Inactivation of NF-κB p65 (RelA) in liver improved insulin sensitivity and inhibited cAMP/PKA pathway

Bilun Ke1,2, Zhiyun Zhao2, Xin Ye2, Zhanguo Gao3, Vincent Manganiello4, Bin Wu1,* and Jianping Ye2,*

1 Department of Digestive Disease, Third Affiliated Hospital, Sun Yet-sen University, Guangzhou 510630, China
2 Antioxidant and Gene Regulation Laboratory, Pennington Biomedical Research Center, Louisiana State University System, 6400 Perkins Road, Baton Rouge, LA 70808, USA
3 Collaborative Innovation Center of Molecular Diagnosis and Laboratory Medicine in Henan Province, School of Laboratory Medicine, Xinxiang Medical University, Xinxiang 453003, China.
4 Pulmonary Cardiovascular Branch, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20892, USA.

*Correspondence:
Jianping Ye: e-mail: yeji@pbrc.edu; phone: (225)763-3163; Fax: (225)763-3030
Bin Wu (wubin6@mail.sysu.edu.cn)

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Abstract

The transcription factor 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 liver of C57BL/6 mice through inactivation of p65 gene, which was achieved by crossing floxed p65 and Alb-cre mice to generate L-p65-KO mice. KO mice did not exhibit any alterations in growth, reproduction and body weight on a Chow diet. However, the mice exhibited an improvement in systemic insulin sensitivity on a high fat diet (HFD). Hepatic insulin sensitivity was enhanced in the mice 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. The increase was a result of 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 TNF-α. Body composition, food intake, energy expenditure, systemic and hepatic inflammation were not significantly altered in KO mice on HFD. These data suggest that NF-κB may inhibit hepatic insulin sensitivity by up-regulating cAMP through suppression of PDE3B gene transcription.
INTRODUCTION

The transcription factor NF-κB (nuclear factor kappa B) is a master regulator of inflammation. It is required for expression of pro-inflammatory cytokines, such as IL-1β and IL-6. In the cytosol, NF-κB is associated with the inhibitor protein IκBα (Kappa B inhibitor alpha), 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 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 the skeletal muscle (5) or adipose tissue (6). Although IKKβ has been studied extensively in different tissues in transgenic mice, the mechanism remains unknown for its action in insulin resistance.

In the liver-specific studies, IKKβ overexpression was found to inhibit insulin sensitivity through induction of IL-6 expression (4), and IKKβ knockout was found to protect insulin sensitivity through inhibition of IL-1β expression (3). Although both studies suggest a role of transcriptional regulation by NF-κB in the mechanism of IKKβ action, the detail remains unknown as the downstream genes are different in the two studies. In addition, IKKβ regulates insulin sensitivity through transcription-independent mechanism of IRS-1 serine phosphorylation.
The relative significance of transcription-dependent and -independent mechanisms remains unknown for the IKKβ activity. Inactivation of NF-κB is an approach to address this issue. NF-κB is a heterodimer protein formed by two subunits p65 (RelA) and p50 (NF-κB 1). The transcriptional activity of NF-κB 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, p65\(^{−/−}\)), 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 on Chow and high fat diets. The mechanistic studies were conducted with a focus on cAMP/PKA pathways to understand the metabolic effects of NF-κB.

**RESEARCH DESIGN AND METHODS**

**Generation of L-p65-KO mice**

The loxP p65 mice were generated in C57BL/6 gene background as described elsewhere (10). Alb-cre mice in the C57BL/6 genetic background (Alb-cre mice, Stock # 003574) were purchased from Jackson Laboratory (Bar Harbor, ME). L-p65-KO (p65\(^{E}{\text{ff}}\) Cre\(^{+/−}\)) mice were generated by crossing the floxed-p65 mice with Alb-cre mice (Stock number 003574, Jackson Laboratory). Floxed-p65 littermates (p65\(^{E}{\text{ff}}\)) were used as wild type (WT) control for knockout mice (p65\(^{−/−}\)). The study was conducted in male mice at the animal facility of the Pennington Biomedical Research Center. The mouse housing environment includes a 12:12-hr light-dark cycle, constant room temperature (22–24°C), free access to water and diet. The mice were fed
Chow diet (5% w/w or 11% calories in fat, 5001, Labdiet, St. Louis, MO) or the high fat diet (HFD, 36% w/w or 58% calories in fat, D12331, Research Diets, New Brunswick, NJ). HFD feeding was started at 8 wks in age to generate a diet-induced obese (DIO) 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 (IACUC) at the Pennington Biomedical Research Center.

**Cell culture**

The human hepatoma cell line HepG2 was purchased from the American Type Culture Collection (ATCC) (HB-8065, Manassas, VA 20110). Primary hepatocytes were made from mice at 6-10 wks in age using a protocol as described elsewhere (11). HepG2 cells and the primary hepatocytes were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum at 37 °C in a 5% CO₂ incubator. The cells were treated with TNF-α in serum-free DMEM containing 0.25% fatty acid-free bovine serum albumin. A ssIκBα cell line was made through stable transfection of HepG2 cells with a ssIκBα-pBABE expression vector.

**Body weight and composition**

Body weight and composition were measured every 2 wks. Body composition was measured using quantitative nuclear magnetic resonance (NMR, Minispec Mn10 NMR scanner, Brucker, Canada, Milton ON, Canada) as described previously (12). In the test, conscious and unrestrained mice were individually placed 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 wks. The average daily food intake was determined over 3 days by the net reduction in diet weight with exclusion of spilled food. Food intake was expressed in g/mouse/day.

**Energy expenditure**

Energy metabolism was monitored in mice after 4 wks 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. Oxygen consumption (VO$_2$), carbon dioxide production (VCO$_2$), spontaneous physical activity and food intake were recorded daily. Energy expenditure (EE: kcal/kg/h) was calculated with data on day 5 using the formula $EE = [3.815 + 1.232 \times VCO_2/VO_2] \times VO_2 \times 0.001$ (13). Energy expenditure data was normalized with body lean mass.

**ITT, GTT and PTT**

Intraperitoneal insulin tolerance testing (ITT) was performed in mice (15 wks on HFD) by insulin injection (0.75 U/kg body weight, I9278, Sigma) after 4 h fasting. Intraperitoneal glucose tolerance testing (GTT) was performed in mice (14 wks on HFD) using glucose (2 g/kg body weight) after overnight fasting. Intraperitoneal pyruvate tolerance testing was performed in mice (24 wks on HFD) with intraperitoneal injection of 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, 180 min after injection. Data were expressed in blood glucose concentration (mg/dl).

**Western blotting**

Liver was collected from mice after 16 wks on HFD and examined for gene expression. Whole cell lysates were prepared from liver tissue/cells and used in Western blotting according to the
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 (Santa Cruz, CA). Antibodies to p-Akt (T308, ab38449), p-Akt (S473, ab66138),
p-CREB (S133, ab32096), 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 ribosome 18S RNA, an
endogenous control. Primers and probes were purchased from Applied Biosystems (Carlsbad,
CA). These included IL-1β (Mm00434228_m1), IL-6 (Mm00446190_m1), MCP-1 (Mm00441242_m1), and
IκBa (Mm00477798_m1), PDE3B (Mm00691635_m1), PEPCK (Mm00440636_m1) and G6Pase (Mm00839363_m1).
Sequence of SYBR green primers for human PDE3B is 5'-AAATTCTGGAGGTGGAAATG-3' (Product No: KSPQ12012G,
H_PDE3B_1, NM_000922, Sigma). The primer was used with SYBR Green Master Mix
(4309155, Applied Biosystems) for PDE3B mRNA in HepG2 cells.

Luciferase reporter assay

PDE3B-luciferase reporter (-5.1/-3.4 SX-luciferase PGL3) was described elsewhere (14). The
vectors for p65-pcDNA, ssIkBa-pBABE and corresponding control plasmids were described
elsewhere (15). The transfection was conducted in HepG2 cells using Lipofectamine 2000
(11668019, Grand Island, NY 14072). The luciferase assay was performed at 48 h using a 96-
well luminometer with the dual-luciferase substrate system (E1960, Promega, Fitchburg, WI).
SV40 (Simian Virus 40 Renilla Luciferase) was used at 0.1 μg/well as an internal control. Each experiment was repeated at least three times.

**Electrophoretic mobility shift assay (EMSA)**

The nuclear extract was made from HepG2 cells and the EMSA test 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’-GCATTAGGGTCTTCCCATATA-3’); site c (5’-TAAAGATGAGAGTCCCATGCC-3’). The authentic NF-κB (human IL-6 kappa B site) and Sp1 probes were described elsewhere (16; 17). In oligonucleotide competition and antibody supershift experiments, a 50-fold excess of unlabeled oligonucleotide probe and 2 μg of IgG were used, respectively.

**Plasma cytokine and insulin**

After overnight fasting, blood was collected from mice on HFD for 18 wks through retro-optical bleeding. Plasma was isolated and used in cytokine assays for IL-1β, IL-6 and TNF-α using a multiplex kit (MCYTOMAG-70K, 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) was measured in the plasma using a standard enzymatic assay (TR71121, TR70121; Thermo Fisher Scientific Inc., Middletown, VA).

**cAMP assay**

cAMP level was determined using an ELISA kit (ADI-900-067, Enzo life science Co.). cAMP concentrations were presented with a fold change over WT control.
**Statistical Analysis.** Statistical analysis was performed using two-tailed, unpaired Student’s t-test in the study. $p < 0.05$ 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 is dependent on the p65 subunit. P65 inactivation is expected to decrease expression of inflammatory genes in hepatocytes. In this study, L-p65-KO mice ($p65^{ff}$, 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 ($p65^{ff}$) were used as WT controls. 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 (IκBα, IL-1β and IL-6) was decreased in hepatocytes from the 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 in tests including ITT, GTT, body weight, body composition, food intake, energy expenditure, physical activity, and substrate utilization (Data not shown). The data suggest that KO does not influence insulin sensitivity and energy balance in lean mice. On HFD (58% calories in fat), insulin sensitivity was significantly improved in obese KO mice as indicated by 50% reduction in fasting insulin, and enhanced tolerance to insulin or glucose (Fig.
2, A-C). The data suggest that NF-κB inactivation in hepatocytes significantly improves systemic insulin sensitivity in L-p65-KO mice in obese, but not in 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. In the study, pyruvate tolerance was performed to determine hepatic gluconeogenesis, a process inhibited by insulin. Insulin sensitivity is inversely associated with glucose elevation in the pyruvate tolerance test. 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 threonine 308 and serine 473 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 mechanism of reduced gluconeogenesis. Both genes were decreased in expression in the KO liver (Fig. 3C). Liver weight was not significantly altered in KO mice on HFD (Fig. 3D), although plasma alanine aminotransferase (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 is dependent on activation of cAMP/PKA pathway. In the cAMP/PKA pathway, cAMP elevation leads to activation of serine kinase PKA, which phosphorylates the transcription factor CREB on serine 133. After phosphorylation, CREB promotes gluconeogenesis through induction of transcription of PEPCK and G6pase. CREB
phosphorylation was examined in liver of mice after overnight fasting. The phosphorylation signal was significantly reduced in KO liver (Fig. 4A). The reduction was associated with a decrease in intracellular cAMP (Fig. 4B) and an increase in PDE3B expression (Fig. 4, C and D). PDE3B is an intracellular enzyme that decreases cAMP level in hepatocytes through catalyzing cAMP hydrolysis. Since cAMP concentrations are determined by relative rates of synthesis and degradation in cells, the increased expression of PDE3B is most likely responsible for the decreased cAMP content and cAMP/PKA signaling in liver tissue of KO mice.

**Regulation of PDE3B by NF-κB**

The data above suggest that PDE3B may play an important role in the molecular mechanisms underlying the improved insulin sensitivity in L-p65-KO mice. The PDE3 family contains two members encoded by distinct genes: PDE3A and PDE3B (18). The isoform (PDE3A) is relatively highly expressed in the cardiovascular system, and the B isoform (PDE3B) is relatively highly expressed in hepatocytes and adipocytes. The mRNA data suggests that PDE3B gene transcription may be up-regulated after NF-κB inactivation. To test the possibility, NF-κB was activated in HepG2 cells by TNF-α treatment. PDE3B mRNA was examined in the system at multiple time points, and a decrease was observed after TNF-α treatment for 0.5 h (Fig. 5A). The TNF-effect was blocked when NF-κB activity was inhibited by IκBα (Fig. 5B). The experiment was performed in an IκBα cell line that was made in HepG2 cells through stable transfection with a ssIκBα expression vector. In the stable cell line, the basal level of PDE3B mRNA was increased, and TNF-α was unable to inhibit PDE3B expression (Fig. 5B). The TNF-α activity was tested in the primary hepatocytes of L-p65-KO mice. The inhibition was observed in WT cells, but not in KO cells (Fig. 5C), suggesting that NF-κB is required for the TNF-α activity.
These data suggest that NF-κB inhibits PDE3B expression in hepatocytes in the TNF-α signaling pathway.

**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 in the EMSA assay through a competition against a classical NF-κB probe (Fig. 6B). All three sites competed with the classical probe, but with different efficiency. The site b exhibited the strongest completion, suggesting that it has the highest binding affinity to NF-κB protein. A radiolabelled probe was made from the site b, and further tested for NF-κB interaction in a supershift assay. The DNA-protein complex was shifted by the antibody to NF-κB p50 subunit, but not by the antibodies to c-JUN or Sp1 (Fig. 6B), suggesting that the probe specifically interacted with NF-κB protein. The function of NF-κB sites was tested in the gene promoter in the luciferase reporter assay. The PDE3B promoter was inhibited by p65 in cotransfection, and the inhibition was blocked by IκBα in cotransfection (Fig. 6C). These data suggest that NF-κB inhibits PDE3B transcription through DNA-binding sites in the gene promoter.

**Energy balance and systemic inflammation**

Energy balance was monitored in KO mice by checking body weight, body composition, food intake and metabolic rate. Those parameters were not altered in L-p65-KO mice on HFD (Fig. 7, A-E) or Chow diets (Fig. 7F). Systemic inflammatory status was examined by measuring plasma
proinflammatory cytokines in mice on HFD. The cytokines include TNF-α, IL-6, IL-1β, IL-10, IL-15 and VEGF. The cytokine levels were not altered in KO mice in non-fasted or fasted conditions (Fig. 7, G and H). These data suggest that p65 inactivation in liver does not alter the energy balance and systemic inflammatory status in mice.

**DISCUSSION**

This study provides evidence that systemic insulin sensitivity is enhanced by liver-selective NF-κB inactivation in DIO mice, suggesting a role of hepatic NF-κB in the regulation of insulin sensitivity. The observation is consistent with those reported for hepatic IKKβ activities in the over expression and knockout studies (3; 4). However, the molecular mechanism of NF-κB action is different from those reported for IKKβ. IKKβ 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 IKKβ models. In addition, the cytokines are controversial in the pathogenesis of insulin resistance since their activities are not observed in the induction of insulin resistance in all studies (19). Although NF-κB was reported to mediate the IKKβ activity, the role of PDE3B was not found at the downstream of NF-κB in those early studies (3; 4). The current study suggests that PDE3B is a new target gene of NF-κB in the regulation of insulin sensitivity. Our data suggests that the 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 which contains 11 phosphodiesterase (PDE1–11) gene families. PDE3B is one of the genes that mediate the crosstalk of insulin and glucagon in
the regulation of energy metabolism (18; 20). PDE3B inhibits glucagon activity through down-regulation of cAMP. Glucagon stimulates glucose production in liver through activation of the cAMP/PKA pathway. Insulin activates PDE3B to inhibit the glucagon activity. There is little information about PDE3B in human. In mice, global inactivation of PDE3B increases energy expenditure through an effect in adipose tissue (21). The inactivation led to insulin resistance in the KO mice due to increased 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 cAMP/PKA pathway (24). However, the molecular mechanism was unknown for the PDE3B inhibition before this report. NIK (NF-κB–inducing kinase) and IKKε are two serine kinases in the signaling pathway of TNF-α (25). Although both of them are activated by TNF-α, the two serine kinases were reported to have opposite activities in the regulation of cAMP signaling pathway. NIK up-regulated the pathway by direct phosphorylation of CREB protein in a study of glucagon signaling in liver (26). IKKε down-regulated the pathway through induction of PDE3B activity in a study of IKKε in adipocytes (27), in which IKKε was found to phosphorylation PDE3B protein. The current study suggests that TNF-α may enhance 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. The NF-κB activity was required for TNF-α inhibition of PDE3B expression. NF-κB inhibition by ssIkBα over expression or p65 gene inactivation eliminated the TNF-α activity. PED3B expression was increased in liver under NF-κB inactivation in 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 proved in the reporter assay of the PDE3B gene promoter.
The detail mechanism of NF-κB action remains unknown after binding to the PDE3B promoter DNA. The NF-κB activity may involve recruitment of a corepressor to the gene promoter, or sequestration of coactivators from other activators in the gene promoter. Other activator in the gene promoter includes CREB in the PDE3B gene promoter (14). Inhibition of PDE3B by NF-κB leads to the 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 the increased ALT (alanine aminotransferase). ALT is found mainly in the liver, but also in smaller amounts in the kidneys, heart, muscles, and pancreas. Low levels of ALT are found in the blood of normal conditions. When the liver is damaged, it releases ALT into the bloodstream to make plasma ALT go up. ALT was measured to determine liver damage in L-p65-KO mice. NF-κB is known to inhibit cell apoptosis. After NF-κB inactivation, hepatic apoptosis may go up to cause some liver damage as indicated by ALT elevation.

In summary, this study provides evidence that NF-κB inactivation in hepatocytes improved hepatic insulin sensitivity and down-regulated cAMP/PKA signaling. The decreased cAMP levels were observed with the enhanced PDE3B activity in the hepatocytes of L-p65-KO mice. The enhanced PDE3B activity 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 3 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
down-regulation of PDE3B transcription in hepatocytes. The study suggests that glucagon signaling activity may be reduced in L-p65-KO mice. This possibility remains to be tested.

Author contribution: B.K, Z.Z, X.Y, and Z.G conducted the experiments. V.M, B.W, and J.Y designed the study and prepared the manuscript. All authors read and approved the final manuscript.

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LEGENDS

Fig. 1 Decreased inflammation in liver of L-p65-KO mice on a Chow diet. A. 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. In the bar figures, values are the mean ± SEM (n=3). ** P<0.001 by Student’s t-test.

Fig. 2 Systemic insulin sensitivity in L-p65-KO mice. A. Fasting insulin. Blood was collected from mice following overnight fasting after 18 wks on HFD (n=7-8). B. ITT testing. The mice were subject to peritoneal injection of insulin (0.75 units/kg) following 4 h fasting after 15 wks on HFD (n=10-12). Blood glucose was determined at the indicated time points. C. GTT testing. The mice was injected with glucose (2 g/kg) into the peritoneal cavity following overnight fasting after 14 wks on HFD (n=10-12). The data represents mean ± SEM. * p<0.05.

Fig. 3 Hepatic insulin action in L-p65-KO mice. A. Pyruvate tolerance. Pyruvate (2 g/kg) was administrated (i.p.) in mice following overnight fasting after 24 wks on HFD (n=8-10). B. Insulin signaling in hepatocytes. Primary hepatocytes were prepared from 8 wk old mice and cultured in vitro. After 15 min of insulin treatment, phosphorylation of Akt at T308 and S473 was examined in cell lysate by Western blotting. C. mRNA expression of gluconeogenic genes in hepatocytes of KO liver (n=6). D. Liver weight. Liver was collected from mice on HFD for 16 wks and used in weight analysis (n=10-12). E. Liver function. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were tested using plasma collected from mice after 4 wks on HFD (n=7). The bar figure presents data of mean ± SEM. * p<0.05.
**Fig. 4 Reduced cAMP content and signaling in liver of L-p65-KO mice.** Liver was collected from mice on HFD for 16 wks and used in this experiment. A. Phosphor-CREB. The phosphorylation was determined via Western blotting. B. cAMP levels in liver tissue. C. PDE3B protein in liver tissue. D. PDE3B mRNA expression in liver of HFD-fed mice (n=5). The bar figure presents data of mean ± SEM. **P<0.01.

**Fig. 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 IκBα. PDE3B expression was determined after TNF-α treatment of HepG2 cells that were stably transfected by a ssIκBα 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 minutes *in vitro*. The bar figure presents data of mean ± SEM (n=3). * p<0.05.

**Fig. 6 Inhibition of PDE3B gene promoter by NF-κB.** A. NF-κB binding sites in 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 PED3B was radiolabeled and used as a probe in the supershift assay with antibodies to p50 (α p50), c-JUN (α Jun) and Sp1 (α Sp1). C. Inhibition of PDE3B gene promoter by p65. The PDE3B luciferase reporter was co-transfected with a p65 or ssIκBα expression vectors (µg) in HepG2 cells. The luciferase activity
was normalized with the internal control (Renilla luciferase). The bar figure presents data of
mean ± SEM (n=3).

**Fig. 7 Energy balance and inflammatory cytokines.** Energy balance was determined by
analysis of body weight, body composition, energy metabolism, and food intake in the mice. A.
intake over three days in the metabolic cage at 14 wk on HFD. E. Energy expenditure. The test
was performed after 4 wk on HFD and the data was normalized with body lean mass. F. Body
weight on Chow diet. G. Plasma cytokines in non-fasted mice. H. Plasma cytokines in overnight
fasted mice. Data is expressed as mean ± SEM (n=9-12). *P<0.05, **P<0.001 by Student’s t-test.
A. p65 protein in liver

B. NF-kB targets in hepatocytes
Fig. 2 Systemic insulin sensitivity in L-p65-KO mice on HFD
Fig. 3 Insulin signaling and glucogenic gene in improved in L-p65-KO mice on HFD.
A. Reduced PKA activity

B. Reduced cAMP

C. PDE3B protein

D. PDE3B mRNA

Fig. 4. cAMP pathway is down in liver of L-p65-KO mice
Fig. 5 Regulation of PDE3B by NF-κB

A  PDE3B inhibition by TNF-α

B  TNF-α in sIκBα cells

C  TNF-α in p65-KO hepatocytes
A. NF-kB binding sites in mouse PDE3B gene promoter

B. NF-kB binding

C. Promoter inhibition by p65

Fig. 6 Inhibition of PED3B promoter by p65
Fig. 7 Energy balance and inflammation.

A Body weight

B Body fat

C Body lean mass

D Food intake

E Energy expenditure

F Body weight on Chow

G Non fasting

H Fasting