To my family and grit
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ROLE OF ZINC AND METAL TRANSPORTER SLC39A14 IN MURINE SKELETAL MUSCLE DURING INFLAMMATION

By

Jinhee Kim

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Inflammation increases the risk of metabolic diseases and conditions such as atrophy of skeletal muscle. Thus, the prevention of inflammation is critical. Zinc is a nutritionally essential metal and has important roles in growth, development, and immune function. Muscle represents the largest pool of body zinc. Zinc metabolism is controlled by zinc transporters. Among 24 zinc transporters, ZIP14 exhibits the greatest upregulation in response to acute inflammation as induced by lipopolysaccharides (LPS). However, the role of muscle zinc and ZIP14 during inflammation has not been investigated. To investigate the influence of ZIP14 on muscle zinc metabolism, whole-body Zip14 knockout (KO) and wild-type (WT) mice were used. Inflammation was induced either through injection with LPS or by long-term feeding of a high-fat diet (HFD).

Deletion of Zip14 resulted in gastrocnemius muscle atrophy and chronic inflammation. Zinc is necessary during inflammation. Therefore, insufficient zinc concentrations in muscle may lead to accelerated inflammatory responses. As expected, elevated ZIP14 expression was shown in gastrocnemius muscle of mice as a result of both LPS-induced and HFD-treated inflammation models. Muscle zinc concentrations increased in the LPS-induced model. In addition, greater amounts of orally administered $^{65}$Zn was detected in gastrocnemius muscle of WT mice after
LPS injection compared to Zip14 KO mice. Therefore, acute inflammation caused zinc accumulation in gastrocnemius muscle of WT mice, suggesting LPS alters zinc metabolism and facilitates ZIP14-mediated zinc uptake in muscle.

Muscle ZIP14-mediated zinc uptake plays a significant role in reducing inflammatory responses in muscle and protecting muscle atrophy. One mode of action is through modulation of Nuclear Factor-kappaB (NF-κB) and other signaling pathways that control inflammation. The results also suggest that ZIP14-mediated zinc transport may have protective effects for muscle metabolism in inflammatory responses as initiated by the metabolic endotoxemia that initiates metabolic diseases and proinflammatory states such as aging. Taken together, it is possible that the unique muscle phenotype found in Zip14 KO mice may be a result of inflammation-induced muscle atrophy, which leads to muscle structural deficits and impaired muscle performance.
CHAPTER 1
INTRODUCTION

Skeletal Muscle Function and Classification

Skeletal muscle is the largest organ in the body and comprises approximately 40% of total body weight. In humans, skeletal muscle contains primarily water (75%), proteins (20%), and other components (5%) such as inorganic salts, minerals, fat and carbohydrates (1). Muscle proteins are highly involved in maintaining muscle mass via protein synthesis and degradation pathways. Disrupted muscle protein metabolism can lead to muscle dystrophy including atrophy or hypertrophy, and this may consequently contribute to muscle-related diseases.

Skeletal muscle is composed of muscle fibers called myofibers or muscle cells. The single muscle fiber is comprised of myofibrils and myofilaments. Skeletal muscle fibers have been classified by the following criteria: (1) color of muscle fibers (red vs. white) which represent myoglobin content based on the intensity of the color red, (2) contractile properties, (3) which metabolic functions are more dominant, either glycolytic or oxidative properties, (4) degree of fatigability, (5) calcium handling by sarcoplasmic reticulum (2).

Skeletal Muscle and Contraction

Contractile activities of skeletal muscle are regulated during exercise (3). Skeletal muscle consists of muscle fibers which become a part of the motor system. The muscle fibers or myofilaments are primarily classified on a basis of contractile activity and metabolic function (4). During contraction, skeletal muscle is affected by two different types of muscle fiber structures: striated or striped fibers. In particular, the striated muscle fibers are primarily involved in contractions (5). Skeletal muscle fibers can also influence metabolic changes. The muscle fibers are categorized into glycolytic (slow twitch), or oxidative (fast twitch) fibers according to metabolic functions. It is important to note that each skeletal muscle type exhibits
different metabolic properties. For instance, gastrocnemius muscle consists of both glycolytic and oxidative fibers while soleus muscle displays only oxidative fibers. Gastrocnemius and soleus muscles are commonly used to study specific functions in response to metabolic states and inflammatory processes (6).

Furthermore, disruptions of myofilament structures by starvation or inflammatory responses are mediated through regulation of different types of muscle fibers (7,8). Muscle length also contributes to the contractile function by controlling concentric and eccentric muscles, corresponding to shortening and lengthening, respectively (9). Therefore, skeletal muscle contraction is associated with different types of muscle fibers and muscle lengths which respond to muscle injury or inflammation. In terms of injured muscle fibers, different types of contraction affect the injury process. In particular, eccentric (lengthening) contraction is more likely to occur in injured muscle fibers when compared to concentric (shortening) and isometric contractions. The injury leads to an impairment of excitation and contraction coupling in the muscle fibers and consequently results in muscle weakness (10).

**Skeletal Muscle and Cytokine Secretion**

Skeletal muscle is involved in a variety of biological processes, including energy production, secretion of regulatory mediators, neuroendocrine function, and immune function. During inflammation, skeletal muscle secretes metabolic signaling molecules, growth factors, and cytokines. For instance, cytokines such as IL-6 (Interleukin-6), LIF (leukemia inhibitory factor), BDNF (brain-derived neurotrophic factor), and irisin are identified as myokines when produced and released from contracting skeletal muscle. The secreted cytokines then act as intercellular messengers locally or systemically. They bind to specific receptors or mediate signal transduction via intracellular messengers and transcription factors.
IL-6 was the first cytokine designated as a myokine and was thought to have both pro- and anti-inflammatory responses during inflammatory modulation (11,12). Previous evidence demonstrated that human skeletal muscle secretes IL-6 in a TNFα-independent fashion and highly increased IL-6 gene expression occurs during low glycogen levels found in response to contraction, suggesting that IL-6 may play a crucial role in metabolic process rather than in the inflammatory response itself (13,14). Supporting these findings, deletion of the IL-6 gene in mice leads to late-onset obesity and glucose intolerance (15). Muscle-derived IL-6 enhanced basal and insulin-stimulated glucose uptake and translocation of muscle-specific glucose transporter 4 (GLUT4) by activation of AMPK and insulin signaling (16). Hence, IL-6 acts as an energy sensor in response to low glycogen levels in skeletal muscle (17). Thus, muscle cells with a high capacity to secrete myokines may have a direct link to metabolic factors which contribute to the production and release of muscle-derived proteins during contraction.

As a secretory organ, skeletal muscle acts as an autocrine, paracrine or endocrine mediator (3,18). The secreted muscle-derived proteins can in turn influence metabolic organs such as liver, adipose tissue, and brain. This secretory machinery directly or indirectly permits the skeletal muscle to communicate between distal organs and contributes to the maintenance of homeostasis (19).

**Zinc Homeostasis Is Regulated by Zinc and Zinc Transporters**

Zinc homeostasis is influenced by physiological stimuli such as pathogens, toxic materials, environmental exposure, and nutritional status. To maintain cellular zinc homeostasis, most zinc transporters are involved in handling of zinc status and regulating metabolic signaling cascades in a tissue-specific manner (20). There are two zinc transporter protein families. There are 10 members of the ZnT (solute carrier, SLC30) family and 14 members of the ZIP (Zrt- and Irt-like protein, SLC39) family. ZnT transporters are associated with decreasing intracellular zinc
levels through zinc efflux from the cytoplasm into either an organellar lumen or the extracellular space, while ZIP transporters increase intracellular zinc levels by zinc influx into the cell cytoplasm from organellar lumen or extracellular spaces (21). Each transporter is believed to transport zinc for site-specific functions (20,22).

**Zinc and Zinc Transporters Are Mediated by Modulation of Cytokine Signaling during Inflammation**

Zinc status and zinc transporters impact the immune system by modulating cytokine expression and secretion during inflammatory responses (23). Altered zinc homeostasis results from both acute and chronic inflammation (24). The acute inflammation can be caused by mediating agents of pathogens such as lipopolysaccharide (LPS), as well as injured tissues, or irradiation (25,26). Sepsis, which is one of the most prevalent diseases worldwide, is an acute inflammation and contributes to weakened immune function (27,28). On the other hand, persistent mild inflammation related to pathogens or stress from unbalanced diet treatments leads to a low-grade chronic systemic endotoxemia (29-31). For example, this has been observed in high fat diets or zinc deficiency.

A previous study demonstrated that zinc transporter 14 (Zip14) is up-regulated in specific tissues of mice treated with LPS to mimic the pro-inflammatory conditions of innate immunity (32). This response did not occur in IL-6 knockout mice, suggesting Zip14 is regulated by IL-6. It was also documented that Zip14 mRNA expression was induced in response to turpentine-induced sterile sepsis in liver of mice (32).

Consistent with these data, cecal ligation and puncture (CLP)-induced inflammation, which is an experimental mouse model of sepsis, is involved in increased Zip14 expression in the spleens and livers of mice. This acute inflammatory response is mitigated with dietary zinc supplementation (33). In agreement with these findings, recent research indicated that Zip8
induction occurs during sepsis and may control expression of cytokines (34,35). Similarly, Zip6 deficiency is also associated with higher susceptibility for inflammation in immune cells following LPS challenge. Furthermore, the Zip6 loss leads to elevated pro-inflammatory responses by upregulating immune cell activation following LPS administration (36,37). These immune responses are indicated through IL-6 promoter activation, suggesting the potential link between modulatory effects of zinc and cytokine signaling pathways during endotoxemia (36,37). Therefore, zinc status and zinc transporters are both involved in complex immune reactions during inflammation (32).

**Zip14 Expression Is Induced in Response to LPS Challenge**

Zip14 was first identified during adipocyte differentiation in mouse 3T3-L1 cells. Zip14 mRNA expression, determined by northern blot analysis and q-PCR assay, was highly elevated at early time points of differentiation in pre-adipocyte cells (38). The mouse Zip14 gene was identified with two variants, a shorter sequence (2,174 bp) and a longer sequence (3,660 bp) of the coding region (GenBank no. NM144808).

To investigate specific tissue abundance of Zip14 during inflammation, Zip14 transcript expression was screened in multiple tissues of wild type (WT) mice after LPS administration. Zip14 mRNA expression is highly expressed in skeletal muscle along with white adipose tissue (WAT) in WT mice following LPS administration (39). Consistent with this observation, zinc concentrations in skeletal muscle and WAT were elevated in female WT mice during LPS-induced inflammation. However, Zip14 KO mice display no alteration of zinc concentration in skeletal muscle while the zinc concentration in WAT increased at 18 h after LPS challenge (39). This difference may provide insight into the importance of a tissue-specific ZIP14-dependent mechanism of zinc redistribution.
ZIP14 Is Required for LPS-Induced Responses in Metabolic Organs

Zip14 could be involved in modulation of the inflammatory response in metabolic organs such as the liver, small intestine, and adipose tissue. Specifically, Zip14 KO mice had greater zinc accumulation in the small intestine, exhibited impaired barrier function, and had reduced tight junction transmembrane proteins located on the basolateral membrane of enterocytes (40). Due to the increased intestinal permeability, Zip14 KO mice at a steady state exhibits low-grade systemic inflammation. In addition, a recruitment of zinc to WAT (white adipose tissue) of WT mice after LPS administration is concomitant with an elevation of pro-inflammatory cytokine expression. IL-6 production by the WAT was significantly increased after LPS injection which coincided with induction of the JAK2/STAT3 pathway and enhanced leptin secretion in the WAT of Zip14 KO mice. Deletion of Zip14 resulted in a hypertrophy in the WAT of mice and altered lipid metabolism; perhaps through reduced adipocyte differentiation (41). Reduced ZIP14 expression produced in 3T3L1 adipocytes by Zip14 siRNA inhibition increased zinc retention in endosome-like vesicles. These data collectively suggest that a decrease of cellular zinc availability by trapped zinc in vesicles of Zip14 KO mice leads to ZIP14-related defects in these tissues during acute endotoxemia. Overall, altered zinc distribution in Zip14 KO mice leads to organ dysfunction of small intestine and WAT by exhibiting different metabolic patterns of zinc processing in response to acute inflammation or metabolic endotoxemia.

ZIP14 Is Necessary for Glucose Metabolism and Insulin Sensitivity

Cytokines mediate inflammatory responses, and these responses influence nutrient-sensing pathways, in particular, glucose metabolism and insulin resistance (42). In normal conditions, the liver increases basal hepatic glucose production via gluconeogenesis, and skeletal muscle enhances glucose uptake with insulin stimulation. This feedback loop is controlled to maintain systemic energy homeostasis. However, impairment of glucose uptake and insulin
sensitivity can be caused by inflammation mediated by pro-inflammatory cytokine activation (43). This suggests that glucose metabolism may have a link to cytokine-related zinc dyshomeostasis.

Previous evidence showed acute endotoxemia impairs glucose metabolism in the livers of Zip14 KO mice. Liver glucose levels and liver-specific glucose transporter 2 (GLUT2) expression were significantly increased following LPS challenge in the livers of Zip14 KO mice (39). Consistent with this observation, the phosphorylation of insulin receptor (IR) was up-regulated with higher insulin and hypoglycemia after LPS challenge in Zip14 KO mice (39). On the other hand, Zip14 KO in adipose tissue of mice attenuated the insulin signaling pathway during inflammation. LPS-induced inflammation led to reduced phosphorylation of IR and subsequently decreased expression of the down-stream mediators AKT and mTOR (41). Therefore, abnormal zinc metabolism by tissue-specific effects produced by ZIP14 during endotoxemia affects glucose metabolism and insulin sensitivity by controlling phosphorylation of IR. This suggests ZIP14 may be a link between inflammation and glucose metabolism by regulating insulin signaling.

**Zinc Transporters Are Involved in Metabolic Endotoxemia-Induced Inflammation in Skeletal Muscle**

Zinc transporters may play a critical role in skeletal muscle during metabolic endotoxemia. Recent evidence demonstrated that the zinc influx transporter 7 (ZIP7) in skeletal muscle cells may be involved in regulating glucose metabolism and glycemic control through the reduction of glucose transporter 4 (GLUT4) and glycogen synthesis. This suggests ZIP7 may have an impact on skeletal muscle insulin resistance (44). Another study showed an influence of the zinc efflux transporter 7 (ZnT7) during high fat diet (HFD)-induced insulin resistance in murine skeletal muscle. After HFD feeding for 10 weeks, ZnT7 KO mice showed impaired
glucose tolerance and hyperglycemia alongside a reduction in insulin sensitivity in primary skeletal myotubes isolated from the ZnT7 KO mice (45).

Long term outcomes of a HFD treatment lead to metabolic endotoxemia (46). A previous study showed that HFD-induced inflammation resulted in dysregulated inflammatory cytokine expression and insulin resistance in skeletal muscle of mice and further chronic low-grade inflammation (47). For example, over 24 weeks of HFD-feeding resulted in significantly induced pro-inflammatory cytokines and NF-kB activation. This was concurrent with obesity and organ adiposity, perhaps as a consequence of metabolic and immune dysfunction. More interestingly, body weight gain and fat accumulation began at 6 weeks and increased gradually by 15-20 weeks of HFD treatment. This suggests that the 15-20 week duration of the study is critical to generating metabolic endotoxemia (43,48). It is most relevant to point out that Zip14 expression is up-regulated by pro-inflammatory factors. With regard to a relationship between zinc transporters and metabolic endotoxemia, long-term treatment with HFD may lead to low-grade chronic inflammation and contribute to Zip14 up-regulation and an alteration of zinc metabolism in skeletal muscle.

**Zinc Transporters Are Involved in Aging-Associated Inflammation in Skeletal Muscle**

Aging is a complex process and is also regarded as a causal factor in metabolic endotoxemia (36). It is well-known that zinc metabolism is influenced by aging and age-related inflammation. Zinc status is decreased in the elderly and may subsequently lead to immune dysfunction (49). Furthermore, zinc deprivation with age is concurrent with an increase of pro-inflammatory cytokines, a condition referred to as inflammatory aging or immunosenescence (50,51). Studies have shown a relation between zinc transporter regulation and the immune aging process. Elevation of pro-inflammatory cytokine expression influenced by Zip6 was evident in
age-associated zinc deficient status (36). In addition, significant reduction of the cytokine expression was observed in aged mice fed a zinc supplementation diet (52).

Taking into account the association between elevated inflammatory pathways with age, an age-related influence of ZIP14 may occur and ZIP14 expression may increase with age in skeletal muscle. In aged mice, ZIP14 expression in skeletal muscle was significantly increased and displayed greater inflammatory responses. Insulin resistance was increased with age in skeletal muscle of both WT mice and Zip14 KO mice, suggesting impaired insulin metabolism may indicate due to lack of zinc accumulation. As a chronic inflammatory marker, serum IL-6 levels were greater in aged Zip14 KO mice compared to aged WT mice. These results suggest that aging accelerates phenotypic defects of deletion of Zip14 and concurrent impaired zinc metabolism (53).
CHAPTER 2
MATERIALS AND METHODS

Animals and LPS Treatments

Whole-body Zip14$^{-}$ (KO) and wild-type (C57BL/6/129S5) female and male mice were bred at University of Florida in animal care service facilities. Mice were given free access to tap water and a commercial chow diet (Teklad LM-485 Mouse/Rat Sterilizable Diet; ENVIGO 7012) with zinc 63 mg/kg and manganese 93 mg/kg. Mice were maintained on a 12 h light/dark cycle, at a constant temperature and humidity. For injection, these animals were injected intraperitoneally (i.p.) with lipopolysaccharide (LPS; 2 mg/kg body weight) derived from E. coli serotype 055:B5 (Sigma-Aldrich) for experimental groups or an equal amount of saline for control groups. Zip14 KO and WT mice were age-matched and divided into treatment groups: saline-injected control group and LPS-injected groups over the time course (3, 6, 9, 18, 24, and 48 h). Mice were killed using isoflurane anesthesia. Blood was collected by cardiac puncture and kept in blood collection tubes (Capiject, Terumo Medical, Elkton, MD). The supernatant (serum) was obtained after centrifugation at 3000 x g; 15 min at 4°C. All animal treatments and procedures were approved by the University of Florida Institutional Animal Care and Use Committee (IACUC).

Dietary Treatments

Female and male WT and Zip14 KO mice were fed with control Chow diet (17 kcal% fat; 63 mgZn/kg; ENVIGO 7012) for various lengths of time. Alternatively, a HFD diet (60 kcal% fat, Research Diets #D12492) containing zinc 39 mg/kg was fed for 16 weeks. Mice were given the diet and tap water ad libitum and individually housed in shoebox cages at 12:12 light-dark cycle. Body weight and diet consumption were calculated every week.
**Dietary Zinc Supplementation**

Female WT and Zip14 KO mice were treated with diets containing adequate zinc (ZnA; 30 mg Zn/kg) or supplement of zinc (ZnS; 180 mg Zn/kg) diet (Research Diets). Mice were given tap water *ad libitum* and individually housed in shoebox cages. After 4 days of acclimation with the zinc adequate (ZnA) diet, the mice were aged-matched and randomly divided into two groups: ZnA and ZnS groups. The dietary zinc treatments were given for 7 days. At the end of the diet treatment, the mice groups were subdivided into two subgroups: LPS injected and saline-injected mice as a control group (n = 4-5 per group). The injection was given 18h before sacrifice.

**Genotyping of Mice**

Genomic DNA was extracted from each mouse tail using a ZyGem-PrepGEM kit. Mice were screened by Platinum Taq polymerase chain reaction (Invitrogen) as described previously (54). To identify Zip14 KO mice, PCR primer sequences amplified a 164–base pair (bp) DNA fragment from the wild-type allele and a 469-bp DNA fragment from the disrupted allele. For identifying Zip14 KO mice, PCR primers were forward primer Zip14, 5’-TGCCTGGGCACATAGAATGC-3’ and reverse primer Zip14, 5’-GCAGCGCATCGCCTTCTATC-3’ and for WT identification were forward primer, 5’-TCATGGACCGCTATGGAAAG-3’ and reverse primer 5’-GTGTCGAGCGGTATCAACAGAGAG-3’. The PCR products were visualized by electrophoresis in 1% agarose gels.

**Muscle Tissue Preparation from Mice**

All mice were anesthetized with isoflurane to harvest serum and muscle tissues. Gastrocnemius muscles from both legs from each animal were rapidly removed and frozen in
liquid nitrogen and kept in a -80°C freezer until used for experimental purposes. In some experiments, soleus muscles were removed and stored identically.

**Determination of Metal Levels in Serum and Muscle Tissues**

Each tissue sample was weighed on an analytical balance and wet-acid digested with concentrated nitric acid at 90°C for 3 h. Serum was diluted in 1:5 with Milli-Q® water and muscle tissues were diluted 1:2-1:4 with Milli-Q® water. Total zinc and manganese concentrations were measured by flame atomic absorption spectrophotometry (AAS) or microwave plasma-atomic emission spectrometry (MP-AES). The zinc and manganese contents were represented as micrograms zinc per gram tissue. Muscle non-heme iron (NHI) was measured by the ferrozine method as described previously (55).

**RNA Isolation and Quantitative Real-Time PCR**

Total RNA was isolated from excised muscle using TRI Reagent® (Molecular Research Center, Inc.) and treated with Turbo DNA-free reagent (Ambion). Isolated total RNA concentrations and purity were measured with a Nanodrop 1000 spectrophotometer or Nanodrop one (Thermo Fisher). The relative mRNA levels of 24 zinc transporters, MT1, TNF-α, IL-6, IL-1β, and iNOS were determined by quantitative real-time PCR (qPCR) using One Step PCR Master Mix Reagents or Two Step PCR Master Mix for TaqMan analysis (Applied Biosystems). qPCR assays for IL-10, TLR4 and GLUT4 were performed with SYBR Green method (Applied Biosystems) after cDNA synthesis by using High-Capacity cDNA Archive method (Applied Biosystems). The primer sequences for the experiments were used from previous studies (41,54). A glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer sequence was used as an endogenous control for mRNA normalization (56).
**Protein Isolation and Western Blot**

The muscle tissues were homogenized in 500 μl non-denaturing lysis buffer (10 mM HEPES, 0.5 mM EDTA, 0.5 mM EGTA, 10% Glycerol) containing EDTA-free protease and phosphatase inhibitor (AG Scientific) and 1 mM phenylmethylsulfonyl fluoride (Sigma Aldrich). The muscle homogenates were prepared by Bullet Blender® (Next Advance) using 0.9-2.0mm diameter stainless steel beads. The lysates were centrifuged at 13,000 X g for 20 min at 4°C and the lysate (supernatant) was collected to measure the protein. Concentrations were measured with the bicinchoninic acid (BCA) protein assay (Pierce) reagents according to manufacturer’s instructions. The same amount of protein (50 μg) was resolved by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane (GE healthcare Bio-sciences). Equal loading of the blots was confirmed with Ponceau red staining. The blots were blocked with 5% nonfat dry milk for 1 h at room temperature and were probed overnight with the following primary antibodies: pNF-kBp65/NF-kBp65, pSTAT3/STAT3, pMAPK/MAPK, MyD88, pIR/IR, pAKT/AKT, pGS/GS, pGSK-3/GSK-3, pPKA/PKA, and pFOXO1/FOXO1 (Cell Signaling Technology), β-Tubulin (Abcam). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control. After washing with Tris-buffered saline (TBS) containing 0.05% Tween-20, four times for 5 min each, the membranes were incubated with goat anti-rabbit IgG antibody conjugated with Horseradish Peroxidase (GE Healthcare) for 1 h. Then, the blots were washed four times for 5 min and developed with chemiluminescent Pierce ECL detection kit (Super Signal West Pico, Thermo Fisher). The blots were visualized by a FluorChem E Imager (Protein Simple).

**Mouse ZIP14 Antibody Purification**

The affinity-purified mouse ZIP14 antibody was prepared using SulfoLink (Pierce) according to manufacturer’s instruction, followed by peptide competition to confirm reactivity,
specificity of the polyclonal antibody against ZIP14. The peptide CNSELDGKAPGTD was used to generate in-house mouse ZIP14 antibodies in rabbits as described previously (32).

**Cytokine Measurement: Enzyme-Linked Immunosorbent Assay (ELISA) and Luminex**

Muscle homogenates were used the mouse IL-6 ELISA by using Ready-SET-Go kits (eBioscience) following the manufacturer’s instructions. The absorption was determined at 450 nm. Multiplex assay using Luminex® reagents was used to measure TNF-α, IL-6, IL-1β, MCP-1, GM-CSF, IL-10, and VEGF in serum and supernatant from gastrocnemius muscle homogenates from LPS-treated WT and Zip14 KO mice compared to control (Millipore Sigma Luminex®).

**Histopathology of Formalin-Fixed Skeletal Muscle**

Fresh gastrocnemius muscles were excised rapidly and fixed in 10% formalin for 24 h at room temperature and then kept in PBS at 4°C. Paraffin-embedded sections were prepared and mounted for experimental purposes. The muscle cross-section sections (5 µm) were stained with Hematoxylin and Eosin (H&E, Santa Cruz) and visualized with a bright-field light microscopy (Zeiss). Images of H&E stained muscle cross-sections were taken at x40 magnification using a microscope and a digital camera.

**Transcriptome Profiling**

Gastrocnemius muscles from female Zip14 KO and female WT mice were added to lysis buffer RLT, and the lysate was purified using RNeasy fibrous tissue reagents (Qiagen) with on-column DNase digestion (Qiagen). The isolated total RNA was evaluated for quality and quantity spectrophotometrically using Nanodrop One (Thermo Scientific) as above and RNA integrity number (RIN) was measured with an Agilent 2100 Bioanalyzer. Mouse Clariom S array (Thermo Fisher) profiling was performed at the University of Florida Interdisciplinary Center for Biotechnology Research Gene Expression Core. Briefly, 150 ng total RNA was converted to double-stranded cDNA and then to biotin-labeled cRNA using Affymetrix GeneChip® WT
Terminal Labeling Kit. 15 μg of labeled cRNA was hybridized to Affymetrix GeneChip®
Hybridization Kit following the manufacturer’s instruction.

**Coomassie Blue Gel Staining**

Gastrocnemius muscle proteins were separated by gel electrophoresis in 10% poly-
acrylamide gel electrophoresis (PAGE) using GelCode® Blue Stain Reagent (Thermo Scientific)
according to the manufacturer’s instruction.

**Proteomics by SOMAscan Assay**

SOMAscan (Slow Off-rate Modified Aptamer) proteomics analysis was conducted with
serum from female WT and female Zip14 KO mice following LPS (2 mg/kg i.p.) injection for 18
h using established serum proteomic procedures (57).

**Blood Glucose Measurement**

Fasting blood glucose was measured from tail vein of mice using a OneTouch®
UltraMini® glucometer with OneTouch® Ultra blue strips during HFD feeding study. One drop of
the blood was put onto the glucometer strip and immediately read. Serum blood glucose was also
measured by this method.

**Oral Glucose Tolerance Test (OGTT) and Insulin Tolerance Test (ITT)**

HFD-treated mice were fasted for approximately 14 h (overnight) and gavaged orally
with D-glucose (3 mg/kg body weight). For the insulin tolerance test (ITT), mice were fasted 4-5
h and insulin (0.75 unit/kg body weight) was administered. Glucose levels were measured from
tail vein blood of mice before and at 15, 30, 60, 90, and 180 min after oral gavage or i.p.
administration. The absolute values of the blood glucose concentration (mg/dL) were reported.

**Cell Culture and LPS Treatment**

Murine myoblast cell line, C2C12, were obtained from American Type Culture
Collection (ATCC, Mannassas, VA) and cultured in Dulbecco’s modified Eagles’ medium
(DMEM) culture medium with 10% fetal bovine serum (FBS), penicillin, and streptomycin (Corning, NY) according to the manufacturer’s instructions. Cells were passaged up to four times to avoid fully confluence and collected for experimental purposes. For experiments, C2C12 cells were stimulated with LPS (1 mg/ml) for 3, 6, 9, and 18 h and saline was used as a control. Cell lysates were washed with PBS and collected in lysis buffer for protein quantification. To measure Zn concentrations, cells were collected in PBS and centrifuged with 3000 x g for 10 min and the pellets were digested with HNO₃ for 3 h at 90 °C before use of flame AAS or MP-AES.

**Statistical Analysis**

The experiments were performed with three or four mice per group, and data were represented as mean ± SEM. Statistical analysis used Student’s t-test to compare either sex or genotype differences. Multiple comparisons for Two-Way and Three-Way were conducted by analysis of variance (ANOVA) followed by Tukey post hoc test using JMP Pro13 Program (SAS version) and GraphPad Prism 5 (GraphPad software). P < 0.05 was considered as statistically significant.
CHAPTER 3
CHARACTERISTICS OF ZIP14 EXPRESSION AND EVALUATION OF METABOLIC CONSEQUENCES IN SKELETAL MUSCLE DURING ACUTE INFLAMMATION

Results

**Gastrocnemius Muscle Zinc in Female WT Mice was Accumulated During LPS-induced Inflammation**

Previous studies demonstrated that an LPS injection alters zinc metabolism and facilitates ZIP14-mediated zinc uptake in liver, small intestine, and adipose tissue of female WT mice (39-41). To examine the process of Zip14-mediated zinc uptake during inflammation, LPS (2 mg/kg B.W., i.p.) was injected to both male and female wild-type (WT) mice. Two different types of skeletal muscles from WT mice were harvested, specifically, gastrocnemius muscle (glycolytic, slow twitch fiber) and soleus muscle (oxidative, fast twitch fiber).

Changes in serum zinc concentrations following LPS injection were assessed by AAS or MP-AES. As shown in Fig. 3-2A, serum zinc concentrations were significantly reduced at 3 h post i.p. LPS injection and remained decreased for 18 h after the LPS-injection in both male and female WT mice. At a steady state, soleus muscle (23 – 25 µg Zn/g tissue) had a concentration of zinc that was three times greater than that of gastrocnemius muscle (5 - 8 µg Zn/g tissue) in both male and female WT mice. Acute inflammation caused zinc accumulation in gastrocnemius muscle of female WT mice.

Notably, zinc concentrations in the gastrocnemius muscle of female mice dramatically increased at 18 h after LPS injection but these of male mice did not show any change (Figure 3-2B). Soleus muscle zinc concentrations in both male and female mice did not exhibit differences over time following LPS administration (Figure 3-2C).

To identify the most stable reference gene for qPCR analysis, three commonly used reference genes (GAPDH, TBP, and 18s) were measured in gastrocnemius muscle of WT mice
following LPS injection. Based upon uniform expression, GAPDH was selected as the most stable reference gene upon LPS treatment (Figure 3-1).

As the greatest zinc accumulation in gastrocnemius muscle of female WT mice at 18 h post LPS injection was in, we performed a screening of 24 zinc transporters to investigate expression of the zinc transporters is altered in female gastrocnemius muscle during LPS-induced inflammation. We checked transcript levels of the ZIP families (SLC39A1-SLC39A14) and ZnT families (SLC30A1-SLC30A10). Among transcripts of 14 ZIPs, zinc transporter Zip14 showed the highest expression (27-fold). This was followed by a 2-3 fold induction in mRNA expression of Zip7 and Zip8 upon LPS administration (Figure 3-2D). The screen of ZnT transcripts measured that ZnT2 exhibited the greatest expression and a slight elevation of ZnT5 expression in female gastrocnemius muscle after LPS injection (Figure 3-2E). Overall, Zip14 and ZnT2 in female gastrocnemius muscles of WT mice exhibited the greatest mRNA expression among ZIPs and ZnTs families, respectively.

**Inflammatory Responses were Increased in Both Female and Male of WT Mice During LPS-Induced Inflammation**

Because of the increased Zip14 (SLC39A14) and ZnT2 (SLC30A2) mRNA abundance following LPS, we compared this response in gastrocnemius muscle from female and male WT mice. Interestingly, Zip14 transcripts were increased at 18h post LPS injection in both male and female gastrocnemius muscle of WT mice (Figure 3-3A). Consistent with this, ZnT2 expression was increased throughout LPS administration in both female and male of WT mice. Male ZnT2 expression was more abundant than female ZnT2 expression during LPS-induced inflammation (Figure 3-3B). Similarly, metallothionein 1 (MT1) mRNA expression was increased in both female and male of WT mice after LPS injection (Figure 3-3C). We further examined IL-6 expression at 18 h post LPS injection a market of inflammation in gastrocnemius muscle of
female and male of WT mice. IL-6 mRNA expression was higher in LPS-treated gastrocnemius muscle from female and male of WT mice (Figure 3-3C).

**Deletion of Zip14 Showed Shortened Legs and Muscle Atrophy in Gastrocnemius Muscle of Female and Male Mice.**

Zinc transporter Zip14 may be critical to inflammation and its zinc recruitment by muscle may contribute to an immune response. Previous studies demonstrated that Zip14 expression was highly elevated during LPS-induced inflammation in liver and white adipose tissue of mice. Therefore, we performed further experiments by using whole body Zip14 knock-out (KO) mice to investigate a role(s) for ZIP14 in gastrocnemius muscle of female and male mice in response to LPS administration.

In this study, we found that gastrocnemius muscle of Zip14 KO mice showed shortened legs and muscle atrophy in both female and male mice (Figure 3-4A and D). The ratios of gastrocnemius muscle to body weight were significantly reduced in Zip14 KO mice compared to WT mice in both sexes (Figure 3-4B and E). There was no sex difference in respects to muscle phenotypic defects in mice. In addition, both sexes of Zip14 KO mice exhibited atrophic muscle fibers (Figure 3-4C and F). Although both female and male of Zip14 KO mice showed phenotypic defects, female Zip14 KO mice exhibited a greater response of inflammatory signaling pathway components (Figure 3-4G). This suggests that female Zip14 KO mice may be more susceptible to inflammation.

**Muscle Zip14-Mediated Zinc Uptake was Exhibited in Gastrocnemius Muscle of Female Mice During LPS-Induced Inflammation.**

As both female and male of Zip14 KO mice exhibited muscle atrophy of gastrocnemius muscle, next, we examined whether sex differences are in muscle Zip14-mediated zinc accumulation and. Both female and male of WT and Zip14 KO mice were administered LPS for
18 h to measure zinc concentration. We chose 18 h LPS administration showed a peak in muscle zinc elevation in WT mice of that time (Figure 1B).

A previous study demonstrated hypozincemia as an indication of inflammation in female WT mice, but that response was not shown in female Zip14 KO mice during LPS-induced inflammation (39). We found there is a sex difference in response to LPS in female and male Zip14 KO mice. Interestingly, male Zip14 KO mice exhibited hypozincemia while female KO mice did not (Figure 3-4A and 4B). Consistent with this, muscle zinc concentrations displayed in a sex-dependent response to LPS. Gastrocnemius muscle zinc concentration in female WT mice was significantly elevated while female Zip14 KO mice showed only a 50% increase in zinc uptake after 18h LPS injection. However, gastrocnemius muscle zinc concentration of male WT and Zip14 KO mice was not changed by LPS injection (Figure 3-5C and 4D).

**ZIP14-Mediated Zinc Uptake was Significantly Increased in Gastrocnemius Muscle of Female Mice During LPS-Induced Inflammation.**

To more closely investigate a role of Zip14 in gastrocnemius muscle of mice during inflammation, we administered LPS (2 mg/kg BW) to female WT and Zip14 KO mice over time (3, 6, 9, 18, 24, and 48 h). Considering that Zip14 expression was steadily increased 18 h post LPS injection, we added the time points of 24 h and 48 h to find the maximum Zip14 mRNA abundance. In line with muscle zinc accumulation after LPS injection, Zip14 mRNA expression peaked at 18 h after LPS administration and decreased sharply after that (Figure 3-7A). In addition, ZIP14 expression was very elevated by 18 h post LPS injection (Figure 3-7B). Thus, the 18 h for LPS injection is appropriate to investigate how ZIP14-mediated zinc uptake and muscle zinc accumulation during acute inflammation response in muscle. As another measure of zinc uptake $^{65}\text{Zn}$ into gastrocnemius muscle of WT and Zip14 KO mice administered $^{65}\text{Zn}$ by oral gavage followed by an LPS i.p. injection. Uptake $^{65}\text{Zn}$ into muscle was slightly increase by
LPS in WT mice. In contrast, $^{65}$Zn uptake was significantly decreased in Zip14 KO mice after LPS treatment (Figure 3-7C). Non-heme iron in LPS-treated or KO mice was not different (Figure 3-7D).

**Deletion of Zip14 Augmented Inflammatory Responses in Gastrocnemius Muscle of Female Mice in Response to LPS.**

To examine ZIP14 involvement during inflammation, expression of inflammation-related genes such as *Mt1*, *Tnfa*, *Il-6*, and *iNOS* were measured. Both *Mt1*, *Tnfa* and *Il-6* mRNA expression were induced in LPS-injected WT and KO mice (Figure 3-8A - C). However, *iNOS* mRNA expression did not show any difference at 18 h post LPS injection (Figure 3-8D). An anti-inflammatory cytokine IL-10 mRNA level showed no difference between two groups of WT and Zip14 KO mice (data not shown).

To understand the potential relevance of Zip14 to inflammatory signaling activation, signaling mediators were screened including NF-kB, MAPK, and STAT3 pathways. LPS stimulated the secretion of pro-inflammatory cytokines through phosphorylation of STAT3 and MAPK over time. However, there was no observation of ZIP14-mediated involvement of the phosphorylation of STAT3 and MAPK signaling in WT and Zip14 KO gastrocnemius muscle. NF-κB activation was measured over time after LPS administration in gastrocnemius muscle from female WT and Zip14 KO mice. Interestingly, female Zip14 KO mice exhibited much greater NF-κB activation than WT mice, and this expression remained 3 to 9 h post LPS administration (Figure 3-9A). Consistent with *Il-6* mRNA induction, IL-6 protein was elevated after LPS injection (Figure 3-9B).

To test whether IL-6 activation is induced by Toll like receptor 4 (TLR4)-dependent signaling, TLR4 mRNA expression in gastrocnemius muscle was measured following LPS
administration and showed no significant difference in TLR4 mRNA expression in WT and Zip14 KO muscle (data not shown).

**Zip14 KO has Greater Insulin Sensitivity in Gastrocnemius Muscle at a Steady State and Reduced Insulin Signaling During LPS-Induced Endotoxemia.**

To determine an association between muscle ZIP14 expression and insulin signaling pathway, selected target proteins including muscle glycogen metabolism and insulin signaling pathways were examined in gastrocnemius muscle of WT and Zip14 KO mice. Zip14 KO mice elicited an increase on insulin receptor (IR) phosphorylation in muscle compared to control mice at a steady-state condition. Consistent with this finding, a downstream regulator of IR, expression of the inactive form of glycogen synthase kinase-3β (GSK-3β) protein was greater in unstimulated muscle of Zip14 KO mice (Figure 3-10A). LPS administration showed reduced muscle insulin signaling in both gastrocnemius muscle of WT and Zip14 KO mice, however, did not show a significant difference on IR and GSK3β phosphorylation of Zip14 KO mice compared to WT mice. Similarly, no significant difference obtained in muscle GSK3β expression between genotypes after LPS stimulation.

To investigate the possibility of Zip14 function and muscle atrophy as mentioned above, FoxO1 phosphorylation was determined by an immunoblot assay (Figure 3-10B). At a steady state, decreased (inactive) FoxO1 phosphorylation was shown in muscle of Zip14 KO mice compared to WT mice and probably due to a dysregulation of Akt/FoxO1-depedent signaling induced by insulin receptor. This present result suggests that increased level of dephosphorylated (active) FoxO1 may induce muscle atrophy in Zip14 KO mice.

In LPS-treated groups, Zip14 KO mice exhibited a reduction of phosphorylated (inactive) FoxO1 expression at 18 h-post LPS while no significant changes obtained over time course LPS administration.
Altered Muscle Zinc Metabolism on Gene Expression Profiles

To determine the profile of mouse gene expression and the molecular pathogenesis of muscle zinc metabolism during inflammation, we used microarray-based transcriptome profiling from female gastrocnemius muscle of LPS-injected and saline-injected WT and Zip14 KO mice.

Microarray-based transcriptome profiling using Affymetrix Mouse Clariom S Array (Thermo Fisher) was used and CEL format files were then processed based on SST and RMA algorithms. RMA was used for normalization and background correction. The mouse genome (Clariom_S_Mouse.mm10) database was used to select differentially expressed genes changed from four experimental groups (WT Con, WT LPS, KO Con, KO LPS). Gene-level fold change (FC) was set as FC < -2 or >2 and P-value < 0.05 (Figure 3-12).

A total of 2239 differentially expressed genes by genotype and treatment in the transcriptome profile were reviewed. As noted, the first step in gene expression analysis is the generation of hierarchical clustering (58). To determine the gene expression pattern, hierarchical clustering was conducted with 2239 genes, whose expression changed by genotype and treatment (Figure 3-13). Most muscle RNAs were responsive to expression, and many genes were altered by genotype and treatment.

Similar analyses of gene expression profiling were then conducted to investigate logical relations between the data sets. Of 2239 genes, 677 were shared by the Zip14 KO and WT mice with a fold-change (FC) > 2 or < -2 (P < 0.05) (Figure 3-14). Venn diagrams display the differentially expressed genes identified in muscle transcriptome profiles (Figure 3-15). This data directly supports the earlier observations that deletion of Zip14 in muscle may cause muscle atrophy and attenuate inflammatory responses.

To further investigate muscle zinc metabolism during inflammation, muscle tissues from Zip14 KO mice were examined. Samples demonstrated 114 genes whose expression was
increased or decreased compared with muscle samples from WT mice (Figure 3-16A). By LPS treatment, 142 genes whose expression altered shown in volcano plots (Figure 3-16B). The scatter plot in Figure 3-17 illustrates the genetic relationships of 677 genes. Specifically, the 10 up-regulated-regulated genes within each comparison are shown in Tables 3-1, 3-2 and 3-3.

To further explore how deletion of Zip14 in muscle may influence muscle inflammation, pathway analysis was conducted with 677 differentially expressed genes. This revealed top pathways involved in inflammatory responses in motor neuron diseases, muscle structure defects and Parkinson’s disease-related pathways (Figure 3-18).

**Discussion**

The study presented provides evidence and supports the role of muscle ZIP14 during inflammation. Specifically, the research demonstrated that: (1) LPS-induced inflammation accelerates muscle zinc accumulation in the primarily glycolytic gastrocnemius muscle of female mice; (2) Deletion of Zip14 results in gastrocnemius muscle atrophy and enhances muscle atrophy-related gene expression at a steady state; (3) ZIP14-mediated zinc uptake reduces NF-κB activation in the gastrocnemius muscle of female mice after LPS administration. These findings suggest, ZIP14 is both necessary and sufficient for muscle atrophy prevention, and presumably for suppression of NF-κB activation in the gastrocnemius muscle of female mice.

Endotoxin administration has been widely used in animal research to elicit acute inflammation. In this study, LPS (2 mg/kg BW), an established model of inflammation was used. It is sufficient to induce acute inflammation at a low and physiologically relevant dose and to initiate the Zip14-signaling cascade in response to LPS administration. Previous studies have demonstrated that ZIP14-mediated zinc uptake is required to maintain zinc homeostasis for normal functions in liver, small intestine, and white adipose tissues in mice during LPS-induced inflammation (39,40,59). It is noteworthy that female Zip14 KO mice exhibit hypozincemia after
LPS treatment and chronic inflammation at a steady state, whereas male Zip14 KO mice do not. These phenotypes suggest a sex-related response to inflammation. Augmented inflammatory responses in gastrocnemius muscle demonstrated here are due to deletion of Zip14. This is consistent with zinc-specific responses to various inflammation signals such as bacterial infection and zinc deficiency. In the animal model, NF-κB activation was greater in Zip14 KO mice compared to WT mice after LPS administration. This demonstrates that ZIP14-mediated zinc uptake lessens inflammatory activation in the gastrocnemius muscle of female mice.

Muscle atrophy is more pronounced in type I glycolytic gastrocnemius muscles than type I oxidative soleus muscles under cachectic conditions such as sepsis, fasting, and cancer (60). Gastrocnemius muscle Zip14 expression is more responsive to LPS than soleus muscle. For this reason, the gastrocnemius muscle was examined for further experiments. Findings suggest a sex difference in gastrocnemius muscle in response to acute inflammation. However, no sex difference in muscle atrophy and shortened leg length was observed. Both female and male mice demonstrate that ZIP14-mediated zinc transport is essential for muscle growth and development.

Muscle atrophy is a common phenotype observed in various systemic diseases and endotoxemia such as diabetes mellitus, cancer and sepsis (61). Several findings support that muscle atrophy is a result of activation of proteolysis mediating a muscle-specific ubiquitin-proteasome pathway (62). Specifically, muscle-specific ubiquitin ligases, muscle RING finger 1 (MuRF1, TRIM63) and muscle atrophy F-box 32 (MAFbx/Atrogin1) are required for protein degradation and progression of the muscle atrophy. This may explain physiological responses such as muscle wasting and weakness in catabolic states (63). Regulation of muscle protein synthesis and degradation during development has shown an association with insulin-like growth factor-1 (IGF-1)/Akt/Forkhead box O (FoxO) signaling pathways (64,65). Disruption of Akt
signaling induced by IGF-1 leads to an increase (dephosphorylated) of FoxO transcription factors. FoxO transcription factors are involved in initiating the muscle atrophy process and subsequently contribute to the activation of muscle atrophy-related genes (atrogenes) such as the MuRF1 (TRIM63) and Atrogin 1 (F-box 32) (66).

Recent evidence has demonstrated that a recruitment of FoxO1 during muscle atrophy plays a critical role in MuRF1 gene promoter activity. This suggests a direct link between activation of the atrogenes and FoxO transcription factor binding activity (67). Interestingly, metallothionein1 (mt1) mRNA expression was found to be elevated in the muscle of fasted mice and the catabolic condition exhibited a significant induction of MT1 transcripts in triple FoxO 1,3,4 muscle-specific KO mice after fasting when compared to fed-control mice (67). Consistent with this finding, MT1 mRNA was elevated in muscle following LPS administration (Figure 3-9A). This may suggest a potential association between regulation of muscle ZIP14 and Akt-mediated FoxO1 transcriptional activity for muscle atrophy. In addition to the MT1 induction in muscle, pro-inflammatory cytokines expression, including TNF-α and IL-6 mRNA, were increased in muscle wasting (68). In this context, LPS induces pro-inflammatory cytokines in the muscles of mice following a TNF-α-mediated mechanism may trigger muscle atrophy by altering Akt/FOXO signaling. Other aspects of insulin/Akt signaling were impacted in LPS-treated mice, including a significant reduction of insulin receptor substrate-1 (IRS-1) which is a downstream molecule of insulin signaling (69). Therefore, it could be predicted that an impairment of ZIP14 in muscle may contribute to activation of dephosphorylated (active) FoxO1 transcriptional regulation. This may play a role in inducing muscle atrophy through Akt/FoxO1 signaling.
In line with this prospective, membrane damage as a consequence of muscle atrophy is associated with dysregulation of several functional mediators such as calpain (70), dysferlin (71), and MG53 (72,73) in response to inflammatory stimuli. A recent study has demonstrated that muscle-specific MG53 proteins require zinc interaction with binding motifs required for membrane repair (74). This strongly suggests that MG53 may be a key mediator of muscle metabolism and, specifically dependent on ZIP14-mediated zinc transport to a specific binding activity during a LPS-induced endotoxemia model. LPS-treated muscle of Zip14 KO mice may be more susceptible to injury and disruption of muscle fiber growth.

Taken together, it is possible that the unique muscle phenotype found in Zip14 KO mice may be a result of cytokine-induced muscle atrophy, which leads to muscle structural deficits and impaired muscle performance. This finding is in line with previous results obtained from studies involving muscle atrophy mentioned above and appear to be relevant to an understanding of genotype-phenotype (GP) relationships in muscle metabolism (75).
Figure 3-1. Selection of the most stable reference gene for accurate normalization in murine skeletal muscle during LPS-induced inflammation. To test gene expression consistency, three commonly used reference genes (GAPDH, TBP, and 18S) were measured in gastrocnemius muscle from WT mice using qPCR. GADPH was most suitable as a reference gene for murine skeletal muscle after LPS injection to WT mice over time.
Figure 3-2. Effects of LPS injection (2 mg/kg BW, i.p.) on zinc concentrations in both female and male wild-type (WT) mice over a time course (3, 6, 9, and 18 h) and saline used as a control. A) Serum zinc concentrations were significantly reduced in both female and male mice following LPS challenge and remained the same during inflammation. B) Serum IL-6 level was increased after LPS. C) Deletion of Zip14 showed two-fold increase at 18h post LPS injection in gastrocnemius muscle of female mice but no change was shown in gastrocnemius muscle of male mice. D) Soleus muscle did not exhibit any significant differences during LPS-induced inflammation. Reported values were relative to GAPDH. Data were represented as mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001
Figure 3-3. Effects of LPS injection (2 mg/kg BW, i.p.) on cytokine expression in gastrocnemius muscle of female and male wild-type (WT) mice at 18 h after i.p. injection and saline used as a control. A) Zip14 expression was significantly increased in gastrocnemius muscle of both female and male mice after LPS injection. B) Znt2 expression in gastrocnemius muscle was more responsive to male mice at a steady state. After LPS injection, Znt2 expression in female mice showed a 10-fold increase after LPS while male mice exhibited an 80-fold elevation at 18 h post injection. C) Mt1 expression in gastrocnemius muscle increased at 18 h post LPS injection but did not exhibit any sex difference during inflammation. D) Il6 expression in gastrocnemius muscle was increased in both female and male WT mice during LPS-induced inflammation. Reported values were relative to GAPDH. Data were represented as mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001
Figure 3-4. Skeletal muscle atrophy in Zip14 KO mice. A and C) Growth retardation in female and male Zip14 KO mice. B and D) Representative images in gastrocnemius muscle of female and male Zip14 KO mice (photos courtesy of author). E and F) The ratio of gastrocnemius muscle weight to body weight of adult 3-5 months of female and male mice (n = 12 per genotype, mean ± SEM, *P < 0.05). G and H) Histopathology of female and male WT and Zip14 KO gastrocnemius muscle (H&E, original magnification X20). I) Activation of inflammatory pathways of muscle from WT and Zip14 KO mice at a steady state.
Figure 3-5. Zinc concentrations in serum and gastrocnemius muscle in female and male of WT and Zip14 KO mice during LPS-induced inflammation. Female and male of wild-type (WT) and Zip14 KO mice were injected with either LPS (2 mg/kg, i.p.) or saline. A and B) Serum zinc concentrations in female and male WT and Zip14 KO mice after LPS injection. C and D) Muscle zinc concentration in female and male WT and Zip14 KO mice after LPS. Values are mean ± SEM, n = 3-4. * P < 0.05; ** P < 0.01; *** P < 0.001.
Figure 3-6. Zinc and manganese concentrations measured by MP-AES (microwave plasma-atomic emission spectrometer) in gastrocnemius muscles of female and male WT and KO mice during LPS-induced inflammation. LPS was administered (2 mg/kg, i.p.) 18h before sacrifice. A) Zinc concentrations in gastrocnemius muscle. B) Manganese concentrations in gastrocnemius muscle. C) Liver zinc concentration was measured as a control. Values are mean ± SEM, n = 3-4 (three-way ANOVA with Tukey’s post hoc test).
Figure 3-7. ZIP14 expression and zinc concentration in gastrocnemius muscle of female WT and KO mice in LPS-induced inflammation. Female wild-type (WT) and Zip14 KO mice were injected with either LPS (2 mg/kg, i.p.) or saline. A) Zip14 mRNA expression showed a peak at 18 h post injection over time course (3, 6, 9, 18, 24, and 48 h) after LPS injection (mean ± SEM; n = 3 - 4 for each genotype). B) ZIP14 expression was increased over time and exhibited a markedly elevated expression at 18 h after LPS injection. C) Uptake of $^{65}$Zn into muscle in LPS-treated mice following administration of oral radioisotope exhibited impairment in Zip14 KO mice. D) Non-heme iron level was not different. * P < 0.05; ** P < 0.01
Figure 3-8. Effects of LPS injection (2 mg/kg BW, i.p.) on cytokine expression in gastrocnemius muscle of female wild-type (WT) and Zip14 KO mice at 18 h after i.p. injection. A) Mt1 mRNA abundance. B) TNFα mRNA expression. C) Il-6 mRNA expression. D) iNOS mRNA expression in gastrocnemius muscle from female WT and Zip14 KO mice. Values are mean ± SEM, n = 3-4. * P < 0.05; ** P < 0.01
Figure 3-9. Deletion of Zip14 significantly influences NF-κB activation and IL-6 production in gastrocnemius muscle during LPS-induced inflammation and muscle atrophy. Female and male wild-type (WT) and Zip14 KO mice were injected with either LPS (2 mg/kg, i.p.) or saline. A) NF-κB activation shown by western blot. B) IL-6 protein production in muscle by ELISA. Values are mean ± SEM, n = 3 – 4, ** P < 0.01.
Figure 3-10. LPS impaired insulin-related signaling pathways in gastrocnemius muscle of WT and Zip14 KO mice (n = 3 for each genotype). A) Phosphorylation of insulin receptor (IR), AKT, glycogen synthase kinase 3 (GSK3), and glycogen synthase (GS) in gastrocnemius muscle compared to WT mice after LPS. B) Phosphorylation of forkhead box O1 (FOXO1) and protein kinase A (PKA) in LPS-injected gastrocnemius muscle.
Figure 3-11. Proposed mechanism of muscle ZIP14 in a LPS-induced murine inflammation model. In response to inflammatory stimulus such as an LPS, the TLR4 (Toll-like receptor 4) pathway is activated. Inflammatory cytokines are produced as autocrine or paracrine response and activate IL-6R pathways. These lead to activation of NF-κB and STAT3 pathways which upregulate Zip14 transcription.
Figure 3-12. Flowchart of array-based transcriptome profiling in gastrocnemius muscle of WT and Zip14 KO mice after LPS i.p. injection (2 mg/kg BW). Mouse Clariom S Assay (Affymetrix) was used for gene expression profiling.
Figure 3-13. Hierarchical clustering of genes with altered expression levels in female WT and Zip14 KO mice in response to LPS. Four experimental groups were compared to determine gene expressions of 2240 genes detected by the transcriptome profile (n=3-4/group).
Figure 3-14. Data analysis of differentially expressed genes. A) Flowchart representing the pipeline used for transcriptome analysis in muscle of LPS and saline-injected WT and Zip14 KO mice. B) Differentially expressed genes with fold change > 2 or <-2 between the data sets.
Figure 3-15. Venn diagram of the differentially expressed genes between the different comparisons (genotype and treatment). The number in each circle represents the number of differentially expressed genes within each comparison. There were 482 genes differentially expressed between WT Con vs WT LPS, 114 genes between WT Con vs KO Con, and 142 genes between the treated groups, WT LPS vs KO LPS (p-value < 0.05).
Figure 3-16. Volcano plots in different experimental groups. A) Volcano plot displaying differentially expressed genes in muscle tissues within control groups, WT control and KO control. B) Volcano plot displaying differentially expressed genes in muscle tissues within the treated groups, WT LPS and KO LPS.
Treatment comparison

WT LPS vs KO LPS
(142 genes)

Figure 3-16. Continued
Figure 3-17. Scatter plot of fold change (FC) KO LPS/KO Con vs. WT LPS/WT Con
Figure 3-18. A gene network associated with spinal cord injury in muscle is impacted by genotype. Genes presented in red are up-regulated in Zip14 KO Control group. Genes presented in green are down-regulated in Zip14 KO Control group. The intensity of the colors is related to fold change. Genes in white are not in the gene expression profiling list, but they are incorporated into the network pathway.
Table 3-1. The top 10 up-regulated genes in KO vs WT mice

<table>
<thead>
<tr>
<th>WT Con Avg (log2)</th>
<th>KO Con Avg (log2)</th>
<th>Fold Change</th>
<th>Gene Symbol</th>
<th>Description</th>
<th>P-val</th>
<th>FDR</th>
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<tr>
<td>14.94</td>
<td>17.83</td>
<td>7.41</td>
<td>Car3</td>
<td>carbonic anhydrase 3</td>
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<td>0.5141</td>
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<tr>
<td>9.85</td>
<td>12.57</td>
<td>6.63</td>
<td>Mir675; H19</td>
<td>microRNA 675; H19, imprinted maternally expressed transcript</td>
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<td>0.7567</td>
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<td>7.86</td>
<td>9.99</td>
<td>4.41</td>
<td>Sparc</td>
<td>secreted acidic cysteine rich glycoprotein</td>
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<tr>
<td>10.07</td>
<td>11.91</td>
<td>3.58</td>
<td>Pfkfb3</td>
<td>6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3</td>
<td>0.000</td>
<td>0.5215</td>
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<tr>
<td>5.42</td>
<td>7.05</td>
<td>3.09</td>
<td>Vmn1r116</td>
<td>vomeronasal 1 receptor 116</td>
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<td>0.0043</td>
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<tr>
<td>10.3</td>
<td>11.79</td>
<td>2.8</td>
<td>Hspb7</td>
<td>heat shock protein family, member 7 (cardiovascular)</td>
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<td>6.83</td>
<td>8.31</td>
<td>2.79</td>
<td>Gm10663</td>
<td>predicted gene 10663 [Source:MGI Symbol;Acc:MGI:3642419]</td>
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<td>12.5</td>
<td>13.88</td>
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<td>Dcn</td>
<td>decorin</td>
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<td>12.73</td>
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<td>7.25</td>
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<td>Gm13242</td>
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Table 3-2. The top 10 up-regulated genes in KO vs WT mice treated with LPS

<table>
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<tr>
<th>WT LPS Avg (log2)</th>
<th>KO LPS Avg (log2)</th>
<th>Fold Change</th>
<th>Gene Symbol</th>
<th>Description</th>
<th>P-val</th>
<th>FDR P-val</th>
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<td>10.79</td>
<td>13.48</td>
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<td>predicted gene 10717 [Source:MGI Symbol;Acc:MGI:3642031]</td>
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<td>0.967</td>
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<td>15.23</td>
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<td>Gm10800</td>
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<td>11.58</td>
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<td>Hp</td>
<td>haptoglobin</td>
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<td>0.918</td>
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<td>6.93</td>
<td>8.54</td>
<td>3.06</td>
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<td>RIKEN cDNA 1700129C05 gene</td>
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<td>0.922</td>
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<tr>
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<td>9.03</td>
<td>2.96</td>
<td>Gm765</td>
<td>predicted gene 765</td>
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<td>0.967</td>
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<td>7.34</td>
<td>8.9</td>
<td>2.94</td>
<td>Smek2</td>
<td>SMEK homolog 2, suppressor of mek1 (Dictyostelium)</td>
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<td>0.967</td>
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<td>6.27</td>
<td>7.72</td>
<td>2.74</td>
<td>V1rd18; V1rd19</td>
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<td>8.62</td>
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<td>Olfr1502</td>
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<td>WT Con Avg (log2)</td>
<td>WT LPS Avg (log2)</td>
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<td>Gene Symbol</td>
<td>Description</td>
<td>P-val</td>
<td>FDR</td>
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<tr>
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<td>140.49</td>
<td>Pdk4</td>
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<td>Serpina3n</td>
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<td>41.79</td>
<td>Cdkn1a</td>
<td>cyclin-dependent kinase inhibitor 1A (P21)</td>
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CHAPTER 4
PROTEOMIC PROFILING BY SOMA SCAN IN SERUM OF ZIP14 KNOCK-OUT MICE
DURING LPS-INDUCED INFLAMMATION

Background

Mouse models have been used to better understand mechanisms of inflammation and are thought to be a useful tool in the study human diseases. Genetically engineered mouse models are particularly useful. Serum has been used extensively for proteomic profiling to identify biomarkers of human diseases related to inflammation and has ultimately provided novel findings for diagnostics and therapeutics. This platform has been marketed to provide extensive coverage and detection of serum proteins.

The initial intent of this proteomic analysis was to evaluate differentially expressed proteins from lysates of muscle tissue from the WT and Zip14 KO mice. Because of the trial nature of the agreement between ICBR and Soma Logic, we were asked to use serum rather than muscle lysate samples (Figure 4-1).

Results

To comprehensively profile the mouse serum proteome, we applied an approach based on SOMA (Slow Off-rate Modified Aptamers) scan profiling (Figure 4-2). Equal volumes of serum from LPS-injected WT and Zip14 KO mice and saline injected WT and Zip14 KO mice were used. Twelve individual serum samples were analyzed. A total of 1139 serum proteins were identified with SOMAmers (Slow off-rate modified aptamers), which bind human serum proteins (or predicted murine homologues) by SOMA scan methodology. After all four experimental groups were compared, a total of 275 proteins were revealed to have a statistically significant change in abundance as determined by Kruskal-Wallis one-way ANOVA (P < 0.05).

To assess whether the data were normally distributed, scatter plots of mean relative fluorescent units (RFU) were generated (Figure 4-3A-D). The scatter plots were derived from
serum of Zip14 KO versus WT mice (Figure 4-3B), WT, LPS-treated versus WT mice (Figure 4-3C), and WT, LPS-treated versus Zip14 KO mice (Figure 4-3D).

The distribution of 66 significantly abundant proteins between all treatment groups was shown in the Venn diagram and Heat map (Figure 4-4 and 4-5). These serum proteins were upregulated over 1.5 fold in KO, WT, LPS, KO, LPS compared to WT in each group. Protein names in each group are listed in Table 4-1.

The results from proteomics revealed that there were 12 differentially expressed proteins in the KO/WT groups: NXPH1, CK2-A1:B, MIC-1, SARP-2, PTN, Mn SOD, SP-D, PDGF-CC, RSPO3, Semaphorin 3A, WISP-3, and FGF-8A. Among these 12 serum proteins, the WT mice shared the most differentially expressed protein (surfactant protein D; SP-D) with the KO, KO-LPS and LPS-treated WT mice. This demonstrated that SP-D may be an important immune modulator after infection or an inflammatory process.

To further characterize biological significance of the SP-D serum proteins, we measured whether SP-D transcripts were expressed in gastrocnemius muscle and the lung of Zip14 KO and LPS-treated WT mice. SP-D has been reported to be a lung immunomodulator (76). As expected, SP-D transcripts were more abundant in lung than in the gastrocnemius muscle of mice (Figure 4-6A and B). SP-D mRNA expression was increased in the lungs of LPS-treated WT mice and in Zip14 KO mice, but to a lesser extent. Of note is the 15-fold increase in Zip14 mRNA in lung of the WT mice (Figure 4-6C). These data suggest that SP-D may be produced in other tissues in mice with deleted Zip14. Of considerable significance is the unexpected finding that LPS reduced Sp-d mRNA abundance in lung (Figure 4-6B).
The other potential serum protein which may be involved in the muscle atrophy of Zip14 KO mice is Semaphorin 3A (Sema3A). Somascan data demonstrated that this protein could potentially be secreted from muscle in Zip14 KO mice.

**Discussion**

This is the first study to characterize serum proteins influenced by Zip14 during LPS-induced inflammation using SOMA (slow off-rate modified aptamers) scan proteomic profiling. Our proteomics data revealed that serum levels of SP-D are increased in the KO mice compared to the WT mice following LPS treatment. Having shown that SP-D modulates inflammation in some tissues, SP-D levels in the serum of mice may be influenced by tissue inflammation in the muscle after LPS or due to chronic low-grade inflammation by deletion of Zip14. Of note is that serum SP-D is increased during acute and chronic inflammation in mice (77). It has been documented that SP-D serves as a biomarker of lung inflammation (78-80). Deletion of SP-D induced reactive oxygen species (ROS) in lung macrophages and increased NF-kB activity. This suggests that SP-D may play a pivotal role in regulating the NF-kB pathway (81). In addition to involvement in lung inflammation, SP-D is expressed in human vascular smooth muscle cells (SMCs) and binds to LPS (82). In a previous study, SP-D mRNA was detected in cultured human smooth muscle cells (SMCs) after LPS treatment (2 ng/ml) for 2-24h. SP-D mRNA showed a peak at 12 h along with induction of the proinflammatory cytokine IL-8 in that model (83). The data are consistent with the model (Figure 4-7). SP-D may be secreted from lung cells of Zip14 KO mice during LPS-induced inflammation by activating NF-kB signaling pathway.

Another potential mediator may be Sema3A protein. Sema3A is a secreted protein which acts as a guide to the axons of the nervous system (84). A previous study showed that this protein was elevated in motor neurons of patients with Amyotrophic lateral sclerosis (ALS). Amyotrophic lateral sclerosis (ALS) is a neurodegenerative condition which results from
degenerating a capacity to control voluntary muscles. It leads to muscle atrophy, limb paralysis, stiff muscles, shortness of breath, and respiratory failure (85). This is also known as motor neuron disease (MND) or Lou Gehrig’s disease. There are two forms of the ALS. The common form is sporadic (90-95%) and is not associated with genetic inheritance. The other type, (5-10%) is a familial-type due to family history. This disease is late-onset disease and usually appears between the age of 50-65 (86).

Worldwide continued efforts are needed to develop more effective ALS treatments such as drugs or nutritional interventions. Therefore, zinc could be of potential importance to ALS patients and may protect against muscle atrophy and stiffness at the early stage of disease.
Figure 4-1. Coomassie blue staining in gastrocnemius muscle of WT and Zip14 KO mice after LPS administration (n=3/group).
Figure 4-2. Experimental plan for proteomic profiling by SOMA scan in serum of WT and Zip14 KO mice during LPS-induced inflammation. We performed proteomic profiling by using SOMAscan proteomics of serum of WT and Zip14 KO mice following 18 h of LPS i.p. injection. This proteomics approach was aimed at identifying potential serum biomarkers influenced by ZIP14 during inflammation. The serum samples from WT and Zip14 KO mice after LPS or saline (control) were used in this analysis.
Figure 4-3. Identification of differently abundant proteins in four experimental groups. A) When all four experimental groups were compared, a total of 275 proteins were revealed statistically significant changes in abundance from all 1129 proteins by Kruskal-Wallis one-way ANOVA (P < 0.05). To determine the most promising candidate serum biomarkers influenced by ZIP14 detection during inflammation, we selected those proteins with fold changes over 1.5 for further analysis. B) Scatter plot of mean relative fluorescent units (RFU) in Zip14 KO vs WT. C) Scatter plot of mean relative fluorescent units (RFU) in WT mice treated by LPS vs WT. D) Scatter plot of mean relative fluorescent units (RFU) in WT mice treated by LPS vs KO.
Figure 4-4. Venn diagram of differentially abundant proteins in serum of mice from three experimental groups (KO, WT LPS, KO LPS) compared to WT by SOMAscan proteomics. When all four experimental groups were compared, a total of 275 proteins had statistically significant changes in abundance from all 1129 proteins detected using a Kruskal-Wallis one-way ANOVA (P < 0.05). A total of 66 serum proteins were upregulated in the groups indicated.
Table 4-1. Identification of the 66 serum proteins from the treatment groups that were upregulated by over 1.5 fold change (FC).

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<tr>
<th>Groups</th>
<th>Total</th>
<th>Protein names</th>
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<tr>
<td>KO/WT, KOLPS/WT, WTLPS/WT</td>
<td>1</td>
<td>SP-D</td>
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<tr>
<td>KO/WT, KOLPS/WT</td>
<td>3</td>
<td>CK2-A1:B, WISP-3, PDGF-CC</td>
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<td>FSTL1 BDNF ANNEXIN I CD30 INTEGRIN A1B1 NID2 ALPHA ENOLASE AMGO2 HSP 70</td>
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<tr>
<td></td>
<td></td>
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<td></td>
<td></td>
<td>A IRF1 HSP 90A/B GOT1 HTRA2 KEAP1 YES RAC3 H31 NIDOGEN</td>
</tr>
<tr>
<td>KO/WT</td>
<td>8</td>
<td>RSPO3 PTN SEMAPHORIN 3A MN SOD FGF-8A SARP-2 MIC-1 NXPH1</td>
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<td>PPASE IL-13 RA1 IL-17 RC PIM1 HXK2</td>
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Figure 4-5. SOMAscan proteomic assay of the 66 proteins in serum of mice from the groups that were upregulated by over 1.5 fold change. Heat map and hierarchical clustering of 12 proteins with statistically significant in serum of mice. Samples from three female mice per group were analyzed.
Table 4-2. The top 12 of serum proteins were shown normalized to WT

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Significant targets having a fold change (FC) > 1.5 in Zip14 KO mice and P < 0.05
Figure 4-6. SP-D mRNA expression in gastrocnemius muscle and lung of female WT and Zip14 KO mice in LPS-induced inflammation. A) SP-D mRNA in gastrocnemius muscle. B) SP-D mRNA in lung. C) Zip14 mRNA in lung. LPS was administered 18h (2 mg/kg, i.p.) before sacrifice. n = 3-4, ** P < 0.01
Figure 4-7. Proposed model for a mode of ZIP14 and expression of SP-D in lung of mice during LPS-induced inflammation and release of the protein into the systemic circulation. SP-D may be secreted from lung cells of Zip14 KO mice during LPS-induced inflammation by activating NF-kB signaling pathway.
CHAPTER 5
EFFECTS OF HIGH FAT DIET (HFD)-INDUCED CHRONIC INFLAMMATION ON ZIP14 IN SKELETAL MUSCLE

Background

In a previous study in our lab (unpublished data), female WT and Zip14 KO mice were given a high fat diet (60% kcal from fat, Research diets D12492) for six weeks to investigate whether dietary fat was capable of inducing low-grade chronic inflammation. Glucose intolerance and insulin resistance were evident as effects of this HFD feeding in both genotype mice. However, at transcript level, muscle Zip14 expression showed no significant difference between chow- and HFD-fed groups of WT mice; although zinc concentration in gastrocnemius muscle was unchanged by HFD feeding. This observation led to further investigation of muscle ZIP14 may be increased during chronic inflammation induced by long-term HFD treatment.

It has demonstrated that male and female muscle fibers in HFD-fed mice showed different responses (87). For example, muscle fibers exhibited a different response to HFD treatment in order to cause skeletal muscle atrophy via proteolysis pathway in oxidative soleus muscle (88). This indicated an alteration of muscle fiber switching between slow-type fibers and fast-type fibers that metabolize fat and glucose in different ways. It is relevant because zinc metabolism is affected by disruption of glucose homeostasis and is followed by endotoxemia. Given this finding, muscle zinc and ZIP14 may be involved in chronic inflammation and reacts differently in a sex-dependent manner.

Results

In line with roles of muscle zinc and ZIP14 during acute inflammation induced by LPS, we examined to determine effects of chronic inflammation through a long-term high fat diet for 16 weeks in both female and male WT and Zip14 KO mice (Figure 5-1). This is a well-known research model for metabolic endotoxemia to study diet-induced obesity and dietary fat sensing
pathways. The aim of this study is to provide an insight into the interaction of muscle zinc and dietary fat metabolism during chronic inflammation.

After long-term HFD feeding, both male and female WT and KO mice significantly gained body weights (Figure 5-2A). Unexpectedly, HFD-fed female KO mice showed a shortened survival rate (Figure 5-2B). To further investigate effects of HFD feeding, oral glucose tolerance test (OGTT) and insulin tolerance test (ITT) were performed. Female Zip14 KO mice exhibited glucose intolerance after 16 week-HFD treatment. HFD-fed Zip14 KO mice did not show glucose intolerance (Figure 5-3 A-C). To investigate the association between glucose metabolism and mortality, glucose metabolism-related targets were checked by western blot (Figure 5-4 D). Zip14 KO mice showed glucose impairment after HFD treatment.

In light of the elevated muscle ZIP14 expression during LPS-induced inflammation, a HFD for 16 weeks led to low-grade inflammation and increased ZIP14 expression in gastrocnemius muscle of WT mice (Figure 5-4A). However, muscle zinc and manganese concentrations did not exhibit accumulation after HFD treatment (Figure 5-4B-C). Non-heme iron concentration after HFD feeding was not changed (5-4D).

**Discussion**

HFD-induced obesity resulted in dysregulated zinc metabolism, which led to glucose impairment and chronic low-grade inflammation in skeletal muscle (89). ZIP14 may be involved in metabolic endotoxemia and influence fat oxidation induced by long-term high fat diet (HFD) treatment, thus impairing zinc metabolism in gastrocnemius muscle of mice.

**Zip14** KO mice exhibited a more pronounced inflammatory response by showing low-grade chronic inflammation (metabolic endotoxemia) compared to WT mice (40,41,90). It has been established that HFD increases triglyceride concentrations and induces insulin resistance by inhibiting GLUT4 expression (91). HFD feeding may lead the free fatty acid (FFA)
concentration which promotes TLR4 signaling in a similar way of LPS induction, therefore possibly leading to metabolic endotoxemia. An impairment of a regulatory role in muscle Zip14 by activating the pathophysiologic role of FFAs may be involved in HFD-induced insulin resistance and impairment of glucose metabolism by mediating inflammatory pathways.

Collectively, ZIP14 may play an important role in mediating fatty acid oxidation during HFD-induced metabolic endotoxemia. This suggests that dietary free fatty acids (FFAs) may initiate pro-inflammatory cytokine signaling to induce Zip14 regulatory mechanisms in skeletal muscle.
Figure 5-1. A mouse model of diet-induced inflammation. Mice were fed a high-fat diet or chow diet for 16 weeks to induce chronic inflammation. WT and Zip14 KO mice were used (n=4/group).
Figure 5-2. Physiological change and survival rate in chow- and HFD-fed WT mice and KO mice. A) Effects of HFD on body weight gain in both HFD-fed WT mice and KO mice. B) HFD treatment for 16 weeks showed decreased survival rate in KO mice group.
Figure 5-3. Effects of HFD on glucose metabolism. A-C) Oral glucose tolerance test (OGTT) were performed. D) Glucose metabolism-related targets were checked by western blot (n=3-4/group).
Figure 5-4. Metal concentrations in muscle after HFD treatment. A) Muscle ZIP14 expression was increased in HFD-fed group. B) Muscle zinc concentration after HFD treatment was not changed. C) Muscle manganese concentration did not change by HFD treatment, but KO mice at a steady state showed increased than WT mice. D) Non-heme iron (NHI) concentration after HFD was not altered.
CHAPTER 6
CONCLUSION

The overall goal of this study was to define a role of Zip14 in mediating immune responses in skeletal muscle during acute (induced by LPS injection) and chronic (induced by feeding a high-fat diet) inflammation and determine regulatory mechanisms and cytokine pathways controlled by Zip14 in skeletal muscle.

The findings in this study provided an insight into the importance of muscle zinc in terms of muscle atrophy and inflammation. Muscle zinc has a protective role against atrophy and aids in reducing inflammatory responses. ZIP14 was shown to contribute to zinc uptake in skeletal muscle. Inflammation in skeletal muscle caused altered zinc metabolism, which resulted in enhanced proinflammatory cytokine production, such as increased interleukin-6 (IL-6), and a phenotypic defect shown as muscle atrophy. Elevation of IL-6 is sufficient to induce muscle-wasting cachexia via regulation of inflammatory signaling and protein degradation pathways. Thus, muscle zinc is required for the normal function and prevention of chronic inflammation-related diseases. In addition, ZIP14 ablation causes dysregulation of genes that are linked to muscle atrophy.

This study may contribute to fields of muscle diseases and can be translated into clinical studies converging on human diseases research in skeletal muscle. In particular, ZIP14-mediated zinc uptake in muscle may potentially be beneficial to aging and Parkinson’s disease in clinical studies.
LIST OF REFERENCES


BIOGRAPHICAL SKETCH

Jinhee Kim was born in Daegu, South Korea. Jinhee received her Bachelor of Science degrees in applied biosciences and chemistry education at Kyungpook National University in Daegu, South Korea in 2011. She moved to Seoul to attend Seoul National University in Seoul, South Korea where she earned her master’s degree in agricultural biotechnology. In 2014, she came to Gainesville, Florida to pursue her PhD program for nutritional sciences in Food Science and Human Nutrition Department at the University of Florida. She started to work in Dr. Cousins’ Lab and focused on the fields of nutritional biochemistry. She received her Ph.D. from the University of Florida in the summer 2018.