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MKP-2 Deficiency Leads to Lipolytic and Inflammatory Response to Fasting in Mice

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Abstract

The liver plays a crucial role in maintaining homeostasis for lipid and glucose. Hepatic lipid synthesis is regulated by nutritional signals in response to fasting and refeeding. It is known that overnutrition regulates MAPK-dependent pathways that control lipid metabolism in the liver by activating MAPK phosphatase-2 (MKP-2). Uncertainty still exists regarding the regulatory mechanisms and effects of MKP-2 on hepatic response to fasting. We investigated the effect of fasting on the expression of MKP-2 and the impact on hepatic inflammatory response to feeding a high-fat diet (HFD). In this study, we show that fasting stress led to an upregulation of hepatic MKP-2 expression and a corresponding decrease in phosphorylation of p38 MAPK in mouse livers. We discovered that hepatic steatosis brought on by fasting is not effective in MKP-2-deficient livers due in part to a decrease in lipolysis and GLUT2 expression. In response to refeeding a chow or HFD, MKP-2 exhibited differential regulation of hepatic inflammatory cytokines including IL-1β. It has been demonstrated that the mitochondrial carrier uncoupling protein 2 (UCP2) plays a significant role in immune function. We discovered that MKP-2 negatively controls the expression of the UCP2 protein in the liver, modulating the expression of inflammatory cytokines. These results lend credence to the idea that upregulation of MKP-2 is a physiologically relevant response and may help the liver better utilize hepatic lipids while fasting. Collectively, these findings show that MKP-2 modulates lipolysis and hepatic inflammatory response in response to alterations in nutritional status, such as excess nutrients and fasting.

Keywords: Cytokines, Fasting, Liver inflammation, Mitogen-activated protein kinase, Protein tyrosine phosphatase, Uncoupling protein-2

Introduction

The incidence of obesity is increasing worldwide, affecting ~33% of adult Americans [1,2]. Overnutrition and obesity are closely linked to a number of metabolic diseases including type 2 diabetes (T2D), atherosclerosis, heart disease, and fatty liver disease [2,3]. The causes of obesity are complex ranging from physiological, hereditary, social, and environmental factors [4]. Interventions against obesity such as exercise, genetic manipulations, and pharmacological treatments have limited effectiveness for many patients with obesity [5]. The imbalance of energy intake and expenditure contributes significantly to the development of obesity and is being affected by signals from many peripheral tissues [6]. Therefore, the identification of ways to improve major insulin-responsive tissue function to increase the pliability of these tissues in response to stress signaling may improve the pathogenesis of obesity and its associated disorders. Although many human studies have demonstrated the beneficial effects of fasting in improving obesity and its complications [5,7], the fundamental mechanisms are unclear. Dysregulated inflammatory responses are important components of overeating and obesity that induce injury in major insulin-responsive tissues including the liver [8]. Consumption of large amounts of

calories such as a high-fat diet leads to hyperglycemia and enhanced serum free fatty acids. The liver is a central organ impacted by inflammation that results from metabolic stress [9].

The liver plays a major role in glucose and lipid metabolism and is comprised of many different cells including hepatocytes, hepatic stellate cells, Kupffer cells, and cholangiocytes, and so on [10]. The liver is impacted by numerous types of stress including the western diet, exotoxins, and overuse of anti-inflammatory medication [9,10]. It has been reported that globally there are approximately two million deaths due to liver disease worldwide every year [9]. Liver injury activates the natural killer cells and Kupffer cells to release cytokines that play an important role in the adaptive immune response and stimulate the elimination of harmful pathogens [11]. However, lipid accumulation can induce liver damage and inflammation [12]. The mitogen-activated protein kinases (MAPKs), serine/threonine kinases expressed in the liver play an important role in regulating lipid accumulation and inflammation [13,14].

Studies have shown that in response to various hepatic cellular stresses, p38 MAPK promotes the progression of hepatic inflammation [13,14]. Overnutrition stimulates ERK1/2 pathway thereby stimulating the development of inflammation and fibrosis. It was reported that phosphorylated JNK1/2 plays a pro-inflammatory role in enhancing the production of inflammatory factors together with activation and infiltration of macrophages into the liver tissue [15]. The MAPK phosphatases (MKPs) counteract the MAPKs by direct dephosphorylation [16]. However, how the MKPs shift the balance towards MAPK phosphorylation to mediate the beneficial effects of fasting on obesity remains unclear.

In rodents, fasting has been reported to mitigate inflammation, fat mass and many components of metabolic disorders [17,18]. Intermittent fasting has been shown to decrease the expression levels of IL-6, IL-1β, and TNFα [17]. Other studies using the brain demonstrated that fasting and refeeding a high-fat diet reduces learning abilities by stimulating caspase-1 [19]. Whether fasting reduces and refeeding a high-fat enhances the activities of inflammatory mediators remains elusive [18]. It has been reported fasting induces differential regulation of p38 MAPK and ERK activation in different regions of the brain in response to energy metabolism [20]. On the other hand, fasting has been shown to increase the effectiveness of anti-cancer action of tyrosine kinase inhibitors by enhancing inhibition of MAPK signaling [21].

The goal of this study is to determine the effect of MKP-2 deficiency on hepatic metabolic response to fasting and refeeding-induced inflammatory conditions associated with poor eating habits. MKP-2 global knockout mice have been genetically characterized elsewhere [22].

Materials and Methods

Reagents and antibodies

All reagents were purchased from standard chemical vendors. The following antibodies phospho-p38 MAPK (#4511s), phospho-ERK1/2 (#9101s), p38 MAPK (#9228s), ERK1/2 (#4696s), beta-actin (#8457s), α-tubulin (#2125s), and UCP2 (#89326s), were obtained from Cell Signaling Technology. MKP-2 (#610850) was obtained from BD Biosciences.

Animal studies

The University of Alabama in Huntsville Institutional Animal Care and Use Committee approved all animal studies and experiments were conducted according to the NIH's Guidelines on the Use of Laboratory Animals. MKP-2 global knockout mice have been genetically characterized elsewhere [22]. These mice were kindly provided by Dr. Robin Plevin, University of Strathclyde, United Kingdom. The fasting and refeeding experiments included six to seven male *Mkp-2*$^{+/+}$ and *Mkp-2*$^{-/-}$ mice in each treatment group. The treatment groups included mice fed *ad libitum* with rodent lab diet (Lab supply, Nothlake, TX) or fasted for 48 h prior to sacrifice. For the refeeding experiments, *Mkp-2*$^{+/+}$ and *Mkp-2*$^{-/-}$ mice were fasted for 48 h and refed either a chow (custom low fat purified rodent diet) or high-fat diet (#112252, Dyets, Bethlehem, PA) for 2 h *ad libitum* prior to sacrifice. We used eight to twelve weeks old male mice for all the fasting and refeeding experiments. All mouse works were conducted between 7:00 AM (light cycle) and 1:00 AM (dark cycle), including fasting, refeeding, and sacking mice. Consumption of large amounts of calories such as a high-fat diet leads to hyperglycemia and enhanced serum free fatty acids. Also, generally, rodents have the propensity to break their fasting by overeating high-calorie diets [23].

Therefore, refeeding a high-fat diet stimulates systemic inflammation.

RNA extraction and real-time quantitative PCR analysis

RNA was isolated from the liver tissue of *Mkp-2*$^{+/+}$ and *Mkp-2*$^{-/-}$ mice using an RNeasy kit (Qiagen, CA, USA) according to the manufacturer's instructions. A total of 1 µg RNA was reverse transcribed to generate cDNA using a reverse transcriptase PCR kit (Applied Biosystems, CA, USA). Real-time quantitative PCR was performed in triplicate with the Applied Biosystems 7500 Fast RT-PCR system and, SYBR Green gene expression master mix with the following primer pairs: IL-1β, 5'-GACGGACCCCAAAAGATGAAGG-3' and 3'-GAGGTGCTGATGTACCAGTTGG-5'; GLUT2, 5'-ACGGT CTTCACGTTGGTCTC-3' and 3'-GAAGGCCACAAAGCCAAATA-5'; TLR2, 5'-TGGCTCAAATCCTGGTTGAC-3' and 3'-GAGAAGGG CACAGCAGACTC-5'; CXCL1, 5'-GCCTATCGCCAATGAGCTG-3' and 3'-GAACCAAGGGAGCTTCAGG-5'; CXCL10, 5'-ATCCCGAG CCAACCTTC-3' and 3'-CTTAGATTCCGGATTCAGACAT-5'; 18S,

5′-ACCGCAGCTAGGAATAATGGA-3′ and 3′-GCCTCAGTTCCGAA AACCA-5′.

All relative gene expression levels were analyzed using the ΔCt method and normalized to 18S. Gene expression master mix from Applied Biosystems were used. For genotyping, PCR analysis was employed using the following primers: WT forward primer 5′-CTTCAGACTGTCCCAATCAC-3′. WT reverse primer 5′-GACTCTGGATTTGGGGTCC-3′. KO forward primer 5′-TGACTAGGGGAGGAGTAGAAGGTGGC-3′. KO reverse primer 5′-ATAGTGACGCAATGGCATCTCCAGG- 3′.

Measurement of blood biochemical parameters and hepatic lipids

Male *Mkp-2*$^{+/+}$ and *Mkp-2*$^{-/-}$ mice aged between 8 to 12 weeks old were used for the measurement of fasting plasma glucose concentrations by a glucometer (CareTouch Blood Glucose Monitoring System). Hepatic triglycerides and alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined using colorimetric assay kits (Cayman Chemical, Michigan) according to the manufacturer's protocol.

Histological analysis of tissue samples

Liver tissues were isolated from male chow-fed and fasted male *Mkp-2*$^{+/+}$ and *Mkp-2*$^{-/-}$ mice and then fixed in 4% paraformaldehyde in PBS and processed for paraffin sections and stained with hematoxylin and eosin.

Immunoblotting

Tissues were isolated from male *Mkp-2*$^{+/+}$ and *Mkp-2*$^{-/-}$ mice at the end of the studies and snap frozen in liquid nitrogen and kept in – 80°C. Liver tissue was homogenized in RIPA buffer (25 mM Tris. HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% NP-40, 0.1 % SDS, 1.0 % sodium deoxycholic acid), supplemented with protease and phosphatase inhibitors (5 µg/ml leupeptin, 5 µg/ml aprotinin, 1 µg/ ml pepstatin A, 1 mM PMSF, 1 mM benzamidine, 1 mM Na_3VO_3, and 10 mM NaF). Homogenates were lysed for 30 min on the shaker at 4°C prior to clarification at 20,800 g for 30 min at 4°C. Protein concentrations were determined by Pierce BCA Protein Assay kit (Pierce, Rockford, IL) [22]. Protein lysates were resolved by SDS-PAGE and transferred to nitrocellulose membranes, and then incubated with phospho-specific antibodies followed by enhanced chemiluminescence or fluorescent detection. All immunoblotting bands were quantified using Bio-Rad ImageLab software.

Statistical analysis

All data represent the mean ± SEM. Differences between groups were assessed using a student's t-test or analysis of variance (ANOVA) with Bonferroni's post-test for multiple comparisons using GraphPad Prism 9.5.1 statistical software.

Results

Metabolic response to refeeding HFD in MKP-2-deficient mice after 48-h fast

Mkp-2$^{+/+}$ and *Mkp-2*$^{-/-}$ mice were fasted for 48 h and referred to either a chow or high-fat diet for 2 h to initially determine the effect of fasting and refeeding on body weight, liver weight, adipose tissue weight, spleen weight, and blood glucose levels. *Mkp-2*$^{-/-}$ mice exhibited comparable body weight in the fasted and refed for 2 h with HFD to those of *Mkp-2*$^{+/+}$ mice **(Figure 1A)**. *Mkp-2*$^{-/-}$ mice exhibited small but significant reduction in body weight in the fed state and when refed chow diet for 2 h compared with *Mkp-2*$^{+/+}$ mice. **(Figure 1A)**. The liver is the main component of the inflammatory response and the target organ for many dietary liver diseases [24]. The spleen is a key organ in controlling the immune response related to inflammation [25]. Further analysis showed that when fasted for 48 h, *Mkp-2*$^{-/-}$ mice displayed a small but significant decrease in liver weight compared with *Mkp-2*$^{+/+}$ mice **(Figure 1B)**. Because of the liver's function in metabolism, and considering that the *Mkp-2*$^{-/-}$ mice body's weight decreased under fasting (**Figure 1A**), their liver weights must equal in order for the liver to function properly. However, the liver weights between *Mkp-2*$^{+/+}$ and *Mkp-2*$^{-/-}$ mice were comparable under fed and 2 h chow or HFD refed conditions **(Figure 1B)**. No difference in blood glucose levels in the fed, fasted or 2 h refed chow or HFD in *Mkp-2*$^{+/+}$ and *Mkp-2*$^{-/-}$ mice **(Figure 1C)**. This is consistent with our previous studies, where chow-fed *Mkp-2*$^{+/+}$ and *Mkp-2*$^{-/-}$ mice exhibited comparable fasting blood glucose levels [26]. When fasted for 48 h, *Mkp-2*$^{-/-}$ exhibited a small but significant increase in spleen weight compared with wild type controls **(Figure 1D)**. This may be due to an increase in splenic proliferation. However, the spleen weights were similar in *Mkp-2*$^{+/+}$ and *Mkp-2*$^{-/-}$ mice in fed, fasted or 2 h refed chow or HFD states **(Figure 1D)**. Although *Mkp-2*$^{-/-}$ mice are leaner under fed and chow refed conditions **(Figure 1A)** this has no impact on white adipose tissue weight **(Figure 1E)**. These findings suggest that MKP-2 deficiency results in reduced body weight and 48 h of fasting did not exacerbate the phenotype.

Fasting increases *Mkp-2* expression in the livers of mice

By evaluating the levels of *Mkp-2* mRNA expression in the livers of mice using fasting- refeeding paradigm, changes in expression were measured to determine whether *Mkp-2* is metabolically regulated. **Figure 2A** shows an experiment in which *Mkp-2*$^{+/+}$ were fasted for 48 h and refed for 2 h with either a chow or HFD diet. Liver tissues were assayed by quantitative real-time PCR for *Mkp-2* mRNA expression. *Mkp-2* mRNA expression levels were low under fed conditions (**Figure 2A**). However, *Mkp-2* mRNA levels were significantly increased ~3 fold in liver after a 48-h fast (**Figure 2A**). Refeeding for 2 h with chow diet decreased *Mkp-2* mRNA to below prefasting levels

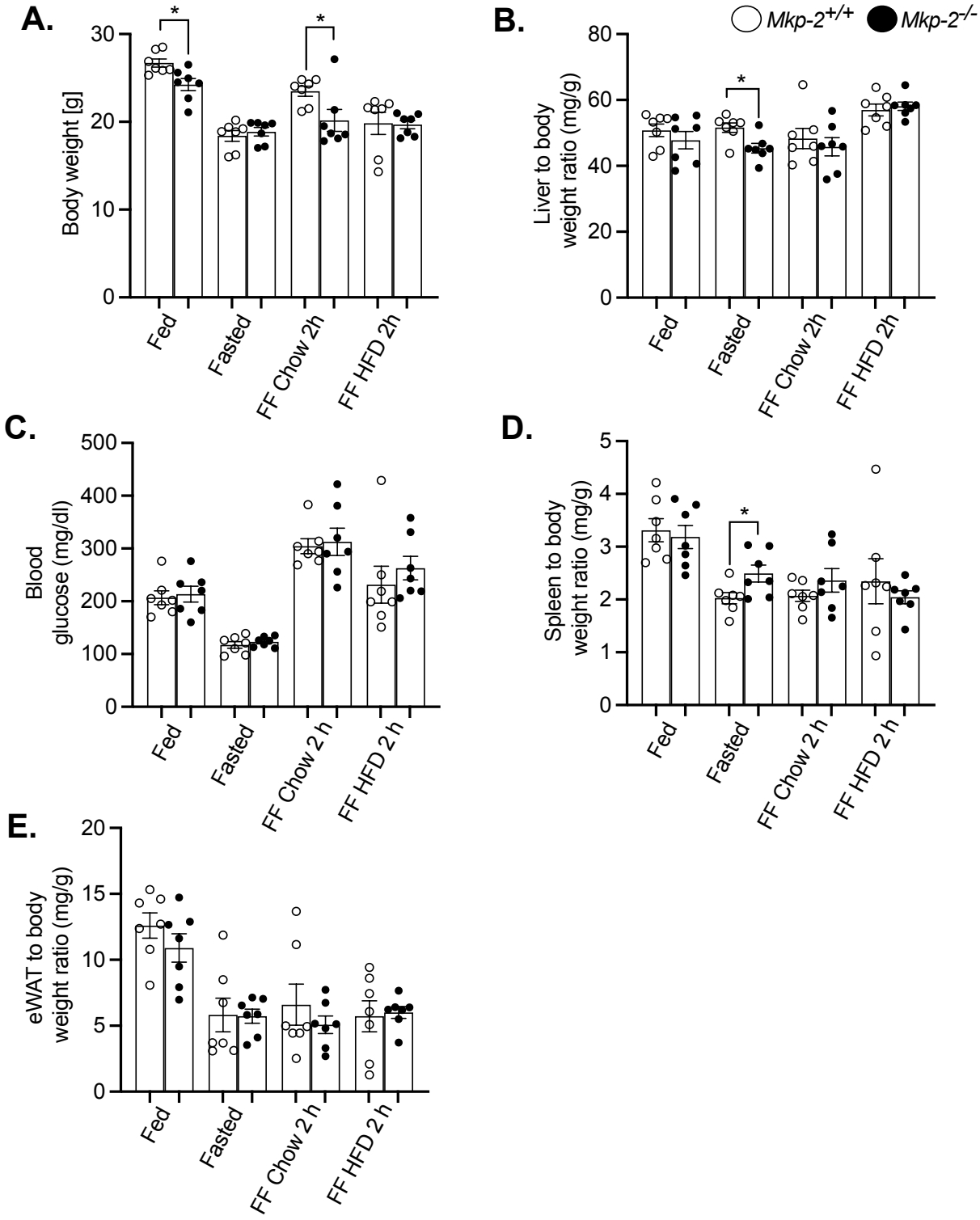

Figure 1. Metabolic response to refeeding a HFD in MKP-2-deficient mice after 48-h fast. (A) Body weight of fasted, chow- or high-fat diet fed male *Mkp-2*$^{+/+}$ and *Mkp-2*$^{-/-}$ mice (**B**) Liver to body weight ratio (**C**) Fasting blood glucose (**D**) Spleen to body weight ratio (**E**) WAT body weight ratio. *Mkp-2*$^{+/+}$ and *Mkp-2*$^{-/-}$ mice (n= 7 per genotype). Data are represented as mean ± SEM; *; p <0.05, as determined by analysis of variance (ANOVA) with Bonferroni's post-test for multiple comparisons. FF; Refed. Open bars, *Mkp-2*$^{+/+}$ mice; closed bars, *Mkp-2*$^{-/-}$ mice.

(**Figure 2A**). Interestingly, refeeding for 2 h with HFD after the 48-h fast increased hepatic *Mkp-2* mRNA levels above the fed state levels, however, this was not statistically significant (**Figure 2A**). To validate the MKP-2 mRNA data we examined the protein expression of MKP-2 using immunoblotting under the same conditions. Consistent with the MKP-2 mRNA expression, MKP-2 protein expression significantly increased ~2 fold in the liver after a 48-h fast (**Figures 2B and 2C**). These data confirmed the MKP-2 mRNA expression. Additionally, the phosphorylation of p38 MAPK decreases concurrently with the rise in *Mkp-2* levels during fasting **(Figures 2D and 2F).** These results demonstrate that *Mkp-2* is induced during

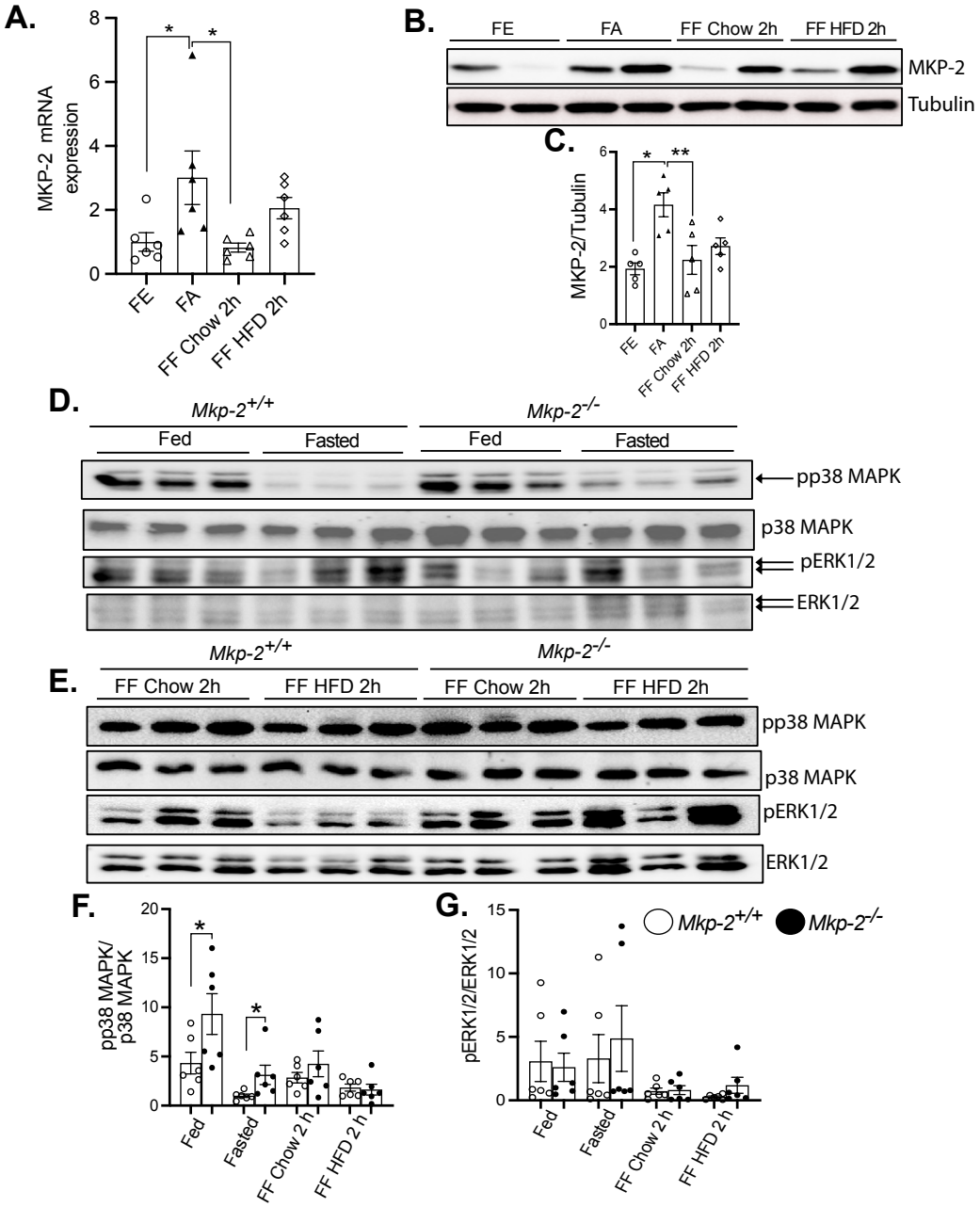

Figure 2. Fasting increases *DUSP4* (MKP-2) expression in the livers of mice. mRNA and protein expression of hepatic MKP-2 from fasted and chow- and high-fat diet refed wild type livers. Liver lysates from fasted and chow- and high fat diet fed *Mkp-2*$^{+/+}$ and *Mkp-2*$^{-/-}$ mice were analyzed by immunoblotting (**B, C, D, E, F and G**) MKP-2, pp38 MAPK, pERK1/2. Representative immunoblots were exposed to enhanced chemiluminescence (ECL) and quantitated by densitometry for the levels of MKP-2/β-actin (**B** and **C**) phospho-p38 MAPK/p38 MAPK, phospho-ERK1/2/ERK1/2. Results represent n = 5-6 per genotype and data shown are the mean ± SEM; *; p <0.05 as determined by analysis of variance (ANOVA) with Bonferroni's post-test for multiple comparisons. FE: Fed; FA: Fasted; FF: Refed. Open bars, *Mkp-2*$^{+/+}$ mice; closed bars, *Mkp-2*$^{-/-}$ mice.

fasting in the livers of wild type mice and is associated with down-regulation of the activity of p38 MAPK.

Since fasting increased the expression of *Mkp-2* in mouse livers, it was important to understand how fasting's metabolic response affected the activation of MAPKs. Immunoblot analysis confirmed the deletion of MKP-2 in a major metabolic organ, liver. As expected, *Mkp-2*$^{-/-}$ mice exhibited >80% reduction in MKP-2 protein in the liver (**Figure S1**). We fasted *Mkp-2*$^{-/-}$ and *Mkp-2*$^{+/+}$ mice for 48 h and refed for 2 h with either a chow or HFD diet. When MKP-2 levels rose in *Mkp-2*$^{+/+}$ mice while they were fasting, p38 MAPK and but not ERK phosphorylation also reduced at the same time (**Figures 2D-2G**). In the fed state, we found enhanced hepatic phosphorylation of p38 MAPK but not ERK in *Mkp-2*$^{-/-}$ compared with *Mkp-2*$^{+/+}$ mice (**Figures 2D-2G**). Refeeding a chow diet (2 h) after the 48 h fast returned hepatic phosphorylation of p38 MAPK in *Mkp-2*$^{-/-}$ mice to levels comparable to the non-fasted levels in *Mkp-2*$^{+/+}$ (**Figures 2D-2G**), however, ERK phosphorylation returned to levels above the non-fasted levels (**Figures 2D-2G**). Furthermore, there was no difference in p38 MAPK and ERK phosphorylation between the two genotypes after refeeding with chow diet (2 h) (**Figures 2D-2G**). After 48 h fast, refeeding a HFD for 2 h reduced hepatic phosphorylation of p38 MAPK below non-fasted levels in both *Mkp-2*$^{-/-}$ and *Mkp-2*$^{+/+}$ mice, however, ERK phosphorylation returned to above non-fasted levels (**Figures 2D-2G**). These findings show that fasting induces downregulation of hepatic p38 MAPK phosphorylation in *Mkp-2*$^{+/+}$ mice, which is consistent with elevated hepatic MKP-2 expression. It appears that the effect of MKP-2 on p38 MAPK phosphorylation is more pronounced under basal conditions compared with fasting, suggesting under fasting that there may be MKP-2-independent pathways that regulate p38 MAPK. This is the first study to demonstrate that stress brought on by fasting increased MKP-2 expression and MAPK regulation in mouse livers.

Resistance to fasting-induced hepatic steatosis in MKP-2-deficient mice

Studies have shown that fatty acids transported to the liver serve as a substitute source of energy during fasting [27]. We next examine the effects of MKP-2 deficiency on fasting-induced hepatic steatosis. We fasted *Mkp-2*$^{+/+}$ and *Mkp-2*$^{-/-}$ mice for 48 h. In the fed state, the livers of *Mkp-2*$^{-/-}$ and *Mkp-2*$^{+/+}$ mice were comparable (**Figure 3A, left panel**). Interestingly, after 48 h fast, the livers of *Mkp-2*$^{-/-}$ mice displayed protection from the development of fasting-induced hepatic steatosis compared with *Mkp-2*$^{+/+}$ control mice as analyzed by hematoxylin and eosin staining (**Figure 3A, right panel**). This is consistent with reduced liver weight in *Mkp-2*$^{-/-}$ mice (**Figure 1B**). Consistent with protection from hepatic steatosis, hepatic triglycerides (TGs) were significantly reduced in *Mkp-2*$^{-/-}$ compared with *Mkp-2*$^{+/+}$ mice (**Figure 3B**). To determine the effects of fasting on liver injury in MKP-2-deficient mice, we examined the serum levels of AST and ALT. In the fed state, *Mkp-2*$^{-/-}$ exhibited reduced AST and ALT compared with *Mkp-2*$^{+/+}$ mice (**Figures 3C and 3D**). Interestingly, fasted *Mkp-2*$^{-/-}$ mice displayed a dramatic reduction in serum levels of AST (~50%) and ALT (~70%) compared with *Mkp-2*$^{+/+}$ mice (**Fig. 3C and D**). This is consistent with protection from hepatic steatosis observed in *Mkp-2*$^{-/-}$ mice. GLUT2 is the principal glucose transporter in humans and mice [28]. To determine the effect of MKP-2 deficiency on glucose uptake in the liver we measured the gene expression of GLUT2. In the fed state, *Mkp-2*$^{-/-}$ and *Mkp-2*$^{+/+}$ mice showed comparable GLUT2 mRNA expression levels in the liver (**Figure 4A**). However, in the fasted state *Mkp-2*$^{-/-}$ exhibited significantly reduced hepatic GLUT2 mRNA expression levels compared with *Mkp-2*$^{+/+}$ controls (**Figure 4A**). This suggests that the uptake of newly synthesized glucose in *Mkp-2*$^{-/-}$ mice was reduced thereby limiting substrates for hepatic lipid synthesis and hence protection from fat accumulation in the liver.

One of the earliest responses of lipid metabolism to fasting is peripheral lipolysis, which explains the elevated serum levels of fatty acids [29]. A key regulator of lipolysis, the LIPE gene encodes hormone-sensitive lipase (HSL) [30]. The expression of LIPE mRNA in the EWAT of fed and fasted *Mkp-2*$^{-/-}$ and *Mkp-2*$^{+/+}$ mice was measured using quantitative real- time PCR analysis in order to evaluate the lipolytic activity in adipose tissue. The weight of EWAT in fed and fasted *Mkp-2*$^{-/-}$ and *Mkp-2*$^{+/+}$ mice were comparable (**Figure 1E**). The adipose LIPE mRNA levels in the fed state in *Mkp-2*$^{+/+}$ and *Mkp-2*$^{-/-}$ mice were comparable (**Figure 4B**). However, after a 48 h fast, LIPE mRNA levels significantly increase in *Mkp-2*$^{+/+}$ mice, however, in *Mkp-2*$^{-/-}$ mice, LIPE expression was significantly reduced compared with *Mkp- 2*$^{+/+}$ controls (**Figure 4B**) suggesting reduced adipose lipolysis. Consequently, *Mkp-2*$^{-/-}$ mice exhibit protection from fasting-induced lipolysis (**Figure 3**). Next, we examined adipose triglyceride lipase (ATGL) known to be a critical regulator of lipolysis. The adipose ATGL mRNA levels in the fed state in *Mkp-2*$^{+/+}$ and *Mkp-2*$^{-/-}$ mice were comparable (**Figure 4C**).

Consistent with the LIPE data, after 48 h fast, the expression of adipose ATGL was significantly reduced in *Mkp-2*$^{-/-}$ mice compared with *Mkp-2*$^{+/+}$ counterparts (**Figure 4C**). We further examined the PLIN1 adipose mRNA levels. The adipose PLIN1 mRNA levels in the fed state in *Mkp-2*$^{+/+}$ and *Mkp-2*$^{-/-}$ mice were comparable **(Figure 4D)**. We found that PLIN1 was significantly reduced in *Mkp-2*$^{-/-}$ mice compared with *Mkp-2*$^{+/+}$ mice after 48 h fast (**Figure 4D**). These findings demonstrate that MKP-2 deficient are protected from fasting-induced hepatic steatosis and that decrease in expression of key adipose lipolytic enzymes and hepatic GLUT2 contributes to the reduced fat accumulation in the liver and MKP-2 in part is an important regulator of these lipolytic genes.

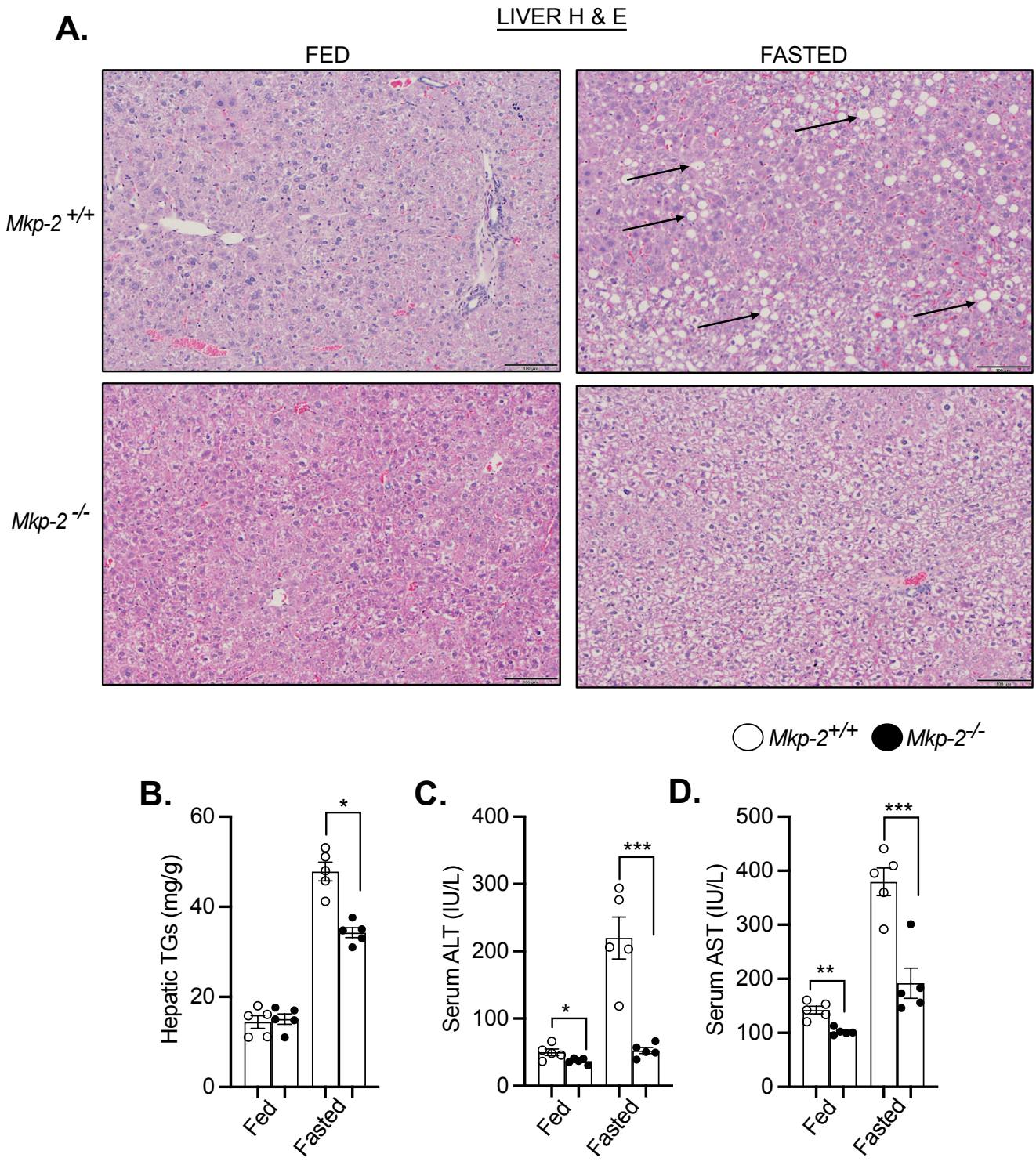

Figure 3. Protection from fasting-induced hepatic steatosis in MKP-2-deficient mice. Representative hematoxylin and eosin staining of liver sections of chow-fed (left panel) and fasted (right panel) *Mkp-2*$^{+/+}$ and *Mkp-2*$^{-/-}$ mice (**A**) (n = 5 per genotype). Arrows show lipid droplets. (100 x Magnification). (**B**) Hepatic triglycerides (TG) (**C**) Serum alanine amino- transferase (ALT), (**D**) Serum aspartate aminotransferase (AST) from chow-fed and fasted *Mkp-2*$^{+/+}$ and *Mkp-2*$^{-/-}$ mice (n = 5 per genotype). Data shown are the mean ± SEM; *; p <0.05,**; p <0.01, ***; p <0.0001, as determined by analysis of variance (ANOVA). Open bars, *Mkp-2*$^{+/+}$ mice; closed bars, *Mkp-2*$^{-/-}$ mice.

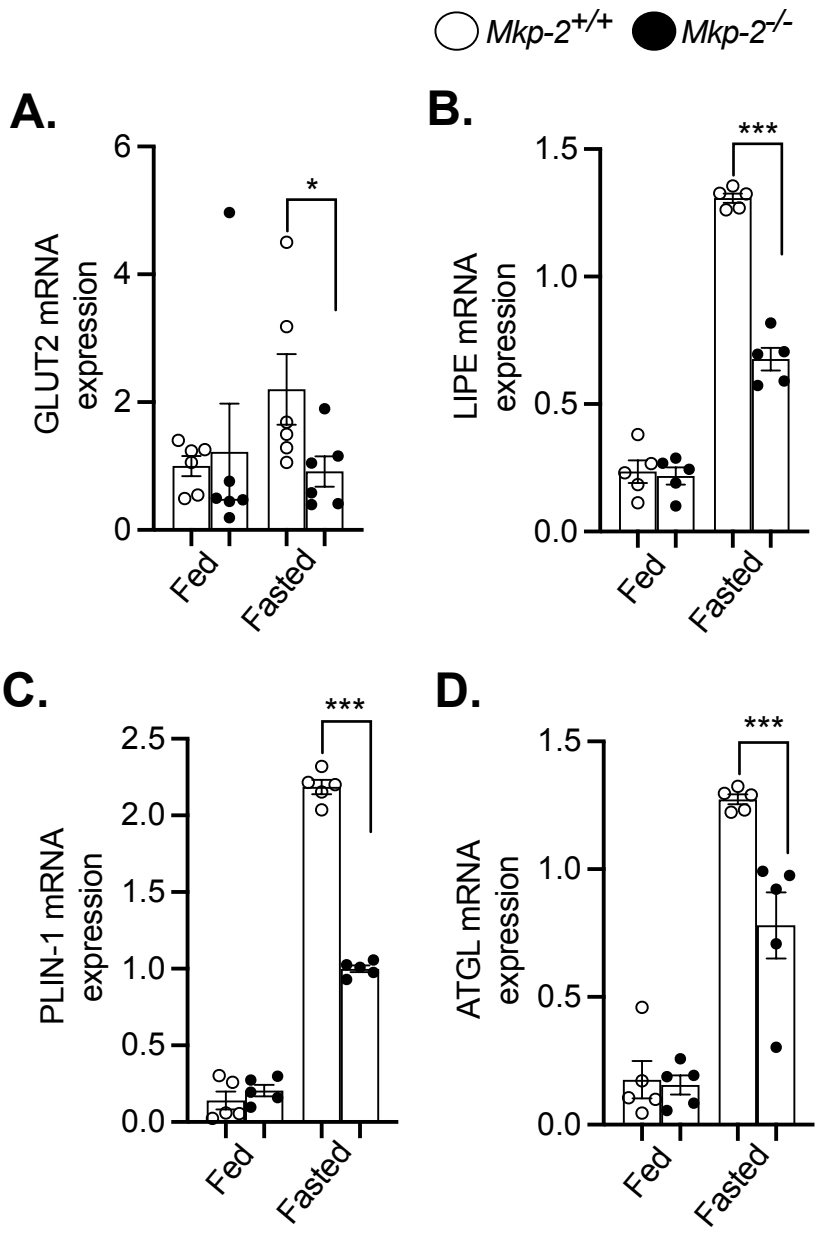

Figure 4. Reduced fasting-induced lipolysis in MKP-2-deficient mice. Hepatic mRNA expression of GLUT2 **(A)**, Adipose mRNA expression of LIPE **(B)**, PLIN1 **(C)** and ATGL **(D)** from chow-fed and fasted *Mkp-2*$^{+/+}$ and *Mkp-2*$^{-/-}$ mice (n = 5 per geno- type). Data shown are the mean ± SEM; *; p <0.05, **; p <0.01, ***; p <0.0001, as determined by analysis of variance (ANOVA). Open bars, *Mkp-2*$^{+/+}$ mice; closed bars, *Mkp-2*$^{-/-}$ mice.

Altered hepatic inflammatory gene expression in response to refeeding a HFD in MKP-2-deficient mice

Next, we sought to determine the effects of refeeding a chow or HFD on pro- and anti- inflammatory gene expression in the livers of MKP-2-deficient mice. Interleukin 1 beta (IL-1β) is involved in many inflammatory diseases including obesity and liver diseases [31]. One of the major families of pattern recognition receptors, toll-like receptors (TLRs) trigger signaling cascades that control inflammatory responses [32]. Chemokines play a part in coordinating the influx of immune cells to the site of damage or inflammation [33]. We fasted *Mkp-2*$^{+/+}$ and *Mkp-2*$^{-/-}$ mice for 48 h. The levels of IL-1β was decreased in the fasted state compared with fed conditions in the livers of *Mkp-2*$^{+/+}$ mice (**Figure 5A**). However, in the fasted state, hepatic IL-1β mRNA levels were significantly increased in *Mkp-2*$^{-/-}$ mice compared with *Mkp-2*$^{+/+}$ mice (**Figure 5A**). Upon 2 h refed with chow or HFD, the livers of *Mkp-2*$^{-/-}$ mice

displayed significantly elevated levels of IL-1β compared with *Mkp-2*$^{+/+}$ mice (**Figure 5A**). The hepatic TLR2 mRNA levels in the fed, fasted or 2 h refed chow in *Mkp-2*$^{+/+}$ and *Mkp-2*$^{-/-}$ mice were comparable **(Figure 5B)**. However, following 2 h refed with HFD the levels of hepatic TLR2 mRNA levels were enhanced compared with prefasting levels and 2 h chow refed groups in the *Mkp-2*$^{+/+}$ mice **(Figure 5B)**. Although the hepatic TLR2 mRNA levels were reduced in *Mkp-2*$^{-/-}$ mice after refeeding with HFD for 2 h but this was not significant compared with *Mkp-2*$^{+/+}$ mice **(Figure 4B)**. Next, we examined the CXC chemokine family of proinflammatory mediators. Our analysis revealed that in the fed, fasted or 2 h refed with chow the mRNA levels of CXCL1 and CXCL10 were comparable in the livers of *Mkp-2*$^{+/+}$ mice **(Figures 5C and 5D)**. However, refeeding with a HFD decreased CXCL1 expression in *Mkp-2*$^{+/+}$ mice **(Figure 5C)**. The livers of *Mkp-2*$^{-/-}$ mice tended to reduce CXCL10 expression in the fed, fasted and 2 h refed with HFD compared with *Mkp-2*$^{+/+}$ mice (**Figure 5D**). These findings imply that in response to refeeding a chow or HFD, MKP-2 exhibits differential regulation of hepatic inflammatory cytokines.

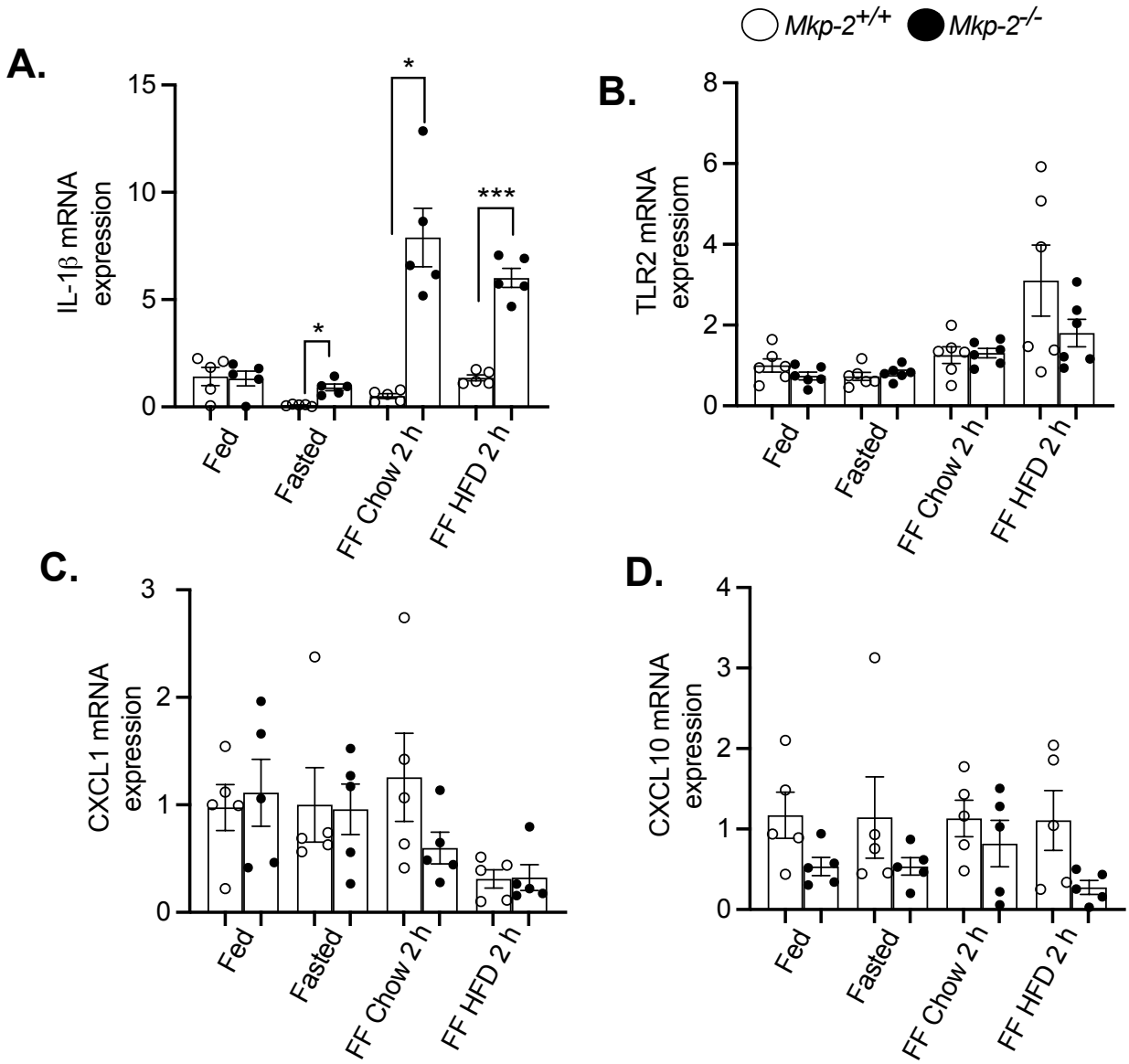

Figure 5. Altered expression of hepatic inflammatory genes in response to refeeding a HFD in MKP-2-deficient mice after 48-h fast. mRNA expression of hepatic inflammatory genes from fasted and chow- and high-fat diet refed *Mkp-2*$^{+/+}$ and *Mkp-2*$^{-/-}$ mice, IL-1β (**A**), TLR2 (**B**), CXCL1 (**C**) CXCL10 (**D**) mRNA. Results represent n = 5-6 per genotype and data shown are the mean ± SEM; *; p <0.05, ***; p <0.0001 as determined by analysis of variance (ANOVA) with Bonferroni's post-test for multiple comparisons. FF; Refed. Open bars, *Mkp-2*$^{+/+}$ mice; closed bars, *Mkp-2*$^{-/-}$ mice.

MKP-2 deficiency reduced uncoupling protein-2 to mediate the altered expression of inflammatory cytokines

In order to understand the mechanism of altered expression of inflammatory cytokines in *Mkp-2*$^{-/-}$ mice, we determined the hepatic protein expression of uncoupling protein-2 (UCP- 2), a mitochondrial carrier protein and key player in stimulation of immune cells [34]. We fasted *Mkp-2*$^{+/+}$ and *Mkp-2*$^{-/-}$ mice for 48 h and refed a chow or HFD for 2 h. In the fed, fasted, and 2 h chow or HFD refed states, we found comparable levels of UCP-2 protein expression levels in the livers of *Mkp-2*$^{+/+}$ mice **(Figures 6A-6C)**. Interestingly, *Mkp-2*$^{-/-}$ mice exhibited significantly reduced hepatic UCP-2 protein expression levels in the fed, fasted, and 2 h HFD refed compared with *Mkp-2*$^{+/+}$ mice **(Figures 6A-6C)**. Many cell types secrete cytokines and various studies demonstrated that this is dependent on the levels of UCP2 expression in these cells [35]. UCP2 knockout mice exhibited enhanced serum levels of IL-1β, IL-6 and interferon-γ (IFN-γ) in response to listerial infection [35]. In this study, we showed that increased IL-1β expression in the livers of *Mkp-2*$^{-/-}$ (**Figure 5A**) that is consistent with reduced UCP2 expression. Other studies have demonstrated that UCP2 knockout displayed enhanced resistance to bacterial infection [35]. These results indicate that MKP-2 modulates the expression of pro- and anti-inflammatory cytokines by negatively regulating UCP2 protein expression in the liver.

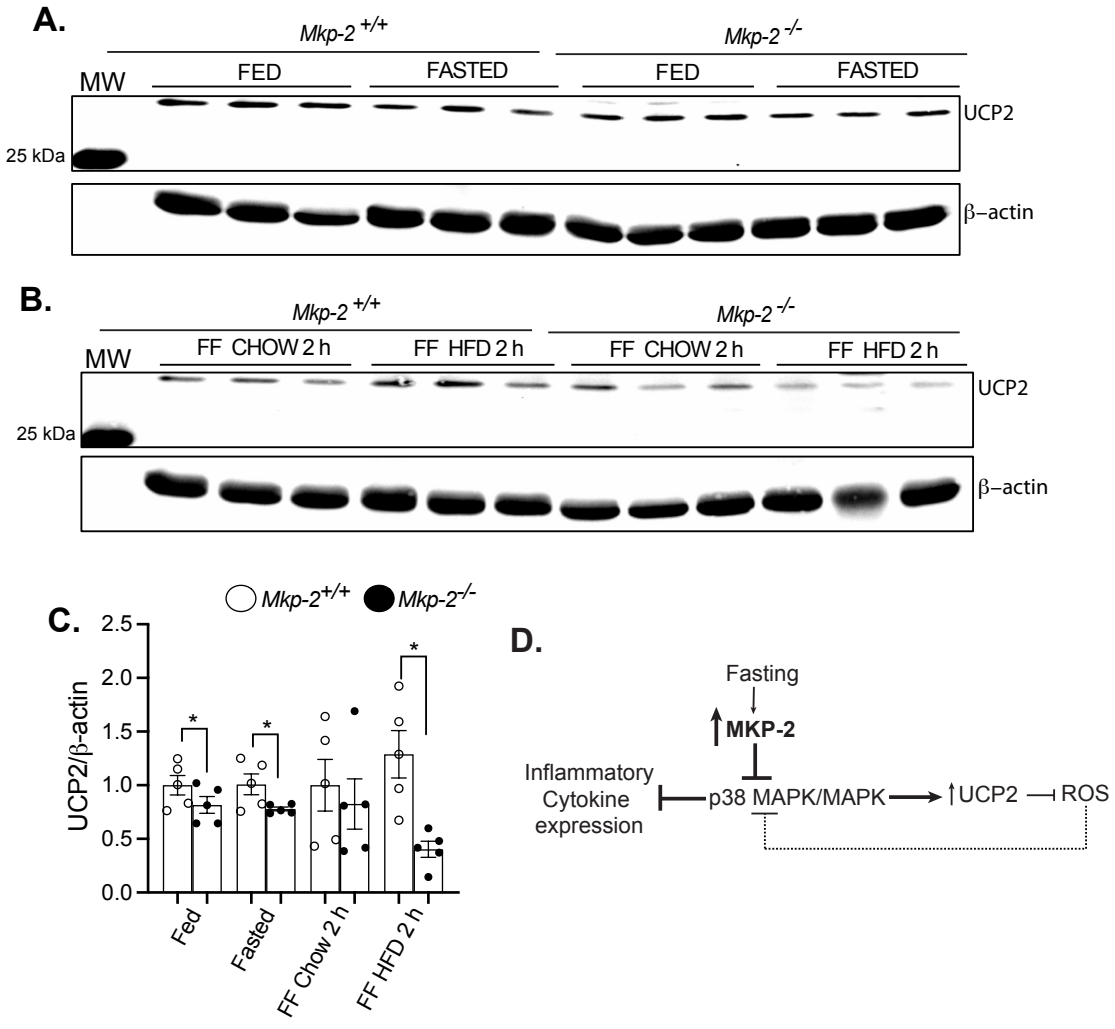

Figure 6. Reduced hepatic protein expression of UCP2 in response to refeeding a HFD in MKP-2-deficient mice after 48-h fast. Liver lysates from fasted and chow- and high-fat diet refed *Mkp-2*$^{+/+}$ and *Mkp-2*$^{-/-}$ mice were analyzed by immunoblotting (**A and B**). Immunoblots were determined using fluorescent imaging and quantitated by densitometry for the levels of UCP2/β-actin (**C**). In the liver, fasting activates MKP-2 expression which serves to regulate p38 MAPK/UCP2-dependent pathways to control inflammatory cytokine expression. Activated p38 MAPK decreases UCP2 expression. As a feedback mechanism, UCP2 down-regulation enhances ROS-dependent p38 MAPK activation (**D**). Results represent n = 5 per genotype and data shown are the mean ± SEM; *; p <0.05, as determined by analysis of variance (ANOVA) with Bonferroni's post-test for multiple comparisons. FF; Refed. Open bars, *Mkp-2*$^{+/+}$ mice; closed bars, *Mkp-2*$^{-/-}$ mice.

Discussion

Previous work demonstrated that MKP-2 is upregulated in major insulin-responsive tissues in response to excess nutrients and plays an important role in liver metabolism [26]. Here, we evaluated the effect of MKP-2 deficiency on hepatic metabolic and inflammatory responses using fasting-refeeding paradigm. In this study fasting induced a significant increase in the expression of Mkp-2 mRNA and protein, while refeeding with chow diet significantly attenuated the expression compared with HFD. After fasting, hepatic p38 MAPK phosphorylation was downregulated, which is consistent with increased *Mkp-2* expression. Few other studies have demonstrated elevated ERK and p38 MAPK phosphorylation in the paraventricular nucleus and increased ERK phosphorylation in the hypothalamic arcuate nucleus of fasted wild type mice. Similar to this, in rat hepatocytes and macrophages, fasting led to ERK and JNK activation. Together, these findings show that fasting stress causes the expression of MKP-2 in the liver as well as selective activation and/or downregulation of p38 MAPK, ERK, and JNK in various tissues and cell types.

Although the immune system responds to insults by injury or infection, there are studies that suggest excess nutrients stimulate the immune system in metabolically active tissues such as the liver as part of the physiological response [7,36,37]. Also, it has been shown that MKP-2 is a negative regulator of JNK and p38 MAPK in macrophages and that it inhibits the expression of proinflammatory cytokines in response to LPS [38]. Our studies demonstrate that fasting activates MKP-2 expression that plays an important role in modulating the innate immune response by regulating the hepatic gene expression of inflammatory cytokines including IL-1β (**Figure 5A**). Interestingly, *Mkp-2*$^{-/-}$ mice exhibited reduced protein levels of UCP2 in the liver, which prompts us to discover a pathway by which MKP-2 negatively regulates IL-1β by antagonizing UCP2 in response to fasting and refeeding. Refeeding with either chow or HFD upregulated hepatic IL-1β expression in *Mkp-2*$^{-/-}$ mice. In addition, some studies have reported that fatty acids induce UCP2 expression [39,40] and here we showed reduced accumulation of fat in the fasted livers of *Mkp-2*$^{-/-}$ mice that is associated with decreased UCP2 protein expression. The mechanisms of how UCP2 increases IL-1β expression remain unclear. In this study we demonstrated for the first time that downregulation of UCP2 protein expression upregulated IL-1β levels in the liver suggesting that MKP-2 in part mediates the effects of UCP2 on IL-1β expression. Refeeding with an HFD-induced increased hepatic TLR2 expression in *Mkp-2*$^{+/+}$ mice in comparison with *Mkp- 2*$^{-/-}$ mice refed with a chow diet. **Table 1** showed that HFD contained higher cornstarch compared with chow diet in **Table 2**. This is consistent with a previous study that showed enhanced hepatic TLR2 in response to dietary carbohydrate [41]. However, this was attenuated in MKP-2 deficient mice. Similarly, TLR2 knockout mice exhibited reduced hepatic CXCL1 and CXCL10 [41] and this is consistent with decreased CXCL1 and CXCL10 in MKP-2 deficient mice [31]. It has been shown that activation

Table 1. The nutrient content of high-fat diet used in the study.

Ingredient	kcal/gm	grams/kg	kcal/kg
Casein	3.58	200	716.00
L-Cystine	4	3	12
Sucrose	3.6	68.8	275.20
Cornstarch	3.6	125	475.00
Dyetrose	3.8	125.00	475.00
Soybean Oil	9	25	225.00
t-Butylhydroquinone	0	0.005	0
Cellulose	0	50	0
Lard	9	245	2205.00
Vitamin Mix #300050	3.92	10	39.20
Choline Bitartrate	0	2	0
Salt Mix #210088	1.6	10	16.00
Dicalcium Phosphate	0	13	0.00
Calcium Carbonate	0	5.5	0.00
Potassium Citrate H20	0	16.5	0.00
		773.805	**3963.400**

Table 2. The nutrient content of chow diet used in the study.

Ingredient	kcal/gm	grams/kg	kcal/kg
Casein	3.58	200	716
L-Cystine	4	3	12
Sucrose	4	350	1400
Cornstarch	3.6	315	1134
Dyetrose	3.8	35	133
Soybean Oil	9	25	225
t-Butylhydroquinone	0	0.005	0
Lard	9	20	180
Cellulose	0	50	0
Mineral Mix #210088	1.6	10	16
Dicalcium Phosphate	0	13	0
Calcium Carbonate	0	5.5	0
Potassium Citrate H2O	0	16.5	0
Vitamin Mix # 300050	3.92	10	39.2
Choline Bitartrate	0	2	0
		1055.005	**3855.2**

of p38 MAPK in macrophages in response to LPS decreased UCP2 expression and increased production of mitochondrial ROS [42]. In a model of taxol-induced mitochondrial stress in human melanoma cells, another study showed enhanced p38 MAPK activation-mediated increase in mitochondrial ROS production [43]. Our data suggest a model in which fasting activates MKP-2 expression in the liver, which controls the expression of inflammatory cytokines via p38 MAPK/UCP2-dependent pathways (**Figure 6D**).

Metabolic syndrome is closely associated with the development of hepatic steatosis [3,44]. The dysregulation of fatty acid metabolism by the liver is mainly responsible for the accumulation of fat in the liver. Our data demonstrate that MKP-2-deficient mice were protected from fasting-induced hepatic steatosis. Furthermore, reduced liver damage was observed in fasted MKP-2-deficient mice as evident by lower serum ALT and AST levels. Our results reveal that changes in hepatic fat metabolism that resulted in resistance to hepatic steatosis in MKP- 2-deficient mice impact glucose uptake in the liver. *Mkp-2*$^{-/-}$ mice exhibited reduced GLUT2 hepatic gene expression, a key glucose transporter in hepatocytes. This suggests that the reduced accumulation of fat in the liver stimulated decreased glucose uptake through reduced GLUT2 expression thereby limiting glucose as substrate for processes such as lipogenesis.

More importantly, *Mkp-2*$^{-/-}$ mice exhibited reduced adipose expression of key lipolytic genes LIPIE, ATGL and PLIN1. The decreased lipolysis in fasted *Mkp-2*$^{-/-}$ mice results in reduced hepatic absorption of fatty acids, which explains protection in hepatic steatosis. All of the research done points to the possibility that blocking MKP-2 could offer a fresh approach to treating metabolic disease. Anti-sense strategies for targeted inhibition of hepatic MKP-2 in obese patients may be useful in treating hepatic steatosis. Whether MKP-2 is overexpressed in human type 2 diabetes is still unknown. We showed recently that hepatic MKP-2 protein expression levels are upregulated in obese/NASH humans [26], indicating that there is a relationship between MKP-2 expression and fat mass. Furthermore, it is unclear which tissue serves as a major conduit for MKP-2 impact on metabolism. Metabolic analysis of MKP-2 tissue-specific deleted mice will be necessary for future work.

Conclusions

Our findings showed that the hepatic inflammatory response to fasting is altered by MKP-2 deficiency. These results lend credence to the idea that upregulation of MKP-2 is a physiologically relevant response and may help the liver better utilize hepatic lipids while fasting. According to these findings, MKP-2 expression is increased in response to alterations in nutritional cues, such as fasting and an abundance of nutrients. Upregulation of MKP-2 while fasting would enhance lipolysis and promote the development of fatty liver disease. This would offer therapeutic intervention strategies for the management of fatty liver disease.

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Disclosures

No potential conflicts of interest relevant to this article were reported.

Author Contributions

A.L. conceived, designed, and wrote the manuscript. S.J, G.W., J.S., I.S, N.G., C.S., M.W., and U.P. performed experiments. S.J. and A.L. analyzed data. K.M. edited, designed, and revised the manuscript.

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