--pi glutathione S-transferase

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氨基酸限制與解毒酵素--Pi 型 glutathione S-transferase

活性與表現

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中文摘要

氨基酸除了作為蛋白質的生合成原料，及提供組織修補材料外，其生理濃度之變化亦可扮演調控基因表現之訊號。本研究以初代肝細胞培養模式，利用分子生物技術探討氨基酸對於解毒酵素-π屬穀胱甘肽硫轉移酶(π form of glutathione S-transferase, GSTP)表現的作用機轉。結果顯示，無論在 0.5 mmol/L L-methionine / 0.2 mmol/L L-cysteine (高含硫氨基酸[HSAA])或 0.1 mmol/L L-methionine / 0.1 mmol/L L-cysteine(低含硫氨基酸[LSAA])培養液，GSTP 蛋白質皆隨培養時間而增加，然而在 LSAA 比 HSAA 有較高的 GSTP 蛋白質；在第六天，LSAA 處理下，相較 HSAA 的 GSTP 蛋白質約增加 94%。Dexamethasone 會抑制 GSTP 蛋白質表現，然而 dexamethasone 的抑制現象，並不會改變含硫氨基酸限制下增加 GSTP 蛋白質的表現，可知氨基酸調節 GSTP 蛋白質表現與 dexamethasone 無關，而且此調節現象亦與 insulin 無關。在 GSTP 酵素活性方面結果與蛋白質表現一致，LSAA 比 HSAA 有較高的 GSTP 酵素活性，而 GSTP 的誘發只發生在甲硫氨酸限制的培養液，其他氨基酸限制下則不影響，因此在第二年，我們則進一步探討甲硫氨酸限制對於 GSTP 表現的作用機轉，結果顯示，GSTP mRNA 和蛋白質表現皆隨著甲硫氨酸限制時間而增加；而且甲硫氨酸限制下 8 至 24 小時會刺激 extracellular signal-regulated kinase (ERK)蛋白質，而不影響 c-Jun NH_2-terminal kinase (JNK)和 p38 蛋白質；Electrophoretic mobility shift assay (EMSA)也顯示，甲硫氨酸限制後 24 小時會增加 AP-1 活化；而使用 PD98059 (ERK 抑制劑)則會破壞此 AP-1 的結合作用，並降低 GSTP 基因的表現。由以上結果可知，含硫氨基酸限制會增加 GSTP 基因表現，而甲硫氨酸限制下上調 GSTP 基因表現與 ERK-AP-1 訊號途徑有關。

Part Ⅰ

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Nutrient-Gene Interactions

Sulfur Amino Acid Restriction Induces the π Class of Glutathione S-Transferase Expression in Primary Rat Hepatocytes[1]

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ABSTRACT The regulation of genes by amino acids is attracting increasing attention. In the present study, we investigated the restriction of expression of the π class of glutathione S-transferase (GST Yp) by sulfur amino acids. Hepatocytes isolated from male Sprague-Dawley rats were cultured with L-15–based medium containing low (LSAA; 0.1 mmol/L L-methionine and 0.1 mmol/L L-cysteine) or high (HSAA; 0.5 mmol/L L-methionine and 0.2 mmol/L L-cysteine) amounts of sulfur amino acids for up to 6 d. Cellular protein contents did not differ between LSAA- and HSAA-treated cells over the entire period. In contrast, glutathione concentrations were suppressed by the LSAA medium and on d 6 were only 20% of those of HSAA-treated cells ($P < 0.05$). As shown by immunoblot analysis, GST Yp protein levels were greater in LSAA-treated cells than in HSAA-treated cells ($P < 0.05$). The induction of GST Yp by L-methionine and L-cysteine restriction was not affected by insulin and dexamethasone, but the latter suppressed GST Yp expression ($P < 0.05$). LSAA increased GST Yp mRNA levels and GST activity toward ethacrynic acid ($P < 0.05$). GST Yp induction occurred only in cells with a limited supply of L-methionine; restriction of L-isoleucine, L-leucine, L-lysine, and L-phenylalanine had no significant effect. In contrast with the induction of GST Yp, the expression of the GST isoforms Ya and Yb was not changed by amino acid restriction. In conclusion, hepatic GST Yp gene expression is upregulated by a limited availability of sulfur amino acids.   J. Nutr. 135: 1034–1039, 2005.

KEY WORDS: • sulfur amino acids • glutathione S-transferase Yp • gene expression
• primary hepatocytes • rats

Amino acids have multiple functions, i.e, they act as gluconeogenic substrates, as regulators of protein turnover, as neurotransmitters, and as precursors of signal transducers (1). A sufficient amino acid supply is required for normal cell growth and physiologic responses. When the amino acid supply is limited, several physiologic functions change through the regulation of the expression of numerous genes; such changes help an organism adapt to amino acid limitation. Growth retardation is one of the physiologic changes that occur with dietary protein restriction (2) and may be attributed in part to the overexpression of insulin-like growth factor binding protein 1 (IGFBP-1),[3] which reduces the available concentration of insulin-like growth factor (3).

Gene transcription in response to amino acid starvation has been well studied in bacteria, such as *Escherichia coli*, and in eukaryotic yeast. In yeast, 2 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 (4,5). In mammals, however, the molecular mechanisms of gene regulation by amino acid starvation are still poorly understood (3,6,7). Candidate genes whose expression is regulated by amino acid starvation include those involved in amino acid metabolism, such as asparagine synthetase (8) and the cationic amino acid transporter cat-1 (9), and those involved in the regulation of cell growth, such as c-*myc*, c-*jun* (10), and C/EBP homologous protein (CHOP) (11). Genes involved in drug metabolism may also be modulated during protein malnutrition. In rats fed a protein-energy–deficient diet, protein levels of hepatic cytochrome P_{450} 3A, 1A2, 2E1, and 2C1 were reduced by >50% (12,13), and such a reduction could be completely or partly normalized by simply replenishing the diet with L-cysteine or L-methionine (12). In contrast with the suppression of cytochrome P_{450} expression, glutathione S-transferase (GST) Ya2/3/5 and Yb1 mRNA levels in rat livers were increased by protein-energy restriction, and this increase was also normalized by L-cysteine or L-methionine supplementation (14).

GST is a phase II drug-metabolizing enzyme that catalyzes the conjugation of glutathione (GSH) with a variety of electrophilic xenobiotics and facilitates their excretion. GST is composed of 6 distinct gene families: 5 cytosolic groups (α, μ, π, θ, and σ) and one microsomal form (κ) (15). The families participate in a similar reaction in detoxification but have different substrate affinity. The class μ and θ GSTs participate in GSH conjugation with benz[*a*]anthracene epoxides and

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polycyclic aromatic hydrocarbons and also influence the production of oxidative DNA damage (16). There is growing interest in the physiologic properties of the π class of GST (GST Yp), not only because of its drug detoxification activity but also because of its association with cell transformation, which may relate to carcinogenesis (17,18). GST Yp activity was used to evaluate the potency of chemoprevention agents in benzo[a]pyrene-induced cancer (19).

As stated, the induction of hepatic GST Ya2/3/5 and Yb1 mRNA levels by protein-energy restriction is reversed by simple L-cysteine or L-methionine replenishment (14). This suggests that L-cysteine or L-methionine deficiency acts to upregulate the transcription of various GST genes. It is of interest therefore to know whether the expression of other GST isozymes is also modulated in response to sulfur amino acid restriction. In the present study, we examined the activity and expression of GST Yp in primary rat hepatocytes cultured in an amino acid–restricted medium.

MATERIALS AND METHODS

Materials. Sodium selenite, methionine, cysteine, ethacrynic acid, HEPES, and type VII rat tail collagen were obtained from Sigma Chemical. Insulin, transferrin, fetal bovine serum, and penicillin-streptomycin solution were obtained from Gibco Laboratory. Collagenase was purchased from Worthington Biochemica. Percoll was from Amersham Biosciences. Trizol was ordered from Invitrogen.

Cell isolation and culture. Male Sprague-Dawley rats were purchased from the National Laboratory Animal Center and were used for hepatocyte isolation at age 7–8 wk. Rats were treated in compliance with the NIH guidelines (20). Hepatocytes were isolated by a 2-step collagenase perfusion method as described previously (21). Cell viability was >90% as determined by trypan blue exclusion. The isolated hepatocytes were suspended in L-15 cell culture medium containing 18 mmol/L HEPES, 5 mg/L transferrin, 5 μg/L selenium as sodium selenite, 1 g/L galactose, 1 × 10^5/L penicillin, 100 mg/L streptomycin, and 2.5% fetal bovine serum. The cells were plated on 60-mm plastic tissue culture dishes (Falcon) precoated with rat tail collagen VII at a density of 2.5 × 10^6 cells per dish; the dishes were incubated in a 37°C humidified incubator in an air atmosphere. Cell attachment on the culture dish was achieved 4 h after plating, after which time the medium was changed. Thereafter, the medium was changed once daily, and the cells were cultured up to 6 d.

In Expt. 1, we investigated the effect of sulfur amino acid restriction on GST Yp expression. In this study, cells were cultured in either the L-15 control medium containing 0.5 mmol/L L-methionine and 0.2 mmol/L L-cysteine (high sulfur amino acid [HSAA] medium) or the L-15 medium containing 0.1 mmol/L L-methionine and 0.1 mmol/L L-cysteine (low sulfur amino acid [LSAA] medium). In addition, we examined the effect on GST Yp expression of insulin (5 mg/L) and dexamethasone (1 μmol/L), 2 commonly supplemented growth factors that are capable of modulating gene expression. For all treatments, cells were harvested at 24, 48, 96, and 144 h after plating.

In Expt. 2, 4 essential amino acids in addition to L-methionine and L-cysteine (L-isoleucine, L-leucine, L-lysine, and L-phenylalanine) were tested to examine whether GST Yp expression is also regulated by nonsulfur-containing amino acids. In this study, hepatocytes were exposed to either an L-15 control medium (0.5 mmol/L Met, 0.2 mmol/L Cys, 1 mmol/L Ile, 1 mmol/L Leu, 0.5 mmol/L Lys, 0.75 mmol/L Phe) or a medium restricted in a single amino acid (i.e., 0.02 mmol/L each of L-methionine, L-cysteine, L-isoleucine, L-leucine, L-lysine, or L-phenylalanine) for up to 4 d. The use of 0.02 mmol/L L-methionine was adapted from the physiologic serum concentration in rats fed a low-protein diet (3).

SDS-PAGE and immunodetection. Cells were washed twice with cold PBS and were then harvested in 500 μL of 20 mmol/L potassium phosphate buffer (pH 7.0). Cell homogenates were centrifuged at 10,000 × g for 30 min at 4°C. The resultant supernatant fluid was then ultracentrifuged at 105,000 × g for an additional 1 h. Protein concentrations were measured with a Coomassie plus protein assay reagent kit (Pierce). Equal amounts of cytosolic proteins of each sample were applied to 10% SDS-PAGE gels and were transferred electrophoretically to polyvinylidene fluoride membranes as described by Towbin et al. (22). Nonspecific binding sites on the membranes were blocked at 4°C overnight with 50 g/L nonfat dry milk in buffer containing 15 mmol/L Tris and 150 mmol/L NaCl (pH 7.4). The membranes were then incubated with antibodies against GST Yp (Transduction Laboratories), Ya, Yb (Oxford Biomedical Research), or carbonic anhydrase III (CA III; kindly provided by Dr. Suzanne Hendrich, Iowa State University). After incubation with the peroxidase-conjugated secondary antibody, color was developed by adding hydrogen peroxide and tetrahydrochloride diaminobenzidine as peroxidase substrates.

Northern blot analysis. Total RNA was extracted by using Trizol reagent. A cDNA probe was prepared by RT-PCR as described previously (23). Two pairs of oligonucleotide primers (forward: 5′-TTCAAGGCTCGCTCAAGTCCAC-3′; backward: 5′-CTTGAT-CTTGGGGCGGGCACTG-3′) were designed on the basis of the published sequence of GST Yp (23,24). The PCR conditions were set as follows: denaturing at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min for 35 cycles followed by a 7-min extension at 72°C. The band corresponding to the GST Yp DNA fragment was labeled with α-^{32}P-dCTP through use of an NEBlot kit (New England Biolabs) and was used as a probe. For Northern blot analysis, 20 μg of each RNA sample was separated electrophoretically on a 1%-agarose gel containing 6% formaldehyde and was then transferred to HyBond N$^+$ membrane as previously described (23). The membrane was prehybridized for 2 h at 42°C in a solution containing 10X Denhardt's reagent (0.2% Ficoll, 0.2% polyvinylpyrolidone, 0.2% bovine serum albumin), 5X SSPE (750 mmol/L NaCl, 50 mmol/L NaH$_2$PO$_4$, 5 mmol/L EDTA), 20 g/L SDS, 50% formamide, and 100 mg/L of single-stranded sheared salmon sperm DNA. The membrane was then hybridized in the same solution with an α-^{32}P-labeled GST Yp cDNA probe at 42°C overnight. After the wash, autoradiography was performed by exposing the membrane to SuperRx X-ray film (Kodak) at −80°C with an intensifying screen. The bands on the X-ray film were measured with an AlphaImager 2000 (Alpha Innotech).

Biochemical assays. GST activity was determined according to the method of Habig et al. (25) with the use of ethacrynic acid as the substrate because of its better specificity for the Yp class (26). Samples for intracellular GSH determination were prepared by adding 1 mL of 5% perchloric acid containing 2.5 mmol/L phenanthroline to each plate. The plates were scraped and the homogenates were centrifuged at 10,000 × g for 10 min. After iodoacetic acid derivation and fluoro-2,4-dinitrobenzene color development, the acid-soluble GSH and glutathione disulfide were determined by HPLC as described by Reed et al. (27).

Statistical analysis. Data were analyzed by one-way ANOVA, and Tukey's test was used to test the significance of the effect of culture time or growth factors in each sulfur amino acid–treated group. A two-way ANOVA was used to test the interaction of sulfur amino acids and growth factors on the GST Yp protein level. The LSAA and HSAA groups were compared using Student's t test. Differences with P-values < 0.05 were considered significant. All statistical analyses were performed with commercially available software (SAS Institute).

RESULTS

Effect of sulfur amino acids on GST Yp protein level. Regardless of the sulfur amino acid content of the medium, GST Yp protein expression increased with time of incubation (**Fig. 1**). However, GST Yp expression increased sooner in cells treated with LSAA medium, and on d 6, the relative GST Yp level was 94% higher in LSAA-treated cells than in HSAA-treated cells.

Effect of growth factors on GST Yp protein expression. We then studied whether the effect of sulfur amino acids on GST Yp expression is influenced by insulin and dexamethasone, 2 commonly supplemented growth factors that modulate gene expression. GST Yp levels were suppressed by dexameth-
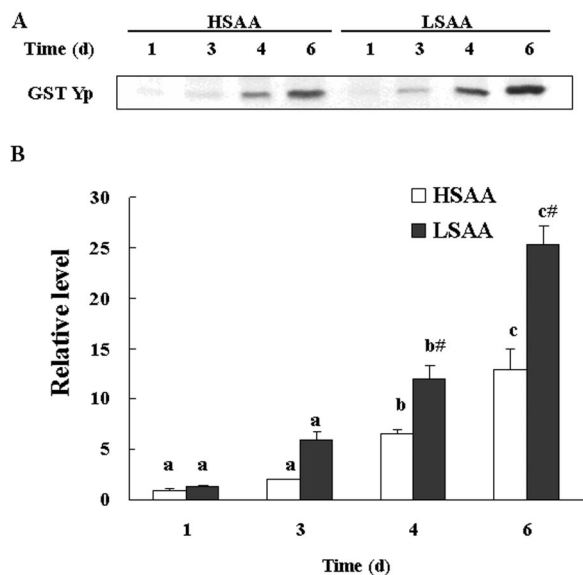
A

B

FIGURE 1   Effect of sulfur amino acid restriction on protein levels of GST Yp in primary rat hepatocytes. (*A*) After the 4-h attachment period, cells were left to incubate in the control medium (HSAA: 0.5 mmol/L L-methionine and 0.2 mmol/L L-cysteine) or were switched to the LSAA (0.1 mmol/L L-methionine and 0.1 mmol/L L-cysteine) for up to 6 d. For each lane, 5 μg of cytosol protein was separated on 10% SDS-polyacrylamide gels and an immunoblot assay was performed. (*B*) Protein was quantitated by densitometry, and the level on d 1 for the HSAA-treated cells was regarded as 1. Each value represented the mean $\pm$ SD, $n = 4$ independent experiments. [abc]Groups in the same medium not sharing a letter differ, $P < 0.05$. [#]Different from HSAA at that incubation time, $P < 0.05$.

asone (**Fig. 2**). However, the suppression by dexamethasone did not change the upregulation of GST Yp by sulfur amino acid restriction, suggesting that the modulation of GST Yp expression was independent of dexamethasone. Insulin did not affect GST Yp expression.
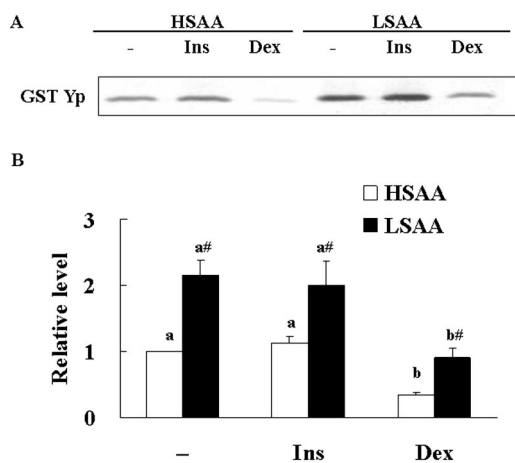
A

B

FIGURE 2   Effect of insulin and dexamethasone on the sulfur amino acid–mediated change in protein levels of GST Yp. Hepatocytes were cultured with HSAA or LSAA medium in the absence of growth factors (–) or in the presence of insulin (Ins) or dexamethasone (Dex), respectively, for 6 d. (*A*) Immunoblot assay of GST Yp protein levels. (*B*) Protein was quantitated by densitometry, and the level on d 6 for the HSAA-treated cells without growth factor was regarded as 1. Values are means $\pm$ SD, $n = 3$. [ab]Groups in the same medium not sharing a letter differ, $P < 0.05$. [#]Different from HSAA at that incubation time, $P < 0.05$.

GST Yp mRNA level and enzyme activity.   Northern blots revealed that GST Yp mRNA expression was consistent with the protein expression. mRNA levels increased with time up to d 6 and were greater in cells treated with LSAA in the absence of insulin and dexamethasone than in those treated with HSAA (**Fig. 3**). mRNA expression was suppressed by dexamethasone but was not influenced by insulin. At d 6, GST enzyme activity was significantly higher in cells incubated in LSAA medium than in those incubated in HSAA medium [59.1 $\pm$ 15.3 vs. 43.8 $\pm$ 7.2 nmol/(min·mg protein)].

Changes in cellular protein and GSH contents.   Regardless of the concentration of L-methionine and L-cysteine, cellular protein contents decreased with time (**Table 1**). In the absence of insulin and dexamethasone, cellular protein contents after incubation in HSAA or LSAA medium for 6 d were 36 and 45% lower, respectively, than in freshly isolated hepatocytes (d 0). Insulin helped to some extent to maintain protein contents during the first 3 d of incubation in both HSAA and LSAA media. Dexamethasone, however, did not affect the decrease in protein content that occurred in the absence of growth factors.

Because both L-cysteine and L-methionine, which is converted to L-cysteine via the transsulfuration pathway, are limiting amino acids for GSH synthesis, changes in hepatic GSH contents were examined. With the HSAA medium, the intracellular GSH concentration increased gradually and reached a maximum at d 3 and then decreased (**Fig. 4**). Compared with the initial level, GSH concentrations were 100% higher on d 3 (62.6 compared with 30.6 nmol/mg protein). In the cells treated with LSAA medium, GSH decreased gradually throughout the culture period. At d 6, only 18% of the GSH measured on d 0 remained.

Effect of amino acid restriction on the expression of GST isozymes.   Increases in GST Yp protein (**Fig. 5***A*) and mRNA levels (Fig. 5*B*) occurred solely in cells with a limited L-methionine supply. Restriction of L-cysteine, L-isoleucine, L-leucine, L-lysine, or L-phenylalanine had only minor or no effects on the expression of this detoxification enzyme. GST Yp protein expression increased dose dependently with greater L-methionine restriction (Fig. 5*C*).

Compared with the upregulation of GST Yp by L-methionine restriction, the Ya and Yb isozymes of GST were not affected by any of the amino acids tested (Fig. 5*A*). In addition, amounts of CA III, an abundant cytosolic protein in male rat livers, were not changed by limiting the supply of L-methionine or that of L-isoleucine, L-leucine, L-lysine, or L-phenylalanine.

DISCUSSION

Several lines of evidences indicate that amino acids not only act as protein and neurotransmitter precursors but also play a crucial role in controlling gene expression (28). In the
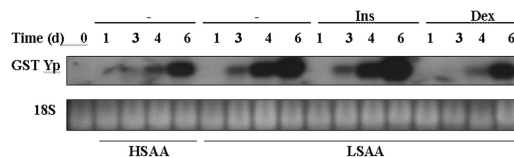

FIGURE 3   mRNA levels of GST Yp in primary rat hepatocytes. Cells were cultured in either HSAA or LSAA medium in the absence of growth factors (–) or the presence of insulin (Ins) or dexamethasone (Dex), respectively, for up to 6 d. Values are means $\pm$ SD, $n = 3$ or 4. [ab]Groups not sharing a letter differ, $P < 0.05$.

TABLE 1

Changes of cellular protein contents of hepatocytes incubated in the normal L-15 medium (HSAA) or in the sulfur amino acids restricted-medium (LSAA)[1,2]

	Time, d				
	0	1	3	4	6
			mg protein/plate		
HSAA					
−	1.47 ± 0.38a	1.41 ± 0.37ab	1.03 ± 0.20bcy	1.02 ± 0.08bcxy	0.94 ± 0.16c
Ins	1.47 ± 0.38a	1.65 ± 0.24a	1.40 ± 0.17ax	1.33 ± 0.26ax	0.84 ± 0.13b
Dex	1.47 ± 0.38a	1.42 ± 0.14a	1.11 ± 0.17abxy	0.97 ± 0.17by	0.85 ± 0.07b
LSAA					
−	1.47 ± 0.38a	1.46 ± 0.22a	1.28 ± 0.23abxy	1.14 ± 0.25abcxy	0.80 ± 0.23c
Ins	1.47 ± 0.38ab	1.61 ± 0.19a	1.38 ± 0.20abx	1.17 ± 0.25bcxy	0.77 ± 0.15c
Dex	1.47 ± 0.38a	1.51 ± 0.31a	1.11 ± 0.27abcxy	1.02 ± 0.22bcxy	0.82 ± 0.18c

[1] Hepatocytes isolated from 7- to 8-wk-old Sprague-Dawley rats were cultured with HSAA or LSAA medium in the absence of growth factors (−) or in the presence of insulin (Ins) or dexamethasone (Dex), respectively, for 6 d.

[2] Values are means ± SD, $n = 5$.

abc Groups in the same medium not sharing a letter differ, $P < 0.05$.

xy Groups at the same incubation time not sharing a letter differ, $P < 0.05$.

present study, we showed that the expression of the π class of GST, a phase II detoxification enzyme, was upregulated when hepatocytes were cultured in a sulfur amino acid–restricted medium and that such modulation was independent of dexamethasone and insulin. Furthermore, the lack of effect of the other essential amino acids tested, e.g., L-isoleucine, L-leucine, L-lysine, and L-phenylalanine, suggests that the induction of this GST isozyme is likely sulfur amino acid specific.

Amino acids are essential for maintaining normal cell function. In the face of amino acid starvation, adaptation occurs, which enhances amino acid biosynthesis by upregulating asparagine synthetase expression (29) and retards the growth rate by enhancing IGFBP expression (3). Drug metabolism in liver tissues is also modulated by feeding an energy-restricted diet (30). In contrast with the suppression of cytochrome P_{450} activity, GST Ya2/3/5 and Yb1 mRNA expression are upregulated by such malnutrition. Moreover, the upregulation of GST isozymes is diminished by replenishing the diet with

cysteine or methionine, a finding that suggests the critical role of sulfur amino acids in the regulation of GST expression by protein-energy restriction (14). In the present study, we showed further that the expression of the Yp isozyme of GST in rat hepatocytes is enhanced by limiting the supply of sulfur amino acids in the culture medium. Although the upregula-
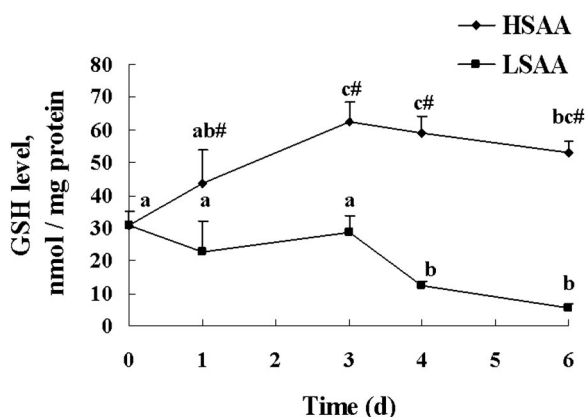

FIGURE 4 GSH contents of rat hepatocytes supplemented with different levels of sulfur amino acids. Cells were incubated in either HSAA or LSAA medium for up to 6 d. GSH contents were determined by HPLC. Values are the means ± SD, $n = 5$. abcGroups in the same medium not sharing a letter differ, $P < 0.05$. #Different from LSAA at that incubation time, $P < 0.05$.
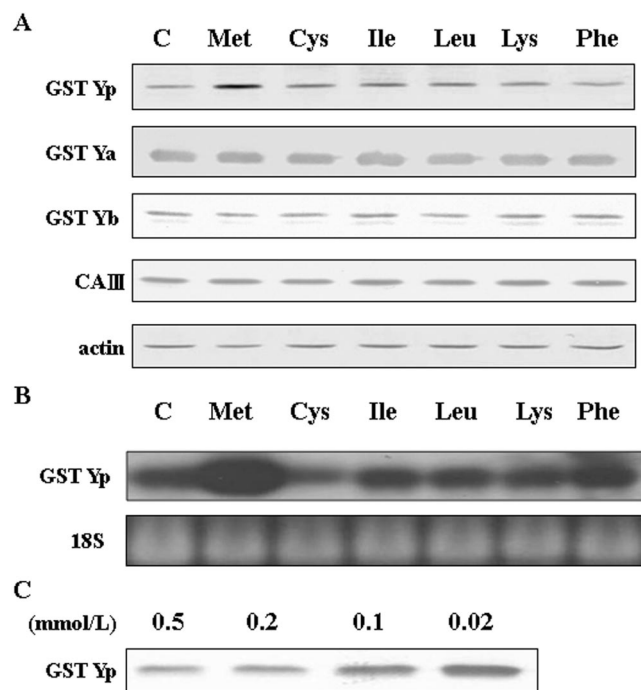

FIGURE 5 The effect of amino acid restriction on the expression of GST isoforms and CA III in primary rat hepatocytes. (*A*) After a 4-d exposure to the L-15 control medium (–) or the medium restricted in L-methionine (Met), L-cysteine (Cys), L-isoleucine (Ile), L-leucine (Leu), L-lysine (Lys), or L-phenylalanine (Phe) (0.02 mmol/L each), GST Ya, Yb, and Yp, and CA III were immunostained by antibody-linked peroxidase activity. (*B*) Northern blot analysis of GST Yp mRNA. (*C*) Dose-dependent change in GST Yp protein level of cells cultured in 0.02–0.5 mmol/L L-methionine for 4 d.

tion of Yp expression occurred in cells cultured in medium restricted in L-methionine, but not in that restricted in L-cysteine, it must be emphasized that an effect of L-cysteine restriction on the regulation of GST Yp expression cannot be excluded. This lack of effect may be explained by the ability of hepatocytes to convert L-methionine to L-cysteine via the transsulfuration pathway. This newly synthesized L-cysteine acts to minimize the shortage of L-cysteine in the cell culture medium. Furthermore, L-cysteine in the medium was likely oxidized to L-cystine, thereby decreasing the availability of L-cysteine to cells. By the HPLC method (28), there was 11.4 ± 3.9% L-cysteine remaining after 24 h medium preparation ($n = 3$). These limitations make it difficult to determine precisely the actual effect of L-cysteine on Yp expression in this study.

The Yp isozyme of GST, little or none of which is found in normal rat liver, is continuously expressed after cell isolation, and this expression is modulated by various medium constituents, such as dexamethasone (21,31). In addition, we reported previously that FBS had a positive effect on GST Yp induction (32). In this study, we further demonstrate that the availability of sulfur amino acids modulates the expression of GST Yp in primary rat hepatocytes. Hepatocytes undergo dedifferentiation in primary culture. The induction of this detoxification enzyme seems to be the result of dedifferentiation, and this association may enhance cell survival after isolation. The expression of GST Yp is highly inducible by chemical carcinogens and is commonly used as the biochemical marker during hepatocarcinogenesis (33). The strong association of GST Yp expression with neoplastic cells has been regarded as a survival mechanism, which provides for the enhanced proliferation in a toxic environment (34).

In the present in vitro study, only GST Yp and not Ya or Yb was upregulated in response to sulfur amino acid restriction. This discrepancy in expression among the GST isozymes indicates that the gene regulation of GST Yp likely differs from that of Ya and Yb. Gene regulation involves a cascade of molecular events that activate the transcription factors, which in turn stimulate gene expression. In the GST Ya promoter/enhancer regions, 2 important DNA consensus sequences, an aryl hydrocarbon response element and an antioxidant response element (ARE) (35,36), were identified as being responsible for the induction of transcription when hepatocytes are exposed to various xenobiotics and prooxidants, including hydrogen peroxide, menadione, and *tert*-butyl hydroquinone (a phenolic antioxidant) (35).

Evidence suggests that the Nrf and small Maf protein families of transcription factors bind to the ARE and induce downstream genes (37,38). Activation of Nrf-1,2 binding to the ARE was shown to be important in the upregulation of hepatic GST Ya and Yb mRNA in protein-energy–malnourished rats (14). The activation of phosphatidylinositol 3-kinase caused by GSH depletion was also shown to be essential for ARE-mediated GST Ya2 induction in H4IIE hepatoma cells cultured in methionine- and cysteine-deprived medium (39). In the present study, the GST Ya level was not changed in LSAA-treated hepatocytes even when the cellular GSH content was 20% that of the HSAA-treated cells. This raises the possibility that activation of the ARE by GSH depletion is cell-type specific or that the extent of GSH depletion in hepatocytes is insufficient to activate ARE-mediated GST Ya expression.

Compared with that for GST Ya, evidence for the molecular regulation of GST Yp transcription is limited. Although there are ARE-like elements in the promoter region of GST Yp, their role in GST Yp transcription is not yet fully under-

stood. The lack of impairment of oltipraz- or 3H-1,2-dithiole-3-thione-induced GST Yp mRNA expression in Nrf2 knockout mice (40,41) suggests that GST Yp expression is not mediated by the Nrf2-ARE pathway. However, Ikeda et al. (42) reported that Nrf2 binding to ARE-like binding sites is involved in mouse GST Yp gene induction. Rat GST Yp can be activated by Nrf2-mediated induction in H4IIE hepatoma cells, but not in normal liver cells (43). Instead, GST Yp enhancer I (GPEI), which is located at −2.5 kb, may play a key role (44). GPEI contains 2 phorbol 12-O-tetradecanote 13-acetate response element (TRE)-like elements that have activating protein (AP)-1–like binding sites (45), and both are required for the basal and inducible expression of GST Yp (46,47). For instance, the TRE is essential for the induction of GST Yp transcription by 3,4,5,3′,4′-penta-chlorinated biphenyl in primary hepatocytes (46). The inhibition of GST Yp mRNA and protein expression by dexamethasone that occurred in the present study (Figs. 2 and 3) was also thought to occur via the AP-1 pathway because dexamethasone acts as an antagonist of AP-1 transcription factor (31). Although the actual molecular mechanism of the sulfur amino acid induction of GST Yp gene expression is not clear, TRE is not likely the sole answer because dexamethasone seems to be ineffective in blocking the upregulation of sulfur amino acid restriction (Fig. 2). Pathways other than the glucocorticoid-suppressed AP-1 binding to TRE, such as GSH depletion-activated ARE and other unidentified factors, are possible candidates.

CHOP and asparagine synthetase (AS) are 2 of the most studied mammalian genes whose expression is regulated by amino acids. The finding that leucine restriction induces CAT activity of reporter constructs containing a CHOP or an AS promoter region supports the involvement of response elements and transcriptional factors in the amino acid–induced transcription of CHOP and AS (29,48). Even so, the pattern of CHOP and AS expression in response to amino acid restriction differs somewhat. CHOP is strongly induced by methionine deprivation and only slightly induced by deprivation of histidine, cysteine, or asparagine. AS, however, is consistently induced in response to deprivation of any one of these amino acids (49). The discrepancy in CHOP and AS expression in response to amino acid restriction indicates that the regulation of the CHOP and AS genes differs to some extent. Because GST Yp induction occurred solely with sulfur amino acid restriction and not with restriction of L-leucine, L-isoleucine, L-lysine, or L-phenylalanine, the mechanism of regulation of GST Yp by sulfur amino acids is probably different from the mechanism of regulation of CHOP and AS.

In mammals, several physiologic functions involved in the defense of or adaptation to amino acid starvation are adjusted through the regulation of the expression of numerous genes. The results of the present study clearly indicate that GST Yp gene expression is upregulated in primary rat hepatocytes by L-methionine and L-cysteine restriction. The lack of response to the other amino acids tested suggests that such an effect is probably sulfur amino acid specific. Further study is required of the molecular mechanisms involved in the regulation of GST Yp gene expression by L-methionine and L-cysteine.

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Part Ⅱ

新完成結果

Materials and Methods

Cell isolation and culture. Male Sprague-Dawley rats were used for hepatocyte isolation in age between 7 to 8-weeks-old. Hepatocytes were isolated by a two-step collagenase perfusion method as described previously (1). The isolated hepatocytes were suspended in L-15 cell culture medium, containing 18 mmol/L HEPES, 5 mg/L transferrin, 5 µg/L selenium as sodium selenite, 1 g/L galactose, 1 X 10^5/L penicillin, 100 mg/L streptomycin, and 2.5% fetal bovine serum. Cell attachment on the culture dish was achieved 4 h after plating, and then changed to fresh medium. Thereafter, the medium was changed once each day and cells were cultured for 8, 24, 48, and 72h.

The effect of methionine restriction on the GSTP expression was investigated. In this study, cells were cultured in the L-15 control medium containing 0.5 mmol/L methionine (control) or in the medium containing 20 µmol/L methionine (Met). For all treatments, cells were harvested at 8, 24, 48, or 72 h post plating. Cells were exposed to 20 µmol/L methionine for 24h in the presence or absence of ERK inhibitor (PD98059).

RNA Isolation and RT-PCR. Total RNA was extracted by Trizol reagent according to the manufacturer's instructions. RNA extracts were suspended in nuclease-free water and frozen at -70 ^{o}C until the RT-PCR analyses were performed according to the method of Demeule et al. (2). The PCR products were separated by 1% agarose gel electrophoresis and visualized by UV light in the presence of ethidium bromide.

SDS-PAGE and Western blot. Cells were washed twice with cold PBS and were harvested in 500 µL of 20 mmol/L potassium phosphate buffer (pH 7.0). Supernatants were centrifuged at 10,000 × g for 30 min at 4°C. Equal amounts of proteins from each sample were applied to 10% SDS-polyacrylamide gels and electrophoretically transferred to polyvinylidene fluoride membranes. To measure the expression of GSTP and actin, the membranes were blocked at 4°C overnight with 50 g/L nonfat dry milk solution and then incubated with antibodies against GSTP or actin. 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, the membranes were incubated overnight at 4°C with anti-JNK1, anti-ERK1/2, and anti-p38 MAPK or anti-phospho-activated JNK1, ERK1/2, and p38 MAPK antibodies. The bands were detected by using an enhanced chemiluminescence kit.

Preparation of nuclear extract. Cell homogenates were centrifuged at 2000g for 5 min. The supernatant was discarded, and the cell pellet was allowed to swell on ice for 15 min after the addition of 200 µL of hypotonic buffer containing 10 mmol/L HEPES, 10 mmol/L KCl, 1 mmol/L MgCl$_2$, 1 mmol/L EDTA, 0.5 mmol/L DTT, 4 ug/ml leupeptin, 20 µg/ml aprotinin, 0.5% Nonidet P-40, and 0.2 mmol/L phenylmethylsulfonyl fluoride. After centrifugation at 6000g for 15 min, pellets containing crude nuclei were resuspended in 50 µL of hypertonic buffer containing 10 mmol/L HEPES, 400 mmol/L KCl, 1 mmol/L MgCl$_2$, 1 mmol/L EDTA, 0.5 mmol/L DTT, 4 ug/ml leupeptin, 20 µg/ml aprotinin, 10% glycerol, and 0.2 mmol/L phenylmethylsulfonyl fluoride and incubated for an additional 30 min on ice. The nuclear extracts were then obtained by centrifugation at 10000g for 15 min and were frozen at -80 °C until the electromobility gel shift assay (EMSA) was performed.

Electromobility gel shift assay. EMSA was performed according to our previous study (3). The LightShift^{TM} Chemiluminescent EMSA Kit and synthetic biotin-labeled double-stranded AP-1 consensus oligonucleotides (5'-CGCTTGATGACTCAGCCGGAA-3') were used to measure the effect of methionine on AP-1 nuclear protein-DNA binding activity. 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% TBE-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.

Results and Discussion

Methionine restriction on GSTP protein and mRNA level. Primary hepatocytes

were incubated with either a L-15 control medium containing 0.5 mmol/L methionine (control) or in the medium containing 20 µmol/L methionine (Met) for 8, 24, 48, and 72h. As shown in Fig. 1A, GSTP protein expression showed a time-dependent increase with the incubation period. However, the increase of GSTP expression was sooner in cells treated with methionine restriction medium as compared to those with control medium. Northern blot assay revealed GSTP mRNA expression was consistent with the protein level as described. GSTP mRNA level was time-dependently increased up to 72h (Fig. 1B) and the increase of GSTP mRNA level was sooner in cells treated with methionine restriction medium as compared to those with control medium.

Methionine restriction and AP-1 activation. Upon treatment with methionine restriction medium, the DNA binding activity of AP-1 nuclear protein increased time dependently compared with that of the control cells (Fig. 2A). After pretreatment with the ERK inhibitor PD98059, however, the increase in AP-1 binding to DNA induced by methionine restriction was inhibited (Fig. 2B).

Methionine restriction and MAPK kinases. Immunoblot analysis showed that the phosphorylation of ERK, but not of JNK and p38, was stimulated after 8 to 48h of methionine restriction medium (Fig. 3). We finally determined whether the up-regulation of GST protein expression by methionine restriction is suppressed in the presence of ERK inhibitor. As noted, PD98059 pretreatment suppressed the expression of GSTP protein (Fig. 4A) and GSTP mRNA (Fig. 4B).

Conclusion

The up-regulation of methionine restriction on GSTP gene expression is related to ERK-AP-1 pathway.

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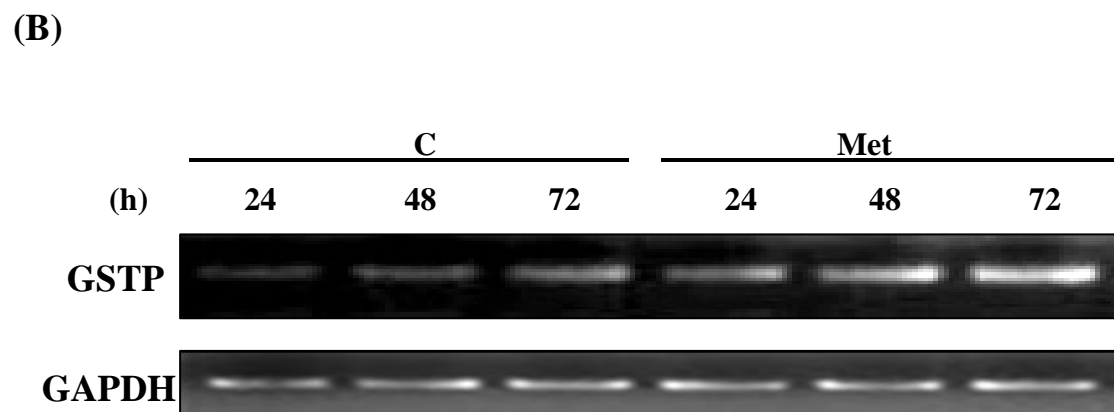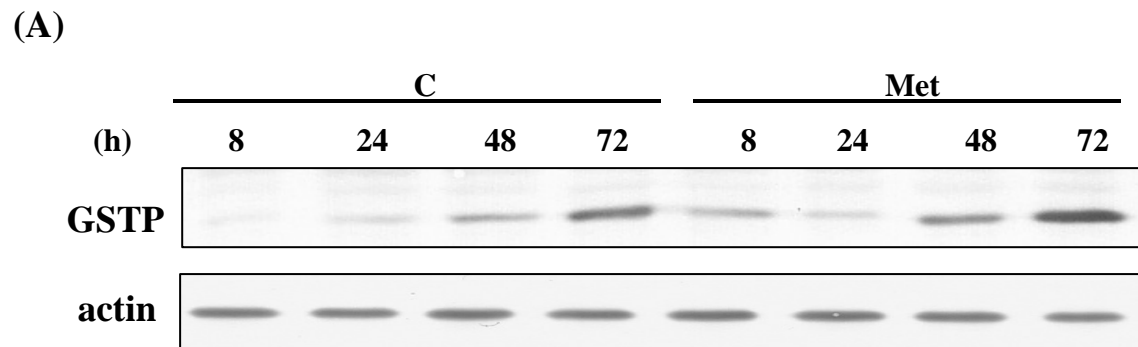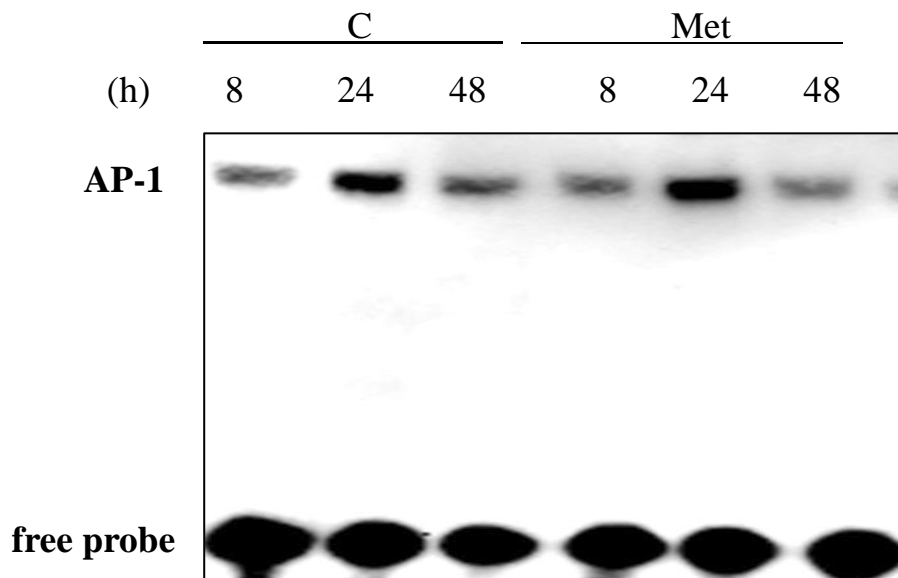
(A)

(B)

Fig 1. (A) GSTP protein was determined by immunoblot assay. (B) GSTP mRNA levels was determined by RT- PCR.
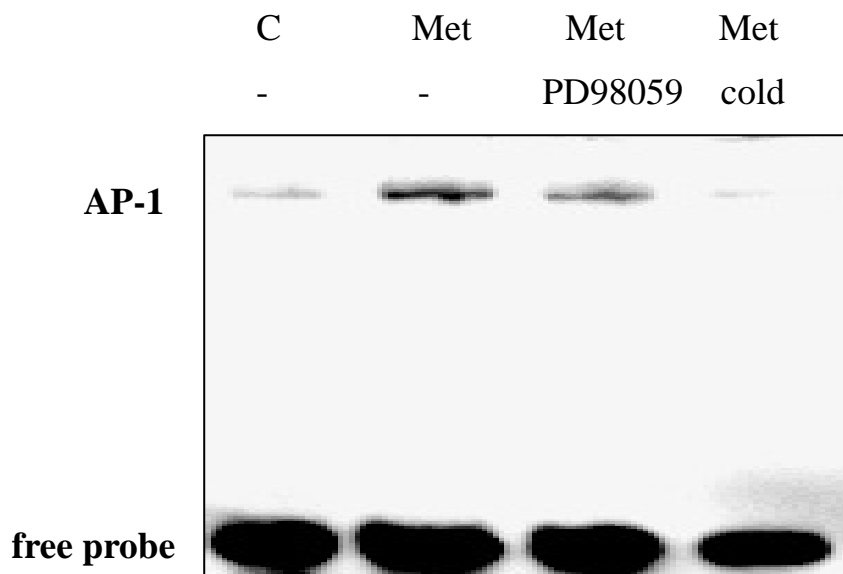
(A)

(B)

Fig 2. (A) Cells were treated with 20 μmol/L methionine for 8, 24 and 48h, and then nuclear extracts were prepared to measure the AP-1 nuclear protein DNA binding activity by EMSA. (B) Cells were exposed to 20 μmol/L methionine for 24h in the presence or absence of ERK inhibitor.
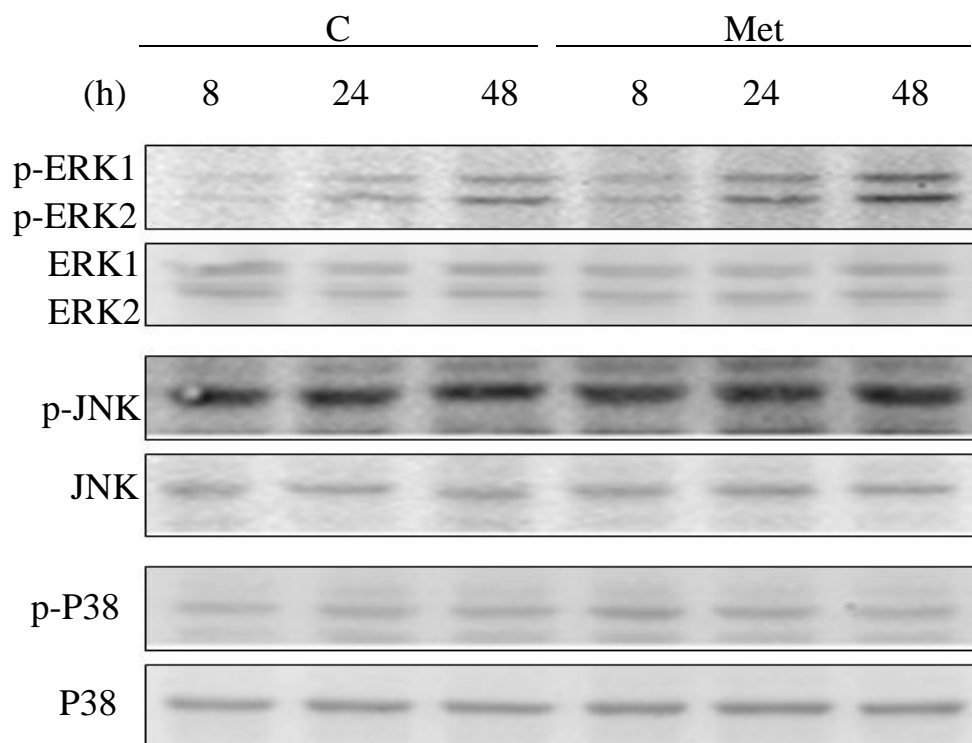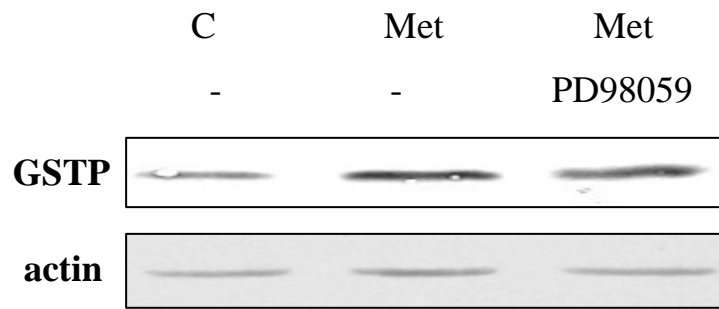
Fig 3. Cells were treated with 20 µmol/L methionine for 8, 24, and 48h.
Activation of ERK1/2 , JNK, and p38 MAPK was assessed by
immunoblot analysis of the phosphorylated forms (p-) of the
MAPK in whole cell lysates.

(A)

	C	Met	Met
	-	-	PD98059

GSTP

actin

(B)

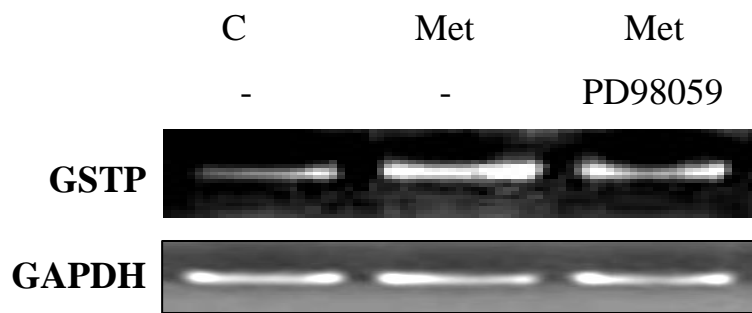|	C	Met	Met
	-	-	PD98059

GSTP

GAPDH

Fig 4. ERK inhibitors suppress methionine restriction induced GSTP protein. (A) and mRNA (B) in primary hepatocytes. Cells were incubated with 20 μmol/L methionine for 72 h in the presence or absence of 20 μmol/L PD98059 for first 24 h.