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Journal of Nutritional Biochemistry 13 (2002) 625–633

THE JOURNAL OF Nutritional Biochemistry

Dietary supplementation with cysteine prodrugs selectively restores tissue glutathione levels and redox status in protein-malnourished mice[1]

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Received 3 April 2002; received in revised form 21 May 2002; accepted 24 May 2002

Abstract

Protein malnutrition (PM) is a major health problem in the world. PM compromises antioxidant defense in the body. In particular, PM decreases tissue glutathione (GSH) levels. A high protein diet was found to restore tissue GSH levels in animal studies, however it is not recommended for the early phase of PM rehabilitation. Therefore, using dietary supplementation to restore tissue GSH without giving a high protein diet may be an adjunct therapy that helps improve antioxidant status during the early rehabilitation of PM. In this study, we systematically compared the efficacy of dietary supplementation of four cysteine prodrugs: *N*-acetylcysteine, L-2-oxo-4-thiazolidine-carboxylate, methionine, and GSH, on tissue GSH in mice fed a protein-deficient (0.5%) diet. Results showed that dietary supplementation of cysteine prodrugs to PM mice restored GSH levels in liver, lung, heart and spleen, but not in colon. GSH and GSSG levels in brain and kidney were not affected by cysteine prodrug or PM. Supplementation also restored the redox status in liver and heart (based on GSH/GSSG), and in liver and spleen (based on GSSG/2GSH reduction potential). This suggests that the restoration of GSH levels and redox status by cysteine prodrugs are tissue-specific, and that the two indicators of redox status are not always interchangeable. However, all four prodrugs exhibited similar GSH-enhancing capacities, showing no prodrug-specificity as seen in cell culture studies. In conclusion, this study provided information that may be useful in a clinical setting where a short-term oral supplementation of cysteine prodrugs is necessary for the early rehabilitation of PM patients.

Keywords: Protein malnutrition; Antioxidant; GSH; GSSG; Cysteine prodrugs; Reduction potential

1. Introduction

Protein malnutrition (PM) is a major health issue worldwide. It is prevalent in children in developing countries [1]. In developed countries, PM often is secondary to chronic diseases such as AIDS and cancer, and affects mostly the hospitalized and the elderly [2]. Tissue antioxidant levels are decreased substantially in PM, and the decrease has been associated with increased sensitivity to infection and reactive oxygen species (ROS)-induced tissue damage [3,4].

Glutathione (GSH, γ-glutamylcysteinylglycine) is the most abundant non-protein thiol in mammalian cells. It plays an important role in antioxidation of ROS, detoxification of xenobiotics, and immune functions [5,6]. Tissue GSH concentrations were decreased in PM, which was correlated with increased susceptibility to oxidative stress and lung oxygen toxicity [7,8]. Decreased levels of blood GSH and increased incidences of opportunistic infection were also found in PM children [4,9]. In contrast, restoration of tissue GSH levels protected against oxygen toxicity in PM mice [8].

Decreased tissue GSH levels could be restored after PM animals were switched from a protein-deficient to a protein-rich diet [10]. However, starting PM rehabilitation with a protein-rich diet may impose metabolic stress due to body's adaptation to the catabolic PM state [11,12]. Thus, it is desirable to develop an adjunct therapeutic strategy that restores tissue GSH without feeding a high protein diet for the early stage of PM rehabilitation. Dietary supplementation of cysteine prodrugs appears to be an important candidate for such a strategy.

Cysteine is the limiting amino acid for GSH synthesis [13]. A shortage in cysteine was found in PM children, which led to a decreased rate of GSH synthesis that was

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[1] Supported in part by the OARDC Hatch Fund to T.M.B., National Institutes of Health Grant NS38315 to T.M.B.

0955-2863/02/$ – see front matter
PII: S0955-2863(02)00218-8

characteristic of this population [9,14]. However, since cysteine is rapidly oxidized and is also toxic at high concentrations, it is not practical to use cysteine as a supplement [15]. Instead, cysteine prodrugs may be a more suitable choice to restore tissue GSH concentrations.

Cysteine prodrugs have been used to elevate GSH level *in vitro* and *in vivo* [16–23]. For example, N-acetylcysteine (NAC) is a cysteine prodrug with multiple therapeutic uses. The physiological benefits of NAC supplementation have been attributed to its GSH-enhancing ability [24]. Similarly, L-2-oxo-4-thiazolidine-carboxylate (OTC) and methionine supplementation increased tissue GSH levels following their intracellular conversion to cysteine, via 5-oxo-L-prolinase [25] and the transulfuration pathway [26], respectively. Lastly, because oral supplementation of GSH itself also increased liver GSH levels and cysteine concentrations in portal blood plasma, GSH could be used as a cysteine prodrug [27].

Notably, most previous *in vivo* studies have administered cysteine prodrugs by either intraperitoneal or subcutaneous injection. In a few studies where oral doses of GSH and OTC were used, tissue GSH concentrations were increased transiently (3–6 hr) but returned to baseline within 24 hr [8,27–29]. Thus, the efficacy of various cysteine prodrugs as short-term (e.g. 1 week) dietary supplements on tissue GSH enhancement has not been compared *in vivo*. Therefore, this study was designed to systematically compare the efficacy of short-term dietary supplementation of the cysteine prodrugs: NAC, OTC, methionine and GSH, on tissue GSH restoration in mice fed a protein deficient (0.5%) diet.

In addition to tissue GSH levels, tissue redox status has also been proposed to play a crucial role in normal cellular function [30,31]. Tissue redox status may be reflected by the GSSG/2GSH related redox state in the tissue. Traditionally, redox state refers to the ratio of the interconvertible oxidized and reduced form of a specific redox couple such as GSSG/2GSH [31]. The redox state of GSSG/2GSH is defined by the half-cell reduction potential and the reducing capacity of this couple [31]. Hence, in this study we compared effects of the cysteine prodrugs on tissue redox status. Two important indicators were used: the GSH/GSSG ratio and the GSSG/2GSH reduction potential. The GSH/GSSG ratio is simple to calculate and widely used. The reduction potential, on the other hand, is derived from the Nernst equation and believed to be more comprehensive [31,32].

2. Methods and materials

2.1. Materials

NAC, OTC, methionine, GSH, oxidized glutathione (GSSG) and all other reagents used for GSH and GSSG assay were obtained from Sigma Chemical Company (St. Louis, MO). AIN-93G [33] purified rodent powder diet (Dyets Inc., Bethlehem, PA) contained 0.5% (deficient), 15% (normal) or 30% (high) protein (Table 1). All 3 diets were formulated to be isocaloric. In the supplemented groups, cysteine prodrugs (59.5 mmol/kg) were mixed in the diet so that the amount of sulfur amino acid was equal to that in a normal protein diet. Since supplementation of cysteine prodrugs also increased the nitrogen level in the diet, one extra treatment group was included to determine whether the potential effect of cysteine prodrugs was due to the nitrogen content. In particular, a 0.5% protein diet group was supplemented with alanine so that the diet was isonitrogenous with that in the GSH-supplemented group.

Table 1
Diet composition[a]

Ingredient	Dietary Protein		
	0.5%	15%	30%
		g/kg diet	
Casein[b]	5.9	176.1	352.1
Cornstarch	594.5	421.4	242.4
Dyetrose	132.0	132.0	132.0
Sucrose	100.0	100.0	100.0
Cellulose	50.0	50.0	50.0
Soybean oil	70.0	70.0	70.0
t-Butylhydroquinone	0.014	0.014	0.014
Salt mix[c]	35.0	35.0	35.0
Vitamin mix[c]	10.0	10.0	10.0
L-Cystine	0.1	3.0	6.0
Choline Bitartrate	2.5	2.5	2.5

[a] AIN-93G (1) purified rodent powder diet, supplied by Dyets Inc., Bethlehem, PA.

[b] Amino acid (*g*)/casein (*kg*): Arginine 36, Alanine 26, Aspartic acid 65, Cysteine 4, Glutamic acid 208, Glycine 18, Histidine 26, Isoleucine 48, Leucine 88, Lysine 74, Methionine 26, Proline 117, Phenylalanine 50, Serine 54, Threonine 38, Tyrosine 53, Tryptophan 12, Valine 57.

[c] Mineral mixture/Vitamin mixture: AIN-93G.

CD-1 male mice (N = 64, 4 weeks) were obtained from Harlan Company (Indianapolis, IN) and used in accordance with animal protocols approved by the Ohio State University Institutional Laboratory Animal Care and Use Committee.

2.2. Study design

A total of eight treatment groups were utilized in this study. One group of mice (n = 8) was fed a 0.5% protein diet for 3 weeks to serve as the PM group. Two groups (n = 8) were fed either a 15% or 30% protein diet for 3 weeks to serve as the normal and high protein group, respectively. The remaining 5 groups (n = 8) were fed a 0.5% protein diet for 2 weeks, and then were randomly assigned to receive a 0.5% protein diet supplemented with NAC, OTC, methionine, GSH or alanine for 1 week.

Food intake and body weight were measured every other day. At the end of the study, liver, lung, colon, heart, spleen, brain and kidney were rapidly removed from animals following cervical dislocation. All tissues were rinsed with cold saline (< 4°C), immediately frozen in liquid nitrogen

and stored at −80°C for later GSH and GSSG determination. Within 2 weeks of tissue collection, GSH and GSSG assay was conducted to minimize the possible oxidation/degradation of GSH.

2.3. Sample preparation

Tissues were homogenized on ice using a stainless steel homogenizer (PowerGen 700, Fisher Scientific) in 10 volumes of 5% metaphosphoric acid. The homogenization was performed in acidic solution so that tissue proteins, including enzymes, would be precipitated. The purpose was to minimize GSH degradation and oxidation that might occur during the process of analysis. The homogenate was then centrifuged (Beckman Avanti J-25) at 10,000 × g and 4°C for 15 min. The supernatant was analyzed for GSH and GSSG using HPLC-EC.

2.4. HPLC chromatography

The determination of GSH and GSSG was achieved by using a method described by Melnyk *et al.* with slight modification [34]. Briefly, an HPLC-EC (EAS, Inc., Chelmsford, MA) was used with a reverse phase C18 column (5 μm; 4.6 × 150 mm, MCM, Inc., Tokyo, Japan). Supernatant obtained from sample preparation was diluted (1:10 v/v) with mobile phase composed of 10 mM sodium phosphate monobasic monohydrate, 0.05 mM ion-pairing agent octane sulfonic acid and 2% methanol (v/v). The mobile phase was adjusted to pH 2.7 with 8.67 mol/L (85%) phosphoric acid. Flow rate was set at 1.0 ml/min. GSH and GSSG were quantified using the 5200A Coulochem II electrochemical detector and the 5010 dual analytical cell (ESA, Inc., Chelmsford, MA). Sample vials were placed in a temperature-controlled (4°C) tray to minimize potential artifact in GSH assay. GSH eluted at the 4th minute while GSSG eluted at the 10th minute after sample was auto-injected into the system. The specificity of GSH and GSSG peak and the reliability of the assay were determined by adding GSH and GSSG standard of known concentration. GSH and GSSG standards were run routinely (10% of the total runs) to ensure the validity of the standard curves.

2.5. Nernst equation

Tissue reduction potential, based on the GSSG/2GSH couple, was calculated from the Nernst equation [31,32]:

$$E_{hc} = -240 - (59.1/2) \log ([GSH]^2/[GSSG]) \text{ mV}$$

at 25°C, pH 7.0

This equation was used in the study to estimate the GSSG/2GSH reduction potential. The unit of GSH and GSSG concentration was mmol/L, according to Schafer and Buettner [31]. To derive the GSH and GSSG concentration, samples were first homogenized with 1:10 (w/v) dilution in 5% metaphosphoric acid. After centrifugation, the supernatant was further diluted 1:10 (v/v) in HPLC mobile phase before it was analyzed. The final concentrations were expressed as per mg protein after protein concentrations in the tissues were determined.

2.6. Statistical analysis

Data were expressed as mean ± SEM. SEM indicated the variation of individual groups. Pooled variance was used in the analysis since the variances appeared to be homogeneous after the test of homogeneity of variances. One-way ANOVA was used to compare the means as we were interested in determining whether the cysteine prodrugs would restore GSH levels and GSSG/2GSH reduction potentials, and whether the prodrugs would differ in their capacities to restore the two parameters. Tukey-HSD test was used for multiple comparisons. Differences between groups were considered significant at $p < 0.05$.

3. Results

3.1. Body weight

Body weight loss is characteristic of PM in humans and animals [2]. Therefore, body weight was measured once every two days in this study to determine the degree of PM. The results indicated that mice fed a protein-deficient diet for 3 weeks suffered significant weight loss (Fig. 1). Increasing the dietary protein level from 15% to 30% did not change the body weight. No difference in food intake was observed among these three groups (data not shown). Dietary supplementation of cysteine prodrugs did not change food intake or body weight (data not shown).

3.2. Tissue weight

Although tissue weights had been found to be decreased in PM mice [35], the effect of cysteine prodrug supplementation to PM mice on tissue weight was unknown. Results of this study demonstrated that supplementation of cysteine prodrugs to PM mice significantly increased tissue weight in liver (NAC:1.04 g, GSH:1.20 g vs PM: 0.76 g), but not in extrahepatic tissues (Table 2). In addition, tissue weights in the PM group were consistently lower than those in the normal or high protein group, which was supported by previous research [35]. The decrease in tissue weight ranged from 10% in brain to nearly 60% in liver.

3.3. Tissue GSH concentration

Tissue GSH levels were measured to determine the effects of PM and cysteine prodrug supplementation on the antioxidant status. The results indicated that PM decreased GSH levels in liver, lung, colon, heart and spleen (Fig. 2A).

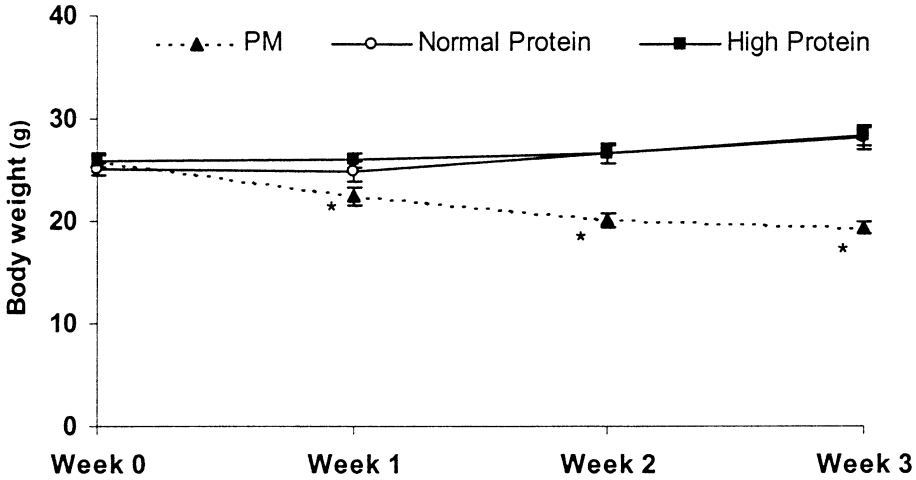

Fig. 1. Mice lost body weight after 3 weeks of PM diet. Body weights were measured at wk 0, 1, 2 and 3. Results (mean ± SEM, n = 8) with different superscripts were statistically different ($p < 0.05$). PM: 0.5g/100g protein diet; Normal protein: 15g/100g protein diet; High protein: 30g/100g protein diet.

Tissue GSH was not further increased when dietary protein level was doubled from 15% to 30%.

Dietary supplementation of cysteine prodrugs to PM mice selectively restored GSH concentration in liver, lung, heart and spleen. The increase of GSH in liver was four-fold. Alanine supplementation did not change tissue GSH, indicating the GSH enhancement was not associated with the extra nitrogen that was present in the cysteine prodrugs (Fig. 2A). GSH levels in colon was increased by cysteine prodrugs, but was not restored to the normal levels. Brain and kidney GSH remained stable regardless of dietary supplementation or protein levels (data not shown).

3.4. Tissue GSSG concentration

Glutathione disulfide, or GSSG, is also a key player in the antioxidant status and cellular function [26]. Hence, tissue GSSG levels were measured to determine the effect of PM and cysteine prodrug supplementation. Results demonstrated that PM decreased tissue GSSG in lung, colon, heart and spleen (Fig. 2B). On the other hand, dietary supplementation of cysteine prodrugs to PM mice selectively increased GSSG in lung, colon and heart. The increase of GSSG was three-fold in lung.

Doubling the dietary protein level from 15% to 30% did not change tissue GSSG levels. Alanine supplementation to PM mice did not alter tissue GSSG either (Fig. 2B).

3.5. Tissue GSH/GSSG ratio

Tissue GSH/GSSG ratio was determined because it is indicative of antioxidant and redox status [31,36]. Our results showed that PM significantly decreased the GSH/GSSG ratio by approximately 80% in liver (Table 3). This suggests that PM liver is more oxidative than normal liver. In contrast, PM lung was less oxidative than normal lung based on the GSH/GSSG ratio.

Table 2
Wet tissue weights (g) of mice in the PM, Normal protein, High protein and Supplemented groups[a,b]

Diet[c]	Liver	Lung	Colon	Heart	Spleen	Kidney	Brain
PM	0.76 ± 0.05	0.19 ± 0.01	0.12 ± 0.01	0.14 ± 0.02	0.04 ± 0.00	0.26 ± 0.01	0.45 ± 0.02
PM + NAC	1.04 ± 0.09[a]	0.18 ± 0.01	0.12 ± 0.02	0.15 ± 0.01	0.05 ± 0.00	0.25 ± 0.01	0.45 ± 0.01
PM + OTC	0.99 ± 0.07[a]	0.17 ± 0.01	0.11 ± 0.01	0.14 ± 0.01	0.04 ± 0.00	0.25 ± 0.01	0.45 ± 0.01
PM + MET	1.01 ± 0.04[a]	0.17 ± 0.01	0.12 ± 0.01	0.14 ± 0.01	0.04 ± 0.00	0.24 ± 0.01	0.46 ± 0.01
PM + GSH	1.20 ± 0.07[a]	0.19 ± 0.01	0.13 ± 0.01	0.14 ± 0.01	0.04 ± 0.00	0.27 ± 0.01	0.46 ± 0.01
PM + ALA[d]	0.78 ± 0.01	0.18 ± 0.00	0.12 ± 0.01	0.14 ± 0.01	0.04 ± 0.00	0.26 ± 0.01	0.46 ± 0.01
Normal Protein	1.79 ± 0.08[b]	0.23 ± 0.01[a]	0.22 ± 0.01[a]	0.18 ± 0.01[a]	0.09 ± 0.01[a]	0.47 ± 0.02[a]	0.50 ± 0.02[a]
High Protein	1.82 ± 0.11[b]	0.23 ± 0.02[a]	0.22 ± 0.01[a]	0.19 ± 0.02[a]	0.10 ± 0.01[a]	0.50 ± 0.02[a]	0.50 ± 0.02[a]

[a] Results expressed as mean ± SEM ($n = 8$).

[b] Results were analyzed by 2-way ANOVA test. When an overall F ratio was significant, Tukey-HSD test was used. Within each column, values with different superscripts (none, a, b) were significantly different from one another ($P < 0.05$).

[c] PM: 0.5g/100g protein diet; Normal Protein: 15g/100g protein diet; high Protein: 30g/100g protein diet.

[d] Alanine (ALA) was added so that the diet 0.5% + Alanine was isonitrogenous with the diet 0.5% + GSH.

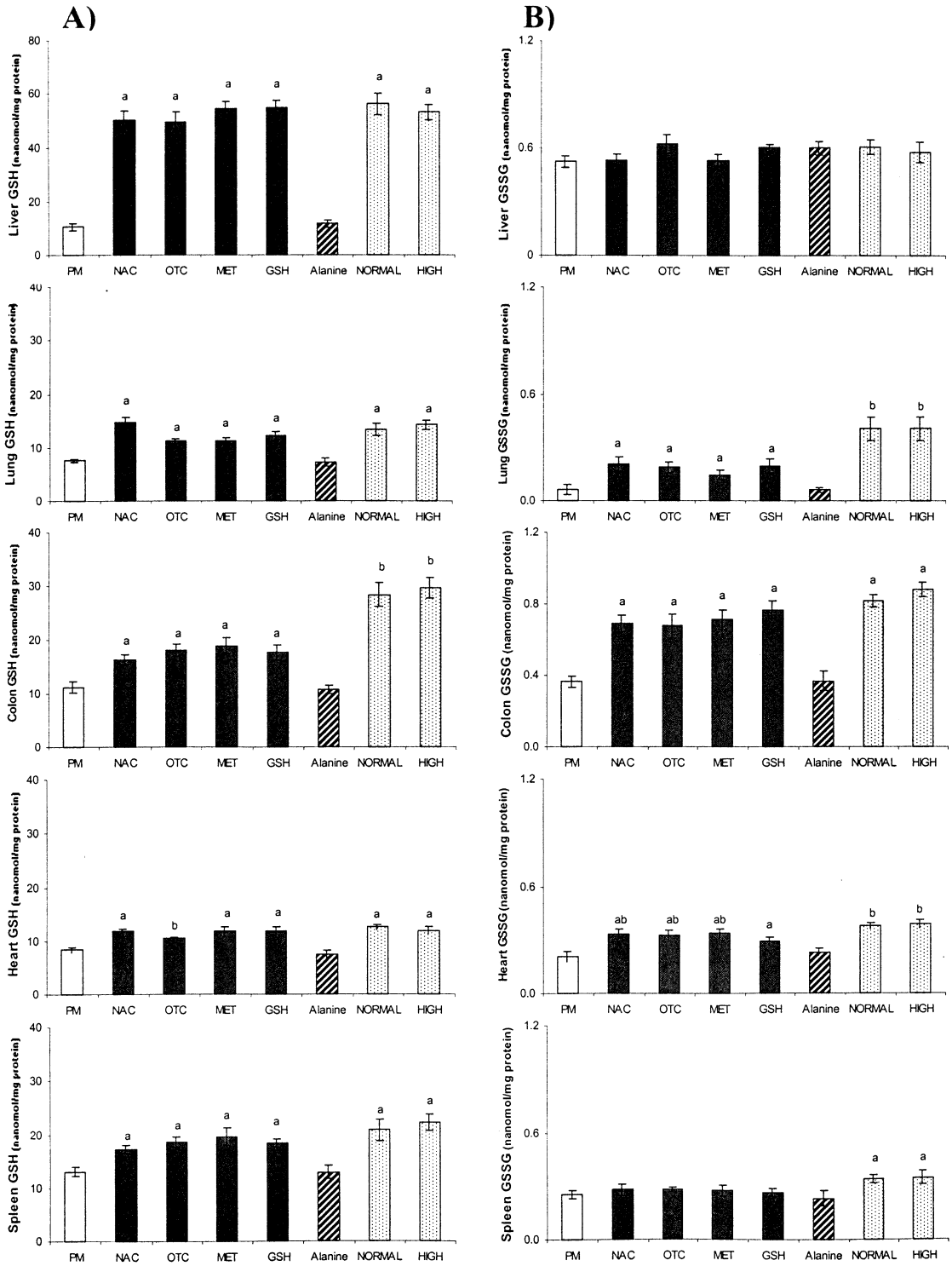

Fig. 2. A. Effect of dietary protein levels and cysteine prodrug supplementation on tissue GSH (the Y-axis of the liver graph differs from the others). B. Effect of dietary protein levels and cysteine prodrug supplementation on tissue GSSG. Three groups of mice (n = 8) were fed a PM, Normal or High protein diet for 3 weeks, while the rest groups (n = 8) were fed a PM diet for 2 weeks before they were supplemented with one of the following: *N*-acetylcysteine (NAC), L-2-oxo-4-thiazolidine-carboxylate (OTC), methionine, GSH or alanine at 59.5 mmol/kg diet. Bars (mean ± SEM, n = 8) with different superscript letters (a, b, c) were significantly ($P < 0.05$) different. PM: 0.5g/100g protein diet; NAC: PM + NAC; OTC: PM + OTC; MET: PM + MET; GSH: PM + GSH; Alanine: PM + Alanine; Normal: 15g/100g protein diet; High: 30g/100g protein diet.

Dietary supplementation of cysteine prodrugs to PM mice exhibited tissue-specificity in its effect on GSH/GSSG ratio. In particular, cysteine prodrug supplementation to PM mice restored the GSH/GSSG ratio in liver and heart, but not in lung. Interestingly, the GSH/GSSG ratio was not affected by PM in colon, spleen, kidney and brain.

Table 3
Tissue GSH/GSSG ratio of mice in the PM, Normal protein, High protein and Supplemented groups[a,b]

Diet[c]	Liver	Lung	Colon	Heart	Spleen	Kidney	Brain
PM	20.4 ± 1.9	136.6 ± 12.1[a]	34.2 ± 4.3	47.6 ± 4.3	54.8 ± 4.5	56.4 ± 6.6	52.6 ± 2.8
PM + NAC	106.7 ± 5.9[a]	82.7 ± 5.6[b]	21.7 ± 0.9[a]	34.4 ± 2.0[a]	59.5 ± 3.7	48.7 ± 4.8	49.2 ± 2.7
PM + OTC	96.5 ± 7.1[a]	64.2 ± 4.7[bc]	26.2 ± 1.9[a]	32.2 ± 1.9[a]	65.4 ± 5.8	56.6 ± 4.1	53.5 ± 2.7
PM + MET	78.2 ± 3.8[b]	80.8 ± 6.2[b]	28.1 ± 2.4[a]	30.3 ± 2.4[a]	66.5 ± 10.2	57.1 ± 6.2	51.9 ± 3.4
PM + GSH	95.7 ± 4.5[a]	60.9 ± 7.6[bc]	22.3 ± 1.4[a]	43.6 ± 1.4	58.5 ± 7.7	46.9 ± 2.0	48.4 ± 1.7
PM + Alanine[d]	18.2 ± 1.2	126.7 ± 7.9[a]	33.5 ± 4.2	43.2 ± 4.2	58.1 ± 8.2	50.2 ± 5.4	53.1 ± 1.9
Normal Protein	91.5 ± 5.4[a]	35.2 ± 4.9[c]	33.7 ± 2.5	34.7 ± 2.5[a]	60.6 ± 6.4	43.9 ± 6.1	49.4 ± 2.2
High Protein	94.3 ± 2.4[a]	39.5 ± 4.5[c]	36.6 ± 2.4	31.1 ± 2.4[a]	63.2 ± 3.7	47.7 ± 2.7	54.9 ± 3.1

[a] Results expressed as mean ± SEM ($n = 8$).
[b] Results were analyzed by 2-way ANOVA test. When an overall F ratio was significant, Tukey-HSD test was used. Within each column, values with different superscripts (none, a, b) were significantly different from one another ($P < 0.05$).
[c] PM: 0.5g/100g protein diet; Normal Protein: 15g/100g protein diet; high Protein: 30g/100g protein diet.
[d] Alanine was added so that the diet 0.5% + Alanine was isonitrogenous with the diet 0.5% + GSH.

3.6. Tissue GSSG/2GSH reduction potential derived from Nernst equation

The GSSG/2GSH reduction potential takes into consideration the correct stoichiometry of GSSG's reduction to GSH, and is believed to be a more comprehensive indicator of redox status [31]. Therefore, GSSG/2GSH reduction potential was derived from the Nernest Equation to determine the effects of PM and cysteine prodrug supplementation. Our results indicated that PM changed the GSSG/2GSH reduction potential in a tissue-specific manner (Table 4). In particular, PM significantly increased the reduction potential in colon, spleen and liver, but decreased it in lung. Notably, PM had no effect on the reduction potentials in heart, kidney and brain. Lastly, dietary supplementation of cysteine prodrugs to PM mice restored the GSSG/2GSH reduction potentials in liver and spleen only, but not in lung and colon.

4. Discussion

This study was designed to systematically compare the efficacy of short-term dietary supplementation of cysteine prodrugs on tissue GSH levels in mice fed a protein deficient diet. The results indicated that short-term (1 week) supplementation of cysteine prodrugs as a dietary component to PM mice restored GSH concentrations in liver, lung, heart and spleen. Supplementation increased colon GSH levels, but not to the ones comparable to normal controls. Interestingly, neither PM nor cysteine prodrugs affected GSH levels in brain or kidney. Thus, dietary supplementation of cysteine prodrugs restored GSH levels in a tissue-specific manner. However, all prodrugs exhibited similar GSH enhancement effects, showing no prodrug-specificity as has been demonstrated previously in cell culture studies [37,38]. In addition, cysteine prodrugs restored the redox status in liver and heart (based on GSH/GSSG ratio), and in liver and spleen (based on GSSG/2GSH reduction potential). Thus, the effect of these prodrugs on redox status was tissue-specific and dependent on the redox status indicator that was used.

In cell culture studies, the efficacy of cysteine prodrugs on GSH enhancement varies. For example, although addition of NAC [23], OTC [17,20] and methionine [22] to the medium significantly increased intracellular GSH levels,

Table 4
Tissue redox potential associated with GSSG/2GSH couple[a,b]

Diet[c]	Liver	Lung	Colon	Heart	Spleen	Kidney	Brain
PM	−202 ± 2.3	−219 ± 3.2	−197 ± 3.2	−205 ± 2.5	−208 ± 2.0	−212 ± 1.9	−212 ± 4.2
PM + NAC	−242 ± 2.6[a]	−220 ± 1.6	−196 ± 1.7	−206 ± 1.5	−215 ± 1.7[a]	−214 ± 2.6	−215 ± 3.4
PM + OTC	−239 ± 2.0[a]	−214 ± 1.3	−200 ± 1.8	−204 ± 1.3	−216 ± 1.6[a]	−215 ± 4.6	−211 ± 2.8
PM + MET	−244 ± 1.7[a]	−215 ± 1.2	−201 ± 2.5	−204 ± 1.8	−217 ± 2.3[a]	−213 ± 1.3	−214 ± 2.8
PM + GSH	−243 ± 1.7[a]	−213 ± 1.2	−197 ± 2.0	−209 ± 1.8	−217 ± 1.2[a]	−213 ± 1.6	−211 ± 1.9
PM + Alanine[d]	−200 ± 3.0	−221 ± 1.5	−197 ± 2.4	−203 ± 3.5	−209 ± 2.2	−210 ± 2.7	−212 ± 2.3
Normal Protein	−242 ± 2.3[a]	−207 ± 1.5[a]	−209 ± 2.7[a]	−207 ± 1.2	−218 ± 1.4[a]	−211 ± 1.9	−210 ± 2.6
High Protein	−243 ± 1.3[a]	−205 ± 1.8[a]	−211 ± 2.1[a]	−205 ± 1.6	−220 ± 2.1[a]	−212 ± 1.5	−213 ± 2.9

[a] Results expressed as mean ± SEM ($n = 8$).
[b] Results were analyzed by 2-way ANOVA test. When an overall F ratio was significant, Tukey-HSD test was used. Within each column, values with different superscripts (none, a, b) were significantly different from one another ($P < 0.05$).
[c] PM: 0.5g/100g protein diet; Normal Protein: 15g/100g protein diet; high Protein: 30g/100g protein diet.
[d] Alanine was added so that the diet 0.5% + Alanine was isonitrogenous with the diet 0.5% + GSH.

they were taken up by cells at varying rates, leading to different extents of GSH enhancement. It has also been reported that the capacity of rat hepatocytes to uptake and metabolize OTC was substantially lower than that for methionine, which was correlated with a less significant increase of GSH in the OTC group [37]. In addition, the rates of conversion to cysteine in cells were also different among the prodrugs. Cysteine was found more rapidly available from NAC than from methionine or OTC in rat hepatocytes [20,38]. In our study, however, short-term dietary supplementation of cysteine prodrugs did not show significant differences in their GSH enhancing/restoring capacity. The difference in uptake and conversion rates of cysteine prodrugs to cysteine in the cells may have a less significant impact on GSH enhancement/restoration when the supplementation period is one week. In the end, how much (but not how fast) cysteine prodrug can be utilized by the tissue and converted to cysteine may be more important in the 1-week supplementation. This appears to be supported by the finding that GSH production accounted for 80% of both NAC and OTC metabolism, although the rates of uptake by tissue and conversion to cysteine were significantly different between the two prodrugs [20].

This study also demonstrated that the cysteine prodrug enhancement of GSH was tissue-specific. This could be due to the tissue-specific efficiency of cysteine prodrug metabolism that was discovered in both *in vivo* and *in vitro* studies. For example, methionine could be actively converted to cysteine via the cystathionine pathway in isolated hepatocytes, but not in renal cells [39]. In addition, radiolabeled NAC was reported to be taken up by different tissues at varying rates and levels [21]. Taken together, tissue-specificity of cysteine prodrugs on GSH enhancement/restoration is possibly caused by varying capacities of prodrug uptake and cysteine conversion in different tissues, which did not seem to change with the length of supplementation (1 day or 1 week) or the choice of experiment model (cell culture or animal study).

One of the interesting findings in this study was that PM also induced tissue-specific changes in GSSG levels. In particular, PM decreased GSSG concentrations in lung but not in liver. One possible explanation for the decrease in lung GSSG comes from previous research where the activity of lung GSSG reductase was increased in PM mice [40]. As a result, GSSG might be reduced back to GSH at a higher rate, thus decreasing GSSG levels in the lung. In contrast, liver GSSG levels remained constant in PM although GSH levels were decreased significantly. This might be associated with increased oxidative stress found in PM liver by previous researchers [9]. Another possible factor is the GSSG efflux in the liver, which is believed to be an energy-requiring process [41]. This GSSG efflux could be disrupted in PM, resulting in a relative accumulation of GSSG in the liver.

Tissue GSH concentration alone is an important indicator of antioxidant status. On the other hand, GSH/GSSG ratio takes into account the dynamic balance between antioxidants and oxidants, and thus is widely used to estimate tissue redox status [31]. However, the GSH/GSSG ratio has its limitations because the correct stoichiometry for GSSG's reduction to GSH is not reflected in the ratio. Therefore, the GSSG/2GSH reduction potential, calculated from the Nernst equation, is proposed to be a comprehensive indicator of redox status since the GSH concentration is entered as a squared term in the equation [31,32].

In this study, tissue redox status based on GSH/GSSG ratio may not always agree with that based on the GSSG/2GSH reduction potential. For instance, cysteine prodrugs increased colon GSH in PM mice; however, the increase in colon GSSG was more prominent. As a result, the GSH/GSSG ratio was decreased. However, the colon reduction potential was unchanged. The apparent discrepancy can be attributed to the Nernst equation, where the elevated (although not yet restored) colon GSH levels were entered into the equation as a squared term and thus offset the more significant increase in GSSG [32]. Therefore, these two indicators are not interchangeable in certain situations.

GSSG/2GSH reduction potential was suggested to be associated with the cell biological status [31]. Specifically, proliferation was correlated with a reduction potential of −240 mV, while −200 mV was associated with differentiation, and −170 mV with apoptosis. In this study, proliferation, differentiation or apoptosis was not determined. However, it was observed that the normal liver had a reduction potential of –240 mV and an average weight of 1.79 g. In PM liver, the numbers were −200 mV and 0.76 g. Supplementation of cysteine prodrugs to PM mice not only restored the GSSG/2GSH reduction potential in liver, but also increased the tissue weight (NAC: 1.04 g, GSH: 1.20 g vs PM: 0.76 g). Although it was not clear whether the increase in liver mass was due to increased proliferation, it would certainly be interesting to investigate this in the future, as increased intracellular GSH induced by NAC supplementation was correlated with enhanced cell proliferation *in vitro* [42,43]. It was also noticed that the Nernst equation was based on 25°C and a neutral pH. Therefore, the GSSG/2GSH reduction potentials calculated might not reflect the true tissue redox status *in vivo.* However, it is a valid method to compare the relative redox status among different groups [31].

GSH and GSSG levels in the brain and kidney remained constant regardless dietary protein levels and supplementation status, suggesting redox status was not changed even in PM. This may be protective for the brain where ROS is believed to be generated at high rates due to the large amount of oxygen consumed in this organ [44]. Although we have not explored the mechanisms that may lead to the stable levels of GSH and GSSG in the brain, previous researchers have demonstrated that GSH, along with cysteine prodrugs, can be transported intact across the blood brain barrier and into the brain [45]. Thus, it is possible that the brain could either acquire GSH directly from blood, or

synthesize it from the cysteine prodrugs, although its capacity to synthesize GSH has been a matter of debate [19,46]. On the other hand, the constant GSH and GSSG levels in kidney might be related to its ability to utilize a number of disulfides by transforming them into GSH precursors [39]. Kidney was also reported to remove about half of the GSH exported by the liver, presumably due to action of gamma-glutamyl transpeptidase and dipeptidases [47]. Therefore, kidney might have sufficient supply of GSH precursors even in the PM situation because of its role in GSH turnover and its ability to utilize disulfides.

In summary, PM decreases tissue GSH levels, and increases ROS-mediated tissue damage and risk of opportunistic infections. Feeding a high-protein diet to PM subjects during early rehabilitation may impose metabolic stress and have other adverse effects. Therefore, restoring tissue GSH levels and redox status without giving a high-protein diet may be a useful adjunct therapeutic strategy during the early stage of PM rehabilitation. Our study showed that dietary supplementation of cysteine prodrugs to PM mice restored tissue GSH levels and redox status in a tissue-specific manner. On the other hand, all four cysteine prodrugs exhibited similar GSH-enhancing capacities, showing no prodrug-specificity. These data therefore provide critical information and insight for the future clinical application of short-term dietary supplementation of cysteine prodrugs in early PM rehabilitation.

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