Effects of moderate hyperhomocysteinaemia induced by 4 weeks methionine-enriched diet on metabolite profile and mesenteric artery function in rats

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Methionine is an essential amino acid and methyl donor for most transmethylation reactions in mammals. The product of transmethylation reactions is homocysteine, which is associated with enhanced risk for CVD. The aim of this study was to analyse metabolic and vascular functional consequences of a methionine-enriched diet in rats. The dose of methionine was chosen to reflect the range of over-nutrition in man. We quantified plasma levels of homocysteine, asymmetrical dimethylarginine and adenosine, determined methionine and its metabolites in tissues and blood plasma and assessed relaxation of mesenteric arteries toward acetylcholine and sodium nitroprusside. A methionine-enriched diet for 4 weeks elevated homocysteine levels in plasma 2-fold and in spleen by 70 %. The level of S-adenosylhomocysteine was increased in liver only, while methionine and S-adenosylmethionine were unchanged in all organs studied. Plasma adenosine and asymmetrical dimethylarginine levels were unchanged, as were vessel relaxations. A 2-fold elevation of plasma homocysteine, which is assigned a risk indicator for cardiovascular events, did not impair mesenteric artery vasodilatation during 4 weeks of a methionine-rich diet. Furthermore, asymmetrical dimethylarginine and adenosine, which have been shown to be changed in more severe degrees of hyperhomocysteinaemia, remained unaltered.

Homocysteine: Asymmetrical dimethylarginine: Amino acids: Vessel function

Methionine is an essential amino acid with a recommended daily intake of 1–2 g per d for human individuals. Most methylation reactions in the body, e.g. methylation of RNA and DNA or synthesis of creatine or adrenaline, rely on methionine metabolism. In the respective pathway, methionine is coupled to ATP leading to formation of S-adenosylmethionine (SAM), which is the main methyl donor in mammals. A product of the methyl transfer reactions is S-adenosylhomocysteine (SAH), which can be further catabolized to homocysteine by SAH-hydrolase. Homocysteine is remethylated by a vitamin B₁₂- and folate-dependent pathway to methionine or metabolized via formation of cystathionine with serine to α-ketobutyrate and cysteine or secreted into the plasma.

Because methionine metabolism is the only known source for homocysteine in mammals, excessive methionine uptake might lead to the various pathophysiological consequences associated with hyperhomocysteinaemia. Elevated plasma homocysteine concentration (hyperhomocysteinaemia) is regarded as an independent risk indicator for cardiovascular events and is associated with renal dysfunction, neuronal diseases and complications in pregnancy. Elevated plasma homocysteine may be caused by enzyme deficiencies or by malnutrition, e.g. vitamin depletion or a high methionine intake.

Several theories have been put forward to explain the pathomechanisms associated with hyperhomocysteinaemia. Under in vitro conditions, homocysteine can interact with various enzymes, leading to an inhibition of the respective enzymatic reaction. This has been described for tyrosinase, histidine ammonia-lyase or dimethylargininase (DDAH). Inhibition of DDAH may consequently lead to accumulation of asymmetrical dimethylarginine (ADMA), which is an inhibitor of endothelial nitric oxide synthase (eNOS). Elevated ADMA diminishes production of nitric oxide and contributes to endothelial dysfunction, making elevated plasma ADMA levels a risk factor for CVD. Adenosine is another known regulator of vessel diameter. Extracellular adenosine concentrations might be lowered in hyperhomocysteinaemia due to a reversed SAH-hydrolase reaction. Because this bidirectional pathway favours intracellular production of SAH, if homocysteine concentrations are increased, this may result in net consumption of adenosine and hence lowered adenosine concentrations. Under those conditions, increased homocysteine concentrations may be reflected by elevated SAH plasma levels. Changes of tissue SAH concentrations are an index for disorders in transmethylation metabolism, which, on one hand, may result in hypermethylation (more SAH is...

Abbreviations: ADMA, asymmetrical dimethylarginine; DDAH, dimethylargininase; eNOS, endothelial nitric oxide synthase; INOS, inducible nitric oxide synthase; MET, methionine-enriched diet; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; SDMA, symmetrical dimethylarginine.

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formed) and, on the other hand, in hypomethylation (SAH inhibits various methyltransferases)\(^{(24,25)}\).

A previous study in human subjects has addressed short-term effects (1–7 d) of methionine or methionine-enriched diet (MET) on homocysteine plasma levels\(^{(26)}\). It was found that plasma homocysteine was unaltered. The present study, conducted in rats, was designed to explore the impact of elevated methionine supplementation over a longer period of time and to assess metabolic consequences more comprehensively with regard to different organs, in addition to the commonly used plasma measurements. With regard to homocysteine-related diseases reported in literature we chose to analyse kidney\(^{(7)}\), heart\(^{(6)}\) and brain\(^{(8)}\). In addition, liver was analysed, because of its special importance for homocysteine metabolism, which includes a second remethylation pathway with betaine as methyl donor and a particularly high activity of the enzymes involved in homocysteine metabolism\(^{(5)}\). Measurements of spleen were included, because this organ is a major blood storage. Furthermore, the potential cardiovascular risk as mediated via homocysteine pathogenicity was addressed by measurement of plasma concentrations of adenosine and dimethylarginine (symmetric and asymmetric). Cholinergic mesenteric artery relaxation was quantitated as a model of endothelium-dependent vessel function. An effect of homocysteine feeding on cholinergic aorta relaxation has been shown in literature\(^{(27)}\). We decided to use a methionine, not a homocysteine, feeding model, as the concentrations of homocysteine in food are far lower than those of methionine\(^{(28)}\). The methionine supplementation was chosen to reflect the upper range that may be consumed additionally during over-nutrition in human individuals.

Methods

Animals and tissue preparation

The experimental project was approved by the University Commission on Animal Experiments with regard to the animal welfare regulations of Germany, which conform to National Institutes of Health guidelines. A written permission for the study was issued by the German local authorities (24-9168-11-1-2004-16).

Male Wistar rats (Charles River Laboratories, Sulzfeld, Germany) were fed either a normal (control, n 9) or MET (n 9) with 4 g/l methionine (Sigma, Taufkirchen, Germany) in the drinking water ad libitum (approximately 40 ml/d). The rationale for choosing this condition was to reflect a 3–4-fold increased methionine intake, as it might occur in human individuals due to over-nutrition. The regular pellet diet (Smiff, Soest, Germany) contained 0.35 g methionine per 100 g and was given to both groups of rats ad libitum (approximately 20 g/d). A duration of 4 weeks feeding was chosen to induce steady state hyperhomocysteinemia\(^{(29)}\).

After 4 weeks, animals were killed by CO\(_2\) exposure, blood was drawn from the vena cava in Li-heparin tubes (Sarstedt, Nuernbrecht, Germany) and plasma was separated from corpuscles immediately after collection by centrifugation at 2000 g. Leucocytes were removed and erythrocytes were washed with 0.9 % NaCl solution and stored at −80°C for analysis of homocysteine. Liver, heart, spleen, kidney and brain were immediately frozen in liquid N\(_2\) and stored at −80°C for analysis of metabolites. Additionally, tissue samples of liver and lung were frozen at −20°C for western blot analysis. For test of vascular function, the mesentry and attached tissue was removed and placed into ice-cold physiological saline solution containing 119 mmol/l NaCl, 4.7 mmol/l KCl, 1.17 mmol/l MgSO\(_4\), 1.18 mmol/l KH\(_2\)PO\(_4\), 25 mmol/l NaHCO\(_3\), 5.5 mmol/l glucose, 0.027 mmol/l EDTA and 2.5 mmol/l CaCl\(_2\).

Isometric force measurement

Vessel function was determined on a Mulvany myograph (Power Lab/400, AD-Instruments, Spechbach, Germany) connected to a PC by interface Model 410A (Danish Myo Technologies, Aarhus, Denmark) using mesenteric arterial vessels\(^{(30)}\). The software Chart v4.1.2 (AD Instruments, Spechbach, Germany) was used for data acquisition. Freshly isolated mesenteric arteries were cleared from surrounding adipose tissue and cut into segments of about 2 mm width. The rings were carefully mounted in a computer-linked force transducer placed in a 10 ml organ chamber (Mitu-toyo, Kawasaki, Japan) filled with physiological saline solution. The organ bath was warmed to 37°C and continuously bubbled with 5 % CO\(_2\) and 95 % O\(_2\) to maintain pH at 7.4. Rings were equilibrated with a resting tension equivalent to that obtained by exposure to an intraluminal pressure of 100 mm Hg. Maximum contraction was obtained by exposure of vessel rings to K-enriched physiological saline solution, containing 123.7 mmol/l KCl, 1.17 mmol/l MgSO\(_4\), 1.18 mmol/l KH\(_2\)PO\(_4\), 25 mmol/l NaHCO\(_3\), 5.5 mmol/l glucose, 0.027 mmol/l EDTA and 2.5 mmol/l CaCl\(_2\). Vasorelaxation was induced with 7.5 µmol/l noradrenalin. Endothelium-dependent vasorelaxation of mesenteric arteries was determined using acetylcholine (1 to 3 µmol/l) and endothelium-independent relaxation using sodium-nitrprusside (0.3 to 3 µmol/l). EC\(_{50}\)-values were calculated from these concentration-response curves.

Western blot analysis of endothelial nitric oxide synthase and inducible nitric oxide synthase

Proteins were isolated by chloroform/methanol precipitation and protein content was determined by amido black method as published\(^{(31)}\). Identical amounts of protein were used for each lane. For western blot analysis of eNOS from lung and liver, proteins were separated by 7.5 % SDS-PAGE. For analysis of inducible nitric oxide synthase (iNOS), proteins were separated on a 5 % SDS-polyacrylamide gel. Separated proteins were transferred to nitrocellulose membranes (Amer-sham, Munich, Germany) by electro blotting. Anti-eNOS and anti-iNOS were purchased from BD Pharmingen (Heidelberg, Germany) and anti-β-tubulin was purchased from Sigma (Taufkirchen, Germany). eNOS and iNOS were detected by enhanced chemiluminescence (ECL western blotting detection reagents (Amersham, Munich, Germany). Horseradish peroxidase-conjugated goat anti-rabbit IgG and sheep anti-mouse IgG were purchased from Dianova (Hamburg, Germany). Protein expression was calculated as intensity of blotting bands relative to β-tubulin.
**Liquid chromatography–tandem MS analysis of arginine, asymmetrical and symmetrical dimethylarginine**

ADMA and symmetrical dimethylarginine (SDMA) were determined together with arginine by liquid chromatography–tandem MS as described previously[32]. In brief, samples (50 μl) were precipitated with 100 μl acetonitrile and supernatants were derivatized with 100 μl 1 M-HCl in butanol (20 min, 65°C) after evaporation. Deuterated ADMA (2 μmol/l; d5-ADMA) and 50 μmol/l deuterated arginine (d1-arginine) were added as internal standards prior to precipitation. Butyl ester derivatives of arginine, ADMA and SDMA were analysed in the electron spray ionisation (ESI) positive mode. The daughter ions m/z 70, 214 and 228 were scanned for arginine, ADMA and SDMA, respectively. The daughter ions with m/z ratios of 77 and 220 were scanned for the internal standards d1-arginine and d5-ADMA, respectively. Peak area ratio was determined and concentrations (μmol/l) were calculated according to calibration curves.

**HPLC analysis of homocystine**

Plasma total homocysteine levels were measured by HPLC with a ready-to-use kit (Immundiagnostic, Bensheim, Germany) after reduction of disulfide bounds. HPLC analysis was performed on a Waters Alliance liquid chromatography module (Waters, Eschborn, Germany) with a Merck-Hitachi fluorescence detector (Merck, Darmstadt, Germany) working at excitation/emission wavelengths of λex 385 nm and λem 515 nm. Separation was carried out on a Novapak RP-C18 column (Waters). Tissue and erythrocyte sample preparations were as follows. Tissue samples were homogenized with a half-automated Potter homogenizer in ice-cold 0·1 mol/l perchloric acid. Precipitated proteins were removed by centrifugation for 15 min at 20 000 g and 4°C. Samples were neutralized immediately after homogenization with 1 mol/l K2PO4. Erythrocytes (500 μl) were disrupted with 300 μl 0·1 mol/l perchloric acid and then immediately neutralized.

**HPLC analysis of plasma adenosine and S-adenosylhomocysteine**

HPLC analysis was based on 1,N6-etheno-derivatization of the adenine body. Derivatization and HPLC conditions were adapted from Haink & Deussen[33] with few modifications. In 200 μl plasma samples, proteins were precipitated with 100 μl perchloric acid (1 mol/l) and separated by centrifugation for 15 min at 20 000 g and 4°C. Samples were neutralized with K2PO4 (1 mol/l) and precipitated potassium perchlorate was removed by centrifugation for 15 min at 20 000 g and 4°C. From the supernatant, 143 μl were added to 51 μl citrate-phosphate-buffer (containing 0·1 mol/l citric acid and 0·2 mol/l Na2HPO4, pH 4). Derivatization was started by addition of 6 μl chloracetalddehyde (40 %; Merck). HPLC analysis was performed on the Waters Alliance liquid chromatography module connected to a Merck Hitachi fluorescence detector (λex 280 nm, λem 410 nm). Separation was carried out on an XTerraMS-C18 column (4·6 x 50 mm, particle size 5 μm; Waters) using a linear phosphate-buffer/acetonitrile gradient from 2 to 70 % acetonitrile over 15 min) with ion-pair reagent pentanesulfonic acid (10 μmol/l) at pH 5. Retention times were 4·8 min for SAM and 8·9 min for SAH.

**Statistical analysis**

The experiment number necessary to detect intergroup differences was estimated by power analysis (α = 0·05; β = 0·8). Data are presented as mean values with their standard errors of the mean. Distribution of data was evaluated by Kolmogorov–Smirnov test. To assess differences of mean values between groups Student’s t test for unpaired data was used (two-sided). Correlation coefficient r was calculated according to Pearson in the methionine feeding group. A value of P < 0·05 was considered to indicate a statistically significant difference. Statistics were performed using SPSS for Windows (version 12.0; SPSS Inc.).

**Results**

**Plasma concentrations of cardiovascular risk indicators**

Rats consumed on average 20 g pellets per d and drank on average 40 ml water. Calculated average methionine intake was 70 mg per d in the control group and 230 mg per d in the high methionine group, respectively (3·3-fold elevated intake). Plasma concentrations of risk indicators for disturbed
cardiovascular functions are summarized in Table 1. Plasma homocysteine concentration was doubled \((P<0.05)\) in animals fed MET. Plasma concentrations of ADMA were not altered significantly by the MET. However, plasma concentrations of SDMA were significantly elevated in rats fed MET. There was no significant change of arginine plasma level. Plasma concentrations of SAH and adenosine were not significantly altered by the MET.

**Effects on vessel function**

Examined vessel segments did not differ significantly between rats fed MET or a normal methionine diet with regard to length \((control\ 1-7 \ (SEM\ 0.2)\ mm,\ MET\ 1-7 \ (SEM\ 0.2)\ mm)\) and internal diameter \((control\ 316 \ (SEM\ 64) \ \mu m,\ MET\ 308 \ (SEM\ 54) \ \mu m)\). Acetylcholine and sodium nitroprusside, respectively, evoked the typical concentration-related vasorelaxations in the mesenteric vessel preparations. Half maximal relaxations were obtained at \(7.19 \ (SEM\ 0.78 \times 10^{-5})\) mol/l for acetylcholine and \(7.24 \times 10^{-5} \ (SEM\ 1.38 \times 10^{-5})\) mol/l for sodium nitroprusside in vessels from control rats. In animals receiving MET, the endothelium dependent \((7.49 \times 10^{-8} \ (SEM\ 1.66 \times 10^{-8})\) mol/l) and independent \((4.43 \times 10^{-5} \ (SEM\ 0.90 \times 10^{-5})\) mol/l) vasorelaxations were similar to those assessed in the control group. eNOS expression was similar in lung and liver extracts of rats fed a control diet (Fig. 1). iNOS expression was lower than eNOS expression in rats fed MET as compared with animals fed the control diet.

**Tissue concentration of homocysteine and its precursors**

Tissue levels of homocysteine largely differed among organs of control rats. Highest homocysteine tissue levels were found in liver \((34.2 \ (SEM\ 5.0)\ nmol/g)\) and lowest levels were found in kidney \((8.6 \ (SEM\ 1.3)\ nmol/g)\) (Fig. 2). MET had no significant effect on homocysteine levels of liver, heart, spleen and kidney. However, the homocysteine level of spleen was significantly \((P<0.05)\) elevated in rats that had received MET. Homocysteine level of erythrocytes was unaltered in rats fed MET \((1-4 \ (SEM\ 0.5)\ \mu mol/l)\) as compared with control animals \((1-3 \ (SEM\ 0.5)\ \mu mol/l)\). To address relationships between plasma and spleen homocysteine, the respective correlations were calculated using the data from individual experiments. Within the methionine group, homocysteine was elevated in plasma and spleen, but no significant correlation existed \((r=-0.177;\ P=0.512)\). There was also no significant correlation between erythrocyte and spleen homocysteine levels \((r=-0.144;\ P=0.596)\).

While 4 weeks of MET was associated with enhanced homocysteine plasma levels (Table 1), no significant elevation of plasma methionine was found in animals fed MET \((71 \ (SEM 14) \ \mu mol/l)\) compared with the control group \((50 \ (SEM 6) \ \mu mol/l)\). Tissue concentrations of methionine, SAM, SAH and the ratio of both substances, often referred to as ‘transmethylation potential’, are summarized in Table 2. From the various organs studied, only the spleen showed a significantly elevated methionine concentration after MET. Spleen also showed the highest methionine level among the studied organs, followed by kidney. Under control conditions, SAM level was highest in liver and approximately 50 % lower in heart, spleen and kidney. Brain SAM level was only one-third of that in liver. MET did not significantly change SAM levels in any organ studied. Control SAH level was highest in liver followed by kidney \((40 \ %)\ lower\). Brain, heart and spleen showed SAH levels in the order of 10 to 20 % of those found in liver. MET further enhanced liver SAH level, but did not change SAH levels of other organs studied. Despite the increase of liver SAH, the transmethylation potential was not significantly decreased in this organ. The sum of essential amino acids (valine, leucine, isoleucine, threonine, methionine, phenylalanine, lysine, tryptophan) was significantly elevated in plasma of rats fed MET (Table 1), but unchanged in organ tissues (data not shown).

**Discussion**

High methionine uptake has been discussed controversially as a potential risk for elevation of plasma homocysteine \((2,6,14,35)\). In human subjects, a single application of a high methionine dose \((0.1 \ g/kg)\) resulted acutely in increased plasma homocysteine \((14)\). However, a diet containing twice the daily intake \((2·1 \ g/d\ instead\ of\ 1·1 \ g/d)\) in volunteers showed no significantly elevated plasma homocysteine after 1 week \((26)\). Because the rat model permits the assessment of organ metabolites in addition to plasma levels, this approach may provide interrelations of methionine and homocysteine metabolism and thus add important information to human studies. In the present study, 4 weeks of MET resulted in a 2-fold elevated homocysteine plasma level in male Wistar rats. Our experiments reflect conditions like those found in moderate hyperhomocysteinemia, e.g. observed during malnutrition \((6)\).

One important question to be addressed in the present study is whether the doubling of plasma homocysteine over 4 weeks of MET resulted in changes of endothelium-dependent mesenteric artery function as a standard model of arterial vessel relaxation \((30)\). We found no significant difference between the vessel function of mesenteric arteries of animals fed MET compared with controls. Other studies \((36)\) found significantly altered arteriolar function in rats in response to 4 weeks of MET. However, it should be noted, that these authors used far higher feeding doses of methionine \((1 \ g/kg\ body\ weight\ per\ d)\), which resulted in a higher increase in plasma homocysteine (from 7·1 to 23·6 \ \mu mol/l), as compared
with the present study. The methionine dose applied in the present study was chosen to reflect the upper limit of human malnutrition (3–4-fold elevation of methionine uptake). Thus, pathophysiological effects reported in the earlier study(36) are unlikely to result from malnutrition.

We tested for an influence of the diet on eNOS expression in lung and liver and iNOS expression in lung, but did not find any altered expression. Homocysteine was described to stimulate the expression of iNOS in macrophages in vitro(37). We were unable to prove this effect in vivo. We conclude from our study that 2-fold elevation of plasma homocysteine induced by MET (3–3-fold elevation of methionine uptake) has no impact on endothelium-dependent mesenteric artery relaxation and no pronounced effects on eNOS and iNOS expression.

As many of the proposed effector mechanisms of hyperhomocysteinemia are based on direct interactions of homocysteine with enzymes inside cells, e.g. inhibition of DDAH by homocysteine leading to an elevated concentration of ADMA, we have tested whether the homocysteine content in erythrocytes, spleen, brain, liver, kidney and heart was altered after 4 weeks of MET. A significant elevation of tissue homocysteine was only found in spleen. Because spleen is a major blood storage, we considered whether homocysteine present in plasma, erythrocytes or leucocytes might have contributed to the augmented homocysteine concentration in this organ. However, correlations of homocysteine concentrations in plasma and spleen and in erythrocytes and spleen did not reveal a significant relationship. Thus, stored plasma or erythrocytes cannot explain elevated spleen homocysteine. Because homocysteine concentrations of leucocytes were not measured in the present study, leucocytes remain a possibility to explain the elevation of homocysteine levels in spleen. In this respect, it is noted here that cultured mononuclear blood cells have been described to exhibit an enhanced secretion of homocysteine under conditions of elevated methionine concentration(38).

Disorders in transmethylation metabolism are another commonly described effector mechanism of hyperhomocysteinemia. Although SAM and SAH may decrease shortly after administration of methionine(39), in long-term experiments, as performed here, elevations of both metabolites are expected(34). The increase of the transmethylation product SAH in liver after methionine feeding (Table 2) may be explained by the following two alternatives: first, by substrate saturation of SAH-hydrolase(3); second, by reversed action of this bidirectional enzyme, when homocysteine concentrations are raised (40). Liver homocysteine concentrations were not significantly altered. Thus, an enhanced synthesis of SAH from adenosine and homocysteine is unlikely and the enhanced SAH level most likely indicates an accelerated methionine metabolism via transmethylation reactions and a rate-limitation of this pathway by activity of SAH-hydrolase.

Elevated homocysteine has been associated with elevated levels of ADMA, because homocysteine may inhibit DDAH, an ADMA catabolizing enzyme(16,17). As shown in Table 1, the mean ADMA plasma concentration was higher in the methionine group as compared with control animals, but this difference did not reach statistical significance. Another link between homocysteine and arginine metabolism is the transmethylation of arginine to SDMA or ADMA(41). As SDMA plasma concentrations were significantly elevated in the methionine enriched-fed animals compared with the control group, an enhanced methylation of arginine might explain the trend of ADMA elevation rather than inhibited ADMA degradation via DDAH. The fact that ADMA was not significantly changed in the current study is compatible...
with the finding that acetylcholine-induced relaxation of mesenteric arteries was also unchanged after 4 weeks of an enriched methionine diet, which caused plasma homocysteine levels to double.

**Study limitations**

Methionine supply in the present study was scaled to reflect conditions of maximal dietary intake of human individuals. However, results obtained in experimental models should be treated with caution. When methionine is supplied in drinking water, the food uptake could be lowered, which might lead to a shortage of essential amino acids. This effect might have influenced the results of the study, as a high methionine diet is typically not induced by enhanced uptake of the pure substance methionine but by over-nutrition, comprising a mix of various amino acids. Because we did not find an alteration of total amino acid content of plasma and of examined tissues, it is unlikely that MET disturbed food uptake in our study.

The present study was conducted in the rat, which has served as a model for studying homocysteine metabolism before. As shown in Table 1, plasma homocysteine level in the rat is considerably lower than that in man (6 to 12 μmol/l). Therefore, absolute levels should not be compared.

Taken together, we have found a significant, 2-fold elevation of plasma homocysteine following 4 weeks of MET. However, no alterations in the mesenteric artery vessel dilator function were observed. Furthermore, no alterations of risk indicators such as elevated plasma ADMA concentration, elevated plasma SAH or lowered plasma adenosine concentration were evident. We conclude that a 2-fold elevation of plasma homocysteine induced by 4 weeks of MET had only a small impact on tissue metabolites related to methionine and no significant impact on mesenteric artery dilatory function.

**Acknowledgements**

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**References**


**Table 2.** Methionine, S-adenosylmethionine (SAM), S-adenosylhomocysteine (SAH) and transmethylation potential (SAM/SAH) in various tissues under control conditions and 4 weeks of methionine-enriched diet (nmol/g)††

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*Mean value was significantly different from control (*P* < 0.05).
†† For details of diets and procedures, see Methods.


