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Yanthrawaduge Savanie Sulakshana Devisirini Fernando

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ROLE OF MKP-2 IN GLUCOSE METABOLISM AND STRESS-INDUCED INSULIN RESISTANCE

by

YANTHRAWADUGE SAVANIE SULAKSHANA DEVSIRINI FERNANDO

A THESIS

Submitted in partial fulfillment of the requirements for the degree of Master of Science in
The Department of Biological Sciences to
The School of Graduate Studies of
The University of Alabama in Huntsville

HUNTSVILLE, ALABAMA

2021
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THESIS APPROVAL FORM

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We, the undersigned members of the Graduate Faculty of The University of Alabama in Huntsville, certify that we have advised and/or supervised the candidate on the work described in this dissertation. We further certify that we have reviewed the dissertation manuscript and approve it in partial fulfillment of the requirements for the degree of Master of Science in Biology.

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ABSTRACT

The School of Graduate Studies
The University of Alabama in Huntsville

Degree Masters College Biological Sciences.

Name of Candidate Yanthrawaduge Savanie Sulakshana Devisirini Fernando.

Title Role of MKP-2 in glucose metabolism and stress-induced insulin resistance

The function of MKP-2 in metabolism and metabolic diseases remains largely unknown. In obese conditions mitogen-activated protein kinase (MAPK) pathway get dysfunctional and MAP kinase phosphatases (MKPs) play major roles in regulating the activities of MAPKs. We examined the regulation of MKP-2 expression in vivo, under physiological and pathological conditions, in the liver and other insulin responsive tissues using diet-induced obesity models using novel MKP-2 knockout mice. Also, we examined MKP-2 expression in vitro using insulin sensitive cell lines. We found that MKP-2 is expressed in insulin-sensitive tissues/cells and overexpression may delay muscle cell differentiation. Our novel data demonstrate that under high fat-diet feeding conditions MKP-2 knockout mice are insulin sensitive and resistant to diet-induced obesity compared to MKP-2 wild type mice. We demonstrate that, in obese conditions, MKP-2 is overexpressed in the liver resulting in inactivation of MAPKs and thereby promoting development of obesity, fatty liver and metabolic dysfunction.

Abstract Approval: Committee Chair

Department Chair Paul G. Wolf 5/18/2021

Graduate Dean 5/19/2021
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<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinases 1 and 2</td>
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<td>HFD</td>
<td>High fat diet</td>
</tr>
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<td>JNK</td>
<td>c-Jun-N-Terminal kinases 1,2,3</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
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<tr>
<td>NAFLD</td>
<td>Non-alcoholic fatty liver disease</td>
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<tr>
<td>PTKs</td>
<td>Protein tyrosine kinases</td>
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<tr>
<td>PTPs</td>
<td>Protein tyrosine phosphatases</td>
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<tr>
<td>WT</td>
<td>Wild type</td>
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DEDICATION

I would like to thank my family and friends for the constant moral support, love and care extended towards me. I want to especially thank my hardworking and loving parents, Sunil Fernando and Lathika Perera, and my brother, Saveen Fernando for guiding me through life’s toughest obstacles with love and care. I express my special thanks and gratitude to my husband, Pemith Mendis for the emotional support, encouragement, and for pushing me to be the best I can be. Also, my son, Jaylon Mendis for all the love and care given during this time. Most importantly, I thank the Almighty God for the strength, guidance and countless blessings bestowed on me.

This thesis is dedicated to my Grandfather, Yanthrawaduge John Luther Fernando, who laid the cornerstone to my education career by inspiring me to achieve great heights.
CHAPTER ONE

INTRODUCTION

1.0 Introduction

1.1 Obesity

The prevalence of obesity is increasing worldwide and has led to increased risk for cardiovascular disease, type 2 diabetes, non-alcoholic fatty liver disease, and even cancers (Roth Flach and Bennett, 2010). There is substantial amount of evidence in the literature which shows a correlation between high fat intake and obesity in both mice and humans (Ozdener et al., 2014; Daoudi et al., 2015; Sayed et al., 2015; Khan et al., 2017). Control of energy intake and expenditure contributes significantly to the development of obesity and is being affected by signals from many peripheral tissues. Consequently, a wide range of peripheral signals originating from different organs contributes to the regulation of body weight and energy expenditure. Thus, many studies are ongoing to identify molecules that participate in the regulation of metabolism. Mitogen-activated protein kinases (MAPKs) are established physiological regulators of metabolic homeostasis and MAPK dysfunction promotes metabolic disease (Manieri and Sabio, 2015). Under pathophysiological conditions such as obesity it has been shown that the activity of MAPKs become dysfunctional (Lawan and Bennett, 2017).
1.2 Protein tyrosine phosphatases

Protein tyrosine phosphorylation play critical roles in fundamental biological processes. The cellular equilibrium of protein tyrosine phosphorylation is achieved through the actions of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs) (Kyriakis and Avruch, 2012). The mitogen-activated protein kinases (MAPKs) are activated through extracellular and intracellular stimuli and are involved in regulation of processes such as gene expression, protein translation, protein stability, protein localization and enzyme activity (Caunt and Keyse, 2013). Also, they are essential in regulating growth and differentiation and they facilitate multiple cellular pathways involved in cell proliferation, differentiation, cell survival, metabolism, and cell death (Wada and Penninger, 2004; Caunt and Keyse, 2013)

1.3 Mitogen-activated protein kinases (MAPKs)

The MAPK pathway comprise a conserved family of enzymes that regulate a wide range of physiological processes in cells including proliferation, development, immune functions, apoptosis and metabolic homeostasis (Gaestel, 2016). In the liver MAPKs are essential to regulate metabolism and there are three major subgroups of them in mammals, which include the extracellular signal-regulated kinases 1 and 2 (ERK ½), c-Jun-N-Terminal kinases 1, 2, and 3 (JNK1/2/3) and p38α/βδ/γ MAP kinases. The ERKs, JNKs, and p38 MAPK regulate functions such as proliferation, apoptosis, and differentiation. The MAPKs signaling reacts to growth factors, peptide hormones, stress and infection and alterations in
this signal cascade can leads to disease such as cancers, diabetes, atherosclerosis, and immune disorders (Kyriakis and Avruch, 2012); (Gehart et al., 2010; Lawan et al., 2012).

1.3.1 c-Jun-N-Terminal kinases (JNKs) pathway

The JNK and p38 are stress responsive MAPKs and ERK1/2 in hepatic metabolism is activated by growth factors. These MAPKs are activated when the MAPK kinases (MKKs) phosphorylate threonine and tyrosine residues on MAPKs. In mammals, MAPKs get inactivated when they are dephosphorylated by a group of dual-specificity phosphatases (DUSPs) which are known as MAPK phosphatases (MKPs) (Caunt and Keyse, 2013; Lawan and Bennett, 2017). The JNK1 and 2 isoforms are known to be expressed in the liver but not the JNK3 isoform. In obese conditions JNK is activated in the liver, because of the overexpression of proinflammatory cytokines (Lawan and Bennett, 2017).

There are studies done that demonstrated liver specific JNK deficient mice display increased insulin resistance (Sabio et al., 2009). In addition, when both the JNK1 and JNK2 isoforms are deleted in the liver of mice, the results showed enhanced glucose and insulin tolerance, increased hepatic insulin action, and reduced fasting blood glucose when fed with high-fat-diet (Vernia et al., 2014). However, deletion of hepatic JNK2 alone enhanced insulin sensitivity, which suggested that JNK2 contributes more to the progression of obesity-induced hepatic insulin sensitivity rather than JNK1 (Bogoyevitch et al., 2010; Lawan and Bennett, 2017). Therefore, this suggests that these two isoforms have different substrate selectivity. However, it is unclear what specific substrates which are phosphorylated differently in the liver that induce these unique outcomes on hepatic glycemic control. Also, studies done using diet-induced obese rats has shown that inhibition of JNK reduced
autophagy and increased hepatic insulin resistance, which means when the JNK1 is overexpressed in obese state it promotes NAFLD (Sabapathy, 2012; Yan, Gao and Zhang, 2017).

1.3.2 p38 MAPK pathway

There are four types of p38 MAPK isoforms which are the alpha, beta, gamma, delta and they are expressed differently in different tissues. For example, p38alpha is expressed in many tissues but scarce in the brain whereas p38beta is the major isoform produced in many tissues (Beardmore et al., 2005). Also, p38delta is expressed only in fewer tissues such as neutrophils and endocrine glands. Moreover, p38 gamma is produced in all tissues and is abundantly present in muscle tissues (Manieri and Sabio, 2015).

It has been shown that several p38 MAPKs are important in metabolic processes. For example, two important transcription factors known as transcriptional PPAR gamma coactivator-1 alpha (PGC1α) and CREB involved in gluconeogenesis are downstream targets of p38 MAPK (;(Puigserver et al., 2001; Cao et al., 2005; Manieri and Sabio, 2015) (Deak et al.,). The p38 MAPKs phosphorylate PGC1α and CREB and enhance their activities which leads to the activation of glucogenic genes which transcribe phosphoenolpyruvate carboxykinase kinase (PEPCK) and glucose-6-phosphatase (Deak et al.,). Also, experiments done using inhibitors and p38-targeting short hairpin RNA, have shown a possibility that p38 MAPK might be involved in modulating gluconeogenesis (Cao et al., 2005). Moreover, p38 MAPK is also known to co-activate other transcription factors such as PPARα, FOXO1, and CCAAT-enhancer-binding protein alpha which are important
in glucose homeostasis (Juge-Aubry et al., 1999; Xu et al., 2005; Manieri and Sabio, 2015) (Qiao et al., 2006). Although these results point p38 MAPK kinases as regulators of glucose homeostasis in the liver, more studies need to be done to define its specific function.

1.3.3 Extracellular signal-regulated kinases (ERKs) pathway

The extracellular-regulated kinase 1,2 (ERK) pathway is the most widely characterized among members of this family of kinases. They participate in the Ras-Raf-MEK-ERK signal transduction cascade which is important in regulating functions such as cell growth, cell adhesion, cell cycle progression, cell migration, cell proliferation, and cell survival (Roskoski, 2012). It is mainly activated by mitogens and growth factors and involves a receptor-mediated activation of a small G-protein for its initiation of signal cascade (Juntila et al., 2007). When the ERK 1,2 isoforms are activated they can translocate to the nucleus and activate several transcription factors. For example, c-Fos, ATF-2, ELK-1, c-Jun, c-Myc, and Ets-1. Also, ERK 1and 2 are known to phosphorylate cytoplasmic and nuclear kinases such as MNK1, MNK2, MAPKAP-2, RSK, and MSK1,2 (Roux and Blenis, 2004; Zebisch et al., 2007) (Juntila et al., 2007). Collectively, these studies showed that both JNK and p38 MAPK are important mediators of metabolic signaling that regulates insulin resistance, glucose and lipid homeostasis and energy metabolism.

1.4 MAP kinase phosphatases (MKPs)

The mitogen activated protein kinase phosphatases (MKPs) control MAPK activation by direct dephosphorylation of their regulatory threonine and tyrosine residues by MAPK
phosphatases (MKPs). In mammals there are 10 structurally distinct dual specific phosphatases (DUSPs) that acts to terminate the activity of MAPKs signaling within a defined subcellular location. The MKPs or DUSPs consist a C-terminal domain and a N-terminal non-catalytic domain which contains a MAP kinase interaction motif (KIM) (Keyse, 2010; Seternes, Kidger and Keyse, 2019).

These DUSPs are known to have unique features as they respond differently to MAPKs depending on substrate specificity, subcellular distribution, and factors regulating induction. For example, MKP-1 which is a nuclear DUSP of type I class is selective for all three MAPKs but MKP-3 which is a cytosolic DUSP of type II class is selective for ERK only (Keyse, 2010; Lawan et al., 2012).

These MKPs are grouped into three subfamilies based on the sequence homology, subcellular localization, and substrate specificity. The first group is mitogen and stress-inducible nuclear MKPs which consists of DUSP-1/MKP-1, DUSP-2(PAC1), DUSP4.MKP-2 and DUSP-5 (Theodosiou and Ashworth, 2002; Owens and Keyse, 2007). The second group is cytoplasmic ERK specific MKPs and contains DUSP6/MKP-3, DUSP7/MKP-X and DUSP9/MKP-4. The third group has JNK/p38 specific phosphatases which includes DUSP8(M3/6), DUSP10/MKP-5 and DUSP16/MKP-7. These are present in the cell nucleus and cytoplasm (Keyse, 2008).

1.4.1 MAP kinase phosphatase-2 (MKP-2)

MAP kinase phosphatase-2 (MKP-2) belongs to the class I DUSP and is located in the nucleus (Al-Mutairi et al., 2010; Caunt and Keyse, 2013). The MKP-2 which is also known as DUSP4 is associated with regulation of ERK, or JNK depending on the cell type and it
can bind to p38 MAPK too (Lawan et al., 2012). However, the in vitro studies in macrophages have shown that MKP-2 can dephosphorylate ERK and JNK but is not effective for p38 MAPK (Al-Mutairi et al., 2010).

The MKP-2 is known to be 43 kDa in size and it is upregulated in response to growth factors, phorbol 12-myristate13-acetate (PMA), oxidative stress, UV light, and lipopolysaccharide (LPS) (Jiao, Tang and Zhang, 2015). Many MKPs are associated to regulation of diseases and any changes in their expression leads to diseases such as gluconeogenesis, insulin resistance, and diabetes (Jiao, Tang and Zhang, 2015). In addition, the function of MKP-2 is important when developing cancer, as it is linked to ovarian cancer, oesophagogastric rib metastasis, and pancreatic tumors. Also, MKP-2 shows higher expression in liver cancers (Lawan et al., 2012). In a study done to observe the interaction between macrophage and adipocyte, it was demonstrated that MKP-2 regulates and can inhibit inflammatory activation of macrophages (Jiao, Tang and Zhang, 2015; Seternes, Kidger and Keyse, 2019).

Also, MKP-2 showed roles in cellular apoptosis and senescence. A study done using macrophages from MKP-2 knockout mice (MKP-2<sup>−/−</sup>) has shown an increase in the activation of JNK and p38 MAPK (Al-Mutairi et al., 2010). In addition, the increased levels of IL-6, IL-12, TNFα and PGF<sub>2α</sub> observed suggested that MKP-2 can alter innate immune response. Moreover, the macrophages derived from the bone marrow of these knockout mice were more prone to get infected with the intracellular parasite Leishmania Mexicana (Al-Mutairi et al., 2010)
1.5 Role of MKP-2 in cellular signaling

1.5.1 Regulation of inflammatory response

There is evidence that MKP-2 is essential in providing protection from inflammatory arthritis. Rheumatoid arthritis is a disease that causes persistent pain, inflammation, and stiffness of the joints in patients, that leads to destruction of joints and disability (Schroeder et al., 2019). The MAPK signaling pathway is linked to this disease and is known to activate all three MAP kinases: ERK, p38 MAPK, and JNK. When the MKP-2 is knocked out, the mice showed increased inflammation in their front and rear paws compared to the wild type mice (Schroeder et al., 2019). This increased inflammation was caused by the increased number of neutrophils in the joints and enhanced release of cytokines (Schroeder et al., 2019).

1.5.2 Regulation of cell cycle progression and apoptosis

Also, MKP-2 which is a DUSP4 of type I response to ERK and JNK kinases and have shown an important role in protection against apoptosis and in cellular senescence. Studies investigated the effects of MKP-2 global deletion on MAPK-mediated signaling and cellular proliferation. The results from this study demonstrate for the first time a critical non-redundant role for MKP-2 in regulating cell cycle progression and apoptosis in vivo (Lawan et al., 2011).

1.5.3 Importance in muscle cell differentiation

It is identified that Dusp4 is essential in muscle cell differentiation and is involved in regulating MAP kinase signaling. The intracellular signaling and extracellular signaling
together controls skeletal muscle hypertrophy and atrophy. When muscle fiber size increases due to the increase of protein synthesis, development or increased activity it is called muscle hypertrophy. In contrast, when protein synthesis is reduced, and the strength of contraction and endurance reduce it leads to muscle atrophy. Some causes of muscle atrophy are aging, limb immobilization, cachexia, corticosteroid use, and denervation. Some of the known mechanisms that initiate muscle wasting cascade are transcriptional activation of the ubiquitin E3 ligases, MuRF1, and MAFbx to muscle atrophy conditions (Haddock et al., 2019).

1.5.4 Metabolic regulation

The MAPKs are known to regulate metabolism by phosphorylating transcription factors and cytosolic signaling proteins. Therefore, we can predict the MKPs are essential in controlling metabolic homeostasis and cell signaling. Although there are studies done to show how the MKP-1 and MKP-3 control MAPK signaling in the liver, to regulate lipid metabolism and gluconeogenesis, there is not much evidence on how MKP-2 function in hepatic metabolism (Lawan and Bennett, 2017).

1.6 Metabolic pathways related to obesity

1.6.1 Phosphoinositide 3-kinase (PI3K)/Akt pathway

One of the complications in obesity is developing type II diabetes and regarded as a metabolic disease. It is characterized by the chronic hyperglycemia caused by a decrease in insulin sensitivity. Most therapies available currently for diabetic hyperglycemia increase the insulin
sensitivity or introduce exogenous insulin but this does not ensure long-term glycemic
control (Huang et al., 2018). One of the pathways studied related to obesity and type II
diabetes is Phosphoinositide 3-kinase (PI3K)/Akt pathway which was discovered in 1985. It
is an important signal pathway involved in mediating growth factor signals involved in
growth as well as cellular processes such as glucose homeostasis, lipid metabolism, protein
synthesis, cell proliferation, and survival (Whitman et al., 1985; Huang et al., 2018).
PI3Ks belong to a family of lipid kinases which phosphorylate phosphatidylinositol and are
divided into three classes. Its known that PI3Ks get activated when receptor tyrosine kinases
(RTKs) and G-protein-coupled receptors (GPCR) are activated in response to ligands,
growth factors, cytokines and hormones. Once activated class I PI3Ks phosphorylate
phosphatidylinositol 4,5-biphosphate (PIP2) to phosphatidylinositol 3,4,5-trisphosphate
(PIP3) and activates signal cascades downstream which leads to the activation of AKT. The
negative regulator of the PI3K, phosphatase and tensin homologue (PTEN), deactivate PI3K
by dephosphorylating PIP3 to PIP2(Franke et al., 1997; Huang et al., 2018).
There are three isoforms of AKTs known based on the differences of their serine/threonine
residues. The AKT1 is expressed ubiquitously in many tissues, AKT2 is expressed in insulin
sensitive tissues, and AKT3 is mainly expressed in the testes and brain. The total activation
of Akt is controlled by two key phosphorylation mechanisms which are PI3K dependent.
One is the phosphorylation of Threonine 308 (AKT1) residue in the kinase domain by
phosphoinositide-dependent protein kinase 1 (PDK1). Second is the phosphorylation of
serine 473 (AKT1) residue in the carboxy-terminal regulatory domain by mTOR complex 2
(mTORC2). Moreover, similar phosphorylation mechanisms occur in AKT2 (T309 and
S474), and AKT3 (T305 and S472). The activation of Akt is known to regulate many cellular
mechanisms through downstream effectors (Hawkins and Stephens, 2016). The activated Akt is known to regulate glucose and lipid metabolism. For example, activated AKT2 promotes translation of glucose transporter 4 (GLUT4). Also, it converts glucose to glucose 6-phosphate by stimulating hexokinase (Huang et al., 2018).

1.6.2 Insulin signaling pathway

Insulin resistance is the state where the sensitivity of target cells to respond to ordinary insulin levels is reduced, triggering failure of downstream metabolic actions. Development of insulin resistance is identified as main cause of Type II diabetes in obese patients. Some of the known conditions that contribute to insulin resistance are TNFα and free fatty acids, that regulates many protein kinases that target along the insulin pathway (Boura-Halfon and Zick, 2009).

When insulin binds to its receptor, it activates the intrinsic tyrosine kinase activity of receptor (IRK). The activated receptor IRK phosphorylates Tyr residues in target proteins such as insulin receptor substrates (IRS-1 to 6), Shc proteins, Cbl, p60dok, APS, and Gab-1. Also, the activation of receptor IRK is known to propagate three major signaling pathways (Saltiel and Kahn, 2001). One is the phosphatidylinositol 3-kinase (PI3K) cascade which leads to metabolic functions of insulin. Second is the MAP kinase pathway which regulates cell growth. Third is the Cbl/CAP pathway which mediates glucose transport by activating GTP-binding protein TC10 and translocating CIP4/Gappex-5 complex to the plasma membrane (Lodhi et al., 2007; Boura-Halfon and Zick, 2009).
Upon insulin stimulation, the downstream effector of IRS protein, PI3K associates with Tyr-phosphorylated IRS protein. This catalyze the formation of phosphatidylinositol 3,4,5-triphosphate and stimulate the activation of phosphoinositide-dependent kinase (PDK-1) which leads to the activation of protein kinase B (PKB) also known as AKT, mammalian target of rapamycin (mTOR), p70 S6 kinase (S6K1), and PKC. Thus, it leads glucose transportation, protein synthesis and glycogen synthesis (Bandyopadhyay et al., 1997; Boura-Halfon and Zick, 2009).

Many studies have shown that obesity promotes insulin resistance and chronic low-grade inflammation. This is related to the conditions that in obesity it increases the release of free fatty acids, glycerol and hormones from adipose tissue such as leptin, adiponectin, endothelin-1. Also, increased levels of proinflammatory cytokines like TNFα, IL-1β, IL-6 and macrophages in the adipose tissue leads to insulin resistance and obesity (Wellen and Hotamisligil, 2005).
Purpose of Study and Hypothesis

Thesis objectives

The overall goal of this thesis is to elucidate the role of hepatic MKP-2 in glucose and lipid metabolism. Members of the MKP family have been shown to play diverse roles in metabolism. MKP-2 is an inducible phosphatase known to be upregulated in response to growth factors, hormones, and lipopolysaccharide (LPS) (Al-Mutairi et al., 2010; Lawan et al., 2011). Recently, the understanding of MKP-2 function has been advanced due to the development of mouse knockout models, which has resulted in the discovery of novel roles for MKP-2 in the regulation of sepsis, infection and cell-cycle progression that are distinct from those of other MKPs (Al-Mutairi et al., 2010; Lawan et al., 2011). However, many functions of MKP-2 still await to be characterized. Excitingly, one study showed 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. Another study suggests that MKP-2 regulate macrophage-adipocyte interaction (Jiao, Tang and Zhang, 2015). However, the physiological function of MKP-2 in metabolism has not been characterized. It is not clear how MKP-2 is involved in metabolism and in disease conditions such as obesity, fatty liver disease. An understanding of the signaling networks that regulate glucose and lipid metabolism could provide us with attractive new targets for drug design to treat metabolic diseases. Preliminary results from our lab indicated that MKP-2 expression is responsive to changes in nutritional cues. Also, MKP-2 whole-body deficient mice are protected from diet-induced obesity.
Taken together these observations suggest a role for MKP-2 in metabolic regulation. Therefore, I aim to examine MKP-2 expression in major insulin-responsive tissues and cells and also identify MKP-2 specific substrates in these cell lines and tissues. Also, I aim to define the expression and function of MKP-2 in vivo under physiological and pathophysiological conditions using high fat diet (HFD) feeding paradigms.
CHAPTER TWO

METHODS

2.0 Materials and Methods

2.1 Reagents and Antibodies

All reagents were purchased from standard chemical vendors. The following antibodies were used. Phospho-p38 MAPK (9215S), phospho-ERK1/2 (9101S), Phospho-JNK 1/2 (4668S), α-Tubulin, β-Actin from Cell Signaling Technology. MKP-2 antibody (Sc-17821) were obtained from Santa Cruz Biotechnology and BD BioSciences.
Table 2.1 Primary and secondary Antibodies

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<th>Catalogue</th>
<th>Dilution</th>
<th>Secondary</th>
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</tr>
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2.2 Animal studies

The University of Alabama Institutional Animal Care and Use Committee approved all animal studies. The novel MKP-2 wildtype (MKP-2\(^{+/+}\)) and MKP-2 knockout (MKP-2\(^{-/-}\)) mice (kindly provided by Robin Plevin, University of Strathclyde, U.K.). Mice lacking MKP-2 have been genetically characterized previously (Mashael et al, 2010, Lawan et al, 2011). For in vivo studies under normal and pathological conditions, three weeks old male mice were maintained on either a custom high fat (60% kcal) purified rodent diet DN 112252 (Dyets Inc) or regular chow diet for 16 to 24 weeks and their body weight was measured every week. For genotyping, PCR analysis was employed using the following primers: WT forward primer 5’-CTTCAGACTGTCCCAATCAC-3’. WT reverse primer 5’-GACTCTGGATTTGGGTCC-3’. KO forward primer 5’-TGACTAGGGGAGGAGTAGAAGGTGCC-3’. KO reverse primer 5’-ATAGTGACGCAATGCGATCTCCAGG- 3’. For fasting and refeeding experiments, animals were either fed chow diet ad libitum, fasted for 24 hours or fasted for 24 hours and refed for 3 hours.

2.3 Glucose tolerance test

Glucose tolerance tests (GTTs) were performed on male MKP-2 wild type and MKP-2 knockout mice fed chow or HFD for 16 to 24 weeks. Mice were put in clean cages with new bedding and were fasted overnight for 16 hours. Then their basal blood glucose levels were measured by tail massaging using a glucometer. Next, mice were injected intraperitoneally with glucose according at 2 g/kg body weight. Blood glucose levels were measured using the time points 0, 15, 30, 60, 90, and 120 minutes.
2.4 Insulin tolerance test

Insulin tolerance tests (ITTs) were performed on MKP-2 WT and MKP-2 KO fed either chow or HFD for 16 to 24 weeks. Mice were kept in clean cages and were fasted for 5 hours. First, their basal blood glucose levels were measured using a glucometer and a drop of blood was taken from their tail. Then they were injected intraperitoneally with 0.75 mU/g human (Humlin R; Elly and Company, Indianapolis, IN). The blood glucose levels were measured at the time points 0, 15, 30, 60, 90, and 120 minutes.

At the end of the studies, animals were sacrificed, and their liver tissues were obtained. After tissues were removed, they were washed using PBS and immediately snap frozen in liquid nitrogen. Then the samples were kept at -80°C until use for further analysis.

2.5 Immunoblotting

Liver tissue from MKP-2 WT and MKP-2 KO mice was homogenized using RIPA buffer (25mM Tris-HCl at pH 7.4, 150mM NaCl, 5mM EDTA, 1% NP-40, 0.1% SDS, 1% sodium deoxycholic acid), which was supplemented with protease and phosphatase inhibitors. Next the homogenates were lysed on a shaker at 4°C for 30 minutes. Then the lysates were clarified at 20,800 g for 30 min at 4°C. Protein concentrations were measured using a BCA assay kit. Afterwards 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to resolve the lysates. Then it was transferred to a nitrocellulose membrane and was incubated with phospho-specific antibodies followed by enhanced chemiluminescence or fluorescent detection using ChemiDoc MP imaging system (BioRad).
2.6 RNA extraction and real-time PCR analysis

The liver tissue from MKP-2 wild type and MKP-2 KO mice were used to isolate total RNA using RNeasy mini kit from Qiagen. Then RNA concentrations were measured using a spectrophotometer. Next, the RNA was reverse transcribed to construct cDNA using a Taqman gene expression kit (Applied Biosystems). Real-time quantitative PCR was performed using a qPCR machine (Applied Biosystems).

2.7 Cell culture and Anisomycin stimulations

The C2C12 myoblast cell line was cultured as described (Lawan et al 2018) and maintained in Dulbecco’s modified Eagle’s medium (DMEM). The media was supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin-streptomycin. The cells were grown at 37°C in a humidified incubator provided with 5% CO2. New media was added every two to three days and when the cells reached 70-80% confluency they were used for experiments. Firstly, the 80% confluent C2C12 myoblasts were plated on a six well plate and was grown until they were 60% confluent. Next, Cell were serum starved in serum free media overnight. Cells were stimulated with anisomycin (5 μM) at 0, 15,30, 60, 120, and 240 minutes. At the end of the experiment, protein extraction was performed using a RIPA buffer supplemented with protease and phosphatase inhibitors. The protein concentration was measured using a BCA assay kit, and 10% SDS page was used to separate 20 μg of total protein. Next, the separated protein was transferred to a nitrocellulose membrane, blocked with 5% Bovine serum albumin, and was incubated with primary antibody. Then enhanced chemiluminescence was used to detect the image using ChemiDoc MP imaging system.
2.8 C2C12 differentiation

The C2C12 immortalized mouse myoblast cell line was purchased and maintained in Dulbecco’s modified Eagle’s medium (DMEM). The media was supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin-streptomycin. The cells were grown at 37°C in a humidified incubator provided with 5% CO₂. New media was added every two to three days and when the cells reached 70-80% confluency they were used for experiments. When the C2C12 myoblasts reached 80% confluency they were plated on a sixty-millimeter plates and was grown until they were 60% confluent. Next, they were brough to basal levels by adding serum free media for overnight. Then the C2C12 myoblasts were changed to differentiation media and were differentiated over a 10-day time period. The protein was extracted using RIPA buffer supplemented with proteases and phosphatase inhibitors. On the first day cells were lysed immediately after adding differentiation media into the control (0hour), then after 1 hour, 2 hour, and 4 hours. Then the protein was extracted for day 1, 2, 3, 4, 5, 6, 8, and 10. Next, the protein concentration was measured using a BCA assay kit, and 10% SDS page was used to separate 20µg of total protein. Next, the separated protein was transferred to a nitrocellulose membrane, blocked with 5% Bovine serum albumin, and was incubated with primary antibody in 1:1000 dilution and secondary antibody in 1:3000 dilution. Then the chemiluminescence was used to detect the image using ChemiDoc MP imaging system.

The murine hepatocyte AML12 cell line was purchased from ATCC, VA, and was maintained in growth media. The base medium used was DMEM:F12 medium supplemented
with 10% Fetal bovine serum, 10 µg/mL Insulin, 5.5 µg/mL Transferrin, 5 ng/mL Selenium, 40 ng/mL Dexamethasone and 1% Penicillin/Streptomycin antibiotics.

The cells were grown at 37°C with 5% CO₂ and new media was added every two-three days. Once the cells reach 80% confluence, they were placed in a six well plate on regular media. When the cells reach 60% confluence, they were brough to basal levels by adding serum free media overnight. Next, they were induced using Anisomycin using the time points 0, 30, 60, 120, 240 and 360 minutes. The protein was lysed from the cells using RIPA buffer supplemented with proteases and phosphatase inhibitors. After the protein concentration was determined, 10% SDS page gel electrophoresis was performed to separate the protein. The Western blotting procedure was performed to detect the protein expression.

The mouse embryonic fibroblast cell line 3T3-L1 was purchased from ATCC, VA and maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% Fetal calf serum and 1% penicillin-streptomycin. The cells were grown at 37°C with 5% CO₂ and cells were used for experiments within two or three days at 50-60% confluency. The cells were kept in serum free media overnight and were stimulated with Anisomycin. The time points used were 0, 30, 60, 120, 240 and 360 minutes. Next, the cell protein was extracted using RIPA buffer supplemented with proteases and phosphatase inhibitors. Once the protein concentration was determined, the SDS page gel electrophoresis and western blotting was performed. The primary antibody in 1:1000 dilution and secondary antibody in 1:5000 dilution was used. The florencece antibodies were used to detect the images using ChemiDoc.
2.9 Data Analysis

All data are presented as mean±s.e.m. Protein levels were compared using student’s unpaired two-tailed t test (Graphpad Prism 9) comparison of physiologic parameters were performed using one- and two-way analysis of variance (ANOVA). Post hoc test were performed using Dunnett’s test and Bonferroni’s multiple comparison test. A value of p<0.05 was considered statistically significant.

2.10 Hematoxylin-and-Eosin staining and Oil Red O staining

When collecting the tissues from mice, a small amount of different tissues including liver were saved after cleaning them in PBS. Then these samples were kept in 1x formalin solution. The liver tissues collected from chow fed and high fat diet fed wildtype and knockout mice were analyzed for the Hematoxylin-and-Eosin (H & E) staining and Oil Red O staining. This was done with the help of our collaborators from UAB histology core and Patty J Williams.
CHAPTER THREE

RESULTS

3.0 Results

Introduction

MAPKs are essential proteins in the regulation of many physiological functions including cell proliferation, survival, differentiation, cell death and metabolism (Gaestel M, 2016). The MAPK phosphatase (MKPs) are the most relevant phosphatases which inactivate MAPK. MKPs specifically inactivate the MAPKs by direct dephosphorylation of MAPK. The role of MKP-2 in metabolic function and disease has not been discovered. This study utilized a novel DUSP4 (MKP-2) knockout mouse kindly provided by Dr. Robin Plevin, University of Strathclyde, United Kingdom. The aim of the experiments in this chapter were to examine the regulation of expression of MKP-2 in insulin-sensitive cell lines and tissues. First, assessed MKP-2 expression during differentiation in C2C12 myoblasts, induction in AML12 (mouse hepatocyte cell) and 3T3-L1 (mouse fibroblasts). Second, determine whether MKP-2 expression is modulated under physiological and pathological conditions in vivo. Third, determine the impact of MKP-2 deletion in response to high fat diet on glucose metabolism.
3.1 MKP-2 protein expression during differentiation in myoblasts and MAPK phosphorylation

3.1.1 MKP-2 is down-regulated during differentiation in myoblasts

To assess the metabolic role of MKP-2 in myoblast differentiation, C2C12 were induced to differentiation. 60% confluent C2C12 myoblasts were grown in serum free media overnight to bring basal levels of signaling. They were changed to differentiation media and were differentiated over a 10-day time period. Whole cell lysates were prepared at different time points after induction of differentiation and analyzed by immunoblotting for MKP-2 expression. The data presented in figure 3.1.1 shows that MKP-2 protein expression was detectable in the undifferentiated myoblasts, maintained during the first few hours up to day 2 and then dramatically decreased by day 3 up to day 10 (Figure 3.1.1). By day 10, MKP-2 protein expression was barely detectable. These results show that MKP-2 is down-regulated during differentiation in myoblasts. These results are consistent with a recent study where Haddock et al showed that overexpression of MKP-2 delays muscle cell differentiation. Differentiation is accompanied by a metabolic switch from glycolysis to fatty acid oxidation (Malandraki-Miller et al., 2019). Since insulin regulates many physiological functions including differentiation and metabolism (Petersen and Shulman, 2018), it is conceivable that MKP-2 might regulate some of insulin’s actions in a MAPK-dependent manner.
Figure 3.1.1: MKP-2 protein expression is downregulated in the C_{2}C_{12} myoblasts during differentiation.

(A) Immunoblots for MKP-2 protein expression (upper panel) and (B) actin antibodies were used to detect the expression of beta-actin as a loading control (lower panel).
3.1.2 MAPK phosphorylation during differentiation in myoblasts

As mentioned earlier, the C2C12 myoblasts were lysed at different time points after being induced for differentiation. Then phosphorylation of MAPKs was evaluated by immunoblotting using phospho-specific antibodies as mentioned in the methods section. Figure 3.1.2 shows that p38 MAPK and JNK1/2 phosphorylation were downregulated during differentiation. This is consistent with a study that showed knockdown p38 MAPK inhibited myoblast differentiation (Lovett A.F. et al, 2010). However, inactivation of p38 MAPK and JNK was not observed when MKP-2 was upregulated during early stages of differentiation. This could be due to compensatory effects of other MKPs that target these MAPKs in these cells.
Figure 3.1.2: Phosphorylation of p38 MAPK and JNK during C2C12 myoblasts differentiation.

(A) phosphorylation of p38 MAPK and loading control for p38 MAPK. (B) phosphorylation of JNK1/2 and loading control for JNK1/2.
3.2 Induction of MKP-2 expression and MAPK phosphorylation in response to anisomycin stimulations in C2C12, AML12 and 3T3-L1 cells

In order to characterize the kinetics of induction of MKP-2 in insulin sensitive cells lines, the expression of MKP-2 was examined over a time course of 0-360 minutes. C2C12, AML12 and 3T3-L1 cells were grown to confluency in 6-well plates and were serum starved overnight before incubation with anisomycin (5 μM). Whole cell lysates were analyzed by immunoblotting for MKP-2 expression.

3.2.1 Induction of MKP-2 expression in the AML12 cells in response to anisomycin stimulation.

To examine induction of MKP-2 expression after adding a stress inducing activator, AML12 cells were grown in serum free media overnight and 5μM anisomycin was added to cells over a time period of 0 to 360 minutes. Next, the cells were lysed, and protein was extracted. Then immunoblotting was used to detect the MKP-2 expression. Figure 3.2.1 showed high basal level of MKP-2 in AML12. However, the expression of MKP-2 dramatically increased in response to anisomycin after 30 minutes and this was sustained for up to 360 minutes (Fig. 3.2.1). Beta actin was used as a loading control. These results established that anisomycin is capable of activating the endogenous expression of MKP-2 in AML12 cells.
Figure 3.2.1: MKP-2 expression in the AML12 cells after stimulation with anisomycin.

(A) Immunoblotting was performed using 20μg of protein to detect MKP-2 expression. (B) Beta Actin was used as a loading control.
3.2.2 Phosphorylation of MAPKs in AML12 cells in response to anisomycin stimulation

Having established the kinetics of induction of MKP-2 in AML12 cells, the phosphorylation of MAPKs were examined. The AML12 cells were grown in 6-well plates in serum free media overnight to bring basal level signaling. Cells were incubated with 5μM anisomycin over a time course of 0 to 360 minutes. Cells were lysed and protein was extracted. Immunoblotting was used to determine the phosphorylation status of p38 MAPK, JNK1/2 and ERK1/2. Figure 3.2.2 showed that the basal phosphorylation level of p38 MAPK was low in unstimulated cells. However, in response to anisomycin the phosphorylation dramatically increased after 30 minutes, and this was sustained up to 360 minutes. (Fig. 3.2.2). In unstimulated cells, the basal level of JNK1/2 and ERK1/2 phosphorylation were low (Fig. 3.2.2). However, the phosphorylation of JNK1/2 and ERK dramatically increased after 30 minutes of incubation with anisomycin, and then reduced after 60 minutes (Fig. 3.2.2). This reduced JNK1/2 and ERK1/2 phosphorylation continued over the duration of the time course. This corelates with enhanced MKP-2 expression in AML12 cells (Fig.3.2.1). These results suggest that MKP-2 contribute to the dephosphorylation of JNK1/2 and ERK1/2 in AML12 cells.
Figure 3.2.2: Phosphorylation of p38 MAPK, JNK1/2 and ERK1/2 in AML12 cells after stimulation with anisomycin.

(A) phosphorylation of p38 MAPK. (B) p38 MAPK loading control. (C) phosphorylation of JNK1/2. (D) JNK1/2 loading control.
3.2.3 Induction of MKP-2 expression in 3T3-L1 cells in response to anisomycin stimulation.

To examine the induction of MKP-2 expression after adding a stress inducing activator, 3T3-L1 cells were grown in serum free media overnight and 5μM anisomycin was added to cells over a time period of 0 to 360 minutes. Next, the cells were lysed, and protein was extracted. Then immunoblotting was used to detect MKP-2 expression. Figure 3.2.3 showed high basal level of MKP-2 in 3T3-L1. However, the expression of MKP-2 dramatically increased in response to anisomycin after 30 minutes and this was sustained for up to 360 minutes (Fig. 3.2.3). Beta actin was used as a loading control. These results established that anisomycin is capable of activating the endogenous expression of MKP-2 in 3T3-L1 cells.
Figure 3.2.3: MKP-2 expression in the 3T3-L1 cells after stimulating with Anisomycin.

(A) Immunoblotting was performed using 20 μg of protein to detect MKP-2 expression. (B) Beta Actin was used as a loading control.
3.2.4 Phosphorylation of MAPKs in 3T3-L1 cells in response to anisomycin stimulation.

Having established the kinetics of induction of MKP-2 in 3T3-L1 cells, the phosphorylation of MAPKs were examined. The 3T3-L1 cells were grown in serum free media overnight to bring basal level signaling. Then cells were incubated with 5μM anisomycin over a time course of 0 to 360 minutes. At each time point the cells were lysed and protein was extracted. Once the protein concentration was determined, immunoblotting techniques was used to determine the phosphorylation of p38 MAPK, JNK1/2 and ERK1/2. Figure 3.2.4 showed high basal level of MKP-2 in 3T3-L1 cells. However, the expression of MKP-2 increased in response to anisomycin after 30 minutes and this was sustained for up to 120 minutes before returning to basal levels (Fig. 3.2.4). Beta actin was used as a loading control. These results established that anisomycin is capable of activating the endogenous expression of MKP-2 in 3T3-L1 cells.
Figure 3.2.4: Phosphorylation of p38 MAPK and JNK1/2 in the 3T3-L1 cells after stimulation with anisomycin.

(A) phosphorylation of p38 MAPK. (B) p38 MAPK loading control. (C) phosphorylation of JNK1/2. (D) JNK1/2 loading control.
3.3. Characterization of the metabolic effects of MKP-2 deletion under physiological and pathophysiological conditions \textit{in vivo}.

3.3.1 Overexpression of MKP-2 in obesity

The previous section of the results described the kinetics of induction of MKP-2 in response to anisomycin in insulin responsive cell lines; C\textsubscript{2}C\textsubscript{12}, AML12 and 3T3-L1 cells and established the endogenous expression of MKP-2 in response to anisomycin in these cells. It also demonstrated the moderate effects of MKP-2 in regulation MAPK signaling in these cells. One study suggests that MKP-2 regulate macrophage-adipocyte interaction (35), and another that MKP-2 is upregulated during skeletal muscle atrophy (36). However, the physiological function of MKP-2 in metabolism \textit{in vivo} has not been characterized. The aims of this section were to examine whether MKP-2 contributes to metabolic function especially in relation to liver metabolism. Firstly, determine whether MKP-2 expression is modulated under physiological and pathophysiological conditions \textit{in vivo} by examining the expression of MKP-2 in wild type MKP-2 mice after chow (control) and high-fat diet (HFD) feeding. Secondly, by examining the effects of MKP-2 deletion on phosphorylation of MAPKs in the liver. Thirdly, by establishing the development of obesity and fatty liver following HFD feeding in MKP-2 knockout and MKP-2 wild type mice.

The MKP-2 wildtype (WT) and MKP-2 knockout (KO) mice were maintained on regular chow diet and high fat diet separately for a period of 16 to 24 weeks. Body weights were monitored every week throughout the duration of the study. At the end of the study, mice were fasted overnight for 16 hours and their tissues were collected. The liver tissue was homogenized using RIPA buffer and the protein concentration was measured. The protein
samples were diluted to 1μg/μL in sample buffer and was kept in -80°C freezer until used. MKP-2 expression was determined by immunoblotting. The data presented in figure 3.1.1 (panel A and B) demonstrated that under chow feeding the expression of MKP-2 was barely detectable in wild type liver. However, MKP-2 expression levels were significantly elevated (> 90%) compared with chow fed liver following HFD feeding (Fig. 3.1.1; panel C). These results demonstrated that MKP-2 is overexpressed in the liver under conditions of obesity.
Figure 3.3.1: MKP-2 overexpression in High Fat Diet fed Wildtype liver

(A) MKP-2 expression in wildtype chow and high fat diet fed liver. (B) Beta-actin was used as loading control. (C) Illustrate quantification by densitometry, expressed as mean±s.e.m. Statistical analyses by student’s t tests; * p<0.001 in comparison with control group. N=5 per group.
3.3.2 MKP-2 protein expression in Chow and High Fat Diet Wildtype EWAT

Similarly, as mentioned in section 3.3 protein was extracted from epididymal white adipose tissue (EWAT) by homogenizing the tissue in RIPA buffer. The proteins samples were diluted to 1µg/µL in sample buffer and was used for SDS-Page gel electrophoresis. Afterwards, following western analysis technique MKP-2 protein expression was examined. The data presented in figure 3.3.2 showed that under chow feeding MKP-2 protein expression was very low. However, upon HFD feeding MKP-2 expression levels were significantly elevated (~ 3-fold; p<0.01). These results demonstrated that MKP-2 is overexpressed in the white adipose tissue in obesity.
Figure 3.3.2 MKP-2 expression in chow and high fat diet fed wildtype EWAT.

(A) MKP-2 expression in wildtype chow fed and high fat diet fed EWAT. (B) Beta-actin was used loading control. (C) Illustrate quantification by densitometry, expressed as mean±s.e.m. Statistical analyses by student’s t tests; ** p<0.01 in comparison with control group. N=4 per group.
3.3.3 Phosphorylation of MAPKs in high fat diet MKP-2 wildtype and MKP-2 knockout liver.

The MKP-2 wildtype and knockout mice were maintained on regular chow diet and high fat diet separately. Once they reached 24 weeks, they were fasted overnight for 16 hours and their tissues were collected. The liver tissue was homogenized using RIPA buffer and the protein concentration was measured. The protein samples were diluted to 1μg/μL in sample buffer and was kept in -80°C freezer until used. MAPKs expression was detected following SDS- Page gel electrophoresis and Immunoblotting. The western analysis for phosphorylated ERK1/2 showed that in high fat diet as pERK1/2 was downregulated in wildtype and was upregulated in knockout mice in figure 3.3.3.1.

Similarly, the phosphorylation of p38 MAPK was examined in the livers of MKP-2 WT and MKP-2 KO mice fed a chow diet. The data presented in figure 3.3.3.2 showed that enhanced phosphorylation of p38 MAPK in MKP-2 KO livers. Moreover, as shown in figure 3.3.3.3, the phosphorylation of p38 was upregulated in high fat diet fed knockout mice compared to the wildtype. These results indicate that deficiency of MKP-2 in the liver results in concomitant upregulation of p38 MAPK, demonstrating that functional loss of MKP-2 in this mouse model.
Figure 3.3.3.1 ERK1/2 phosphorylation in high fat diet fed mice liver.

(A) phosphorylation ERK1/2 in HFD-fed MKP-2 WT and MKP-2 KO mice. (B) ERK1/2 was used as loading control. (C) Illustrate quantification by densitometry, expressed as mean±s.e.m. Statistical analyses by student’s t tests; * p<0.05 in comparison with control group. N=5 per genotype.
Figure 3.3.3.2 p38 MAPK phosphorylation in chow fed mice liver.

(A) phosphorylation of p38 MAPK in chow fed MKP-2 WT and MKP-2 KO mice. (B) p38 MAPK was used as loading control. (C) Illustrate quantification by densitometry, expressed as mean±s.e.m. Statistical analyses by student’s t tests; * p<0.05 in comparison with control group. N=5 per genotype.
Figure 3.3.3.3 p38 MAPK phosphorylation in High fat diet fed mice liver.  

(A) phosphorylation of p38 MAPK in high fat diet fed MKP-2 WT and MKP-2 KO mice.  
(B) p38 MAPK was used as loading control.  
(C) Illustrate quantification by densitometry, expressed as mean±s.e.m. Statistical analyses by student’s t tests; * p<0.05 in comparison with control group. N=4 per genotype.
3.4 Growth curves, plasma glucose concentration, GTT and ITT in chow and HFD male mice.

3.4.1 Resistance to diet-induced obesity in male MKP-2 KO mice

Three weeks old wildtype and knockout mice were kept on regular chow diet (control) or high fat diet (containing 60% kcal fat) for 16 to 24 weeks. Their body weight was taken every week during this period. Under chow diet, MKP-2 wildtype and MKP-2 knockout mice had comparable weights (Fig. 3.4.1; panel A). The slight increase in body weight in MKP-2 WT compared with MKP-2 KO was not statistically significant under chow diet. Under HFD feeding, at three weeks of age both MKP-2 wildtype and MKP-2 KO mice had comparable weight. However, during the course of the study MKP-2 WT mice gained significantly more weight compared with MKP-2 KO mice. (fig. 3.4.1; panel B). So, under HFD feeding conditions MKP-2 wildtype mice had a significant increase in their weight gain compared to the MKP-2 knockout mice. These results demonstrated that mice lacking MKP-2 expression are resistant to diet-induced weight gain.
(A) Male weight gain Chow diet

- MKP-2 WT
- MKP-2 KO

(B) Weight gain on HFD

- MKP-2 WT
- MKP-2 KO

P < 0.001
Figure 3.4.1 Resistance to diet-induced obesity in male MKP-2 KO mice.

(A) Analysis of body weight of male MKP-2 wild type and knockout mice on a regular chow diet. (B) Analysis of body weight of male MKP-2 wild type and knockout mice on a high fat diet. Data represent mean±s.e.m. ***p<0.001 as determined by ANOVA with Bonferroni posttest for multiple comparisons. (C) High fat diet fed wild type and knockout C57Bl/6 mice. Data represent n= 5-11 mice/genotype.
3.4.2 Plasma glucose concentration levels of Chow fed, and high fat diet fed mice after fasting overnight

The MKP-2 wild type and knockout mice either fed with regular Chow diet or high fat diet were used to measure the plasma glucose levels. Once they reached 16 to 18 weeks, they were changed to new cages with new bedding and were starved for 16 hours. A glucometer was used to measure the basal blood glucose levels by tail massaging. The data were recorded immediately and plotted using Graphpad Prism 9. Under chow feeding conditions, the fasting blood glucose levels were comparable between MKP-2 WT and MKP-2 KO mice (Fig. 3.4.2). However, MKP-2 KO mice fed a HFD exhibit significantly reduced fasting blood glucose levels compared with MKP-2 WT mice, demonstrating improved glycemia in MKP-2 KO mice.
Figure 3.4.2 Fasting plasma glucose concentration levels of Chow- and high fat diet-fed MKP-2 knockout mice.

The 16 to 18 weeks old mice were fasted overnight for 16 hours and the fasting blood glucose levels were measured. Data represent n=5-10 mice/genotype. (A) fasting blood glucose of MKP-2 chow fed mice. (B) fasting blood glucose of MKP-2 high fat diet fed mice.
3.4.3 Glucose tolerance in chow-fed Male MKP-2 KO mice

Glucose tolerance tests (GTTs) were performed on male MKP-2 wild type and MKP-2 knockout mice fed chow diet for 16 to 24 weeks. Mice were put in clean cages with new bedding and were fasted overnight for 16 hours. Then their basal blood glucose levels were measured by tail massaging using a glucometer. Next, mice were injected intraperitoneally with glucose according at 2 g/kg body weight. Blood glucose levels were measured using the time points 0, 15, 30, 60, 90, and 120 minutes using a glucometer. After the glucose solution was injected mice in both types of diet showed increase levels of blood glucose measurements. Then after 30 minutes it started decreasing and after two hours the blood glucose levels reached the normal level. Analysis of the data revealed that chow fed MKP-2 knockout mice were glucose tolerant compared with their MKP-2 counterparts (Fig. 3.4.3).
Data represent n=5 mice/genotype. (A) Fasting blood glucose levels of chow fed male MKP-2 wildtype and knockout mice. (B) Area under the curve data analysis for panel (A) data. Mean ± SEM. *P < 0.05; **P < 0.01; as determined by ANOVA with Bonferroni posttest for multiple comparisons.

**Figure 3.4.3** Chow fed MKP-2 knockout mice are glucose tolerant.
3.4.4. Glucose tolerance in HFD-fed male MKP-2 KO mice

Glucose tolerance tests (GTTs) were performed on male MKP-2 wild type and MKP-2 knockout mice fed high fat diet for 16 to 24 weeks. Mice were put in clean cages with new bedding and were fasted overnight for 16 hours. Then their basal blood glucose levels were measured by tail massaging using a glucometer. Next, mice were injected intraperitoneally with glucose according at 2 g/kg body weight. Blood glucose levels were measured using the time points 0, 15, 30, 60, 90, and 120 minutes using a glucometer. The blood glucose levels of the mice raised after the glucose injections on both diet types after 30 minutes. The MKP-2 wildtype mice showed increased levels of blood glucose and this phenotype remained even after two hours (Fig. 3.13). In contrast, the MKP-2 knockout mice displayed glucose tolerance and the blood glucose levels decreased at the end of two-hour period (Fig. 3.13). These results showed that, HFD-fed mice MKP-2 KO mice showed improved glucose homeostasis.
Figure 3.4.4 Glucose tolerance in HFD-fed MKP-2 KO mice.

Data represent n=5-10 mice/genotype. (A) Fasting blood glucose levels of high fat diet fed male MKP-2 wildtype and knockout mice. (B) Area under the curve data analysis for panel (A) data. Mean ± SEM. *P < 0.05; **P < 0.01, as determined by ANOVA with Bonferroni posttest for multiple comparisons. N=9-10 mice/genotype.
3.4.5 Insulin sensitivity in chow-fed male MKP-2 KO mice

Insulin tolerance tests (ITTs) were performed on MKP-2 WT and MKP-2 KO mice who were fed with regular chow diet 16 to 24 weeks. Mice were kept in clean cages and were fasted for 5 hours. First, their basal blood glucose levels were measured using a glucometer and a drop of blood was taken from their tail. Then they were injected intraperitoneally with 0.75 mU/g human (Humlin R; Elly and Company, Indianapolis, IN). The blood glucose levels were measured at the time points 0, 15, 30, 60, 90, and 120 minutes. The data showed that chow fed male MKP-2 KO mice developed increased insulin sensitivity compared to wildtype mice. After injecting insulin, the blood glucose levels went down for both types of diet, but it increased and reached normal levels in wildtype mice (Fig. 3.4.5). However, in knockout mice the blood glucose levels after insulin injection remained low after two hours (Fig. 3.4.5) These results showed that chow fed MKP-2 KO mice are insulin sensitive.
Figure 3.4.5 Chow-fed male MKP-2 KO mice exhibit insulin sensitivity.

The data showed that lower levels of blood glucose in knockout mice after two hours of insulin injection. Data represent n=5-10 mice/genotype. Mean ± SEM. *P < 0.05; **P < 0.01, as determined by ANOVA with Bonferroni posttest for multiple comparisons. N=5 mice/genotype.
3.4.6 Insulin sensitivity in male high fat diet-fed MKP-2 KO mice

Insulin tolerance tests (ITTs) were performed on MKP-2 WT and MKP-2 KO high fat diet mice who were 16 to 24 weeks old. Mice were kept in clean cages and were fasted for 5 hours. First, their basal blood glucose levels were measured using a glucometer and a drop of blood was taken from their tail. Then they were injected intraperitoneally with 0.75 mU/g human (Humlin R; Elly and Company, Indianapolis, IN). The blood glucose levels were measured at the time points 0, 15, 30, 60, 90, and 120 minutes. The data showed that high fat diet fed male MKP-2 KO mice developed increased insulin sensitivity compared to wildtype mice. After injecting insulin, the blood glucose levels went down for both wildtype and knockout, but it increased and reached normal levels in wildtype mice. However, in knockout mice the blood glucose levels after insulin injection remained low after two hours. Thus, it showed that high fat diet fed MKP-2 KO mice are insulin sensitive.
Figure 3.4.6 High fat diet-fed male MKP-2 KO mice exhibit insulin sensitivity.

The data showed that lower levels of blood glucose in knockout mice after two hours of insulin injection. Data represent n=5-10 mice/genotype. Mean ± SEM. *P < 0.05; as determined by ANOVA with Bonferroni posttest for multiple comparisons. N=9-10 mice/genotype.
3.5 Representative Oil Red O staining and H&E staining, liver to body weight ratio, fat mass ratio, and mRNA expression in PPARγ, sterol regulatory element-binding protein 1c (SREBP1c) and SREBP2

3.5.1 Protection from hepatic steatosis in HFD-fed MKP-2 knockout mice

Three weeks old mice were weaned and kept on chow or high fat diet for 16 to 24 weeks. After the GTTs or ITTs experiments were completed, they were given time for recovery. Then after fasting overnight the mice were sacrificed as described in the methods section. Once the tissues were collected, they washed with PBS and snap frozen in liquid nitrogen. The samples were kept in a -80°C freezer until taken for use. A small amount of tissues including liver were saved on 1x formalin solution. Liver tissues from HFD-fed MKP-2 wildtype and MKP-2 knockout mice were analyzed for hematoxylin and eosin (H&E), and Oil red O staining. MKP-2 KO mice are resistant to diet-induced obesity figure 3.4.2., are insulin sensitive (Fig. 3.4.6). The data presented in figure 3.5.1.1 and 3.5.1.2 demonstrated that MKP-2 KO mice were protected from the development of hepatic steatosis following HFD feeding as shown by the H&E and Oil Red O staining. Consistent with the protection from hepatic steatosis, MKP-2 KO mice exhibit decreased liver weight when fed a HFD (figure 3.5.1.3). Also, MKP-2 KO mice exhibited reduced fat mass compared with wild type mice (figure 3.5.1.4). Consistent with protection from fatty liver, the expression of mRNAs for PPARγ, sterol regulatory element-binding protein 1c (SREBP1c) and SREBP2 were significantly reduced in the livers of MKP-2 KO mice (figure 3.5.1.5). This is consistent with resistance to diet-induced obesity, glucose tolerance and insulin sensitivity phenotype in male MKP-2 KO. These results that suggest MKP-2 overexpression in liver of mice may contribute to the development of obesity, insulin resistance and T2D. In
obesity, it also suggests that MKP-2 upregulation promotes hepatic steatosis and lipogenesis.

Figure 3.5.1.1 H&E staining in chow- and high fat diet-fed male MKP-2 WT and MKP-2 KO liver.

The liver tissues were collected, and Hematoxylin-and-Eosin staining were done to identify the accumulation of fat in the liver. Left top panel represent the high fat diet fed MKP-2 KO mice liver. Left bottom panel shows the high fat diet fed MKP-2 wildtype liver. Top right panel displays chow fed MKP-2 KO liver. Top bottom shows chow fed MKP-2 WT liver. N=5 mice/genotype.
Figure 3.5.1.2 Oil Red O staining in high fat diet fed wildtype mice and knockout mice.

The liver tissues were collected, and Oil Red O staining was performed. Top panel shows the high fat diet fed MKP-2 KO mice protected from fatty liver accumulation. Bottom panel shows high fat diet fed MKP-2 WT mice liver that have more fatty liver. N= 5 mice/genotype.
Figure 3.5.1.3 Liver to body ratio of high fat diet fed mice.

Data shows the liver to body weight ratio. N=9-11 mice/genotype. Data represent n=9-11 mice/genotype. Mean ± SEM. *P < 0.05 as determined by student t tests.

Figure 3.5.1.4 Fat mass ratio of high fat diet fed mice.

Data shows the fat mass in MKP-2 wildtype and MKP-2 knockout mice. N=9-11 mice/genotype. Data represent n=9-11 mice/genotype. Mean ± SEM. **P < 0.01 as determined by student t tests.
**Figure 3.5.1.5** Liver mRNA expression PPARγ, SREBP1c and SREBP2 from high fat diet fed MKP-2 KO mice.

The RNA was extracted from liver tissues of high fat diet fed mice, then amplified to cDNA using regular PCR. Next RT-PCR was performed to measure the mRNA expression of genes related to lipid metabolism. Data represents the mRNA expression of PPARγ, sterol regulatory element-binding protein 1c (SREBP1c) and SREBP2. Data represent n=6 mice/genotype. Mean ± SEM. *P < 0.05; **P < 0.01, as determined by student t tests.
Figure 3.5.1.6 Model of MKP-2 in obesity.

In obesity MKP-2 is overexpressed resulting in inactivation of MAPKs thereby promoting the development of obesity and hepatic steatosis.
3.6 Phosphoinositide 3-kinase (PI3K)/Akt pathway

The phosphoinositide 3-kinase (PI3K)Akt pathway is one of the metabolic pathways related to obesity and Type II diabetes. It is involved in cellular process like glucose homeostasis, lipid metabolism, protein synthesis, cell proliferation and survival. The activation of Akt protein is controlled by the phosphorylation of Threonine 308 residue and serine 473 residue. It’s known that activated Akt promotes translation of glucose transporter 4 (GLUT4). In liver, glucose is utilized for gluconeogenesis and glycogenolysis in the fasting state. In the fed state of the liver, the PI3K/Akt pathway reduces hepatic glucose production and glycogenolysis. This lead increases synthesis of glycogen and fatty acid, which is stored and used in other tissues. In this experiment we aimed to determine the phosphorylation and activation of insulin-mediated Akt pathway in MKP-2 wildtype and knockout mice.

3.6.1 Insulin-mediated Akt activation in the liver of Chow diet fed mice

The chow fed mice were kept in new cages and fasted overnight for 16 hours to bring the basal level of signaling. Then they were injected with 0.2U/kg insulin by injecting intraperitoneally. Once injected the mice were euthanized after 15 minutes and the liver tissues were collected immediately. As mentioned in section 3.3 protein was extracted from liver tissues by homogenizing in RIPA buffer. The protein samples were diluted to 1μg/μL in sample buffer and was used for immunoblotting. As shown in figure 3.6.1. the western analysis indicates that phosphorylation of Akt (Ser 473) is upregulated in MKP-2 knockout mice compared to the wildtype mice. This upregulation in phosphor-Akt corelates with our previous results, that MKP-2 KO mice are insulin sensitive compared to the wildtype mice.
This result may suggest a potential role of MKP-2 in insulin-mediated regulation of Akt in the liver that could be studied further in future.

Figure 3.6.1 Insulin-mediated Akt activation in the liver of Chow diet fed mice.

(A) Phosphorylation of Akt Serine 473 residue. (B) Loading control Akt.
CHAPTER FOUR

DISCUSSION AND CONCLUSIONS

4.0 Discussion

MAP kinase phosphatase-2 (MKP-2) is a type 1 dual specificity phosphatase (DUSP) that regulates the activities of the MAPKs, JNK and ERK in vitro (Lawan et al., 2012, Seternes et al., 2019). The activities of ERK, JNK and p38 MAPK has been implicated in the development of insulin resistance and pathogenesis of obesity and diabetes in mice and humans (Lawan et al, 2017). However, the metabolic function of this particular MKP family member are largely unknown. This study utilized a novel MKP-2 knockout mouse and assessed the consequences of deficiency at the level of both MAPK regulation and in relation glucose and lipid metabolism, and insulin resistance and obesity. MKP-2 is widely expressed MKP which shows significant sequence identity with MKP-1 and PAC1. Therefore, it may be expected that MKP-2 have overlapping functions particularly with MKP-1.
The induction of MKP-2 was achieved using established agonist that induce this class of protein: anisomycin (Lawan et al., 2012). This study used one \textit{in vitro} model of stress-induced insulin resistance in AML12 and 3T3-L1 cells: produced by anisomycin. The induction of MKP-2 was observed 30 minutes after stimulation in AML12 and 3T3-L1 cells, although the basal level of MKP-2 expression was high in unstimulated cells. Studies that will examine the mechanisms of MKP-2 induction would demonstrate whether MAPKs are involved in these cells. In serum stimulated fibroblasts the kinetics of p-ERK1/2 dephosphorylation coincides with the induction of MKP-2 (Lawan et al, 2011). It has been shown that p-ERK2 dephosphorylation coincides with the induction of MKP-1 (Alessi et al 1993). In this study the induction of MKP-2 by anisomycin moderately dephosphorylate ERK and JNK in these cells.

The mitogen activated protein kinases (MAPKs) are important in many physiological processes in cells such as proliferation, development, immune functions, apoptosis, and metabolic homeostasis (Gaestel, 2016). Initial experiments found that MKP-2 expression was down-regulated during myoblast differentiation. These results are consistent with a recent study where Haddock et al showed that overexpression of MKP-2 delays muscle cell differentiation (Haddock et al, 2019). Differentiation is accompanied by a metabolic switch from glycolysis to fatty acid oxidation (Magadum A, and Engel, 2018). Since insulin regulates many physiological functions including differentiation and metabolism (Magadum A, and Engel, 2018), it is conceivable that MKP-2 might regulate some of insulin’s actions during differentiation.

One study showed 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 (Al-Mutairi et al, 2010). Another study suggests that MKP-2 regulate macrophage-adipocyte interaction (Jiao et al, 2015). We investigated how MKP-2 expression is modulated in insulin responsive tissues in vivo under normal and pathological conditions using MKP-2 wildtype mice and MKP-2 whole body deficient mice. The data showed that MKP-2 expression increased in the liver and white adipose tissues of wild type mice fed a high fat diet suggesting that overexpression of MKP-2 in obesity promotes development of obesity.

Interestingly, the phosphorylation of p38 MAPK and ERK1/2 MAPKs revealed that they were upregulated in the livers of chow-fed and -high fat diet MKP-2 knockout mice respectively. This is the first study that indicated p38 MAPK as an MKP-2 substrate in vivo. The current study showed that MKP-2 knockout mice were protected from diet induced obesity (Figure 3.4.1). This leads to improved hepatic metabolism and inhibition of accumulation of fat in the liver (Figure 3.5.1). The mRNA expression levels measured in liver tissues for PPARγ, SREBP1C, and SREBP2 demonstrated low levels in the high fat diet fed knockout mice as compared with MKP-2 WT. These data corroborate that in obese conditions MKP-2 KO mice showed improved hepatic lipid metabolism, low levels of triglyceride accumulation, and are protected from hepatic steatosis. Studies have also shown that MKP-1 whole-body deficiency protects from development of diet-induced obesity and protection for hepatic steatosis (Wu et al 2006). However, MKP-2 KO mice were insulin sensitive and glucose tolerant compared with their wild type counterparts under both chow and HFD feeding conditions (Figure 3.4.4). Further experiments are needed to fully understand the mechanisms of insulin sensitivity and glucose tolerance in MKP-2 KO mice. These results demonstrate an important role for MKP-2 in glucose and lipid metabolism. We
hypothesize that in obese conditions, MKP-2 expression is upregulated, and this promotes development of obesity and metabolic dysfunction.

In conclusion, MKP-2 is expressed in insulin-sensitive tissues and is down-regulated during differentiation. This study demonstrates that in pathological condition such as obesity MKP-2 is overexpressed in the liver, concomitant to disruption in MAPKs activity. Therefore, leading to development of obesity and resulting to fatty liver accumulation in the liver. Thus, further studies of the role of hepatic MKP-2 will give insights how MKP-2 can be used as an inhibitor to prevent hepatic steatosis, fatty liver, and consequently prevent from developing nonalcoholic fatty liver disease.
APPENDIX A

TO: Ahmed Lawan

SUBJECT: Notice of Approval with Modifications

Principal Investigator: Ahmed Lawan

Approval Date: May 6, 2019

UAB Approval Number and Proposal Titles:

<table>
<thead>
<tr>
<th>Year.Code.ShortTitle</th>
<th>Principal Investigator</th>
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<tr>
<td>2019.B084.Metabolism</td>
<td>Ahmed Lawan</td>
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The application described above has been reviewed by the UAH Animal Care and Use Committee. The following modifications are required for approval:

1) A plan for how animals will be shipped to the Vanderbilt Metabolic Phenotyping Center.
2) A Biosafety risk assessment
3) Relevant Biosafety Protocols
4) Letter of Approval from the UAH Biosafety Committee,

I believe that the Biosafety committee is meeting May 17, and that you already have an application submitted to them. I look forward to hearing from you later this month.

Approvals will be in effect for three years. If you have any significant amendments to make (change in PI, change in number or species, significant change in protocol, etc.) please let me know as soon as possible.

Best regards,

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REFERENCES


