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FOXO1-dependent Up-regulation of MAP Kinase Phosphatase 3 (MKP-3) Mediates Glucocorticoid-induced Hepatic Lipid Accumulation in Mice

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Abstract

Long-term treatment with glucocorticoids (GCs) or dysregulation of endogenous GC levels induces a series of metabolic diseases, such as insulin resistance, obesity and type 2 diabetes. We previously showed that MAP kinase phosphatase-3 (MKP-3) plays an important role in glucose metabolism. The aim of this study is to investigate the role of MKP-3 in GC-induced metabolic disorders. Dexamethasone (Dex), a synthetic GC, increases MKP-3 protein expression both in cultured hepatoma cells and in the liver of lean mice. This effect is likely mediated by forkhead box protein O1 (FOXO1) because disruption of endogenous FOXO1 function by either interfering RNA mediated FOXO1 knockdown or overexpression of a dominant negative FOXO1 mutant blocks Dex-induced upregulation of MKP-3 protein. In addition, overexpression of FOXO1 is sufficient to induce MKP-3 protein expression. MKP-3 deficient mice are protected from several side effects of chronic Dex exposure, such as body weight gain, adipose tissue enlargement, hepatic lipid accumulation, and insulin resistance. The beneficial phenotypes in mice lacking MKP-3 are largely attributed to the absence of MKP-3 in the liver since only hepatic insulin signaling has been preserved among the three insulin target tissues (liver, muscle and adipose tissue).

Keywords

hepatosteatosis; dexamethasone; obesity; insulin resistance; lipogenesis

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1. Introduction

Endogenous GCs are steroid hormones secreted by the cortex of adrenal gland and exert their actions on multiple organ systems through the glucocorticoid receptor (GR) that exists in almost all cell types. GCs and their synthetic analogs have been widely prescribed as medications for their anti-inflammatory and immunosuppressive properties (1). GC drugs have been found effective to treat numerous diseases like rheumatoid arthritis, asthma, allergy, autoimmune diseases, and organ transplant rejection. However, chronic GC treatment is associated with many metabolic disorders, such as hepatosteatosis, insulin resistance, hyperlipidemia, hypertension, and hyperglycemia (2, 3). Elevated endogenous GCs cause Cushing syndrome and patients are also featured with metabolic side effects commonly found with prolonged GC therapy (4, 5). Among these GC-related metabolic disorders, fat accumulation in the liver has been considered as an independent risk factor for the development of insulin resistance (6-8). Therapies that can attenuate the side effects of GCs will greatly benefit numerous patients who are depending on these medications.

MAP kinase phosphatase 3 (MKP-3), which is also known as dual specificity protein phosphatase 6 (DUSP6), belongs to the dual specificity protein phosphatase family (9). These phosphatases inactivate members of the mitogen-activated protein (MAP) kinase family members (ERK, JNK, p38) by dephosphorylating both the phosphoserine/threonine and phosphotyrosine residues (9, 10). MKP-3 specifically dephosphorylates extracellular signal-regulated kinases (ERK1/2) to attenuate MAP kinase signaling and MKP-3^{-/-} mice display enhanced basal ERK1/2 phosphorylation (11-13). Growth factors including insulin downregulate MKP-3 expression through a MEK/ERK dependent feed-forward mechanism (14-16). The phosphoinositide 3-kinase (PI3K)/mammalian target of rapamycin (mTOR) signaling pathway is also involved in growth factor-induced phosphorylation and degradation of MKP-3 (17). We were the first to report that MKP-3 expression is significantly increased in the liver of both diet-induced obese (DIO) and genetically obese (ob/ob) mice and MKP-3 is a critical player in glucose homeostasis by promoting hepatic gluconeogenesis (18-20). The effect of MKP-3 on hepatic glucose output is implemented through dephosphorylation and activation of FOXO1, a forkhead transcription factor with a well-established role in turning on the gluconeogenic program (19, 21, 22). In addition to promoting gluconeogenesis, FOXO1 also increases glycogenolysis in the liver, elevates hepatic very low-density lipoprotein (VLDL) production and decreases liver insulin sensitivity (23-26). Cytoplasmic retention of FOXO1 by Akt-mediated phosphorylation on threonine 24, serine 256 and serine 319 is the major mechanism for insulin to repress gluconeogenesis in liver cells, subsequently leads to FOXO1 ubiquitination and degradation (21). MKP-3 interacts with FOXO1 and promotes its nuclear translocation by dephosphorylation on serine 256 (19, 20). Knocking down MKP-3 in the liver of DIO and ob/ob mice is sufficient to attenuate obesity-related hyperglycemia and improve systemic insulin sensitivity (19).

Dex, a widely used synthetic GC, has been reported to increase FOXO1 expression in muscle and pancreatic β cells (27-29). Interestingly, we found that Dex induces expression of both FOXO1 and MKP-3 in cultured rat hepatoma Fao cells (18, 19). In addition, Dex has a synergistic effect with MKP-3 on increasing gluconeogenic gene expression and promoting

gluconeogenesis both in Fao cells and in the liver of lean mice upon acute treatment. These data indicate that MKP-3 may be a downstream mediator for Dex induced metabolic disorders. In this study, we investigated the role of FOXO1 in Dex-induced MKP-3 expression in cultured hepatoma cells and in the liver of lean mice. Furthermore, we evaluated the role of MKP-3 in the metabolic disorders caused by chronic Dex treatment by using MKP-3^{-/-} mice.

2. Materials and Methods

2.1 Reagents and cells

MKP-3 promoter luciferase constructs were provided by Dr. Stephen M Keyse (University of Dundee, Dundee, Scotland). AdFOXO1 was provided by Dr. Pere Puigserver (Dana Farber Cancer Institute, Boston, MA). AdshFOXO1 was provided by Dr. Marc Montminy (Salk Institute, La Jolla, California). AdFOXO1 256 was provided by Dr. Henry Dong (University of Pittsburgh, Pittsburgh, PA). Dex, Dex 21-phosphate disodium salt and Ru486 were purchased from Sigma (St Louis, MO). MKP-3, IRS1, SCD1 and anti-goat IgG-HRP antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Akt, phospho-Akt T308, FOXO1, β -actin, anti-mouse IgG-HRP and anti-rabbit IgG-HRP antibodies were purchased from Cell Signaling (Danvers, MA). Phospho-IRS1 S307 antibody was purchased from Millipore (Bedford, MA). Tubulin antibody was purchased from Abcam (Cambridge, MA). HEK 293A cells were purchased from Invitrogen (Life Technologies, Carlsbad, CA). For in vitro studies in cultured liver cell lines, commonly used mouse (Hepa1-6) and rat (Fao) hepatoma cells were used. Hepa1-6 cells were provided by Dr. Gokhan Hotamisligil (Harvard School of Public Health, Boston, MA). Fao cells were provided by Dr. Zhidan Wu (Novartis Institutes for Biomedical Research). Mouse Ultrasensitive Insulin ELISA kit was purchased from ALPCO Diagnostics (Salem, NH). Humulin R was purchased from Eli Lilly and Company (Indianapolis, IN).

2.2 Cell treatments

For Dex treatment, Hepa1-6 cells were incubated in serum-free medium for 16 hours followed by 2.5 μ M Dex for 2 hours. For Ru486 treatment, Hepa1-6 cells were incubated in serum-free medium for 16 hours, pretreated with 10 μ M Ru486 or DMSO for 1 hour, then treated with 2.5 μ M Dex or vehicle plus 10 μ M Ru486 or DMSO for 2 hours. For adenovirus-mediated gene overexpression or knockdown, Hepa1-6 cells were infected for fifty-four hours, and then incubated in serum-free medium overnight before being harvested or treated with vehicle or Dex.

2.3 RNA extraction and real-time PCR analysis

RNA samples were extracted using the TRIZOL® reagent from Invitrogen according to the manufacturer's manual. For real-time PCR analysis, random hexamers were used for reverse transcription. Real-time PCR analysis was performed in a 15 μ l reaction in 96-well clear plates using Power SYBR® Green RT-PCR Reagents on an ABI thermal cycler Step-One Plus (Life Technologies). Reactions contained 1x Power SYBR® Green PCR Master Mix (Life Technologies), 300nM forward primer, 300nM reverse primer, and 20ng cDNA sample. PCR conditions were: 50°C for 2min followed by 95°C for 10min for 1 cycle, and

then 95°C for 15sec followed by 60°C for 1min for 40 cycles. The real time PCR data was analyzed by 2-delta delta CT method using 28S as the reference. The sequences of the primers are as the following:

28S forward, TTCACCAAGCGTTGGATTGTT;
 28S reverse, TGTCTGAACCTGCGGTTTCCT;
 PPAR γ forward, GGAAGACCACTCGCATTCCTT
 PPAR γ reverse, TCGCACTTTGGTATTCTTGGAG
 FAS forward, GGCTCTATGGATTACCCAAGC;
 FAS reverse, CCAGTGTTTCGTTCCCTCGGA;
 SCD1 forward, CCTACGACAAGAACATTCAATCCC;
 SCD1 reverse, CAGGAAGCTCAGAAGCCCAAAGC;
 ACC1 forward, CGGACCTTTGAAGATTTTGTTCAGG;
 ACC1 reverse, GCTTTATTCTGCTGGGTGAACTCTC;
 ACC2 forward, GGAAGCAGGCACACATCAAGA;
 ACC2 reverse, CGGGAGGAGTTCTGGAAGGA;

2.4 Immunoprecipitation and western-blot analysis

To prepare cell lysates, Hepa1-6 cells were washed with ice-cold PBS once and lysed with lysis buffer supplemented with protease inhibitors. To prepare liver lysates, livers were immediately frozen in liquid nitrogen, pulverized into powder and homogenized in lysis buffer supplemented with protease inhibitors. To immunoprecipitate MKP-3 from protein lysates, thirty microliters of Exactra D immunoprecipitation matrix (Santa Cruz Biotechnology) slurry were used to pre-clear lysates at 4°C for 30min. Then forty microliters of MKP-3 antibody-bound Exactra D immunoprecipitation matrix slurry were added to pull down MKP-3. For direct western blot analysis, one hundred micrograms of protein lysate from each sample were used. Following PAGE on 4-12% gel (Bio-Rad Laboratories, Hercules, CA), the resolved proteins were transferred onto PVDF membranes. Membranes were blocked in 1% BSA/1xTBST or 5% milk/1xTBST for 1hr followed by incubation with the appropriate primary antibodies (MKP-3 Ab, 1:500; FOXO1 Ab, 1:1000; pAkt T308 Ab, 1:1000; Akt Ab, 1:1000; tubulin AB 1:10000). After thorough wash, membranes were incubated with appropriate horseradish peroxidase-linked secondary antibodies diluted 1:2000 for 1hr in 5% milk/ 1x TBST. Protein signals were detected by ECL western blotting detection reagent (Perkin Elmer, Waltham, MA) after thorough wash on the Alpha-Inotech fluorochem imaging system (Alpha Innotech Corporation, San Leandro, CA). Blots were quantified with Image J software (National Institutes of Health, Bethesda, MD).

2.5 Mice maintenance and treatments

All animal experiments were approved by the Institutional Animal Care and Use Committee of Rhode Island Hospital. MKP-3^{-/-} mice on a mixed background of C57BL/6 and 129 were purchased from the Jackson Laboratory and backcrossed to C57BL/6 wild type mice for 6

generations. For Dex injection, 9-10-week old MKP-3^{-/-} or wild type mice were randomized to four groups with equal body weights and postprandial glucose levels. Dex or 0.9% saline was injected intraperitoneally (i.p.) daily at the dose of 15mg/kg or 50mg/kg for designated time. Body weights were measured weekly. At the end of studies, body weights were measured after an overnight fast and mice were sacrificed by CO₂ asphyxiation. Tissue samples were rapidly dissected, weighed and frozen for further analysis.

2.6 Indirect calorimetry

Oxygen consumption (VO₂), carbon dioxide production (VCO₂), and food intake were measured individually for 24 hours using the comprehensive lab animal monitoring system (Columbus Instruments, Columbus, OH) after one-day of acclimation. During the experiment, mice had free access to food and water. Energy expenditure was calculated using the following formula: $VO_2 \times (3.815 + 1.232 \times RQ)$, and normalized to (body mass)^{0.75}.

2.7 Insulin tolerance test and hepatic insulin signaling

For insulin tolerance test (ITT), 27-week old male mice were treated with Dex (15mg/kg) for 12 weeks, fasted for 6 hours, and injected with insulin at the dose of 1U/kg by i.p.. Blood glucose levels were measured at 0, 15, 30, 45, 60 and 90 minutes post injection. For hepatic insulin signaling, 27-week old female mice were treated with Dex (15mg/kg) for 16 weeks, fasted overnight, and anesthetized with ketamine/domitor. Insulin was injected through portal vein at the dose of 0.5U/kg. Livers were dissected five minutes after injection and immediately frozen for immunoblot analysis.

2.8 Measurement of triglyceride (TG) and glycogen contents

The content of hepatic TG was determined using homogenates of liver. Frozen tissues were pulverized in liquid nitrogen, weighed, homogenized in ethanol, vortexed, centrifuged, and the supernatant was collected for measurement. TG standard (Sigma) or samples were mixed with the reaction buffer (100mM Tris, pH7.4, 1mM MgCl₂, 0.05mM ATP, 0.2U/ml horse radish peroxidase, 1U/ml glycerol phosphate oxidase, 2U/ml glycerol kinase, 25U/ml lipase, and 0.05mM Amplex red) and incubated for 30 minutes at 37°C. For measurement of hepatic glycogen content, protein was precipitated with 10% SDS and trichloroacetic acid, and then centrifuged. The supernatant was mixed with 4 volumes of methanol and incubated at -80°C for 30 minutes. After centrifugation, glycogen pellet was redissolved in 50μl of 150mM sodium acetate (pH4.6), followed by addition of 3.3μl of 20mg/ml amyloglucosidase and incubated at 37°C for 2 hours. Glycogen standards were simultaneously treated with amyloglucosidase for 2 hours. 5μl digested glycogen standards or samples were incubated with 100μl reaction buffer (100mM Tris, pH7.4, 1mM MgCl₂, 0.2U/ml horse radish peroxidase, 0.2U/ml glucose oxidase and 0.05mM Amplex red) at 37°C for 30 minutes. The fluorescence was read at excitation 530nm/emission 590nm using Synergy 4 plate reader (BioTek Instruments, Winooski, VT).

2.9 Transfection of Fao cells and luciferase assay

Fao cells were transfected with Fugene HD reagent (Roche Diagnostics) upon 50% confluency. The DNA:Fugene HD ratio was 1:2.5. The MKP-3 promoter wild type or

mutant firefly luciferase expression plasmid was co-transfected with FOXO1 or TORC2 expression plasmid. Renilla luciferase expression plasmid was also co-transfected as an internal control. Twenty-four hours after transfection, Fao cells were incubated overnight in serum-free RPMI1640 medium containing vehicle or 100ng/ml insulin. Forty-eight hours after transfection, cells were rinsed once in PBS and lysed in passive lysis buffer by two freeze-thaw cycles. Luciferase assay was performed using the Dual-Glo luciferase assay kit from Promega (Madison, WI). The results were expressed as relative luciferase activity by normalizing firefly luciferase activity to renilla luciferase activity.

2.10 Statistical analysis

Results are presented as mean $\pm$ SEM. Statistical significance was determined at $P < 0.05$. Student's t-test was used to compare differences between two groups. Two-way ANOVA and Bonferroni posttests were used to analyze multiple experimental groups. Error bars represent mean $\pm$ standard errors.

3. Results

3.1 Dex increases MKP-3 and FOXO1 protein expression in hepatoma cells

We previously reported that MKP-3 protein expression is increased in the liver of obese rodents and it promotes hyperglycemia through stimulating hepatic gluconeogenesis (19). Expression of MKP-3 can be upregulated by Dex. GCs also induce hyperglycemia and the main mechanism is through increasing gluconeogenesis in the liver. It is interesting to investigate whether MKP-3 plays any role in GC-mediated metabolic disorders. To address this question, cultured mouse hepatoma Hepa1-6 cells were treated with Dex to assess the mechanism of Dex-induced MKP-3 expression. Dex significantly increased MKP-3 protein expression (Figure 1A-B). Dex also significantly increased FOXO1 protein expression (Figure 1A-B). The effects of Dex on upregulating MKP-3 and FOXO1 protein are mediated through GR because treatment with Ru486, a well-established antagonist of GR, completely reversed MKP-3 and FOXO1 protein expression to the basal levels (Figure 1C-D). A forkhead transcription factor-binding element, not GC response element, was identified in the MKP-3 promoter. Dex can't activate MKP-3 promoter directly (data not shown). This prompted us to hypothesize that FOXO1 could be the factor mediating Dex-induced MKP-3 expression (30). Indeed, co-expression of FOXO1 and MKP-3 promoter luciferase reporter construct significantly increased activity of firefly luciferase, indicating that FOXO1 activates MKP-3 promoter transcription (Figure 1E). TORC2, a gluconeogenic transcription co-activator, was not able to activate MKP-3 promoter. Mutation of six nucleotides in the forkhead binding element significantly blunted the effect of FOXO1 on MKP-3 promoter transcription (Figure 1E). The effect of FOXO1 on activating MKP-3 promoter can also be attenuated by insulin treatment (Figure 1F).

3.2 FOXO1 is the mediator of Dex-induced MKP-3 protein expression in hepatoma cells

To examine whether FOXO1 has any effect on endogenous MKP-3 protein expression, FOXO1 was overexpressed in Hepa1-6 cells via adenovirus-mediated gene transfer. As shown in figure 2A-B, MKP-3 protein expression was significantly increased by FOXO1 overexpression. To determine whether FOXO1 is necessary for Dex to induce MKP-3

protein expression, the function of endogenous FOXO1 was disrupted by two approaches: knocking down by interfering RNA and inactivation by a dominant-negative mutant. FOXO1 knockdown in Hepa1-6 cells significantly decreased basal MKP-3 protein expression and attenuated Dex-induced MKP-3 upregulation (Figure 2C-D). Overexpression of FOXO1²⁵⁶, a dominant negative mutant that worked more potently than FOXO1 interfering RNA, markedly reduced basal MKP-3 protein level and completely abolished Dex-induced MKP-3 protein expression (Figure 2E-F). These results indicate that FOXO1 is required for Dex to increase MKP-3 protein expression in Hepa1-6 cells.

3.3 Dex increases MKP-3 and FOXO1 protein expression in the liver of lean mice

To further dissect the physiological relevance of FOXO1 in mediating Dex-induced MKP-3 upregulation, the effect of Dex on MKP-3 protein expression was examined in the liver of lean mice. After treatment with Dex for 28 days at the dose of 15mg/kg, MKP-3 protein expression level was significantly increased by 8-fold compared to mice treated with vehicle (Figure 3A-B). The upregulation of MKP-3 protein was accompanied by hyperinsulinemia since fasting plasma insulin level was increased by 4.7-fold compared to mice treated with vehicle (Figure 3C). To determine whether Dex can induce FOXO1 expression in the liver of mice and whether upregulation of FOXO1 occurs prior to upregulation of MKP-3, Dex was injected at the dose of 15mg/kg and livers were collected at 4h, 8h and 24 days post injection. Dex treatment also drastically increased FOXO1 protein expression 8 hours after Dex administration (Figure 3D-E). FOXO1 protein expression level went down to baseline 28 days post injection when MKP-3 protein expression level started to increase (Figure 3A, 3D). These results indicate that FOXO1 is likely a mediator of Dex-induced MKP-3 protein expression in the liver of lean mice because its up-regulation occurs earlier than that of MKP-3. When endogenous hepatic FOXO1 function was impaired by overexpression of FOXO1²⁵⁶ through tail vein injection of adenovirus, MKP-3 protein expression was significantly reduced in the liver of lean mice (Figure 3F-G).

3.4 MKP-3 deficiency in mice prevents Dex-induced body weight gain and hepatic lipid accumulation

To study whether MKP-3 deficiency can protect mice from developing Dex-induced metabolic disorders, wild type (WT) or MKP-3^{-/-} mice received daily Dex injection for seven weeks at the dose of 15 mg/kg. Dex injection significantly increased body weight of WT male mice after five weeks and the body weight increase became more obvious at six and seven weeks of injection (Figure 4A) compared to vehicle treated WT male mice. In contrast, no difference was observed between vehicle and Dex-treated MKP-3^{-/-} male mice (Figure 4A). Similar results were obtained in female mice (Figure 4B). This indicates that absence of MKP-3 is sufficient to prevent Dex-induced body weight gain. Dex injection significantly increased weight of epididymal adipose tissue in both WT and MKP-3^{-/-} mice (Figure 4C). MKP-3 deficiency partially protected Dex-induced adiposity since the epididymal adipose tissue of Dex-treated MKP-3^{-/-} mice is 36% lighter than that of Dex-treated WT mice. Dex injection also significantly increased weight of liver in both WT and MKP-3^{-/-} mice (Figure 4D). To determine the cause of the lean phenotype in MKP-3^{-/-} mice, food intake and energy expenditure were measured. MKP-3^{-/-} mice have significantly increased energy expenditure despite increased food intake (Figure 4E-F). Liver TG content

of Dex-treated WT mice was significantly higher than that of vehicle injected WT mice (Figure 5A-B). In vehicle-treated mice, absence of MKP-3 in the liver was sufficient to reduce hepatic TG content by 50% compared to WT mice. Furthermore, MKP-3 deficiency completely abolished Dex-induced hepatic TG accumulation (Figure 5A). It is interesting to note that liver weight of Dex-treated MKP-3^{-/-} mice is not different from that of Dex-treated WT mice despite a 63% reduction in TG content. This indicates that other component(s) in the liver of MKP-3^{-/-} mice must be increased by Dex injection. Liver is the main storage site of glycogen and our data showed that Dex treatment increased liver glycogen content by 3.1-fold in WT mice but by 5.4-fold in MKP-3^{-/-} mice (Figure 5C-D). This translates into a 60% higher glycogen content in MKP-3^{-/-} mice treated with Dex compared to WT mice with the same treatment, which may explain why there is no difference in liver weight between the two groups.

3.5 The effect of Dex on induction of lipid synthesis genes is blunted by MKP-3 deficiency

To explore the mechanism of lower liver TG content in MKP-3^{-/-} mice compared to WT mice upon chronic Dex treatment, we examined the expression of several lipid synthesis genes in the liver by real-time PCR analysis, including peroxisome proliferator-activated receptor gamma (PPAR γ), fatty acid synthase (FAS), stearoyl-Coenzyme A desaturase 1 (SCD1), acetyl-CoA carboxylase 1 (ACC1) and acetyl-CoA carboxylase 2 (ACC2). Chronic Dex treatment significantly increased hepatic PPAR γ (2.6-fold), FAS (6.1-fold), SCD1 (2.9-fold) and ACC1 (2.4-fold) mRNA levels in WT mice compared to vehicle treated controls (Figure 6A-D). Among these four genes, Dex only significantly upregulated FAS (4.6-fold) mRNA level in MKP-3^{-/-} mice and expression levels of PPAR γ , SCD1 and ACC1 mRNA remained unchanged (Figure 6A-D). Despite the fact that Dex did not increase ACC2 mRNA expression, MKP-3^{-/-} mice have significantly lower ACC2 gene expression compared to WT mice in response to Dex treatment (Figure 6E). Immunoblot analysis of SCD1 and ACC2 protein levels confirmed the conclusion that MKP-3 deficiency lowered the expression of lipogenic enzymes (Figure 6F). These results indicate that MKP-3 deficiency most likely represses Dex-induced lipogenesis in the liver.

3.6 MKP-3^{-/-} mice are protected from Dex-induced insulin resistance

We previously reported that hepatic MKP-3 knockdown by adenovirus-mediated expression of interfering RNA in DIO mice improves systemic insulin sensitivity (19). To evaluate whether MKP-3 deficiency protects mice from Dex-induced insulin resistance, insulin tolerance test was performed. As shown in figure 7A, chronic Dex treatment induced systemic insulin resistance in WT mice as reflected by unchanged blood glucose levels upon insulin injection up to 90 minutes. In contrast, blood glucose levels of Dex treated MKP-3^{-/-} mice started to decrease 30 minutes after insulin injection and remained significantly lower than those of Dex treated WT mice at 45, 60 and 90 minutes post insulin injection (Figure 7A). Systemic insulin tolerance was undistinguishable between vehicle treated MKP-3^{-/-} mice and WT mice. At the above mentioned three time points, blood glucose levels of Dex-treated MKP-3^{-/-} mice are similar to those of vehicle treated mice which demonstrates a nearly complete protection from Dex-induced insulin resistance.

To investigate whether MKP-3 deficiency preserves insulin sensitivity by maintaining the integrity of insulin signaling in insulin target tissues in response to chronic Dex exposure, liver, epididymal adipose tissue and muscle were collected from MKP-3^{-/-} and WT mice five minutes after insulin injection through portal vein and frozen immediately in liquid nitrogen. Phosphorylation status of Akt, a critical component of insulin signaling, was examined. The phosphorylation level of Akt on threonine 308 (T308) was decreased by 64% after normalization to Akt protein in the liver of Dex-treated WT mice compared to vehicle-treated WT mice (Figure 7B-C) whereas it was increased by 2-fold in the liver of Dex-treated MKP-3^{-/-} mice compared to vehicle treated MKP-3^{-/-} mice. Compared to Dex-treated WT mice, the phosphorylation level of Akt on T308 is 6.6-fold higher in the liver of Dex-treated MKP-3^{-/-} mice (Figure 6B-C). Surprisingly, absence of MKP-3 was not sufficient to preserve insulin signaling in epididymal adipose tissue and muscle (data not shown). These results indicate that MKP-3 deficiency most likely protects mice from Dex-induced insulin resistance through preserving hepatic insulin signaling.

4. Discussion

In our previous publications, we reported that MKP-3 and Dex have a synergistic effect on promoting transcription of gluconeogenic genes and hepatic glucose output through acute overexpression or knockdown of MKP-3 in liver cells and in the liver of lean mice (18, 19). In this study, we identified MKP-3 as a novel potentiating factor of Dex-induced hepatic lipogenesis and enlargement of fat mass. MKPs play important roles in cell proliferation and differentiation through regulation of MAP kinase signaling. Our work has connected MKP-3 to both glucose and lipid homeostasis in relation to GC treatment. Publications from other laboratories also show crosstalk between Dex and other MKPs. Dex has been reported to induce expression of MKP-1 and MKP-4, which are involved in Dex-repressed glucose uptake in 3T3-L1 adipocytes (31). Many publications show that the anti-inflammatory effect of Dex is partially mediated through induction of MKP-1 expression in vitro and in vivo (32-35). These results indicate that members of the dual specificity protein phosphatase family act as more than regulators of mitogenesis.

GR controls transcription of target genes both directly by interaction with glucocorticoid regulatory elements (GRE) and indirectly by cross-talking with other transcription factors such as FOXO1 and HNF4 α . GCs can also act through a variety of nongenomic signaling pathways (36-38). The effect of Dex on inducing MKP-3 expression requires the integrity of GR since the effect can be completely abolished by a GR antagonist. Dex treatment does not directly activate the -6.4kb MKP-3-Luc reporter construct and GRE is not found in the promoter of MKP-3 (data not shown). This result indicates that Dex indirectly induces MKP-3 expression. Several transcription factor binding sites have been identified in the promoter of MKP-3, including sites for forkhead transcription factors, the Ets family of transcription factors, NF- κ B, pre-B-cell leukaemia transcription factor 1-related homeobox factors, the sex-determining region Y-box containing factor SOX5, regulatory factor X1 and hepatic nuclear factor 1 (30). It has been reported that Dex activates FOXO1 transcription in β cells (39). Forkhead transcription factor binding site is present in MKP-3 promoter and our data indicate that Dex also induces FOXO1 expression in hepatoma cells. Therefore we focused on investigating the role of FOXO1 in regulating MKP-3 expression. We first

demonstrated that FOXO1 stimulates MKP-3 promoter transcription and overexpression of FOXO1 significantly increases endogenous MKP-3 expression in hepatoma cells. Consistent with these results, knockdown of FOXO1 or overexpression of FOXO1 256 significantly decreases MKP-3 protein level in hepatoma cells. Furthermore, Dex also induces FOXO1 protein expression in the liver of lean mice, which occurs prior to the upregulation of MKP-3 protein expression, and FOXO1 256 overexpression significantly decreases MKP-3 protein expression. These results suggest that FOXO1 is likely the mediator of Dex-induced MKP-3 expression in vitro and in vivo.

The effect of GC on promoting gluconeogenesis has been extensively studied and the underlying molecular mechanism is well established. In contrast, the mechanism of GC on inducing fatty liver has not been well characterized. Nonalcoholic fatty liver can be a result of excessive lipogenesis, decreased fatty acid oxidation, decreased secretion of VLDL particles, or a combination of more than one causes. It has been reported that GCs contribute to hepatosteatosis through a combination of increased fatty acid synthesis and decreased fatty acid β oxidation (40, 41). In our study, administration of Dex at 15mg/kg significantly increases hepatic TG content and expression of lipogenic genes. MKP-3 deficiency is sufficient to prevent mice from Dex-induced fatty liver. Respiratory exchange ratio and plasma TG levels are not altered in WT mice after 7-week treatment of Dex (data not shown), indicating unchanged whole body lipid oxidation. Therefore, Dex most likely promoted TG accumulation through de novo lipogenesis in the liver though we can't exclude the possibility of decreased hepatic fatty acid oxidation. This hypothesis is supported by increased lipogenic enzyme expression and absence of MKP-3 blunts the effect of Dex on promoting lipogenic enzyme expression. The reason that we did not observe decreased energy expenditure upon Dex treatment is perhaps we used a much lower dose than the previous publication, which administered mice at a dose of 100mg/kg (40).

In humans, it is well recognized that long term GC therapy induces body weight gain and insulin resistance (42). In our experiment, Dex treatment significantly increases body weight and adiposity as well as induces insulin resistance. In addition to fatty liver, MKP-3^{-/-} mice are also protected from developing the above-mentioned metabolic side effects induced by chronic Dex treatment. It seems that the dose of administration is important for the phenotype. One recent study treated mice with Dex at the dose of 5mg/kg for 7 weeks and reported no change of body weight in lean mice (43). The authors actually showed decreased body weight in high fat diet-fed mice after the 7-week treatment. In our hand, administration of Dex at 5mg/kg for seven weeks did not change body weight of lean mice either, which is consistent with literature.

In summary, we identified MKP-3 as a downstream component of Dex signaling. Many metabolic side effects associated with chronic Dex treatment can be attributed to induction of MKP-3 expression. The effect of Dex on inducing MKP-3 expression is dependent on FOXO1. Our results are highly interesting since the crosstalk between MKP-3 and Dex in regulation of lipid metabolism is a novel finding and the results may help to design therapeutic approaches to alleviate metabolic side effects of long term GC administration, particularly hepatosteatosis and central obesity.

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Abbreviations

MKP-3	MAP kinase phosphatase 3
Dex	Dexamethasone
FOXO1	forkhead box protein O1
GC	glucocorticoid
DUSP6	dual specificity protein phosphatase 6
MAPK	mitogen-activated protein kinase
ERK	extracellular signal-regulated kinase
PI3K	phosphoinositide 3-kinase
mTOR	mammalian target of rapamycin
DIO mice	diet-induced obese mice
VLDL	very low-density lipoprotein
WT	wild type
TG	triglyceride
PPARγ	peroxisome proliferator-activated receptor gamma
FAS	fatty acid synthase

SCD1	stearoyl-Coenzyme A desaturase 1
ACC1	acetyl-CoA carboxylase 1
ACC2	acetyl-CoA carboxylase 2

Highlights

- We examined the mechanism of Dex-promoted MKP-3 protein expression in cultured liver cells and in the liver of lean mice.
- We demonstrated that FOXO1 is required for Dex-induced MKP-3 protein upregulation.
- Global MKP-3 deficiency protected mice from developing Dex-induced metabolic disorders.

Figure 1. Dexamethasone increases the expression level of MKP-3 and FOXO1 protein in Hepa1-6 cells

A. MKP-3 and FOXO1 protein levels in Hepa1-6 cells treated with vehicle (veh) or dexamethasone (Dex). **B.** Quantification of immunoblots in A. The mean of three replicates was used and results shown are representative of three independent experiments. **C.** MKP-3 and FOXO1 protein levels in Hepa1-6 cells treated with Veh or Dex in the presence/absence of glucocorticoid receptor antagonist Ru486. **D.** Quantification of immunoblots in C. The mean of two replicates was used and results shown are representative of three independent experiments. **E.** FOXO1 activates MKP-3 promoter in Fao cells. The mean of three replicates was used and results shown are representative of three independent experiments. Luc, luciferase; MKP-3-Luc (mutant), six nucleotides were mutated in the forkhead binding element. **F.** Insulin represses the effect of FOXO1 on MKP-3 promoter in Fao cells. *, $P < 0.05$ as indicated; **, $P < 0.01$ as indicated.

Figure 2. FOXO1 is the mediator of Dex-induced MKP-3 protein expression in Hepa1-6 cells

A. The effect of FOXO1 overexpression on MKP-3 protein level. **B.** Quantification of A. **C.** Knocking down FOXO1 by interfering RNA attenuates Dex-induced MKP-3 protein expression. **D.** Quantification of C. **E.** The dominant negative FOXO1 (FOXO1 Δ 256) abolishes Dex-induced MKP-3 protein expression. **F.** Quantification of E. For 2B, 2D and 2F, the mean of two replicates was used and results shown are representative of three independent experiments. AdGFP, adenovirus expressing green fluorescent protein; AdFOXO1, adenovirus overexpressing FOXO1; AdshGFP, adenovirus overexpressing interfering RNA against GFP; AdshFOXO1, adenovirus overexpressing interfering RNA against FOXO1; Ad FOXO1 Δ 256, adenovirus overexpressing FOXO1 Δ 256. *, $P < 0.05$ as indicated; **, $P < 0.01$ as indicated.

Figure 3. Effect of Dex on MKP-3 protein expression in the liver of lean mice

A. MKP-3 protein levels in the liver of mice treated with Dex for 28 days (28D). **B.** Quantification of A (n=4 per group). **C.** Fasting plasma insulin levels of mice in A (n=4 per group). **D.** FOXO1 protein levels in the liver of mice (n=3 per group) treated with Dex for 4h, 8h and 28 days. **E.** Quantification of D at 8h time point (n=3 per group). **F.** Disruption of endogenous FOXO1 function decreases MKP-3 protein expression in the liver of lean mice (n=3 per group). **G.** Quantification of F (n=3 per group). Veh, vehicle; Dex, dexamethasone 15mg/kg; 4h, four hours; 8h, eight hours; 28D, 28 days. *, P<0.05 as indicated; **, P<0.01 as indicated; ***, P<0.001 as indicated.

Figure 4. MKP-3 deficiency renders mice resistant to Dex-induced body weight and tissue weight gain

A. Growth curves of wild type (WT) and MKP-3^{-/-} male mice upon 7-week of Dex treatment (n=4-5 per group). Dex treatment was initiated when mice were 9 weeks old. **B.** Growth curves of WT and MKP-3^{-/-} female mice upon 7-week of Dex treatment (n=3-4 per group). **C-D.** Weights of epididymal adipose tissue and liver from male mice after 7 weeks of Dex treatment (n=4-5 per group). **E.** Food intake of male mice after 7 weeks of Dex treatment (n=3-6 per group). **F.** Energy expenditure of male mice after 7 weeks of Dex treatment (n=3-6 per group). Veh, vehicle; Dex, Dexamethasone; \$, P<0.05; \$\$, P<0.01 WT Dex vs WT Veh. #, P<0.05; ##, P<0.01 WT Dex vs MKP-3^{-/-} Dex. *, P<0.05; **, P<0.01; ***, P<0.001 as indicated.

Figure 5. MKP-3 deficiency alleviates Dex-induced hepatosteatosis

A. Liver triglyceride contents of male mice after 7 weeks of Dex treatment (n=4-5 per group). **B.** Histology of livers from vehicle or Dex-treated wild type (WT) or MKP-3^{-/-} mice stained with oil red O. **C.** Liver glycogen contents of male mice after 7 weeks of Dex treatment (n=4-5 per group). **D.** Histology of livers from vehicle or Dex-treated wild type (WT) or MKP-3^{-/-} mice stained according to periodic acid schiff (PAS) protocol. *, P<0.05; **, P<0.01; ***, P<0.001 as indicated.

Figure 6. MKP-3 deficiency attenuates Dex-induced upregulation of genes involved in lipid synthesis

Hepatic mRNA levels of PPAR γ (A), FAS (B), SCD1 (C), ACC1 (D) and ACC2 (E) were measured by real-time PCR analysis and shown as the fold change relative to wild type mice treated with vehicle (n=4-5 per group). F. Hepatic protein levels of ACC2 and SCD1.

Tubulin was used as the loading control. *, P<0.05; **, P<0.01; ***, P<0.001 as indicated.

Figure 7. MKP-3 deficiency prevents mice from developing Dex-induced insulin resistance

A. Insulin tolerance test with mice treated with vehicle (Veh) or dexamethasone (Dex), n=5-6 per group. **B.** Insulin signaling in the liver of mice treated with vehicle (Veh) or dexamethasone (Dex). **C.** Quantification of B. \$, P<0.05; \$\$, P<0.01 WT Dex vs WT Veh. #, P<0.05; ##, P<0.01 WT Dex vs MKP-3^{-/-} Dex; *, P<0.05; **, P<0.01; ***P<0.001, as indicated.