogen peroxide (100 nM) and palmitic acid (100 μM), and NIK activity was measured by in vitro kinase assays 2 h after the treatments. In a few experiments (Fig. 6g), Flag-tagged NIK was overexpressed in hepatocytes by adenoviral infection and immunopurified with antibody to Flag. HEK293 cells were transfected with plasmids expressing Myc-tagged CREB or 13S-A; cell extracts were boiled for 3 min to inactivate kinases and then immunoprecipitated with antibody to Myc. Immunopurified NIK was mixed with CREB or 13S-A and subjected to in vitro kinase assays.

Glucose production was normalized to the total hepatocyte protein concentrations. In a few experiments, the HFD groups were pretreated with a combination of TNF-\(\alpha\) (10 ng ml\(^{-1}\)), hydrogen peroxide (100 nM) and palmitic acid (100 μM) for 4 h before the glucose production assays.

Nuclear extract preparation. Liver tissues or hepatocytes were homogenized in a lysis buffer (20 mM HEPES, 1 mM EDTA, 250 mM sucrose, 10 μM \(\mu\)M aprotinin, 10 μg ml\(^{-1}\) leupeptin, 1 mM PMSF, 1 mM Na\(_2\)VO\(_4\), 0.2 mM benzamidine and 0.5 mM DTT; pH 7.4) and centrifuged sequentially at 1,100g and 4,000g (at 4 °C). Pellets were resuspended in a high-salt solution (20 mM HEPES, 420 mM NaCl, 0.2 mM EDTA, 0.5 mM DTT, 1 mM PMSF and 1 mM Na\(_2\)VO\(_4\); pH 7.9), and protein concentrations were measured using Bio-Rad protein assay kits.

HeptG2 cell transfection. CREB and NIK plasmids (1 μg) were incubated with 12 μl polyethylenimine (1 mg ml\(^{-1}\)) in 100 μl serum-free DMEM for 15 min and added to a HeptG2 cell suspension (6 × 10\(^5\) cells in 0.5 ml serum-free DMEM). Cells were plated into 12-well plates, incubated at 37 °C for 4 h and then grown in DMEM supplemented with 8% FBS.

Statistical analyses. Data are presented as means ± s.e.m. Differences between groups were analyzed with a two-tailed Student's t-test. P < 0.05 was considered statistically significant.