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FOXO1-dependent up-regulation of MAP kinase phosphatase 3 (MKP-3) mediates glucocorticoid-induced hepatic lipid accumulation in mice

Bin Feng, Qin He, Haiyan Xu *

Hallett Center for Diabetes and Endocrinology, Rhode Island Hospital, Warren Alpert Medical School of Brown University, Providence, RI 02903, USA

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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).

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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 (Rhen and Cidlowski, 2005). 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 (Vegiopoulos and Herzig, 2007; Andrew et al., 2002). Elevated endogenous GCs cause Cushing syndrome and patients are also featured with metabolic side effects commonly found with prolonged GC therapy (Chanson and Salenave, 2010; Mazziotti et al., 2011). 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 (Kotronen et al., 2008; Marchesini et al., 1999; Nguyen-Duy et al., 2003). 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 (Camps et al., 2000). 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 (Camps et al., 2000; Dickinson and Keyse, 2006). 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 (Fjeld et al., 2000; Zhao and Zhang, 2001; Maillet et al., 2008). Growth

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.

* Corresponding author. Address: Division of Endocrinology, Warren Alpert Medical School of Brown University, 55 Claverick St., Rm 318, Providence, RI 02903, USA. Tel.: +1 401 444 0347; fax: +1 401 444 3784.

E-mail addresses: bfeng@lifespan.org (B. Feng), qinh123d@gmail.com (Q. He), hxu@lifespan.org (H. Xu).

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factors including insulin downregulate MKP-3 expression through a MEK/ERK dependent feed-forward mechanism (Feng et al., 2012; Jurek et al., 2009; Marchetti et al., 2005). 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 (Bermudez et al., 2008). 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 (Xu et al., 2005; Wu et al., 2010; Jiao et al., 2012). 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 (Wu et al., 2010; Daitoku et al., 2003; Puigserver et al., 2003). 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 (Nakae et al., 2001; Kamagate et al., 2008; Matsumoto et al., 2007, 2006). 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 (Daitoku et al., 2003). MKP-3 interacts with FOXO1 and promotes its nuclear translocation by dephosphorylation on serine 256 (Wu et al., 2010; Jiao et al., 2012). 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 (Wu et al., 2010).

Dex, a widely used synthetic GC, has been reported to increase FOXO1 expression in muscle and pancreatic β cells (Zhao et al., 2009; Smith et al., 2010; Chen et al., 2011). Interestingly, we found that Dex induces expression of both FOXO1 and MKP-3 in cultured rat hepatoma Fao cells (Xu et al., 2005; Wu et al., 2010). 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 Δ256 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 h followed by 2.5 μM Dex for 2 h. For Ru486 treatment, Hepa1-6 cells were incubated in serum-free medium for 16 h, pretreated with 10 μM Ru486 or DMSO for 1 h, then treated with 2.5 μM Dex or vehicle plus 10 μM Ru486 or DMSO for 2 h. 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 μl 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 1× Power SYBR® Green PCR Master Mix (Life Technologies), 300 nM forward primer, 300 nM reverse primer, and 20 ng cDNA sample. PCR conditions were: 50 °C for 2 min followed by 95 °C for 10 min for 1 cycle, and then 95 °C for 15 s followed by 60 °C for 1 min 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, TGTCTGAACCTGCGGTTCCT;
PPARγ forward, GGAAGACCACTCGCATTCCTT
PPARγ reverse, TCGCACTTTGGTATTCTTGGAG
FAS forward, GGCTCTATGGATTACCCAAGC;
FAS reverse, CCAGTGTTCGTTCCTCGGA;
SCD1 forward, CCTACGACAAGAACATTCAATCCC;
SCD1 reverse, CAGGAACTCAGAAGCCCAAAGC;
ACC1 forward, CGGACCTTTGAAGATTTTGTCAGG;
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 preclear lysates at 4 °C for 30 min. 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/1×TBST or 5% milk/1×TBST for 1 h 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

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