2012

NRF2 AS A NUTRIENT SENSITIVE TRANSCRIPTION FACTOR

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NRF2 AS A NUTRIENT SENSITIVE TRANSCRIPTION FACTOR

By

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A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE

REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

IN

PHARMACOLOGY AND TOXICOLOGY

UNIVERSITY OF RHODE ISLAND

2012

DOCTOR OF PHILOSOPHY DISSERTATION

OF

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2012

ABSTRACT

Obesity and diabetes are the most prevalent metabolic disorders. It is estimated that more than 86% of US adults would be overweight or obese and more than 50% obese by the year 2030. Non Alcoholic Fatty Liver Disease (NAFLD) has also been referred to as hepatic manifestation of insulin resistance. NAFLD or steatosis is defined as lipid accumulation exceeding 5% by weight in hepatocytes in the absence of substantial alcohol intake. The prevalence of NAFLD is estimated to be between 17% and 40% of the world population, known to afflict obese and normal weight persons and children as well. Epidemiological and clinical studies demonstrate changed pharmacokinetic and –dynamic parameters of drugs and environmental toxicants in models of NAFLD and cause drug induced liver injury (DILI) which can be attributed to altered expression of Phase I, Phase II drug metabolizing enzymes and drug transporter expression in human and rodent models of NAFLD. NAFLD also causes propogation of insulin resistance complicating metabolic syndrome. Due to its increasing prevalence throughout the world, understanding mechanisms and consequences of NAFLD and identifying novel targets to reverse this condition are of increasing clinical relevance.

Nuclear factor-E2 related factor 2 (Nrf2) belongs to leucine zipper based loop-helix-loop family of transcription factors which plays an important role in activation of a battery of antioxidant genes, thus protecting the cells from

oxidative stress which is either causality or a consequence that aggravates a number of disorders, an important condition being NAFLD. Nrf2 is sequestered in the cytoplasm via sulfhydryl based interactions with Kelch like ECH-associated protein 1 (Keap1); oxidative stress disrupts these interactions and phosphorylation based changes in Nrf2 protein aid its translocation into the nucleus where it binds to antioxidant response element (ARE) and initiates transcription of the antioxidant gene battery which includes but not limited to NAD(P)H:quinine oxidoreductase 1 (NQO1), hemoxygenase (HO1), glutamine cysteine ligase catalytic and regulatory subunit (GCLC and GCLM). Hepatic clearance is a predominant mechanism for excretion of chemicals, such as hormones and drugs. Several families of transporters localized to sinusoidal and canalicular membrane of hepatocytes drive bile flow and biliary excretion. Nrf2 also regulates expression of ATP-binding cassette (ABC) superfamily of membrane proteins, efflux organic anions into bile (e.g. Abcc2) and from hepatocytes into blood (e.g. Abcc3 and 4), which contribute to hepatic clearance of various endogenous and exogenous chemicals.

Diet (caloric restriction, CR) and exercise are the recommended therapeutic intervention to treat NAFLD and reverse hepatic fat accumulation. Beneficial effects of CR are attributed to activation of Sirtuin 1, a NAD (+)-dependent protein deacetylase which activates gluconeogenic and fatty acid oxidation genes expression via coactivator; Peroxisome proliferator-activated receptor gamma coactivator 1-alpha. The important regulatory

roles of SIRT1 in both lipid and glucose metabolism make it an important molecule in hepatic mechanisms in development of steatosis.

Resveratrol, an antioxidant compound and a potent Sirt1 activator also activates Nrf2 in certain in vitro models as determined by an increase in the expression of prototypical Nrf2 target genes. Additionally, Nrf2 has been implicated in protection against development of cancer in mice undergoing CR. Ability of CR mimetics or Sirt1 activators to activate Nrf2-Keap1 pathway and scant but significant evidence of Nrf2 in nutrient regulation demand an indepth analysis of the Nrf2-Keap1 pathway in the nutrient deprived conditions.

A possible role of Nrf2 in adipogenesis, adipocyte differentiation as well as glucose tolerance has been suggested in mouse models, however, very little is known about Nrf2 signaling pathway in nutrient deprived states. Very little is known about effect of nutritional status on biliary clearance mechanisms or bile flow.

The purpose of study performed in **MANUSCRIPT I** is to elucidate the effect of fasting pathways on Nrf2-Keap1/ARE pathway. Few studies exist regarding how fasting affects transporter or the nuclear receptor-regulated pathways which regulation expression. Fasting effects on the Nrf2-Keap1 signaling pathway, which is responsive to oxidative stress and altered redox status have not been elucidated. The observations presented in this study prove that 1) fasting increases Abcc expression through Nrf2- and Sirt1-

dependent mechanisms, 2) cAMP/PKA activators increase ARE activation via Nrf2- and Sirt1-dependent mechanisms *in vitro*, 3) Nrf2 expression is co-ordinately regulated with Pgc-1α and Sirt1 expression and 4) obesity/steatosis diminishes fasting-mediated Abcc induction.

In summary, **MANUSCRIPT-I** demonstrates that fasting and PKA activators increase mouse Abcc2-4 expression in liver by Nrf2- and Sirt1-related mechanisms in mouse liver and hepatocytes. Additionally, 8-Br-cAMP and PKA activating compounds increase Nrf2 target gene and ABCC2-4 mRNA expression in human hepatocytes. Our data illustrate an undescribed role for Nrf2 as a "fasting" responsive transcription factor that is activated via cAMP/PKA-Sirt1 upstream mechanisms.

CR alters activity and expression of various biotransformation enzymes such as Sult2a1, Cyp2b10, Ugt1a1, CYp4a14 in liver. However, to our knowledge, no studies have shown how CR actually affects drug transporter expression in livers of obese mice, which have hepatic steatosis and better mimic the population most likely to undergo intervention. The purpose of study performed in **MANUSCRIPT II** hypothesized that CR would reverse the NR and drug transporter expression changes previously observed in obesity-induced hepatic steatosis. Given the well-defined association of Nrf2 with Abcc transporter induction, emphasis was placed on the Nrf2 pathway, but other NRs that have been described to regulate transporter expression were also evaluated. Our data herein illustrate that CR differentially regulates NR, biotransformation enzyme, and transporter

expression in livers from C57Bl/6 (WT) and $Lep^{ob/ob}$ (OB) mice. Furthermore, AMPK and Sirt1 activators differentially modulated transporter and NR expression in lean and steatotic hepatocytes.

To summarize, **MANUSCRIPT II** demonstrates that activation of CR pathways can only partly reverse the changes in drug metabolizing enzyme and transporter expression occurring due to fat deposition in the liver. Leptin appears to play an important role in upstream regulation of pathways that relay CR signals to ultimately change DME and DT expression. The above observations also indicate that the inability of CR to reverse certain DME and DT changes may indicate towards altered disposition and pharmacokinetics of metabolites and xenobiotics. Data also shows that CR decreases the expression and activity of Nrf2-ARE regulatory pathway and target gene expression.

Small non-coding RNAs, microRNAs have been recently demonstrated to provide an additional layer of regulatory control in development, homeostasis and pathology. miRNA expression and regulation are being explored in detail as potential targets and possible biomarkers in various diseases, specifically metabolic syndrome and cancer. Various miRNAs such as miR34a, miR122, miR370, miR221, miR33a, let-7 have been proven to be important regulators of fatty acid oxidation and lipid synthesis regulators such as Sirt1, Pgc-1α, Srebp1c, Lxr. In **MANUSCRIPT III** we demonstrate differential susceptibility of a mouse model, constitutively

overexpressing Nrf2 (Keap1-KD mice) to CR. We attribute these changes to novel changes in the miRNA regulatory circuit.

. We demonstrate that the enhanced susceptibility to CR in Nrf2 overexpressing mice could be partly associated with differential changes in miRNA expression such as miR34a, miR370, miR122 and miR144, especially those known to be involved in regulation of lipogenic as well as fatty acid oxidation genes.

In summary, the studies presented herein demonstrate Nrf2-ARE pathway is a nutrient sensitive pathway that is: (a) activated upon fasting via Sirt1-Pgc-1α dependent mechanisms, (b) negatively regulated upon CR and AMPK pathway, (c) plays a role in development of obesity, and (d) regulates expression of miRNA regulatory circuit thereby changing susceptibility to weight loss and fatty acid metabolism, (e) is a potential target to alleviate metabolic syndrome.

ACKNOWLEDGEMENTS

Successful completion of this milestone in my graduate studies would have not been possible without the motivation and support from my professional colleagues as well as social well wishers. I would like to take this opportunity to extend my sincere gratitude towards them for helping me achieve my goal.

With an immense sense of gratitude and respect, I would like to thank Dr. Angela Slitt for introducing me to the field of scientific research. Without her continuous support; scientific as well as financial, motivation to improve and persuasion to make use of the right opportunities for progress, I would not have been able to accomplish this task. Thank you Dr. Slitt, for your confidence in me, believing in my scientific ideas and helping me bring those ideas into experimental successes. You will always inspire me to be the best I can……and more.

I extend my gratitude towards my dissertation committee members, Dr. Zahir Shaikh, Dr. Bingfang Yan, Dr. Kathleen Melanson, Dr. Roberta King and Dr. Frederick Vetter for validating and supporting the scientific proposal and the results obtained.

My success would not have been possible without the unquestioned and complete support from my parents, Mr. Raghavendra Kulkarni and Mrs.

Vidya Kulkarni and my brother Sujay Kulkarni. Appa and Amma, your constant, everlasting support and belief in me motivate me to achieve greater heights, professionally and personally. You have taught me innumerous values that I cannot even begin to describe…..I hold them close to me forever. A special thank you to my younger brother Sujay; your excitement and happiness in the small achievements I made along my graduate career, motivated and inspired me to do better and aim higher!

I would not have been able to complete my graduate studies without the constant support of my present and past lab members. Ajay Donepudi, Vijay More, Maneesha Paranjpe; I greatly value your mutual support throughout the years, both in the lab and outside...I could not have asked for better professional colleagues to begin my research career with. Thank you very much for helping me through the thick and thin of lab experiences! A special thank you to Rajani Kaimal for all the support and motivation. A heartfelt thank you to my Joyce Macwan and Surag Gohel for tolerating my ups and downs and for their advice on a day-to-day basis……you have been the best roomies ever!

Last but not the least, I would like to thank everyone College of Pharmacy at URI, for being a part of successful completion of my dissertation!

DEDICATION

My parents: the best mentors, friends and support. It is to them that I dedicate the following dissertation, for encouraging me, motivating me and being my pillars of strength……

Gurur Brahma Gurur Vishnu,

Gurur Devo Mahesh Varah.

Guru Shakshat Para Brahma,

Tasmai Shri Guruve Namah.

PREFACE

This dissertation was prepared according to the University of Rhode Island 'Guidelines for the Format of Theses and Dissertations' standards for Manuscript format. This dissertation consists of five manuscripts that have been combined to satisfy the requirements of the department of Biomedical and Pharmaceutical Sciences, College of Pharmacy, University of Rhode Island.

MANUSCRIPT I: Fasting Induces Nuclear Factor E2-Related Factor 2 and ATP-Binding Cassette Transporters via Protein Kinase A and Sirtuin-1 in Mouse and Human Models.

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

MANUSCRIPT II: Effect of Caloric Restriction and AMPK Activation on Nuclear Receptor, Biotransformation Enzyme, and Transporter Expression in Livers of Lean and Obese mice.

This manuscript has been prepared for submission to 'Journal of Pharmacology and Experimental Therapeutics' as an original research communication.

MANUSCRIPT III: Differential expression of micro-RNAs regulating lipid metabolic genes enhances susceptibility to caloric restriction in models of constitutive Nrf2 overexpression.

This manuscript has been prepared for submission to 'Pharmaceutical Research' as an original research communication.

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Submitted to Antioxidant Redox Signaling for publication

Original Research Communication

Fasting Induces Nuclear Factor E2-Related Factor 2 and ATP-Binding Cassette Transporters via Protein Kinase A and Sirtuin-1 in Mouse and Human

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Word Count: 3916

References: 53

Greyscale Illustrations: 2

Color Illustrations: 6

Abstract

Aim: The purpose of this study was to determine how fasting modulates Nrf2 activity and ABCC expression in liver, with specific focus on cAMP-protein kinase A (PKA) and Sirtuin-1 (Sirt1) dependent mechanisms. ATP-binding Cassette (ABC) transport proteins (ABCC2-4) are essential for chemical elimination from hepatocytes and biliary excretion. Nuclear factor-E2 related-factor 2 (Nrf2) is a transcription factor that mediates ABCC induction in response to chemical inducers and liver injury. However, a role for Nrf2 in the regulation of transporter expression in non-chemical models of liver perturbation is largely undescribed.

Results: Here we show that fasting increased Nrf2 target gene expression through Nrf2- and Sirt1 –dependent mechanisms. In intact mouse liver, fasting induces Nrf2 target gene expression by at least 1.5 to 5 fold. In mouse and human hepatocytes, treatment with 8-Br-cAMP, a cAMP analogue, increased Nrf2 target gene expression and antioxidant response element activity, which was decreased by the PKA inhibitor, H-89. Moreover, fasting induced Nrf2 target gene expression was abolished in liver and hepatocytes of Sirt1 liver-specific null mice and Nrf2-null mice. Additionally, leptin deficient (OB) obese mice were more resistant to fasting effects on Nrf2 target gene induction compared to lean mice.

Innovation: Nrf2 activation by oxidative stress is well described, yet the influence of basic metabolic processes on Nrf2 activation remain largely undescribed.

Conclusion: The current data points toward a novel role of nutrient status in regulation of Nrf2 activity and the antioxidant response, and indicates that cAMP/PKA and Sirt1 are upstream regulators for fasting-induced activation of the Nrf2-ARE pathway.

INTRODUCTION

Members of the ATP-binding cassette (ABC) superfamily efflux organic anions from hepatocytes into bile (e.g. Abcc2) and blood (e.g. Abcc3 and 4). Increased Abcc2 expression increases biliary excretion of organic anion (e.g. DBSP), whereas increased Abcc3 expression reverses vectorial excretion of glucuronide conjugates into blood (29). Genetic and pharmacological Nuclear factor-E2 related factor 2 (Nrf2) activation can upregulate Abcc2-4 expression in liver (29,38).

Nrf2 belongs to the basic leucine zipper (loop-helix-loop) family of transcription factors, and is tethered in the cytoplasm as a complex with Kelch like associated protein-1 (Keap1) as reviewed by Kobayashi et al. (31) . Upon activation, Nrf2 translocates to nucleus and activates gene transcription by binding to Antioxidant Response Element (ARE). Nrf2 is responsive to cellular redox status alterations (41) and is largely described to mediate induction of xenobiotic metabolism and cytoprotection genes, such as Nadph:quinone oxidoreductase (Nqo1) and glutamate-cysteine ligase, catalytic subunit (Gclc), in response to oxidative stress and change in cellular redox status (27).

Nutrient homeostasis in liver relies on redox status. During fasting, decreased glucose and increased pyruvate concentrations increase the NAD+/ NADH ratio, initiating a cascade of events that activate

gluconeogenesis (46). Fasting increases glucagon, which increases intracellular 3'-5'-cyclic adenosine monophosphate (cAMP) content (33), activates protein kinase A (PKA), and modulates transcription of multiple genes in liver that maintain glucose homeostasis, metabolism, and transport (9). cAMP/PKA cascade activates downstream expression of gluconeogenic genes, restoring blood glucose levels to normal (5). Short-term fasting induces gluconeogenesis via mTOR/TORC2 pathway which is inhibited during longer fasting intervals by Sirtuin1 (NAD-dependent deacetylase, Sirt1) switching to the use of free fatty acids as precursors for glucose production via deacetylation and activation of PPAR gamma coactivator alpha (Pgc-1α) and Forkhead Box O1 (Foxo1). (20,36,46).

Along with gluconeogenic genes, fasting induces transcription of some genes involved in biotransformation (e.g. Cyp2b10, CYP2C11, CYP2E1, CYP2B1/2) and transport (e.g. Slco1b2) in coordination with nuclear receptors (6,10). However, little is known about effect of nutritional status on biliary clearance mechanisms or bile flow. Few studies exist regarding how fasting affects transporter or the nuclear receptor-regulated pathways which regulation expression. Fasting effects on the Nrf2-Keap1 signaling pathway, which is responsive to oxidative stress and altered redox status have not been elucidated. Some evidence suggests a possible role for Nrf2 in lipid and glucose homeostasis (3,43), very little is known about how fasting affects the Nrf2-Keap1 pathway. To date, no studies have

addressed how Sirt1 regulates Nrf2 activity and subsequent gene expression *in vivo*.

The purpose of this study was to identify whether 1) fasting increases Abcc expression through Nrf2- and Sirt1-dependent mechanisms, 2) cAMP/PKA activators increase ARE activation via Nrf2- and Sirt1-dependent mechanisms *in vitro*, and 3) how obesity impacts fasting-mediated Abcc induction. The current study determines whether fasting and pharmacological activation of the fasting response pathway increases Nrf2 activity and upregulates Abcc2-4 expression through cAMP/PKA-Sirt1-dependent mechanisms. Data herein present an undescribed role for Nrf2 as a "nutrient sensitive" responsive transcription factor that is regulated via Sirt1-dependent mechanisms *in vivo*, and illustrate that in obesity, hepatic clearance mechanisms are differentially affected by fasting.

EXPERIMENTAL PROCEDURES

***Animals and treatments*.** Adult male C57Bl/6 and OB mice (n=8/group), Nrf2-null (n=6/ group) (24) and ARE-hPAP reporter mice (n=3/ group) (26) were fed Harlan Teklad LM-485 Mouse/Rat Sterilizable Diet (Harlan Laboratories, IN,USA) *ad libitum* or were food withheld for 24 hrs. Adult male Sirt1-LKO (44) and Sirt-WT mice (n=3) were fed standard chow *ad libitum* or were food deprived (fasted) for 18 hrs. Blood and livers were

collected between 9-11 am for all individual fasting experiments. Livers from Sirt1 overexpressing (Sirt1-OE) fed or fasted mice were obtained from J. Rodgers and P.Puigserver from a previously described study (47). Serum glucose (Cayman Chemicals, Ann Arbor, MI), triglyceride (TG) (Pointe scientific, MI, USA) and free fatty acid (NEFA) concentrations (Wako diagnostics, VA), hepatic and serum cAMP (Cell Signaling Technologies, Danvers, MA, USA), serum bile acids (Bioquant Kits, San Diego, CA, USA) were determined according to manufacturer's instructions.

Determination of biliary excretion of disulfobromophthalein (DBSP): Adult, male, C57Bl/6 (n=5 per group) were fed standard chow *ad libitum* or were food withheld for 18 hrs. DBSP, purchased from SERB Laboratories (Paris, France) was injected i.p. (150 µmol/kg/5ml) and 45 minutes after injection gallbladders were collected. Bile DBSP content (µmol/ µl bile/kg body weight) was determined spectrophotometrically at 580 nm after alkalinization with 0.1N NaOH.

***Cell Lines and Treatments*.** Huh-7 cells were treated with 0.5-2.0 mM 8-Bromoadenosine-3', 5'-Cyclic Adenosine Monophosphate (8-Br-cAMP) (Sigma Aldrich, St.Louis, MO, USA) or media alone for 24 hrs. Huh-7 cells were cultured to 85% confluence and transiently transfected with an ARE–luciferase (0.1 µg) (14) and pRL-CMV (0.01 µg) reporter constructs using lipofectamine-plus reagent (Invitrogen, CA, USA). 24 hrs after transfection,

cells were treated with 0.1mM or 1.0mM 8-Br-cAMP for 24 hrs (15) . Luciferase activity was measured using a Dual Luciferase Assay (Promega Corp., MD, USA), and Firefly Luciferase Relative Light Units (RLUs) were normalized to Renilla Luciferase RLUs.

Plasmid transfections and endogenous gene expression analysis. All plasmids used were obtained from Addgene plasmid repository (Cambridge, MA). Huh-7 cells were transiently transfected with (0.5-1.0μg) Flag-Pgc-1α, 75ng Flag-Nrf2 and 50ng Myc-Sirt1 expression plasmids or (50-100ng) Myc-Sirt1, 75ng Flag-Nrf2 and 0.5μg Flag-Pgc-1α expression plasmids using Lipofectamine LTX-plus reagent (Invitrogen, CA, USA) for 24 hrs. Cells were then treated with 1.0mM 8-Br-cAMP for 24 hrs. Nrf2 and Pgc-1α mRNA expression was measured using real time quantitative PCR, with expression being normalized to 18S rRNA expression.

Immunoprecipitation (IP) Assay. Huh-7 cells were cultured in 6 cm dishes and transiently transfected with 3.0μg Flag-tagged Nrf2, with or without 0.75μg cMyc-tagged Sirt1 plasmid for 24 hrs using Lipofectamine LTX-plus reagent (Invitrogen, CA, USA). After 24 hrs, cells were treated with 1.0mM 8-Br-cAMP for 45 mins and lysed with buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% NP-40). 1mg protein lysate was incubated with Anti-FlagM2 magnetic beads (Sigma Aldrich, St.Louis, MO) overnight in lysis buffer. After incubation, beads were washed with Tris-Buffered Saline, pH

7.4, mixture boiled with SDS samples buffer, and immunoprecipitates were separated on a 10% SDS-PAGE followed by immunoblotting against Pgc-1α (Abcam, Cambridge, MA), FlagM2 and c-myc (Sigma Aldrich, St.Louis, MO).

Primary hepatocyte isolation, culture, and treatment. Primary mouse hepatocytes were obtained from adult C57Bl/6, ARE-hPAP and SirtLKO mice as described previously (54) . Primary human hepatocytes (Cellzdirect™, NC, USA) were maintained in William's E Media supplemented with Cellzdirect ™ recommended supplements. Hepatocytes were treated with DMSO vehicle or 1.0mM 8-Br-cAMP dissolved in DMSO for 24 hrs.

mRNA Quantification. Total RNA was isolated from tissue and cells using TRIzol reagent (Invitrogen, CA, USA). mRNA expression was quantified by Branched DNA signal amplification assay or real time quantitative PCR, with expression being normalized to 18S rRNA expression. At the end of treatment, primary human hepatocytes were lysed with Lysis Mixture™ (Panomics Inc.,Freemont, CA) according to manufacturer's protocol and stored at -80°C until analysis. mRNA expression was measured using Quantigene® Discover Kit (Panomics Inc.,Freemont, CA). Target gene expression was normalized to ß-actin expression.

Western blot and immunohistochemistry. Proteins were electrophoretically separated by SDS-PAGE and transferred to PVDF membrane. Membranes were blocked with 5% Non-fat dry milk in phosphate-buffered saline with 0.05% Tween-20 overnight, and then incubated with appropriate concentrations of primary and secondary antibodies. The blots were incubated in ECL-Plus western blot detection reagent. Bands were visualized using autoradiography film and quantified using Image Quant Software (GE Healthcare and Life Sciences, N J, USA). Details are described in (39) and Abcc2 staining immunostaining of liver sections was performed as described (4).

Nrf2 binding Assay. Nuclear extracts were isolated from livers using a TF Procarta nuclear extraction kit (Panomics Inc, Freemont,CA, USA) and protein concentrations were measured by a BCA protein assay (Pierce, Rockford, IL, USA). Nrf2 binding to a prototypical ARE consensus sequence was quantified using a Procarta TF custom array (Affymetrix, Santa Clara,CA) according to manufacturer's instructions. Data was acquired by a Luminex Bio-plex^{TM} 200 Array reader with Luminex 100 X-MAP technology, and data were acquired using Bio-Plex Data Manager Software Version 5.0 (Bio-Rad Laboratories, Hercules, CA, USA). All data was normalized to TFIID binding activity.

NAD+/NADH Assay. Tissue NAD+/NADH was measured using NAD/NADH assay kit (BioVision Inc., CA,USA) according to manufacturer's protocol.

***Statistics*.** Statistical significance of differences was determined by Duncan's factorial ANOVA test. $P<0.05$ was considered statistically significant. Groups without a common letter are significantly different.

RESULTS

Fasting increases Abcc2-4 expression in liver and hepatocytes. First, it was determined whether fasting modulates biliary clearance via induction of Abcc transporters expression and activity. Fasting increased excretion of DBSP, a prototypical organic anion that undergoes biliary excretion via Abcc2 (29), into gallbladder bile by ~3 fold compared to fed mice (Fig. 1A). Fasting correspondingly increased liver Abcc2-4 mRNA levels (Fig. 1B) and Abcc2-4 protein expression (Fig. 1C) compared to mice fed *ad libitum*. Abcc induction in liver was time dependent, and as length of fasting increased, induction declined (Supp. Fig. 2 and 3). 8-Br-cAMP, a cAMP analogue that activates cAMP/PKA pathway, increased expression of Abcc2-4 by 6, 1.75 and 1.5 fold in primary mouse hepatocytes (Fig.1D). Additionally, 8-Br-cAMP induced ABCC2-4 expression by 5, 2, and 1.25 fold, in Huh-7 hepatocarcinoma cells (Fig. 1E); indicating that fasting

induced activation of Nrf2 was conserved in human-derived cells. In line with these data, several upstream activators of fasting pathway, glucagon, epinephrine, and 8-Br-cAMP, increased ABCC2-4 expression in primary human hepatocytes (Fig. 1F).

Nqo1 is considered to be a prototypical Nrf2 target gene in mouse and human (30). As shown in Figure 2A, fasting not only increased mRNA levels of Nqo1, but also Nrf2 itself in mouse livers by at least 1.5 fold over fed controls (Fig. 2A). Fasting correspondingly increased Nqo1 protein expression by ~3 fold over fed controls (Fig. 2B). Moreover, 8-Br-cAMP, a PKA activator, increased Nqo1 expression by 2 fold in primary mouse hepatocytes (Fig. 2C). Additionally, PKA activators increased NQO1 expression in primary human hepatocytes (Fig. 2D). Similarly, Huh-7 cells treated with 8-Br-cAMP demonstrated a 2 fold increase in NQO1 mRNA expression accompanied by increased NQO1 protein expression in cytosols of these cells (Fig. 2E).

To further explore the molecular mechanism underlying the fasting induced activation of Nqo1 and ABCC2-4, we examined subcellular localization of Nrf2 before and after 8-Br-cAMP treatment. As shown in Figure 2D, increase in ABCC2-4 and NQO1 expression was preceded by increased nuclear accumulation of Nrf2 at 1 and 3 hrs post treatment in mouse hepatocytes along with increased cytosolic Nqo1 expression (Fig. 2D), as

well as by increased Nrf2 binding in Huh-7 cells (Supp. Fig. 4). The observations indicate that activation of fasting pathways induces Nrf2, Nqo1, Abcc2-4 mRNA and protein expression in mouse and human *in vitro* and *in vivo* models.

Fasting induces Nrf2 activation and Abcc2-4, and Nqo1 expression in WT in part through Nrf2-dependent mechanisms. To determine whether fasting induces expression of Nrf2 target genes through Nrf2 dependant mechanisms, relative Nqo1 and Abcc expression in WT and Nrf2-null fasted mice was quantified. Fasting increased Nqo1, Abcc2-4 expression at 24 hrs in Nrf2 WT mice, whereas expression in livers of Nrf2-null mice was significantly lower (Fig. 3A). The relative mRNA fold increase in Nqo1 mRNA was higher than Abcc2-4, which is consistent with a previous observation (2,55). Fasting also increased Abcc2 protein staining in livers of C57Bl/6 mice, but not in livers of Nrf2 null mice (Fig. 3B). Further, fasting increased Abcc2, Abcc3, Nqo1 protein expression (Fig. 3C). This increase was accompanied by an increased Nrf2 binding to consensus ARE sequence in liver nuclear extracts from Nrf2 WT fasted mice (Fig. 3D).

To identify whether changes in mRNA and protein expression of Abccs during fasting contributed to phenotypic changes in metabolites and substrates, we quantified serum and liver cAMP in Nrf2 WT and Nrf2 null fed and fasted mice because cAMP is a described substrate for Abcc4 (11,34). Fasting increased serum cAMP levels in WT mice by ~1.77 fold

whereas they remained unchanged in sera of Nrf2 null mice, whereas liver cAMP levels in Nrf2 WT mice decreased by 48% upon 24 hr fasting, but remained unchanged in the Nrf2 null mice (Fig. 3D), which corresponds to the observed lack of Abcc4 induction (Fig. 3A).

To further test the possibility that Nrf2 induces expression of its targets through ARE, ARE-hPAP reporter transgenic mice possessing a human alkaline phosphatase reporter downstream of ARE consensus sequence (26) were fasted hPAP mRNA expression was determined. Fasting increased hPAP and Nqo1 mRNA expression in livers of ARE-hPAP mice by 8 and 5 fold compared to fed controls (Fig. 3F). hPAP mRNA expression was somewhat more inducible than Nqo1 mRNA expression, which is consistent to a previous publication (53). 8-Br-cAMP increased hPAP mRNA expression by about 2.5 fold in primary mouse hepatocytes isolated from ARE-hPAP mice (Fig. 3G). These results indicate that fasting induces Nrf2, Nqo1 and Abcc2-4 in part through Nrf2. Other transcription factors such as CAR, PPARα also regulate expression of Abcc2-4 (15). However, Nrf2-null mice exhibited dampened induction of Abcc2-4 indicating that fasting induction of Abcc2-4 occurs via some Nrf2 dependent mechanisms.

8-Br-cAMP activates cAMP/PKA pathway upstream of Nrf2-ARE activation. To identify a connection between cAMP/PKA signaling pathway

and Nrf2 activation, induction of ARE-driven luciferase reporter was analyzed in Huh7 cells treated with 8-Br-cAMP, glucagon and epinephrine. As shown in Fig. 4A and Supp. Fig. 5, all three treatments increased ARE-driven luciferase activity by about 1.5-2.5 fold. Primary ARE-hPAP mouse hepatocytes treated with 8-Br-cAMP showed increased hPAP expression, which was muted upon treatment with H89 (Fig. 4B). 8-Br-cAMP treatment increased Nrf2, Nqo1, Abcc2 and 4 mRNA expression in primary hepatocytes isolated from Nrf2-WT, but not in Nrf2-null hepatocytes (Fig. 4C and 4D). Induction of Nrf2, Nqo1, Abcc2, and Abcc4 expression by 8-Br-cAMP was blunted by H89 in Nrf2-WT hepatocytes, but not in Nrf2-null hepatocytes (Fig. 4C and 4D).

Induction of NAD+/NADH is one mechanism that activates Sirt1. Liver NAD+/NADH levels were decreased by ~50% in Nrf2-null compared with Nrf2 WT mice (Fig. 4E). Fasting resulted in a greater fold increase in liver NAD+/NADH ratio in Nrf2-null mice as compared to the WT mice (Fig. 4E), but as seen from Fig. 3D, Nrf2 null mice did not demonstrate the changes in cAMP levels as seen in the Nrf2 WT mice. Lack of Nrf2 did not affect Pgc-1α and Pepck expression in these mice (Supp. Fig. 6), indicating that fasting induced expression of gluconeogenic genes is not Nrf2 dependant. However, even though upstream pathways of fasting were activated in Nrf2 null mice, Abcc2-4 and Nqo1 expression was muted indicating that induction of these target genes occurs via Nrf2 dependant mechanisms.

In primary mouse hepatocytes AMPK activators AICAR, MET and NAD+ failed to induce Nrf2 and Nqo1, further suggesting a role for cAMP/PKA instead of the AMPK pathway (Fig. 4F). Total AMPKα and p-AMPKα levels decreased in livers of WT mice, but were unchanged in Nrf2-null livers, further suggesting that AMPK activation was not upstream to Nrf2 activation (Fig. 4G). These observations indicate that cAMP/PKA induces expression of ARE-containing genes via Nrf2-dependent mechanisms.

Fasting induced Abcc2-4 and Nrf2-target gene expression is Sirt1-dependent in vivo. In addition to cAMP/PKA signaling pathway, previous studies have indicated that Sirt1 is a critical mediator of fasting-induced expression and activation of Pgc1-α (47), as well as other fasting physiologies such as gluconeogenesis, fatty acid oxidation, and ketogenesis (7). To test whether Sirt1 plays a role in fasting induced activation of Nrf2, liver-specific Sirt1-null mice were fasted and expression of Abcc2-4 determined. Absence of functional Sirt1 in liver minimally affected basal expression of genes measured (Fig. 5A). Upon fasting, Nrf2 and Abcc2-4 mRNA expression were induced in livers of control mice, but not in Sirt1LKO mice (Fig. 5A). Fasting increased Nqo1 expression to a similar degree in Sirt1-WT and Sirt1-LKO mice. Conversely, adenoviral over-expression of Sirt1 enhanced fasting induced Nrf2 and Abcc2-4 expression (Fig. 5B).

Nrf2 expression increases co-ordinately with increasing Sirt1 and Pgc-1α in vitro.

In order to identify whether the Sirt1-Pgc-1α cascade acts upstream of the Nrf2-ARE pathway during fasting, Nrf2 and Pgc-1α mRNA expression was quantified in Huh7 cells transfected with either constant or varying amounts of Nrf2 ,Sirt1 and Pgc-1α. As depicted in Fig. 6C and 6D, Nrf2 expression was coordinately regulated with Pgc-1α. Nrf2 expression increased by 5-7 fold (Fig. 6C) and 1.5-2 fold (Fig. 6D) with increasing concentration of Sirt1 and Pgc-1α expression plasmid, respectively. 8-Br-cAMP increases this fold induction of Nrf2 further in both cases (Fig. 6C and 6D). Thus, Sirt1-Pgc-1α cascade potentially acts upstream of Nrf2-ARE pathway, increasing transcription of Nrf2 and possibly its activity. IP assays were performed on Huh7 cells transfected with Flag-Nrf2/cMyc-Sirt1 or Flag-Nrf2 only and treated with 8-Br-cAMP to determine whether Sirt1 or Pgc-1α possibly could act as transcriptional activators. 8-Br-cAMP induced cMyc protein expression indicating and induction of Sirt1 protein (Supp. Fig. 7) as well as FlagM2 protein in the immunoprecipitate indicating an increase in Nrf2 expression (Fig. 6F) Immunoprecipitation of Flag- tag by Anti-Flag M2 antibody resulted in coimmunoprecipitation of endogenous Pgc-1α along with Flag-Nrf2 (Fig. 6F) indicating that Pgc-1α could be a coactivator of Nrf2-ARE transcriptional pathway. It is also interesting to observe that this coimmunoprecipitation was barely detectable in cells which were not

transfected with Sirt1 expression plasmid (Fig. 6F). Sirt1 (c-Myc antibody) is also co-immunoprecipitated along with Nrf2 (Fig.6F).

Above observations indicate that Pgc-1α acts as a positive upregulator of Nrf2 expression and Nrf2 transcriptional pathway with activity of Pgc-1α partly dependent on presence of Sirt1.

Effect of fasting in obese mice. Leptin-deficient obese mice ($Lep^{ob/ob}$, OB) were fasted to determine whether similar fasting induction of Nrf2 activity and Abcc2-4 upregulation occurs. A differential fasting response was observed in OBs compared to C57BL/6 mice with regard to serum chemistry (Supp. *Table 1)*. In fed mice, hepatic mRNA Abcc2-4 expression was elevated in OB mice, consistent with a previous report (13). Fasting did not increase Abcc2 and 3 in livers from OB mice, and Abcc4 mRNA expression was decreased compared to fed OB mice (Fig. 7). In general, obesity blunted fold induction of Abcc2-4, Nrf2 and Nqo1 as the observed fold induction was lower in obese mouse livers.

DISCUSSION

Studies herein demonstrate that fasting upregulates Nrf2 activity and increases mouse and human ABCC2-4 expression in liver via cAMP/PKA-Sirt1 dependant mechanisms. Hepatic transport mechanisms are integral to liver function to maintain systemic hormone and second messenger

levels. There are only a handful of published observations that document effects of fasting on hepatic clearance mechanisms.

Nrf2 is best described for regulating xenobiotic detoxification and clearance mechanisms, such as glutathione synthesis and conjugation enzymes, Phase-II biotransformation enzymes, and Abcc transporters (30). As an oxidative stress sensor; it is activated by reactive oxygen species, as well as antioxidants. A majority of studies demonstrate a classical function of Nrf2 in mediating cytoprotective response in response to xenobiotic insult or disease, but few identify whether Nrf2 participates in basic metabolic functions. Herein, we demonstrate that fasting response upregulates Nrf2-ARE activation, subsequent target gene mRNA, and protein expression, along with corresponding increased Nrf2-ARE binding in both mouse and human models.

It is interesting to consider why Nrf2 activation and hepatic clearance mechanisms are upregulated during food deprivation. Fasting causes a metabolism shift from lipid storage to lipid oxidation for energy generation, resulting in fat mobilization from adipose to liver, and consequently increasing lipid peroxidation products (1). Lipid breakdown in fasting preferentially increases arachidonic acid, stearic acid and docosahexanoic acid, which are probable substrates for Abcc3 (12,32). A consequence of fasting is hepatic lipid accumulation and break down, as well as, increased

bilirubin, bile acids, and cyclic nucleotides, which are all substrates for Abcc2-4. Fasting increases hepatic cAMP (33), cGMP (37), bilirubin (22), UGT expression (35), lipid peroxidation products, bile acids (45), bile flow (49), and forms glutathione conjugates, which depletes cellular glutathione (1) . Fig. 8 illustrates how fasting may increase Nrf2 and the ARE to enhance hepatic clearance mechanisms of endogenous metabolites through Abcc2-4 upregulation. For example, mouse and human ABCC2 are known to be high affinity transporters for bilirubin-glucuronide and contribute to bilirubin excretion to bile (25). Fasting increases bilirubin glucuronidation (51) and Abcc2 induction could be in response to increase bilirubin clearance. Second, as ABCC2-4 contribute to bile acid efflux from hepatocytes, induction might aid in decreasing the increased bile acid load that occurs with fasting. Third, cAMP and cGMP are substrates for Abcc4 (52), and our data herein illustrate that serum and liver cAMP levels corresponded with Abcc4 mRNA expression. Abcc4 induction during food deprivation may be a response to control intracellular cyclic nucleotide concentrations within liver to better regulate the fasting response. Fasting is known to increase NAD+/NADH and GSSG/GSH ratios within liver (21). Use of GSH in conjugating reactions also increases GSH conjugates, which are also ABCC2-4 substrates. Moreover, upregulation of GSH synthesis restores redox balance and can enhance bile flow. Altered NAD+ and NADH levels in Nrf2-null mice reflect the inability to cycle NADH to NAD+; required for glycolytic processes, which might be compensated by

alternative pathways as indicated by a higher fold increased NAD+/NADH ratios upon fasting. This observation is consistent with a recent observation that PKA activates Sirt1 in NAD+ independent mechanisms (19). However, the role of NAD+ in activating Sirt1 cannot be overlooked. Lastly, fasting mobilizes fats from adipose tissue to liver resulting in fasting-induced steatosis. As Nrf2 has been implicated in promoting steatosis, perhaps early Nrf2 activation during fasting is one of the cellular responses to aid this process and promote energy conservation in the form of TG storage (42).

Downstream regulation of Abcc2-4 by Nrf2 is well described, yet upstream signaling pathways are less described. Insulin activates Nrf2 via PI3K, mTOR and Akt pathways in diabetic models (40,57). However, pathways contributing to fasting- dependant Nrf2 activation and subsequent target gene expression have not been described. To date two *in vitro* studies illustrate that cAMP regulates Abcc3 and 4 expression (8,48). Presently, cAMP/PKA pathway activators (e.g. epinephrine, glucagon,cAMP analogs, and food deprivation) increased ARE activity Nrf2 target gene expression in primary human and mouse hepatocytes, as well as mouse liver. H89, a well described PKA inhibitor attenuated 8-Br-cAMP induction of ARE activation and Nrf2 target gene upregulation, *in vitro*, further supporting a role for the cAMP/PKA pathway. Moreover, AMPK activators decreased Nrf2 target gene expression and were not increased in fasting. Thus, the

data support a role for cAMP/PKA cascade as a regulator of Nrf2-ARE activation.

An additional aspect of the current study was to identify factors downstream of cAMP/PKA cascade, which mediate activation of Nrf2- ARE pathway. The cAMP/PKA cascade activates CREB, which is also known to interact with Nrf2, and synergistically increase Nrf2 target gene transcription (50) . Because Pgc-1α is a central transcriptional co-activator to multiple nuclear receptors (15), we hypothesized that fasting might also modulate Nrf2 via Sirtuin 1- Pgc-1 related mechanisms *in vivo*. Epigenetic mechanisms, such as acetylation/deacetylation by p300/cbp protein on lysine residues in the Neh1 domain of Nrf2 as well as phosphorylation/ dephosphorylation at serine 40 and tyrosine 568 modulate Nrf2-ARE binding (27,28,50). Sirt1 can decrease Nrf2 binding to ARE *in vitro* (28). However, to date, no studies have addressed how Sirt1 regulates Nrf2 activity and subsequent gene expression *in vivo*. The data herein illustrate that fasting mediated Nrf2 activation and Abcc upregulation is Sirt1 dependent.

Sirt1 and Pgc-1α act in concert to activate gluconeogenesis pathways in the hepatocytes (46). Mice expressing Sirt1 in liver induced Nrf2 target gene mRNA expression upon fasting. However, fasting induction of Nrf2 target gene expression (including Abcc2-4) was muted in livers of mice with hepatocyte-specific Sirt1 deletion. Moreover, fasting generally increased

liver Abcc2-4 mRNA expression with Sirt1 overexpression. The above observations indicate towards a probable role of Sirt1 as an upstream regulator of Nrf2-ARE pathway.

Leptin resistance is hallmark of obesity (56), which is modeled in OB mice (23). Conditions of obesity and steatosis increase liver Nrf2 and Abcc2-4 expression in mice, indicating endogenous metabolites can modulate their expression (13,39). Upon fasting, OB mice displayed a muted response to fasting, with lower Abcc2-4 and Nqo1 induction observed. Some models of obesity, such as rats fed a high fat diet, are described as unable to activate fasting pathway over lean models (16).

In summary, fasting and PKA activators increased mouse Abcc2-4 expression in liver by Nrf2- and Sirt1-related mechanisms in mouse liver and hepatocytes. Additionally, 8-Br-cAMP and PKA activating compounds increased Nrf2 target gene and ABCC2-4 mRNA expression in human hepatocytes. Our data illustrate an undescribed role for Nrf2 as a “fasting” responsive transcription factor that is activated via cAMP/PKA-Sirt1 upstream mechanisms.

ACKNOWLEDGEMENTS:

The authors thank Drs. Joseph T. Rodgers and Pere Puigserver (Dana-Farber Cancer Institute, Harvard Medical School) for sharing liver tissue,

Dr. Curtis D. Klaassen (University of Kansas Medical School) and Dr. Jeff Chan (UC-Irvine) for sharing Nrf2-null mice. We thank Dr. Michael Goedken for histopathology consultation, as well as, Vijay More, and Maneesha Paranjpe for technical assistance.

FOOTNOTES

List of Abbreviations:

Multidrug resistance-associated proteins (Mrps), Nuclear factor-E2 related-factor 2 (Nrf2), Sirtuin-1 (Sirt1), Kelch like associated protein-1 (Keap1), Nadph:quinone oxidoreductase (Nqo1), glutamate-cysteine ligase, catalytic subunit (Gclc), Antioxidant Response Element (ARE), PPAR gamma coactivator-1 alpha (Pgc-1α), 3'-5'-cyclic adenosine monophosphate (cAMP), Protein Kinase A (PKA), 8-Bromoadenosine-3', 5'-Cyclic Adenosine Monophosphate (8-Br-cAMP),

Financial Support: This work was supported by National Institute of Health [1R01ES016042], and in part, by Rhode Island IDeA Network of Biomedical Research Excellence [Award # P20RR016457-10] from the National Center for Research Resources, National Institute of Health, National Institute of Health [5K22ES013782], and the Rhode Island Foundation.

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FIGURE LEGENDS:

FIGURE I-1: Fasting induces Abcc2-4 expression *in vitro* and *in vivo*. (A) Increased biliary DBSP excretion upon fasting. DBSP (150μmol/kg/5ml, i.p) was injected into C57Bl/6 mice (n=5 per group) either fed *ad libitum* or fasted for 18 hrs. 45 min after injection, gallbladders were collected and biliary DBSP content was determined spectrophotometrically after appropriate dilution and alkalinization of bile with 0.1N NaOH. The data is represented as average ± SEM μmol of DBSP/ μl bile/ kg body weight. **(B) Induction of Abcc 2-4 expression in mouse liver after a 24 hr fast-** Total RNA was isolated from livers of C57Bl/6 mice either fed *ad libitum* or fasted for 24 hrs. Relative Abcc 2- 4 mRNA expression was quantified by real-time quantitative PCR. The data is represented as average ± SEM fold change over the fed controls. All data has been normalized to 18S rRNA. **(C) Induction of Abcc2-4 protein expression in mouse liver after 24 hour fasting-** Crude membrane proteins were isolated from livers of C57Bl/6 mice either fed ad libitum or fasted for 24 hrs. Abcc2-4 protein expression was quantified by western blotting. **(D) Induction of Abcc 2-4 mRNA expression in primary mouse hepatocytes by 8-Br-cAMP-** Primary mouse hepatocytes were treated with DMSO or 8-Br-cAMP (1mM) for 24 hrs. Total RNA was isolated 24 hrs after treatment and Abcc 2- 4mRNA expression was quantified using real time quantitative PCR. The data is represented as average ± SEM (n=3) fold change over the fed controls. All data is normalized to 18S rRNA.

Groups without a common letter are significantly different. **(E) Time dependant expression of ABCC 2-4 mRNA in Huh-7 cells by 8-Br-cAMP-** Huh-7 cells were treated with1.0mM 8-Br-cAMP for 0, 3, 6, 12, 24 and 48 hrs with media as the control (n=3) . Total RNA was isolated and ABCC 2-4 mRNA expression was quantified using the Branched DNA Signal Amplification assay. The data is represented as average RLU per 10µg total RNA ± SEM (n=3). **(F) Induction of ABCC 2-4 mRNA expression in primary human hepatocytes by 8-Brc-AMP-** Primary human hepatocytes were treated with media, DMSO, 8-Br-cAMP (0.1 and 1mM), epinephrine (10 and 100 µM) or glucagon (0.1 and 1 µM) for 24 hrs. At the end of 24 hrs cells were lysed using Lysis Mixture™. mRNA levels of ABCC 2-4 were quantified using Branched DNA signal amplification assay . The data is represented as relative mRNA expression per 25 µl lysate normalized to beta actin from three human hepatocyte donors.

FIGURE I-2: Fasting induces Nrf2 target gene expression *in vitro* and *in vivo*. (A) Induction of Nqo1 and Nrf2 mRNA expression in mouse liver after 24 hr fast- Total RNA was isolated from livers of C57Bl/6 mice either fed *ad libitum* or fasted for 24 hrs. Gene expression of Nqo1 was quantified by real-time quantitative PCR. The data is represented as average ± SEM fold change over the fed controls. All data has been normalized to 18S rRNA. **(B) Induction of Nqo1 protein expression in mouse liver after 24 hour fasting-** Cytosolic proteins were isolated from

livers of C57Bl/6 (n=3) mice either fed ad libitum or fasted for 24 hrs. Nqo1 protein expression was quantified by western blotting. The data is representative of three individual protein quantifications. **(C) Induction of Nqo1 and Nrf2 mRNA expression in primary mouse hepatocytes by 8-Br-cAMP-** Primary mouse hepatocytes were treated with DMSO or 8-Br-cAMP (1mM) for 24 hrs. Total RNA was isolated 24 hrs after treatment and Nqo1 and Nrf2 mRNA expressionwas quantified using real time quantitative PCR. The data is represented as average ± SEM (n=3) fold change over the fed controls. All data is normalized to 18S rRNA. Groups without a common letter are significantly different. **(D) Induction of NQO1 mRNA expression in primary human hepatocytes by 8-Brc-AMP-** Primary human hepatocytes were treated with media, DMSO, 8-Br-cAMP (0.1 and 1mM), epinephrine (10 and 100 µM) or glucagon (0.1 and 1 µM) for 24 hrs. At the end of 24 hrs cells were lysed using Lysis Mixture™. mRNA levels of NQO1 was quantified using Branched DNA signal amplification assay . The data is represented as relative mRNA expression per 25 µl lysate normalized to beta actin from 3 human hepatocyte donors. **(E) Time dependant increase in Nqo1 and Nrf2 protein expression in Huh7 cells by 8-Br-cAMP-** Huh-7 cells were treated with1.0mM 8-Br-cAMP for 0, 3, 6, 12, 24 hrs with media as the control (n=3). mRNA levels of NQO1 was quantified using Branched DNA signal amplification assay. The data is represented as fold change in Nqo1 mRNA expression (n=3) . Nqo1 protein levels were determined by western blotting with gapdh as the

loading control. Nuclear Nrf2 protein levels were measured by western blotting with laminB1 as loading control.

FIGURE I-3: Fasting increases Abcc2-4, Nqo1 expression in Nrf2 dependant manner. (A)Fasting for 24 hrs induces the expression of Nrf2 target genes in wild type C57Bl/6 mice livers but not in Nrf2 null mice livers- Total RNA was isolated from livers of C57Bl/6 (n=6) and Nrf2-null (n=6) mice either fed *ad libitum* or fasted for 24 hrs. Nqo1and Abcc 2-4 gene expression was quantified by real time quantitative PCR. The data is represented as average ± SEM (n=6) fold change over the fed controls. **(B) Immunohistochemical staining of Abcc2-** Cryosections were incubated with anti-Abcc2 antibody followed by incubation with AlexaFluor-conjugated secondary antibodies (green). **(C) Induction of Abcc2-4 protein expression in Nrf2-WT but not in Nrf2-null mouse liver after 24 hour fasting-** Crude membrane proteins were isolated from livers of C57Bl/6 (n=2) and Nrf2 null (n=2) mice either fed ad libitum or fasted for 24 hrs. Nqo1, Abcc2, Abcc3 and beta actin protein expression was quantified by western blotting. The data is representative of three individual protein quantifications. **(D) Fasting for 24 hrs decreases liver cAMP while increasing serum cAMP levels in Nrf2 WT but not in Nrf2 null mice.** Serum bile acids and serum and liver cAMP levels were determined from serum and livers obtained from C57Bl/6 (n=4) and Nrf2 null (n=4) mice fasted for 24 hrs. Data is represented as average ± SEM (n=4) fold change

relative to control. **(E) Fasting increases binding of Nrf2 to its consensus sequence-** Liver nuclear extracts were prepared from control and fasted C57Bl/6 mice using Procarta TF nuclear extraction kit and analysis for transcription factor binding was determined using a 9 plex Procarta TF binding assay. The results obtained are represented as data normalized to TFIID. Data is expressed as Fluresence Intensity/10 µg total liver nuclear extract normalized to WT fed controls. $P<0.05$ was considered statistically significant. **(F) Fasting increases hPAP and Nqo1 mRNA in livers of ARE-hPAP mice-** Total RNA was isolated from livers of C57Bl/6 (n=3) and ARE-hPAP (n=3) mice either fed ad libitum or fasted for 24 hrs. Nqo1 and human alkaline phosphatase (hPAP) mRNA expression was quantified by real time quantitative PCR. The data is represented as average ± SEM (n=3) fold change over the fed controls. All data has been normalized to 18S rRNA. **(G) 8-Br-cAMP induces hPAP mRNA expression in primary mouse hepatocytes-** ARE-hPAP mouse hepatocytes were treated with 1mM 8-Br-cAMP for 24 hrs. Total RNA was isolated and ARE-hPAP mRNA expression was quantified using RT^2 -PCR. Target gene expression was normalized to 18s rRNA. Groups without a common letter are significantly different.

<u>FIGURE I-4:</u> PKA pathway acts as upstream regulator of Nrf2-ARE pathway. (A) 8-Br-cAMP induces Antioxidant Response Element (ARE) in Huh-7 cells- Huh7 cells were cultured to 85% confluence and

transiently transfected with an Antioxidant Response Element –luciferase reporter construct (100ng) for 24 hrs. The cells were then treated with 0.1mM and 1.0mM 8-Br-cAMP for 24 hrs. Luciferase activity was measured by dual luciferase assay with renilla luciferase as a control. The data is expressed as average fold induction in activity of the ARE-luciferase construct ± SEM (n=4). **(B) H89 inhibits 8Br-cAMP induced mRNA expression of hPAP-** ARE-hPAP mouse hepatocytes were treated with 1mM 8-Br-cAMP with or without H89 (10uM) for 24 hrs. Total RNA was isolated and hPAP, expression was quantified using RT^2 -PCR. Target gene expression was normalized to 18s rRNA. **8-Br-cAMP induced (C) Nrf2, Nqo1 and (D) Abcc2 and 4 mRNA expression is muted by H89 co-treatment in Nrf2 WT, but not Nrf2 null hepatocytes-** Primary mouse hepatocytes obtained from $Nrf2^{+/+}$ or $Nrf2^{-/-}$ mice were treated with DMSO or 8-Br-cAMP (1mM) with or without H89 (20 μM) for 24 hrs. Total RNA was isolated 24 hrs after treatment and Abcc2 mRNA expression was measured using real time quantitative PCR. The data is represented as average ± SEM (n=3) fold change over the fed controls. All data is normalized to 18S rRNA. $P<0.05$ was considered statistically significant. Groups without a common letter are significantly different. **(E) Fasting induced upregulation of hepatic NAD+/NADH levels occurs to the greater extent in Nrf2 null mice-** NAD+/NADH ratio was quantified in livers obtained from C57Bl/6 mice fasted for 24 hrs according to manufacturer's protocol. The data is represented as average ± SEM (n=4)

fold change over the fed controls. **(F) AMPK activators do not induce expression of Nrf2 target genes-** Primary mouse hepatocytes were treated with control, AICAR (0.5mM), NAD+ (5mM) or metformin (1mM) for 24 hrs. Total RNA was isolated 6 hrs after treatment and mRNA expression of Nqo1 and Nrf2 was quantified using real time quantitative PCR. The data is represented as average ± SEM (n=3) fold change over the fed controls. All data is normalized to 18S rRNA. Groups without a common letter are significantly different. **(G) Total AMPKα and p-AMPKα is attenuated in Nrf2 WT and Nrf2 null mouse livers upon fasting-** Proteins lysates were obtained from livers of C57Bl/6 (n=2) and Nrf2 null (n=2) mice either fed ad libitum or fasted for 24 hrs. Total AMPK and p-AMPKα and gapdh protein expression was quantified by western blotting.

FIGURE I-5: Changes in Nrf2-ARE pathway during fasting are Sirt1 dependant. (A) Sirt1LKO mice demonstrate resistance to effects of fasting- Total RNA was isolated from livers of SirtWT (n=5) and Sirt1LKO (n=3) mice either fed ad libitum or fasted for 24 hrs. Gene expression of Nrf2, Abcc 2-4 was quantified by real time quantitative PCR. The data is represented as average ± SEM fold change over the fed controls. All data has been normalized to 18S rRNA. $P<0.05$ was considered statistically significant. Groups without a common letter are significantly different. **(B) Sirt1OE mice demonstrate enhanced induction of Abcc2-4 and Nrf2 target genes upon fasting-** Total RNA was isolated from livers of Sirt1OE

(n=8) mice either fed ad libitum or fasted for 19 hrs. Gene expression of Nrf2, Abcc 2-4 was quantified by real time quantitative PCR. The data is represented as average ± SEM fold change over the fed controls. Groups without a common letter are significantly different. All data is normalized to 18S rRNA. $P<0.05$ was considered statistically significant. Groups without a common letter are significantly different.

FIGURE I-6: Nrf2 expression increases co-ordiantely with Pgc-1α and Sirt1 expression. (A) Nrf2 mRNA coordinately increases with increasing activation of Sirt1- Huh-7 cells were cultured to 85% confluence and transiently transfected (50-100ng) Myc-Sirt1, 75ng Flag-Nrf2 and 0.5μg Flag-Pgc-1α expression plasmids using Lipofectamine LTX-plus reagent (Invitrogen, CA, USA) for 24 hrs. The cells were then treated with 1.0mM 8-Br-cAMP for 24 hrs. Endogenous Nrf2 and Pgc-1α mRNA expression was quantified by real time quantitative PCR. The data is represented as average ± SEM (n=3) over control All data has been normalized to 18S rRNA. **(B) Nrf2 mRNA coordinately increases with increasing Pgc-1α-** Huh-7 cells were cultured to 85% confluence and transiently transfected with (0.5-1.0 μg) Flag-Pgc-1α, 50ng Myc-Sirt1, 75ng Flag-Nrf2 and 0.5μg Flag-Pgc-1α expression plasmids for 24 hrs. The cells were then treated with 1.0mM 8-Br-cAMP for 24 hrs. Nrf2 and Pgc-1α mRNA expression was quantified by real time quantitative PCR. The data is represented as average ± SEM (n=3) over control All data has been

normalized to 18S rRNA. **(C and D) Pgc-1α co-immunoprecipitates with Nrf2 in a Sirt1 dependent manner-** Huh-7 cells were cultured to 85% confluence in 6cm dishes and transiently transfected with 3.0μg Flag-tagged Nrf2, with or without 0.75μg cMyc-tagged Sirt1 plasmid for 24 hrs. At the end of 24 hrs, cells were treated with 1.0mM 8-Br-cAMP for 45 mins and then lysed using lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% NP-40). 1mg of the protein lysate was incubated with Anti-FlagM2 magnetic beads overnight in lysis buffer and immunoprecipitates were separated on SDS-PAGE and immunoblotted for Flag-M2 and endogenous Pgc-1α. Gapdh was used as protein loading control.

FIGURE I-7: Fasting effects are generally blunted in obese mice. Total RNA was isolated from livers of C57Bl/6 (n=8) and OB (n=8) mice either fed ad libitum or fasted for 24 hrs. Gene expression of Nrf2, Nqo1, Abcc 2- 4 was quantified by real time quantitative PCR. The data is represented as average ± SEM (n=8) fold change over the fed controls. All data has been normalized to 18S rRNA.

FIGURE I-8: Proposed model for fasting induced activation of Nrf2-ARE pathway and expression of downstream target genes. Fasting increases circulating glucagon and epinephrine levels activating cAMP/PKA cascade which eventually increases pepck and Pgc-1α expression and also activates Sirt1; thus gluconeogenesis. cAMP/PKA also induces Nrf2

binding to ARE in a Sirt1 dependant mechanisms leading to an increase in Nqo1, Abcc2-4 expression. It is hypothesized fasting will result in the production or accumulation of metabolic intermediates, which could be eliminated from hepatocytes via Abcc2-4. Fasting increases NAD+/NADH ratios, cAMP, bile acids, lipid peroxidation, glucuronide and glutathione (GSH) conjugation, and decreases GSH levels within the hepatocyte. GSH and glucuronide conjugates are substrates for Abcc2-4, some lipid peroxidation products are substrates for Abcc3. Abcc4 transports cyclic nucleotides, such as cAMP and cGMP. Overall, fasting could increase the hepatic efflux of metabolite conjugates and intermediates resulting from induction of the fasting response to maintain cellular homeostasis.

FIGURE I-1:

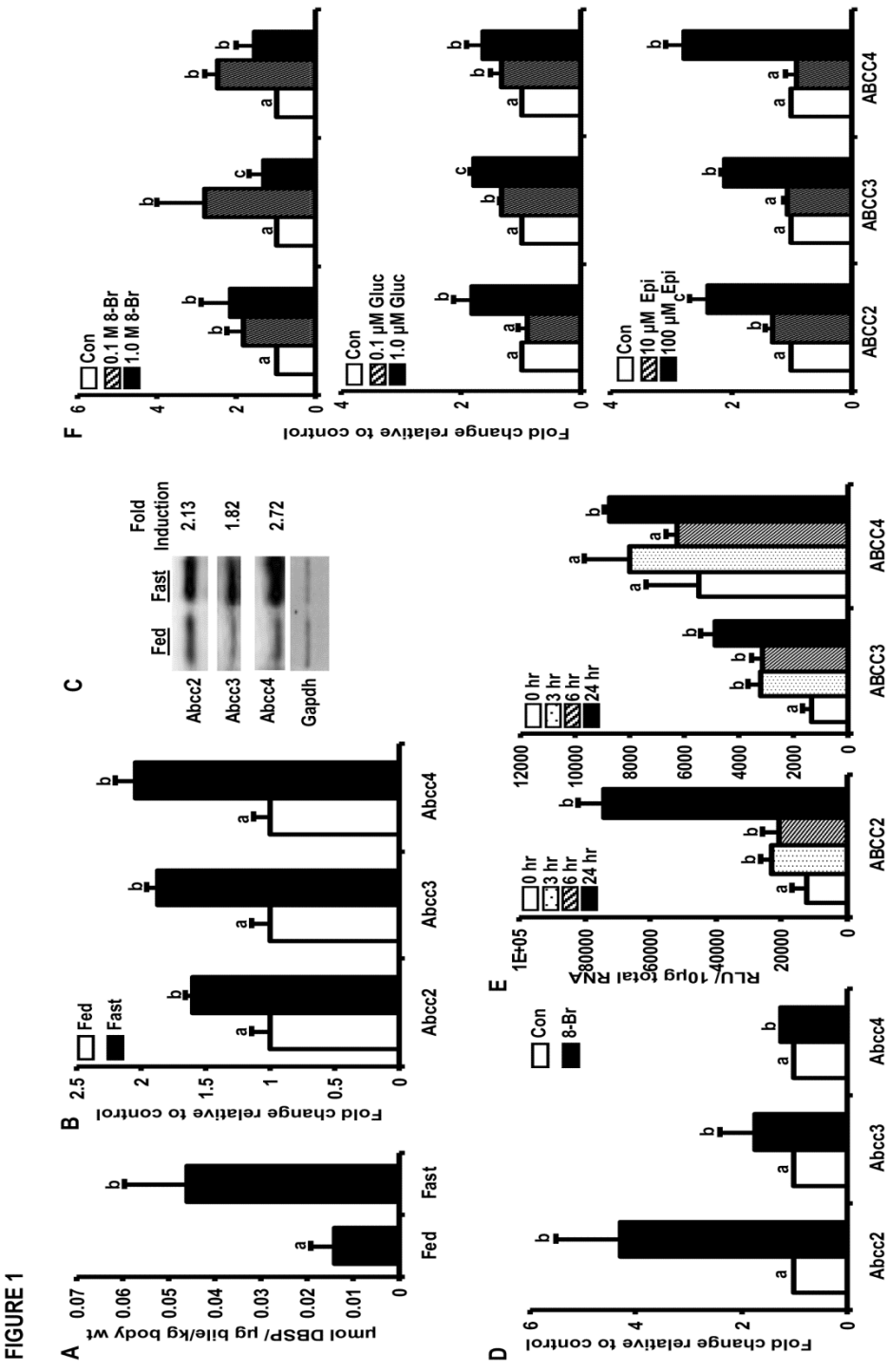


FIGURE 1
A
μmol DBSP/ μg bile/kg body wt
Fed
Fast
B
Fold change relative to control
Fed
Fast
Abcc2
Abcc3
Abcc4
C
Fed
Fast
Fold Induction
Abcc2
2.13
Abcc3
1.82
Abcc4
2.72
Gapdh
D
Fold change relative to control
Con
8-Br
Abcc2
Abcc3
Abcc4
E
RLU/ 10μg total RNA
0 hr
3 hr
6 hr
24 hr
ABCC2
ABCC3
ABCC4
F
Fold change relative to control
Con
0.1 M 8-Br
1.0 M 8-Br
Con
0.1 μM Gluc
1.0 μM Gluc
Con
10 μM Epi
100 μM Epi
ABCC2
ABCC3
ABCC4

FIGURE I-2:

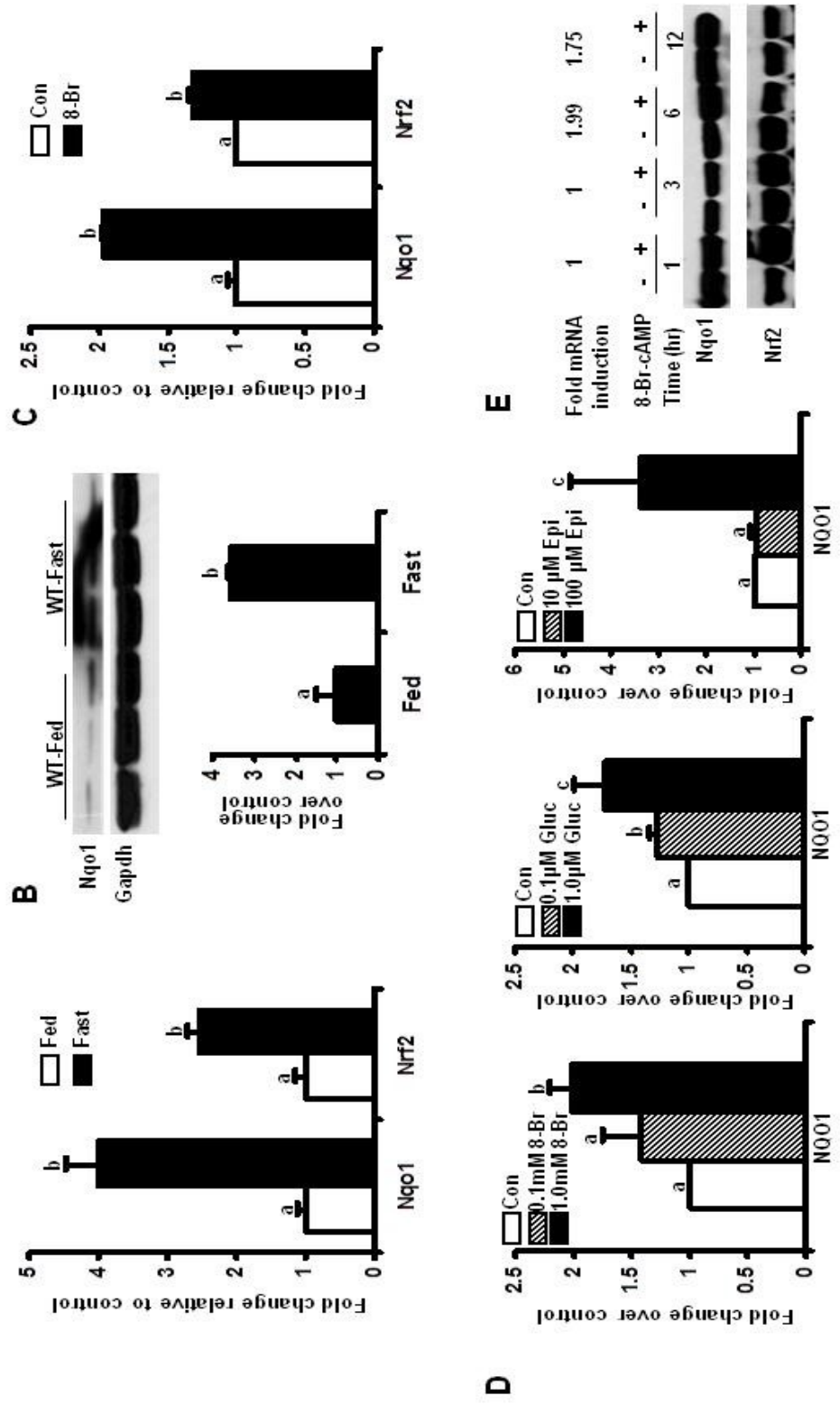

A
Fed
Fast
Fold change relative to control
Nqo1
Nrf2
B
WT-Fed
WT-Fast
Nqo1
Gapdh
Fold change over control
Fed
Fast
C
Con
8-Br
Fold change relative to control
Nqo1
Nrf2
D
Con
0.1mM 8-Br
1.0mM 8-Br
Fold change over control
NQO1
Con
0.1μM Gluc
1.0μM Gluc
Fold change over control
NQO1
Con
10 μM Epi
100 μM Epi
Fold change over control
NQO1
E
Fold mRNA induction
8-Br-cAMP
Time (hr)
Nqo1
Nrf2

FIGURE I-3:

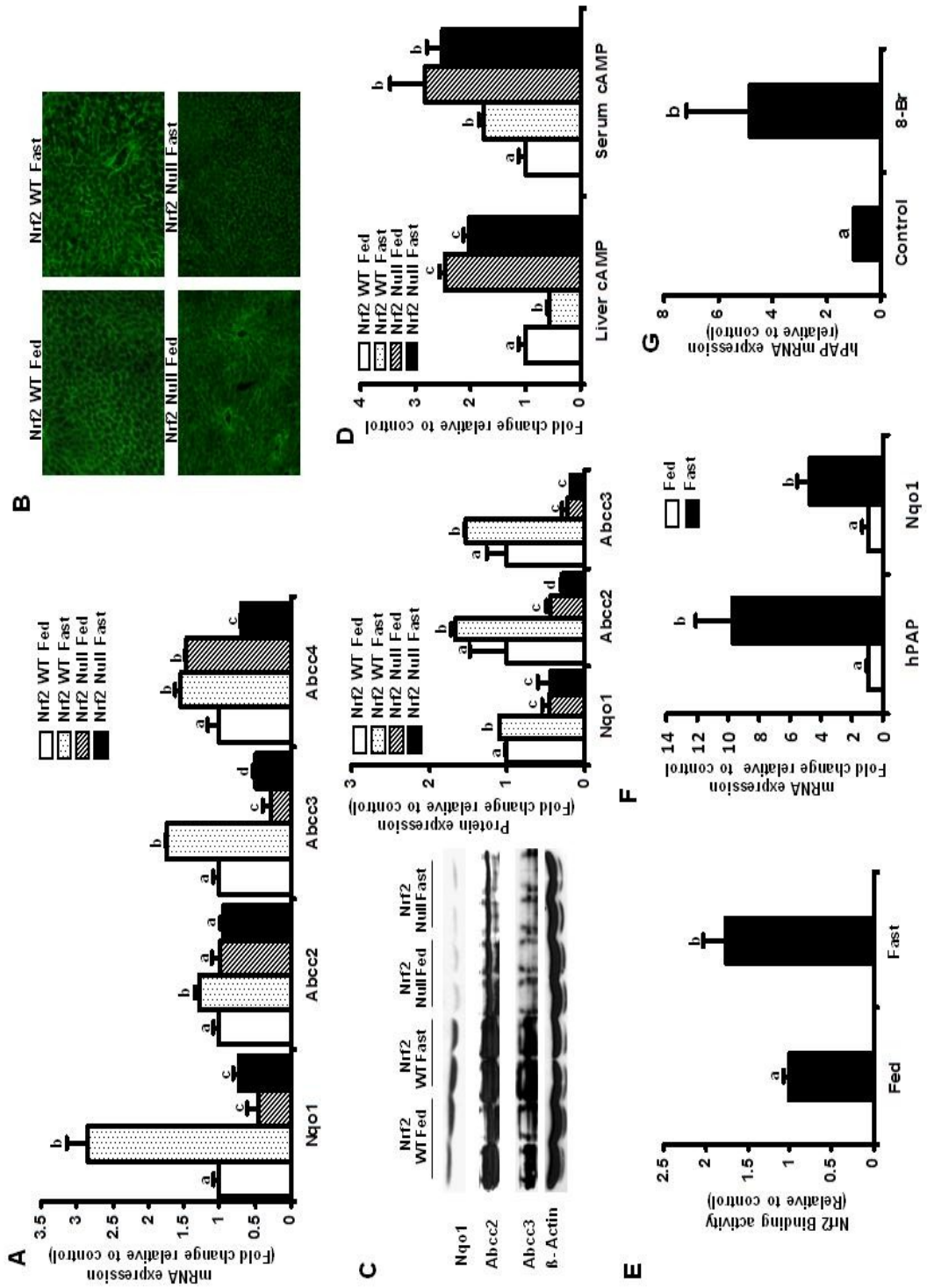

A
mRNA expression
(Fold change relative to control)
Nrf2 WT Fed
Nrf2 WT Fast
Nrf2 Null Fed
Nrf2 Null Fast
Nqo1
Abcc2
Abcc3
Abcc4
B
Nrf2 WT Fed
Nrf2 WT Fast
Nrf2 Null Fed
Nrf2 Null Fast
C
Nrf2 WT Fed
Nrf2 WT Fast
Nrf2 Null Fed
Nrf2 Null Fast
Nqo1
Abcc2
Abcc3
ß-Actin
D
Fold change relative to control
Liver cAMP
Serum cAMP
Protein expression
(Fold change relative to control)
Nqo1
Abcc2
Abcc3
E
Nrf2 Binding activity
(Relative to control)
Fed
Fast
F
mRNA expression
Fold change relative to control
Fed
Fast
hPAP
Nqo1
G
hPAP mRNA expression
(relative to control)
Control
8-Br

FIGURE I-4:

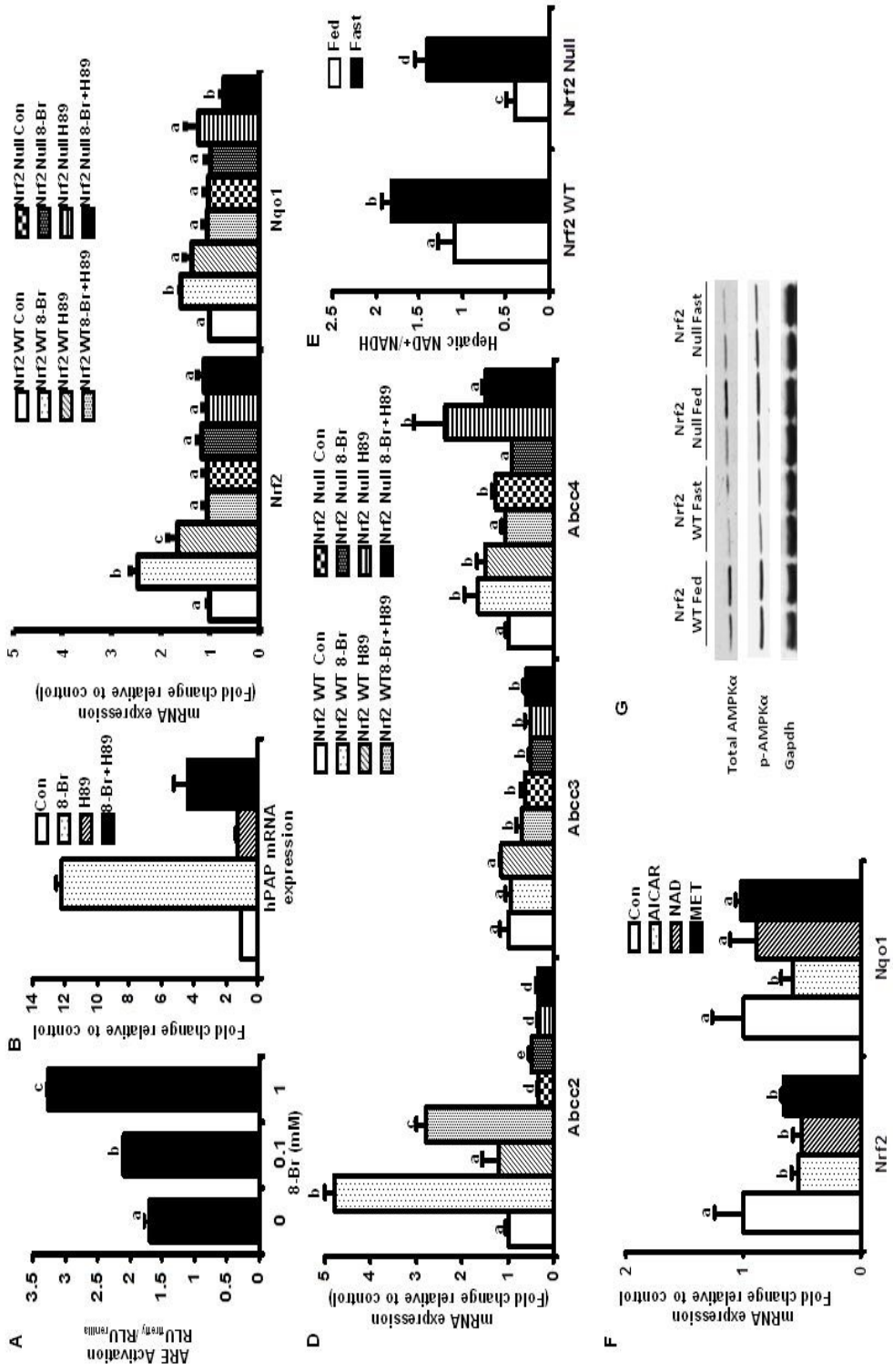

A
ARE Activation
RLU_treatg/RLU_media
8-Br (mM)
B
Fold change relative to control
Con
8-Br
H89
8-Br+H89
hPAP mRNA expression
mRNA expression (Fold change relative to control)
Nrf2 WT Con
Nrf2 WT 8-Br
Nrf2 WT H89
Nrf2 WT8-Br+H89
Nrf2 Null Con
Nrf2 Null 8-Br
Nrf2 Null H89
Nrf2 Null 8-Br+H89
Nrf2
Nqo1
D
Abcc2
Abcc3
Abcc4
E
Hepatic NAD+/NADH
Fed
Fast
Nrf2 WT
Nrf2 Null
F
Con
AICAR
NAD
MET
Nrf2
Nqo1
G
Nrf2 WT Fed
Nrf2 WT Fast
Nrf2 Null Fed
Nrf2 Null Fast
Total AMPKα
p-AMPKα
Gapdh

FIGURE I-5:

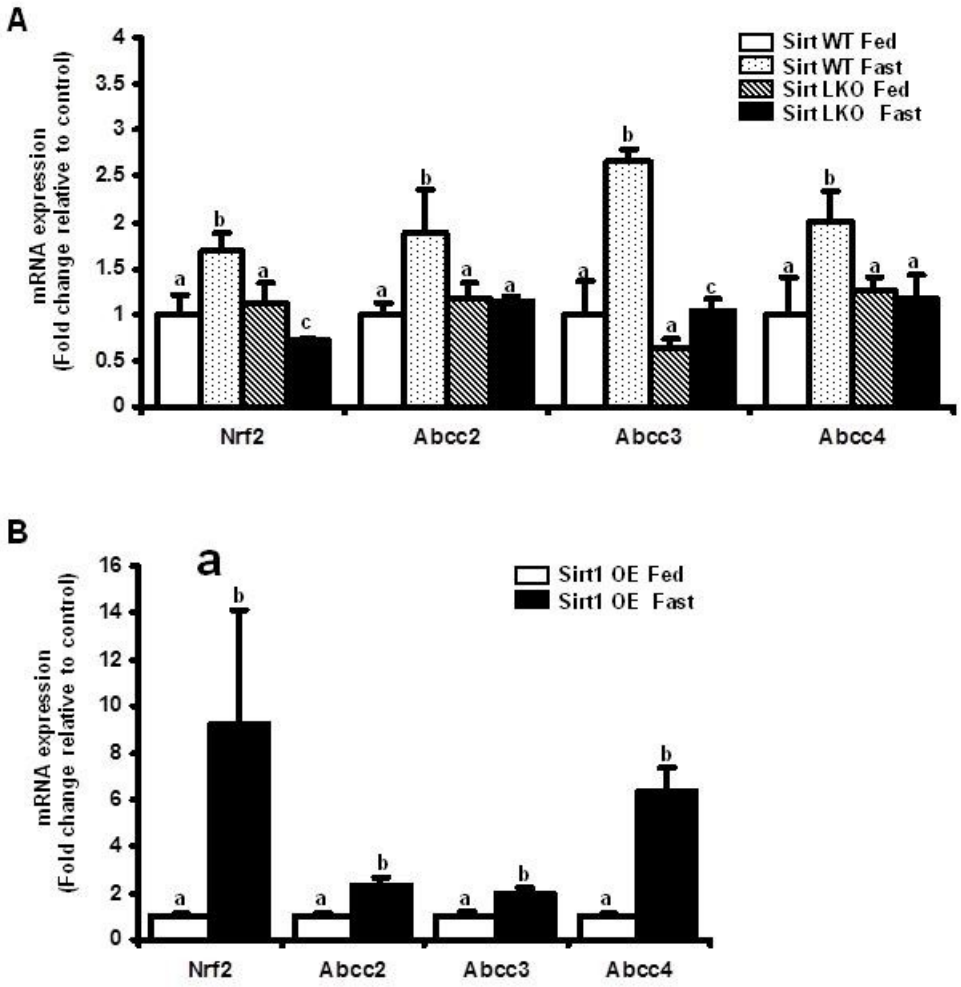

A
mRNA expression
(Fold change relative to control)
Sirt WT Fed
Sirt WT Fast
Sirt LKO Fed
Sirt LKO Fast
Nrf2
Abcc2
Abcc3
Abcc4
B
mRNA expression
(Fold change relative to control)
Sirt1 OE Fed
Sirt1 OE Fast
Nrf2
Abcc2
Abcc3
Abcc4

FIGURE I-6:

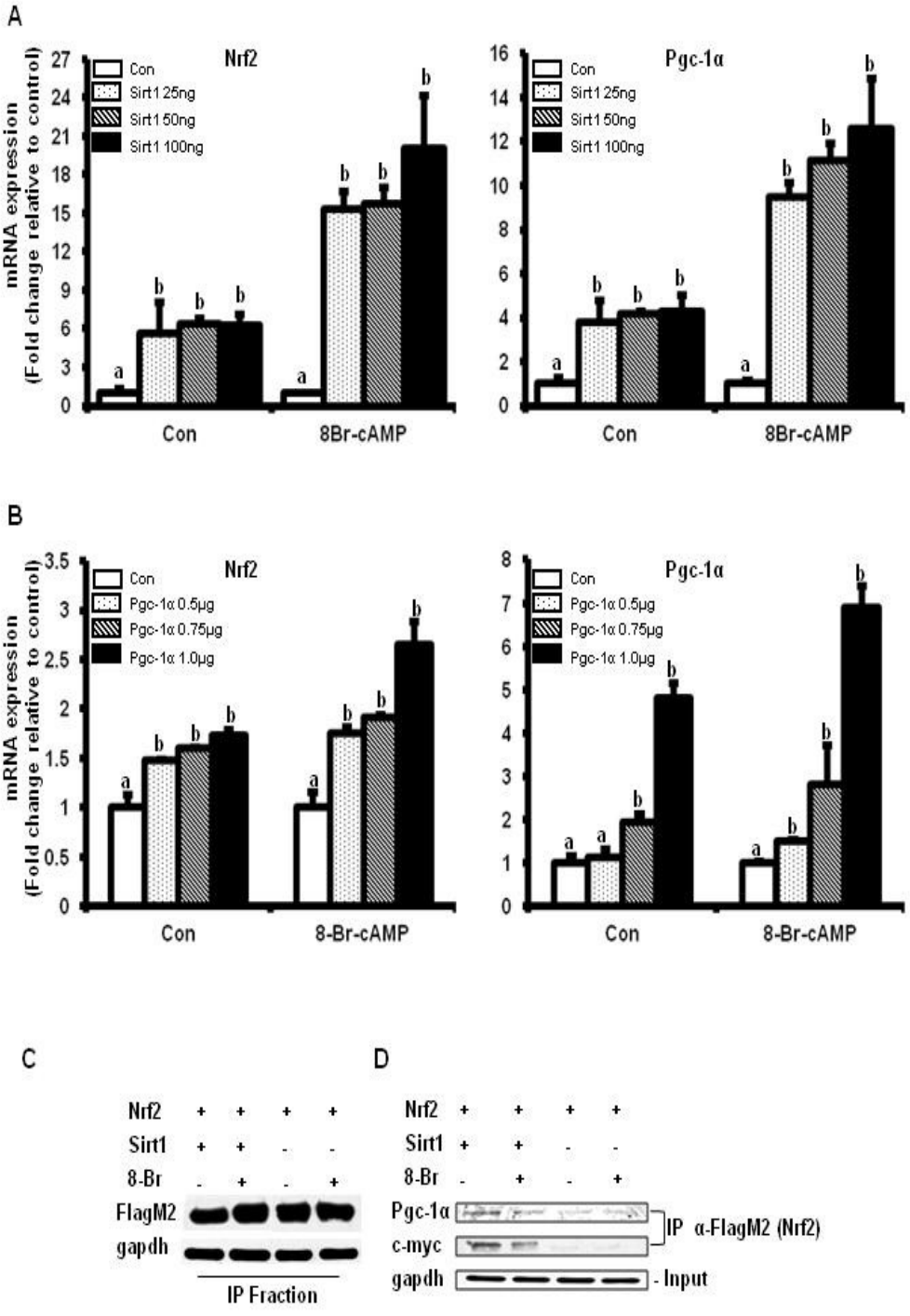

A
Nrf2
Con
Sirt1 25ng
Sirt1 50ng
Sirt1 100ng
mRNA expression
(Fold change relative to control)
Con
8Br-cAMP
Pgc-1α
B
Nrf2
Con
Pgc-1α 0.5μg
Pgc-1α 0.75μg
Pgc-1α 1.0μg
mRNA expression
(Fold change relative to control)
Con
8-Br-cAMP
Pgc-1α
C
Nrf2
Sirt1
8-Br
FlagM2
gapdh
IP Fraction
D
Nrf2
Sirt1
8-Br
Pgc-1α
c-myc
gapdh
IP α-FlagM2 (Nrf2)
Input

FIGURE I-7:

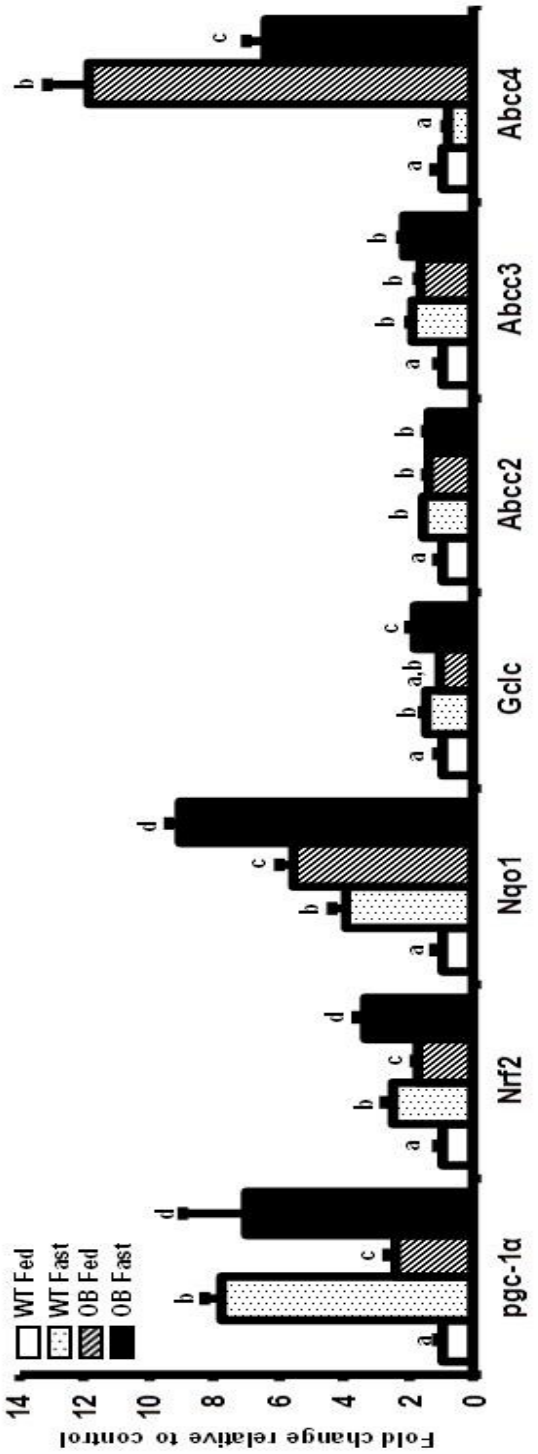
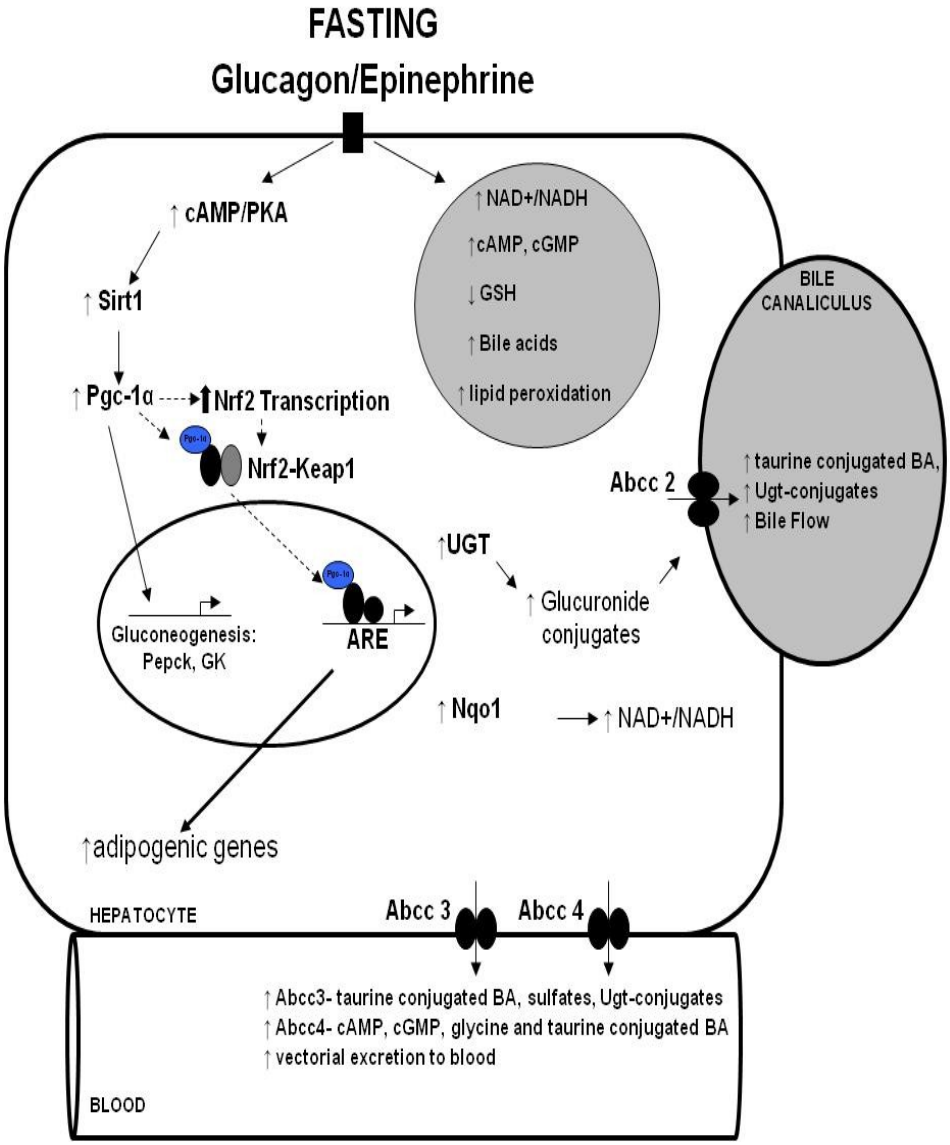

Fold change relative to control
0
2
4
6
8
10
12
14
WT Fed
WT Fast
OB Fed
OB Fast
pgc-1α
Nrf2
Nqo1
Gclc
Abcc2
Abcc3
Abcc4

FIGURE I-8:


FASTING
Glucagon/Epinephrine
↑ cAMP/PKA
↑ Sirt1
↑ Pgc-1α
↑ Nrf2 Transcription
Nrf2-Keap1
↑ NAD+/NADH
↑cAMP, cGMP
↓ GSH
↑ Bile acids
↑ lipid peroxidation
BILE CANALICULUS
Abcc 2
↑ taurine conjugated BA,
↑ Ugt-conjugates
↑ Bile Flow
↑UGT
↑ Glucuronide conjugates
Gluconeogenesis: Pepck, GK
ARE
↑ Nqo1
↑ NAD+/NADH
↑adipogenic genes
HEPATOCYTE
Abcc 3
Abcc 4
↑ Abcc3- taurine conjugated BA, sulfates, Ugt-conjugates
↑ Abcc4- cAMP, cGMP, glycine and taurine conjugated BA
↑ vectorial excretion to blood
BLOOD

SUPPLEMENTARY INFORMATION:

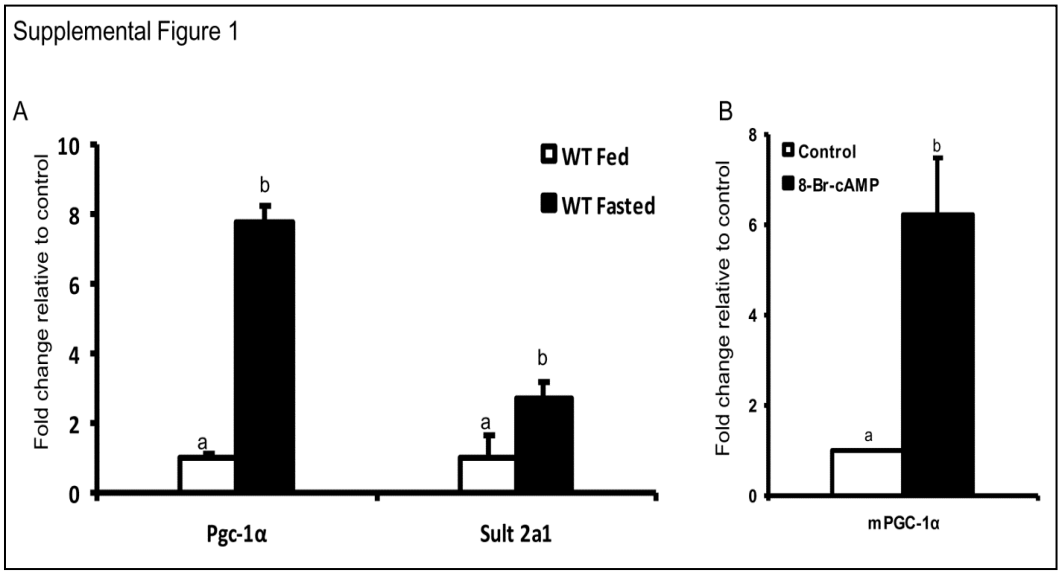


FIGURE 1: **Induction of Pgc-1α, Pepck and Sult2a1 mRNA expression upon activation of fasting pathway.** Adult C57Bl/6 (n=8/group) mice were fed *ad libitum* or fasted for 24 hours. **(A) 24 fasting increases Pgc-1α, Pepck and Sult2a1 mRNA expression-** Total RNA was isolated from livers and gene expression of Pgc-1α and Sult2a1 was quantified by real time quantitative PCR. The data is represented as average ± SEM (n=8) fold change over the fed controls. All data has been normalized to 18S rRNA. $P<0.05$ was considered statistically significant. Groups without a common letter are significantly different. **(B) Induction of Pgc-1α mRNA expression in primary mouse hepatocytes by 8-Br-cAMP-** Primary mouse hepatocytes were treated with DMSO or 8-Br-cAMP (1mM) for 24 hours. Total RNA was isolated 24 hours after treatment and Abcc 2-4mRNA expression was quantified using real time quantitative PCR. The data is represented as average ± SEM (n=3) fold change over the fed

controls. All data is normalized to 18S rRNA. Groups without a common letter are significantly different.

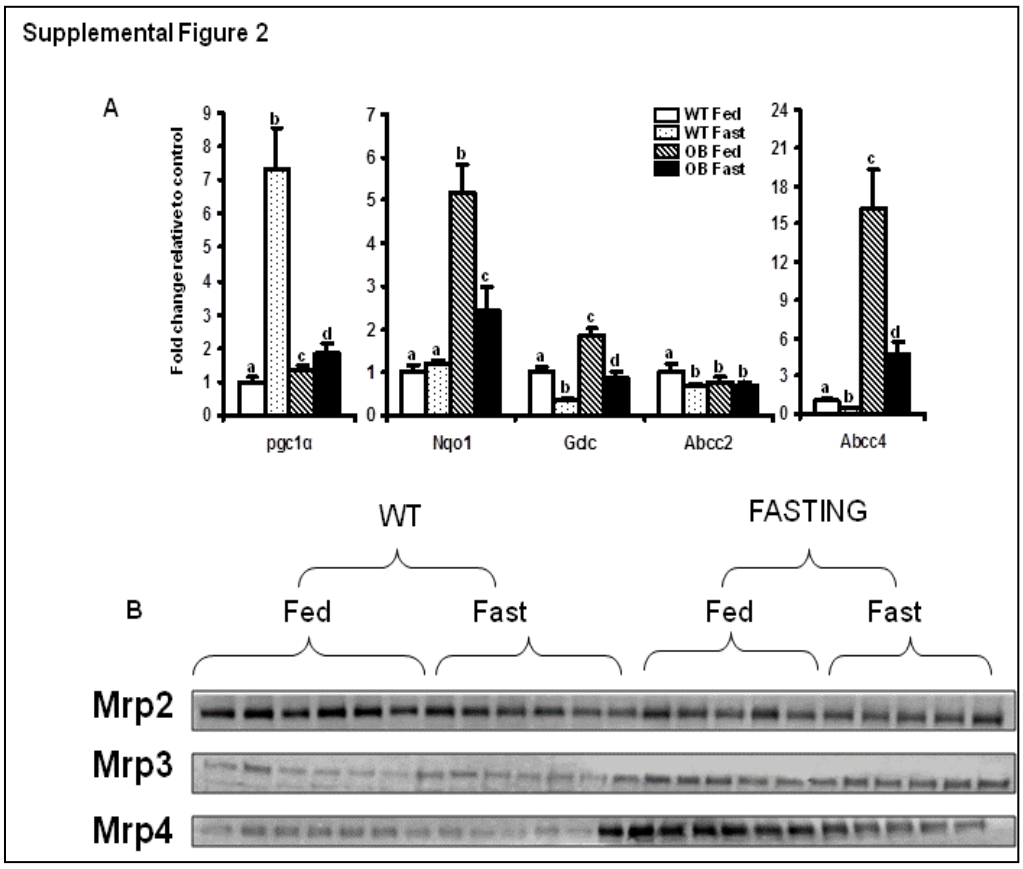


FIGURE 2: **Pgc-1α, Abcc2-4, Nqo1, Gclc and Nrf2 expression decreases in mouse liver after 30 hour fasting.** Adult C57Bl/6 or OB (n=6/group) mice were either fed *ad libitum* or fasted for 30 hours. **(A) 30 hour fasting decreases mRNA expression of Pgc-1α, Abcc2-4, Nqo1, Gclc and Nrf2 -** Total RNA was isolated from livers and gene expression of Pgc-1α, Abcc2-4, Nqo1, Gclc and Nrf2 was quantified by real time quantitative PCR. The data is represented as average ± SEM (n=6) fold change over the fed controls. All data has been normalized to 18S rRNA.

P<0.05 was considered statistically significant. Groups without a common letter are significantly different. **(B) 30 hour fasting decreases expression of Abcc2-4 protein-** Crude membrane proteins were isolated from livers of C57Bl/6 or OB (n=8) mice either fed *ad libitum* or fasted for 30 hours. Protein expression of Abcc2-4 was quantified by western blotting.

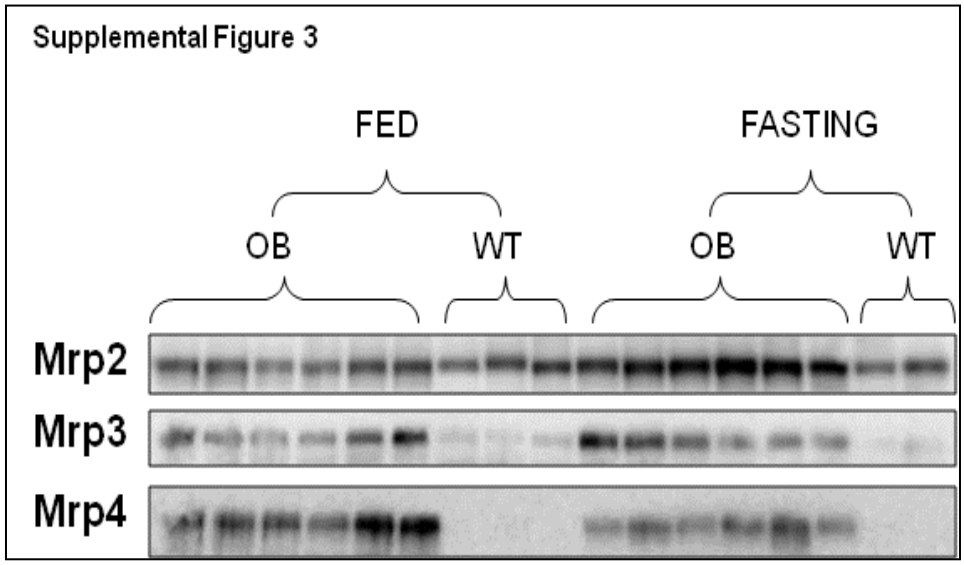


FIGURE 3: **Abcc2-4 protein expression is abolished in mouse liver after 48 hour fasting.** Adult C57Bl/6 or OB mice (n=6/group) were either fed *ad libitum* or fasted for 48 hours. Crude membrane proteins were isolated from livers of C57Bl/6 (n=8) mice either fed *ad libitum* or fasted for 48 hours. Protein expression of Abcc2-4 was quantified by western blotting.

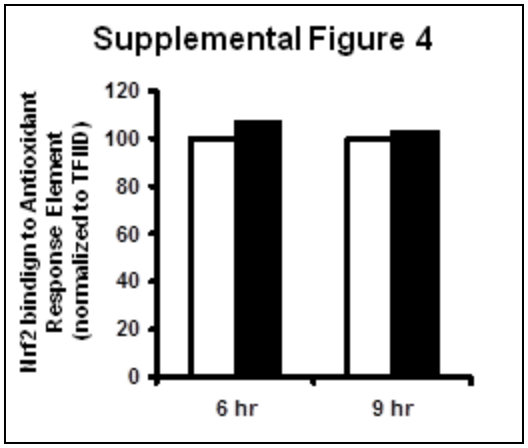


FIGURE 4: **(A) 8-Br-cAMP increases Nrf2 binding to its consensus sequence in Huh-7 cells.** Huh-7 cells were treated with1.0mM 8-Br-cAMP for 6 hours with media as the control. Liver nuclear extracts were prepared from control and treated Huh-7 cells using Procarta TF nuclear extraction kit and analyzed for Nrf2 binding using a 9 plex Procarta TF binding assay. The results obtained are represented as data normalized to TFIID. Data is expressed as Fluresence Intensity/10 µg total liver nuclear extract normalized to untreated Huh-7 cells. **(B) Time dependant mRNA expression of NQO1 in Huh-7 cells by 8-Br-cAMP-** Huh-7 cells were treated with1.0mM 8-Br-cAMP for 0, 3, 6, 12, 24 and 48 hrs with media as the control. Total RNA was isolated and mRNA expression of NQO1 was quantified using Branched DNA signal amplification assay. The data is represented as average RLU per 10µg total RNA ± SEM (n=3).

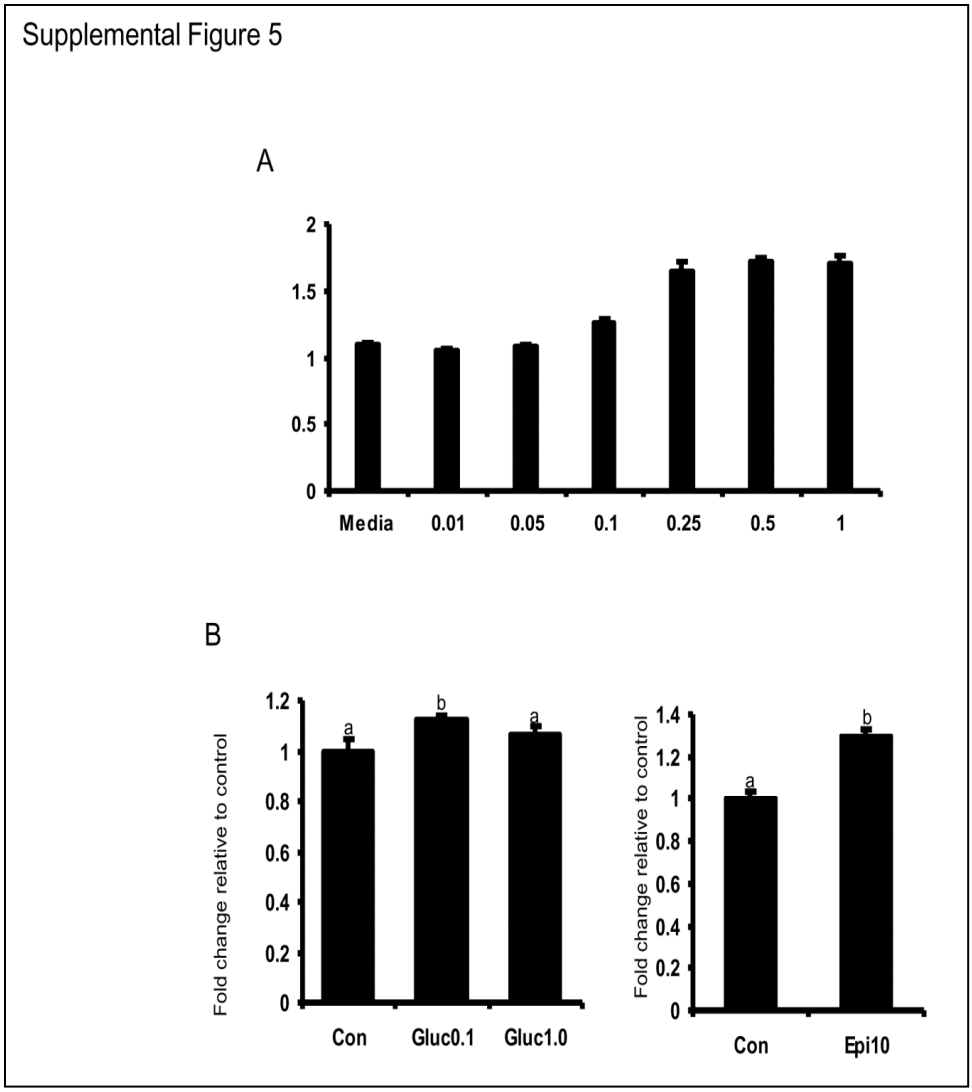


FIGURE 5: **Activation of antioxidant response element (ARE) in Huh-7 cells by (A) 8-Br-cAMP, and (B) glucagon and epinephrine.** Huh7 cells were cultured to 85% confluence and transiently transfected with Antioxidant response element –luciferase construct (100ng) and the empty vector (100ng) along with a renilla control construct (10ng) for 24 hours. The cells were then treated with 0.01- 2.0 mM 8-Br-cAMP, 0.1 μM or 1.0 μM glucagon or 10 μM epinephrine for 24 hours. The luciferase activity was measured by dual luciferase assay with renilla luciferase as a control. The

data is expressed as average fold induction in activity of the ARE-luciferase construct ± SEM (n=4).

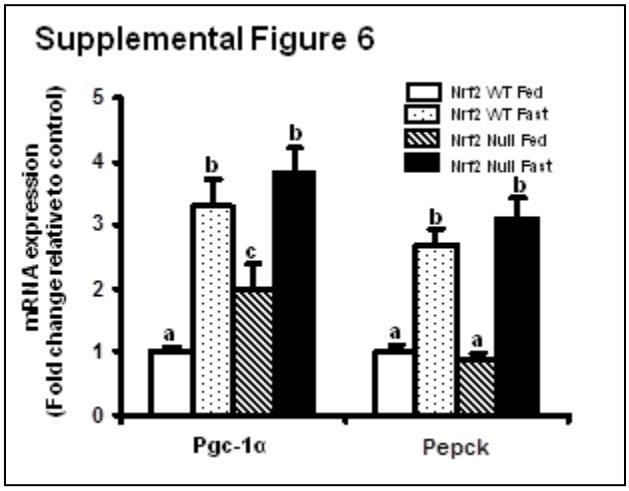


FIGURE 6: **Fasting induces Pgc-1α and pepck expression to a similar extent in Nrf2 WT and Nrf2 null mice-** Total RNA was isolated from livers of Nrf2 WT (n=6) and Nrf2 null(n=6) mice either fed ad libitum or fasted for 24 hrs. Gene expression of Pgc-1α and pepck was quantified by real time quantitative PCR. The data is represented as average ± SEM (n=6) fold change over the fed controls. All data has been normalized to 18S rRNA.

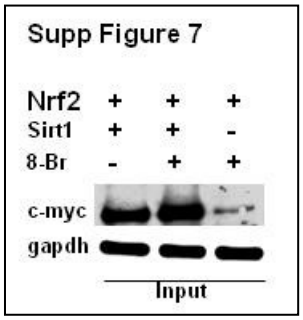


FIGURE 7: **8-Br-cAMP induces expression of Sirt1 in Huh-7 cells.** Huh-7 cells transiently transfected with (0.5-1.0μg) Flag-Pgc-1α, 75ng Flag-Nrf2 and 50ng Myc-Sirt1 expression plasmids or (50-100ng) Myc-Sirt1, 75ng Flag-Nrf2 and 0.5μg Flag-Pgc-1α expression plasmids using

Lipofectamine LTX-plus reagent (Invitrogen, CA, USA) for 24 hrs. Cells were then treated with 1.0mM 8-Br-cAMP for 24 hrs. After 24 hours, cells were lysed with lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% NP-40). 25µg protein lysate was then subject to immunoblot analysis for anti-cMyc tag protein.

TABLE I-1:

Effect of 24 hour fasting on body weight, liver-to-body weight ratios and biochemical profile: Adult C57Bl/6 and ob/ob mice (n=8/group) were fed (*ad libitum*) or fasted 24 hours. Average liver weights, liver-to-body weight ratios, serum glucose (mg/dl), serum and liver triglycerides (mg/dl) and serum NEFA (mmol/dl) were determined. $P<0.05$ was considered statistically significant. Groups without a common letter are significantly different.

Strain	Fed/ Fasted	Average liver wt	Average liver (g)/body weight (g)	Serum Glucose (mg/dl)	Serum Triglycerides (mg/dl)	Liver Triglycerides (mg/gm liver wt)	Serum NEFA (mmol/dl)	Liver NEFA (mEq/gm liver wt)
C57Bl/6	Fed	1.08 ± 0.02^{a}	0.049 ± 0.0006^{a}	166 ± 5.48^{a}	40.38 ± 6.11^{a}	39.5 ± 3.15^{a}	0.037 ± 0.002^{a}	0.040 ± 0.002^{a}
	Fasted	0.77 ± 0.01^{b}	0.042 ± 0.0004^{b}	142 ± 5.27^{b}	25.86 ± 08^{b}	60.3 ± 6.91^{b}	0.049 ± 0.003^{b}	0.039 ± 0.007^{b}
ob/ob	Fed	2.40 ± 0.06^{a}	0.062 ± 0.0009^{b}	535 ± 27.4^{c}	49.93 ± 5.49^{b}	99.2 ± 9.25^{b}	0.067 ± 0.004^{b}	0.038 ± 0.002^{b}
	Fasted	2.20 ± 0.01^{b}	0.057 ± 0.0008^{b}	195 ± 19.0^{b}	45.00 ± 2.18^{b}	125.3 ± 10.2^{b}	0.082 ± 0.006^{b}	0.047 ± 0.002^{b}

MANUSCRIPT II

To be submitted to 'Journal of Pharmacology and Experimental Therapuetics'.

Effect of Caloric Restriction and AMPK Activation on Nuclear Receptor, Biotransformation Enzyme, and Transporter Expression in Livers of Lean and Obese mice

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RUNNING TITLE: Caloric Restriction and Transporters

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Number of text pages:35

Number of tables:2

Number of Figures:8

References:

Number of words:

Abstract:250

Introduction:756

Discussion:

Non-standard Abbreviations: Abcc, ATP-Binding Cassette, sub-family C; AhR, aryl hydrocarbon receptor; AL, *ad libitum*, CR, caloric restriction, CAR, constitutive androstane receptor; Creb, cAMP response element binding protein; Cyp, Cytochrome P450; Fxr, Farnesoid x receptor; Gclc, glutamate-cysteine ligase, catalytic subunit; Gsta1, Glutathione *S*-transferase a1; Ho-1, Heme oxygenase 1; Nqo1, NADH quinone oxidoreductase; Nrf2, Nuclear

factor (erythroid-derived 2)-like 2; NR, nuclear receptor; Oatp, Organic anion transporting polypeptide (Oatp), PGC1α, peroxisome proliferator-activated receptor-γ coactivator-1α; PPAR, Peroxisome proliferator-activated receptor; Pepck, Phosphoenolpyruvate carboxykinase; PXR, Pregnane X receptor; RXR, Retinoid X receptor; Srebp-1c, Sterol regulatory element binding protein 1c; Sod1, Superoxide dismutase 1

Keywords: Transporter, Nuclear Factor E2-Related Factor 2, NFE2L2, nuclear receptor

ABSTRACT

Nuclear receptors (NR), CAR, PXR, FXR, PPARα, and Nrf2 are transcriptional regulators of transporter expression in liver in coordination with Phase-I and -II biotransformation enzymes. NAFLD markedly alters hepatic transporter expression in mice. Caloric restriction (CR) is a common regimen to treat NAFLD and proven model to activate Sirtuin1 deacetylase activity in liver conferring protection against steatosis. Purpose of this study was to expand on currently negligible information to evaluate whether CR affects hepatic transporter expression in livers from lean mice and “reverse” transporter expression changes observed with steatosis. Adult lean (C57Bl/6) and obese (ob/ob, OB) mice were fed *ad libitum* or placed on a 40% (kCal) reduced diet. CR decreased hepatic triglyceride levels in both mice, but not to the same degree in livers from OB mice. CR decreased Oatp1a1, Oatp1b2 (~90%) as well as Bsep, Bcrp (~40%) mRNA and protein expression in lean but not OB mice. CR increased Abcc2 mRNA expression by ~2 fold in OB livers only. However, protein expression increased in both lean and OB mice, reversing basal downregulation of Abcc2 in OB mice to a level comparable to lean fed controls. Steatotic livers from OB mice show increased Abcc1, 3, 4 and 5 mRNA expression. CR decreased Abcc3 protein expression in lean mice but increased it in OB livers. CR did not alter Abcc1, 4 and 5 mRNA expression in lean mice but significantly decreased their expression in OB mouse livers. Abcc4 protein expression increased in lean and OB mice upon CR. In steatotic livers from

OB mice; AhR, PXR, NFκβ, Nrf2, CREB, CAR and PPAR binding to respective RE increased by 147-275% but FXR binding decreased by 60%. This increased binding in hepatic nuclear fractions from OB mice was reversed by CR and mirrored by respective target gene expression. In summary, CR can “reverse” expression of some transporters in liver, but not all. Overall, these data indicate a potential to restore hepatic disposition changes associated with obesity through CR.

INTRODUCTION:

Non-Alcoholic Fatty Liver Disease (NAFLD) or steatosis is defined as lipid accumulation exceeding 5% by weight in hepatocytes in the absence of substantial alcohol intake (McCullough, 2006), often with increased hepatic triglyceride accumulation. Metabolic syndrome is considered to be the underlying cause of NAFLD (Browning and Horton, 2004). It is estimated that more than 86% of US adults will be overweight or obese, and more than 50% obese by the year 2030 (Wang et al., 2008). In the United States, the prevalence of NAFLD alone, or in combination with increased liver enzymes in serum, was 3.1% and 16.4% among adults, respectively (Browning et al., 2004; Lazo et al., 2011). With no intervention, NAFLD can progress to Non-alcoholic steatohepatitis (NASH), which is characterized by increased hepatic inflammation and cellular injury, and then cirrhosis, which permanently scars the liver (Angulo and Lindor, 2002).

Epidemiological and clinical studies demonstrate changed pharmacokinetic and –dynamic parameters of some drugs in obese subjects (Abernethy and Greenblatt, 1986; Fideleff et al., 2006; Jain et al., 2011). NAFLD and NASH are also associated with altered pharmacokinetics of some drugs (e.g ezetimibe, acetaminophen) as well as, altered endogenous metabolite levels, such as cholesterol and bilirubin (Barshop et al., 2011; Schrieber et al., 2011; Simonen et al., 2011; Kumar et al., 2012). For example, acetaminophen-glucuronide concentration in plasma and urine was higher in children with NAFLD (Barshop et al., 2011),

which is similar to what has been reported in db/db mice that exhibit hepatic steatosis (More et al., 2012). This likely occurs because Phase-I, -II biotransformation enzyme, and drug transporter expression is altered compared to non-steatotic livers (Slitt et al., 2003; Lickteig et al., 2007; Fisher et al., 2009; Xu et al., 2012).

Diet (caloric restriction, CR) and exercise are the recommended therapeutic intervention to treat NAFLD and reverse hepatic fat accumulation (2002; Deng et al., 2007; Larson-Meyer et al., 2008; Vuppalanchi and Chalasani, 2009; Zelber-Sagi et al., 2011). For example, a regimen of diet and exercise decreased hepatic steatosis and serum lipids in overweight subjects with NAFLD (Larson-Meyer, 2008). Also, vigorous physical activity was associated with decreased adjusted odds of having NASH in adults with NAFLD (Kistler et al., 2011). CR decreases systemic glucose levels, increases fatty acid oxidation, and decreases the fat-to-body weight ratio (Frame et al., 1998; Mahoney et al., 2006; Bruss et al., 2010).

Beneficial effects of CR are attributed to of Sirtuin 1 (Sirt1), a deacetylase that activates gluconeogenic and fatty acid oxidation gene expression via deacetylation and upregulation of Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (Pgc-1α), a transcriptional co-activator (Canto and Auwerx, 2009; Jeninga et al., 2010). The AMP-Kinase (AMPK) secondary messenger pathway is an upstream activator of the Sirt-Pgc1-α cascade during CR. AMPK activates Sirt1 deacetylase in

response to changes in redox status (NAD+/NADH ratio) of the cell (Hou et al., 2008; Lan et al., 2008; Canto et al., 2009). Steatotic livers exhibit decreased AMPK pathway activity in rodents and reversal of this activity reverses fatty liver (You et al., 2008; Ha et al., 2011; Zheng et al., 2011). Interestingly, CR alters activity and expression of of various biotransformation enzymes such as Sult2a1, Cyp2b10, Ugt1a1, CYp4a14 in liver (Zhang et al.; Maglich et al., 2004; Corton and Brown-Borg, 2005). However, to our knowledge, no studies have shown how CR actually affects drug transporter expression in livers of obese mice, which have hepatic steatosis and better mimic the population most likely to undergo intervention.

Nuclear factor-E2-related factor 2 (Nrf2) is a transcription factor, which upregulates cytoprotective mechanisms in response oxidative stress and drug-induced liver injury, via upregulation of antioxidant enzymes (e.g. Superoxide dismutase, Sod-1; Glutamate cysteine ligase catalytic subunit, Gclc), biotransformation enzymes (e.g. Glutathione S-transferase a1, Gsta1; Heme oxygenase 1, Ho-1; Nad(p)h:oxidoreductase 1, Nqo1) and ATP Binding Cassette transporters (Multidrug resistance-associated protein, Mrp2) (Itoh et al., 2003; Maher et al., 2007). Multiple studies have shown an association between Nrf2 activation and ATP Binding Cassette (Abcc) transporter induction (CITE). Moreover, Nrf2 binding and target gene expression is increased in steatotic livers (Cheng et al., 2008; Hardwick et al., 2010; Kay et al., 2011; Xu et al., 2011).

In the present study we hypothesized that CR would reverse the NR and drug transporter expression changes previously observed in obesity-induced hepatic steatosis (Cheng et al., 2008; Xu et al., 2012). Given the well-defined association of Nrf2 with Abcc transporter induction, emphasis was placed on the Nrf2 pathway, but other NRs that have been described to regulate transporter expression were also evaluated. Our data herein illustrate that CR differentially regulates NR, biotransformation enzyme, and transporter expression in livers from C57Bl/6 (WT) and $Lep^{ob/ob}$ (OB) mice. Furthermore, AMPK and Sirt1 activators differentially modulated transporter and NR expression in lean and steatotic hepatocytes.

METHODS

Animals and treatments. Mice were housed in a temperature-, light-, and humidity-controlled environment in cages with corn cob bedding. The mice were fed Harlan Teklad LM-485 Mouse/rat sterilizable diet (Harlan Laboratories, Madison, WI) *ad libitum*. Adult male C57BL/6 (WT) and $Lep^{ob/ob}$ (OB) (B6.V-Lep^{ob}/J, stock no. 0000632) mice were purchased from Jackson Labs (Bar Harbor, ME). After acclimation for 2 weeks, mice were transferred on to a purified diet (AIN93-G) obtained from Test Diet, IN, USA and allowed to acclimatize for a period of 2 weeks. The average caloric consumption was calculated for each mouse over a period of 10 days. WT (n=7) and OB mice (n=7) were fed *ad libitum* for 10 weeks. Alternatively, WT (n=10) and OB mice (n=10) were fed a 40% reduced caloric diet for 10 weeks with access to water *ad libitum*. Body weight and food consumption were monitored at least once per week for the entire study. The study was terminated when weights were constant for several weeks. The study was carried at University of Rhode Island and IACUC approved.

RNA Isolation and mRNA Quantification. Total RNA was isolated from the collected livers by phenol-chloroform extraction with RNAzol B reagent (Tel-Test Inc., Friendswood, TX) according to the manufacturer's instructions. RNA concentration was determined by measuring UV absorbance at 260 nm using NanoDrop™ and the integrity was confirmed by formaldehyde gel electrophoresis. The total RNA samples were stored at

-80°C until further use for analysis. The total RNA samples were analyzed for mRNA quantification using Branched DNA signal amplification assay (Quantigene 1.0 assay), obtained from Panomics Inc., CA, USA. RNA obtained from hepatocyte treatment was quantified using RT^2-PCR methods as described in (Xu et al., 2011)

Tissue homogenate, membrane, and cytosol preparation. Liver homogenates were obtained by homogenization (1:9, tissue:buffer ratio) with RIPA (Tris 50mM, NaCl 150 mM, SDS 0.1 % and TritonX-100 1.0%) and short centrifugation at 14000xg to remove the debris. For transporter western blots, liver membrane and cytosol fractions were obtained as previously described (Aleksunes et al., 2006; Cheng et al., 2008). Briefly, tissues were homogenized 150mM sucrose/10mM tris-HCl (ST) buffer (1:9 ratio), pH 7.5, using a Potter Elevejem motorized homogenizer. Homogenates were centrifuged at 100,000 x g for 1 hour at 4°C. The resulting supernatant (cytosolic fraction) was saved and pellets were re-suspended in ST buffer (Cheng et al., 2008; More and Slitt, 2011).

Western Blotting. 50µg protein lysates were solubilized in Laemmli buffer containing β-mercaptoethanol and electrophoretically separated by SDS-PAGE (8% for Mrps and 10% for Oatps, 12% for cytosolic, nuclear and homogenate samples) at 200V for 50 min and transferred onto a PVDF membrane at 100V for 30-45 min or at 75V for 1.5 hours. The membrane was blocked overnight with 5% Non fat dry milk (NFDM) in phosphate-buffered saline with 0.05% Tween 20 (PBS/T). After blocking, the

membrane was incubated with primary antibodies diluted in 5%NFDM in PBS/T or TBS/T for 2 hours and subsequently with corresponding horseradish peroxidase labeled secondary antibodies also diluted in 5%NFDM in PBS/T as mentioned in table 3. The blots were incubated in Pierce ECL-Plus western blot detection reagent (Thermo Fisher Scientific, Rockford, IL,USA) and exposed to X-ray film for developing and visualization. The blots were quantified using ImageQuant software (Bio-Rad, Hercules, CA). Oatp1a4 and 2b1 western blots were attempted, but not successful. Details of the antibodies used are as previously published (More and Slitt, 2011).

Transcription factor binding assay. Nuclear extracts were isolated from livers using a TF Procarta nuclear extraction kit (Panomics Inc, CA, USA) and protein concentrations were measured with BCA protein assay (Pierce, Rockford, IL, USA). The resulting fractions were checked for enrichment of nuclear proteins by western blot with Lamin B1 antibody. Nrf2, AhR, Pxr, Fxr, binding to respective consensus sequences was quantified using a Procarta TF custom array (Affymetrix, CA) using 10µg nuclear extract protein per sample according to manufacturer's instructions and published work (Xu et al., 2012). Ppar assays in the kit determine the binding of TFs to the consensus Ppar cis-response elements and does not differentiate between DNA binding of Pparα from that of other Ppar family members, including Pparβ and Pparγ. Hence, data for Ppar binding has not been

presented. Samples were analyzed using a Luminex Bio-Plex 200 array reader with Luminex 100 xMAP technology, and data were acquired using Bio-Plex Manager software (version 5.0). Data was acquired by a Luminex Bio-plex^{TM} 200 Array reader with Luminex 100 X-MAP technology, and data were acquired using Bio-Plex Data Manager Software Version 5.0 (Bio-Rad). All data was normalized to TFIID binding activity.

Primary mouse hepatocyte isolation. Primary mouse hepatocytes were obtained from adult WT and OB mice livers using a two-step collagenase perfusion; 1x10^6 cells/well in 2 mL completed medium (MEM supplied with 10% FBS) were seeded on collagen-coated 6-well plates. After cell attachment (~4 h), they were cultured in serum-free MEM containing 1% ITS supplement (Invitrogen, CA). Approximately 24 hours post-plating, hepatocytes were treated with control (media or 0.01% DMSO), AICAR (0.5mM), NAD+ (5mM) or metformin (1mM) for 6 hours. Total RNA was isolated from TRIzol reagent (Invitrogen, CA) according to the manufacturer's instructions.

Heatmap and cluster analysis. mRNA expression of genes quantified *in vivo* and *in vitro* were plotted on a heatmap using the R software environment (R Foundation for Statistical Computing, Vienna, Austria). The genes in WT and OB CR groups were normalized to their respective ad

libitum controls and the fold changes relative to control were used for heatmap analysis.

Statistics. The statistical significance of differences was determined by Duncan's factorial ANOVA test. Letters different from each other represent a statistical difference between groups ($p \leq 0.05$).

RESULTS

CR decreases body weight, hepatic lipid and glutathione content in WT and OB mice. At 9 weeks, CR decreased average body weight by 30% and 12% in WT and OB mice, respectively (Fig. 1A). CR decreased the average liver weight and liver-to-body weight ratio in both genotypes, with a greater decrease in WT mice (Table 1). CR also decreased serum TG (50% in WT and 11% in OB), serum glucose (50% in WT and 20% in OB), and increased Non-esterified fatty acid (NEFA) levels (1.6 fold in WT and 2 fold in OB). Additionally, CR decreased micro and macrovesicular vacuolation in both WT and OB mice, but more so in WT mice. Similarly, glucagon expression was stimulated in OB mice undergoing CR (Table 2). CR significantly decreased serum insulin levels in both WT and OB mice (80% and 40% respectively), and leptin levels which were undetectable in OB mouse serum. CR decreased hepatic glutathione (GSH) by 60% in WT and 30% in OB mice (Fig. 1C). Overall, OB mice were relatively more resistant to CR than WT mice with regard to weight loss and reversal of steatosis, which is consistent with a previous observation (Sloan et al., 2011).

CR increases expression of gluconeogenic genes and gluconeogenic hormones, decreases expression of lipogenic genes and insulin levels. CR induces glucagon secretion to increase mitochondrial biogenesis and upregulate glucose production. This well characterized

response results in the induction of genes needed to increase gluconeogenesis (Bordone and Guarente, 2005). Therefore, genes known to be regulated by CR were measured to further confirm response to the food restriction. CR increased expression of gluconeogenic genes Pgc-1α and Pepck in WT mouse livers (2 and 3.5 fold, respectively), however this increase was blunted in OB mouse livers (Fig. 1D), which has been previously described (ref). Conversely, mRNA expression of the lipogenic master regulator, Srebp-1c, was decreased in WT mouse livers but remained unchanged in OB mouse liver after CR (Fig. 1D).

Effect of CR on Phase I biotransformation enzyme expression in livers of WT and OB mice. Phase I biotransformation enzymes are typically downstream target genes for NRs (Waxman, 1999; Xu et al., 2005) and are often measured as indirect markers of NR activation/repression. Therefore, several Cyps described to be regulated via NR activation were measured in WT and OB mice fed AL or after CR. Consistent with previous observations (Cheng et al., 2008), Cyp2b10, Cyp3a11 and Cyp4a14 basal expression was higher in OB mice compared to WT mice (Fig. 2A). In contrast, Cyp7a1 and Cyp7b1 basal expression was lower in OB mouse livers as compared to WT mice (Fig. 2A). CR increased expression of Cyp2b10 and Cyp4a14 mRNA expression in WT but to a lower extent in OB mouse livers, whereas Cyp3a11 mRNA expression remained unchanged. In contrast, CR

significantly decreased expression of Cyp7b1 in both WT and OB mouse livers (Fig. 2A). CR did not affect Cyp7a1 mRNA expression in WT mice, but increased Cy7a1 mRNA expression in livers from OB mice (Fig. 2A).

Effect of CR on Antioxidant and Phase II biotransformation enzyme expression in livers of WT and OB mice. Previous work by our group and others have shown that Nrf2 and Nrf2 target gene expression is increased in livers of OB mice (Hardwick et al., 2010; Xu et al., 2012). Compared to WT, OB mouse livers had increased Gclc, Gsta1, Nqo1, and Ho-1 mRNA expression (Fig. 2B). Consistent with hepatic GSH concentrations, CR decreased expression of Gclc in both WT and OB mouse livers (Fig. 2B). CR significantly increased Nqo1, Sod1 and Gsta1 in livers of WT mice. In livers from OB mice, CR decreased Gsta1 expression, but not Nqo1 and Sod1 expression (Fig. 2B). Ho-1 expression was equivalent in livers of WT mice fed ad libitum or on CR, whereas CR decreased Ho-1 by about 50% in livers of OB mice.

Effect of CR on Oatp expression in livers of WT and OB mice. Consistent with previous observations , Oatp1a1 and 1a4 expression was lower in livers of WT than OB mice and Oatp1b2 expression was comparable between the two genotypes (Fig. 3A). CR decreased Oatp1a1 and 1b2 mRNA, but not 1a4 mRNA expression in livers of WT mice (Fig. 3A) (Zhang et al., 2010). Oatp1a1 and 1b2 expression remained

suppressed in livers OB mice even after CR. Lastly, Oatp1a1 protein expression was relatively undetectable in livers from OB mice, consistent with Cheng et al., 2008. Oatp1a1 protein expression was undetectable in livers from WT and OB mice after CR (Fig. 3B).

Effect of CR on Abc transporter expression in livers of WT and OB mice. OB mice had lower Abcb11 and Abcc2 expression and equivalent Abcg2 expression compared to livers from *ad libitum* fed WT mice (Fig. 4A and 4B). As depicted in Fig. 4A, CR decreased mRNA and protein expression of Abcb11 and Abcg2 in livers from WT mice, but not in the OB mice (Fig. 4A and 4B). On the other hand, Abcc2 expression was similar in WT mice fed ad libitum or under CR, whereas CR increased Abcc2 mRNA expression (Fig. 4A). CR increased Abcc2 protein expression in both WT and OB livers.

Abcc1, 3-6 mRNA expression was increased in livers of OB compared to *ad libitum* fed WT mice (Fig. 4A), consistent with previous reports (Cheng et al., 2008; Hardwick et al., 2011). In WT mouse livers, Abcc3 mRNA and protein expression decreased upon CR, with no observable change in Abcc1 and 5 mRNA expression (Fig. 4A and 4B). Conversely, Abcc4 mRNA was unaltered, but protein expression increased upon CR in WT mouse livers (Fig. 4A and 4B). CR decreased Abcc1 and 5 expression in OB mouse livers. Abcc3 mRNA expression decreased upon CR, however this change did not translate into decreased protein

expression and Abcc4 mRNA and protein expression remained unchanged in OB mouse livers upon CR (Fig. 4A and 4B). CR did not alter Abcc6 expression in either WT or OB mice.

Transcription factor binding activity in WT and OB mouse livers upon CR. Transcription factor binding to prototypical consensus sequences was also determined in nuclear fractions isolated from liver (Fig. 5). Ahr, Creb, and Pxr binding was increased in liver nuclear fractions from OB mice compared to WT mice. In WT mice, CR slightly decreased Ahr, Fxr, and Nrf2 binding and did not affect Creb or PXR binding. In OB mice, CR markedly decreased AhR, Creb, and Nrf2 binding.

To determine whether prototypical targets of these transcription factors were affected similarly by CR and the potential differences between the WT and OB livers on CR, simples heatmap was created (Fig. 5B). As seen from the heatmap created, the prototypical target genes of Car, Ahr, Fxr, Pxr and Nrf2 were regulated similarly in WT livers. However, in the OB livers, effect of CR was not consistent amongst the prototypical targets of transcription factors such as Car, while in other cases the target gene expression was opposite in OB livers as compared to WT such as in case of Nrf2. These observations are consistent with the transcription factor binding data as seen in (Fig. 5A).

Effect of AMPK and Sirt1 activators on Abcc and NR induction in hepatocytes obtained from WT and OB mice. AMPKinase pathway is an important CR signal transduction pathway upstream of transcription factor activation. Hence, primary mouse hepatocytes were isolated from 11-week old WT and OB mice and treated with pharmacological Ampk (AICAR) and Sirt1 (NAD+) activators (Figure 6). Abcc2 and 3 mRNA expression was increased about 3 fold in hepatocytes isolated from OB mice compared to WT mice (Fig. 3A), which is similar to what has been observed in intact livers from WT and OB mice of the same age (Cheng et al., 2008). Treatment with AMPK activators (AICAR, NAD+) significantly increased mRNA expression of Abcc2 and Abcc3 in WT mouse hepatocytes (Fig. 6A). As opposed to lean hepatocytes, AICAR and NAD+ treatment decreased Abcc2 and 3 mRNA expression in steatotic hepatocytes, thus reversing their expression back to normal levels. The effect of AICAR and NAD+ treatment on NR expression was also evaluated (Fig. 6B). Car mRNA expression was increased about 50% above control in hepatocytes isolated from OB mice compared to WT mice (Fig. 3A), which is similar to what was observed in intact livers from WT and OB mice of the same age (Xu et al., 2012). Fxr, Nrf2, and Pxr expression was equivocal between WT and OB mice, which is different from what was observed in intact livers from WT and OB mice of the same age (Xu et al., 2012). AICAR and NAD+ treatment increased CAR, decreased Nrf2, and did not change PXR expression in WT hepatocytes. AICAR and NAD+ treatment increased

CAR, decreased Nrf2, and did not change PXR expression in WT hepatocytes. FXR was decreased by AICAR, but not NAD+. In OB hepatocytes, CAR expression was not affected by either treatment, AICAR decreased FXR, but NAD+ did not. AICAR and NAD+ treatment decreased Nrf2 mRNA expression. PXR expression was decreased by AICAR, but not NAD+ treatment.

DISCUSSION

The following study demonstrates that CR affected the expression of drug transporters in conjunction with alterations in NR and NR-target gene expression in conjunction with weight loss and decreased liver lipid content. As CR is known to activate Ampk and Sirt1 dependent pathways, the work herein demonstrated that AICAR and NAD+ can modulate transporter and NR expression in hepatocytes from WT and OB mice. Treatment with AICAR and NAD+ indicated that AMPK and Sirt1 are likley upstream regulators of NR and Abcc transporter expression. Lastly, our work illustrates that CR produced differential gene expression patterns in the lean or obese condition for both hepatocytes and in mice.

Current study demonstrates that CR affects expression of some Phase-I, -II, and transporters in liver. In lean mice, mRNA expression of Cyp2b10, Cyp4a14 increased (as previously published by (Corton et al., 2004; Zhang et al., 2010)) whereas Cyp7b1 decreased while Cyp7a1 and CYp3a11 remain unchanged. mRNA expression of phase II enzymes, Gsta-1, Nqo1 and Sod1 increased while that of Gclc decreased and Ho1 mRNA expression remained unchanged. CR decreased mRNA expression of Oatp1a1, Oapt1a4 and Oatp1b2 with proteins following a similar trend as mRNA. Our observations in part agree with previously published data by (Zhang et al., 2010). mRNA expression of efflux transporters of the ABC family did not follow a specific pattern. While Abcb4 and Abcc4 mRNA

expression increased upon CR, with Abcc2 expression showing an increasing trend. Abcb11, Abcc3, Abcg2 expression decreased and Abcc1, Abcc5 and Abcc6 expression remained unchanged in the lean mice upon CR. On the other hand, Abcc2 protein expression demonstrated an increase along with Abcc4 in the lean CR livers. Abcc3 and Abcg2 protein expression mirrored the mRNA expression showing a decrease in expression upon CR.

In OB mouse livers, CR did not cause significant changes in mRNA expression of Phase-I enzymes as compared to lean livers. Cyp4a14 and Cyp7a1 mRNA expression increased while Cy2b10, Cyp3a11 remained unchanged and Cyp7b1 mRNA expression decreased upon CR. In obese CR livers, Gsta-1, Gclc, Ho1 mRNA expression decreased while Nqo1 and Sod1 expression remained unchanged. mRNA and protein expression of Oatp1a1, Oatp1a4 and Oatp1b2 remained unchanged in the obese livers upon CR. CR did not change mRNA expression of Abcb4, Abcb11, Abcc4 and Abcc6, whereas it decreased the expression of Abcc1, Abcc3, Abcc5, Abcg2 and resulted in an increased mRNA expression of Abcc2. Protein expression in these livers followed the mRNA expression pattern; Abcc2 with an increase, Abcc4 with no change and Abcg2 with a decrease in expression respectively while Abcc3 protein expression remained unchanged although mRNA expression demonstrated a decrease. Observations with transporter expression indicate towards a complex upstream multiple pathway regulation in models of CR.

Biotransformation enzymes and transporters are regulated by various nuclear receptors and transcription factors such as Pxr, Fxr, Car, Nrf2, Ahr (Xu et al., 2005). To identify hierarchal relationhip and co-ordinate regulation between different drug metabolizing enzymes, transporters and nuclear receptors, preliminary cluster analysis on mRNA expression obtained from *in vivo* and *in vitro* studies was performed. CR had a differential effect on expression of many genes of interest in the OB mice as compared to the WT mouse livers. However, in both OB and WT mice, genes of interest clustered in similar groups indicating that absence of leptin interferes with the effects of CR. As seen in Fig. 8, genes that were similarly regulated in both WT and OB mouse livers included Pparα, Car, Nrf1, Pepck, Sod1, Cyp2b10, Cyp3a11, Cyp4a14, Cyp7a1, Oatp1a4, Abcc2, Abcb4. mRNA expression of Abcc5, Ho1, Abcc6, Keap1, Gsta1, Nqo1, Pgc-1α were positively upregulated in the WT mouse livers by CR, were either unchanged in OB mice or decreased in expression. On the other hand CR decreased Nrf2, Abcb11, Oatp1b2, Fabp, shp, Hnf1α, Cyp1a1, Gclc, Abcg2, Abcc3, lrh, Srebp1, Abcc1, Cyp7b1, Oatp1a1 in the WT mouse livers but increased or remained unchanged in expression in the OB mouse livers.

CR induced activation of Transcriptional Pathways regulating transporter expression:

Activation of metabolic pathways during CR is mediated physiologically by various cytokines and hormones; leptin, insulin, glucagon being considered pivotal in relaying important intracellular signals. CR is known to increase serum glucagon, decrease insulin as well as reverse the increases in leptin levels (Takemori et al., 2011). AMPK pathway is regarded as one of the major secondary pathways delegating intracellular signals of CR, inhibiting gluconeogenic pathway activity and increasing fatty acid oxidation as well as glucose uptake into cells. Pparα and γ regulate pathways responsible for fatty acid oxidation and synthesis respectively (Tontonoz and Spiegelman, 2008; Pyper et al., 2010). PPAR alpha is activated in models of CR via ligand dependant and independent activation by Pgc-1 α via Protein Kinase A and AMPKinase secondary messenger pathways (Rodgers et al., 2008). Higher expression of Pparα in steatotic livers could be a result of fatty acid- ligand dependant activation of Pparα. Previous studies have demonstrated Pparα to be the downstream target of AMPK activation via leptin (Lee et al., 2002; Suzuki et al., 2007). In the study herein, CR likely activated Pparα as observed by increased mRNA expression of Pparα target gene Cyp4a14 in WT mouse liver along with an important coactivator Pgc-1α (Maglich et al., 2004; Corton and Brown-Borg, 2005). With leptin signaling mutated in livers of OB mice, the signal transduction is hampered and hence Cyp4a14, Pcg-1α (Fig. 1C and 2A) were not activated in these livers. Consistent with these observations, CR increased Abcc4 expression in livers of wild type mice indicating Pparα as

an upstream regulator of Abcc4 expression during CR. Endogenous metabolites such as cAMP and cGMP being substrates for Abcc3 and Abcc4 (Chandra et al., 2005; Sassi et al., 2008), the increase in expression of Abcc4 could attributed to increased concentrations of these substrates owing to activation of PKA and AMPK pathways during CR (Jeninga et al., 2010). In steatotic OB livers, higher fold activation of Pparα does not translate to increased Abcc4 activation upon CR which may be due to inefficient response in these livers to activation of fatty acid oxidation pathways, indicating as mentioned earlier a resistance to CR induced weight loss and steatosis reversal.

Apart from metabolic pathways, activation of Pgc-1α during CR marks an important step in mediating biotransformation pathways too. Pgc-1α is a known co-activator of Car (Ding et al., 2006), Fxr (Zhang et al., 2004), Hnf4α (Louet et al., 2002), Pxr (Eloranta et al., 2005) pathways. In WT mice, CR induced mRNA expression of Car, Cyp2b10. Car is known to activate Abcc2 by certain xenobiotic compounds (Aleksunes and Klaassen, 2012). As observed in Fig. 4A, Abcc2 expression follows a pattern similar to Car and Cyp2b10 as well as gets clustered with Car in the hierarchal tree (Fig. XX). Similarly, Pparα, Car, Nrf1, Pepck, Sod1, Cyp2b10, Cyp3a11, Cyp4a14, Cyp7a1, Oatp1a4, Abcc2 all cluster together indicating that AMPK-Pgc-1α-Pparα cascade activation occurs upstream of Car, Pxr and Fxr activation. Our observations in WT mice reflect previously published work (Zhang et al., 2010).

Nrf2-ARE pathway is an upstream regulator of various Phase-II metabolizing enzymes as well as Abc transporters along with a battery of genes responsive to oxidative stress. It has been previously reported that CR restriction induces Nrf2 expression (Pearson et al., 2008). The observations made in the above study differed from previously made observations by , but in agreement with recently published data by (Zhang et al., 2010). Along with mRNA, binding of Nrf2 decreased in both WT and OB mouse livers correlates with the decrease in liver triglycerides, NEFA, and an overall decrease in steatosis. Genes which clustered together with Nrf2 include Abcb11, Oatp1b2, Hnf1α in WT mouse livers. Previous reports indicate that, in some models Nrf2 is a positive regulator of Abcb11 (Xu et al., 2010), interacts with Hnf1α and also regulates Oatp1b2 (Tanaka et al., 2009). Nrf2 also interacts with Ahr (Wakabayashi et al., 2010) increasing Ahr activity and target gene expression. Ahr binding co-ordinately decreases with Nrf2 binding both WT and OB livers along with decrease in Cyp1a1 expression, indicating that Nrf2-Ahr cascade regulates the expression of genes clustered together with Nrf2.

From correlation studies and the heatmap analyses, it is clear that effect of CR on WT and OB livers is different. Model of steatotic liver used in this study being the $Lep^{ob/ob}$, leptin deficient mice, importance of leptin axis in regulating downstream effects of CR on expression of transcription factors and drug processing genes is evident. Serum leptin along with adiponectin levels are known to correlate with the severity of steatosis (Lebensztejn et

al., 2009; Ayonrinde et al., 2011). Hepatic and central leptin resistance is known to cause insulin resistance and obesity (Scarpace and Zhang, 2009). CR and exercise being AASLD recommended regimen to reverse NAFLD, the observations with leptin deficient models demonstrates a potential difference in the steatotic human population response to drug metabolizing enzyme and transporter changes upon CR. While fat content of the liver significantly decreases in the steatotic liver upon CR, the biotransformational pathways are not completely reversed in these models. With a significant portion of obese-steatotic population undergoing CR the altered metabolic pathways may indicate an altered vectorial disposition or/and pharmacokinetics of drugs, endogenous metabolites and environmental toxicants. Endogenous metabolites such as glucuronide conjugates of bile acids, bilirubin, cAMP/cGMP, glutathione conjugates and lipid peroxidation products such as arachidonic acid form substrates for the ABC transporters (Klaassen and Aleksunes, 2010). While CR in WT/lean liver alters the expression of these transporters to potentially accommodate the metabolite concentrations in the hepatocytes, OB/steatotic livers do not demonstrate expression changes. Inability to achieve these transporter changes could indicate towards increased levels of metabolites in the hepatocyte which could have adverse outcomes. As an example, previous studies have published an increase in serum bilurubin levels and bile acid flow upon CR (Ferland et al., 1989; De Guzman et al., 2012). Bilirubin and bile acid conjugates form substrates for Abcc2. In WT livers CR while there

in an increase in Abcc2 expression, there is no change in Abcc2 expression in the OB livers indicating a potentially higher concentration of these metabolites in the liver. Unchanged Abcc2 and Abcg2 expression may indicate towards a higher bile acid level in livers of OB mice, which potentially indicates an aberrant vectorial disposition of these substrates. Inability of steatotic liver to reverse Abcc2 and Abcg2 expression upon CR may have important consequences on disposition and accumulation of drugs and xenobiotics transported by these membrane proteins.

All the above observations indicate that activation of CR pathways can only partly reverse the changes in drug metabolizing enzyme and transporter expression occurring due to fat deposition in the liver. Leptin appears to play an important role in upstream regulation of pathways that relay CR signals to ultimately change DME and DT expression. The above observations also indicate that the inability of CR to reverse certain DME and DT changes may indicate towards altered disposition and pharmacokinetics of metabolites and xenobiotics.

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FIGURES LEGENDS

FIGURE II-1: ***Effect of 40% kCAL reduced calorie diet in WT (C57Bl/6) and Lep$^{ob/ob}$ (OB) mice.*** (A) Decreased body weight and percent body weight in WT and OB mice after CR. During 10 weeks of 40% reduced caloric diet (CR), body weight of *ad libitum* fed (n=7) and CR (n=10) mice (both WT and OB) was determined every week. The percent body weight changes were calculated with the body weight at initial time point considered as 100% and each mouse being its own control. The data is represented as average ± SEM (n=7 for controls and n=10 for CR) body weight or percent body weight change over the weeks of CR. (B) Morphological changes in livers of WT and OB mice upon CR. Adult WT and OB mice either fed *ad libitum* (n=7) or 40% reduced caloric diet (CR) (n=10) for 10 weeks. Livers were collected, fixed in 10% buffered formalin, paraffin embedded, sectioned, and stained with hematoxylin and Eosin. (C) Induction of Pgc-1α, pepck, Pparα, Car and downregulation of Srebp-1 mRNA expression in mouse liver after 10 weeks CR. Total RNA was isolated from livers of *ad libitum* (AL) fed controls and mice undergoing CR and gene expression of Pgc-1α, pepck, Pparα, Car and Srebp-1 was quantified by Branched DNA signal amplification assay (Quantigene 1.0 assay), obtained from Affymetrix Inc., CA, USA. The data is represented as average ± SEM (n=7 for AL and n=10 for CR) fold change over the fed controls. $P<0.05$ was considered statistically significant. Groups without a common letter are significantly different. (D) Effect of CR on hepatic

glutathione levels in WT and OB mice. Hepatic glutathione levels were measured in livers of WT and OB AL and CR mice using the GSH-Glo™ kit (Promega Corp., MD, USA) according to manufacturer's protocol. The data is represented as average ± SEM (n=7 for AL and n=10 for CR) uM GSH / mg liver weight. $P<0.05$ was considered statistically significant. Groups without a common letter are significantly different.

FIGURE II-2: ***Effect of CR on expression of certain Phase I and II metabolizing enzymes in WT and OB mouse livers.*** **(A) Changes in expression of Phase I metabolizing enzymes in WT and OB mouse livers upon CR**. Total RNA was isolated from livers of *ad libitum* (AL) fed controls and mice undergoing CR and gene expression of Cyp2b10, Cyp3a11, Cyp4a14, Cyp7a1, Cyp7b1 was quantified by Branched DNA signal amplification assay (Quantigene 1.0 assay), obtained from Affymetrix Inc., CA, USA. The data is represented as average ± SEM (n=7 for AL and n=10 for CR) fold change over the fed controls. $P<0.05$ was considered statistically significant. Groups without a common letter are significantly different. **(B) Changes in expression of Phase II metabolizing enzymes in WT and OB mouse livers upon CR**. Total RNA was isolated from livers of *ad libitum* (AL) fed controls and mice undergoing CR and gene expression of Gclc, Gsta-1, Ho1, Nqo1, Sod1 was quantified by Branched DNA signal amplification assay (Quantigene 1.0 assay), obtained from Affymetrix Inc., CA, USA. The data is represented as average ± SEM (n=7

for AL and n=10 for CR) fold change over the fed controls. P<0.05 was considered statistically significant. Groups without a common letter are significantly different

FIGURE II-3: ***Effect of CR on liver uptake transporter (A) mRNA and (B) protein expression in WT and OB mice.*** Total RNA was isolated from livers of *ad libitum* (AL) fed controls and mice undergoing CR and gene expression of Oatp1a1, Oatp1a4 and Oatp1b2 was quantified by Branched DNA signal amplification assay (Quantigene 1.0 assay), obtained from Affymetrix Inc., CA, USA. The data is represented as average ± SEM (n=7 for AL and n=10 for CR) fold change over the fed controls. P<0.05 was considered statistically significant. Groups without a common letter are significantly different. Membrane proteins were isolated from livers of AL and CR WT and OB mice. Protein expression of Oatp1a1, Oatp1b2 was quantified by western blotting. The data is representative of three individual protein quantifications.

FIGURE II-4: ***Effect of CR on liver efflux transporters mRNA and protein expression in WT and OB mice.*** Total RNA was isolated from livers of *ad libitum* (AL) fed controls and mice undergoing CR and gene expression of Abcb4, Abcb11, Abcc1-6, Abcg2 was quantified by Branched DNA signal amplification assay (Quantigene 1.0 assay), obtained from Affymetrix Inc., CA, USA. The data is represented as average ± SEM (n=7

for AL and n=10 for CR) fold change over the fed controls. P<0.05 was considered statistically significant. Groups without a common letter are significantly different. Membrane proteins were isolated from livers of AL and CR WT and OB mice. Protein expression of Abcc2-4 and Abcg2 was quantified by western blotting. The data is representative of three individual protein quantifications.

FIGURE II-5: ***Effect of CR on (A) transcription factor activity and (B) relative expression patterns of prototypical target genes of transcription factors in livers of WT and OB mice*** **. (A) CR changes the transcription factor binding activity in livers of WT and OB mice.** Liver nuclear extracts were prepared from control and fasted WT and obese mice using Procarta TF nuclear extraction kit and analysis for Ahr, Creb, Fxr, Nrf2 and PXr binding was determined using a 9 plex Procarta TF binding assay. The data is represented as average ± SEM (n=7 for AL and n=10 for CR) fold change over the fed controls. P<0.05 was considered statistically significant. Groups without a common letter are significantly different. **(B) CR differentially affects expression of prototypical target genes of transcription factors in WT and OB mouse livers.** Heatmaps were created for visual pattern analysis of Cyp2b10, Cyp3a11, Cyp4a14, Cyp7a1, Oatp1a1,Oatp1a4, Abcc1-6, Abcg2, Nqo1, Ho1, Gclc, Gcta1, Sod1 in both WT and OB livers upon CR using R statistical software. The gene

expression was analysed as fold change of WT and OB CR mice over respective AL groups.

FIGURE II-6: ***Effect of AMPK pathway activation on transcription factor expression in WT and OB hepatocytes.*** **(A) mRNA quantification of transcription factor expression.** Primary hepatocytes were obtained from (male, 11 weeks old) WT and OB mouse livers. Primary mouse hepatocytes were treated with control, AICAR (0.5mM), NAD+ (5mM) or metformin (1mM) for 6 hours. Total RNA was isolated 6 hours after treatment and mRNA expression of Car, Fxr, Nrf2, and Pxr was quantified using real time quantitative PCR. The data is represented as average ± SEM (n=3) fold change over the fed controls. All data is normalized to 18S rRNA. Groups without a common letter are significantly different. **(B) AMPK activators differentially alter mRNA expression of various transcription factors in WT and OB mice.** Heatmaps were created for visual pattern analysis of Nqo1, Gclc, Abcc2, Abcc3, Nrf2, Car, Pxr and Fxr in both WT and OB livers upon CR using R statistical software. The gene expression was analysed as fold change of WT and OB CR mice over respective AL groups.

FIGURE II-7: ***Activation of CR pathways in part reverses drug metabolizing enzyme and transporter changes caused due to steatosis.*** Caloric restriction via activation of AMPK pathway alters

expression and activity of Car, Nrf2, Fxr while Pxr activity remains unaffected. These changes in transcription factor activity changes the expression of various Phase I and II biotransformation enzymes and transporters. Steatotic liver demonstrates an altered transcription factor activity which upon CR is only partly reversed as seen in B.

FIGURE II-1:

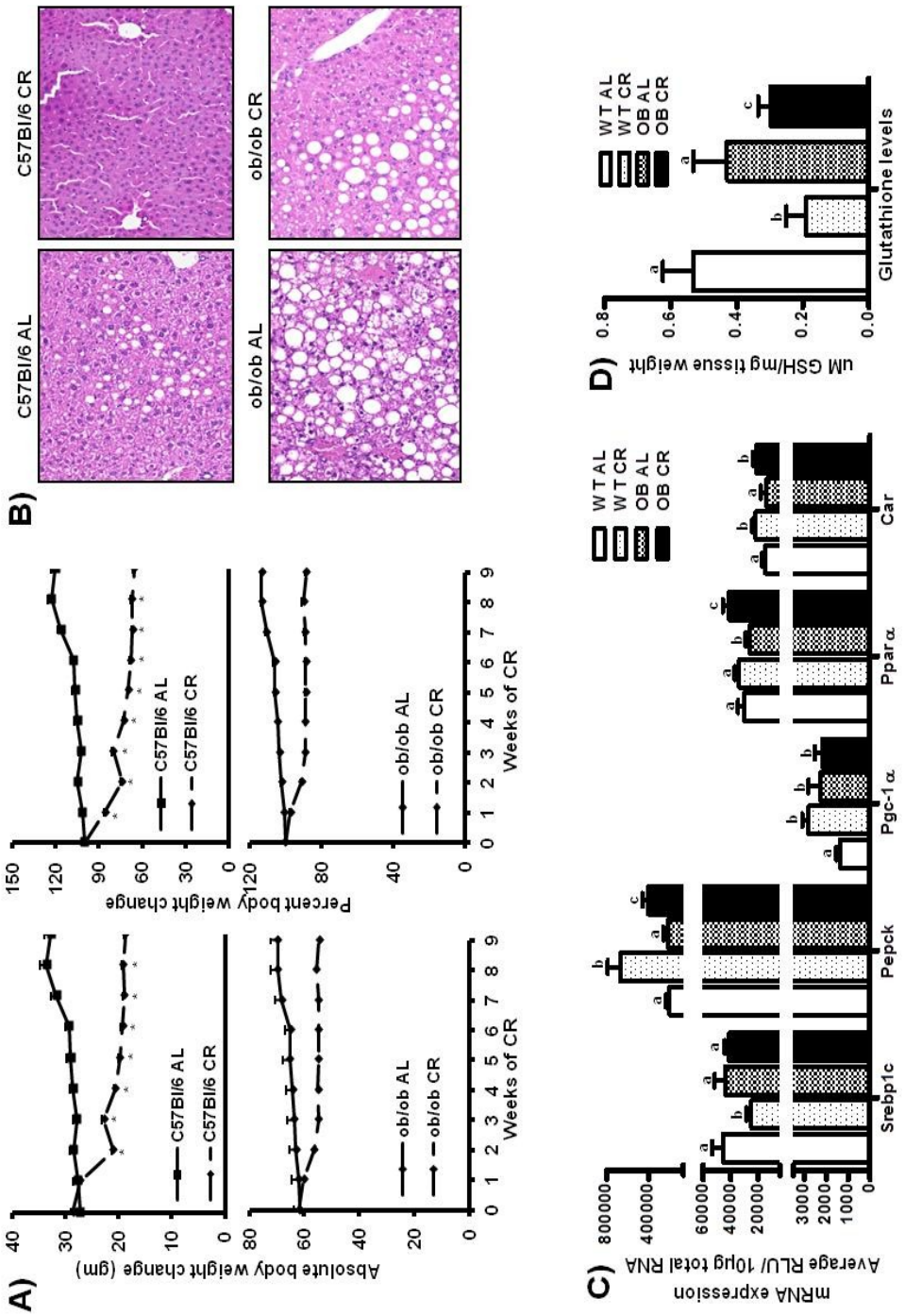

A)
Absolute body weight change (gm)
Percent body weight change
C57Bl/6 AL
C57Bl/6 CR
ob/ob AL
ob/ob CR
Weeks of CR
B)
C57Bl/6 AL
C57Bl/6 CR
ob/ob AL
ob/ob CR
C)
mRNA expression
Average RLU/ 10µg total RNA
WT AL
WT CR
OB AL
OB CR
Srebp1c
Pepck
Pgc-1α
Pparα
Car
D)
uM GSH/mg tissue weight
Glutathione levels

FIGURE II-2:

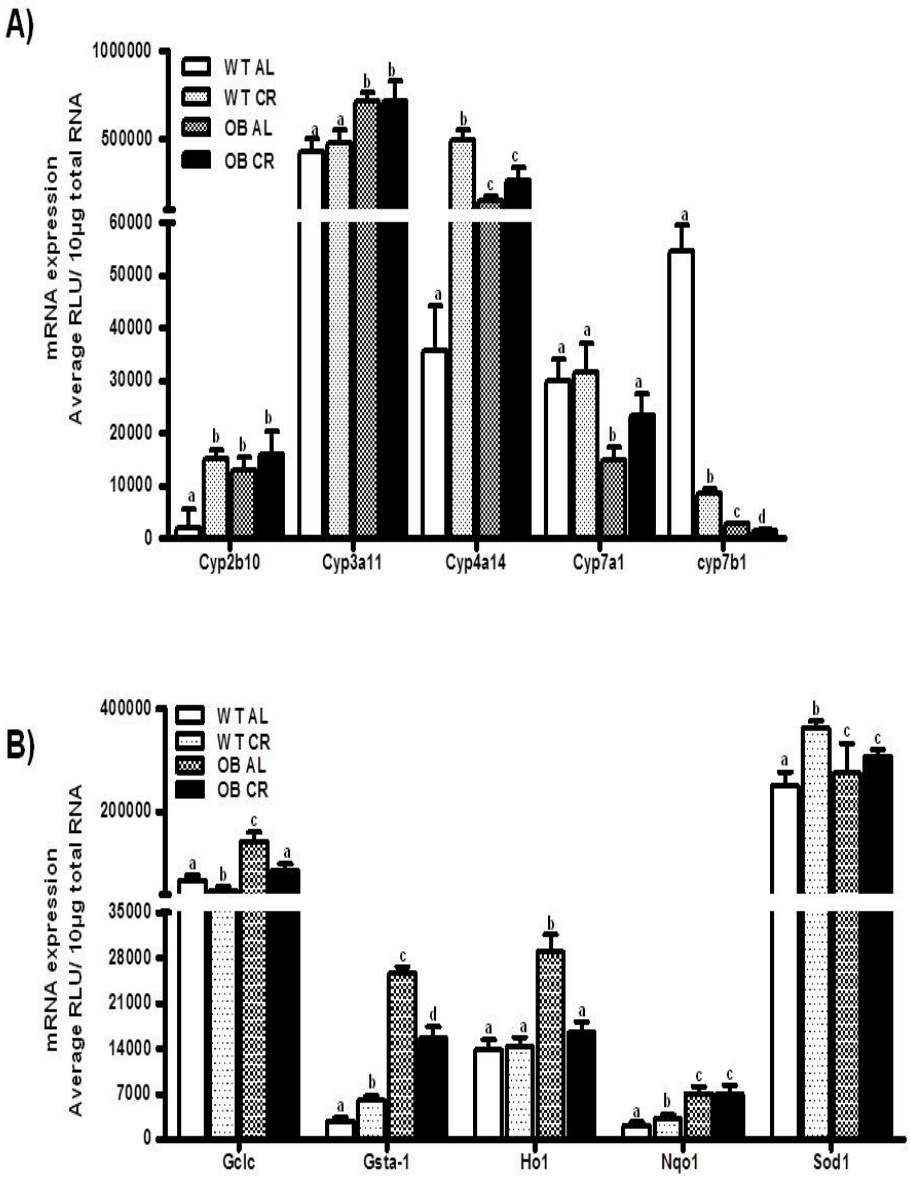

A)
mRNA expression
Average RLU/ 10µg total RNA
WT AL
WT CR
OB AL
OB CR
1000000
500000
60000
50000
40000
30000
20000
10000
0
Cyp2b10
Cyp3a11
Cyp4a14
Cyp7a1
cyp7b1
B)
mRNA expression
Average RLU/ 10µg total RNA
WT AL
WT CR
OB AL
OB CR
400000
200000
35000
28000
21000
14000
7000
0
Gclc
Gsta-1
Ho1
Nqo1
Sod1

FIGURE II-3:

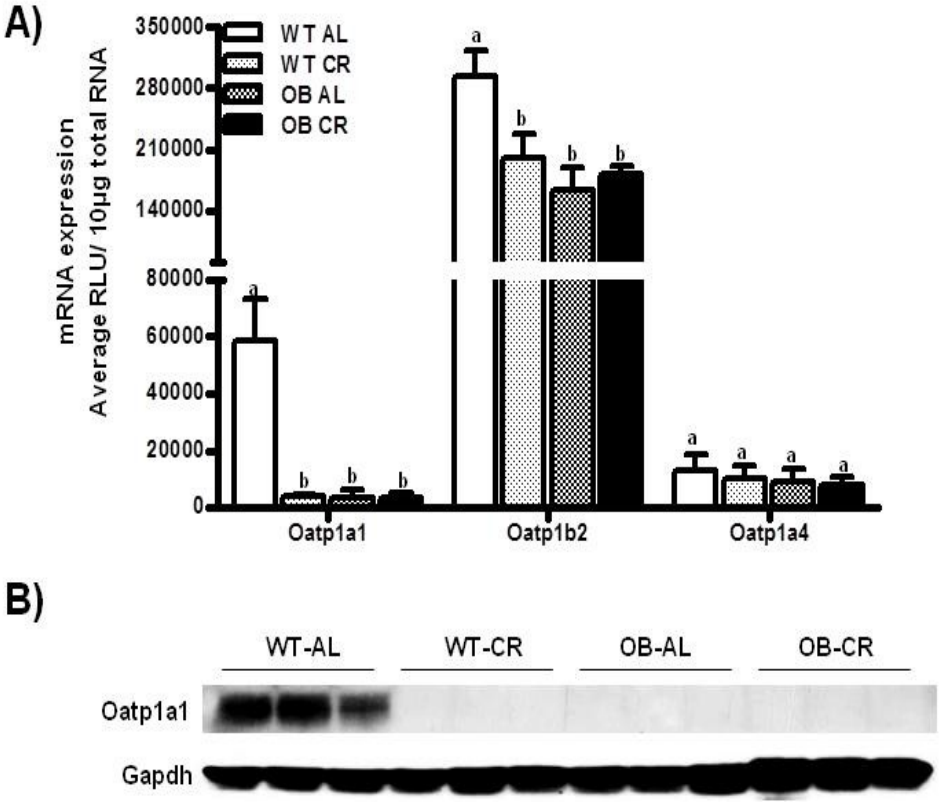

A)
mRNA expression
Average RLU/ 10µg total RNA
350000
280000
210000
140000
80000
60000
40000
20000
0
WT AL
WT CR
OB AL
OB CR
a
b
b
b
a
b
b
b
a
a
a
a
Oatp1a1
Oatp1b2
Oatp1a4
B)
WT-AL
WT-CR
OB-AL
OB-CR
Oatp1a1
Gapdh

FIGURE II-4:

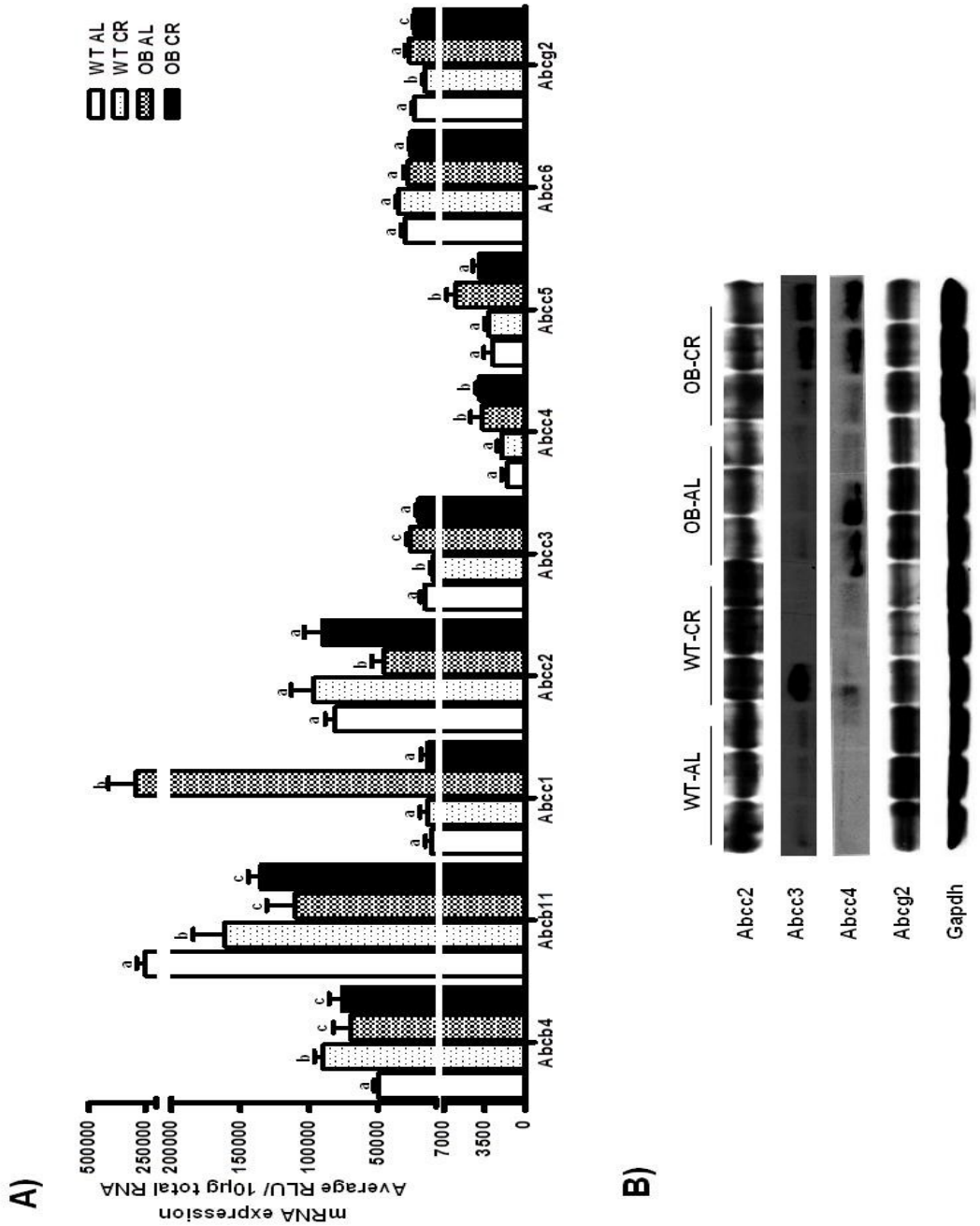

A)
WT AL
WT CR
OB AL
OB CR
mRNA expression
Average RLU/ 10µg total RNA
500000
250000
200000
150000
100000
50000
7000
3500
0
Abcb4
Abcb11
Abcc1
Abcc2
Abcc3
Abcc4
Abcc5
Abcc6
Abcg2
B)
WT-AL
WT-CR
OB-AL
OB-CR
Abcc2
Abcc3
Abcc4
Abcg2
Gapdh

FIGURE II-5:

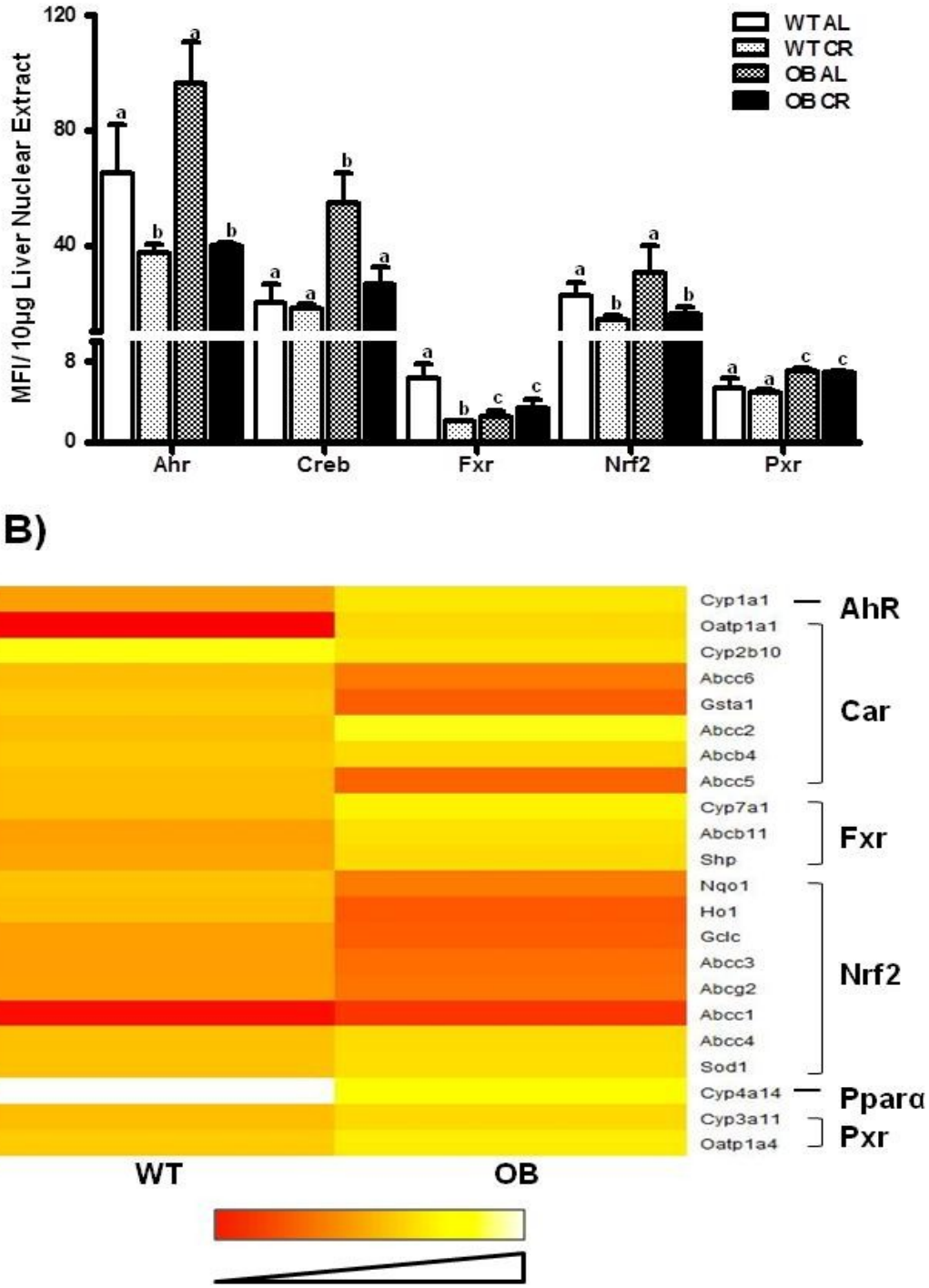

MFI/10µg Liver Nuclear Extract
120
80
40
8
0
WT AL
WT CR
OB AL
OB CR
Ahr
Creb
Fxr
Nrf2
Pxr
B)
Cyp1a1
Oatp1a1
Cyp2b10
Abcc6
Gsta1
Abcc2
Abcb4
Abcc5
Cyp7a1
Abcb11
Shp
Nqo1
Ho1
Gclc
Abcc3
Abcg2
Abcc1
Abcc4
Sod1
Cyp4a14
Cyp3a11
Oatp1a4
AhR
Car
Fxr
Nrf2
Pparα
Pxr
WT
OB

FIGURE II-6:

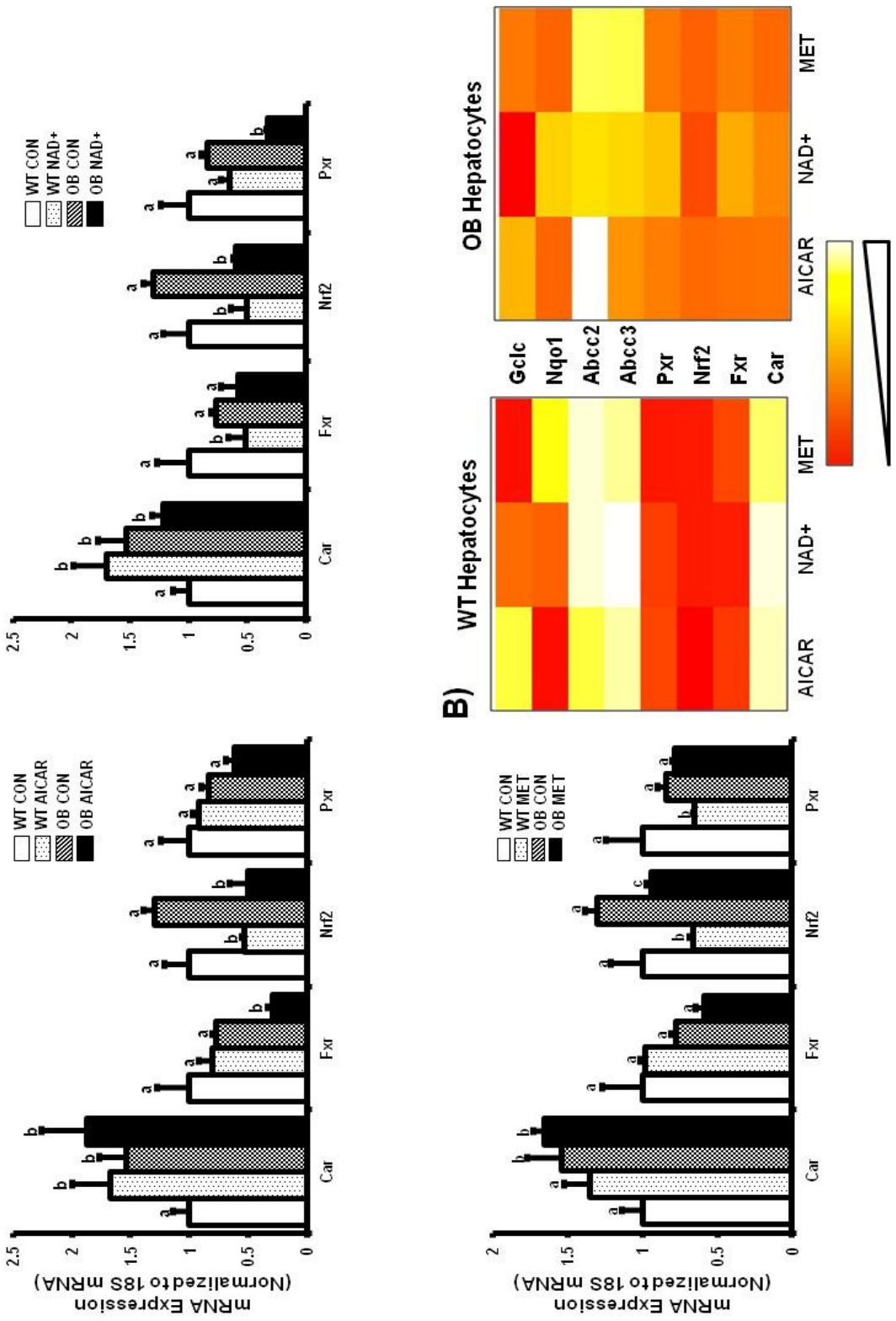
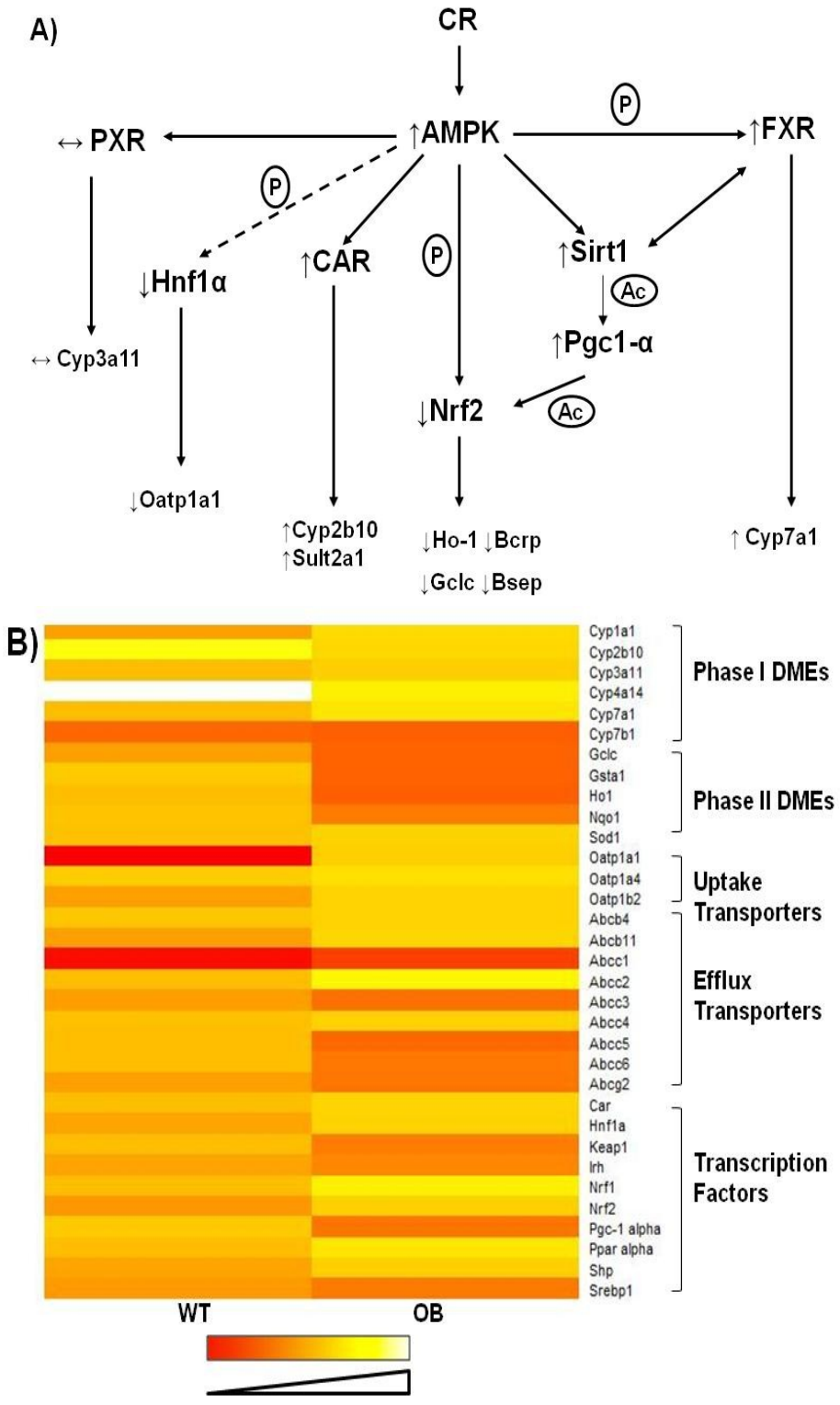

WT CON
WT NAD+
OB CON
OB NAD+
WT AICAR
OB AICAR
WT MET
OB MET
mRNA Expression
(Normalized to 18S mRNA)
Car
Fxr
Nrf2
Pxr
B)
WT Hepatocytes
OB Hepatocytes
Gclc
Nqo1
Abcc2
Abcc3
Pxr
Nrf2
Fxr
Car
AICAR
NAD+
MET

FIGURE II-7:


A)
CR
↔ PXR
↑AMPK
P
↑FXR
P
↓Hnf1α
↑CAR
P
↑Sirt1
Ac
↔ Cyp3a11
↑Pgc1-α
↓Nrf2
Ac
↓Oatp1a1
↑Cyp2b10
↑Sult2a1
↓Ho-1 ↓Bcrp
↓Gclc ↓Bsep
↑ Cyp7a1
B)
Cyp1a1
Cyp2b10
Cyp3a11
Cyp4a14
Cyp7a1
Cyp7b1
Phase I DMEs
Gclc
Gsta1
Ho1
Nqo1
Sod1
Phase II DMEs
Oatp1a1
Oatp1a4
Oatp1b2
Uptake Transporters
Abcb4
Abcb11
Abcc1
Abcc2
Abcc3
Abcc4
Abcc5
Abcc6
Abcg2
Efflux Transporters
Car
Hnf1a
Keap1
Irh
Nrf1
Nrf2
Pgc-1 alpha
Ppar alpha
Shp
Srebp1
Transcription Factors
WT
OB

TABLES

TABLE II-1: Effect of CR on body weight, liver-to-body weight ratios and biochemical profile: Adult C57Bl/6 and ob/ob mice (n=7-10/group) were fed (*ad libitum*) or put on 40% reduced caloric diet for 9 weeks. At the end of 9 weeks, mice were euthanized. Average liver weights, liver-to-body weight ratios, serum glucose (mg/dl), serum and liver triglycerides (mg/dl) and serum NEFA (mmol/dl) were determined. $P<0.05$ was considered statistically significant. Groups without a common letter are significantly different.

Strain	AL/CR	Average liver wt	Average liver (g)/body weight (g)	Serum Glucose (mg/dl)	Serum Triglycerides (mg/dl)	Serum NEFA (mmol/dl)
C57Bl/6	AL	1.385 ± 0.0554	0.041856 ± 0.000670	202.48 ± 8.15	66.81 ± 6.82	0.031 ± 0.0022
	CR	0.663 ± 0.0203	0.035472 ± 0.000977	109.79 ± 7.45	33.96 ± 1.14	0.051 ± 0.0025
ob/ob	AL	5.172 ± 0.406	0.072843 ± 0.00427	184.33 ± 8.82	71.37 ± 5.65	0.028 ± 0.0026
	CR	2.286 ± 0.129	0.041881 ± 0.002186	222.47 ± 19.07	64.11 ± 2.77	0.059 ± 0.0038

TABLE II-2: Effect of CR on serum metabolic hormones: Adult C57Bl/6 and ob/ob mice (n=7-10/group) were fed (*ad libitum*) or put on 40% reduced caloric diet for 9 weeks. At the end of 9 weeks, mice were euthanized. Average serum leptin, glucagon, resistin, and insulin levels were determined. $P<0.05$ was considered statistically significant. Groups without a common letter are significantly different.

Strain	AL/CR	Insulin	glucagon	leptin	resistin
C57Bl/6	AL	2121.70 ± 484.25	NA	10486.6 ± 2376.48	14355.8 ± 1679.58
	CR	388.58 ± 57.30	NA	267.22 ± 56.78	18178.371 ± 1672.10
ob/ob	AL	17834.15 ± 3240.94	43.98 ± 4.67	NA	20875.02 ± 1549.58
	CR	6735.686 ± 830.59	85.268 ± 24.09	NA	25365.84 ± 2645.83

MANUSCRIPT III

To be submitted to 'Pharmaceutical research'

Differential expression of micro-RNAs regulating lipid metabolic genes enhances susceptibility to caloric restriction in models of constitutive Nrf2 overexpression.

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RUNNING TITLE: Caloric Restriction and Transporters

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Number of text pages:32

Number of tables: 1

Number of Figures:4

References:

Number of words:

Abstract: 250

ABSTRACT

Studies demonstrate that changes in miRNA expression alter etiology and development of steatosis. CR reverses steatosis by increase in fatty acid oxidation genes such as Pparα, Cpt1a via activation of Sirt1/Pgc-1α cascade while downregulating expression of lipogenic master regulators Srebp1c, Pparγ and their target genes. Nuclear factor E2-related factor 2 (Nrf2)/Kelch like ECH associated Protein 1 (Keap1) pathway is a key antioxidant gene regulatory pathway with altered activity in steatotic conditions. However, very little is known about the interactions of Nrf2/Keap1 and CR activated pathways. The purpose of this study was to determine the effect of Nrf2/Keap1 pathway on expression of miRNAs important in regulating lipid metabolic genes upon CR. Keap1 WT and KD mice were maintained on 40%CR for 6 weeks and the lipid metabolic gene expression (Pparα, Cpt1a, Pgc1α, Sirt1 and Srebp1c, Fas, Acc1) were quantified. Keap1 knockdown enhanced the effect of CR on induction of fatty acid oxidation genes while increasing fold inhibition of lipid synthetic genes. miRNA 34a, 370, let-7b, 144, 221 were significantly affected by alteration in Nrf2/Keap1 pathway activity as well as CR. Thus, the data presented in this study indicate an important regulatory role of Nrf2/Keap1 pathway in miRNA expression as well as potential novel mechanism for the differential effect of CR in Keap-1KD mice.

INTRODUCTION

Small non-coding RNAs, microRNAs have been recently demonstrated to provide an additional layer of regulatory control in development, homeostasis and pathology (1). microRNAs, 19-22 nucleotides long, act as gene silencers with the RNA induced silencing complex (RISC) and modestly regulate gene expression (2). Mammalian microRNAs do not necessarily exhibit complete homology with the target mRNA and along with classically known cleavage of target mRNA, they can also affect mRNA stability (1). Due to partial homology, a single miRNA is shown to regulate expression of multiple target mRNAs (3). miRNA expression and activity is altered in various disease conditions including metabolic syndrome (4). miRNA expression and regulation are being explored in detail as potential targets and possible biomarkers in various diseases, specifically metabolic syndrome and cancer (5, 6).

Non alcoholic fatty liver disease (NAFLD) is referred to as the hepatic manifestation of metabolic syndrome (7, 8) and encompasses various conditions of fat deposition and inflammation of the liver. Steatosis or fatty liver comprises of excessive accumulation of fat in the hepatocytes. Prevalence of NAFLD is estimated to be between 3.1% and 16.4% among adults, respectively (9, 10), known to afflict obese and normal weight persons and children as well with consequences such as altered pharmacokinetics of xenobiotics and endogenous metabolites, drug

induced liver injury (DILI) and propagation of insulin resistance complicating metabolic syndrome.

Through various *in vitro* and *in vivo* studies, miRNA have been shown to alter etiology and development of steatosis. miR122 has been shown to be involved in regulation of cholesterol and triglyceride synthesis, silencing miR122 drastically decreased cholesterol synthesis, decreased fatty acid levels and triglyceride load on the liver in mice fed a high fat diet (11, 12). Patients with NAFLD demonstrated increased circulating miR122 levels along with miR34a (13). miR34a is another miRNA that has been shown to regulate important aspects of fatty acid oxidation via silencing Sirtuin1 (Sirt1), a master regulator of energy homeostasis in the cell (14). Sirt1 is known to inhibit miR34a via histone deacetylation, while positively upregulating FXR expression (15). miR33a has been shown to be important for its regulation of Srebp expression and hence steatotic development (16, 17). miR221 has been demonstrated to be important in development of liver fibrosis and hepatocellular carcinoma (18). Let7 miRNA increases insulin resistance via modulating insulin receptor (InsR) expression along with glut4 expression in skeletal muscle (19, 20). Knockdown of Let-7 miRNA prevented ectopic fat deposition in liver along with build up of adipose tissue in mice on a high fat diet (21).

American Association for Study of Liver Disease (AASLD) recommends caloric restriction and exercise to reverse steatosis/fat load on

the liver . Beneficial effects of CR are attributed to activation of Sirt1 which activates gluconeogenic and fatty acid oxidation genes expression via coactivator; Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (Pgc-1α) (22, 23). Sirt1 activates the nuclear receptor Pparα via acetylation of Pparα coactivator (Pgc1-α) and upregulation of fatty acid oxidation genes such as Cpt1a (24, 25). At the same time CR activation of Sirt1/Pgc-1□ cascade inhibits expression of lipogenic factors and regulators such as Srebp1c, Lxr and their target genes Fas, Acc1, Acot1, Fabp4, Scd1.

Among various transcription factors altered in steatosis, Nuclear factor E2-Related factor 2 (Nrf2) pathway has been shown to be increased in mouse and human models of NAFLD. Nrf2 belongs to the basic leucine zipper family of transcription factors, tethered into the cytoplasm via a complex with Kelch like ECH-associated Protein 1 (Keap1), which upon activation translocates to the nucleus and activates gene transcription (26, 27). Nrf2 is an important transcription factor known for its role in xenobiotic metabolism and oxidative stress response (28, 29). Nrf2 regulates expression of genes important in metabolism and oxidative stress response such as (Nqo1), Heme Oxygenase (HO-1) and Glutathione S-Transferase (30). Nrf2 acts via inhibition of LXRα dependant increases in Srebp1 expression and thus decreased lipogenesis (31). Shin et al., 2009 and Tanaka et al., 2008 demonstrated increased hepatic lipid accumulation in absence of Nrf2 in mice basally or upon high fat diet feeding (32, 33).

More recently, studies also identify Nrf2 as a positive regulator of lipogenic genes such as Pparγ, Scd-1, Srebp1, Cebpβ, basally or upon exposure to high fat diet (34-36). Recent studies by Xu et al,. 2012 demonstrates an increased expression and activity of Nrf2 in leptin deficient obese mice (37). Despite being controversial, evidence points towards an important role for Nrf2 in either ameliorating or exacerbating steatosis.

Recent studies have demonstrated that anti-carcinogenic, protective activity of Nrf2 in models of caloric restriction (38). However, no studies determine the effect of caloric restriction on Nrf2 and its target gene expression. Furthermore, no studies present an account of effect of altered Nrf2 activity and susceptibility to CR. No studies outline whether CR induced fatty acid oxidation and diminished lipogenesis involves a change in Nrf2 activity.

In the present study, we demonstrate differential susceptibility of a mouse model, constitutively overexpressing Nrf2 (Keap1-KD mice) to CR. We attribute these changes to novel changes in the miRNA regulatory circuit. We demonstrate that the enhanced susceptibility to CR in Nrf2 overexpressing mice could be partly associated with differential changes in miRNA expression, especially those known to be involved in regulation of lipogenic as well as fatty acid oxidation genes. We present novel data potentially indicating an important regulatory role for Nrf2-Keap1 pathway in small RNA expression.

MATERIALS AND METHODS:

Animals and treatments: Keap1-KD mice were obtained from laboratory of Dr.Curtis Klaassen at the Kansas University Medical Center,Kansas, USA bred at the animal facility in Fogarty Hall, University of Rhode Island. The average caloric consumption were calculated for each mouse on a purified diet (AIN93-G) obtained from Testdiet, IN,USA over a period of 10 days. C57Bl/6 and Keap-1KD mice (16 weeks age) were each divided into 2 groups of n=5 and n=6. One group of each genotype (n=5) was maintained as a control group which was fed ad libitum (AL) over the length of the experiment. The other group of each phenotype (n=6) was put on a 40% reduced caloric diet (CR) for a period of 10 weeks with ad libitum access to water. Body weight and food consumption was monitored at least weekly throughout the study. The study was terminated when weight loss plateau was reached (After 7 weeks CR). Mice were sacrificed; blood and livers were collected. Livers were snap frozen in liquid nitrogen and stored in -80°C until further analysis. Serum glucose (Cayman Chemicals, Ann Arbor, MI), serum and liver triglyceride (Pointe scientific, MI, USA) and free fatty acid concentrations (Wako diagnostics, VA) were determined by spectrophometeric assay kits.

RNA Isolation and mRNA Quantification: Total RNA was isolated from the collected livers by phenol-chloroform extraction with RNAzol B reagent (Tel-Test Inc., Friendswood, TX) according to the manufacturer's

instructions. RNA concentration was determined by measuring UV absorbance at 260 nm using NanoDrop™ and the integrity were confirmed by formaldehyde gel electrophoresis. mRNA expression was quantified by RT^2-PCR, with expression normalized to 18S rRNA expression.

miRNA quantification: Total RNA enriched for small nucleotide fraction was isolated from livers obtained from AL and CR C57Bl/6 and Keap1-KD mice using the miRNA easy mini kit (Qiagen Inc, MD, USA). 1µg total RNA was used for miRNA cDNA preparation using RT^2 miRNA first strand kit (Qiagen Inc, MD, USA) and miR34a, miR370, miR122, miR221, miR144 expression was measured by by RT^2-PCR. The miRNA expression was normalized to mean expression levels of Snord65, Snord66 and Snord85.

Tissue homogenate preparation: Liver tissue homogenate was obtained by homogenization with RIPA (Tris 50mM, NaCl 150 mM, SDS 0.1 % and TritonX-100 1.0%) and short centrifugation to remove the debris. The protein concentration of the samples will be measured according to the manufacturer's instruction using BCA protein assay (Thermo Fisher Scientific, Rockford, IL, USA).

Western Blotting: Proteins in the obtained membrane preparations will be solubilized in Laemmli buffer and β-mercaptoethanol and electrophoretically separated on a tris-polyacrylamide gel (10% for Sirt1 and Fas) at 200V for 50 min and transferred onto a PVDF membrane at 100V for 30-45 min or at

75V for 1.5 hours. The membrane will be blocked overnight with 2% Non fat dry milk (NFDM) in phosphate buffered saline with 0.05% Tween 20 (PBS/T or TBS/T). After blocking, the membrane will be incubated with appropriate concentrations of primary antibodies diluted in 2%NFDM in PBS/T or TBS/T for 2 hours and subsequently with corresponding horseradish peroxidase labeled secondary antibodies also diluted in 2%NFDM in PBS/T or TBS/T. The blots will then be incubated in ECL-Plus western blot detection reagent and the blots will be exposed on X-ray films. The blots will be quantified using ImageQuant software. Results obtained will be tested for statistical significance.

miRNA array analysis: PCR arrays (mouse genome V2.0) (SABiosciences, Frederick, MD,USA) were used to determine expression of miRNA in pooled liver RNA samples isolated using miRNA easy kit (Invitrogen Corp, MD, USA) as per manufacturers instructions. Data analysis was performed with the web-based software package for the miRNA PCR array system. (http://www.sabiosciences.com/pcr/arrayanalysis.php).

Statistics: The statistical significance of differences will be determined by Duncan's factorial ANOVA test or a standard student T-test. $P<0.05$ will be considered statistically significant. Groups without a common letter will be considered significantly different.

RESULTS

Constitutive overexpression of Nrf2 enhances susceptibility to weight loss and fat mobilization upon CR: To determine whether constitutive overexpression of Nrf2 changes susceptibility to weight loss and fat mobilization, C57Bl/6 and Keap1-KD mice were either fed *ad libitum* or out on 40%CR for 6 weeks. As seen in Fig. 1A, Keap1-KD mice fed *ad libitum* gained less weight over time than the C57Bl/6 counterparts. While C57Bl/6 mice fed *ad libitum* gained approximately 15% of their initial weight at the end of the study, Keap-1 KD mice gained significantly lower percentage (8%) of their initial weight at the starting point (week0) (Fig. 1A). Upon CR, both C57Bl/6 and Keap1-KD mice lost approximately 43% of their initial weight (Fig. 1A).

Caloric restriction increases expression of fatty acid oxidation genes to a higher fold in Keap1-KD mouse livers- To identify whether the hallmark fatty acid oxidation genes are activated upon CR, mRNA expression of Sirt1, Pgc1α, Ppar α was quantified along with Cpt1a. Basally, Keap1KD mouse livers demonstrated an elevated expression of Cpt1a and Ppar α over the C57Bl/6 controls (Fig. 2A). As observed in Fig.2A, CR restriction induced the expression of Sirt1, Pgc1 α, Ppar α in livers of both C57Bl/6 and Keap1-KD mice. The induction of Pgc1α, Pparα was the highest in livers of Keap1-KD mice which were calorically restricted (Fig. 2A) presenting a novel observation and potentially an effect of Keap1

knockdown increasing the inducibility of the Sirt1/Pgc1α cascade in these livers.

Constitutive overexpression of Nrf2 increases CR induced downregulation of lipogenic gene expression in mouse livers- CR is known to classically down regulate the expression of gene involved in biosynthesis of fats. Hence, mRNA expression of genes such as Srebp1c, Lxr and the target genes such as Fas, Acc1, Fabp4 and Scd1. As seen from Fig. 2B, in C57Bl/6 mice, CR downregulated expression of lipogenic gene expression regulators, Srebp1c Lxr to ~50% of the *ad libitum* fed controls. Target genes regulated by these transcription factors such as, Fas, Fabp4, Acc1 were also downregulated significantly over the fed controls (Fig. 2B). Basal expression of some of the lipogenic genes such as Acc1, Fab4, Fas and Scd1 was lower in the Keap1-KD mice as compared to *ad libitum* fed C57Bl/6 mice (Fig.2B). Upon CR, the expression of these lipogenic genes was not altered in Keap1-KD mice, even though the gene downregulation of the regulatory Srebp1c was significantly lower in the Keap1-KD mouse livers over the calorically restricted C57Bl/6 mice (Fig. 2B).

Keap1-KD enhances the effect of caloric restriction on Nrf2 target gene expression- To determine whether CR has an effect of Nrf2 target gene expression, we quantified the mRNA expression of Nrf2 and its target

genes Nqo1 and Gclc in C57Bl/6 and Keap1-KD mice on CR. As seen in Fig. 2C, CR induces expression of Nqo1 (~1.6 fold) and downregulates the expression of Nrf2 and Gclc (~30% and 79% respectively) in C57Bl/6 mice. Basal expression of Nrf2 target genes, Nqo1 and Gclc was significantly higher in Keap1-KD mouse livers, which is consistent with previous publications (39). Absence of Keap1 did not significantly alter the effect of CR on Nrf2 mRNA expression (~69% downregulation over the *ad libitum* Keap1-KD controls). However, knockdown of Keap1 significantly enhanced the effect of CR on downregulation of Gclc (80% in C57Bl/6 CR versus 60% in Keap1-KD CR mouse livers) and induction of Nqo1 (~1.6 fold in C57Bl/6 CR versus 1.4 fold in Keap1-KD CR mouse livers) (Fig. 2C).

Caloric restriction significantly alters expression of miRNA important in regulating fatty acid oxidation and lipogenic genes- To determine if the induction of fatty acid oxidation genes upon CR is dependent upon altered levels of miRNA regulatory circuit, miRNA expression of various miRNAs found in the mouse genome were quantified in Keap-1 WT CR and Keap1-KD CR pooled samples. CR differentially regulated the expression of miRNAs as shown in Table 1A and 1B.

Working on the observation further, miR34a, miR370, let-7b, miR122 and miR221 was quantified. As seen in Fig.3A, Keap1 knockdown significantly decreased basal expression of miR34a, miR370 and let-7b. This observation is consistent with the higher basal expression of Cpt1a and

Pparα (Fig. 2B). Caloric restriction significantly decreases expression of miR34a, let7b and miR144 in C57Bl/6 mouse livers (Fig. 3A). Keap1-KD did not significantly affect the decrease in miR144 expression upon CR, while it enhanced the decrease in expression of miR34a (Fig. 3A). On the other hand, CR resulted in downregulation of miR370 in Keap1-KD mouse livers while it remained unchanged in C57Bl/6 mice. On the other hand CR decreased let7b expression in C57Bl/6 mice while remaining unchanged in Keap1-KD mice (Fig. 3A).

Knockout of Nrf2 reverses basal miRNA expression pattern changes in mouse liver - To determine whether basal miRNA expression changes were due to a change in Nrf2 expression in the Keap1-KD mouse model, miRNA expression was quantified in Nrf2 KO male mice put on 40% caloric restriction. As seen in Fig. 3B, Nrf2 KO mice showed a reversal of basal expression changes as compared to Keap1-KD mice. miR34a and let7b while being downregulated in Keap1-KD mice, remained unchanged basally (Fig. 3B), whereas miR144 and miR221 demonstrated an opposite basal expression pattern in Nrf2 KO and Keap1-KD mice as compared to C57Bl/6 mice. miR370 however, was significantly downregulated in both Nrf2 KO mice as well as Keap1-KD mice, with a higher fold downregulation in the Nrf2 Kos (Fig.3B). CR in this cohort of mice did not alter miR 34a, let7b expression (Fig. 3B) while miR221 was induced by ~3 fold. CR however, increased the expression of miR34a, miR144 and miR221 in Nrf2 KO

mouse livers, while in Keap1-KD mice their expression was either decreased (miR34a, miR144) or remained unchanged (miR221). These observations indicate towards as novel role of Nrf2 as well as Keap1 in regulating expression of miRNAs important in lipid metabolism as well as a differential effect of CR in Keap1-KD versus Nrf2 KO mice.

Caloric restriction decreases expression of certain RISC complex genes- Differences in the expression of RISC complex components along with miRNAs could also affect target mRNA gene expression in Keap1-KD mice and potentially explain the basal level differences in expression of certain genes in these mice. Hence, mRNA expression of RISC complex components such as Dicer, Drosha, exportin 5 was determined by RT^2-PCR. Although no significant differences were observed in basal expression of these genes in Keap1-KD mouse livers as compared to C57Bl/6 controls, the effect of CR was different in each genotype. As seen in Fig. 4, CR decreased expression of Exportin5 and Drosha in C57Bl/6 mice. RISC complex factors in Keap1-KD mice on the other hand remained unchanged upon CR (Fig. 4), indicating a potential role for Keap1 in regulating CR dependent changes in RISC complex factor expression.

DISCUSSION

There has been an increase in the number of studies focusing on the miRNA regulatory pathways as potential gene therapy candidates and biomarkers for pathological conditions such as diabetes, cancer, NAFLD (40). The purpose of this study was to identify whether constitutive over expression of Nrf2 by knockdown of Keap1, changes the effect of caloric restriction (CR) on expression of fatty acid oxidation and lipogenic genes. This study also aims at identifying whether CR alters expression changes expression of miRNAs that have been demonstrated to regulate the expression of lipid metabolic and biosynthetic genes. This study presents novel data about presence or absence of Nrf2 and Keap1 changing miRNA expression, and how these changes cause a differential effect of CR has also been studied.

Epigenetic regulation has emerged as an important contributor to downstream effects of CR. Preliminary studies have demonstrated that CR intervention causes DNA methylation changes at loci of gene important in insulin signaling and weight control in human adipose tissue (41) and these changes are being considered for biomarker studies during weight loss (42). Other studies have demonstrated a change in DNA methylation machinery such as DNA methyl transferases (dnmt3a) and methylation on oncogenes such as p53 and H-Ras (43). CR has also been shown to change histone acetylation (44) via changes in expression of histone acetyl transferases and histone deacetylases, specifically Sirt-1 models *in vitro*

and *in vivo*. Scant but convincing data demonstrates the importance of miRNA regulation in effects of CR. Hence, it is important to address the regulatory role of Nrf2-Keap1 pathway in basal and CR induced changes miRNA expression and downstream alterations in lipid metabolic genes.

The effect of miRNA expression in the current study can be divided into two parts: miRNAs basally altered by both Keap1 and Nrf2 knockdown as well as CR (miR34a, miR370, Let-7b*, miR221); miRNAs altered only by CR (miR144). As observed from results obtained in this study, CR effects the expression of miR34a, miR370, miR 122, Let7-b in mouse livers. miR34a known to regulate and be regulated by Sirt1(15), is down regulated upon CR in WT mouse livers corresponding to an increase in Sirt1 mRNA expression along with Pgc-1α. As observed miR34a expression is lower in Keap-1KD livers to begin with (Fig. 3A) and decreases further upon CR with a correspondingly higher increase in Sirt1 and Pgc-1α mRNA expression. On the other hand, CR increases miR34a expression in Nrf2 null mouse livers indicating and important effect of Nrf2 on regulating miR34a expression upon activation of CR pathways such as AMPKinase pathway (45). These results indicate an important effect of Keap1 knockdown as well as absence of Nrf2 on the regulation of miR34a expression and hence, potentially Sirt1 and Pgc-1α expression. Similarly, Keap1 knockdown basally decreased expression of miR370 and let-7b*. miR370 is known to target the 3'UTR of Cpt1a (46) and decrease fatty acid oxidation while increasing triglyceride build up. In the same study, a dominant negative

form of c-jun increased miR370 expression along with Srebp1c mRNA expression while decreasing Cpt1a mRNA expression, a pattern which is opposite to the observations made in the Keap1 knockdown livers in this study. This potentially indicates towards a positive correlation between c-Jun and Keap1. Nrf2 null mouse livers however, do not demonstrate an increase in miR370 expression, which indicates towards the importance of Keap1 and not Nrf2 in miR370 regulation. It has been indicated that Keap1 and c-Jun act as negative regulators of Nrf2 activity (47), thus indicating that Keap1 and c-Jun directly or indirectly via Nrf2 regulate the expression of miR370 and by association, miR122 (46). Let-7 family of miRNAs is known to affect the expression of genes insulin pathway such as insulin receptor and Glut4. Let-7 family also enhances the expression of hemoxygenase-1 (HO-1) an important Nrf2-Keap1 target gene important in anti-oxidant response by inhibiting Bach1 repressor *in vitro* (48). In this study, let-7b* expression is down regulated in Keap1-KD mice while it remains unaffected in Nrf2 null mice basally. These preliminary observations imply a potential negative feedback regulation of Let-7b expression by Nrf2 or simply a negative regulatory effect of Nrf2. CR did not alter the expression of Let7b in Keap1-KD as well as Nrf2 null mice indicating a Nrf2-Keap1 independent mechanism of Let-7b regulation by CR.

miR144 is downregulated by CR. Increased miR144 is associated with oxidative stress in red blood cells (49) and a decreased Nrf2 expression

and activity. Basally, miR144 was downregulated in Keap1-KD mouse livers over wild types while being increased in expression in the Nrf2 null mouse livers (Fig. 3B). In line with these observations, Nqo1 expression increases in Keap1-KD mouse livers basally and higher upon CR (Fig. 2C). These preliminary observations indicate a potential negative, inhibitory regulatory action of Nrf2 on miR144 expression.

The outcome of Keap-1 KD on effect of CR primarily indicates towards a higher efficiency of Keap-1KD mice in weight loss and fatty acid oxidation over the wild types. The miRNA regulatory circuit is differentially expressed in moouse models of decreased Keap1 or Nrf2 activity along with a differential effect of CR on either genotype. Increasing evidence suggests an important regulatory role of Keap1-Nrf2 pathway in development and progression of steatosis, obesity-diabetes and metabolic syndrome associated adverse outcomes.

A novel observation is the effect of CR on the miRNA processing complex as well as the upstream processors of miRNA processing such as the RISC complex. Basal expression of the components was not affected by Keap-1 knockdown. CR decreased the expression of drosha and exportin5 in control mice while remaining unchanged in Keap1-KD mice. These preliminary observations indicate absence of Keap-1or increased abundance of Nrf2 as a regulator of RISC complex components. One study has published a decrease in dicer expression upon aging (50), making mice more susceptible to oxidative stress. Keap1-KD mice do show an

increasing trend in expression of these RISC components which might explain the ability of Keap-1KD mice to antagonize oxidative stress better via increased expression of Nrf2 target genes and a decreased expression of miRNAs regulating their expression, such as miR144.

With miRNA gene therapy being investigated as potential treatments to alleviate metabolic syndrome, the miRNAs effecting and affected by the Nrf2-Keap1 regulatory pathway potentially represent important targets for further investigation.

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FIGURE LEGENDS:

FIGURE III-1: Keap-1KD mice are gain less weight on purified rodent diet and are more susceptible to weight loss upon CR as compared to C57Bl/6 mice. (A) Decreased body weight and percent body weight in Keap1 WT and Keap1-KD mice after CR. During 6 weeks of 40% reduced caloric diet (CR), body weight of *ad libitum* fed (n=5) and CR (n=5) mice (both WT and OB) was determined every week. The percent body weight changes were calculated with the body weight at initial time point considered as 100% and each mouse being its own control. The data is represented as average ± SEM (n=5) body weight or percent body weight change over the weeks of CR. (D) Effect of CR on serum glucose, triglycerides and free fatty acid levels in Keap WT and Keap1-KD mice during active weight loss. Serum glucose, triglyceride and free fatty acid levels were quantified from Keap1 WT and Keap1-KD mice, 3 weeks into 40% CR. Blood was collected by cheek pouch bleed, serum isolated and

used for quantification. The data is represented as average ± SEM (n=5) fold change in serum biochemical factors over the ad libitum fed controls. P<0.05 was considered statistically significant. Groups without a common letter are significantly different.

FIGURE III-2: Keap1 knockdown enhances the activation of fatty acid oxidation genes and inhibition of lipogenic genes upon CR: (A) Effect of CR on fatty acid oxidation gene expression in Keap1 WT and Keap1-KD mouse liver: After 6 weeks of 40% reduced caloric diet (CR), total RNA was isolated from *ad libitum* and CR Keap1 WT and Keap1-KD mouse livers. mRNA expression of Sirt1, Pparα, Cpt1a, Acot1 and Pgc-1αwas quantified by RT^2-PCR. The data is represented as average ± SEM (n=5) fold changes in expression over the controls. (B) Effect of CR on lipogenic gene expression in Keap1 WT and Keap1-KD mouse liver: After 6 weeks of 40% reduced caloric diet (CR), total RNA was isolated from *ad libitum* and CR Keap1 WT and Keap1-KD mouse livers. mRNA expression of Srebp1c, Lxr, Fas, Acc1, Scd1 was quantified by RT^2-PCR. The data is represented as average ± SEM (n=5) fold changes in expression over the controls. (C) Effect of CR on Nrf2 target gene expression in Keap1 WT and Keap1-KD mouse liver: After 6 weeks of 40% reduced caloric diet (CR), total RNA was isolated from *ad libitum* and CR Keap1 WT and Keap1-KD mouse livers. mRNA expression of Nqo1, Gclc and Nrf2 was

quantified by RT^2-PCR. The data is represented as average ± SEM (n=5) fold changes in expression over the controls.

FIGURE III-3: Effect of constitutive over-expression and knockdown of Nrf2 on miRNA expression in mouse livers upon CR: (A) Effect of CR on miRNA expression in livers of Keap1 WT and Keap1-KD mouse livers: RNA fraction enriched for small RNAs was isolated from livers of ad libitum fed and 40% CR, Keap1 WT and KD and miRNA expression of miR34a, miR370, Let-7b, miR144, miR221 and miR122 was quantified by RT^2-PCR. The data is represented as average ± SEM (n=4) fold changes in expression over the controls. (B) Effect of CR on miRNA expression in livers of Nrf2 WT and Nrf2 null mouse livers: Small RNA fraction enriched for small RNAs was isolated from livers of ad libitum fed and 40% CR, Nrf2 WT and Nrf2 null and miRNA expression of miR34a, miR370, Let-7b, miR144, miR221 and miR122 was quantified by RT^2-PCR. The data is represented as average ± SEM (n=4) fold changes in expression over the controls.

FIGURE III-4: Effect of constitutive over-expression and knockdown of Nrf2 on RISC complex component expression in mouse livers upon CR: After 6 weeks of 40% reduced caloric diet (CR), total RNA was isolated from *ad libitum* and CR Keap1 WT and Keap1-KD mouse livers. mRNA expression of Dicer, Exportin 5 and Drosha was quantified by RT^2-

PCR. The data is represented as average ± SEM (n=5) fold changes in expression over the controls.

TABLES:

TABLE III-1: Differential (A) increase and (B) decrease in expression of miRNAs in livers of C57Bl/6 and Keap1-KD mice upon CR: RNA fraction enriched for small RNAs was isolated from livers of ad libitum fed and 40% CR, Keap1 WT and KD and miRNA expression was quantified from pooled RNA samples from WT CR and KEap-1KD CR mice by RT^2-PCR using Mouse Genome V2.0 miRNA array from SABiosceinces as per manufacturer's insturtions. Data analysis was performed with the web-based software package for the miRNA PCR array system. (http://www.sabiosciences.com/pcr/arrayanalysis.php). Data is presented as fold increase or decrease in miRNA expression of Keap-1KD CR pooled samples over the Keap-1 WT CR poled sample.

FIGURE III-1:

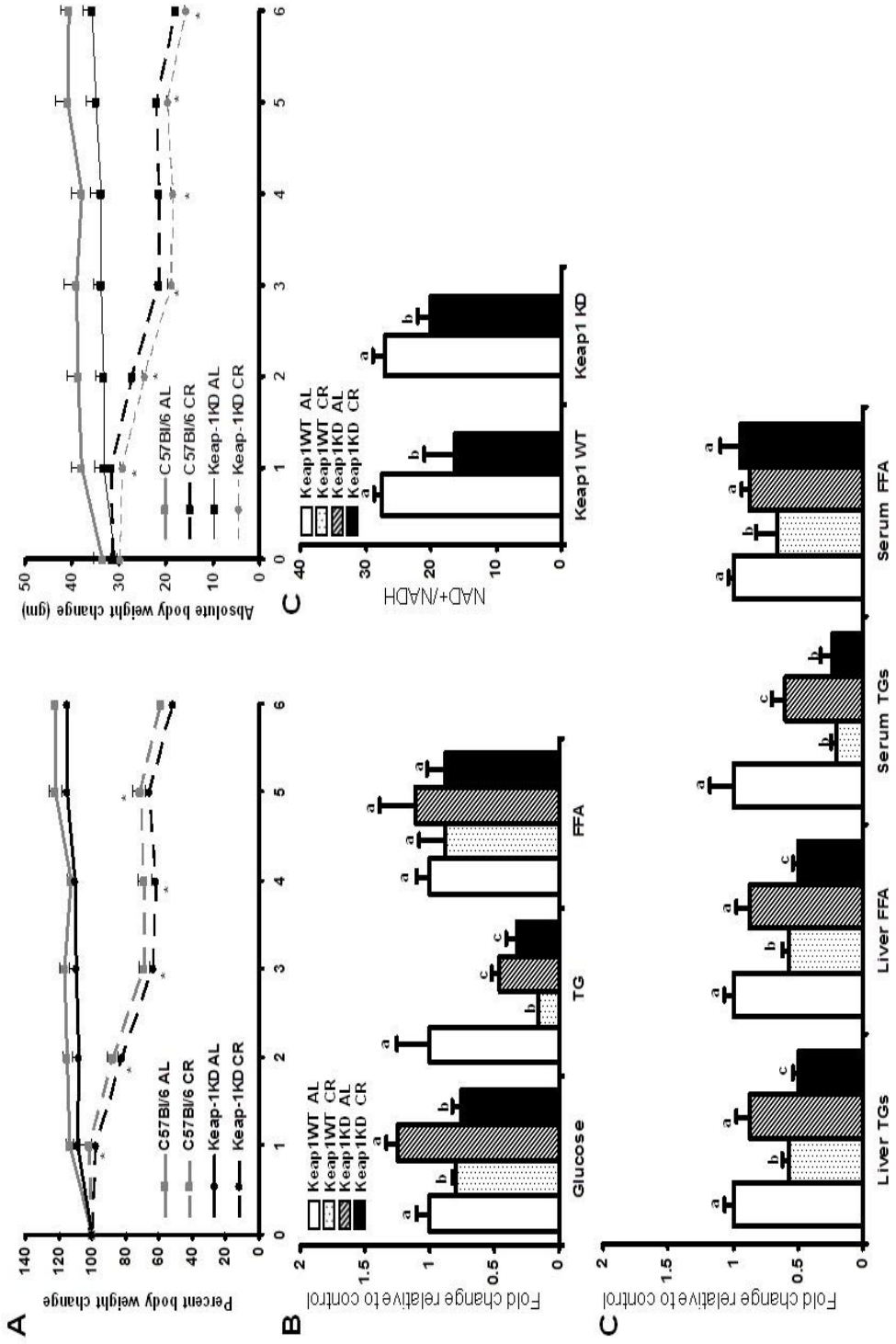
A
Percent body weight change
C57Bl/6 AL
C57Bl/6 CR
Keap-1KD AL
Keap-1KD CR
Absolute body weight change (gm)
B
Fold change relative to control
Keap1WT AL
Keap1WT CR
Keap1KD AL
Keap1KD CR
Glucose
TG
FFA
C
NAD+/NADH
Keap1 WT
Keap1 KD
Liver TGs
Liver FFA
Serum TGs
Serum FFA

FIGURE III-2:

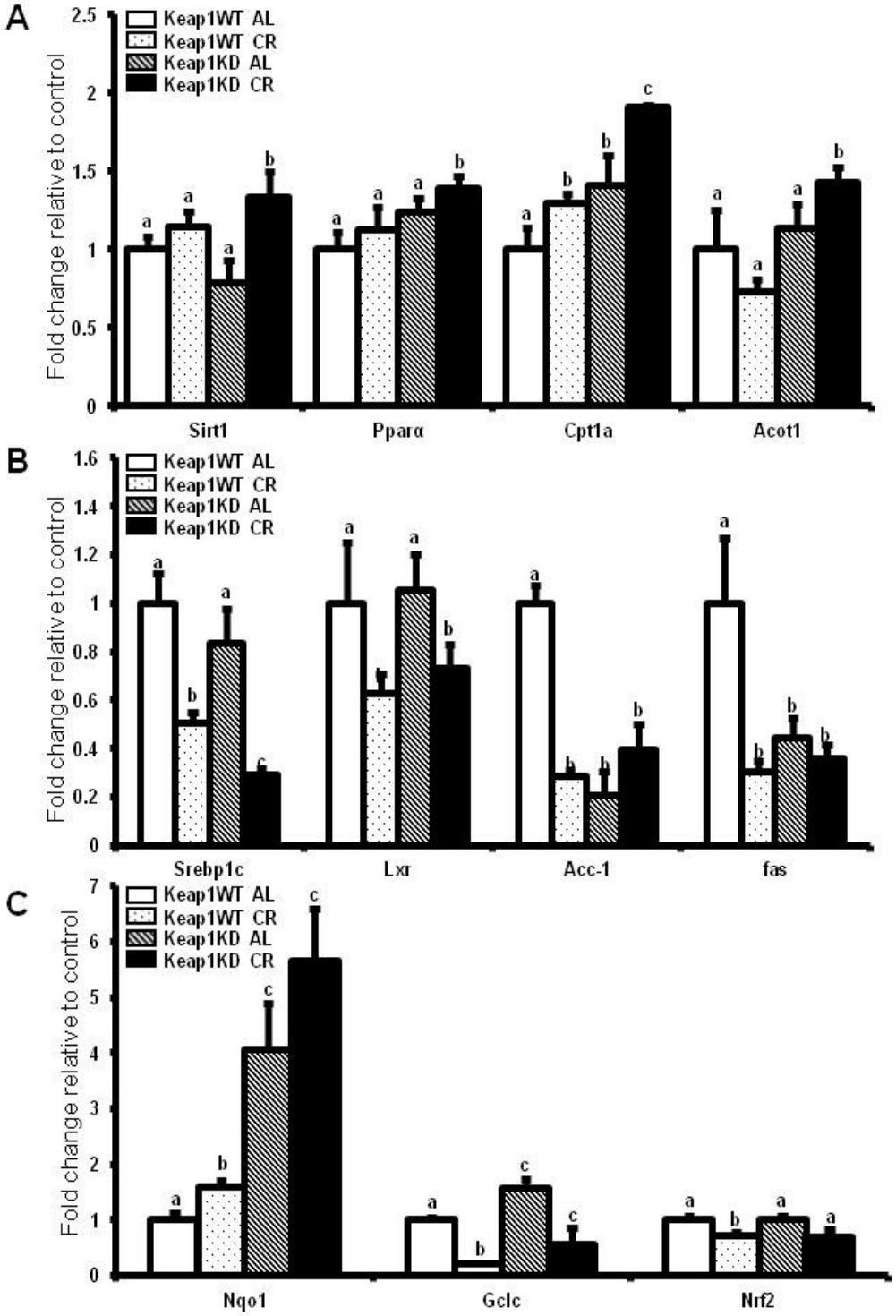
A
Keap1WT AL
Keap1WT CR
Keap1KD AL
Keap1KD CR
Fold change relative to control
Sirt1
Pparα
Cpt1a
Acot1
B
Srebp1c
Lxr
Acc-1
fas
C
Nqo1
Gclc
Nrf2

FIGURE III-3

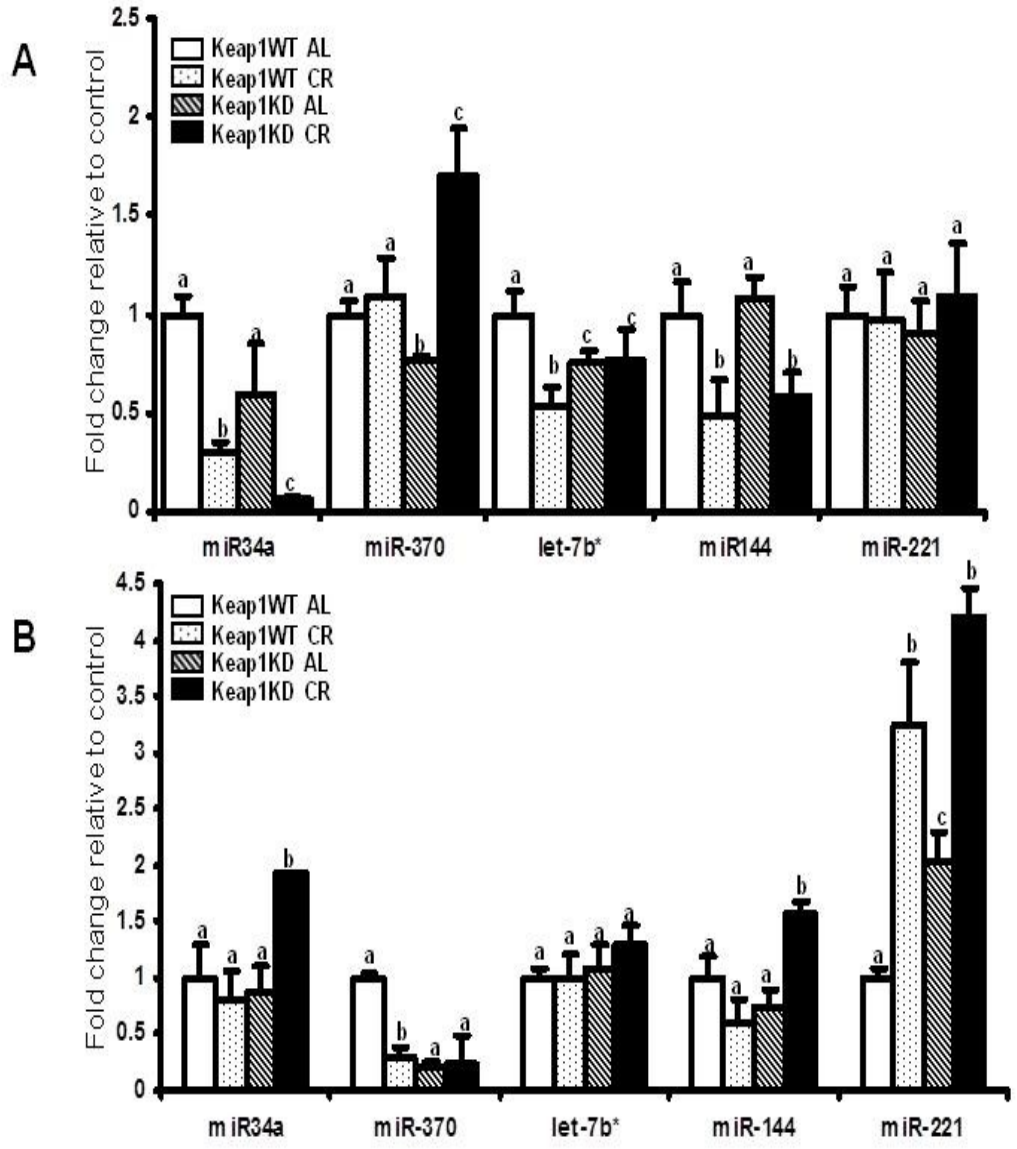
A
Keap1WT AL
Keap1WT CR
Keap1KD AL
Keap1KD CR
Fold change relative to control
2.5
2
1.5
1
0.5
0
miR34a
miR-370
let-7b*
miR144
miR-221
B
Keap1WT AL
Keap1WT CR
Keap1KD AL
Keap1KD CR
Fold change relative to control
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
miR34a
miR-370
let-7b*
miR-144
miR-221

FIGURE III-4:

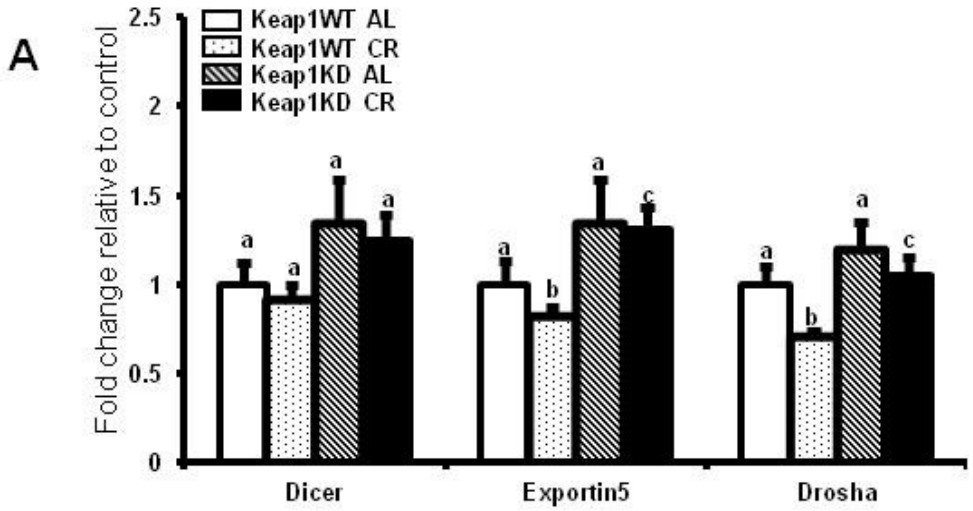
A
Keap1WT AL
Keap1WT CR
Keap1KD AL
Keap1KD CR
Fold change relative to control
2.5
2
1.5
1
0.5
0
a
a
a
a
a
b
a
c
a
b
a
c
Dicer
Exportin5
Drosha

TABLE III-1:

A

Identity	Fold change
mmu-miR-193	3.36
mmu-miR-149	3.20
mmu-miR-434-5p	3.73
mmu-miR-34b-5p	8.33
mmu-miR-205	10.61
mmu-miR-455	3.60
mmu-miR-343	3.20
mmu-miR-692	102.54

B

Identity	Fold change
mmu-miR-184	-4.63
mmu-miR-135a	-4.18
mmu-miR-146b	-14.55
mmu-miR-802	-6.07
mmu-miR-665	-4.32
mmu-miR-383	-4.24
mmu-miR-200b*	-4.21
mmu-miR-449b	-4.57
mmu-miR-667	-4.63
mmu-miR-150*	-5.11
mmu-miR-411*	-5.91
mmu-miR-466j	-6.42
mmu-miR-485*	-11.26
mmu-miR-92a*	-6.69
mmu-miR-1898	-5.06
mmu-miR-1899	-4.06