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Obesity reduces methionine sulphoxide reductase activity in visceral adipose tissue

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Abstract
Visceral obesity is linked to insulin resistance and cardiovascular disease. A recent genetic study indicated that the gene locus for the anti-oxidant defense enzyme methionine sulphoxide reductase A (MsrA) is positively associated with the development of visceral adiposity. This work tested the hypothesis that Msr activity is diminished in visceral fat as a result of obesity. It used two animal models of obesity, wild-type rats fed a high-fat (45% of calories from fat) diet and Zucker rats fed a 10% fat calorie diet. The data indicate that MsrA activity was selectively reduced by ~ 25% in the visceral adipose, but not subcutaneous adipose or liver, of both rat models as compared to control, wild type rats receiving a 10% fat calorie diet. MsrB activity was similarly reduced only in visceral fat. The data indicate that Msr activity is reduced by obesity and may alter oxidative stress signalling of obesity.

Keywords: *Oxidative stress, adipose, oxidative damage, Zucker, visceral*

Introduction

Obesity is a societal problem that affects over one third of the American population as a whole, with an even greater proportion of obese individuals belonging to specific geographic or ethnic groups [1]. Obesity raises the risk for multiple diseases including diabetes, cardiovascular disease and cancer (for recent reviews see [2–6]). On mechanistic levels, these sequelae of obesity result in part as the result of elevations in inflammation and oxidative damage [7–10]. Several studies demonstrate that levels of circulating lipid peroxidation markers are positively correlated with adipose mass [8,11–13].

While elevated oxidative damage results from an increase in the production of reactive oxygen species (ROS), elevated levels of oxidative damage may also occur through decreases in the detoxification of ROS or detoxification of oxidative damage products. With regards to obesity, levels of the electrophile detoxification enzyme glutathione S-transferase A4 (GSTA4) are reduced in adipose tissue of obese mice [14–16]. This class of GST has high activity towards the lipid peroxidation product, *trans*-4-hydroxy-2-nonenal (HNE), and not surprisingly elevated HNE-protein adducts are observed in the adipose tissue of these same obese mice. GSTA4 null mice are heavier than wild type mice and have a greater percentage body fat than their wild-type counterparts [15]. These data indicate that a decrease in oxidative damage defense enzymes may play a part in the development of obesity.

Recently, population-based genome-wide association studies reported that the *MSRA* locus is linked to visceral adiposity in a European cohort but not in a Japanese cohort [17–19]. *MSRA* encodes the methionine sulphoxide reductase A (MsrA) protein, a 26 kD, oxidative damage defense protein that catalyses the reduction of methionine-*S*-sulphoxide epimer to methionine on oxidized proteins [20,21]. Sub-cellular localization studies indicate that MsrA is found in the cytosol and in the mitochondria [22,23].

Mammalian MsrB, on the other hand, contains four members, MsrB1, MsrB2, MsrB3A and MsrB3B [24]. In contrast to MsrA, the MsrB enzymes catalyse the reduction of the methionine-*R*-sulphoxide epimer [20].

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While MsrB2, MsrB3A/B and MsrA contain active site cysteines, MsrB1 utilizes an active site selenocysteine and has a higher specific activity than other MsrB enzymes [20].

The formation of methionine sulphoxide (METO) residues upon proteins has functional effects. Data derived from *in vivo* and *in vitro* studies indicate that over-expression of Msr proteins increases resistance to oxidative stressors whereas inhibition of Msr activity enhances susceptibility to oxidative stress insult. Known biochemical targets of methionine sulphoxidation include the calcium-regulated protein calmodulin and the high-density lipoprotein constituent, apolipoprotein A1 (ApoA1) [25–28]. METO-modification of ApoA1 reduces cholesterol trafficking and elevated levels of METO-modified ApoA1 are observed in individuals with type I diabetes [29,30]. MsrB1 and METO were recently shown to regulate the TRPM6 ion channel [31]. While conflicting reports exist regarding the role of MsrA and lifespan, MsrA null mice have higher sensitivity to oxidative stress than control mice [32,33]. These data demonstrate that methionine oxidation has functional targets with physiological and potentially pathological outcomes.

Because of this relationship of the *MSRA* locus to obesity, the importance of Msr's to reversing oxidant injury and the data indicating that obesity decreases levels of the anti-oxidant defense enzyme GSTA4, we tested the hypotheses that obesity decreases Msr activity in adipose and liver. In this work, we utilized two commonly-used rat models of obesity, a high-fat fed rat and the Zucker (fa/fa) obese rat. Our data indicate that Msr activities are reduced selectively in the visceral adipose tissue but not subcutaneous adipose or liver of obese rats.

Materials and methods

Animals

All experiments were performed in accordance with the NIH guidelines for use of live animals and were approved by the Institutional Animal Care and Use Committee of the USDA/Agricultural Research Service, Grand Forks Human Nutrition Research Center. Eight-week old, male, Zucker (fa/fa) fat rats and their wild type (WT) counterparts were purchased from Harlan Laboratories (Indianapolis, IN). Following arrival animals were placed on a defined AIN 93 with lard (Dyets, Inc. Bethlehem, PA) used as a fat source. Zucker rats were maintained on the AIN 93 diet containing 10% fat calories from lard; whereas wild type animals were distributed into two feeding groups: (1) AIN93 diet containing 10% fat calories from lard and (2) diet containing 45% fat calories from lard. Diets were isocaloric. Six rats were included in each group. All diets were vitamin and mineral replete. Fatty acid composition of the lard was determined by gas chromatography following derivatization to fatty acid methyl esters [34]. Diet composition data are provided in Tables I and II. Animal weights and feeding amounts were recorded and provided in the supplementary data. Animals were kept on these diets for 6 weeks before euthanasia. Animals were euthanized by administration of anaesthetic (ketamine 100 mg/kg and xylazine 13 mg/kg) followed by cardiac puncture. Livers and visceral adipose tissue (peri-renal) and sub-cutaneous adipose tissue (inguinal) were removed and frozen in liquid N_2 and stored at −80°C until use.

Triglyceride determinations

Pieces of liver (~200 mg) were homogenized in a 20-fold (v/wt) chloroform:methanol (2:1) solution in a glass vial. The homogenate was centrifuged at 1800 g at 4°C for 10 min to pellet any insoluble material. The supernatant was kept, transferred to a clean glass vial and dried under nitrogen. The dried sample was resuspended in 1 mL of ethanol. The triglyceride content of the sample was determined using a COBAS Integra Analyser with triglyceride assay kit (Hoffman-LaRoche).

Determination of MsrA activity

MsrA activity in liver and adipose tissue was determined by following the reduction of dabsyl Met-*S*-(O) to dabsyl Met as described by Minetti et al. [35] and Moskovitz et al. [36] with modifications. Briefly, samples were homogenized (1 g liver/4 mL buffer; 1 g adipose/2 mL buffer) in 25 mM KH_2PO_4, pH 7.5 containing 1 mM 1,4-dithioerythritol (DTE) at 4°C. The homogenate was centrifuged at 46 000 × g (r_{avg}) for 30 min at 4°C; the supernatant was used for the assay. Protein concentration in the liver supernatant was determined by the BCA method (Pierce); for adipose, protein was determined by the Micro BCA method (Pierce) with the addition of 1% (final concentration) SDS [37]. The reaction mixture (200 µL) contained 25 mM KH_2PO_4, pH 7.5; 10 mM $MgCl_2$; 30 mM KCl; 20 mM DTE; 0.25 mM dabsyl Met-*S*-(O); and supernatant (0.02 mL for liver and 0.04 mL

Table I. Diet composition.

Ingredient	45% Fat calories (g/kg diet)	10% Fat calories (g/kg diet)
Casein	237.95	192.68
L-Cystine	3.57	2.89
Corn Starch	281.98	570.81
Sucrose	118.98	96.34
Lard	241.52	43.35
AIN 93G Mineral Mix	41.64	33.72
AIN 93 Vitamin Mix	11.90	9.63
Choline Bitartrate	2.97	2.41
Cellulose	59.49	48.17
Total grams	1000	1000

Table II. Fatty acid composition of lard.

	Mole %	
Name	Mean	SD*
C14:0	1.20	0.04
C14:1n-5	0.00	0.00
C16:0	21.06	0.49
C16:1n-7	2.58	0.22
C18:0	11.57	0.04
C18:1n-9	37.13	0.66
C18:2n-6	19.99	0.19
C18:3n-6	0.61	0.25
C18:3n-3	1.37	0.14
C20:1n-9	1.13	0.15
C20:2n-6	0.98	0.17
C20:3n-6	0.34	0.06
C22:0	0.19	0.06
C20:4n-6	1.09	0.18
C22:1n-9	0.05	0.04
C22:2n-6	0.00	0.00
C24:0	0.00	0.00
C22:4n-6	0.12	0.03
C24:1n-9	0.00	0.00
C22:5n-6	0.19	0.01
C22:5n-3	0.22	0.00
C22:6n-3	0.19	0.00
Total	100.00	

*Sample analysis was performed in triplicate.

for adipose). Capped tubes were incubated at 37°C. After 60 min the reaction was stopped by the addition of 200 µL ice-cold 100% ethanol containing 30 mM diamide [azodicarboxylic acid bis(dimethylamide)]. The tubes were then placed at −20°C overnight. The samples were then centrifuged at 10 000 rpm for 10 min at 4°C and the supernatant carefully removed and stored at −20°C until HPLC analysis. As shown by Minetti et al. [35], this reaction (as well as the MsrB reaction, see below) is linear with respect to time and protein concentration. With our modifications incorporated, we verified the linearity of the reactions over time and protein concentration (unpublished data).

Determination of MsrB activity

MsrB activity was determined as outlined above except 0.25 mM dabsyl Met-*R*-(O) was used as the substrate and the reaction was incubated for 15 min at 37°C. For liver samples, 0.01 mL supernatant was used and for adipose samples, 0.02 mL supernatant was used.

HPLC

HPLC determination of dabsyl Met was done by the method of Minetti et al. [35] and Moskovitz et al. [36] with minor modifications. Briefly, a 4.6 × 150 mm column (YMC ODS-AQ 5 µm; Waters Corp., Milford, MA) was equilibrated at 50°C with 0.14 M sodium acetate containing 0.5 mL triethylamine/L, pH 6.1. The buffer (buffer A) was made by combining 0.14 M acetic acid (containing 0.5 mL triethylamine/L) and 0.14 M sodium acetate (containing 0.5 mL triethylamine/L) to pH 6.1. Buffer B was made by combining 700 mL buffer A and 300 mL acetonitrile. Buffer C was made by combining 300 mL buffer A and 700 mL acetonitrile. By using a linear gradient with buffer B (start) and buffer C (end), dabsyl Met-*R*/*S*-(O) was eluted at ~7.9 min and dabsyl Met at ~10.1 min as monitored at 436 nm. The column was then re-equilibrated with buffer C prior to the next injection.

Preparation of L-methionine-S-sulphoxide and L-methionine-R-sulphoxide

The two diastereomers of L-methionine sulphoxide were prepared according to the method described by Lavine [38] as modified by Uthus [39].

Immunoblot analysis of MsrA and MsrB1

Samples were homogenized by sonication in ice-cold buffer consisting of sodium phosphate (20 mM, pH 7.4) containing diethylenetriaminepentaacetic acid (DEPA, 1 mM DTPA) to chelate redox active metals, 2,6-di-tert-butyl-4-methy phenol (0.05 mM; BHT) to inhibit lipid peroxidation, 0.5% Triton X-100 and dithiothreitol (1 mM, DTT). For liver, buffer was added at 10:1 (volume:weight) while adipose was 4:1 owing to the lower protein content in adipose. Protein content was determined by Bio-Rad Protein Dye Kit using BSA as the standard.

Samples were electrophoresed by SDS-PAGE under denaturing conditions using 4–20% gradient polyacrylamide gels (Invitrogen, Carlsbad, CA). Proteins were transferred to PVDF membrane. After blocking the membrane with tris-buffered saline (TBS) containing 5% non-fat dry milk (TBSM) for 2 h at room temperature or overnight at 4°C, the blots were incubated with primary antibody overnight at 4°C with shaking. Rabbit anti-MsrA antibody (1:500; Millipore, Billerica, MA) and MsrB1 (1:2000; Biovendor, Candler, NC) were diluted in TBS with 0.2% Tween-20 (TBST) with 5% non-fat dry milk (TBSTM).

Secondary antibody, goat anti-rabbit-horseradish peroxidase (Promega, Madison, WI) was diluted 1:4000 in TBSTM. Incubation was either 2 h at RT or overnight at 4°C. Following washing, the membrane was incubated in enhance chemiluminescent horseradish peroxidase substrate (ECL; Pierce, Rockford, IL). Digital images were acquired using a UVP BioSpectrum system (UVP, Upland, CA). Following stripping and re-blocking, membranes were probed with mouse anti-β-actin (1:5000; Cell Signaling Technologies, Danvers, MA) followed by

goat-anti-mouse horseradish peroxidase (1:5000; Promega, Madison, WI).

Immunoblot analysis of METO-modified proteins

Tissue content of methionine sulphoxide protein was determined by immunoblot using anti-METO-modified protein antibodies (Cayman Chemical; Ann Arbor, MI). A control METO-modified protein was provided. Specificity of the antibody was determined through competition of the signal by blocking the antibody with purified *L*-methionine sulphoxide that contained equal amounts of the *R* and *S* sulphoxide epimers. Samples were homogenized by sonication in buffer (see above) containing DTT (100 mM). Buffer was bath-sonicated and sparged with argon to reduce oxygen content prior to use.

Statistical analyses

Data are presented as the mean ± standard deviation. Data were compared between groups using a one-way ANOVA with Bonferroni post tests (GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego, CA) with significance defined as $p < 0.05$.

Results

Recent genetic studies indicate that the *MSRA* gene locus is involved with the development of central adiposity. In order to examine the relationship of obesity with MsrA/B activity, we examined two commonly-used rat models of obesity, the Zucker rat (lacking functional leptin receptors) and a high-fat (45% of calories) fed wild-type rat and compared them to wild-type rats fed a 10% fat-calorie diet as the control. As shown in Figure 1, there was no difference in total body mass between wild type 10% fat-fed or 45%-fat fed animals, an observation perhaps owing to the fact that the diets were isocaloric. On the other hand, the Zucker rats were significantly heavier than both other groups. The 45% fat-fed WT rats and Zucker rats had higher peri-renal fat content per kg body mass (1B) and higher hepatic triglyceride levels (1C) than control, WT rats, characteristics of elevated adiposity and hepatic steatosis. Zucker rats had a greater liver mass

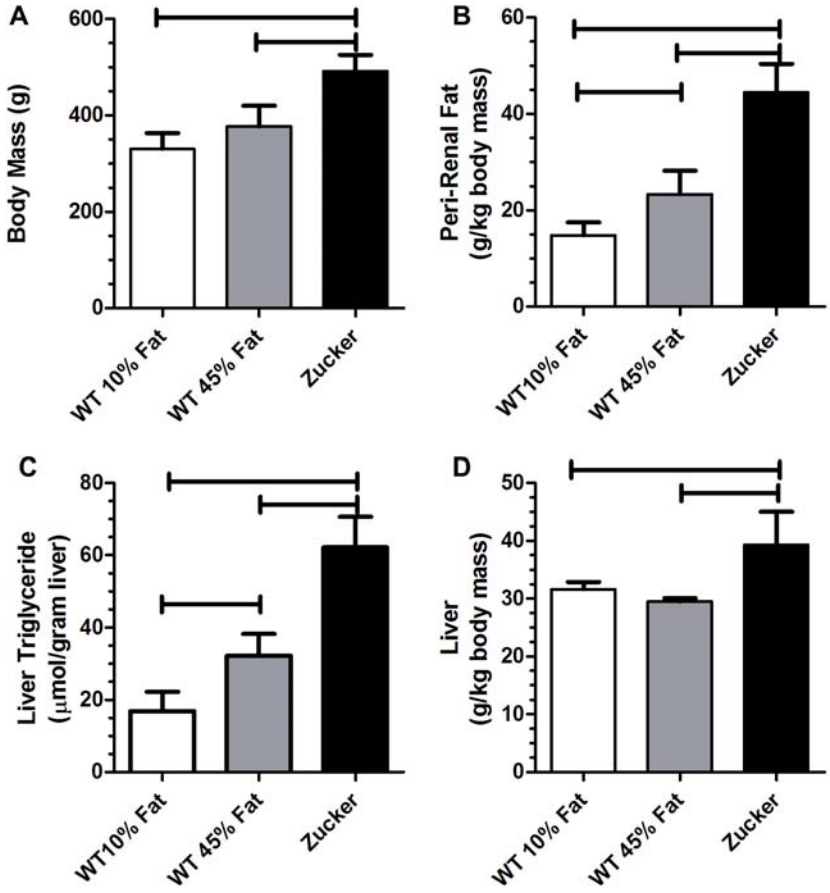

Figure 1. Elevated hepatic steatosis and fat deposition in rat models of obesity. Adult, male wild type (WT) were fed a 10% fat calorie or a 45% fat calorie diet for 6 weeks. Adult, male Zucker rats were fed a 10% fat calorie diet for 6 weeks. Zucker rats had a greater body mass (A) than the WT 10% and WT 45% groups. Peri-renal adipose content (B) and hepatic triglyceride content (C) were elevated in both models of obesity. Hepatic mass was elevated only in the Zucker group (D). Data are the mean ± SD ($n = 6$). Bars connecting the columns indicate significant differences ($p < 0.05$) between those groups. Data were compared using a one-way ANOVA with Bonferroni post-test.

than the other groups (1D). Peri-renal fat was utilized as our target visceral adipose tissue.

We analyzed Msr activities in adipose using the dabsyl derivatives of the *S*-METO epimer as a substrate for MsrA activity and the dabsyl derivative of the *R*-METO epimer as the substrate for MsrB activity (Figure 2). MsrA activity was significantly lower in the visceral adipose tissue in the high-fat fed (28% decrease) and the Zucker rat (25% decrease) vs the control (Figure 2A). MsrB activity was significantly lower (23%) in the visceral adipose of the Zucker rat vs the control (Figure 2B). Subcutaneous adipose showed disparate results in that there was a 45% increase in MsrA activity in the Zucker rats over controls with no difference in MsrA activity in the high-fat fed animals vs controls (Figure 2C). There were no difference in

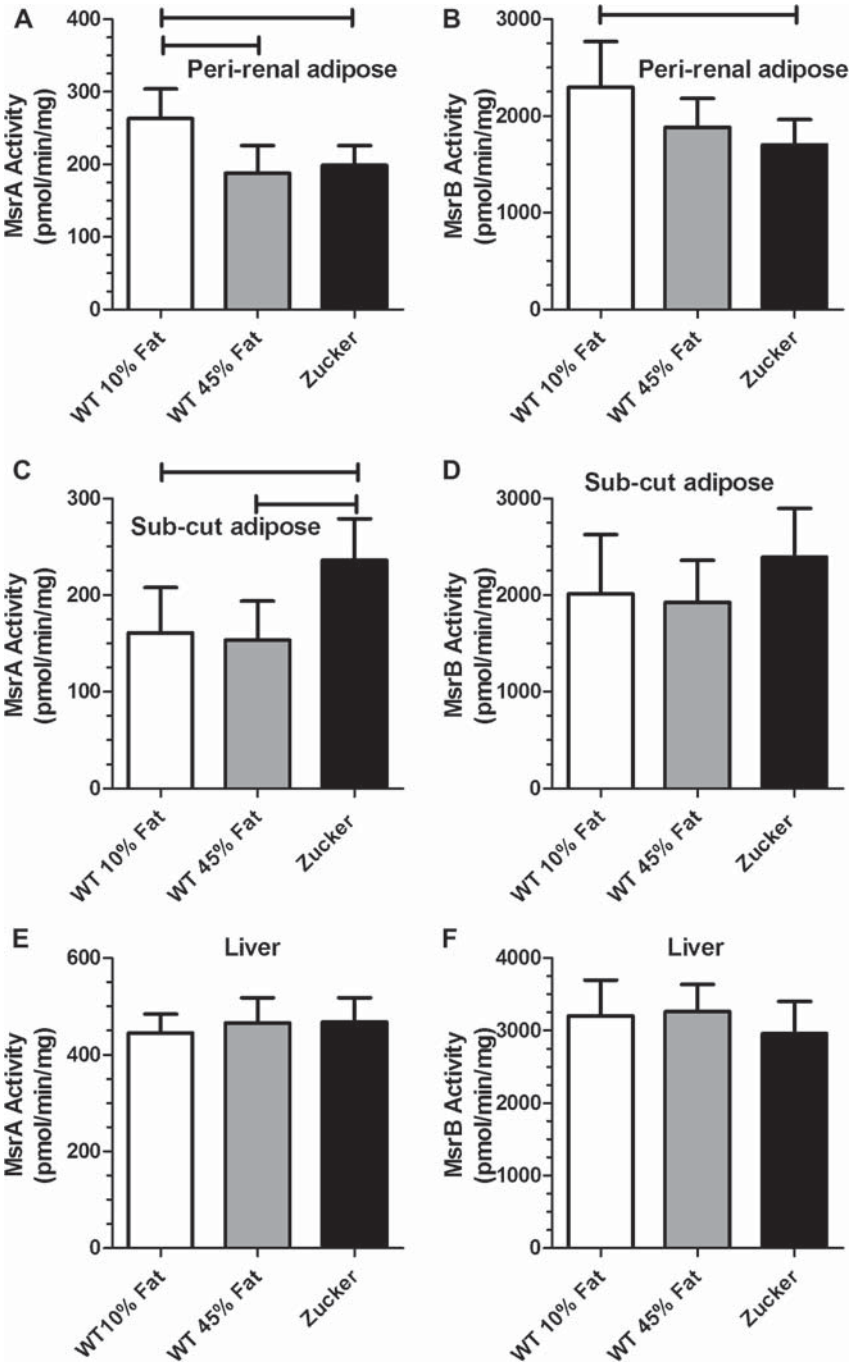

Figure 2. Methionine sulphoxide reductase activity is selectively reduced in visceral (peri-renal) adipose tissue. Adult, male wild type (WT) were fed on a 10% fat calorie or a 45% fat calorie diet for 6 weeks. Adult, male Zucker rats were fed a 10% fat calorie diet for 6 weeks. (A, B) MsrA and MsrB activities were reduced in peri-renal adipose as a result of obesity. (C, D) MsrA but not MsrB activity was elevated in the subcutaneous adipose of Zucker rats whereas no change in Msr activity was observed in liver (E, F). Data are the mean ± SD ($n = 6$). Bars connecting the columns indicate significant differences ($p < 0.05$) between those groups. Data were compared using a one-way ANOVA with Bonferroni post test.

MsrB activities in subcutaneous adipose between the groups (Figure 2D). There were no changes in MsrA or MsrB activities in liver between the obese and control groups (Figures 2E and F). Comparison of total tissue levels of MsrA and MsrB activities within the different tissues of the control group demonstrated significant differences in MsrA activity in the order of liver > visceral adipose > subcutaneous adipose and MsrB activity of liver > visceral adipose = subcutaneous adipose.

We determined the extent to which the reduction in Msr activities was the result of decreases in MsrA/B content in the tissue by immunoblot analysis with anti-MsrA and anti-MsrB1 antibodies using β-actin as a normalizing protein. For MsrA analysis, an immunoreactive protein band of the correct size (~26 kDa) was present (Figure 3). In some cases, a protein band of ~50 kDa was also observed that likely is the dimeric form of MsrA. Both forms were quantified. In contrast to our data showing a decrease in activity, our immunoblot data indicate no change in MsrA protein content, suggesting that there is a decrease in the specific activity of the enzyme in peri-renal adipose. MsrB1 protein content was also evaluated by immunoblot (Figure 4). We chose to examine MsrB1 as this enzyme has the highest specific activity of the MsrB enzymes owing to the selenocysteine active site [24]. There was no difference in MsrB1 levels between groups except a decrease in MsrB1 content was observed in the peri-renal adipose tissue of the Zucker rat vs the wild-type control.

Since decreases in MsrA and MsrB activities were observed, our next hypothesis was that obesity increases METO protein content. Utilizing a commercially available antibody against METO modified proteins, we observed a major anti-METO immunoreactive protein of ~50 kDa in liver via immunoblot analysis along with using a METO-positive control protein (data not shown). However, the specificity of the antibody towards METO-modified proteins in liver was questionable. While the control protein signal could be ablated by competition of the antibody with purified METO (200 mM), the METO-protein from liver could not be competed away. Given these results, analysis of METO-proteins was not pursued further.

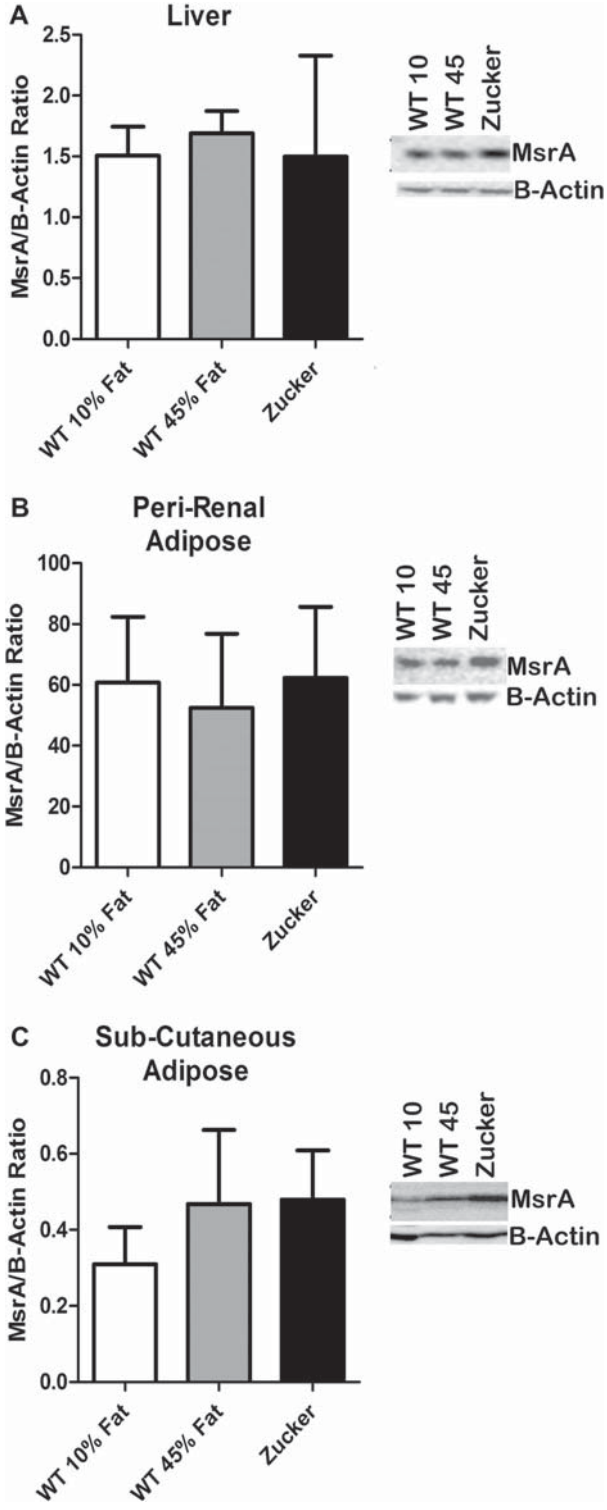

Figure 3. MsrA protein content is not altered by obesity. Adult, male wild type (WT) were fed on a 10% fat calorie or a 45% fat calorie diet for 6 weeks. Adult, male Zucker rats were fed a 10% fat calorie diet for 6 weeks. Liver (A) and adipose depots (B, C) were analysed for MsrA content by immunoblot and the data normalized to β-actin signal. Representative blots are shown. Signal was quantified and shown in corresponding bar graphs; 100 μg, 20 μg and 17 μg of protein were loaded per lane for liver, peri-renal adipose and subcutaneous adipose, respectively. No differences were observed within tissue types. Data are the mean ± SD ($n = 6$). Data were compared using a one-way ANOVA with Bonferroni post-test.

Discussion

Recent data indicate that the *MSRA* gene locus is positively correlated with visceral obesity. This observation is important since methionine sulphoxide reductases are one of only a few classes of enzymes that are able to reverse oxidative modifications of a protein. Methionine oxidation is a potentially reversible process that allows for regulation of enzymatic activity and protein–protein interactions. Thus, in the context of methionine sulphoxidation, oxidative modification of proteins can be viewed as a cell-signalling event. Recent studies indicate that oxidative stress is a causal factor in

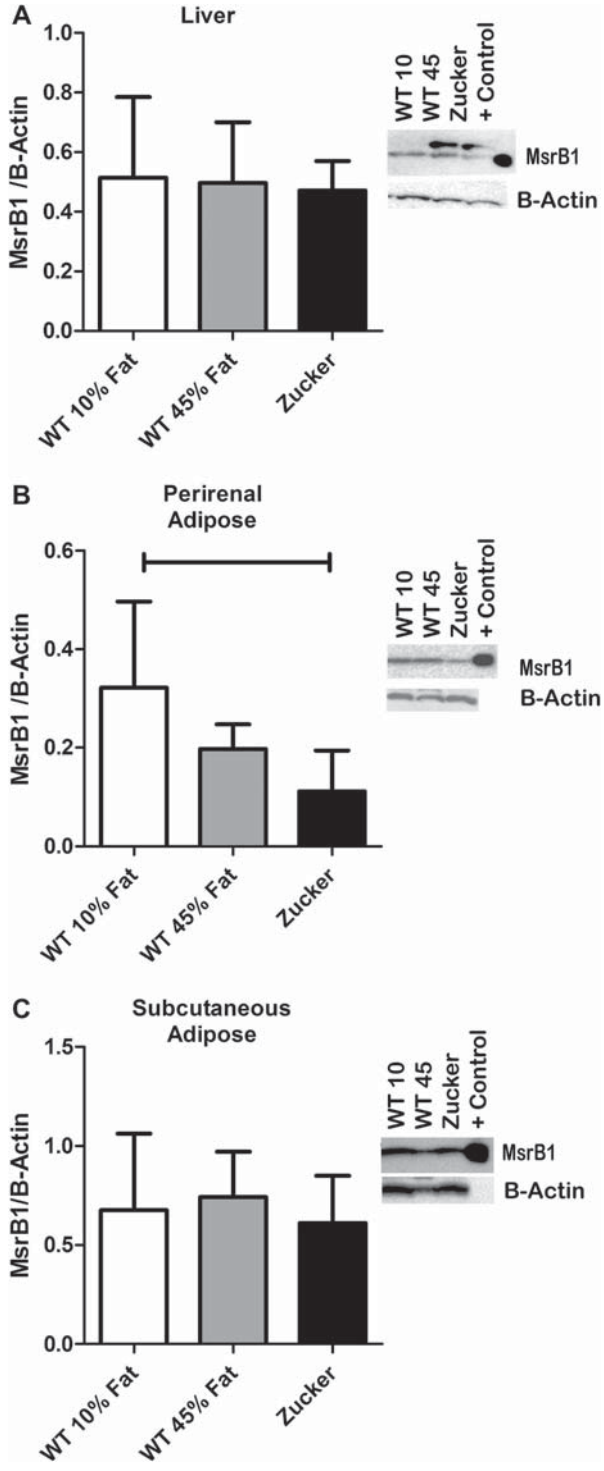

Figure 4. MsrB1 protein is reduced in peri-renal adipose of Zucker rats. Adult, male wild type (WT) were fed on a 10% fat calorie or a 45% fat calorie diet for 6 weeks. Adult, male Zucker rats were fed a 10% fat calorie diet for 6 weeks. Liver (A) and adipose depots (B, C) were analysed for MsrB1 content by immunoblot and the data normalized to β-actin signal. Representative blots are shown. Signal was quantified and shown in corresponding bar graphs; 50 μg, 10 μg and 15 μg of protein were loaded per lane for liver, peri-renal adipose and subcutaneous adipose, respectively. Data are the mean ± SD ($n = 6$). Bars connecting the columns indicate significant differences ($p < 0.05$) between those groups. Data were compared using a one-way ANOVA with Bonferroni post-test.

the development of obesity as well as a downstream effector. For these reasons, we tested the hypotheses that obesity decreases Msr activity.

Surprisingly, little or no data exist regarding the Msr activity or Msr proteins in adipose. Our data demonstrate that the development of obesity yields a decrease in MsrA activity in visceral adipose in both obesity models and a decrease in MsrB in the visceral adipose of the Zucker rat. Thus, our data confirm that there is a link between visceral adiposity and MsrA. The extent to which changes in body fat composition are temporally related to alterations in Msr is not known. However, our data indicate that moderate changes may be sufficient since the decrease in Msr activities were comparable between the Zucker rat and the 45% fat-fed wild type rat. Since obesity can exist with insulin resistance and frank diabetes, more detailed studies are needed to define the extent to which these two physiologic changes affect Msr and other oxidant defense enzymes.

Our data point to a decrease in the specific activity of MsrA in peri-renal adipose tissue of obese animals. These results are similar to those of Petropoulos et al. [21] in which a significant decrease in MsrA activity was found without a decrease in MsrA protein content in aged rats. The active site of MsrA contains a cysteine residue critical for activity. Thus, it is possible that this cysteine undergoes oxidation in peri-renal adipose. Since our activity assays are performed with reductant present, the reduction in specific activity in MsrA may be irreversible. The reduction in total MsrB activity in the peri-renal adipose of Zucker rats may also be in part owing to decreases in MsrB1 protein content. Our work did not address other MsrB enzymes. However, MsrB1 does contain an active site selenocysteine, in contrast to MsrA, that is also highly susceptible to oxidative modification.

Our findings indicate that obesity causes adipose-specific changes in Msr activity. We noted an increase (46%) in MsrA in the subcutaneous adipose of the Zucker animals. Our immunoblot analysis did not indicate a significant elevation of MsrA protein in the subcutaneous adipose of the Zucker animals vs the wild-type. These data are in apparent contrast to the data obtained from the peri-renal adipose. The mechanisms underlying this finding are unclear. One possibility is that there exist in the subcutaneous adipose, unlike peri-renal adipose, greater pools of inactivated MsrA protein in the wild-type groups (10% fat and 45% fat) than in the Zucker. Further proteomic analysis of Msr proteins in adipose tissue is needed.

Our findings support the observations that subcutaneous and visceral adipose are physiologically and biochemically distinct and can have different levels of resident cell types in the obese state [40–44]. Little data exist, however, as to the differences in oxidative stress defense enzymes in subcutaneous adipose and visceral adipose. Furthermore, the changes in Msr activity must be considered in the context of the anatomy

and cell biology adipose tissue itself. Adipose tissue is a combination of cell types that include stromal vascular cells, adipocytes, mesenchymal stem cells, and in the obese state, elevated levels of macrophages [40,45,46]. Our studies did not determine the cell-type source of the Msr activity. Furthermore, Msr's have distinct subcellular localization that our studies were not able to address, and we are cognizant that MsrB activity likely is the contribution of at least three separate isoforms. Subsequent work is needed to determine the adipose cell types that undergo changes in oxidative damage defense enzymes, those cells that have changes in oxidative damage endpoints, e.g. potentially elevated METO-proteins, and the identity of the METO-proteins.

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Declaration of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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