Inactivation of NF-κB p65 (RelA) in Liver Improves Insulin Sensitivity and Inhibits cAMP/PKA Pathway

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The transcription factor nuclear factor-κB (NF-κB) mediates inflammation and stress signals in cells. To test NF-κB in the control of hepatic insulin sensitivity, we inactivated NF-κB in the livers of C57BL/6 mice through deletion of the p65 gene, which was achieved by crossing floxed-p65 and Alb-cre mice to generate L-p65-knockout (KO) mice. KO mice did not exhibit any alterations in growth, reproduction, and body weight while on a chow diet. However, the mice on a high-fat diet (HFD) exhibited an improvement in systemic insulin sensitivity. Hepatic insulin sensitivity was enhanced as indicated by increased pyruvate tolerance, Akt phosphorylation, and decreased gene expression in hepatic gluconeogenesis. In the liver, a decrease in intracellular cAMP was observed with decreased CREB phosphorylation. Cyclic nucleotide phosphodiesterase-3B (PDE3B), a cAMP-degrading enzyme, was increased in mRNA and protein as a result of the absence of NF-κB activity. NF-κB was found to inhibit PDE3B transcription through three DNA-binding sites in the gene promoter in response to tumor necrosis factor-α. Body composition, food intake, energy expenditure, and systemic and hepatic inflammation were not significantly altered in KO mice on HFD. These data suggest that NF-κB inhibits hepatic insulin sensitivity by upregulating cAMP through suppression of PDE3B gene transcription.

The transcription factor nuclear factor-κB (NF-κB) is a master regulator of inflammation. It is required for expression of proinflammatory cytokines, such as interleukin (IL)-1β and IL-6. In the cytosol, NF-κB is associated with the inhibitor protein κB inhibitor α (IκBα), which controls nuclear translocation of NF-κB. Degradation of IκBα leads to NF-κB activation for transcriptional regulation of gene expression. IκBα degradation is initiated by serine kinase IκB kinase-β (IKKβ), which phosphorylates IκBα at serine residues to induce ubiquitination-mediated degradation in proteasomes. The roles of IKKβ were studied in the pathogenesis of insulin resistance in global and tissue-specific transgenic mice. Those studies suggested that IKKβ deficiency (IKKβ−/−) protected mice from obesity-induced insulin resistance (1), although the same result was not observed in a subsequent study by a different group (2). Tissue-specific effects of IKKβ provide a mechanism for the discrepancy. The phenotypes of tissue-specific IKKβ transgenic mice suggest that IKKβ contributes to insulin sensitivity when it is activated in liver (3,4) or myeloid cells (3) but not in skeletal muscle (5) or adipose tissue (6). Although IKKβ has been studied extensively in various tissues in transgenic mice, the mechanism remains unknown for its action in insulin resistance.

In liver-specific studies, IKKβ overexpression was found to inhibit insulin sensitivity through induction of IL-6 expression (4), and IKKβ knockout (KO) was found to protect insulin sensitivity through inhibition of IL-1β expression (3). Although both studies suggested a role of transcriptional regulation by NF-κB in the mechanism of IKKβ action, the details remain unknown because the downstream genes were different in the two studies. In addition, IKKβ regulates insulin sensitivity through a transcription-independent mechanism of insulin receptor substrate-1.
serine phosphorylation (7,8). The relative significance of transcription-dependent and -independent mechanisms remains unknown for the IKKB activity. Inactivation of NF-kB is an approach to addressing this issue. NF-kB is a heterodimer protein formed by two subunits p65 (RelA) and p50 (NF-kB 1). The transcriptional activity of NF-kB is determined by the subunit p65, which contains an activation domain. Whole-body p65 inactivation leads to embryonic lethality (9), which does not allow phenotypic analysis. In this study, we inactivated p65 gene in liver (L-p65-KO) and examined insulin sensitivity in a comprehensive phenotypic study.

L-p65-KO mice were made by crossing floxed-p65 mice with Alb-cre mice. The phenotypic study included analysis of insulin sensitivity and energy balance in mice fed either a chow diet or high-fat diet (HFD). The mechanistic studies were conducted with a focus on cAMP/protein kinase A (PKA) pathways to understand the metabolic effects of NF-kB.

**RESEARCH DESIGN AND METHODS**

**Generation of L-p65-KO Mice**

LoxP p65 mice were generated on a C57BL/6 genetic background as described elsewhere (10). Alb-cre mice on the C57BL/6 genetic background (stock number 003574) were purchased from The Jackson Laboratory (Bar Harbor, ME). L-p65-KO (p65fl Cre+/-) mice were generated by crossing the floxed-p65 mice with Alb-cre mice. Floxed-p65 littermates (p65flfl) were used as the wild-type (WT) control for KO mice (L-p65-KO). The study was conducted in male mice at the animal facility of the Pennington Biomedical Research Center. The mouse housing environment included a 12-h light-dark cycle, constant room temperature (22–24°C), and free access to water and diet. The mice were fed the chow diet (5% weight for weight or 11% calories from fat, 5001; LabDiet, St. Louis, MO) or HFD (36% weight for weight or 58% calories from fat, D12331; Research Diets, New Brunswick, NJ). HFD feeding was started at 8 weeks of age to generate a diet-induced obese model. All procedures were performed in accordance with the National Institutes of Health guidelines for the care and use of animals and were approved by the Institutional Animal Care and Use Committee at the Pennington Biomedical Research Center.

**Cell Culture**

The human hepatoma cell line HepG2 (HB-8065) was purchased from the American Type Culture Collection (Manassas, VA). Primary hepatocytes were made from mice at 6–10 weeks of age using a protocol described elsewhere (11). HepG2 cells and the primary hepatocytes were maintained in DMEM supplemented with 10% FBS at 37°C in a 5% CO2 incubator. The cells were treated with tumor necrosis factor-α (TNF-α) in serum-free DMEM containing 0.25% fatty acid–free BSA. An sslkBax cell line was made through stable transfection of HepG2 cells with an sslkBax-pBABE expression vector.

**Body Weight and Composition**

Body weight and composition were measured every 2 weeks. Body composition was measured using quantitative nuclear magnetic resonance (NMR) (Minispec Mn10 NMR scanner, Brucker, Milton, ON, Canada) as described previously (12). In the test, conscious and unrestrained mice were placed individually in a small tube and then placed in the NMR analyzer. Fat and lean mass were recorded within 1 min.

**Food Intake**

Food intake was determined manually for individually housed mice on HFD for 14 weeks. The average daily food intake was determined over 3 days by the net reduction in diet weight with exclusion of spilled food. Food intake is expressed in grams per mouse per day.

**Energy Expenditure**

Energy metabolism was monitored in mice after 4 weeks on HFD using the indirect calorimetry system (Comprehensive Laboratory Animal Monitoring System; Columbus Instruments, Columbus, OH). Mice were kept in the metabolic chamber for 6 days. VO2, VCO2, spontaneous physical activity, and food intake were recorded daily. Energy expenditure in kilocalories per kilogram per hour was calculated with day 5 data using the following equation: energy expenditure = [3.815 + 1.232 × VCO2/VO2] × VO2 × 0.001 (13). Energy expenditure data were normalized with body lean mass.

**Insulin, Glucose, and Pyruvate Tolerance Testing**

An intraperitoneal insulin tolerance test (ITT) was performed in mice (15 weeks on HFD) by insulin injection (0.75 units/kg body weight, I9278; Sigma-Aldrich) after 4 h of fasting. An intraperitoneal glucose tolerance test (GTT) was performed in mice (14 weeks on HFD) using glucose (2 g/kg body weight) after overnight fasting. An intraperitoneal pyruvate tolerance test (PTT) was performed in mice (24 weeks on HFD) using pyruvate (2 g/kg body weight) after overnight fasting. Blood glucose was measured in tail vein blood using the FreeStyle blood glucose monitoring system (Therasense, Phoenix, AZ) at 0, 30, 60, 120, and 180 min after injection. Data are expressed as blood glucose concentration (mg/dL).

**Western Blotting**

Livers were collected from mice after 16 weeks on HFD and examined for gene expression. Whole-cell lysates were prepared from liver tissue/cells and used in Western blotting according to protocols described elsewhere (12). Antibodies to NF-κB p65 (sc-8008), NF-κB p50 (sc-114X), c-JUN (sc-1694X), Sp1 (sc-59X), and CREB (sc-7583X) were purchased from Santa Cruz Biotechnology (Dallas, TX). Antibodies to p-Akt (threonine T) 308, ab38449), p-Akt (serine S) 473, ab6138), p-CREB (S133, ab32096), phosphodiesterase-3B (PDE3B) (ab125675), β-actin (ab6276), and tubulin (ab7291) were obtained from Abcam (Cambridge, MA). Tubulin and β-actin were used as internal controls.
Quantitative Real-Time RT-PCR

Total mRNA was extracted from liver tissue or cells using TRIzol reagent following the manufacturer’s protocol (Invitrogen, Carlsbad, CA). TaqMan Universal PCR Master Mix (4304437; Applied Biosystems, Carlsbad, CA) was used to quantify gene mRNA in the RNA extracts using the ABI 7900 machine. Target mRNA was normalized to ribosomal 18S RNA, an endogenous control. Primers and probes were purchased from Applied Biosystems. These included IL-1β (Mm00434228_m1), IL-6 (Mm00446190_m1), MCP-1 (Mm00441242_m1), IgG1 (Mm00477998_m1), PDE3B (Mm00691635_m1), PEPCk (Mm00440636_m1), and G6Pase (Mm00839363_m1). The sequence of SYBR Green primers for human PDE3B is 5'-AAACCTGAGGTGGAAATGTC-3' (product number KSPQ12012G, H_PDE3B_1, NM_000922; Sigma-Aldrich). The primer was used with SYBR Green Master Mix (430155; Applied Biosystems) for PDE3B mRNA in HepG2 cells.

Luciferase Reporter Assay

PDE3B-luciferase reporter (−5.1/−3.4 SX-luciferase PGL3) is described elsewhere (14). The vectors for p65-cDNA, ssIkBα-pBABE, and corresponding control plasmids are also described elsewhere (15). The transfection was conducted in HepG2 cells using Lipofectamine 2000 (11668019; Life Technologies, Grand Island, NY). The luciferase assay was performed at 48 h using a 96-well luminometer with the dual-luciferase substrate system (E1960; Promega Corporation, Fitchburg, WI). SV40 (simian virus 40 Renilla luciferase) 0.1 μg/well was used as an internal control. Each experiment was repeated at least three times.

Electrophoretic Mobility Shift Assay

The nuclear extract was made from HepG2 cells, and the electrophoretic mobility shift assay (EMSA) was conducted as described elsewhere (15). The DNA probes for NF-κB binding sites in the mouse PDE3B gene promoter were synthesized according the following sequences: site a (5′-ACACTGGGGATTTGACCTCTA-3′), site b (5′-GCAATTAGGGTCTTCCATATA-3′), and site c (5′-TAAAGATGAGTGAGTCATG-3′). The authentic NF-κB (human IL-6-κB site) and Sp1 probes are described elsewhere (16,17). In oligonucleotide competition and antibody supershift experiments, a 50-fold excess of unlabeled oligonucleotide probe and 2 μg IgG were used, respectively.

Plasma Cytokine and Insulin

After overnight fasting, blood was collected from mice on HFD for 18 weeks through retro-optical bleeding. Plasma was isolated and used in cytokine assays for IL-1β, IL-6, and TNF-α using a multiplex kit (MCTOMAG-70K; EMD Millipore). Plasma insulin was measured using a multiplex kit of mouse metabolic hormones (MMHMAG-44K; EMD Millipore).

Liver Function

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were measured in the plasma using a standard enzymatic assay (TR71121, TR70121; Thermofisher Scientific Inc., Middletown, VA).

cAMP Assay

cAMP level was determined using an ELISA kit (ADI-900-067; Enzo Life Sciences, Inc.). cAMP concentrations were presented as fold change in nmol/g over WT control.

Statistical Analysis

Statistical analysis was performed using two-tailed, unpaired Student t test. P < 0.05 was considered significant. Results are presented as mean ± SEM.

RESULTS

Decreased Inflammation in Liver of L-p65-KO Mice

The transcriptional activity of NF-κB depends on the p65 subunit. Inactivation of p65 is expected to decrease expression of inflammatory genes in hepatocytes. In this study, L-p65-KO mice (p65(fl/fl), Alb-Cre(+/−)) were generated by crossing floxed-p65 mice with Alb-cre mice. Analysis of the phenotype was conducted in male L-p65-KO mice, and floxed-p65 littermates (p65(fl/+/)) were used as WT controls. The p65 protein was examined in the liver tissues to verify the gene KO; p65 was reduced by 90% in the liver tissue of KO mice relative to that of WT controls (Fig. 1A). Expression of NF-κB target genes (IkBα, IL-1β, and IL-6) was decreased in hepatocytes from KO mice (Fig. 1B). Primary hepatocytes were prepared and used in the gene expression studies to exclude the activities of macrophages (Kupffer cells). The results in Fig. 1 suggest that NF-κB function is inactivated in the hepatocytes of L-p65-KO mice.

Enhanced Systemic Insulin Sensitivity in L-p65-KO Mice on HFD

Analysis of the phenotype was first performed in L-p65-KO mice on a chow diet. No phenotypic change was observed by ITT and GTT, body weight, body composition, food intake, energy expenditure, physical activity, and substrate use (data not shown). The data suggest that KO does not influence insulin sensitivity and energy balance in lean mice. In obese KO mice on HFD (58% calories from fat), insulin sensitivity was significantly improved as indicated by a 50% reduction in fasting insulin and

Figure 1—Decreased inflammation in liver of L-p65-KO mice on a chow diet. A: The p65 protein in liver tissue. Proteins were determined by Western blotting. B: Expression of mRNA of NF-κB target genes in primary hepatocytes. The primary hepatocytes were treated with TNF-α (20 ng/mL) for 2 h to induce expression of the indicated genes. Data are mean ± SEM (n = 3). **P < 0.001 by Student t test.
enhanced tolerance to insulin and glucose (Fig. 2A–C). The data suggest that NF-κB inactivation in hepatocytes significantly improves systemic insulin sensitivity in L-p65-KO mice in obese but not lean conditions.

**Enhanced Hepatic Insulin Sensitivity in L-p65-KO Mice on HFD**

Liver insulin sensitivity was examined to understand the improved systemic insulin sensitivity in KO mice on HFD. Pyruvate tolerance was studied to determine hepatic gluconeogenesis, a process inhibited by insulin. Insulin sensitivity was inversely associated with glucose elevation in PTT. The tolerance was enhanced in L-p65-KO mice for a smaller increase in blood glucose (Fig. 3A). Phosphorylation of Akt was examined to determine insulin signaling activity in hepatocytes. Akt phosphorylation at T308 and S473 was enhanced in KO mice (Fig. 3B). Expression of gluconeogenic genes phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) was examined in hepatocytes to determine the mechanism of reduced gluconeogenesis, and both were decreased in the KO liver (Fig. 3C). Liver weight was not significantly altered in KO mice on HFD (Fig. 3D), although plasma ALT was elevated in KO mice (Fig. 3E). These data suggest that hepatic insulin sensitivity is improved in L-p65-KO mice on HFD.

**Reduced cAMP Activity in Liver of L-p65-KO Mice**

Insulin action is antagonized by effects of glucagon in the control of hepatic gluconeogenesis. Glucagon activity depends on activation of the cAMP/PKA pathway. In the cAMP/PKA pathway, cAMP elevation leads to activation of serine kinase PKA, which phosphorylates the transcription factor CREB on S133. After phosphorylation, CREB activates the cAMP/PKA pathway, cAMP elevation leads to activation of cAMP-dependent transcription factors like CREB on S133. After phosphorylation, CREB activates the transcription of gluconeogenic genes like PEPCK and G6Pase.

**Inhibition of PDE3B Gene Promoter by NF-κB**

As a transcription factor, NF-κB regulates gene expression through direct interaction with the gene promoter DNA. Potential NF-κB binding sites were searched in the PDE3B gene promoter through analysis of the nucleotide sequence. Three sites were identified in the mouse PDE3B gene promoter (Fig. 6A). Interaction of those sites with NF-κB was tested with EMSA through a competition against a classical NF-κB binding site. Site b exhibited the strongest competition, suggesting that NF-κB binds to the PDE3B gene promoter. A radiolabeled probe was made from site b and further tested for NF-κB binding.

**Figure 2—Systemic insulin sensitivity in L-p65-KO mice.** A: Fasting insulin. Blood was collected from mice following overnight fasting after 18 weeks on HFD (n = 7–8). B: ITT. After 15 weeks on HFD, mice received insulin 0.75 units/kg i.p. following 4 h of fasting (n = 10–12). Blood glucose was determined at the indicated time points. C: GTT. After 14 weeks on HFD, mice received glucose 2 g/kg i.p. following overnight fasting (n = 10–12). Data are mean ± SEM. *P < 0.05. ip, intraperitoneal.
interaction in a supershift assay. The DNA-protein complex was shifted by the antibody to the NF-κB p50 subunit but not by the antibodies to c-JUN or Sp1 (Fig. 6B), suggesting that the probe specifically interacted with the NF-κB protein. The function of NF-κB sites was tested in the gene promoter of the luciferase reporter assay. The PDE3B promoter was inhibited by p65 in cotransfection, and the inhibition was blocked by IκB in cotransfection (Fig. 6C). These data suggest that NF-κB inhibits PDE3B transcription through DNA-binding sites in the gene promoter.

**DISCUSSION**

This study provides evidence that systemic insulin sensitivity is enhanced by liver-selective NF-κB inactivation in diet-induced obese mice, suggesting a role of hepatic NF-κB in the regulation of insulin sensitivity. The observation is consistent with that reported for hepatic IKKb activities in overexpression and KO studies (3,4); however, the molecular mechanism of NF-κB action is different from those reported for IKKb. IKKb was reported to inhibit insulin sensitivity through induction of IL-1β (3) or IL-6 expression (4). The relative significance of the two cytokines is unknown in the IKKb model. In addition, the cytokines are controversial in the pathogenesis of insulin resistance because their activities are not observed in the induction of insulin resistance in all studies (19). Although NF-κB was reported to mediate IKKb activity, the role of PDE3B was not found downstream of NF-κB in those early studies (3,4). A novel finding of the current study is that PDE3B...
is a target gene of NF-κB in the regulation of insulin sensitivity. The data suggest that increased PDE3B after NF-κB inactivation contributes to insulin sensitivity in L-p65-KO mice, which involves a reduction in cAMP activity.

This study demonstrates that NF-κB is a transcriptional repressor of the PDE3B gene. PDE3B belongs to a large and complex superfamily of 11 phosphodiesterase (PDE1–11) gene families. PDE3B is one of the genes that mediates the cross talk of insulin and glucagon in the regulation of energy metabolism (18,20). PDE3B inhibits glucagon activity through downregulation of cAMP. Glucagon stimulates glucose production in liver through activation of the cAMP/PKA pathway, and insulin activates PDE3B to inhibit the glucagon activity. Little information exists about PDE3B in humans, but in mice, global inactivation of PDE3B increases energy expenditure through an effect in adipose tissue (21), and this inactivation leads to insulin resistance in the KO mice due to increased

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**Figure 5**—Inhibition of PDE3B expression by NF-κB. A: PDE3B inhibition after NF-κB activation by TNF-α treatment. PDE3B mRNA was examined in HepG2 cells after TNF-α treatment (20 ng/mL) (n = 3). B: Inhibition of TNF-α activity by IkBα. PDE3B expression was determined after TNF-α treatment of HepG2 cells that were stably transfected by an ssIkBa expression vector. C: TNF-α effect in p65-KO cells. PDE3B mRNA was examined in primary hepatocytes of L-p65-KO mice following TNF-α treatment for 30 min in vitro. Data are mean ± SEM (n = 3). *P < 0.05.

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**Figure 6**—Inhibition of PDE3B gene promoter by NF-κB. A: NF-κB binding sites in mouse PDE3B gene promoter. The sites were identified by sequence analysis. B: Characterization of the NF-κB sites a, b, and c in gel shift assays. NF-κB was activated in hepatocytes by TNF-α, and the nuclear extracts were used in EMSA. In the competition assay, cold probes of NF-κB sites were used to compete with the radiolabeled authentic NF-κB probe. NF-κB site b of PDE3B was radiolabeled and used as a probe in the supershift assay with antibodies to p50, c-JUN, and Sp1. C: Inhibition of PDE3B gene promoter by p65. The PDE3B-luciferase reporter was cotransfected with a p65 or an ssIkBa expression vector (μg) in HepG2 cells. The luciferase activity was normalized with the internal control (Renilla luciferase). Data are mean ± SEM (n = 3). **P < 0.01. ATG, adenine thymine guanine; bp, base pair; luc, luciferase.
cAMP activity in liver (22). Inhibition of PDE3B by a chemical inhibitor (cilostazol) was reported to reduce obesity and improve insulin sensitivity in db/db mice (23). In 3T3-L1 adipocytes, TNF-α inhibits PDE3B expression to increase the activity of the cAMP/PKA pathway (24). However, the molecular mechanism was unknown for the PDE3B inhibition before the present report. NIK (NF-κB–inducing kinase) and IKKe are two serine kinases in the signaling pathway of TNF-α (25). Although both are activated by TNF-α, they were reported to have opposite activities in the regulation of the cAMP signaling pathway. NIK upregulated the pathway by direct phosphorylation of the CREB protein in a study of glucagon signaling in liver (26). IKKe downregulated the pathway through induction of PDE3B activity in a study of IKKe in adipocytes (27) in which IKKe was found to phosphorylate the PDE3B protein. The current study suggests that TNF-α enhances the pathway activity through inhibition of PDE3B expression by activation of NF-κB. We observed that NF-κB inhibited the gene promoter activity of PDE3B and found that NF-κB activity was required for TNF-α inhibition of PDE3B expression. NF-κB inhibition by sIL1Rα overexpression or p65 gene inactivation eliminated the TNF-α activity. PDE3B expression was increased in liver under NF-κB inactivation in the L-p65-KO mice. NF-κB binding sites were identified in the PDE3B gene promoter using oligonucleotide competition and antibody-mediated supershift assays in EMSA. The inhibitory activity of NF-κB was proven in the reporter assay of the PDE3B gene promoter. The detailed mechanism of NF-κB action remains unknown after binding to the PDE3B promoter DNA, but NF-κB activity may involve recruitment of a co-repressor to the gene promoter or sequestration of co-activators from other activators in the gene promoter. Other activators in the gene promoter include CREB in the PDE3B gene promoter (14). Inhibition of PDE3B by NF-κB leads to increased cAMP activity, which provides a new mechanism for TNF-α induction of the cAMP/PKA pathway.

Liver damage may occur in L-p65-KO mice as indicated by increased ALT levels. ALT is found mainly in the liver but is also found in smaller amounts in the kidneys, heart, muscles, and pancreas. Low levels of ALT are found in the blood under normal conditions. When the liver is damaged, it releases ALT into the bloodstream; thus ALT level was measured to determine liver damage in L-p65-KO mice. Because NF-κB is known to inhibit cell apoptosis, inactivation of NF-κB should cause hepatic apoptosis to increase and result in liver damage, as indicated by ALT elevation.

In summary, this study provides evidence that NF-κB inactivation in hepatocytes improves hepatic insulin sensitivity and downregulates cAMP/PKA signaling. The decreased cAMP levels were observed with the enhanced PDE3B activity in the hepatocytes of L-p65-KO mice, which is most likely a result of elevated gene transcription.
in the absence of NF-κB activity. NF-κB was found to inhibit the PDE3B gene promoter through three DNA binding sites. The study suggests a new molecular mechanism for inflammation in the pathogenesis of insulin resistance in which NF-κB promotes cAMP signaling activity through downregulation of PDE3B transcription in hepatocytes. The study also suggests that glucagon signaling activity may be reduced in L-p65-KO mice, a possibility that remains to be tested.

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