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Activation of Nrf2 Is Required for Up-Regulation of the π Class of Glutathione *S*-Transferase in Rat Primary Hepatocytes with L-Methionine Starvation

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ABSTRACT: Numerous genes expression is regulated in response to amino acid shortage, which helps organisms adapt to amino acid limitation. The expression of the π class of glutathione (GSH) *S*-transferase (GSTP), a highly inducible phase II detoxification enzyme, is regulated mainly by activates activating protein 1 (AP-1) binding to the enhancer I of GSTP (GPEI). Here we show the critical role of nuclear factor erythroid-2-related factor 2 (Nrf2) in up-regulating GSTP gene transcription. Primary rat hepatocytes were cultured in a methionine-restricted medium, and immunoblotting and RT-PCR analyses showed that methionine restriction time-dependently increased GSTP protein and mRNA expression over a 48 h period. Nrf2 translocation to the nucleus, nuclear proteins binding to GPEI, and antioxidant response element (ARE) luciferase reporter activity were increased by methionine restriction as well as by L-buthionine sulfoximine (BSO), a GSH synthesis inhibitor. Transfection with Nrf2 siRNA knocked down Nrf2 expression and reversed the methionine-induced GSTP expression and GPEI binding activity. Chromatin immunoprecipitation assay confirmed the binding of Nrf2 to the GPEI. Phosphorylation of extracellular signal-regulated kinase 2 (ERK2) was increased in methionine-restricted and BSO-treated cells. ERK2 siRNA abolished methionine restriction-induced Nrf2 nuclear translocation, GPEI binding activity, ARE-luciferase reporter activity, and GSTP expression. Our results suggest that the up-regulation of GSTP gene transcription in response to methionine restriction likely occurs via the ERK-Nrf2-GPEI signaling pathway.

KEYWORDS: *L-methionine, π class of glutathione S-transferase, GPEI, Nrf2, primary hepatocytes*

INTRODUCTION

L-Methionine, a sulfur-containing essential amino acid, plays an important role in energy metabolism, immune responses, antioxidation, and nerve function.[1−3] It acts as an efficient methyl donor in the methylation of DNA and proteins and is a precursor of cysteine.[4] Accumulating evidence indicates that shortage of the L-methionine supply not only limits global protein synthesis but also triggers a stress signal that induces the transcription of several genes, such as methionine adenosyltransferase, C/EBP homologous protein (CHOP), and asparagine synthetase, which helps cells to adjust several physiologic functions involved in the adaptation to amino acid limitation.[5,6] Amino acid response elements have been characterized in the promoter region of the CHOP and asparagine synthetase genes, and the activating transcription factors (ATF) and CCAAT/enhancer binding protein (C/EBP) families including ATF2, ATF3, ATF4, ATF5, and CHOP are known to be involved in nutrient deprivation-induced gene transcription.[7−10] In addition, an increase in the protein level can also be explained by elevating mRNA stability in response to amino acid availability.[11] The signaling pathways that mediate the regulation of gene expression in response to amino acid availability include GCN2 kinase, mitogen-activated protein kinases (MAPK), and mammalian target of rapamycin (mTOR).[12]

Glutathione (GSH) *S*-transferase (GST), a phase II detoxification enzyme, catalyzes the conjugation of GSH with a variety of electrophilic xenobiotics and facilitates their excretion. Eight classes of GST isozymes have been identified in mammals, including α, μ, ω, π, σ, θ, ζ, and κ.[13] The expression of several GST isozymes is inducible by a variety of chemicals, including carcinogens, dietary lipids, and phytochemicals.[13] When mice are fed a protein-free diet, levels of the α3 form of GST (GSTA3) and the π1 form of GST (GSTP1) in liver are increased, whereas normal contents are preserved in rats fed a protein-free diet replenished with either L-methionine or L-cysteine.[14] These findings suggest that the expression of certain GST genes can be modulated depending on cellular L-methionine status.[14] Recently, we demonstrated that GSTP gene expression is up-regulated in rat primary hepatocytes by methionine and cysteine restriction but not by restriction of leucine, isoleucine, phenylalanine, or lysine.[15] This finding suggests that this induction of GSTP expression in hepatocytes is specific for sulfur amino acids.

GSTP is essentially absent from normal hepatocytes but is highly inducible by a variety of chemicals and is expressed at elevated levels in spontaneously occurring neoplastic lesions.[16] GSTP is of importance to cell physiology and cancer research because of its role in detoxifying carcinogens, activating antineoplastic prodrugs, and metabolizing chemotherapeutic agents and its involvement in the regulation of the cell cycle and apoptosis.[17,18] GSTP strongly affects human susceptibility to several cancers, asthma, and neurodegenerative diseases.[19] The

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inducibility of GSTP is generally attributed to the existence of a strong enhancer named GSTP enhancer I (GPEI), which has two inverted 12-*O*-tetradecanoylphorbol 13-acetate response (TRE)-like elements (5′-TCAGTCActaTGATTCAgca-3′) in the 5′ upstream region.[20] Activator protein 1 (AP-1), which consists of several closely related proteins belonging to the Jun and Fos family, is well recognized to be the major transcription factor responsible for the up-regulation of GSTP expression through binding to the GPEI.[21]

Nuclear factor erythroid-2-related factor 2 (Nrf2) is a basic leucine zipper (bZip) transcription factor with a "cap 'n' collar" structure. Under basal conditions, Nrf2 is retained in the cytoplasm by Kelch-like ECH-associated protein 1 (Keap1), which acts as a substrate adapter of a Cullin 3 based E3 ubiquitin ligase complex and results in rapid degradation of Nrf2 through the proteasome pathway.[22] The Keap1−Nrf2 complex is disrupted in response to several electrophilic compounds and antioxidants, after which free Nrf2 quickly translocates into the nucleus, forms a heterodimer with small Maf, and binds to the antioxidant response element (ARE). The ARE is found in the promoter of many detoxifying and antioxidant enzyme genes, including glutamate cysteine ligase catalytic subunit (GCLC), heme oxygenase 1, NAD(P)H/quinone oxidoreductase, and the α and μ forms of GST.[23−25] In addition to the α and μ forms of GST, the role of Nrf2 in regulating the GSTP gene seems to be dependent on animal species. In humans and mice, the importance of Nrf2 on this GST isozyme has been well demonstrated.[26,27] In rats, however, whether Nrf2 binds to GPEI and regulates GSTP transcription remains unclear. To date, only limited reports have indicated that Nrf2/MafK binds to GPEI and is responsible for GSTP induction in H4IIE rat hepatoma cells.[28]

It is well demonstrated that GSH depletion invokes impaired cellular redox homeostasis, which in turn activates Nrf2 nuclear translocation and up-regulates ARE-harboring antioxidant/phase II detoxification gene transcription.[29] Methionine is a precursor for cysteine formation by the methionine transsulfuration pathway in liver. Because cysteine is a substrate for glutathione synthesis, we hypothesize that methionine restriction leads to GSTP expression in normal rat hepatocytes which is likely to be associated with Nrf2 activation caused by GSH depletion. In this study, rat primary hepatocytes were cultured in a medium restricted in L-methionine or in a medium supplemented with L-buthionine sulfoximine (BSO), a GSH synthesis inhibitor. The role of Nrf2 in regulating the expression of GSTP in response to methionine restriction and the mechanisms involved were investigated.

MATERIALS AND METHODS

Chemicals. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless specified otherwise. Type I rat tail collagen was purchased from BD Biosciences (San Jose, CA). Type I collagenase was purchased from Worthington Biochemical (Lakewood, NJ). Percoll was purchased from Amersham Biosciences (Uppsala, Sweden). Sulfur amino acid (SAA) omitted Leibovitz L-15 medium and fetal bovine serum were purchased from HyClone (Logan, UT). Small interfering RNA (siRNA) against Nrf2 and ERK2 were purchased from Dharmacon (Lafayette, CO). Trizol was purchased from Invitrogen (Carlsbad, CA). RNase inhibitor, oligo dT, dNTP, and Moloney murine leukemia virus RT were purchased from Promega. Taq polymerase and Nanofectin transfect reagent were purchased from PAA (Pasching, Austria).

Cell Isolation and Culture. Male Sprague-Dawley rats at 7−8 weeks old were obtained from the BioLASCO Experimental Animal Center (Taipei, Taiwan) and were used for hepatocyte isolation. For the use of animals in the study, ethical approval was obtained from Institutional Animal Care and Use Committee of China Medical University (Protocol No. 97-140-N) and rats were treated in compliance with the Guide for the Care and Use of Laboratory Animals.[30] Hepatocytes were isolated by a two-step collagenase perfusion method as described previously.[31] The isolated hepatocytes were suspended in L-15-based culture medium (SAA-omitted Leibovitz L-15 plus 0.5 mmol/L L-methionine and 0.2 mmol/L L-cysteine) containing 18 mmol/L HEPES, 5 mg/L transferrin, 5 μg/L sodium selenite, 1 g/L galactose, 1×10^5 U/L penicillin, 100 mg/L streptomycin, and 2.5% fetal bovine serum. Cells (1×10^6) were planted on 3 cm plastic culture dishes precoated with type I rat tail collagen and incubated in a 37 °C humidified incubator in an air atmosphere. Twenty-four hours after isolation, hepatocytes were incubated in L-15-based medium (Con), L-15-based medium plus 200 μmol/L BSO), or a methionine-restricted L-15 medium (−Met; SAA-omitted Leibovitz L-15 plus 0.02 mmol/L L-methionine and 0.2 mmol/L L-cysteine) for the indicated time. The L-methionine and L-cysteine supplements were freshly prepared. The medium was changed once daily. Hepatocytes cultured in L-methionine restriction or BSO medium up to 48 h did not affect the cell viability (data not shown).

SDS−PAGE and Immunoblotting. Cells were harvested with 20 mmol/L potassium phosphate buffer (pH 7.4), and the cell lysates were sonicated and centrifuged at 10000*g* for 20 min at 4 °C. Protein concentrations of supernatants were measured with the Bradford protein assay kit (Pierce, Rockford, IL), and samples were stored at −20 °C. Proteins were separated by 10% SDS−PAGE and were electrophoretically transferred to polyvinylidene fluoride membranes. After the nonspecific binding sites were blocked, the membranes were incubated with GSTP (BD Transduction Laboratories, Lexington, NY), Nrf2 (Santa Cruz Biotechnology, Santa Cruz, CA), ERK, and phospho-ERK (Thr202/Tyr204) antibodies (Cell Signaling Technology, Beverly, MA) (1:1000 dilution). After incubation with the horseradish peroxidase conjugated secondary anti-rabbit or anti-mouse IgG (1:2000 dilution), the immunoblots were detected by use of an enhanced chemiluminescence kit (Perkin-Elmer Life Science, Boston, MA) and the image intensities of specific bands were quantified with an LAS-4000 Mini Fujifilm imaging system.

Preparation of Nuclear Extracts. Nuclear extracts were prepared according to a previously published method.[32] Cells were washed twice with ice-cold PBS and then scraped gently from the dishes with 1 mL of PBS. The cells were centrifuged at 2000*g* for 5 min, and the pellets were swollen with 200 μL of hypotonic buffer containing 10 mmol/L HEPES, 10 mmol/L KCl, 1 mmol/L EDTA, 1 mmol/L $MgCl_2$, 0.5 mmol/L DTT, 0.2 mmol/L PMSF, 4 mg/L leupeptin, 20 mg/L aprotinin, and 0.5% NP-40 (pH 7.4). The cell suspensions were incubated for 15 min in an ice bath and were then centrifuged at 6000*g* for 15 min at 4 °C. The supernatants were saved as cytosol fractions, and the pellets containing crude nuclei were resuspended in 50 μL of hypertonic buffer containing 10 mmol/L HEPES, 400 mmol/L KCl, 1 mmol/L $MgCl_2$, 0.5 mmol/L DTT, 0.2 mmol/L PMSF, 4 mg/L leupeptin, 20 mg/L aprotinin, 10% glycerol, and 0.2 mmol/L EDTA (pH 7.4) and incubated for 30 min in an ice bath. The samples were centrifuged at 10000*g* for 15 min to obtain nuclear proteins.

Electromobility Gel Shift Assay. EMSA was performed to measure the effect of methionine restriction on Nrf2-GPEI DNA binding activity.[32] The synthetic biotin-labeled double-stranded GPEI consensus oligonucleotide (5′-AGTAGTCAGTCACTATGATTCAGCAAC-3′) and LightShift Chemiluminescent EMSA kit (Pierce, Rockford, IL) were used. Unlabeled double-stranded GPEI sequence and mutant double-stranded oligonucleotide (5′-AGTAGTCAGTCACTAgacTTCAGCAAC-3′) were also used to confirm the specific binding. Four micrograms of nuclear protein, poly(dI-dC), and biotin-labeled double-stranded GPEI oligonucleotide 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−boric acid−EDTA polyacrylamide gel and was then electrotransferred to a Hybond-N+ nylon membrane (GE Healthcare, Buckinghamshire, U.K.). The membrane was incubated with streptavidin−horseradish peroxidase, and the nuclear protein−DNA bands were developed by using an enhanced chemiluminescence kit (Thermo).

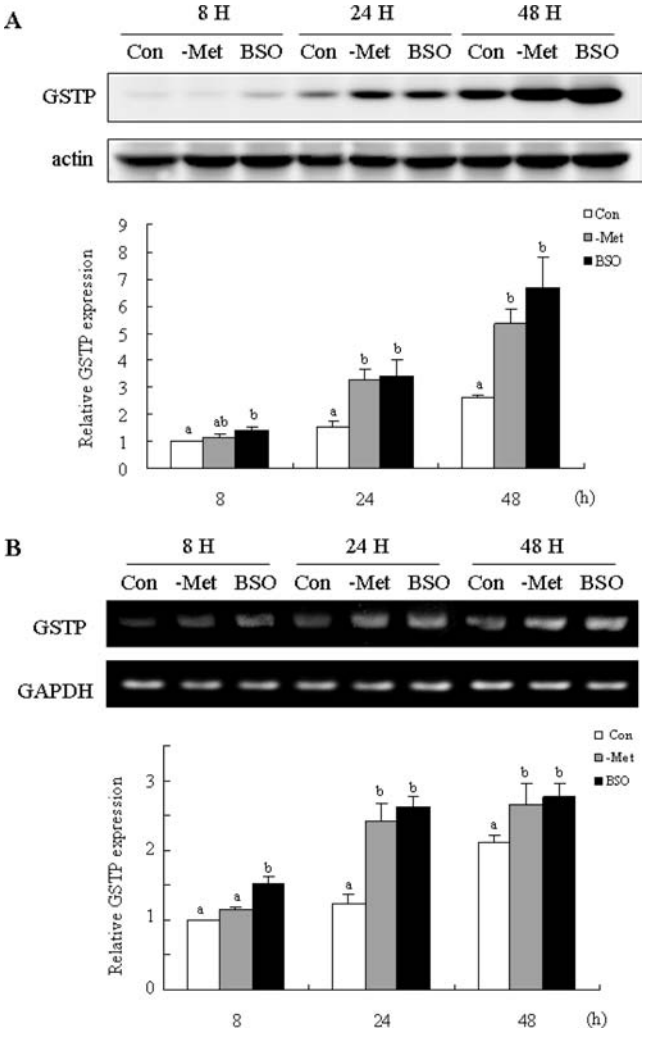

Figure 1. Methionine restriction and BSO treatment induce the expression of GSTP protein (A) and mRNA (B) in rat primary hepatocytes. Twenty-four hours after isolation, cells were incubated in an L-15-based control medium (Con, 0.5 mmol/L L-methionine), a methionine-restricted L-15 medium (−Met, 0.02 mmol/L L-methionine), or the control medium plus BSO (200 μmol/L) for 8, 24, and 48 h. The protein and mRNA value for cells treated with L-15-based control medium for 8 h was set at 1. Values are the mean ± SD, n = 3. For letters a and b, values of treatments not sharing a common letter differ significantly, $P < 0.05$. BSO indicates L-buthionine sulfoximine. GSTP indicates the π class of glutathione S-transferase.

RT-PCR. Total RNA isolation and reverse transcription were performed as described previously.[32] For PCR, the cDNA samples were mixed with PCR master mixture containing 4 mmol/L $MgCl_2$, 2.5 units of Taq polymerase, and forward and reverse primers to a total volume of 50 μL. The sequences of the primers were as follows: for GSTP (forward, 5′-TTCAAGGCTCGCTCAAGTCCAC-3′; reverse, 5′-CTTGATCTTGGGGCGGGCACTG-3′); for Nrf2 (forward, 5′-GAGACGGCCATGACTGATTT-3′; reverse, 5′-TGGGTCTCCGTAAATGGAAG-3′); for GAPDH (forward, 5′-GACGTGCCGCCTGGAGAAA-3′; reverse, 5′-GGGGGCCGAGTTGGGATAG-3′). The PCR reactions were performed as follows: 5 min at 94 °C; 30 cycles of 40 s at 94 °C, 40 s at 60 °C, and 120 s at 72 °C; and a final extension for 7 min at 68 °C. The PCR amplicons were separated on 2% agarose gels containing 40 mmol/L Tris, 20 mmol/L glacial acetic acid, and 2 mmol/L EDTA, and the relative densities of the PCR products were detected by using an image scan and analysis system (Alpha Innotech) and were quantitated by use of Image Gauge software (Fujifilm, Tokyo, Japan).

Table 1. Effects of Methionine Restriction and BSO on Cellular GSH Content[a]

treatment	time (h)	GSH (nmol/mg protein)	GSH/GSSG
	0	51.4 ± 12.2 a	246 ± 62 a
Con	4	42.1 ± 4.7 a,x	213 ± 55 a,x
	8	45.3 ± 12.1 a,x	195 ± 71 a,x
	24	89.9 ± 23.8 b,x	233 ± 5 a,x
−Met	4	34.6 ± 3.2 ab,y	214 ± 48 a,x
	8	22.8 ± 1.1 b,y	76 ± 25 b,y
	24	19.4 ± 1.5 b,y	128 ± 33 ab,y
BSO	4	11.7 ± 5.2 b,z	110 ± 64 ab,x
	8	6.4 ± 0.8 b,y	46 ± 36 b,y
	24	5.3 ± 5.3 b,y	38 ± 27 b,z

[a]Twenty hours after attachment, hepatocytes were treated with methionine restriction (−Met) or BSO for the indicated time periods. Values are the mean ± SD, n = 3. For letters a−c, values of the same medium over the 24 h incubation period not sharing the same letter differ significantly, $p < 0.05$. For letters x−z, values of the same time period not sharing the same letter differ significantly, $P < 0.05$.

Reporter Plasmid Constructs. A 2713-bp fragment of the GSTP gene promoter was inserted into the *Mlu*I and *Nhe*I sites of pTA-SEAP/Luc vector (pTA-PGST-2713) as described previously.[33] In addition, a reporter plasmid with the GPEI fragment was constructed by inserting the 2713- to 2605-bp segment of the GSTP gene promoter into pTASEAP/Luc vector and was designated as pTA-GPEI. A reporter (pGL3-2xARE/Luc) contained double repeats of double-stranded oligonucleotides that spanned the Nrf2 binding site, 5′-TGACTCAGCA-3′, which was kindly given by Dr. B. S. Wung (Department of Applied Microbiology, National Chiayi University, Chiayi, Taiwan).

Transient Transfection and Luciferase Activity Assay. Rat primary hepatocytes (1×10^6) were plated into 3 cm dishes and cultured for 16 h. The cells were then transiently transfected for 8 h with 1 μg of pTA-PGST-2713, pTA-GPEI, or pGL3-2xARE luciferase reporter plasmid and 0.1 μg of β-galactosidase expression plasmid by using Nanofectin. After transfection, the cells were cultured with methionine restriction or BSO containing medium for an additional 24 h. For luciferase assays, the cell lysate was first mixed with a luciferase substrate solution (Promega), and the resulting luciferase activity was measured by using a microplate luminometer (TROPIX TR-717, Applied Biosystems). β-Galactosidase activity was measured by an enzyme assay system (Promega) according to the manufacturer's instructions. For each experiment, luciferase activity was normalized to β-galactosidase activity.

Transient Transfection of siRNA. Twenty-four hours after isolation, rat primary hepatocytes were transiently transfected for 16 h with 50 nmol/L each of ERK2 or Nrf2 siRNA SMARTpool by using DharmaFECT siRNA transfection reagents (Thermo) according to the manufacturer's instructions. The four siRNAs against the rat ERK2 gene are (1) ACACUAAUCUCUCGUACAU, (2) AAAAUAAGGUGCCGUGGAA, (3) UAUACCAAGUCCAUUGAUA, and (4) UCGAGUUGCUAUCAAGAAA. The four siRNAs against the rat Nrf2 gene are (1) GAACACAGAUUUCGGUGAU, (2) AGACAAACAUUCAAGCCGA, (3) GGGUUCAGUGACUCGGAAA, and (4) AGAAUAAAGUUGCCGCUCA. A nontargeting control-pool siRNA (NTC) was also tested and was used as the negative control. Cells were then incubated in L-15-based medium, L-15-based medium plus 200 μmol/L BSO (+BSO), or in a methionine-restricted L-15 medium for the indicated time.

ChIP Analysis. ChIP analysis was performed by using the EZ-ChIP chromatin immunoprecipitation kit (Millipore, Bedford, MA) according to the manufacturer's instructions. Briefly, cells were fixed in 1% formaldehyde at 37 °C for 10 min and then 2.5 mol/L glycine solution was added to stop the cross-linking reaction. After the cells were washed twice with ice-cold PBS containing 1 mmol/L PMSF, 1 mg/L aprotinin, and 1 mg/L pepstatin, the cells were harvested in 1% SDS lysis buffer, sonicated on ice at 50% amplitude (40 kJ) by use of a Sonics VibraCell Ultrasonic Processor (Newtown, CT), and

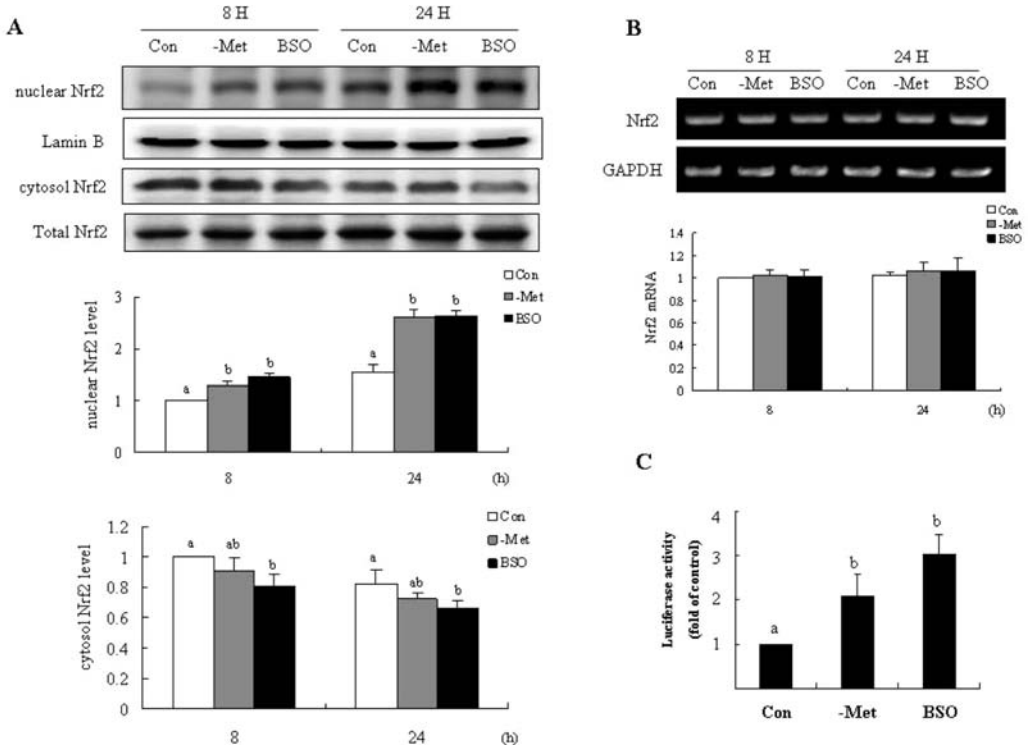

Figure 2. Nrf2 activation by methionine restriction and BSO. After the 24 h attachment period, hepatocytes were treated with an L-15-based control medium (Con), a methionine-restricted L-15 medium (−Met, 0.02 mmol/L L-methionine), or the L-15-based control medium plus 200 μmol/L BSO for 8 and 24 h. (A) Nrf2 expression in the nuclear, cytosolic, and whole cell fractions was determined by immunoblot analysis. (B) Nrf2 mRNA level was determined by RT-PCR. The value for cells treated with L-15-based control medium for 8 h was set at 1. (C) Cells were transfected with the pGL3-2xARE construct and were then treated with the control, −Met, or BSO medium for 24 h. Values are the mean ± SD, $n = 3$. For the letters a and b, values of treatments not sharing the same letter differ significantly, $P < 0.05$.

centrifuged for 10 min at 13000*g*. The supernatant chromatin fraction was diluted 10-fold with ChIP dilution buffer and precleared with 80 μL of salmon sperm DNA in a protein A−agarose slurry (50% by wt). The precleared chromatin fraction was incubated with either anti-Nrf2 or preimmune serum for 16 h at 4 °C with gentle rotation. Immunocomplexes were mixed with protein A−agarose slurry for 1 h at 4 °C and washed sequentially with low-salt immune complex wash buffer, next with high-salt immune complex wash buffer, then with LiCl immune complex wash buffer and, finally, twice with 10 mmol/L Tris-HCl/1 mmol/L EDTA buffer (pH 8.0). Complexes were eluted from protein A−agarose by adding elution buffer, and cross-linking was reversed by heating to 65 °C for 4 h. DNAs were purified by proteinase K digestion and phenol/chloroform extraction. The specific GSTP GPE I regions were amplified by PCR by using the following primer sequences 5′-TGGTAAATGGATAAACTG-3′ and 5′-CAGATAAACAGGAAACTC-3′. The amplified DNAs were electrophoresed on 2% agarose gels.

Statistical Analysis. Statistical analysis was performed with SAS statistical software (Cary, NC). The significance of the difference among mean values was determined by one-way analysis of variance followed by Tukey's test, and the difference between mean values was determined by Student's *t* test. $P < 0.05$ was taken to be statistically significant.

RESULTS

Methionine Restriction Induces GSTP Expression. Compared with cells cultured in the normal L-15 medium, hepatocytes incubated in the L-methionine-restricted medium expressed higher levels of GSTP protein (Figure 1A) and mRNA (Figure 1B) over the 48 h incubation period ($P < 0.05$). To confirm whether methionine restriction affected the GSTP gene expression associated with GSH depression, rat primary hepatocytes were cultured in an L-15-based medium plus 200 μmol/L BSO, which is a GSH synthesis inhibitor that reduces cellular GSH production and raises oxidative stress. Similar to the changes noted in the state of L-methionine restriction, BSO treatment up-regulated GSTP protein and mRNA expression over the 48 h period ($P < 0.05$) (Figure 1).

Changes of Intracellular GSH Content. As shown in Table 1, GSH content was time-dependently decreased in cells treated with BSO and the trend was similar to that in cells cultured in L-methionine restriction medium. After 4 h of incubation in the L-methionine-restricted medium or BSO-containing medium, cells had significantly lower GSH content than cells incubated in control medium ($P < 0.05$). A similar trend was found for the GSH/GSSG ratio, an indicator of cellular redox status ($P < 0.05$). Thus, the up-regulation of GSTP expression in response to methionine restriction seems to be associated with GSH depletion, which subsequently enhances cellular oxidative stress.

Methionine Restriction Activates Nrf2 and ARE-Luciferase Activity. Nrf2 is a key transcription factor that is known to be sensitive to changes in cellular redox status. This raises the possibility that Nrf2 activation is involved in the GSTP induction by L-methionine restriction. We therefore examined whether methionine restriction activates Nrf2 by measuring Nrf2 nuclear translocation (Figure 2A). As shown, when cells were cultured with L-methionine-restricted medium for 8 or 24 h, the nuclear Nrf2 protein level was greater than that in cells with L-15-based medium ($P < 0.05$). Similarly, an increase in Nrf2 nuclear translocation also occurred with BSO treatment ($P < 0.05$). Over the 24 h incubation period, Nrf2 mRNA levels were unchanged in all groups (Figure 2B). In addition, hepatocytes were transfected with the luciferase reporter

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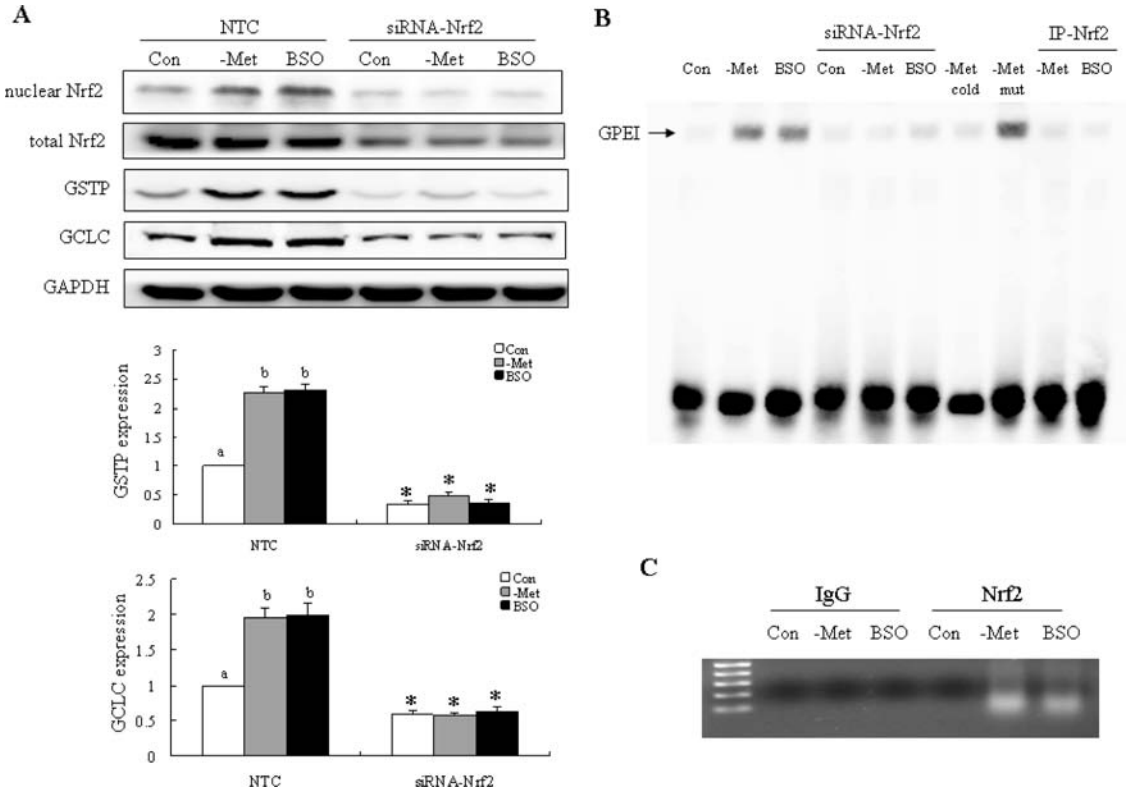

Figure 3. Nrf2 plays a role in methionine restriction-induced GSTP protein expression. Twenty-four hours after isolation, hepatocytes were transfected with Nrf2 siRNA for 16 h and were then treated with an L-15-based control medium (Con), a methionine-restricted L-15 medium (−Met), or the L-15-based control medium plus 200 μmol/L BSO for an additional 16 h (Nrf2 protein) or 24 h (GCLC and GSTP expression). Control cells were transfected with a nontargeting siRNA construct (NTC). The Nrf2, glutamate cysteine ligase catalytic subunit (GCLC), and GSTP protein levels were measured by immunoblot analysis (A), and the DNA binding activity of GPEI was measured by EMSA (B). After the 24 h attachment period, hepatocytes were treated with Con, −Met, or the BSO-contained medium for 16 h. Nrf2 binding to the GPEI was determined by ChIP assay as described in Materials and Methods (C). For letters a and b, values of treatments not sharing the same letter differ significantly, $P < 0.05$, $n = 3$. The asterisk (∗) indicates siRNA-Nrf2 vs NTC by Student's t test, $P < 0.05$.

gene which under the control of the double ARE repeats exhibited a significant increase in transcriptional activity after exposure to methionine restriction and BSO ($P < 0.05$) (Figure 2C).

Effect of Methionine Restriction on GSTP Expression Is Nrf2-Dependent. To further investigate the role of Nrf2 in GSTP induction, we tested an Nrf2 knockdown model by using a siRNA SMARTpool system. The knocking down efficiency of the Nrf2 siRNA SMARTpool system was confirmed by Western blot assay. Compared with control cells, Nrf2 siRNA ablated the increase in the nuclear Nrf2 level induced by methionine restriction and BSO (Figure 3A). Furthermore, in addition to blocking Nrf2 nuclear translocation, Nrf2 siRNA reversed the increase in GSTP expression. We also examined the change in the expression of GCLC, whose transcription is known to be regulated by Nrf2 (Figure 3A). As shown, both methionine restriction and BSO increased the GCLC level ($P < 0.05$), and this increase in GCLC disappeared when Nrf2 was silenced by siRNA transfection. Next, EMSA with the GPEI probe showed that methionine limitation and BSO increased DNA−protein binding activity (lanes 2 and 3, Figure 3B). Knocking down of Nrf2 reversed the methionine-restriction- and BSO-induced GPEI binding activity to the nuclear proteins (lane 5 vs lane 2 and lane 6 vs lane 3). To verify whether Nrf2 binds to GPEI, nuclear protein samples in which Nrf2 was excluded by immunoprecipitation with anti-Nrf2 antibody were used for EMSA. As noted, anti-Nrf2 antibody pretreatment diminished the binding of nuclear proteins to GPEI oligonucleotides (lanes 9 and 10).

Next, a ChIP assay was performed to further confirm the binding of Nrf2 to GPEI. The anti-Nrf2 antibody precipitated the GPEI from the chromatin of cells treated with methionine restriction or BSO, but this change was not noted in control cells (Figure 3C). This result clearly indicates that Nrf2 was bound to the GPEI in the cells expressing high levels of GSTP.

ERK as an Upstream Activator of the Methionine Restriction-Induced Nrf2 Activation. Nrf2 activation is reported to be under the regulation of mitogen-activated protein kinase, phosphatidylinositol 3-kinase (PI3K)/Akt, and protein kinase C.[34] ERK, but not JNK or P38, is known to be activated by restricting the L-methionine supply.[32] We next investigated whether ERK is involved in Nrf2 activation and GST gene transcription. Compared with that in the control cells, higher ERK2 phosphorylation resulted in cells responding to methionine restriction (Figure 4A). ERK2 activation was noted at 8 h and lasted until 24 h ($P < 0.05$). The role of ERK2 in methionine restriction-induced GSTP protein expression was investigated as well. As shown in Figure 4B, ERK2 silencing blocked methionine restriction and BSO-induced activation of ERK2 phosphorylation and ablated the induction of GSTP protein expression ($P < 0.05$). EMSA revealed that ERK2 siRNA attenuated the methionine-restriction- and BSO-induced Nrf2 binding to GPEI oligonucleotides (Figure 4C).

We further used cells transfected with luciferase reporter constructs harboring the GPEI (pTA-GPEI, 2713- to 2605-bp) (Figure 5A), the entire 2713-bp fragment of the GSTP gene

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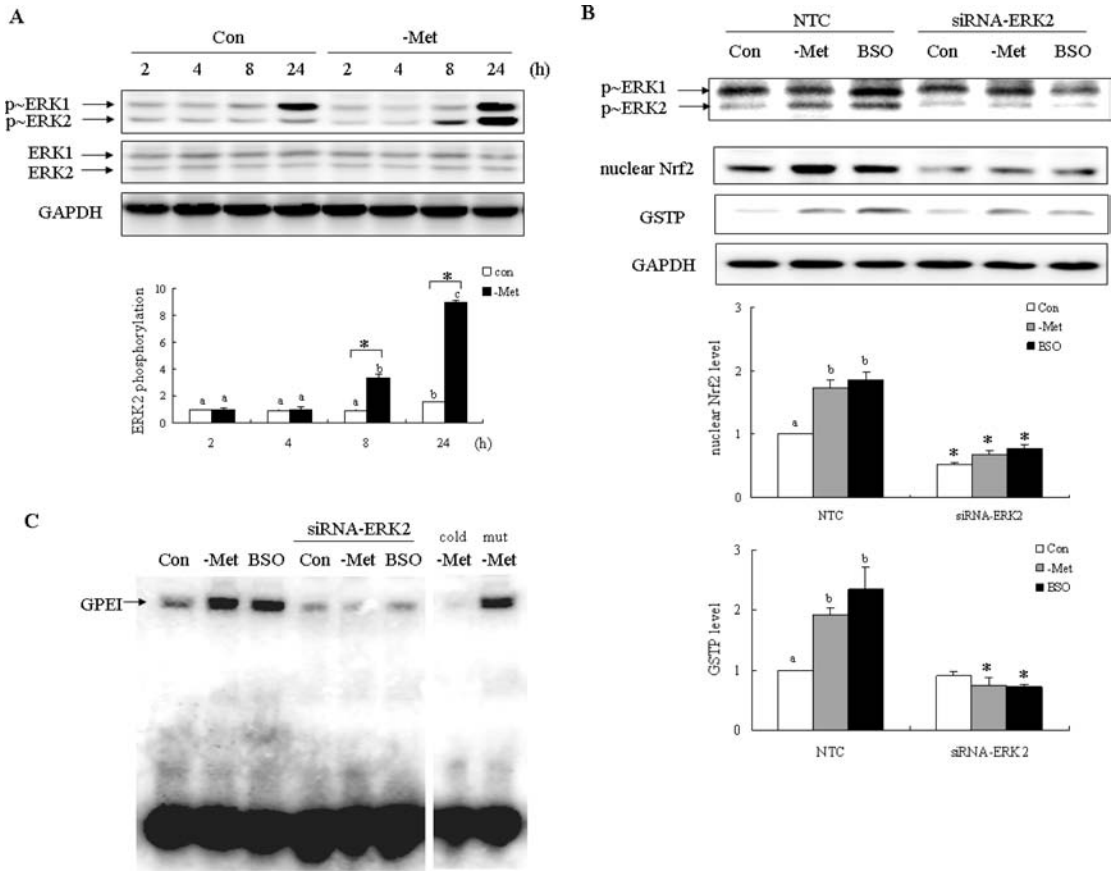

Figure 4. ERK acts as an upstream activator of Nrf2. Hepatocytes were treated with an L-15-based control medium (Con) or a methionine-restricted L-15 medium (−Met) for the indicated time. ERK phosphorylation was determined (A). For gene silencing, hepatocytes were transfected with the ERK2 siRNA (siRNA-ERK2) or a nontargeting siRNA construct (NTC) for 16 h and were then incubated with the control, −Met, or BSO medium for an additional 16 h. The phosphorylation of ERK, Nrf2 nuclear translocation, and GSTP expression were measured by immunoblot analysis (B), and the DNA binding activity of GPEI was measured by EMSA (C). Values are the mean ± SD, $n = 3$. For letters a−c, values of treatments not sharing the same letter differ significantly, $P < 0.05$. The asterisk (∗) indicates Con vs −Met or siRNA-ERK2 vs NTC by Student's t test, $P < 0.05$.

promoter (pTA-PGST-2713) (Figure 5B), or the ARE (pGL3-2xARE) (Figure5C) to determine the specificity of methionine restriction for these sequences. As shown, methionine restriction and BSO treatment increased luciferase activity of reporters harboring the GPEI, the full length of the PGST promoter, and 2x-ARE ($P < 0.05$). When hepatocytes were transfected with ERK2 or Nrf2 siRNA, the increases in reporter activities by methionine restriction and BSO were abolished ($P < 0.05$). Taken together, these findings suggest that ERK signaling likely acts as an upstream activator of Nrf2 and that this subsequently mediates GSTP gene transcription.

DISCUSSION

In response to protein or amino acid deficiency, the global protein synthesis rate in cells decreases through a reduction in translation initiation and elongation[35] and the protein degradation rate increases through the induction of macroautophagy.[36] However, the expression of certain genes, such as amino acid transporters, enzymes, and transcriptional factors, is up-regulated by restricting protein or amino acid availability. Among the genes up-regulated, the working molecular mechanism of asparagine synthase, CHOP, and IGFBP1 induction in mammals cells has been well investigated.[12] For instance, induction of IGFBP1 by leucine limitation involves both transcriptional activation and mRNA stabilization.[37] Overexpression of IGFBP1 inhibits the mitogenic and metabolic effects of the insulin-like growth factors 1 and 2 and is responsible for growth inhibition in response to prolonged feeding of a protein-deficient diet.[38] Induction of the phase II detoxification enzyme GST including A3/5, M1, and P1 in liver tissue has also been reported in rats fed a protein-deficient diet or a methionine-deficient diet.[13,39,40] In a previous work, we reported that GSH depletion-dependent activation of ERK-AP-1 signaling is responsible for the induction of GSTP resulting from L-methionine restriction.[32] The results of the present study further indicate that Nrf2 is activated by a limited methionine supply in an ERK-dependent manner and that this subsequently prompts Nrf2 binding to the enhancer GPEI of the GSTP gene.

Reactive oxygen species (ROS) are a ceaseless outgrowth of aerobic metabolism and determine the cellular redox state. Controlling redox homeostasis is critical for many cellular functions. Once the balance between oxidation and reduction is overwhelmed toward oxidation, oxidative stress is resulted. Redox imbalance has been implicated in the etiology and progression of insulin resistance, atherosclerosis, cancer, and neurodegenerative diseases.[41,42] However, ROS also act as signaling molecules for the modulation of antioxidant action, drug metabolism, and energy metabolism, which helps organisms to adapt to environmental changes and chemical insults.[43] MAPKs, PI3K/Akt, and protein kinase C have been demonstrated to be activated under oxidative stress.[44] Sensing and adaptation to redox changes can be mediated through cysteine residues in various proteins and in the GSH molecule.[45] Through transsulfuration, methionine

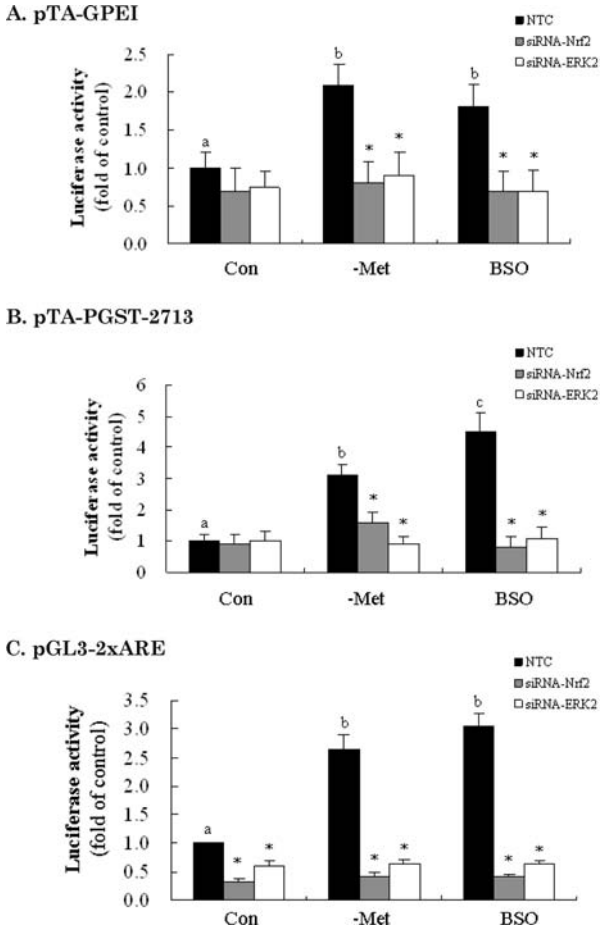

Figure 5. ERK2 and Nrf2 silencing reduced the activity of reporters harboring the GSTP enhancer I (GPEI) (A), 2713-bp promoter of GSTP (B), or 2xARE (C). Eight hours after isolation, hepatocytes were pretransfected with the Nrf2 siRNA, ERK2 siRNA, or a nontargeting siRNA construct (NTC) for 8 h and were then co-transfected with the indicated reporter plasmid for an additional 8 h. Cells were then treated with an L-15-based control medium (Con), methionine-restricted L-15 medium (−Met), or 200 μmol/L BSO-containing medium for 24 h. The cells were lysed and luciferase activity was measured. Values are the mean ± SD, $n = 3$. For letters a–c, values of cells transfected with NTC not sharing the same letter differ significantly, $P < 0.05$. The asterisk (*) indicates siRNA vs NTC by Student's t test, $P < 0.05$.

is converted into cysteine, the key substrate for GSH synthesis.[46] Accordingly, a short supply of methionine limits de novo synthesis of GSH and increases ROS production in hepatocytes.[32] In the present study, the increase in GSTP expression caused by methionine restriction was similar to that resulting from treatment with BSO (Figure 1A), which depletes cellular GSH to a level similar to that in methionine-restricted cells. Moreover, GSTP induction by methionine restriction is partially reversed by pretreating with the GSH donors GSH ethyl ester and *N*-acetylcysteine.[32] These findings support the notion that GSH depletion plays, at least in part, a role in up-regulating the expression of this phase II detoxification enzyme.

Activation of the GSTP gene is well demonstrated to be regulated mainly by the GPEI enhancer element, which contains the TRE-like binding site.[20] ERK and its downstream target AP-1 are thought to be the most important signaling pathway that is responsible for GSTP induction by various phytochemicals and carcinogens. Diallyl disulfide and diallyl trisulfide, the two most abundant organosulfur compounds in garlic oil, and butein, a flavonoid rich in hops, safflower, and wax apple, up-regulate GSTP expression in primary rat hepatocytes in association with ERK-AP-1 activation.[47,48] In primary rat cardiocytes, neuregulin-stimulated GSTP-1 expression can be down-regulated by the ERK inhibitor PD98059.[49] Although p38 and JNK are also activators of AP-1, these kinases are less important in the case of GSTP induction. Instead, JNK has been reported to be negatively regulated by GSTP expression through JNK and GSTP interaction.[50,51] In this study, knockdown of ERK2 by siRNA transfection and by addition of PD98059 to cells attenuated the GSTP induction by methionine restriction (Figure 4), indicating the importance of ERK signaling in GSTP expression.

Although activation of the rat GSTP gene is mediated in large part by the binding of AP-1 to the GPEI enhancer, evidence indicates that activity of GPEI can be regulated by transcription factors other than AP-1.[52] Indeed, the activation of the MAPK pathway via ROS or the electrophilic-mediated stress response activates the bZIP transcription factors Nrf2 and NFκB as well. Diallyl sulfide stimulates a transient increase in ROS and activates the ERK kinase, resulting in Nrf2 nuclear translocation and heme oxygenase 1 gene transcription in rat liver.[53] Overexpression of heme oxygenase 1 in human renal cancer cells is associated with activation of the Ras-Raf-ERK pathway, which prompts Nrf2 binding to the gene promoter.[54] Although the GPEI is recognized by the Nrf2-MafK heterodimer and up-regulates GSTP expression during hepatocarcinogenesis,[28,55] whether Nrf2 plays the same role in normal rat hepatocytes remains unclear.

Nrf2 is recognized to bind to the ARE, which was initially characterized to have a consensus core sequence TGACnnnGCA.[56] It is well demonstrated that Nrf2 binding to the ARE is essential for the up-regulation of antioxidant and detoxification enzyme genes, including heme oxygenase 1, NAD(P)H quinone oxidoreductase, glutamate cysteine ligase catalytic and modifier subunits, gastrointestinal GSH peroxidase, and UDP-glucuronyl transferase under oxidative stress.[57] The sequence homology between the ARE core sequence and the TRE-like element in GPEI (TGATTCAGCA) suggested the possibility that, other than AP-1, Nrf2 likely binds to GPEI and thus enhances GSTP transcription. In the present study, methionine restriction as well as BSO treatment increased nuclear Nrf2 translocation (Figure 2A) and the activity of reporters harboring GPEI and the ARE (Figure 2C). Moreover, EMSA and ChIP assay confirmed that nuclear Nrf2 bound to GPEI when cells were cultured in a medium with a restricted methionine supply or that contained BSO (Figure 3B and Figure 3C). Moreover, when Nrf2 expression was silenced by siRNA, both methionine-restriction- and BSO-induced nuclear Nrf2 translocation and GSTP protein expression (Figure 3A) were attenuated. These findings strongly support the mechanism that methionine restriction initiates oxidative stress, activates Nrf2 translocation into the nucleus, and enhances Nrf2 binding to GPEI and that this subsequently up-regulates GSTP gene transcription in rat hepatocytes.

Under basal conditions, Nrf2 is retained in the cytosol by binding to Keap1.[22] The Keap1−Nrf2 complex is disrupted in response to oxidative stress and several electrophilic antioxidants, and free Nrf2 is translocated to the nucleus, where it binds to the ARE sequences of the target gene promoter in conjunction with small Maf proteins. Therefore, Nrf2 is regarded as an oxidative stress effector responsible for up-regulating the transcription of many antioxidant genes.[58] Several signaling kinases including protein kinase C, MAPK, and PI3K/Akt have been reported to act as upstream activators that phosphorylate Nrf2.[58] In addition, thiol

oxidation of Keap1 cysteine residues by ROS leads to Nrf2 activation.[45] In this study, an increase of ERK2 phosphorylation and Nrf2 nuclear translocation concomitantly resulted from methionine restriction and BSO treatment (Figure 4A). When ERK2 was silenced by siRNA transfection, methionine-restriction- and BSO-induced Nrf2 nuclear translocation (Figure 4B), GPEI binding activity to Nrf2 (Figure 4C), and GSTP protein expression (Figure 4B) were suppressed. Moreover, the luciferase activity of reporter genes constructed with the entire GSTP promoter (2713-bp fragment), GPEI only (2713- to 2605-bp), or the ARE (pGL3-2xARE) was inhibited by ERK2 silencing (Figure 5). Thus, the evidence shows that ERK is an upstream enzyme that activates Nrf2-dependent GSTP transcription in hepatocytes in response to methionine availability.

In conclusion, our results strongly suggest that induction of GSTP gene transcription by methionine restriction in rat hepatocytes is ascribed to GSH depletion, which activates the ERK-Nrf2 signaling pathway and drives Nrf2 binding to the GPEI in the GSTP promoter region. Such an increase of phase II metabolizing enzyme GSTP expression might reflect the compensatory effect of protein and/or methionine deficiency on the detoxification capacity in liver.

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Notes

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ABBREVIATIONS USED

ARE, antioxidant response element; ChIP, chromatin immunoprecipitation assay; ERK, extracellular signal-regulated kinase; GCLC, glutamate cysteine ligase catalytic subunit; GSTP, π class of glutathione *S*-transferase; GPEI, π class of glutathione *S*-transferase enhancer I; Keap1, Kelch-like ECH-associated protein; Nrf2, nuclear factor erythroid-2-related factor 2; ROS, reactive oxygen species; siRNA, small interference RNA

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