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NRF AS AN OXIDATIVE STRESS AND NUTRIENT RESPONSIVE TRANSCRIPTION FACTOR IN CALORIE RESTRICTION

By

LAURA ARMSTRONG

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY
IN

PHARMACOLOGY AND TOXICOLOGY

UNIVERSITY OF RHODE ISLAND

2015

DOCTOR OF PHILOSOPHY DISSERTATION

OF

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2015

ABSTRACT

Metabolic syndrome (MetS) can classically be defined by physiological factors, clinical factors, and metabolic factors that are responsible for increased risk of cardiovascular diseases (CVD) and type 2 diabetes (T2D). The International Diabetes Federation has released the most recent definition of MetS, defined by increased waist circumference and two of the following factors: increased serum triglycerides or decreased HDL-cholesterol, increased fasting blood glucose, or hypertension. MetS is also profoundly defined by chronic low-grade inflammation. This proinflammatory state can develop as a result adipocyte hypertrophy and hyperplasia that is responsible for increased secretion of glycerol, free fatty acids, and secretion of adipocytokines. States of adipose tissue dysregulation due to obesity can directly affect systemic inflammation, and the progression to diseased states of insulin resistance and MetS. Adipose tissue does not only contribute to the pathophysiology through proinflammatory signaling, but can alter systemic lipid content by releasing circulating free fatty acids (FFAs) that accumulate in liver, induce insulin resistance in skeletal muscle, and impair pancreatic β-cell functions, all contributing to the potential development of severe metabolic diseases. Most importantly though, oxidative stress has been suggested to be an early event in the pathophysiology and manifestation of chronic metabolic disorders in humans including MetS, T2D, coronary artery disease, and hypertension.

The excess storage of lipids in white adipose tissue (WAT), known as obesity, results in oxidative stress and infiltration of macrophages that can alter WAT metabolism. WAT is a metabolically active organ that is essential for proper maintenance of systemic energy balance. WAT is a storage depot for triglycerides, and through hormonal-signaling is responsible for the release of energy to be utilized by tissues, such as, liver and skeletal muscle. In addition to lipid metabolism and mobilization, WAT is responsible for glucose homeostasis, and has major endocrine functions involving secretion of hormones, cytokine, and transcription factors. Due to the major functions of WAT, it is suggested that WAT plays an important role in the development of obesity-related diseases—requiring greater knowledge and understanding of WAT development, signaling pathways, and its role in systemic diseases.

As stated previously, oxidative stress is a unifying characteristic of factors that contribute to MetS, and it is well established that increased lipid content and metabolism can contribute to localized oxidative stress. Metabolic stress within WAT can be defined as the balance between antioxidants and oxidative stress—and publications have begun to demonstrates its effects on adipose tissue, as it relates to metabolic diseases. Another form of oxidative stress is achieved through the generation of reactive oxygen species (ROS) by environmental toxicants. This form of chemical-induced oxidative stress can also be considered important in WAT due to the highly lipophilicity and deposition of persistent organic pollutants in adipose tissue, and the growing concern of these chemicals to act as obesogens. Within this dissertation,

Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) is addressed as a potential responsive transcription factor to both metabolic stress and nutrient status within a cell, and chemical-induced oxidative stress by persistent lipophilic environmental toxicants in WAT.

Nrf2 is a nuclear transcription factor that is known to regulate expression of antioxidant proteins in response to stimulation by oxidative stress or inflammation. Nrf2 is still studied for its cytoprotective effects, but has recently been shown to regulate genes involved in lipid metabolism. Therefore, this overlap in the regulation of antioxidant response and lipid metabolism through antioxidant response element (ARE) activation provides a potential model for the interplay between oxidative stress and metabolic diseases. Our lab has been able to establish the importance of Nf2 in models of steatosis, calorie restriction in the liver, and most recently in obesity and adipogenesis.

The purpose of the study performed in MANUSCRIPT I is to elucidate the role of Nrf2 in calorie restriction and identify a potential mechanism of Nrf2-dependent lipid regulation involved in weight loss and decreased fat storage. Few studies exist that have shown Nrf2 activation in adipose tissue (AT), and even fewer studies demonstrate the response of Nrf2 to nutrient status. The data presented in this study demonstrate that 1) The ARE, Nrf2, and classical downstream target expression is induced by calorie restriction in WAT, 2) Nrf2 induction bolsters the antioxidant capacity *in vivo* and *in vitro*, 3) Key regulators of calorie restriction and WAT lipid metabolism are induced by an

Nrf2-dependent pathway, 4) Important enzymes involved in lipid uptake, lipogenesis, and lipolysis are Nrf2-dependent genes, and 5) CR mimetics and Nrf2 activators can decrease lipid content of adipocytes.

In summary, MANUSCRIPT I, demonstrates the importance of increased antioxidant capacity to alleviate factors involved in the development of MetS, such as: glucose intolerance, hypertriglyceridemia, and obesity. The most significant finding is the ability of Nrf2 to respond to the nutrient status of the organism in order to regulate signaling pathways related to energy storage and mobilization.

Nrf2 has a novel role in its response it metabolic stressors, nutrient overload, or nutrient deprivation, but the classical activation of Nrf2 by chemical-induced oxidative stress can be reconsidered in understanding how environmental contribute to development of fat mass or obesity. Many environmental toxicants are known to stimulate the ARE/Nrf2 signaling pathway, and it has been considered a hallmark of induction of oxidative stress. Most recently though, environmental chemicals are more persistent in the environment and more lipophilic, therefore being deposited in lipophilic tissues such as AT. The ability of these compounds to activate Nrf2 may not result in an antioxidant response, but can potentially alter the homeostasis of WAT and cause AT dysfunction. AT dysfunction is the hallmark of many diseases, and can be directly to related to obesity. In MANUSCRIPT II, 2,2',4,4',5-pentabromodiphenyl ether (BDE-99) is studied for its ability to induce adipogenesis and lipid development in models of pre-adipocyte differentiation.

The ability of the compound to act as an obesogen in WAT, allows for Nrf2 to be studied in a mechanism of chemical-induced regulation of lipid metabolism. Therefore, the purpose of MANUSCRIPT II, was to demonstrate that environmental toxicants can alter Nrf2 expression and subsequent oxidative stress response to alter lipid metabolism in WAT. The observations in this study support 1) The role of BDE-99 in the development of excess lipids during adipocyte differentiation, and 2) A potential mechanism of decreased Nrf2-signaling during differentiation.

To summarize, MANUSCRIPT II, demonstrates the ability of an environmental chemical to alter WAT metabolism and contribute to adiposity, through a potential Nrf2-dependent regulation of antioxidant capacity or lipid metabolism directly.

Lastly, MANSCURPT III, studies the ability of perinatal deltamethrin exposure to alter WAT metabolism. The focus of this study was to demonstrate that an environmental compound exposure during gestation and alter WAT metabolism and result in phenotypic changes. The observations in the study demonstrate 1) Nrf2 can be induced by chemical-exposure in WAT, 2) Perinatal-exposure to deltamethrin has a hermetic effect on gene expression that does not result in phenotypic changes as adults, 3) Deltamethrin could epigenetically reprogram gene expression, and potentially be involved in the development of susceptibility to obesity or metabolic diseases related to WAT metabolism.

In summary, MANUSCRIPT III, represents a perinatal exposure model in which Nrf2 is downregulated in WAT and results in a subsequent down-regulation of genes involved adipogenesis, fat metabolism, and cytokine expression.

In conclusion, this dissertation establishes a link between antioxidant response/oxidative stress and WAT metabolism through the ARE/Nrf2 signaling pathway and continues to demonstrate that Nrf2 activation is a hormetic effect and the timing, disease model, and length or intensity of activation need to be considered in order to maintain homeostasis of the vital signaling pathways regulated by Nrf2.

AKNOWLEDGEMENTS

The completion of my Ph.D. and graduate studies at the University of Rhode Island would not have been possible without the drive from my advisor, the support and motivation from my colleagues, and the continual push from friends and family. I want to first acknowledge all those that have contributed to my graduate school career and dissertation and extend my utmost gratitude; I really wouldn't have been able to do it without every individual statement or action of support. Secondly, I must thank Dr. Angela Slitt for her continued and unwavering guidance and belief in my abilities as a scientist. She not only provided the necessary support all-around to guide me through my dissertation, but continually pushed me further as an intellectual. I want to extend my gratitude towards my dissertation committee members, Dr. Bingfang Yan, Dr. Kathleen Melanson, Dr. Disa Hatfield, and Dr. Eric Harstad supporting my continually growing set of scientific data and aiding in creating the greater picture. Without my committee this expansive subject area might not have been focused, but in having my committee endure multiple meetings and data reviews I was able to achieve a clear and concise contemplation of data that began 5 years ago. My committee was integral for me in understanding the breadth of my research for multiple disciplines and proving to myself the significance of my research.

A major lesson I learned within my program is that research is defined by discussing data, analyzing data, and verifying your results; and for that I am thankful and extend my deepest gratitude to the individuals that I interacted

with every day and were my sounding board throughout my graduate school career. These include lab members past and present: Dr. Jialin Xu, Wei Wei, Dr. Supriya Kulkarni, Dr. Ajay Donepudi, Dr. Vijay More, Dr. Prajakta Shimpi, Dr. Deanna Salter, Dr. Maneesha Paranjpe. Also, an extended thanks to all my URI colleagues, especially: Aseel Eid, Kyle Scully, Chrissy Dao, Swapnil Malekar, Chris Octeau, Zhengxi Wei, and Craig Irving. You all have specifically played a role during my dissertation through intense scientific conversations to everyday jokes and laughs we've shared to push ourselves forward.

Lastly, my deepest gratitude goes out to my immediate and extended family and friends who stood by me throughout these past 5 years. The extended support and the number of people I was able to make proud through the successful completion of my dissertation makes me grateful for the immense understanding everyone had of my absence from family events, annual trips, daily outings, and the multiple moving days. A special thank you to my "personal assistant"/boyfriend during a time when anyone else would of given up; I am thankful every day you could see past the stress. I have achieved one of my many goals in life because of the overwhelming support that surrounds me. Thank you everyone for contributing to my dissertation.

DEDICATION

To my parents, LuAnne and Richard Armstrong, for their unwavering faith in me and their continual support to achieve more, and to my two brothers, Rick and Michael, who have been the best mentors any younger sister could ask for. Without my immediate and extended family I would not have persevered through the last 5 years.

Thank you for giving me the opportunity to make you all proud.

PREFACE

This dissertation was prepared in manuscript format according to the University of Rhode Island Graduate School guidelines. This dissertation consists of three manuscripts that satisfy the requirements of the department of Biomedical and Pharmaceutical Sciences, College of Pharmacy, University of Rhode Island.

MANUSCRIPT I: The role of Nrf2 activation via calorie restriction in white adipose tissue: The cross-talk between antioxidant signaling and lipid metabolism.

This manuscript has been prepared for submission to '*Antioxidants & Redox Signaling'* as an original research communication.

MANUSCRIPT II: BDE-99 increases lipid accumulation throughout differentiation in 3T3-L1 and human pre-adipocytes *in vitro.*

This manuscript has been prepared for submission to '*Toxicological Sciences*'.

MANUSCRIPT III: Effects of Developmental Deltamethrin Exposure on White Adipose Tissue Gene Expression. (J Biochem Mol Toxicol. 2013 Apr;27(4):251-2.)

This manuscript has been published in '*Journal of Biochemical and Molecular Toxicology'.*

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MANUSCRIPT I

Prepared for submission to the journal *Antioxidants & Redox Signaling*

Original Research Communication

Calorie restriction induces Nuclear Factor E2 Related Factor 2 (Nrf2) in white adipose tissue: Antioxidant signaling and lipid metabolism.

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Abbreviated Title: Nrf2 and Calorie Restriction

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Word Count: (6,000): 5,916

Abstract (250 words): 365

Introduction: 902

References(75): 42

Greyscale Illustrations (8-10): 10 + 5 Supplemental Figures

Color Illustrations: 1

Abstract

Aim: The purpose of this study was to determine if calorie restriction (CR) contributes to an increased antioxidant capacity and to determine the role of Nrf2 activation in WAT; with specific focus on Nrf2-dependent and –independent mechanisms *in vivo*, and AMPK and Nrf2 activation *in vitro*. Nuclear factor-E2 related-factor 2 (Nrf2) is a well-established inducible transcription factor resulting in transactivation of antioxidant response and cytoprotective genes. CR, considered part of the primary management of metabolic syndrome by International Diabetes Federation (IDF), is defined as a total reduction in energy intake without nutrient deprivation. Adipose tissue (AT) is an important endocrine organ that has recently been directly related to the pathophysiology of metabolic syndrome through altered metabolism and nutrient handling. Oxidative stress has most recently been shown to be an important mediator of adipocyte dysfunction and dysfunctional adipose tissue. Therefore, this study addresses the importance of Nrf2 and antioxidant signaling in achieving homeostatic energy balance in white adipose tissue (WAT) *in vivo* and in *vitro*.

Results: Data demonstrate that the antioxidant response element (ARE) and Nrf2 are inducible in WAT by calorie restriction. CR induction of antioxidant response is Nrf2-dependent, and Nrf2 contributes to signal transduction pathways related to adipocyte metabolism, specifically lipid metabolism. In WAT from ARE-hPAP mice, CR activated the ARE, and induced Nrf2 and downstream target gene expression. Antioxidant capacity was Nrf2-

dependent in WAT of female mice, and significant changes in protein expression of p-Ampk, Pparγ, and Lpl related to lipid metabolism were observed. Lipogenesis and β-oxidation protein expression was not altered in Nrf2KO mice, but mRNA expression of *Hsl,* a lipolysis enzyme, was transcriptionally activated in WT-CR and not in Nrf2KO mouse model. *In vitro* adipocyte models illustrate that CR mimetics and Nrf2 activators induce antioxidant gene expression, cytosolic antioxidant capacity, and decrease triglycerides.

Innovation: This work illustrates mechanisms by which CR bolsters antioxidant capacity in the adipose tissue. These findings establish the importance of Nrf2-mediated antioxidant response in CR and WAT metabolism.

Conclusion: The current data support a novel role of nutrient status in the regulation of Nrf2 and the antioxidant response in WAT, and point toward Nrf2 as a factor to combat oxidative stress and regulate lipid metabolism in WAT.

INTRODUCTION

Adipose tissue (AT) has been underestimated in its function to regulate metabolism and has been recently described as a vital endocrine organ. AT is composed of adipocytes, pre-adipocytes, macrophages, and endothelial cells that are key components in the maintenance of systemic energy balance (Kusminski and Scherer, 2012). AT is the primary organ for lipid storage in the form of triglycerides and release of free fatty acids (FFAs) to maintain energy homeostasis and respond to whole-body energetic needs (Rutkowski *et al.*, 2015); therefore, adipocytes have a higher burden of free-fatty acids (FFA) during normal physiological conditions compared to other cells (Kusminski and Scherer, 2012). Due to this primary metabolic function of AT, adipocytes tolerate a high reactive oxygen species (ROS) load that cannot be sustained by other cells or organs without apoptosis (Kusminski and Scherer, 2012). It has been discussed that the electron-transport chain (ETC) is the major producer of ROS in adipocytes, with the production of superoxide anion radicals ($O_2{\bullet}^{-}$) and downstream secondary messenger, hydrogen peroxide (H_2O_2) (Kusminski and Scherer, 2012). As reviewed by Kusminski, excess caloric-intake is characterized by increased ROS production by the ETC and an overload of oxidative stress associated with obesity(Kusminski and Scherer, 2012). Increased oxidative stress associated with obesity leads to a dysfunctional antioxidant response, as demonstrated by a down-regulation of antioxidants such as, superoxide dismutase 2 (*Sod2*), glutathione peroxidase (*GPx*), *catalase* in mouse model of metabolic syndrome (Furukawa *et al.*,

2004), and glutathione-S-transferase (*GSTA4*) in obese and insulin-resistant humans (Curtis *et al.*, 2010). WAT is also known for its ability to expand, a function that is considered important in the protection against metabolic abnormalities (Kusminski and Scherer, 2012). It has been demonstrated *in vitro* that moderate ROS can lead to hypertrophic adipocytes due to decreased expandability which is reversible by treatment with antioxidants; therefore, changes in oxidative metabolism may act as signals involved in determining lipid pool size and tissue mass in metabolic diseases (Carriere *et al.*, 2003). Excess ROS production is considered in the pathophysiology of diseases, specifically metabolic syndrome, and changes in the antioxidant capacity of AT can be assessed in order to determine beneficial mechanisms to combat these diseases that are highly correlated to dysfunctional AT.

Calorie restriction (CR) is considered part of the primary management of metabolic syndrome by International Diabetes Federation (IDF) and is defined as a total reduction in energy intake without nutrient deprivation. Dietary intervention in humans is the most effective therapy to combat metabolic syndrome (Lopez-Legarrea *et al.*, 2013). The underlying mechanism of metabolic syndrome and an effective pharmacological target have not been identified in order to treat the disease as a whole. The IDF has defined metabolic syndrome as an individual having central obesity and two of the four precursors to disease: dyslipidemia (increased triglycerides or lowered HDL-cholesterol), insulin-resistance (increase fasting blood glucose), or hypertension (increased blood pressure). Oxidative stress and dysfunctional

AT can be linked to each of these states in human studies (Bremer *et al.*, 2011; Bremer and Jialal, 2013; Furukawa, Fujita, Shimabukuro, Iwaki, Yamada, Nakajima, Nakayama, Makishima, Matsuda and Shimomura, 2004; Roberts and Sindhu, 2009; Vincent and Taylor, 2006). The RESMENA study, in which a novel dietary strategy was compared to the American Heart Association (AHA) guidelines for dietary components, revealed that dietary total antioxidant capacity (TAC) was the predominant factor in decreased body weight, BMI, and fat mass (Lopez-Legarrea, de la Iglesia, Abete, Bondia-Pons, Navas-Carretero, Forga, Martinez and Zulet, 2013). Dietary TAC compared to other dietary aspects is most relevant to decrease in markers of obesity and metabolic syndrome (Abete *et al.*, 2010; Lopez-Legarrea, de la Iglesia, Abete, Bondia-Pons, Navas-Carretero, Forga, Martinez and Zulet, 2013; Puchau *et al.*, 2009). Therefore, understanding the mechanism by which CR maintains energy homeostasis in AT without production of excess ROS may be essential in determining an effective pharmacological treatment of metabolic syndrome.

CR has been shown to transcriptionally induce a battery of antioxidant genes in liver accompanied by Nrf2 expression, and this antioxidant response was attenuated in Nrf2KO mice (Pearson *et al.*, 2008). Nrf2 was demonstrated to be inducible in WAT through Keap1-KD and pharmacological activators (Xu *et al.*, 2012). The transcription factor has been classically defined to regulate the basal and inducible expression of genes, and is negatively regulated by Kelch-like ECH-associated protein 1 (Keap1) due to sequestration in the cytoplasm (Klaassen and Reisman, 2010; Niture *et al.*, 2010). During periods

of cellular stress, by endogenous or exogenous increases in ROS and electrophiles, Nrf2 translocates to the nucleus, where it heterodimerizes with small Maf protein and activates the antioxidant response element (ARE) (Nguyen *et al.*, 2009; Niture, Kaspar, Shen and Jaiswal, 2010). Activation of the ARE has been characterized to regulate genes involved in antioxidant response, and most notably Nrf2 is known to induce Phase I- and II- drug metabolizing enzymes and transporters for the elimination of potentially harmful xenobiotics (Klaassen and Reisman, 2010). More recently, in both liver and WAT Nrf2 expression has been shown to be directly related to lipid content and body weight changes. Our laboratory was able to show that constitutive activation of Nrf2 by Keap1-KD *in vivo* enhanced the reduction of liver triglycerides and non-esterified fatty acids (NEFAs) via CR by upregulation of β-oxidation genes (Kulkarni *et al.*, 2013), and constitutive Nrf2 activation also repressed HFD-induced obesity and lipid accumulation in WAT *in vivo* (Xu, Kulkarni, Donepudi, More and Slitt, 2012). A previous study showed that the prototypical Nrf2 activating compound, CDDO- Imidazolide, increased weight loss in HFD-mice (Shin *et al.*, 2009). There was also a positive relationship between hyperlipidemia and Nrf2 activation (Xu, Kulkarni, Donepudi, More and Slitt, 2012) suggesting that this well-described transcriptional regulator of the antioxidant response may have a potential role in the regulation of oxidative stress and lipid homeostasis in WAT.

The purpose of this study was to identify whether 1) CR can induce the ARE and Nrf2-dependent gene expression in WAT, 2) WAT weight loss and lipid

homeostasis is Nrf2-dependent, and whether CR mimetics and Nrf2 activators can directly alter the antioxidant capacity of adipocytes to regulate WAT mass. Data presented herein describes CR induction of the Nrf2 pathway and antioxidant response in WAT, as well as the role of Nrf2 in decreased fat mass upon CR.

RESULTS

CR induces the antioxidant response transcriptionally in WAT of mature ARE-hPAP mice, and Nrf2 induction is age-dependent. The 40% CR was effective, as illustrated by a 36% loss of body weight (BW) (Fig. 1A), as well as WAT weight and 53.5 mg/dL decrease in blood glucose levels after 5 weeks of CR compared to *ad libitum* (AL) (Supplemental figure 1; Fig. 1B). Induction of the ARE, measured by human alkaline phosphatase *(hPAP)* expression, was only achieved in WAT (2-fold increase), whereas *Nrf2* and downstream targets, *Nqo1* and *Gclc*, were all induced greater than 2-fold in both WAT and skeletal muscle (SKM) (Fig. 1C). For this CR paradigm, ARE-hPAP and Nrf2 targets were not significantly altered (Fig 1C), although decreased liver weight was observed (Supplemental Figure 1). Data from a second cohort of ARE-hPAP mice separated by age, Mature (average age of 16-wks ± 25.5) versus Old (average age of 97-wks ± 16), illustrated that *Nrf2* and Nrf2-target genes were inducible in the cohort of mature age, without induction of the ARE (Fig. 2A), but not inducible by CR in old age even with induction of the ARE (Fig. 2B). The difference in expression and induction of genes may be due to large standard deviation in age of second cohort of mature ARE-hPAP mice.

CR reduction in body weight and WAT is not Nrf2-dependent in both male and female mice. To determine if Nrf2 is an integral signaling pathway in CR and if the reduction of WAT weight is Nrf2-dependent, male and female Nrf2KO mice were placed on a 40% and 25% calorie-restricted diet, respectively. BW and WAT weight were measured. In both male and female

Nrf2KO mice on CR, BW and WAT weights were not significantly different from wild-type (WT) mice CR (Fig . 3 A and 3B). *Ad libitum* Nrf2KO mice have a decreased initial body weight and WAT weight compared to WT (Fig. 3B); these data are consistent with previous publications that demonstrate the importance of Nrf2 for adipocyte differentiation (Hou *et al.*, 2012; Pi *et al.*, 2010). CR decreased body and WAT weight similarly in male and female WT and Nrf2KO mice CR increased *Nrf2* and *Nqo1* mRNA expression in WT, but not Nrf2KO WAT (Fig. 3B and 3C), which *Nrf2* and *Nqo1* gene expression in WT WAT was similar to that observed in the ARE-hPAP mice (Fig. 1C); with *Nrf2 and Nqo1* expression being induced by at least 2-fold respectively (Fig. 3C).

CR induces the antioxidant response in WAT and serum antioxidant capacity in vivo. CR doubled Gclc mRNA in WAT of both male and female mice, but not in Nrf2KO mice (Fig 4A), whereas catalase mRNA expression was increased similarly (2-3-fold) in both WT-CR and Nrf2KO-CR (Fig. 4B). Subsequently, serum antioxidant capacity (AOC) was measured by ORAC assay in order to assess whether CR bolstered the antioxidant capacity in WAT. Male and female serum AOC was determined after 5 and 6 weeks of CR, respectively. CR increased AOC in male and female WAT (Fig 5A and B), but this induction was only significantly Nrf2-depdendent in females, with AOC in Nrf2KO being similar to WT-AL fed females (Fig. 5B).

Sod1 increased protein and activity by calorie restriction in female mice is Nrf2-indepednent, and proposed to be a potential WAT factor in systemic glucose homeostasis and hepatic gluconeogenesis. Induced oxidative stress in obese and diabetic mice has been shown to decrease Sod1 in WAT (Furukawa, Fujita, Shimabukuro, Iwaki, Yamada, Nakajima, Nakayama, Makishima, Matsuda and Shimomura, 2004), and models of Sod1 activation have decreased glucose intolerance and lipid peroxidation caused by HFD. To determine whether Sod1 is regulated via Nrf2 upon CR and contributes to changes in systemic glucose metabolism: Sod1 mRNA, protein, and enzyme activity was measured. Glucose tolerance (GTT) and pyruvate tolerance tests (PTT) were carried out in WT and Nrf2KO mice. Nrf2 was observed to contribute to an increase in *Sod1* mRNA upon CR (Fig. 6A), but post-transcriptional changes may be responsible for significantly induced protein expression in both WT and Nrf2KO mice (Fig. 6B). Lastly, CR induced the enzymatic activity of Sod1 in WT-CR and Nrf2KO-CR mice, supporting protein expression observations (Fig. 6C). Blood glucose levels were measured throughout the study and there was no difference in response to CR in the absence of Nrf2, with no significant difference between WT-CR and Nrf2KO-CR blood glucose levels (Figure 6D). Subsequently, CR increased glucose tolerance in WT mice, but Nrf2KO mice basally had significant increase glucose tolerance compared to WT-AL (Fig. 6E). Hepatic gluconeogenesis was induced in both WT and Nrf2KO mice, as demonstrated by an increased pyruvate tolerance, and to the same extent (Fig. 6F).

Therefore, Sod1 was shown to be induced without Nrf2 activation, and beneficial effects on glucose metabolism were not dependent on Nrf2, suggesting a role of Sod1 in preventing oxidative stress that contributes to glucose intolerance and decreased gluconeogenesis in models of obesity.

p-Ampk and Pparγ lipid regulation is dysregulated at the transcriptional and protein level in Nrf2KO mice in vivo altering transcriptional regulation of lipid uptake and lipolysis genes. The major transcription factors of CR, p-Ampk, and adipogenesis and lipid metabolism, Pparγ, were measured to assess whether CR induced changes in AMP:ATP ratio and altered lipid metabolism are Nrf2-dependent. Ampk activation by phosphorylation was shown to be Nrf2-dependent in both male and female mice, but only female WT mice had a significant induction in protein expression upon calorie restriction (Fig. 7B). Both male and female mice demonstrated a significant increase in Pparγ with CR, but only female mice showed an Nrf2-dependent response (Fig. 7B). Lpl and Cd36, involved in lipid uptake from the serum, did show consistent protein expression between male and female mice and Nrf2 regulation, except that both males and females did not show a significant increase in Lpl upon calorie restriction (Fig. 7B). Protein expression of these key factors in lipid mobilization did not reflect their mRNA expression or transcriptional activation by Nrf2 (Fig. 7A). Therefore, there may be alterations in post-transcriptional modifications or protein turnover in calorie

restriction and Nrf2KO model. Lastly, *Hsl* transcriptional activation by CR was demonstrated to be Nrf2-dependent (Fig 8A). This reflects another downstream target of Pparγ.

Induction of β-oxidation genes is not Nrf2-dependent, while negative feed-back for induction of lipogenic enzymes is dysregulated in Nrf2KO mice. Key enzymes involved in both lipogenesis and fatty acid β-oxidation were measured to further assess the physiological response of WAT to CR and potential role of Nrf2. β-oxidation was not altered by Nrf2KO, as demonstrated by 2-fold or greater induction of transcriptional regulators, *Pgc-1α* and *Pparα*, and enzymes involved in the initial steps of β-oxdiation, *Acadm* and *Eehad* (Fig 9C). Genes involved in lipogenesis were not consistently transcriptionally activated by Nrf2. *Srebp1c* and *Fas* were induced in both WT- and Nrf2KO-CR mice by 7.3- and 6.5-fold and 9- and 8-fold, respectively (Fig. 9A). Other key enzymes in lipogenesis in WAT, *Acc-1* and *Scd-1*, were shown to be induced upon CR by 16- and 6.4-fold, but Nrf2 acts as a coactivator for their transcriptional activation as demonstrated by a significantly decreased induction in Nrf2KO-CR mice, 10.5- and 3.7-fold, respectively (Fig. 9A). All mRNA expression was analyzed in female mouse models of CR, but Nrf2-coactivation of Acc upon CR was reflected at the protein level in both male and female mice (Fig. 9B).

Calorie restriction mimetic, AICAR, and Nrf2 activators, oltipraz and sulforaphane induce the antioxidant response in vitro and decrease lipid load of mature adipocytes. Two well accepted models of differentiated mature adipocytes, 3T3-L1 pre-adipocytes and MEFs were used to study the effect of calorie restriction and Nrf2 activation *in vitro*. AICAR and oltipraz induced *Nrf2* and transcriptional expression of downstream genes *Nqo1* and *Gclc* in mature 3T3-L1 pre-adipocytes, and sulforaphane induced only *Nqo1* significantly at the mRNA level (Fig10A). In mature 3T3-L1 pre-adipocyte model mRNA expression correlated with induction of cellular antioxidant capacity and Nqo1 protein expression (Fig. 10B and 10C). p-Ampk was induced by AICAR and oltipraz at 6 and 12 h post-treatment, and Nqo1 remained induced (Fig. 10C). Total AMPK was increased at 12 h and 24 h post-treatment (Supplemental Fig 3). Sulforaphane induced both p-Ampk and Nqo1 at 6 h, but protein expression did not remain stable (Fig. 10C). RLU quantification of all blots was determined (n=1) and represented in Supplemental Figure 3, in addition to total Ampk and β-Actin blots as controls. All 3 treatments induced the Nrf2-dependent antioxidant response cascade; therefore, in order to study if Nrf2 activation contributes to decrease in lipid content of adipocytes the lipid profile of mature MEFs and 3T3-L1 pre-adipocytes was examined. Both models showed significant decrease in triglycerides with Ampk and Nrf2 activation. Triglyceride concentration was decreased significantly in MEFs and 3T3-L1 model by Aicar, 28% and 39%, oltipraz 19% and 42.5%, and sulforaphane 38%, respectively (Fig. 11A). In

preliminary MEF experiments Sirt1720, a calorie restriction mimetic through activation of Sirt1, showed no significant decrease in triglycerides (Fig. 11A). Total lipid content, measured by oil red o staining, was reduced with 48 h treatment of AICAR, oltipraz, and sulforaphane in 3T3-L1 pre-adipocytes (Fig 11B).

DISCUSSION

Nrf2 is a transcription factor historically studied for its cytoprotective effects, and has recently been demonstrated to be a nutrient responsive factor and a key regulatior of lipid metabolism. In the present study *Nrf2* was demonstrated to be transcriptionally induced in WAT by CR *in vivo* resulting in upregulation of antioxidant signaling. Other studies have only demonstrated an increase in Nrf2 upon CR by measurement of Nrf2, Nqo1, and other downstream targets' activation in liver (Fu and Klaassen, 2014; Pearson, Lewis, Price, Chang, Perez, Cascajo, Tamashiro, Poosala, Csiszar, Ungvari, Kensler, Yamamoto, Egan, Longo, Ingram, Navas and de Cabo, 2008). CR was demonstrated to induce Nrf2 activation in WAT by subsequent activation of the ARE, *Nqo1* and *Gclc*; therefore, this study supports our laboratory's findings that the ubiquitously expressed transcription factor responds to nutrient stimulus in multiple metabolically active tissues (Kulkarni, Armstrong and Slitt, 2013; Kulkarni *et al*., 2014; Xu, Kulkarni, Donepudi, More and Slitt, 2012). In addition, CR-activation of Nrf2 was demonstrated to be physiologically important in inducing total serum antioxidant capacity. TAC in humans has been negatively correlated to increased central fat and obesity in both males and females, with reduced TAC by 5-7% and 6-10%, respectively, with females having a greater reduction in TAC (Chrysohoou *et al*., 2007). Therefore, more recent studies have focused on the effect of increased TAC through dietary interventions (Lopez-Legarrea, de la Iglesia, Abete, Bondia-Pons, Navas-Carretero, Forga, Martinez and Zulet, 2013; Puchau, Zulet, de

Echavarri, Hermsdorff and Martinez, 2009). Specifically, dietary TAC intake was a major factor in body weight, waist circumference, and fat mass decreases in the REMENA-S study (Lopez-Legarrea, de la Iglesia, Abete, Bondia-Pons, Navas-Carretero, Forga, Martinez and Zulet, 2013). The induction of Nrf2 in WAT by calorie restriction contributed to an overall systemic induction of TAC without correlation to increased weight loss in mice, which differs from human data. Further studies need to address localized TAC levels and the direct role of WAT regulation in systemic TAC.

It is previously reported that oxidative stress is directly related to obesity, glucose intolerance, and insulin resistance—specifically superoxide anion radicals ($O_2 \bullet^-$) are a common feature across models of insulin resistance (Hoehn *et al.*, 2009) and decrease in WAT Sod1 is associated with murine models of severe obesity, hyperlipidemia, and insulin resistance(Furukawa, Fujita, Shimabukuro, Iwaki, Yamada, Nakajima, Nakayama, Makishima, Matsuda and Shimomura, 2004). Also, WAT oxidative stress has been demonstrated to effect insulin resistance with alterations in ROS linked to changes in insulin sensitivity in 3T3-L1 preadiocytes (Kusminski and Scherer, 2012). In our study we were able to demonstrate that Nrf2 was not necessary to enhance glucose tolerance and hepatic gluconeogenesis, and Sod1 induction, a major enzyme responsible in reducing cellular oxidative stress, was not Nrf2-dependent; therefore, it can be suggested that Sod1 may be a key enzyme in maintaining glucose homeostasis. This notion is supported by previously published data that demonstrate: the deleterious effects of Sod1 on

glucose homeostasis and lipid metabolism (Wang *et al.*, 2012), the beneficial effects of Sod1 overexpression to decrease glucose intolerance and lipid peroxidation caused by a high-fat diet (Liu *et al.*, 2013), and the induction of Sod1 in ob/ob mice by rosiglitazone to ameliorate insulin insensitivity (Takazawa *et al.*, 2009). The post-transcriptional modification or alteration in protein degradation is noted in our study due to discrepancies between Nrf2 transcriptional regulation of Sod1 and Sod1 protein and activity. This observation should be further investigated as another study has demonstrated the same regulation of Sod1 in WAT by exercise training (Sakurai *et al.*, 2009).

CR and P-Ampk have been previously studied in the context of Ampk as an energy sensor of intracellular adenosine levels and is activated by increased in AMP:ATP or ADP:ATP levels that can be due to cellular stress, low nutrients, and prolonged exercise (Mihaylova and Shaw, 2011). Ampk is responsible for regulating catabolic and anabolic pathways by phosphorylation of proteins and has been reviewed to regulate metabolic transcription factors or enzymes in fatty acid synthesis, inactivation of Srebp1 and ACC in liver, or lipolysis, HSL and ATGL in adipose, to restore ATP levels (Mihaylova and Shaw, 2011). An activator of AMPK is AICAR, which mimics AMP binding to the Ampkγ subunits (Mihaylova and Shaw, 2011). In the present study we were able to demonstrate that calorie restriction induced phosphorylation of Ampk *in vivo*, and p-Ampk was inducible by AICAR (6 hr exposure), oltipraz (12 hr exposure), and sulforaphane (24 hr) *in vitro*. In vitro modeling in pre-adipocytes demonstrated a potential cross-talk between Ampk activation and

Nrf2 activation, in which AICAR induced p-Ampk and Nqo1 at 6 and 12 hrs, and oltipraz subsequently induced Nqo1 prior to its induction of p-Ampk. Recent publications support a cross-talk between Ampk and Nrf2 in proinflammatory macrophages treated with an Ampk activator, in which the data demonstrate that Nrf2 activation is Ampk-dependent and the anti-inflammatory effect of the Ampk activator is Nrf2-dependent (Mo *et al.*, 2014). The data presented *in vivo* demonstrated that Ampk phosphorylation was not induced by calorie restriction in Nrf2KO mice in both sexes. Therefore, the response to metabolic stressors in metabolically active tissues, specifically WAT in the present study, may be dependent on the cross-talk between Ampk and Nrf2. Additional studies should be developed to determine which nutrient responsive factor is upstream in the signaling cascade or if they are coordinately regulated in states of metabolic stress.

The study of key regulatory pathways in adipocyte metabolism of both male and female mice contributes to the understanding of the role of Nrf2 in lipid homeostasis and the relationship between oxidative stress and lipid metabolism. In WAT transcriptional activation of Nrf2 is responsible for CR-induction of Pparγ, a master regulator of adipogenesis and lipid metabolism. Pparγ maintains metabolic homeostasis in adipose tissue, in which it is predominately expressed, that systemically protects against lipid overload in alternate tissues and maintains a balanced secretion of adipocytokines, predominantly involved in systemic insulin sensitivity (Janani and Ranjitha Kumari, 2015). Pparγ is known for its transcriptional regulation of *Lpl* and

Cd36, genes directly involved in the uptake of lipids from serum, and the regulation of genes in lipogenesis or the stimulation of storage of fatty acids in mature adipocytes and WAT expandability(Janani and Ranjitha Kumari, 2015). Our findings provide *in vivo* data through CR-induced Nrf2 activation that supports previous findings that Nrf2 activates the *Pparγ* promoter activity *in vitro* (Pi, Leung, Xue, Wang, Hou, Liu, Yehuda-Shnaidman, Lee, Lau, Kurtz and Chan, 2010). Nrf2 was demonstrated to be involved in the transcriptional activation of both *Lpl* and *Cd36*, either independently or as a coactivator, respectively.

In addition to Nrf2 regulation of Pparγ and *Lpl, Hsl* gene expression was dependent on CR-induced Nrf2 activation, and initial *in vitro* data support the role of Nrf2 activation on activation of lipolysis upon CR (data not presented, Supplemental Figure 2). Nrf2 activation was also shown to contribute to the activation of key enzymes involved in lipid storage, Acc-1 and Scd-1, which demonstrate the role of Nrf2 activation in adipose tissue expandability. Lastly, CR-induction of β-oxidation in adipocytes was not Nrf2-dependent, but it is well-established that Pgc1-α is a master regulator of cellular energy metabolism and under normal conditions has a low expression level in WAT, but with increased mitochondrial biogenesis and oxidative metabolism Pgc1-α activation may be the predominant activator of downstream β-oxidation gene expression (Liang and Ward, 2006). Therefore, our models of CR and utilization of the Nrf2KO mouse model demonstrated not only the role of Nrf2 in CR-induced changes in lipid uptake and lipolysis, but other potential key

regulators in WAT metabolism that are responsive to CR specifically related to oxidative metabolism.

The study not only demonstrated CR-induced Nrf2 activation *in vivo*, but the ability of the CR mimetic AICAR and Nrf2 activators, oltipraz and sulforaphane, to increase cellular antioxidant capacity and decrease lipid content in two *in vitro* models of mature adipocytes. The *in vitro* findings further support a direct interplay between oxidative stress or antioxidants and lipid metabolism. As stated previously all compounds were able to activate both p-Ampk and Nqo1, with varying degrees of induction, and produce equivalent physiological effects as seen in our *in vivo* modeling of CR. Our data specifically support previously published data that have demonstrated oltipraz, a small molecule activator of Nrf2, and sulforaphane, an organic phytochemical inhibitor of Nrf2 degradation, attenuates fat gain in high-fat diet fed mice (Yu *et al.*, 2011) and inhibit lipid accumulation in MEFs (Xu, Kulkarni, Donepudi, More and Slitt, 2012), respectively. These data further represent the concept that Nrf2 is protective in various cell types (liver, lung, GI tract, kidney, skin, neurons, and macrophages), and the ubiquitously expressed protein coordinately regulates not only ARE-driven gene expression, but cell type-specific protective genes, which results in a bolstered local and systemic defense system (Lee *et al.*, 2005).

Recent publications support our overall finding that Nrf2 is inducible in WAT and plays a pivotal role in adipocyte metabolism to alter systemic pathophysiology of disease models. Specifically, global and adipose-specific

Nrf2 deficiency correlated to a reduction in WAT mass and suppression of pro-lipogenic and inflammatory genes and proteins, but resulted in worsened characteristics of metabolic syndrome (Xue *et al.*, 2013). At the other extreme, constitutive Nrf2 activation in leptin-deficient mice also impaired lipid accumulation in adipocytes and resulted in worsened insulin resistance and hepatic steatosis by, reduction in insulin-signaling molecules in adipose and increase gene expression of pro-lipogenic factors in liver (Xu, Kulkarni, Donepudi, More and Slitt, 2012). Our model demonstrated an overall beneficial effect of Nrf2 activation by calorie restriction to alleviate metabolic stress and contribute to decreased lipid storage in WAT and liver (Supplemental Figure 4). The notion that the benefits of Nrf2 activation are hormetic (Maher and Yamamoto, 2010; Taguchi *et al.*, 2010) is equally supported by our findings and previous publications; therefore, making it a difficult systemic drug target in diseased states. Overall, the study was able to show that tissue-specific regulation of Nrf2 in adipose tissue can be described as a mechanism to combat metabolic diseases associated with oxidative stress and excess lipids in the presence of a healthy leptin-axis, and identified the potential therapeutic benefit of Ampk and Nrf2 cross-talk.

INNOVATION

The data presented show a novel role of Nrf2 in calorie restriction related to WAT metabolism, and further contribute to potential mechanisms related to recently published data supporting the beneficial effects of antioxidant-rich dietary components and the overall beneficial effects of induced antioxidant capacity in calorie restriction. These novel findings can provide insight into future pharmacological targets for the underlying oxidative stress in multiple precursors of metabolic syndrome, and subsequent metabolic diseases. The ability to induce the ARE and subsequent Nrf2 gene expression in WAT of ARE-hPAP mouse model has never been described.

MATERIALS AND METHODS

Animals and treatments. Three animal models were used for the following animal studies: ARE-hPAP transgenic reported mice from Dr. Jeff Johnson's laboratory (Johnson *et al.*, 2008), C57Bl/6 (wild-type, WT) mice purchased from Charles River Laboratories (Frederick, MD), and Nrf2KO mice shared from Dr. Curtis D. Klaassen's laboratory (University of Kansas Medical School). All animal studies and procedures were carried out in accordance with and approved by the Institutional Animal Care and Use Committee at the University of Rhode Island. Adult 16-week old (n=9) ARE-hPAP transgenic reporter mice, and adult 14-week old male and female C57Bl/6 (n=10, n=16) and Nrf2KO (n=15, n=22) mice were monitored for food intake for 2 weeks, and average daily calorie intake was determined based on consumption of normal chow—Harlan Teklad LM-485 Mouse/Rat Sterilizable Diet (Harlan Laboratories, Indianapolis, IN) for 1 week, followed by purified and nutrient-rich chow—AIN-93G Growth Purified Diet (TestDiet, St. Louis, MO) during week 2. At 18 weeks and 16 weeks, respectively, ARE-hPAP and C57Bl/6 and Nrf2KO mice were separated equally into two groups: *ad libitum* and calorie restricted (40% of daily average calorie intake for males, and 25% for females). Calorie restricted mice were fed AIN-93G Growth Purified Diet based upon food weight equivalent to initial calculated daily calorie intake. All cohorts received 6.62-7.72 kCal/day during calorie restriction (Supplemental Figure 5). All cohorts were maintained on experimental diets (*ad libitum* or calorie restricted) for 5 to 6 weeks prior to sacrifice. Body weight (BW) was

measured weekly and blood glucose (BG) was measured every 2 weeks via tail vein bleed with Bayer® Contour glucometer and corresponding blood glucose test strips. Prior to necropsy BW and BG were measured, and at time of necropsy white adipose tissue (WAT) and liver weights were measured. All tissues collected (liver, WAT, and skeletal muscle) were snap frozen in liquid nitrogen and stored at -80°C.

Glucose and Pyruvate Tolerance Tests (GTT and PTT). After a 16-hr fast both male and female cohorts received an i.p. injection of 2 g/kg of glucose (Sigma-Aldrich, St Louis, MD) for the GTT. Pyruvate was administered as an i.p, injection of 2 g/kg of sodium pyruvate (Sigma) following an 18-hr fast for both male and female cohorts. Fasting blood glucose concentrations were taken prior to the injections and 15, 30, 60, and 120 minutes post-injection. All glucose concentrations were measured via tail vein bleed using a Bayer® glucometer following IACUC protocol.

Media, Reagents, and Chemicals. Dulbecco's Modified Eagle Medium (DMEM) high glucose (4.5 g/L) with L-glutamine (Lonza), DMEM/F-12 (Gibco), Fetal Bovine Serum (FBS, Gibco), and penicillin-streptomycin (pen-strep) was purchased from ThermoFisher Scientific (Waltham, MA). Oltipraz was purchased from LKT laboratories (#04578, St Paul, MN) and Sirt1720 is a Selleck Chemicals, LLC compound (#51129, Pittsburg, PA). All other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO): Dimethyl Sulfoxide (DMSO), 5-Aminoimidazole-4-carboxamide 1-β-D-

ribofuranoside (AICAR), L-Sulforaphane, 3-isobutyl-1-methylxanthine (IBMX), Dexamethasone (DEX), human insulin solution, and Oil Red O, specifically.

Primary mouse embryonic fibroblast (MEF) isolation, differentiation, and treatment. Primary MEF isolation from ARE-hPAP mice and differentiation to mature adipocytes was performed as described (Xu *et al.*, 2015). Following differentiation, day 9 mature MEFs were treated with DMEM containing 10% FBS and 1% pen-strep supplemented with DMSO (0.1%), AICAR (500 μM), Oltipraz (100 μM), or Sirt1720 (10 μM) for 4 days with media changed 2 days post-treatment. MEFs were analyzed for lipid development, lipid content, and mRNA expression.

3T3-L1 pre-adipocyte cell culture, differentiation, and treatment. Mouse 3T3-L1 pre-adipocytes (ATCC® CL-173™) were obtained from American Type Culture Collection (ATCC, Manassas, VA) and were cultured following ATCC protocol utilizing. 3T3-L1 pre-adipocytes were induced and differentiated according to previously described protocol (Shin, Wakabayashi, Yates, Wakabayashi, Dolan, Aja, Liby, Sporn, Yamamoto and Kensler, 2009; Xu, Kulkarni, Donepudi, More and Slitt, 2012). Briefly, 48 h post-confluent cells were induced by induction media (Maintenance media (DMEM, 10% FBS, and 1 % p/s) supplemented with 0.5 mM IBMX, 0.2 mM DEX, and 10 μg/mL insulin) for 48 h and then differentiated only in the presence of insulin (10 ug/mL) for 6 days. At day 8, cells are considered mature with the formation of lipid droplets and maintained in DMEM supplemented with 10 %

FBS, and 1% pen-strep (maintenance media). Mature 3T3-L1 pre-adipocytes were treated at Day 8 with maintenance media supplemented with DMSO (0.1%), AICAR (500 μM), Oltipraz (100 μM), or Sulforaphane (10 μM) for 24 and 48 h. 3T3-L1 pre-adipocytes were analyzed for lipid development, lipid content, mRNA and protein expression, and antioxidant capacity.

Cellular Lipid Development—Oil Red O Staining. Mature MEF and 3T3-L1 pre-adipocytes were stained for lipid development in 12-well plates (n=3 per treatment). Following treatment the media was removed and cells were gently washed with PBS and fixed with 10% acetate-buffered formalin (ThermoFisher Scientific, Waltham, MA) for 1 h. Oil Red O stock solution (0.6%) was prepared in 100% isopropanol. Oil Red O working solution was made directly prior to staining by combining Oil Red O stock solution and DI water 3:2 v/v, allowing the solution to sit for 10 mins, and then filtering the solution. Following fixation, the cells were washed and stained with Oil Red O working solution for 15 mins. The cells were then rinsed with water until the water rinsed clear. Cells were stored and imaged in DI water. Oil Red O images were taken at 20x magnification (EVOS, Life Technologies, Carlsbad, CA), and Oil Red O staining was quantified by ImageJ software (National Institute of Health) following the previous published protocol (Mehlem *et al.*, 2013).

Lipid Isolation and Quantification—Triglycerides. Triglyceride concentrations from MEF and 3T3-L1 cells were measured from 1 mL PBS lysates (6-well plates; n=3/group) collected after 48 h treatment. 200 μl of

lysate was transferred to 15 mL screw-capped tube containing 3.75 mL chloroform-methanol (2:1, v/v) solution. Water (0.5 mL) was added and centrifuged at 3000 rpm for 5 min at room temperature. The chloroform-methanol layer containing lipids was transferred to glass tube and evaporated to dryness in a fume hood overnight. The completely dried lipid residue was resuspended in 200 ul of 1% Triton X-100 in 100% ethanol. Extracted lipids and serum were measured for triglyceride content using the microtiter protocol for Triglycerides GPO-Reagent Kit (Pointe Scientific, Canton, MI). Briefly, standard and samples (3 µl) were run in triplicate on a 96-well plate by adding 250 µl of reconstituted triglyceride reagent, and absorbance was read at 540 nm on Spectramax M2 (Molecular Devices, Sunnyvale, CA). Cellular triglyceride content was expressed as a ratio of triglyceride concentration (mg/dL) to total protein concentration (mg/dL) of lysates determined by DC™ Protein Assay (Bio-Rad Laboratories, Hercules, CA).

Messenger RNA quantification. Total RNA was isolated from tissues or cells using TRIzol reagent (Invitrogen, Carlsbad, CA) following manufacturer protocol, and quantified following cDNA synthesis (Transcriptor First Strand cDNA Synthesis Kit) via LightCycler 480® SYBR Green I Master chemistry (Roche Applied Sciences, Indianapolis, IN). All gene primers (Supplemental table 1) were utilized at a final concentration of 0.5 µM with a final concentration of cDNA template of 50-60 ng.

Oxygen Radical Antioxidant Capacity Assay (ORAC). Whole blood was collected at time of necropsy and incubated on ice for 2 h prior to

centrifugation at 8,000 xg at 4°C for . Serum was collected and stored at -80°C. 3T3-L1 cytosolic cellular fractions were collected from NE-PER Nuclear and Cytoplasmic Extraction Kit (ThermoFisher Scientific, Waltham, MA) following the manufacturer protocol. Mouse serum and 3T3-L1 media and cytosolic cellular fractions were diluted 1:500 and 1:100, respectively. The ORAC assay protocol by BioTek® (citation) was optimized for the Spectramax M2 (Molecular Devices, Sunnyvale, CA) using sensitivity=6 and bottom-reader for a 96-well clear-bottom plate. Trolox standards and samples were pipetted in replicate (25 µl) with 150 µl of 2 µM fluorescein and fluorescence (485nm, 528nm) was measured over 1 h every minute immediately following addition of the radical generator 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH). The total AUC was calculated on GraphPad Prism Software (La Jolla, CA).

Western blotting. Western blots were used for identification and quantification of WAT and 3T3-L1 pre-adipocyte proteins from total cell lysates in RIPA buffer. Protein was electrophoretically separated by SDS-polyacrylamide gel electrophoresis. The gel was transblotted on PVDF membrane (Bio-Rad Laboratories, Hercules, CA) at 100 V for 1 h. The membrane was blocked for 1 h (room temperature) with 5% non-fat dry milk in tris-buffered saline with 0.1% Tween 20 (TBS-T). The membrane was then incubated with primary antibody in 5 % BSA in TBS-T at 4°C overnight. After washing, the membrane was incubated with species-specific HRP-conjugated secondary antibody for 1 h at room temperature. Specific antibody information

and conditions are provided in Supplemental Table 2. The blots were incubated in Clarity™ Western ECL Blotting Substrate (Bio-Rad Laboratories, Hercules, CA) and were visualized using autoradiography film. Protein bands were quantified using ImageJ (National Institute of Health) and all bands were normalized to corresponding β-Actin protein quantification.

Statistical Significance. Student's T-test was performed to determined statistical significance between ARE-hPAP mice fed *ad libitum* or CR, unless otherwise stated ($p<0.05$*, $p<0.01$**, $p<0.001$***, $p<0.0001$****). Statistical significance was determined between WT-AL, WT-CR, Nrf2KO-AL, and Nrf2KO-CR for both male and female cohorts by one-way ANOVA followed by Newman's Keuls post-hoc test. Each letter represents a statistically significant difference between all groups. For *in vitro* statistical analysis one-way ANOVA followed by Dunnett's Test was performed to determine significant differences between control (DMSO) and treatments ($p<0.05$*, $p<0.01$**, $p<0.001$***, $p<0.0001$****), unless otherwise stated.

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Figure Legends:

Figure 1. ARE and Nrf2-target genes are induced with weight loss via calorie restriction. ARE-hPAP mice were fed *ad libitum* (n=4) or 40% calorically restricted (n=5) for 5 weeks. A) Body weight (g) was monitored weekly. B) Blood glucose (mg/dL) was measured via tail vein bleed using a Bayer® glucometer week 2,4 and 5. C) All metabolically active tissues at time of necropsy were analyzed for mRNA expression via qPCR. Significance was determined by Student t-tests between AL and CR cohorts (p<0.05*, p<0.01**).

Figure 2. Mouse ARE and Nrf2-target genes are not transcriptionally activated in old age. Two cohorts of ARE-hPAP mice were fed either *ad libitum* or 40% calorically restricted: mature age mice had an average age of 16 wks±25.5 and old age mice had an average age of 97 wks±16. A) WAT from mature aged mice (n=) and B) WAT from old age mice (n=) were analyzed for mRNA expression via qPCR. Significance was determined by Student t-tests between AL and CR cohorts (p<0.05*, p<0.01**, p<0.001***).

Figure 3. Decreased body weight and WAT due to calorie restriction is not Nrf2-dependent. Both C57Bl/6 and Nrf2KO 16-week old mice were fed *ad libitum* or 40% calorically restricted for 5 weeks (males) or 25% calorically restricted for 6 weeks (females). A) Male and female body weights (g) were measured weekly. B) At time of necropsy male and female WAT weight was measured (g, 0.01) and C) mRNA expression of Nrf2 and Nqo1 was measured

via qPCR. A one-way ANOVA followed by Newman-Keuls post-hoc was used to determine statistical significance between all groups within each separate cohort (male and female) (each letter represents a difference in significance, a-d).

Figure 4. **Calorie restriction induces genes involved in antioxidant response in WAT.** *Gclc* and *Catalase*, important antioxidant enzymes, were measured in male and female mice fed *ad libitum* or calorically restricted (40%, and 25% CR, respectively) were measured by qPCR. A) *Gclc* mRNA expression compared to WT-AL. B) *Catalase* mRNA expression compared to WT-AL. Statistical significance was determined by One-way ANOVA followed by Newman Keuls post-hoc.

Figure 5. **Serum total antioxidant capacity in both male and female mice calorically restricted**. Total antioxidant capacity was measured in serum of both (A) male and (B) female mice fed *ad libitum* or calorically restricted (40% and 25%, respectively) by ORAC assay and expressed as Trolox equivalents (μM) determined from standard curve of Trolox antioxidant capacity. Statistical significance was determined by one-way ANOVA followed by Newman Keuls post-hoc.

Figure 6. **Nrf2 is not needed for the induction of WAT Sod1 protein and activity by calorie restriction in female mice.** WAT collected from the female cohort (*ad libitum* or 25% calorically restricted) was analyzed for mRNA expression, protein levels, and enzymatic activity of Sod1. A) Sod1 mRNA

expression by qPCR was normalized to B2M and represented as fold-change compared to WT-AL B) Sod1 protein was determined in whole cell lysates by western blot and expressed as RLU normalized to Gapdh C) Sod1 activity was measured by a commercially available kit (Biovision, Milpitas, CA) and represented as the % inhibition of xanthine oxidase. The sample absorbance was normalized to individual sample blanks and % inhibition was calculated according to kit protocol. Induction of WAT Sod1 may directly contribute to decreased blood glucose levels, glucose tolerance, and hepatic gluconeogenesis. Blood glucose concentrations were taken every 2-weeks and at time of necropsy and glucose tolerance test (GTT) was performed at week 4 and pyruvate tolerance test (PTT) was performed at week 5 of calorie restriction. Blood samples were taken via tail vein bleed and glucose concentrations were measured by a Bayer® glucometer following IACUC protocol D) Average blood glucose levels (mg/dL) are represented over the course of the study E) Glucose concentrations were taken prior to i.p. injection of 2g/kg glucose and 15, 30, 60, and 120 minutes post-injection to measure glucose tolerance F) Glucose concentrations were taken prior to i.p. injection of 2g/kg sodium pyruvate and 15, 30, 60, and 120 minutes post-injection to indirectly measure hepatic gluconeogenesis.

Figure 7. p-Ampk and Pparγ lipid regulation is dysregulated at the transcriptional and protein level in Nrf2KO mice *in vivo* altering transcriptional regulation of lipid uptake genes. WAT from male and female mice fed *ad libitum* or calorically restricted were analyzed for mRNA

expression and protein levels to assess the role of CR and Nrf2 in p-Ampk, Pparγ, Lpl, and Cd36 expression. A) Transcriptional regulation of *Pparγ*, *Lpl*, and *Cd36* was determined by measurement of relative mRNA normalized to B2M expression, and represented by fold change compared to WT-AL. B) All proteins were visualized by western blot and quantified using ImageJ analysis. Quantification of protein was normalized to β-actin levels and expressed as RLU (n=3). Statistical significance was determined by one-way ANOVA followed by Newman Keuls post-hoc.

Figure 8. **Nrf2-dependent induction of rate-limiting enzyme of lipolysis, hormone sensitive lipase (*Hsl*).** *Hsl* gene expression was measured in both male and female cohorts by qPCR to determine the transcriptional activation upon CR.

Figure 9. **Negative feedback for induction of lipogenic enzymes does not occur in Nrf2-KO mice, but β-oxidation genes are responsive in both cohorts.** (A) Lipogenic and (B) β-oxidation gene expression was measured in the female cohort via qPCR and represented as fold change compared to WT-AL. (C) Both male and female protein expression of Acc was measured to confirm transcriptional regulation by Nrf2. Average protein band intensity per group (n=3) are represented by RLU measurement in ImageJ and normalized to β-actin expression.

Figure 10. **Nrf2 and downstream targets are inducible in cultured adipocytes resulting in increased antioxidant capacity.** 3T3-L1 pre-

adipocytes were differentiated to mature adipocytes and treated at Day 9 in the presence of AICAR (500 μM), Oltipraz (100 μM), and Sulforaphane (10 μM) for 24 hr prior to isolation. A) mRNA expression of *Nrf2*, *Nqo1*, and *Gclc* was measured via qPCR (n=3) as fold change compared to WT-AL B) NE-PER extraction was performed to obtain the cellular cytosolic fraction, and an ORAC assay was performed to measure total antioxidant capacity represented at Trolox equivalents (μM), determined from a Trolox standard curve. C) p-Ampk and Nqo1 protein was measured in whole cell lysate RIPA extractions at 6 hr, 12 hr, and 24 hr to demonstrate time-dependent induction, and are represented by western blot. Refer to Supplemental figure 3 for quantification and total AMPK and β-actin protein expression.

Figure 11. Nrf2 inducing agents decrease lipid content and in cultured adipocytes. Mature 3T3-L1 pre-adipocytes and MEFs were treated with DMSO (0.1%), AICAR (500 μM), oltipraz (10 μM), sulforaphane (10 μM), and Sirt1720 (10 μM), Day 8-10 and Day 9-13, respectively. A) Triglyceride concentration (mg triglyceride/ g protein) was determined following 48 h treatment and B) total neutral lipid content was visualized by Oil Red O staining after 24 and 48 h treatments. A Dunnett's test was used to analyze significance of treatment compared to control ($p<0.05$*, $p<0.0001$****).

Supplemental Figure 1. Total tissue weights of ARE-hPAP mice fed *ad libitum* or CR. WAT and liver tissue were collected at time of necropsy from ARE-hPAP mice fed *ad libitum* or 40% CR. Accurate weights (0.00g) were obtained prior to snap freezing A) Average WAT weight (g) B) Average liver weight (g). Statistical significance was determined by Student's T-test, $p<0.001$*** $p<0.0001$****.

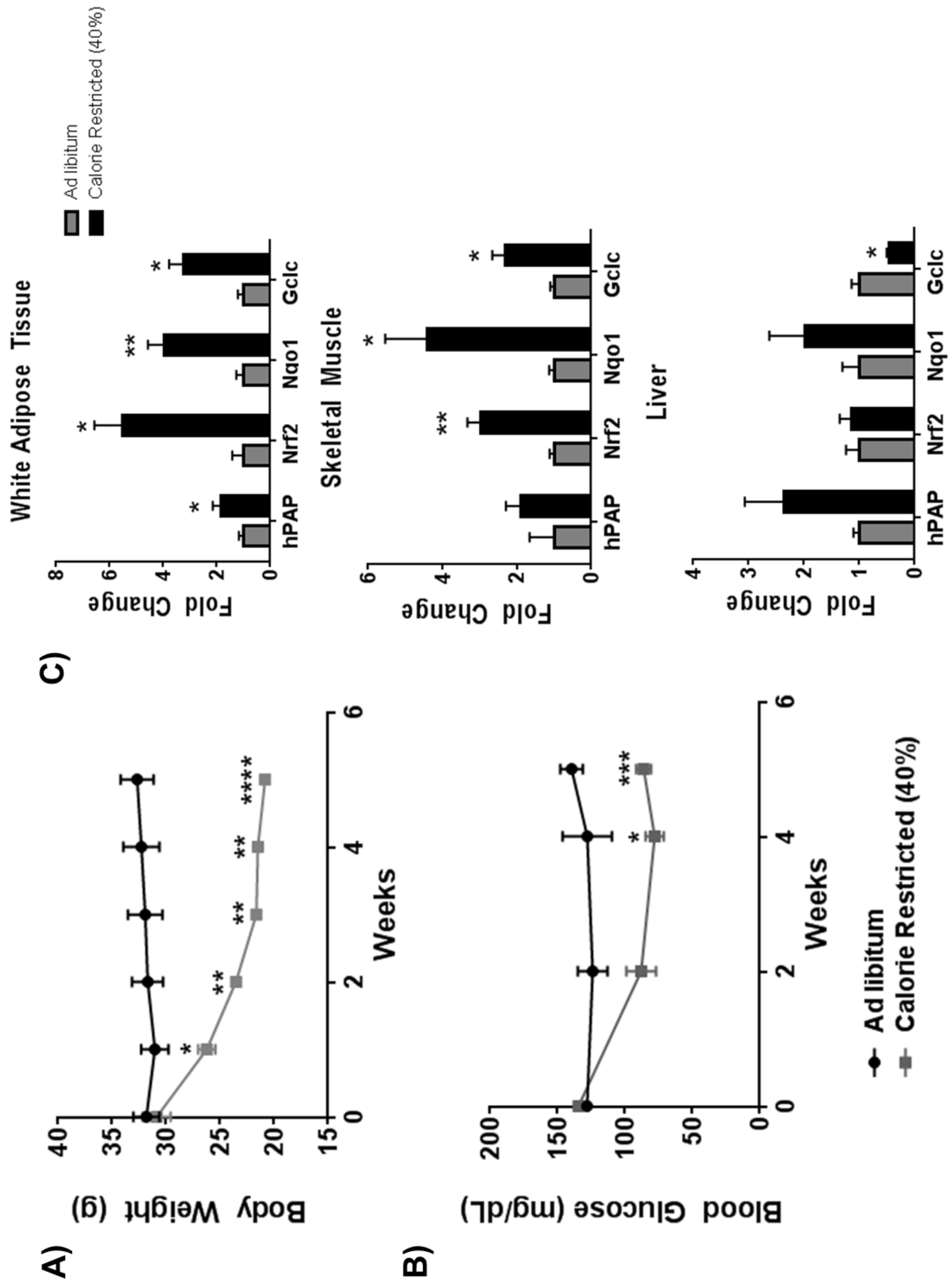
Supplemental Figure 2. Primary human mesenteric adipocytes treated with Ampk and Nrf2 activators demonstrated induced lipolysis ex vivo. Primary human adipocytes were used to determine if treatment with chemical activators, used in in vitro CR studies, stimulated lipolysis. Primary human adipocytes were isolated from the mesenteric depot from an individual donor and incubated in the presence of adenosine deaminase (ADA, Roche, Indianapolis, IN), phenylisopropyl adenosine (PIA) and a chemical activator of Ampk or Nrf2 (n=3/treatment of biological replicates). ADA and PIA are used to standardize any variations in adenosine levels, and ADA (1 units/mL) + PIA (20nM) is considered basal lipolytic rate due to inhibition of adenosine. Positive control, isoproterenol (10^{-6} M), was performed alongside treatments for direct measurement of stimulated lipolytic rates. Isoproterenol significantly stimulated lipolysis with an average glycerol release of 367.6 nM ($p<0.0001$). Glycerol release was measured in the presence of AICAR (500 μM), Oltipraz (100 μM), and Sulforaphane (10 μM) (n=9/treatment—biological replicates performed in triplicate). Statistical significance was determined by one-way ANOVA followed by Dunnett's Test ($p<0.01$** and $p<0.0001$****).

<u>Supplemental Figure 3.</u> Protein expression was measured in 3T3-L1 pre-adipocytes following 6, 12, and 24 hr exposures. A) p-Ampk:AMPK and Nqo1 protein quantification via ImageJ (n=1) normalized to β-Actin B) Total Ampk levels quantified via ImageJ B) β-Actin and total Ampk levels represented via western blot.

<u>Supplemental Figure 4.</u> Decreased triglyceride content in liver due to calorie restriction. Overall decrease in liver weights was measured, and the metabolically active tissue was analyzed for changes in lipid content due to 40% calorie restriction in male and 25% calorie restriction in female cohorts for 5-6 weeks. A) Liver weights were measured at time of necropsy (0.00 g) prior to snap-freezing of tissue. Lipid extraction was performed on liver PBS lysates to measure B) triglyceride (mg triglyceride / g tissue) content and C) Serum triglycerides were measured directly.

<u>Supplemental Figure 5.</u> Average daily calorie intake during calorie restriction in *in vivo* models. Male ARE-hPAP and Male WT and Nrf2KO mice were subjected to a 40% calorically restricted diet. Female WT and Nrf2KO mice were only subjected to a 25% calorically restricted diet, but their daily caloric intake was not significantly different from the male cohorts. A) Food intake was measured by total feed weight consumed and converted into calorie intake (3.89 kcal/g—purified chow) and represented as average kcal/day. B) Calorie intake per day during calorie restriction was determined by a 40% (males) or 25% (females) decrease in average total Kcal/day consumption.

Figure I-1.



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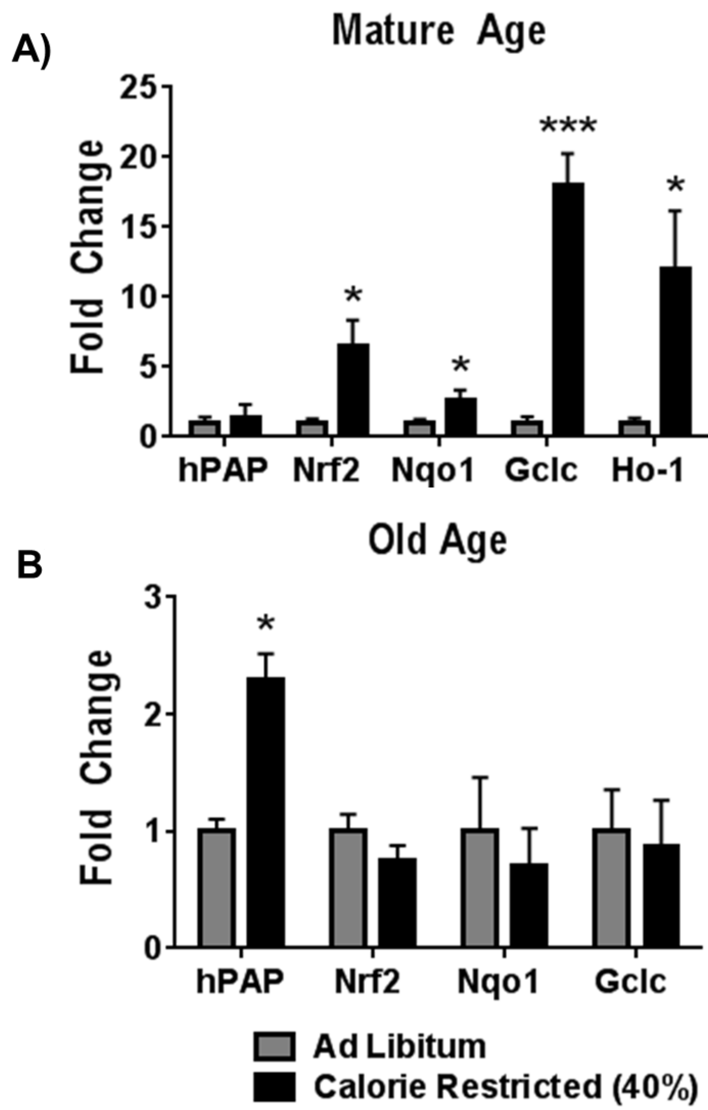

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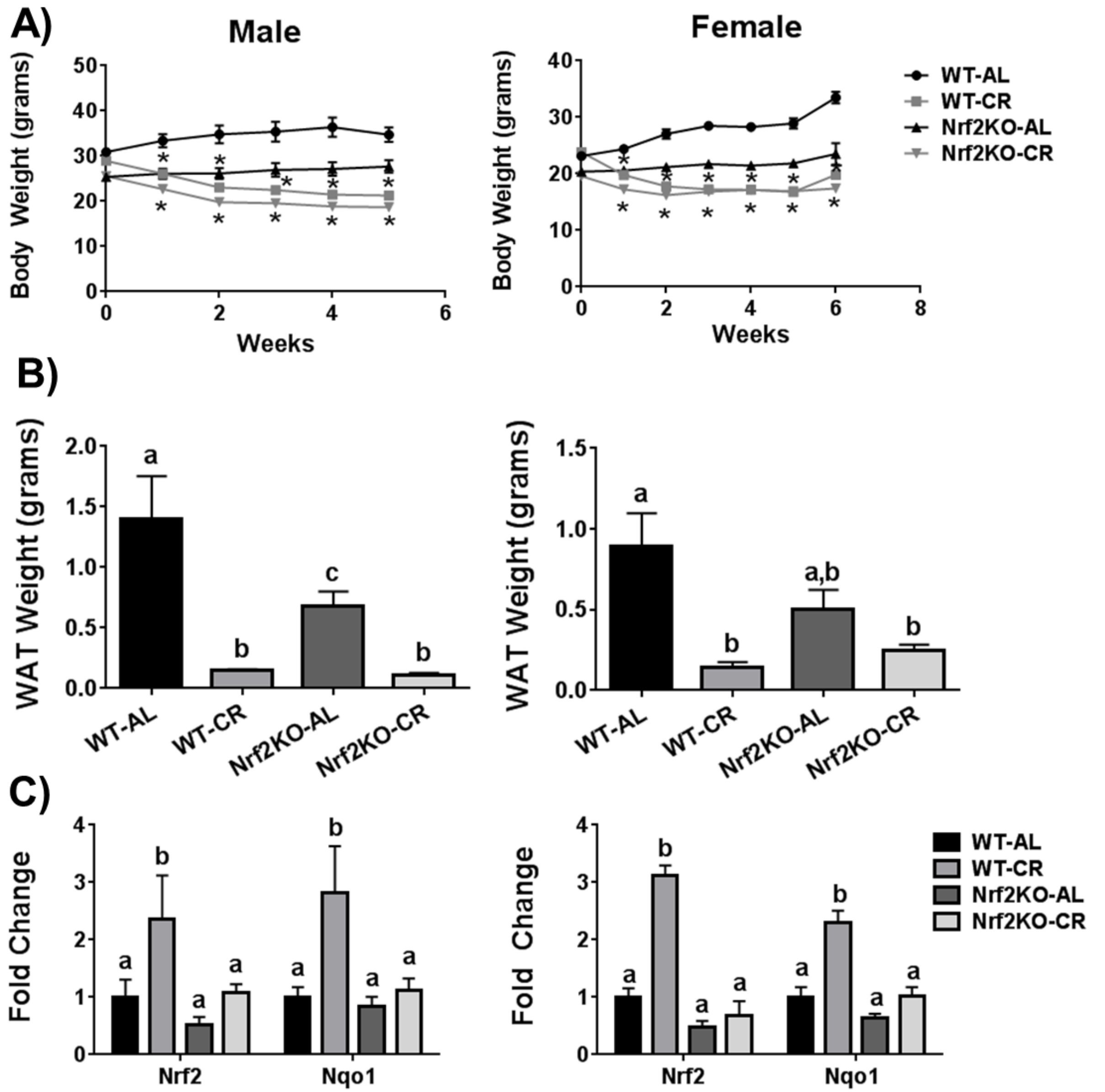

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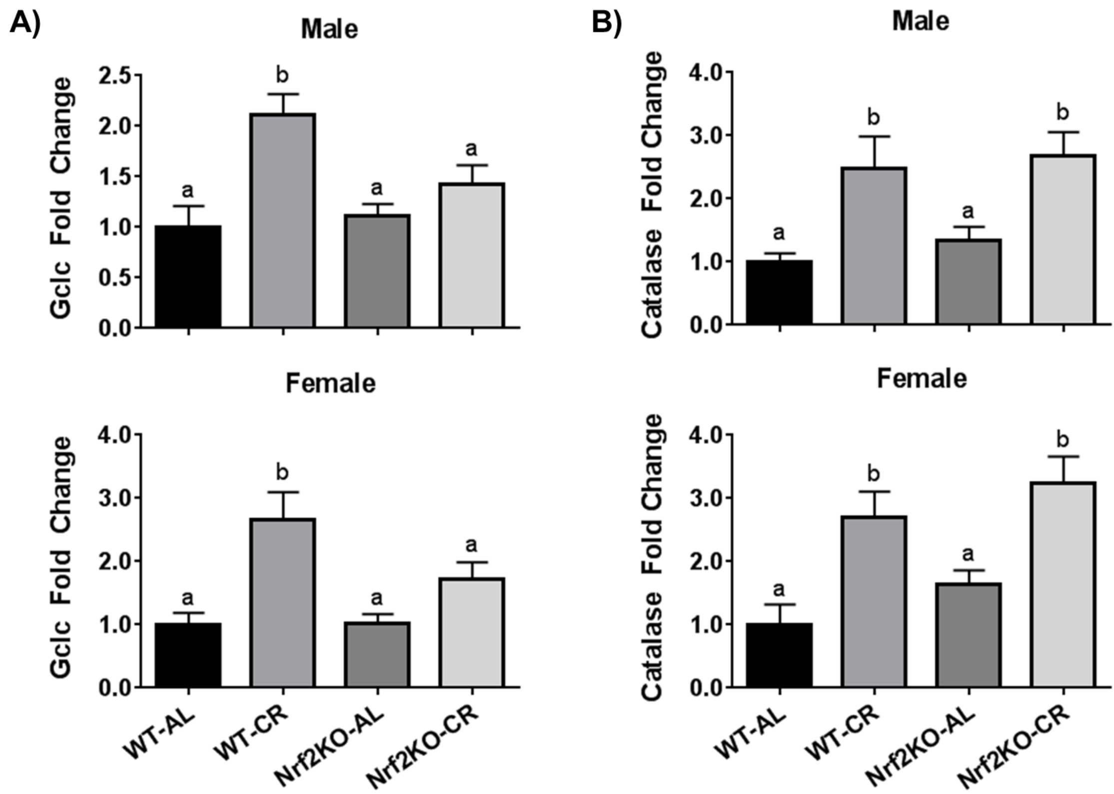

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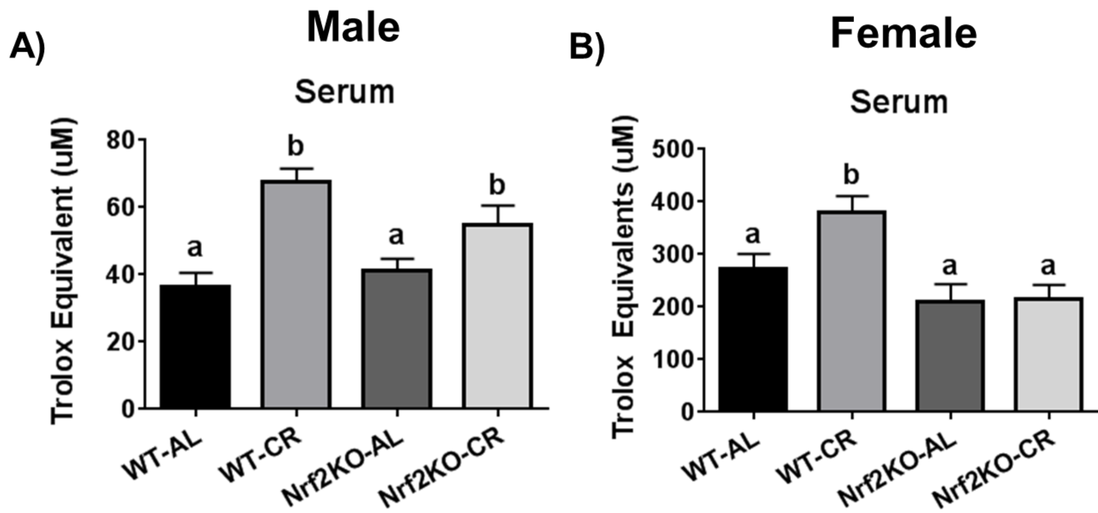

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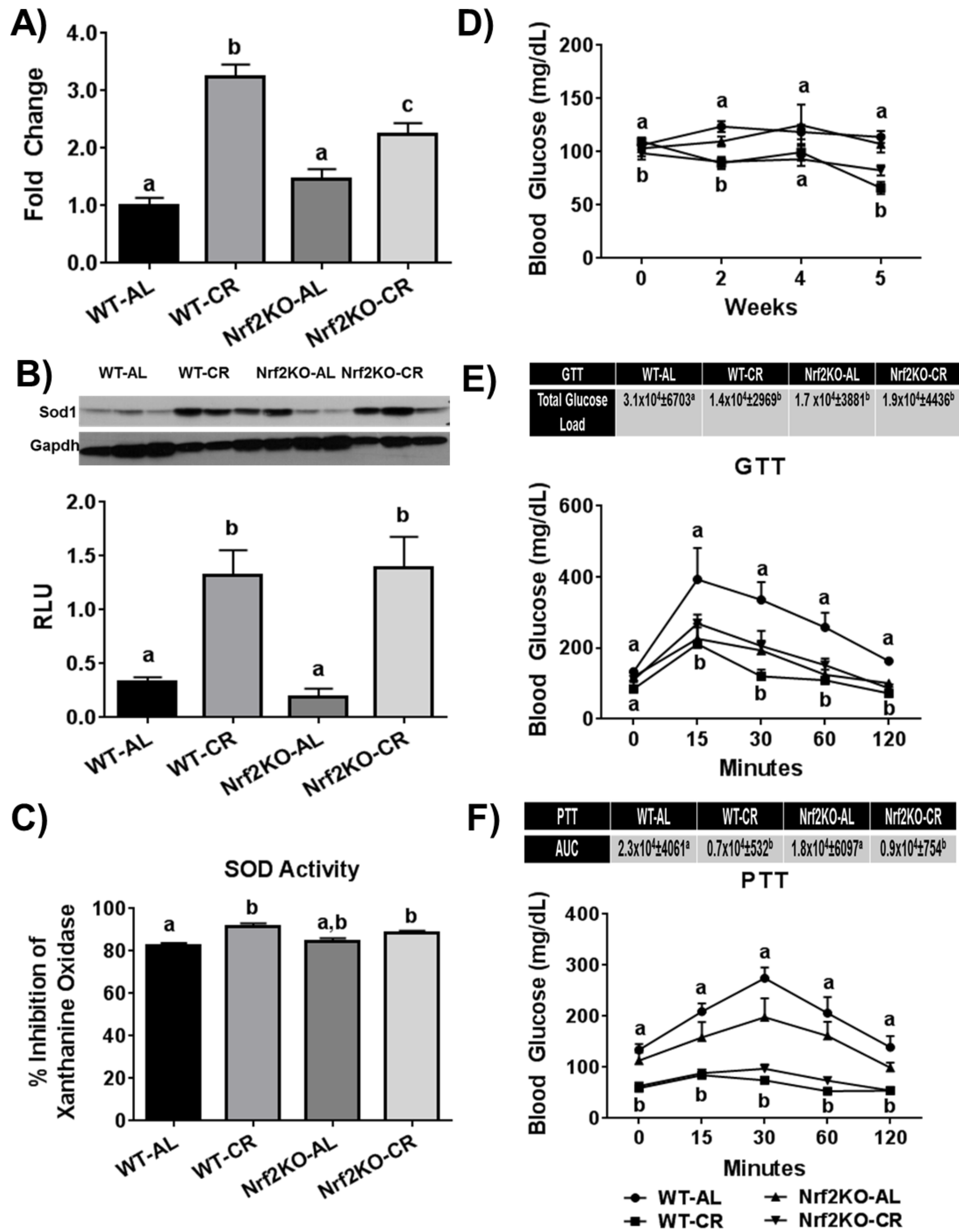
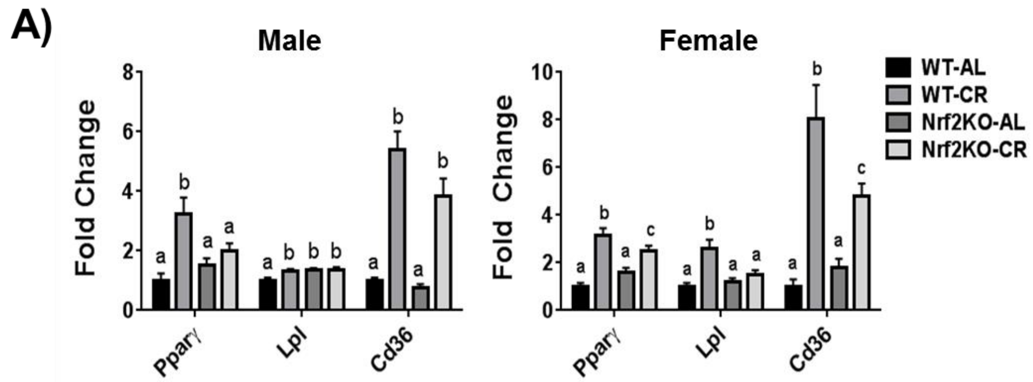
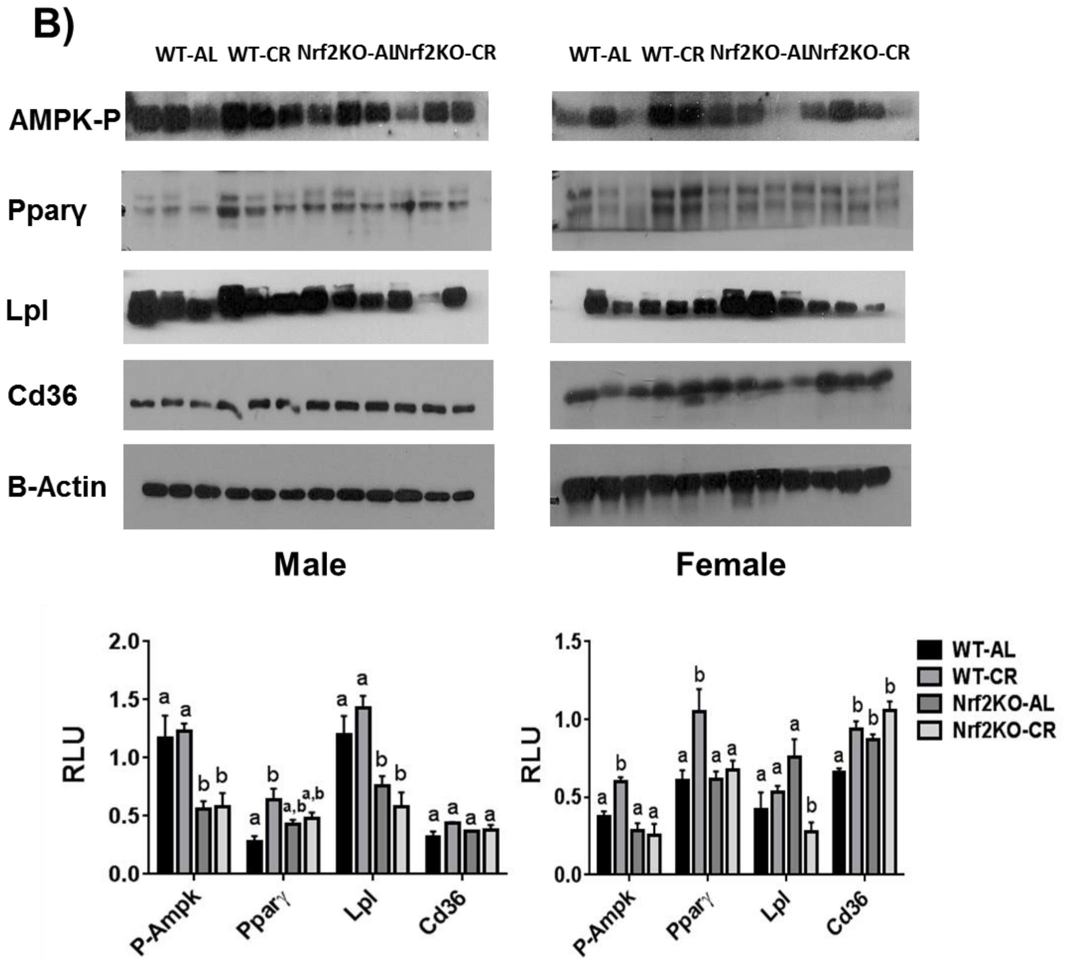


GTT	WT-AL	WT-CR	Nrf2KO-AL	Nrf2KO-CR
Total Glucose Load	$3.1 \times 10^4 \pm 6703^a$	$1.4 \times 10^4 \pm 2969^b$	$1.7 \times 10^4 \pm 3881^b$	$1.9 \times 10^4 \pm 4436^b$

PTT	WT-AL	WT-CR	Nrf2KO-AL	Nrf2KO-CR
AUC	$2.3 \times 10^4 \pm 4061^a$	$0.7 \times 10^4 \pm 532^b$	$1.8 \times 10^4 \pm 6097^a$	$0.9 \times 10^4 \pm 754^b$

Figure I-7.





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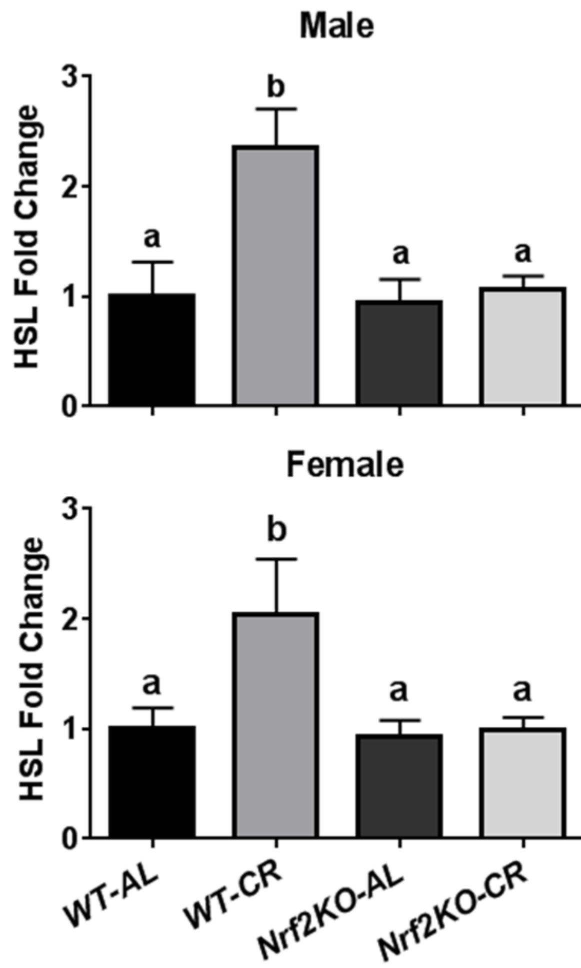

Figure I-9

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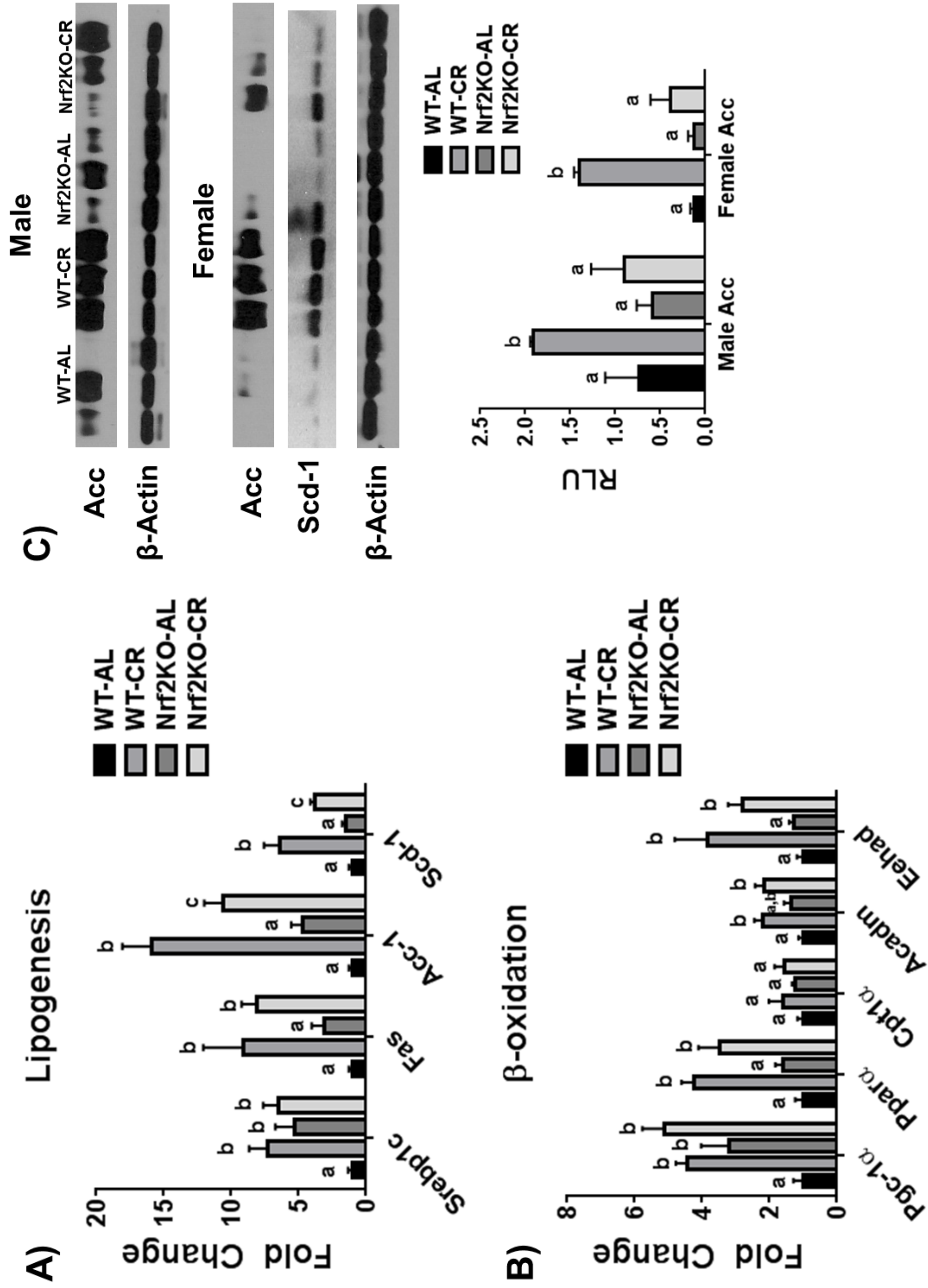

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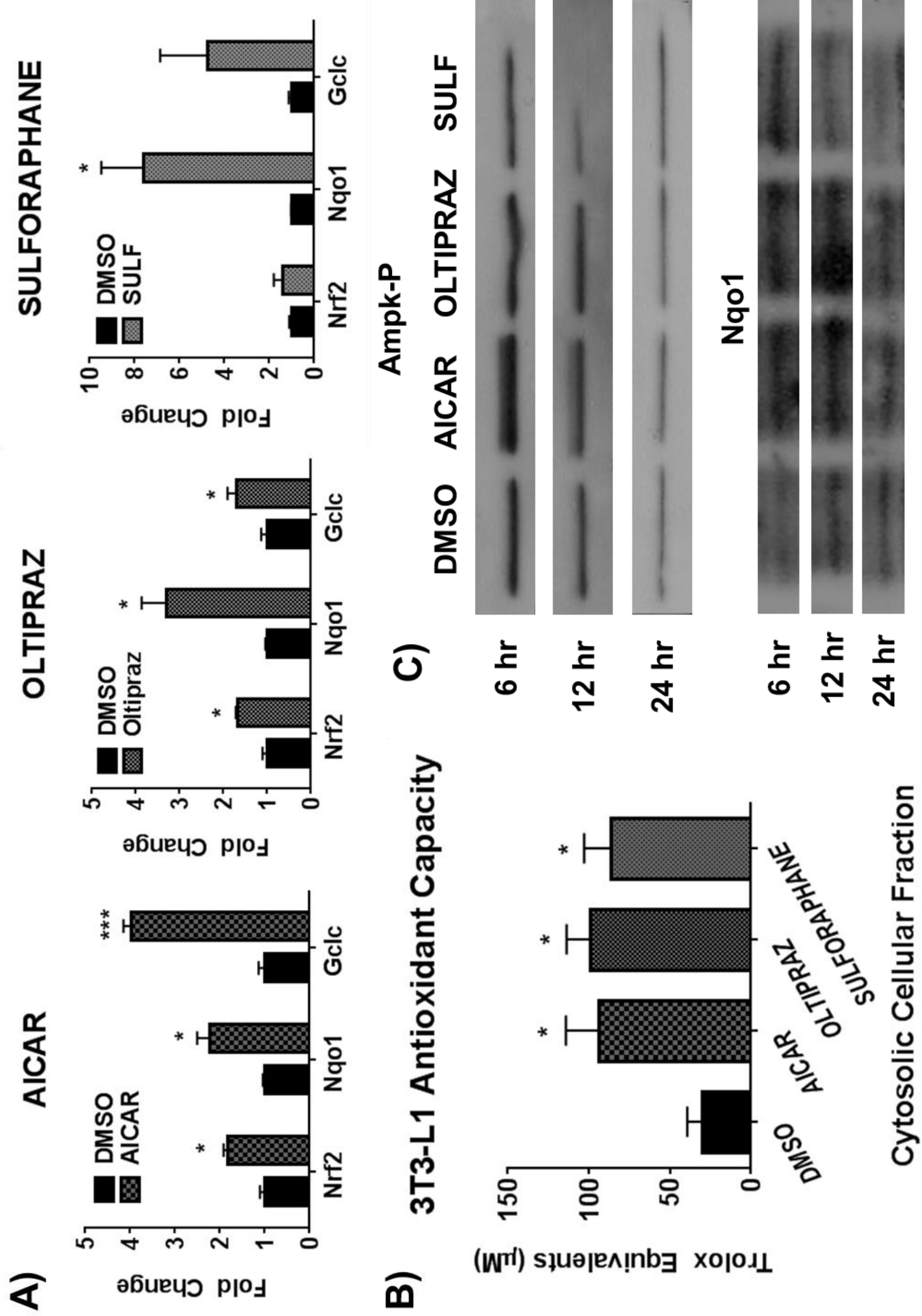

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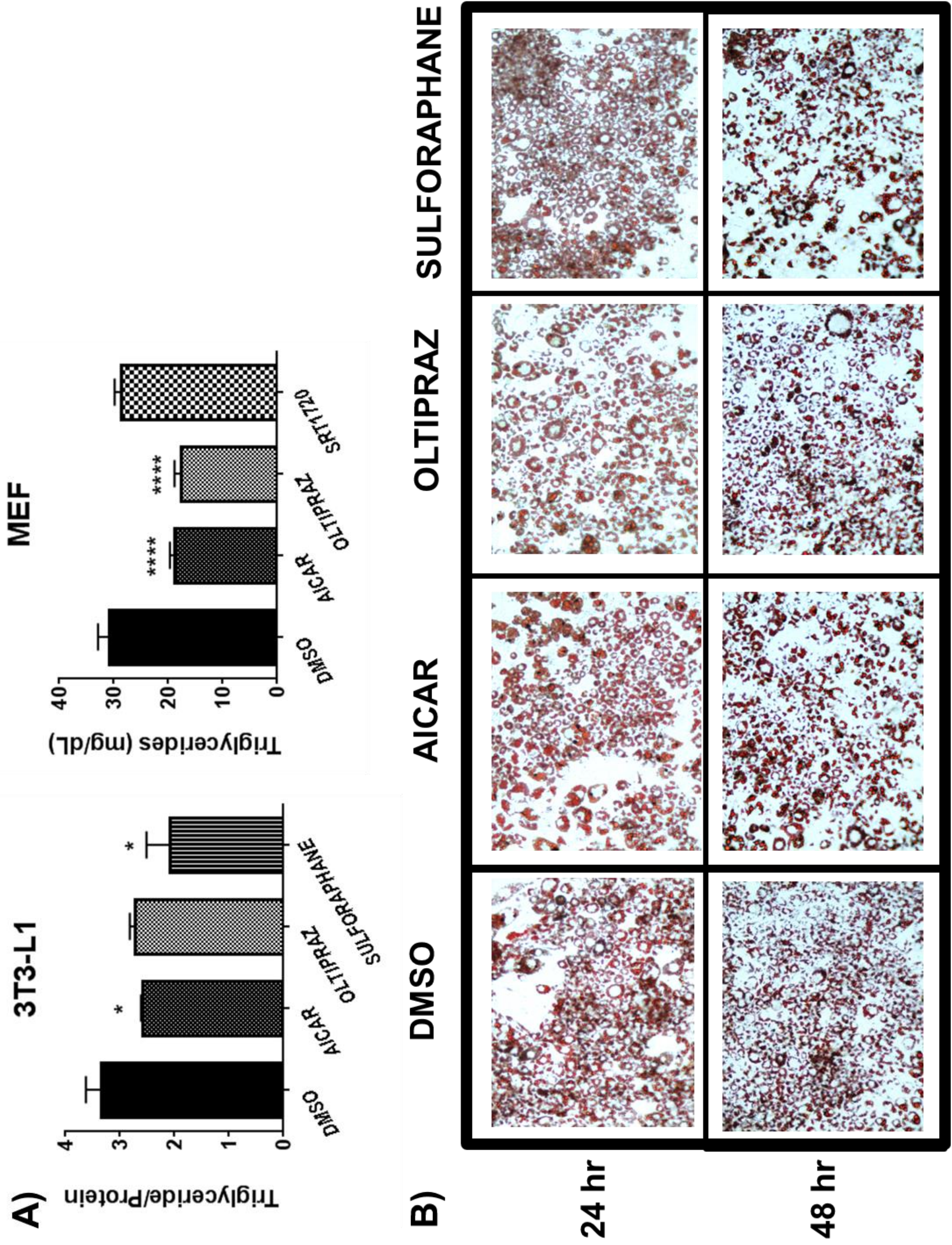

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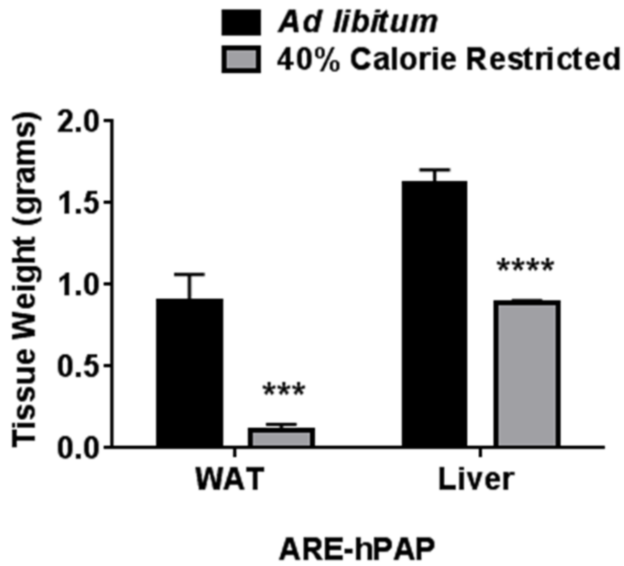

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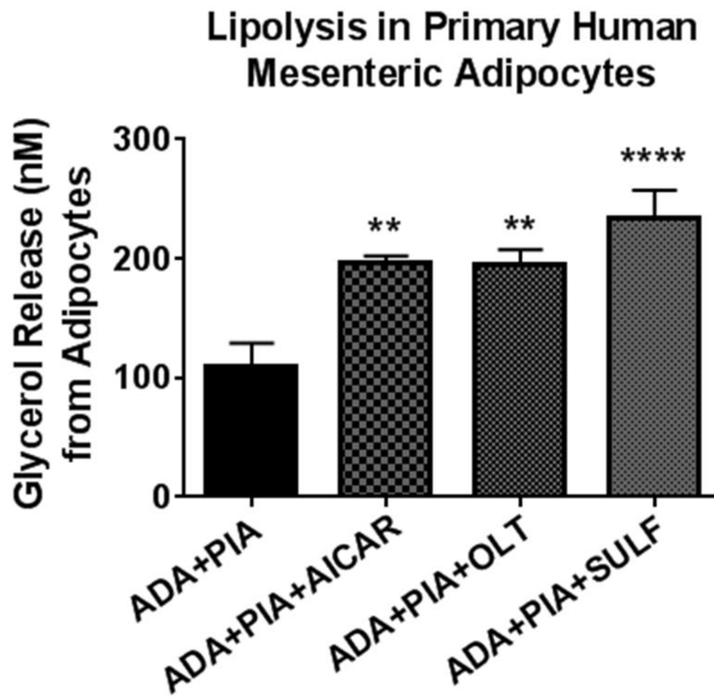

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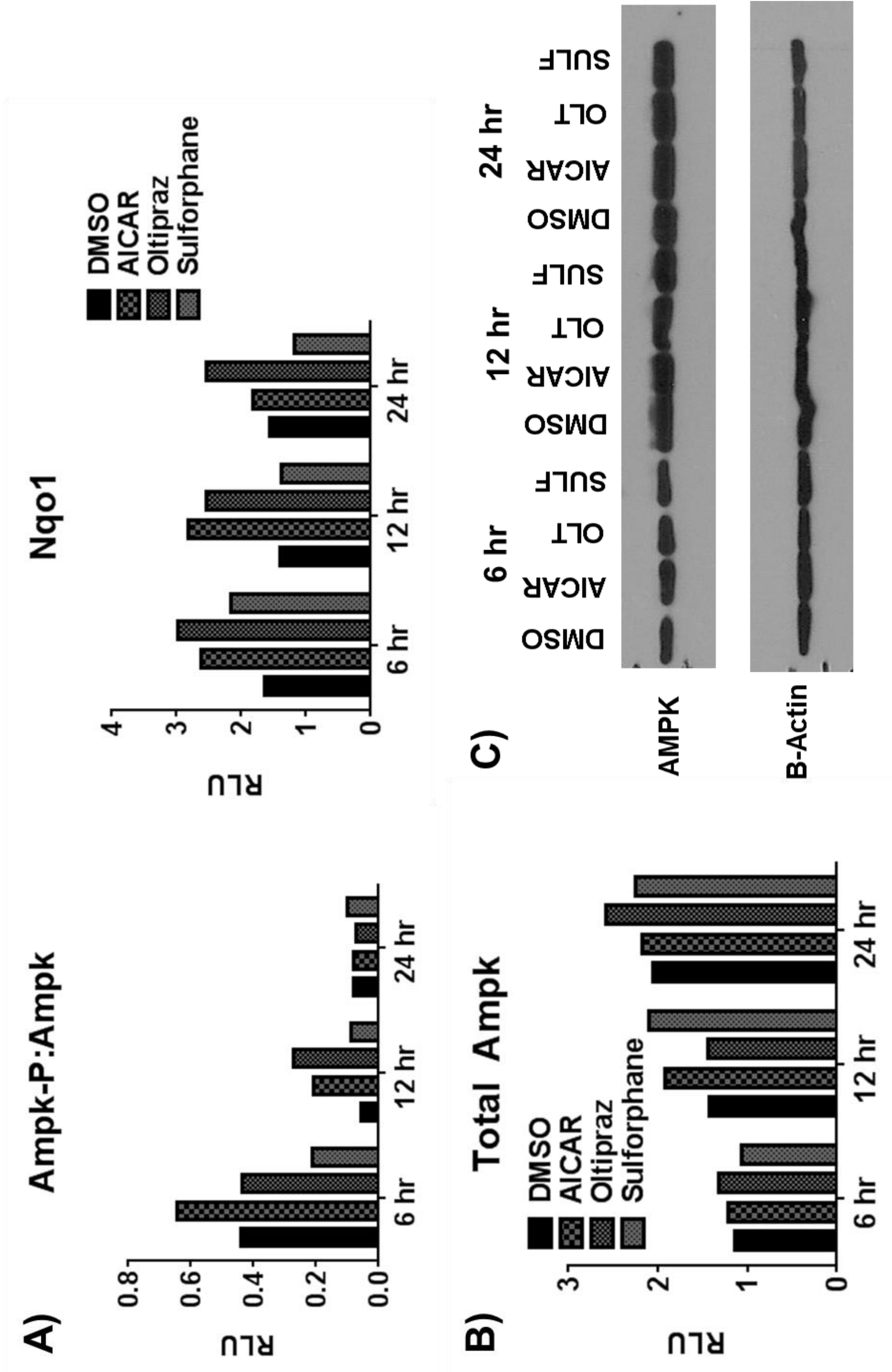

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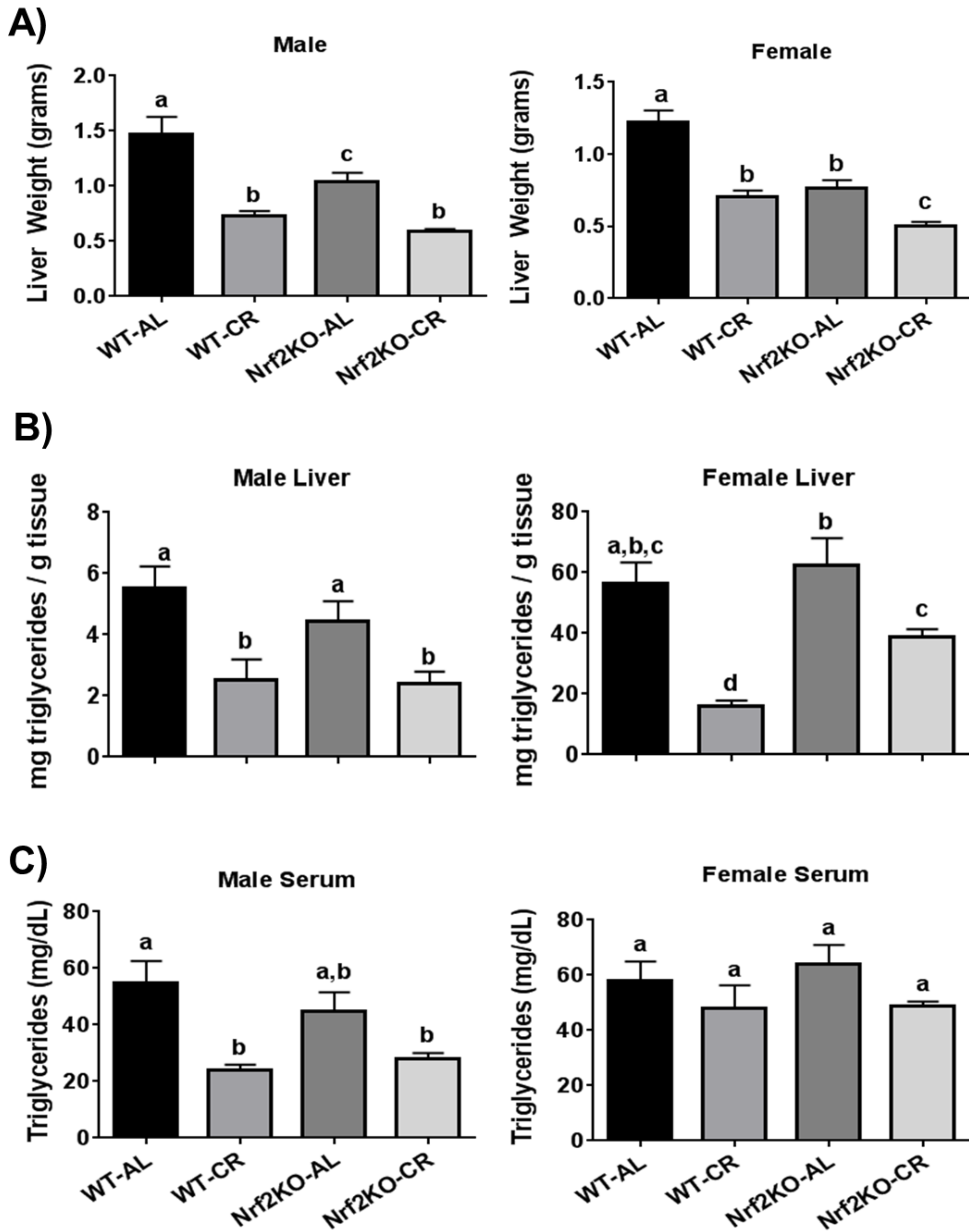

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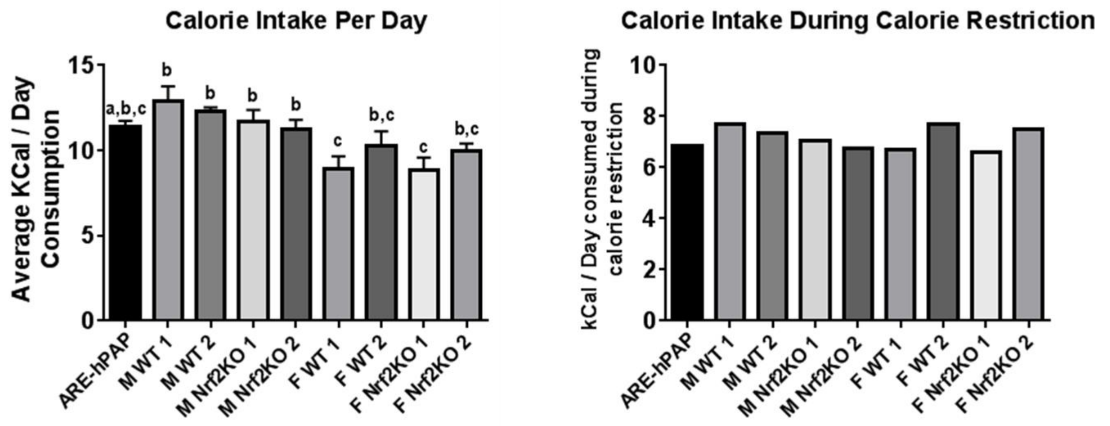

MANUSCRIPT II

This manuscript is prepared for submission to the journal *Toxicological Sciences*

BDE-99 increases lipid accumulation throughout differentiation in 3T3-L1 and human pre-adipocytes *in vitro*

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Abbreviated Title: BDE-99 increases lipids in pre-adipocytes

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Word Count:

Abstract: 137 (250)

Intro: 705 (750)

Discussion: 831 (1500)

References: 17

Greyscale Illustrations: 3

Color Illustrations: 4

Abstract

Flame retardants, specifically polybrominated diphenyl ethers (PBDEs), are chemical compounds widely used for industrial purposes and household materials. NHANES data indicates that nearly all Americans have trace amounts of PBDEs in serum, with even higher levels associated with occupational exposure (foam recyclers and carpet installers). PBDEs are known to bioaccumulate in the environment due to their lipophilicity and stability, and more importantly have been detected in human adipose tissue. The present study examined whether the PBDE congener, BDE-99 (0.2 μM-20 μM) enhances adipogenesis of pre-adipocyte cell models *in vitro* by increased lipid accumulation. 3T3-L1 mouse pre-adipocytes and human visceral pre-adipocytes were analyzed for increased lipid development. The second most abundant congener found in human adipose tissue, BDE-99 (20 μM), increased chemically-induced pre-adipocyte differentiation in both mouse and human *in vitro* models.

Introduction

Polybrominated diphenyl ethers (PBDE) are a class of brominated flame retardants that were part of the largest marketed group of flame retardants in 2004 due to their effectiveness and low cost (Birnbaum and Staskal, 2004). There are 209 congeners of PBDEs, with the most prevalent being BDE-47,-99,-100,-153, and -154 (Birnbaum and Staskal, 2004). PBDEs are found in electronics, textiles, and polyurethane foam, with major routes of exposure being inhalation and ingestion through indoor air /dust, outdoor air, animal food, and aquatic biota world-wide (Hoppe and Carey, 2007). PentaBDE was a commercially used flame retardant that is predominantly found in polystyrene foams, accounting for 30% of the weight of the foam, and was essentially only used in the Americas (Birnbaum and Staskal, 2004). Due to the lipophilic and highly stable nature of PBDEs, concern has grown over their bioaccumulation in the environment; as evidenced by their persistence in the environment via air/dust for 2 days, water for 2 months, and soil for 6 months (Birnbaum and Staskal, 2004). A remarkable decline in PBDE exposure has not been noted since initial bans of use of pentaBDE in 2008; which, could potentially be due to the persistence of the compounds in products prior to the ban, new products made from recycled materials, and continued presence in food sources (Tung *et al.*, 2014). PBDEs are detected in human adipose tissue, breast milk, birds, mammals, and fish (Hoppe and Carey, 2007), and it is noted in a 2003-2004 study of a cohort in New York City that the BDE-47 and BDE-99 were the most prevalent congeners found in human subcutaneous fat depots with a mean concentration of 399 ng/ g lipid weight (Johnson-Restrepo *et al.*, 2005). BDE-99 has been shown to be a well absorbed congener, and therefore preferred sites of deposition are lipophilic tissues (Birnbaum and Staskal, 2004).

The persistence of PBDEs and their potential effects on the environment and human health has been brought to the forefront by the US EPA, who identified PBDEs a priority human health concern (Tung, Boudreau, Wade and Atlas, 2014). Due to the deposition of the most abundant congeners in WAT (BDE-47 > BDE-99 > BDE-100 > BDE -153) (Johnson-Restrepo, Kannan, Rapaport and Rodan, 2005) and the increased knowledge of interactions between persistent organic pollutants (POPs), predominantly endocrine disruptors, and adipose tissue (AT) (La Merrill *et al.*, 2013), recent research has begun to focus on the effects of PBDEs on AT metabolism. It has been well established that PBDEs act as endocrine disruptors by decreasing T_4 levels or identified as agonists/antagonists of estrogen receptors (Hoppe and Carey, 2007).

AT is metabolically active and essential for normal lipid homeostasis. Adipocytes can be stimulated by insulin to store excess energy in the form of triglycerides, and can release those stores by lipolysis to meet energetic needs during periods of metabolic stress (e.g. fasting and calorie restriction) (La Merrill, Emond, Kim, Antignac, Le Bizec, Clement, Birnbaum and Barouki, 2013). Due to the nature of the composition of AT, persistent organic pollutants (POPs) can not only alter metabolic pathways in mature adipocytes, but can influence the differentiation of pre-adipocytes. Pre-adipocytes are fibroblasts within WAT that are induced through hormonal signals and a specific signal-transduction cascade to differentiate (Kamstra *et al.*, 2014). The study of effects of POPs on the differentiation of pre-adipocytes is directly relevant to developmental models, insulin sensitivity, and obesity related to WAT metabolism. Therefore, there are several publications that address BDE-47 effects on pre-adipocyte differentiation (Kamstra, Hruba, Blumberg, Janesick,

Mandrup, Hamers and Legler, 2014), but none that address the second major congener BDE-99.

Oral administration of a BDE-47 and BDE-99 mixture to rats altered insulin signaling with an increase in isoproterenol-stimulated lipolysis and decrease in insulin-stimulated glucose oxidation that resembled metabolically obese and non-insulin-dependent diabetic individuals (Hoppe and Carey, 2007). Body burden of other BDEs are also implicated in the pathogenesis of diabetes and metabolic syndrome (Lim *et al.*, 2008). Therefore, studies using PBDE mixtures and the predominant congener BDE-47 for effects on adipogenesis have been of recent interest. The mechanism by which PBDE mixtures enhance adipogenesis in WAT is not clear, and considering that the most prevalent congeners in human WAT are BDE-47, BDE-99, and BDE-153 (Johnson-Restrepo, Kannan, Rapaport and Rodan, 2005), this paper focuses on determining the effect and mechanism by which BDE-99 alone can contribute to an enhanced adipogenic effect.

Materials and Methods

Chemicals and Reagents. A neat 2,2',4,4',5-Pentabromodiphenyl ether preparation (BDE-99, catalog# ; lot#) was purchased (AccuStandard®,Inc., New Haven, CT, and stock solution was prepared to 100 μM in DMSO. Dulbecco's Modified Eagle Medium (DMEM) high glucose (4.5 g/L) with L-glutamine (Lonza), DMEM/F-12 (Gibco), Fetal Bovine Serum (FBS, Gibco), and penicillin-streptomycin (pen-strep) was purchased from Thermo Fisher Scientific (Waltham, MA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO): Dimethyl Sulfoxide (DMSO) , 3-isobutyl-1-methylxanthine (IBMX), Dexamethasone (DEX), and human insulin solution, specifically.

3T3-L1 pre-adipocyte cell culture, differentiation, and treatment. Mouse 3T3-L1 pre-adipocytes (ATCC® CL-173™) were obtained from American Type Culture Collection (ATCC, Manassas, VA) and were cultured according to ATCC protocol. 3T3-L1 pre-adipocytes (passage 6) were induced and differentiated according to a previously described protocol (Shin *et al.*, 2009; Xu *et al.*, 2012). Briefly, 48 h post-confluent cells (day 0) were induced by induction media (DMEM, 10% FBS, IBMX, DEX, insulin (10 μg/mL)) for 48 h and then differentiated only in the presence of insulin (10 μg/mL) for 6 days. Initial treatment of cells with vehicle (DMSO) or BDE-99 (20 μM) was performed from Day 0-2 (induction) and Day 2-8 (post-induction). All subsequent experiments, cells were treated with vehicle (DMSO) or BDE-99 (20 μM) throughout the course of differentiation, Day 0-8, and media was changed every 2 days.

Human pre-adipocyte cell culture, differentiation, and treatment. Poietics^{TM} human visceral pre-adipocytes (Cat #: PT-5005 Lot #: 0000313366, Lonza, Walkersville, MD) were passaged once and differentiated according to manufacturer's instructions for use. Cells were seeded at a density of 8700 cells/well in a 48-well plate using prepared Preadipocyte Growth Medium-2. Treatment with BDE-99 (20 µM) was carried out in the absence and presence of prepared Adipocyte Differentiation Medium (prepared media did not contain indomethacin) at day 0 and cells were stained for lipids or collected for mRNA analysis on day 11.

Oil Red O Staining and Lipid Quantification. 3T3-L1 and human pre-adipocytes were stained for lipid development (n=3). Cells were stained with Oil Red O at day 4, day 6, or day 8 following previously published protocol (Armstrong et al.). Oil Red O images were taken at 10x magnification (EVOS, Life Technologies, Carlsbad, CA), and Oil Red O staining was quantified by isopropanol (100%) extraction followed by absorbance measured at 510 nm on Spectramax M2 (Molecular Devices, Sunnyvale, CA) and ImageJ software (National Institute of Health) following the previous published protocol (Mehlem *et al.*, 2013).

Western blot. Western blots were used for identification and quantification of 3T3-L1 pre-adipocyte proteins from total cell lysates in RIPA buffer. Protein was electrophoretically separated by SDS-polyacrylamide gel electrophoresis. Primary antibodies for Fatty Acid Synthase (C20G5) (Fas, #3180, Cell Signaling, Beverly, MA) and Fatty acid binding protein 4 (D25B3) (Fabp4, #3544, Cell Signaling) were prepared in 5 % BSA in TBS-T at a 1:3000 dilution and incubated at 4°C overnight.

Secondary antibody, anti-rabbit (Sigma-Aldrich) was carried out for 1 hr at room temperature. The blots were incubated in Clarity™ Western ECL Blotting Substrate (Bio-Rad Laboratories, Hercules, CA) and visualized using autoradiography film.

mRNA expression and quantification of Nrf2. Total RNA was isolated from tissues or cells using TRIzol reagent (Invitrogen, Carlsbad, CA) following manufacturer protocol, and quantified following cDNA synthesis (Transcriptor First Strand cDNA Synthesis Kit) via LightCycler 480® SYBR Green I Master chemistry (Roche Applied Sciences, Indianapolis, IN). All Nrf2 gene primers were utilized at a final concentration of 0.5 μM with a final concentration of cDNA template of 50-60 ng.

Results

BDE-99 induces Fas and Fabp4 during induction (Day 0-2) and post-induction (Day 2-8) and demonstrated exposure during induction was not needed to induce lipid accumulation in pre-adipocytes. 3T3-L1 pre-adipocytes were induced in the presence of hormonal stimulators (DEX, IBMX, and insulin) with and without BDE-99, and pre-adipocytes were induced and were treated with or without BDE-99 during insulin-stimulated differentiation. Fas and Fabp4 protein levels were observed to be induced during induction (Fig 2A), 3.6 and 6.6-fold, respectively (Supplemental Figure 1). Only Fas was significantly induced (1.5-fold), by post-induction treatment with BDE-99 (Fig. 2A and Supplemental Figure 1). Increased Fabp4 protein during induction could signify increased fatty acid content prior to differentiation. Quantification of western blots via ImageJ is shown in Supplemental Figure 1. Post-induction BDE-99 treatment, in the presence of insulin, stimulated increased lipid development without chemical exposure during hormonal induction (Fig 2B and 2C), suggesting a direct involvement in the regulation of WAT lipogenesis.

BDE-99 increases lipid development in differentiating pre-adipocytes. 3T3-L1 pre-adipocytes were differentiated to mature adipocytes with or without BDE-99 for 6 and 8 days. Oil red O staining was utilized to assess lipid accumulation in maturing adipocytes. Figure 3A, demonstrates that BDE-99 increases lipid content during differentiation compared to vehicle (DMSO), and when quantified both day 6 and 8 have a significant increase in Oil Red O staining (Figure 3B). An increase in lipid staining compared to DMSO was similar in both 2 and 20 μM treatments (Figure 4A). Quantification of the dose-dependent changes in lipid development revealed

increased in lipid staining for cells treated with 2 and 20 μM BDE-99, but not 0.2 μM. The induction was not concentration dependent (Figure 4B).

BDE-99 treatment increases lipid development in human visceral pre-adipocytes during differentiation. In order to determine whether BDE-99 also has effects in human derived-cells we determined whether BDE-99 treatment increased lipid development using human visceral pre-adipocytes. Human visceral pre-adipocytes (Lonza) were treated with vehicle or BDE-99 (20 μM) in the absence or presence of differentiation media. Figure 5A illustrates that lipid development is enhanced in both non-differentiated and differentiated pre-adipocytes, as depicted by an increase in Oil Red O staining with BDE-99 treatment compared to DMSO. Staining was quantified by Oil Red O absorbance and a significant increase of staining was shown in both non-differentiated and differentiated cells (Figure 5B); staining was further confirmed by ImageJ quantification of images of Oil Red O staining in which differentiated cells had a 1.9-fold increase and non-differentiated cells had a greater 2.7-fold increase in total lipid content (Figure 5C).

BDE-99 decreases Nrf2 expression during adipogenesis. Nrf2 gene expression is increased throughout the course of adipocyte differentiation, and BDE-99 (20 μM) decreases the basal level expression of Nrf2 throughout the course of differentiation (Figure 6). Therefore, BDE-99 may not be acting directly on key pathways of adipogenesis, but may alter expression of Nrf2 that has previously shown to be important in adipocyte differentiation in mouse embryonic fibroblasts (Pi *et al.*, 2010).

Discussion

PBDEs have been identified as a primary human health concern by the U.S. EPA. PBDEs have been shown to have toxicity potential in in vivo models, including altered thyroid hormone levels and morphological effects in thyroid, liver and kidney (Kalantzi *et al.*, 2011), and non-Hodgkin's lymphoma (Kalantzi, Geens, Covaci and Siskos, 2011) and metabolic syndrome (Lim, Lee and Jacobs, 2008) have been associated with PBDE exposure in humans. These observations are even more concerning, as measurements of PBDE levels were assessed in the human population over the last decade (Johnson-Restrepo, Kannan, Rapaport and Rodan, 2005; Malarvannan *et al.*, 2013; Sjodin *et al.*, 2008; Stapleton *et al.*, 2008) with increasing exposures noted and persistence/bioaccumulation of compounds in the environment (Birnbaum and Staskal, 2004). Human levels of PBDEs and specific congeners are not consistent across all studies. PBDE exposure differs between populations, specifically concentrations of PBDEs in human matrices of European populations, exhibited lower exposure levels than U.S. populations predominantly due to the decreased usage of BFRs in these countries (Malarvannan, Dirinck, Dirtu, Pereira-Fernandes, Neels, Jorens, Gaal, Blust and Covaci, 2013). Not only do exposure levels differ, but U.S. populations have demonstrated a difference in distribution of PBDE congeners (Kalantzi, Geens, Covaci and Siskos, 2011). PentaBDE is a mixture that is predominantly used in the United States and is composed primarily of tri- and hepta-BDEs(Kalantzi, Geens, Covaci and Siskos, 2011); therefore, research within the U.S. has focused on congeners BDE-47 and BDE-99 due to population and environmental levels. Our findings utilizing BDE-99 predominately represent exposure specific to the U.S. population by exposure to pentaBDE (Kalantzi, Geens, Covaci and Siskos, 2011).

BDE-47 has been demonstrated to induce adipogenesis in models of 3T3-L1 pre-adipocyte differentiation (Kamstra, Hruba, Blumberg, Janesick, Mandrup, Hamers and Legler, 2014; Tung, Boudreau, Wade and Atlas, 2014), but the mechanism of action of BDEs needs to be studied further. A mixture of PBDEs representative of the North American body burden and BDE-47 alone were shown to have dose-dependent increased in aP2 and perilipin protein and significant increase in mRNA expression by day 8, which is representative of increased lipid droplet size and development demonstrated through increased lipid accumulation by both treatments (Tung, Boudreau, Wade and Atlas, 2014). This supports our observations that the congener BDE-99 also contributes to increased lipid development in differentiating pre-adipocytes. The findings also demonstrate that exposure during initial induction of pre-adipocytes is not needed to increase total lipid staining, as was previously shown with modest changes to mRNA expression during the first 2 days of differentiation (Kamstra, Hruba, Blumberg, Janesick, Mandrup, Hamers and Legler, 2014).

An interesting finding and contribution to understanding the mechanism of BDE-induced adipocyte differentiation and lipid accumulation was BDE-47 and BDE-99 have poor Pparγ binding activity determined by their relative potency (determined from IC_{50} and K_d values) (Fang *et al.*, 2015). The only active ligands of Pparγ were hydroxylated metabolites of BDE-47, and 5-OH-BDE-99 (K_d=30 μM)(Fang, Webster, Ferguson and Stapleton, 2015). Therefore, no significant induction of Pparγ transcript by BDE-47 (Tung, Boudreau, Wade and Atlas, 2014), and induction of Pparγ2 mRNA without down-stream target gene expression (Kamstra, Hruba, Blumberg, Janesick, Mandrup, Hamers and Legler, 2014) in 3T3-L1 cells demonstrates that Pparγ activation may not be the predominant transcription factor involved in induction of lipid development. Preliminary studies of Pparγ protein

expression showed that the transcription factor was not up-regulated in our exposure model, but the γ2-subunit was induced with treatment without Cepbα induction (Supplemental Figure 2). Interestingly, Pparγ expression was significantly increased by Day 8 by a PBDE mixture (Tung, Boudreau, Wade and Atlas, 2014), suggesting other potential congeners or metabolites are involved in its regulation. Our subsequent finding of decreased Nrf2 expression with BDE-99 treatment supports a potential hypothesis of decreased adipogenesis with increased lipid development, resulting in hypertrophic adipocyte development. It has previously been demonstrated that Nrf2KO MEFs have decreased ability to differentiate (Pi, Leung, Xue, Wang, Hou, Liu, Yehuda-Shnaidman, Lee, Lau, Kurtz and Chan, 2010), and without Pparγ and Cebpα induction, BDE-99 does not contribute to increased differentiation. Therefore, further studies must be carried out to determine what lipogeneic pathway is targeted by BDE-99 treatment to increase lipid development throughout differentiation.

BDE-99 was studied due to its prevalence in human adipose tissue, and more specifically because of its defined metabolism and disposition in rat and mouse models (Chen *et al.*, 2006). Chen et al. demonstrated that 85% of BDE-99 was absorbed by single-p.o. and 34-38% remained in tissues, with significant disposition in adipose and minimal decay of levels over 10 days, and significant increase in concentration with 10-day daily doses (Chen, Lebetkin, Sanders and Burka, 2006). In addition to concentrated exposure of BDE-99 exposure in adipose tissue, it was demonstrated that BDE-99 was also more extensively metabolized than BDE-47, which is excreted by day 10 (Chen, Lebetkin, Sanders and Burka, 2006). Therefore, the effect of BDE-99 exposure on adipose tissue is an essential to understand

potential human health outcomes, in which BDE-99 metabolites may need to be addressed for their toxicity also.

Our study is the first to translate BDE-99 effect on adipogenesis and lipid development to a human model. The findings support that human pre-adipocytes develop increased lipid development upon BDE-99 exposure in both non-differentiated cells, and differentiating cells. This research provides additional data supporting the understanding of POP exposure to cause dysregulation of insulin homeostasis, obesity, and hepatic steatosis,(Ruzzin *et al.*, 2010) and the toxicological importance of adipose tissue (La Merrill, Emond, Kim, Antignac, Le Bizec, Clement, Birnbaum and Barouki, 2013). Therefore, these human *in vitro* data can be utilized in order to determine if increased BDE-99 concentrations in adipose tissue contribute to insulin resistance, diabetes, obesity, and overall metabolic syndrome, with a mechanistic approach.

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Figure 1. *In vitro* pre-adipocyte differentiation paradigm with treatment of environmental toxicant BDE-99.

Figure 2. BDE-99 induces Fas and Fabp4 during induction (Day 0-2) and post-induction (Day 2-8) and it was demonstrated that exposure during induction of pre-adipocytes was not needed to induce lipid accumulation. 3T3-L1 pre-adipocytes were treated with BDE-99 (20 μM) during induction (Day 0-2) and post-induction (Day 2-8). A) Induced and post-induction pre-adipocytes treated with BDE-99 were lysed in RIPA buffer and Fas and Fabp4 protein levels were determined by western blot B) Post-induced pre-adipocytes were stained with Oil Red O, images were taken at 10x magnification C) Images were quantified using ImageJ to determine induction of lipid content (n=2/treatment).

Figure 3. Increased lipid development throughout differentiation of 3T3-L1 pre-adipocytes treated with BDE-99. 3T3-L1 cells were differentiated in the presence of DMSO (0.1%, control) or BDE-99 (20 μM). A) Treated 3T3-L1 cells were stained for the development of neutral lipids by Oil Red O at Day 6 and Day 8. B) BDE-99 dependent lipid development was determined through quantification of 5 separate Oil Red O images taken at 10x magnification per well (n=3 wells/treatment group). Significance was determined by individual Student's t-test compared to control at each time point ($p<0.01$**, $p<0.0001$****).

Figure 4. Lower doses of BDE-99 *in vitro* elicit the same enhancement of lipid development in 3T3-L1 pre-adipocytes. 3T3-L1 cells were differentiated in the presence of DMSO (0.1%, control) or BDE-99 (0.2, 2, or 20 μM). A) Treated 3T3-L1 cells were stained with Oil Red O at Day 8. B) Enhancement of lipid development during differentiation was quantified by ImageJ utilizing 5 separate Oil Red O images

taken at 10x magnification per well (n=3 wells/treatment group). A Dunnett's test was used to determined significance compared to control ($p<0.01$**, $p<0.001$***).

Figure 5. Human visceral pre-adipocytes responded to BDE-99 treatment equally, with increased lipid content in mature adipocytes. Human visceral pre-adipocytes were treated with BDE-99 (20 μM) with and without Adipocyte Differentiation Media (ADM) for 11 days. A) Cells were stained with Oil Red O on Day 11 for the accumulation of neutral lipids in matured adipocytes and images were taken at 10x, 20x (not shown), and 40x magnification (EVOS, Life Technologies). B) Oil Red O was abstracted from the fixed cells with 100% isopropanol, and absorbance of each well was measured at 510 nm to quantify lipid staining. C) ImageJ software was utilized to quantify 5 images from each well (n=3 wells/treatment group) to confirm quantification of Oil Red O staining. Significance was determined by Student's t-test compared to control for both non-differentiated and differentiated staining ($p<0.05$*, $p<0.01$**).

Figure 6. BDE-99 decreases transcriptional expression of Nrf2 throughout differentiation. 3T3-L1 cells were treated with DMSO (0.1%, control) or BDE-99 (20 μM) from Day 0 to Day 8 of differentiation, and total RNA was collected at Day 4, Day 6, and Day 8. Nrf2 mRNA expression was quantified via qPCR and normalized to housekeeping gene B2M. Student's T-test was used to determine the statistical significance between treatments for each individual time point ($p<0.05$*).

Supplemental Figure 1. Quantification of Fas and Fabp4. Fas and Fabp4 were measured to assess the lipogenic pathway in BDE-99-treated 3T3-L1 cells and fatty acid content. Band intensity of each protein was measured via ImageJ (n=1). Fas and Fabp4 protein was normalized to β-Actin, and expressed as Relative Luminescence Units (RLU).

Supplemental Figure 2. Protein detection of adipogenic transcription factors in BDE-99-treated 3T3-L1 cells. 3T3-L1 cells were exposed to 20 μM BDE-99 during day 0-2 and day 2-8 of adipocyte differentiation, and whole cell lysates were obtained via RIPA extraction. A) Western blots of Pparγ and Cebpα.

Figure II-1.

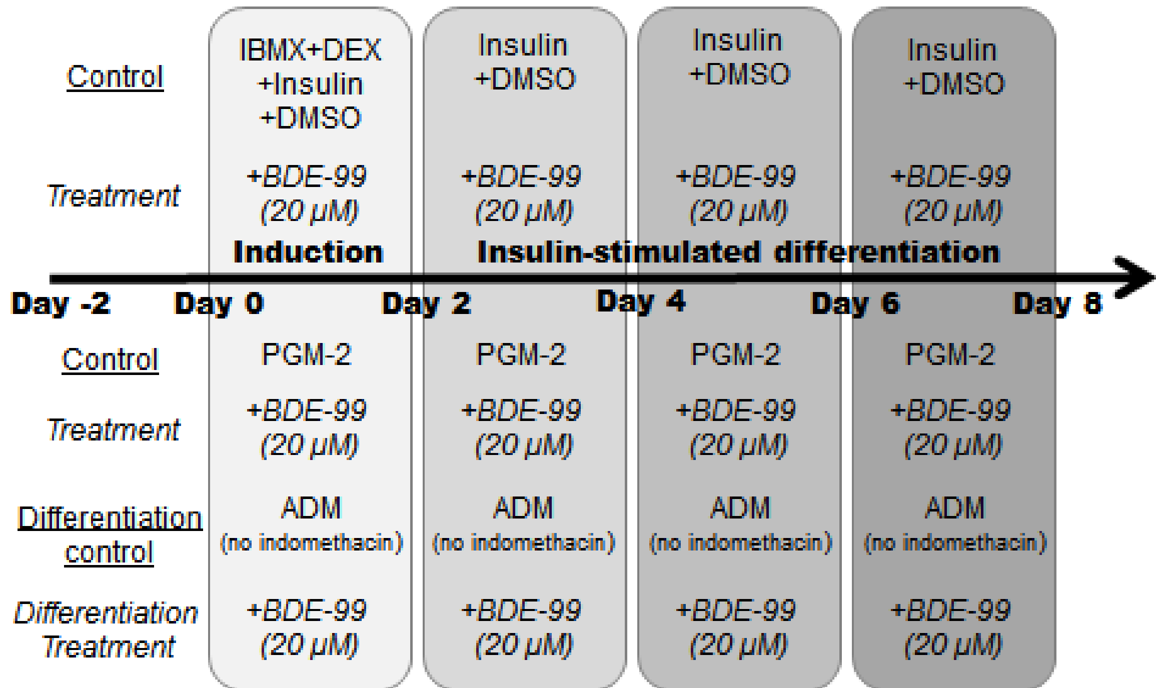

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Figure 2.

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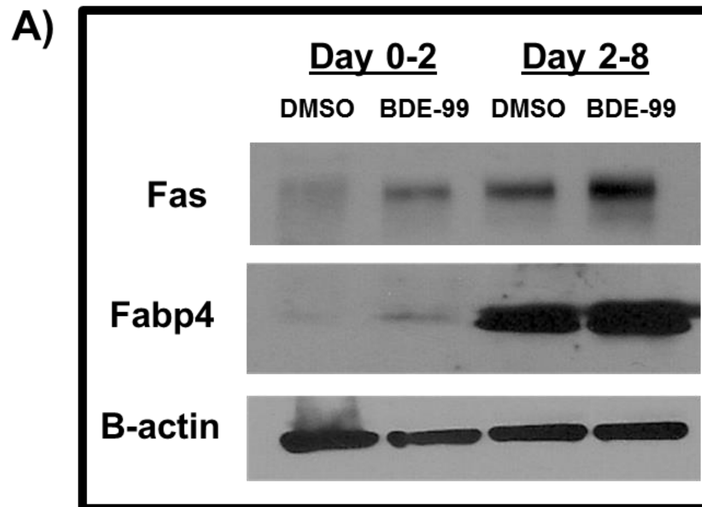

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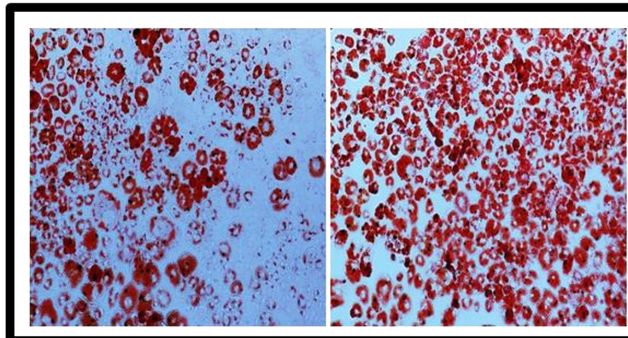

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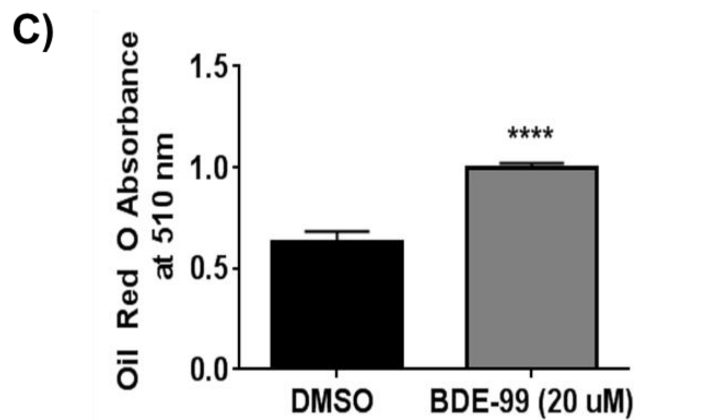

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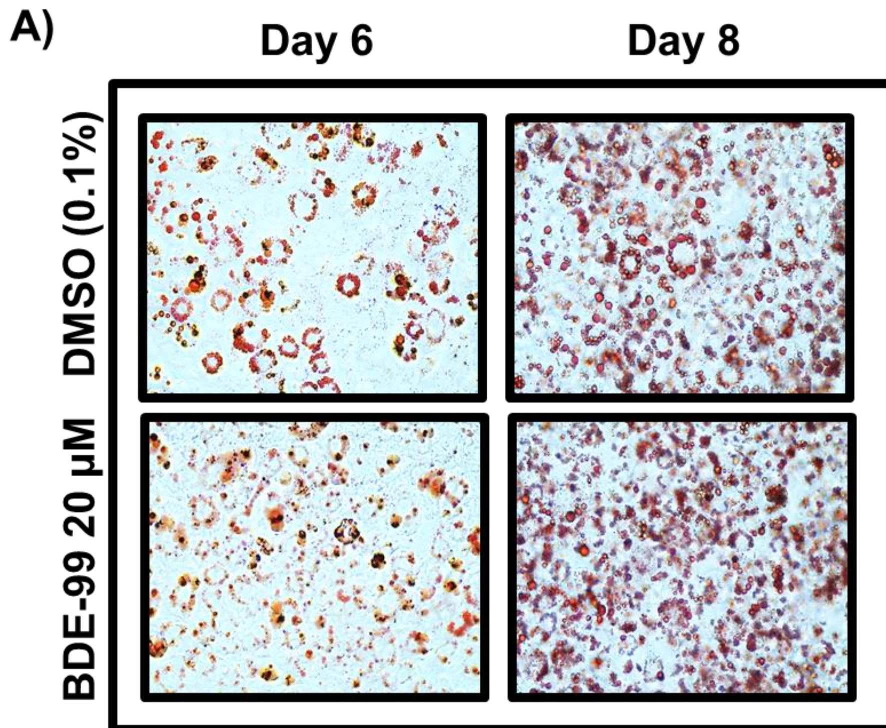

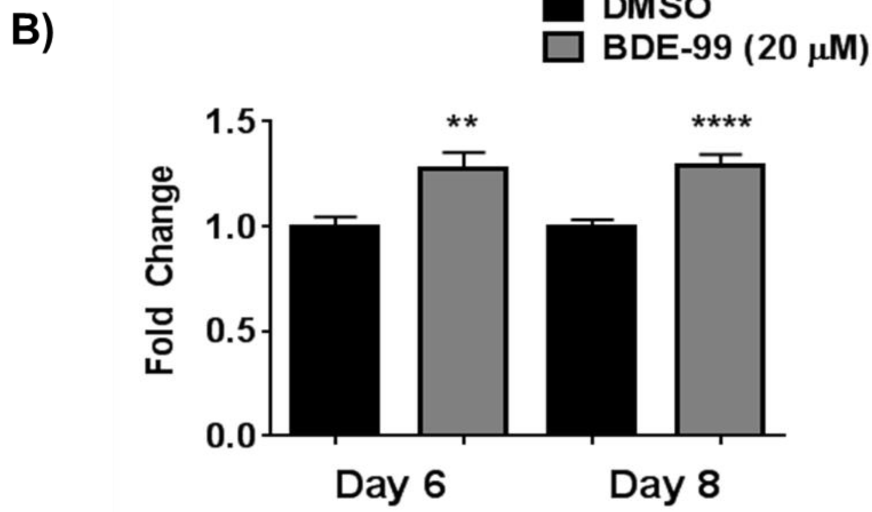

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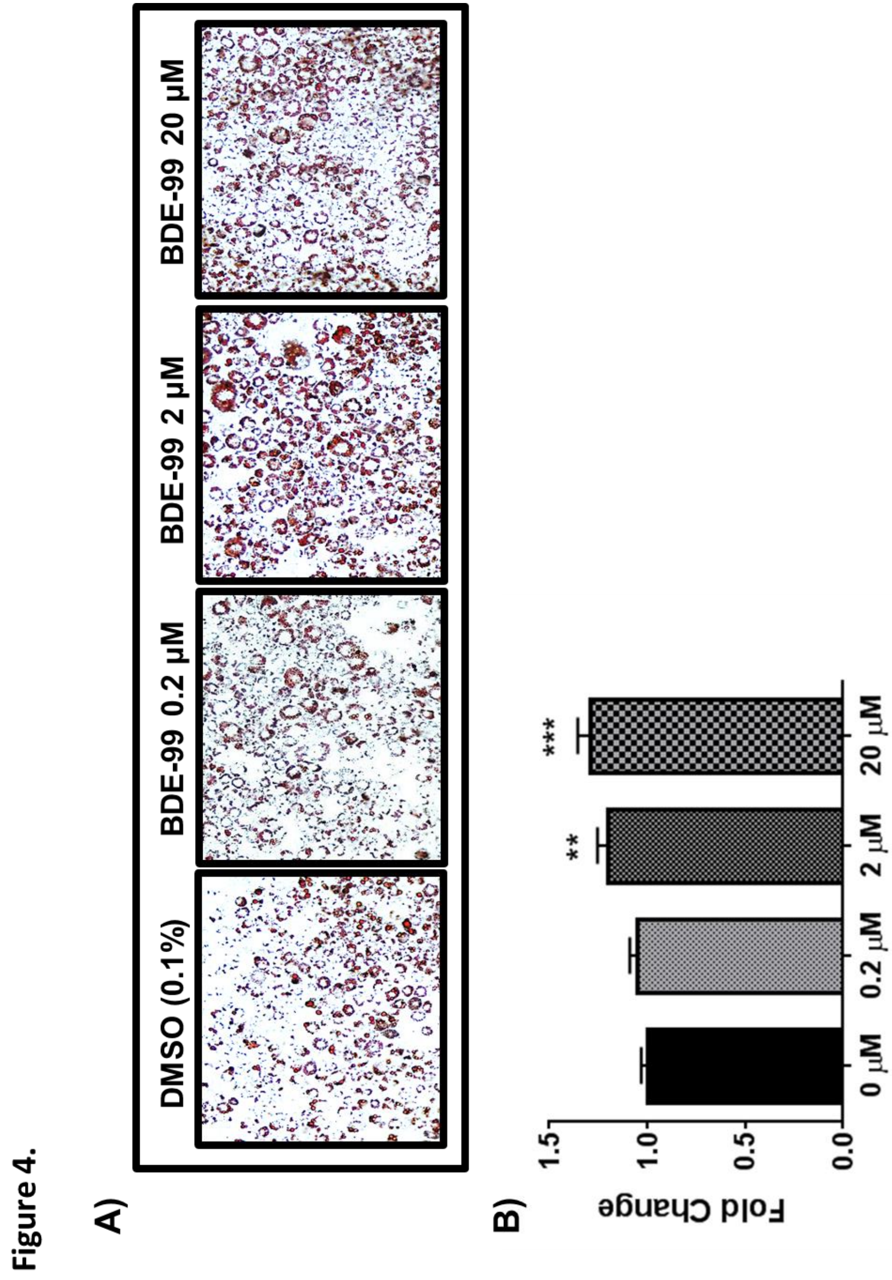

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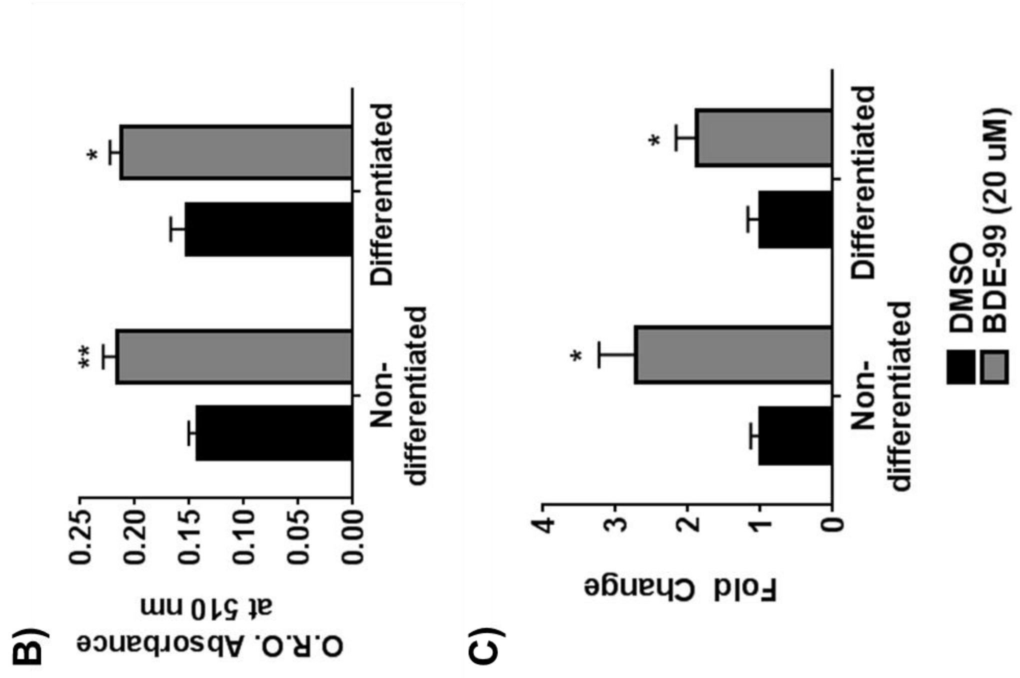

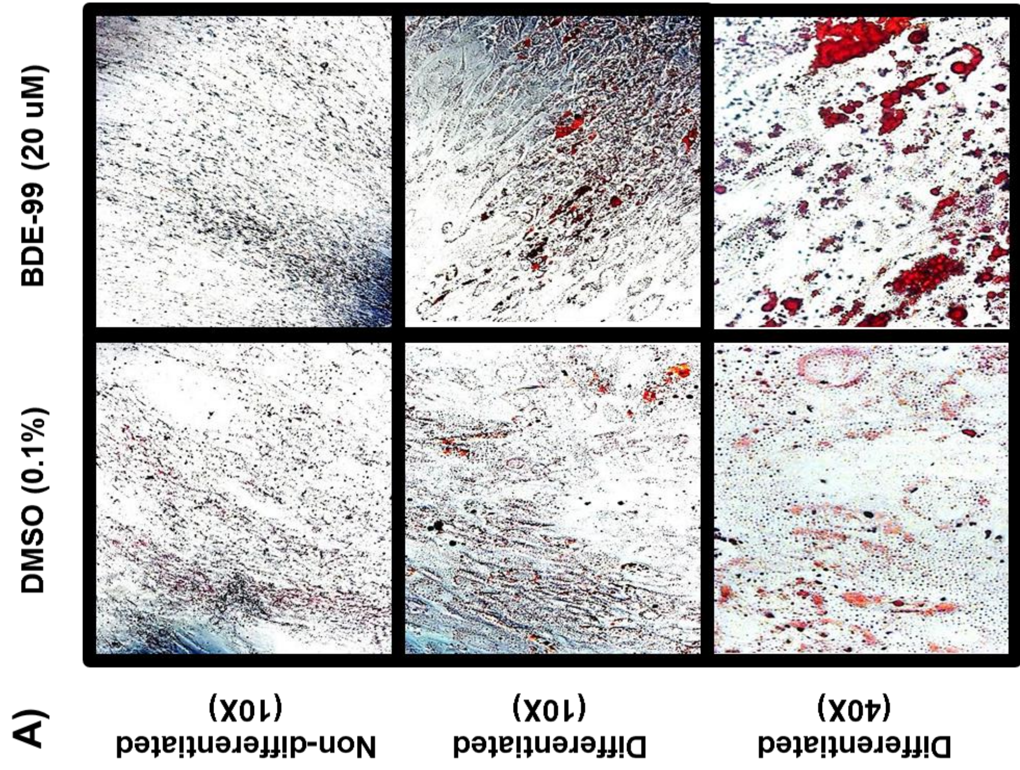

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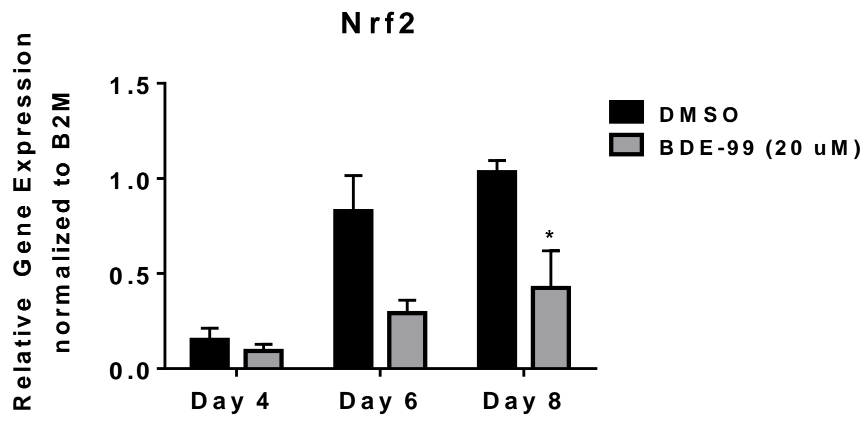

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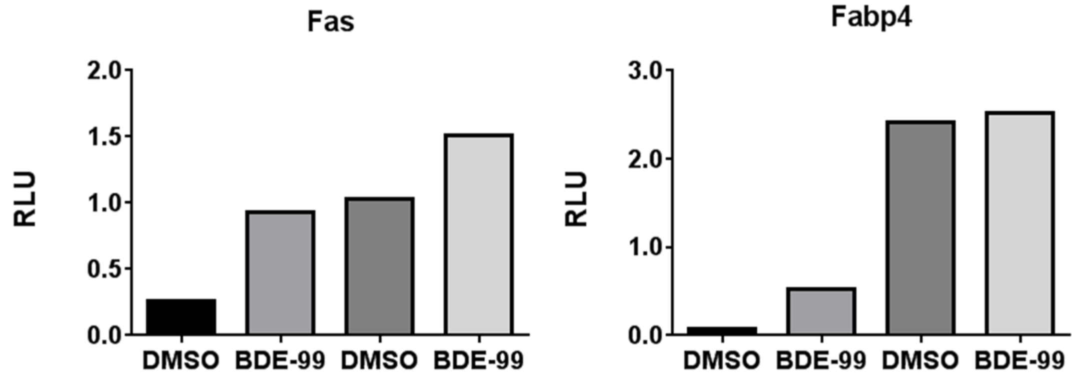

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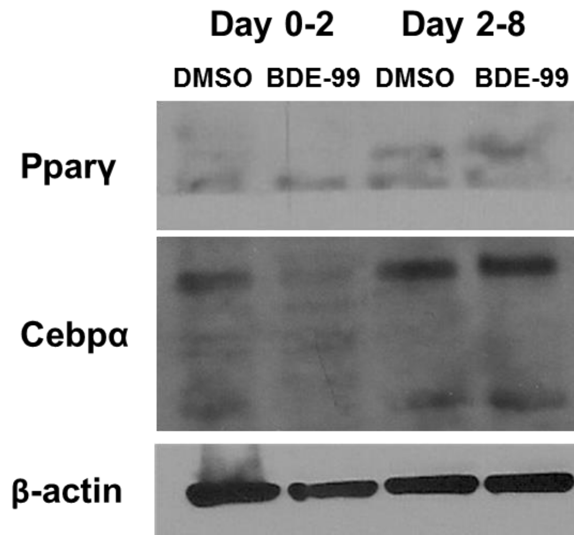

MANUSCRIPT III

Accepted for publication in *The Journal of Biochemical and Molecular Toxicology*, in press 2013.

Title page

Effects of Developmental Deltamethrin Exposure on White Adipose Tissue Gene Expression

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Abstract: 180 words (150)

Introduction: 490 words (550)

Discussion: 953 words (550)

Figures: 3

Tables: 0

References: 28

Running title: Developmental Deltamethrin and Adipose Tissue

Keywords: Deltamethrin, White Adipose Tissue (WAT), Adipogenesis, Perinatal, Pyrethroid

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ABSTRACT

Deltamethrin, a type II pyrethroid, is a widely used insecticide. The purpose of this study was to determine whether perinatal deltamethrin exposure altered the expression of adipogenic and lipogenic genes in white adipose tissue (WAT) in adult pups. C57BL/6 pregnant mice were administered 0, 1, or 3 mg/kg of deltamethrin orally every 3 days throughout gestation and lactation. Offspring were weaned on postnatal day 25 and WAT was collected from 5 month old male mice. Perinatal deltamethrin exposure decreased the mRNA expression of adipogenesis related transcription factors Pparγ, Cebpα and lipogenic genes Srebp1c, Acc-1, Cd36, Lpl, Scd-1; along with Nrf2 and target genes Nqo1 and Gclc at the 1 mg/kg treatment. Cytokine expression of Fas/Tnf-R and Cd209e at the 1 mg/kg treatment was significantly decreased and expression of Tnf, Cd11c, and Fas/Tnf-R was decreased at the 3 mg/kg treatment. Developmental deltamethrin exposure did not overtly affect body weight or adipose weight, but decreased mRNA expression of specific genes that may potentially disrupt normal adipogenesis and lipid and glucose metabolism if the offspring are challenged by changes in diet or environment.

INTRODUCTION

Pyrethroids are synthetic chemicals modeled after the naturally occurring pyrethrins, found in chrysanthemums (1). Type I and type II pyrethroids are commonly used as potent and effective insecticides for agricultural and public health applications (2) and can easily enter the exoskeleton of insects. These axonic poisons cause paralysis, and ultimately death of the organism by keeping sodium channels open in the neuronal membranes. Type II pyrethroids, such as deltamethrin, are defined by an α-cyano group that is known to produce a longer-lasting inhibition of voltage-activated sodium channels (1,2). Pyrethroid pesticides are often thought of as "safer" alternatives to the more toxic organophosphates because of their low mammalian toxicity. This relatively low toxicity is attributed to a combination of efficient detoxification mechanisms in mammals (3) and lower sensitivity of ion channels (2). However, metabolic detoxification mechanisms are not fully developed in the very young, potentially increasing susceptibility to pyrethroids in this population (4). This is particularly crucial because pyrethroid use has increased tremendously since the cancellation or reduction in the use of many organophosphorus pesticides (5). In recent years, significant levels of pyrethroid metabolites, including those of deltamethrin, have been found in the urine of pregnant women and children (6-9). Furthermore, deltamethrin, which is widely used to control malaria vectors, has also been detected in the breast milk of South African women (10). The latter data pose questions regarding the safety of deltamethrin, as developmental neurotoxicity and other adverse

developmental effects are currently being studied in these susceptible populations (1).

Adipose tissue is a lipophilic tissue that is metabolically active and essential for the proper maintenance of systemic energy balance. Adipogenesis requires a highly regulated cascade of transcription factors, including C/EBP family and Pparγ, which regulate the differentiation of preadipocytes to adipocytes (11). Most recently, this cascade of transcription factors has been proven to be regulated by Nrf2, a well-defined transcription factor of oxidative stress (12,13). The three major functions of adipose tissue are lipid storage and mobilization, glucose homeostasis, and endocrine function, involving secretion of hormones, cytokines, and transcription factors (11,14). Changes in the expression of transcription factors involved in adipocyte differentiation during development can lead to dysfunction in the metabolic and endocrine functions of white adipose tissue (WAT), as demonstrated by gene knock-down, knock-out, and induction studies (11,14). When characterizing deltamethrin as a safe pesticide, The World Health Organization (WHO) observed lower body weight gain in adult rats that were orally administered the pesticide, decreased mean fetal weights with a perinatal high dose of 10 mg/kg body weight, and a dose-related decrease in body weight of postnatal mice (15). Despite increased research on the developmental toxicology of pyrethroids, to our knowledge, no studies have addressed whether exposure to pyrethroids during developmental stages affects adipose tissue development and homeostasis. Therefore, the purpose

of this study was to address the potential metabolic effects of perinatal deltamethrin exposure in white adipose tissue, to address previously described changes in body weight during development.

MATERIALS AND METHODS

Animals

Eight week old female and male C57BL/6J mice purchased from Jackson Laboratory (Bar Harbor, ME) were used. Mice were maintained on a 12:12 light/dark cycle with food and water available *ad libitum*. All procedures were conducted in accordance with the NIH Guide for Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at Robert Wood Johnson Medical School.

Treatment

10 week old female C57BL/6J mice were individually housed and mated with C57BL/6J males. Upon identification of a vaginal plug, males were removed and single-housed female mice were administered 0 (control), 1, or 3 mg/kg body weight deltamethrin (ChemService, West Chester, PA), dissolved in corn oil and mixed with peanut butter (~ 100 mg; Skippy Creamy Peanut Butter) every 3 days throughout gestation and lactation as described previously (16-18). Mice were monitored to ensure total consumption of the

treatment dose, which generally occurred within 10 min. This administration method reduces handling stress associated with injections during pregnancy and most closely mimics human oral exposure conditions (16,17).

The dose selected in this study is 1/4 of the developmental NOAEL (19). Unfortunately, the rapid metabolism of pyrethroids has made dose extrapolation difficult. There have been no comparative studies in rodent and humans to determine half-lives of the parent compound, but separate studies have presented the half-life of deltmethrin, in humans and rats, to appear to be in a similar range of several hours. It should be noted that recent pharmacokinetic modeling in rats predicted that humans would experience a higher brain concentration of deltamethrin compared to rodents, most likely because humans do not have plasma carboxylesterase activity (20).

The offspring were weaned at postnatal day (PND) 25, and once weaned; offspring received no additional exposure to deltamethrin. The 5 month time point was selected because tissues were being shared from a cohort from a study being conducted by Dr. Richardson's group for the primary purpose of developmental deltamethrin effects on behavior. Those cohorts had been assessed for neurobehavioral changes and 5 months was determined to be an appropriate time of necropsy for Dr. Richardson's study. As 5 months represents adulthood and mature adipose tissue, white adipose tissue was evaluated for changes in expression of genes related to adipogenesis, lipid synthesis, glucose uptake, and inflammation.

Prior to sacrifice, blood glucose concentrations were obtained via tail vein nick and measured using a TRUEtrack™ glucometer. Animals were not fasted prior to sacrifice. Mice were sacrificed by CO_2 inhalation followed by cardiac puncture for blood collection at 5 months of age. Blood was centrifuged at 14,000 × *g* for 10 min to obtain serum. White adipose tissue from the abdomen was removed and snap frozen in liquid nitrogen. Samples were stored at -80°C until shipment to University of Rhode Island on dry ice. Dosing of mice and sample collection was carried out by the laboratory of Dr. Jason Richardson at the University of Medicine and Dentistry of New Jersey, Piscataway, NJ.

RNA Extraction

Perinatal exposure to deltamethrin causes postnatal behavioral changes in adult male, but not female, offspring (Richardson et al., submitted). For this reason, adult male mice were selected for the current investigation. Total RNA was isolated from 50-100 mg frozen WAT from the male 5 month cohort via homogenization in Trizol™ lysis buffer (Life Technologies, Grand Island, NY) followed by chloroform-isopropanol extraction. The RNA concentration was determined by measuring UV absorbance of the sample at 260 nm using NanoDrop™ (Wilmington, DE) and RNA integrity was confirmed by the presence of distinct 18S and 28S bands using formaldehyde-agarose gel electrophoresis. RNA samples were stored at -80°C until utilized.

Quantigene Plex 2.0 Assay

Total RNA was applied to the Quantigene Plex (Affymetrix, Santa Clara, CA) and hybridized overnight at 54˚C with specific mRNA capture beads and capture probes. The following day, the samples were hybridized with pre-amplifier and a biotinylated label probe for 1 h at 50˚C followed by application of streptavidin conjugated phycoerythrin detection probe for 30 min at room temperature. The plex was analyzed on a Luminex Bio-Plex 200 array reader with Luminex 100 xMAP technology (Austin, TX). mRNA expression was normalized to the Rpl13a housekeeping gene, which was not significantly different between treatment groups. Only male gene expression in adipose tissue was determined in an attempt to reduce gender variability as a confounding factor

qPCR mRNA Quantification

qPCR was utilized measure the mRNA gene expression of important genes in WAT that were not available on the Quantigene Plex used. Total RNA was utilized as a template to make complementary DNA via polymerase chain reaction (PCR) following the specified protocol for Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Sciences Cat. #: 04897030001). The cDNA was amplified and quantified for gene expression levels using LightCycler 480® SYBR Green I Master chemistry (Roche Applied Sciences) for qPCR determination of Nrf2, Nqo1, and Gclc. Rpl13a was used as the housekeeping gene for normalization.

Statistical Analysis

Statistical analyses of data were performed using a one-way analysis of variance (ANOVA) and further analyzed by a Dunnett's Test *post hoc* test to determine significant different between control and each treatment group. $P < 0.05$ was considered statistically significant. Unless otherwise stated, all data were presented as mean ± SE of 9 animals (control) and 8 animals (1 mg/kg, 3mg/kg doses). Statistica v.9 (StatSoft, Tulsa, OK) was used for statistical analysis.

RESULTS

Effect of developmental exposure on physiological parameters and the expression of genes related to glucose homeostasis in WAT

The physiological data collected prior to necropsy showed no significant differences in the body weight (BW) between pups that were exposed to vehicle or deltamethrin during development (Fig. 1A), and blood glucose concentration was similar between both treatments compared to control (Fig. 1B). The expression of a predominant insulin-responsive gene and two essential glucose transporters in WAT were determined using Luminex 100xMAP technology. Figure 1C shows that there were some differences between the gene expression of insulin responsive genes and glucose transport genes, Irs-1, Glut4, and Glut2, in WAT. Developmental deltamethrin (1 mg/kg) exposure decreased Glut4 (31%), and both deltamethrin doses significantly decreased Glut2 mRNA (83%, 82%). No changes in Irs-1 mRNA expression were observed. These data are supported by the blood glucose measurements that were also found to be unchanged with perinatal deltamethrin exposure.

Effect of developmental deltamethrin exposure on expression of genes related to adipogenesis and lipogenesis in WAT

There were no significant differences in body weight between the male pups that were exposed to vehicle or deltamethrin during development at 5

months of age (Fig 1A), but because of the significant decrease in mRNA expression in glucose transport the primary functions of adipose tissue, development of adipocytes and lipid transport and metabolism should be described. Expression levels of lipogeneic genes in WAT and the well described regulators of adipogenesis were determined using Luminex 100xMAP technology. Significant decreases in the expression of lipogenic genes Srebpf1 (28%), Acc-1 (44%), Fabp4 (19%), Cd36 (21%), Lpl (22%), and Scd-1 (22%) were observed in the 1mg/kg treatment group compared to control. Furthermore, no changes in the expression of any of the lipogeneic genes measured were observed in the 3 mg/kg treatment group (Fig. 2A). The relative gene expression of adipogenesis regulators (Pparγ, Cebpα, and Cebpβ) is illustrated in Figure 2B. Perinatal deltamethrin exposure (1 mg/kg) decreased Pparγ and Cebpα expression in WAT 28% and 32%, respectively, compared to vehicle controls. Cebpβ expression in WAT was similar for all groups. A potential further up-stream regulator of adipogenesis and lipid metabolism in adipose issue is Nrf2 (13,21). Nrf2 and its target genes Nqo1 and Gclc were measured by qPCR, using LightCycler 480® SYBR Green chemistry and instrumentation, and presented in Figure 2C. Nrf2, Nqo1, and Gclc were all significantly down-regulated at the 1 mg/kg treatment. Nrf2 was decreased to 27%, Nqo1 13%, and Gclc 11% of the control. The decreased mRNA of the down-stream target genes of Nrf2 suggests the decreased protein level or activity of Nrf2, which if decreased could potentially explain the down-regulation of gene expression by perinatal deltamethrin exposure.

Effect of developmental deltamethrin exposure on cytokine mRNA expression in WAT

Tnf, Ccl-2, Cd11c, Fas/Tnf-R, Cd209e, and Il4 cytokine levels in WAT were measured in 5 month old male pups that were exposed to deltamethrin via perinatal exposure. Significant decreases in cytokine levels for both treatment groups were observed when compared to control group (Fig. 3). Tnf levels decreased 47% in the 3 mg/kg treatment group, compared to the control group. Tnf expression was similar between control pups and pups exposed to the 1 mg/kg developmental deltamethrin dose. Cd11c expression was only decreased in the 3 mg/kg group by 66% compared to control. Tnf-R expression was markedly decreased 70% (1 mg deltamethrin/kg) and 75% (3 mg deltamethrin/kg) when compared to the control group. Cd209e levels were decreased to 39% of control in the 1 mg/kg treatment group, but were similar to controls in the 3 mg deltamethrin/kg group. There were no significant differences in Ccl-2 and Il4 cytokine levels compared to the control in either the 1 mg/kg or 3 mg/kg treatment groups.

DISCUSSION

Deltamethrin and other pyrethroids have been established as safe pesticides based on data showing decreased toxicity in mammals from rapid metabolism to non-toxic or less-toxic forms of the parent compound (3). Many pharmacokinetic and dynamic studies have elucidated that a portion of orally administered pyrethroids partition into fatty tissues, in which they persist for at least 3 weeks (3,22,23). Deltamethrin can be detected in the adipose tissue of rats with a half-life of 5-6 days (24), and is most persistent in the body fat of animal models (3). The retention of these highly lipophilic pesticides in metabolically active adipose tissue should increase the spectrum of research from not only neurotoxicity, but to the possibility of metabolic effects of pyrethroid exposure, especially in susceptible populations. In this study, we showed that developmental deltamethrin affects adipogenesis and lipid homeostasis at the transcriptional level, decreasing the expression of some genes in the offspring of dams exposed to 1 mg/kg every three days. Previous toxicological studies noted non-dose-dependent reduction in body weight gain in adult rats and dogs following short-term exposure (0.1-10 mg/kg), and decreased fetal weight in rabbits at 16 mg/kg perinatal exposure (15). In a reproductive study on deltamethrin using rats, Abdel-Khalik *et al.* (25) observed a significant dose-dependent difference ($p<0.01$) in mean fetal weights and retardation of fetal growth at all doses tested (1, 2.5, 5 mg/kg). These observations of retarded development of body mass correlate to the transcriptional down-regulation in gene expression for the major transcription

factors of adipogenesis, Pparγ and Cebpα, which regulate adipocyte development and maintain adipocyte phenotype through regulation of downstream targets Cd36, Lpl, and Glut4. A significant down-regulation of cytokines excreted by WAT that are involved directly in the T-cell response (Tnf, Fas/Tnf-R, Cd209e), has a direct influence on the development of adipocytes. Decreased levels of these cytokines, potentially may contribute to an altered primary immune response of dendritic cells (Cd209e), initiation of programmed cell-death by recruitment of caspase (Fas/Tnf-R), or alteration of macrophage Tnfα secretion, which could impact adipocyte differentiation and proliferation. The expression of fatty-acid uptake genes, Fabp4, Cd36, and Lpl, regulated by Pparγ, were down-regulated, consistent with an overall metabolic change in gene expression for the pathway. The down-regulation of Srebp1c, a transcription factor abundant in WAT that regulates and maintains lipid homeostasis through downstream targets Acc-1 and Scd-1, contributes further to alterations in the metabolic state of WAT in the developing mice. The reported non-monotonic dose-response of changes in gene expression cannot be explained by previous observations or our physiological findings.

The physiological data herein describing cohorts of developmentally exposed male mice conflicts with previously reported toxicological observations for reproductive and developmental toxicity studies in rats, which reported significant changes in body weight and WAT mass (15). No significant difference in body weight and liver weight was observed between vehicle and deltamethrin-treated groups in multiple cohorts of 4, 5, and 11-12

months of age male mice (data not shown). However, the finding that developmental deltamethrin exposure increases locomotor activity may confound the interpretation of potential body weight changes and metabolic phenotypic changes (Richardson et al., personal communication), which is why the significant changes in regulators of adipogenesis and lipid metabolism at the mRNA level suggests potential underlying epigenetic effects.

Little is known regarding the effect of deltamethrin on glucose homeostasis, metabolic, or immune response. Our data suggest that developmental deltamethrin exposure had no marked effect on glucose homeostasis based on blood glucose concentrations, but significant changes in the relative gene expression of Glut4 and Glut2 in WAT are noted. Glut4 is a major glucose transporter in WAT that if down-regulated will decrease the insulin-responsive uptake of glucose into the cells, but the significant down-regulation of Glut2 at both treatment doses, major glucose transporter in the liver, has the potential to affect glucose transport between adipose tissue, plasma, and potentially liver. A decrease in the glucose transport genes may alter the deposition of glucose. Early insulin-resistance has been linked to adipose tissue dysfunction (26) and decreased WAT expandability is associated with increased risk for diabetes or glucose intolerance (21,27,28). Thus, deltamethrin might affect the normal response to high-fat diet challenge or treatment with thiazolidinedones, which promote adipogenesis to improve insulin resistance.

The transcriptional down-regulation of genes essential for adipose development and lipid metabolism in offspring after perinatal exposure to 1 mg/kg dose of deltamethrin provides data that supports the potential for metabolic effects of deltamethrin exposure during development with regard to WAT expandability. Nrf2, normally known for its role in oxidative stress, has recently been shown to be a regulator of adipose development and function (12). Nrf2 has been shown to be an upstream regulator of Pparγ and Cebpα, in which Nrf2 knock-out in MEF cells decreased lipid accumulation, decreased Pparγ and Cebpα expression at the protein, along with down-stream target genes (13). Therefore, the decreased expression of Nrf2 and its target genes at the mRNA level can serve as initial evidence that Nrf2 may be involved in the gene expression changes related most specifically to the transcription factors Pparγ and Cebpα, and therefore, lead to changes in glucose and lipid homeostasis. These significant changes in gene expression at the transcriptional level may correlate to an epigenetic regulation in the offspring that may eventually lead to altered responses of WAT to environmental toxicants (i.e. obesogens, endocrine disruptors) or changes in diet (i.e. high-fat diet), however these changes did not manifest in significant alteration in physiological parameters. To summarize, perinatal deltamethrin exposure did not result in measurable changes in body weight in male offspring at 5 months of age, but did decrease the expression of some genes related to adipogenesis, lipogenesis, and inflammation in WAT that may potentially be under the regulation of alterations in Nrf2 activity.

FOOTNOTES

This work was supported by grants from National Institute of Health [4R01ES016042, 5K22ES013782, R01ES015991, T32ES007148, P30ES005022]; and National Center for Research Resources [5P20RR016457-11] and Institute for General Medical Science [P20 GM103430-11] components of the National Institutes of Health (NIH). Additional support was provided by a graduate fellowship to AB by Bristol-Myers Squibb. This work was presented, in part, at the Annual Society of Toxicology meeting held March 12-15, 2012, in San Francisco, California and at the Northeast Society of Toxicology meeting held October 19, 2012 at Salve Regina University in Newport, Rhode Island. Drs. Slitt, Richardson, and Aleksunes are recipients of the NIEHS Outstanding New Investigator Scientist (ONES) Award. This study represents collaborative effort of these investigators resulting from the ONES program and is an invited submission highlighting work performed by ONES recipients.

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FIGURE LEGENDS

Figure 1. Body weight was used to describe any potential metabolic effects of perinatal deltamethrin exposure, along with blood glucose concentration and mRNA gene expression of key factors in insulin response of WAT to describe potential effects on glucose homeostasis. Body weights of 5 month old male mice were taken prior to sacrifice. (A) Average body weight (g) of each treatment group expressed as a mean BW ± SEM (n=6-7). (B) Blood glucose level taken at time of necropsy of 5 mo. adult male mice expressed as a mean BG ± SEM (n=5-6). mRNA gene expression data in white adipose tissue of 5 month old adult male mouse pups from dams exposed to 0, 1, or 3 mg delatmethrin/kg every three days during getation and lactation. Total RNA was isolated from white adipose tissue and mRNA levels were quantified by Quantigene Plex 2.0 assay (Affymetrix). All gene expression data were normalized to Rpl13a (no significant change in gene expression) and are expressed as mean ± SEM (n=8-9). (A) Insulin responsive and glucose transport: Irs-1, Glut-4, Glut-2 mRNA expression. mRNA gene expression data in white adipose tissue of 5 month old adult male mouse pups from dams exposed to 0, 1, or 3 mg deltamethrin/kg every three days during gestation and lactation. * and # represents statistical difference between control and treatment doses ($p<0.05$ and $p<0.005$, respectively).

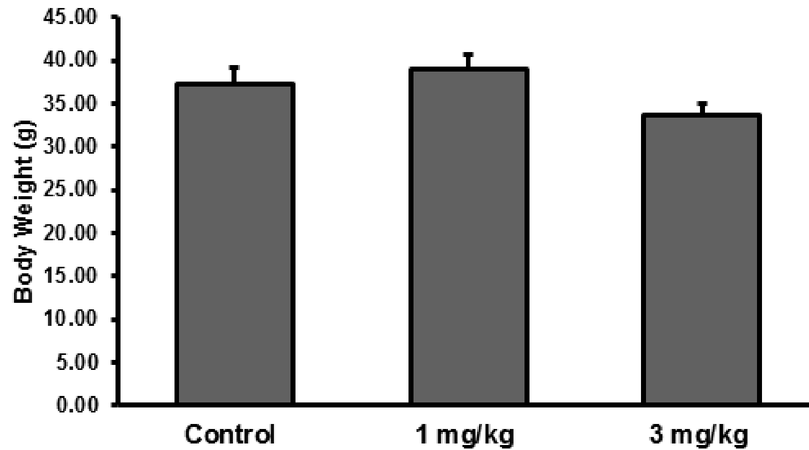
Figure 2. mRNA gene expression data in white adipose tissue of 5 month old adult male mouse pups from dams exposed to 0, 1, or 3 mg deltamethrin/kg every three days during gestation and lactation. Total RNA was isolated from white adipose tissue and mRNA levels were quantified by Quantigene Plex 2.0 assay (Affymetrix). All gene expression data were normalized to Rpl13a (no significant change in gene expression) and are expressed as mean ± SEM (n=8-9). (A) Regulators of

adipogenesis: Ppar□, Cebp□, Cebp□ mRNA expression (B) Lipogenic genes:Srebp1, Acc-1, Fabp4, Cd36, Lpl, Scd-1 mRNA expression. mRNA gene expression for Nrf2, Nqo1, and Gclc was quantified by Lightcycler 480 SYBR green qPCR method. (C) Nrf and target genes Nqo1 and Gclc mRNA expression. *and # represents statistical difference between control and treatment doses (p<0.05 and p<0.005, respectively).

Figure 3. mRNA gene expression of relevant cytokines in white adipose tissue of 5 month old adult male pups from dams exposed to 0, 1, or 3 mg deltamethrin/kg every three days during gestation and lactation. Total RNA was isolated from white adipose tissue and mRNA levels were quantified by Quantigene Plex 2.0 assay (Affymetrix). All gene expression data were normalized to Rpl13a (no significant change in gene expression) and are expressed as mean ± SEM (n=8-9) Cytokines: Tnf, Ccl-2, Cd11c, Fas/Tnf-R (M1 macrophage markers) and Cd209e (M2 macrophage marker). * and **#** represents statistical difference between control and treatment doses (p<0.05 and p<0.005, respectively).

Figure III-1.

Figure 1. (A)



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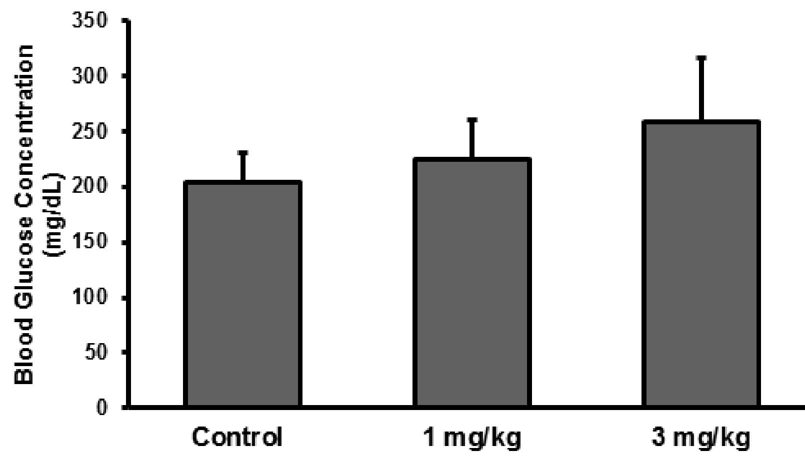

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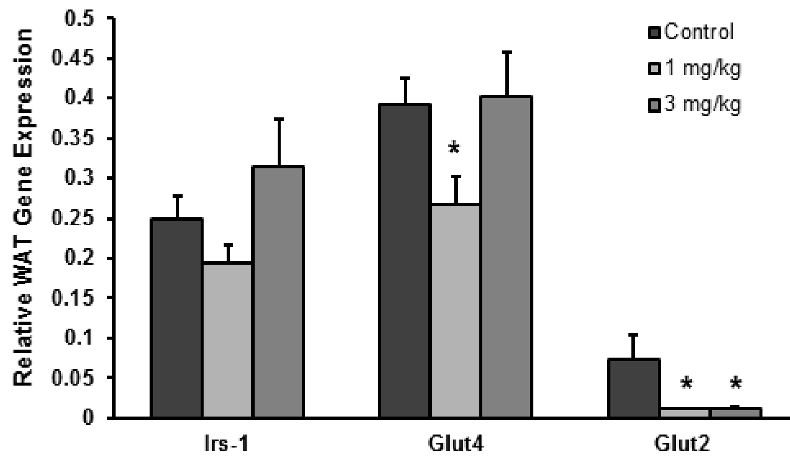
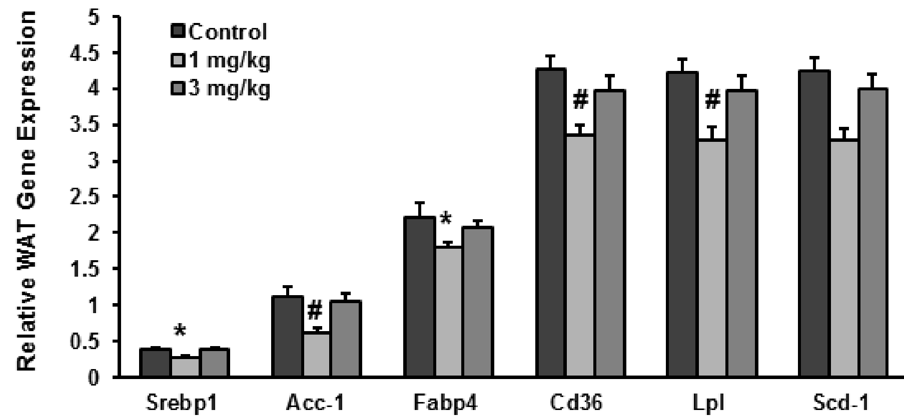


Figure III-2.

Figure 2.

(A)



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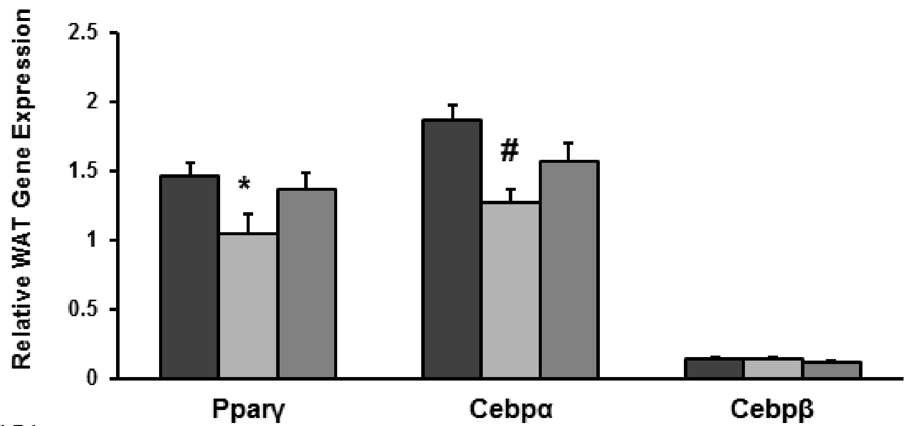

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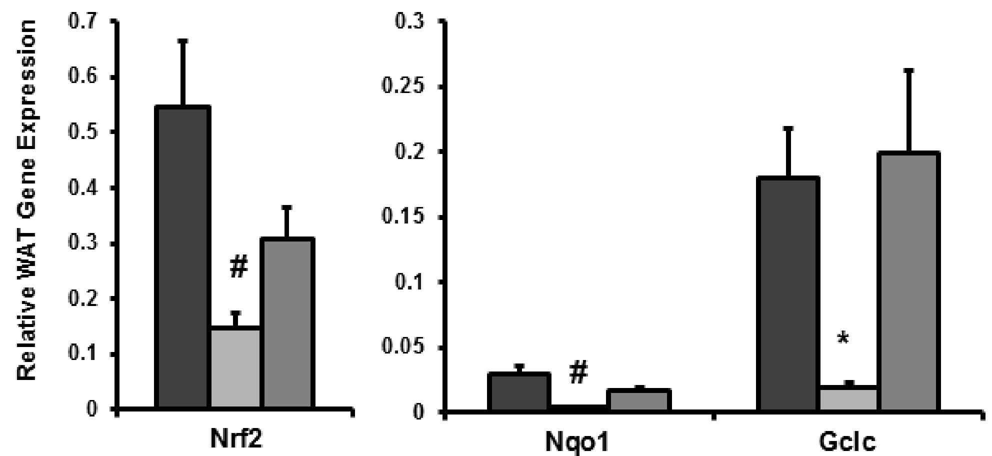
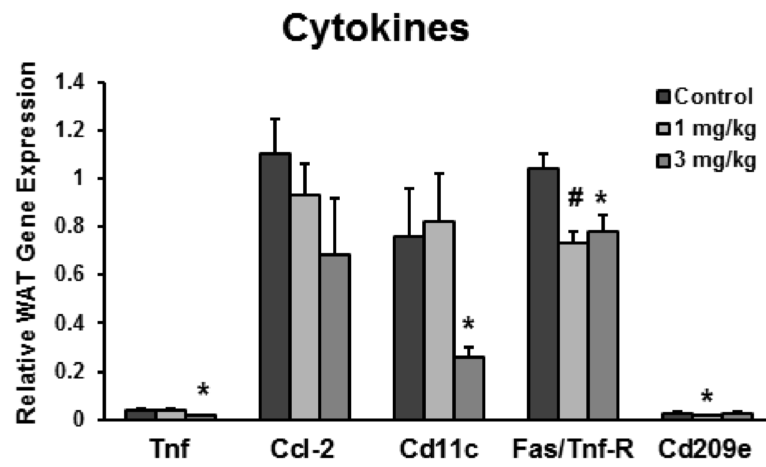


Figure III-3.

Figure 3.



CONCLUSION

Obesity has previously been defined at the dysregulation of energy balance, in which nutrient intake exceeds energy expenditure; therefore excess nutrients are stored in WAT (Christodoulides *et al.*, 2009). The primary role of WAT is to store excess FFAs in the form of triglycerides, and because of this WAT is physiologically able to handle greater FFA and oxidative stress loads than other metabolically active tissues (Kusminski and Scherer, 2012). Therefore, WAT is known for its expandability—which has been suggested to be directly related to the pathophysiology of diseases related to overall metabolic syndrome (Christodoulides, Lagathu, Sethi and Vidal-Puig, 2009). AT expandability is recognized as an adaptive response to excess nutrient intake, thus preventing lipid deposition and toxicity in alternate organs (Rutkowski, Stern and Scherer, 2015). Expandability can be considered through two mechanisms: hypertrophy and hyperplasia. Hypertrophy is the expansion of fat mass due to an increase in adipocyte size, and hyperplasia is due to increased adipocyte number or differentiation (Rutkowski, Stern and Scherer, 2015). The relative turnover of mature adipocytes or induction of adipocyte differentiation in adults is considered low (Spalding *et al.*, 2008). Therefore, the effects of environmental exposures, Deltamethrin and BDE-99, and the beneficial effects of dietary restriction or CR on Nrf2 expression and WAT lipid metabolism can be summarized considering the changes in expandability of WAT.

Hyperplasia within AT can be considered directly related to the ability of pre-adipocytes to differentiate to adipocytes. Hyperplasia is essential for AT expandability, but because of low turnover may not be directly to human exposure models. Therefore, much of the research considers obesity as being directly related to hypertrophy of adipocytes (La Merrill, Emond, Kim, Antignac, Le Bizec, Clement, Birnbaum and Barouki, 2013). The two environmental chemicals presented in this dissertation support the importance of AT expandability and the potential chemical effects that can result in AT dysfunction due to hypertrophy. **MANUSCRIPT III** demonstrated that regulation of WAT expandability can be altered during development. Deltamethrin significantly decreased adipogenic, lipogenic, and glucose uptake gene expression in adult mice exposed perinatally. This did not result in a phenotypic change, but with a HFD-challenge could demonstrate the physiological relevance of inhibited AT expandability. **MANUSCRIPT II** findings showed that the environmental compound BDE-99 can increase AT hypertrophy *in vitro* in both mice and humans. This was demonstrated by no significant findings in AT differentiation, but a marked increase in lipogenic gene expression and lipid deposition in BDE-99 treated cells. This manuscript supports previous findings and studies that identify compounds as obesogens (La Merrill, Emond, Kim, Antignac, Le Bizec, Clement, Birnbaum and Barouki, 2013). Lastly, **MANUSCRIPT I** exemplified CR as an effective treatment to decrease WAT mass and lipid content through mechanisms directly related to

the antioxidant response in order to achieve homeostasis where energy intake does not exceed energy expenditure.

Most significantly, all three manuscripts support a novel role for Nrf2 regulation in WAT expandability and lipid homeostasis. Environmental exposures of deltamethrin and BDE-99 that predispose or contribute to AT hypertrophy, respectively, were correlated to decreased Nrf2 gene expression. While, AT homeostasis achieved by CR was shown to induce Nrf2 gene expression and key pathways of lipid metabolism were demonstrated to be Nrf2-dependent. This dissertation supports the role of Nrf2 to achieve AT homeostasis, maintain lipid homeostasis, and prevent against AT hypertrophy that can result in obesity and metabolic syndrome.

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