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Journal of Nutritional Biochemistry 19 (2008) 362–370
Journal of Nutritional Biochemistry

RESEARCH ARTICLES

Dietary methionine effects on plasma homocysteine and HDL metabolism in mice

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Received 31 October 2006; accepted 17 May 2007

Abstract

The effects of dietary manipulation of folate and methionine on plasma homocysteine (Hcy) and high-density lipoprotein cholesterol (HDL-C) levels in wild-type and apolipoprotein-E-deficient mice were determined. A low-folate diet with or without folate and/or methionine supplementation in drinking water was administered for 7 weeks. Fasted Hcy rose to 23 μM on a low-folate/high-methionine diet, but high folate ameliorated the effect of high methionine on fasted plasma Hcy to ~10 μM. Determination of nonfasted plasma Hcy levels at 6-h intervals revealed a large diurnal variation in Hcy consistent with a nocturnal lifestyle. The daily average of nonfasted Hcy levels was higher than fasted values for high-methionine diets but lower than fasted values for low-methionine diets. An acute methionine load by gavage of fasted mice increased plasma Hcy 2.5 h later, but mice that had been on high-methionine diets had a lower fold induction. Mice fed high-methionine diets weighed less than mice fed low-methionine diets. Based on these results, two solid-food diets were developed: one containing 2% added methionine and the other containing 2% added glycine. The methionine diet led to fasted plasma Hcy levels of >60 μM, higher than those with methionine supplementation in drinking water. Mice on methionine diets had >20% decreased body weights and decreased HDL-C levels. An HDL turnover study demonstrated that the HDL-C production rate was significantly reduced in mice fed the methionine diet.

Keywords: Homocysteine; HDL turnover; Folate

1. Introduction

Homocysteine (Hcy) is a metabolite of methionine (specifically the methyl donor *S*-adenosylmethionine) and precursor of cysteine biosynthesis [1]. A 1995 meta-analysis of 27 primarily case–control studies concluded that a 5-μM increment in total plasma homocysteine after reduction (tHcy) increases the odds ratio for coronary artery disease (CAD) by 1.6 and 1.8 for men and women, respectively [2]. A more recent meta-analysis found a more modest effect, such that subjects with a 25% lower tHcy level have an 11% decreased risk for ischemic heart disease [3]. This meta-analysis also found that retrospective studies have odds ratios larger than those of prospective studies, indicating that elevated tHcy could be a result of, rather of than a cause of, cardiovascular disease [3]. The normal levels of plasma tHcy in humans are 7–14 μM, and

Abbreviations: CAD, coronary artery disease; *Cbs*, cystathionine-β-synthase; FCR, fractional catabolic rate; Hcy, homocysteine; HDL-C, high-density lipoprotein cholesterol; HiF/HiM, high folate/high methionine; HiF/LoM, high folate/low methionine; LoF/HiM, low folate/high methionine; LoF/LoM, low folate/low methionine; MTHFR, methylenetetrahydrofolate reductase; PR, production rate; tHcy, total plasma homocysteine after reduction.

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doi:10.1016/j.jnutbio.2007.05.005

hyperhomocysteinemia due to genetic causes or vitamin deficiency is defined as tHcy>15 μM. The most common genetic cause of hyperhomocysteinemia is polymorphism in the methylenetetrahydrofolate reductase (MTHFR) gene, which yields a thermolabile enzyme [4]. The frequency of homozygotes for this allele is about 12% in the White population, and homozygosity for this allele is associated with increased tHcy. A recent meta-analysis of 40 studies found that homozygotes had a 16% increased odds ratio for cardiovascular disease; however, this increased odds ratio was not observed in locations with high-folate diets [5]. This is explainable by a large gene–diet interaction, as increased tHcy is observed only in homozygotes for the thermolabile MTHFR allele that have low plasma folate levels [6]. It is well known (and used as the basis for clinical measurement) that tHcy levels rise acutely after an oral methionine load, although there are mixed results about the chronic effects of dietary methionine and/or protein on plasma tHcy in humans [7–10].

In numerous mouse studies, high-methionine and/or vitamin-deficient diets have been used to raise tHcy levels and to look for effects on atherosclerosis, thrombosis and vascular reactivity. It had also been shown that different strains of mice respond to different levels of plasma tHcy 2.5 h after an acute oral methionine load [11]. There have been also previous indications that altered Hcy metabolism can have an effect on high-density lipoprotein (HDL) levels. Cystathionine-β-synthase (*Cbs*)-deficient mice with highly elevated tHcy levels have decreased plasma high-density lipoprotein cholesterol (HDL-C) levels [12,13]. Mice hemizygous for MTHFR deficiency have a mild elevation of tHcy levels and decreased levels of plasma apolipoprotein (apo) A-I, the major HDL protein [14]. A high-methionine diet yielding a moderate increase in plasma tHcy, but not a high-methionine/low-folate diet yielding a robust increase in plasma tHcy, was reported to lower total plasma cholesterol in wild-type C57BL/6 mice [15].

In the current mouse study, we examined the effects of chronic methionine feeding on many parameters, including body weight, blood counts, plasma cholesterol and HDL-C levels. We also measured tHcy levels in fasted and nonfasted mice at various times during the day and after an acute methionine load. We found that chronic feeding of a high-methionine diet led to a significant decrease in plasma HDL-C; in an HDL turnover study, this decrease was associated with a decreased HDL-C production rate (PR).

2. Materials and methods

2.1. Mice and diets

ApoE-deficient and wild-type mice, both on C57BL/6 background, were purchased from Jackson Laboratory (Bar Harbor, ME). Male mice were used exclusively throughout this study. In addition to a normal chow diet (PicoLab 20-5053 rodent chow), the following specifically designed synthetic diets were used (all from Harlan Teklad): (a) TD 97095 — a low-folate/low-methionine (LoF/LoM) diet containing 6% fat and 19.5% casein (wt/wt); (b) TD 98400 — a "GLY" diet containing 6% fat, 19.5% casein and 2 mg/kg folate, supplemented with 2% glycine; and (c) TD 98399 — a "MET" diet, which is identical to the GLY diet but with 2% methionine instead of 2% glycine. The exact formulations for these diets are shown in Table 1. The actual levels of folate and methionine in all diets were chemically determined by Covance (Madison, WI). In some studies, methionine and folate were supplemented in drinking water in different concentrations and changed weekly. A 25-g male C57BL/6 mouse is reported to eat 2.62 g of food and to drink 2.86 g of water daily (Jackson Laboratory Mouse Phenome Database: http://phenome.jax.org/pub-cgi/phenome/mpdcgi%3Frtn%3Ddocs/home). We used these values in order to estimate the daily intake of folate and methionine in mice on various diets. In order to obtain fasted plasma samples, food was removed and methionine-containing and/or folate-containing water was exchanged for normal

Table 1
Synthetic diet compositions

	Folate deficient (TD 97095[a]; g/kg)	GLY (TD 98400[a]; g/kg)	MET (TD 98399[a]; g/kg)
Casein (vitamin free)	195	195	195
Methionine			20
Glycine		20	
Sucrose	345	325	325
Corn starch	306	301	301
Soybean oil	60	60	60
Cellulose	50	50	50
Mineral mix (TD 94046)	35	35	35
Calcium phosphate	4	4	4
Ethoxyquin	0.012	0.012	0.012
Vitamin mix (TD 40060)		10	10
Ascorbic acid	1.02		
Vitamin B_{12}	0.03		
Biotin	0.0005		
Calcium pantothenate	0.066		
Choline citrate	3.5		
Inositol	0.11		
Menadione	0.05		
Niacin	0.1		
Pyridoxine HCl	0.022		
Riboflavin	0.022		
Thiamine HCl	0.022		
Vitamin A palmitate	0.04		
Vitamine D_3	0.0044		
Vitamin E acetate	0.242		
Measured folate content (mg/kg)[b]	0.065	2	2
Measured methionine content[b]	4.3	4.3	24.3

[a] Harlan Teklad catalog number.
[b] For comparison, PicoLab 20-5053 rodent chow was measured to contain 2.5 mg/kg folate and 4.2 g/kg methionine.

water on the evening before sacrifice. In the morning, the animals were bled from the retro-orbital plexus under Metofane anesthesia. Acute methionine loading was achieved by oral gavage of methionine solution to deliver 320 mg of methionine per kilogram of body weight. Two and a half hours later, the mice were anesthetized with Avertin, and blood was removed from the left ventricle during sacrifice by exsanguination. This blood was used to determine plasma Hcy and other lipid and hematological parameters after methionine loading.

2.2. Hcy assay

Total plasma Hcy was measured using an enzymatic assay that was adapted from prior studies [16–18]. Twenty-microliter aliquots of plasma or standards were reduced by dilution with 44 μl of K_3PO_4 (0.1 M, pH 7.4), 10 μl of dithioerythritol (0.2 M) and 1 μl of pentostatin (2′-deoxycoformycin; 0.1 M; Pfizer, Ann Arbor, MI). The mixture was boiled for 10 min and centrifuged. The supernatant was decanted and cooled on ice water. The following were added to 45 μl of the reduced sample: 23 μl of K_3PO_4 (0.1 M, pH 7.4), 1 μl of pentostatin (0.1 M), 1 μl of [8-^{14}C]adenosine (1 mM, 52 mCi/mmol) and 10 μl of *S*-adenosyl-L-homocysteine-hydrolase (13 mU/μl; Sigma, St. Louis, MO). The reaction was incubated for 30 min at 37°C and terminated by the addition of 10 μl of formic acid (2 M). Five microliters of unlabeled *S*-adenosyl-L-homocysteine (10 mM) was added as a carrier. The samples were boiled for 10 min and centrifuged for 10 min at maximum speed in a microfuge. A 25-μl aliquot of the reaction was spotted, together with adenosine and *S*-adenosyl-homocysteine as markers, on cellulose chromatogram sheets (Kodak, Rochester, NY), dried and developed for 3 h in a chamber with ethanol:acetic acid:water (64:1:35, vol.%). The sheets were air dried, and spots containing labeled adenosine and *S*-adenosyl-homocysteine were marked under UV light. The spots were cut out, and radioactivity was determined by liquid scintillation counting. L-homocysteine (Sigma) solutions were used as standards, and this assay yielded linear correlations between Hcy input and ^{14}C counts per minute recovered.

2.3. Plasma lipids and HDL-C values

Plasma triglycerides and cholesterol were determined using commercial kits (Sigma), which were adapted for 96-well plates. HDL-C was determined after separation from non-HDL-C by two-step density ultracentrifugation. In the first step, 30 μl of plasma was overlaid with 30 μl of phosphate-buffered saline in a thick-walled 200-μl tube, and very-low-density lipoprotein was floated up by spinning for 3 h at 70,000 rpm in an S100AT3 rotor (Beckman). The bottom 30 μl was transferred to a clean tube and mixed with 30 μl of a 1.12-g/ml KBr solution, resulting in a solution with a final density of 1.063 g/ml, which was spun for 16 h at 70,000 rpm. The bottom half (with a density of >1.063) that contains HDL and lipid-free protein was used for cholesterol determination by the assay described above. Hematological values were determined using routine clinical assays.

2.4. Cholesterol absorption

Cholesterol absorption was determined as previously described [19]. Briefly, mice were housed singly in metabolic cages and gavaged orally with a mixture of [^{14}C]cholesterol and nonabsorbable [^{3}H]sitostanol in olive oil. Feces were collected for a 24-h period, and the ^{14}C/^{3}H ratio in the feces and gavage mixture was used to calculate the percentage of cholesterol absorption, as previously described [19].

2.5. HDL turnover

HDL was prepared from wild-type mice fed a chow diet by sequential density ultracentrifugation using the fraction d=1.063–1.21 g/ml. HDL was labeled in its core with [^{3}H]cholesteryl oleyl ether, as previously described [20], by the use of rabbit lipoprotein-deficient plasma as a source of cholesterol ester transfer protein. The labeled HDL was injected into the tail vein, and blood samples were obtained at various time points from the retro-orbital plexus under Metofane anesthesia. Plasma radioactivity was counted for each sample and normalized to radioactivity at the 5-min (initial) time point. The decay curve for each mouse was fitted by nonlinear regression to a two-phase exponential decay, with plateau set to zero (analysis performed using GraphPad Prism software). HDL-C fractional catabolic rates (FCRs) for slow decay curves were used to calculate HDL-C PRs using the formula: PR=(FCR×HDL-C

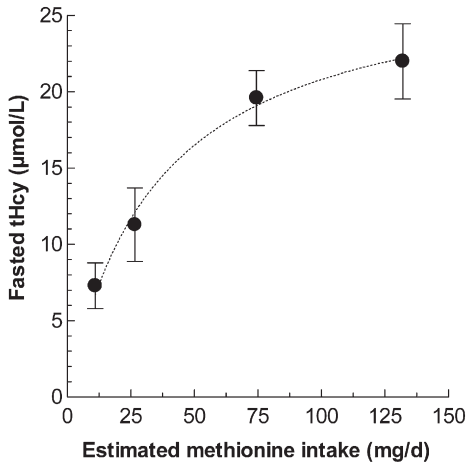

Fig. 1. Effect of varying dietary methionine levels on fasting plasma Hcy levels in apoE-deficient mice. Chow diet, calculated to provide 12.5 μg/day folate, was supplemented with drinking water containing various levels of methionine to yield daily methionine intakes as shown. Groups of five mice were fed each diet for 4 weeks, fasted overnight with plain drinking water and bled for the determination of fasting plasma Hcy. Values are presented as mean±S.D., and the line was determined by nonlinear regression analysis.

Table 2
Approximate daily folate and methionine intakes per mouse on different diets

Diet	Abbreviation	Folate (μg/day)	Methionine (mg/day)
Chow		6.6	11
Low folate/low methionine	LoF/LoM	0.17	11.3
Low folate/high methionine	LoF/HiM	0.17	125.7
High folate/low methionine	HiF/LoM	28.8	11.3
High folate/high methionine	HiF/HiM	28.8	125.7
Glycine	GLY	5.24	11.3
Methionine	MET	5.24	63.7

Values calculated are based on daily food intake and daily water intake of 2.62 g and 2.86 ml, respectively.

concentration×plasma volume), with or without normalization for body weight. Plasma volume was estimated to be 3.85% of body weight.

3. Results

The effects of dietary methionine and folate on plasma tHcy in 8-week-old apoE-deficient mice were investigated. It was first determined whether dietary methionine (during a 4-week period) affected fasting plasma tHcy levels in chow-diet-fed male apoE-deficient mice. The chow diet was analyzed to contain 2.5 mg/kg folate and 4.2 g/kg methionine, resulting in an approximate daily intake of 6.6 μg of folate and 11 mg of methionine per mouse. Three increasing amounts of methionine were fed chronically by methionine addition to drinking water. The mean fasting plasma tHcy in mice without methionine supplementation was 7.3±1.5 μM, and increasing methionine in the diet resulted in increasing concentrations of fasting plasma tHcy, with an estimated methionine intake of 132 mg/day leading to a fasting tHcy of 22±2.5 μM (Fig. 1). Nonlinear regression of these data yields a B_{max} of 28 μM tHcy (r^2=.90), suggesting that this is the theoretical limit of the fasting level of tHcy that could be obtained in these mice on a normal folate diet by methionine supplementation in drinking water.

To investigate the effects of both folate and methionine, we used a low-folate diet that was analyzed to contain 0.065 mg/kg folate and 4.3 g/kg methionine (a methionine content similar to the level in the chow diet). This diet was supplemented with drinking water containing either: (a) no addition to yield a LoF/LoM diet; (b) 4% (wt/vol) methionine to yield a low-folate/high-methionine (LoF/HiM) diet; (c) 10 mg/L folate to yield a high-folate/low-methionine (HiF/LoM) diet; or (d) 10 mg/ml folate plus 4% methionine to yield a high-folate/high-methionine (HiF/HiM) diet. The estimated daily intakes of folate and methionine for these four diets are shown in Table 2. Mice were fed these four diets chronically from 9 to 16 weeks of age (8–10 per group), and their tHcy levels, after an overnight fast and after replacing the folate/methionine-supplemented drinking water with plain water, are shown in Table 3. The two groups on low-methionine diets (HiF/LoM and LoF/LoM) had mean fasting tHcy levels of 9.3 and 12.6 μM, respectively. The LoF/HiM diet dramatically increased mean fasting plasma tHcy to 22.8 μM. However, the fasting hyperhomocysteinemia caused by the LoF/HiM diet was ameliorated by feeding high amounts of folate in the HiF/HiM diet, lowering mean fasting tHcy levels to 10.0 μM.

Fasting tHcy levels may not be representative of average tHcy levels in mice with free access to food and water; thus, plasma tHcy levels were monitored every 6 h over 1 day (Fig. 2). Mice are nocturnal and, thus, we observed a large diurnal variation in nonfasted plasma tHcy levels, with higher values at night and lower values during the day. Interestingly, fed tHcy values were greater than fasting values for both high-methionine diets, whereas fed tHcy

Table 3
Effects of the test diets on tHcy levels and other parameters

	tHcy fasted (μM)	tHcy eff (μM)	tHcy gav (μM)	Body weight (g)	Spleen (mg)	Total cholesterol (mg/dl)
LoF/LoM (n=8)	12.6±2.5	5.7±1.2	104±16	28.9±2.0	78±23	589±175
LoF/HiM (n=9)	22.8±6.8	37±10	113±31	23.6±1.6	76±20	533±96
HiF/LoM (n=9)	9.3±2.5	4.5±0.9	81±25	27.9±2.5	85±12	471±109
HiF/HiM (n=10)	10.0±3.5	15±4.0	49±15	25.1±1.0	72±16	516±58
P values						
ANOVA	<.0001	<.0005	<.0001	<.0001	ns	ns
Newman–Keuls test						
LoF/HiM vs. LoF/LoM	<0.001	<0.001	ns	<0.001		
HiF/LoM vs. LoF/LoM	ns	ns	<0.05	ns		
HiF/LoM vs. LoF/HiM	<0.001	<0.001	<0.05	<0.001		
HiF/HiM vs. LoF/LoM	ns	ns	<0.001	<0.001		
HiF/HiM vs. LoF/HiM	<0.001	<0.01	<0.001	ns		
HiF/HiM vs. HiF/LoM	ns	ns	<0.01	<0.01		

Newman–Keuls posttest was performed only when the overall ANOVA test was significant.
Hcy eff=effective diurnal mean of tHcy level; Hcy gav=tHcy 2.5 h after methionine loading.

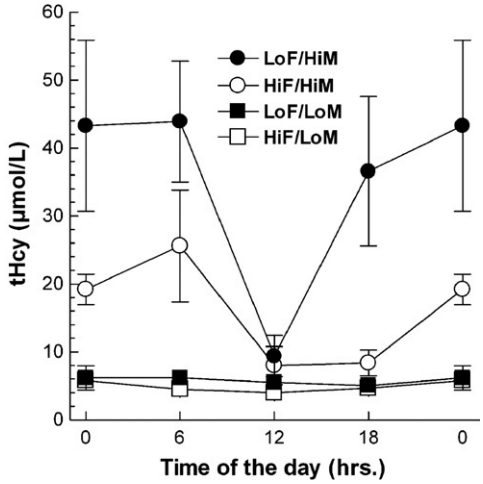

Fig. 2. Diurnal variation in nonfasted plasma Hcy levels. Mice (n=3 per diet) on the four test diets were bled at midnight (0 h) and at 6-h intervals for the determination of plasma Hcy levels. The lights in the room were on from 0700 to 1900 h. Values are presented as mean±S.D. LoF/HiM=low folate/high methionine; HiF/HiM=high folate/high methionine; LoF/LoM=low folate/low methionine; HiF/LoM=high folate/low methionine.

values were less than fasting tHcy levels for both low-methionine diets (compare Fig. 2 to Table 3). By averaging diurnal tHcy values at midnight, 0600 h, noon and 1800 h, mean fed tHcy values were calculated for the four experimental diets, which we have called effective tHcy levels and are a better reflection of chronic tHcy levels than are fasted tHcy values (Table 3). The effective tHcy levels were 4.5±0.9 µM (HiF/LoM), 5.7±1.2 µM (LoF/LoM), 37±10 µM (LoF/HiM) and 15±4.0 µM (HiF/HiM). The most striking difference between fasted and effective tHcy values was observed in the HiF/HiM-diet-fed group, where fasting values were roughly similar to the values for the LoF/LoM and HiF/LoM groups, while effective values were about threefold higher than the values for the LoF/LoM and HiF/LoM groups. The lack of significance between the effective tHcy of the HiF/HiM group and the effective tHcy of the LoF/LoM and HiF/LoM groups is only due to a small sample size and the conservative nature of the analysis of variance (ANOVA) Newman–Keuls multiple comparison posttest, as a similar test comparing data from just these three diets found the effective tHcy levels of mice on the HiF/HiM diet to be different from the effective tHcy levels of mice on both the LoF/LoM and the HiF/LoM diets (P<.01), as did t tests for the HiF/HiM diet versus both the LoF/LoM and the HiF/LoM diets (P<.02).

After obtaining fasting blood samples, mice on the four test diets were subjected to oral gavage with 320 mg/kg methionine and bled 2.5 h later at the time of sacrifice. These postgavage blood samples were used to obtain plasma tHcy values after oral methionine loading. There were dramatic increases in plasma tHcy after oral methionine loading for mice on all four test diets, with values of >100 µM detected for both folate-deficient diets (Table 3). The fold increase in tHcy levels comparing methionine-loaded to fasted samples is shown in Fig. 3. Regardless of the folate content of the diet, the fold increase in tHcy levels after methionine loading was almost twice as great (~8-fold) for mice fed the two low-methionine diets than for mice fed the two high-methionine diets (~4.5-fold). Therefore, chronic methionine intake resulted in an adaptive response that limited the induction fold in tHcy levels after an acute methionine load.

The different diets had no significant influence on spleen weight, total cholesterol (Table 3), hemoglobin, hematocrit, complete blood count or differential blood count (values of the latter not shown) in these apoE-deficient mice. The LoF/HiM diet did, however, lead to a significant decrease in body weight compared to the other three diets (a 18% decrease vs. the LoF/LoM diet; Table 3). Despite this effect on body weight, all mice were healthy looking and well groomed.

Based on these results, we designed two new diets in which either 2% methionine or glycine, as a control amino acid, was milled into a solid-food diet containing 2.0 mg/kg folate, which we designated as MET and GLY diets, respectively. These diets are considerably easier to use compared to methionine supplementation in water, which requires weekly mixing. We fed these diets to groups of male wild-type and apoE-deficient mice starting at 7 weeks of age for a period of 5 weeks. The mice on GLY diets had mean fasted Hcy levels of ~5 µM. The MET diet led to significantly higher mean fasted Hcy levels of 90 and 68 µM for wild-type and apoE-deficient mice, respectively (Fig. 4A). Body weights were reduced by >20% in both strains of mice fed the MET diet (Fig. 4B), similar to the body weight reduction observed in mice supplemented with 4% methionine in their drinking water (Table 3). One possible explanation for this finding would be that the mice did not like the taste of the MET diet and ate less food. However, this was not the case, as we found no significant

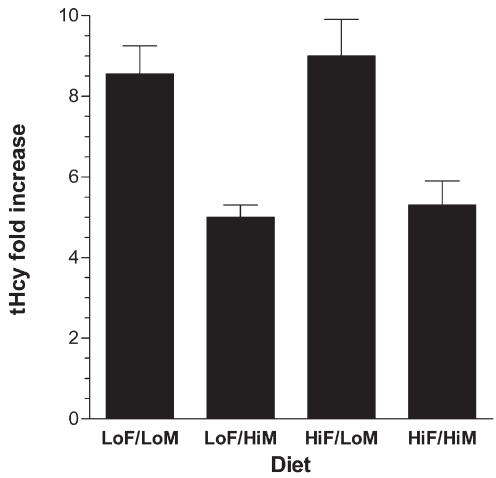

Fig. 3. Fold increase in plasma Hcy levels in mice after an acute oral methionine load compared to fasting Hcy levels. Hcy levels were determined from fasted plasma and plasma taken 2.5 h after oral gavage with 320 mg/kg methionine. Fold increases (presented as mean±S.D.) are shown (n=5 per diet).

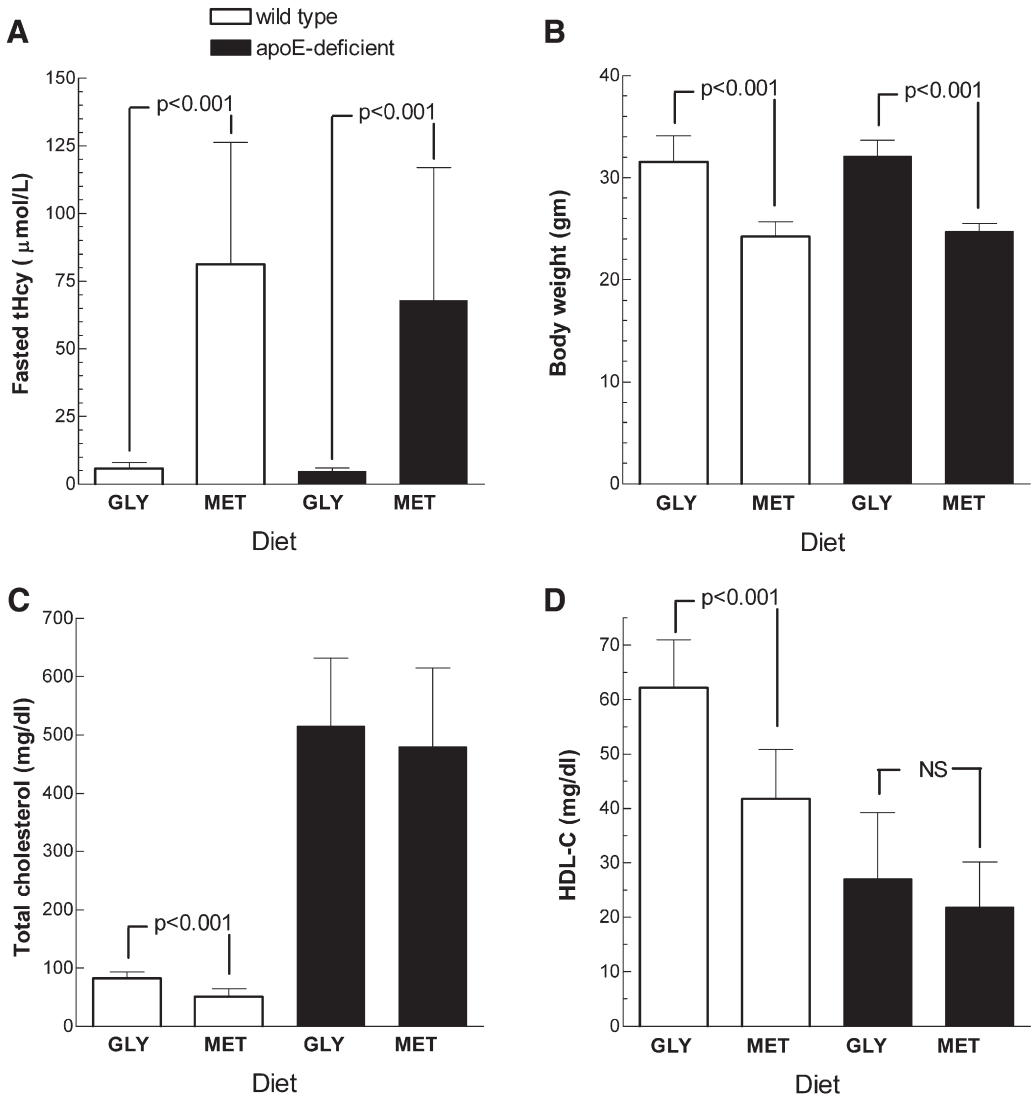

Fig. 4. MET and GLY diet effects on wild-type and apoE-deficient mice. (A) Fasted Hcy levels in wild-type (open bars) and apoE-deficient (closed bars) mice on the GLY and MET diets, as indicated (n=9–13 per group, mean±S.D.; P values shown are from two-tailed t tests of the diet effect within each mouse type). (B) Body weights in wild-type and apoE-deficient mice on the GLY and MET diets (analysis as described in A). (C) Total plasma cholesterol in wild-type and apoE-deficient mice on the GLY and MET diets (analysis as described in A). (D) HDL-C in wild-type and apoE-deficient mice on the GLY and MET diets (analysis as described in A).

difference in the amount of food consumed when we compared mice fed the MET diet (3.58+0.57 g/day; n=5) and mice fed the GLY diet (3.70+0.58 g/day; n=5), which was assessed over a 24-h period in 12-week-old mice housed in metabolic cages. As expected, plasma total cholesterol levels were ~500 mg/dl in apoE-deficient mice and <100 mg/dl in wild-type mice (P<.001 for apoE-deficient mice vs. wild-type mice, by ANOVA posttest; Fig. 4C). Wild-type mice fed the MET diet had 37% lower total cholesterol levels than mice on the GLY diet (P<.001), and the same trend was observed in apoE-deficient mice, albeit not statistically significant. Since most of the plasma cholesterol in wild-type mice is carried on HDL, we assayed HDL-C levels in these samples as well. The MET diet led to a highly significant 33% reduction in HDL-C levels in wild-type mice and a 25% reduction in HDL-C levels in apoE-deficient mice, which was not statistically significant (Fig. 4D). As previously observed [21], HDL-C levels were reduced in apoE-deficient mice compared to wild-type mice (P<.01 for apoE-deficient mice vs. wild-type mice, by ANOVA posttest).

Cholesterol absorption and HDL turnover studies were performed to follow up on the MET-diet-induced reduction in HDL-C in wild-type mice. Cholesterol absorption was measured by a dual-isotope gavage method using [^{14}C] cholesterol and the poorly absorbed [^{3}H]sitostanol in two independent experiments, each with five wild-type male mice on each diet. Pooling data from both experiments, mice on the GLY and MET diets had 80.0±5.1% and 82.8±10.1% mean cholesterol absorptions, respectively (not significant). HDL turnover was assayed by intravenous injection of [^{3}H]

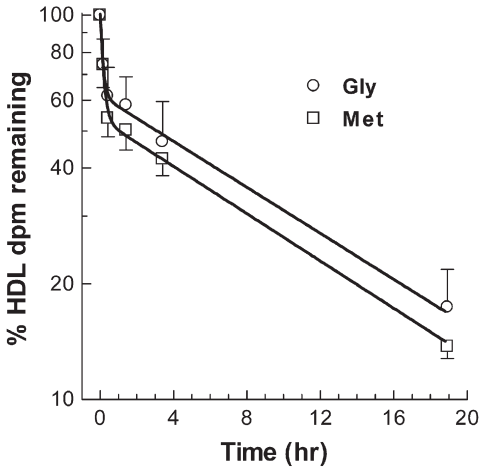

Fig. 5. HDL turnover in wild-type mice fed the GLY diet or the MET diet. [^{3}H]Cholesteryl-oleyl-ether-labeled HDL was injected into groups of mice fed the GLY diet (open squares; n=8) or the MET diet (open circles; n=10). Clearance of plasma ^{3}H dpm (disintigrations per minute) was followed over time (normalized to the 5-min postinjection time point; mean±S.D.). The data fitted a two-phase exponential decay model.

cholesteryl-oleyl-ether-labeled HDL into five wild-type male mice each on the GLY and MET diets. Since the ether linkage of cholesteryl oleyl ether cannot be cleaved, the label is not readily releasable from tissues and, thus, the plasma disappearance of the label is reflective of HDL turnover (Fig. 5). The turnover of the labeled HDL in each mouse was fitted to a two-phase exponential decay by nonlinear regression. The initial rapid phase had average FCRs of 9.0 and 6.1 pools/h for mice on the GLY and MET diets, respectively (ns). This initial rapid phase of decay is probably due to plasma HDL equilibration with interstitial fluid and, thus, we calculated the HDL-C FCR by using the second and slower phases of HDL clearance. The slow-phase HDL FCRs were 0.070 and 0.071 pools/h for mice on the GLY and MET diets, respectively (ns; Table 4). As before, the MET diet led to a significant reduction in body weight (a 24% reduction; Table 4). Furthermore, mean HDL-C levels were reduced by 24%, from 94.4 mg/dl on the GLY diet to 71.8 mg/dl on the MET diet. Using HDL-C values, as well as calculated plasma volumes and body weights, we calculated HDL-C PRs of 2.53 and 1.91 μg/h/g body weight for mice on the GLY and MET diets, respectively (P=.019; Table 4). Thus, the 24% decrease in HDL-C levels was reflected by a significant 25% decrease in HDL PR adjusted per gram of body weight and a significant 45% reduction in HDL transport rate not adjusted by body weight (Table 4).

4. Discussion

This study focuses on the effects of feeding mice a high-methionine diet. At a normal level of dietary folate, a high-methionine diet led to decreased body weight, increased plasma tHcy and decreased plasma HDL-C. Most Hcy studies in mice and many studies in humans have used fasting samples for the determination of plasma tHcy. For mice on low-methionine diets, we observed that fasted plasma tHcy levels were much higher than effective tHcy levels averaged over 24 h in fed mice. However, after an overnight fast, the mice had not had a major feeding period for over 1 day, since mice eat primarily at night. We have observed that this fasting regimen led to a 10–15% decrease in body weight and, thus, these mice were in a catabolic state. We speculate that endogenous protein turnover during this catabolic state could lead to increased plasma methionine, compared to the fed state of these mice on low-methionine diets, and that this might account for increased Hcy in fasted mice on these diets. Similar to what has been observed in mice fed the low-methionine diets, tHcy levels are also reported to be higher in human subjects who had fasted for at least 6 h than in subjects who had a recent meal [22]. In contrast, fasted tHcy levels were lower than the effective levels for mice fed the two high-methionine diets, since effective tHcy levels were greatly influenced by plasma levels of methionine in the fed state on these diets. Another finding of interest is that the high-folate diet masked the effect of the high-methionine diet on fasted tHcy levels (10 μM), but had less effect in masking the effect of the high-methionine diet on effective tHcy levels averaged over 24 h (15 μM).

Chronic feeding of the high-methionine diet, independent of dietary folate, diminished the fold induction of tHcy levels after oral methionine loading, compared to fasting values. Although tHcy levels were high for all mice after the oral methionine load, the induction fold was about half as large in mice chronically fed either of the high-methionine diets. This apparently adaptive response to chronic high-methionine diet could possibly be due to up-regulation in enzymes that metabolize Hcy (e.g., *Cbs*). It is of interest that of five mouse strains subjected to oral methionine loading, C57BL/6, the strain used in the current study, was the most responsive and yielded the highest levels of tHcy after methionine loading [11].

We observed a very strong diurnal variation in plasma tHcy levels for mice on the high-methionine diets, with the highest levels observed during the dark cycle, when mice are more active and eat most of their food. A much weaker

Table 4
Methionine effects on HDL turnover

	Body weight (g)	HDL-C (mg/dl)	HDL-C FCR (pools/h)	HDL-C PR (μg/h)	HDL-C PR (μg/h/g)
GLY	31.8±1.8	94.4±6.8	0.070±0.010	81±12	2.53±0.33
MET	24.1±1.4	71.8±4.8	0.071±0.010	45±4	1.91±0.27
P	<.0001	.0003	ns	.001	.019

All values are presented as mean±S.D. (five mice per group).
P values are based on two-tailed t test.
ns=not significant.
HDL-C FCR values are based on a slow-phase turnover, as described in the text.

diurnal effect has been observed in human subjects with decreased plasma tHcy levels at night [23]. In addition, we observed a striking difference in fasted tHcy levels based on whether methionine was added to drinking water or milled into food. For example, the fasting tHcy for apoE-deficient mice on the LoF/HiM diet (water-supplemented diet) was ~23 μM (Table 3), while the fasting tHcy for apoE-deficient mice on the solid-food MET diet (containing more folate and less methionine than the LoF/HiM water-supplemented diet) had much higher tHcy levels of ~68 μM (Fig. 4A). This could be due to the different chemical stability of methionine in water versus solid-food-supplemented diets, or to differences in methionine absorption or metabolism; however, the precise reason for this effect is not known.

Dietary methionine has been reported to have only minor effects on fasted plasma tHcy in humans. In one study based on a diet questionnaire, subjects who were in the top quartile of methionine intake, compared to subjects in the bottom quartile, actually had lower fasted plasma tHcy, arguing against a hyperhomocysteinemia effect of dietary methionine [8]. Another study found that methionine dietary supplementation did lead to a trend of increased plasma tHcy levels in men, with plasma levels of 18 μM tHcy being achieved with 75 mg/kg/day methionine supplementation [9]. Interestingly, vegans, who have less dietary protein intake than meat eaters, had higher fasted plasma tHcy levels, although the might be due to their low plasma levels of vitamin B_{12}, a cofactor for Hcy metabolism [10]. Since all of the above human studies used fasted plasma samples, one might ask if this is the best type of plasma sample that can be used in the determination of tHcy levels in humans. Further human studies are needed to resolve this issue, but based on our mouse studies, obtaining both a fasting sample and a post-methionine-load sample may prove to be the most informative. It would be interesting to ascertain whether the ratio of postload Hcy to fasting Hcy is influenced by the pattern of dietary methionine intake in humans and if this ratio is affected by vitamin intake and common MTHFR polymorphism.

We observed a significant effect of the MET diet on decreasing HDL-C in wild-type mice, which was associated with a decreased PR, rather than an increased turnover rate. A similar decrease in plasma HDL was observed in *Cbs*$^{-/-}$ mice that have profound hyperhomocysteinemia [12,13]. Additionally, total plasma cholesterol levels were reported to decrease by 27% in wild-type C57BL/6 mice fed a high-methionine diet [15]; although HDL-C levels were not reported, we speculate that increased tHcy due to dietary methionine may lead to the down-regulation of one of the key players in HDL production, such as apoA-I, ABCA1 or LCAT. Supporting this idea, *Cbs*$^{-/-}$ mice were shown to have decreased LCAT mRNA and plasma activities [13]. Recently, a gene array study of liver RNA from mildly hyperhomocysteinemic hemizygous *Mthfr*$^{+/-}$ mice revealed decreases in apoA-I and apoA-IV mRNA, compared to wild-type mice [14]. In addition, 30–40% decreases in plasma apoA-I were detected in *Mthfr*$^{+/-}$ mice, as well as in *Cbs*$^{+/-}$ mice, implying a Hcy effect, although, curiously, plasma HDL-C levels were not reported in *Mthfr*$^{+/-}$ mice [14]. Furthermore, this paper also reported inverse correlations between plasma tHcy and both plasma apoA-I levels and HDL-C in a cohort of men with CAD [14]. ApoA-I levels in HepG2 hepatoma cells were reduced by the incubation of cells with 5 mM Hcy, but this dose is clearly out of the range of physiological levels [14], weakening the significance of this finding. Endoplasmic reticulum stress has been shown to be involved in the Hcy regulation of gene expression in both cultured endothelial cells and HepG2 cells [15], but again, this is a response is millimolar levels of Hcy. We speculate that the effects of Hcy on reducing HDL-C and an implied reduction in reverse cholesterol transport may play a role in the association of elevated tHcy with atherosclerosis and CAD, but whether this mechanism is, in fact, involved in atherosclerosis remains to be determined.

Acknowledgments

This work was supported by grant PO1 HL54591 from the National Institutes of Health (to J.D.S.) and by grant Me 1507/1-1 from the Deutsche Forschungsgemeinschaft (to M.M.).

We thank Jan L. Breslow for his support, discussion and critical reading of the manuscript.

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