Original Article

Cystine supplementation sustains plasma mercaptalbumin levels in rats fed low-protein diets more effectively than methionine

Yukimi Yano,[1] Chihiro Maeda,[1] Ichiro Kaneko,[2] Yukiko Kobayashi,[1] Wataru Aoi,[1] and Masashi Kuwahata[1,*]

[1]Department of Nutrition Science, Graduate School of Life and Environmental Sciences, Kyoto Prefectural University, Shimogamo-hangi-cho, Sakyo-ku, Kyoto 606-8522, Japan
[2]Department of Applied Nutrition, Tokushima University Graduate School of Biomedical Sciences, 3-18-15 Kuramoto, Tokushima 770-8503, Japan

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We recently reported that dietary cystine maintained plasma mercaptalbumin levels in rats fed low-protein diets. The present study aimed to compare the influence of low-protein diets supplemented with cystine and methionine, which is another sulfur amino acid, on plasma mercaptalbumin levels in rats. Male Sprague–Dawley rats were fed a 20% soy protein isolate diet (control group), 5% soy protein isolate diet (low-protein group) or 5% soy protein isolate diet supplemented with either methionine (low-protein + Met group) or cystine (low-protein + Cyss group) for 1 week. The percentage of mercaptalbumin within total plasma albumin of the low-protein + Met group was significantly lower than that of the control and low-protein + Cyss groups. No significant differences in the mRNA levels of tumor necrosis factor-α, interleukin-6, interleukin-1β, and cyclooxygenase 2 in blood cells were observed between the low-protein + Met and low-protein + Cyss groups. Treatment with buthionine-(S,R)-sulfoximine, an inhibitor of glutathione synthesis, did not influence the percentage of mercaptalbumin within total plasma albumin in rats fed the low-protein diet supplemented with cystine. These results suggest that supplementation with cystine may be more effective than that with methionine to maintain plasma mercaptalbumin levels in rats with protein malnutrition. Cystine might regulate plasma mercaptalbumin levels via the glutathione-independent pathway.

***Key Words*: mercaptalbumin, cystine, methionine, low-protein diet, rat**

Albumin is one of the major proteins synthesized in the liver, and it is the most abundant protein in the plasma of healthy individuals. The albumin molecule contains 35 cysteine residues and forms 17 intramolecular disulfide bonds.[1] Thus, albumin has a single free cysteine residue at the 34th position from the N-terminal (Cys34). The albumin molecule having a free thiol in Cys34 is referred to as mercaptalbumin (MA), which is the reduced form of albumin.[2] On the other hand, albumin molecules in which Cys34 forms a mixed disulfide with small thiol compounds and sulfinic/sulfonic acid, which is the oxidized form of albumin, are referred to as nonmercaptalbumin-1 (NA-1) and NA-2, respectively. In healthy adults, MA accounts for 70% to 80%, NA-1 accounts for 20% to 30%, and NA-2 accounts for approximately 2% to 5% of total plasma albumin.[2] The percentage of MA and NA within total plasma albumin decreases and increases, respectively, during the course of several pathological conditions, such as diabetes mellitus,[3] diabetic nephropathy,[4] chronic liver failure,[5] chronic kidney disease,[6] hyperlipidemia,[7] and inflammatory bowel disease,[8] as well as in surgical and dialysis patients.[9,10] These alterations in the oxidized/reduced state of plasma albumin may be attributed to the oxidative stress accompanying these diseases.[11]

A previous study reported that protein malnutrition induced a low-grade inflammatory state in rats, as evidenced by elevated serum levels of tumor necrosis factor (TNF)-α, interleukin (IL)-6 and IL-1β, and reduced serum levels of albumin.[12] Our recent data indicated that in rats fed low-protein diets, the percentage of MA within total plasma albumin decreased, while that of NA-1 increased, along with the decrease in plasma albumin concentration.[13–15] Furthermore, a recent study showed that NA-1 triggered an inflammatory response in leukocytes.[16] The modulation of the oxidized/reduced state of plasma albumin in rats with protein malnutrition might be associated with the induction of systemic inflammation.

Our recent studies suggested that the oxidized/reduced state of plasma albumin may reflect both the quantity and quality of dietary protein. Furthermore, we found that dietary proteins containing higher levels of cystine (Cyss) were effective in sustaining the percentage of MA within total plasma albumin in rats fed low-protein diets.[17] Cyss is the oxidized dimer of Cys, which is an essential substrate for the synthesis of glutathione. Glutathione is an antioxidative tripeptide that plays an important role in maintaining cellular homeostasis.[18] Although glutathione protects macromolecules from oxidative stress, its influence on the oxidized/reduced state of plasma albumin in rats fed low-protein diets is unknown.

Cys can be produced through the transsulfuration pathway from L-Met degradation.[19] In our recent study, the level of plasma Met was increased in rats fed a low-protein diet supplemented with Cyss.[17] This might be attributable to the Met-sparing effect of Cyss.[20] Met might be an important amino acid in regulating the oxidized/reduced state of plasma albumin. However, the effect of supplementation with Met on the oxidized/reduced state of plasma albumin in rats fed low-protein diets is unknown. The aim of this study was to compare the effects of Cyss and Met on the oxidized/reduced state of plasma albumin and the gene expression of inflammation-related molecules in blood cells of rats fed low-protein diets. Soy protein isolate (SPI) contains a small amount of sulfur amino acids, and consists of equal amounts of Cyss and Met.[21] In this study, SPI was used as a protein source. Furthermore, we investigated whether hepatic glutathione plays a role in the regulation of the oxidized/reduced state of plasma albumin by dietary Cyss.

*To whom correspondence should be addressed.
E-mail: kuwahata@kpu.ac.jp

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Materials and Methods

Animal experiments. The animal facilities and protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Kyoto Prefectural University. Male Sprague–Dawley rats (4 weeks old; weight, approximately 80 g) were housed under a 12-h light/dark cycle with *ad libitum* access to a 20% SPI diet and water for an acclimatization period of 1 week.

In experiment 1, rats were divided into four groups and fed a 20% SPI diet (control group, $n = 6$), 5% SPI diet [low-protein (LP) group, $n = 6$], or 5% SPI diet supplemented with either 0.165% Met (LP + Met group, $n = 6$) or 0.165% Cyss (LP + Cyss group, $n = 6$) *ad libitum* for 1 week (Table 1). The levels of Met and Cyss in each supplemented diet were comparable to those in the 20% SPI diet.[21] Dietary intake and body weight were measured daily during the experimental period. Blood was drawn from the tail vein and centrifuged to obtain plasma for analysis of the albumin concentration and the oxidized/reduced state of albumin. After 1 week of the dietary treatments, the rats were euthanized by deep anesthesia with isoflurane (Mylan, Canonsburg, PA) and terminal exsanguination. Blood was drawn from the abdominal aorta for preparation of total RNA. A small amount of blood was centrifuged to obtain plasma for the amino acid analysis. The liver was perfused with saline containing heparin (10 U/ml) and rapidly removed. Both plasma and liver samples were stored at −80°C until analysis.

In experiment 2, rats were fed a 5% SPI diet for 1 week. Then, the rats were refed with the 5% SPI diet supplemented with 0.165% Cyss and received either distilled water (control group, $n = 3$) or distilled water containing 30 mM D,L-buthionine-(S,R)-sulfoximine (BSO, Santa Cruz, CA; BSO-treated group, $n = 3$) for 3 days. During this period, rats in the control group were given the same amount of the diet consumed by rats in the BSO-treated group. Blood was drawn from the tail vein and centrifuged to obtain plasma. After the dietary treatments, the rats were euthanized by deep anesthesia with isoflurane and terminal exsanguination. The liver was perfused with saline containing heparin (10 U/ml) and rapidly removed. The liver samples were stored at −80°C until analysis.

Amino acid composition by capillary electrophoresis electrospray ionization time-of-flight mass spectrometry. Plasma (100 µl each) was added to 0.9 ml of ice-cold methanol containing 10 µM methionine sulfone as an internal standard. The mixtures were vortexed with 1 ml of chloroform and 0.4 ml of ice-cold Milli-Q water. After centrifugation at 2,300 × *g* for 5 min at 4°C, the supernatant was centrifugally filtrated through 5-kDa cut-off filters (Millipore, Bedford, MA) at 9,100 × *g* for 4 to 5 h to remove proteins. The filtrate was centrifugally concentrated in a vacuum evaporator, dissolved in Milli-Q water, and analyzed by capillary electrophoresis electrospray ionization time-of-flight mass spectrometry (CE-TOFMS).

The CE-TOFMS analysis was performed using an Agilent CE system combined with a TOFMS (Agilent Technologies, Palo Alto, CA). Each metabolite was identified in reference to internal standards (H3304-1002, Human Metabolome Technology, Inc., Tsuruoka, Japan) for *m*/*z* and migration time, and quantified by peak area.

Measurement of plasma albumin. The plasma albumin concentration was determined using the bromocresol green method.[22] The albumin concentration was measured using an ALB-P kit (FUJIFILM, Kanagawa, Japan). Plasma samples were analyzed using a DRI-CHEM 4000i automated clinical chemistry analyzer (FUJIFILM).

Analysis of the oxidized/reduced state of albumin. High-performance liquid chromatography (HPLC) was performed as described previously.[13] The HPLC system comprised a #3023 autosampler, #3101 pumps, and a #3213 fluorescence detector (excitation wavelength, 280 nm; emission wavelength, 340 nm) in conjunction with an S-MC system controller (all from Shiseido, Tokyo, Japan). An ES-502N ion-exchange column (Shodex-Asahipak, Showa Denko K.K., Kawasaki, Japan) was used. Plasma samples were diluted 10-fold with saline. The diluted samples were filtered using a 0.45-µm polyvinylidene difluoride membrane (Millipore, Billerica, MA) and subjected to HPLC. The sample injection volume was 10 µl. Measurements were conducted using solvent gradient elution with increasing concentrations of ethanol (0% to 10%) in 50 mM sodium acetate and 400 mM sodium sulfate (pH 4.85) at a flow rate of 1.0 ml/min.

Preparation of total RNA and quantitative reverse transcription polymerase chain reaction. Blood samples were used for the preparation of total RNA. The total RNA was extracted using NucleoSpin RNA Blood Midi (MACHEREY-NAGEL, Duren, Germany) according to the manufacturer's protocol. Livers were homogenized in ISOGEN II reagent (Nippon Gene, Tokyo, Japan), and total RNA was extracted according to the manufacturer's protocol. First-strand complementary DNA was synthesized using M-MLV reverse transcriptase (Invitrogen Corp., Carlsbad, CA). Real-time polymerase chain reaction (PCR) (LightCycler 96, Roche Diagnostics GmbH, Mannheim, Germany) was conducted using FastStart Essential DNA Green Master (Roche Diagnostics GmbH) and specific primers for albumin (forward, 5'-AAGCCTGGGCAG TAGCTCGT-3'; reverse, 5'-CCAACAGGTCGCCGTGACAG-3'), tumor necrosis factor α (TNF-α) (forward, 5'-GGAGCCCAT

Table 1. Ingredient composition of the diets fed to rats (g/kg diet)

Component	20% SPI	5% SPI	5% SPI +Met[§]	5% SPI +Cyss[¶]
Soy protein isolate	200	50	50	50
Methionine	—	—	1.65	—
Cystine	—	—	—	1.65
Cornstarch	457	557	557	557
Sucrose	228	278	276	276
Rapeseed oil	35	35	35	35
Soybean oil	15	15	15	15
Cellulose	20	20	20	20
Vitamin mixture[†]	10	10	10	10
Mineral mixture[‡]	35	35	35	35

[†]AIN-93G vitamin mixture. [‡]AIN-93G mineral mixture. [§,¶]The level of Met or Cyss in the supplemented diets was comparable to that in the 20% SPI diet. SPI, soy protein isolate; Cyss, cystine.

TTGGGAACTTCT-3'; reverse, 5'-GGGGCCTCCAGAACT CCA-3'), IL-6 (forward, 5'-TTGGGACTGATGTTGTTG-3'; reverse, 5'-TGTGGGTGGTATCCTCTGT-3'), IL-1β (forward, 5'-GCTGTGGCAGCTACCTATGTCTTG-3'; reverse, 5'-AGG TCGTCATCATCCCACGAG-3'), cyclooxygenase (COX) 2 (forward, 5'-GCTCATACTGATAGGAGAGACGA-3'; reverse, 5'-TGGAACTGCTGGTTGAAAAG-3'), and 18s rRNA (forward, 5'-GGGAGGTAGTGACGAAAAATAACAAT-3'; reverse, 5'-TTG CCCTCCAATGGATCCT-3').

Quantification of total glutathione in liver. Livers were homogenized in 5% sulfosalicylic acid. Homogenates were centrifuged at 8,000 × *g* for 10 min at 4°C. The resulting supernatant fraction was subjected to glutathione quantification. The glutathione level was determined by using a total glutathione quantification kit (Dojindo Molecular Technologies Inc., Rockville, MD) according to the manufacturer's protocol. The protein concentration was determined using a BCA protein assay reagent (Pierce, Rockford, IL).

Statistical analyses. Data are expressed as the mean ± SD. The Student's *t* test was used to analyze the differences between two groups. Statistical analysis for multiple comparisons was performed using one-way analysis of variance followed by Tukey–Kramer's post-hoc test or Scheffe's *F* test. Data analysis was performed using Statcel3 software (OMS Publishing Inc., Tokyo, Japan), and p values <0.05 were considered to be statistically significant.

Results

Food intake, body weight, and plasma free amino acids in rats fed low-protein diets. In experiment 1, food intake in the control group was increased when compared to the other groups fed low-protein diets (Fig. 1A). After 1 week of dietary treatments, the body weight in the control group was significantly increased when compared to the other groups (Fig. 1B). However, no significant differences in body weight were observed among the three groups given the low-protein diets. Most of the plasma indispensable amino acid levels in the three groups fed the low-protein diets were decreased when compared to the control group (Table 2). In contrast, no significant differences in the level of plasma Phe were observed among the groups, and the level of plasma His in the LP group was significantly increased when compared to the other groups. The level of plasma Met in the LP + Met group was significantly increased when compared to the LP and LP + Cyss groups. The level of plasma Cys was significantly decreased in the LP group when compared to the other groups.

Plasma albumin and hepatic albumin gene expression in rats fed low-protein diets. The plasma albumin concentration was significantly lower in the LP group than in the control group (Fig. 2A). However, no significant difference in plasma albumin concentration was observed between the control and LP + Met groups. The plasma albumin concentration in the LP + Cyss group was significantly higher than in the control group. The percentages of MA within total plasma albumin in both the LP and LP + Met groups were significantly lower than in the control group (Fig. 2B). However, the percentage of MA within total plasma albumin in the LP + Met group was significantly higher than in the LP group. On the other hand, no significant difference in the percentage of MA within total plasma albumin was seen between the control and LP + Cyss groups. Typical HPLC profiles for each group are shown in Fig. 2C. The level of hepatic albumin mRNA in the control group did not differ significantly from the level in the other three groups (Fig. 2D). However, the level of hepatic albumin mRNA in both the LP and LP + Met groups was significantly lower when compared to the LP + Cyss group.

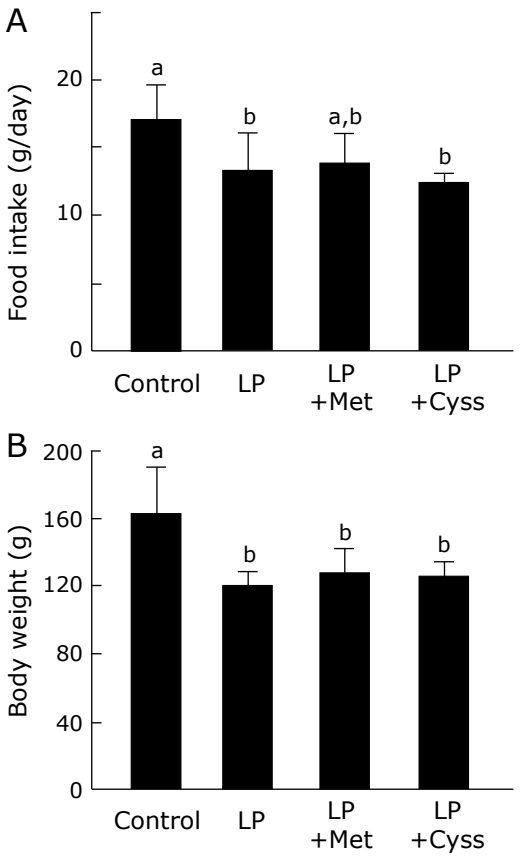

Fig. 1. Food intake and body weight in rats fed a 20% protein diet, 5% protein diet, or 5% protein diet supplemented with either methionine (Met) or cystine (Cyss). Rats were fed experimental diets containing 20% soy protein isolate (SPI) diet (control group, n = 6), 5% SPI diet [low-protein (LP) group, n = 6] or 5% SPI diet supplemented with either 0.165% Met (LP + Met group, n = 6) or 0.165% Cyss (LP + Cyss group, n = 6) for 1 week. Dietary intake was measured during the experimental period (A). Body weight was measured after 1 week of the dietary treatments (B). Values represent the mean ± SD. Data were analyzed by one-way analysis of variance followed by Tukey–Kramer's post-hoc test. Means not sharing the same letter are significantly different from each other (p<0.05).

Levels of mRNAs encoding TNF-α, IL-6, IL-1β, and COX2 in blood cells of rats fed low-protein diets. To investigate whether the intake of a low-protein diet is related to the induction of systemic inflammation, we assessed the gene expression in blood cells. The levels of mRNAs encoding TNF-α, IL-6, IL-1β, and COX2 in blood cells were significantly higher in the LP group than in the other groups (Fig. 3). No significant differences in the levels of these mRNA were observed among the control, LP + Met and LP + Cyss groups.

Hepatic glutathione content in rats fed low-protein diets. Glutathione is a cysteine-containing endogenous antioxidant. The level of total glutathione was significantly lower in the LP group than in the control group (Fig. 4). On the other hand, the level of total glutathione was significantly higher in both the LP + Met and LP + Cyss groups than in the control group.

Effect of BSO treatment on the oxidized/reduced state of plasma albumin in rats fed a low-protein diet supplemented with Cyss. In experiment 2, after feeding of the low-protein diet for 1 week, rats were refed the low-protein diet supplemented with Cyss and received either distilled water or distilled water containing BSO, an inhibitor of glutathione synthesis, for 3 days. The level of total glutathione was significantly lower in the

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Table 2. Plasma amino acid concentrations (μmol/L)

Amino acid	Control group	LP group	LP + Met group	LP + Cyss group
Val†	317 ± 56[a]	199 ± 22[b]	162 ± 28[b]	137 ± 18[b]
Leu†	213 ± 40[a]	145 ± 11[a,b]	128 ± 36[b]	107 ± 18[b]
Ile†	172 ± 33[a]	95 ± 8[b]	95 ± 23[b]	83 ± 14[b]
Met†	35 ± 6[a,b]	24 ± 5[a,c]	45 ± 8[b]	12 ± 3[c]
Trp†	115 ± 14[a]	47 ± 9[b]	63 ± 11[b]	63 ± 20[b]
Thr†	640 ± 82[a]	368 ± 42[b]	61 ± 19[c]	82 ± 22[c]
Lys†	731 ± 50[a]	442 ± 28[b]	338 ± 110[b]	330 ± 62[b]
Phe†	62 ± 11	60 ± 4	55 ± 12	44 ± 8
His‡	92 ± 10[a]	160 ± 19[b]	71 ± 1[a]	75 ± 5[a]
Ala†	766 ± 39	1,056 ± 267	1,029 ± 143	1,104 ± 204
Pro†	361 ± 40	275 ± 107	272 ± 44	218 ± 41
Gly†	214 ± 15[a]	341 ± 47[b]	377 ± 62[b]	386 ± 15[b]
Ser†	563 ± 80[a]	909 ± 64[b]	453 ± 104[a]	515 ± 43[a]
Cys†	3 ± 0[a]	1 ± 0[b]	3 ± 0[a]	3 ± 1[a]
Asn†	155 ± 17[a]	70 ± 6[b]	68 ± 15[b]	69 ± 10[b]
Gln†	948 ± 95	888 ± 110	1,012 ± 91	1,038 ± 125
Tyr†	139 ± 13[a]	47 ± 2[b]	48 ± 18[b]	69 ± 14[b]
Arg‡	262 ± 41[a]	139 ± 1[b]	166 ± 22[b]	149 ± 27[b]
Asp†	18 ± 4	43 ± 13	43 ± 11	47 ± 21
Glu†	95 ± 23	127 ± 12	154 ± 47	107 ± 24

Rats were fed a 20% SPI diet (control group), 5% SPI diet (LP group), or 5% SPI diet supplemented with Met (LP + Met group) or Cyss (LP + Cyss group) for 1 week. Free amino acids in the plasma were analyzed by capillary electrophoresis electrospray ionization time-of-flight mass spectrometry. Amino acid levels represent the means ± SD (n = 3). Data were analyzed by a Tukey–Kramer's post-hoc test† or a Scheffe's F test‡. Means not sharing the same letter are significantly different from each other (p<0.05). SPI, soy protein isolate; Cyss, cystine.

liver of the BSO-treated group than in the control group (Fig. 5A). No significant difference in the level of hepatic albumin mRNA was observed between the control and BSO-treated groups (Fig. 5B). In the same way, no significant differences in the plasma albumin concentration (Fig. 5C) and the percentage of MA within total plasma albumin (Fig. 5D) were seen between the control and BSO-treated groups. Typical HPLC profiles for each group are shown in Fig. 5E.

Discussion

The results of the present study revealed that supplementation with sulfur amino acids attenuates the decrease in the percentage of MA within total plasma albumin and represses the increase in the expression of mRNAs encoding proinflammatory cytokines and COX2 in the blood cells of rats fed low-protein diets. The attenuating effect of Met was weaker than that of Cyss. These results suggest that supplementation with Cyss may be more effective than Met in improving the oxidized/reduced ratio of plasma albumin under a state of protein malnutrition.

In the present study, supplementation with Cyss had no significant effect on either food intake or body weight in rats fed low-protein diets. Previous studies have reported that various factors may influence the efficacy of controlling body weight with dietary cyst(e)ine.[23–27] The effects of dietary cyst(e)ine supplementation on body weight control might depend on the nutritional status, age and strain of the experimental animals.

A previous study reported that the activity of hepatic cystathionine β-synthase, which is an enzyme in the transsulfuration pathway, increased in response to the dietary SPI level when an SPI was used as a protein source.[28] Decreased activity of the conversion of Met to Cys might have caused the difference seen in the plasma MA level between the LP + Met and LP + Cyss groups. In the present study, no significant difference in the level of plasma Cys was observed between the LP + Met and LP + Cyss groups. However, the level of Cys in hepatocytes, rather than that in plasma, might be important for maintaining the plasma MA level. Future studies are needed to evaluate the relationship between hepatocellular Cys and plasma MA levels.

A previous study reported that supplementation with Cys increased plasma albumin concentrations in rats fed a Met-restricted diet prepared from an amino acid mixture.[25] Moreover, another study reported that both the serum albumin concentration and the level of hepatic albumin mRNA were significantly higher in rats fed a 10% SPI diet supplemented with Cyss than in those fed the 10% SPI diet.[29] It also reported that no significant differences in the levels of either serum albumin and hepatic albumin mRNA were seen between rats fed the 10% SPI diets with and without Met supplementation. Although the mechanism remains unclear, the data in the present study are consistent with the results of these previous studies. We previously reported a relationship between the plasma MA level and the fractional synthesis rate of albumin in the liver.[14] The percentage of MA within total plasma albumin as well as the plasma albumin concentration might also be regulated by Cyss supplementation partly via hepatic albumin gene expression in rats fed low-protein diets.

In recent years, a relationship between the oxidized/reduced state of plasma albumin and inflammation has been suggested. A recent study reported that the percentage of NA within total plasma albumin was significantly correlated with the plasma levels of inflammatory markers in patients with cirrhosis.[16] Moreover, it was also indicated that human NA-1 induced the expression of mRNAs encoding inflammatory cytokines and eicosanoid-generating enzymes in peripheral blood mononuclear cells from healthy donors. The presence of circulating NA-1 may induce systemic inflammation by triggering the activation of peripheral leukocytes. As in the previous studies,[13–15,17] the

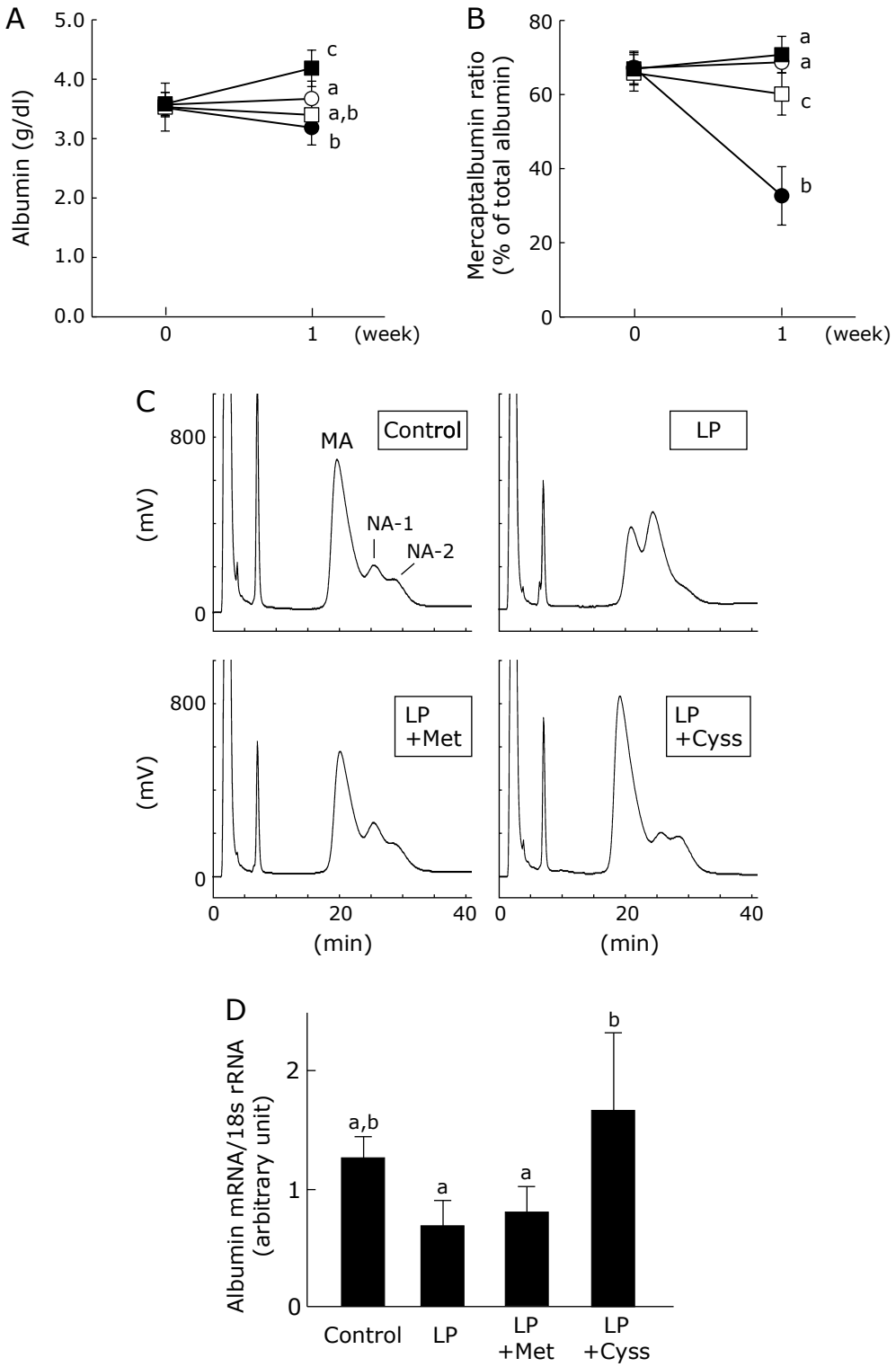

Fig. 2. Plasma albumin concentration, the percentage of MA within total plasma albumin, and expression of hepatic albumin mRNA in rats fed a 20% protein diet, 5% protein diet, or 5% protein diet supplemented with either methionine (Met) or cystine (Cyss). Rats were fed experimental diets containing 20% soy protein isolate (SPI) diet (control group, $n = 6$), 5% SPI diet (low-protein (LP) group, $n = 6$) or 5% SPI diet supplemented with either 0.165% Met (LP + Met group, $n = 6$) or 0.165% Cyss (LP + Cyss group, $n = 6$) for 1 week. Blood was drawn from the tail vein and centrifuged to obtain plasma. The plasma albumin concentration was measured (A) (control group, open circles; LP group, closed circles; LP + Met group, open squares; LP + Cyss group, closed squares). The percentage of MA within total plasma albumin was analyzed by high-performance liquid chromatography (B, C) (control group, open circles; LP group, closed circles; LP + Met group, open squares; LP + Cyss group, closed squares). The level of albumin mRNA in the liver was analyzed by real-time PCR and normalized to 18s rRNA (D). Values represent the mean ± SD. Data were analyzed by one-way analysis of variance followed by Tukey–Kramer's post-hoc test or Scheffe's *F* test. Means not sharing the same letter are significantly different from each other ($p<0.05$). MA, mercaptalbumin; NA, nonmercaptalbumin.

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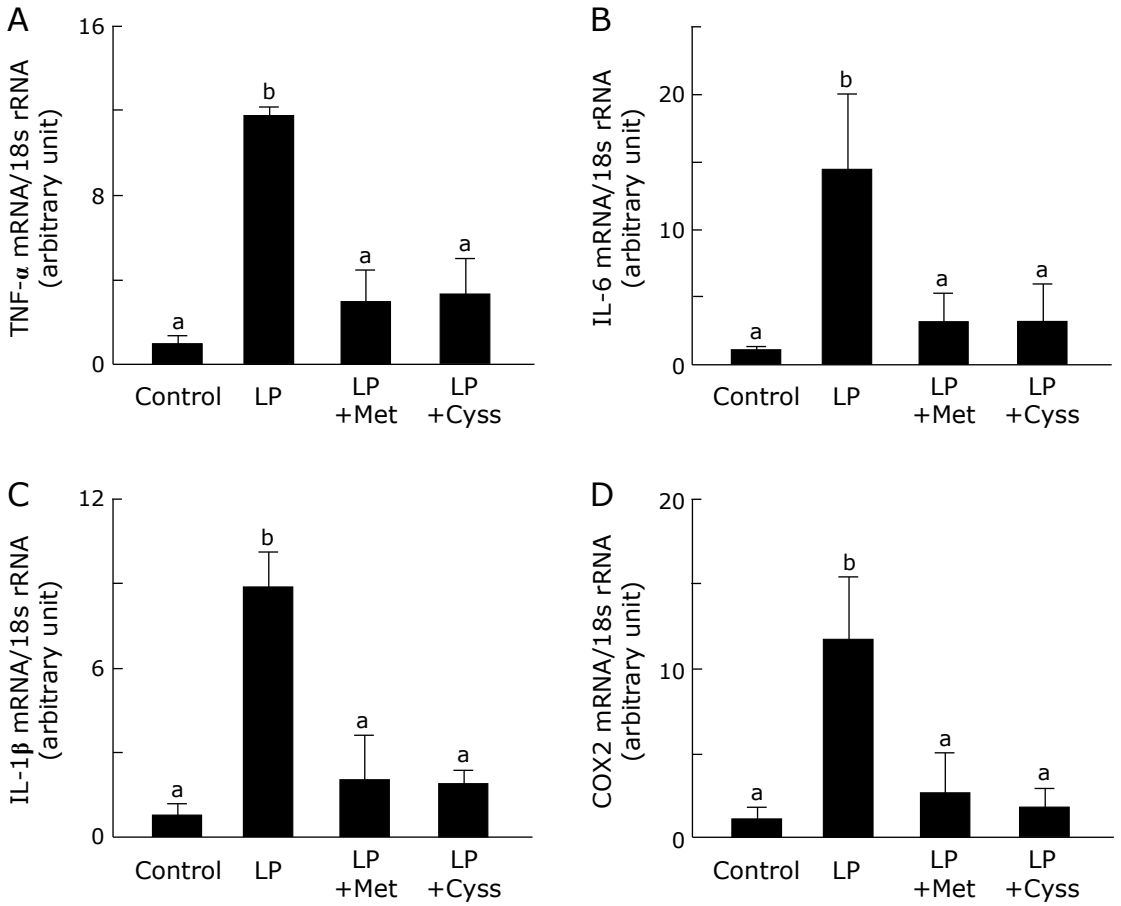

Fig. 3. Expression of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin-1β (IL-1β), and cyclooxygenase 2 (COX2) mRNAs in blood cells prepared from rats fed a 20% protein diet, 5% protein diet or 5% protein diet supplemented with either methionine (Met) or cystine (Cyss). Rats were fed experimental diets containing 20% soy protein isolate (SPI) diet (control group, $n = 3$), 5% SPI diet [low-protein (LP) group, $n = 3$] or 5% SPI diet supplemented with either 0.165% Met (LP + Met group, $n = 3$) or 0.165% Cyss (LP + Cyss group, $n = 4$) for 1 week. The expression levels of mRNAs encoding TNF-α (A), IL-6 (B), IL-1β (C), and COX2 (D) were analyzed by real-time PCR and normalized to that of 18s rRNA. Values represent the mean ± SD. Data were analyzed by one-way analysis of variance followed by Tukey–Kramer's post-hoc test or Scheffe's *F* test. Means not sharing the same letter are significantly different from each other ($p<0.05$).

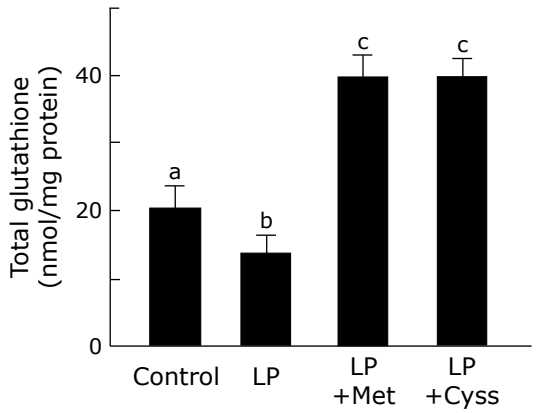

Fig. 4. Level of total glutathione in the liver of rats fed a 20% protein diet, 5% protein diet, or 5% protein diet supplemented with either methionine (Met) or cystine (Cyss). Rats were fed experimental diets containing 20% soy protein isolate (SPI) diet (control group, $n = 4$), 5% SPI diet [low-protein (LP) group, $n = 4$] or 5% SPI diet supplemented with either 0.165% Met (LP + Met group, $n = 4$) or 0.165% Cyss (LP + Cyss group, $n = 4$) for 1 week. Total glutathione level and the protein concentration in liver homogenates were determined. Values represent the mean ± SD. Data were analyzed by one-way analysis of variance followed by Tukey–Kramer's post-hoc test. Means not sharing the same letter are significantly different from each other ($p<0.05$).

results of the present study showed that the percentage of MA within total plasma albumin decreased, while that of NA-1 increased, in the LP group. Furthermore, the expression of mRNAs encoding proinflammatory cytokines and COX2 was increased in the blood cells of the LP group. On the other hand, no differences in the expression levels of these mRNAs were seen between the LP + Met and LP + Cyss groups, although the percentage of MA within total plasma albumin was significantly lower in the LP + Met group than in the LP + Cyss group. Previous studies indicated that the production of proinflammatory cytokines in various diseases was attenuated by supplementation with Cys or its precursor.[30–33] Such effects may have been due to the function of glutathione and hydrogen sulfide, which are synthesized using Cys as a substrate. In this study, we cannot exclude the influence of factors other than the albumin molecule, which might limit the interpretation of our findings. Future studies should investigate the important factors that attenuate the production of proinflammatory cytokines in blood cells, and the direct effects of albumin molecules prepared from the plasma of rats fed a low-protein diet on blood cells.

Cys plays important roles in antioxidative defense via glutathione production. Previous studies indicated that the level of hepatic glutathione decreased in rats fed low-protein diets and a sulfur amino acid-deficient diet.[34,35] In this study, the level of hepatic glutathione was significantly lower in the LP group than

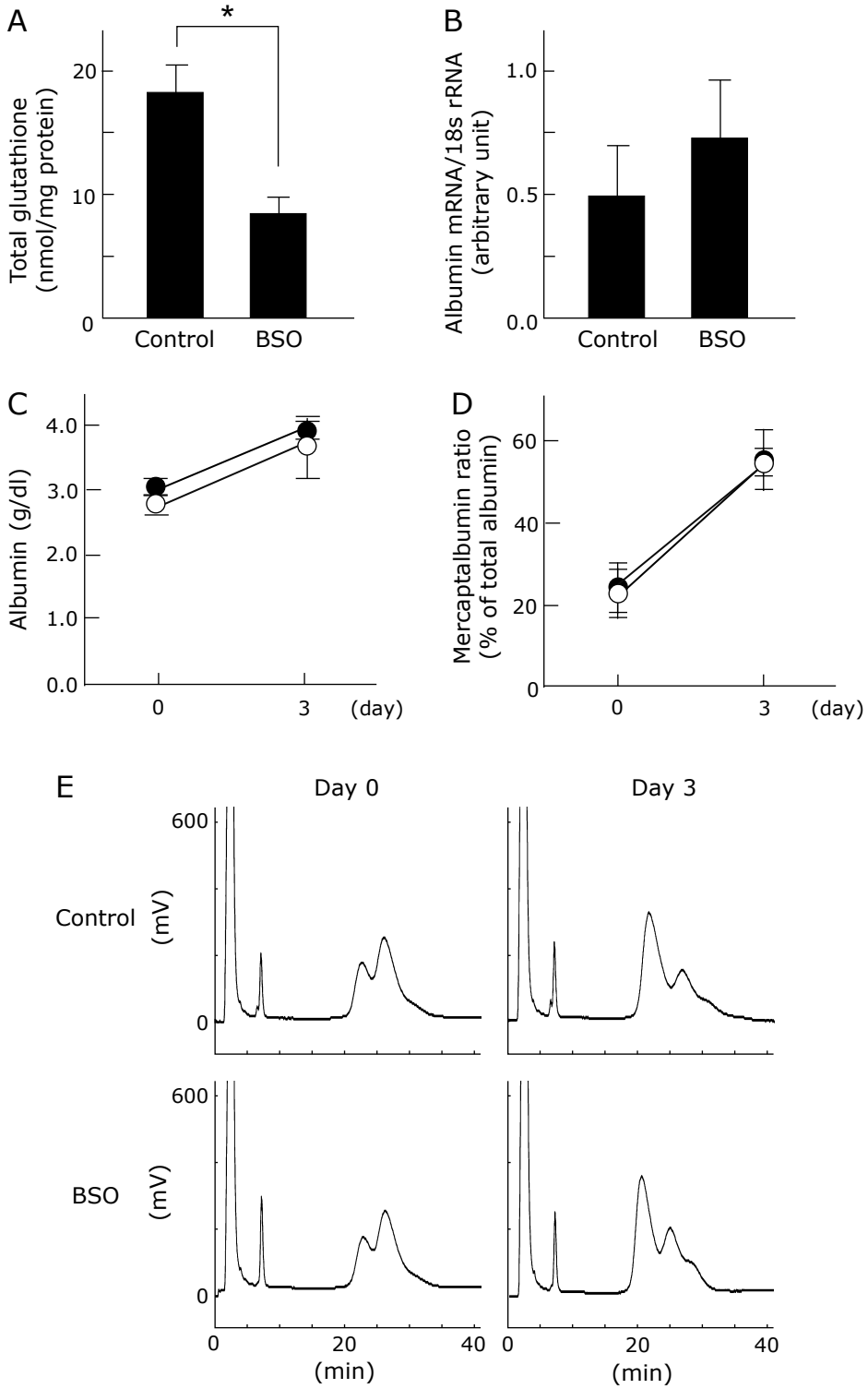

Fig. 5. Level of hepatic total glutathione, expression of hepatic albumin mRNA, plasma albumin concentration, and percentage of MA within total plasma albumin in rats treated with or without buthionine-sulfoximine (BSO). Rats were fed the low-protein diet for 1 week. Then, rats were refed with the low-protein diet supplemented with Cyss and received either distilled water (n = 3) or distilled water containing BSO (n = 3) for 3 days. Total glutathione level and the protein concentration in liver homogenates were determined (A). The level of albumin mRNA in the liver was analyzed by real-time PCR and normalized to 18s rRNA (B). The plasma albumin concentration was measured (C) (control group, open circles; BSO-treated group, closed circles). The percentage of MA within total plasma albumin was analyzed by high-performance liquid chromatography (D, E) (control group, open circles; BSO-treated group, closed circles). Values represent the mean ± SD. Data were analyzed by Student's t test. *p< 0.05 vs the corresponding the control group.

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in the control group, which was the same as in previous studies. On the other hand, the level of hepatic glutathione in both the LP + Met and LP + Cyss groups was higher than in the control group. This result seems to be influenced by decreases in the level of hepatic proteins in rats fed low-protein diets. At a minimum, supplementation of low-protein diets with either Met or Cyss increased the hepatic glutathione level, which was the same as the plasma MA level. However, the percentage of MA within total plasma albumin increased by supplementation with Cyss in rats fed a low-protein diet and treated with an inhibitor of glutathione synthesis. A recent study showed that supplementation with glutathione to a low-protein diet only reversed the percentage of MA within total plasma albumin to an extent similar to that observed with Cyss supplementation.[36] The authors of that study suggested that the glutathione supplemented to the low-protein diet would primarily serve as a source of Cys rather than exert antioxidative activity. The percentage of MA within total plasma albumin might be regulated by dietary Cyss via the glutathione-independent pathway. On the other hand, in this study, we did not examine the effect of BSO treatment on the expression of mRNAs encoding proinflammatory cytokines and COX2 in the blood cells of rats fed a Cyss-supplemented diet. Future studies should investigate the relationship between glutathione levels in the liver and plasma and the production of proinflammatory cytokines in blood cells.

In conclusion, to sustain the plasma MA level in rats fed low-protein diets, Cyss supplementation seemed to be more effective than Met supplementation. However, in experiment 1, the molar content of the thiol groups in the supplemented Cyss and Met was not the same. Since the quality of a food protein is assessed by the ratio of the content (in grams) of the restricted amino acids in the evaluated protein to the content (in grams) of the amino acids in the reference protein,[37] the same amounts (in grams) of Cyss and Met were added to the experimental diets. As a result, there was no significant difference in the molar contents of thiols ingested between the LP + Met and LP + Cyss groups (154 ± 23 μmol/day and 171 ± 9 μmol/day). Nevertheless, it would also be important to evaluate each experimental diet supplemented with equal molar contents of thiols in the Cyss and Met. Furthermore, we could not clarify whether this effect was caused by cyst(e)ine or its metabolites, which might limit the interpretation of our findings. In our preliminary study, the percentage of MA within total plasma albumin in rats fed the low-protein diets could not be sustained by supplementation with taurine, which is a Cys metabolite (unpublished results). Future studies should clarify the detailed mechanism underlying the regulation of the oxidized/reduced state of plasma albumin by Cyss. A decline in plasma MA levels is found in protein malnutrition and several pathological conditions, and it might induce mild systemic inflammation, thus aggravating the malnutrition states. Supplementation with Cyss might be an effective nutritional treatment under conditions of protein malnutrition.

Author Contributions

YY, CM, and IK, acquisition of data; YK, WA, and MK, study concept and design; MK, obtained funding and study supervision.

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Abbreviations

BSO	D,L-buthionine-(S,R)-sulfoximine
CE-TOFMS	capillary electrophoresis electrospray ionization time-of-flight mass spectrometry
COX	cyclooxygenase
Cyss	cystine
HPLC	high-performance liquid chromatography
IL	interleukin
LP	low-protein
MA	mercaptalbumin
NA	nonmercaptalbumin
PCR	polymerase chain reaction
SPI	soy protein isolate
TNF	tumor necrosis factor

Conflict of Interest

No potential conflicts of interest were disclosed.

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