Cysteine Supplementation Reverses Methionine Restriction Effects on Rat Adiposity: Significance of Stearoyl-Coenzyme A Desaturase

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Running title: Cysteine supplementation, SCD1 and adiposity

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ABSTRACT

Stearoyl-coenzyme A desaturase-1 (SCD1) is a key enzyme in fatty acid and energy metabolism, but little is known about its nutritional regulation. Dietary methionine restriction in rats decreases hepatic Scd1 mRNA and protein, increases energy expenditure, and decreases fat-pad mass/body-weight% (FM/BW%). In humans, plasma concentrations of the methionine product, cysteine, are associated with obesity. To determine which consequences of methionine-restriction are mediated by decreased cysteine availability, we monitored obesity-related variables in 4 dietary groups for 12 weeks: control-fed (CF), methionine-restricted (MR), MR supplemented with 0.5% L-cysteine (MR+Cys) and CF+Cys rats. MR lowered weight gain and FM/BW% despite higher food intake/weight than CF, and lowered serum cysteine. Hepatic Scd1 expression was decreased, with decreased serum SCD1 activity indices (calculated from serum fatty acid profile), decreased serum insulin, leptin and triglycerides, and higher adiponectin. Cysteine supplementation (MR+Cys) essentially reversed all these phenotypes, and raised serum cysteine but not methionine to CF levels. Adding extra cysteine to control diet (CF+Cys) increased serum taurine but did not affect serum cysteine, lipids, proteins or total weight gain. FM/BW% and serum leptin were modestly decreased. Our results indicate that anti-obesity effects of MR are caused by low cysteine, and that dietary sulfur amino acid composition contributes to SCD1 regulation.

SUPPLEMENTARY KEYWORDS

Sulfur amino acids; diet; fat mass; food intake; weight gain; insulin; leptin; adiponectin; fatty acid profile; desaturase indices; taurine
BACKGROUND

Accumulating evidence suggests that the type and quantity of dietary proteins affects body weight. In humans, high protein diets and leucine supplementation promote weight loss, improve body composition and decrease insulin when used in weight loss programs (1). These effects may be related to enhanced satiety and increased energy expenditure (2). On the other hand, high habitual protein intake is epidemiologically associated with greater BMI (3) and body fat (3, 4). Rats fed high protein diets develop insulin resistance (5), and type 1 diabetic patients on a high protein diet have reduced insulin sensitivity compared to those with lower protein intake (6). Specifically, higher intake of animal-derived protein is associated in large cross-sectional studies with greater BMI and higher prevalence of obesity (7-9). Prospective studies show that subjects with higher animal protein consumption gain more weight over time (10) and are more likely to develop diabetes (11, 12). The possibility that specific amino acid constituents mediate metabolic effects of different protein types is likely, but has not been clearly documented.

Methionine, an essential sulfur-containing amino acid mainly ingested in animal-derived foods (13), is a potential mediator of adverse effects of excess animal protein intake (14), and, at high intake levels, is associated with increased BMI and cardiovascular risk (15). Methionine is the precursor of homocysteine, a non-proteinogenic amino acid that can in turn be converted to cysteine via the intermediate, cystathionine (16). Although cysteine is the precursor of two sulfur compounds with antioxidant properties, taurine and glutathione (16), plasma total cysteine (tCys) levels are positively associated with body fat mass (17), obesity (18), hypercholesterolemia (19) and metabolic syndrome (20) in humans.
Methionine restriction (MR) is a recognized intervention which reduces fat mass and enhances insulin sensitivity in rats via a host of changes in enzymes, adipokines and transcription factors involved in lipid and energy metabolism (21-23). In particular, adiponectin, which is a specific inverse predictor of diabetes incidence in humans (24), is markedly elevated by MR (23). Moreover, MR rodents exhibit a hypermetabolic phenotype (23, 25, 26), linked to profound suppression of hepatic stearoyl CoA desaturase-1 (SCD1) (21, 25), a δ-9 fatty acid desaturase that has been identified as a key regulator of lipid and energy metabolism (27). However, it is not known whether these effects are mediated via decrease in methionine itself, or one of its downstream amino acid products which are profoundly altered by MR (28).

Serum tCys and methionine levels are both lower in MR rats (28). Since epidemiologic studies link plasma concentrations of tCys rather than methionine with hypercholesterolemia and obesity (17-19), we hypothesized that lower cysteine synthesis in MR rats is responsible for their decreased adiposity. To identify the role of lowered cysteine levels in the phenotype of MR rats we examined the anthropometric and metabolic consequences of supplementing MR rats with cysteine for 12 weeks. We also investigated whether excess cysteine intake on top of a control diet with adequate methionine content would promote obesity.
METHODS

Animal husbandry, diets and sampling

The study was approved by Orentreich Foundation for Advancement of Science, Inc’s. Institutional Animal Care and Use Committee and performed in accordance with the National Institutes of Health guidelines for use of animals in research laboratories.

Four-week-old male Fischer-344 rats from Taconic Farms (Germantown, NY) were maintained one rat per cage in a controlled 12 h light/dark cycle with free access to water and a standard diet (LabDiet 5001; PMI Nutrition International, LLC, Brentwood, MO) for 2 weeks. The rats were then randomly assigned to one of 4 dietary groups consuming chemically defined AIN-76-based diets with protein replaced by different amino acid mixtures (Dyets Inc.; Bethlehem, PA) for 12 weeks: 1) Control-fed (CF, 0.86% methionine); 2) methionine restricted (MR, 0.17% methionine); 3) CF supplemented with 0.5% L-cysteine (CF+Cys); and 4) MR supplemented with 0.5% L-cysteine (MR+Cys). Certificates of analysis indicated that the CF+Cys and MR+Cys diets contained 0.453% and 0.476% (by weight) cysteine, respectively. The unsupplemented control and MR diets were devoid of cysteine and other sulfur amino acids apart from methionine as described previously (29). To compensate for the reduced amino acid content in the MR diet, glutamic acid content was raised on an equal weight basis. Food and water were provided ad libitum.

Body weight was registered at baseline and weekly throughout the experiment. Food intake was measured weekly by weighing residual food at the end of a 48 hour period, and the average intake over 24 hours was calculated. At baseline and after 6 weeks and 12 weeks on the dietary regimen, non-fasting blood samples were collected from the subclavian vein after anaesthetizing the rats using an E-Z Systems apparatus (Palmar, PA).
After completing the dietary regimens for 12 weeks, the rats were euthanized by Euthanex E-Z Systems. White adipose tissue fat pads (inguinal, epididymal, peri-intestinal, retroperitoneal), interscapular brown fat (obtained by dissecting away the white superficial fat and harvesting the deeper brown fat between the shoulder blades), liver, and skeletal (quadriceps) muscle were immediately harvested, weighed, frozen in liquid nitrogen, and stored at −80°C.

**Serum biochemistry**

Liquid chromatography tandem mass spectrometry was used to analyze total homocysteine (tHcy), methionine, cystathionine, tCys and total glutathione (tGSH) concentrations (30). Taurine and glutamate were measured by a modification of a previously described liquid chromatography tandem mass spectrometry method (28).

Serum concentrations of glucose, lipids, albumin and protein were measured on a Beckman Coulter AU400 semi-automated clinical chemistry analyzer (31). Commercially available rat ELISA kits were used to measure leptin (Assay Design, Ann Arbor Michigan), IGF-1 (IDS, Fountan Hills, AZ), insulin (Alpco, Salem, NH) and adiponectin (Millipore, Billerica, MA).

Fatty acid profiles in serum total lipids were assayed by gas liquid chromatography with flame ionization detection at AS Vitas, Oslo Innovation Center, Oslo, Norway (www.vitas.no). The method has been validated for quantification of polyunsaturated fatty acids according to US FDA Guidance Document on Bioanalytical Method Validation. In a separate pre-validation study, efficacy of the direct transmethylation step has been found to be 96-104 % for the lipid classes cholesterol esters, triglycerides, and phospholipids. Fatty acid content was calculated based on the area % of peaks and response factors relative to stearic acid (C18:0).
Estimated indices of desaturase and elongase enzyme activities

Desaturase and elongase activity indices were estimated as product/precursor ratios of fatty acids in serum (32). SCD1-16 activity was estimated as 16:1n-7/16:0, SCD1-18 activity as 18:1n-9/18:0, Δ⁶-desaturase (D6D) activity as 18:3n-6/18:2n-6; Δ⁵-desaturase (D5D) activity as 20:4n-6/20:3n-6; and elongase activity as 18:0/16:0 (32).

Gene expression profiling

Total RNA was isolated from inguinal white adipose tissue, liver and quadriceps muscle tissue using Qiagen’s RNeasy kits. RNA concentrations were determined spectrophotometrically and their integrity was assessed by agarose gel electrophoresis. Messenger RNA (mRNA) was transcribed into cDNA using a High-Capacity cDNA Reverse Transcription Kit in a Perkin-Elmer (Wellesley, MA) GeneAmp PCR System 9600 as previously described (21). Multiplexed PCR was conducted in a StepOnePlus Real-Time PCR System with the prepared cDNA and commercially available TaqMan primer-probe sets (Life Technologies, Carlsbad CA). Gene expression was assessed by the comparative CT (ΔΔCT) method with β-actin as the reference gene. β-actin CT values were similar in tissues from each group, and efficiencies for β-actin and the genes of interest in the multiplexed reactions were greater than 90% and comparable to each other.

Statistical methods

Data is presented as mean (±SEM). Groups were compared by one way ANOVA followed, where relevant (P ANOVA <0.05), by pairwise comparisons: MR vs CF, MR+Cys vs CF, CF+Cys vs CF, and MR vs MR+Cys. All tests were 2-tailed and P-values <0.05 were considered significant. Analyses were performed using the Statistical Package for Social Sciences 12.0 for Windows (SPSS, Chicago. IL).
RESULTS

Weight gain, body composition and food intake

MR markedly lowered weight gain, as previously reported (21, 22, 28), despite food intake/body weight being consistently higher than CF group throughout the study (Figure 1A, B). Supplementing the MR diet with cysteine (MR+Cys) completely rescued weight gain despite decreasing food intake/body weight to CF levels, but no increase in weight gain above CF levels was observed in the CF+Cys group. Food intake/body weight in the CF+Cys group tended to be lower than CF throughout the study, the difference being significant at weeks 3 and 7 (Figure 1A, B).

Fat pad mass expressed as a percent of body weight was reduced in all depots from MR rats, except for brown adipose tissue mass which was larger than in CF rats (Figure 1C). Compared to the MR group, MR+Cys rats featured increased white adipose tissue fat pad mass % to, or towards, control levels, and reduced brown fat mass. In contrast, cysteine supplementation of the control diet (CF+Cys) promoted a modest but significant reduction of white adipose tissue mass in all fat depots with no effect on brown fat mass (Figure 1C).

Overall, supplementing the MR diet with cysteine restored weight gain and adiposity to control levels, whereas adding cysteine to the control diet tended to decrease food intake and modestly decreased adiposity with no effect on total weight gain.

Serum glucose, lipid and protein parameters

Table 1 shows serum metabolites at baseline and after 12 weeks on the diets. MR had mixed favorable and unfavorable effects on serum lipids. Triglycerides and total cholesterol were lowered compared to control, confirming previous reports (21, 22). However, LDL-C was approximately 25% higher, and HDL-C was approximately 60% lower in MR compared to CF.
The addition of cysteine to the MR diet (MR+Cys) prevented the triglyceride-lowering and LDL-elevation induced by MR. LDL-C was reduced in MR+Cys to levels below those observed in MR and CF rats.

Total serum proteins and serum albumin were decreased in MR rats, and were restored by cysteine supplementation (MR+Cys) to control levels. No difference in any of the serum lipid or protein parameters was observed between the CF and CF+Cys groups.

In summary, supplementation of the MR diet with cysteine prevented most of the MR effects on serum lipids and protein markers, whereas addition of cysteine to the control diet had no effect on these parameters.

**Serum fatty acid profile**

The fatty acid profile in total serum lipids was determined after 12 weeks of dietary intervention. Marked differences were noted between the MR and CF groups, which were essentially blunted by cysteine (MR+Cys) (*Table 2*). MR lowered the levels of monounsaturated fatty acids palmitoleic (16:1,n-7) and oleic acids (18:1,n-9), and raised stearic acid (18:0), with consequent decreases in estimated indices of SCD1-16 and SCD1-18 desaturase activities (*Figure 2*). These effects were reversed by cysteine supplementation of the MR diet (MR+Cys). Furthermore, cysteine supplementation of the MR diet markedly lowered estimated indices of D5D, D6D and elongase activities (*Figure 2*).

MR also raised the omega 6 (n-6) polyunsaturated fatty acids gamma-linoleic acid (18:3, n-6) and docosadienoic acid (22:2, n-6), and reduced eicosadienoic acid (20:2, n-6). These effects were also reversed by cysteine. In contrast, cysteine supplementation of the control diet produced no changes in the fatty acid profile (*Table 2*).
Serum hormones and adipokines

Serum hormones and adipokines were measured at baseline and after 6 and 12 weeks of dietary intervention (Figure 3). As previously observed (21, 23), MR significantly decreased serum concentrations of insulin, leptin, IGF-1 and raised adiponectin compared to CF. All these effects were reversed by cysteine (MR+Cys) at 12 weeks. Compared to the CF group, CF+Cys rats had lower serum leptin at 6 and 12 weeks (Figure 3), consistent with the marginal reduction in fat pad mass (Figure 1). Paradoxically, the CF+Cys group also had lower adiponectin levels compared to CF rats.

Thus cysteine reversed the MR effects on serum insulin, IGF-1, leptin and adiponectin, while adding cysteine to the control diet lowered serum leptin.

Serum sulfur amino acids and glutamate

Serum sulfur amino acids and glutamate were measured at baseline and at 6 and 12 weeks on the diets. As previously reported (28) and shown in Figure 4, MR markedly decreased serum methionine, cystathionine, tCys, and taurine compared to CF, and elevated tHcy. Serum glutamate was not different in MR compared to CF rats. No difference in serum tGSH was observed among the dietary groups at any time-point (p ANOVA at 12 weeks= 0.64; data not shown).

Supplementation of the MR diet with cysteine (MR+Cys) restored plasma tCys and cystathionine to control levels, but only partly raised taurine levels and partly lowered tHcy. After 12 weeks of intervention, serum tHcy in MR+Cys group (25.5±2.2 μmol/L) was lower (p<0.001) compared to MR (37.7±3.4 μmol/L), but remained higher (p=0.016) than CF (18.2±0.6 μmol/L). Serum methionine was not raised by adding cysteine to the MR diet (Figure 4).
No consistent difference in sulfur amino acid levels were observed between the CF+Cys and CF groups, except for an approximate 25% and 15% increase of serum taurine at 6 weeks and 12 weeks respectively (p≤0.005 at both time points; Figure 4). Notably, serum tCys was not higher in CF+Cys vs CF after 6 or 12 weeks on the diets (Figure 4).

In summary, supplementing the MR diet with cysteine restored serum tCys, cystathionine, tHcy and taurine to or towards control levels, with no effect on methionine. Adding cysteine to a control diet failed to increase serum tCys but raised serum taurine.

**Gene expression profile**

We examined changes in expression of some genes known to be responsive to MR (Figure 5) (21). Consistent with previous reports, MR enhanced hepatic expression of peroxisome proliferator activated receptor g (Pparg), and markedly suppressed Scd1. Cysteine (MR+Cys) reversed or partly reversed these effects. However, cysteine supplementation of the control diet (CF+Cys) had no effect on either gene.

In contrast to hepatic changes, Scd1 expression was greatly induced in white adipose tissue from MR rats, as previously observed (21). Cysteine (MR+Cys) lowered adipose tissue Scd1 expression to control levels, and further suppressed adipose tissue Scd1 expression below control values when added to the control diet (CF+Cys). Supplementing the control diet with cysteine (CF+Cys) also decreased the expression of cytochrome c oxidase subunit IV (Cox4i1), Pparg and fatty acid binding protein 4 (Fabp4) in adipose tissue.

In skeletal muscle, cysteine reversed a 2-fold up-regulation of uncoupling protein 3 (Ucp3) induced by MR, and also modestly decreased expression of carnitine palmitoyl transferase 1b (Cpt1b) (CF+Cys vs CF and MR+Cys vs MR) and Cox4i (MR+Cys vs MR).
DISCUSSION

To study the potential link between anti-obesity effects of MR in rats (21, 23, 28), and the epidemiologic association of plasma tCys with obesity (17, 18), we investigated whether cysteine supplementation of either MR or control diets would increase fat accumulation in rats. Cysteine supplementation had different effects depending on the underlying diet. Our results indicate that reduced cysteine supply secondary to MR is the major factor underlying the anti-obesity effects of MR, as these effects were essentially reversed by cysteine. However, cysteine supplementation of a control diet did not promote further fat mass gain, and in fact, tended to decrease adiposity.

Cysteine supplementation of MR diet: MR+Cys versus MR rats

Low-methionine dietary rodent models feature lower weight gain and fat mass%, higher energy expenditure and insulin sensitivity, concomitant with decreased adipocyte size and profound inhibition of hepatic Scd1 expression (21-23, 25, 26). This phenotype is independent of energy intake as demonstrated by pair-feeding (23), and by our observation that MR rats consumed more food per gram body weight than CF rats. Decreased BMI despite higher energy intake and adequate dietary protein was also observed in adults on a MR diet for treatment of cancer (33). It has also been suggested that vegetarianism is a “mild” form of MR (14, 34), which may partly explain the lower weight gain and diabetes incidence among vegetarians (7, 8, 10-12), in whom sulfur amino acid intake is typically ~40-70% of omnivores (35).

The present study extends previous findings of decreased hepatic Scd1 mRNA and protein levels in MR rodents (21, 25), by demonstrating decreased serum concentration of the SCD1 products palmitoleic and oleic acids, with concomitant reduction in estimated SCD1 activity indices in MR rats. The observed reduction in serum TG and MR rats is also consistent with
Scd1 suppression (36), as synthesis of monounsaturated fatty acids by SCD1 is a prerequisite for their incorporation into triglycerides (27). Cysteine supplementation blocked MR effects on Scd1 expression, SCD1 indices, weight gain, fat mass%, and serum triglycerides and leptin. Serum insulin was higher, and adiponectin markedly lower in MR+Cys compared to MR rats, suggesting that cysteine may prevent the enhanced insulin sensitivity of MR rats (23). Consistent with this, high plasma tCys was recently reported in insulin resistant subjects (37).

In mice, SCD1 activity index correlates with BMI and fat mass% (38). The reduced adiposity in MR rats and its reversal by cysteine paralleled the changes in Scd1 expression and function, suggesting that SCD1 changes mediate these phenotypes. A shift from lipogenesis towards β-oxidation via activating hepatic AMP-activated protein kinase underlies the hypermetabolism induced by hepatic SCD1 inhibition (27, 39). Further, cysteine reduced skeletal muscle CPT1b mRNA, which is translated into the rate-limiting enzyme in transport of long-chain fatty acid into mitochondria for β-oxidation (40). Cysteine also reversed the MR-induced up-regulation of muscle Ucp3. Mice over-expressing Ucp3 are hypermetabolic and lean, at least partly due to uncoupling of substrate oxidation from ATP production (41), although whether UCP3 has a physiological function in regulating energy expenditure is uncertain (42). Overall, cysteine induced transcriptional changes that favored fatty acid partitioning away from β-oxidation.

The possible contribution of the integrated stress response to the SCD1 and fat mass changes of MR rats deserves investigation. Integrated stress response, triggered by numerous factors including imbalance or deprivation of essential amino acids, involves phosphorylation of the α-subunit of the eukaryotic initiation factor 2 complex (eIF2α). Phosphorylated eIF2α induces subsequent changes in transcription and translation of genes involved in adaptations to dietary stress (43). It was recently shown that MR rats feature enhanced eIF2α phosphorylation in
conjunction with up-regulation of various components of the integrated stress response (44). The same pathway was found to mediate both SCD1 suppression and decreased abdominal fat mass in response to leucine deprivation (45). This raises the possibility that the changes in SCD1 and fat mass observed in MR rats, and their reversal by cysteine, may be mediated by elements of the integrated stress response.

One limitation of this study is that SCD1 indices were calculated from fatty acid profiles of total serum lipids, rather than of VLDL, which better reflects hepatic Scd1 expression (32). However, measuring fatty acid profile in specific lipid fractions is particularly indicated in human studies in which SCD1 indices are used to infer gene expression (32, 46). In the present study, Scdl mRNA was measured in liver and white adipose tissue and the serum fatty acid profile clearly coincided with hepatic gene expression. In contrast to liver, adipose tissue showed enhanced Scd1 expression in MR rats, an effect that was reversed by cysteine supplementation. Cysteine also markedly decreased the activity indices of elongase, D5D and D6D. Although these serum indices have been linked to BMI, serum lipids and risk of metabolic syndrome (47, 48), it has been shown that, unlike SCD1 index, they are of little value in predicting corresponding indices or enzyme gene expression in the liver (32).

Both serum methionine and tCys are decreased in MR rats (28). Cysteine blocked the effects of MR without increasing serum methionine. This suggests a relatively direct effect of cysteine rather than a methionine-sparing action of cysteine (49), and implicates lowered cysteine as the cause of reduced adiposity in MR. However, cysteine is the precursor of many biologically active compounds including taurine, GSH, and sulfate (16). A recent study showed decrease in weight gain and in hepatic lipogenesis in rats treated with an inhibitor of the rate-limiting enzyme in GSH synthesis, γ-glutamylcysteine synthetase (50). We observed no difference in
plasma tGSH between the groups, but MR rats feature hepatic GSH depletion (51) similar to that observed with γ-glutamylcysteine synthetase blockade (50). Thus changes in hepatic GSH synthesis may have contributed to the metabolic impacts of MR and cysteine supplementation.

**Cysteine supplementation of Control diet: CF+Cys vs CF rats**

Cysteine supplementation of the control diet did not induce obesity. Compared to the CF group, CF+Cys rats showed no difference in serum tCys, or in hepatic SCD1 expression or activity indices, and had slightly lower fat mass%. We postulate that this response may have resulted from the marginally decreased food intake in CF+Cys rats, as observed by others (52, 53). Kawayami et al supplemented the same dose of L-cysteine used in the present study (0.5%) to 4 diets of different protein quality and quantity, and found that cysteine reduced or tended to reduced food consumption (53). Further, cysteine enhanced weight gain only in groups where plasma tCys was increased above the unsupplemented group (53). Similarly, in the present study, supplementary cysteine enhanced SCD1 function and adiposity only in the model in which plasma tCys increased from subnormal (MR) to normal (MR+Cys) levels. Cysteine supplementation in CF+Cys rats neither increased tCys nor adiposity above CF levels, and thus failed to replicate the elevated plasma tCys that is linked to human obesity (17, 18). This may result from the role of the liver, demonstrated in rodents (54), in disposing of excess dietary cysteine via conversion to taurine, as shown by raised serum taurine in CF+Cys rats.

**Perspectives**

It is tempting to speculate that the lean phenotype associated with low serum tCys in MR rats (28) and the human obesity associated with elevated plasma tCys (17, 18) are related processes. However, the evidence for this remains limited, and different mechanisms may be involved. Our data from rats indicates that MR increases energy expenditure by triggering a
cellular response in which lipid synthesis and degradation pathways are simultaneously activated, initiating energy-consuming "futile cycles" (22). In contrast, mice fed a high cystine diet feature decreased energy expenditure (Elshorbagy et al, unpublished), but whether the molecular pathways mirror those of MR remains to be determined. Further, in the present study the effect of dietary cysteine on SCD1 function and fat mass accretion clearly showed a ceiling effect that stopped at control values for these parameters. This is at odds with human findings of a linear dose-response relation between tCys and body fat extending well into the obese range of BMI and fat mass (17, 18). The discrepancy could reflect a species difference in body weight response to excess dietary cysteine, or could alternatively indicate that high tCys in humans is not driven by excess cysteine intake.

Apart from the recognized induction of SCD1 in response to glucose oversupply to activate de novo lipogenesis (55), available data on nutritional regulation of SCD1 is limited. Our observation that SCD1 expression and activity indices are strongly responsive to dietary sulfur amino acid composition, with corresponding impact on adiposity and metabolic profile, is a key finding linking dietary protein quality with SCD1 regulation. Whether moderate inter-individual differences in sulfur amino acid intake, as observed between vegetarians and omnivores (35), might influence weight gain and metabolic risk (10, 12) through an effect on SCD1 function deserves investigation.
ACKNOWLEDGEMENTS

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AUTHOR CONTRIBUTIONS

Amany K. Elshorbagy: Concept, study design, statistical analysis and interpretation, preparation of the manuscript.

Maria Valdivia-Garcia: Development of liquid chromatography tandem mass spectrometry method for taurine and glutamate assay, conduction of all mass spectrometry assays.

Dwight A. L. Mattocks: Conduction of body and tissue data collection as well as gene expression analysis, review of the manuscript.

Jason D. Plummer: Primary contact person regarding formulation of the cysteine supplemented diets as well as conduction of body and tissue data collection.

A. David Smith: Concept, study design, critical revision of the manuscript.

Christian A. Drevon: Study design related to fatty acid metabolism, critical revision of the manuscript.

Helga Refsum: Concept, study design, critical revision of the manuscript.

Carmen E. Perrone: Study design, coordination of MR studies, conduction of MR experiment, tissue collections and serum hormone assays and critical revision of the manuscript.

ALL AUTHORS HAVE READ AND APPROVED THE FINAL VERSION OF THE MANUSCRIPT.
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FIGURE LEGENDS

Figure 1. Weight gain, food intake, and weight of adipose tissues and liver in CF (control-fed), CF+Cys (control-fed supplemented with cysteine), MR (methionine-restricted), and MR+Cys rats. Data represents mean ± SEM; N=8 per group. 

A: Weight gain after 12 weeks as a percent of initial body weight; B: Food intake/ 24 hours normalized to body weight; C: Fat pad and liver weights as a percent of body weight after 12 weeks. All parameters show significant differences by ANOVA (p<0.05). Pairwise comparisons are presented only for CF vs. each of the other groups, and for MR vs. MR+Cys.  

(*) = p<0.05 compared to CF  

(**)= p≤0.001 compared to CF  

(#) = MR+Cys significantly different from MR, p<0.05,  

(##)=MR+Cys significantly different from MR, p≤0.001.  

For pairwise comparisons in food intake see text.

Figure 2. Estimated indices of desaturase and elongase activities in CF (control-fed), CF+Cys (control-fed supplemented with cysteine), MR (methionine-restricted), and MR+Cys rats after 12 weeks. Data represents mean for 6 rats per group. All indices show significant differences by ANOVA (p<0.05).  

(*) = p<0.05 compared to CF  

(**)= p≤0.001 compared to CF  

(#) = MR+Cys significantly different from MR, p<0.05,  

(##)=MR+Cys significantly different from MR, p≤0.001.
Figure 3. Changes in serum hormones and adipokines over 12 weeks in CF (control-fed), CF+Cys (control-fed supplemented with cysteine), MR (methionine-restricted), and MR+Cys rats. Data represents mean ± SEM; N=8 per group. All adipokines studied showed significant differences by ANOVA (p<0.05). For relevant pair-wise comparisons, see text. One outlier value was excluded from the CF+Cys 6-week insulin data.

Figure 4. Changes in serum sulfur amino acids and glutamate over 12 weeks in CF (control-fed), CF+Cys (control-fed supplemented with cysteine), MR (methionine-restricted), and MR+Cys rats. Data represents mean ± SEM; N=8 per group. All amino acids illustrated show significant group differences by ANOVA (p<0.05). For pair-wise comparisons, see text.

Figure 5. Gene expression profile in CF (control-fed), CF+Cys (control-fed supplemented with cysteine), MR (methionine-restricted), and MR+Cys after 12 weeks of dietary intervention, in liver, inguinal white adipose tissue and quadriceps muscle. Data represents mean ± SEM; N=8 per group.

(*) = p<0.05 compared to CF

(**)= p≤0.001 compared to CF

(#) = MR+Cys significantly different from MR, p<0.05,

(##)=MR+Cys significantly different from MR, p≤0.001.
Table 1. Serum concentration of glucose, lipids and proteins in rats fed different diets

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<td>Baseline</td>
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<td>2.83 ± 0.23* ##</td>
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</tr>
<tr>
<td>Baseline</td>
<td>0.56 ± 0.03</td>
<td>0.56 ± 0.03</td>
<td>0.53 ± 0.03</td>
<td>0.52 ± 0.07</td>
</tr>
<tr>
<td>Final</td>
<td>0.61 ± 0.02</td>
<td>0.57 ± 0.02</td>
<td>0.67 ± 0.07</td>
<td>0.56 ± 0.02</td>
</tr>
<tr>
<td>Total-C, mM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>2.22 ± 0.02</td>
<td>2.13 ± 0.03</td>
<td>2.28 ± 0.05</td>
<td>2.26 ± 0.28</td>
</tr>
<tr>
<td>Final</td>
<td>2.87 ± 0.11</td>
<td>2.85 ± 0.05</td>
<td>1.82 ± 0.05**</td>
<td>1.72 ± 0.05**</td>
</tr>
<tr>
<td>LDL-C, mM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>0.29 ± 0.01</td>
<td>0.30 ± 0.01</td>
<td>0.33 ± 0.02</td>
<td>0.32 ± 0.04</td>
</tr>
<tr>
<td>Final</td>
<td>0.27 ± 0.01</td>
<td>0.26 ± 0.01</td>
<td>0.36 ± 0.02**</td>
<td>0.21 ± 0.01** ##</td>
</tr>
<tr>
<td>HDL-C, mM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>1.36 ± 0.01</td>
<td>1.31 ± 0.02</td>
<td>1.40 ± 0.04</td>
<td>1.40 ± 0.18</td>
</tr>
<tr>
<td>Final</td>
<td>1.66 ± 0.07</td>
<td>1.60 ± 0.04</td>
<td>0.96 ± 0.03**</td>
<td>0.87 ± 0.03**</td>
</tr>
<tr>
<td><strong>Proteins</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein, g/L</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>56.4 ± 0.4</td>
<td>56.7 ± 0.3</td>
<td>57.2 ± 0.4</td>
<td>56.8 ± 0.4</td>
</tr>
<tr>
<td>Final</td>
<td>67.6 ± 0.7</td>
<td>66.8 ± 0.5</td>
<td>58.1 ± 0.9**</td>
<td>69.7 ± 0.6##</td>
</tr>
<tr>
<td>Albumin, g/L</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>29.8 ± 0.2</td>
<td>29.6 ± 0.3</td>
<td>29.6 ± 0.3</td>
<td>29.2 ± 3.6</td>
</tr>
<tr>
<td>Final</td>
<td>33.7 ± 0.2</td>
<td>33.6 ± 0.3</td>
<td>28.5 ± 0.4**</td>
<td>35.1 ± 0.2**##</td>
</tr>
</tbody>
</table>

(1) Baseline= immediately prior to start of experimental diets; final=after 12 weeks on the diets. Data represents mean ± SEM; N=7-8 per group. CF (control-fed), CF+Cys (control-fed supplemented with cysteine), MR (methionine-restricted), and MR+Cys (methionine-restricted supplemented with cysteine). C, cholesterol.

(2) (*) = p<0.05 compared to CF
(***)= p<0.001 compared to CF
(#) = MR+Cys significantly different from MR, p<0.05,
(##)=MR+Cys significantly different from MR, p<0.001.
Table 2. Serum fatty acid profile in total lipids in rats fed different diets

<table>
<thead>
<tr>
<th>Fatty Acid (FA)</th>
<th>CF</th>
<th>CF + Cys</th>
<th>MR</th>
<th>MR + Cys</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Saturated</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C14:0 (myristic)</td>
<td>0.50 ± 0.03</td>
<td>0.51 ± 0.07</td>
<td>0.30 ± 0.02**</td>
<td>0.61 ± 0.07##</td>
</tr>
<tr>
<td>C15:0 (pentadecanoic)</td>
<td>0.16 ± 0.01</td>
<td>0.16 ± 0.01</td>
<td>0.12 ± 0.02**</td>
<td>0.22 ± 0.03##</td>
</tr>
<tr>
<td>C16:0 (palmitic)</td>
<td>17.86 ± 0.40</td>
<td>18.01 ± 0.75</td>
<td>15.98 ± 0.59**</td>
<td>19.76 ± 0.92##</td>
</tr>
<tr>
<td>C17:0 (hepadecanoic)</td>
<td>0.17 ± 0.01</td>
<td>0.17 ± 0.01</td>
<td>0.15 ± 0.01*</td>
<td>0.17 ± 0.02#</td>
</tr>
<tr>
<td>C18:0 (stearic)</td>
<td>8.65 ± 0.64</td>
<td>8.45 ± 0.87</td>
<td>10.95 ± 0.52**</td>
<td>6.25 ± 0.91##</td>
</tr>
<tr>
<td>C20:0 (arachidic)</td>
<td>0.17 ± 0.01</td>
<td>0.16 ± 0.00</td>
<td>0.23 ± 0.02**</td>
<td>0.18 ± 0.02##</td>
</tr>
<tr>
<td>C22:0 (behenic)</td>
<td>0.17 ± 0.01</td>
<td>0.19 ± 0.02</td>
<td>0.36 ± 0.02**</td>
<td>0.18 ± 0.02##</td>
</tr>
<tr>
<td><strong>Monounsaturated</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C16:1 (palmitoleic)</td>
<td>1.80 ± 0.17</td>
<td>1.58 ± 0.33</td>
<td>0.82 ± 0.11**</td>
<td>1.58 ± 0.63##</td>
</tr>
<tr>
<td>C18:1, t6-t11</td>
<td>1.44 ± 0.22</td>
<td>1.29 ± 0.17</td>
<td>0.99 ± 0.10**</td>
<td>1.29 ± 0.14##</td>
</tr>
<tr>
<td>C18:1, n9 (oleic)</td>
<td>12.48 ± 0.97</td>
<td>12.41 ± 1.71</td>
<td>8.55 ± 1.14**</td>
<td>15.29 ± 1.72##</td>
</tr>
<tr>
<td>C18:1, n11</td>
<td>1.84 ± 0.10</td>
<td>1.74 ± 0.18</td>
<td>0.94 ± 0.04**</td>
<td>1.79 ± 0.24##</td>
</tr>
<tr>
<td>C20:1, n9 (eicosenoic)</td>
<td>0.19 ± 0.01</td>
<td>0.17 ± 0.02</td>
<td>0.19 ± 0.05</td>
<td>0.18 ± 0.01</td>
</tr>
<tr>
<td><strong>Polyunsaturated; n-3</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C18:3, n3 (α-linolenic)</td>
<td>0.28 ± 0.06</td>
<td>0.30 ± 0.03</td>
<td>0.26 ± 0.05</td>
<td>0.41 ± 0.08##</td>
</tr>
<tr>
<td>C20:5, n3 (eicosapentaenoic)</td>
<td>0.08 ± 0.01</td>
<td>0.07 ± 0.01</td>
<td>0.06 ± 0.00</td>
<td>0.07 ± 0.01</td>
</tr>
<tr>
<td>C22:5, n3 (docosapentaenoic)</td>
<td>0.08 ± 0.01</td>
<td>0.07 ± 0.01</td>
<td>0.06 ± 0.00*</td>
<td>0.07 ± 0.01#</td>
</tr>
<tr>
<td>C22:6, n3 (docosahexaenoic)</td>
<td>1.04 ± 0.11</td>
<td>0.98 ± 0.13</td>
<td>1.12 ± 0.13</td>
<td>0.56 ± 0.10##</td>
</tr>
<tr>
<td><strong>n-6</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C18:2, n6 (linoleic)</td>
<td>21.44 ± 2.71</td>
<td>22.41 ± 1.58</td>
<td>23.79 ± 2.13</td>
<td>30.85 ± 2.93##</td>
</tr>
<tr>
<td>C18:3, n6 (γ-linolenic)</td>
<td>0.40 ± 0.04</td>
<td>0.37 ± 0.03</td>
<td>0.50 ± 0.07**</td>
<td>0.30 ± 0.03##</td>
</tr>
<tr>
<td>C20:2, n6 (eicosadienoic)</td>
<td>0.38 ± 0.02</td>
<td>0.36 ± 0.03</td>
<td>0.29 ± 0.02**</td>
<td>0.43 ± 0.04##</td>
</tr>
<tr>
<td>C20:3, n6 (dihomo-γ-linolenic)</td>
<td>0.35 ± 0.06</td>
<td>0.31 ± 0.03</td>
<td>0.28 ± 0.04*</td>
<td>0.42 ± 0.05##</td>
</tr>
<tr>
<td>C20:4, n6 (arachidonic)</td>
<td>21.23 ± 2.39</td>
<td>21.19 ± 2.81</td>
<td>23.70 ± 2.31</td>
<td>12.15 ± 2.33##</td>
</tr>
<tr>
<td>C22:2, n6 (docosadienoic)</td>
<td>0.08 ± 0.01</td>
<td>0.08 ± 0.01</td>
<td>0.11 ± 0.01**</td>
<td>0.06 ± 0.01##</td>
</tr>
</tbody>
</table>
(1) After 12 weeks on the experimental diets. Data represents mean ± SEM, g/100g fatty acid methyl ester. CF (control-fed), CF+Cys (control-fed supplemented with cysteine), MR (methionine-restricted), and MR+Cys. N=6 per group. All fatty acids showed significant differences by ANOVA (p<0.05), except C20:5, n-3. Pairwise comparisons are presented only for CF vs each of the other groups, and for MR vs MR+Cys.

(*) = p<0.05 compared to CF
(**)= p≤0.001 compared to CF
(#)= MR+Cys significantly different from MR, p<0.05,
(##)=MR+Cys significantly different from MR, p≤0.001.
Figure 1
Figure 2

SCD-1-16 index
C16:1n-7/C16:0

SCD-1-18 index
C18:1n-9/C18:0

D5D index
C20:4n-6/C20:3n-6

D6D index
C18:3n-6/C18:2n-6

Elongase index
C18:0/C16:0

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Figure 3

- Insulin, ng/mL
- IGF-1, µg/mL
- Leptin, pg/mL
- Adiponectin, µmol/L
Figure 4

**Methionine, µmol/L**

**tHcy, µmol/L**

**tCys, µmol/L**

**Cystathionine, µmol/L**

**Taurine, µmol/L**

**Glutamate, µmol/L**

Baseline  6w  12w