

**ADIPOCYTE PROTEIN CARBOXYLATION AND OXIDATIVE STRESS IN
OBESITY-LINKED MITOCHONDRIAL DYSFUNCTION AND INSULIN
RESISTANCE**

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JESSICA MARIE CURTIS

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DR. DAVID A BERNLOHR, ADVISOR

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DEDICATION

This thesis is dedicated to the people I love and who love me, namely my family and friends that have supported me throughout this journey.

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ABSTRACT

Carbonylation is the covalent, non-reversible modification of the side chains of cysteine, histidine and lysine residues by lipid peroxidation end products such as 4-hydroxy- and 4-oxononanal. The antioxidant enzyme glutathione S-transferase A4 (GSTA4) catalyzes a major detoxification pathway for such reactive lipids but its expression was selectively down regulated in the obese, insulin resistant adipocyte resulting in increased protein carbonylation. The effects of such modifications are associated with increased oxidative stress and metabolic dysregulation centered on mitochondrial energy metabolism. Mitochondrial functions in adipocytes of lean or obese GSTA4 null mice were significantly compromised compared to wild type controls and were accompanied by an increase in superoxide anion. Silencing GSTA4 mRNA in cultured adipocytes resulted in increased protein carbonylation, increased mitochondrial ROS, dysfunctional state 3 respiration and altered glucose transport and lipolysis.

To address the role of protein carbonylation in the pathogenesis of mitochondrial dysfunction quantitative proteomics was employed to identify specific targets of carbonylation in GSTA4-silenced or overexpressing 3T3-L1 adipocytes. GSTA4-silenced adipocytes displayed elevated carbonylation of several key mitochondrial proteins including the phosphate carrier protein, NADH dehydrogenase 1 alpha subcomplexes 2 and 3, translocase of inner mitochondrial membrane 50, and valyl-tRNA synthetase. Elevated protein carbonylation is accompanied by diminished complex I activity, impaired respiration, increased superoxide production and a reduction in membrane potential without changes in mitochondrial number, area or density. These results suggest protein carbonylation plays a major instigating role in mitochondrial dysfunction and may be a linked to the development of insulin resistance in the adipocyte.

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CHAPTER ONE

Adipocyte Metabolism, Insulin Resistance & Mitochondrial Function

Jessica Curtis wrote this chapter in its entirety.

I. Metabolic Syndrome and Insulin Action in Obesity-linked Type II Diabetes

Excess food consumption and physical inactivity inevitably lead to weight gain and in extreme cases, obesity. Increased adiposity associated with poor nutritional choices in combination with genetic predisposition triggers the development of insulin resistance in peripheral tissues, such as adipose, liver and muscle. This pre-diabetic state leads to hyperglycemia, as these tissues fail to translate insulin's signal to uptake glucose from the blood and suppress hepatic glucose output. In response to high blood glucose, pancreatic β -cells produce and secrete more insulin. If β -cells fail to compensate for increased demand, organs are exposed to toxic levels of glucose, referred to as glucotoxicity. This is a destructive environment for many tissues, including β -cells, altering functionality, proliferation and apoptosis. Significant reductions of insulin secretion caused by the destruction of β -cells marks the transition from insulin resistance to overt Type 2 Diabetes (T2D) (1,2). Over 90% of type 2 diabetics are overweight or obese, indicating excess weight is a risk factor for the development of insulin resistance and T2D (3). Insulin resistant individuals also face an increased risk of heart disease and stroke. This cluster of diseases is collectively referred to as metabolic syndrome (4). Weight loss does increase insulin sensitivity (5,6), but unfortunately, lifestyle modifications have proven difficult for many overweight insulin resistant individuals. Therefore, understanding the biochemical mechanisms behind insulin resistance and T2D is an important step in developing therapeutic intervention strategies.

Insulin action varies slightly in each insulin responsive tissue but generically acts to promote glucose uptake and inhibit energy-distributing processes such as lipolysis and gluconeogenesis (As reviewed in (7)). Insulin is produced and secreted by pancreatic β -cells in response to postprandial elevation of blood glucose. When insulin binds to the insulin receptor, it initiates a signaling cascade responsible for changes in protein synthesis and glucose uptake. Insulin stimulates the inducible glucose transporter (GLUT4) to translocate to the plasma membrane, facilitating glucose uptake into fat and muscle cells for oxidation and storage. In the liver, insulin is not required for glucose uptake, and its primary signaling function is to inhibit gluconeogenesis and stimulate synthesis of glycogen, a branched chain polymer used to store glucose. Upon glycogen saturation (~5% liver mass), the liver converts glucose into fatty acids for export as lipoproteins. These lipoproteins circulate and are taken up predominately by the adipocyte to be stored as triglyceride. Insulin action in the adipocyte is also responsible for inhibition of lipolysis, the process whereby free fatty acids (FFAs) are liberated from the triacylglyceride droplet for use as an energy source by the liver, muscle, pancreas, and brain during glucose deprivation (8,9). Insulin resistance is characterized by loss of these responses, resulting in high blood glucose and fatty acid concentrations.

Development of insulin resistance and T2D is the result of metabolic dysfunction in multiple tissues, and it remains controversial which tissue is responsible for the initial defect in insulin signaling. According to the adipocentric theory, adipose tissue insulin resistance is the first phase of T2D (10). Some studies suggest that muscle is responsible for ~80% of glucose disposal (11,12), however mice without the insulin receptor in the muscle maintain insulin sensitivity (12), whereas mice lacking the insulin-responsive glucose transporter GLUT4 specifically in the adipocyte develop systemic insulin resistance (13). Therefore, while adipocyte clearance of blood glucose may be relatively low, it is still essential to maintain whole body insulin sensitivity.

As an adipocyte becomes insulin resistant, often consequent of a high fat diet, it fails to clear glucose from the blood and without insulin's anti-lipolytic signal continues to efflux FFAs (Figure 1). This leaves other tissues exposed to high concentrations of glucose and FFAs. The muscle and liver are infiltrated with FFAs, and the metabolic pathways responsible for processing FFAs (β -oxidation and lipoprotein synthesis) are overwhelmed, leading to ectopic intracellular storage of lipids in myocytes and hepatocytes. The increase in lipids is followed by increases of lipid metabolites, such as long chain acyl-CoA, diacylglycerol (DAG) and ceramides, which interfere with insulin signaling and causes insulin resistance in the muscle and liver (14). Increased FFA delivery and defective

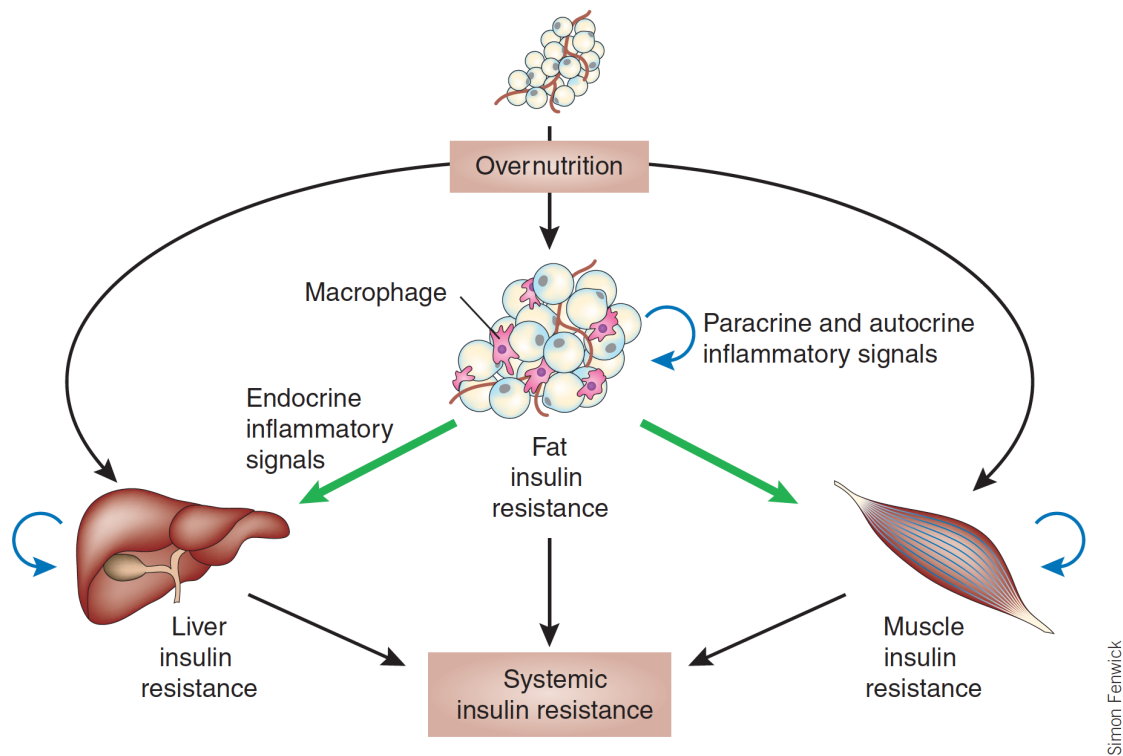


Figure 1. Adipocentric view of the development of obesity-linked insulin resistance. Accumulation of excess lipid in the adipocyte as a consequence of overnutrition leads to inflammation in the adipose tissue, signaling for recruitment and activation of resident adipose tissue macrophages, which propagate the proinflammatory environment and contribute to local insulin resistance. Cytokine and fatty acid secretion from the adipose tissue act in paracrine and autocrine manner to influence the insulin sensitivity of other insulin responsive tissues such as muscle and liver.

Image courtesy of Nature Medicine © 2006 (15).

fatty acid metabolism are collectively referred to as lipotoxicity, which causes the upregulation of inflammatory signaling and promotes a diabetogenic environment.

Lipotoxicity, inflammation and adipokines have a large role in the development of insulin resistance in adipocytes and peripheral tissues. Cytokines (peptide hormones) secreted by the adipocytes are referred to as adipokines. These molecules may act in endo-, para- or auto-crine manner to signal to other cells in the body (16,17). The antidiabetogenic adipokines adiponectin and leptin are produced exclusively by the adipocyte, whereas other cytokines, such as tumor necrosis factor (TNF)- α , may be produced by other cell types, such as resident adipose tissue macrophages. Leptin signals to the brain to suppress appetite, and obese humans have high circulating leptin levels indicating leptin resistance (18). Adiponectin has anti-inflammatory properties, and its expression is reduced in obese insulin resistant individuals (19,20). Pro-inflammatory adipokines, such as interleukin (IL)-1, IL-6, TNF α and monocyte chemoattractant protein (MCP)-1 have increased expression with obesity-induced insulin resistance and play a crucial role in the development of T2D (21,22). Adipocyte expression of MCP-1 is a component of the pro-inflammatory signaling responsible for the infiltration and activation of macrophages to obese adipose tissue (22). IL-6 and TNF α are hypersecreted from activated macrophages and obese adipocytes in adipose tissue and propagate chronic inflammation (23). Increased expression of these inflammatory markers are linked to the pathogenesis of insulin resistance and

T2D. Recent evidence demonstrates that $\text{TNF}\alpha$ signaling inhibits adiponectin expression and blocks insulin-stimulated tyrosine phosphorylation of both the insulin receptor and insulin receptor substrate 1 (IRS1), crucial components of the insulin-signaling pathway (24,25). This mechanism contributes to the increased lipolysis observed with insulin resistance.

As an insulin responsive tissue, the liver also has an important role in maintaining blood glucose and lipid concentrations. Hepatic insulin resistance results in unregulated gluconeogenesis and glycogenolysis resulting in hyperglycemia (26). FFAs released from adipocytes are packaged with cholesterol into lipoproteins by the liver. Lipoproteins, such as very low-density lipoproteins (VLDL; 'bad cholesterol') and high-density lipoproteins (HDL; 'good cholesterol'), are transported through the blood to the adipocyte, where they are broken down and stored as triglycerides in the lipid droplet. Expression of lipoprotein lipase (LPL), an enzyme important for lipoprotein breakdown, is downregulated with obesity-induced insulin resistance leading to elevated serum lipoproteins (27). Excess circulating VLDLs heighten the risk of vascular complications associated with obesity and metabolic syndrome, such as atherosclerosis, stroke, and cardiac infarction.

The hyperglycemic and lipotoxic conditions that develop with insulin resistance, in combination with impaired fatty acid metabolism, cause lipid metabolites to

amass in the liver (28,29). Intracellular lipid metabolites, such as ceramide, DAG or acyl-CoA, trigger serine kinase signaling pathways that directly interfere with insulin signaling by decreasing tyrosine phosphorylation of IRS2 (30). Protein kinase C (PKC)- ϵ is a player in this cascade and is directly activated by DAG (31). This indicates that it is not necessarily obesity that causes insulin resistance, but the increase of intracellular lipid metabolites. To test this hypothesis, patients exhibiting extreme hepatic insulin resistance and lipid content were fed a hypocaloric diet (1200kcal/day, 3% fat) (6). After 8 weeks, these patients had an 81% reduction in hepatic lipid content, and insulin sensitivity returned to normal levels. These changes were attributed to decreased hepatic glucose production, primarily by diminishing gluconeogenesis, as insulin-stimulated peripheral glucose uptake was unchanged.

Skeletal muscle is a major end point for dietary sugars and fat as it is capable of oxidizing both glucose and fatty acids for use as an energy source. Expression of the insulin-stimulated glucose transporter, GLUT4, does not change with insulin resistance, but glucose uptake is one of the first processes affected by insulin resistance, indicating a signaling obstruction. The defect of glucose uptake is strongly correlated with both excess FFA and insulin resistance (32). Fatty acids directly interfere with insulin signaling in the muscle and impair glucose uptake and glycogen synthesis, contributing substantially to the development of insulin resistance and T2D (30). FFAs activate multiple kinases in the muscle: JNK, IKK,

and PKC θ (31,33). These serine kinases phosphorylate IRS-1, which targets it for degradation and inhibits tyrosine phosphorylation. Without tyrosine phosphorylation the insulin-signaling cascade is halted before PI 3-Kinase activation (34); indicating that excess fatty acids inhibit propagation of insulin effects.

Excess circulating FFAs observed with obesity cause upregulation of genes involved in fatty acid β -oxidation in the skeletal muscle. As fatty acid oxidation increases, a surplus of acyl-CoA and reducing equivalents (ie. FADH₂) are generated. These substrates feed into the TCA cycle and the electron transport chain, respectively. However, these mitochondrial metabolic pathways are ill-equipped to handle excess substrate flux because FFAs do not stimulate upregulation of proteins involved in these processes (35). With deficient mitochondria, the surplus of FFAs is unable to be fully processed and lipid intermediates build up. When the mitochondria are congested with lipid intermediates, both structural and metabolic alterations occur. This is known as mitochondrial dysfunction (36,37). As both consequence and cause of dysfunction, mitochondrial production of reactive oxygen species (ROS) is elevated, beginning with increased superoxide anion production by the electron transport chain at complexes I and III. Some research suggests that ROS have a causal role in the development of insulin resistance (38), but mitochondrial function has been predominately studied in muscle and brown adipose tissue.

This thesis aims to advance the understanding of mitochondrial function in the white adipocyte.

II. Mitochondrial Metabolism in the Adipocyte

White adipose tissue (WAT) is responsible for coordinating storage and distribution of energy metabolites. Glycolytic metabolism of glucose to pyruvate allows for carbon entry into the mitochondrion and subsequent processing through the tricarboxylic acid (TCA) cycle. This process generates reducing equivalents NADH and FADH₂ that funnel into the electron transport chain (ETC, Figure 2). Upon entry into complex I or complex II, respectively, electrons are passed through complexes III and IV and ultimately used to generate water from molecular oxygen. All the while, protons are pumped into the inter membrane space, generating an electrochemical gradient. Complex V, the F₀F₁ ATP Synthase, uses the membrane potential to drive its molecular motor and generate ATP. Theoretically, each glucose molecule that enters the cell produces 36-38 ATP molecules, in actuality inefficiency and proton leakage cause this number to be approximately 30 ATP molecules per glucose (39).

The ETC draws electrons not only from glucose, but also fatty acid and amino acid catabolism. Fatty acids enter the adipocyte either by diffusion or facilitated transport and are activated by acyl-CoA synthetases, which catalyze the ATP-dependent formation of fatty acyl-CoA (40). Once acylated, the activated fatty acid can enter the mitochondrion for oxidation in a carnitine-dependent manner. Fatty acyl-CoA transiently associates with carnitine for transport across the inner mitochondrial membrane (IMM) by the carnitine palmitoyl transferase. Upon entry

into the mitochondrial matrix, the fatty acyl-CoA enters β -oxidation, a series of oxidation and cleavage reactions that ultimately produce FADH_2 , acetyl-CoA and a fatty acyl-CoA that is two carbons shorter. The FADH_2 produced can then enter the ETC for ATP generation, acetyl-CoA can be used in the TCA cycle for generation of reducing equivalents, and the remaining acyl-CoA is further oxidized. Alternatively, acetyl-CoA, which is also produced from the decarboxylation of pyruvate, can be used as the starting material for fatty acid and triglyceride synthesis. The fate of adipocyte fuels is intricately regulated based on availability and demand of the entire body. While aspects of glucose and lipid metabolism occur in the cytoplasm or endoplasmic reticulum, essential components are located in the mitochondrion.

However, the importance of mitochondrial function in the white adipose tissue (WAT) is largely underappreciated, as mitochondria have predominately been studied in brown adipose tissue (BAT), liver, and muscle. This is most likely due to relatively low levels of mitochondria in WAT and their presumed unimportance to lipid storage. Though, in the last decade it has been observed that adipogenesis instigates mitochondrial biogenesis and remodeling, implying that differentiated white adipocytes require enhanced mitochondrial function (41). In fact, the diversity of white adipocyte mitochondrial proteome is even broader than that of muscle mitochondria (42). The additional expression of mitochondrial proteins is thought to support the adipocyte's unique roles in fatty acid synthesis

(43), adiponectin production and secretion (44), and insulin signaling (45). The synthesis and secretion of the insulin-sensitizing adipokine adiponectin was positively correlated to expression of nuclear respiratory factor 1 (Nrf1), an important mediator of mitochondrial biogenesis (44). Similarly, when expression of the key mitochondrial DNA transcription factor Tfam was reduced in 3T3-L1 adipocytes, oxygen consumption and ATP synthesis were reduced, and surprisingly, insulin-stimulated GLUT4 translocation and subsequent glucose uptake was blocked. However, AKT phosphorylation was elevated, suggesting mitochondrial function effects insulin-stimulated glucose uptake down stream of AKT (45). These results imply that mitochondrial function is explicitly linked to other metabolic responsibilities of the adipocyte.

Differences in mitochondrial function have also been observed between specific adipose depots. The oxidative capacity of visceral adipose tissue is reported to be higher than subcutaneous adipose tissue in rats due to increased mitochondrial density, mtDNA and enzyme activity (46). However, when normalized to mtDNA, mitochondrial respiration in visceral adipose tissue from obese humans was actually lower than from subcutaneous depots (47). This could be due to loss of mitochondria function associated with obesity or simply differences in rat and human physiology. Indeed, decreased mitochondrial content has been observed with insulin resistance, but the role of mitochondrial function in the pathology of T2D is still unclear (48,49).

While it is unknown whether mitochondrial dysfunction in the adipocyte is causal or consequent to impaired insulin signaling, mice lacking the insulin receptor specifically in fat cells have elevated expression of mitochondrial genes involved in fatty acid oxidation and oxidative phosphorylation (50), increased PGC-1 α expression and extended lifespan (51), implying that loss of insulin signaling is actually beneficial to mitochondrial function. Induction of mitochondrial oxidative stress via proinflammatory cytokine treatment leads to the sequential activation of apoptosis signal-regulating kinase 1 (ASK1) and JNK, which serine phosphorylates insulin receptor substrate 1 (IRS1), inhibiting insulin-mediated tyrosine phosphorylation of IRS1 and blocking the insulin signaling pathway (52). These lines of evidence suggest metabolic dysregulation may occur through multiple mechanisms.

Regulation of mitochondrial function is a complex process involving biogenesis, fission and fusion, and autophagy. The mitochondrial genome consists of 13 proteins and 24 RNA molecules involved in protein synthesis (53), therefore, a majority of mitochondrial proteins are encoded by nuclear DNA and require import across the outer and inner mitochondrial membrane (reviewed by (54)). Nuclear-encoded proteins are synthesized as precursors with mitochondrial targeting sequences. The translocase of the outer mitochondrial membrane (TOM) complex and mitochondrial import (MIM) proteins allow entry of these

precursor proteins into the intermembrane space. The proteins are distributed further based on their hydrophobicity, structure and final destination. Translocases of the inner mitochondrial membrane (TIM) proteins move proteins into the matrix and if required the presequence is cleaved, although many proteins contain embedded targeting sequences. The reliance upon protein import to supply the mitochondrion with functional components presents many opportunities for error. Expression of mitochondrial import proteins (MIPs) is markedly reduced in the diabetic heart (55) and diminished expression of MIPs leads to compromised mitochondrial integrity and initiation of apoptosis (56). The pathways that control expression of mitochondrial proteins are not entirely defined, but several regulators of mitochondrial biogenesis are known.

The coordination of mitochondrial biogenesis relies upon transcriptional coregulation of gene expression in response to metabolic changes. Dysregulation of these factors by cellular signaling is thought to contribute to the pathogenesis of metabolic disorders (57). The proposed master regulator of mitochondrial biogenesis is peroxisome proliferator-activated receptor 1 alpha (PGC-1 α), whose role was first discovered in brown adipose tissue (58). Upon exposure to cold, expression of PGC-1 α increases considerably in brown fat and muscle of mice, causing elevated transcriptional activity of the nuclear receptor peroxisome proliferator-activated receptor gamma (PPAR γ) and subsequent expression of uncoupling protein 1 (UCP1), a thermogenic protein expressed exclusively in

BAT. Activation of the nuclear receptor PPAR γ leads to adipogenesis and insulin sensitization (59); as such, it provides a useful pharmacologic target for the treatment of insulin resistance and T2D. Transgenic expression of PGC-1 α in white adipocytes triggers expression of UCP1 and elevates mtDNA content. Fatty acid oxidation and respiratory capacity are also elevated by transgenic expression of PGC-1 α in human adipocytes (60) leading many to believe that the cure for obesity may be to increase fat utilization by expressing BAT-specific proteins in WAT.

As a master regulator of mitochondrial biogenesis, PGC-1 α controls expression of mitochondrial proteins along with activity and expression of other transcription factors (61). For example, PGC-1 α is a co-activator of NRF-1, controlling expression of NRF-1 itself and expression of Tfam, an essential mitochondrial transcription factor (62). Co-regulation of oxidative phosphorylation genes by PGC-1 α is accomplished with NRF-1 and NRF-2 in conjunction with nuclear hormone estrogen-related receptor alpha (63).

An important upstream regulator of PGC-1 α activity and mitochondrial biogenesis is endothelial nitric oxide synthase (eNOS). Expression of eNOS is downregulated in genetic and diet-induced models of obesity by TNF- α , as obese TNF receptor 1 knockout mice have normal expression of eNOS and mitochondrial biogenesis (64). Therefore, the transcriptional control of

mitochondrial biogenesis is subject to intra- and extracellular signaling. The complete details of PGC-1 α regulation are not entirely understood, but post-translational modifications such as phosphorylation and acetylation have critical roles in translating metabolic cues (65).

Another mechanism controlling mitochondrial density is selective autophagy, or mitophagy (66). Autophagy can be non-selective, such as in response to nutritional cues of starvation, to initiate a recycling process of intracellular proteins and organelles to provide cellular energy. Alternatively, mitophagy provides the selective autophagic removal of dysfunctional mitochondria. This targeted degradation serves as a quality control mechanism by removing dysfunctional mitochondria. This is a relatively new field of study and the instigating molecular signals remain largely unknown, but the basic principles involve the formation of a double-layered isolation membrane that surrounds components to be recycled, called an autophagosome. Once the autophagosome completely encapsulates the contents it fuses with a lysosome, and the contents are degraded and salvaged.

Before complete removal of dysfunctional mitochondria, fission is used to sequester damaged constituents and divide mitochondria into manageable pieces for isolation membranes to surround (67). Regulation of mitochondrial fission in mammalian cells is accomplished through mitochondrial fission protein

1 (Fis1) and dynamin-related protein 1 (DRP1) (68). Fis1 is thought to recruit DRP1 from the cytosol at a frequency proportional to the rate of fission. Selective fission produces two daughter mitochondria, one with intact membrane potential and another with low membrane potential that possibly contains dysfunctional enzyme complexes and damaged mtDNA (67). The dysfunctional fragment also contains less OPA1, a protein required for mitochondrial fusion, an event that combines together membranes of separate mitochondria resulting in shared matrix constituents. If OPA1 is present at high enough concentrations in the dysfunctional mitochondrial fragment, it can fuse with healthy mitochondria and avoid degradation (67). If mitochondria fail to recover membrane potential, depolarized mitochondria are flagged for removal.

The precise cues initiating mitophagy are unclear but both cytoplasmic and mitochondrial proteins are involved. In response to mitochondrial damage, PTEN-induced putative kinase protein 1 (PINK) localizes to the outer mitochondrial membrane (OMM). PINK1 is selectively stabilized on dysfunctional mitochondria and acts to recruit and activate the E3 ubiquitin ligase Parkin, which uses ubiquitination as a signal for mitophagy (69,70). Other signaling pathways exist to activate mitophagic elimination of impaired mitochondria from within the mitochondria. These pathways are induced when OMM proteins bind to autophagy proteins located on the isolation membrane. The OMM proteins include autophagy-related protein 32 (Atg32) and NIP3-like protein X (NIX) which

associate with the autophagosome through interaction with microtubule-associated protein 1A/1N-light chain (LC3) present on the isolation membrane (71,72). This tethering allows for the mitochondria to be drawn into the forming autophagosome and degraded. The explicit events initiating mitophagic signaling are unknown, but regardless of the source, loss of membrane potential is commonly associated with the induction of mitophagy.

Many factors can contribute to the reduction of mitochondrial membrane potential. The potential gradient is generated predominately by the electron transport chain (ETC). As electrons are funned through complexes I-IV, protons are pumped into the inner membrane space. The activity of inner membrane transport proteins such as the uncoupling proteins or the phosphate carrier can influence membrane potential, and complex V, the F_0F_1 ATP synthase, exploits the electrochemical gradient to drive its molecular motor. However, these biochemical operations typically propel the ETC to rebuild the electrochemical gradient. If functional capacity decreases due to events including but not limited to oxidative damage or reduced enzyme expression, decreased mitochondrial substrate flux through the ETC can contribute to low membrane potential. Impaired membrane integrity can also cause protons to leak back into the mitochondrial matrix. Hence, many events can trigger membrane depolarization and initiation of mitophagy.

The balance between creation and destruction of mitochondria relies on integrated signaling networks to transmit cues about the cellular environment and respond to changes in mitochondrial integrity. The mitochondria are in constant cycles of fission and fusion, adapting to the metabolic requirements of the cell. However, sometimes mitochondrial function is overwhelmed or compromised, which can cause cellular damage, ranging from production of reactive species, failure to generate ATP, or initiation of apoptosis by release of cytochrome c. Therefore, maintaining a healthy population of mitochondria is essential to both proper cellular function and survival.

III. Oxidative Stress and Antioxidants

Aerobic respiration is invariably associated with the production of partially reduced reaction oxygen species (ROS). Under physiological conditions, ROS production is balanced by antioxidant detoxification, controlling the oxidation and reduction, or redox, environment. While there is a strong literature base to support the role of ROS in cellular signaling (as reviewed in (73)), elevation of ROS production without concomitant increases to antioxidant capacities is responsible for damage to cellular components such as lipids, proteins, and DNA. Accumulation of oxidative stress is linked to pathological alterations of metabolism and even cell death (74-77).

ROS are the product of incomplete oxygen reduction in electron transfer reactions, including NADPH oxidase, xanthine oxidase, lipoxygenase, and cytochrome p-450 reactions in the endoplasmic reticulum (78). Significant concentrations of ROS are produced in the mitochondria from complexes I and III of electron transport (Figure 2). One-electron reduction of molecular oxygen (O_2) generates superoxide ($O_2^{\cdot-}$). This short-lived species is rapidly dismutated to hydrogen peroxide (H_2O_2) by the antioxidant enzyme superoxide dismutase (SOD). Mitochondrial (MnSOD) and cytoplasmic (Cu/Zn-SOD) isoforms of SOD are extremely efficient ($k = 1.6 \times 10^9 \text{ M}^{-1}\cdot\text{s}^{-1}$), speeding up the reduction by $\sim 10^4$ -fold compared to uncatalyzed reactions (79). Due to this, few other molecules

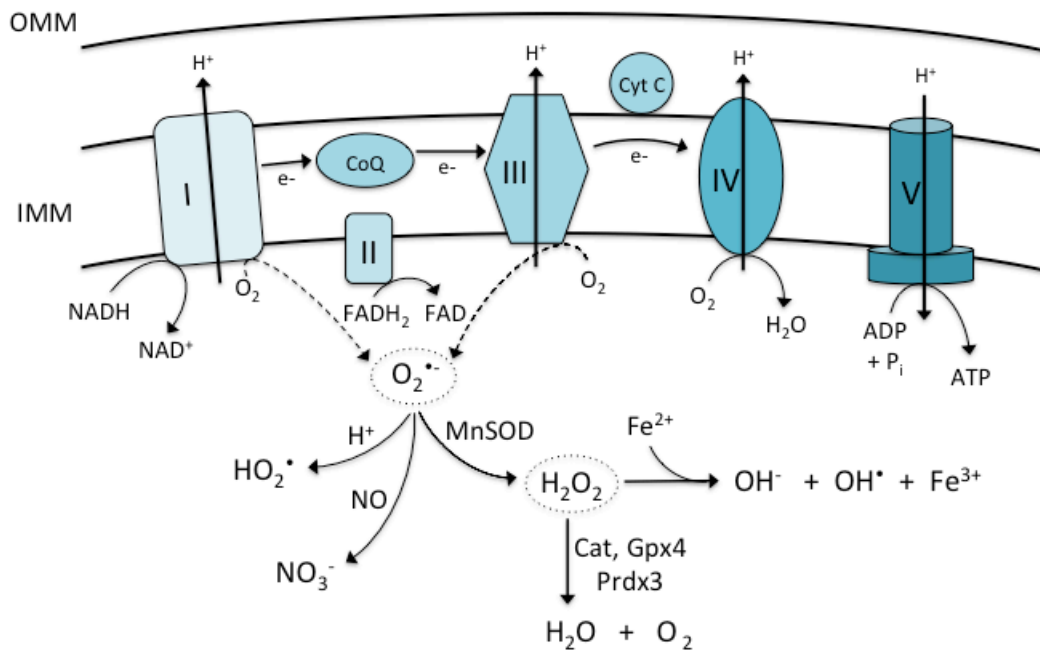


Figure 2. Mitochondrial Electron Transport, Superoxide Generation and Antioxidant Detoxification. Detailed in text. Abbreviations not in text: Cat, catalase; Gpx4, glutathione peroxidase 4; Prdx3, peroxyredoxin 3.

have the opportunity to react with $O_2^{\bullet-}$, and the majority of $O_2^{\bullet-}$ is converted to H_2O_2 .

However, with a reaction rate constant of $6.7 \times 10^9 \text{ M}^{-1}\cdot\text{s}^{-1}$ nitric oxide (NO^{\bullet}) is capable of competing with SOD for substrate access to $O_2^{\bullet-}$ producing the highly reactive anion peroxynitrite ($ONOO^-$) (80). NO^{\bullet} is generated enzymatically from the conversion of L-arginine to citrulline in low concentrations for signaling purposes by the endothelial nitric oxide synthase (eNOS) (81) or in much higher concentrations by the inducible isoform (iNOS) as an oxidative defense against bacterial infections (82). A mitochondrial isoform (mtNOS) also exists, allowing for localized production of $ONOO^-$ at the site of electron leak from the ETC (83). Peroxynitrite can directly modify substrates (nitration) or decompose into reactive nitrogen species capable of protein, lipid and DNA oxidation (84). In the aqueous phase, $ONOO^-$ rapidly reacts with carbon dioxide to form carbonate and nitrogen dioxide radicals. The conjugate acid of $ONOO^-$, peroxynitrous acid ($ONOOH$), produces hydroxyl and nitrogen dioxide radicals via homolysis. This bond dissociation reaction occurs predominately in phospholipid bilayers, where radicals initiate oxidative and nitrative modification of lipids.

Nitric oxide is not generally present in high concentrations so the majority of superoxide is dismutated to H_2O_2 by SOD. Hydrogen peroxide is a potent oxidizer without any unpaired electrons and is capable of diffusing across lipid

bilayers. Antioxidant enzymes such as catalase, glutathione peroxidase, and peroxidoredoxin reduce H_2O_2 to H_2O . Alternatively, H_2O_2 can participate in metal-catalyzed reactions, such as Fenton or Haber-Weiss reactions, to produce the extremely toxic hydroxyl radical ($\cdot\text{OH}$) (85). The majority of $\cdot\text{OH}$ production arises from reactions with iron and copper and to a lesser extent with other metals such as chromium and cobalt (86). Even though the vast majority of iron is chelated to hemoglobin or other iron storage proteins, excess cellular $\text{O}_2^{\cdot-}$ causes iron liberation from iron-containing molecules (eg. ferritin) (85). Superoxide also inactivates [4Fe-4S] cluster-containing enzymes, which exist as [2Fe(II) 2Fe(III)-4S]. Oxidation of one Fe(II) ion to Fe(III) by $\text{O}_2^{\cdot-}$ yields 3Fe(III) ions in the cluster, release of Fe(II) ion and H_2O_2 production (85). Ferrous iron can then be oxidized by H_2O_2 to yield Fe(III), $\cdot\text{OH}$ and OH^- . The hydroxyl radical can also be formed by the Haber-Weiss reaction in which $\text{O}_2^{\cdot-}$ reacts with H_2O_2 (87).

Hydroxyl radical toxicity stems from its highly reactive nature and inability to be enzymatically detoxified. The half-life of $\cdot\text{OH}$ in aqueous solutions is less than 1 ns (88), causing reaction substrates to be based on proximity to radical formation. Substrates include protein side changes, nucleic acids, and lipids. DNA oxidation by $\cdot\text{OH}$ produces over 100 different products and is linked to mutagenesis and carcinogenesis. Severity of DNA damage is often assessed by the presence of 8-hydroxyguanine (8-OH-G) (89).

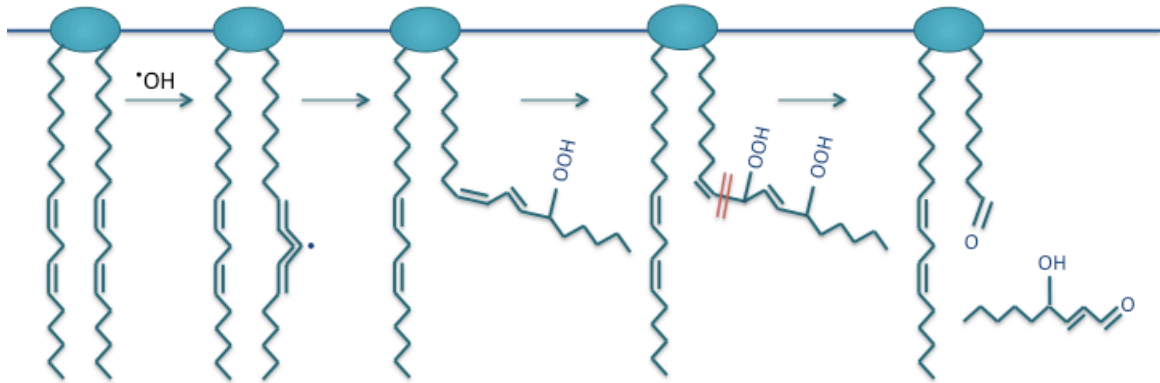


Figure 3. Peroxidation of polyunsaturated acyl chains of glycerophospholipids. Acyl chains are susceptible to free-radical reaction at bis-allyl sites, to form a free radical intermediate, which further reacts with molecular oxygen to generate hydroperoxide derivatives. The abstraction of allylic hydrogen at C-8 forms a radical intermediate. The radical migrates to the C10 position. Oxygenation yields an unstable di-hydroperoxide derivative that undergoes non-enzymatic Hock cleavage at C9 & C10 to yield aldehyde fragments.

The hydroxyl radical also initiates peroxidation of polyunsaturated acyl chains of glycerophospholipids and triacylglycerol (Figure 3). Bis-allylic sites on these acyl chains are subject to electrophilic attack by hydroxyl radicals, forming delocalized-radical intermediates (90,91). Serial oxidation and radical migration generate unstable hydro- and di-hydroperoxide derivatives that quickly undergo Hock cleavage to produce α , β -unsaturated aldehydes such as acrolein, 4-hydroxy- and 4-oxo *trans*-2,3-nonenal (4-HNE, 4-ONE). These reactive lipid species can be metabolized through oxidative or reductive enzyme catalyzed reactions, but the predominant detoxification route is glutathionylation via glutathione *S*-transferase A4 (GSTA4) and subsequent cellular export of the glutathionylated lipid through the facilitated transporter RLIP76 (92,93). If electrophilic end products of lipid peroxidation escape detoxification, they can react with the protein side chains of cysteine, histidine, and lysine residues, in a reaction termed protein carbonylation (Figure 4). The sulfhydryl, imidazole and ϵ -amino groups of these amino acids, respectively, participate in nucleophilic Michael addition to the partial-positive C3 of 4-HNE and similar lipid aldehydes (94). Protein carbonylation is a non-reversible chemical modification, and as these residues are commonly important for catalysis, carbonylation can cause alterations to protein function, especially inactivation of thiol-dependent enzymes, and promote degradation.

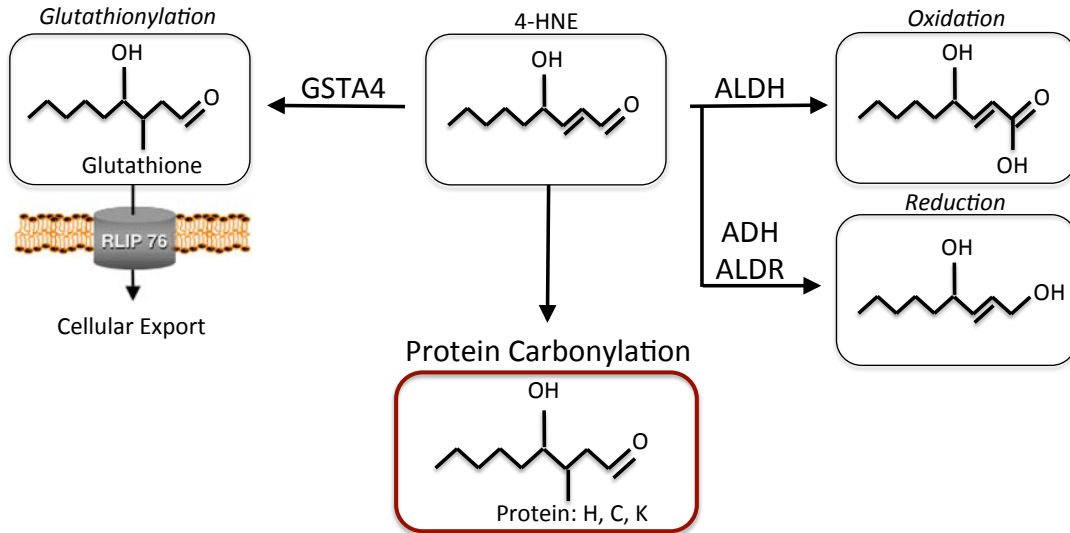


Figure 4. Detoxification of Lipid Aldehydes and Protein Carbonylation. 4-HNE and other α , β -unsaturated aldehyde products of lipid peroxidation are subject to detoxification reactions. Oxidation or reduction of aldehydes occurs enzymatically by aldehyde dehydrogenase (ALDH), alcohol dehydrogenase (ADH), and aldehyde reductase (ALDR), which produce a less reactive molecule. However, the predominant route of detoxification is glutathionylation via GSTA4 and subsequent export out of the cell. If aldehydes escape these pathways, they can participate in Michael addition reactions, irreversibly modifying amino acid side chains of histidine (H), cysteine (C) or lysine (K).

Image modified from (95).

While 4-HNE production is certainly not limited to the mitochondria, the double membrane structure and abundant ROS supply serve as an excellent source of lipid peroxidation reaction substrates. 4-HNE is the most abundant aldehyde products of lipid peroxidation (96). Increased 4-HNE concentrations, which can range from 0.2-1.5 nM *in vivo* (97), cause mitochondrial targeting of GSTA4 (98,99). The activity of GSTA4 is selective for products of lipid peroxidation and has high catalytic efficiency for 4-HNE ($k > 3 \times 10^6 \text{ M}^{-1}\cdot\text{s}^{-1}$) (100). In fact, conjugation of 4-HNE to glutathione is the primary detoxification route, and GSTA4 is recognized as the predominant enzyme responsible for glutathionylated-HNE (GSHNE) production (93,101). While this reaction can occur spontaneously, it is significantly enhanced by GSTA4 catalysis (102).

GSTA4 expression is regulated by the antioxidant response. Elevations of ROS and accumulation of carbonylated proteins lead to transcriptional upregulation of antioxidant response element (ARE)-containing genes (103). A critical regulator of ARE genes is nuclear factor erythroid 2-related factor 2 (Nrf2), whose activity is regulated by Kelch-like ECH-associated protein 1 (Keap1) (104). Keap 1 sequesters Nrf2 in the cytoplasm until it is degraded by the proteasome or released for nuclear translocation. Keap1 is a cysteine-rich protein, and modification of C273 and C288 by electrophiles such as 4-HNE causes the dissociation of the Keap1-Nrf2 complex (105,106). Upon release, Nrf2 accumulates in the nucleus and induces expression of ARE-driven genes such

as GSTA4 and components of glutathione synthesis, resulting in increased antioxidant capacity. These events contribute to reduction of oxidative stress.

Interestingly, the expression of GSTA4 is selectively downregulated in the white adipose tissue of obese insulin-resistant C57Bl/6 mice (107). As a result, protein carbonylation is also elevated ~3-fold relative to lean mice. The C57Bl/6 mouse strain is genetically more susceptible to diet-induced obesity, hyperinsulinemia, hyperglycemia and hypertension than most other mouse backgrounds and thus serves as a good model of human metabolic syndrome (108-110). With the discovery that protein carbonylation was elevated in the adipose tissue with obesity, it was hypothesized that protein targets of carbonylation could play a role in the development of obesity-link diabetes. To determine the specific proteins involved, previous studies in the Bernlohr lab conducted by Paul Grimsrud characterized targets of protein carbonylation in adipose tissue from high-fat fed C57Bl/6 mice. The results of this work identified a broad range of protein targets with functions varying from antioxidant and stress response, signal transduction, and carbohydrate and lipid metabolism (107).

One of the most abundant proteins in the adipose tissue, adipocyte fatty acid binding protein (AFABP, FABP4 or aP2), was identified in the proteomic screen for carbonylated proteins. *In vitro* studies confirmed covalent 4-HNE modification of AFABP at C117 (111) and reported a 10-fold loss of affinity for fatty acid

substrates when AFABP is bound to 4-HNE (107). Estimates predict 6-8% of AFABP in obese adipose tissue is modified with 4-HNE. The cellular impact of modification is unclear and currently only correlated with obesity and insulin resistance.

The development of insulin resistance is a multi-factorial process, involving diet, genetics and many other factors. With an aim to understand the impact of protein carbonylation, GSTA4 null mouse models have been developed. Original studies characterizing GSTA4 null mice reported higher 4-HNE concentrations and a 40-80% loss of glutathione conjugating activity towards 4-HNE, depending on the tissue (92). Expression of other antioxidant enzymes such as GSTA3, catalase, SOD1, SOD2, and glutathione peroxidase were upregulated in the livers of GSTA4 null mice, but even so they remained hypersensitive to oxidative stress, as evidenced by lower survival rates in response to paraquat administration, which causes formation of superoxide. The authors concluded that these mice would be “useful for the study of degenerative conditions in which 4-HNE is postulated to be a contributing factor” (92).

Later studies by this same group reported GSTA4 null mice had age-dependent development of obesity and subsequent insulin resistance in the 129/sv background (97). Expression of acetyl-CoA carboxylase (ACC), a key enzyme in fatty acid biosynthesis, was significantly elevated, contributing to increased fat

accumulation. Surprisingly, GSTA4 null mice on a C57Bl/6 background did not have elevated 4-HNE levels in tissues and remained lean. Wildtype 129/sv mice are significantly less prone to developing diet-induced obesity and insulin resistance relative to C57Bl/6 mice (110). However, it is likely that GSTA4 null mice on the C57Bl/6 background have a higher capacity for compensatory upregulation of other 4-HNE metabolizing pathways.

Another unexpected phenotype of the GSTA4 null mice is that they live ~14% (100 days) longer than wildtype mice on average (112). This is a surprising finding because orthologous studies in *C. elegans* concluded that loss of 4-HNE conjugating enzymes shortened lifespan and overexpression extended lifespan (113-115). The longevity studies were performed on the C57Bl/6 background due to favorable aging characteristics including a fairly long lifespan and resistance to tumor formation (116). The phenomenon of increased lifespan in GSTA4 null mice was explained by increased Nrf2 activation and upregulation of antioxidant gene expression. Purportedly, the loss of GSTA4 leads to an initial increase of 4-HNE, releasing Nrf2 from Keap1, leading to sustained upregulation of ARE-driven genes.

IV. Current Objectives

The association among oxidative stress, protein carbonylation and disease is clear, but the molecular mechanisms remain uncertain. Downregulation of GSTA4 and increased protein carbonylation is concurrent with obesity-linked insulin resistance in the adipocyte. Oxidative stress is suggested to be causally linked to insulin resistance in the adipocyte (38) and expression of mitochondrial oxidative phosphorylation genes is downregulated with human obesity (117). It is conceivable that loss of mitochondrial function resulting in increased oxidative stress could impair insulin action in the adipocyte, but why is mitochondrial function reduced to begin with?

The central hypothesis of this research is that downregulation of GSTA4 by inflammatory factors in the adipose tissue leads to protein carbonylation-induced mitochondrial dysfunction and ROS production. Metabolic alterations associated with these changes compound the primary effects of oxidative protein carbonylation and initiate a feed forward cycle that propagates cellular dysfunction. These events are contributing factors to the development of insulin resistance in the adipocyte.

The studies presented herein aim to answer the following questions: What is the impact of reduced GSTA4 expression on adipocyte mitochondria function? How does the adipocyte respond to changes in mitochondrial function? And what

mitochondrial proteins are carbonylated in the adipocyte? Using a combination of genetic, metabolic, and proteomic assays, this thesis investigates the role of protein carbonylation on mitochondrial function in relationship to GSTA4 expression in the adipocyte.

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CHAPTER 2

Downregulation of Adipose Glutathione S-Transferase A4 Leads to Increased Protein Carbonylation, Oxidative Stress, and Mitochondrial Dysfunction

Curtis, J. M.,* Grimsrud, P. A.,* Wright, W. S.,* Xu, X., Foncea, R. E., Graham, D. W., Brestoff, J. R., Wiczer, B. M., Ilkayeva, O., Cianflone, K., Muoio, D. E., Arriaga, E. A., and Bernlohr, D. A. (2010). *Diabetes Journal* **59**, 1132-42.

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* JMC, PAG & WSW have equal contributions to this research.

Jessica Curtis performed experiments in their entirety from figures 4B, 5, S1, 7B, & 7D, and prepared samples for experimental collaborations in figures 5F, 5I, S2, & 7.

OBJECTIVE - Peripheral insulin resistance is linked to an increase in reactive oxygen species (ROS) leading in part to the production of reactive lipid aldehydes that modify the side chains of protein amino acids in a reaction termed protein carbonylation. The primary enzymatic method for lipid aldehyde detoxification is via glutathione S-transferase A4-dependent glutathionylation. The objective of this study was to evaluate the expression of GSTA4 and the role(s) of protein carbonylation in adipocyte function.

RESEARCH DESIGN AND METHODS – GSTA4 silenced 3T3-L1 adipocytes and GSTA4 null mice were evaluated for metabolic processes, mitochondrial function and reactive oxygen species production. GSTA4 expression in human obesity was evaluated using microarray analysis.

RESULTS - GSTA4 expression is selectively down regulated in adipose tissue of obese insulin resistant C57BL/6J mice and in human obesity-linked insulin resistance. TNF α treatment of 3T3-L1 adipocytes decreased GSTA4 expression and silencing GSTA4 mRNA in cultured adipocytes resulted in increased protein carbonylation, increased mitochondrial ROS, dysfunctional state 3 respiration and altered glucose transport and lipolysis. Mitochondrial functions in adipocytes of lean or obese GSTA4 null mice were significantly compromised compared to wild type controls and was accompanied by an increase in superoxide anion.

CONCLUSIONS - These results indicate that down regulation of GSTA4 in adipose tissue leads to increased protein carbonylation, ROS production and mitochondrial dysfunction and may contribute to the development of insulin resistance and type 2 diabetes.

INTRODUCTION

Obesity-linked type 2 diabetes and its associated health complications are major human health concerns (1) and recent studies have implicated increased levels of reactive oxygen species such as superoxide anion, hydrogen peroxide, peroxynitrite and hydroxyl radicals as major contributing factors (2-4). Excess ROS are causally linked to insulin resistance in adipocytes (5) and alteration of adipokine secretion in a manner that promotes insulin resistance in peripheral tissues (2,6,7). As adipose metabolism plays a substantial role in regulating whole body insulin sensitivity (8), evaluating the conditions that lead to oxidative stress in adipocytes is an important goal.

Whereas various ROS react with all cellular components, the hydroxyl radical mediated peroxidation of polyunsaturated acyl chains of glycerophospholipids is particularly harmful as it results in the formation of lipid peroxidation products considered second messengers of oxidative stress (9). Peroxidated acyl chains are unstable and undergo nonenzymatic Hock cleavage generating a family of reactive α , β -unsaturated aldehydes (10). Such reactive lipid aldehydes, including *trans*-4-hydroxy-2-nonenal (4-HNE), covalently modify protein and DNA, activate cellular stress response systems (11) and the transcription factors Nrf2 and Tfam (12,13). In the case of protein modification, the process is generically termed protein carbonylation and often results in loss of function (14).

Using proteomic profiling, we have previously shown that high-fat fed obese, glucose intolerant C57Bl/6J mice exhibit ~2-3-fold increased adipose protein carbonylation compared to lean controls (15). In addition, obese animals exhibited a ~4 fold decrease in the abundance of glutathione S-transferase (GST) A4 in adipose tissue. GSTA4 catalyzes the glutathionylation of α , β -unsaturated aldehydes to produce a conjugation product that is transported from the cell (16). Our studies herein focus on the role of GSTA4 as an antioxidant enzyme linking protein carbonylation to metabolic dysfunction. We reveal the unanticipated finding that the expression of GSTA4 is down regulated by TNF α in 3T3-L1 adipocytes and mitochondria from GSTA4 silenced 3T3-L1 cells, or from adipose tissue of GSTA4 null or obese C57Bl/6J mice accumulate ROS and have compromised respiration. Metabolically, this results in impaired glucose and lipid homeostasis suggesting that TNF α -induced down regulation of GSTA4 is a major determinant linking inflammation with oxidative stress and insulin resistance.

RESEARCH DESIGN AND METHODS

Animals. C57BL/6J mice were placed on a normal chow (~4% fat by weight, Teklad) or a high fat diet (~35% fat by weight, F3282, BioServ Inc) at weaning (17). Mice were housed on a 12-hour light/dark cycle and fed ad libitum with continual access to water. At 12 to 16 weeks of age, mice were sacrificed by cervical dislocation, tissues were harvested, frozen in liquid nitrogen and stored at -80° C until further processing. Primary adipocytes were isolated from fresh epididymal fat pads as described previously (18). Mixed strain mice B6;129S5-GSTA4^{Gt(neo)⁶¹⁹Lex} were purchased from the Mutant Mouse Regional Resource Centers (UC Davis, CA). GSTA4 heterozygous mice were out bred to C57/BL6J mice and the resulting heterozygous progeny were interbred to generate GSTA4 null and wild type mice. The University of Minnesota Institutional Animal Care and Use Committee approved all experiments.

Generation of GSTA4 silenced adipocytes. 3T3-L1 fibroblasts were transduced with lentivirus carrying shRNA as described previously (19). shRNA sequences directed toward GSTA4 mRNA were purchased from Ambion Inc. Each oligo and the reverse complement were synthesized, annealed and the dsDNA ligated into pENTR/U6 cloning vector (Invitrogen Corp.) according to manufacturer's protocol. Cell lines used for experiments contained the following shRNA sequences: Scr control -

AGTACTGCTTACGATACGGTGTGCTGTCCGTATCGTAAGCAGTACT;

GSTAA4 Kd –

GGTATATAGATCCCAGGAGTGTGCTGTCCTCCTGGGATCTATATACC.

Protein carbonylation. Protein carbonylation was detected using EZ-link Biotin Hydrazide (Pierce) as described previously (15) with slight modifications. PVDF membranes were blocked in Odyssey® Blocking Buffer, biotinylated proteins were detected with DyLight™ 800 Conjugated Streptavidin and visualized using an Odyssey® Infrared Imager.

Mitochondrial isolation, respiration, and matrix superoxide. 3T3-L1 adipocytes were scraped and incubated for 20 minutes on ice in 20 mM Tris (pH 6.8), 1 mM EDTA containing protease inhibitors. Cells were lysed with 40 strokes of a dounce homogenizer and the resulting homogenate supplemented with a final concentration of 220 mM mannitol and 70 mM sucrose. For mitochondrial isolation from adipose tissue, epididymal fat pads were minced, washed in ice-cold KRH, and lysed with 1:5 w/v of isolation buffer (20 mM Tris-HCL, 220 mM mannitol, 70 mM sucrose, 1 mM EDTA, pH 7.4 supplemented with protease inhibitors) by dounce homogenization. Homogenates were centrifuged at 700 x g to remove nuclei, unbroken cells and the lipid cake. Mitochondria were recovered by centrifugation at 12,000 x g.

Oxidative respiration was assessed in isolated mitochondria as described

previously (20) using a FOXY-R Oxygen Sensor (Ocean Optics Inc., Dunedin, FL). Isolated mitochondria were incubated at room temperature in 10 mM HEPES (pH 7.4), 125 mM KCl, 5 mM MgCl₂ and 2 mM K₂HPO₄ supplemented with 5 mM pyruvate and 5 mM malate to stimulate state 2 respiration. State 3 respiration was measured after the addition of 0.5 mM ADP and oxygen consumption rate was normalized to mitochondrial protein. TPP-HE was used to detect superoxide in isolated mitochondria as described previously (21).

Analysis of human GSTA4 expression. Microarray analysis of human genes expressed in omental and subcutaneous adipose tissue was reported by Cianflone and colleagues (22). Dataset analysis was conducted using the Significant Analysis of Microarrays procedure (23) version 3.02 available at <http://www-stat.stanford.edu/~tibs/SAM>. Statistical analyses were conducted using the statistical package SAS, version 9.1.3 (SAS Institute, Inc., Cary, NC).

Statistical analysis. All values are expressed as mean +/- SEM. Statistical significance was determined using the two-tailed Student's t-test assuming unequal variances or where appropriate, a two-way ANOVA with Bonferroni or Holm-Sidak post-hoc analysis. *P* values < 0.05 are considered significant (*) with an increased significance of *P* value < 0.01 indicated (**).

Chemical reagents. Fatty acid-free bovine serum albumin (BSA) was purchased

from Sigma. [1-³H]-palmitate was purchased from Perkin Elmer and [1-¹⁴C]-acetate was purchased from Amersham Corp. Recombinant mouse TNF α was purchased from R&D systems. NAD⁺/NADH Quantification Kit purchased from BioVision. Antibodies against A-FABP and E-FABP were produced in house. Other antibodies were obtained from commercial sources: β -actin (Sigma); C/EBP β , CD36, LPL, and PPAR γ (Santa Cruz); Mitofilin (Novus Biologicals). Antibodies to GLUT1 and GLUT4 were kind gifts of Xiaoli Chen (University of Minnesota).

Quantitative RT-PCR. Expression of mRNAs was measured by quantitative RT-PCR. Total RNA was isolated from tissue, fibroblasts or adipocytes using Trizol reagent (Invitrogen Corp.) according to manufacturer's protocol. RNA was treated with DNase I and cDNA was synthesized using iScript cDNA synthesis kit (BioRad). Amplification was monitored with iQ SYBRgreen Supermix and the MyiQ detection system (BioRad). Primer sequences used to amplify and detect target transcripts can be found in the original publication of this work (24). Cell culture conditions. 3T3-L1 fibroblasts were maintained and differentiated into adipocytes as described previously (25). The culture media was supplemented with 1 μ g/ml blasticidin until induction of differentiation. Differentiation was induced by addition of DMEM containing 10% fetal bovine serum (FBS), 0.5 mM methylisobutylxanthine, 0.25 μ M dexamethasone, 170 μ M insulin and 5 μ M troglitazone for 48 h. The methylisobutylxanthine, dexamethasone and

troglistazone were removed after two days and the insulin was removed after four days. Differentiated 3T3-L1 cells were maintained in DMEM with 10% FBS and used in experiments six to twelve days after induction.

Immunoblot analysis. 3T3-L1 adipocytes were sonicated in homogenization buffer (50 mM Tris, pH 7.4, 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 1 mM NaP207) supplemented with protease inhibitor cocktail (Roche, Cat. No. 11836170001), 2 µg/mL pepstatin, and phosphatase inhibitors I and II (Sigma). For GLUT1 and GLUT4 analysis, cells debris was removed by low speed centrifugation. The supernatant was centrifuged at 100,000 x g for 1 hr at 4° C to isolate the membrane fraction and this pellet was resuspended in 2 M urea, 1.4% SDS, 14 mM Tris (pH 6.8), and 100 mM DTT. Equal amounts of protein were separated by SDS-PAGE, transferred to PVDF immobilon-FL (Millipore) membranes, blocked in Odyssey® Blocking Buffer (LI-COR Biosciences). Membranes were incubated with infrared conjugated secondary antibodies and visualized using Odyssey® Infrared Imager (LI-COR Biosciences).

Metabolic profiling. GSTA4 Kd and Scr 3T3-L1 adipocytes (day 7) were pre-incubated for 24 h with 50 µM L-carnitine in fresh culture media. Cell monolayers were washed with ice-cold PBS and rapidly frozen with liquid nitrogen. Lysates were sonicated in H₂O and centrifuged at 14,000 x g for 15 min at 4° C. The infranatant was collected for analysis of organic acids and acylcarnitines by

quantitative mass spectrometry. Organic acids were assayed as previously described, using a Trace Ultra GC coupled to a trace DSQ MS operating under Excalibur 1.4 (Thermo Fisher Scientific) (26,27).

In-gel protein digestion and mass spectrometry. Proteins from GSTA4 Kd and Scr adipocytes were separated by SDS-PAGE. The gel was stained with SYPRO Ruby (Invitrogen) and the protein bands of interest cut out and prepared as described previously (28). Gel pieces were incubated in 10 mM DTT, 100 mM NH_4HCO_3 for 45 minutes at 56° C then incubated with 55 mM iodoacetic acid, 100 mM NH_4HCO_3 for 30 minutes in the dark at room temperature to block free sulfhydryl groups, rehydrated with NH_4HCO_3 , dried with acetonitrile and solvent evaporated. The gel pieces were digested with 12.5 ng/ μL Trypsin Gold (Promega) in 50 mM NH_4HCO_3 pH 8.5, 1 mM CaCl_2 overnight at 37° C. Peptides were recovered and washed with 20 mM NH_4HCO_3 followed by three washes with 50% acetonitrile/5% formic acid. The recovered peptides plus all washes were pooled and concentrated to dryness. The samples were desalted using a C18 zip tip, eluted with 80% acetonitrile, 0.1% TFA in water, dried and reconstituted in 0.1% formic acid for analysis.

Peptide analysis was performed as described previously (29) using a LTQ linear ion trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA) auto sampled on a Paradigm MS4 system (Michrom Bioresources, Inc., Auburn, CA).

Briefly, the peptide mixtures were concentrated and desalted on a precolumn (0.15 x 50 mm, 400- μ l volume) and then loaded onto a C18 in-line analytical capillary column (75 μ m internal diameter x 12 cm). The peptides were eluted over a linear gradient of 10-40% acetonitrile in 0.1% formic acid over 60 minutes at a flow rate of ~250 nL/minute followed by isocratic elution at 80% acetonitrile in 0.1% formic acid. The four most intense precursor ions were selected for MS/MS. Proteins were identified using SEQUEST (30) and X! Tandem (31,32). The search was done using a FASTA combined human, mouse rat library containing greater than 263,677 entries. The search parameters using SEQUEST consisted of fragment ion mass tolerance of 1.00 Da, parent ion tolerance of 0.80 Da and 2 trypsin miscleavages allowed. Scaffold (version Scaffold- 01_05_14, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm (9) and contained at least 2 identified peptides.

β -Oxidation. 3T3-L1 adipocytes were incubated for 24 h in DMEM and 10% FBS + 1 mM L-carnitine and subsequently serum-starved for 1 h in KRH buffer containing 5.4 mM glucose, 1 mM L-carnitine, and 0.1% FA-free BSA. β -Oxidation was initiated upon addition of 400 μ M [1 - 14 C]-palmitate (0.8 μ Ci/mL) buffered with 0.7% fatty acid-free BSA and incubated for 60 min at 37 $^{\circ}$ C and 5% CO₂ (33). Media and cells were transferred to glass vials containing a center well

filled with 400 μL 1 M NaOH. 200 μL 70% perchloric acid was injected into the glass vials capped with a rubber stopper and incubated for 1 hr at room temperature. Center wells containing trapped CO_2 were transferred to liquid scintillation fluid and $^{14}\text{CO}_2$ content was determined. The remaining acidified sample was centrifuged at 13,000 x g at 4 °C and the supernatant containing acid soluble metabolites was added to liquid scintillation fluid and determined as ^{14}C -labeled acid soluble metabolites.

Lactate determination. GSTA4 Kd and scrambled 3T3-L1 adipocytes were incubated 24 hours in DMEM containing 0.5% FBS. Media was collected and L(+)-lactate concentrations were determined using the Lactate Assay Kit (BioVision, Mountain View, CA) according the manufacturer's instructions.

2-deoxyglucose transport assay. 2-Deoxyglucose transport was measure as described previously with varying concentration of insulin (19).

ATPase activity. ATPase activity was assayed spectrophotometrically at 340 nm by coupling the production of ADP to NADH oxidation with pyruvate kinase and lactate dehydrogenase (34). Briefly, freshly isolated mitochondria were resuspended in 1 ml of 50 mM Tris HCl, pH 7.4, 120 mM NaCl, 5 mM KCl, 1 mM MgSO_4 , 1 mM CaCl_2 and 10 % glycerol, supplemented with 1% digitonin. After 20 min on ice with gentle mixing, samples were centrifuged at 250,000 x g for 1 hr at

4 °C. Solubilized mitochondria (300 x g) were added to 1.3 ml of 100 mM MgATP, 2 mM MgCl₂, 50 mM KCl, 0.2 mM EDTA, 0.23 mM NADH, 1mM PEP, 1.4 unit pyruvate kinase, and 1.4 unit lactate dehydrogenase. To determine F₀F₁-specific ATPase activity, oligomycin (7 µg/ml) was pre-incubated with mitochondrial samples for 1 hr at room temperature.

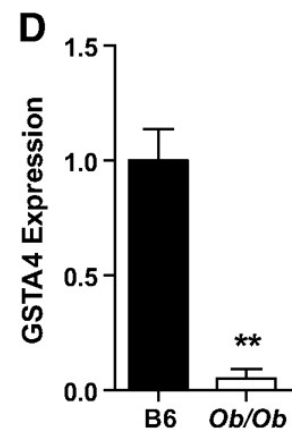
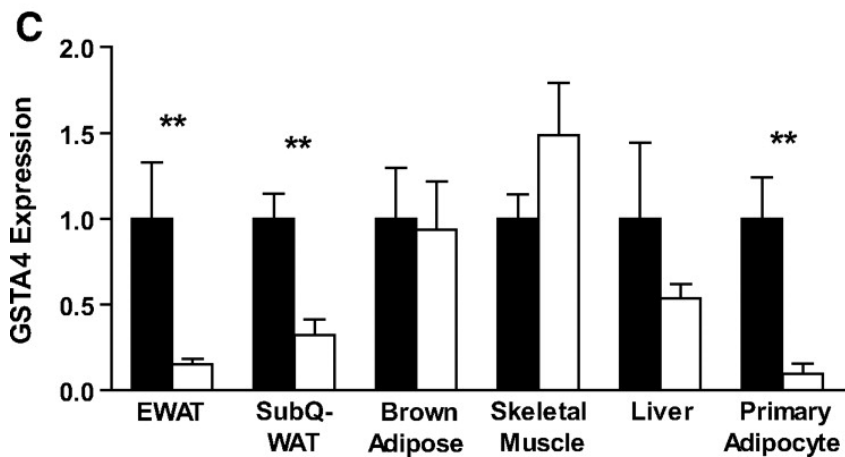
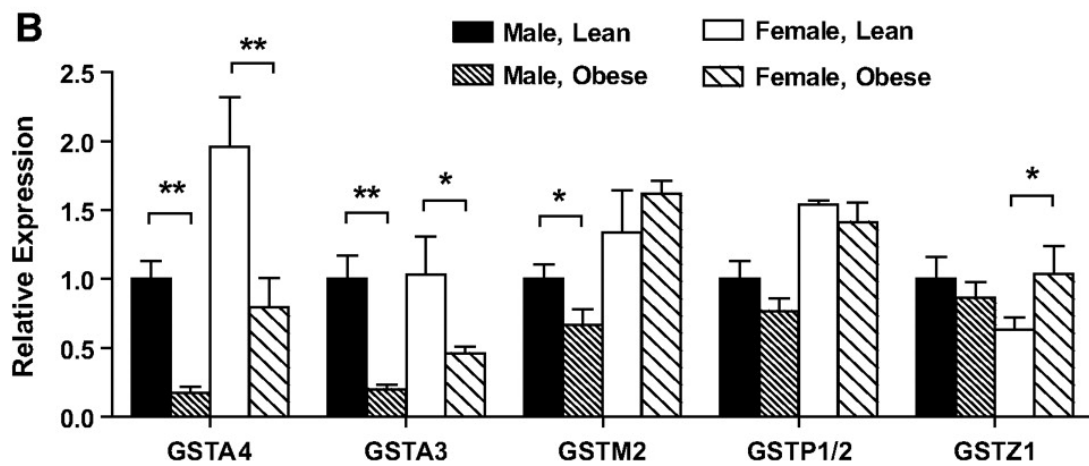
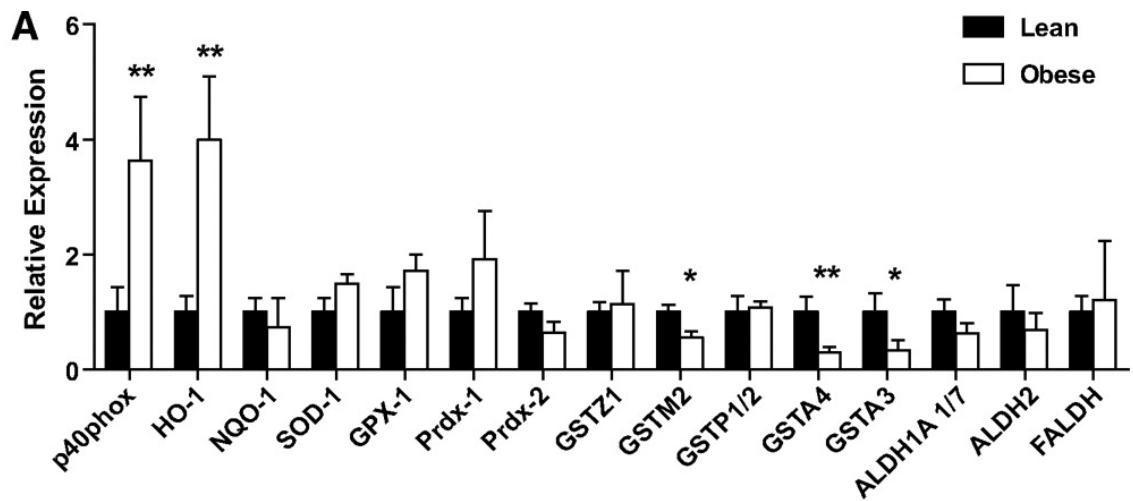
Lipolysis assay. To evaluate lipolysis, GSTA4 Kd and Scr 3T3-L1 adipocytes were washed with phosphate-buffered saline (PBS) and incubated at 37° C in KRH supplemented with 2% fatty acid-free BSA and 5 mM glucose with or without 20 µM forskolin and/or insulin (5 or 50 µM). Media was collected after one hour and the non-esterified fatty acids determined using the NEFA-HR (2) assay according to the manufacturer's instructions (Wako Diagnostics, Mountain View, CA, USA).

Mitochondrial DNA quantification. To determine mitochondria DNA (mtDNA) levels, total genomic DNA was isolated with DNAzol reagent (Invitrogen, Carlsbad, CA), and the amount of mtDNA was compared with nuclear DNA. mtDNA copy number was measured by means of quantitative PCR of mtDNA genes cytochrome C oxidase II and cytochrome b and compared to the nuclear gene, uncoupled protein-2 as described (35,36). mtDNA copy number was calculated by taking the absolute value of Δ Ct values between groups (Scr vs. Kd).

RESULTS

To evaluate the expression of genes linked to oxidative stress and 4-HNE metabolism quantitative RT-PCR (qPCR) was performed on mRNA isolated from gonadal white adipose tissue of lean or obese male C57Bl/6J mice (Figure 1A). Adipose tissue from obese animals exhibited increased expression of p40 phox subunit of NADPH oxidase and heme oxygenase (HO-1) and trends toward increased expression of superoxide dismutase 1 (SOD-1), glutathione peroxidase 1 (GPX-1) and peroxyredoxin (PRDX)-1: all consistent with an antioxidant stress response (2). In contrast, the expression of multiple isoforms of the GST family (A4, A3, M2) was decreased in obese mice compared to lean controls. Most notably, the expression of GSTA4 decreased ~3-4 fold consistent with microarray analysis of lean and obese C57Bl/6J mice where GSTA4 expression was profiled amongst the most highly regulated transcripts (37). The expression of other 4-HNE metabolizing enzymes including several aldehyde dehydrogenases (ALDH1A 1/7, ALDH2 and FALDH) was not significantly altered with obesity (Figure 1A). GSTA3 and GSTA4 expression was also decreased in obese female mice (Figure 1B) relative to lean controls. Interestingly, the basal GSTA4 expression in adipose tissue of lean female mice was ~2-fold greater than in lean male mice such that obese female mice express GSTA4 at levels comparable to that expressed by lean male animals (Figure 1B).

Figure 1. Expression of oxidative stress responsive genes in adipose tissue in obesity. C57Bl/6J mice were fed either a low fat or high fat diet for 9-12 weeks and mRNA isolated from the indicated tissue. (A) Expression of the indicated genes in epididymal WAT analyzed by qPCR (Lean, n=8; Obese, n=7). NQO-1, NADPH quinone oxidoreductase 1. (B) Expression of GST isozymes in WAT of lean (L) and obese (O) male (M) and female (F) mice (ML, n=8; MO, n=7; FL, n=5; FO, n=4). (C) Expression of GSTA4 in various tissues or cells from lean and obese male mice (n=8). (D) Expression of GSTA4 in C57Bl/6J mice (B6) relative to that in adipose tissue of ob/ob mice. TATA-box binding protein (TBP); transcription factor II E (TFIIE). * P < 0.05; ** P < 0.01.



To determine the tissue specificity of GSTA4 down regulation, qPCR was performed on mRNA isolated from epididymal white adipose tissue (EWAT), subcutaneous white adipose tissue (SubQ-WAT), brown adipose tissue, liver and gastrocnemius muscle of lean and obese male mice (Figure 1C). While decreased GSTA4 expression was observed in both visceral and subcutaneous white adipose tissue, no significant change was observed in other insulin-responsive tissues analyzed. Since adipose tissue contains multiple cell types, adipocytes were separated from stroma in EWAT by collagenase digestion and exhibited a ~95% decrease in GSTA4 expression in the adipocyte fraction. Reduced expression of GSTA4 was not limited to high fat fed C57Bl/6J mice. GSTA4 mRNA expression was also significantly decreased in adipose tissue from ob/ob animals (Figure 1D) and GSTA4 expression is down regulated ~ 10-fold in adipose tissue of high fat fed BTBR mice relative to low fat fed lean littermates (<http://www.diabetes.wisc.edu>; (38)) indicating that the expression of GSTA4 is markedly reduced in a variety of metabolic and genetic models of obesity and insulin resistance.

GSTA4 expression in murine and human systems. To investigate the mechanism underlying reduced GSTA4 expression in insulin resistant adipocytes, we assessed GSTA4 mRNA expression in response to a variety of hormones and metabolites utilizing the 3T3-L1 cell culture system. Treatment of 3T3-L1 adipocytes with the pro-inflammatory cytokine tumor necrosis factor-alpha

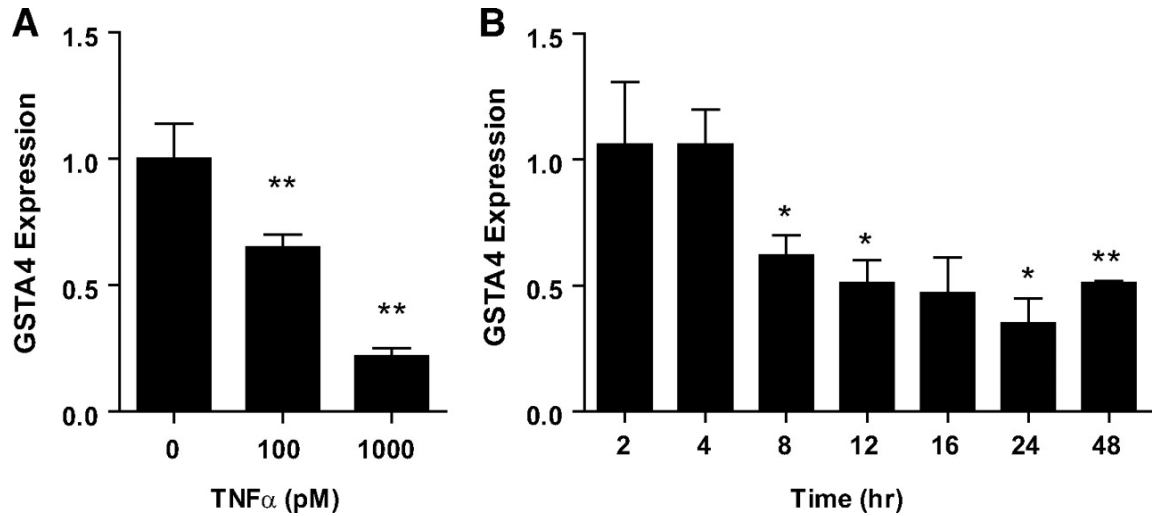


Figure 2. Effect of TNF α treatment on GSTA4 expression in 3T3-L1 adipocytes. qPCR analysis of GSTA4 mRNA expression in day 8 3T3-L1 adipocytes normalized to TFIIIE as a function of (A) TNF α level after 24 hours or (B) time of treatment with 1 nM TNF α . * P < 0.05; ** P < 0.01 relative to control samples.

(TNF α) resulted in a time and concentration dependent decrease in GSTA4 expression. After 24 hr treatment, GSTA4 mRNA expression was reduced 40% with 100 pM TNF α and 80% with 1 nM TNF α (Figure 2A). No further reduction of GSTA4 expression was obtained with 10 nM TNF α (data not shown). The reduced GSTA4 expression was observed as early as 8 hours with 1 nM TNF α treatment (Figure 2B).

To assess the expression of GSTA4 in human obesity and insulin resistance, we evaluated GSTA4 expression in omental (OM) and subcutaneous (SC) adipose from obese diabetics and non-diabetics and compared it to lean counterparts using microarray analysis (22). Interestingly, GSTA4 expression in adipose tissue of humans was decreased in the obese insulin resistant population relative to lean and obese insulin sensitive individuals (Figure 3A) and was verified using real-time PCR (results not shown). Moreover, the decrease in GSTA4 mRNA was evident in both the subcutaneous and omental fat depots. There was no correlation between GSTA4 expression and body mass index (Figure 3B) but a statistically significant negative correlation between GSTA4 levels and HOMA-IR (homeostatic model assessment of insulin resistance) (Figure 3C). Evaluation of GSTA3 expression revealed no relationship to HOMA-IR or body mass index (results not shown).

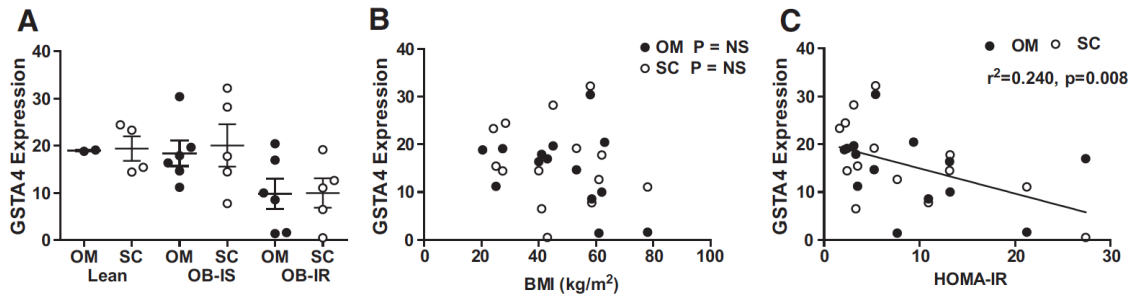


Figure 3. Expression of human GSTA4 in obesity and insulin resistance. (A) Relative expression of GSTA4 in omental (OM) and subcutaneous (SC) white adipose tissues of patients characterized as lean insulin sensitive (Lean), obese insulin sensitive (OB-IS) or obese insulin resistant (OB-IR). Data are expressed using means \pm SEM. (B) Correlation of GSTA4 mRNA expression in OM or SC adipose with patient body mass index (BMI) in kg/m². (C) Correlation of GSTA4 mRNA expression in OM or SC adipose with HOMA-IR, calculated as [insulin (uU/mL) x glucose (mmol/L)]/22.5. Each data point represents one individual.

Generation of *GSTA4* silenced adipocytes. Since *GSTA4* is central to 4-HNE detoxification and metabolism and carbonylation is increased with obesity (15,39), we evaluated whether decreased *GSTA4* expression directly leads to increased protein carbonylation. To that end, 3T3-L1 fibroblasts were transduced with shRNA directed against *GSTA4* mRNA or nonspecific scrambled control sequence to establish *GSTA4* knockdown (Kd) and Scrambled (Scr) cell lines. Although several distinct shRNA sequences were analyzed for *GSTA4* silencing, one line with ~60-70% decrease in *GSTA4*, comparable to that observed in the animal system, was chosen for detailed assessment (Figure 4A), some measures were confirmed in other silenced cell lines. While *GSTA3* was not expressed in the preadipocytes at an appreciable level, its expression increased during preadipocyte differentiation (data not shown) and was upregulated ~ 2-fold in *GSTA4* knockdown adipocytes compared to Scr adipocytes (Figure 4A).

GSTA4 knockdown 3T3-L1 cells differentiated normally and expressed adipocyte marker proteins peroxisome proliferator-activated receptor γ (PPAR γ), CCAAT/enhancer binding protein β (CEBP β), adipocyte fatty acid-binding protein (A-FABP/aP2), b-actin, and lipoprotein lipase (LPL) to the same extent as Scr cells. Interestingly, expression of the fatty acid translocase CD36 was upregulated ~2-fold in the *GSTA4* Kd adipocytes (Figures 4B).

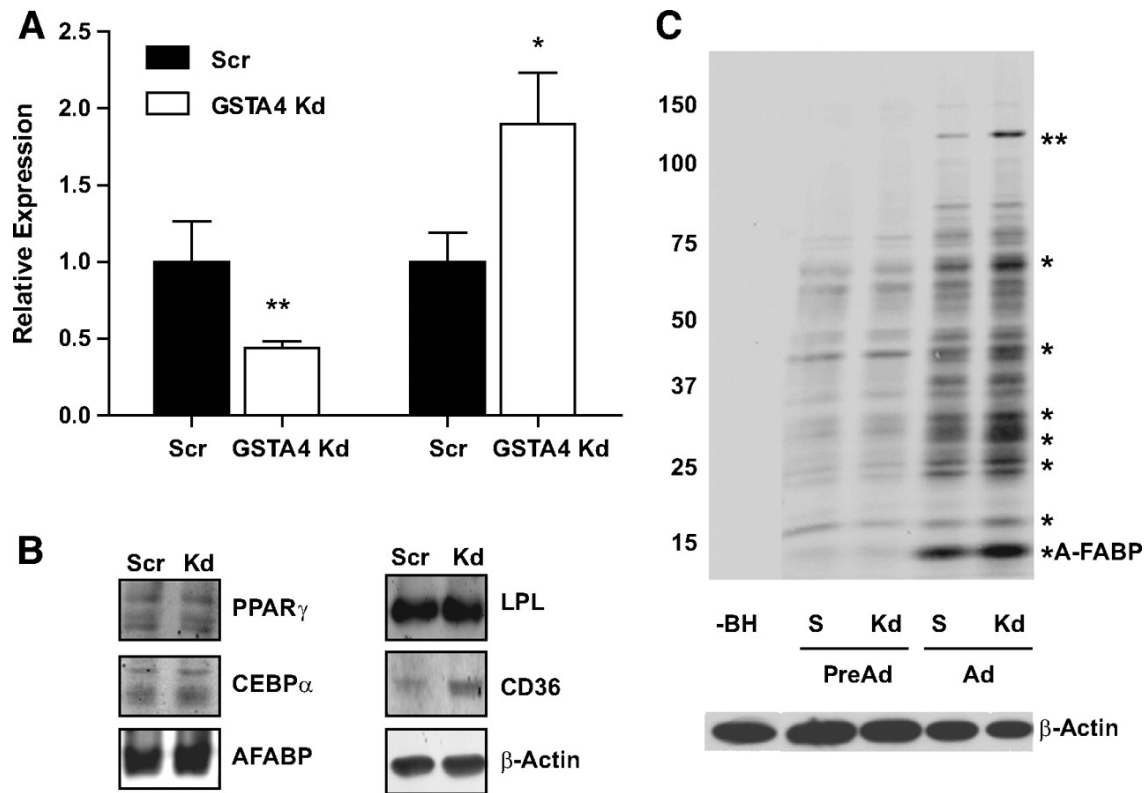


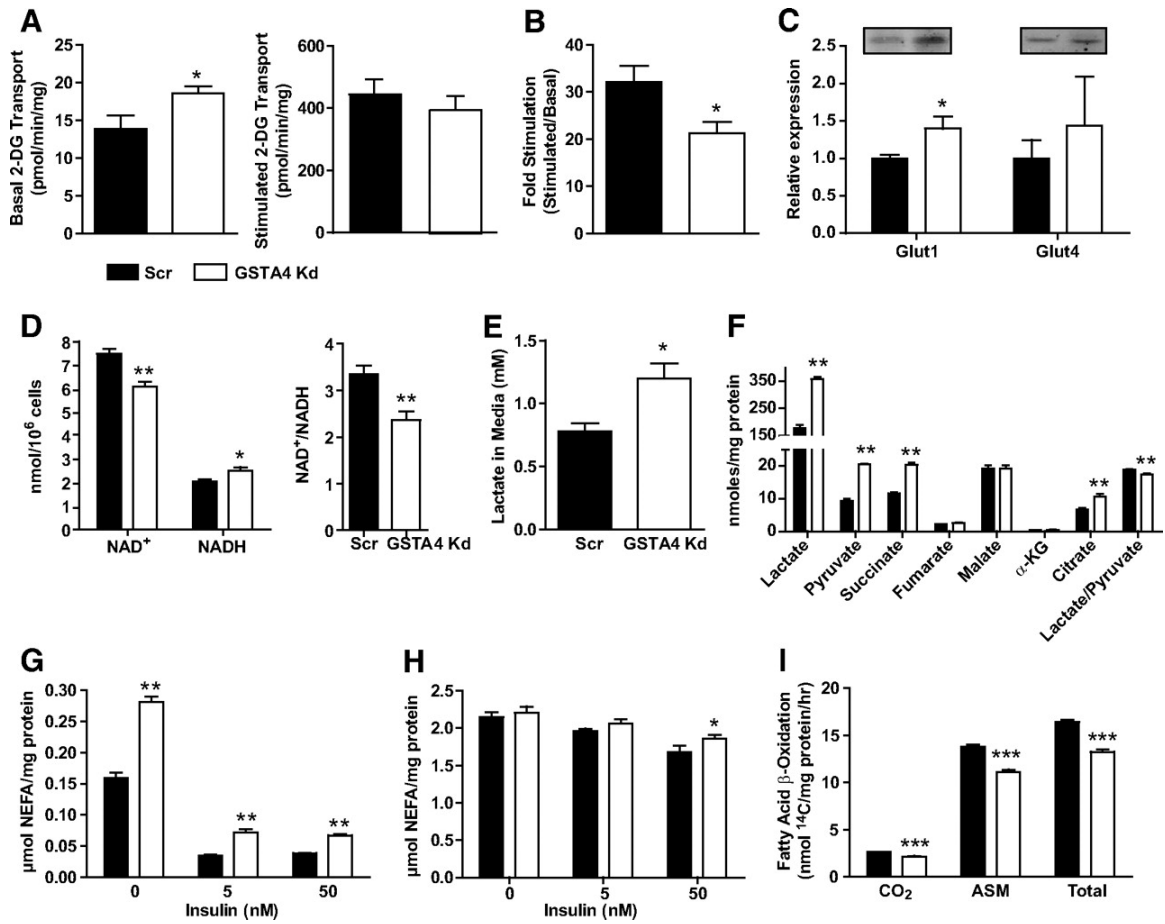
Figure 4. GSTA4 silencing and protein carbonylation in 3T3-L1 adipocytes. (A) Relative levels of GSTA4 and GSTA3 mRNA were quantified by qPCR (n=6 per group). GSTA4 expression was normalized to TFIIE and GSTA3 expression was normalized to 36B4. (B) Expression of adipogenic marker proteins (n=6 per group). (C) Protein carbonylation in GSTA4 Kd and Scr control cells. Protein bands found to have increased carbonylation in the GSTA4 Kd adipocytes are indicated (*). The ~145 kDa band (**) was digested and subjected to LC-ESI MS/MS for protein identification detailed in Table S2. S; Scrambled, -BH; minus biotin hydrazide. Figure 4C is a composite where the -BH lane has been moved from the same digital image (at the same exposure) to be adjacent to the experimental lanes.

Protein carbonylation in GSTA4 knockdown and Scr control cells was assessed by biotin hydrazide modification (Figure 4C) (15). Adipocytes exhibited a ~3-4-fold increase ($P < 0.01$) in total protein carbonylation relative to preadipocytes for both GSTA4 knockdown and Scr cell lines ($n = 4$). Increased carbonylation of specific proteins was noted in GSTA4 Kd relative to Scr control adipocytes. The prominent band near ~15 kDa in adipocytes has previously been identified as the adipocyte fatty acid binding protein (aP2) (15). Another protein at ~145 kDa (**) exhibited a ~2-3-fold increase ($P < 0.01$) in carbonylation in the GSTA4 Kd cells. This band was excised from the gel, digested with trypsin and the peptides sequenced by liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI MS/MS). The resultant peptides (nine unique peptides representing 8% sequence coverage) identified the protein as xanthine dehydrogenase (Data not shown).

Altered glucose and lipid metabolism in GSTA4 silenced adipocytes. To determine the influence of increased protein carbonylation on adipocyte function we examined a variety of metabolic parameters linked to glucose and lipid metabolism. GSTA4 Kd adipocytes exhibited a significant increase in basal glucose transport resulting in a net decrease in insulin-stimulated hexose uptake (Figures 5A and 5B). Western analysis indicated increased expression of the basal glucose transporter GLUT1 (Figure 5C), but not the insulin-responsive glucose transporter, GLUT4. Consistent with increased hexose metabolism,

Figure 5. Glucose and lipid metabolism in GSTA4 Kd and Scr adipocytes.

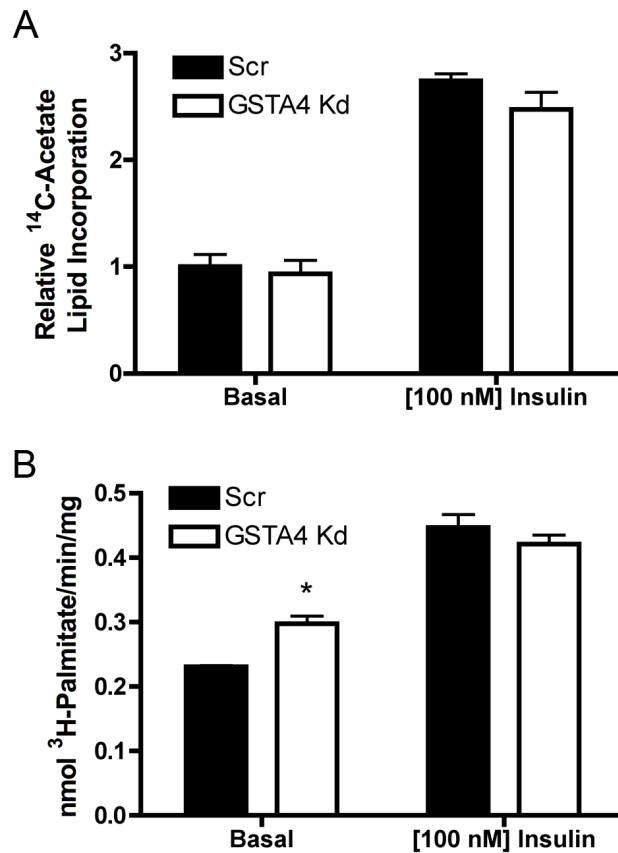
(A) 2-deoxyglucose (2-DG) transport in GSTA4 Kd and Scr adipocytes under basal (left) and 100 nM insulin stimulated (right) conditions. (B) Fold stimulation of hexose transport in GSTA4 Kd and Scr 3T3-L1 adipocytes (n=9). (C) Expression of glucose transporters GLUT1 and GLUT4 (n=6 per group). (D) NAD⁺, NADH and NAD⁺/NADH in day 7 GSTA4 Kd and Scr adipocytes (n=6). (E) L(+)-Lactate in the medium of GSTA4 Kd and Scr cells (n=6). (F) Organic acids from GSTA4 Kd and Scr adipocyte cell lysates. Basal (G) and forskolin-stimulated (H) lipolysis in Scr and GSTA4 Kd 3T3-L1 adipocytes. (I) β -Oxidation of [14C]-palmitate in Scr and Kd adipocytes (n=6). Panels G & H analyzed by two-way ANOVA with Bonferroni post-hoc analysis. * P < 0.05; ** P < 0.01.



analysis of reduced coenzyme levels revealed that the ratio of NAD⁺/NADH was significantly lower in GSTA4 Kd cells (Figure 5D) and the culture medium of GSTA4 knockdown cells was slightly acidic (results not shown) due to a 65-70% increased level of L (+)-lactate compared to scrambled control cells (Figure 5E).

Since tricarboxylic acid cycle (TCA) enzymes are targets of protein carbonylation and are inactivated by the addition of 4-HNE (40,41), GSTA4 knockdown and Scr adipocytes were assayed for small organic acids by direct injection tandem mass spectrometry (26). GSTA4 Kd adipocytes had significantly increased ($P < 0.01$) levels of intracellular lactate, pyruvate, succinate, and citrate (Figure 5F). The ratio of lactate to pyruvate was also significantly decreased in GSTA4 knockdown adipocytes relative to Scr control cells and in sum suggested that multiple steps in the tricarboxylic acid cycle may be compromised due to the silencing of GSTA4 and increased protein carbonylation.

To profile lipid metabolism in GSTA4 silenced 3T3-L1 adipocytes, we evaluated ¹⁴C-acetate incorporation into lipid pools as a measure of *de novo* lipogenesis. GSTA4 Kd adipocytes exhibited no difference in *de novo* lipogenesis under basal or insulin stimulated conditions relative to scrambled control cells (Figure S1A). However, consistent with elevated CD36 expression (Figure 4B), fatty acid uptake measured by ³H-palmitate influx was slightly, but significantly increased under basal (but not insulin stimulated) conditions in GSTA4 Kd adipocytes

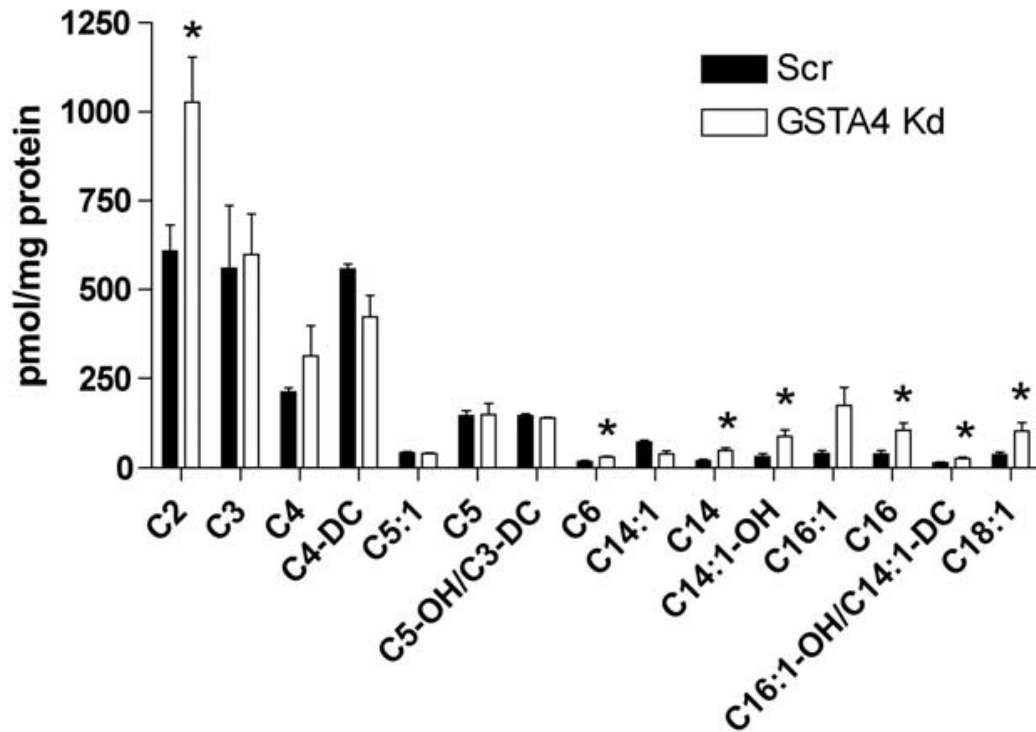


Supplemental Figure 1: Lipid Metabolism in GSTA4 Kd and Scr 3T3-L1 Adipocytes. (A) Basal and insulin stimulated *de novo* lipogenesis. Cells were serum starved for 2 h in KRH plus 5 mM glucose, stimulated with insulin during last 20 min, and incubated with 5 mM ^{14}C -acetate. Chloroform: methanol extraction was performed, and incorporation of ^{14}C into the organic layer (lipid) was measured. (B) Fatty acid uptake was assayed after 2 h serum starvation plus 0.1% fatty acid-free BSA, with or without a 20 min insulin stimulation. 10 mM ^3H -palmitate was added and washed away after 1 min. Wash buffer contained 0.2 mM phloretin, an analog that inhibits fatty acid uptake.

(Figure S1B). Although both basal glucose and fatty acid transport are increased in GSTA4 Kd cells, total lipid storage was unchanged. We therefore analyzed lipolysis of non-esterified fatty acids from GSTA4 Kd and Scr adipocytes. Silencing GSTA4 led to a ~50% increase in basal lipolysis, even in the presence of insulin (Figure 5G). Insulin suppression of forskolin-stimulated lipolysis was slightly blunted in GSTA4 Kd adipocytes (Figure 5H). These results were confirmed in separate GSTA4 Kd 3T3-L1 cell lines generated from different shRNA sequences (data not shown).

To assess mitochondrial β -oxidation, the conversion of radiolabeled palmitate to CO_2 and soluble metabolites was evaluated (33). GSTA4 knockdown adipocytes exhibited decreased oxidation of [1- ^{14}C]-palmitate as determined by the production of ^{14}C -labeled CO_2 (complete oxidation) and ^{14}C -labeled acid soluble metabolites (ASM, incomplete oxidation) (Figure 5I). Mass spectrometry based analyses were employed to detect changes in the acyl-carnitine profile of GSTA4 silenced adipocytes (Figure S2) and revealed that acetyl-carnitine (C2) and several other long chain acyl-carnitine species were elevated in GSTA4 Kd cells.

Silencing of GSTA4 expression leads to impaired respiration. Given the altered glucose metabolism and TCA cycle intermediates, we assessed mitochondrial respiration in GSTA4 Kd and control cells. GSTA4 silencing had a significant effect ($p=0.017$) on oxygen consumption rate as assessed by two-way



Supplemental Figure 2: Acyl-carnitine profiling. GSTA4 knockdown (Kd) and scrambled (Scr) 3T3-L1 adipocytes (day 7) were pre-incubated for 24 h with 50 μ M L- carnitine, washed with ice-cold PBS and rapidly frozen. Cells were scraped and sonicated in 500 μ L of H₂O and soluble lysates analyzed for acyl carnitine content by quantitative mass spectrometry (26). * $p < 0.05$

ANOVA with Bonferroni post-hoc analysis. Whereas control 3T3-L1 adipocytes exhibited robust state 2 respiration and an increase in state 3 respiration following addition of ADP, GSTA4 Kd mitochondria displayed ~2-fold decrease in state 2 respiration and no increase in oxygen consumption following addition of ADP (Figures 6A-C). As mitochondrial dysfunction is often coupled with increased reactive oxygen species generation, we evaluated superoxide anion production in isolated mitochondria (21). Silencing GSTA4 resulted in a ~3-fold increase in superoxide production in the mitochondrial matrix (Figure 6D). However, whole cell ROS evaluated by the fluorescent probe chloromethyl-H₂DCFDA was not changed in GSTA4 knockdown compared to Scr control cells (data not shown), suggesting that increased oxidative stress is centered on the mitochondrion and not a property of the entire cell.

In the muscle, insulin resistance is characterized by not only loss of mitochondrial function, but also mitochondria protein and DNA leading to reduced numbers of functional organelles (42,43). As such, we evaluated markers of mitochondrial biogenesis at the mRNA, protein and DNA level. The expression of the key transcription factors Nrf1 and Tfam as well as the central co-factor PGC1 α are all reduced in GSTA4 silenced adipocytes as well as the expression of mitochondrial proteins Cox IV and cytochrome c (Figure 7A and B). Interestingly, MnSOD and UCP2 expression were not affected by GSTA4 silencing. In the GSTA4 knockdown cells, eNOS expression was down regulated ~ 50% suggesting that

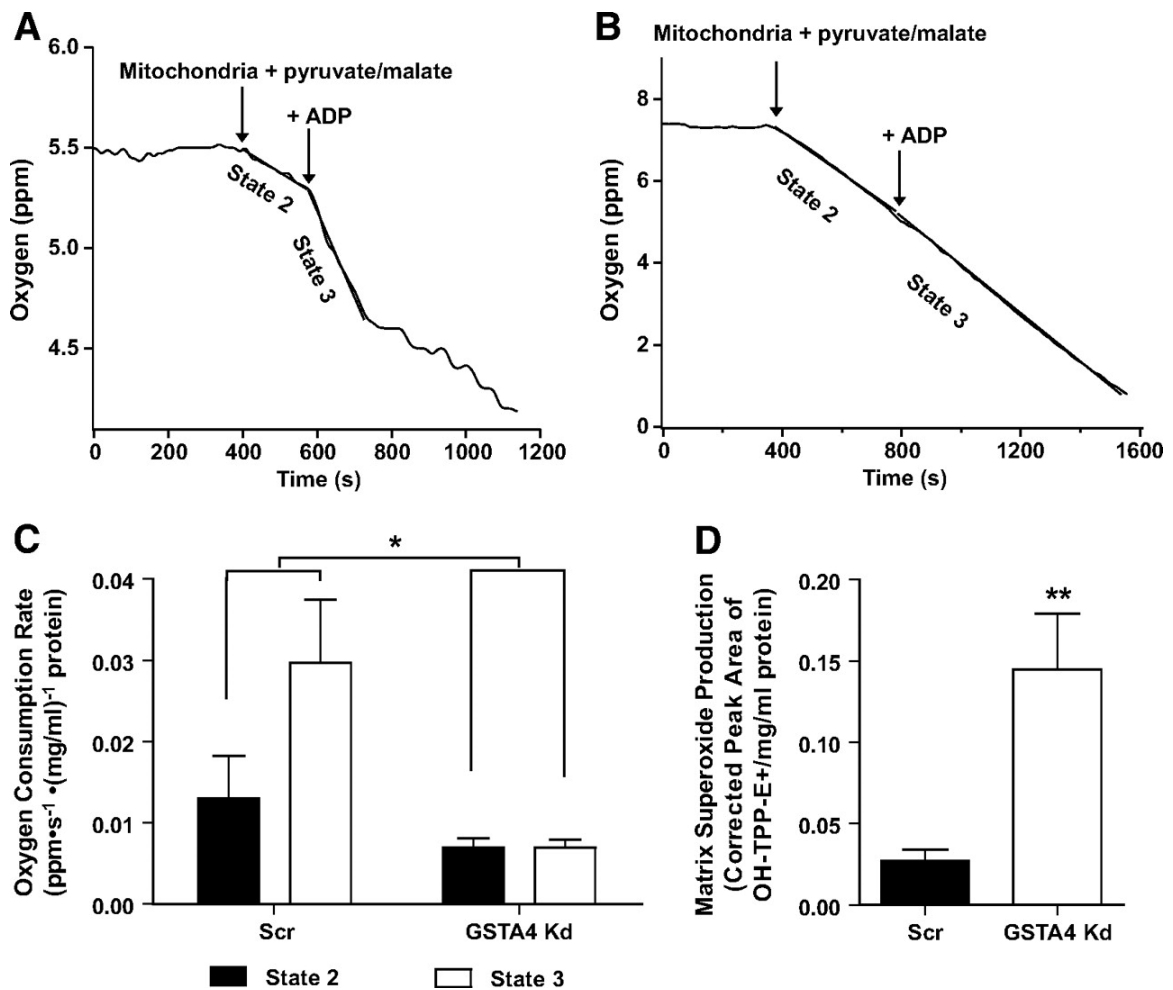


Figure 6. Mitochondria function in GSTA4 Kd and Scr 3T3-L1 adipocytes.

Mitochondrial oxygen consumption in Scr (A) or GSTA4 Kd (B) adipocytes. (C) Oxygen consumption rates for Scr and GSTA4 Kd adipocytes (n=3). Statistics calculated by two-way analysis with Bonferroni post-hoc analysis. (D) Mitochondrial matrix superoxide production in GSTA4 Kd and Scr adipocytes (n=3).

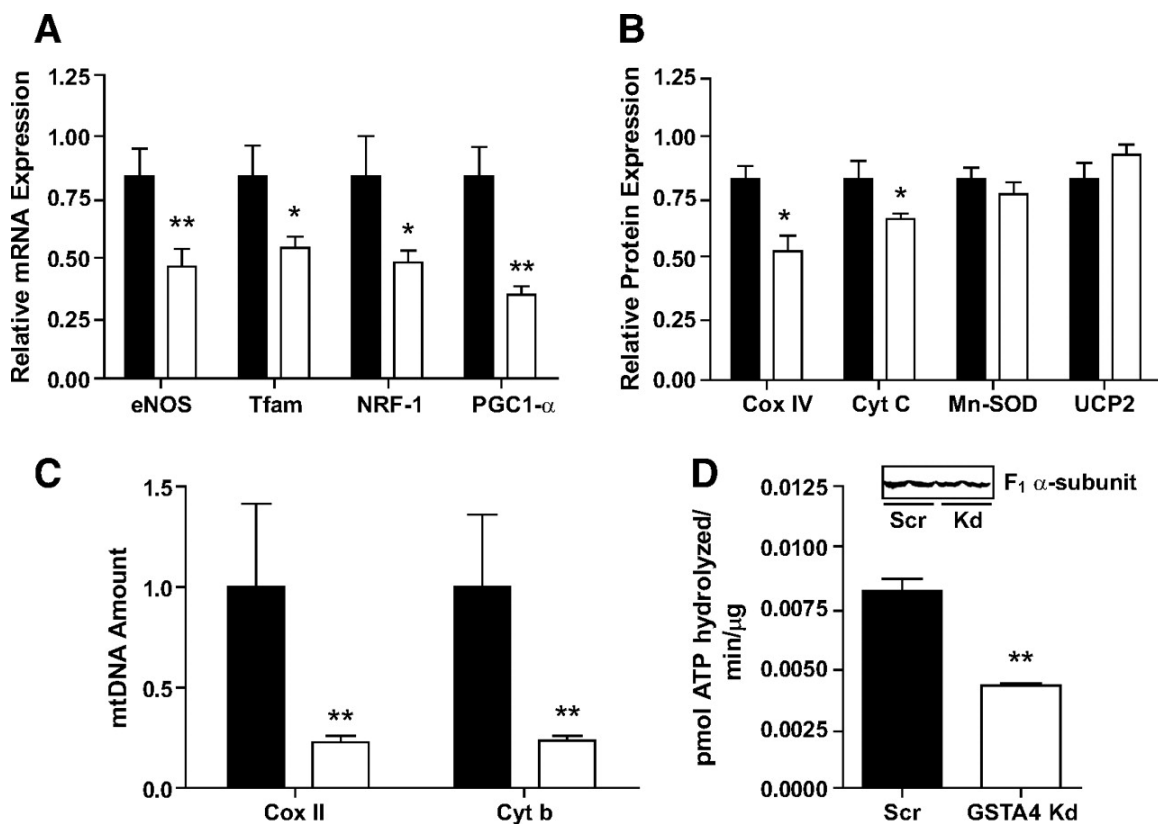
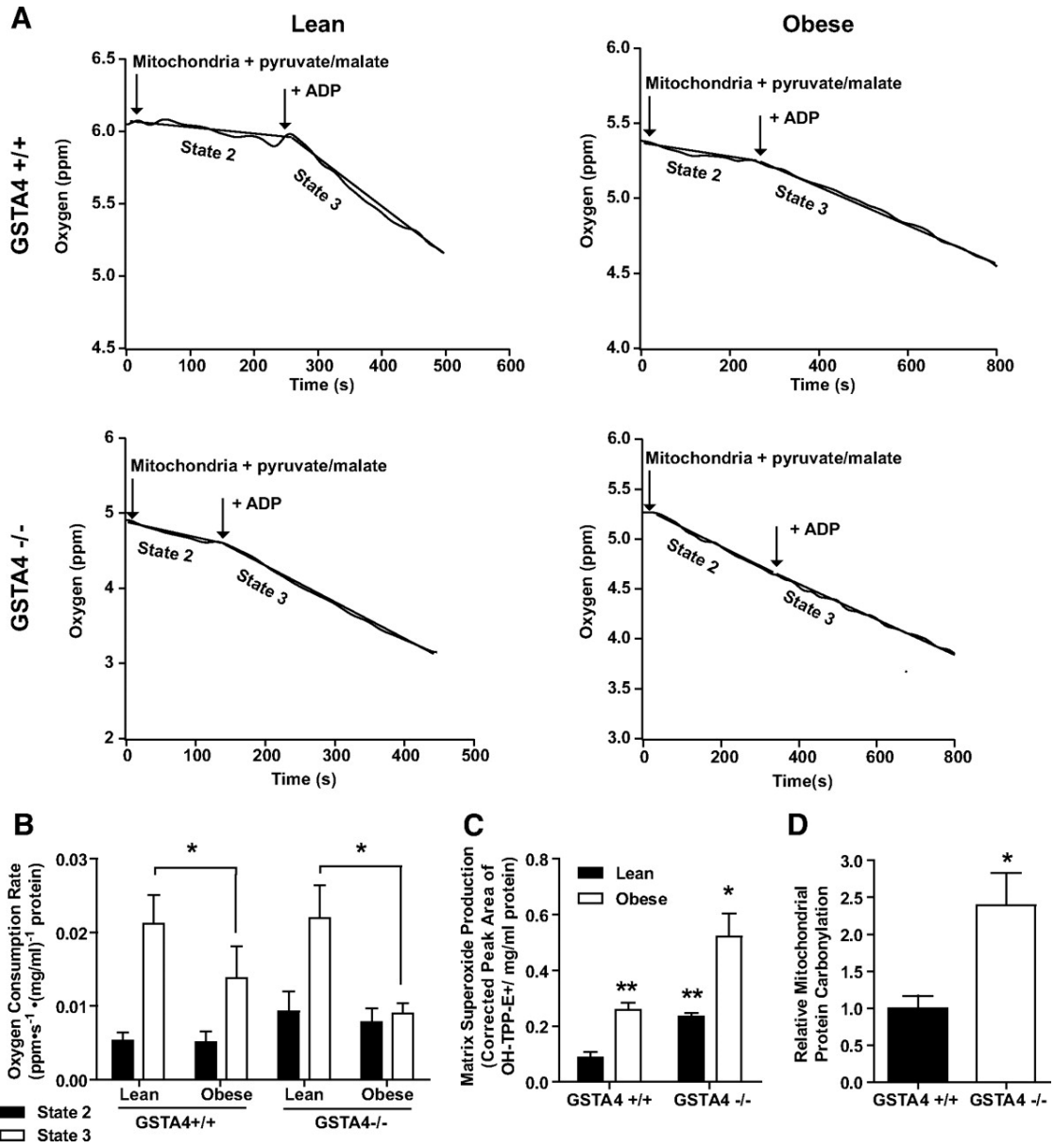


Figure 7. Expression of genes and proteins linked to mitochondrial biogenesis. (A) Expression of transcription factors and target mRNA in GSTA4 silenced (open bars) and scrambled (closed bars) adipocytes (n=6). (B) Mitochondrial protein expression in GSTA4 Kd and Scr adipocytes (n=3-6). (C) Expression of COX II and Cytb DNA relative to UCP2 DNA in GSTA4 Kd and Scr adipocytes (n=6). (D) Activity of F₀F₁ ATPase in GSTA4 silenced and scrambled adipocytes and level of ATP synthase alpha- subunit protein (n=3). * P < 0.05; ** P < 0.01

increased protein carbonylation initiates a cascade of events that links to the entire mitochondrial biogenesis pathway (43). Consistent with this, the abundance of the COX II and Cyt b (mitochondrial) genes relative to UCP2 (nuclear) gene was significantly reduced (Figure 7C). Paralleling the reduction in state 3 respiration, Figure 7D shows that the activity of the ATP Synthase was reduced 50% in the GSTA4 silenced adipocytes.

To determine if mitochondrial dysfunction in the GSTA4 silenced 3T3-L1 cells was also exhibited in animal systems, respiration was evaluated in mitochondria isolated from male GSTA4 null (-/-) mice and wild type (+/+) littermates maintained on standard chow (lean) or high fat diet (obese). Lean or obese GSTA4 null mice exhibited no significant difference in body weight relative to wild type controls (34.2 +/- 3.3 g versus 38.6 +/- 4.0 g, chow diet; 45.6 +/- 6.9 g and 49.2 +/- 9.0 g, high fat diet). No changes in fasting glucose or insulin levels were found between wild type and GSTA4 null mice (data not shown). Figures 8A & 8B show that in wild type C57Bl/6J mice, the lean to obese transition results in little change in state 2 respiration but a decrease in state 3 respiration, coincident with the down regulation of GSTA4. Interestingly, GSTA4 null mice exhibited a trend towards increased state 2 respiration relative to wild type animals. The state 3 oxygen consumption rate of mitochondria from lean GSTA4 null mice was comparable to wild type, however, similar to mitochondria from GSTA4 silenced adipocytes, mitochondria from obese GSTA4 null mice displayed no increase in

Figure 8. Mitochondrial function and expression in adipose tissue from C57Bl/6J and GSTA4 -/- mice. Mitochondria were isolated from EWAT of 4-5 month-old mice maintained on a standard chow (Lean) or high fat (Obese) diets. (A) Mitochondrial oxygen consumption in lean and obese wild-type (+/+) and GSTA4 null (-/-) adipose. The mitochondrial protein concentrations were 0.22 (lean +/+), 0.23 (obese +/+), 0.20 (lean -/-) and 0.23 (obese -/-) mg/mL. (B) Oxygen consumption rates for all four groups (n =3). Statistics calculated using two-way ANOVA for each state with Holm-Sidak post-hoc analysis. Variation due to loss of GSTA4 in State 2: $p=0.1$; variation due to obesity in State 3: $p=0.03$ (C) Mitochondrial matrix superoxide production (n=3). Statistics calculated using two-way ANOVA with Bonferroni post-hoc analysis. Effect of obesity: $p=0.0009$; effect of GSTA4: $p=0.0018$. (D) Quantitation of mitochondrial protein carbonylation in EWAT from lean wild type and GSTA4 null mice (n=6). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.



oxygen consumption in response to ADP. Accompanying the changes in respiration, adipocyte mitochondria from wild type mice produced 2-3 fold more matrix superoxide in the obese state compared to lean controls. Adipocyte mitochondria from GSTA4 null mice exhibited markedly increased matrix superoxide in the lean state compared to wild type controls and an even greater level of superoxide in the obese state (Figure 8C). These findings are consistent with published reports that defects in oxidative phosphorylation leads to increased matrix superoxide (44) and are likely consequence of elevated protein carbonylation in the mitochondria of EWAT from GSTA4 null mice (Figure 8D). Unlike the GSTA3 up regulation in the GSTA4 Kd 3T3-L1 cell culture model, there was no significant change in expression of GSTA3 or other glutathione S-transferases or dehydrogenases linked to 4-HNE metabolism in the GSTA4 null mice (Figure S2A).

DISCUSSION

The work described herein profiles the systemic metabolic events associated with down regulation of GSTA4 that initiate with carbonylation of cellular proteins. Protein carbonylation is a chemical event with multiple protein targets alkylated under conditions of increased oxidative stress, and the biological outcomes in a cell or tissue are not likely to be based solely on one specific protein modification event (45). As such, a systems-wide approach is needed to characterize functional consequences of carbonylation. Our previous work characterized carbonylated polypeptides in adipose tissue using proteomic methods (15) and this report extends those findings to metabolic outcomes. The results presented identify GSTA4 as a key determinant of adipocyte protein carbonylation and mitochondrial function and suggest that its activity may be a protective factor against metabolic dysfunction.

In humans, GSTA4 is down regulated in omental and subcutaneous depots of obese, insulin resistant but not obese, insulin sensitive individuals (Figure 3). Similarly, GSTA4 is down regulated specifically in adipose tissue from obese, insulin resistant C57Bl/6J mice (15,37), in ob/ob animals (Figure 1D) or in TNF α -treated 3T3-L1 adipocytes (Figure 2). Previous work by Awasthi, Zimniak and colleagues has shown GSTA4 null mice have increased levels of 4-HNE, reduced antioxidant capacity, increased apoptosis and JNK activation (11,46). Although GSTA4 expression was down regulated in obese female mice its basal

expression was more robust than in males (Figure 1B) raising the possibility that increased levels of GSTA4 protect female mice from the harmful effects of lipid peroxidation products and may contribute to the attenuated insulin resistance characteristics observed in female C57Bl/6J mice (47).

While silencing GSTA4 in adipocytes also led to overall increased protein carbonylation, one protein target identified was xanthine dehydrogenase (Figure 3D). *In vitro*, xanthine dehydrogenase is converted from its dehydrogenase form to its oxidoreductase form by modification of cysteine residues (48). Such modification of xanthine dehydrogenase by 4-HNE or other lipid aldehydes could lead to an increased oxidoreductase activity of the enzyme and the production of superoxide anion, amplifying oxidative stress signaling. Interestingly, xanthine dehydrogenase is known to be converted to xanthine oxidoreductase in response to mitochondrial damage *in vivo* (49), providing a link between mitochondrial dysfunction and oxidative stress.

Consistent with elevated lactate, the increased basal glucose transport by GSTA4 knockdown adipocytes could be a compensatory response to diminished mitochondrial function. In muscle cells, mitochondrial dysfunction has previously been linked to increased basal glucose transport (50) and obese human type 2 diabetic patients also exhibit increased basal 2-deoxyglucose transport in skeletal muscle cells (20,51). Increased GLUT1 expression in GSTA4 Kd

adipocytes (Figure 5C) is consistent with previous reports of increased glucose transport via GLUT1 during treatment of epithelial cells with inhibitors of mitochondrial oxidative phosphorylation (52) as well as reports of oxidative stress-induced increases in basal glucose transport in adipocytes (3). Increased basal glucose transport may provide sufficient ATP for metabolism and signaling since the levels of total and phospho-AMPK were not altered in the GSTA4 silenced adipocytes (data not shown).

Insulin resistance is correlated with increased basal lipolysis (53) and GSTA4 knockdown adipocytes exhibited ~50% increased basal lipolysis in the presence or absence of insulin (Figure 5G). Surprisingly, GSTA4 knockdown adipocytes had no change in total triglyceride accumulation (results not shown) potentially due to increased fatty acid uptake. Consistent with this, the expression of CD36 protein, a plasma membrane fatty acid- transporter whose expression is induced by the 4-HNE responsive transcription factor Nrf2 (54) was significantly increased in the GSTA4 Kd adipocytes (Figure 4B).

The increase in key organic anion intermediates suggests that the flux through the tricarboxylic acid cycle is attenuated. Previous proteomic data has identified multiple TCA cycle enzymes as well as complexes I-IV of the oxidative phosphorylation machinery as targets of carbonylation (45). The decrease in [1-¹⁴C]-palmitate oxidation (Figure 5I) indicates that multiple steps in lipid oxidation

and/or metabolism of acetyl CoA is reduced in the GSTA4 silenced cells. This is supported by mass spectrometry data that shows elevated levels of several acyl-carnitine species (Figure S1) and increased acetyl-carnitine suggests that acetyl-CoA is accumulating. As such, increased protein carbonylation in the mitochondrion is likely to lead to decreased functions such as tricarboxylic acid cycle and fatty acid oxidation (40,55).

A major finding of this study is that respiration is markedly altered in GSTA4 Kd adipocytes (Figure 6) and in adipocytes of both lean and obese GSTA4 null mice (Figure 8). In the cell culture system, silencing of GSTA4 led to diminished oxygen consumption (Figure 6C) and increased NADH levels (Figure 5D) suggesting electron transfer through the electron transport chain is impaired. Moreover, there was no increase in oxygen consumption in response to ADP in mitochondria from the GSTA4 silenced adipocytes. Consistent with this, the activity of ATP synthase was decreased $\sim 50\%$ in the GSTA4 silenced adipocytes. These observations may be due to a combination of factors including increased proton leakage across the inner mitochondrial membrane, carbonylation of Complex V proteins affecting the ability to couple the proton gradient to ATP production, the carbonylation of the adenine nucleotide translocator (ANT), or carbonylation-dependent changes in the abundance of critical proteins linked to state 3 respiration. Indeed, under certain conditions 4-HNE can facilitate proton leak in other cell types through ANT or uncoupling

proteins (56,57).

In the GSTA4 null adipocytes, changes in both state 2 and state 3 respiration were similar, but not identical to the 3T3-L1 GSTA4 knockdown system. In the animal model, mitochondria from lean wild type C57Bl/6J mice exhibit robust state 2 respiration that increased with the addition of ADP. Obese C57Bl/6J mice exhibited no change in state 2 respiration relative to lean counterparts, but had attenuated ADP-coupled oxygen consumption, potentially due to the down regulation of GSTA4. In GSTA4 null animals, mitochondria from lean and obese mice exhibited a trend towards increased state 2 respiration compared to the wild type animals, possibly due to increased proton leakage. Indeed, previous studies by Eschetay et al. (56) have suggested that carbonylation of uncoupling proteins may under certain circumstances lead to increased proton leak thereby providing for increased state 2 respiration. Similar to the GSTA4 silenced cells, mitochondria from obese GSTA4 null exhibited virtually no increase in respiration with ADP (Figures 8A & B).

Associated with down regulation of GSTA4 is decreased expression of eNOS, Nrf1, PGC1a and Tfam, critical regulators of mitochondrial biogenesis (Figure 7). Indeed, GSTA4-silencing resulted in loss of mtDNA and decreased expression of CoxII and Cyt c. Work by Nisoli and colleagues have focused on eNOS as the key regulator of TNF α action (43) and that if eNOS activity is lost from adipocytes,

the entire program of mitochondrial activation and biogenesis is affected (58). These data suggest that down regulation of GSTA4 is upstream of eNOS regulation and may be mechanistically regulated by protein carbonylation.

In sum, the results presented herein focus on the role of GSTA4 as an antioxidant enzyme primarily responsible for elimination of reactive electrophiles from adipocytes. The results herein suggest a model whereby down regulation of GSTA4 by pro-inflammatory cytokines results in increased protein carbonylation, altered glucose and lipid metabolism, decreased mitochondrial β -oxidation, TCA cycle activity, electron transport and respiration. Thus, protein carbonylation in white adipose tissue may provide a molecular mechanism linking increased oxidative stress to metabolic dysfunction associated with insulin resistance.

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CHAPTER 3

Protein Carbonylation and Adipocyte Mitochondria Function

Jessica M. Curtis, Wendy S. Hahn, Matthew D. Stone, Margaret A. Donoghue, Eric K. Long, Anibal G. Armien, Edgar Arriaga, Timothy J. Griffin, and David A. Bernlohr¹. (2011). *In preparation*.

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Carbonylation is the covalent, non-reversible modification of the side chains of cysteine, histidine and lysine residues by lipid peroxidation end products such as 4-hydroxy- and 4-oxononenal. The antioxidant enzyme glutathione S-transferase A4 (GSTA4) catalyzes a major detoxification pathway for such reactive lipids but its expression is selectively down regulated in the obese, insulin resistant adipocyte resulting in increased protein carbonylation. The effects of such modifications are associated with increased oxidative stress and metabolic dysregulation centered on mitochondrial energy metabolism. To address the role of protein carbonylation in the pathogenesis of mitochondrial dysfunction quantitative proteomics was employed to identify specific targets of carbonylation in GSTA4-silenced or overexpressing 3T3-L1 adipocytes. GSTA4-silenced adipocytes displayed elevated carbonylation of several key mitochondrial proteins including the phosphate carrier protein, NADH dehydrogenase 1 alpha subcomplexes 2 and 3, translocase of inner mitochondrial membrane 50, and valyl-tRNA synthetase. Elevated protein carbonylation is accompanied by diminished complex I activity, impaired respiration, increased superoxide production and a reduction in membrane potential without changes in mitochondrial number, area or density. These results suggest protein carbonylation plays a major instigating role in mitochondrial dysfunction and may be a linked to the development of insulin resistance in the adipocyte.

INTRODUCTION

Obesity is a positive risk factor for the development of insulin resistance and diabetes (1). Increased adiposity is often associated with chronic inflammation, altered glucose and lipid metabolism and oxidative stress (2). Inflammatory cytokines such as tumor necrosis factor alpha (TNF α) and interleukin-6 (IL-6) are produced by inflammatory macrophages that infiltrate adipose tissue and are elevated in the obese, insulin resistant state. Increased oxidative conditions in adipose tissue have been associated with increased reactive oxygen species (ROS) that may be causal in the development of insulin resistance (3).

The mitochondrion is a major source of reactive oxygen species. The transfer of electrons to molecular oxygen from the electron transport chain at sites other than Complex IV produces superoxide anion as a primary ROS species. The antioxidant enzyme manganese superoxide dismutase is abundantly expressed in mitochondria and efficiently metabolizes superoxide to hydrogen peroxide that is further reduced by enzymes such as catalase, glutathione peroxidase, and peroxiredoxin. However, in the presence of ferrous iron, hydrogen peroxide will oxidize to form hydroxide and hydroxyl radical. The hydroxyl radical initiates peroxidation of polyunsaturated acyl chains of glycerophospholipids and triacylglycerol leading to the production of α , β -unsaturated aldehydes such as 4-hydroxy- and 4-oxo *trans*-2,3 nonenal (4-HNE, 4-ONE) that are highly reactive towards nucleophilic addition (4). These reactive lipid species can be

metabolized through oxidative or reductive enzyme catalyzed reactions, but the predominant detoxification route is glutathionylation via glutathione S-transferase A4 (GSTA4) and subsequent cellular export of the glutathionylated lipid (5,6). If the aldehyde escapes detoxification, it can react with the protein side chains of cysteine, histidine, and lysine residues, in a reaction termed protein carbonylation.

Inflammatory cytokines down regulate GSTA4 expression in cultured white adipocytes and GSTA4 expression is similarly down regulated *in vivo* in white adipose tissue of obese insulin resistant C57Bl/6J mice. The down regulation of GSTA4 is specific for white fat (visceral and subcutaneous depots) and does not occur in brown fat, liver or muscle (7,8). Our previous studies indicated that the level of protein carbonylation is elevated in the obese adipocyte and is likely due to the downregulation of GSTA4 that occurs upon the transition from the lean, insulin sensitive state to the obese, insulin resistant condition. Such events lead to mitochondrial dysfunction and disruption of a number of metabolic pathways including β -oxidation, electron transport and the citric acid cycle. Despite the wide body of information concerning the metabolic effects of protein carbonylation in the adipocyte mitochondrion, the identification of mitochondrial targets has not been carried out. To that end, the current study was initiated to characterize mitochondrial targets of protein carbonylation and investigate how their protein function is altered. To specifically focus on protein carbonylation

events linked to regulation of GSTA4, we generated GSTA4 silenced and over expressing 3T3-L1 adipocyte cell lines and evaluated protein carbonylation via proteomic methods. We report here the identification of several differentially-carbonylated mitochondrial proteins and evaluate the potential functional impact of such modifications on mitochondrial function.

EXPERIMENTAL PROCEDURES

Materials – The pcDNA plasmid containing aP2 promoter and intron/polyA was kindly provided by Dr. Ormond MacDougald (University of Michigan, USA). Rabbit monoclonal anti-HA antibody was obtained from Cell Signaling Technologies (Danvers, MA). Anti β -actin mouse monoclonal and PiC (SLC25A3) mouse polyclonal antibodies was obtained from Sigma-Aldrich (St. Louis, MO). NDUFA3 mouse polyclonal antibody was obtained from Abcam (Cambridge, MA). IR-conjugated secondary antibodies used for immunoblotting were obtained from Odessey Imaging System (Licor Bioscience, Lincoln, NE). Other commercial materials were of the highest available quality.

Cell culture – 3T3-L1 cells were differentiated for 8 days using the standard methylisobutyl xanthine, dexamethasone, insulin protocol (9) supplemented with 1 μ g/mL troglitazone. GSTA4 silenced (Kd) and scrambled (Scr) control adipocytes were generated as described previously (7). aP2-HA-GSTA4 over expressing cells were generated by ligating the 8.2 kb mouse aP2/FABP4 promoter sequence upstream of the GSTA4 coding region containing a N-terminus hemagglutinin (HA) tag followed by the rabbit beta-globin intron/ polyA signal. The construct in pcDNA3.1 or pcDNA3.1 alone was transfected into 3T3-L1 fibroblasts using Effectene transfection reagent (Qiagen, Valencia, CA) according to manufacturers instructions and pooled cells selected for stable incorporation using 400 μ g/ml Geneticin (Gibco/Invitrogen, Carlsbad, CA) for 8

days. The culture media was supplemented with 1 $\mu\text{g/ml}$ blasticidin for GSTA4 Kd and Scr cells or 400 $\mu\text{g/ml}$ geneticin for aP2-HA-GSTA4 and pcDNA cells.

GSTA4 mRNA measurements – Adipocytes were lysed in Trizol (Invitrogen, Carlsbad, CA) and RNA was isolated according to manufacturer's protocol. cDNA was prepared using iScript cDNA Synthesis Kit (BioRad Hercules, CA) and the relative expression of GSTA4 mRNA measured by real-time PCR Syber Green Detection using the My iQ iCycler (Biorad, Hercules, CA). GSTA4 mRNA expression was normalized to TFIIE and reported relative to control groups using the $\Delta\Delta\text{Ct}$ method (10).

Enrichment of mitochondrial protein carbonyls with biotin hydrazide conjugation and avidin affinity chromatography – Crude mitochondria containing endoplasmic reticulum fragments were isolated in triplicate from each of the four 3T3-L1 adipocyte cell lines (GSTA4 kd and control, aP2-HA-GSTA4 and control) by differential centrifugation (11). Mitochondrial pellets were dissolved in biotin hydrazide coupling buffer (100 mM sodium acetate, 20 mM NaCl, 1% SDS, pH 5.5) and centrifuged at 13,000 x g for 10 min at 15° C. Mitochondria from three independent experiments were pooled and modified with biotin hydrazide. Biotin hydrazide (5 mM, Pierce, Rockford, IL) was added to 5 mg of mitochondrial proteins for 2 hours at room temperature to specifically modify aldehyde groups. Derivatized protein was diluted 1:10 in ice-cold phosphate-buffered saline (PBS)

and dialyzed against the same buffer at 4° C to remove excess biotin hydrazide. Avidin affinity columns (monomeric avidin agarose, Pierce, Rockford, IL) were washed with PBS, and 2 mM D-biotin was added to block non-reversible biotin binding sites. Excess biotin was removed with the addition of 0.1 M glycine, pH 2.8 and the column re-equilibrated into PBS. Samples were applied, the columns extensively washed and the bound biotinylated proteins eluted with 3 mL 0.1 M glycine, pH 2.8. The eluted proteins were neutralized with the addition of 750 µL of 2.5 M Tris, pH 8.5 and stored at -20° C until further processed.

iTRAQ™ peptide labeling and Nanoflow LC-MS/MS – 100 micrograms of affinity-captured biotinylated protein was denatured with SDS to 0.5%, reduced with 5 mM tris(2-carboxyethyl)phosphine, and free sulfhydryls modified with 20 mM methylmethane thiosulfonate. The solution was diluted 10-fold with buffer containing 1 mM CaCl₂ and proteins digested overnight at 37° C with 2 µg trypsin (Promega, Madison, WI). The resultant peptides were acidified with formic acid and purified by solid phase extraction using 3 mL MCX cartridges (Waters, Milford, MA).

For proteomic analysis, peptides were reconstituted in iTRAQ™ dissolution buffer and labeled with isobaric iTRAQ™ tags (Applied Biosystems, Carlsbad, CA) according to manufacturer's protocol. After labeling, the four samples were combined, re-purified with MCX cartridges, and further fractionated with strong

cation exchange (SCX) HPLC as described (12). SCX fractions having an average absorbance of at least 10 milliunits at 215 nm were selected (18 total) and processed for mass spectrometry.

Reversed-phase nanoHPLC and mass spectrometry was performed as previously described (12). Briefly, fragmentation was performed in optimized PQD mode (13) with normalized fragmentation energy of 31 and an activation time of 100 ms. Tandem mass spectra were acquired with 2 microscans with automatic gain control settings of 30,000 charges or 100 ms. Raw spectral data were extracted and converted to mzXML format using MSconvert from ProteoWizard. Data were evaluated using SEQUEST v27.0 against a concatenated forward and reversed mouse database (derived from UniProt mouse canonical and isoform from Aug. 2010) appended with common contaminants from <http://www.thegpm.org/crap/index.htm> totaling 47932 entries. Precursor and fragment ion tolerances were set at 1 and 0.8 amu. Semi-tryptic specificity was used with up to 2 missed cleavage sites. A fixed modification of 144.102 amu was set for Lys and peptide N-termini. Variable modifications included 15.9949 on Met and 394.2039 (4-HNE modification) on Cys, His, and Lys. Candidate identifications were organized and peptide probabilities (14) calculated using Scaffold v3 (www.proteomesoftware.com). Protein identifications were filtered to <1% false discovery rate by using the following filter criteria: 10 ppm precursor mass tolerance, >5% peptide probability, and at least 2 unique

peptides per protein identification. Relative iTRAQ™ reporter ion ratios and p-values were calculated for proteins with 3 or more matched spectra using LTQ-iQuant software (15). For comparison of experimental samples, GSTA4 Kd and aP2-HA-GSTA4 data were normalized to respective controls. Protein subcellular localization was determined with GO terms using <http://www.uniprot.org> and verified manually.

Adipocyte respiration – Adipocyte respiration was analyzed with the XF24 Extracellular Flux Analyzer (Seahorse Biosciences, Billerica, MA). 3T3-L1 fibroblasts were plated on V7 microplates coated with 0.2% gelatin, and differentiated into adipocytes. Eight days post differentiation, cells were washed and incubated at 37° C without CO₂ in bicarbonate-free DMEM containing 1.0 mM sodium pyruvate, 2.0 mM glutamax, and 25 mM D-glucose. During the assay, cell monolayers were exposed to 1 µg/ml oligomycin, 2.5 µM carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), and 4 µM antimycin A. Respiration rates calculated as previously described (16,17).

Electron microscopy – Adipocytes were grown in 6-well dishes on chemically resistant Thermanox coverslips (Ted Pella, Redding, CA). Eight days post-differentiation the cells were fixed on ice with 1 mL of 2.5% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) in 0.1 M sodium cacodylate overnight at 4° C. Cells on thermanox coverslips were washed three times with

0.1 M sodium cacodylate buffer and post fixed with 1% osmium tetroxide (Electron Microscopy Sciences, Hatfield, PA). An additional set of samples were treated with 1% tannic acid, postfixed with 1% osmium tetroxide, dehydrated with ethanol and embedded with Embed 812 resin (Electron Microscopy Sciences, Hatfield, PA). Embedded samples were sectioned on a Leica UC6 Ultramicrotome (Leica Microsystems, Vienna, Austria) stained, and analyzed using a JEOL 1200 EX II transmission electron microscope (JEOL LTD, Tokyo, Japan). Images were obtained using a Veleta 2K x2K camera with iTEM software (Olympus SIS, Munster, Germany). Seven cells were selected randomly and the number of mitochondria was counted in each cells. Perimeter and area were measured for 10 randomly selected mitochondria in each cell, using iTEM software (Olympus SIS, Munster, Germany).

Fluorescence microscopy – 3T3-L1 cells were differentiated on chambered 1.5 borosilicate coverglass (Lab Tek, Thermo Fisher Scientific, Rochester, NY) and adipocytes incubated with 50 nM tetramethylrhodamine methylester perchlorate (TMRM, Molecular Probes, Invitrogen, Carlsbad, CA) at 37° C for 30-90 min before washing and mounting in Hank's buffered salt solution for visualization. All images were captured at 37° C using a DeltaVision PersonalDV system (Applied Precision, Inc, Issaquah, WA) at 100x with identical settings, deconvolved, and projected with equal numbers of z-slices. For quantification of TMRM fluorescence intensity, cells were stained as described and imaged at 60x

on a Nikon Biostation IM that allowed time-lapse imaging of multiple points. Equal numbers of z-slices were projected and quantified in ImageJ (NIH).

Superoxide anion detection – Superoxide was detected in isolated mitochondria with triphenylphosphonium hydroethidine (TPP-HE) as described previously (18) but with modifications to internally control for membrane potential dependent probe accumulation using rhodamine 123 (19).

Enzyme assays – 3T3-L1 adipocytes were lysed in 50 mM NaPO₄, pH 6.5 containing 1 μM diethylene triamine pentaacetic acid, 250 μM butylated hydroxytoluene and protease inhibitors and centrifuged at 3,000x g for 5 minutes at 4° C. Each sample was diluted to a final protein concentration of 0.1 mg/mL in 50 mM sodium phosphate buffer pH 6.5 containing 1 mM diethylenetriaminepentaacetic acid, 250 μM BHT, 1 mM glutathione and 1 μM 4-HNE at room temperature. After two minutes, the reaction was stopped by acidification using a final concentration of 0.1% formic acid (20) and 20 μL of the reaction mixture analyzed by LC-MS/MS using an Agilent 1100 HPLC system coupled to an AB-Sciex API 2000Qtrap tandem mass spectrometer. Liquid chromatography was performed using 2.1 mm X 100 mm Agilent Zorbax Eclipse Plus C18 column with a 1.8 micron particle size. Glutathionylated-HNE (GS-HNE) was detected using negative mode electrospray ionization and multiple reaction monitoring. Authentic GS-HNE was synthesized as a standard and the

transition ions at 306.1 and 272.2 amu used for analysis as reported previously (20). GS-HNE was quantified by stable isotope dilution with GS-HNE-d3 as internal standard (Cayman Chemical, Ann Arbor, MI).

Complex I Activity was assessed as described previously but adapted to a microplate format (21). Briefly, Complex I transfers electrons from NADH to decylubiquinone that subsequently delivers them to dichlorophenylindole (DCIP). Reduction of DCIP is monitored spectrophotometrically at 600 nm. Isolated mitochondria were resuspended in 10 mM tris, pH 7.6 and assayed in buffer containing 25 mM KH_2PO_4 , 3.5 mg/ml fatty acid-free BSA, 60 μM DCIP, 70 μM decylubiquinone, and 1.0 μM antimycin A. The reaction was initiated with the addition of 0.2 mM NADH, and absorbance was measured over a period of 5 minutes.

Statistical Analysis – All values are expressed as mean \pm SEM. Statistical significance was determined using the two-tailed Student *t* test assuming unequal variances. *P* values ≤ 0.05 are considered significant (*) with an increased significance of *P* value ≤ 0.01 indicated (**).

RESULTS

Obesity-linked insulin resistance is correlated with mitochondrial dysfunction and occurs coincident with cytokine-mediated down regulation of key antioxidant genes, particularly that encoding GSTA4. GSTA4 catalyzes the glutathionylation of reactive aldehydes such as 4-HNE and 4-ONE and functions in an antioxidant capacity reducing oxidative stress and its downstream metabolic effects due to carbonylation. Previous results have demonstrated that selective loss of GSTA4 protein results in increased total protein carbonylation and mitochondrial dysfunction yet the mitochondrial targets of such modification have not been identified (7). To address the role of protein carbonylation in adipocyte mitochondrial dysfunction we utilized a strategy based on loss or gain-of-function of GSTA4. Because cytokine-treated cells exhibit a wide spectrum of effects and we wanted to focus exclusively on protein carbonylation, we developed cell lines with either reduced or augmented levels of GSTA4. We previously utilized a lentiviral-derived stable 3T3-L1 cell line harboring a shRNA targeting GSTA4 that reduces GSTA4 mRNA expression by ~ 70%, similar to the extent down regulated in murine adipose tissue of high fat fed mice. To produce an adipocyte cell line over expressing GSTA4 we stably transfected 3T3-L1 fibroblasts with HA-tagged GSTA4 under the control of the fat-specific promoter, aP2. Several cell lines were derived and one exhibiting stable elevated expression of HA-GSTA4 (as evaluated by immunoblotting for HA; antibodies monoselective for the murine GSTA4 isoform are not available) was selected for further study. The

aP2-HA-GSTA4 cells had approximately 8-fold higher mRNA expression of GSTA4 compared to control cells (Figure 1A) and expressed HA-GSTA4 protein similarly (Figure 1B). To verify the HA-tag had no impact on the ability of GSTA4 to metabolize 4-HNE, the *in vitro* enzyme activity was assessed by monitoring the synthesis of glutathionylated-HNE (GS-HNE) in a glutathione and 4-HNE dependent manner. Using cellular extracts from over expressing (aP2-HA-GSTA4) and control adipocytes (pcDNA), GS-HNE synthesis was measured by LC-MS/MS. Over expressing adipocytes exhibited approximately 5-fold more GS-HNE production relative to controls (Figure 1C) indicating the HA-GSTA4 retains activity in the presence of the HA epitope.

Loss of GSTA4 in cultured 3T3-L1 adipocytes or in adipose tissue from high fat fed C57Bl/6J mice leads to elevated mitochondrial carbonylation, diminished respiration and altered metabolic flux through the tricarboxylic acid cycle and β -oxidation pathways (7). In addition, obesity-induced insulin resistance in humans positively correlates with protein carbonylation indicating that increased oxidative stress leads to increased levels of carbonylated proteins (22). Because the mitochondrial electron transport chain is the major site of ROS synthesis, we utilized an unbiased proteomics approach focused on adipocyte mitochondria to define specific targets of protein carbonylation. We hypothesized that due to the reduced detoxification of reactive aldehydes in GSTA4 silenced (Kd) adipocytes and increased 4-HNE glutathionylation in aP2-HA-GSTA4 cells that we would be

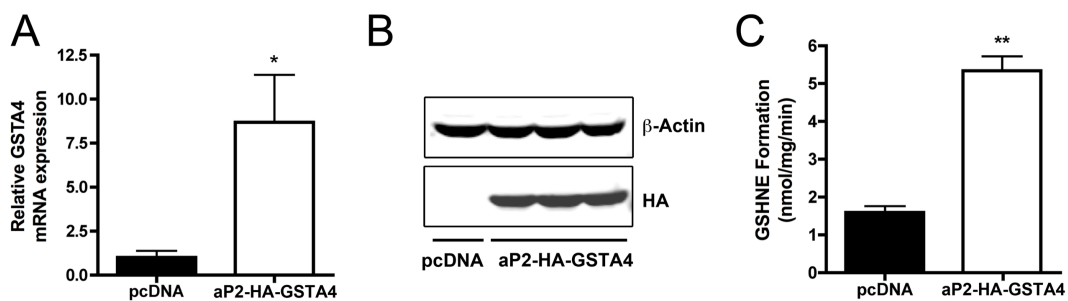
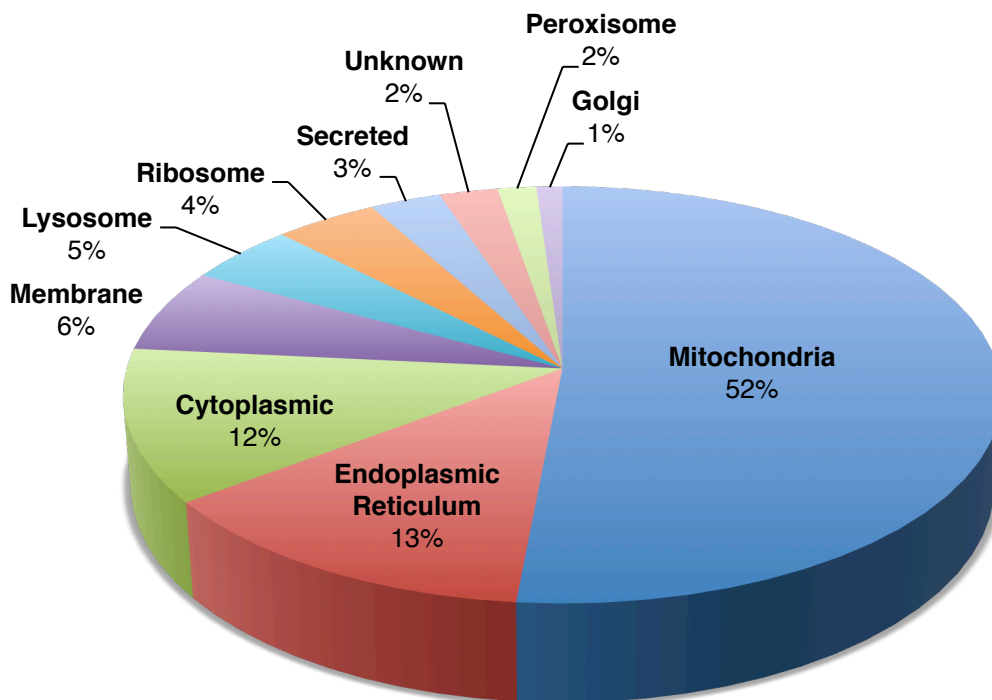


Figure 1. Expression and functionality of aP2-HA-GSTA4 in 3T3-L1 adipocytes. *A.* Relative mRNA expression of GSTA4 in control (pcDNA) and over expressing (aP2-HA-GSTA4) adipocytes normalized to TFIIE. *B.* Expression of HA-GSTA4 (anti-HA) relative to β -actin (anti-actin) in separate over expressing cell lines. *C.* *In vitro* GS-HNE formation assay (n = 3; * p < 0.05; ** p < 0.01).

able to identify critical carbonylation targets in the mitochondrion that may be causal for the pathophysiology of the cells. To test this hypothesis, mitochondrial protein fractions from GSTA4 silenced, over expressing, and cognate control cell lines were subjected to free-carbonyl modification with biotin hydrazide, avidin enrichment chromatography, iTRAQ labeling and mass spectrometric analysis. Over 370 proteins were identified from the proteomic analysis, and approximately 52% of those proteins exhibit mitochondrial localization. This represents approximately 20% of all mitochondrial proteins (23). Since the endoplasmic reticulum and mitochondrion contains sites of interaction defined by the linking proteins Mmm1/Mdm10/Mdm12/Mdm34 (24), it was not surprising that our fractionation led to identification of some non-mitochondrial proteins. Indeed, ~13% of total carbonylated proteins were annotated as being endoplasmic reticular in origin. Other cellular locations represented include cytoplasm (12%), membrane bound (6%) and lysosomal (5%). A complete proteomic summary of the resolved proteins can be found in Supplemental Table 1. The mitochondrial pathways most represented in the dataset are those linked to oxidative phosphorylation (19%), branched chain amino acid catabolism (28%), tricarboxylic acid cycle (33%), and propanoate metabolism (20%) (Table 1). Other pathways with 10% coverage or more include fatty acid, amino acid and ketone body metabolism (data not shown). When the carbonylation level of mitochondrial proteins in GSTA4 silenced relative to over expressing cells was quantitated, a small number of differentially modified polypeptides were identified.

Localization of Carbonylated Proteins



Supplemental Figure 1: Subcellular localization summary of identified targets of carbonylation. Sorted by predominant localization according to Uniprot GO Terms.

TABLE 1**Pathway Analysis of Carbonylated Mitochondrial Proteins in Adipocytes**

Pathway	Pathway Coverage	Carbonylated Proteins
Tricarboxylic Acid Cycle	19/58	<u>ACO2</u> , <u>CS</u> , <u>DLD</u> , <u>DLST</u> , <u>FH</u> , <u>IDH1</u> , <u>IDH2</u> , <u>IDH3A</u> , <u>IDH3G</u> , <u>MDH1</u> , <u>MDH2</u> , <u>OGDH</u> , <u>PC</u> , <u>PCK2</u> , <u>SDHA</u> , <u>SDHB</u> , <u>SUCLA2</u> , <u>SUCLG1</u> , <u>SUCLG2</u>
Oxidative Phosphorylation	32/165	<u>ATP5A1</u> , <u>ATP5B</u> , <u>ATP5C1</u> , <u>ATP5F1</u> , <u>ATP5H</u> , <u>ATP5I</u> , <u>ATP5O</u> , <u>ATP6V1A</u> , <u>COX15</u> , <u>COX4I1</u> , <u>COX5A</u> , <u>COX5B</u> , <u>CYC1</u> , <u>MT-ATP8</u> , <u>MT-CO2</u> , <u>NDUFA2</u> , <u>NDUFA3</u> , <u>NDUFA4</u> , <u>NDUFA10</u> , <u>NDUFB4</u> , <u>NDUFB5</u> , <u>NDUFC2</u> , <u>NDUFS1</u> , <u>NDUFS3</u> , <u>NDUFS7</u> , <u>PPA2</u> , <u>SDHA</u> , <u>SDHB</u> , <u>UQCRB</u> , <u>UQCRC1</u> , <u>UQCRC2</u> , <u>UQCRFS1</u>
Branched Chain Amino Acid Catabolism	31/111	<u>ACAD9</u> , <u>ACAD10</u> , <u>ACADL</u> , <u>ACADM</u> , <u>ACADS</u> , <u>ACADSB</u> , <u>ACADVL</u> , <u>ACAT1</u> , <u>ALDH2</u> , <u>ALDH1B1</u> , <u>ALDH4A1</u> , <u>ALDH6A1</u> , <u>BCAT2</u> , <u>BCKDHA</u> , <u>DBT</u> , <u>ECH1</u> , <u>ECHS1</u> , <u>HADH</u> , <u>HADHA</u> , <u>HADHB</u> , <u>HIBCH</u> , <u>HMGCL</u> , <u>HSD17B4</u> , <u>HSD17B10</u> , <u>IVD</u> , <u>MCCC1</u> , <u>MCCC2</u> , <u>MUT</u> , <u>OXCT1</u> , <u>PCCA</u> , <u>PCCB</u>
Propanoate Metabolism	26/130	<u>ACAD9</u> , <u>ACAD10</u> , <u>ACADL</u> , <u>ACADM</u> , <u>ACADS</u> , <u>ACADSB</u> , <u>ACADVL</u> , <u>ACAT1</u> , <u>ACSL1</u> , <u>ALDH2</u> , <u>ALDH1B1</u> , <u>ALDH4A1</u> , <u>ALDH6A1</u> , <u>ECH1</u> , <u>ECHS1</u> , <u>HADHA</u> , <u>HADHB</u> , <u>HIBCH</u> , <u>IVD</u> , <u>LDHA</u> , <u>MUT</u> , <u>PCCA</u> , <u>PCCB</u> , <u>SUCLA2</u> , <u>SUCLG1</u> , <u>SUCLG2</u>

Proteins underlined are pathway specific targets of carbonylation while others are represented in 2 or more pathways.

TABLE 2**Differentially Modified Targets of Carbonylation in Adipocyte Mitochondria**

Accession Number	Protein Symbol	Protein Name	GSTA4 Kd vs Scr		aP2-HA-GSTA4 vs pcDNA		GSTA4 Kd vs aP2-HA-GSTA4	
			Fold	p value	Fold	p value	Fold	p value
Q8VEM8	MPCP	Phosphate carrier protein, mitochondrial	2.59	2.5E-06	-2.06	6.9E-05	5.33	< E-15
Q9CQ91	NDUA3	NADH dehydrogenase (ubiquinone) 1 alpha subunit 3	1.62	2.9E-01	-2.95	2.8E-06	4.77	3.3E-06
Q9D880	TIM50	Mitochondrial import inner membrane translocase subunit TIM50	1.06	8.8E-01	-2.40	1.7E-03	2.56	4.0E-02
Q9CQ75	NDUA2	NADH dehydrogenase (ubiquinone) 1 alpha subunit 2	-1.02	9.4E-01	-2.21	2.0E-13	2.17	9.3E-06
Q3U2A8	SYVM	Valyl-tRNA synthetase, mitochondrial	1.02	9.3E-01	-1.82	1.6E-10	1.85	4.7E-02
Q9DCU6	RM04	39S ribosomal protein L4, mitochondrial	-1.68	2.1E-04	-1.24	2.3E-02	-1.35	2.0E-04
P84091	AP2M1	Adaptor-related protein complex 2, mu 1 subunit	-2.52	8.0E-15	1.00	9.9E-01	-2.51	1.7E-05
P22315	HEMH	Ferrochelatase, mitochondrial	-2.23	2.4E-09	1.32	5.1E-01	-2.95	4.0E-02
Q8JZN7	MIRO2	Mitochondrial Rho GTPase 2	-2.66	3.0E-13	1.22	3.5E-01	-3.23	9.8E-15
Q8R127	SCPDH	Saccharopine dehydrogenase (putative)	-2.36	1.3E-11	1.52	1.6E-01	-3.58	1.1E-10

Values represented as fold change with positive numbers indicating elevated carbonylation and negative numbers designating decreased carbonylation relative to the compared cell type. Data in the GSTA4 Kd vs aP2-HA-GSTA4 column have been normalized to respective controls. P-values are calculated with ratio data from 3 or more matched spectra.

Carbonylated target data in Table 2 are presented as fold change with positive numbers indicating elevated carbonylation and negative numbers designating decreased carbonylation relative to the compared cell type. These targets include mitochondrial phosphate carrier, two subunits of NADH dehydrogenase (Complex I of mitochondrial electron transport), translocase of the inner mitochondrial membrane 50, and valyl tRNA synthetase. Comparison of the relative carbonylation level in GSTA4-silenced vs. scrambled cells and to that in the aP2-HA-GSTA4 vs. pcDNA cells suggested that the largest number of differences were measured in the aP2-HA-GSTA4 vs. pcDNA cells.

Silencing GSTA4 in 3T3-L1 adipocytes leads to loss of ADP-coupled (State 3) respiration in isolated mitochondria (7). However, because these cells have compensatory changes in substrate level phosphorylation and fatty acid uptake, it was important to assess their *in situ* respiratory capacity. This was determined using the XF24 extracellular flux analyzer with cultured adipocytes. Similar to studies with isolated mitochondria, GSTA4 silenced cells had reduced respiratory capacity relative to control adipocytes (Figure 2A). Respiration rates were calculated as a function of changes in the oxygen consumption rate (OCR) with the indicated treatments, Figure 2B (16,17). The results of the respiration studies indicated that GSTA4 Kd adipocytes had approximately 25% less basal and coupled respiration. Surprisingly, GSTA4 over expression did not significantly affect OCR relative to controls (Figure 2 C, D), suggesting that 3T3-L1 adipocytes

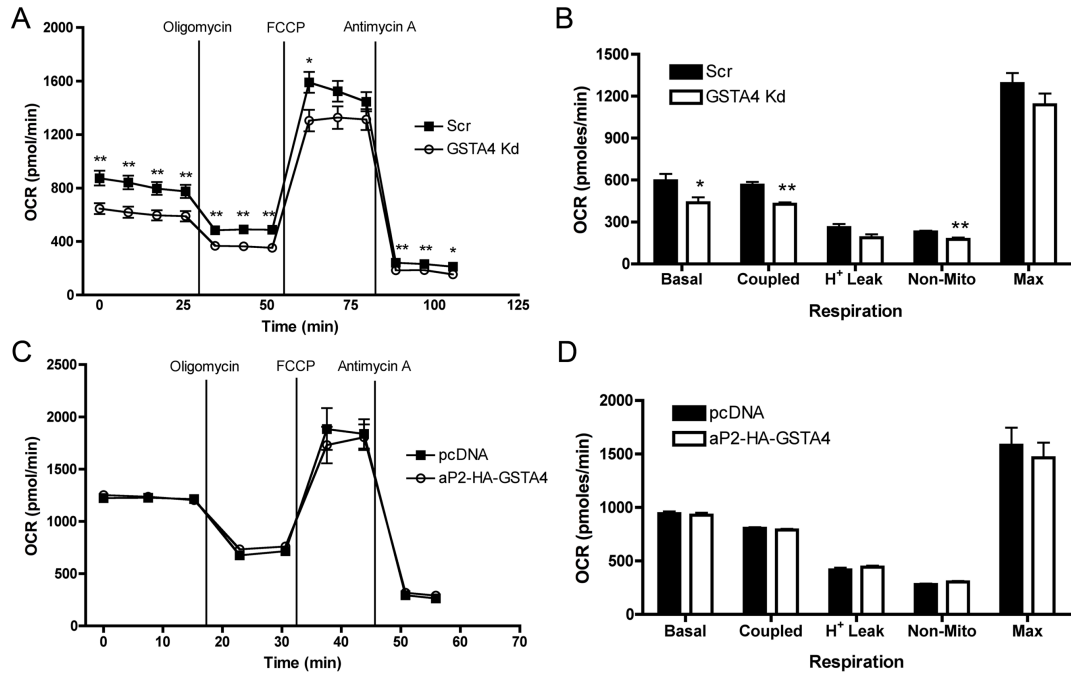


Figure 2. Oxygen consumption rates in GSTA4 silenced and over expressing adipocytes. A & C. Cellular oxygen consumption rates (OCR) for (A) scrambled control (Scr), GSTA4 knockdown (Kd), (C) control (pcDNA) and GSTA4 over expressing (aP2-HA-GSTA4) adipocytes as measured by XF24 Extracellular Flux Analysis. Oligomycin (50 μ g/ml), FCCP (4 μ M), and antimycin A (2.5 μ M) were injected at the indicated time points. B & D. Respiration rates as determined from A & C. (n = 5-10; * p < 0.05; ** p < 0.01).

have sufficient expression of endogenous GSTA4 to prevent carbonylation induced mitochondrial dysfunction.

Mitochondrial respiratory efficiency is inversely proportional to superoxide anion production, where inhibition of complex I led to increased superoxide (25). As two complex I subunits were identified in our proteomic analysis of mitochondrial carbonylation and this complex is a significant site of ROS production, we analyzed superoxide produced in isolated mitochondria. The accumulation of the superoxide probe, TPP-HE, across the inner mitochondrial membrane is driven by membrane potential thus introducing a bias in product formation. To evaluate superoxide levels in a membrane potential-independent manner, we utilized rhodamine 123 (R123) that accumulates in the mitochondria based on membrane potential but has no interaction with superoxide (19) as a normalization control. TPP-HE based analysis of membrane potential in the presence of R123 demonstrated a significant increase in superoxide anion production in GSTA4 Kd adipocytes but no change with GSTA4 over expression (Figure 3A).

The most prominent differentially-carbonylated protein identified was the mitochondrial inner membrane phosphate carrier (PiC) (Table 2). The mitochondrial phosphate carrier is a co-transporter of protons and inorganic phosphate across the inner mitochondrial membrane (26) and likely works in conjunction with the F_0F_1 ATPase to facilitate efficient ATP generation in

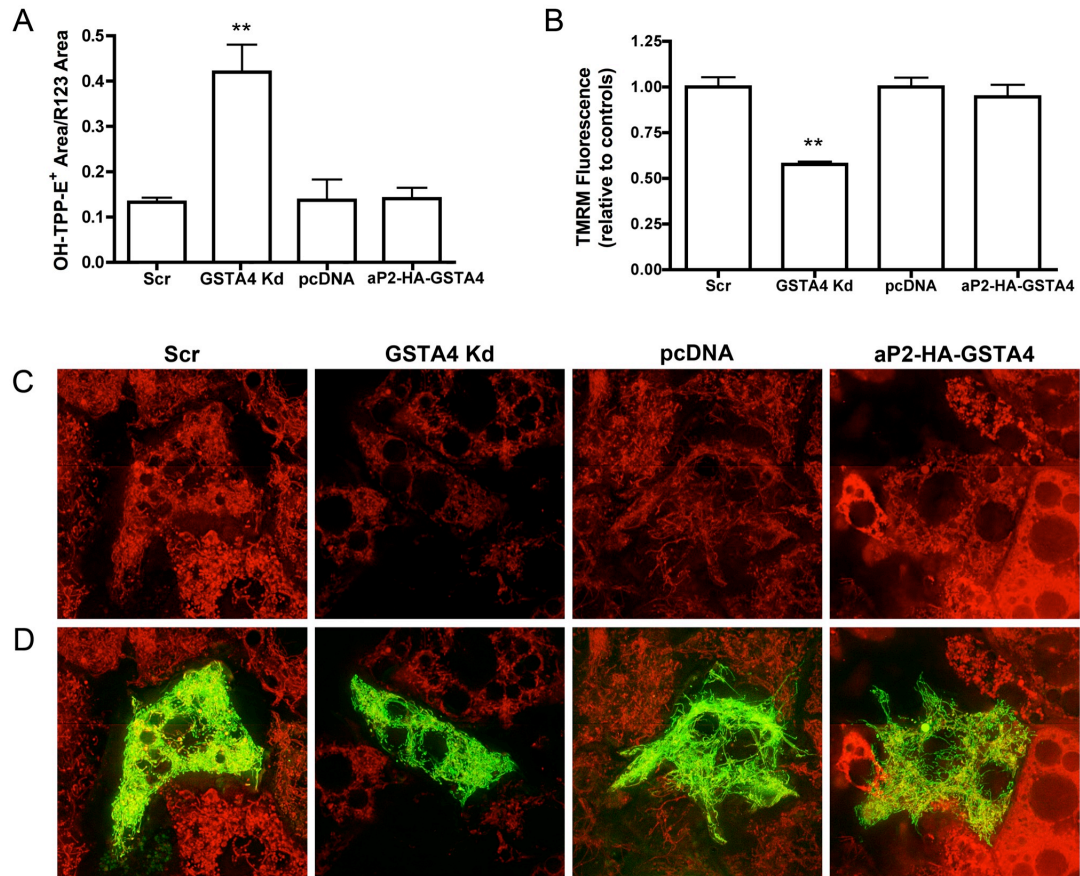


Figure 3. Mitochondrial superoxide production and membrane potential.
 A. Mitochondrial superoxide anion production normalized to R123 membrane potential control (n = 3). B. TMRM fluorescence using ImageJ (n = 10 frames).
 C. TMRM staining of 3T3-L1 adipocytes. D. Merge of TMRM with mitochondrial targeted GFP localization control. (** p < 0.01).

response to the electrochemical gradient across the inner membrane (27). Since the PiC was a significant target of protein carbonylation (Table 2) and coupled respiration was impaired in both whole cell and isolated mitochondrial experiments (Figure 2, (7)), the membrane potential was assayed in live cells using tetramethylrhodamine methyl ester (TMRM), a cationic probe specific for mitochondrial membrane potential. Silencing GSTA4 lead to diminished membrane potential relative to control and over expressing adipocytes under basal conditions (Figure 3 C, D) whereas the over expression of GSTA4 did not confer any changes in membrane potential relative to control cells. Additionally, no change in PiC protein abundance was observed (Figure 4A). Loss of mitochondrial membrane potential is often associated with cytochrome c (Cyt C) release and the induction of the apoptotic response (28,29), therefore localization of Cyt C was analyzed between GSTA4 silenced and over expressing cells. There was no increase in Cyt C release in the GSTA4 silenced cells relative to controls or decrease in aP2-HA-GSTA4 over expressing cells (Figure 4 B, C), indicating the apoptosis pathway had not been triggered by changes in GSTA4-dependent protein carbonylation despite the loss of activity.

Our previous studies utilizing GSTA4 silenced adipocytes demonstrated reduced mitochondrial respiration, mitochondrial DNA (mtDNA) copy number and expression of several key transcription factors regulating mitochondrial biogenesis (7). In order to determine whether mitochondrial dysfunction was a

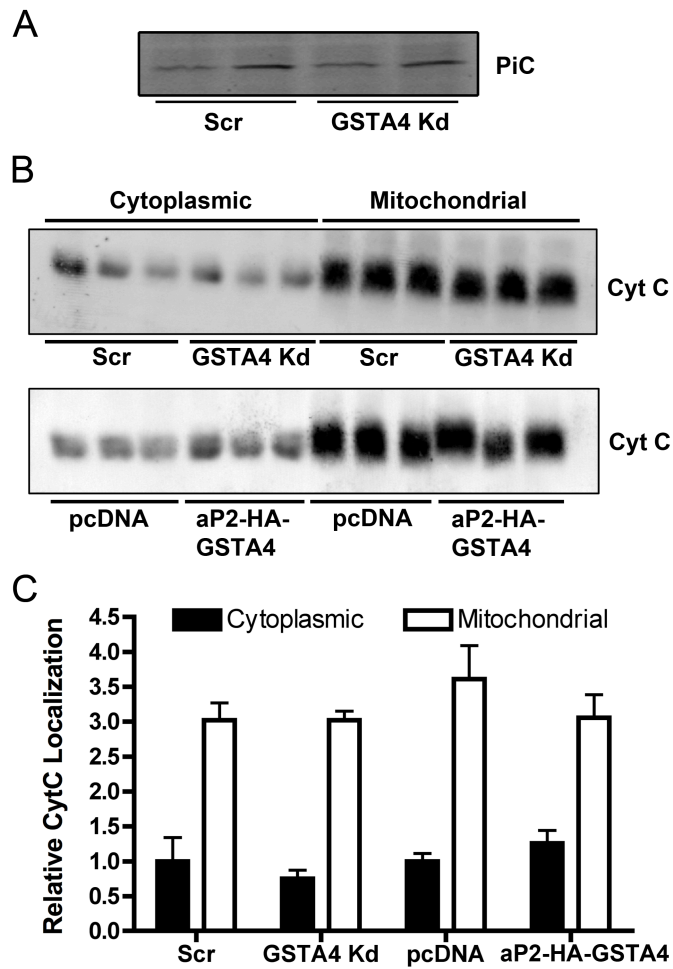


Figure 4. PiC Protein Abundance and Cytochrome C localization in GSTA4 silenced and over expressing 3T3-L1 adipocytes. *A.* Immunoblot analysis of PiC. *B.* Immunolocalization of Cyt C protein in 3T3-L1 adipocyte cytoplasmic and mitochondrial fractions. *C.* Quantitation of immunoblots from of *B.*

product of decreased number of mitochondria or decreased mitochondrial activity, electron microscopy was employed. Using differentiated adipocytes from GSTA4 silenced, over expressing, and control cell lines, mitochondrial number, area and perimeter were evaluated (Figure 5). Despite a ~ 75% reduction in mtDNA content in GSTA4 silenced adipocytes there was no difference in mitochondrial number, area or volume (Figure 5 E-G). On a qualitative basis, mitochondria appeared to be irregularly shaped in GSTA4 silenced cells compared to either scrambled or over expressing adipocytes and the cristae were less well organized. These results suggest that decreased mitochondrial function is not linked to changes in mitochondrial number. Consistent with this, expression of autophagy markers ATG5, ATG6, ATG9 and ATG13 were not different between the cells lines nor was the phosphorylation of S6 kinase, an mTOR target (results not shown).

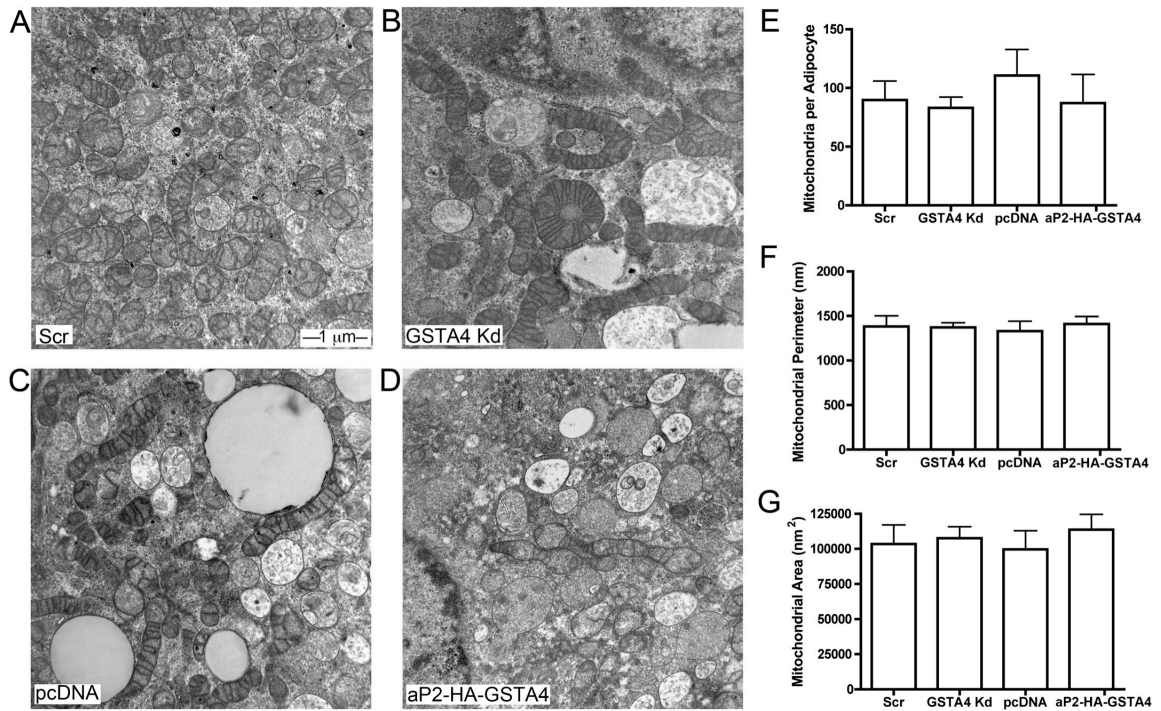


Figure 5. Mitochondrial number and area by electron microscopy. *A-D.* Electron micrographs at 30,000x magnification of scrambled (A), GSTA4 Kd (B), pcDNA (C), and aP2-HA-GSTA4 (D) 3T3-L1 adipocytes. *E-F.* Quantitation of mitochondrial number (E), perimeter (F), and area (G) from multiple fields (n= 70-100 each).

To investigate whether carbonylation affects Complex I activity was assessed from mitochondria isolated from each of the cell lines. The transfer of electrons from NADH to dichlorophenolindophenol via the Complex I dependent transfer of electrons to decylubiquinone was evaluated spectrophotometrically (21). As shown in Figure 6A, the reduction of GSTA4 expression is associated with a ~10% decrease in Complex I enzyme activity, whereas adipocytes with higher expression of GSTA4 exhibited a ~ 10% increase in Complex I activity. No change in NDUFA3 protein expression was observed between any of the cell lines (Figure 6B).

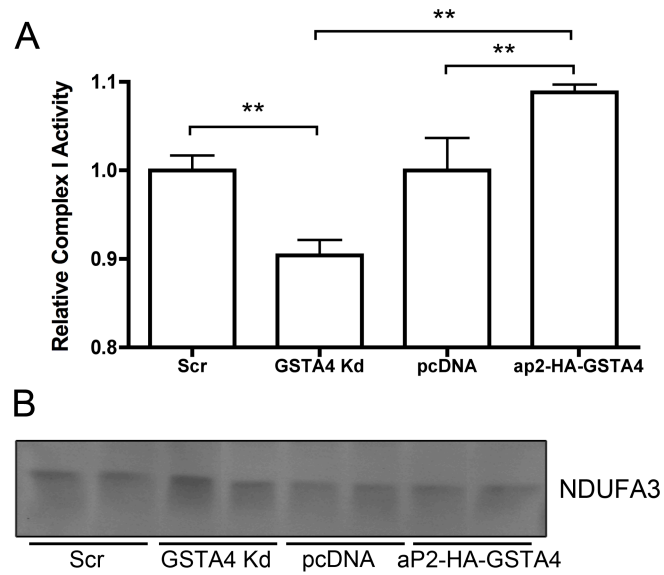


Figure 6. Complex I activity in isolated mitochondria. *A.* The Complex I dependent reduction of dichlorophenolindolephenol was monitored spectrophotometrically at 595 nm in isolated mitochondria (n = 4; ** p < 0.01). *B.* Protein expression of NDUFA3 Complex I subunit in mitochondrial fractions (representative blot, n = 6).

DISCUSSION

Oxidative stress and the production of reactive oxygen species have been strongly implicated as causal in the development of obesity-linked insulin resistance and a variety of studies have reported that anti-oxidants may have beneficial effects attenuating the characteristics of the metabolic syndrome (3,30-32). Linked to increased oxidative stress and the production of hydroxyl radicals is the hydroperoxidation of unsaturated acyl chains of membrane lipid and triglyceride. Lipid hydroperoxides undergo Hock cleavage to produce a family of α,β unsaturated hydroxylated aldehydes that by virtue of their electron withdrawing carbonyl and hydroxyl functions are subject to Michael addition reactions centering on carbon 3 of the lipid producing a covalent protein-lipid adduct. Such alkylation, referred to as protein carbonylation, has been demonstrated in a variety of oxidative systems including inflammation, neurodegeneration and cancer (33-35). Since the mitochondrial electron transport chain is a major site of ROS production mitochondrial carbonylation is thought to contribute substantially to oxidative damage, although this hypothesis has not been rigorously tested. The objective of this study was to utilize a proteomic strategy to identify carbonylation targets in adipocyte mitochondria linked to down regulation of glutathione *S*-transferase A4. It should be noted that the biotinylated peptides carrying the carbonyl modification are not identified. The reason for this is unclear but may be because they do not resolve during chromatography or they fragment in a manner that precludes their identification

via sequence database searching. Methods are in development to isolate and analyze such alkylated peptides to determine the site(s) and stoichiometry of carbonylation. Additionally, while both amino acid side chain oxidation and carbonylation with lipid aldehydes occurs, studies indicate that side chain alkylation is ~ 10-fold more prevalent than direct side chain oxidation (36).

Our previous studies have established that protein carbonylation is elevated in the adipose tissue of obese, insulin resistant mice and in obese humans and identified soluble protein targets of carbonylation (7,22,37). Increased protein carbonylation has been mechanistically linked to TNF α -dependent down regulation of the antioxidant enzyme GSTA4. In both murine and human adipose tissue the major soluble carbonylation target protein was the fatty acid binding protein FABP4 and FABP5 and carbonylation of murine FABP4 on Cys117 in the ligand binding cavity results in loss of fatty acid binding activity. However, biochemically, the major site of metabolic dysfunction was in mitochondrial metabolism suggesting that carbonylation of mitochondrial targets may be causative for changes in oxidative metabolism. To address this we developed adipocyte models with variable expression of GSTA4 as a means to identify and evaluate the role of protein carbonylation in the pathogenesis of obesity-linked insulin resistance. Coupling the enrichment of carbonylated mitochondrial proteins with quantitative proteomics, we identified differentially modified proteins between our cellular models of oxidative stress. Surprisingly, only a handful of

proteins were differentially carbonylated suggesting that the changes in metabolism may be focused on just a small number of metabolic loci.

Other published studies using exogenous 4-HNE treatment of cells transfected with GSTA4 have shown that localization of GSTA4 to the mitochondria (38) protects against oxidative damage (39) and resultant apoptosis (40). Proteomic analysis demonstrated that GSTA4 over expressing adipocytes had less mitochondrial carbonylation relative to GSTA4 silenced cells (Table 2 & Supplemental Table 1) but functional analysis shows this did not confer large changes to respiration, superoxide production, membrane potential or complex I activity relative to control cells. This suggests that endogenous levels of GSTA4 are sufficient for protection against carbonylation-induced functional changes to adipocyte metabolism under normoxic conditions. Carbonylated proteins are frequently targeted for proteasome-dependent degradation (41). Proteins whose relative level of carbonylation is decreased in the GSTA4 silenced cells relative to the HA-GSTA4 over expressing cells (Table 2) may represent targets that are selectively proteolyzed resulting in decreased abundance. This does not pertain to the phosphate carrier or NDUFA3; their abundance did not differ between cellular states (Figures 4 and 6, respectively).

Among the important targets of carbonylation implicated in mitochondrial dysfunction, two Complex I subunits have been identified: NDUFA2 and

NDUFA3. These proteins are accessory subunits of NADH:ubiquinone oxidoreductase that transfers electrons from NADH to ubiquinol: cytochrome c oxidoreductase (complex III) (42). This large, 1 MDa complex consists of 45 subunits and NDUFA2 is located on the peripheral arm of complex I in the inner mitochondrial matrix where NADH binding occurs (43). Its expression in white adipose tissue is positively correlated with peroxisome proliferator-activated receptor- γ coactivator-1 α (PGC-1 α) expression, a potent mediator of mitochondrial biogenesis, and energy expenditure (44). NDUFA3 fractionates with proteins connecting the peripheral arm to the membrane embedded domain and is one of 31 subunits of complex I without an established primary function (42). Defects in complex I assembly are a common cause of mitochondrial disorders (43,45) and mutations in complex I alone are responsible for 23% of all childhood respiratory chain defects in humans (46). Mutations in NDUFA2 affecting complex I activity frequently lead to infant mortality (47). Since proper complex I function requires intricate coordination of subunits, it is possible that post-translational modifications such as carbonylation could disrupt this process. In fact, micromolar concentrations of 4-HNE are linked to loss of mitochondrial respiration through inhibition of complex I (48). In this study, carbonylation of complex I subunits is associated with impaired activity (Figure 6), reduced membrane potential (Figure 3B) and diminished respiration (Figures 2A-B).

A second possible impact carbonylation of complex I subunits may have is on the generation of superoxide. Complex I is a major site of mitochondrial superoxide production and under normal metabolic conditions approximately 1-3% of electrons passing through complex I are lost as superoxide (49-51). Carbonylation of NDUFA2 or NDUFA3, located near the NADH binding site, may interfere with the ability of complex I to efficiently transfer electrons from NADH to complex III leading to elevated superoxide generation (Figure 3A). Consistent with this, the production rate of superoxide by Complex I is inversely proportional to the NAD^+/NADH ratio (52) which is reduced in the GSTA4 silenced adipocytes (7).

One of the central functions of the mitochondrion is the generation of ATP by F_0F_1 ATP synthase (Complex V). This enzyme is part of a super-complex known as the ATP synthasome, which consists of the adenine nucleotide translocase (ANT), the mitochondrial phosphate carrier (PiC) and the ATP synthase (27,53). ANT exchanges ATP for ADP across the inner mitochondrial membrane and PiC co-transporters inorganic phosphate (P_i) and protons into the inner mitochondrial matrix. Through physical association these complexes centralize the production of ATP by bringing the synthase together with ADP and P_i . With proper substrate availability, the F_0F_1 molecular motor exploits the proton gradient established by complexes I-IV of the electron transport chain to generate ATP. Both ANT and several Complex V subunits were identified as carbonylated (Table S1) but there

was no significant difference in the amount of carbonylation between variable models of oxidative stress. However, PiC was differentially carbonylated to the greatest extent between the various cell lines (Table 2). The PiC plays a significant role in ATP synthesis as down regulation of PiC in INS-1 β -cells leads to blunted ATP production (54). Interestingly, even with reduced respiration and mitochondrial membrane potential (Figures 2 & 3), phosphorylation of AMP-activated kinase, a cellular indicator of the AMP:ATP ratio, is unaltered in GSTA4 silenced adipocytes (Curtis and Bernlohr, unpublished) likely due to increased basal glucose uptake (7). This suggests that GSTA4 silenced cells have adapted their metabolism to rely on substrate level phosphorylation as a result of reduced mitochondrial function. As oxygen consumption is coupled to ATP production, the impaired respiration observed in GSTA4 silenced adipocytes (Figure 2A-B) may in part be due to carbonylation of PiC interfering with its ability to transport phosphate or protons or associate with the ATP synthasome.

An alternative function for the PiC is as a component of the mitochondrial permeability transition pore (MPTP) (55-57) suggesting a larger role for the PiC in the regulation of mitochondrial membrane potential. Opening of the MPTP uncouples the proton gradient and induces apoptosis by releasing Cyt C. Over expression of PiC induces apoptosis whereas PiC knockdown leads to reduced Cyt C release and apoptosis (56). In this study there were no changes in PiC abundance or relative cytochrome c localization between GSTA4 silenced and

over expressing cells (Figure 4) indicating the amount of oxidative damage observed in GSTA4 silenced adipocytes is insufficient to trigger apoptosis. Symport of protons and phosphate across the inner mitochondrial membrane by PiC uncouples the proton gradient but is an electroneutral event. The PiC contains several sulfhydryl residues that are essential for its primary function (58) and thiol reagents, such as N-ethylmaleimide modify cysteine residues and inhibit symport activity (59). Additionally, dithiol crosslinking causes homodimerization of PiC and formation of a non-specific anion channel (60). These observations suggest that protein carbonylation of the PiC may either affect its symport activity and/or ability to function as a component of the MPTP.

Carbonylation of mitochondrial proteins did not change the number or size of the mitochondrial pool (Figure 5) despite down-regulation of key mitochondrial-encoded proteins and transcription factors regulating mitochondrial biogenesis, such as PGC-1 α , and Tfam1 (7). While highly variable, ultrastructure analysis via electron microscopy suggests disorganization of the cristae and irregular shaping in mitochondria from GSTA4 silenced adipocytes relative to those from control or GSTA4 over expressing cells. Mitochondrial depolarization, which occurs in GSTA4 silenced adipocytes, is an important signal for mitophagic clearance (as reviewed by (61)); however, evaluation of autophagy signaling proteins ATG5/6/9/13 and S6K (data not shown) indicate that such cellular clearance mechanisms have not been activated.

In summary, mitochondrial protein carbonylation is regulated via the expression and activity of GSTA4 in the adipocyte. The transition from lean to obese leads to cytokine-mediated down regulation of GSTA4 expression and concomitant modification of mitochondrial proteins. Such protein modification results in impaired mitochondrial respiration, increased superoxide production, reduced membrane potential and decreased complex I activity. Specific targets of protein carbonylation such as complex I subunits and the PiC may contribute significantly to mitochondrial dysfunction and play an important role in regulating adipocyte metabolism in the obese, insulin resistant adipocyte.

ABBREVIATIONS

GSTA4, Glutathione S-transferase A4; ROS, reactive oxygen species; 4-HNE, 4-hydroxynonenol; PiC, mitochondrial phosphate carrier; NDUFA2/3, NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 2/3; GSHNE, glutathione-conjugated HNE; OCR, oxygen consumption rate; Cyt C, cytochrome c; mtDNA, mitochondrial DNA; TNF- α , tumor necrosis factor alpha; HA, hemagglutinin; Kd, knockdown; Scr, scrambled; PBS, phosphate buffered saline; SDS, sodium dodecyl sulfate; HPLC, high-performance liquid chromatography; PQD, pulsed Q collision induced dissociation; DMEM, Dubelco's modified eagle medium; TMRM, tetramethylrhodamine methylester; TPP-HE, triphenylphosphonium hydroethidine; R123, rhodamine 123; liquid chromatography tandem mass spectrometry; ER, endoplasmic reticulum; GFP, green fluorescent protein; MPTP, mitochondrial permeability transition pore; NAD/NADH, nicotinamide adenine dinucleotide; ATP, adenosine triphosphate; PGC-1 α , peroxisome proliferator-activated receptor gamma coactivator - 1 alpha; FCCP, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone; DCIP, dichlorophenylindole.

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Supplemental Table 1

Targets of Protein Carbonylation: Categorized by Subcellular Localization; Sorted by GSTA4 Kd vs aP2-HA-GSTA4

Accession	Symbol	Protein names	GSTA4 Kd vs Scr		aP2-HA-GSTA4 vs pcDNA		GSTA4 Kd vs aP2-HA-GSTA4	
			Fold Change	p-value	Fold Change	p-value	Fold Change	p-value
Mitochondria								
Q8VEM8	MPCP	Phosphate carrier protein, mitochondrial	2.593	2.50E-06	-2.056	6.86E-05	5.331	<E-15
Q9WUR9	KAD4	Adenylate kinase isoenzyme 4, mitochondrial	1.998	6.66E-01	-2.521	2.53E-02	5.037	3.72E-01
Q9CQ91	NDUFA3	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 3	1.617	2.94E-01	-2.950	2.84E-06	4.770	3.30E-06
Q8K1Z0	COQ9	Ubiquinone biosynthesis protein COQ9, mitochondrial	1.325	6.91E-01	-2.008	9.70E-02	2.660	2.50E-01
Q9D880	TIM50	Mitochondrial import inner membrane translocase subunit TIM50	1.064	8.82E-01	-2.403	1.69E-03	2.557	3.98E-02
Q8BGT5	ALAT2	Alanine aminotransferase 2	1.349	6.17E-01	-1.882	1.37E-01	2.540	3.22E-01
P63030	BR44L	Brain protein 44-like protein	1.233	7.19E-01	-2.029	6.19E-02	2.502	2.98E-01
Q99MN9	PCCB	Propionyl-CoA carboxylase beta chain, mitochondrial	1.017	9.78E-01	-2.339	3.30E-02	2.378	3.68E-01
Q9CQ75	NDUFA2	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 2	-1.018	9.37E-01	-2.212	1.96E-13	2.172	9.29E-06
Q9CQN1	TRAP1	Heat shock protein 75 kDa, mitochondrial	1.276	7.69E-01	-1.668	2.46E-01	2.129	5.02E-01
O35857	TIM44	Mitochondrial import inner membrane translocase subunit TIM44	1.328	6.07E-01	-1.565	1.33E-01	2.078	3.16E-01
Q8CHT0	AL4A1	Delta-1-pyrroline-5-carboxylate dehydrogenase, mitochondrial	1.214	7.87E-01	-1.663	5.56E-01	2.019	5.96E-01
Q925I1	ATAD3	ATPase family AAA domain-containing protein 3	1.255	6.00E-01	-1.560	3.98E-01	1.958	3.66E-01
Q99KE1	MAOM	NAD-dependent malic enzyme, mitochondrial	1.142	6.28E-01	-1.695	3.82E-05	1.936	8.08E-02
Q3U2A8	SYVM	Valyl-tRNA synthetase, mitochondrial	1.019	9.31E-01	-1.816	1.61E-10	1.851	4.72E-02
Q9CQA3	DHSB	Succinate dehydrogenase [ubiquinone] iron-sulfur subunit, mitochondrial	1.225	7.33E-01	-1.461	3.32E-01	1.790	5.04E-01
P54071	IDHP	Isocitrate dehydrogenase [NADP], mitochondrial	-1.029	9.74E-01	-1.790	5.00E-01	1.740	7.18E-01
Q8BX10	PGAM5	Serine/threonine-protein phosphatase PGAM5, mitochondrial	1.132	8.51E-01	-1.538	2.84E-01	1.740	5.38E-01
Q9D0M3	CY1	Cytochrome c1, heme protein, mitochondrial	-1.390	4.82E-01	-2.349	3.86E-02	1.690	4.17E-01
Q9QZA0	CAH5B	Carbonic anhydrase 5B, mitochondrial	1.531	3.32E-02	-1.077	8.69E-01	1.649	4.41E-01
Q9CZS1	AL1B1	Aldehyde dehydrogenase X, mitochondrial	-1.053	8.85E-01	-1.716	6.23E-03	1.630	1.79E-01
Q9D404	OXSM	3-oxoacyl-[acyl-carrier-protein] synthase, mitochondrial	1.081	8.71E-01	-1.501	4.90E-01	1.623	6.39E-01
Q06185	ATP5I	ATP synthase subunit e, mitochondrial	-1.001	9.98E-01	-1.580	1.15E-01	1.579	4.32E-01
O08756	HCD2	3-hydroxyacyl-CoA dehydrogenase type-2	-1.009	9.79E-01	-1.581	6.71E-02	1.567	4.04E-01
Q8BH59	CMC1	Calcium-binding mitochondrial carrier protein Aralar1	1.088	8.26E-01	-1.423	5.34E-01	1.548	5.21E-01
Q9DBL1	ACDSB	Short/branched chain specific acyl-CoA dehydrogenase, mitochondrial	-1.014	9.78E-01	-1.551	2.50E-01	1.529	6.07E-01
Q9CW42	MOSC1	MOSC domain-containing protein 1, mitochondrial	-1.022	9.65E-01	-1.558	1.61E-01	1.525	5.18E-01
Q9QZD8	DIC	Mitochondrial dicarboxylate carrier	-1.156	8.54E-01	-1.711	3.66E-01	1.481	7.26E-01
Q9CZU6	CISY	Citrate synthase, mitochondrial	-1.086	8.38E-01	-1.592	3.59E-01	1.466	6.05E-01
Q8RON6	HOT	Hydroxyacid-oxoacid transhydrogenase, mitochondrial	-1.052	9.15E-01	-1.526	2.65E-01	1.450	4.62E-01
Q99JR1	SFXN1	Sideroflexin-1	-1.013	9.71E-01	-1.469	5.06E-01	1.450	5.88E-01
P51174	ACADL	Long-chain specific acyl-CoA dehydrogenase, mitochondrial	-1.037	9.40E-01	-1.487	3.89E-01	1.434	6.36E-01

Accession	Symbol	Protein names	GSTA4 Kd vs Scr		aP2-HA-GSTA4 vs pcDNA		GSTA4 Kd vs aP2-HA-GSTA4	
			Fold Change	p-value	Fold Change	p-value	Fold Change	p-value
Q8QZS1	HIBCH	3-hydroxyisobutyryl-CoA hydrolase, mitochondrial	1.327	6.38E-01	-1.080	9.04E-01	1.433	6.88E-01
P00405	COX2	Cytochrome c oxidase subunit 2	1.189	8.88E-01	-1.175	8.85E-01	1.397	8.80E-01
Q9DC70	NDUS7	NADH dehydrogenase [ubiquinone] iron-sulfur protein 7, mitochondrial	-1.152	7.45E-01	-1.602	7.26E-02	1.391	4.47E-01
P38060	HMGCL	Hydroxymethylglutaryl-CoA lyase, mitochondrial	-1.169	8.29E-01	-1.604	1.60E-01	1.373	7.58E-01
P42125	ECI1	Enoyl-CoA delta isomerase 1, mitochondrial	1.036	9.43E-01	-1.316	4.39E-01	1.363	6.60E-01
P47738	ALDH2	Aldehyde dehydrogenase, mitochondrial	-1.206	7.78E-01	-1.640	3.09E-01	1.359	7.65E-01
Q9CZW5	TOM70	Mitochondrial import receptor subunit TOM70	-1.067	9.52E-01	-1.443	4.32E-01	1.353	8.19E-01
P70404	IDHG1	Isocitrate dehydrogenase [NAD] subunit gamma 1, mitochondrial	-1.002	9.97E-01	-1.348	6.45E-01	1.346	7.66E-01
Q91VR2	ATPG	ATP synthase subunit gamma, mitochondrial	-1.135	7.49E-01	-1.527	2.58E-01	1.346	6.26E-01
Q71RI9	KAT3	Kynurenine--oxoglutarate transaminase 3	-1.180	7.50E-01	-1.584	1.14E-01	1.342	7.15E-01
Q91YP2	NEUL	Neurolysin, mitochondrial	-1.147	5.70E-01	-1.522	9.06E-04	1.328	2.74E-01
Q64133	AOFA	Amine oxidase [flavin-containing] A	-1.239	7.90E-01	-1.642	4.66E-01	1.326	8.42E-01
Q8BGH2	SAM50	Sorting and assembly machinery component 50 homolog	-1.230	5.91E-01	-1.618	3.48E-01	1.315	7.55E-01
Q8VCW8	ACSF2	Acyl-CoA synthetase family member 2, mitochondrial	-1.008	9.89E-01	-1.325	4.94E-01	1.314	7.54E-01
Q99J99	THTM	3-mercaptopyruvate sulfurtransferase	-1.113	8.00E-01	-1.446	3.68E-01	1.299	6.75E-01
Q99KI0	ACON	Aconitate hydratase, mitochondrial	-1.084	9.00E-01	-1.403	6.26E-01	1.294	8.21E-01
Q8BH04	PCKGM	Phosphoenolpyruvate carboxykinase [GTP], mitochondrial	-1.056	9.17E-01	-1.365	5.88E-01	1.293	7.38E-01
Q8K0D5	EFGM	Elongation factor G, mitochondrial	-1.068	9.00E-01	-1.381	1.05E-01	1.293	7.16E-01
P50544	ACADV	Very long-chain specific acyl-CoA dehydrogenase, mitochondrial	1.065	8.79E-01	-1.207	6.49E-01	1.286	6.99E-01
Q99MR8	MCCA	Methylcrotonoyl-CoA carboxylase subunit alpha, mitochondrial	-1.019	9.65E-01	-1.310	4.36E-01	1.285	7.11E-01
Q3V3R1	C1TM	Monofunctional C1-tetrahydrofolate synthase, mitochondrial	1.042	9.05E-01	-1.225	4.81E-01	1.276	6.91E-01
P52196	THTR	Thiosulfate sulfurtransferase	-1.007	9.89E-01	-1.271	3.91E-01	1.262	7.16E-01
P35487	ODPAT	Pyruvate dehydrogenase E1 component subunit alpha, testis-specific form, mitochondrial	-1.131	8.01E-01	-1.427	4.16E-01	1.261	7.65E-01
P67778	PHB	Prohibitin	-1.091	8.84E-01	-1.375	6.00E-01	1.261	8.18E-01
P47934	CACP	Carnitine O-acetyltransferase	-1.094	8.83E-01	-1.376	5.57E-01	1.258	7.83E-01
Q9D2G2	ODO2	Dihydrolipoylysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial	-1.066	9.16E-01	-1.323	6.37E-01	1.241	8.19E-01
Q9CRB9	CHCH3	Coiled-coil-helix-coiled-coil-helix domain-containing protein 3, mitochondrial	-1.100	8.31E-01	-1.361	4.74E-01	1.237	7.61E-01
Q8JZQ2	AFG32	AFG3-like protein 2	1.057	9.33E-01	-1.166	6.72E-01	1.233	8.18E-01
Q9DB20	ATPO	ATP synthase subunit O, mitochondrial	-1.083	8.81E-01	-1.335	6.01E-01	1.233	8.00E-01
P19783	COX41	Cytochrome c oxidase subunit 4 isoform 1, mitochondrial	-1.277	6.19E-01	-1.569	1.37E-01	1.228	7.56E-01
P38647	GRP75	Stress-70 protein, mitochondrial	-1.074	8.95E-01	-1.316	6.66E-01	1.225	8.38E-01
Q9Z0X1	AIFM1	Apoptosis-inducing factor 1, mitochondrial	-1.118	8.32E-01	-1.367	4.98E-01	1.223	7.93E-01
Q8CAQ8	IMMT	Mitochondrial inner membrane protein	-1.099	8.55E-01	-1.336	6.43E-01	1.216	8.37E-01
Q99JY0	ECHB	Trifunctional enzyme subunit beta, mitochondrial	-1.029	9.63E-01	-1.226	6.70E-01	1.192	8.51E-01
Q9CZ83	RM55	39S ribosomal protein L55, mitochondrial	-1.079	9.45E-01	-1.277	8.25E-01	1.184	9.35E-01

Accession	Symbol	Protein names	GSTA4 Kd vs Scr		aP2-HA-GSTA4 vs pcDNA		GSTA4 Kd vs aP2-HA-GSTA4	
			Fold Change	p-value	Fold Change	p-value	Fold Change	p-value
Q03265	ATPA	ATP synthase subunit alpha, mitochondrial	-1.121	8.47E-01	-1.322	6.07E-01	1.179	8.54E-01
Q8K009	AL1L2	Aldehyde dehydrogenase family 1 member L2, mitochondrial	-1.064	9.53E-01	-1.253	7.88E-01	1.177	9.24E-01
P12787	COX5A	Cytochrome c oxidase subunit 5A, mitochondrial	-1.363	8.01E-02	-1.588	2.05E-06	1.165	4.12E-01
Q05920	PYC	Pyruvate carboxylase, mitochondrial	-1.146	8.01E-01	-1.325	6.51E-01	1.156	8.73E-01
Q91VD9	NDUS1	NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial	-1.060	9.11E-01	-1.215	6.28E-01	1.146	8.48E-01
P19536	COX5B	Cytochrome c oxidase subunit 5B, mitochondrial	1.271	8.24E-01	1.111	9.11E-01	1.144	9.42E-01
P97807	FUMH	Fumarate hydratase, mitochondrial	-1.102	7.75E-01	-1.261	7.50E-01	1.144	8.80E-01
Q61941	NNTM	NAD(P) transhydrogenase, mitochondrial	-1.186	5.92E-01	-1.343	4.88E-01	1.132	8.26E-01
Q8BH95	ECHM	Enoyl-CoA hydratase, mitochondrial	-1.194	6.78E-01	-1.340	4.70E-01	1.122	8.71E-01
Q9CQQ7	AT5F1	ATP synthase subunit b, mitochondrial	-1.165	7.78E-01	-1.292	6.23E-01	1.109	8.98E-01
P20108	PRDX3	Thioredoxin-dependent peroxide reductase, mitochondrial	1.101	8.47E-01	-1.005	9.92E-01	1.107	8.71E-01
P63038	CH60	60 kDa heat shock protein, mitochondrial	-1.185	7.48E-01	-1.307	5.83E-01	1.103	9.08E-01
P51881	ADT2	ADP/ATP translocase 2	-1.665	5.04E-01	-1.821	2.33E-01	1.093	9.27E-01
Q32MW3	ACO10	Acyl-coenzyme A thioesterase 10, mitochondrial	-1.048	9.28E-01	-1.145	8.41E-01	1.093	9.40E-01
Q8JZN5	ACAD9	Acyl-CoA dehydrogenase family member 9, mitochondrial	-1.001	9.97E-01	-1.090	8.40E-01	1.089	8.96E-01
P62897	CYC	Cytochrome c, somatic	-1.237	7.72E-01	-1.344	6.54E-01	1.086	9.33E-01
P05202	AATM	Aspartate aminotransferase, mitochondrial	-1.120	8.56E-01	-1.215	7.27E-01	1.085	9.33E-01
O08749	DLDH	Dihydrolipoyl dehydrogenase, mitochondrial	-1.131	8.07E-01	-1.207	7.63E-01	1.067	9.44E-01
Q61578	ADRO	NADPH:adrenodoxin oxidoreductase, mitochondrial	-1.070	9.15E-01	-1.140	7.47E-01	1.065	9.46E-01
Q9CQC7	NDUB4	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 4	-1.064	7.57E-01	-1.133	7.02E-01	1.065	7.49E-01
P35486	ODPA	Pyruvate dehydrogenase E1 component subunit alpha, somatic form, mitochondrial	-1.278	5.17E-01	-1.356	5.54E-01	1.061	9.35E-01
Q9CXT8	MPPB	Mitochondrial-processing peptidase subunit beta	1.161	8.69E-01	1.100	8.89E-01	1.056	9.71E-01
Q9WTP6	KAD2	Adenylate kinase 2, mitochondrial	-1.200	7.52E-01	-1.262	6.47E-01	1.052	9.53E-01
Q9CQ62	DECR	2,4-dienoyl-CoA reductase, mitochondrial	-1.226	6.72E-01	-1.269	6.94E-01	1.035	9.68E-01
Q91ZA3	PCCA	Propionyl-CoA carboxylase alpha chain, mitochondrial	-1.452	3.91E-01	-1.500	5.07E-01	1.033	9.68E-01
P03930	ATP8	ATP synthase protein 8	-1.410	7.03E-01	-1.452	5.23E-01	1.030	9.84E-01
Q9D855	QCR7	Cytochrome b-c1 complex subunit 7	-1.325	6.30E-01	-1.348	6.86E-01	1.017	9.87E-01
Q9QZ23	NFU1	NFU1 iron-sulfur cluster scaffold homolog, mitochondrial	-1.117	4.81E-01	-1.136	5.93E-01	1.017	9.48E-01
P41216	ACSL1	Long-chain-fatty-acid-CoA ligase 1	-1.240	7.24E-01	-1.255	6.79E-01	1.012	9.89E-01
P53395	ODB2	Lipoamide acyltransferase component of branched-chain alpha-keto acid dehydrogenase complex, mitochondrial	-1.056	9.19E-01	-1.057	9.31E-01	1.000	1.00E00
Q8K411	PREP	Presequence protease, mitochondrial	-1.240	7.06E-01	-1.233	5.07E-01	-1.006	9.93E-01
P24270	CATA	Catalase	-1.066	9.26E-01	-1.054	9.44E-01	-1.011	9.92E-01
Q61586	GPAT1	Glycerol-3-phosphate acyltransferase 1, mitochondrial	-1.168	8.53E-01	-1.153	8.71E-01	-1.013	9.94E-01
Q920A7	AFG31	AFG3-like protein 1	-1.380	5.98E-01	-1.357	5.67E-01	-1.017	9.87E-01
Q9JH15	IVD	Isovaleryl-CoA dehydrogenase, mitochondrial	-1.255	6.32E-01	-1.230	7.24E-01	-1.020	9.81E-01
Q8CGK3	LONM	Lon protease homolog, mitochondrial	-1.187	6.11E-01	-1.163	8.55E-01	-1.020	9.82E-01
O35459	ECH1	Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase, mitochondrial	-1.448	2.53E-01	-1.417	1.13E-01	-1.021	9.66E-01

Accession	Symbol	Protein names	GSTA4 Kd vs Scr		aP2-HA-GSTA4 vs pcDNA		GSTA4 Kd vs aP2-HA-GSTA4	
			Fold Change	p-value	Fold Change	p-value	Fold Change	p-value
Q9D6R2	IDH3A	Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial	-1.219	7.06E-01	-1.186	7.61E-01	-1.029	9.76E-01
Q9DCW4	ETFB	Electron transfer flavoprotein subunit beta	-1.419	4.07E-01	-1.375	5.24E-01	-1.032	9.62E-01
Q9WTP7	KAD3	GTP:AMP phosphotransferase, mitochondrial	-1.320	6.06E-01	-1.270	7.23E-01	-1.039	9.70E-01
P16332	MUTA	Methylmalonyl-CoA mutase, mitochondrial	-1.471	4.06E-01	-1.412	6.47E-01	-1.042	9.72E-01
Q60597	ODO1	2-oxoglutarate dehydrogenase, mitochondrial	-1.317	6.36E-01	-1.262	6.99E-01	-1.043	9.69E-01
Q8K2B3	DHSA	Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial	-1.125	8.53E-01	-1.069	9.17E-01	-1.053	9.63E-01
Q9DCS3	MECR	Trans-2-enoyl-CoA reductase, mitochondrial	-1.680	3.10E-01	-1.590	1.52E-01	-1.057	9.45E-01
P45952	ACADM	Medium-chain specific acyl-CoA dehydrogenase, mitochondrial	-1.288	6.65E-01	-1.216	7.19E-01	-1.060	9.50E-01
Q8BMS1	ECHA	Trifunctional enzyme subunit alpha, mitochondrial	-1.254	6.88E-01	-1.146	8.19E-01	-1.095	9.23E-01
Q9CQH3	NDUB5	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 5, mitochondrial	-1.231	4.31E-01	-1.123	8.40E-01	-1.096	8.23E-01
Q9D0K2	SCOT1	Succinyl-CoA:3-ketoacid-coenzyme A transferase 1, mitochondrial	-1.130	7.20E-01	-1.023	9.67E-01	-1.104	8.86E-01
Q9Z219	SUCB1	Succinyl-CoA ligase [ADP-forming] subunit beta, mitochondrial	-1.311	7.09E-01	-1.187	7.83E-01	-1.105	9.36E-01
Q8K370	ACD10	Acyl-CoA dehydrogenase family member 10	-1.460	5.66E-01	-1.319	7.26E-01	-1.107	9.27E-01
P32020	NLTP	Non-specific lipid-transfer protein	-1.204	8.23E-01	-1.082	8.76E-01	-1.112	9.29E-01
O35129	PHB2	Prohibitin-2	-1.243	6.93E-01	-1.116	8.14E-01	-1.114	8.95E-01
Q9CR68	UCRI	Cytochrome b-c1 complex subunit Rieske, mitochondrial	-1.383	4.28E-01	-1.233	5.07E-01	-1.122	8.24E-01
P48962	ADT1	ADP/ATP translocase 1	-1.181	7.28E-01	-1.052	9.33E-01	-1.123	8.95E-01
Q60936	ADCK3	Chaperone activity of bc1 complex-like, mitochondrial	-1.132	8.03E-01	-1.007	9.89E-01	-1.124	7.78E-01
Q91VT4	CBR4	Carbonyl reductase family member 4	-1.238	6.96E-01	-1.093	8.50E-01	-1.132	8.87E-01
Q60931	VDAC3	Voltage-dependent anion-selective channel protein 3	-1.341	4.39E-01	-1.179	7.57E-01	-1.138	8.65E-01
Q8QZT1	THIL	Acetyl-CoA acetyltransferase, mitochondrial	-1.516	5.99E-01	-1.313	7.54E-01	-1.154	9.00E-01
Q9CZ13	QCR1	Cytochrome b-c1 complex subunit 1, mitochondrial	-1.428	6.06E-01	-1.211	8.04E-01	-1.180	8.98E-01
Q8BIJ6	SYIM	Isoleucyl-tRNA synthetase, mitochondrial	-1.553	3.63E-01	-1.283	5.38E-01	-1.211	7.87E-01
Q64433	CH10	10 kDa heat shock protein, mitochondrial	-1.569	5.62E-01	-1.273	6.70E-01	-1.233	8.71E-01
Q9CR62	M2OM	Mitochondrial 2-oxoglutarate/malate carrier protein	-1.236	6.15E-01	1.004	9.93E-01	-1.241	7.49E-01
Q922Q1	MOSC2	MOSC domain-containing protein 2, mitochondrial	-1.372	6.41E-01	-1.102	8.82E-01	-1.245	8.66E-01
P08249	MDHM	Malate dehydrogenase, mitochondrial	-1.398	5.94E-01	-1.120	8.46E-01	-1.248	8.30E-01
Q9Z218	SUCB2	Succinyl-CoA ligase [GDP-forming] subunit beta, mitochondrial	-1.755	1.42E-01	-1.389	2.33E-01	-1.264	2.63E-01
Q61425	HCDH	Hydroxyacyl-coenzyme A dehydrogenase, mitochondrial	-1.637	3.23E-01	-1.284	6.36E-01	-1.275	7.89E-01
Q8BKZ9	ODPX	Pyruvate dehydrogenase protein X component, mitochondrial	-1.370	2.55E-01	-1.069	8.91E-01	-1.282	7.04E-01
Q9D051	ODPB	Pyruvate dehydrogenase E1 component subunit beta, mitochondrial	-1.465	5.96E-01	-1.140	8.42E-01	-1.285	8.03E-01
Q62425	NDUA4	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 4	-1.804	1.11E-02	-1.403	1.60E-01	-1.286	5.95E-01
Q91WS0	CISD1	CDGSH iron-sulfur domain-containing protein 1	-1.054	8.81E-01	1.247	7.05E-01	-1.315	7.48E-01
P08074	CBR2	Carbonyl reductase [NADPH] 2	-1.749	1.04E-01	-1.316	4.19E-01	-1.329	5.63E-01

Accession	Symbol	Protein names	GSTA4 Kd vs Scr		aP2-HA-GSTA4 vs pcDNA		GSTA4 Kd vs aP2-HA-GSTA4	
			Fold Change	p-value	Fold Change	p-value	Fold Change	p-value
Q9EQ20	MMSA	Methylmalonate-semialdehyde dehydrogenase [acylating], mitochondrial	-1.481	5.70E-01	-1.111	8.66E-01	-1.333	8.01E-01
Q9R112	SQRD	Sulfide:quinone oxidoreductase, mitochondrial	-1.440	2.83E-01	-1.075	8.31E-01	-1.340	4.69E-01
Q9DCU6	RM04	39S ribosomal protein L4, mitochondrial	-1.677	2.11E-04	-1.239	2.28E-02	-1.353	1.97E-04
P26443	DHE3	Glutamate dehydrogenase 1, mitochondrial	-1.415	5.51E-01	-1.035	9.61E-01	-1.367	7.83E-01
Q8BJ03	COX15	Cytochrome c oxidase assembly protein COX15 homolog	-1.259	5.02E-01	1.086	7.86E-01	-1.368	3.39E-01
P52825	CPT2	Carnitine O-palmitoyltransferase 2, mitochondrial	-1.776	3.28E-01	-1.293	6.77E-01	-1.373	7.67E-01
Q99KR7	PPIF	Peptidyl-prolyl cis-trans isomerase F, mitochondrial	-1.644	1.26E-02	-1.183	6.34E-01	-1.390	2.59E-01
Q921G7	ETFD	Electron transfer flavoprotein-ubiquinone oxidoreductase, mitochondrial	-1.213	7.59E-01	1.147	8.39E-01	-1.392	7.75E-01
P99029	PRDX5	Peroxiredoxin-5, mitochondrial	-1.496	5.99E-01	-1.063	9.39E-01	-1.407	7.93E-01
Q9DCX2	ATP5H	ATP synthase subunit d, mitochondrial	-1.209	6.35E-01	1.195	7.03E-01	-1.444	6.04E-01
Q9D0S9	HINT2	Histidine triad nucleotide-binding protein 2, mitochondrial	-1.214	8.13E-01	1.190	7.94E-01	-1.445	8.05E-01
Q9JIY5	HTRA2	Serine protease HTRA2, mitochondrial	-1.389	3.49E-01	1.044	8.85E-01	-1.450	4.84E-01
Q9Z210	LETM1	LETM1 and EF-hand domain-containing protein 1, mitochondrial	-1.611	5.73E-01	-1.106	8.57E-01	-1.456	7.20E-01
P56480	ATPB	ATP synthase subunit beta, mitochondrial	-1.488	5.97E-01	-1.011	9.90E-01	-1.472	7.76E-01
Q60932	VDAC1	Voltage-dependent anion-selective channel protein 1	-1.632	5.22E-01	-1.056	9.25E-01	-1.546	6.67E-01
Q99LC5	EFTA	Electron transfer flavoprotein subunit alpha, mitochondrial	-1.442	5.31E-01	1.080	9.08E-01	-1.557	6.78E-01
Q8BFR5	EFTU	Elongation factor Tu, mitochondrial	-1.529	5.79E-01	1.031	9.55E-01	-1.576	6.90E-01
P50136	ODBA	2-oxoisovalerate dehydrogenase subunit alpha, mitochondrial	-1.666	5.10E-01	-1.035	9.74E-01	-1.609	7.76E-01
Q60649	CLPB	Caseinolytic peptidase B protein homolog	-2.020	3.09E-01	-1.255	2.91E-01	-1.610	5.49E-01
Q07417	ACADS	Short-chain specific acyl-CoA dehydrogenase, mitochondrial	-1.621	3.77E-01	-1.002	9.95E-01	-1.618	3.92E-01
Q8BMF4	ODP2	Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex, mitochondrial	-1.667	3.18E-01	-1.009	9.88E-01	-1.651	6.18E-01
O35855	BCAT2	Branched-chain-amino-acid aminotransferase, mitochondrial	-1.614	1.07E-01	1.023	9.67E-01	-1.651	4.11E-01
Q9DB77	QCR2	Cytochrome b-c1 complex subunit 2, mitochondrial	-1.570	3.86E-01	1.058	9.54E-01	-1.661	6.99E-01
Q8R404	QIL1	Protein QIL1	-1.088	8.37E-01	1.534	2.84E-01	-1.670	4.21E-01
Q3TQB2	FXRD1	FAD-dependent oxidoreductase domain-containing protein 1	-1.761	8.70E-03	-1.049	7.25E-01	-1.678	1.38E-01
Q9WUM5	SUCA	Succinyl-CoA ligase [GDP-forming] subunit alpha, mitochondrial	-1.458	5.70E-01	1.158	8.13E-01	-1.689	6.49E-01
Q9DCT2	NDUS3	NADH dehydrogenase [ubiquinone] iron-sulfur protein 3, mitochondrial	-1.957	5.00E-03	-1.157	8.07E-01	-1.691	5.29E-01
P85094	ISC2A	Isochorismatase domain-containing protein 2A, mitochondrial	-1.841	1.70E-04	-1.084	7.86E-01	-1.698	2.13E-01
Q6PB66	LPPRC	Leucine-rich PPR motif-containing protein, mitochondrial	-1.586	4.06E-01	1.091	9.21E-01	-1.730	6.81E-01
Q9D172	ES1	ES1 protein homolog, mitochondrial	-1.072	7.81E-01	1.616	6.21E-01	-1.733	5.71E-01
Q9D023	BR44	Brain protein 44	-2.004	3.61E-01	-1.147	7.98E-01	-1.747	6.52E-01
Q9Z110	P5CS	Delta-1-pyrroline-5-carboxylate synthase	-1.747	3.63E-01	1.005	9.92E-01	-1.756	5.61E-01
Q60930	VDAC2	Voltage-dependent anion-selective channel protein 2	-1.822	6.84E-02	-1.024	9.47E-01	-1.779	3.31E-01
P00015	CYC2	Cytochrome c, testis-specific	-1.520	3.11E-01	1.175	8.07E-01	-1.786	3.72E-01
P58281	OPA1	Dynamin-like 120 kDa protein, mitochondrial	-1.793	3.62E-01	-1.003	9.94E-01	-1.787	5.55E-01
Q91VM9	IPYR2	Inorganic pyrophosphatase 2, mitochondrial	-2.144	-1.11E-15	-1.191	7.60E-01	-1.800	3.41E-01

Accession	Symbol	Protein names	GSTA4 Kd vs Scr		aP2-HA-GSTA4 vs pcDNA		GSTA4 Kd vs aP2-HA-GSTA4	
			Fold Change	p-value	Fold Change	p-value	Fold Change	p-value
Q9Z1J3	NFS1	Cysteine desulfurase, mitochondrial	-1.602	3.76E-01	1.129	8.07E-01	-1.809	3.77E-01
Q91ZE0	TMLH	Trimethyllysine dioxygenase, mitochondrial	-1.562	4.95E-01	1.170	8.21E-01	-1.827	6.28E-01
Q78IK4	APOOL	Apolipoprotein O-like	-1.899	3.56E-01	-1.020	9.78E-01	-1.862	6.49E-01
Q3ULD5	MCCB	Methylcrotonoyl-CoA carboxylase beta chain, mitochondrial	-2.060	3.70E-01	-1.079	9.10E-01	-1.909	6.59E-01
Q9CQ54	NDUC2	NADH dehydrogenase [ubiquinone] 1 subunit C2	-1.881	1.96E-01	1.059	8.86E-01	-1.991	2.70E-01
Q64521	GPDM	Glycerol-3-phosphate dehydrogenase, mitochondrial	-1.905	3.20E-01	1.111	8.86E-01	-2.117	5.29E-01
P29758	OAT	Ornithine aminotransferase, mitochondrial	-1.892	3.42E-01	1.149	8.82E-01	-2.174	5.33E-01
P50516	VATA	V-type proton ATPase catalytic subunit A	-1.909	1.72E-01	1.201	5.66E-01	-2.293	9.33E-02
Q8K1R3	PNPT1	Polyribonucleotide nucleotidyltransferase 1, mitochondrial	-1.387	6.38E-01	1.712	4.47E-01	-2.375	2.94E-01
Q9D6M3	GHC1	Mitochondrial glutamate carrier 1	-1.840	1.18E-01	1.307	3.62E-01	-2.405	1.48E-01
Q9CXW2	RT22	28S ribosomal protein S22, mitochondrial	-1.550	6.01E-01	1.859	1.61E-01	-2.881	3.87E-01
P22315	HEMH	Ferrochelatase, mitochondrial	-2.234	2.39E-09	1.321	5.07E-01	-2.951	3.99E-02
Q8JZN7	MIRO2	Mitochondrial Rho GTPase 2	-2.660	3.03E-13	1.215	3.46E-01	-3.233	-9.77E-15
Q8R127	SCPDH	Probable saccharopine dehydrogenase	-2.356	1.26E-11	1.518	1.62E-01	-3.576	1.10E-10
Q99LC3	NDUAA	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 10, mitochondrial	-2.308	2.76E-01	1.726	4.00E-01	-3.984	1.84E-01
Endoplasmic Reticulum								
Q9DBG6	RPN2	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 2	1.037	8.87E-01	-2.467	5.60E-08	2.558	5.77E-04
Q01405	SC23A	Protein transport protein Sec23A	1.339	2.73E-03	-1.876	1.78E-15	2.512	1.97E-09
P13516	ACOD1	Acyl-CoA desaturase 1	1.628	4.41E-01	-1.527	2.91E-01	2.485	2.64E-01
Q91X78	ERLN1	Erlin-1	1.152	5.08E-01	-1.971	2.18E-07	2.271	2.64E-03
P46978	STT3A	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit STT3A	1.097	9.21E-01	-1.719	2.84E-01	1.886	6.52E-01
Q01853	TERA	Transitional endoplasmic reticulum ATPase	1.239	3.61E-01	-1.404	9.24E-02	1.740	1.95E-06
Q8K297	GT251	Procollagen galactosyltransferase 1	-1.099	6.89E-01	-1.847	1.39E-02	1.680	1.09E-01
Q8BFZ9	ERLN2	Erlin-2	1.024	9.48E-01	-1.626	2.95E-02	1.666	1.02E-01
P08113	ENPL	Endoplasmin	1.013	9.84E-01	-1.434	5.14E-01	1.453	6.95E-01
O08795	GLU2B	Glucosidase 2 subunit beta	-1.186	7.47E-01	-1.713	9.08E-02	1.445	5.34E-01
Q922F8	PDIA6	Protein disulfide-isomerase A6	-1.089	9.12E-01	-1.558	2.02E-01	1.431	6.61E-01
P08003	PDIA4	Protein disulfide-isomerase A4	-1.066	8.83E-01	-1.439	3.91E-01	1.351	6.24E-01
Q9DCN2	NB5R3	NADH-cytochrome b5 reductase 3	-1.122	8.66E-01	-1.465	2.33E-01	1.306	7.46E-01
Q8BHN3	GANAB	Neutral alpha-glucosidase AB	-1.100	8.65E-01	-1.426	5.18E-01	1.296	7.63E-01
O70503	DHB12	Estradiol 17-beta-dehydrogenase 12	-1.143	2.48E-01	-1.407	3.65E-01	1.232	6.02E-01
Q91W90	TXND5	Thioredoxin domain-containing protein 5	1.152	6.78E-01	-1.057	9.23E-01	1.218	7.32E-01
Q61595	KTN1	Kinectin	-1.122	5.79E-01	-1.362	4.58E-01	1.214	7.47E-01
Q80W54	FACE1	CAAX prenyl protease 1 homolog	-1.189	6.99E-01	-1.441	6.96E-01	1.212	8.85E-01
P24369	PPIB	Peptidyl-prolyl cis-trans isomerase B	-1.006	9.85E-01	-1.202	7.61E-01	1.194	7.74E-01
Q3UBX0	TM109	Transmembrane protein 109	-1.141	8.42E-01	-1.357	7.28E-02	1.189	8.11E-01
P09103	PDIA1	Protein disulfide-isomerase	-1.128	8.36E-01	-1.323	6.44E-01	1.173	8.69E-01
Q60716	P4HA2	Prolyl 4-hydroxylase subunit alpha-2	1.176	8.82E-01	1.008	9.65E-01	1.167	9.04E-01

Accession	Symbol	Protein names	GSTA4 Kd vs Scr		aP2-HA-GSTA4 vs pcDNA		GSTA4 Kd vs aP2-HA-GSTA4	
			Fold Change	p-value	Fold Change	p-value	Fold Change	p-value
Q60715	P4HA1	Prolyl 4-hydroxylase subunit alpha-1	-1.464	2.89E-01	-1.676	6.80E-02	1.145	6.51E-01
P35564	CALX	Calnexin	-1.070	7.48E-01	-1.170	7.96E-01	1.094	8.92E-01
P61620	S61A1	Protein transport protein Sec61 subunit alpha isoform 1	-1.637	1.92E-03	-1.675	7.64E-02	1.023	9.55E-01
Q91YQ5	RPN1	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 1	-1.302	6.65E-01	-1.321	5.77E-01	1.015	9.88E-01
P19324	SERP1	Serpin H1	-1.456	5.20E-01	-1.464	4.72E-01	1.005	9.95E-01
O35887	CALU	Calumenin	-1.060	9.08E-01	-1.019	9.61E-01	-1.040	9.60E-01
Q3UVK0	ERMP1	Endoplasmic reticulum metalloproteinase 1	-1.264	7.19E-01	-1.148	8.02E-01	-1.101	8.83E-01
P20029	GRP78	78 kDa glucose-regulated protein	-1.285	6.78E-01	-1.162	7.56E-01	-1.105	9.09E-01
P56395	CYB5	Cytochrome b5	-1.455	5.80E-01	-1.222	7.35E-01	-1.191	8.75E-01
Q99PL5	RRBP1	Ribosome-binding protein 1	1.135	7.69E-01	1.383	5.73E-01	-1.218	7.61E-01
Q9Z247	FKBP9	Peptidyl-prolyl cis-trans isomerase FKBP9	-1.088	8.05E-01	1.122	7.73E-01	-1.221	7.25E-01
P37040	NCPR	NADPH--cytochrome P450 reductase	-1.126	8.88E-01	1.113	7.96E-01	-1.254	6.56E-01
P27773	PDIA3	Protein disulfide-isomerase A3	-1.335	5.83E-01	1.018	9.81E-01	-1.359	7.81E-01
Q9DBS1	TMM43	Transmembrane protein 43	-1.821	3.32E-02	-1.309	8.17E-01	-1.392	8.01E-01
Q91V01	MBOA5	Lysophospholipid acyltransferase 5	-1.207	8.14E-01	1.317	3.88E-01	-1.590	6.77E-01
Q9QY76	VAPB	Vesicle-associated membrane protein-associated protein B	-1.682	4.45E-01	-1.024	9.66E-01	-1.643	6.83E-01
Q5XKN4	JAGN1	Protein jagunal homolog 1	-1.426	2.08E-01	1.201	7.89E-01	-1.713	5.78E-01
P33267	CP2F2	Cytochrome P450 2F2	-3.055	1.19E-12	-1.405	8.01E-09	-2.174	3.00E-04
Q9JKR6	HYOU1	Hypoxia up-regulated protein 1	-1.974	2.55E-01	1.188	7.54E-01	-2.344	4.32E-01
Q9EP69	SAC1	Phosphatidylinositol phosphatase SAC1	-1.561	3.73E-01	1.848	4.03E-01	-2.886	1.53E-01
P63242	IF5A1	Eukaryotic translation initiation factor 5A-1	-2.193	3.34E-02	1.590	2.87E-03	-3.487	9.17E-06
O54998	FKBP7	Peptidyl-prolyl cis-trans isomerase FKBP7	-3.360	3.55E-02	1.451	5.32E-01	-4.875	1.01E-01
Cytoplasm								
Q9WVK4	EHD1	EH domain-containing protein 1	1.414	3.52E-01	-2.749	1.78E-03	3.886	1.96E-02
O88844	IDHC	Isocitrate dehydrogenase [NADP] cytoplasmic	1.176	8.25E-01	-1.943	2.44E-01	2.285	3.96E-01
P97930	KTHY	Thymidylate kinase	-1.021	9.42E-01	-1.668	3.91E-02	1.634	2.32E-01
P16858	G3P	Glyceraldehyde-3-phosphate dehydrogenase	-1.087	8.77E-01	-1.590	2.23E-01	1.462	6.02E-01
Q8CI94	PYGB	Glycogen phosphorylase, brain form	1.054	8.08E-01	-1.353	8.93E-02	1.427	9.87E-02
Q9DBJ1	PGAM1	Phosphoglycerate mutase 1	1.066	7.27E-01	-1.333	1.91E-01	1.420	1.20E-01
O55137	ACOT1	Acyl-coenzyme A thioesterase 1	-1.157	2.72E-01	-1.576	1.64E-01	1.362	4.30E-01
Q91VJ2	PRDBP	Protein kinase C delta-binding protein	1.069	8.38E-01	-1.253	7.16E-01	1.340	6.81E-01
Q63918	SDPR	Serum deprivation-response protein	-1.127	7.92E-01	-1.501	5.01E-01	1.332	7.55E-01
P19096	FAS	Fatty acid synthase	1.233	2.40E-01	-1.078	8.10E-01	1.329	4.75E-01
Q8CGN5	PLIN1	Perilipin-1	-1.221	5.13E-01	-1.579	4.07E-02	1.293	5.96E-01
P14152	MDHC	Malate dehydrogenase, cytoplasmic	-1.170	8.19E-01	-1.498	2.12E-01	1.281	7.21E-01
P17156	HSP72	Heat shock-related 70 kDa protein 2	-1.212	5.51E-01	-1.420	4.22E-01	1.172	8.10E-01
P61982	1433G	14-3-3 protein gamma [Cleaved into: 14-3-3 protein gamma, N-terminally processed]	-1.383	2.23E-01	-1.593	2.44E-01	1.152	8.27E-01
Q8C1B7	SEPT11	Septin-11	-1.064	9.60E-01	-1.182	7.94E-01	1.110	9.48E-01
Q9CPY7	AMPL	Cytosol aminopeptidase	-1.230	6.99E-01	-1.355	5.88E-01	1.101	8.93E-01

Accession	Symbol	Protein names	GSTA4 Kd vs Scr		aP2-HA-GSTA4 vs pcDNA		GSTA4 Kd vs aP2-HA-GSTA4	
			Fold Change	p-value	Fold Change	p-value	Fold Change	p-value
O70456	1433S	14-3-3 protein sigma	1.033	9.40E-01	-1.050	8.88E-01	1.085	9.16E-01
Q80UG5	SEPT9	Septin-9	-1.028	9.53E-01	-1.106	6.95E-01	1.076	9.03E-01
P11499	HS90B	Heat shock protein HSP 90-beta	1.007	9.79E-01	-1.004	9.92E-01	1.011	9.85E-01
P14824	ANXA6	Annexin A6	-1.376	7.75E-01	-1.379	7.04E-01	1.002	9.99E-01
P09411	PGK1	Phosphoglycerate kinase 1	-1.295	4.45E-01	-1.186	6.58E-01	-1.092	8.02E-01
Q9CQV8	1433B	14-3-3 protein beta/alpha	-1.132	7.64E-01	-1.030	9.48E-01	-1.099	8.99E-01
Q00612	G6PD1	Glucose-6-phosphate 1-dehydrogenase X	-1.240	4.02E-01	-1.122	8.05E-01	-1.105	8.49E-01
P10126	EF1A1	Elongation factor 1-alpha 1	-1.267	6.97E-01	-1.133	7.97E-01	-1.118	8.93E-01
P35700	PRDX1	Peroxiredoxin-1	-1.130	8.59E-01	1.018	9.75E-01	-1.150	8.82E-01
P24549	AL1A1	Retinal dehydrogenase 1	-1.045	9.66E-01	1.108	8.79E-01	-1.158	9.27E-01
O54984	ASNA	ATPase Asna1	-1.348	4.77E-01	-1.156	1.04E-02	-1.166	7.47E-01
P06151	LDHA	L-lactate dehydrogenase A chain	1.041	9.74E-01	1.261	5.54E-01	-1.211	9.07E-01
P54310	LIPS	Hormone-sensitive lipase	-1.232	6.61E-01	-1.004	9.91E-01	-1.227	7.60E-01
P34914	HYES	Epoxide hydrolase 2	-1.342	3.96E-01	-1.093	8.13E-01	-1.228	6.56E-01
P26039	TLN1	Talin-1	-1.382	7.69E-01	-1.123	6.91E-01	-1.230	8.44E-01
Q8R164	BPHL	Valacyclovir hydrolase	-1.491	1.18E-01	-1.208	8.07E-01	-1.234	7.85E-01
Q9D8N0	EF1G	Elongation factor 1-gamma	-1.341	5.38E-01	-1.073	8.92E-01	-1.249	8.03E-01
O08528	HXK2	Hexokinase-2	-1.493	4.32E-01	-1.154	7.61E-01	-1.294	7.33E-01
Q61171	PRDX2	Peroxiredoxin-2	-1.374	4.35E-01	-1.048	9.65E-01	-1.311	8.33E-01
Q8BTM8	FLNA	Filamin-A	-1.316	4.34E-01	1.007	9.79E-01	-1.326	6.45E-01
Q8BFZ3	ACTBL	Beta-actin-like protein 2	-1.428	6.11E-02	-1.064	8.86E-01	-1.342	6.33E-01
P17751	TPIS	Triosephosphate isomerase	-1.770	4.56E-01	-1.287	7.64E-01	-1.375	8.27E-01
P13707	GPDA	Glycerol-3-phosphate dehydrogenase [NAD+], cytoplasmic	-1.750	2.54E-06	-1.271	4.78E-01	-1.377	4.84E-01
Q922Q4	P5CR2	Pyrroline-5-carboxylate reductase 2	-1.218	5.09E-01	1.176	7.06E-01	-1.432	6.20E-01
P04117	FABP4	Fatty acid-binding protein, adipocyte	-1.750	3.14E-01	-1.196	7.76E-01	-1.463	7.27E-01
P14211	CALR	Calreticulin	-1.809	2.50E-01	-1.129	7.86E-01	-1.603	5.60E-01
P49817	CAV1	Caveolin-1	-1.763	4.25E-01	-1.049	9.36E-01	-1.680	6.40E-01
P42208	SEPT2	Septin-2	-1.412	2.48E-01	1.243	6.61E-01	-1.755	4.29E-01
P62737	ACTA	Actin, aortic smooth muscle	-1.478	2.60E-01	1.225	6.58E-01	-1.811	3.92E-01
Q9QXS1	PLEC	Plectin	-1.440	5.58E-03	1.264	6.26E-01	-1.819	2.71E-01
P51863	VA0D1	V-type proton ATPase subunit d 1	-1.777	2.02E-02	1.057	8.75E-01	-1.879	2.86E-01
Q9QZ88	VPS29	Vacuolar protein sorting-associated protein 29	-1.213	6.23E-02	1.562	5.01E-01	-1.894	2.99E-01
P17742	PPIA	Peptidyl-prolyl cis-trans isomerase A	-1.647	4.56E-01	1.212	8.50E-01	-1.997	6.73E-01
P07901	HS90A	Heat shock protein HSP 90-alpha	-2.058	2.53E-02	1.165	7.07E-01	-2.397	3.14E-06
P08752	GNAI2	Guanine nucleotide-binding protein G	-2.713	4.98E-03	-1.051	9.33E-01	-2.582	2.63E-01
P63028	TCTP	Translationally-controlled tumor protein	-2.423	2.99E-01	1.072	8.74E-01	-2.597	4.47E-01
O08807	PRDX4	Peroxiredoxin-4	-1.979	2.02E-01	1.439	3.72E-01	-2.849	2.66E-01
Q78PY7	SND1	Staphylococcal nuclease domain-containing protein 1	-1.981	1.92E-01	1.649	2.61E-01	-3.266	6.15E-02
P05064	ALDOA	Fructose-bisphosphate aldolase A	-2.608	5.65E-04	1.614	1.33E-02	-4.211	3.80E-04
Q9R1T4	SEPT6	Septin-6	-2.812	5.12E-05	1.744	3.54E-01	-4.904	2.98E-02

Accession	Symbol	Protein names	GSTA4 Kd vs Scr		aP2-HA-GSTA4 vs pcDNA		GSTA4 Kd vs aP2-HA-GSTA4	
			Fold Change	p-value	Fold Change	p-value	Fold Change	p-value
Golgi Apparatus								
Q8CF93	GLT13	Polypeptide N-acetylgalactosaminyltransferase 13	1.011	9.88E-01	-2.029	1.54E-01	2.052	5.65E-01
Q9CQX2	CYB5B	Cytochrome b5 type B	1.112	7.25E-01	-1.174	6.81E-01	1.306	6.83E-01
Q8CG76	ARK72	Aflatoxin B1 aldehyde reductase member 2	-1.266	7.21E-01	-1.613	2.67E-01	1.275	7.85E-01
Q9D1D4	TMEDA	Transmembrane emp24 domain-containing protein 10	-1.276	6.93E-01	-1.026	9.33E-01	-1.244	7.40E-01
Lysosome								
O89023	TPP1	Tripeptidyl-peptidase 1	-1.141	8.71E-01	-1.947	1.78E-01	1.706	6.27E-01
P16675	PPGB	Lysosomal protective protein	-1.193	7.11E-01	-1.953	1.69E-01	1.637	5.78E-01
P51150	RAB7A	Ras-related protein Rab-7a	-1.033	9.60E-01	-1.315	6.52E-01	1.274	8.25E-01
P20060	HEXB	Beta-hexosaminidase subunit beta	-1.017	9.76E-01	-1.293	6.49E-01	1.271	7.99E-01
P29416	HEXA	Beta-hexosaminidase subunit alpha	-1.313	7.11E-01	-1.481	4.16E-01	1.128	9.21E-01
O35114	SCRIB2	Lysosome membrane protein 2	1.106	7.47E-01	1.003	9.87E-01	1.103	7.90E-01
P50429	ARSB	Arylsulfatase B	-1.121	8.73E-01	-1.216	3.63E-01	1.086	8.99E-01
Q3TCN2	PLBL2	Putative phospholipase B-like 2	-1.313	1.18E-01	-1.383	9.46E-02	1.053	8.13E-01
P12265	BGLR	Beta-glucuronidase	-1.220	7.35E-01	-1.267	6.37E-01	1.039	9.62E-01
Q9WV54	ASAH1	Acid ceramidase	1.155	6.81E-01	1.204	6.60E-01	-1.042	9.35E-01
Q8BFR4	GNS	N-acetylglucosamine-6-sulfatase	-1.273	6.27E-01	-1.180	7.60E-01	-1.079	9.34E-01
P18242	CATD	Cathepsin D	-1.516	6.02E-01	-1.342	5.92E-01	-1.130	9.10E-01
Q8CIF6	SIDT2	SID1 transmembrane family member 2	-1.166	4.96E-01	-1.020	9.64E-01	-1.143	8.37E-01
O09159	MA2B1	Lysosomal alpha-mannosidase	-1.238	6.67E-01	-1.039	9.15E-01	-1.192	7.94E-01
O35448	PPT2	Lysosomal thioesterase PPT2	-1.383	5.39E-01	-1.064	8.83E-01	-1.300	7.71E-01
Q9CQF9	PCYOX	Prenylcysteine oxidase	-1.808	2.52E-01	-1.275	7.63E-01	-1.418	7.83E-01
P17047	LAMP2	Lysosome-associated membrane glycoprotein 2	-3.685	6.42E-02	1.521	1.41E-01	-5.607	1.77E-02
Ribosome								
P62862	RS30	40S ribosomal protein S30	1.663	3.39E-01	-2.370	2.82E-01	3.940	2.84E-01
P62751	RL23A	60S ribosomal protein L23a	1.316	5.42E-01	-1.925	1.08E-02	2.533	1.14E-01
P62717	RL18A	60S ribosomal protein L18a	1.176	8.27E-01	-2.015	1.93E-01	2.368	4.42E-01
P12970	RL7A	60S ribosomal protein L7a	1.389	7.16E-02	-1.629	2.83E-01	2.262	1.88E-01
P47962	RL5	60S ribosomal protein L5	-1.087	7.63E-01	-2.126	7.72E-06	1.957	4.44E-02
P27659	RL3	60S ribosomal protein L3	-1.065	1.08E-01	-1.745	1.95E-01	1.638	2.42E-01
P61358	RL27	60S ribosomal protein L27	1.391	3.43E-01	-1.165	2.37E-01	1.621	2.72E-02
P35979	RL12	60S ribosomal protein L12	-1.163	7.19E-01	-1.611	1.58E-01	1.385	5.93E-01
P62984	RL40	Ubiquitin-60S ribosomal protein L40	-1.269	5.44E-01	-1.750	3.66E-01	1.380	7.44E-01
P62830	RL23	60S ribosomal protein L23	1.047	9.03E-01	-1.252	5.86E-01	1.311	6.97E-01
P47911	RL6	60S ribosomal protein L6	1.051	8.83E-01	-1.081	4.71E-01	1.137	7.56E-01
O70251	EF1B	Elongation factor 1-beta	1.042	9.57E-01	-1.042	7.52E-01	1.085	9.15E-01
P60867	RS20	40S ribosomal protein S20	-1.503	1.20E-01	-1.448	4.10E-01	-1.038	9.51E-01
P53026	RL10A	60S ribosomal protein L10a	-1.938	4.16E-01	-1.833	3.35E-01	-1.057	9.67E-01
P47963	RL13	60S ribosomal protein L13	-1.338	5.98E-01	-1.186	7.21E-01	-1.128	8.88E-01
O55142	RL35A	60S ribosomal protein L35a	-1.836	4.93E-02	-1.186	6.29E-01	-1.548	5.10E-01

Accession	Symbol	Protein names	GSTA4 Kd vs Scr		aP2-HA-GSTA4 vs pcDNA		GSTA4 Kd vs aP2-HA-GSTA4	
			Fold Change	p-value	Fold Change	p-value	Fold Change	p-value
Secreted								
Q9WVJ3	PGCP	Plasma glutamate carboxypeptidase	1.756	3.64E-01	-1.565	2.52E-01	2.748	2.95E-01
P01887	B2MG	Beta-2-microglobulin	1.222	7.58E-01	-1.182	7.62E-01	1.445	5.63E-01
Q60994	ADIPO	Adiponectin	-1.217	8.25E-01	-1.667	3.49E-01	1.370	7.78E-01
Q920A5	RISC	Retinoid-inducible serine carboxypeptidase	-1.157	8.04E-01	-1.549	3.21E-01	1.339	7.02E-01
Q9CYD3	CRTAP	Cartilage-associated protein	1.054	8.73E-01	-1.175	5.80E-01	1.238	7.05E-01
P01027	CO3	Complement C3	-1.011	9.74E-01	-1.115	7.99E-01	1.103	8.48E-01
Q05793	PGBM	Basement membrane-specific heparan sulfate proteoglycan core protein	-1.314	5.70E-01	-1.313	3.18E-01	-1.000	9.99E-01
Q8CC88	K0564	Uncharacterized protein KIAA0564 homolog	-1.504	2.68E-01	-1.358	5.23E-01	-1.108	9.03E-01
P07356	ANXA2	Annexin A2	-1.127	8.41E-01	1.050	9.18E-01	-1.184	8.22E-01
P00687	AMY1	Alpha-amylase 1	-1.643	4.51E-01	-1.106	8.82E-01	-1.486	6.83E-01
Q6GQT1	A2MP	Alpha-2-macroglobulin-P	-1.793	3.26E-01	1.003	9.91E-01	-1.797	1.79E-01
Membrane Bound								
Q91ZX7	LRP1	Prolow-density lipoprotein receptor-related protein 1	-1.184	6.66E-01	-1.442	2.01E-01	1.218	7.66E-01
Q9DC37	MFSD1	Major facilitator superfamily domain-containing protein 1	1.147	7.28E-01	-1.860	1.53E-01	2.134	3.58E-01
P62835	RAP1A	Ras-related protein Rap-1A	1.501	8.61E-02	-1.419	1.62E-01	2.130	5.00E-15
Q9EP72	CO024	UPF0480 protein C15orf24 homolog	1.049	9.72E-01	-1.625	4.53E-01	1.705	7.89E-01
P62492	RB11A	Ras-related protein Rab-11A	1.062	9.27E-01	-1.504	4.93E-01	1.597	6.32E-01
Q6P5F7	TTYH3	Protein tweety homolog 3	-1.090	8.85E-03	-1.651	5.16E-02	1.515	1.01E-01
Q9DCZ4	APOO	Apolipoprotein O	-1.003	9.96E-01	-1.274	4.60E-01	1.270	7.79E-01
Q68FD5	CLH	Clathrin heavy chain 1	1.245	7.85E-01	-1.009	9.91E-01	1.255	8.15E-01
P97300	NPTN	Neuroplastin	-1.016	9.54E-01	-1.207	3.37E-01	1.188	5.36E-01
Q9R1J0	NSDHL	Sterol-4-alpha-carboxylate 3-dehydrogenase, decarboxylating	-1.153	6.13E-01	-1.351	3.15E-01	1.172	7.75E-01
P61027	RAB10	Ras-related protein Rab-10	-1.247	7.15E-01	-1.348	3.91E-01	1.081	9.25E-01
O54724	PTRF	Polymerase I and transcript release factor	-1.356	7.03E-01	-1.339	5.83E-01	-1.012	9.90E-01
P62874	GBB1	Guanine nucleotide-binding protein G	-1.354	1.29E-01	-1.217	4.67E-01	-1.113	8.18E-01
P13595	NCAM1	Neural cell adhesion molecule 1	-1.305	3.64E-02	-1.141	6.89E-01	-1.144	6.49E-01
P11152	LIPL	Lipoprotein lipase	-1.388	5.68E-01	-1.209	7.57E-01	-1.148	8.89E-01
P18572	BASI	Basigin	-1.201	8.12E-01	-1.044	9.30E-01	-1.151	8.93E-01
Q9D7N9	APMAP	Adipocyte plasma membrane-associated protein	-1.341	6.97E-01	-1.141	8.40E-01	-1.175	8.93E-01
Q9Z1Q2	ABHGA	Abhydrolase domain-containing protein 16A	-1.297	6.29E-01	-1.052	2.98E-01	-1.233	7.00E-01
O70423	AOC3	Membrane primary amine oxidase	-1.415	6.96E-02	-1.130	3.39E-01	-1.253	3.42E-01
Q8BH64	EHD2	EH domain-containing protein 2	-1.295	7.12E-01	1.034	9.28E-01	-1.339	7.65E-01
P54116	STOM	Erythrocyte band 7 integral membrane protein	-1.016	9.58E-01	1.705	2.45E-06	-1.732	1.72E-01
Q8C1E7	T120A	Transmembrane protein 120A	-1.924	1.50E-01	1.164	8.34E-01	-2.239	4.52E-01
P84091	AP2M1	AP-2 complex subunit mu	-2.517	-7.99E-15	-1.002	9.94E-01	-2.512	1.73E-05
Q60714	S27A1	Long-chain fatty acid transport protein 1	-2.885	4.25E-03	1.334	4.45E-01	-3.849	3.59E-02
Peroxisome								
Q2TPA8	HSDL2	Hydroxysteroid dehydrogenase-like protein 2	1.070	9.38E-01	-1.262	6.71E-01	1.350	8.28E-01

Accession	Symbol	Protein names	GSTA4 Kd vs Scr		aP2-HA-GSTA4 vs pcDNA		GSTA4 Kd vs aP2-HA-GSTA4	
			Fold Change	p-value	Fold Change	p-value	Fold Change	p-value
Q99LB2	DHRS4	Dehydrogenase/reductase SDR family member 4	-1.403	4.01E-01	-1.780	3.20E-01	1.269	5.89E-01
P51660	DHB4	Peroxisomal multifunctional enzyme type 2	-1.284	6.92E-01	-1.402	4.64E-01	1.092	9.11E-01
Q921H8	THIKA	3-ketoacyl-CoA thiolase A, peroxisomal	-1.459	6.36E-01	-1.528	6.43E-01	1.047	9.39E-01
Q9QXE0	HACL1	2-hydroxyacyl-CoA lyase 1	-1.683	2.96E-01	-1.562	2.66E-01	-1.078	9.29E-01
P98192	GNPAT	Dihydroxyacetone phosphate acyltransferase	-1.351	3.15E-05	1.144	6.38E-01	-1.546	1.37E-01
Other								
P06467	HBAZ	Hemoglobin subunit zeta	-1.064	9.09E-01	-1.431	3.42E-02	1.346	5.55E-01
Q5RKZ7	MOCS1	Molybdenum cofactor biosynthesis protein 1	-1.192	3.30E-01	-1.170	4.88E-01	-1.019	9.58E-01
Q9R099	TBL2	Transducin beta-like protein 2	-1.708	1.68E-01	-1.557	4.16E-01	-1.097	9.21E-01
P40142	TKT	Transketolase	-1.327	2.61E-01	1.438	1.49E-02	-1.908	1.19E-04
P70696	H2B1A	Histone H2B type 1-A	-2.130	6.35E-02	1.306	7.14E-01	-2.782	3.02E-01

CHAPTER 4

Conclusions and Perspectives

Jessica Curtis wrote this chapter.

In the last 50 years, America has had unprecedented change in the abundance and availability of high carbohydrate and high-fat foods. While in previous centuries overindulgence was an indication of prosperity, current popular lifestyles have led to the precipitous decline of weight management in a majority of Americans. The USDA estimates that caloric intake has increased 20% in the last several decades (1), and CDC data confirm that this rise has not been equally matched with energy expenditure (2). Three out of four Americans are now classified as overweight or obese and an astonishing 33% of adults are considered obese (3). There are significant health risks associated with obesity, and as the American population plumps up, likewise will the healthcare costs. Excess medical care costs warranted by comorbidities of obesity such as cardiovascular disease, hepatic steatosis and diabetes, collectively referred to as metabolic syndrome, are estimated to be \$130 billion annually, which represents roughly 1/3 of all healthcare costs (4). While weight loss provides obvious cosmetic incentives, it more importantly improves metabolic health. However, many people struggling with weight management look for pharmacological or surgical solutions. The development of successful treatment methods relies upon a basic understanding of the pathogenesis of metabolic syndrome.

The regulation of whole body insulin sensitivity relies upon metabolic coordination between β -cells, adipose tissue, liver and muscle. In an effort to define the role of adipose tissue biology in the development of obesity-linked diabetes, this

dissertation has explored metabolic regulation by oxidative protein modification in the adipocyte. The foundations behind this investigation were the observations that insulin resistance and obesity are linked with elevated oxidative stress, protein carbonylation and reduced GSTA4 expression in the adipocyte (5). Indeed, many studies have reported on the interrelationship between oxidative stress and disease (6-9). My research goals were to isolate the contribution of GSTA4 to adipocyte metabolism and determine how GSTA4 is involved in the development of insulin resistance. The initial hypothesis was that downregulation of GSTA4 in the adipocyte leads to protein carbonylation and accumulation of inactivating protein modifications, interfering with adipocyte glycemetic control.

While no overt alterations of insulin signaling or glucose tolerance were observed with the loss of GSTA4 (Figure 1), many metabolic hallmarks of diabetes were present, including increased lipolysis and basal glucose uptake, impaired cellular respiration and increased ROS production. Microarray data from human studies suggested that regulation of GSTA4 expression is explicitly linked to the degree of insulin sensitivity in obese subjects (Chapter 2, Figure 3). One of the major differences between obese insulin-sensitive and obese insulin-resistant individuals is chronic inflammation characterized by increased resident adipose tissue macrophages and elevated expression of proinflammatory adipokines and cytokines, like TNF- α (10-12). Correspondingly, TNF- α treatment of 3T3-L1 adipocytes led to downregulation of GSTA4 expression (Chapter 2, Figure 2).

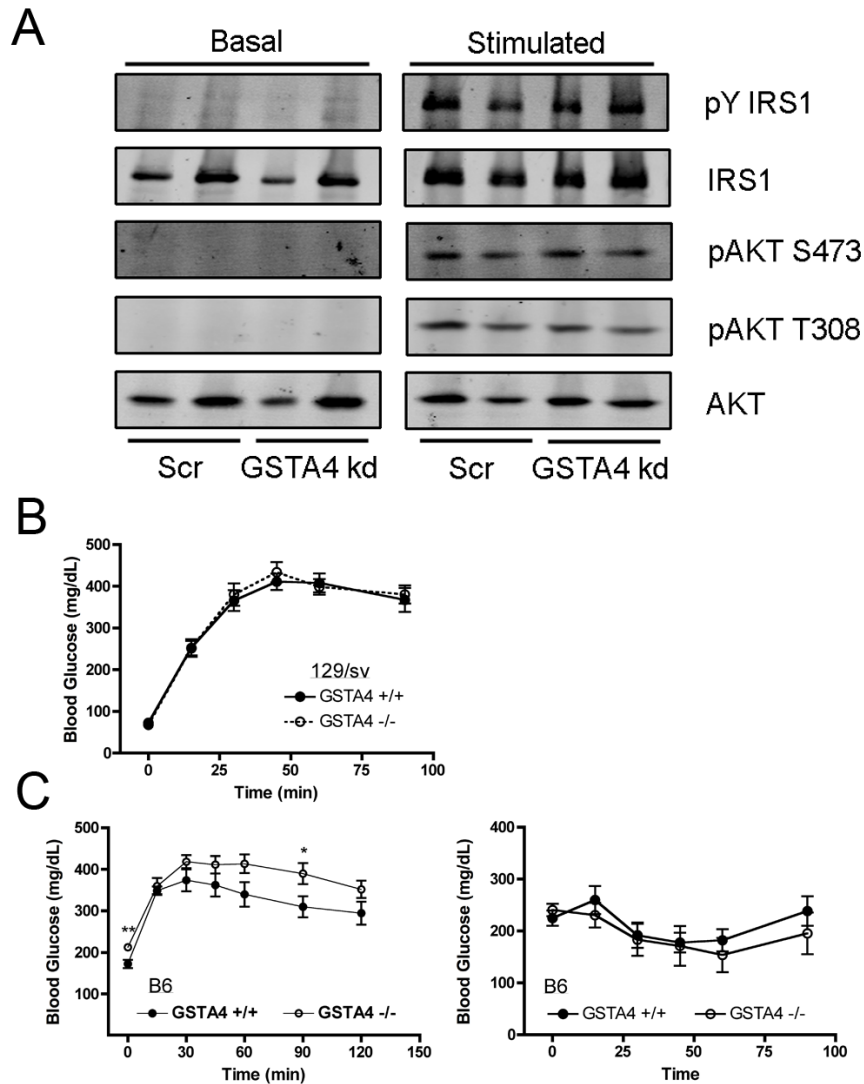


Figure 1. Insulin signaling and glucose tolerance in the absence of GSTA4.

A. Immunoblot analysis of insulin signaling in basal or 50 nM insulin stimulated conditions in GSTA4 knockdown (Kd) and Scrambled (Scr) 3T3-L1 adipocytes. B. Glucose tolerance in high fat-fed wildtype (+/+) and GSTA4 knockout (-/-) mice on congenic 129/sv background strain. C. Glucose (left) and insulin (right) tolerance in high fat fed wildtype (+/+) and GSTA4 knockout (-/-) mice on congenic C57Bl/6 background strain.

Further studies in the Bernlohr lab have similarly concluded that treatment of cultured adipocytes with other proinflammatory cytokines also decreased GSTA4 expression (unpublished), indicating that the inflammatory environment of the adipose tissue is directly involved in regulation of GSTA4 expression.

As outlined in previous chapters, the impact of protein carbonylation was most obvious in the mitochondria. Oxidative metabolism in the mitochondria consumes 98% of inhaled oxygen and is actually the driving force behind the human requirement for oxygen (8). In turn, the majority of cellular oxidative stress originates from the mitochondria. Under physiological conditions, approximately 3% of cellular oxygen is converted to superoxide by electron transfer reactions in the mitochondria (13-15). Disturbances to mitochondrial function contribute to additional free radical production. With improper antioxidant capacities, radical species participate in a feed-forward loop, whereby ROS-induced lipid peroxidation in the absence of GSTA4 yields protein carbonylation. These oxidative modifications then impair mitochondrial function propagating ROS production.

Characterization of GSTA4 silenced adipocytes detailed a multitude of metabolic alterations suggesting that protein carbonylation results in decreased mitochondrial function (Chapter 2). However, the extent of this dysfunction was not great enough to cause changes to adipogenesis, insulin signaling, or cell

survival. In fact, the cells seem to have shifted from reliance upon mitochondrial ATP generation in favor of substrate level phosphorylation. FOF1 ATP Synthase activity is reduced (Figure 2A) while basal glucose transport is increased (Chapter 2), leading to no change in AMPK activation, an intracellular AMP:ATP sensor (Figure 2B). One of the striking results of GSTA4 ablation was a complete loss of ADP-coupled respiration and a concurrent 5-fold increase of superoxide production in both cell and animals (Chapter 2, Figure 6,8). Because mitochondrial function is the result of both density and efficiency, markers of mitochondrial mass were assessed. Expression of transcriptional factors regulating mitochondrial biogenesis were significantly downregulated in GSTA4 silenced adipocytes, as was the amount of mitochondrial DNA (Chapter 2, Figure 7). However, upon further examination with electron microscopy, no change in number or size of mitochondria was observed (Chapter 3, Figure 5). This finding demonstrated that use of mitochondrial DNA and expression of biogenesis genes are not good indicators of mitochondrial mass and that functional changes in GSTA4-silenced adipocytes are consequent to impaired metabolism as opposed to decreased number of mitochondria.

The quantitative proteomic identification of mitochondrial targets of carbonylation was performed to identify potential contributors of mitochondrial dysfunction in the GSTA4-silenced adipocytes (Chapter 3). Identification of proteins without differential carbonylation status between models with variable GSTA4 expression

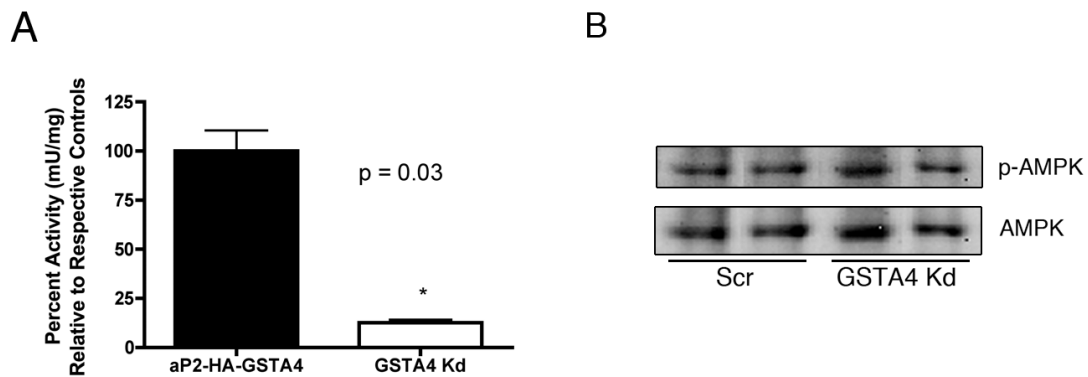


Figure 2. F₀F₁ ATPase activity and AMPK activation. *A.* F₀F₁ ATPase activity in isolated mitochondria from GSTA4 knockdown and over expressing adipocytes. GSTA4 Kd adipocytes display an 85% decrease in activity relative to GSTA4 over expressing adipocytes. *B.* Immunoblot analysis of AMPK activation. Phosphorylation of AMPK is an intracellular indicator of AMP:ATP levels. No changes were observed between GSTA4-silenced (Kd) and control (Scr) adipocytes.

could be considered as background or basal carbonylation targets. However, when considered for pathway analysis, pathways most affected by carbonylation include the tricarboxylic acid cycle, oxidative phosphorylation, branched chain amino acid catabolism, and propanoate metabolism (Chapter 3, Table 1). Taking these proteins into consideration allows for interpretation of the global impact of protein carbonylation, whereas differentially modified proteins are more likely to have a greater independent impact on mitochondrial function.

Interestingly, only a small number of polypeptides were differentially carbonylated, most notably the phosphate carrier protein and two subunits of complex I of electron transport. Further analysis of these proteins indicated that increased carbonylation is associated with impaired function. For example, the phosphate carrier plays a role in regulation of membrane potential through transport of phosphate and protons across the inner mitochondrial membrane or through association with the mitochondrial permeability transition pore. The carbonylation of the phosphate carrier is associated with decreased membrane potential in GSTA4 silenced adipocytes (Chapter 3, Figure 3). Likewise, carbonylation of complex I subunits is also associated with a 10% reduction of electron transport activity (Chapter 3, Figure 6).

There are several interesting directions in which this project could proceed. The identification of the mitochondrial phosphate carrier as a significant target of

protein carbonylation in GSTA4-silenced cells indicated a central role for this protein in the development of oxidative stress and mitochondrial dysfunction. However, there is not currently any information on the site of modification. To this aim, PiC could be tagged and overexpressed in cultured adipocytes and immunoprecipitated after treatment with oxidative agents such as hydrogen peroxide or inflammatory cytokines such as $TNF\alpha$. From this point, the immunoprecipitate can be probed for carbonylation via western blot or mass spectrometry. Using this specifically enriched sample for mass spectrometry may allow for sites of modification to be more readily observed.

Because GSTA4-silenced adipocytes have a multitude of metabolic disturbances, they may not all be due to changes of function for a single protein. For example, the mitochondrial import protein TIMM50 was also identified as a significant target of protein carbonylation in the GSTA4-silenced cells, possibly indicating dysfunctional mitochondrial protein import. The observation that expression of mitochondrial biogenesis genes is down is perplexing in the face of mitochondrial oxidative stress and no change of mitochondrial number. Perhaps it is possible that dysregulation of the mitochondrial protein import machinery leads to decreased function and subsequent buildup of nuclear-encoded mitochondria-targeted polypeptides. This could then negatively regulate transcription of mitochondrial genes.

Along these same lines, it is curious that alterations of membrane potential, respiration, and ROS production do not lead to mitochondrial degradation. As mitochondrial number remains unchanged, it is likely that increases of protein carbonylation also interfere with the activity of autophagosome formation and lysosomal degradation, effectively inhibiting selective removal of dysfunctional mitochondria by mitophagy. While several markers of autophagy were unchanged in these adipocytes, specific markers of mitophagy have not been evaluated. Mitophagy is inexplicably linked to the fission and fusion of mitochondria, and these events should be considered in relationship to protein carbonylation. I hypothesize that loss of GSTA4 and oxidative stress are linked to decreased removal of dysfunctional mitochondria mediated by fission events and mitophagy.

One of the most important questions about protein carbonylation regards causality. Such a large number of proteins can be modified that interpreting data can be complicated. For instance, PGC-1 α is downregulated by loss of GSTA4. Therefore, changes to mitochondrial gene expression are most likely a secondary (or higher) effect of protein carbonylation. Establishing the primary consequences of reduced GSTA4 expression could be pursued with rescue experiments using GSTA4 null or silenced adipocytes overexpressing PGC-1 α or permutations thereof.

The culmination of this work portrays GSTA4 as a crucial mediator of redox balance in the adipocyte. The downregulation of GSTA4 associated with obesity-linked inflammation is responsible for initiating a series of oxidative protein modifications resulting in alterations of cellular energy metabolism and impaired mitochondrial efficiency. These studies have established the important role of antioxidant protection against protein carbonylation in the adipocyte and lend insight to the contributing factors of obesity-linked diabetes.

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