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Journal of Nutritional Biochemistry 15 (2004) 666–671

THE JOURNAL OF Nutritional Biochemistry

Effects of intravenous betaine on methionine-loading–induced plasma homocysteine elevation in rats

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Received 10 December 2003; received in revised form 22 April 2004; accepted 28 May 2004

Abstract

An intravenous methionine-loading model was characterized, and the suppressive effect of betaine on plasma homocysteine elevation induced by methionine loading was examined in rats. The plasma homocysteine concentrations significantly increased 5–120 minutes after 0.34 mmol/kg of methionine loading and then returned to the baseline within 240 minutes. Betaine was then intravenously administered at the same time as the methionine loading. The total increment of plasma homocysteine was assessed using the positive incremental area under the plasma homocysteine concentration curve over the 240-minute post–methionine-loading period ($\Delta AUC_{0\text{-}240}$).

Betaine reduced $\Delta AUC_{0\text{-}240}$ dose-dependently: 81% of the control by 1.7 mmol/kg of betaine and 33% by 3.4 mmol/kg. The effects of glycine and methylglycine, analogues of betaine, were also investigated. As observed for betaine, methylglycine decreased $\Delta AUC_{0\text{-}240}$ to 44% of the control, whereas glycine showed no significant effect on $\Delta AUC_{0\text{-}240}$, indicating that methyl groups of betaine and dimethylglycine were necessary to suppress plasma homocysteine elevation. These results suggest that betaine contributes to the suppression of plasma homocysteine elevation by promoting homocysteine metabolism, and seems to work as a methyl donor.

Keywords: Betaine; Homocysteine; Methionine loading; Methyl group; Rats

1. Introduction

Betaine (trimethylglycine) is a natural component that is widely found in most living organisms and that has been consumed for ages. In the living body, it is an endogenous catabolite of choline [1]. Since three methyl groups are contained in its structure, betaine is presumed to work as a methyl donor and to play an important role in homocysteine metabolism [2]. Homocysteine, a sulfur-containing amino acid, is a metabolite of methionine. Previous studies have shown that high plasma homocysteine concentrations are a potential risk factor for arterial sclerosis and cardiovascular diseases, with no clear-cut threshold [3–6]. There are two major pathways for homocysteine metabolism: 1) remethylation to methionine by 5-methyltetrahydrofolate-homocysteine methyltransferase (MFHT; EC 2.1.1.13), and 2) metabolism to cystathionine by cystathionine β-synthase (CBS; EC 4.2.1.22) [7]. Methylenetetrahydrofolate-reductase (MTHFR; EC 1.1.1.68) catalyzes conversion of 5, 10-methylentetrahydrofolate (MeTHF) to methyltetrahydrofolate (MTHF) and also plays a significant role in the regulation of the intravital homocysteine level [7]. Folic acid, vitamin B_{12}, and vitamin B_6 regulate these pathways, and supplementation of these nutrients lowers the intravital homocysteine level [8–12]. In addition to these nutrients, betaine is also essential for the metabolic pathway of homocysteine driven by betaine–homocysteine methyltransferase (BHMT; EC 2.1.1.5), providing homocysteine with a methyl group for remethylation to methionine. Thus, it is strongly expected that supplementation with betaine would promote metabolism of homocysteine and reduce the intravital homocysteine level. In fact, betaine supplementation is effective for reducing plasma homocysteine levels in humans [13,14] and in homocystinuria patients with MTHFR deficiency [15]. Although it is commonly known that betaine plays an important role in homocysteine metabolism, the relevance of the supplementation of betaine and the intravital homocysteine level has not re-

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0955-2863/04/$ – see front matter
doi:10.1016/j.jnutbio.2004.05.004

ceived much attention except in a few studies. In this study, we examined the suppressive effect of betaine on the elevation of the plasma homocysteine level.

As a typical hyperhomocysteinemia model, the folate-deficient model in rats, in which homocysteine remethylation is inhibited by the deficiency, is commonly used [16,17]. However, there are two major disadvantages to this model: 1) it takes ≥4 weeks to induce hyperhomocysteinemia via folate deficiency, and 2) the general state of animals gets considerably worse during the test period; for example, it causes severe anemia. We therefore characterized an intravenous methionine loading model as a concise hyperhomocysteinemia model in rats and tested the effect of betaine on the post–methionine-loading elevation of plasma homocysteine. In addition, the contribution of methyl groups to the suppressive effect on plasma homocysteine elevation was examined using betaine and its analogues. It is conceivable that prevention of elevation of the homocysteine level is the most prominent factor lowering the risk for arterial sclerosis and cardiovascular diseases, although the reduction of the elevated homocysteine level is also effective. The present study is important for estimating whether betaine reduces homocysteine levels and prevents arterial sclerosis and cardiovascular diseases related to homocysteine from occurring.

2. Methods and materials

2.1. Chemicals

All chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) unless otherwise noted.

2.2. Animals

All experimental protocols were approved by the Prime Minister's Office in Japan (No. 6 of March 27, 1980). Male Wistar rats weighing 150–200 g (Japan SLC Ltd., Hamamatsu, Shizuoka, Japan) were used throughout this study. They were housed individually in a stainless steel cage (W150 × L210 × H170 mm) at 22 ± 2°C with a 12-hour dark/light cycle (light: 6:00 to 18:00) and fed a commercial laboratory diet (Certified Diet MF, Oriental Yeast, Co., Tokyo, Japan). Water was available *ad libitum*.

2.3. Methionine loading and sample preparation

After a 1-week acclimation period and 18-hour fasting, rats were intravenously injected with methionine at a dose of 0.34 (low-Met) or 0.67 (high-Met) mmol/kg. Betaine, methylglycine, and glycine were also administered intravenously at the same time as methionine loading. All chemicals were dissolved in saline (Otsuka Pharmaceutical Co., Tokyo, Japan). In one experiment, rats received an intravenous treatment alone without chemicals. Approximately 250 μL of blood was taken from the jugular vein under diethyleter anesthesia immediately before and at 5, 15, 30, 60, 120, and 240 minutes after methionine loading. Blood samples were promptly collected into a polypropylene tube containing heparin-Na and centrifuged at 5000 × *g* for 10 minutes. The supernatant was separated as a plasma sample and kept frozen at −80°C until analysis.

2.4. Analytical methods

The plasma homocysteine concentration was determined according to previous methods [18] with slight modifications. Plasma samples (100 μL) were treated with 10 μL of 10%(v/v) tri-*n*-butylphosphine in dimethylformamide for 30 minutes at 4°C. The mixture was chilled and mixed with 100 μL of 10% trichloroacetic acid and 1 mmol/L Na_2EDTA, and centrifuged at 15,000 × *g* for 5 minutes at 4°C. The supernatant (20 μL) was separated and treated with 8 μL of 0.75 mol/L sodium hydroxide, 50 μL of 0.11 mol/L borate buffer (pH10.5), 4 mmol/L disodium dihydrogen ethylenediamine tetraacetate, and 20 μL of 1 mg/mL ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate (Sigma, St. Louis, MO, USA) for 60 minutes at 60°C. After incubation, the mixture was chilled on ice. Homocysteine in aliquots was analyzed using an HPLC system consisting of a Shimadzu LC-10AD pump, a Shimadzu Chromatopac C-R6A recorder and a Shimadzu RF-10AXL fluorescence detector (excitation at 385 nm, emission at 515 nm) (Shimadzu Co., Kyoto, Japan). Separation was carried out using a Wakosil-II 5C18 column (4.6 × 250 mm) (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Analysis was performed under isocratic conditions (0.1 mol/L acetate buffer with 2% methanol, pH 4.0: 0.1 mol/L phosphate buffer with 5% methanol, pH 6.0 = 1:1) at a flow rate of 1.0 mL/min. The concentration of homocysteine was calculated using DL-homocysteine as a standard.

Increments of plasma homocysteine concentration (ΔHcy) were calculated according to the following equation: ΔHcy = postloading concentration − preloading concentration.

The area under the ΔHcy − time curve from 0 to 240 minutes ($\Delta AUC_{0\text{-}240}$) was calculated using the linear trapezoidal rule [19,20]. Individual preloading plasma homocysteine concentrations were used as baseline for the calculation of $\Delta AUC_{0\text{-}240}$.

2.5. Statistical analysis

Comparisons between two or more groups were performed using the Student *t* test and one-way analysis of variance followed by the Bonferroni/Dunn multiple range test, respectively, with StatView J. 5.0 for Windows (SAS Institute Inc., Cary, NC, USA).

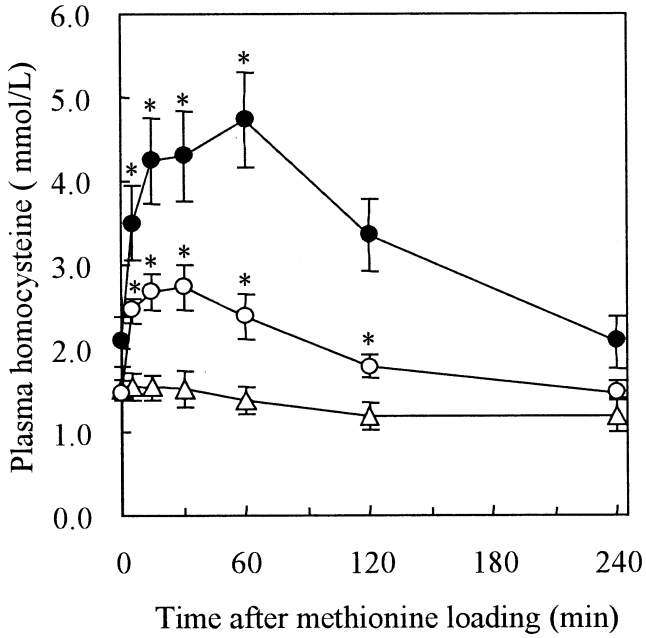

Fig. 1. Time course of plasma homocysteine after methionine loading. Values are means ± SEM, $n = 4$ for blank (□), $n = 5$ for low-Met (methionine 0.34 mmol/kg) (○) and high-Met (methionine 0.67 mmol/kg) (●).* Significantly different from initial value (0 min), Student t test for within-subject comparisons ($P < 0.05$).

3. Results

3.1. Change in plasma homocysteine after methionine loading

Methionine was intravenously administered to the rats and plasma homocysteine concentrations were measured at various time points. The levels of homocysteine elevated immediately (5 minutes) after administration, and peak concentrations were observed at 30 minutes in the low-Met group and at 1 hour in the high-Met group, with a mean increment of 1.26 μmol/L and 2.65 μmol/L, respectively (Fig. 1). In these methionine-treated groups, the plasma homocysteine concentrations significantly exceeded the preloading level for 5–120 minutes and then returned to the baseline concentration within 240 minutes. The coefficient of variation (CV) ranges of plasma homocysteine concentration for all time points after methionine loading are 2.8–15.5% in the low-Met group and 26.7–33.2% in the high-Met group. No significant change was observed in the homocysteine levels of the blank group (no methionine; vehicle only) up to 240 minutes after methionine loading. The general state of rats did not change after methionine loading in both the control and the two methionine-treated groups.

The low-Met dose was adopted for subsequent studies for the following two reasons: 1) substantial elevation of the plasma homocysteine concentration was induced, and 2) the individual variation of increments of the plasma homocysteine level was less in the low-Met group.

3.2. Effect of betaine on the elevation of homocysteine and contribution of the methyl group to the effect

The increments of the plasma homocysteine concentration after methionine loading and the positive incremental area under the plasma homocysteine concentration curve over the 240-minute post–methionine-loading period ($\Delta AUC_{0\text{-}240}$) are shown in Tables 1 and 2, respectively.

In the low-Bet group (betaine 1.7 mmol/kg), the time course of the plasma homocysteine increment is slightly low compared with the control group, although there was no significant difference at any time point. Increments of the plasma homocysteine concentration in the high-Bet group (betaine 3.4 mmol/kg) were significantly lower than those in the control group and low-Bet group at 15, 30, and 60 minutes after methionine loading. Furthermore, betaine reduced $\Delta AUC_{0\text{-}240}$ dose-dependently: 81% of the control in the low-Bet group and 33% in the high-Bet group. These results showed that betaine suppressed plasma homocysteine elevation after methionine loading.

To investigate the correlation between the number of methyl groups and the suppressive effect on plasma homocysteine elevation, the increments of plasma homocysteine concentration and $\Delta AUC_{0\text{-}240}$ were compared among the control, high-Bet, methylglycine (MGly) and glycine (Gly) groups. Betaine, methylglycine, and glycine have three, one, and no methyl groups, respectively. The time course of the plasma homocysteine increment in the Gly group (glycine 3.4 mmol/kg) was similar to that in the control group, except that the increment of the plasma homocysteine was significantly higher at 5 minutes. No significant difference was observed between

Table 1
Effects of betaine and its analogs on the increments of plasma homocysteine

Time after methionine loading (min)	Increments of plasma homocysteine (μmol/L)				
	Control ($n = 5$)	Low-Bet ($n = 8$)	High-Bet ($n = 6$)	MGly ($n = 11$)	Gly ($n = 8$)
5	0.98 ± 0.068	1.08 ± 0.12	0.73 ± 0.065	0.58 ± 0.048	1.66 ± 0.18*†
15	1.18 ± 0.056	0.97 ± 0.050	0.68 ± 0.068*	0.73 ± 0.057*	1.77 ± 0.16†
30	1.18 ± 0.11	0.89 ± 0.061	0.58 ± 0.089*	0.59 ± 0.046*	1.34 ± 0.087†
60	0.66 ± 0.087	0.52 ± 0.048	0.11 ± 0.051*	0.31 ± 0.054	0.60 ± 0.059
120	0.11 ± 0.059	0.075 ± 0.019	N.I.	0.011 ± 0.0078	0.10 ± 0.048
240	0.064 ± 0.045	0.024 ± 0.013	N.I.	0.006 ± 0.0048	0.051 ± 0.031

Methionine was intravenously administered to rats at a dose of 0.34 mg/kg. A quantity of 1.7 mmol/kg of betaine (low-Bet), 3.4 mmol/kg of betaine (high-Bet), 3.4 mmol/kg methylglycine (MGly), 3.4 mmol/kg glycine (G), or vehicle (Control) was administered simultaneously with methionine loading. Concentrations of plasma homocysteine were measured at various time points, and post–methionine-loading increments of plasma homocysteine were calculated. Values are means ± SEM.

*,† Significantly different from control and high-Bet, respectively, within the particular time point (Bonferroni/Dunn's multiple range test, $P < 0.05$).

N.I. = no increment observed.

MGly (methylglycine 3.4 mmol/kg) and high-Bet groups up to 240 minutes. In the high-Bet and MGly groups, $\Delta AUC_{0\text{-}240}$ declined significantly to 33% and 44% of the control, respectively, whereas the difference in $\Delta AUC_{0\text{-}240}$ between Gly and control groups was not significant.

These results showed that the presence of methyl groups was essential to the suppressive effect on plasma homocysteine elevation.

4. Discussion

This study was conducted to characterize an intravenous methionine-loading model in rats and to examine the suppressive effect of betaine and its analogues on the methionine-loading–induced elevation of the plasma homocysteine level. In the present study, we established a model for temporary hyperhomocysteinemia in rats by intravenous methionine loading. The methionine loading test is clinically used to identify latent abnormalities in homocysteine metabolism [4]. In the methionine-loading model reported previously, methionine was administered orally in humans [21] and in rats [22], and intraperitoneally in rats [23]. Individual differences throughout the process of methionine absorption seem to occur in these administration routes. The intravenous methionine loading characterized in this study is useful, in that this expected individual difference is avoided. Moreover, we followed the time course of plasma homocysteine concentrations after methionine loading up to 240 minutes, when the plasma homocysteine level returned to the baseline value. The total increment of the plasma homocysteine level, $\Delta AUC_{0\text{-}240}$, as well as the increment for a particular time was determined. This resulted in an accurate assessment. Our methionine-loading model induced hyperhomocysteinemia in an extremely short time, and the general state of rats did not change with the methionine loading. These findings show that our model has the advantages of assessing plasma homocysteine variation accurately and relieving the stress of experimental animals.

Table 2
Comparison of ΔAUC_{0-240} between betaine and its analogs

Group	ΔAUC_{0-240} (mmol · min · L^{-1})	% Control
Control ($n = 5$)	91.8 ± 10.4[a]	—
Low-Bet ($n = 8$)	74.3 ± 5.5[b]	80.9
High-Bet ($n = 6$)	30.6 ± 4.8[c]	33.3
MGly ($n = 11$)	40.1 ± 3.6[c]	43.7
Gly ($n = 7$)	101.7 ± 6.8[a]	110.8

Values are means ± SEM.

[abc]Values with different superscripts are significantly different (Bonferroni/Dunn multiple range test, $P < 0.05$).

In this study, methionine-loading–induced elevation of the plasma homocysteine level was shown to be suppressed by intravenously administered betaine. Betaine provides its methyl group to homsycteine through the intermediation of BHMT, which is specially contained in the liver [24]. Therefore, intravenously administered betaine seems to be transferred to the liver and played its role as a substrate for BHMT there. This result implies the hypothesis that orally ingested betaine suppresses plasma homocysteine elevation when it is absorbed into the blood. Indeed, it has been reported that orally ingested betaine was detected in plasma [25]. The hepatic content of betaine is related to dietary levels of choline, a precursor of betaine [26], and choline intake would also induce a suppressive effect of plasma homocysteine. Hence, daily ingestion of betaine is expected to prevent elevation of plasma homocysteine.

An increased plasma homocysteine concentration is a strong independent risk factor for arterial sclerosis and cardiovascular diseases [3,4,6]. It has been shown that hyperhomocysteinemia is also related to the incidence of some other diseases, such as Alzheimer dementia and cancer [27–29]. Supplementation with folic acid and vitamin B_{12} is effective for the descent of high-level plasma homocysteine

[8,11,12] and is thought to be useful in preventing these diseases. On the other hand, it is critically important to prevent the occurrence of hyperhomocysteinemia. Our study potently indicates that betaine makes a substantial contribution to the prevention of these diseases through the avoidance of plasma homocysteine elevation.

As a methyl donor, betaine plays an essential role in the metabolic pathway of homocysteine driven by BHMT. It is noteworthy that methylglycine has a betaine-like structure containing one methyl group, and is also expected to be used by BHMT. In this study, the suppressive effect on plasma homocysteine elevation of betaine and its analogues, methylglycine and glycine, is shown to be correlated with the presence or absence of methyl groups. This correlation suggests that the suppressive effect of betaine is expressed through the remethylation pathway of homocysteine driven by BHMT. Betaine and homocysteine is included in the complicated metabolic pathway with many reactions [7]. Methylglycine might be demethylated, converted to MTHF and participate in the MFHT-related metabolic pathway of homocysteine [30]. Further studies will be needed to clarify the alteration in BHMT activity by application of betaine and its analogues.

In the metabolic pathway of homocysteine mediated by folic acid, conversion from tetrahydrofolate (THF) to MeTHF is accompanied by the synthesis of glycine from serine [7]. There is a hypothesis that an excess level of glycine might inhibit this reaction, which would result in an interference in the conversion of THF to MeTHF. Also, remethylation of homocysteine will be blocked. On the other hand, glycine-*N*-mehyltransferase (EC 2.1.1.20) regulates the S-adenosylmethionine/S-adenosylhomocysteine (SAH) ratio. This regulation leads to the other hypothesis that an excess level of glycine might be used by glycine-*N*-methyltransferase and result in a high level SAH followed by an increase in homocysteine synthesis. These hypotheses are in agreement with our results that the plasma homocysteine concentration increased several minutes after methionine loading in the Gly group.

In summary, intravenously injected betaine suppresses plasma homocysteine elevation in the intravenous methionine loading model characterized in this study and seems to work as a methyl donor. Considering the previous evidence that orally ingested betaine was absorbed into the blood, it is strongly suggested that betaine can suppress homocysteine by oral ingestion.

Acknowledgment

We thank Ms. Akiko Tamura, Central Research Laboratory, Fancl Co. (Yokohama, Japan), for her helpful discussions regarding this study.

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