THE ROLE OF MITOCHONDRIALLY DERIVED OXIDATIVE STRESS IN THE DEVELOPMENT OF INSULIN RESISTANCE IN THE ADIOPCTYE

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CHAPTER ONE

Obesity Induced Insulin Resistance and Oxidative Stress

Written by Dalay Olson and edited by Amy Hauck.

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Scope of the Problem

The number of people living with type-2-diabetes (T2D) is increasing at an alarming rate. In 2014, approximately 9% of the adult population worldwide was suffering from diabetes, with greater than 90% suffering from the type-2 form of the disease [1]. Perhaps even more alarming, T2D, once a disease one that only occurred in adults, is now appearing in a growing number of children [2]. The World Health Organization estimates that by 2030 diabetes will be the 7th leading cause of death in the world, as it increases ones risk of death two-fold over age matched people living without the disease [3].

One prominent risk factor for the development of T2D and insulin resistance is obesity. 90% of type-2-diabetics are overweight or obese, highlighting this striking link between the two diseased states [4]. In the United States alone it is estimated that the majority of our adult population is overweight, and that approximately one third can be categorized as obese [2]. Although genetics does contribute to T2D and obesity, both diseases tend to occur as a result of excess food consumption coupled with decreased physical activity.

This dramatic increase in the diabetic population worldwide has triggered an interest in defining molecular mechanisms underlying obesity induced T2D and insulin resistance.

Insulin resistance is a physiological response to nutrient overload and occurs progressively over time as insulin sensitive tissues begin responding less-and-less to circulating insulin levels. To compensate, the pancreas produces supra amounts of insulin in an attempt to drive glucose out of the blood stream and into the peripheral tissue beds [5]-[7]. T2D occurs as a result of a significant peripheral insensitivity to insulin or failure of the β-cell to produce insulin [8]. Because insulin is the most potent anabolic hormone, deregulation in its production, coupled with peripheral insensitivity, inevitably leads to elevated levels of plasma glucose. Prolonged exposure to supra physiological levels of plasma glucose, a condition referred to as glucotoxicity, can result in damage to blood vessels, kidneys, eyes and nerves [9]-[13]. Under more extreme conditions, sustained glucotoxicity can necessitate limb amputation and result in blindness.

To date, many unanswered questions remain regarding specific mechanisms essential for propagation of insulin resistance and T2D. One reason for this lack in understanding is that T2D is a disease that affects multiple organ systems within the body. Therefore, treatment will most likely require a multipronged approach targeting multiple tissues beds simultaneously. Currently, Metformin is the most widely prescribed anti-diabetic agent in clinical practice. Metformin is thought to function partially through inhibition of gluconeogenesis in the liver, as well as through activation of AMPK, which has been shown to participate in

insulin *independent* glucose uptake in muscle and adipose tissue [14]. Metformin's use together with insulin-sensitizers like pioglitazone or troglitizone is one multipronged approach used to activate insulin sensitive and insulin independent pathways of glucose transport simultaneously within the body.

Insulin and Glucose Disposal

Under normal conditions glucose absorption, into the bloodstream from the intestine, circulates and is sensed by the pancreatic β-cells. In the β-cell, glucose is transported down its concentration gradient via the insulin independent glucose transporter 2 (GLUT2), which is constitutively expressed in the plasma membrane. As glucose is metabolized intracellularly, ATP levels rise and ATP sensitive potassium (K^+) channels close in response [15]-[17]. Blocking the ability of K^+ to exit the cell depolarizes the membrane and activates voltage gated calcium (Ca^{2+}) channels. Calcium entering the cell, then mobilizes the fusion and exocytosis of vesicles containing insulin with the plasma membrane. Circulating insulin then binds to insulin receptors found on the surface of insulin sensitive tissues and activates intracellular signaling cascades that facilitate glucose uptake for utilization and storage. There are three main tissues within the body that respond to insulin—liver, muscle and adipose.

The liver constitutively expresses GLUT2 at its plasma membrane. GLUT2 is an insulin independent glucose transporter that facilitates movement of glucose into

and out of the cell based on the glucose concentration gradient. In the liver, GLUT2 transports glucose into the hepatocyte when systemic glucose levels are high during the fed state and transports glucose out of the hepatocyte and into the bloodstream to replenish the falling glucose levels during the fasted state. In hepatocytes insulin does not stimulate the movement GLUT2 to the plasma membrane, but rather activates hexokinase, the enzyme responsible for converting glucose to glucose-6-phosphate. This phosphorylation event keeps intracellular glucose levels low in order to promote sustained glucose influx from the bloodstream during the fed state. Once in the hepatocyte, glucose can be oxidized for energy or stored as glycogen. When glycogen stores are replete and energy demands are low, excess glucose is converted to fatty acids for export into circulation in soluble, vesicular like structures known as lipoproteins. The membrane of lipoproteins are studded with apo-proteins that work to facilitate the docking and release of fatty acids from the lipoprotein into peripheral tissues.

Additionally, a second important role of insulin in the liver is to suppress gluconeogenesis by favoring the production and activity of the enzymes involved in glycolysis (hexokinase, phosphofructrokinase, pyruvate kinase and pyruvate dehydrogenase), while simultaneously inhibiting the activity of those involved in gluconeogenesis (fructose-1,6-diphosphatase, phosphoenolpyruvate carboxylase and pyruvate carboxylase). Under insulin resistant conditions gluconeogenesis

proceeds uninhibited contributing in an even further increase in blood glucose levels. Therefore, one major target of diabetes treatment is to inhibit gluconeogenesis in an attempt inhibit an even further elevation of circulating glucose levels.

On the other hand, muscle and adipose tissue respond to insulin by activating a phosphorylation cascade that ultimately results in mobilizing the intracellular glucose transporter GLUT4 to the plasma membrane where it is able to transport glucose into the cell (Fig. 1).

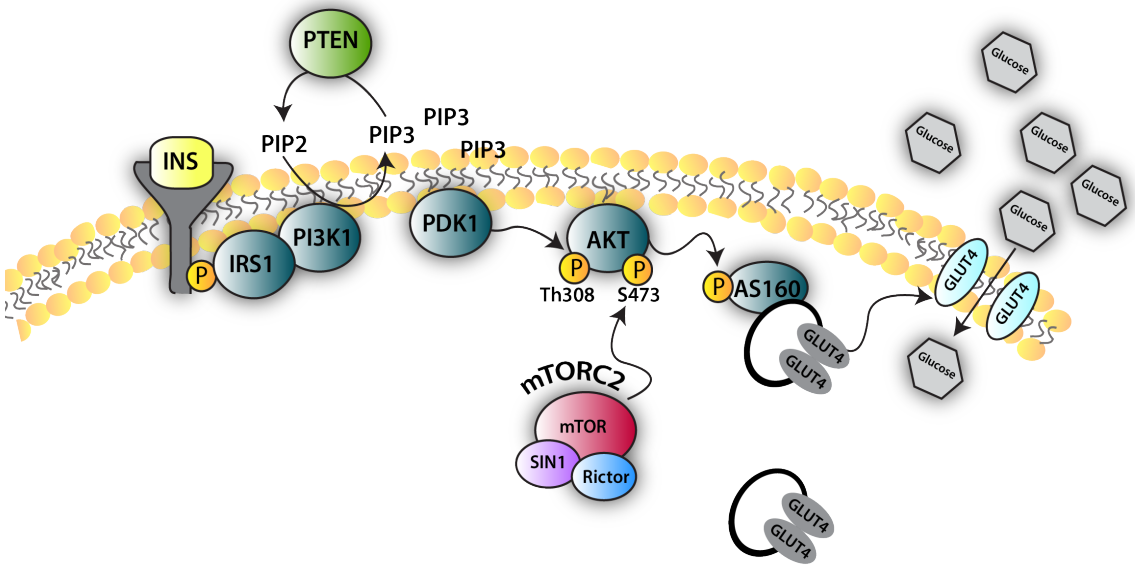

Figure 1. The Insulin Signaling Cascade. The binding of insulin to the insulin receptor activates a phosphorylation cascade involving IRS1, PI3K1, PDK1, mTORC2, AKT and AS160. Through a series of phosphorylation events AS160 is activated and aids in mobilizing glucose transporters to the plasma membrane to allow glucose entry into the cell. PTEN is a phosphatase that dephosphorylates PIP3 effectively terminating the insulin-signaling cascade.

Abbreviations: Insulin (INS), Insulin receptor substrate 1 (IRS1), Phosphatidylinositol-3-kinase 1 (PI3K1), protein kinase 3-phosphoinositide dependent protein kinase-1 (PDK1), Mammalian target of rapamycin complex 2 (mTORC2) and Protein Kinase B (AKT)

Briefly, the binding of insulin to the insulin receptor activates auto-phosphorylation of the heterotetrameric insulin receptor complex. Tyrosine phosphorylation of the insulin receptor recruits and phosphorylates insulin receptor substrate 1 (IRS1). Phosphorylated IRS1 then interacts with a subunit of phosphatidylinositol-3-kinase 1 (PI3K1) which allows for its targeting to the plasma membrane. At the membrane, PI3K1 phosphorylates phosphatidylinositol (4,5) bisphosphate (PIP_2) forming PIP_3. Increased levels of PIP_3 recruits several other enzymes like protein kinase 3-phosphoinositide dependent protein kinase-1 (PDK1) to the plasma membrane. Once recruited, PDK1 phosphorylates AKT at Th308. Additionally, AKT is also phosphorylated at a second site, S473 by a protein complex known as the mammalian target of rapamycin complex 2 (mTORC2), fully activating AKT [18].

Fully activated AKT then phosphorylates and activates AS160, a Rab GTPase, which facilitates the translocation and fusion of intracellular vesicles containing glucose transporter 4 (GLUT4) with the plasma membrane [19]. Membrane bound GLUT4 transports glucose down its concentration gradient into the cell in both muscle and adipose tissue (Fig. 1). As glucose and insulin levels fall, GLUT4 transporters are removed from the plasma membrane and are stored within the cytoplasm until insulin levels rise again.

Intracellular glucose within the muscle is either oxidized for energy production or stored as glycogen. Glycogen pools are found in both liver and muscle cells. Although small, glycogen storage sites play a critical role in providing cells with a rapid source of energy. However, compared with adipose tissue, the amount of energy that can be stored as glycogen is trivial. Therefore, only a fraction of glucose entering the cell is converted to glycogen. Instead, the majority of the glucose is typically oxidized or shunted to adipose tissue where it is converted to triglyceride.

Adipose tissue, on the other hand, is capable of oxidizing glucose and converting it to triglyceride through de-novo lipogenesis. Another important role for insulin within adipose tissue, is the suppression of lipolysis [20], [21]. During the fed state, when glucose levels tend to be high, the binding of insulin to adipocytes inhibits the release of stored fatty acids. Importantly, this suppression is lost in people with T2D and results in uncontrolled fatty acid efflux from the adipocyte. Elevated circulating levels of free fatty acids, not only increases ones risk of cardiovascular disease, but is known to propagate insulin resistance itself.

Although previously underappreciated, adipose tissue plays a major role in initiating and propagating systemic insulin resistance. While previous studies suggest that muscle is responsible for approximately 80% of all postprandial glucose disposal, muscle specific deletion of the insulin receptor does not affect

insulin sensitivity [22], [23]. However, adipocyte specific deletion of the glucose transporter, GLUT4, results in insulin resistance [24]. Therefore, although the disposal rate of glucose into the adipose tissue may be lower, it plays a significant role in regulating and maintaining whole body insulin sensitivity. The next section will be dedicated to adipose tissue specific drivers of insulin resistance.

Drivers of Insulin Resistance in Adipose Tissue

Adipose tissue is composed of multiple cellular subtypes including adipocytes, macrophages, pre-adipocytes and endothelial cells. It has long been appreciated that adipose tissue, under obese conditions, is characterized by a state of chronic, low-grade inflammation. During the transition from lean to obese the population of pro-inflammatory immune cells increases, occupying up to 40% of the adipose tissue itself [25]. Additionally, pro-inflammatory cells communicate in a paracrine fashion with the adipocyte through secretion of cytokines like TNF-α, IFN-γ, IL-1β and IL-6 [25]. Increased concentrations of these markers in adipose tissue positively correlates with insulin resistance in humans and rodents [26].

Anatomically, adipose tissue is typically deposited either intra-abdominally or peripherally. Intra-abdominal, or visceral adipose tissue, is composed of adipose depots that surround organs within the abdominal cavity. Conversely, subcutaneous adipose tissue is categorized as adipose accumulating directly

beneath the skin. Previously, increases, specifically, in intra-abdominal obesity have been correlated with decreased insulin sensitivity, increased circulating triglycerides and concomitant decreases in high-density lipoprotein levels [27], [28]. Consistent with this observation, in humans, after adjusting for BMI, individuals with increased visceral adipose tissue mass also had a greater level of insulin resistance, whereas individuals with an increase only in subcutaneous adipose tissue mass showed a decrease risk for insulin resistance [27].

Interestingly, although obesity and metabolic syndrome are tightly linked, there remains a subset of obese individuals who maintain insulin sensitivity [29], [30]. These metabolically healthy, but obese individuals confirm that obesity and metabolic disease can be uncoupled [31]. Indeed, there are many mouse models that de-couple obesity from insulin resistance and have been crucial tools for understanding metabolic disease [32]-[34]. Importantly, evidence from these models has indicated that, in general, insulin sensitivity is maintained under obese conditions when the adipose tissue does not transition to a pro-inflammatory state. Conversely, insulin resistance is positively correlated with inflammation, particularly within visceral adipose depots [35], [36]. As such, it has been suggested that the presence or absence of pro-inflammatory cells within adipose tissue is a good indicator of whole body insulin sensitivity and metabolic health (Fig. 2)

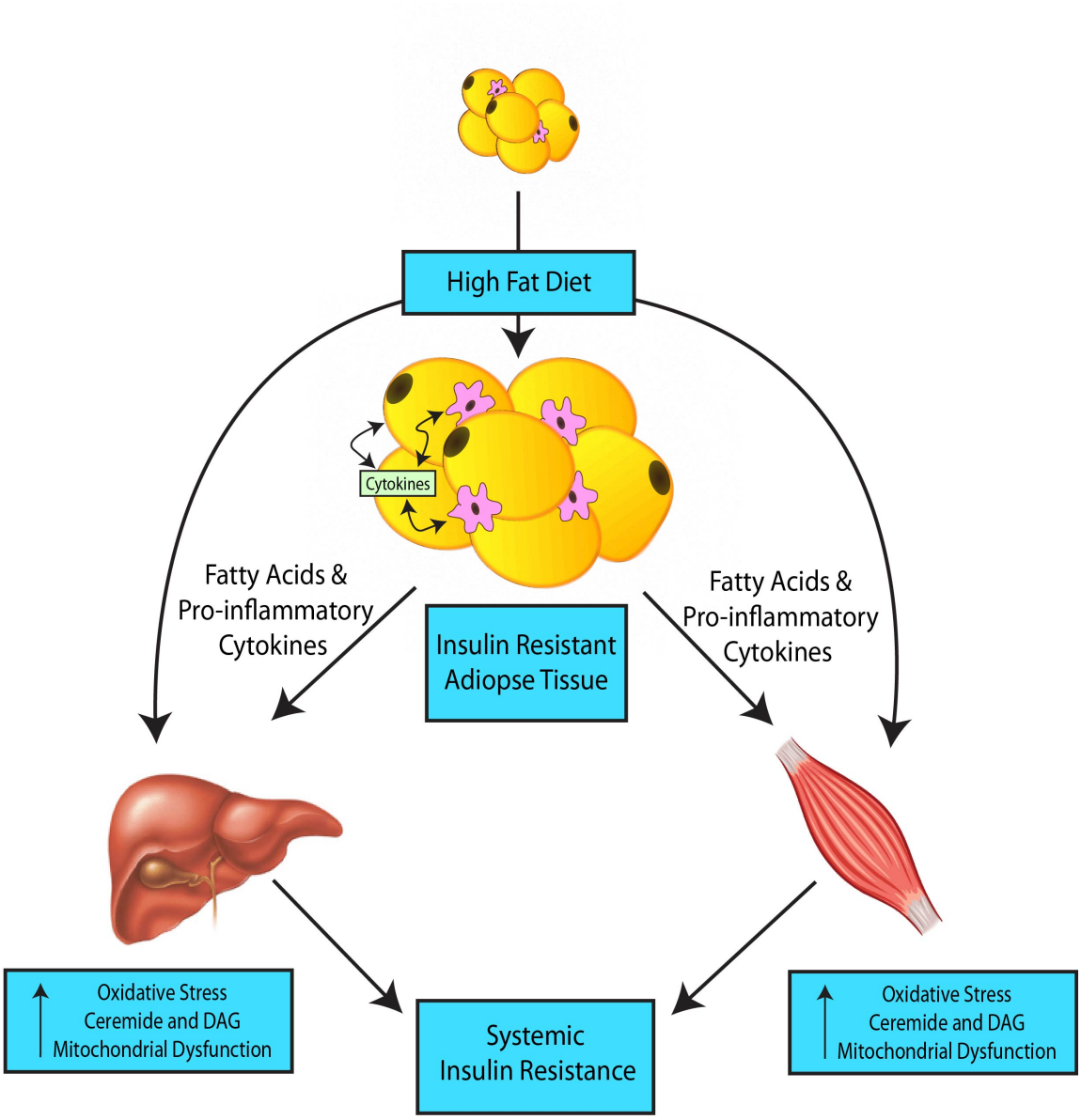

Figure 2. Adipose tissue induced insulin resistance. Overnutrition drives adipose tissue expansion and pro-inflammatory macrophage (mϕ) accumulation within adipose tissue. Release of fatty acids from insulin resistant adipocytes as well as pro-inflammatory cytokines from both mϕ and adipocytes promote local and peripheral insulin resistance.

A second common characteristic of insulin resistant adipose tissue is decreased suppression of lipolysis leading to increased fatty acid efflux from the adipocyte. Locally, free fatty acids (FFA) can bind to toll-like receptors found on resident macrophages, within the adipose tissue, polarizing or priming the macrophage to a more pro-inflammatory state (M1 state), in turn promoting the recruitment of other macrophages and monocytes as well [37], [38].

Systemically, increases in circulating fatty acids, originating form adipose tissue, can induce insulin resistance in both muscle and liver. In the liver, exposure to high levels of FFA promotes the accumulation of ectopic fat. Under high-fat fed conditions, the liver begins to accrue excess lipids, storing it within lipid droplets in the hepatocytes. Metabolism of FFA within the liver form metabolites such as ceremide and diacylglycerol (DAG) [39]. Increases in these metabolites results in activation of signaling cascades like protein kinase C epsilon (PKC-ε), a known driver of insulin resistance within hepatic tissue [17], [39]-[41] (Fig. 2).

Similarly, in muscle, increases in intracellular accumulation of DAG and ceremide can decrease PI3K signaling, driving insulin resistance [42]. Several studies have shown that myocyte insulin resistance can affect the expression of mitochondrial genes and protein levels of several electron transport chain subunits, resulting in decreased mitochondrial function, size, density and DNA content [43]. Additionally, infiltration of fatty acids into muscle can overwhelm the

cell and induce decreases in mitochondrial respiratory capacity, ATP production and increase oxidative stress [40].

Recently, substantial evidence from obesity models in rodents, as well as human studies, support a mechanistic link between inflammation, circulating free fatty acids and oxidative stress [44]-[49]. Indeed, inflammation-induced oxidative stress is now thought to be a dominant driver of insulin resistance within the adipose tissue, although the underlying mechanisms outlining subcellular localization as well as signaling mechanisms of ROS remain poorly understood. As such, much recent work has been driven by the hypothesis that obesity induced inflammation of the adipose tissue leads to increased oxidative stress and insulin resistance.

Indeed, studies using KKAy mice, a genetic model of hyperphagy, revealed elevated levels of hydrogen peroxide selectively in the visceral adipose tissue with no change in oxidative stress in liver, skeletal muscle or aorta at seven weeks of age [50]. Moreover, adipose tissue from these mice, exhibited increased transcript levels of multiple NADPH oxidase subunits including $gp91^{phox}$, the cytosolic component $p47^{phox}$, and $p67^{phox,}$ with no change in transcript levels in liver or skeletal muscle [50]. Consistent with this observation, Pu.1, a transcription factor that has been shown to be responsible for increasing transcription of NADPH oxidase subunits is up-regulated [50]. Extending this analysis, Curtis *et al.* have shown that Gsta4, an important mitochondrial

antioxidant, is also down regulated in visceral adipose tissue of obese, insulin resistant humans, but not obese insulin sensitive individuals, suggesting that the decreased expression is linked to metabolic disease rather than obesity, per se. The molecular mechanisms that lead to the decreased expression of Gsta4 is likely due to increased inflammatory cytokines, however specific pathways underlying their link have not been well defined.

Although fatty acid signaling and inflammation are two important mechanisms known to induce insulin resistance, I will focus on the production and signaling outcomes of oxidative stress for the remainder of this chapter.

Oxidant Production and Methods of Detoxification

Classically, oxidative stress is defined as an imbalance between the production of oxidants and the presence and activity of the antioxidant defense system. Although the term oxidative stress encompasses many forms of reactive oxidants, the production and signaling mechanisms of reactive oxygen species are the best characterized [51], [52].

Within the cellular environment three main reactive oxygen species dominate: superoxide anion ($^{\bullet}O_2^-$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($^{\bullet}OH$). All ROS forms are generated under basal conditions as products of various metabolic functions within the cell. Due to their high reactivity, each ROS is capable of non-enzymatically modifying proteins, DNA, RNA, carbohydrates

and lipids in their biological environment. This quality enables them to act as signaling molecules in metabolic pathways, and in fact, modest production of ROS is required for many cellular functions, including adipogenesis [53]-[56]. The high reactivity of these species also renders them dangerous to the cell at high levels. As such, the production and metabolism of ROS is tightly controlled by antioxidant enzymes whose role is to reduce the pool of ROS such that oxidative damage to cellular machinery is minimized.

There are several metabolic systems that produce ROS as a consequence of normal enzymatic function. For example, xanthine oxidase, lipoxygenases, cyclooxygenases, nitric oxide synthases and the NADPH oxidase have all been shown to catalyze ROS production [57]. Beyond individual ROS-producing enzymes, the mitochondria contribute greatly to ROS pools, particularly under diseased states and in response to inflammatory stimuli. Strikingly, there are eight known enzymes of ROS production within the mitochondria alone [58], [59].

Within the mitochondria, electron leak from the electron transport chain is the most well studied source of ROS. Under physiological conditions NADH and $FADH_2$ oxidation by Complex's I and II, respectively, liberate electrons that are passed between numerous carriers, releasing energy that is used to pump protons into the inter-mitochondrial membrane space. The electrochemical potential created by the proton gradient is then utilized by the F_oF_1 ATP

synthase, coupling its energy release to the formation of ATP. However, compared to cardiac or skeletal muscle, adipocytes have very little work function and thus, maintain high levels of ATP mostly for triacylglycerol synthesis. Moreover, under conditions where ATP levels are high and NADH oxidation is needed for glucose metabolism to facilitate triacylglycerol synthesis, electrons can leak from the electron transport chain (ETC), most likely at Complexes I and III, to form superoxide anion.

The one electron reduction of molecular oxygen occurs virtually instantaneously as electrons leak from the ETC. Due to the unpaired electron, superoxide anion is unable to pass through lipid membranes and as a result, if not enzymatically detoxified, will react and modify biomolecules in its immediate environment. To prevent accumulation of superoxide anion, the mitochondria contain high levels of superoxide dismutase (SOD) for production of hydrogen peroxide (**Figure 3**).

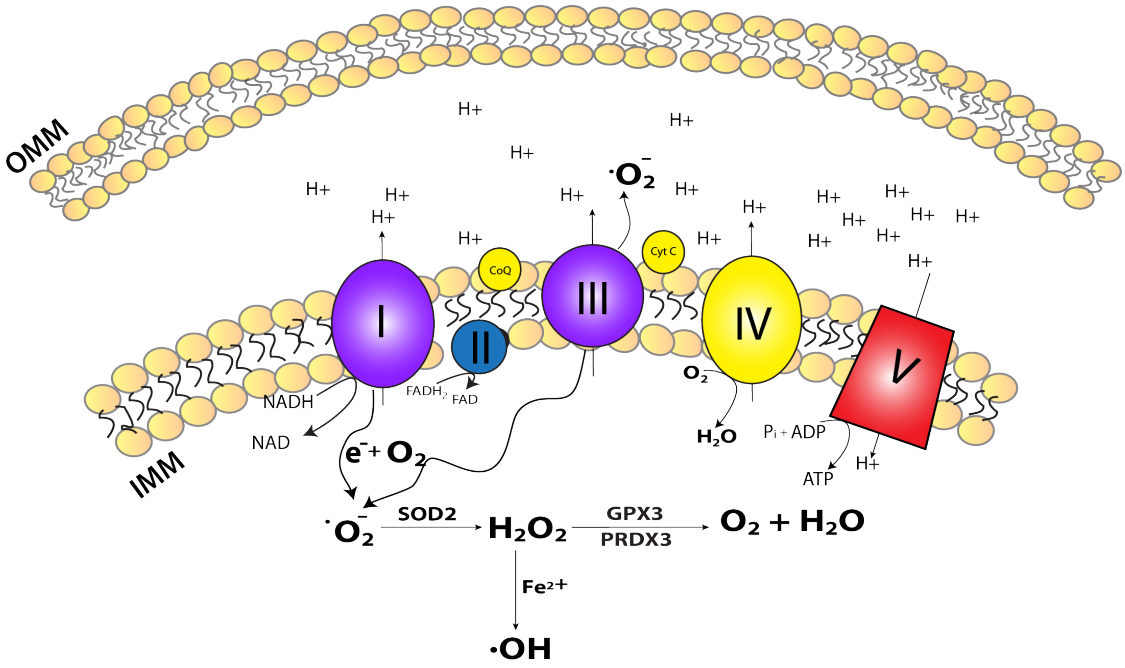

Figure 3. Mitochondrially derived oxidants and the antioxidant system. Reducing equivalents NADH and $FADH_2$ feed electrons into complexes I and II of the electron transport chain (ETC) respectively. Electrons passed between complexes I, III and IV release energy that is harvested to pump protons into the inner membrane space between the outer mitochondrial membrane (OMM) and the inner mitochondrial membrane (IMM) creating an electrochemical gradient. H^+ ions are then dissipated through complex V and the energy released fuels the production of ATP from ADP and inorganic phosphate. Under normal and diseased conditions electrons are capable of escaping the ETC and reacting with molecular oxygen to form superoxide anion. Superoxide anion is metabolized to hydrogen peroxide by superoxide dismutase 2 (SOD2). Hydrogen peroxide is then either metabolized by antioxidants glutathione peroxidase-3 (Gpx3), peroxiredoxin-3 (Prdx3) or it is converted to the hydroxyl radical (•OH) through Fenton chemistry.

Hydrogen peroxide (H_2O_2) is the least reactive and most stable of the three major ROS forms. Due to its chemical nature, hydrogen peroxide is capable of both transmembrane diffusion as well as facilitated movement through aquaporins [60], [61]. As such, its diffusibility allows it to traverse multiple compartments within the cell and interact with a wide variety of biomolecules distal from its site of formation. Thus, unlike superoxide anion that reacts within its immediate environment, the combined qualities of stability and diffusibility allow for a much broader functional boundary for H_2O_2.

In general, H_2O_2 follows one of three paths in the adipocyte: detoxification by antioxidant enzymes, generation of the hydroxyl radical and direct oxidation of biomolecules. There are many enzymes that metabolize hydrogen peroxide to water and molecular oxygen including peroxidredoxin and glutathione peroxidase (Fig. 3) [62]. These enzymes are present at high levels in the mitochondria, but are also found throughout other cellular compartments and function to regulate the pool of H_2O_2, preventing it from reaching toxic levels.

In cells with high levels of H_2O_2 (potentiated by superoxide dismutase) and a diminished capacity for its metabolism (attenuated glutathione peroxidase, peroxiredoxin), free iron(II) can readily oxidize hydrogen peroxide to the hydroxyl radical through Fenton chemistry [63]. The hydroxyl radical is the most reactive ROS. Unlike superoxide anion and H_2O_2, it is not readily metabolized and is only

consumed through the removal of hydrogen atoms from molecules in its immediate environment, resulting in propagation of radical formation through oxidation of neighboring biomolecules. In adipocytes, one common substrate for radical attack is the fatty acyl groups of membrane phospholipid or triacylglycerol. It is this mechanism that links hydroxyl radical formation to the production of lipid aldehydes and therefore protein carbonylation.

Additionally, if H_2O_2 is not metabolized by antioxidants, it can oxidize a variety of targets, and even at low levels is capable of oxidizing sulfur atoms of either cysteine or methionine residues, thereby altering protein function and affecting signaling pathways. The next section of this chapter will be dedicated to outlining the outcomes of side chain oxidation by hydrogen peroxide.

Side Chain Oxidation by H_2O_2

Alterations in the redox status of proteins by ROS also plays an important role both physiologically as well as under diseased states [46], [64]-[66]. More specifically, hydrogen peroxide is thought to play the largest role in altering the redox state of proteins. Even at low levels, H_2O_2 can oxidize a variety of targets within the cell altering protein function and signaling pathways. Hydrogen peroxide is capable of oxidizing sulfur atoms of either cysteine or methionine containing proteins. Although methionine oxidation is known to occur, its

physiological effects on protein function have not been extensively studied. Therefore, we will place more emphasis on cysteine oxidation instead.

It is estimated that approximately 10% of all cysteine residues within the proteome are redox sensitive [46]. Additionally, the sulfur atom found on cysteine residues can exist as a thiolate anion ($-S^-$), a free sulfhydryl (-SH), sulfenic acid (-SOH), sufinic acid ($-SO_2H$), sulfonic acid (SO_3H) and as a disulfide (-S-S) [46]. For a given cysteine residue, each oxidation state can have profound effect on protein conformation, intermolecular interactions, intrinsic activity and degradation [67]. Although hydrogen peroxide is capable of oxidizing free sulfhydryls of cysteine residues, this reaction is unfavorable under physiological conditions. On the other hand, hydrogen peroxide is much more reactive with the thiolate anion. Under physiological conditions the pKa of solvent exposed cysteine residues is about 8.3 and therefore, most cysteine residues exist in the protonated form [66], [67]. Interestingly, because not all proteins contain cysteine residues in the thiolate form, this offers a level of targeted specificity for oxidation by hydrogen peroxide [46], [67]. Unlike other oxidation states, sulfenic acid can be reduced by the enzyme thioredoxin consuming free glutathione [66], [68]. Although the effects of ROS are primarily discussed under diseased states, it is important to note that redox state switching is a process that occurs both physiologically and pathologically.

Perhaps the most well characterized physiological redox switch occurs in a family of protein phosphatases [69], [70]. Nearly all tyrosine phosphatases contain conserved redox sensitive cysteine residues that become oxidized in the presence of low levels of hydrogen peroxide. Phosphatase oxidation temporarily decreases activity of the enzyme allowing for propagation of phosphorylation signaling cascades. In the adipose tissue, binding of insulin to the insulin receptor initiates not only the insulin signaling cascade, but also the activation of the NADPH oxidase [70]. Upon activation, the NADPH oxidase releases superoxide into the extracellular compartment of the cell. Superoxide dismutase present on the outer membrane converts superoxide to hydrogen peroxide. Increases in extracellular hydrogen peroxide levels create a favorable concentration gradient allowing for its movement into the cell [46]. Intracellular hydrogen peroxide has been shown to oxidize the tyrosine protein phosphatase PTEN to a sulfenic acid on conserved cysteine residues, temporarily rendering it inactive. Inactivation of PTEN under insulin stimulating conditions is favorable to the cell because it amplifies the insulin signal by allowing for its propagation to occur uninhibited [70], [71]. Because the formation of sulfenic acids is readily reversible, PTEN does not remain permanently inactivated. Enzymes like thoredoxin are able to reduce these oxidized cysteines, recycling the protein back to its active state effectively terminating the phosphorylation cascade [70]-[72].

Just as oxidation can occur under physiological conditions protein oxidation also occurs under pathological conditions as well [73]. Increased oxidative stress is a hallmark feature of diseased states impacting virtually all tissues and organ systems within the body. Because the onset of most diseased states correlates with age, the process of aging itself is thought to induce disease. In the 1950s Denham Harman proposed the “free radical theory of aging.” His theory suggests that the accumulation of free radicals as a result of prolonged oxidative metabolism can cause irreversible damage to DNA, RNA, and proteins [74], [75]. Chronic exposure to increased oxidative stress can lead to accumulation of inactive, irreversibly oxidized proteins [67].

As previously mentioned, specific residues, mainly cysteines, found on proteins are capable of becoming oxidized in the presence of ROS. Moreover, exposure to chronic oxidative stress or to acute high levels of oxidative stress can result in irreversible protein oxidation. Sulfenic acids are capable of undergoing two additional rounds of oxidation by hydrogen peroxide forming sulfinic acid and sulfonic acid respectively [46], [67]. Importantly, unlike sulfenic acid, neither sulfinic acid nor sulfonic acid oxidation can be enzymatically reversed. As a result it is believed that these oxidation states typically target proteins for degradation. Additionally, because sulfur groups found on cysteines are many times required for catalytic activity, their oxidation thought to result in protein

inactivation [67]. Therefore, it is not oxidation per se that is damaging, but rather the degree to which proteins become oxidized.

One potential target of oxidation that has recently been suggested is the mammalian target of rapamycin complex 2 (mTORC2). mTORC2 oxidation will be a major focus of Chapter 3, and therefore, a brief introduction to the protein complex will be outlined here.

Mammalian Target of Rapamycin and Associated Complexes

Mammalian target of rapamycin (mTOR) serves as the base of two different protein complexes within the cell, mammalian target of rapamycin complex 1 (mTORC1) and mammalian target of rapamycin complex 2 (mTORC2). mTORC1 is composed primarily of mTOR, mammalian lethal with SEC13 protein 8 (mLST8) and regulatory-associated protein of mTOR (raptor), while mTORC2s core components are mTOR, mammalian stress-activated map kinase-interacting protein 1 (SIN1) and rapamycin-insenstive companion of mTOR (rictor). Within the cell, mTORC1 regulates multiple important signaling pathways affecting protein synthesis, lipid synthesis, lysosome biogenesis, autophagy and energy metabolism. mTOR is a protein that is potently inhibited by rapamycin and was originally identified in budding yeast during a genetic screen for rapamycin resistance [76]-[80]. Since the discovery of rapamycin, its immunosuppressive and anti-proliferative effects has gained attention in the world of cancer biology.

To date, rapamycin is FDA approved to be used as an immunosuppressant during organ transplantation, a chemotherapy agent in soft-tissue and bone sarcomas and as a restenosis preventative post angioplasty [81]. Over the past few decades a bourgeoning field of research has emerged investigating the role of mTOR and its accessory proteins in cell growth, proliferation and survival.

Interestingly, although mTORC1 is rapamycin sensitive, mTORC2 is only responsive to rapamycin following prolonged treatment [82]-[84]. It is thought that this extended treatment inhibits any newly formed mTOR from associating with the accessory proteins that make up mTORC2, effectively inhibiting its function. Compared to mTORC1, much less is understood about the physiological functions of mTORC2. For the remainder of this section I will focus on mTORC2, specifically, its components, substrates, physiological effects and potential redox sensitivity.

In 2005, mTORC2 was identified as the elusive kinase responsible for phosphorylating AKT at S473, a critical site involved in insulin signaling. Using a variety of cell lines Sarbassov, *et al.* showed that silencing of rictor results in decreased phosphorylation of AKT at S473 with no changes in downstream targets of mTORC1 [18]. Additionally, because mTOR is a major component of mTORC1 and mTORC2, silencing of mTOR also resulted in decreased S473 phosphorylation on AKT as well as decreased phosphorylation of T389 on S6K1,

a downstream marker of mTORC1. Over the past decade a new branch of research has emerged focusing on understanding the contributions of mTORC2 to disease.

As previously stated, mTORC2 is composed of mammalian target of rapamycin mTOR, mSIN1 and rictor. mTOR is a member of a family of protein kinases termed phosphatidylinositol 3-kinase-related-kinase (PIKK). mTORC2 has been shown to modulating actin organization, rRNA maturation and insulin signaling. However, mTORC2s critical role in insulin signaling is perhaps best characterized.

mTOR is a serine, threonine kinase that contains many evolutionarily conserved domains important for kinase activity as well as protein-protein interactions. mTOR interacts with a wide variety of proteins that make up two distinct complexes, mTORC1 and mTORC2. mTOR itself is modulated by phosphorylation through its four serine phosphorylation sites. Although not completely understood, mTOR phosphorylation seems to play a role in modulating kinase activity. For example, phosphorylation at S1261 has been shown to increases mTORC1 kinase activity without affecting complex formation in tissue [85]. Additionally, S2481 autophosphorylation increases following insulin stimulation and correlates with increased mTORC2 activity [86].

mSIN1 is another component of mTORC2 and plays a critical role in maintaining mTORC2 activity [87], [88]. Whole body knockout of mSIN1 disrupts mTORC2 formation and is embryonic lethal, highlighting its developmental importance. Previous work suggests an association between mSIN1 and rictor, and both appear to play important roles in maintaining mTORC2 stability and activity [89]-[91]. Alternative splicing of mSIN1 generates five isoforms, two of which contain pleckstrin domains, that are capable of binding lipids and targeting to mSIN1 to the plasma membrane [87], [92]. Pleckstrin containing splice variants mSIN1 and mSIN1β within mTORC2 both respond to insulin by phosphorylating AKT at S473. It has recently been suggested that the pleckstrin domain found on these isoforms aids in plasma membrane association allowing for AKT-mTORC2 interactions.

Rictor is the third major component of mTORC2 and its presence is required for complex stability. Tissue specific deletions of rictor all show decreases in S473 phosphorylation of AKT, suggesting decreased complex activity [90], [93], [94]. One additional layer of rictor regulation is through phosphorylation. Rictor contains 28 identified phosphorylation sites, most of which are found clustered within the C-terminal half of the protein [95]. To date the function and signaling mechanisms of most of these phosphorylation sites remains unknown.

Similar to mSIN1, whole body knockout of rictor results in disruption of mTORC2 and is embryonic lethal [96]. As a result, tissue specific deletions have been made to further understand the role of rictor within mTORC2. Muscle specific knockout of rictor results in decreased insulin stimulated glucose uptake due decreased phosphorylation of AS160, a critical protein involved in GLUT4 translocation to the plasma membrane (Fig. 1) [97]. Adipose specific deletion of rictor results in insulin resistance characterized by decrease glucose uptake as a result of suppressed GLUT4 translocation, increased β-cell mass, hyperinsulinemia and increased tissue mass. Additionally, rictor deletion negatively affects complex formation and activity of mTORC2.

More recently it has been suggested that mTORC2 may be redox sensitive. Work from Wang *et al.* revealed decreased association of mTORC2 in a hepatic Sirt1 deficient mouse model that is characterized by increased oxidative stress and insulin resistance. [98] This paper was the first to suggest that increases in ROS production could result in disruption of mTORC2, inhibiting is ability to phosphorylate AKT, potentially driving insulin resistance. Currently no additional publications exist supporting this observation. However, work presented here supports this hypothesis showing, in a model of increased oxidative stress, decreased mTORC2 association, S473 phosphorylation as well as rictor oxidation. Our work has focused on the oxidation of rictor as a potential node

regulating mTORC2 association. Additionally, with 37 cysteine residues, rictor is an attractive target for cysteine oxidation.

Current Objectives

Currently it is clear that there exists an association between oxidative stress, obesity and inflammation within the context of insulin resistance. While oxidative stress has been appreciated as a precipitating factor in the development of insulin resistance and T2D, it is not clear weather oxidative stress is a causal or casual factor. Using multiple models of obesity induced insulin resistance I will highlight a plausible source of oxidative stress originating within the mitochondria due to a down regulation in multiple mitochondrial antioxidants. The underlying question this thesis aims to answer is can mitochondrially derived oxidative stress cause insulin resistance, and if so, what is the molecular mechanism? To answer this, our lab has generated an adipocyte Peroxiredoxin-3 (prdx3) knockdown cell line. Because Prdx3 is the primary antioxidant responsible for metabolizing upwards of 90% of the hydrogen peroxide produced within the mitochondria it provides an ideal system to evaluate the contribution of mitochondrially derived oxidative stress to insulin resistance *independent* of inflammation.

Unlike other reactive oxygen species produced within the cell, the diffusibility and stability of hydrogen peroxide allows it to interact with a variety of cellular

proteins outside of its site of production. As previously mentioned, one critical interaction between hydrogen peroxide and proteins is through side chain oxidation. This work will highlight the role of protein oxidation, and its ability to alter signaling pathways that modulate insulin sensitivity within the adipocyte. More specifically I will focus on the previously underappreciated redox sensitivity of mTORC2, whereby oxidation of one of its subunits, rictor, is associated with decreased complex stability and activity effectively inducing insulin resistance.

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CHAPTER TWO

High Fat Diet Induces Changes in Adipose Tissue *trans*-4-Oxo-2-Nonenal and *trans*-4Hydroxy-2-Nonenal Levels in a Depot-Specific Manner

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Dalay Olson performed experiments in their entirety from figures 6-7

Summary

Protein carbonylation is the covalent modification of proteins by α,β-unsaturated aldehydes produced by non-enzymatic lipid peroxidation of polyunsaturated fatty acids. The most widely studied aldehyde product of lipid peroxidation, *trans*-4-hydroxy-2-nonenal (4-HNE), is associated with obesity-induced metabolic dysfunction and has demonstrated reactivity toward key proteins involved in cellular function. However, 4-HNE is only one of many lipid peroxidation products and the lipid aldehyde profile in adipose tissue has not been characterized. To further understand the role of oxidative stress in obesity-induced metabolic dysfunction, a novel LC-MS/MS method was developed to evaluate aldehyde products of lipid peroxidation and applied to the analysis of adipose tissue. 4-HNE and *trans*-4-oxo-2-nonenal (4-ONE) were the most abundant aldehydes present in adipose tissue. In high fat fed C57Bl/6J and *ob/ob* mice the levels of lipid peroxidation products were increased 5-11 fold in epididymal adipose, unchanged in brown adipose but decreased in subcutaneous adipose tissue. Epididymal adipose tissue of high fat fed mice also exhibited increased levels of proteins modified by 4-HNE and 4-ONE while subcutaneous adipose tissue levels of these modifications were decreased. High fat feeding of C57Bl/6J mice resulted in decreased expression of a number of genes linked to antioxidant biology selectively in epididymal adipose tissue. Moreover, TNFα treatment of 3T3-L1 adipocytes resulted in decreased expression of GSTA4, GPx4, and Prdx3 while up regulating the expression of

SOD2. These results suggest that inflammatory cytokines selectively down regulate antioxidant gene expression in visceral adipose tissue resulting in elevated lipid aldehydes and increased protein carbonylation.

Keywords: adipose, obesity, oxidative stress, 4-HNE, 4-ONE, gene expression

Introduction

Obesity is characterized by increased adiposity and frequently results in the development of insulin resistance and type 2 diabetes [1-3]. Obesity-induced insulin resistance is accompanied by a number of molecular changes in adipose tissue [4-7] including increased macrophage infiltration, particularly in the visceral depots, increased ER stress and mitochondrial dysfunction [8, 9]. Of particular interest is increased oxidative stress that results in elevated protein carbonylation that occurs coincident with the development of insulin resistance [4, 5, 10-13]. Given the importance of cysteine, lysine and histidine (protein carbonylation targets) in catalysis, proteins modified by reactive aldehydes are typically inactivated and may be targeted for degradation. However, some signaling systems are activated upon carbonylation [13-17]. Protein carbonylation is believed to play a major role in altering mitochondrial respiration and metabolic capacity [4, 18, 19] and recently Curtis et al., have identified the mitochondrial phosphate carrier and two subunits of Complex I (NDUFA2 and NDUFA3) as critical carbonylation targets using the 3T3-L1 model cell system [10].

Reactive α,β-unsaturated aldehydes responsible for protein carbonylation are products of lipid peroxidation that result from oxidative modification of polyunsaturated fatty acids (PUFA) [20]. These α,β-unsaturated aldehydes are believed to play a major role in onset and progression of disease [15]. *Trans*-4-hydroxy-2-nonenal (4-HNE) is the most widely studied α,β-unsaturated aldehyde.

4-HNE is moderately reactive and freely diffuses within the cell and across membranes [13, 21, 22]. A number of studies have demonstrated that 4-HNE is cytotoxic, depletes glutathione, induces mitochondrial dysfunction, and targets some enzymes for degradation in a number of cell culture systems [6, 14, 19, 23]. In addition, 4-HNE has been linked to obesity-induced insulin resistance [7, 24].

While 4-HNE has received a great deal of attention, a number of other α,β-unsaturated aldehydes result from lipid peroxidation [25]. The aldehyde profile resulting from lipid peroxidation depends on the PUFA composition of the tissue in question. As such, the cellular consequences of lipid peroxidation depend largely on the profile of aldehydes produced, as they vary to a great degree in rates of formation and metabolism, diffusibility, and reactivity. For example, *In vitro* studies have shown that lipid peroxidation of linoleic acid, the most abundant PUFA in adipose tissue, results primarily in production of 4-HNE and *trans*-4-oxo-2-nonenal (4-ONE), suggesting that 4-ONE may be an important effector of oxidative stress in adipose tissue [26].

A variety of Phase I and Phase II antioxidant enzymes have been implicated in enzymatic detoxification of reactive lipid aldehydes [5]. Aldehyde dehydrogenase has been considered a key antioxidant enzyme catalyzing the oxidation of aldehydes to the corresponding carboxylic acid and in addition a variety of

glutathione peroxidases and peroxiredoxins have been suggested to play major roles in lipid hydroperoxide reduction, preventing formation of lipid aldehydes [27-29]. The key Phase II enzyme responsible for Michael adduction of lipid aldehydes is to glutathione is glutathione S-transferase A4 and mice lacking GSTA4 exhibit increased protein carbonylation and metabolic dysfunction [10].

Despite numerous studies showing increases in protein carbonylation and mitochondrial dysfunction as a result of diet-induced obesity, the specific aldehydes responsible for these processes have not been identified [4, 5, 10-12]. To this end, a novel method was developed and validated for quantitation of individual aldehydes in adipose tissue of high fat-fed C57Bl/6J or *ob/ob* mice. We report herein that high fat fed and *ob/ob* mice exhibited increased levels of 4-HNE and 4-ONE in epididymal white adipose tissue (EWAT). In contrast, feeding a high fat diet resulted in significant decreases in subcutaneous white adipose tissue (SAT) 4-HNE and 4-ONE content without any alteration in aldehyde content of brown adipose tissue. Consistent with these results, 4-HNE/4-ONE Michael adducts were increased in EWAT of high fat-fed mice, but decreased in SAT of high fat-fed mice. Furthermore, EWAT of *ob/ob* and high fat-fed mice displayed general decreases in expression of genes encoding key metabolic enzymes while expression of these enzymes remained relatively unaltered in SAT of *ob/ob* and high fat-fed mice.

Materials and Methods

Differentiation of 3T3-L1 adipocytes and cytokine treatment. 3T3-L1 fibroblasts grown to confluence and differentiated using the standard dexamethasone, methylisobutylxanthine and insulin protocol [31]. Differentiation was assessed by triglyceride accumulation and the expression of adipocyte marker proteins such as the adipocyte fatty acid binding protein, hormone sensitive lipase and the insulin-stimulatable glucose transporter. On day 8 of differentiation cells were treated with 1nmol/L TNFα (R&D systems, Minneapolis) for 24 hours and subsequently harvested for RNA analysis.

Anti-HNE Michael adduct polyclonal primary antibody was purchased from Calbiochem (No. 393207), a subsidiary of EMD Millipore (Billerica, MA). Goat polyclonal anti-actin (No. sc-1616) was purchased from Santa Cruz Biotechiology (Santa Cruz, CA). Horseradish peroxidase-linked rabbit anti-goat IgG antibody (No. NB710-H) was purchased from Novus Biologicals (Littleton, CO). Restore Plus Western Blot Stripping Buffer (No. 46430), SuperBlock Blocking Buffer (No. 37536), and SuperSignal West Pico Chemiluminescent Substrate (No. 34087) were purchased from Thermo Scientific (Rockford, IL).

Animal Protocol. Wild-type C57Bl/6J mice were weaned and maintained on either a high fat (20% protein, 35.5% fat, 36.3% carbohydrate; Bioserv, Frenchtown, NJ) or chow diet at 3 weeks of age. Ob/ob mice were purchased at 10 weeks of age and maintained for 2 weeks on normal chow diet prior to

sacrifice. At 12 weeks of age (9 weeks on diet for high fat and chow fed controls), animals were sacrificed and selected tissues were removed and flash frozen in liquid nitrogen. Frozen tissues were stored at -80° C prior to analysis. All samples were prepared for analysis less than 1 month after dissection and storage. The University of Minnesota Institutional Animal Care and Use Committee approved all animal procedures.

Derivatization of Aldehydes and Solid Phase Extraction. 100 mg of adipose tissue was homogenized in 50 mM sodium acetate buffer containing 5 mM aminooxyacetic acid with 250 μM BHT and 500 μM DTPA at pH 5.0, spiked with deuterated internal standards, and incubated for 1h on ice. Derivatization resulted in oxime formation as shown in Figure 1. After derivatization, samples were vortexed briefly and centrifuged at 10,000g for 10 minutes. Strata-X-AW columns (60 mg sorbent) were conditioned with 1 mL methanol and equilibrated with 1 mL 100 mM sodium acetate, pH 5.3. The aqueous phase of samples was loaded and columns were washed with 1 mL 100 mM sodium acetate buffer, pH 5.3 followed by 1 mL methanol. Columns were dried briefly under vacuum, and derivatized aldehydes were eluted with 1 mL 5:95 NH_4OH:MeOH. Eluates were dried to a film under nitrogen, re-suspended in 125 μL MeOH and stored at -80° C prior to analysis by LC-MS/MS. Immediately preceding LC-MS/MS analysis, samples were diluted to 250 μL with water in order to bring the concentration of aldehyde derivatives in the sample within the linear range validated for analysis.

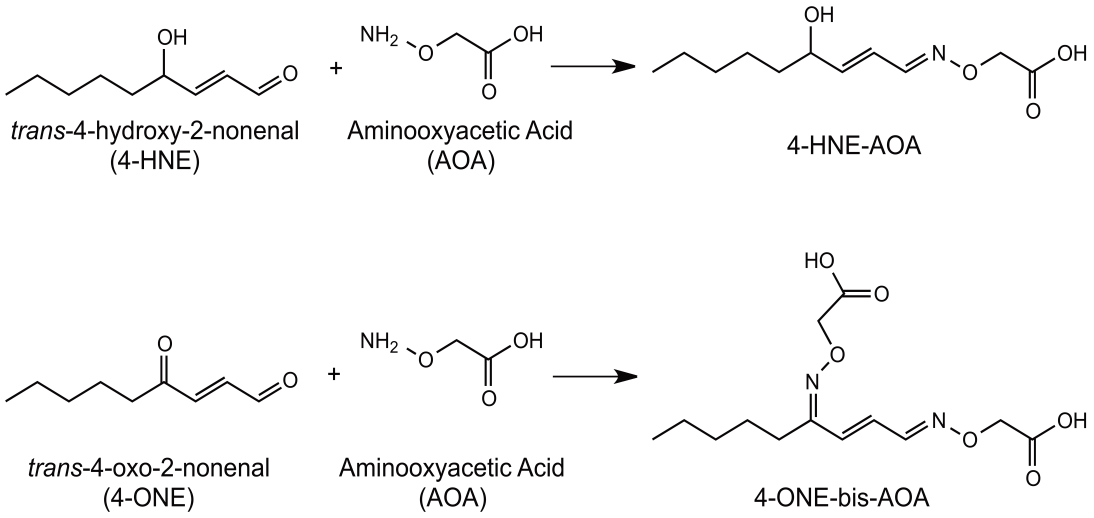

Figure 1. Derivatization of Aldehydes With Aminooxyacetic Acid. Schematic representation of the reaction between aminooxyacetic acid and either 4-HNE or 4-ONE resulting in oxime formation.

Liquid Chromatography-Tandem Mass Spectrometry. LC-MS/MS was carried out on an Agilent 1100 HPLC coupled to an AB/Sciex API 4000Qtrap mass spectrometer. Chromatography was performed using a 100 mm x 2.1 mm Agilent Zorbax Eclipse plus C18 column with a 3.5 micron particle size using a gradient elution. Solvent A was 0.1% formic acid in water and Solvent B was acetonitrile containing 0.1% formic acid. The column was equilibrated at 95:5 A:B. After sample injection, the mobile phase was held at 95:5 for 3 minutes after which time solvent B was increased to 100% using a linear gradient from 3-15 minutes. Solvent B was held at 100% for 3 minutes, followed by equilibration at 95% solvent A. A representative chromatogram of synthetic standards is depicted in Figure 2.

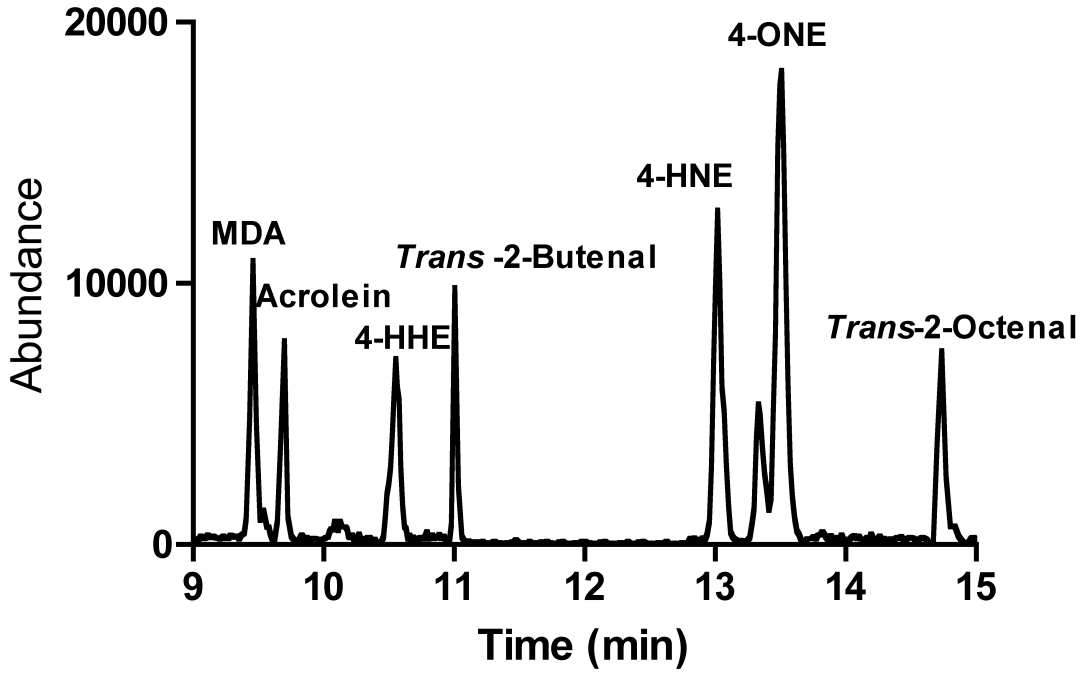

Figure 2. Standard Aldehyde Chromatogram. A mixture of synthetic aldehydes was derivatized using aminooxyacetic acid and separated via LC-MS/MS as described in Materials and Methods. The resolved lipids were analyzed using a AB/Sciex API 4000Qtrap mass spectrometer. Multiple reaction monitoring (MRM) conditions were developed using direct infusion tandem mass spectrometry.

MRM transitions are listed in Table 1. Analyses were performed using electrospray ionization with an ionspray voltage of -4500V. Collision energy for all analytes was -15V, with the exception of malondialdehyde (-10V). Stable isotope dilution was used for quantitation of aldehydes in tissue samples and final quantities were normalized by weight of tissue homogenized. Values are presented as nanograms of aldehyde per gram of tissue.

Analyte	MRM 1 (m/z)	MRM 2 (m/z)	MRM 3 (m/z)
4-HNE	228.1 / 75.1	228.1 / 99.1	228.1 / 136.3
4-ONE	299.3 / 75.2	299.3 / 149.3	299.3 / 225.1
4-HHE	186.3 / 75.2	186.3 / 94.2	186.3 / 110.1
Malondialdehyde	217.0 / 75.2	217.0 / 67.2	217.0 / 141.2
Trans-2-Octenal	198.0 / 75.2	198.0 / 122.0	198.0 / 124.1
Trans-2-Butenal	142.1 / 75.2	142.1 / 66.3	142.1 / 68.1
Acrolein	128.1 / 75.2	128.1 / 52.2	128.1 / 54.2
4-HNE-d3	231.1 / 75.2	231.1 / 102.1	231.1 / 139.3
4-ONE-d3	302.3 / 75.2	302.3 / 152.3	302.3 / 228.1

Table 1. Multiple Reaction Monitoring Conditions

Adipose Tissue Aldehyde Screening. 100 mg of EWAT was prepared and analyzed for eight potential products of lipid peroxidation: malondialdehyde, acrolein, *trans*-2-butenal, 4-HHE, *trans*-2-hexenal, 4-HNE, 4-ONE, and *trans*-2-octenal. 4-HNE, 4-ONE, and MDA were detectable in all samples, with *trans*-2-octenal present at low levels in some samples. The abundance of 4-HNE was similar to that of 4-ONE but much greater than MDA, The absolute levels of 4-HNE and 4-ONE were ~300-500 ng/g tissue while MDA was present at ~20 ng/g tissue (data not shown). Based on abundance and far greater reactivity of 4-HNE and 4-ONE as compared to MDA and *trans*-2-octenal, validation parameters were acquired for 4-ONE and 4-HNE.

Method Validation Parameters. 50 mg of tissue homogenate was spiked with varying concentrations of 4-ONE and 4-HNE with a constant amount of 4-HNE-d_3 and 4-ONE-d_3. Endogenous levels of 4-HNE and 4-ONE were determined in quadruplicate in homogenate spiked only with deuterated internal standards, and subtracted from each sample. The linear range of this assay was 20 fmol to at least 1000 fmol for both 4-HNE and 4-ONE (Figure 3). Intra-day precision was assayed at six concentrations (10, 20, 50, 100, 250, and 1000 fmol) in quadruplicate. Precision, as determined by percent relative standard deviation (%RSD), was within the acceptable range (<20%) from 20 -1000 fmol (Table 2). Accuracy was determined at four concentrations (20, 50, 250, and 1000 fmol) in quadruplicate and was within the acceptable range at 50, 250, and 1000 fmol,

but not at 20 fmol (Table 3). Based on the results of these validation parameters, lower limit of quantitation was defined as 50 fmol based on acceptable values for accuracy, precision and linearity [32].

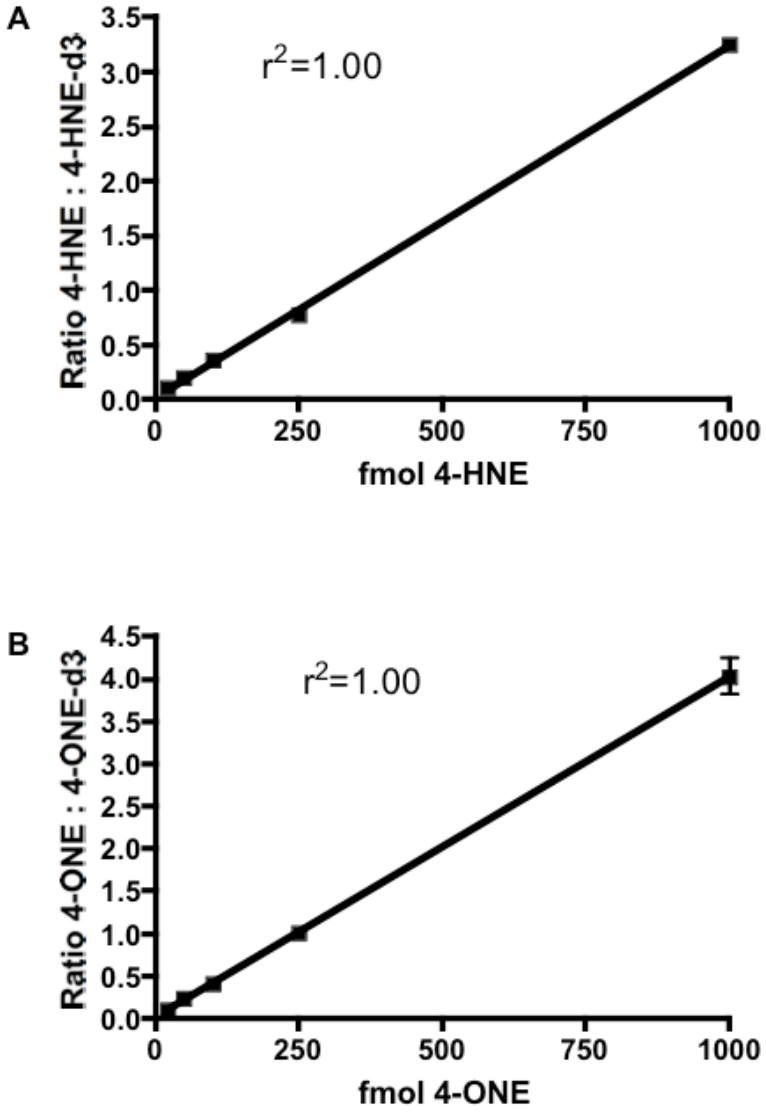

Figure 3. Calibration Curves for AOA-derivatized 4-HNE and 4-ONE. The indicated amounts of 4-HNE (A) and 4-ONE (B) were incubated with 50 mg adipose tissue homogenate and derivatized with aminooxyacetic acid. 4-HNE and 4-ONE oxime derivatives were extracted, separated and quantified by stable isotope dilution using 4-HNE-d_3 and 4-ONE-d_3, respectively. Linearity was assessed using linear regression analysis.

fmol on column	4-HNE	4-ONE
10	---	---
20	8.56	2.41
50	10.34	9.45
100	6.77	4.75
250	2.97	7.58
1000	9.70	5.08

Table 2. Intra-day Precision (%RSD)

fmol on column	4-HNE	4-ONE
20	65	73
50	110	118
250	99	106
1000	94	111

Table 3. Accuracy (% Authentic Standard)

Measurement of 4-HNE/4-ONE Protein Adducts. 250 mg of EWAT or SAT was homogenized in pH 5.5 sodium acetate buffer containing 250 μM BHT using an electronic homogenizer. Samples were vortexed briefly and centrifuged at 3800 rpm for 10 minutes at 4° C. The protein concentration was determined by the bicinchoninic acid protein quantitation assay (Sigma-Aldrich, St. Louis, MO). EWAT samples (20 μg of protein per sample) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 12-20% gradient polyacrylamide gel, while SAT samples were separated on a 10% polyacrylamide gel. After separation, proteins were transferred to Immobilon-FL membranes (Millipore, Darmstadt, Germany) and reduced for 1h with 50 mM sodium borohydride in phosphate-buffered saline (PBS). Membranes were then blocked using LI-COR Odyssey Imaging Systems (LI-COR Biosciences, Lincoln, NE) blocking buffer for 45 minutes prior to overnight incubation at 4° C with the anti-4-HNE/4-ONE Michael adduct antibody. Membranes were washed 4 times with PBS containing 0.2% Tween-20 (PBS-T), then incubated with LI-COR goat anti-rabbit IR-800 secondary antibody for 1h at room temperature. Membranes were washed 4 times with PBS-T and visualized using a LI-COR Odyssey imaging system.

For normalization, membranes were stripped with Restore Plus western blot stripping buffer for 7 minutes. Membranes were washed 4X with PBS-T and blocked with Superblock blocking buffer for 30 minutes prior to overnight incubation at 4° C with 1:1000 goat polyclonal anti-actin antibody. Membranes

were washed 4X with PBS-T, then incubated for 1h with 1:50,000 HRP-linked rabbit anti-goat polyclonal antibody. Membranes were washed 4X with PBS-T and incubated with HRP substrate for 5 minutes. Bands were detected by autoradiography.

Quantitative Real Time PCR (qPCR). Total RNA was extracted from 3T3-L1 cells and tissue using TRIzol reagent according to manufacturer's protocol (Invitrogen, Grand Island, NY). Both subcutaneous and epidiymal adipose tissue were harvested from age matched lean, ob/ob and high fat fed mice. Real-time amplification was performed using a Bio-Rad MyiQ thermocycler using iQ SYBR Green Supermix and recommended thermocycler parameters (Bio-Rad, Hercules, CA). Gene expression assays were performed for glutathione peroxidase 4 (Gpx4), peroxiredoxin 3 (Prdx3), manganese superoxide dismutase (SOD2), glutathione-S-transferase A4 (GSTA4), and aldehyde dehydrogenase 2 (ALDH2) (Primer sets shown in Table 4). Relative gene expression comparisons were carried out using transcription factor 2E (TFIIE) as an endogenous control.

Statistical Analyses. All statistical analyses were done using unpaired two-tailed student's t-tests with significance level set at $p<0.05$.

Target	Forward and Reverse Primers
SOD2	Fw: 5'-GCGAGGAGAAGTACCACGAG Re: 5'-GCTTGATAGCCTCCAGCAA
Gpx4	Fw: 5'-GCTGTGCGCGCTCCAT Re: 5'-CCATGTGCCCGTCGATGT
Prdx3	Fw: 5'-GCAGCTGCGGGAAGGTTGCT Re: 5'-TGCTGGGTGACAGCAGGGGT
GSTA4	Fw: 5'-CGATGGGATGATGCTGACACA Re: 5'-CACTGGGAAGTAACGGGTTTTAGC
TFIIE	Fw: 5'-CAAGGCTTTAGGGGACCAGATAC Re: 5'-CATCCATTGACTCCACAGTGACAC
ALDH2	Fw: 5'-TTTATCCAGCCCACCGTGTTC Re: 5'-CAAGCCCATACTTAGAATCATTGG

Table 4. qRT-PCR Primer Sequence

Results

Quantitation of 4-HNE and 4-ONE in Adipose Tissue. To evaluate the levels of aldehydes in adipose tissue, high fat fed and chow fed C57Bl/6J and *ob/ob* mice were sacrificed and EWAT and SAT adipose depots harvested. Samples were homogenized, derivatized with aminooxyacetic acid and analyzed via liquid chromatography tandem mass spectrometry. In EWAT, levels of 4-HNE were increased >5-fold in high fat fed (HF) C57Bl/6J and chow fed ob/ob mice as compared to chow fed controls (LF) ($p<0.05$) (Figure 4A). Similarly, 4-ONE levels were significantly increased >10-fold in adipose tissue of high fat fed C57Bl/6J and ob/ob mice as compared to chow fed controls ($p<0.05$) (Figure 4A). In contrast, 4-HNE and 4-ONE were significantly decreased in SAT of high fat fed versus chow fed mice (Figure 4B). 4-ONE and 4-HNE were not significantly decreased in SAT of *ob/ob* mice, although levels were reduced to approximately 50% of control levels (Figure 4B). 4-ONE and 4-HNE levels did not differ in brown adipose tissue as a result of high fat diet (Figure 4C).

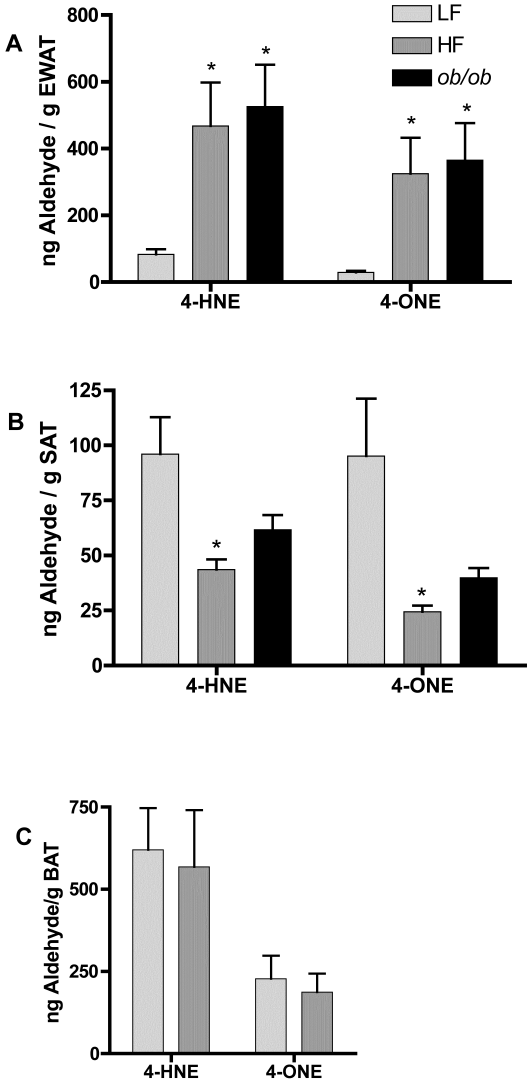

Figure 4. Adipose Tissue Levels of 4-HNE and 4-ONE. Adipose tissue from C57Bl/6J mice fed either chow (LF) or high fat (HF) diet for 9 weeks post-weaning or *ob/ob* leptin deficient mice was harvested and analyzed for 4-HNE and 4-ONE levels. (A) epididymal adipose tissue (n=6 for chow fed mice, n=10 for high fat fed mice, n=6 for *ob/ob* mice), (B) subcutaneous adipose tissue (n=7 for chow and high fat fed mice, n=6 for *ob/ob* mice), (C) and brown adipose tissue (n=7 for chow and high fat fed mice). *Denotes statistically significant difference between control and experimental groups with $p<0.05$ as determined by an unpaired student's t-test.

4-HNE/4-ONE Protein Adduct Analysis. Given the changes in reactive aldehydes in the various adipose depots we assessed the levels of protein carbonylation in the various adipose depots of high fat fed mice compared to chow fed controls using an antibody directed towards 4-HNE/4-ONE protein Michael adducts. Due to the reduction step used in this assay, 4-ONE and 4-HNE protein adducts are both reduced to 1,4-dihydroxynonene adducts and are indistinguishable [33]. Similar to the increased aldehydes in EWAT, high fat-fed mice exhibited increased levels of 4-HNE/4-ONE protein adducts compared to chow fed mice (Figure 5A). These results agree with previous reports that protein carbonylation is increased in EWAT in response to high fat diet [4, 11]. In contrast, 4-HNE/4-ONE Michael adducts were significantly decreased in SAT in high fat-fed mice as compared to chow fed mice (Figure 5B). These results are consistent with the observation that 4-HNE and 4-ONE levels are decreased in SAT of high fat-fed and *ob/ob* mice.

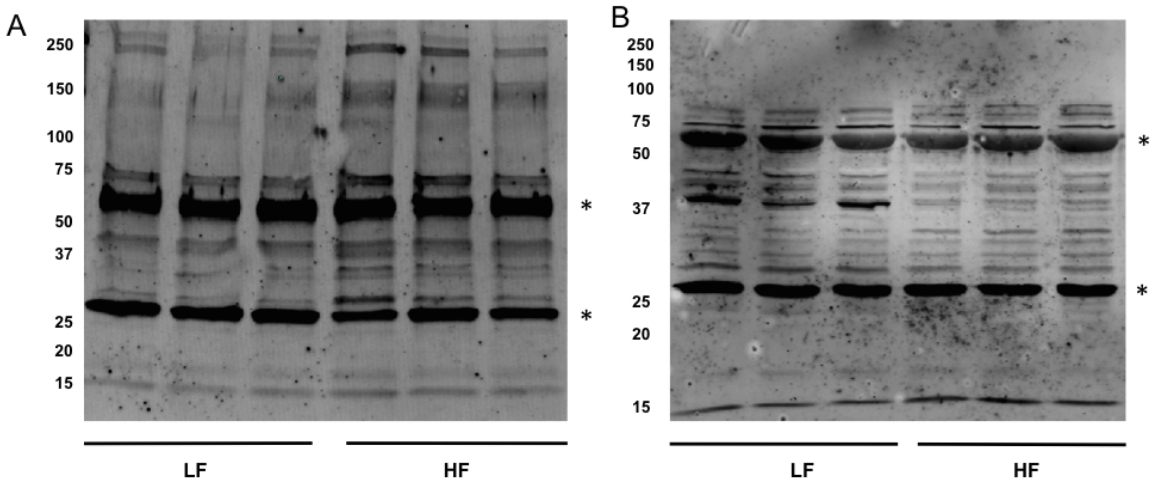

Figure 5. 4-HNE and 4-ONE Protein Adducts in Visceral and Subcutaneous Adipose Tissue. 20 μg of total protein from EWAT (A) or SAT (C) was separated by SDS-PAGE, transferred to PVDF membranes and treated with an antibody specific for 4-HNE/4-ONE Michael adducts. The heavily carbonylated bands at ~ 30 kDa and ~ 67 kDa (*) represent serum proteins and are not included in the data analysis. Quantified bands for EWAT (B) and SAT (D) were normalized to actin and represented as bar graphs. *Denotes a statistically significant difference ($p<0.05$) between low fat and high fat-fed groups.

Expression Levels of Antioxidant Genes in Adipose Tissue. Because lipid aldehydes are formed via hydroxyl radical mediated oxidation of polyunsaturated fatty acyls that are regulated by a series of antioxidant enzymes, epididymal and subcutaneous adipose tissue of chow and high fat fed C57Bl/6J and *ob/ob* mice were analyzed for expression of a panel of antioxidant genes using quantitative real time PCR. Adipose tissue from high fat fed and *ob/ob* mice exhibited significantly decreased expression of GSTA4 and Prdx3 in EWAT. EWAT from high fat fed mice showed significantly decreased expression of Gpx4 (Figure 6A) and Gpx3 (results not shown). Interestingly, the level of GPx1 did not change in response to high fat feeding (results not shown). SOD2 expression did not change significantly as a result of high fat diet, but was significantly increased in EWAT of *ob/ob* mice (Figures 6A, C). ALDH2 and Gpx4 expression levels were significantly decreased in EWAT while they showed no change in expression in SAT of high fat fed as compared to chow fed mice (Figures 6A, B). However, ALDH2 expression was decreased in both EWAT and SAT while Gpx4 expression levels remained unchanged in ob/ob mice as compared to wild-type controls (Figures 6C, D). In subcutaneous adipose tissue, GSTA4 expression was significantly decreased in *ob/ob* mice with a trend toward decreased expression in high fat fed mice, consistent with previous studies (Figures 6B, D). In contrast to EWAT, Prdx3, Gpx4 and Gpx3 (data not shown) expression did not change significantly in SAT of high fat fed and ob/ob mice (Figures 6B, D). However, Gpx4 showed a trend toward increased expression in SAT of ob/ob

mice (Figure 6D). SOD2 expression was not significantly changed in SAT of either high fat fed or *ob/ob* mice (Figures 6B, D).

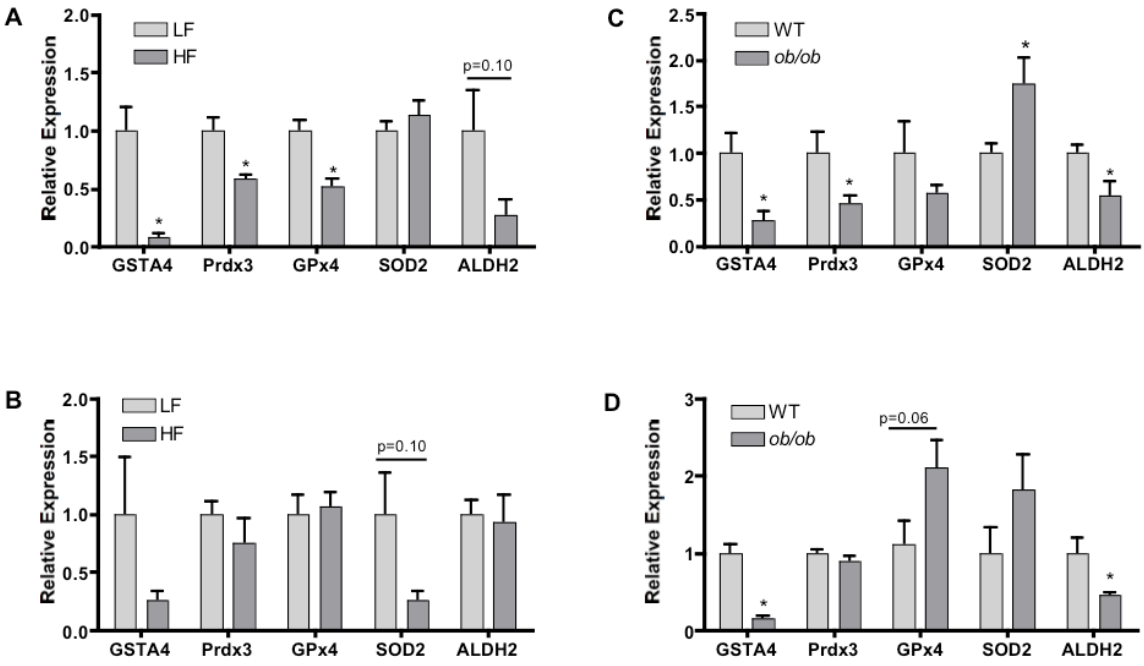

Figure 6. Expression of Genes Linked to Antioxidant Biology in Adipose Tissue. Relative expression of GSTA4, Prdx3, GPx4, SOD2, and ALDH2 in EWAT (A) and SAT (B) of high fat fed (HF) and chow fed (LF) C57Bl/6J mice measured by qRT-PCR. Relative expression of GSTA4, Prdx3, GPx4, SOD2, and ALDH2 in EWAT (C) and SAT (D) of wild-type C57Bl/6J and *ob/ob* mice measured by qRT-PCR. *Denotes a statistically significant difference ($p<0.05$) between control and experimental groups for each gene analyzed.

Since visceral adipose depots are known to be infiltrated with inflammatory macrophages preferentially compared to subcutaneous depots we assessed the effect of inflammatory cytokines on antioxidant gene expression using the 3T3-L1 cell culture system. Figure 7 demonstrates that treatment of 3T3-L1 adipocytes with 1nM of TNFα for 24 h led to the down regulation of GSTA4, Prdx3, GPx4 while increasing the expression of SOD2. As such, a plausible mechanistic basis for the decreased expression of antioxidant genes in EWAT inflammatory cytokine-mediated down regulation.

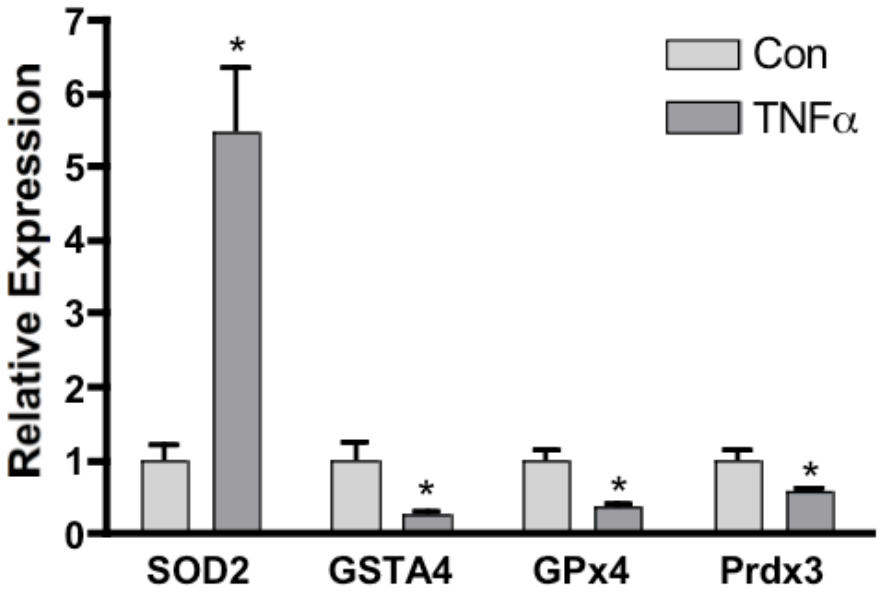

Figure 7. Regulation of Gene Expression in 3T3-L1 Adipocytes by TNFα. 3T3-L1 day 8 adipocytes were incubated for 24 h with 1 nmol/L TNFα and harvested for analysis of SOD2 GSTA4, Gpx4, Prdx3, expression via qRT-PCR. *Denotes a significantly significant difference ($p<0.05$) between control and treatment groups for each gene analyzed.

Discussion

Obesity-induced insulin resistance and resultant type 2 diabetes is strongly linked to oxidative stress and mitochondrial dysfunction [4, 5, 10-12, 24]. Also associated with obesity is a state of chronic low-grade inflammation characterized by increased macrophage infiltration to adipose tissue and resultant increases in pro-inflammatory cytokines such as TNF-α, IL-6 and IL-1β [8, 34, 35]. In studies of insulin resistance, protein carbonylation is often used as a biomarker of oxidative stress in adipose and other tissues and correlates positively with metabolic dysfunction [4, 5, 10-12, 24]. While immunodetection of protein carbonylation provides information regarding total oxidative state of the tissue, it does not provide specific information as to the composition of aldehydes responsible for modification but have largely been assumed to be due to 4-HNE [15, 36-38]. The effects of 4-HNE on cellular systems and the correlation between 4-HNE modifications of proteins and disease have been well defined [6, 17, 23, 36, 39-49].

In adipose tissue, linoleic acid is the most abundant PUFA, representing 15-40 mole-percent of total fatty acid [50, 51]. Based on these levels, it is very likely that lipid peroxidation products of linoleic acid are the driving force behind oxidative stress-induced protein carbonylation. Indeed, one study suggests that 4-substituted alkenals are major products of lipid peroxidation in adipose tissue of mice fed a high fat diet as determined by derivatization and colorimetric

analysis [6]. The results were attributed to 4-HNE despite the inability of this method to distinguish distinct 4-substituted alkenals [52]. As 4-ONE is a major lipid peroxidation product of linoleic acid, it is possible that the results obtained via this method represent 4-ONE, or a mixture of 4-HNE and 4-ONE [26].

Application of a novel LC-MS/MS method confirmed the hypothesis that 4-HNE and 4-ONE are the major products of lipid peroxidation in adipose tissue, and that both increase in abundance in EWAT of high fat fed or *ob/ob* mice. While obesity-induced increases in oxidative stress and lipid peroxidation are widely accepted, the finding that 4-ONE is abundant in adipose tissue is a novel observation. Reactive oxygen species production occurs largely in mitochondria [53] and radicals responsible for peroxidation of PUFA are short-lived, this process likely occurs in mitochondrial membranes and in triglyceride droplets closely neighboring mitochondria. These spatial considerations are important in that mitochondrial protein carbonylation is increased in visceral adipose tissue in response to over nutrition, and isolated mitochondria of animals fed high fat diet display considerable alterations in mitochondrial respiration [4, 10]. As 4-ONE is >100-fold more reactive than 4-HNE, 4-ONE may selectively modify mitochondrial proteins and act as a major effector of oxidative stress-induced mitochondrial dysfunction [21, 22]. Interestingly, RAW 264.7 and primary peritoneal macrophages did not produce detectable levels of 4-HNE or 4-ONE (data not shown). In contrast, primary adipocytes and 3T3-L1 adipocytes did

produce both 4-HNE and 4-ONE (data not shown) suggesting that adipocytes may be the primary producers of lipid aldehydes in adipose tissue.

Consistent with the reactivity differences between 4-ONE and 4-HNE, a recent report by Picklo *et al.* showed that 4-ONE is a more potent inducer of mitochondrial uncoupling (IC_{50} = 5μM) than 4-HNE (IC_{50} > 100μM) [18]. Assuming aldehydes are diffusible within the entire adipocyte, the concentration of 4-HNE and 4-ONE in adipose tissue is estimated to be ~2-5 μM. This estimate suggests that 4-ONE is present at concentrations sufficient to induce mitochondrial uncoupling while 4-HNE levels are ~20-50 fold lower than the IC_{50} for mitochondrial uncoupling.

Previous studies have documented changes in antioxidant gene expression in visceral and subcutaneous adipose tissue depots individually, but direct comparisons are sparse [57, 58]. The results of RT-PCR analysis showed that GSTA4, ALDH2 and Prdx3 gene expression decreased significantly in EWAT of high fat fed C57Bl/6J and *ob/ob* mice compared to chow fed mice. GSTA4 is the primary glutathione-S-transferase responsible for glutathionylation of 4-HNE and 4-ONE, and is known to be significantly down regulated with obesity [12]. This down regulation directly contributes to increased levels of 4-HNE and 4-ONE by reducing metabolic capacity [59-61]. In addition, decreased GPx4 and Prdx3 in EWAT of high fat fed mice are linked to decreased hydrogen peroxide and lipid

hydroperoxide metabolism allowing for greater production 4-HNE and 4-ONE [62, 63]. Specifically, hydrogen peroxide accumulation provides substrate for Fenton chemistry-mediated production of hydroxyl radical, which induces lipid peroxidation. In conjunction, decreased lipid hydroperoxide metabolism results in accumulation of these unstable intermediates, allowing for greater degradation of oxidized PUFA via Hock cleavage to 4-HNE and 4-ONE [20]. Interestingly, SOD2 levels were increased in EWAT of *ob/ob* mice suggesting that superoxide anion is converted more efficiently to hydrogen peroxide. In conjunction with decreases in the capacity to metabolize hydrogen peroxide, this accumulation may contribute to increased production of hydroxyl radicals and accumulation of lipid hydroperoxides. These results provide the basis for the model that over nutrition leads to metabolic dysregulation in EWAT resulting in decreased metabolic capacity of both hydrogen peroxide and lipid aldehydes resulting in accumulation of oxidative stress products.

In contrast to EWAT, high fat fed C57Bl/6J and *ob/ob* mouse SAT exhibited only a trend towards decreased expression of Prdx3, and SOD2. Consistent with previous reports, GSTA4 levels were decreased significantly [4, 11]. Interestingly, ALDH2 expression was decreased in EWAT and SAT of ob/ob mice, which is consistent with the increases in lipid aldehydes observed in EWAT, but not in SAT. The antioxidant gene expression profile in EWAT is consistent with increased lipid peroxidation, but does not adequately explain the

decreases in lipid aldehydes observed in SAT or the dramatic differences between the two tissues. However, metabolism of products of oxidative stress is complex, with multiple classes of both phase I and phase II enzymes contributing to biotransformation of a variety of classes of molecules. Alternatively, the mechanisms by which subcutaneous adipose tissue responds differently to high fat diet than visceral adipose tissue may be linked to decreased inflammatory macrophages present in subcutaneous fat relative to visceral depots [64, 65]. Interestingly, levels of 4-HNE and 4-ONE along with protein carbonylation were decreased in subcutaneous adipose tissue. Many reports suggest that increased visceral adipose tissue correlates more strongly with metabolic disease than subcutaneous adipose tissue [54, 55, 56]. Decreased levels of 4-HNE and 4-ONE suggest that subcutaneous tissue may undergo a robust antioxidant response to high fat feeding, resulting in decreased oxidative stress.

Obesity is associated with a chronic low-grade inflammatory state characterized by macrophage infiltration into adipose tissue [66]. Macrophage infiltration of visceral adipose depots is greater than in subcutaneous depots and may drive the metabolic dysfunction associated with obesity [65]. These macrophages produce and secrete large amounts of proinflammatory cytokines, such as tumor necrosis factor α (TNFα) [67]. Treatment of 3T3-L1 adipocytes with TNFα resulted in decreased expression of GSTA4, GPx4, and Prdx3. In contrast, SOD2 expression increased dramatically. These changes are consistent with

increased production, and decreased metabolism, of H_2O_2, providing substrate for production of hydroxyl radical and subsequent lipid peroxidation. These results provide evidence that inflammation may be responsible for propagation of oxidative stress in EWAT exposed to activated macrophages [65]. Moreover, since SAT is not infiltrated with macrophages to the same extent as is EWAT, oxidative stress has been suggested to be less critical for SAT depots [65]. Moreover, several antioxidant genes such as Prdx3 and ALDH2 were not down regulated in SAT in response to high fat feeding suggesting that the decreased aldehydes and protein carbonylation may be due to antioxidant enzyme action. Further characterization of free radicals and other reactive oxygen species in conjunction with inflammatory signaling pathways may provide further evidence regarding the differences in disposition of EWAT and SAT in response to over nutrition.

Abbreviations

EWAT – epididymal white adipose tissue; SAT – subcutaneous white adipose tissue; 4-HNE – *trans*-4-hydroxy-2-nonenal; 4-ONE – *trans*-4-oxo-2-nonenal; 4-HNE-d_3 – trans-4-hydroxy-2-nonenal-9,9,9-d_3; 4-O NE-d_3 - trans-4-oxo-2-nonenal-9,9,9-d_3 MDA – malondialdehyde; 4-HHE – trans-4-hydroxy-2-hexenal; GS-HHE – glutathionyl trans-4-hydroxy-2-hexenal; PUFA – polyunsaturated fatty acids; GSTA4 – glutathione-S-transferase A4; Gpx4- glutathione peroxidase 4; GPx3 – glutathione peroxidase 3; Prdx3 – peroxiredoxin 3; SOD2 – manganese superoxide dismutase; ALDH2 – aldehyde dehydrogenase 2; AOA – aminooxyacetic acid; BHT – butylated hydroxytoluene; DTPA – Diethylenetriaminepentaacetic acid; LLOQ – lower limit of quantitation; RSD – relative standard deviation; LF – low fat diet; HF – high fat diet

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CHAPTER THREE

Rictor Oxidation and Disruption of the Mammalian-Target of Rapamycin Complex 2 (mTORC2) Links Mitochondrial Oxidative Stress to Insulin Resistance in Adipocytes

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In preparation

Dalay Olson performed experiments in their entirety from figures 1, 2,3 (A, B, F), 4 (A-E), 5, 6 and 7.

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Summary

Oxidative stress has been implicated as an initiating factor in the development of obesity-induced insulin resistance and is correlated with down regulation of Peroxiredoxin-3 (Prdx3), a primary mitochondrial antioxidant responsible for converting mitochondrial hydrogen peroxide (H_2O_2) to water and oxygen. Prdx3 knockout mice exhibit whole-body insulin resistance while Prdx3 transgenic animals remain insulin sensitive when placed on a diet high in saturated fat. Silencing of Prdx3 (Prdx3 KD) in 3T3-L1 adipocytes provides mechanistic insight into the role of adipose oxidative stress and insulin resistance. Prdx3 KD cells exhibit a two-fold increase in H_2O_2, decreased insulin signaling, reduced insulin-stimulated glucose transport, attenuated phosphorylation of the mTORC2 substrate S473AKT, as well as increased protein side chain oxidation. Importantly, the decrease in glucose uptake can be rescued by pre-treatment with the antioxidant N-acetyl-cysteine (NAC). The changes in insulin sensitivity occur independently from endoplasmic reticulum stress as revealed by no detectable changes in BIP, phospho-eIF2α or phospho-JNK protein. Cysteine oxidation analysis indicates that rictor, a component of mTORC2, shows increased oxidation in Prdx3 KD cells. Co-immunoprecipitation analysis reveals decreased association between rictor and mTOR, implying reduced activity of mTORC2, which can be rescued with a 24 hour pretreatment of NAC. Taken together, these data suggest a novel mechanism whereby increased mitochondrial oxidative stress leads to cysteine oxidation of rictor and decreased

association with mTOR, effectively inhibiting signaling capabilities of mTORC2, thus driving insulin resistance.

Introduction

Obesity is a precipitating factor in the development of insulin resistance and is characterized by a state of chronic, low grade inflammation, impaired glucose metabolism and oxidative stress [1]-[4]. Pro-inflammatory cytokines such as tumor necrosis factor alpha (TNFα) and interleukin -6 (IL-6) released from classically activated macrophages recruited to obese adipose tissue is elevated during insulin resistance [1]-[7]. A large body of evidence has linked adipose tissue derived inflammation and oxidative stress as key determinants of insulin resistance. However, the underlying contribution of oxidative stress to insulin resistance, *independent* of inflammation, remains underdeveloped.

Classic markers of insulin resistance include decreased capacity for oxidative phosphorylation, impaired antioxidant expression, increased oxidative stress and protein oxidation [8]. Although the term oxidative stress encompasses many forms of reactive oxidants, the production and signaling mechanisms of reactive oxygen species (ROS) are the best characterized, and while cells are capable of producing ROS at many sites, mitochondria are thought to be a major contributor [9].

During the process of oxidative phosphorylation electrons are passed between complexes of the electron transport chain with the final electron acceptor being molecular oxygen, resulting in the formation of water. Under basal conditions a

small fraction of escaped electrons can react with molecular oxygen to form superoxide anion.

Within mitochondria, superoxide anion is quickly metabolized to hydrogen peroxide (H_2O_2) by manganese superoxide dismutase (SOD2). H_2O_2, under normal conditions, is metabolized to water and molecular oxygen by several antioxidant enzymes. However, under conditions favoring oxidative stress, H_2O_2 is capable of oxidizing a wide variety of proteins, lipids and DNA found both within and outside of the mitochondria [2], [10], [11]. Specifically, H_2O_2 is capable of modifying sulfur atoms of methionine and cysteine containing proteins and, because cysteine residues are typically found within active sites, their oxidation often renders proteins inactive [3], [12], [13]. Previously, H_2O_2 has been shown to oxidize thioredoxin, resulting in the release and auto-phosphorylation of its interacting protein, apoptosis signal-regulating kinase 1 (ASK1). Accumulation of activated ASK1 leads to phosphorylation of the stress sensitive c-Jun N-terminal kinase (JNK) [14]. In addition to activating nuclear factor kappa B (NF-κB), JNK is responsible for phosphorylating serine 307 on IRS1, signaling for its degradation, effectively attenuating insulin signaling [15].

Results from our lab suggest that increases in oxidative stress occurs within visceral adipose tissue of obese, insulin resistant mice, due to a selective down regulation of several key mitochondrial antioxidant enzymes responsible for

metabolizing H_2O_2 [16]. One antioxidant that is consistently down-regulated *in vivo* is peroxiredoxin-3 (Prdx3) [16].

The peroxiredoxin family of enzymes consists of six distinct isoforms and is one of the most ancient and abundant families of antioxidant proteins [17]. Peroxiredoxins are capable of reducing peroxynitrite, lipid peroxides as well as H_2O_2 through the oxidation of specific cysteine residues found on each isoform [18], [19]. Although the mitochondria contain many antioxidant enzymes, Prdx3 is unique in that it is the only antioxidant whose isoform is not found anywhere outside of the mitochondria [20]. Whole body knockout of Prdx3, in mice, results in increased white adipose tissue accumulation, oxidative stress, adipokine disregulation and whole body insulin resistance [21]. Furthermore, transgenic mice over expressing Prdx3 are protected from high-fat diet induced insulin resistance, highlighting the critical role that oxidative stress plays in preserving insulin sensitivity [1]-[4], [22]. Moreover, in the adipose tissue of obese, insulin resistant humans, Prdx3 is transcriptionally down-regulated [1]-[7], [23]. Although it is clear that Prdx3 plays an important role in maintaining metabolic homeostasis, the underlying pathways driving insulin resistance within adipose tissue have not been thoroughly investigated. Moreover, while inflammation induced oxidative stress can result in insulin resistance it is not known whether mitochondrially derived oxidative stress *alone* is sufficient to induce insulin resistance in adipocytes.

By knocking down Prdx3 in 3T3-L1 cells we have generated an adipocyte-specific model where mitochondrially derived oxidative stress results in insulin resistance *independent* of inflammation. Interestingly, the insulin resistance was also independent of endoplasmic reticulum stress (ER-stress) and the mitochondrial unfolded protein response (mito-UPR). Instead, the major driver appears to be oxidation and disassociation of the mammalian target of rapamycin complex 2 (mTORC2) leading to decreased phosphorylation of S473-AKT, decreased glucose uptake and insulin resistance. Importantly, insulin sensitivity as well as mTORC2 formation can be restored with pretreatment of N-acetyl cysteine (NAC), a potent antioxidant, implicating oxidative stress as the major trigger for insulin resistance in this model.

Methods

Materials and Chemicals. Mouse monoclonal anti-Prdx3 antibody (sc-59661), mouse polyclonal anti-PPAR-γ (sc-7196), rabbit polyclonal anti-GLUT1 antibody (sc-7903), rabbit polyclonal, phospho-Y989IRS1 antibody (sc-17200-R), rabbit polyclonal anti-BiP antibody (sc-13968) and goat polyclonal anti-mTOR antibody (sc-1549) were all purchased from Santa Cruz Biotechnology. Rabbit polyclonal phospho-S307IRS1 antibody (2381), rabbit polyclonal anti-phospho-S473AKT antibody (9271S), rabbit polyclonal phospho-T308AKT (9275), rabbit polyclonal phospho-eIF2α antibody (9721), rabbit polyclonal anti-eIF2α antibody (9722), rabbit monoclonal phospho-JNK (Cell Signaling, 4668), rabbit monoclonal anti-JNK (9252S) and rabbit monoclonal anti-rictor antibody (9476) were purchased from Cell Signaling Technology (Beverly, MA). Rabbit monoclonal anti-CLPP antibody (ab124822), rabbit polyclonal anti-LONP1 antibody (ab103809), mouse monoclonal anti-ATP5a antibody (ab14748) were all purchased from Abcam, Cambridge, MA. Mouse monoclonal anti-AKT antibody (610861) was purchased from BD Transduction. Rabbit polyclonal anti-IRS1 antibody (06-248) and anti-HNE Michael (393207) was purchased from Calbiochem (MilliPore, Bellerica, MA). Mouse monoclonal anti-β-actin was purchased from Sigma. IRDye secondary antibodies were obtained from Odyssey Imaging System (LiCor Bioscience, Lincoln, NE).

3T3-L1 differentiation and treatment. 3T3-L1 fibroblasts were grown to confluence and differentiated using the standard dexamethasone, methylisobutylxanthine and insulin protocol [8], [24]. On day 7 of differentiation cells were treated with 1nmol/L TNFα (R&D systems, Minneapolis, MN) or 1nmol/L Dexamethasone (Sigma) for 24 hours and subsequently harvested for RNA analysis. For insulin stimulation cells were treated with 100nM porcine insulin for 30 minutes on day 8 of differentiation prior to cell harvest.

Animal protocol and insulin stimulation. Wild-type C57Bl/6J mice were weaned and maintained on either a high fat (20.5% protein, 36% fat, 35.7% carbohydrate; Bioserv, Frenchtown, NJ) or chow diet at 3 weeks of age. Ob/ob mice were purchased at 10 weeks of age and maintained for 2 weeks on normal chow diet prior to sacrifice. At 12-15 weeks of age (9-12 weeks on diet for high fat and chow fed controls), animals were sacrificed and selected tissues were removed and flash frozen in liquid nitrogen. Frozen tissues were stored at -80°C until analyzed. 15 week old C57Bl/6J mice maintained on a high fat or normal chow diet were fasted for 6 hours and injected with 1.0 unit insulin/kg sterile porcine insulin for 30 minutes prior to sacrifice.

Quantitative real time PCR (qPCR). Total RNA was extracted from 3T3-L1 cells and mouse tissue using TRIzol reagent according to manufacturer's protocol (Invitrogen, Grand Island, NY). Both subcutaneous and epididymal adipose tissue were harvested from age matched lean, ob/ob and high fat fed mice. Real-time amplification was performed using a Bio-Rad MyiQ thermocycler using iQ SYBR Green Supermix and recommended thermocycler parameters (Bio-Rad, Hercules, CA). Gene expression assays were performed for glutathione peroxidase 4 (Gpx4), glutathione peroxidase 3 (Gpx3) peroxiredoxin 3 (Prdx3), manganese superoxide dismutase (SOD2), glutathione-S-transferase A4 (GSTA4), and aldehyde dehydrogenase 2 (ALDH2) (Primer sets shown in Table 4). Relative gene expression comparisons were carried out using transcription factor 2E (TFIIE) as an endogenous control.

Immunoblotting. Cells were harvested, lysed, sonicated for 10 seconds, and centrifuged at 16,000g for 10 minutes on day 8-9 of differentiation in a hypotonic buffer. Supernatant was collected, assessed for protein content and frozen at -80°C for future use.

Generation of peroxidredoxin 3-silenced adipocytes. 3T3-L1 fibroblasts were transduced with lentivirus carrying short hairpin RNA (shRNA) as described previously [9], [25]. The shRNA clones directed toward prdx3 (TRCN0000120672) and enhanced green fluorescence protein (eGFP) were purchased from the University of Minnesota Genomic Center. The full hairpin sequence used for the Prdx3:
CCGGCCACAGGCTTTGTAATTCTAACTCGAGTTAGAATTACAAAGCCTGTGGTTTTTG.

Hydrogen peroxide measurements. On day 8 of differentiation cells were harvested, sonicated and spun at 10,000g for 10 minutes in 150 µl of 0.25M sodium phosphate buffer containing 0.1%Triton-X. Supernatant was removed and assay was performed as recommended by the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen, Grand Island, NY, USA).

Glutathione measurements. Cells were grown in six-well plates as described previously [2], [10], [11], [24]. Cells were washed twice in ice-cold PBS, scraped, sonicated and centrifuged prior to the assay. The supernatant was collected, deprotonated and assayed for oxidized and total glutathione by the Glutathione Assay Kit according to manufacturer's instructions (Cayman Chemical, Ann Arbor, MI). The glutathione levels were normalized to protein content determined by bicinchoninic acid assay (Sigma Aldrich, St. Louis, MO).

Membrane potential measurement. Mitochondrial proton motive force was measured by tetramethylrhodamine methyl ester (TMRM) staining (Invitrogen). Briefly, cells were washed with PBS and incubated in 1 ml of KRH buffer (pH 7.4) with a 50 nM final concentration of TMRM for 30 min. Then cells were washed with PBS and harvested into 300 µl of KRH buffer. Samples (200 µl each) were loaded into 96-well plates, and fluorescence was measured using a microplate reader with excitation at 531 nm and emission at 572 nm. Fluorescence units were normalized to protein content for each sample.

Aldehyde measurement. As described previously, cells were homogenized in 50mM sodium acetate buffer containing 5mM amino-oxyacetic acid with 250μM BHT and 500μM DTPA at pH 5.0, spiked with deuterated internal standards and incubated for 1h on ice. Samples were vortexed, centrifuged, run over Strata-X-AW columns before analysis by liquid chromatography-tandem mass spectrometry (LC-MS/MS) on an AB/Sciex API 4000Qtrap mass spectrometer. Stable-isotope dilution was used for quantitation of aldehydes [3], [12], [13], [16].

Cellular respiration and proton efflux. Cellular respiration and proton efflux were analyzed using the XF24 Analyzer and system software (Seahorse Bioscience, Billerica, MA). Detailed procedure is described elsewhere[14], [26]. Similar conditions and inhibitor concentrations were used in all experiments.

Mitochondrial imaging. Cells were seeded and differentiated in six-well plates containing 25-mm round glass coverslips coated with 2% gelatin. Mitochondria were labeled by incubating cells with 300 nM MitoTracker Green FM (30 min, 37ºC) in KRH buffer supplemented with 0.5% BSA. Then, cells were washed and kept in KRH buffer and observed under and Olympus Deltavision Personal DV microscope with a ×100/1.4 oil objective at 25°C. Fourty five 0.25 μm thick Z-slices were obtained. Image acquisition and deconvolution was performed using SoftWoRx version 4.1.2 and image processing and data obtension was done using ImageJ on volume reconstituted images. Experiments were performed five independent times and 15-20 cells per condition were recorded. Increases in the number of mitochondria per cell, together with a decrease in the mean mitochondrial volume were considered fission criteria as reported previously [15], [27].

2-Deoxyglucose uptake assay. Uptake assays were performed on day 8 of differentiation. With a subset of cells, 50 μM N-acetyl-cysteine was added to complete media 24 hours prior to uptake experiments. Detailed methods have

been previously published[16], [25].

Lactate determination. Day 7 differentiated adipocytes were incubated for 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.

Cell number determination. On day 8 of differentiation cells were trypsinized in 0.5ml Trypsin. Once lifted, 1ml of complete medium was added to each well. 200uL were transferred to a new tube containing 500uL of PBS and 300uL of 0.4% Trypan blue. 20uL of this sample were then loaded into a hemocytometer and counted. All quadrants were counted 2x for each sample. For cellometer experiments cells were trypsinized, centrifuged to pellet cells and re-suspended in 200µL of isolation buffer containing lipidtox, 4% paraformaldehyde and 0.05% saponin on day 8 of differentiation. Cell suspension was diluted 1:100 and 20µL of diluted sample was added to the loading chamber. Samples were measured in duplicates with an n=6 for each treatment group.

Biotin-switch method for detecting oxidized cysteines. Mitochondria were isolated from cells by differential centrifugation as described previously.[16], [28] Oxidized cysteines were as described previously with several modifications [16], [17], [29]. Briefly, proteins were precipitated with 20% TCA, re-suspended, free thiols blocked with n-ethylmaleimide (Sigma Aldrich, St. Louis, MO), reduced with sodium arsenite, labeled with biotin-maleimide (Sigma Aldrich, St. Louis, MO), separated by SDS-PAGE and detected using IR-conjugated streptavidin (Li-Cor Biosciences, Lincoln, NE).

Co-immunoprecipitiation (Co-IP) and Immunoprecipitation (IP). Cells were lysed on day 8 of differentiation in 0.3% CHAPS in PBS (Co-IP) or hypotonic buffer

(IP), sonicated for 10 seconds (IP only), centrifuged at 16,000g for 10 minutes and supernatant was quantified by BCA. For animal experiments, cytoplasmic fractions from visceral adipose tissue were collected through differential centrifugations and concentrated using Amicon Ultra 15mL centrifugal filters. Concentrated samples were quantified by BCA. Equal amounts of protein (1-1.5mg) for all experiments were added to protein G beads (co-IP) or protein A beads (IP) along with anti-mTOR antibody (Co-IP) or anti-rictor antibody (IP). Control IgG was added to one set of lysates for each given experiment. Lysates were rotated overnight at 4°C. Following day beads were pelleted and washed 5x with 0.3% CHAPS in PBS buffer (Co-IP) or hypotonic buffer (IP). Beads were re-suspended in 4x DNS and boiled for 5 minutes in preparation for western blotting.

Dimedone labeling. On day 8 of differentiation cells were treated with complete media containing 10mM dimedone for 2 hours prior to cell harvest. Oxidized proteins were detected using a rabbit polyclonal anti-cysteine sulfenic acid, (07-2139) antibody that recognizes dimedone labeled proteins from MilliPore, Bellerica, MA.

Membrane fractionation. Cells were harvested in 250mM Sucrose, 1mM EDTA, 10mM Trips pH 7.2, homogenized, sonicated and spun at 500xg. The supernatant was spun at 100,000 x g for 1 hour and separated. The pellet (membrane proteins) was re-suspended in the same volume as the supernatant (soluble proteins) fraction and equivalent volumes were loaded and separated by SDS-PAGE.

Statistical Analyses. All statistical analyses were done using unpaired two-tailed student's t-tests with significance level set at $p<0.05$.

Results

A growing body of evidence suggests that adipose tissue derived oxidative stress is a precipitating factor in the induction of insulin resistance [18], [19], [30]-[33]. However, detailed mechanisms outlining the origins and contributions of oxidative stress are still unclear. Here we extensively show that adipose tissue, from multiple mouse models of obesity induced insulin resistance, exhibit a selective transcriptional down regulation of the mitochondrial antioxidants. Mitochondrial antioxidants Glutathione S-transferase alpha 4 (Gsta4), Peroxiredoxin-3 (Prdx3), Glutathione peroxidase 4 (Gpx4) and Aldehyde dehydrogenase 2 (Aldh2) were all down regulated in visceral adipose tissue of both high fat fed C57Bl/6J mice as well as leptin deficient *ob/ob* mice (Fig. 1A and 1B). On the other hand, cytoplasmic isoforms of these antioxidants show little-to-no change in expression levels (Data not shown).

Additionally, treatment of differentiated, 3T3-L1 adipocytes with either dexamethasone (DEX) or TNFα, two commonly used methods to induce insulin resistance in adipocytes, reveals a similar transcriptional down-regulation of mitochondrial antioxidants. DEX treatment results in significant down regulation of Sod2, Gpx4 and Gsta4 (Fig. 1C) while TNFα induced a down-regulation of Prdx3, Gpx4, Aldh2 and Gsta4, as well as an increase in Sod2 (Fig. 1D). Together the animal and cellular data suggest that under insulin resistant conditions, the mitochondria are generating a significant pool of reactive oxygen

species (ROS) due to depletion of antioxidant capacity. Furthermore, the increased expression of Sod2 coupled to decreased expression of Prdx3, Gpx4, Aldh2 and Gsta4 suggests that H_2O_2 could be the ROS accumulating in highest concentration.

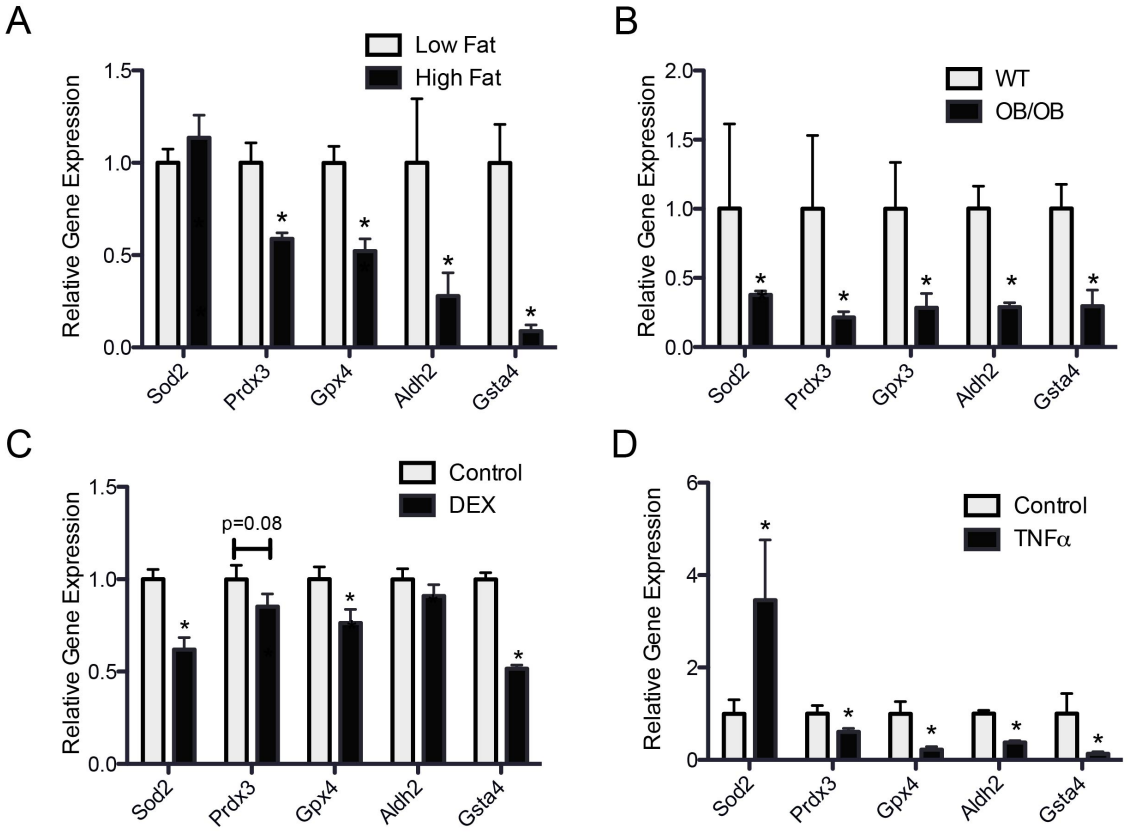

Figure 1. Obesity induced insulin resistance results in transcriptional down regulation of mitochondrial specific antioxidant enzymes. A-D show relative gene expression analysis for mitochondrial antioxidant enzymes Sod2, Prdx3, Gpx4, Aldh2 and Gsta4. A, Visceral adipose tissue from high-fat fed and low-fat fed mice. B, Visceral adipose issue from leptin deficient (*Ob/Ob*) and wild type (WT) mice. C, Dexamethasone (DEX) treated 3T3-L1 adipocytes. D, TNFα treated 3T3-L1 adipocytes *P<0.05

Previous work has highlighted the important biological role of mitochondrial antioxidants in whole body systems. Prdx3 deficient mice show increased levels of mitochondrial ROS, mitochondrial dysfunction, adipokine deregulation and impaired glucose homeostasis, while transgenic Prdx3 mice show protection from high fat diet induced insulin resistance [20]-[22]. Although these studies are fail to uncouple inflammation from oxidative stress, they do provide evidence in favor of mitochondrial ROS as a regulator of insulin sensitivity. Therefore, hypothesizing that mitochondrially derived ROS could drive insulin resistance, independent of inflammation, we developed a knockdown cell line of Prdx3 to evaluate insulin sensitive signaling mechanisms affected by ROS in adipocytes.

Prdx3 silenced 3T3-L1 adipocytes (Prdx3 KD) display a significant decrease in both transcript and protein levels of Prdx3 compared to controls (Fig. 2A). Prdx3 KD cells differentiate normally exhibiting similar protein levels of both peroxisome proliferator-activated receptor gamma (PPAR-γ) and adipocyte fatty acid binding protein (AFABP), two common markers of differentiation, between cell lines (Fig. 2B). Light microscope images and oil-red-o staining show an increase in accumulation of triglyceride within the Prdx3 KD adipocytes (Fig. 2C and 2D). It is known that adipocyte differentiation and cell division is modulated by increased levels of ROS [23], [34]. During differentiation, increases in hydrogen peroxide concentrations originating from the mitochondria play a critical role in differentiation and cell division in adipocytes [34]. Therefore, it is not surprising

that Prdx3 KD cells showed an increase in hyperplasia as well as a decrease in adipocyte diameter on day 8 of differentiation. This was confirmed using two independent methods to quantify cell number, a hemocytometer and a cellometer, to demonstration an increase in cell number in Prdx3 KD cells compared to control (Fig. 2E). Moreover, cell diameter measurements taken by the cellometer reveal a decrease in cell size in the Prdx3 KD cells (Fig. 2F). Together these data show that Prdx3 KD results in increased oxidative stress, increased cellularity and increases in lipid accumulation with no change in markers of differentiation.

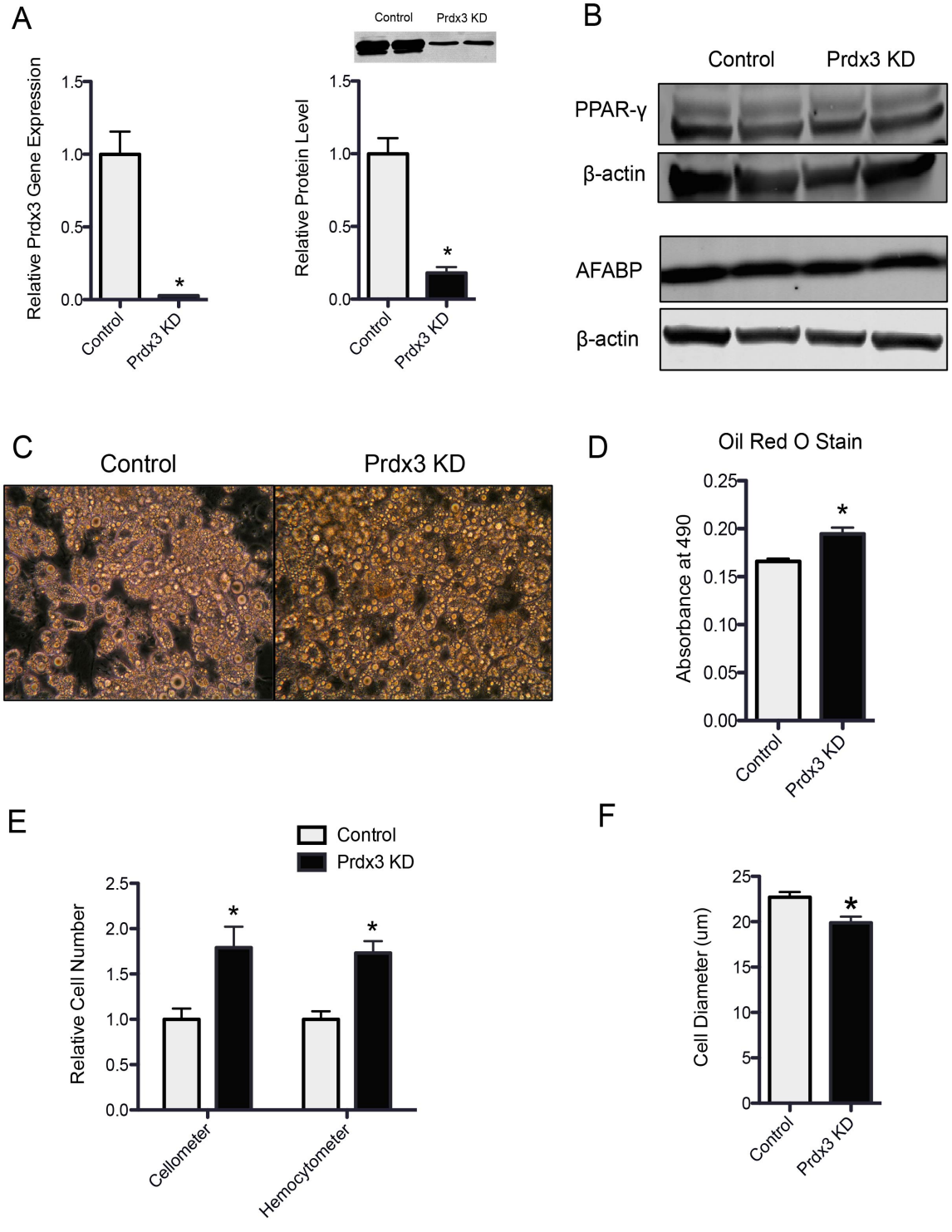
A
Relative Prdx3 Gene Expression
1.5
1.0
0.5
0.0
*
Control
Prdx3 KD
Control
Prdx3 KD
Relative Protein Level
1.5
1.0
0.5
0.0
*
Control
Prdx3 KD
B
Control
Prdx3 KD
PPAR-γ
β-actin
AFABP
β-actin
C
Control
Prdx3 KD
D
Oil Red O Stain
Absorbance at 490
0.25
0.20
0.15
0.10
0.05
0.00
*
Control
Prdx3 KD
E
Control
Prdx3 KD
Relative Cell Number
2.5
2.0
1.5
1.0
0.5
0.0
*
*
Cellometer
Hemocytometer
F
Cell Diameter (um)
25
20
15
10
5
0
*
Control
Prdx3 KD

Figure 2. Pre-adipocytes deficient in Prdx3 differentiate normally and display hyperplasia. A, transcript and protein levels of Prdx3 in the Prdx3 KD cell line compared to control. B, Western blotting for PPAR-γ and AFABP in Prdx3 KD and control cells. C, Differentiation of Prdx3 KD and control cells with light microscope D, Oil-red-O stain E, Cell count using hemocytometer and cellometer. F, Cell diameter. *P<0.05

Prdx3 is the primary and most reactive antioxidant responsible for metabolizing H_2O_2 within the mitochondria [18]. As expected, silencing Prdx3 in 3T3-L1 adipocytes results in an approximately two-fold increase in H_2O_2 levels present in whole cell lysate compared to control cells (Fig. 3A). Additionally, Prdx3 KD cells displayed an increase in oxidized glutathione, a common marker of oxidative stress (Fig. 3C). Importantly, knockdown of Prdx3 did not change the antioxidant expression of Sod2, Gpx4 or Gsta4 although levels of the phase I antioxidant, Aldh2, were significantly decreased (Fig. 3B). However, because Aldh2 detoxifies lipid aldehydes that form further downstream of hydrogen peroxide, its affects on hydrogen peroxide pools is considered minimal. Since H_2O_2 was increased and Aldh2 expression levels were decreased, we hypothesized that other downstream reactive species would be increased as well.

Increased hydrogen peroxide levels can result in protein side chain oxidation. Mitochondrial cysteine oxidation was measured using the biotin-switch method to detect cysteine residues on proteins that have been oxidized to sulfenic acids. The biotin-switch method works by labeling all sulfenic acids with a biotin-maleamide moiety. Proteins modified with the biotin-maleimide are then detected using a streptavidin IR conjugated antibody. Prdx3 KD cells exhibit a significant increase in mitochondrial protein oxidation compared to control cells (Fig 3D).

In addition, it is well understood that, in the presence of ferrous iron, H_2O_2 can react to form the hydroxyl radical. Oxidation of lipids by the hydroxyl radical can ultimately form a family of reactive α,β-unsaturated aldehydes capable of covalently modifying proteins through a process termed protein carbonylation. Indeed, Prdx3 KD cells showed an increase in free aldehyde levels compared to control. Using mass spectrometry we detected increased free levels of the highly reactive aldehyde, 4-hydroxy-hexnal (4-HHE) in Prdx3 KD cells (Fig. 3E). Free 4-HHE levels were significantly elevated in the Prdx3 KD cells while free levels of 4-hydroxy-nonenal (4-HNE) did not change, nor could we detect increases in protein carbonylation using an antibody against 4-HNE (Fig. 3F). Although increases in 4-HNE were not present by western blot or mass spectrometry we do detect an approximately two-fold increase in the free levels of 4-HHE. Due to its high reactivity we predict that accumulation of 4-HHE would result in increased protein carbonylation, supporting what is observed in adipose tissue from obese insulin resistant mice and humans [35]-[37].

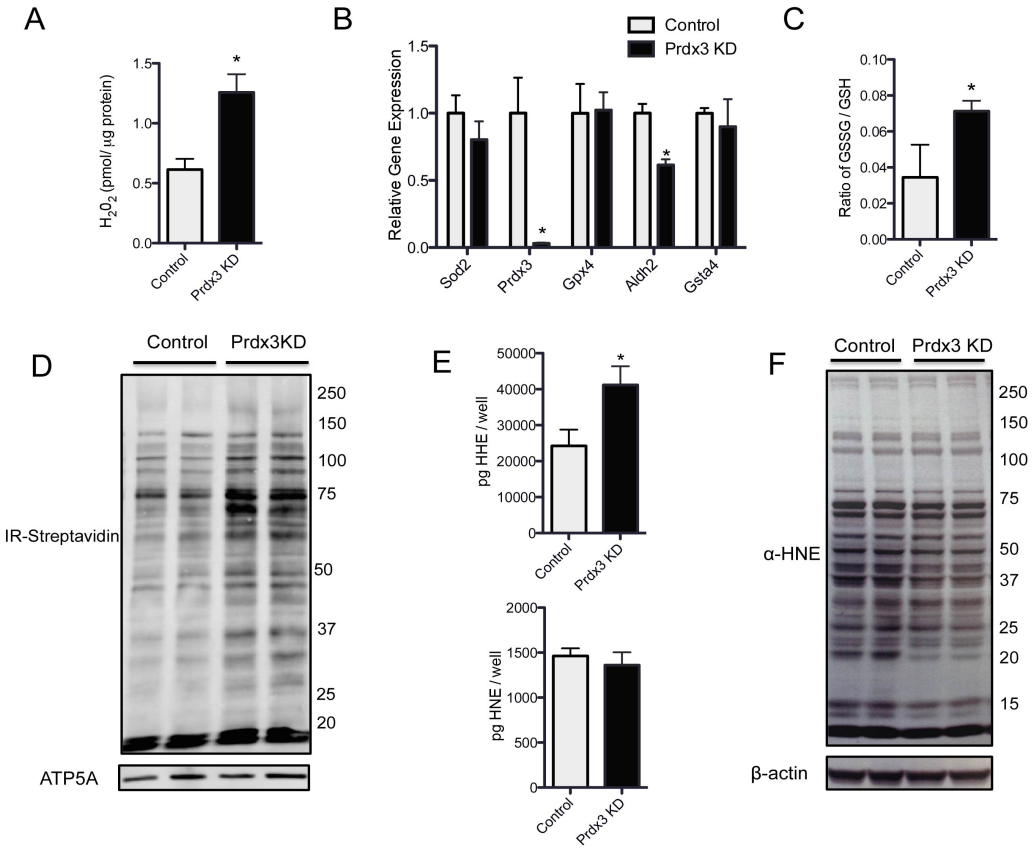

Figure 3. Prdx3 deficiency results in increased oxidative stress. A, Hydrogen peroxide levels normalized to µg protein. B, Relative gene expression of mitochondrial antioxidants. C, Ratio of oxidized to reduced glutathione levels. D, Oxidized mitochondrial proteins using the biotin-switch method. E, Intracellular levels of free aldehydes 4-HNE and 4-HHE. F, Protein carbonylation western blot using an antibody specific for 4-HNE /4-ONE Michael adducts. *P<0.05

Mitochondria play a central role in regulating multiple metabolic pathways, and perturbations in its normal function can have damaging effects culminating in metabolic disease. Mitochondrial dysfunction has long been associated with type-2-diabetes-mellitus (T2DM) and insulin resistance in muscle, liver and adipose [38]-[40]. Although many factors can contribute to the induction of mitochondrial dysfunction, oxidative stress is known to play an important role in driving decreases in ATP production, oxygen consumption and increased membrane potential [33].

In addition to increases in H_2O_2, Prdx3 KD cells exhibit many common defining features of mitochondrial dysfunction. Mitochondrial respiration was assessed in intact, differentiated adipocytes using a Seahorse XF Analyzer. Real time measurements of oxygen consumption rate (OCR) revealed deficiencies in maximal respiration when stimulated with the uncoupling agent carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP). Additionally, Prdx3 KD cells showed decreases in coupled respiration, a measurement that correlates with ATP production (Fig. 4A and 4B).

Prdx3 KD cells also displayed an increase in membrane potential as measured using the membrane potential dependent dye tetramethylrodamine methyl ester (TMRM) a characteristic associated with decreased mitochondrial function and respiration (Fig. 4C). Under conditions of decreased mitochondrial capacity, cells

rely heavily on glycolysis to meet ATP demands. Increased flux through glycolysis produces lactate as an endpoint and increases in lactate levels have been measured within plasma of insulin resistant humans [41]. Prdx3 KD cells display an approximately seven-fold increase in lactate secretion into the media as well as increased GLUT1 protein level compared to control cells (Fig. 4D and 4E). GLUT1 up-regulation is another typical characteristic cellular response to oxidative stress, as H_2O_2 has been shown to activate transcription factors that function to up-regulate stress sensitive genes like Glut1 [42].

Because Prdx3 KD cells exhibited signs of mitochondrial dysfunction, we were interested in evaluating mitochondrial content and network morphology. Previous work using 3T3-L1 adipocytes revealed that treatment with TNFα results in mitochondrial fragmentation [5]. Prdx3 KD cells also appear to show mitochondrial fragmentation as evaluated by an increase in mitochondrial number and by a decrease in mean mitochondrial volume (Fig. 4F 4G). Enlarged region of the mitochondrial reticulum reveal a punctate appearance and decreased association between mitochondria in Prdx3 KD adipocytes (Fig. 4F). Together these data show that knockdown of Prdx3 in adipocytes is sufficient to drive mitochondrial dysfunction, mimicking what is seen *in vivo*.

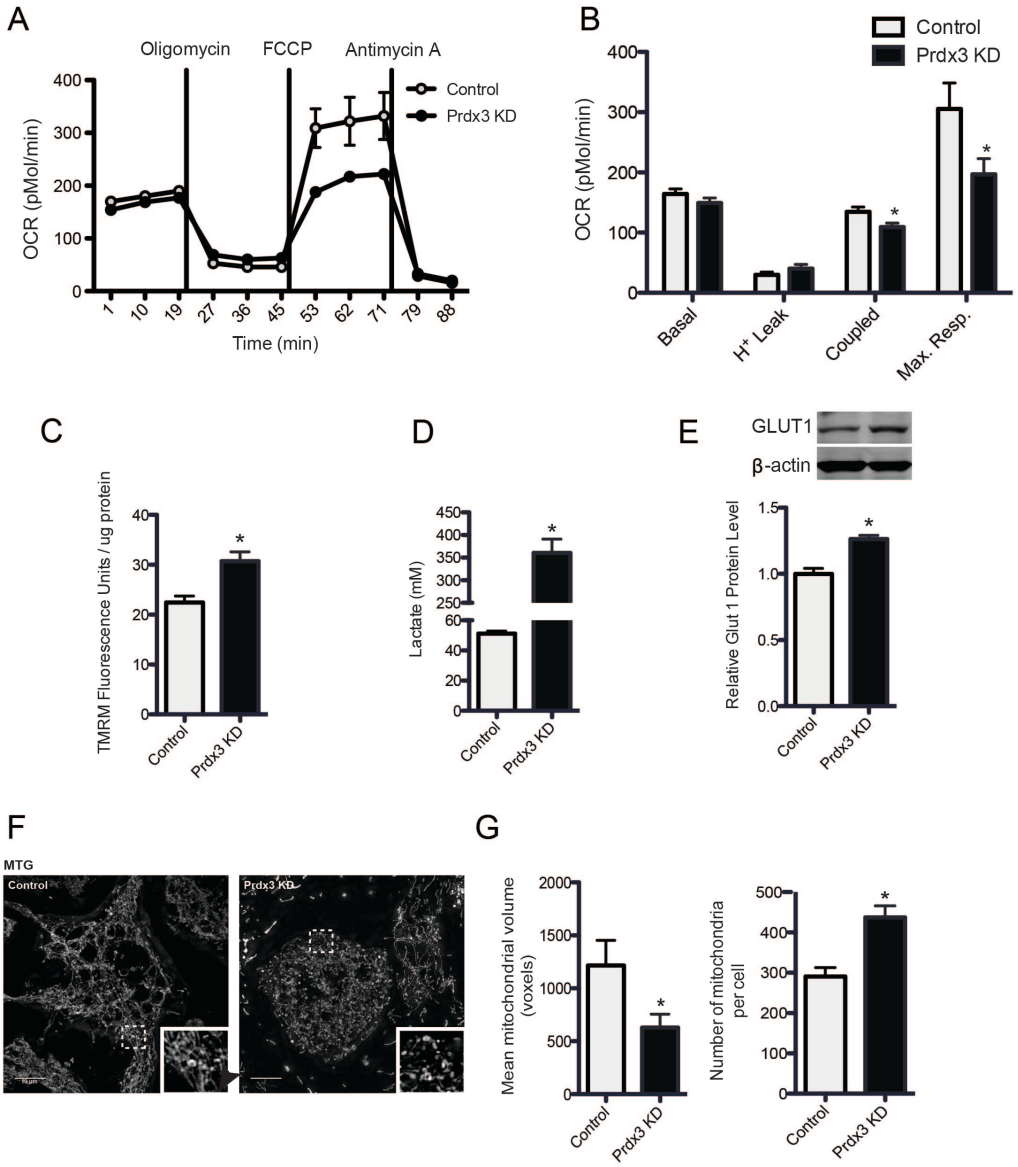
A
Oligomycin
FCCP
Antimycin A
Control
Prdx3 KD
OCR (pMol/min)
Time (min)
B
Control
Prdx3 KD
OCR (pMol/min)
Basal
H+ Leak
Coupled
Max. Resp.
C
TMRM Fluorescence Units / ug protein
Control
Prdx3 KD
D
Lactate (mM)
Control
Prdx3 KD
E
GLUT1
β-actin
Relative Glut 1 Protein Level
Control
Prdx3 KD
F
MTG
Control
Prdx3 KD
G
Mean mitochondrial volume (voxels)
Control
Prdx3 KD
Number of mitochondria per cell
Control
Prdx3 KD

Figure 4. Prdx3 KD cells develop mitochondrial dysfunction. A, Cellular oxygen consumption rates (OCR) for Prdx3 KD and control cells measured by XF24 extracellular flux analysis. Oligomycin (50μg/ml), carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP, 4μM) and antimycin A (2.5μM) were injected at indicated time points. B, Respiration rates as determined from A. C, Protein levels of GLUT1 relative to β-actin. D, Lactate secretion present in the media 24 hours after re-feeding. E, TMRM fluorescence units normalized to μg of protein. F, Epi-fluorescence images of mitochondria stained with MitoTracker Green FM. Enlarged region of image highlights mitochondrial fission in Prdx3KD compared to control cells. G,Quantificaiton of mean mitochondrial volume and mitochondrial number per cell. *P<0.05

Oxidative stress and inflammation have long been implicated as drivers of insulin resistance within adipose tissue of obese humans and mice [37], [43]. However, it has remained unclear whether oxidative stress was causative or casually associated with insulin resistance. The Prdx3 KD cell line provides a method to evaluate mitochondrially derived oxidative stress and its contribution to insulin resistance independent of inflammation. Importantly, loss of Prdx3 in 3T3-L1 adipocytes resulted in decreased propagation of the insulin-signaling cascade. Western blotting analysis revealed deficiencies in the phosphorylation of S473-AKT as well as increased basal and stimulated phosphorylation of S307-IRS1 (Fig. 5A-5C), mimicking what is seen in epididymal adipose tissue from high fat fed mice (Fig. 5D). Prdx3 KD cells exhibited a significant decrease in total protein levels of IRS1 under basal and stimulated conditions. Previous work has shown that phosphorylation at S307-IRS1 can occur as a result of increases in oxidative stress [44]. Additionally, because phosphorylation at S307-IRS1 targets it for degradation, decreases in total protein levels of IRS1 is expected [44]. Decreased levels of IRS1 in Prdx3 KD cells contributed to the increased phosphorylation at Y989-IRS1, suggesting a compensation mechanism attempting to preserve robust insulin signaling in these cells. No significant changes in basal or stimulated T308-AKT phosphorylation were observed (Fig. 5A).

Deficiencies in activation of key phosphorylation events following insulin stimulation translated to deficiencies in insulin stimulated glucose uptake in Prdx3 KD cells. No significant changes in basal glucose uptake were seen between cell lines although an increase in both GLUT1 expression and protein was observed in Prdx3 KD cells. Previously, it has been shown that N-acetyl-cysteine (NAC), a commonly used antioxidant capable of metabolizing oxidants as well as replenishing glutathione pools, is capable of partially rescuing insulin resistance in TNFα or DEX treated 3T3-L1 adipocytes [45]. Excitingly, twenty-four hour pretreatment with NAC resulted in complete rescue of the glucose uptake deficiencies in Prdx3 KD cells, thereby emphasizing the role of oxidative stress as a mediator of insulin resistance in adipocytes.

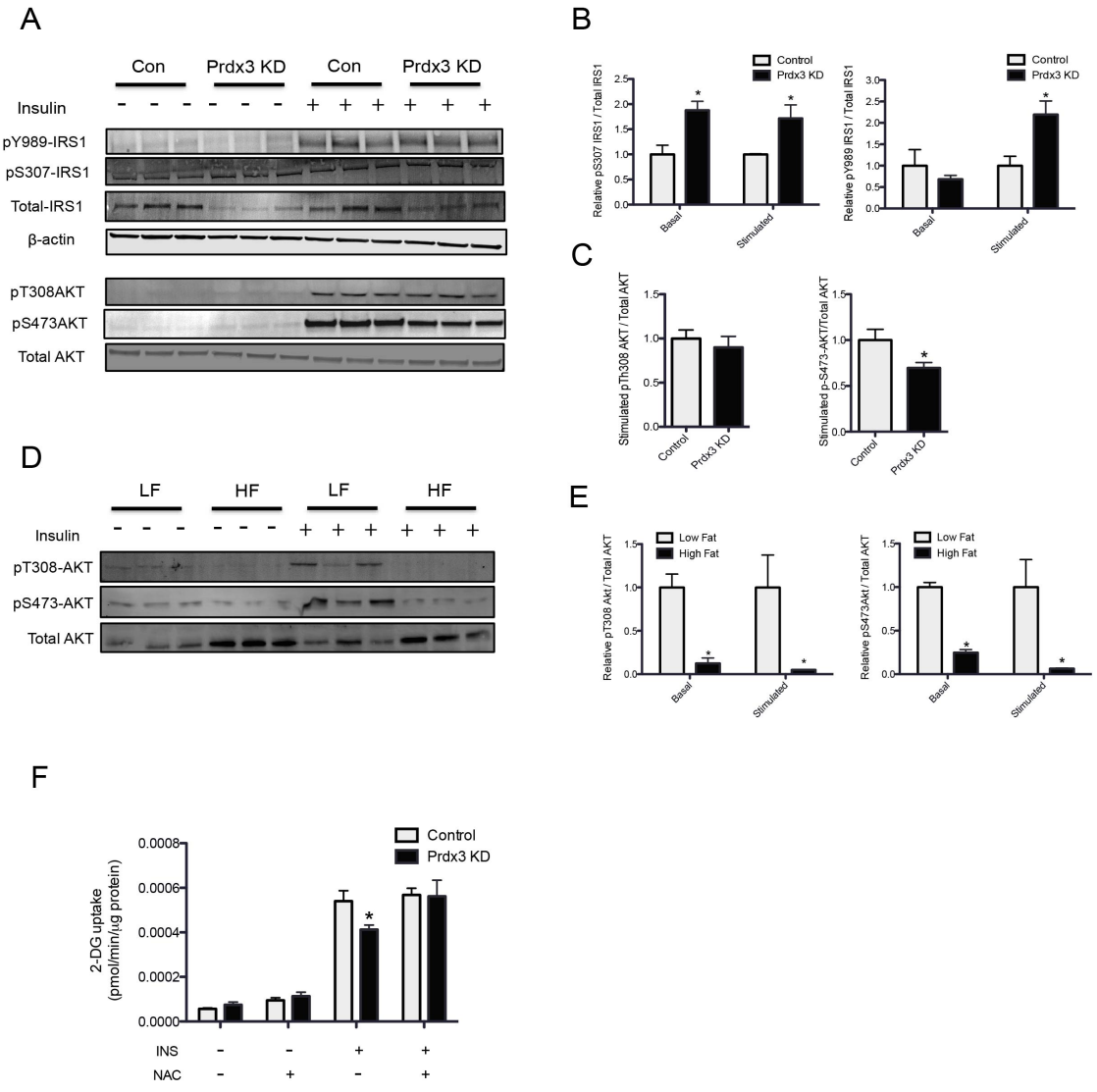
A
Con
Prdx3 KD
Con
Prdx3 KD
Insulin
pY989-IRS1
pS307-IRS1
Total-IRS1
β-actin
pT308AKT
pS473AKT
Total AKT
B
Control
Prdx3 KD
Relative pS307 IRS1 / Total IRS1
Relative pY989 IRS1 / Total IRS1
Basal
Stimulated
C
Stimulated pTh308 AKT / Total AKT
Stimulated p-S473-AKT/Total AKT
Control
Prdx3 KD
D
LF
HF
LF
HF
Insulin
pT308-AKT
pS473-AKT
Total AKT
E
Low Fat
High Fat
Relative pT308 Akt / Total AKT
Relative pS473Akt / Total AKT
Basal
Stimulated
F
Control
Prdx3 KD
2-DG uptake
(pmol/min/μg protein)
INS
NAC

Figure 5. Prdx3KD cells display insulin resistance that can be rescued with NAC. A, Representative western blots of phosphorylated Y989-IRS1, S307-IRS1, T308-AKT, S473-AKT for Prdx3 KD and control cells with (+) or without (-) insulin. Due to changing levels of IRS1 between the knockdown and control cells, β-actin is included as an additional control. B, Quantification of relative phosphorylation of S307IRS and Y989IRS1 to total IRS1 present in Prdx3 KD and control cells under basal and insulin stimulated conditions. C, Quantification of relative phosphorylation of S473-AKT and T308-AKT to total AKT present in Prdx3 KD and control cells under insulin stimulated conditions. D, Representative western blot of phospho-T308-AKT, phospho-S473-AKT and total AKT in animals fed a high fat or chow diet for 15 weeks with (+) or without (-) insulin stimulation. F, Transport of 2-deoxyglucose in Prdx3 KD and control in the presence or absence of insulin and NAC. *P<0.05

It is well established that mitochondrial dysfunction and ER-stress are two major drivers of insulin resistance and occur simultaneously in many diseased states [46]. Interestingly, although Prdx3 KD cells are insulin resistant and display mitochondrial dysfunction, they do not display ER-stress activation. No changes in the canonical ER-stress markers binding immunoglobulin protein (BIP), phospho-eukaryotic translation factor 2A (eIF2α) or phospho-c-Jun N-terminal kinase (JNK) were observed in KD cells lines compared to control (Fig. 6A). Moreover, Prdx3 KD cells show no transcriptional activation of activating transcription factor 6 (ATF6), activating transcription factor 4 (ATF4) and spliced X-box protein 1 (sXBP1), common downstream markers of ER-stress (Fig. 6B).

Another, perhaps less understood, outcome of mitochondrial dysfunction is activation of the mitochondrial unfolded protein response (mito-UPR). Previous work has shown a close association in the activation of ER-stress and the mito-UPR in the face of mitochondrial dysfunction and it has been suggested that overnutrition can result in accumulation of unfolded proteins in the mitochondria [47]-[50]. However, the mitochondrial dysfunction in Prdx3 KD cells is not sufficient to induce activation of the mito-UPR. There were no differences in protein content of CLP protease (CLPP) and LON protease (LONP), two markers of the mito-UPR (Fig. 6C). Together these data suggest that the insulin resistance induced by Prdx3 KD is not a result of activation of either ER-stress or

the mito-UPR and point to another molecular driver of insulin resistance within the Prdx3 KD cells.

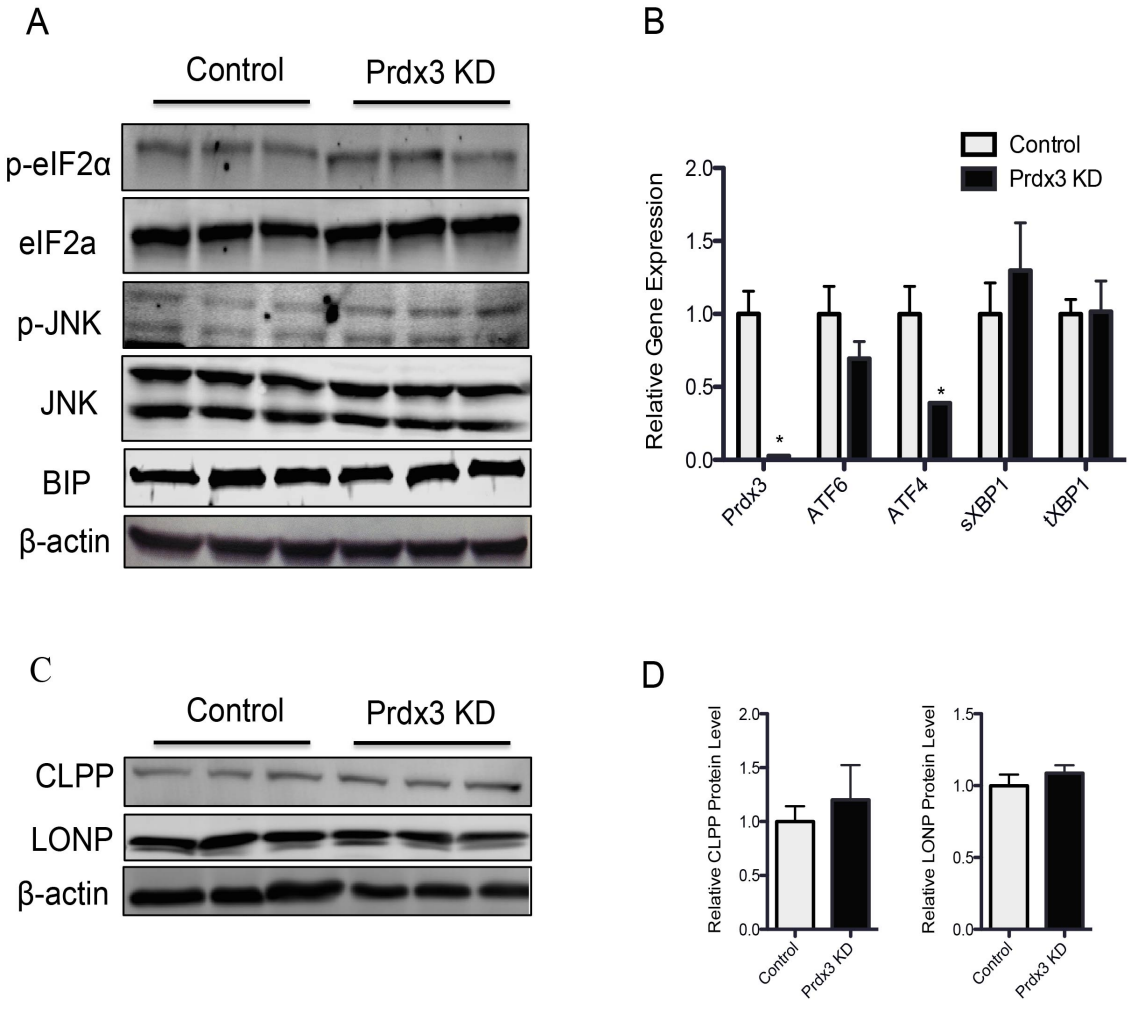

Figure 6. Prdx3 KD cells do not exhibit endoplasmic reticulum stress (ER stress) or activation of the mitochondrial unfolded protein response (mitoUPR). A, Representative western blots for p-eIF2a, p-JNK and BIP are shown relative to their respective control. B, Relative ER-stress transcriptional targets, ATF6, ATF4,sXBP1 and tXBP1 in Prdx3 KD and control cells. All transcript levels are normalized to TFIIE. C, Representative western blots for mitoUPR markers CLPP and LONP are shown relative to β-actin. *P<0.05

mTORC2 is a cytoplasmic protein complex consisting of rictor, mammalian target of rapamycin (mTOR), and mammalian stress-activated map kinase-interacting protein 1 (mSIN1). In response to insulin, mTORC2 propagates insulin signaling by phosphorylating AKT at S473. Phosphorylation at S473-AKT allows for full activation of AKTs downstream signaling, and results in the mobilization of glucose transporters to the plasma membrane. Decreased association between members of mTORC2 inhibits activity and results in decreased S473-AKT phosphorylation and glucose uptake [51].

Previous work has revealed decreased association of components of mTORC2 under conditions of oxidative stress. Using liver specific Sirt1 knockout mice as a model of oxidative stress, Wang *et al*. showed decreased association between members of mTORC2 [52]. Additionally, treatment of HEK293 kidney cells with hydrogen peroxide revealed decreased S473-AKT phosphorylation as well as decreased complex formation of mTORC2 [52]. Because Prdx3 KD cells exhibited significantly decreased phosphorylation at S473-AKT, we hypothesized that mTORC2 activity and complex formation were impaired.

Co-immunoprecipitation assays revealed decreased association between two major components of mTORC2, rictor and mTOR, in both Prdx3 KD cells as well as in high-fat fed animals compared to their respective controls (Fig 7A and 7C). Importantly we found that twenty-four hour pretreatment with NAC was able to

rescue complex formation in the Prdx3 KD cell lines, suggesting that increased ROS, is driving insulin resistance through destabilization of mTORC2 (Fig. 7B). Together these data suggest that in adipocytes, mTORC2 stability is regulated by the cellular redox state and that increases in oxidative stress may drive insulin resistance through oxidation and destabilization of mTORC2.

The increased mitochondrial oxidation profile (Fig. 3C) within Prdx3 KD suggested that multiple proteins were oxidized. Because changes within mTORC2 stability were observed, we were specifically interested in investigating the susceptibility of mTORC2 to oxidation as an explanation for the decreased phosphorylation of S473-AKT. Each subunit within mTORC2, mTOR, rictor and mSIN1, plays an important role in maintaining the integrity of the complex. Deletion of any one of the three subunits results in decreased association and activity of mTORC2. Adipose tissue specific deletion of rictor results in decreased mTORC2 formation that is associated with hyperglycemia and insulin resistance [53]. Of the three subunits, rictor contains the highest percentage of cysteine residues and therefore, was selected as a likely potential oxidation target.

To identify overall oxidation of rictor we labeled all oxidized cysteine residues with dimedone, a cyclic, diketone that is known to readily react with oxidized cysteines found on proteins. Immunoprecipitation of dimedone-labeled rictor

revealed increased oxidation in the Prdx3 KD cells with no change in total levels of rictor (Fig. 7D). Together these data support the hypothesis that oxidation of rictor results in dissociation and decreased activity of mTORC2, ultimately leading to insulin resistance in Prdx3 KD cells.

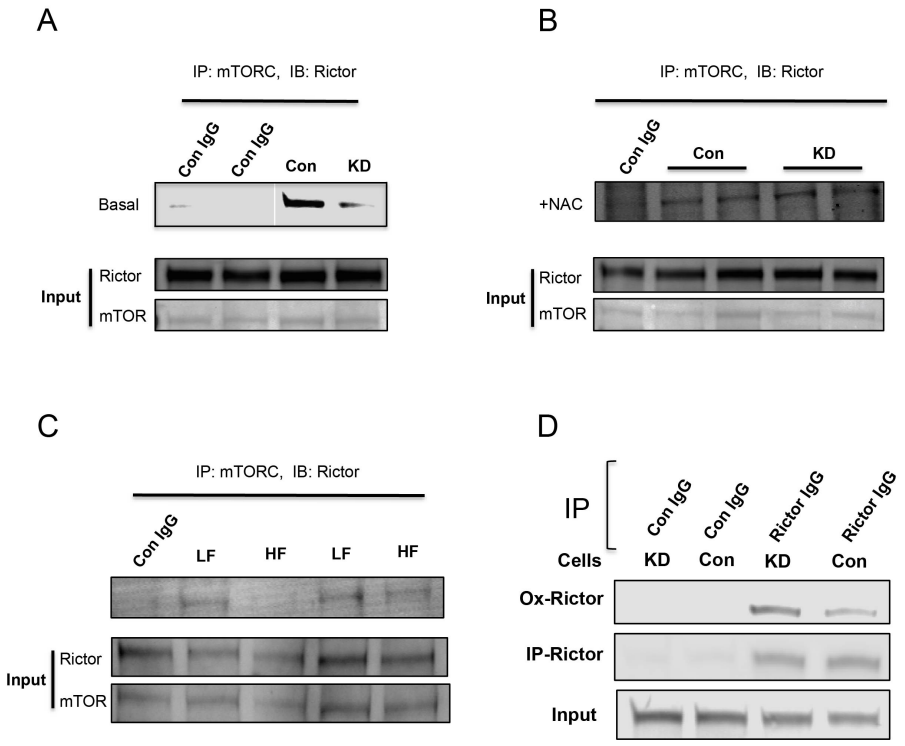

Figure 7. Insulin resistance in Prdx3 KD cells is associated with increased rictor and mitochondrial oxidation and decreased mTORC2 formation. A, Immunoprecipitation with an antibody against mTOR followed by immunoblotting with an antibody against rictor. B, Co-immunoprecipitation of mTOR and rictor following 24-hour pretreatment with NAC in Prdx3 KD and control cells. C, Immunoprecipitation with an antibody against mTOR followed by immunoblotting with an antibody against rictor in high-fat fed and control mice. D, Immunoprecipitation of dimedone-labeled rictor

Discussion

The aim of this work was to evaluate the role of oxidative stress and its contribution to insulin resistance in adipocytes. Here we have comprehensively investigated the previous observation that obesity induced insulin resistance results in a mitochondrial specific antioxidant down-regulation [16]. More specifically, both high-fat fed mice, *ob/ob* mice as well as two cultured adipocyte models of insulin resistance show a mitochondrial specific, antioxidant down-regulation. Prdx3 is the primary antioxidant responsible for metabolizing H_2O_2, protecting the mitochondria from oxidative damage. Furthermore, loss of Prdx3 is correlated with obesity, diabetes and multiple forms of cancer [16]. Whole body knockout of Prdx3 results in insulin resistance characterized by decreased glucose tolerance and insulin sensitivity, while transgenic mice overexpressing Prdx3 are protected from high-fat diet induced glucose intolerance and insulin resistance [21], [22]. Together, these data not only point to the mitochondria as an important generator of ROS, but also highlight the importance of Prdx3 and its contribution to whole body insulin sensitivity. Importantly our results here provide evidence supporting and extending what is known about Prdx3 by showcasing how increased levels of H_2O_2 is able to negatively affect insulin sensitivity though protein oxidation and dissociation of mTORC2.

Because oxidative stress is a hallmark feature of obese, insulin resistant adipose tissue, we were interested in identifying critical metabolic pathways that are

regulated by oxidative stress, independent of inflammation, within the adipocyte itself. Knockdown of Prdx3 in adipocytes provides a tool that not only mimics what is observed in diseased states, but also it allows us to mechanistically study the direct effects of mitochondrially generated oxidative stress on adipocyte insulin sensitivity. Importantly, our results show that silencing Prdx3 in adipocytes results in increased oxidative stress and insulin resistance. This effect is mediated by rictor oxidation, decreased mTORC2 activity and subsequent decreases in S473-AKT phosphorylation.

Complex formation of mTORC2 plays an important role in maintaining the kinase activity required to phosphorylate S473-AKT. Although whole body knockouts of rictor, mSIN1 or mTOR are embryonic lethal, adipose specific deletion, as well as muscle specific deletion of rictor is characterized by insulin resistance, hyper-insulinemia, decreased S473-AKT phosphorylation and impaired glucose uptake [54]-[60]. Furthermore, these results emphasize that the deficiencies in insulin sensitivity are a direct result of decreased mTORC2 association [51], [61]. In addition to other models that highlight mTORC2 as a redox sensor, it has recently been reported that lipolytic products within adipocytes can reduce the association between rictor and mTOR resulting in a decrease in mTORC2 association and insulin resistance. It was not clear which subunit of mTORC2 were effected by the lipolytic products. However, these data showcase the importance of complex stability in maintaining activity and insulin sensitivity.

Co-immunoprecipitaiton experiments in Prdx3 KD cells revealed decreased association between mTOR and rictor suggesting impaired complex formation. Importantly, mTORC2 formation can be rescued following a twenty-four hour pretreatment with NAC, an antioxidant that is known to decrease oxidative stress by directly metabolizing reactive oxygen species as well as replenishing glutathione levels. Moreover, in Prdx3 KD cells, NAC is able to rescue the deficit seen in insulin stimulated glucose uptake. Rescue of both mTORC2 formation and glucose uptake in the Prdx 3KD cells provides evidence in support of oxidative stress as a driver of insulin resistance, mediated at least in part, through decreased mTORC2 formation.

Within proteins, cysteine residues make up approximately 2% of amino acid residues [62]. Although underrepresented, cysteine residues are often found within active sites of proteins and their oxidation can many times render the protein inactive [63]. Additionally, it is estimated that 10% of cysteine residues are redox sensitive [2]. The biotin switch method used to detect cysteine oxidation revealed increased protein oxidation in isolated mitochondria in Prdx3 KD cells compared to controls. This result supports the hypothesis that in Prdx3 KD cells, H_2O_2 modifies cysteine residues on proteins that could be involved in modulating proper insulin signaling.

Compared to mTOR and mSIN1, rictor, with 37 cysteines, contains the highest percentage of cysteine residues. Because of its high cysteine content and its importance in stabilizing mTORC2, we decided to investigate rictor as a likely target of oxidation the Prdx3 KD cells. Immunoprecipitation of dimedone-labeled rictor revealed that rictor is indeed more highly oxidized in the Prdx3 KD cells compared to controls, supporting the hypothesis that rictor oxidation may be affecting the stability of mTORC2. Interestingly, amino-acid alignment of rictor between humans and mice revealed that all 37 cysteines are conserved, suggesting evolutionary importance. In 2002, TOR2 components; Tor (mTOR orthologue), Avo1 (SIN1 ortholouge), as well as Avo3 (rictor ortholouge), were identified in budding yeast [64]. Sequence analysis within conserved domains of rictor revealed one conserved cysteine residue, C728, between yeast and humans. Could C728-rictor be a critical cysteine involved in redox regulation of mTORC2? Although an attractive target, further experimentation is needed to speculate on the significance of this conserved residue. In the future, work identifying oxidized cysteine residues on rictor in the Prdx3 KD cells will need to be done. Additionally, future site-directed mutagenesis experiments could potentially reveal important cysteine residues required for either protein-protein interaction or catalytic function of mTORC2, thus providing important regulatory insight into a central insulin signaling nodes within the adipocyte.

In summary, our results highlight the importance of Prdx3 in regulating the oxidative stress environment in adipocytes. These data together support a mechanism where increased H_2O_2 levels drive insulin resistance through destabilization of mTORC2, ultimately resulting in decreased glucose uptake. Furthermore, our studies have identified rictor, a critical component of mTORC2, as novel target of protein oxidation. Although it is unclear at this point which residues become oxidized, this observation provides insight into a potentially underappreciated molecular mechanism contributing to insulin action.

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CHAPTER FOUR

Conclusions and Perspectives

Dalay Olson wrote this chapter

Diabetes mellitus was originally documented thousands of years ago. The name, translated from the Greek words *siphon* and *sweet,* comes from the observation that urine of people suffering this disease was sweet. This devastating and deathly disease, now known as Type-1-diabetes, is characterized by hyperglycemia and lack of functional insulin producing cells.

Today there remains a population of individuals who suffer from Type-1-diabetes, but far and away, the majority of people diagnosed with diabetes suffer from Type-2-diabetes (T2D). T2D is characterized by a severe resistance to insulin, hyperglycemia and β-cell death. Obesity is the best predictor of Type-2-diabetes as over 90% of obese subjects experience resistance to insulin [1]. With over one third of our adult population now considered obese, T2D is a disease that is growing at an alarming rate.

Although T2D has been studied for years, the underlying mechanisms outlining the development of obesity induced insulin resistance remain poorly understood. Currently, it is well established that adipose tissue inflammation plays a major role in the induction of insulin resistance [2]-[4]. However, it is not known whether oxidative stress, a common outcome of inflammation and a characteristic of insulin resistant adipose tissue, is a causative or a casual factor in its etiology.

Therefore, the aim of my work was to identify whether adipocyte specific oxidative stress could result in insulin resistance. Furthermore, based on work presented here, it is now clear that the mitochondria are a major source of oxidative stress. High-fat fed animals, as well as *ob/ob* mice, two well established models of obesity induced insulin resistance, show a mitochondrial specific decrease in antioxidant expression within visceral adipose tissue. Importantly, this decrease in antioxidant expression can be recapitulated in tumor necrosis factor alpha (TNFα) or dexamethasone (DEX) treated 3T3-L1 cells, two different adipocyte cell culture models of insulin resistance (Chapter 3, Fig. 1). Both the *in vivo* and *in vitro* experiments suggest that mitochondria within adipocytes may be generating oxidative stress due to decreased antioxidant expression resulting in the induction of insulin resistance.

To test this hypothesis we developed a Peroxiredoxin-3 deficient (Prdx3 KD) adipocyte cell line. The Prdx3 KD cell line not only mimic what is seen *in vivo,* but also provides us with a model to study the outcomes of mitochondrially derived oxidative stress. Since it is known that Prdx3 deficient mice are insulin resistant and that Prdx3 transgenic mice maintain insulin sensitivity when challenged with a high-fat diet, we hypothesized that Prdx3 KD in 3T3-L1 adipocytes would mimic what is seen *in vivo,* resulting in an insulin resistance phenotype [5], [6].

Importantly, Prdx3 KD cells do display increased oxidative stress, mitochondrial dysfunction and insulin resistance. Based on previous literature suggesting an important role of endoplasmic reticulum stress (ER-stress) in the induction of insulin resistance we sought to investigate the contribution of ER-stress in this model [7]. Interestingly, we observe no activation of ER-stress (Chapter 3, Fig.6) nor the mitochondrial unfolded protein response, a less characterized outcome of oxidative stress, suggesting that insulin resistance was occurring via an alternative mechanism. Additionally, these data may also be useful for understanding molecular mechanisms that precede activation of ER-stress and the mito-UPR.

As an alternative explanation to explain the decrease in insulin sensitivity, we began investigating the potential role of protein oxidation in mediating insulin resistance. Because phosphorylation of serine 473 on AKT (S473-AKT) was significantly decreased in the Prdx3 KD cells, this pointed to mTORC2 as a potential target of oxidation. Furthermore, this hypothesis is supported by the observation that increased hydrogen peroxide levels in both liver and kidney cells can negatively affects mTORC2 stability and activity [8]. Excitingly, Prdx3 KD cells exhibit insulin resistance that is characterized by increased rictor oxidation and is mediated by mTORC2 dissociation (Fig. 1). Additionally, mTORC2 stability and glucose uptake can be rescued with a 24-hour pretreatment with N-acetyl cysteine (NAC), an antioxidant responsible for scavenging radicals and

replenishing glutathione levels. Together these experiments suggest that increases in oxidative stress can result in decreased association of mTORC2 leading to a decrease in glucose uptake and insulin resistance.

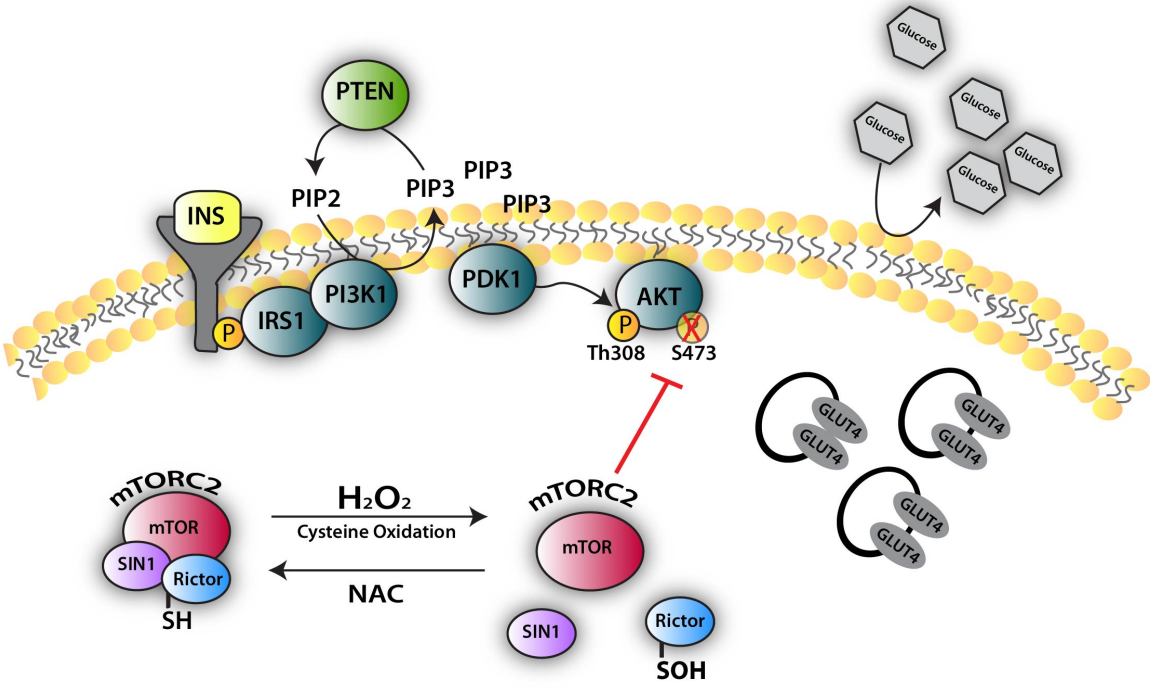

Figure 1. Insulin stimulated glucose uptake is blunted in Prdx3 KD cell due to increased cysteine oxidation of rictor, decreased mTORC2 formation and impaired S473-AKT phosphorylation. Decreases in mTORC2 formation can be rescued with 24 hour pretreatment with NAC.

In the future there are many exciting directions for this project. First, because we have evidence to support that rictor oxidation could be mediating insulin resistance in Prdx3 KD cells, an important next step will be identifying which cysteine residues are being oxidized. Identification of site-specific residues that have been oxidized can now be done using mass spectrometry. Prdx3 KD and control lysate samples could be subjected to the biotin-switch method used to label oxidized cysteine residues and mass spectrometry could be done to identify specific sites of cysteine modification [9], [10].

Briefly, free sulfhydryl groups on proteins are blocked with an alkylating agent like iodoacitamide. Oxidized cysteines are then reduced to free sulfhydrls by a reducing agent like sodium arsinite. The reduced sulfhydrls are then biotylated with a biotin-maleamide moiety allowing for protein enrichment. Lastly, rictor would be immunoprecipitated out of the labeled lysate and run on a western blot. The enriched rictor band of interest could then be excised and analyzed using mass spectrometry to identify specific cysteine residues that have been modified by the maleamide. To identify the biological implications of each oxidized cysteine residue on rictor, site directed mutagenesis experiments would need to be performed. Results from these experiments could outline a novel regulatory mechanism for rictor and perhaps, one day this oxidative marker could be used in clinical practice as an early predictor of insulin resistance.

Additionally, non-targeted mass spectrometry approaches to identify proteins that are oxidized in the Prdx3 KD cell lines is another direction for this project. Since oxidative stress can affect a multitude of intracellular proteins, we do not expect rictor to be the only oxidation target. Therefore we fully expect many other proteins within the cell to be oxidized. This model provides a great tool in which to study proteins within common pathways that are susceptible to oxidative damage in the context of insulin resistance and could open up new avenues of research in the future.

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