RESEARCH ARTICLE

Methionine restriction up-regulates the expression of the pi class of glutathione *S*-transferase partially *via* the extracellular signal-regulated kinase-activator protein-1 signaling pathway initiated by glutathione depletion

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Understanding the molecular events underlying gene regulation by amino acids has attracted increasing attention. Here, we explored whether the mechanism by which methionine restriction affects the expression of the π class of glutathione *S*-transferase (GSTP) is related to oxidative stress initiated by glutathione (GSH) depletion. Rat primary hepatocytes were cultured in an L-15-based medium in the absence or presence of 200 μM L-buthionine sulfoximine (BSO) or in a methionine-restricted L-15 medium supplemented with 20 μM L-methionine up to 72 h. BSO and methionine restriction time-dependently induced GSTP mRNA and protein expression in a similar pattern accompanied by a decrease in the cellular GSH level. The phosphorylation of extracellular signal-regulated kinase (ERK), but not of c-Jun NH_2-terminal kinase and p38, was stimulated by methionine restriction and BSO. Electromobility gel shift assay showed that the DNA-binding activity of nuclear activator protein-1 (AP-1) increased in cells exposed to methionine restriction or BSO. With the ERK inhibitor FR180204, AP-1 activation and GSTP expression were abolished. Moreover, the induction of GSTP by methionine restriction and BSO was reversed by GSH monoethyl ester and *N*-acetylcysteine. Our results suggest that methionine restriction up-regulates GSTP gene expression, which appears to be initiated by the ERK-AP-1 signaling pathway through GSH depletion in rat hepatocytes.

Keywords:
Buthionine sulfoximine / Glutathione / L-methionine / π class of glutathione *S*-transferase / Primary hepatocytes

1 Introduction

A lesser susceptibility to carcinogen insult is known to be partially related to higher tissue levels of phase II biotransformation enzymes. Glutathione *S*-transferase (GST) catalyzes the conjugation of glutathione (GSH) with a variety of electrophilic xenobiotics and facilitates their excretion. In mammals, eight GST isozymes have been identified: A (α),

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Abbreviations: AARE, amino acid response element; **ARE,** antioxidant-responsive element; **AP-1,** activator protein-1; **ARE,** antioxidant-responsive element; **ATF,** activating transcription factor; **BSO,** L-buthionine sulfoximine; **EMSA,** electromobility gel shift assay; **ERK,** extracellular signal-regulated kinase; **GCS,** γ-glutamylcysteine synthase; **GSH,** glutathione; **GSH-EE,** GSH monoethyl ester; **GST,** glutathione *S*-transferase; **GSTP,** π class of GST; **GSTA,** α class of GST; **GSTM,** μ class of GST; **GPE I,** GSTP enhancer I; **JNK,** c-Jun NH_2-terminal kinase; **MAPK,** mitogen-activated protein kinase; **NAC,** *N*-acetylcysteine; **Nrf2,** nuclear factor E2-related factor 2; **ROS,** reactive oxygen species; **TNF-α,** tumor necrosis factor-α; **TRE,** 12-*O*-tetradecanoylphorbol-13-acetate response element

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M (μ), O (ω), P (π), S (σ), T (θ), Z (ζ), and K (κ) [1, 2]. Among those, the π class of glutathione *S*-transferase (GSTP) attracts much attention not only because of its action in drug detoxification but also because of its possible roles in cell transformation [3, 4]. Compared with the other GST isozymes, GSTP is more effective in the detoxification of the electrophilic α,β-unsaturated carbonyl compounds that are generated by radical reactions of lipids [5]. Because it is highly inducible during carcinogenesis, GSTP expression is regarded as an important determinant of cancer susceptibility and as a reliable marker of tumorigenesis [6]. The inducibility of GSTP is generally attributed to the existence of a strong enhancer named GSTP enhancer I (GPE I), which has two 12-*O*-tetradecanoylphorbol-13-acetate response-like elements (TRE), in the 5′ upstream region [7–9]. Multiple transcriptional factors, mainly activator protein-1 (AP-1), that bind to the TRE are responsible for the up-regulation of GSTP expression [10]. A number of cellular stresses and cytotoxic chemicals engage extracellular signal-regulated kinase (ERK), c-Jun NH_2-terminal kinase (JNK), and p38 kinase, which in turn activate AP-1 [11].

The tripeptide GSH (γ-glu-cys-gly) is an important cellular antioxidant and also is the most abundant cellular thiol. GSH has multiple functions, including protection of cells from the toxic effects of reactive oxygen species (ROS), participation in the detoxification by the GST enzymes, serving as a storage and transport form of cysteine, and promotion of the thiol forms of proteins [12]. Moreover, GSH protects cells from the toxic effects of ROS. The primary determinants of cellular GSH levels are thought to be the activity of the GSH biosynthetic enzymes, mainly γ-glutamylcysteine synthase (GCS), and the availability of substrates. L-cysteine and also L-methionine, which is converted to L-cysteine *via* the transsulfuration pathway in the liver, are limiting amino acids for GSH synthesis in hepatocytes [12]. A limited supply of L-cysteine decreases the synthesis of GSH and, thus, leads to an increase in cellular ROS production [13].

Gene expression in response to amino acid starvation has been well studied in bacteria, such as *Escherichia coli*, and eukaryotic yeast. In yeast, two control processes were shown, *i.e.* a specific control process, which is regulated by the specific amino acid end products, and a general control process, which is activated by a deficiency of any single amino acid such as leucine, histidine, and arginine [14, 15]. A similar adaptation mechanism to amino acid adequacy is present in mammals. Candidate genes whose expression is up-regulated by amino acid starvation include those involved in amino acid metabolism, such as insulin-like growth factor binding protein 1 [16] and asparagine synthetase [17], and those involved in the regulation of cell growth, such as c-*myc*, c-*jun* [18], and C/EBP homologous protein [19]. In addition, genes involved in drug metabolism are potent candidates whose expression is regulated by protein malnutrition. For instance, mouse liver contents of the GSTA3 and GSTP1 were increased with a protein-free diet, whereas normal contents were preserved with a protein-free diet replenished with either L-methionine or L-cysteine [20]. Moreover, mRNA levels of GSTA2/3/5 and GSTM1 in rat livers were increased by protein-calorie restriction, and this increase could be normalized by supplementation with L-cysteine or L-methionine [21].

In a previous study, we showed that GSTP gene expression is up-regulated in primary rat hepatocytes by L-methionine and L-cysteine restriction [22]. Interestingly, this increase of GSTP is not noted in hepatocytes by limiting other amino acids, including leucine, isoleucine, lysine, or phenylalanine. This finding suggests that a sulfur amino acid-specific mechanism exists in the up-regulation of this phase II detoxification enzyme. However, the molecular mechanisms involved in the regulation of GSTP gene expression by L-methionine and L-cysteine are not clearly elucidated. It has been shown that the activation of phosphatidylinositol 3-kinase caused by GSH depletion was essential for ROS-mediated GSTA2 induction in H4IIE hepatoma cells cultured in methionine- and cysteine-deprived medium [23]. This finding raises the possibility that the up-regulation of GSTP in response to L-methionine and L-cysteine restriction is related to the oxidative stress of low cellular GSH. To verify this hypothesis, we cultured primary hepatocytes in a medium restricted in L-methionine supply or in a medium containing L-buthionine sulfoximine (BSO, an inhibitor of γ-glutamylcysteine synthtase) and then determined GSTP expression and the activation of mitogen-activated protein kinase (MAPK) signaling.

2 Materials and methods

2.1 Materials

L-methionine, L-cysteine, ethacrynic acid, BSO, GSH monoethyl ester (GSH-EE), *N*-acetylcysteine (NAC), tumor necrosis factor-α (TNF-α), and type I rat tail collagen were obtained from Sigma Chemical (St. Louis, MO, USA). FR180204 was obtained from Calbiochem (La Jolla, CA, USA). Fetal bovine serum, sulfur amino acids-omitted Leibovitz L-15, and penicillin-streptomycin solution were obtained from Gibco Laboratory (Grand Island, NY, USA). Collagenase was purchased from Worthington Biochemical (Lakewood, NJ, USA). Percoll was from Amersham Biosciences (Uppsala, Sweden). Trizol was ordered from Invitrogen (Carlsbad, CA, USA). Antibodies against JNK and phospho-JNK were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against ERK, phospho-ERK (Thr202/Tyr204), p38, and phospho-p38 (Thr180/Tyr182) were purchased from Cell Signaling Technology (Beverly, MA, USA).

2.2 Cell isolation and culture

Male Sprague-Dawley rats were purchased from Charles River Laboratories (BioLASCO Taiwan) and were used for hepatocyte isolation when aged 7–8 wk old. Rats were treated in compliance with the *Guide for the Care and Use of Laboratory Animals* [24]. Hepatocytes were isolated by a two-step collagenase perfusion method as described previously [25]. Cell viability was >90% as determined by trypan blue exclusion. The isolated hepatocytes were suspended in L-15-based (sulfur amino acids-omitted Leibovitz L-15 plus 0.5 mM L-methionine and 0.2 mM L-cysteine) culture medium containing 18 mM HEPES, 5 mg/L transferrin, 5 μg/L selenium as sodium selenite, 1 g/L galactose, 1×10^5 U/L penicillin, 100 mg/L streptomycin, and 2.5% fetal bovine serum. Cells were plated on 35-mm plastic tissue culture dishes (Falcon, Franklin Lakes, NJ, USA) precoated with rat tail collagen type I at a density of 1×10^6 cells *per* 3 cm dish; the dishes were incubated in a 37°C humidified incubator in an air atmosphere. After 4 h, the cells were changed to fresh L-15-based culture medium. Twenty hours after attachment, hepatocytes were incubated in L-15-based medium, L-15-based medium plus 200 μM BSO (+BSO), or in a methionine-restricted L-15 medium (–Met, sulfur amino acids-omitted Leibovitz L-15 plus 0.02 mM L-methionine and 0.2 mM L-cysteine) for the indicated time. The L-methionine and L-cysteine supplements were freshly prepared. The medium was then changed once daily and cells were additionally cultured up to 72 h. Cells cultured in the L-15-based medium without BSO were regarded as the control. Inhibition of ERK activity by FR180204 at a concentration of 10 μM was performed 2 h before the treatment with methionine restriction or BSO. For antioxidant treatments, 2 mM NAC or GSH-EE were co-cultured with BSO and methionine restriction for 24 h.

2.3 SDS-PAGE and immunostaining

Cells were washed twice with cold PBS and were then harvested in 500 μL of 20 mM potassium phosphate buffer (pH 7.0). Supernatants were centrifuged at $10\,000 \times g$ for 30 min at 4°C. Protein concentrations were measured with a coomassie plus protein assay reagent kit (Pierce Chemical Rockford, IL, USA). Five micrograms protein of each sample was applied to 10% SDS-PAGE gels and was electrophoretically transferred to polyvinylidene fluoride membranes. The nonspecific binding sites on the membranes were blocked at 4°C overnight with 50 g/L nonfat dry milk in 25 mM Tris/150 mM NaCl buffer, pH 7.4. The blots were then incubated with primary antibodies against GSTP (Transduction Laboratories, Lexington, KY, USA), GSTA (α class of GST, Oxford Biomedical Research, Oxford, MI, USA), GSTM (μ class of GST, Oxford Biomedical Research), GCS heavy subunit (Abcam, Cambridge, UK), actin or α-tubulin (Sigma Chemical). After incubation with the horseradish peroxidase-conjugated secondary antibody, color was developed by adding hydrogen peroxide and tetrahydrochloride diaminobenzidine as peroxidase substrates. For the detection of MAPK phosphorylation, the membranes were incubated overnight at 4°C with antibodies against JNK1, phospho-JNK1, ERK1/2, phospho-ERK1/2, p38, or phospho-p38. The bands were detected by using an enhanced chemiluminescence kit (Perkin Elmer Life Science, Boston, MA, USA).

2.4 Electromobility gel shift assay

Hepatocytes were washed twice with cold PBS followed by scraping from the dishes with PBS. Cell homogenates were centrifuged at $2000 \times g$ for 5 min. The cell pellet was allowed to swell on ice for 15 min after the addition of 200 μL of hypotonic buffer containing 10 mM HEPES, 10 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, 0.5 mM DTT, 0.5% Nonidet P-40, 4 mg/L leupeptin, 20 mg/L aprotinin, and 0.2 mM phenylmethylsulfonyl fluoride. After centrifugation at $6000 \times g$ for 15 min, pellets containing crude nuclei were resuspended in 50 μL of hypertonic buffer containing 10 mM HEPES, 400 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, 0.5 mM DTT, 10% glycerol, 4 mg/L leupeptin, 20 mg/L aprotinin, and 0.2 mM phenylmethylsulfonyl fluoride and incubated for an additional 30 min on ice. The nuclear extracts were then obtained by centrifugation at $10\,000 \times g$ for 15 min and were frozen at −80°C until the electromobility gel shift assay (EMSA) was performed. EMSA was performed according to our previous study [26]. The LightShift™ Chemiluminescent EMSA Kit (Pierce Chemical) and synthetic biotin-labeled double-stranded AP-1 consensus oligonucleotides (5′-CGCTTGATGACTCAGCCGGAA-3′) were used to measure the effect of methionine restriction and BSO on AP-1 nuclear protein-DNA binding activity. Unlabeled double-stranded AP-1 oligonucleotide and a mutant double-stranded oligonucleotide were used to confirm the protein-binding specificity. Two micrograms of nuclear protein, poly(dI-dC), and biotin-labeled double-stranded AP-1 oligonucleotides, were mixed with the binding buffer to a final volume of 20 μL and were incubated at room temperature for 30 min. The nuclear protein–DNA complex was separated by electrophoresis on a 6% Tris-borate-EDTA polyacrylamide gel and was then electrotransferred to a Hybond-N^+ nylon membrane. The membrane was treated with streptavidin-horseradish peroxidase, and the nuclear protein-DNA bands were developed by using an enhanced chemiluminescence kit.

2.5 RNA isolation and RT-PCR

Total RNA was extracted by using Trizol reagent. RNA extracts were suspended in nuclease-free water and were

frozen at −20°C until the RT-PCR analysis was performed [27]. Briefly, 0.2 μg of total RNA was reverse transcribed with M-MMLV reverse transcriptase in a 20 μL final volume of the reaction buffer containing 25 mM Tris-HCl (pH 8.3), 50 mM $(NH_4)_2SO_4$, 0.3% β-mercaptoethanol, 0.1 g/L bovine serum albumin, 5 mM $MgCl_2$, and 1 mM each of deoxynucleotide triphosphate, 2.5 units RNase inhibitor, and 2.5 mM oligo(dT). For the synthesis of complementary DNA, reaction mixtures were incubated for 15 min at 45°C and were stopped by denaturing the reverse transcriptase at 99°C for 5 min. To these complementary DNA samples, PCR master mix containing 4 mM $MgCl_2$, 2.5 units Taq polymerase, and forward and reverse primers was added to a total volume of 30 μL. The sequences for the RT-PCR primers were as follows: for GSTP (forward: 5′-TTCAAGGCTCGCT-CAAGTCCAC-3′; reverse: 5′-CTTGATCTTGGGGCGGG-CACTG-3′); for glyceraldehydes-3-phosphate dehydrogense (forward: 5′-GACGTGCCGCCTGGAGAAA-3′; reverse: 5′-GGGGGCCGAGTTGGGATAG-3′). The PCRs were performed as follows: 5 min at 94°C; 35 cycles for 40 s at 94°C, 40 s at 60°C, and 120 s at 72°C; and a final extension for 5 min at 68°C. The PCR amplicons were then electrophoresed in 1%-agarose gels containing 1× TAE buffer (40 mM Tris, 20 mM glacial acetic acid, and 2 mM EDTA).

2.6 Biochemical assays

GST activity was determined by using ethacrynic acid as the substrate for GSH conjugation because of its better specificity for the π class isozyme [28]. Samples for intracellular GSH determination were prepared by adding 1 mL of 5% perchloric acid containing 2.5 mM phenanthroline to each plate. The plates were scraped and the homogenates were centrifuged at $10\,000 \times g$ for 10 min. After iodoacetic acid derivation and fluoro-2,4-dinitrobenzene color development, the acid-soluble GSH and GSH disulfide were determined by HPLC as described by Reed *et al.* [29].

Cell viability was determined by the 3-(4,5-dimethylthiazol-2yl)-2,5- diphenyltetrazolium bromide method as described previously [30]. Results indicated that up to 72 h of BSO treatment had no effect on cell survival (95 ± 3.6% of the control cells at 72 h). For cells with L-methionine restriction, cell viability at 48 and 72 h was 94 ± 2.8% and 78 ± 2.8%, respectively, that of the control cells.

2.7 Statistical analysis

All statistical analyses were performed with commercially available software (SAS Institute, Cary, NC, USA). Data were analyzed by means of one-way ANOVA, and the significant difference among treatment means was assessed by use of Tukey's test. The recovery of cellular GSH by antioxidants was compared by using Student's *t*-test. A value of $p < 0.05$ was considered to be significant.

3 Results

3.1 Effect of methionine and BSO on the GSTP protein level

The immunoblot assay showed that GSTP protein expression increased over the 72-h incubation period in a time-dependent manner in all groups (Fig. 1A). However, the increase in GSTP expression in cells treated with 0.02 mM L-methionine was greater than that in cells cultured in a normal L-15 medium containing 0.5 mM L-methionine ($p < 0.05$). Depletion of the cellular GSH level by BSO resulted in a change in GSTP protein expression similar to that with methionine restriction. At 72 h, the GSTP protein level in the methionine restriction and BSO groups was increased 1.8- and 2.8-fold, respectively, that of the control cells ($p < 0.05$) (Fig. 1B). In addition to GSTP, the expression of two other GST isozymes GSTA and GSTM and one GSH-related enzyme GCS over the 48-h incubation period was determined in this study as well (Fig. 1C). Compared with GSTP, the expression of GSTA and GSTM was not influenced by methionine restriction and BSO. GCS is a heterodimer consisting of a heavy and a light chain, and the expression of the heavy chain was greater in cells treated with methionine restriction and BSO than that in control cells.

3.2 GSTP mRNA level and enzyme activity

By RT-PCR, the changes in GSTP mRNA levels were consistent with those noted for protein expression. Regardless of the medium used, mRNA expression increased time dependently up to 72 h. mRNA levels were greater in cells exposed to methionine restriction or BSO than in control cells (Fig. 2A). A similar finding was noted for changes in GSTP enzyme activity by using ethacrynic acid as the substrate for GSH conjugation. Compared with that in control cells, GSTP enzyme activity to ethacrynic acid was 26 and 38% higher with methionine restriction or BSO treatment, respectively (Fig. 2B).

3.3 Changes of cellular GSH and protein contents

The intracellular GSH concentration in the control cells increased during the first 24 h and then decreased gradually up to 72 h (Fig. 3). In contrast, in cells incubated in a methionine-restricted medium, the GSH content dramatically decreased during the first 24 h (31.9 *versus* 6.5 nmol/mg protein; $p < 0.05$) and then remained constant over the next 48 h. A similar depletion of cellular GSH content from 31.9 to 4.2 nmol/mg protein also resulted from BSO treatment. Seventy-two hours after treatment, the GSH level was 13.8 and 4.4% in cells cultured with methionine restriction or BSO, respectively ($p < 0.05$). Although hepatic GSH level dramatically decreased in cells treated with methionine restriction and BSO, the changes of total cellular protein

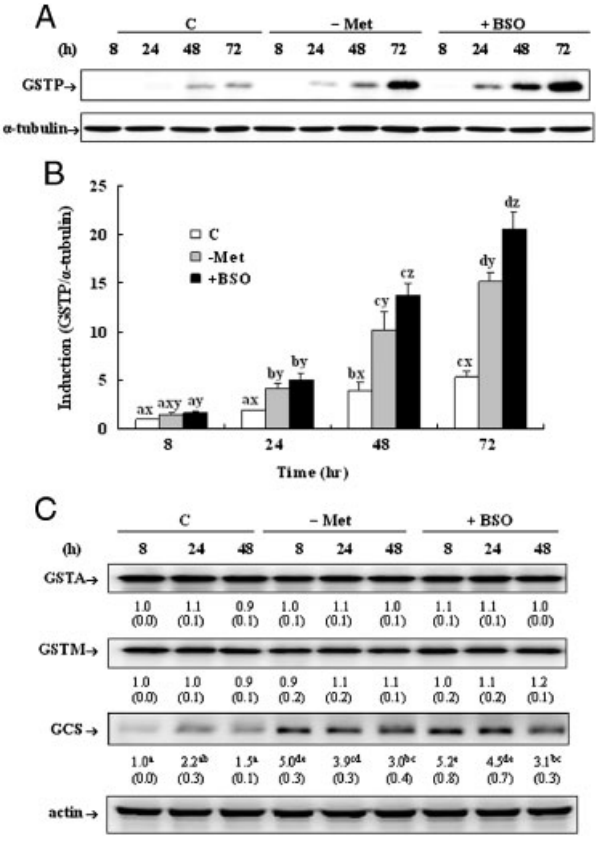

Figure 1. Methionine restriction and BSO induce expression of the GSTP in hepatocytes. After the 24-h attachment period, cells were incubated in an L-15-based control medium ((C) 0.5 mM L-methionine), a methionine-restricted L-15 medium (−Met, 0.02 mM L-methionine), or the L-15-based control medium plus BSO (200 μM) for 8, 24, 48, and 72 h. Expression of GSTP (A), GSTA, GSTM, and GCS heavy subunit (C) was determined by immunostaining. The protein was quantified by densitometry, and the level in control cells was set 1. Values are means (SD), $n = 3$. Means without a common letter differ, $p < 0.05$. Changes of GSTP protein level were normalized to the amount of α-tubulin (B). The level in control cells was regarded as 1. Values are means ± SD, $n = 4$. ^{abcd}Treatments in the same medium with different incubation times not sharing a common letter differ significantly, $p < 0.05$. ^{xyz}Treatments at the same incubation time not sharing a common letter differ significantly, $p < 0.05$.

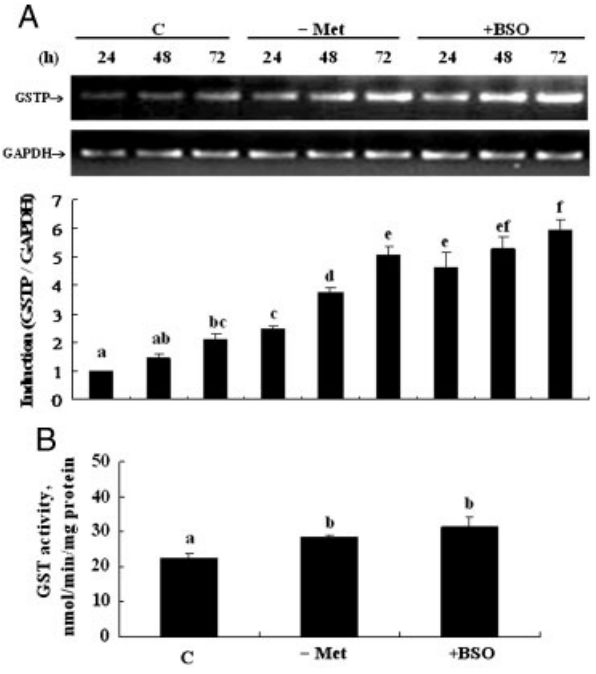

Figure 2. Changes in the GSTP mRNA expression and enzyme activity by L-methionine restriction (–Met) and +BSO treatment. Hepatocytes were incubated in a medium with 0.5 mM methionine (C), 0.02 mM methionine (–Met), or 0.5 mM methionine plus 200 μM +BSO for 24, 48, and 72 h. (A) RT-PCR assay of GSTP mRNA levels. The level in control mRNA was set 1. (B) GST activity at 72 h was measured using ethacrynic acid as substrate. Values are means ± SD, $n = 3$. Treatments not sharing a common letter differ significantly, $p < 0.05$.

content in all three groups showed a similar time-dependent decrease manner (Table 1). After 72-h incubation, a 33, 36, and 33% protein loss was found in control, in cells with methionine restriction or BSO, respectively.

3.4 Activation of MAPKs

MAPK signaling pathways, including JNK, ERK, and p38, are upstream mediators of AP-1. The phosphorylation of each MAPK was determined by immunoblot analysis and the results showed that ERK1/2 phosphorylation in the methionine restriction and BSO groups was noted at 8 h and time-dependently increased up to 24 h and then decreased (Fig. 4). By comparison, ERK1/2 phosphorylation in control cells was initially induced at 24 h, and the extent of activation was less than that noted in both GSH-depletion groups. No difference in JNK or p38 phosphorylation was detected among the three groups.

3.5 AP-1 activation

The activation of nuclear AP-1 by methionine restriction and BSO was measured by EMSA (Fig. 5A). Compared with that in control cells, the DNA-binding activity of AP-1 was increased in cells treated with methionine restriction (−Met) and BSO (+BSO), which was similar to that in cells treated with TNF-α, a well-known AP-1 activator (Fig. 5A). Specificity of the DNA–protein interaction for AP-1 was demonstrated by a competitive assay with 100-fold excess of unlabeled double-stranded oligonucleotide (cold) and also with a mutant double-stranded oligonucleotide (mut). In the presence of the ERK inhibitor FR180204, the activation of AP-1 by methionine restriction and BSO was abolished. To demonstrate the importance of the ERK-AP-1 pathway on GSTP induction, we then determined whether FR180204

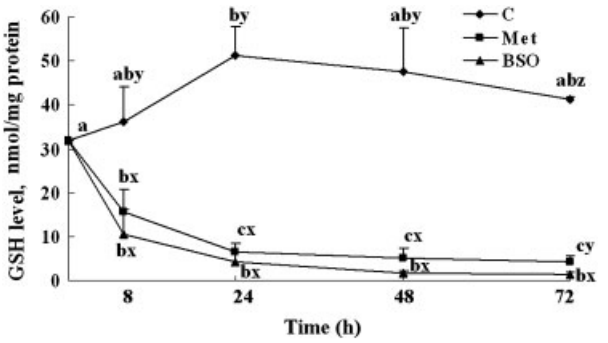

Figure 3. Depletion of cellular GSH contents by L-methionine restriction (–Met) and +BSO treatment. Over the 72-h treatments, GSH contents were determined by HPLC. Values are the means ± SD, $n = 3 \sim 5$. [abc]Groups in the same medium with different incubation times not sharing a common letter are significantly different, $p < 0.05$. [xyz]Treatments at the same incubation time not sharing a common letter differ significantly, $p < 0.05$.

Table 1. Changes of cellular protein content[a,b)]

Treatments	Time (h)		
	24	48	72
	mg protein/10^6 cells		
C	1.53 ± 0.16[a)]	1.28 ± 0.06[a,b)]	1.15 ± 0.15[b)]
–Met	1.51 ± 0.16[a)]	1.39 ± 0.18[a,b)]	1.10 ± 0.14[b)]
+BSO	1.46 ± 0.09[a)]	1.37 ± 0.07[a)]	1.15 ± 0.01[b)]

a) After the 24-h attachment period, hepatocytes were incubated in a medium with 0.5 mM methionine (C), 0.02 mM methionine (–Met), or 0.5 mM methionine plus 200 μM buthionine sulfoximine (+BSO) for up to 72 h.

b) Values are means ± SD, $n = 3$–4.

a,b) Treatments in the same medium with different incubation times not sharing a common letter differ significantly, $p < 0.05$. Cellular protein contents at time 0 was 1.71 ± 0.14 mg/10^6 cells.

suppressed the up-regulation of GST protein expression by methionine restriction and BSO. As noted in Fig. 5B, in the presence of FR180204, induction of the expression of this phase II detoxification enzyme disappeared.

3.6 Effect of antioxidants on GSTP

Two GSH precursors (NAC and GSH-EE) were tested to demonstrate that GSH depletion is crucial for the up-regulation of GSTP expression by methionine restriction and BSO. As noted, in the absence of NAC and GSH-EE, GSH contents were significantly decreased by methionine restriction and BSO ($p < 0.05$; Fig. 6A). With GSH-EE co-treatment, the cellular GSH level was significantly increased in both the methionine restriction and BSO groups ($p < 0.05$). The recovery of GSH by NAC, however, was noted

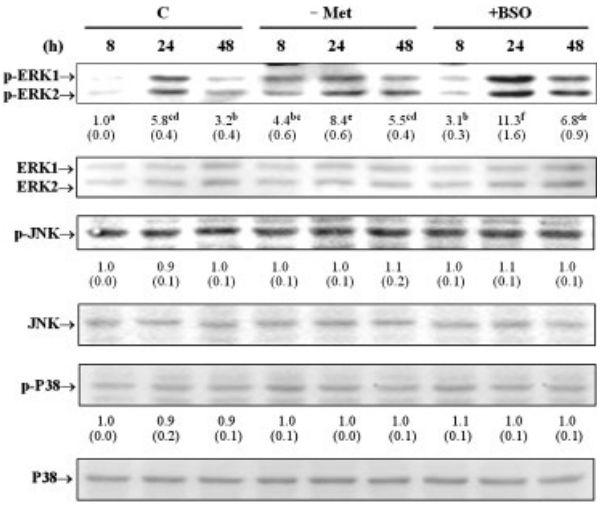

Figure 4. Activation of ERK, JNK, and p38 by methionine restriction and BSO. After the 24-h attachment period, hepatocytes were treated with an L-15-based control medium (C), a methionine-restricted L-15 medium (–Met), or the L-15-based control medium plus +BSO for 8, 24, and 48 h. The protein was quantified by densitometry, and the level in control cells was set 1. Values are pooled results of both ERK1 and ERK2 and are expressed as means (SD), $n = 3$. Means without a common letter differ, $p < 0.05$.

only in the methionine restriction group ($p < 0.05$) and not in the BSO group. Consistent with these changes in GSH content, ERK phosphorylation (Fig. 6B) and GSTP protein induction (Fig. 6C) was reversed by GSH-EE in both the methionine restriction and BSO groups and by NAC in the methionine restriction group.

4 Discussion

It has been known since the early 1970s that hepatic drug metabolism capacity can be modulated by feeding rats a protein-calorie restricted diet [31]. Later, the expression of several GST isozymes, including GSTA3 and GSTP1, was shown to be up-regulated in mouse livers under protein malnutrition. Moreover, this increase could be diminished by replenishing the diet with L-methionine and L-cysteine, which suggests a critical role of sulfur amino acids in GST expression [20]. Recently, we further reported that the expression of GSTP in rat hepatocytes is induced by limiting the supply of L-methionine and L-cytseine, but not by limiting other amino acids, including lysine, leucine, isoleucine, or phenylalanine [22]. This finding suggests that a sulfur amino acid-specific mechanism exists in the up-regulation of this phase II detoxification enzyme. In the present study, we showed that the up-regulation of GSTP gene expression *via* L-methionine restriction is likely through a GSH depletion-activated ERK-AP-1 signaling pathway.

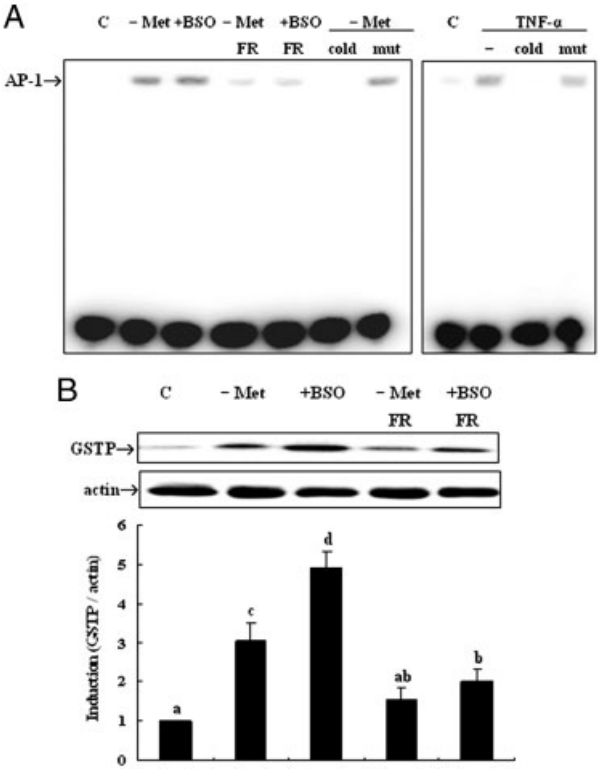

Figure 5. FR180204 suppression of AP-1 activity (A) and GSTP protein expression (B). After the 24-h attachment period, hepatocytes were incubated in the control L-15-based medium (C), the control medium plus 200 μM +BSO, a methionine-restricted L-15 medium (–Met) for 24 h, or the control medium plus 1 ng/mL TNF-α for 1 h. Hepatocytes were pretreated with the ERK inhibitor FR180204 (FR) for 2 h before methionine restriction or BSO treatment. The DNA-binding activity of AP-1 was measured by EMSA, and GSTP levels were measured by immunoblot assay. Unlabeled double-stranded AP-1 oligonucleotide (cold) and a mutant double-stranded oligonucleotide (mut) were added for the specificity assay. The protein was quantified by densitometry, and the level in control cells was set 1. Values are means ± SD, $n = 3$. Means without a common letter differ, $p < 0.05$.

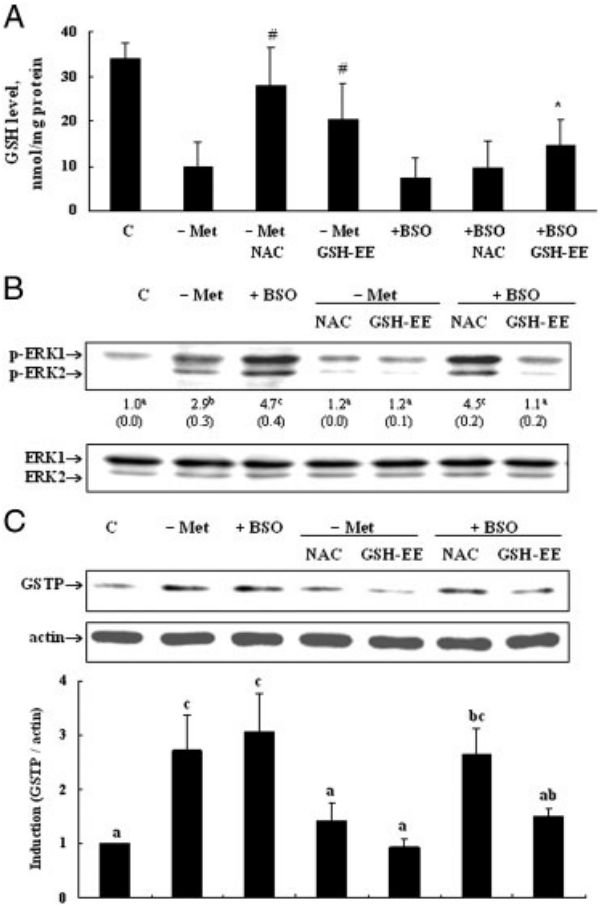

Figure 6. NAC and GSH-EE repletion of GSH contents (A) and suppression of ERK phosphorylation (B) and GSTP protein expression (C) in hepatocytes. After the 24-h attachment period, cells were allowed to incubate in the control L-15-based medium (C) or were switched to a methionine-restricted L-15 medium (–Met, 0.02 mM L-methionine) or to a control L-15-based medium containing 200 μM +BSO in the presence or absence of 2 mM NAC or 2 mM GSH-EE for an additional 24 h. The protein was quantified by densitometry, and the level in control cells was set 1. Values are pooled results of both ERK1 and ERK2 and are expressed as means (SD), $n = 3$. Means without a common letter differ, $p < 0.05$. The GSH levels are means ± SD, $n = 3 \sim 4$. $^{\sharp}p < 0.05$ *versus* Met alone group by Student's *t*-test. $^{*}p < 0.05$ *versus* BSO alone group by Student's *t*-test.

Amino acids modulate gene expression at the level of DNA transcription, mRNA translation, and also protein turnover [32, 33]. In the case of responding to essential amino acids sufficiency, translation initiation can be up-regulated or down-regulated by regulating the activity of eukaryotic initiation factors and the phosphorylation of eukaryotic initiation factor 4E-binding protein 1 and protein S6K1, which affects the formation of a translationally competent 80S ribosome [34]. In addition, the up-regulation of C/EBP-homologous protein in response to L-leucine restriction is mainly at the transcriptional level and is determined by the binding of ATF2 and ATF4 at the amino acid response element (AARE) (5′-ATTGCATCA-3′) [35, 36]. The activation of ATF4 binding to AARE is also responsible for the induction of arginine/lysine transporter cat-1 in C6 rat glioma cells during cysteine, methionine, and glutamine starvation [37]. L-cysteine and also L-methionine, which is converted to L-cysteine *via* the transsulfuration pathway, are limiting amino acids for GSH synthesis in hepatocytes [12]. A shortage of L-cysteine and/or L-methionine supply decreases cellular GSH contents and increases oxidative stress and in turn activates redox-sensitive transcriptional factors, including AP-1, NF-κB, and nuclear factor E2-related factor 2 (Nrf2) [38]. Therefore, it is possible that L-methionine restriction up-regulates GSTP gene expression *via* a GSH depletion-dependent pathway.

To examine this possibility, we used BSO to inhibit GSH synthesis and to mimic the GSH depletion that results from

methionine restriction. As noted, over the entire 72 h, the decrease in the cellular GSH content in cells treated with 0.02 mmol/L L-methionine was similar to that in cells treated with 200 μmol/L BSO (Fig. 3). Moreover, GSTP induction by methionine restriction was similar to that resulting from BSO treatment (Fig. 1A). These findings support the notion that GSH depletion plays, at least in part, a role in up-regulating the expression of this detoxification enzyme.

The expression of GSTP is known to be highly inducible and is largely attributed to the existence of a strong enhancer termed GPE I, which has two TRE in the 5′ upstream region [7–9]. TRE is essential for the induction of GSTP transcription by 3,4,5,3′,4′-penta-chlorinated biphenyl in primary hepatocytes [8]. Because of the existence of AP-1-like binding sites in the TRE, multiple transcriptional factors, mainly AP-1, that bind to TRE are responsible for the up-regulation of GSTP expression [10]. In the present study, the parallel response of ERK phosphorylation (Fig. 4) and AP-1-binding activity to DNA (Fig. 5A) and the inhibition of FR180204 on GSTP induction (Fig. 5B) strongly support that the ERK-AP-1 pathway plays a key role in the up-regulation of GSTP gene transcription by methionine restriction.

ERK is regarded as an oxidative stress-sensitive signaling pathway [41]. To further examine whether oxidative stress resulting from GSH depletion is responsible for GSTP induction, we tested the effects of two antioxidants, GSH-EE and NAC. GSH-EE hydrolyzed by esterases helps to restore intracellular GSH [42]. NAC functions not only as an antioxidant to scavenge oxygen-derived free radicals but also as a precursor of L-cysteine [43]. Although NAC can act as an antioxidant directly, most NAC is deacetylated extracellularly before uptake by hepatocytes [44]. Meanwhile, cysteine is used as a substrate for cellular GSH synthesis. Therefore, NAC protection of hepatocytes against oxidative damage is likely associated with its effectiveness in GSH synthesis. By comparison, GSH depletion resulting from methionine restriction was partially reversed by both NAC and GSH-EE (Fig. 6A). In the BSO group, only GSH-EE resulted in the recovery of cellular GSH. Parallel with the recovery of GSH content, ERK activation and GSTP induction by methionine restriction and BSO was suppressed (Figs. 6B and C). These findings suggest that GSH depletion by methionine restriction presumably results in oxidative stress that in turn activates the ERK-AP-1 signaling pathway and finally up-regulates GSTP expression.

In addition to AP-1, the participation of the redox-sensitive transcription factor Nrf2 in rat GSTP expression cannot be excluded. Evidence indicates that activation of Nrf2 binding to the antioxidant-responsive element (ARE) is essential for the up-regulation of heme oxygenase-1, NAD(P)H quinone oxidoreductase, and GSTA2 under oxidative stress [23, 45, 46]. In addition, Ikeda *et al.* (2002) reported that androgen-induced GSTP expression was dependent on Nrf2 activation in a knockout mouse model [47]. However, it needs to be addressed why, instead of the existence of a conserved ARE in the mouse GSTP gene promoter, there are only ARE-like elements (−1776 to −1768 bp, 5′-ATTCCACCA-3′, and −1970 to −1978 bp, 5′-ACTGCCTCA-3′) identified in rat GST (Genbank DQ102708). Although a later study by Ikeda *et al.* (2004) reported that Nrf2/MafK can bind to GPE I and is responsible for GSTP induction during early carcinogenesis in H4IIE rat hepatoma cells [48], evidence of the actual role of Nrf2 in modulating rat GSTP gene transcription is limited. In this study, Nrf2 activation as measured by nuclear Nrf2 contents was noted to increase in cells treated with methionine restriction or BSO (data not shown), which suggests that Nrf2 is activated when cellular GSH is depleted. It will be interesting to further examine whether Nrf2 binding to ARE-like elements is also responsible for GSTP up-regulation by methionine restriction and BSO.

In conclusion, the mechanism by which methionine restriction up-regulates GSTP gene expression appears to be associated with GSH depletion, which in turn activates the ERK-AP-1 signaling pathway. Under amino acid starvation, cells accommodate themselves to the changed environment through the up-regulation of a diversity of genes in order to survive. The increase in GSTP expression may afford cells the ability to protect against toxicant insults under a condition such as methionine deficiency and/or protein malnutrition.

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