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

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

**Research Design And Methods** – GSTA4 silenced 3T3-L1 adipocytes and GSTA4 null mice were evaluated for metabolic processes, mitochondrial function and reactive oxygen species production. GSTA4 expression in human obesity was evaluated using microarray analysis.

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

**Conclusions** - These results indicate that down regulation of GSTA4 in adipose tissue leads to increased protein carbonylation, ROS production and mitochondrial dysfunction and may contribute to the development of insulin resistance and type 2 diabetes.
Obesity-linked type 2 diabetes and its associated health complications are major human health concerns (1) and recent studies have implicated increased levels of reactive oxygen species such as superoxide anion, hydrogen peroxide, peroxynitrite and hydroxyl radicals as major contributing factors (2-4). Excess ROS are causally linked to insulin resistance in adipocytes (5) and alteration of adipokine secretion in a manner that promotes insulin resistance in peripheral tissues (2; 6; 7). As adipose metabolism plays a substantial role in regulating whole body insulin sensitivity (8), evaluating the conditions that lead to oxidative stress in adipocytes is an important goal.

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

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

RESEARCH DESIGN AND METHODS

Animals. C57BL/6J mice were placed on a normal chow (~4% fat by weight, Teklad) or a high fat diet (~35% fat by weight, F3282, BioServ Inc) at weaning (17). Mice were housed on a 12-hour light/dark cycle and fed ad libitum with continual access to water. At 12 to 16 weeks of age, mice were sacrificed by cervical dislocation, tissues were harvested, frozen in liquid nitrogen and stored at -80°C until further processing. Primary adipocytes were isolated from fresh epididymal fat pads as described previously (18). Mixed strain mice B6;129S5-GstA4 Gt(neo) 619 Lex were purchased from the Mutant Mouse Regional Resource Centers (UC Davis, CA). GSTA4 heterozygous mice were out bred to C57/BL6J mice and the resulting heterozygous progeny were interbred to generate GSTA4 null and wild type mice. The University of Minnesota Institutional Animal Care and Use Committee approved all experiments.

Generation of GSTA4 silenced adipocytes. 3T3-L1 fibroblasts were transduced with lentivirus carrying shRNA as described previously (19). shRNA sequences
directed toward GSTA4 mRNA were purchased from Ambion Inc. Each oligo and the reverse complement were synthesized, annealed and the dsDNA ligated into pENTR/U6 cloning vector (Invitrogen Corp.) according to manufacturer’s protocol. Cell lines used for experiments contained the following shRNA sequences: Scr control - AGTACTGTTACGATACGGTGTGCTGT CCGTATCGTAAGTACT; GSTAA4 Kd – GGTATATAGATCCCAGGAGTGTGCTGT CCTCCTGGGATCTATATACC.

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

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

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

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

**Statistical analysis.** All values are expressed as mean +/- SEM. Statistical significance was determined using the two-tailed Student’s t-test assuming unequal variances or where appropriate, a two-way ANOVA with Bonferroni or Holm-Sidak post-hoc analysis. P values < 0.05 are considered significant (*) with an increased significance of P value < 0.01 indicated (**).
(PRDX)-1: all consistent with an antioxidant stress response (2). In contrast, the expression of multiple isoforms of the GST family (A4, A3, M2) was decreased in obese mice compared to lean controls. Most notably, the expression of GSTA4 decreased ~3-4 fold consistent with microarray analysis of lean and obese C57Bl/6J mice where GSTA4 expression was profiled amongst the most highly regulated transcripts (24). The expression of other 4-HNE metabolizing enzymes including several aldehyde dehydrogenases (ALDH1A 1/7, ALDH2 and FALDH) was not significantly altered with obesity (Figure 1A). GSTA3 and GSTA4 expression was also decreased in obese female mice (Figure 1B) relative to lean controls. Interestingly, the basal GSTA4 expression in adipose tissue of lean female mice was ~2-fold greater than in lean male mice such that obese female mice express GSTA4 at levels comparable to that expressed by lean male animals (Figure 1B).

To determine the tissue specificity of GSTA4 down regulation, qPCR was performed on mRNA isolated from epididymal white adipose tissue (EWAT), subcutaneous white adipose tissue (SubQ-WAT), brown adipose tissue, liver and gastrocnemius muscle of lean and obese male mice (Figure 1C). While decreased GSTA4 expression was observed in both visceral and subcutaneous white adipose tissue, no significant change was observed in other insulin-responsive tissues analyzed. Since adipose tissue contains multiple cell types, adipocytes were separated from stroma in EWAT by collagenase digestion and exhibited a ~95% decrease in GSTA4 expression in the adipocyte fraction. Reduced expression of GSTA4 was not limited to high fat fed C57Bl/6J mice. GSTA4 mRNA expression was also significantly decreased in adipose tissue from ob/ob animals (Figure 1D) and GSTA4 expression is down regulated ~ 10-fold in adipose tissue of high fat fed BTBR mice relative to low fat fed lean littermates (http://www.diabetes.wisc.edu/; (25)) indicating that the expression of GSTA4 is markedly reduced in a variety of metabolic and genetic models of obesity and insulin resistance.

**GSTA4 expression in murine and human systems.** To investigate the mechanism underlying reduced GSTA4 expression in insulin resistant adipocytes, we assessed GSTA4 mRNA expression in response to a variety of hormones and metabolites utilizing the 3T3-L1 cell culture system. Treatment of 3T3-L1 adipocytes with the pro-inflammatory cytokine tumor necrosis factor-alpha (TNFα) resulted in a time and concentration dependent decrease in GSTA4 expression. After 24 hr treatment, GSTA4 mRNA expression was reduced 40% with 100 pM TNFα and 80% with 1 nM TNFα (Figure 2A). No further reduction of GSTA4 expression was obtained with 10 nM TNFα (data not shown). The reduced GSTA4 expression was observed as early as 8 hours with 1 nM TNFα treatment (Figure 2B).

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

**Generation of GSTA4 silenced adipocytes.** Since GSTA4 is central to 4-HNE detoxification and metabolism and carbonylation is increased with obesity (15; 26), we evaluated whether decreased GSTA4 expression directly leads to increased protein carbonylation. To that end, 3T3-L1 fibroblasts were transduced with shRNA directed against GSTA4 mRNA or nonspecific scrambled control sequence to establish GSTA4 knock down (Kd) and Scrambled (Scr) cell lines. Although several distinct shRNA sequences were analyzed for GSTA4 silencing, one line with ~60-70% decrease in GSTA4, comparable to that observed in the animal system, was chosen for detailed assessment (Figure 4A), some measures were confirmed in other silenced cell lines. While GSTA3 was not expressed in the preadipocytes at an appreciable level, its expression increased during preadipocyte differentiation (data not shown) and was upregulated ~ 2-fold in GSTA4 Kd adipocytes compared to Scr adipocytes (Figure 4A).

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

Protein carbonylation in GSTA4 Kd and Scr control cells was assessed by biotin hydrazide modification (Figure 4C) (15). Adipocytes exhibited a ~3-4-fold increase ($P < 0.01$) in total protein carbonylation relative to preadipocytes for both GSTA4 Kd and Scr cell lines ($n = 4$). Increased carbonylation of specific proteins was noted in GSTA4 Kd relative to Scr control adipocytes. The prominent band near ~15 kDa in adipocytes has previously been identified as the adipocyte fatty acid binding protein (aP2) (15). Another protein at ~145 kDa (**) exhibited a ~2-3-fold increase ($P < 0.01$) in carbonylation in the GSTA4 Kd cells. This band was excised from the gel, digested with trypsin and the peptides sequenced by liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI MS/MS). The resultant peptides (nine unique peptides representing 8% sequence coverage) identified the protein as xanthine dehydrogenase (Table S2).

**Altered glucose and lipid metabolism in GSTA4 silenced adipocytes.** To determine the influence of increased protein carbonylation on adipocyte function we examined a variety of metabolic parameters linked to glucose and lipid metabolism. GSTA4 Kd adipocytes exhibited a significant increase in basal glucose transport resulting in a net decrease in insulin-stimulated hexose uptake (Figures 5A and 5B). Western analysis indicated increased expression of the basal glucose transporter GLUT1 (Figure 5C), but not the insulin-responsive glucose transporter, GLUT4. Consistent with increased hexose metabolism, analysis of reduced coenzyme levels revealed that the ratio of NAD$^+$/NADH was significantly lower in GSTA4 Kd cells (Figure 5D) and the culture medium of GSTA4 Kd cells was slightly acidic (results not shown) due to a 65-70% increased level of L (+)-lactate compared to scrambled control cells (Figure 5E).

Since tricarboxylic acid cycle (TCA) enzymes are targets of protein carbonylation and are inactivated by the addition of 4-HNE (27; 28), GSTA4 Kd and Scr adipocytes were assayed for small organic acids by direct injection tandem mass spectrometry (29). GSTA4 Kd adipocytes had significantly
increased \( (P < 0.01) \) levels of intracellular lactate, pyruvate, succinate, and citrate (Figure 5F). The ratio of lactate to pyruvate was also significantly decreased in GSTA4 Kd adipocytes relative to Scr control cells and in sum suggested that multiple steps in the tricarboxylic acid cycle may be compromised due to the silencing of GSTA4 and increased protein carbonylation.

To profile lipid metabolism in GSTA4 silenced 3T3-L1 adipocytes, we evaluated \(^{14}\text{C}-\text{acetate} \) incorporation into lipid pools as a measure of \textit{de novo} lipogenesis. GSTA4 Kd adipocytes exhibited no difference in \textit{de novo} lipogenesis under basal or insulin stimulated conditions relative to scrambled control cells (results not shown). However, consistent with elevated CD36 expression (Figure 4B), fatty acid uptake measured by \(^3\text{H}-\text{palmitate} \) influx was slightly, but significantly increased under basal (but not insulin stimulated) conditions in GSTA4 Kd adipocytes (results not shown). Although both basal glucose and fatty acid transport are increased in GSTA4 Kd cells, total lipid storage was unchanged.

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

To assess mitochondrial \( \beta \)-oxidation, the conversion of radiolabeled palmitate to \( \text{CO}_2 \) and soluble metabolites was evaluated (30). GSTA4 Kd adipocytes exhibited decreased oxidation of [1-\(^{14}\text{C}\)]-palmitate as determined by the production of \(^{14}\text{C}\)-labeled \( \text{CO}_2 \) (complete oxidation) and \(^{14}\text{C}\)-labeled acid soluble metabolites (ASM, incomplete oxidation) (Figure 5I). Mass spectrometry based analyses were employed to detect changes in the acyl-carnitine profile of GSTA4 silenced adipocytes (Figure S1 which can be found in an online appendix at http://diabetes.diabetesjournals.org) and revealed that acetyl-carnitine (C2) and several other long chain acyl-carnitine species were elevated in GSTA4 Kd cells.

\textbf{Silencing of GSTA4 expression leads to impaired respiration.} Given the altered glucose metabolism and TCA cycle intermediates, we assessed mitochondrial respiration in GSTA4 Kd and control cells. Mitochondria were isolated from GSTA4 Kd and Scr adipocytes and oxygen consumption was evaluated. GSTA4 silencing had a significant effect \( (p=0.017) \) on oxygen consumption rate as assessed by two-way ANOVA with Bonferroni post-hoc analysis. Whereas control 3T3-L1 adipocytes exhibited robust state 2 respiration and an increase in state 3 respiration following addition of ADP, GSTA4 Kd mitochondria displayed \(~2\)-fold decrease in state 2 respiration and no increase in oxygen consumption following addition of ADP (Figures 6A-C). As mitochondrial dysfunction is often coupled with increased reactive oxygen species generation, we evaluated superoxide anion production in isolated mitochondria (21). Silencing GSTA4 resulted in a \(~3\)-fold increase in superoxide production in the mitochondrial matrix (Figure 6D). However, whole cell ROS evaluated by the fluorescent probe chloromethyl-\( \text{H}_2\text{DCFDA} \) was not changed in GSTA4 Kd compared to Scr control cells (data not shown), suggesting that increased oxidative stress is centered on the mitochondrion and not a property of the entire cell.

In the muscle, insulin resistance is characterized by not only loss of mitochondrial function, but also mitochondria protein and DNA leading to reduced levels of functional organelles (31; 32). As such, we evaluated markers of mitochondrial biogenesis at the mRNA, protein and DNA
level. The expression of the key transcription factors Nrf1 and Tfam as well as the central co-factor PGC1α are all reduced in GSTA4 silenced adipocytes as well as the expression of mitochondrial proteins Cox IV and cytochrome c (Figure 7A and B). Interestingly, MnSOD and UCP2 expression were not affected by GSTA4 silencing. In the GSTA4 knockdown cells, eNOS expression was down regulated ~ 50% suggesting that increased protein carbonylation initiates a cascade of events that links to the entire mitochondrial biogenesis pathway (32). Consistent with this the abundance of the COX II and Cyt b (mitochondrial) genes relative to UCP2 (nuclear) gene was significantly reduced (Figure 7C). Paralleling the reduction in state 3 respiration, Figure 7D shows that the activity of the ATP Synthase was reduced 50% in the GSTA4 silenced adipocytes.

To determine if mitochondrial dysfunction in the GSTA4 silenced 3T3-L1 cells was also exhibited in animal systems, respiration was evaluated in mitochondria isolated from male GSTA4 null (-/-) mice and wild type (+/+) littermates maintained on standard chow (lean) or high fat diet (obese). Lean or obese GSTA4 null mice exhibited no significant difference in body weight relative to wild type controls (34.2 +/- 3.3 g versus 38.6 +/- 4.0 g, chow diet; 45.6 +/- 6.9 g and 49.2 +/- 9.0 g, high fat diet). No changes in fasting glucose or insulin levels were found between wild type and GSTA4 null mice (Figure S3B). Figures 8A & 8B show that in wild type C57Bl/6J mice, the lean to obese transition results in little change in state 2 respiration but a decrease in state 3 respiration, coincident with the down regulation of GSTA4. Interestingly, GSTA4 null mice exhibited a trend towards increased state 2 respiration relative to wild type animals. The state 3 oxygen consumption rate of mitochondria from lean GSTA4 null mice was comparable to wild type, however, similar to mitochondria from GSTA4 silenced adipocytes, mitochondria from obese GSTA4 null mice displayed no increase in oxygen consumption in response to ADP.

Accompanying the changes in respiration, adipocyte mitochondria from wild type mice produced 2-3 fold more matrix superoxide in the obese state compared to lean controls. Adipocyte mitochondria from GSTA4 null mice exhibited markedly increased matrix superoxide in the lean state compared to wild type controls and an even greater level of superoxide in the obese state (Figure 8C). These findings are consistent with published reports that defects in oxidative phosphorylation leads to increased matrix superoxide (33) and are likely consequence of elevated protein carbonylation in the mitochondria of EWAT from GSTA4 null mice (Figure 8D). Unlike the GSTA3 up regulation in the GSTA4 Kd 3T3-L1 cell culture model, there was no significant change in expression of GSTA3 or other glutathione S-transferases or dehydrogenases linked to 4-HNE metabolism in the GSTA4 null mice (Figure S3A).

**DISCUSSION**

The work described herein profiles the systemic metabolic events associated with down regulation of GSTA4 that initiate with carbonylation of cellular proteins. Protein carbonylation is a chemical event with multiple protein targets alkylated under conditions of increased oxidative stress, and the biological outcomes in a cell or tissue are not likely to be based solely on one specific protein modification event (34). As such, a systems-wide approach is needed to characterize functional consequences of carbonylation. Our previous work characterized carbonylated polypeptides in adipose tissue using proteomic methods (15) and this report extends those findings to metabolic outcomes. The results presented identify GSTA4 as a key determinant of
adipocyte protein carbonylation and mitochondrial function and suggest that its activity may be a protective factor against metabolic dysfunction.

In humans, GSTA4 is down regulated in omental and subcutaneous depots of obese, insulin resistant but not obese, insulin sensitive individuals (Figure 3). Similarly, GSTA4 is down regulated specifically in adipose tissue from obese, insulin resistant C57Bl/6J mice (15; 24), in ob/ob animals (Figure 1D) or in TNFα-treated 3T3-L1 adipocytes (Figure 2). Previous work by Awasthi, Zimniak and colleagues has shown GSTA4 null mice have increased levels of 4-HNE, reduced antioxidant capacity, increased apoptosis and JNK activation (11; 35). Although GSTA4 expression was down regulated in obese female mice its basal expression was more robust than in males (Figure 1B) raising the possibility that increased levels of GSTA4 protect female mice from the harmful effects of lipid peroxidation products and may contribute to the attenuated insulin resistance characteristics observed in female C57Bl/6J mice (36).

While silencing GSTA4 in adipocytes also led to overall increased protein carbonylation, one protein target identified was xanthine dehydrogenase (Figure 3D). *In vitro*, xanthine dehydrogenase is converted from its dehydrogenase form to its oxidoreductase form by modification of cysteine residues (37). Such modification of xanthine dehydrogenase by 4-HNE or other lipid aldehydes could lead to an increased oxidoreductase activity of the enzyme and the production of superoxide anion, amplifying oxidative stress signaling. Interestingly, xanthine dehydrogenase is known to be converted to xanthine oxidoreductase in response to mitochondrial damage *in vivo* (38), providing a link between mitochondrial dysfunction and oxidative stress.

Consistent with elevated lactate, the increased basal glucose transport by GSTA4 Kd adipocytes could be a compensatory response to diminished mitochondrial function. In muscle cells, mitochondrial dysfunction has previously been linked to increased basal glucose transport (39) and obese human type 2 diabetic patients also exhibit increased basal 2-deoxyglucose transport in skeletal muscle cells (20; 40). Increased GLUT1 expression in GSTA4 Kd adipocytes (Figure 5C) is consistent with previous reports of increased glucose transport via GLUT1 during treatment of epithelial cells with inhibitors of mitochondrial oxidative phosphorylation (41) as well as reports of oxidative stress-induced increases in basal glucose transport in adipocytes (3). Increased basal glucose transport may provide sufficient ATP for metabolism and signaling since the levels of total and phospho-AMPK were not altered in the GSTA4 silenced adipocytes (data not shown).

Insulin resistance is correlated with increased basal lipolysis (42) and GSTA4 Kd adipocytes exhibited ~50% increased basal lipolysis in the presence or absence of insulin (Figure 5G). Surprisingly, GSTA4 Kd adipocytes had no change in total triglyceride accumulation (results not shown) potentially due to increased fatty acid uptake. Consistent with this, the expression of CD36 protein, a plasma membrane fatty acid-transporter whose expression is induced by the 4-HNE responsive transcription factor Nrf2 (43) was significantly increased in the GSTA4 Kd adipocytes (Figure 4B).

The increase in key organic anion intermediates suggests that the flux through the tricarboxylic acid cycle is attenuated. Previous proteomic data has identified multiple TCA cycle enzymes as well as complexes I-IV of the oxidative phosphorylation machinery as targets of carbonylation (34). The decrease in [1-14C]-
palmitate oxidation (Figure 5I) indicates that multiple steps in lipid oxidation and/or metabolism of acetyl CoA is reduced in the GSTA4 silenced cells. This is supported by mass spectrometry data that shows elevated levels of several acyl-carnitine species (Figure S1) and increased acetyl-carnitine suggests that acetyl-CoA is accumulating. As such, increased protein carbonylation in the mitochondrion is likely to lead to decreased functions such as tricarboxylic acid cycle and fatty acid oxidation (27; 44).

A major finding of this study is that respiration is markedly altered in GSTA4 Kd adipocytes (Figure 6) and in adipocytes of both lean and obese GSTA4 null mice (Figure 8). In the cell culture system, silencing of GSTA4 led to diminished oxygen consumption (Figure 6C) and increased NADH levels (Figure 5D) suggesting electron transfer through the electron transport chain is impaired. Moreover, there was no increase in oxygen consumption in response to ADP in mitochondria from the GSTA4 silenced adipocytes. Consistent with this, the activity of ATP synthase was decreased ~ 50% in the GSTA4 silenced adipocytes. These observations may be due to a combination of factors including increased proton leakage across the inner mitochondrial membrane, carbonylation of Complex V proteins affecting the ability to couple the proton gradient to ATP production, the carbonylation of the adenine nucleotide translocator (ANT), or carbonylation-dependent changes in the abundance of critical proteins linked to state 3 respiration. Indeed, under certain conditions 4-HNE can facilitate proton leak in other cell types through ANT or uncoupling proteins (45; 46).

In the GSTA4 null adipocytes, changes in both state 2 and state 3 respiration were similar, but not identical to the 3T3-L1 GSTA4 knockdown system. In the animal model, mitochondria from lean wild type C57Bl/6J mice exhibit robust state 2 respiration that increased with the addition of ADP. Obese C57Bl/6J mice exhibited no change in state 2 respiration relative to lean counterparts, but had attenuated ADP-coupled oxygen consumption, potentially due to the down regulation of GSTA4. In GSTA4 null animals, mitochondria from lean and obese mice exhibited a trend towards increased state 2 respiration compared to the wild type animals, possibly due to increased proton leakage. Indeed, previous studies by Brand and colleagues have suggested that carbonylation of uncoupling proteins may under certain circumstances lead to increased proton leak thereby providing for increased state 2 respiration (45). Similar to the GSTA4 silenced cells, mitochondria from obese GSTA4 null exhibited virtually no increase in respiration with ADP (Figures 8A & B).

Associated with down regulation of GSTA4 is decreased expression of eNOS, Nrf1, PGC1α and Tfam, critical regulators of mitochondrial biogenesis (Figure 7). Indeed, GSTA4-silencing resulted in loss of mtDNA and decreased expression of CoxII and Cyt c. Work by Nisoli and colleagues have focused on eNOS as the key regulator of TNFα action (32) and that if eNOS activity is lost from adipocytes, the entire program of mitochondrial activation and biogenesis is affected (47). These data suggest that down regulation of GSTA4 is upstream of eNOS regulation and may be mechanistically regulated by protein carbonylation.

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

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REFERENCES


FIGURE LEGENDS

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

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

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

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

Figure 5. Glucose and lipid metabolism in GSTA4 Kd and Scr adipocytes. (A) 2-deoxyglucose (2-DG) transport in GSTA4 Kd and Scr adipocytes under basal (left) and 100 nM insulin stimulated (right) conditions. (B) Fold stimulation of hexose transport in GSTA4 Kd and Scr 3T3-L1 adipocytes (n=9). (C) Expression of glucose transporters GLUT1 and GLUT4 (n=6 per group). (D) NAD⁺, NADH and NAD⁺/NADH in day 7 GSTA4 Kd and Scr adipocytes (n=6). (E) L(+)-Lactate in the medium of GSTA4 Kd and Scr cells (n=6). (F) Organic acids from GSTA4 Kd and Scr adipocyte cell lysates. Basal (G) and forskolin-stimulated (H) lipolysis in Scr and GSTA4 Kd 3T3-L1 adipocytes. (I) β-Oxidation of [14C]-palmitate in Scr and Kd adipocytes (n=6). Panels G & H analyzed by two-way ANOVA with Bonferroni post-hoc analysis. * P < 0.05; ** P < 0.01.
**Figure 6. Mitochondria function in GSTA4 Kd and Scr 3T3-L1 adipocytes.** Mitochondrial oxygen consumption in Scr (A) or GSTA4 Kd (B) adipocytes. (C) Oxygen consumption rates for Scr and GSTA4 Kd adipocytes (n=3). Statistics calculated by two-way analysis with Bonferroni post-hoc analysis. (D) Mitochondrial matrix superoxide production in GSTA4 Kd and Scr adipocytes (n=3).

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

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