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Characterization of the mouse white adipose tissue redox environment and associations with perinatal environmental exposures to bisphenol A and high-fat diets☆☆

Kari Neier^a, Elizabeth M. Marchlewicz^a, Leah D. Bedrosian^a, Dana C. Dolinoy^{a,b}, Craig Harris^{a,b,*}

^aDepartment of Environmental Health Sciences, University of Michigan School of Public Health, Ann Arbor, MI, USA

^bDepartment of Nutritional Sciences, University of Michigan School of Public Health, Ann Arbor, MI, USA

Abstract

White adipose tissue (WAT) plays an important role in obesity pathophysiology. Redox signaling underlies several aspects of WAT physiology; however, the thiol redox environment of WAT has not yet been fully characterized. Dietary and endocrine disrupting chemical (EDC) exposures during development can transiently impact the cellular redox environment, but it is unknown whether these exposures can reprogram the WAT thiol redox environment. To characterize the WAT thiol redox environment, we took a descriptive approach and measured thiol redox parameters using high-performance liquid chromatography in mouse mesenteric (mWAT), gonadal (gWAT) and sublingual (sWAT) depots. Cysteine (CYSS:CYS) and glutathione (GSSG:GSH) redox potentials (E_h) were more oxidizing in gWAT and sWAT than mWAT. Increased body weight, relative WAT weight and age were associated with oxidizing GSSG:GSH E_h in mWAT in a sex-specific manner. Body weight and relative WAT weight were also positively associated with GSSG:GSH E_h in sWAT. We carried out a second mouse study with perinatal exposures to bisphenol A (BPA) and Mediterranean and Western high-fat diets (HFDs) to determine whether early-life chemical and dietary factors have long-lasting impacts on mWAT redox parameters. Mice exposed to Mediterranean HFD or BPA had more oxidizing GSSG:GSH mWAT E_h than controls, with more pronounced differences in females. These findings suggest an important role for the thiol redox environment in WAT physiology. Observed sex-specific and depot-specific differences in thiol redox parameters are consistent with known WAT physiology. Lastly, mWAT GSSG:GSH E_h may be reprogrammed by developmental exposure to HFDs and EDCs, which may have implications for obesity risk.

☆☆Disclosure summary. The authors have nothing to disclose.

*Corresponding author at: 1415 Washington Heights, School of Public Health Department of Environmental Health Sciences, Ann Arbor, MI 48109. Tel.: +1 734 936 3397; fax: +1 734 763 8095. charris@umich.edu (C. Harris).

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jnutbio.2019.01.005>.

Keywords

Obesity; Endocrine disruptors; Glutathione; Developmental programming; Redox potentials; White adipose tissue

1. Introduction

Obesity remains a major concern for public health. It has been estimated that more than one third of US adults are obese and 17% of US children are obese, with total health care costs approaching \$200 billion for obesity and obesity-related conditions [1,2]. The pathophysiology of obesity is multifactorial, and an emphasis has recently been placed on understanding the role of white adipose tissue (WAT), which has been recognized to have multiple important metabolic and endocrine functions in addition to energy storage [3]. WAT's role in the pathophysiology of obesity and its related conditions varies based on its depot location in the body. Increased visceral WAT (vWAT), or WAT found in the abdominal cavity, is strongly associated with an increased risk of cardiovascular disease and insulin resistance, while increased subcutaneous WAT (sWAT) is thought to play a protective role against metabolic syndrome [4]. Furthermore, WAT exhibits depot-dependent differences in physiological functions [5–7]. In general, vWAT has higher metabolic activity, whereas sWAT has increased capacity for long-term energy storage and increased adipokine secretions [4]. sWAT is also characterized by increased differentiation of preadipocytes compared to vWAT, which is thought to be due to a difference in preadipocyte cell populations among depots [5–7]. sWAT's increased capacity for preadipocyte differentiation and energy storage are considered to be key mechanisms of its protection against lipotoxicity [4,8].

Several important WAT physiological functions have been found to be under control of redox signaling [9–12]. Redox signaling is cell signaling mediated through the oxidative posttranslational modification of regulatory proteins, through a process that is initiated by reactive oxygen species (ROS) and reversibly attenuated by cellular antioxidants, such as glutathione (GSH), and a variety of related redox regulatory enzymes [13–15]. Thus, the redox status of GSH and other reactive thiols, such as cysteine (CYS), can reveal insights into redox signaling and the overall redox environment. Another critical indicator of the redox environment is protein S-glutathionylation (S-glut). Protein S-glut is a reversible oxidative modification on CYS residues, is responsive to the redox environment and plays a crucial role in redox signaling [16]. An example of an essential WAT function regulated by redox signaling is adipocyte differentiation, which is stimulated in part by ROS produced from mitochondria [11]. Thiol redox status may also differ by WAT depot; a study in humans indicated that vWAT has higher levels of GSH than sWAT [17]. This same study also reported that GSH was depleted in both vWAT and sWAT of obese individuals. Furthermore, lower protein S-glut levels in perirenal adipose have been linked with obesity [18], but this relationship has not yet been explored in other WAT depots. Therefore, the WAT thiol redox environment likely plays a role in influencing physiology across depots and in the pathophysiology of obesity. However, the redox environment of WAT across depots has still not been fully characterized.

Redox signaling is also a crucial factor driving developmental processes, such as proliferation and differentiation during organogenesis [19]. Environmental and nutritional exposures are capable of altering the redox environment to disrupt redox signaling during development [20,21], but it is unknown whether alterations in the redox environment induced by early-life exposures can be “reprogrammed” to an altered redox state that persists across the life course. The developmental origins of health and disease hypothesis posits that early-life environment influences health and disease in adulthood [22–24]. Perinatal exposures to endocrine disrupting chemicals (EDCs), including bisphenol A (BPA), and nutritional factors have been demonstrated to have the capability to reprogram metabolism and influence susceptibility to metabolic disease in adulthood [25–29]. Furthermore, developmental exposure to BPA has been linked with increased markers of oxidative stress in fat in animal studies and systemically in humans [30], indicating a potential for developmental exposures to impact redox signaling. The ability for developmental exposures to Mediterranean and Western high-fat diets (HFDs) to alter markers of oxidative stress is largely unknown, but direct consumption of Mediterranean diets is associated with protection against oxidative stress, whereas direct consumption of Western diets is associated with increased oxidative stress [31–34]. Given the physiologic importance of redox signaling in WAT, if the WAT redox environment can be “reprogrammed” by developmental exposures to BPA and Mediterranean and Western HFDs, there could be implications for obesity susceptibility.

In this study, we sought to determine whether developmental exposures to BPA and two different HFDs, Mediterranean and Western, can influence the WAT redox environment in adulthood. However, the mouse WAT redox environment has not yet been fully described. Furthermore, the measurement of redox potentials (E_h) in combination with GSH/GSSG and Cys/CySS concentrations provides a more realistic and reliable methodology for comparison of redox conditions across different tissues and exposure conditions, with more positive E_h values indicating an increasingly more oxidized environment [35]. Thus, we first employed a descriptive mouse study to (a) optimize a method for measuring redox parameters in WAT, (b) characterize the redox environment across different WAT depots and (c) determine whether WAT redox status is associated with obesity-related phenotypes. We collected WAT tissue from two vWAT depots (mesenteric and gonadal) and one sWAT depot (subinguinal) from adult male and female lean and overweight/obese mice. We then utilized high-performance liquid chromatography (HPLC) to measure thiol concentrations, GSH and CYS E_h , and protein S-glut and compared these parameters across depot, sex, relative WAT weight and body weight in order to characterize the WAT redox environment. In a second study, we employed an established longitudinal mouse model of perinatal exposures to combinations of BPA and Mediterranean and Western HFDs. We utilized the same HPLC methodology to compare the WAT redox environment in mesenteric vWAT from 10-month-old offspring that were perinatally exposed to BPA and HFDs *versus* controls.

2. Material and methods

2.1. Animals

2.1.1. Experimental design—Mice used in this study were either A^{vy}/a or a/a mice obtained from a colony of viable yellow agouti (A^{vy}) mice, which has been maintained for over 220 generations with forced heterozygosity for the A^{vy} allele on the male side resulting in an isogenic background 93% identical to C57BL/6J strain [36]. Comprehensive descriptions of the A^{vy} strain can be found elsewhere [37–40]. Briefly, “wild-type” a/a mice are black in color, whereas mice that are heterozygous for the A^{vy} allele (A^{vy}/a) range in coat color from brown to yellow depending on epigenetic markers present in the cryptic promoter region of the Agouti gene [38,41–43]. In addition to coat color phenotype, A^{vy}/a mice also have adult-onset obesity and type II diabetes, with increasing incidence correlating with increased proportion of yellow fur.

The initial mouse study carried out to characterize WAT Eh utilized 10 male and 10 female mice — half of which were a/a and the other half A^{vy}/a — that ranged in age from 12 to 27 weeks (Fig. 1A). These mice were randomly selected from a pool of available A^{vy} colony mice maintained at the University of Michigan and fed Laboratory Rodent Diet 5L0D (PicoLab) at the time of the study. A subsequent longitudinal mouse study was carried out on a/a mice to evaluate whether WAT Eh could be altered by perinatal exposures to BPA and HFDs (Fig. 1B). The HFDs and BPA exposures that were used in this study are fully described in a previously published study [44]. Virgin a/a females (F_0) were randomly assigned to one of six experimental diets: (a) 7% corn oil control (based on AIN-93G), (b) Mediterranean HFD, (c) Western HFD, (d) 7% corn oil control+50 μ g BPA/kg chow, (e) Mediterranean HFD+50 μ g BPA/kg chow or (f) Western HFD+50 μ g BPA/kg chow. After 2 weeks on the experimental diets, a/a females were mated with A^{vy}/a males. Dams remained on their respective diets throughout gestation and lactation until weaning at PND21, resulting in a perinatal exposure period for the F_1 offspring. Dams were sacrificed for tissue collection 4 days postweaning. The largest and healthiest a/a male and female pups from each litter were weaned onto 7% corn oil control chow and followed until 10 months of age, which is roughly equivalent to middle age in mice [45]. Litters were generated until there were 10–12 males and females per experimental group. All mice were treated humanely, following the guide for the care and use of laboratory animals, and all procedures performed on animals were carried out under an animal protocol that was approved by the University of Michigan’s Institutional Animal Care and Use Committee.

2.1.2. Tissue collection—In both studies, mice were sacrificed for tissue collection *via* CO₂ asphyxiation, followed by double pneumothorax and cardiac puncture. Body weights were measured (Mettler Toledo) just prior to euthanasia. Sacrifices of 10-month-old F_1 females were standardized so that all females were necropsied during estrus, as detected *via* vaginal cytology [46]. From the mice in the first study (Fig. 1A), three aliquots of WAT were collected from each of three different depots: subinguinal (sWAT), gonadal fat pad (gWAT) and mesenteric (mWAT). For sWAT, the maximum amount of subcutaneous fat tissue available in the hind leg area was removed and weighed. For gWAT, the entire gonadal fat pads were removed, and reproductive tract organs were trimmed away from the fat. For

mWAT, the internal organs were removed, and WAT was cut away from the stomach, intestines, spleen and pancreas. From 10-month-old mice that were included in the longitudinal mouse study on perinatal exposures (Fig. 1B), only one aliquot of mWAT was collected using the same procedure as the colony mice. Entire WAT depots were weighed (Mettler Toledo) prior to aliquoting into tubes for collection, and then approximately 5 mg of WAT tissue was placed in 1× HPLC preservation buffer containing perchloric acid (Fisher Scientific), boric acid (Sigma-Aldrich) and an internal standard (γ -glutamyl glutamate) and then flash frozen in liquid nitrogen before storing at -80°C .

Dam mWAT, blood and serum were collected to characterize the perinatal environment of the 10-month-old offspring and to attempt to identify maternal factors that may influence developmental programming of the WAT redox environment. Blood was also collected from dams at sacrifice after cardiac puncture, and $\sim 150\ \mu\text{L}$ of blood was placed into a tube containing $150\ \mu\text{L}$ of 2× HPLC preservation buffer, flash frozen and stored at -80°C . Serum was obtained from dams at sacrifice by centrifuging whole blood collected in microcentrifuge tubes at $2000\times g$ for 10 min. In preparation for 8-isoprostane analysis, 0.005% BHT was added to serum samples to prevent oxidation during storage. Serum was then stored at -80°C prior to further analysis.

2.2. Measurement of redox parameters

2.2.1. Intracellular thiol concentrations and E_h —Reverse-phase HPLC was used to quantify CYSS:CYS and GSSG:GSH redox couples in WAT, and the Nernst equation was used to calculate intracellular E_h . The protocol followed is detailed elsewhere [47], although minor adjustments were made to the protocol to optimize it for adipose tissue. Briefly, WAT that was collected and stored in 1× HPLC buffer comprised of 5% perchloric acid (Sigma-Aldrich, St. Louis, MO, USA), 0.2 M boric acid (Sigma Aldrich, St. Louis, MO, USA) and $10\ \mu\text{M}$ γ -glutamyl-glutamate (Sigma-Aldrich, St. Louis, MO, USA) was thawed on ice and homogenized using a microtip sonicator. A chloroform:methanol (ThermoFisher Scientific, Waltham, MA, USA) (1:2) extraction was performed on homogenized WAT, and $300\ \mu\text{L}$ of the aqueous layer was removed and derivatized with the fluorophore dansyl chloride (Sigma-Aldrich, St. Louis, MO, USA) as described in Harris and Hansen (2012) [47]. A Waters 2695 separations module with a Supelcosil LC-NH2 (Sigma-Aldrich, St. Louis, MO, USA) column was used to separate each thiol and the internal standard, and they were detected at excitation wavelengths of 335 and 518 nm using a Waters 2475 detector. Mobile phase buffer was set at a flow rate of 1.0 ml/min using a gradient of two mobile phases: (a) 80% methanol (ThermoFisher Scientific, Waltham, MA, USA) and 20% water and (b) 62.5% methanol, 12.5% glacial acetic acid (ThermoFisher Scientific, Waltham, MA, USA) and 214mg/mL sodium acetate trihydrate (Sigma-Aldrich, St. Louis, MO, USA) in water. Peaks were analyzed using Waters Empower Software; peaks for CYSS, CYS, GSSG and GSH were normalized to the internal standard and to sample protein concentrations as measured *via* BCA assay (ThermoFisher Scientific, Waltham, MA, USA) in order to estimate intracellular thiol concentrations. Due to the formation of disulfides as their primary oxidized states, calculation of intracellular E_h for GSSG/GSH and CYSS/CYS redox couples using the Nernst equation requires that absolute thiol concentrations be entered into the equation. Precise measurement of intracellular volume needed for this calculation is not routinely

possible when measuring tissues and mixed cell populations. To circumvent this obstacle, empirical measurements of cytosolic cell volumes and their respective protein concentrations were made in various monocultures and used to establish a conversion factor that, when applied, adequately equates protein content to cell volume [19,48,49]. The Nernst equation [Eqs. (1) and (2)] was used to calculate E_h , with $E_0 = -264$ mV and $RT/nF = 30$.

Nernst equation for calculating GSSG:GSH E_h

$$E_h = -264 + 30 * \log \frac{[GSSG]}{[GSH]^2} \quad (1)$$

Nernst equation for calculating CYSS:CYS E_h

$$E_h = -264 + 30 * \log \frac{[CYSS]}{[CYS]^2} \quad (2)$$

2.2.2. Protein S-glut—WAT protein S-glut was measured by adding 150 μ l 15.4 mg/ml dithiothreitol (DTT; Sigma-Aldrich, St. Louis, MO, USA) in water to the protein pellet after chloroform: methanol extraction in order to cleave disulfide bonds between GSH and protein, resulting in free GSH in solution. To derivatize the solution, 150 μ l of 2 $\times$ HPLC buffer (10% perchloric acid, 0.4 M boric acid and 20 μ M γ -glutamyl-glutamate) was added to the DTT-protein solution and then derivatized with dansyl chloride in the same manner as described above. Derivatized samples were also analyzed *via* HPLC and fluorescence detection as described above, and resulting S-glut concentrations were normalized to protein concentrations as measured using a BCA assay.

2.3. Serum 8-isoprostane

Frozen serum samples were transported to Cayman Chemical (Ann Arbor, MI, USA) on dry ice in one batch. Measurement of 8-isoprostane was carried out using serum collected from dams at sacrifice using a competitive enzyme immunoassay (EIA) kit (no. 516351). The EIA was carried out as specified in the Cayman Chemical protocol, with sensitivity 2.7 pg/ml and an intraassay % coefficient of variation (CV) of 9.5%. Briefly, this protocol uses the competitive binding of 8-isoprostane and a tracer (8-isoprostane acetylcholinesterase) to 8-isoprostane-specific rabbit antiserum binding sites. Tracer levels are constant in all samples, while 8-isoprostane is sample-specific, so the amount of unbound tracer is inversely proportional to the sample's 8-isoprostane concentration. A tracer substrate, Ellman's reagent, is added to reaction wells, turning yellow when acted on by the tracer. Intensity of the yellow color indicates the amount of unbound tracer; 8-isoprostane levels are quantified as the inverse of tracer concentration.

2.4. Statistical analyses

All statistical analyses were carried out using R version 3.3.1 (www.R-project.org). CVs were calculated for technical replicates (biological samples from the same WAT depot of the same mouse) by dividing the standard deviation by the mean. The mean of the three

technical replicates for each depot from each mouse was used in subsequent analyses. The distributions of S-glut and thiol concentrations tended to be skewed, and therefore, these outcomes were log-transformed in all analyses. To compare measures across the three WAT depots, a one-way analysis of variance (ANOVA) was carried out with a *post hoc* Tukey test. Univariate analyses between continuous predictor variables (*e.g.*, body weight, depot weight, age) and WAT redox parameters were carried out using a Pearson correlation test. Univariate analyses between dichotomous variables (*e.g.*, sex, genotype) and WAT redox parameters were carried out using a Student's *t* test. Multivariate analyses were performed using multiple linear regression. Analyses were stratified by sex if an interaction term containing sex and another variable was significant in preliminary models.

3. Results

3.1. Within-depot variability

To characterize within-depot variability of WAT redox parameters, we calculated CVs for three samples of each WAT depot (sWAT, gWAT and mWAT) from each individual mouse from the first study (Fig. 1A). CVs were calculated for *Eh* and S-glut (protein oxidation as protein-S-S-glutathione), as well as for GSH, GSSG, CYS and CYSS concentrations (Table 1). CVs were calculated from three replicates, obtained by taking measurements in three samples from each WAT depot from each individual mouse. Average CVs fell below 1 in most cases. CVs were lower for *Eh* than for intracellular thiol concentrations and S-glut, indicating that *Eh* are more homogenous within WAT depots and that thiol concentrations and S-glut have substantial within-depot variability. CVs were also lower for mWAT than gWAT and sWAT for some measures, indicating that measurements were less variable in mWAT. The most notable differences in CVs across WAT depots were for CYS measures in mWAT compared to gWAT; CVs for intracellular CYS concentrations and CYSS:CYS *Eh* were lower for mWAT than for gWAT ($P=.003$ and $P=.01$, respectively). GSSG:GSH *Eh* also had lower CVs for mWAT than sWAT ($P=.05$).

3.2. Redox potentials and S-glut levels differ by WAT depot

A comparison of redox parameters across WAT depots revealed striking differences between the mWAT redox environment and the gWAT and sWAT redox environments. mWAT had significantly more reducing GSSG:GSH *Eh* (-195.5 mV) and CYSS:CYS *Eh* (-149.95 mV) than both gWAT (-160.7 mV and -100.81 mV, respectively) and sWAT (-165.3 mV and -109.03 mV, respectively) (Fig. 2A and B). The reducing GSSG:GSH *Eh* in mWAT were accompanied by increased levels of GSH, but not GSSG, relative to gWAT and sWAT (Fig. 2D), indicating that mWAT's relatively reducing GSSG:GSH *Eh* were due primarily to elevated GSH concentrations in mWAT. Both CYS and CYSS intracellular concentrations were increased in mWAT relative to the other two depots, but this difference was more statistically significant for CYS (Fig. 2E). Thus, the relatively reducing CYSS:CYS *Eh* in mWAT were due to higher levels of both CYS and CYSS. S-glut levels were also higher in mWAT than in gWAT and sWAT (Fig. 2C).

3.3. The redox environment of sWAT is sexually dimorphic

WAT is sexually dimorphic; it contains both estrogen and androgen receptors and is also capable of steroid synthesis [50,51]. Thus, we compared redox parameters of male and female WAT across all three depots to determine whether thiol redox parameters differed by sex. Redox parameters in gWAT did not differ by sex, and only intracellular CYSS concentrations differed by sex in mWAT, with the males exhibiting highly variable CYSS levels ($138.92 \pm 46.0 \mu\text{M}$ in females vs. $1904.68 \pm 720.8 \mu\text{M}$ in males, $P=.01$). On the other hand, sWAT measures significantly differed by sex with respect to several measures: GSH, GSSG, CYSS, GSSG:GSH *Eh* and S-glut (Fig. 3). Mean sWAT GSSG:GSH *Eh* were significantly more reducing in females (-176.2 mV) compared to males (-154.4 mV) ($P=.01$), and females had increased sWAT S-glut levels compared to males ($P=.01$) (Fig. 3A and C). The reducing GSSG:GSH *Eh* in sWAT of females were accompanied by increases in intracellular concentrations of both GSH and GSSG relative to males (Fig. 3B), suggesting that female sWAT utilizes more GSH than male sWAT.

3.4. Body weight and adiposity are associated with mWAT and sWAT redox parameters

We next sought to determine whether the WAT redox environment was altered by phenotypic traits, such as body weight, relative adipose depot weight and age. We performed a univariate analysis to identify factors associated with redox parameters in each of the three mouse WAT depots. Because of the potential for sex to modify the relationship between WAT redox environments and phenotypic traits, all analyses were stratified by sex. Several mWAT and sWAT thiol redox parameters were associated with body weight and relative WAT depot weight, but gWAT redox parameters were not associated with body weight, relative depot weight, age or genotype (Supplemental Table 2). Increased body weight and increased relative depot weight were associated with oxidizing GSSG:GSH *Eh* in male and female sWAT and mWAT (Fig. 4A, B, D and E). The observed relationships between oxidizing GSSG:GSH *Eh* and increased body weight and depot weight were stronger in female mWAT than in male mWAT but were stronger in male sWAT than in female sWAT, providing further evidence that the WAT redox environment is sexually dimorphic in nature. In both male and female mWAT, GSSG:GSH *Eh* also became slightly more oxidizing with age (Fig. 4C and F). A similar trend was also observed in sWAT, but the association was not statistically significant ($P>.10$).

CYSS:CYS *Eh* were also associated with body weight and relative depot weight in sWAT and mWAT (Supplemental Table 2). CYSS:CYS *Eh* were more oxidizing in mWAT with increased body weight and depot weight in both males ($P=.01$ and $P=.02$, respectively) and females ($P=.007$ and $P=.0006$, respectively), but sWAT CYSS:CYS *Eh* were more oxidizing only with body weight and only in females ($P=.02$). There was also a relationship between oxidizing CYSS:CYS *Eh* and increased age in mWAT from males ($P=.03$) but not females. WAT S-glut levels differed by body weight and depot weight but only in males (Supplemental Table 2). In male sWAT, increased body weight and depot weight were associated with modestly decreased S-glut levels ($P=.05$ and $P=.08$, respectively), whereas increased body weight and depot weight were associated with increased S-glut levels in mWAT ($P=.01$ and $P=.02$, respectively).

Few redox parameters were significantly different across genotype even though A^{VY}/a mice weighed more and had higher relative depot weights than a/a mice (Supplemental Tables 1 and 2). Male A^{VY}/a mice had more oxidizing mean sWAT GSSG:GSH Eh than male a/a mice ($P=.02$), and female A^{VY}/a mice had oxidizing mean sWAT CYSS:CYSS Eh and increased mean mWAT protein S-glutathionylation than female a/a mice ($P=.04$ and $P=.03$, respectively). However, there was no statistically significant difference in mWAT GSSG:GSH Eh or mWAT CYSS:CYSS Eh in A^{VY}/a versus a/a mice. Overall, the relationships between body weight and relative depot weight and WAT redox parameters were stronger than the relationship between genotype and WAT redox parameters, particularly in mWAT.

3.5. mWAT redox parameters are associated with developmental exposures and maternal factors

To examine whether mWAT redox parameters in 10-month-old offspring are associated with developmental exposures to BPA and HFDs and/or maternal redox parameters, we first performed a univariate analysis to identify factors associated with 10-month-old adult offspring mWAT redox parameters. Offspring mWAT GSSG:GSH Eh were associated with maternal redox parameters in a sex-specific manner. Oxidizing maternal mWAT GSSG:GSH Eh were associated with *oxidizing* mWAT GSSG:GSH Eh in males ($P=.003$), whereas oxidizing maternal mWAT GSSG:GSH Eh were associated with *reducing* mWAT GSSG:GSH Eh in females ($P=.04$) (Fig. 5A, Supplemental Table 3). Maternal mWAT S-glut also had sexually dimorphic relationships with offspring mWAT GSSG:GSH Eh . Increased maternal mWAT S-glut was associated with *oxidizing* Eh in females ($P=.002$) and *reducing* Eh in males, although the relationship in males was not statistically significant ($P=.14$) (Fig. 5B). Furthermore, offspring mWAT S-glut was associated with maternal factors only in males; oxidizing maternal mWAT GSSG:GSH and CYSS:CYSS Eh were both associated with decreased S-glut levels in male offspring mWAT ($P=.06$ and $P=.06$, respectively). With respect to measures in maternal blood and serum, oxidizing maternal blood CYSS:CYSS Eh were associated with reducing mWAT GSSG:GSH Eh in males ($P=.03$) but not in females (Fig. 5C). On the other hand, increased maternal serum 8-isoprostane was associated with oxidizing GSSG:GSH Eh in mWAT from female offspring ($P=.02$) but not males (Fig. 5D). Notably, offspring CYSS:CYSS Eh did not exhibit any significant relationships with maternal redox parameters. Together, these results support the hypothesis that maternal redox environment may influence the redox environment of mWAT in adult offspring.

Perinatal exposures to BPA and Mediterranean HFD resulted in more oxidizing mWAT GSSG:GSH in offspring, with males and females exhibiting slightly different effects (Fig. 6). Males exposed to a Mediterranean HFD had more oxidizing mWAT GSSG:GSH Eh than control males, regardless of whether they were also exposed to BPA ($P=.04$). BPA had a stronger impact on mWAT in females; females exposed to control diet + BPA and Mediterranean HFD + BPA had more oxidizing mWAT GSSG:GSH Eh than females exposed to control diet without BPA and Mediterranean HFD without BPA ($P=.03$ and $P=.01$, respectively). Exposure to a Western HFD, with or without BPA, resulted in mean mWAT GSSG:GSH Eh that were similar to those of controls for both males and females. The only impact of Western HFD exposure was on CYSS:CYSS mWAT Eh in female offspring, which were more reduced in Western HFD-exposed females than control females

($P=.07$) (Supplemental Table S3). Perinatal exposure to HFD and/or BPA did not impact mWAT CYSS:CYS Eh , nor did it impact mWAT S-glut. Therefore, developmental exposures to BPA and Mediterranean HFD were associated with altered mWAT GSSG:GSH and CYSS:CYS Eh , but not S-glut, in a sex-specific manner.

To confirm that associations between 10-month-old offspring mWAT GSSG:GSH Eh and maternal redox parameters and perinatal exposures were not confounded by effects of offspring body weight or relative mWAT weight, a multiple linear regression (MLR) was carried out to control for these variables (Table 2). Because body weight and relative mWAT depot weight are highly correlated with one another, only relative mWAT weight was included in the model. Among 10-month offspring of both sexes, relative mWAT weight had a stronger univariate correlation with GSSG:GSH Eh than body weight ($P=.02$ vs. $P=.37$, respectively; Supplemental Table S3). The MLR results demonstrated that the mWAT redox environment was associated with maternal environment and perinatal exposure to BPA and HFDs in a sex-specific manner. In males, maternal mWAT GSSG:GSH Eh were the most significant predictor of mWAT GSSG:GSH Eh ($P=.05$); oxidizing maternal mWAT Eh were associated with oxidizing mWAT Eh in male offspring. In females, maternal serum 8-isoprostane was the most significant predictor of mWAT GSSG:GSH Eh ($P=.01$), with increased serum 8-isoprostane associated with oxidizing GSSG:GSH Eh in female offspring mWAT. Exposure to Mediterranean HFD was also correlated with oxidizing mWAT GSSG:GSH Eh in females in the MLR model ($P=.08$). Although not statistically significant at $P>.10$, the MLR analyses indicated that 10-month mWAT GSSG:GSH Eh tended to be more oxidized in BPA exposed males and females ($P=.11$ and $P=.14$, respectively). The results of these analyses indicated that both maternal redox environment and perinatal exposures to BPA and HFDs were associated with altered mWAT redox profiles even while controlling for relative mesenteric fat weight.

4. Discussion

Redox signaling is known to play an important role in WAT physiology, including differentiation and metabolism [9–12]. In this study, we investigated whether developmental exposures to an EDC, BPA and two different HFDs, Mediterranean and Western, could influence the WAT redox environment in adulthood and found that perinatal exposure to Mediterranean HFD and BPA was associated with more oxidizing mWAT in adulthood. We employed a descriptive study to characterize the mouse WAT redox environment by measuring thiol concentrations, Eh and S-glut across three different WAT depots in male and female adult mice, and investigated whether these parameters were associated with age, body weight and relative fat weight. Our findings demonstrated depot- and sex-specific differences in thiol Eh , and associations between oxidizing Eh and obesity phenotypes in mWAT and sWAT.

Some WAT redox parameters exhibited high within-depot variability, while others were more stable. CVs were calculated among three replicates per depot per mouse to characterize within-depot variability for each parameter. CVs indicated that within-depot variability was low for GSSG:GSH and CYSS:CYS Eh but was higher for measures of individual thiol concentrations. S-glut levels had a moderate amount of variation across

samples, indicating that this measure may be somewhat heterogeneous across tissues as well. Additionally, mWAT exhibited significantly lower CVs than sWAT and gWAT with respect to Eh , indicating that mWAT may have a more homogenous redox environment. Overall, these findings indicate that Eh are a more stable measure when characterizing the WAT redox environment than concentrations of individual thiols and S-glut levels and that mWAT was more homogenous than sWAT and gWAT [3].

Our findings demonstrated that the thiol redox environments of mouse gWAT and sWAT were significantly different than that of mWAT, which may be reflective of differences in physiology across WAT depots. Oxidizing thiol Eh accompany differentiation of preadipocytes to adipocytes [52]; thus, our findings that gWAT and sWAT had more oxidizing CYSS:CYS and GSSG:GSH Eh indicate that these depots may have increased adipocyte differentiation compared to mWAT. Oxidizing redox environments are also associated with increased adipogenesis [52], which is a known feature of sWAT [7] and may be a feature of gWAT. The more reducing Eh observed in mWAT, along with the increased thiol concentrations, may be linked with mWAT's increased metabolic capacity. Studies have demonstrated that mWAT is more involved in metabolism of fatty acids than other WAT depots, potentially due to direct blood flow from the hepatic portal vein [4]. These results were consistent with known differences in physiology between sWAT and mWAT. Notably, the redox environment of gWAT was more similar to sWAT than to mWAT in the mice used in this study even though mWAT and gWAT are both occasionally grouped together as vWAT. Our study indicates that the redox environment is drastically different between mWAT and gWAT and sWAT. The common use of the term vWAT can obscure mechanistic study of adipose pathophysiology by combining (or not differentiating between) two very different adipose depots: mWAT and gWAT. Future studies should take these differences into consideration to avoid misinterpretation of findings.

The GSSG:GSH and CYSS:CYS Eh obtained for WAT in this study, especially in sWAT and gWAT, were generally more oxidizing than previously reported in other tissue types. For example, hepatic GSSG: GSH Eh in untreated mice have been reported to range between approximately -240 mV and -300 mV, and heart GSSG:GSH Eh have been reported to range between approximately -205 mV and -260 mV [53,54], compared to -165.3 mV for sWAT and -160.7 mV for gWAT observed in the present study. We observed that mean mWAT GSSG: GSH Eh were -195.5 mV, similar to values reported by Li et al. (2002) for lung, colon and heart [54].

In the present study, we observed a significant positive correlation between oxidizing mWAT and sWAT GSSG:GSH Eh and increased body weight and relative WAT weight. One possible explanation for this relationship is that adipogenesis is associated with an oxidizing redox environment [52], and mice with higher body weight and WAT weight may have an increased rate of adipogenesis. Additionally, there is a large body of literature linking obesity with oxidative stress [55–57]. Many studies have provided evidence that systemic oxidative stress and inflammation are associated with obesity, and some studies have suggested that this originates from WAT or the resident macrophages in WAT [58–60]. Our observations that WAT redox parameters were associated with body weight and WAT weight led us to expect that there would be significant differences in $A^{V\gamma/a}$ mice, which are typically

overweight or obese, and *a/a* mice, which are typically lean. However, we observed few differences in redox parameters by genotype. This could be due to unique WAT redox status in *A^{VY}/a* mice, or it could be due to a large amount of variability in body weight, WAT weight and redox parameters in the *A^{VY}/a* mice used in this study. The *A^{VY}/a* mice used in this study had a wide variety of coat colors, ranging from brown to mottled to yellow. Yellow mice are more obese than mottled and brown mice [38], and therefore, there was a wide range of body weights represented in the *A^{VY}/a* group of mice used for this study. The use of a wide range of coat colors and body weights was intentional in an attempt to determine whether genotype or phenotype had a larger influence on WAT redox parameters. Although some WAT redox parameters were still associated with the presence of the *A^{VY}* allele (e.g., sWAT CYSS:CYS *Eh* in females and mWAT S-glut levels in females), most relationships were diminished compared to the relationships observed between body weight and relative WAT depot weight, suggesting that phenotype likely plays a larger role in WAT redox status than genotype with respect to the *A^{VY}* allele.

Our findings that mWAT *Eh* was more oxidizing with increased age are consistent with other studies that have suggested a link between aging and increasing tissue oxidation [61]. It should be noted that although we observed a relationship between age and mWAT oxidation in colony mice, this relationship is less clear when comparing mWAT from 10-month-old control offspring from our longitudinal mouse study to colony mice that were aged 12–27 weeks (3–6 months). 10-month-old offspring had mean mWAT GSSG:GSH *Eh* of –213.6 mV for females and –211.6 mV for males, and the *a/a* colony mice that were aged 12–27 weeks had mean mWAT GSSG:GSH *Eh* of –198.6 mV for females and –192.3 mV for males. However, the 10-month-old control offspring are not directly comparable to the colony mice used in this study because the colony mice were on a diet containing soy, while the control mice were on a soy-free diet. Soy has estrogenic properties and therefore could influence redox parameters of WAT [62,63]. Interestingly, gWAT redox parameters were not associated with body weight, relative depot weight or age. The reason for this relative stability of thiol parameters in gWAT is not clear, but it is possible that gWAT's role in protecting the reproductive tract requires more stability in these measures. The findings in our study are consistent with the theories that WAT is more oxidizing with increased body weight, body fat and age, but more work is needed to fully characterize the underlying physiology.

This study is the first to examine whether the WAT redox environment is influenced by developmental exposures to HFDs and/or environmental chemicals, or other maternal factors. Our results indicate that perinatal exposure to Mediterranean HFD may reprogram mWAT GSSG:GSH *Eh* to be more oxidizing in 10-month-old offspring, and this effect appeared to be stronger in females than in males. The effect of perinatal exposure to Mediterranean HFD remained significant in females when controlling for other factors that we found to have an impact on mWAT GSSG:GSH *Eh*, including relative depot weight, providing further evidence that mWAT GSSG:GSH may be reprogrammed by maternal diet. Surprisingly, mice perinatally exposed to a Western HFD did not exhibit alterations in mWAT thiol redox parameters. However, other maternal factors were associated with oxidizing mWAT GSSG:GSH *Eh* in 10-month-old offspring, including maternal mWAT GSSG:GSH *Eh*. Perinatal exposure to BPA appeared to have modest effects on mWAT

GSSG:GSH *Eh*; females exposed to BPA and those exposed to Mediterranean HFD + BPA had more oxidizing mean GSSG:GSH *Eh* than control females, but BPA had nonsignificant ($P > .10$) effects on mWAT GSSG:GSH when controlling for other factors in an MLR model. Taken together, our findings demonstrate an association between maternal factors, including perinatal dietary and EDC exposures, presenting the possibility that WAT redox environment, and particularly GSSG:GSH *Eh*, may be reprogrammed during development.

Our findings revealed sexual dimorphism in WAT redox environments both in baseline levels and in changes following developmental exposures. The redox profile of sWAT was significantly different in males *versus* females. Associations between increased relative depot weight and oxidized GSSG:GSH *Eh* were more pronounced in mWAT for females and sWAT for males. Furthermore, alterations in mWAT redox parameters following perinatal BPA and Mediterranean HFD exposures were more apparent in females than in males. WAT physiology is known to be sexually dimorphic, especially with respect to estrogen receptor expression and function [64,65]. Therefore, the sex-specific redox effects observed in this study suggest that redox regulation either may play a role in or may be a consequence of sexually dimorphic characteristics of WAT.

The findings presented in this study suggest that the redox environment, and particularly GSSG:GSH *Eh*, is associated with overweight/obese phenotypes and aging and may be reprogrammed by diet, EDC exposure and maternal factors during development. However, additional studies are needed to fully elucidate the role of the WAT redox environment in physiology and pathology. For example, measurement of WAT *Eh* and protein S-glutathionylation across time in the same individual throughout the life course in lean and obese mouse models would shed more light on whether oxidizing WAT *Eh* are causative or a result of aging and obesity. Additional concurrent measurements in WAT would also provide insights into the role of thiol *Eh* in WAT physiology and how WAT *Eh* are programmed and reprogrammed during development, including but not limited to expression and activities of glutathione reductase, peroxidase, transferase, synthetase and transporters. More comprehensive measures of protein oxidation would also provide insights into whether differences observed in S-glut are really due to increased protein oxidation in the cell. Although differences in total S-glut can serve as an additional useful indicator of overall cellular redox state, only a detailed evaluation of the specific redox-sensitive proteins that are oxidized and reduced would lend itself to a more mechanistic understanding of its significance. It should be noted that the calculation of *Eh* in this study relied on an assumption of protein content per cellular cytoplasmic volume and that this assumption remained constant across depots and individuals. Adipocytes have been demonstrated to vary widely in size [66]; however, this size variation is primarily due to the size of the lipid droplet, not the cytoplasm. Thus, there may be some variation in the protein per cytoplasmic volume across WAT depots and individuals, but we expect this variation to be relatively small. Further study is warranted given the potential benefit of understanding the role of WAT *Eh* in obesity and metabolic syndrome pathophysiology.

5. Conclusion

In this study, we present a method for measuring thiol *Eh*, intracellular thiol concentrations and protein S-glut in WAT samples obtained from *in vivo* studies. The utility of these measures, particularly GSSG:GSH *Eh*, was demonstrated by the striking differences across WAT depots and their associations with body weight, WAT weight and aging in mice. Our findings also suggested a potential for mWAT GSSG:GSH *Eh* to be reprogrammed by developmental exposures to HFDs and EDCs, as well as maternal redox parameters. This study begins to scratch the surface on the possible roles for thiol *Eh* and S-glut in WAT physiology and pathophysiology. Additional studies are needed to further our understanding of the pathophysiology of obesity and metabolic syndrome, including influences of developmental exposures to HFDs and EDCs on WAT.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Experimental design. (A) A^{vy} strain colony mice were bred with forced heterozygosity through the male line, resulting in approximately half a/a “wild-type” black-coated offspring and half A^{vy}/a offspring, with coat colors ranging from yellow to mottled to brown, and varying risks of adult-onset obesity. Ten females (5 a/a and 5 A^{vy}/a) and 10 males (5 a/a and 5 A^{vy}/a) were sacrificed at 12 to 27 weeks of age for collection of WAT from the inguinal subcutaneous depot (sWAT), the gonadal depot (gWAT), and the mesenteric depot (mWAT). Three sections of each WAT depot were collected from each mouse and were subsequently used for measurement of reactive thiols *via* HPLC. (B) Virgin female a/a mice were exposed to HFDs and BPA through the chow starting 2 weeks prior to mating and ending at weaning, resulting in a perinatal exposure spanning from preconception to weaning. One male and one female a/a mouse from each litter were followed to 10 months of age, at which time mWAT was collected for HPLC measurement of reactive thiols.

Fig. 2.

Differences in *Eh*, S-glut levels and thiol levels across WAT depots. One-way ANOVAs were used to compare data across the three WAT depots. Data in box plots are represented as medians, with whiskers representing the upper and lower quartiles. Data in bar graphs are represented as means. *** $P < .001$ vs. gonadal, ** $P < .01$ vs. gonadal, ^ $P < .10$ vs. gonadal, ### $P < .001$ vs. subcutaneous, # $P < .05$ vs. subcutaneous, & $P < .10$ vs. subcutaneous.

Fig. 3.

Differences in sWAT redox environment between males and females. Student's *t* tests were performed to compare sWAT *E_h*, intracellular GSH concentrations and S-glut between males and females. Data in box plots are represented as medians, with whiskers representing the upper and lower quartiles. Data in bar graphs are represented as means. **P*<.05, ***P*<.01.

Fig. 4.

Correlation between GSSG:GSH *E_h* and body weight, relative depot weight and age.

Univariate analyses were performed *via* Pearson's correlation coefficient for each WAT depot. Analyses were stratified by sex. sWAT is in green, with plus (+) symbols representing each data point; gWAT is in black, with circles representing each data point; and mWAT is in red, with triangles representing each data point.

Fig. 5.

Correlation between mWAT GSSG:GSH Eh of 10-month-old offspring and select maternal parameters. Univariate analyses were performed *via* Pearson's correlation coefficient for 10-month-old offspring mWAT GSSG:GSH Eh. Analyses were stratified by sex. Males are in blue with triangles for each data point, whereas females are in red with circles for each data point.

Fig. 6.

Differences in 10-month-old offspring mWAT GSSG:GSH *Eh* and intracellular GSH concentrations across exposure groups. Multiple linear regression was used to compare each group back to the control group. Student's *t* tests were used to compare +BPA and -BPA groups within each of the three diets (e.g., Med -BPA vs. Med +BPA). Data represented as means, with error bars representing standard errors of the means. ^*P*<.10, **P*<.05, ***P*<.001, #*P*<.05 vs. Med.

Table 1

Coefficients of variation for measures across WAT depots

WAT depot	GSSG:GSH		CYSS:CVS		S-glut	
	Mean CV	CV range	Mean CV	CV range	Mean CV	CV range
Gonadal	0.13	0.02–0.39	0.47	0.10–1.51	0.62	0.04–1.58
Subcutaneous	0.16	0.05–0.41	0.35	0.04–0.67	0.61	0.19–1.37
Mesenteric	0.09*	0.01–0.37	0.22**	0.01–0.83	0.72	0.09–1.70

WAT depot	GSH		GSSG		CVS		CYSS	
	Mean CV	CV range	Mean CV	CV range	Mean CV	CV range	Mean CV	CV range
Gonadal	0.77	0.07–1.87	0.55	0.10–1.14	1	0.63–1.38	0.85	0.45–1.35
Subcutaneous	0.76	0.12–1.48	0.58	0.07–1.17	0.85	0.17–1.40	0.81	0.32–1.33
Mesenteric	0.64	0.03–1.36	0.66	0.11–1.31	0.62***	0.08–1.40	0.70	0.10–1.41

CVs were compared across WAT depot using one-way ANOVA and *post hoc* Tukey tests. *N* = 10 males and 10 females total, with three aliquots of WAT taken from each depot from each mouse.* *P* = .05 compared to subcutaneous.

**

* *P* = .01 compared to gonadal.

* *P* = .003 compared to gonadal.

Table 2

Multiple linear regression predicting mWAT GSSG:GSH Eh in 10-month-old offspring

Variable	Estimate	P value	R ²	Model P value
Females				
Mediterranean diet	24.2	.08 *	0.51	.002 ***
Western diet	16.9	.22		
BPA	21.2	.14		
MedxBPA	-4.1	.83		
WestxBPA	-26.7	.18		
Relative mesenteric fat weight	7.6	.03 **		
Maternal mWAT EhGSH	-0.1	.55		
Maternal mWAT EhCYS	0.0	1		
Maternal mWAT S-glut	13.6	.35		
Maternal serum 8-isoprostane	0.0085	.01 ***		
Males				
Mediterranean diet	15.4	.18	0.23	.07 *
Western diet	11.22	.34		
BPA	18.9	.11		
MedxBPA	-12.56	.44		
WestxBPA	-8.38	.61		
Relative mesenteric fat weight	4.4	.7		
Maternal mWAT EhGSH	0.4	.05 **		
Maternal mWAT S-glut	-4.7	.7		
Maternal blood EhCYS	-0.11	.36		

Multiple linear regression analyses were performed to analyze the relationship between variables and outcomes. S-glut and thiol concentrations were log-transformed to adjust for skewed distributions. *N*=9–12/sex/group.

*
P .1.

**
P .05.

P .01.