1-1-2013

Linking Environmental Toxicant Exposure To Diabetes Susceptibility

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LINKING ENVIRONMENTAL TOXICANT EXPOSURE TO DIABETES SUSCEPTIBILITY

by

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DISSERTATION

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

2013

MAJOR: PATHOLOGY

Approved by:

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Advisor Date

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DEDICATION

I would like to dedicate this work to my children, Tristan, Kieran and Ryley. You have been my inspiration, my cheerleading squad and my comedy team. The support and encouragement that I received from each of you has been, without a doubt, the driving force that kept me going through some of my worst days. Without you guys life would be non-existent. I want each of you to know how much it has meant to me to come home to your laughter, your love, and yes, even the arguing every night. You guys made this possible and I love you so very much.

And to my grandfather, Donald Moore, you believed in me when even I didn't believe in myself. You knew I would make it one day, I just wish you weren't watching me from above and could have been here to see it.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my mentor, Dr. Todd Leff. Todd, you have been an excellent mentor and I will be forever grateful for the inordinate amount of patience and understanding you have shown me throughout the years. I have grown and developed as both a scientist and a person with your guidance and tutelage.

To my committee members: Dr. James Granneman, Dr. John Reiners Jr., Dr. Shijie Sheng and Dr. Paul Stemmer. You have always been incredibly supportive and have done a wonderful job in guiding and advising me in the direction of my studies and experiments. Thank you.

To all who have offered feedback and advice along the way, thank you for taking the time with me, I sincerely appreciate it. Especially Dr. Rajesh Amin; Raj, you are an invaluable font of knowledge, as well as a friend, thank you so much for everything.

To the many friends and colleagues I have met along this journey; Melissa, Megan, Jill, Shelly, Angela S., Angie A., Mary, Angela D., Nagat, Judy, YunHee, Anelia, Lilijana, Debbie H., Douglas and so many more. Thank you for listening to me vent and being there with support, the Rum Runners are on me next time.

Thank you to the faculty from SVSU's Biology Department; especially Dr. Richard Trdan, Dr. David Stanton and Dr. Gary Lange. For being the sparks that lit the flames that burst into the fire of passion that drives my research and ambitions to this day, thank you. I would not be where I am today if you had not pushed me beyond my limits then.

I would like to thank my family for their unwavering love and support; I wouldn't be here if not for you guys. I know how much you guys have sacrificed for me and the pursuit of my career and I will be forever grateful.

And, finally, I would like to thank my "sailing family"; John and Caroline, Bill, Dick, Brian, Roy and Diana, Judi, Toni, Kent and Patty. I don't know how I got so lucky to meet such a great group of people, I have been truly gifted with the friendships and camaraderie that you guys have given so freely; fair winds and following seas, my friends.

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LIST OF ABBREVIATIONS

>……..……..Greater than

<……Less than

%β……percent beta-cell function

AAS……..……..……Atomic Absorption Spectrometry

ACC……Acetyl CoA Carboxylase

AF1…….....…N-terminal receptor-dependent transactivation domain

AGE……..…Advanced Glycation End-products

AhR……..……..……aryl hydrocarbon receptor

AICAR.....AMP analog, 5-aminoimidazole-4-carboxamid-1-β-D-ribofuranoside

Akt……Protein Kinase B

AMP…….....……adenosine monophosphate

AMPK……..……AMP-activated Protein Kinase

ANOVA…….....……Analysis of Variance

Ar……..Arsenic

AUC…….....……..…….....……..Area Under Curve

BCS…..……Bovine Calf Serum

β-cell…….....…….....beta-cell

BPA……..……Bisphenol A

BSA…….....……Bovine Serum Albumin

Ca……Calcium

cAMP…….....……cyclic adenosine monophosphate

Cd……Cadmium

cDNA……complementary deoxyribonucleic acid

CO_2……Carbon dioxide

Con……Control

CPT-1……Carnitine Palmitoyl Transferase-1

CRE……cAMP Response Element

CREB……cAMP Response Element Binding protein

CYP 1A1……Cytochrome P450, Family 1, Subfamily A, Polypeptide 1

δ-ALA……delta-aminolevulinic acid

δ-ALAD……delta-aminolevulinic acid dehydratase

δ-ALAS……delta-aminolevulinic acid synthetase

DDE……1,1-*bis*-(4-chlorophenyl)-2,2-dichloroethene

DDT……1,1,1-trichloro-2,2-di(4-chlorophenyl)ethane

Dex……Dexamethasone

dH_2O……distilled water

dL……deciliter

DMC-UHL……Detroit Medical Center – University Hospital Laboratories

DMEM……Dulbecco's Modified Eagle Medium

DMSO……dimethyl sulfoxide

DNA……deoxyribonucleic acid

ECL……Enhanced Chemiluminescence

EDTA……[[2-(*Bis*-carboxymethyl-amino)-ethyl]-carboxymethyl-amino] acetic acid

ELISA……Enzyme-Linked ImmunoSorbent Assay

EtOH……Ethanol

FA……Fatty Acid

FBG……Fasting Blood Glucose

FBS……Fetal Bovine Serum

Fe……Iron

FFAs……Free Fatty Acids

FoxO1……Forkhead box protein O1

G6Pase……Glucose-6-phosphatase

GAPDH……Glyceraldehyde-3-phosphate dehydrogenase

GRE……Glucocorticoid Response Element

H & E……Hematoxylin & Eosin

HDAC3……Histone Deacetylase 3

HF diet……high fat diet

Hg……Mercury

hIAPP……human Islet Amyloid Polypeptide (mouse)

HNF4-α……Hepatocyte Nuclear Factor 4-alpha (NR2A1)

HOMA2-IR……Homeostatic Model Assessment 2 – Insulin Resistance

IAPP……Islet Amyloid Polypeptide

ICP-MS……Inductively Coupled Plasma – Mass Spectrometry

INS-2……Insulin-2 promoter

IR……Insulin Resistance

IRE……Insulin Response Element

IRS-1……Insulin Receptor Substrate-1

IRS-2……Insulin Receptor Substrate-2

JNK……..…….....….c-Jun N-terminal Kinases

mRNA…….....……...….messenger ribonucleic acid

MCA……Malonyl CoA

mV……..…millivolts

mm……millimeter

Na…….....……Na-treated, control

NaAc…….....Sodium Acetate

NCoR…….……Nuclear Co-Repressor

NF-κB……..……Nuclear Factor kappa-light-chain-enhancer of activated B cells

NHANES……...…...…....National Health and Nutritional Examination Survey

NIH……National Institutes of Health

nM……...……..nanomolar

$O^{2\bullet-}$……..……superoxide radicals

OGTTs…….……...……Oral Glucose Tolerance Tests

OTC…….....Freezing media from TissueTek for tissue preservation

p38 MAPK…….....……p38 Mitogen-Activated Protein Kinase

pACC-β……phospho-Acetyl CoA Carboxylase-beta

pAMPK-α……...…….....phospho-AMP-activated Protein Kinase-alpha

PAS……Periodic-Acid Schiff

Pb……Lead, Pb-treated

PbAc …….....Lead Acetate

PBDEs……Polybrominated diphenylethers

PBS…….....……Phosphate Buffered Saline

PCBs……Polychlorinated biphenyls

PCR……Polymerase Chain Reaction

PD or PD168641…….....AhR antagonist, 3'-methoxy-4-nitroflavone

PEPCK……Phosphoenolpyruvate carboxykinase

PFCs……Perfluorinated compounds

PGC1-α……PPAR-γ-co-activator-1-alpha

PKA ……Protein Kinase A

PKC……Protein Kinase C

POPs……Persistent Organic Pollutants

PPIA……Peptidylprolyl Isomerase A or Cyclophilin A

PRH ……Primary Rat Hepatocytes

qRT-PCR……quantitative real-time polymerase chain reaction

RBC……Red Blood Cells

RD13004……Research Diets 45% calories from fat diet

Re……Rhenium

Rev-erb-α……also known as NR1D1

ROREs……Rev-erb-α/ROR-α Response Elements

ROS……Reactive Oxygen Species

SAPK……Stress Activated Protein Kinases

SDS-PAGE……Sodium dodecyl sulfate – polyacrylamide gel electrophoresis

SEM……Standard Error of the Mean

SOD……Superoxide Dismutase

SubQ……subcutaneous

tACC-β ……..……total- Acetyl CoA Carboxylase-beta

tAMPK-α ……..……total- AMP-activated Protein Kinase-alpha

TBS….....……Tris-Buffered Saline

TBS-T……....……..……..Tris-Buffered Saline with Tween 20

TCDD……..……2,3,7,8 – tetrachlorodibenzo-*p*-dioxin

TGs ……Triglycerides

TNF-α……...……Tumor Necrosis Factor-alpha

µg……..micrograms

µm……..micrometer

µM……..micromolar

US……..United States

VLDL……..……....……Very Low Density Lipids

w/v…….....…….weight per volume

WSU-SOM…….....……Wayne State University – School of Medicine

ZDF…….Zucker Diabetic Fatty (rat)

Zn……...Zinc

Chapter 1 – Introduction

Endocrine disruptors, xenobiotics, environmental obesogens, persistent organic pollutants and environmental toxicants are all terms used to describe chemicals in the environment that affect human and/or animal health. Some are naturally occurring substances (e.g. mercury, lead, and radon), but the majority are synthetic compounds (e.g. Bisphenol A, phthalates, pesticides and dioxins) introduced into the environment by man, only to later discover their deleterious effects on humans and wildlife.

Many naturally occurring (non-synthetic) environmental toxicants fall under the classification of heavy metals (e.g. lead, mercury, arsenic and cadmium). They are considered to be persistent environmental contaminants, found in their most basic elemental forms and can't be further broken down or destroyed. Multiple health effects are associated with heavy metal exposure. Arsenic has been shown to cause weight loss, central nervous system deficits, respiratory and reproductive system complications, and diabetes and lung cancer [1-4]. Mercury exposure has been shown to affect renal, respiratory, cardiovascular, gastrointestinal and central nervous systems [5]. Lead (Pb) exposure has been associated with loss of cognitive function, renal and central nervous system impairments, hypertension, anemia and thyroid hormone imbalances [6-16]. Although the sources of some of these heavy metals, such as Pb, have been eliminated (e.g. from paints and gasoline), other Pb-containing sources like industrially contaminated soils and turn-of-the-century housing, prevalent in urban areas like Detroit, remain a significant exposure risk throughout residents' lifetimes [17-20].

Synthetic, or man-made toxicants, are numerous and pose risks for diseases associated with exposure [21]. Synthetic toxicants include, but are not limited to,

polybrominated diphenylethers (PBDEs), polychlorinated biphenyls (PCBs), phthalates, organochlorinated pesticides (e.g. DDT), Bisphenol A (BPA), perfluorinated compounds (PFCs) and dioxin (2,3,7,8-tetrachlorodibenzo-*p*-dioxin, TCDD) or dioxin-like compounds. PBDEs are flame retardant chemicals known to impair liver and thyroid function as well as cause cancer [22-24]. Phthalates increase flexibility in plastics products, are used to add fragrances to everyday items, and are known to cause testicular dysgenesis, asthma and have recently been linked to thyroid dysfunction, obesity and diabetes [25-29]. Dioxins are a by-product of plastics manufacturing, they are known to affect whole-body energy metabolism and interfere with thyroid function as well as cause peripheral insulin resistance, cancer and diabetes [30-42].

An important and unresolved question in the environmental health field is whether exposure to common environmental toxicants, such as dioxin and heavy metal like Pb, increase the risk of developing diabetes, especially in those populations subjected to concomitant risk factors such as obesity. Currently, 11.3% of adults in the US are diabetic and NIH reports indicate steady increases in diabetes diagnoses since 1990 [43]. Obesity, which is found in greater than one third of the US population (72 million people), is strongly associated with diabetes and the metabolic syndrome [44]. High obesity rates may be attributed to the social/behavioral stressors of physical inactivity and over-nutrition, yet this issue can be viewed from the perspective of potential pathological interactions between different classes of environmental stressors - chemical (exposure to environmental toxicants) and social (lifestyle behaviors leading to obesity).

With a vast array of literature describing its deleterious effects, dioxin is one of the most studied environmental toxicants. Links have been established between dioxin exposure and peripheral insulin resistance, however, there was no evidence of elevated blood glucose levels or fasting hyperglycemia [35, 39, 42, 45-47]. The development of insulin resistance is correlated with an increased risk of developing diabetes and is a clinical symptom of type 2 diabetes mellitus as are elevated blood glucose levels and fasting hyperglycemia. Data suggests that exposure to dioxin may result in the suppression of hepatic glucose production causing hypoglycemia due to increased insulin levels and/or transcriptional suppression of phosphoenolpyruvate carboxykinase (PEPCK) gene expression [30, 34, 38, 39, 47]. While dioxin exposure is not prevalent in human populations, the mechanism of action is well established having been found to be primarily mediated by activation of the aryl hydrocarbon receptor (AhR) [48-54].

In contrast to dioxin, the mechanism of action for the physiological effects of Pb exposure is less well established, however, human exposures are much more widespread. Pb is of particular interest because there is some evidence that exposure affects metabolism and data presented herein suggests that chronic Pb exposure contributes to development of diabetes in obese rats. The Pb-obesity interaction is a relevant issue in large sections of the US population where environmental stressors of over-nutrition (taking in more calories than the body needs) and an inactive or sedentary lifestyle are common and co-exist with exposure to persistent environmental toxicants, such as Pb. This is certainly the case in the City of Detroit where obesity and diabetes rates are above the national average, and Pb exposure is relatively common (described in more detail in the Background).

The overall goal of our lab is to examine the possibility that exposure to environmental toxicants increases the susceptibility and/or severity of diabetes in metabolically and nutritionally stressed populations. The goal of this PhD project is two-fold: 1) characterize the effect and determine the molecular mechanism of dioxin exposure on hepatic gluconeogenic gene expression and 2) characterize the effect of Pb exposure on diabetes risk in metabolically stressed rodents and identify the molecular and cellular mechanisms by which it affects metabolic balance. These are initial steps in a broader research objective to determine if there is a cooperative interaction between environmental toxicant exposure and additional stressors from the physical and social environment that promote metabolic instability and disease.

Results from this work could have a significant impact on how we view the interaction of environmental toxicants with behavioral and lifestyle stressors in humans, and may compel others to carry out epidemiologic studies to assess the risks of toxicant exposure in metabolically and nutritionally stressed populations. The *in vivo* models developed here may prove useful in developing therapies to reduce the deleterious effects of Pb exposure in at-risk humans. Finally, the *in vitro* systems that I establish will be of general utility for research on the interaction of other environmental toxicants and metabolically stressed individuals.

Chapter 2 – Background

2.1 – Diabetes

Diabetes is a leading cause of death and disability in the United States, affecting over 10% of the adult population. More alarming than the diagnosed diabetes percentage is the estimation that 26% of the adult population has impaired fasting blood glucose, defined as fasting blood glucose levels between normal and diabetes (>100mg/dL - <125mg/dL) [55]. These estimations point to greater than one quarter of the population having, or being likely to develop, diabetes [56]. The incidence of diabetes has been increasing steadily, with the number of diagnosed cases increasing approximately 2.5-fold between 1990 and 2005 [56]. Although the causes of the high incidence and increasing rate of diabetes are not well understood, multiple environmental factors have been suggested to contribute to disease susceptibility. In particular, environment/lifestyle factors such as over nutrition (caloric overload) and physical inactivity (sedentary lifestyle) are believed to increase susceptibility to diabetes. These factors are hypothesized to contribute to the parallel rise in obesity (estimated to be 32% in 2007), which contributes to diabetes susceptibility in complex and poorly understood ways.

Research studies have enhanced the appreciation of the integral involvement of molecular mechanisms such as oxidative stress and inflammation in the development of insulin resistance and diabetes [57-59]. One of the earliest detectable precursors of type 2 diabetes, and a known contributor to the development of hyperglycemia, is insulin resistance [60]. Hyperglycemia has been linked to the presence of oxidative stress as well as increased production of reactive oxygen species (ROS), all of which

can exacerbate insulin resistance [60-62]. Studies have shown that levels of oxidative stress biomarkers are altered in diabetics and people who are insulin resistant [63-65].

The mechanisms by which hyperglycemia induces oxidative stress are not fully understood. One possible scenario is that changes in cellular metabolism caused by hyperglycemia alter mitochondrial function leading to overproduction of superoxide radicals ($O^{2\cdot-}$) by the mitochondrial electron transport chain [58, 59, 62, 66-74]. Likewise, the mechanisms by which elevated ROS increase insulin resistance and other diabetes-related parameters are poorly understood. Possible links between ROS and insulin resistance include increased levels of advanced glycation end-products (AGE), increased polyol and/or hexosamine pathway fluxes, and activation of specific protein kinase C (PKC) isoforms. Hyperglycemia has been shown to induce each of the aforementioned processes; even a postprandial acute rise in glucose levels can induce these processes [60, 61].

Increased ROS production can also stimulate activation of inflammatory signals (tumor necrosis factor-α [TNF-α]) and stress-response genes (JNK/SAPK, p38 MAPK, NF-κB), and inflict oxidative damage that interferes with the insulin signaling pathway [62, 70, 72, 75-92]. ROS presence is increased in diabetes [57, 60, 93] and obesity [58, 94], where the presence of ROS has been coupled with a chronic low-level inflammatory condition [62]. It has been demonstrated that diabetics exhibit an abnormal response to hyperglycemia with reduced antioxidant enzyme expression [95, 96]. The reduction in antioxidant enzyme expression does not allow the cell to detoxify the ROS adequately, resulting in elevated levels of oxidative stress, cellular damage and inflammatory mediators, all of which can interfere with insulin signaling.

There are regional variations in diabetes rates suggesting that exposure to specific localized environmental conditions may also influence disease susceptibility. Although there are many reasons for regional variations in disease rates, one possibility is that specific environmental contaminants present in some areas increase susceptibility to certain diseases. Environmental toxicants such as dioxin and heavy metals such as lead (Pb) have been shown to cause oxidative stress both *in vivo and in vitro* [32, 97]. Exposure to Pb and dioxin has been shown to influence metabolism and may, therefore, increase susceptibility to Type 2 diabetes in exposed individuals [39, 57, 98-100]. Given the relationship between oxidative stress and diabetes, exposure to environmental toxicants capable of causing oxidative stress is likely to increase diabetes susceptibility.

2.2 – Glucose Production and Utilization

A significant portion of my thesis work is focused on the effects of environmental toxicants on glucose homeostasis in general and hepatic glucose metabolism in particular. The following is a brief overview of the relevant physiologies.

The balance between glucose production and utilization is regulated by a network of hormones, neural pathways, and metabolic signals. Insulin plays a pivotal role in this process. In the fasting state, insulin secretion is low, which leads to increased gluconeogenesis in the liver and kidneys and increased glucose generation by the breakdown of liver glycogen. In the fed state, insulin released from pancreatic β-cells reverses this process by inhibiting glycogenolysis and gluconeogenesis, enhancing peripheral glucose uptake and utilization, and lipogenesis and protein synthesis. The

net result is that excess glucose is converted to glycogen, triglycerides (TGs), and protein. When more glucose is present in liver cells than can be metabolized or stored as glycogen, insulin causes the excess glucose to be converted into free fatty acids (FFAs). These FFAs are packaged as TGs in VLDL, transported in the blood in this form, and deposited as fat in adipose tissue.

2.3 – Gluconeogenesis and Regulation of Gluconeogenic Gene Expression

Phosphoenolpyruvate carboxykinase (PEPCK) is the rate-limiting enzyme in the gluconeogenesis pathway, its regulation is controlled primarily at the level of gene transcription and it is central to the management of hepatic glucose production during fasting [101]. The crucial role of PEPCK in controlling the temporal fluctuations in hepatic glucose output, and the fact that its regulation occurs mainly at the level of transcription, has made the PEPCK promoter one of the most highly studied transcriptional regulatory regions in the eukaryotic genome. The signaling pathways and many of the transcription factors and cofactors that mediate the hormonal regulation of PEPCK gene transcription have been identified and a large number of molecular tools are available for studying the transcriptional regulation of this gene. The combination of its central role in the regulation of hepatic glucose metabolism, and our detailed understanding of how its transcriptional activity is regulated, make the PEPCK gene an ideal candidate for deciphering the molecular effects of environmental toxicants on the regulation of hepatic glucose production [101]. The regulatory mechanisms that control the transcriptional activity of the PEPCK gene in response to metabolic signals are complex and full description is beyond the scope of this background section. Only

those specific regulatory mechanisms that control PEPCK gene transcription that are relevant to this project will be discussed.

Between meals and during a fast, the liver contributes to blood glucose level maintenance by increasing gluconeogenesis and glycogenolysis. The increase in gluconeogenesis under these conditions is mediated by glucagon, and to some degree glucocorticoids. Glucagon stimulates PEPCK gene transcription by activation of the PKA signaling pathway and the stimulation of the transcription factor CREB (Fig. 1, *Fasted*). CREB stimulates PEPCK gene expression via direct action on the PEPCK promoter as well as by stimulating expression of PPARγ co-activator 1-α (PGC1-α). PGC1-α is a well-characterized transcriptional co-factor for many metabolic genes [102] and functions as a transcriptional co-activator for HNF4-α, a key component of the PEPCK transcriptional complex [102, 103].

After a meal, glucagon levels fall and insulin levels rise, setting off a chain of events that result in the down-regulation of PEPCK gene expression. Without glucagon present, PKA is no longer stimulated and CREB activation declines. As CREB activity falls, its stimulatory effect on both PEPCK and PGC1-α is reduced. The loss of PGC1-α also reduces the transcriptional activity of HNF4-α, which together contributes to a reduction of PEPCK transcription. Finally, insulin activates Akt, which directly phosphorylates forkhead box protein O1 (FoxO1), causing its nuclear exclusion (Fig. 1, *Fed*). The loss of FoxO1 from the nucleus further contributes to the down-regulation of PEPCK expression by insulin. The overall effect of these events on PEPCK gene transcription leads to a decrease in hepatic glucose production. External signals, such as exposure to environmental toxicants that alter the activity of any of these signaling

molecules or transcription factors and co-factors could have a significant effect on hepatic glucose production and whole body glucose homeostasis.

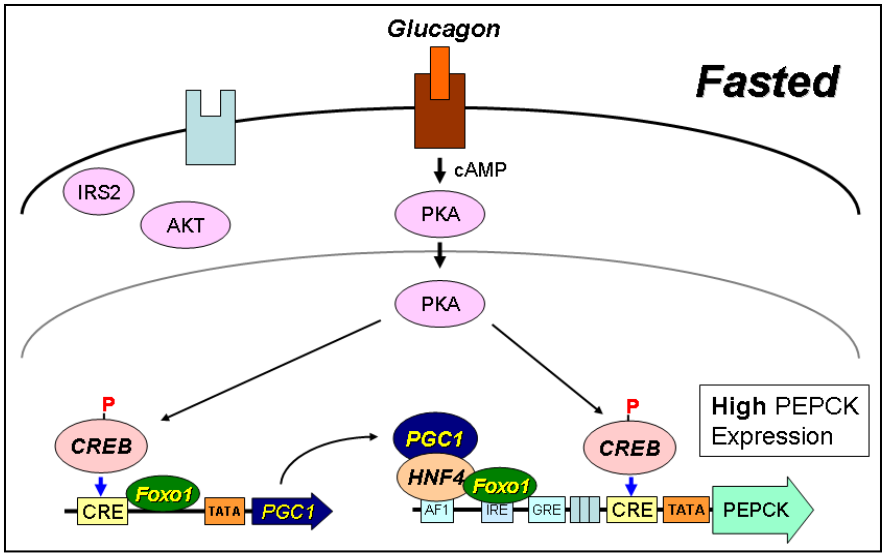

Fig. 2.1 – Regulation of PEPCK gene transcription.
In the fasted state, PEPCK is stimulated by glucagon (top), and in the fed state is suppressed by insulin (bottom).

2.3 – Toxicants Known to Affect Diabetes Susceptibility

The incidence of diabetes in the US population is at an all-time high and is continuing to rise each year. Although little progress has been made in slowing the growth of the disease, great strides have been made in our understanding of the molecular causes of diabetes and its associated metabolic abnormalities such as insulin resistance, obesity, hyperlipidemia and hypertension. One result of this increased knowledge is an appreciation of the important role that oxidative stress plays in the development of the disease [57-59]. Many toxicants, and in particular metals, are known to induce oxidative stress in exposed cells and organisms (reviewed in more detail below). Given the clear role of oxidative stress in diabetes it seems reasonable to ask if exposure to environmental toxicants, especially those with pro-oxidant activity, could increase susceptibility to diabetes and related metabolic disorders.

This is an especially relevant question if one considers the pervasiveness of environmental toxicants in industrialized countries. Taken together, these issues can be rephrased into a simple, experimentally approachable question, does exposure to specific environmental toxicants increase diabetes susceptibility or severity. There is some evidence that general exposures to environmental toxicants can affect diabetes susceptibility. For example, a recent report demonstrated that individuals living in the vicinity of known toxic waste sites were more likely to be hospitalized for diabetes then individuals living further from such sites [104]. While a specific toxicant cannot be implicated in this study, it supports the general idea that toxicant-induced environmental stress increases the risk of developing diabetes.

Obviously, all toxicants do not affect physiology by the same molecular mechanism. Different toxicants affect expression of the distinct sets of genes, and alter the activities of different enzymes. Even with regard to the effect of metals on diabetes, there are wide differences in which aspects of diseases are affected by exposure to different toxicants. Cadmium (Cd) has been shown to increase G6Pase, fructose-1,6-bisphosphatase, PEPCK and total hepatic glucose output both *in vivo* and *in vitro* [105-108]. Arsenic (Ar) has been shown to induce changes in expression of genes related to Type 2 diabetes and many diabetics have been shown to have high body burdens of Ar [109-112]. Mercury (Hg), Pb, Cd and Ar have all been shown to induce oxidative stress and the formation of ROS [106, 113-124], which have been associated with increased insulin resistance [60-62].

Persistent organic pollutants (POPs) and organochlorinated pesticides like PCBs and DDT exposure were found to be significantly associated with diabetes diagnoses when NHANES data were evaluated by Everett et.al. [125]. A 2009 study of Great Lakes sport fishermen found that, of those subjects that were diabetic, they also possessed much higher body burdens of both PCBs and *p,p'*-diphenyldichloroethene (DDE, a congener of DDT) [126]. Finally, Vasiliu et.al. found a statistically significant relation between female diabetics and blood levels of PCBs, but not with male diabetics [127].

More recently, studies have found an association between increased waist circumference, insulin resistance and blood levels of phthalates, indicating a link not only between phthalate exposure and diabetes, but also between phthalates and obesity [29, 128, 129]. Bisphenol A (BPA) exposure has been linked to an increased

risk of obesity [130] as well as adiponectin inhibition (leading to a loss of insulin sensitivity), hyperinsulinemia, insulin resistance and glucose intolerance in rodents [131-133]

Because many of the known environmental toxicants are bioaccumulative, and exposure can occur repeatedly throughout life, the effects of exposure may not be fully evident until later in life. While it is known that toxicants are capable of causing effects alone, scientists are just beginning to explore multiple toxicant exposures, and evidence thus far demonstrates greater detrimental effects in co-exposure scenarios. Overall, it is clear from the findings presented above (which are representative of a larger body of data), that in general exposure of the population of environmental toxicants is likely to have a significant impact on metabolic disease susceptibility.

2.4 – Dioxin

My initial foray into exploring mechanisms by which environmental toxicants affect metabolic regulation examined the effects of dioxin on hepatic gluconeogenesis (described in Chapter 4, below). The following is a brief summary of background information on dioxin designed to provide the necessary information for this part of the study.

Dioxins are a group of widely disseminated, persistent environmental contaminants that can have effects on whole-body energy metabolism [30-34]. In both *in vitro* and *in vivo* models, dioxin exposure has been shown to increase superoxide dismutase (SOD) activity and oxidative stress enzymes, and to cause peripheral insulin resistance [32, 35, 36, 134]. Peripheral insulin resistance is correlated with an

increased risk of developing diabetes and is a clinical symptom of type 2 diabetes mellitus [135-137].

2.4.1 – Dioxin and Diabetes

There is clear evidence that dioxin exposure increases the risk of developing insulin resistance, a condition that often precedes and always accompanies Type 2 diabetes. In *in vivo* models subjected to a high fat sucrose diet, obesity and insulin resistance were observed although the animals did not develop full-scale diabetes [36]. Similar findings have been reported in human subjects. A recent analysis of diabetes parameters in human epidemiological studies conducted on Vietnam veterans from Operation Ranch Hand, as well as persons subjected to accident or work-related dioxin exposures, patients showed a strong positive correlation between dioxin exposure, hyperinsulinemia and insulin resistance [31, 33, 35-37, 138, 139]. Interestingly, these same subjects did not demonstrate abnormal fasting blood glucose levels, fasting hyperglycemia and were not diabetic [31, 33, 35, 37, 138], suggesting that one of the effects of dioxin exposure may be to suppress hepatic glucose output.

This would be consistent with observations from dioxin treated rats where hypoglycemia was observed that was caused, at least in part, by reduced hepatic glucose production [30, 47, 140]. Together, these findings suggest that dioxin inhibits hepatic glucose production by suppressing expression of gluconeogenic genes. Such results have indeed been reported for the hepatic gluconeogenic genes PEPCK and glucose-6-phosphatase (G6Pase) [30, 34, 38, 39, 47, 140], although not all studies were in agreement with these observations [99]. Although its effects on metabolism

are obviously not well understood, dioxin is a clear example of an environmental toxin that modulates metabolism in a way that could interact negatively with other deleterious environmental conditions (e.g. over-nutrition and inactivity) to alter susceptibility to diabetes.

2.4.2 – Aryl hydrocarbon Receptor Primarily Mediates Dioxin Effects

The effects of dioxins are primarily mediated by activation of the aryl hydrocarbon receptor, AhR [48-54]. However, my data (described in this thesis) and a previously published study have suggested an alternative mechanism whereby dioxin may be suppressing PEPCK and G6Pase gene expression, via activation of AMPK [34]. AMPK is a cellular energy-sensing protein that is activated when AMP levels within the cell become high [141]. The ability of AMPK to suppress the key gluconeogenic genes PEPCK and G6Pase demonstrates its importance as a regulator of hepatic glucose production and as a potential target of dioxin. While the effects of dioxin on AMPK and energy metabolism are not clearly understood, AMPK regulates a variety of metabolic pathways and its activation by an environmental toxicant would constitute a mechanistic link between toxicant exposure and the regulation of metabolism.

2.4.3 – Hypotheses and Aims of Dioxin Project

It is clear that dioxins are a persistent environmental contaminant with the ability to influence glucose metabolism and possibly lead to alterations in diabetes susceptibility.

Because of this possibility, the following hypotheses were formed: (1) Exposure to dioxin alters hepatic gluconeogenic gene expression. (2) Dioxin-mediated AMPK activation contributes to the suppression of hepatic glucose production. These hypotheses were tested by two specific aims: (1) Characterization of the effect of dioxin on hepatic gluconeogenic gene expression. (2) Determination of the molecular mechanism(s) by which dioxins alter PEPCK gene expression.

2.5 – Lead (Pb)

The majority of this thesis is focused on the effects of Pb exposure on diabetes susceptibility in obese rodents (described in Chapters 5 and 6, below). The following is a brief summary of background information on Pb designed to provide the necessary information for this part of the study.

Pb is a persistent environmental contaminant that is found in high levels in many urban and industrial areas. Although it has been studied extensively in relation to cognitive and behavioral effects, little attention has been given to its potential involvement in metabolic diseases such as diabetes. The Pb-obesity interaction is a relevant topic in large sections of the US population where environmental stressors of over-nutrition (taking in more calories than the body needs) and an inactive or sedentary lifestyle, are common and co-exist with exposure to persistent environmental toxicants, such as Pb. This is the case in the city of Detroit where obesity and diabetes rates are above the national average, and Pb exposure is relatively common (as described below).

In spite of the fact that there are no epidemiological studies directly addressing the possibility that Pb exposure enhances diabetes severity, there are several lines of evidence, in addition to my findings in obese rats, suggesting that Pb exposure promotes metabolic instability and diabetes.

2.5.1 – Pb Exposure in the Human Population

Although Pb exposure and blood lead levels have generally declined over the past decade, Pb body burdens continue to rank as major health concerns, particularly in urban areas and especially in young children and adolescents [142]. Sources of Pb exposure include pre-1950s housing (e.g. over 38,000,000 homes), lead based paint available until the 1970s, and contaminated soil in areas surrounding existing superfund hazardous waste sites, neighborhood industrial activities, and pre-existing conditions, such as the former presence of Pb smelters in urban neighborhoods. The highly industrialized areas with existing Pb contamination largely contain urban under-privileged/under-served populations. In the city of Detroit for example, 6.4% of children under the age of 6 were classified as having elevated blood lead levels (> 10 µg/dL) by the Michigan Department of Human Health in 2003 [143].

Pb is a ubiquitous contaminant with long half-life *in vivo* (10 to 38 months depending on exposure, blood and bone levels), which could increase the long-term effects of a limited exposure to Pb. It is found in nature as a divalent cation [144], and has no known biological function in humans. Pb has been shown to interfere with Fe, Zn^{2+}, Ca^{2+} & Ca^{2+} -regulated events [145, 146], and to bind phospholipids thereby

altering membrane permeability [147]. Any of these actions could have multiple and widespread secondary effects on many aspects of cellular and organismal physiology.

2.5.2 – Pb Causes Oxidative Stress, a Risk Factor for Diabetes

Like many toxicants, Pb induces oxidative stress *in vitro* and *in vivo*, a known risk factor for the development of insulin resistance and diabetes [57, 93]. The ability of Pb to catalyze oxidative reactions and generate reactive oxygen species (ROS) has been demonstrated in numerous studies [97]. Although the mechanism by which Pb acts as a pro-oxidant is not fully understood, it is due, at least in part, to direct inhibition of the heme biosynthetic pathway enzyme δ-aminolevulinic acid dehydratase (δ-ALAD). Inhibition of δ-ALAD leads to elevated levels of the enzyme's substrate, δ-aminolevulinic acid (δ-ALA), a known pro-oxidant that stimulates ROS production, lipid peroxidation, oxidative stress and is present in the blood and urine of Pb-exposed individuals [148]. Pb also directly inhibits glutathione reductase, and binds protein sulfhydryls, thereby contributing further to oxidative stress in cells and tissues. Markers of oxidative stress can be detected in blood, RBCs, serum, and urine of Pb exposed individuals [97]. Recent studies revealed a strong association of blood Pb with markers of oxidative stress in the general population, and suggested that oxidative stress should be considered in the development of Pb-mediated diseases, even among people with relatively low environmental exposure to Pb (i.e. <10 μg/dL) [142].

Pb-induced oxidative stress is relevant to my hypothesis that Pb exposure promotes the formation of diabetes. Oxidative stress is thought to promote the diabetic state by direct effects on cellular signaling pathways that influence insulin signaling.

Subjects exposed to Pb have increased ROS production, oxidative stress induction and elevated concentrations of δ-ALA in their blood and urine [120, 149-151]. One proposed mechanism is that ROS activate intracellular signaling through JNK/SAPK, p38 MAPK and NF-κB. JNK activation results in serine phosphorylation and inhibition of IRS (Insulin Receptor Substrate) 1 and 2. IRS1 and IRS2 are required for downstream signaling through additional serine/threonine kinases and their phosphorylation by JNK results in decreased insulin signaling and insulin resistance (for a review see [57]).

2.5.3 – Previous Studies on the Effect of Pb on Metabolic Parameters in Rodents

Although there is little recent work that addresses the effect of Pb on metabolism in animal models, three papers published in the 1970s and 80s suggested that Pb exposure could induce hepatic insulin resistance in rats. Singhal et al. [152] reported that male rats treated with Pb acetate for 45 days (IP injection), showed a dose-dependent increase in amounts of the gluconeogenic enzymes PEPCK and G6Pase in the liver. Consistent with these Pb-induced increases in gluconeogenic gene expression, this study also reported a significant, dose-responsive increase in fasting blood glucose. A similar finding was reported by Stevenson et al. in young female rats exposed to 20, 40 or 80 ppm Pb for 56 days in drinking water [153]. These animals also showed a Pb dose-responsive increase in PEPCK and G6Pase enzyme levels in the liver and significantly elevated fasting blood glucose. Finally, Whittle et al. demonstrated that even small increases in blood lead levels from a control level of 7.9±1.4 µg/dL to 13.8±1.3 µg/dL, for 42 days caused a significant increase in expression of hepatic gluconeogenic enzymes in rats [154].

When taken together, these data suggest an interesting possibility that Pb exposure induces hepatic gluconeogenic gene expression, which would contribute to elevated blood glucose levels. However, these studies are somewhat dated and limited in scope - they did not include direct measurements of gene expression, and examined only minimal physiological parameters related to diabetes. Updated techniques may also allow for the investigation of the molecular mechanism behind the deleterious effects of Pb.

2.5.4 – The Potential Link Between Pb and Rev-erb-α

Emerging evidence indicates that components of the circadian clock machinery can regulate expression of metabolic genes, including PEPCK, G6Pase and PGC1-α [155]. This evidence was further support by data gathered from mice with mutated *Clock* genes showing abnormal gluconeogenic gene expression [156-158]. However, the mechanism that links metabolic gene expression to circadian clock rhythms remains poorly understood.

Recently, studies have shown that heme functions as an endogenous ligand for the orphan nuclear receptor Rev-erb-α (NR1D1), a known component of the circadian clock machinery [159]. Rev-erb-α is highly expressed in multiple tissues including liver, and functions as a heme-dependent transcriptional repressor [160-163]. Heme binding to Rev-erb-α suppresses the transcription of Rev-erb-α target genes including PEPCK, G6Pase and PGC1-α [155, 157, 158, 161, 162]. Thus, in the absence of heme, the gluconeogenic program is elevated [132]. The well described effect of Pb in the inhibition of heme biosynthetic enzymes [164-167] (described above) raises the

possibility that Pb exposure will contribute to elevated gluconeogenesis by reducing the suppressive effect of Rev-erb-α on gluconeogenic gene expression.

2.5.5 – Hypotheses and Aims of Pb Project

The overall goal of the Pb portion of my PhD project is to fully characterize the effect of Pb exposure on diabetes risk in metabolically stressed (obese) rodents, and to identify the molecular and cellular mechanisms by which it affects metabolic balance. The specific goals were to establish *in vivo* and *in vitro* models to study the physiological and molecular mechanisms by which Pb affects insulin sensitivity and glucose homeostasis. This was an initial step in a broader research objective to determine if there is a cooperative interaction between environmental toxicant exposure and additional stressors from the physical and social environment that promote metabolic instability and disease.

Together, these ideas formed the underlying hypothesis of my project, that in combination with metabolic stresses like obesity, Pb exposure increases diabetes susceptibility and severity. The work was divided into two specific aims: (1) Characterize the extent to which Pb exposure promotes diabetes in two well-established rodent models of metabolic disease (Zucker Diabetic Fatty [ZDF] rats and human Islet Amyloid Polypeptide [hIAPP] transgenic mice) and determine the dependence of Pb-induced diabetes on obesity and diet composition, (2) Characterize the mechanism by which Pb alters hepatic insulin sensitivity and gluconeogenesis.

Data generated from this work could have a significant impact on how we view the interaction of environmental toxicants with other behavioral and lifestyle stressors in

humans, and may provide the impetus to carry out epidemiologic studies to assess the risks of toxicant exposure in metabolically & nutritionally stressed populations. If such a link was observed in the human population, the animal models developed here may prove useful in developing therapies to reduce the deleterious effects of Pb exposure. Finally, the model systems established in this study will be of general utility for research on the interaction of other environmental toxicants and metabolically stressed individuals.

Chapter 3 – Materials and Methods

3.1 – Zucker Diabetic Fatty Rats

Female Zucker Diabetic Fatty rats (ZDF-Lepr^{fa}/Crl; stock 370 (OBESE)) 6 weeks of age, were purchased from Charles River Laboratories, Portage, Michigan. Animal protocol (#A 07-16-08) for ZDF rats was approved by the Institutional Animal Care and Use Committee at Wayne State University.

Two groups (n=4) of female ZDF fa/fa rats were treated with 0.05% w/v Pb acetate (PbAc) in the drinking water, which has been previously determined to generate a blood lead level of approximately 40-50 μg/dL in rats [154]. Control animals received equimolar acetate in drinking water in the form of Na acetate (NaAc).

Animals were housed on paper bedding for the duration of the study because animals can ingest corncob bedding during a fast, which can alter fasting blood glucose measures. All animals were fed a standard chow diet (Purina 5008) from weeks 0 – 20, then switched to a high fat diet (RD 13004, 45% calories from fat diet, Research Diets, Inc.) from week 20 through termination (week 25). Food and water were offered ad lib. Body weights and food consumption were measured weekly. Food consumption was assessed by weighing the amount of food provided at the beginning of each week and subtracting the amount remaining at the end of the week. While this method is not as accurate a measure of food consumption as would be obtained in a metabolic cage, it can reveal gross differences in nutritional intake.

Fasting blood glucose (FBG) measures were obtained from whole blood using a Hemocue glucometer at weekly intervals (from the saphenous vein) after a 6 hour fast beginning at 4:00 am, until week 20 (start of high fat diet) when they were taken bi-

weekly. After the fasting blood glucose measurement was completed, a total of 400µL of blood was collected and mixed with 3.73µL of 0.5M EDTA, to attain a final concentration of 4.67mM EDTA per sample, placed on ice immediately and spun in 4°C centrifuge at 8,500g for 20 minutes. Plasma was removed from packed RBCs and placed into new 0.5mL centrifuge tube in 25µL aliquots. Both plasma and packed RBCs were place into storage at -80°C to be utilized for further metabolic measures (See Table 2.1 for a list of metabolic measures).

Oral glucose tolerance tests (OGTTs) were conducted on each animal once every four weeks after a 6 hour fast beginning at 4:00 am. Animals were given a bolus of 50% glucose equivalent to 2mg/g body weight, by oral gavage and blood glucose was measured using a Hemocue glucometer from 5µL of blood removed from the saphenous vein. Time points were at 0 (prior to glucose bolus), 15, 30, 60 and 120 minutes.

Blood Pb levels in these samples were determined using standard assay protocols for measuring patient blood Pb levels at Detroit Medical Center University Laboratories by atomic absorption spectrometry. Packed red blood cells were resuspended to a final volume of 2mL in 0.9% physiological saline and measured on a Hitachi Z-2000 with Zeeman background correction.

Animals were sacrifice at 25 weeks following a 6 hour fast/ 2 hour re-feeding protocol by CO_2 asphyxiation and exsanguination by cardiac puncture. Relevant tissues were removed, subdivided and placed in 4% buffered formalin at 4ºC, flash-frozen or frozen in OTC (See Table 2.1 for a list of tissues).

3.2 – hIAPP Transgenic Mice

Dr. Steven E. Kahn (Dept. of Veterans Affairs, Puget Sound Health Care System, Seattle, WA) generously supplied hIAPP transgenic male C57 Black 6 (C57Bl6) mice [168]. Females, 5-6 weeks old C57Bl/6J mice (stock no. 000664) and males, 6 weeks old DBA/2J (stock no. 000671) were purchased from Jackson Laboratories (Bar Harbor, ME). Animal protocol (#A 08-15-10) for hIAPP mice was approved by the Institutional Animal Care and Use Committee at Wayne State University.

Four hIAPP transgenic male C57Bl6 mice were bred with four female C57Bl/6J mice. Female progeny were weaned at 3 weeks of age, tail-clipped for genotyping and ear notched for identification. Female progeny found to be transgene positive were then bred to male DBA/2J mice. DBA/2J background was utilized to provide a mouse model with increased insulin secretion, as insulin and islet amyloid polypeptide (IAPP) are co-secreted this model was expected to experience increased amyloid plaque formation (discussed further in Chapter 6). Male progeny were weaned at 3 weeks of age, tail-clipped for genotyping and ear notched for identification. Transgenic male progeny were separated and utilized for this study as previous studies observed a negative influence of estrogen on expression of the hIAPP transgene [169, 170] . Breeding and genotyping were conducted in the laboratory of Dr. Paul Stemmer.

Four groups of male hIAPP mice were established. Two groups (n=16) were treated with 0.02% w/v PbAc (“high treated”) or equimolar NaAc (“high control”) in the drinking water. The other two groups (n=12) were treated with 0.005% w/v PbAc (“low treated”) or equimolar NaAc (“low control”) in the drinking water. Animals began PbAc or NaAc treatment at 10 weeks of age.

Animals were housed on paper bedding for the duration of the study because animals can ingest corncob bedding during a fast, which can alter fasting blood glucose measures. All animals were fed a standard chow diet (Purina 5009) until weaned, then placed on a high fat chow diet (Autoclavable Mouse Breeder Diet 5021, Purina) containing 9% fat w/w (45% calories from fat) through termination (week 45). Food and water were offered ad lib. Body weights and food consumption were measured weekly. Food consumption was assessed by weighing the amount of food provided at the beginning of each week and subtracting the amount remaining at the end of the week. While this method is not as accurate a measure of food consumption as would be obtained in a metabolic cage, it can reveal gross differences in nutritional intake.

Fasting blood glucose (FBG) levels were measured with a Hemocue glucometer from whole blood obtained at bi-weekly intervals (from the saphenous vein) after an 8 hour fast beginning at 8:00 pm. A total of 200µL of blood was collected and mixed with 18.6µL of 0.05M EDTA to attain a final concentration of 4.67mM EDTA per sample and placed on ice immediately. A 20µL aliquot of whole blood was separated for blood Pb level measurements and the remaining sample was spun in 4°C centrifuge at 2,500g for 20 minutes as soon as possible. Plasma was removed from packed RBCs and placed into new 0.5mL centrifuge tubes in 25µL aliquots. Both plasma and packed RBCs were place into storage at -80°C to be utilized for further metabolic measures (See Table 2.1).

Oral glucose tolerance tests (OGTTs) were conducted on each animal, to establish baseline (time 0) glucose tolerance and at 6 months and 9 months of treatment after an 8 hour fast begun at 8:00 pm. Animals were given a solution of 30%

glucose equivalent to 1.5mg/g body weight, administered as a bolus via balled gavage needle. Blood glucose was measured using a Hemocue glucometer from 5µL of whole blood removed from the saphenous vein. Time points were 0 (prior to glucose bolus), 15, 30, 60 and 120 minutes.

Blood Pb levels were assessed by Dr. Paul Stemmer and Steven Jones using the ICP-MS facilities in the Biochemistry Department at Wayne State University School of Medicine. See section 3.5 below for specific protocol.

Sacrifice at 45 weeks was by CO_2 asphyxiation followed by cervical dislocation and exsanguination by cardiac puncture. Relevant tissues were removed by sterile technique, washed with a solution of ice cold PBS (1x) plus 0.01M EDTA, subdivided, then either placed in 4% buffered formalin at 4°C, freeze-clamped or flash-frozen in OTC (See Table 2.1 for a list of tissues).

Table 2.1 – Post-Termination Testing and Tissue Protocols

Blood parameters		
Blood Pb levels	Termination (rats) Biweekly (20µl, mice)	DMC-UHL (packed RBCs) ICP-MS facility at WSU SOM (whole blood)
Fasting blood glucose	Weekly (4 µl)	Glucometer (Hemocue) from whole blood
Insulin	Weekly (20 µl)	Elisa (Millipore)
Glucose tolerance (OGTT)	Every 4 weeks (rats) 0, 6 mo, 9 mo (mice)	Glucometer (Hemocue) from whole blood
Liver Tissue Parameters		
Liver histology	At termination	H & E, Masson's Trichrome, Periodic Acid Schiff (fixed sections)
Pancreatic histology	At termination	H & E, Masson's Trichrome, Aldehyde Fuchsin, Congo Red (mice only) (fixed sections)
Triglyceride content	At termination	TG assay (kit from Cayman Chemicals)
PEPCK, G6Pase, PGC1-□ (mRNA & protein)	At termination	qRT-PCR and western blots; from frozen liver
Additional tissue samples for future analysis		
Liver samples for RNA profiling	At termination	From frozen liver tissue
Pancreas	At termination	Frozen in OTC for histology
Skeletal Muscle (gastroc. & soleus)	At termination	Flash- frozen for RNA and protein analysis
Adipose: subQ (inguinal), abdominal (retroperitoneal)	At termination	Flash-frozen

3.3 – Genotyping

Genotyping of hIAPP mice was done using DNA extracted from tail clip, using a protocol provided by Dr. Rebecca L. Hull's laboratory (see reference [168]) and the previously published protocol by Laird et.al. [171]. Briefly, 0.5 cm of clipped mouse tail was digested with protease overnight, and DNA was ethanol precipitated from the clear supernatant after centrifugation. Genotyping was carried out by Ms. NamHee Shin in Dr. Paul Stemmer's lab.

3.4 – Tissue Processing and Histological Staining

Tissues were fixed with 4% buffered formalin at 4°C; paraffin embedded, sliced in 5µm sections, affixed to glass slides and allowed to dry. Slides were then stained with Hematoxylin and Eosin, Aldehyde Fuchsin, Periodic Acid-Schiff, Congo Red or Masson's Trichrome, all histological supplies were purchased from American MasterTech Supplies.

3.4.1 – Deparaffinization and Dehydration

To remove paraffin prior to staining, slides were put through 2 changes of 100% Xylene for 3 minutes each, followed by 2 changes of 100% EtOH for 3 minutes each, 2 changes of 95% EtOH for 3 minutes each, one change of 75% EtOH for 3 minutes, one change of 50% EtOH for 3 minutes and running tap water for 1 minute. Slides were then treated with the staining protocol of choice followed by dehydration and mounting.

To dehydrate after conclusion of staining, slides were dipped into 95% EtOH, followed by 3 changes of 100% EtOH, once into 1:1 100% EtOH:100% Xylene and 2

changes of 100% Xylene. Coverslips were mounted with ClearMount (American MasterTech) and allowed to dry for 72 hours undisturbed.

3.4.2 – Hematoxylin and Eosin Staining

After deparaffinization, the American MasterTech protocol for Harris' Hematoxylin and Eosin (Item# KTHNEPT) procedure was followed. Both pancreas and liver slides were placed in Harris' Hematoxylin for 5 minutes followed by running water rinse for 1 minute. Slides were then dipped into Differentiating Solution 3 times, followed by running water rinse for 1 minute. Slides were placed in Bluing Solution for 35 seconds, rinsed in running water for 3 minutes then moved to 70% EtOH for 1 minute. After 60 seconds in Eosin Y Stain, slides were dehydrated, coverslips mounted and dried.

3.4.3 – Aldehyde Fuchsin Staining

After deparaffinization, the American MasterTech protocol for Aldehyde Fuchsin (Item# STAFU) procedure was followed. Pancreas sections were placed in Aldehyde Fuchsin Stain for 2 hours, rinsed with 70% EtOH, placed in Light Green Counterstain for 3 minutes then dehydrated, coverslips mounted and dried.

3.4.4 – Periodic Acid-Schiff Staining

After deparaffinization, the American MasterTech protocol for Periodic Acid-Schiff (Item# KTPAS) procedure was followed. Liver sections were placed in 0.5% Periodic Acid for 7 minutes, rinsed quickly in distilled water then placed in Schiff's Solution for 15 minutes. Slides were removed from Schiff's Solution, rinsed in running water for 5

minutes, placed in Modified Mayer's Hematoxylin for 2 minutes then rinsed in running water for 3 minutes. Slides were then placed in to Light Green Counterstain for 10 minutes, rinsed with 70% EtOH, dehydrated, coverslips mounted and dried.

3.4.5 – Congo Red Staining

After deparaffinization, the American MasterTech protocol for Congo Red (Item# KTCRE) procedure was followed. Pancreas sections from hIAPP mice were placed in Congo Red Amyloid Stain for 2 hours, differentiated in 1% Sodium Hydroxide for 30 seconds then rinsed in running water for 5 minutes. Slides were then placed in to Modified Mayer's Hematoxylin for 3 minutes, rinsed in running water for 1 minute, dehydrated, coverslips mounted and dried.

3.4.6 – Masson's Trichrome Staining

After deparaffinization, the American MasterTech protocol for Masson's Trichrome (Item# KTMTR) procedure was followed. Pancreas and liver sections were placed in to Bouin's Fluid and left overnight at room temperature. The following day, they were left in running tap water until the tissue was colorless. The slides were then placed into Weigert's Hematoxylin for 5 minutes, rinsed in running water, placed in Biebrich Scarlet-Acid Fuchsin for 15 minutes and rinsed in distilled water. They were placed into Phosphomolybdic/Phosphotungstic Acid for 15 minutes, then moved directly to Aniline Blue Stain for 10 minutes, rinsed in distilled water, placed in 1% Acetic Acid for 5 minutes, dehydrated, coverslips mounted and dried.

3.5 – Blood Pb Analyses

The protocol for blood Pb analyses was designed and carried out by Steven Jones, under the supervision of Dr. Paul Stemmer using the ICP-MS facilities in the Biochemistry Department at Wayne State University School of Medicine.

Whole blood was collected from the saphenous vein and 20µL transferred to Eppendorf tubes and stored at -80°C. Samples were diluted with two volumes of 0.1% TritonX-100 added to the storage tube to ensure the protein was dissolved. The clear samples then were further diluted with 17 volumes of 2% nitric acid spiked with 1 ppb rhenium (Re) for a final dilution of 20 fold. Solutions were clear of obvious precipitates prior to addition of the nitric acid and proteins that were soluble in the triton solution precipitated upon the addition of the nitric acid. The precipitated protein samples were incubated with the nitric acid for 24 hours then diluted for analysis.

Precipitates were pelleted by centrifugation and supernatants removed to a fresh tube. Samples from animals that had not received Pb were diluted 3 fold while samples from Pb-treated animals were diluted 20 fold for a final dilution of 60 or 400 fold respectively. Standards containing 0, 0.1, 0.3, 0.5, 0.8 and 1.0 ppb Pb in 2% nitric acid with 1 ppb rhenium were run before and after analysis of the samples. The analysis was carried out using a PerkinElmer SCI EX ICP-MS (ELAN 9000). The Pb and Re standards were purchased from Acros Organics. The Pb was 2% in HNO_3 and the Re was 1 mg/ml in water.

3.6 – Microscopy

Histological imaging was completed utilizing a Leica D4000b Bright-field Microscope (Fisher Scientific) with Leica Digital Imaging Software (Fisher Scientific) housed at the Wayne State University Department of Anatomy and Cell Biology Imaging and Histopathology Core.

3.6 – Triglyceride Assay

Triglyceride Colorimetric Assay Kit (Item# 10010303, Cayman Chemicals) was used according to manufacturer's instructions to assess tissue triglyceride levels. Briefly, 350mg of liver tissue from both ZDF rats and hIAPP mice was homogenized in 1x Standard Diluent Assay Reagent, centrifuged at 10,000g and 4ºC, supernatant transferred to new tubes and stored on ice. Samples were diluted 1:9 in Standard Diluent Assay Reagent (per protocol recommendations for tissue samples). 10µL per sample were loaded in duplicate and read at 540nm absorbance on VersaMax plate reader with SoftMax Pro 3.0 software (Molecular Devices). Regression analysis of data was completed with GraphPad Prism 5.

3.7 – Insulin ELISA

Rat/Mouse Insulin ELISA Kit (Catalog# EZRMI-13K, EMD Millipore) was used according to manufacturer's instructions to assess plasma insulin levels in both ZDF rats and hIAPP mice. Briefly, frozen plasma samples from both ZDF rats and hIAPP mice were thawed, centrifuged at 2,500g at 4ºC, and the supernatants transferred to new tubes and stored on ice. Duplicate 20µL samples were loaded into the prepared

96-well plate and combined with 80µL of Detection Antibody and allowed to incubate for 2 hours. After the incubation, the plate was washed, 100µL of Enzyme Solution added and incubated for 30 minutes. After a series of 6 final washes, 100µL of Substrate Solution was added, followed by 100µl of Stop Solution. The samples were then read at both 450nm and 590nm absorbance on VersaMax plate reader with SoftMax Pro 3.0 software (Molecular Devices). Regression analysis of data was completed with GraphPad Prism 5.

3.8 – Cell Culture

Primary rat hepatocytes (PRH) were obtained from Wayne State University's Institute for Environmental Health Sciences Cell Culture Core. PRH cells were plated at a density of 9 x 10^5 cells per well on collagen-coated (Rat tail collagenase, Invitrogen) 6-well plates (Denville), in 10nM insulin-containing serum-free DMEM (HyClone) and allowed to recover overnight. PRH cells were changed to insulin-free DMEM 2 hours prior to initiation of treatment protocols.

Human hepatoma (HepG2) cells were cultured in 100mm dishes with high glucose DMEM (HyClone), supplemented with 10% FBS and 1% (v/v) penicillin/streptomycin. HepG2 cells were plated at a density of 1 x 10^5 cell per well in 6-well plates for gene expression experiments.

FAO rat hepatoma cells, cultured in RPMI 1640 (Invitrogen) supplemented with 10% FBS and 1% (v/v) penicillin/streptomycin. FAO cells were plated at a density of 4.5 x 10^5 cell per well in 6-well plates for gene expression experiments and 7.75 x 10^5 in 60mm dishes for western blot experiments.

H4IIe rat hepatoma cells, cultured in high glucose DMEM (HyClone) supplemented with 5% FBS, 5% BCS and 1% (v/v) penicillin/streptomycin. H4IIe cells were plated at a density of 6 x 10^4 cell per well in 6-well plates for gene expression experiments. All cell cultures were maintained at 37ºC with a 5% CO_2 atmosphere.

3.9 – Western Blotting

Cells were harvested with RIPA buffer, sonicated and cleared (centrifuged for 30 minutes at 13,000g and 4°C). SDS-PAGE gels (pre-cast, Pierce) were loaded with 50µg protein per lane, one lane with 5uL Easy-Run REC-pre-stained marker (Fisher Scientific) and run for 1 hour at 40mA. Transfer to nitrocellulose membrane was carried out overnight at 4ºC and 40mA in a Bio-Rad Mini Protean 3 transfer device. Nitrocellulose membranes were removed from transfer apparatus, Ponceau stained to confirm transfer of proteins, washed with TBS-T, blocked with 5% milk in TBS-T for 2 hours followed by overnight incubation with phospho-AMPK^{Ser79}, phospho-ACC^{Thr172} (1:1000 in 5% BSA in TBS), total-AMPK or total-ACC (1:1000 in 5% BSA in TBS-T) antibodies (Cell Signaling Technologies). After 3 washes in 1x TBS-T buffer, membranes were incubated with goat-anti-rabbit secondary antibody (1:1000 in 5% milk in TBS-T, Cell Signaling Technologies) at room temperature for 1 hour. TBS-T was utilized for washes and ECL (Pierce) was utilized for chemiluminescence. Membranes were stripped, re-blocked and incubated for loading control with GAPDH.

3.10 – Gene Expression Analyses

3.10.1 – In Vivo Gene Expression

Total RNA was extracted a small piece of flash-frozen liver tissue using SpinSmart RNA Purification System with on-column tissue homogenizer kit (CM-610-50, Denville Scientific). RNA quality and quantity was assessed by Nanodrop absorbance at 260/280 (Thermo Scientific) and mRNA was reverse-transcribed using High-Capacity cDNA Reverse Transcription Kit (Applied BioSystems). A total of 100ng of cDNA was analyzed in a 20µL qRT-PCR reaction (TrueAmp SYBR Green, Smart Bioscience, Inc.) with 300 nM of primers. Gene expression data were normalized to the house keeping gene peptidylprolyl isomerase A (PPIA) using the 2-delta-delta C(T) method ($2\text{-}\Delta\Delta C^t$) described by Livak and Schmittgen [172]. Sequences for primers used for qRT-PCR are listed in Table 2.2.

Table 2.2 – Primer Sequences Used for qRT-PCR

	Forward (5'-3')	Reverse (5'-3')
hIAPP Mouse Primers		
mG6Pase	5'-CTGTCTGTCCCGGATCTACC-3'	5'-GCGCGAAACCAAACAAGAAG-3'
mPEPCK	5'-AAGCGGATATGGTGGGAACT-3'	5'-CTGCGGCCAGGTATTTCTTC-3'
mPGC1-α	5'-ACGCGGACAGAATTGAGAGA-3'	5'-GTTTCGTTCGACCTGCGTAA-3'
mPPIA	5'-GGTCCTGGCATCTTGTCCAT-3'	5'-ATGCCTTCTTTCACCTTCCCA-3'
ZDF Rat Primers		
	Forward (5'-3')	Reverse (5'-3')
rG6Pase	5'-TGAATCACTTCCGGTCTCCTGTCC-3'	5'-TCCCGATCCTTCCTTCTCTTCCTAG-3'
rPEPCK	5'-GGGGTGCATGAAAGGCCGCA-3'	5'-ATCCGCATGCTGGCCACCAC-3'
rPGC1-α	5'-GAGAGGCAGAAGCAGAAAGCAATTG-3'	5'-CATCATCCCGCAGATTTACGGTG-3'
rPPIA	5'-CGGCTGATGGCGAGCCCTTG-3'	5'-GCCAAATCCTTTCTCCCCAGTGC-3'

3.10.2 – In Vitro Gene Expression

Cells were washed with cold PBS after removal of media, lysed with direct application of Tri reagent (Sigma) and placed on ice. Total RNA was extracted from cell lysates, mRNA quality and quantity was assessed by Nanodrop absorbance at 260/280

(Thermo Scientific) and mRNA was reverse-transcribed using High-Capacity cDNA Reverse Transcription Kit (Applied BioSystems). Gene expression was analyzed by semi-quantitative PCR using conditions optimized for each individual primer set. A total 100ng of cDNA, 10nM dNTPs (Denville), 1x Choice *Taq* (Denville) and 200 nM of gene-specific primers (Integrated DNA Technologies, Inc.) were analyzed in a 20µL reaction (MyCycler, Bio-Rad). Each primer was run for a specific number of cycles: PEPCK (23 cycles) and PPIA (23 cycles). Primers were designed to span exon junctions to minimize signal from potential genomic DNA contamination. Final PCR products were run on a 1.5% agarose gel (0.5µg/mL EtBr) in 1x TAE at 130mV for 80 minutes and imaged with a ChemiDoc XRS (Bio-Rad). Band densities were analyzed using the Image J software program and a specific sized selection box that limited the number of pixels were analyzed. Gene expression data were normalized to the house keeping gene PPIA. Primer sequences used for semi-quantitative PCR are listed in Table 2.3.

Table 2.3 – Primer Sequences Used for Semi-Quantitative PCR

	Forward (5'-3')	Reverse (5'-3')
rPEPCK:	5'-GCAGGCTGGCTAAGGAGGAA-3'	5'-GGCCAGGTTGGTTTTCCCAC-3'
rPPIA:	5'-GTCGCGTCTGCTTCGAGCTGTTTG-3'	5'-GTGTGAAGTCACCACCCTGGCACA-3'

3.11 – HOMA2-IR Analyses

Homeostatic Model Assessment 2 (HOMA2-IR) calculator was accessed on 30 June 2013 from: http://www.dtu.ox.ac.uk/index.php?maindoc=/homa/. HOMA2-IR is a computer modeling program that calculates a corrected nonlinear estimation of insulin resistance (IR) and percent beta-cell function (% β-cell function). A number of empirical equations (nonlinear) are utilized by the HOMA2-IR computer program to predict numerical values from paired fasting blood glucose and insulin samples. Paired fasting

blood glucose and insulin measurements from weeks 0, 14, 26, 40 and 45 were converted to mmol/L and pmol/L, respectively. Data are estimated numerical values for the calculations of IR and % β-cell function.

3.12 – Statistical Analyses

Statistical analyses, given in detail in figure legends, were performed with GraphPad Prism 5 and SPSS. Data are presented as mean ± SEM. Statistical significance between two groups was determined by unpaired t-test. Comparison among groups was performed using two-way repeated measures ANOVA or two-way mixed-design ANOVA and corrected for violation of sphericity with the Greenhouse-Geisser epsilon.

Chapter 4 – Dioxins

4.1 – Hypotheses and Aims of Dioxin Project

It is clear that dioxins are a persistent environmental contaminant with the ability to influence glucose metabolism and possibly lead to alterations in diabetes susceptibility.

Because of this possibility, the following hypotheses were formed: (1) Exposure to dioxin alters hepatic gluconeogenic gene expression. (2) Dioxin-mediated AMPK activation contributes to the suppression of hepatic glucose production. These hypotheses were tested by two specific aims: (1) Characterize the effect of dioxin on hepatic gluconeogenic gene expression. (2) Determine the molecular mechanism(s) by which dioxins alter PEPCK gene expression.

4.2 – Characterize the Effect of Dioxin on Hepatic Gluconeogenic Gene Expression

As described in the background section, dioxin exposure in humans has been linked to changes in diabetes-related metabolic parameters [35, 36, 45, 138, 173]. However, its specific effect on hepatic glucose production (a key element in whole-body

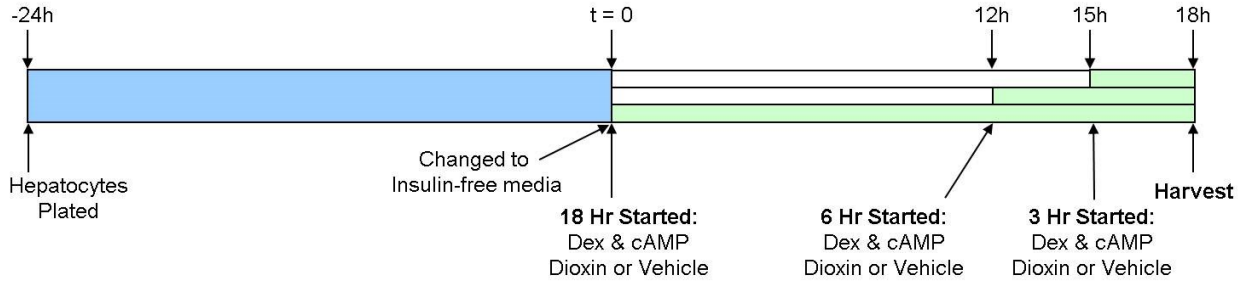

Fig. 4.1 – Experimental paradigm utilized for all gene expression studies.
Cells were plated at t = -24 hours and changed to insulin-free media when 18 hour treatment was begun at t = 0, 6 hour treatment was begun at t = 12 and 3 hour treatment was begun at t = 15.

glucose homeostasis) is not clearly understood. To determine if dioxin affects expression of the key genes involved in hepatic glucose production, primary rat hepatocytes (PRH) and FAO rat hepatoma cells were treated with dioxin and mRNA levels of gluconeogenic genes (PEPCK and G6Pase) were measured. Treatment times (Figure 4.1) and doses were established in a series of pilot optimization experiments (data not shown). As seen in Figure 4.2, 10nM dioxin was sufficient to suppress both PEPCK and G6Pase by greater than 2-fold at 6 hours and by 4- and 10-fold respectively at 18 hours over the 3 hour vehicle control. In contrast, expression of CYP1A1 (a known AhR-regulated dioxin target gene) was strongly stimulated by dioxin treatment, confirming the presence of dioxin in the cells (Figure 4.2). These results demonstrate the ability of dioxin to suppress PEPCK and G6Pase, two key gluconeogenic genes involved in hepatic glucose homeostasis. Results from FAO cells (data not shown) mirrored those obtained from PRH cells.

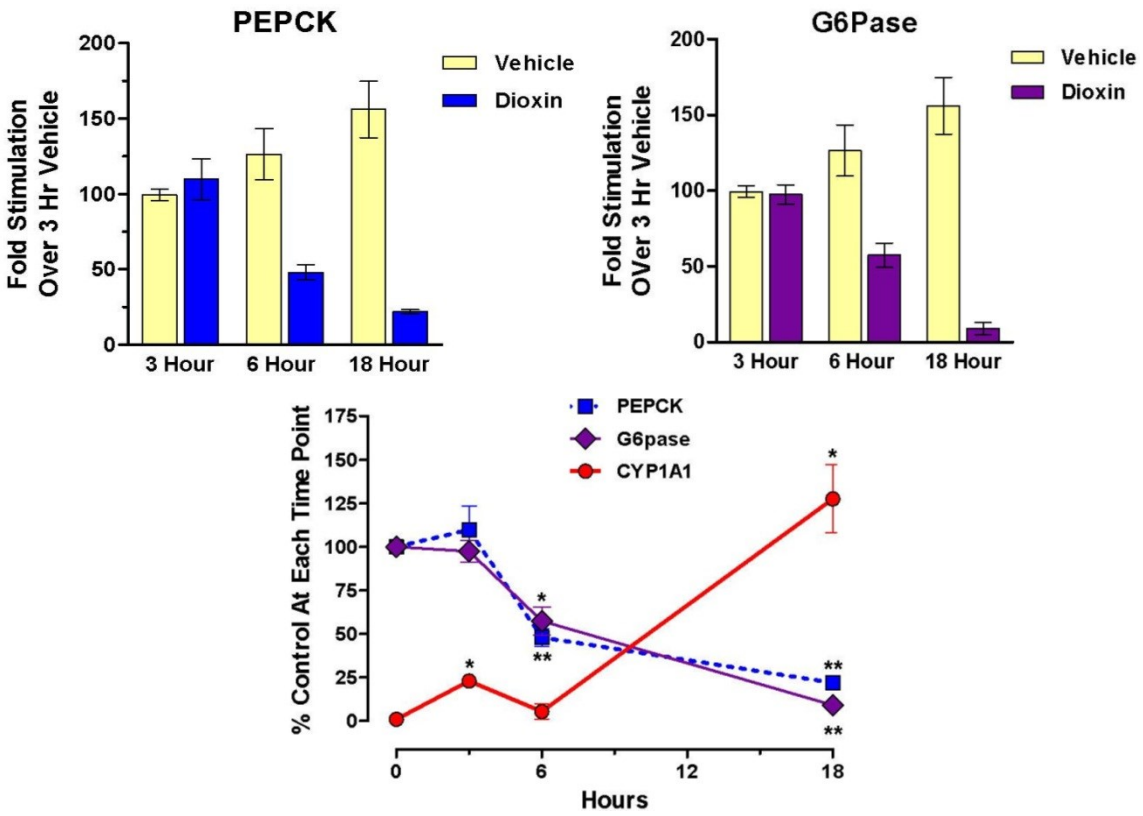

Fig. 4.2 – Dioxin exposure leads to increased suppression of both PEPCK and G6Pase over time.
PRH cells in insulin-free media were exposed to DMSO or 10nM dioxin as well as 1μM dex plus 1mM cAMP for 3, 6 and 18 hours and changes in gene expression were assessed by qRT-PCR. Data analyzed by students T test and are the means +/- SEM of three independent experiments, *p<0.05, **p<0.005.

4.3 – Determine the Molecular Mechanism by Which Dioxin Alters PEPCK Gene Expression

4.3.1 – Effect of AhR Inhibition on Dioxin-Mediated PEPCK Gene Suppression

The effects of dioxin are primarily mediated by activation of the Aryl hydrocarbon receptor (AhR) [48-54]. To determine if the dioxin-mediated suppression of PEPCK gene expression is dependent on activation of AhR, a pharmacological antagonist of the AhR, PD168641 (3'-methoxy-4-nitroflavone) was utilized [174, 175]. PRH cells were pretreated with 1μM PD168641 for 15 minutes prior to addition of 1μM dex and 1mM

cAMP and either DMSO or 10nM dioxin. Results demonstrated that PD168641 significantly inhibited the AhR at 24 hours, as evidenced by the nearly complete suppression of the stimulatory effect of dioxin on CYP1A1 mRNA levels, in the presence of PD168641 (Figure 4.3, right panel). In contrast, the suppression of PEPCK and G6Pase gene expression was essentially unaffected by the presence of PD168641, demonstrating that activation of the AhR was not the mechanism whereby dioxin suppressed gluconeogenic gene expression.

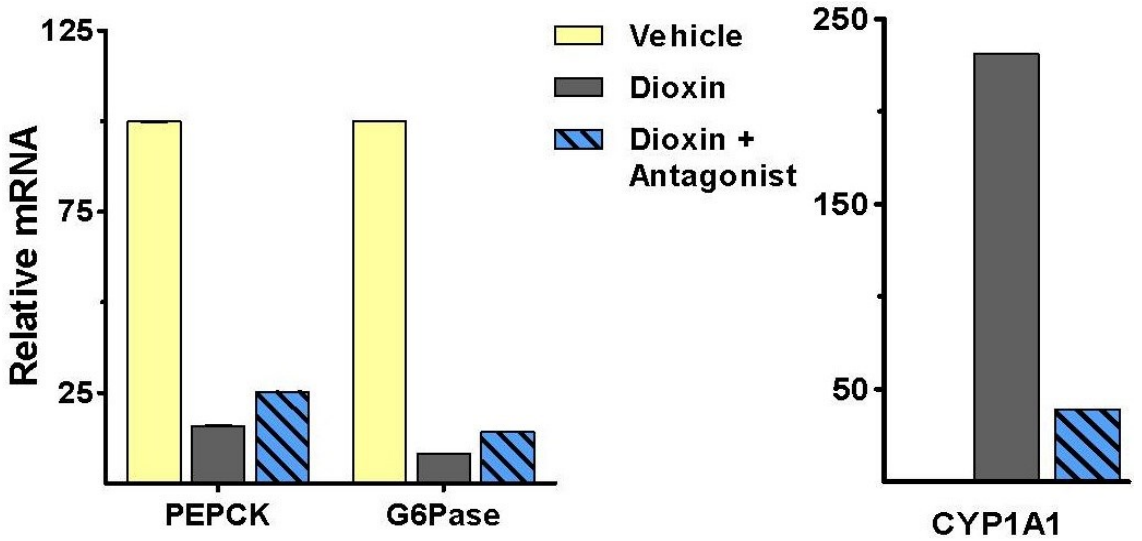

Fig. 4.3 – Effect of AhR inhibition on dioxin-mediated suppression of PEPCK gene expression.
PRH cells were pre-treated with PD 168641 (AhR antagonist) for 15 minutes. Cells were then subjected to treatment groups consisting of: Vehicle = DMSO, Dioxin = 1mM cAMP + 1μM dex + 10nM dioxin, Dioxin + Antagonist = 1mM cAMP + 1μM dex + 10nM dioxin + 1μM PD 168641 for 24 hours. Data are means of 2 independent experiments.

4.3.2 Dioxin is a Mild AMPK Activator

Most of the effects of dioxins are mediated by activation of the AhR. However, my findings and a previous study have suggested an alternative mechanism whereby dioxins may be suppressing PEPCK gene expression, via activation of AMPK [34].

AMPK is activated by phosphorylation and is known to mediate a down regulation of PEPCK gene expression [176, 177]. AMPK can be pharmacologically activated in PRH and other hepatoma cell lines by the AMP analog, 5-aminoimidazole-4-carboxamide-1-ß-D-ribofuranoside (AICAR) [178]. To determine if dioxin activates AMPK, (PRH) cells were exposed to either 50nM dioxin or 500µM AICAR for 1, 2 and 4 hours (see Figure 4.4 for experimental time line) and then analyzed by western blot. Results demonstrated that AMPK is phosphorylated and mildly activated in the presence of 50nM dioxin at 1, 2 and 4 hours (Figures 4.5 & 4.6). Secondarily, a downstream target of AMPK, Acetyl CoA Carboxylase (ACC), is also phosphorylated in cells exposed to dioxin, causing it to be inactivated [179]. These data demonstrate that dioxin activates AMPK, and suggests that its effects on gluconeogenic gene expression are mediated by this mechanism.

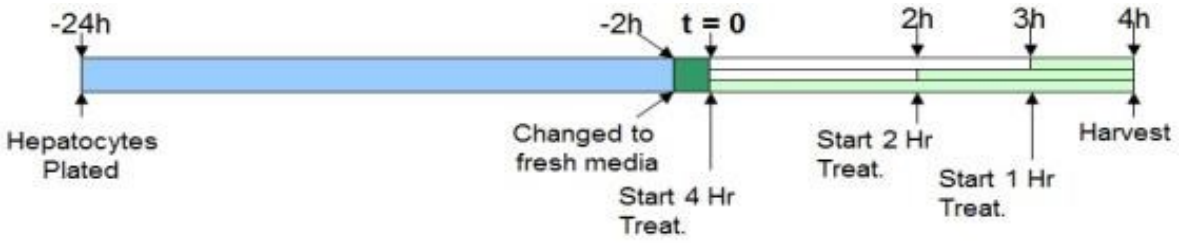

Fig. 4.4 – Experimental paradigm utilized for all protein expression studies.
Cells were plated at t = -24 hours and changed to insulin-free media two hours prior to beginning dioxin exposure. Cells were treated with 1µM dex and 1mM cAMP and either 50nM dioxin or 500uM AICAR.

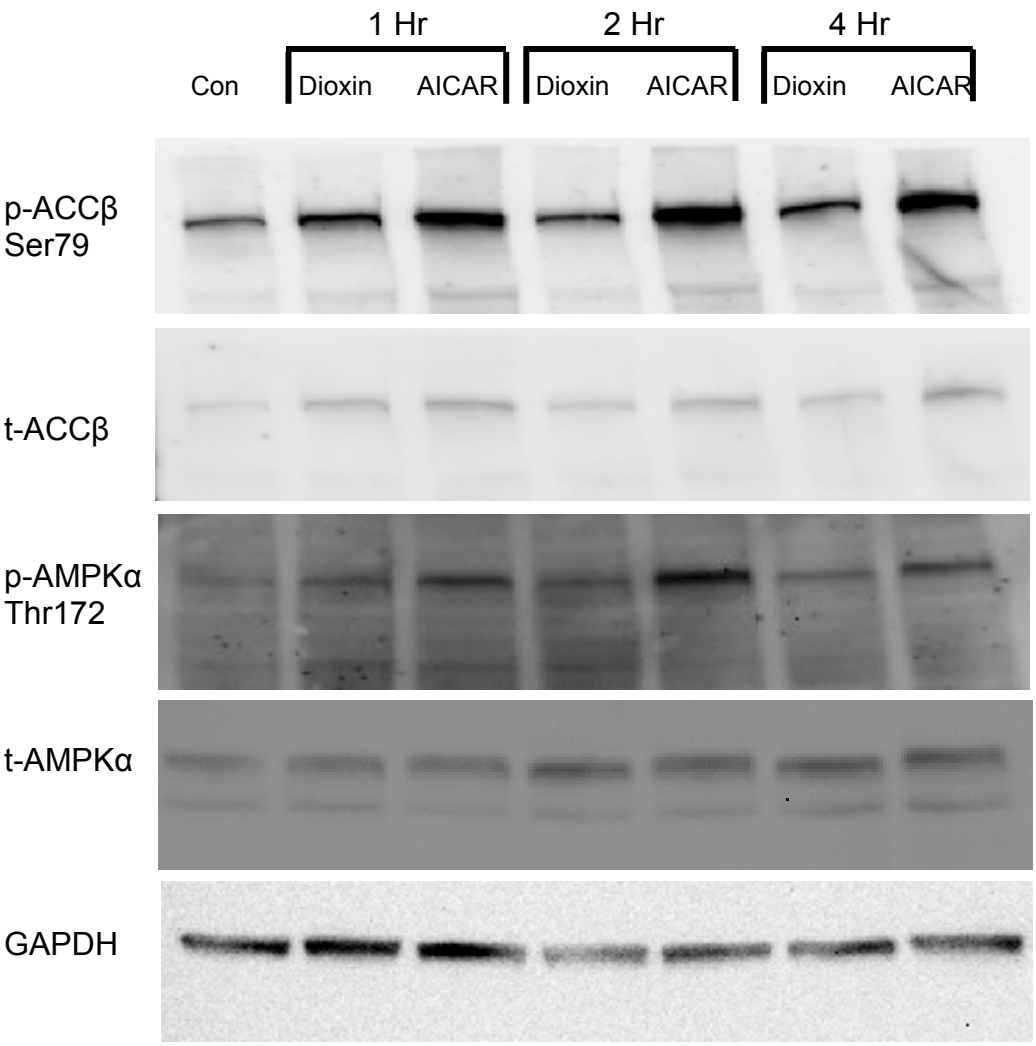

Fig. 4.5 – Dioxin is a mild AMPK activator.
Western blot showing changes in phosphorylation after dioxin or AICAR exposure. PRH cells treated for 1, 2 and 4 hours with either 50nM dioxin or 500μM AICAR. p-ACC = phospho-ACC, t-ACC = total ACC, p-AMPK = phospho-AMPK, t-AMPK = total AMPK.

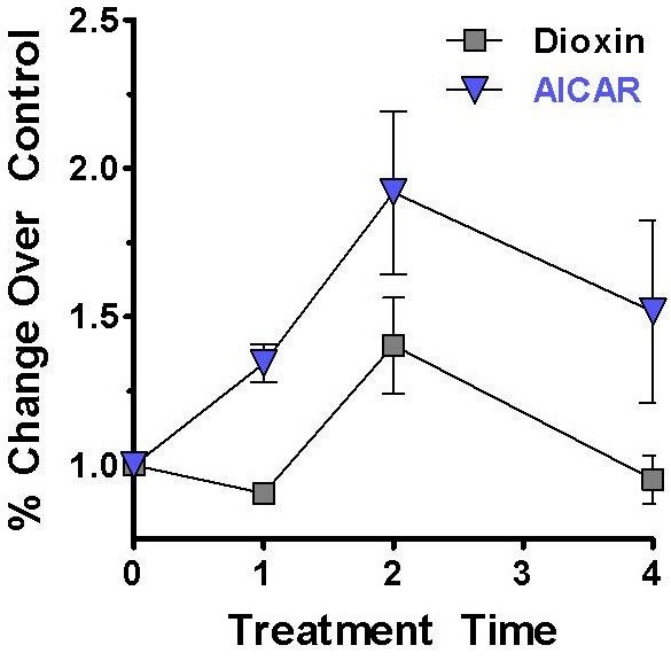

Fig. 4.6 – Quantification of AMPK activation by dioxin. AICAR is a known AMPK activator. PRH cells treated for 1, 2 and 4 hours with either 50nM dioxin or 500μM AICAR. There is a "nearly significant" ($p = 0.0505$) stimulation by dioxin observed at 2 hours. Data are the means +/- SEM of three independent experiments.

4.4 – Conclusions

The effects of dioxins are primarily mediated by activation of the aryl hydrocarbon receptor, AhR [48-54]. However, my data (figures 4.5 and 4.6) and a previously published study have suggested an alternative mechanism whereby dioxin may be suppressing PEPCK and G6Pase gene expression, via activation of AMPK [34]. AMPK is a cellular energy-sensing protein that is activated when AMP levels within the cell become high [141]. AMPK can be artificially activated in primary rat hepatocytes and other hepatoma cell lines by the pharmacological reagent, 5-aminoimidazole-4-carboxamide-1-ß -D-ribofuranoside (AICAR) resulting in AMPK phosphorylation. Acetyl CoA Carboxylase (ACC) stimulates the conversion of Aceyl CoA to Malonyl CoA (MCA)

which is a substrate for fatty acid (FA) synthesis and a potent inhibitor of the mitochondrial FA transporter carnitine palmitoyltransferase 1 (CPT1) [180]. Phosphorylated AMPK is known to phosphorylate and inactivate ACC, this leads to decreased fatty acid synthesis. Phosphorylated AMPK is also known to cause suppression of PEPCK and G6Pase gene expression, which would reduce hepatic glucose production [178]. Hepatic glucose production regulation by AMPK has been attempted in the clinical setting with the anti-diabetic drug metformin [181, 182]; this drug has been proposed to lower hepatic glucose production in diabetic patients via AMPK activation [183, 184]. The ability of AMPK to suppress the key gluconeogenic genes PEPCK and G6Pase demonstrates its importance as a regulator of hepatic glucose production and as a potential or possible target of dioxin.

We observed that dioxin treatment stimulated the AMPK signaling pathway (figures 4.5 and 4.6), which is known to have a suppressive effect on PEPCK gene expression [178]. The pharmacological reagent Compound C (6-[4-(2-Piperidin-1-yl-ethoxy)-phenyl)]-3-pyridin-4-yl-pyrrazolo-[1,5-a]-pyrimidine) has been shown to inhibit AMPK activation and could be utilized to ascertain if the suppressive effects of dioxin on PEPCK and G6Pase gene expression are mediated via dioxin-induced AMPK phosphorylation [182, 185].

Previously published studies suggested that while dioxin exposure increased peripheral insulin resistance it did not appear to cause fasting hyperglycemia or elevated hepatic glucose output [35, 39, 42, 45-47]. In concordance with these findings we observed that dioxin treatment caused a strong suppression of the expression of genes for the key gluconeogenic enzymes PEPCK and G6Pase (figures 4.2 and 4.3).

However, the effects of dioxin in our *in vitro* system appear to be mediated independently of the AhR (figure 4.3). There is a consensus among researchers that dioxin-induced toxicity, while primarily mediated via the AhR, may function via an AhR-independent pathway [186-188]. A recently published study demonstrated the ability of dioxin to suppress expression of the checkpoint protein Mad2 in AhR-deficient mice, suggesting a novel dioxin-induced AhR-independent signaling mechanism at work [189]. Additionally, AhR-independent effects of dioxin-induced toxicity include the ability of dioxin to induce expression of several mouse glutathione S-transferase genes that do not contain xenobiotic response elements (XREs), previously thought to be a necessity in eliciting a dioxin response [190]. And several independent studies have shown that dioxin targets T cells via both AhR-dependent and AhR-independent mechanisms resulting in suppression of the immune system [191-196].

While the effects on hepatic glucose production, AMPK and energy metabolism are not clearly understood, previous studies and my data suggest that, in addition to interacting with its well-characterized receptor AhR, dioxin may function in an AhR-independent manner to target and activate the metabolic kinase, AMPK. AMPK regulates a variety of metabolic pathways and its activation by an environmental toxicant would constitute a mechanistic link between toxicant exposure and the regulation of metabolism.

Chapter 5 – Lead Exposure Promotes Diabetes Development in Obese Rats

As described in the background section, little recent work addresses the effect of Pb on metabolic parameters *in vivo*. The data that do exist, when taken together, suggest that Pb exposure alters liver function, hepatic gluconeogenic gene expression and glycemia [152-154]. However, they do not address the potential pathological interactions between Pb exposure and concomitant risk factors such as obesity, which is strongly associated with diabetes and the metabolic syndrome [44]. The Pb-obesity interaction is a relevant issue in large sections of the US population where environmental stressors of over-nutrition (taking in more calories than the body needs) and an inactive or sedentary lifestyle are common and co-exist with exposure to persistent environmental toxicants, such as Pb.

The overall goal of the *in vivo* portion of my PhD work is to characterize the effect of Pb exposure on diabetes risk in metabolically stressed (obese) rodents. To explore the specific hypothesis that Pb exposure increases diabetes susceptibility in obese individuals, two well-established rodent models of obesity were utilized: the Zucker Diabetic Fatty (ZDF) rat and the high fat fed, human islet amyloid polypeptide (hIAPP), transgenic mouse (presented in Chapter 6). The experimental approach is designed to reproduce, as far as possible, effects of Pb exposure in the human population, where obesity and diabetes risk factors are common.

5.1 – Zucker Diabetic Fatty (ZDF) Rat Model

One of the standard rodent models used in diabetes research to study the physiology of obesity-related type 2 diabetes is the Zucker Diabetic Fatty (ZDF) rat. Originally derived from a colony of rats at Eli Lilly Research Laboratories and named after the scientists that discovered the fatty mutation, the ZDF (fa/fa) rat was established from a colony that had the diabetic characteristics of obesity and fasting hyperglycemia [197]. The genetic lesion that causes hyperphagia and obesity in ZDF rats is a mutation in the leptin receptor. Rats homozygous for this mutation (fa/fa) will become obese when fed regular chow. A specially formulated high fat diet leads to development of Type 2 diabetes in fa/fa animals. ZDF rats that are heterozygous (fa/+) tend to be lean and are often utilized as a non-diabetic control.

The diabetes phenotype differs in male vs. female ZDF rats. There are several advantages in utilizing female ZDF rats instead of the more commonly used male ZDF rats. Feeding commercially available rodent diets, such as Purina 5008, to female ZDF rats induces a prolonged insulin resistant but non-diabetic state. However, when fed the RD13004 high fat diet from Research Diets, Inc., they develop fulminate diabetes within approximately 1 month [198]. The progression of the female ZDF toward fulminate diabetes on the RD13004 diet is very well characterized and highly reproducible.

Unlike the male ZDF, the female possesses more robust β-cell compensation even though they share similar levels of insulin resistance. The β-cell compensation allows for maintenance of blood insulin levels for a much longer time frame and extends the time course of diabetes development. They can be initiated into this progressive

pathway at 6 weeks and be maintained in this insulin resistant state for up to 25 weeks of age. This large span of time provides an extended period in which these animals are obese, insulin resistant and non-diabetic, providing an excellent opportunity to observe subtle effects of Pb exposure on the rate of diabetes progression during the experimental time frame (Figure 5.1).

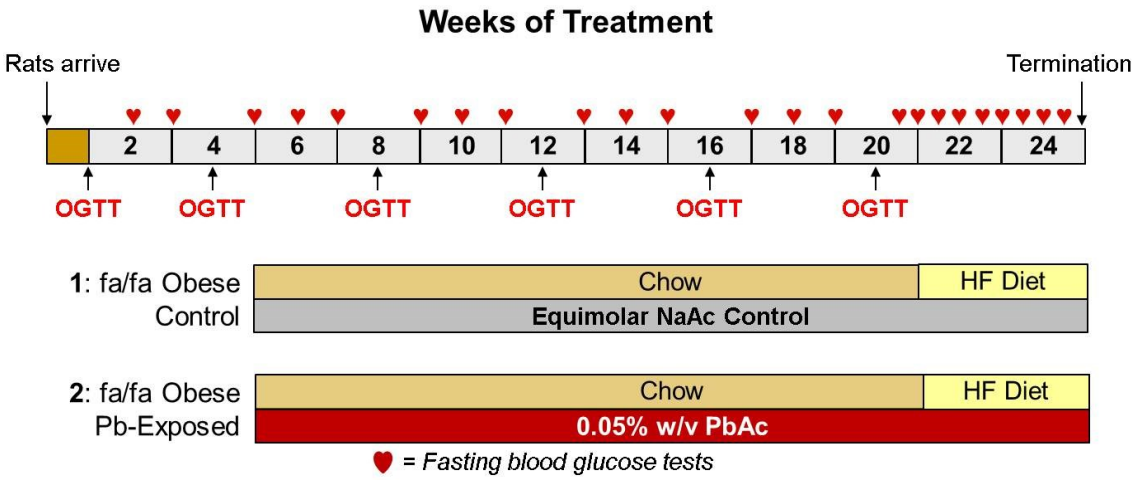

Fig. 5.1 – Experimental time frame for ZDF rats.
Rats arrived and were allowed to acclimate for one week. Weekly fasting blood glucose draws and monthly oral glucose tolerance tests (OGTTs) were performed as indicated. RD13004 high fat diet feeding was initiated at 20 weeks.

5.2 – ZDF Experimental Setup

The ZDF experiment consisted of two groups (n=4) of 6 week old female fa/fa rats, the characteristics of which were described in the previous paragraphs. Rats arrived and were allowed one week to acclimate prior to initiation of experimental protocols. One group was provided 0.05% w/v Pb acetate (PbAc) in drinking water (an amount previously determined to generate a blood lead level of approximately 40-50 µg/dL in rats [154]). The control group was provided an equimolar amount of NaAc in drinking water to balance the acetate intake of the two groups. Animals were fed a

standard chow diet (Purina 5008) until week 20, when they were switched to the RD13004 high fat diet (45% calories from fat diet) from Research Diets, Inc. Food and water were offered ad lib.

Body weight and food intake were measured weekly, blood was drawn weekly from fasted animals for blood glucose level determination. Oral glucose tolerance tests (OGTTs) were performed every 4 weeks. After 25 weeks of exposure, animals were sacrificed by CO_2 asphyxiation and exsanguination via aortic puncture. Immediately preceding termination, rats were fasted for 6 hours and then allowed a 2 hour re-feeding period. Pancreatic and liver tissues were removed and portions were placed in 4% buffered formalin at 4ºC, flash-frozen or frozen in OTC for histology and molecular analyses. Additional endpoint measures included plasma insulin and liver triglycerides.

5.3 – Basic Parameters of the ZDF Model

Pb exposure did not affect body weight (Figure 5.2) or food intake (Figure 5.3). To assess blood Pb levels generated by 0.05% w/v PbAc in drinking water (an amount previously determined to generate a blood lead level of approximately 40-50 µg/dL in rats [154]), Pb levels in packed red blood cells were measured by atomic absorption spectrometry at Detroit Medical Center – University Laboratories. The average blood Pb level of the Pb-treated rats, at termination, was determined to be 49µg/dL (Figure 5.4). The findings that there were no body weight or food intake differences suggest that there were no major effects of Pb on appetite or activity even at relatively high levels of exposure.

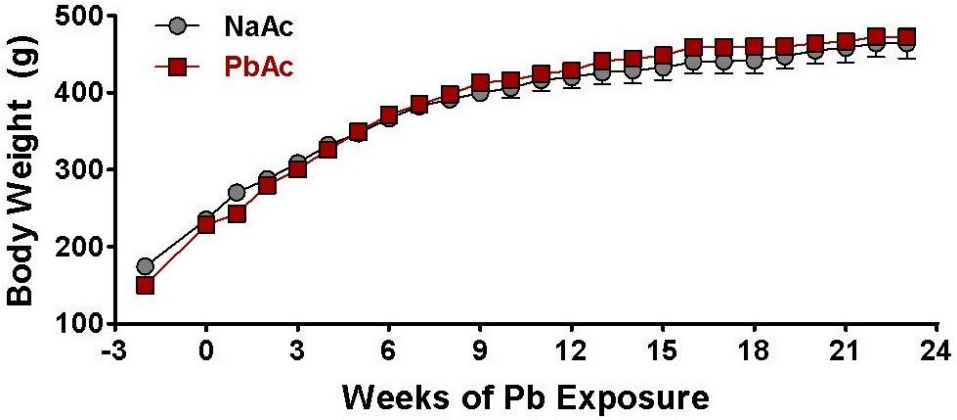

Fig. 5.2 – Body weight measures for ZDF rats.
There was no difference in body weight between groups. Data are means +/- SEM, weeks 0-8: both groups n=4, Weeks 9-25: Na n=4, Pb n=3.

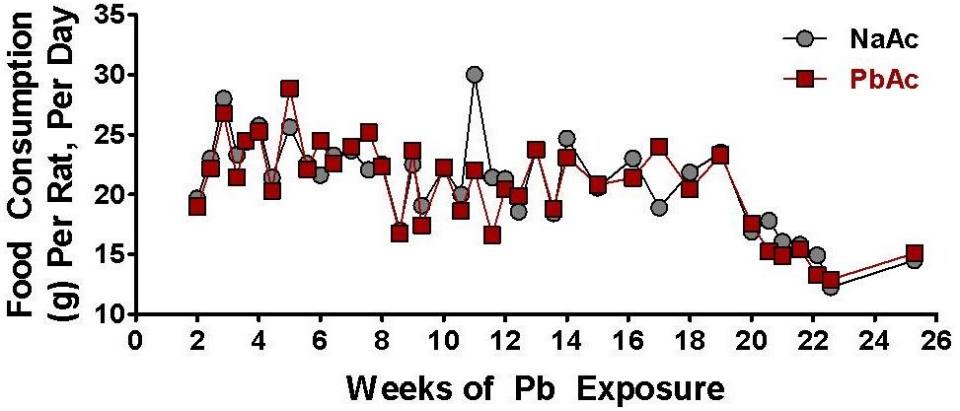

Fig. 5.3 – Food consumption measures for ZDF rats.
There was no difference in food consumption between groups. Data are means, +/- SEM, weeks 0-8: both groups n=4, Weeks 9-25: Na n=4, Pb n=3.

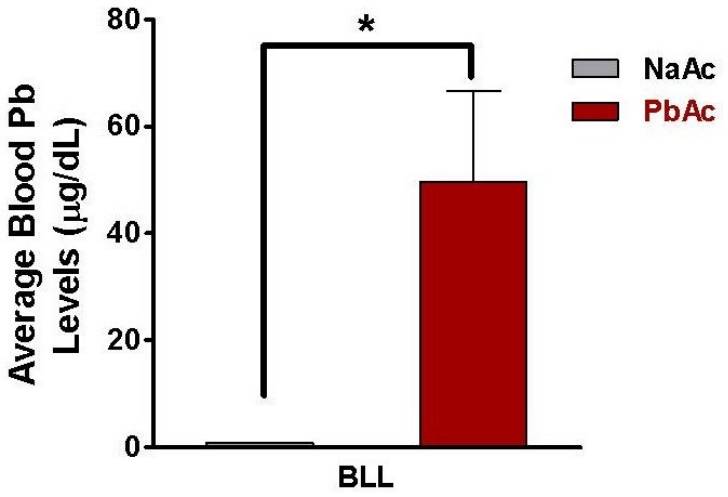

Fig. 5.4 – Blood Pb levels for ZDF rats at termination. ZDF rats treated with 0.05% w/v PbAc or equimolar NaAc in drinking water. Termination BLL average was 49µg/dL. Analysis completed by students T test. Data are means with SEM, * $p<0.05$, Na n=4, Pb n=3.

5.4 – Pb Exposure Effects on Metabolic Parameters

Elevated fasting blood glucose is one of the hallmarks of type 2 diabetes. Although female ZDF fa/fa rats fed a chow diet develop insulin resistance, they are able to maintain relatively normal blood glucose levels due to efficient compensation of insulin output by the endocrine pancreas. However, as shown in Fig. 5.5, exposure to Pb induced a persistent fasting hyperglycemia starting at 10 weeks exposure, compared to control rats who maintained near normal levels of fasting blood glucose throughout the study. Repeated measures ANOVA calculations demonstrated a significant deviation in fasting blood glucose levels between the Pb and control groups beginning at 8 weeks of exposure through termination.

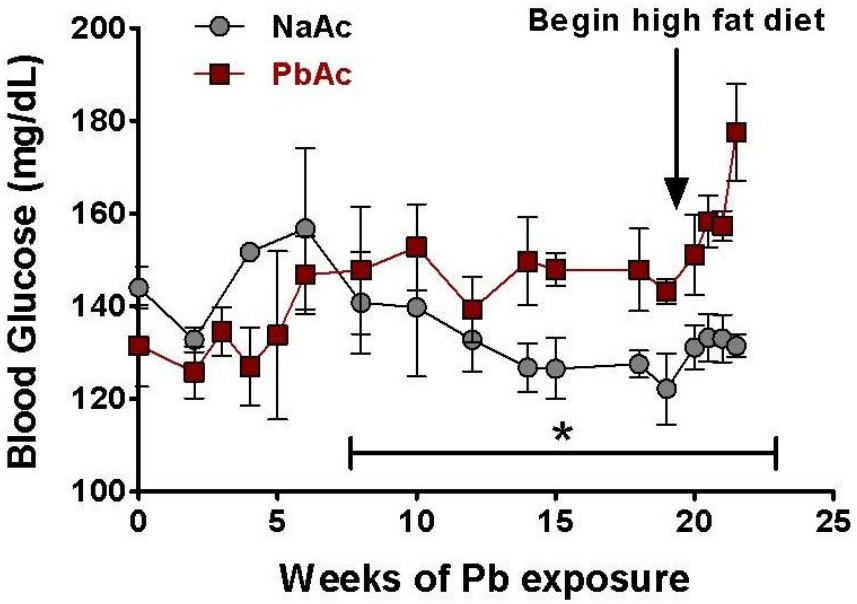

Fig. 5.5 – Pb exposure causes fasting hyperglycemia in obese rats.
The Pb group started with lower fasting glucose levels (due to chance), but from week 8, their glucose levels rose above the control group. The high fat diet uncovers an elevated susceptibility to diabetes in the Pb group. Analysis completed by repeated measures ANOVA and corrected for violation of sphericity with the Greenhouse-Geisser epsilon demonstrated significant hyperglycemia in Pb group from week 8 through termination. Data are means +/- SEM, *p<0.05, weeks 0-8: both groups n=4, Weeks 9-25: Na n=4, Pb n=3.

Another hallmark of diabetes is glucose intolerance, the inability to clear postprandial glucose from the circulation. Monthly OGTTs performed after an overnight fast showed a striking progressive glucose intolerance in Pb-exposed rats, but not in control rats (Figures 5.6 and 5.7). The fasting hyperglycemia and glucose intolerance observed in the Pb treated animals clearly demonstrates that Pb exposure has a profound negative effect on glucose homeostasis in obese rats. There are multiple mechanistic possibilities for how Pb might alter glucose metabolism, including reduced peripheral insulin sensitivity, defective glucose-mediated insulin secretion by the pancreas, and/or elevated hepatic glucose production.

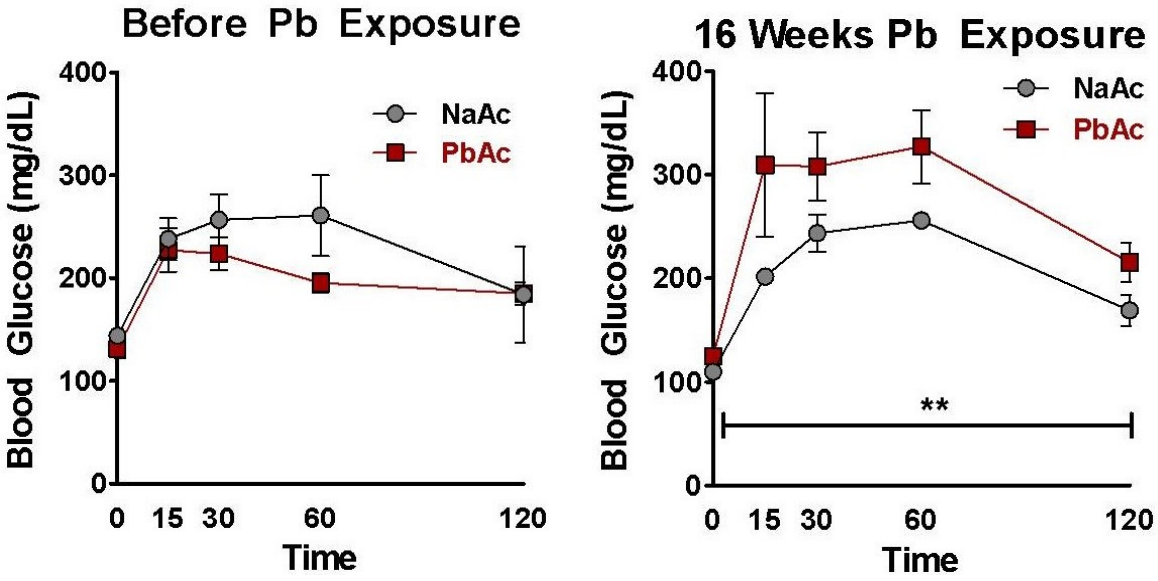

Fig. 5.6 – Pb exposure causes glucose intolerance.
Oral glucose tolerance tests (OGTTs) in control (NaAc) and Pb-treated (PbAc) female ZDF fa/fa rats after exposure to Pb for 0 or 16 weeks. Both groups had similar glucose tolerance before Pb exposure, while at 16 weeks, the Pb exposed rats were glucose intolerant compared to control rats. Glucose tolerance at 16 weeks was significantly different by ANOVA, **p<0.005. Data are means, +/- SEM, before Pb exposure: n=4 both groups, 16 Weeks: Na n=4, Pb n=3.

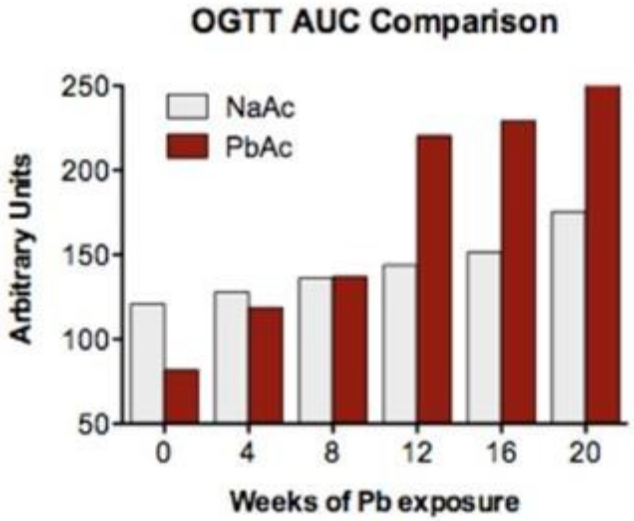

Fig. 5.7 – Progressive glucose intolerance caused by Pb exposure.
The area under the curve (AUC) was calculated for each of the OGTT assays performed on the ZDF rats. The data shows a progressive glucose intolerance in the Pb exposed rats, while the control rats maintained a similar glucose tolerance throughout the course of the study. Data are means, weeks 0-8: both groups n=4, weeks 12-20: Na n=4, Pb n=3.

To examine the possibility that Pb directly affects regulation of glucose production in the liver, changes in hepatic gluconeogenic gene expression in ZDF rats was examined. Upon termination, mRNA was isolated from a small sample of flash-frozen liver tissue. Changes in gluconeogenic gene expression were quantified by two-step qRT-PCR with sequence-specific primers for PEPCK, PGC1-α and PPIA. As shown in Fig 5.8, these data revealed that expression of the hepatic PEPCK gene was elevated in Pb-exposed animals. Elevated PGC1-α mRNA is consistent with the PEPCK results as PGC1-α functions as a transcriptional co-activator for this gene. Overall, the gene expression results are consistent with the possibility that the fasting hyperglycemia observed in the Pb-exposed animals is due, at least in part, to abnormally elevated hepatic glucose output. It is important to note that the animals were sacrificed at termination after a fasting re-feeding protocol (described in methods), so that in this context, the elevated gluconeogenic gene expression could be related, at least in part, to a deficiency in the insulin-mediated suppression of gluconeogenic gene expression. In other words, these findings may be indicative of hepatic insulin resistance.

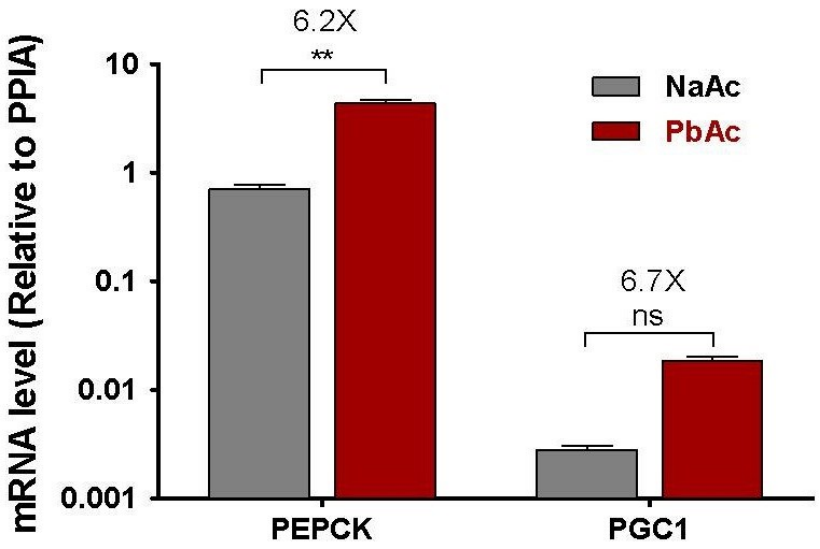

Fig. 5.8 – Pb exposure induces gluconeogenic gene expression. Changes in gluconeogenic gene (mRNA) expression were quantified by qRT-PCR with sequence-specific primers for PEPCK and PGC1-α. Data are means relative to housekeeping gene PPIA +/- SEM, presented on a log axis, Na n=4, Pb n=3.

To examine the possibility that Pb exposure affects pancreatic insulin production, insulin levels were determined in plasma samples from multiple time points throughout the study. Insulin ELISA results from the ZDF rats show a mild decrease in blood insulin levels in the Pb-treated rats from approximately 8 weeks of treatment through termination (Figure 5.9). While these findings are consistent with a potential defect in the ability of the pancreas to produce insulin, the differences between the control and treated groups were small and not statistically significant. Insulin ELISA results from fasting/re-feeding termination samples showed no difference between the control and treated groups (data not shown). Together these findings indicate that while there may be some pancreatic deficiency relative to the control group, Pb exposure did not cause pancreatic failure or a major defect in the ability of islets to produce insulin.

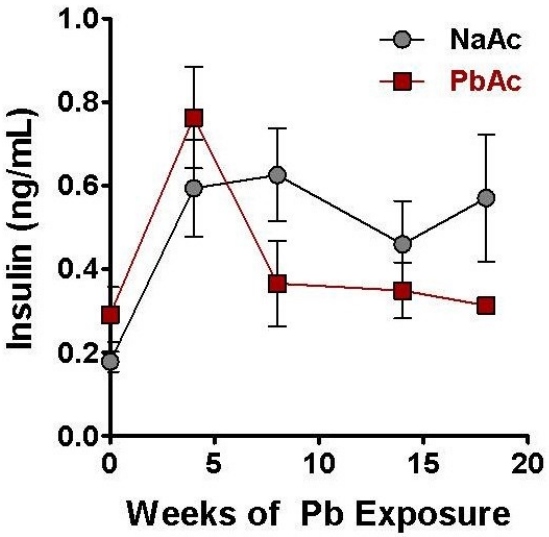

Fig. 5.9 – Pb exposure does not affect insulin production.
Plasma insulin levels measured by ELISA show decreased levels in Pb-treated rats from approximately week 8 forward. Data are means +/- SEM, Weeks 0-8: both groups n=4, Weeks 12-20: Na n=4, Pb n=3.

A small sample of flash-frozen liver tissue was ground and analyzed for triglyceride content by colorimetric ELISA assay to determine lipid content. Results revealed that the Pb-treated ZDF rats had accumulated significantly more triglycerides in their liver than the control animals (Figure 5.10). The data demonstrates that Pb exposure promotes accumulation of triglycerides within the liver tissue consistent with the development of hepatic steatosis.

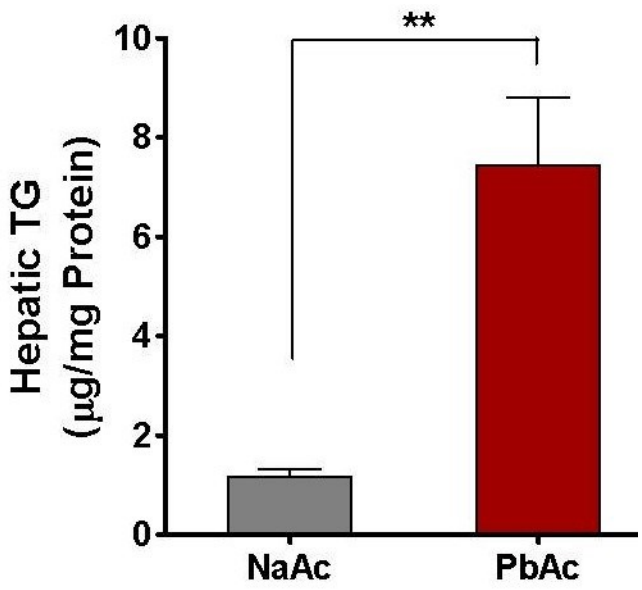

Fig. 5.10 – Pb exposure causes increased triglyceride accumulation. Liver tissue triglycerides were measured by colorimetric assay. Data are means +/- SEM, analyzed by students t test, **p < 0.005, Na n=4, Pb n=3.

5.5 – Pb Exposure Effects on Histological Parameters

To determine if Pb exposure affected liver morphology, a portion of the liver from each animal was subjected to standard histological analyses. Hematoxylin and eosin (H & E) staining demonstrated that both control and Pb-treated rats have normal hepatic lobule structure (Figures 5.11 and 5.12), showing that Pb does not affect gross liver morphology.

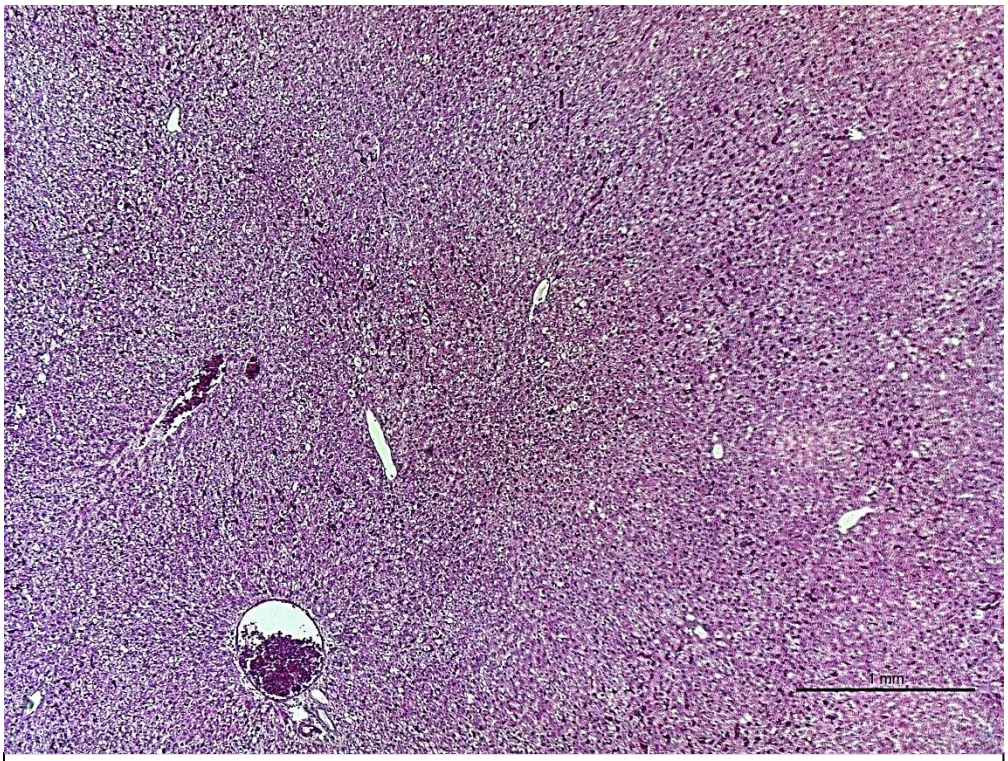

Fig. 5.11 – Control ZDF Liver H & E.
Normal hepatic lobular structure is intact. Magnification 5x, bar = 1mm.

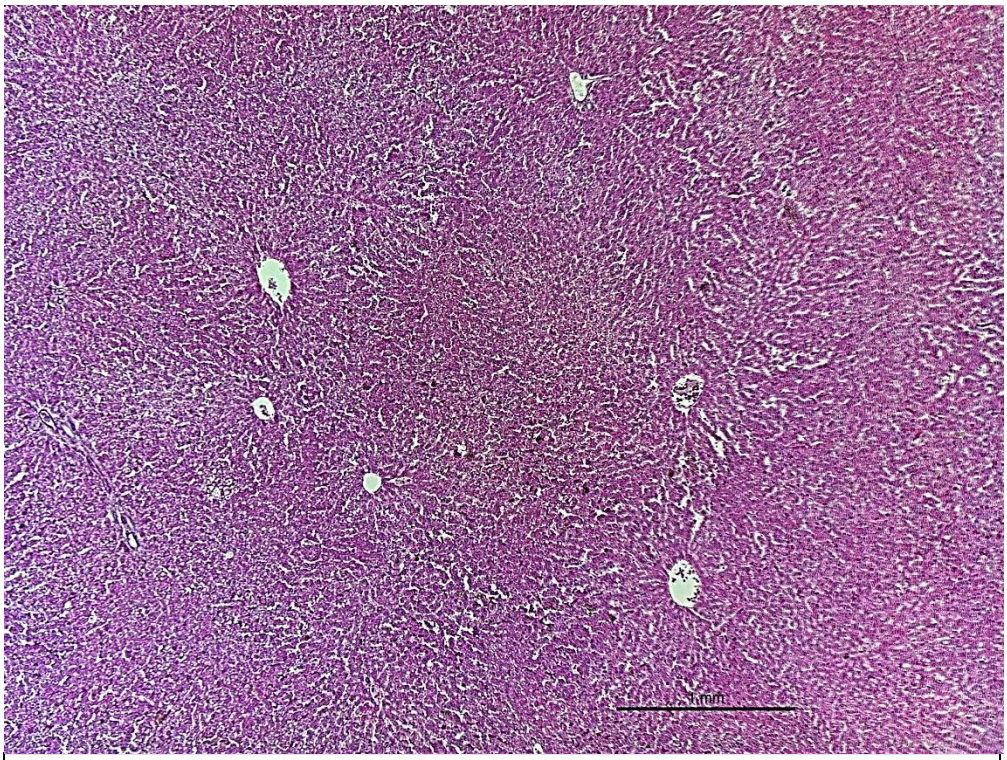

Fig. 5.12 – Pb-treated ZDF Liver H & E.
Normal hepatic lobular structure is intact. Magnification 5x, bar = 1mm.

Having determined that Pb exposure did not alter hepatic morphology, I sought to determine if it had any effect on glycogen content or distribution. Periodic Acid-Schiff stain is commonly utilized to detect glycogen in histological sections. A specimen that is PAS-positive will show glycogen (carbohydrates) as pink/magenta to purple/red in color. PAS staining of prepared liver slides, while not quantitative, revealed that Pb exposure caused a generalized depletion of hepatic glycogen, as evidenced by the decreased level of PAS-positive staining in the Pb-treated liver slide (Figures 5.13 and 5.14).

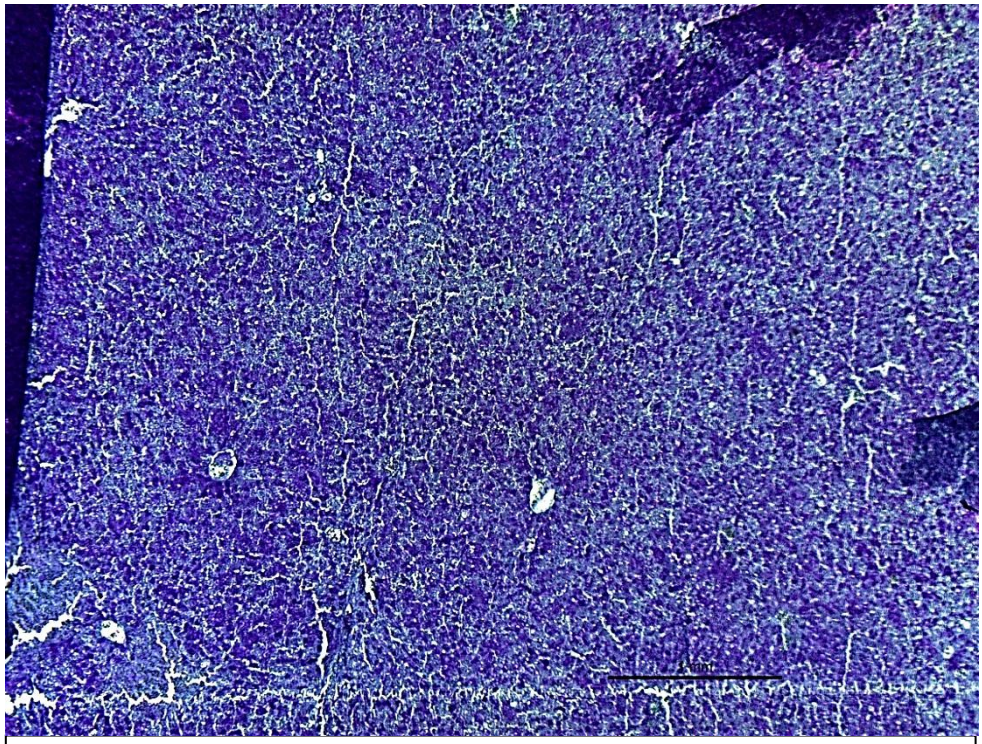

Fig. 5.13 – Control ZDF Liver PAS.
Note the numerous PAS-positive glycogen deposits (purple coloration) in the liver of the control rat. Magnification 5x, bar = 1mm.

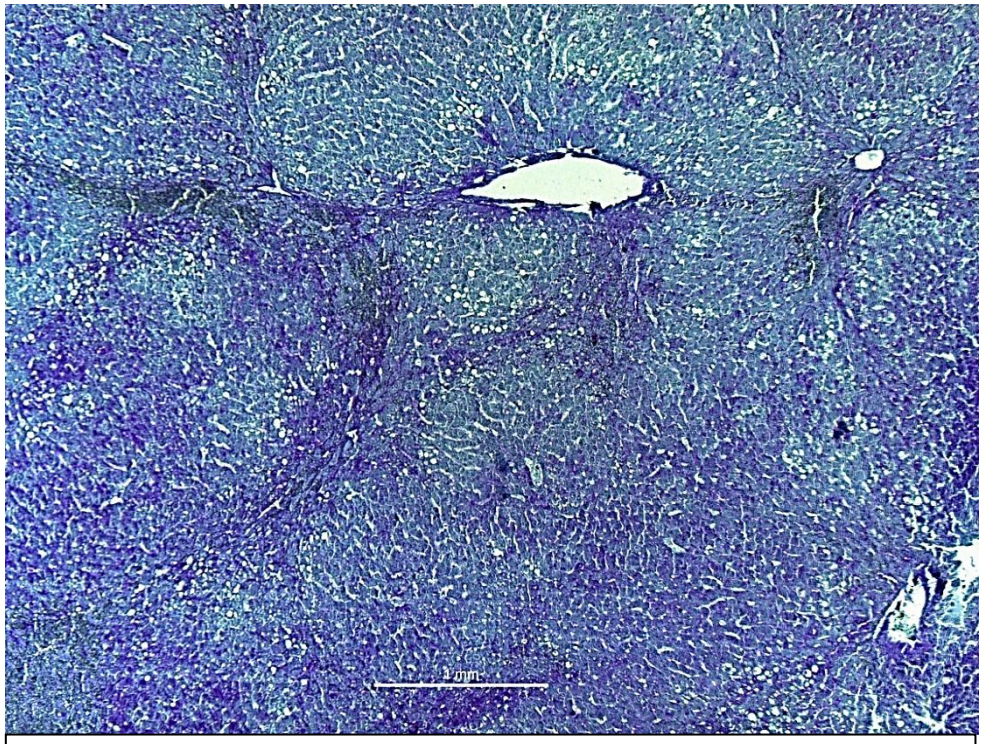

Fig. 5.14 – Pb-Treated ZDF Liver PAS.
Evidence of glycogen depletion after Pb exposure. Note the decrease in PAS-positive staining in the liver of the Pb-treated rat. Magnification 5x, bar = 1mm.

To determine if Pb exposure affected pancreatic morphology, a portion of the pancreas from each animal was subjected to standard histological analyses. While the quality of the specimens was not ideal due to the embedding technique utilized, H & E staining demonstrated that Pb exposure did have an effect on islet morphology. Islets from control rats (n = 4) appeared normal, with a round shape and a well-defined border (Figure 5.15). However, islets from Pb-treated rats (n = 3) appeared to be somewhat larger in size and did not display a well-defined border (Figure 5.16). Overall, the subjective appearance of the islets from the Pb exposed animals suggested a general degradation of normal islet architecture, which is consistent with the possibility that these islets have at least a minor degree of compromised endocrinological function. Staining for specific hormones was not an option due to the poor quality of the specimens.

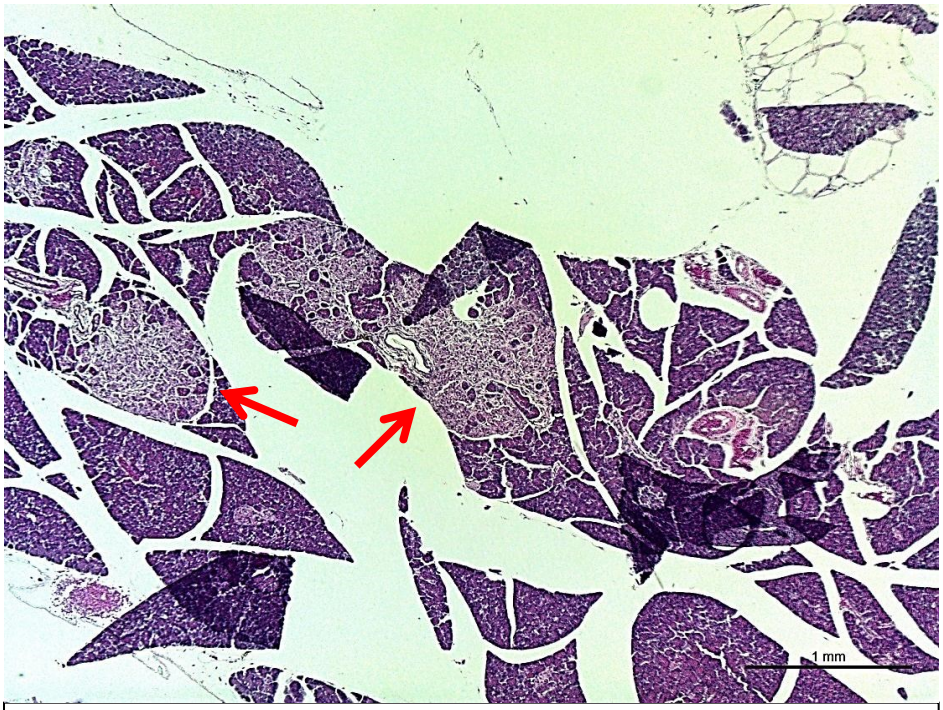

Fig. 5.15 – Control ZDF Pancreas H & E.
Islets are lighter stained, round areas, denoted by the red arrows. Control animal islet physiology appears normal. Magnification 5x, bar = 1mm.

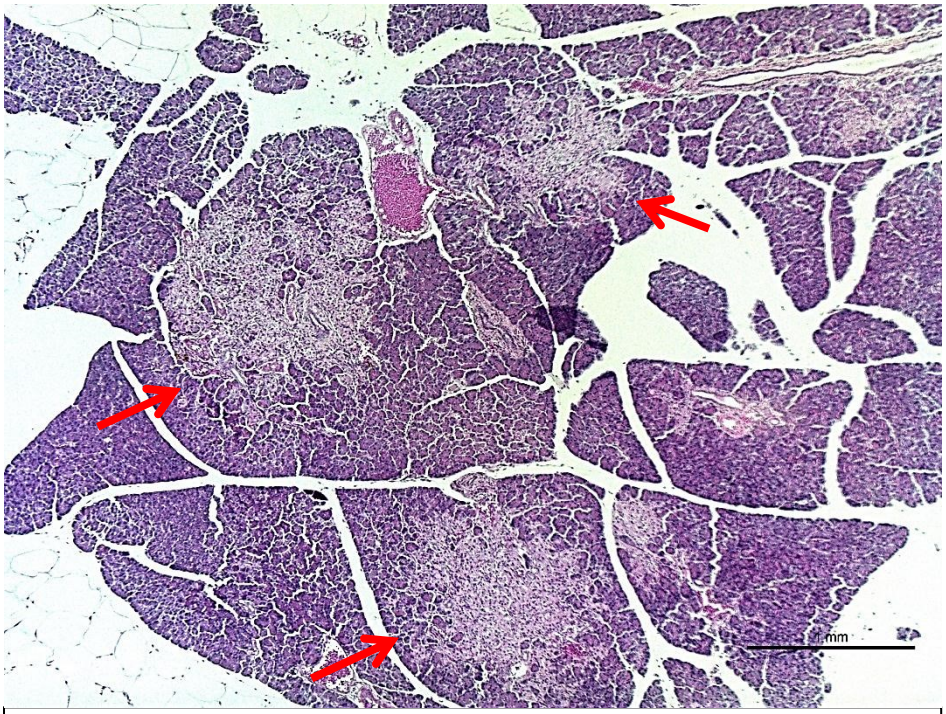

Fig. 5.16 – Pb-Treated ZDF Pancreas H&E.
Islets are lighter stained areas, denoted by the red arrows. Note perimeter changes and damaged architecture of islets in Pb-treated animal, this islet physiology appears abnormal. Magnification 5x, bar = 1mm.

Noting the alterations in islet morphology in Pb-treated animals, I wanted to determine if Pb exposure caused an increased presence of β-cells in pancreatic islets. Aldehyde Fuchsin is commonly used to distinguish β-cells from α-cells and δ-cells within the islets of pancreatic tissue samples as β-cells stain a blue to violet or purple punctate in color. Although these specimens are not ideal, and therefore the β-cells are not easily quantified, there appeared to be increased Aldehyde Fuchsin staining in Pb exposed samples (Figures 5.17 and 5.18). These findings, while not striking, are suggestive of β-cell hyperplasia in the Pb-treated animals and are consistent with the apparent larger size of islets observed in the H&E stained sections.

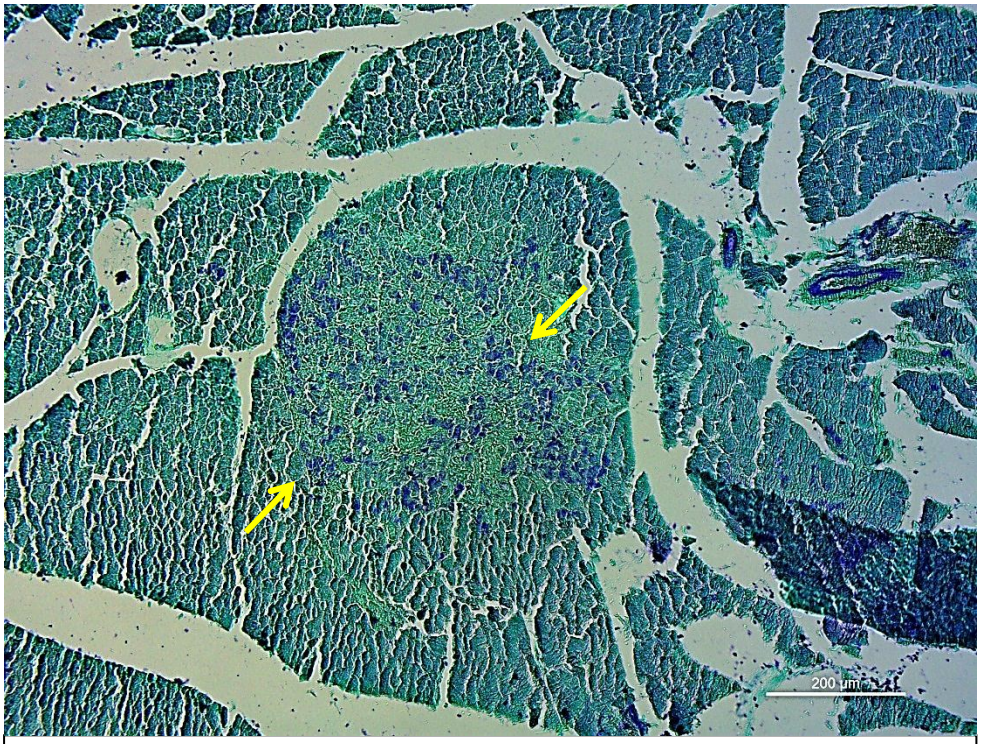

Fig. 5.17 – Control ZDF Pancreas Aldehyde Fuchsin.
Yellow arrows indicate Aldehyde Fuchsin-positive β-cells. Magnification 10x, bar = 200µm.

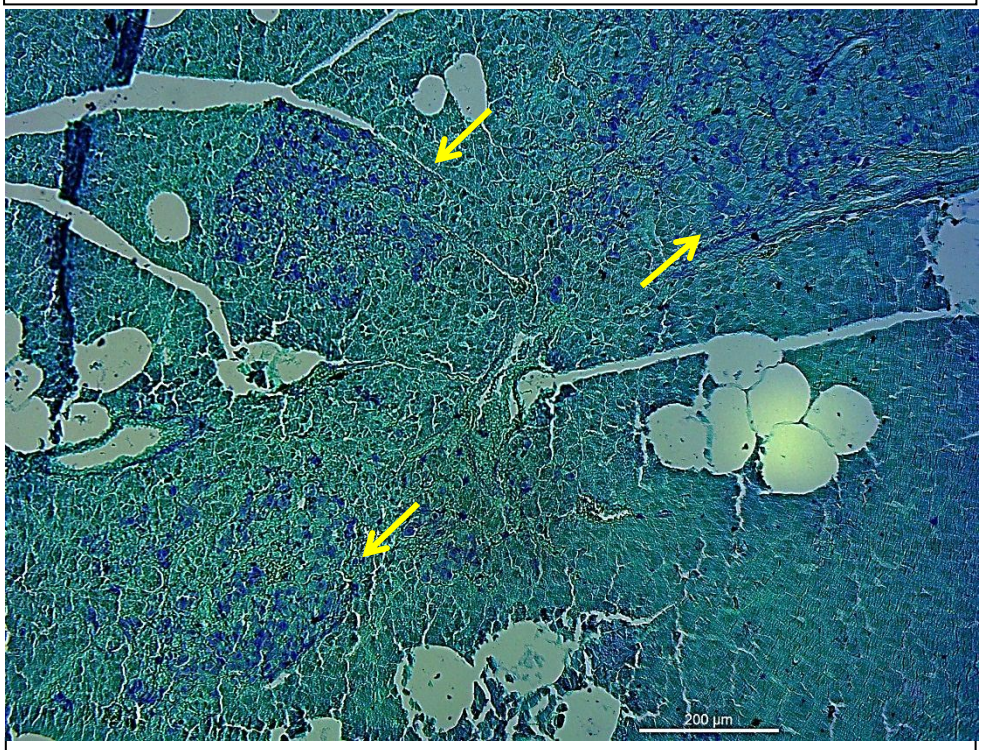

Fig. 5.18 – Pb-Treated ZDF Pancreas Aldehyde Fuchsin.
The appearance of a greater number of Aldehyde Fuchsin-positive β-cells (yellow arrows) is suggestive of a greater degree of β-cell hyperplasia in the Pb-treated animals. Magnification 10x, bar =200µm.

5.6 – Additional Histological Observations

Liver tissue was examined for fibrosis with Masson's Trichrome stain, but there was no discernible difference between the control and Pb-treated animals (data not shown). Pancreatic tissue was also examined for fibrosis with Masson's Trichrome Stain, and while there was some fibrosis present, there was no discernible difference in the degree of fibrosis between the control and Pb-treated groups (data not shown).

5.7 – Discussion of ZDF Study

It is clear from the data presented here, that Pb exposure promotes the development of diabetes in obese rats, specifically causing the development of fasting hyperglycemia and glucose intolerance. Of additional interest is the observation that when the animals were switched to a diabetogenic high fat diet at week 20 the Pb-exposed rats showed a dramatic increase in fasting hyperglycemia, suggesting that extended exposure to the toxicant made the animals more susceptible to the deleterious effects of the further metabolic stress of a high fat diet. These striking findings provide strong support for the hypothesis that, in metabolically stressed rodents, Pb exposure increases susceptibility to diabetes and promotes the progressive development of metabolic abnormalities.

Although many factors can contribute to fasting hyperglycemia, a major contributor is often elevated hepatic glucose output [199, 200]. In accordance with the possibility that Pb directly affects the regulation of glucose production in the liver, gene expression data revealed that PEPCK and PGC1-α expression were elevated in Pb-exposed animals. These findings at the mRNA level are consistent with previously

published results from somewhat similar experiments with Pb exposed rats elevated activity levels of PEPCK were observed (described in more detail in the background section) [152-154]. The dysregulation of the insulin-mediated suppression of hepatic gluconeogenic gene expression could be a major contributor to the glucose intolerance observed in this animal study and suggests hepatic insulin resistance.

Consistent with this possibility is the observation of hepatic steatosis in the Pb-treated animals. Fatty liver is frequently observed in insulin resistant humans and animal models and is associated specifically with hepatic insulin resistance [201-203]. The hepatic lipid accumulation in our animals may be secondary to the hyperglycemia we observed, which has been shown to be a "trigger" that stimulates *de novo* synthesis of hepatic lipids [204, 205]. Also consistent with Pb-induced metabolic dysfunction centered on the liver, is the observation that livers from Pb exposed rats were glycogen depleted (Figures 5.13 and 5.14). Glycogen depletion has been associated with elevated fasting blood glucose [200, 206], and may also be consistent with hepatic insulin resistance.

Examination of gross pancreatic morphology using H & E staining revealed Pb-related disturbances in islet organization. Control rat islets appeared normal, with a round shape and a reasonably definable border. Pb-treated rat islets lacked the round shape seen in the control animals and had less well defined borders. Although suggestive of pancreatic dysfunction, we did not observe a significant difference in blood insulin levels suggesting that these abnormalities were not severe enough to block β-cell compensation in these animals. Beta-cell compensation in Pb-treated animals was confirmed by a greater prevalence of Aldehyde Fuchsin positive β-cells.

Our results do not rule out the possibility that Pb affects peripheral insulin resistance in these animals. Unfortunately, a direct measurement of peripheral insulin sensitivity in intact animals is difficult and was beyond the technical scope of our lab and my thesis. If there is peripheral insulin resistance in these animals, it would contribute to the observed hyperglycemia, which was present despite normal insulin levels.

These data demonstrate for the first time that Pb promotes the development of Type 2 diabetes in a rat model of obesity. I identified Pb-induced defects in both hepatic glucose output and in postprandial glucose clearance. Both of these are insulin mediated processes and it seems likely that Pb is interfering in the insulin signaling pathway, at least in liver, and possibly also in muscle. The data also identified key histological aspects of Pb-exposure, including islet abnormalities and increased β-cell compensation.

A key question that the data do not address is if the pro-diabetic effect of Pb is dependent on obesity or other metabolic stresses. The experiments conducted in the hIAPP transgenic mouse model (described in the next chapter) were designed to examine this possibility and to characterize the specific effects of Pb exposure in a high fat diet-induced rodent model of Type 2 diabetes. The gene expression results are consistent with previously published animal studies and yield a potential molecular mechanism to be explored *in vitro* (described in Chapter 7).

Chapter 6 – Lead Exposure Causes Metabolic Abnormalities in a Humanized Mouse Model of Pancreatic Amyloidosis

The Pb-obesity interaction is a relevant issue in large sections of the US population where environmental stressors of over-nutrition and an inactive sedentary lifestyle are common, and co-exist with exposure to persistent environmental toxicants, such as Pb.

The overall goal of the *in vivo* portion of my PhD work is to characterize the effect of Pb exposure on diabetes risk in metabolically stressed (obese) rodents. To explore the specific hypothesis that Pb exposure increases diabetes susceptibility in obese individuals, two well-established rodent models of obesity were utilized: the Zucker Diabetic Fatty (ZDF) rat (presented in Chapter 5) and the high fat fed, human islet amyloid polypeptide (hIAPP), transgenic mouse, presented in the current chapter. The experimental approach for the animal studies is designed to reproduce the effects of Pb exposure in the human population, where obesity and diabetes risk factors are common.

6.1 – human Islet Amyloid Polypeptide (hIAPP) Mouse Model

In contrast to the ZDF rat, which possesses a genetic predisposition to obesity and diabetes due to hyperphagia, the hIAPP mouse is a high fat diet-induced transgenic rodent model that expresses the human islet amyloid polypeptide gene. The hIAPP transgenic mouse was created to mimic a common pathophysiology of human Type 2 diabetes, pancreatic amyloidosis [207, 208]. Amyloid deposits accumulate in pancreatic

islets in the vast majority of people with type 2 diabetes. Amyloid is produced by β-cells of the islets and is co-secreted with insulin in response to changes in blood glucose [209-211]. And accumulation of amyloid plaques in pancreatic islets is associated with the progression of diabetes, including characteristic loss of beta-cell mass and function [207, 208]. The amyloid deposits that form in the pancreas are generated specifically from a pancreatic-specific amyloid called islet amyloid polypeptide (IAPP). The amyloidogenic potential of IAPP is dependent on the sequence of the peptide, and the sequence of the protein produced in humans, non-human primates and cats is of the amyloidogenic form. The sequence of the gene in most rodents, including mice, is different and produces an islet amyloid protein that does not form plaques [212, 213]. Because rodent IAPP is non-amyloidogenic, they do not experience the same amyloid-dependent pancreatic dysfunction observed in humans, even in the face of similar metabolic stresses (i.e. peripheral insulin resistance). Creation of hIAPP transgenic rodents allowed for expression of the human IAPP gene and amyloid aggregation & deposition in the pancreas under certain dietary conditions [169, 214].

The hIAPP transgenic mouse strain is maintained in a C57Bl6 background [215-217]. This mouse expresses the transgene specifically in beta cells (under the control of the rat INS2 promoter) but apparently does not develop diabetes, hyperglycemia or a pancreatic insufficiency [214]. However, when the hIAPP transgene is expressed in C57Bl6/DBA-2 F1 mice, pancreatic amyloid deposition and an elevated susceptibility to Type 2 diabetes occurs. Previous studies observed a negative influence of estrogen on expression of the hIAPP transgene [169, 170], so only male C57Bl6/DBA-2 F1 hIAPP-heterozygous transgenic mice were used in our study.

Because previously published data demonstrated that hIAPP transgenic mice required a minimum of 12 months on a high fat diet to develop pancreatic amyloid plaques, the experimental time frame for the hIAPP transgenic mice was lengthened from that of the ZDF rats (See Figure 6.1) [215]. This also allowed an extended period to observe what may be subtle effects of Pb exposure on the rate of diabetes progression.

6.2 – hIAPP Experimental Setup

Four groups of male hIAPP mice were established (the characteristics of which were described in the previous paragraphs). Amounts of PbAc utilized in the hIAPP transgenic mouse study were based on previously published data [154], and the blood lead levels generated in my own ZDF rat experiment described above. Two groups

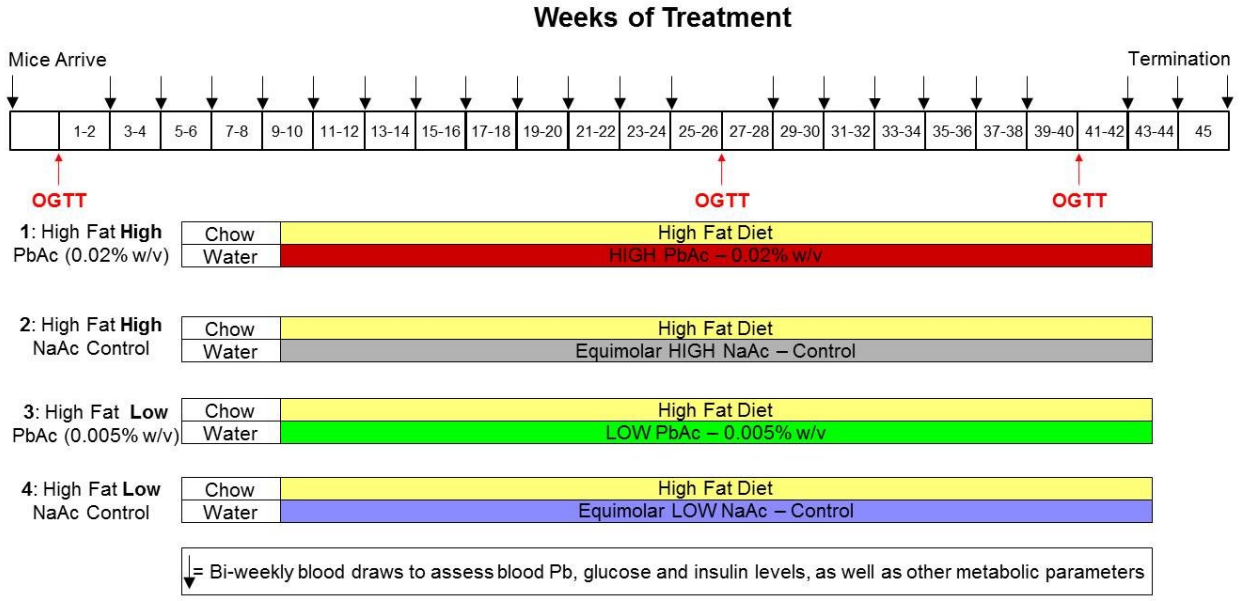

Fig. 6.1 – Experimental time line for hIAPP Mice.
At 10 weeks of age, mice were placed on the high fat diet and treatment with Pb was initiated. Bi-weekly fasting blood glucose draws and bi-annual OGTTs were performed as shown above. Both high dose groups (n=16), both low dose groups (n=12).

(n=16) were treated with 0.02% w/v PbAc ("high treated") or equimolar NaAc ("high control") in the drinking water. The other two groups (n=12) were treated with 0.005% w/v PbAc ("low treated") or equimolar NaAc ("low control") in the drinking water. Male heterozygous hIAPP transgenic mice were started on the feeding/exposure protocol at 10 weeks of age. All animals were fed a standard chow diet (Purina 5009) until weaned, then placed on a high fat chow diet (Autoclavable Mouse Breeder Diet 5021, Purina) containing 9% fat w/w, (45% calories from fat) through termination (week 45 of the study, 55 weeks of age). Food and water were offered ad lib.

Body weights and food consumption were measured weekly; blood was drawn bi-weekly from fasted animals for blood glucose level determination. Oral glucose tolerance tests (OGTTs) were performed before the initiation of treatment to establish baseline, and then repeated at 26 weeks and 40 weeks of treatment to look for changes in glucose tolerance. After 45 weeks of exposure, animals were sacrificed following an 8 hour fast by CO_2 asphyxiation followed by cervical dislocation and exsanguination by cardiac puncture. Relevant tissues were removed and portions placed in 4% buffered formalin at 4°C, freeze-clamped in liquid nitrogen or frozen in OTC for histological and molecular analyses. Additional endpoint measures included plasma insulin and liver triglycerides.

6.3 – Basic Parameters of the hIAPP Transgenic Model

Pb exposure did not affect body weight (Figures 6.2 and 6.3) or food intake (Figures 6.4 and 6.5). Blood lead levels were assessed by ICP-MS at Wayne State University School of Medicine's Biochemistry Department (See Chapter 3 for protocol).

The average blood lead levels for the high treated animals, exposed to 0.02% w/v PbAc, was 41.5 µg/dL at termination (Figure 6.6). The average blood lead levels for the low treated animals, exposed to 0.005% w/v PbAc, was 21.5 µg/dL at termination (Figure 6.7). The lack of significant differences in body weight or food intake suggests that there were no major effects of Pb on appetite or activity with these levels of exposure.

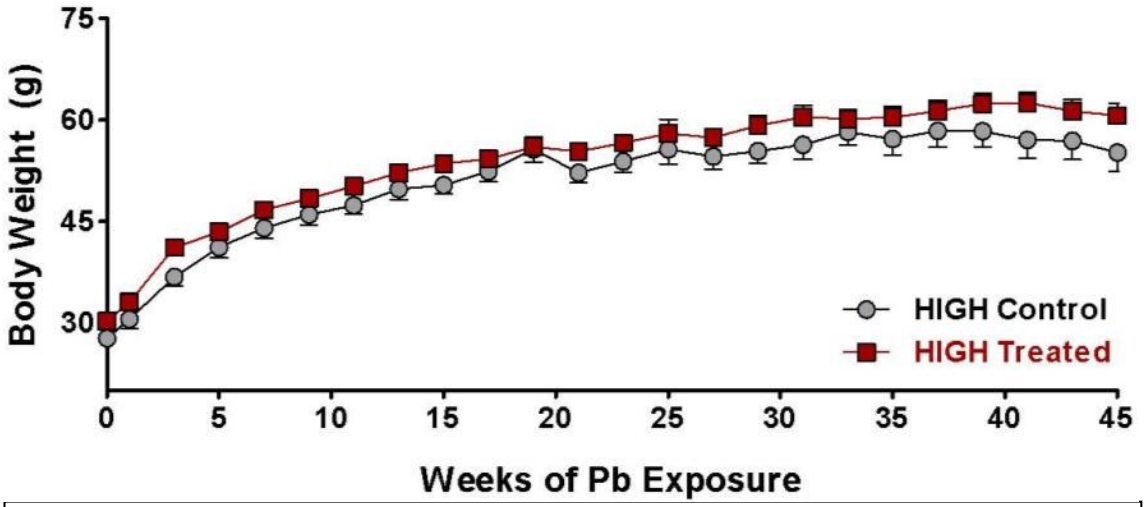

Figure 6.2 – Body weight measures for hIAPP high dose (0.02% w/v) group.
Data are means +/- SEM. High control: Weeks 0-16 n=11, weeks 17-34 n=15, weeks 35-40 n=13, weeks 41-43 n=12, weeks 44-45 n=11. High treated: Weeks 0-20 n=16, weeks 21-28 n=15, weeks 29-44 n=14, weeks 44-45 n=13.

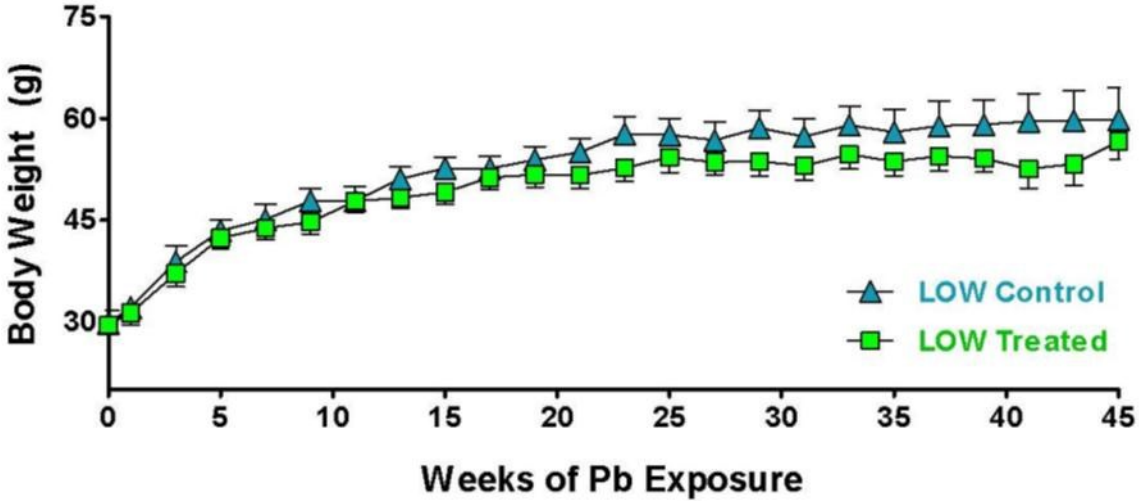

Figure 6.3 – Body weight measures for hIAPP low dose (0.005% w/v) group.
Data are means +/- SEM. Low control: Weeks 0-21 n=12, weeks 22-23 n=11, weeks 24-29 n=10, weeks 30-35 n=9, weeks 36-40 n=8, weeks 41-45 n=7. Low treated: Weeks 0-16 n=12, weeks 17-42 n=11, weeks 43-44 n=10, weeks 44-45 n=8.

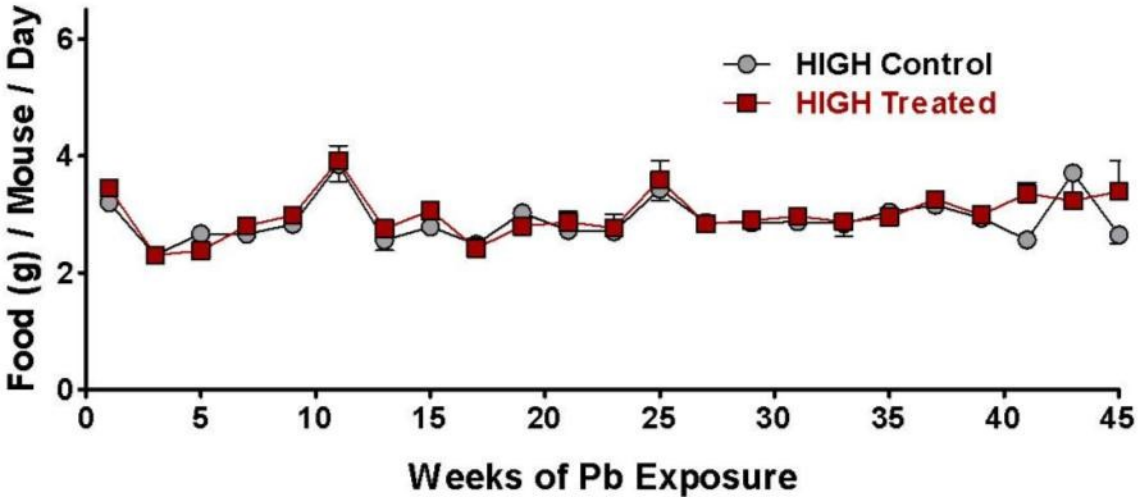

Fig. 6.4 – Food consumption measures for hIAPP high dose (0.02% w/v) group.
High control: Weeks 0-16 n=11, weeks 17-34 n=15, weeks 35-40 n=13, weeks 41-43 n=12, weeks 44-45 n=11. High treated: Weeks 0-20 n=16, weeks 21-28 n=15, weeks 29-44 n=14, weeks 44-45 n=13. Data are means +/- SEM.

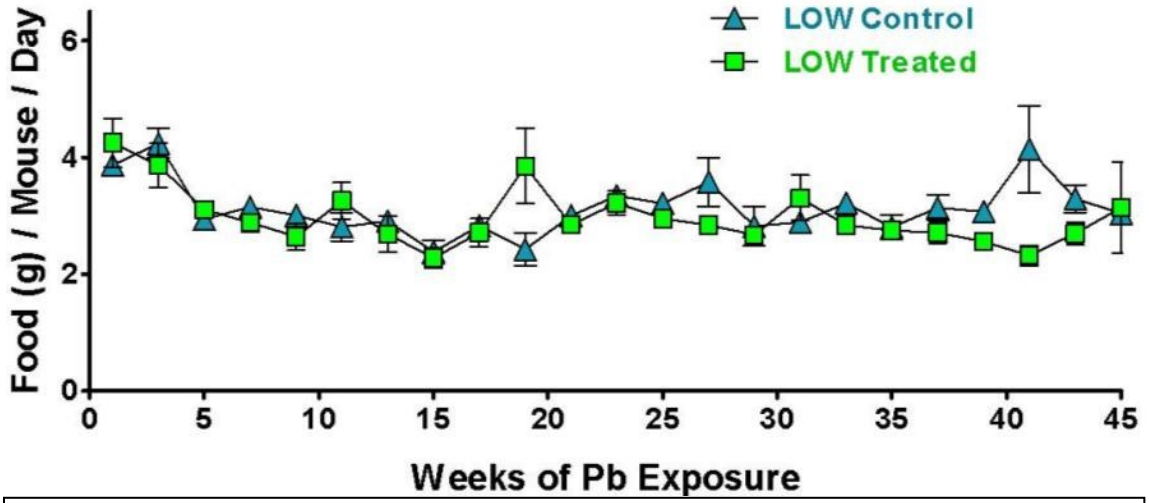

Fig. 6.5 – Food consumption measures for hIAPP low dose (0.005% w/v) group.
Data are means +/- SEM. Low control: Weeks 0-21 n=12, weeks 22-23 n=11, weeks 24-29 n=10, weeks 30-35 n=9, weeks 36-40 n=8, weeks 41-45 n=7. Low treated: Weeks 0-16 n=12, weeks 17-42 n=11, weeks 43-44 n=10, weeks 44-45 n=8.

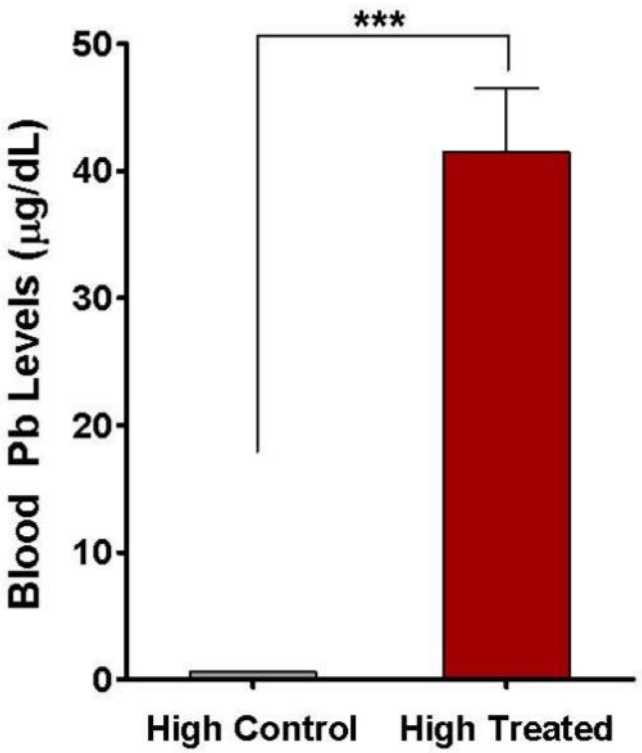

Fig. 6.6 – Blood Pb levels at termination of hIAPP high dose (0.02% w/v) group. Analysis completed by students T test. Data are means with SEM, high control n=11, high treated n =13. *** $p < 0.0002$.

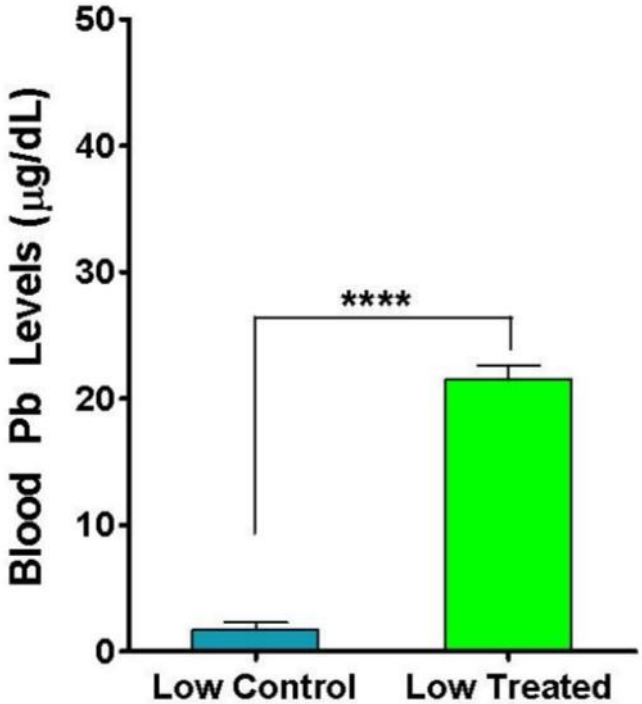

Fig. 6.7 – Blood Pb levels at termination of hIAPP low dose (0.005% w/v) group. Analysis completed by students T test. Data are means with SEM, low control n=7, low treated n =8. **** $p < 0.0001$

6.4 – Pb Effects on Metabolic Parameters in hIAPP Transgenic Mice

An elevation in fasting blood glucose is a characteristic of Type 2 diabetes. Because the hIAPP transgenic mice were bred on to a DBA/2 background, they had a predisposition to develop insulin resistance, but are able to retain relatively normal blood glucose levels due to insulin hypersecretion in response to elevated blood glucose levels [218]. As shown in Figures 6.8 and 6.9, Pb exposure induced a persistent fasting hyperglycemia from 8 weeks of treatment, in both the high and low exposure groups as compared to the near-normal fasting blood glucose levels throughout the study of both control groups.

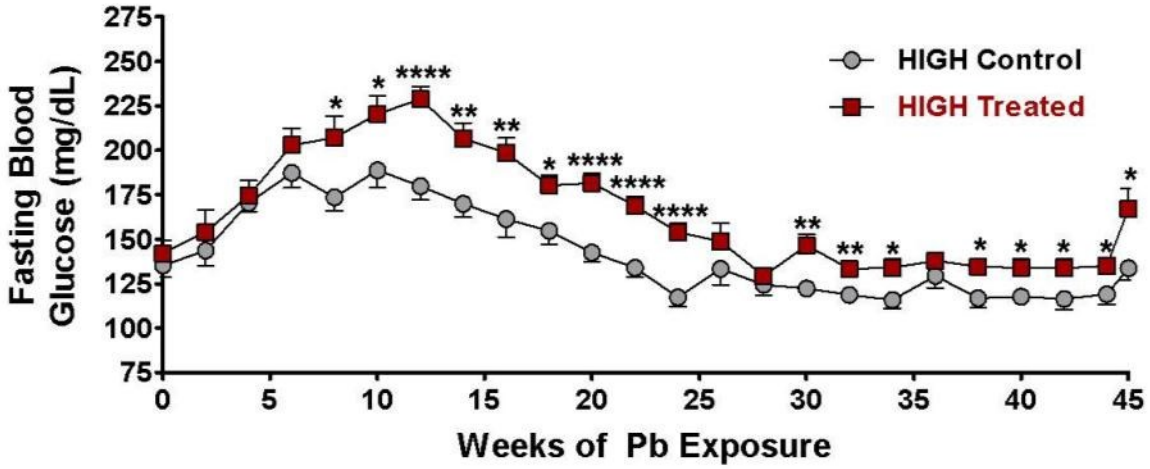

Fig. 6.8 – Fasting blood glucose measures for hIAPP high dose (0.02% w/v) group.
Both groups began with normal fasting blood glucose levels (due to chance), but from week 8, the Pb-treated (high treated) group rose above the high control group. Analyses by students T test, data are means +/- SEM. *p<0.05, **p<0.005, ****p<0.0001.
High control: Weeks 0-16 n=11, weeks 17-34 n=15, weeks 35-40 n=13, weeks 41-43 n=12, weeks 44-45 n=11.
High treated: Weeks 0-20 n=16, weeks 21-28 n=15, weeks 29-44 n=14, weeks 44-45 n=13.

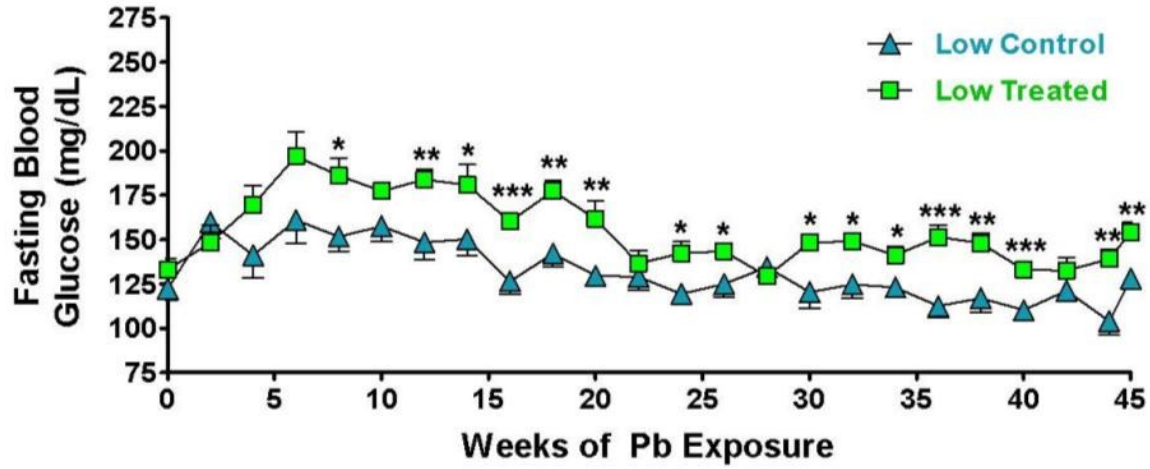

Fig. 6.9 – Fasting blood glucose measures for hIAPP low dose (0.005% w/v) group.
Both groups began with normal fasting blood glucose levels (due to chance), but from week 4, the Pb-treated (low treated) group rose above the low control group. Analyses by students T test, data are means +/- SEM. * p<0.05, ** p<0.005, *** p<0.0005
Low control: Weeks 0-21 n=12, weeks 22-23 n=11, weeks 24-29 n=10, weeks 30-35 n=9, weeks 36-40 n=8, weeks 41-45 n=7.
Low treated: Weeks 0-16 n=12, weeks 17-42 n=11, weeks 43-44 n=10, weeks 44-45 n=8.

Glucose intolerance, the inability to clear postprandial glucose from the circulation, is another characteristic of diabetes. Complete OGTTs were performed after an 8 hour fast to establish a baseline (Time 0) and repeated at 26 weeks of treatment to observe changes in glucose tolerance. Glucose tolerance was similar in all groups before the initiation of Pb exposure, but after 26 weeks of Pb exposure, the high dose group was more glucose intolerant compared to the controls, (Figure 6.10). The low dose group behaved similarly; after 26 weeks of exposure, the Pb-treated group was glucose intolerant compared to the low control group although this difference did not achieve statistical significance (Figure 6.11).

The increased glucose intolerance in the low dose animals is more clearly visualized when the data are rearranged to directly compare, the effect of time on glucose tolerance within a single treatment group. OGTT data of the low control at 0 and 26 weeks were plotted on one graph, while the same was done for the low treated (Figure 6.12). When viewed in this manner it is clear that 6 months of Pb exposure induced glucose intolerance. The fasting hyperglycemia and glucose intolerance observed in both the high and low Pb-treated mice resembles that seen in the ZDF rats and further supports the idea that Pb exposure has a profound negative effect on glucose homeostasis in metabolically stressed rodents. As stated previously, there are multiple mechanistic possibilities for how Pb might alter glucose metabolism, including reduced peripheral insulin sensitivity, defective glucose-mediated insulin secretion by the pancreas, and/or elevated hepatic glucose production.

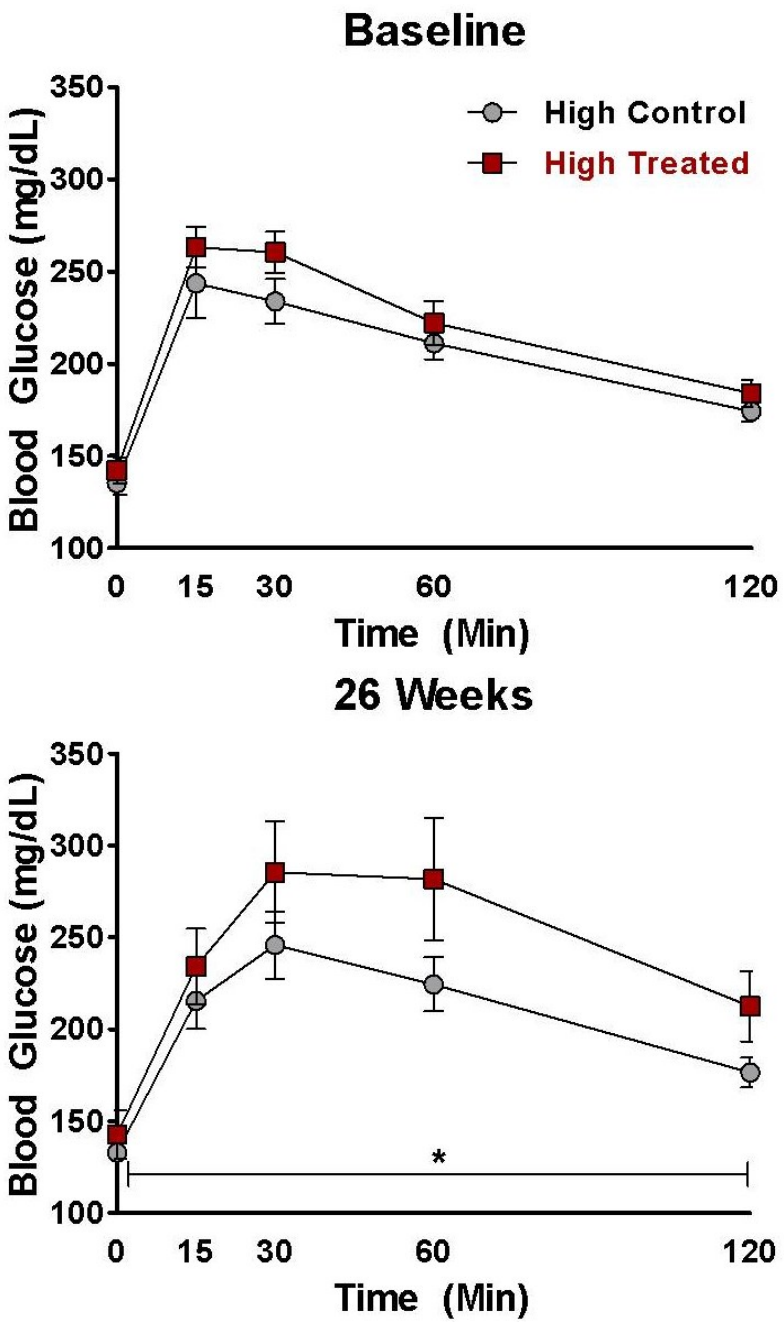

Fig. 6.10 – Pb exposure causes glucose intolerance in high dose group.
Oral glucose tolerance tests in hIAPP high treated (0.02% w/v) or high control after exposure to Pb for 0 or 26 weeks. The OGTT curves from the baseline measure are not different, while at 26 weeks, the high Pb-treated group is glucose intolerant compared to the high control group. Time and treatment had an effect on glucose tolerance at 26 weeks as determined by ANOVA, *p<0.01. Data are means +/- SEM. Baseline: n=16 both groups. 26 weeks: n=15 both groups.

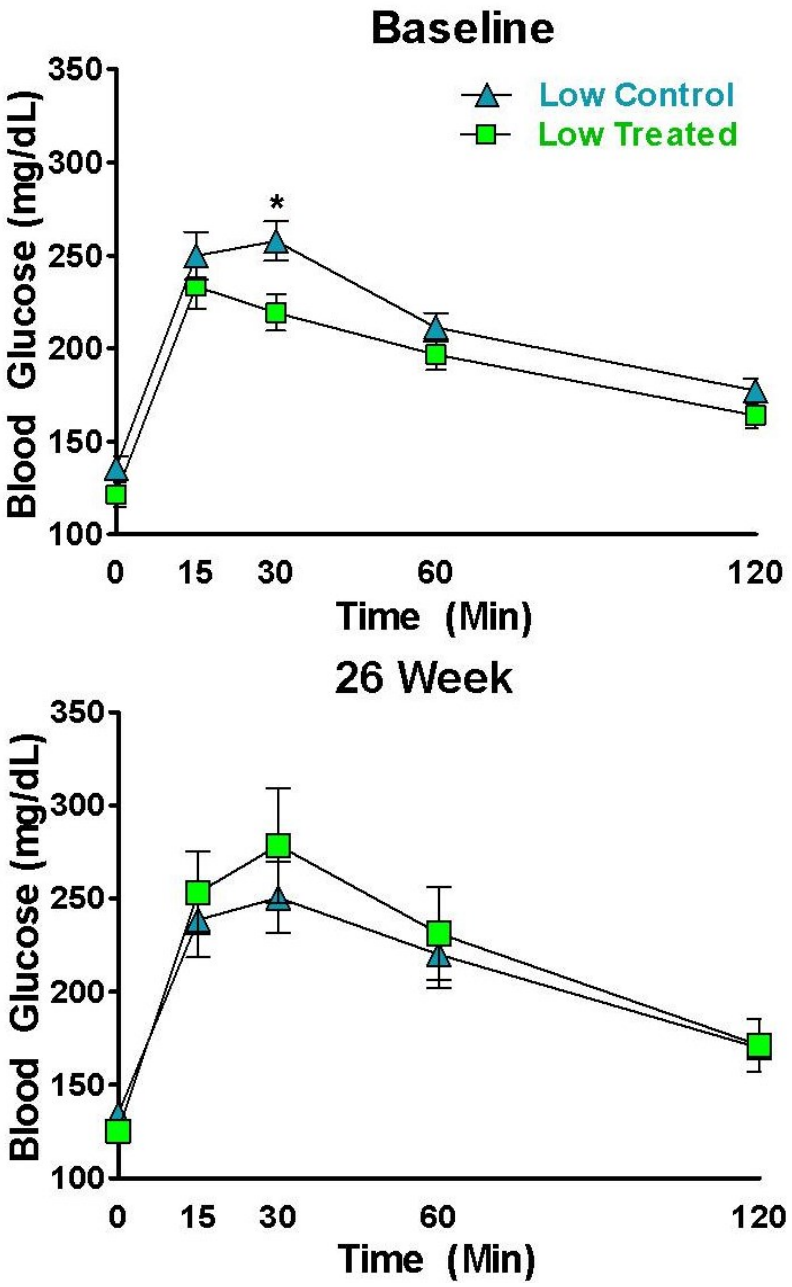

Fig. 6.11 – Pb exposure causes glucose intolerance in low dose group.
Oral glucose tolerance tests in hIAPP low treated (0.02% w/v) or low control after exposure to Pb for 0 or 26 weeks. The OGTT curves from the baseline differ only at 30 minutes (*p<0.01), while at 26 weeks, the low Pb-treated group is glucose intolerant compared to the low control group. Data are means +/- SEM. Baseline: n=12 both groups. 26 Weeks: low control n=10, low treated n=11.

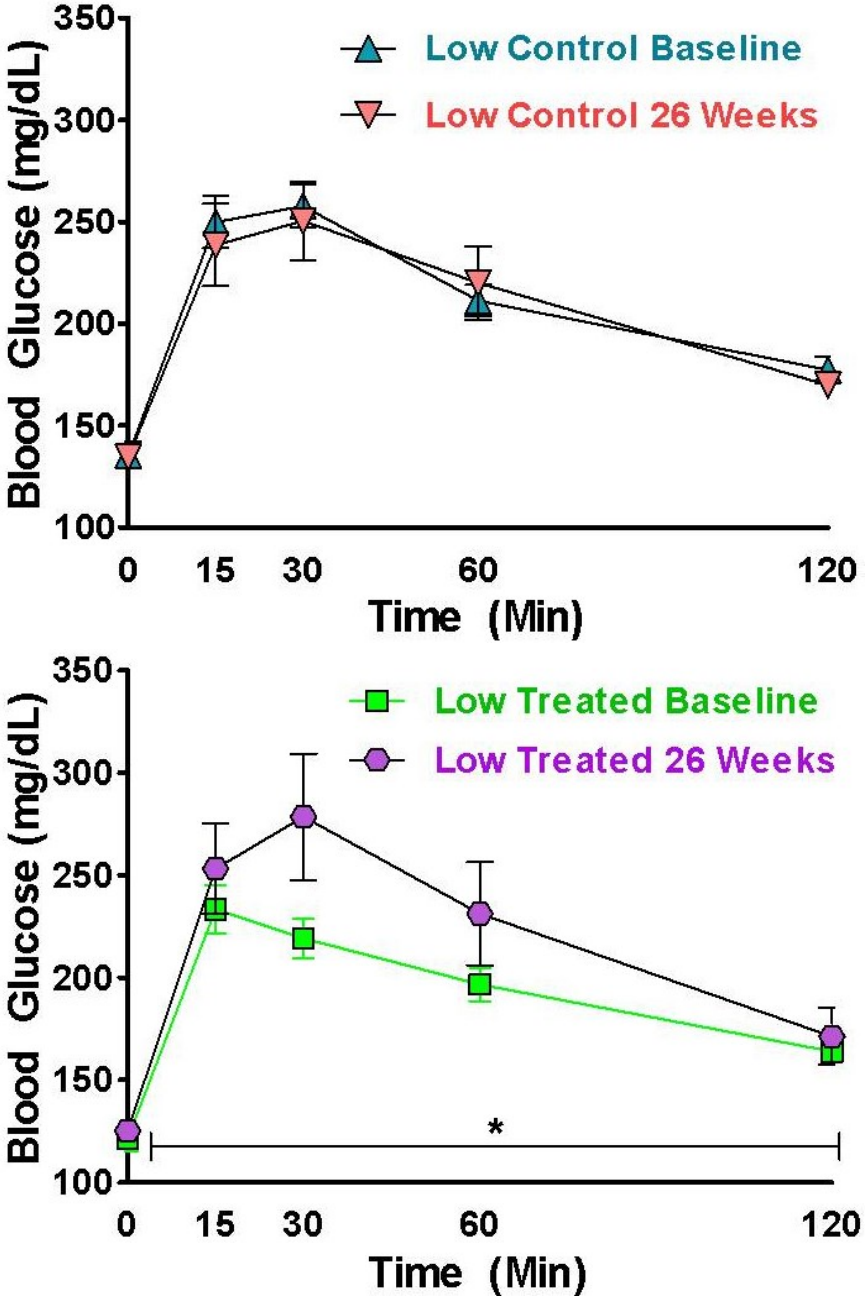

Fig. 6.12 – Data rearranged for direct comparison of effect of time on glucose tolerance.
Oral glucose tolerance tests in hIAPP low treated (0.02% w/v) or low control after exposure to Pb for 0 or 26 weeks. Time of treatment had an effect on glucose tolerance at 26 weeks in the Pb treated group (*$p<0.01$) as demonstrated by ANOVA. Data are means +/- SEM. Baseline: n=12 both groups. 26 Weeks: low control n=10, low treated n=11.

To examine the possibility that Pb directly affects regulation of glucose production in the liver, changes in hepatic gluconeogenic gene expression in hIAPP transgenic mice were examined. Upon termination, mRNA was isolated from a small sample of flash-frozen liver tissue. Changes in gluconeogenic gene expression were quantified by two-step qRT-PCR with sequence-specific primers for PEPCK, G6Pase, PGC1-α and PPIA. These data showed that there was no significant difference in expression of any genes between Pb-exposed or control animals in either the high or low dose groups (Figures 6.13). The gene expression results from the hIAPP mice differ from those observed in the ZDF rats. However, this is consistent with previously published data demonstrating that DBA/2 mice respond to elevated glucose by compensatory hypersecretion of insulin, and have been shown to have higher than normal plasma insulin levels during a fast, which could reduce hepatic gene expression levels [218]. A significant difference between the ZDF rat experiment and the mouse experiment, in this regard, was that the rats were sacrificed at termination after a fasting re-feeding protocol (described in methods), while the hIAPP mice were sacrificed after an 8 hour fasting-only protocol.

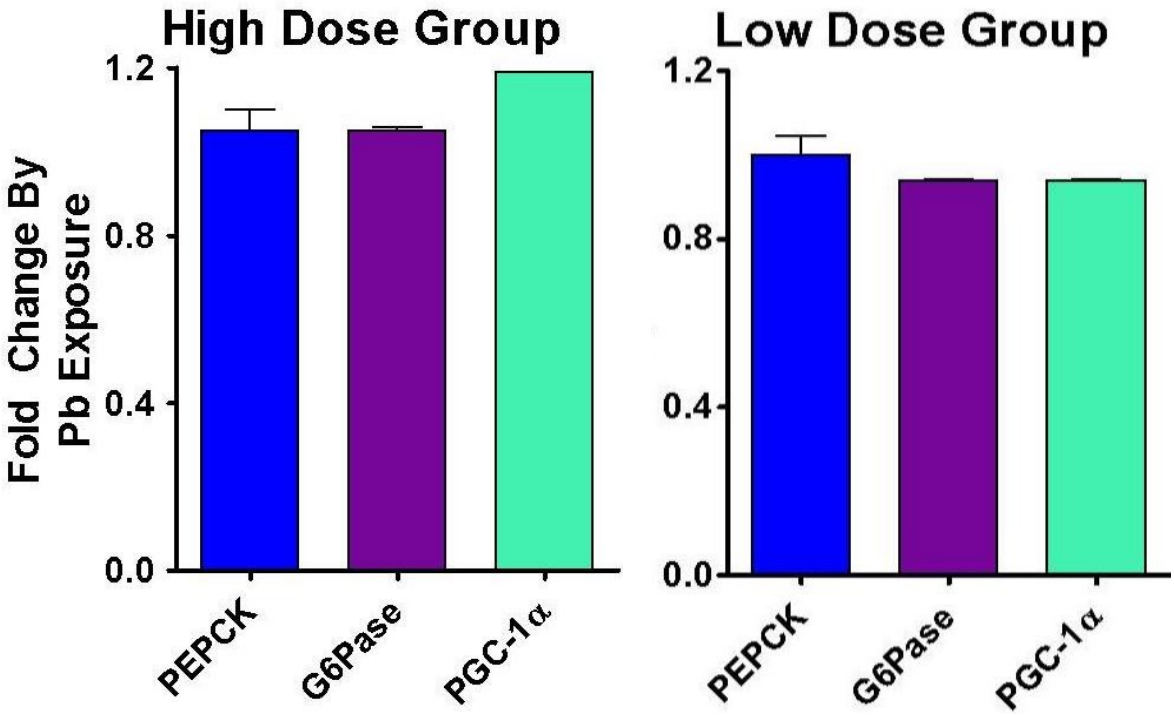

Fig. 6.13 – Pb exposure does not affect gluconeogenic gene expression in hIAPP mice. No changes in gluconeogenic gene (mRNA) expression were observed in either the high or low dose groups. Gene expression for PEPCK, G6Pase, PGC1α and PPIA was quantified by qRT-PCR. Data are means with SEM relative to PPIA. High control n=11, high treated n=13, low control n=7, low treated n=8.

To examine the effects of Pb exposure on pancreatic insulin production, insulin levels were determined in plasma samples from multiple time points throughout the study. There was also an assessment of insulin levels during the 9 month OGTT at both the 0 and 60 minute time points. Insulin ELISA results from the hIAPP mice did not show differences between Pb and control groups. All groups displayed increasing insulin levels until week 40, when insulin levels decreased (Figure 6.14). The insulin ELISA results from the 40 week OGTT 0 and 60 minute samples showed that the high treated animals not only appear to hypersecrete insulin when faced with a glucose challenge, but that they appear to have higher basal levels of insulin as well (Figure 6.15). The low dose mice did not show the expected increase in insulin levels after a glucose challenge. Although the reason for this is not clear, it is clear that like the ZDF rats, Pb exposure did not cause a significant reduction in the capacity of the pancreas to produce insulin. Although not directly measured, these results are consistent with the possibility that Pb induces deficiency in peripheral insulin sensitivity.

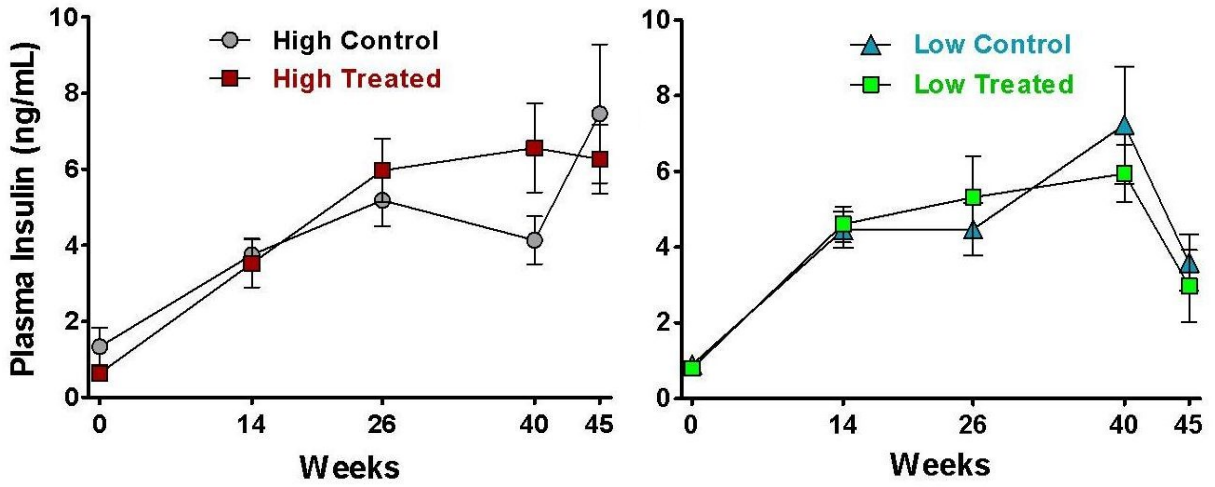

Fig. 6.14 – Pb exposure does not affect insulin production in hIAPP mice.
Plasma insulin measured by ELISA showed increasing levels in all groups until week 40. After week 40, both the low and high treated groups' insulin levels decreased. Data showed no statistical significance by ANOVA and are means +/- SEM. Week 0 and 14: High control and treated n=16, Low control and treated n=12. Week 26: High control and treated n=15, Low control n=10 low treated n=11. Week 40: High control n=13, high treated n=14, low control n=8, low treated n=10. Week 45: High control n=11, high treated n=13, low control n=7, low treated n=8.

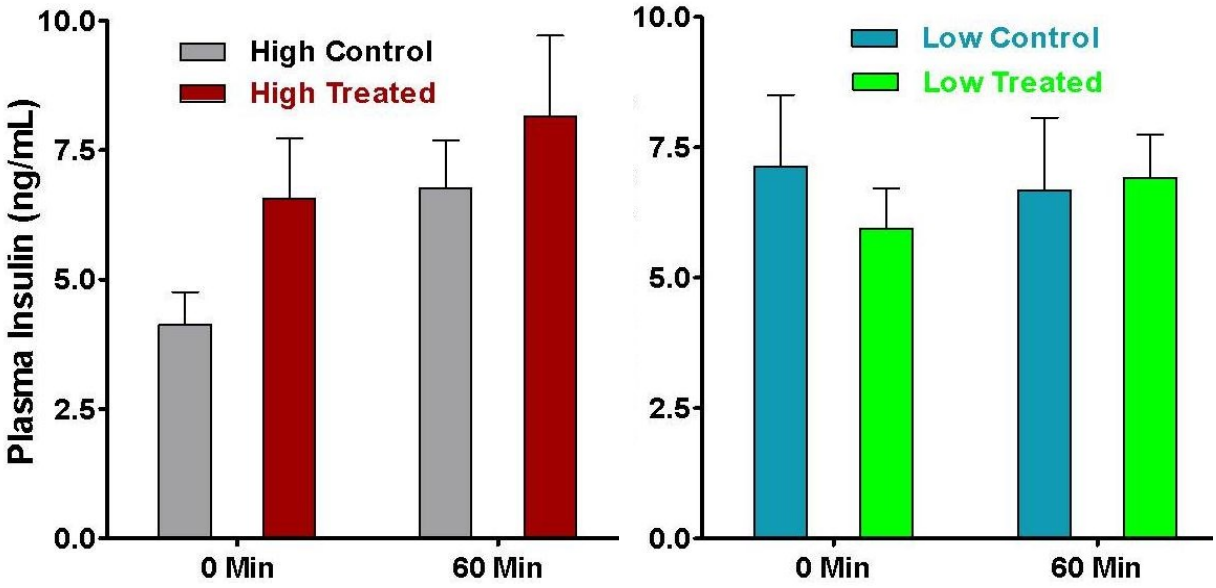

Fig. 6.15 – Pb exposure does not affect insulin production after glucose challenge.
Plasma insulin measured at 40 weeks, before (0 Min) and after (60 Min) glucose bolus showed that high treated mice began with higher basal levels of insulin than control mice. While both groups' insulin levels increase proportionally, the high Pb-treated mice appear to hypersecrete insulin. The low dose mice show no significant difference at 0 and 60 minutes. Data showed no statistical significance by students T test. Data are means +/- SEM. 40 Weeks: High control n=13 high treated n=14, low control n=8, low treated n=10.

A small sample of flash-frozen liver tissue was analyzed for triglyceride content. These findings revealed that in the high treated animals, Pb exposure induced an accumulation of triglycerides in the liver (Figure 6.16). Similar findings were observed in the low dose animals, although the differences were not statistically significant in this group. Overall however, these data are strongly consistent with the findings from the ZDF rats; clearly demonstrating that Pb exposure induces hepatic steatosis in rodents.

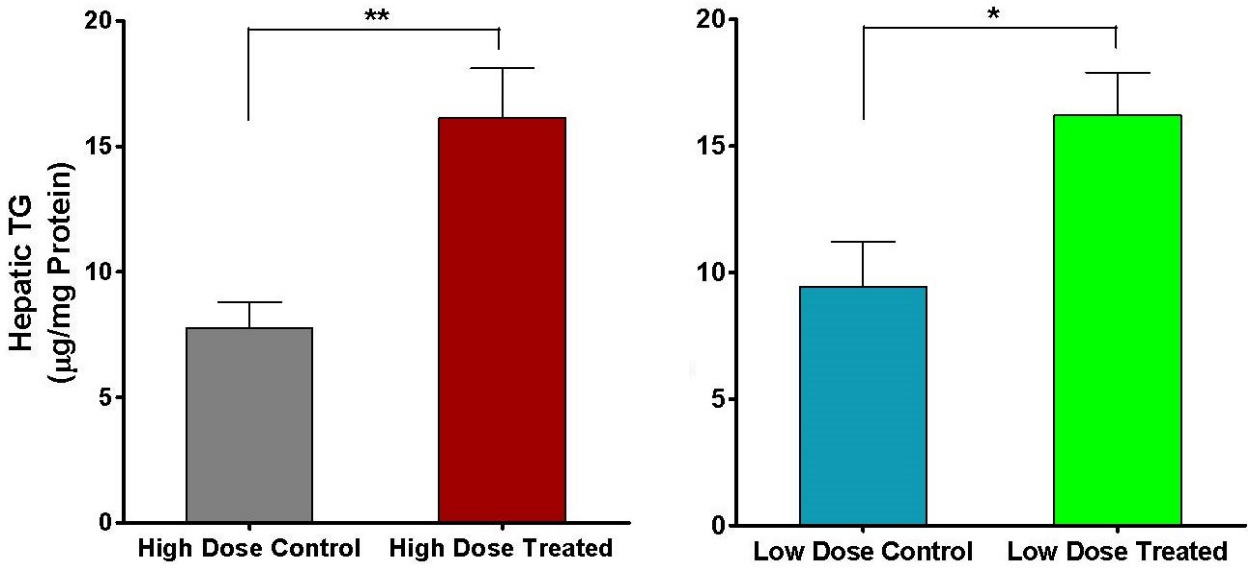

Fig. 6.16 – Pb exposure causes increased triglyceride accumulation in hIAPP mice.
Liver triglycerides measures showed both high and low dose treated mice had greater accumulations of triglycerides within the liver tissue. Data analyzed by students T test. Data are means +/- SEM *p<0.05, **p<0.005. High control n=11, high treated n=13, low control n=7, low treated n=8.

6.5 – Pb Exposure Effects on Histological Parameters

To determine if Pb exposure affected liver morphology, a portion of the liver from each animal was subjected to standard histological analyses. Hematoxylin and eosin (H & E) staining demonstrated that Pb exposure induced the accumulation of lipid droplets in liver. This occurred at both doses and is consistent with the triglyceride assay presented above. However, other than the lipid droplets, all of the mice had normal hepatic lobule structure (Figures 6.17, 6.18, 6.19 and 6.20), showing that Pb does not have a significant effect on gross liver morphology.

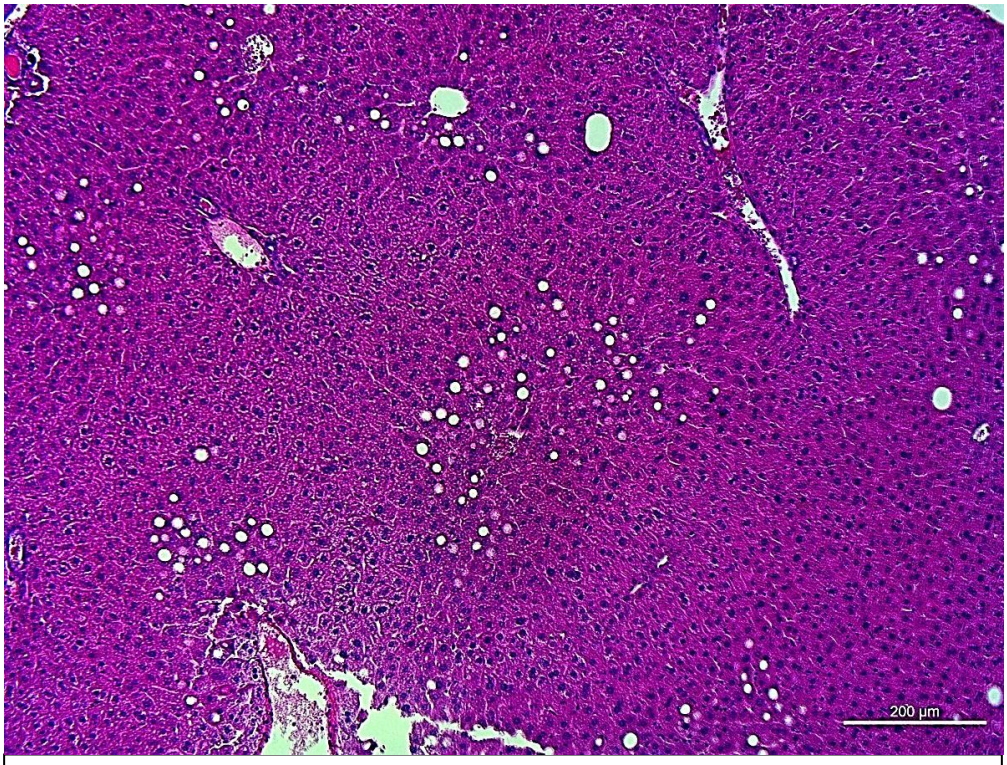

Fig. 6.17 – High control liver H & E.
Normal hepatic lobular structure is intact. Magnification 10x, bar = 200µm.

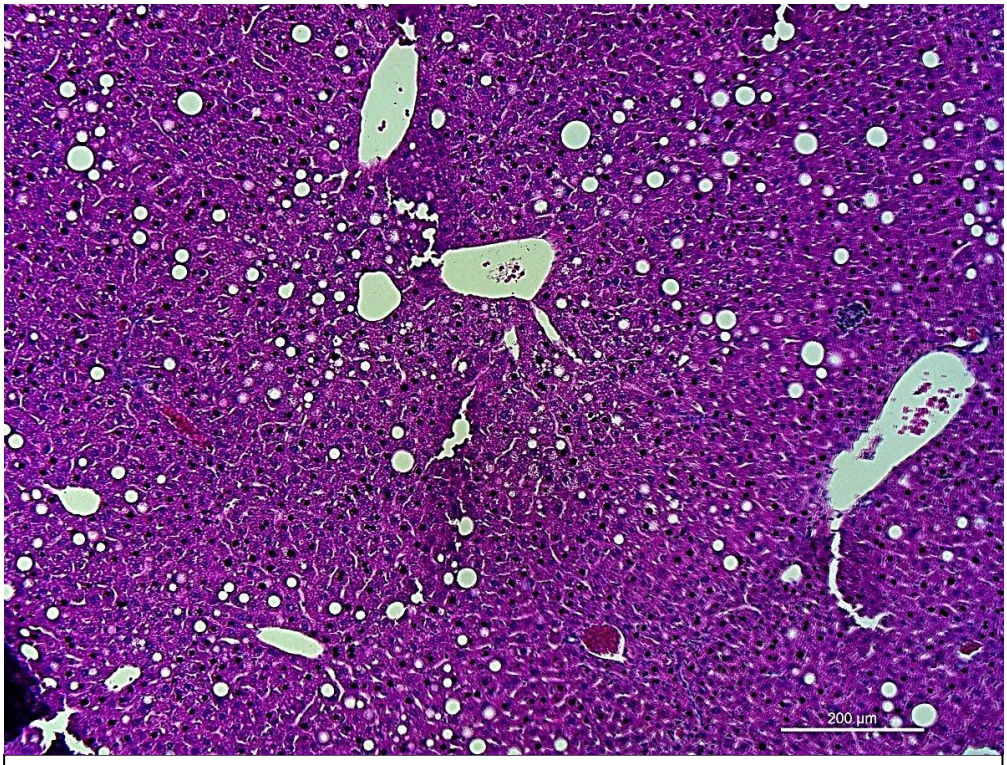

Fig. 6.18 – High treated liver H & E.
Lipid droplets are visible within the hepatic tissue of high treated mice. Magnification 10x, bar = 200µm.

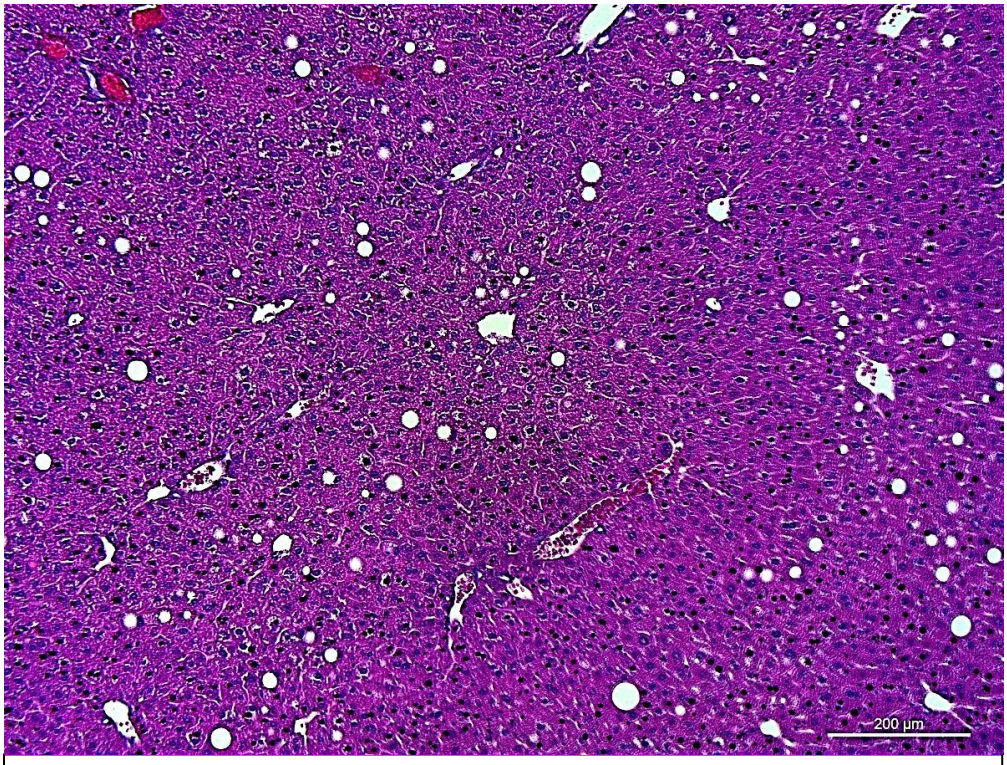

Fig. 6.19 – Low control liver H & E.
Normal hepatic lobular structure is intact. Magnification 10x, bar = 200µm.

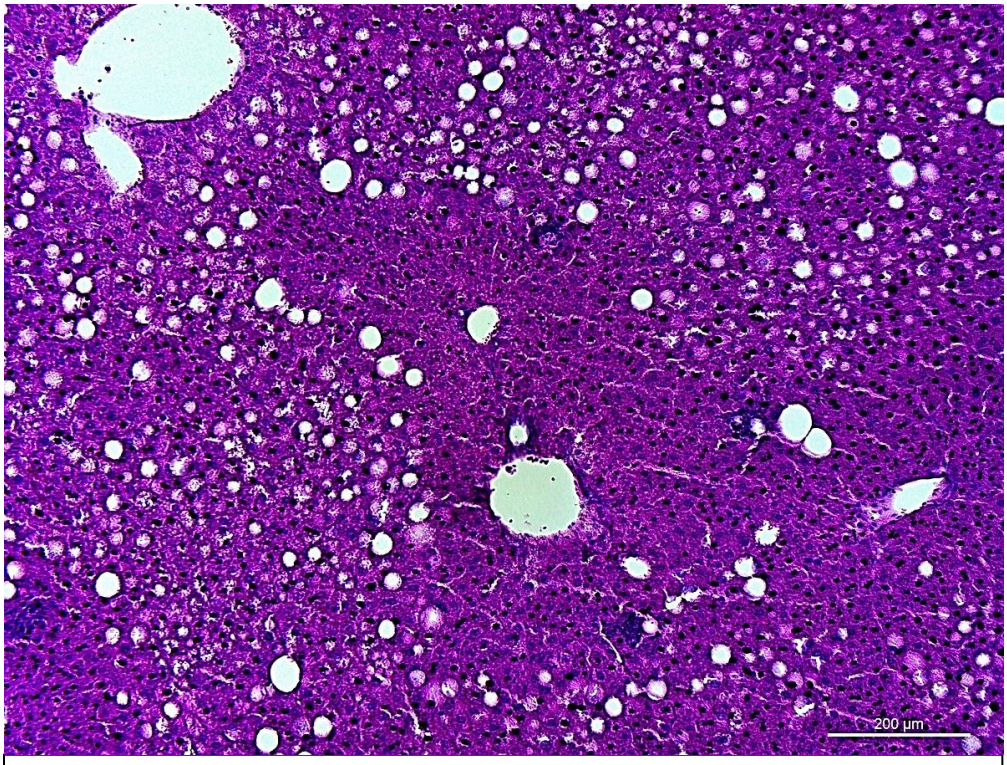

Fig. 6.20 – Low treated liver H & E.
Lipid droplets are visible within the hepatic tissue of low treated mice. Magnification 10x, bar = 200µm.

Periodic Acid-Schiff (PAS) stain was used to determine if Pb had any effect on hepatic glycogen content or distribution. A specimen that is PAS-positive will show glycogen (carbohydrates) as pink/magenta to purple-violet/red staining. PAS staining revealed that liver from Pb-exposed animals contained less glycogen than control animals, as evidenced by the decreased level of PAS-positive staining in both the high and low Pb-treated mouse liver slides (Figures 6.21, 6.22, 6.23 and 6.24). Glycogen depletion was further confirmed in the low dose animals by diastase digestion followed by PAS staining (Figures 6.23 and 6.24). Diastase digestion is often used to confirm the presence of glycogen in tissue samples, as diastase digestion removes carbohydrates from tissues, thereby resulting in negative PAS staining in tissues that had previously been PAS-positive.

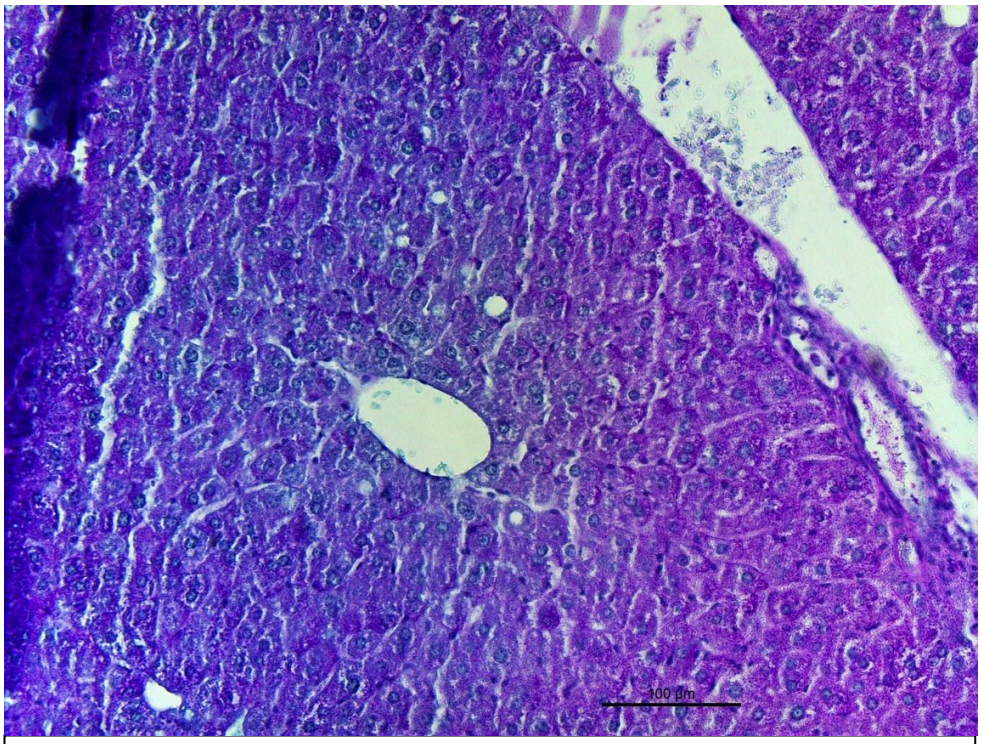

Fig. 6.21 – High control liver PAS.
Numerous PAS-positive glycogen deposits (purple-violet staining) are present in the control mouse liver. Magnification 20x, bar = 100µm.

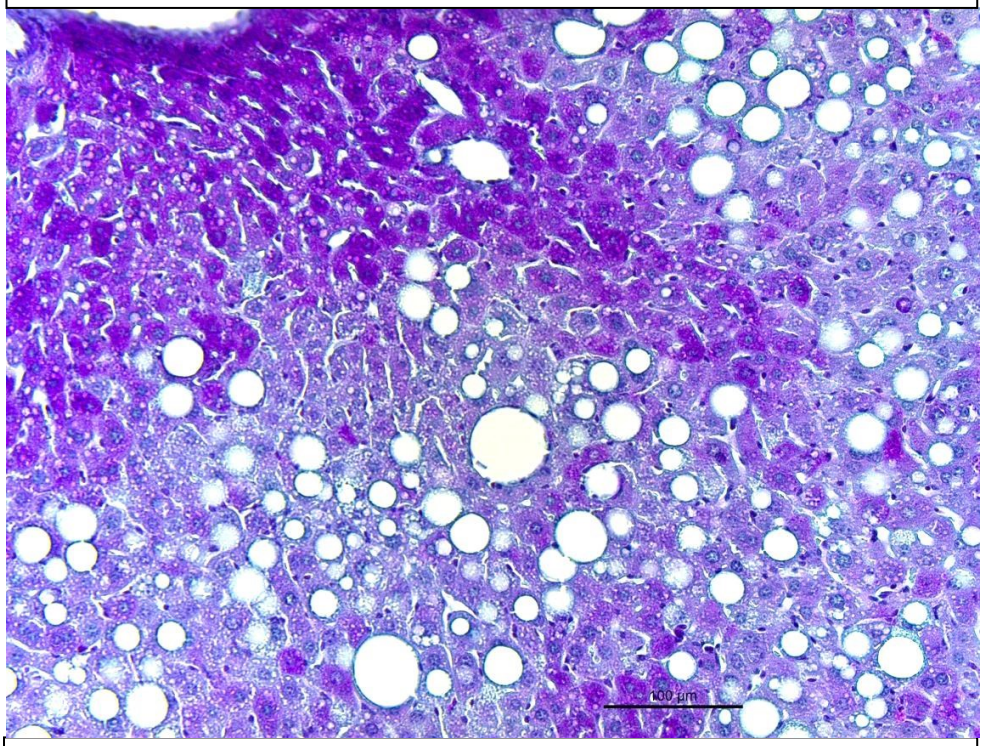

Fig. 6.22 – High treated liver PAS. Glycogen depletion after Pb exposure as evidenced by decreased PAS-positive staining (purple-violet coloration) in the Pb-treated liver. Magnification 20x, bar = 100µm.

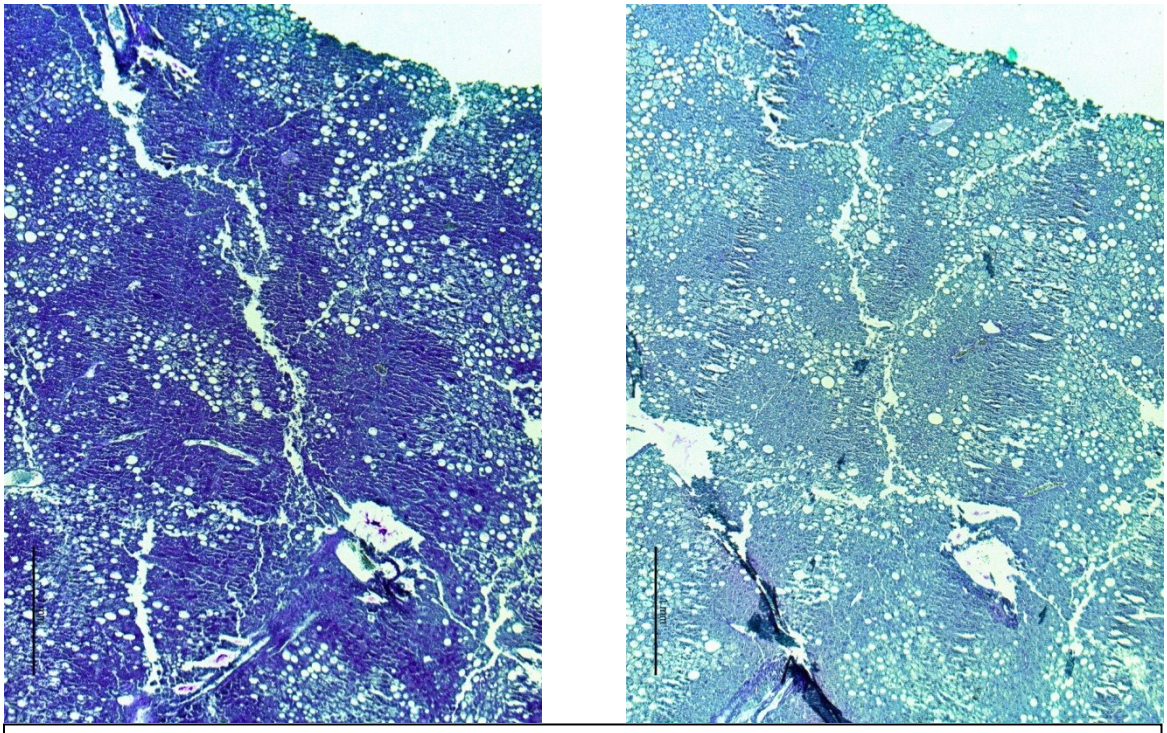

Fig. 6.23 – Low control liver PAS. Numerous PAS-positive glycogen deposits (purple-violet staining) are present in the undigested sample (left). But when digested (right), there is a dramatic loss of PAS-positive staining. Magnification 5x, bar = 1mm.

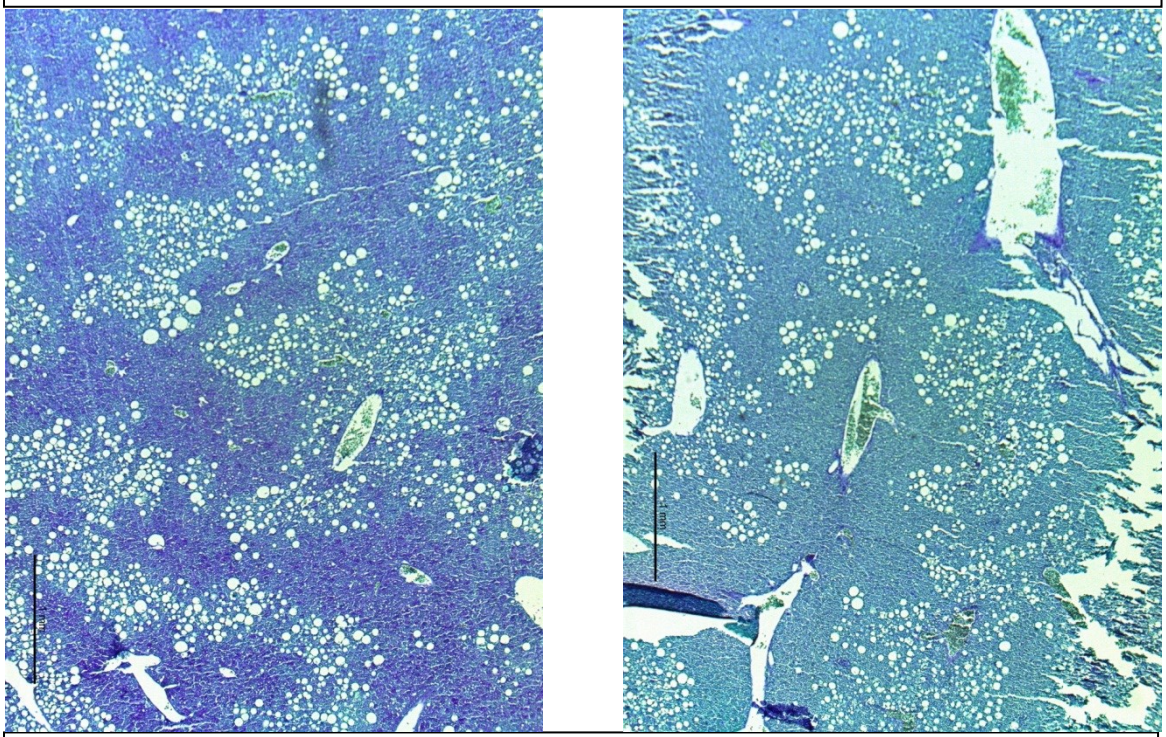

Fig. 6.24 – Low treated liver PAS. Glycogen depletion after Pb exposure as evidenced by decreased PAS-positive staining (purple-violet coloration) in the undigested (left) sample. Digestion (right) does not result in the same dramatic changes as seen in the control mouse above. Magnification 5x, bar = 1mm.

To determine if Pb exposure affected pancreatic morphology, a portion of the pancreas from each animal was subjected to standard histological analyses. H & E staining demonstrated that Pb exposure does have an effect on islet morphology. Both high and low control mouse islets appear normal, with a round shape and a well-defined border (Figures 6.25 and 6.27). However, both high and low Pb-treated mouse islets are much larger in size than in control animals (Figures 6.26 and 6.28).

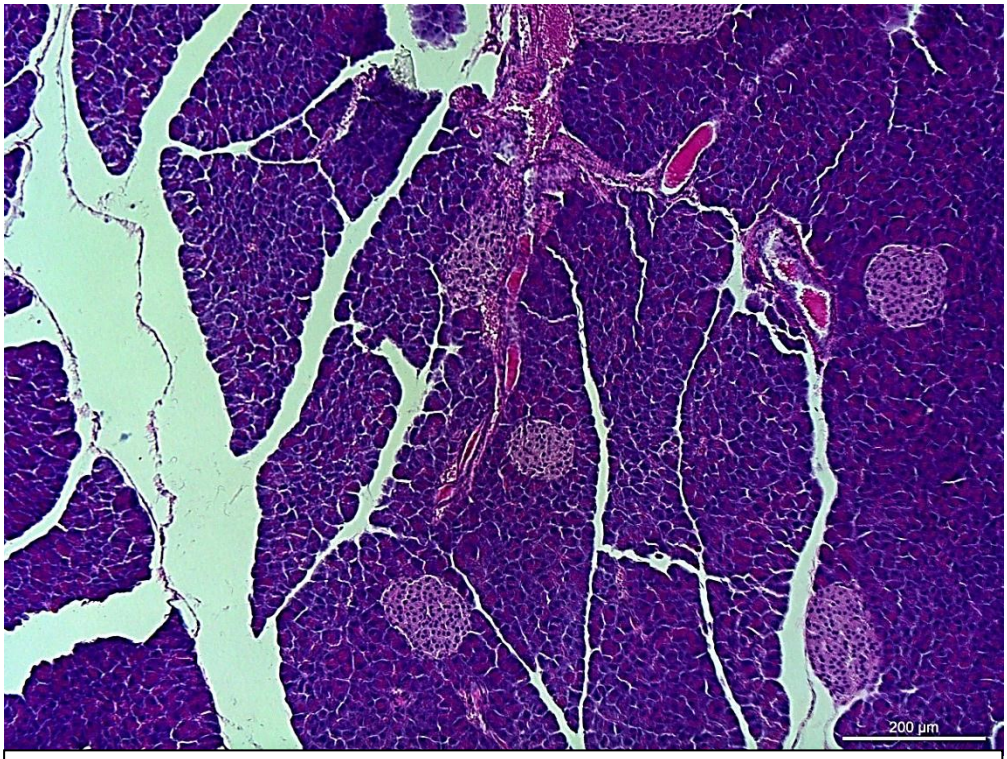

Fig. 6.25 – High control pancreas H & E.
Islets are lighter stained, round areas. Control mice appear to have normal islet physiology. Magnification 10x, bar = 200µm.

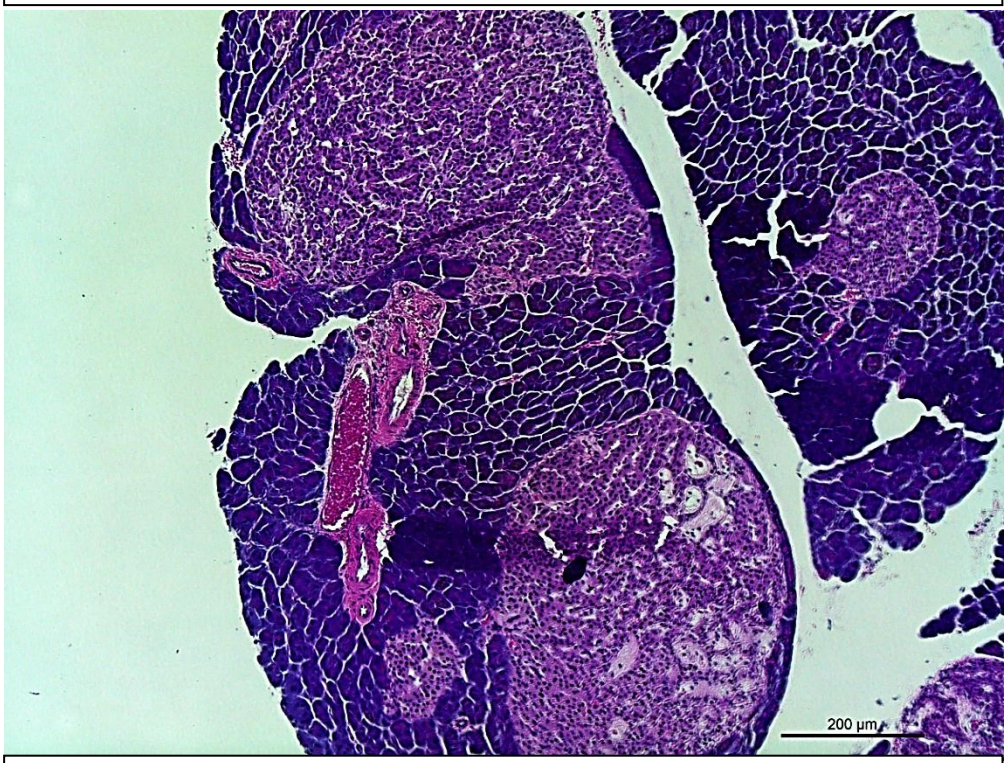

Fig. 6.26 – High treated pancreas H & E.
Islets are lighter stained areas. Pb-treated mice appear to have hypertrophied islets. Magnification 10x, bar = 200µm.

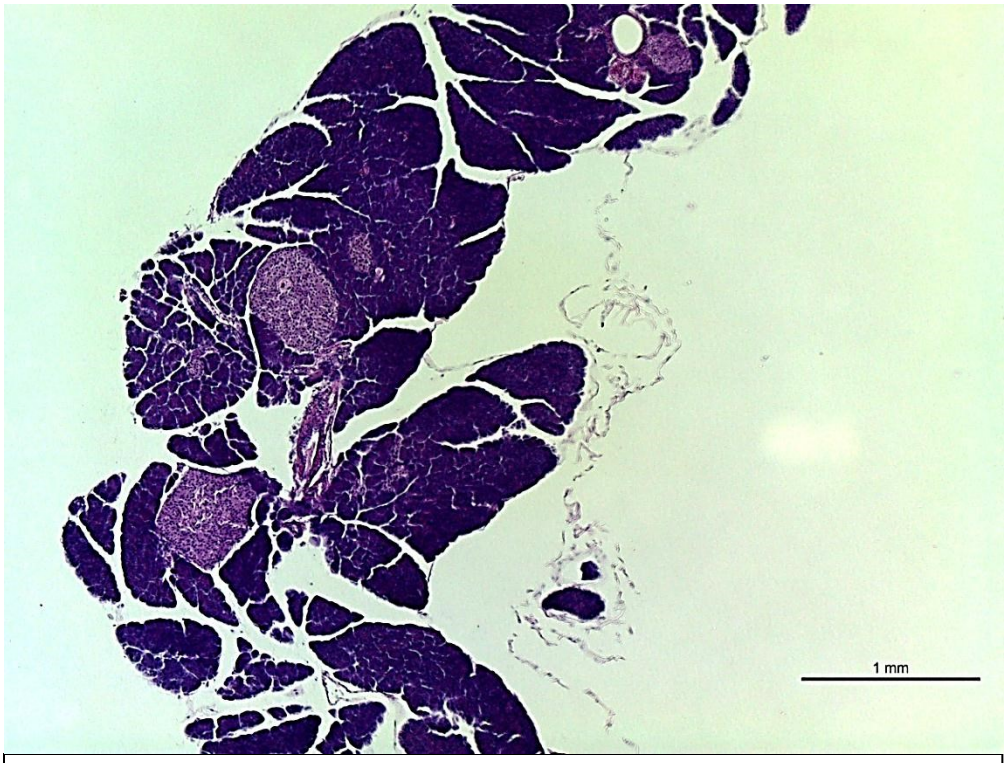

Fig. 6.27 – Low control pancreas H & E.
Islets are lighter stained, round areas. Control mice appear to have normal islet physiology. Magnification 5x, bar = 1mm.

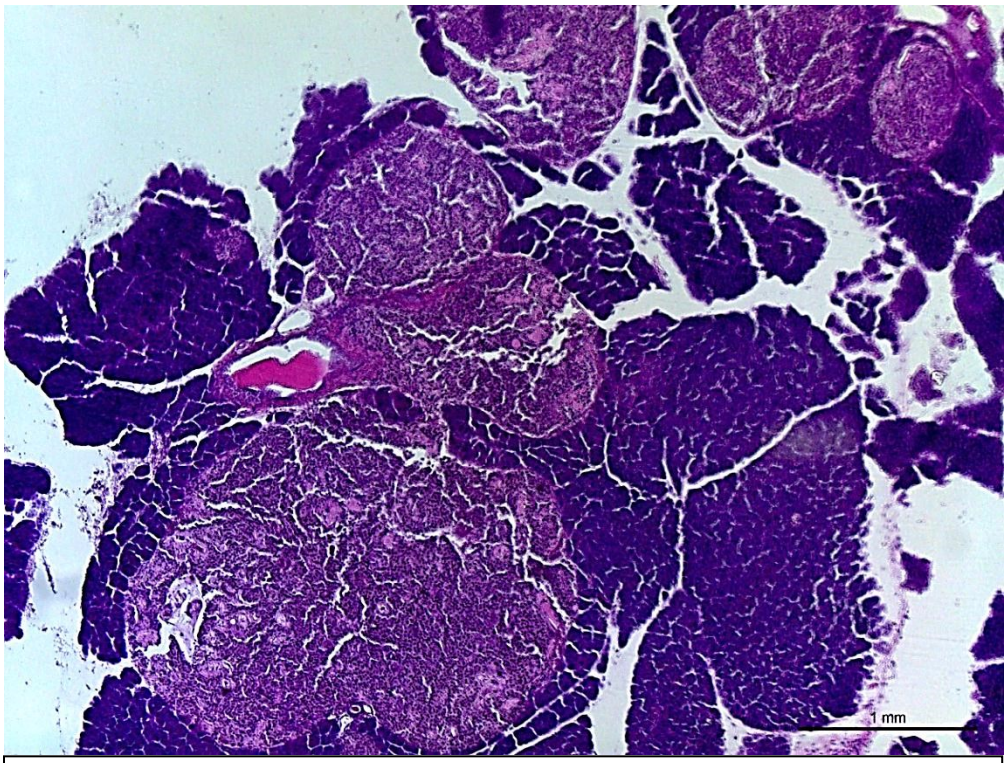

Fig. 6.28 – Low treated pancreas H & E.
Islets are lighter stained areas. Pb-treated mice appear to have hypertrophied islets. Magnification 5x, bar = 1mm.

Differences in islet sizes were quantified by measuring islet diameter and dividing them into 3 categories: small (<400µm), medium (>400µm - < 800µm) and large (>800µm). Figures 6.29 and 6.30 illustrate the distribution of islet sizes in control and treated groups. While the control groups show a higher proportion of islets in the smaller size categories, the Pb-treated groups show distributions strongly skewed to the larger size categories. These findings clearly demonstrate that Pb exposure induces islet hypertrophy. In addition, the subjective appearance of the islets from the Pb exposed mice indicates a general degradation of islet architecture, which is consistent with the possibility that these islets have at least some degree of compromised endocrinological function.

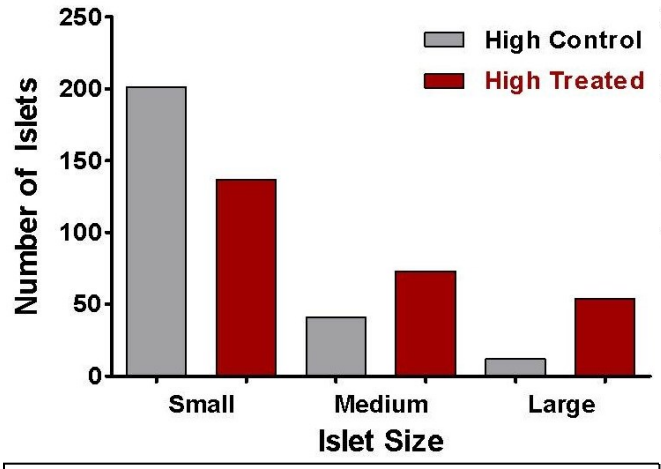

Fig. 6.29 – Pb exposure affects islet size distribution in high dose group. Islets were counted and divided based on size. High Pb-treated mice had many larger islets, while the high control mice had many smaller size islets. Total number of islets assayed: 254-high treated and 264-high control.

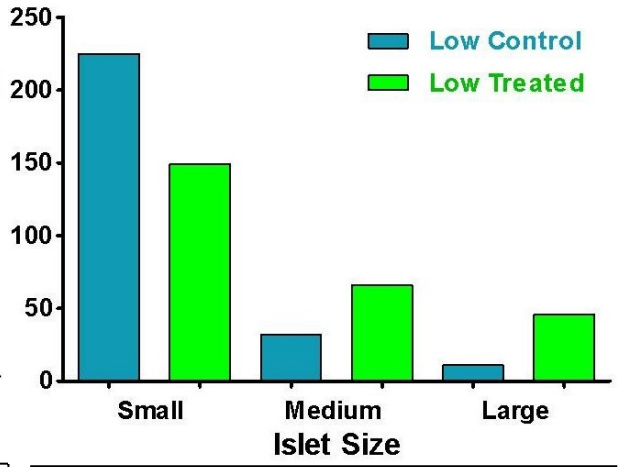

Fig. 6.30 – Pb exposure affects islet size distribution in low dose group. Islets were counted and divided based on size. Low Pb-treated mice had many larger islets, while the low control mice had many smaller size islets. Total number of islets assayed: 261-low treated and 265-low control.

Noting the Pb-mediated alterations in islet morphology, I wanted to determine if Pb exposure affected the prevalence of β-cells in pancreatic islets. Aldehyde Fuchsin is commonly used to detect islets and β-cells in pancreatic tissue samples as blue to violet or purple punctate in color. Although not easily quantified, the density of dark blue punctate staining appeared to be increased in the Pb exposed animals. However, because the Pb-treated islets are much larger, both groups appear to have the same proportion of β-cells (Figures 6.31, 6.32, 6.33 and 6.34). These findings, while not striking, suggest that the increased islet size in Pb-treated mice is related to Pb-dependent processes occurring within the islets of these mice.

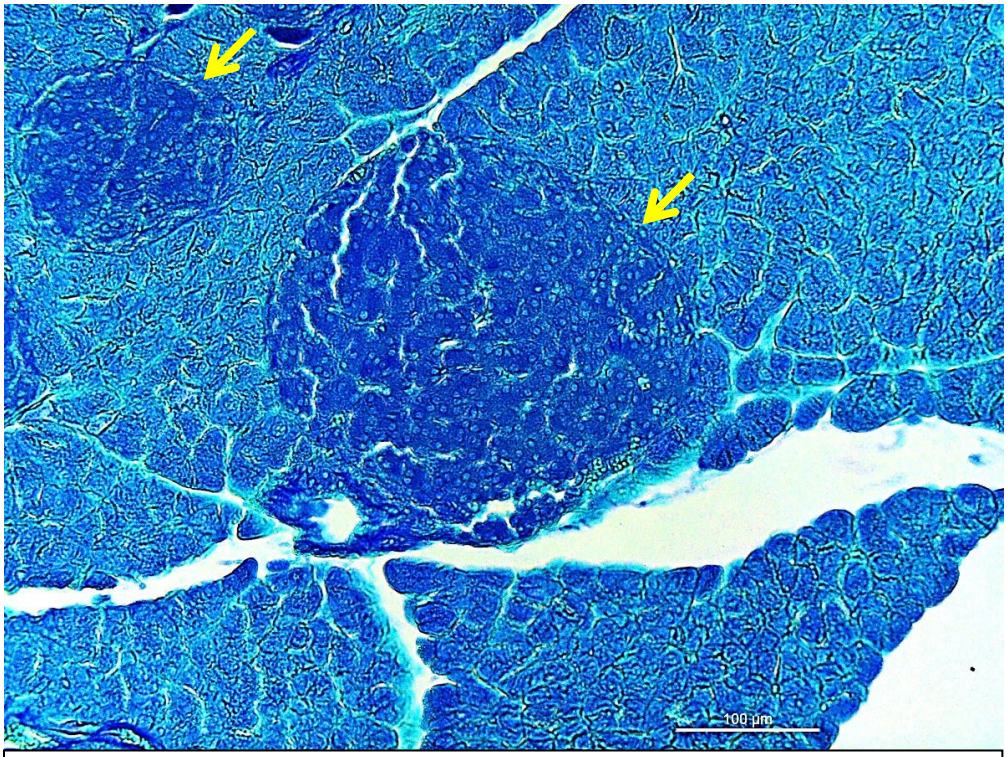

Fig. 6.31 – High control pancreas Aldehyde Fuchsin.
Arrows indicate location of islets, and Aldehyde Fuchsin-positive β-cells. Magnification 20x, bar = 100µm.

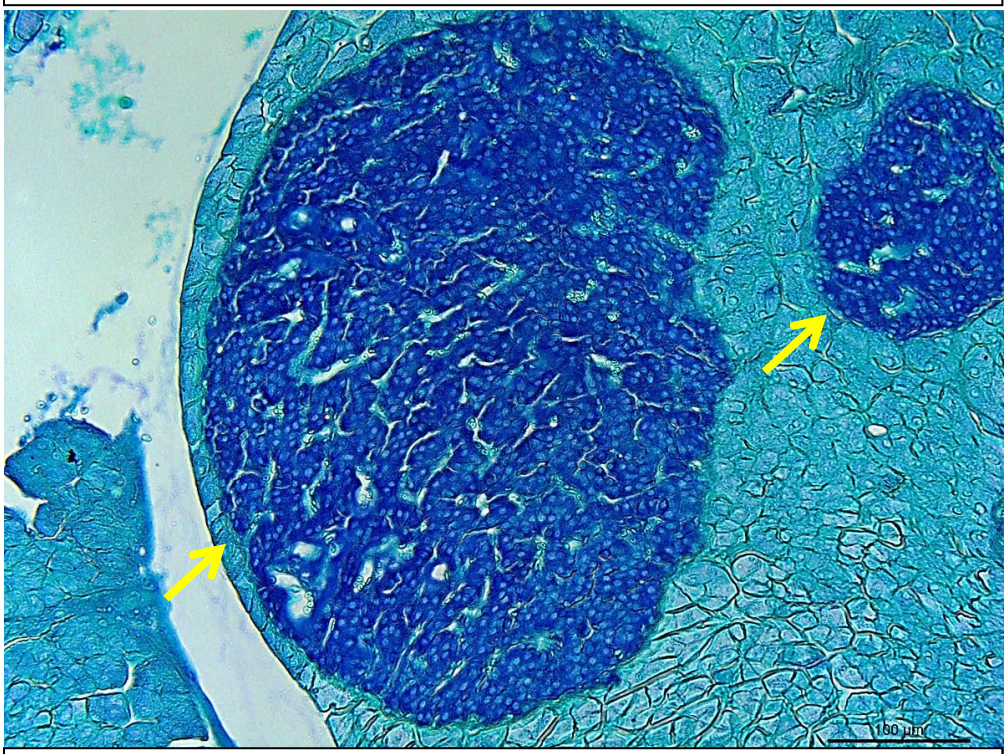

Fig. 6.32 – High treated pancreas Aldehyde Fuchsin.
Arrows indicate location of islets, and Aldehyde Fuchsin-positive β-cells. Magnification 20x, bar = 100µm.

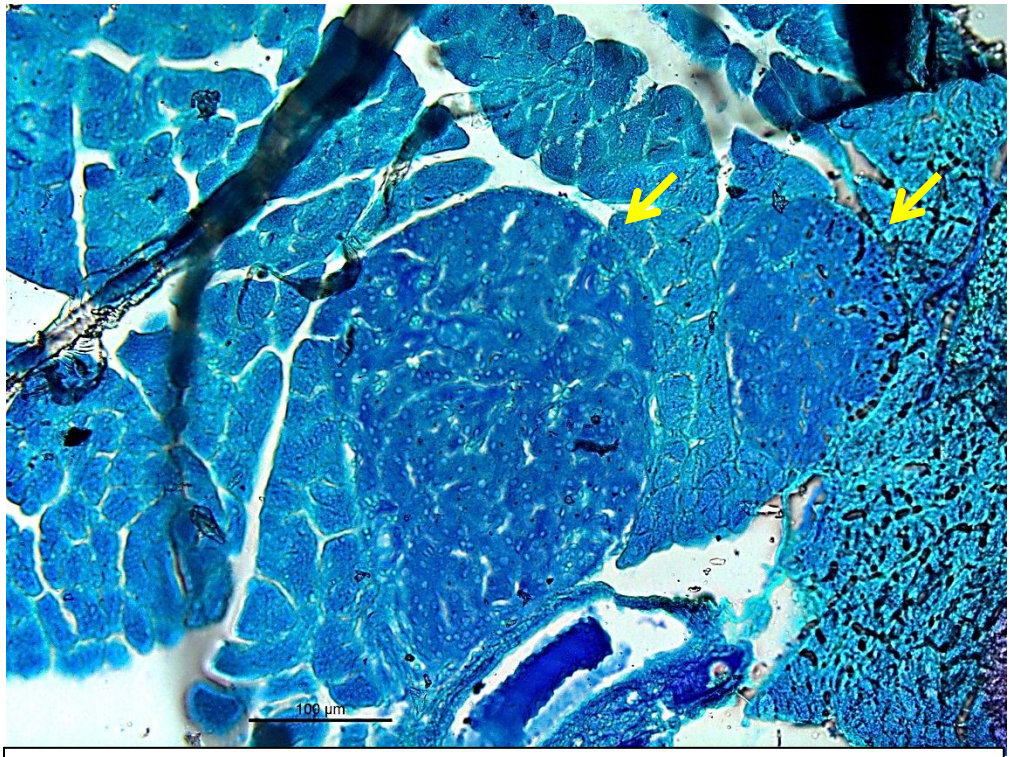

Fig. 6.33 – Low control pancreas Aldehyde Fuchsin.
Arrows indicate location of islets, and Aldehyde Fuchsin-positive β-cells. Magnification 20x, bar = 100µm.

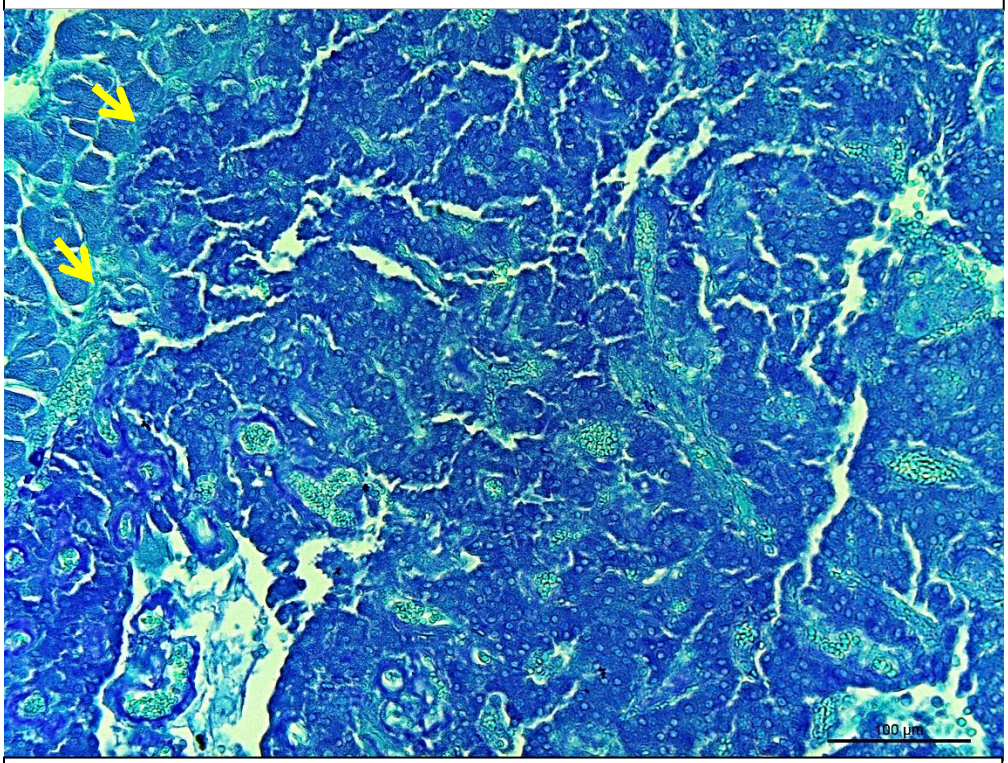

Fig. 6.34 – Low treated pancreas Aldehyde Fuchsin.
Arrows denote the edge of the islet (which is too large for the field of view) and Aldehyde Fuchsin-positive β-cells. Magnification 20x, bar = 100µm.

6.6 – Pb Exposure Causes Increased Amyloid Plaque Formation

The transgenic mouse model utilized in this study was specifically created to more closely mimic the human form of diabetes, including amyloid plaque formation. To determine the extent of amyloidosis within the pancreatic islets of the hIAPP transgenic mice, a specialized stain for amyloid was utilized. Congo Red is commonly used to detect amyloid in pancreatic tissue samples, it appears as a bright pink/red stain in a bright field microscope; while polarized light shows it as an apple-green birefringence. As is evident from the photos below, both the high and low treated mice had significantly greater amyloid accumulation within the pancreatic islets (Figures 6.35, 6.36, 6.37 and 6.38).

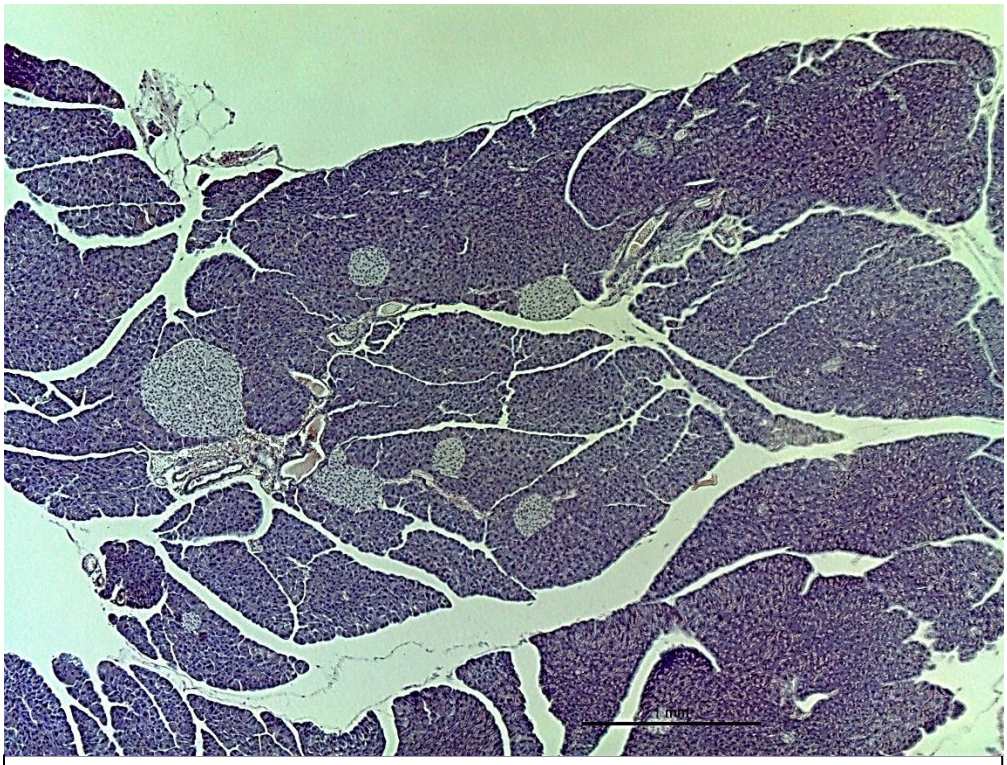

Fig. 6.35 – High control pancreas Congo Red, bright field.
Lighter stained areas are islets, pink inside islets is amyloid. Control mice had very little amyloid. Magnification 5x, bar = 1mm.

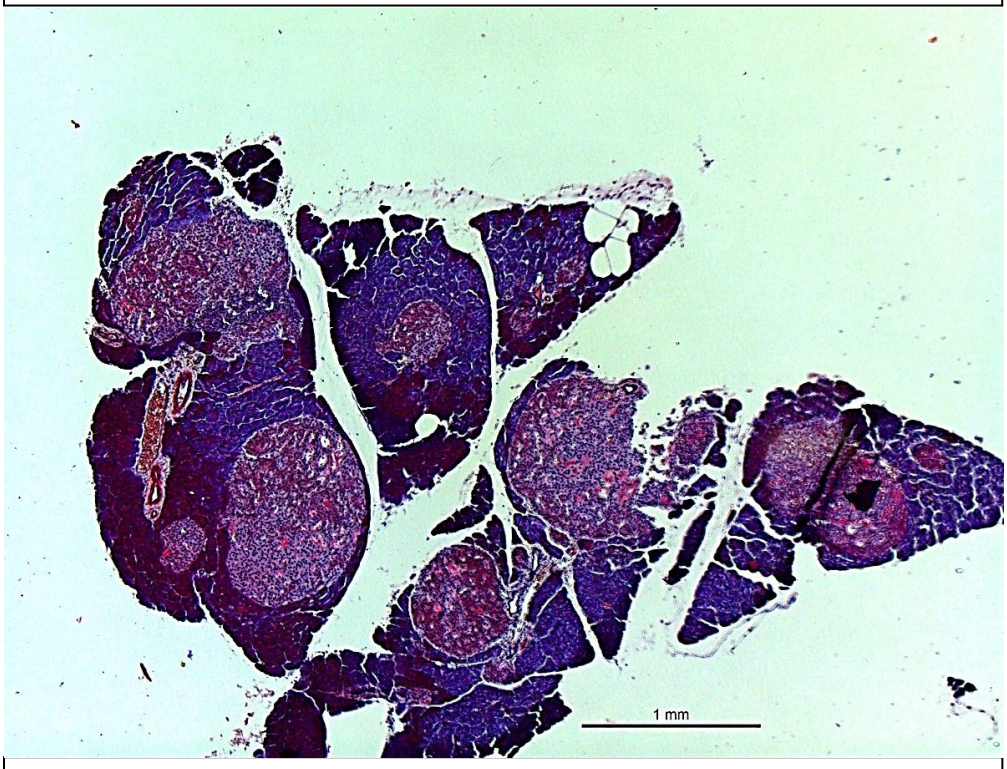

Fig. 6.36 – High treated pancreas Congo Red, bright field.
Bright pink and red areas are amyloid positive inside of islets. Treated mice had significantly more amyloid depositions. Magnification 5x, bar = 1mm.

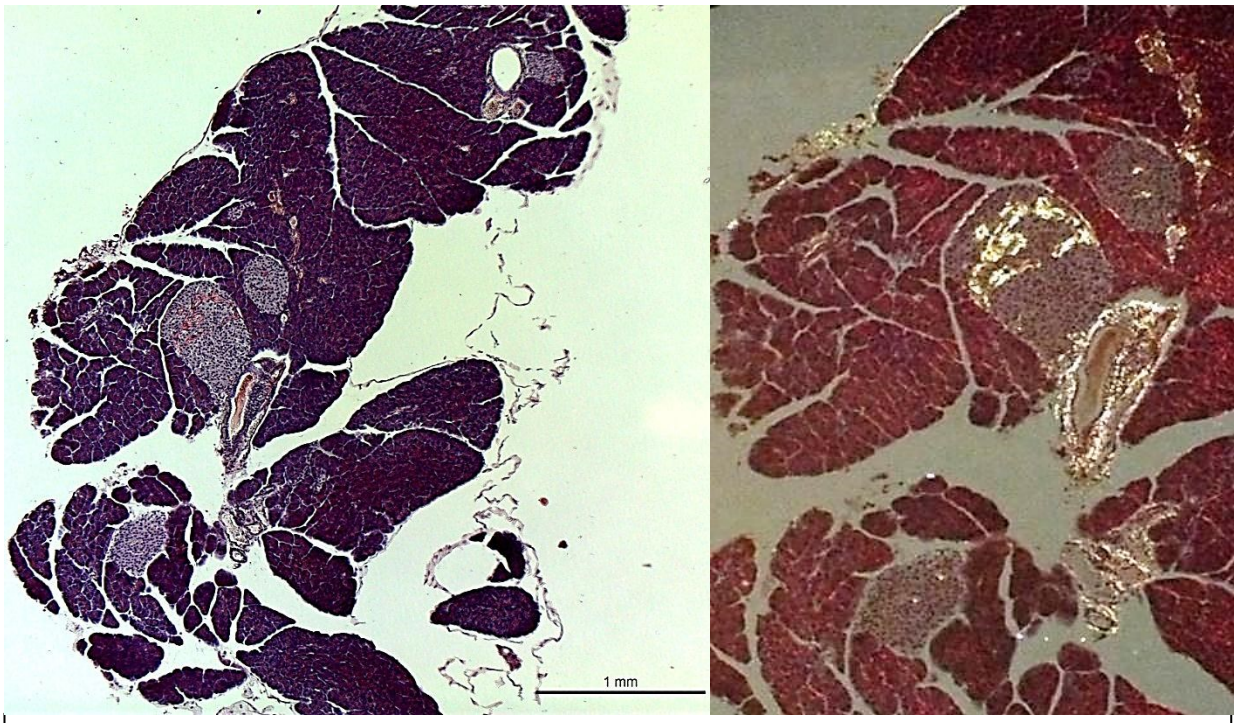

Fig. 6.37 – Low control pancreas Congo Red, bright field (left) and polarized (right).
Lighter stained areas are islets; pink inside islets is amyloid (left). Apple-green bi-refringence inside islets is amyloid deposits. Control mice had very little amyloid. Magnification 5x, bar = 1mm (left), magnification 10x (right).

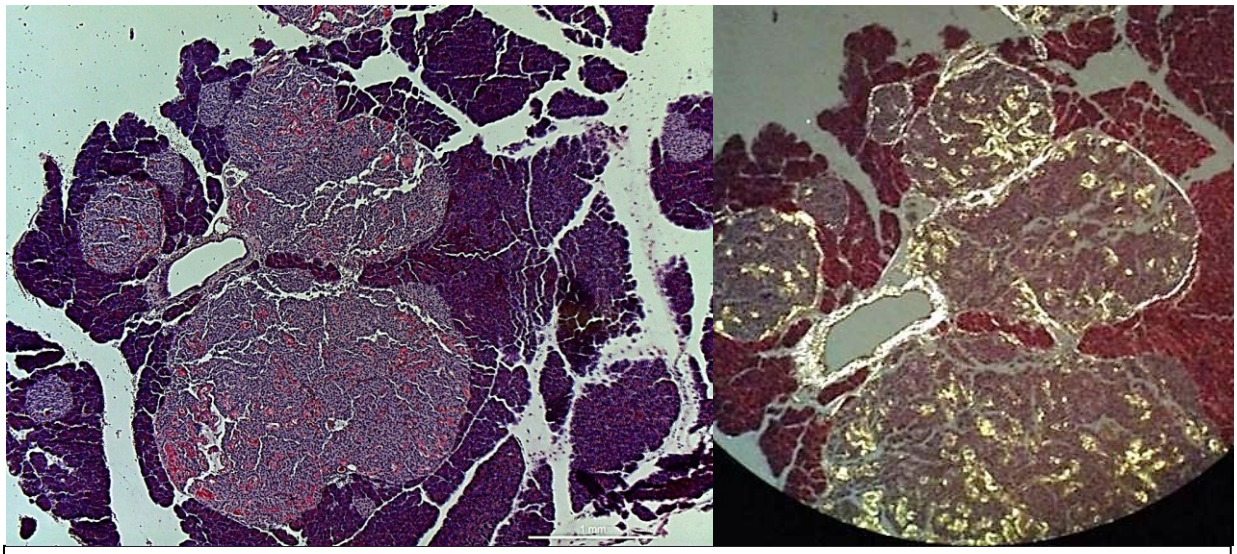

Fig. 6.38 – Low treated pancreas Congo Red, bright field (left) and polarized (right).
Lighter stained areas are islets; bright pink-red inside islets is amyloid (left). Apple-green bi-refringence inside islets is amyloid deposits. Treated mice had significantly more intra-islet amyloid. Magnification 5x, bar = 1mm (left), magnification 10x (right).

Quantification of the portion of the islet covered with amyloid was carried out by dividing islets into 4 categories: no amyloid, low = <1/3 amyloid coverage, medium = >1/3-<2/3 amyloid coverage and high = >2/3 amyloid coverage (Figures 6.39 and 6.40). These findings are striking and clearly demonstrate that Pb-exposure exacerbates amyloid plaque formation in pancreatic islets.

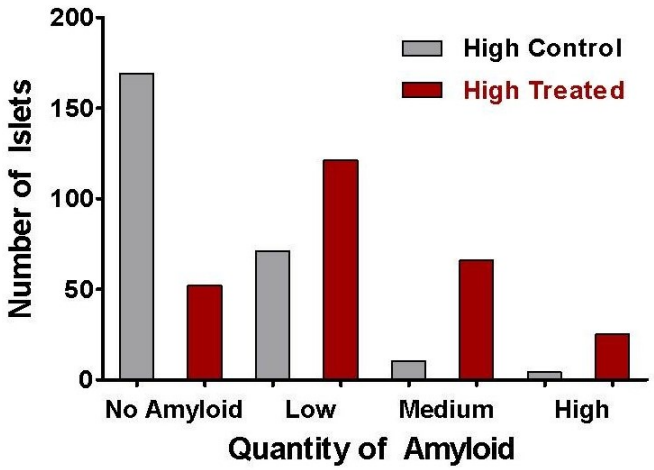

Fig. 6.39 – Pb exposure increases amyloid plaque formation in high dose group. Islets were assessed based on surface area filled with amyloid. High Pb-treated mice had significantly fewer islets with no amyloid than the high control mice. Total number of islets assayed: 254-high treated and 264-high control.

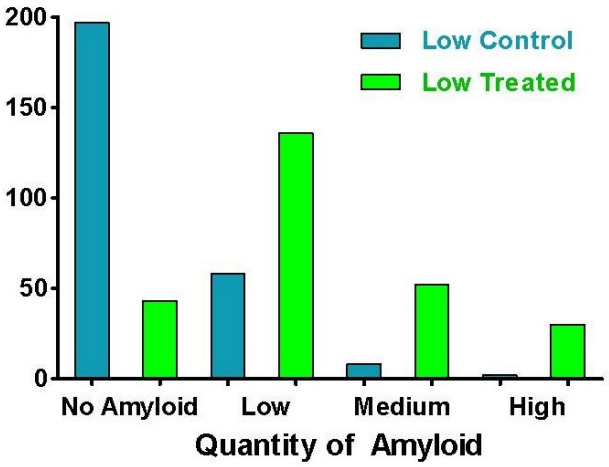

Fig. 6.40 – Pb exposure increases amyloid plaque formation in low dose group. Islets were assessed based on surface area filled with amyloid. Low Pb-treated mice had significantly fewer islets with no amyloid than the low control mice. Total number of islets assayed: 261-low treated and 265-low control.

6.7 – Pb Exposure Affects Liver: Body Weight Ratios

Upon termination and dissection of organs, I noted that livers from control animals appeared larger than livers from Pb-exposed animals. This observation was confirmed when the whole excised livers were weighed and liver weights graphed as percent of body weight (Figure 6.41). Both the high and low Pb-treated mice had significantly lower liver-to-body weight ratios than the control mice from both groups. This was an unexpected finding, especially considering the observed Pb-induced hepatic steatosis (Figure 6.16), which is usually associated with increased liver weight.

It is well-known that there is a negative correlation between liver size and age [219-221] and it is possible that Pb exposure accelerated this age-related effect on liver size, although the mechanism by which this might occur remains obscure.

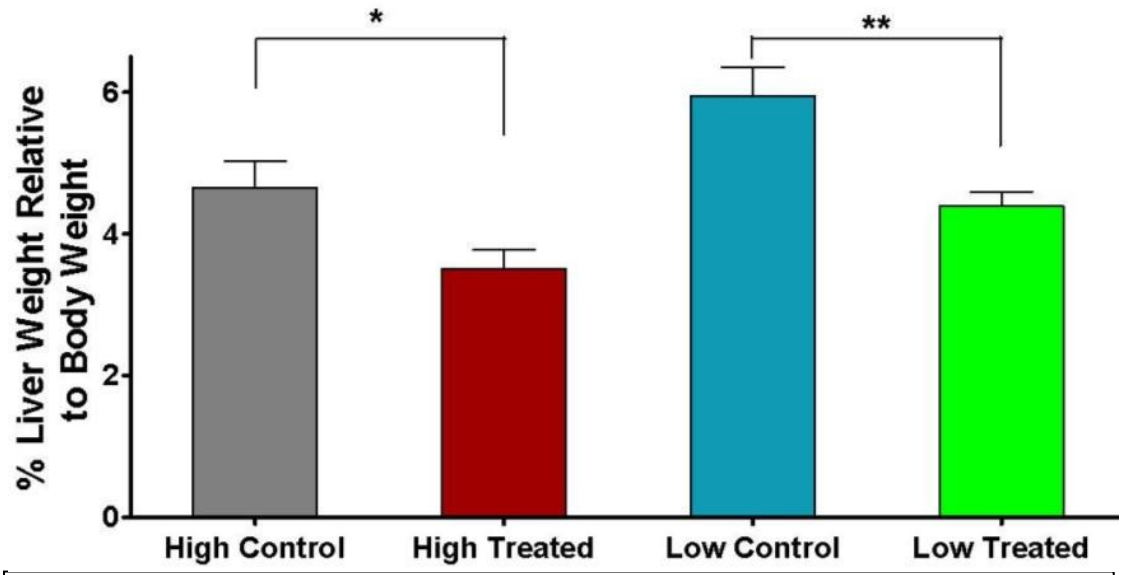

Fig. 6.41 – Pb exposure affects liver: body weight ratios in hIAPP mice.
Whole liver weights obtained at sacrifice showed that both the high and low Pb-treated mice had significantly lower liver-to-body weight ratios compared to controls. Data analyzed by students T test. Data are means +/- SEM *p<0.05, **p<0.005. High control n=11, high treated n=13, low control n=7, low treated n=8.

6.8 – Pb Exposure, Whole-Body Insulin Resistance and β-Cell Function

Our lab is not equipped to assess insulin sensitivity by direct measure, so I utilized an assessment tool that has been widely used in human epidemiological studies, but is given mixed acceptance when applied to animal studies. The homeostatic model assessment (HOMA) is a technique utilized to estimate insulin resistance and beta-cell function from insulin and fasting blood glucose samples obtained at the same time. The original HOMA-IR technique, developed in 1985, was utilized extensively, particularly in clinical and epidemiological studies [222]. However, the 1985 model did not consider differences in peripheral and hepatic insulin sensitivity, alterations in insulin secretion or hepatic glucose output when fasting blood glucose measured above 180 mg/dL, or the presence of circulating pro-insulin. To take into consideration all of these differences, a modified version of the analysis, termed HOMA2-IR, was created [223]. The HOMA2-IR model utilizes a computer modeling program that calculates a corrected nonlinear estimation of insulin resistance (IR) and percent beta-cell function (% β-cell function). A number of empirical equations (nonlinear) are utilized by the HOMA2-IR computer program to predict numerical values from paired fasting blood glucose and insulin samples. I downloaded the HOMA2-IR calculator and utilized the fasting blood glucose and insulin measures from weeks 0, 14, 26, 40 and 45 of treatment to view estimated numerical values for the calculations of IR and % β-cell function between treated and control groups [224].

As demonstrated in Figures 6.42 and 6.43, all groups experienced progressively increasing insulin resistant and beta-cell function. However, there appears to be a trend toward greater peripheral insulin resistance in the high treated group. This is not,

however a confirmation of insulin resistance and it did not reach statistical significance. The β-cell graph is consistent with not seeing differences in insulin levels and changes in β-cell function are directly associated with insulin sensitivity. Increased beta-cell function is indicative of decreased peripheral insulin sensitivity and may correlate with β-cell hyperplasia.

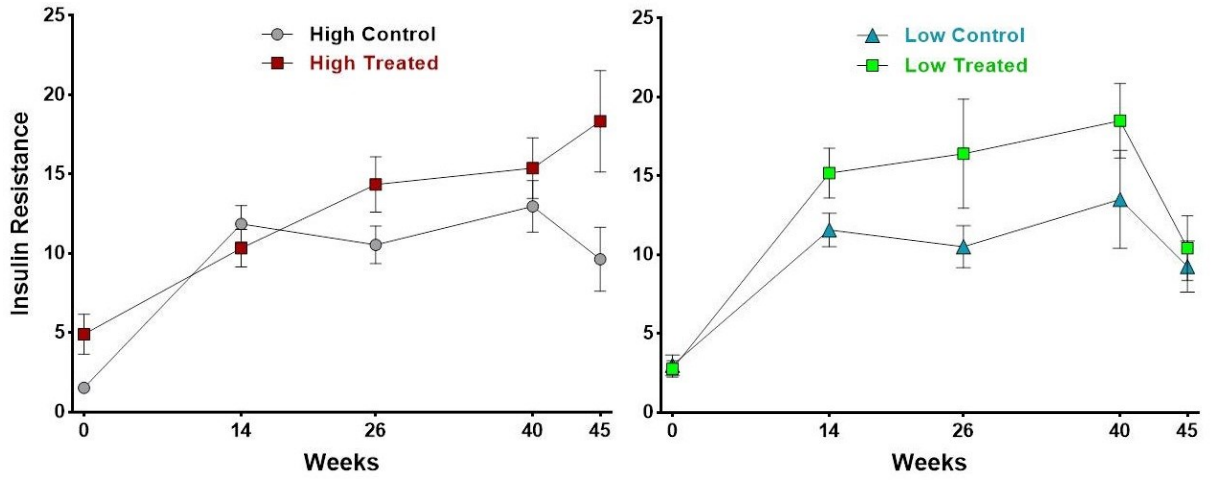

Fig. 6.42 – Pb exposure affects whole-body insulin resistance in hIAPP mice.
Whole-body insulin resistance as calculated by HOMA2-IR (units are arbitrary) demonstrated that all groups experienced progressively increasing insulin resistance (IR≤1.0 = non-insulin resistant, IR>2.7 = insulin resistant). Data are means +/- SEM. Weeks 0 and 14: High control and treated n=16, Low control and treated n=12. Week 26: High control and treated n=15, Low control n=10 low treated n=11. Week 40: High control n=13, high treated n=14, low control n=8, low treated n=10. Week 45: High control n=11, high treated n=13, low control n=7, low treated n=8.

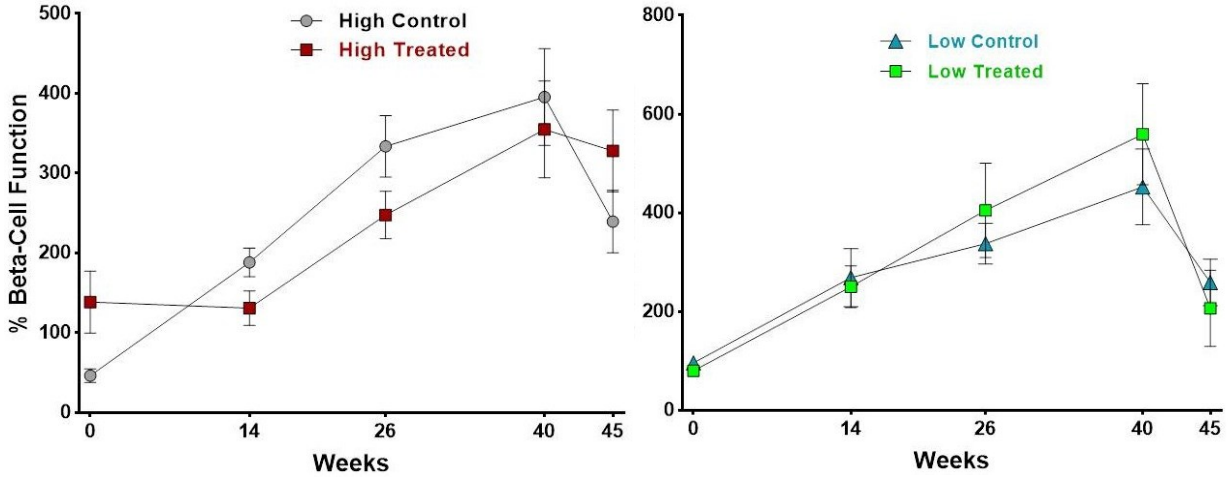

Fig. 6.43 – Pb exposure affects Beta-cell function in hIAPP mice.
Percent beta-cell function, as calculated by HOMA2-IR (%***B***=100 = normal beta-cell function, %***B***>100 = increased beta-cell function). Data are means +/- SEM. Weeks 0 and 14: High control and treated n=16, Low control and treated n=12. Week 26: High control and treated n=15, Low control n=10 low treated n=11. Week 40: High control n=13, high treated n=14, low control n=8, low treated n=10. Week 45: High control n=11, high treated n=13, low control n=7, low treated n=8.

6.9 – Additional Histological Observations

Liver tissue was examined for fibrosis with Masson's Trichrome stain, but there was no discernible difference between the control and Pb-treated in either the high or low dose groups (data not shown). Pancreatic tissue was also examined for fibrosis with Masson's Trichrome Stain, and while there was some fibrosis present, there was no discernible difference in the degree of fibrosis between the control and Pb-treated in either the high or low dose groups (data not shown).

6.10 – Discussion of hIAPP Study

The experiments conducted in the hIAPP transgenic mouse model were designed to determine if Pb-exposure was pro-diabetic in animals with high fat diet-induced obesity, and to specifically determine if Pb-exposure exacerbated the islet amyloid deposition characteristic of advanced Type 2 diabetes in humans.

It is clear from the data presented here, that Pb exposure, even at 0.005% w/v, in combination with the high fat diet, promotes the development of diabetes in hIAPP transgenic mice, specifically causing the development of fasting hyperglycemia and glucose intolerance over time.

Even though the hIAPP mice did not experience the same Pb-mediated elevations in gluconeogenic gene expression as the ZDF rats, this may be accounted for by differences in termination procedures as well as differences in the physiology of the two rodents. The ZDF rats were sacrificed under a fasting/re-feeding protocol that would reveal defects in insulin-mediated suppression of hepatic gluconeogenesis. The hIAPP mice, on the other hand, were sacrificed after a simple overnight fast (no re-feeding period), and liver gene expression levels would be more indicative of the inter-meal state, rather than the post-prandial state. In addition, I observed that basal insulin levels were higher in hIAPP mice (Figures 6.14 and 6.15) than in ZDF rats (Figure 5.9) which could potentially cause a background suppression of hepatic gene expression that overpowered any effects of Pb-exposure. While the dysregulation of the insulin-mediated suppression of hepatic gluconeogenic gene expression observed in ZDF rats was not seen in the hIAPP mice; the combination of elevated insulin and blood glucose levels in the hIAPP mice is suggestive of hepatic insulin resistance.

Consistent with this possibility is the observation of hepatic steatosis in the Pb-treated animals. Fatty liver is frequently observed in insulin resistant humans and animal models and is associated specifically with hepatic insulin resistance [201-203]. The hepatic lipid accumulation in both models may be secondary to the hyperglycemia we observed, which has been shown to be a “trigger” that stimulates *de novo* synthesis

of hepatic lipids [204, 205]. Also consistent with Pb-induced metabolic dysfunction centered on the liver, is the observation that livers from Pb exposed rats were glycogen depleted (Figures 6.21, 6.22, 6.23 and 6.24). Glycogen depletion has been associated with elevated fasting blood glucose [200, 206], and may also be consistent with hepatic insulin resistance. Although not necessarily indicative of hepatic insulin resistance, the HOMA2-IR assessment (Figures 6.42 and 6.43) demonstrated progressive overall insulin resistance and increased beta-cell function in the hIAPP mice.

And, while it may seem contradictory, the significant differences in liver-to-body weight ratios actually accentuate the data showing that Pb exposure affects normal hepatic function. It is well-known that there is a negative correlation between liver size and age [219-221]. Decreases in hepatic blood flow are common with age and studies have shown that decreased ability to clear toxins has caused drug overdoses in the elderly [219, 220, 225]. It is possible that the age-related effect on hepatic blood flow decreased clearance of Pb from the liver and exacerbated its effects on hepatic physiology, including the age-related decrease in liver size.

Examination of gross pancreatic morphology by use of H & E staining revealed Pb-induced islet hypertrophy in both high and low treatment groups. This may be related in part to the strikingly higher level of amyloid deposition in exposed animals. The data also suggest an increase in beta-cell numbers in the Pb-treated groups, which may be indicative of classic islet compensation in response to mounting global insulin resistance, which clearly occurred in the exposed mice (Figures 6.42 and 6.43). Amyloid accumulations are suggestive of pancreatic dysfunction, which is somewhat contrary to the observation that insulin levels were not substantially different in Pb-

exposed vs. control mice. As with the ZDF rats, it appears that while there are striking Pb-induced islet abnormalities, these alterations are not enough to cause pancreatic failure, at least within the timeframe of these experiments.

Our results do not rule out the possibility that Pb affects peripheral insulin resistance in these mice. Unfortunately, a direct measurement of peripheral insulin sensitivity in intact animals is difficult and was beyond the technical scope of our lab and my thesis. However, the calculations provided by HOMA2-IR for insulin resistance and beta-cell function demonstrated a trend in increasing whole-body insulin resistance that may be accompanied by peripheral insulin resistance.

My findings demonstrate for the first time that Pb promotes the development of diabetes in a high fat fed transgenic mouse model. I identified Pb-induced defects in both hepatic glucose output and in postprandial glucose clearance. Both of these are insulin mediated processes and it seems likely that Pb is interfering with insulin signaling, at least in liver, and possibly also in muscle. The data also identified key histological aspects of Pb-exposure, including islet abnormalities and increased β-cell compensation. Additionally, the data showed striking Pb-induced exacerbations of amyloid plaque formations in pancreatic islets. These findings demonstrate that, in metabolically stressed rodents, Pb exposure increases susceptibility to diabetes and promotes progressive development of metabolic abnormalities.

Chapter 7 – Exploring the Molecular Mechanism of Pb Action

The *in vivo* data presented above demonstrate that Pb exposure causes fasting hyperglycemia, glucose intolerance and elevated hepatic gluconeogenic gene expression. The dysregulation of the insulin-mediated suppression of hepatic gluconeogenic gene expression and hepatic insulin resistance could be major contributors to these *in vivo* observations. To examine the effects of Pb on hepatic physiology I developed a set of hepatic cell culture systems that reflect the *in vivo* consequences of Pb exposure. The overall goal of the *in vitro* studies using these systems was to characterize the molecular mechanisms by which Pb alters hepatic insulin sensitivity and gluconeogenesis. The two aims for this portion of my thesis were to: 1) *determine if Pb exposure alters gluconeogenic gene by interfering with insulin signaling and* 2) *determine if Pb exposure alters gluconeogenic gene expression by interfering with Rev-erb-α activity.*

7.1 – Development of the Model Cellular System

The *in vivo* data described in chapters 5 and 6 suggest that one of the metabolic effects of Pb exposure in obese rats and mice is to suppress the ability of the liver to regulate glucose production. Pb concentrations which correlate with blood Pb levels observed in Pb-exposed individuals were utilized in an effort to make this *in vitro* system more clinically relevant [226-229]. In order to decipher the molecular mechanism(s) responsible for the hepatic effects of Pb, and begin development of the *in vitro* model systems, the effect of Pb exposure on hepatic gluconeogenic genes in cultured primary

rat hepatocytes (PRH) and in two rat hepatoma cell lines were assessed. Extensive optimization experiments in all three cell systems were carried out to determine hormonal conditions, treatments times and Pb exposure levels that generated effects on gluconeogenic gene expression that were most consistent with the *in vivo* findings (data not shown).

As will be described in more detail below, the results from these experiments were consistent with the animal studies in that treatment of liver cells with Pb increased expression of the two key gluconeogenic genes (PEPCK and G6Pase), as well as the transcriptional co-activator PGC1-α, which is a strong transcriptional activator of gluconeogenic gene expression in liver. This effect was seen both on basal gene expression (Section 7.2) and in the context of insulin mediated suppression of gene expression (7.3).

7.2 – Pb Stimulates Basal Expression of Key Gluconeogenic Genes

The rat hepatoma cell line FAO was used to examine the effect of Pb on basal levels of gluconeogenic gene expression. The cells were cultured in serum containing media in the presence of low levels of insulin. After a 2 hour serum deprivation period (during which insulin levels were maintained at 1nM) cells were treated with 5μM PbAc or equimolar NaAc for 8 hours. Cells were washed, lysed by direct application of Tri reagent and placed on ice. After mRNA isolation, first-strand cDNA synthesis was conducted and changes in gluconeogenic gene expression were quantified by qRT-PCR with primers for PEPCK, G6Pase, PGC1-α and the housekeeping gene, PPIA. As shown in Figure 7.1, exposure to Pb induced the expression of genes encoding key

rate-limiting enzymes involved in glucose biosynthesis (PEPCK and G6Pase) and of the PGC1-α gene that encodes a transcriptional co-activator that plays a key stimulatory role in PEPCK gene transcription.

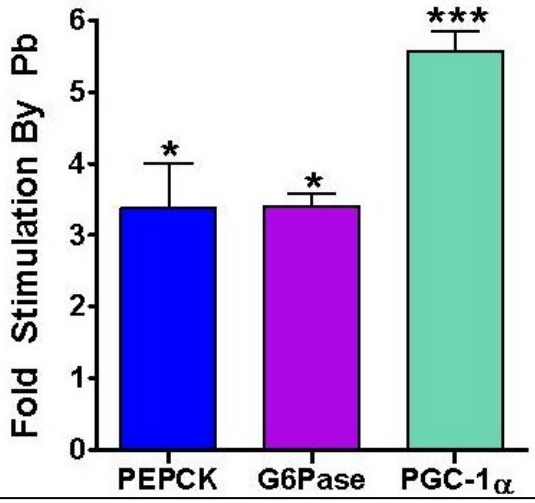

Fig. 7.1 – Pb stimulates the expression of key genes involved in hepatic glucose output in FAO cells.
Cells were treated with 5μM PbAc or equimolar NaAc in the presence of 1nM insulin. Changes in expression of key genes (mRNA) were quantified by qRT-PCR with sequence-specific primers. Data are the mean +/- SEM of three independent experiments. Values are normalized to PPIA; p*<0.05, p***<0.001.

The data from the FAO cells was supported by collaborative profiling data generated by Dr. Raymond Novak and Dr. Lowell Overton, Wayne State University Institute of Environmental Health Sciences (Figure 7.2). The profiling experiments were conducted in PRH cells treated for 24 hours with 1μM Pb (left panel) and 0.5μM or 5.0μM Pb (right panel) in the presence of the indicated amount of insulin (0, 0.1nM, 1nM, 10nM). These data confirm that Pb exposure stimulates mRNA expression of a key gluconeogenic co-factor, PGC1-α, as seen in FAO cells. It was noted that PGC1-α gene expression was stimulated in the presence of Pb, but only when insulin was present.

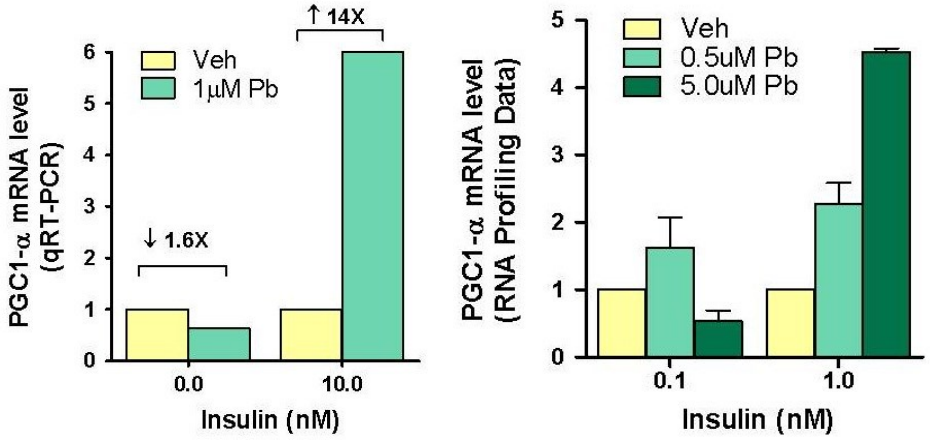

Fig. 7.2 – Pb stimulates the expression of PGC1-α in the presence of insulin.
Primary rat hepatocytes were treated with 1μM (left panel) or with the indicated amount of Pb (right panel) for 24 hours in the presence of the indicated amount of insulin. In the left panel, note the suppressive effect of insulin on PGC1-α expression in the absence of Pb. The data in the right panel (from a separate RNA profiling experiment) are normalized to the vehicle (no Pb) control at each insulin concentration. Data for left panel are preliminary and were not repeated. Data for right panel are means +/- SD for two experiments. *Data courtesy of Dr. Raymond Novak and Dr. Lowell Overton; unpublished.*

7.3 – Pb Blocks Insulin-Mediated Down-Regulation of PEPCK Gene Expression

Based on data from both FAO cells and preliminary profiling showing that PGC1-α gene expression was stimulated by Pb, but only when insulin was present (Figures 7.1 and 7.2), I proposed that the primary effect of Pb on liver glucose metabolism might be to oppose the well-known insulin-mediated down-regulation of gluconeogenic gene expression. To examine this, two well-established *in vitro* cellular models for hepatic insulin signaling pathways related to glucose production were utilized, primary rat hepatocytes and H4IIe rat hepatoma cells (the parent cell line of the FAO cells used above). The pharmacological reagent dexamethasone (Dex) is a glucocorticoid mimic that is utilized *in vitro* to stimulate PEPCK gene expression. In this in vitro system, the effect of insulin on gluconeogenic gene expression is more readily observed in the presence of dexamethasone. H4IIe cells were serum-starved for 12 hours then treated

with 500nM dexamethasone (Sigma), 500nM dexamethasone + 10nM Insulin, 10nM Insulin alone, or vehicle for 4 hours, in the presence of either 50μM PbAc or equimolar NaAc. Cells were washed with cold PBS after removal of media, lysed with direct application of Tri reagent and placed on ice. After mRNA isolation, first-strand cDNA synthesis was conducted and utilized in semi-quantitative PCR with primers specific for rat PEPCK and housekeeping gene, PPIA. Final PCR products were analyzed by agarose gel electrophoresis and quantitative image analysis using a ChemiDoc XRS imaging system. Band densities were analyzed using the NIH Image J program (described in further detail Section 3.10.2).

Figure 7.3 shows the timeline of the experiment, the compiled quantitative data from three experiments (bar graph) and representative agarose gels of semi-quantitative PCR runs. Figure 7.4 is a subset of the same data from Figure 7.3 assembled for illustrative purposes, to allow an easier comparison between Na-treated and Pb-treated groups.

The data demonstrate that Pb exposure blocks the suppressive effects of insulin on PEPCK gene expression. It is also important to note that the ability of Pb to block insulin's suppressive effect occurs even in the absence of dexamethasone (last pair of bars in Fig. 7.4).

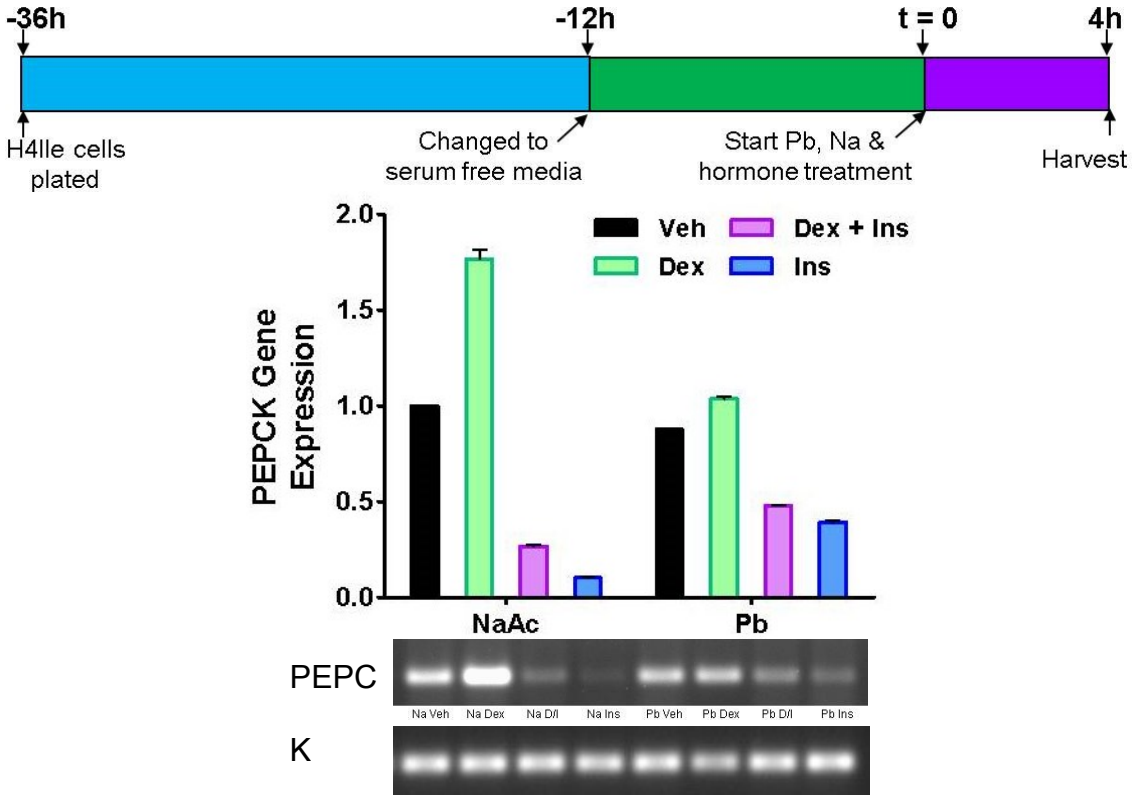

Fig. 7.3 – Pb blocks insulin-mediated down-regulation of PEPCK gene expression.
H4IIe cells treated with 500nM Dex, 500nM Dex + 10nM Insulin, 10nM Insulin or Vehicle (DMSO, PBS & dH_2O) for 4 hours plus either 50μM PbAc or 100μM NaAc. Data are means +/- SEM of three independent experiments.

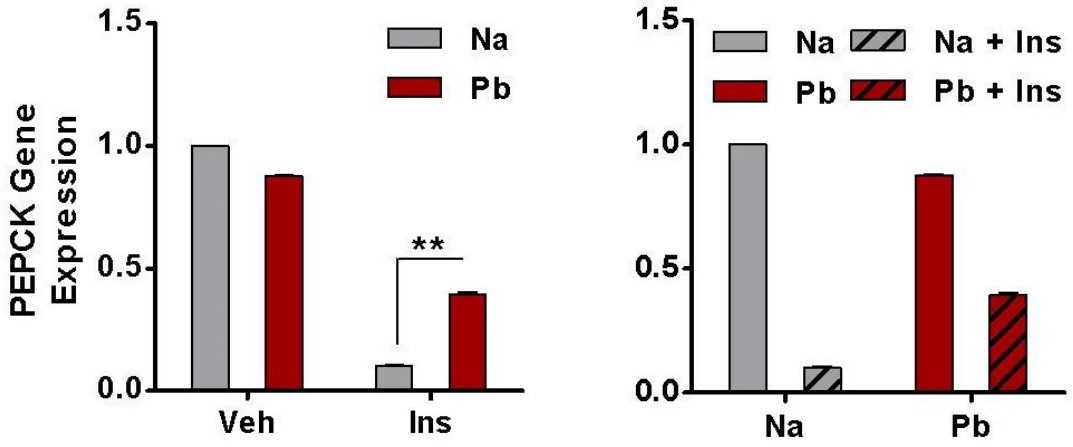

Fig. 7.4 – Subset of data from Figure 7.3.
Clearly demonstrates the ability of Pb to block insulin-mediated down-regulation of PEPCK gene expression. H4IIe cells treated with 10nM Insulin or Vehicle (DMSO, PBS & dH_2O) for 4 hours plus either 50μM PbAc or 100μM NaAc. Data are means +/- SEM of three independent experiments. P values relative to PPIA control, ** $p < 0.001$

Similar experiments in primary hepatocytes showed analogous results. PRH cells were plated on collagen-coated plates in insulin-containing media and allowed to recover overnight. Cells were subjected to a 2 hour incubation in insulin-free media and then treated with 50μM PbAc or equimolar NaAc and either PBS (Veh) or 10nM insulin for 24 hr. Semi-quantitative PCR carried out as described above generated the results shown in Figure 7.5. Pb blunted the insulin-mediated suppression of PEPCK gene transcription in PRH cells in a manner similar to that seen in H4IIe cells in Figure 7.3 and in the right hand panel of Figure 7.5.

These gene expression results are consistent with the observation made by our colleagues Ray Novak and Lowell Overton (WSU IEHS) who kindly provided data from an experiment in primary hepatocytes showing the insulin mediated Akt phosphorylation was suppressed by Pb (Figure 7.6). As Akt mediates the insulin signal leading to suppression of FoxO1, which is required for PEPCK, G6Pase and PGC1-α expression

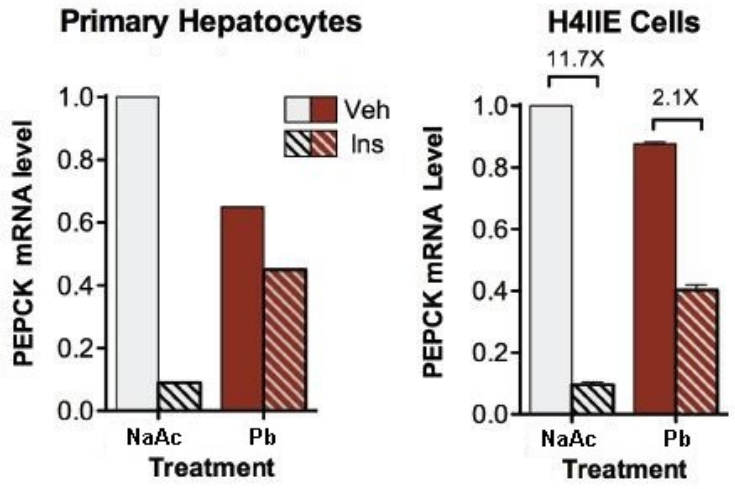

Fig. 7.5 – Pb blunts the ability of insulin to suppress PEPCK gene expression. Primary rat hepatocytes (left) were treated with 50μM PbAc or equimolar NaAc and either PBS (Veh) or 10nM insulin for 24 hr. PEPCK mRNA levels were measured by qRT-PCR with sequence-specific primers. Data for PRH cells are preliminary and were not repeated. The same experiment was conducted in H4IIe rat hepatoma cells (right) except that treatment time was for 4 hours. In both cell systems, Pb strongly blunted the suppressive effects of insulin. Data for H4IIe cells are the mean +/- SEM of three independent experiments.

(see Figure 2.1). These findings suggest a possible mechanism by which Pb exposure alters insulin-mediated changes in gene expression.

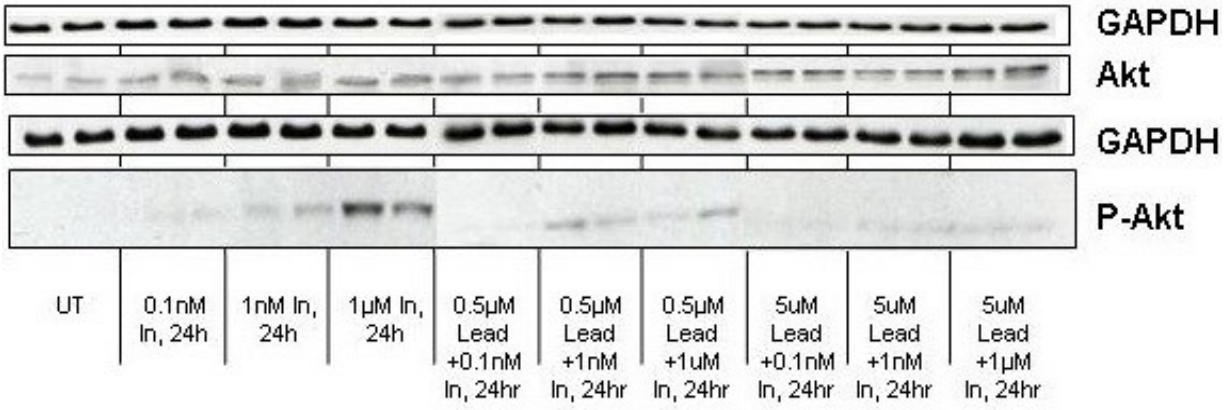

Fig. 7.6 – Pb inhibits Akt phosphorylation in presence of insulin.
Primary rat hepatocytes were treated with increasing concentrations of insulin for 24 hours, which resulted in a dose-responsive increase in Akt phosphorylation (left part of figure). This insulin-mediated phosphorylation of Akt was strongly suppressed by the inclusion of 0.5μM Pb in the media and completely blocked by 5μM Pb. *Data were preliminary, not repeated, and are courtesy of Dr. Raymond Novak and Dr. Lowell Overton; unpublished.*

7.4 – Pb, Rev-erb-α and Effects on Basal PEPCK Gene Expression

To examine the effects of Pb exposure on basal PEPCK gene expression in relation to the orphan nuclear receptor Rev-erb-α, H4IIe rat hepatoma cells were utilized. Emerging evidence indicates that components of the circadian clock machinery can regulate expression of metabolic genes, including PEPCK, G6Pase and PGC1-α [155]. This evidence was further supported by data gathered from mice with mutated Clock genes showing abnormal gluconeogenic gene expression [156-158]. However, the mechanism that links metabolic gene expression to circadian clock rhythms remains poorly understood.

Recently, studies have shown that heme functions as an endogenous ligand for the orphan nuclear receptor Rev-erb-α, a known component of the circadian clock

machinery [159]. Rev-erb-α is highly expressed in multiple tissues including liver, and functions as a transcriptional repressor [160, 163]. When heme is bound, it facilitates the interaction of Rev-erb-α with the nuclear receptor corepressor-histone deacetylase 3 (NCoR-HDAC3) complex [160-162]. When heme binds to Rev-erb-α it initiates recruitment of the NCoR-HDAC3 complex and suppresses the transcription of Rev-erb-α target genes including PEPCK, G6Pase and PGC1-α [161]. It is well established that Pb interferes with heme biosynthesis by direct inhibition of δ-aminolevulinic acid synthetase (δ-ALAS), δ-aminolevulinic acid dehydratase (δ-ALAD) and causing an accumulation of the known pro-oxidant, δ-ALA [164-167]. Taken together, these observations suggest the following model (Figure 7.7). Pb exposure leads to suppression of heme biosynthesis and a reduction in intracellular heme levels. The reduction in intracellular heme leads to reduced ability of Rev-erb-α to suppress gene transcription and thereby an increase in the expression of PEPCK, G6Pase and PGC1-α. The resulting increase in gluconeogenic gene expression will lead to elevated hepatic glucose production, contributing to the pathogenesis of diabetes.

The overall experimental strategy to test this model is divided into three stages. First, establish a system in hepatoma cell lines and primary hepatocytes in which we can confirm the previously reported effects of heme on PEPCK and G6Pase transcriptional activity. Commercially available well-characterized reagents to artificially alter intracellular heme levels were used to establish the experimental parameters. Succinyl acetone (Sigma D1415) to suppress heme biosynthesis and hemin, (Sigma H9030) to increase intracellular heme levels were used as previously described [159, 161]. Once this system was established it was to be used to examine the specific role

of Rev-erbα in regulation of PEPCK and G6Pase gene transcription. This was to be accomplished using a combination of luciferase reporter constructs containing highly specific Rev-erb-α DNA binding sites (ROREs) upstream of a minimal promoter, as well as PEPCK and G6Pase promoter/reporter constructs that we already have available in the lab. The ability of Pb to reduce the suppressive activity of Rev-erb-α on PEPCK and G6Pase gene transcription would constitute evidence in support of the model described above.

Extensive optimization experiments in multiple cell lines (H4IIe hepatoma, CHO, NIH 3T3 and HepG2 cells) were conducted to elicit an effect of Pb on Rev-erb-α. While I was able to observe an effect from exogenous heme on luciferase reporter gene activity, the results were inconsistent; and, to date, I have been unable to see a clear effect of Pb on Rev-erb-α activity (data not shown).

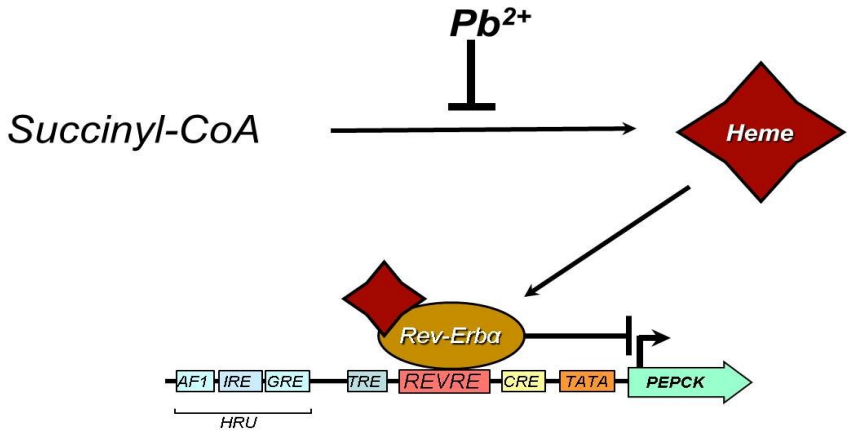

Fig. 7.7 – Rev-erb-α model for how Pb stimulates PEPCK gene expression. Based on recently published data demonstrating the link between Rev-erb-α and its ligand, heme; along with the ability of Pb to suppress heme biosynthesis.

7.5 – Discussion of In Vitro Studies

PEPCK gene expression is controlled by multiple transcription factors and co-factors. Some of the transcription factors that play key roles in PEPCK gene expression are FoxO1, Rev-erb-α and the co-activator, PGC1-α [141, 230-233]. Hepatic insulin signals involving Akt-mediated phosphorylation and subsequent translocation of FoxO1 outside of the nucleus plays a central role in regulation of PEPCK gene transcription [234-236]. The transcriptional rate of PEPCK gene plays a major role in regulating hepatic glucose production and output, and its suppression by insulin plays a key role in the maintenance of normal plasma glucose levels. Therefore, in an effort to characterize the molecular mechanism of Pb in the liver, the *in vitro* system was optimized to observe maximal effects of Pb on PEPCK.

Although I was able to show clearly that Pb exposure *in vitro* caused an elevation in PEPCK gene expression and a reduction in the ability of insulin to suppress PEPCK expression, I was not successful in identifying the exact molecular mechanism by which this occurred. The two proposed mechanisms; blocking insulin signaling upstream of Akt, and reducing the suppressive effects of Rev-erb-α by blocking production of its ligand heme, are still both viable possibilities.

The combination of data presented here related to alterations in gluconeogenic gene expression and insulin signaling in PRH and rat hepatoma cells after Pb exposure suggests that a major consequence of Pb in the liver is hepatic insulin resistance. Figure 7.8 is proposed as a model for the role of Pb involvement in insulin signaling.

As described above, the results from these experiments are consistent with the animal studies; *in vitro* treatment of liver cells with Pb either induced the expression of

genes that are required for hepatic glucose production (PEPCK, G6Pase, and PGC1-α), or blocked the ability of insulin to down-regulate these genes. The dysregulation of the insulin-mediated suppression of hepatic gluconeogenic gene expression could be a major contributor to the hyperglycemia observed in the animal studies and further supports the supposition that Pb exposure causes hepatic insulin resistance. Together, these observations suggest that Pb exposure interferes with insulin signaling, inducing a hepatic insulin resistance that compromises the ability of the liver to control glucose production and promotes the development of hyperglycemia, consistent with *in vivo* observations.

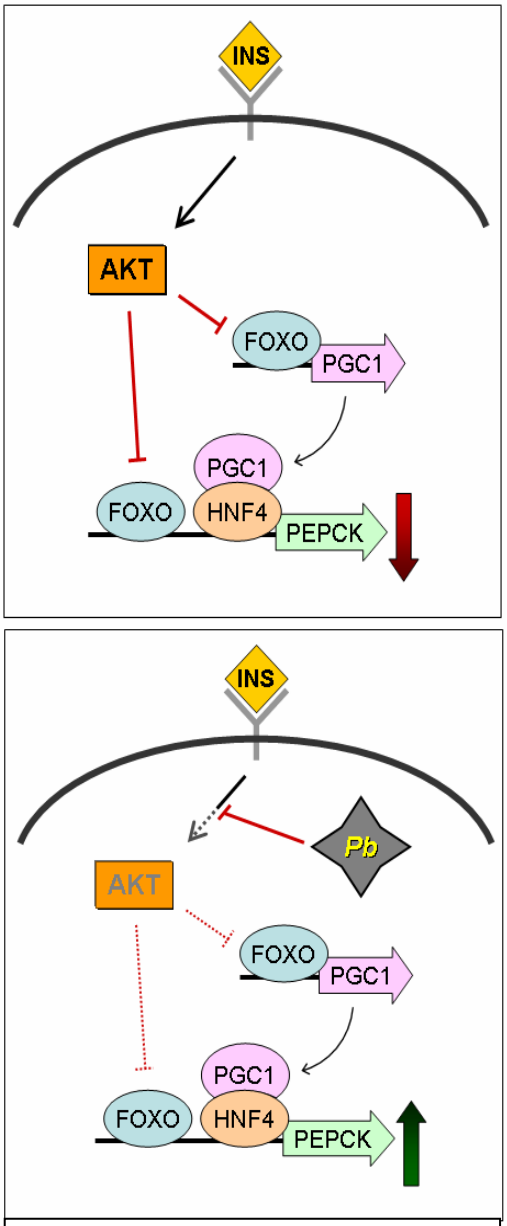

Fig. 7.8 – Hypothetical model of how Pb may inhibit insulin signaling.
This model is based on preliminary data of how Pb functions to alter insulin signaling and hepatic gluconeogenesis in the liver.

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ABSTRACT

LINKING ENVIRONMENTAL TOXICANT EXPOSURE TO DIABETES SUSCEPTIBILITY

by

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August 2013

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An important and unresolved question in the environmental health field is whether exposure to common environmental toxicants, such as dioxin and heavy metals like Pb, increase the risk of developing diabetes, especially in combination with other common metabolic stressors such as obesity.

Previous studies suggested that dioxin exposure increased peripheral insulin resistance but did not appear to cause fasting hyperglycemia or elevated hepatic glucose output. In concordance with those findings we observed that dioxin treatment caused a strong suppression of the expression of the key hepatic gluconeogenic genes PEPCK and G6Pase. However, this suppression was not solely mediated by the dioxin (AhR) receptor as we observed that dioxin treatment stimulated the AMPK signaling pathway, which is known to have a suppressive effect on PEPCK gene expression.

Although Pb exposure and blood Pb levels have declined over the past decade, the interaction between obesity and Pb exposure is a relevant issue in large sections of the US population where environmental and lifestyle factors co-exist with exposure to persistent environmental toxicants, such as Pb. We characterized the effect of Pb

exposure on diabetes risk in metabolically stressed rodents and attempted to identify the *in vitro* mechanisms by which Pb affects metabolic balance.

These findings demonstrate for the first time that, in metabolically stressed rodents, Pb exposure promotes the development of Type 2 diabetes. In ZDF rats we identified Pb-induced defects in hepatic glucose output and postprandial glucose clearance as well as key histological aspects of Pb-exposure, including islet abnormalities and increased β-cell compensation. And in hIAPP transgenic mice we demonstrated that Pb exposure, in combination with a high fat diet, caused the development of fasting hyperglycemia and glucose intolerance as well as Pb-induced islet hyperplasia and striking pancreatic amyloid plaque formation. *In vitro* data from cultured hepatocytes clearly showed that Pb exposure either induced the expression of genes that are required for hepatic glucose production (PEPCK, G6Pase, and PGC1-α), or blocked the ability of insulin to down-regulate those genes, which would contribute to the development of hyperglycemia *in vivo*. Together, these observations suggest that Pb exposure interferes with insulin signaling, inducing a hepatic insulin resistance that compromises the ability of the liver to control glucose production.

Understanding the cooperative interaction between toxicant exposure and additional physical and social stressors that may promote metabolic instability and disease will be of enormous significance in delineating disease/toxicant etiology as well as establishing earlier interventions for those populations most at risk.

AUTOBIOGRAPHICAL STATEMENT

Education

2001 – 2005	B.S. in Biological Sciences, Chemistry Minor from Saginaw Valley State University
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2005	Mamie Hamilton Student Tutor of the Year Award, SVSU
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