

RECEPTOR CROSS TALK IN ARSENIC-IMPAIRED FAT METABOLISM

by

Diana Yesica Garciafigueroa

Licenciatura en Química de Alimentos, Universidad Autónoma de México, Mexico, 1998

Maestria en Ciencias Médico Biológicas, Instituto Politécnico Nacional, Mexico, 2003

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This dissertation was presented

by

Diana Yesica Garciafigueroa

It was defended on

December 9th, 2013

and approved by

Dissertation Advisor: Aaron Barchowsky, PhD, Professor, Department of Environmental and Occupational Health, Graduate School of Public Health, University of Pittsburgh

Claudette St. Croix, PhD, Assistant Professor, Department of Environmental and Occupational Health, Graduate School of Public Health, University of Pittsburgh

Bruce Pitt, PhD, Professor and Chairman, Department of Environmental and Occupational Health, Graduate School of Public Health, University of Pittsburgh

Robert O 'Doherty, Associate Professor, Division of Endocrinology and Metabolism, School of Medicine, University of Pittsburgh

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Diana Y Garciafigueroa, PhD

University of Pittsburgh, 2013

ABSTRACT

Metabolic syndrome is a major public health concern that increases cardiovascular risk from dysfunctional lipid and glucose metabolism. Many factors, including genetics and environmental factors, such as inorganic arsenic, contribute to increase the risk for the disease. However, the mechanisms for this correlation are not well understood. This study tested the main hypothesis that arsenic alters proper lipid storage, remodeling and metabolic changes in adipose tissue through specific activation of membrane receptors in adipocytes. Mice exposed to arsenic through drinking water demonstrated that circulating levels of insulin and tryacylglycerides were affected. Moreover, arsenic stimulated adipose tissue remodeling and angiogenesis in epididymal fat pads, as well as redistribution of fat in ectopic tissues.

In a model of cultured adipocytes, arsenic induced loss of lipid droplets and expression of lipid-coated protein perilipin (PLIN-1). Lipolytic activity was also stimulated by arsenic within 24 hour of exposure and sustained after 3 days, which was indicative of aberrant lipid storage. The prevention of arsenic effects through pertussis toxin sensitive pathways strongly suggested that a G_i -linked receptors mediates arsenic toxicity. Further investigation revealed that the G-protein coupled receptors (GPCR) endothelin-1 receptor A (EDNRA) and B (EDNRB) were activated by arsenic. The selective blocking of EDNRA with BQ610, but not EDNRB with BQ788, partially prevented the loss of fat droplets. In contrast, both, blocking EDNRA and EDNRB prevented the increase in glycerol release by arsenic. Activation of the receptor tyrosine kinase (RTK) EGFR

in response to arsenic was not mediated by Src family or oxidant stress. However, arsenic exposure induced a transient association of G α i protein- EGFR. These results indicated a novel mechanism through which arsenic stimulates cross talk between EDNRA-EGFR.

In conclusion, these studies demonstrated that arsenic impairs proper functionality and metabolism of adipose tissue by stimulation of GPCR-RTK cross communication. The result of this dissertation contributes to better understand the etiology and pathophysiology of metabolic diseases. The advanced understanding of the role of environmental exposure in the etiology of diseases, such as diabetes mellitus, atherosclerosis and hypertension, seen the large population exposed to arsenic will aid in developing strategies to greatly reduce the burden of disease.

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PREFACE

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LIST OF ABBREVIATIONS

ANP	Natriuretic peptide
AT	Adipose Tissue
AT ₁ R	Angiotensine II type I receptor
ATGL	Adipose triglyceride lipase
BAT	Brown Adipose Tissue
BMI	Body Mass Index
C/EBP- α	CCAAT/enhancer –binding protein- α
cAMP	cyclic Adenosine monophosphate
CDS	Chanarin Dorfman Syndrome
ChREBP	Carbohydrate response element binding protein
DAG	Diacylglycerol
DMA	Dimethyl arsenic
DNL	De novo lipogenesis
EDNRA	Endothelin receptor A
EDNRB	Endothelin receptor B
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor receptor
ET-1	Endothelin-1
FFAs	Free Fatty Acids
GPCRs	G-protein coupled proteins
GSH	Glutathione
HDAC	Hystone deacetylase
HDL	High Density Lipoprotein
HSL	Hormone sensitive lipase
IHD	Ischemic heart disease
IRS-1	Insulin receptor substrate-1
ISGT	Insulin-stimulated glucose transport
LD	Lipid droplet
LDL	Low Density Lipoprotein
MAG	Monoacylglycerol
MetS	Metabolic Syndrome

MGL	Monoglycerol lipase
MMA ^{III}	Monomethylarsonous acid
MMA ^V	Monomethylarsonic acid
MSC	Mesenchymal stem cells
NEFAs	No esterified fatty acids
NLSDM	Neutral lipid storage disease
NO	Nitric oxide
PAI-1	Plasminogen activator inhibitor-1
PI-3K	Phosphatidylinositol-3 kinase
PKA	Phospho kinase A
PLC	Phospholipase C
PNLA2	Patatin-like phospholipase domain containing protein-2
PPAR γ	Peroxisome proliferator-activated receptor γ
Ptx	Pertussis toxin
PVD	Peripheral vascular disease
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
S1P1	Sphingosine phosphatase receptor 1
SAM	S-adenosylmethionine
-SH	Sulfhydryl group
SREBP-1c	Sterol regulatory element binding protein 1-c
T II DM	Type II diabetes mellitus
TAG	Triacylglycerides
TNF- α	Tumor Necrosis factor α
UCP-1	Uncoupling protein-1
VCAM-1	Vascular cell adhesion protein
VEGF	Vascular endothelium growth factor
VEGFR1	Vascular endothelium growth factor receptor 1
VEGFR2	Vascular endothelium growth factor receptor 1
VLDL	Very low Density Lipoprotein
WAT	White Adipose Tissue
WHO	World Health Organization

CHAPTER 1.0 INTRODUCTION

1.1.ARSENIC OVERVIEW

1.1.1 ARSENIC DISTRIBUTION IN THE ENVIRONMENT

Arsenic is a ubiquitous metalloid and ranks 20th in abundance in the Earth's crust. It is formally classified as metalloid, meaning that it displays some properties of both metal and a nonmetal. Arsenic exists in nature in three allotropic forms, α (yellow), β (black), γ (grey), of the metallic state and in a number of ionic forms. Arsenic is present as +5, +3 and -3, in which the element is able to form both organic or inorganic compounds in the environment and within the human body (Jomova et al.,2010). In the crust of earth, the natural arsenic minerals are Orpiment (As_2S_3), Arsenopyrite (FeAsS), Realgar (As_4S_4), and Niccolite (NiAs). Arsenic within sedimentary rocks and soils is found in combination with hydroxides, sulfides, iron and manganese. The concentration of arsenic is smaller in rocks compared with that in soils, where inorganic species of arsenic are predominant (Rehman and Naranmandura.,2012).

Humans have been exposed to arsenic throughout the course of history and its associated medicinal properties associated have been well recognized (Ghosh et al.,2009). Human exposure mainly occurs via ingestion of contaminated water, due to the physical associations of arsenic with geological formations that leaches into ground water supplies through natural process (Druwe and Vaillancourt.,2010). There are biological activities and natural factors including soil-water reactions and weather, that produce mobilization of arsenic from rocks and soil towards

groundwater and this arsenic mobilization increases the chances of arsenic availability in the environment. The polluted drinking water may be derived from any of the water reservoirs which include rivers, lakes or aquifers (Rehman and Naranmandura.,2012).

1.1.2.ROUTES OF EXPOSURE

Groundwater contamination by arsenic and other metals has impacted the health of populations worldwide. Some examples of contamination have occurred in Bangladesh and West Bengal, where more than 43 millions people have been exposed to arsenic in the drinking water (Jomova et al.,2011). Arsenic can be solubilized in groundwater depending on pH, redox conditions, temperature and solution composition. Sediments with slow flushing rates, mineralized and mine areas (like gold deposits), volcanogenic sources and thermal springs significantly contribute to arsenic in water supplies (Nordstrom.,2002). Moreover, there are two main environmental conditions that can lead to high arsenic: first, closed basin in arid-semi arid climates; and second strongly reducing aquifers often composed of alluvial sediments with low sulfate concentrations. Contamination of the drinking water by arsenic can also occur due to the reduction and oxidation of arsenic- containing soils and water by microorganism or dissimilatory arsenate-reducing prokaryotes (Watanabe and Hirano.,2012).

According to the World Health Organization for 1990, 43 % of the world's population do not have adequate sanitation and 22 % do not have clean drinking water. The rising demand for sanitary water as the world population increase often cannot be met by surface water supplies. This has led to increase dependence on ground-water resources. The recent increase utilization of ground water in India and Bangladesh has caused detrimental problems to health and numerous

other occurrences worldwide have been reported (Table 1.) Higher ground water arsenic concentrations are frequently associated with sedimentary deposits. In the United States, this occurs in the mountains of some western states including New Mexico, Arizona, Nevada, Southern California, Idaho and Nebraska. In other countries like Taiwan, Mexico, Chile, Argentina, Bangladesh, and Inner Mongolia arsenic occurs at high levels ranging from several hundred to over 1000 $\mu\text{g/L}$ (Brown and Ross, 2002). In 2001 the EPA set the maximum contaminant level (MCL) that regulates the concentration of arsenic in public water supplies to 10 $\mu\text{g/litter}$ from the 50 $\mu\text{g/litter}$ standard established in 1942 (Smith et al., 2002).

Table 1 Worldwide occurrences of arsenic in ground water.

GLOBAL ARSENIC CONTAMINATION IN GROUND WATER			
Country/ region	Potential exposed population	Concentration ($\mu\text{g}/\text{liter}$)	Environmental conditions
Bangladesh	30,000,000	<1 to 2,500	Natural; alluvial/deltaic sediments with high phosphate,* organics
West Bengal, India	6,000,000	<10 to 3,200	Similar to Bangladesh
Vietnam	>1,000,000	1 to 3,050	Natural; alluvial sediments
Thailand	15,000	1 to >5,000	Anthropogenic; mining and dredged alluvium
Taiwan [†]	100,000 to 200,000	10 to 1,820	Natural; coastal zones, black shales
Inner Mongolia	100,000 to 600,000	<1 to 2,400	Natural; alluvial and lake sediments; high alkalinity
Xinjiang, Shanxi	>500	40 to 750	Natural; alluvial sediments
Argentina	2,000,000	<1 to 9,900	Natural; loess and volcanic rocks, thermal springs; high alkalinity
Chile [‡]	400,000	100 to 1,000	Natural and anthropogenic; volcanogenic sediments; closed basin lakes, thermal springs, mining
Bolivia [§]	50,000	–	Natural; similar to Chile and parts of Argentina
Brazil	–	0.4 to 350	Gold mining
Mexico	400,000	8 to 620	Natural and anthropogenic; volcanic sediments, mining
Germany	–	<10 to 150	Natural: mineralized sandstone
Hungary, Romania	400,000	<2 to 176	Natural; alluvial sediments; organics
Spain [¶]	>50,000	<1 to 100	Natural; alluvial sediments
Greece [#]	150,000	–	Natural and anthropogenic; thermal springs and mining
United Kingdom ^{**}	–	<1 to 80	Mining; southwest England
Ghana	<100,000	<1 to 175	Anthropogenic and natural; gold mining
USA and Canada	–	<1 to >100,000	Natural and anthropogenic; mining, pesticides, As_2O_3 stockpiles, thermal springs, alluvial, closed basin lakes, various rocks

From Nordstrom D.K. "Worldwide Occurrences of Arsenic in Ground Water.2002 Reprinted with permission from AAAS."

On average there is more exposure to arsenic from drinking water than from food, however food is still a significant source. Fish and seafood contain high amounts of arsenic in form of arsenobetaine and arsenocholine, both of which contain the organic form of arsenic and are harmless (Watanabe and Hirano.,2012). In the United States, the average adult intake of arsenic

range of 1-20 µg/day. Arsenobetaine is the major form of arsenic in marine animals, nevertheless it is considered non toxic under conditions of human consumption. The studies available indicate that arsenobetaine is not mutagenic, immunotoxic, or embryotoxic (Hughes et al.,2011). Arsenocholine is considered a metabolic precursor of arsenobetaine in marine animals. After administration of labeled arsenocholine, it is absorbed and transformed to arsenobetaine with no degradation to inorganic arsenic, methyl arsenic (MA) or dimethyl arsenic (DMA). It is non embryotoxic in rats and it causes clastrogenic effects but only at very high levels (Borak et al., 2007). Recently, another types of organic arsenic have been identified in seafood: arsenosugars and arsenolipids. Arsenosugars are detected in seaweed and to a lesser extent in marine mollusks. Studies *in vitro* suggested that arsenosugars are less toxic than both inorganic arsenic and trivalent methylated arsenic metabolites. Arsenolipids, which are present in fish oil, have had fewer toxicological studies than arsenosugars (Hughes et al.,2011). Consumption of fish and seafood contribute relatively small share of dietary inorganic arsenic. In contrast, the most significant dietary source of inorganic arsenic include uncooked rice, grains and flours (Borak et al., 2007).

The presence of arsenic in the air is relative low compared to other sources such as food or water. The European Commission reports that arsenic (almost entirely as inorganic arsenic) ranges 0-1 ng/m³ in remote areas, 0.2-1.5 ng/m³ in rural areas, 0.5-3 ng/m³ in urban areas and 50 ng/m³ close to industrial sites. Overall air contributes less than 1% of total arsenic exposure (Hughes et al.,2011). Yet, in 1980, the International Agency for Research on Cancer (IARC) determined that inorganic arsenic was a human lung carcinogen based on studies on occupational exposure. Workers manufacturing arsenical pesticides and workers inhaling arsenic

in copper smelters shown increased lung cancer mortality and dose-response relationship based on air concentrations of arsenic.

Color pigments used in the cosmetic industry to produce eye-shadows, commonly contain toxic elements, such as arsenic. The arsenic particles can be water soluble and may undergo percutaneous absorption through the wet skin. When it reaches the circulatory system via percutaneous absorption, arsenic may represent a potential risk of carcinogenesis (Komova et al.,2010).

1.1.3. ARSENIC METABOLISM

Arsenic methylation has been thought of as a detoxification process for converting As(III) into less toxic end products. Various species of plants, animals and fungi have the ability to biomethylate arsenic into methylated metabolites (Rehman and Naranmandura;2012). There is an important variation between species and among human population in the rate and extent of methylation (Hughes;2002).

The classical pathway of arsenic methylation, which is most generally accepted, was proposed by Challenger in 1945. Inorganic arsenic is metabolized by a sequential process involving a two-electron reduction of pentavalent arsenic, followed by oxidative methylation to pentavalent organic arsenic (Fig 1). This pathway implies that the pentavalent arsenic metabolite monomethylarsonic acid (MMA^{V}) is the result of oxidative methylation of inorganic As III followed by reduction of monomethylarsonous acid (MMA^{III}), the trivalent methylated metabolite. Further oxidative reactions include methylation of MMA^{III} to form dimethylarsinic acid (DMA^{V}) which in turn is reduced to dimethylarsinous acid (DMA^{III}) (Hughes;2002,

Hughes et al., 2011, Del Razo et al., 2001. In contrast, several researches reported that DMA^{V} is immediately excreted in the urine in an unchanged form after oral and parenteral administration in mice and rats. Similarly, the oral administration of MMA^{V} in goats, sheep, mice and humans produced the excretion of MMA^{V} in urine in its unmodified form. Moreover, most of the administered arsenic was recovered in urine and feces at 12 or 24 h after dosing in an unmodified form (Rehman and Naranmandura; 2012). The fact that pentavalent arsenic metabolites are hardly metabolized in mammals suggests that the classical pathway should be reconsidered.

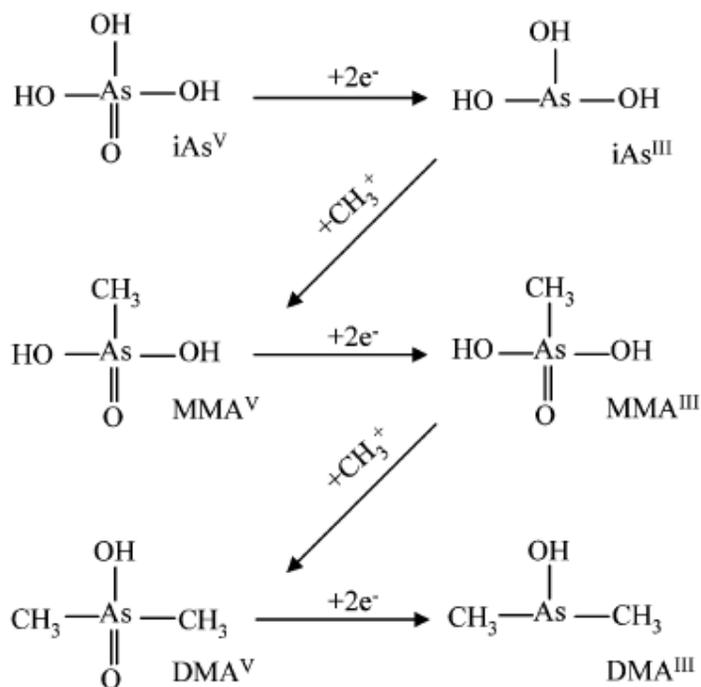


Figure 1 Oxidative methylation pathway for inorganic arsenic in mammals.

From Rehman and Naranmandura. 2012 "Arsenic metabolism and thioarsenicals". Reprinted with permission of Elsevier.

An alternative mechanism is one proposed by Hayakawa, where the reduction of arsenic can occur in the presence of a thiol such as glutathione (GSH) or other endogenous reductants like thioredoxin. The proposed pathway begins with the oxidation of trivalent methylated metabolites MMA^{III} and DMA^{III} to produce pentavalent metabolites MMA^V and DMA^V. The reaction is enzymatic and requires S-adenosylmethionine (SAM) as a methyl donor and is catalyzed by the methyltransferase AS₃ MT (Hughes;2002, Hughes et al.,2011, Del Razo et al.,2001). The reaction implies that methylation of arsenic requires a reductant either simultaneously or after methylation. In the absence of GSH, a sulfhydryl group (-SH) on AS₃MT may act as the reductant. This group is then reduced by glutaredoxin and GSH (Naranmandura et al; 2006).

1.1.4. HEALTH EFFECTS OF ARSENIC

Numerous epidemiological studies have associated chronic exposure to As(III) with increased prevalence of skin (Walton et al.,2004) and internal cancer as bladder, kidney, liver and lung (Izquierdo-Vega et al.,2005, Druwe and Vaillancourt;2010). Even though, much of the carcinogenic effects has been study in the past decades epidemiological studies demonstrate that As(III) exerts other adverse effects including peripheral vasculature, cardiovascular system and endocrine dysfunctions (Izquierdo-Vega et al.,2005), hepatomegaly, respiratory system dysfunction, black foot disease and cerebrovascular diseases (Walton et al.,2004).

1.1.4.1. Arsenic and cancer

Inorganic arsenic is classified by the International Agency for Research on Cancer (IARC) and the US Environmental Protection Agency (EPA) as a know human carcinogen (Hughes,2002).

Occupational exposure to arsenic compounds occurs primarily among smelter workers, workers engaged in the production and use of arsenic-containing pesticides, vineyard, ceramic glass – making, and wood preservation (Tchounwou et al., 2003, Pershagen, 1981). Cancer has developed in individuals exposed to arsenic through occupational exposure via inhalation, or naturally contaminated drinking water (Hughes et al., 2002). Inhalation of arsenic triggered tumors primarily in lungs, while oral exposure to arsenic develops mainly tumors in skin, but also in bladder, liver and kidney in residents in the endemic areas of chronic arsenicism (Pysher et al., 2008, Chen et al.; 1992). Also human and rodent studies indicate that arsenic can potentially act as co-carcinogen or tumor promoter/progressor (Nelson et al., 2007). The mechanism may involve the promotion of oxidative stress, in which the antioxidant capacity of the living organism is overwhelmed by ROS, resulting in molecular damage to proteins, lipids and most significantly DNA (Jomova et al., 2011).

1.1.4.2. Arsenic and cardiovascular disease

An extensive review from Wang et al., (2010) emphasized results obtained on several epidemiological studies performed in Taiwan. Long-term exposure to arsenic is associated with subclinical and clinical cardiovascular-related outcomes such as carotid atherosclerosis, peripheral vascular disease (PVD), ischemic heart disease (IHD) and cerebrovascular disease showing a dose-response relationship. In India, individuals exposed to elevated arsenic levels showed PVD, noncirrhotic portal fibrosis, nasal septum perforation, bronchitis, and polyneuropathy (Tchouwou et al., 2003). While evidence showing the association of arsenic and CVD at high exposure levels has been established, less consistent results were obtained at low-

moderate levels of arsenic in drinking water. Studies conducted in diverse geographic areas including Japan, Slovakia, Spain, Hungary and USA reported positive association with CVD and arsenic exposure, although the magnitude of association was modest and the pooled relative risk was small (Moon et al.,2012). Although the association between arsenic and CVD was not significant at lower exposure levels, other outcomes were indeed relevant. Arsenic exposure was related to markers of endothelial dysfunction and vascular inflammation (Wu et al.,2012, Moon et al.,2012). The levels of cell adhesion markers such as VCAM-1 and PAI-1 were positively correlated with arsenic. The effect of arsenic exposure on soluble VCAM-1 suggested an increased risk of clinical CVD. PAI-1 increase in human microvascular endothelial cells may lead to reduced fibrinolytic activity. The PAI-1 levels were significantly increased in patients with blackfoot disease, a peripheral vascular occlusive disease related to arsenic exposure from drinking water (Wu et al.,2012).

1.1.4.3. Arsenic and adipose tissue

The atherogenic and carcinogenic effects of arsenic are well established. Recently, arsenic has been shown to disrupt glucose homeostasis. Inorganic arsenic is relatively potent inhibitor of several enzymes involved in glucose metabolism, such as α -ketoglutarate dehydrogenase, succinyl-CoA synthase, and piruvate dehydrogenase (Paul et al., 2007). Arsenic inhibits insulin-stimulated glucose uptake (ISGU) by interfering with the PKB/Akt signaling, thus preventing translocation of Glut 4 in adipocytes (Walton et al.,2004). In contrast, other studies reported that acute stress induced by exposure to toxic concentrations of arsenic was associated with p38 or phosphorylated PKB/Akt-mediated increase in ISGU. Taken together, the effect of arsenic on

glucose metabolism is dose dependent (Bazuine et al.,2003, Paul et al., 2007). Studies in adipocyte-progenitor cells have demonstrated the negative impact of arsenite at high concentrations ($\geq 5 \mu\text{M}$). Arsenite induced growth inhibition, G2-M arrest and apoptotic death in Mesenchymal Stem Cells (MSCs)- the multipotency stem cells able to differentiate into bone, cartilage, tendon or adipocytes (Yadav et al.,2010). Similarly, Cheng et al, demonstrated that arsenic trioxide promoted MSC senescence before it caused an effect on cell apoptosis and death. More importantly, this study indicated that low concentrations of arsenic disrupt the adipogenic and osteogenic differentiation determination, most likely by decreasing the two key transcript factors of adipogenesis: CCAAT/enhancer –binding protein- α (C/EBP α) and Peroxisome proliferator-activated receptor γ (PPAR γ). Previous reports also demonstrated that arsenic decreased expression of fat cell specific and adipogenic genes in C3H 10T1/2 culture cells. Arsenic–treated cells induced phenotypic reversion of differentiated adipocytes and reduction of fatty acid–binding protein (aP2) (Wauson et al.,2001).

1.2. METABOLIC SYNDROME

1.2.1 DEFINITION

The Metabolic Syndrome (MetS) is defined as a cluster of metabolic abnormalities that increase the risk for cardiovascular diseases. The complexity of a “syndrome” implies that the specific etiology of the disease is not clear. Although there are divergent criteria for the identification of MetS, they all tend to agree that the components of MetS include: abdominal obesity, atherogenic dyslipidemia, hypertension, insulin resistance with glucose intolerance (Table 2)

(Alessi et al.,2006, Hung et al.,2005, Grundy et al.,2004). The WHO definition and the European Group for the Study of Insulin Resistance agree that they include either glucose intolerance or insulin resistance as an essential component. In contrast, the National Cholesterol Education Program's Adult Treatment Panel III (NCEP: ATP III) did not include the insulin resistance component, but focused on identifying individuals at higher risks of CVD that extended beyond low density lipoproteins (LDL) and obesity (Eckel et al.,2005, Cornier et al.,2008). A high prevalence of MetS is noted with increase in age (Srinivasan et al.,2013). 30% of men and women between 40 and 59 years of age and 40% of individuals 60 years of age or older are currently classified as having metabolic syndrome. The metabolic syndrome has been documented in younger populations as well. Estimates from NHANES suggest that over 6% of adolescents (9% male, 4% female) between 12 and 18 years of age meet criteria for the metabolic syndrome (Eckel et al.,2005).

- a) Atherogenic dyslipidemia. The condition consists of elevation of triglycerides and, small LDL particles and reduced high density lipoproteins (HDL) cholesterol. The elevated concentrations of Very low density lipoproteins (VLDL) and LDL contributing to increase total apo B, thus triggering atherogenic dyslipidemia. The small LDL particles penetrate more easily through arterial walls, resulting in atherogenic modification and reduction of HDL levels, therefore increasing the risk factor for the atherogenic process (Day,2007). Epidemiological studies have revealed the contribution of LDL to the development of atherosclerosis and HDL to its prevention. Together they are considered a risk factor in the development of MetS.
- b) Hypertension. This condition represents a major risk factor for mortality and morbidity worldwide. Risk factors for development of the disease include high salt intake, increased body mass index (BMI), genetic predisposition and exposure to psychosocial stress (Abhyankar et

al.,2012). There is strong evidence that indicates a relationship between insulin resistance and hypertension. Insulin is a vasodilator when given intravenously to people of normal weight, with secondary effects on sodium reabsorption in the kidney. Nevertheless, in the context of insulin resistance, the vasodilatory effect of insulin can be lost, but the renal effect on sodium reabsorption is preserved (Eckel et al.,2005). Some investigators believe that high blood pressure does not play as large a role as other MetS components (Grundy et al.,2004).

Table 2 Metabolic Syndrome definitions.

Components	WHO (modified) ^{13,14}	EGIR ¹⁵	NCEP (ATP III) ¹⁶	AACE (modified) ¹⁷	IDF ^{18,19}	AHA/NHLBI ²⁰
IR*	Presence	Presence				
IFG (FPG) or IGT (2h PG) mmol/L	≥ 6.1 ≥ 7.8 or T2DM	≥ 6.1	≥ 6.1	≥ 6.1 > 7.8 (not T2DM)	≥ 5.6 or Rx	≥ 5.6 or Rx
Waist (cm)** or WHR	WHR > 0.9 (> 0.85)	≥ 94 (≥ 80)	> 102 (> 88)		≥ 94 (≥ 80)***	> 120 (> 88)***
BMI (kg/m ²)	> 30			≥ 25		
BP (mmHg)	≥ 140/90	≥ 140/90	≥ 130/85	≥ 130/85	≥ 130/85 or Rx	≥ 130/85 or Rx
TG (mmol/L)	≥ 1.7	> 2.0	≥ 1.7	≥ 1.7	≥ 1.7 or Rx	≥ 1.7 or Rx
HDL-C (mmol/L)	< 0.9 (1.0)	< 1.0	< 1.04 (<1.29)	< 1.04 (< 1.29)	≤ 1.03(≤ 1.29) or Rx	≤ 0.9 (≤ 1.1) or Rx
Number of components for diagnosis	<i>IR or IFG or IGT plus ≥ 2 others from: central obesity (using WHR +/or BMI), ↑ BP, dyslipidaemia (↑ TG +/- ↓ HDL-C) or Microalbuminuria</i>	<i>IR plus ≥ 2 others from: central obesity (waist circumference), IFG, ↑ BP, dyslipidaemia (↑ TG +/- ↓ HDL-C)</i>	≥ 3 of the components above	Diagnosis depends on clinical judgement based on risk factors/features of insulin resistance	<i>Central obesity (waist circumference) plus 2 other components. Waist circumference defined for different ethnic groups</i>	≥ 3 of the components above

Key: WHO = World Health Organization; EGIR = European Group for the Study of Insulin Resistance; NCEP (ATP III) = National Cholesterol Education Program (Adult Treatment Panel III); AACE = Association of American Clinical Endocrinologists; IDF = International Diabetes Federation; AHA/NHLBI = American Heart Association/National Heart, Lung and Blood Institute; BMI = body mass index; BP = blood pressure; FPG = fasting plasma glucose; HDL = high-density lipoprotein cholesterol; IAAT = intra-abdominal adipose tissue; IFG = impaired fasting glucose; IGT = impaired glucose tolerance; IR = insulin resistance; PG = plasma glucose; TG = triglycerides; WHR = waist:hip ratio; Rx = receiving treatment

*IR: defined by sex- and cohort-specific top 25% distribution of fasting insulin conc. in non-diabetic population.
 ** 80 cm = 30.5 ins, 88 cm = 35 ins, 94 cm = 37 ins, 102 cm = 40 ins. *** in Europids. (*italics*) values in females

From Day C. (2007). The metabolic syndrome, or what you will: definitions and epidemiology. Reprinted with permission of SAGE.

c) Insulin resistance. Insulin is a hormone that has effects on amino acid uptake, protein synthesis, proteolysis, adipose tissue triglyceride lipolysis, muscle and adipose tissue (AT) glucose uptake, muscle and liver glycogen synthesis, and endogenous glucose uptake (Cornier et al.,2008, Bazuine et al.,2003). Individuals with insulin resistance showed impaired glucose uptake in muscle and adipose tissue metabolism or tolerance by an abnormal response to glucose challenge, elevated fasting glucose levels and/or hyperglycemia or reduction in insulin action after iv administration of insulin. These events lead to glucose intolerance and Type 2 diabetes in predisposed individuals (Day, 2007). An important contributor to the development of insulin resistance is the increased concentration of free fatty acids (FFAs). One of the effects of insulin in AT is blocking lipolysis activity. Thus, when insulin resistance develops, the increase activity in lipolysis released triacylglycerol molecules and produce more fatty acids, that in turn can also inhibit the antilipolytic effect of insulin (Eckel et al.,2005, Dandona et al.,2004). Studies have demonstrated that an increase in FFA in normal subjects to levels comparable to those in the obese, results in the induction of oxidative stress, inflammation, and change in vascular reactivity (Dandona et al.,2004). Insulin resistance generally rises with increasing body fat content (Grundy et al.,2004.), but in some populations (South Asians) insulin resistance may occur with BMI < 25 kg/m² or impair production and secretion of adipose-derived proteins, including leptin, resistin and adiponectin (Kondo et al.,2002).

d) Abdominal obesity. In the context of increasing prevalence of obesity worldwide, an excess of fat is considered a risk factor in the development of insulin resistance and the MetS. Obesity has a big impact in the regulation of glucose and lipid metabolism (Vigoroux et al.,2011). It is important to note that not all overweight or obese individuals are metabolically unhealthy and

patients of normal weight can be insulin resistant. Mechanistically, a distinction between a large waist due to increases in subcutaneous fat versus visceral fat is debated. Increases in intra-abdominal or visceral AT will trigger higher rate of flux of FFA to the liver (Eckel et al.,2005). Visceral adiposity can be measured by waist circumference, waist-hip ratio, or radiographic scans, and it correlates well with insulin resistance and other features of metabolic syndrome (Miranda et al.,2004, Gustafson ,2010). In contrast, an increase in subcutaneous fat would release FFA into the systemic circulation and avoid further effects on hepatic metabolism (glucose production, lipid synthesis or secretion of prothrombotic proteins) (Eckel et al.,2005). Ectopic fat could be secondary to a defect of fat cell proliferation or a failure of adipocytes to increasing their size, leading to redistribution of fat storage (Alessi et al.,2006).

1.3. ADIPOSE TISSUE

1.3.1. ADIPOSE TISSUE DEVELOPMENT AND DIFFERENTIATION

AT is mainly composed of white adipocytes that have the function of energy storage and brown adipocytes specialized in thermogenesis. However, in normal human adults, only white adipose tissue (WAT) is present while the brown is almost completely absent (Large et al.,2004, Gustafson et al.,2009).

Generally, adipocytes arise from mesenchymal stem cells (MSC) by a sequential pathway of differentiation developing from ectoderm or mesoderm into undifferentiated precursors that can differentiate into several lineages including adipocytes, myogenic and osteogenic lineages (Vigouroux et al.,2011, Rodeheffer et al.,2008). However not all white adipocytes progenitors

are equivalent as distinct subpopulations have been identified in fat from different body locations. Recently, it was demonstrated that a subpopulation of adipocytes in white and brown fat tissue arise from bone marrow progenitor cells (BMP). These cells proved to be highly accumulated in visceral rather than subcutaneous fat and in female rather than male mice. The difference in pattern expression of BMP-derived adipocytes may explain, adipose depots heterogeneity and metabolism of adipocytes with aging, adiposity and gender (Majka et al.,2010).

The differentiation of preadipocytes into mature adipocytes involves multiple changes including growth arrest, and clonal expansion, as well as early and terminal differentiation. Adipogenesis is controlled by transcription factors that activate the expression of hundreds of adipogenic genes responsible for the mature phenotype. The first stages require suppression of growth-related factors including tumor suppressors, p21 and p18 (Moldes et al.,2003). Next, upon hormonal induction growth-arrested preadipocytes synchronously re-enter the cell-cycle and undergo several rounds of cell division, this stage is known as mitotic clonal expansion (Lefterova and Lazar, 2009). Clonal expansion is followed by activation of factors that induce the expression of adipocyte phenotype, including PPAR γ , C/EBP α family and SREBP1 (Moldes et al.,2003, Gustafson et al., 2009,Ali et al.,2013). During adipogenesis, early expression of C/EBP- β promotes the expression of C/EBP α and PPAR γ – the central transcriptional regulator of differentiation. The later stages of differentiation are characterized by expression of C/EBP α and (Christodoulides et al.,2008, Wakao et al.,2010). Late differentiation is also be characterized by intracellular accumulation of lipid vesicles (Fink et al.,2011, Christodoulides et al.,2008, Wakao et al.,2010).

1.3.2. AT METABOLISM

Most energy reserves in human body are stored in adipocytes as triacylglycerides (TAG). During food intake, excess of dietary non-esterified fatty acids (NEFAs) need to be esterified to chemically inert TAG, which are subsequently stored in cytosolic lipid droplets (LDs) of adipocytes in a process called lipogenesis. During starvation or increased energy demand, TAG reserves are mobilized by their hydrolytic cleavage (Lass et al., 2011). A highly regulated balance of lipid synthesis and hydrolysis in WAT and other tissues will maintain energy homeostasis of the entire body. In contrast, imbalances of these processes have been linked to metabolic disorders including obesity, lipodystrophies, T II DM and MetS (Zimmerman et al., 2009).

WAT is the most efficient organ for storing excessive amounts of circulating FA or releasing them during energy demand. Therefore, adipocytes are well equipped with the proper enzymatic machinery, proteins and binding factors for regulating lipid metabolism (Frühbeck et al., 2004). Interestingly, in the past decades AT has emerged as an endocrine organ involved in multiple homeostatic processes including blood pressure control, immunity, vascular diseases, and insulin sensitivity (Vigouroux et al., 2011, Ali et al., 2013). Moreover the recent identification of many bioactive proteins secreted by AT called adipokines, revealed an underestimated function of WAT. Adipokines including adiponectin (AdipoQ), leptin, visfatin, etc have the ability to influence lipid and glucose metabolism not only locally but also in skeletal muscle and liver. Some adipokines or factors can also have an effect on appetite regulation or on inflammation and vascular biology, like tumor necrosis factor α (TNF α), interleukin 6 (IL-6), interleukin 8 (IL-8), angiotensinogen, prostaglandins, resistin (Ali et al., 2013).

1.3.3. DE NOVO LIPOGENESIS (DNL)

The term DNL refers to the biosynthesis of lipids from non lipid precursors mainly from glucose but also from amino acids and ethanol. Ethanol substrates produce acetyl-CoA during their catabolism, therefore they are susceptible to conversion into fatty acids in the intermediary metabolism (Schutz et al.,2004). FA biosynthesis occurs to a greater extent in rodents compared to human and is a minor contributor to whole body lipid storage. DNL occurs essentially in all cells, however AT and liver are the major sites (Lodhi et al.,2012, Large et al.,2004). In the liver, TAG secretions is considered minor (1 g/day) compared to the daily amount of TAG available from oral intake (> 80 g/day in Western countries). A high carbohydrate diet demonstrated to increase by 2-4 fold lipogenesis in the liver (Lexetier et al., 2003). Lipogenesis is considered less active in human than in rats. The first attempts to clarify such as difference focus on nutritional conditions, however the results indicated that lipogenic capacity was not related to difference in diet (Lexetier et al.,2003). Instead, it was proposed that sterol regulatory element binding protein 1c (SREBP-1 c) was a key transcription factor that control the expression of lipogenic pathway (Lexetier et al.,2003, Kersten,2001). Further studies revealed that lipogenic enzyme activities are partially regulated by post-translational mechanisms but the main control is at the transcriptional level. The transcription of lipogenic enzymes is fully stimulated by both high insulin and glucose concentration and is under transcriptional control of SREBP-1 c and carbohydrate response element binding protein (ChREBP). SREBP-1c controls the activity of glucokinase, the first enzyme of the glycolytic pathway whereas lipogenic genes such as fatty acid synthase and acetyl-CoA carboxylase require both SREBP-1c and ChREBP to be fully induced (Ferré and Foufelle, 2010).

Recent studies in rodents demonstrated that DNL in AT is downregulated in obesity and alteration in lipogenesis is an important contributor to systemic insulin resistance and metabolic disease. (Eissing et al,2012). Palmitate (C16:1 n7), a relevant product of FA synthesis, appears to mediate the insulin-sensitizing effect of DNL in murine AT and liver (Eissing et al.,2012 , Lodhi et al.,2012).

1.3.4 LIPOLYSIS

Lipolysis is a property of mature, differentiated adipocytes. The main purpose is to contribute substantially to daily energy use in the form of TAG breakdown. The lipolytic phase of fasting follows the postprandial and glycogenolytic phases. Lipolysis occurs earlier in fasting infants, which generates ketone molecules after an overnight fast. In adults it is gradually activated after one or more days of fasting. However, the flux of FA may be greater after an overnight fast. In contrast, lipolysis is suppressed by postprandial hyperinsulinemia. For instance in adults an increase in plasma insulin of 4-5 $\mu\text{U/ml}$ produces a 50% decline of FFA concentration and turnover (Wang et al.,2008).The process of lipolysis involves several steps, that includes the hydrolysis of TAG into diacylglycerols (DAG) and then into monoacylglycerol (MAG). The final step forms 3 moles of FFA and 1 mole of glycerol per mole of completely hydrolyzed TAG (Large,2004, Juan et al; 2005). The main lipases identified in the hydrolysis of TAG are called adipose triglyceride lipase (ATGL), which selectively breaks-down TAG to DAGs and non-esterified fatty acids (NEFA). The hormone sensitive lipase (HSL) is a multifunctional enzyme capable of hydrolyzing a variety of acylester like TAG, DAG and MAG. The third lipase

involved is the monoglyceride lipase (MGL) which cleaves MAG into glycerol and NEFA(Lass et al.,2011, Brasaemle et al.,2007).

1.3.4.1. Hormone sensitive lipase (HSL)

The HSL a serine esterase that is activated by phosphorylation on several serine sites including ser 563, ser659 and ser660. HSL is a multifunctional enzyme that possesses TG, diglyceride (DG), cholesterol ester and retinyl ester hydrolase activities. Nevertheless in WAT it has a high selectivity for TGs and DG (Langin and Arner, 2006). HSL is found freely floating in the cytosol of unstimulated adipocytes (Kolditz and Langin,1999, Brasaemel.,2007). It is highly expressed in WAT and BAT, however is also present at low concentrations in muscle, testis, steroidogenic tissues and pancreatic islets. The HSL structure is composed of an NH₂-terminal lipid binding region, an α/β hydrolase fold domain including a catalytic triad, and a regulatory module that contains all the phosphorylation sites regulating of enzyme activity (Zechner et al.,2012). There are at least 5 serine residues that can be phosphorylated by PKA, AMPK and MAPK (Zimmerman et al.,2008). HSL was initially discovered in WAT of mammals as an enzyme which is induced by fasting and catecholamines (Lass et al.,2011). The rate limiting function of HSL in the catabolism of TAG was challenged by studies in HSL null mice (Haemmerle et al., 2002). Contrary to all expectations HSL *null* mice maintain normal body weight and accumulation of TAG was not apparent. In contrast, HSL *null* mice exhibited reduced WAT mass and were resistant to dietary induced obesity. TG synthesis was reduced in adipose tissue with increased necrotic cell death and macrophage infiltration (Holm et al.,2003, Wang et al.,2008, Zimmerman et al.,2009). Basal lipolysis was increased, fasting lipolysis was normal but

there was an increased expression of uncoupling protein-1(UCP-1) which is normally very abundant in BAT. These discoveries lead to the recognition that ATGL plays a greater role in basal or unstimulated lipolysis, whereas HSL is required for maximal catecholamine-stimulated lipolysis (Brasaemle et al.,2007, Tansey et al.,2004, Lafontain., 2008, Pidoux et al.,2001).

1.3.4.2.Adipose triglyceride lipase (ATGL)

ATGL officially named PNPLA2 (Patatin-like phospholipase domain containing protein-2) is also known as desnutrin, iPL2 ζ or TTS-2.2 (Wang et al.,2008, Schweiger et al.,2006). It is highly abundant in WAT and BAT and to a lesser extent in testis, cardiac muscle and skeletal muscle. ATGL mRNA expression was first detected at Day 4 post differentiation in murine 3T3 L1 adipocytes, maximum expression was observed on Day 6 (Zimmerman et al.,2004). In contrast to HSL regulation, ATGL is not a target for PKA-mediated phosphorylation, but it is localized on the surface of cytosolic LD and other intracellular membranes. Upon stimulation ATGL binds to its co-factor CGI-58 for full hydrolase activity and is activated up to 20 times more than in basal conditions (Quiroga et al.,2012). The mechanism underlying ATGL activation by CGI-58 is poorly understood, but it has been suggested that ATGL binds to CGI-58 and enzyme activation can be observed with isolated ATGL and CGI-58 (Zimmerman et al.,2009). Mutant forms of CGI-58 (See table 3) have been identified as causative for Chanarin-Dorfman syndrome (CDS), a disorder in which affected individuals lose their ability to activate AGTL, producing a mistargeting of CGI-58 from lipid droplet to the cytosol and TG accumulation in multiple tissues (Schweiger et al.,2006. Langin ,2008).

The importance of ATGL has become more relevant following the results obtained from models of ATGL deficient mice (*Atgl*- ko). These animals were characterized by enlarged fat depots and

significantly reduced TAG hydrolysis (80 %) and extreme cold sensitivity (Zimmerman et al.,2009). In addition, *Atgl*-ko mice have reduced FA release from WAT and reduce lipolytic activity, which in turn reduces levels of plasma FA. (Zimmerman et al.,2009, Schweiger et al.,2006).In non-adipose tissue *Atgl* deficiencies are more severe, it produces a massive accumulation of TAG in essentially all tissues of the body, especially in cardiac and skeletal muscle, testis, kidney and pancreas (Haemmerle et al.,2006). Little information is available for mutations in the human ATGL gene (See Table 3). There have only been 5 reported cases of individuals affected by this autosomal recessive disorder, named Neutral Lipid Storage Disease (NLSDM) (Fischer et al.,2007). Individuals with the disease suffer from a systemic TG accumulation, miopathy as well as accumulation of TG in leukocytes. They may also suffer cardiac abnormalities and hepatomegaly (Fisher et al., 2007, Kobayashi et al.,2008).

1.3.4.3.Monoglyceride lipase (MGL)

This lipase is considered the rate-limiting enzyme for the breakdown of monoacylglycerol (MAG) and is the final step in lipolysis. MGL is ubiquitously expressed and is found in the cytosol, the plasma membrane and LDs (Quiroga et al.,2012, Zechner et al.,2012). The protein contains 303 amino acids and belongs to the large group of α/β hydrolases and esterases. The active site contains a catalytic triad (Ser 122, Asp 239, and His 269) in which the nucleophilic serine is located within a GXSXG consensus sequence (Young and Zechner.,2013). Studies with an *Mgl*-deficient mouse model revealed an accumulation of MAG species in the brain, which suggests that MGL plays an important role for the breakdown of MAG not only in WAT but in other non-adipose tissues (Lass et al.,2011). Moreover, MGL-inhibition brought especially

attention, since it was found that MGL activity had a pivotal role in endocannabinoid signaling, diminishing cannabinoid signaling in the brain (Quiroga et al.,2012, Lass et al,2011).MGL is the major enzyme in the catabolism of 2-arachidonylglycerol (2-AG), an important endocannabinoid that regulates multiple physiological process including motor function, pain, appetite cognition, emotional behavior, and immunity (Young and Zechner.,2013).

1.3.4.4. Perilipin

Perilipins are a family of phosphoproteins that predominantly coat the surface of lipid droplets in BAT and WAT (Wang et al.,2008, Tansey et al.,2004). Three protein isoforms of Perilipin have been described that arise from the translation of alternatively splice mRNA. Perlipin 1 (58kDa) and Perilipin 2 (46 kDa) share 406 amino acids but differ in the phosphorylation PKA sites (Tansey et al.,2004, Large et al.,2004). Perilipin 1 is the main protein coating lipid droplets during adipocyte differentiation, as it displaces ADRD from the droplet surface. Plin 1 is phosphorylated on at most 6 different PKA sites (aa # 81, 223, 277, 434, 492 and 517 in the mouse sequence) whereas Plin 2 has only 3 amino terminal PKA sites. However it is unclear whether all the sites in Plin 1 are phosphorylated after activation of PKA. Several studies shown that one or more of the three N-terminal PKA sites serines are critical for lipolysis catalyzed by HSL (Braceamle, 2007, Tansey , 2004).

It appears that Plin 1 has a dual function acting as a barrier to suppress basal or constitutive lipolysis and enhancing PKA–stimulated lipolysis. PKA-mediated phosphorylation of Plin is required for stimulated lipolysis; the translocation of HSL to the LD is dependent of Plin, but

independent of phosphorylation of Plin1. Recently, it was demonstrated that close cooperation of HSL and Plin1 in lipolysis, implying a physical interaction. Among these two proteins, HSL physically interacts with the N-terminal region located between aa141 and 200 of Plin1, as well as with the C-terminal region located between aa 406 and 408. These results suggest that this interaction between HSL-Plin1 might have a dual function to facilitate the movement of HSL in close proximity to the LD as well as to prevent HSL from hydrolysis TAG (Shen et al., 2009).

Under basal state, Plin1 associates with the protein CGI-58 and restricts the access of cytosolic HSL to the lipid droplet. Once PKA phosphorylates Plin1, CGI-58 dissociates from Plin1, which in turn recruits HSL at the surface of the LD. This reversible binding of CGI-58 to Plin1 could represent an indirect PKA-dependent mechanism to regulate ATGL (Zechner et al.,2012). The importance of CGI-58 regulating lipolysis was demonstrated in models of mutant Plin1 in humans. Mutants of Plin1 (L404fs and V398 fs) fail to bind CGI-58, leading to unrestrained lipolysis, partial lipodystrophy, hypertriglyceridemia and insulin resistance (Zechner et al.,2012). Moreover, numerous studies have explored the role of Plin in Plin1^{-/-} models. Plin1 null mice fed a higher food diet, Plin1 null animals were resistant to diet-induced obesity but not to glucose tolerance (Tansey et al., 2001). A similar study using knockout mice exhibited a decrease in adipose tissue mass by 60-70 % and greater muscle content. The decrease in fat mass was explained as a result of constitutively high basal adipocyte lipolysis in absence of the protective coating of Plin1 LD (Miyoshi et al.,2010). Interestingly, the mice were resistant to β -adrenergic stimulation which suggesting that Plin1 acted as a protective barrier in absence of stimulation, but it is essential for proper stimulation of to lipolysis. Moreover, studies in glucose response reported that Peri null mice exhibited an increased tendency to develop glucose intolerance and peripheral insulin resistance (Large et al., 2004, Wang et al., 2008, Tansey et al., 2004). In

humans, there is little information related to mutations in PLIN genes. Two different heterozygous PLIN1 frame shift mutations (p.L404VfsX158 and p.V398GfsX166) have been identified. Patients carrying the mutation suffer from partial lipotrophy, insulin resistant diabetes, hypertriglyceridemia and liver steatosis. Subcutaneous AT also showed an increased in fibrosis with macrophage infiltration and adipocytes of decreased size (Vigouroux et al., 2011).

Table 3 Genetic abnormalities of lipolysis and related pathways in mice and humans.

Gene	Lipolysis	Other WAT phenotypes	Non-WAT signs
<i>Human and mouse phenotypes</i>			
LPL	NA	NA	Elevated levels of chylomicrons and VLDL; pancreatitis
CGI-58 (gene ABHD5)	NA	NA	Multivisceral TG storage; hepatosplenomegaly, lipid myopathy; ichthyosis
ATGL (gene PNPLA2)	Human: NA mouse: reduced (basal & β -adrenergic)	Human: non-obese ; mouse: moderate obesity; low circulating FFA level	Human (four patients) lipid myopathy, electrocardiographic abnormalities, small stature and mass, cardiomyopathy and diabetes (one patient); lipid vacuoles in leukocytes. Mouse: lipid cardiomyopathy with heart failure, testicular and renal steatosis, cold intolerance, insulin sensitivity and glucose tolerance
Glycerol kinase	NA	Elevated circulating glycerol and FFA levels	Human: often asymptomatic; in some, psychomotor retardation and failure to thrive with ketoacidotic episodes. Mice: death at 4 days
<i>Mouse phenotypes</i>			
β -Adrenergic receptors (inactivation of β 1, 2 and 3)	Normal basal; suppression of β -adrenergic response	Obesity (increased with age)	BAT, unilocular fat droplets and cold intolerance
HSL	Increased basal; suppressed β -adrenergic response	Lean with adipocyte hypertrophy; marked inflammatory infiltrate in WAT. Mild decrease in FFA	Lipid degeneration in adrenal cortex; male infertility; insulin sensitivity and glucose tolerance despite diminished first phase of insulin secretion
PLIN	Increased basal; resistance to β -adrenergic stimulation	Lean and obesity-resistant; low adipocyte diameter	Insulin resistance; glucose intolerance
Adipophilin	Normal (in differentiated embryonal fibroblasts)	Unchanged (mass & diameter)	Resistance to hepatic steatosis
Caveolin-1	NA	Resistant to diet-induced obesity. Possible cholesterol metabolic abnormalities	Dilated seminal vesicles; thickened urinary bladder; BAT hypertrophic but inactive with cold intolerance; insulin resistance and reduction of insulin receptor concentration
FABP4 (aP2)	Decreased	Sensitive to diet-induced obesity	Glucose tolerant; insulin sensitive; normal FFA concentration
DGAT-1	Overexpression associated with increased lipolysis	Deficiency: Lean, resistant to diet-induced obesity, low adipocyte diameter	Deficiency: Insulin sensitivity, glucose tolerance, increased energy expenditure
DGAT-2	NA	Severely decreased WAT mass; low circulating FFA level	Loss of normal skin barrier function, dehydration and neonatal death
Stearoyl-CoA desaturase 1 (SCD-1)	NA	Obesity-resistant	Energy expenditure and beta-oxidation increased; abnormal cutaneous barrier function
CD36	Normal	Loss of high affinity FA uptake. Elevated fasting FFA level	Grossly normal. Loss of high affinity FA uptake in muscles. Dysgeusia for fatty foods
FATP1 (Slc27a1)	NA	Normal fat mass and adiponectin production	Reduced skeletal muscle FA-derived metabolite levels and protection against insulin resistance in high fat diet-induced obesity
FATP4 (Slc27a4)	NA	Embryonal fibroblast differentiation <i>in vitro</i> resembles that of normal controls	Cell-autonomous restrictive dermatopathy with 8-fold increase in transepidermal water loss and neonatal death
Aquaporin 7	NA	Obesity, adipocyte hypertrophy, glycerokinase activity increased	Insulin resistance
PEPCK	NA	Overexpression results in obesity	

From Wang, S., Soni, K.G., Semache, M., Casavant, S., Fortier, M., Pan, L., Mitchell, G.A. "Lipolysis and the integrated physiology of lipid energy metabolism". Molecular genetics and metabolisms 95:117-126. Reprinted with permission from Elsevier.

1.3.5.REGULATION OF LIPOLYSIS

The most studied model of lipolysis is that activated by catecholamines. The catecholamines, epinephrine and norepinephrine bind to β subtypes adrenoreceptors. This receptor signals through a stimulatory G protein (G_s) to activate adenylyl cyclase. Upon stimulation adenylylase catalyzes the formation of cyclic AMP (cAMP) (Brasamele et al.,2007, Pidoux et al.,2011). The binding of cAMP to the regulatory subunits of PKA produces their dissociation from the catalytic subunit, leading to PKA activation (Ariotti et al.,2002, Schweiger et al.,2006, Miyoshi et al.,2006, Holm, 2003). In contrast α_2 adrenoreceptors are coupled with inhibitory G (G_i) protein that inactivate adenylylase and decrease PKA activation (Langin, 2000). Human adipocytes express significant levels of β_1, β_2 and α_2 adrenergic receptors. Endogenous ligands, epinephrine and norepinephrine activate both classes of receptors which indicate a α_2/ β balance in regulating lipolysis and energy (Valet et al.,2000).Therefore, the net effect of catecholamines on AT is dependent on the functional balance between β and α_2 (Koldits and Langin., 2010). Moreover, epinephrine and norepinephrine have a higher affinity for α_2 than β , which suggests an important role of α adrenergic pathway in control of lipolysis in subcutaneous WAT (Langin, 2006). β -3 AR subtype has a main role in lipolytic and thermogenic responses in murine adipocytes, however the importance of this subtype is less clear at least in healthy humans (Tansey et al.,2004, Langin, 2006). β -3 activity is increased on visceral adipose tissue due to an increase in receptor numbers in obese subjects. Accordingly, the elevated rate of lipolysis in visceral fat is due to increase number and activity of β -3 AR as well as reduced activity of α_2 .AR (Carpuso et al.,2012).

To a lesser extent, other lipogenic factors that contribute to lipolysis are atrial –natriuretic peptide (ANP), tumor necrosis factor (TNF- α), and growth hormone (Moro et al.,2004, Langin, 2006,). In contrast, the primary antilipolytic hormone that controls fat mobilization is insulin. Lipolysis in healthy subjects is extremely sensitive to insulin. Inhibition of lipolysis by insulin and insulin-like growth factor-1 (IGF-1) in humans is activated through stimulation of the phosphodiesterase PDE-3, which in turn reduces the levels of cAMP (Kolditz and Lagin,2010).

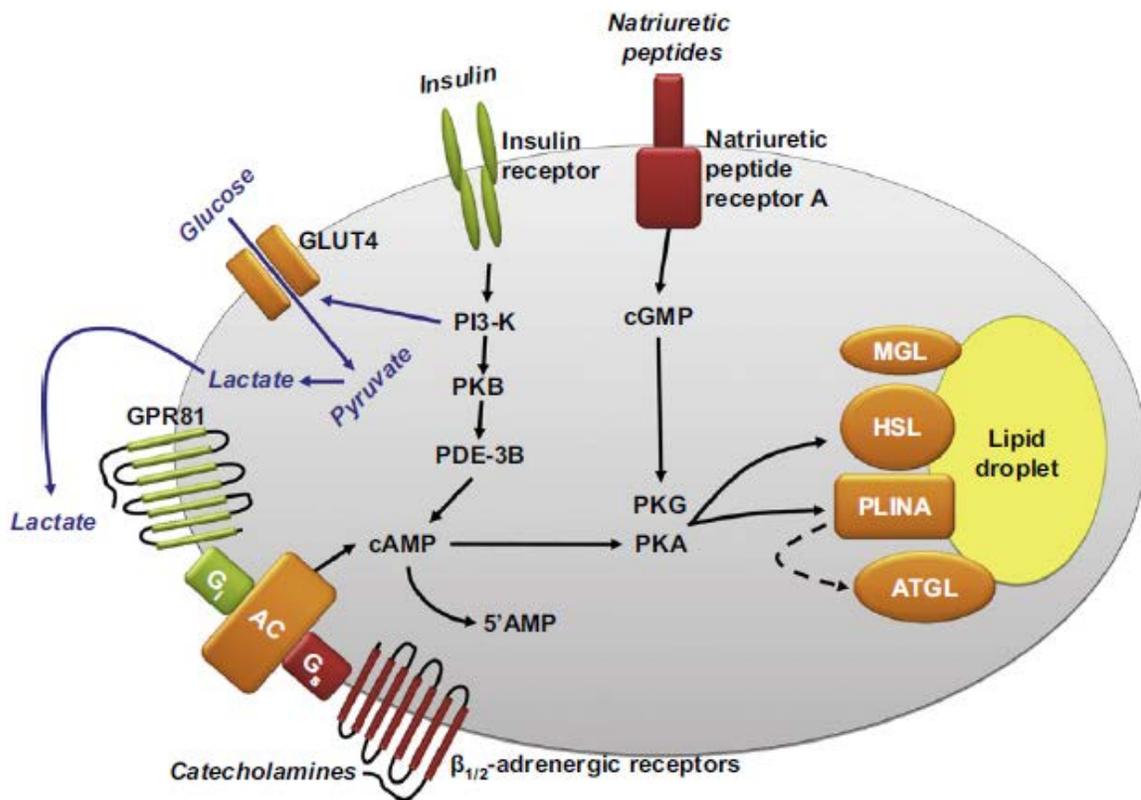


Figure 2 Control of human adipocyte-lipolysis.

From Langin D. "Adipose tissue lipolysis revisited (again!):Lactate involvement in insulin antilipolytic action. 2010 Reprinted with permission of Elsevier.

1.4. G PROTEIN COUPLED PROTEINS (GPCRS)

1.4.1.OVERVIEW

GPCRs or members of the seven-transmembrane receptors (7TMR) are integral proteins that constitute the largest group of cell surface receptors in the human genome (Shukla et al.,2011, Violin et al.,2007). These receptors bind a wide variety of ligands which include hormones, peptides, neurotransmitter and lipids (Whalen et al.,2011). Activation of G-receptors signaling starts by coupling an agonist to the receptors and conformational changes in the receptors. Then, the receptors promotes its interaction with heterotrimeric G proteins ($G\alpha\beta\gamma$), that in turn will activate a variety of downstream effectors, including Phospholipase C (PLC), adenylyate cyclase and ion channels to generate second messengers (cAMP) (Shukla et al.,2011, Selbie et al 1998). As one activated receptor couples to multiple G-proteins, its activation may not necessarily lead to a direct effect on a particular signaling pathway, but rather amplifies the response produced by a separate coincident signal within the same cell or tissue. A model for the cross-talk of two GPCR requires that the signal transduction of one receptor can be modified by that of other receptor (Selbie et al;1998, Hur et al.,2001).

1.4.2. GPCR REGULATING LIPOLYSIS

Hormones which bind to GPCR stimulate lipolysis by activating adenylyate cyclase, that in turn increases production of cAMP (Langin and Arner, 2006). In humans β 1 and β 2 AR are the most important stimulators of lipolysis, while factors that bind to G_i -protein coupled receptors

inhibited adenylate cyclase, as a result cAMP levels are reduced. α -2 AR are the most dominant receptors that reduce lipolysis.

Insulin can inhibit adenylyl cyclase and internalize β -ARs in human adipocytes. This alternative mechanism has been proposed to contribute to insulin induced lipolytic catecholamine resistance (Mauriege et al.,1988). Nicotinic acid (niacin) used as lipid-lowering agent exerts its hypolipidemic action by inhibition of lipolysis. Niacin inhibits lipolysis through PUMA-G , a Gi/o protein receptor (Lafontan, 2004).

1.4.3 GPCR REGULATING ADIPOGENESIS

1.4.3.1 Sphingosine phosphatase 1 (S₁P₁)

S₁P₁ is a signal mediator participating in the regulation of multiple cellular process and disease such as cancer, CVD, wound healing, atherosclerosis, immunity and asthma. S₁P can couple a family of five GPCR (S₁PR 1-5) or intracellular targets like prohibitin2, TNF receptor-associated factor 2 (TRAF2) and histone deacetylase (HDAC) (Liu et al.,2011). A study using adipose tissue-derived mesenchymal stem cells (MSC) demonstrated that S₁P stimulated differentiation of MSC to smooth muscle cells. The presence of S₁P enhanced two smooth muscle markers proteins, α -SMA and trangelin in a concentration–dependent fashion (Nicheri et al.,2009).

1.4.3.2. Angiotensin II

The vascular effects of Ang II are mediated by activation of two heptahelical GPCR: Ang II type I receptor (AT₁R) and type II (AT₂R); AT₁R interacts with multiple heterotrimeric G-

proteins, including $G_{q/11}$, G_{12} , G_{13} and G_i to produce signaling molecules such as inositol triphosphate, diacylglycerol, reactive oxygen species (ROS) and Nitric Oxide (NO). It activates receptor tyrosine kinases (PDGF, EGFR, Insulin receptor) and serine threonine kinases (Muniyappa et al.,2012). Recent clinical studies suggested that blockage of the rennin-angiotensin system (RAS) contribute to lower the risk for development of TII DM. Also, the blockage of AT_1R and angiotensin-converting enzyme (ACE) improve serum levels of cholesterol and TAG (Saiki et al.,2006). In adipose tissue, it has been reported that adipocytes contains a complete RAS and angiotensin that regulate preadipocytes maturation and differentiation (Matsushita et al.,2006).

In vitro studies in MSC-adipocytes have indicated that endogenous Ang II is increased in MSC committed to adipogenic differentiation and that Ang II activity at the AT_2 receptor inhibits the differentiation of MSC to adipocytes. In contrast, AT_1 receptor activity may have stimulatory effects (Matsushita et al.,2006). However, the role of AT in adipogenesis is still controversial, since other groups have found contradictory results. Janke et al; showed that stimulation of AT_1R by Ang II reduced adipose conversion in human preadipocytes. Moreover, it was demonstrated that mitogenic GPCR including AT_1 able to trigger autophosphorylation of growth factor receptor tyrosine kinases (RTK), in a phenomenon call RTK transactivation. It has been shown that AT_1 can transactivate the receptors for PIGF, EGFR and IGF (Elbaz et al.,2000).

1.4.3.3. Endothelin-1 (ET-1)

Endothelial cells are known to produce vasoactive mediators such as endothelin (ET) to maintain hemodynamic responses. Among the ET family, the bioactivity of ET-1 is mediated through potent vasoconstrictor and proinflammatory action, but also it has been implicated in the

pathogenesis of hypertension and vascular disease (Hsieh et al., 2012). ET-1 is a vascular polypeptide primarily secreted by endothelial cells. ET-1 binds to two heptahelical transmembranal receptors, ET type A receptor (EDNRA) and EDNRB, both mediating signaling pathways like PLC, PKC, calmodulin, PLA₂ or Phosphatidylinositol -3-kinase (PI-3K) (Juan et al.,2006, van Harmelen et al.,2008). ET receptors belong to the superfamily of rhodopsin-like proteins comprising seven transmembrane spanning regions. EDNRA signals through G_{αs} to increase cAMP formation, while EDNRB attenuates forskolin-stimulated cAMP synthesis via G_{αi}. As members of GPCR, endothelin receptors are regulated by post-translational modification that affect their structure and activity (Cramer et al.,2001).

Several studies suggest that ET-1 may play a role in the regulation of metabolic events. For instance, there has been establish a correlation with elevated levels of plasma ET-1 an diseases such a as type 2 diabetes, obesity and hypertension (Juan et al.,2005, 2007, Usui et al., 2005, Chai et al.,2012). Moreover, hypertriglyceridemia and hyperinsulinemia have been suggested to trigger ET-1 released in humans (Juan et al.,2005). Studies in 3T3-L1 adipocytes also shown that chronic ET-1 treatments lead to heterologous desensitization of metabolic and mitogenic actions of insulin. ET-1 caused Insulin receptor substrate-1 (IRS-1) degradation and a decrease insulin-stimulated glucose transport (ISGT) via G_{αq/11} deactivation and PI3-kinase (Ishibashi et al.,2001, Usui et al.,2005). Similarly results where ET-1 treatment impairs ISGT were reported in rat adipocytes as well as in healthy subjects, leading to insulin resistance (Chien et al.,2011). ET-1 and cAMP/catecholamine can stimulate synergistic induction of IL-6 expression in culture cells, while ET-1 plus epinephrine induces a significant increase in circulating IL-6 levels in mice. Both of those effects contribute to adipocyte inflammation (Chai et al.,2012).

In adipocytes, ET-1 may also contribute to metabolic disease by modulation of lipolysis and elevated fatty acid levels release (Chien et al.,2011). Long-term treatment of adipocytes with ET-1 in vitro directly stimulates human adipocytes lipolysis (van Harmelen et al.,2008). Nonetheless, the mechanism underlying this activity is still unclear. One study using differentiated adipocytes from abdominal subcutaneous fat from human volunteers suggested that ET-1 promoted systemic insulin resistance and inhibited anti-lipolytic activity of insulin. However this effect proved to have regional responsiveness since ET-1 counteracted insulin response in omental but not in subcutaneous fat via EDNRB stimulation (van Harmelen et al., 2008). In contrast, other studies suggested that ET-1 increased lipolysis mostly by EDNRA but not EDNRB in human adipocytes (Ericksson et al., 2008). Studies in rodent adipocytes and 3T3-L1 adipocytes also shown that the effect of ET-1 on lipolysis had been mediated through EDNRA and downstream activation of ERK 1/2 (Ericksson et al., 2008, Juan et al.,2005, 2006).

1.5 RECEPTORS CROSS TALK IN REGULATION OF LIPOLYSIS

1.5.1 EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR)

The signaling network composed of EGF and their receptors regulates the proliferation and differentiation of many tissues types. Deregulation of this system is a significant factor in the progression of several human cancer, including neoplasm of the brain, lung, breast, ovary, pancreas and prostate (Riese and Stern, 1998, Pagano and Calvo, 2003; Adachi et al.,1994). The EGF family is composed for at least eight different members: EGF itself, transforming growth factor alpha (TGF- α), heparin binding epidermal growth factor-like factor (HB-EGF),

amphiregulin (AR), epiregulin (EPR), betacellulin (BTC), the neuregulins or heregulins (NRGs), and neuregulin-2s (NRG-2s) (Riese and Stern, 1998, Singh and Harris, 2005). Its receptor EGFR (ErB1) is a member of the ErbB family of receptor tyrosine kinase (RTK) activity Type I, which also includes erbB2, erbB3 and erbB4 (Darcy et al 1999, Bublil et al., 2010). The human forms of these receptors range in size from 1,210 to 1,343 amino acids. They consist of cysteine-rich extracellular domain, a single membrane-spanning domain, and a large cytoplasmic domain composed of a tyrosine kinase (TK) domain and several tyrosine residues that are phosphorylated upon receptor activation (Riese and Stern, 1998). Binding of a specific ligand to one of the erbB receptors triggers the formation of specific receptors homo and heterodimers. EGFR activation induces receptor dimerization, activation of TK activity, autophosphorylation and subsequent phosphorylation of downstream signaling molecules (Filosto et al., 2011, Bublil et al., 2010, Goldkorn et al., 1998). The activation of EGFR triggers numerous downstream signaling pathways including PLC γ and its downstream calcium and PKC-mediated cascades, ras activation leading to various MAPK, other small activation such as rho and rac, multiple STAT isoforms, and heterotrimeric G proteins, phospholipid-directed enzymes PI3 kinase and PLD and src (Singh and Harris, 2005) (Fig 3).

Beside canonical activation, stimulation of EGFR can also be triggered without its ligand and without ligand-supported dimerization, an event referred to as transactivation or cross-activation (Filosto et al., 2011). EGFR transactivation mediates several downstream signaling cascades such as ERK activation, Ras/ MAPK signaling cascades. These events are mediated by pathways involving second messengers (Ca²⁺, diacylglycerol) as well as protein kinases, non-receptor TK, matrix metalloproteinases (MMP) and reactive oxygen species (ROS) (Makki et al., 2013). EGFR transactivation can be triggered after activation of GPCR including the

lysophosphatidic acid, α and β -adrenergic, muscarinic cholinergic, angiotensin, thrombin and bradykinin receptors, and cytokine receptors, such as the growth hormone and prolactin receptors (Roudabuch et al.,2000).

EGFR alone has been correlated with metabolic diseases like Type II diabetes (TII D). In a cohort study of women with a wide range of BMI values and insulin sensitivity, it was demonstrated that subjects with insulin resistance had lower EGFR protein abundance. Comparatively, ErbB1 reduction correlates with ATGL reduction and adipogenic genes. These results suggest that reduction in ErbB1 expression, refrain lipolysis and ultimately protect the organism against dyslipidemia and insulin resistance (Roger et al.,2012).

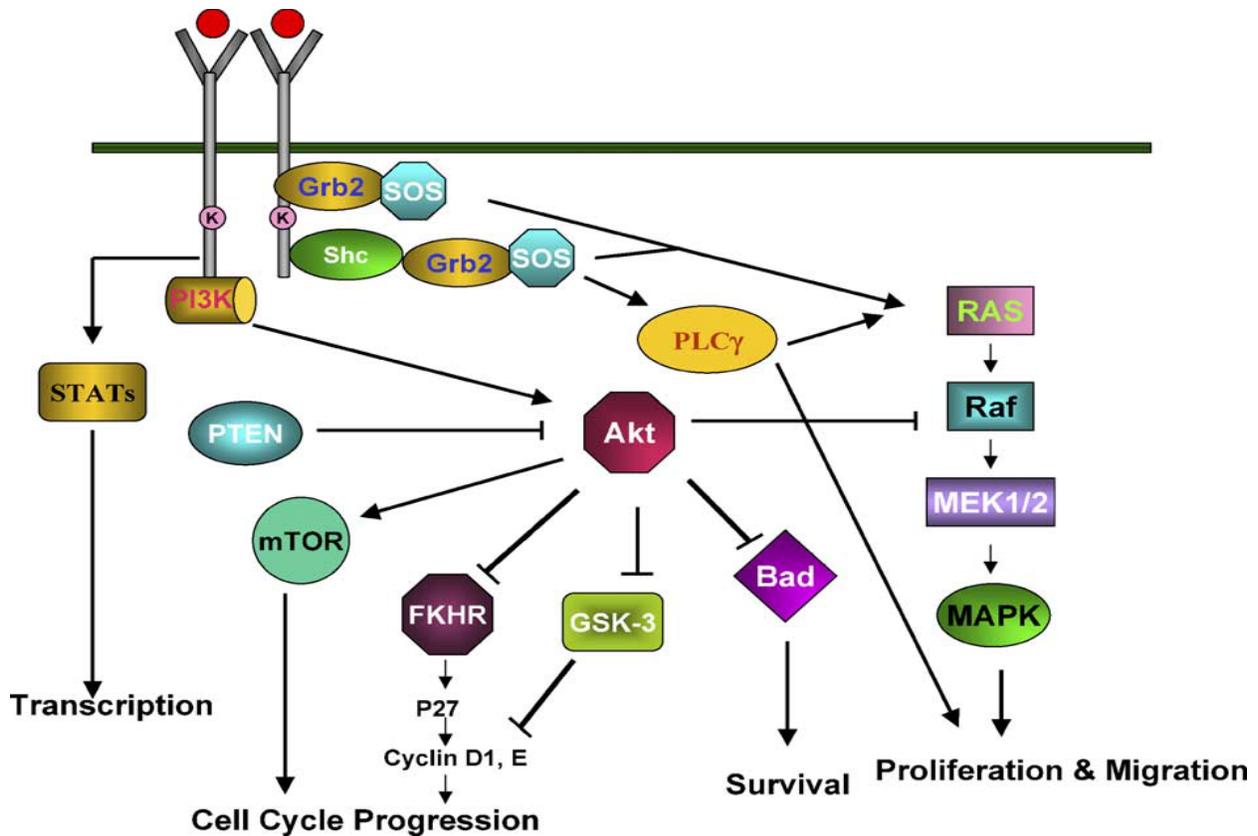


Figure 3 Signaling network activated by ligand dependent activation of EGFR.

From Singh and Harris (2005). "Autocrine, paracrine and juxtacrine signaling by EGFR ligands". Reprinted with permission of Elsevier.

1.6. SUMMARY AND GLOBAL HYPOTHESIS SCOPE

There is compelling evidence that chronic exposure to inorganic arsenic- mostly by contaminated water- is associated with increased risk for metabolic syndrome (MetS) that may advance to diabetes mellitus, atherosclerosis, hypertension and other metabolic diseases. Adipose tissue (AT) plays an essential role in development of insulin resistance which is considered a key factor

of MetS. Epidemiological and experimental studies indicated that inorganic arsenic and its metabolites impair insulin signaling in multiple tissues including, AT, liver and skeletal muscle. Moreover, AT is an endocrine organ that has the ability to expand or regress according to metabolic requirements. However, a positive balance in energy or loss of fat pads may be detrimental for adipocyte homeostasis. In this context, it has been demonstrated that arsenic inhibits adipogenesis and AT function in several studies. Nevertheless, the mechanisms underlying arsenic lipotoxicity are not well understood. Therefore, **the objective of this research was to identify the pathogenic mechanism for arsenic dysfunction of AT and lipid metabolism.**

The global hypothesis of this work is that As(III) impairs adipocyte maturation and metabolism through activation of receptor signaling. To investigate this hypothesis, the study was divided in three specific aims:

- 1) Characterize the effects of arsenic on AT phenotype, morphology, lipid storage, and gene expression in vivo.
- 2) To demonstrate that arsenic activates specific G protein-coupled receptor (GPCR) signaling pathways to impair lipid metabolism and lipid droplet formation, in human mesenchymal stem cell-derived adipocytes.
- 3) To demonstrate that As(III) stimulates specific GPCR-receptor tyrosine kinase (RTK) interactions to impair lipid storage.

The elucidation of the molecular mechanism underlying arsenic toxicity will contribute to better understand and prevent arsenic-related metabolic disease. The scheme in Fig 4 summarize our working hypothesis for arsenic-stimulation of dysfunctional insulin and lipid metabolism.

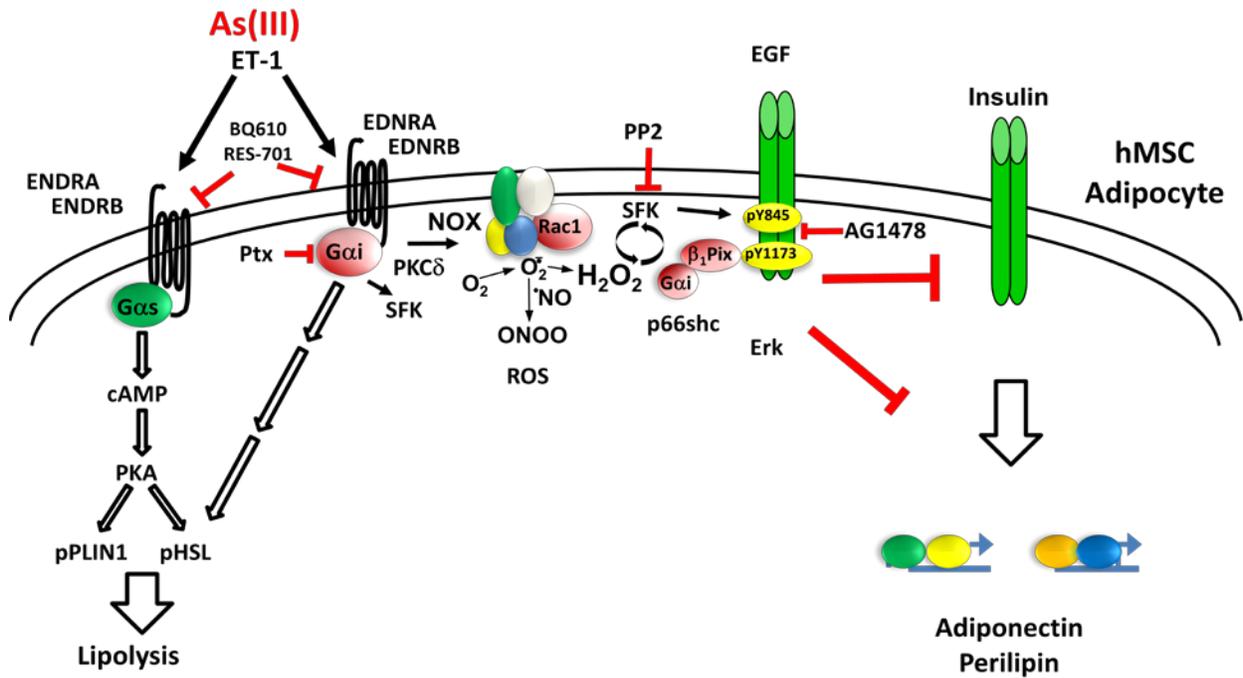


Figure 4 Scheme of As(III)-impairment of adipocyte function.

CHAPTER 2.0 METHODS AND MATERIALS

2.1 IN VIVO MOUSE EXPOSURE

Mouse exposures were performed in agreement with institutional guidelines for animal safety and welfare under the supervision of the University of Pittsburgh Department of Laboratory Animal Research. Groups of 8 five to six wk old male C57BL/6 Tac mice (Taconic Farms, Hudson NY) were exposed for 5 wk to 0 or 100 µg/L sodium arsenite (ThermoFisher, Pittsburgh PA) in their drinking water. This exposure is representative of a moderate human drinking water arsenic exposure lasting 2-3 years. Fresh As(III) solutions were provided three times per week to maintain effective concentrations of As(III). At the end of the exposure period, the mice were euthanized with CO₂ and epididymal fat and tibialis anterior muscle was collected for histological examination and measurement of protein expression. Epididymal fat was isolated since it is one of the largest visceral adipose depots in rodents and change in visceral fat is a risk factor for humans to develop cardiovascular and metabolic diseases. Skeletal muscle composition was evaluated to determine whether As(III) exposure causes lipid redistribution and pathogenic ectopic lipid deposition.

2.2 CELL CULTURE

2.2.1 HUMAN MESENCHYMAL STEM CELLS

Adipose tissue-derived primary human mesenchymal stem cells (hMSCs) from young female donors (Lifeline Cell Technology, Frederick MD; passages 4-8) were grown to confluence in StemLife MSC Medium. The hMSCs used in these studies were from two separate lots derived

from different donors. As(III) responses were comparable in cells from both donors, as well as in hMSCs derived from a male donor that were not used in these experiments. The experimental paradigm is shown in Fig 4. At confluence, cells were seeded onto glass coverslips (immunofluorescence) or into 12 well plates (protein or RNA extraction). Differentiation was initiated by change to AdipoLife DfKt-1 adipogenesis medium for 48 hours. The cells were then maintained in AdipoLife Maintenance Medium, which was changed every 3 days until cells were fully differentiated (day 9). On days 9, 10, and 11, 0 or 1 μ M As(III) was added to provide 72, 48, and 24 h exposures, respectively, and determine the time course for arsenic effects in lipid storage and metabolism, as well as gene or protein expression. All cell cultures were harvested 12 days after differentiation.



Figure 5 Experimental design for cell culture experiments.

The scheme shows the time line for hMSC culture and differentiation, followed by exposure to arsenic in the absence and presence of inhibitors. Controls were differentiated cells that received no treatments for the 12 days post induction

2.3 TREATMENTS

2.3.1 INHIBITOR TREATMENTS

On culture day 9, adipose-derived MSCs were treated with BQ610 or BQ788 (competitive antagonists of ENDRA and ENDRB respectively; Enzo-Life Sciences, Farmingdale NY) 30 minutes before adding As(III). Other inhibitors added 30 minutes before As(III) included VPC 23019 (S1PR1/3 competitive antagonist: Avanti Polar Lipids, Inc., Alabaster AL) or L158,809 (AGTR1 competitive antagonist: a kind gift from Merck Research Laboratories). Ptx (Sigma Aldrich, St Louis MO) was added to the cells the night of culture day 8 before medium change and As(III) addition on day 9.

2.4 PROTEIN ISOLATION

2.4.1 Tissue

Epididymal fat tissue was homogenized in modified RIPA buffer (Table 4) supplemented with protease inhibitors (Calbiochem Inc) and sodium orthovanadate. Then, fat was extracted by rotation at 4°C for two hours. The samples were centrifuged at 13,000g and the supernatant collected for protein determination. Protein concentration was determined by a modified Bradford assay (Pierce Coomassie Plus Reagent , Thermo Fisher) using BSA as a standart.

2.4.2. Cultured cells

Cells were rinsed twice in Stop Buffer (supplemented with protease inhibitors (Calbioche Inc) and sodium orthovanadate), then scraped with a rubber police scraper in SDS Lysis Buffer (Table 4). Cells were sonicated at 50 % amplitude 3 times, followed by centrifugation at 13, 000 rpm for 5 min. Protein concentrations in all samples were determined using a modified Bradford assay (Pierce Coomassie Plus Reagent (ThermoFisher)).

2.5 WESTERN BLOT

Cell lysates were resolved by SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, MA). All proteins were resolved on a 4-12% Bis –Tris polyacrylamide gel (Invitrogen, Carlsbad, CA). A prestained molecular weight ladder was resolved simultaneously with proteins (Invitrogen, Carlsbad, CA). Membranes were blocked in either 5% non-fat milk or 5% BSA for 1 h at room temperature and incubated overnight at 4°C with the primary antibodies (Table 4). After washing the membranes three times and gently shaking with TTBS, secondary antibodies (Table 5) were added for one hour before 3 additional TTBS washes. The proteins were visualized using enhanced chemiluminescence (Perkin-Elmer, Boston, MA) and quantification was performed using Image J software v1.38 x (National Institutes of Health, Bethesda, MD) and normalized to the band density of β -actin in the same sample.

2.6 MEASUREMENT OF SERUM PROTEINS

Blood samples from C57BL/6 mice exposed to arsenic as describe above were collected in heparine tubes with a 23 G syringe. Samples were centrifuge at 1000 rpm for 20 min at 4°C, supernatant was transfer to new tubes and freeze at – 80C° until processing. Samples were analyzed at Lipid, Lipoprotein, and Glucose Metabolism Core at University of Cincinnati for total triglycerides, Nonesterified fatty acids (NEFA), adiponectin, glucose and insulin levels.

Table 4 Primary antibodies.

Antibody	Supplier	Dilution	Protocol
CD31	Santa Cruz Biotechnology	1:100	IF
VEGFR2	Cell Signaling	1:300	IF
pERK	Cell Signaling	1:1000	W
ERK	Cell Signaling	1:1000	W
Y1173	Cell Signaling	1:1000	W
EGFR	Cell Signaling	1:1000	W
EDNRA	Abcam	1:1000	W
EDNRB	Abcam	1:1000	W
Perilipin	Cell Signaling	1:2000	W /IF
β -actin	Sigma	1:20,000	W

Table 5 Secondary antibodies.

Antibody	Source	Dilution	Protocol
Anti-rabbit -HRP	Goat	1:5000	W
Anti-mouse –AP	Donkey	1:5000	W
Anti-rat Alexa 488	Goat	1:1000	IF
Anti-rabbit Alexa 488	Goat	1:1000	IF
Hoechst		1:100	IF
Nile Red		300 nm	IF

Table 6 List of Buffers.

Buffer	Formulation	Protocol
Stop Buffer	10mM Tris HCL, pH 7.4, 10mM EDTA, 5mM EGTA, 0.1M NaF, 0.2M sucrose, 100microM orthovanadate, 5mM pyrophosphate, protease inhibitors	W
SDS Lysis Buffer	20 mM Tris, pH 7.5, 1% SDS	W
TTBS	10mM Tris-HCL, pH 8.0, 150mM NaCl, 0.05% Tween-20	W
PBS	0.136M NaCl, 0.00268M KCl, 0.008M sodium Phosphate, 0.002M potassium phosphate	W/IF
Cell fixative	2.5 % paraformaldehyde	IF
MOPS	1mM MOPS, 1mM Tris Base, 69.3 mM SDS, 20.5 mM EDTA.	W
Towbins Buffer	25 mM Tris, 192 mM glycine, 20% methanol, 0.01% SDS	W
RIPA Buffer	50 mM Tris, pH 7.6, 150 mM NaCl, 1 mM EDTA, 10 mM NaF, 1% Triton X-100, 0.1% SDS	W
PBT	0.2 % Triton X-100, 0.1 % BSA in PBS	IF
PBG Buffer	0.5 % BSA, 0.14 % Glycine in PBS	IF
PBGT Buffer	0.05 % Triton- X-100 in PBG Buffer	IF

2.7 LIPOLYSIS ASSAY

Cell culture medium was collected from each experiment, centrifuged at 13,000g, and supernatant collected and stored at -80 °C until assayed for glycerol content. Glycerol levels were measured using a colorimetric enzyme immunoassay (Glycerol Cell-Based Assay Kit (Cayman Chemical Company, Ann Arbor MI)) according to the manufacturer's instructions. All measurements were performed in duplicate samples from each culture and quantified against a standard curve.

2.8 cAMP DETERMINATION

cAMP levels were quantified in adipose-derived hMSC at day 12 post-induction using a EIA cAMP kit (Cayman Chemicals) according to the manufacturer instructions. Cells were pre-treated with 10 µM IBMX (Sigma Aldrich) as phosphodiesterase inhibitor 4 hr before As (III) exposure. 10 nM Isoproterenol for 90 min was used as a positive control. Cells were treated with 1 µM for 30 to 4 hr. Confluent cells were incubated for 20min at room temperature with 1 ml of 0.1 M HCl for every 35 cm² (120 µl for 12 well plates). Cells were scrapped with a policeman scrapper and transferred to a 1.5 ml tube. Lysed was centrifuged at 1,000x g for 10 min at 4 °C and the supernatant transferred to a clean tube. Supernatant was acetylated by addition of 100 µl 4m KOH and 25 µl acetic anhydride. Vortex for 15 sec, followed by addition of 25 µl of 4 M KOH and vortex. Standard curved and samples were prepared according to the instructions and final product was measured by absorbance at 405 and 420 nm.

2.9 MICROSCOPY AND QUANTITATIVE IMAGING

2.9.1 Histological analysis

Adipose tissue from mouse was collected for histological analysis after 5 weeks of As(III) exposure. Tissue was fixed in 4% paraformaldehyde (Polysciences Inc) for 24-48h at 4 °C followed by paraffin embedding. Tissue sections were deparaffinized and rehydrated in graduate solution of EtOH. Tissues were stained for Hematoxin and Eosin according to standard methodologies. Images were captured at 20x magnification using brightfield illumination on an inverted Nikon Diaphot microscope. Skeletal muscle analysis was performed with frozen, fixed section of tibialis muscle that were stained with H&E.

2.9.2 Immunofluorescence analysis

Cells .Adipose derived-hMSC were grown on glass coverslips. At day 9 post-induction cells were treated with As(III) and /or inhibitors according to the Fig 3. At day 12 cells were paraformaldehyde –fixed (Polyscience, Inc), followed by 3 times rinse with PBS. Cells were permeabilized 15 min (0.2 %Triton X-100, 4% BSA in PBS solution) and then blocked in 20% normal goat immune in PBS pure serum (Thermo –Fisher Scientific) for 1 h at room temperature. Primary antibodies were diluted in PBT (Table 4), overnight at 4°C in the dark. After four times rinsed in PBT, cells were incubated 1 h at room temperature with secondary antibody. Cells were rinsed with PBS and stained with a solution of 300 nM Nile Red (Across, New Jersey, USA) for 10 min at room temperature in the dark, to measure neutral lipid droplets. Nuclei were stained using Hoechst dye in PBS for 10 min and rinsed with PBS. Coverslips were

mounted in microscope slide using Fluoromount (Sigma-Aldrich). Four fields of cells from each coverslip were imaged at 40x using an Olympus Fluoview 1000 Confocal Microscope. The percent of thresholded pixel density per unit area for Nile Red or perilipin staining was calculated using MetaMorph (Molecular Devices, Downingtown PA) software and normalized to the percent thresholded pixel density for DAPI staining. Mean values were calculated for fluorescence on four coverslips of cells from two separate experiments and averaged to provide values for each treatment group.

Adipose tissue. Mice from 5 weeks exposure to As(III) was fixed by immersion in 2 % paraformaldehyde EM grade (Polyscience Inc) in PBS for at least 2 hours. Next, tissue was transferred to PBS and storage at 4°C until processed. Tissue was cut into approximately 1 mm³ pieces and stained in situ. The staining procedure was performed similarly to cell-stained with the following changes. All rinses were for 15 min each and all rinses /incubation were mixed on a rotator. Tissue was rinsed 3 times with PBG, followed by 1hr permeabilization in PBG buffer containing 0.1 % Triton X-100. Then blocked for 2 hours at room in 5 % normal goat immunopure serum in PBGT buffer. Primary antibodies (Table 4) were diluted in PBGT buffer and incubated overnight at 4°C in the dark. After incubation, tissue was rinsed four times in PBGT and incubated with the appropriate secondary antibody (Table 5) in combination of Hoechst dye overnight at 4°C in the dark. The third day, tissue was rinsed with PBS, then coverslipped using Fluoromount (Sigma-Aldrich). Slides were viewed at 40 X on a Fluoroview 1000 confocal microscope (Olympus, Center Valley, PA). 4-5 confocal stacks of 2 µm each were processed using MetaMorph (Molecular Devices, Downingtown PA) software and normalized to the percent thresholded pixel density for DAPI staining.

2.10 QUANTITATIVE RT-PCR

Total RNA was isolated using TRIzol® reagent (Invitrogen) according to manufacturer's instructions. Briefly, cells were lysed in TRIzol® reagent and incubated for 5 min at room temperature. After transferring the sample to a tube, chloroform was added and samples were mixed vigorously for 15 s. Samples were incubated at room temperature for 2-3 min before centrifuging at 11,000g for 15 min at 4°C. The aqueous layer was transferred to a new tube, isopropanol was added, and samples were incubated for 10 min at room temperature before centrifuging at 11,000g for 15 min at 4°C. The supernatant was discarded and the pellet was washed with 75% ethanol and centrifuged at 7,500g for 5 min at 4°C. The pellet was dried for some minutes using vacuum and resuspended in 25-50 µL RNase/DNase-free water. The RNA was quantified by measuring absorbance (260 nm) and was reverse transcribed to cDNA.

cDNA levels were amplified by real-time PCR using the MJ Research Opticon 2 (Bio-Rad Laboratories, Hercules, CA)) with specific primers. Thermal cycling was performed by incubating at 50°C for 2 min and 95°C 10 min followed by 40 cycles of 95°C for 15 s and 57°C for 1 min. Gene expression of Adipo Q, Plin 1 and the house keeping gene RPL13 A were quantified using standard curves for both the mRNAs of interest as well as RPL13A. Target transcript levels were normalized to RPL13A transcript levels to determine the pg of normalized products per ml of reaction.

2.11 IMMUNOPRECIPITATION

Adipocytes were grown and differentiated at day 12 post induction before harvest. Cells were rinse 2x with cold Stop Buffer keeping the cells on ice (Table 6) and scrapped in cold Lysis

Buffer. Lysates were collected into a 1.5 bullet tube and sheer 5x with a 25-30 gauge needle. Extracts were centrifuge at 13,000g for 5 min/ 4 °C and supernatant were transfer to a screw tube. Equal amounts of protein (200 µg/ml) were incubated overnight against EGFR antibody on rotator at 4°C. The next day protein complex was incubated with A/G beads for 3-4 h at 4°C on rotator. Extracts were centrifuge at 7500g for 5 min and supernatant was remove, pellet was rinse 3x with HNTG buffer. In the final rinse, 50 µL of 2x loading buffer was added and boiled for 5 min. Extracs were vortex and supernatant was loaded onto a gel.

2.12 STATISTICS

Standard unpaired t-tests were used to test significance between control and single treatment groups. One-way analysis of variance (ANOVA) was used to identify significant differences ($p < 0.05$) between multiple treatment groups and controls. The degree of significance between groups was compared using Bonferoni's or Newman-Keuls post-hoc tests. All statistics were performed using GraphPad Prism, v 5.02 software (GraphPad Software, San Diego, CA). Data are presented as means \pm SEM of quantified values or fold control

CHAPTER 3.0 ARSENIC INDUCED REMODELING AND REDISTRIBUTION OF LIPIDS *IN VIVO*

3.1 ABSTRACT

Exposure to chronic and low levels of arsenic in the drinking water has been associated with a great number of diseases including different types of cancer, cardiovascular disease, and metabolic changes. The most common determinants for developing metabolic syndrome are adipose tissue expansion and insulin resistance. As(III) has been recently linked with diabetogenic effects in adipose tissue and pancreas, but the underlying mechanisms for pathogenic effects in these tissues remain unclear. To evaluate the hypothesis that arsenic exposure promotes changes in lipid and glucose metabolism and remodeling in adipose tissue, CB57BL/6 mice were exposed for 5 weeks to sodium arsenite in the drinking water before collection of epididymal fat and serum. Systemically, the only change in fasting serum lipid levels was a decrease in triglycerides. There was an increased level of insulin, but no change in fasting glucose levels. At the tissue level, however, there was a reduced number of small adipocytes in male mice, an increased conversion of white to brown adipose tissue in females, and increased tissue angiogenesis. There was increased perivascular ectopic fat deposition in the skeletal muscle of As(III)-exposed mice, relative to controls. As(III) effects on adipose gene expression supported the tissue remodeling and suggested altered mitochondrial function and metabolism. These results together with changes in circulating triglycerides, insulin, and ectopic fat deposition may be indicative of early stages of insulin resistance, where more insulin is required in adipose tissue, skeletal muscle and liver to transport and retain metabolites.

3.2 INTRODUCTION

Animals, seed plants, and fungi store excessive amounts of energy substrates in the form of intracellular triacylglyceride (TAG). In mammals, TAG are stored in adipose tissue (AT) fat droplets and are mobilized as the primary source of energy during periods of food deprivation. Either a deficiency or a surplus of AT disturbs its fat buffering capacity, resulting in a mismatch between energy availability and TAG storage capacity (Sudgen et al., 2009, Scherer, 2006). The buffering capacity of AT can be exceeded during chronic over nutrition, which results in spillover of lipids from AT and their pathological accumulation in ectopic tissues like skeletal muscle, liver and pancreatic β -cells. Metabolites derived from this ectopic lipid accumulation impair insulin action in peripheral tissue and insulin production by the pancreas in a process termed lipotoxicity (Itoh et al., 2013). Some studies have demonstrated that obese adipose tissue is characterized by adipose hypertrophy, followed by increased angiogenesis, immune cell infiltration and extracellular matrix (ECM) overproduction. These changes may increase pro-inflammatory adipokines during the progression of chronic inflammation. The pro-inflammatory stage in AT is similar of that produced in atherosclerotic vascular walls, also known as vascular remodeling, which is mostly mediated through complex interactions among vascular endothelial and smooth muscle cells, lymphocytes and monocyte-derived macrophages. Vascular remodeling is an adaptive process in response to long-term changes in hemodynamic conditions and lipid metabolism (Itoh et al., 2013). Therefore, dynamic changes seen in obese adipose tissue can be referred as AT remodeling, where stromal cells change dramatically in number and cell type during AT expansion (Suganami and Ogawa, 2010, Itoh et al., 2011).

The concept of AT remodeling refers to the turnover of cells within AT and the renovation of the ECM in response to requirements for growth and expansion, changes in hormonal homeostasis, aging, or pathologies such as cancer cachexia or lipodystrophies (Jeong et al.,2010). The expansion of AT can be supported either by neovascularization (hyperplasia) or by dilation and remodeling of existing capillaries (adipose hypertrophy) (Christianens and Lijnen.,2010). The process of neovascularization or angiogenesis has been extensively reported in adipose tissue (Corvera and Gealekman, 2013,Rupnick et al.,2002). Angiogenesis is the main mechanism involved in physiological and pathological neovascularization in adults (Maharaj et al.,2006). Adipose tissue exhibits angiogenic activity and release a diverse group of pro- and anti-angiogenic factors. Pro-angiogenic factors include vascular endothelial growth factor (VEGF), PlGF, fibroblast growth factor (FGF-2), angiopoietin (Ang1 and 2), leptin (Cae et al.,2011, Kitade et al.,2006), plasminogen activator inhibitor-1(PAI-1) (Crandall et al.,2001), TNF α , tissue factor (TF), MMPs and cathepsins (Ouchi et al.,2003, Fukumura et al.,2003). The anti-angiogenic factors include, adiponectin (Yamauchi and Kawawaki., 2008), thrombospondin (TSP)-1, TSP-2, ADAM and ADAMs family members (Fukumura et al.,2003). Among the different types of pro-angiogenic factors, VEGF has a determinant role in regulating physiological angiogenesis as in wound healing, or pathological angiogenesis as in tumor vascularization and ocular diseases (Maharaj et al., 2006). In mammals, seven VEGF ligands have been recognized so far: VEGF –A, B, C , D, E, F and PlGF. These ligands are recognized and coupled to three different receptor tyrosine kinase receptors, which as known as VEGFR-1,2 and 3 and the neuropilins (NP-1 and NP-2)(Olsson et al.,2006, Ottrock et al.,2007). VEGF-A and VEGF -B are responsible for most of adipose tissue's angiogenesis capacity, although VEGF-B is also implicated in ECM degradation via regulation of PAI-1 (Christiaens and Lijnen., 2010).

VEGF-A binding to VEGFR2 is highly expressed in AT and its expression increases significantly during adipocyte differentiation. Recently, it has been reported that VEGF-A exerts metabolic effects, and its expression levels can control energy metabolism. VEGF-A overexpression in adipose tissue protected transgenic mice against diet-induced obesity and insulin-resistance (Elias et al.,2012). Other studies suggested that VEGFR1 and VEGFR2 have opposite effects in cold-induced models of angiogenesis. VEGFR2 blockage abrogated angiogenesis and significantly impaired non-shivering thermogenesis capacity. In contrast, VEGFR1 blockage resulted in adipose vascularity and non-shivering thermogenesis capacity in mice (Xue et al.,2009).

As(III) is known to induce both neovascularization and angiogenesis (Soucy et al., 2003,2005) through direct effects on vascular endothelial cells (Straub et al., 2009) and increased tissue expression of VEGF (Soucy et al., 2004). As(III) has dose dependent effects on endothelial cells with low concentrations stimulating proliferation (Barchowsky et al., 1996) and angiogenesis (Soucy et al., 2003), but higher concentrations cause cell death (Barchowsky et al., 1996) and inhibit angiogenesis (Soucy et al., 2003, Roboz et al., 2000).

While these studies demonstrate the potential for As(III) to induce or inhibit angiogenesis, effects on angiogenesis in the context of tissue remodeling other than tumors has not been explored and little is known As(III) impact on the vasculature in the adipose tissue remodeling. Thus, we used an *in vivo* murine model of environmentally-relevant arsenic exposure to evaluate the hypothesis that low exposure As (III) promotes remodeling and phenotypic changes in AT that would support remodeling and altered energy metabolism.

3.3 RESULTS

Arsenic effects on systemic metabolic markers. AT secretes a large number of adipocytokines, growth factors, and hormones that participate in the metabolism of lipids and glucose. To evaluate whether these factors were affected after As(III) treatment, we quantified serum levels of adipocytokines, lipid profiles and hormones after 6 hours of fasting in mice that were exposed to 100 µg/ml As(III) in their drinking water for 5 wk. Among the parameters evaluated, we found a significant decrease in serum TAG (18 %) compared to the control group (Fig 6). Other markers related to lipid metabolism, such as cholesterol, lipoproteins or FFAs, did not change (data not shown). Similarly, the circulating levels of the two principal adipocytokines, adiponectin and leptin, were not affected by As(III) exposure. However, the insulin levels in the arsenic group were increased 30% with respect to the control (Fig 6B), without altering the circulating levels of glucose (Fig 6C). The data suggested a global insulin resistance with a possible increased storage or catabolism of TAG.

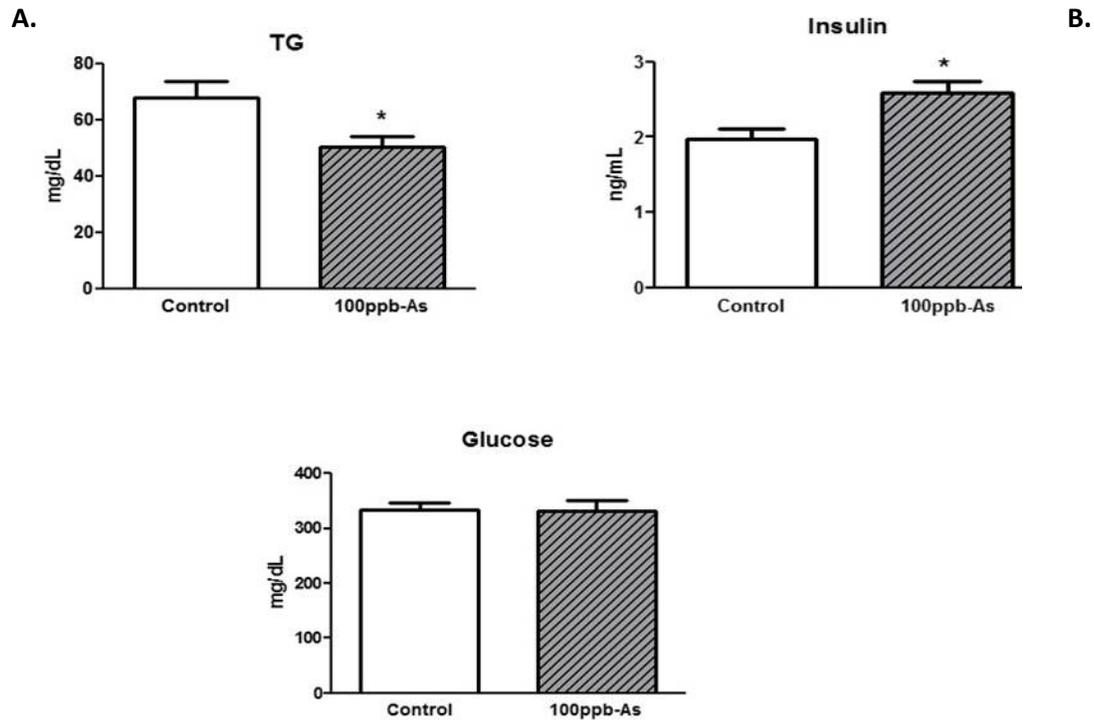


Figure 6 Arsenic induced metabolic changes in mice.

Male mice were exposed for 5 weeks to 100 $\mu\text{g/L}$ (ppb) As(III) in drinking water. Blood was collected after 6 hr fasting and serum levels of TAG, glucose, and insulin were determined. * designates significant difference at $p < 0.05$, $n = 12$ for A and $n = 8$ for B and C.

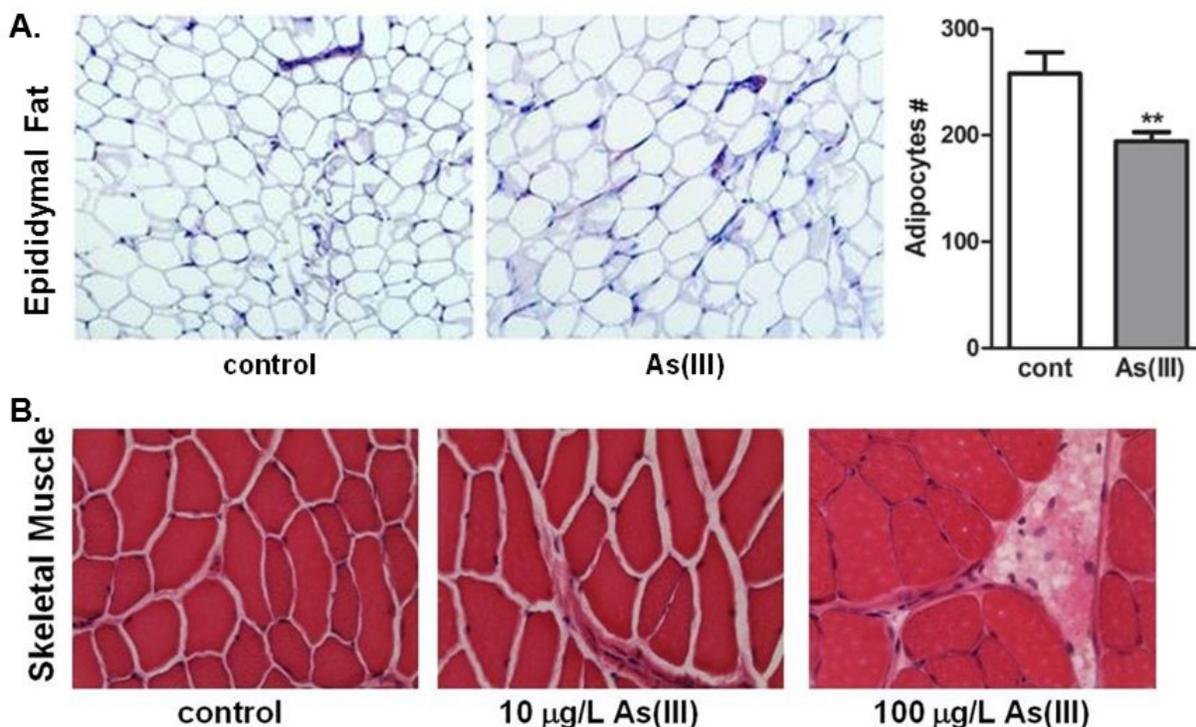


Figure 7 As(III) effects on adipose tissue and ectopic fat deposition in skeletal muscle.

Male mice were exposed to 0 (control), 10, or 100 μg/L As(III) in drinking water for 5 wk. At the end of exposure, epididymal fat and tibialis anterior muscle were collected for histological analysis (H&E stain of paraformaldehyde fixed, paraffin embedded or cryopreserved thin sections, respectively). **A.** Morphology of epididymal fat in control and As(III)-exposed mice showing relative size of adipocytes captured at 20x magnification. The graph presents mean ± SEM number of adipocytes measured in three separate microscopic fields from 8 mice in each group **B.** Cross sections of tibialis anterior muscle compare morphology of muscle fibers and perivascular fat droplet deposition (20x magnification). The images are representative of muscle images taken from eight mice in each group.

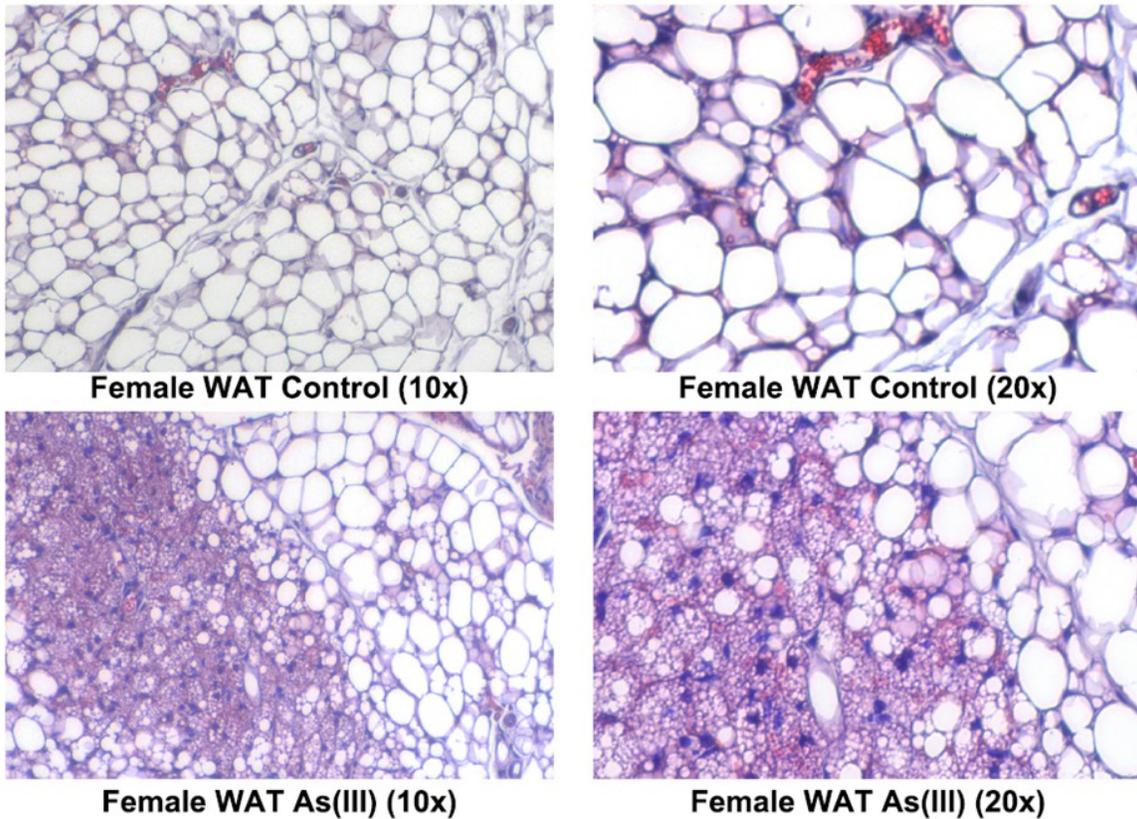


Figure 8 As(III) effects on the conversion of WAT into brown-like fat.

Female mice were exposed for 2 wk to 100 $\mu\text{g/L}$ As(III) in their drinking water. After euthanizing, abdominal fat pads were removed, fixed, and paraffin sections were stained with H&E. Images were captured at 10x to show the extent of change and 20x to show more detail of the phenotypic shift in the adipose tissue. Areas of “beige” fat were observed in 5/5 As(III)-exposed mice and 0/5 controls.

Arsenic stimulates AT-remodeling. The potential for AT to expand or regress is substantial. In addition, types of fat (i.e. white or brown) and different fat deposits (e.g. epididymal or subcutaneous) undergo differential remodeling and interconversion. Recent reports demonstrated that adipocytes are surrounded by fibrous and connective tissue, collagen, nerves and blood vessels. To some extent the participation of the vast network of blood vessels and vascularization maintains AT plasticity. Impact of As(III) exposure on AT morphology and histologic composition was examined in mice exposed to 0 or 100 µg/L As(III) for 5 weeks and ectopic fat deposition was examined in skeletal muscle. Histological analysis of epididymal fat (WAT) in male mice indicated adipocyte hypertrophy in the As(III) exposed mice, relative to control (Fig. 7A). This was confirmed by demonstrating that the number of adipocytes per 20x microscopic field was decreased in As(III) exposed mice, relative to controls. Consistent with the change in adipose tissue lipid storage, histological examination of tibialis anterior muscle demonstrated ectopic lipid deposition with pronounced perivascular fat (Fig. 7B). There were no signs of overt As(III)-induced cell or tissue death in either of the tissues.

In contrast to the effects of As(III) on WAT in male mice, As(III) promoted conversion of female abdominal WAT to “beige” or brown-type fat (Fig 8). Adipocytes in brown fat are derived from myogenic-lineage progenitors. However, “beige” adipocytes in WAT are derived from mesenchymal progenitors, but share the thermogenic properties and histologic character of brown fat (e.g. high level of mitochondria to give brown coloration and high vascularization) (Wu et al., 2012). It is not clear whether “beige” cells represent a represent conversion of the WAT cells or arise from a different differentiation program. It is important to note that the control and arsenic exposed females were much more active and had much less abdominal fat

than their male counterparts. This suggested that they burn fat more than store it; consistent with a higher level of brown fat.

Examination of the intrascapular brown fat (BAT) in both males (Fig 9) and females again revealed that As(III) caused tissue remodeling without any overt toxicity. The BAT appeared denser, more deeply colored, and, as in the WAT, more vascularized (luminal structures with red blood cells (Fig 9)). The fat droplets in both male and female BAT from the As(III)-exposed mice were smaller relative to the sex-matched controls. However, the droplets in female control mice were smaller than male controls and comparable in size to those seen in the male exposed mice. This, again, may reflect the high level of activity in the females relative to the male mice.

The As(III)-induced adipose vascularity that was evident in histological analysis of WAT and BAT was quantified by imaging the abundance of fluorescent antibody binding to CD31/PECAM (Fig 10) or VEGF receptor-2 (VEGFR2) (Fig 11) in WAT. PECAM is an endothelial cell specific adhesion molecule and VEGFR2 is the major tyrosine kinase receptor for VEGF that promotes angiogenesis and vascular remodeling. WAT excised from male mice that were exposed to As(III) for 2 wk showed a 45% increase in PECAM and a 68% increase in of VEGFR2 stained vessels. The increase in PECAM staining was supported by a 1.5-fold increase in PECAM mRNA extracted from male WAT (Table 8). In addition, VEGF mRNA expression was increased nearly 2-fold in WAT of from both male and female mice, and there was a 3-4 fold increase in VEGF mRNA in male and female BAT (Tables 7 and 8). These data suggested that As(III)-induced AT- remodeling was associated with a strong angiogenic stimulus.

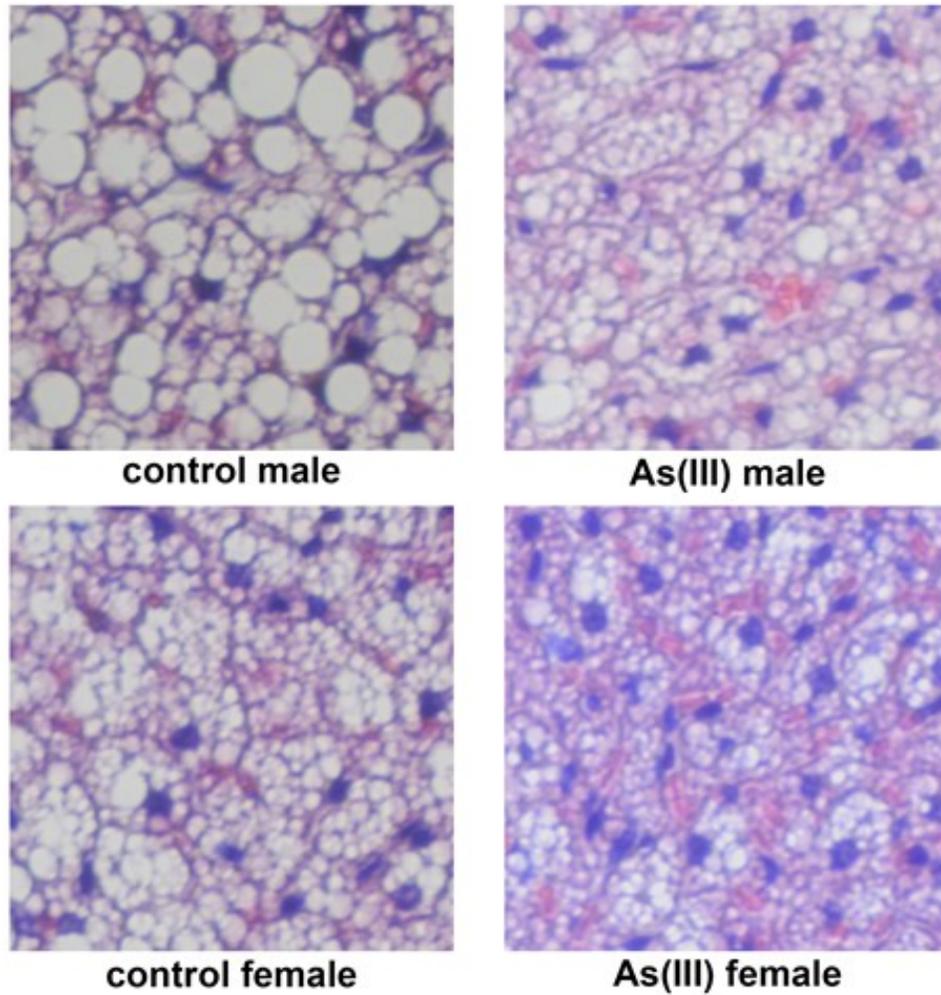


Figure 9 As(III) effects on brown fat composition.

Male and female mice were exposed for 2 weeks to 100 $\mu\text{g/L}$ As(III) in drinking water.

Intrascapular brown fat was excised, fixed, and paraffin-embedded sections were stained with

H&E. Images were captured at 20x and are representative of images from 8 mice in each group.

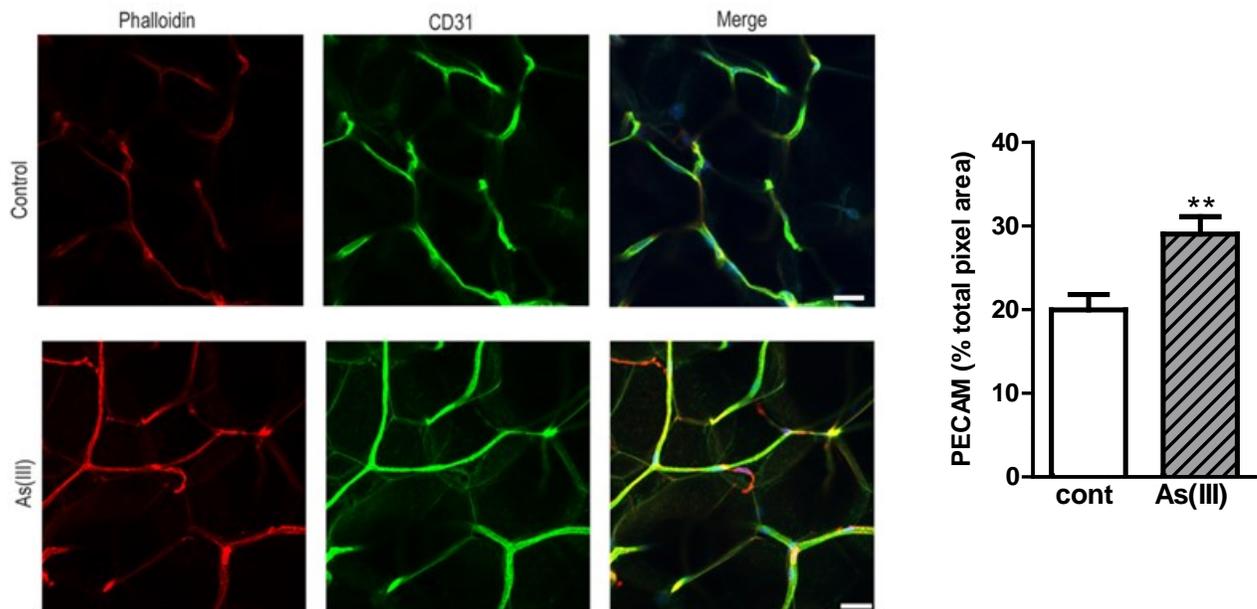


Figure 10 As(III)-induced increase in vascularization of WAT.

Male mice were exposed to 0 or 100 $\mu\text{g/L}$ As(III) for 2 wk and then WAT was excised and prepared for immunofluorescent staining. Immunofluorescence of F-actin binding phalloidin (Red), anti-PECAM (Green) and DAPI-stained nuclei (Blue) was performed in images of four fields per cover slip were captured at 40x (scale bars = 50 μm) and the thresholded fluorescence quantified and averaged. The data in the graphs present mean \pm SEM percentage of positive pixels normalized to DAPI staining. Data were analyzed by t-test for differences between groups ($P < 0.01$).

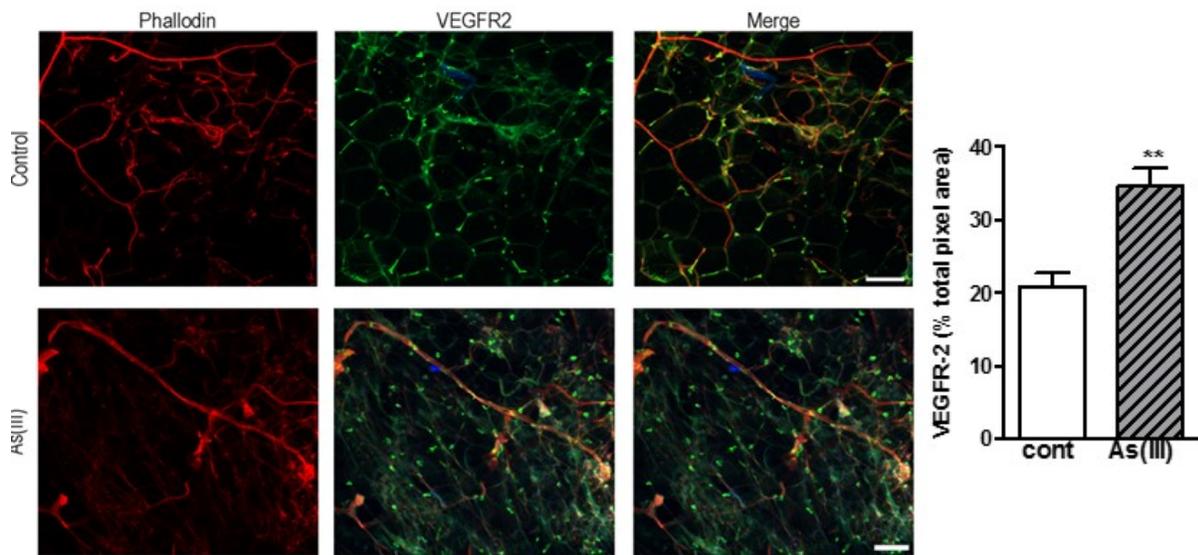


Figure 11 Arsenic increases expression of VEGFR2 positive vessels.

Immunostaining analysis of epididymal fat stained for phalloidin (Red), VEGFR2 (Green) and nuclei (Blue). Images of four fields per cover slip were captured at 40x (scale bars = 50 μ m) and the thresholded fluorescence quantified and averaged. The data in the graphs present mean \pm SEM percentage of positive pixels normalized to DAPI staining. Data were analyzed by t-test for differences between groups ($P < 0.01$).

Arsenic effects on AT gene expression. Quantification of gene transcript and protein expression in WAT (Table 7) and BAT (Table 8) excised from male and female mice exposed to 0 or 100 µg/L As(III) for 2 wks revealed that As(III) promoted adipose phenotypes with decreased cholesterol efflux, enhanced mitochondrial uncoupling, and, as mentioned above, increased angiogenesis. There were distinct tissue and sex differences in the gene responses that were concomitant with the observed histological differences in the tissues. For example, transcript levels for Pgc-1α, a master transcriptional regulator of lipid metabolism, mitochondrial biogenesis and vascularization, were increased significantly in male WAT, but only trended upwards in female WAT (Table 7). However, as might be expected for the more mitochondrial dense BAT, there was about a 10-fold increased Pgc-1a transcript level in BAT relative to WAT and As(III) increased this level in both sexes. These transcript differences were reflected at the level of protein expression in male WAT (Fig 12). However, the As(III)-induced increase in Pgc-1a transcript in BAT did not appear to increase the already greater level protein expression, relative to WAT (Fig 9). The transcript differences were consistent with tissue changes (Fig 9), although it is interesting that the As(III)-induced transcript level in the female WAT was not greater to support the increased “browning”.

In addition to the increased levels of Pgc-1a expression, there were patterns of increased expression of other mitochondrial related genes that would support increased inefficient metabolism. Uncoupling protein-1 (Ucp1) is a mitochondrial protein that is highly expressed in brown fat and is responsible for uncoupling electron transfer from energy generation to generate heat. Ucp1 transcript levels were very low in WAT, as expected, but increased in response to As(III) exposure. However, there was a very strong sex difference in WAT Ucp1 transcript levels with the females showing a higher basal level, but no As(III) induction relative to the

males. In contrast, the nearly 10,000-fold higher Ucp1 transcript levels in BAT were induced by As(III) in both sexes with a higher level of induction in the females. Again this is reflected at the levels of protein expression (Fig 12) and tissue histology (Fig 8). Overall, the female mice demonstrate a higher level of As(III) responsiveness in expression of other WAT mitochondrial genes (e.g. NRF-1, mtTFA, Cidea) that would support the increased browning of the tissues (Fig 9). It is interesting that there was no increased expression of the constitutive mitochondrial pore protein VDAC in the female WAT (Table 7).

There was increased expression of PEPCK, the rate limiting enzyme in gluconeogenesis, that was more pronounced in As(III) exposed female mice. PEPCK expression is suppressed by insulin (Xiong et al., 2011) and the slight induction in the As(III)-exposed mice may indicate, along with the slight increase in circulating insulin (Fig 6) that the tissues have increased insulin resistance.

ATP binding cassette transporter A1 (ABCA1) is an important regulator of cholesterol and phospholipid efflux in many cell types and normally transfers non-esterified cholesterol to apolipoprotein-A1 (Le Lay et al 2003). However, in the adipocytes that are cholesterol depots, ABCA1 acts as a cholesterol sensor that transfers cholesterol from the plasma membrane to an enriched layer coating the fat droplets (Le Lay et al 2003). As(III) exposure essentially doubled ABCA1 mRNA levels in both types of fat and in both sexes (Tables 7 and 8). However, the exposure reduced ABCA1 protein by 50% in both male WAT and BAT (Fig 12). The discordance of mRNA and protein changes might be expected, since Le Lay et al., demonstrated that large increases in ABCA1 mRNA during differentiation result in only modest increases in protein and phospholipid efflux. Nonetheless, the As(III) induced decrease in protein may have resulted in a change to the lipid droplet coat that would favor the larger cell size seen in Fig 9. If

the protein is decreased in other cell types, one would expect retention of cholesterol consistent with ectopic fat deposition (Fig 9).

Perilipins are critical regulators of the lipid droplet coat integrity and decreased perilipin content elevates the basal rate of lipolysis (Brasaemle, 2007). As(III) exposure dramatically reduced protein expression of PLIN1, the major perilipin in WAT, in male WAT (Fig 13) indicating that despite the larger size of the adipocytes (Fig 7A), there was a functional deficit in lipid storage capacity. Again, this would support ectopic fat deposition.

Table 7 Comparison of As(III) effects on transcript expression in male and female WAT.

Gene	Male (mean + SEM fg/ml product, n=8)			Female (mean + SEM fg/ml product, n=8)			M vs F (p value)
	control	As(III)	p value	control	As(III)	p value	
mitochondria							
Pgc-1 α	0.18 \pm 0.03	0.35 \pm 0.08	p<0.05	0.15 \pm 0.02	0.24 \pm 0.04	p>0.05	p>0.05
Ucp1	0.01 \pm 0.004	0.04 \pm 0.009	p<0.05	3.40 \pm 0.60	4.21 \pm 1.26	p>0.05	p<0.01
NRF-1	0.72 \pm 0.04	0.97 \pm 0.20	p>0.05	0.60 \pm 0.06	1.10 \pm 0.10	p<0.01	p>0.05
mtTFA	0.62 \pm 0.09	0.76 \pm 0.08	p>0.05	0.34 \pm 0.03	0.73 \pm 0.08	p<0.01	p>0.05
Cidea	0.14 \pm 0.03	0.12 \pm 0.03	p>0.05	2.0 \pm 0.64	0.70 \pm 0.16	p<0.01	p<0.01
Vdac	3.28 \pm 0.58	3.34 \pm 0.68	p>0.05	3.11 \pm .51	5.27 \pm 0.98	p>0.05	p>0.05
Vascular							
PECAM	9.53 \pm 0.97	14.35 \pm 2.85	p<0.05	6.93 \pm 0.87	10.85 \pm 1.60	p>0.05	p>0.05
VEGF	2.39 \pm 0.21	4.48 \pm 1.19	p<0.05	3.02 \pm 0.12	5.44 \pm 0.47	p<0.05	p>0.05
FN1	4.42 \pm 0.75	7.75 \pm 1.15	p<0.05	5.16 \pm 1.33	12.03 \pm 2.17	p<0.01	p>0.05
Metabolic							
PEPCK	38.64 \pm 5.04	87.78 \pm 17.77	p<0.05	47.46 \pm 6.51	120.58 \pm 25.13	p<0.01	p<0.01
Cholesterol transport							
ABAC1	58.26 \pm 8.80	116.35 \pm 18.93	p<0.01	56.13 \pm 0.49	101.97 \pm 23.52	p<0.01	p>0.05

Quantitative RT-PCR analysis of AT mRNA expression. The each transcript value is quantified against a standard curve amplified DNA specific to the indicated gene and then values are normalized against transcript for the constitutive gene RPL41. Statistical differences between exposure groups and sexes were determined by 2-way ANOVA followed by Bonferroni's post-hoc test.

Table 8 Comparison of As(III) effects on transcript expression in male and female BAT.

Gene	Male (mean + SEM fg/ml product, n=8)			Female (mean + SEM fg/ml product, n=8)			M vs F (p value)
	control	As(III)	p value	control	As(III)	p value	
mitochondria							
Pgc-1 α	1.51 \pm 0.13	2.84 \pm 0.43	p<0.05	1.60 \pm 0.13	5.65 \pm 0.70	p<0.01	p<0.01
Ucp1	1,696 \pm 200	2,222 \pm 258	p<0.05	2107 \pm 174	4730 \pm 588	p<0.01	p<0.01
NRF-1	0.95 \pm 0.01	1.26 \pm 0.13	p<0.05	1.28 \pm 0.14	2.20 \pm 0.21	p<0.01	p<0.01
mtTFA	1.39 \pm 0.16	2.35 \pm 0.30	p<0.01	1.71 \pm 0.12	2.10 \pm 0.30	p<0.01	p<0.05
Cidea	175 \pm 18	244 \pm 20	p<0.05	170 \pm 16	243 \pm 19	p<0.05	p>0.05
Vdac	12.71 \pm 1.38	15.33 \pm 0.94	p>0.05	12.82 \pm 1.19	15.04 \pm 2.17	p>0.05	p>0.05
Vascular							
PECAM	2.83 \pm 0.15	2.46 \pm 0.14	p>0.05	2.27 \pm 0.19	2.72 \pm 0.20	p>0.05	p>0.05
VEGF	0.38 \pm 0.01	5.49 \pm 0.12	p<0.01	1.07 \pm 0.03	3.64 \pm 0.08	p<0.01	p>0.05
FN1	2.19 \pm 0.07	2.36 \pm 0.05	p>0.05	2.23 \pm 0.12	4.33 \pm 0.08	p<0.05	p<0.05
Metabolic							
PEPCK	32.79 \pm 0.36	34.19 \pm 0.45	p>0.05	26.47 \pm 1.36	74.31 \pm 3.76	p<0.01	p<0.01
Cholesterol transport							
ABCA1	51.36 \pm 7.13	77.55 \pm 7.86	p>0.05	63.39 \pm 5.85	127.16 \pm 7.49	p<0.01	p<0.01

Quantitative RT-PCR analysis of AT mRNA expression. The each transcript value is quantified against a standard curve amplified DNA specific to the indicated gene and then values are normalized against transcript for the constitutive gene RPL41. Statistical differences between exposure groups and sexes were determined by 2-way ANOVA followed by Bonferroni's post-hoc test.

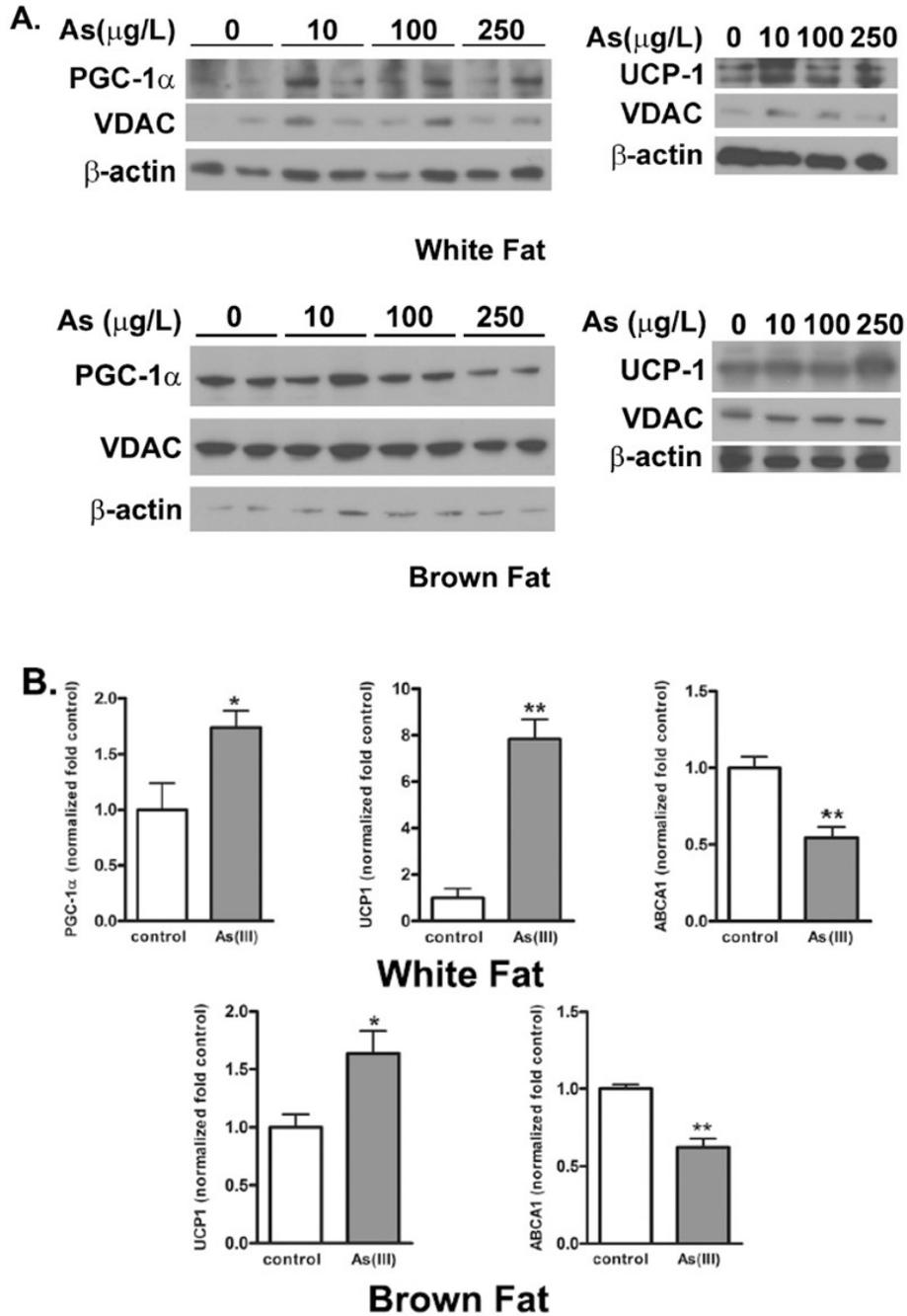


Figure 12 Effects of As(III) on AT protein expression.

A. Male mice were treated with the indicated amounts of As(III) in their drinking water for 2 weeks. Total protein was isolated from AT and analyzed for expression by Western. **B.** Data

mean + SEM of indicated protein from control or 100 µg/L As(III) exposed mice normalized to β-actin and analyzed by t-test designates significance from control (* p<0.05, ** p<0.01, n = 4).

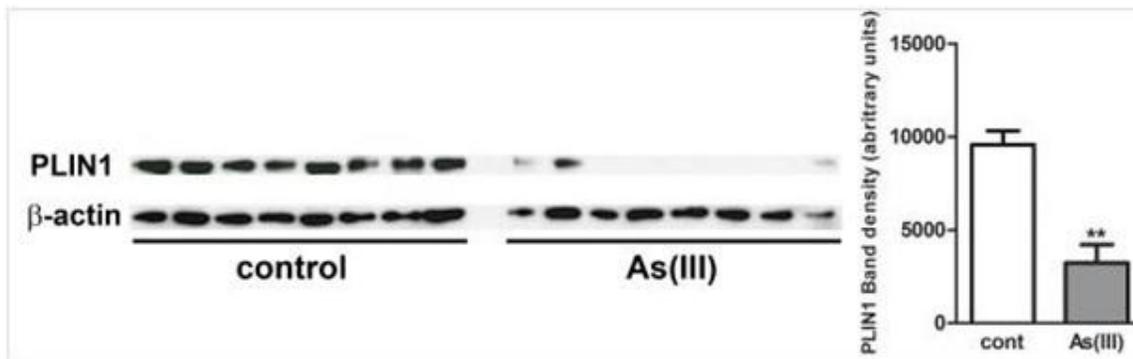


Figure 13 As(III) fat droplet coat protein expression.

Male mice were exposed to 0 (control) or 100 µg/L As(III) in drinking water for 5 wk. At the end of exposure, epididymal fat was collected and total protein extracted for Western analysis of PLIN1 expression. The immunoblots show PLIN1 and β-actin expression of perilipin with protein in each lane isolated from individual mice. The graph presents mean + SEM of the relative band density of perilipin normalized to β-actin. (** represents significance at p<0.01 as determined by t-test).

3.4 DISCUSSION

Chronic exposure to arsenic, usually via drinking water contamination with inorganic arsenic, has been associated with an increased risk for insulin resistance and changes in glucose uptake in adipocytes (Bazuine et al.,2003). The main goal of this study was to test the hypothesis that environmental doses of As(III) had a negative impact in lipid and glucose metabolism in a murine model *in vivo*. It is generally agreed upon that in early stages of obesity and insulin resistance, there is an increase in the flux of TAG as a result of stimulated lipolysis in absence of its principal inhibitor: insulin. In addition, numerous studies have indicated a diabetogenic effect in adipocytes and pancreatic cells exposed to arsenic. Therefore, we expected to find an increase in TAG in serum. In contrast, we found a reduction in the levels of circulating levels of TG in animals exposed to As(III) (Fig 6A).Consequently, our results were more in agreement with previous reports addressing arsenic exposure. Inorganic arsenic exposure lowered serum TAG in a low fat diet (LFD) and high fat diet (HFD) in a concentration-dependent manner. One of the proposed mechanisms suggested that if when is an impaired TAG synthesis in the liver- one of the main organisms implicated in lipogenesis- then there would be a decrease in the amount of TAG secretion for transport to the AT (Paul et al., 2011). This mechanism seems plausible, considering other studies have demonstrated that environmental levels of arsenic promoted endothelial cell dysfunction and remodeling in the liver vasculature in mice (Straub et al.,2007). The latter report strongly suggested that arsenic exposure can impair liver function and promote an imbalance in TAG synthesis.

In addition, it has been reported that the mechanism underlying the impairment of glucose and lipid metabolism in obesity involves perturbation in the production of adipokines that modulate

insulin sensitivity such adiponectin (Desai et al.,2013). Our results indicated that there were no substantial changes in circulating adipokines, or in any of lipid metabolism products. Nevertheless, the circulating levels of insulin, demonstrated an increase of 30% in arsenic – group compared to the control. Insulin stimulated glucose uptake (ISGU) by skeletal muscle and adipose tissue is a key process responsible for the normalization of postprandial blood glucose levels (Paul et al., 2007). Although the precise mechanism for diabetogenic effect of arsenic are still undefined, there is strong evidence that arsenic and its metabolites impair ISGU and result in insulin resistance (Fu et al., 2010). Therefore it is plausible that one of the early changes produced by arsenic is the impairment of insulin signaling in AT.

In the current study, As(III) caused remodeling of epididymal adipose tissue with enhanced adipocyte size and ectopic lipid deposition in muscle (Fig 7). As(III) also inhibited expression of perilipin *in vivo* (Fig 13), which would increase lipolysis and reduce the adipocyte capacity to store lipid. The pattern of ectopic intramyocellular lipid storage seen in Fig 7B is observed in both obese individuals and patients with lipodystrophies. The abundance of intramyocellular fat may be an earlier sign of insulin resistance and impaired metabolism than free fatty acids in serum (Sell, Dietze-Schroeder et al. 2006; Gustafson, Hammarstedt et al. 2007; Vigouroux, Caron-Debarle et al. 2011). Redistribution of adipose lipids to perivascular fat accumulation occurs in the pathogenesis of cardiomyopathies, coronary artery disease, and atherosclerosis (Ouwens, Sell et al. 2010; Turer, Hill et al. 2012). Thus, remodeling of adipose tissue and redistribution of lipid deposition may combine with As(III) inhibition of adipogenesis (Klei, Garciafigueroa et al. 2013) to support a novel mechanism for the pathogenesis of As(III)-induced metabolic and cardiovascular diseases.

The capacity of adipocytes to alter their metabolic phenotype to BA-like type is known as “browning”. Typical browning involves the appearance of multilocular adipocytes cluster that are dispersed among unilocular WAT (Lee et al., 2013). The images shown in Fig 8 demonstrate this pattern of multilocular adipocyte in female WAT exposed to arsenic. Furthermore, gene expression (Table 7) in WAT females also confirmed remodeling of WAT into BAT-like adipocytes. In agreement with previous studies that indicated under certain circumstances, such as β -adrenergic or chronic PPAR γ agonist stimulation, WAT depots can increase expression of “brownish fat” specific proteins (Petrovic et al., 2010).

The sex- differences found in remodeling of both BAT and WAT (Table 7 and 8) may be explained by hormonal and metabolic differences. The metabolic rate per kilogram AT is reported to be higher in women than in men and this could be due to increase expression of various genes involved in mitochondrial function, including UCP-1 (Fuente-Martin et al., 2013). In a study that screened for expression of 271 gene transcripts that regulate weight and obesity, it was reported that gender had a great influence on AT expression of 88 genes, which persist after normalizing for fat mass (Vigueri et al., 2012). AT depots also respond to gonadal steroids. Estrogen exerts its effect through estrogen receptor α and β (ER α and ER β). Both receptors are expressed in AT and skeletal muscle regulating glucose metabolism and insulin signaling. In WAT ERs are highly expressed in mitochondria, suggesting important influence on cell metabolism (Gorres et al., 2011).

Angiogenesis plays an active role in modulation of adipogenesis and obesity which is regulated by adipokines and blood vessels (Xue et al., 2009). Most of the studies in AT are intended to elucidate the molecular mechanism underlying tissue expandability, obesity and its association with metabolic diseases. However there is little to no data that considers how the environment

may impact lipid metabolism and function. Here we demonstrated that chronic exposure to inorganic As promoted pro-angiogenic factors that mediated remodeling of AT (Fig 10 and 11). AT remodeling might be indicative of failure of an adaptative mechanism under cellular or tissue stress. Chronic or sustained conditions may turn in disease tissue remodeling (Itoh et al.,2011). Furthermore, As(III) promotes capillarization and vascular remodeling of endothelial cells in the liver (Straub et al 2007). Liver sinusoidal endothelial cells (LSEC) suffer capillarization, an event that precedes vascular remodeling after 2 weeks exposure to 250 ppb of As. In addition, markers of capillarization like PECAM and laminin-1 proteins were increased (Straub et al.,2007). Arsenic also induced vascular remodeling stimuli, such as increased VEGF expression in smooth muscle cells (Soucy et al., 2005). Moreover Matsumoto et al., (2001) demonstrated that during liver development, VEGFR2 signaling was necessary for endothelial cells to undergo cell multiplication and migration. Studies in preadipocytes indicate that VEGFR2 signaling is critical for modulation not only in angiogenesis and tissue growth but also in control of preadipocytes differentiation. The blocking of VEGFR2 produced a decrease in angiogenesis but also in adipogenesis program. In addition, VEGFR2 –VEGF did not directly mediate adipose tissue formation but act in a paracrine manner (Fukumura et al., 2009).

Most studies related with obesity and adipocyte expansion suggest that hypoxia is a key mediator factor of vascular growth and remodeling. In response to hypoxia, AT produces hypoxia–induced factor 1 (HIF-1 α) that regulates angiogenesis and vasculogenesis (Cao, 2007). In agreement, accumulating reports in the literature support the hypothesis that angiogenesis can accelerate adipogenesis through release of cytokines (Park et al., 2010) such as leptin(Cao et al.,2001) and PAI-1(Crandall et al.,2001). Nevertheless, Halberg et al., (2009) showed that during AT expansion, hypoxic conditions increased the levels of HIF-1 α , but failed to induce the expression

of proangiogenic factors. VEGF mRNA decreased, while HIF1- α stimulated fibrotic program like Lysyl oxidase (LOX), collagen I and II. Comparatively, the ablation of ARNT (HIF-1 β) using ARNT^{-/-} mice or HIF-1 α ^{-/-} mice shown reduced fat formation and protection from a high fat diet (HFD) against obesity and insulin resistance (Jiang et al., 2011). In agreement with these findings, it is unlikely that in our model As(III) stimulated angiogenesis through activation of HIF-1 α and then increased VEGF levels. However, the increased expression of PGC-1 α (Table 7 and Fig 11), that cooperates with HIF-1 α in inducing VEGF may also explain the observed increase in WAT VEGF (Table 7). PGC-1 α is able to induce VEGF gene expression independently of HIF-1 α activity (Shoag and Arany, 2009). PGC-1 α coactivates the orphan nuclear receptor EER α that in turn activates VEGF gene in cultured muscle cells and skeletal muscle *in vivo* (Arany et al., 2008, Shoag and Arany, 2009). As shown in Table 7, the levels of *Pgc1 α* in WAT exposed to arsenic where increase, especially in males. Over expression of *Pgc1 α* may be correlated with increased neovascularization and remodeling observed in male fat pads (Fig 10 and 11). Our results showed that As(III) increased the vascular markers as CD31 and VEGFR2 (Fig 10 and 11 respectively and Table 7). Since both markers were increased in a similar manner, the increase most likely reflected the expansion of the vessels rather than being drivers of the angiogenesis.

CHAPTER 4.0 ARSENIC-STIMULATED LIPOLYSIS AND ADIPOSE REMODELING IS MEDIATED BY G-PROTEIN COUPLED RECEPTORS

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D. Yesica Garciafigueroa¹, Linda R. Klei¹, Fabrisia Ambrosio², and Aaron Barchowsky^{1,3}

¹. Department of Environmental and Occupational Health, University of Pittsburgh Graduate School of Public Health, Pittsburgh, PA, 15219. ². Department of Physical Medicine and Rehabilitation, University of Pittsburgh School of Medicine, Pittsburgh PA, 15219 ³. Vascular Medicine Institute, University of Pittsburgh School of Medicine, Pittsburgh PA, 15219

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4.1 ABSTRACT

Arsenic in drinking water promotes a number of diseases that may stem from dysfunctional adipose lipid and glucose metabolism. Arsenic inhibits adipocyte differentiation and promotes insulin resistance; however, little is known of the impacts of and mechanisms for arsenic effects on adipose lipid storage and lipolysis. Based on our earlier studies of arsenic signaling mechanisms for vascular remodeling and inhibition of adipogenesis, we investigated the hypothesis that arsenic acts through specific adipocyte G-protein coupled receptors to promote lipolysis and decrease lipid storage. We first demonstrated that 5 wk exposure of mice to 100 $\mu\text{g/L}$ of arsenic in drinking water stimulated epididymal adipocyte hypertrophy, reduced the adipose tissue expression of perilipin (a lipid droplet coat protein), and increased perivascular ectopic fat deposition in skeletal muscle. Incubating adipocytes, differentiated from adipose-derived huMSC, with arsenic stimulated lipolysis and decreased both Nile red positive lipid droplets and perilipin expression. Arsenic-stimulated lipolysis was not associated with increased cAMP levels. However, pre-incubation of adipocytes with the G_i -inhibitor, *Pertussis* toxin, attenuated As(III)-stimulated lipolysis and lipid droplet loss. Antagonizing G_i -coupled endothelin-1 type A and B receptors (EDNRA/EDNRB) also attenuated arsenic effects, but antagonizing other adipose G_i -coupled receptors that regulate fat metabolism was ineffective. The endothelin receptors have different roles in arsenic responses, since only EDNRA inhibition prevented arsenic-stimulated lipolysis, but antagonists to either receptor protected lipid droplets and perilipin expression. These data support a role for specific G-protein coupled receptors in arsenic signaling for aberrant lipid storage and metabolism that may contribute to the pathogenesis of metabolic disease caused by environmental arsenic exposures.

4.2 INTRODUCTION

Arsenic (As(III)) is a ubiquitous naturally occurring metalloid found in drinking water that poses health risks to more than 2% of the world population. Chronic As(III) exposure increases the risk of a number of cancers and chronic non-cancer diseases, including cardiovascular, pulmonary, and metabolic diseases (Parvez, Chen et al. 2010; Hughes, Beck et al. 2011; Abhyankar, Jones et al. 2012; Maull, Ahsan et al. 2012; Moon, Guallar et al. 2012). Even low to moderate As(III) exposures may increase the risk of cardiovascular disease (Chen, Graziano et al. 2011; Moon, Guallar et al. 2012) and potentially enhance insulin resistance in metabolic disease (Gribble, Howard et al. 2012; Maull, Ahsan et al. 2012). The etiology and pathogenic mechanisms of As(III)-promoted metabolic dysfunction and toxicity remain undefined, but may relate to impacts on adipose tissue remodeling and function.

Adipose tissue was believed to be an inert tissue, but is now recognized as a critical endocrine organ that is essential for control of energy metabolism, insulin sensitivity, and appropriate lipid storage. It is a dynamic tissue responsive to and responsible for hormonal, inflammatory, and metabolic interactions (both homeostatic and pathogenic) with other organs (Turer, Hill et al. 2012). Paradoxically, both excess adipose tissue in obesity and loss of adipose tissue in lipodystrophies contribute to metabolic diseases and pathogenic consequences of ectopic lipid storage in non-adipose tissues (Gustafson, Hammarstedt et al. 2007; Vigouroux, Caron-Debarle et al. 2011; Turer, Hill et al. 2012). Hypertrophic expansion of adipose tissue resulting from excess lipid storage suppresses adipose regeneration and causes dysfunctional ectopic storage of lipid in liver, heart, and skeletal muscle (Gustafson, Hammarstedt et al. 2007; Turer, Hill et al. 2012). Conversely, lack or loss of adipose tissue and the ability of adipose tissue to store lipid result in metabolic and oxidative stress, as well as ectopic lipid storage and systemic

inflammation (Gustafson, Hammarstedt et al. 2007). While the severity of metabolic disease caused by disrupted lipid storage varies, it is evident that factors that chronically impair proper lipid metabolism and storage greatly enhance risk for cardiovascular, liver, impaired skeletal muscle composition and metabolism, and development of diabetes (Gustafson, Hammarstedt et al. 2007; Vigouroux, Caron-Debarle et al. 2011; Turer, Hill et al. 2012).

A significant number of human lipodystrophies have genetic origins with mutations found in genes coding for enzymes regulating lipid storage or lipolysis, as well as proteins that regulate lipid droplet formation and maintenance (Vigouroux, Caron-Debarle et al. 2011). Lipid storage and lipolysis are highly regulated by lipases, fatty acid binding proteins, and proteins coating the lipid droplets, such as perilipin (PLIN1) (Bezaire, Mairal et al. 2009; Kolditz and Langin 2010). The canonical pathway for physiological lipolysis involves Gs protein-coupled receptor-mediated (e.g. β -adrenergic stimulation) increase of intracellular cAMP levels that stimulate protein kinase A (PKA) phosphorylation of hormone sensitive lipase and perilipin. This signaling allows activated lipase access to cleave stored lipid droplet triglycerides (Soeder, Snedden et al. 1999; Kolditz and Langin 2010). In addition, perilipin expression regulates lipid droplet size and basal lipolytic rate. Lipolysis increases as perilipin expression decreases, as seen in a number of pathologic conditions and following chronic TNF α -stimulated wasting (Bezaire, Mairal et al. 2009; Kolditz and Langin 2010). Perilipin loss raises basal lipolysis, but impairs stimulated lipolysis.

Autocrine, paracrine, hormonal, and possibly environmental factors impact the critical lipid storage balance. Insulin is the major hormonal factor promoting both lipid storage and glucose homeostasis. However, hypertrophic expansion of mature adipocytes, inflammation, and tissue injury increases levels of ligands for a number of G-protein coupled receptors (GPCR) whose

activation disrupts insulin signaling, inhibits adipogenesis, and promotes lipolysis (Janke, Engeli et al. 2002; Bhattacharya and Ullrich 2006; Mogi, Iwai et al. 2006; Tomono, Iwai et al. 2008; van Harmelen, Eriksson et al. 2008; Eriksson, van et al. 2009). Obese tissues and mature adipocytes paradoxically increase lipolysis and suppress adipose regeneration in part by increasing paracrine action of the secreted peptides, endothelin-1 and angiotensin II (Janke, Engeli et al. 2002; Juan, Chang et al. 2006; Tomono, Iwai et al. 2008; van Harmelen, Eriksson et al. 2008; Eriksson, van et al. 2009). These peptides are ligands for Gi and Gq-coupled GPCRs (EDNRA/EDNRB and AGTR1/AGTR2, respectively) that, when stimulated, regulate adipocyte differentiation and adipose tissue maintenance (Elbaz, Bedecs et al. 2000; Janke, Engeli et al. 2002; Tomono, Iwai et al. 2008; van Harmelen, Eriksson et al. 2008; Iwai, Tomono et al. 2009). In addition, stimulation of AGTR2 may trans-inactivate insulin signaling (Elbaz, Bedecs et al. 2000; Iwai, Tomono et al. 2009) and chronic stimulation of EDNRA decreases insulin sensitivity by decreasing expression of insulin receptor and insulin receptor substrates to (van Harmelen, Eriksson et al. 2008). Endothelin-stimulated lipolysis is seen predominantly in visceral fat and may be mediated by EDNRB (van Harmelen, Eriksson et al. 2008). Chronic elevation of endothelin in obese tissue, however, may also promote lipolysis through EDNRA (Eriksson, van et al. 2009). Additional GPCRs that regulate adipocyte function and lipid storage include the sphingosine-1-phosphate receptors (S1PR1/S1PR2) (Nincheri 2009; He, H'ng et al. 2010). S1PR1 was also shown to mediate As(III)-stimulated oxidant production and remodeling of vascular endothelial cells (Straub, Klei et al. 2009).

As(III) promotes metabolic dysfunction, metabolic-associated diseases, and impairs insulin responsiveness (Paul, Devesa et al. 2008; Chen, Graziano et al. 2011; Gribble, Howard et al. 2012; Maull, Ahsan et al. 2012; Moon, Guallar et al. 2012). As(III) inhibits differentiation of

stem cells into adipocytes (Wauson, Langan et al. 2002; Cheng, Qiu et al. 2011; Klei, Garciafigueroa et al. 2013) and disrupts insulin signaling that stimulates glucose uptake (Walton, Harmon et al. 2004; Paul, Devesa et al. 2008). The impact of As(III) on lipid storage and lipolysis has not been investigated, nor have the potential mechanisms that underlie these pathogenic metabolic actions. We recently found that *Pertussis* toxin (Ptx)-sensitive and endothelin-1 GPCRs (EDNRA/EDNRB) mediate a significant portion of the anti-adipogenic effect of As(III) (Klei, Garciafigueroa et al. 2013). Therefore, we investigated the hypothesis that As(III)-activates specific GPCR signaling pathways in adipocytes to stimulate lipolysis and reduce lipid storage capacity. The following studies provide support for this hypothesis and elucidate the roles of EDNRA and EDNRB signaling in mediating the effects of arsenic on adipocyte lipid storage and metabolism.

4.3 RESULTS

As(III) stimulates adipocyte lipolysis and reduces perilipin-coated lipid droplets. The effects of As(III) on lipid mobilization was investigated in a model of cultured human adipocytes differentiated from primary adipose-derived hMSC. The cells were treated with As(III) in the late stages of differentiation when the cells contained pronounced perilipin and Nile Red positive lipid droplets. Within 24 h of exposure, As(III) maximally increased lipolysis, as measured by release of glycerol from the exposed cells relative to control cells (Fig. 14). The increased lipolysis was followed by a progressive loss of lipid droplets (Nile Red staining) and perilipin coating the lipid droplets that was significant by 72 hours of exposure (Fig. 15). More detailed Western analysis demonstrated significant loss of perilipin by 48 h (Fig. 16) and this loss of protein paralleled a loss of approximately 74 % of PLIN1 transcript. There was also a decrease of approximately 72% of ADIPOQ (adiponectin) transcript, a marker for mature adipocytes (Fig. 16 B). This indicated a programmatic transcriptional change in the adipocytes rather than a selective effect of arsenic on PLIN1 expression. Dose response comparisons demonstrated that the threshold for these effects of As(III) on adipocytes was between 0.1 and 0.2 μM and exposures to greater than 2.5 μM caused toxicity (data not shown).

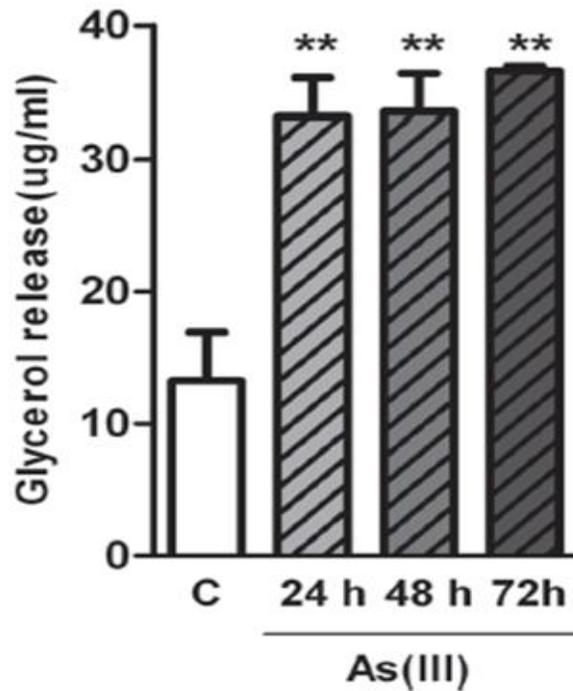


Figure 14 As(III) increases lipolysis in adipocytes.

Adipocytes differentiated from hMSC were incubated with or without 1 μ M As(III) for 72 h and medium above the cells was sampled at the indicated times for glycerol content as a measure of lipolysis (control was sampled at 72 h). Released glycerol was measured using a colorimetric assay. Data are presented as mean \pm SEM glycerol released in three separate cultures and from two independent experiments (each sample assayed in duplicate). Data were analyzed for significance by ANOVA and Newman-Keuls post-test for differences between groups (** $p < 0.01$ from control).

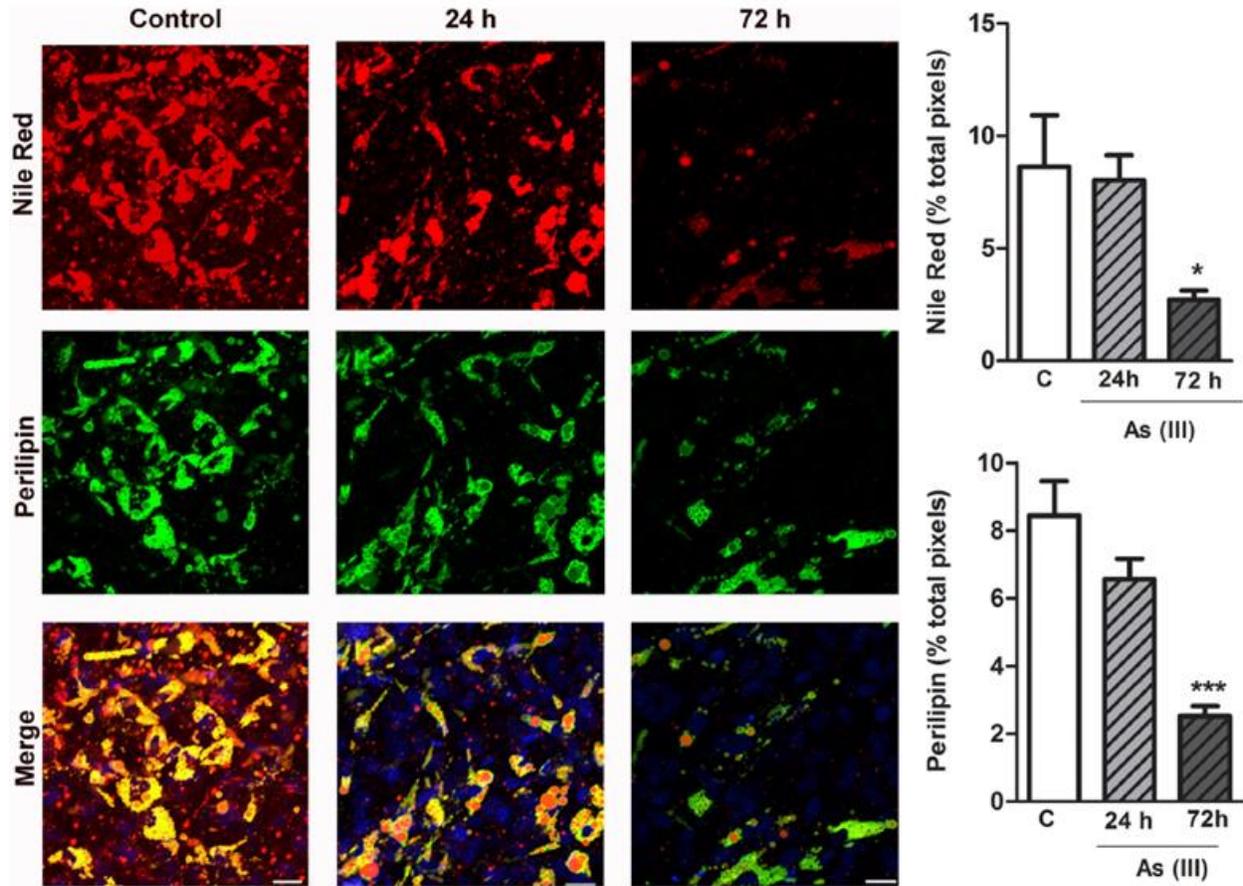


Figure 15 As(III) causes progressive loss of adipocyte perilipin-coated lipid droplets.

Adipocytes were differentiated and grown on glass coverslips and then treated with 1 μ M As(III) for the indicated times. The cells were fixed and stained for neutral lipid-droplets (Nile Red), perilipin (green), and nuclei (DAPI blue) content. Images of four fields per cover slip were captured at 40x (scale bars = 50 μ m) and the thresholded fluorescence quantified and averaged. The data in the graphs present mean \pm SEM percentage of positive pixels normalized to DAPI staining in four separate cultures representative of two separate experiments. Data were analyzed by ANOVA and Newman-Keuls post-test for differences between groups (* $p < 0.05$ and *** $p < 0.001$ from control).

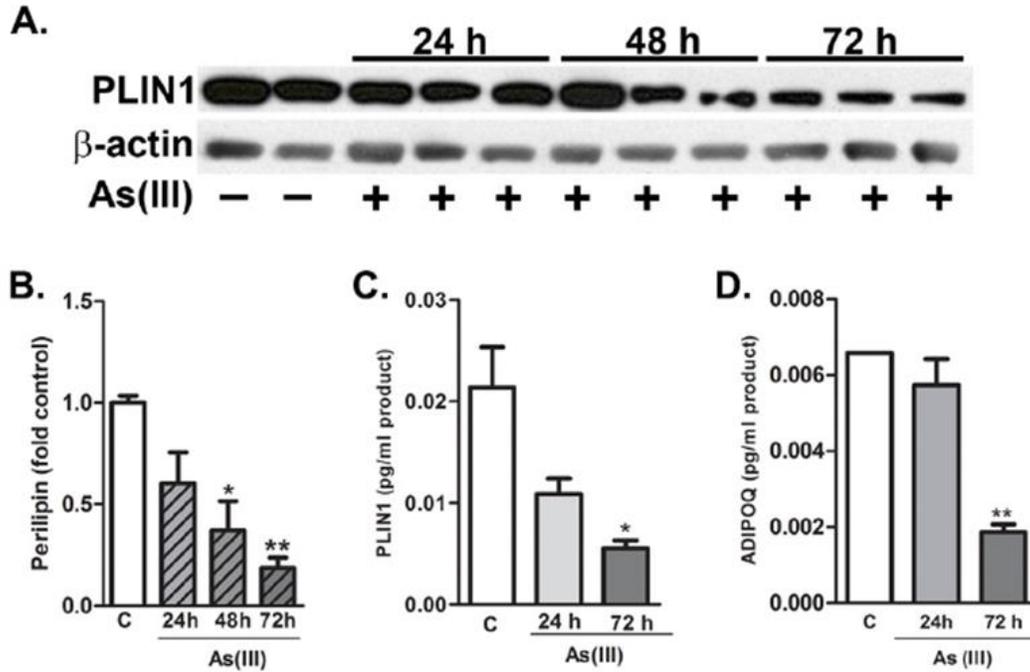


Figure 16 As(III) inhibits perilipin expression.

Adipocytes were cultured in the absence or presence of 1 μ M As(III) for 24, 48 or 72h before extraction or protein or RNA. **(A and B)** Total protein extracts were probed by Western analysis for perilipin and β -actin. **(B)** The graph presents mean \pm SEM fold band densities relative to control for perilipin immunoblots normalized to β -actin from three separate cultures in two independent experiments. **(C AND D)** Total RNA was probed for PLIN1, ADIPOQ, or RPL13 transcript levels by qRT-PCR. The data are presented as mean \pm SEM of the pg/mL of PCR product normalized to the housekeeping gene RPL13. Data were analyzed by one-way ANOVA and Bonferroni's post hoc test (* p < 0.05 and ** p < 0.01 relative to untreated cells).

GPCR in As(III)-stimulated lipolysis and loss of perilipin-coated droplets. Physiologic lipolysis is stimulated through Gs-coupled GPCR signaling and increased AMP levels and PKA activity (Juan, Chang et al. 2006; Vigouroux, Caron-Debarle et al. 2011) or Gi/Gq GPCR-mediated inhibition of insulin-stimulated lipid storage (Mogi, Iwai et al. 2006; van Harmelen, Eriksson et al. 2008; Vigouroux, Caron-Debarle et al. 2011). We recently demonstrated that a major portion of As(III)-inhibited hMSC differentiation into adipocytes was mediated by Ptx-sensitive GPCRs (Klei, Garciafigueroa et al. 2013). To test whether similar receptors mediated As(III) effects on adipocytes, differentiated cells were incubated with Ptx overnight before incubation with As(III) for 72 h. Ptx treatment completely prevented As(III)-stimulated loss of lipid loss droplets (Fig 18) and 50% (Western analysis, Fig 17A) - 87% (quantitative immunofluorescence, Fig 18) loss of perilipin protein expression. Similarly, Ptx attenuated As(III)-induced repression of PLIN1 and ADIPOQ transcript levels (Fig. 17B). Functionally, Ptx treatment attenuated As(III)-stimulated lipolysis (Fig. 17C). In contrast to stimulating lipolysis through Gi-coupled signaling, As(III) exposure of 30 min to 4 h or for 24 h (data not shown) did not stimulate Gs-coupled signaling for increased intracellular cAMP levels relative to isoproterenol, a positive control for Gs-coupled receptor activation (Fig 19).

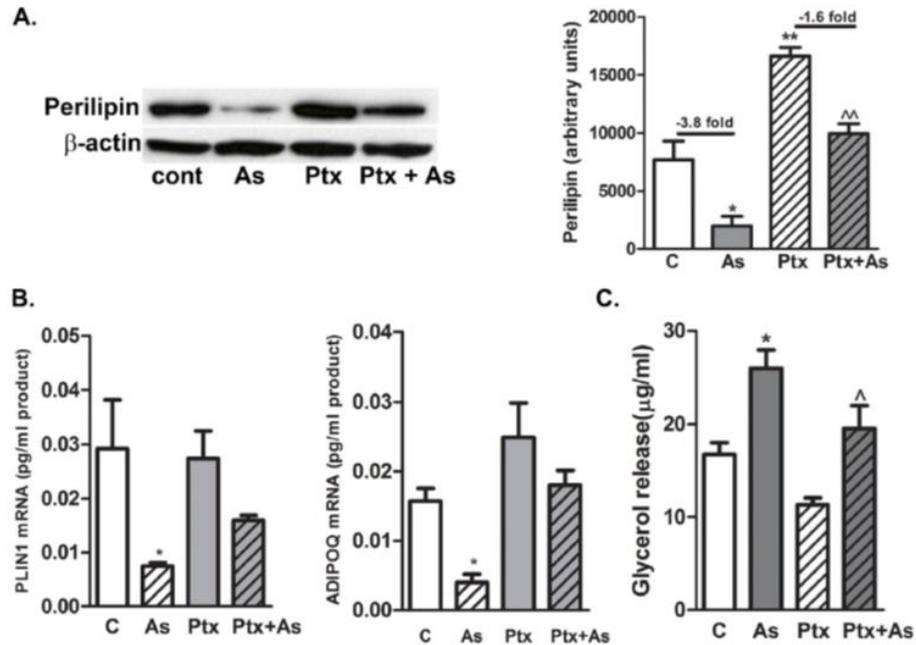


Figure 17 Effect of Ptx on As(III)-exposed adipocytes.

Ptx (1 μ M) was added to the indicated groups of differentiated adipocytes for 24 h before 1 μ M As(III) was added. After 72 h, medium was collected for glycerol measurements and the cells were harvested for protein and RNA isolation. **(A)** Total proteins from cell lysates were probed by Western analysis for perilipin and β -actin levels and the graph presents densitometric analysis from six cultures in each group (mean \pm SEM of fold difference from untreated cells (control)). **(B)** Total RNA was assayed by qRT-PCR for PLIN1, ADIPOQ or RPL13 transcript levels. Data are mean \pm SEM of at least 2 independent experiments. **(C)** Released glycerol was quantified and data are presented as mean \pm SEM μ g of glycerol released per ml of medium in three separate cultures and from two independent experiments (each sample assayed in duplicate). Data was analyzed by one-way analysis ANOVA and Bonferroni's post-test for significance (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

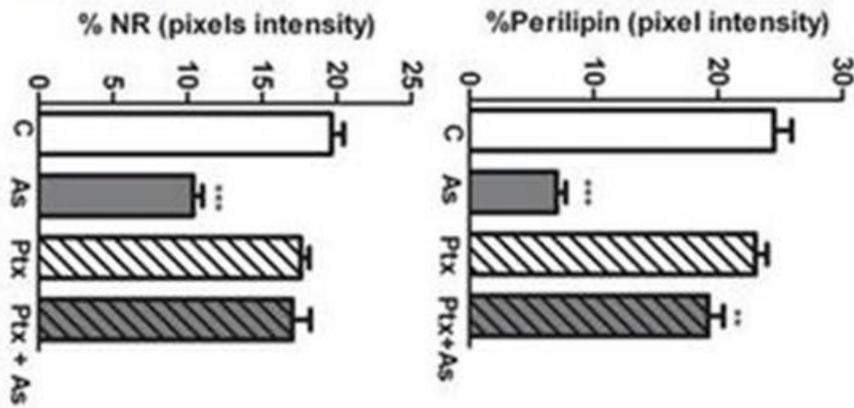
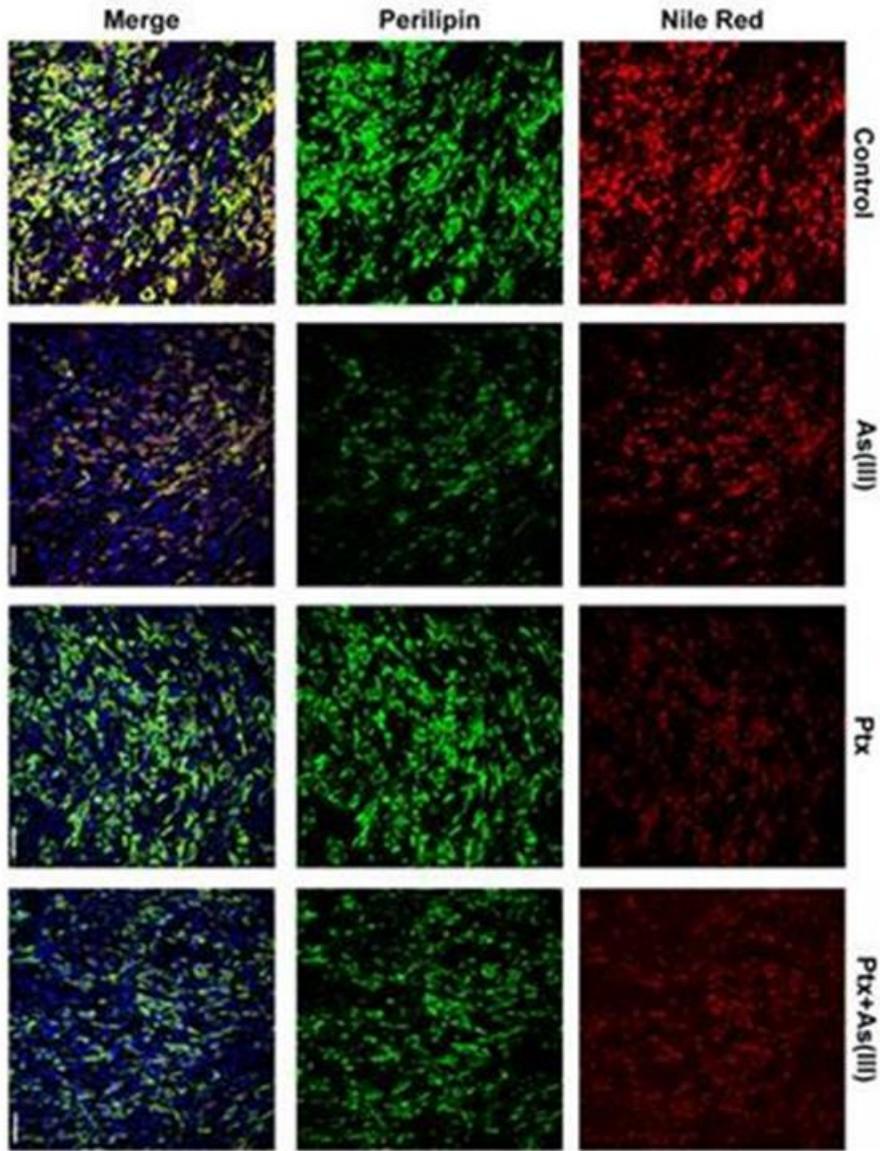


Figure 18 Ptx prevents As(III)-stimulated loss of lipid droplets and perilipin.

Ptx (1 μ M) was added to the indicated groups of adipocytes grown on glass coverslips for 24 h before 1 μ M As(III) was added. After 72 h, the cells were fixed and stained for triglyceride content (Nile Red), perilipin (green), or nuclei (blue). Images of four fields per cover slip were captured at 40x (scale bars = 50 μ m) and the thresholded fluorescence quantified and averaged. The data in the graphs present mean \pm SEM percentage of positive pixels normalized to DAPI staining in four separate cultures representative of two separate experiments. Data were analyzed by ANOVA and Newman-Keuls post-test for differences between groups (** $p < 0.01$ and *** $p < 0.001$ relative to control).

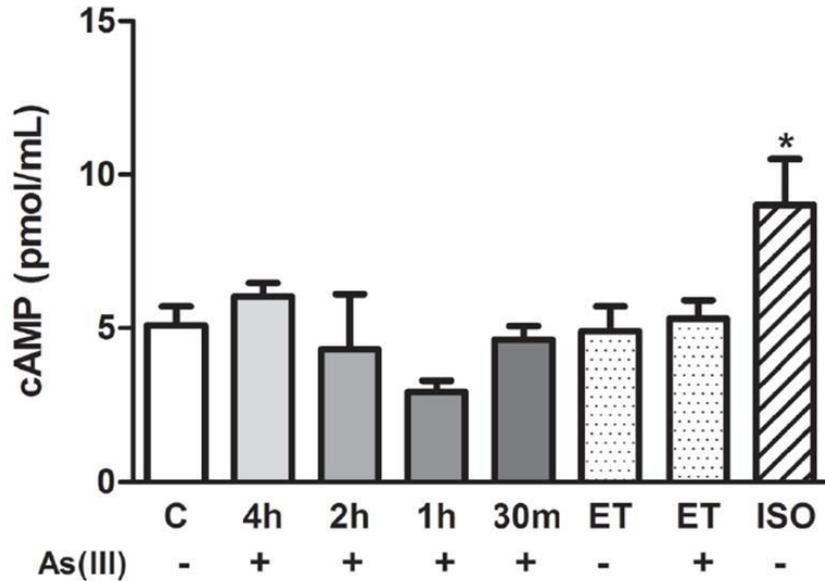


Figure 19 As(III) does not increase intracellular cAMP levels.

Adipocytes were incubated with 1 μ M As(III) for the time indicated, 100 nM endothelin-1 for 4 h, or 10 nM isoproterenol (positive control) for 1.5 h. At the end of exposure, cells were harvested, lysed, and cAMP levels were quantified using a colorimetric enzyme immunoassay, according to manufacturer's instructions. Data are presented as mean \pm SEM cAMP of three separate cultures (each sample assayed in duplicate) from two independent experiments. Data were analyzed by ANOVA and Newman-Keuls post-test for differences between groups.

As(III) stimulates endothelin-1 receptors to cause adipocyte dysfunction.

Ptx selectively ADP-ribosylates Gi proteins to block Gi-coupled receptor signaling. There are at least three types of Gi-linked receptors that affect adipocyte lipolysis, including S1PR1/3, AGTR1, and ENDRA/ENDRB (Bhattacharya and Ullrich 2006; Tomono, Iwai et al. 2008; van Harmelen, Eriksson et al. 2008; Hashimoto, Igarashi et al. 2009). To identify which receptors mediate the As(III) effects, we pre-treated adipocytes with specific antagonists before and during three day incubations in the presence or absence of As(III). As seen in Fig 20 A-B, antagonizing ENDRA with BQ610 attenuated As(III)-induced loss of lipid droplets and perilipin expression. In contrast, antagonizing ENDRB with BQ788 reduced basal perilipin expression and there was no further reduction by As(III) (Fig. 20 B). Blocking either ENDRA or EDNRB prevented As(III)-stimulated lipolysis (Fig. 20 C). Antagonizing S1P1 or AGTR1 with VPC 23019 or L-158,809, respectively, did not prevent As(III) effects on lipolysis (data not shown) or perilipin expression (Fig 21).

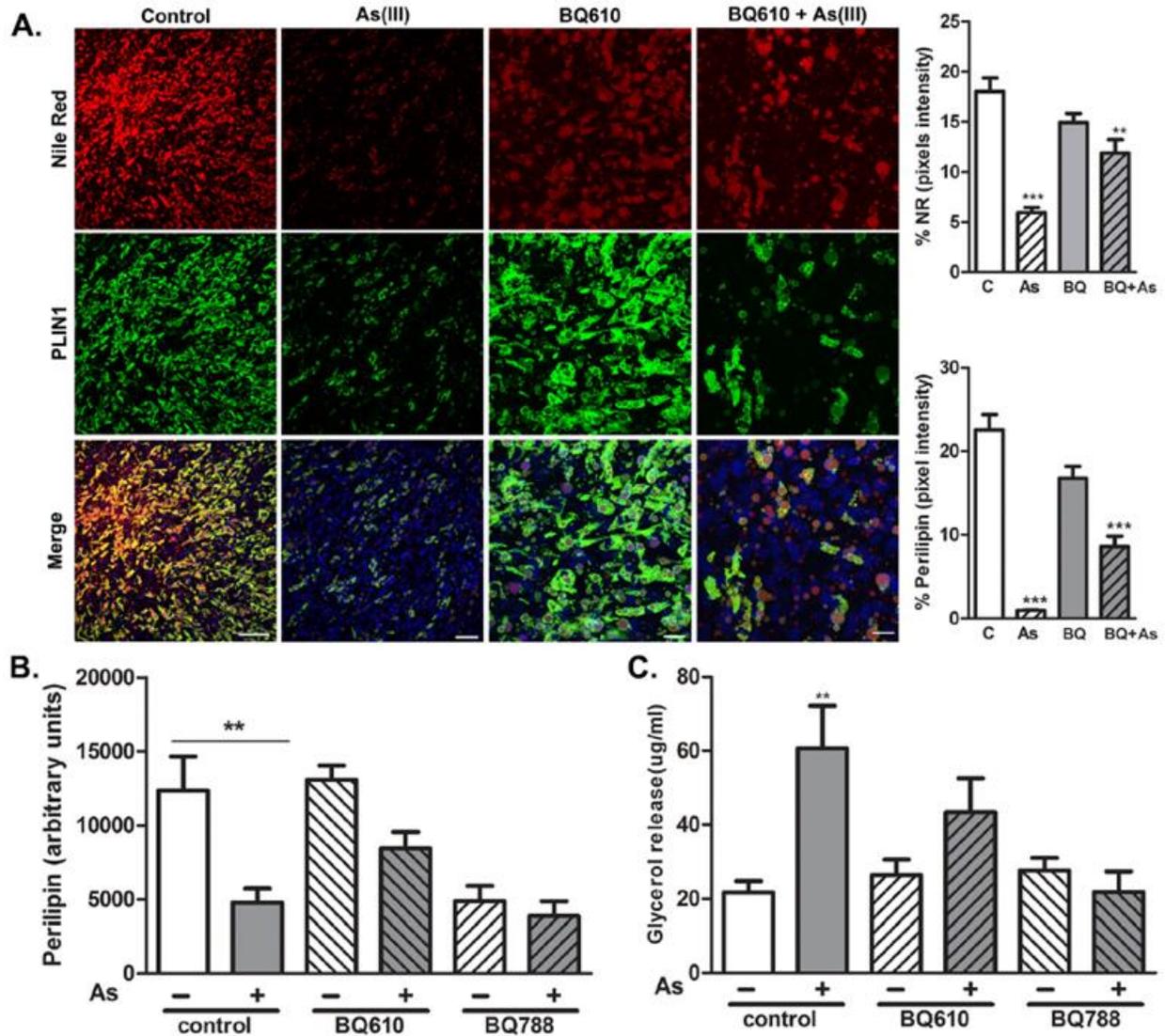


Figure 20 Endothelin-1 receptors mediate As(III)-stimulated adipocyte dysfunction.

Adipocytes grown on coverslips (A) or in 12 well culture plates (B, C) were incubated for 30 min in the absence or presence of ENDRA antagonist BQ610 (1 μ M) or ENDRB antagonist BQ788 (1 μ M), and then incubated with 1 μ M As(III) for 72h. (A) Coverslips were processed for imaging of triglycerides with Nile red, perilipin (green), and nuclei (blue). Images of four fields per cover slip were captured at 40x (scale bars = 50 μ m) and the thresholded fluorescence

quantified and averaged. The data in the graphs present mean \pm SEM percentage of positive pixels normalized to DAPI staining in three separate cultures representative of two separate experiments. In the experiment in well plates, culture medium was collected and total protein extracts prepared from cell lysates. **(B)** Protein extracts were analyzed by Western analysis with antibodies to perilipin or β -actin. Data represents mean \pm SEM of band density of extracts from 3 independent cultures in two separate experiments. **(C)** Released glycerol was quantified and data are presented as mean \pm SEM of glycerol ($\mu\text{g/ml}$ medium) released three separate cultures and from two independent experiments (each sample assayed in duplicate). Data was analyzed by one-way analysis ANOVA with Newman-Keuls post-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) versus control.

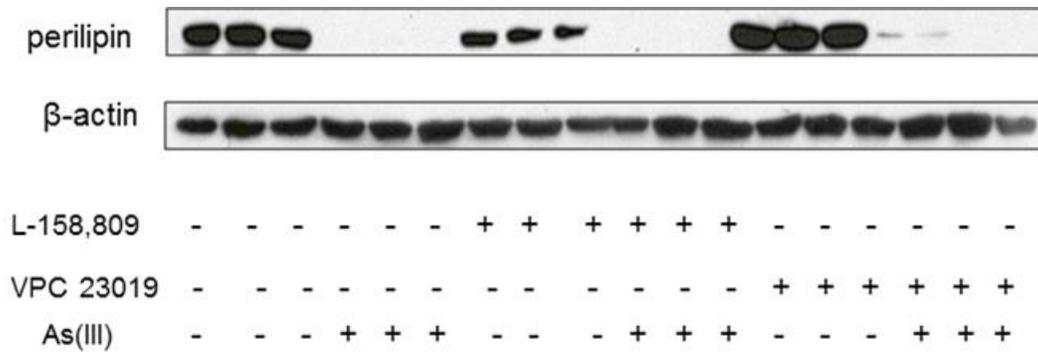


Figure 21 As (III) does not suppress perilipin by stimulating the S1PR1 or AGTR1.

Adipocytes were incubated with 1 μ M of either the S1PR1/3 inhibitor VPC23019 or the AGTR1 inhibitory L 158,809 for 30 min before adding 1 μ M As(III). The cells were then incubated for 72 h before isolating total proteins. Western analysis with antibodies specific for perilipin or β -actin was used to measure protein abundance. Each lane contains protein from a separate cell culture

4.4 DISCUSSION

The data presented support the hypothesis that As(III)-stimulated lipid mobilization and inhibited lipid storage is mediated or initiated in part by activating Gi-coupled receptor signaling. Stimulating Ptx-inhibitable receptors accounted for almost all of As(III)-induced loss of lipid droplets and a majority of As(III)-stimulated lipolysis and decreased expression of lipid coat protein. The inhibitor studies suggest that endothelin GPCR receptors mediate a majority of the Ptx-inhibitable responses, however, it is evident that other non-identified receptors and signaling pathways contribute to As(III) inhibition of normal adipose tissue metabolism and fat distribution. The hypothetical signaling scheme for As(III) effects on adipogenesis and adipocyte function is shown in Fig 22.

Imbalances in fat storage, either excess energy storage in obesity or fat loss in lipodystrophies, results in metabolic disorders with impaired regulation of glucose and lipid metabolism in both adipose and non-adipose tissues (Sell, Dietze-Schroeder et al. 2006; Gustafson, Hammarstedt et al. 2007; Ouwens, Sell et al. 2010; Vigouroux, Caron-Debarle et al. 2011). As(III) exposure is associated with increased metabolic disease risk or an inability to control metabolic syndromes (Paul, Devesa et al. 2008; Chen, Graziano et al. 2011; Gribble, Howard et al. 2012; Maull, Ahsan et al. 2012; Moon, Guallar et al. 2012). As(III) inhibited expression of perilipin *in vivo* (Fig13 in Chapter 3) and in cultured cells (Figs 15 and 16), which indicates a reduced capacity of adipocytes to store lipid. Increased lipolysis following decreased perilipin expression is seen in a number of pathologic conditions (Bezaire, Mairal et al. 2009; Kolditz and Langin 2010). Decreased perilipin expression raises the basal rate of lipolysis, but impairs stimulated lipolysis (Bezaire, Mairal et al. 2009; Vigouroux, Caron-Debarle et al. 2011). This perilipin-dependent

shift in basal lipolysis may explain the apparent new steady state of lipolysis seen in the As(III) exposed adipocytes (Fig 14).

There are several GPCR-mediated mechanisms that regulate adipogenesis, lipolysis, lipid metabolism, lipid storage. As(III) did not increase intracellular levels of cAMP indicating that it does not activate the Gs-coupled physiological pathway for stimulating lipolysis for energy production. However, all of the effects of arsenic on adipocyte lipid metabolism and storage measured in these studies were attenuated by Ptx treatment. Depending on the endpoint measured and assay used in the measurements, Ptx ribosylation of Gi proteins prevented 50-87% of As(III) effects. This is consistent with our previous observations of Ptx attenuation of As(III)-inhibited adipogenesis (Klei, Garciafigueroa et al. 2013). It is evident, however, that stimulating Gi-protein coupled signaling is not responsible for all of As(III) actions on the adipocytes and it is likely that other signaling pathways contribute to the full impact of As(III) on adipocytes and adipose tissues. This is separation of pathways is similar to angiogenic and remodeling As(III) effects on endothelial cells being mediated by activation of S1PR1 receptors that is independent of As(III)-induced stress responses (Straub, Klei et al. 2009).

Suppression of adipogenesis and adipose tissue maintenance by Ptx-sensitive Gi-coupled receptors is well established (Shinohara, Murata et al. 1992; Hauner, Petruschke et al. 1994; Janke, Engeli et al. 2002; Tomono, Iwai et al. 2008; van Harmelen, Eriksson et al. 2008). Gi-coupled receptors, such as AGTR1 (Elbaz, Bedecs et al. 2000; Janke, Engeli et al. 2002) or EDNRA/EDNRB (van Harmelen, Eriksson et al. 2008; Eriksson, van et al. 2009) oppose increases in cAMP and block insulin signaling that suppresses lipolysis. As with receptor-mediated As(III) inhibition of hMSC differentiation into adipocytes (Klei, Garciafigueroa et al. 2013), endothelin-1 receptor activation accounted for most, but not all of Ptx protection from

As(III)-stimulated lipolysis, loss of lipid droplets, and perilipin repression. Endothelin-1 produced and released by endothelial cells in the adipose tissue vasculature stimulates lipolysis and inhibits insulin-stimulated lipid storage (Juan, Chuang et al. 2007; van Harmelen, Eriksson et al. 2008; Eriksson, van et al. 2009). Endothelin-1 release and action increases more than 2.5 fold in obese humans, but its impact on lipolysis and adipose remodeling are greater in visceral fat, relative to subcutaneous fat (van Harmelen, Eriksson et al. 2008). This is consistent with our observation that As(III) acting through endothelin receptors decreases perilipin expression and possibly lipid storage in epididymal fat, but increased ectopic fat deposition in muscle. However, even though no role was found for other major Gi-coupled receptors that regulate adipose function, such as AGRT1 and SIPR1, there are clearly other unidentified Gi-coupled/Ptx-inhibitable receptors that mediate As(III) effects.

The subtype of endothelin receptor that regulates endothelin-1-stimulated lipolysis and insulin resistance is not clear with reports of the receptors acting differentially *in vivo* and in different cell culture models. In human adipocytes differentiated from progenitor cells isolated from visceral but not subcutaneous fat, endothelin-1 induced the expression of EDNRB and stimulated the receptor to cause a sustained loss on insulin receptor and its downstream repression of lipolysis (van Harmelen, Eriksson et al. 2008). In contrast, EDNRA was found responsible for endothelin-1 stimulated lipolysis and repression of adiponectin expression in freshly isolated primary human adipocytes and in differentiated mouse 3T3-Li adipocytes, respectively (Juan, Chuang et al. 2007; Eriksson, van et al. 2009). In our model of human adipocytes differentiated from hMSC isolated from visceral fat, we found that EDNRA mediates As(III)-stimulated lipolysis, loss of lipid droplets, and repression of perilipin expression. Antagonizing EDNRB inhibited perilipin expression to such an extent that further inhibition by As(III) was not

observed. However, antagonizing EDNRB was equally as effective as antagonizing EDNRA in preventing As(III)-induced lipolysis without affecting basal lipolysis (Fig 20). This suggests that the As(III)-stimulated Gi-mediated pathways for lipolysis and transcriptional repression are separate. It is important to note that EDNRA and EDNRB signaling is often cooperative with the formation of heterodimeric signaling complexes (Zuccarello, Boccaletti et al. 1999; Watts 2010), suggesting that As(III) may be acting through a complex of EDNR to repress perilipin expression. In addition, we found that siRNA knockdown of either receptor affected As(III) effects on the expression of the other receptor in differentiating adipocytes (Klei, Garciafigueroa et al. 2013). This adds to the complexity of dissecting the roles of the individual receptors in As(III)-stimulated effects.

In conclusion, the data support a pathogenic pathway for As(III)-stimulated adipose remodeling and redistribution of adipose deposition that is substantially mediated by selective Gi-coupled receptor stimulation. As we found previously, Ptx-inhibition of all Gi-receptor signaling was more effective than antagonizing any individual Gi-receptor in protecting against As(III) effects and that the type of Gi-receptor mediating As(III) effects are tissue specific (Straub, Klei et al. 2009; Klei, Garciafigueroa et al. 2013). Blocking the endothelin-1 receptors attenuated As(III) lipolytic actions, but the decreased efficacy relative to Ptx suggests that additional Gi-proteins might mediate portions of the As(III) effects. These selective receptor-mediated effects may contribute to the molecular pathogenesis of As(III)-promoted adipose tissue remodeling and insulin resistance observed in As(III) induced metabolic and cardiovascular diseases.

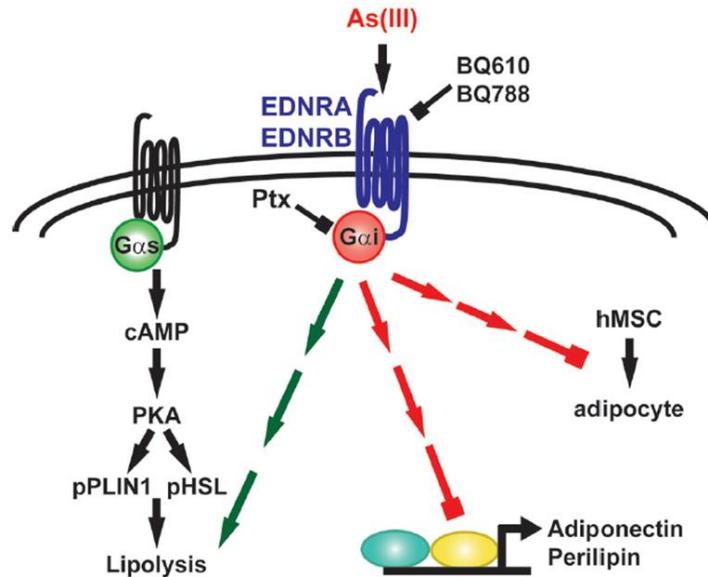


Figure 22 Signaling scheme for endothelin-1 receptor mediated effects of arsenic on adipocytes.

As(III) stimulates endothelin receptors (EDNRA/EDNRB) to increase lipolysis and decrease expression of lipid droplet coat protein, perilipin. In addition, the scheme includes the signaling for As(III) inhibition of differentiation of human mesenchymal stem cells to adipocytes (Klei, Garciafigueroa et al. 2013). The broken arrows indicate the multiple steps in the signaling cascade between receptor activation and lipolysis or effects on transcription or differentiation

CHAPTER 5.0 ARSENIC STIMULATES RECEPTORS CROSS COMMUNICATION

5.1 ABSTRACT

We observed that arsenic inhibits stem cell differentiation into adipocytes (Klei et al., 2013) and demonstrated in Chapter 4 that arsenic promotes lipolysis and dysfunctional lipid storage in adipocytes. These actions required Gi protein-coupled signaling that is partially mediated by endothelin receptor subtypes (EDNRA/ERNRB). In the current studies, we further examined the GPCR mediated actions of arsenic to elucidate other receptor actions that fully account for arsenic-stimulated adipocyte dysfunction. Based on literature reports of EDNR regulation of lipolysis and adipose tissue remodeling, we hypothesized that full stimulation of lipolysis involved coordinated interactions between arsenic-stimulated EDNRA or other GPCR with the receptor tyrosine kinase (RTK) epidermal growth factor receptor (EGFR). We found that the selective EGFR kinase inhibitor AG1478 prevented arsenic-stimulated loss of fat droplets and PLIN1, implicating EGFR in arsenic-promoted adipocyte dysfunction. Arsenic also increased signaling by phosphorylated and total ERK, a downstream effector of EGFR. Combined inhibition of EDNRA and EGFR was highly effective in preventing arsenic actions. Immunoprecipitation of EGFR signaling complexes revealed that arsenic increased G α i protein interactions with EGFR, suggesting that EGFR may use G α i as a platform to cross-talk with EDNRA or other GPCRs. However, arsenic-stimulated transactivation of EGFR appeared to be independent of the conventional oxidant promoted signaling through Src family kinase activity.

5.2 INTRODUCTION

The observations in Chapter 4 and the recent report of arsenic acting through Gi-coupled receptor signaling to inhibit adipogenesis (Klei et al., 2013) suggested that other receptors may be involved in promoting arsenic dysfunction in adipocytes. Literature reports also indicated that more Gi-coupled protein signaling regulates adipocyte function. Chronic activation of Gi-coupled AGTR1, as well as EDNRA/EDNRB, promotes metabolic disease (Vinson 2007; Tomono, Iwai et al. 2008), causes insulin resistance (van Harmelen, Eriksson et al. 2008), inhibits adipocyte differentiation (Tomono, Iwai et al. 2008; van Harmelen, Eriksson et al. 2008), and stimulates lipolysis (van Harmelen, Eriksson et al. 2008). However, AGTR1 was not involved in arsenic inhibited adipogenesis (Klei et al., 2013) or PLIN1 expression (Chapter 4). In obesity, endothelin released from adipose tissue inhibits MSC differentiation to adipocytes, stimulates lipolysis, and promotes insulin resistance (van Harmelen, Eriksson et al. 2008; Eriksson, van et al. 2009). In addition, there is significant cooperation between EDNR and EGFR in inhibiting adipogenesis, promoting lipolysis, and opposing insulin signaling (Bhattacharya and Ullrich 2006).

EGFR is one of four members of the Erb B RTK family. In addition to EGFR (ErbB1), this subfamily consists of: Her-2/neu/ErbB2, HER-3/Erb3 and HER-4/ErbB4. The receptors exist as inactive monomers. Upon binding to ligands, such as epidermal growth factor (EGF) and transforming growth factor- α , the receptors undergo conformational changes that facilitate homo or heterodimerization (Pao and Miller., 2005, Rogers et al., 2012). Activation of EGFR stimulates Akt and signal transducer and activator of transcription (STAT) and the MAP kinase (Erk) pathways (Pao and Miller., 2005). Alternatively, the EGFR can be activated by stimuli that

do not directly interact with EGFR ectodomain, in a process called transactivation or cross-communication (Glisic et al., 2012, Rodaoudabush et al.,2000). The stimulation can be triggered by GPCR ligands, other RTK agonists, cytokines and cell adhesion elements (Gschwind et al.,2001). Examples of GPCR ligands that can transactivate EGFR include lysophosphatidic acid (LPA) acting on the SPR1, angiotensin II acting through AGTR1 and ET-1 acting on EDNRA (Roudabush et al., 2000, Pyne and Pyne, 2011). One mechanism of transactivation GPCR-mediated activation of the zinc-dependent disintegrin and metalloproteinase family (ADAM) that cleaves to activate the EGFR ligand proheparin-binding-EGF (HB-EGF) (Prenez et al.,1999). GPCR ligands can also activate cytoplasmic non-receptor tyrosine kinases, such as c-Src and Pyk-2 that catalyze tyrosine phosphorylation of the EGFR and other RTK independently of HB-EGF release (Luttrell et al.,1999, Pyne and Pyne, 2011). The GPCR agonists induce a rapid and transient activation of the Src family members, often through increased H₂O₂ formation, to phosphorylate EGFR at Tyr-845 (Rozenfurt, 2007). In a similar manner, arsenic acts through NADPH oxidase mediated H₂O₂ formation to directly increase Src substrate phosphorylation (Barchowsky, Roussel et al. 1999).

ET-1-mediated activation of MAPK modulates cellular responses, such as hypertrophy, growth, proliferation, and cell survival in different cells types (Hsieh et al.,2012). Moreover, ET-1 stimulates transactivation of EGFR in ovarian cancer cells, vascular smooth muscle cells, or rat cardiac fibroblast, to activate MAPK. (Kasanabe et al., 2004, Hsieh. et al., 2012). In cultured astrocytes, ET-1 induced transactivation of EGFR and prevents the ligand-induced internalization of the EGFR (Glisic et al.,2012). As indicated above, EDNRs transactivate adipocyte EGFR to increase Erk activity such that its signaling suppresses AKT signaling

(Bhattacharya and Ullrich 2006). This transactivation of signaling decreases adipocyte insulin responsiveness and promotes lipolysis (Bhattacharya and Ullrich., 2006).

Arsenic activates EGFR in different cell lines *in vitro* including lung epithelial cells, human keratinocytes HaCat cells (Cooper et al.,2004), Reznikova et al.,2009), and human uroepithelial cells (Simeonova et al., 2002), as well as *in vivo* lung tumor samples (Andrew et al., 2009). Furthermore, there is evidence that arsenic treatment increases EGFR tyrosine phosphorylation in PC12 cells, bronchial epithelial cells, and bladder epithelial cells (Cooper et al., 2004). Similarly As₂O₃ is known to activate cellular signaling pathways such as ERK, Jun and p38 kinase pathways (Liu and Huang., 2006). *In vitro* and *in vivo* studies indicate that arsenic stimulation of c-Src is involved in transactivation and stabilization of EGFR (Simeonova and Luster 2002) or that Src family kinase combine with EGFR signaling to affect cells differentiation (Patterson and Rice 2007).

The role of EGFR signaling in adipocytes has been extensively studied; however it is not well understood. Early reports demonstrated that EGF inhibited the differentiation of preadipocytes (isolated from inguinal or epididymal fat pads) and reduced their capacity to accumulate triglycerides. Interestingly, EGF had no inhibitory effect on cells once differentiation was completed (Serreno et al.,1987, Pagano and Calvo.,2003). In accordance with these results, Adachi et al.(1994), confirmed that EGF inhibited adipogenesis when EGF was present at the beginning of the differentiation. However EGF may promote triglyceride synthesis and adipogenesis in differentiated cells. The latest reports suggested that EGFR (erB1) is downregulated in mature adipocytes to allow differentiation compared to non-differentiated cells (Pagano and Calvo.,2003). *In vivo*, EGFR activation in adipose stromal vascular cells (MSC) caused low grade inflammation and promoted insulin resistance (Prada, Ropelle et al. 2009).

Selective inhibition of EGFR reverses insulin resistance and improves glucose tolerance impaired by feeding mice high fat diets (Prada et al., 2009).

We recently demonstrated that arsenic produced loss of fat droplets and activation of lipolysis was only partially mediated by EDNRA (Chapter 4; Garciafigueroa et al.,2013). Based on the observations in the literature of arsenic induced transactivation of EGFR and the reported interaction of EDNR and EGFR inhibiting adipocyte differentiation and function, we hypothesized that arsenic stimulated EGFR transactivation may provide the majority of its effects on lipid storage. The studies in this Chapter address this hypothesis and explore mechanisms for arsenic stimulated linkage between GPCR and EGFR signaling.

5.3 RESULTS

EGFR RTK activity is required for arsenic impaired adipocyte function. Activation of EGFR has been highly correlated with arsenic exposure in several models. To evaluate whether arsenic alters adipocyte differentiation by activation of EGFR, hMSC-derived adipocytes were treated with 1 μ M arsenic for 72 hours. As shown in Chapter 4, arsenic decreased the lipid droplet and PLIN1 expression (Fig 15, 16). Immunostaining of neutral lipid (Nile Red) and lipid-coated protein Perilipin confirmed a 2.5 and 2.9 fold loss of droplets respectively, in adipocytes following arsenic exposure (Fig 23).Pretreatment of adipocytes with AG1478, a selective inhibitor of EGFR, attenuated arsenic inhibitory effects as lipid content and PLIN1 were decreased by 1.6 or 1.5 fold, respectively compared with cells treated with AG1478 alone (Fig 23). Western analysis of total protein extracts from the adipocytes in the different treatment

groups indicated that AG1478 completely blocked the effect of arsenic on PLIN1 expression (Fig 24). However, measurement of the glycerol content of medium above the cells indicated that AG1478 was ineffective in preventing arsenic-stimulated lipolysis.

Co-incubation of EGFR and ENDRA inhibitors prevented arsenic-stimulated loss of fat droplets

To evaluate the cooperation between one or more receptors implicated in arsenic-induced lipotoxicity, adipocytes were preincubated at day 9 post-differentiation with AG1478 and the EDNRA inhibitor prior to exposure to arsenic for 72 h. Both immunostaining and Western blot analysis showed that arsenic activates the receptors and they cooperate to reduce lipid droplet content and coat protein expression (Fig 25).

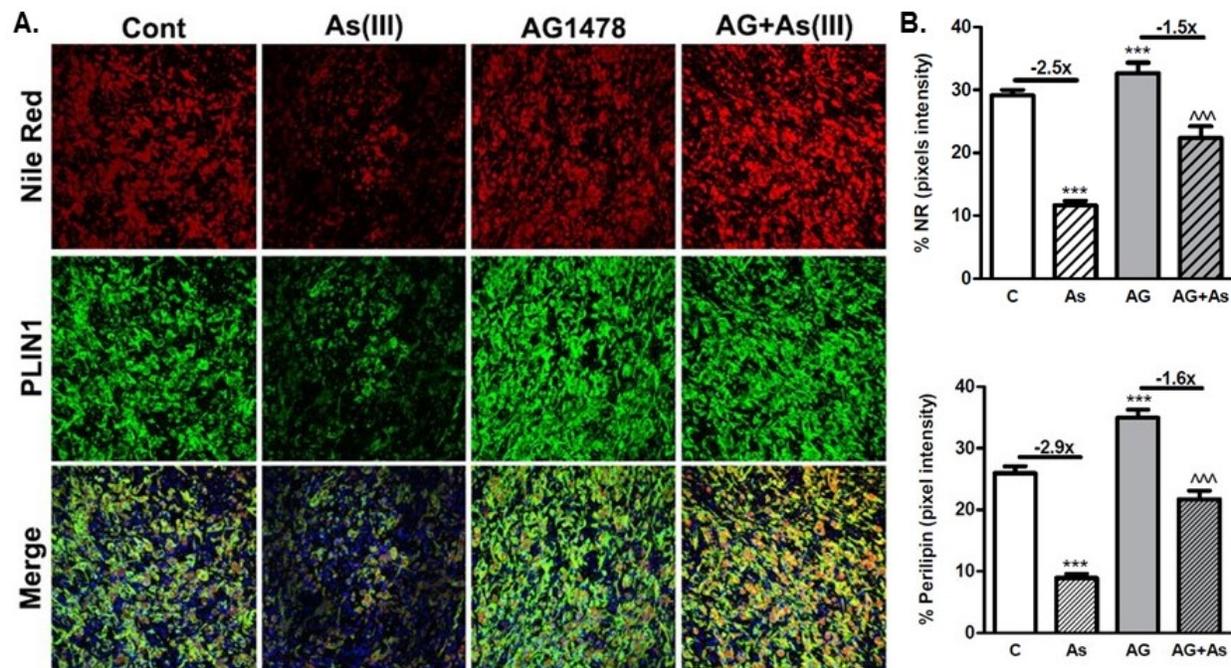


Figure 23 EGFR-inhibition attenuates As(III)-loss of fat droplets.

MSC-derived adipocytes were grown and differentiated in coverslips for 12 days. On day 9, cells were pretreated with 1 μ M AG1478 (a EGFR selective inhibitor), before arsenic exposure. Inhibitors and arsenic solution was incubated for 72 hr before cell fixation. Cells were paraformaldehyde- fixed and stained for Nile Red (Red), Perilipin (Green) and DAPI(blue). **A.** Images are representative of four field per coverslips (n=4 per group) taken at 40x in a Confocal Microscope (scale bars = 50 μ m) and then thresholded fluorescence quantified and averaged. **B** The graphs represent mean \pm SEM percentage of positive pixels normalized to DAPI staining. Data were analyzed by ANOVA and Newman-Keuls post-test for differences between groups (* p< 0.05 and *** p<0.001 from control).

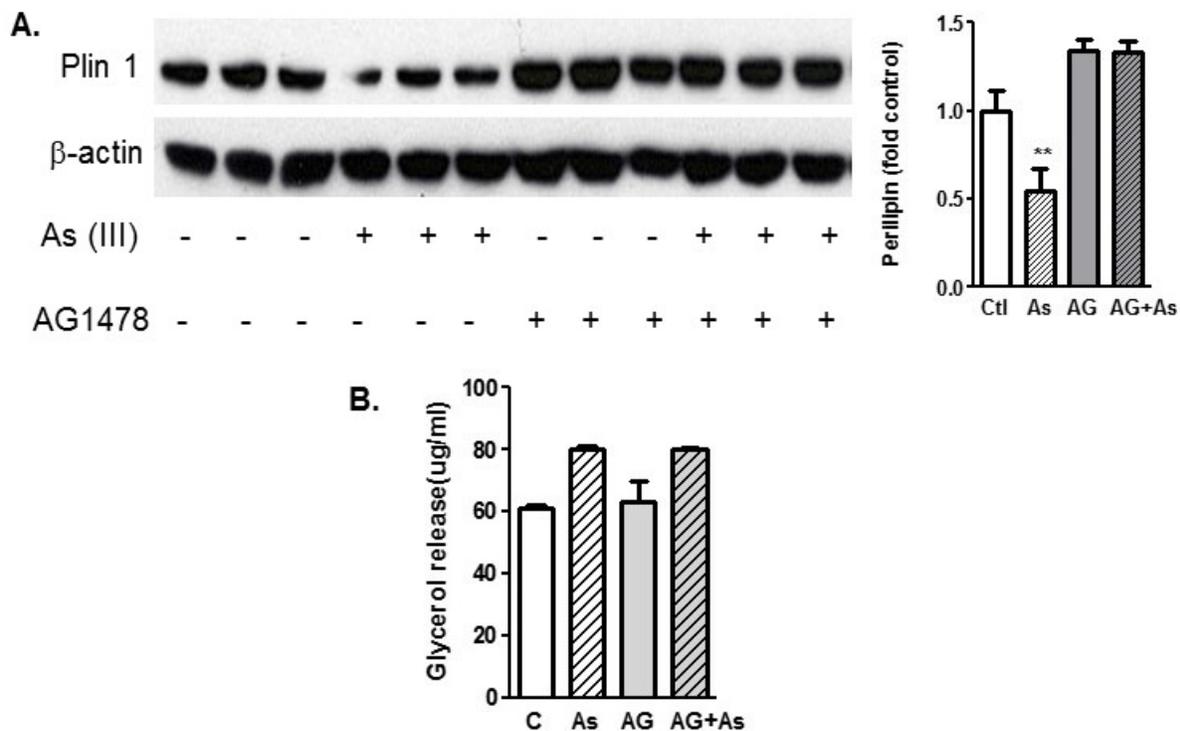


Figure 24 As(III)-stimulated loss of lipid droplets is attenuated after AG1478 treatment.

MSC were grown and differentiated for 9 days before pretreatment with AG1478 and then As(III). Cells were collected at day 12 post-differentiated and equal amounts of protein were resolved by SDS –PAGE and immunoblotted as described in Material and Methods. The graph presents mean \pm SEM of PLIN1 normalized to β -actin. Data were analyzed by ANOVA and Newman-Keuls post-test for differences between groups (* $p < 0.05$ and *** $p < 0.001$ from control)

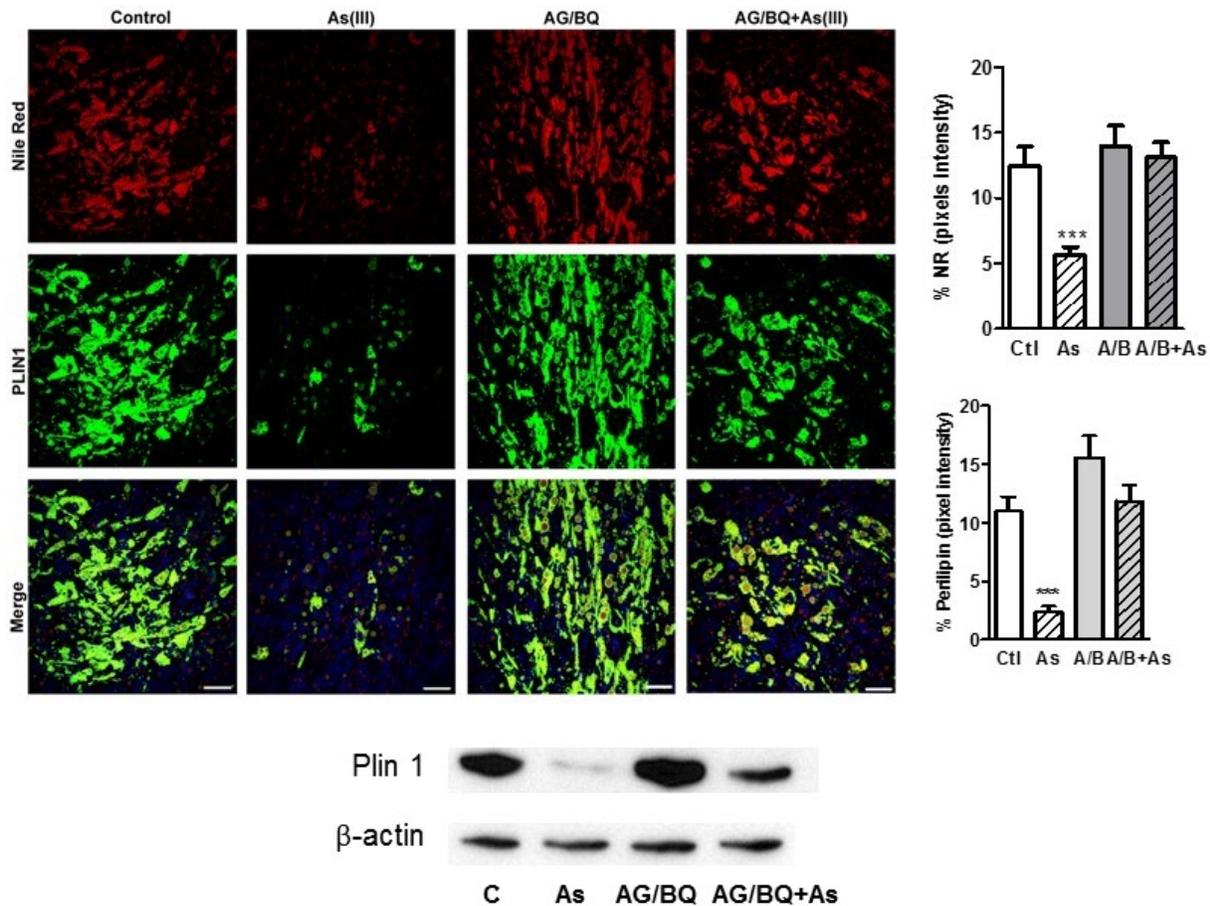


Figure 25 EGFR and EDNRA mediate arsenic-induced lipotoxicity.

MSC-derived adipocytes were grown and differentiated for 12 days. At day 9 post differentiation, cells were pretreated with both 1 μ M of EGFR tyrosine kinase inhibitor AG1478 and the EDNRA BQ610 before exposure to 1 μ M arsenic until day 12. A. Adipocytes were paraformaldehyde-fixed, and stained for PLIN1 (green), Nile Red (red) and nuclei (DAPI blue). Images were capture at 40X (scale bar =50 μ m) and are representative of four coverslips per group and four field per coverslip. Images were quantified for thresholded fluorescence and averaged. The graphs represent mean \pm SEM percentage of positive pixels normalized to DAPI staining. Data were analyzed by ANOVA and Newman-Keuls post-test for differences between groups (* $p < 0.05$ and *** $p < 0.001$ from control).

Arsenic activates EGFR signaling in adipocytes.

It is well established that arsenic maintains cell proliferation in keratinocytes and other cancerous cells by activating EGFR and downstream effectors, such as like ERK 1/ 2 and Cyclin D1 (Patterson et al., 2007), however activation of ERK1/2 following arsenic exposure in adipocytes has not been explored. To assess whether arsenic signals through EGFR to activate Erk, total protein extracts from adipocytes pretreated with AG1478 prior to arsenic exposure, were probed by Western analysis for phosphorylated ERK relative to total enzyme. Results demonstrated that adipocytes exposed to arsenic have an increased in phospho/total ERK, which was attenuated by AG1478. These findings suggest that arsenic stimulates phosphorylation of ERK via EGFR as a mechanism in the loss of lipid droplets (Fig 26). Of note, the total levels of total ERK protein were slightly increased following arsenic exposure, and decreased in cells pretreated with AG1478. The increased levels of ERK after arsenic were previously reported in Beas -2B human bronchial epithelial cells (Andrew et al.,2009).

Activation of Src or oxidant generation are not involved in arsenic-stimulated loss of fat droplets

Src activation was previously been shown to activate EGFR and ERK signaling cascades. To evaluate whether Src family was implicated in arsenic stimulation of EGFR that inhibits adipocyte function, adipocytes were pretreated with PP2, a selective Src family kinase inhibitor, prior to adding arsenic and impacts on PLIN1 levels were evaluated after 72 h. The results showed that inhibition of Src did not attenuate arsenic-stimulated decreases in PLIN1 expression (Fig 27 A). Alternatively, adipocytes were preincubated with Tempol, a superoxide scavenger, before arsenic treatment to evaluate whether oxidative stress was involved in arsenic signaling for coat protein loss. As in seen in Fig 27B, Tempol also failed to prevent the loss of PLIN1

expression in arsenic-exposed adipocytes. The gp91 ds-tat an inhibitory peptide of Nox2 as well as Nox1A inhibitor were used to evaluate whether arsenic stimulates these proteins to activate EGFR. Arsenic stimulates Nox-based oxidase activity in vascular cells to induce remodeling of liver sinusoidal endothelial cells (Straub et al.,2009). However in adipocytes, the results shown that arsenic did not stimulated these proteins to mediate EGFR activation (Fig 28).

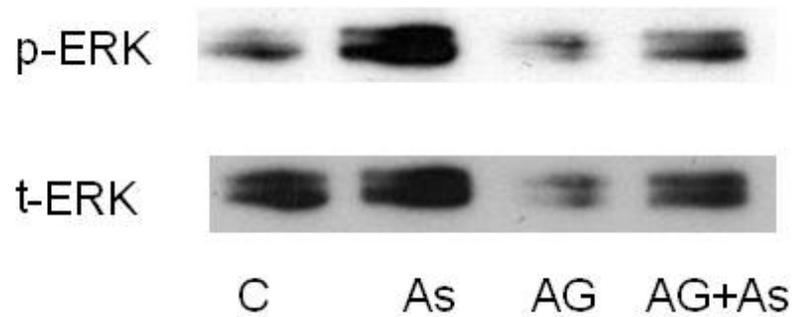


Figure 26 Arsenic-induced activation of ERK is prevented by inhibition of EGFR.

MSC derived adipocytes were grown and differentiated for 9 days, pretreated with 1 μ M AG1478 30 minutes before 1 μ M As(III). Cells were incubated to day 12 post-differentiation and collected for total protein. Samples were resolved by SDS-PAGE and blotted for phosphorylated ERK and total ERK. Blot is representative of two independent experiments.

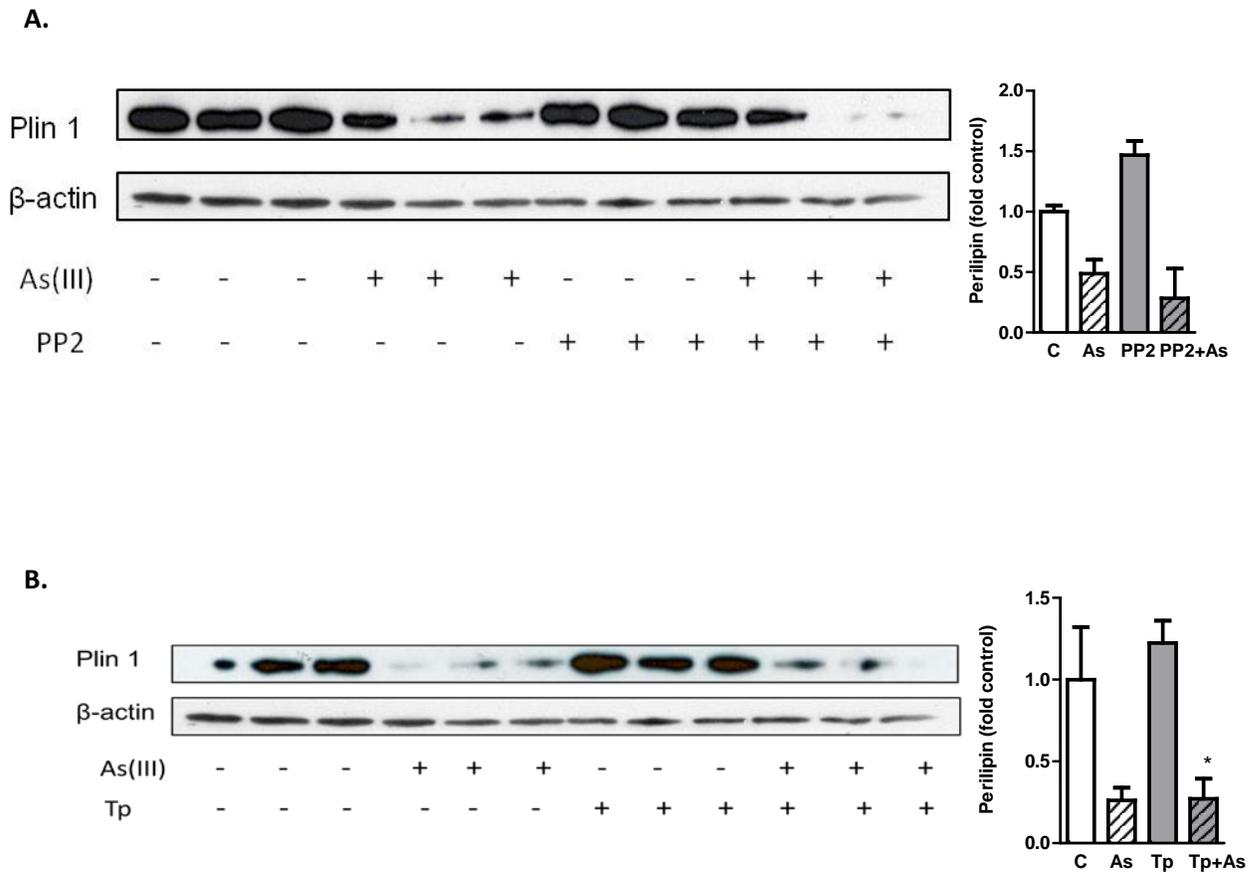


Figure 27 Arsenic –induced activation of EGFR does not involve Src family kinases or generation of reactive oxygen species.

MSC-derived adipocytes differentiated for 9 days were pretreated with **A)** 1 μ M PP2 and **B)** 1 μ M Tempol for 30 min before arsenic as described in Materials and Methods. Cells were harvested at day 12 post-differentiation and probed for Plin1 and β actin. The graphs represent mean \pm SEM of fold band densities relative to control for Perilipin immunoblots normalized to β -actin.

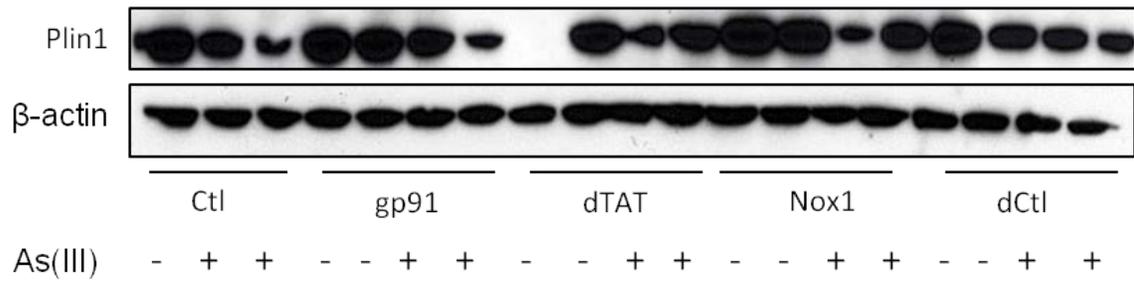


Figure 28 Activation of EGFR is not mediated by Nox proteins.

MSC-derived adipocytes differentiated for 9 days were pretreated with gp91 or Nox1 inhibitors for 30 min before arsenic as described in Materials and Methods. Cells were harvested at day 12 post-differentiation and probed for Plin1 and β actin.

Arsenic stimulates *EGFR interaction with Gi*.

To investigate the potential involvement of $G_{\alpha i}$ in arsenic activation of EGFR in adipocytes, cells were treated with arsenic for 4 or 24 h or the EGFR ligand, EGF(10 nM) for 20 min. and then extracted for protein. The protein extracts were immunoprecipitated with antibody against EGFR and proteins complexes were separated by electrophoresis and probed for $G_{\alpha i}$ followed by Western Blot analysis. As seen in Fig 29, there was an increase in $G_{\alpha i}$ associating with EGFR after arsenic exposure at both 4 h or 24 h. It is important to note that EGF stimulation of EGFR causes rapid degradation of the receptor and that arsenic has been shown to inhibit this degradation (Andrew et al.,2009). Thus the amount of EGFR varies with treatment and there is a possible increased abundance in arsenic treatments of 4 h and possible loss at 24 hours. The amount of EGFR in the immunoprecipitates of EGF stimulated cells was below the Western detection, but substantial amounts of G_i were present indicating a very large increase relative to the arsenic-induced increase.

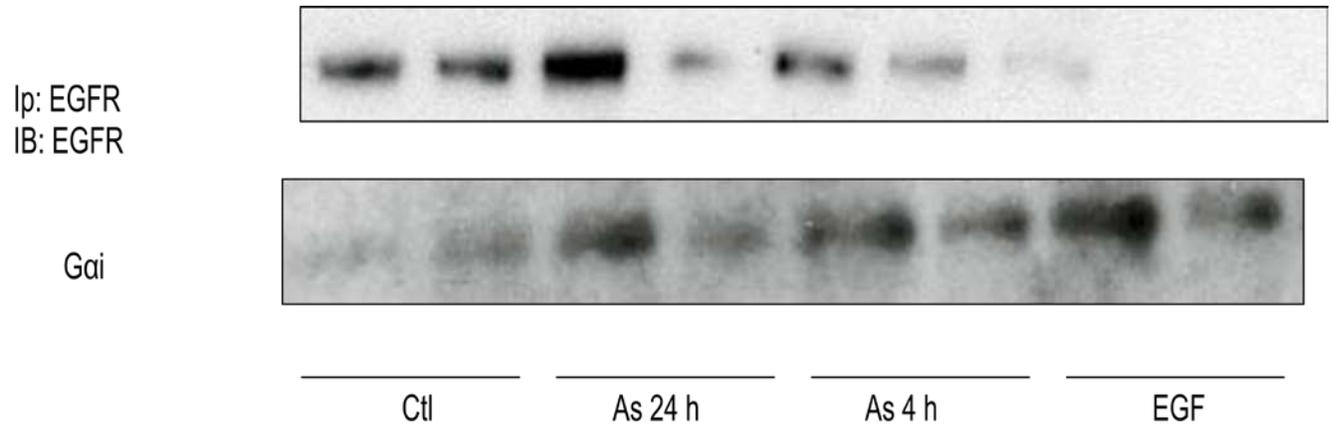


Figure 29 Gαi association with EGFR is increased by arsenic.

hMSC derived-adipocytes grown at day 12 post-differentiation were treated for 1 or 4 h with 1 μ M As(III) or 30 minutes with 10 nM EGF before harvesting. Equal amounts of total protein extracts were immunoprecipitated with antibody against EGFR. Immunoprecipitated complexes were separated by electrophoresis and probed for Gαi or EGFR content.

5.4 DISCUSSION

EGF and EGFR signaling have controversial roles in adipogenesis and regulation of adipocyte functions. While EGF appears to inhibit differentiation in preadipocytes, other studies suggested that it enhances differentiation in adipose cells already committed (Serreno et al., 1987). Our studies demonstrated for the first time that adipocyte EGFR is activated indirectly by arsenic, independent of the receptor ligand EGF. Although other studies have demonstrated that EGFR can be transactivated by certain physical or chemical stimuli, including arsenic, none of them address the question in adipocytes. Here we showed that EGFR contributes to arsenic-stimulated loss of fat droplets in MSC-derived adipocytes by non-canonical activation. We demonstrated in a model of MSC-derived adipocytes the determinant role of GPCR activation in arsenic-mediated loss of lipid droplets and activation of lipolysis. We identified ET-1 receptor A (EDNRA) as the principal receptor activated by arsenic (Garciafigueroa et al., 2013). However, it remains possible that additional GPCR are implicated since inhibition of EDNRA did not completely prevent the effects of arsenic. As shown in Fig 25, the combined inhibition of EDNRA and EGFR prevented arsenic-induced lipotoxicity. In agreement with our findings, a study in 3T3 L1 cells demonstrated that co-treatment with EGF and ET-1 caused an anti – adipogenic response, probably by repressing of C/EBP α and PPAR γ expression (Bhattachaya et al., 2006). The inhibitory effect also produced attenuation of Akt phosphorylation and increased ERK1/2 phosphorylation (Bhattachaya et al., 2006).

The concept of RTK transactivation has been extensively studied. It has been recognized that the process of transactivation can be directional between RTKs and GPCRs. In the case of GPCR transactivation, the RTK uses a G-protein to induce activation of signaling pathways. This

process, also known as RTK hijacking, is different from RTK transactivation, in which the G protein functions upstream of the RTK (Pyne and Pyne, 2011). GPCR transactivation may result from two distinct mechanisms depending on the nature of the GPCR-RTK partner. The first mechanism implies that GPCR transactivation results from synthesis and secretion of a cognate ligand of the transactivated GPCR. In the second mechanism, the transactivation of GPCR by RTK occurs in a ligand independent manner, and involves the formation of GPCR-RTK complexes and sometimes phosphorylation of the transactivated GPCR (Delcourt et al.,2007). Our results indicate that arsenic induces a G α i-EGFR complex (Fig 29). The findings suggest that the mechanism underlying arsenic-stimulated loss of fat droplets is by GPCR transactivation by EGFR in a ligand independent manner rather than RTK transactivation. Our conclusion is based in the facts that: 1) Src family or oxidative stress did not activate EGFR in response to arsenic (Fig 27, 28), even if previous studies demonstrated that c-Src activity was necessary for arsenic-induced EGFR and ERK activation (Simeonova et al.,2002). 2) EGFR activation does not involve the autophosphorylation sites Tyr¹¹⁷³ and Tyr⁸⁴⁵ (data not shown); although there was increased expression of total EGFR. 3) The results obtain in Fig 25 strongly suggested that there is a cooperative mechanism between ENDRA-EGFR in arsenic-stimulated loss of lipid droplets, that was inhibited by selective receptor inhibitors.

In summary, this data suggested a new cooperative mechanism through which arsenic induced dysfunctional lipid metabolism through activation of GPCR-RTK partners. Nevertheless, the mechanism remain unclear, therefore more characterization of this cross-talk will be necessary.

6.0 CONCLUSIONS

The last decades of obesity research have produced a better understanding of adipose tissue function and its role in pathophysiology. The concept of AT as a mere energy-storage organ has totally changed to the identification of AT as an active endocrine tissue (Frühbeck et al., 2004). The endocrine activities of AT include regulation of body weight homeostasis, glucose and lipid metabolism, reproduction, immunity, blood pressure control, fibrinolysis, coagulation, and angiogenesis. Despite and improved awareness of the important role in homeostasis, the role of environmental influences on the pathogenic disruption of this role in producing metabolic related diseases are underappreciated. More specifically, the work presented in this dissertation research advances the mechanistic understanding of the pathogenic mechanisms of arsenic-promoted disease and the signaling pathways stimulated to disrupt AT maintenance and function.

6.1 ARSENIC EFFECTS IN ADIPOSE TISSUE *IN VIVO*

Fat expansion is considered as highly deleterious with deregulation of glucose and lipid metabolism, leading to increased cardiovascular diseases. Loss of fat pads due to genetic or acquired causes proved to be also a risk for CVD and metabolic function (Vigouroux et al., 2011). Environmental toxicants such as inorganic arsenic are highly correlated with increased risk for metabolic disease and CVD. Epidemiological studies indicate the diabetogenic role of arsenic, even though its implications are still in debate (Diaz –Villaseñor et al., 2007).

Although the role of arsenic in DM is controversial, it is known that it alters ISGU (Paul et al., 2007) and differentiation of stem cell to adipocytes (Klei et al., 2013), suggesting that arsenic interferes with maturation and function of AT. The data presented in Chapter 3 demonstrated that an environmental exposure to arsenic disrupted insulin sensitivity and altered proper lipid storage *in vivo*. The insulin and lipid dysfunction observed here were accompanied by phenotypic changes WAT and BAT in both male and female mice. However, in females there was more evident remodeling of WAT to brown-like adipocytes, characterized by multilocular cells and high levels of UCP-1 protein. In male WAT, remodeling of adipocytes was also present and there was induction of angiogenesis program. The results obtained here sustain the observation of arsenic-impairment of insulin, and that arsenic produces changes in the structure of AT. Moreover, the information obtained from vascular protein analysis confirmed that during remodeling, AT requires growth of its vascular network. Most literature reports indicate that during AT expansion the process of angiogenesis is very active, to provide oxygen supply, delivery and removal of nutrients, and for transit of cells (Corvera and Gealekman.,2013). Here we demonstrated that an environmental factor, such as arsenic, induced the angiogenic program and increase the size of adipocytes.

The studies not only demonstrated that arsenic produce structural changes in AT, but also functional changes. First, the levels of perilipin, the main protein-coating lipid droplets, were decreased in arsenic-treated mice. *In vivo* and *in vitro* studies indicate that reduced levels or absence of perilipin results in increased basal lipolysis, since perilipin functions as a barrier between stored neutral lipids and lipases (Miyoshi et al., 2006). Second, the loss of TAG was accompanied by redistribution of fat droplets in ectopic tissues like skeletal muscle, which indicated that WAT was not able to maintain proper lipid and energy storage. Third, the results

in Chapter 4 (Fig 13), confirmed that loss of perilipin by arsenic increased basal lipolysis. In sum, the capacity of AT to store TAG was impaired after moderate arsenic exposure *in vivo*. These findings expand the knowledge of current mechanisms that explain metabolic diseases and propose an alternative mechanism beside obesity by which metabolic diseases are affecting a great portion of the population.

6.2 ARSENIC-STIMULATED LIPOLYSIS AND ADIPOSE REMODELING IS MEDIATED BY G-PROTEIN COUPLED RECEPTORS.

Arsenic inhibits the differentiation of adipocytes. In previous studies, Klei et al., 2013 demonstrated that low doses of arsenic were able to inhibit the differentiation of hMSCs into mature adipocytes. MSCs could proliferate and differentiate into specific functional cell types, including osteocytes, adipocytes, and chondrocytes (Cheng et al., 2011). Under specific cocktail of growth factors and agonist, MSC are able to differentiate within 12-18 days into mature adipocytes. When low dose –non cytotoxic dose of arsenic (1 μ M) was present at the induction or during the early stages of differentiation, MSC were unable to differentiate and form lipid droplets. The results in this study indicated that arsenic produced a decreased transcriptional promoter of differentiation (PPAR γ and C/EBP α) and increased transcriptional repressors. It was proposed that a portion of deleterious impact of arsenic on adipogenic program was mediated through activation of a GPCR, particularly endothelin-1 receptors (Klei et al., 2013).

To further elucidate the effect of arsenic in cultured cells, a model of MSC- derived adipocytes was used. Here, MSC were differentiated into adipocytes. Once the cells entered the late phase of differentiation (and lipid droplets were visible), they were treated with a low dose of arsenic. The

results obtained in Chapter 4 indicated that arsenic impaired lipid metabolism and functionality in cells that are already committed to generate adipocytes. This inhibition appeared to be mediated by a similar GPCR-mediated mechanism as implicated in inhibition of differentiation. The results obtained in Chapter 3 strongly suggested that arsenic affected proper storage of lipids by loss of fat droplets in WAT of mice exposed through drinking water, but exporting them to non-adipose tissue. In Chapter 4, we confirmed in cultured adipocytes this effect of arsenic. Adipocytes exposed to non-cytotoxic dose of arsenic between 24 to 72 hours, showed a time dependent loss of fat droplets. By 72 h of arsenic exposure, the amount of lipid droplets measured by protein expression of PLIN1 (Western Blot) and immunostaining (Neutral lipids and protein-coated lipid droplet) decreased substantially. In the same way, the transcript levels of adipogenic factors, Plin1 and Adipo Q were significantly decreased after 72h. AdipoQ is a protein specifically secreted from adipose tissue. This adipokines had proved to have antidiabetogenic and antiatherogenic effects. Indeed, a decrease in the circulating levels of adiponectin by genetic and environmental factors has been shown to contribute to the development of diabetes and metabolic syndrome (Kadowaki et al., 2006). Therefore, the effect of arsenic on AdipoQ by itself would contribute to development of metabolic syndrome, since adipokines affect a large number of other tissues, such as the liver, the brain, the reproductive system, pancreatic β -cells and the vasculature (Scherer, 2006). As mentioned above, basal lipolysis activity was stimulated after arsenic treatment, which indicated dysfunctional storage of TAG. Lipogenesis is a property of mature, differentiated adipocytes. Mature adipocytes have massive TAG storage capacity compared to any other tissue. Conversely, a decrease of adipocyte differentiation, which is associated with reduction of the lipid droplet volume, will transiently be accompanied by a net increase in lipolysis (Wang et al., 2008). Similarly, the reduction of lipid

droplets observed after arsenic treatment (Fig 14 and 15) was accompanied with increased lipolysis activity. Of notice, the stimulation of lipolysis occurs at 24 hr and was sustained within 3 days, while the changes in perilipin expression took at least 48h. These results clearly indicated that mature adipocyte function was affected by arsenic.

Previous reports that investigated the mechanism of action of arsenic indicated that it mostly acts through a Ptx-inhibited mechanism. In liver endothelial cells, arsenic requires the $G_{i/o}$ linked S1PR1 to induce oxidant signaling for endothelial cell capillarization and vascular remodeling (Straub et al., 2009). In MSCs, Klei et al., (2013) demonstrated that arsenic inhibited differentiation in a Ptx-sensitive manner and that a GPCR was involved. Therefore to test the hypothesis that arsenic stimulated aberrant lipid storage was through a GPCR, adipocytes were pretreated with Ptx before cells were exposed to arsenic. The result obtained here confirmed that Ptx totally abolish the effect of arsenic in adipocytes (Fig 16 and 17), indicating that arsenic alters lipid metabolism by a GPCR. Further investigation of the mechanism of arsenic-activation of GPCR in adipocytes, showed that endothelin-1 receptors (ETRs) were implicated in loss of fat droplets. Endothelin A receptor (EDNRA) inhibition produced a partially prevention of lipid loss. These results were evaluated by expression of Plin1 and quantification of positive staining in adipocytes treated with BQ610, a selective inhibitor of EDNRA. Although EDNRA plays a great role in arsenic-stimulation of dysfunctional lipid metabolism, endothelin receptor B (EDNRB) also moderate arsenic effects. The inhibition of EDNRB blocked lipolysis activity rather than protecting perilipin expression (Fig 20 B and C). These differences in activities of the endothelin receptors have been address in the past. For instance, Eriksson et al., (2009) showed in human adipocytes isolated from visceral fat that EDNRA mediated the effect of ET-1 on lipolysis. Cheng et al.,(2006), also found that ET-1 caused lipolysis in rat adipocytes through

stimulation of EDNRA and activated the ERK pathway. In contrast, other studies showed that ET-1 impaired insulin signaling and stimulated lipolysis in human adipocytes by stimulation of EDNRB. This effect was noted only in visceral fat, but not in subcutaneous adipocytes. Although mainly expressed in vascular tissue, the EDNRA and EDNRB are also expressed in variety of other tissues. ET-1 impairs ISGU and suppress the expression of the insulin receptor, and insulin receptor substrate-1 in rat adipocytes (Juan et al.,2006). ET-1 directly modulates lipolysis and may contribute to the elevated FA levels in obesity patients with insulin resistance and hypertension (Chien et al., 2011). In summary, these results are the first to demonstrate that dysfunctional lipid storage and metabolism induced by arsenic are mediated at least by one GPCR, the EDNRA in adipocytes. Involvement of ETRs in lipolysis and insulin metabolism has been address before, however in this study was demonstrated that the activation of ETRs was not mediated by its ligand ET-1. In contrast, ETRs were stimulated by low dose of arsenic in human adipocytes.

6.3 RECEPTORS CROSS TALK IN ARSENIC-LOSS OF FAT DROPLETS.

Signal transduction initiated by both GPCR and receptor tyrosine kinases (RTKs) is mediated by specific kinase-dependent cascades. GPCR and RTKs can activate a common set of signaling molecules and they do not operate in an isolated fashion (Delcourt et al.,2007). Mitogenic GPCRs have the ability to trigger autophosphorylation of growth factor RTKs, a phenomenon referred to as RTK transactivation. For instance, the type 1 angiotensin receptor can transactivate the receptors for PDGF, EGF, and IGF-1 (Elbaz et al., 2000). In Chapter 4, it was demonstrated that arsenic acts through activation of EDNRA to impair lipid storage on adipocytes. However it

was noticed that this GPCR was not acting alone, in contrast other receptors might be involved in arsenic effect.

EGFR is a member of a family of four structurally similar RTKs. EGFR are expressed in a variety of tissues of epithelial, mesenchymal, and neuronal origin where they play fundamental roles in development, proliferation, and differentiation (Singh and Harris, 2005). Numerous studies have demonstrated that arsenic activates this RTK in different cell lineages. Based on this data, it was hypothesized that EGFR can be the RTK that is activated by arsenic, to mediate the loss of fat droplets found in Chapter 3 and 4. Moreover, it was hypothesized that EGFR cross-talk with EDNRA to disrupt lipid storage in adipocytes. In Chapter 5, the participation of EGFR in arsenic-stimulated loss of fat droplets was confirmed. The data also suggested that arsenic activated an EGFR-EDNRA interaction. The selective inhibition of EGFR with AG1478 before arsenic treatment shown in Fig 23 indicated that when this RTK was inactive the amount of lipid droplets were similar to the control. These results were evaluated by both expression of perilipin and quantification of positive perilipin and neutral lipid staining. Then, when EGFR and EDNRA were inhibited with AG1478 and BQ610 respectively, the amount of lipid droplets after arsenic exposure was unaffected. This effect was similar to that found with Ptx suggesting that inhibition of Gi proteins helps to prevent the lipotoxic effects of arsenic in adipocytes.

One of the main downstream signaling proteins activated by EGFR is ERK. This ERK activation regulates cell proliferation, survival, differentiation, migration among others (Makki et al., 2013). It was shown that signaling through MAPKs, ERK1/2 especially, in response to GPCR agonists can be mediated through transactivation of the EGFR. The analysis of ERK expression in adipocytes pretreated with EGFR inhibitor before arsenic exposure indicated that EGFR stimulation in turn activates ERK signaling cascade (Fig 26). However the mechanism of

arsenic stimulation of EGFR and consequently activation of ERK remain unclear. EGFR can be transactivated via Src family kinase activation, as well as oxidant stress (Filosto et al., 2011). Nevertheless the studies performed with selective inhibition of Src (PP2) or oxidant species (Tempol, Nox1 inhibitor, or Nox 2 inhibitor) did not prevent the loss of droplets stimulated by arsenic. This indicated that neither the Src family kinases nor oxidant stress is implicated in activation of EGFR. However, in Chapter 5 it was demonstrated that after arsenic exposure, there is a transient association of Gai protein- EGFR that disappears over the time. This finding indicates that Gai protein serves as a platform to activate EGFR and most likely activates in turn EDNRA. This reverse transactivation phenomenon, known as GPCR high jacking, has been reported recently. In this scenario, RTK uses a G protein to induce activation of signaling pathways. In RTK transactivation a G-protein functions upstream of the RTK, however here, RTK transactivate the GPCR (Pyne and Pyne., 2011). These novel results potentially reveal a new cooperative mechanism through which arsenic stimulates cross talk between a GPCR and RTK to produce dysfunction and aberrant storage of lipids in adipocytes. However, more extensive characterization of this interaction and proof that it is essential in the etiology of arsenic-promoted AT dysfunction *in vivo* will be required to confirm the significance of the interaction in arsenic-induced metabolic disease.

In conclusion, this dissertation demonstrated mechanisms for arsenic-stimulated loss of lipid droplets *in vivo* and *in vitro* and neovascularization and remodeling of adipose tissue, as well as redistribution of TAG to ectopic tissues. Moreover it was demonstrated for the first time that arsenic induced lipotoxicity through stimulation of cross talk between EGFR-EDNRA signaling. The studies contribute to understanding pathogenic mechanisms in metabolic diseases like DM, atherosclerosis, and obesity. In addition, they advance the understanding the pathophysiology of

these diseases following environmental exposures, and elucidate alternative etiologies for arsenic-promoted disease. Confirmation of the important impact of this disease promotion in the large population of individuals exposed to arsenic will improved public health by indentifying novel strategies to prevent or treat these environmentally-derived diseases.

6.4. SUMMARY AND FUTURE DIRECTIONS

In summary, this dissertation research found that low levels of arsenic stimulated aberrant lipid storage characterized with loss of lipid droplets, ectopic redistribution of fat in tissues, and activation of basal lipolysis. *In vivo* studies also demonstrated that loss of TAG was accompanied by increased level of circulating insulin, neovascularization and remodeling of adipose tissue. The mechanism underlying arsenic lipotoxicity appeared to be mediated by activation of GPCR-RTK partners: EDNRA-EGFR. Elucidating this mechanism contributed to the understanding of the pathophysiology of environmentally-metabolic diseases.

Future directions of this work should explore the signaling mechanisms underlying the proposed receptor cross-talk and further characterize the pathogenic signal pathways activated downstream of the receptor interaction. In addition, future work should address the question of how arsenic activates either of these receptors. It is know that GPCR signal capacity is fine-tuned by post-translational modification. Palmitoylation of conserved cysteine residues in the tail region of many GPCR is a target for modulation of functional activity (Cramer et al., 2001). These regulatory cysteines may be direct targets of arsenic or arsenic may alter may alter the reversible palmytoylation or other modification (e.g. oxidation or nitrosation) of these cysteines critical to

allosterically affect receptor activity, interacting G proteins or internalizing chaperones. These possible mechanisms will be explore

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